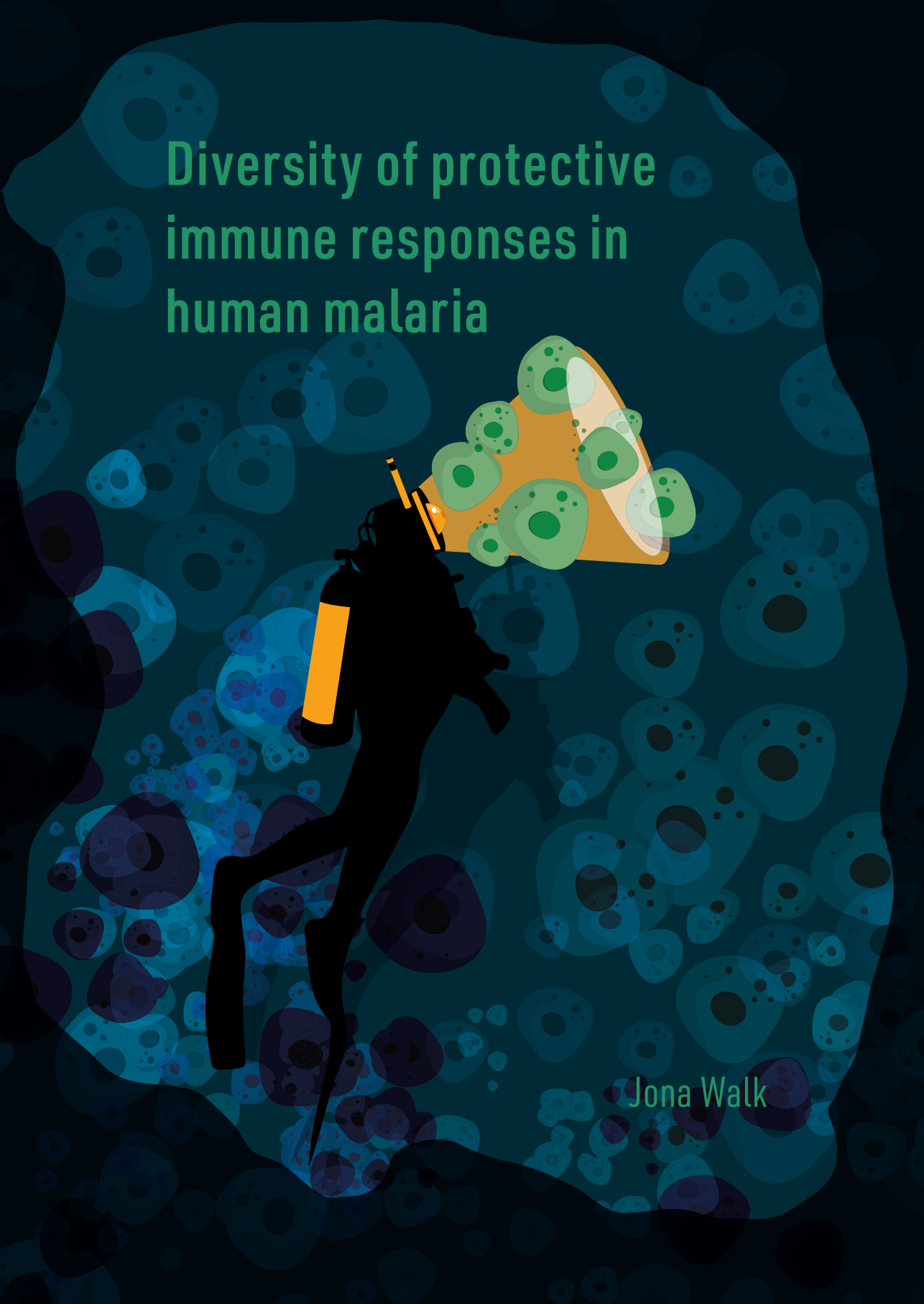
Diversity of protective immune responses in human malaria



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About the cover

You are balanced just a few meters above the water, the starry night sky above you and the dark waves below. Your fins dangle over the edge of the boat, your weight rests solely on your heels. You preform your very last checks: your BCD is inflated, your octopus is secure, your computer and compass are fixed and ready. You put on your mask and take your first breath of dry, compressed air through your regulator. Finally you turn on your lamp and hold it tightly to your chest as you plunge into the pitch-black water below.

Beneath the waves the ocean is calm and tranquil, but completely dark. Only the small bundle of light from your lamp reveals the magnificent corals and abundance of life that surrounds you. Here you can observe the immense complexity of the underwater world only where your light falls, one square meter at a time. You have entered a vast ecosystem that depends on the interactions between millions of parts, from single-celled plankton to the symbiosis between coral polyps and photosynthetic algae, from schools of fish to migrating manta rays, everything is interconnected. Yet our limited human eyes are fated to study its components one by one.

In your head you have a plan for what you will do next; the maps were drawn on whiteboards and the procedures agreed upon in advance. But in practice you never know exactly what you are going find underwater, so you must fall back on your training and trust in your buddies as you explore the unknown.

In this thesis I present a few small pieces of the diverse immune responses that lead to protection in human malaria. They are observations from different trials and distinct experiments that often study only a few components at a time. Where I could I have filled in the gaps with the vast body of research published by many other centers. Yet much of this complex but amazing immunological world remains, for now, incompletely understood and ready for the next wave of explorers.

Contents

CHAPTER 1

General Introduction

The single celled malaria parasite *Plasmodium* is one of the most consequential pathogens in human history. It has been found to infect a variety of mammals and birds, including the common ancestor of both humans and chimpanzees, and it is clear that the battle against this disease has shaped the very evolution of our species [1-3].

Documentation of the impact of this disease can be found throughout history, from ancient Chinese medical texts to records found in Egypt and Greece [4]. Early humans were likely infected predominantly with *Plasmodium vivax* and *Plasmodium malariae* [5], which caused chronic, debilitating infections that diminished both the duration and quality of life [4]. Then, most likely sometime during the last ten thousand years, a new sub-species of *Plasmodium* appeared in humans, *Plasmodium falciparum* [6]. Unlike the other human *Plasmodia*, *Plasmodium falciparum* caused acute, severe illness and high mortality even in previously healthy individuals. Periodic outbreaks of this parasite were devastating. At the height of their global dominance in the 19th century, the human *Plasmodium* parasites likely killed one out of every ten people on Earth [4].

However, in the twentieth century the tide began to change. Thanks in large part to an extensive WHO campaign in the 1950's and 1960's to use vector control strategies like dichlorodiphenyltrichloroethane (DDT) and new antimalarial drugs like chloroquine, countries with seasonal malaria transmission, like The Netherlands, were able to eradicate the disease. Unfortunately, the same success has not been achieved in large parts of South America, South-East Asia and sub-Saharan Africa, where especially the deadly *Plasmodium falciparum* parasite continues to be responsible for a significant proportion of child mortality.

Malaria control: successes and failures

According to the World Health Organization (WHO) there were approximately 219 million cases of malaria in 2017 [12], a 50% reduction since the Roll Back Malaria initiative began in 2000. This reduction is generally attributed to increased use of vector control strategies, including insecticide-treated bednets (ITNs) and indoor residual spraying (IRS) [13-15] and to improved access to and use of rapid diagnostic tests (RDTs) and artemisinin-combination therapy (ACT) [13, 16, 17]. RDTs have improved diagnostics in remote areas where skilled microscopists and complex molecular techniques are unavailable, and treatment of asymptomatic individuals in these communities has helped to reduce disease transmission [18-21].

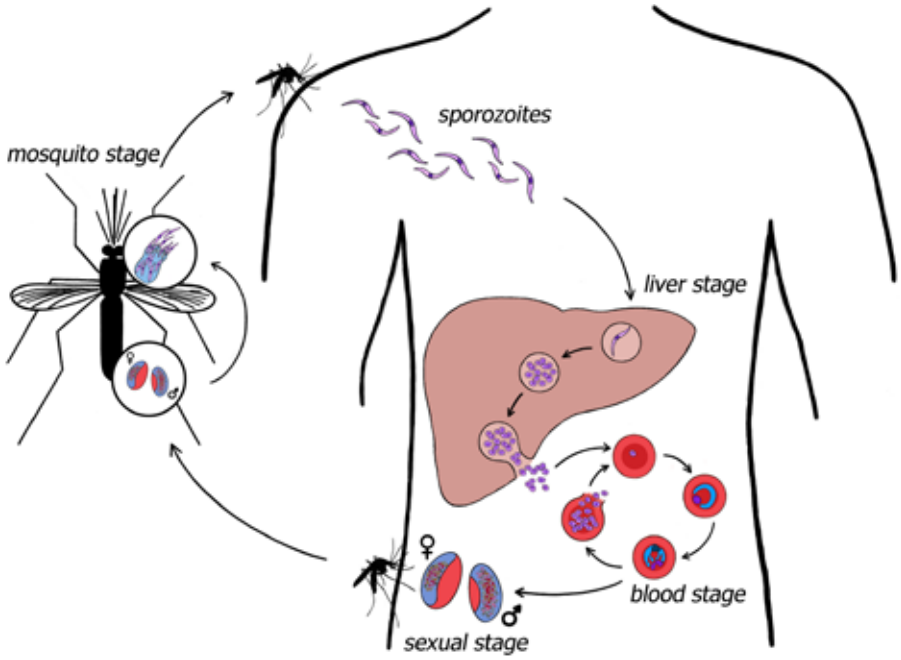

Figure 1: *Plasmodium falciparum* life cycle. An infected female *Anopheles* mosquito injects 10-200 **sporozoites** into the skin. Sporozoites travel through the skin to enter the capillaries and make it into the larger blood vessels to reach the liver [7]. Each sporozoite that reaches the liver traverses several hepatocytes before finally establishing infection in one. During the **liver stage** sporozoites develop within a hepatocyte to about 30,000 merozoites [8]. After 5-7 days of development and multiplication in the liver, **blood stage** merozoites are released into the circulation where they proceed to invade and further replicate in erythrocytes. During each 48 hour replication cycle a new generation of 16-32 merozoites is released from each infected red blood cell [9]. During each cycle a small fraction of merozoites commit to **sexual stage** development, maturing into either a female or male gametocytes over the course of 10-12 days [10]. Fully matured gametocytes, taken up by another feeding *Anopheles* mosquito can initiate **mosquito stage** infection. In the mosquito midgut gametocytes emerge from the erythrocyte as gametes, and male gametes adhere to and penetrate female gametes to generate zygotes [11] that give rise to a new progeny of sporozoites.

Unfortunately, between 2015 and 2017 this trend leveled off and though certain countries and regions continued to make great strides in combating malaria, a resurgence of cases in other areas means there has been no global progress over the past three years [12, 22] (figure 2). Though this is mostly the result of a failure to further implement vector control and community treatment programs [22], newly emerging problems such as insecticide resistance and parasite drug resistance will further complicate malaria control over the next decade.

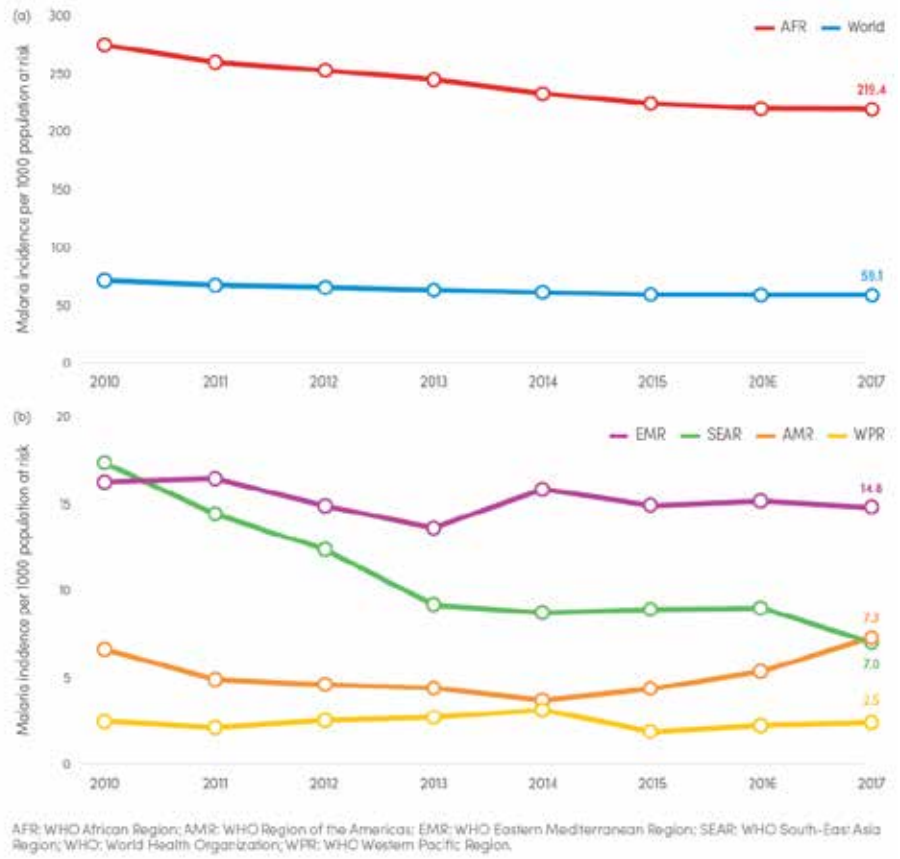

Figure 2: Yearly incidence of malaria in cases per 1000 population at risk, globally (A) and per WHO region (B) from 2010 to 2017. World Health Organization estimates, source [12].

In 2017 the world spent 3.1 billion US dollars on funding for malaria control and elimination, and the United States Centers for Disease Control (CDC) has estimated the annual costs of malaria in endemic areas (including cost of drugs, travel and medical expenses, and lost days of work) to be in excess of 12 billion USD [23]. From a clinical perspective, providing adequate care is complicated by the enormous number of malaria cases [12]. Inadequate access to health care and delay to treatment are important factors in malaria mortality, especially in young African children. Vaccines are considered to be among the most cost-effective medical interventions. In addition to providing clinical protection for individuals, an effective malaria vaccine would be a valuable additional tool to support the reduction of transmission and the eventual eradication of the disease.

Malaria vaccination strategies: diverse approaches to a complicated problem

The complexity of the malaria life cycle presents many opportunities for vaccine-induced immunity to act. These approaches can be divided into three types of vaccines: pre-erythrocytic, blood stage and sexual stage. Pre-erythrocytic vaccines target sporozoites either in the skin and bloodstream [7] or during their development in the liver [8]. Vaccines that induce anti-sporozoite antibodies immobilize sporozoites in the skin or during their transit in the blood vessels [24] and prevent infection of the liver. Other approaches target developing liver stages. Though clinically silent, there is increasing evidence for activation of local cellular immune responses in the liver [25-27], and several vaccination strategies seek to strengthen this immunity [28, 29]. The goal of this type of vaccine is to prevent symptomatic infection in humans completely.

Blood stage vaccines aim to block merozoite invasion and further replication in erythrocytes after their release from the liver. Vaccine-induced protection against these stages is mediated by both antibodies that inhibit red blood cell invasion [30] and cellular immune responses [30-33]. The goal of these vaccines is to reduce parasitemia and thereby diminish clinical symptoms.

Vaccines that target the sexual parasite stages represent a unique approach as they strive to induce antibody responses against the gametocytes or gametes. As such, they do not reduce infection or clinical symptoms in humans, instead they prevent transmission from humans to mosquitoes and have their effect on population level. To date, several antigens have been demonstrated to induce antibodies capable of blocking this transmission [11, 34-36].

The studies described in this thesis concentrate on the induction of pre-erthrocytic, protective immunity. The liver stage is an attractive target for vaccines because it represents a bottleneck in parasite development and precedes the onset of clinical symptoms. Furthermore, when sterilizing liver stage immunity is achieved, the absence of any blood stage parasites also disrupts human to mosquito transmission [37-39].

RTS,S: the first malaria vaccine

Adapted from L.A. van der Schans, J. Walk et al. [40]

Over the past decade's most malaria vaccine research has concentrated on developing a so-called 'sub-unit' malaria vaccine that consists of a single parasite protein. One such vaccine, RTS,S/AS01 (Mosquirix®), was developed by GlaxoSmithKline

Biologicals in collaboration with the United States army and supported by the Bill and Melinda Gates Foundation. After 30 years of research this vaccine received a positive advice from the European Medicines Agency (EMA) [41], however the WHO has advised large pilot studies be conducted to better determine safety, implementation and efficacy of the vaccine in routine care [42]. These trials are currently underway but it will be several years before the data are available.

The RTS,S vaccine consists of part of the circumsporozoite protein (CSP), the main surface protein of the sporozoite stage, coupled to the surface antigen of the Hepatitis B-virus (HbsAg). This vaccine was designed to generate an immune response against the sporozoite stages, blocking parasite development prior to onset of the blood stage responsible for clinical symptoms [43, 44], a so-called pre-erythrocytic vaccine.

In a large multicenter trial in seven African countries, four vaccinations in children 5-17 months old gave 36.3% (95%-CI: 31.8%-40.5%) protection against clinical malaria during the trial period of 48 months. However, the protective efficacy declined quickly during follow-up [45], and this rapid loss of protection remains an important point of discussion. A long term follow-up study running seven years after RTS,S vaccination showed an efficacy of only 4.4% (95%-CI: -17.0% – 21.9%) [46]. Questions about vaccine safety also remain. The multicenter efficacy study found a statistically significant increase in cases of meningitis in the vaccination group and increased mortality in girls. A relationship with RTS,S could not be ruled out [47, 48].

Large pilot studies will create a better picture of both the efficacy and safety of RTS,S. However, for the purpose of malaria elimination the WHO has set the goal for a malaria vaccine that provides at least 75% efficacy lasting at least two years [49], making it clear that other vaccines must be developed.

Controlled Human Malaria Infections for vaccine development: a balancing act between safety and scientific value

One of the most important tools for the further development and testing of malaria vaccines is the Controlled Human Malaria Infection (CHMI) model [50-52]. CHMI involves exposing healthy volunteers to infectious malaria parasites either by exposure to sporozoite stages via infected mosquito bites [53-56] or direct injection [57-61], or through intravenous injection of blood stage parasites [62, 63]. In these studies normally 100% of unvaccinated controls develop patent parasitemia, meaning a high statistical power can be achieved with a small sample size [50, 64]. Over the last decade, CHMI has become increasingly standardized between trials and study centers [52]. Nevertheless, studies have observed significant variability in progression of parasitemia [54, 56], and questions remain about the comparability of trials and CHMI centers. These issues of

variability and reproducibility are important to address when comparing data from trials testing different malaria vaccines.

To ensure safety after infection, volunteers are followed up closely by study physicians, including daily blood screening for malaria parasites using either microscopy, long considered the gold-standard diagnostic test, or quantitative PCR (qPCR). Standardization of thick blood smears in 2004 improved the comparability of CHMI outcomes between trials and study centers [54], and treatment based on microscopy ensures cure for infected volunteers before any risk of serious symptoms. However, volunteers generally have submicroscopic parasitemia for several days before they become thick blood smear positive [51, 54, 65]. Despite an excellent safety profile, this parasitemia does cause mild to moderate malaria symptoms like malaise and headache in most, and severe symptoms in about half of volunteers [54, 56, 66]. Reducing this period of sub-patent parasitemia might further diminish clinical symptoms, but this has not yet been established. Ideally, CHMI study design would be optimized to minimize the height and duration of parasite exposure without losing scientific value.

Whole sporozoite vaccines: an attractive alternative approach

To date several vaccination techniques have focused on generating liver stage immunity, including viral-vectored subunit vaccines [29] and live-attenuated sporozoite vaccines [28]. So far only live-attenuated sporozoites have been able to induce high levels of sterile protection in CHMI models [67, 68].

For almost 50 years studies have shown that live sporozoites weakened by exposure to radiation can induce protective pre-erythrocytic immunity [69]. In early studies subjects exposed to the bites of >1000 infected and irradiated mosquitoes showed high protection against a CHMI with a non-attenuated *P. falciparum* parasite of the same strain [70, 71]. More recently, Sanaria, Inc, a United States based biotech company has manufactured GMP-compliant, cryopreserved, aseptic radiation attenuated sporozoites designated PfSPZ Vaccine. Depending on the regimen this vaccine provides 80-90% short term (3 weeks) and over 50-70% long term (24-59 weeks) protection against homologous CHMI [53, 67, 72], but this level of protection requires intravenous administration of a total of $1.35x10^6$ sporozoites.

A far more efficient means of inducing pre-erythrocytic immunity is through the administration of live, non-attenuated sporozoites under cover of drug prophylaxis that targets the blood stages without affecting liver stage development. This Chemoprophylaxis and Sporozoite (CPS) Immunization has been successfully administered by bites from infected mosquitoes [65, 68] or by intravenous injection of aseptic, cryopreserved sporozoites (Sanaria® PfSPZ-CVac) [73]. Three immunizations

with bites from 10-15 infected mosquitoes [65, 68, 74] or i.v. injection of 50,000 sporozoites [73] resulted in >90% protection, with 4 of 6 volunteers still protected after 2.5 years in a long term follow-up study [75]. This makes CPS at least 20x more efficient than irradiated sporozoites. The enhanced immunogenicity likely results from the longer parasite development, with full liver stage progression and even a brief exposure to blood stages [76, 77].

Whole sporozoite vaccines: obstacles to implementation

Despite their high efficacy, both irradiated sporozoites and CPS immunization have important disadvantages. Radiation randomly damages parasite genes, generating 'non-replicating' sporozoites. Radiation dose is key, as radiation-killed sporozoites are non-immunogenic and, conversely, insufficiently attenuated parasites will cause clinical disease [70, 71, 78]. Inevitably this results in a heterogeneous population, where only a fraction of the sporozoites are capable of invading hepatocytes. On the other hand, a downside of CPS immunization is that it uses live sporozoites capable of causing disease unless chemoprophylaxis is used correctly. In contrast, sporozoite attenuation by genetic modification, in which one or more genes essential to liver stage development are knocked out, has the potential to create a homogenous, intrinsically attenuated parasite. Theoretically, these genetically attenuated parasites (GAPs) have the dual advantage of being safe without drug cover and of progressing until a well-defined developmental endpoint. Indeed rodent studies suggested that GAPs have increased immunogenicity compared to radiation attenuated sporozoites (RAS) [77, 79, 80], but human immunogenicity studies have not been performed.

A second challenge in the development of all pre-erythrocytic vaccines is cross strain protection. *P. falciparum* parasites show an enormous genetic diversity [81-83]. Indeed genetic polymorphisms in target antigens have been shown to decrease the efficacy of single-protein *P. falciparum* vaccines [84-86]. Theoretically genetic diversity may be less challenging for whole sporozoite vaccines to overcome as they represent a broader antigen repertoire that could increase the chances for generating functional, cross-strain immunity. Yet early studies on RAS [87] and CPS [88] immunization indicate that protection against heterologous *P. falciparum* strains is also more difficult to achieve than against a homologous parasite. However, these studies used relatively low immunization dosages that were insufficient for full homologous protection, and immunization with higher sporozoite numbers may increase the chances of heterologous protection.

Protective immunity: the picture remains incomplete

A complete picture of how immunity against malaria is generated will be important for further vaccine development. However, the interaction of the *Plasmodium* parasite

with the human immune system is complex. Data from chronically exposed populations indicate that immunity to *P. falciparum* develops more slowly than to most bacterial or viral pathogens. While a majority of people become protected from severe disease after multiple infections over several years, true sterilizing protective immunity is rare in endemic areas. Antibodies against blood stage parasites have a major role in this protection, suppressing replication and keeping parasitemia relatively low [89-92].

This type of acquired immunity is very different from the sterile immunity that pre-erythrocytic vaccines strive to achieve. Indeed there are significant differences in immunity between naturally exposed and CPS immunized people [93, 94]. Immune responses after protective whole sporozoite immunization have been extensively examined in mice and humans, but predictive correlates of protection remain elusive. RAS immunization has been shown to induce CD8+ T cell responses targeting liver stage antigens and is generally thought to protect via this mechanism [67, 72, 95, 96]. On the other hand, strong induction of CD4+ T cells correlating with protection has been observed in CPS immunization [74], and RAS, GAP and CPS immunization have all been shown to induce functional antibodies capable of blocking sporozoite liver invasion [67, 97-99]. These studies are further complicated by significant heterogeneity in responses between individuals. To date the relative importance of these adaptive immune responses, or whether protection results from a combination of these effectors, remains unknown.

Studies on protective immunity have focused almost exclusively on T and B cell responses against *P. falciparum*. Though activation of the innate immune system has been demonstrated [100-103], these have been studied primarily in the context of disease pathology and not as potential effectors after immunization. For a long time, innate responses were considered to lack any memory, but more recently the perception of the role and effector function of innate immunity has shifted. A number of studies have shown that innate cells can undergo sustained functional changes after infections allowing them to respond more effectively to a second infection, called 'trained' innate immunity [104-110].

These findings may have implications for vaccinology. Both monocytes [107] and NK cells [108] can be 'trained' by vaccination with *Bacillus Calmette-Guerin* (BCG), a widely used vaccine against tuberculosis. BCG strongly increases production of IFN-γ[106, 107], IL-6 [107] and IL-1 [111] when immune cells are restimulated with other pathogens, an effect shown to persist for months [106]. Changes in epigenetic regulation form the basis of this type of innate immune memory in monocytes [112, 113]. Mouse models have shown that trained immunity provides clinically relevant protection against infections, including *Staphylococcus aureus* sepsis [113] and lethal

Candida albicans [114] infection, but this has not been demonstrated in humans. There is also some data suggesting that malaria infection and CPS immunization cause changes in monocyte phenotype [103] and induce memory-like NK cell responses [74, 115, 116], though the latter are T cell dependent. Taken together these data support a further exploration of innate immune responses in the context of malaria and malaria vaccines.

Tissue resident immunity: location, location, location

In evaluating protective immunity against malaria liver stages in humans, it is important to consider that the relevant responses might be confined to the liver's unique anatomy and distinctive immunological properties [117-119]. The mechanism by which tissue-specific T cell immunity is achieved has received increasing attention in recent years [120, 121]. In the past decade, a new population of memory T cells, tissue-resident memory T cells (T_{RM}), has been shown to persist in non-lymphoid tissues including the skin, gastro-intestinal tract, brain, lung, kidney and liver [121-123]. A broad range of studies in mice and humans show that these cells remain localized in the tissue of the initial immune response and suggest that they provide immediate, font-line protective immunity against reinfection [124-127].

Considering that whole-sporozoite vaccines provide liver stage protection, it is very possible that these cells play an important role. When both blood and hepatic T cells are analyzed in mouse and non-human primate studies with liver stage malaria vaccines, it becomes apparent that peripheral blood cell responses do not quite represent and/or correlate with T cell responses in the liver [67, 95, 128]. This represents an important challenge for malaria vaccine research. These cells remain inaccessible in human studies complicating the identification of relevant effector functions and correlates of protective liver stage immunity.

Thesis aims

Current malaria vaccine strategies are insufficient for the level of protection needed for eradication. Whole sporozoite immunization may provide superior protection, but further research is needed to establish proof of principle for heterologous efficacy and improve upon it. Better heterologous protection will require that we increase both the breadth of the immune response and its magnitude at the critical site, in the intrahepatic compartment. In this thesis we evaluate the current status of heterologous protection after sporozoite immunization and then add new observations to further complete the picture of heterologous immunity.

In part 1 we begin by optimizing the safety and assessing the reproducibility of the Controlled Human Malaria Infection model to help us in answering proof-of-principle

questions about the protective efficacy of whole sporozoite vaccines. In **chapter 2** we strive to answer the lingering question of the reproducibility of CHMIs within and between study centers by investigating the effect of mosquito sporozoite load on their infectivity for CHMI volunteers. In **chapter 3** we focus on maximizing the safety and tolerability of the model by evaluating the impact of treatment based on prospective qPCR diagnostics on the duration of parasitemia and symptoms after CHMI. We then use the CHMI model for clinical trials on the protective efficacy of two immunization strategies. In **chapter 4** we assess the safety and potential protective efficacy of a new genetically-attenuated whole-sporozoite vaccine that arrests during early liver stage against homologous sporozoite infection. In **chapter 5** we evaluate a CPS immunization regimen optimized for homologous protection for its ability to provide protection against genetically distinct parasite strains.

In part 2 we move on to studying an aspect of the immune system that has been overlooked in the evaluation of vaccine induced memory. Here we zoom in on NK cells and monocytes, two cell types of the innate immune system only recently found to possess memory-like characteristics, and we evaluate the role of these unique memory types (also known as 'trained immunity') in sporozoite infection. In **chapter 6** we examine NK cell activation in naïve volunteers during a Controlled Human Malaria Infection, to determine how their heterogeneity prior to infection determines their activation in response to parasitemia. In **chapter 7** we move to monocytes and establish whether *Plasmodium falciparum*, like other pathogens, can induce memory-like changes in monocyte cytokine responses. Finally in **chapter 8** we evaluate whether these trained immune responses, known to be induced by the BCG-vaccine actually provide any clinically relevant protection against a *Plasmodium falciparum* infection.

In the final part 3 this thesis we shed light on the picture of liver immunity, thus far underrepresented despite its importance, as all the preceding chapters have focused on immune measurements in the blood. In **chapter 9** we evaluate the comparability of circulating and tissue-resident memory CD8+ T cells, drawing on data from many different disease models in animal and human studies, with the goal of determining whether we can extrapolate observations in circulating lymphocytes to the hepatic immune system. Finally, in **chapter 10** we use hepatocytes and intra-hepatic lymphocytes from healthy human donors to characterize the capacity of tissue-resident lymphocytes to produce interferon-γ and the ability of IFN-γ to protect against liver-stage parasite development.

Utilizing innate immune memory and targeting cellular immune responses in the liver where they count may be attractive steps in improving heterologous protective immunity in malaria vaccination.

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PART 1

Immunogenicity and efficacy of whole sporozoite vaccines

CHAPTER 2

Mosquito infectivity and parasitemia after Controlled Human Malaria Infection

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Abstract

Controlled Human Malaria Infection (CHMI) has become an increasingly important tool for the evaluation of drugs and vaccines. CHMI has been demonstrated to be a reproducible model; however, there is some variability in time to onset of parasitemia between volunteers and studies. At our center, mosquitoes infected with *Plasmodium falciparum* by membrane feeding have variable and high salivary gland sporozoite load (mean 78,415; range 26,500 – 160,500). To determine whether this load influences parasitemia after CHMI, we analyzed data from thirteen studies. We found no correlation between the sporozoite load of a mosquito batch and time to parasitemia or parasite density of first-wave parasitemia. These findings support the use of infected mosquito bite as a reproducible means of inducing *P. falciparum* infection and suggest that within this range, salivary gland sporozoite load does not influence the stringency of a CHMI.

Controlled human malaria infection (CHMI) by the bites of *Plasmodium falciparum* infected, laboratory reared *Anopheles* mosquitoes has been used to study the infection since the 1980's. In recent years CHMI has become highly standardized and is used to assess the efficacy of anti-malarial drugs and vaccines prior to large scale field trials. This has been possible in part because of the high reproducibility of CHMI studies within and between centers [1]. Nevertheless, there is variability in time to detectable parasitemia between studies and between centers [2, 3]. As in any biological system, there is also significant variability between batches of infected mosquitoes used for CHMI, most notably in the number of salivary gland sporozoites. Recently, CHMI centers have increasingly started using intravenous injection of cryopreserved *P. falciparum* sporozoites to initiate infection [4, 5], in part because it is easier to standardize dosage. However, using the natural route of infection via the mosquito still has advantages over intravenous injection. Most importantly, it includes the immune response in the skin, which likely plays a role in both vaccine induced protection [6] and the response to primary infection [7].

To test whether differences in salivary gland sporozoite load between mosquito batches influenced the outcomes of CHMI studies at our center, we collected data from all past clinical trials since 2007 in which malaria naïve volunteers were challenged with bites from five NF54 strain *P. falciparum* infected *Anopheles* mosquitoes, as described previously [3]. In these studies, mosquito batch percent infectivity and sporozoite load were quantified one day prior to CHMI, by dissection of salivary glands from a sample of ten mosquitoes per batch. Dissected salivary glands were pooled and homogenized using a glass grinder; sporozoites were quantified in a counting chamber using phase-contrast microscopy. The mean salivary gland sporozoite number per mosquito was calculated. After the malaria infection, volunteers were followed-up once to three times daily from day 6 after infection until three days after treatment of parasitemia. Quantitative real-time PCR (qPCR) was performed on blood samples either prospectively as the primary diagnostic test or retrospectively if thick blood smear was used as primary diagnostic test [8, 9].

We analyzed data from thirteen CHMIs taking place between 2007 and 2016, involving 75 malaria naïve volunteers (table 1). The mean sporozoite load of the mosquito batches was variable, between 26,500 and 160,500 (mean 78,415; 95% CI 59,627 – 97,204). We found no correlation between the mean sporozoite load of the batch used and the time to parasitemia detectable by qPCR (Spearman r = 0.10), figure 1A. Day 7 parasitemia can be used as a reliable proxy measurement for liver parasite burden: parasite density on day 7 after challenge correlates strongly with the mean parasitemia of the first wave of parasites to emerge from the liver (n=50; Spearman

r=0.90; p<0.0001), when anti-malarial treatment is initiated after the first peak. There was no correlation between mosquito sporozoite load and parasitemia on day 7 after CHMI (Spearman r = 0.05), figure 1B. Forty-nine volunteers (65%) were exposed to exactly five mosquitoes, the rest required a second exposure because either a mosquito was unfed or a fed mosquito was uninfected. As unfed mosquitoes may still have probed, possibly transmitting sporozoites, we performed a second analysis adjusting for the total infected mosquito exposure. Here, there was also no correlation with time to parasitemia (Spearman r = 0.17) or day 7 parasitemia (Spearman r = 0.06).

In CHMIs where the number of injected sporozoites is precisely controlled, as in intravenous injection studies, increasing the sporozoite number has an effect on prepatent period; injection of 50 or 3200 sporozoites resulted in a prepatent of 13.3 and 11.2 days, respectively [4]. However, this analysis shows that between our CHMI trials there is no difference in infectivity to volunteers depending on salivary gland sporozoite load of the mosquito batch used. In contrast, a recent study found that the probability of malaria transmission to humans decreased when mosquitoes had sporozoite loads of less than 1000 [10]. However, only mosquitoes with 1-10, 11-100, 101-1000 and >1000 sporozoites were compared, a much lower load than the mosquitoes used in our analysis. Taken together these observations suggest that above a certain threshold an increased number of sporozoites in the salivary glands no longer increases the number of sporozoites transmitted.

In the past, findings on the association between the mosquito salivary gland load of *P. falciparum* and the number of sporozoites injected by a salivating mosquito have been contradictory, with some studies finding a correlation [11] and others not [12, 13]. In these studies, mosquitoes were typically induced to salivate onto glass cover slips or into capillary tubes. The number of sporozoites released during salivation was shown to be between 1 and nearly 1000 sporozoites per mosquito, even when a much greater number was present in the salivary glands [13, 14]. More recently, studies with live imaging of fluorescent parasites have confirmed that only a small number of sporozoites are present in the mosquito salivary ducts and that each mosquito injects tens to hundreds of sporozoites during a feed [15, 16]. However, these types of studies have always been confined to rodent malaria models. Modeling of parasitemia after CHMI has calculated that an infected mosquito transmits an average of 21 sporozoites that successfully infect the liver [17], but studies directly linking the mosquito sporozoite load and liver parasite burden *P. falciparum* are lacking.

The current data support the idea that the number of sporozoites injected by a feeding mosquito is independent of the number in the salivary glands, at high infection intensities. It is important to emphasize that all mosquito batches used in these studies

had salivary gland sporozoite loads far exceeding that found in the field (usually less than 10,000) [18]. Other CHMI centers either have mosquito salivary gland sporozoite loads that are much lower or in the same range as the studies presented here [10, 19, 20]. Irrespective of mosquito sporozoite load, most of these studies have pre-patent periods similar to those at our center [9, 19, 20].

A weakness of this analysis is that it used only pooled mean mosquito batch sporozoite counts. In future studies qPCR can be applied to analyze individual mosquito sporozoite loads in relation to volunteer pre-patent periods. In such a study it would also be interesting to generate mosquitoes with more variable sporozoite loads by titrating gametocyte concentration in their blood meal.

In conclusion we demonstrate that the infectivity of mosquitoes to humans in CHMI studies at our center is independent of the salivary gland sporozoite load of the mosquito batch used. This finding supports the use of infected mosquito bite as a reproducible means of inducing *P. falciparum* infection, and suggests that at high levels of infectivity increased salivary gland sporozoite load does not increase the stringency of a mosquito challenge.

Table 1: CHMI studies used in this analysis

	Year	Trial registration number	Number of volunteers	SPZ prevalence (% mosquitoes infected)	SPZ intensity (mean number of SPZ per mosquito)
Study 1	2007	NCT00442377	5	80	31500
Study 2	2008	NCT00509158	18	100	72800
Study 3	2009	NCT00757887	5	96.5	88000
Study 4	2010	NCT01002833	4	100	69000
Study 5	2011	NCT01218893	5	100	79500
Study 6	2012	NCT01422954	4	100	98250
Study 7	2012	NCT01627951	5	100	101250
Study 8	2012	NCT01728701	5	93.8	75800
Study 9	2012	NCT01728701	4	100	98000
Study 10	2015	NCT02080026	5	100	74000
Study 11	2015	NCT02098590	2	90	26500
Study 12	2015	NCT02098590	3	100	44300
Study 13	2016	NCT02692963	10	100	160500
Total			**75**		

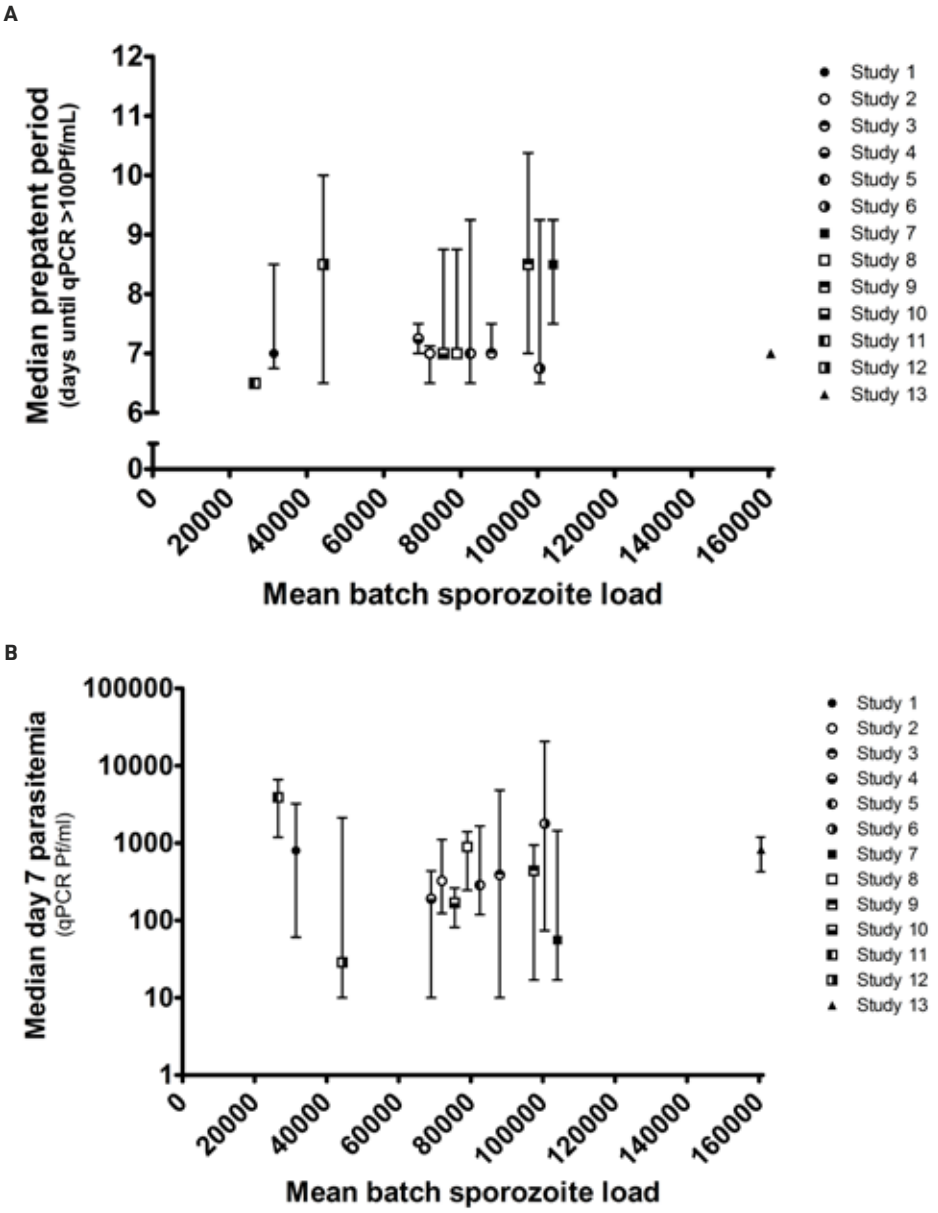

Figure 1: Correlation between *P. falciparum* mosquito infection and infectivity to humans. The mean salivary gland sporozoite load determined by dissection of a sample of ten mosquitoes compared to (A) the time to parasitemia detectable by PCR (>100 parasites per milliliter) and (B) the height of parasitemia on day 7 post infection. Points and error bars show the median and interquartile range.

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CHAPTER 3

Diagnosis and treatment based on quantitative PCR after Controlled Human Malaria Infection

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Abstract

Background

Controlled Human Malaria Infection (CHMI) has become well-established in the evaluation of drugs and vaccines. Anti-malarial treatment is usually initiated when thick blood smears are positive by microscopy. Here, we explore the effects of using the more sensitive qPCR as the primary diagnostic test.

Methods

1,691 diagnostic blood samples were analysed by microscopy and qPCR from 104 volunteers (55 malaria naïve and 60 having received Chemoprophylaxis and Sporozoite immunization) who were challenged by five mosquitoes infected with *Plasmodium falciparum* sporozoites of the NF54 strain.

Results

Retrospective analysis of different qPCR criteria for diagnosis and treatment, showed that once daily qPCR (threshold100 parasites/ml) had 99% sensitivity and 100% specificity, shortened the median prepatent period from 10.5 to 7.0 days after CHMI when compared to twice daily measurement of thick blood smears (threshold 4,000 parasites/ml) and was associated with 78% decrease of adverse events before initiation of treatment. Trial outcome related to infection and protective efficacy remained unchanged.

Conclusion

The use of qPCR as primary diagnostic test in CHMI decreases symptoms as well as parasitemia while obviating the need for twice daily follow-up. The implementation improves safety while reducing the clinical burden and costs without compromising the evaluation of protective efficacy.

Background

Controlled human malaria infection (CHMI) has proven to be a valuable tool to evaluate the efficacy of drugs and vaccines and to study the pathogenesis of clinical malaria. These challenge trials have become highly standardized [1] and are considered a critical step in the clinical development of pre-erythrocytic malaria vaccines [2].

Traditionally, volunteers are followed after CHMI by once to three times daily thick blood smears, and anti-malarial treatment is initiated immediately once two or more parasites are detected by microscopy. In 2004, a standardized protocol for CHMI thick blood smears was introduced using a threshold of 4,000 parasites/ml to improve the comparability of study outcomes between centres [3]. Volunteers generally develop submicroscopic parasitaemia for several days before they become thick smear positive. The more sensitive quantitative PCR (qPCR) with a detection limit of 20 parasites/ml was introduced for retrospective analysis feeding a statistical model for more detailed estimation of important parasite parameters including liver load and asexual parasite maturation and multiplication rates [4, 5].

Over the past decade, CHMIs have been performed in over 300 healthy volunteers at Radboud university medical center (Radboudumc), the 'Harbour Hospital' in Rotterdam or Leiden University Medical Center (LUMC). Despite an acceptable safety profile, CHMIs inevitably cause mild to moderate malaria symptoms such as headache, myalgia and malaise in almost all volunteers, and severe (grade 3) symptoms in about half of volunteers [3, 6]. Moreover, there have been three serious adverse cardiac events shortly after treatment for parasitemia that have remained incompletely understood [7, 8]. As clinical malaria symptoms are only associated with asexual blood stages, a shorter duration of parasitaemia may reduce the number and severity of adverse events, thereby further minimizing risks and volunteer burden. In addition, treating volunteers before (severe) symptoms occur, would simplify the conduct and follow-up, thereby lowering costs.

In this retrospective study, different thresholds for qPCR diagnostics were analysed in relation to prepatent period and occurrence of adverse events as well as effects on assessment of protective efficacy.

Methods

Study volunteers

Retrospective qPCR data were collected from nine CHMI trials performed at the Radboud university medical center (Radboudumc), the 'Harbour Hospital' in Rotterdam or the Leiden University Medical Center (LUMC) between 2007 and 2012 [9-15], table 1.

All study subjects were healthy female and male volunteers between the age of 18 and 35 years exposed to bites of five *P. falciparum* NF54 strain infected *Anopheles stephensi* mosquitoes. Prior to challenge infection, 55 volunteers were malaria naïve and 60 had received Chemoprophylaxis and Sporozoite (CPS) immunization. CPS-immunization was administered via infected mosquito bites at different dosages under chloroquine or mefloquine prophylaxis, as described previously [10-14].

Prior to inclusion, study volunteers were medically screened as described previously [13] and provided written informed consent. All clinical trials were approved by the Radboudumc Committee on Research Involving Human Subjects (CMO) or the Central Committee on Research Involving Human Subjects (CCMO) of the Netherlands.

Parasitological data

Treatment was initiated after CHMI when a thick blood smear was found positive for parasites. Thick smears were made twice or three times daily and read according to a standard protocol [11]. In short, a slide was considered positive if after reading the number of fields equivalent to 0.5uL of blood at least two parasites were seen (a threshold of 4 parasites per µL), and positivity was confirmed by a second independent reader.

qPCR assessment was performed according to previously published protocols [16]. qPCR was performed retrospectively from samples taken twice per day from day 5 until day 15 after challenge and once per day from day 16 until day 21.

Recording of adverse events

Subjects were asked to keep a diary recording symptoms while followed up for adverse events (AEs) on an outpatient basis once or twice daily starting on day 5 after challenge infection until day 21. Adverse events were collected until end of study visits either on day 28 or day 35 after challenge, depending on the study. An adverse event was defined as any undesirable symptom occurring after challenge infection. AEs were defined as grade 1, no interference with daily activity; grade 2, some interference with daily activity; or grade 3, requiring bed rest. The following symptoms were solicited: fever, headache, malaise, fatigue, myalgia, arthalgia, nausea, vomiting, chills, diarrhoea and abdominal pain.

Statistical analysis

Depending on the study, qPCR data was analyzed using Microsoft Excel (version 2007) for Windows or using a specialized electronic Case Report Form program (Hermsen Computer Services) created for Radboudumc CHMI trials. Data was combined using Microsoft Excel 2007 for Windows and statistical analysis was performed using IBM SPSS Statistics 22 for Windows.

Table 1: Summary of data included in the analysis. Data was included from all malaria naïve or CPS-immunized volunteers undergoing challenge infection with bites from five mosquitoes infected NF54 since 2007.

					Pre-patent period[1]		
	Year	Number of volunteers	CPS-immunization	Patent parasitemia	Median	Range	References
Study 1	2007	10	3 x 12-15 mosquitoes	0/10	-	-	Roestenberg and McCall 2009
		5	-	5/5	9	7 - 10.5	
Study 2[2]	2007	18	-	18/18	10.5	9 - 12.5	
Study 3	2009	6	3 x 12-15 mosquitoes[3]	2/6	16.8	15 - 18.6	Roestenberg 2011
		4	-	4/4	8.5	7.5 - 10.5	
Study 4	2010	5	-	4/5	10.6	10.6 - 11	Teirlinck and Roestenberg 2013
Study 5	2011	5	3 x 15 mosquitoes	1/5	12	-	Bijker and Teirlinck 2014
		9	3 x 10 mosquitoes	1/9	12	-	
		10	3 x 5 mosquitoes	5/10	11	9 - 15	
		5	-	5/5	9.5	9 - 13.5	
Study 6	2011	5	3 x 15 mosquitoes	0/5	-	-	Bijker and Bastiaens 2013
		5	-	5/5	12.5	9.5 - 12.5	
Study 7	2012	15	3 x 8 mosquitoes	5/15	12	11 - 14	Bijker and Schats 2014
		4	-	4/4	8.5	7 - 12	
Study 8	2012	5	-	5/5	10.5	9 - 10.5	
Study 9	2012	5	-	5/5	12	10.5 - 16	Bastiaens 2015

[1] Only volunteers with patent parasitemia included. In all studies pre-patent period is defined as time to positive thick blood smear.

[2] Volunteers received three immunization with a candidate malaria vaccine but were unprotected from challenge infection.

[3] Rechallenge of CPS-immunized volunteers from Study 1, 2.5 years after immunization and malaria naive controls.

Results

Fifty-five malaria naïve volunteers in nine trials received a challenge infection with bites from five NF54 infected mosquitoes. Geometric mean parasitaemia curves generated from retrospective qPCR data were similar between trials, figure 1A. These volunteers received anti-malarial treatment at positive thick blood smear at a median of 10.5 days post-challenge (range 7.0-16.0). Based on the retrospective qPCR data, initiating treatment based on qPCR can gradually decrease the duration of parasitaemia, depending on the treatment threshold and blood sampling frequency used, figure 1B. When two consecutive positive qPCR measurements above 500 parasites per milliliter are used as a criterion to initiate treatment, volunteers are treated at a median of nine days post CHMI. When only a single positive qPCR is required to initiate treatment, the mean day of treatment decreases further. Using the conservative threshold of 100 parasites per millilitre blood, the median duration of parasitemia would decrease by 3.5 days.

All solicited adverse events that were possibly, probably or definitively related to the CHMI occurring between day 5 post-infection and the end of the study were collected. Fifty-five percent of all adverse events and 39% of severe adverse events occurred prior to the initiation of anti-malarial treatment (Figure 1C). Importantly, only 22% of the total adverse events and 13% of grade 3 adverse events before treatment occurred before parasitaemia reached 100 parasites/ml (Figure 1D).

Once daily blood sampling for qPCR (threshold of 100 Pf/ml), instead of twice daily sampling, did not influence the median treatment day, figure 1B. Five volunteers (9%) would have been treated 24 hours earlier when sampling for qPCR twice daily. However, the mean number of adverse events before treatment increased only minimally when once daily sampling was used, figure 1C.

CPS immunization induces dose-dependent protection against CHMI [12]. Partial protection was determined by time to parasitaemia and mean parasite density of the first wave, as estimation of the liver parasite load [4]. Since both parameters depend on the method of parasite detection and treatment threshold used, it was retrospectively assessed whether the proportion of volunteers with partial protection changed with qPCR sampling once daily and initiation of treatment based on a single qPCR above 100 parasites per millilitre. Table 2 shows that differences in pre-patent period and mean parasitaemia of the first wave for 10 partially protected volunteers and controls [11, 12] gave similar outcomes when using microscopy or qPCR.

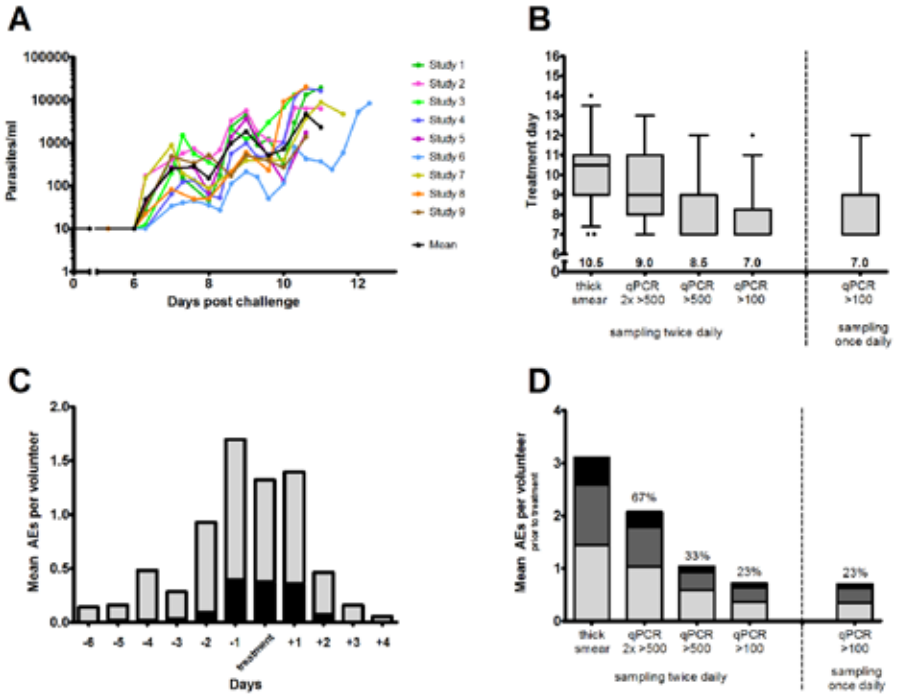

Figure 1: Parasitaemia at different thresholds of qPCR and association with adverse events. (A) Mean parasitaemia by qPCR from a total of 55 malaria naïve volunteers undergoing CHMI by five NF54 infected mosquito bites in 9 trials. **(B)** Day of positive thick smear or positive qPCR at different parasite density thresholds as starting day of curative treatment. Box-and-whisker plots show the median, first and third quartiles and 5-95th percentiles. Numbers above the x-axis are median treatment days. **(C)** The mean number of adverse events per volunteer occurring prior to thick smear positivity compared to different parasite thresholds for initiation of treatment . Percentages above the bars show the percentage of total AEs that occur relative to thick smear. Black = grade 3; dark gray = grade 2; light gray = grade 1.

A tentative diagnostic replacement of microscopy by qPCR requires a reliable test outcome. A total of 778 retrospective qPCR tests have been performed in 35 fully protected volunteers without a single qPCR above 100 parasites/ml. In the same studies, performed between 2010 and 2012, 107 qPCR standard curves were generated using serial dilutions of blood samples with known parasite densities, diluted from isolated ring stages whose concentration had been determined by microscopy. At densities of 20, 50 and 100 parasites per millilitre, the parasitaemia in these samples was correctly quantified (less than 5% deviation between duplo samples) in 63% (57/107), 87% (93/107) and 96% (103/107) of the samples, respectively. With recent introduction of a new standardized reagents mix for the DNA extraction in 2014 (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche Diagnostics), 81 of 82 standard

curve samples with 100 parasites per millilitre and 79 of 82 samples with 50 parasites per millilitre were correctly measured. The combined data indicate that qPCR with threshold of 100 parasites per millilitre can be reliably used for diagnosis in the CHMI model, with a sensitivity of 99% and a specificity of 100%.

Table 2: Partial protection after CPS immunization as detected by thick smear or retrospective qPCR. Differences between mean pre-patent periods were determined by Mann-Whitney U test in 10 partially protected and 9 control volunteers after CPS immunization [11, 12]. Parasitemia of the first parasite wave was estimated by determining the geometric mean parasitemia from 6.5 until 8.0 days after challenge. Differences in the mean parasitemia of the first peak was determined by an independent samples t-test.

	Number	Pre-patent period (days)			Parasitemia 1st peak (log)		
		Mean	SD	p value	Mean	SD	p value
POSITIVE THICK SMEAR[1]							
CPS-immunized (partially protected)[2]	10	12.2	1.85	0.006	1.00	0.56	0.02
Controls (unprotected)	9	9.7	2.05		2.07	1.07	
POSITIVE qPCR[3]							
CPS-immunized (partially protected)[2]	10	9.6	2.06	0.035	1.10	0.67	0.04
Controls (unprotected)	9	7.9	1.83		1.99	1.06	

[1] Threshold of 4000 parasites/ml and twice daily blood sampling

[2] Only volunteers with patent parasitemia included in the analyis

[3] Threshold of 100 parasites/ml and once daily blood sampling

Discussion

This retrospective qPCR analysis shows that the duration of blood stage parasitaemia in CHMI volunteers can be shortened by 3.5 days compared to thick blood smear if a treatment threshold of 100 parasites per millilitre is used. This threshold has a sensitivity of 99% and a specificity of 100%.

Shortening the duration of parasitaemia in volunteers after CHMI has several potential advantages. Most importantly, an increase in safety as malaria symptoms are related to the height and duration of parasitaemia, and the potential to greatly decrease the burden for volunteers. Over half the adverse events after CHMI occur prior to thick smear positivity. This analysis shows that anti-malarial treatment of volunteers when parasitaemia reaches 100 parasites per millilitre will lead to a 78% reduction in the number of adverse events occurring before treatment. Presumably, treatment of volunteers at lower parasitaemia will also lead to a decrease in adverse events occurring after treatment.

If prospective qPCR diagnostics are introduced with a low threshold (100 parasites per millilitre), once daily blood sampling will suffice without the need for a second sample within 24 hours, as there appears to be only a slight effect on the duration of parasitaemia and/or the number of adverse events. Five volunteers (9%) would have been treated 24 hours earlier when sampling for qPCR twice daily. Notwithstanding, we still favour once daily sampling considering the great burden of twice daily blood sampling and the absence of a significant increase in the number of adverse events at that very low parasitaemia. Shortening the duration of parasitemia and decreasing the frequency of blood sampling will significantly reduce the follow-up of CHMI volunteers. Given the intensive visit schedule for volunteers, requiring multiple personnel and safety laboratory evaluations, the reduced follow-up period will substantially simplify the conduct of these trials, which will also lower CHMI costs.

However, these benefits should not compromise the scientific value of the trial. This study shows that using these diagnostic criteria will not impede the ability to discriminate the delay in parasitaemia and/or reduction in mean first wave parasitemia as proxy for parasite liver stage development that occurs when a vaccine provides partial pre-erythrocytic protection. Therefore, using once daily qPCR with 100 parasites per millilitre threshold will likely provide a similar primary outcome of protective vaccine efficacy in prospective studies. However, the standard deviations of both mean time to parasitaemia and mean parasitaemia in the vaccination groups increased in this analysis. Consequently, when a relatively smaller difference is anticipated between vaccinees and controls, use of these qPCR criteria may require an increase in sample size to obtain sufficient statistical power.

Evaluation of qPCR data from 35 CPS-immunized and protected volunteers shows that since the introduction of the current qPCR method at Radboudumc, LUMC and the Harbour Hospital in 2010, no immunized and fully protected volunteers developed a positive qPCR after challenge above 100 parasites per millilitre. Using this qPCR method, parasites can be detected at a threshold of 50 parasites/ml with about 96% sensitivity and at 100 parasites/ml with 99% sensitivity. Therefore, the test clearly has sufficient accuracy for diagnostic purposes at these centres. A possible hazard of using a single positive qPCR as a criterion to initiate treatment is the risk of false-positives by cross-contamination or accidental sample switching, especially since treatment will now often be initiated in the absence of clinical symptoms. To minimize this risk, it is important to set up quality control steps not only within the qPCR test but in the conduct and logistics of the qPCR as well. Prior to a CHMI study, qPCR standards are generated and validated, and the same standard is used throughout an entire study. In order to ensure comparability of CHMI data between centres it will be a logical next step to standardize the PCR assay, or make commercially available *P. falciparum* qPCR standards.

