CONSTRUCTING PROTECTION AND IMMUNE RESPONSES AGAINST MALARIA

STUDIES ON WHOLE SPOROZOITE IMMUNIZATION APPROACHES

ELSE BIJKER

Constructing protection and immune responses against malaria

Studies on whole sporozoite immunization approaches

Colofon

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CHAPTER 1

General introduction

Malaria remains one of the most important infectious diseases causing significant burden worldwide, and an effective vaccine will be indispensable to fight this disease. However, in order to design and develop such a vaccine we need to improve our understanding of protective immunity. This thesis describes work that was carried out on a unique model for studying *Plasmodium falciparum* infections: the Controlled Human Malaria Infection (CHMI) model. In this model healthy volunteers are infected with malaria under controlled circumstances, providing for an effective methodology to investigate protection and immune responses. When CHMI is applied in conjunction with antimalarial prophylaxis, this becomes a highly efficient protocol to induce sterile protection: Chemoprophylaxis and Sporozoites (CPS) immunization.

This general introduction provides a brief historical overview of the fight against malaria, the current strategies and challenges in malaria control, and the status of malaria vaccine development. The chapter concludes with a description of the aims and outline of this thesis.

An estimated 198 million cases and 584.000 deaths were caused by malaria in 2013, mainly amongst young children in sub-Saharan Africa (1). Malaria is caused by *Plasmodium* parasites, which are transmitted by mosquitoes. Six species of the *Plasmodium* parasite are known to cause malaria in humans: *P. falciparum, P. vivax, P. ovale, P. malariae, P. knowlesi,*

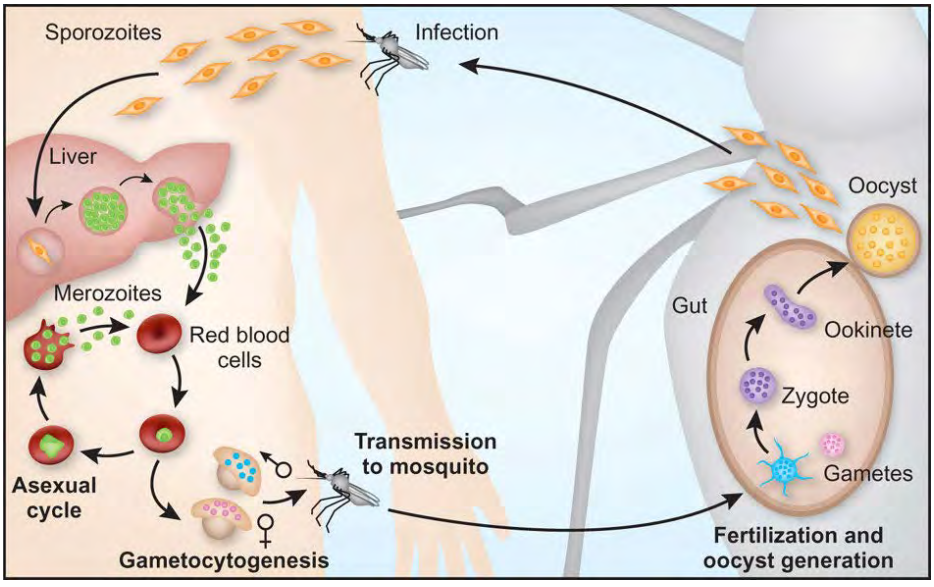

Figure 1 The *Plasmodium falciparum* life cycle. Vaccines are being developed against all life cycle stages of the malaria parasite: sporozoites, liver stages, asexual blood stages and sexual forms. Figure reprinted from reference (9) with permission from Nature Publishing Group.

and *P. cynomolgi* (2, 3). *Plasmodium* parasites are transmitted to humans through the bites of infected female *Anopheles* mosquitoes. The injected sporozoites move to the liver where they invade hepatocytes, then mature and multiply. The liver stage of the *Plasmodium* life cycle is clinically silent. *P. vivax* and *P. ovale* can form dormant liver forms called hypnozoites, which can reactivate after several months or even years (4). Once the infected hepatocytes burst open, merosomes containing merozoites are released into the blood stream and symptoms may occur. If not treated promptly and adequately, *P. falciparum* and to a lesser extent *P. vivax* infections may result in severe anemia, cerebral malaria, acute respiratory distress syndrome, organ failure, or death. Some blood-stage parasites develop into gametocytes, which cause infection of a mosquito when it takes a blood meal from the human host. Subsequently, sporozoites develop in the salivary glands of the infected mosquito, completing the life cycle of the *Plasmodium* parasite (Figure 1). While 40% of the world's population is at risk from *P. vivax* and 21-27% of severe malaria cases in Southeast-Asia and India are caused by it (5-8), *P. falciparum* remains responsible for the vast majority of the morbidity and mortality worldwide and is therefore the focus of this thesis.

The battle against malaria: a historical perspective[1]

In 1955, the WHO launched the 'Global malaria eradication program', a campaign based on the use of dichloride-diphenyl-trichlorethane (DDT) to combat malaria mosquitoes, and the drug chloroquine for treatment and prevention of infections in humans. With this dual strategy, malaria was successfully eradicated in Europe and in large areas of subtropical Asia and Latin America (10). In the Netherlands the last case of endemic malaria occurred in 1959, after which the WHO in 1970 officially declared the country 'malaria free' (11). In Sub-Saharan Africa, this campaign was only implemented in Ethiopia, South Africa and Zimbabwe. It was not expected that the campaign would yield results in other areas because of high entomological inoculation rates and poor infrastructures (12). In 1969, global malaria-eradication was concluded to be unrealistic: the campaign was halted, and treatment of individual cases became the new focus (13).

In the following years, the global situation rapidly deteriorated. The number of malaria-related hospital admissions in Africa increased two to three-fold in the 1980s, and infection with *P. falciparum* was the cause of mortality in 30% of the children in sub-Saharan Africa at that time (14). An important cause for the worsening of the situation was the emergence and

[1] Parts of this introduction were previously published in 1) Bijker EM, Sauerwein RW. Malariavaccins: de huidige stand van zaken. [Malariavaccines: an update. In Dutch]. *Tijdschrift voor infectieziekten*, 2014; 2) Bijker EM, Sauerwein RW. *Plasmodium falciparum* whole-parasite malaria vaccines. In: *Malaria Vaccine Development: Over 40 Years of Trials and Tribulations*. Corradin G, Engers H (Eds). Future Science Group, London, UK, 149–162 (2014) and 3) Bastiaens GJ, Bijker EM, Sauerwein RW. On the way to eradicating malaria. *Ned Tijdschr Geneeskd* 2012; 156:A4095.

spread of chloroquine-resistant parasites and DDT-resistant mosquitoes. In addition, political instability in the areas where malaria was endemic (15), the emerging HIV-epidemic (16, 17) and possibly climate change (18) resulted in a crisis in the control of malaria by the end of the 1990s (19).

By the beginning of the new millennium, political interest and willingness to combat malaria were again renewed, probably strengthened by an increased understanding of its significant socio-economic effects (20). This resulted in an international funding impulse: funds available for malaria control increased to 2.7 billion dollars in 2013 (1). Although still significantly less than the estimated 5.1 billion dollars considered to be annually needed for adequate control (1), this was an important step forward. Implementation of WHO-recommended control measures over the past 10-15 years resulted in a decrease of malaria morbidity and mortality: the number of clinical malaria cases decreased from 226 million in 2000 to 198 million in 2013 and the number of deaths decreased from 881.000 to 584.000 (1). However, it is evident that malaria remains a major burden in the poorest countries of the world, keeping millions of people in vicious cycles of disease, reduced productivity, income loss and poverty (20).

Current cornerstones and future challenges of malaria control

Adequate diagnosis and prompt treatment with effective drugs is the first cornerstone of malaria control and elimination. However, treatment of fever episodes with antimalarials without confirming the diagnosis often occurs, and this can accelerate the emergence of drug-resistant parasites (19). Hence adequate diagnostic tools are crucial. Microscopy is the gold standard, but implementation can be problematic, especially in rural clinics, because it requires well-trained staff, good quality microscopes and reagents and, usually, a power-source for the microscope. Alternatively, there are a number of user-friendly, sufficiently sensitive and specific rapid diagnostic tests (RDTs) available, which are based on the detection of either *P. falciparum* or *P. vivax* specific or pan-malaria antigens (21). Quantification of parasitemia is however not possible with RDTs, and the vulnerability of the tests to high temperatures and humidity is a concern.

Artemisinin-combination therapy is the first-line treatment of choice for uncomplicated *P. falciparum* malaria, and intravenous or intramuscular artesunate is the recommended therapy for severe malaria (22). Yet, an important problem is the progressive development of *Plasmodium*-resistance against existent drugs. Its urgency is emphasized by reports demonstrating clinical artemisinin-resistance in Southeast Asia (23, 24). Resistance to both chloroquine and sulfadoxine–pyrimethamine has emerged in this area and subsequently spread to Africa (25). Continuous investment in drug discovery and development will be crucial to increase the portfolio of effective and affordable drugs and overcome this threat.

The second cornerstone of malaria control is targeting the vector, i.e. the mosquito, by indoor insecticide spraying and the use of insecticide-treated mosquito nets (ITNs). Per 1000 children, approximately 5.5 lives can be saved annually if ITNs are adequately used (26). Approximately half of the population at risk had access to an ITN in 2013 and 90% of them slept under it, indicating that the efforts to encourage ITN use have been effective and continuous supply of new nets is needed (1). Indoor spraying of walls with DDT is an adequate way to kill *Anopheles* mosquitoes and an effective intervention that significantly reduces malaria incidence, partially because of the longevity (6-12 months) of the effect after spraying (27). Other insecticides work for a significantly shorter period, 2 to 6 months, requiring these compounds to be sprayed more than twice every year. Until an alternative with similar efficacy and cost-effectiveness becomes available, the WHO accepts DDT despite its negative environmental effects (27). However, the emerging resistance of *Anopheles* mosquitoes poses a serious threat to the effectiveness of both ITNs and insecticide spraying (28). Furthermore, there is increasing evidence of the change in feeding behavior of *Anopheles* mosquitoes from mainly indoors to mainly outdoors (29), which would render these interventions fruitless.

A number of novel methods are being evaluated to handle the changes in *Anopheles* behavior and the development of insecticide resistance: (a) application of fungi that are pathogenic for mosquitoes but harmless for humans (30); (b) microbial larvicides combined with existing interventions such as ITNs (31); (c) odor traps containing synthetically fabricated odors that attract female *Anopheles* mosquitoes (32). In addition, research efforts are being directed towards the generation of genetically modified mosquitoes that cannot transmit *Plasmodium* parasites (33). Although conceptually attractive, this remains a complex approach with considerable risks inherent to the introduction of a genetically modified organism into the environment, which need careful evaluation.

The third cornerstone of malaria control that is currently pursued is intermittent preventive treatment. Approximately 28% of pregnant women in sub-Saharan Africa are infected with *P. falciparum.* They are at a high risk of developing severe complications such as maternal anemia and low birth weight of the baby (34). When all pregnant women in an endemic area were preventively treated with sulfadoxine–pyrimethamine during the 2nd and 3rd trimester, the risk of infections and complications was reduced significantly (35). In infants, preventive treatment with long-acting antimalarials such as sulfadoxine–pyrimethamine resulted in a reduced incidence of malaria episodes (36). Resistance against sulfadoxine–pyrimethamine is however widespread (37), which implies that these drugs should not be used for this purpose any longer. Artemisinin-combination drugs might be good and safe alternatives, but the search for other drugs and drug-combinations remains a priority.

Bearing these three cornerstones in mind, effective malaria control and elimination require a tailor-made approach for each endemic area by using specific interventions that take

local transmission characteristics into account. The efficiency of *Plasmodium* transmission between humans and mosquitoes varies per area and is dependent, amongst other things, on population density, climate and geographic factors (38), making malaria transmission a highly dynamic process (39).

A crucial element of any elimination strategy will be a vaccine, since current interventions appear insufficient to eliminate malaria.

Vaccine strategies

Vaccination is one of the most cost-effective interventions in public health: it resulted in the eradication of smallpox and substantial reduction in morbidity and mortality by several other infectious diseases (40, 41). For malaria, however, no vaccine is licensed yet, although a partially effective first-generation vaccine is expected to become available in the near future (42). Design and development of an effective malaria vaccine are hampered by the complexity of the parasite lifecycle, the high degree of antigenic variation and polymorphism, the lack of correlates of protection and poor understanding of mechanisms of protective immunity and host-parasite interactions. This underlines the need for models to study and explain protection against malaria and then to improve rational vaccine design and development.

Malaria vaccines are categorized by the different stages of the parasite life cycle that are targeted (43):

1. *Pre-erythrocytic stage vaccines* are directed against sporozoites that are injected by mosquitoes and/or against the subsequent liver stages
2. *Blood-stage vaccines* are directed against the asexual blood stages that cause the symptoms and complications of malaria.
3. *Transmission blocking vaccines* target sexual and/or sporogonic stages that cause transmission of the parasite from the human host to the mosquito.

Vaccines may be composed of attenuated whole organisms or subunits based on single antigens or combinations of antigens. After the discovery of smallpox inoculation by Jenner in 1796, the concept of whole organism immunization was adopted for clinical vaccine development against a number of viral and bacterial pathogens, including measles virus, mumps virus, yellow fever virus, poliovirus, varicella-zoster virus, rotavirus, rubella virus, adenovirus, *Mycobacterium tuberculosis*, *Salmonella typhi* and *Vibrio cholera*. Presently, the majority of effective and licensed vaccines is based on live-attenuated or killed whole organisms.

The emerging technical feasibility to generate recombinant malaria proteins in 1983 (44, 45) raised expectations for the rapid development of subunit vaccines. Advantages of

such vaccines are the relatively easy production, up-scaling, storage and transportation, consistency of the end product and detailed knowledge and characterization of the immune epitope. Disadvantages, however, include (i) poor immunogenicity of individual antigens, ii) antigenic diversity of target proteins and (iii) insufficient breadth and coverage of the induced immune response based on a single or limited number of antigens. Induction of a strong and long-lasting immune response against soluble protein is dependent on an adjuvant. For use in humans only alum, Adjuvant System (AS) from GlaxoSmithKline Biologicals (GSK) and MF59 (Novartis) are available. Expression of the antigen by viral vectors is an alternative approach to induce strong, mainly cellular, immune responses.

Attenuated live whole organism vaccination has several advantages over subunit vaccination. These include natural invasion of the host and expression of (almost) all antigens and pathogen-associated molecular patterns to stimulate the innate and adaptive immune system (46). Critical for successful whole organism vaccination is the separation of virulence from the induction of protective immunity, i.e. sufficient attenuation of the pathogen to not cause illness while still inducing protective immune responses.

Testing vaccines: Controlled Human Malaria Infection trials

Vaccine candidates that show promising results in pre-clinical testing can progress in the developmental pipeline. Before testing these products in malaria-endemic areas, the CHMI model offers a unique possibility to efficiently test efficacy in a small group of healthy malaria-naïve volunteers. These volunteers are exposed to infectious *P. falciparum* sporozoites by the bites of *Anopheles* mosquitoes or to an inoculum of blood-stage parasites. This enables investigators not only to assess a vaccine's efficacy at early stages of clinical testing but also to study immune responses and identify immune correlates of protection (47).

Already in 1917 Julius Wagner-Jauregg performed deliberate infections of humans with *Plasmodium* parasites for the treatment of neurosyphilis (48). To induce malaria, he drew blood from a patient with parasites, injected this blood under the skin of the back of a paretic patient and rubbed some drops upon scarifications that he made on the upper arm (48). The patients benefited from the induced fever, Wagner-Jauregg received the Nobel Prize for his invention in 1927 and this 'malaria treatment' was used for neurosyphilis until antibiotics were discovered (49, 50). Since the 1960s, experimental human malaria infections are again performed, but now mainly in order to assess novel vaccines and drugs. In a number of immunization studies performed by the University of Maryland in the 1970's volunteers were immunized and challenged by bites from infected mosquitoes (51-53). These mosquitoes had fed on volunteers who served as 'gametocyte donors': after inoculation with the malaria parasite, they received small doses of quinine to avoid complications and induce sexual parasite stages. This method of generating gametocytes and infected mosquitoes

was complex and unpredictable, resulting in irregular availability of infected mosquitoes for immunizations (52). After it became possible to culture *P. falciparum in vitro* in 1986, mosquitoes could be infected by feeding on gametocyte-containing blood through so-called membrane feeders (54-56). This significantly increased the feasibility and reproducibility of experimental infections.

In addition to the application of CHMI as a testing platform for new vaccines and therapies, this model can also be used to study clinical responses to a first or second malaria infection (57-59), and to investigate more fundamental questions. These questions can comprise immunological issues, such as complement activation (60), systemic cytokine responses (61) and the association of immune responses with parasite growth (62), but also pathophysiological topics including iron homeostasis (63), thrombocytopenia and endothelial cell activation (64). Moreover, parasitological questions concerning transcription of *P. falciparum* variant surface antigens (65) and gametocyte commitment and maturation (66) can be addressed by using CHMI.

In the Netherlands, CHMI studies have been performed at the Radboud university medical center with more than 300 subjects since 1998. Extensive expertise has been built on all aspects of these trials. The first CHMI studies were used to optimize the model, i.e. the number of infected bites (58), the clinical follow-up protocol, thick smear diagnostics (67) and polymerase chain reaction for quantification of parasitemia (68). Consequently, bites from 5 mosquitoes infected with the NF54 strain of *P. falciparum* was established as consistently inducing 100% infection in malaria-naïve volunteers (69). In the following years, newly isolated *P. falciparum* strains were investigated for CHMI, establishing NF135.C10 as a heterologous strain that can be used to investigate protection after immunization with NF54 (70). The variety of opportunities offered by the CHMI model is exploited in this thesis in order to improve our understanding of protection against malaria. These include i) the availability of both sporozoite and blood-stage challenge, ii) the availability of heterologous strains, iii) the possibility to vary the number of infected bites, iv) the possibility to investigate immune responses in exposed subjects, and v) the opportunity to follow these subjects during their illness and recovery.

Pre-erythrocytic vaccine strategies

Pre-erythrocytic stage vaccines are directed against sporozoites and/or against the subsequent liver stages. Theoretically, an effective pre-erythrocytic vaccine has the potential to prevent both clinical disease and transmission. The parasite load in this stage is relatively low, i.e. tens to hundreds of parasites, in contrast to the parasite load in the asexual blood stage, which can be several million parasites. This makes the pre-erythrocytic stage an attractive target in the life cycle and these vaccines have shown the most promising results in both experimental

and field settings until today. Pre-erythrocytic vaccines based on whole sporozoites have shown superior efficacy in experimental settings and are the focus of this thesis.

Pre-erythrocytic subunit vaccine approaches

Remarkable few of the more than 2000 antigens that are expressed in the pre-erythrocytic stage have been investigated as subunit vaccines. Most research has been performed on the circumsporozoite protein (CSP), which is dominantly expressed on the surface of the sporozoite and crucial for binding to hepatocytes (71, 72). Antibodies against CSP reduce sporozoite mobility and prevent traversal and invasion of hepatocytes (73). Moreover, CSP is expressed in infected hepatocytes, and can thereby serve as a target for cytotoxic T cells (74). The candidate vaccine that is tested most extensively, and the only vaccine that has progressed to a phase-III trial is RTS,S, which consists of recombinant *P. falciparum* CSP fused with hepatitis B surface antigen formulated in the AS01 adjuvant from GSK. RTS,S induces both CD4 T cells and antibodies and protects 50% of vaccinated subjects from developing blood-stage infection after CHMI (75). Protection in phase-II studies is consistently approximately 50% and a large phase-III trial was started in May 2009 in 11 research centers in seven African countries (76). The inclusion of 15,460 infants was completed in January 2011, and preliminary results from the first 12 months of follow-up show 26-50% protection against developing clinical disease (77, 78). Analysis of pooled data from phase II studies indicates that vaccine efficacy wanes quickly to zero after 3 years (76). Moreover, efficacy appears to be inversely related to transmission-intensity, with almost no efficacy in areas with high transmission (76). In summary, RTS,S does not yet fulfill the goal set in the *Malaria Vaccine Technology Roadmap* to license a first-generation vaccine with at least 50% protective efficacy during at least 3 years in 2015 (79). But the immense efforts that were put in this phase-III trial are monumental in malaria vaccine development. Since the studies have shown that vaccination can induce a certain degree of protection in endemic countries, this is an important milestone that will enable future vaccine studies in endemic areas (80). In 2015 all analyses from the RTS,S phase-III trial will be completed, and policy will be developed on possible implementation. Amongst other things, cost-effectiveness will be an important issue in this decision process.

In addition to soluble *Plasmodium* protein-adjuvant combinations, viral vectors are currently investigated as antigen delivery platform. Antigens presented by a viral vector are expressed in host cells, often leading to a strong T cell response. Such a T cell response will presumably be important against the intracellular liver stages. Multiple studies indeed show a strong specific T cell response after heterologous prime-boost immunizations, in which different viral vectors are combined with each other, with DNA or with recombinant protein vaccines (81, 82). When human adenovirus strains are used as vectors, the vaccine is neutralized by pre-existing antibodies, and loses its immunogenicity (83). Therefore, a

chimpanzee adenovirus (ChAd63) has been developed, in which thrombospondin related adhesion protein (TRAP) was incorporated next to other multiple epitopes (ME). Vaccination of healthy volunteers with this ChAd63 ME-TRAP vaccine followed by a modified vaccinia virus Ankara (MVA) boost with the same antigen, resulted in 21% protection against CHMI, correlating with a CD8 T cell response (84). Clinical trials with this vaccine in Africa are currently ongoing.

Whole sporozoite approaches

Several regimes of attenuated sporozoite immunization have been tested in humans and animals under controlled clinical or laboratory conditions and repeatedly proven to induce (almost) complete protection. In order to attenuate sporozoites for immunization purposes, several approaches have been applied, including attenuation by radiation, genetic modification and concomitant administration of antimalarial drugs.

RADIATION ATTENUATED SPOROZOITES (RAS)

In 1941 it was shown for the first time that immunity against the pre-erythrocytic stages of *Plasmodium* can be induced; repeated infection with UV radiation-attenuated *P. gallinaceum* sporozoites induced partial protective immunity (i.e. reduced mortality) to sporozoite challenge in chickens (85). Radiation of *Plasmodium*-infected mosquitoes induces DNA damage in sporozoites to such a degree that they are able to invade the host hepatocytes, but fail to mature and multiply, thereby preventing the onset of blood-stage parasitemia. In contrast, dead sporozoites did not induce protection, demonstrating the critical balance between radiation dose for sufficient attenuation and persistence of sporozoite infectivity. Efficacy of this protocol using gamma-irradiation of sporozoites was subsequently confirmed in a number of animal models including rodents (86) and non-human primates (87, 88). Administration of radiation attenuated live sporozoites became the proof of concept of immunization against malaria.

In humans, sterile protection after administration of irradiated *P. falciparum* sporozoites by mosquito bites was first demonstrated in a small number of volunteers at the University of Maryland, the US Navy and the Stateville Correctional Center, Joliet, IL, USA in the 1970s (89, 90) and subsequently in studies carried out by the Biomedical Research Institute/ Naval Medical Research Center and the Walter Reed Army Institute for Research. Systematic analysis of studies performed in the period between 1970-1990 showed that protection against a homologous challenge infection could be induced in 93% of volunteers by bites of >1,000 *P. falciparum*-infected and irradiated mosquitoes (i.e. in 33 of 35 challenges in 14 volunteers) (91). Usage of <1,000 infected mosquitoes resulted in only 40% protection. In a small number of volunteers (i.e. 6), longevity of protection was shown for up to 42 weeks after immunization (91).

RAS-induced protection appears to be cross-strain but species-specific as tested in only 4 subjects; while *P. falciparum* RAS immunization protected against a heterologous strain, 2 subjects challenged with *P. vivax* were not protected (91). While antibody responses seem to contribute to RAS-induced protection (92, 93), rodent and non-human primate studies have shown the requirement of CD8 T cell responses for recognition and clearance of infected hepatocytes (Figure 1) (94). In humans, the exact immune mechanism of protection against malaria induced by whole parasite immunization remains to be elucidated, but most likely a combination of several effectors is required, including CD4 T cells (95) and CD8 T cells (96) in addition to humoral immunity.

GENETICALLY ATTENUATED PARASITES (GAP)

Sporozoites can be genetically attenuated using reverse-genetic methods. When one or more liver-stage specific genes are deleted, the parasite is programmed to die at a specific point during liver-stage development. A Genetically Attenuated Parasite (GAP) preparation consists of a uniform, homogenous population of parasites with a characterized attenuation phenotype, which is an advantage over RAS. While in theory a risk of GAP vaccination could be a reversal of the knockout parasite to wild type and thereby breakthrough blood-stage infection, this is virtually impossible since *P. falciparum* is a haploid organism in the pre-erythrocytic and blood stages, excluding the occurrence of recombination in the human host.

A number of different GAPs arrested at the liver stage have been developed in rodent malaria models, including parasites that lack genes which are crucial for the parasitophorous vacuole, genes involved in fatty acid synthesis and a gene involved in (post-) transcriptional regulation of early pre-erythrocytic genes (97, 98). Immunization of mice with these GAPs showed arrest at different stages of development in the liver. A comprehensive analysis of attenuation approaches for sporozoite immunization in rodent models showed a relationship between the degree of liver-stage development and protection; in order to reach similar levels of protection, immunization approaches with late liver-stage arresting parasites required lower doses than early and mid liver-stage arrest (Figure 2) (99). In these rodent models, occurrence of blood-stage parasitemia during immunization appeared to have a negative effect on the induction of immunity, stressing the need for a balanced and targeted arrest of the parasite (99).

In human volunteers, only one GAP candidate vaccine, that lacks both p52 and p36, genes essential for the formation of the parasitophorous vacuole, has been tested in a Phase I trial, showing a breakthrough infection in one out of six volunteers (100). This emphasizes the need for thorough preclinical evaluation of any GAP candidate, including (i) evaluation in both *P. yoelii* and *P. berghei* for breakthrough and protective efficacy in different murine strains including outbred mice; and (ii) *in vitro* evaluation of liver-stage development of the *P. falciparum* equivalent GAP in human hepatocytes (98). Probably, removal of multiple

genes required for independent cellular processes essential to liver-stage development will be needed to ensure complete liver-stage arrest.

CHEMOPROPHYLAXIS AND SPOROZOITES (CPS)

An alternative strategy to induce sterile protection in humans is the use of an immunization protocol combining chemoprophylaxis and live wild-type sporozoites (CPS). In a proof of concept study published in 2009, ten subjects received a standard prophylactic regimen of chloroquine for three months, during which period they were exposed to 12-15 *P. falciparum* infected mosquito bites three times, at monthly intervals. When subsequently exposed to a

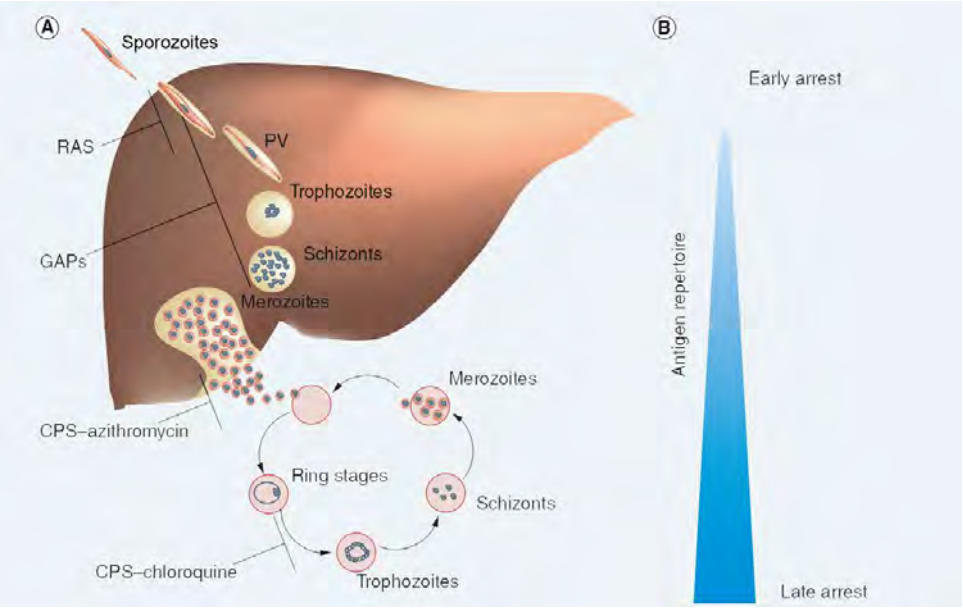

Figure 2 Developmental arrest of parasites in different whole sporozoite immunization methods. Radiation Attenuated Sporozoites (RAS) arrest early in the liver stage, before the establishment of a parasitophorous vacuole (PV). In genetically attenuated parasites (GAP) one or more specific genes are removed, resulting in arrest of the parasite at the point where this gene becomes essential, which can be anywhere in the liver stage, depending on the selected gene (98). In ChemoProphylaxis and Sporozoites (CPS) immunization, wild type sporozoites are administered. The moment of arrest therefore depends on the selected prophylactic drug. Azithromycin, for example, targets the apicoplast: not affecting liver-stage load, but preventing blood-stage parasitemia to occur (101). Chloroquine does not affect sporozoites, nor liver stages: the parasite fully develops in the liver and only arrests in the first blood-stage cycle, where chloroquine kills the trophozoites (102). As illustrated by the triangle on the right, later developmental arrest results in exposure to a broader antigen repertoire (99). *Adapted from Figure 1 from reference (99) with permission from Elsevier.*

standard challenge infection of five *P. falciparum*-infected mosquito bites, 10/10 subjects were sterilely protected (102). In a subset of the volunteers this immunity was shown to be long lasting (103). CPS-immunization, requiring only a total of 45 infected mosquito bites, is therefore more than 20 times more efficient than RAS where >1,000 *P. falciparum*-infected mosquitoes are needed. This may be explained by: i) increased breadth and magnitude of the induced immune response, due to higher antigenic load and broader antigenic repertoire including late liver-stage and early blood-stage antigens; ii) known immune modulating effects of chloroquine.

The striking efficiency of CPS-immunization raises a number of questions and opens numerous exciting avenues for further research. For example, this approach could be developed into an implementable vaccination strategy if sporozoites could be administered by needle and syringe. And, possibly even more important, CPS-immunization can be used as a model to investigate a number of issues related to anti-malarial protection. These include the lifecycle-stage specificity of the protective immune responses, the induction of immune responses and their association with protection, the role of chloroquine, and protection against heterologous strains. This thesis aims to address a number of these important issues in order to improve our understanding of protection and immune responses against malaria induced by whole sporozoite immunization.

Blood-stage vaccine strategies

Blood-stage vaccines target asexual stages that appear in the blood after the liver stage. These products aim to reduce clinical disease and control parasitemia. Clinical studies of blood-stage vaccines have mainly focused on subunit vaccines, but the development of such vaccines is hampered by the high degree of polymorphism of target antigens and a great diversity of *P. falciparum* strains in malaria-endemic areas (80). As a result of this, an immune response against a specific protein of one strain often does not protect against other strains.

Vaccines have been developed against antigens on the surface of infected erythrocytes and merozoites. Eight antigens have been investigated in clinical trials: Apical Membrane Antigen 1 (AMA-1), Merozoite Surface Protein 1, 2 and 3 (MSP1-3), Glutamate rich Protein (GLURP), Erythrocyte-Binding Antigen 175 (EBA 175), Ring-infected Erythrocyte Surface Antigen (RESA) and Serine Repeat Antigen (SERA). In residents of malaria-endemic areas, antibodies against these antigens are associated with clinical protection. However, clinical vaccine trials have until now only shown partial protection at best (104). Strain-specific efficacy, however, can be up to 64% (105).

Asexual blood-stage parasites multiply exponentially in the blood and escape from the immune system in several ways. Development of a sterilely protective blood-stage subunit vaccine is therefore unlikely to be successful. In the search for an effective vaccine it will be important to identify new blood-stage antigens that are sufficiently immunogenic,

but little polymorphic. The recently discovered reticulocyte-binding protein Homologue 5 (PfRH5) appears to fulfill these criteria. Anti-PfRH5 antibodies efficiently inhibit blood-stage multiplication of heterologous strains *in vitro* (106). Alternatively, combining different variants in a polyvalent vaccine would be a sensible strategy.

Transmission blocking vaccines

Transmission blocking vaccines (TBVs) are directed against sexual and/or sporogonic stages and thus aim to prevent transmission of the parasite to the mosquito and subsequent spread into the human population. Since 2007, malaria elimination is back on the global agenda and this resulted particularly in an increased interest in TBVs (107, 108). Biological efficacy of induced antibodies can be tested in the laboratory by adding test or control antibodies to an infected blood meal for *Anopheles* mosquitoes that feed on a glass membrane feeder (109). The transmission-blocking effect of the antibodies is determined by comparing parasite development in test and control mosquitoes. Only subunit TBVs have been developed. Target proteins on the surface of sexual and sporogonic stages for transmission-blocking antibodies are Pfs25, Pfs28, Pfs230 and Pfs48/45 (110, 111). Initial clinical studies with soluble Pfs25 show that it is difficult to find an appropriate antigen-adjuvant combination that induces robust transmission blocking immunity without causing too many side effects (110). Up until now, no TBVs have progressed to phase-II trials.

Aims & outline of this thesis

The overall aim of this thesis is to improve our understanding of protection and immune responses against malaria using the chemoprophylaxis and sporozoites (CPS) immunization model. Inoculating malaria naïve individuals under chloroquine prophylaxis with live sporozoites has been shown to induce higher levels of protection than any other vaccination method in an experimental setting. We now use this model to advance our understanding of anti-malarial immunity induced by whole sporozoite immunization. The possibilities offered by the CHMI model allow us to explore and test a diverse range of issues including life cycle stage specificity of protective immunity, the minimum immunization dose that still induces protection, induction of cellular immune responses and correlates of protection, protection against a heterologous *P. falciparum* strain, the possibility of using alternative chemoprophylactic drugs for CPS, and an alternative way of administering sporozoites in order to replace the mosquito as method of inoculation—all of these together will aid the development of an effective vaccine.

The objective of ***Chapter 2*** is to provide a detailed description of the complex CHMI trials. This will help to understand the context and the work processes described in the other chapters of this thesis, and it shows how these CHMI studies are designed and carried out to

produce valuable scientific knowledge. The heuristics used in this chapter are from Science Technology and Society studies (STS), an interdisciplinary field that combines anthropology, philosophy, history and sociology to study the production of scientific knowledge and the role of science and technology in society. More precisely, the CHMI studies will serve as a strategic research site to study the role of trust and control and their interaction in clinical trials.

The highly efficient induction of protection by CPS-immunization, compared to naturally acquired immunity and other immunization or vaccination protocols, raises many questions, one of which concerns the role of the administrated chloroquine. Chloroquine is a highly versatile drug, known not only for its antimalarial activity, but also for a number of immune-modulating effects including improvement of cross-presentation (112-114). The objective of ***Chapter 3*** is to review and reflect on the use of chloroquine to modulate the immune system in order to potentially increase protection. In a next step, we will use *P. berghei* rodent models to investigate a potentially beneficial effect of chloroquine on sporozoite immunization; this is described in ***Chapter 4***. In ***Chapter 5***, we explore the option of translating chemoprophylaxis and parasite exposure into a field intervention with the aim of inducing protective immune responses.

In ***Chapters 6, 7, 8, 9,*** and ***10*** results from four clinical CPS trials are presented. Since individuals are exposed to pre-erythrocytic as well as blood-stage parasites during CPS-immunization, the protective immune responses could be directed against either of these stages. Resolving this question is important because of the traditional lifecycle stage-specific distinction between vaccines designed to induce blood-stage *versus* pre-erythrocytic protection (115). The objective of ***Chapter 6*** is to explore the *Plasmodium* lifecycle stage-specificity of the CPS-induced protective immune responses.

Immune mechanisms and correlates of protection against malaria are elusive, and their identification would greatly facilitate clinical development of new vaccines (116, 117). Both cellular and humoral immune responses are induced by whole sporozoite immunization (102, 118), but animal studies indicate that especially T cells are crucial (119-121). In CPS-immunized human volunteers, *P. falciparum* specific pluripotent CD4 T cells were previously identified as possible correlate of protection, but it remained unclear whether these responses were rather a marker of parasite exposure (102). To address this issue, a dose-titration CPS-immunization study is conducted in ***Chapter 7***, to generate a differentially protected cohort allowing for the analysis of immune memory responses and their association with protection. ***Chapter 8*** further explores the induction and differentiation of cellular immune responses using *ex vivo* analysis of lymphocytes directly after sporozoite immunization.

So far CPS-immunization trials and most RAS studies have investigated homologous protection only, i.e. protection against the same *P. falciparum* strain that was used for immunization. In endemic areas however, infections are caused by multiple, genetically and

antigenically different strains (122-124). This huge diversity in strains is considered one of the main reasons why immunity develops only slowly in the field (125). In order for CPS to remain valuable as an immunization strategy, heterologous protection needs to be present, and this is studied in ***Chapter 9***.

The unprecedented efficiency of CPS may be related to or dependent on the anti-malarial drug used during immunization. ***Chapter 10*** therefore investigates safety, immunogenicity and protective efficacy of CPS-immunization with mefloquine compared to chloroquine.

The complex infrastructure, advanced skills and expertise required for parasite culture, mosquito breeding and infection of mosquitoes through membrane feeding restricts the availability of CHMI and CPS-immunization by mosquito bites to a few specialized centers. Replacing the infected mosquito as method of inoculation would greatly extend the implementability of CHMI and CPS-immunization worldwide. Therefore, we will investigate an alternative approach to infect healthy volunteers in ***Chapter 11***: administration of aseptic, purified and cryopreserved sporozoites by needle and syringe.

Chapter 12 explores tolerance as an alternative protection mechanism against primary parasite infection. There are three conceptually distinct ways for a host to protect itself against invading pathogens: i) resistance (i.e. limiting pathogen load), ii) tolerance (i.e. limiting the damage caused by the pathogen) and iii) avoidance (126, 127). For semi-immune adults in malaria-endemic areas, the first two mechanisms appear to go hand in hand: parasite density as well as symptoms and complications are controlled and limited (125, 128). Their individual contribution, however, is hard to disentangle. Therefore, innovative approaches are required. Cross-sectional data from residents from malaria-endemic areas suggest that so called "disease maps" may be a useful tool. Since longitudinal datasets are obtained in CHMI trials, we use these in ***Chapter 12*** to assess tolerance in healthy malaria-naïve subjects using disease space.

Finally, the findings of this thesis are placed in context in ***Chapter 13,*** and future directions and avenues for further research are discussed.

The cover of this book depicts the wall of a house in Tirana, Albania. When Edi Rama was elected major of this city in 2000, he decided to paint a large number of Tirana houses in bright colors and bold geometric designs. "It is not a matter of what color you may want this or that building, because that would be a question of trying to add up all the tastes and find the golden mean, which would be a grey", *he said. I believe that the same is fruitful for science: a wide variety of approaches, 'bright colors' and 'bold designs'.*

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CHAPTER 2

Controlled human malaria infection trials: how tandems of trust & control construct scientific knowledge

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Abstract

Controlled Human Malaria Infections (CHMIs) are clinical trials in which healthy volunteers are deliberately infected with malaria under controlled conditions. CHMIs are complex clinical trials: many different groups and institutions are involved, and several complex technologies are required to function together. This functioning together of technologies, people and institutions is under special pressure because of potential risks to the volunteers.

In this paper two medical researchers and an STS scholar use CHMIs as a strategic research site to study the use of control, the role of trust and the interactions between trust and control in the construction of scientific knowledge. The authors argue that tandems of trust and control play a central role in the successful execution of clinical trials and the construction of scientific knowledge. More specifically, two aspects of tandems of trust and control will be highlighted: tandems are sites where trust and control coproduce each other; and tandems link the personal, the technical and the institutional domains.

Understanding how tandems of trust and control make scientific experiments work and enable the construction of scientific knowledge, results in setting an agenda for both clinical trial research and social studies of science.

Introduction

In this paper we will explore how trust and control play a central role in the production of scientific knowledge, using the example of Controlled Human Malaria Infections (CHMIs).[1] CHMIs are clinical trials in which healthy volunteers are deliberately infected with malaria under controlled conditions, for example to test the efficacy of potential vaccines or to investigate immunological questions (1). Clinical trials are similar to other scientific experiments, but for one crucial characteristic—they involve people as objects of research. This makes them into a strategic research site for studying the particular aspects of experimenting that we want to focus on: the use of control and the role of trust in the production of credible knowledge. This analysis will, we hope, contribute to the understanding that science-studies scholars have of scientific experiments in general.

Clinical trials have been studied from a social science and STS (science, technology & society studies) perspective since the 1990s. The first line in this body of research investigates the role of the pharmaceutical industry in clinical trials. Research and industry are increasingly intertwined—in almost all domains of research, but certainly and perhaps mostly in medicine—resulting in a potential problem of introducing a bias in trial design, conduct of clinical trials, interpretation of results and publication strategies (2-7). Some of this research broadens its scope to analyze the resulting political economy of the health care system and the implications of a globalizing clinical trial 'industry' (8, 9). This industrialization and commercialization of medical research is particularly visible in the increasingly prominent role of contract research organizations, or CROs, in the conduct of clinical trials (10-12). A second line of research focuses on the participants or subjects, often called 'volunteers', in clinical trials, and asks questions about volunteers' motivation to participate, the effects of participation on their health, and the economic and educational background of these volunteers (13-18). The third line of social science research on clinical trials addresses issues of risk and vulnerability—mostly relating to the volunteers, but also about the trials' scientific quality as potentially compromised by their complex and industrialized organization (18-21). Almost all of this research concerns drug trials: the testing of drugs on patients or healthy volunteers. These trials are often distinguished in three types: phase-I trials are to test the drug's safety on healthy volunteers (often a small number of tens to hundreds); phase-II trials are for testing the efficacy of the drug on patients (usually some hundreds); and phase-III trials test the drug's therapeutic effect (with generally thousands of patients).