Andrews et al. first demonstrated the increased sensitivity of qPCR compared to thick smear, and recognized that qPCR could be used to initiate earlier treatment, at a threshold of 1000 parasites per millilitre [17]. However, recent advances in qPCR methodology, such as the use of an automated system for extraction, has improved sensitivity at low parasite densities. The current analysis shows that this has made it possible to lower the treatment threshold much further. Likewise, other CHMI study centres have also repeatedly shown that qPCR first becomes positive 2-4 days before thick blood smear when both are determined [18-21]. Similarly, studies assessing blood stage drugs or vaccines have already begun to use qPCR as a primary outcome, and have confirmed its sensitivity and specificity [22]. In 2014 Kamau et al. analysed parasitological data from 16 subjects undergoing CHMI in two trials. They also showed that qPCR is positive two to seven days before thick smear [23]. Based on their analysis, the authors recommend treatment after CHMI after two (not necessarily consecutive) positive qPCRs of which one is above 2,000 parasites per millilitre. This threshold was chosen to assess parasite multiplication rates requiring at least two replication cycles. For evaluation of pre-erythrocytic vaccines, however, a treatment threshold of 100 parasites per millilitre will be sufficiently adequate. This analysis shows that different qPCR thresholds can be chosen to assess the duration of parasitaemia. For example, using two consecutive positive qPCRs above 500 parasites per millilitre as a threshold, prolongs the median pre-patent period to 9 days. Different qPCR treatment thresholds will therefore lead to different durations of parasitaemia. In this way, CHMI can be made a fit-for-purpose model matching the diagnostic qPCR protocol with the considered primary endpoints.

Although retrospective analyses should be interpreted prudently in general, the predictive value of this study can likely be met with confidence since retrospective qPCR data have been remarkably consistent over time between CHMI trials, and CHMI centres [3]. Therefore, PCR may be preferred for diagnosis and treatment when evaluating the protective efficacy of pre-erythrocytic vaccines [19].

Conclusions

After CHMI, qPCR becomes positive on average 3.5 days before thick blood smear. This analysis shows that depending on the threshold used, treatment based on qPCR diagnostics can greatly reduce the pre-patent period and the number of adverse events occurring before treatment. Furthermore, these data demonstrate for the first time that qPCR has sufficient sensitivity and specificity to use 100 parasite per millilitre as a treatment threshold without affecting trial outcome related to infection and pre-erythrocytic protective efficacy. Therefore, the implementation of these diagnostics would improve safety while reducing the clinical burden and costs without compromising the evaluation of protective efficacy.

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CHAPTER 4

Safety, immunogenicity and preliminary efficacy of the genetically attenuated sporozoite vaccine PfSPZ-GA1 against controlled human malaria infection; a double-blind placebo controlled clinical trial

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Abstract

Genetically engineered *Plasmodium berghei* sporozoites (SPZ) lacking the genes b9 and slarp induced sterile protection against malaria in mice. Consequently, PfSPZ-GA1 Vaccine, a *Plasmodium falciparum* (Pf) identical double knock-out (Pf*Δb9Δslarp*), was generated as the first genetically attenuated malaria parasite vaccine. Groups of volunteers were immunized by direct venous inoculation (DVI) with cryopreserved PfSPZ-GA1 Vaccine, PfSPZ Vaccine (radiation attenuated PfSPZ) or normal saline (NS) placebo in a multi-center phase 1 trial. Once dose-escalation safety was established, volunteers were randomized double blind to receive three immunisations of PfSPZ-GA1 Vaccine ($9.0x10^5$ or $4.5x10^5$ PfSPZ, N=13 each), control PfSPZ Vaccine ($4.5x10^5$ PfSPZ, N=13) or NS at 8-week intervals, followed by exposure to mosquito bite controlled human malaria infection (CHMI). This trial is registered at clinicaltrials.gov number NCT03163121. Immunisations with PfSPZ-GA1 Vaccine were well tolerated without break-through blood stage infections. Post-CHMI, 3/25 volunteers from both PfSPZ-GA1 groups were sterilely protected and the remaining 17/22 showed a patency ≥9 days (median patency in controls 7 days, range 7-9). All volunteers in the PfSPZ Vaccine control group developed parasitemia (median patency 9 days; range 7-12). Immunized groups exhibited a significant, dose-related increase in anti-Pf circumsporozoite protein (CSP) antibody levels and Pf-specific IFN-γ-producing T cells. Although no definite conclusion can be drawn on the potential strength of protective efficacy of PfSPZ-GA1 Vaccine, the favourable safety profile and induced immune responses by PfSPZ-GA1 Vaccine warrant further clinical evaluation.

Introduction

A recent resurgence in *Plasmodium falciparum* (Pf) malaria cases after years of control, underscores the need for a highly efficacious vaccine for elimination [1]. The Pf circumsporozoite protein (CSP) subunit vaccine RTS,S/AS01E (Mosquirix, GlaxoSmithKline) is the only malaria vaccine to move beyond phase 3 clinical trials, though it provides only short-term and partial clinical vaccine efficacy (VE) [2].

In the past decade, there has been a growing interest in attenuated whole Pf sporozoite (PfSPZ) vaccines based on the idea that this whole organism immunization will be able toinduce the protection needed against the breadth of antigens present in the parasite. The first approach to immunizing humans with radiation attenuated PfSPZ was developed almost 50 years ago [3]. Recently, methods have been developed to purify and cryopreserve radiation attenuated, metabolically active, aseptic PfSPZ that meet regulatory standards for parenteral injection. This product, Sanaria® PfSPZ Vaccine, is injected by direct venous inoculation (DVI) and has been administered to 1595 subjects aged 5 months to 65 years in 20 clinical trials in the U.S., Europe, and Africa. It has an excellent safety and tolerability profile in multiple double blind, placebo-controlled trials without significant differences in adverse events between vaccinees and normal saline controls [4-6], Sanaria unpublished data). PfSPZ Vaccine provides high-level protection against short term (3 weeks) and long term (24-59 weeks) homologous controlled human malaria infection (CHMI) with the Pf NF54 strain by mosquito bite [7-9], about 50% protection at 8 months after heterologous mosquito bite (7G8) CHMI [10] and approximately 50% protection by time to event analysis against malaria in the field [6].

Radiation induces random DNA damage in the parasite genome, generating a heterogeneous non-replicating population of PfSPZ. These PfSPZ invade hepatocytes, partially develop, and then arrest at an early stage in the liver. As an alternative to radiation based attenuation, genetic modification generates a homogeneous formulation of PfSPZ which stop development in the liver at a well-defined point. In rodent models immunization with genetically attenuated malaria SPZ can induce similar, or even better, levels of protective immunity compared to radiation attenuated malaria SPZ [11]. The intrinsic and irreversible nature of the genetic attenuation greatly reduces safety risks during manufacture of PfSPZ. Consequently, several liver-arresting genetically attenuated Pf parasites have been generated [12-14], two of which have been tested for safety in volunteers by mosquito bite [13, 15].

We engineered attenuated PfSPZ by deletion of two genes encoding *slarp* and *b9,* each governing independent and critical processes for successful liver-stage development [14]. Pf double-knock-out (Pf*Δb9Δslarp*) SPZ were capable of invading primary human hepatocytes *in vitro*, but arrested growth early after invasion and were not detected at day 2-7 after infection, similar to PfSPZ Vaccine. Pf*Δb9Δslarp* parasite development was fully abrogated in the liver of humanized mice [14]. SPZ of the equivalent rodent *Plasmodium berghei* attenuated parasite (Pb*Δb9Δslarp*) also showed aborted liver stage development while retaining the capacity to induce fully protective immunity in both the BALB/c and C57BL/6 mouse models [14]. This preclinical data justified formulation and clinical assessment of Pf*Δb9Δslarp*.

Manufacture of aseptic, purified, cryopreserved Pf*Δb9Δslarp* PfSPZ (Sanaria® PfSPZ-GA1 Vaccine) was performed in compliance with Good Manufacturing Practice [12]. We report the first-in-human evaluation of PfSPZ-GA1 Vaccine. We tested safety and immunogenicity of PfSPZ-GA1 Vaccine and subsequently examined the protective VE against a homologous CHMI with wild-type (WT) Pf (NF54) and compared this to a previously tested regimen of PfSPZ Vaccine.

Materials and methods

Production of PfSPZ-GA1

The genetically attenuated Pf NF54 parasite, Pf*Δb9Δslarp* [14] lacks two genes, *b9* and *slarp*, which are vital for liver stage development [14]. Master and working cell banks were generated from the clone Pf NF54 parasite, Pf*Δb9Δslarp*, filed under a US FDA Master File and Investigational New Drug (IND) application, resulting in the product hereafter referred to as PfSPZ-GA1 Vaccine. PfSPZ-GA1 parasites were tested sensitive to the antimalarial drugs chloroquine, mefloquine, artemether/lumefantrine, atovaquone/proguanil and pyrimethamine.

Manufacture of PfSPZ-GA1 Vaccine bulk product followed the identical manufacturing schema of PfSPZ (NF54) Vaccine [12] except for several tests of vialed final product that were specific to PfSPZ-GA1 Vaccine. These tests included a PCR test for identity that confirmed the genetic signature of PfΔb9Δslarp [14], the potency assay that documented 3 day parasites that developed in HCO4 cells *in vitro* and the 6 day safety assay that confirms the absence of late stage developing parasites *in vitro.* The manufacturing process generated aseptic *Anopheles stephensi* mosquitoes that were infected with Pf*Δb9Δslarp* [14]. PfSPZ were harvested, purified, vialed, cryopreserved and shipped in liquid nitrogen vapor phase (LNVP) at -150°C to -196°C. On the day of administration,

vials of PfSPZ-GA1 Vaccine were thawed and diluted using phosphate buffered saline and human serum albumin to the correct dose in a sterile environment.

Study design

The study was designed as a multi-center phase 1, open label, dose escalating trial to assess safety, tolerability and immunogenicity of PfSPZ-GA1. In the initial, open-label safety stage of the trial (stage A), single escalating doses of PfSPZ-GA1 were administered by DVI to three groups of healthy adults at the Leiden University Medical Center (LUMC). Group A1 (n=3) was inoculated with 1.35 x 10^5 PfSPZ of PfSPZ-GA1 Vaccine, group A2 (n=3) was inoculated with 4.5 x 10^5 PfSPZ of PfSPZ-GA1 Vaccine and group A3 (n=13) was inoculated with 9.0 x 10^5 PfSPZ of PfSPZ-GA1 Vaccine. For this first, proof-of-concept study, a dose of 1.35 x 10^5 PfSPZ of PfSPZ-GA1 Vaccine was chosen because this is the lowest dose at which PfSPZ Vaccine has shown to induce levels of protective immunity [9], whereas after three doses of 9.0 x 10^5 PfSPZ Vaccine >90% VE was to be expected. This is based on a study where three doses of 4.5 x 10^5 PfSPZ induced >80% protection [7]. In the follow-on efficacy stage of the trial (stage B) a total of 48 volunteers were included at LUMC (n=24) and Radboud University Medical Center (RUMC) (n=24) with double blind randomization over four study groups. Groups 1 and 2 received three immunisations with PfSPZ-GA1 Vaccine at doses of 9.0x10^5 (n=13) and 4.5x10^5 (n=13) PfSPZ. Group 3 received three immunisations with the control PfSPZ Vaccine at a dose of 4.5x10^5 PfSPZ (n=13) and Group 4 was injected three times with normal saline (NS) as placebo (n=9, Figure 2). All immunisations in stage B were administered at 8 week intervals. Three weeks after the final immunisation, all stage B volunteers were exposed to 5 bites of Pf NF54-infected *A. stephensi* mosquitoes according to previously described procedures to assess VE [16].

The clinical trial was conducted under a US FDA IND application and was approved by the central committee for research involving human subjects in The Hague (CCMO; NL56657.000.16). It was performed in the Netherlands under a licence from the Dutch Ministry of Infrastructure and Environment (Ministerie van Infrastructuur en Milieu; IenM) for deliberate release of genetically modified organisms (IM-MV 15-004 and IM-MV 15-009). The study was registered at clinicaltrials.gov (NCT03163121).

Participants

A total of sixty-seven healthy malaria-naive male and female volunteers aged 18-35 years were recruited for the study. All included volunteers were in good health as assessed by medical history, physical examination, general chemistry and haematology evaluation and an electrocardiogram (ECG). All included volunteers provided informed consent and females were counselled to use adequate contraception. A detailed list of inclusion and exclusion criteria is provided in the supplementary data.

Randomisation and masking

In stage B of the trial, participants were randomly assigned to one of the groups according to a randomisation list prepared by the study head pharmacist. Randomisation was stratified per study site. The investigator, site personnel and the sponsor were masked to treatment assignment. The site pharmacist or qualified employees were not masked and prepared the assigned vaccines.

Procedures

Volunteers were immunized by a trained nurse administering 0.5 mL of the vaccine by DVI through a 25 gauge needle. They were observed for 30 minutes following every immunisation. Local adverse events and pain scores were assessed immediately. In stage A, volunteers visited the trial facility daily from day 6 to day 21 after every immunisation to report adverse events and to collect blood samples for assessment of parasites by qPCR (Figure 2). During the immunisation period, all volunteers were treated with a curative regiment of atovaquone/proguanil when qPCR was positive for malaria, or at day 28 after immunisation. Complete blood counts and general chemistry labs were performed on day 6, 14, 21, 30 and 35 after immunisation. Platelet counts, lactate dehydrogenase and highly sensitive troponin T tests were performed daily to detect possible myocarditis in an early stage, in line with previously established protocols [17, 18]. Blood samples for immunological assays were taken at baseline, day 6, 14, 21, 28, 35, 100 and 188. In stage B of the clinical trial, visits were on day 14 after every immunization and the day prior to immunisation for safety assessments. Three weeks after the third and final immunization volunteers were exposed to the bites of 5 mosquitoes infected with the homologous Pf NF54 strain (CHMI). All mosquitoes were checked for a blood meal and infectivity by dissection [16]. Further details on the CHMI with Pf are reported in Supplementary Table S2. After CHMI volunteers visited the trial center on a daily basis from day 6 to day 21. All volunteers were treated with a curative regiment of atovaquone/proguanil if they were qPCR positive, or alternatively at day 28 after CHMI. Final visits took place at day 35, 100 and 188 after CHMI. Blood samples for immunological analysis were taken before and 14 days after each immunisation, before CHMI and at days 6, 14, 21, 35, 100 and 188 after CHMI (Figure 2).

Outcomes

The primary objective of the study was to investigate the safety and tolerability of PfSPZ-GA1 Vaccine, by analysis of 1) the presence of blood stage parasites after inoculation and 2) the frequency and magnitude of adverse events. A secondary objective was the VE of PfSPZ-GA1 Vaccine against mosquito bite CHMI with Pf NF54 sporozoites, as assessed by the presence or absence of parasitemia after CHMI.

Adverse events

Solicited and unsolicited adverse events after DVI were recorded at every visit until 35 days after immunisation. Solicited local adverse events were tenderness, induration, bruising/extravasated blood, erythema, swelling, pain and pruritis. Solicited systemic adverse events were fever, rash, urticaria, pruritis, edema, headache, fatigue, malaise, chills, myalgia and arthralgia. All volunteers were instructed to fill out a diary card listing daily temperature and any adverse events up to day 35 after immunisation. Causality of all adverse events was assessed by the investigators as definitely related, probably related, possibly related, unlikely related or not related to the study procedures. In dichotomous analysis the latter two were regarded as "unrelated" and the first three categories as "related". All adverse events were graded as mild (grade 1), moderate (grade 2), severe (grade 3) or serious (grade 4). Review of all safety data by an independent Safety Monitoring Committee was performed at 28 days after each immunisation in stage A, before continuing dose escalation to the next group and on day 28 after CHMI in stage B.

Blood stage parasitemia

To examine if PfSPZ-GA1 were fully attenuated and incapable of establishing a blood stage infection, blood samples were monitored for parasites by qPCR [19-21]. Blood samples were considered negative if no signal was detected in 50 cycles or the Pf load was <100 Pf/ml. Any sample with a load >100 Pf/ml was considered positive. Parasite densities were determined with the use of a trendline of standardized control samples between 20 Pf/mL and 10^6 Pf/mL.

Immunology

Exploratory endpoints included immune responses following immunisation with PfSPZ-GA1 Vaccine. Antibodies were detected by enzyme-linked immunosorbent assay (ELISA) against PfCSP [22]. Cellular immune responses were analysed using peripheral mononuclear cell (PBMC) samples obtained one day before the first immunization and 21 days after the third immunization. Cells were isolated using heparin CPT tubes according to previously published protocols [22]. After thawing, cells were stimulated as described previously [23]. In short, PBMCs were cultured at $2.5x10^6$ cells/mL in a final volume of 200uL per well in RPMI 1640 (Dutch Modification; Gibco) with 5mg/ml gentamicin (Centraform), 100mM pyruvate (Gibco), 200mM glutamax (Gibco) and 10% heat-inactivated pooled human A+ serum (Sanquin, Nijmegen, The Netherlands). Cells were stimulated with purified NF54 schizonts (PfRBC) or uninfected red blood cells (uRBC) at a concentration of $2.5x10^6$ RBC/mL for 24 hours. Brefeldin A (10µg/mL; Sigma-Aldrich) and monansin (2µM; eBioscience) were added during the last 4 hours of stimulation. Cells were stained with fixable viability dye labeled with eFlour780 (eBioscience), CD3-PE-Dazzle549 (Biolegend; clone OKT3), CD4-FITC (Biolegend;

clone OKT4), CD8-AlexaFluor700 (Biolegend; clone HIT8A), pan-γδTCR-PE (Beckman Coulter; clone IMMU510), and CD56 PerCP-Cy5.5 (Biolegend; clone HCD56), for 30 minutes at 4 degrees. Cells were subsequently permeabilized using Foxp3 fixation/ permeabilization buffer (eBioscience) and stained for intracellular cytokines with IFN-γ-PE-Cy7 (Biolegend; clone 4S.B3), IL-2-BrilliantViolet510 (Biolegend; clone MQ1-17H12) and TNF-γ-AlexaFluor647 (Biolegend; clone MAb11). Analysis was performed using a Gallios flow cytometer (Beckman Coulter) and Flow Jo software (version 10.0.8 for Apple OS). Background cytokine production after stimulation with uRBC were subtracted from PfRBC responses. On an individual level, we defined IFN-γ-responders as those volunteers with a percentage increase in IFN-γ-producing cells greater than twice the standard deviation of all pre-immunization samples.

Statistical analysis

The presence of blood stage parasites after inoculation with PfSPZ-GA1 Vaccine and the frequency and magnitude of adverse events after immunization were primary endpoints. Presence of blood stage parasites after immunization was a stopping criterium. Adverse events were evaluated by tabulating according to intention to treat analysis. The proportion of volunteers in each group who reported mild, moderate or severe adverse events was calculated and analysis was primarily descriptive. The secondary endpoint of the study was the presence of parasitemia (by qPCR) after CHMI with the (WT) Pf NF54 strain in stage B of the study. Differences between groups were evaluated by log rank test.

Differences in immunological parameters between groups were assessed by comparing mean values between the groups using one-way ANOVA when comparing several groups or a two-tailed student's t-test or non-parametric equivalents. Paired tests were used if pre-exposure values were compared with post-exposure values, unpaired tests were used if comparisons were made between groups. For discrete variables, the chi-squared test or Fisher's exact test was used (two-tailed). All statistical analyses were performed with SPSS version 23.

Results

Study population

In total 124 malaria-naïve adults were screened for participation in the study from the 1st of May until the 28th of November 2017. Nineteen volunteers were selected as volunteers in stage A of the study and 48 were selected for stage B. In addition, six back-up volunteers were enrolled in stage B to replace any dropouts before immunisation. One volunteer withdrew informed consent after the second immunization in stage B of

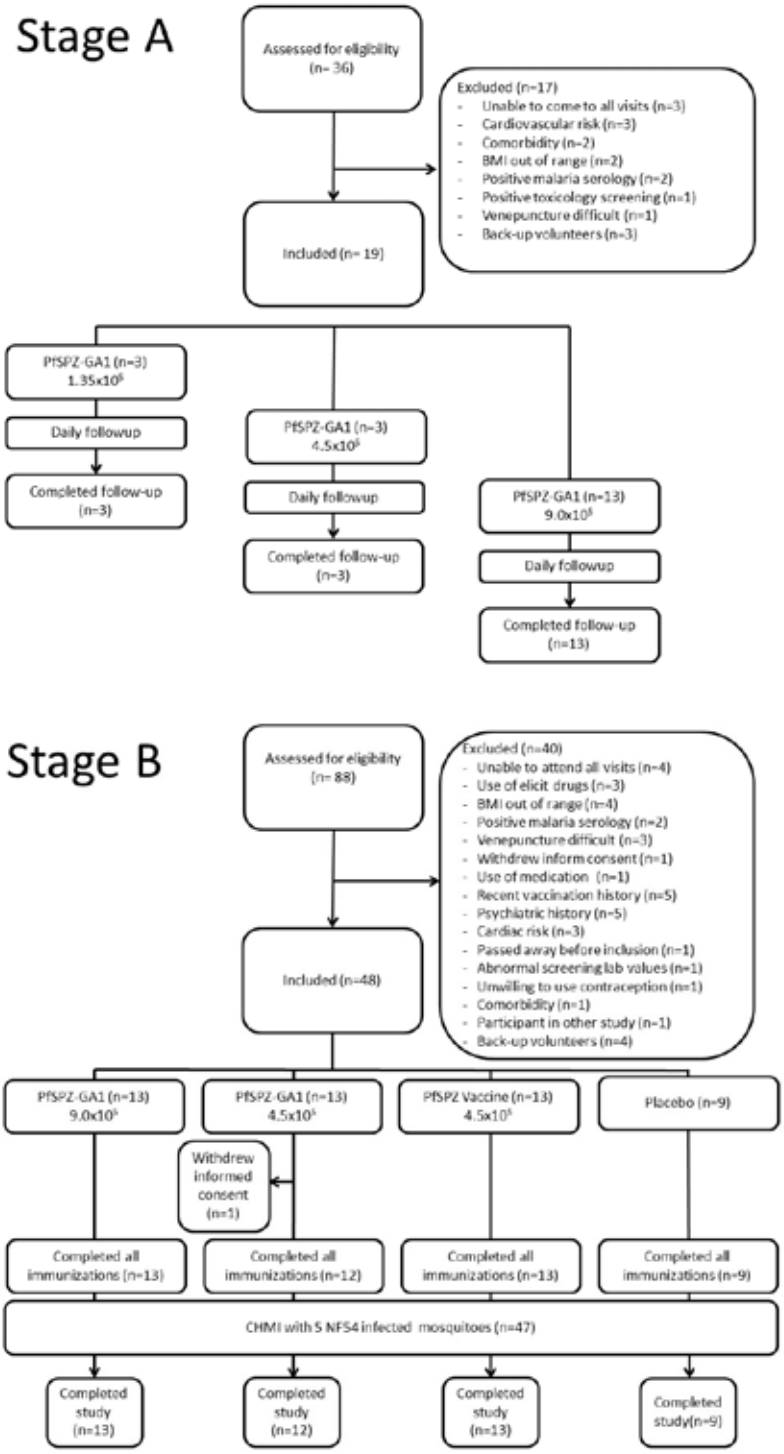

Figure 1: Study flow chart

the trial for reasons unrelated to the trial, all others completed follow-up (Figure 1). In total 34 of 67 (51%) were males. Mean age of the volunteers was 23 years old (SD 4, range 18-34), mean body mass index (BMI) was 23.5 kg/m^2 (standard deviation (SD) 3.0, range 18-30) (Table 1).

Safety results

No serious adverse events occurred during this trial. None of the blood samples taken for blood-stage infection at any time point after DVI of $1.35x10^5$ or $4.5x10^5$ or $9.0x10^5$ PfSPZ of PfSPZ-GA1 Vaccine in stage A and after any of three immunizations with $4.5x10^5$ or $9.0x10^5$ PfSPZ of PfSPZ-GA1 Vaccine in stage B were positive for parasite DNA. Blood samples were tested for erythrocytic stage parasites by quantitative polymerase chain reaction (qPCR) every day from day 6-21 after immunization and on day 28 in stage A and on day 14 after each immunization in stage B (Figure 2).

Table 1: Volunteer demographics

		STAGE A		STAGE B		
		Group A1 start	**Group A2 start**	**Group A3**		**Total**
Number of Volunteers		3	3	13	48	67
Age (years)	Mean	23	25	23	23	23
	SD	3	3	5	3	4
	Median	23	26	23	23	23
	Min, Max	20, 26	20, 29	18, 34	18, 33	18, 34
Sex	Male	1	2	5	26	34
	Female	2	1	8	22	33
BMI	Mean	25.3	23.9	23.6	23.3	23.5
	SD	0.7	2.7	2.9	3.1	3.0
	Median	25.1	24.9	23.6	22.5	23.4
	Min, Max	25, 26	18, 26	19, 29	18, 30	18, 30

All immunizations with PfSPZ-GA1 Vaccine and PfSPZ Vaccine were well tolerated and there were no significant differences in incidence or severity of adverse events between vaccine and placebo groups in stage B. A total of 66 related adverse events were reported after immunization (Table S2). DVI was successful after a single needle stick in 93% of injections (151/162 injections) and after 3 attempts all but one DVI was successful. Volunteers reported no or mild pain during injection, only one volunteer reported severe pain once for a few seconds during needle insertion. Bruising after DVI was the most commonly reported local adverse event, occurring in 7/67 (10%) of volunteers. Headache and fatigue/malaise were the most frequently reported systemic

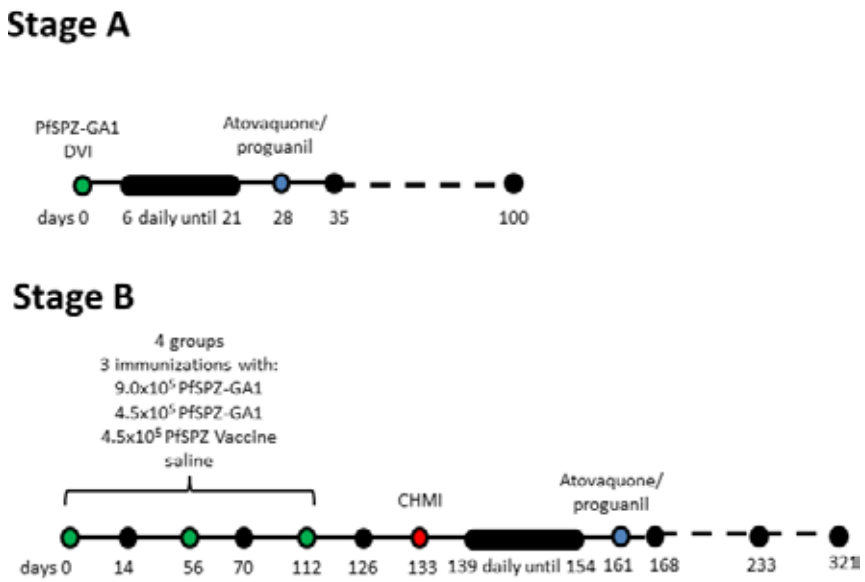

Figure 2: Study design. In Stage A study volunteers were immunized by direct venous inoculation (DVI) with either 1.35x10^5, 4.5x10^5 or 9.0x10^5 PfSPZ of PfSPZ-GA1 Vaccine (n=3, 3 or 13 respectively, green circle) after which blood samples were taken on a daily basis from day 6 until day 21 (multiple black circles). At day 28 (blue circle) all volunteers were treated with a curative regimen of atovaquone/proguanil. Final visits were at day 35 and 100. In Stage B study four groups of volunteers were immunized 3 times (green circles) with either 4.5x10^5 or 9.0x10^5 PfSPZ of PfSPZ-GA1 Vaccine or 4.5x10^5 PfSPZ of PfSPZ Vaccine or saline placebo by DVI (n=13 or 13, 13 and 9 respectively), with blood samples taken for blood stage parasitemia at day 14 after every immunization. CHMI by mosquito bite with WT Pf NF54 (red circle) was performed 3 weeks after the final immunization, after which daily follow up was performed from day 139-154. All volunteers received curative treatment with atovaquone/proguanil (blue circle) and came for three final follow up visits.

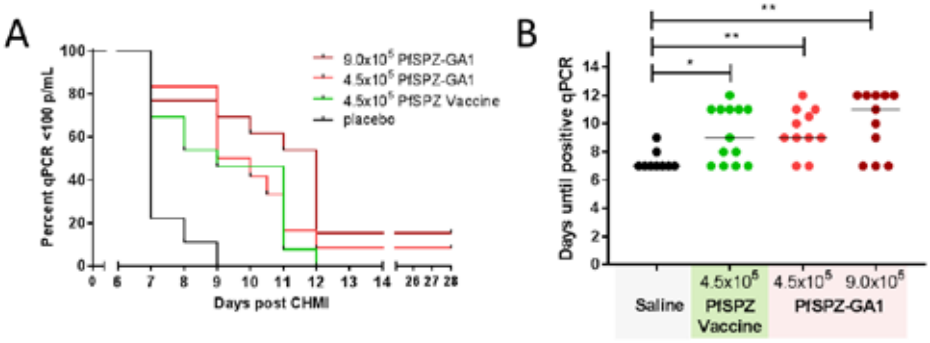

Figure 3: Kaplan-Meier showing number of volunteers without blood stage parasitemia as measured by qPCR between 0-28 days after CHMI (panel A, log-rank p=0.0003) and days until patency (qPCR >100 p/mL, panel B), for the 9.0x10^5 PfSPZ-GA1 Vaccine (dark red), 4.5x10^5 PfSPZ-GA1 Vaccine (bright red), 4.5x10^5 PfSPZ Vaccine (green) and placebo group (black). Lines indicate median. Panel B shows significance by Mann-Whitney: * p<0.05, ** p<0.01.

adverse event (reported by 31 and 14 volunteers respectively) in both intervention and placebo groups, of which three events were severe. One severe unsolicited adverse event probably related to immunization occurred when a volunteer experienced a vasovagal reaction during immunization. There were no clinically significant laboratory abnormalities.

The most common adverse event after CHMI with Pf WT NF54 was headache (52% of volunteers) and fatigue (51%). One volunteer (placebo group) reported severe chills two days after atovaquone/proguanil treatment for blood test positive Pf malaria. Two volunteers (placebo group and PfSPZ Vaccine group) reported severe dizziness on the first and third day of atovaquone/proguanil treatment. All adverse events resolved without sequelae.

There were two cases of mild (grade 1) highly sensitive (hs-) troponin T elevation to a maximum of 19 ng/ml (reference <14ng/ml) 10-12 days after CHMI at the time when blood samples were positive for Pf by qPCR, one ($9.0x10^5$ PfSPZ of PfSPZ-GA1 Vaccine group) deemed probably related and one ($4.5x10^5$ PfSPZ of PfSPZ-GA1 Vaccine) deemed possibly related to CHMI. Both volunteers were asymptomatic and ECG did not show abnormalities. Both volunteers were treated with atovaquone/proguanil on the first day of troponin elevation at which time blood samples were positive for Pf. The hs-troponin T levels decreased to normal range within a day and volunteers experienced no sequelae.

Protective efficacy against CHMI

To obtain a preliminary measure of PfSPZ-GA1 VE, the immunized volunteers in the stage B study underwent CHMI with Pf NF54 WT parasites by mosquito bite. The volunteers were monitored on a daily basis and blood samples were tested for the presence of parasites by qPCR (Figure 2). Although the primary endpoint of proportion protected was not significantly different between any vaccine groups and the placebo control group, all vaccine groups showed a significant delayed time to positive qPCR as compared to the placebo (Figure 3, Log rank test p=0.0003). All volunteers in the placebo group developed parasitemia with a median of 7 days post CHMI (7 volunteers at day 7, one at day 8, one at day 9). All 13 volunteers immunized with the control $4.5x10^5$ PfSPZ of PfSPZ Vaccine developed parasitemia with a median delay of 2 days (median 9, range 7-12 days, compared with placebo Mann-Whitney p=0.0078). Post-CHMI, 3/25 volunteers from both PfSPZ-GA1 groups were sterilely protected. Immunization with $4.5x10^5$ PfSPZ of PfSPZ-GA1 Vaccine resulted in 11/12 volunteers developing blood stage parasitemia with a median delay of 2 days (median day 9, range 7-12 days, compared with placebo Mann-Whitney p=0.0005). In the highest dose PfSPZ-GA1 group, 11/13 volunteers became qPCR positive with a median 4 day delay (median

prepatent period 11 days, range 7-12 days, compared with placebo Mann-Whitney p=0.0018). This study was not powered to detect significant differences in time to positive qPCR between vaccine groups.

Immunogenicity

All immunized groups showed a significant increase in antibody levels against PfCSP between pre-immunization and pre-CHMI timepoints (p<0.0001 paired t-test overall, data not shown). Immunization with 4.5x10^5 PfSPZ of PfSPZ Vaccine and PfSPZ-GA1 Vaccine induced similar anti-PfCSP antibody levels whereas immunization with 9.0x10^5 PfSPZ of PfSPZ-GA1 Vaccine produced significantly higher anti-PfCSP titers (Figure 4A, one-way ANOVA Tukey post-hoc mean diff 5,573 95%CI 332-30,823 p=0.04).

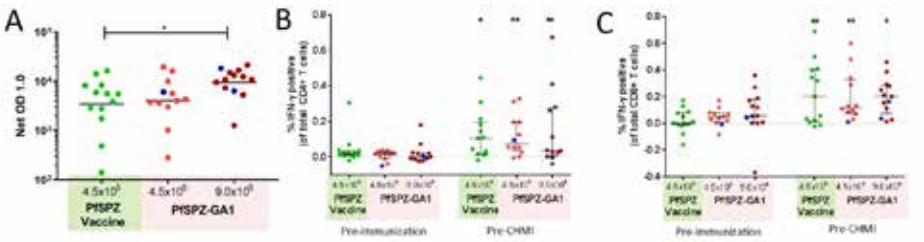

Figure 4: Anti-PfCSP antibody levels for the 9.0x10^5 PfSPZ-GA1 Vaccine (dark red), 4.5x10^5 PfSPZ-GA1 Vaccine (bright red), 4.5x10^5 PfSPZ Vaccine (green) groups (panel A) 14 days after the final immunization. Fully protected volunteers shown in blue, lines indicate geomeans. One way ANOVA post-hoc Tukey: * p<0.05.
Number of IFNγ-producing CD4+ (panel B) and CD8+ (panel C) T cells before immunization and the day before CHMI for the 9.0x10^5 PfSPZ-GA1 Vaccine (dark red), 4.5x10^5 PfSPZ-GA1 Vaccine (bright red), 4.5x10^5 PfSPZ Vaccine (green) and placebo group (black). Fully protected volunteers displayed in blue. Lines indicate medians with interquartile range. T-test of pre-CHMI data with pre-immunisation data:* p<0.05, ** p<0.01.

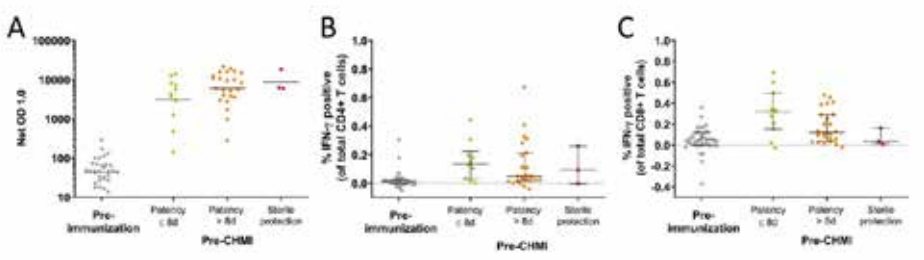

Figure 5: Relationship between anti-PfCSP antibody levels (panel A, lines indicate geomean) and % of IFN-γ-producing CD4+ or CD8+ T cells (panel B and C respectively, lines indicate median and interquartile ranges) and the protection status of volunteers. Protection grouped by day of positive qPCR (patency) ≤ 8 or >8 or "sterile" if qPCR negative until day 28.

All immunized groups exhibited a significant increase in CD4+ and CD8+ T cells producing IFN-γ upon stimulation with Pf infected red blood cells (PfRBC, Figure 4D+E) as compared to baseline. In total, 35% and 32% of all immunized volunteers were IFN-γ-responders for CD4+ or CD8+ T cells, respectively, with 46% of volunteers showing an increase in at least one subset. There were no significant differences in cellular responses between the vaccine groups and PfRBC IL-2 and TNF-α responses post immunization which were all low (data not shown).

In order to examine if there was a correlation between an increase in antibody or cellular responses and protection, data from immunized individuals were segregated based on protection status. The anti-PfCSP antibody levels correlated significantly with time until positive qPCR-based blood stage patency (Pearson correlation r=0.32 95%CI -0.01 to 0.59, p=0.02 R^2=0.1; Figure S1, 5A). However, cellular responses did not correlate with protection, although there was trend towards lower IFN-γ-producing CD8+ T cells in fully protected individuals (one-way ANOVA for patency ≤8 days, >8 days and full protection, p=0.05, post-hoc Tukey ns). Whether this reflects a compartmental shift of CD8+ T cells to non-lymphoid tissues, as observed in animal models [15], will require further study.

We thus demonstrate that immunisation with three doses of $4.5x10^5$ PfSPZ of PfSPZ Vaccine and PfSPZ-GA1 induced similar anti-PfCSP antibody responses, and that there was a dose dependent increase in anti-PfCSP responses after immunisation with $9.0x10^5$ PfSPZ of PfSPZ-GA1. Moreover, anti-PfCSP antibody responses correlated with protection.

Discussion

Here we report the first-in-human administration and efficacy data of the live, injectable, non-replicating, genetically attenuated PfSPZ, PfSPZ-GA1 Vaccine. PfSPZ-GA1 Vaccine was safe and well tolerated and no blood stage infections were observed in 45 volunteers after 97 injections totalling more than $6x10^7$ PfSPZ administered by DVI. PfSPZ-GA1 Vaccine was immunogenic and induced both significant antibody and CD4+ and CD8+ T cell responses with a potency analogous to the comparator PfSPZ Vaccine. Homologous CHMI through the bites of wild type (Pf NF54)-infected mosquitoes three weeks after the last immunization resulted in 3 fully protected individuals, and significant delays in time to patency in 17/25 volunteers at both dosages of PfSPZ-GA1 Vaccine. The delay in time to patency correlated with increased anti-PfCSP antibody responses.

This is the first study to show that an injectable, double gene-deletion attenuated parasite vaccine is safe and immunogenic in humans. Previously, two genetically attenuated parasites (GAP) have undergone safety evaluation in healthy volunteers in an experimental setting through the bites of infected *Anopheles* mosquitoes [13, 24]. One GAP, lacking the two genes *p52* and *p36*, showed a blood stage infection in a single volunteer after being exposed to 200 infectious bites [24]. The breakthrough blood parasites were confirmed as having the *p52* and *p36* gene deletion genotype, indicating that deletion of these two genes was not sufficient to result in a complete growth arrest in the liver stage. This same incomplete attenuation phenotype (at high infection doses) had also been observed in rodent malaria parasites lacking the same genes [25]. In a subsequent study, a novel GAP was analyzed which additionally included a deletion of the *slarp* gene, also referred to as *sap1*. This triple knock-out GAP (Pf*Δp52Δp36Δsap1*) was administered to healthy volunteers through the bites of 150-200 infectious *Anopheles* mosquitoes and no breakthrough blood infections were observed [13]. No protective efficacy studies of the triple knock-out GAP have been reported yet. In contrast to Pf*Δp52Δp36Δsap1*, PfSPZ-GA1 Vaccine was manufactured as a vialed product in compliance with FDA and EMA regulations for investigational products and administered as an injectable vaccine.

We found a significant delay in patency up to day 12 post CHMI as compared to a 7-9 day patency in controls, reflecting a 2-log reduction in parasites released from the liver in volunteers vaccinated with both doses of PfSPZ-GA1. However, the interpretation of the VE data is complicated by the unexpected low efficacy of the PfSPZ Vaccine reference group. The dose of the control PfSPZ Vaccine was chosen based on a previous study in which 3 doses of $4.5x10^5$ PfSPZ of PfSPZ Vaccine by direct venous inoculation (DVI) protected 13 of 15 volunteers from mosquito bite CHMI 3 weeks after the last immunisation, and 8 of 14 volunteers from CHMI 24 weeks after the last immunisation [7]. The same dose was selected for PfSPZ-GA1 in Group 1, to enable a comparison of the two vaccines immunogenicity. As anticipated when designing the trial, the PfSPZ Vaccine group allows us to put the VE results in perspective of previous trials with the PfSPZ Vaccine as reference. This difference in VE of PfSPZ Vaccine between the study in the U.S. and our study may be explained by either i) differences in the stringency of the CHMI or ii) differences in the immunogenicity of PfSPZ Vaccine in the two studies. With regard to the first possibility, both studies used mosquito-bite based CHMI at three weeks after the last immunization. Mosquito bite CHMI is more similar to natural infections as it includes the possibly relevant SPZ skin stage where antibodies against SPZ may have an effector function [26, 27]. However, in the Epstein et al. study the 3D7 clone of the NF54 strain of Pf was used in the CHMI, whereas in this study we used the NF54 strain of Pf. Small genotypic differences between these two strains have been identified [28], however it remains unclear whether these are relevant and

result in differences in prepatent period as observed in independent clinical trials [29]. Unfortunately, direct comparisons have not been performed. Because PfSPZ-GA1 was created in a NF54 background, we would expect the NF54 CHMI to be more homologous. In addition, the primary parasitologic outcome variable differed between the trials (qPCR in The Netherlands, thick smear in the U.S.) prepatent periods cannot be directly compared, making it difficult to assess challenge stringency. However, for both strains 5 mosquito bites are needed to achieve a virtually 100% infection rate and ultimately cannot account for the lower than expected VE. Thus, we do not think that differences in stringency of CHMI can explain the difference in VE between the two studies. Moreover, we have broad experience with this mosquito bite CHMI model, including studies in which we show 100% VE by the chemoprophylaxis with sporozoites approach against homologous Pf NF54 CHMI [30-32].

Both PfSPZ Vaccine and PfSPZ-GA1 Vaccine were immunogenic and induced anti-PfCSP antibodies and PfRBC-specific T cells. There was a positive association between anti-PfCSP antibodies and the protection status of the volunteers as measured by prepatent period. However, less than half of volunteers had significant induction of T cell responses. This is in contrast with other PfSPZ Vaccine studies [8, 9] in which typically most volunteers show induction of CD4+ T cell responses, although CD8+ T cell responses have been variable. Possibly a difference in the *in vitro* cell stimulus (PfRBC versus PfSPZ) could also explain this difference and therefore in future studies with GAP vaccines it would be of importance to also compare these two stimuli. However, anti-PfCSP antibody levels in our study were also significantly lower than those found in the Epstein et al. study [7] after immunization with PfSPZ Vaccine using the same schedule and dose of $4.5x10^5$ PfSPZ (median level (net OD 1.0) at 2 weeks after the third dose of 19,044 vs 5,465).

Based on these data we consider a lower vaccine immunogenicity of PfSPZ Vaccine compared to the Epstein et al. study to be a likely explanation for the decreased VE of PfSPZ Vaccine observed in our study [7]. However, the true cause of the decreased immunogenicity remains unclear. Retrospective evaluation did not reveal any procedural complications or deviations from established protocols in vaccine transport, storage or administration. In addition, the trial in our study was performed in two centers, with different teams performing vaccine preparation and administration and yet both showed similar immunogenicity results. Vaccine-lot specific problems also do not seem a likely explanation. The same lot of PfSPZ Vaccine was used in a clinical trial in Germany where immunization with 3 doses of $9.0x10^5$ PfSPZ of PfSPZ Vaccine by DVI protected >80% of vaccinees against heterologous CHMI of cryopreserved PfSPZ Challenge administered by DVI at 3 and 9.5 weeks after the last dose of vaccine (Personal communication, B. Mordmüller). While we observed that PfSPZ-

GA1 Vaccine appeared to be as immunogenic as PfSPZ Vaccine, at an equivalent dose, the unexpected low VE of the PfSPZ Vaccine comparator limits our ability to draw firm conclusions on the VE of PfSPZ-GA1 Vaccine. However, given the suboptimal VE of PfSPZ-GA1, the next generation genetically attenuated PfSPZ vaccines should aim at enhanced potency either by increasing dose or potentially through an arrest later in the liver stage.

This study demonstrates that a genetically attenuated, live parasite vaccine, PfSPZ-GA1, can be safely administered to malaria naïve volunteers by DVI. Genetically attenuated PfSPZ have advantages over other whole PfSPZ vaccination strategies because they can improve the safety and consistency of manufacturing. While future studies will be needed to establish the potential protective efficacy of PfSPZ-GA1 Vaccine, the confirmation that it is immunogenic and fully attenuated with a favourable safety and tolerability profile, strongly supports the feasibility of genetically attenuated vaccines in practice and paves the way for future testing and further development of such vaccines.

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Competing interests

The investigators are employees of Radboudumc, LUMC and Sanaria. The board of directors of the academic institutes were not involved in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication. KCN, YA, TM, TLR, BKLS and SLH are salaried, full-time employees of Sanaria, the developer and sponsor of Sanaria PfSPZ Vaccine and PfSPZ-GA1 Vaccine. SLH and BKLS also have financial interests in Sanaria. CJJ, SMK and RWS are inventors on a patent of PfΔ*b9*Δ*slarp*. The patent application has been assigned to RUMC, LUMC and Sanaria. All other authors declare no competing interests.

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Supplementary materials

Materials and methods. Inclusion and exclusion criteria

Table S1: Number of adverse events after immunisation

Table S2: Mosquito infectivity and bite numbers used for CHMI for the different study arms

Figure S1: Correlation of anti-PfCSP antibody titer with prepatent period

Supplementary materials:

Materials and methods

Inclusion criteria

1. Subject is aged ≥ 18 and ≤ 35 years and in good health.
2. Subject has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
3. Subject is able to communicate well with the investigator, is available to attend all study visits.
4. Furthermore, the subject will remain within the Netherlands or within reasonable travelling distance from the Radboudumc from day -1 till day +28 after each parasite exposure. After CHMI, subjects have to be reachable by phone (24/7) from day -1 until day 35.
5. Subject agrees to inform his/her general practitioner (GP) about participation in the study and to sign a request to release by the GP, and medical specialist when necessary, any relevant medical information concerning possible contra-indications for participation in the study.
6. Subject agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period and for a defined period thereafter according to Sanquin guidelines (3 years minimum, depending on serology).
7. Non-pregnant, non-lactating females of reproductive potential (i.e., have a uterus and are neither surgically sterilized nor post-menopausal) should agree to use adequate contraception and not to breastfeed for the duration of study.
8. Subject agrees to refrain from intensive physical exercise (disproportionate to the subjects' usual daily activity or exercise routine) for twenty-one days following each immunization and during the malaria challenge period.
9. Subject has signed informed consent.

Exclusion criteria

1. Any history, or evidence at screening, of clinically significant symptoms, physical signs or abnormal laboratory values suggestive of systemic conditions, such as cardiovascular, pulmonary, renal, hepatic, neurological, dermatological, endocrine, malignant, haematological, infectious, immune-deficient, psychiatric or other disorders, which could compromise the health of the volunteer during the study or interfere with the interpretation of the study results. These include, but are not limited to, any of the following:
   a. Body weight <50 kg or Body Mass Index (BMI) <18.0 or >30.0 kg/m2 at screening
   b. A heightened risk of cardiovascular disease, defined as:

i. An estimated ten-year risk of fatal cardiovascular disease of ≥5% at screening, as determined by the Systematic Coronary Risk Evaluation (SCORE);
ii. History, or evidence at screening, of clinically significant arrhythmia's, prolonged QT-interval or other clinically relevant ECG abnormalities; or
iii. a positive family history of cardiac events in first or second degree relatives (according to the system used in medical genetics) <50 years old.

c. Functional asplenia, sickle cell trait/disease, thalassaemia trait/disease or G6PD deficiency.
d. History of non-febrile seizure at any time prior to study onset, even if no longer on medication.
e. Positive HIV, HBV or HCV screening tests.
f. Chronic use of i) immunosuppressive drugs, ii) antibiotics, iii) or other immune modifying drugs within three months prior to study onset (excluding inhaled and topical corticosteroids and incidental use of oral anti-histamines) or expected use of such during the study period.
g. History of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past five years.
h. Any history of treatment for severe psychiatric disease by a psychiatrist in the past year.
i. History of drug or alcohol abuse interfering with normal social function in the period of one year prior to study onset, positive urine toxicology test for cocaine or amphetamines at screening or prior to infection or positive urine toxicology test for cannabis prior to infection.

2. For female subjects: breastfeeding, or positive urine pregnancy test at screening or prior to immunization or prior to CHMI.
3. Any history of malaria, positive serology for *P. falciparum,* or previous participation in any malaria (vaccine) study or CHMI.
4. Known hypersensitivity to or contra-indications (including co-medication) for use of atovaquone/ proguanil or artemether/lumefantrine, or history of severe (allergic) reactions to mosquito bites.
5. Receipt of any vaccinations in the 3 months prior to the start of the study or plans to receive any other vaccinations during the study period or up to 8 weeks thereafter.
6. Participation in any other clinical study in the 30 days prior to the start of the study or during the study period.
7. Being an employee or student of the department of Medical Microbiology or Infectious Diseases of the Radboudumc or the LUMC.
8. Any other condition or situation that would, in the opinion of the investigator, place the subject at an unacceptable risk of injury or render the subject unable to meet the requirements of the protocol or would compromise the integrity of the data.

Table S1: Number of volunteers reporting solicited local and systemic adverse events possibly, probably or definitely related to immunization. Percentages are given in parenthesis. Data collected until 35 days after each immunization. No rash, induration, edema, chills or arthralgia were reported.

			PfSPZ-GA1 Vaccine			PfSPZ Vaccine	Placebo
		Grade	1.35x10^5	4.5x10^5	9.0x10^5	4.5x10^5	
			n=3	n=16	n=26	n=13	n=9
Local	Tenderness	1	0 (0)	0 (0)	2 (8)	1 (8)	0 (0)
	Bruising	1	1 (33)	0 (0)	3 (12)	3 (23)	0 (0)
	Erythema	1	0 (0)	0 (0)	0 (0)	0 (0)	1 (11)
	Swelling	1	0 (0)	0 (0)	1 (4)	2 (15)	0 (0)
	Pain	1	0 (0)	0 (0)	2 (8)	1 (8)	1 (11)
	Pruritis	1	0 (0)	0 (0)	2 (8)	0 (0)	1 (11)
Systemic	Fever	1	0 (0)	1 (6)	1 (4)	1 (8)	0 (0)
		2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		3	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)
	Headache	1	0 (0)	5 (31)	11 (42)	4 (31)	3 (33)
		2	1 (33)	1 (6)	2 (8)	2 (15)	1 (11)
		3	0 (0)	0 (0)	0 (0)	1(8)	0 (0)
	Fatigue/malaise	1	1 (33)	2 (13)	3 (12)	2 (15)	1 (11)
		2	0 (0)	1 (6)	1 (4)	0 (0)	1 (11)
		3	0 (0)	0 (0)	1 (4)	0 (0)	1 (11)
	Myalgia	1	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)
		2	0 (0)	0 (0)	2 (8)	0 (0)	1 (11)
		3	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)

Table S2: Mosquito infectivity and bite numbers used for CHMI for the different study arms

	Mosquito infectivity		Infection		
	Percent	# Sporozoites/ mosquito	Number of sessions median (range)	# Infected bites median (range)	# Uninfected bites median (range)
PfSPZ-GA1 9 x10^5	100%	106,000	1 (1-3)	5	0 (0-2)
PfSPZ-GA1 4.5 x10^5			1 (1-2)	5	0 (0-2)
PfSPZ Vaccine 4.5 x10^5			1 (1-2)	5	0 (0-1)
Placebo			1 (1-2)	5	0 (0-3)

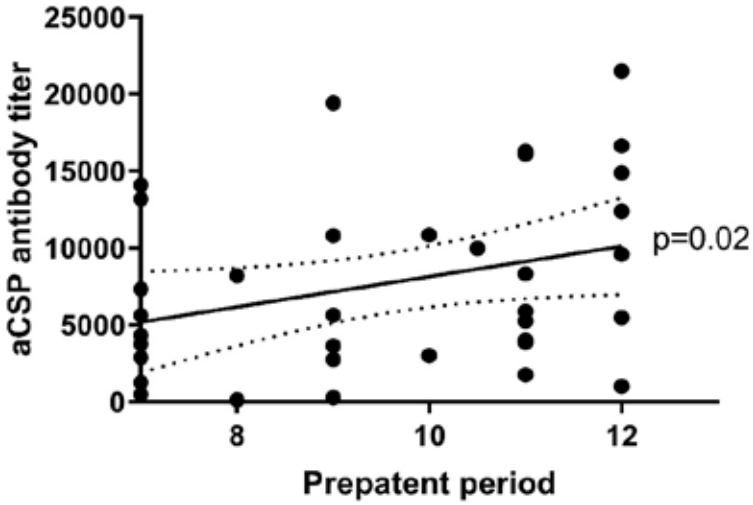

Figure S1: Correlation of anti-PfCSP antibody titer with prepatent period (Pearson correlation p=0.02, r=0.32)

CHAPTER 5

Modest heterologous protection after *Plasmodium falciparum* sporozoite immunization: a double-blind randomized controlled clinical trial

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Abstract

Background

A highly efficacious vaccine is needed for malaria control and eradication. Immunization with *Plasmodium falciparum* NF54 parasites under chemoprophylaxis (CPS-immunization) induces the most efficient long-lasting protection against a homologous parasite. However, parasite genetic diversity is a major hurdle for protection against heterologous strains.

Methods

We conducted a double-blind, randomized controlled trial in 39 healthy participants, of NF54-CPS-immunization by bites of 45 NF54-infected (n=24 volunteers) or uninfected mosquitoes (placebo; n=15 volunteers) against a Controlled Human Malaria Infection (CHMI) with the homologous NF54 or the genetically distinct NF135.C10 and NF166.C8 clones. Cellular and humoral immune assays were performed as well as genetic characterization of the parasite clones.

Results

NF54 CPS-immunization induced complete protection in 5/5 volunteers against NF54 challenge infection at 14 weeks post-immunization, but sterilely protected only 2/10 and 1/9 volunteers against NF135.C10 and NF166.C8 challenge infection, respectively. Post-immunization plasma showed a significantly lower capacity to block heterologous parasite development in primary human hepatocytes compared to NF54. Whole genome sequencing showed that NF135.C10 and NF166.C8 have amino acid changes in multiple antigens targeted by CPS-induced antibodies. Volunteers protected against heterologous challenge were among the stronger immune responders to *in vitro* parasite stimulation.

Conclusions

Although highly protective against homologous parasites, NF54 CPS-induced immunity is less effective against heterologous parasite clones both *in vivo* and *in vitro*. Our data indicate that whole sporozoite-based vaccine approaches require more potent immune responses for heterologous protection.

Background

Malaria has a significant impact on human health and economic welfare worldwide, causing over 200 million cases of disease and nearly half a million deaths in 2015 [1]. Though a significant decrease in malaria deaths has been observed in the last 15 years [1], the emergence of insecticide-resistant mosquitoes [2] and drug-resistant parasites [3] are threatening malaria control efforts and underscore the need for a highly effective vaccine.

While naturally acquired immunity likely never results in sterile protection against the parasite [4], generation of long-lasting and sterilizing immunity against malaria is the goal of pre-erythrocytic vaccine approaches. So far, only one sub-unit vaccine, RTS,S (Mosquirix, Glaxo Smith Kline), has been recommended for defined clinical application [5]. This vaccine is based on a major sporozoite surface protein, the circumsporozoite protein (CSP), and has shown to induce a short-term 30-50% efficacy in reducing the incidence of clinical malaria in endemic areas, as well as in the Controlled Human Malaria Infection (CHMI) model [6-8]. Sterilizing immunity can be induced by attenuated whole sporozoite approaches. Immunization with radiation-attenuated sporozoites requires bites of at least 1000 infected mosquitoes to induce sterile protection in 50% of volunteers [9], or a total dose of 675k cryopreserved sporozoites injected intravenously for full homologous protection [10]. In contrast, bites by only 30-45 malaria-infected mosquitoes [11, 12] or 150k intravenously injected cryopreserved sporozoites [13] in the ChemoProphylaxis and Sporozoite (CPS) regimen induces complete sterile protection against the homologous parasite. CPS-induced protection can last for at least 2.5 years, showing unprecedented efficiency and sustainability of the protective immune response [14]. Specifically, strong effector memory T cell responses are induced [11, 12], as well as memory B-cell and antibody responses targeting pre-erythrocytic stage antigens with functional activity against homologous sporozoites, inhibiting parasite development in liver cells *in vitro* and *in vivo* in human liver-chimeric mice [15-17].

Despite these advances, a major hurdle for the induction of protection against heterologous strains is the well-known genetic diversity of *Plasmodium falciparum*, allowing parasite evasion and reducing protective efficacy. More recently, immunization with radiation attenuated sporozoites provided 53% protection against a heterologous challenge [18]. There is also incidental evidence for CPS-induced heterologous protection in the CHMI model [19], but this has not been systematically addressed.

Here we describe the first double-blind, placebo-controlled CHMI trial of NF54-CPS-immunization by a total of 45 *P. falciparum* NF54-infected mosquitoes followed by a challenge infection with either *P. falciparum* NF135.C10 clone from Cambodia or NF166. C8 clone from Guinea. Parasites were characterized by whole genome sequencing and CPS-induced cellular and humoral responses were analyzed.

Methods

Study design and participants

This single center, double-blind, randomized, placebo-controlled trial was conducted at the Radboud university medical center (Nijmegen, The Netherlands) between February and November 2015. Study participants were healthy male and female volunteers (18-35 years old) with no history of malaria and screened for eligibility including a complete medical and family history, physical examination, blood hematological and biochemical parameters, and serology for human immunodeficiency virus (HIV), hepatitis B and C and the asexual stages of *P. falciparum* as previously described [20]. All candidate participants provided written informed consent at the screening visit.

Study approval

The study was approved by the Central Committee for Research Involving Human Subjects of The Netherlands (CCMO NL48732.091.14) and conducted according to the principles outlined in the Declaration of Helsinki and Good Clinical Practice standards. This trial is registered at ClinicalTrials.gov, identifier NCT02098590.

Procedures

All included study subjects (n=41, Figure 1) received chloroquine in a prophylactic dose (i.e. a loading dose of 300mg of chloroquine on each of the first two days, followed by 300mg once a week), for a total duration of 13 weeks. While under chloroquine prophylaxis, study groups 1, 2 and 3 received three immunizations with bites of 15 *P. falciparum* NF54-infected *Anopheles stephensi* mosquitoes. Groups 4, 5 and 6 received three mock-immunizations with bites of 15 uninfected mosquitoes.