The clinical trials that we investigate in this article are of a different character than those studied in the literature we reviewed above. Instead of administering a drug that has never been tested on humans to healthy volunteers (Phase-I trials), or assessing the efficacy of such a new drug on patients (Phase-II and III trials), in CHMI trials healthy volunteers are

1 See list of abbreviations at the end of the chapter.

made into patients, by infecting them with the malaria parasite that also causes millions of natural infections every year. Hereby, pathophysiological and immunological mechanisms of the infection can be studied. Most CHMI trials are not (yet) interesting for pharmaceutical industry, and the trials are carried out by university researchers in university hospitals.[2] The trial subjects are mostly, but not necessarily, medical students, who receive a fee of some 1000-2000 euros; the numbers are typically small—10-40 volunteers per trial. Though most of the world's malaria patients are in Africa and South-East Asia, it is crucial that CHMIs are done with 'malaria-naive' volunteers, and thus in a country like The Netherlands.[3]

Not only are CHMIs different from the trials reported in the existing STS literature, also the questions we ask in this article are different. We ask how trust and control play a role in the construction of scientific knowledge and the execution of scientific experiments, and we consider CHMIs as a particularly good research site to ask that question. If the previously reviewed literature brings STS to clinical trial research, our article brings CHMI trials to STS.

In CHMI trials, volunteers are made ill as part of the trial design. Their being infected with malaria, a potentially lethal disease, does demand very high levels of trust and control. This was the starting point for our research—CHMIs seem to present a 'hard case' for studying trust and control in Harry Collins' (22: 142) sense: "if one wants to prove a general thesis, you endeavor to prove it for the case where the thesis seems least likely to hold." But, even though this formed the starting point of our project, we will not exclusively focus on the relations between the volunteers and the researchers. We will argue that 'tandems of trust & control' play a central role in the construction of scientific knowledge much more generally—also in the relations between researchers themselves, in the relations between researchers and instruments, in the relations between researchers and institutions, and in other scientific experiments than clinical trials.

Trust has been a central concept in understanding the construction of scientific knowledge, as well as in analyzing the social functioning of science in society. Without scientists trusting each other, there would be no positive production of knowledge, since they would get stuck in an experimenters' regress, as Collins (23: 130) describes: "the competence of experimenters and the integrity of experiments can only be ascertained by examining *results*, but the appropriate results can only be known from competently performed experiments, and so forth. Other ways of testing for the competence and integrity of experiments, such as 'tests of tests', turn out to need 'tests of tests of tests'—and so on" (emphasis in the original). This is closely related to the key role of tacit knowledge and the scientist's skills in performing an

2 The CHMIs that are reported on in this article are investigator-initiated and have been funded by the Netherlands Organization of Scientific Research (NWO), the European Union, and the Bill & Melinda Gates Foundation.

3 'Malaria-naive' is a term widely used in malaria research to indicate that a subject was never infected with malaria (1).

experiment. Independent replication of an experiment cannot resolve an issue, since no two experiments can ever be exactly the same in all details and so the status of an experiment as replication has to be negotiated. Thus, "for any finding to be accepted, scientists cannot be utter skeptics. (…) At any given moment, *some* knowledge must be taken on faith, if science is to proceed as a social institution." (24: 15) For the functioning of science in society, it is crucial that non-scientists trust scientists and the results of their scientific work. Much recent work in STS relates to this issue: research about different forms of expertise, about new forms of scientific and technological democracy, and about participation of citizens, consumers and patients in science and technology. All these studies contribute, in different ways and with different foci, to our understanding how societal trust in scientists, scientific institutions and scientific knowledge is crucial for the functioning of science in society (25-29). In this paper we do not address these trust issues at the societal scale, but rather focus on the role of trust at the micro level of the clinical trial.

It is an almost trivial statement to say that control is crucial for the production of scientific knowledge through experiments. It is the proper and clever control of experimental circumstances that creates the laboratory conditions in which new knowledge can be generated; and, *vice versa,* scientific controversies are often about whether the experimental variables have been properly controlled. In the case of clinical trials, a textbook even states explicitly: "you must maintain control of the study at all times" (30: 249). As we will explore in our detailed analysis of CHMIs, in practice this is less straightforward than it may seem in theory. Explicating the various meanings of control will, we think, be fruitful to help trial researchers reflect on their practices and possibly improve them, and to yield insights in the construction of scientific knowledge more generally.

After our analysis of CHMIs in terms of trust and control, we will conclude this article by arguing more specifically that it is the *interplay* of trust and control that makes the CHMIs work; these 'tandems of trust & control' make the social construction of scientific knowledge happen. Tandems of trust & control are specific combinations of these two, in which they work together, coproduce each other or partly substitute each other.

The empirical research for this paper is a participant-observation study, and was carried out in two research hospitals—Radboud university medical center in Nijmegen and Leiden University Medical Center, both in The Netherlands—where the first and second author conduct(ed) CHMIs. Unlike most anthropological lab studies, where an anthropologist first gains access to the lab and then gradually picks up some tasks and adds 'participation' to 'observation', it was the other way around in this study. The first author of this paper is a clinical researcher, who became interested in science-studies questions: so, gradually she added 'observation' to 'participation' in her way of being in the lab. She has a degree in medicine and is being trained as a researcher in clinical trials, parasitology and immunology. The last author was trained as a physicist and now is professor of sociology of science and

technology. His field is indeed the study of social processes in science and technology, but he has no direct experience with medical research or clinical trials. The second author is medical specialist in microbiology and professor of medical parasitology and one of the internationally leading experts in malaria vaccine research. His being fully socialized in the CHMI community provided a crucial role in the CHMI design and check on the observations and interpretations that our participant-observer made. He is the principal investigator of the CHMIs.

The first author's being a young medical researcher allowed her to approach questions about clinical trials with fresh curiosity, since she was not yet completely socialized into the CHMI community. The approach she followed is similar to Leon Anderson's 'analytic autoethnography', for which he proposes five key features: "(1) complete member researcher (CMR) status, (2) analytic reflexivity, (3) narrative visibility of the researcher's self, (4) dialogue with informants beyond the self, and (5) commitment to theoretical analysis" (31: 378). For the particular questions we are addressing in this paper we consider the third characteristic of "narrative visibility of the researcher's self" less important and thus have minimized such self-presence in the text.

Control

Different forms of control seem to play a crucial role in the making of scientific knowledge. We will distinguish two types of control—methodological and institutional—and we will show that both types can play out in three 'locations': control of people, of machines, and of institutions. The 'methodological' type is primarily internal to the trial and exerted by the researchers (and, we shall argue, by many other actors within the trial), the 'institutional' is external and exerted by actors in the institutional environment of the trials. Examples of methodological control are the use of a control group, inclusion-exclusion criteria for research subjects, processes of blinding and randomization, and sophisticated biostatistics. Institutional control is, for example, the requirement for a data monitoring system or GCP training of trial staff.

Methodological control is at the core of doing scientific experiments, and indeed of doing any scientific work. The more precisely a research question is formulated, the better the design of experiments or the selection of observational data can be controlled. All standard scientific methodologies—from interviewing to statistical analysis, from hermeneutical text analysis to coding of interviews, and from thick smear analysis to Polymerase Chain Reaction (PCR) analysis (which we will both describe below)—are meant to control the otherwise unruly world in the laboratory or in 'the wild'. The better the control, the more trustworthy (although less surprising) the knowledge that is produced.

The standard account in medical textbooks about the history of clinical trials is that

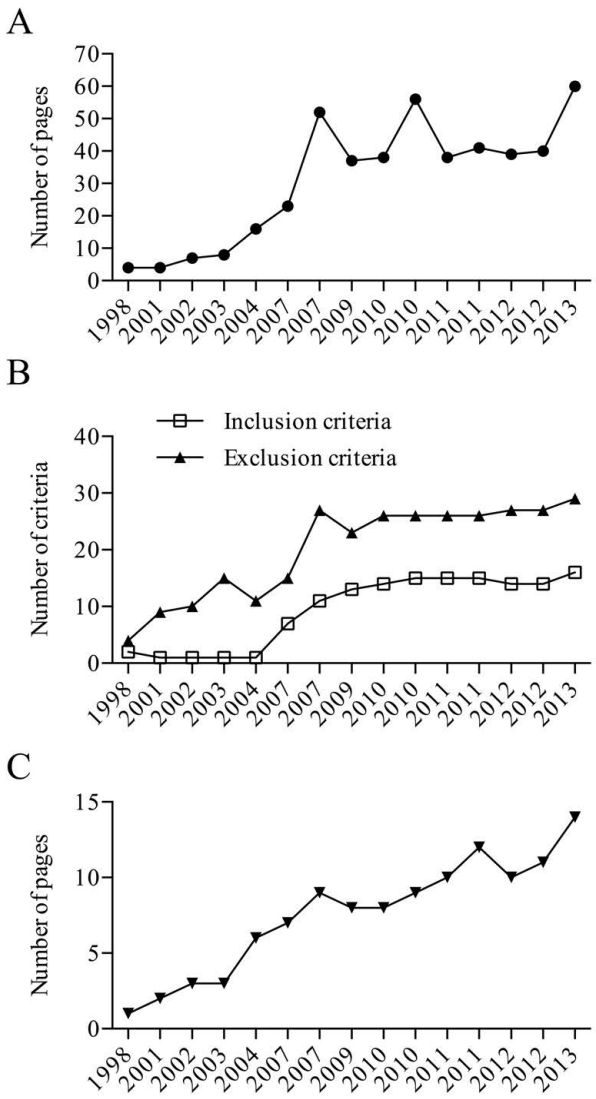


Figure 1 Increasing control, 1998-2013, A: Number of pages of Clinical Trial Protocol. B: Number of in- and exclusion criteria. C. Number of pages in Volunteer Information.

methodological improvements were introduced to reduce the subjectivity of the interpretation of the results, and to increase the scientific validity of clinical trial results (32, 33). In the first half of the 20th century, increasing distrust in the personal judgments of medical professionals was one of the factors that led to stepping-up standardization and regulation of clinical trials (34, 35). Moreover, a number of detrimental cases during clinical trials led to further increased control. For example, the death of a 24-year old healthy volunteer in an asthma trial in 2001 at the Johns Hopkins University led to changes including "more resources; new procedures;

more training for investigators and for IRB members, chairs, and staff; and the appointment of a vice dean for clinical investigation to oversee the process" (36: 719). It seems fair to say that by this kind of methodological and institutional interventions, clinical trials have become one of the most controlled ways of doing scientific research.

The design of a clinical trial, with all its stipulated methods and procedures, can be seen as a framework or recipe that has to be executed during the trial. Over the past decades both the overall trial designs and the included procedures and methods have become more and more detailed, in order to exert more control. To illustrate this: the clinical trial protocol for the first CHMI study, which was performed at Radboudumc in 1999, was only 4 pages long, while the protocol for the 2013 study is 60 pages long (see Figure 1A). At the same time, the numbers of in- and exclusion criteria have increased from 2 and 4, to 16 and 29 respectively (Figure 1B), and the volunteer information sheet was expanded from 1 to 14 pages (Figure 1C).

As stated in the previously cited textbook on clinical trial medicine: "There are several key aspects of 'good clinical practice'. The first is the concept of *being in control."* And further specified: "You must have systems in place to detect problems when they occur. You must have, in other words, the quality systems and standard operating procedures (SOPs). (…) [And] if problems are detected, you must take corrective action to ensure that the problem is abated and that the way you do business (processes) is modified as is reasonable to avoid the problem in the future." (30: 243). Such 'Standard operating procedures' (SOPs) thus are increasingly used to explicate methodologies. As illustration of the kind of procedures that are described in these documents, supplementary table 1 provides a list with the SOPs from the parasite lab. Similar sets of SOPs are available for mosquito-related activities (46 SOPs), general procedures (e.g. good documentation practices; 8 SOPs), clinical activities (18 SOPs) and immunological and molecular methods (17 SOPs).

But who is exerting control? Evidently the textbook requires the researcher to be in control. But we will describe that many other actors have opportunities (and are sometimes expected) to control the conduct and outcome of the trials. Lab technicians are crucial in controlling the growth of mosquitos and the timely infection with parasites. Trial subjects or volunteers are supposed to be controlled by the trial design and the researchers, but sometimes take over and control the outcome of the trial in unintended ways, as we will describe below. And there is the Data and Safety Monitoring Board (DSMB) as a form of institutional control that is mandatory for some trials. Such a DSMB assesses safety and efficacy data while the trial is ongoing and can advise the sponsor about terminating a trial based on these data. As we will discuss, CHMIs are not legally required to have a DSMB; but the researchers decided to have one in order to increase trust by the ethical review board and the scientific community.

Trust

If a variety of social groups exert some control and if, as we will show below, sometimes control measures have unintended consequences, why then is there no stalemate of conflicting control efforts? We will argue that trust—of different actors in each other, but also by actors in instruments, various elements of the clinical trial machinery and in the institutions involved—is crucial, and makes the trial succeed, in alliance with control.

It may seem that trust is thus invoked as a residual explanation—as what is left when control cannot explain what is happening.[4] We do want, however, to propose a more positive—say, stand-alone—concept of trust, so that in the final section of this article we can develop a *tandem* of trust & control that is conceptually symmetrical. As a general starting point we consider, with McEvily *et al.* (37), trust as the willingness to accept vulnerability, based on positive expectations about another's intentions and behavior (if about persons), about the regular functioning of organizational structures and rules (if about social institutions), and about the truth of the laws of science and the regularity of natural phenomena (if about machines, instruments and the natural world). Trust is thus partly a positive expectation that the world will 'act' in certain predictable ways, and it also involves a willingness to contribute to the relationship by being vulnerable, by a "leap of faith" (37: 93). Humans cannot function in this world without trust: not in the social world, and not in the physical world.

As we mentioned previously, the concept of trust has been used in explaining the social construction of scientific knowledge, though mostly at what we will call the macro level of the society and the meso level of the scientific community. Steven Shapin (38) answers questions at a macro level about societal trust in science by showing how this was generated at a meso level by trust relations between gentlemen scientists in the 17th century. Harry Collins (39) analyzed how trust allows scientists to continue their work without questioning everything all the time (see also Collins (40)). Collins' work is almost exclusively focused on the meso level, describing the role of trust between scientists in the scientific community. Our analysis of clinical trials is primarily based on an analysis of trust at the micro level, of actors within the experiment of the trial.

Shapin first cites the generally acknowledged distinction between *amoral* trust and *morally consequential* trust. Amoral trust is trust "in the fulfillment of inductively generated expectations about events in the world" (38: 8). We, for example, trust the sun to rise every day, and malaria researchers almost as much trust the mosquitoes to bite when they are put in a cage on the skin of the volunteers. This is trust that the world will continue to 'behave' as it always has behaved. In the case of *morally consequential* trust, a breach of that trust could lead to blaming the one who made the breach. After citing this distinction, Shapin's key argument is that the two types of trust are not that radically distinct in the construction

4 We thank one of the (anonymous) reviewers for highlighting this point.

of scientific knowledge. After all, the trust in the mosquitoes' biting is built on other people *saying* that mosquitoes always bite in such circumstances: "insofar as our factual knowledge is built up through assent to what we have been told, the two, allegedly distinct, notions of trust both belong within the same moral frame, the second routinely visible as such, the first routinely not." (38: 8).

Collins adds another distinction that we find useful for our analysis—between passive and active trust. In his work on gravitation wave science and scientists Collins argues that trust is often passive: scientists unconsciously trust the results of earlier experiments performed by others, as well as the materials, machines and computers in their experiments. Only when there are reasons for distrust, trust becomes active. Jill Fisher (20) has analyzed the role of trust in clinical trials, especially focusing on the pharmaceutical industry's mistrust of the clinicians and human subjects in drug-testing trials. Starting point of her analysis is the observation that pharmaceutical industry typically does not trust researchers and trial subjects to execute the trials in such a way that the industry's economic interests are served. Researchers are expected "to follow study protocols, not to have scientific insights or ethical misgivings about the studies (…) collect data honestly and thoroughly." (20: 406-407) And trial subjects are expected to be compliant, even if they do not directly benefit and even if they experience adverse effects. So here is a case where there were previous instances of the passive trust being violated, probably at a personal level and at isolated instances; this then resulted in active trust requirements being formulated, also at an institutional level; but then, Fisher argues, the general climate of distrust was too strong and recourse to control was necessary, which took shape in strict monitoring schemes. Even though the type of trials discussed by Fisher is very different from the CHMIs, her example of closely relating (mis) trust and control is illuminating. She distinguishes personal and institutional trust, to which we want to add a third type.

For our understanding of the functioning of the CHMIs, we will distinguish three types, or rather locations, of trust—trust between persons, trust in machines and materials, and trust in institutions—mirroring the control of people, of machines, and of institutions that we introduced previously. We thus follow a similar course as Sally Wyatt (28) and colleagues who analyzed the development of trust relationships in people, data and machines at different moments and in different ways in the case of online genetic research.

First, interpersonal trust between actors can be based on perceived intentions or on perceived abilities (41), as clearly exemplified in the triangle of clinical investigator–PI–clinical supervisor. The PI has the final and overall responsibility, but has delegated for example clinical responsibility to the clinical supervisor who is an experienced infectious disease specialist. All daily activities are carried out by the clinical investigator, who is thus supervised by both the PI and the clinical supervisor (on clinical decisions specifically). Both the PI and the clinical supervisor need to trust the clinical investigator's ***abilities***. Both

supervisors also need to trust the clinical investigator's *intentions* and integrity: will she perform all activities as required and not modify data or conceal adverse events to produce better results? The clinical investigator, on the other hand, also needs to trust the intentions and capacities of both supervisors, in order for the supervision to be successful. Similar trust relations, based on perceived or assumed abilities and intentions, exist between the PI and the clinical supervisor.

Second, researchers also need to trust the machines and instruments they use. If things go smoothly, this trust becomes taken for granted and thus implicit, or with Collins' term passive. But often enough there are hiccups—the machine for parasite detection is not giving the expected signal, or there is increased and unexplainable mosquito mortality—an example we will describe below. In such cases trust has to be restored actively to be able to continue.

Thirdly, researchers need to trust the relevant institutions and vice versa. As the PI in an asthma study where a healthy volunteer died after administration of a drug to investigate pathophysiology of asthma said: "responsibility for protection of patients in research activities is collective and systemic in nature" (36: 718). The PI did not intend "to deny or diminish his own role in ensuring the safety of research subjects" and he expressed "deep concern and sorrow regarding the death." But he underlined that clinical trial participants trust the various institutions that are involved in designing, approving and conducting the clinical trials.[5] Both interpersonal trust and institutional trust in the regulatory authorities and the research institution where the study takes place have indeed been shown to be a reason for patients to participate and cooperate with clinical research (42, 43).

Controlled Human Malaria Infection trials (CHMIs)

In 1917, Julius Wagner-Jauregg was the first to perform deliberate infections with malaria parasites, as treatment for neurosyphilis patients. These patients benefitted from the fever that was induced by the malaria parasites. The number of parasites in the blood could be controlled by targeted administration of antimalarial drugs. Wagner-Jauregg received the Nobel Prize for his discovery, but malaria-treatment became obsolete when antibiotics were discovered. In the 1960s, experimental malaria infections were used to test antimalarial drugs in healthy prison inmates in the US. The development of protocols for *in vitro* culture of malaria parasites in 1976 allowed for more routine execution of human malaria infections. Between 1985 and 2012, a total of more than 1400 subjects were experimentally infected with malaria (44, 45)

Initially, these deliberate infections were known as EHMIs: Experimental Human

5 See also the letter by Daniel A. Kracov, Counsel to Alkis Togias, M.D., to Martin H. Cohen, Food and Drug Administration, 16 July 2001. http://www.hopkinsmedicine.org/press/2001/july/togiasletter.htm; last retrieved on 19 June 2014.

Malaria Infections. During a meeting in Amsterdam in June 2011, sponsored by the European Malaria Vaccine Development Association, clinical malaria researchers from the Netherlands, USA, UK, Germany, Switzerland and Tanzania collectively decided that a new term with a more positive connotation was desirable. Someone proposed the word 'controlled', arguing that the deliberate human malaria infections were executed under controlled circumstances and with standardized protocols. The proposal was immediately embraced[6]. From that day onwards, Experimental Human Malaria Infections were called Controlled Human Malaria Infections (CHMI), without any change in the actual procedures or protocols at that point in time.[7]

Table 1 'Relevant Social Groups' in CHMI trials

Sponsor[1]	Person or entity who initiates the clinical trial.
Principal investigator	Is responsible and accountable for conducting the clinical trial, for the treatment and evaluation of the participants and for the integrity of the research data and results.
Clinical investigators	Medical doctors who conduct the clinical trial in practice.
Lab technicians	Perform several tasks during CHMI: serology tests, blood safety measurements, reading thick smears, performing PCR analysis, etc.
Nurses	Assist clinical investigators.
Volunteers	Clinical trial participants (also called 'subjects')
Funders	Provide funding[2].
Institutional review board	Institution responsible for conducting the medical ethics review.
Biological evaluators	Researchers who are responsible for the measurement of biological outcomes, such as parasitemia or immunological readouts.
Clinical supervisors[3]	Medical doctors (infectious diseases specialists) responsible for safety, clinical follow-up and treatment of the volunteers.
Safety monitor	Independent physician based in study site. Volunteers advocate and sounding board for investigators regarding safety of the volunteers.
Data Safety Monitoring Board (DSMB)	A group of independent experts who monitor participant safety and outcomes while a clinical trial is going on.

6 Source: notes from EMVDA workshop *Optimization and Standardization of Human Experimental Malaria Infections.* Amsterdam, 22 and 23 June 2011; made by first author.
7 Until 2012, CHMI-trials were performed in only six clinical centers worldwide. However, since cryopreserved parasites 'in a bottle' have become available, many more centers worldwide can now perform CHMIs.

What makes these CHMI trials work? We shall try to answer this question by analyzing CHMIs as socio-technical machineries. CHMIs are complex systems of people, instruments, animals, institutions and protocols, which produce scientific knowledge. In our analysis of the machinery of CHMIs we will implicitly follow the heuristics of the "social construction of technology" (46, 47). We will meet many social groups that can be found in any scientific research laboratory, though some may be specific for clinical trials (see table 1). We trace the chronology of the trial, and by focusing on the various relevant social groups we shall describe the role of trust and control in conducting the CHMIs.

Medical ethics assessment

In the Netherlands, there are 24 accredited Institutional Review Boards (IRBs) and a Central Committee on Research involving Human Subjects (CCMO), which can perform the medical ethics assessment of clinical trials.[8] The majority of studies is reviewed by IRBs, but certain types of complex research are by law required to be reviewed by the CCMO.[9] The law does not require CHMI trials to be reviewed by the CCMO, but after the occurrence of a cardiac Serious Adverse Event (SAE)[10], the local IRB requested that CHMI studies be reviewed by the CCMO.

The CCMO reviews whether the CHMI trial protocols are in accordance with the specifications laid down in the Dutch law. The CCMO will assess the ethical question of a proper balance between risk and benefit, i.e. the burden to the subjects and the scientific value of the research. CCMO's main focus is the safety of the research subjects. It is therefore required that the investigators describe the procedures aimed at ensuring the safety

8 For more on the CCMO, see http://www.ccmo.nl/en/reviewing-committee-mrec-or-ccmo. (last retrieved on March 29, 2015)

9 Ibidem

10 Definition of SAE (source: CCMO-website): "an undesired medical event which occurred to a research subject that does not necessarily have a causal link to the treatment and:
- is fatal, and/or;
- is life-threatening for the research subject, and/or;
- makes hospital admission or an extension of the admission necessary, and/or
- causes persistent or significant invalidity or work disability, and/or
- manifests itself in a congenital abnormality or malformation"

The cardiac event in this case was defined as an SAE because the subject was admitted to the hospital. She experienced chest pain after she had been treated for the induced malaria infection. The details of this SAE have been described in a case report (48). From the abstract of this article: "She recovered quickly and her follow-up was uneventful. Whether the event was related to the study procedures such as the preceding vaccinations, malaria infection or antimalarial drugs remains elusive. However, the relation in time with the experimental malaria infection and apparent absence of an underlying condition makes the infection the most probable trigger. This in striking contrast, however, with the millions of malaria cases each year and the fact that such complication has never been reported in the literature. The rare occurrence of cardiac events with any of the preceding study procedures may even support a coincidental finding."

of the volunteers as comprehensively as possible. These procedures include, for example, determining the sensitivity of the parasite for antimalarial drugs, regular assessment of routine laboratory values during follow-up, but also a specific safety protocol in case of cardiac complications.

For the medical ethics evaluation it is important for the researchers to invest in and maintain open communication with the secretariat of the CCMO. It will help, when timelines are tight, that some 'thinking along' is offered by the CCMO's secretariat. The CCMO Secretary may sometimes call to inform the investigators that the CCMO may raise additional questions in the up-coming committee meeting. If these issues can quickly be addressed before the CCMO meets, and then presented by the Secretary, an additional round of questions is avoided. Once all questions are answered to the satisfaction of the CCMO, the trial is approved. In our conclusion, we will critically reflect on this good relationship between researchers and CCMO.

After approval by the CCMO, the study centers where the study is carried out perform another check of the study: permission from the board of directors is required for execution in their center. At the Radboudumc, this check includes the volunteer information, the data management plan, a monitoring plan, a risk assessment and a check whether the involved researchers have followed the obligatory GCP course. After approval by the study center, official procedures can commence.

Trial preparation

Already before CCMO approval is obtained, preparation of the trial has to start. The days for screening, for infection and for follow-up visits are planned. Based on this planning, the mosquito- and parasite cultures are prepared.

The Radboudumc insectary produces approximately 15,000 mosquitoes per week. Mosquito eggs are distributed in plastic containers filled with water, in a climate-controlled room (Photograph 1). The eggs develop into larvae, then pupae and mosquitoes in approximately 2 weeks. Before the pupae develop into mosquitoes, a net is placed over the containers to prevent the mosquitoes from escaping. The mosquitoes are then collected and kept in a cage until they are infected with parasites. Parasites are cultured in glass flasks (Photograph 2). Twice a day, the medium with waste materials is removed, and fresh culture medium is added. In order to infect mosquitoes, they need to take a blood meal containing parasites via a "membrane feeder" (Photographs 3 and 4).

The trial schedule, once fixed, creates a long chain of interconnected and interdependent actions. Enough infected mosquitoes must be available on the infection day. The infected mosquitoes have to be 14-21 days old, since younger mosquitoes are not infectious, and older mosquitoes are less eager to bite and the risk of mosquito-mortality increases. Complicated planning is required to have sufficient batches of high quality mosquitoes and parasites on

the planned dates.

During a clinical trial in 2011, the malaria unit technicians noticed increased mosquito mortality. The reasons for this were not clear: protocols were followed strictly and had not been changed recently.[11] In order to secure a sufficient number of infected mosquitoes, extra batches were bred and infected. Furthermore, the technicians tried to trace the cause of the mortality. They checked with the blood bank whether the blood-withdrawing methods or the collection tubes had changed, but this was not the case. They also considered whether chemicals, used in the building activities on the roof of the central animal facility where the malaria unit is located, might have caused the problem. Another option was that the tap water, which is used to breed to mosquitoes, was contaminated or that there was a problem with the ventilation of the climate rooms. But these options were ruled out when mortality was shown to have increased in both the old and new units in separate buildings. In the end, the cause of the increased mortality was never discovered, and the technicians found a way to circumvent the problem by using very young mosquitoes. The incident made the technicians and the scientists aware that, in our words and using Collins' distinction, they had been *passively* trusting the heparin tubes, the ventilation system and the tap water for many years. After developing the new method to work around this problem, an *active* trust was built up in the mosquito breeding facilities.

Photograph 1
Mosquito breeding climate room. The technician is collecting mosquitoes with a 'mosquito-vacuum-cleaner' into the grey box.

Recruitment and screening of volunteers

After CCMO approval, the clinical investigators start recruiting and screening volunteers. These clinical trial participants are called 'volunteers', because they make the choice to participate and have the right to terminate their participation at any point in time. But the fact that they receive financial

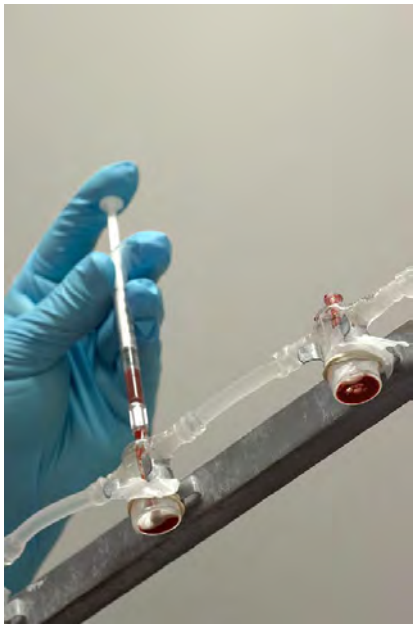
11 As the head of the mosquito unit reported: "More than 25-50% of the mosquitoes die within 48 hours, and I don't have a good feeling with the remaining mosquitoes. The infectious mosquitoes that are needed for the first immunization of the EHMI9 study are now under pressure." (e-mail from the head of the mosquito unit, 8Apr2011).

reimbursement for their participation may diminish their autonomy in making the decision.[12] Calling them volunteers strengthens the notion that subjects are in control of their own involvement. It also suggests that society can trust that they independently and deliberately decide to participate in clinical research. Both in the communication to volunteers and in scientific publications, the terms 'volunteer' and 'subject' are used alternatingly; we will do that here as well.

Photograph 2 Parasite culture.

The recruitment process consists of four steps. 1) Distribution of advertisements on flyers and posters; 2) Comprehensive information package; 3) An information meeting; 4) The medical screening. Taken together, for a study with 30 subjects, approximately 240 have to show interest during the first phase of the recruitment trajectory. In the screening, volunteers are selected by applying in- and exclusion criteria, listed in the protocol as approved by the CCMO. In summary, participants are healthy, 18-35 years old, male or female. In practice the mean age has been 21.5 years, with a range of 18-32. The male-to-female ratio is on average 1:2. Most importantly, participants have to be very healthy to participate in the trial. Laboratory results outside the normal range, a deviant electrocardiogram, family history of cardiac disease, drug- or excessive alcohol use are all reasons for exclusion of a subject. They also have to be 'malaria-naïve': i.e. never had malaria. This is assessed by taking a detailed travel-history and by testing for antibodies against the malaria parasite. It is essential that the volunteers are malaria-naïve, since pre-existing immunity against the malaria parasite would interfere with the effect of the controlled infection and thus the result of the trial. During the trial, the subjects are not allowed to leave the country; The Netherlands becomes the

12 For participating in a single CHMI with approximately 25 follow-up visits the volunteers receive 1000 euros.



Photograph 3 Injection of blood into mini-feeder.

investigator's laboratory.[13]

This selection process results in a group of subjects that is distinctly different in comparison to most of the subjects that have been studied in STS research of clinical trials. There is one similarity with phase-I drug trials: that the subjects are healthy volunteers and cannot expect personal health benefit. But on all other accounts the CHMI selection process produces a quite different group of subjects. Unlike the 'professional guinea pigs' that Roberto Abadie (17) describes, CHMI volunteers can only participate once (because of the requirement of being malaria-naïve) and thus cannot be financially dependent on CHMIs for their livelihood. There is no 'informal economy' with volunteers living off CHMIs, as studied by Jill Fisher (8). CHMI volunteers do receive financial compensation, but this is a one-time addition to their regular budget, rather than a form of income.[14] Since the recruitment efforts are targeted to university students, because of positive experience with their flexibility, motivation and reliability, the large majority of volunteers turn out to be students. However, unlike in the early history of medical student volunteers (15), the CHMI students typically do not have a student-teacher relationship with the researcher, nor are awarded study credits. This different profile of the CHMI volunteers has implications for the balance between trust and control, as we will discuss below.

Trust is of central importance in any medical relationship between doctor and patient (49), but to trust a doctor who is performing a clinical trial is a different matter than to trust your own physician—if only because other interests are at stake than only your own health. Within clinical research a further differentiation is useful. While treatment-trials offer a potential benefit to the participants—or at least to their fellow sufferers with the same disease in the future—this is not the case in trials with healthy volunteers, such as phase-I trials

13 The infected volunteers will not be a source of secondary infections because of very early treatment when the transmission stages have not been formed yet.

14 In one instance, the parents of a student did not like him participating in the CHMI and offered him the same amount of money if he would withdraw. He did not do so. Then, later during the trial, his father once visited one of the monitoring sessions in the hospital and intensively interacted with the researchers, nurses and volunteers. He left with "now I understand why you do it", and was reconciled with his son's participation.

and CHMIs: there is no intended health benefit for them. In CHMIs, the trust relationship between volunteers and researchers can thus not be the same as in treatment trials or in common patient-doctor relations. Why do the volunteers trust the investigators; why would

Photograph 4
Feeding of mosquitoes on midi-feeder.

they trust someone who infects them with malaria? The investigators need to establish and to maintain such trust by the volunteers (see also Burgess (50)).

The investigators generate trust amongst the volunteers by informing them comprehensively, by explicating their good intentions, their expertise and abilities, and also how they are checked and controlled by the independent physician and the ethical review. For example, they explain the scientific background, the research questions, the study design, and the expected burden and possible risks. Pictures of the parasite culture, the mosquito breeding and the antimalarial drugs are shown during the information evening. They tell about the extensive experience, the team of "world-class technicians" behind the scenes and the good results that have been obtained in similar studies by the Radboudumc. The investigators emphasize that they will be always reachable and that the health and safety of the subjects is their primary concern. They further explain how the supporting technologies (such as the mosquito breeding and the microscopic parasite identification) help to control the parasites and thus the malaria infection in the volunteers. In other words, the researchers specifically explicate the various control-mechanisms—of persons, institutions and machines—that function in a CHMI, and thus seek to generate trust. Moreover, the investigators aim to remain very transparent during the trial about what is going on. Every once in a while the researchers and nurses discuss the greater goal of the project, and share the excitement about the possible results of the trial. In sum, the volunteers are more treated as partners in the research than as object of research.[15] Some of the volunteering medical students indicate that

15 See also the discussion about promoting research participation by recognizing a broader motivation in participants by Williams *et al.* (13, 51, 52)

one reason to participate is to better understand the inner workings of a clinical trial.

This hotel-housing of CHMI volunteers resembles the in-house confinement used in many drug trials (8, 17, 18, 20), but is distinctly different. In the CHMI case the aim is not to control the subjects' behavior in detail, as described by Fisher (18) in her risk analysis of phase I trials. Rather, the purpose is to have the volunteers in a known location at close distance to the hospital. While staying in the hotel, the volunteers are free to do what they want—from going to their lectures to using the gym. They do receive instructions like 'only one glass of alcohol per day' and 'sleep well', but this is more done after fully informing them about the details of the trial and then giving them control over their own day-to-day affairs—it thus is more a matter of trusting them than controlling them.

And volunteers have, of course, other kinds of control too: they legally have the right to withdraw their informed consent at any time and for any reason. After a volunteer is infected this right remains the same, but they are then obliged to take antimalarial treatment to cure the infection before they can actually withdraw.[16]

A different story evolves if the volunteers cuts communication and disappears. Such exertion of control by a volunteer happened in a CHMI trial in 2010 in Oxford when a volunteer, who had been included and infected, did not turn up for the first follow-up visit, and was only found after an extended search in the Netherlands.[17]

The trial

One day before the infection, all subjects are checked again for eligibility and then officially 'included' in the trial. One or more alternate volunteers who passed screening are available as substitutes for volunteers who withdraw or fall ill just before the start of the study. Once the trial starts, events of trust and control can be seen everywhere. We will describe the main course of the trial without each time explicitly labeling such control and trust occurrences, until in the next section we concentrate on the process of data gathering and monitoring.

On infection day all volunteers come to the central animal facility of the Radboud University. Infection day starts early because the mosquitoes are more eager to bite in the morning. While in the field mosquitoes bite in the evening, in the lab their day-night scheme is reversed for the convenience of the technicians. Mosquitoes are also starved overnight to further increase their eagerness to bite. The infection procedure takes place in a designated room in the insectary with a double-door barrier along with a blower system to prevent flight across entryways. For each volunteer a small cage, containing five mosquitoes, has been prepared. This cage is placed between the forearms of the volunteer (Photograph 5), a towel is placed over the cage (because mosquitoes bite better in the dark), and mosquitoes are allowed to bite for ten minutes. Afterwards, the volunteers return to a waiting room.

16 If volunteers withdraw after being infected, they receive the same malaria treatment and have to fulfill the same post-treatment safety checks as volunteers who complete trial participation.

17 See http://www.bbc.co.uk/news/uk-england-11572862 (last retrieved on March 29, 2015)

Technicians dissect all mosquitoes to check whether they have (1) fed on the volunteer and (2) were infected with malaria-parasites. If the answer to both questions is affirmative, the subject has received an infectious bite. Otherwise, the volunteer has to undergo an additional round, until exactly five infectious mosquitoes have bitten.

After the infection a 5-day period of relative rest follows, because the parasites replicate in the human liver cells during this time, and symptoms don't occur. From day 5 onwards, the subjects have to visit the trial center 1-3 times per day. At each visit, the subjects are asked questions about their complaints and symptoms, they show the diary they are keeping, and blood samples are drawn. Several blood parameters are tested. Most importantly, immediately

Photograph 5
Exposure of human volunteer to bites of *Anopheles* mosquitoes.

after each visit all blood samples are checked for parasites with a standardized test.

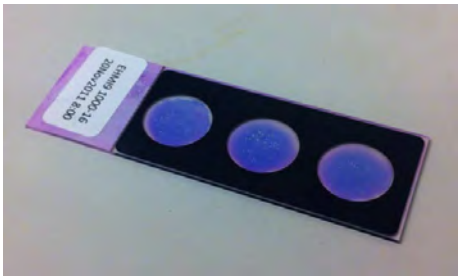
When parasites are detected in the blood of a subject, this person is immediately called back to the trial center for treatment. The subject takes the first treatment dose at the trial center and goes home. Usually, most symptoms of the malaria infection occur on the next day. Common complaints are headache, fatigue, muscle pain, and fever. Nausea, abdominal pain, joint pain and vomiting might also occur, but are more rare. Complaints typically don't last for more than two or three days. The second and third doses of medication are handed over to the volunteer during the subsequent morning visits for the volunteer to take at home, 24 and 48 hours after the first dose. There are two more follow-up visits in the following three weeks.

At each visit, the clinical investigators record adverse events (AEs) that are reported by the subjects. All AEs are first recorded in the paper Case Report Form, and subsequently transferred to the electronic clinical data management system (CDMS). All AEs are described in safety reports regularly during the trial.

The blood samples are prepared into so-called 'thick smears' according to an SOP.

Briefly, glass slides are used, with three wells with a standardized diameter (Photograph 6). On each well, a fixed amount of blood is pipetted to create a standardized thickness and surface area. The slides are then stained with Giemsa staining solution and assessed under a standard light microscope. The number of High Power fields to be screened is calculated using the diameter of the ocular lens, resulting in the reading of a fixed volume of blood. All parasites seen need to be confirmed by a second microscopist. Thick smears are the worldwide gold standard for malaria diagnosis.

In addition to the thick smears for parasite detection, several safety parameters are tested during the follow-up visits, such as routine hematology and biochemistry values. Since the above described cardiac event occurred in an experimental vaccine-trial, several cardiovascular markers are now measured too (48).



Photograph 6 Thick blood smear.

CHMIs have been performed in a handful of different trial centers around the world. There were differences in outcome that led to a discussion about methodologies in the different sites. The time-to-thick-smear-positivity, for example, was a few days longer in Oxford than in Radboudumc. First it was thought that this was because of superior microscopic diagnostics in Radboudumc. Therefore, the SOP was further optimized and adjusted to the kind of microscope used, to make sure that all centers investigated exactly the same amount of blood. But even after the implementation, time-to-diagnosis by microscopy remained longer in Oxford. Therefore, samples from subjects in both centers were directly compared by Polymerase Chain Reaction (PCR), a more sensitive detection method. The conclusion of this comparison was that development of the parasites in Oxford was actually slower. When a trial in Radboudumc was done with the same parasite-strain from Oxford (3D7, a clone from the strain used in Nijmegen, NF54), time to diagnosis was longer in Radboudumc too, showing that the parasite was the critical variable. This is an example in which extra control (in the form of a SOP) allowed for the creation of new trust (amongst two research centers about the quality of their microscopy). We will return to this below.

Data monitoring

Reviewing the ways in which trust and control figure in carrying out CHMI's, we mostly followed a chronological order through the trial process. We will now discuss in some detail the process of clinical trial monitoring to investigate the various control measures that are built into the trial process and their relation to questions of trust. Implementation

of monitoring is a crucial part of 'Good Clinical Practice (GCP)', which is a prerequisite for trials with medicinal products and increasingly recommended for other trials as well. Monitoring entails a number of activities, which are meant to verify that:

a) The rights and wellbeing of human subjects are protected.

b) The reported trial data are accurate, complete, and verifiable from source documents.

c) The conduct of the trial is in compliance with the currently approved protocol/ amendment(s), with GCP, and with the applicable regulatory requirement(s). (53: 31)

The term 'monitoring' is generally used to describe on-site monitoring, where an independent monitor visits the trial site to check whether the research is carried out appropriately, and assesses some documents such as the *curricula vitae* and training records of the staff, and relevant SOPs. A monitor typically is a consultant from an independent CRO with a biomedical background, who is hired to perform the data monitoring. Data monitoring has been obligatory for trials that investigate a medicinal product for a while, but the Netherlands Federation of University Medical Centres (NFU) recently issued a report in which data monitoring is recommended to be obligatory for all clinical trials (54). Radboudumc, where the CHMIs are carried out, has already implemented this guideline. An important part of monitoring is *source data verification*. In the situation where data are initially collected in paper source documents and then transferred to a clinical data management system (CDMS), the monitor will check whether the data in the CDMS are the same as those in the source documents.

In the NFU guideline, the amount of source data verification that is required depends on the estimated risk of the trial; when subjects in a trial are exposed to low risk, only 10% of patients case report files (CRFs) have to be checked, while for a high risk trial, all data have to be verified. Thus, according to the NFU, higher risk asks for more control. One of the most important source documents in CHMI trials is the paper CRF, in which the investigator makes notes during each follow up visit; e.g. symptoms and complaints, blood pressure and temperature. Other source documents are the diaries kept by volunteers, and forms with mosquito data from the infection day. From these source documents the investigators enter data on primary and secondary outcomes into the CDMS. From this system, data will later be extracted for analysis.

However, the transfer of data from source documents to the data management system is only one link in the chain that generates results (see Figure 2). Before data are noted in a source document, they have to be collected. In the case of symptoms, this means questioning the volunteer; in the case of blood pressure and temperature, it means measuring these variables. But the data monitor is not standing next to the investigator while these measurements are performed, and thus cannot check whether the right questions are asked to the subject, or if the value is measured correctly and recorded properly in the source document. In order to make sure that measurements are performed correctly, protocols and SOPs are generated.

All people involved in the clinical trial are obliged to study the appropriate SOPs, to make sure they will perform their tasks accordingly. However, protocols are no guarantee for the proper execution of a task (55-57). Also recording of AEs, however much protocolled or standardized, remains in part a skilled, professional (others might call it: subjective) exercise.

After a trial has been completed, and all data have been checked by the monitor and found to be in agreement with the source documents, the data management system is locked:

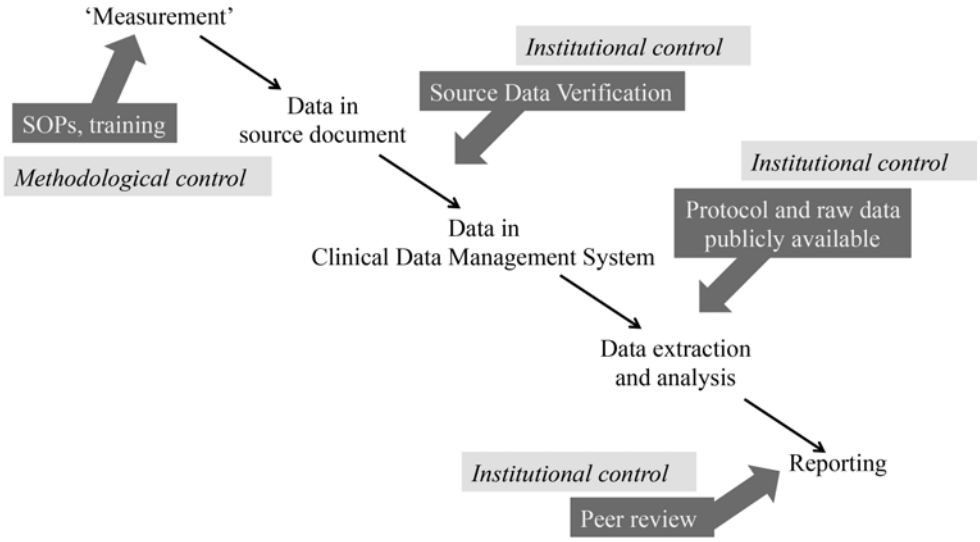


Figure 2 The process of data-generation and different locations of control.

data can no longer be added, changed or removed. The final data are thus controlled. However, what happens after the data management system is locked? The data are exported and analyzed in programs such as Excel, Graphpad Prism or SPSS, leaving plenty of opportunities to affect the interpretation and presentation of the data. As any experienced researcher will recognize, the same set of data could result in quite different (though it is assumed: not contradictory) results and conclusions when analyzed by different people. To increase control over this part of the process, more and more journals are requiring the registration of the clinical trial in a publicly accessible database before inclusion of the first subject (58). Thus journals and readers can check whether the research questions, outcomes and methods of analysis are in agreement with the original plan. Furthermore, journals are increasingly formulating policies about making raw data files available, so that other researchers can repeat the analysis independently (59).[18]

18 For a concrete example, see PLOS: www.plos.org/plos-data-policy-faq/ (last retrieved om April 7, 2015)

The last step in the generation of scientific knowledge by clinical trials is the transfer of the results of data analysis into scientific papers. Here, peer-review is the institutionalized control of the quality of scientific data.

The example of data management in CHMIs shows that control is exerted in many locations. Within the trial research community there is a strong focus on data transfer into the CDMS as location where control should be executed. The degree of control here seems to be nearly complete: the transfer of every single digit from the source document to the CDMS is checked and approved. But there are many other locations of control—with various degrees of strictness. The publicly available trial registration is such a location; comparing the original protocol in this database with the published article provides a measure of control that is certainly less strict than the data monitor's checking of the CDMS, but more than peer-review typically exerted in the pre-repository era. We also identified the consulting-room as a possible location for control: is the clinical investigator making proper use of the blood pressure meter? The degree of control seems much lower here, though SOPs are increasingly applied in order to increase control, as we will further discuss below.

In one CHMI trial, the non-Dutch sponsor requested a new regime for controlling the CRF-CDMS transfer: the paper case report files needed to be written in English "to match the CDMS". The sponsor intended to increase the control, since a non-Dutch speaking monitor would then be able to check exactly whether the paper file matched the CDMS. The outcome, however, is probably less rather than more control. Under this new regime something is left out of the CRF and therefore impossible to be checked: the translation step that the clinical investigator makes while talking to the volunteer in Dutch and making her notes in English. Where in the old regime a Dutch-and-English speaking monitor could check both this translation step and the transfer of data into the CDMS, the translation step is not accounted for in the new regime. So, in this case an intervention to increase the degree of control actually resulted in less control.

So, the data monitoring process involves a variety of control measures, some exerted by persons (e.g. the data monitor), some by institutional means (e.g. journals requiring the deposition of data in a publicly accessible repository), and others by machines (e.g. a blood pressure meter). They have complex relations to trust. In some cases they are installed to compensate for lack of trust and are meant to create more trust (e.g. the CRF in English rather than Dutch language), some are only possible because supported by underlying trust (e.g. peer review of the final reporting). It is these intricate relations between trust and control that we now turn to.

Tandems of trust & control make the socio-technical trial machinery work

In this section we will argue that an important key to understanding CHMIs, clinical trials, and indeed scientific experiments in general, is the *interplay of control and trust*. Such a combined conceptualization of trust and control is not entirely new. Researchers in organizational studies have explored the "trust-control nexus" to map "the similarities and differences between trust-based and control-based modes of control" and asked whether "these modes of governance go hand in hand" and "does control chase out trust or does trust diminish the need for control?" (60: 260). We will now ask similar questions about the production of scientific knowledge and about the various relationships in carrying out CHMIs and other clinical trials. We will especially elaborate the distinction between trust in and control of *persons, machines, and institutions*.

Often, trust and control are considered in contrasting ways: trust tends to involve personal relationships, generally asks for some symmetry in them, and is commonly associated with subjectivity; control involves rules and protocols, often is hierarchical, and is associated with objectivity and standardization. Ted Porter thus proposes trust in quantification as an alternative to personal trust between scientists, and argues that standards became increasingly required by regulatory authorities in the 20th century as a response to distrust in these scientists' expertise (35). More specifically about medical research, another historian, Harry Marks (61), noted that increasing distrust in medical practice in combination with a growing emphasis on efficiency led to increased standardization and regulation in clinical practice and medical research.

We want to argue, however, that trust and control are not just alternatives, which substitute for each other when one does not suffice, but that they actually work in conjunction to make clinical trials and, more generally, scientific research work. We follow Guido Möllering's (62: 284) plea for "a *duality* perspective, which entails that trust and control each assume the existence of the other, refer to each other and create each other, but remain irreducible to each other." This will take our discussion of trust and control beyond the discussions in the clinical trial literature where this relationship is primarily considered in the context of industry-researcher and researcher-subject interactions.

When the CCMO trusts the sponsor, when the subjects trust the investigators, when the investigators trust the technicians—in all cases such trust is not merely based on personal relationships, on the trusted persons' good character or the color of their eyes. Such trust rests on the assumption that there are institutions and social structures in place that regulate the behavior of the other actors who are to be trusted. These social structures comprise the institute where the study takes place, the scientific infrastructure, the larger scientific community and society as a whole. Actors assuming the presence of these structures creates order and thereby control (62). Trust benefits from knowing that there is some measure of

control. Trust works in tandem with control—in this case trust in people with control by institutions.

Control, similarly, cannot exist without trust. Even when institutions, social structures and control measures are in place, actors still need to trust each other to comply with the rules since they cannot possibly monitor all other persons' behavior. The CCMO, for example, ultimately trusts the sponsor's abilities and intentions, because any contract or protocol leaves room for interpretation, and not every improper use can possibly be fully controlled. And data monitoring as a form of control builds on trusting that the researcher properly notes the data into the original paper file, whether in English or in Dutch.

Another example of such a tandem of trust & control could be seen at work in the previously described riddle of the difference in time-to-thick-smear-positivity between Oxford and Nijmegen. A new SOP to control the microscopy practices in the Radboudumc and Oxford labs worked in tandem with confirmed trust in the constancy of the parasite strain and other natural variables. So, in this case control of people combined with trust in the (institutional) world.

We want to highlight two aspects of tandems of trust & control. The first is that tandems of trust & control are sites where trust and control coproduce each other; the second is that they link the personal, the technical and the institutional domains. Trust and control thus do not only occur together and presuppose each other—they also produce each other (62). However, the relationship between the two elements in these tandems of trust & control is not unidirectional or unequivocal. Often they enhance each other (more control through the microscopy SOP yielded more trust by the Oxford and Nijmegen researchers in their basic materials and work processes), but sometimes one can partially substitute the other (more trust in measuring equipment might lead to less need for monitoring control of the data). Hence our use of the rather ambiguous term "tandem": trust and control coproduce each other, but they can—within that tandem—also partly replace each other. Trust and control are bound together as the two riders on a tandem bicycle, including their ambivalent relationship: the idea is that both riders peddle and that their combined power moves the bike forward extra swiftly, but it is also possible that one freewheels a little (or even applies the brakes…) so that the other needs to push extra hard.

The investigators, for example, build and maintain trust by volunteers in the clinical trial's socio-technical machinery by explicating the control measures: the frequent follow-up visits, the testing of the drug-sensitivity of the parasite and the ethical review by an independent committee. The control measures produce the trust that volunteers have in the CHMI. Similarly, the sponsor builds and maintains trust by the CCMO, by the clinical supervisor and by the larger scientific community by implementing a variety of controls: the installation of a DSMB (though legally not required), an audit by an independent external party, and registration of the trial in a publicly accessible database.

Control thus creates trust, but this is not a simple positive association where more control always results in more trust, and thus in an unequivocally positive result. At a meso level, Collins (23) showed that when lack of trust between scientists would result in more and more control, an experimenter's regress would follow and bring the production of scientific knowledge to a standstill. This also applies to the micro level of the scientific experiment. Machines and instruments are validated, checked and calibrated regularly, but at some point one needs to trust the validation and calibration methods for it not to result in an endless exercise of tests of tests. Similarly, CHMI staff members have to read and understand SOPs and work instructions, and sign a training record form indicating that they did so. This formal documentation is important for the records, but is only meaningful because of an appropriate balance between trust and control: rather than making them sign the form every month, the staff members are trusted to keep their knowledge up-to-date. So, control can produce trust, but more control does not always produce more trust.

Trust also produces control. The first mechanism by which this happens was already identified by Georg Simmel (63: 348): "the trust we receive contains an almost compulsory power, and to betray it requires thoroughly positive meanness". Here Simmel examined interpersonal trust and stressed its moral dimension: "In the confidence of one man in another lies as high a moral value as in the fact that the trusted person shows himself worthy of it" (*ibidem).* Bestowing interpersonal trust on another person has a disciplining effect on that person, and thus results in exerting some degree of control. In the CHMIs, the interpersonal trust between the volunteers and the investigators thus enables methodological control: it helps to maintain the strict conditions that are required for a proper trial. The researchers bestow trust upon the subjects, for example by including them as much as possible in the process of the trial. Then typically the subjects will come to the check-ups on time, not drink more than one glass of alcohol, and generally follow the instructions.

The second aspect of tandems of trust & control that we want to highlight is that they typically link the domains of persons, machines and institutions. Volunteers typically trust the socio-technical machinery of the trial, including the scientific instrumentation, the institutional infrastructure of CCMO + principal investigator + hospital institution, and the individual researchers they are interacting with. It is partly on the basis of this trust that they are willing to let themselves be controlled. The cases of the weight-lifting volunteer and of the Oxford-Nijmegen discrepancies in time-to-thick-smear-positivity both could be better understood by including the role of machines (e.g. microscopes and biochemical lab equipment). Another example, about interaction between the sponsor and the CCMO, illustrates how interpersonal trust can produce institutional control. In the Netherlands, the sponsor is free to choose any IRB for ethical evaluation and approval of the clinical trial. The CHMI sponsor submits studies for assessment to the CCMO, even though this national committee could be considered 'more heavy' and possibly 'more strict' than the average

local IRB. The CHMI sponsor trusts the CCMO. Based on many years of experience, he knows the members to be scientifically capable, to assess the protocol critically but fairly, to ask pertinent questions and to make reasonable demands. *Vice versa* he will readily provide them with all documentation, answer all questions that arise, and candidly discuss difficulties in the trial design. Interpersonal trust between sponsor and CCMO staff thus supports and perhaps to some degree even substitutes institutional control of the trial by the CCMO. This is, however, not without risk as Adam Hedgecoe (19) describes. We will discuss this issue at a more general level in the final section.

Implications for trial design and science studies

We have argued that both control and trust are crucial for the socio-technical machinery of clinical trials to function—not just as alternatives, but in tandem coproducing each other. This, then, sets two further agendas—first, a design-experimentation agenda for clinical trials; and, second, a research agenda for the sociology of scientific knowledge.

If control and trust work in conjunction to make the socio-technical machinery of the clinical trial work, can researchers then also strategically shape them, to improve the functioning of a CHMI? Can clinical researchers decide where and how to locate control, which institutions and people to trust, and where to apply one to enable the other? An example from our fieldwork suggests that this is the case. After an SAE in 2013[19] the researchers decided to change the CHMI protocol and increase control by adjusting the treatment criteria. Rather than treating subjects at the time that parasites are detected by thick smear, the investigators decided to replace thick smear analysis by PCR as primary treatment criterion since the latter is a more sensitive method and thus leading to earlier treatment. The increased control of this adjusted treatment paradigm will lead to lower parasitemia and is expected to result in fewer complaints and possible complications. This helped to restore trust by the CCMO in the CHMI protocol. The CCMO approved the next trial. In this tandem of trust & control, a change in the control machinery led to an increase in institutional trust.

So, the shift from thick smear to PCR meant more control and resulted in more trust; but it came at a cost. The PCR-instigated treatment has a scientific disadvantage, because information about the development and kinetics of parasitemia is obtained over a shorter and thus less informative period since volunteers are treated earlier. So the design decision to change the protocol entailed a trade-off: less scientific output against higher safety. This is one example in which a conscious and strategic change in the tandem of trust & control (though these words were not used at the time) had direct effects on the production of scientific knowledge.

19 Acute myocarditis occurred in a volunteer participating in a CHMI trial. This SAE is described in detail by Van Meer *et al*. (64).

Trying to control everything, as the clinical trial textbook suggests, does not work: protocols and standards cannot cover everything. In designing experiments and trials, as well as in running them, researchers must decide how to balance control and trust. One could, as it were, perform a 'sensitivity analysis'[20] to assess where and how trust and control could be best applied: one could do thought experiments to see how the machinery might work differently when designed with differently attuned tandems of trust & control. Some critical reflection on the trend of increased control, which we mentioned before (see figure 1), might thus lead to rebalancing the tandem of trust & control and consciously investing more in trust relations. Investing in the trust relation between, for example, the ethical committee and researchers by creating semi-public scientific meetings between committee members and researchers—rather than restricting the interactions to documents, forms and check-boxes—could be one way of countering the trend of over-regulation and production of an illusion of security through control.

The multi-domain aspect of tandems of trust & control is particularly useful. Hedgecoe (19) describes a severe drug trial disaster at London's Northwick Park Hospital in 2006, in which six volunteers fell unexpectedly and seriously ill. They were sent to an intensive care unit and eventually all survived, but at least one of them never completely recovered. The ensuing press coverage made this trial "world famous". An Expert Scientific Group, which investigated the event, concluded that the trial, "however unfortunate for the individuals effected, did what it was designed to do"—reveal the toxicity of a drug. In contrast, many public press media "insisted that given the severity of what went wrong, someone must be at fault" (19: 60). Hedgecoe's analysis is that both the expert group and the newspapers are wrong and that something happened that is similar to Snook's (65) "practical drift". In his analysis of the shooting down of two US helicopters over Iraq by 'friendly fire', Snook shows how in an incremental process the day-to-day practice gradually drifted away from the written guidelines.[21] Hedgecoe concludes that something similar happened in the trial in Northwick Park Hospital. His central observation is that good personal relations between the research ethics committee and the researchers "can, on occasion, serve as the basis of pathological decision making." (19: 63).

Hedgecoe's observation can be read as a critical warning about the good personal relationships we described as existing between the CCMO and CHMI researchers. It is exactly a similarly good relationship between ethical reviewers and researchers that in the Northwick Park Hospital trial caused the "organizational deviance [to be] normalized within and by the culture shared between the part of the company running the research and the Research Ethics Committee which approved the trial." (19: 74). We now want to suggest that

20 We thank Koen Dechering, CEO of the Radboudumc spin-off TropIQ, for this formulation.

21 Snook's concept of practical drift has been taken up more widely in STS studies of risk and vulnerability (66, 67). Diana Vaughan's (68) study of the Challenger disaster makes a similar argument about "normalization of deviance."

this drift and organizational deviance could happen because the tandem of trust & control in the Northwick Park case became too mono-dimensionally personal. Maintaining, and if necessary increasing, the multi-domain aspect of tandems of trust & control—incorporating technological and institutional means of trust and control in addition to personal trust and control—will make such drift processes less likely to happen. The requirement that trials in Nijmegen—in addition to the CCMO approval—seek permission by the Radboudumc Board of Directors, as we mentioned at the end of the section on medical ethics assessment, is an extra institutional control that helps to guard against possible 'drifting' unchecked by the good personal trust relationship between the CCMO secretary and the researcher.

The second implication of our argument can be translated into a research agenda for the social studies of science and technology. The construction of scientific knowledge can, we want to suggest, be understood in terms of tandems of trust & control: by tracing how specific combinations of trust and control shape scientific knowledge during its construction. We will, as a first illustration, discuss two examples.

Wyatt et al. (69) analyze how the use of self-reported data in scientific research affects several methodological and epistemological questions, such as informed consent by subjects, ownership of research data, representativeness of samples, validity of data, and generalizability of findings. One of their cases is *23andMe*, a large and well-known company offering genetic testing online. The tests are not just offered to their web-site visitors, but the accumulated self-reported data from these consumers is used by *23andMe* for scientific research. Quoting a report in WIRED Magazine, Wyatt and colleagues suggest that this online use of self-reported data bypasses "centuries of epistemology in favor of a more Googley kind of science" (69: 137, 70).

It is crucial for *23andMe* to build up a trust relationship with its consumers. After persuading them to "part with their money and saliva", the company needs to exert control over these customers/subjects to transform them "into research participants, willing to share information about themselves, not just once, but regularly and often." (69: 145). Because of the exclusively online relationship with these customers/subjects/participants, such control can only be exerted through trust. In this tandem of trust & control, several institutional and technological means can be identified. First, *23andMe* obtained ethical approval from an (albeit commercial) IRB and presents this proudly on its website to generate trust—not only by its customers, but also by the scientific community. Second, Wyatt and co-authors demonstrate how *23andMe* tries to build this trust through a variety of 'material, literary and social technologies' (71), such as rhetorical techniques on the website and in surveys, the (claimed) use of encryption and firewall technologies to guarantee privacy, and of course its genetic tests. By use of this tandem of trust & control, *23andMe* seeks to circumvent problems of self-reported data such as quality of data, verifiability of data, and representativeness of sample, and thus to produce credible scientific knowledge.

We have shown how tandems of trust & control allow the complex clinical trials with malaria-infected human volunteers to produce scientific knowledge. This insight from studying CHMIs can, we have argued, also be extrapolated to other scientific experiments. *Vice versa,* our science-studies analysis of CHMIs can inform and inspire changes and improvements in the design and execution of clinical trials by strategically modifying tandems of trust & control.

List of abbreviations

3D7	Clone of NF54 *Plasmodium falciparum* strain
AE	Adverse Event
CCMO	Central Committee on Research Involving Human Subjects
CDMS	Clinical Data Management System
CEO	Chief executive officer
CHMI	Controlled Human Malaria Infection
CRF	Case Report Form
CRO	Contract Research Organization
DSMB	Data and Safety Monitoring Board
GCP	Good Clinical Practice
HIV	Human Immunodeficiency Virus
IRB	Institutional Review Board
LDH	Lactate Dehydrogenase
NF54	Nijmegen *falciparum* 54
NFU	Netherlands Federation of University Medical Centres
NWO	Netherlands Organization for Scientific Research
PCR	Polymerase chain reaction
SAE	Serious Adverse Event
SCORE	Systematic COronary Risk Evaluation
SCOT	Social Construction of Technology
SOP	Standard Operating Procedure
STS	Science, Technology & Society studies

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Photography: ZorginBeeld / Frank Muller

Supplementary table 1 Standard Operating Procedures (SOP's) in the Parasite Lab, Radboud university medical center, October 2014

Number	SOP Title
RMW401	Preparation of *Plasmodium falciparum* culture medium
RMW403	Preparing human serum for culturing and transmission of *Plasmodium falciparum*
RMW406	Giemsa staining of blood films
RMW407	Gametocyte production
RMW413	Preparation of blood with *Plasmodium falciparum* gametocytes for the prefeed
RMW414	Preparation of blood with *Plasmodium falciparum* gametocytes for sporozoite production
RMW415	Preparing erytrocytes for culturing and transmission of *Plasmodium falciparum*
RMW416	Preparation of sodium bicarbonate solution
RMW417	Preparation of Giemsa stock, phosphate buffer and Giemsa working solutions
RMW422	Cryopreservation of *Plasmodium falciparum parasites*
RMW423	Retrieval of *Plasmodium falciparum* parasites
RMW424	Checking maturity of gametocytes
RMW426	Collecting culture material for MSP/Mycoplasma PCR
RMW427	Sterility test of the medium RPMI1640
RMW428	Cleaning and decontamination of culture flasks
RNW013	Mycoplasma detection in *Plasmodium falciparum* cultures by PCR
RNW014	Genotyping *Plasmodium falciparum* strains by PCR

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CHAPTER 3

Empowering malaria vaccination by drug administration

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Abstract

Although significant progress has been made in clinical development, a protective malaria vaccine remains elusive. Here we review some of the immune subversive mechanisms used by the *Plasmodium* malaria parasite and propose a potentially effective strategy to achieve complete protection that may serve as a blue print for clinical usage. The premise is to modulate the immune response with drugs that neutralize suppressive functions and potentiate protective responses. Chloroquine may be a first attractive candidate facilitating protective cellular immune responses by improving cross-presentation and reducing suppressive regulatory T cell responses.

Introduction

Both natural and experimental exposure to malaria parasites can lead to development of protective immunity, providing a strong foothold for the development of a vaccine (1-5). The development of a malaria vaccine has been a continuous effort over the past half century (6). Several strategies are being followed, aiming at the sporozoite, liver, blood and/or sexual transmission stages (Figure 1). The main efforts have been concentrated on development of a sporozoite / liver-stage vaccine against *P. falciparum,* on the basis of the observation that potent protection could be achieved by inoculation with irradiated non-replicating but metabolically active sporozoites (4).

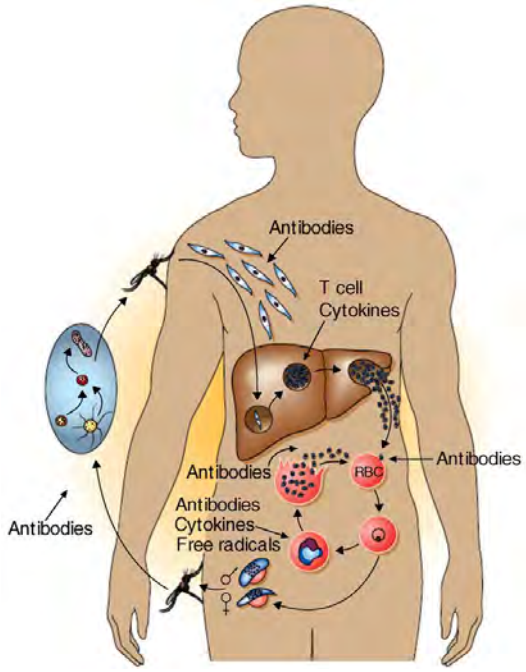

Figure 1 *Plasmodium* lifecycle. *P. falciparum* malaria is caused by a protozoan parasite that has a complex multi-stage lifecycle involving both intracellular and extracellular stages in human host and mosquito vector. Malaria infection starts by the bite of an infected *Anopheles* mosquito that inoculates sporozoite forms. Via the bloodstream they reach the liver and invade, mature and multiply in hepatocytes. Once released by infected hepatocytes into the bloodstream as pathogenic asexual forms they start to multiply in invaded red blood cells. A small fraction of blood stage parasites are committed to become sexual forms and mature into gametocytes that are responsible for transmission to mosquitoes. Mature gametocytes are ingested by blood-feeding mosquitoes and differentiate after a number of transitions into sporozoites and migrate to the salivary gland. At each mosquito bloodmeal these motile parasites are injected into the human host resulting in the spread of the parasite and associated disease in the population. Unlike sporozoites and gametocytes, which are clinically silent, only asexual stages of the life cycle are responsible for clinical symptoms, complications and the possibility of death. Figure is reproduced with permission from Richie TL and Saul A: Progress and challenges for malaria vaccines. *Nature* 2002, 415(6872): 694–701 (Nature Publishing Group).

The field of malaria vaccinology has followed the traditional vaccine approach: expose the host to a malaria antigen or antigens and, in the case of subunit vaccines, maximally stimulate

the immune response using adjuvants, immune-stimulatory compounds or self-adjuvanting delivery systems such as viral vectors or virus-like particles. No vaccine based on this paradigm has worked well, however, for inducing high grade protection against malaria in humans, and the majority of candidates have failed. Different formulations of a number of antigens have been tested in Phase 1 trials and only about a dozen candidates have been evaluated in Phase 2 clinical field trials (WHO. Date Accessed 19th April 2010. Malaria Vaccine Rainbow Tables; URL: http://www.who.int/vaccine_research/links/Rainbow/en/index.html). The best vaccine to date, RTS,S, does not provide long term protection against infection but delays patency and reduces clinical severity. After several field trials demonstrating roughly 50% protection as measured by delays in the time to acquiring parasitemia or clinical malaria, RTS,S is now undergoing testing in a Phase 3 multicenter trial in Africa (7, 8). Although a milestone in itself and potentially a welcome tool in the combat against malaria, it is clear that better vaccine efficacies will be required in particular for the purpose of malaria elimination (9). One rational approach would be to counteract the immune-modulating effects of the parasite that result in slow or partial induction of protection and effective memory responses. Co-administration of drugs with immune-modulating properties may be a strategy to meet this objective.

Natural acquisition and evasion of malaria immunity

Malaria parasites generate strong immune responses, and a degree of protective immunity can be acquired through natural exposure, although the mechanisms of protection are poorly understood (1, 2). The development of this immunity is marked initially by the ability to control the clinical symptoms associated with parasitemia, allowing the individual to tolerate significant parasite densities without overt disease. The type of clinical immunity, typically developing in children, is followed by resistance to parasitemia, such that older children and adults no longer experience high densities of asexual forms in the blood. However, sterile immunity is never observed in naturally exposed populations; adults living in endemic areas often harbor parasites albeit at low densities, and will promptly re-acquire infections if cured through the administration of antimalarial drugs.

Where and how invading *Plasmodia* are recognized and processed by the immune system is not well understood, but probably involves dendritic cells [DCs] with extracellular and intracellular pattern recognition receptors [PRRs]. PRR signal transduction determines the nature of the DC response, and is modulated by the PRRs involved, antigen dose, duration of exposure and microenvironment. Both TLR-dependent [TLR2, TLR4 and TLR9] as well TLR-independent [NALP3 inflammasome] pathways are involved in *Plasmodium* recognition but major differences between human and murine immune systems hamper conclusive interpretation (10-13). Activation of DCs preferentially leads to their maturation

followed by induction of T effector cells or it may lead to tolerogenic responses by induction of regulatory T cells [Tregs]. The ratio of effector and regulatory responses may influence the risk of productive malaria infection and clinical disease (14). Protection is accomplished by stage-specific host effector responses and seems to require both cellular and humoral components. While specific antibodies are primarily important against sporozoites and blood stages, distinct cellular responses are required for protection against liver-stage parasites (2). There is substantial evidence that cytotoxic lymphocytes recognize intra-hepatic parasites and that interferon-γ [IFN-γ] plays an essential role in protection. However, cellular mechanisms are also induced by infected red blood cells [iRBCs] and may control blood stages (3, 15)**.**

Thus, malaria manifests itself initially as an acute infectious disease in susceptible persons but evolves into a chronic infection with acquisition of partial immunity. The human immune system gradually controls acute clinical disease, but *Plasmodia* are adapted to prevent complete elimination, establishing a lowgrade, chronic infection in the majority of hosts. This clinical immunity is difficult to acquire and wanes once exposure is withheld. Thus frequent parasite exposure is required to ensure continuity of protection. The efficiency of mounting a protective response is challenged by the morphologically and antigenically distinct lifecycle stages, the existence of genetic and antigenic diversity as well as the parasite's ability to modulate immune responses to its own survival benefit (1, 16).

Malaria parasites can manipulate recognition by DCs and can compromise the induction of effective immune responses by stimulating Tregs or by other immunosuppressive or immunodiversionary tactics (14, 16, 17)**.** However, the picture in human and animal studies is inconsistent and the outcome depends on the DC subset as well as the model of parasites and animals studied. While DC maturation may be inhibited or stimulated, the capacity to activate human T cells is generally impaired. Results depend on the subset of DCs studied and the ratio of iRBC to DCs; low ratios are stimulatory while high ratios inhibit or result in apoptosis (16, 17). Reduced DC function may also be the result of immune inhibition across parasite lifecycle stages as shown in a murine model where asexual stages in the circulation inhibit the generation of effective $CD8^+$ T cell responses targeting liver stages (18). The DCs exhibit poor maturation and a shifted cytokine profile from primary IL-12 to IL-10 production upon blood stage exposure. In the *P. berghei* model, reduced $CD8^+$ T cell priming is caused by impairment of the cross-presentation by DCs normally essential for antigen presentation by the exogenous route (19). Also in humans, inhibition of pre-erythrocytic T cell responses by blood stages is suggested by *in vitro* studies comparing lymphocytes from Duffy antigen-positive individuals [exposed to *P. vivax* blood stages] and Duffy-negative individuals [no exposure to blood stages] (20). These data fit the epidemiological observation that naturally acquired immunity fails to prevent re-infection even in areas with high infection rates. $CD8^+$ responses are relatively low in individuals from such areas and these effector mechanisms appear incapable of eliminating parasites from the population (21, 22). Thus, DCs that are

influenced by blood stages might affect clearance of liver stages upon re-infection.

$CD4^+CD25^+Foxp3^+$ Tregs are crucial for maintenance of self-tolerance and control of damaging pro-inflammatory responses induced by pathogens including malaria parasites. Expansion of Treg-populations induced by *Plasmodia* is generally seen in both humans and animal models but the functional consequences remain equivocal (14, 16). Outcomes of functional studies show large variation and, similar to studies of DCs, relate to the model and circumstances chosen. Human Treg activation, however, generally points towards lower pro-inflammatory responses and facilitation of blood stage infection (14). The balance between Th1 effector cell and Treg responses may determine the clinical presentation. Strong inflammatory responses may overwhelm suppressive Tregs and leave effector T cells unabated with possibly severe clinical symptomatology in an effort to control parasitemia. On the contrary strong Tregs responses dampen inflammation and symptoms but permit parasitemia and suppress the magnitude of T memory responses possibly owing to competition for IL-2 (14).

Better than nature

Chronic exposure to malaria blood stage parasites may help to explain the slow generation of an effective immune response under natural conditions. Support for this concept comes from rodent studies, where sterile protection against malaria can be achieved by the inoculation of intact sporozoites while treating the animals concomitantly with chloroquine, a drug that kills parasites in the asexual blood stage but not in the pre-erythrocytic liver stage (23). A proof-of-concept clinical study with a similar protocol in volunteers who had not been previously exposed to malaria likewise showed sterile protection against an experimental *P. falciparum* malaria infection (5). This approach exposed volunteers' immune systems to the full course of intra-hepatic development combined with a very brief blood stage parasitemia abrogated by chloroquine. The high degree of protection achieved by a relatively miniscule dose, that is a total of 45 infectious mosquito bites over a period of 3 months, is remarkable. Such inoculation rates may approach the levels in areas of periodical intense transmission in Africa, but sterile protection is not seen under natural conditions. A possible explanation for this difference is the very brief and extremely low parasite density achieved in the blood owing to the killing effect of chloroquine. Parasitemia in nonimmune Africans will, at the minimum, increase to the threshold of clinical symptoms that is at least 1000-fold higher compromising any protection that may result from exposure to sporozoites and liver-stage parasites.

Previously it has been shown that radiation-attenuated sporozoites induce >90% protection in humans (4). Irradiation of infectious mosquitoes disrupts the gene expression of sporozoites, which remain capable of hepatocyte invasion but are no longer capable of

complete liver-stage maturation or progression to the pathogenic blood stage. However, this generally requires 1000 bites by irradiated mosquitoes during five or more immunization sessions which is a strikingly 20-fold lower potency than the 45 bites in the human model using chloroquine. Similar results are found in mouse studies (23)**.**

Many hypotheses could be generated why intact sporozoites provide substantially better protection than radiation-attenuated sporozoites. These include [i] improved homing to the liver or other sites such as lymph nodes where antigen presentation occurs, [ii] larger antigen yield per sporozoite due to unrestricted asexual reproduction in the liver, [iii] expression of late liver-stage /early blood stage antigens, which are not expressed by irradiated sporozoites. Although these hypotheses are plausible and should be tested, they beg the question why natural exposure to a few mosquito bites does not provide equivalent protection to the experimental model under chloroquine. What other mechanisms might be operative?

Low parasitemia may induce protective effector mechanisms as shown by Pombo *et al*. where repeated intravenous injections of low numbers of parasites followed by a curative treatment before patency resulted in protection against a subsequent blood stage challenge administered without curative drugs (3). More recently, it was shown in a murine model that subpatent blood stage infection with genetically attenuated blood stage parasites likewise provides complete protection apparently through both humoral and cellular immune responses (24). By contrast, high parasitemia as observed in the field may inhibit the development of protective immunity and may relate to inhibition of cross-presentation required for the induction of cytotoxic T cells (18-20).

A cost-effective vaccine requires efficient induction of protective immunity over a short period of time and should therefore perform better than nature.

A fresh perspective for failing vaccines

Malaria vaccine development faces a variety of scientific challenges and some of these are addressed by ongoing subunit vaccine initiatives (8, 25, 26). The most advanced among several recombinant protein-adjuvant combinations is RTS,S, a virus-like particle displaying recombinant circumsporozoite protein [CSP] on its surface, expressed together with recombinant hepatitis B surface antigen. The vaccine is formulated with the proprietary adjuvant ASO2A later replaced by its liposomal form ASO1B. Several clinical trials have been conducted in endemic populations including young children age 1–4 years with follow-up up to 45 months. Both anti-CSP antibodies and $CD4^+$ T cell responses show correlation with the observed partial protection. It has proven difficult, however, to achieve significant improvements through combination with other antigens (27).

More recently, vaccine platforms designed to induce cytotoxic T lymphocyte responses targeting hepatic stage parasites through gene-based approaches have achieved partial

protection in human volunteers challenged by infected mosquito bites. For example, in a heterologous vaccination strategy, approximately 25% protection was observed both after priming volunteers with a chimpanzee-derived adenovirus vector encoding the TRAP antigen then boosting with a modified vaccinia virus Ankara [MVA] vector also encoding TRAP (AVS Hill, oral presentation, 5th MIM Pan-African Malaria Conference, Nairobi, Kenya; November 2009), and after priming with naked DNA and boosting with a human-derived adenovirus vector [serotype 5] with both platforms encoding CSP and AMA1 (I Chuang *et al.* poster presentation, given at Malaria: New Approaches to Understanding Host-Parasite Interactions, Keystone Symposia on Molecular and Cellular Biology, Copper Mountain, Colorado; April 2010). Whether the modest protection achieved by viral vectors can be improved by the addition of antigens is not yet known.

One approach to enhance the protection afforded by subunit vaccines is to combine recombinant protein in adjuvant with viral vectors. The former induces primarily antibodies and helper T cell responses, while the latter induce strong cell mediated immunity including cytotoxic CD8+ T cells. Combined into heterologous regimens, these two approaches might powerfully activate both the humoral and cellular arms of the immune system, thereby enabling the destruction of multiple parasite stages (28). However, all subunit vaccines must overcome the challenge of antigenic heterogeneity and the difficulty of protecting a genetically heterogeneous population.

Intact parasites attenuated by irradiation (4), genetic modification (29) or by co-administration of antimalarial drugs (3, 5, 23)**,** show a superior degree of protection to subunit vaccines in animal studies and in controlled experimental human infections. Presenting potentially hundreds of antigens to the immune system, these approaches are designed to provide 'something for everyone' thereby circumventing the limitations of genetic restriction by broadening the antigenic repertoire. A radiation-attenuated sporozoite vaccine is currently undergoing clinical testing, while a genetically attenuated sporozoite vaccine will soon follow into the clinic. Although it is too early to judge the success of these approaches, some may be confronted with the generation of immune suppressive mechanisms normally used by wild type parasites thereby limiting their ability to protect. A major challenge may be to redress such malaria-associated suppressive mechanisms.

Co-administration of chloroquine may enhance protection induced by sporozoite inoculation owing to lowering of parasitemia and/or its immune-modulating effects. Short-course treatment of mice with chloroquine improves the priming of naïve $CD8^+$ T cell responses against soluble antigens *in vivo* (30). Cross-presentation of soluble viral antigens to specific $CD8^+$ T cell clones *in vitro* is improved when DCs are pulsed with the antigen in the presence of chloroquine. Furthermore, if hepatitis B virus vaccine [HBV] responders are further boosted together with a single dose of chloroquine a substantial increase of HBV-specific $CD8^+$ T cells is observed (31). Presentation of soluble viral antigens to specific $CD8^+$

T cell clones by DCs is greatly improved in the presence of chloroquine, which prevents endosomal acidification, and seems to promote the transfer of endocytosed material into the cytosol. The net effect will depend on the routing and processing conditions such as acidification of the endosomal compartment (32).

There may be more drug choices to explore for co-administration during vaccinaton. Chemotherapeutic drugs have shown potential benefits for the immune response against tumors (33, 34)**.** It has for instance been shown that low dose cyclophosphamide selectively depletes Tregs in both animal models and cancer patients with resulting enhancement of T cell functions. The potential benefits and safety risks of low dose cytostatic drugs in healthy volunteers should be carefully considered. The combined data show that orchestration of the antigen presenting pathways by drug modulation might tailor the immune response to a desired profile. The functionality of the increased numbers of Tregs observed in human and animal malaria is not clear but there is an inverse correlation during acute disease between Tregs and malaria-specific memory responses (35). Antigen presentation by immature or partially mature DCs conditions the emergence of Tregs. To activate Tregs, TLR9 signaling

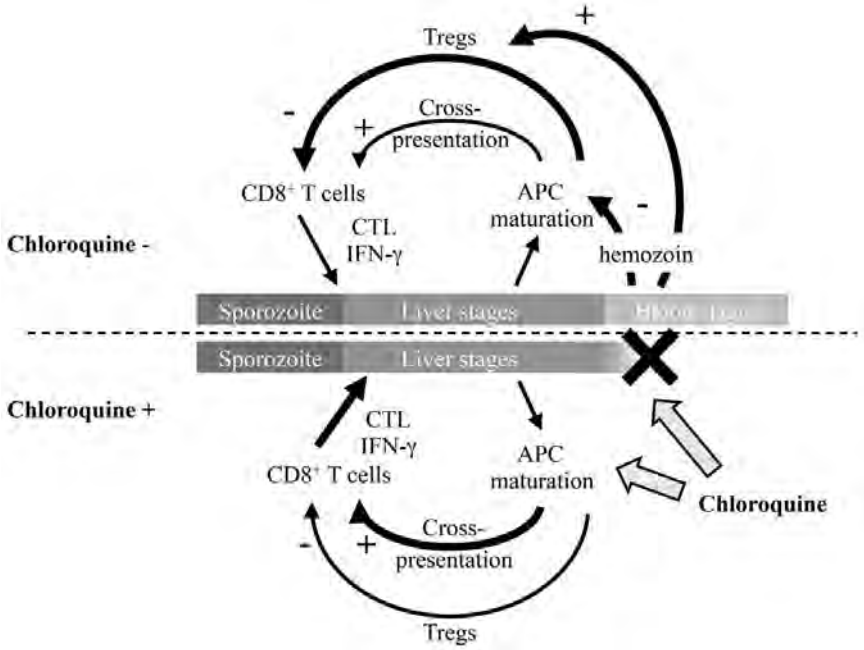

Figure 2 Model for modulation of cellular responses by chloroquine during malaria. Blood stage parasites inhibit the development of liver-stage immunity via antigen presenting cells and hemozoin. This results in upregulation of Tregs and therefore less effective establishment of $CD8^+$ T cell responses. Chloroquine prevents the development of blood stage parasitemia, thereby diminishing the immune-evasing action of these parasites. Furthermore, it inhibits Treg induction and improves cross-presentation, therefore leading to a more effective $CD8^+$ T cell response. CTL = cytotoxic T lymphocyte.

in dendritic cells is required and mediated by hemozoin, a digestion product of hemoglobin produced by *Plasmodium* that is involved in TLR9 activation (11). Since chloroquine abrogates hemozoin-mediated cytokine production, this drug might inhibit this evading mechanism leading to a more effective establishment of immunological memory (36) (Figure 2).

Does combining a parenterally administered vaccine with orally administered chloroquine or other immunomodulatory drug hold any practical application as a novel vaccination strategy? Given the long half-life of chloroquine, a single administration might maintain effective plasma levels throughout the induction phase of an immune response, making it possible to administer single doses of co-drug and vaccine as the immunization procedure, although the effectiveness of this reduced-dose approach would need to be tested. In the RUNMC study, the vaccine component consisted of intact *P. falciparum* sporozoites, presenting a potentially insurmountable safety concern (5). However, both radiation-attenuated and genetically attenuated sporozoite approaches should demonstrate very low levels of breakthrough blood stage infection, if they occur at all, and thus could serve as ideal partners for an immunomodulatory co-drug. A first step would be to test these combinations in order to determine the minimal protective dose of both vaccine and co-drug, as well as the fewest number of immunizations needed to achieve high grade protection. If chloroquine or another antimalarial were chosen, this approach could provide an added level of safety since it would treat any emergent blood stage infections even if the sporozoite vaccine were only partially attenuated. As long as the vaccine strain was completely sensitive to the co-drug, both immune modulation and protection against breakthrough blood stage infections could be achieved at the same time. Practical application will depend on the selected drug, drug half-life and vaccination regime.