Volunteers were followed on an outpatient basis from days 6 to 10 after each immunization. Blood was examined daily, including hemocytometry, white-blood cell counts, lactic acid dehydrogenase (LDH) and highly sensitive troponin-T. From day 7 to 9, blood was also checked for malaria parasites by thick blood smear microscopy and quantitative real-time PCR (qPCR) was performed retrospectively (after study de-blinding) as described previously [21, 22].

Fourteen weeks after discontinuation of chloroquine prophylaxis, all participants were exposed to bites of five *P. falciparum* infected *Anopheles stephensi* mosquitoes (Table 1). Subjects of groups 1 and 4 were challenged with the heterologous NF135.C10 clone; groups 2 and 5 with the heterologous NF166.C8 clone; groups 3 and 6 with the homologous NF54 strain. Mosquitoes were examined to verify that a blood meal was taken and the presence of sporozoites in mosquito salivary glands was confirmed by dissection. If insufficient infected mosquitoes had taken a blood meal, subjects were exposed to additional mosquitoes.

After challenge infection, subjects visited the clinical trial site twice daily from day 6 to day 15 and once daily from day 16 until day 21. Blood was drawn for parasitological assessments at every visit and safety laboratory measurements were performed once daily as described above. The following symptoms were solicited: fever, headache, malaise, fatigue, myalgia, arthralgia, nausea, vomiting, chills, diarrhea and abdominal pain. All signs and symptoms (solicited and unsolicited) were recorded and graded by the attending physician as follows: mild (easily tolerated), moderate (interferes with normal activity), or severe (prevents normal activity), or in case of fever grade 1 (>37·5°C – 38·0°C), grade 2 (38·1°C – 39·0°C) or grade 3 (>39·0°C).

Subjects were treated with a curative regimen of 1000mg atovaquone and 400mg proguanil once daily for three days, when parasitemia above the treatment threshold (100 parasites per milliliter of blood) was detected by qPCR [22]. Subjects that remained qPCR negative were presumptively treated with the same regimen 28 days after challenge infection. Complete cure was confirmed by two consecutive negative qPCR measurements after treatment.

Randomization and masking

All study subjects, in two time-separated cohorts, were randomly allocated to one of the six study groups using a computer-generated list of random numbers (Microsoft Excel 2007, Redmond, WA, USA), with study groups stratified equally over each cohort. Randomization was prepared by two independent investigators and was stored securely, in sealed opaque envelops with restricted access. Subjects, investigators and primary outcome assessors were masked to group assignment.

During the study, the homologous (NF54) challenge strain infection of the second cohort was delayed as there was no sufficiently infected batch of mosquitoes available (>40% infected; according to our standard operating procedures). Challenge strain blinding had to be lifted for this group (6 subjects), allowing them to be challenged two weeks later. The investigators remained blinded to immunization allocation of all participants and to the challenge strain allocation of the NF135.C10 and NF166.C8 groups until the

end of the study. All study subjects and immunology assessors remained blinded during the entire study. The partial de-blinding procedure was documented and reported to the Central Committee for Research Involving Human Subjects of The Netherlands (CCMO).

Primary study outcome

The primary outcome was pre-patent period: time to parasitemia (a single qPCR measurement with a parasite density greater than 100 parasites per milliliter of blood) in subjects after challenge infection. Study sample size was determined in order to be able to detect a difference in pre-patent of three days between the immunization and control groups (with α=0.05, power=0.90).

Parasites

NF54 is a longstanding and well-characterized strain isolated several decades ago from a person with airport malaria near Schiphol Airport (Amsterdam, The Netherlands) and likely originating from West Africa [23]. The NF135.C10 clone originated from a clinical isolate in Cambodia [24]. The NF166.C8 clone originated from a patient after a recent visit to Guinea (West Africa) [25] .

Parasite culture and generation of infected mosquitoes

Plasmodium falciparum NF54, NF135.C10 and NF166.C8 asexual and sexual blood stages were cultured in a semi-automated culture system [26-29]. *Anopheles stephensi* mosquitoes for immunizations and challenge infections were reared in the Radboud university medical center insectary (Nijmegen, The Netherlands) according to standard operating procedures. Infected mosquitoes were obtained by standard membrane feeding on gametocyte cultures of the different strains as described previously [29].

For *in vitro* sporozoite infectivity assays, salivary glands from infected *Anopheles stephensi* mosquitoes were hand dissected and collected in complete William's B culture medium without serum. Salivary glands were homogenized in a homemade glass grinder and the number of *P. falciparum* sporozoites was counted in a Bürker-Türk counting chamber using phase contrast microscopy [15].

Plasma samples

Citrated plasma samples were collected from 24 CPS-immunized volunteers at different time points using citrated vacutainer cell preparation tubes (CPT vacutainers; Becton Dickinson). Samples collected 11-14 days before the first CPS-immunization (pre-immunization) and one day before challenge infection (18 weeks after the last immunization; post-immunization) were used for analysis of malaria-antigen specific antibody levels and assessment of functional activity *in vitro*. Plasma samples were stored in aliquots at -20 °C and re-thawed no more than three times.

Prior to use for *in vitro* sporozoite infectivity assays in primary human hepatocytes, citrated plasma aliquots were heat-inactivated for 30 minutes at 56 °C and spun down at 13,000 rpm for 5 minutes at room temperature.

PBMC isolations and cryopreservation

Venous whole blood was collected in CPT vacutainers at different time points. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples, cryopreserved and stored as described previously [11]. Briefly, PBMCs were isolated by centrifugation, washed in ice-cold phosphate buffered saline (PBS), and counted in 0·1% Trypan blue with 5% Zap-o-Globin II Lytic Reagent (Beckman Coulter) to assess cell viability. Cells were cryopreserved at a concentration of 10^7 PBMCs/ml in ice-cold fetal bovine serum (FBS, Gibco) containing 10% dimethylsulfoxide (DMSO, Merck, Germany) using Mr. Frosty freezing containers (Nalgene). Subsequently, PBMC samples were stored in vapor-phase nitrogen. PBMC samples collected 11-14 days before the first CPS-immunization (pre-immunization) and one day before challenge infection (18 weeks after the last immunization; post-immunization) were used for *in vitro* PBMC restimulation experiments and flow cytometric analysis.

Humoral immunological assays

Malaria antigen-specific antibody levels

Specific antibodies to *P. falciparum* circumsporozoite protein (CSP) (3D7 full-length protein, obtained from Genova Biotechniques Pvt. Ltd. in Hyderabad, India) in citrated anti-coagulated plasma samples were determined by a standardized enzyme-linked immunosorbent assay (ELISA) at indicated time points as previously described. Antibody levels were expressed as arbitrary units (AU) in relation to a pool of 100 sera from adults living in an area in Tanzania where malaria is highly endemic (HIT), with this positive control set at 100 AU [12] see also Supplementary information S1 for detailed information [17]. ELISA data analysis was performed with Auditable Data Analysis and Management System for ELISA (ADAMSEL, version 1.1) as previously described [17]. Post-immunization plasma samples were corrected for baseline responses for CSP.

In vitro sporozoite infectivity assay of primary human hepatocytes

To test CPS-induced functional antibody activity against sporozoite development, fresh primary human hepatocytes were isolated and cultured from patients undergoing partial hepatectomy as described in Supplementary information S2. Briefly, viable hepatocytes ($5*10^4$ hepatocytes/well) in complete William's B medium were seeded into 96-well flat-bottom plates (Falcon, 353219) coated with 0·056 mg/ml rat tail collagen I per well (Roche Applied Science, 11179179-001), and cultured at 37 °C in an atmosphere of 5% CO_2.

Two to three days after seeding of hepatocytes, batches of fresh *P. falciparum* NF54, NF135.C10 or NF166.C8 sporozoites in Williams B medium were pre-incubated with heat-inactivated naive human control serum (10% final concentration) and heat-inactivated pre- or post- CPS-immunization plasma at a final concentration of 10% for 30 minutes on ice (final concentration of serum/plasma in each sample: 20%). Sporozoites pre-incubated with an anti-CSP monoclonal antibody (mAb 2A10, 10 µg/ml final concentration, MR4; MRA-183A) served as a positive control. Sporozoites in the presence of 20% heat-inactivated naive human control serum served as standard control. $5*10^4$ of pre-incubated sporozoites were added to 96-well plates containing monolayers of primary human hepatocytes in triplicate. Five to six days post-infection, the number of *P. falciparum* infected hepatocytes was assessed by staining for *P. falciparum* Heat shock protein (Hsp)-70 and indirect immuno-fluorescence analysis using a Leica DMI6000B inverted microscope as described in Supplementary information S3 and S4.

Cellular immunological assays

For the assessment of *P. falciparum*-specific cellular immune responses, pre- and post-immunization PBMCs from CPS-immunized volunteers whom received NF135.C10 and NF166.C8-challenge infection, were re-stimulated *in vitro* with cryopreserved *P. falciparum* NF54-infected erythrocytes (*Pf*RBCs) as described previously [11, 14] and described in detail in Supplementary information S5. Briefly, after thawing, PBMCs in complete culture medium (final concentration of $10*10^6$cells/ml) were stimulated *in vitro* in duplicate with either 10^6 cryopreserved NF54 *Pf*RBCs or 10^6 uninfected erythrocytes (uRBC; negative control) for 24h at 37 °C with 5% CO_2. Fluorochrome-labeled monoclonal antibody to CD107a was added for the duration of the stimulation. During the last four hours, 10 µg/ml Brefeldin A and 2 µM monensin were added, and 10 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml ionomycin were added to positive control wells.

After 24 hours of stimulation in total, cells were stained with a Live/Dead fixable dead cell stain dye and fluorochrome-labeled antibodies against the surface markers CD3, CD4, CD8, gamma delta T cell receptor and CD56, and against the intracellular cytotoxic marker granzyme B and the cytokine IFN-ɣ Samples were kept cold and dark in 1% paraformaldehyde (PFA) in PBS until measured by flow cytometry on the same day. Both time points for each volunteer were thawed, stimulated and stained within the same experimental round. Samples were acquired using a 10-colour Gallios flow cytometer (Beckman Coulter), and single stained cells were run every round for compensation. Data analysis was performed using FlowJo software (Version 10·0·8, Tree Star). uRBC responses were subtracted from *Pf*RBC-specific responses for every volunteer for each time point, and post-immunization responses were corrected for pre-immunization responses.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 5, GraphPad Software Inc., California, USA). Differences in prepatent period by qPCR between groups were determined by Log-Rank test. Differences in antibody levels across the immunization groups were analyzed with One-way ANOVA with Bonferroni's multiple comparison post-hoc correction. For analysis of *in vitro* sporozoite infectivity data, differences were tested using the paired Student's t-test, except when comparing between immunization groups, when a one-way ANOVA with Bonferroni's multiple comparison post-hoc correction was used. A p-value of <0·05 was considered significant.

Genetic analysis of parasites

Genomic DNA was obtained from the three study strains and 2.3 µg each were submitted for Illumina sequencing. The resulting fastq reads (150 bp) were aligned to the *P. falciparum* 3D7 reference genome (v.3, plasmoDB) using bowtie2 software (bowtie-bio.sourceforge.net). Single nucleotide polymorphisms (SNPs) were called with samtools and bcf/vcftools software (samtools.sourceforge.net). An alignment of all SNPs (with bcftools quality score > 100) was used to compare the study strains to others from Ghana, Guinea, Kenya, Cambodia, Thailand and Vietnam in a combined analysis of genetic variation, as described by Campino el al. [30]. Furthermore, genes that were identified to elicit a humoral immune response in CPS-immunization were examined [16]. This panel of 11 pre-erythrocytic genes including CSP and LSA-1, complemented by MAEBL, was checked for amino acid changes in the translated protein.

Results

Protective efficacy of CPS-immunization against heterologous challenge

Forty-one volunteers were included into the study and twenty-four immunization and fifteen placebo-control volunteers completed the clinical trial, and were included in the per protocol analysis. One volunteer withdrew after being unable to attend the study visits and one volunteer was excluded due to a concomitant condition (Figure 1). Baseline characteristics of the study population are shown in Table 1.

As previously observed, NF54 CPS-immunization induced sterile protection against a homologous NF54 challenge in five out of five volunteers (Figure 2A) [11, 12, 31]. In contrast, two out of ten volunteers were sterilely protected after challenge with NF135. C10, with six out of ten volunteers showing a prolonged pre-patent period compared to mock-immunized controls (more than two times the standard deviation of controls)

(Figure 2B). After NF166.C8 challenge, one out of nine NF54-immunized volunteers was fully protected, while eight out of nine volunteers showed no delay to patency (Figure 2C). There was also no difference in mean day 7 parasitemia (representing the liver parasite burden) between immunized and controls challenged with NF166.C8 (Supplementary Figure 1). In line with a previous study [25], NF135.C10 and NF166.C8 show higher infectivity than NF54. Overall, NF54-CPS immunization induces only modest protection against heterologous clones.

Table 1: Baseline characteristics of subjects included in the analysis.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Number of participants (n=39)	**10**	**9**	**5**	**5**	**5**	**5**
Sex						
Male	3 (30%)	2 (22%)	2 (40%)	3 (60%)	2 (40%)	5 (100%)
Female	7 (70%)	7 (78%)	3 (60%)	2 (40%)	3 (60%)	0 (0%)
Age (years)	21.5 (1.8)	21.1 (2.6)	22.4 (1.6)	20.1 (1.3)	20.4 (2.5)	22.6 (3.1)
BMI (kg/m2)	21.8 (2.3)	21.4 (1.7)	22.0 (3.1)	20.6 (2.0)	24.8 (3.3)	23.2 (1.7)
***P. falciparum* strain**						
Immunization	NF54	NF54	NF54	uninfected	uninfected	uninfected
Challenge	NF135.C10	NF166.C8	NF54	NF135.C10	NF166.C8	NF54

Data are n (%), mean (SD). BMI=body-mass index

As marker for the induction of immune responses, antibody levels to the major sporozoite vaccine-target antigen CSP [7, 8] were measured. All volunteers showed a post-immunization CSP antibody titer as determined by ELISAs and corrected for baseline (median 3·7 AU); IQR 2·9-4·5), which did not significantly differ between groups (Group 1: 24·7 (IQR 10·4-45·2); Group 2: 24·7 (IQR 17·0-37·1) and Group 3: 74·1 (IQR 20·9-187·0)).

In vitro inhibition of intra-hepatic sporozoite development by CPS-induced antibodies

Next, functional antibody activity to inhibit sporozoite development in primary human hepatocytes *in vitro* was tested. Post-immunization plasma from all 24 NF54 CPS-immunized volunteers significantly reduced homologous intra-hepatic sporozoite development ($p<0.0001$; Figure 3A). However, inhibition of intra-hepatic NF135.C10 and NF166.C8 development was significantly lower ($p<0·01$), with median percentages inhibition of 40·7% (IQR 29·6-59·1), 26·9% (IQR 15·6-35·0) and 31·0% (IQR 22·6-43·2) for NF54, NF135.C10, NF166.C8 sporozoites, respectively (Figure 3B). Intra-

hepatic development of both heterologous clones was equally inhibited by NF54 CPS-immunization induced antibodies (Figure 3B). While these *in vitro* data reflect the clinical outcome *in vivo* at group level, individual inhibition *in vitro* did not correlate with *in vivo* pre-patent periods and/or parasitemia (data not shown).

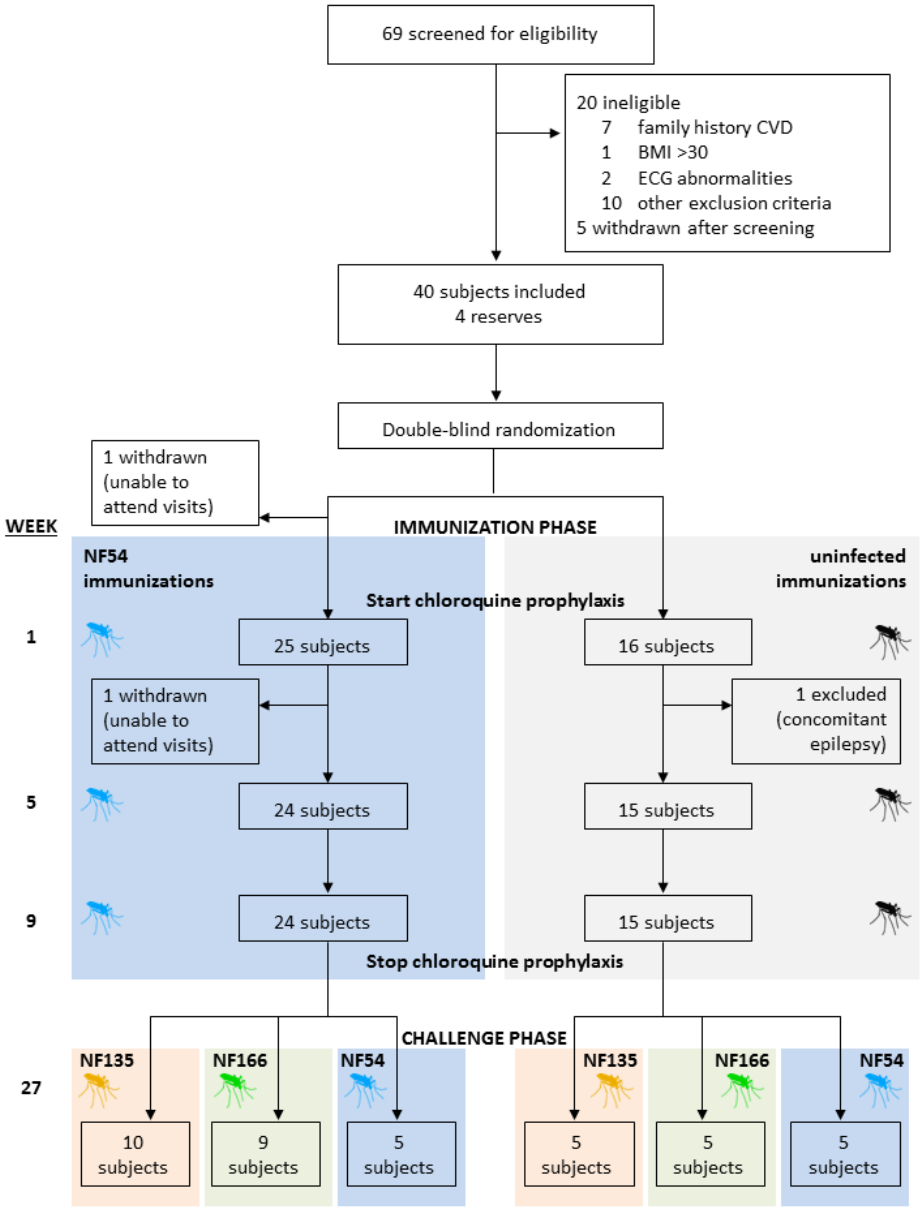

Figure 1: Clinical trial profile.
CVD = cardiovascular disease; BMI = body mass index; ECG = electrocardiogram

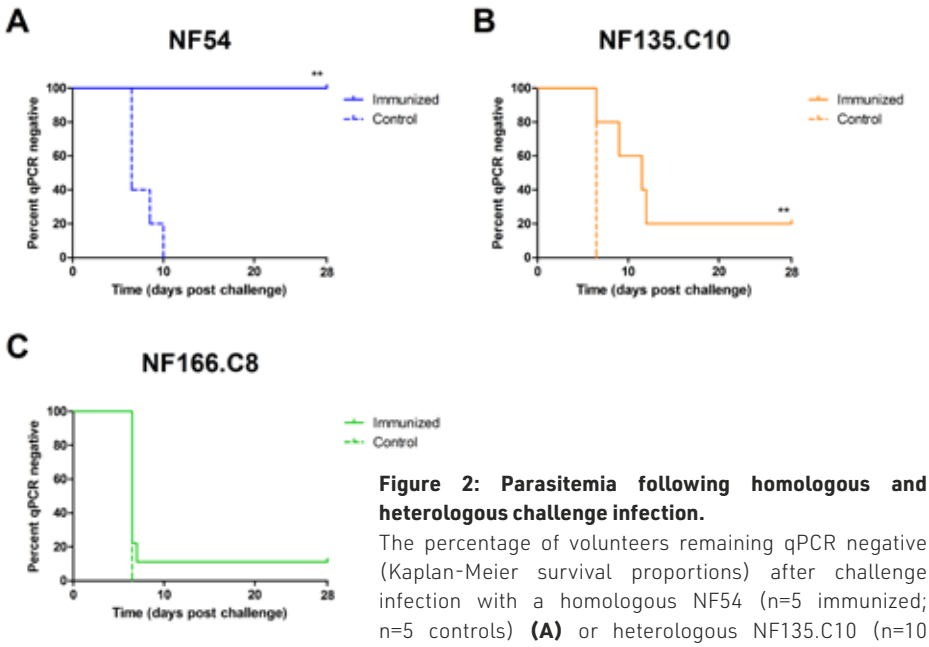

Figure 2: Parasitemia following homologous and heterologous challenge infection.
The percentage of volunteers remaining qPCR negative (Kaplan-Meier survival proportions) after challenge infection with a homologous NF54 (n=5 immunized; n=5 controls) **(A)** or heterologous NF135.C10 (n=10 immunization; n=5 controls) **(B)** or NF166.C8 (n=9 immunized; n=5 controls) **(C)** strain is shown. Solid lines represent CPS-immunized volunteers and dotted lines represent placebo-control immunized volunteers. ** p<0.01 as determined by Log-rank (Mantel Cox) Test.

Genetic diversity of NF54, NF135.C10 and N166.C8 challenge strains

The genetic diversity among *P. falciparum* mutants has been shown to be a strong factor in parasite evasion of protective immune responses. Whole genomes of the parasite clones used in this study were sequenced. High quality Single Nucleotide Polymorphisms (SNPs) were called with about equal numbers for NF135.C10 and NF166.C8 (Supplementary Table 1; Supplementary Figure 3). All polymorphisms were used to infer a phylogeny including isolates from Southeast Asia and East and West Africa [30]. As expected, NF54 and NF166.C8 were very similar to West African isolates, while NF135.C10 showed more resemblance to other Southeast Asian strains (Figure 4). We next compared amino acid changes of twelve genes encoding target antigens for CPS-induced antibodies as previously described [16]. With the exception of EIF3A, all examined proteins showed amino acid changes in either NF135.C10 or NF166.C8 with respect to NF54 (Table 2). Remarkably, in contrast to their relative geographical distances, eleven out of twelve proteins in NF166.C8 and eight out of twelve in NF135.C10 were different compared to NF54.

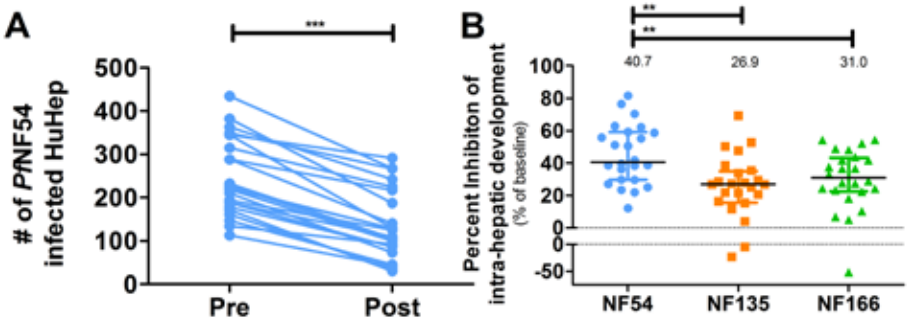

Figure 3: Neutralizing effect of CPS-induced antibodies on *in vitro* sporozoite functionality of homologous and heterologous *P. falciparum* strains.

A. The number of primary human hepatocytes infected by homologous NF54 sporozoites in the presence of Pre- or Post-immunization plasma in all n=24 CPS-immunized volunteers was determined by microscopy. **B.** *P. falciparum* NF54, NF135.C10 or NF166.C8 sporozoites were pre-incubated with Pre- or Post-immunization plasma from CPS-immunized volunteers and the percent inhibition of intra-hepatic development of NF54, NF135.C10 or NF166.C8 was calculated for Post- compared to Pre-immunization plasma for each individual volunteer and presented as squares (NF135.C10), triangles (NF166.C8) or circles (NF54). Data are shown as the mean of triplicate measurements for each individual volunteer (Figure 3A) or the median of all data points with an interquartile range (Figure 3B). Differences in the percent inhibition of intra-hepatic development between parasite strains were tested using One-way ANOVA with Bonferroni's multiple comparison correction.

Humoral and cellular markers of protection

Next, we tested a number of previously established markers that associate with exposure and homologous protection after CPS-immunization [11, 32]. Figure 5 shows the distribution of antibody-mediated inhibition of intra-hepatic sporozoite development as well as the cellular markers interferon (IFN)-γ and granzyme B in $CD4^+$ and $CD8^+$ cells in all immunized volunteers undergoing heterologous challenge. Remarkably, CD107a expression in particularly $CD4^+$ T cells was not increased as previously found (data not shown) [11]. Two out of three heterologous protected volunteers were among the highest antibody-mediated inhibitors of *in vitro* heterologous intra-hepatic development (Figure 5, orange square: NF135.C10-challenged; green triangle: NF166.C8-challenged), with the highest numbers of IFN-γ-producing $CD8^+$ T cells against *Pf*RBC. The third protected volunteer showed only average neutralizing antibody responses and IFN-γ-producing $CD4^+$ and $CD8^+$ T cells, but had a high number of granzyme B^+ $CD8^+$ T cells (orange upside down triangle: NF135.C10-challenged). The group of NF135.C10 volunteers with a prolonged pre-patent period did not distinguish themselves from the unprotected volunteers.

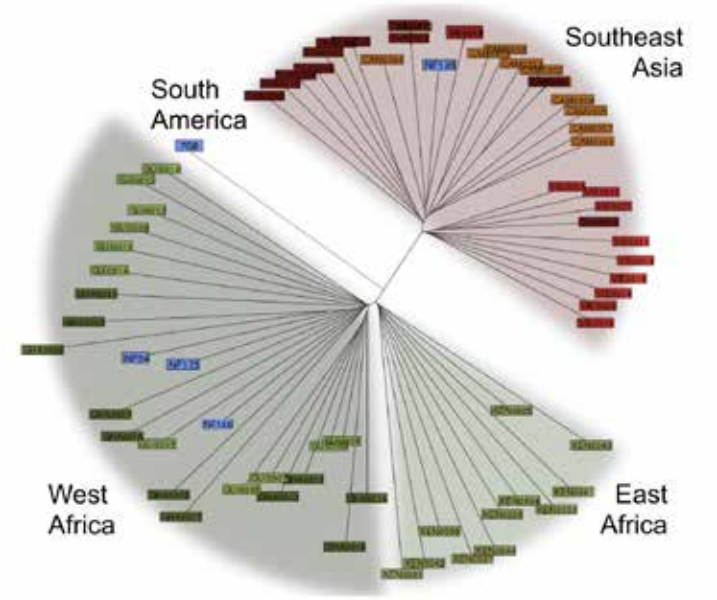

Figure 4: Whole-genome sequencing shows genetic variations between study strains.
Phylogenetic positions of the three *P. falciparum* strains (NF54, NF135.C10 and NF166.C8) used in the study relative to other known *P. falciparum* strains. Whole-genome sequencing was used to infer relatedness to *P. falciparum* strains from different areas [30] . Asian strains, THA, Thailand (dark red). VIE, Vietnam (light red). CAM, Cambodia (orange). East Africa represented by KEN, Kenya. West African strains, GUI, Guinea (light green) and GHA, Ghana (dark green). NF strains and 7G8 (blue).

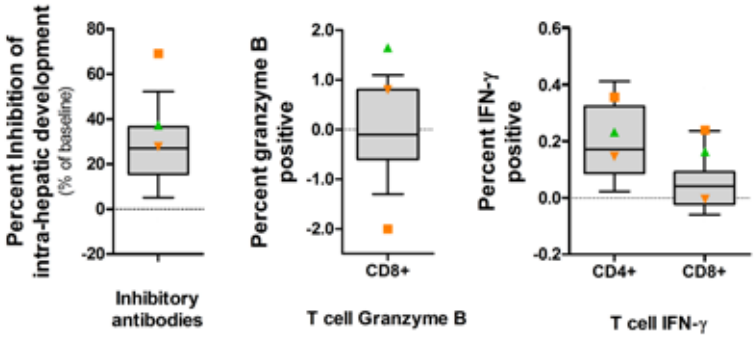

Figure 5: Analysis of *in vitro* intra-hepatic sporozoite development inhibition by CPS-induced antibodies, cellular responses and protection status *in vivo*.
CPS-induced antibody-mediated inhibition of *in vitro* challenge strain intra-hepatic development and cytotoxic and cytokine-producing T cell responses to NF54 infected RBCs are shown. The 10th and 90th percentile of each response in all (n=19) CPS-immunized volunteers that received a heterologous challenge infection are shown as grey box-and-whisker plots. The green triangle represents one out of nine CPS-immunized volunteers sterilely protected against NF166.C8 challenge infection, while the orange square and upside down triangle represent the two out of ten CPS-immunized volunteers with sterile protection against NF135.C10 challenge infection.

Table 2: Amino acid changes in genes involved in the humoral immune response after CPS immunization.

Alterations in gene and protein sequences, based on whole genome sequencing, for 12 genes from NF135.C10 and NF166.C8 were compared to NF54. Single nucleotide polymorphisms and small insertions/deletions are shown together as sequence changes with expected changes in the *in silico* translated protein sequence (counting altered amino acids). Changes in CSP protein sequence are indicated with single-letter amino acid code.

	***Pf*NF135.C10**		***Pf*NF166.C8**	
Gene	**Sequence changes**	**Protein changes**	**Sequence changes**	**Protein changes**
PF3D7_1036400 LSA-1	6	4	14	9
PF3D7_0108300 Conserved unknown	21	12	15	8
PF3D7_1033100 AdoMetDC/ODC	10	9	9	8
PF3D7_0108300 MSP2	13	7	8	7
PF3D7_0304600 CSP	6	6 A98G S301N K317E E318Q N321K A361E	7	7 S301N K314Q K317E E318K N321K K322T E357Q
PF3D7_0509400 RNApol	6	4	9	6
PF3D7_0630600 Conserved unknown	7	2	8	3
PF3D7_0502400 MSP8	2	1	4	3
PF3D7_1147800 MAEBL	2	0	3	1
PF3D7_0703700 Conserved unknown	0	0	1	1
PF3D7_0829000 Conserved unknown	0	0	1	1
PF3D7_1212700 EIF3A	1	0	1	0

Discussion

This randomized, controlled clinical trial shows that CPS-immunization with *P. falciparum* NF54 sporozoites induces modest sterile protection against challenge infection with genetically distinct *P. falciparum* parasites. In line with these clinical observations, antibodies induced by NF54 CPS-immunization inhibit intra-hepatic development of both homologous and heterologous sporozoites *in vitro,* but less efficiently inhibit heterologous clones. *P. falciparum* specific IFN-y and granzyme B T cell responses are also induced, corroborating previous studies, though unlike previous studies, there was no clear induction of CD107a responses [11, 12]. We have previously shown that PBMCs from NF54 exposed volunteers have equivalent responses to homologous and heterologous *Pf*RBC stimulation [24]. Here, volunteers protected against heterologous challenge show relatively high cellular and/or antibody responses. However, none of these individual markers predicts protection.

In this study heterologous protection was assessed against a primary challenge infection, in a double-blind manner, strongly reducing any potential for bias. However, this study lacks the power to discriminate the low protective efficacy seen in the NF166.C8 group with statistical significance. Furthermore, although we chose to test two geographically diverse heterologous strains, we do not know how representative these are for the total genetic diversity for *P. falciparum* in the field.

Plasmodium falciparum parasite strain diversity is a major impediment to the development of effective, sterilizing immunity [33]. Indeed, previous studies with single-protein vaccines show that antigen polymorphisms decrease vaccine efficacy [34-36]. However, genetic diversity may be less challenging for whole sporozoite vaccines representing a broader antigen repertoire that could increase the chances for generating functional, cross-strain immunity. In fact, immunization with radiation-attenuated sporozoites by 1000 mosquitoes [37] or with a total of 1.35×10^6 sporozoites administered intravenously [38] has been shown to induce 80% protection against challenge with the heterologous challenge strain 7G8 (IMTM-22 isolate from Brazil) at three weeks post-immunization, rapidly waning to only 10% protection at 24 weeks. Doubling the dose of intravenous sporozoite immunization to a total of 2.7×10^6 showed an adjusted 53% efficacy against 7G8 at 33 weeks [18]. These data indicate that induction of heterologous protection requires substantial high immunization dosages, which puts constraints on vaccine manufacturing and costs. In contrast, the CPS regimen shows a 10-20 fold higher efficiency for homologous protection likely because volunteers are exposed to the full pre-erythrocytic cycle and the initial phase of blood stage [39]. Previously, two out of thirteen NF54 CPS-immunized volunteers receiving a sub-optimal immunization dose, were protected against a re-challenge infection

with NF135.C10 at fourteen months after the last immunization [19]. Both studies with radiation-attenuated and CPS-immunization showed higher homologous than heterologous protective efficacy. However, none of these studies used an immunization regimen sufficient for >90% homologous protective efficacy. The current study is the first randomized, controlled trial with a whole sporozoite immunization regimen sufficient for complete homologous protection, testing primary heterologous challenge infection eighteen weeks after the last immunization [11].

Despite 100% protection against homologous challenge, we obtain only a modest 10-20% protection against heterologous strains. The observed difference in protection against homologous and heterologous infections may be explained by i) unequal distribution of induced immune responses between study groups; however, anti-CSP antibody titers show a clear consistency and similarity between study groups; ii) differences in numbers of inoculation sporozoites or stringency of the challenge infection. The former is unlikely as mosquito salivary gland infections were similar between the generated strain batches (Supplementary Table 2).

As NF135.C10 and NF166.C8 show higher sporozoite infectivity compared to NF54 [25], it may be more difficult to protect against the NF135.C10 and NF166.C8 clones. However, intra-hepatic sporozoite development of NF135.C10 and NF166.C8 is equipotently inhibited by a functional anti-CSP mAb, 2A10 (Supplementary Figure 2; IC50 concentrations of 2·5 ug/mL (95% CI 0·58-11 ug/mL), 3·5ug/mL (95% CI 1·1-11ug/mL) and 0·88ug/mL (95% CI 0·26-2·9ug/mL)), which makes this explanation less likely; iii) genetic variation between the challenge parasites, shown to be considerable with amino acid changes in either NF135.C10 or NF166.C8 in eleven out of twelve target proteins for CPS-induced antibodies [16]. We consider decreased immune efficacy against genetically diverse parasite strains as the most likely explanation for the modest protection against heterologous parasites.

As the three out of nineteen volunteers with sterile protection against heterologous challenge tend to show more potent responses to previously identified immune markers [11, 15, 32], decreased efficacy against heterologous parasites may be overcome by stronger immune responses. Improvement of the strength of cellular and antibody responses may be achieved by altering immunization regimens. For instance, this may be achieved by increasing the immunization liver-stage antigen load, by raising the NF54 sporozoite immunization dose, as has been done with radiation attenuated sporozoites [18, 40]. Alternatively, increased liver-stage infectivity with a parasite such as NF135.C10 likely increases the antigen load without the need for increasing the number of sporozoites administered. A mixture of parasite strains, or sequential immunizations with

different strains are alternative options but will complicate the product manufacturing or practical application. The latter approach might be more efficient, as heterologous sporozoites would evade strain-specific neutralizing immunity, thereby increasing the liver parasite burden at second and third immunizations. These studies should aim to find the optimal balance between the desired induction of cross-stage immunity and related adverse events that may occur. Taken together, our findings highlight the need to further explore the immunological basis of cross-strain protection against *P. falciparum*, to improve existing whole-sporozoite immunization strategies.

Conclusions

These data demonstrate that despite providing complete protection against homologous challenge infection, CPS immunization with the NF54 strain provides only modest sterile protection against the genetically distinct NF135.C10 and NF166.C8 clones. Our immunological and parasite sequencing data suggest that genetic variation between the strains reduces the efficiency of antibodies to block heterologous parasites. Since protected volunteers tended to be higher immune responders, this study underscores the need for whole sporozoite vaccination regimens increase the height or breadth of immune responses to achieve heterologous protection.

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Conflict of interest

The authors declare that they have no competing interests.

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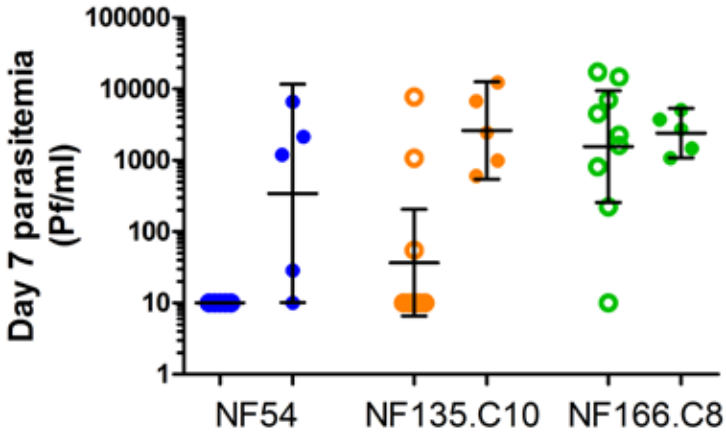
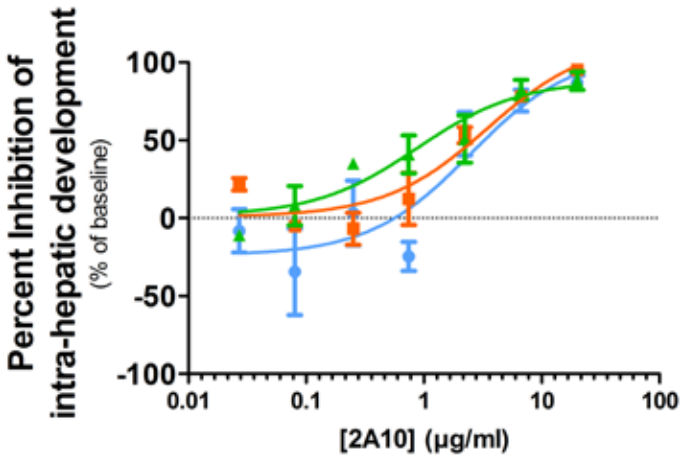
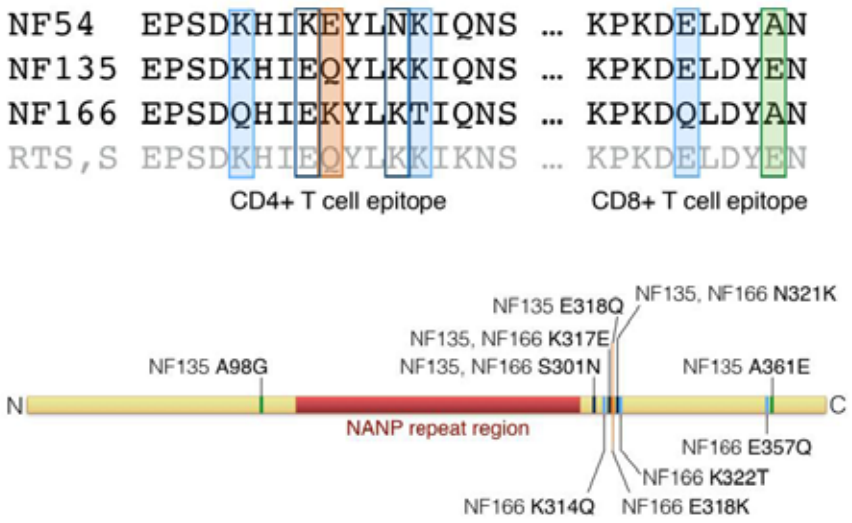
Supplementary Figures



Supplementary Figure 1: First-wave parasitemia after challenge.
Parasitemia on day 7 post challenge in immunized (open circles) and control (closed circles) volunteers. The line and error bars show the geometric mean and 95% CI interval.



Supplementary Figure 2: Inhibition of *in vitro* homologous and heterologous intra-hepatic sporozoite development in primary human hepatocytes by mAb 2A10.
P. falciparum NF54 (blue), NF135.C10 (orange) and NF166.C8 (green) sporozoites in the presence of 10% heat-inactivated naive human control serum were pre-incubated with 3-fold serial dilutions of the 2A10 monoclonal antibody (0.027-20 µg/ml), targeting the repeat region of the circumsporozoite protein (CSP), and added to primary human hepatocyte cultures. Six days post-infection, the number of *P. falciparum* infected hepatocytes was assessed as described in Supplementary information S4 and S5.



Supplementary Figure 3: Amino acid changes in CSP.

Supplementary Table 1: Whole-genome sequencing statistics.

Strain	Mio read pairs	Median coverage (excl. apicoplast and mitochondrial DNA)	% genome with min. 5x coverage	SNPs	Indels
NF54	3.5	20	94.7	–	–
NF135.C10	4.8	25	95.0	13,352	3863
NF166.C8	5.6	30	95.6	12,418	3566

Supplementary Table 2: Mosquito salivary gland infectivity and sporozoite load of the three clones. Mean mosquito salivary gland infectivity and sporozoite load determined one day prior to challenge infection by dissecting a sample of 10 mosquitoes per strain.

	No. volunteers	Sporozoite load	Percent infectivity
Cohort 1			
NF54	4	26,500	90
NF135.C10	8	18,000	100
NF166.C8	6	59,500	100
Cohort 2			
NF54	6	44,300	100
NF135.C10	7	23,500	80
NF166.C8	8	17,000	90

Supplementary information:

Protocol: malaria antigen-specific IgG ELISA (S1)

Levels of malaria antigen-specific antibodies in plasma were determined by a standardized enzyme-linked immunosorbent assay (ELISA) and expressed as arbitrary units (AU) in relation to a pool of 100 sera from adults living in an area in Tanzania where malaria is highly endemic [12].

Polystyrene flat-bottom 96 well plates (ThermoScientific, NUNC™ Maxisorp, 439454) were coated overnight at 4 °C with 0.5 µg/ml circumsporozoite protein (CSP, Genova) in PBS. Subsequently, plates were washed four times with PBS, blocked with 5% milk in PBS for 1 h at room temperature and again washed four times with PBS. Citrated plasma samples were diluted 1:50 in PBS containing 0.05% Tween20 and 1% milk (PBST/1% milk), analyzed in duplicate and a three-point 1:3 dilution series was carried out for each plasma sample. Both time points from each volunteer were measured on the same plate. As a standard and positive control, a plasma pool obtained from 100 Tanzanian hyper-immune adults living in a highly malaria-endemic area (HIT) was diluted 1:200 in PBST/1% milk and included on every plate in a seven-point 1:2 dilution series in duplicate. After 3h incubation with plasma samples at room temperature, plates were washed four times with PBST and then four times with PBS. Bound malaria antigen-specific IgG antibodies were detected by incubation with a 1:40,000 dilution of horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-human IgG antibody (P0214, Dako Denmark) for 1h at room temperature, followed by washing four times with PBST and PBS. Plates were developed with HRP substrate (tetra-methyl-benzidine, tetu-bio laboratories) at room temperature. The reaction was stopped using 0.2M H_2SO_4 after 13 minutes. Spectrophotometrical absorbance at 450 nm was measured using the iMark Microplate Absorbance Reader (Bio-Rad). ELISA data analysis was performed with Auditable Data Analysis and Management System for ELISA (ADAMSEL, version 1.1) as previously described [17]. Antibody levels were calculated in relation to the positive control (HIT plasma pool), which was set at 100 arbitrary units (AU).

Protocol: Isolation and cultivation of primary human hepatocytes (S2)

Fresh primary human hepatocytes were isolated from patients undergoing partial hepatectomy as described. Remnant liver tissue, varying 1-5cm^3 in size, was transferred into a 1000 ml sterile glass containing 100 ml UW (Bristol-Meyers-Squibb, 1148162) immediately after isolation from the patient and transported to the laboratory. The liver tissue was put on sterile bandage gauze and perfused via any vessel (venous or portal vein) with 500 ml of oxygenized HBSS medium (Gibco, 14170-088) supplemented with 10mM HEPES (Gibco, 15630-056) and 0.64 mM EDTA (Invitrogen, 15575-038) to inactivate proteases. Subsequently, the liver tissue was perfused with 500 ml of

oxygenized HBSS medium supplemented with 10mM HEPES, followed by perfusion with 100 ml of oxygenized HBSS medium supplemented with 10mM HEPES, 0.75 mg/ml $CaCl_2$ and low concentrations of collagenase (3,333 units per 50ml). Next, the liver tissue was perfused with 100 ml of oxygenized HBSS medium supplemented with 10mM HEPES, 0.75 mg/ml $CaCl_2$ and high concentrations of collagenase (13,333 units per 50ml). This buffer was re-used for perfusion for maximum 20 minutes until the liver tissue became very soft. Subsequently, the liver tissue was transferred into a petri dish containing 40 ml cold DMEM medium (Gibco, 31885-023) supplemented with 10% FBS (Gibco, 10270) to inactivate collagenase activity. The liver tissue was cut into small pieces and clouds of hepatocytes flowing into the medium could be observed. Medium containing hepatocytes was transferred into a 50ml Falcon tube over a 100 µm cell strainer (Falcon, 352360) and meanwhile, another 40 ml of cold DMEM supplemented with 10% FBS was added to the small liver pieces. Medium was again collected and run over a 100 µm cell strainer. Subsequently, primary human hepatocytes were centrifuged at 10g with low brake for 5 minutes at 4 °C. Hepatocyte pellets were washed in cold DMEM without serum and again centrifuged at 10g with low brake for 5 minutes at 4 °C. This step was repeated until the supernatant looked clear. Subsequently, cells were resuspended in 30 ml complete William's B medium, consisting of William's E medium with Glutamax (Gibco, 32551-087) supplemented with 10% heat-inactivated human serum, 1% insulin/transferrin/selenium (Gibco, 41400-045), 1% sodium pyruvate (Gibco, 11360-036), 1% MEM-NEAA (Gibco, 1140-035), 1% Fungizone Antimycotic (Gibco, 15290-018), 2% penicillin/streptomycin (Gibco, 15140-122) and 1.6 µM dexamethasone, and 15 ml each was carefully stacked upon 25ml of 25% Percoll in 50 ml Falcon tubes. Tubes were centrifuged at 2,000 RPM without brake for 2 minutes at 4 °C. Supernatant containing dead cells was carefully removed and the pellet was resuspended in 5-10 ml complete William's B medium and counted at a 1:2 dilution in Trypan blue. Viable primary human hepatocytes were diluted to a final concentration of $0.5x10^6$ hepatocytes/ml in complete William's B medium and 5.10^4 primary human hepatocytes were seeded into each well in black 96 well plates (Falcon, 353219) coated with 0.056 mg/ml rat tail collagen I/well (Roche Applied Science, 11179179-001). Hepatocytes were cultured at 37 °C in an atmosphere of 5% CO_2, medium was refreshed the next morning (100 µl/well complete William's B medium) and then every two days.

Protocol: *In vitro* sporozoite infectivity assay of primary human hepatocytes (S3)

Two to three days following seeding of primary human hepatocytes into 96 well plates, the neutralizing activity of CPS-induced antibodies on *in vitro* sporozoite invasion was assessed in *in vitro* sporozoite infectivity assays with primary human hepatocytes.

P. falciparum NF54, NF135.C10 or NF166.C8 sporozoites were pre-incubated with 10% heat-inactivated naive control human serum and 10% heat-inactivated plasma from CPS-immunized volunteers (either Pre- or Post-immunization plasma) for 30 minutes on ice. Sporozoites pre-incubated with 20% heat-inactivated non-immune human serum in the presence or absence of 10 µg/ml of monoclonal anti-CSP antibody [41] served as a positive and standard control, respectively.

$5{\cdot}10^4$ sporozoites were added to 96 well plates containing monolayers of $5{\cdot}10^4$ primary human hepatocytes in triplicates, centrifuged at 3,000 RPM for 10 minutes at RT with a low brake (Eppendorf Centrifuge 5810 R) and incubated for 3 hours at 37 °C in 5% CO_2. After incubation, medium containing sporozoites was gently removed to remove non-invaded sporozoites and subsequently, 100 µl/well complete William's B medium was added to each well. Plates were kept at 37°C in 5% CO_2 and medium of primary human hepatocytes was refreshed every two days. Five to six days after infection of primary human hepatocytes with *P. falciparum* sporozoites, wells were gently washed three times with PBS and fixed with 4% paraformaldehyde for 15-20 minutes at room temperature. After fixation, wells were gently washed three times with PBS and 200 µl PBS was added to each well. Plates were stored at 4 °C in the dark until immuno-fluorescent staining of infected primary human hepatocytes. Investigators carrying out the *in vitro* sporozoite infectivity assay of primary human hepatocytes were blinded to the volunteer identification codes and their respective outcomes during immunization and following challenge infection.

Protocol: Immunofluorescent analysis of *P. falciparum*-infected primary human hepatocytes (S4)

The number of primary human hepatocytes harbouring *P. falciparum* parasites was assessed by indirect immuno-fluorescence analysis. Firstly, aldehyde groups in hepatocyte cultures were blocked with 100 µl/well 0·1M glycine for 10 minutes at room temperature to prevent autofluorescence and hepatocytes were gently washed three times with 200 µl/well PBS. Subsequently, non-specific protein binding sites in the wells were blocked with 100 µl/well 10% FBS in PBS for 20 minutes at room temperature and wells were gently washed three times with 200 µl/well PBS. Non-invaded or adhering sporozoites were stained with 50 µl/well of an anti-CSP antibody conjugated with FITC (1:50 diluted in 10% FBS in PBS) for 2 hours at room temperature in the dark. Hepatocytes were gently washed three times with 200 µl/well PBS and subsequently permeabilized for 5 minutes at room temperature with 1% Triton X-100 (Sigma T9284, diluted in PBS). Primary human hepatocytes infected with viable parasites were stained intracellularly with 50 µl/well of anti-*P. falciparum* Hsp-70 rabbit polyclonal antibody (StressMarQ BioSciences, SPC-186D; 1:75 dilution in 10% FBS in PBS) for 2 hours at room temperature in

the dark. Hepatocytes were gently washed three times with 200 µl/well PBS and subsequently stained with a secondary antibody and a nuclei stain (Alexa Fluor 594 goat-anti-rabbit antibody and DAPI nuclei stain, 1:200 and 1:100 diluted in 10% FBS in PBS, respectively). Hepatocytes were gently washed three times with 200 µl/well PBS and finally, 200 µl PBS/well was added to each well. Plates were stored at 4 °C in the dark until fluorescent microscopic analysis.

Overview images of each well were captured using a Leica DMI6000B inverted microscope and adjusted for brightness and/or contrast using the ImageJ 1.48v program (NIH, USA). Channels (red, green and blue) were split and a macro that was made for automatically counting of NF54, NF135 or NF166 parasites in primary human hepatocytes, was run on the picture from the red channel. To check the sensitivity of the macro, selected wells were also manually counted for the presence of parasites using the ImageJ 1.48v program (NIH, USA). Differences between automatical and manual counts of no more than 10% were accepted.

Protocol: *In vitro* PBMC restimulation with *Pf*RBCs and flow cytometry staining (S5)

Pre- and post-immunization PBMCs from CPS-immunized volunteers whom received NF135.C10 and NF166.C8-challenge infection were restimulated *in vitro* with cryopreserved *P. falciparum* NF54-infected erythrocytes (*Pf*RBCs) as described previously [11, 14]. PBMCs were thawed and washed twice with Dutch Modified RPMI 1640 (Gibco) and counted in 0·1% Trypan blue with 5% Zap-o-Globin II Lytic Reagent (Beckman Coulter) to assess cell viability. Cells were resuspended in complete culture medium (Dutch Modified RPMI 1640 containing 2 mM glutamine, 1mM pyruvate and 0·05 mM gentamycin and 10% human A+ serum (Sanquin, Nijmegen) at a final concentration of $10*10^6$cells/ml. $0·5*10^6$ PBMCs/well were transferred into 96-well round-bottom plates and stimulated *in vitro* in duplicate with either 10^6 cryopreserved NF54 *Pf*RBCs or 10^6 uninfected erythrocytes (uRBC) for 24h at 37 °C with 5% CO_2. During the last four hours, 10 µg/ml Brefeldin A (Sigma-Aldrich) and 2 µM monensin (eBioscience) were added, and 10 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml ionomycin (Sigma-Aldrich) were added to positive control wells. After 24 hours of stimulation in total, cells were harvested and stained. PBMCs were first stained with Live/Dead fixable dead cell stain dye eF780 (eBioscience) and after washing, cells were stained with antibodies against the surface markers CD3 ECD (Beckman Coulter; clone UCHT1), CD4 V500 (BD Horizon; clone RPA-T4), CD8 AF700 (BioLegend; clone HIT8A), gamma delta T cell receptor PE (Beckman Coulter; clone IMMU510) and CD56 PerCP/Cy5·5 (BioLegend; clone HCD56). Cells were washed again, fixed in Foxp3 fixation/permeabilization buffer (eBioscience) and stained with antibodies against the intracellular cytotoxic marker granzyme B FITC (BioLegend; clone GB11) and the cytokine IFNα PECy7 (BioLegend;

clone 4S.B3). After washing cells in permeabilization buffer, samples were kept cold and dark in 1% paraformaldehyde (PFA) in PBS until measured by flow cytometry on the same day. Both time points each volunteer were thawed, stimulated and stained within the same experimental round. Samples were acquired using a 10-colour Gallios flow cytometer (Beckman Coulter), and single stained cells were run every round for compensation. Data analysis was performed using FlowJo software (Version 10·0·8, Tree Star). uRBC responses were substracted from *Pf*RBC-specific responses for every volunteer on every time point, and post-immunization responses were corrected for pre-immunization responses.

PART 2

Innate immune memory in malaria

CHAPTER 6

Activatory receptor NKp30 predicts NK cell activation during Controlled Human Malaria Infection

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Jona Walk and Robert W. Sauerwein

Abstract

Natural killer (NK) cells are known to be activated during malaria infection, exhibiting both cytokine production and cytotoxic functions. However, NK cells are heterogeneous in their expression of surface activatory and inhibitory receptors which may influence their response to malaria parasites. Here, we studied the surface marker profile and activation dynamics of NK cells during a Controlled Human Malaria Infection in 12 healthy volunteers. Although there was significant inter-patient variability in timing and magnitude of NK cell activation, we found a consistent and strong increase in expression of the activatory receptor NKp30. Moreover, high baseline NKp30 expression was associated with NK cell activation at lower parasite densities. Our data suggest that NKp30 expression may influence the NK cell response to *P. falciparum*, explaining inter-patient heterogeneity and suggesting a functional role for this receptor in malaria.

Introduction

Malaria infection in humans activates a broad cellular immune response involving monocytes, T cells, B cells and NK cells. NK cells may play a functional role in protection against *Plasmodium falciparum*, as certain NK cell receptor genotypes are associated with decreases in malaria susceptibility and pathology (reviewed in [1]). During the pathological blood stage of *P. falciparum* infection, circulating NK cells display a dual functional role, i.e. cytokine production [2-5] and killing of infected blood cells both via antibody-independent [6-8] and antibody-dependent cytotoxicity [9, 10]. Their relative contribution to protection remains unknown.

NK cells are often considered a homogenous, unchanging population, but multicolored flow cytometry and mass cytometry have revealed that NK cells actually consist of many distinct populations, differing in their functionality against specific diseases [11-14]. Artavanis-Tsakonas et al. previously demonstrated that in malaria naïve donors a specific subpopulation of NK cells expressing the lectin-type receptor NKG2A are the main IFN-γproducers in response to *P. falciparum*-infected RBC [15]. Most studies determining the NK cell response against *P. falciparum* demonstrate that there is large inter-donor variability [16, 17]. We hypothesized that this heterogeneity might at least in part be explained by differences in NK cell phenotype prior to infection.

To date most data on responsiveness of NK cells to *P. falciparum* has been obtained from *ex vivo* stimulation experiments or case-control studies in endemic areas. We took advantage of the Controlled Human Malaria Infection model to evaluate the activation and function of different NK cell subsets at multiple time points during a malaria infection. Our data show *in vivo* NK cell activation in all donors with an upregulation of IFN-γ and granzyme B production. There was indeed a significant variability both in the timing and magnitude of the NK cell response, and increased baseline receptor expression of NKp30 predicted a more rapid *in vivo* NK cell activation.

Materials and Methods

Clinical trials

Study 1 was a single-center, open-label clinical trial in 12 malaria naïve individuals conducted at the Radboud university medical center (Nijmegen, The Netherlands) from May until June 2018. Study volunteers provided written informed consent and were screened as described previously [18]. The trial was approved by the Central Committee on Research Involving Human Subjects (CCMO; NL63552.091.17) of the Netherlands, performed according to the Declaration of Helsinki and Good Clinical Practice and prospectively registered at ClinicalTrials.gov (NCT03454048). Volunteers were infected by the bites of five *P. falciparum* 3D7 strain-infected *Anopheles* mosquitoes, and followed up for parasitemia twice daily starting on day 6 post infection. Parasitemia was assessed by thick blood smear and qPCR. Volunteers were treated with a sub-optimal dose of piperaquine when parasitemia reached density detectable by thick blood smear or 5000 parasites/milliliter by qPCR, and received curative treatment if recrudescent parasitemia occurred.

Study 2 was a single-center randomized placebo controlled malaria vaccine trial (CCMO NL39541.091.12; NCT01728701) published previously [19]. Only study subjects that received placebo vaccination followed by CHMI were included in the current analysis. In short, volunteers received bites from five *P. falciparum* NF54 strain-infected *Anopheles* mosquitoes, and were followed up for parasitemia twice daily starting on day 5 post infection. Parasitemia was assessed by thick blood smear and/or qPCR, and volunteers received curative treatment with atovaquone/proguanil, either when parasitemia reached levels detectable by microscopy (n=5) or after two consecutive qPCRs >500 parasites/milliliter (n=4).

Whole blood NK cell phenotyping

In study 1, 100µL fresh EDTA blood was stained directly with a pre-prepared and antibody mixture containing: CD3-AlexaFluor700 (Biolegend; clone OKT3), pan-γδTCR-PE (Beckman Coulter; clone IMMU510), CD56-Brilliant Violet(BV)421 (Biolegend; clone HCD56), CD16-APC-eFluor780 (eBiosciences; clone CB16), CD69-PerCP-Cy5.5 (Biolegend; clone FN50), NKp30-APC (Biolegend; clone P30-15), NKG2D-Brilliant Violet(BV)510 (Biolegend; clone 1D11), NKG2A-PEVio770 (Miltenyi Biotec; clone REA110), and CD57-FITC (Biolegend; clone HCD57). A single mixture was prepared one day before the first time point, aliquotted per time point and stored in the dark until use. Samples were stained at 4°C in the dark for 30 minutes, followed by erythrocyte lysis with 1mL FACS Lysis buffer (BD Biosciences) for exactly 5 minutes. Samples were centrifuged and then washed with 0.5% Bovine Serum Albumin (BSA) in PBS. Cell pellets were resuspended in 100µL 1% paraformaldehyde (PFA) and analyzed on a

Gallios flow cytometer (Beckman Coulter). At each time point, staining and fixation was completed within 4 hours of blood draw and flow cytometry was performed the same day using identical acquisition settings and a standardized protocol. CD69 was used as a marker for lymphocyte activation after CHMI, as described earlier [20, 21].