Conclusion

In malaria where immune diversionary mechanisms are a primary immune-evasion strategy, co-administration of immune-modulating drugs along with the vaccine may be required in order to achieve high grade protection. The hypothesis of vaccination with drug co-administration assumes that the vaccine component mimics the natural pathogen in terms of subverting the host immune responses. This is a reasonable assumption in the case of whole organism vaccines such as attenuated sporozoites. This approach might be less applicable to a subunit vaccine based on discrete single antigens, although even in this instance there may be immunodominant immune responses [repeat motifs, for example, that if suppressed would allow more protective, subdominant responses to emerge]. The current armamentarium of immunomodulatory agents used to affect the immune system including cytostatic drugs could have potential as co-agents and be screened for activity. Clearly dose and duration of

treatment will be important, not too high and not too low, to strike the balance to interfere with the pathogen's adaptation strategy without preventing the host immune system from responding adequately and safely.

Chloroquine may be the first opportunity to test and this could be done by comparing the administration of chloroquine with that of less immune-modulating antimalarials, or by giving chloroquine with irradiated or genetically attenuated sporozoites to see if the potency of the vaccine is increased. Despite more than 30 years of chloroquine resistance in Africa, this antimalarial drug is still widely available and used for presumed treatment. Although responsible for increasing numbers of treatment failures, one may hypothesize that the immune-modulating effects of chloroquine may still have some contribution to development of clinical protection.

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CHAPTER 4

Studying the effect of chloroquine on sporozoite-induced protection and immune responses in Plasmodium berghei malaria

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Abstract

Background

Sporozoite immunization of animals and humans under a chemo-prophylactic cover of chloroquine (CPS-CQ) efficiently induces sterile protection against malaria. In humans, CPS-CQ is strikingly more efficient than immunization with radiation attenuated sporozoites (RAS), and we hypothesize that this might be partially due to CQ. CQ, an established anti-malarial drug, is also well known for its immune modulating properties including improvement of cross-presentation. We therefore aimed to investigate whether co-administration of CQ during sporozoite immunization improves cellular responses and protective efficacy in *Plasmodium berghei* models.

Methods

We performed a number of experiments in selected complimentary *P. berghei* murine models in Balb/cByJ and C57BL/6j mice. First, we studied the effect of CQ administration on the induction of protection and immune responses by RAS immunization. Next, we investigated the effect of CQ on the induction of circumsporozoite (CS) protein-specific $CD8^+$ T cells by immunization with *P. berghei* parasites expressing a mutant CS protein. Finally, we performed a direct comparison of CPS-CQ to CPS with mefloquine (MQ) an antimalarial with little known immune modulating effects.

Results

When CQ was co-administered during immunization with graded numbers of RAS, we observed no increase in frequencies of total memory $CD8^+$ T cells or CS protein-specific $CD8^+$ T cells. Also parasite-specific cytokine production and protection remained unaltered. Replacement of CQ by MQ for CPS immunization resulted in significantly reduced percentages of IFNγ producing memory T cells in the liver ($p=0.01$), but similar protection.

Conclusions

Our data do not provide evidence for a direct beneficial effect of CQ on the induction of sporozoite-induced immune responses and protection in *P. berghei* malaria models. Alternatively, the higher efficiency of CPS compared to RAS might be explained by an indirect effect of CQ through limiting blood-stage exposure after immunization or to increased antigen exposure and therefore improved breadth of the immune response.

Background

Whole sporozoite immunization approaches such as chloroquine chemoprophylaxis and sporozoites (CPS-CQ) and radiation-attenuated sporozoites (RAS) efficiently induce protection in murine malaria models (1-3). In humans CPS-CQ is about 20 times more efficient than RAS, requiring bites from a total of 45 versus 1000 mosquitoes, respectively (4-6). Moreover, long-lasting immune responses after CPS-CQ immunization in studies with mice (7) and healthy human volunteers (8) go together with protracted protection. Several murine studies have demonstrated the essential role of $CD8^+$ T cells in sporozoite-induced pre-erythrocytic immunity (2, 9-14). Generation of these $CD8^+$ T cells against pre-erythrocytic antigens requires cross-priming by dendritic cells (15).

CQ has since its discovery in 1934 been used widely and successfully as antimalarial, until resistance developed (16), and has more recently been explored for treating cancer and viral infections (17, 18). Interestingly, CQ was also shown to enhance cross-presentation of soluble antigens and non-replicating influenza virus *in vitro* (19, 20). Moreover, *in vivo* cross-priming of naïve $CD8^+$ T cells with soluble ovalbumin was more effective in CQ–treated compared to untreated mice (21), and CQ improved the induction of influenza-specific cytolytic T cells in mice (20). In humans, co-administration of CQ with a hepatitis B vaccine booster significantly increased the number of virus-specific IFNγ-producing $CD8^+$ T cells (19).

We hypothesized that CQ, which affects endosomal acidification and the degradation and transport of antigens to the cytosol (22, 23), could favor cross-presentation of pre-erythrocytic *Plasmodium* antigens and thereby contributed to the efficient induction of immune responses and protection by CPS-CQ immunization. We first studied the effect of CQ on immunization by RAS, an established immunization model relying on $CD8^+$ T cell responses, thus potentially benefiting from improved cross-presentation. $CD8^+$ T cells recognizing the immunodominant circumsporozoite (CS) protein can mediate protective immunity (24), so we next investigated the effect of CQ on the induction of CS-specific $CD8^+$ T cells by immunization with *P. berghei* parasites that express a mutant CS protein containing the model SIINFEKL H-$2K^b$ epitope. Finally, we performed a direct comparison of CPS-CQ to CPS with mefloquine (MQ) an antimalarial with little known immune modulating effects (25-27), not including improvement of cross-presentation. Akin to CQ, MQ induces arrest of early blood-stage parasites without an effect on pre-erythrocytic parasite stages, allowing full liver-stage development and brief exposure to early blood stages. By performing experiments in these selected complimentary *P. berghei* murine models we aimed to explore the effect of CQ on protection and T cell responses after whole sporozoite immunization.

Methods

Mice and parasites

Balb/cByJ and C57BL/6j mice (6 to 8 weeks old) were purchased from Elevage-Janvier (Le Genest Saint Isle, France). These mouse strains were selected based on extensive experience with these strains for malaria immunization studies (28). We used *P. berghei* (ANKA) wild type parasites and *P. berghei* CS5M parasites in which the endogenous CS gene had been replaced with a modified circumsporozoite gene expressing the H-2Kb restricted SIINFEKL (15). Sporozoites were obtained by dissection of the salivary glands of infected female *Anopheles stephensi* mosquitoes 21-29 days after a blood meal on infected mice. All animal studies and procedures performed in the Netherlands were approved by the Ethical Committee on Animal Research of the Radboud University Nijmegen (RU-DEC 2009-179, 2009-225, 2010-115, 2010-135). Mice were housed at the Central Animal Facility in Nijmegen and received a standard diet and water *ad libitum*. All animal procedures in the United States of America were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University (Protocol Number MO10H167) and followed the National Institutes of Health guidelines for animal housing and care.

Immunization schedules, sporozoite challenge and assessment of protection

Mice were immunized with one (Balb/cByJ; . S1A) or two to three (C57BL/6j; Figure S1B) intravenous (iv) injections of *P. berghei* RAS (16krad, Gammacel 1000 ^{137}Cs) at weekly intervals or with one intradermal injection of *P. berghei* CS^{5M} RAS (Figure S1C). Dose de-escalation of RAS immunization was performed in order to obtain a suboptimal RAS dose to detect possible beneficial effects of CQ (Figure S1A and S1B). In RAS experiments, RAS-CQ groups received either CQ prophylaxis (chloroquine diphosphate, Sigma-Aldrich) for 10 days (Balb/cByJ –1040µg base/day oral) or 17 days (C57BL/6j –800µg base/day intraperitoneal). Efficacy of these prophylactic regimens was established in pilot studies, and they were chosen because of their closest resemblance to the human CPS-protocol. Alternatively, mice were given two subcutaneous injections of 500µg CQ base, 2h before and 6h after each immunization, because this particular regimen was previously shown to improve cross-presentation (Figure S1B)(20, 21).

Furthermore, mice under CQ or MQ prophylaxis were immunized three times at weekly intervals by intravenous administration of 20,000 wild-type *Pb*SPZ (CPS immunization; Figure S1D). For CPS immunization, CQ (diluted in PBS) and MQ (diluted in DMSO/water for injection) were given orally for 24 consecutive days at dosage 1040µg base/day (CQ) or 350µg base/day (mefloquine hydrochloride, Sigma-Aldrich) starting from the first day of *Pb*SPZ administration.

Challenge infections were performed by intravenous injection of 10,000 or 50,000 sporozoites around four or eleven weeks after the end of CQ/MQ prophylaxis. Giemsa-

stained blood smears were screened for parasitized red blood cells every other day from days 3-14 and finally on day 21 after challenge. Protection was defined as the absence of blood-stage parasites until day 21 post-challenge (Figure S1A, S1B and S1C).

Ex vivo memory phenotyping and in vitro IFNγ responses against sporozoites and blood-stage parasites

Mice were euthanized by isoflurane inhalation after intravenous injection of 50 units heparin. Spleen and liver were collected after perfusion of the liver with 10ml PBS. Cell suspensions of liver and spleen were made by passage of the organs through a 70-µm nylon cell strainer (BD Labware). Liver cells were re-suspended in 35% Percoll (GE Healthcare) and centrifuged at 800g for 20min. Liver and spleen erythrocytes were lysed using 5 min incubation on ice in a lysing solution of ammonium chloride. After erythrocyte lysis, hepatic mononuclear cells (HMC) and splenocytes were re-suspended in RPMI 1640 medium.

Five-color staining of HMC and splenocytes was performed using monoclonal antibodies purchased from Biolegend: Pacific blue-conjugated anti CD3 (17A2), Peridinin Chlorophyll Protein (PerCP)-conjugated anti CD4 (RM4.5), Alexa fluor 700-conjugated anti CD8a (53-6.7), fluorescein isothiocyanate (FITC)–conjugated anti-CD44, allophycocyanin (APC)– or phycoerythrin-Cy7 (PE-Cy7)-conjugated anti-CD62L (MEL-14). Briefly, 10^6 cells were re-suspended in cold assay buffer (PBS supplemented with 0.5% bovine serum albumin (Sigma-Aldrich)) and incubated for 30min at 4°C with the monoclonal antibodies. Cells were fixed with Fix & Perm medium A (Invitrogen) and collected in an assay buffer for measurement.

For the detection of parasite-specific cytokine production, HMC and splenocytes ($5x10^5$ cells/well) were co-cultured in complete RPMI 1640 culture medium (29) in the presence of *P. berghei* cryopreserved sporozoites (*Pb*SPZ - $5x10^4$/ml) or infected red blood cells (*Pb*iRBC - $5x10^6$/ml). Exposure to salivary gland preparations from uninfected mosquitoes and uninfected red blood cells (uRBC - $5x10^6$/ml) were used as respective negative controls. Cells were stimulated at 37°C/5%CO2 for 24 hours and Brefeldin A (Sigma-Aldrich) was added during the last four hours (10µg/ml final concentration). As positive control, PMA (100ng/ml) and Ionomycin (1.25µg/ml) (Sigma-Aldrich) were added simultaneously along with Brefeldin A. Cells were harvested after 24-hours *in vitro* stimulation and stained with monoclonal antibodies against CD3, CD4, CD8a and CD44 as indicated above. Fixed and permeabilized cells were stained with PE-conjugated anti-IL-2 (JES6-5H4) and APC-conjugated anti-IFNγ (XMG1.2) in Fix & Perm medium B (Invitrogen) at 4°C for 30min. Flow cytometry was performed on a 9-color Cyan ADP (Beckman Coulter) and data analysis was performed using FlowJo software (version 9.1; Tree Star), using a gating strategy as described previously (7).

Quantification of SIINFEKL specific CD8⁺ T cells

Prior to intradermal immunization with 20.000 *P. berghei* CS^{5M} RAS, C57BL/6j mice received 2*10^3 CD45.1+OT-1 cells and the RAS-CQ group received a 10-days CQ treatment (1040µg base/day – oral). Expansion of CD45.1$^+$CD8$^+$ SIINFEKL cells in liver and spleen was assessed by flow cytometry ten days after immunization as described previously (15) (Figure S1C).

Data analysis and statistics

Difference in protection between two groups was tested with a Fisher's exact test. Overall comparisons between immunized and naïve groups were performed using the Kruskal-Wallis test. Direct comparisons between two groups (RAS versus RAS-CQ or CPS-CQ versus CPS-MQ) were performed by Mann-Whitney U test. For the analysis of cytokine production, background responses to salivary glands and uRBC were subtracted from *PbSPZ* and *Pb*iRBC responses, respectively, for each individual mouse. All statistical analyses were performed using PRISM software version 5.0 (Graphpad, San Diego, CA). A p-value of ≤0.05 was considered statistically significant.

Results

Effects of chloroquine on RAS immunization

First, we investigated whether administration of a prophylactic regimen of CQ improved CD8$^+$ memory T cell responses induced by RAS immunization in C57BL/6j mice. *Ex vivo*

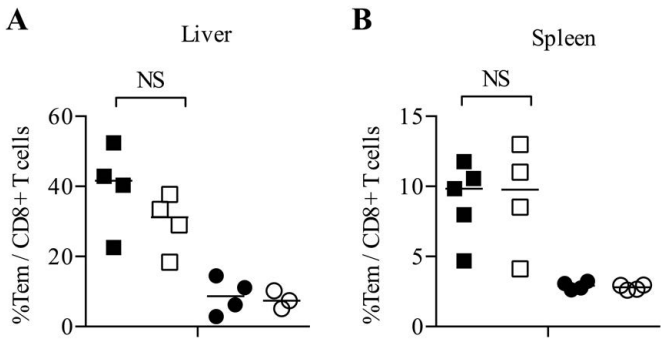

Figure 1 Frequencies of CD8$^+$ Tem cells following RAS immunization of C57BL/6j mice under CQ cover. Percentages of CD8$^+$ T cells with effector memory phenotype (CD44$^+$CD62L$^-$ Tem) were measured one day before challenge by direct *ex vivo* staining in the liver and spleen of RAS immunized mice (filled squares), RAS immunized and CQ treated mice (open squares), naïve mice (filled circles) and naïve-CQ mice (open circles). Horizontal lines represent group medians. NS=not significant.

analysis showed that percentages of $CD8^+$ T cells with an effector memory phenotype ($CD44^+CD62L^-$; Tem) in the liver were 4-5 fold higher a day before challenge (C-1) in immunized compared to naïve mice (p=0.011). However, the percentage of $CD8^+$ Tem cells was similar in RAS versus RAS-CQ mice (Figure 1A). Similar patterns were observed in the spleen with 3-fold increased Tem levels at C-1 (p=0.007, Figure 1B).

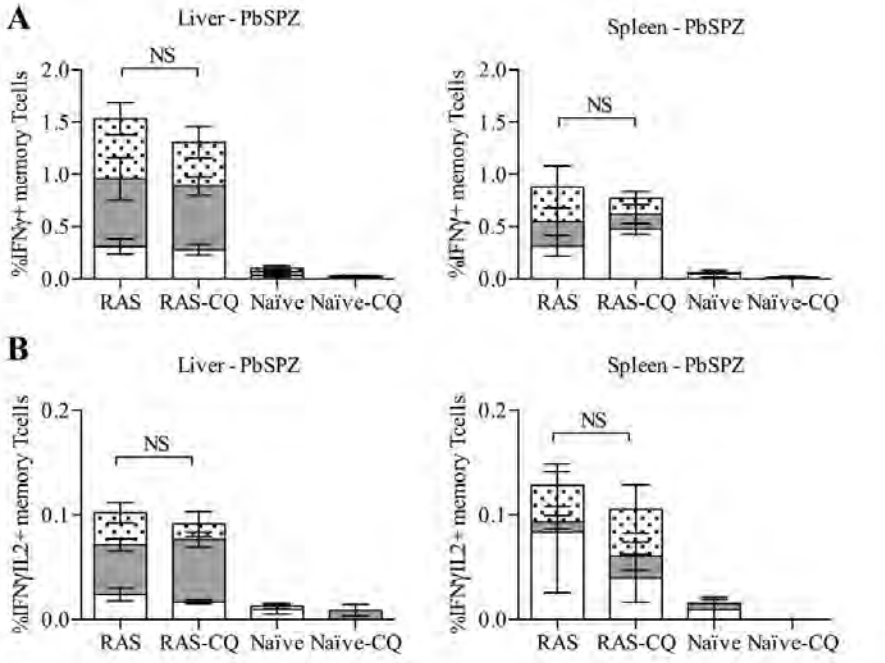

Figure 2 Sporozoite specific cytokine responses following RAS immunization under CQ cover. Percentages of IFNγ (A) and IFNγ and IL-2 (B) producing memory T cells were measured in RAS (n=5) RAS-CQ (n=4), naïve control (n=5) and CQ-control (n=4) mice, a day before challenge by intracellular staining after *in vitro* re-exposure of liver and spleen cells to *P. berghei* sporozoites (*Pb*SPZ). Responses of $CD4^+$ T cells (open area), $CD8^+$ T cells (grey area) and $CD4^-CD8^-$ T cells (dotted area) relative to total memory T cell responses are presented. Background responses to salivary glands were subtracted from *Pb*SPZ responses for each individual mouse. Error bars represent standard error of the mean. NS= not significant.

In vitro re-exposure of immune cells to *Pb*SPZ showed high levels of IFNγ producing memory T cells in both liver and spleen of RAS-immunized mice (p=0.003 and 0.027, respectively), which were not increased by additional CQ administration (Figure 2A). Similar observations were made for hepatic and splenic pluripotent memory T cells producing both IFNγ and IL-2, with a major contribution of $CD8^+$ cells in the liver (Figure 2B). In summary, additional CQ

administration affected neither frequency of RAS-induced CD8$^+$ Tem cells, nor sporozoite-specific cytokine production by T cells.

Next, we investigated the effect of CQ on CD8$^+$ T cell responses against a SIINFEKL H-2K^b restricted epitope integrated in the circumsporozoite (CS) protein; an established target protein of protective immunity in mice and humans (24, 30). In line with the results above, mice immunized with RAS whilst under CQ cover showed similar percentages of hepatic and splenic CS-specific CD8$^+$ T cells compared to untreated mice (Figure 3).

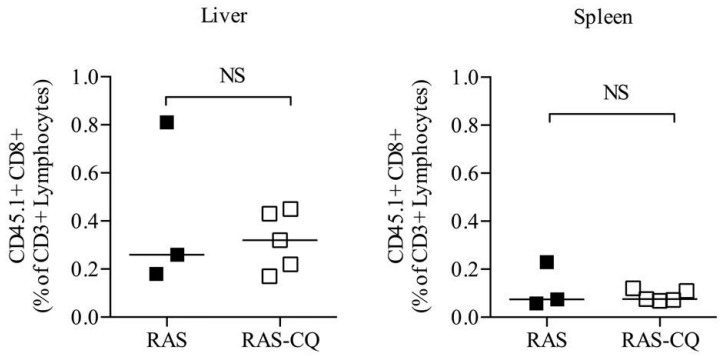

Figure 3 Frequencies SIINFEKL specific CD8+ T cells following RAS immunization under CQ cover. Prior to RAS intradermal immunization with (RAS-CQ) or without chloroquine (RAS), mice received injection of SIINFEKL-specific CD8$^+$ T cells. Ten days after a single immunization, expansion of CD45.1$^+$CD8$^+$ SIINFEKL-specific cells was determined in the liver and spleen of both immunized groups.

To evaluate a potential effect of CQ on RAS protective efficacy, groups of Balb/cByJ and C57BL/6j mice were immunized with graded numbers of RAS, then challenged and monitored for parasitemia. In both mice strains, reduction of immunization dose resulted in a stepwise decrease in protection that was not influenced by either a prophylactic regimen of daily CQ, or two low doses of CQ at 2h before and 6h after each immunization (Table 1). We also did not observe a difference in pre-patent period between RAS and RAS-CQ groups (data not shown). The CQ administered during immunization had no effect on the challenge infection, since all control mice that received CQ-prophylaxis showed the same pre-patent period as untreated naïve mice.

Comparing chloroquine to mefloquine prophylaxis for CPS immunization

Next, we studied immune responses and protection after CPS-CQ and CPS-MQ immunization. CD8$^+$ Tem levels (Figure 4), IFNγ production upon *in vitro* restimulation with *Pb*SPZ or *Pb*iRBC and pluripotent T cells producing both IFNγ and IL-2 (Figure 5) were significantly increased in immunized compared to control mice.

Table 1 Effect of chloroquine on RAS protective efficacy

				No. protected/ no. challenged	
Immunization	**Mouse strain**	**Immunization dose (x10^3 RAS PbSPZ)**	**Inoculations (n)**	**Chloroquine -**	**Chloroquine +[#]**
RAS	Balb/cByJ	1	1	14/16 (88)	13/16 (81)
		0.5	1	4/10 (40)	4/10 (40)
		0.3	1	5/10 (50)	2/10 (20)
	C57BL/6j	10	3	5/5 (100)	4/5 (80)
		4	3	4/5 (80)	4/5 (80)
		1	3	0/5 (0)	1/5 (20)
		4	2	15/23 (65)	14/23 (61)[##]
None	Balb/cByJ	N/A	N/A	1/6 (16)	0/6 (0)
	C57BL/6j	N/A	N/A	0/10 (0)	0/15 (0)

[#]Mice received chloroquine prophylaxis for 10 or 17 days, with the exception of the experiment indicated with [##], where mice received two injections of CQ, 2h before and 6h after each immunization.

Although not significant, there was a trend for higher CD8$^+$ Tem levels in CPS-CQ compared to CPS-MQ in both liver and spleen (p=0.08 and 0.16, respectively; Figure 4). T cells isolated from the liver of CPS-CQ mice showed higher IFNγ responses after *in vitro* re-exposure to

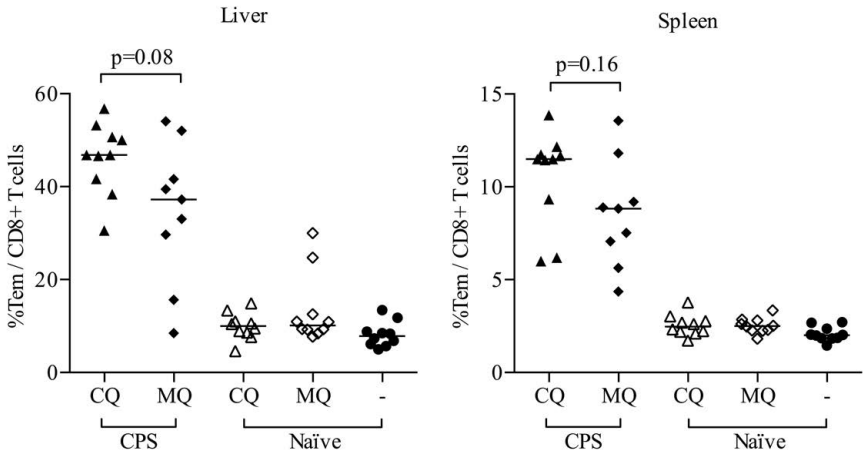

Figure 4 Frequencies of CD8$^+$ Tem cells following sporozoite immunization under CQ or MQ cover. Percentages of CD8$^+$ T cells with effector memory phenotype (CD44$^+$CD62L$^-$ Tem) were measured by direct *ex vivo* staining a day before challenge (C-1) in the liver and spleen of C57BL/6j mice immunized with sporozoites under CQ cover (filled triangle) or MQ cover (filled diamonds), naïve-CQ mice (open triangles), naïve-MQ mice (open diamonds) and untreated naïve mice (filled circles). Horizontal lines represent group medians.

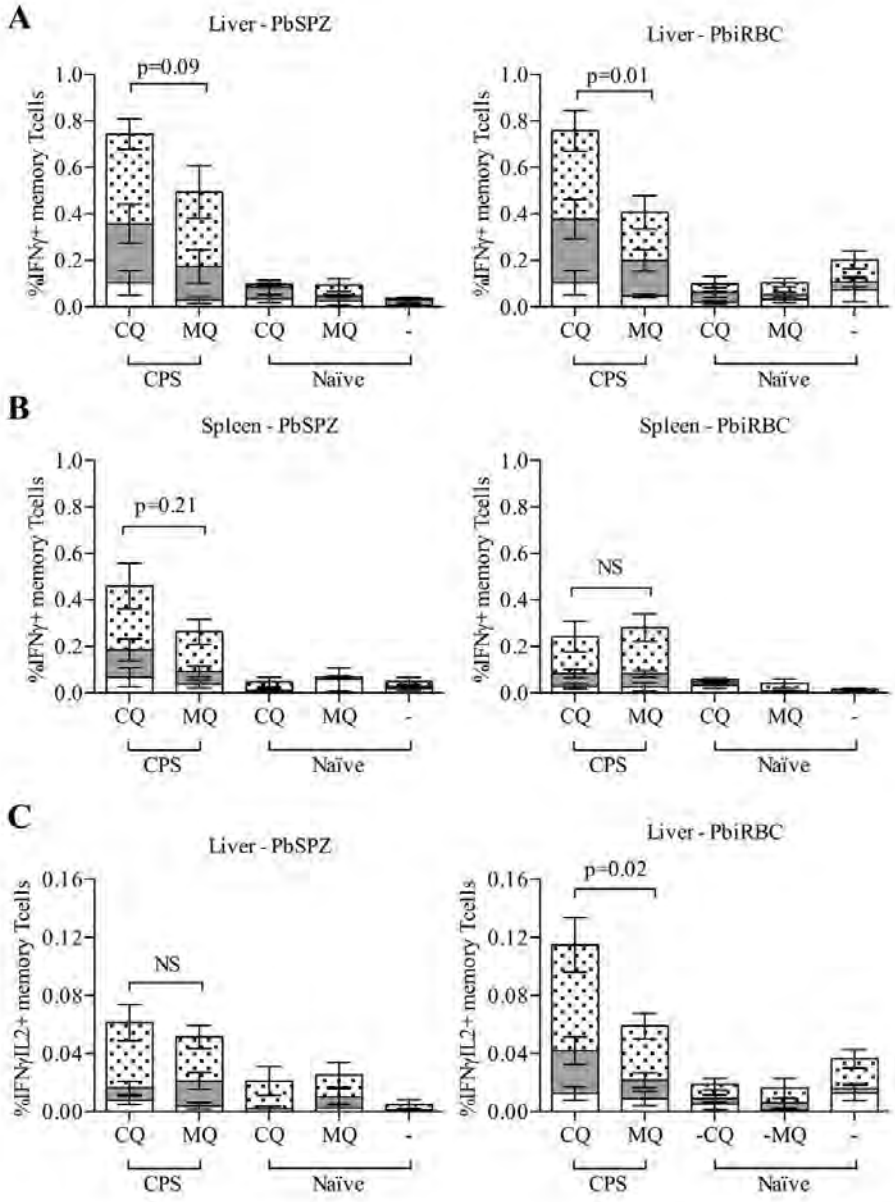

Figure 5 Sporozoite specific cytokine responses following sporozoite immunization under CQ or MQ cover. Percentages of IFNγ producing memory T cells were measured in CPS-CQ (n=10), CPS-MQ (n-9), naïve-CQ (n=10), naïve-MQ (n=10) and no-drug naïve controls (n=10) a day before challenge by intracellular staining after re-exposure of (A) liver and (B) spleen cells to *P. berghei* sporozoites (*Pb*SPZ) or infected red blood cells (*Pb*iRBC). Responses of $CD4^+$ T cells (open area), $CD8^+$ T cells (grey area) and $CD4^-$

CD8$^-$ T cells (dotted area) relative to total memory CD3$^+$ T cell responses are presented. (C) Hepatic IFNγ and IL-2 responses of memory T cells after re-exposure to *Pb*SPZ and *Pb*iRBC are presented similarly. Background responses to salivary glands and uninfected red blood cells were subtracted from *Pb*SPZ and *Pb*iRBC responses respectively for each individual mouse. Error bars represent standard error of the mean. NS = not significant.

*Pb*iRBC (p=0.01) and a trend for higher IFNγ responses to *Pb*SPZ re-exposure (p=0.09; Figure 5A). In the spleen, a similar trend of higher IFNγ responses in the CPS-CQ group was observed upon re-exposure to *Pb*SPZ but not *Pb*iRBC (Figure 5B). Following *in vitro* re-exposure to *Pb*iRBC but not *Pb*SPZ, the percentage of hepatic pluripotent memory T cells producing both IFNγ and IL-2 was significantly higher in the CPS-CQ group compared to the CPS-MQ group (p=0.02, Figure 5C). Thus, CPS-CQ resulted in somewhat higher specific cytokine responses compared to CPS-MQ.

Finally, mice were challenged by intravenous administration of 10,000 sporozoites five or ten weeks after the second booster (day 50 or 100) or with 50,000 sporozoites at day 50. At day 50, 100% of CPS-CQ (13/13) and CPS-MQ (21/21) immunized mice were protected against challenge with low (10K) or high (50K) sporozoite dose. All control mice including CQ (10/10) and MQ (10/10) prophylaxis groups developed blood-stage parasitemia. Postponement of challenge to day 100 resulted in 90% protection (9/10) in both the CPS-CQ and CPS-MQ group (Table 2).

Altogether, there was no difference in protection after CPS immunization with either CPS-CQ or CPS-MQ, although we observed increased cellular responses after whole sporozoite immunization under CQ cover.

Table 2 Protection by sporozoite immunization under CQ or MQ cover[#]

	Day 50 (x10^3 PbSPZ challenge)		**Day 100 (x10^3 PbSPZ challenge)**
	10	**50**	**10**
CPS-CQ	3/3 (100)	10/10 (100)	9/10 (90)
CPS-MQ	11/11 (100)	10/10 (100)	9/10 (90)
Naïve-CQ	0/5 (0)	0/5 (0)	-
Naïve-MQ	0/5 (0)	0/5 (0)	-
Naïve	0/5 (0)	0/5 (0)	0/5 (0)

[#]No. protected / No. challenged (% protection)

Discussion

Addition of CQ to a *P. berghei* RAS immunization protocol improves neither protection nor parasite-specific $CD8^+$ T cells responses. Only slightly reduced T cell responses and similar protective efficacy are found when CPS-CQ is compared to CPS-MQ. We did not study the effect of CQ on cross-presentation *in vitro*, nor did we perform detailed mechanistic antigen presentation studies (31). Alternatively, we decided to investigate more functional readouts such as parasite-specific $CD8^+$ T cell responses and protection from challenge infection to assess potential immune enhancing effects of CQ. These combined *P. berghei* data do not provide evidence for significant improvement of whole sporozoite immunization in the presence of CQ and therefore indicate that CQ is not responsible for the strikingly higher efficiency of CPS-CQ compared to RAS in humans.

Improved cross-presentation resulting in increased IFNγ production by $CD8^+$ T cells has been shown in *in vitro* studies where dendritic cells were pulsed with soluble viral antigen in the presence of CQ (19). In mice, CQ was shown to enhance cross-presentation of soluble OVA to OT-I cells both *in vitro* and *in vivo* and to improve specific $CD8^+$ T cell responses after alum-OVA immunization (21). An effect of CQ on OVA cross-presentation was observed upon administration of 20μg but not 200μg protein (21), suggesting that immunomodulatory effects of CQ are only beneficial under suboptimal immunization conditions. In our experiments, RAS immunization both with and without CQ induces strong cellular responses with similar contribution of $CD8^+$ T cells, which translates to equal protection levels. Even after down-titration of RAS immunization dose, which is associated with decreasing protection, clear improvement by CQ remains undetected.

In humans, a single administration of CQ during Hepatitis B booster vaccination significantly improved $CD8^+$ T cell response (19). But despite several reports of enhanced immune responses by CQ in mice and men (19, 21, 23, 32), only one study has reported improved protection; mice immunized with a heat-inactivated influenza virus showed improved survival rates after challenge infection (20). The significance of improved immune responses for protection thus remains to be further explored.

One cannot assume that improvement of cross-presentation by CQ is applicable to any soluble protein or peptide, or antigens presented in the form of a whole sporozoite. Improvement of cross-presentation of SIINFEKL peptide ($OVA_{257-264}$) and inactivated influenza virus by CQ in mice have been demonstrated (20, 21). In our study however, CQ administration during sporozoite immunization with SIINFEKL expressing *P. berghei* sporozoites showed no increase of SIINFEKL-specific $CD8^+$ T cells, suggesting that presentation pathways and effects of CQ might differ between pathogens or antigens.

CPS-CQ immunization of C57BL/6j mice requires relatively high and lengthy drug prophylaxis to prevent development of *P. berghei* infection (K. Nganou-Makamdop, unpublished data). As a result, CPS-CQ mice cumulatively receive much more CQ than the

two doses of 800µg chloroquine diphosphate salt that were previously shown to improve cross-presentation (21). We therefore assessed in a separate experiment the effect of this low CQ-dose regimen on RAS immunization but did not find higher protection levels compared to RAS alone, indicating that the choice of CQ dose was not crucial.

Immune modulating effects of MQ have been reported but do not include cross-presentation (25-27, 33). We cannot formally exclude that MQ in CPS immunization regimes may have similar properties as CQ. Both CQ and MQ are lysosomotropic agents that limit endosomal acidification (22), which for CQ is known to result in inhibition of lysosomal enzymes that require an acidic pH to function and the fusion of endosomes with lysosomes (34). As such CQ, but not MQ, has been widely used to study the role of endosomal acidification in cellular processes (35). CQ has also been studied extensively for its inhibitory effect on autophagy (17), but a recent publication suggests that MQ has similar effects on autophagy (36). Because both endosomal acidification and autophagy might influence antigen presentation, the effect of MQ on these processes may result in immune modulating effects just as is the case for CQ.

In the absence of evidence for a direct immune-modulating effect of CQ during whole sporozoite immunization, we cannot rule out that both CQ and MQ might instead contribute to the efficient induction of protection in an indirect way. A review of rodent sporozoite immunization studies demonstrates the importance of optimal exposure to the entire repertoire of liver stage antigens as occurs during CPS-CQ and CPS-MQ, with reduced protective efficacy if liver stage development is halted by drugs or in the case of RAS or genetically attenuated parasites (37). Furthermore, some reports show a negative effect of blood-stage parasites on induced pre-erythrocytic $CD8^+$ T cell responses by interfering with dendritic cell function (1, 38). By limiting exposure to blood stages during CPS immunization, CQ and MQ might thus have an indirect positive effect on pre-erythrocytic immunity.

Conclusions

We did not find evidence of improved immune responses or protective efficacy by CQ in the *P. berghei* model. Instead, the higher efficiency of CPS compared to RAS in humans might be explained by an indirect effect of CQ through limiting blood-stage exposure after immunization or to an improved breadth of the immune response as a result of increased antigen exposure. In the absence of a clear immune enhancing effect of CQ here, future work is needed to assess whether these findings can be translated to human settings.

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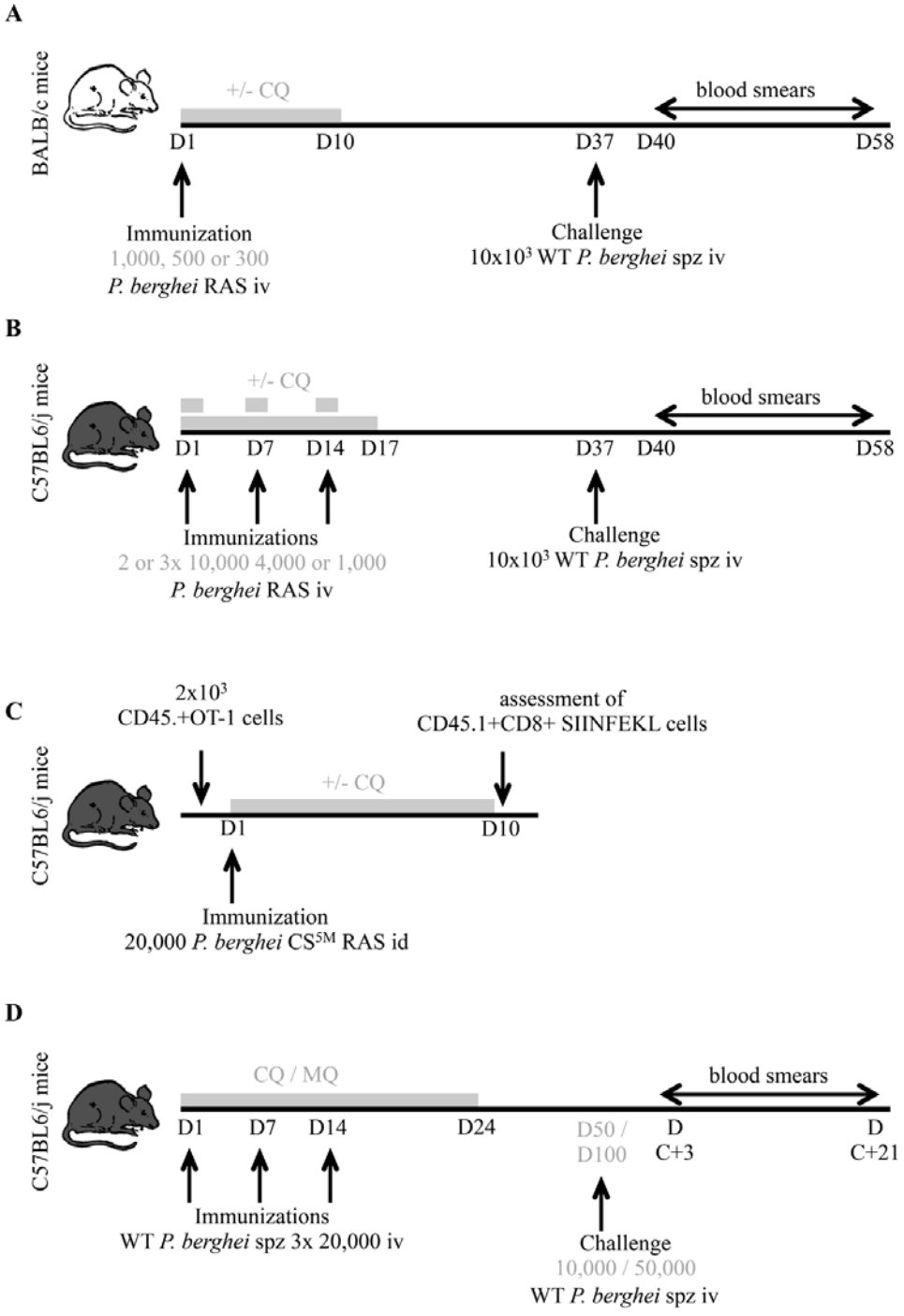
Supplementary Figure 1 (next page): Study designs. The effect of chloroquine (CQ) on immune responses and protection by whole sporozoite immunization was tested in a number of *P. berghei* models. Balb/cByJ (A) and C57BL/6j mice (B) received intravenous (iv) immunizations with radiation attenuated sporozoites (RAS; immunization dose in grey), with or without additional administration of CQ (grey bars). All mice were challenged by iv injection of $10x10^3$ wild type (WT) *P. berghei* sporozoites (spz), and followed up with blood smears for the detection of parasites. (C) C57BL/6j mice were immunized with *P. berghei* CS5M RAS after receiving CD45.1+OT-1 cells, with or without CQ prophylaxis (grey bar). Expansion of CD45.1+CD8+ SIINFEKL cells in liver and spleen was assessed by flow cytometry 10 days after immunization. (D) C57BL/6j mice were immunized by iv administration of WT *P. berghei* spz while receiving either CQ or mefloquine (MQ) prophylaxis, then challenged after 50 days with either 10x103 or $50x10^3$ WT *berghei* spz or after 100 days with $10x10^3$ WT *berghei* spz, and followed up with blood smears for the detection of parasites


A
BALB/c mice
+/- CQ
blood smears
D1
D10
D37
D40
D58
Immunization
1,000, 500 or 300
P. berghei RAS iv
Challenge
10x10³ WT P. berghei spz iv
B
C57BL6/j mice
+/- CQ
blood smears
D1
D7
D14
D17
D37
D40
D58
Immunizations
2 or 3x 10,000 4,000 or 1,000
P. berghei RAS iv
Challenge
10x10³ WT P. berghei spz iv
C
C57BL6/j mice
2x10³
CD45.+OT-1 cells
assessment of
CD45.1+CD8+ SIINFEKL cells
+/- CQ
D1
D10
Immunization
20,000 P. berghei CS5M RAS id
D
C57BL6/j mice
CQ / MQ
blood smears
D1
D7
D14
D24
D50 /
D100
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C+3
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C+21
Immunizations
WT P. berghei spz 3x 20,000 iv
Challenge
10,000 / 50,000
WT P. berghei spz iv

CHAPTER 5

Enhancement of naturally acquired immunity against malaria by drug use

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Abstract

Combination of chemoprophylaxis with chloroquine and so-called 'controlled human malaria infections' has been shown to induce sustained and fully protective immunity against malaria in experimental settings. This opens possibilities of translating this approach into an effective and applicable strategy for the field. We review the different ways in which antimalarial drugs have been used for prevention of malaria in endemic settings and discuss the possibilities and challenges of applying a strategy of drug use and naturally acquired infection in the field.

Introduction

Malaria remains one of the most important infectious diseases worldwide, causing almost 655,000 deaths per year, of which the majority are children under five years of age. Intense malaria control interventions during the past decade have successfully established a reduction of more than 50% in either confirmed cases of malaria or malaria admissions and deaths in eleven countries of the WHO African region (1). However, increases in the number of malaria cases in 2009 in Rwanda, Sao Tome and Principe and Zambia, which previously reported reductions illustrate the fragility of the current successes. This underlines the need for additional and innovative strategies.

Availability of an effective vaccine would greatly contribute to malaria control and elimination. It is well known that clinical immunity is acquired in endemic areas after a number of years and a sufficient number of naturally acquired infections (2). The search for malaria-vaccines against *Plasmodium falciparum* has been pursued for decades, with the main focus on the development of subunit-vaccines, but with limited success. Twenty candidate vaccines are currently under clinical investigation but only one product, RTS,S, has progressed into a phase 3 field trial having recently shown encouraging indications of protection in an interim evaluation (3, 4).

One of the shortcomings of subunit-vaccines is the inability to appropriately address the significant antigenic diversity of target epitopes and the often-observed poor immunogenicity of the soluble parasite derived proteins used. Against that background a whole-parasite approach may perform better. Indeed, immunization with sporozoite forms has consistently been shown to induce >90% protection in rodents and humans in experimental set-ups (5, 6).

Transmission intensity varies greatly in sub-Saharan Africa where individuals can be subjected to up to 10 infectious bites per night at certain periods of the year. Here we explore the idea that using medicines together with naturally acquired infection could result in the induction of protective immunity. We will review the different ways in which antimalarial drugs have been used for prevention of malaria in endemic areas and we will reflect on the possibilities and challenges of applying a strategy of drug use together with naturally acquired infection in the field (see Table for an overview of the interventions discussed in this review).

Drug applications for prevention of malaria in endemic areas

Millions of travellers visit malaria-endemic areas for short periods of time while using chemoprophylaxis for prevention of malaria. Such practice has never been considered as a realistic strategy for endemic populations. Reasons include lack of sustainability, problems with acceptance by the community, risk of emerging drug resistance and concerns about

impairment of the development of natural immunity (7).

Nevertheless, the potential effects of drug administration on development of clinical protection in natural settings have been explored in a number of studies. Different recipes of chemoprevention have been applied, including mass drug administration in the general population and application of drugs in specific risk groups including children, infants and pregnant women (7).

Intervention	***Definition***
Chemoprophylaxis	Administration of a drug in such a way that its blood concentration is maintained above the minimum inhibitory level
Mass Drug Administration (MDA)	Administration of a full therapeutic course of an anti-malarial drug to a whole population at risk
Intermittent Preventive Treatment (IPT)	Administration of anti-malarial treatment to a population at risk at specified time points, regardless of whether or not they are infected
Controlled Human Malaria Infections (CHMI) under chemoprophylaxis	Exposure of healthy malaria-naïve volunteers to *Plasmodium* infected mosquito-bites, whilst taking prophylactic drugs
Immunization by drug use and natural infection	Exposure to parasites under drug-cover in malaria-endemic areas

Table Overview of interventions

Mass drug administration (MDA) involves the prescription of antimalarial drugs to whole populations without screening for the presence of parasitemia. This can be done either directly, when a curative dose of the antimalarial drug is given to an entire population; or indirectly, when the antimalarial is added to food, usually to salt.

Several studies have been conducted since the late 1950's, which show that MDA does substantially reduce the incidence of clinical malaria and parasite prevalence but that the impact of MDAs was transitory (8). Evaluation and interpretation of the true effects are likely compromised by missing a substantial proportion of *P. falciparum* infections due to the limited sensitivity of microscopic parasite detection (9). Submicroscopic parasitemia and frequent asymptomatic parasite carriage widely exist in endemic areas, particularly in those with very low endemicity. However, it is currently unknown what the effects on the immune responses are and how these individuals manage to control malaria.

Chemoprophylaxis is the administration of a drug in such a way that its blood concentration is maintained above the minimum inhibitory level. Travellers who visit malaria-endemic areas use chemoprophylaxis to prevent malaria, and in endemic areas it has been applied for specific risk groups, such as children. In children, it can effectively reduce

overall mortality and clinical malaria attacks; it also improves mean hemoglobin levels, reduces severe anaemia and improves school attendance (10).

To further increase adherence and sustainability and reduce the risk of inducing drug-resistance, a more targeted approach has been developed. Intermittent preventive treatment (IPT) is the administration of a full course antimalarial treatment at specified time points without parasite screening. This IPT restricts the use of antimalarials to specific risk groups at specified time points. The idea is that parasite exposure will be less to undesired subtherapeutic drug-concentrations while concomitantly allowing a more effective generation of natural immunity in the intermittent periods between two doses of IPT (11, 12). When administered in the existing health system during routine visits, for example during pregnancy or at infant-vaccinations, costs can be reduced and sustainability increased (7).

IPT was initially investigated in the context of pregnant women (IPTp), and subsequently extended to infants and children (IPTi, IPTc). IPTp with sulfadoxine–pyrimethamine (SP), administered two or three times during the second and third trimesters, effectively reduces disease burden and adverse outcomes of malaria in pregnancy by substantially reducing placental malaria [relative risk (RR) 0.48; 95% confidence interval (CI) 0.35-0.68], low birth weight (RR, 0.71; 95% CI, 0.55-0.92) and anaemia (13). Moreover, IPTp is readily implementable and cost effective (14, 15). The WHO therefore recommends IPTp with SP for areas with high or moderate transmission, and many countries have substantially scaled up delivery of IPTp, although coverage is still inadequate (16). IPTi has been shown to be safe in infants, with a protective efficacy of ~30% against clinical malaria and 21% against the risk of anaemia from the first dose until 12 months of age (17). Unfortunately, levels of SP resistance are on the rise in many areas, requiring adaptation of SP regimes. This further underlines the need for new drugs or drug combinations and for innovative interventions.

Immune responses in the context of reduced parasite exposure

More than fifteen years ago it was suggested that interventions that reduce malaria transmission and thus the level of exposure may interfere with the acquisition of natural immunity (2, 18). This hypothesis, however, is difficult to address in the absence of an established immune correlate of clinical protection against malaria. More than two decades ago, a number of studies made attempts to investigate the effects of chemoprophylactic measures on humoral and/or cellular immune responses. The effect of insecticide-treated bed net use on antimalarial immunity has been investigated separately, showing inconclusive results that we will not explore further here (19-21).

While the majority of studies on humoral responses show a decrease in antibodies to malaria antigens after chemoprophylaxis (22-28), this may simply represent less parasite exposure rather than an actual loss of protective immunity. Similar results were obtained in a

number of IPT studies; IgG levels against crude *P. falciparum* lysate were significantly lower in Ghanian children after a single dose of SP compared to a placebo, without any evidence of rebound malaria (29). In Senegal, a slightly decreased antibody response was measured in children receiving IPTc, when compared to non-treated controls (30). In contrast, a study in Mozambique showed no significant difference in antibody responses between children receiving IPTi with SP and placebo-treated controls. Interestingly, one exception was observed here in that IgG and IgG1 responses to *P. falciparum* apical membrane antigen (AMA)-1 and/or merozoite surface protein (MSP)-1_{19} at ages 5, 9, and 24 months were actually significantly increased in the SP group (31). Therefore, these authors concluded that IPTi reduces the incidence of illness while allowing the development of naturally acquired antibody responses.

Relatively few studies have been conducted on cellular immune responses after chemoprophylaxis. These point towards higher lymphoproliferative responses and interferon (IFN)-γ production in the presence of a lower overall exposure to parasites (27, 32).

Clinical rebound after chemoprophylactic interventions

One could argue that incidences of malaria morbidity and mortality are actually more relevant than immune responses after drug-based preventive measures. Prophylaxis with various drugs has been tested for children in many studies in Africa, Asia, Central America and the Pacific. Assessments showed that rebound malaria generally occurred from three months to one year after discontinuation of chemoprophylaxis, which was given for a period of three months to five years in infants aged less than one year up to children aged five to ten years. Different end points were used to assess rebound malaria (10).

Of twelve studies investigating rebound-malaria after termination of chemoprophylaxis, nine did not show increased clinical malaria or parasitemia (10). For example, data from the famous Garki project in Nigeria, where pyrimethamine prophylaxis was combined with insecticide spraying, show large reductions in infant and child mortality rates during the intervention without increased morbidity or mortality afterwards (33). Similar results were obtained in another Nigerian study that investigated the effects of chloroquine-prophylaxis in children (26). Furthermore, when dapsone-pyrimethamine was administered to Gambian children for two years between their 3rd and 5th birthdays, there was no increase in clinical malaria after cessation of the prophylaxis (28). The same lack of rebound-malaria was shown in a study using pyrimethamine or chlorproguanil in 2–9-year-old children in Liberia (34). Finally, a non-immune adult population in Irian Jaya (West Papua), Indonesia, having used chloroquine for a year, initially showed an increased incidence of *P. falciparum* parasitemia in the post-chloroquine group but did not show any significant difference in time to first parasitemia and clinical malaria incidence in the complete 28-week follow-up period (35).

Few studies have shown a significant increase in clinical malaria (36, 37). Taken weekly, dapsone-pyrimethamine effectively reduced the risk of clinical malaria and severe anaemia when given to Tanzanian infants between 2 and 12 months of age. Once stopped, however, the group of treated children showed a significantly higher incidence rate of clinical malaria compared to the placebo group (38). Gambian children receiving dapsone-pyrimethamine between the ages of six months and five years during the transmission season developed an increased risk of clinical malaria in the year after stopping chemoprophylaxis. There was, however, a beneficial effect of a 15% increase in survival rates during the overall surveillance period up until the age of seven years (39).

The perceived fear of a clinical rebound effect was one of the arguments for limiting the presumptive drug use to high risk groups and/or to specified time points. Drug levels that fall below inhibitory concentrations in-between IPT gifts would allow for limited parasite exposure and may, therefore, lead to building of immunity. Indeed, the first IPTi study actually showed a reduction in malaria incidence for a much longer period than could have been expected as a direct effect of the drug, suggesting that the desired enhanced acquisition of immunity did occur (11). Unfortunately, this effect has not been reproduced in later studies. In some occasions, even a small increase in malaria incidence was observed after cessation of the intervention (40, 41) but, as was the case for chemoprophylaxis, most studies did not show a change in malaria incidence after the end of the intervention (17, 29, 42-44).

There are large methodological differences between these studies, including age group, drug choice, dosage, frequency and duration of administration, malaria endemicity and clinical evaluation. Variation in these parameters prevents the possibility to draw unequivocal conclusions regarding the effects of drug use on the acquisition of natural immunity. The combined data, however, suggest that there is insufficient empirical evidence to support the earlier concept that chemoprophylaxis or IPT, when given in field conditions, substantially impairs the development of clinical immunity. Occasional breakthrough infections seem to be equally effective or even better at inducing protective immunity compared to uncontrolled parasite exposure. Since antibody responses are consistently reported to be lower following chemoprophylactic interventions, cellular immune responses may be responsible for this clinical immunity.

Protection by controlled human malaria infections under chemoprophylaxis

In contrast to data from a variety of field studies, there is strong experimental evidence that a combination of chemoprophylaxis and so-called ‘controlled human malaria infection’ (CHMI) (45) can induce fully protective immune responses. Efficient induction of sterile protection against malaria can be achieved in rodents by inoculation of intact sporozoites

under chemoprophylaxis (46, 47). In an analogous proof-of-concept CHMI study, malaria-naïve adult volunteers received 12–15 *P. falciparum*-infected mosquito bites once a month for three months under chloroquine prophylaxis. In a subsequent challenge infection with five infected mosquitoes at three months post-immunization and discontinuation of chloroquine, immunized volunteers were completely protected. When re-challenged after >2 years, the majority of these volunteers was still fully protected against a CHMI. Long-lasting cellular immune responses, more specifically multifunctional effector memory T-cells that produce both IFN-γ and interleukin-2 upon *ex vivo* stimulation, are associated with protection (48-50).

The difference in the efficiency of inducing full protection is striking when compared to the situation in endemic countries where it may take years to acquire clinical protection. So what is the critical difference between CHMI and chemoprophylactic interventions in the field? Several differences may contribute to, or account for, these results: i) In the experimental studies, challenge infection was performed by a homologous strain, while the genetic diversity of *Plasmodium* in the field is immense. Therefore, observed protection may be strain-`specific; however, we do not favour this explanation, since protection from a heterologous challenge has been shown after immunization with irradiated sporozoites by mosquito bites (6); ii) Asexual parasites have been shown to suppress immune responses in rodent studies (51); therefore, the presence of (submicroscopic) parasitemia might be accountable for a compromised induction of protective immune responses in field studies; iii) When hepatitis B virus (HBV) vaccine responders are boosted with hepatitis B envelope protein vaccine with or without a single dose of chloroquine, a substantial increase in HBV-specific $CD8^+$ T-cells is observed in the individuals receiving chloroquine (52). Some have argued that exposure to *Plasmodium*-infected mosquito bites induces malaria-specific regulatory T-cells in the skin and, therefore, parasite-specific immunotolerance, which blocks vaccine efficacy. Chloroquine may inhibit this induction of regulatory T-cells and, therefore, enhance the acquisition of immunity (53). As such, the known immune-modulating effects of the drug chloroquine may be, at least partially, responsible for the efficient induction of immunity in the CHMI model (54). A clinical trial where immunization with CHMI under chloroquine is compared to another antimalarial drug that does not have these immune-modulating effects, for example mefloquine, will provide clarification on this hypothesis (54).

Innovative application of drug use in the field

Now that the potential for induction of complete and sterile protection has been demonstrated under conditions of CHMI, one may consider the translation of this to a practical application under field conditions. Evidence for a proof-of-concept may be obtained from a study in an area with a short but intensive transmission season that approaches similar inoculation rates

as CHMI (46). This will allow for high exposure to pre-erythrocytic antigens while blood-stage infections are controlled. If transmission is low or virtually absent between the malaria seasons, one can evaluate the possibility of induced protective efficacy in the next season.

For safety reasons, alternatives to chloroquine monotherapy have to be used because of the widespread chloroquine-resistance. Recently, immunization with sporozoites of the rodent malaria *Plasmodium berghei* in combination with azithromycin, pyrimethamine or primaquine, resulted in a high protective efficacy. Interestingly, when animals received azithromycin prophylaxis during sporozoite exposure, lower liver loads and superior protection were observed (5). The combination of azithromycin and chloroquine has demonstrated synergistic effect against parasite growth *in vitro* (55) and has shown substantially improved clinical and parasitological outcomes compared to azithromycin or chloroquine monotherapy *in vivo* (56). In treatment trials with azithromycin–chloroquine in Africa, this drug combination showed non-inferiority compared to mefloquine (57). Both drugs have been safely administered in all trimesters of pregnancy. Since the antimalarial activity of chloroquine is pleiotropic, drug resistance may be due to different mechanisms, each amenable to reversal by drug combination (58). This opens up exciting possibilities of combining azithromycin and chloroquine as an effective drug combination for immunization by drug use and naturally acquired infections. In this way, even in areas with chloroquine resistance, the antimalarial effects of both drugs and the possibly beneficial immune-modulating effects of chloroquine could be combined. Another interesting candidate might be dihydroartemisinin–piperaquine, which is an effective first-line treatment for *P. falciparum* malaria in both adults and children (59, 60).

In conclusion, this concept of drug use and naturally acquired infections may be used in the road map towards innovative use of antimalarial drugs for the control or even elimination of malaria. Once field trials are as effective as the promising data from CHMI studies, the concept can hopefully be translated to an effective and applicable strategy for endemic populations.

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CHAPTER 6

Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity

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Abstract

Volunteers immunized under chloroquine chemoprophylaxis with *Plasmodium falciparum* sporozoites (CPS) develop complete, long-lasting protection against homologous sporozoite challenge. Chloroquine affects neither sporozoites nor liver-stages, but kills only asexual forms in erythrocytes once released from the liver into the circulation. Consequently, CPS-immunization exposes the host to antigens from both preerythrocytic and blood stages, and induced immunity might target either of these stages. We therefore explored the life cycle stage specificity of CPS-induced protection.

Twenty-five malaria-naïve volunteers were enrolled in a clinical trial, fifteen of whom received CPS-immunization. Five immunized subjects and five controls received a sporozoite challenge by mosquito bites, whereas nine immunized and five control subjects received an i.v. challenge with *P. falciparum*-infected erythrocytes. The latter approach completely bypasses preerythrocytic stages, enabling a direct comparison of protection against either life cycle stage.

CPS-immunized subjects (13 of 14) developed anti-circumsporozoite antibodies, whereas only one volunteer generated minimal titers against typical blood-stage antigens. IgG from CPS-immunized volunteers did not inhibit asexual blood-stage growth *in vitro*. All CPS-immunized subjects (5 of 5) were protected against sporozoite challenge. In contrast, nine of nine CPS-immunized subjects developed parasitemia after blood-stage challenge, with identical prepatent periods and blood-stage multiplication rates compared with controls. Intravenously challenged CPS-immunized subjects showed earlier fever and increased plasma concentrations of inflammatory markers D-dimer, IFN-γ, and monokine induced by IFN-γ than i.v. challenged controls.

The complete lack of protection against blood-stage challenge indicates that CPS-induced protection is mediated by immunity against preerythrocytic stages. However, evidence is presented for immune recognition of *P. falciparum*-infected erythrocytes, suggesting memory responses unable to generate functional immunity.

Introduction

Malaria remains one of the most common and severe infectious diseases, with an estimated 216 million cases and 655,000 deaths annually (1). The malaria parasite *Plasmodium falciparum* is responsible for most of these cases, particularly in sub-Saharan Africa. *P. falciparum* sporozoites are transmitted to humans by the bites of infected *Anopheles* mosquitoes. Sporozoites migrate from the skin to the liver, where they invade hepatocytes, develop and multiply. Approximately six days after invasion, hepatocytes rupture and merozoites are released into the bloodstream where they multiply in 48-hour cycles of erythrocyte invasion, replication, erythrocyte rupture, and release of infectious merozoites. These asexual blood-stage parasites cause the clinical symptoms of malaria. To fight malaria, an effective vaccine is urgently needed. Development of vaccines generally has been stage-oriented, specifically targeting preerythrocytic or asexual blood stages of the parasite (2).

In the controlled human malaria infection model, we previously showed that immunization of healthy malaria-naïve volunteers while they are taking chloroquine prophylaxis with *P. falciparum* sporozoites via infected mosquito bites [chemoprophylaxis and sporozoites (CPS)-immunization], induces long-lasting sterile protection against a homologous challenge infection (3, 4). The unprecedented efficacy of the CPS-immunization model is represented by the low dose sufficient to induce protection, i.e., three times 12–15 infected mosquito bites, compared with 1,000 bites required in the irradiated sporozoite approach (5).

Chloroquine kills only developing blood stages of *P. falciparum*, without affecting sporozoites or liver stages (6). This results in transient low-level blood-stage parasitemia during CPS-immunization (3). Consequently, the host's immune system will be exposed to a relatively broad repertoire of antigens, including sporozoite, liver-stage, and early blood-stage antigens. Humoral and cellular immune responses are induced against both sporozoites and blood stages (3, 7). In addition, many antigens are shared between these stages (8), leaving open the possibility that the observed protection may be mediated by immune responses against either of these parasite life cycle stages or a combination thereof (9). The absence of parasitemia after challenge infection and the predominant induction of preerythrocytic antibodies suggest that preerythrocytic immunity primarily is responsible for protection, although a possible requirement for immune responses against asexual stages cannot be ruled out (3). Indeed, previously it was shown that exposure to very low densities of blood stages may induce protection in the controlled human malaria infection model (10). In this study, protected subjects displayed strong parasite-specific T-cell proliferation and IFN-γ production (10). Moreover, CPS-immunized volunteers also exhibited strong IFN-γ responses upon *in vitro* restimulation with infected erythrocytes (7).

To explore the possible role of immunity against pre-erythrocytic- and/or blood-stage in protection, CPS-immunized volunteers were subjected to either a *P. falciparum* sporozoite

or asexual blood-stage challenge. Because the latter approach completely bypasses the liver stages, any protection seen would indicate that blood-stage immunity may contribute to CPS-induced protection.

Results

Twenty-five out of 42 screened subjects (median age 21 years; range 19–32) were included in the study (Figure S1). Fifteen volunteers were immunized according to the CPS protocol as described previously (3). Briefly, while taking chloroquine prophylaxis, volunteers (groups 1 and 2) were exposed to bites of 15 *P. falciparum*-infected mosquitoes (8 mosquitoes with the NF54 strain and 7 mosquitoes with the 3D7 clone) at monthly intervals for a period of three months. Control volunteers (groups 3 and 4) received chloroquine prophylaxis only. One subject in group 1 withdrew consent after the third immunization for reasons unrelated to the trial.

After the first immunization, 14 of 15 subjects (groups 1 and 2) developed transient low blood-stage parasitemia, as retrospectively detected by quantitative real time PCR (qPCR). The geometric mean of peak parasitemia was 1,378 parasites/mL [95% confidence interval (CI) 456-4,165 parasites/mL; Figure 1]. Thick smears remained negative except in two subjects (one each in groups 1 and 2), who developed a positive thick smear on day 7. Their peak parasitemia was 14,454 and 6,761 *P. falciparum*/mL. Both the severity and frequency

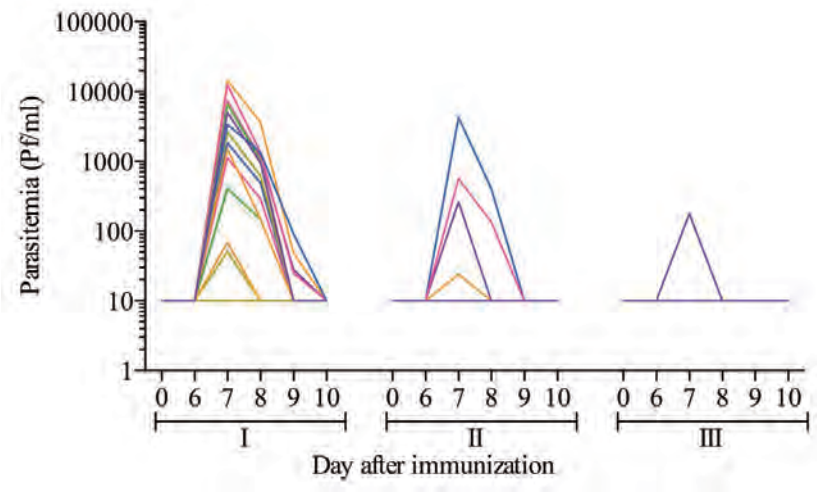

Figure 1 Blood-stage parasitemia during CPS-immunization. Blood-stage parasitemia was measured from day 6 until day 10 after the first (I), second (II) and third (III) immunization by qPCR. Each line represents an individual subject (n=15); values shown as 10 on the logarithmic scale were negative.

of adverse events (AEs) were similar to those in the other subjects, and chloroquine plasma concentrations were within the prophylactic range (53 and 56 µg/L). These two subjects were treated promptly with atovaquone/proguanil and continued study participation according to protocol. All subjects in groups 1 and 2 reported solicited AEs (mean duration, 1.0 ± 0.11 days) after the first immunization. The most common AEs were headache (13/15 subjects), and fever and nausea (both in 8/15 subjects). Four subjects experienced a grade 3 AE (headache n=2, malaise n=2; mean duration 1.8 ± 0.6 days), which all occurred between days 7 and 10 after the first immunization and were considered probably related to the immunization.

After the second immunization, four subjects developed parasitemia by qPCR (geometric mean peak parasitemia, 351 parasites/mL; 95% CI 43-2,857; Figure 1), whereas thick smears remained negative. Two subjects experienced mild or moderate AEs. After the third immunization, only one subject showed blood-stage parasitemia (178 parasites/mL; Figure 1) and three subjects experienced mild AEs. No serious AEs occurred during the trial.

Antibody levels against the circumsporozoite protein (CSP), apical membrane antigen 1 (AMA-1) and glutamate-rich protein (GLURP) were measured before CPS-immunization and before challenge. CPS-immunized subjects (13/14) showed induction of anti-CSP antibodies (at least a twofold increase in antibody titer), whereas only a single subject (group 1) showed a minimal increase in AMA-1 and GLURP antibody titers (Table 1). IgG was isolated from plasma of all immunized subjects at baseline and before challenge-infection. *In vitro* blood-stage growth inhibition assay (GIA) did not show an inhibitory effect of purified IgG on blood-stage parasite growth in any of the subjects (Table 1).

Table 1 Antibody titers and *in vitro* growth inhibition[#]

		I-7	**C-1**	**Δ [(C-1)-(I-7)]**	**p-value**
Antibody titer (AU)	**CSP**	0.91 (0; 2.15)	24.3 (15.2; 48.5)	24.3 (10.8; 47.3)	0.006
	AMA-1	0.12 (0.08; 0.17)	0.11 (0.09; 0.17)	0.00 (-0.03; 0.01)	0.35
	GLURP	1.23 (1.01; 2.39)	1.06 (0.90; 3.39)	-0.13 (-0.35; 0.14)	0.46
Growth inhibition (%)		-3.3 (-7.1;-1.1)	-8.6 (-10.0; -5.46)	-4.4 (-6.0; 1.8)	0.03

[#]*Antibody titers against CSP, AMA-1 and GLURP, and in vitro growth inhibitory activity of isolated IgG in all CPS-immunized subjects, before immunization (I-7) and on the day before challenge (C-1). Data are expressed as median (25;75 percentile). Differences between time points were tested using a paired t-test.*

The minimum therapeutic plasma chloroquine concentration is 30 µg/L (11) and its reported half-life varies from 5 to 58 days (11, 12). To ensure sufficient clearance of chloroquine in view of the very low blood-stage challenge dose, the challenge infection was conducted 17 weeks after the last chloroquine dose, corresponding to 21 weeks after the

last immunization. Group 1 (n=9) and group 3 (n=5) received a blood-stage challenge by i.v. administration of 3D7 asexual parasites. Group 2 (n=5) and 4 (n=5) were subjected to a sporozoite challenge using five mosquitoes infected with 3D7 sporozoites (13). There was no difference in parasitemia between CPS-immunized group 1 and control group 3; both groups became thick smear positive with a median prepatent period of 8.0 days (range 7.0–8.3 days and 8.0–8.3 days, respectively; p=0.83). Likewise, the prepatent period by qPCR was similar

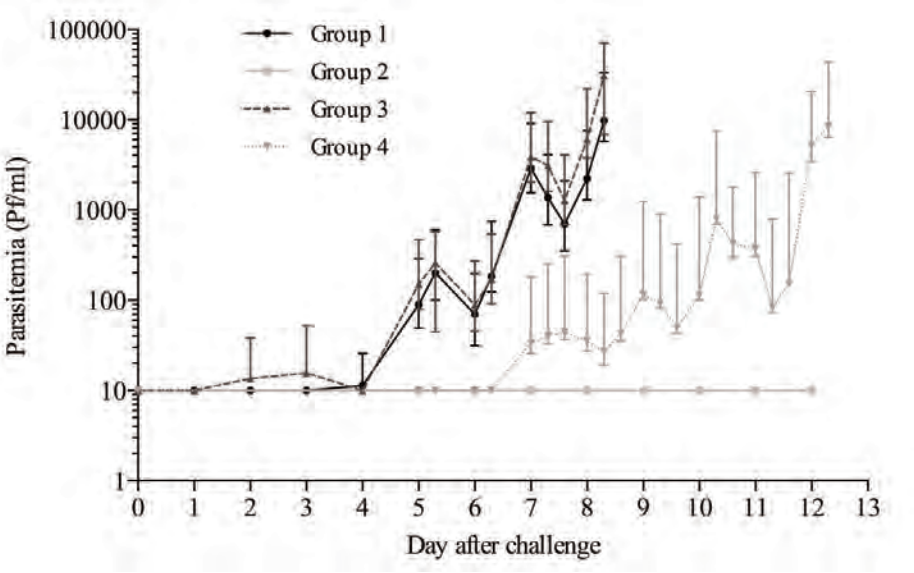

Figure 2 Parasitemia after challenge as assessed by qPCR. Geometric mean parasite density ±95% CI, from day of inoculation until the day of treatment after blood-stage challenge [black line, group 1, CPS-immunized (n=9); dashed dark grey line, group 3, controls (n=5))] or sporozoite challenge [light gray line, group 2, CPS-immunized (n=5); dashed light gray line, group 4, controls (n=5)]. Values shown as 10 on the logarithmic scale were negative.

in both groups (median 5.0 days; range 3.0–5.3 days and 2.0–6.3 days, respectively; p=0.41; Table 2). Furthermore, there was no statistically significant difference in multiplication rates of blood-stage parasites between the CPS-immunized subjects and naïve controls [median 8 (range 6–18) and 14 (range 7–24), respectively; p=0.19; Table 2 and Figure 2].

In group 2, challenged with sporozoites, four of five CPS-immunized subjects remained negative throughout the follow-up period by both thick smear and qPCR. One CPS-immunized subject, however, showed a positive qPCR at day 21 post challenge (457 parasites/mL; determined retrospectively). Controls in group 4 all became thick smear positive with a median prepatent period of 12.3 days (range 9.3–12.3; multiplication rate 10, range 5–23).

Parasitemia in group 4 (sporozoite-challenged controls) developed approximately four days later compared with group 3 (blood stage-challenged controls; day 5 vs. 9 by qPCR, p=0.01, and day 8 vs. 12.3 by thick smear, p=0.01; Table 2). The range in prepatent periods was significantly smaller in group 3 (8.0–8.3 days) than in group 4 (9.3–12.3 days). The parasite multiplication rate in the blood of control subjects was similar in those receiving either a blood-stage or sporozoite challenge (14 (7–24) vs. 10 (5–23) respectively; p 0.57).

Table 2 Protection against blood-stage versus sporozoite challenge by CPS immunization

	Challenge	Protection (%)	Prepatent period (days) median (range)		Blood-stage parasite multiplication rate median (range)
			Thick smear	PCR	
CPS	Sporozoite	5/5 (100)	N/A	N/A*	N/A
	Blood-stage	0/9 (0)	8.0 (7.0-8.3)	5.0 (3.0-5.3)	8 (6-18)
Control	Sporozoite	0/5 (0)	12.3 (9.3-12.3)	9.0 (7.0-10.0)	10 (5-23)
	Blood-stage	0/5 (0)	8.0 (8.0-8.3)	5.0 (2.0-6.3)	14 (7-24)

**One subject became PCR positive on day 21 after challenge.*

All 19 unprotected volunteers reported solicited AEs considered possibly or probably related to the challenge (mean number of AEs per subject, 6.4; mean duration: 1.4 ± 0.1 days), including headache, fever and nausea as the most common symptoms (Table S1). The peak of AEs occurred later in subjects who received a sporozoite challenge, concordant with the later onset of parasitemia, but there was no difference in accumulative duration of AEs compared with blood stage-challenged controls (Figure 3 A1 and A2 p=0.24). In contrast, protected subjects showed significantly fewer AEs: three of five experienced mild or moderate AEs (mean number/subject, 1.4; mean duration: 0.3 ± 0.1 days; p=0.002 compared with unprotected subjects; Figure 3A2).

Lymphocyte counts decreased after challenge in all unprotected subjects (Figure 4A), as did platelet counts (Figure 4B), with the exception of one volunteer. Platelet counts declined below the lower limit of normal (150 x 10^9/L) in 9 of 19 unprotected subjects (mean lowest value 132 ± 10 x 10^9/L). D-dimer concentrations were elevated in all thick smear-positive subjects (n=19; mean peak concentration 3,908 ± 650 ng/mL; Figure 4C). All abnormal laboratory values normalized without complications; bleeding or thrombotic complications were not detected in any of the subjects.

There was a remarkable difference in occurrence of fever and AEs between blood stage-challenged groups 1 and 3, although curves of developing parasitemia were identical (Figures 2 and 3). CPS-immunized subjects in group 1 developed fever at a significantly earlier time point than controls in group 3 (mean first day of temperature ≥37.5 °C, day 7.25

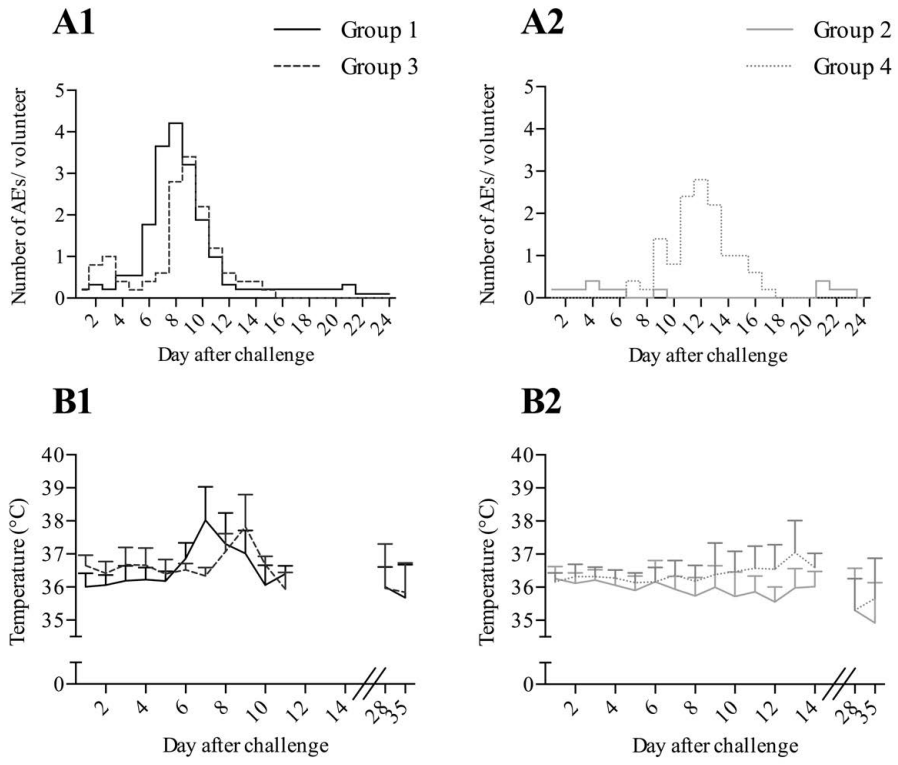

Figure 3 AEs and temperature after challenge. AEs and body temperature were recorded daily after challenge. Blood-stage challenge (A1) and sporozoite challenge (A2): mean number of possibly or probably related solicited AEs per subject. Blood-stage challenge (B1) and sporozoite challenge (B2): temperature (mean +SEM). Black line, group 1 (CPS-immunized, blood-stage challenge; n=9); dashed dark gray line, group 3 (controls, blood-stage challenge; n=5); light gray line, group 2 (CPS-immunized, sporozoite challenge; n=5); dashed light gray line, group 4 (controls, sporozoite challenge; n=5).

vs. day 8.5; p=0.002; Figure 3B1), concordant with an earlier mean decline in lymphocytes (Figure 4A; $p < 0.01$) and increase in D-dimer concentrations (mean first day of D-dimer >1,000 ng/mL: day 8.0 vs. day 9.0; p=0.05; Figure 4C).

We next investigated plasma concentrations of IFN-γ, a key mediator of cellular immunity in malaria (14), and monokine induced by IFN-γ (MIG), a downstream mediator in the IFN-γ pathway (15). Figure 4 D and E shows distinct increases of both IFN-γ and MIG plasma concentrations upon blood-stage challenge at 2-3 days earlier in group 1 compared with group 3 ($p < 0.001$).

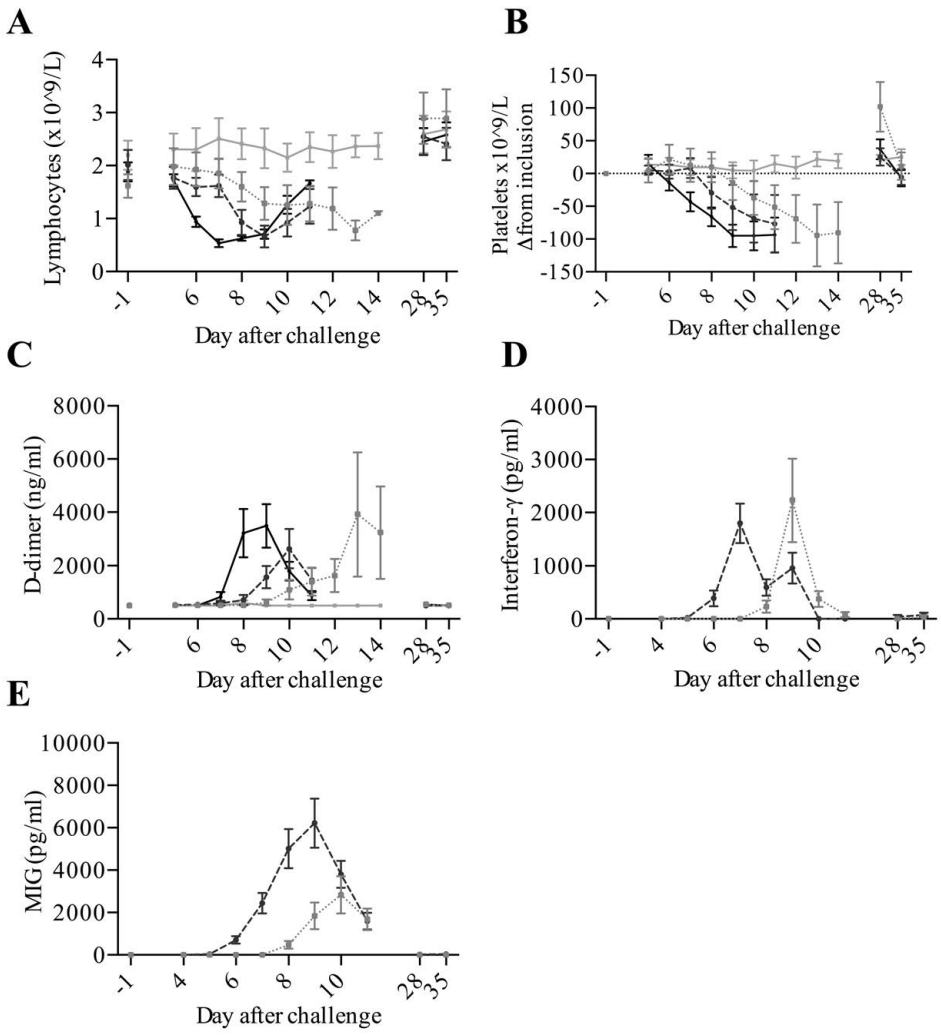

Figure 4 Hematological and inflammatory markers after challenge. (A) Peripheral lymphocyte counts (x10^9/L); (B) Platelet counts (difference from value at inclusion, x10^9/L); (C) D-dimer (ng/mL); (D) IFN-γ (pg/mL); (E) MIG (pg/mL). Black line, group 1 (CPS-immunized, blood-stage challenge; n=9); dashed dark gray line, group 3 (controls, blood-stage challenge; n=5); light gray line, group 2 (CPS-immunized, sporozoite challenge; n=5); dashed light gray line, group 4 (controls, sporozoite challenge; n=5). Data are shown as mean ± SEM.

Discussion

This study shows that sporozoite immunization by *P. falciparum*-infected mosquito bites of human subjects while taking chloroquine chemoprophylaxis (CPS-immunization) does not protect against an i.v. administered blood-stage challenge infection. The presence of transient low-level parasitemia during CPS-immunization is sufficient to induce immune recognition of asexual forms, as indicated by an earlier increase of IFN-γ and MIG after blood-stage challenge. However, these responses apparently are insufficient to confer any functional blood-stage immunity. In contrast, complete protection is obtained against a sporozoite challenge by mosquito bites as described before (3, 4).

The previously observed absence of detectable parasitemia in CPS-immunized subjects after mosquito challenge suggested predominance of preerythrocytic immunity, but asexual stage immunity might have contributed to protection (3). In the present study, however, the complete lack of any sign of clinical and/or parasitological protection against even an unphysiologically low blood-stage infection [<2,000 ring forms, i.e. 20 fold lower than an estimated average of 40,000 merozoites released from a single infected hepatocyte (16)], suggests the complete absence of any functional blood-stage immunity. This is supported by the lack of antibodies against blood-stage antigens after CPS-immunization in all but one volunteer, and the absence of *in vitro* growth-inhibitory activity of IgG isolated from CPS-immunized subjects. The single volunteer who developed detectable, although very low, AMA-1 and GLURP antibody levels was the only subject who experienced qPCR-detectable blood-stage parasitemia after all three immunizations. One immunized subject developed parasitemia on day 21 after sporozoite challenge, as retrospectively detected by qPCR. The blood-stage parasite multiplication rate after controlled human malaria infection in malaria-naïve subjects is 10.9 on average, but may be as low as 2 (17). With a multiplication rate of 2, a load of 457 parasites/mL on day 21 would be the result of an estimated 9,000 merozoites released from the liver (i.e., one infected hepatocyte). Given the total lack of *in vivo* protection from blood-stage challenge and *in vitro* growth-inhibitory activity of IgG in all immunized subjects, the delayed prepatency in this volunteer most likely was caused by either a profound reduction in liver-stage burden or a prolonged liver stage and therefore delayed release of merozoites into the blood.

Although clinical immunity and control of blood-stage parasitemia are acquired with repeated parasite exposure in endemic populations, the occurrence of sterile protection mediated by sporozoite/liver stages alone has not been confirmed (18). Hence, this study is a unique and unambiguous demonstration of induction of sterile preerythrocytic immunity generated against nonattenuated wild-type *P. falciparum* sporozoites. Sterile protection induced by immunization with irradiated sporozoites that arrest early after liver cell invasion also most likely is based on preerythrocytic immunity (5). In this situation, asexual forms never occur, and the apparent lack of blood-stage immunity was shown in the 1970s in a

single irradiated sporozoite-immunized volunteer challenged with blood-stage parasites (19).

Blood stage-challenged subjects in our study showed neither a delay in the prepatent period nor a reduction in asexual multiplication rate compared with naïve controls. This is remarkable because several studies have shown that protective immunity to *P. falciparum* blood stages can be induced readily after very few infections (10, 20): i) Adult patients treated with prolonged *P. falciparum* infections for neurosyphilis in the 1920s and 1930s showed clear evidence of clinical and parasitological immunity during a second asexual blood-stage infection by a decrease in frequency of fever and parasitemia (20), ii) Repeated administration of ±30 *P. falciparum*-infected erythrocytes followed by early treatment with atovaquone/proguanil induced protection against blood-stage challenge (10) in three of four subjects, although a potential effect of residual atovaquone blood levels could not be ruled out (21). A plausible explanation for the absence of blood-stage protection might be the short duration and low grade of parasitemia as a result of the use of chloroquine and hence insufficient exposure to blood-stage antigens. Induction of protective immunity against blood stages requires several cycles of parasite replication and sufficient duration of parasitemia (20, 22). Even in the trial by Pombo et al (10), in which subjects were immunized with unphysiologically low numbers of blood-stage parasites, treatment was initiated only after 8 or 14 days, allowing at least four replication cycles and therefore sufficiently long exposure to blood-stage antigens. This stands in contrast to the CPS-immunization protocol, in which prophylactic levels of chloroquine constantly are present, preventing a full blood-stage replication cycle of parasites to occur. Thus, although the occurrence of low parasitemia during CPS-immunization might benefit the induction of preerythrocytic immunity as a result of the expression of cross-stage antigens, it clearly is insufficient to induce a functional protective immune response against blood stages.