PBMC isolation and cryopreservation

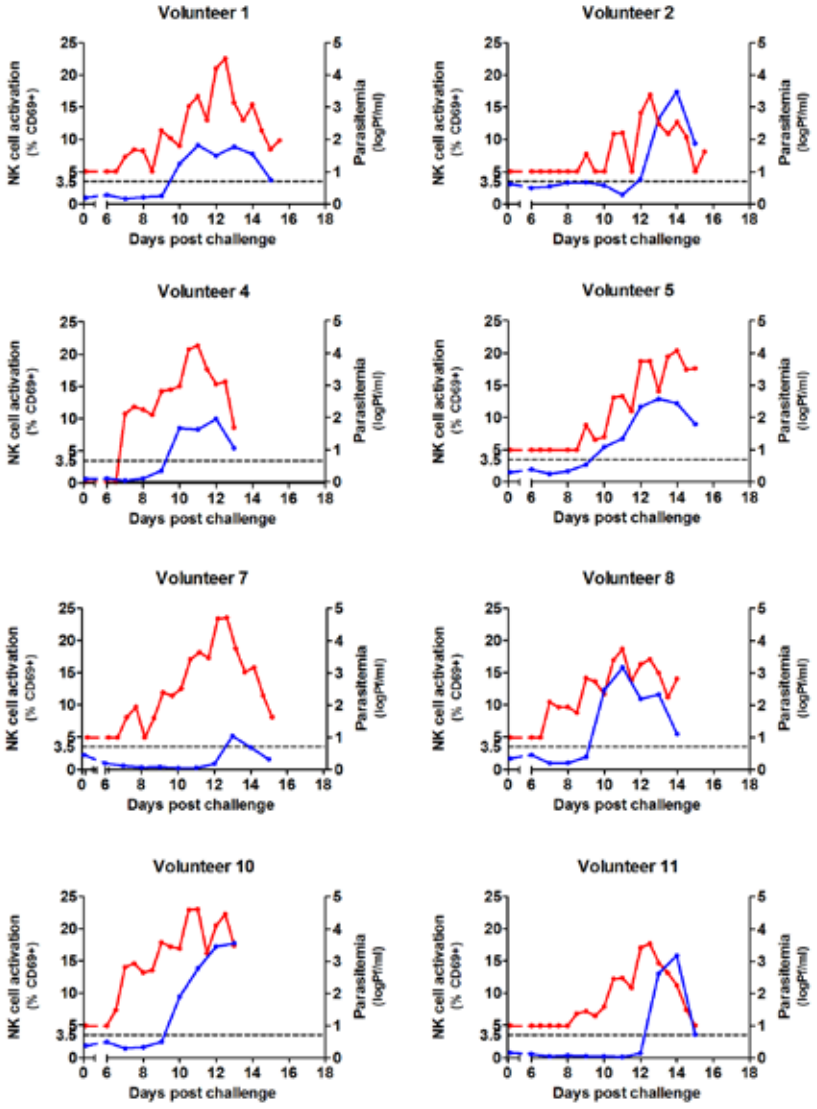
In study 2, blood samples for peripheral blood mononuclear cell (PBMC) isolation were taken pre-challenge, 3 days after antimalarial treatment and 35 days after challenge infection. Isolation and cryopreservation was performed as described previously [22]. In short, PBMCs were isolated from citrate anti-coagulated blood using vacutainer cell preparation tubes (CPT; BD Diagnostics) by density gradient centrifugation. Cells were washed four times in ice-cold phosphate buffered saline (PBS), counted using 0·1% Trypan blue with 5% Zap-o-Globin II Lytic Reagent (Beckman Coulter), cryopreserved at a concentration of 10×10^6 cells/ml in ice-cold fetal calf serum (Gibco)/10% DMSO (Merck), and stored in vapor-phase nitrogen.

PBMC thawing and re-stimulation

Immediately prior to use, cells were thawed and washed twice in Dutch-modified RPMI 1640 (Gibco/Invitrogen). Cell viability was assessed by counting in 0·1% Trypan blue with 5% Zap-o-Globin II Lytic Reagent (Beckman Coulter) to assess cell viability. PBMCs were cultured at $2.5x10^6$ cells/ml in RPMI 1640 (Dutch Modification; Gibco) with 5mg/ml gentamycin (Centraform), 100mM pyruvate (Gibco), 200mM glutamax (Gibco), supplemented with 10% heat-inactivated pooled human A+ serum (obtained from Sanquin Bloodbank, Nijmegen, The Netherlands) at a final volume of 200µL in 96-wells plates. Cells were stimulated with purified *Plasmodium falciparum* NF54 schizonts or uninfected red blood cells at a concentration of $5x10^6$ RBC/ml. After 3 hours, Brefeldin A (10µg/mL; Sigma-Aldrich) and monansin (2µM; eBioscience) were added to culture. After another 3 hours (6 hours total stimulation) cells from two stimulation replicates ($1.0x10^6$ cells total) were combined, washed and stained with Fixable Viability Stain 700 (BD Biosciences) for 30 minutes. After washing with PBS, cells were stained with extracellular antibodies, CD3-AlexaFluor700 (Biolegend; clone OKT3), CD56-PE (Biolegend; clone HCD56), CD16-APC-eFluor780 (eBiosciences; clone CB16), NKG2A-PEVio770 (Miltenyi Biotec; clone REA110) and CD57-APC (Biolegend; clone HCD57) for 30 minutes at 4°C in the dark. Cells were washed and fixed with Foxp3 fixation/permeabilization buffer (eBioscience). After washing with permeabilization buffer (eBioscience) cells were stained for intracellular cytokines with IFN-γ-PE-Dazzle (Biolegend; clone 4S.B3) and granzyme B-FITC (Biolegend; clone GB11). After another wash with permeabilization buffer, cells from two staining replicates ($2.0x10^6$ cells total) were taken up in 200µL 1% paraformaldehyde (PFA) and analyzed on a Gallios flow cytometer (Beckman Coulter) the next day.

Data analysis and statistics

Flow cytometry data was analyzed using Flow Jo software (version 10.0.8 for Apple OS). Statistical analysis was performed using GraphPad Prism (version 5.03 for Windows). Gating strategy and representative plots are shown in supplementary figure 4 (whole blood) and supplementary figure 5 (PBMCs).



Results

Heterogeneity in NK cell activation after CHMI

After malaria infection, NK cell activation as defined by upregulated CD69 expression was determined daily from day six post-infection until three days after antimalarial treatment (supplementary figure 1). In study #1 the first activation of NK cells in a number of volunteers was observed one day after the first appearance of parasitemia detectable by qPCR (figure 1A).

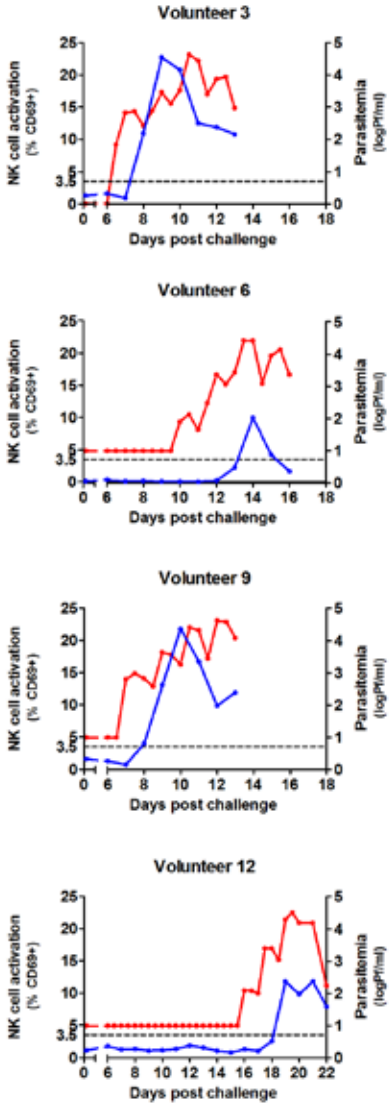


Figure 1: Kinetics of parasitemia and NK cell activation during Controlled Human Malaria Infection. NK cells were analyzed by flow cytometry daily in whole venous blood from 12 volunteers undergoing Controlled Human Malaria Infection. Antimalarial treatment was initiated when parasite densities reached levels detectable by microscopy. Each graph shows the activation of NK cells (defined by CD69 surface expression) from day 6 post infection until day 3 after antimalarial treatment (blue line, left axis). The same graph shows parasitemia measured by qPCR from day 6 after infection, until day 3 after antimalarial treatment (red line, right axis). NK cell activation is first seen one to two days after the first appearance of parasitemia. Each graph represents the data gathered for a single volunteer (n=12).

In the absence of parasitemia, up to 3.5% of NK cells expressed CD69, therefore >3.5% CD69 expression was considered significant NK cell activation above background (figure 2A). There was indeed a significant heterogeneity in the timing of first NK cell activation, ranging from 1 day after the first appearance of parasitemia (i.e. volunteer 5) to 5 days after parasitemia (volunteer 7). This may be partially explained by differences in starting parasite density. Parasitemia (prior to the initiation of antimalarial treatment) correlated strongly with the degree of NK cell activation (Spearman p=0.0017; figure 2A). However, this does not explain the diversity entirely, as some volunteers have significant NK cell activation (defined as CD69 expression >3.5%) at very low circulating parasitemia, such as volunteer 5, while others require very high parasitemia before NK cells become activated, such as volunteer 7. This circulating parasite density prior to NK cell activation was highly variable between volunteers (mean 4,798 Pf/ml, range 25-26,152 Pf/ml), suggestive for a host-dependent factor.

Baseline NKp30 expression predicts activation after CHMI

NK cell activation is dependent on a delicate balance between activatory- and inhibitory receptors, and the expressed receptor profile may relate to the observed heterogeneity during CHMI. Therefore, we next determined whether the expression of activatory receptors NKp30 or NKG2D, the inhibitory receptor NKG2A or the differentiation marker CD57 predicted an individual's response to CHMI. Indeed, higher baseline NK cell NKp30 expression correlated with activation at lower parasitemia (linear regression p=0.047; figure 2B). NKp30 and NKG2D were expressed on nearly 100% of NK cells for all volunteers (supplementary figure 1).

NKp30 was strongly upregulated during CHMI (pre-challenge vs. day of antimalarial treatment: mean MFI 3,145 vs. 4,913, Wilcoxon matched-pairs signed rank test p=0.0010; figure 2C), while the upregulation of NKG2D was marginal (pre-challenge vs. day of antimalarial treatment: mean MFI 5,268 vs. 5,916, Wilcoxon matched-pairs signed rank test p=0.043; figure 2C). The increase in NKp30 expression was proportional to total NK cell activation at antimalarial treatment (linear regression p=0.041; figure 2D).

NK cells can be divided into distinct populations representing levels of differentiation based on their expression of CD56, CD16, NKG2A and CD57 [12], and a previous study suggested NKG2A+ NK cells are more responsive to *P. falciparum in vitro* [15]. We sought to determine whether this may result from differential expression of NKp30. However, while baseline expression of NKp30 varied between CD56dimNKG2A+ and CD56dimNKG2A- subsets (figure 3A; supplementary figure 2), all NK cell subsets showed an upregulation of NKp30 (figure 3A). Furthermore, we did not see any differences in activation as defined by CD69 upregulation between the CD56dim subsets,

though there was significantly more activation of the CD56dim subset compared to the CD56bright subset (figure 3B-C).

NK cell subsets upregulate CD69, IFN-γ and granzyme B during CHMI

As there appears to be little activation of the CD56bright NK cell subset during the course of infection, we wanted to determine the ability of both the CD56brightCD16- and CD56dimCD16+ subsets to produce granzyme B and IFN-γ and degranulate during infection, using isolated and cryopreserved peripheral blood mononuclear cells (PBMCs) from study #2 (supplementary figure 3). We found that both subsets increase production of granzyme B and IFN-γ and show improved degranulation during infection (figure 4A-C; supplementary figure 4).

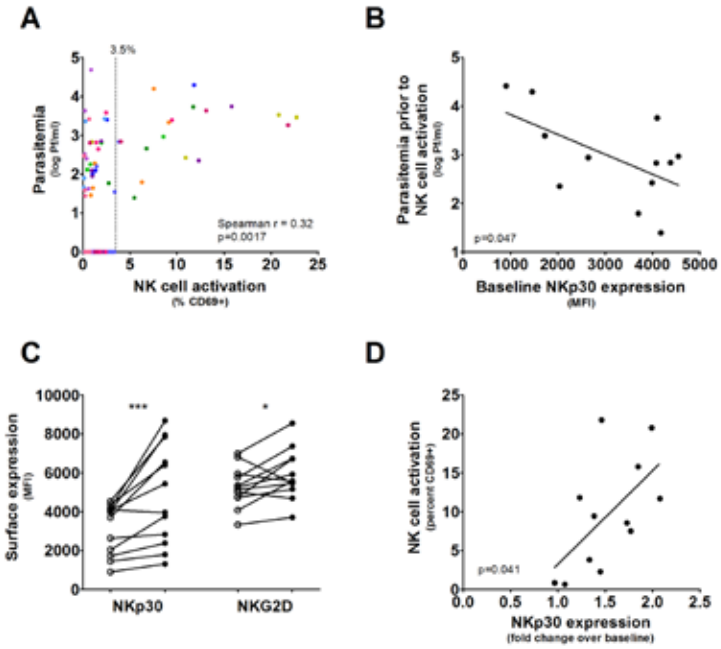


Figure 2: NKp30 predicts rapid NK cell activation during CHMI. (A) The graph shows NK cell activation correlated with parasitemia in all post-challenge but pre-treatment blood samples, where each color represents the samples from an individual volunteer. Blood samples without parasites showed up to 3.5% NK cell CD69 expression. **(B)** For each volunteer (n=12) the maximum parasitemia measured by qPCR in prior to or at the moment of first NK cell activation measured in whole blood was determined. Increased baseline NK cell NKp30 surface expression determined by Mean Fluorescent Intensity correlated with NK cell activation at lower parasitemia. Line and p-value are the result of a linear regression analysis. **(C)** Surface expression of the activatory receptors NKp30 and NKG2D determined on total NK cells for each volunteer prior to malaria infection (open circles) and on the day of antimalarial treatment (closed circles). P-values are the result of Wilcoxon matched-pairs signed rank test; * p<0.05; **p<0.01; ***p<0.001. **(D)** The fold change in NKp30 expression (determined by MFI) between measurements at baseline and antimalarial treatment for each volunteer were correlated to NK cell CD69 expression at on the day of antimalarial treatment. Line and p-value are the result of a linear regression analysis.

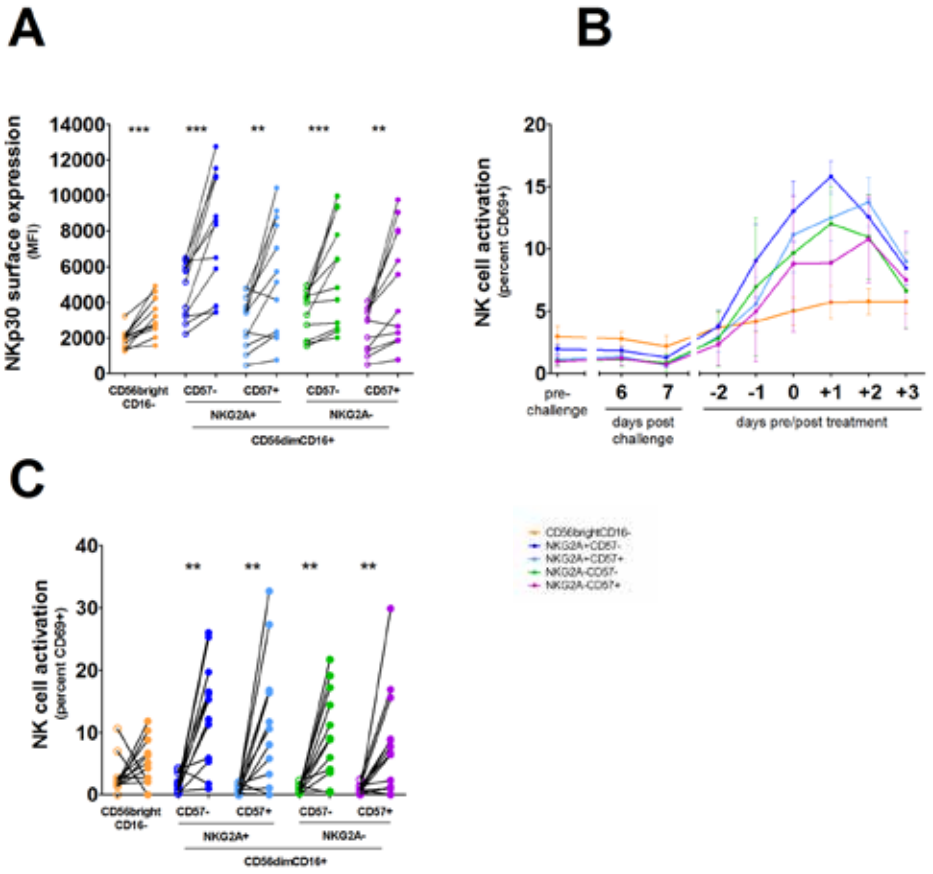


Figure 3: NKp30 expression and activation kinetics on NK cell subsets. NK cells were analyzed by daily flow cytometry in whole venous blood from 12 volunteers undergoing Controlled Human Malaria Infection. Antimalarial treatment was initiated when parasite densities reached levels detectable by microscopy. **(A)** Total NK cells were divided into five subpopulations based on their surface expression of CD56, CD16, NKG2A and CD57: $CD56^{bright}$CD16- (orange), $CD56^{dim}$CD16+NKG2A+CD57- (dark blue), $CD56^{dim}$CD16+NKG2A+CD57+ (light blue), $CD56^{dim}$CD16+NKG2A-CD57- (green), and $CD56^{dim}$CD16+NKG2A-CD57+ (purple). Surface expression of NKp30 for each NK cell subset prior to malaria infection (open circles) compared with NKp30 expression on each NK cell subset on the day of antimalarial treatment (closed circles). P-values are the result of Wilcoxon matched-pairs signed rank test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. (**B**) The graph shows the mean and error of NK cell CD69 surface expression on each subset per day in 12 volunteers. **(C)** Surface expression of CD69 was determined for each NK cell subset prior to malaria infection (open circles) and on the day of antimalarial treatment (closed circles). P-values are the result of Wilcoxon matched-pairs signed rank test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

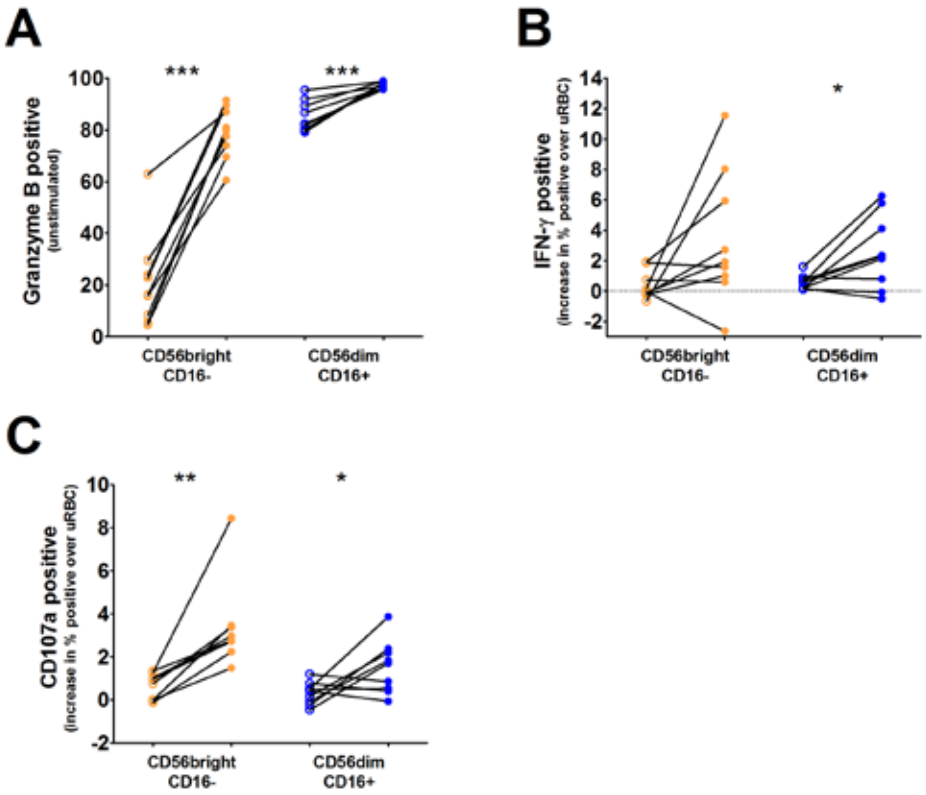


Figure 4: IFN-γ, granzyme B and degranulation responses per NK cell subset. Cryopreserved PBMCs from 9 volunteers taken before challenge (open circles) and three days after antimalarial treatment (closed circles) were thawed and stimulated for 6 hours with *Pf*-infected red blood cells (PfRBC) or uninfected RBC (uRBC). Total NK cells were divided into two subpopulations based on their surface expression of CD56 and CD16. **(A)** Intracellular granzyme B content (% cells positive) determined by flow cytometry in unstimulated NK cells (those incubated with uninfected RBC cultures) at both time points. **(B)** Intracellular IFN-γ production (% cells positive) in response to PfRBC stimulation at both time points, IFN-γ production after PfRBC stimulation was corrected for production in response to uRBC. **(C)** CD107a staining (% cells positive) in response to PfRBC stimulation at both time points, CD107a staining after PfRBC stimulation was corrected for production in response to uRBC. For all three graphs open circles are pre-infection time points and closed circles are day 3 post antimalarial treatment. P-values are the result of paired samples t-test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Discussion

These data show that NKp30 is a marker for the NK cell response during a Controlled Human Malaria Infection, and suggests a possible functional role in the response to infected red blood cells. We demonstrate that the expression of this receptor at baseline relates to individual NK cell responses to *P. falciparum in vivo*. Furthermore we show that NK cell activation during the course of infection is linked to an increase in NKp30 expression.

Both NKp30 and NKG2D have been shown to increase expression during NK cell activation [14, 23], however, during CHMI the magnitude of NKp30 upregulation of is particularly pronounced compared to NKG2D. It has previously been demonstrated *in vitro* that NKp30 binds to the *P. falciparum* protein *Pf*EMP1 leading to NK cell activation [24]. This supports our finding that NK cells with higher resting NKp30 expression are more sensitive to activation at lower parasitemia. However, it is important to note that other *in vitro* studies suggest that *Pf*EMP1 may be dispensable [25] and that MDA5 signaling may be essential [26] for NK cell activation in response to *Pf*RBC. Therefore multiple mechanisms may be involved in NK cell activation during malaria.

This is the first study with longitudinal daily samples from the initial phase of a malaria infection as parasites emerge from the liver that suggests an important role for NKp30. We thereby measured CD69 expression directly in patient blood samples, without re-stimulation, remaining close to the induced *in vivo* phenotype of an early natural infection. Furthermore, we show that baseline NKp30 expression is linked to a more rapid NK cell activation during subsequent infection.

Population based studies conducted in sub-Saharan Africa have identified a single nucleotide polymorphism (SNP) in the promoter for the NCR3 gene that encodes NKp30 that is associated with an increased number of clinical, uncomplicated malaria episodes in individuals over 5 years old [27-29]. The combined data are highly suggestive for a potential functional role of NKp30-mediated NK cells in malaria. In our study we do not detect differences in time to parasitemia, maximum parasitemia or parasite multiplication rate between those with high NKp30 expression and those with low NKp30 expression (data not shown). However, an important limitation of this study is that it was not designed to measure an effect on control of blood stage parasite replication. Even in volunteers with very rapid NK cell activation, this occurred only two days before the initiation of antimalarial treatment. This period between NK cell activation and drug treatment would be too short to measure an effect on parasite multiplication. Instead, cohort studies in endemic areas are better suited to answer this question. Future studies in endemic areas could determine NKp30 expression on NK cells at the beginning of a

malaria season and during follow-up visits, and correlate this with number of clinical malaria episodes.

Broad inter-donor variability in the activation of NK cells in response to *P. falciparum* has been described in multiple studies [16, 17, 21]. Our current finding suggest that baseline NK cell phenotype can play a role in this diversity. However, other immunological factors, including other activatory and inhibitory receptors not studied here, interactions with other immune cells and cytokine production likely also contribute to the NK cell response. Furthermore, parasitological factors, such as the initial starting parasitemia and parasite multiplication rate may also affect host response.

The phenotypic diversity of NK cells has been a topic of extensive study during the last decade [13, 14]. Since the first discovery of NK cell memory in murine CMV infection [30], specific NK cell phenotypes have been identified as the main responders in human EBV [11], CMV [31] and HIV infection [14, 32] as well. Similarly, studies suggested that NKG2A+ NK cells, specifically respond to *P. falciparum* [15, 16]. Interestingly, this does not appear to be the case during controlled human malaria infection *in vivo*.

Nevertheless, the finding that NKp30 expression predicts the response to CHMI, underscores the potential importance of NK cell phenotype in our susceptibility to disease. The diversity of the NK cell repertoire has been implicated in the risk of HIV acquisition [14, 33], and viral infections in turn have been shown to change its composition [34-37]. Our study suggests that NK cell phenotype affects the response to a *P. falciparum* infection.

The current study was limited to analysis of CD56, CD16, NKG2A, CD57, NKp30 and NKG2D. In contrast, data from studies on other diseases using cytometry by time-of-flight (CyTOF) have suggested there may be more than 100,000 NK cell phenotypes, each characterized by a distinct combination of surface receptors [13]. Furthermore the expression of diverse killer cell immunoglobulin-like receptors (KIRs) plays an important role in NK differentiation and function [12]. Therefore, it is likely that additional receptors, or combinations of inhibitory and activatory receptors, are also important for the interaction between NK cells and *P. falciparum* parasites. Future studies looking at a larger number of receptors and cytokines could unravel both these effects in more detail.

In conclusion, this study is the first to identify the expression the NK cell activatory receptor NKp30 as a marker that predicts a rapid NK cell response to parasitemia and suggest a potential role for this receptor in NK cell functionality against *P. falciparum*.

Acknowledgements

We would like to thank all the trial volunteers and the staff of the Radboud university medical center who were involved in the study, in particular Manon Alkema, Guido Bastiaens, Gerdie de Jong, Maurits van der Meer and Isaie Reuling for conducting the clinical trials. We also thank Marga van de Vegte-Bolmer, Wouter Graumans and Rianne Stoter for the generation and cryopreservation of the *P. falciparum*-infected red blood cells, and Anieck Lomans and Marleen Meij for their assistance in setting up the flow cytometry analyses.

Conflicts of Interest

The authors declare no conflicts of interests.

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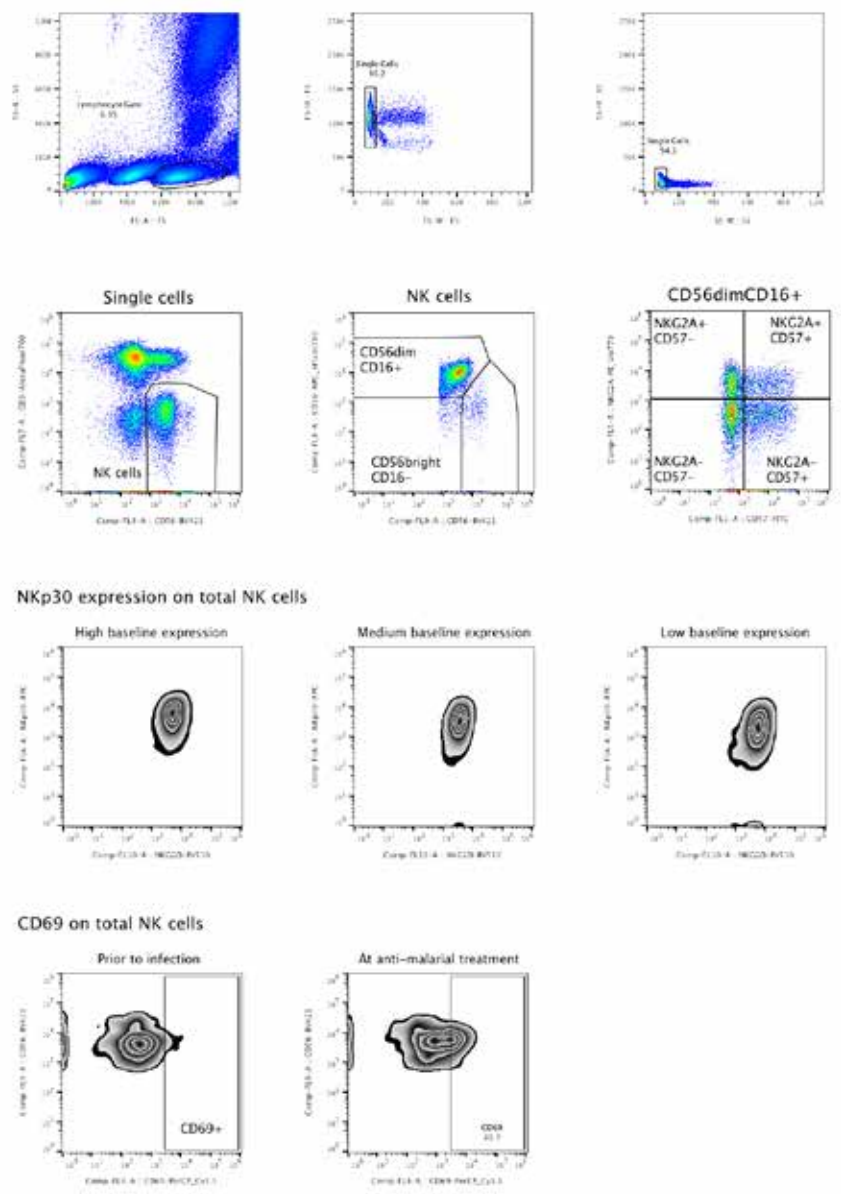
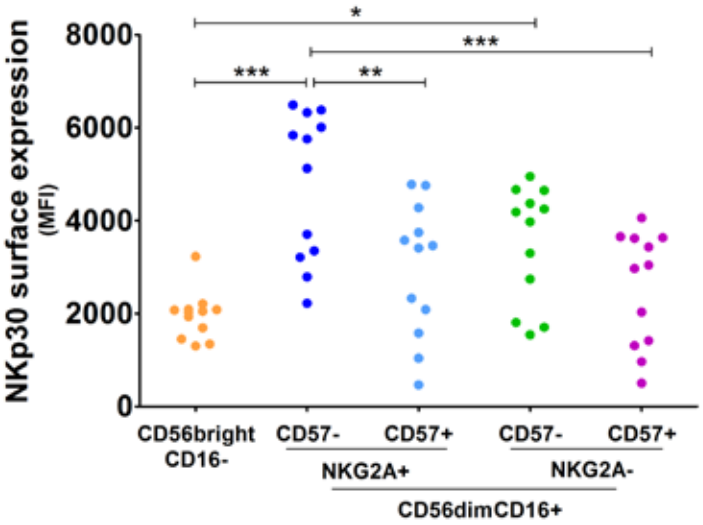
Supporting Information

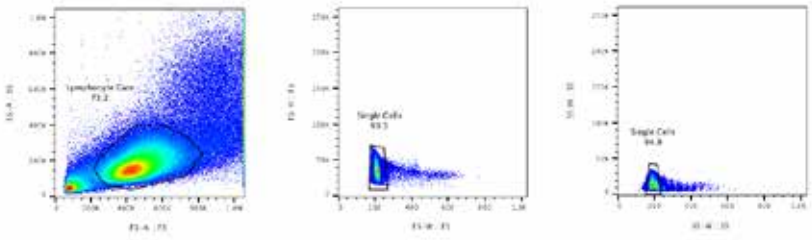


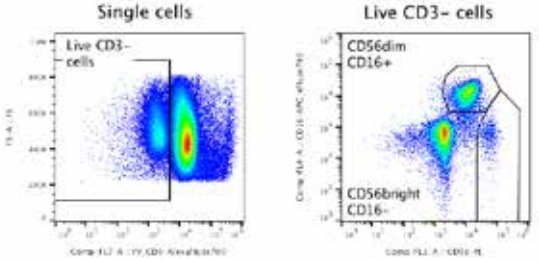
Supplementary figure 1: Gating strategy whole blood NK cell flow cytometry. Lymphocytes were gated from fresh whole blood based on forward- and side-scatter characteristics. Duplets were excluded from the analysis. NK cells were identifies as CD3-CD56+ cells, and further divided into CD56dimCD16+ and CD56brightCD16-. CD56dimCD16+ NK cells were further split into four populations based on NKG2A and CD57 expression. In all samples NKp30 was expressed on nearly all NK cells and quantified based on mean fluorescent intensity (MFI). CD69 was expressed on a subset of cells, only during parasitemia, and quantified as percent positive cells.



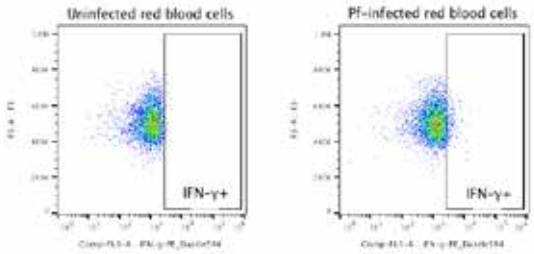
Supplementary figure 2: NKp30 expression on NK cell subsets at baseline. NK cells were analyzed by daily flow cytometry in whole venous blood from 12 volunteers undergoing Controlled Human Malaria Infection. Antimalarial treatment was initiated when parasite densities reached levels detectable by microscopy. Total NK cells were divided into five subpopulations based on their surface expression of CD56, CD16, NKG2A and CD57: CD56brightCD16- (orange), CD56dimCD16+NKG2A+CD57- (dark blue), CD56dimCD16+NKG2A+CD57+ (light blue), CD56dimCD16+NKG2A-CD57- (green), and CD56dimCD16+NKG2A-CD57+ (purple). Surface expression of NKp30 was determined for each NK cell subset prior to malaria infection at baseline. P-values are the result of Friedman test with Dunn's multiple comparison test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Supplementary figure 3: Gating strategy PBMC stimulation flow cytometry. Lymphocytes were gated from cryopreserved PBMCs based on forward- and side-scatter characteristics. Duplets were excluded from the analysis. Viability dye negative, CD3- cells were selected. From live CD3- cells NK cells were identified as either CD56dimCD16+ and CD56brightCD16-. Granzyme B was expressed on a subset of NK cells at all timepoints. The gate was defined using total live CD3- cells, as they show a clear negative and positive population, examples of this gate, and NK cell populations with a high- and low number of granzyme B positive cells are shown. IFN-γ and CD107a were expressed on a subset of cells only after stimulation and quantified as percent positive cells.

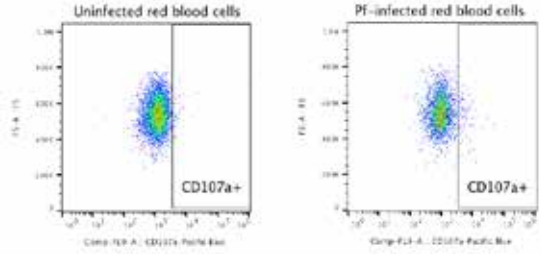




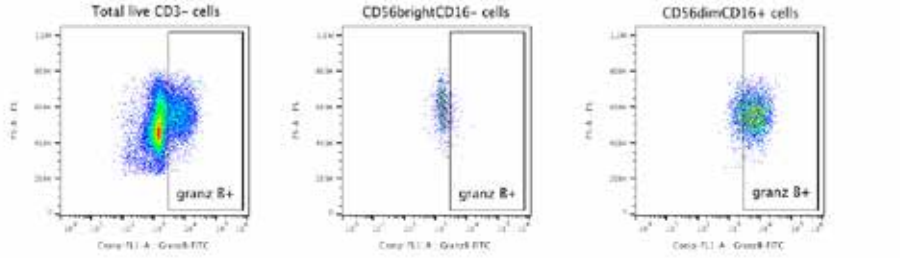
IFN-γ expression in CD56dimCD16+ NK cells

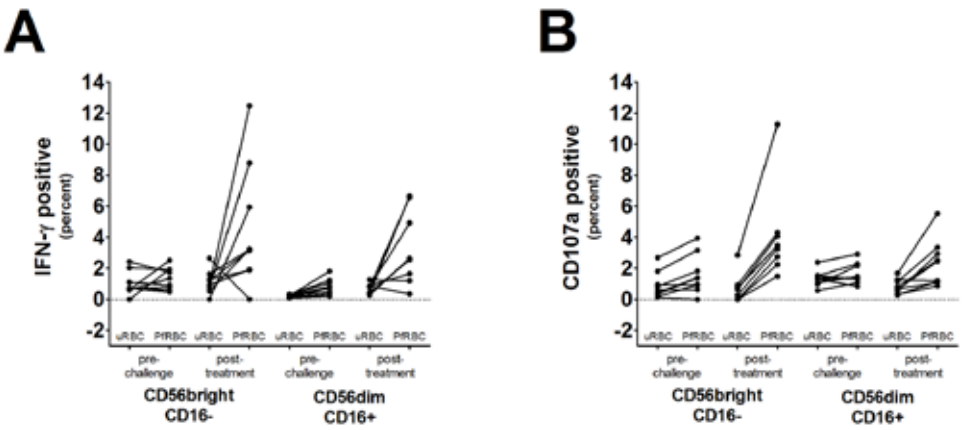


CD107a expression in CD56dimCD16+ NK cells



Granzyme B expression





Supplementary figure 4: IFN-γ production and degranulation in response to *P. falciparum.* Cryopreserved PBMCs from 9 volunteers taken before challenge and three days after antimalarial treatment were thawed and stimulated for 6 hours with *Pf*-infected red blood cells (PfRBC) or uninfected RBC (uRBC). Total NK cells were divided into two subpopulations based on their surface expression of CD56 and CD16. **(A)** Intracellular IFN-γ production (% cells positive) in response to PfRBC and uRBC stimulation at both time points **(B)** Degranulation defined as CD107a staining (% cells positive) after PfRBC and uRBC stimulation at both time points.


6

CHAPTER 7

Controlled Human Malaria Infection induces long-term functional changes in monocytes

Accepted for publication in *Frontiers in Molecular Biosciences*

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* contributed equally

Abstract

Innate immune memory responses (also termed '*trained immunity*') have been described in monocytes after BCG vaccination and after stimulation *in vitro* with microbial and endogenous ligands such as LPS, β-glucan, oxidized LDL, and monosodium urate crystals. Whether clinical infections are also capable of inducing a trained immunity phenotype has not been studied. We evaluated whether *Plasmodium falciparum* infection can induce innate immune memory by measuring monocyte-derived cytokine production from five volunteers undergoing Controlled Human Malaria Infection. Monocyte responses followed a biphasic pattern: during acute infection, monocytes produced lower amounts of inflammatory cytokines upon secondary stimulation, but 36 days after malaria infection they produced significantly more IL-6 and TNF-α in response to various stimuli. Furthermore, transcriptomic and epigenomic data analysis revealed a clear reprogramming of monocytes at both timepoints. *Plasmodium falciparum* challenge induced long-term changes of H3K4me3 at the promoter regions of inflammatory genes that remain present for several weeks after parasite clearance. These findings demonstrate an epigenetic basis of trained immunity induced *in vivo* by Controlled Human Malaria Infection.

Introduction

Throughout our life, the immune system continually encounters a wide array of microorganisms and other danger signals. Until recently, exposure to microbial ligands or infectious agents was thought to induce immune memory exclusively in T and B lymphocytes. However, long-lasting changes in the innate immune compartment have also been described after exposure to various microorganisms [1, 2]. Epigenetic reprogramming underlies these effects [3-5], and it can result in either decreased pro-inflammatory cytokine production (e.g. after LPS exposure), a process known as 'tolerance', or increased cytokine production seen after exposure to BCG [6], or β-glucan [5], known as 'trained immunity' (recently reviewed in [7]). Trained immunity is thought to underlie some clinical observations of heterologous protective effects after vaccination, including the reduction in all cause infant mortality after BCG and measles vaccination [8].

Acute malaria infection is also associated with activation of monocytes through TLR-dependent responses [9, 10]. Interestingly, it was demonstrated recently that *in vitro* exposure to *Plasmodium falciparum* or the parasite pigment haemozoin increased monocyte cytokine responses to restimulation with a TLR2 agonist [11], suggesting that *Plasmodium* may induce trained immunity. However, whether physiological concentrations of *P. falciparum* can induce trained immunity *in vivo* has not been explored. Here we determined monocyte responses in five healthy volunteers undergoing controlled human malaria infection [12], and demonstrate that even at very low parasite densities *P. falciparum* induces functional changes in monocytes lasting at least one month, suggestive of trained immunity. Transcriptomic and epigenomic analyses reveal that these changes occur on a genome-wide scale at both the RNA level and H3K4me3 histone marks.

Methods

Clinical Trial

Five healthy, malaria-naive male and female volunteers were screened as described previously [13] and enrolled as controls in an open-label vaccine trial (registered under NCT02080026). The trial was approved by the Central Committee on Research Involving Human Subjects (CCMO NL48301.091.14) of the Netherlands. Volunteers were infected at the Radboud university medical center (Nijmegen, The Netherlands) in May 2015 by bites from 5 *P. falciparum* NF54-strain infected *Anopheles stephensi* mosquitoes according to established protocols [14]. Parasitemia was assessed by

prospective, daily qPCR from day 6 until day 21 post infection, and treatment was initiated when parasitemia reached 100 parasites/milliliter, as reported previously [15].

Isolation and stimulation of PBMCs and monocytes

Blood was collected in ethylene-diamine-tetra-acetic acid (EDTA) vacutainers (Becton Dickinson). Peripheral blood mononuclear cell and monocyte isolation and stimulation was performed as described previously [1]. In short, PBMCs were separated using Ficoll-Paque (GE healthcare, UK) density centrifugation and monocytes were isolated using CD14+ selection magnetic separation (MACS; Miltenyi Biotech). For culture, cells were suspended in RPMI (Roswell Park Memorial Institute medium; Invitrogen) supplemented with 50 µg/mL gentamicin (Centrafarm), 2 mM Glutamax (GIBCO), and 1 mM pyruvate (GIBCO). PBMCs were cultured at $2.5x10^6$/mL and monocytes at $0.25x10^6$/mL in a final volume of 200µL/well in a 96-well plate. Cells were stimulated with RPMI, sonicated *Mycobacterium tuberculosis* H37Rv (5 µg/mL), heat-killed *Candida albicans* (1×10^6/mL, strain UC820) or *Staphylococcus aureus* (1×10^6/mL clinical isolate), *Salmonella typhymurium* ($1x10^6$/mL, clinical isolate), *Escherichia coli* LPS (10 ng/mL, serotype 055:B5, Sigma-Aldrich), or phytohemagglutinin (PHA, 10 ng/mL, Sigma-Aldrich) for 24 hours or 7 days.

In vitro monocyte training

Cells were isolated from buffy coats from healthy adult donors (n=6), obtained from Sanquin Bloodbank (Nijmegen, The Netherlands). Experiments were performed using previously published protocols [16]. In short, from total PBMCs, monocytes were isolated using a high-density hyper-osmotic Percoll gradient. Monocytes were seeded at 0.1x10^6 cells/well in flat-bottom 96-well plates and allowed to adhere for 1 hour. Non-adherent cells were washed away with PBS, and monocytes were incubated with schizont and trophozoïte-stage *P. falciparum* NF54-strain-infected red blood cells or uninfected red blood cells for 24 hours. After washing, monocytes were allowed to rest in medium (see above) for 5 days. After 5 days they were restimulated with *Escherichia coli* LPS (10 ng/mL, serotype 055:B5, Sigma-Aldrich) for 24 hours.

Cytokine measurements

Cytokine measurements were performed in the supernatants collected after 24 hours or 7 days using commercial ELISA kits from R&D Systems (TNF-α, IL-1β, IL-10, IL-17 and IL-22; Minneapolis, MN, USA) or Sanquin (IL-6, IFN-γ; Amsterdam, the Netherlands), as previously described [1].

Leukocyte differentiation counts

Absolute lymphocyte and monocyte counts were determined directly in EDTA whole blood by the Department of Laboratory Medicine of the Radboud university medical center (Nijmegen, The Netherlands), using a Sysmex XE-5000-14 analyzer.

Statistical analysis

Analyses were performed in GraphPad Prism 5.03 for Windows.

Total RNA Extraction and cDNA Synthesis for RNA-seq

Total RNA was extracted from CD14+ monocytes using the QIAGEN RNeasy RNA extraction kit (QIAGEN, Netherlands) and on-column DNaseI treatment. Utilizing riboZero rRNA removal kit (Illumina) ribosomal RNA was removed from total RNA. RNA was then fragmented into approximately 200bp fragments by incubation for 3 min at 95C in fragmentation buffer (200 mM Tris-acetate, 500 mM Potassium Acetate, 150 mM Magnesium Acetate [pH 8.2]). First strand cDNA synthesis was performed using SuperScript III (Life Technologies), followed by synthesis of the second cDNA strand, recruiting DNA polymerase and DNA ligase (NEB). Libraries for RNA-sequencing were prepared using the KAPA hyperprep kit (KAPA Biosystems), according to the manufacturer's protocol.

Chromatin Immunoprecipitation

CD14+ monocytes were fixed with 1% formaldehyde (Sigma) at a concentration of approximately 10 million cells per ml. Fixed cell preparations were sonicated for 7 cycles (30 sec on, 30 sec off) using a Diagenode Bioruptor Pico sonicator. Around 1 million cells were mixed with dilution buffer, protease inhibitor cocktail (Roche) and 1mg of H3K4me3 antibody (Diagenode) and incubated overnight at 4C with rotation. Pre-washed protein A/G magnetic beads mixture, diluted in 0.15% SDS and 0.1% BSA, added to the chromatin/antibody mix and incubated for 60 min at 4C. Beads were washed with 400µl wash buffer for 5 min at 4C (with rotation). Five rounds of washes were done. After wash, chromatin was eluted using elution buffer. Eluted chromatin mixed with 8 µl 5M NaCl, 3µl proteinase K, and samples were incubated for 4 hours at 65C with rotation. Decrosslinked samples were purified using Qiaquick MinElute PCR purification Kit (Qiagen) and eluted in 20 µl EB. Purified immunoprecipitated chromatin was used for library preparation.

Library Preparation for Next Generation Sequencing

Illumina libraries for next generation sequencing was done using the Kapa Hyper Prep Kit (Roche), according to the manufacturer's protocol. Briefly, for end repair and A-tailing double stranded DNA, was incubated with end repair and A-tailing buffer

and enzyme for 30 min at 20C and then for 30 min at 65C. Subsequently, adapters were ligated using DNA ligase, and Illumina NextFlex adapters, specific for each sample, for 15 min at 20C. Post-ligation cleanup was performed using Agencourt AMPure XP reagent and adapter-ligated samples were eluted in 20 ml elution buffer. Libraries were amplified by mixing 2x KAPA HiFi Hotstart ReadyMix and 10x Illumina library amplification primer mix and using PCR for 10 cycles. Amplified PCR products were purified using the QIAquick MinElute PCR purification kit (Qiagen). Utilizing E-gel, 300bp fragments selected from each sample. Correct size selection was approved by BioAnalyzer analysis. Sequencing experiments were performed in Illumina NextSeq 500 machine in the paired end fashion.

RNA-Seq Data Analysis

To infer gene expression levels, RNA-seq reads were aligned to the hg38 human reference transcriptome using STAR [17]. Genes with fewer than 50 reads on average in all time points (baseline, day9 and day36) were excluded from downstream analysis. Utilizing DESeq2 libraries normalized and differentially expressed genes determined [18]. Genes with more than two-fold change between at least two time points, who have the same up- or down-ward trend in all donors, were identified as dynamic genes. In addition, genes with more than two fold change difference between at least two time points and p_{adj} value < 0.05 were designated as significant differentially expressed genes.

ChIP-Seq Data Analysis

Reads from NGS results were mapped to hg38 human reference genome using bowite2 [19]. Duplicated fragments were excluded from further analyses steps. Utilizing MACS2 software ChIP peaks were called on remaining reads with the *p*value of 1e-8 [20]. Called peaks across all samples were merged and reads aligning within merged peaks from each sample were counted using peakstats. Quantified ChIP-seq data were normalized using DESeq2. Peaks with more than 1.5 fold change and padj < 0.05 between at least two of time points were identified as significant differential peaks. For the intersection analysis of ChIP and RNA-seq data, H3K4me3 peaks near the promoter regions of differentially expressed gene were extracted.

Data availability

The RNA and ChIP-seq data set has been submitted to Gene Expression Omnibus (GEO) and can be accessed using accession number GSE137044.

Results

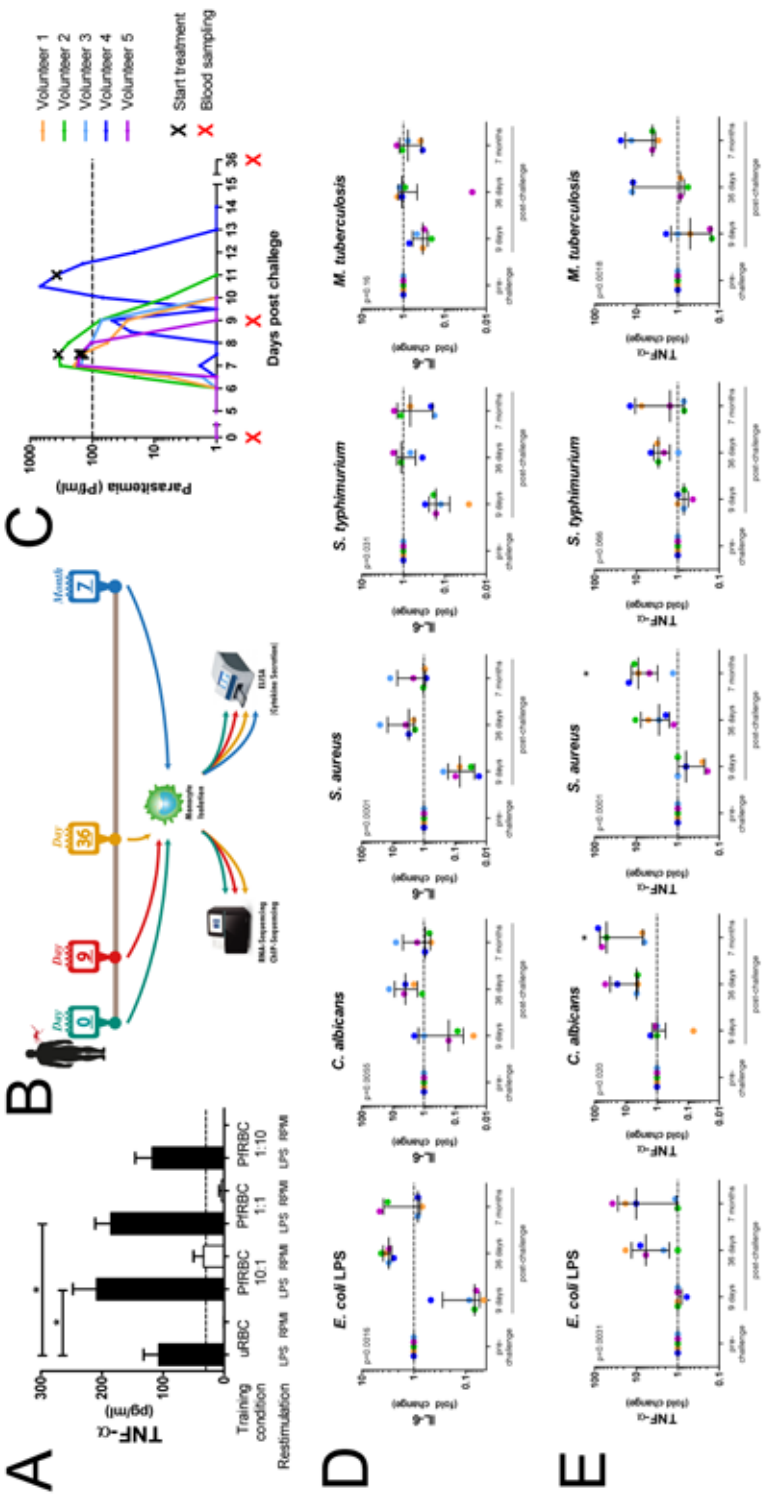
Controlled *in vivo* malaria infection induces trained immunity in monocytes

Monocytes isolated from healthy donors were incubated with different concentrations of *P. falciparum*-infected red blood cells for 24 hours, allowed to rest in culture medium for 5 days, and then restimulated with LPS. There was a dose-dependent increase in TNF-α production upon LPS stimulation when monocytes had been pre-incubated with infected red blood cells (figure 1A).

To investigate whether *in vivo* infection also induced this training effect, five volunteers were infected with *P. falciparum* sporozoites in a controlled manner (figure 1B). All five volunteers developed parasitemia detectable by qPCR after infection (figure 1C). Baseline characteristics and parasitological data for each volunteer are shown in supplementary table 1. CD14+ cells were stimulated with different stimuli pre-infection (baseline), on day 9 after infection, day 36, and 7 months after infection. During parasitemia (day 9) monocytes produced less IL-6 and IL-1β compared to baseline in response to all stimuli (figure 1D and F). In contrast, 36 days after infection, more than 3 weeks after parasite clearance, IL-6 and TNF-α production was significantly increased compared to baseline upon stimulation with LPS, *C. albicans* and *S. aureus* (figure 1D and E). Seven months after infection, IL-6 production returned to baseline, though TNF-α and IL-1β (the latter in case of LPS and *S. aureus* stimulations) production remained increased compared to baseline. Monocytes from most donors did not produce detectable amounts of IL-10 upon stimulation at any time point (data not shown).

Controlled malaria infection induces genome-wide transcriptomic and epigenomic reprogramming in monocytes

RNA-sequencing (RNA-seq) of CD14+ cells isolated from four volunteers at baseline, day 9 and day 36 post challenge (Figure 1B) identified 207 out of 8796 detectable genes that were significant differentially expressed ($p_{adj} < 0.05$ & fold-change > 2) across all time points and volunteers. Heatmap, correlation and principal component analysis of the 207 dynamic genes show a clear distinction between time points, indicating reprogramming of transcription over the time course (figure 2A-C). Nearly all of the up-regulated genes (116) were maximally expressed at day 9. The majority of these were associated with antiviral responses and the type I interferon signaling pathway (figure 2D, upper panel). At day 36, several anti-inflammatory genes are significantly down-regulated (padj <0.05_and FC>2), such as NFKBID, NFKBIZ, DUSP1 & 2 and ATF3 (figure 2D, lower panel) in line with the observed stronger response to a secondary stimulus (figure 1D-F).


A
TNF-α (pg/ml)
Training condition
Restimulation
uRBC
PfRBC 10:1
PfRBC 1:1
PfRBC 1:10
LPS
RPMI
B
Day 0
Day 9
Day 36
Month 7
C
Parasitemia (Pf/ml)
Days post challenge
Volunteer 1
Volunteer 2
Volunteer 3
Volunteer 4
Volunteer 5
Start treatment
Blood sampling
D
E
E. coli LPS
C. albicans
S. aureus
S. typhimurium
M. tuberculosis
IL-6 (fold change)
TNF-α (fold change)
pre-challenge
post-challenge

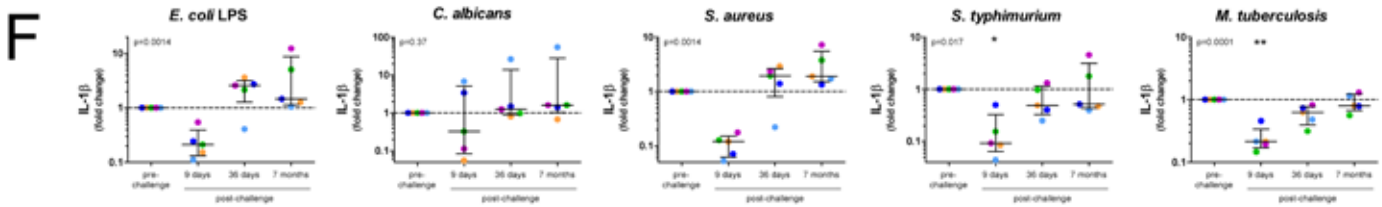
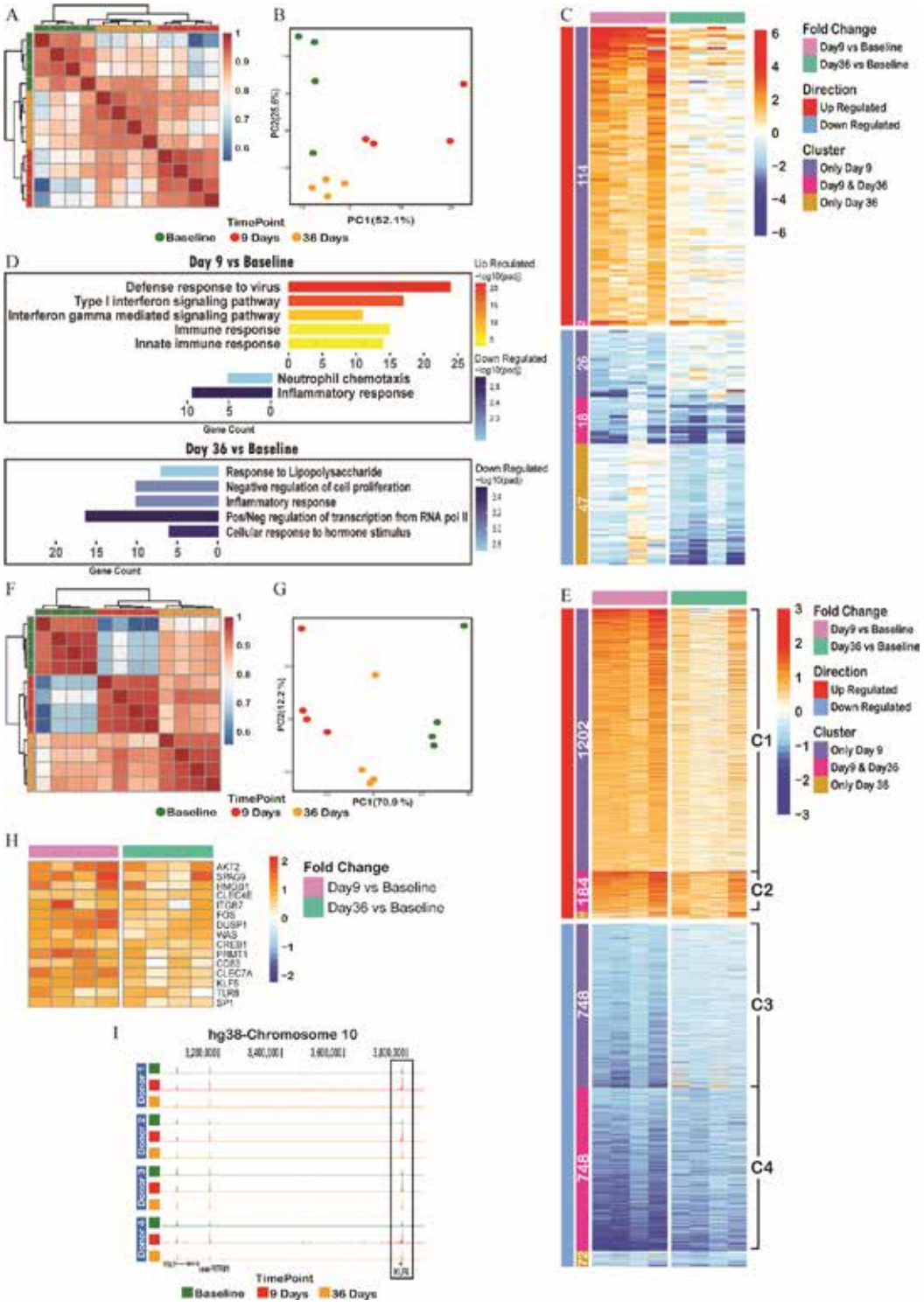


Figure 1: Controlled Human Malaria Infection induces lasting changes in monocytes. (A) Adherent monocytes from 6 healthy donors were seeded at $0.1x10^6$/well and pre-incubated with uninfected red blood (uRBC) cells at a concentration or $1x10^6$/well, or *P. falciparum* infected red blood cells (PfRBC) at a concentration of $1x10^6$/well, $0.1x10^6$/well or $0.01x10^6$/well for 24 hours. After 5 days resting in normal medium, monocytes were restimulated with 10ng/mL LPS (black bars) or RPMI as a negative control (white bars) for 24 hours. The graph shows TNF-α concentrations measured in supernatants by ELISA. P-values are the result of Wilcoxon matched-pairs signed rank test, * $p<0.05$. **(B)** Five healthy volunteers were infected with *P. falciparum* by mosquito bite challenge, blood was sampled at baseline (before infection), and 9 and 36 days, and 7 months after infection. **(C)** The graph shows the parasitemia as determined by twice daily qPCR starting on day 6 after challenge for each individual volunteer. All volunteers were treated when parasitemia reached 100Pf/mL. 4 volunteers were treated on day 7 after challenge and one volunteer on day 11 after challenge. At baseline (before infection), 9 days after challenge, 36 days after challenge and 7 months after challenge, isolated monocytes were stimulated for 24 hours with LPS, *C. albicans*, *S. aureus*, *S. typhimurium*, or *M. tuberculosis*. Graphs show supernatant IL-6 **(D)**, TNF-α **(E)**, and IL-1β **(F)** concentrations determined by ELISA as fold change over the baseline measurements. Dots represent individual volunteers (colors represent each volunteer). P-values shown are the results of Friedman Repeated Measures ANOVA. If time points differed significantly from baseline after correction for multiple testing using Dunn's Multiple Comparison Test this is indicated above the appropriate column. * $p<0.05$, ** <0.01.