Notwithstanding the absence of protection against blood-stage challenge, we do find evidence for immune recognition of blood stages. Previously, we showed that *P. falciparum*-infected erythrocytes elicit release of IFN-γ, mainly from innate cells including natural killer and γδT cells (7, 23, 24). This innate response may be enhanced through and supplemented by adaptive memory T cells producing cytokines (25). In addition, effector memory T cells produce IL-2 and IFN-γ upon *in vitro* restimulation with *P. falciparum*-infected erythrocytes (7). In the present study, CPS-immunized subjects, while unprotected against a blood-stage challenge, showed an earlier *in vivo* peak of plasma IFN-γ in the course of blood-stage infection than naïve controls, despite identical kinetics of developing parasitemia. Early recognition of blood stages by memory cells in these immunized subjects apparently led to an accelerated and enhanced production of IFN-γ and further downstream mediators, including the chemokine MIG (15). MIG may have contributed to the observed earlier lymphocyte recruitment out of the peripheral circulation. The clinical and laboratory signs/symptoms in the unprotected CPS-immunized and blood stage-challenged volunteers (group 1) most

likely represent a shift of inflammatory responses common to malaria (26) to earlier time points compared with the challenged controls. Thus, immune recognition represented by these markers took place at an earlier time point in CPS-immunized individuals compared with naïve volunteers, suggesting the presence of memory responses to asexual blood stages despite the absence of protection.

Mechanisms and target antigens for protective immunity induced by CPS-immunization remain to be unraveled. Although mainly antibodies are important in controlling blood-stage parasitemia (27), rodent and primate studies indicate that $CD8^+$ T-cell responses against parasite liver stages are critically involved in preerythrocytic immunity (28-30). Therefore, detailed analysis of T-cell responses will be the subject of future studies.

Furthermore, this efficient immunization model will enable studies of antigen specificity of cellular and humoral immune responses for identification of potential new antigens or combinations thereof for subunit vaccine candidates. Malaria vaccine development to date has been stage oriented, aimed at targeting either the preerythrocytic or asexual blood stage of the parasite. Vaccines against asexual blood-stage antigens likely will not prevent infection, but instead may reduce parasite densities and provide protection against clinical disease. Preerythrocytic immunization strategies such as CPS-immunization, however, induce sterile protection, thereby preventing blood-stage infection (31).

In conclusion, sporozoite immunization by the CPS protocol may induce sterile protection entirely mediated by immune responses against the preerythrocytic stages of *P. falciparum*. These findings support a continued focus on vaccine development toward preerythrocytic stages, particularly whole-sporozoite approaches.

Materials and Methods

Study Design

We conducted this single-center, open-label study at the Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands) from April 2011 until March 2012 following approval by the Central Committee for Research Involving Human Subjects of The Netherlands (CCMO NL34273.091.10). The study team complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. The trial is registered at ClinicalTrials.gov (NCT01236612). Written informed consent of all volunteers was obtained before screening.

Twenty-five healthy subjects (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 (SI Materials and Methods, Screening of Study Subjects) and randomly assigned to four groups (groups 1, 2, 3 and 4; Figure S1). Fifteen subjects received chemoprophylaxis and sporozoites (CPS)

immunization (groups 1 and 2) as described in detail in SI Materials and Methods. Ten controls (groups 3 and 4) received only chloroquine chemoprophylaxis.

Seventeen weeks after discontinuation of chloroquine prophylaxis, corresponding to 21 weeks after the last immunization, all subjects received a challenge infection. Group 1 (n=9; one lost to follow-up) and control group 3 (n=5) were challenged by i.v. administration of 1,962 viable 3D7 *P. falciparum*-infected erythrocytes (blood-stage challenge), which were derived from a stock produced at the Queensland Institute of Medical Research as described previously (32) and used in numerous studies (10, 32-36). Group 2 (n=5) and group 4 (n=5) were exposed to the bites of five 3D7 *P. falciparum*-infected *Anopheles stephensi* mosquitoes (sporozoite challenge). Subjects and investigators were aware of the study group, whereas primary outcome assessors were kept blinded to the allocation. All volunteers were treated with a curative regimen of antimalarial drugs at time of thick smear positivity, or presumptively on day 21 after challenge if thick smears remained negative.

Study Outcomes

The primary study outcome was time to parasitemia after challenge, as assessed by microscopy (SI Materials and Methods). The prepatent period was defined as the period between challenge and the first positive thick smear. Volunteers were defined as protected from challenge if they remained thick smear negative until day 21. Additionally, parasitemia was measured retrospectively by real-time quantitative PCR (qPCR) (37). Blood-stage parasite multiplication rate was calculated as described previously (17). Assessment of *in vitro* growth inhibition and measurements of antibodies, hematological parameters, MIG and IFN-γ are described in detail in SI Materials and Methods.

Statistical Methods

Statistical analysis was performed using GraphPad Prism 5. The difference in adverse events (AEs) among groups was calculated by unpaired Student *t* test on the accumulative duration of AEs. Differences among groups on the first day of fever (≥37.5°C), first day of D-dimer increase above two times the upper limit of normal (≥1,000 ng/mL), and first day of detectable IFN-γ and MIG were tested by unpaired Student *t* test. Differences among groups in prepatent periods by thick smear and qPCR and blood-stage parasite multiplication rates were tested by the Mann–Whitney test. Differences in antibody-levels and *in vitro* growth inhibition between time points were tested by paired *t* test.

Analysis of lymphocyte kinetics after challenge was performed with SPSS version 18 and based on data obtained at days 5, 6, and 7 (pretreatment). Two regression-type models were fitted to the data. The dependent variable was lymphocyte number, and independent variables were time, treatment, the interaction between time and treatment, and the baseline observation of the dependent variable. The longitudinal character of the data

was accommodated using general least-squares estimation; a heterogeneous, unstructured covariance matrix was assumed.

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Supplementary information Materials and Methods

Screening of study subjects

Forty-two subjects without a history of malaria or residence in a malaria-endemic area in the 6 months prior to study entry were screened for eligibility based on medical and family history, physical examination and general haematological and biochemical tests. Serologic analyses for HIV, hepatitis B, hepatitis C and *P. falciparum* were negative in all subjects. Urine toxicology screening was negative and none of the volunteers was pregnant or lactating. All subjects had an estimated 10-year risk of developing a cardiac event smaller than 5% as estimated by the systematic coronary evaluation system (38).

Preparation of P. falciparum-infected mosquitoes for CPS-immunization

A. stephensi mosquitoes were reared at our insectary and infected by allowing them to feed on cultured gametocytes of *P. falciparum* parasites according to standard procedures as described previously (39). We intended to immunize the subjects with 3D7 parasites only, since this *P. falciparum* strain was to be used for the challenge infections. Initial stocks of 3D7 parasites for immunization and challenge were kindly provided by Adrian Hill, Oxford. However, due to difficulties in culturing 3D7 parasites during the immunization period, we immunized each volunteer with seven 3D7-infected plus eight NF54-infected mosquito bites per immunization instead. The 3D7-strain is a clone of NF54 and both are sensitive to chloroquine. The percentage infected mosquitoes of the batches used for immunization 1, 2 and 3 was 75, 85 and 90 for 3D7 and 95, 90 and 100 for NF54 respectively. Mosquitoes were infected with an average of 5.042 and 109.000 sporozoites for 3D7 and NF54 respectively.

Chemoprophylaxis and sporozoites (CPS) immunization procedure

Chloroquine was administered to all subjects according to a standard prophylactic regimen for a period of 14 weeks (98 days) as described previously by Roestenberg et al (3). While receiving chloroquine, Groups 1+2 (15 subjects) were immunized three times at monthly intervals, starting eight days after the first chloroquine dose. Immunization was performed by exposure to the bites of exactly 15 *P. falciparum*-infected mosquitoes for 15 minutes, twice

briefly interrupted. Following each feeding session, the salivary glands of all blood-engorged mosquitoes were dissected to confirm the presence of sporozoites. All ten control subjects (Groups 3+4) received chloroquine prophylaxis only, but no mosquito bites.

On days 6 to 10 after each immunization we checked all 15 immunized subjects once daily at our outpatient clinical research department. Blood was drawn for thick blood smears, standard haematological markers and retrospective assessment of blood-stage parasitemia by quantitative real-time real-time quantitative PCR (qPCR) using *P. falciparum* standard curves prepared by DNA extraction from titrated samples of ring-infected cells (qPCR, (37)). All signs and symptoms were recorded by the attending physician as mild (grade 1, easily tolerated), moderate (grade 2, interferes with normal activity), or severe (grade 3, prevents normal activity), or in case of fever grade 1 (>37·5–38·0°C), grade 2 (>38·0–39·0°C), or grade 3 (>39·0°C). For safety reasons related to a previously reported cardiac event (40), we measured cardiovascular markers throughout the trial (highly sensitive troponin, platelets, d-dimer, and lactate dehydrogenase). Whenever abnormal, blood samples were checked for the presence of fragmentocytes and von Willebrand-cleaving protease activity, according to previous protocols (4).

Challenge infections

Seventeen weeks after discontinuation of chloroquine prophylaxis, corresponding to 21 weeks after the last immunization, all subjects received a challenge infection. Group 1 (N=9, one lost to follow-up) and control Group 3 (N=5) subjects were challenged by intravenous administration of 3D7 *P. falciparum-infected* erythrocytes (blood-stage challenge), while Group 2 (N=5) and Group 4 (N=5) were exposed to the bites of five 3D7 *P. falciparum*-infected *Anopheles stephensi* mosquitoes (sporozoite challenge).

Subjects were checked daily on an outpatient basis for symptoms and signs of malaria, thick blood smears, hematologic tests and cardiovascular markers. Subjects who received a blood-stage challenge were checked from the first day after challenge onwards, sporozoite-challenged subjects from day 5 onwards. All signs and symptoms (solicited and unsolicited) were recorded as described for the CPS-immunization. At time of thick smear positivity, each subject was treated with a curative regimen of 1000 mg atovaquone and 400 mg proguanil once daily for three days according to Dutch national guidelines. Subjects who remained thick smear negative until day 21 after challenge were presumptively treated with the same curative regimen. Complete cure was confirmed by two consecutive parasite-negative blood smears after treatment.

Blood-stage inoculum

Inocula for blood-stage challenge were derived from a stock of 3D7 *P. falciparum*-infected erythrocytes (blood group O, rhesus negative) produced at the Queensland Institute of Medical

Research as described previously (32). The donor was seronegative or PCR negative, or both, for a panel of parenterally transmissible viruses at the time of donation and one year later, but was IgG positive for EBV and CMV (32). The blood-stage inoculum was cryopreserved in Glycerolyte 57 and stored in liquid nitrogen under temperature-monitored conditions.

To prepare the inocula for intravenous administration, an aliquot of the seed stock was thawed and washed under sterile conditions as described previously (33) using solutions licensed for clinical use and sterile, single-use consumables. Bacterial culture plates of the hood and both aerobic and anaerobic blood culture of the inoculum did not show any bacterial growth. The inoculum was prepared by diluting to the appropriate dose and dispensed aseptically into 5mL syringes for administration. Based on microscopic estimates of the donor's parasite density prior to freezing of blood samples, each inoculum contained 4,289 infected erythrocytes. The inocula were kept on ice during preparation. Alternating between CPS-immunized and control subjects, all volunteers were inoculated intravenously between 103 and 111 minutes after thawing. The number of viable parasites in the inoculum was verified in retrospect by limiting dilution culture and qPCR detection as previously described (33, 37) and the inoculum was calculated to contain 1.962/4,289 (46%) viable/total parasites per subject. This recovery of viable parasites was in the range of what has been reported for the inoculum elsewhere (10, 33, 36).

Sporozoite challenge

A. stephensi mosquitoes fed on cultured gametocytes of *P. falciparum* strain 3D7 were 100% infected with an average of 100.000 sporozoites per mosquito. A total of 10 volunteers from Group 2 (N=5) and Group 4 (N=5) were exposed to bites of five infected *A. stephensi* mosquitoes for ten minutes as described previously (3). One feeding session was sufficient for 5 volunteers, while a second session was required in the remaining 5 volunteers in order to obtain an infectious challenge by exactly five infected mosquitoes in all 10 subjects.

Study outcomes

The primary study outcome was time to parasitemia after challenge, as assessed by microscopy. Sampling started on a daily basis for Group 1+3 (blood-stage challenge) on days 1-4 and continued for all groups post-challenge twice daily on days 5 and 6, thrice daily on days 7-11, again twice daily on days 12-15, and finally once daily on days 16-21. Thick blood smears were made from 15µL of EDTA-anti-coagulated blood, spread over the standardised surface of one well of a three-well glass slide according to harmonized standard protocol for Controlled Human Malaria Infections *(Laurens MB, Roestenberg M, and Moorthy VS; manuscript in preparation).* After drying, wells were stained with Giemsa for 30 minutes. Slides were read at 1000-times magnification by assessing 200 high-power fields, equal to about 0.5µL of blood. The smear was deemed positive if two unambiguously

identifiable parasites were found. Lymphocyte and platelet counts were determined in EDTA anti-coagulated blood with the Sysmex XE-5000. D-dimer concentrations were assessed in citrate plasma by STA-R (Roche, Almere, The Netherlands).

Immunological measurements

Concentrations of malaria antigen-specific antibodies were determined in serially diluted citrate plasma by standardized enzyme-linked immunosorbent assay in NUNC™ Maxisorp plates (Thermo Scientific) coated with 1 µg/ml full-length CSP, AMA-1 (FVO-allele, (41) or GLURP (42) diluted in PBS. ELISAs were developed using biotinylated polyclonal goat anti-human IgG (1/2500), streptavidin-conjugated horseradish peroxidase (1/2000) and Tetramethylbenzidine (all Mabtech). Spectrophotometrical absorbance was measured at 450 nm. Levels of antibody reactivity were analyzed in relation to a pool of sera from adults living in a highly endemic area in Tanzania (3), which was defined to contain 100 arbitrary units (AU) of IgG directed against an antigen. Antibody responses were considered positive when they were increased at least two-fold after immunization, compared to baseline.

Antibodies for growth inhibition assay (GIA) were isolated from citrate plasma that was collected the day before the start of chloroquine prophylaxis and the day before challenge infection. IgG was purified using protein G columns (HiTrapTM Protein G HP, GE Healthcare). Eluted samples were exchanged into RPMI 1640 on a Vivaspin 20, 30kDa molecular mass cutoff ultra-filtration unit (Sartorius Stedim, Germany), concentrated to 20mg/ml, filter-sterilised and stored at -20°C until used. IgG protein concentrations were measured using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The GIA was performed as previously described (41). Briefly, the effect of IgG on *in vitro* parasite growth was evaluated at an IgG concentration of 10 mg/mL. Samples were run in triplicate using alanine-synchronized cultures of *P. falciparum* 3D7 schizonts at an initial parasitaemia of 0.7%. After 40 to 42 hours, parasite growth was assessed by measuring parasite lactate dehydrogenase levels in culture supernatants with the lactate diaphorase APAD substrate system. EDTA (4 mM) was included in every test plate as positive control. Induction of growth inhibitory activity by CPS-immunization was assessed by subtracting for each volunteer the pre-immunization percentage of growth inhibition from the post-immunization value.

Monokine-induced-by-gamma (MIG, CXCL9) and interferon-γ (IFNγ) concentrations in frozen and stored EDTA anti-coagulated plasma samples were determined retrospectively. MIG was measured using a DuoSet® ELISA (R&D systems) and IFNγ was measured using a Ready-SET-Go!® ELISA (eBioscience) according to the manufacturer's recommendations.

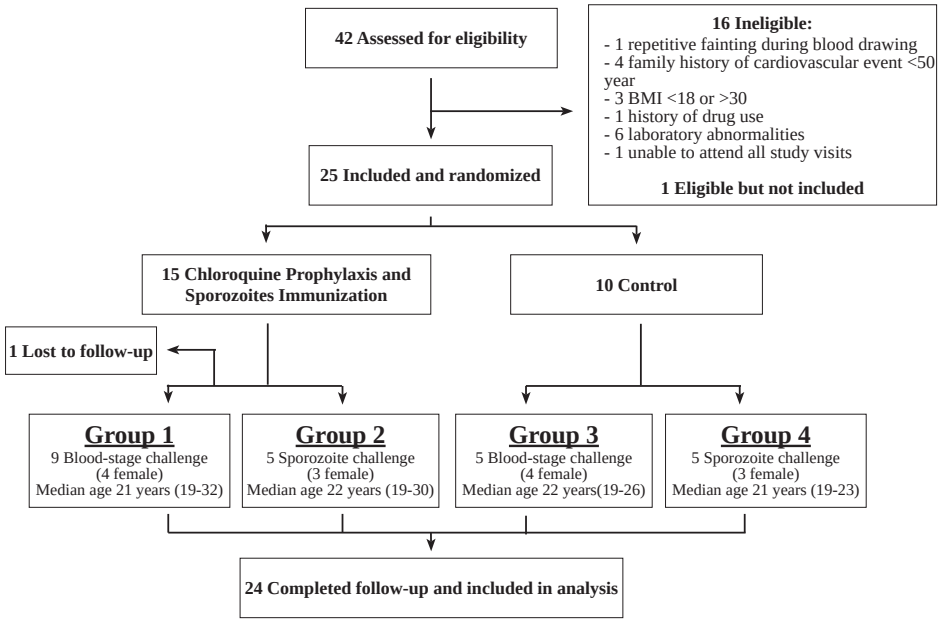

Figure S1 Study flow diagram with relevant baseline data. Fifteen subjects were CPS-immunized by the bites of three times fifteen *P. falciparum*-infected mosquitoes whilst taking chloroquine prophylaxis (Group 1 and 2). Ten control subjects received chloroquine prophylaxis, but no infected mosquito-bites (Group 3 and 4). One subject withdrew informed consent after the immunization for reasons unrelated to the trial. Seventeen weeks after discontinuation of chloroquine prophylaxis nine immunized (Group 1) and five control volunteers (Group 3) received a blood-stage challenge, while five immunized (Group 2) and five control volunteers (Group 4) received a sporozoite challenge by mosquito bites.

Table S1 Possibly or probably related solicited adverse events after challenge infection

	BLOOD STAGE CHALLENGE				SPOROZOITE CHALLENGE			
	CPS-immunized (n=9)		Control (n=5)		CPS-immunized (n=5)		Control (n=5)	
Adverse event	No. of volunteers	Mean duration ± SD (days)	No. of volunteers	Mean duration ± SD (days)	No. of volunteers	Mean duration ± SD (days)	No. of volunteers	Mean duration ± SD (days)
Any								
Abdominal pain	3	1.5 ± 0.4	2	0.6 ± 0.3	0	N/A	1	0.0
Arthralgia	1	1.6	0	N/A	0	N/A	0	N/A
Chills	2	0.8 ± 0.0	0	N/A	0	N/A	1	0.0
Fatigue	3	2.2 ± 4.3	2	2.8 ± 0.3	0	N/A	2	1.9 ± 2.5
Fever	8	1.4 ± 0.6	5	1.6 ± 1.0	1	0.3	4	0.8 ± 0.3
Headache	8	1.6 ± 0.9	5	1.4 ± 1.2	2	1.4 ± 2.2	5	1.4 ± 1.1
Malaise	3	2.3 ± 1.4	0	N/A	0	N/A	2	1.5 ± 0.7
Myalgia	6	1.6 ± 0.9	3	2.9 ± 2.7	0	N/A	1	1.5
Nausea	8	0.9 ± 0.6	4	1.8 ± 1.5	2	0.4 ± 0.6	4	1.1 ± 1.5
Vomiting	4	0.9 ± 0.8	1	1.4	0	N/A	1	0.0
Any	9	1.6 ± 1.5	5	1.7 ± 0.8	3	0.8 ± 1.4	5	1.0 ± 0.8
Grade 3								
Abdominal pain	1	1.8	0	N/A	0	N/A	0	N/A
Arthralgia	0	N/A	0	N/A	0	N/A	0	N/A
Chills	0	N/A	0	N/A	0	N/A	0	N/A
Fatigue	0	N/A	1	3.0	0	N/A	1	3.7
Fever	4	1.3 ± 0.7	0	N/A	0	N/A	2	0.9 ± 0.4
Headache	1	1.0	2	1.2 ± 1.0	0	N/A	1	1.8
Malaise	3	2.3 ± 1.7	0	N/A	0	N/A	1	2.0
Myalgia	0	N/A	0	N/A	0	N/A	0	N/A
Nausea	6	0.9 ± 0.5	1	0.8	0	N/A	1	0.0
Vomiting	4	0.9 ± 0.8	1	1.4	0	N/A	1	0.0
Any	8	1.2 ± 0.9	3	1.5 ± 1.0	0	N/A	3	1.3 ± 1.3

N/A, not applicable

CHAPTER 7

Cytotoxic markers associate with protection against malaria in human volunteers immunized with Plasmodium falciparum sporozoites

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Abstract

Background

Immunization of healthy volunteers under chloroquine chemoprophylaxis by bites from *Plasmodium falciparum (Pf)*-infected mosquitoes (CPS immunization) induces sterile protection against malaria. CPS-induced protection is mediated by immunity against pre-erythrocytic stages, presumably at least partially by cytotoxic cellular responses. We therefore aimed to investigate the association of CPS-induced cytotoxic T cell markers with protection.

Methods

In a double-blind randomized controlled trial (NCT01218893), we performed dose titration of CPS immunization followed by homologous challenge infection in 29 subjects. Immune responses were assessed by *in vitro* restimulation of PBMCs and flow cytometry.

Results

Dose-dependent complete protection was obtained in 4/5 volunteers after immunization with bites from a total of 45, in 8/9 volunteers with 30, and in 5/10 volunteers with 15 *Pf*-infected mosquitoes respectively (OR=5.0; 95% CI 1.5-17). Proportions of CD4 T cells expressing the degranulation marker CD107a and CD8 cells producing granzyme B after *Pf*-restimulation were significantly higher in completely protected subjects (OR=8.4; 95% CI 1.5-123; p=0.011 and OR=11; 95% CI 1.9-212; p=0.004 respectively).

Conclusions

These data underline the efficiency of CPS immunization to induce sterile protection, and support a possible role for cytotoxic CD4 and CD8 T cell responses in pre-erythrocytic immunity.

Introduction

Malaria remains a major public health problem, with an estimated incidence of 207 million clinical cases leading to approximately 627,000 deaths every year (1). *Plasmodium falciparum (Pf)* is the most severe and lethal of five species that can cause malaria in humans. Availability of an effective vaccine will be critical to fight this disease, but currently there is no licensed vaccine available, despite decades of research. Most efforts have focused on the development of subunit vaccines, unfortunately showing only limited protective efficacy (2, 3). Immunization strategies based on whole parasites, however, have repeatedly induced high levels of protection in experimental settings (4-7). Previously we showed that immunization of healthy, malaria-naive subjects, while taking chloroquine chemoprophylaxis, with live sporozoites delivered by 36-45 mosquito bites (ChemoProphylaxis and Sporozoites (CPS) immunization) induces robust, long-lasting sterile protection against *Pf* malaria (8, 9). CPS immunization is about 20 times more efficient than the only alternative approach for complete sterile protection against malaria in humans i.e. immunization with radiation-attenuated *Pf* sporozoites (RAS), requiring bites from >1000 infected and irradiated mosquitoes (4), or intravenous administration of 675,000 sporozoites (10).

CPS-induced protective immunity targets the earliest stages of the parasite lifecycle, i.e. sporozoites and/or liver stages, rather than the subsequently developing asexual blood stages (11). The immune pathways responsible for this pre-erythrocytic protection, however, remain unknown. In murine malaria models, cytotoxic killing of *Plasmodium*-infected hepatocytes appears to play a role in protection, but the exact contribution and mechanism of cytotoxicity remain elusive (12, 13). Also in humans, a role for both cytotoxic CD4 T cells and CD8 T cells has been suggested, but evidence is scarce and largely circumstantial (reviewed by Tsuji et al. (14)). We conducted a double-blind randomized controlled CPS immunization dose titration and challenge study. Subjects, while taking chloroquine prophylaxis, were immunized by bites from a total of 45 (3x15), 30 (3x10) or 15 (3x5) infected mosquitoes followed by a challenge infection, resulting in dose-dependent protection. Next, we explored markers of cytotoxic T cell responses induced by CPS immunization and identified two cytotoxic markers associated with protection.

Materials and Methods

Human ethics statement

All subjects provided written informed consent before screening. The study was approved by the Central Committee for Research Involving Human Subjects of The Netherlands (NL33904.091.10) and complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. ClinicalTrials.gov Identifier: NCT01218893.

Clinical trial design and procedures

A single centre, double-blind study was conducted at the Leiden University Medical Center from April 2011 until April 2012. Healthy subjects between 18 and 35 years of age with no history of malaria were screened as described previously (11). Thirty subjects were randomly divided into four groups using a computer-generated random-number table. Subjects, investigators and primary outcome assessors were blinded to the allocation. All subjects received CPS immunization as described previously (8, 11), but the number of NF54 *Pf* infected versus uninfected mosquitoes varied per group: five subjects received three times bites from 15 infected mosquitoes (Group 1), ten subjects received three times bites from 10 infected and 5 uninfected mosquitoes (Group 2), ten subjects received three times bites from 5 infected and 10 uninfected mosquitoes (Group 3) and five control subjects received three times bites from 15 uninfected mosquitoes (Group 4). Nineteen weeks after the last immunization (fifteen weeks after the last chloroquine dose), all subjects were challenged by the bites of five mosquitoes infected with the homologous NF54 *Pf* strain, according to previous protocols (8, 15). The primary outcome was prepatent period, defined as the time between challenge and first positive thick blood smear. Thick blood smears were prepared and read as described previously (11). For more details about the immunization and challenge procedures and follow-up, see supplementary information.

Immunological methods

Peripheral blood mononuclear cells (PBMCs) were collected on the following time points: before initiation of chloroquine prophylaxis (baseline; B), 27 days after each immunization; I1, I2 and I3 (I1 and I2 are one day before the second and third immunization respectively), the day before and twenty weeks after the challenge infection (C-1 and C+140). For the assessment of *Pf* specific immune responses, PBMCs were restimulated *in vitro* with *Pf* infected red blood cells (*Pf*RBC) as described before (16). Expression of the degranulation marker CD107a, the cytotoxic molecule granzyme B and the cytokine IFNγ by CD4, CD8 and γδ T cells was assessed by flow cytometry. For a detailed description, see supplementary information.

Statistical analysis

The dose-dependent induction of protection was tested by logistic regression using SPSS 20. Comparison of CD107a expression and granzyme B and IFNγ production by T cell subsets between immunized unprotected and protected volunteers after CPS immunization was done per selected cellular response by means of Firth's penalized logistic regression (17, 18), resulting in p-values, odds ratios (OR) related to a change of one interquartile range, and 95% profile likelihood Confidence Intervals (95% CI) for the OR, using R software version 3.0.1 (19), with R packages logistf version 1.21 (20), rms version 4.1-3 (21) and penalized version

0.9-42 (22, 23). The ability of (a combination of) markers to discriminate between protected and unprotected volunteers was assessed with the Area under the Receiver Operator Curve (ROC), based on leave-one-out cross-validation (LOOCV), using the R-software and pROC package version 1.7.1 (24). For further details, see supplementary information.

Results

CPS immunization

Thirty volunteers were included (median age 21 years, range 19–31), out of sixty-three subjects screened for eligibility (Figure S1). Volunteers were randomly assigned to four groups and received CPS immunization by bites from 3x15 (Group 1, n=5), 3x10 (Group 2, n=10) or 3x5 (Group 3, n=10) mosquitoes infected with strain NF54 sporozoites. Control subjects (Group 4, n=5) received chloroquine prophylaxis and bites from 3x15 uninfected mosquitoes. After each consecutive immunization the number of subjects with parasitemia, as retrospectively detected by qPCR, steadily decreased in Group 1 and 2. In Group 3, however, five volunteers still showed parasitemia after the second and third immunization (Figure 1). Remarkably, in four immunized subjects, parasitemia was never detectable by qPCR at any time point (three subjects in Group 2, one in Group 3). One subject from Group 2 withdrew consent after the first immunization for reasons unrelated to the trial, and was excluded from the analysis.

Challenge infection

Nineteen weeks after the last immunization, volunteers were challenged by standard exposure to bites from five homologous strain NF54-infected mosquitoes (5).

Table 1 Protection against challenge infection after CPS immunization.

Group (# of *Pf*-infected mosquitoes used for immunization)	**Protection**		**Day of positivity after challenge (TS+ subjects)[c]**	
	Number[a]	**Percentage[b]**	**Thick smear**	**qPCR**
Group 1 (3x15)	4/5	80 (36.0 – 98.0)	12.0	9.5
Group 2 (3x10)	8/9	89 (54.3 – >99.9)	12.0	8.5
Group 3 (3x5)	5/10	50 (23.7 – 76.3)	11.0 (9.0-15.0)	9.0 (6.5-13.0)
Group 4 (Control)	0/5	0 (0.0 – 48.9)	9.5 (9.0-13.5)	6.5 (6.5-10.5)

[a] Presented as protected/total number of subjects

[b] Presented as % protected (95% CI by modified Wald Method)

[c] Presented as median (min-max).

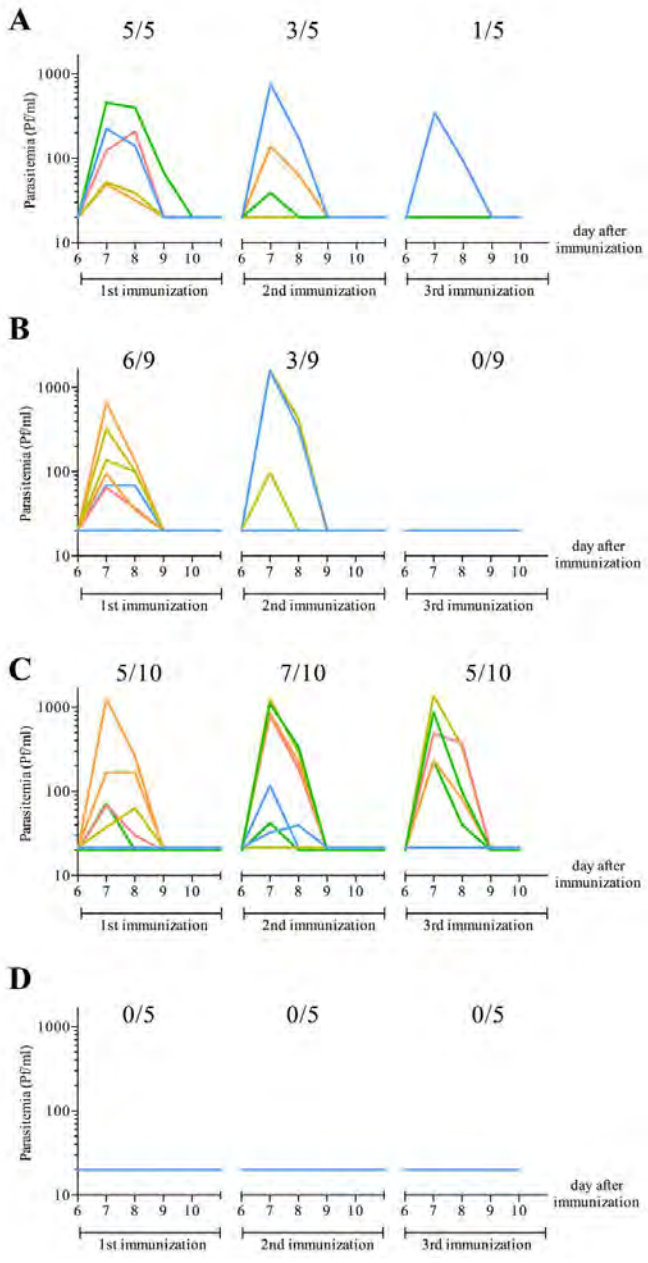

Figure 1 Parasitemia after the first, second and third CPS immunization. Parasitemia was determined once daily by qPCR from day 6 until day 10 after each immunization. Each line represents an individual subject. Panels show data for volunteers from (A) Group 1 (3x15), (B) Group 2 (3x10), (C) Group 3 (3x5) and (D) Group 4 (controls). Values shown as 10 on the log-scale were negative (i.e. half the detection limit of the qPCR: 20 parasites/ml). The number of subjects with a positive qPCR/ total number of volunteers after each immunization are shown below the graphs.

Protection by CPS immunization was dose-dependently induced in four out of five subjects in Group 1, eight out of nine subjects in Group 2 and five out of ten subjects in Group 3, while

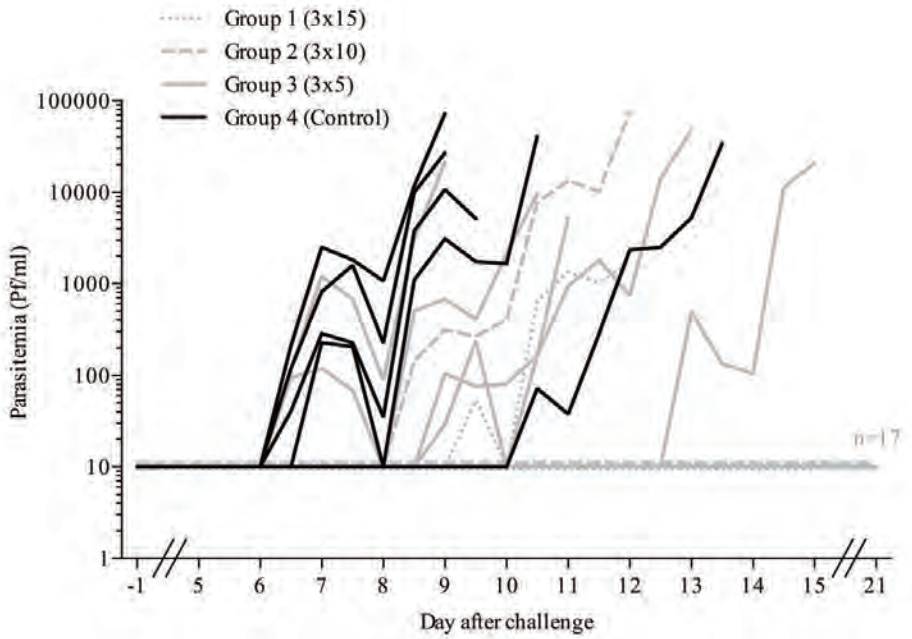

Figure 2 Parasitemia after challenge infection. Parasitemia was assessed retrospectively by real-time quantitative PCR (qPCR) from day 5 after challenge onwards, up until day 21, at two time points per day for TS+ volunteers, and one time point per day for protected volunteers. Each line represents an individual subject. Grey dotted lines show CPS-immunized volunteers from group 1 (3x15; n=5), grey dashed lines subjects from group 2 (3x10; n=9), grey solid lines subjects from group 3 (3x5; n=10) and black lines represent malaria-naive control subjects (n=5). Values shown as 10 on the log-scale were negative. The two TS+ subjects from Group 1 and 2 became qPCR positive on day 8.5 and 9.5 respectively, both became thick smear positive on day 12.0.

all control subjects became thick smear positive (OR=5.0; 95% CI 1.5-17; p=0.01).

The median prepatent period was 2.5 days longer in CPS-immunized unprotected subjects compared to controls, both by thick smear and qPCR. Although not statistically significant (p=0.22 and 0.31 respectively), this delay is suggestive for the presence of partial protection at least in some of the unprotected CPS-immunized subjects (Figure 2 and Table 1). In retrospect, all six volunteers with detectable parasitemia by qPCR after the third immunization were not completely protected from challenge infection, while 17 out of 18 subjects with a negative qPCR after the third immunization were fully protected.

Platelets decreased below reference value ($150x10^9$/L) in eight out of twelve thick smear positive (TS+; i.e. both controls and CPS-unprotected) subjects at any point after challenge (median for all TS+: $134x10^9$/L, range 79 - $213x10^9$/L). D-dimer was elevated in all TS+ subjects after challenge (median peak concentration 2431 ng/mL, range 1014-5000

ng/ml). Parameters normalized in all subjects after treatment without complications. All TS+ subjects experienced solicited adverse events (AEs) during challenge infection consistent with uncomplicated malaria (median number of AEs per subject 9.5 (range 4-14), median duration of each AE 1.1 days (range 0.0-12.3)). As expected, protected subjects presented with less AEs: 15 out of 17 subjects experienced solicited AEs possibly or probably related to the challenge (median number of AEs per subject: 2 (range 0-15), median duration 0.7 days (range 0.00-15.9)). One subject from Group 2 was preliminarily treated with atovaquone/proguanil at day 10.5 after challenge because of unrelated exertional rhabdomyolysis after extensive sports activity (weightlifting) followed by sauna visits. No other severe adverse events (SAE) occurred. One volunteer from Group 1 was treated for reasons unrelated to the trial at day 19. Both these volunteers remained parasite negative by qPCR analysis after the third immunization and at any time point after challenge and were considered protected in further analysis.

Analysis of cytotoxic T cell markers after in vitro Pf-stimulation

Next, we tested a panel of representative cytotoxic T cell markers including surface expression of degranulation marker CD107a, and granzyme B and IFNγ production in CD4, CD8 and γδ-T cells after *in vitro* restimulation with *Pf*-infected red blood cells (*Pf*RBC) in all immunized subjects (Table 2). CPS-immunization induced a significant increase in both the percentage and iMFI of CD107a positive CD4 and γδ-T cells, already after the first immunization up until challenge. Similarly CD8 T cells expressed a significantly higher CD107a iMFI after the second immunization. The proportion of granzyme B positive cells did not change after immunization, but granzyme B iMFI was significantly increased in both CD8 and γδ-T cells, returning to baseline at C-1. Production of IFNγ was induced in all T cell subsets, but most pronouncedly in CD4 and γδ-T cells. There were only weak correlations between cellular responses on C-1 and total blood-stage parasite exposure, as calculated by the sum of parasites/ml after all three immunizations (data not shown, Spearman's rho for all <0.5). None of the responses in the control group changed significantly from baseline at any point of time (Table 2), suggesting that chloroquine alone did not affect *P. falciparum* specific T cell responses.

We next assessed the association of these markers with protection after challenge (Figure 3). Indeed, complete protection associated with the proportion of CD107a positive CD4 T cells (OR=8.4; 95% CI 1.5-123; p=0.011, Figure 3A), the iMFI of CD107a on CD4 T cells (OR=11; 95% CI 1.6-188; p=0.011, data not shown) and granzyme B by CD8 T cells (OR=11; 95% CI 1.9-212; p=0.004, Figure 3E) at C-1. A subgroup analysis of data from Group 3 only confirmed these findings: the proportion of both CD107a positive CD4 T cells and granzyme B positive CD8 T cells were the only markers higher in protected subjects (OR=4.2; 95% CI 0.9-140; p=0.081 and OR=27; 95% CI 1.5-27687; p= 0.019

Table 2 Cytotoxic T-Cell markers, relative to immunization time, for 24 volunteers who received CPS immunizations and 5 controls

	CD4 T cells					CD8 T cells					γδ T cells				
Study group, marker[a]	**B**	**I1**	**I2**	**I3**	**C-1**	**B**	**I1**	**I2**	**I3**	**C-1**	**B**	**I1**	**I2**	**I3**	**C-1**
CPS immunized															
CD107a															
Expression, % , mean	0.26	0.53 ***	0.64 ***	0.59 ***	0.53 ***	0.08	0.09	0.15	0.14	0.19	26.6	36.5 ***	41.2 ***	40.3 ***	33.8 **
iMFI	15.7	38.5 ***	46.0 ***	41.0 ***	37.0 ***	7.6	14.5	19.2 *	17.5 *	20.9 **	4472	6925 ***	7925 ***	7594 ***	6262 **
Granzyme B															
Expression, % , mean	0.25	0.49	0.41	0.38	0.43	0.50	0.89	0.92	1.03	0.94	6.06	7.35	8.27	8.96	8.79
iMFI	1.40	9.80	11.3	7.50	3.24	5.11	71.3 *	40.5	26.7	19.9	2.54	15.3 ***	15.0 ***	12.4 ***	8.18
IFNγ															
Expression, % , mean	0.08	0.43 ***	0.54 ***	0.42 ***	0.47 ***	0.04	0.11	0.13 *	0.08	0.13 **	6.01	13.6 ***	16.3 ***	14.9 ***	12.0 **
iMFI	5.16	32.2 ***	37.7 ***	25.7 *	37.2 ***	2.96	9.8	9.3	6.4	12.5 **	407	1020 **	1173 ***	1029 **	982 *
Controls															
CD107a															
Expression, % , mean	0.25	0.27	0.16	0.20	0.42	0.02	0.06	0.02	0.00	0.05	26.3	22.6	22.6	21.1	29.2
iMFI	15.6	17.2	10.6	13.4	27.3	3.38	7.04	3.28	1.56	7.92	4438	4439	4191	4512	5608
Granzyme B															
Expression, % , mean	0.31	0.18	0.20	0.10	0.60	0.95	0.43	0.96	0.72	1.16	5.68	2.41	1.00	1.73	8.56
iMFI	1.06	4.04	2.98	-0.36	11.1	-23.2	-3.02	14.6	-25.2	25.3	45.1	-77.4	-258	-326	302
IFNγ															
Expression, % , mean	0.09	0.02	0.00	0.02	0.12	0.02	0.02	0.01	0.01	0.07	5.58	3.51	3.40	3.13	7.06
iMFI	4.62	0.86	0.36	0.70	6.98	1.44	0.38	0.22	0.26	5.44	356	161	146	143	486

Plasmodium falciparum infected red blood cell (PfRBC) - specific responses were corrected for the value for the uninfected RBC background. Significance was calculated using 1-way analysis of variance with the Dunnett post hoc test, with the B value serving as a control. *P<0.05, ** P<0.01, ***P<0.001.

Abbreviations: B, baseline; C − 1, 1 day before challenge; I1, 27 days after immunization 1; I2, 27 days after immunization 2; I3, 27 days after immunization 3.

[a] The geometric mean fluorescence intensity (iMFI) is calculated as the percentage of positive cells multiplied by the MFI of this positive population.

respectively). While expression of CD107 on CD4 T cells and granzyme B in CD8 T cells predicted protection with an Area Under the ROC Curve (AUC) of 0.73 (95% CI 0.48-0.98) and 0.81 (95% CI 0.63-0.99) respectively, combining both markers resulted in only a slight improvement of the AUC (0.82, 95% CI 0.61-1).

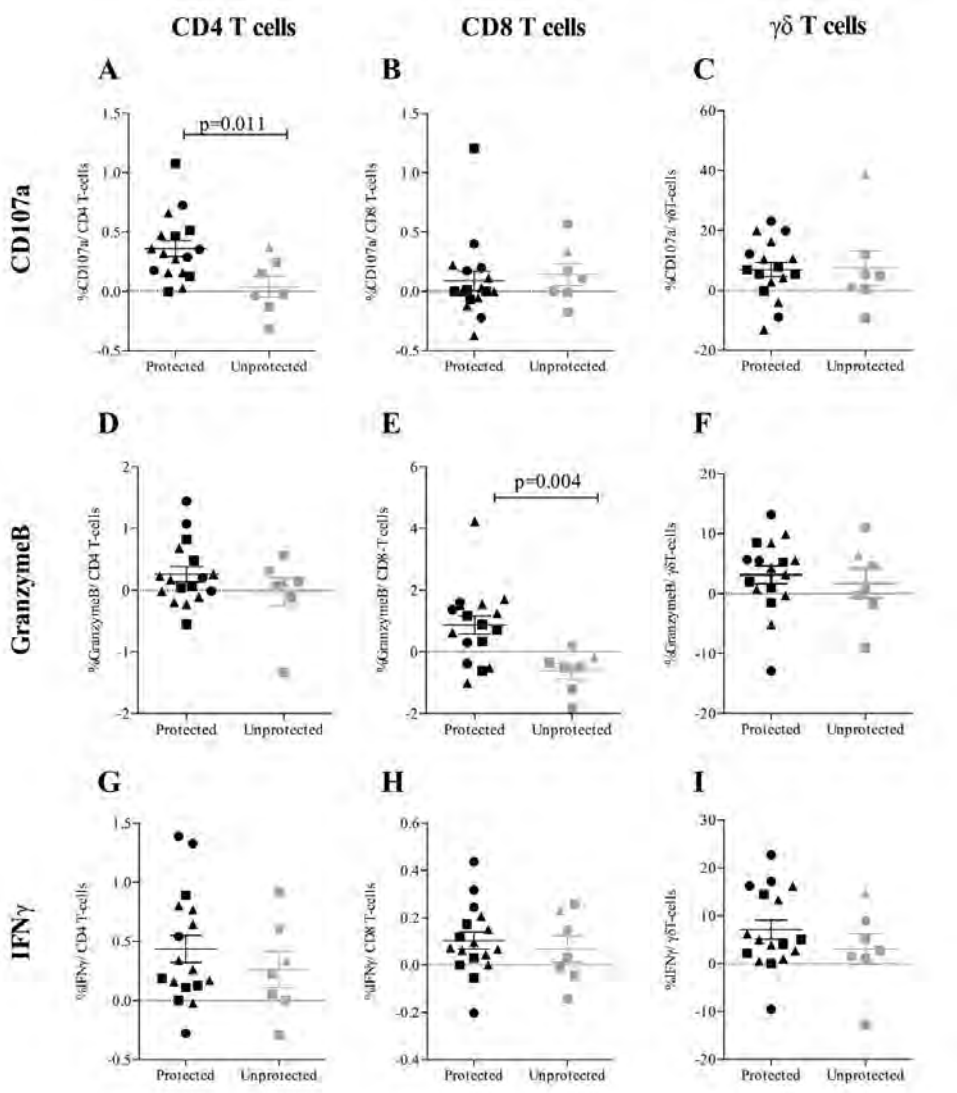

Figure 3 Cytotoxic immune responses upon in vitro PfRBC stimulation at one day before challenge infection (C-1). Each symbol represents a single protected (black symbols) or CPS-immunized unprotected (grey symbols) individual from group 1 (dots), group 2

(triangles) or group 3 (squares). Horizontal bars and whiskers represent means and SEMs. Panels show CD107a+ CD4 (A), CD8 (B) and γδ (C) T cells, granzyme B expression on CD4 (D), CD8 (E) and γδ (F) T cells and IFNγ+ CD4 (G), CD8 (H) and γδ (I) T cells. Values are corrected for uRBC background and for baseline-response before immunization. Background responses to uRBC stimulation were 0.19±0.01, 0.41±0.02 and 0.61±0.05 for CD107a, 1.65±0.50, 15.34±1.46 and 64.56±1.74 for granzyme B and 0.09±0.00, 0.07±0.00 and 0.14±0.01 for IFNγ, on CD4, CD8 and γδT cells respectively (mean ±SEM, calculated for all volunteers on both baseline and C-1). High uRBC granzyme B responses in CD8 and γδ T cells indicate that a significant percentage of these cells contains granzyme B even in a resting situation. uRBC responses did not change significantly from baseline for any of the readouts. The differences between responses of protected and unprotected volunteers in the graphs without a p-value are non-significant. The differences between protected and unprotected volunteers are calculated using logistic regression.

Pf-specific IFNγ production by CD4, CD8 or γδ-T cells could not distinguish protected volunteers (Figure 3G, 3H and 3I). Also pluripotent (IFNγ+IL-2+) effector memory T cell (CD4+ CD62L- CD45RO+) responses, previously shown to be significantly increased by CPS immunization (8), were again induced (p=0.013), but did not differentiate between protected and unprotected volunteers (OR=1.6; 95% CI 0.5-4.9; p=0.41; data not shown).

CD107a expressing CD4 T cells presented as the clearest marker associated with protection, consistently higher in fully protected subjects from I1 onwards (Figure 4A), and independent of immunization dose (Figure 4B). A significant correlation was found between CD107a expression by CD4 T cells after one immunization and prepatent period after challenge-infection in all TS+ (Spearman's rho=0.69; p=0.013, Figure 4C). The proportion CD107a+ CD4 T cells in the control subject who developed parasitemia significantly later than the other controls (i.e. day 13.5 versus day 9-10.5), was at baseline on average 2.8 fold higher than in the other subjects. Possibly, the inherently higher response in this volunteer contributed to delayed pre-patency after challenge.

CD107a+ CD4 T cells expressed proportionally more granzyme B (7.4% versus 0.39% on C-1; p<0.0008) in protected subjects, indicative for their cytotoxic phenotype, and IFNγ (13.3% versus 0.39% on C-1; p<0.0001) than CD4 T cells negative for CD107a (Figure 4D and Figure 4E). CD8 T cells, traditionally considered the cytotoxic subclass of T cells, indeed contained a larger proportion of CD107a positive cells at baseline than CD4 T cells when unstimulated (uRBC); 0.39% versus 0.19% respectively; p<0.0001 (all volunteers). However, the proportion of *Pf*-specific degranulation of CD8 T cells was not notably increased by CPS immunization (p=0.44), in contrast to CD4 T cells (p<0.0001, Figure S2A&B).

Both CD107a expression by CD4 T cells and granzyme B production by CD8 T cells remained significantly elevated up to twenty weeks after the challenge-infection (C+140) (p<0.05 and p<0.01; Figure 5A and 5B), demonstrating longevity of the CPS-induced T cell response.

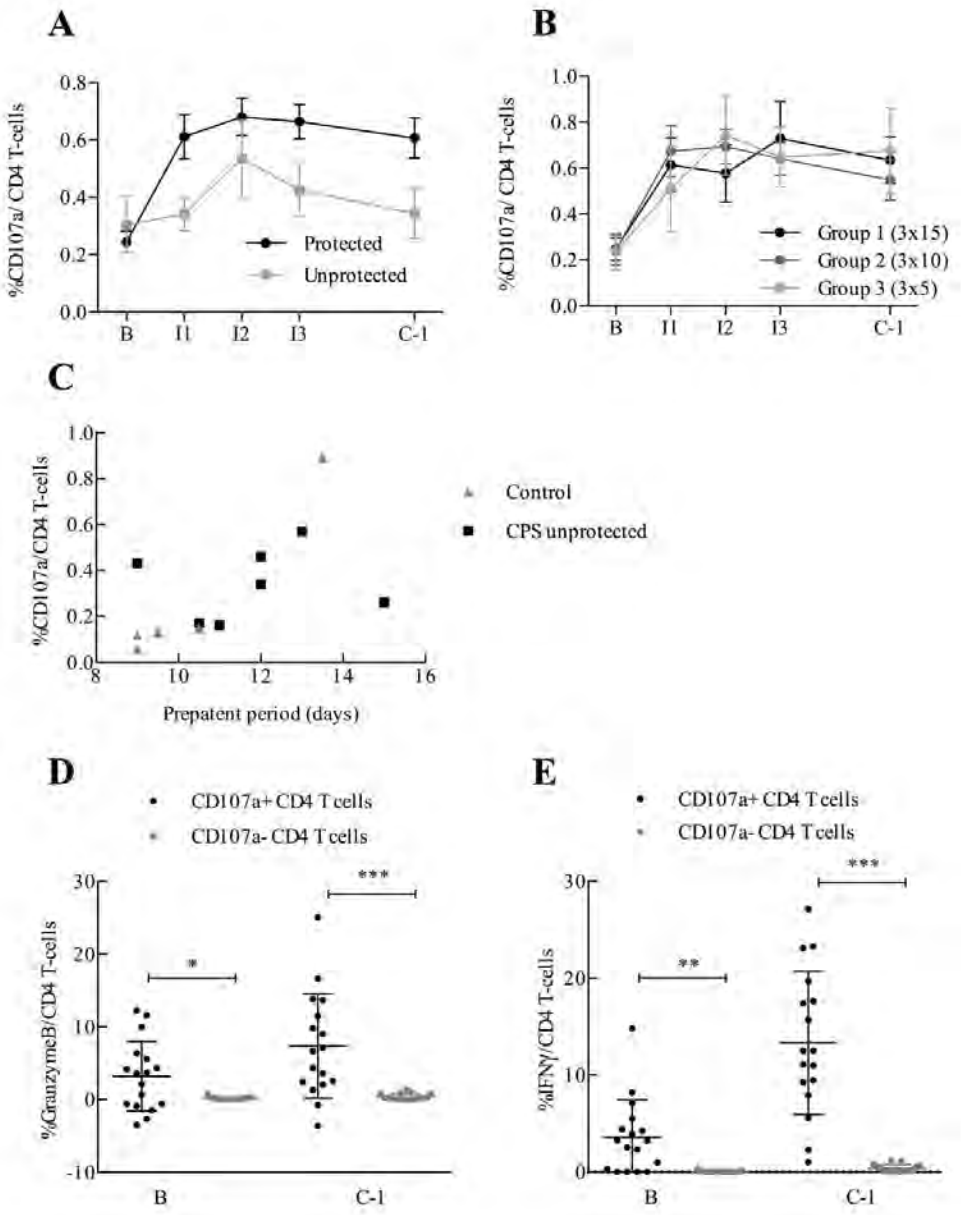

Figure 4 Cytotoxic profile of CPS-induced CD4 T cells. (A+B) Induction of *Pf*-specific CD107a positive CD4 T cells was determined (A) in protected and unprotected CPS-immunized subjects over the course of immunization and (B) in protected subjects separated for each immunization dose. Horizontal bars and whiskers represent mean responses and SEM. (C) The relationship between *Pf*-specific CD107a CD4 T cells on I1 and the prepatent period after challenge for all TS+ volunteers (CPS-immunized and controls).

Within protected CPS-immunized subjects, (D) granzyme B and (E) IFNγ production by CD107a+ (black dots) and CD107a- (grey dots) CD4 T cells was analyzed at baseline (B on x-axis) and after CPS immunization (C-1) in all protected subjects. Horizontal bars show the mean response. All data were corrected for uRBC background for every volunteer at each time point. Abbreviations on the x-axis: B= baseline; I= 27 days after indicated immunization; C-1=one day before challenge. *=p<0.05 **p<0.01 ***p<0.001

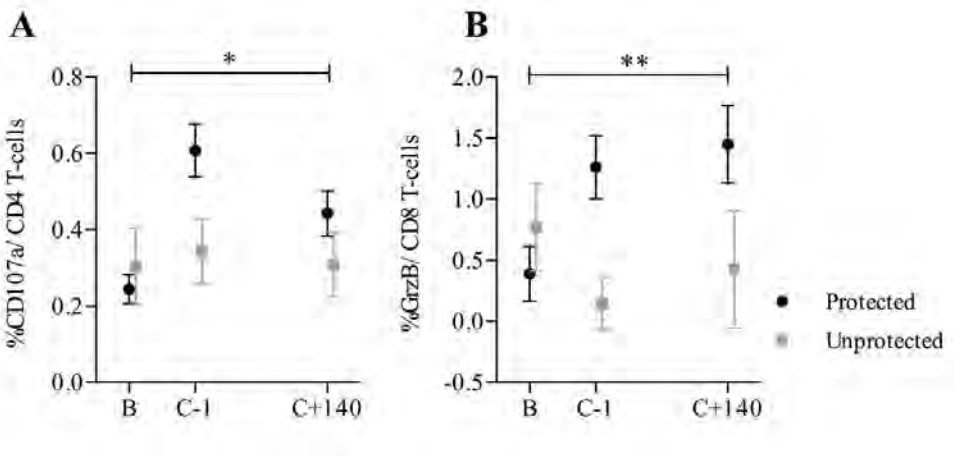

Figure 5 Longevity of cellular immune responses after CPS immunization. *Pf*-specific cellular immune responses (corrected for uRBC background) were assessed in protected (black dots) and unprotected (grey squares) CPS-immunized volunteers before CPS immunization (B), and before (C-1) and 20 weeks after challenge infection (C+140). Data are shown as mean ±SEM for (A) CD107a expression on CD4 T cells and (B) granzyme B production by CD8 T cells. Tests are performed separately for protected and immunized unprotected volunteers, by the repeated measures ANOVA (including all time points before and after immunizations) and the Dunnett's Multiple comparison post test, using B as control column. Only the test results of C+140 compared to baseline for protected volunteers are displayed. For immunized unprotected volunteers, all results were non-significant. *=p<0.05 **p<0.01

Discussion

We show that CPS immunization reproducibly and dose-dependently induces protection against a homologous challenge infection. With exposure to a total number of *Pf* infected mosquito bites as low as 30, CPS immunization still induces 89% protection in healthy volunteers. We furthermore demonstrate that markers of cytotoxic T cell responses are associated with protection against malaria after whole sporozoite immunization.

This study provides further support for the remarkable potency of the CPS-protocol

to induce complete protection by using even lower numbers of *Pf*-infected mosquitoes than before (8). The observed dose-dependent protection is in line with results from RAS immunization trials with sporozoites administered either intravenously by needle and syringe (10) or by bites from irradiated infected mosquitoes (4). Although the delay of patency in unprotected CPS-immunized subjects was not statistically significant, the patterns of parasitemia indicate partial protection in some subjects. The unexpectedly delayed control subject hampered statistical significance but could be considered an outlier, possibly because of the inherently high baseline immune response. The establishment of a sub-optimal CPS immunization regimen inducing protection in 50% of the immunized volunteers with 3x5 mosquito bites will facilitate further studies of protective immune mechanisms against *Pf* malaria.

Our data provide evidence for a role of cytotoxic T cell responses in pre-erythrocytic immunity in humans. Due to obvious practical limitations, we only assessed immune cells in the peripheral blood, which may not necessarily reflect responses in the liver but rather represent a surrogate. The results of this exploratory analysis will have to be confirmed in future trials, and the functional relevance remains to be investigated.

'Classical' cytotoxic CD8 T cells can be activated by malaria antigen on infected hepatocytes via major histocompatibility complex (MHC) class I (25) and are associated with protection in a number of (animal) models (13, 14, 26). CD8 T cells are involved in protection in the murine CPS and RAS models (27-29), but their precise effector mechanisms remain subject of debate. They might either require direct contact with infected hepatocytes (13), or in fact be independent of granzyme B and/or other cytotoxic molecules, suggestive for a more indirect cytokine mediated effect by CD8 T cells (12) or other hepatic immune cells (30). In addition, a functional role for cytotoxic CD4 T cells is also conceivable as these cells can use cytolytic pathways such as granulysin, perforin and granzymes and FAS-L, as shown mostly in viral infections (31, 32). The protective role of CD4 T cells in murine malaria has been suggested, using *in vitro* experiments (33), and *in vivo* depletion (12) or passive transfer (34). Furthermore, functional cytotoxic CD4 T cells, derived from RAS- or synthetic peptide immunized volunteers, are able to lyse autologous B cells pulsed with a peptide from the circumsporozoite protein (35-37). We used surface expression of CD107a (LAMP-1), a marker for cytotoxic degranulation, to phenotypically identify cytotoxic CD4 T cells (31). In order to directly kill a *Pf*- infected hepatocyte, parasite antigens should be presented in the context of MHC class II (MHCII) to the cytotoxic CD4 T cells. Although hepatocytes do not express MHCII in non-inflammatory circumstances, the presence of MHCII on human hepatocytes has been shown in a small number of patients with chronic hepatitis (38) and immune mediated liver disorders (39, 40). Functionally, over-expression of MHCII on hepatocytes in a transgenic mice model showed their capacity for co-stimulation, antigen-presentation and CD4 T cell activation (41). Only indirect evidence suggests that

MHCII expression on mice hepatocytes may play a role in murine malaria (33, 42), and the presence of MHCII on hepatocytes in human malaria has never been studied. Here, we show for the first time that degranulating CD4 T cells are associated with protection in human malaria and already significantly induced after one immunization.

The observed lack of boosting by the second and third immunization may reflect a saturated response of antigen specific memory cells. This raises the possibility that fewer immunizations may be sufficient to induce protection, supported by the increased proportion of volunteers without parasitemia after the second and third immunization in Group 1 and 2. Moreover, the observed longevity of the immune response is in line with long-term protection after CPS immunization in a previous study (9).

The T_H1 cytokine IFNγ has been repeatedly shown to be an important effector molecule in protection against the malaria parasite (43), and the clear induction of T_H1 responses in our study corroborates earlier findings in both animals and humans after whole sporozoite immunization (8, 10, 12, 26, 27). We previously showed that a broad range of both innate and adaptive cellular subsets contribute to CPS-induced *Pf*-specific IFNγ production (16), which is sustained at least up to 2.5 years after immunization (9). IFNγ production alone, however, does not correlate with protection in neither RAS (10) nor our CPS model. Also production of both IFNγ and IL-2 by effector memory CD4 T cells, and IFNγ production by γδ-T cells, although clearly increased in immunized volunteers (8, 16), did not differentiate between protected and unprotected volunteers.

During CPS immunization, four protected subjects did not show parasitemia by qPCR at any measured time point, not even after the first immunization. A possible explanation is that the number of merozoites released from the liver is too low for qPCR detection. A strong primary innate immune response may be responsible for clearing sporozoites and/or killing infected hepatocytes upon first encounter. Previous studies in mice indeed showed that inflammatory cytokines IL-1 and IL-6 block pre-erythrocytic development in mice (16, 44). Alternatively, chloroquine may have contributed to the decreased, i.e. undetectable number of parasites released from the liver either by direct killing, or indirectly by stimulating the immune system.

Antigen recognition and immune cell activation are essential for an effective response. To investigate pre-erythrocytic cellular immune responses, stimulation with cultured *Pf* liver stages would be preferred, but this is currently impossible. We therefore used asexual blood stage parasites for our experiments and although responses to purely pre-erythrocytic antigens may be missed, the majority of potential memory responses are likely detected upon *Pf*RBC stimulation, given the large overlap between liver and blood stage antigens (45). Future antigen screening by stimulation with a comprehensive library of pre-erythrocytic and cross-stage proteins or peptides, and subsequent functional studies focussing on cytotoxic T cells will further identify and delineate the specificity of protective responses (33, 46).

In conclusion, we identified two *in vitro* cellular cytotoxic immune markers that are associated with protection against malaria in a controlled clinical setting. Furthermore, this study confirms the robustness of CPS immunization as a highly efficient and reproducible immunization strategy for complete homologous protection. Further exploration of immune responses induced by CPS immunization will make important contributions to pre-erythrocytic malaria vaccine development and clinical testing.

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Supplementary information

CPS immunization and challenge

All subjects received a standard prophylactic regimen of chloroquine consisting of a loading dose of 300 mg on each of the first two days and then 300 mg once a week for a total duration of 14 weeks. During this period, all subjects were exposed three times to the bites of *Anopheles stephensi* mosquitoes at monthly intervals starting eight days after the first chloroquine dose as described previously (11). All volunteers were exposed to bites from exactly 15 mosquitoes at each session, but the number of NF54 *Pf* infected versus uninfected mosquitoes varied per group: five subjects received three times bites from 15 infected mosquitoes (Group 1), ten subjects received three times bites from 10 infected and 5 uninfected mosquitoes (Group 2), ten subjects received three times bites from 5 infected and 10 uninfected mosquitoes (Group 3) and five control subjects received three times bites from 15 uninfected mosquitoes (Group 4). From day 6 to 10 after each immunization, subjects were checked daily on an outpatient basis and blood was drawn for peripheral blood smears, standard haematological measurements and cardiovascular safety markers and stored for retrospective analysis of parasitemia by quantitative real-time PCR (qPCR) (47).

After the challenge-infection, volunteers were checked twice daily on an outpatient basis from day 5-21 for (un)solicited symptoms and signs. As soon as parasites were detected by thick smear, subjects were treated with a standard curative regimen of 1000 mg atovaquone and 400 mg proguanil once daily for three days, according to Dutch national guidelines. If subjects remained thick smear negative, they were presumptively treated with the same curative regimen on day 21 after challenge infection. Chloroquine levels one day before challenge were measured in EDTA-plasma by liquid chromatography and were below detection limit (5 µg/L) in all volunteers one day before challenge (48).

Retrospectively, parasitemia was quantified on day six until day ten after each immunization and from day five until day 21 after challenge by qPCR using *Pf* standard curves prepared by DNA extraction from titrated samples of ring-infected cells (47). Adverse events (AEs) were recorded as described previously (11).

Platelet counts were determined in EDTA-anticoagulated blood with the Sysmex XE-2100 (Sysmex Europe GmbH, Norderstedt, Germany). D-dimer concentrations were assessed in citrate plasma by STA-R Evolution (Roche Diagnostics, Almere, The Netherlands).

PBMC isolation and cryopreservation

Venous whole blood was collected into citrated vacutainer cell preparation tubes (CPT; Becton and Dickinson) and stored at room temperature for a maximum of 4 hours; PBMCs were isolated by centrifugation and washed four times in ice-cold phosphate-buffered saline (PBS). Cells were counted and cryopreserved at a concentration of 10^7 cells/ml in ice-cold foetal-calf serum (Gibco) containing 10% dimethylsulfoxide (Merck, Germany) using Mr.

Frosty freezing containers (Nalgene). Samples were stored in vapour-phase nitrogen.

In vitro Pf- infected erythrocyte re-stimulation assay

PBMC were thawed, washed twice in Dutch-modified RPMI 1640 (Gibco/ Invitrogen) and counted in 1% trypan blue containing 5% zap-oglobin II Lytic Reagent (Beckman Coulter) using a Neubauer improved bright line counting chamber (Marienfield, Germany); median cell recovery was 80%. PBMCs were *in vitro* re-stimulated with cryopreserved NF54 *Pf*-infected erythrocytes (*Pf*RBC) as described previously (16). Cells were re-suspended in complete culture medium (Dutch-modified RPMI 1640 containing 2 mM glutamine, 1mM pyruvate, 0.05 mM gentamycine and 10% human A+ serum, (Sanquin, Nijmegen) at a final concentration of $2.5x10^6$/ml. PBMC were transferred into polystyrene 96- well round-bottom plates and stimulated in duplicate wells with either $5x10^6$/ml (final concentration) cryopreserved *Pf*RBC or uRBC (uninfected erythrocytes) in a total volume of 110 µl/well for 24 hours at 37°C/ 5%CO_2. For the last four hours, 10 µg/ml Brefeldin A (Sigma-Aldrich) and 2µM monensin (eBioscience) were added, based on pilot experiments. In positive control wells, PMA (50 ng/ml, Sigma-Aldrich) and ionomycin (1 mg/ml, Sigma-Aldrich) were added the last four hours. After a total of 24 hours, cells were harvested and stained.

Flow cytometry analysis

PBMCs were co-incubated during the 24 hour-stimulation with CD107a Pacific Blue (Biolegend, clone H4A3). All cells were transferred to a polystyrene V-bottom plate and washed twice with 200µl PBS. Next, cells were stained with Live/Dead fixable dead cell stain dye aqua (Invitrogen) in 50 µl PBS for 30 minutes at 4°C. After washing with PBS containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich) cells were stained with antibodies against the surface markers CD3 PerCP (Biolegend, clone UCHT1), CD4 ECD (Beckman-Coulter, clone SFCI12T4D11) CD8 APC-H7 (BD Biosciences, clone SK1), γδ-T cell receptor PE (Beckman-Coulter, clone IMMU510) and CD56 APC (eBioscience, clone MEM188) in 50 µl PBS containing 0.5% BSA for 30 minutes at 4°C. Cells were washed again and fixed in Foxp3 fixation/permeabilization buffer (eBioscience). Following a wash step with Foxp3 permeabilization buffer (eBioscience), cells were stained in permeabilization buffer containing granzyme B FITC (Biolegend, clone GB11) and IFNγ PeCy7 (Biolegend, clone 4S.B3). Cells were washed again in permeabilization buffer and kept cold and dark in fixation buffer (1% paraformaldehyde in PBS) until measured by flow cytometry on the same day. For every individual volunteer, all time points were thawed, stimulated and stained within the same experimental round. In a separate experiment, cells from the time points B and C-1 were *in vitro* re-exposed to *Pf* infected erythrocytes and stained for viability, γδ-T cell receptor PE, CD56 PE, CD3 PerCP, CD45RO ECD (Beckman-Coulter, clone mIgG2a), CD62L PeCy7 (Biolegend, clone DREG-56) CD4 Pacific Blue (eBioscience, clone OKT-4)

CD8 AF700 (Biolegend, clone HIT8A), IFNγ FITC and IL-2 APC (eBioscience, clone MQ1-17H12) using the same protocol as described for the other staining panel.

Samples were acquired using a 9-color Cyan ADP (Beckman Coulter), each round using single stained cells for compensation. Per sample, a median of $93.8x10^3$ (range $12.5x10^3$ - $221x10^3$) singlet living lymphocytes were acquired. Data analysis was performed using FlowJo software (version 9.6; Tree Star). A representative example showing the gating strategy is shown in S3. The definition of cell positivity (for cytokines and cytotoxic molecules) was performed automatically, based on the MFI of unresponding PBMCs for each sample separately. Responses to uRBC were subtracted from the response to *Pf*RBC for every volunteer on every time point.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 unless mentioned otherwise. Differences between immunized unprotected and control volunteers in prepatent periods by thick smear and qPCR were tested by Mann-Whitney U test. Induction of cytotoxic immune responses on the time points I1, I2, I3 and C-1 were tested by the repeated measures ANOVA and the Dunnett's Multiple comparison post test, with baseline as control column. Induction of immune responses on 140 days after challenge (C+140) was tested separately for protected and immunized unprotected volunteers, by the repeated measures ANOVA (including all previous time points mentioned above) and the Dunnett's Multiple comparison post test, with baseline as control column. The correlation of CD107a expression by CD4 T cells with the prepatent period, and the correlation of cellular immune responses with cumulative parasitemia during CPS immunization were assessed by non-parametric Spearman correlation. The proportion of CD107a+ CD4 vs CD8 T cells and the production of granzyme B and IFNγ on $CD107a^+$ vs CD107a- CD4 T cells were tested by the paired Student's t-test. For the correlation of CD107a CD4 T cells with prepatent period after challenge, immune re-call responses to *Pf*RBC (corrected for uRBC stimulation background) were tested on the different time points, while for all other tests we assessed the change from baseline (B).

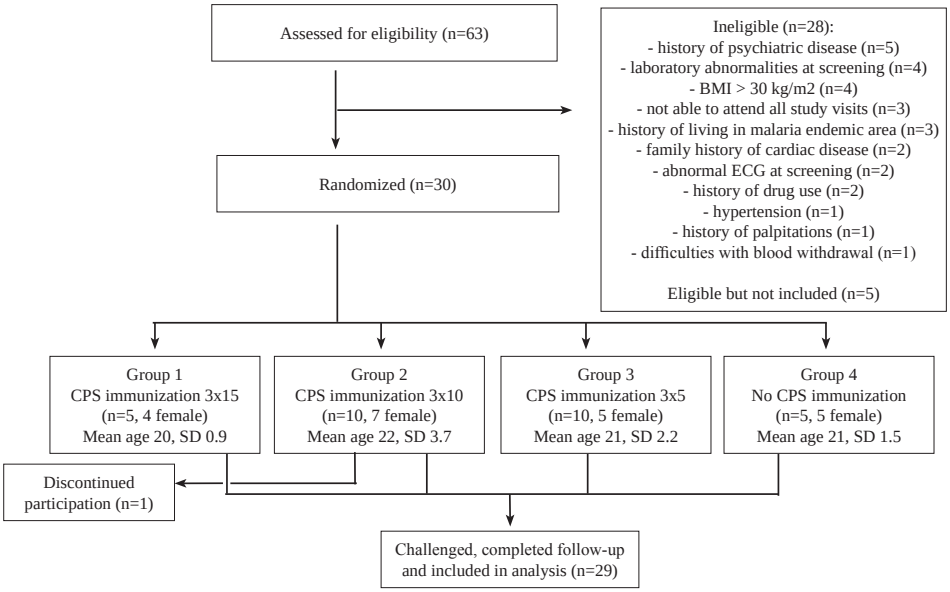

Figure S1 Study flow diagram. Twenty-five subjects were randomly assigned to receive different doses of CPS immunization in a double-blind fashion; five control subjects received bites from uninfected mosquitoes. One subject withdrew informed consent after the first immunization for reasons unrelated to the trial. Twenty-nine subjects received a challenge infection by the bites of five infected mosquitoes fifteen weeks after discontinuation of chloroquine chemoprophylaxis.

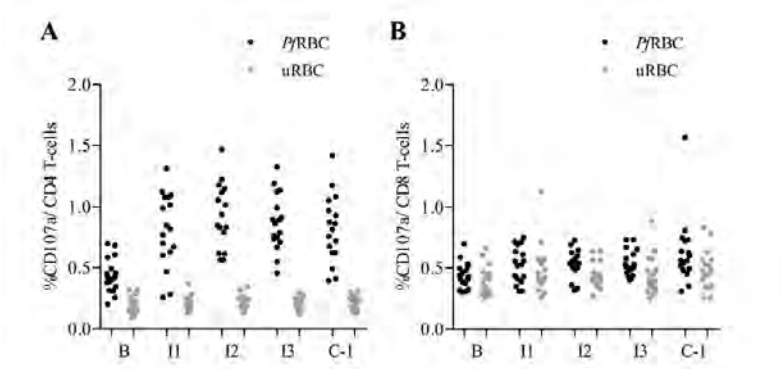

Figure S2 Induction of cytotoxic CD4 and CD8 T cell responses by CPS immunization. CD107a expression was assessed on (A) CD4 T cells and (B) CD8 T cells after stimulation with PfRBC (black dots) and uRBC (grey dots) before, during and after CPS immunization (protected subjects only). B= baseline; I= 27 days after indicated immunization; C-1=one day before challenge.

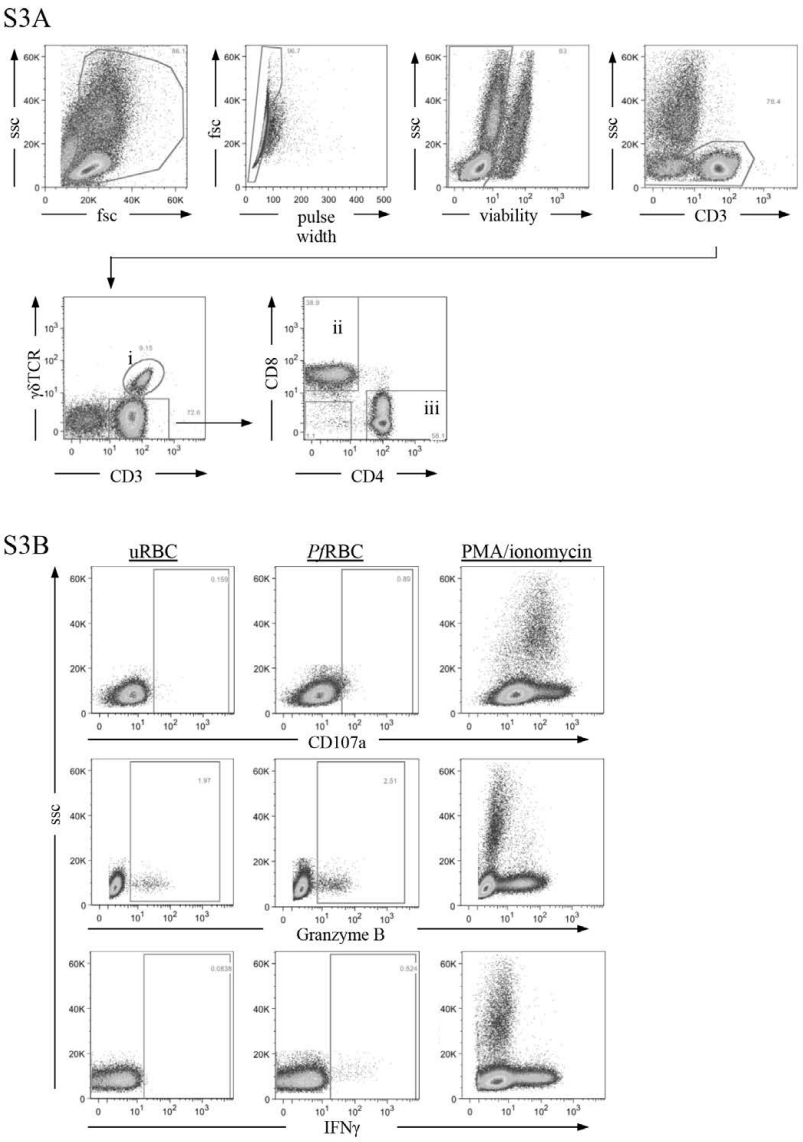

Figure S3 Flow cytometry gating strategy. (A) Representative flow cytometry plots for a uRBC stimulated sample from one volunteer at baseline (before immunization). Singlet viable CD3+ PBMC were subdivided into (i) γδT cells, (ii) CD8 T cells and (iii) CD4 T cells; No additional dump channel for CD14, CD19 and CD20 was used. (B) Gating of CD107a, granzyme B and IFNγ positive cells for uRBC, PfRBC and PMA/ionomycin re-stimulated cells at baseline. For uRBC and PfRBC stimulation CD4 T cells are shown, for PMA/ionomycin total viable PBMCs. Within each sample, gating of marker positive cells was performed automatically, based on the MFI of marker negative cells.

CHAPTER 8

Ex vivo lymphocyte phenotyping during Plasmodium falciparum sporozoite immunization in humans

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Abstract

Immunization of malaria-naïve volunteers under chemoprophylaxis with *Plasmodium falciparum* sporozoites (CPS) efficiently and reproducibly induces sterile protection and thus constitutes an excellent model to study protective immune responses against malaria. Here we performed the first longitudinal assessment of lymphocyte activation and differentiation kinetics during sporozoite immunization in 15 volunteers by *ex vivo* lymphocyte flow cytometry analysis. Both CD4 and CD8 T cells as well as γδT cells, NK cells and CD3+CD56+ cells showed increased activation and proliferation following immunization. Transient induction of the transcription factor T-bet and the cytotoxic molecule granzyme B indicated a role of Th1 responses and cytotoxic T cells in CPS-induced immunity. The absolute number of γδT cells as well as the proportion of granzyme B-containing γδT cells showed a significant and sustained increase. Regulatory T cell (Treg) proliferation was significantly higher after the second immunization in subjects subsequently not protected against challenge infection. These findings indicate an important role for γδT cells, Th1 and cytotoxic responses in whole sporozoite immunization with a possibly suppressive role of Tregs.

Introduction

Sterile protection of healthy volunteers against *Plasmodium falciparum* can be efficiently and reproducibly induced by exposing them to sporozoites while they are taking antimalarial chemoprophylaxis (1-3). This chemoprophylaxis and sporozoites (CPS) immunization protocol provides a unique opportunity to investigate protective immune responses against malaria (3, 4). Cellular memory responses induced by a number of sporozoite immunization approaches including both CPS as well as radiation attenuated sporozoites (RAS), have been investigated by *in vitro* re-stimulation of cells collected post-immunization (1, 4, 5). These studies have provided insight into the composition of *P. falciparum* specific memory responses, indicating an important role of Th1 and cytotoxic responses (1, 4, 5).

Th1 cytokines such as IFN-γ are crucial mediators of protection against malaria (6). In humans, both CPS and RAS immunization induce IFN-γ production by innate and adaptive lymphocytes (4, 5). Additionally, cytotoxic markers, i.e. degranulation of CD4 T cells and granzyme B expression by CD8 T cells, were shown to associate with protection from challenge infection after CPS immunization (3). Pre-clinical data indicate that in addition to CD4 and CD8 T cells, also γδT cells and NK cells can play a role (7-14).

Here we investigated the early kinetics and composition of *ex vivo* immune cell responses in the days after sporozoite exposure, when memory responses are initiated and orchestrated by the interplay between stimulating and suppressive signals. As activation markers we used CD38 and HLA-DR, which were shown to accurately identify virus-specific effector CD8 T cells after yellow fever vaccination, allowing for the quantification of the magnitude of the induced response (15). For the assessment of Th1 lineage commitment (16) we used the transcription factor T-bet, which also controls the generation of cytotoxic CD8 T cells (17). As possible suppressive markers, we focused on programmed cell death-1 (PD-1), which is associated with T cell exhaustion (18) and shown to significantly reduce *Plasmodium*–specific CD8 T cell responses in a rodent model (19) and on regulatory T cells (Tregs) (20).

Materials and Methods

Study subjects and CPS immunization

Nineteen healthy, malaria naïve adult Dutch volunteers (age 18-25 years, median 21) were included in a randomized double-blind controlled CPS immunization trial as described elsewhere (21). In short, five subjects received chloroquine prophylaxis and three times bites from 8 NF54 *P. falciparum* infected mosquitoes (CPS-CQ) at monthly intervals; ten subjects received mefloquine prophylaxis and bites from the same number of mosquitoes (CPS-MQ). Four control subjects received mefloquine prophylaxis and bites from uninfected mosquitoes. There was no significant difference in protection against homologous challenge

infection or recall responses after CPS-CQ or CPS-MQ: 3/5 and 7/10 subjects were protected respectively (21). Therefore, all CPS immunized subjects were pooled for immunological analysis in the current study.

Cell isolation, cryopreservation and staining

Peripheral blood mononuclear cells (PBMCs) were collected and cryopreserved one day before the first immunization (I1-1), 7 and 10 days after the first immunization (I1+7 and I1+10, respectively), one day before the second immunization (I2-1, 27 days after the first immunization), 7 and 10 days after the second immunization (I2+7 and I2+10, respectively) and one day before the third immunization (I3-1, 27 days after the second immunization). PBMCs were isolated, cryopreserved and stored as described previously (3). Immediately prior to use cells were thawed, washed twice in Dutch-modified RPMI 1640 (Gibco/Invitrogen) and counted in 0.1% trypan blue (to assess viability) containing 5% zap-oglobin II Lytic Reagent (Beckman Coulter) using a Neubauer improved bright line counting chamber (Marienfield, Germany). Median recovery of cells directly after thawing was 82%, with a viability of >95%.

Sequential samples from each volunteer were stained simultaneously to avoid influence of day-to-day variation. Fluorochrome-labeled antibodies that were used for flow cytometry are listed in Table S1. From each sample, 10^6 cells per panel were transferred to a 96 well V-bottom plate, washed twice with 200 µl phosphate buffered saline (PBS) and incubated with viability stain for 30 minutes on ice. Next, cells were washed twice with staining buffer (PBS containing 0.5% bovine serum albumin (Sigma)), stained with 50 µl antibody mix diluted in staining buffer for 30 minutes at room temperature, and again washed with staining buffer. For panel II, this was followed by a second staining step with streptavidin. Subsequently, cells were resuspended in 50 µl FoxP3 fixation/permeabilization buffer (eBioscience) and kept on ice for 30 minutes, after which they were washed with FoxP3 Permeabilization buffer (eBioscience). Next, cells were incubated with 50 µl antibody mix for intracellular staining in permeabilization buffer at room temperature for 30 min, followed by another wash with permeabilization buffer. Cells were then resuspended in 200 µl PBS/1% paraformaldehyde and kept at 4°C until acquisition on a Cyan ADP 9-colour flow cytometer (Dako/Beckman Coulter). A viability stain was included in all flow cytometry panels. A median of 217,000 (range 15,443-590,000) singlet living PBMCs were acquired per sample. Flow cytometry data were analyzed using FlowJo v9.7 software; a representative gating strategy for each panel is shown in Figure S1. Lymphocyte counts were determined in EDTA-anti-coagulated blood with the Sysmex XE-2100 (Sysmex Europe GmbH, Norderstedt, Germany). Absolute cell numbers of different lymphocyte subsets were calculated using these lymphocyte counts and percentages (within the Boolean lymphocyte gate, figure S1 [panel II]), obtained by flow cytometry. In this gating strategy we took care to exclude monocytes, based on their

differential SSC. As an additional measure to minimize inclusion of monocytes into this gate, we plotted SSC against either CD3 or CD56 staining, to enable inclusion of lymphocyte blasts, which would otherwise overlap with monocytes if simple FSC/SSC gating had been applied. Integrated median fluorescence intensity (iMFI) of Ki67 in CD4+CD45RO+FoxP3+ regulatory T cells (Tregs) was calculated as the percentage of Ki67+ cells multiplied with the MFI of Ki67 in these cells.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6. Data from sequential samples were tested by repeated measures one-way ANOVA with Dunnett's post-hoc test, comparing all time points to baseline data (I1-1). Relationships between parasitemia and cellular responses were analyzed by Pearson correlation, as were relationships between *ex vivo* responses and re-call responses published previously (21). Two-Way ANOVA with Bonferroni post-test was used to assess whether responses differed between protected and unprotected subjects. Since we aimed to assess the effect of sporozoite immunization on cellular responses rather than the effect of different drugs, we did not compare responses between the CPS-CQ and CPS-MQ groups. A p-value of <0.05 was considered statistically significant.