A
B
C
TimePoint
Baseline
9 Days
36 Days
PC1(52.1%)
Fold Change
Day9 vs Baseline
Day36 vs Baseline
Direction
Up Regulated
Down Regulated
Cluster
Only Day 9
Day9 & Day36
Only Day 36
D
Day 9 vs Baseline
Defense response to virus
Type I interferon signaling pathway
Interferon gamma mediated signaling pathway
Immune response
Innate immune response
Neutrophil chemotaxis
Inflammatory response
Gene Count
Up Regulated
Down Regulated
Day 36 vs Baseline
Response to Lipopolysaccharide
Negative regulation of cell proliferation
Inflammatory response
Pos/Neg regulation of transcription from RNA pol II
Cellular response to hormone stimulus
F
G
E
PC1(70.9 %)
C1
C2
C3
C4
H
AKT2
SPAG9
HMGB1
CLEC4E
ITGB7
FOS
DUSP1
WAS
CREB1
PIBF1
CD83
CLEC7A
KLF6
TLR8
SP1
I
hg38-Chromosome 10
Donor 1
Donor 2
Donor 3
Donor 4
KLF6

< Figure 2: Controlled Human Malaria Infection has long lasting transcriptomic and epigenomic effects on monocytes. (A) Spearman correlation clustering of significant differential expressed (DE) genes between at least one of the time points (207 genes), **(B)** Principal component analysis plot of DE genes over the time course, **(C)** Heatmap of the log2(fold change over the baseline) of DE genes, clustered by their behavior over the time course (differentially expressed on only day 9 or only day 36 or both of these time points), **(D)** Significant gene ontology terms associated with differentially expressed genes on day 9 (upper panel) or day 36 (lower panel), **(E)** Heatmap of log2(fold change over the baseline) of differential ChIP regions, clustered by their behavior over the time course (differential in only day 9 or only day 36 or both of these time points over the baseline H3K4me3 level), **(F)** Spearman correlation clustering of significantly dynamic H3K4me3 histone ChIP peaks between at least one of time points (2984 peaks), **(G)** Principal component analysis plot of dynamic peaks over the time course, **(H)** Heatmap of up-regulated H3K4me3 regions (annotated to their nearest promoter) remained elevated on day 36 with immunogenic functions. **(I)** H3K4me3 histone peak around KLF6 gene promoter, indicating an increase in signal level at day 9, following a decrease on day 36 while still significantly higher than baseline level.

We also performed chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) on histone 3 trimethylation at lysine 4 (H3K4me3), an active mark of promoters, on monocytes from all three timepoints (figure 1B). Unlike the overall RNA transcription data, the overall H3K4me3 ChIP-seq data showed a clear clustering of time point-specific samples (supplementary figure 2). Around 17% (2984/17990) of identified H3K4me3 peaks were dynamic at least between two time points (padj < 0.05, fold-change > 1.5). There were 1386 up- and 1496 down-regulated peaks between baseline and day 9 time points, while 224 up- and 820 down-regulated regions were identified in the comparison of baseline and day 36 samples (figure 2E). Spearman correlation and PCA of these dynamic genes revealed clear differences between samples of different time points (figure 2F and G).

Next, we assessed the extent of retained epigenetic changes over the time course. As illustrated in figure 2E, around 87% (1202/1386) of regions with elevated H3K4me3 on day 9 (cluster C1) slowly lost their elevated marking over time but did not reach baseline levels at day 36. Similarly, the loss of H3K4me3 at day 9 of infection was not entirely restored to base line level at day 36 (figure 2E, clusters C3 & C4). At a small number of genomic sites (184, cluster C2 in figure 2E), H3K4me3 levels remained at significantly (*p*Value = 1.1e-4) higher level as compared to baseline, indicative of epigenetic memory after *P. falciparum* infection.

Importantly, genes with retained elevated H3K4me3 levels at their promoters (cluster C2 in figure 2E) did not show significantly altered transcription levels at day 36, suggesting that these genes are primed for a better immune response and will only be activated upon a secondary stimulation. In this case, the nearest genes linked

to cluster C2 regions are likely to contain inflammatory response related genes. We therefore inspected the list of 184 regions (cluster C2) and found 15 known immune-related genes with elevated H3K4me3 on day 36, reminiscent of primed promoters. As an example, figure 2I shows the H3K4me3 peaks at the KLF6 promoter that is elevated on day 9 and slowly decreased to the level observed on day 36, which is still significantly higher than baseline level.

Taken together, this indicates that *P. falciparum* infection induces trained immunity that is associated with epigenomic changes in monocytes that are maintained for at least several weeks.

Discussion

In the present study, we detect a marked increase in monocyte pro-inflammatory cytokine production to heterologous stimuli five weeks after a Controlled Human Malaria Infection in previously malaria-naïve volunteers. This pattern is similar to trained immunity seen in humans after BCG vaccination [1, 6], and represents the first observation of innate immune memory after infection with a pathogen. These findings are in line with an earlier study that found increased monocyte cytokine responses after *in vitro* exposure to *Plasmodium falciparum* [11]. Here, *P. falciparum* exposure and subsequent monocyte training occurs *in vivo* after a mosquito-transmitted infection at low parasite densities. As the study design allows for longitudinal sampling of individuals at multiple time points before and after infection, CHMI presents an ideal opportunity to study the induction of trained immunity in humans after *in vivo* infection.

Studies comparing *in vitro* LPS tolerance and β-glucan training have suggested that these stimuli induce opposing functional programs [5, 16, 21, 22]. In the present study the changes in monocyte function are biphasic, with a transient tolerance during infection followed by stronger pro-inflammatory response after parasite clearance. Nevertheless, the responses observed after malaria infection are reminiscent of the effect of LPS, which can lead to hyper-responsiveness or tolerance depending on dose and timing [22, 23]. It would be interesting in future studies to determine monocyte responses at additional time points to determine the precise course of these changes.

Another study by Dobbs et al. [24] recently determined monocyte TLR responses in Kenyan children with acute malaria. In contrast to our findings, they found similar cytokine production during the infection and six weeks after treatment, which were both higher than in North American adults. However, these children may have had repeated (asymptomatic) parasitemia or other infections between the time points. Further studies are needed to determine whether natural malaria infections with high and/or prolonged parasitemia also induce trained immunity.

In agreement with the cytokine assays, we observed a genome-wide reprogramming of monocytes in both transcription (RNA-seq) and epigenome (ChIP-seq) levels. Our RNA-seq analysis shows that several inhibitors of the NF-kB and MAPK signaling pathways were significantly down-regulated 36 days after infection, including NFKBID, NFKBIZ, DUSP-1, -2 [25], NR4A2 [26] and tristetraprolin (TTP or ZFP36). It has been demonstrated that TTP targets the AU-rich region (ARE) of TNF-α and IL-6 resulting in mRNA degradation of these genes [27]. Down-regulation of ZFP36 suggests a potential mechanism for the observed priming of monocytes towards a better response against secondary stimuli at this time point.

Among the 15 genes with increased H3K4me3 at day 36 in the C2 cluster, a few are of special interest to the induction of trained immunity. AKT2 is a member of the PI3K/AKT/MTOR pathway that is important in the induction of immunometabolic changes in trained immunity [4]. PRMT1 is an epigenetic enzyme that induces histone methylation, and HMGB1 is an important chromatin protein that regulates transcription and is a mediator of inflammation. Expression of the CLEC4E and CLEC7A receptors was also increased, which is interesting as CLEC7A (also known as dectin-1) is the receptor for the induction of trained immunity by β-glucan [28].

Unexpectedly, several inflammatory response genes such as IL-1β, IL-8 and CCL3 were down regulated at day 9 and 36 (Figure 2C). A plausible explanation of the observed down-regulation of inflammatory response at day 9 is that the initial cytokine response to the infection has ended before day 9 despite the clear parasitemia phenotype at this time point. Contrary to the results from Schrum et. al. [11], we could not identify an increase in H3K4me3 peaks over the promoter region of TNFA on this day, which may reflect differences between *in vitro* and *in vivo P. falciparum* exposure.

This observation of reduced monocyte responsiveness on day 9 is in stark contrast to the increase in pro-inflammatory responses in total PBMCs frequently seen in malaria [9, 10, 29, 30], including in our CHMI model [10]. This suggests that the reduced cytokine production we observe is monocyte specific and/or can be overcome during

the setting of active infection by the presence of and/or interaction with lymphocytes. Indeed, a recent study examined the interplay of T cells and tissue macrophages during infection, and demonstrated the importance of lymphocytes in boosting the induction of trained immunity [31].

This study was limited by its small sample size that does not allow us to perform post-testing adjusted for multiple comparisons. However, most changes occurred in all the 5 volunteers investigated, and are consistent across multiple stimuli and cytokines.

To date only a few pathogens have been shown to induce trained immunity. As such, our observation of innate memory after a common human parasitic disease is significant, and it suggests that trained immunity occurs after the regular encounters with pathogens. We recently showed that BCG vaccine-induced trained immunity increases the pro-inflammatory response to CHMI, offering partial protection in about half of volunteers [32]. Future studies are warranted to determine whether trained immunity is induced in natural malaria or after attenuated sporozoite immunization, and whether it plays any role in protection against repeated infections.

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Supplementary table 1: Characteristics of CHMI volunteers.

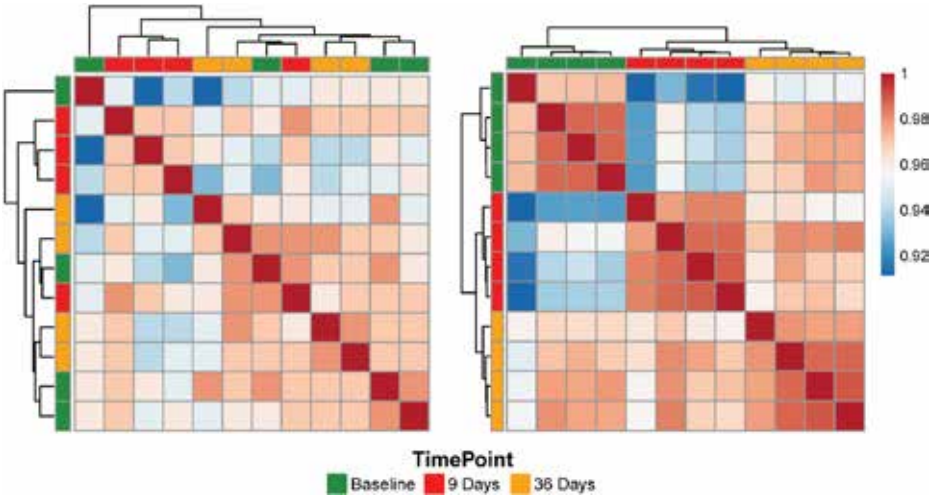
	Age (years)	Gender	Pre-patent period (days)*	Duration of parasitemia (days)**	Maximum parasitemia (Pf/mL)
Volunteer 1	23	Female	7	3.5	197
Volunteer 2	22	Male	7	3.5	353
Volunteer 3	22	Male	7	3.5	153
Volunteer 4	19	Male	10.5	5	690
Volunteer 5	25	Female	7	2	170

* Defined as time until 1st parasitemia >100 Pf/mL.

** Defined as number of days with positive qPCR.

Supplementary table 2: List of Differentially Expressed Genes across RNA-seq Samples

Supplementary table 3: List of Differential H3K4me3 Regions across ChIP-seq Samples



Supplementary figure 1: Spearman correlation of RNA-seq and H3K4me3 ChIP-seq data. Spearman correlation of overall RNA-seq data (8796 genes) (left), along with spearman correlation clustering of overall H3K4me3 ChIP-seq data (right). Overall ChIP-seq peaks (17990 peaks) show a clear clustering of samples from each time point, indicating the epigenome basis of trained immunity phenotype.

CHAPTER 8

Outcomes of Controlled Human Malaria Infection after BCG vaccination

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Abstract

Recent evidence suggests that certain vaccines, including Bacillus-Calmette Guérin (BCG), can induce changes in the innate immune system with non-specific memory characteristics, termed 'trained immunity'. Here we present the results of a randomized, controlled phase 1 clinical trial in 20 healthy male and female volunteers to evaluate the induction of immunity and protective efficacy of the anti-tuberculosis BCG vaccine against a controlled human malaria infection. After malaria challenge infection BCG vaccinated volunteers present with earlier and more severe clinical adverse events, and have significantly earlier expression of NK cell activation markers and a trend towards earlier phenotypic monocyte activation. Furthermore, parasitemia in BCG vaccinated volunteers is inversely correlated with increased phenotypic NK cell and monocyte activation. The combined data demonstrate that BCG vaccination alters the clinical and immunological response to malaria, and form an impetus to further explore its potential in strategies for clinical malaria vaccine development.

Introduction

With nearly 200 million clinical cases and nearly half a million deaths in 2015 [1], malaria remains a major global health problem and there is pressing need for a highly efficacious vaccine. RTS,S (Mosquirix®, GlaxoSmithKline), the only registered malaria vaccine, confers only modest, short-term protection [2]. It is clear that novel and improved malaria vaccine strategies are required for eradication.

To date malaria vaccine research has focused primarily on the induction of strong antibody or T cell responses. However, recent evidence suggests that certain vaccines, including the Bacillus-Calmette Guérin (BCG) developed against tuberculosis, can induce long-term changes in the innate immune system with non-specific memory characteristics. This BCG-induced 'trained immunity' [3] increases pro-inflammatory cytokine responses to other pathogens [4, 5] and is mediated by epigenetic changes in innate immune cells [6, 7]. The clinical relevance of trained innate immunity has been demonstrated in mice, where it reduced mortality of *Staphylococcus aureus* sepsis [6] and *Candida albicans* infection [8].

There is also evidence that BCG administration reduces parasitemia in rodent malaria models [9-12], and in endemic areas BCG vaccination has been associated with reduced malaria-specific mortality [13]. However, any direct evidence for protective efficacy of BCG-induced trained immunity against malaria, or any clinically relevant pathogen, in humans was lacking.

Here we show that a subset of BCG vaccinated healthy volunteers have accelerated, NK cell and monocyte activation that correlates with reduced parasitemia after controlled human malaria infection (CHMI). These findings are consistent with the possibility that BCG vaccination may induce trained immunity with functional activity against another human pathogen *in vivo*.

Methods

Clinical trial

This single-center, single-blinded randomized controlled trial was conducted at the Radboud university medical center (Nijmegen, The Netherlands) from August 2016 until February 2017. Prior to inclusion, study volunteers were medically screened as described previously [14] and provided written informed consent. The trial was approved by the Central Committee on Research Involving Human Subjects (CCMO NL56222.091.15) of the Netherlands, performed according to the Declaration of Helsinki and Good Clinical Practice and prospectively registered at ClinicalTrials.gov (NCT02692963).

Twenty healthy, BCG-naive volunteers (age 18–35 years) without a history of malaria or residence in a malaria-endemic area in the 6 months before study entry were included and randomly assigned to two groups. Male and female volunteers were allocated separately, to ensure an equal distribution between groups. Volunteers had not received any other vaccinations within three months of enrollment. Ten subjects received standard dose (0.1mL of the reconstituted vaccine) of intradermal BCG vaccination (BCG Bulgaria, Intervax) five weeks prior to challenge infection. Ten controls (group 2) received no vaccination.

Five weeks after BCG vaccination, both groups (BCG vaccinated, n = 9; 1 excluded after BCG vaccination and controls, n = 10) were exposed to bites of five *Plasmodium falciparum* NF54 strain infected *Anopheles stephensi* mosquitoes (sporozoite challenge). Details on the challenge infection are provided in supplementary table 3. Subjects and investigators were not blinded, whereas those performing the qPCR analysis were blinded until after the last qPCR data had been collected. qPCR was performed prospectively, once daily from day 6 after CHMI until day 3 after antimalarial treatment, according to previously published protocols [15-17]. All volunteers were treated with a curative regimen of antimalarial drugs (atovaquone/proguanil) once the treatment threshold of 100 parasites/ml blood was exceeded detected by qPCR or presumptively on day 21 after challenge if qPCR remained below treatment threshold.

Recording of adverse events

Subjects recorded clinical symptoms in a diary, from the time of BCG vaccination until 37 days after the CHMI. Both solicited and unsolicited adverse events were recorded after questioning by the investigators at set time points: prior to BCG vaccination, prior to the CHMI, daily from day 6 after infection until 3 days after antimalarial treatment, and on day 37 post CHMI [18, 19]. Adverse events were graded according to criteria defined in the Clinical Trial Protocol: mild (grade 1): awareness of symptoms that are easily tolerated and do not interfere with usual daily activity; moderate (grade 2):

discomfort that interferes with or limits usual daily activity; severe (grade 3): disabling, with subsequent inability to perform usual daily activity, resulting in absence or required bed rest. Relatedness was assessed by the investigator, also on the bases of pre-defined criteria: probable: an adverse event that follows a reasonable temporal sequence from the challenge procedure and cannot be reasonably explained by the known characteristics of the subject's clinical state; possible: an adverse event for which insufficient information exists to exclude that the event is related to the study procedure; not related: an event for which sufficient information exists to indicate that the aetiology is unrelated either because of the temporal sequence of events or because of the subject's clinical state or other therapies.

Oral temperature was measured by volunteers and recorded in the symptom diary every morning and more frequently during symptoms. Tympanic temperature was measured by the study physician at every follow-up visit. Fever was scored as follows: mild (grade 1): 37.6-38.0° Celsius; moderate (grade 2): 38.1-39.0° Celsius; severe (grade 3): ≥39.1° Celsius.

Whole blood flow cytometry

100µL (lymphocytes) or 50µL (monocytes and neutrophils) of fresh EDTA blood was stained directly with antibodies. For lymphocyte analysis samples were stained with CD3-AlexaFluor700 (Biolegend; clone OKT3; catalogue number 317340; final dilution 1:640), pan-γδTCR-PE (Beckman Coulter; clone IMMU510; catalogue number COIM1349; final dilution 1:160), CD56-Brilliant Violet(BV)421 (Biolegend; clone HCD56; catalogue number 318328; final dilution 1:320), CD16-APC-eFluor780 (eBiosciences; clone CB16; catalogue number 47-0168-42; final dilution 1:640), CD69-PerCP-Cy5.5 (Biolegend; clone FN50; catalogue number 310926; final dilution 1:640). For monocyte analysis, samples were stained with a lineage mix containing CD3-PerCP-Cy5.5 (Biolegend; clone HIT3a; catalogue number 300328; final dilution 1:400), CD19-PerCPCy5.5 (Biolegend; clone HIB19; catalogue number 302230; final dilution 1:200) and CD56-PerCP-Cy5.5 (Biolegend; clone HCD56; catalogue number 318322; final dilution 1:100), CD14-FITC (Biolegend; clone HCD14; catalogue number 325604; final dilution 1:80), CD16-PE-Cy7 (Biolegend; clone 3G8; catalogue number 302016; final dilution 1:1280), HLA-DR-APC-Cy7 (Biolegend; clone L243; catalogue number 307618; final dilution 1:160) and CD86-PacificBlue (Biolegend; clone IT2.2).; catalogue number 305423; final dilution 1:100). For neutrophil analysis samples were stained with CD14-PerCP (Biolegend; clone HCD14; catalogue number 325632; final dilution 1:30), HLA-DR-APC (Biolegend; clone L243; catalogue number 307610; final dilution 1:80), CD16-APC-eFluor780 (eBiosciences; clone CB16; catalogue number 47-0168-42; final dilution 1:1280), CD62L-PE-Cy7 (eBioscience; clone DREG-56; catalogue number 25-0629-42; final dilution 1:1280) and CD11b-BV510 (Biolegend; clone ICRF44; catalogue number

301334; final dilution 1:180). Samples were stained for 30 minutes at 4° Celsius (C) in the dark. After staining, erythrocytes were lysed for 5 minutes at 4°C with 1mL BD FACS Lysis buffer, followed by centrifugation. Cell pellets were washed once with 1mL FACS buffer (0.5% Bovine Serum Albumin (BSA) in PBS) and resuspended in PBS with 1% paraformaldehyde (PFA) and analyzed on a Gallios flow cytometer (Beckman Coulter) the same day. Flow cytometry data was analysed using Flow Jo software (version 10.0.8 for Apple OS). The gating strategy and representative plots are shown in supplementary figure 10.

PBMC isolation, cryopreservation and thawing

Blood samples for peripheral blood mononuclear cell (PBMC) isolation were collected at inclusion (incl), prior to challenge (C–1) and 37 and 121 days after challenge infection (C+37, C+121). PBMC were isolated by density gradient centrifugation from citrate anti-coagulated blood using vacutainer cell preparation tubes (CPT; BD Diagnostics). Following four washes in ice-cold phosphate buffered saline (PBS), cells were counted and cryopreserved at a concentration of 10×10^6 cells/ml in ice-cold fetal calf serum (Gibco)/10% DMSO (Merck) using Mr. Frosty freezing containers (Nalgene). Samples were stored in vapour-phase nitrogen. Immediately prior to use, cells were thawed, washed twice in Dutch-modified RPMI 1640 (Gibco/Invitrogen) and counted in 0·1% Trypan blue with 5% Zap-o-Globin II Lytic Reagent (Beckman Coulter) to assess cell viability.

PBMC restimulation

For lymphocyte responses, PBMCs taken at inclusion, C-1, C+37 and C+121 were stimulated with purified NF54 strain schizonts or uninfected erythrocytes. Cells were cultured in RPMI 1640 (Dutch Modification; Gibco) with 5mg/ml gentamycin (Centraform), 100mM pyruvate (Gibco), 200mM glutamax (Gibco), supplemented with 10% heat-inactivated pooled human A+ serum (obtained from Sanquin Bloodbank, Nijmegen, The Netherlands). Anti-CD107a-Pacific Blue antibody (Biolegend; clone H4A3; catalogue number 328624; final dilution 1:400) was added throughout co-culture. Brefeldin A (10µg/mL; Sigma-Aldrich) and monansin (2µM; eBioscience) were added after 20 hours. After an additional 4 hours of stimulation, cells were washed and stained with a fixable viability dye labeled with eFlour780 (eBioscience) for 30 minutes at 4 degrees Celsius. After washing cells were stained with antibodies against surface markers: CD3-ECD (Beckman Coulter; clone UCHT1; catalogue number A07748; final dilution 1:100), CD4-FITC (BD Biosciences; clone SK3; catalogue number 340133; final dilution 1:20), CD8-AlexaFluor700 (Biolegend; clone HIT8A; catalogue number 300920; final dilution 1:2000), pan-γδTCR-PE (Beckman Coulter; clone IMMU510; catalogue number COIM1349; final dilution 1:160), and CD56 PerCP-Cy5.5 (Biolegend; clone HCD56; catalogue number 318322; final dilution 1:100), for 30 minutes at 4 degrees.

Cells were washed and fixed with Foxp3 fixation/permeabilization buffer (eBioscience) for 30 minutes at 4 degrees. After washing with permeabilization buffer (eBioscience) cells were stained for intracellular cytokines with IFN-γ-PE-Cy7 (Biolegend; clone 4S.B3; catalogue number 502528; final dilution 1:200) and granzyme B-AlexaFluor647 (Biolegend; clone GB11; catalogue number 515406; final dilution 1:200) for 30 minutes at 4 degrees. After washing with permeabilization buffer cells were taken up in PBS with 1% PFA. Cells stimulated PMA (10ng/mL; Sigma) and ionomycin (1µg/mL; Sigma) for 4 hours were used as a positive control.

Samples were analyzed on a Gallios flow cytometer (Beckman Coulter) the same day. Flow cytometry data was analysed using Flow Jo software (version 10.0.8 for Apple OS). CD107a and cytokine responses to PfRBC were corrected for uRBC at every time point (thus, defined as percent increase over background), and then corrected for baseline (pre-vaccination) responses. Gating strategy and representative plots are shown in supplementary figure 11.

Circulating cytokines and granzyme B

Plasma concentrations of TNF-α, IL-1β, (detection range 0,98- 4000 pg/mL) IL-6 (0,36-1500 pg/mL) , IL-8 (0,62-2500 pg/mL), IL-10, (2,92-12000 pg/mL) IFN-γ (1,22-5000 pg/mL) and granzyme B (2-10.000 pg/mL) were measured in citrate plasma using a Luminex assay according to the manufacturer's instructions (Milliplex, Merck Millipore, Billerica, MA, USA).

High sensitivity C-reactive protein

Automated hsCRP measurements were performed on citrated plasma samples with immunonephelometry with a Behring Nephelometer Analyzer following the manufacturers' instructions, using reagents and calibrators specifically designed for high sensitivity measurements. The detection limit was 0.16 mg/l.

Malaria specific antibody ELISA

Malaria specific antibody levels were determined by standardized ELISA as described previously [20]. In short, plates were coated with circumsporozoite protein (CSP), Liver Stage Antigen-1 (LSA1) protein or lysed ring stage parasites. Citrated plasma from volunteers was diluted 50x and 150x and analyzed in duplicate. A standard curve was generated by serial 2-fold dilutions of serum from a pool of 100 Tanzanian adults living in an endemic area (HIT serum). ELISA data analysis was performed with Auditable Data Analysis and Management System for ELISA (ADAMSEL, version 1.1). Post-challenge plasma samples were corrected for pre-challenge responses.

Sporozoite invasion assay

HC-04 human hepatoma cells (obtained from MR4) were seeded in collagen coated 96-well plates (coated with 0.056mg/mL for 1 hour; Collagen from Rat Tail, Sigma-Aldrich) at 50,000 cells per well. Sixteen hours after seeding, NF54 *P. falciparum* sporozoites were pre-incubated on ice for 30 minutes with 10% heat-inactivated pre- or post-challenge citrate plasma from volunteers and 10% heat-inactivated serum from non-immune adult. Sporozoites incubated with 10% heat-inactivated serum from highly immune Tanzanian adults and 10% non-immune serum, or 20% non-immune serum served as positive and negative control, respectively. Following pre-incubation, 50,000 sporozoites were added per well in triplicate. Plates were centrifuged at 3,000 rpm for 10 minutes (Eppendorf Centrifuge 5810R) and incubated for 3 hours on 37˚C, 5% CO_2.

After three hours, wells were washed three times with PBS to remove medium, antibodies and non-invaded sporozoites. Subsequently, cells and any extracellular adherent sporozoites were dissociated by incubating with 0.05% trypsin with EDTA (ThermoFisher) for 5 minutes at 37°C, followed by neutralization with an equal volume 10% heat-inactivated human serum in PBS. Cells were transferred into 96 well V-bottom plates, spun down at 1700 rpm for 4 minutes at 4 °C.

Cells were washed with PBS and fixed with Foxp3 fixation/permeabilization buffer (eBioscience). After washing with permeabilization buffer (eBioscience), intracellular sporozoites were stained with FITC-labeled 3SP2 antibody (monoclonal antibody against CSP, published previously [21]) for 30 minutes at 4°C. After washing in permeabilization buffer cells were taken up in 1% paraformaldehyde and analyzed on a Gallios flow cytometer (Beckman Coulter) the same day.

Flow cytometry data was analyzed using Flow Jo software (version 10.0.8 for Apple OS). Live cells were gated based on forward scatter/sideways scatter characteristics and percent invasion was defined as percentage of live cells positive for FITC. Post-challenge samples were compared to pre-challenge samples.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author upon request. A reporting summary for this Article is available as a Supplementary Information file.

Results

BCG vaccination alters the clinical course of *P. falciparum* infection

In a single-blind, randomized controlled clinical trial, 10 healthy BCG- and malaria-naïve volunteers received an intradermal BCG vaccination, while 10 control volunteers received no intervention. A single volunteer was excluded post-vaccination due to a concomitant Epstein-Barr virus infection. Five weeks after vaccination, 9 BCG-vaccinated and 10 control volunteers underwent a Controlled Human Malaria Infection (CHMI) by exposure to bites of five *P. falciparum* (*Pf*) infected female *Anopheles* mosquitoes (supplementary figure 1). Randomization was stratified by gender in order to ensure an equal distribution of male and female volunteers. Other baseline characteristics were similar between groups (supplementary table 1). Study primary endpoints were 1) Frequency and magnitude of adverse events and 2) Time to blood stage parasitemia detectable by qPCR. Study secondary endpoints were 1) Changes in cellular (innate and adaptive) immune responses and 2) Changes in plasma cytokine levels.

All volunteers developed parasitemia as detected by qPCR after challenge infection. Blood samples from 8 out of 9 BCG vaccinated and all controls exceeded the predetermined threshold of 100 parasites per milliliter blood on day 7, which was followed by a curative treatment with atovaqone/proguanil. One BCG vaccinated volunteer became positive on day 9. Interestingly, the variation in day 7 parasitemia was much higher in the BCG vaccinated group (geometric mean: 752 Pf/mL, 95% CI: 217-2602 Pf/mL) than in the controls (geometric mean: 813 Pf/mL, 95% CI 481-1373 Pf/mL) (Levene's test for equality of variances: p=0.005, figure 1A-B).

BCG-vaccinated volunteers developed clinical symptoms of malaria infection at an earlier time point and reported a higher frequency of moderate or severe clinical symptoms than control volunteers (Gehan-Breslow-Wilcoxon Test, p=0.01, figure 1C; supplementary table 2). The moderate and severe adverse event frequency in the BCG vaccinated group was also significantly higher than in historical controls (supplementary figure 2). In line with this finding, BCG vaccinated volunteers presented with a more significant decrease in platelet count (mean relative change BCG group: 0.689, 95% CI: 0.637-0.741; control group: 0.778, 95% CI: 0.703-0.853; student's t test: p=0.05; supplementary figure 3). Moreover, in a subset of BCG vaccinated volunteers, circulating platelets, lymphocytes and neutrophils dropped earlier (figure 1D-F). There was no significant difference in temperature during follow-up (supplementary figure 4).

BCG vaccinated volunteers show memory-like immune responses

To study the kinetics of the immune response to CHMI, whole blood flow cytometry was performed to determine lymphocyte, monocyte and neutrophil activation. Post-BCG

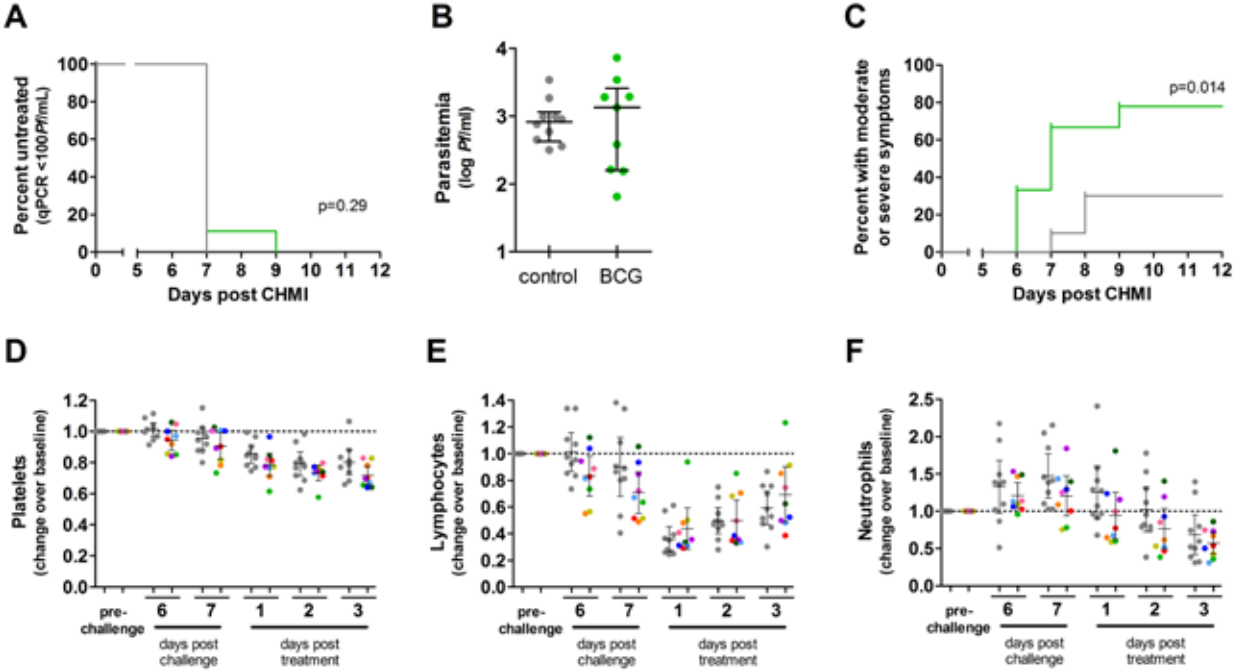

Figure 1: parasitemia, clinical symptoms and laboratory abnormalities after CHMI. Parasitemia was measured by daily qPCR from day 6 after CHMI until the third day after antimalarial treatment. **(A)** The Kaplan-Meier survival curve shows percent of volunteers remaining untreated. 8/9 BCG vaccinated (green) and 10/10 control volunteers (grey) surpassed the treatment threshold of 100 parasites per milliliter, and were treated on day 7 after challenge. 1/9 BCG vaccinated volunteers remained below 100 Pf/mL until day 9. **(B)** All volunteers did have parasitemia detectable by qPCR on day 7 after CHMI. The graph shows log parasites per milliliter on day 7 post CHMI for BCG vaccinated (green) and control (grey) volunteers. **(C)** Adverse events were collected daily. The Kaplan-Meier curve shows the percentage of volunteers experiencing one or more moderate or severe, solicited, symptoms during follow-up, BCG vaccinated volunteers (green) compared to controls (grey). **(D-F)** Absolute platelet, lymphocyte and neutrophil differentiation counts were determined by daily hemocytometry starting on day 6 post challenge. Graphs show relative change in cell counts compared to pre-challenge values in both BCG vaccinated (n=9, each colored dot shows and individual volunteer, colors consistently represent the same volunteers across each graph) and non-BCG vaccinated controls (n=10, grey dots).

vaccination but prior to CHMI and during parasite liver stage (day 5 post-challenge), there was no activation of peripheral blood leukocytes in either group as measured by the expression of the early activation marker CD69 in lymphocytes, or the expression of CD16, the antigen presenting molecule HLA-DR, and the co-stimulatory molecule CD86, in monocytes (figure 2A-G). On day 7, coinciding with the first appearance of blood stage parasites, there was a marked increase in the proportion of $CD56^{dim}$ NK cells expressing CD69 in half of the BCG vaccinated volunteers, absent in the control group (Mann-Whitney U test of vaccinated versus controls: p=0.03, figure 2A). Instead, control volunteers primarily showed immune activation only after treatment. CD69 expression on gamma-delta (γδ) T cells, NKT cells and alpha-beta (αβ) T cells followed a similar pattern, with the same subgroup of BCG vaccinees showing activation on day 7 post-challenge (figure 2B-D). There was no significant increase in CD69 expression on $CD56^{bright}$ NK cells after challenge (supplementary figure 5).

Next, the expression of CD16, HLA-DR and CD86, were determined on $CD14^{+}$ monocytes (figure 2E-G), markers previously shown to increase with monocyte activation during CHMI [22]. The percentage of activated neutrophils, those lacking CD62L with high CD11b expression, was also analyzed (figure 2H). On day 7 after challenge, the same subgroup of BCG vaccinated volunteers showed increased expression of HLA-DR and CD86 on $CD14^{+}CD16^{-}$ monocytes, which was absent in controls.

The 3 BCG vaccinees with the strongest lymphocyte and monocyte activation also responded with early increases in plasma interferon-gamma (IFN-γ) or granzyme B, and inflammatory C-reactive protein (CRP) concentrations (figure 2I-K).

BCG-induced trained immunity correlates with lower parasitemia

We next evaluated whether this altered innate immune phenotype had consequences for control of parasitemia. While at group level this primary endpoint was not statistically met, we identify a subgroup of approximately half of BCG vaccinated volunteers with lower levels of parasitemia after challenge. These effects were correlated with changes in the immune parameters defined as secondary endpoints. Indeed, the subset of BCG-vaccinated volunteers with early lymphocyte and monocyte activation were also those with lower parasitemia within the BCG group (figure 3A-B and supplementary figure 6), and early NK cell CD69 expression and monocyte HLA-DR expression were correlated with decreased parasitemia. In contrast, increased neutrophil activation in BCG vaccinated volunteers was not associated with decreased parasitemia (supplementary figure 7).

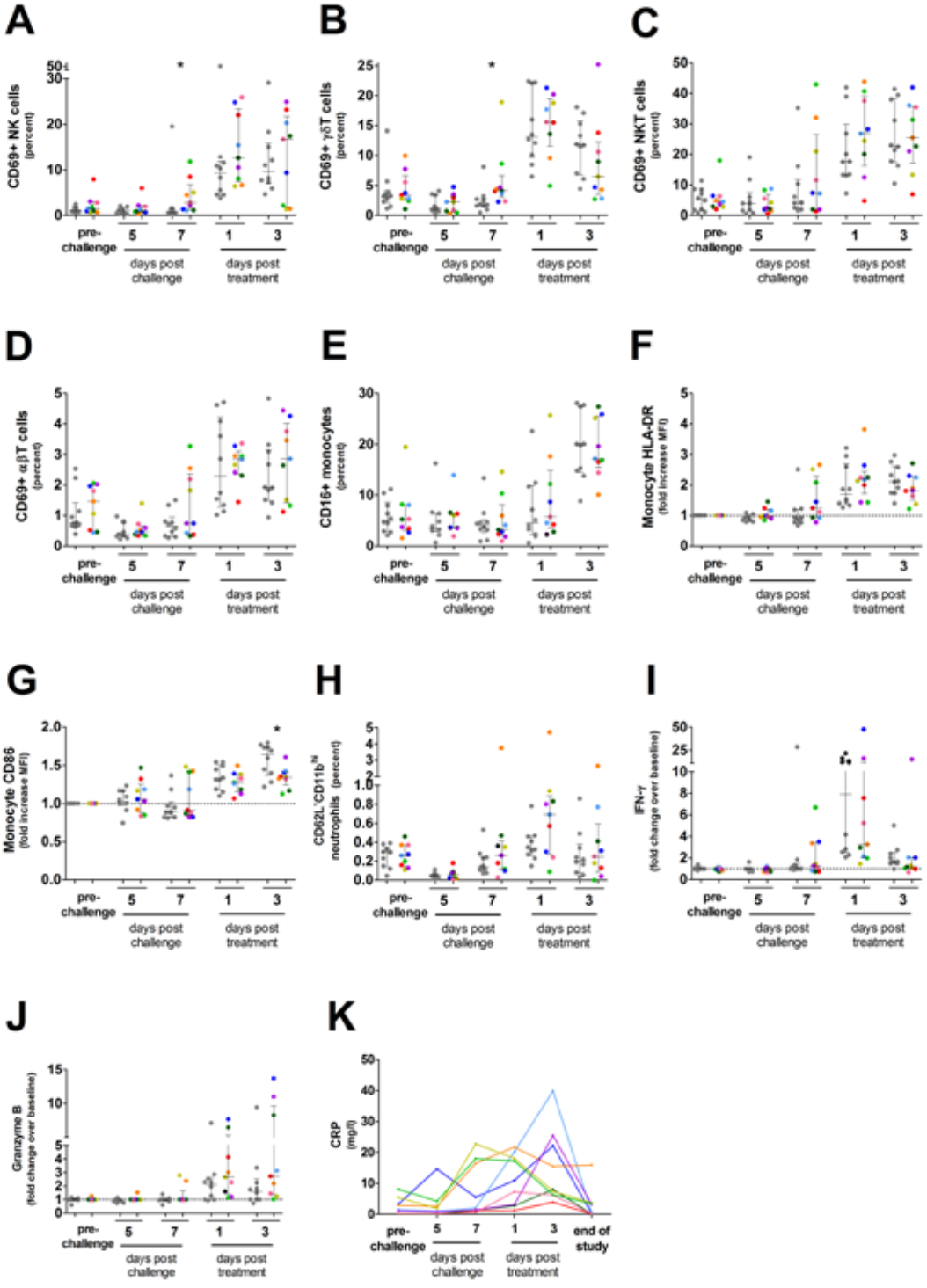
A
CD69+ NK cells (percent)
B
CD69+ γδT cells (percent)
C
CD69+ NKT cells (percent)
D
CD69+ αβT cells (percent)
E
CD16+ monocytes (percent)
F
Monocyte HLA-DR (fold increase MFI)
G
Monocyte CD86 (fold increase MFI)
H
CD62L-CD11b+ neutrophils (percent)
I
IFN-γ (fold change over baseline)
J
Granzyme B (fold change over baseline)
K
CRP (mg/l)
pre-challenge
5
7
1
3
days post challenge
days post treatment
end of study

Figure 2: *in vivo* activation of lymphocytes, monocytes and neutrophils after CHMI. *In vivo* leukocyte activation was determined by direct staining of fresh whole blood with fluorescent antibodies every two days post challenge. Lymphocytes were defined based on forward scatter and sideward scatter characteristics, and duplet events were excluded. **(A)** NK cell activation was defined as the percentage of $CD3^{-}CD56^{dim}CD16^{+}$ live cells expressing CD69. **(B)** γδT cell activation was defined as the percentage of $CD3^{+}\gamma\delta TCR^{+}$ live cells expressing CD69. **(C)** NKT cell activation was defined as the percentage of $CD3^{+}\gamma\delta TCR^{-}CD56^{+}$ live cells expressing CD69. **(D)** αβT cell activation was defined the as percentage of $CD3^{+}\gamma\delta TCR^{-}CD56^{-}$ live cells expressing CD69. **(E)** Monocytes were defined based on forward and side scatter characteristics, and the as $HLA\text{-}DR^{+}CD14^{+}$. Within the monocyte population, cells were then divided into $CD16^{-}$ and $CD16^{+}$ monocytes. **(F-G)** Within the $CD16^{-}$ monocyte population, the relative change in mean fluorescent intensity of HLA-DR and CD86 compared to pre- malaria challenge values was determined. **(H)** Neutrophils were defined based on forward and side scatter characteristics, and then defined as $HLA\text{-}DR^{-}CD14^{-}CD16^{+}CD11b^{+}$. Activated neutrophils were defined as $CD62L^{dim}CD11b^{high}$. **(I-J)** IFN-γ and granzyme B were measured by Luminex assay in citrate plasma taken ever two days. Circulating cytokine levels are corrected for baseline levels (pre-BCG vaccination time point) at each time point. In all graphs the grey dots represent non-BCG vaccinated control group volunteers (n=10), and each colored dot shows an individual BCG vaccinated volunteer (n=9). Statistical analysis are between BCG vaccinated and control volunteers at a single time point, and p-values are the results of Mann-Whitney U test. *p<0.05. **(K)** Circulating CRP levels were measured in citrate plasma are shown for each BCG vaccinated volunteer (colors consistently represent the same volunteers across each graph).

BCG augments *P. falciparum*-induced cytotoxic lymphocyte responses

NK cells stimulated with *P. falciparum*-infected red blood cells (*Pf*RBC) *in vitro* showed no difference in degranulation (defined by CD107a staining), IFN-γ or granzyme B production in BCG vaccinated versus controls prior to challenge infection (figure 4). However, 37 days after challenge infection, NK cells from BCG vaccinated volunteers produced significantly more granzyme B (Mann-Whitney U test: p=0.03) with a tendency towards increased degranulation, compared to controls.

The induction of T cell responses after CHMI was also analyzed in both groups (supplementary figure 8). Again, there were no measurable differences between the groups after BCG vaccination and prior to malaria infection. However, post-CHMI there were more *P. falciparum*-specific CD4+ T cells producing granzyme B in BCG vaccinated volunteers (Mann-Whitney U test: p=0.02). Furthermore, there were trends towards increased degranulation and granyzme B production in γδT cells.

Finally, the induction of *P. falciparum*-specific antibody responses was analyzed in both groups. There were no differences in antibody responses to the immunodominant Circumsporozoite protein (CSP) expressed on sporozoite stages, the Liver Stage Antigen (LSA) expressed on liver stages or total lysate of asexual blood stages (supplementary figure 9A-C). Furthermore, BCG vaccination did not influence the ability of antibodies to block sporozoite invasion into the HC04 hepatoma cell line (supplementary figure 9D).

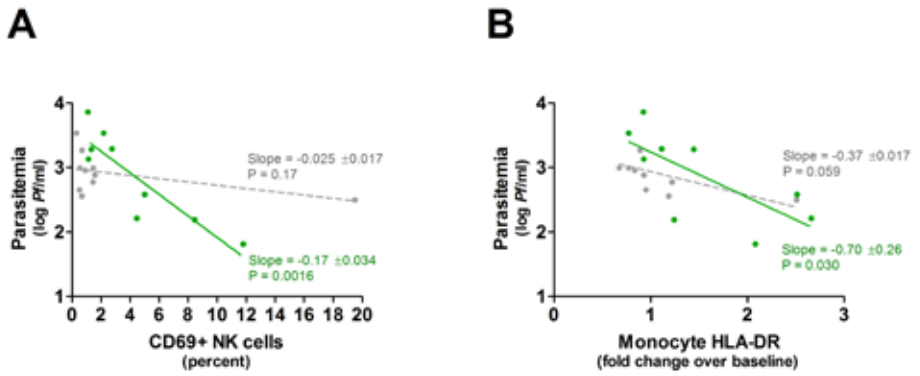

Figure 3: early NK cell and monocyte activation correlates with decreased parasitemia. Correlations between **(A)** NK cell CD69 expression and log parasitemia on day 7 after challenge infection, and **(B)** increase in monocyte HLA-DR MFI and log parasitemia on day 7 are shown for BCG vaccinated (n=9, green) and control (n=10, grey) volunteers. Lines show the result of linear regression analysis for both groups.

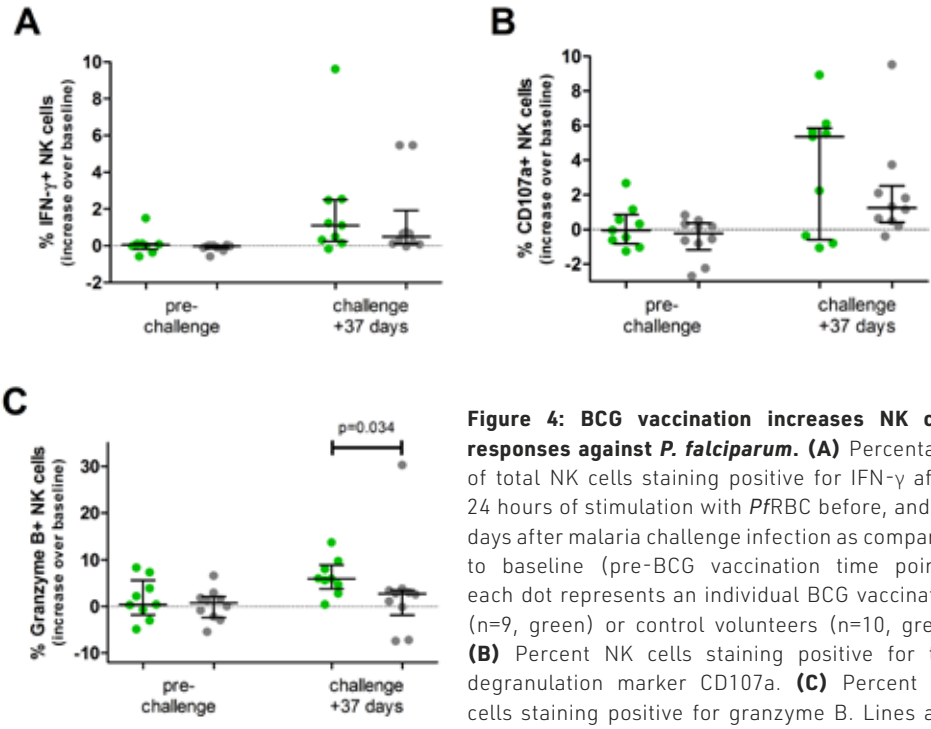

Figure 4: BCG vaccination increases NK cell responses against *P. falciparum*. (A) Percentage of total NK cells staining positive for IFN-γ after 24 hours of stimulation with *Pf*RBC before, and 37 days after malaria challenge infection as compared to baseline (pre-BCG vaccination time point), each dot represents an individual BCG vaccinated (n=9, green) or control volunteers (n=10, grey). **(B)** Percent NK cells staining positive for the degranulation marker CD107a. **(C)** Percent NK cells staining positive for granzyme B. Lines and error bars show median and interquartile range. P values are the result of Mann-Whitney U test. *p<0.05. Stimulation was performed in duplo, with replicates combined for flow cytometry analysis.

Discussion

Here we provide *in vivo* evidence suggestive of the induction of functional trained immunity by BCG vaccination against a heterologous, clinically relevant human pathogen. The existence of trained immunity after BCG vaccination has been previously demonstrated *in vitro* [4, 5, 23] and in murine models [6, 8]. However, translation of such findings into equivalent human responses *in vivo* has so far been limited to a single study where BCG reduced viremia after vaccination with the non-pathogenic, live attenuated yellow fever vaccine [24].

The vaccinated volunteers develop early clinical symptoms and laboratory abnormalities, and earlier and stronger inflammatory responses in a subset of volunteers are associated with lower parasitemia. This altered course of immune activation in BCG-vaccinated individuals sharply contrasts with CHMIs in control volunteers of this and previous studies, where innate immune activation is only detectable just prior to microscopic parasitemia [25-27], i.e. at 3-4 days after parasites emerge from the liver. In addition, the course of clinical symptoms is strikingly different from that seen in other, similar CHMI studies at our center where symptoms are typically absent on day 7 post-challenge [17, 19, 28]. The prompt activation of NK cells in BCG vaccinated volunteers apparently represents a true memory phenotype rather than persistent inflammation, as immediately prior to CHMI there was no difference in activation of peripheral blood lymphocytes between the control and test groups.

Interestingly, the earlier and stronger immune activation markers in half the BCG vaccinated volunteers correlated with a reduced parasitemia in early infection, whereas those with higher parasitemia had no immune activation on day 7 after challenge. This may be due to either reduced release of parasites emerging from the liver or by rapid clearance of blood stage parasites. Indeed, IFN-γ produced by liver lymphocytes in mice suppresses schizont development and subsequent parasitemia [29, 30]. In this study we neither detect peripheral blood leukocyte activation, nor increases in circulating IFN-γ or CRP during the liver stage. However, the contribution of undetected local inflammatory processes in the liver cannot be excluded. More sensitive techniques such as (single-cell) transcriptomic analysis may be needed to study peripheral blood responses during the liver stage. Alternatively, the observed reduction of parasite load after BCG vaccination may be the result of efficient asexual clearance as previously shown in C57BL/6 mice infected with *P. yoelii* [12]. In the current CHMI, estimation of asexual parasite multiplication has not been possible since curative treatment was administered at very low parasite densities. In future studies, this might be addressed by allowing longer duration of parasitemia, or alternatively, by using a blood stage

challenge infection with a low inoculum, which would allow for even longer exposure to blood stage parasites.

The changes in the clinical and parasitological outcomes in BCG vaccinated volunteers are also associated with *P. falciparum*-specific elicited cellular immune responses after CHMI, including an improved *P. falciparum*-induced NK cell granzyme B production and a trend towards increased degranulation. Such memory-like NK cell responses after CHMI have been described previously [31], and found to be T cell dependent. In addition, the number of granzyme B producing CD4+ T cells in response to *P. falciparum* is also increased with a trend towards increased CD4+ T cell IFN-γ production and γδT cell granzyme B production and degranulation. The combined data do suggest that the altered kinetics of immune cell activation in BCG vaccinated volunteers may improve their ability to generate *P. falciparum*-specific responses as has been shown in relation to other vaccines [32]. However, a CHMI with 5 mosquito bites is not likely to induce significant cellular or humoral immunity, and this hypothesis should be tested in a study combining BCG with a malaria vaccine.

A recent study examined the epigenetic and transcriptomic changes in monocytes of healthy volunteers vaccinated with BCG [24], showing genome-wide changes in histone H3 acetylation at lysine 27 (H3K27ac) in 'responding' volunteers. Our study finds functional changes in NK cells as well, confirming previous in vitro observations [23]. This may be the result of increased monocyte activation, as NK cell activity against malaria is partially dependent on monocytes [33]. Whether BCG induces epigenetic changes in NK cells as well should be subject of a future study.

This study is limited by its small sample size and the subsequent lack of sufficient power for comprehensive statistical analysis of all immune responses. However, the fact that strong correlations can be found in such a small sample size is encouraging. It is striking that there is such clear dichotomy between volunteers, with 4 out of 9 'responders', showing accelerated immune responses and a relative decrease in parasitemia. Interestingly, the 'non-responders' in this cohort seem to have increased parasitemia compared to the controls. Other studies with BCG induced trained innate immunity have also identified significant variability in responses between individuals [34]. The small size of this study, however, does prohibit a clear identification of the factors that predict the effect of BCG vaccination, and future larger cohort studies are needed to explore the factors underlying this variation.

For this study the observation period of five weeks was chosen based on evidence of BCG induced protection against malaria in mice at 1-2 months post vaccination [9-12] and BCG induced trained innate immunity in humans at 2 weeks and 3 months post

vaccination [5]. Since the observation period is limited to five weeks, it will be important in future studies to determine the duration of this effect, even more so as the *in vitro* effects seem to persist up to a year after vaccination [4].

Yet even in the possible absence of longer term effects, these findings may still have clinical implications for malaria as BCG vaccination may improve immunity to malaria, before sufficient adaptive immune responses have been generated to prevent (severe) disease. Although its immune modulatory properties have been known for decades [35], BCG is currently facing renewed interest after randomized controlled trials have shown it decreases neonatal mortality due to sepsis and respiratory infections [36, 37]. There is limited data on BCG and the incidence of malaria from observational studies, with one study showing a reduction in malaria mortality in BCG vaccinated infants [13]. Non-specific beneficial clinical effects of BCG vaccination might be explained by trained innate immunity [38, 39], supporting the further exploration of these effects to better inform the place of BCG in vaccination regimens. Though BCG vaccination is common practice in malaria endemic countries as part of the WHO Expanded Programme on Immunization, potential efficacy against malaria and other pathogens underscores the need for investment in timely and correct BCG administration. Epidemiological data and randomized trials suggest revaccination with live-attenuated vaccines such as BCG confers additional protection against all cause mortality [40]. It will be important to determine whether BCG revaccination induces non-specific beneficial effects against malaria. Although BCG revaccination did not reduce malaria morbidity in one study in Guinea-Bissau [41] potential confounding effects of other vaccines, including DTP with known interference with the overall non-specific effects of BCG [42] was not taken into account.

In conclusion, BCG vaccination alters some of the clinical, immunological and parasitological outcomes of malaria infection in a subset of volunteers. Earlier NK cell and monocyte activation in this subset of vaccinated volunteers is consistent with the possibility that induction of trained innate immunity *in vivo* may have functional activity against a heterologous pathogen in humans. These findings may open perspectives and pathways for clinical vaccine development.

Acknowledgements

We would like to thank Jolanda Klaassen, Laura Pelser-Posthumus, Jacqueline Kuhnen and Astrid Pouwelsen for technical assistance with generation of infected mosquitoes and with performing the malaria challenge infection. We also thank Heidi Lemmers, Kiki Schraa, Helga Dijkstra, Michelle Damen, Mark Gresnigt, Martin Jaeger, Berenice Rosler for their assistance with the PBMC isolations and cytokine ELISAs. We thank Prof. Jan Smit for his advice as safety monitor for the clinical trial. Finally, we would like to thank all the volunteers who participated in this trial.

Funding

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Competing interests

The authors declare no competing interests.

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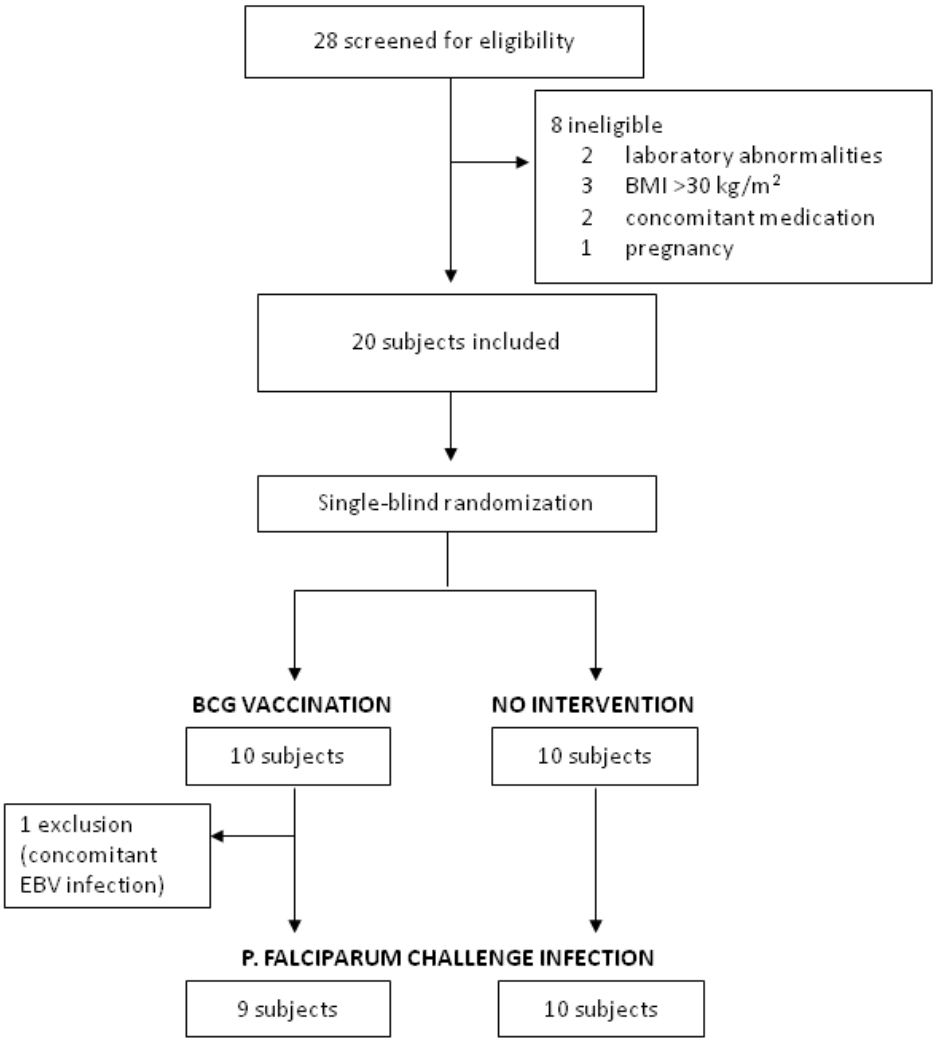
Supplementary Materials



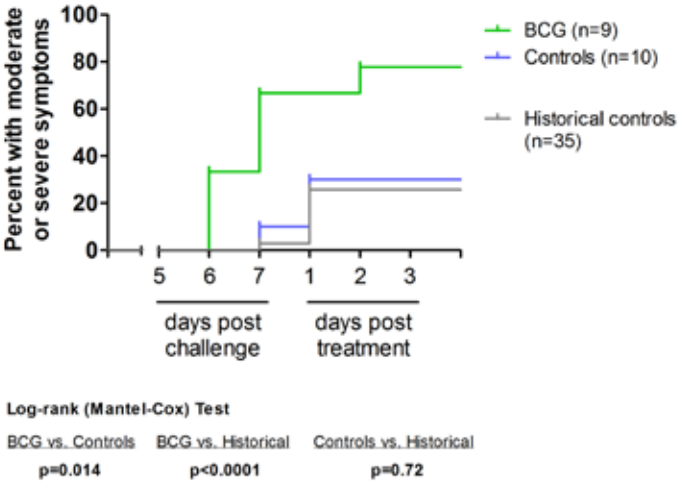
Supplementary figure 1: clinical trial flow chart. BMI = Body Mass Index; EBV = Epstein-Barr Virus

Supplementary table 1: baseline characteristics of study subjects.

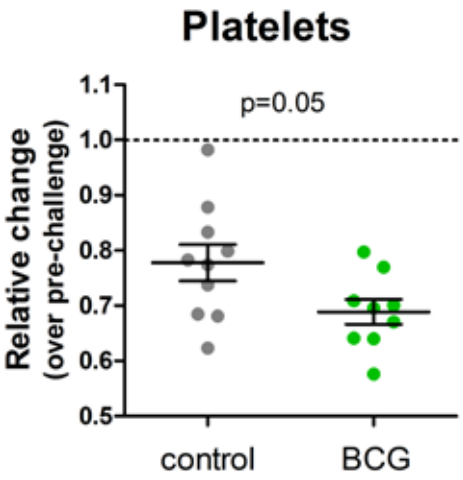
	BCG vaccinated (n=10)	Controls (n=10)	Total (n=20)
Age (years) ± SD	**21.2 ± 2.1**	**23.0 ± 3.2**	**22.1 ± 2.8**
Sex (*n* (%))			
Female	7 (70)	6 (60)	13 (65)
Male	3 (30)	4 (40)	7 (35)
Race (*n* (%))			
Caucasian	10 (100)	9 (90)	19 (95)
Afro-Caribbean	0 (0)	1 (10)	1 (5)

Supplementary table 2: adverse events reported by BCG vaccinated and control volunteers. Solicited adverse events were collected from day 6 after the malaria infection until day 3 after antimalarial treatment. Below the number and percent of BCG vaccinated or control volunteers reporting a specific adverse event is listed, for adverse events occurring before antimalarial treatment and adverse events reported after antimalarial treatment.

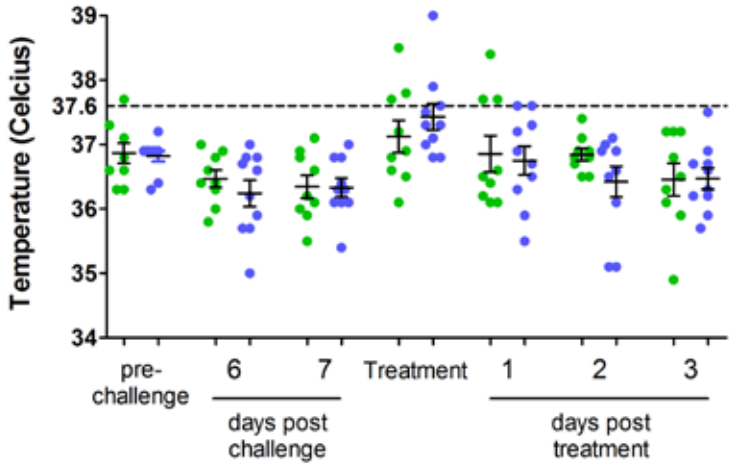
	Pre-treatment		Post-treatment	
	BCG	**Control**	**BCG**	**Control**
Headache	**5 (56%)**	**2 (20%)**	**8 (89%)**	**4 (40%)**
grade 1	4 (44%)	2 (20%)	5 (56%)	2 (20%)
grade 2	1 (11%)	0 (0%)	3 (33%)	2 (20%)
grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Malaise/chills	**1 (11%)**	**0 (0%)**	**1 (11%)**	**1 (10%)**
grade 1	0 (0%)	0 (0%)	0 (0%)	1 (10%)
grade 2	1 (11%)	0 (0%)	1 (11%)	0 (0%)
grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Fever	**3 (33%)**	**1 (10%)**	**5 (56%)**	**3 (30%)**
grade 1	2 (22%)	0 (0%)	3 (33%)	2 (20%)
grade 2	1 (11%)	1 (10%)	2 (22%)	0 (0%)
grade 3	0 (0%)	0 (0%)	0 (0%)	1 (10%)
Myalgia/arthalgia	**2 (22%)**	**1 (10%)**	**3 (33%)**	**3 (30%)**
grade 1	2 (22%)	1 (10%)	2 (22%)	3 (30%)
grade 2	0 (0%)	0 (0%)	1 (11%)	0 (0%)
grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Nausea/vomiting	**4 (44%)**	**0 (0%)**	**5 (56%)**	**1 (10%)**
grade 1	1 (11%)	0 (0%)	3 (33%)	0 (0%)
grade 2	0 (0%)	0 (0%)	1 (11%)	0 (0%)
grade 3	3 (33%)	0 (0%)	1 (11%)	1 (10%)
Abdominal pain	**0 (0%)**	**1 (10%)**	**1 (11%)**	**1 (10%)**
grade 1	0 (0%)	1 (10%)	1 (11%)	0 (0%)
grade 2	0 (0%)	0 (0%)	0 (0%)	1 (10%)
grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Diarrhoea	**1 (11%)**	**0 (0%)**	**3 (33%)**	**1 (10%)**
grade 1	1 (11%)	0 (0%)	3 (33%)	1 (10%)
grade 2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
grade 3	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Fatigue	3 (33%)	1 (10%)	5 (56%)	0 (0%)
grade 1	1 (11%)	0 (0%)	4 (44%)	0 (0%)
grade 2	1 (11%)	1 (10%)	1 (11%)	0 (0%)
grade 3	1 (11%)	0 (0%)	0 (0%)	0 (0%)



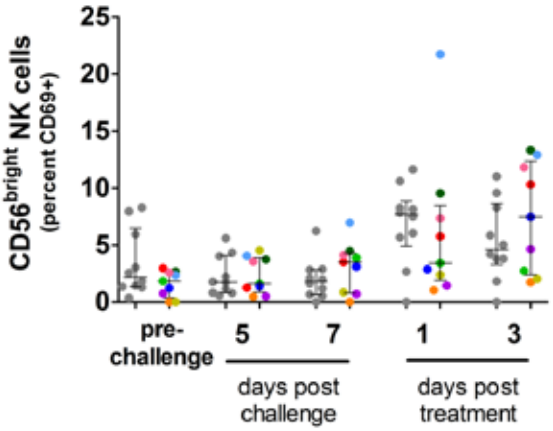
Supplementary figure 2: clinical symptoms in BCG and control volunteers versus historical controls. Adverse events were collected daily. The Kaplan-Meier curve shows the percentage of volunteers experiencing one or more moderate or severe, solicited, symptoms during follow-up on day 5-7 after challenge and day 1-3 after antimalarial treatment. BCG vaccinated volunteers (green) experienced earlier and more moderate/severe symptoms than controls (blue). 35 volunteers participating in other CHMI studies using the same parasite strain and the same treatment criteria (grey) also differed significantly from the BCG vaccinated volunteers but not the controls.



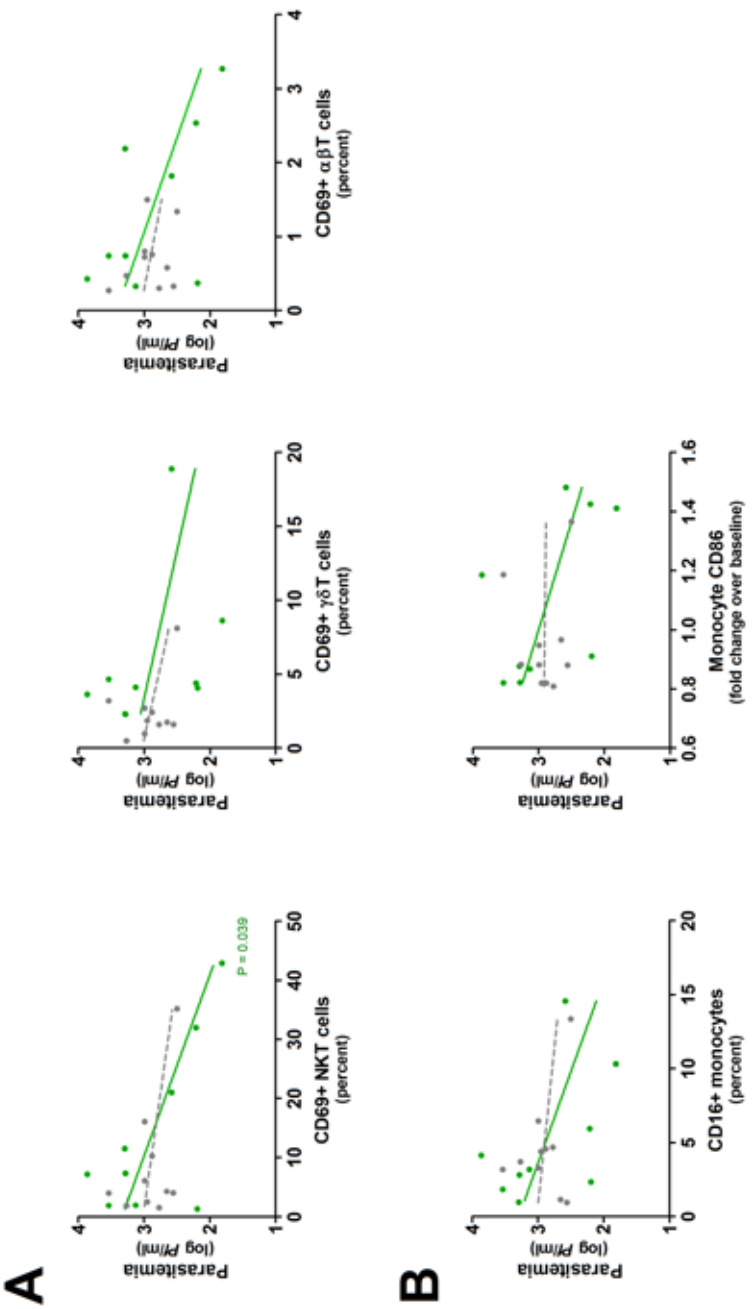
Supplementary figure 3: Platelet decreases during infection in BCG and control volunteers. Graph shows the relative change in circulating platelets between baseline and the lowest measurement during follow-up in BCG vaccinated (green) versus control (grey) volunteers. P-value is the result of a student's t-test.



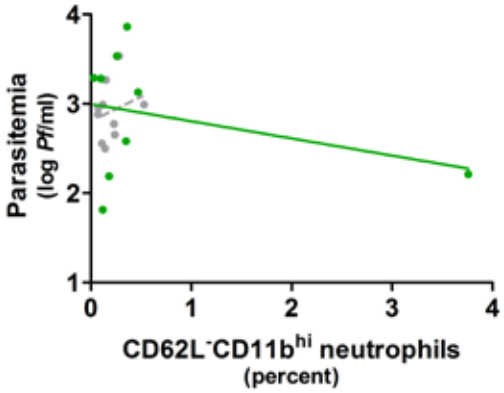
Supplementary figure 4: temperature in BCG vaccinated and control volunteers. Tympanic temperature was measured daily and at moment of antimalarial treatment in BCG vaccinated (green) and control (blue) volunteers. The graph shows temperature on day 6 and 7 post infection, at treatment and on day 1-3 post treatment for all volunteers.



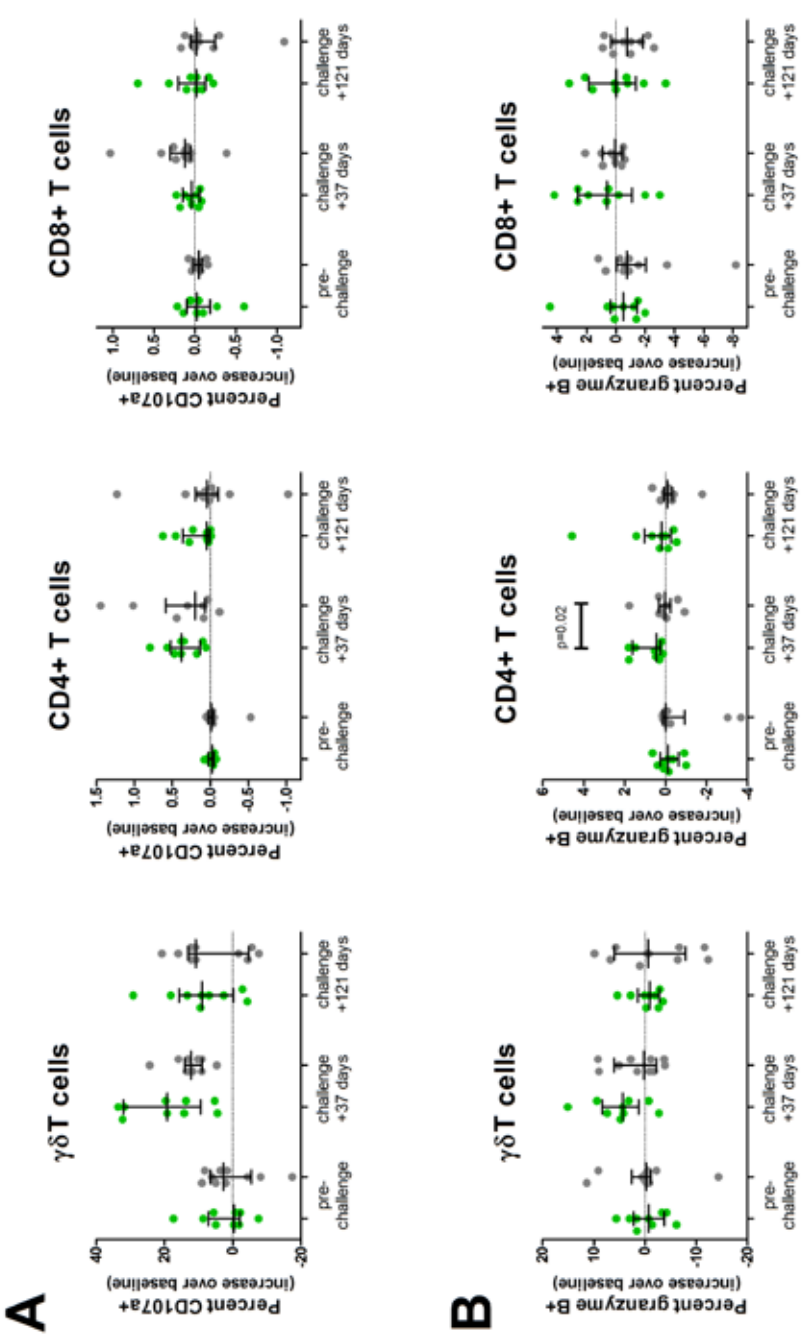
Supplementary figure 5: *in vivo* activation of CD56^{bright}CD16- NK cells. *In vivo* leukocyte activation was determined by direct staining of fresh whole blood with fluorescent antibodies every two days post challenge. Lymphocytes were defined based on forward scatter and sideward scatter characteristics, and duplet events were excluded. NK cell activation was defined as the percentage of CD3^{-}CD56^{bright}CD16^{-} live cells expressing CD69. The grey dots show the non-BCG vaccinated control group volunteers (n=10) and each colored dot shows an individual BCG vaccinated volunteer (n=9). Lines and error bars show the median and interquartile range of each group.

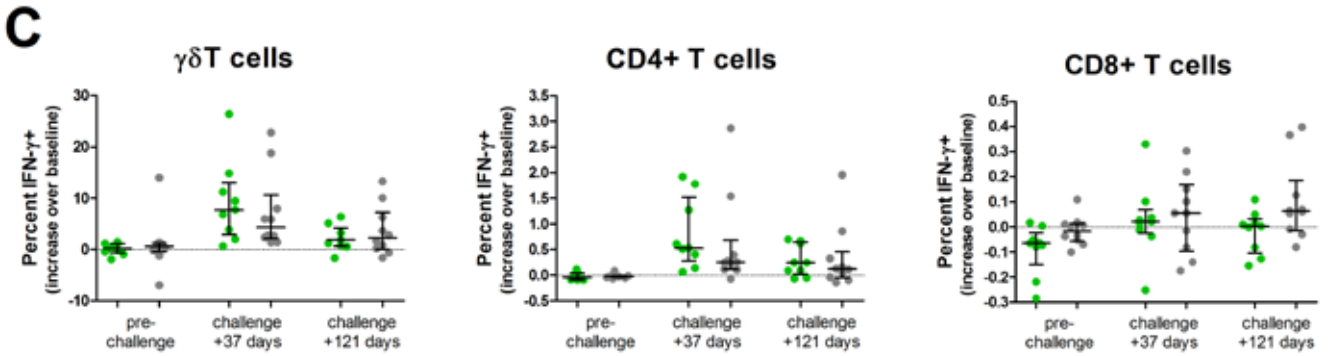


Supplementary figure 6: BCG vaccinated volunteers with accelerated immune activation had lower parasitemia. (A) Correlations between CD69 expression in different lymphocyte subset and log parasitemia on day 7 after challenge infection, **(B)** percentage $CD16^+$ monocytes and increase in $CD16^-$monocyte CD86 MFI and log parasitemia on day 7 are shown for BCG vaccinated volunteers (green) and control volunteers (grey). Lines show the result of linear regression analysis, p-values are shown if $p<0.05$.

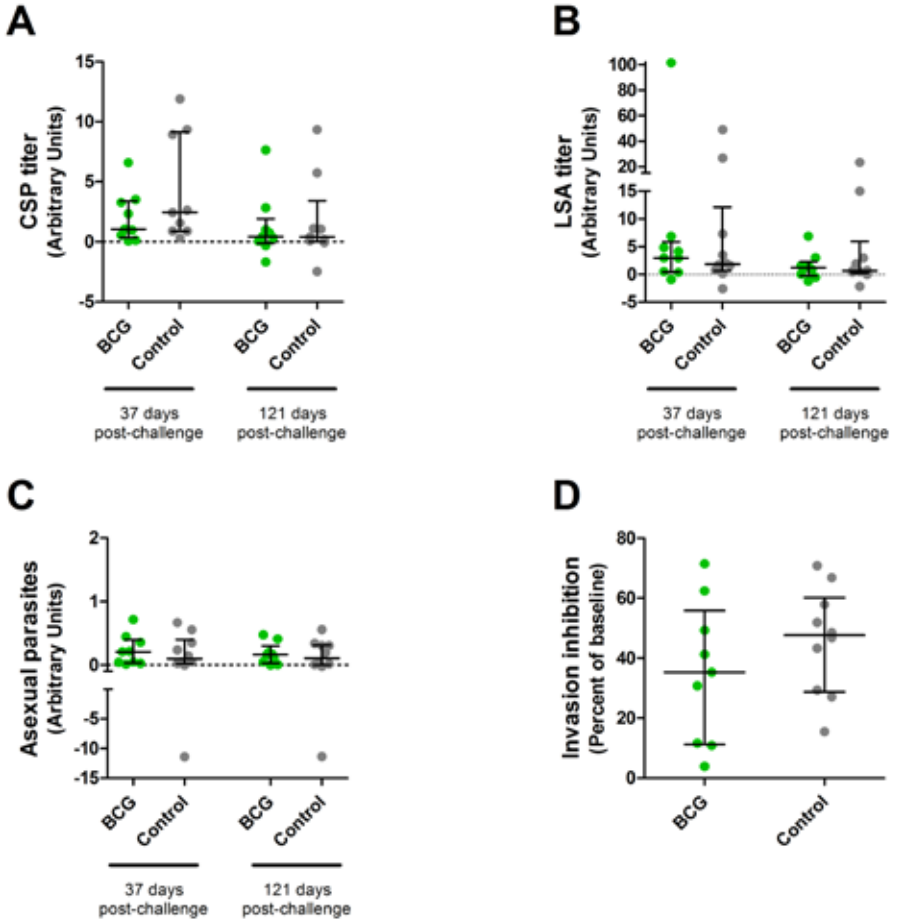


Supplementary figure 7: Neutrophil activation and parasitemia in BCG vaccinated volunteers. Correlations between neutrophil activation, defined as percentage of neutrophils that are CD62L-CD11b^{hi}, and log parasitemia on day 7 after challenge infection are shown for BCG vaccinated (n=9, green) and control (n=10, grey) volunteers. Lines show the result of linear regression analysis for both groups.


A
γδT cells
CD4+ T cells
CD8+ T cells
Percent CD107a+
(increase over baseline)
pre-
challenge
challenge
+37 days
challenge
+121 days
B
γδT cells
CD4+ T cells
CD8+ T cells
Percent granzyme B+
(increase over baseline)
p=0.02



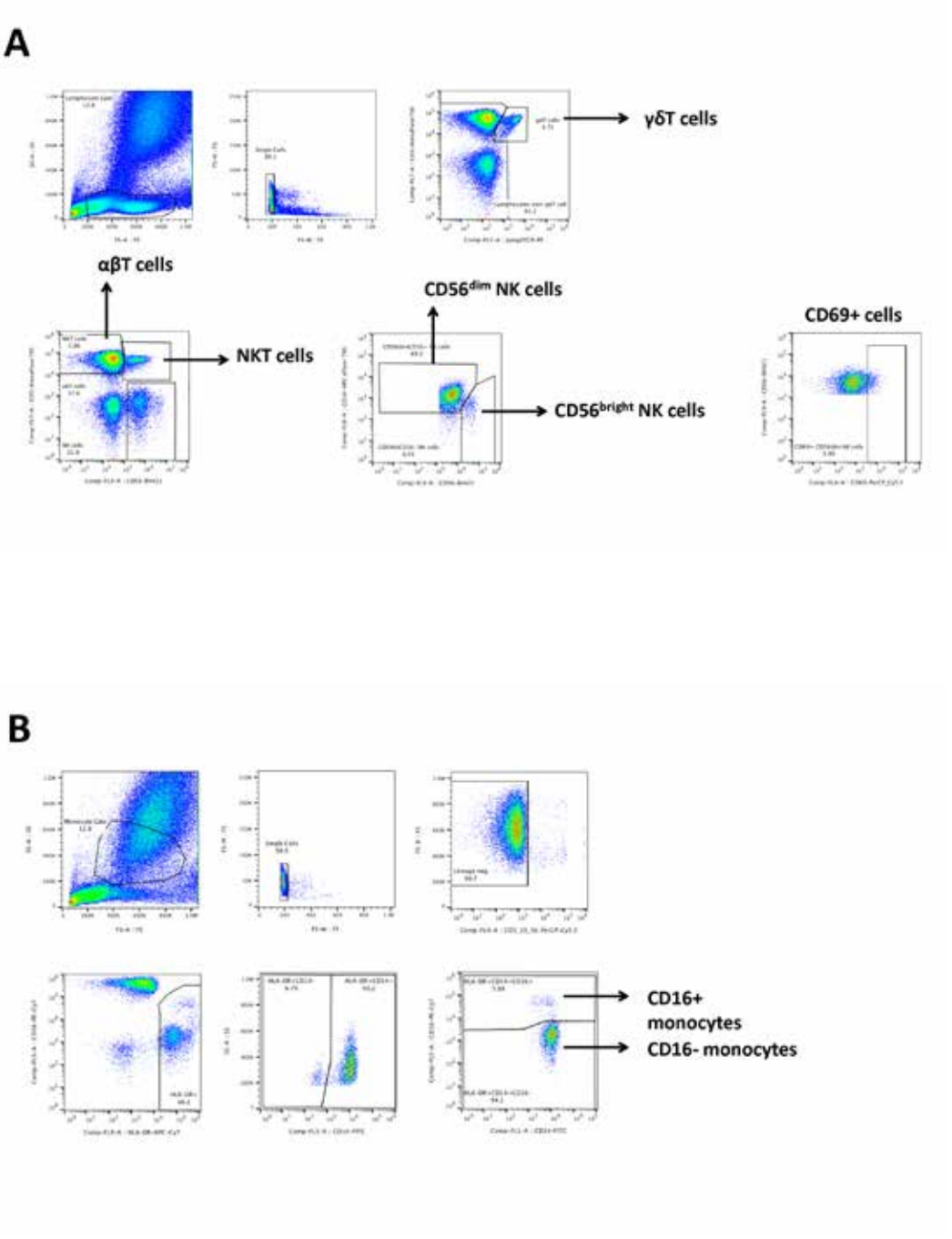
Supplementary figure 8: *P. falciparum*-specific T cell responses in BCG and control volunteers. Percentage of cells staining positive for **(A)** CD107a, **(B)** granzyme B and **(C)** IFN-γ after 24 hours of stimulation with *Pf*RBC before, and 37 and 121 days after malaria challenge infection as compared to baseline (pre-BCG vaccination time point) in BCG vaccinated (green) and control volunteers (grey). Lines and error bars show median and interquartile range. P-values are the result of Mann-Whitney U test.

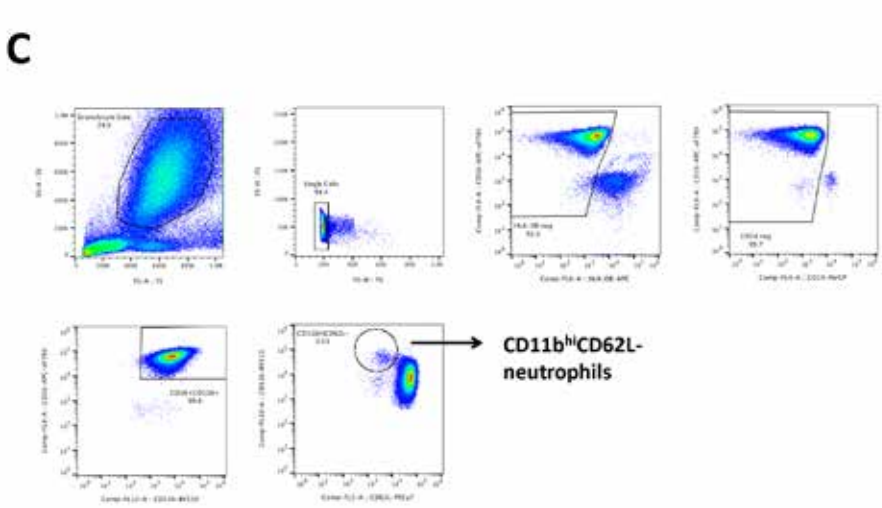


Supplementary figure 9: BCG vaccination does not affect *Pf*-specific antibodies. (A) Antibody titers to circumsporozoite protein (CSP), **(B)** liver stage antigen (LSA) and **(C)** total asexual parasite lysate 37 and 121 days post challenge in BCG vaccinated versus controls. Values are corrected for pre-CHMI levels and shown as percentage of positive control (serum from Tanzanian adults in a highly endemic area). **(D)** Percentage inhibition of sporozoite invasion in HC04 hepatoma cells by plasma taken 37 days post CHMI, controlled for pre-CHMI plasma in BCG vaccinated versus controls.

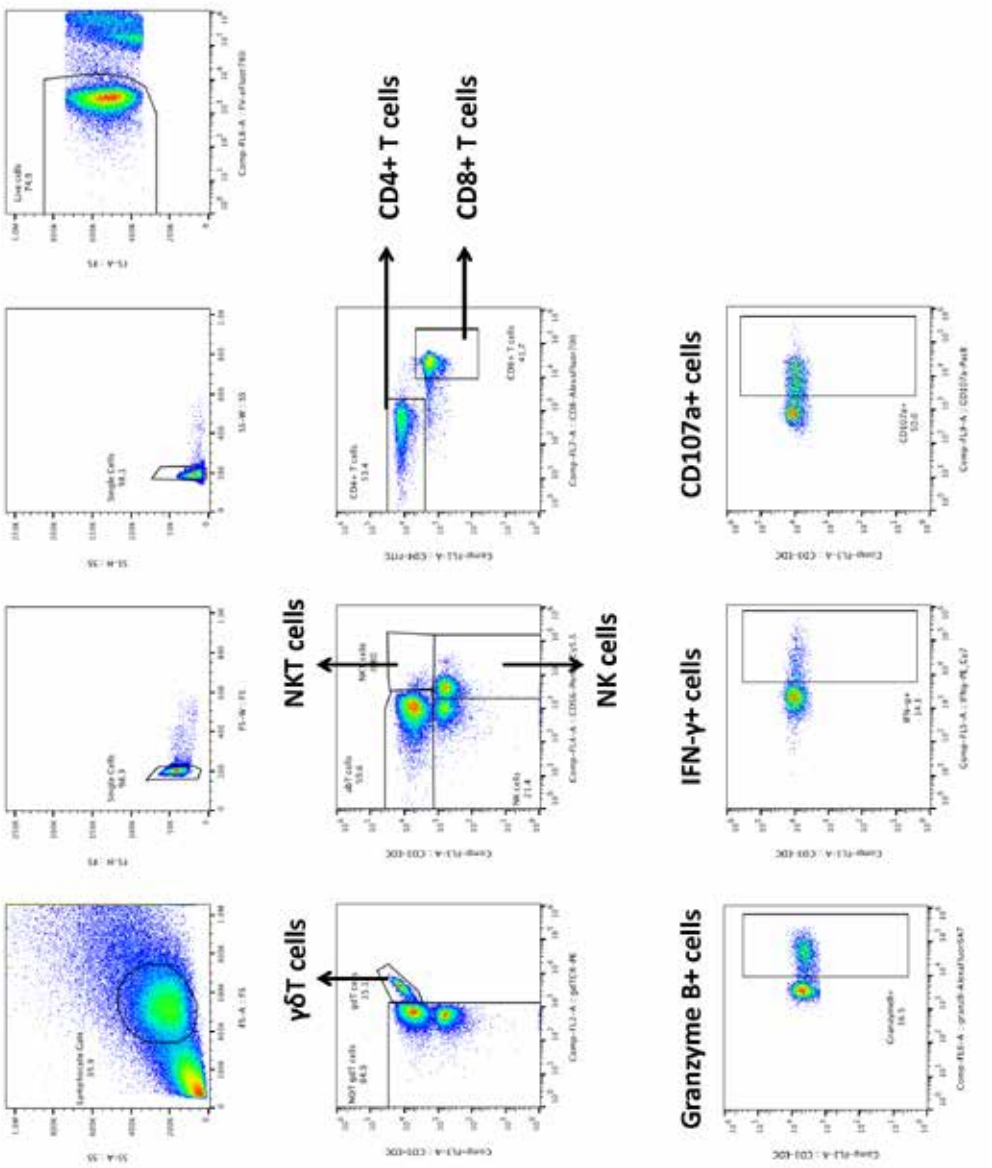
Supplementary table 3: **data on mosquito infection and controlled human malaria infection.** All volunteers were infected with the same batch of *P. falciparum* infected *Anopheles stephansi* mosquitoes. Batch infectivity and mean sporozoite load was determined by dissection of a sample of 10 mosquitoes one day before the challenge infection. All volunteers received exactly 5 bites from infected mosquitoes. Most volunteers required only one or two sessions for a sufficient number of infected mosquito bites, with a single exception who required a third session.

	Mosquito infectivity		Infection		
	Percent	**# Sporozoites per mosquito**	**Number of sessions median (range)**	**# Infected bites median (range)**	**# Uninfected bites median (range)**
BCG group	100%	160,500	1 (1-3)	5	0 (0-1)
Control group			1 (1-2)	5	0 (0-1)


A
γδT cells
αβT cells
NKT cells
CD56dim NK cells
CD56bright NK cells
CD69+ cells
B
CD16+
monocytes
CD16- monocytes



Supplementary figure 10: gating strategy for whole blood flow cytometry of lymphocytes (A), monocytes (B) and neutrophils (C).



Supplementary figure 11: gating strategy *ex vivo P. falciparum* lymphocyte stimulation.

PART 3

Hepatic lymphocytes against malaria liver stages

CHAPTER 9

Can Patrolling Liver-Resident T Cells Control Human Malaria Parasite Development?

Trends Immunol. 2019 Mar;40(3):186-196

Jona Walk, Jorn E. Stok, Robert W. Sauerwein

Abstract

Recently a population of non-recirculating, tissue-resident memory $CD8^+$ T cells have been identified, which seem to serve as key sentinels for invading micro-organisms with enhanced effector functions. In malaria, the liver represents the first site for parasite development before a definite infection is established in circulating red blood cells. Here, we discuss the existing evidence obtained from animal models of a number of diseases and hypothesize that liver-resident memory $CD8^+$ T cells (hepatic T_{RM}) play a critical role in providing protective liver stage immunity against *Plasmodium* malaria parasites. Though observations in human malaria trials are limited to the peripheral blood, we propose recommendations for the translation of some of these findings into human malaria research.

Immunity against Malaria Liver Stages

Malaria remains a significant global health burden, responsible for nearly half a million deaths every year [1]. After deposition in the skin by mosquitoes, the sporozoite stages of the human malaria parasite *Plasmodium falciparum* must mature and multiply in hepatocytes for 6-7 days [2]. Immune responses that control parasite development here can prevent the symptomatic blood stages completely. Although this sterilizing immunity is rarely seen in naturally exposed persons, it has been extensively described after immunization with viral-vectored subunit vaccines [3] or live-attenuated sporozoite vaccines [4]. These vaccines induce functional IgG antibodies [5], $CD4^+$ [6] and $CD8^+$ T cells [7, 8] in the blood of protected individuals. However, to date predictive correlates of protection in humans remain elusive and how exactly immune responses can control liver parasite development remains unknown. Studies in mice and non-human primates found that protective whole sporozoite immunization regimens induced higher numbers of *Plasmodium*-specific $CD8^+$ T cells in the liver than non-protective regimens [7, 9, 10], but this has not been confirmed in humans.

In evaluating protective immunity against malaria liver stages in humans, it is important to consider the liver's unique anatomy and distinctive immunological properties [11-13]. Blood entering the liver via the portal vein or the hepatic artery flows into vessels with a complex architecture called 'sinusoids'. The liver sinusoids have a fenestrated endothelium and the speed of blood flow in these sinusoids is strongly reduced. This allows for thorough scanning by immune cells [11, 13], but also allows hepatotropic pathogens to reach underlying hepatocytes [13]. Due to the large body of foreign, gut-derived antigens delivered via the portal circulation, the liver must also be immunologically tolerant to protect against continual inflammation [14].

The mechanism by which tissue-specific $CD8^+$ T cell immunity is achieved has received increasing attention in recent years. During primary infection, naïve T cells activated by antigen presenting cells (APCs) undergo clonal expansion and migrate into infected tissues to combat infection [15, 16]. However, after pathogen clearance only a fraction of these activated $CD8^+$ T cells survive and differentiate into either central memory T cells (T_{CM}) that circulate or home into secondary lymphoid tissues, or effector memory T cells (T_{EM}) that circulate or enter peripheral tissues [15, 16]. In the past decade, a third population of memory $CD8^+$ T cells, tissue-resident memory T cells (T_{RM}), have been shown to persist in non-lymphoid tissues of mice and humans, including the skin, gastro-intestinal tract, brain, lung, kidney and liver [16-

18]. A broad range of studies in mice -- including parabiosis experiments [19] and skin graft studies [20], as well as in humans -- including the discovery of highly localized T_{RM} in recurrent psoriasis skin lesions [21] and the maintenance of skin immunity after depletion of circulating T cells by Alemtuzumab treatment [22] have been undertaken. Collectively, results show that these cells remain localized in the tissue of the initial immune response. This suggests that they can provide immediate, font-line protective immunity against reinfection.

$CD8^+$ T cells recognize *Plasmodium berghei*-infected mouse hepatocytes [23-26], and recently, *Plasmodium*-specific T_{RM} have been discovered in the livers of immunized, protected mice and non-human primates. These T_{RM} appear to be functionally distinct from *Plasmodium*-specific circulating T_{EM} [25, 27]. Furthermore, when both blood and hepatic T cells are analyzed in mouse and non-human primate studies with whole sporozoite or viral-vectored malaria vaccines, is also apparent that peripheral blood cell responses do not quite represent and/or correlate with $CD8^+$ T cell responses in the liver [7, 28, 29]. Together, these novel findings are a critical consideration for human malaria vaccine studies where immunological evaluations are restricted to circulating leukocytes. Although clearly a major challenge for studies in humans, here we advocate that the specific location and function of hepatic $CD8^+$ T_{RM} cells renders them a key study focus as effector cells in the defense against *Plasmodia.* We recommend that future malaria immunization studies make an attempt to clarify the relative contributions of the two cell types to protection.

Implications of resident memory $CD8^+$ T cell differentiation for malaria parasite liver stages

To become hepatic T_{RM} cells, naïve T cells are either activated in secondary lymphoid organs subsequently homing to the liver, as observed in mice infected subcutaneously with *P. yoelii* or *P. berghei* sporozoites [30-32], or are directly activated in the liver as seen in mice after adoptive transfer of naïve $CD8^+$ T cells reactive to antigens expressed in liver cells [33, 34]. T_{RM} and T_{EM} share T cell receptor (TCR) repertoires, suggesting that they arise from a single progenitor T cell [35]. Studies in mice show that during herpes simplex virus (HSV) infections in the skin [36] and *Listera monocytogenes* infection in the gut [37], early effector T cells are recruited from the spleen and infiltrate infected tissue. In contrast, differentiated circulating effector T cells are unable to migrate to tissues. These early-infiltrating, activated effector T cells differentiate into non-circulating memory T_{RM} cells [36, 37]. Unlike T_{EM}, these cells are characterized by low level expression of the killer cell lectin-like receptor subfamily G member 1 (KLRG1) that is associated with terminal T cell differentiation [36], suggesting that similar to KLRG1- T_{CM}, they can give rise to long-lived T cell populations.

Hepatic T_{RM} isolated from mice and human liver biopsies from either healthy donor livers or healthy tissue removed during liver surgery, express a distinct surface marker profile; like T_{EM} cells they are $CD62L^{low}$ and $CCR7^{-}$, but T_{RM} can be distinguished by the expression of CD69 -- a canonical activation marker of T cell parenchyma residence across many tissues [18, 38], as well as by high expression of the chemokine receptors CXCR3 and CXCR6 [25, 39, 40].

This is relevant because chemotactic signals, such as the production of CXCR3 and CXCR6 ligands by hepatocytes and liver sinusoidal endothelial cells (LSECs), likely attract and keep T_{RM} localized in the liver [36, 40-43]. CXCR6 is associated with T cell liver homing in humans [39] and is required for hepatic T_{RM} maintenance in mice after immunization with radiation attenuated *P. berghei* sporozoites, as CXCR6 knock-out mice do not maintain *P. berghei*-specific hepatic T cells [40]. CXCR3 is also known to be essential for localization of effector $CD8^{+}$ T cells to the epidermis after mice are infected with HSV and to the lung after mice are vaccinated against influenza A [36, 44]. However, though hepatic T cells express high levels of CXCR3 [39] that seem to promote adhesion and transmigration across hepatic endothelium in humans [42], CXCR3 knock-out mice are capable of generating and maintaining *P. berghei*-specific liver $CD8^{+}$ T cell populations after radiation attenuated sporozoite administration [40], suggesting these cells can adhere to hepatic endothelium. Moreover, CD103, a subunit of the αEβ7 integrin expressed by a fraction of T_{RM}, may also play a role in hepatic retention, as it binds adhesion molecule E-cadherin expressed on human hepatocytes [36, 38, 39], potentially keeping these $CD103^{+}$ T_{RM} in the liver.

Interleukin (IL)-15 and Transforming Growth Factor (TGF)-β are able to drive T_{RM} different-iation upon *in vitro* stimulations of human $CD8^{+}$ T cells [39]. IL-15 is constitutively produced in the liver [45]. Culture with IL-15 *in vitro* induces the surface expression of CD69, CXCR3 and CXCR6 on human peripheral blood $CD8^{+}$ T cells [39]. IL-15 signaling has also been implicated in the generation of T_{RM} in various other tissues in both humans and mice [36, 45-49]. However, it remains unclear whether it is an absolute requirement for hepatic T_{RM} production: on the one hand, it was recently found that IL-15 knock-out mice were unable to generate hepatic T_{RM} [50], on the other hand, T cell receptor engagement with CD3 cross-linking antibodies could replace IL-15 signaling when effecting differentiation of human $CD8^{+}$ T cells into a T_{RM} phenotype *in vitro* [39]. TGF-β is required for expression of CD103 and further downregulation of the memory precursor cell marker KLRG1 in cultured human peripheral blood $CD8^{+}$ T cells , and selective knock-out of the TGF-β receptor on mouse T cells prevents CD103 and promotes KLRG1 expression [51].

Hepatic T_{RM} differentiation after IL-15 and TGF-β stimulation is under tight transcriptional control by four transcription factors: T-bet, Eomesodermin (Eomes), Blimp1 and Homolog of Blimp1 in T Cells (Hobit); fully differentiated human liver-resident T_{RM} are characterized as T-bet^{lo}Eomes^{lo}Blimp1^{hi}Hobit^{lo} [38, 39]. However, the sequential expression of transcription factors in the generation of T_{RM} has been primarily delineated in mice. During the first phase of skin-resident T_{RM} differentiation, T-bet and Eomes, important transcription factors for T_{CM} and T_{EM} generation, are down-regulated [16, 18, 39, 52]. Eomes expression is completely abrogated at this stage but T-bet remains present at low concentrations to induce the expression of the IL-15 receptor for T_{RM} survival [53]. Both Blimp1 and Hobit are key transcription factors for the subsequent further development and tissue retention of mouse T_{RM} [54, 55].

It remains to be determined whether these pathways are similar in humans and how their function will be influenced by the liver-specific microenvironment. It is clear, however, that T_{RM} require additional steps for final and functional differentiation after infiltration into non-lymphoid tissue. Local signals appear to drive differentiation; the formation of tissue-specific T_{RM} populations during viral infections, for instance, is promoted by antigen deposition into non-lymphoid tissues [35, 56]. In mice, liver infection with either live-attenuated *Plasmodium berghei* parasites or hepatotropic viruses expressing *P. berghei* antigens is sufficient for the generation of T_{RM} without the need for adjuvant [25, 28, 57]. When the number of administered *P. berghei* sporozoites successfully infecting the liver is reduced (such as after subcutaneous or intramuscular needle injection) fewer hepatic CD8$^+$ T cells are generated [9]. Likewise, when viruses expressing *P. berghei* antigens fail to infect the liver they do not generate robust hepatic T_{RM} in mice [28]. A similar mechanism may govern the generation of *Plasmodium* specific T_{RM} in humans, as needle-based subcutaneous or intramuscular attenuated sporozoite immunizations that result in lower liver infections [58, 59] also provide inferior protective immunity [7-9], but this has not been studied. *P. berhgei* sporozoites infecting the mouse liver induce type 1 interferons (IFN) in hepatocytes and IFN-γ signaling attracts leukocytes to the site of infection [60, 61], but a subsequent differentiation pathway for T_{RM} remains elusive.

Still less is known about how T_{RM} populations are boosted after reinfection. Recently, it was demonstrated that T_{RM} in the mouse reproductive tract [62] and dermis [63] proliferate locally after *in situ* lymphocytic choriomeningitis virus (LCMV) and HSV antigen re-exposure, respectively. T_{RM} proliferation in the liver microenvironment has not been directly studied. However mathematical modeling of the liver and spleen CD8$^+$ T cell frequencies of mice after vaccination with live attenuated malaria parasites suggests that T_{RM} may undergo local boosting, as hepatic T_{RM} apparently increase without expansion of the spleen T_{CM} after repeat immunizations [64].

Table 1: Examples of phenotypic markers expressed on T cell subsets.

Naive T cells	**Central memory T cells (T_{CM})**	**Resident memory T cells (T_{EM})**
$CD45RA^{hi}$ $CD45RO^{lo}$ $CCR7^{+}$ $CD62L^{+}$ $CD27^{+}$	$CD45RA^{lo}$ $CD45RO^{hi}$ $CCR7^{+}$ $CD62L^{+}$ $CD27^{+}$	$CD45RA^{lo}$ $CD45RO^{hi}$ $CCR7^{+}$ $CD62L^{+}$ $CD27^{+/-}$ $CXCR6^{+}$ $CXCR3^{+}$ $CD69^{+}$ $CD103^{+/-}$ $KLRG1^{-}$
Effector T cells (T_{E})	**Effector memory T cells (T_{EM})**	
$CD45RA^{hi/lo}$ $CD45RO^{lo}$ $CCR7^{-}$ $CD62L^{-}$ $CD27^{-}$ $CXCR6^{+/-}$ $CXCR3^{+/-}$	$CD45RA^{lo}$ $CD45RO^{hi}$ $CCR7^{-}$ $CD62L^{-}$ $CD27^{-}$ $CXCR6^{+/-}$ $CXCR3^{+/-}$	

Resident memory T cells functions in the liver

It is likely that local signals not only govern the differentiation and retention of hepatic T_{RM} but also influence their survival and function. Comparisons of T_{RM} transcriptional profiles between various tissues in both humans and mice show that T_{RM} express a core set of genes that distinguishes them from circulating T_{EM} [25, 36, 54, 65-67]. Next to core T_{RM}-specific genes, hepatic T_{RM} must express liver-specific genes necessary for their retention and function on site [54, 68].

Hepatic T_{RM} appear to provide the first line of defense against (re)infection with liver-specific pathogens. In patients with hepatitis B (HBV) and hepatitis C (HCV) virus infection, there is an inverse correlation between virus-specific T_{RM} in the liver and viral load [69-71], suggesting that high numbers of T_{RM} can result in effective immunosurveillance. Several studies in mice also show that hepatic T_{RM} are localized in the vasculature of the hepatic sinusoids [25, 72, 73]; however, based on parabiosis experiments, these cells are unable to recirculate [25, 73]. Instead, intravital microscopy has demonstrated that unlike circulating T_{EM}, these cells migrate through sinusoids in a specific manner; they 'crawl', exhibiting an amoeboid shape, moving slowly and independently from the blood stream at a speed of 10 μm/min [25]. This patrolling behavior occurs even in the absence of antigen recognition [25, 26]. However, this study [26] did not distinguish

between T_{EM} and T_{RM}, in the mouse model of HBV infection used. Nevertheless, it appears that $CD8^+$ T cells found in the liver sinusoids patrol along the vessel until they encounter an infected hepatocyte, which they detect by extending cytoplasmic protrusions through the fenestrae between endothelial cells. Moreover, antigen recognition prompts $CD8^+$ T cell arrest in the sinusoid and activates effector functions including IFN-γ and granzyme production [26]. Slow-moving intra-hepatic $CD8^+$ T cells make contact with both hepatocytes and antigen presenting cells through the fenestrated endothelium [25, 74]. The combined observations strongly support the notion that hepatic T_{RM}, rather than recirculating T_{EM}, act as the primary cell type recognizing and targeting infected hepatocytes, although further experiments are warranted to fully validate this notion.

$CD8^+$ T cells recognize *Plasmodium berghei*-infected hepatocytes via antigen-dependent Major Histocompatibility Complex (MHC)-I presentation [23-26]. Considering that only T_{RM} are capable of effectively patrolling liver sinusoids, it is likely that these are the $CD8^+$ T cells that identify infection. This is supported by the observation that depletion of T_{RM} in mice immunized with radiation-attenuated sporozoites [25] and certain viral-vectored vaccines [28] abrogates protective efficacy against a subsequent *P. berghei* challenge infection. The number of *Plasmodium*-specific liver $CD8^+$ T cells have also correlated with protection against challenge infection with *P. berghei* in mice [9, 10] and *P. knowlesi* in non-human primates [27], and radiation-attenuated sporozoite immunization regimens that are protective against *P. falciparum* in humans induce higher hepatic T_{RM} in non-human primates than non-protective regimens [7, 8, 29]. Together these data suggest that patrolling T_{RM} must be able to 'cover' the entire liver, though additional studies are necessary to demonstrate this.

Regardless, in order to achieve protection, patrolling hepatic T_{RM} must either directly kill infected hepatocytes or effectively recruit other cells to site of infection. Both human and mouse T_{RM} are rapid and effective producers of IFN-γ, Tumor Necrosis Factor (TNF)-α and IL-2, in response to *in vitro* activation [25, 39, 75]. While human hepatic T_{RM} express low concentrations of IFNG and TNFA gene mRNA under homeostatic conditions [38], both human and mouse T_{RM} produce far more IFN-γ, TNF-α and IL-2 per cell upon stimulation than their T_{EM} counterparts [25, 39]. Human T_{RM} have also been found to have increased expression of the activation markers HLA-DR and CD38 on their surface compared to T_{EM}. These observations suggest that T_{RM} may be more responsive to activation signals than T_{EM} [38].

The ability of T_{RM} to attract and activate other immune cells has been demonstrated in the mouse reproductive tract [75]. In an apparent reversed order of traditional innate and adaptive immune responses, activation of LCMV-specific $CD8^+$ T_{RM} by local peptide

deposition, increased dendritic cell (DC) maturation and activation of natural killer (NK) cell populations at the site of infection compared to deposition of an irrelevant peptide [76]. Similarly, activation of HSV-1-specific T_{RM} in mouse skin by deposition of a single peptide induced broad transcriptional changes in skin cells, with an upregulation of 89 genes associated with inflammation and immunity. Most of these transcriptional changes did not occur when IFN-γ-receptor knock-out T_{RM} were activated, demonstrating the response is dependent on IFN-γ signaling [77]. It remains to be shown whether hepatic T_{RM} will be similarly able to activate the large numbers of other lymphocytes such as NK cells or semi-innate lymphocytes like NKT, Mucosal-associated invariant T (MAIT) or γδT cells present in the liver, or induce transcriptional changes in hepatocytes.

T_{RM} seem to be able to act as local scouts with signaling skills, but whether they can directly kill infected hepatocytes remains unclear. While liver T_{RM} can indeed produce granzyme B in both humans and mice [25, 38, 39], their synthesis of perforin is more controversial [38, 39], as is the absolute requirement of Fas- or perforin-mediated cellular cytotoxicity for protection against malaria liver stages protection [78, 79]. Therefore the potential relevance of the cytotoxic effector functions of hepatic T_{RM} requires further clarification. Regardless, these combined observations of T_{RM} in various disease models indicate that these cells can be highly responsive to local pathogens.

Induction of hepatic T_{RM} by vaccination

Liver-resident $CD8^+$ T cells represent a distinct population of T cells that differentiates locally and is uniquely capable of recognizing infected hepatocytes and rapidly responding with IFN-γ, TNF-α and IL-2production [25, 28, 39]. Based on these observations, it is tempting to consider the approach of inducing hepatic T_{RM} as a major objective for malaria vaccination strategies, further discussed in box 1. In fact, immunization studies in mice have long indicated that depletion of $CD8^+$ T cells abrogates protection against pre-erythrocytic stages [10, 80-82]. Recently, such murine studies demonstrated that the hepatic immune response to immunization with radiation-attenuated *P. berghei* sporozoites and viral-vectored vaccines expressing *Plasmodium* antigens [25, 28, 64, 83] is dominated by hepatic T cells with a T_{RM} phenotype. While induction of intrahepatic $CD8^+$ T cell responses has also been shown in non-human primates [7, 27, 29], the picture in human studies is much less clear; some studies find CD8+ T cell responses are important in protection against *P. falciparum* malaria [84, 85], but other clinical studies have identified $CD4^+$ T cells [6], γδT cells [29] and antibodies [5, 86] as correlates of protective immunity against challenge infection. In fact, in many human trials using whole-sporozoite immunization, induced $CD8^+$ T cell responses measured in total peripheral blood mononuclear cell (PBMC) fractions have been documented to be low, even though the volunteers were protected from *P. falciparum* malaria challenge (for example: NCT01441167/NCT02015091 [29] and

NCT02098590 [87]) [29, 87]. However, non-human primate studies have shown that after whole sporozoite immunization the numbers of *P. falciparum* or *P. knowlesi*-specific $CD8^+$ T cells producing IFN-γ, TNF-α or IL-2 is far higher in the liver than peripheral blood [27, 29], indicating that *Plasmodium*-specific $CD8^+$ T cells may be hidden in the liver. These cells may be responsible for protection, as in the mouse studies [25, 28], but they would be excluded from the current PBMC analyses in human trials [8, 29].

Nevertheless, the relative importance of the $CD8^+$ T cell response appears to vary between immunization types, dosages and routes of administration, and even between vaccinees. The importance of $CD8^+$ T cells in immunity has also been challenged by adoptive transfer experiments performed in mice that show that extremely high numbers of *P. berghei*-specific $CD8^+$ T cells must be transferred in order to achieve for complete protection against malaria [25, 88, 89]. Yet in mice vaccinated with radiation-attenuated sporozoites, the numbers of hepatic T_{RM} already begin to wane during the first 100 days after immunization, while protection against malaria is maintained [64]. Similarly, high levels of sterile protection have been described in humans exposed to a *P. falciparum* challenge infection up to 2.5 years post-immunization with live-attenuated sporozoites [29, 90]. The discrepancy might be explained by the fact that adoptive transfer experiments have not yet accounted for the possibility of $CD8^+$ T cells providing protection against challenge infection in combination with *Plasmodium*-specific $CD4^+$ T cells and antibodies.

Therefore, much remains unknown and the role of T_{RM} in protection may be more complex; rather than being the sole effectors against infected hepatocytes, $CD8^+$ T_{RM} may act as sentinels, patrolling sinusoids and responding swiftly with a cytokine alarm upon encountering an infected hepatocyte. These cytokines might inhibit schizont development locally but also attract other effector cells to the liver, and perhaps even induce tissue-wide hepatic changes that further suppress infection. In conjunction, antibodies may play an important role by reducing the liver parasite load, thus making it possible for T_{RM} to provide sterile protection.

With the ongoing efforts of clinical malaria vaccine development specifically aiming to induce hepatic T_{RM} [4, 7, 28, 82], it will be essential to gain a more complete picture of how this subset can contribute to protection against malaria.

$CD8^+$ T_{RM} and control of parasite liver stages: what's next

Plasmodium-specific hepatic $CD8^+$ T cell activity appears to be both antigen specific and MHC-I-dependent in murine models [23-25]. However, the antigen target of these $CD8^+$ T cells remain unknown and therefore there is no data on the kinetics of potential target antigens during the development of *Plasmodium* liver stages. Nevertheless, the unique

location and function of patrolling T_{RM} makes them the most likely cells activated by a secondary *Plasmodium* liver stage infection. Recent studies showed that *P. berghei*-specific T_{RM} isolated from mouse livers degranulated and produced IFN-γ when they were restimulated with *P. berghei in vitro* [25] or were found near liver schizonts *in vivo* in sections of infected mouse livers examined by fluorescence microscopy [28]. Furthermore, numbers of hepatic T_{RM} correlate with protection, which is abrogated by their depletion in mice [25, 28]. Taken together these results suggest that hepatic T_{RM} might be able to control liver stage development (Key Figure, Figure 1). However, this remains speculative and a number of questions remain to be addressed. Moreover, whether or how *Plasmodium*-specific T_{RM} directly kill infected hepatocytes remains unknown. We hypothesize that they may be able to lyse target cells through Fas- or perforin-mediated cellular cytotoxicity, or hepatic T_{RM} may directly control parasite development via cytokines only. T_{RM} are potent IFN-γ and TNF-α producers; total $CD8^+$ T cells or flow-cytometry sorted $CD8^+$ T_{RM} from immunized murine and non-human primate livers produce high levels of IFN-γ in response to *P. berghei, P. knowlesi or P. falciparum* antigen *in vitro* [7, 25, 27-29]. Moreover, there is circumstantial evidence supporting a direct effect of IFN-γ on liver parasites; specifically studies in mice show that IFN-γ production during a primary *P. berghei or P. yoelii* infection decreases the liver parasite burden during a secondary liver stage infection [61, 91], and injection of recombinant IL-12 or IFN-γ reduces or entirely prevents the onset of blood stage infection after sporozoite challenge in rodents [92-94] and non-human primates [95-97]. However, these results remain far from definitive. Future studies should focus on clarifying the effector functions of *Plasmodium*-specific T_{RM} and determining their effectiveness against liver schizonts.

Finally, it is possible that activated T_{RM} recruit other cell types to the site of *Plasmodium*-infected hepatocytes to kill these target cells. Studies in mice show that lymphocytes and Kupffer cells cluster around liver schizonts [28, 60, 98], but the effector functions of these cells have not been clarified. Activated T_{RM} have the ability to issue tissue-wide alerts and recruit other cell types in the mouse skin [77] and reproductive tract [76]. It will be important to determine whether this cell recruitment and/or potential cell to cell interactions occur in hepatic malaria as well. A better understanding of these processes may reveal how *Plasmodium*-specific T_{RM} could target infected hepatocytes throughout the liver to achieve sterile protection [25, 99].

Future Recommendations

To date all data on *Plasmodium*-specific T_{RM} have been generated in murine and non-human primate models with the challenge to explore and possibly translate these data to humans. Malaria candidate vaccine trials conducted in health volunteers face ethical and practical challenges in accessing *P. falciparum*-specific T_{RM} from malaria protected

individuals, which has significantly impeded exploration of this pathway. Recently, significant progress has been made in studying the phenotype and transcriptome of T_{RM} isolated from healthy human liver segments [38, 39]. However, to our knowledge this has never been extended to *P. falciparum* infection and there are still significant gaps in our knowledge that impede extrapolation of murine data to human data to inform rational malaria vaccine development.

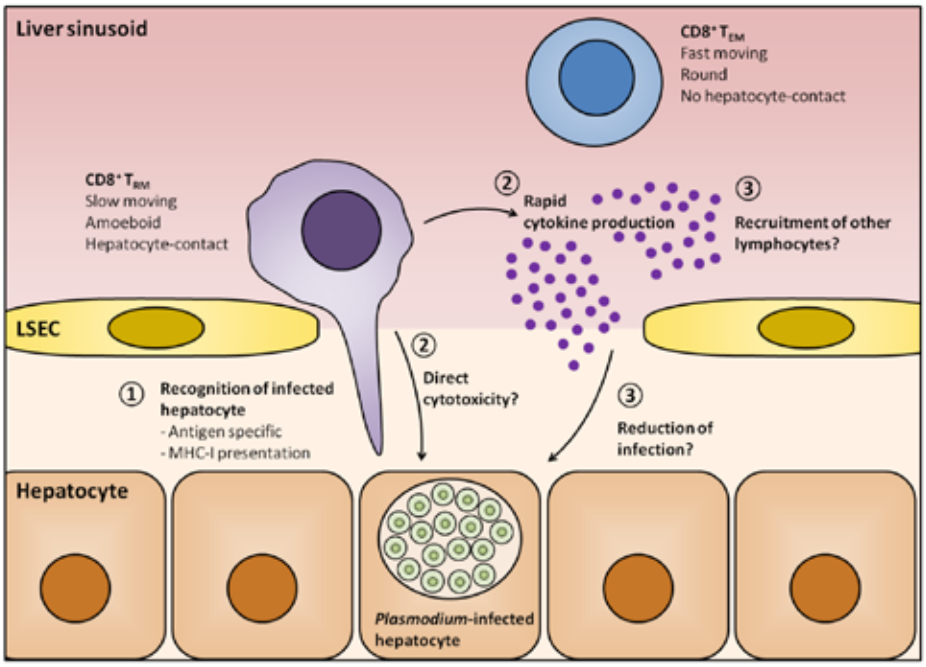


Figure 1: Model proposing a key role for hepatic T_{RM} in controlling human liver stage parasite development. Unlike effector memory T cells (T_{EM}), tissue-resident T cells (T_{RM}) travel slowly and make contact with hepatocytes through fenestrae between liver sinusoidal endothelial cells (LSEC) [25, 26]. (1) *Plasmodium falciparum*-specific T cells subsequently recognize infected hepatocytes via major histocompatibility complex (MHC)-I presented antigens, though the target antigens and their kinetics during the malaria life cycle remain unknown [23-25]. (2) Upon activation these cells rapidly produce large amounts of interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-2 [7, 25, 27, 28], but whether hepatic T_{RM} can directly kill target cells remains unknown. (3) Secreted cytokines such as IFN-γ may be able to inhibit liver stage hepatocyte infection and/or recruit circulating or nearby lymphocytes to the site of infection, but neither process has been established yet.

A first challenge will be to better define the capacity of human T_{RM} to secrete cytokines and/or engage in cytolysis. To date, few functional analyses have been performed on human hepatic T_{RM}. A second challenge for human malaria vaccine trials is the lack of a clear phenotypic marker for liver residency. Matched hepatic and peripheral blood

T cells should also be used to better establish which markers, or combination of markers, are differentially expressed between the two populations. It may be possible to detect activated T cell populations expressing tissue residency markers in the peripheral blood during malaria immunization and to determine whether any of these markers predict the development of liver-stage protective immunity. Alternatively, T_{RM} could be defined based on their unique transcriptional signature. The increasing availability of single-cell level RNA analysis may make it possible to identify low numbers of circulating $CD8^+$ T cells with a liver resident signature, suggesting an expansion of this cell population in the liver after immunization.

Moreover, a humanized mouse model engrafted with human liver and a human immune system could also provide key insights into the functional abilities of human T_{RM} to control *P. falciparum* development. Unfortunately, currently available mouse models do not yet support such systems (reviewed in [100]). However, rapid advancements in this field may make these functional T cell assays possible in the coming future.

Concluding remarks

Several promising malaria immunization strategies are designed to induce protective $CD8^+$ T cells in the liver, but the recent discovery of tissue-resident $CD8^+$ T cells has significantly impacted this field. Hepatic T_{RM} appear to be functionally distinct from circulating T_{EM} and uniquely capable of providing rapid, local protection against secondary infection of the liver. Based on a number of recent studies, we propose that these cells may play a key role in protection against *P. falciparum* liver stages by rapidly secreting pro-inflammatory cytokines after recognition of an infected hepatocyte. Consequently the focus on clinical development of malaria vaccines that induce high total T cell responses may have to redirect to candidate vaccines that generate robust hepatic $CD8^+$ T_{RM}, and future studies are warranted to validate this hypothesis. Accordingly, increasing our understanding of the functional capacities of human hepatic T_{RM} may shed a better light on their critical role in protective liver immunity. Many questions remain and human studies on T_{RM} remain limited to peripheral blood and indirect observations (see Outstanding Questions). Thus finding ways to distinguish circulating T_{RM} from T_{EM} in future studies will be critical to understanding the specific roles of these T cell types in protection against malaria infection as well as other pathogens. Indeed it will be interesting to follow future advances highlighting the roles of tissue-resident lymphocytes during infections.

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CHAPTER 10

Interferon-γ directly inhibits intra-hepatic *Plasmodium falciparum* development

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Abstract

Protective immune responses induced by live sporozoite immunization, including Chemoprophylaxis and Sporozoites (CPS), are considered to act via local cellular immune responses with a major role for interferon-γ (IFN-γ). Here we show that parasite-specific IFN-γ production particularly in CD4+ T-cells, after a single round of CPS-immunization via mosquito bites is significantly higher in volunteers protected against parasitaemia from a second round of immunization. In addition, IFN-γ responses in intra-hepatic innate and adaptive T cells *in vitro* in malaria-naïve volunteers are higher than in circulating T cell counterparts. Finally, IFN-γ alone was able to inhibit >95% of developing liver schizonts in primary human hepatocyte cultures when added during the first 2 days post-invasion. The combined data highlight the potent capacity of local liver IFN-γ production with direct functional inhibition of parasite liver-stage development *in vitro* which may contribute to prevention of clinical parasitaemia.

Introduction

A pre-erythrocytic malaria vaccine that offers sterile protection, completely preventing the development of the pathological blood stages would be an invaluable tool for disease eradication [1]. To date the only registered vaccine that aims to do this is RTS,S (Mosquirix; GlaxoSmithKleine), which gives approximately 30% short-term protection against clinical disease [2, 3].

To achieve sterile protection it is generally considered essential to induce humoral or cellular effector responses that prevent invasion and/or maturation of sporozoites in infected liver cells. Several approaches, including viral-vectored liver antigen vaccines [5, 6], radiation attenuated live sporozoite vaccines [7, 8] and immunization with non-attenuated sporozoites under chemoprophylaxis (CPS immunization) [9, 10] are thought to do so via *Plasmodium*-specific intrahepatic CD4+ and CD8+ T cell responses [5, 12, 13]. Such immunization regimes induce IFN-γ production in multiple lymphocyte populations [13-15], suggestive for a potential key role [7, 14, 16, 17].

To date, most of the functional data on IFN-γ in malaria has been obtained using rodent models. During a primary *P. berghei* or *P. yoelii* infection, schizont development induces type I interferons in infected hepatocytes, which in turn recruits IFN-γ producing lymphocytes [18-20]. This response appears to be able to protect against a secondary liver stage infection [18, 20]. Similarly, injection of IFN-γ or IL-12 prior to sporozoite infection or even during parasite-liver stage development prevents the onset of blood stages in rodents [21-23] or non-human primates [24-26]. However, these functional data have not been translated to humans.

After completion of the regime of three CPS immunizations, >95% of volunteers are fully protected against a homologous challenge [10, 12, 27] with IFN-γ-producing peripheral blood mononuclear cells (PBMCs) in virtual all participants [10, 12, 28]. However, there is great variability in the dynamics of acquired protective immunity during the course of immunization. Roughly half of volunteers do not develop parasitaemia during the first booster [10, 12, 27], indicating complete parasite liver stage arrest after a single immunization. Here, we studied whether heterogeneity of induced *P. falciparum*-specific IFN-γ responses might be a marker for protection. We also investigated the evidence for local intra-hepatic IFN-γ production and its functional involvement in direct parasite killing.

Methods

CPS immunization study

Twenty-four healthy, male and female, malaria naïve volunteers were medically screened and enrolled in a clinical study that has been published previously [12]. The trial was approved by the Central Committee for Research Involving Human Subjects (CCMO; NL48732.091.14), and conducted according to the principles outlined in the Declaration of Helsinki and Good Clinical Practice standards and prospectively registered at ClinicalTrials.gov (NCT02098590).

Volunteers received chloroquine prophylaxis and three immunizations consisting of bites from 15 *P. falciparum* NF54-infected *Anopheles stephansi* mosquitoes, spaced four weeks apart. Quantitative PCR (qPCR) was performed prior to immunization and on days 7, 8 and 9 post immunization, as described previously [29, 30].

Collection of matched blood and liver samples

Ten male and female patients scheduled to undergo surgery to remove primary or metastatic liver tumors (baseline characteristics described in table 1) were included into an observational study conducted between February 2017 and June 2018 at Radboud university medical center in Nijmegen, The Netherlands. The study was approved by the Committee on Human Research for Arhnem-Nijmegen (CMO; 2016-3049) and patients provided written informed consent.

Isolation and cryopreservation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from citrate whole blood taken in CPT vacutainers (BD Biosciences) by density gradient centrifugation. Following four washes with ice cold phosphate-buffered saline, cells were counted in 0·1% Trypan blue with 5% Zap-o-Globin II Lytic Reagent (Beckman Coulter). PBMCs were concentrated at 10^7 cells/mL and frozen in ice cold 90% fetal bovine serum (FBS, Gibco) containing 10% dimethylsulfoxide (DMSO, Merck, Germany) using Mr. Frosty freezing containers (Nalgene), and stored in vapor-phase nitrogen.

Isolation and cryopreservation of human hepatocytes and intrahepatic immune cells

Human hepatocytes (HuHep) were isolated from fresh liver segments obtained from surgeries according to a previously published protocol [12]. In short, liver segments were perfused sequentially with warm (37 degrees) media 1) 500mL HBSS medium (Gibco, 14170-088) supplemented with 10mM HEPES (Gibco, 15630-056) and 0.64 mM EDTA (Invitrogen, 15575-038), 2) 500mL HBSS medium supplemented with 10mM HEPES, and 3) 100mL HBSS medium supplemented with 10mM HEPES, 0.75 mg/ml

$CaCl_2$ and low concentrations of collagenase (3333 units per 50ml). Subsequently liver tissue was perfused with HBSS medium supplemented with 10mM HEPES, 0.75 mg/ml $CaCl_2$ and high concentrations of collagenase (13333 units per 50ml), for 10-20 minutes until soft. Collagenase was inactivated by adding DMEM medium (Gibco, 31885-023) supplemented with 10% FBS (Gibco, 10270) and cells were passed over a 100 µm cell strainer (Falcon, 352360). Cell suspensions were centrifuged at 50g with low brake for 5 minutes at room temperature, and then washed twice with DMEM medium. During each step, supernatants were collected for isolation of intrahepatic immune cells. After the second wash, cell pellets were suspended in 25% Percoll and centrifuged at 150g for 10 minutes. The cell pellet was resuspended in warm DMEM and centrifuged at 50g for 5 minutes. Human hepatocytes in the cell pellet were counted in 0·1% Trypan blue and taken up at a concentration of $6.0x10^6$ cells/mL in warm William's E medium with Glutamax (Gibco, 32551-087). Warm fetal bovine serum containing 20% DMSO was added slowly until cells were at a concentration of $3.0x10^6$ cells/mL (in a final concentration of 10% DMSO). Vials were transferred immediately to Mr. Frosty containers, frozen at -80 degrees Celsius and then transferred to vapor-phase nitrogen.

Intrahepatic immune cells were isolated from supernatants in 50mL tubes. After centrifugation at 600g for 10 minutes at 4 degrees Celsius, cell pellets were washed three times with ice cold PBS. Cell pellets were resuspended in 20 mL 35% Percoll containing heparin 100 IU/mL and centrifuged at 850g for 30 minutes (low acceleration, no brake). Cell pellets were resuspended in 10mL ice cold ACK lysis buffer and incubated at 4 degrees for 10 minutes. 40mL ice cold PBS was added and tubes were centrifuged at 600g for 4 minutes. Cell pellets were washed once with ice cold PBS. Intrahepatic immune cells were counted and cryopreserved according to the same protocol as the PBMCs.

Stimulation of PBMCs with *P. falciparum*

Immediately prior to use, cells were thawed and washed twice in RPMI 1640 "Dutch modification" (Gibco/Invitrogen). Cell viability was assessed by counting as described above. Total PBMCs were cultured in 96-wells plates at $2.5x10^6$ cells/ml in RPMI 1640 (Dutch Modification; Gibco) with 5mg/ml gentamycin (Centraform), 100mM pyruvate (Gibco), 200mM glutamax (Gibco), supplemented with 10% heat-inactivated pooled human A+ serum (obtained from Sanquin Bloodbank, Nijmegen, The Netherlands) at a final volume of 200uL. Cells were stimulated with purified *Plasmodium falciparum* NF54 schizonts or uninfected red blood cells at a concentration of $5x10^6$ RBC/ml. After 20 hours, Brefeldin A (10µg/mL; Sigma-Aldrich) and monansin (2µM; eBioscience) were added to the culture. After 4 hours (24 hours total stimulation) cells from two stimulation replicates ($1.0x10^6$ cells total) were combined, washed and stained with Fixable Viability-eFluor506 (eBiosciences) for 30 minutes. After washing with PBS,

cells were stained with extracellular antibodies, CD19-BV510 (Biolegend; clone), CD3-PE-Dazzle (Biolegend; clone OKT3), CD4-AlexaFluor647 (Biolegend; clone), CD8-AlexaFluor700 (Biolegend; clone), CD16-APC-eFluor780 (eBiosciences; clone CB16), CD56-BV421 (Biolegend; clone), pan-γδTCR-PerCP-Cy5.5 (Beckman Coulter; clone IMMU510), HLA-DR-FITC (Biolegend; clone) for 30 minutes at 4°C in the dark. Cells were washed, fixed and permiabilized with Foxp3 fixation/permeabilization buffer (eBioscience). Then cells were stained for intracellular cytokines with IFN-γ-PE-Cy7 (Biolegend; clone). After another wash with permeabilization buffer, cells from two staining replicates ($2.0x10^6$ cells total) were taken up in 200uL 1% paraformaldehyde (PFA) and analyzed on a Gallios flow cytometer (Beckman Coulter) the next day.

Thawing, culture and infection of primary human hepatocytes

Vials containing $3.0x10^6$ HuHep in 1mL were thawed in a water bath at 37 degrees for exactly 45 seconds, then 1mL warm William's E medium with Glutamax (Gibco, 32551-087) was added to the vial and gently resuspended until the contents were thawed completely. Cells were then transferred immediately to a 50mL tube containing 44mL William's E medium. Maximally three vials of HuHep were added to 44mL William's E. Tubes were centrifuged at 50g with low brake for 5 minutes at room temperature. Cell pellets were suspended in 25% Percoll and centrifuged at 150g for 10 minutes. The cell pellet was resuspended in warm DMEM and centrifuged at 50g for 5 minutes. Human hepatocytes in the cell pellet were counted as described above.

Optimal seeding density for each liver was determined in a separate experiment by seeding triplicate wells with either 60K, 90K or 120K hepatocytes and infecting with *P. falciparum* sporozoites as described below. The seeding density that resulted in the highest number of infected hepatocytes on day 5 post infection was considered optimal.

The human hepatocyte culture and infection protocol has been published previously [12]. In short, HuHep from each liver were seeded at their optimal density in complete William's E medium with Glutamax (Gibco, 32551-087) supplemented with 10% heat-inactivated human serum, 1% insulin/transferrin/selenium (Gibco, 41400-045), 1% sodium pyruvate (Gibco, 11360-036), 1% MEM-NEAA (Gibco, 1140-035), 1% Fungizone Antimycotic (Gibco, 15290-018), 2% penicillin/streptomycin (Gibco, 15140-122) and 1.6 μM dexamethasone, into 96-well flat-bottom plates (Falcon, 353219) coated with 0·056 mg/ml rat tail collagen I per well (Roche Applied Science, 11179179-001), and cultured at 37 °C in an atmosphere of 5% CO_2. Two days post seeding 50K sporozoites were added to each well and the plate was centrifuged at 1000g for 10 minutes without brake. After 3 hours supernatants were removed and cells were washed once.

Human hepatocytes were incubated with 150uL either complete William's E medium or complete William's E medium with 1, 10, 50 or 100 ng/mL recombinant human IFN-γ (Immunkine; Boehringer Ingelheim; lot number 407613). Every day 75uL medium was refreshed except when hepatocytes previously incubated with IFN-γ were switched to normal medium, in that case all medium was removed and cells were washed once.

Immunofluorescent analysis of *P. falciparum* infected hepatocytes

The number of infected hepatocytes was assessed 1 and 5 days after infection by immune-fluorescence as previously described [12]. In short, cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100 (Sigma T9284, diluted in PBS) and stained first with anti-*P. falciparum* Hsp-70 rabbit polyclonal antibody (StressMarQ BioSciences, SPC-186D) and then with goat-anti-rabbit-AlexaFluor594 antibody and DAPI nuclei stain.

Each well was photographed using a Leica DMI6000B inverted microscope and analyzed using ImageJ version 1.47 for Windows. Blue and red channels were split and counted using a macro. DAPI positive spots were counted as hepatocytes and DAPI+AlexaFluor594 positive spots were counted as parasites. The macro was validated in the first experiment by manually recounting the number of infected hepatocytes. The same microscope and macro settings were used for each experiment.

Phenotyping of PBMC and intrahepatic immune cells

Immediately prior to use, cells were thawed and washed twice in RPMI 1640 "Dutch modification" (Gibco/Invitrogen). Cell viability was assessed by counting as described above. $2.0x10^6$ immune cells were stained with fixable Viability-eFluor780 (eBiosciences) for 30 minutes. After washing with PBS, cells were stained with extracellular antibodies, CD3-PE-Dazzle (Biolegend; clone OKT3), CD4-AlexaFluor647 (Biolegend; clone), CD8-AlexaFluor700 (Biolegend; clone), CD56-FITC (Biolegend; clone), pan-γδTCR-PE (Beckman Coulter; clone IMMU510), CD45RO-BV510 (Biolegend; clone), CD62L-PE-Cy7 (Biolegend; clone) and CD69-PerCP-Cy5.5 (Biolegend; clone) for 30 minutes at 4°C in the dark. Cells were taken up in 200uL 1% paraformaldehyde and analyzed by flow cytometry as described above.

IFN-γ production of PBMC and intrahepatic immune cells

Immune cells were thawed and cultured as described above. Cells were stimulated with PMA (10ng/mL; Sigma) and ionomycin (1μg/mL; Sigma) or medium alone in the presence of Brefeldin A (10μg/mL; Sigma-Aldrich) and monansin (2μM; eBioscience) were added to the culture. Anti-CD107a-PacificBlue antibody (Biolegend; clone) was added throughout culture. After 4 hours cells were washed and stained with either an αβT cell panel or and NK cell panel. For the αβT cell panel cells were stained first with

Fixable Viability-eFluor780 (eBiosciences) for 30 minutes, and then with extracellular antibodies against pan-γδTCR-APC-Fire750 (to exclude γδT cells; Biolegend; clone), CD3-PE (Biolegend; clone OKT3), CD4-AlexaFluor647 (Biolegend; clone), CD8-AlexaFluor700 (Biolegend; clone), CD56-PE-Cy7 (Biolegend; clone), CD45RO-BV510 (Biolegend; clone) for 30 minutes at 4°C in the dark. For the NK cell panel cells were stained first with Fixable Viability-700 (eBiosciences) for 30 minutes, and then with extracellular antibodies against CD3-AlexaFluor700 (to exclude all T cells; Biolegend; clone), CD56-PE (Biolegend; clone), CD16-APC-eFluor780 (Biolegend; clone) for 30 minutes at 4°C in the dark. Cells were washed, fixed and permiabilized with Foxp3 fixation/permeabilization buffer (eBioscience). Then cells were stained for intracellular cytokines with IFN-γ-PE-Dazzle594 (Biolegend; clone) and Granzyme B-FITC (Biolegend; clone). After another wash with permeabilization buffer, cells from two staining replicates ($2.0x10^6$ cells total) were taken up in 200uL 1% paraformaldehyde (PFA) and analyzed on a Gallios flow cytometer (Beckman Coulter) the next day.

Results

IFN-γ responses after the first CPS immunization

Induction of *P. falciparum (Pf)*-infected red blood cell (*PfRBC*)-specific IFN-γ responses was measured before a primary and before a boosting CPS immunization in 24 volunteers [12]; volunteers were either qPCR+ (n=15) or qPCR- (n=9) after the booster immunization. PBMCs collected just before the second immunization showed significantly higher IFN-γ production in qPCR- volunteers (Mann-Whitney test p=0.0029; figure 1A), while baseline (pre-immunization I-1) production was comparable between groups.

When cells were analyzed by flow cytometry, qPCR- volunteers showed a higher percentage of IFN-γ producing cells in response to *Pf*RBC stimulation (Mann Whitney test p=0.0008; figure 1B), with significantly more IFN-γ producing CD4+ T cells, γδT cells and NK cells prior to the second immunization (figure 1C).

IFN-γ production in PBMC and intra-hepatic immune cells (IHICs)

We next analyzed the composition of circulating and intra-hepatic immune cells (IHICs) using matched blood and liver samples from surgical resections (table 1). Isolated IHICs showed strong expression of CD69, showing them as true tissue-resident cells (supplementary figure 2).

CD8+ T cells outnumber CD4+ T cells in IHICs in contrast to PBMC, and IHICs contained large populations of NKT and $CD56^{bright}$ NK cells, absent in peripheral blood (figure 2A). Furthermore, IHICs showed an increased capacity to produce IFN-γ (PBMC versus IHIC: mean 27.8% versus 59.4%, paired samples t-test p=0.0046; figure 2B). This increase results from both stronger production of IFN-γ by intrahepatic memory CD4+ and CD8+ T cells and $CD56^{dim}$ NK cells compared to their PBMC counterparts (figure 2C), and from a greater number of IFN-γ producing NKT and $CD56^{bright}$ NK cells within the intrahepatic lymphocyte fraction (figure 2D). Intrahepatic lymphocytes also had increased degranulation capacity but contained less granzyme B than matched peripheral blood samples (supplementary figure 3). γδT cell numbers were low in both peripheral blood and intrahepatic lymphocytes and they could not be properly distinguished in stimulation experiments.

IFN-γ inhibits *P. falciparum* liver maturation

To study its potential direct functional activity, we tested the capacity of IFN-γ to reduce *P. falciparum* invasion and maturation in primary human hepatocytes. We found a dose-dependent inhibition of schizont maturation when IFN-γ was added from 1 day before until 5 days post sporozoite infection with a 50% inhibitory concentration (IC_{50})

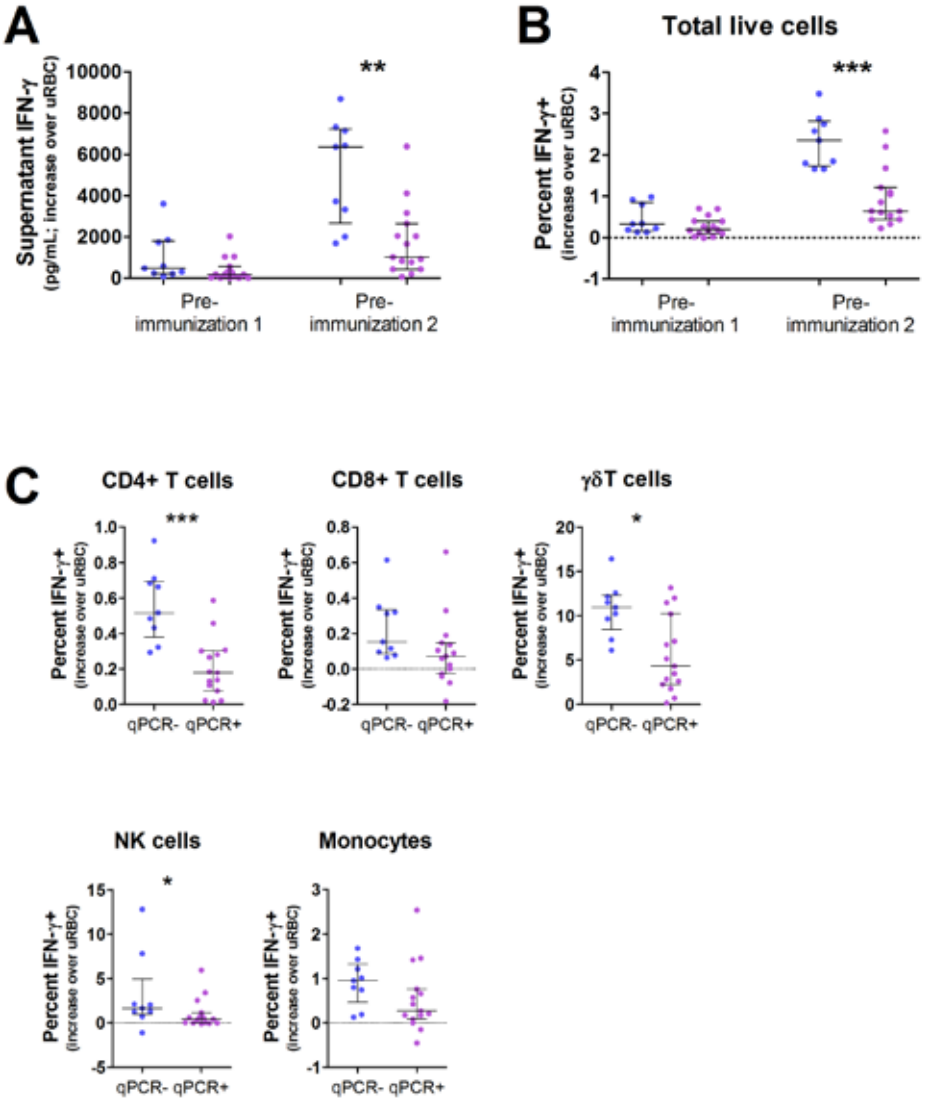

Figure 1: Parasite-specific IFN-γ production in PBMC subpopulations after the first CPS immunization. (A) Concentration of IFN-γ in supernatants from PBMCs of CPS-immunized volunteers (n=24) with (n=15; purple) or without (n=9; blue) parasitaemia after the second immunization, stimulated for 24 hours with *Pf*RBC and corrected for uninfected RBC stimulated background levels. **(B)** Intracellular IFN-γ production determined by flow cytometry in the same samples. **(C)** Proportion of IFN-γ+ cells in CD4+ T cells, CD8+ T cells, γδT cells, NK cells and monocytes. For all graphs each dot represents a single volunteer, lines and error bars represent the median and interquartile range, p-values are the result of Mann-Whitney U test, *p<0.05, *p<0.01, *p<0.001.

Table 1: Clinical characteristics of PBMC and liver donors.

	Age (years)	Gender	Operation indication	Comorbidities	Hemoglobin (mmol/L)[1]	Leukocytes (x10^9/L)[2]	Platelets (x10^9/L)[3]	ALT (U/L)[4]
Patient 1	70	Male	Liver metastasis (rectal carcinoma)	Cardiovascular disease	9.4	8.5	231	N/A
Patient 2	76	Male	Liver metastasis (colon carcinoma)	Atrium fibrillation	8.4	5.7	165	22
Patient 3	57	Female	Liver adenoma	Asthma; obesity	8.3	5.6	238	21
Patient 4	64	Male	Liver metastasis (rectal carcinoma)	Atrium fibrillation	9	5.7	243	25
Patient 5	62	Male	Liver metastasis (colon carcinoma)	Hypertension; hypercholesterolemia	10.1	7.3	323	35
Patient 6	59	Male	Liver metastasis (colon carcinoma)	-	9.3	4.6	290	N/A
Patient 7	50	Female	Liver metastasis (colon carcinoma)	-	7.3	4.5	259	N/A
Patient 8	65	Female	Liver metastasis (atypical carcinoma)	COPD; psoriasis treated with MTX	8.2	11.6	247	N/A
Patient 9	69	Male	Liver metastasis (colorectal carcinoma)	Atrium fibrillation; steato-hepatitis	9.6	7	196	66
Patient 10	58	Male	Liver metastasis (colon carcinoma)	-	9	4.8	217	N/A

1) Reference range: 8.4-10.8 mmol/L (male), 7.4-9.9 mmol/L (female)

2) Reference range: 4.0-11.0*10^9/L

3) Reference range: 150-400*10^9/L

4) Reference range: <45 U/L (male), <35 U/L (female)

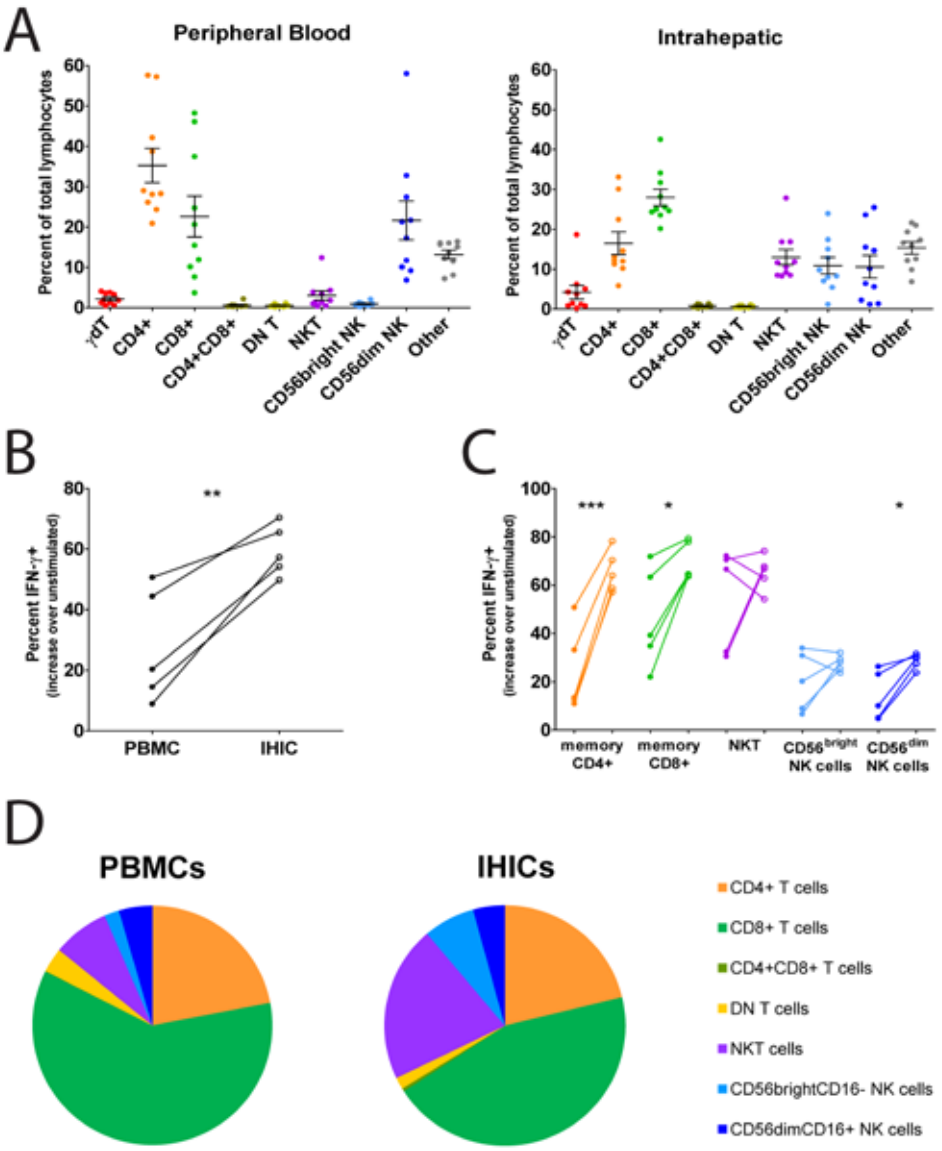

Figure 2: Intrahepatic lymphocytes are strong IFN-γ producers. (A) The relative percentages of each population within the total live lymphocyte gate in purified cell populations from match peripheral blood and liver samples. **(B)** Percentage of cells that are positive for intracellular IFN-γ production after total PBMC or intrahepatic immune cell (IHIC) populations were stimulated for 4 hours with PMA and ionomycin or RPMI alone (negative control) in the presence of Brefeldin A and monansin. Each dot represents a single matched patient sample (n=5), corrected for IFN-γ production in unstimulated (RPMI) wells. **(C)** The percentage IFN-γ producing memory CD4+ T cells, memory CD8+ T cells, NKT cells, $CD56^{bright}$ NK cells and $CD56^{dim}$ NK cells. **(D)** The composition of the IFN-γ response in n=5 paired patient PBMC and IHIC samples. For all graphs p values are the results of paired samples t-test. *p<0.05, **p<0.01, ***p<0.001.

between 10 and 50 ng/mL (figure 3A). We next tested IFN-γ at different time points during parasite liver-stage development showing a stronger inhibition when IFN-γ was added during the first 2 days post-infection (reduction of maturation day 0-2 vs. day 3-5: median 85.3% (range 75.9-94.5%) versus median 31.5% (range 17.4-55.3%), Mann-Whitney test p=0.0022; figure 3B). When IFN-γ was added for 24 hours only prior to infection, there was still a reduction in parasite maturation (median 70.9%, range 47.4-87.5%; figure 3B), while no clear effect of IFN-γ on sporozoite invasion was seen (supplementary figure 4).

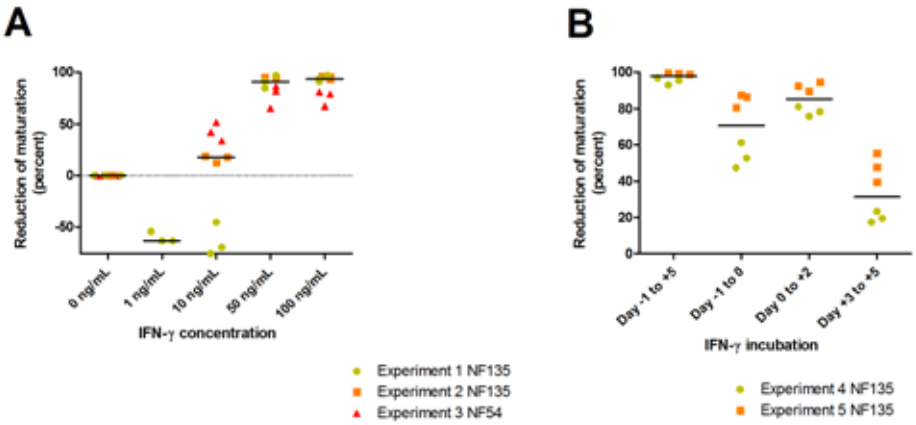

Figure 3: IFN-γ inhibits early *P. falciparum* schizont development. Isolated and cryopreserved primary human hepatocytes were thawed and cultured as a monolayer for 24 hours. **(A)** After 24 hours different concentrations of IFN-γ were added to the culture. 24 hours after IFN-γ addition, hepatocytes were infected with sporozoites of NF135.C10 clone in experiment 1 (yellow) and experiment 2 (orange) or the NF54 strain in experiment 3. Liver stages were allowed to mature for 5 days before cells were fixed and schizonts counted by microscopy. The graph shows the percent reduction in number of maturing schizonts compared to control wells without IFN-γ. Individual dots are triplicate wells from each of three independent experiments. Experiment 2 and 3 were performed using hepatocytes from the same donor and experiment 1 used hepatocytes from a different donor. IFN-γ reduces schizont maturation in a dose-dependent manner, with near complete inhibition at 50ng/ml. **(B)** 48 hours after thawing hepatocytes were infected with NF135.C10 sporozoites. Hepatocytes were cultured in the presence of 50ng/ml IFN-γ from 1 day prior to infection until 5 days post infection (positive control), 1 day before infection until just prior to infection, 3 hours post infection until 2 days post infection or 3 days post infection until 5 days post infection. The graph shows the percent reduction in number of maturing schizonts compared to control wells without IFN-γ. Individual dots are triplicate wells from two experiments. IFN-γ strongly reduces early schizont development but has little effect on late development.

Discussion

Taken together our data support a model for the role of IFN-γ produced by intrahepatic lymphocyte in the inhibition of *P. falciparum* schizont development.

Previous studies showed that mouse hepatic T cells with a tissue-resident phenotype appear to have an enhanced ability to produce IFN-γ and degranulate [31] compared to their peripheral blood counterparts. Two recent studies using unmatched human intrahepatic and blood lymphocytes showed a trend towards increased IFN-γ production in T_{RM} as well, however, the responses were highly variable and not statistically significant [32, 33]. Here we used matched samples, giving us more power to detect differences despite inter-donor variability, confirming that human intrahepatic lymphocytes also have an increased ability to produce IFN-γ and degranulate.

Several recent studies in mice and non-human primates have demonstrated that malaria immunizations can induce hepatic T_{RM} [5, 31, 34], with one study showing these T_{RM} produce IFN-γ near schizonts *in situ* [5]. Our data support this IFN-γ production as a potential mechanism for T_{RM} elimination of infected hepatocytes. However, in mice, both type I and type II interferons appear to play a role [18-20], and a possible role for type I interferons cannot be ruled out in our human hepatocyte model. Alternatively, IFN-γ may prevent schizont maturation by induction of nitric oxide (NO). Indeed, studies in mice [35] and isolated mouse [36] and human [37] hepatocytes have suggested that IL-12 or IFN-γ induced protection against sporozoite infection requires NO, though others indicate that mice deficient in NO production do not have increased parasitemia [38, 39]. Further studies in primary human hepatocytes are required to fully delineate a possible role of both NO and/or type I interferons.

In this study we chose to focus on IFN-γ because it is consistently induced in humans after CPS immunization. However, other cytokines such as TNF-α have also been shown to play a role as well [40-42]. Furthermore it is interesting to note that in these experiments, even with high levels of IFN-γ (100ng/mL) there was no complete inhibition of liver stage development. In each experiment at least a single schizont matured. This too suggests that other mechanisms must contribute to providing complete, sterilizing immunity.

In this study between 10-50ng/mL IFN-γ was required to achieve high levels of inhibition. This is higher than the circulating levels of IFN-γ during experimental and natural malaria infection (around 1ng/mL) [27, 43-45]. However, it is likely that during the intrahepatic response to infection, local IFN-γ levels are much higher, as multiple

studies in mice have now shown that IFN-γ producing lymphocytes cluster around infected hepatocytes [5, 19, 31].

Our inability to access liver-resident T cells from immune individuals after CPS immunization remains a critical impediment to fully understanding the mechanisms and correlates of protection. Here we use a polyclonal stimulus to at least demonstrate that the hepatic T cells are intrinsically capable of producing IFN-γ, and show that they are in fact more effective IFN-γ producers than peripheral blood T cells, even when correcting for differences in naïve versus memory/effector phenotype.

In conclusion, these data again confirm the importance of IFN-γ in the protection induced by CPS-immunization and demonstrate that a rapid induction of cellular IFN-γ responses correlates with fast acquisition of protective immunity. We further demonstrate a possible mechanism for this rapid protection by showing that human liver-resident T cells are capable IFN-γ producers and that IFN-γ is capable of inhibiting sporozoite maturation in multiple donors and parasite strains. These data underscore the importance of this cytokine in liver stage protection.

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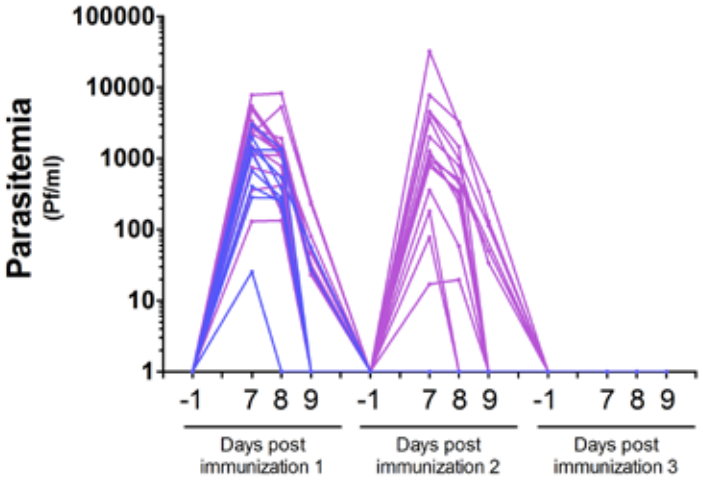
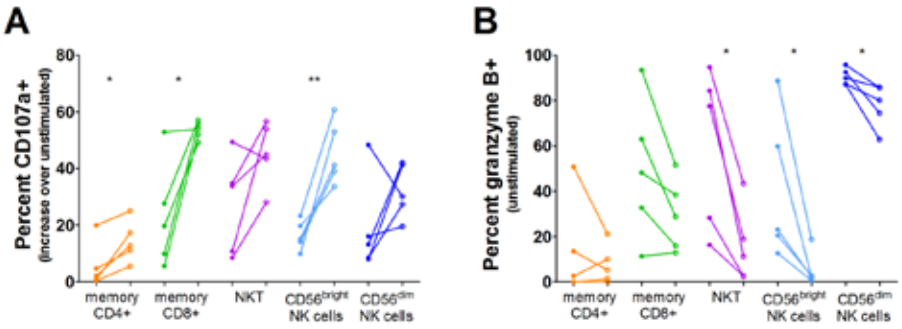
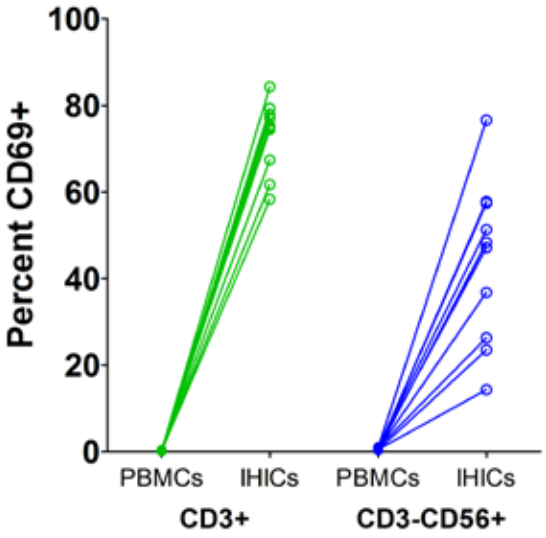


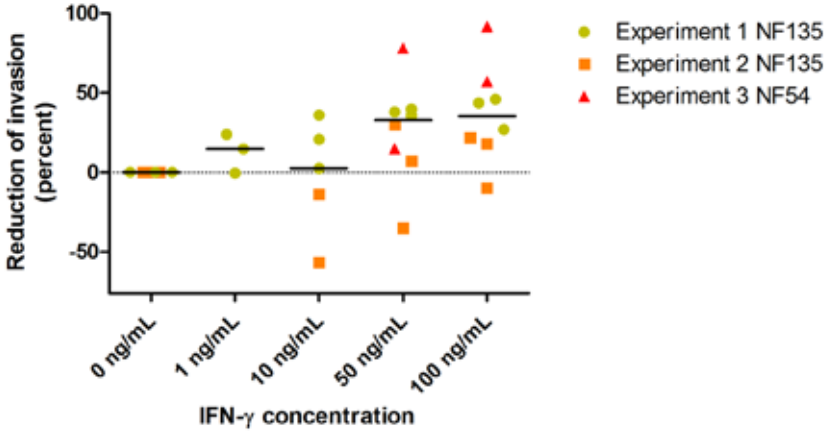
Supplementary figure 1: qPCR from 'fast' and 'slow' responders. 24 healthy volunteers received three immunizations with 15 bites of PfNF54-infected mosquitoes under chloroquine prophylaxis. After the first immunization all 24 volunteers became qPCR positive. After the second immunization 15 volunteers became qPCR positive (purple), and 9 volunteers remained qPCR negative (blue), indicating liver stage protection. The graph shows the Pf parasitemia in parasites per milliliter prior to each immunization (-1) and on days 7, 8 and 9 after each immunization. PBMC samples were taken at immunization 1 -1 day and immunization 2 -1 day.



Supplementary figure 2: Intrahepatic lymphocytes have increased degranulation but contain less granzyme B. (A) After thawing, total PBMC or intrahepatic immune cell (IHIC) populations were stimulated for four hours with PMA and ionomycin or RPMI alone (negative control) in the presence of Brefeldin A, monansin and anti-CD107a-PacficBlue antibody. The graph shows percentage of memory CD4+ T cells (defined as live CD3+γδTCR-CD56-CD8-CD4+CD45RO+ cells), memory CD8+ T cells (defined as live CD3+γδTCR-CD56-CD8+CD4-CD45RO+ cells), NKT cells (defined as live CD3+γδTCR-CD56+ cells), CD56bright NK cells (defined as live CD3-CD56bright cells) and CD56dim NK cells (defined as live CD3-CD56dim cells) that are CD107a positive in PBMC and IHIC populations. Each dot represents a single matched patient sample (n=5), corrected for CD107a staining in unstimulated (RPMI) wells. **(B)** After thawing total PBMC or intrahepatic immune cell (IHIC) populations were cultured for 4 hours in the presence of Brefeldin A and monansin. Cells were stained for intracellular granzyme B production and analyzed by flow cytometry. The graph shows percentage of memory CD4+ T cells (defined as live CD3+γδTCR-CD56-CD8-CD4+CD45RO+ cells), memory CD8+ T cells (defined as live CD3+γδTCR-CD56-CD8+CD4-CD45RO+ cells), NKT cells (defined as live CD3+γδTCR-CD56+ cells), CD56bright NK cells (defined as live CD3-CD56bright cells) and CD56dim NK cells (defined as live CD3-CD56dim cells) that are granzyme B positive in PBMC and IHIC populations. For all graphs p values are the results of paired samples t-test. *p<0.05, **p<0.01, ***p<0.001.



Supplementary figure 3: Intrahepatic cells have a CD69+ tissue-resident phenotype. CD69 surface expression on isolated PBMCs (closed circles) and IHICs (open circles) was analyzed by flow cytometry. Cells were divided into total T cells (CD3+ population; green) and NK cells (CD3-CD56+ population; blue).



Supplementary figure 4: Effects of IFN-γ on sporozoite invasion. Isolated and cryopreserved primary human hepatocytes were thawed and cultured as a monolayer for 24 hours. **(A)** After 24 hours different concentrations of IFN-γ were added to the culture. 24 hours after IFN-γ addition, hepatocytes were infected with sporozoites of NF135.C10 clone in experiment 1 (yellow) and experiment 2 (orange) or the NF54 strain in experiment 3. One day after infection cells were fixed and parasites counted by microscopy. The graph shows the percent reduction in number of invaded parasites compared to control wells without IFN-γ. Individual dots are triplicate wells from each of three independent experiments. Experiment 2 and 3 were performed using hepatocytes from the same donor and experiment 1 used hepatocytes from a different donor.


10

CHAPTER 11

General Discussion

With a complex lifecycle, broad array of target antigens and multiple immune evasion strategies, malaria is a unique disease and developing a vaccine will require unconventional approaches. In this thesis we use the Controlled Human Malaria Infection Model to evaluate the protective efficacy of whole sporozoite immunizations against both homologous and heterologous parasite strains. Then, to better inform the subsequent improvement of these vaccines, we strive to expand the current picture of heterologous immunity by shedding light on two previously underexamined aspects: whether innate immune memory can be harnessed to provide heterologous protection and how tissue immunity might differ in its efficacy against *P. falciparum*.

In this pursuit we try to answer five critical questions:

1. How can we optimize the CHMI model to minimize the risks and maximize the scientific value?

2. Can whole sporozoite immunizations provide sufficient heterologous protection in practice?

3. Does *P. falciparum* infection induce memory in the innate immune compartment?

4. Do innate immune memory responses provide clinically relevant protection against a *P. falciparum* infection?

5. Can the immunogenicity of liver-stage vaccines be assessed using peripheral blood lymphocyte assays?

Controlled Human Malaria Infections: Have we minimized the risks and maximized the value?

Human challenge trials have a long history of both far-reaching contributions to the field of infectious diseases [1-5] and significant ethical dilemmas [6, 7], which require weighing the potential for scientific insight against the risks for trial volunteers. The extraordinary global burden of malaria [8-11] and the difficulties in designing an effective vaccine [12] would seem to support the justification of Controlled Human Malaria Infections. However, international guidelines dictate that human challenge models must meet further criteria, among them, assuring scientific merit and minimizing the burden for volunteers [13-15].

In order to maximize the scientific relevance of CHMI in the assessment of malaria vaccine efficacy, studies must be strictly reproducible within- and between centers. Therefore, challenge stringency must be roughly equal between different trials. The combined data from studies performed at our center show that differences in mosquito batch sporozoite load does not affect prepatent period or parasitemia after infection. Interestingly, studies using intravenous injection of sporozoites also show significant variability [16-21], possibly resulting from differences between parasite batches or administration practices. However, host biological factors may also play larger role than has been recognized to date. It should be noted that our findings do not rule out a relationship with the number of sporozoite injected at lower salivary gland parasite loads. Nevertheless, the finding that mosquito salivary gland sporozoite load does not influence the challenge stringency under CHMI conditions support its scientific validity as means of testing drug- and vaccine efficacy.

Most guidelines on human challenge trials agree that investigators have an ethical obligation to initiate treatment as early as possible, without compromising scientific value [6, 7, 13-15]. For CHMI specifically, this would mean weighing the clinical burden of a longer duration of parasitemia against its added scientific value. Using a retrospective analysis of multiple CHMI studies, we showed that there is a clear decrease in clinical symptoms as the duration of parasitemia post challenge is shortened. We also found that for a study testing the pre-erythrocytic efficacy of a drug or vaccine, parasitemia can be treated at very low densities using qPCR without loss of scientific validity. However, this may not be the case for CHMI studies using endpoints other than sterile protection.

One of the potential weaknesses of chapter 3 was that we analyzed only retrospective data, limiting our ability to draw conclusions about the effect of earlier treatment on adverse events post treatment. We also performed a prospective analysis of the incidence and duration of adverse events in CHMI volunteers treated upon positive thick blood smear (n=18) or 100 parasites per milliliter by qPCR (n=20). There was a 75% reduction in the number of adverse events and a 79% reduction in the duration of adverse events when treating based on qPCR (figure 1A-B), with an especially large decrease in grade 2 and 3 adverse events. Furthermore, those treated based on qPCR had a significantly lower decreases in circulating lymphocytes and platelets (figure 1 C-D). Taken together these findings confirm that earlier treatment at lower circulating parasitemia after CHMI decreases symptoms and laboratory abnormalities associated with infection.

Based on these findings, and our findings in chapter 2 and 3, I would argue that there is no single recipe for the perfect balance between scientific validity and disease burden in CHMI. Instead, CHMI must above all be seen as a 'fit for purpose' model. For some purposes, such as mimicking natural sporozoite inoculation, the model may never suitable [22, 23]. In other cases, such as the evaluation of pre-erythrocytic vaccines as in chapter 4 and chapter 5, the model is highly suitable and trial endpoints do not require prolonged parasitemia, allowing for the very early treatment of volunteers. In contrast, in trials testing blood stage vaccines [24, 25] or even transmission blocking vaccines [26, 27], prolonged parasitemia is crucial to an optimal balance of safety and scientific merit.

An example of these considerations in practice is chapter 8, where one of the primary objectives was to evaluate pre-erythrocytic protection of BCG vaccination against sporozoite infection. Taking into consideration the other primary objective, the assessment of the safety of BCG vaccination prior to Controlled Human Malaria Infection, we chose to treat infection early based on qPCR at very low parasite densities. As a result we can draw no conclusions about the effect of BCG-induced trained immunity on blood stage infection, while the rapid activation of monocytes and NK cells in vaccinated individuals makes it tempting to suggest such an effect could exist. As a consequence, future studies designed to answer that question would have to accept higher and longer parasitemia to balance scientific validity with discomfort for volunteers.

This remains one of the greatest strengths of CHMIs: infectivity is so reproducible and parasitemia can be so accurately quantified that the model can be tailored to such a broad array of study questions.

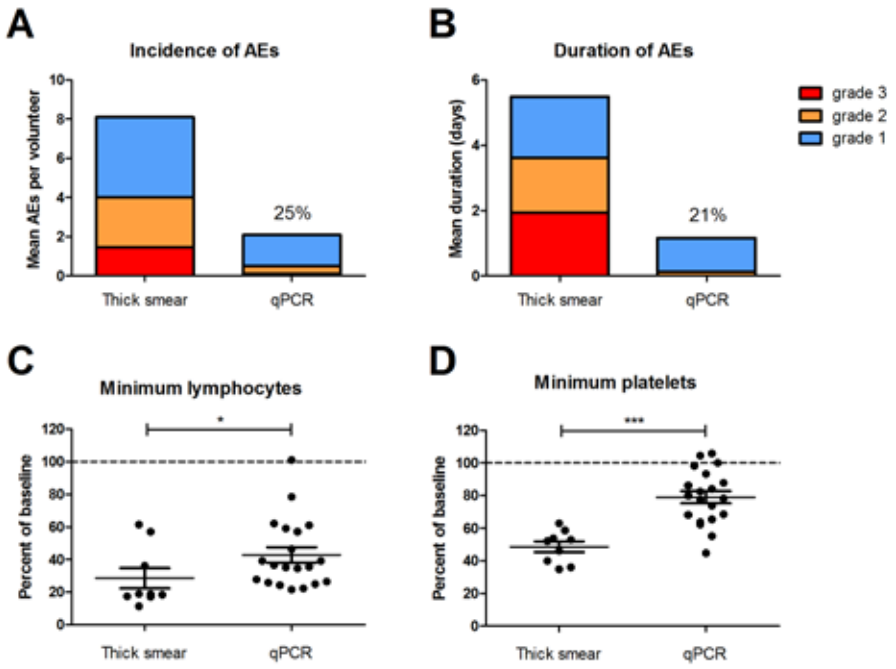


Figure 1: (A) The mean number of adverse events grade 1, 2 and 3 per volunteer before or after treatment in CHMI volunteers treated at positive thick blood smear (n=18) or 100 Pf/ml measured by qPCR (n=20). **(B)** The average total duration of adverse events grade 1, 2 and 3 per volunteer before or after treatment. **(C)** The minimum number of circulating lymphocytes measured after CHMI as percent of baseline values in volunteers post CHMI. **(D)** The minimum number of circulating platelets measured after CHMI as percent of baseline values in volunteers post CHMI.

Whole sporozoite vaccines: Can we generate heterologous protection in practice?

Whole sporozoite vaccines and especially Chemoprophylaxis and Sporozoite (CPS) immunization, remains the most effective means of generating protection against *Plasmodium falciparum* [28-33]. However, it has yet to prove itself as a viable method of immunization and protection in the field.

One significant obstacle to the use of CPS immunization in practice is the need for concurrent administration of a prophylactic agent. Attenuating sporozoites using genetic modification instead of chemoprophylaxis may go a long way to solving this issue [34-40], provided these sporozoites are truly incapable of causing blood stage infection and

provide equivalent protection. In chapter 4 we assessed the safety and efficacy of such a Genetically Attenuated Parasite (GAP), PfSPZ-GA1.

One of the most encouraging findings in chapter 4 was the fact that after 97 injection in 45 volunteers, no GA1-sporozoites were capable of causing blood stage infection. This is in contrast to another study using a GAP that lacked two genes coding for *p52* and *p36*, where the parasite was capable of causing parasitemia in a single volunteer after exposure to 200 infectious bites [41, 42]. Unfortunately our finding is limited by the fact that the administered dose provided insufficient efficacy against CHMI. Therefore future trials will be needed to assess whether GA1 sporozoites are also safe at a dose or regimen that provides sufficient protective efficacy.

The other major obstacle to the application of CPS immunization in practice is the question of protection against genetically distinct, heterologous strains [43]. There is already some evidence from the field that *P. falciparum* genetic diversity impedes vaccine efficacy [44-49]. However, we theorized that genetic diversity would be easier for whole sporozoite vaccines to overcome because the broader range of antigens might increase the chance for cross-protective responses. In chapter 5 we tested this hypothesis but found that even at dosages capable of generating extremely high homologous protection (>95%), CPS immunization with the NF54 strain only provided 10-20% protection against the genetically distinct NF135.C10 and NF166.C8 clones.

Our results present an interesting contrast to findings from studies using Radiation Attenuated Sporozoites (RAS). Cryopreserved RAS produced by Sanaria and designated PfSPZ-Vaccine, have been able to generate over 50% efficacy against heterologous CHMI with the 7G8 strain, and over 30% efficacy against naturally acquired malaria, even at dosages that provide only 70-80% homologous protection [29-31, 33, 50]. This suggests differences in the mechanisms of protection between the two vaccines, but does not resolve the problem of heterologous protection.

Our study clearly revealed that the antibodies induced by NF54 CPS were less effective at inhibiting liver infection by a genetically distinct strain. There is some evidence that T cells may be more cross-reactive [31], possibly because they respond to internal, conserved antigens as seems to be the case for T cells induced by influenza vaccination [51-55]. We were unable to test this in chapter 5, because to date conventional T cell screening methods have failed to accurately quantify *Plasmodium* antigen-specific T cell responses. However, there is sufficient evidence to conclude that current CPS immunization regimens are insufficient for heterologous protection.

At first glance it would seem that this problem could be solved by increasing immunization dosage. Namely, if we generate higher levels of antibody and T cell responses, we increase responses to individual antigens improving the chance of fully protective immunity. Yet, we may be approaching theoretical maximums for sporozoite dosages from a manufacturing perspective, and there is preliminary evidence that suggests further increasing the dosage may not improve vaccine efficacy [56].

However, there are a few alternative options worth exploring in future studies. We may be able to increase the antigen burden without increasing sporozoite dose by using a more infective parasite like NF135.C10 or NF166.C8 [57-59]. There is evidence from mice that suggests that high liver antigen burden increases immune activation, especially in the interferon pathway [60-62], which is likely relevant for the induction of anti-malarial immunity [60, 63-74]. For the vaccine development and manufacturing process this would be the most desirable approach, and should be explored as the next step in CPS immunization.

A more complicated alternative is vaccination with multiple strains. Unfortunately the broad global genetic diversity of *P. falciparum* [75, 76] currently prevents us from generating a multivalent vaccine that represents all strains. However, vaccinating with several strains may sufficiently increase the breadth of immune responses to achieve heterologous protection. Hypothetically, such a vaccine could consist of several genetically different *P. falciparum* strains either administered concurrently or sequentially under chemoprophylaxis. In this case, sequential immunizations with distinct *P. falciparum* strains might have two significant advantages. Firstly, it stands to reason that upon re-vaccination with a heterologous strain a greater number of sporozoites would successfully invade the liver, because of the strain specificity of circulating antibodies we found in chapter 5. A higher liver load could assure boosting of intrahepatic T cell responses after each immunization. Secondly, sequential immunizations with genetically distinct strains might selectively boost immune responses against cross-reactive epitopes. By focusing on increasing liver resident and cross-reactive lymphocyte responses such an approach could improve the quality of the anti-*Plasmodium* immune response. However, this type of vaccine would be more difficult to test, manufacture and license, so may be less desirable than vaccination with a more infectious parasite. A possible alternative is to use genetic modification to generate a single parasite that displays genetically diverse surface antigens, but such 'knock-in' parasites would present their own licensing difficulties.

In any case, there are multiple vaccination strategies that warrant further study. In order to steer vaccine development towards improved heterologous protection, we still need a better understanding of the immune responses underlying this protection. In this thesis

we have tried to fill in two critical gaps in this understanding: the contribution of innate and innate memory responses to heterologous protection and the comparability of liver resident and circulating lymphocytes.

Innate immunity: Does *P. falciparum* induce innate immune memory?

Malaria vaccine development has generally focused on the induction of T cells and antibodies. The contribution of innate responses, where immunological memory was considered to be absent, has largely been restricted to activation and regulation of these adaptive responses. However, the discovery of innate immune memory, also termed trained innate immunity [77-82], after exposure to BCG vaccine and beta-glucan led us to ask whether *P. falciparum* infection also induces memory in the innate immune compartment.

Natural Killer cells

One likely candidate for innate immune memory after malaria is the natural killer cell. Where NK cells were once thought to be a homogenous population of functionally similar cells, we have recently begun to understand that they are much more diverse, with variability in the expression of surface receptors and functions [83-87]. In fact, studies using mass cytometry have shown that NK cells consist of thousands of individual subsets, which may differ in their functionality against disease [88-93]. Both changes in the relative frequency of distinct subsets [94-97] and epigenetic reprogramming of NK cell functionality [86, 87, 98, 99] underly NK cell memory after a primary infection.

We examined whether CHMI had a lasting effect on NK cell function, and found that up to 35 days after infection CD56dimCD16+ NK cell subsets contained more granzyme B than at baseline (figure 2A). We also looked at the effect of CHMI on NK cell phenotype. NK cells can be divided into distinct populations representing levels of differentiation based on their expression of CD56, CD16, NKG2A and CD57 [90], and another study suggested that NKG2A+ NK cells more readily produce IFN-γ in response to *P. falciparum* [100]. We found that CHMI affects the distribution of these subpopulations, with changes persisting 29 and even 64 days after infection (figure 2B-C). Taken together these data provide the first evidence for NK cell memory after malaria.

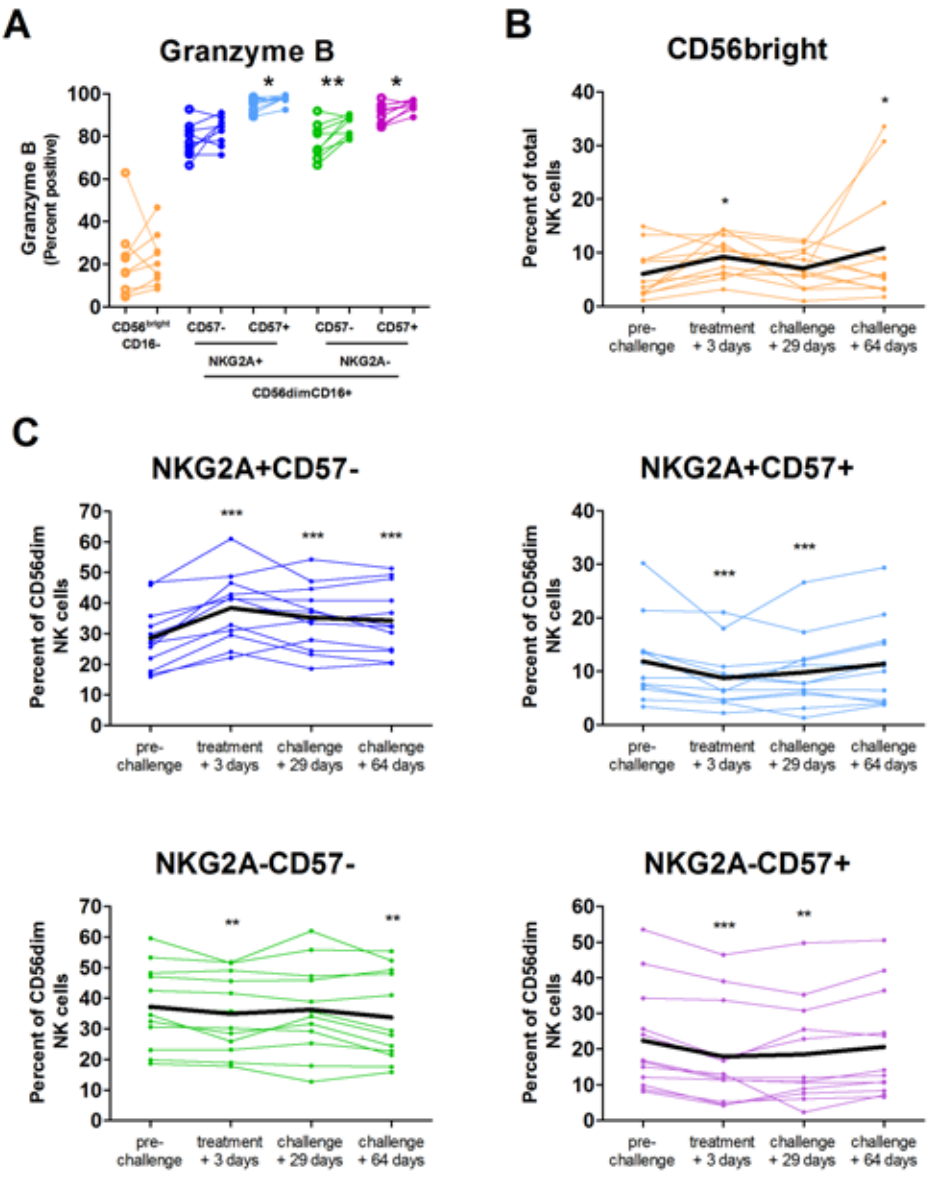


Figure 2: (A) PBMCs from 9 volunteers prior to CHMI (open circles) and 35 days after (closed circles) were divided into five subpopulations, and intracellular granzyme B content (% cells positive) was determined by flow cytometry. P-values are the result of Wilcoxon matched-pairs signed rank test. **(B-C)** In 12 volunteers undergoing CHMI, relative frequencies of each subset were analyzed before CHMI, three days post antimalarial treatment and 29 and 64 days post infection. Colored lines represent matched time points of individual volunteers, black lines represent the mean of all 12 volunteers. P-values are the result of Wilcoxon matched-pairs signed rank test comparing each post infection time point with the pre-infection time point. For all graphs * p<0.05; **p<0.01; ***p<0.001.

This type of 'NK cell memory' may have consequences for subsequent malaria infections. NK cells are capable of controlling both pre-erythrocytic [62, 101] and erythrocytic [102-110] forms of *Plasmodium*, and NK cell receptor genotypes are associated with decreases in malaria susceptibility (reviewed in [111]). Previous studies [100] and our findings in chapter 6 indicate that expression of surface receptors affects NK cell ability to respond to malaria. Therefore, the changes we see in NK cell phenotype and function after a primary *P. falciparum* infection might affect resistance to a subsequent infection, but this remains to be assessed.

As such the clinical relevance of any NK cell memory remains unclear. In CMV infection there is a clear expansion of a specific subset of NKG2C+ NK cells that are responsible for a memory-like anti-viral response [95, 96, 112-115]. Yet in CHMI this does not appear to be the case. As shown in figure 2, there is an expansion of CD56bright and CD56dimNKG2A+CD57- NK cells after CHMI, and these subsets produce more IFN-γ than others when incubated with *Pf*-infected red blood cells. However, during CHMI all subsets upregulate IFN-γ (data not shown), raising questions about the importance of the small increase in NKG2A+CD57- NK cells.

However, this does not exclude the possibility that these changes do make a difference in low grade infections in partially immune individuals in the field, as has been suggested for NKp30 expression [116-118]. Future studies examining the phenotype and function of NK cell responses in at risk individuals in endemic areas might be most suited to answering these questions. In HIV at risk individuals, such a study successfully identified correlations between NK cell diversity and risk of contracting the disease [92].

Regardless of whether NK cells can offer full or partial protection against a second malaria infection, changes in NK cell function and phenotype may still be important for vaccine design. NK cells have previously been shown to play a role in the induction of adaptive immunity [119, 120]. Here we observe NK cell changes at 28 days post CHMI. Future CPS immunization studies should evaluate whether similar changes in NK cell phenotype and function occur, and if so, whether they impact the quality or magnitude of the boosting that occurs after a second sporozoite exposure.

Innate Lymphoid Cells

NK cells are only one of several types of innate lymphocytes that may play a role in protection and pathogenesis of malaria. Innate lymphoid cells (ILCs) are a diverse population of mostly non-cytotoxic, cytokine producing cells, increasingly recognized to play a role in defense and inflammation [121-124]. Like NK cells, ILCs do not express RAG-dependent rearranged T- or B cell receptors [125, 126]. ILCs can be characterized into three subgroups [124, 126, 127]. Group 1 ILCs, like NK cells, produce IFN-γ,

activating macrophages and inducing immunity against viruses, bacteria [128] and parasites [129]. Group 2 ILCs produce IL-4, IL-5 and IL-13 and are thought to play a role in the immune response to helminths [130, 131]. Group 3 ILCs reside at mucosal sites where they can produce IL-22 to promote cell proliferation and regulate Th17 cells in the response to extracellular bacteria [132-135]. ILCs represent only approximately 0.1% of all circulating lymphocytes, though they are more numerous at tissue sites [136-138].

To date very little is known about the role of these cells in the defense against malaria. A study in mice has suggested ILC2s in protect against severe pathology, by demonstrating that recombinant IL-33 mediated expansion of IL-4, IL-5 and IL-13 producing ILC2s reduced the incidence of cerebral malaria during *Plasmodium berghei* ANKA (*Pb*A) infection [139].

ILC1 have been shown to protect against *Toxoplasma gondii* via IFN-γ and TNF [129], and could theoretically defend against *Plasmodium* blood stages via a similar mechanism. A study using blood stage Controlled Human Malaria Infection with *Plasmodium falciparum* found that circulating ILC1s decrease after infection. This suggests these cells may contribute to defense, however, the same studies found that depletion of ILC1s did not affect the course of *Plasmodium chabaudi* infection in mice [140].

We examined the activation and relative frequencies of innate lymphoid cell populations in ten volunteers after mosquito bite Controlled Human Malaria Infection, figure 3. We found that all three ILC subsets are activated during infection, as they upregulate CD69 surface expression, but the magnitude of activation is greatest in the ILC3 subset. Interestingly, the frequency of ILC3s as a proportion of total ILCs actually decreases during CHMI, possibly representing chemotaxis to the tissues. Activation and phenotype distribution returned to baseline levels 35 days after infection.

However, the effective contribution of this activation to the antimalarial immune response remains unclear. Innate lymphoid cells represent an extremely small proportion of circulating lymphocytes and one can wonder whether such a population can have a significant functional effect. Furthermore, patients who do not reconstitute ILCs after stem cell transplantation do not have more infectious complications than those who do [141]. Yet there are two ways ILCs might contribute. Firstly, the more important ILC response may be occurring in the tissues. Studies in mice have shown that though tissue ILCs are static during homeostasis, during infection they are repopulated not only by an influx of cells from the tissue, but also through local expansion [142-148]. Secondly tissue ILCs may play a role in determining the development of the local adaptive response. ILCs are populated into the tissues during fetal development [149-151] and undergo local expansion around birth and early in life [148, 152, 153]. It is

likely that in early life ILCs outnumber adaptive immune cells in the tissues, and this has led some experts to propose that ILCs and the factors they produce may play a role in in conditioning the local immunological milieu [123, 154]. Lymphoid tissue inducer (LTi) cells are already recognized to fulfill this role in the development of secondary lymphoid tissues [155-157], and other ILC types could hypothetically exert an influence on the quantity or quality of adaptive immunity in non-lymphoid tissues.

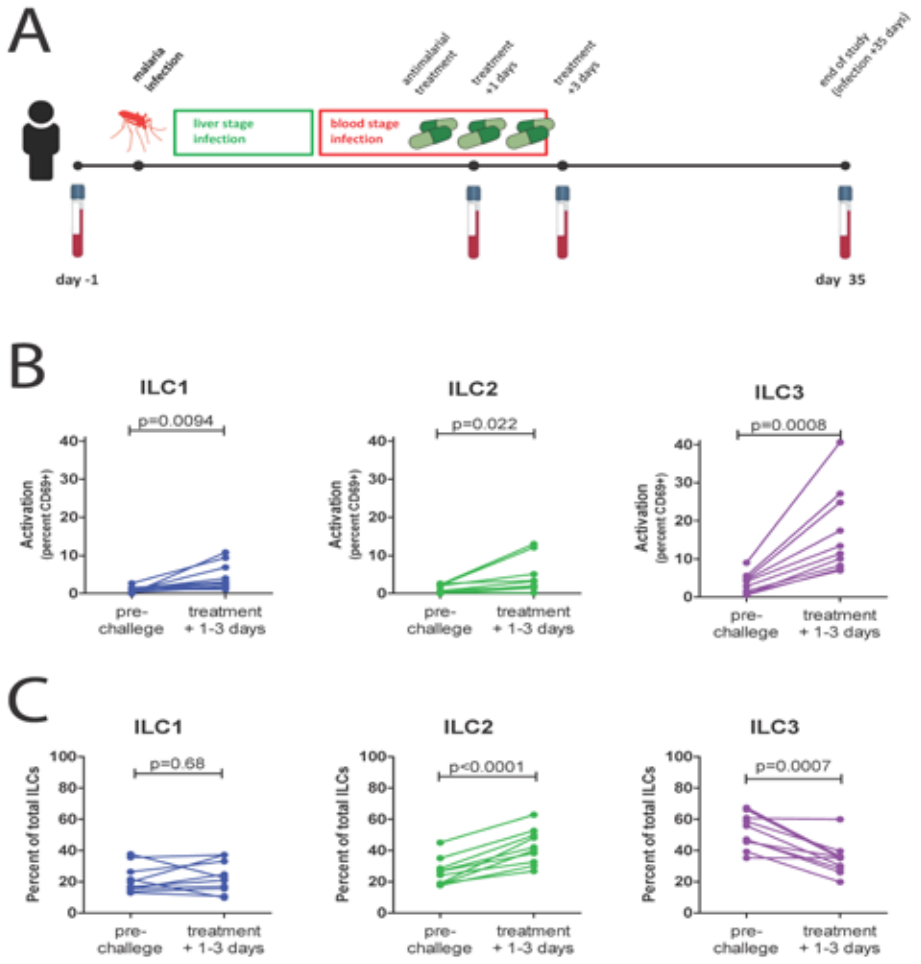


Figure 3: (A) Innate lymphoid cell populations were determined in total PBMC samples from 10 healthy volunteers undergoing Controlled Human Malaria Infection (CHMI) by the bites of 5 *P. falciparum*-infected mosquitoes. Cells were analyzed prior to infection, on day 1 or day 3 after parasites became detectable by thick blood smear and treatment was initiated, and 35 days after infection. Total ILCs were defined as live CD45+lineage-CD127+ lymphocytes and subsets were identified as ILC1 (CRTH2-CD117-), ILC2 (CRTH2+CD117+/-) or ILC3 (CRTH2-CD117+). **(B)** ILC activation was determined by surface expression of the early activation marker CD69, **(C)** and the relative distribution of each sub-population as a percentage of total ILCs is shown.

Both scenarios could suggest a role for innate lymphoid cells in the development of the tissue adaptive immune response to malaria vaccination. Considering our results show clear activation of these cells in early infection and the depletion of active ILC3s from the circulation, future studies examining the response of tissue ILCs are warranted. Though it is difficult to perform such studies directly in humans, experiments where liver ILCs are isolated from waste tissue (using the protocol we describe in chapter 10) and exposed to sera from persons undergoing CHMI could provide further data on the role of ILCs in the induction of anti-malarial immunity.

Monocytes

In chapter 7 we showed that Controlled Human Malaria Infection induces innate immune memory in monocytes, in a manner that resembles the trained immunity induced by BCG vaccination [80, 158-164]. We demonstrated that five weeks post infection, monocytes produced higher levels of IL-1β, TNF-α and IL-6 upon re-stimulation with heterologous pathogens. Though our assay did not allow us to interpret monocyte responses to *P. falciparum* after CHMI, it is not a stretch to consider that the epigenetic changes we observed also affect the monocyte response to a subsequent malaria infection.

Namely, both increased monocyte activation [165] and higher levels of IL-1β, TNF-α and IL-6 [166-168] are associated with protection against parasitemia. Though it seems unlikely they would provide sterilizing protection considering the slow acquisition of protective immunity in chronically exposed populations, these responses may still contribute to partial or clinical protection. Similarly, other studies have associated transcriptional changes in monocyte populations with protection against severe malaria or increased parasitemia [169-173].

A limitation of the data in chapter 7 is that we looked only at cytokine production. We have not studied phagocytosis, another important function of monocytes during malaria [174-183]. There are suggestions that previous exposure increases monocyte phagocytic ability [183, 184], and combined with the acquisition of opsonizing antibodies [177, 179, 183] this could represent a significant response against *P. falciparum* blood stages.

In the future we will need to determine whether such monocyte memory can also be induced in populations in malaria endemic areas, as previous or concurrent exposure to other pathogens, like helminth infections, may affect these responses.

A second critical question is what effect monocyte memory may have on CPS immunization. Like the NK cell memory described above, these monocyte changes persist until at least five weeks post infection, meaning that they are present at a second CPS immunization. We did not observe an increase in IFN-γ production, the cytokine

typically associated with protective immunity after CPS ([32, 50, 60, 62, 68, 69, 72, 74, 167, 185, 186]; chapter 5 and chapter 10). However, higher levels of other pro-inflammatory cytokines could nonetheless influence the quality or quantity of adaptive immune responses induced by immunization. Similarly, it will be interesting to determine whether whole sporozoite vaccines that do not induce blood stage parasitemia, such as PfSPZ-GA1, also induce trained immunity like other live attenuated vaccines [77, 80, 158, 187-196].

Innate immune memory: Clinically relevant in malaria?

These findings, and those of other groups, suggest *Plasmodium falciparum* may induce memory in various innate immune cells. However, the clinical relevance of innate immune memory to a second *P. falciparum* infection had not been established.

Innate immune memory was first observed following *in vitro* stimulation of monocytes [80, 158, 161-163, 197], where epigenetic changes after exposure to BCG or β-glucan [159, 198, 199] strongly increased production of IFN-γ [161, 162], IL-6 [162] and IL-1 [200] upon restimulation. Follow-up studies showed that in mouse models, these increased responses also provided protection against *in vivo* infections [162, 163] including *Staphylococcus aureus* sepsis [198] and lethal *Candida albicans* [201]. There was also circumstantial and indirect evidence that suggested BCG-induced trained immunity could protect against malaria [77, 80, 187, 188, 193-195, 202-206], but the picture remained incomplete with observational studies revealing contradictory findings [188, 194], while mouse studies showed vaccination considerably reduced [200, 207, 208] or prevented [192] malaria.

In chapter 8 we sought to determine whether innate immune memory was clinically relevant for protection against malaria. Our study clearly identified clinical and immunological differences between BCG vaccinated and control volunteers undergoing CHMI, establishing at least that BCG changes the *in vivo* immune response to *P. falciparum*. However the data are less clear regarding the effect of these altered immune responses on parasitemia. It is extremely tempting to theorize that the correlation between rapid activation of immune responses and lower first wave parasitemia after BCG vaccination might mean that these individuals have a lower starting parasitemia and increased control of parasite replication improving their probability of survival. Such a finding could then explain why BCG vaccinated persons have decreased mortality in malaria endemic areas. Though the data in chapter 8 fall far short of supporting such a finding due to the small sample size and the limited

duration of parasitemia, I think these encouraging results certainly warrant further research on two fronts.

Future studies seeking to clarify the contribution of BCG vaccination to protection against malaria should focus on two main questions. Firstly, they need to assess the ability of BCG-induced innate memory to control of blood stage parasite replication. Both NK cells [100, 102-108, 110, 111, 209, 210] and monocytes [184, 211-217] can kill *P. falciparum* infected erythrocytes, and there was preliminary for improved inhibition of blood stage replication in a single BCG-vaccinated volunteer in chapter 8. But such an effect can be better assessed in a blood stage challenge trial where volunteers are inoculated with a very low dose of *P. falciparum* infected erythrocytes allowing for several replication cycles prior to antimalarial treatment [218-221]. Secondly, future trials will need to determine whether a similar effect can be seen in endemic areas with chronic malaria exposure and previous vaccination with BCG.

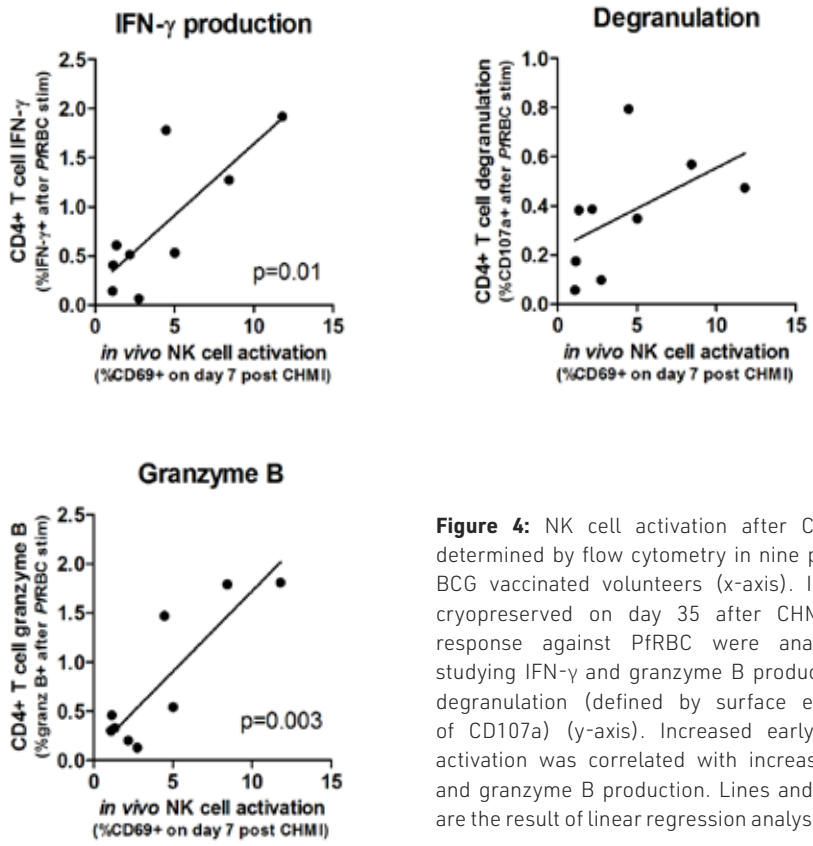


Figure 4: NK cell activation after CHMI was determined by flow cytometry in nine previously BCG vaccinated volunteers (x-axis). In PBMCs cryopreserved on day 35 after CHMI T cell response against PfRBC were analyzed by studying IFN-γ and granzyme B production, and degranulation (defined by surface expression of CD107a) (y-axis). Increased early NK cell activation was correlated with increased IFN-γ and granzyme B production. Lines and p-values are the result of linear regression analysis.

The other key question that remains is the effect of prior BCG vaccination on the induction of *P. falciparum*-specific adaptive immunity. Monocytes [184] and NK cells [120] support and stimulate the induction of adaptive immunity, so one could postulate that innate memory could stimulate adaptive memory. Interestingly we see preliminary evidence of this in the BCG-EHMI study. In chapter 8 we found that BCG vaccinated volunteers had increased CD4+ T cell responses when post-CHMI PBMCs were re-stimulated with *Pf*-infected red blood cells (*Pf*RBCs). Critically, the strength of CD4+ T cell IFN-γ and granzyme B production post infection correlated with the rapid activation of NK cells during early infection, figure 4. This suggests that volunteers with a trained innate immune system were better able to generate immunity to *P. falciparum*.

If it can be validated in clinical study designed and powered to answer this question, it could be a critical finding. One significant problem with malaria vaccine development is the lack of proper immunogenicity and protection in certain populations such as infants [44, 222-227]. If BCG vaccination could prime the immune system towards a pro-inflammatory 'trained' state better able to mount an adaptive response to malaria it may be a step towards overcoming this problem.

Pre-erythrocytic immunity: can we compare peripheral blood with hepatic lymphocytes?

In chapter 9 we sought to establish the comparability of blood and liver lymphocyte responses. Broadly speaking liver resident lymphocytes differ from their circulating counterparts in phenotype, transcriptome and functionality, leading us to conclude that they are not comparable. This immediately presents a significant problem for those of us who seek to understand pre-erythrocytic immunity to *P. falciparum*. There is a large body of evidence suggesting that at least for live attenuated sporozoite vaccines [30, 32, 34, 39, 50, 65, 71, 228-231] and specific viral-vectored vaccines [232-239] protective immunity occurs in the liver. Yet to date all published human analyses have been performed on circulating lymphocytes, and chapter 9 raises questions about the conclusions we can draw from these results. Based on our findings I would argue that it is necessary to interpret these results in the context of the differences between circulating and tissue resident lymphocytes.

In chapter 10 we try to use hepatocytes and intrahepatic lymphocytes to elucidate some of these differences, and highlight a role for IFN-γ in controlling liver-stage infection. Using the same samples we have also managed to go one step further and perform co-cultures of *Pf*-infected human hepatocytes (*Pf*HuHep) with isolated intrahepatic

lymphocytes. We show that *Pf*HuHep can activate innate and semi-innate intrahepatic lymphocytes and induce IFN-γ production and cellular proliferation, figure 5.

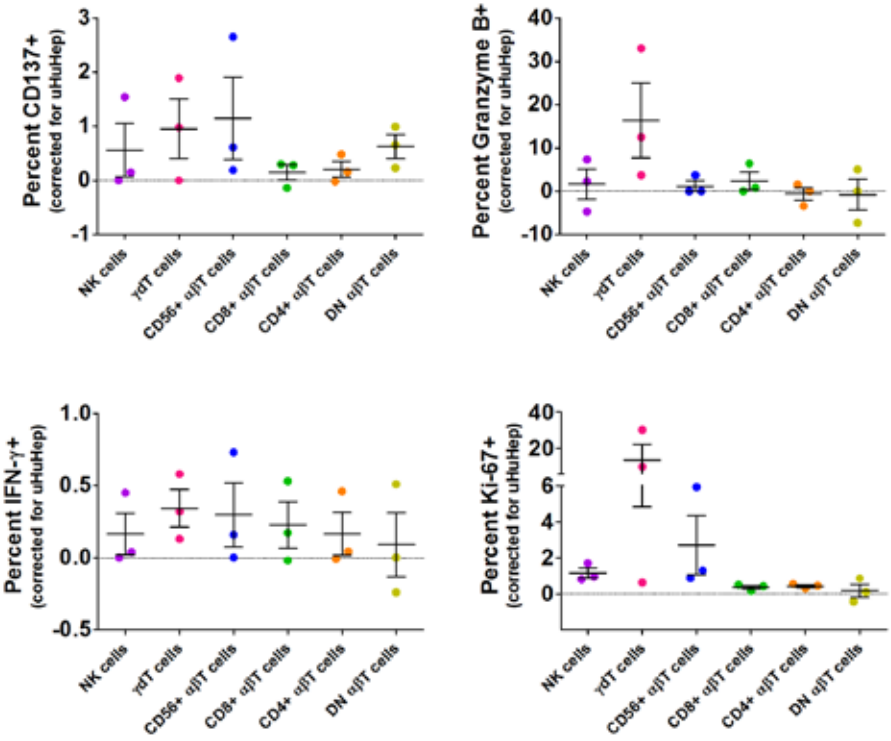


Figure 5: Human hepatocytes were infected with *P. falciparum* sporozoites and cultured for five days. On the fifth day, donor matched intrahepatic immune cells (IHICs) were added to the culture and the co-culture was incubated for 24 hours, with the last 4 hours in the presence of Brefeldin A and monansin. After 24 hours IHIC populations were removed from the culture, stained an analyzed by flow cytometry. The surface expression of the activation marker CD137 was determined, as well as intracellular expression of granzyme B and IFN-γ, and intracellular expression of Ki-67 a marker for cell proliferation.

Unfortunately while this model may help us to understand innate intrahepatic responses to *P. falciparum*, it will not elucidate how CD8+ T cells are generated and maintained in the livers of CPS or otherwise immunized individuals. Many researchers have weighed the risks and scientific rewards of performing fine-needle liver biopsies in humans participating in malaria vaccine trials. However, I would argue against such studies. Though there is undoubtably some value in observing total CD8+ intrahepatic

lymphocyte responses against *P. falciparum* in vaccinated individuals, the number of cells that can be obtained via fine needle biopsy is extremely low. This excludes the possibility for detailed functional or target antigen analysis. When we compare these serious limitations to the very real and serious risks for volunteers, I believe these risks far outweigh the scientific benefits.

Yet this leaves us with the question of how we can gain a better understanding of antigen-specific intrahepatic responses. At the moment there is no ready experimental design and such studies will require the development of new technologies and models. We may be able to design better humanized mice that allow us to study both the human immune system and the human liver, and recent advances in this field are very promising [240]. Other efforts could focus on generating HLA compatible liver and lymphocyte systems, either by HLA matching human hepatocyte donors to volunteers participating in vaccine studies, or using alternative cellular systems such pluripotent stem cells or organoids [241]. However, to our knowledge such a system does not currently exist.

In conclusion, this thesis finds that whole sporozoite immunization strategies such as CPS immunization and genetically attenuated sporozoites do not yet have sufficient efficacy against genetically diverse *P. falciparum* strains for malaria eradication. Though this is a setback, there may still be many ways to improve these vaccines. Immunization strategies striving to improve heterologous protection should focus on two main goals: 1) generating a *broader* immune response, and 2) increasing the *magnitude* of protection where it counts, in the liver.

With regards to the first question, this thesis demonstrates that sporozoite infection induces lasting phenotypical and functional changes in the innate immune compartment. It also finds the first evidence that such responses could contribute to heterologous protective immunity, making the case for the inclusion of such effects in future immunization studies. On the second point, we find that liver-resident lymphocytes are functionally distinct from circulating lymphocytes, meaning that researchers seeking to increase the *P. falciparum*-specific liver resident response must take into account the unique intrahepatic physiological and immunological conditions.

In both areas we find that there may still be a great deal of room to optimize and improve malaria vaccination strategies. Despite decades of research and thousands of publications, it is clear that the immunological picture remains incomplete. Therefore, to further advance the development of truly effective malaria vaccines, future studies must aim to fill in these gaps and not solely focus on efficacy outcomes.

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Appendix

Summary

There were approximately 219 million cases of malaria in 2017, which represented a 50% reduction since the Roll Back Malaria initiative began in 2000. Unfortunately, between 2015 and 2017 this trend leveled off and there has been no global progress in malaria eradication. Vaccines are considered to be among the most cost-effective medical interventions. In addition to protecting the individual, an effective malaria vaccine would be a valuable tool to support the eventual eradication of the disease.

Malaria is caused by a single-celled parasite, *Plasmodium*, which is transmitted to humans by infected mosquitoes. After an infected mosquito bites a human, *Plasmodium* parasites travel from the skin to the liver where they develop and multiply thousands-fold. Parasites then burst out of the liver and into the bloodstream where they continue to replicate in red blood cells; this is the stage that causes disease. A small portion of these parasites develop into male or female gametocytes, which can then infect a new mosquito if it bites the person.

The complexity of the malaria life cycle presents many opportunities for our immune system to attack and kill the parasite. The studies described in this thesis concentrate on vaccines that create an immune response against the parasites developing in the liver. This stage is an attractive target because it represents a so-called 'bottle-neck' where there are only a few parasites in the body. Also, if protection in the liver is completely effective (called 'sterile immunity') no parasites reach the blood. This not only means that a vaccinated person does not get sick with malaria, but that person also cannot infect another mosquito, breaking the cycle of transmission.

The most efficient way to get this kind of protection is by vaccination with live malaria parasites under cover of a drug that kills the blood stage parasites without affecting liver stage development. This is called Chemoprophylaxis and Sporozoite (CPS) Immunization and it has been demonstrated to provide more than 90% short-term protection, with 60% of volunteers still protected after 2.5 years.

This thesis starts by studying the Controlled Human Malaria Infection Model (CHMI), where healthy volunteers are exposed to malaria through bites of infected mosquitoes to test the effectiveness of candidate vaccines. In **chapter 2** we show that CHMI causes malaria with very reproducible results between studies, even though they used different batches of mosquitoes. There is a lot of experience demonstrating the safety of CHMIs, but volunteers develop symptoms like fever and headache if the vaccine does not work and malaria parasites reach their blood. In **chapter 3** we demonstrated that by using

the recently developed quantitative PCR diagnostic test that detects very low levels of parasites in the blood, we can treat such an infection earlier and reduce symptoms in volunteers.

We used Controlled Human Malaria Infections to assess the protective efficacy of a new genetically attenuated malaria vaccine, PfSPZ-GA1, in **chapter 4**. This weakened parasite infects the liver, where it induces a protective immune response, but it cannot develop into the blood stage parasites that cause disease. In **chapter 5** we studied the genetic variability of the *Plasmodium* parasites. We discovered that though CPS immunization is more than 90% effective in protecting against parasites that are genetically similar, it is much less effective (10-20%) in providing protection against parasites with a different genetic background. This means that the next generation of malaria vaccines will need to cause stronger or more diverse immune responses to protect against all strains of the parasite.

Malaria vaccines have traditionally focused on only part of the immune system, so-called 'adaptive immunity', which protects against a single, specific disease. However, we also have an 'innate immune system' that can recognize more diverse pathogens. In **chapter 6** and **chapter 7** we looked at how malaria activates parts of the innate immune system, in order to determine whether we can use this to improve vaccines. In **chapter 8** we put this theory to the test by activating the innate immune system of healthy volunteers using a BCG vaccine (a vaccine against tuberculosis) and then infecting them with *Plasmodium*. We showed that about half of the volunteers that received the BCG vaccine were able to fight malaria more effectively using their innate immune system.

In order to work, a malaria vaccine not only needs to induce a strong immune response, but also an immune response at the right location. If the goal of a vaccine is to kill the parasites in the liver, immune cells must be present in the liver to accomplish this. In **chapter 9** and **chapter 10** we looked at how the immune system in the liver works and find that though there are plenty of active immune cells in the human liver, they are different from the ones we find in the blood. Therefore, in order to better understand how CPS immunization and other such vaccination strategies work we need to study the immune response in the liver, not only in the blood.

In conclusion, we have shown that though CPS immunization, the most efficient malaria vaccine in development to date, is very good at protecting against parasites that are genetically similar, it is far more difficult to protect against genetically diverse parasites. We theorize that in order to provide broader protection, a more diverse immune response that includes aspects of our innate immune system is required. This immunity

must also be concentrated in the right place, namely in the liver. These findings have implications for malaria vaccine design going forward, which should concentrate on optimal stimulation of the immune system in the liver in order to create a strong barrier of cells to stop this deadly parasite before it causes disease.


A

Samenvatting

Er waren ongeveer 219 miljoen gevallen van malaria in 2017, een vermindering van 50% sinds 2000. Helaas is deze trend tussen 2015 en 2017 afgevlakt en is er geen wereldwijde vooruitgang geweest in het uitroeien van malaria. Vaccins zijn een van de meest kosteneffectieve medische interventies. Naast het beschermen van het individu zou een effectief malariavaccin een belangrijk middel zijn om uiteindelijk malaria uit te roeien.

Malaria wordt veroorzaakt door een eencellige parasiet, *Plasmodium*, die door geïnfecteerde muggen wordt overgedragen op mensen. Nadat een geïnfecteerde mug een mens heeft gestoken, gaan *Plasmodium*-parasieten van de huid naar de lever waar ze zich ontwikkelen en zich duizenden keren vermenigvuldigen. Parasieten barsten vervolgens uit de levercellen om in de bloedbaan te komen waar ze zich blijven vermenigvuldigen in rode bloedcellen; dit is het stadium dat ziekte veroorzaakt. Een klein deel van deze parasieten ontwikkelt zich tot mannelijke of vrouwelijke gametocyten, die vervolgens een nieuwe mug kunnen infecteren als deze de persoon steekt.

De complexiteit van de levenscyclus van malaria biedt ons afweersysteem veel kansen om de parasiet tegen te houden. De onderzoeken die in dit proefschrift worden beschreven, concentreren zich op vaccins die een afweerreactie opwekken tegen de parasieten tijdens hun ontwikkeling in de lever. Vaccins tegen dit stadium zijn aantrekkelijk omdat er op dat moment slechts een paar parasieten in het lichaam aanwezig zijn. Daarnaast, als de bescherming in de lever volledig effectief is (ook wel 'steriele immuniteit' genoemd) komen er geen parasieten in het bloed. Dit betekent niet alleen dat een gevaccineerde persoon niet ziek wordt van malaria, maar ook dat die persoon geen nieuwe muggen kan infecteren. Hierdoor wordt cyclus van malaria besmettingen doorbroken.

De meest efficiënte manier om dit soort bescherming te krijgen is door vaccinatie met levende malariaparasieten onder dekking van een medicijn dat de parasieten in het bloedstadium doodt zonder de ontwikkeling in de lever te beïnvloeden. Dit wordt chemoprofylaxe en sporozoïeten (CPS) immunisatie genoemd. CPS immunisatie geeft meer dan 90% bescherming op korte termijn, waarbij 60% van de vrijwilligers na 2,5 jaar nog steeds beschermd is.

Dit proefschrift begint met het bestuderen van het Controlled Human Malaria Infection Model (CHMI). Hierin worden gezonde vrijwilligers onder gecontroleerde omstandigheden blootgesteld aan malaria door beten van geïnfecteerde muggen om zo

de effectiviteit van kandidaat-vaccins te testen. In **hoofdstuk 2** lieten we zien dat CHMI malaria veroorzaakt met zeer reproduceerbare resultaten tussen studies. Er is reeds veel ervaring die de veiligheid van CHMIs bevestigd, maar vrijwilligers ontwikkelen wel symptomen zoals koorts en hoofdpijn als het vaccin niet werkt en malariaparasieten de bloedbaan bereiken. In **hoofdstuk 3** hebben we aangetoond dat we die infectie eerder kunnen behandelen en de symptomen bij vrijwilligers kunnen verminderen door gebruik te maken van de recent ontwikkelde 'kwantitatieve PCR' diagnostische test. Deze test is in staat om ook hele lage aantallen parasieten in het bloed te detecteren.

In **hoofdstuk 4** gebruikten we Controlled Human Malaria Infections om de werkzaamheid van een nieuw genetisch verzwakt malariavaccin, PfSPZ-GA1, te beoordelen. Deze verzwakte parasiet infecteert de lever, waar het een beschermende afweerrespons opwekt, maar het kan zich niet ontwikkelen tot de bloedstadium parasieten die ziekte veroorzaken. In **hoofdstuk 5** hebben we de genetische variabiliteit van de *Plasmodium* parasieten bestudeerd. We ontdekten dat hoewel CPS immunisatie meer dan 90% effectief is tegen parasieten die genetisch hetzelfde zijn, het veel minder effectief is (10-20%) in het opwekken van bescherming tegen parasieten met een andere genetische achtergrond. Dit betekent dat de volgende generatie malariavaccins een sterkere of een meer diverse afweerreactie zal moeten creëren om te bescherming te bieden tegen alle stammen van de parasiet.

Malariavaccins zijn meestal gericht op slechts een deel van het afweersysteem, de zogenaamde 'adaptieve afweer', dat bescherming biedt tegen een enkele, specifieke ziekte. Mensen hebben echter ook een 'aangeboren afweersysteem' dat meer verschillende ziekteverwekkers kan herkennen. In **hoofdstuk 6** en **hoofdstuk 7** hebben we gekeken naar hoe malaria de cellen van het aangeboren afweersysteem activeert, om te bepalen of we dit kunnen gebruiken om vaccins te verbeteren. In **hoofdstuk 8** hebben we deze theorie getest door het aangeboren afweersysteem van gezonde vrijwilligers te activeren met een BCG vaccin (een vaccin tegen tuberculose) en ze vervolgens te infecteren met *Plasmodium*. We toonden aan dat ongeveer de helft van de vrijwilligers die het BCG vaccin kregen malaria effectiever konden bestrijden.

Om te werken moet een malariavaccin niet alleen een sterke afweerreactie opwekken, maar ook een afweerreactie op de juiste locatie. Als het doel van een vaccin is om de parasieten in de lever te doden, moeten afweercellen ook in de lever aanwezig zijn om dit te bereiken. In **hoofdstuk 9** en **hoofdstuk 10** hebben we gekeken naar hoe het afweersysteem in de lever werkt. Wij lieten zien dat er veel actieve afweercellen in de menselijke lever zitten, maar dat deze anders zijn dan die we in het bloed aantreffen. Om beter te begrijpen hoe CPS immunisatie en andere dergelijke vaccins werken moeten we daarom de afweerreactie in de lever bestuderen, niet alleen in het bloed.

Concluderend hebben we aangetoond dat hoewel CPS immunisatie zeer goed beschermt tegen parasieten die genetisch vergelijkbaar zijn, het veel moeilijker is om te beschermen tegen genetisch diverse parasieten. Om een bredere bescherming te bieden is mogelijk een meer diverse afweerrespons vereist die ook gebruik maakt van ons aangeboren afweersysteem. Deze afweer moet ook op de juiste plaats geconcentreerd worden, namelijk in de lever. Deze bevindingen hebben implicaties voor de verdere ontwikkeling van malariavaccins die zou moeten streven naar optimale stimulatie van het afweersysteem in de lever om deze dodelijke parasiet daar te stoppen voordat het ziekte veroorzaakt.


A

Curriculum Vitae

Jona Walk was born on July 6th 1987 in Meyrin Switzerland. She went to primary school first in Pijnacker in The Netherlands before moving to Mandeville (just outside New Orleans) in the United States when she was 7 years old. She graduated high school in Houston, obtaining her International Baccalaureate Diploma from Awty International School. She started her studies at University College in Utrecht, majoring in Biomedical Science with a minor in Cultural Anthropology. Towards the end of her Bachelor study she decided that she wanted to be medical doctor engaged in clinical practice as well as a researcher, and on October 12th 2012 she received her Master's degree in medicine from the University of Utrecht. During her Master's studies she did a research internship in pediatrics looking at immune regulation in RS virus infection, which sparked her interest in how our immune system fights disease.

After graduating, she worked as a clinician in the department of pediatrics at Groene Hart Ziekenhuis in Gouda for a year. However, missing the thrill of fundamental research she applied for a PhD position at Radboud university medical center, under the supervision of Robert Sauerwein, professor of parasitology. During five years of PhD research she conducted four clinical malaria vaccine trials as well as experimental research on cellular immune responses against *Plasmodium falciparum*.

In 2019 she started as a clinician in the department of Internal Medicine at Radboudumc, before being accepted to a residency position in February 2020. She currently works as a resident in Internal Medicine at Canisius Wilhelmina Hospital in Nijmegen and tries to make time occasionally to continue research on how our immune system can be optimized to fight infectious diseases.

PhD Portfolio

COURSES AND WORKSHOPS

2017 Herregistratie BROK (Good Clinical Practice), Radboudumc, Nijmegen
2017 The Art of Presenting Science, Radboudumc, Nijmegen
2016 Scientific Integrity, Radboudumc, Nijmegen
2016 Workshop Controlled Human Infection models, LUMC, Leiden
2014 BROK (Good Clinical Practice), Radboudumc, Nijmegen

SYMPOSIA AND CONFERENCES

2019 Innate Immune Memory, Nijmegen
2019 Organizing committee Dutch Malaria Day conference, Radboudumc, Nijmegen
2018 Netherlands Center for One Health, Utrecht
2018 European Congress of Immunology, Amsterdam
2018 Radboud Science Day, Infectious Diseases and Global Health
2018 American Society for Tropical Medicine and Hygiene, New Orleans, United States
2018 International PfSPZ Consortium meeting, New Orleans, United States
2017 IABS 2nd Human Challenge Trials Conference, Washington D.C., United States
2017 Innate Immune Memory, Cambridge, United Kingdom
2017 Radboud Science Day, Infectious Diseases and Global Health
2016 International PfSPZ Consortium meeting, LUMC, Leiden
2016 Malaria Vaccines for the World, LUMC, Leiden
2016 Radboud Science Day, Infectious Diseases and Global Health
2015 Innate Immune Memory, Cambridge, United Kingdom
2015 Dutch Malaria Day, LUMC, Leiden
2015 International PfSPZ Consortium meeting, Tübingen, Germany
2014 Summer Frontiers, Radboudumc, Nijmegen
2014 NVP Spring Meeting, Utrecht

SCIENTIFIC PRESENTATIONS

2018 Scientific Spring Meeting KNVM & NVMM
Oral presentation: 'BCG vaccination induces a trained innate immune response to CHMI'

2018 American Society for Tropical Medicine and Hygiene, New Orleans, United States
Poster presentation: 'BCG-induced trained immunity in CHMI'
Poster presentation: 'CHMI induces long term changes in monocytes'

2017 IABS 2nd Human Challenge Trials Conference, Washington D.C., United States
Invited oral presentation: 'Managing safety in controlled human malaria infections'

2017 Innate Immune Memory, Cambridge, United Kingdom
Poster presentation: 'BCG vaccination induces a trained innate immune response to CHMI'
Poster presentation: 'CHMI induces trained innate immune responses in healthy volunteers'

2016 Radboud Science Day, Infectious Diseases and Global Health
Oral presentation: 'BCG vaccination and Controlled Human Malaria Infection'

2016 Summer Frontiers, Young Researchers in Immunology Symposium, Nijmegen
Oral presentation: 'Controlled Human Malaria Infections; a model for innate immunity'

2016 Malaria Vaccines for the World, Leiden
Oral presentation: 'Heterologous protection after CPS immunization'

COMMUNICATION TO GENERAL AUDIENCE

2019 Guest in *De Nieuwe Mens*, debate evening LUX Nijmegen

2019 Interview for *De Gelderlander* on malaria vaccines

2017 Interview for *De Kennis van Nu* about the GA1 malaria vaccine trial

2016 Presentation for *Health Valley Event*, networking for businesses in health

2016 Finalist Radboud Talks pitch competition, LUX Nijmegen

2015 Lecture about developments in malaria vaccines for *Physica,* Tiel

TEACHING

2017 – 2019 Guest lectures, Verpleegkunde opleiding, HAN, Nijmegen

2016 – 2019 Lectures and practicals Minor Global Health, Biomedical Science, Radboudumc

2015 – 2019 Guest lectures Master Molecular Life Sciences, HAN, Nijmegen

2016 – 2017 Lectures Capita selecta, Master Geneeskunde, Radboudumc

SUPERVISION

2018 Bachelor internship (12 weeks): 'Effect of IFN-γ on liver stage *P. falciparum* development'

2017 Master thesis (5 weeks): 'Liver resident CD8+ memory T cells; implications for vaccines'

2017 Master internship (6 months): 'Influence of type 1 interferons in malaria immunization'

2017 Master internship (6 months): 'Function of NK cell subtypes during malaria infection'

2016 Bachelor internship (12 weeks): 'Optimization of NK cell flow cytometry during CHMI'

2015 Supervising Radboud Honors Students, writing a research project proposal

List of Publications

1. J. Walk, A.S.M. Dofferhoff, J. van den Ouweland, H. van Daal, R. Janssen. "Vitamin D -contrary to vitamin K -does not associate with clinical outcome in hospitalized COVID-19 patients." *Published as a pre-print, DOI: 10.1101/2020.11.07.20227512.*

2. R. Janssen and J. Walk. "Vitamin K epoxide reductase complex subunit 1 (VKORC1) gene polymorphism as determinant of differences in Covid-19-related disease severity." *Medical Hypotheses.* 2020 Nov;144:110218.

3. R. Janssen, M. Visser, A.S.M. Dofferhoff, C. Vermeer, W. Janssens, J. Walk. "Vitamin K metabolism as the potential missing link between lung damage and thromboembolism in Coronavirus disease 2019." *British Journal of Nutrition*. 2020 Oct 7;1-8.

4. A.S.M. Dofferhoff, I. Piscaer, L.J. Schurgers, M.P.J. Visser, J.M.W. van den Ouweland, P.A. de Jong, R. Gosens, T.M. Hackeng, H. van Daal, P. Lux, C. Maassen, E.G.A. Karssemeijer, C. Vermeer, E.F.M. Wouters, L.E.M. Kistemaker, J. Walk* and R. Janssen*. "Reduced vitamin K status as a potentially modifiable risk factor of severe COVID-19." *Clinical Infectious Diseases*. 2020 Aug 27;ciaa1258.

5. X.Z. Yap, J. Walk, M.B.B.McCall, R.W. Sauerwein. "T cell inhibitory ligand expression at baseline impacts protective immunity in controlled human malaria infection." *Manuscript in preparation.*

6. J. Walk*, F. Keramati*, L.CJ. de Bree, R.J.W. Arts, B. Blok, M.G. Netea, H.G. Stunnenberg, R.W. Sauerwein. "Controlled Human Malaria Infection induces long-term functional changes in monocytes." *Accepted for publication in Frontiers in Molecular Biosciences*.

7. V.A.C.M. Koeken, L.C.J. de Bree, V.P. Mourits, S.J.C.F.M. Moorlag, J. Walk, B. Cirovic, R.J.W. Arts, M. Jaeger, H. Dijkstra, H. Lemmers, L.A. Joosten, C.S. Benn, R. van Crevel, M.G Netea. "BCG vaccination in humans inhibits systemic inflammation in a sex-dependent manner." *Journal of Clinical Investigation*. 2020 Jul 21;133935.

8. M. Roestenberg* and J. Walk*, S.C. van der Boor, M.C.C. Langenberg, M. Hoogerwerf, J. J. Janse, M. Manurung, X. Zen Yap, A. Fabra García, J.P.R. Koopman, P. Meij, E. Wessels, K. Teelen, Y.M. van Waardenburg, M. van de Vegte-Bolmer, G.J. van Gemert, L.G. Visser, A.J.A.M. van der Ven, Q. de Mast, K.C. Natasha, Y. Abebe, T. Murshedkar, T.L. Richie, B.K Lee Sim, C.J. Janse, S.L. Hoffman, S.M. Khan, R.W. Sauerwein.

"Safety, immunogenicity and preliminary efficacy of the genetically attenuated sporozoite vaccine PfSPZ-GA1 against controlled human malaria infection; a double-blind placebo controlled clinical trial." *Science Translational Medicine*. 2020 May 20;12(544).

9. J. Walk and R.W. Sauerwein. "Activatory receptor NKp30 determines NK cell activation during Controlled Human Malaria Infection." *Frontiers in Immunology*. 2019 Dec 10;10:2864.

10. J. Walk, J. Stok and R.W. Sauerwein. "Can patrolling liver-resident T cells control human malaria parasite development." *Trends in Immunology*. 2019 Mar; 40(3):186-196.

11. J. Walk, L.C.M. de Bree, W. Graumans, R. Siebelink-Stoter, G.J. van Gemert, M. van de Vegte-Bolmer, K. Teelen, C.C. Hermsen, R.J.W. Arts, M.C. Behet, F. Keramati, S.J.C.F.M. Moorlag, A.S.P. Yang, R. van Crevel, P. Aaby, Q. de Mast, A.J.A.M. van der Ven, C. Stabell Benn, M.G. Netea, R.W. Sauerwein. "Outcomes of controlled human malaria infection after BCG vaccination." *Nature Communications*. 2019 Feb 20;10(1)874.

12. L.L. van den Hoogen, J. Walk, T. Oulton, I.J. Reuling, L. Reiling, J.G. Beeson, R.L. Coppel, S.K. Singh, S.J. Draper, T. Bousema, C. Drakeley, R.W. Sauerwein, K.K.A. Tetteh, "Antibody responses to antigenic targets of recent exposure are associated with low-density parasitemia in Controlled Human Plasmodium falciparum infections." *Frontiers in Microbiology*. 2019 Jan 16;9:3300.

13. I.J. Reuling, G.M. de Jong, X.Z. Yap, M. Asghar, J. Walk, L.A. van de Schans, R. Koelewijn, A. Färnert, Q. de Mast, A.J. van der Ven, T. Bousema, J.J. van Hellemond, P.J.J. van Genderen, R.W. Sauerwein. "Liver injury in uncomplicated malaria is an overlooked phenomenon: an observational study." *EBioMedicine*. 2018 Oct 36(131-139).

14. J. Walk, G.J. van Gemert, W. Graumans, R.W. Sauerwein, E. Bijker. "Mosquito infectivity and parasitemia after Controlled Human Malaria Infection." *American Journal of Tropical Medicine and Hygiene*. 2018 Jun 98;6(1705-1708).

15. B.K. Sack, S.A. Mikolajczak, M. Fishbaugher, A.M. Vaughan, E.L. Flannery, T. Nguyen, W. Betz, M. Jane Navarro, L. Foquet, R.W.J. Steel, Z.P. Billman, S.C. Murphy, S.L. Hoffman, S. Chakravarty, B.K.L. Sim, M. Behet, I.J. Reuling, J. Walk, A. Scholzen, R.W. Sauerwein, A.S. Ishizuka, B. Flynn, R.A. Seder, S.H.I. Kappe. "Humoral protection

against mosquito bite-transmitted Plasmodium falciparum infection in humanized mice." *NPJ Vaccines*. 2017 Oct 9;2:27. doi: 10.1038/s41541-017-0028-2.

16. R.J. Geerdink, M.P. Hennus, G.H.A. Westerlaken, A.C. Abrahams, K.I. Albers, J. Walk, E. Wesselink, R. Janssen, L. Bont, L. Meyaard. "LAIR-1 limits neutrophil extracellular trap formation in viral bronchiolitis." *Journal of Allergy and Clinical Immunology*. 2017 Oct 16; doi: 10.1016/j.jaci.2017.08.031.

17. J. Walk*, I.J. Reuling*, M.C. Behet*, L. Meerstein-Kessel, W. Graumans, G.J. van Gemert, R. Siebelink-Stoter, M. van de Vegte-Bolmer, T. Janssen, K. Teelen, J.H.W. de Wilt, Q. de Mast, A.J. van der Ven, E.D. Benavente, S. Campino, T.G. Clark, M.A. Huynen, C.C. Hermsen, E.M. Bijker, A. Scholzen, R.W. Sauerwein. "Modest Heterologous protection after Plasmodium falciparum sporozoite immunization: a double-blind randomized controlled clinical trial. *BMC Medicine*. 2017 Sep 13;15(1):168.

18. M.B.B. McCall, L.J. Wammes, M.C.C. Langenberg, G.J. van Gemert, J. Walk, C.C. Hermsen, W. Gaumans, R. Koelewijn, J.F. Franetich, S. Chishimba, M. Gerdsen, A. Lorthiois, M. van de Vegte, D. Mazier, E.M. Bijker, J.J. van Hellemond, P.J.J. van Genderen, R.W. Sauerwein. "Infectivity of Plasmodium falciparum sporozoites determines emerging parasitemia in infected volunteers." *Science Translational Medicine*. 2017 Jun 21;9(395). doi: 10.1126/scitranslmed.aag2490.

19. L.A. van de Schans, J. Walk, I.J. Reuling, R.W. Sauerwein, "Huidige status en ontwikkeling van malariavaccins." *Bijblijven*. 2017 Feb 33;1(62-69).

20. J. Walk*, R. Schats*, M.C. Langenberg, I.J. Reuling, K. Teelen, M. Roestenberg, C.C. Hermsen, L.G. Visser, R.W. Sauerwein. "Diagnosis and treatment based on quatitative PCR after controlled human malaria infection." *Malaria Journal*. 2016 Aug 5; 15(1): 398.

21. J. Walk, P. Dinga, C. Banda, T. Msiska, E. Chitsamba, N. Chiwayula, N. Lufesi, R. Mlotha-Mitole, A. Costello, A. Phiri, T. Colbourn, E.D. McCollum, H.J. Lang. "The use of non-invasive ventillation with bubble CPAP is feasable and improves respiratory physiology in hospitalized Malawian children with acute respiratory failure." *Pediatrics and International Child Health*. 2016 Feb;36(1):28-33.

22. A.J. Stoppelenburg, V. Salimi, M.P. Hennus, R. Huis in 't Veld, M. Plantinga, J. Walk, J. Meerding, L. J. Bont, and M. Boes. "Local IL-17 potentiates early neutrophil recruitment to RSV infected infant lungs." *PLoS One*. 2013 Oct 23;8(10):e78461.

23. T.A.M. Steevels, K. van Avondt, G.H.A. Westerlaken, J. Walk, L.J. Bont, P.J. Coffer, and L. Meyaard, "Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) negatively regulates the oxidative burst in human phagocytes." *European Journal of Immunology*. 2013. Apr;43(5):1297-308. doi: 10.1002/eji.201242916.

24. J. Walk, G.H.A. Westerlaken, N.O. van Uden, M.E. Belderbos, L. Meyaard, L.J. Bont. "Inhibitory receptor expression on neonatal immune cells." *Clinical and Experimental Immunology*. 2012 Aug;169(2):164-71.

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My dearest friends at the parasitology and virology labs, I always tell people who are thinking about doing a PhD: don't pick the research project, pick the research group. Because experiments fail and all research eventually has its dips, but a PhD will be great if you are surrounded by people who motivate you. Your dedication to eliminating malaria was an inspiration every day, and your support, company and the thousands of cups of coffee we drank got me through the tough times. My fellow PhD students, **Roos, Laura, Helmi, Will, Lisette, Fitsum, Felix, Aissata and Chiara**, I could not have picked better people to share (and survive) this experience with, thanks for all the fun times, speciaal biertjes and delicious dinners in Nijmegen. **Matthijs, Katharine, Amanda, Nick, Sanna, Annie, Shehu, Taco, Teun, Meta, Richard**, the post-docs and PIs I could look up to and learn from. Thanks for being a great example of how to do good science. **Matthew,** I really enjoyed our malaria/immunology discussions over beer and dinner, hopefully we will collaborate again in the future without the interference of a global pandemic.
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