Results

Changes in lymphocyte numbers after sporozoite immunization

The number of αβT, NK and CD3+CD56+ cells significantly decreased after the first sporozoite immunization, suggesting redistribution to possible effector sites including liver and/or spleen (Figure 1A, B, D, and E). While these subsets normalized four weeks after exposure, the number of γδT cells remained significantly increased (mean ± SEM $0.07x10^9$/L ± $0.01x10^9$/L, $0.12x10^9$/L ± $0.01x10^9$/L and $0.14x10^9$/L ± $0.02x10^9$/L at I1-1, I2-1 and I3-1, respectively; Figure 1C). In controls, cell numbers did not change significantly at any time point (data not shown).

Activation and proliferation of lymphocytes directly following sporozoite immunization

Both CD4 and CD8 T cells in peripheral blood showed significantly increased proliferation by intracellular Ki67 staining ($p<0.001$ for both CD4 and CD8; Figure 2A and 2B) and activation by increased co-expression of CD38 and HLA-DR ($p<0.001$ for both CD4 and CD8; Figure 2C and 2D) on day 10 after the first sporozoite immunization. Activation strongly correlated with proliferation for both CD4 and CD8 T cells at I1+10 ($r=0.91$ $p<0.0001$ and $r=0.88$ $p<0.0001$, respectively). After the second immunization, CD8 T cell proliferation and

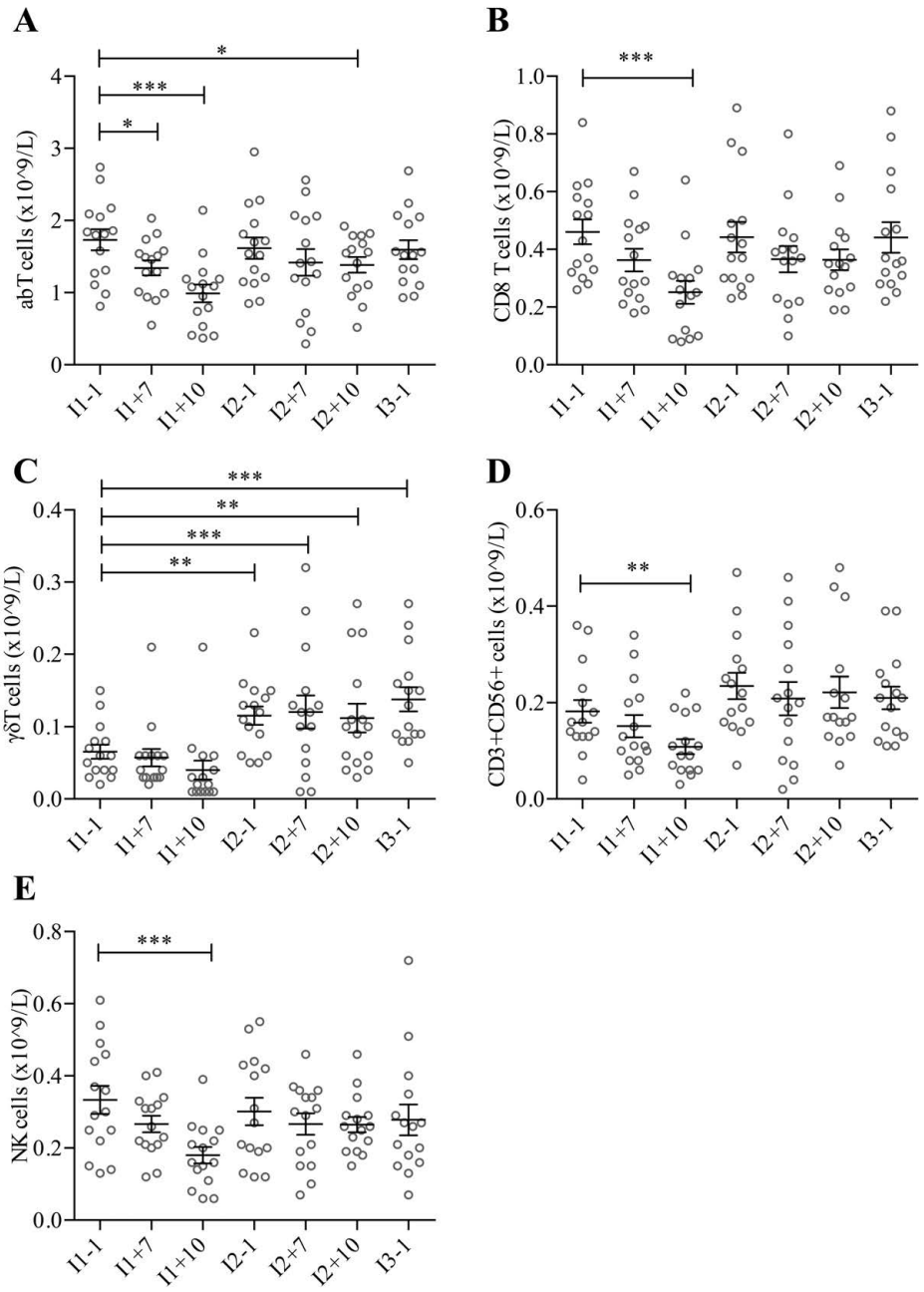

Figure 1 Changes in cell subsets induced by CPS immunization. αβT cells (A), CD8 T cells (B), γδT cells (C), CD3+CD56+ cells (D) and NK cells (E) were assessed one day before the first, second and third immunization (I1-1, I2-1 and I3-1, respectively) and

7 and 10 days after the first and second immunization (I1+7, I1+10, I2+7 and I2+10, respectively). Data are represented as individual values for CPS immunization subjects (n=15) and lines indicate mean±SEM. Data were tested by repeated measures one-way ANOVA with Dunnett's post-hoc test, comparing all time points to baseline data (I1-1). Significant differences are indicated with *=p<0.05, **=p<0.01, ***=p<0.001. *Note different y-axis scales.*

activation did not significantly increase, while CD4 T cells responses were comparable to the first immunization (Figure 2A-D).

Similarly, γδT, NK and CD3+CD56+ cells showed a significant increase in proliferation, but only after the first immunization (all p<0.001; Figure 3A, 3B and 3C). Expression of CD25 did not change on γδT cells over the course of CPS immunization (Figure 3D). Activation of NK and CD3+CD56+ cells, however, was increased after the first immunization. After the second immunization activation of NK cells was again induced, but reduced in CD3+CD56+ cells compared to baseline (Figure 3E and 2F).

In control subjects, we also observed increased proliferation and activation of CD4 and CD8 T cells over the course of immunization by uninfected mosquitoes. The magnitude of these responses, however, was much smaller and analysis of statistical significance was hampered by the small sample size (Figure 2 E-H).

Activation and proliferation of lymphocyte subsets correlate with parasite exposure during immunization rather than protection

Parasite density correlated with proliferation of almost all lymphocyte subsets at I1+10 (CD4 r=0.64 p=0.011, CD8 r=0.56 p=0.03, γδ T r=0.57 p=0.03, CD3+CD56+ cells r=0.56 p=0.03, NK cells: ns). Activation of both CD4 and CD8 T cells (r=0.79 p=0.0005, and r=0.81 p=0.0003, respectively) but not of the other T cell or lymphocyte subsets correlated with parasitemia. Notably, immunized subjects without parasitemia also showed increased responses, mainly CD4 T cell proliferation (Figure 2A-D). Neither proliferation nor activation of any lymphocyte subset during immunization was associated with subsequent protection from challenge infection (not shown).

Induction of regulatory markers during CPS immunization

Proliferation of CD4+CD45RO+FoxP3+ regulatory T cells (Tregs) significantly increased directly following both the first and second immunization and returned to baseline levels after a month (Figure 4A). In contrast to other lymphocyte subsets, proliferation of Tregs was already significantly increased 7 days after immunization, the first day of detectable parasitemia. Neither Treg proliferation nor the number of circulating Tregs correlated with parasitemia (data not shown). There was also no relationship between ex vivo Treg proliferation during immunization and induced memory responses, i.e. de-granulation or

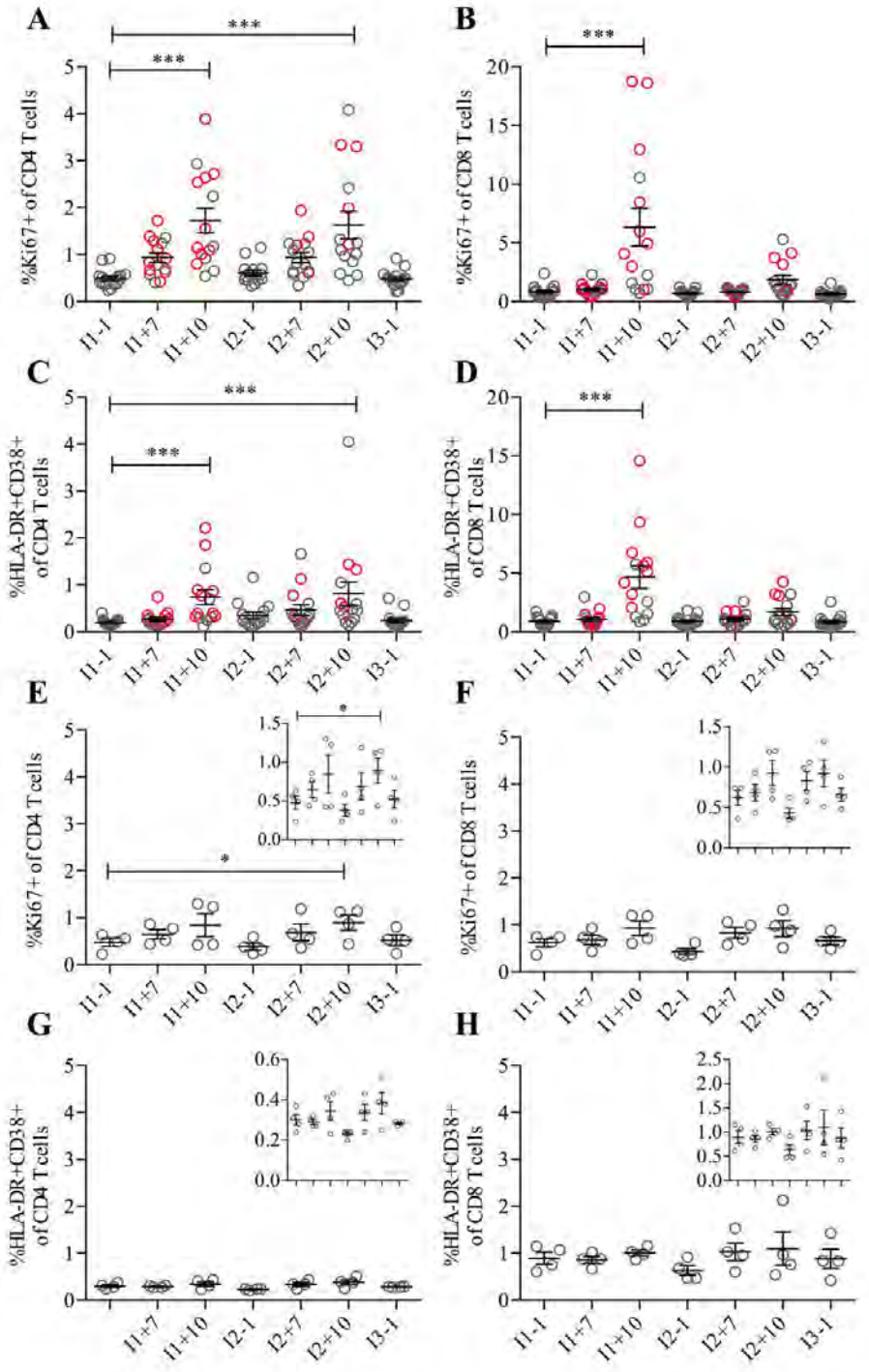
A
%Ki67+ of CD4 T cells
***
***
B
%Ki67+ of CD8 T cells
***
C
%HLA-DR+CD38+ of CD4 T cells
***
***
D
%HLA-DR+CD38+ of CD8 T cells
***
E
%Ki67+ of CD4 T cells
*
*
F
%Ki67+ of CD8 T cells
G
%HLA-DR+CD38+ of CD4 T cells
H
%HLA-DR+CD38+ of CD8 T cells
I1-1
I1+7
I1+10
I2-1
I2+7
I2+10
I3-1

Figure 2 Proliferation and activation of CD4 and CD8 T cells following CPS immunization or uninfected mosquito bites. Proliferation of CD4 (A, E) and CD8 (B, F) T cells was measured by Ki67 expression and activation of CD4 T cells (C, G) and CD8 T cells (D, H) was measured by co-expression of HLA-DR and CD38 one day before the first, second and third immunization (I1-1, I2-1 and I3-1, respectively) and 7 and 10 days after the first and second immunization (I1+7, I1+10, I2+7 and I2+10, respectively) in CPS-immunized subjects (A-D, n=15) or control subjects who received bites from 8 uninfected mosquitoes at each immunization (E-H, n=4). Data are represented as individual values; data from subjects who experienced parasitemia after immunization are indicated as red circles, subjects who remained PCR negative as grey circles; lines indicate mean ± SEM. The inserts in graphs E-H show the same data for control volunteers on a different axis to show the changes over time. Data were tested by repeated measures one-way ANOVA with Dunnett's post-hoc test, comparing all time points to baseline data (I1-1). Significant differences are indicated with **=$p<0.01$, ***=$p<0.001$. Note different scales of y-axes in CD4 versus CD8 graphs.

IFNγ production by CD4 T cells in response to in vitro re-stimulation twenty weeks after the last immunization ((21), data not shown). The degree of Treg proliferation as quantified by integrated MFI of Ki67, however, was significantly higher at I2+10 in subjects not protected against challenge infection ($p<0.0001$; Figure S2). Expression of programmed cell death-1 (PD-1) was transiently increased on both CD4 and CD8 T cells, but returned to baseline levels at I3-1 (Figure 4B and 4C). Expression of PD-1 was not different in unprotected subjects compared to protected subjects at any time point (not shown).

Phenotypic analysis of CD4 and CD8 T cells shows Th1 and cytotoxic commitment

In both CD4 and CD8 T cells, T-bet expression was transiently increased in proliferating cells 10 days after the first immunization, indicating Th1 and cytotoxic commitment, respectively (Figure 5A and 5B, Figure S3). Furthermore, expression of the cytotoxic molecule granzyme B was increased significantly in the activated CD8 T cells after the first immunization (Figure 5C). γδT cells also gained a more cytotoxic phenotype during CPS immunization: the proportion of these cells containing granzyme B increased significantly and remained elevated over the course of CPS immunization (Figure 5D).

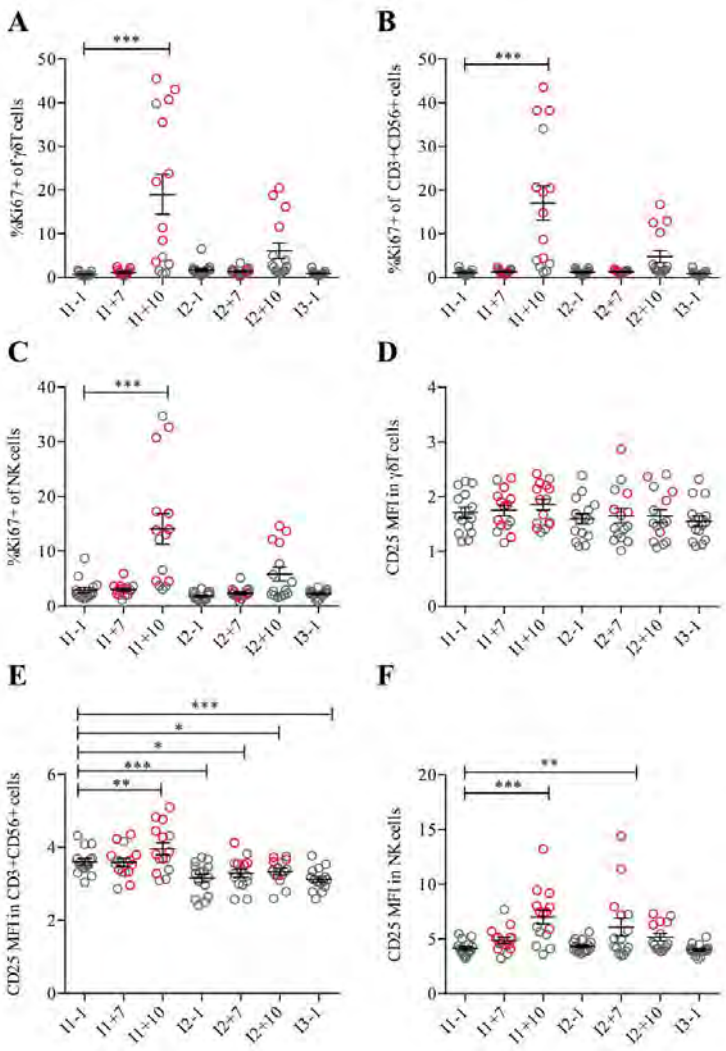

Figure 3 Proliferation and activation of γδT cells, NK cells and CD3+CD56+ cells. Proliferation of γδT cells (A), CD3+CD56+ cells (B) and NK cells (C) was measured by Ki67 expression and activation of γδT cells (D), CD3+CD56+ cells (E) and NK cells (F) was measured by expression of CD25 one day before the first, second and third immunization (I1-1, I2-1 and I3-1 respectively) and 7 and 10 days after the first and second immunization (I1+7, I1+10, I2+7 and I2+10 respectively). Data are represented as individual values for CPS immunization subjects (n=15); data from subjects who experienced parasitemia after immunization are indicated as red circles, subjects who remained PCR negative as grey circles; lines indicate mean ± SEM. Data were tested by repeated measures one-way ANOVA with Dunnett's post-hoc test, comparing all time points to baseline data (I1-1). Significant differences are indicated with *=p<0.05, **=p<0.01, ***=p<0.001.

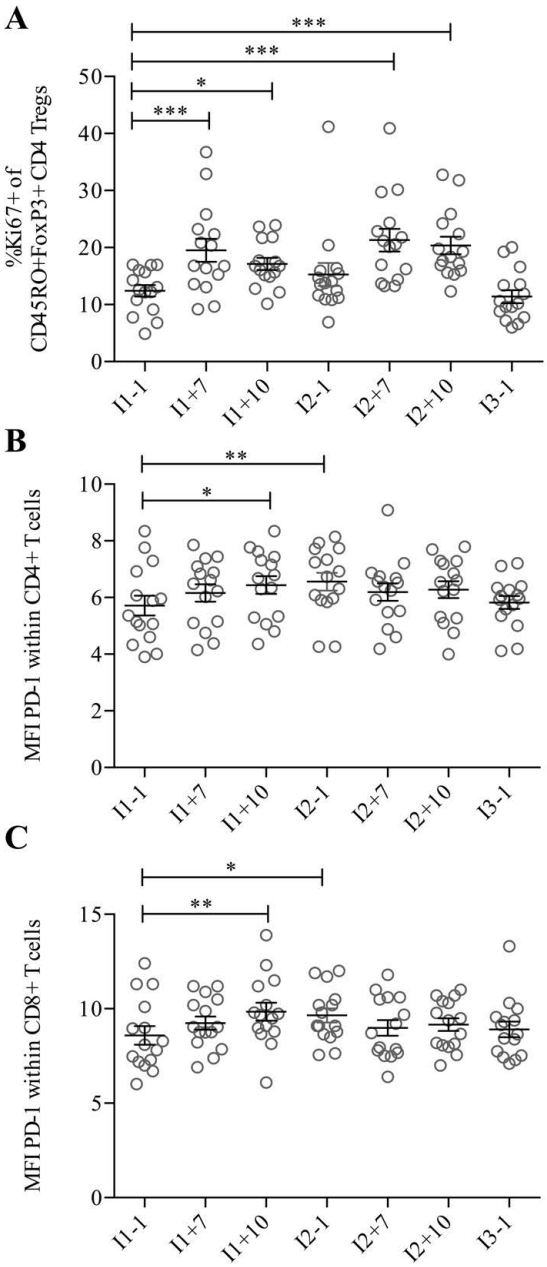

Figure 4 Induction of Tregs and the PD-1 during CPS immunization. Proliferation of CD45RO+FoxP3+ Tregs (A) and PD-1 expression by CD4 (B) and CD8 (C) T cells was assessed one day before the first, second and third immunization (I1-1, I2-1 and I3-1 respectively) and 7 and 10 days after the first and second immunization (I1+7, I1+10, I2+7 and I2+10 respectively). Data are represented as individual values for CPS immunization subjects (n=15) and lines indicate mean±SEM. Data were tested by repeated measures one-way ANOVA with Dunnett's post-hoc test, comparing all time points to baseline data (I1-1). Significant differences are indicated with *=p<0.05, **=p<0.01, ***=p<0.001

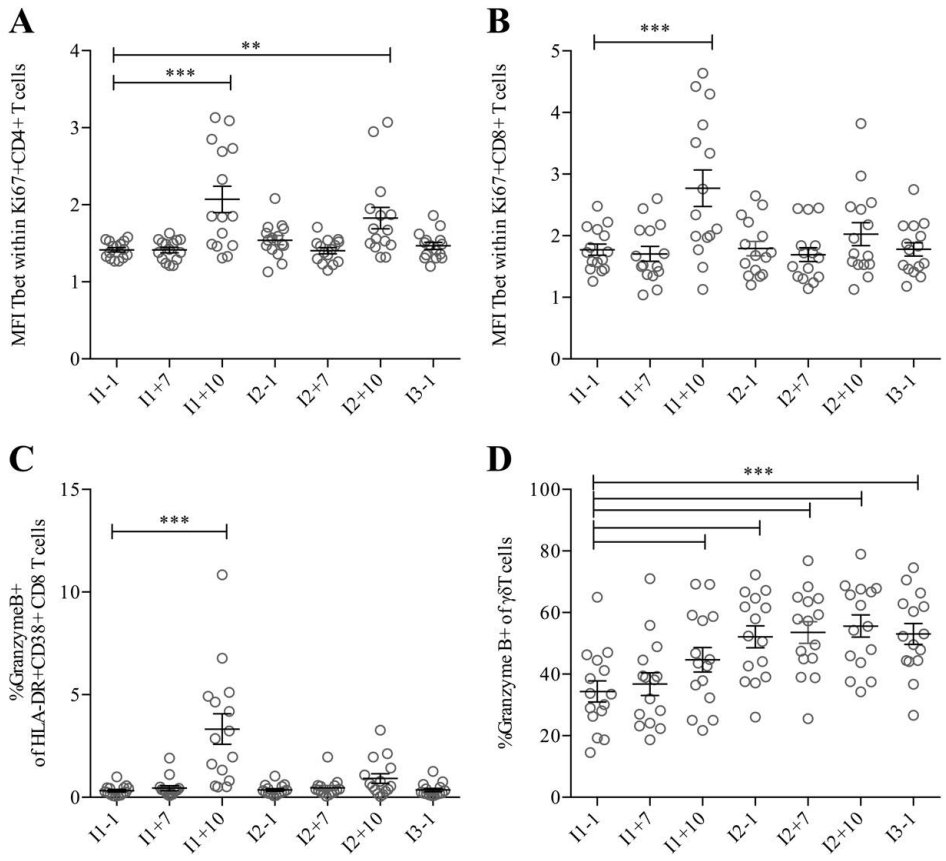

Figure 5 Induction of T cells with a cytotoxic phenotype directly following CPS immunization. T-bet expression of proliferating CD4 T cells (A) and proliferating CD8 T cells (B), and percentage of granzyme B positive activated CD8 T cells (C) and γδT cells (D) were assessed one day before the first, second and third immunization (I1-1, I2-1 and I3-1, respectively) and 7 and 10 days after the first and second immunization (I1+7, I1+10, I2+7 and I2+10, respectively). Data are represented as individual values for CPS immunized subjects (n=15) and lines indicate mean±SEM. Data were tested by repeated measures one-way ANOVA with Dunnett's post-hoc test, comparing all time points to baseline data (I1-1). Significant differences are indicated with *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

Discussion

Our data show that both T cells and other lymphocytes are readily activated shortly after the first sporozoite immunization, with increased T-bet expression in CD4 and CD8 T cells indicative for differentiation towards a Th1 and cytotoxic phenotype. This is in line with previous findings suggesting a contribution of these cells to CPS-induced protection (3, 4) and their induction in other sporozoite-immunization regimens (5, 22). These responses were most prominent at 10 days post CPS, in particular after the first immunization, and returned to baseline four weeks later. Since parasitemia occurred 7 or 8 days after exposure to mosquito bites, these responses could be a result of exposure to pre-erythrocytic stages and/or blood-stages. Indeed, T cells from RAS and CPS immunized subjects recognize both pre-erythrocytic and blood-stage antigens *in vitro* (4, 23). Since activation, particularly of CD4 T cells, also occurs in a number of subjects without parasitemia, pre-erythrocytic exposure alone may be apparently sufficient; however, an effect of very low parasitemia at sub-qPCR densities cannot be formally excluded. Proliferative responses of CD8 T cells and innate cells were lower after the second immunization, probably related to the reduced development of late liver stage parasites and hence parasitemia after subsequent immunizations as a consequence of developing pre-erythrocytic immunity (2, 21).

Target antigens of protective immunity after sporozoite immunization have thus far not been identified. It is therefore not yet feasible to assess whether the observed lymphocyte activation is *P. falciparum*-specific, or the result of an aspecific bystander effect. After smallpox or yellow fever vaccination at least, only a minimal bystander CD8 T cell response has been shown to occur, but the lack of a human challenge model for these infections hampers assessment of the functional significance of these findings (15). Here we established that after CPS immunization neither proliferation nor activation of CD4 or CD8 T cells predicts protection. Studies are ongoing to identify target antigens of CPS-induced protection (Clinicaltrials.gov NCT02080026), which would allow tetramer analysis of T cells in order to shed light on the composition and heterogeneity of the induced responses and possible correlation of individual antigen-specific responses with protection.

As known markers of immune-regulation in malaria, we measured Treg proliferation and PD-1 expression during immunization. Tregs are known to suppress Th1 responses in mice (24-26). Also in humans, Tregs are induced during blood-stage infection following controlled malaria infection, in association with decreased antigen-specific immune responses and pro-inflammatory cytokine production (27). Moreover, FOXP3 mRNA levels during acute disease in Gambian children were inversely correlated with cultured IFNγ responses to *P. falciparum* schizont extract 28 days later (28). We did not find an association between Treg proliferation and Th1 memory responses, but proliferation of Tregs was significantly higher after the second immunization in subjects subsequently not protected against challenge. Although the sample size for this comparison is low, this finding might support the notion

that Tregs have a negative role in the induction of protection by CPS. In fact, the Treg subset was the first measured cell population that significantly proliferated already 7 days after exposure.

PD-1 is up-regulated on CD4 and CD8 T cells upon activation, and indeed increased expression of PD-1 on both CD4 and CD8 T cells concurred with emergence of parasitemia during immunization. PD-1 inhibits cytokine production and cytotoxic functions of T cells (29); in Malian children infected with malaria PD-1 expression on CD4 T cells was associated with T cell dysfunction, and in mice blockade of the PD-1 ligand PD-L1 restored CD4 T cell function (30). However, data on the role of PD-1 are not unambiguous. For example, analysis of PBMCs from healthy humans showed that most CD8 T cells expressing PD-1 are effector memory cells, rather than exhausted cells (31). Our data show that up-regulation of PD-1 expression is not associated with lack of protection, suggesting that immune responses are not compromised via this inhibitory pathway during CPS immunization. Instead, it may rather represent a physiological regulatory mechanism or indicate activation of these cells (32).

The absolute number of circulating γδT cells doubled over the course of two CPS immunizations, in line with previous findings in both CPS and RAS studies (4, 5). In addition, we observed a substantial specific IFNγ production (4) as well as sustained increase in granzyme B content of γδT cells, which supports their potential cytotoxic role in protection. A protective role for γδT cells has been shown in rodents after RAS immunization (12). Whether the expansion of the γδT cell population is an antigen-specific or bystander effect remains to be investigated.

We did observe a positive but non-significant response of CD4 T cell activation after bites from uninfected mosquitoes. Although clearly inconclusive, a mosquito-saliva component might contribute to the efficiency of CPS immunization. In rodent studies, bites from uninfected *Anopheles* mosquitoes resulted in lower liver-stage infection after challenge, concurrent with the induction of a Th1 immune response (33).

CPS-induced protection is mediated by immune responses directed against pre-erythrocytic stages (2) and skin, liver and blood have unique characteristics including distinct immune cell compositions that could be important in the interaction with *Plasmodium* (34). A major shortcoming for human CD8 T cell studies is the fact that peripheral blood is generally the only accessible lymphocyte source, which is obviously not the compartment of major CD8 activity. Alternatively, non-human primate studies can be used to shed light on the role of tissue-resident memory CD8 T cells (35). Indeed, the frequency of *P. falciparum* specific IFNγ producing T cells was shown to be significantly higher in the liver than in peripheral blood of RAS-immunized rhesus macaques (36) and depletion of CD8 T cells abrogates RAS induced immunity (11). Another limitation of our study is that the majority of panels used to assess CD8 T cells did not include markers for the γδ T cell receptor or the semi-invariant

T-cell receptor Vα7.2-Jα33/12/20. Only a minor fraction of CD8 T cells in our volunteers expressed the γδ T cell receptor, and proliferation kinetics of γδ negative CD8 T cells were comparable to those of total CD8 T cells (data not shown). Nevertheless, we can thus not exclude that γδTCR+CD8+ T cells and mucosal-associated invariant T (MAIT) (37) cells possibly included in the CD8 T cell gate might show slightly different patterns in activation, PD-1 and T-bet expression from classic αβ CD8 T cells.

Taken together, we find that different lymphocyte subsets have distinct activation kinetics during whole sporozoite immunization, that T cell activation also occurs in the absence of blood stage parasitemia and that Treg proliferation negatively associates with protection. Moreover, we show that T cell proliferation goes hand in hand with differentiation towards the Th1 and cytotoxic lineages and a particular increase in the cytotoxic potential of γδT cells in a majority of subjects. Once target antigens are identified, future studies using the relatively simple and highly efficient CPS immunization model can be designed to disentangle the regulatory and effector functions of different antigen-specific T cell subsets and their role in protection.

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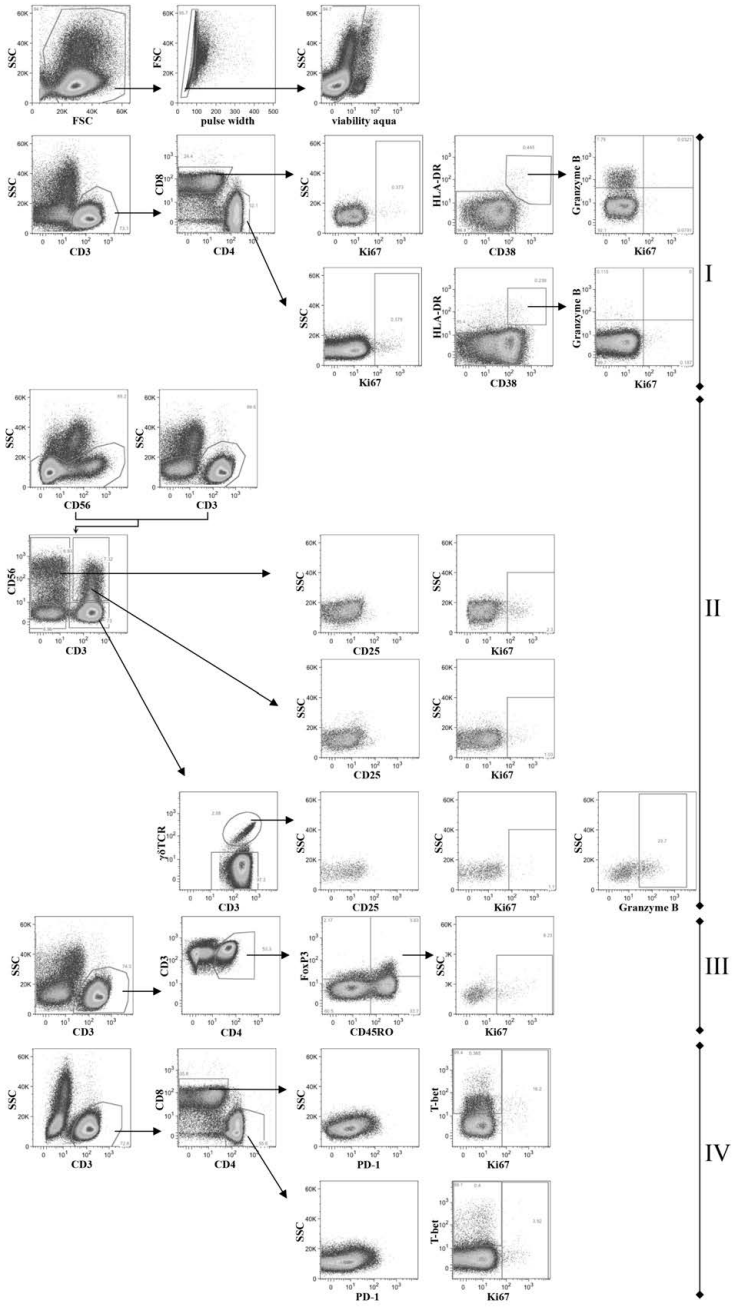

Figure S1
Gating strategy. Representative flow cytometry plots for a baseline sample from one volunteer. Single viable PBMCs (all panels) were stained with four different panels (I-IV). In panel I, proliferation (Ki67+), activation (HLA-DR+ and CD38+) and granzyme B expression of CD4 and CD8 T cells were determined. In panel II, proliferation (Ki67+), activation (CD25+) and granzyme B expression of (CD56+CD3-) NK, CD56+CD3+ (including iNKT cells, classical T cells and MAIT cells) and (CD56-CD3+) γδT cells was assessed. With panel III, proliferation (Ki67+) of (CD4+FoxP3+ CD45RO+) regulatory T cells was determined and with panel IV expression of PD-1 and T-bet by CD4 and CD8 T cells was assessed.

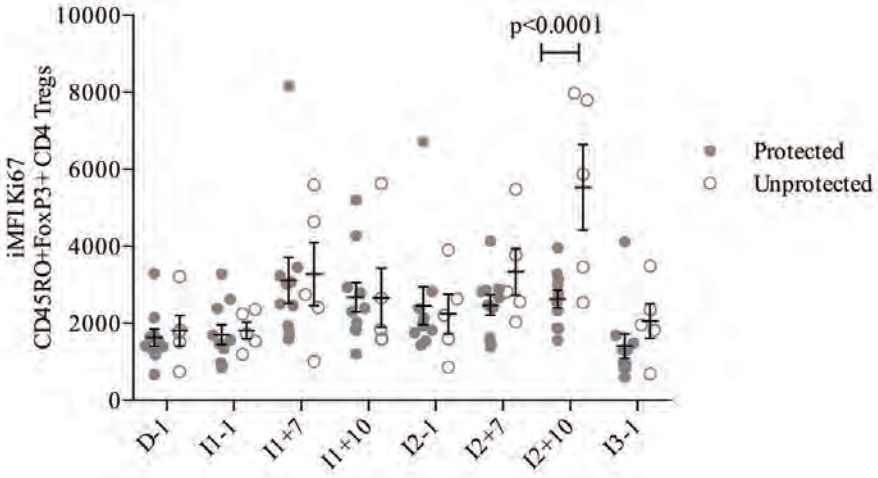

Figure S2 Proliferation of regulatory T cells. The degree of CD4+CD45RO+FoxP3+ Treg proliferation was assessed as Ki67 integrated MFI. Differences between protected (filled circles) and unprotected (open circles) subjects were assessed by two-way ANOVA with Bonferroni post-hoc test for multiple comparison correction.

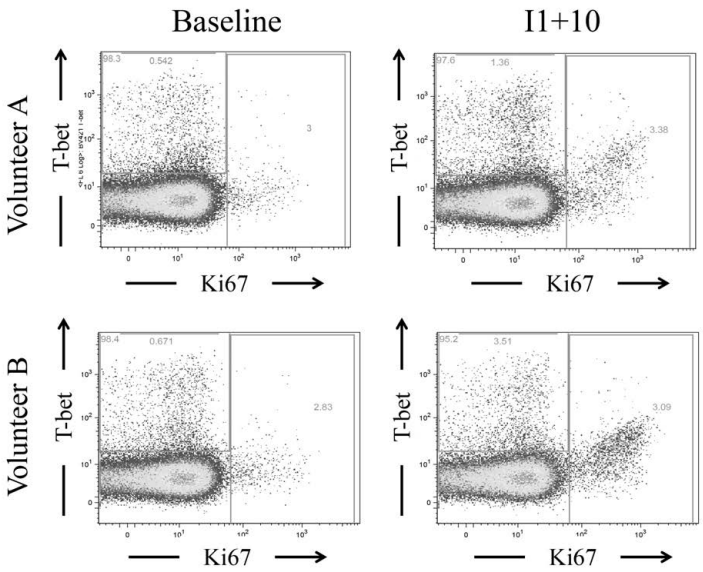

Figure S3 T-bet expression in CD4 T cells. Representative flow cytometry plots for two volunteers at baseline and I1+10 (x-axis Ki67, y-axis T-bet expression).

Supplementary information

Table S1 Antibodies used for flow cytometry

Target	Fluorochrome	Clone	Supplier
All panels			
Fixable viability	Aqua	-	eBioscience
Panel I			
HLA-DR	PE	L243	BioLegend
CD4	PerCP	RPA-T4	BioLegend
CD3	ECD	UCHT1	Beckman Coulter
CD38	PeCy7	HIT2	BioLegend
CD8	APC-H7	SK1	BD Biosciences
Granzyme B	Fitc	GB11	BioLegend
Ki67	AF647	B56	BD Biosciences
Panel II			
γδTCR	PE	IMMU510	Beckman Coulter
CD25	ECD	B1.49.9	Beckman Coulter
CD3	PerCP	UCHT1	BioLegend
CD8	APC-H7	SK1	BD Biosciences
CD56	biotin	HCD56	BioLegend
Streptavidin	eF450	-	eBioscience
Granzyme B	Fitc	GB11	BioLegend
Ki67	AF647	B56	BD Biosciences
Panel III			
CD3	PerCp	UCHT1	BioLegend
CD45RO	ECD	UCHL1	Beckman Coulter
CD4	APC-H7	RPA-T4	BD Biosciences
Ki67	Fitc	B56	BD Biosciences
FoxP3	eF450	PCH101	eBioscience
Panel IV			
CD3	Fitc	OKT3	BioLegend
CD4	PerCP	RPA-T4	BioLegend
PD-1	PeCy7	EH12.2H7	BioLegend
CD8	APC-H7	SK1	BD Biosciences
Ki67	AF647	B56	BD Biosciences
T-bet	BV421	4B10	BioLegend

CHAPTER 9

Heterologous protection against malaria after immunization with Plasmodium falciparum sporozoites

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Abstract

Background

In the controlled human malaria infection (CHMI) model, complete protection in >90% of volunteers against homologous *Plasmodium falciparum* infection has so far only been achieved after immunization with whole parasites. The most efficient regimen is sporozoite immunization of healthy volunteers under chloroquine prophylaxis (CPS immunization), requiring bites from only 30-45 mosquitoes infected with *P. falciparum*-sporozoites. Given the large diversity of *P. falciparum* parasites, it is essential to assess protection against heterologous parasite strains.

Methods

In an open-label follow-up study, 16 volunteers previously CPS-immunized and challenged with *P. falciparum* NF54 (West-Africa) in a dose de-escalation and challenge trial, were re-challenged with clone NF135.C10 (Cambodia) at 14 months after the last immunization (NCT01660854).

Results

Two out of thirteen NF54 protected volunteers previously fully protected against NF54 were also fully protected against NF135.C10, while 11/13 showed a delayed patency [median prepatent period of 10.5 days (range 9.0-15.5) versus 8.5 days in 5 malaria-naïve controls (p=0.0005)]. Analysis of patency by qPCR indicated an estimated 91 to >99% reduction of liver parasite load in 7/11 partially protected subjects. Three volunteers previously not protected against NF54, were also not protected against NF135.C10.

Conclusion

This study shows that CPS immunization can induce heterologous protection for a period of more than one year, which is a further impetus for clinical development of whole parasite vaccines.

Introduction

Malaria remains a tremendous public health problem affecting approximately 40% of the world's population. The global incidence of malaria is estimated to be around 198 million clinical cases resulting in 584.000 deaths (1) most of which are caused by *Plasmodium falciparum*. Since current interventions fail to reduce malaria incidence sufficiently, a vaccine is needed to combat this disease.

Sterile protection against *P. falciparum* malaria can efficiently and reproducibly be achieved in the Controlled Human Malaria Infection (CHMI) setting by repeated inoculation of live sporozoites by bites of laboratory-reared *Anopheles* mosquitoes to healthy malaria-naïve volunteers under chemoprophylaxis: ChemoProphylaxis and Sporozoites (CPS) immunization (2, 3). CPS-induced protection is dose-dependent (3) and was shown in a subset of volunteers to last for more than two years (4). Furthermore, bites from only 30-45 *P. falciparum*-infected mosquitoes are sufficient to induce sterile protection in >90% of subjects, while immunization with radiation-attenuated sporozoites (RAS) requires a minimum of 1,000 *P. falciparum*-infected mosquitoes, or intravenous injection of 675,000 cryopreserved sporozoites (5, 6). So far, CPS immunizations and challenges have been performed with the homologous NF54 strain only, while in malaria-endemic areas there is a large genetic and antigenic diversity of *P. falciparum* strains. This diversity is considered an important reason why naturally acquired immunity is obtained slowly, only after several years of repeated exposure (7). Previously, heterologous protection has been reported in 4/6 RAS-immunized volunteers (5).

Next to the widely used *P. falciparum* strain NF54 and its clone 3D7, NF135.C10 originating from Cambodia has become available for CHMI (8). In this study, volunteers who had previously participated in a NF54 dose de-escalation CPS immunization and challenge trial (9) were re-challenged with NF135.C10 after more than one year.

Materials and Methods

Study design

A single center open label clinical trial was conducted at the Leiden University Medical Center (LUMC) from July 2012 until February 2013. The study was approved by the Central Committee for Research Involving Human Subjects of The Netherlands (NL39414.000.12) and complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. ClinicalTrials.gov Identifier: NCT01660854.

Study participants

Eighteen volunteers from a NF54 CPS dose-de-escalating study (ClinicalTrials.gov

Identifier: NCT01218893; (9) and 8 newly recruited malaria-naïve subjects aged 18-35 years were all screened in July 2012 for eligibility based on medical and family history, physical examination and standard hematological and biochemical measurements (Figure 1).

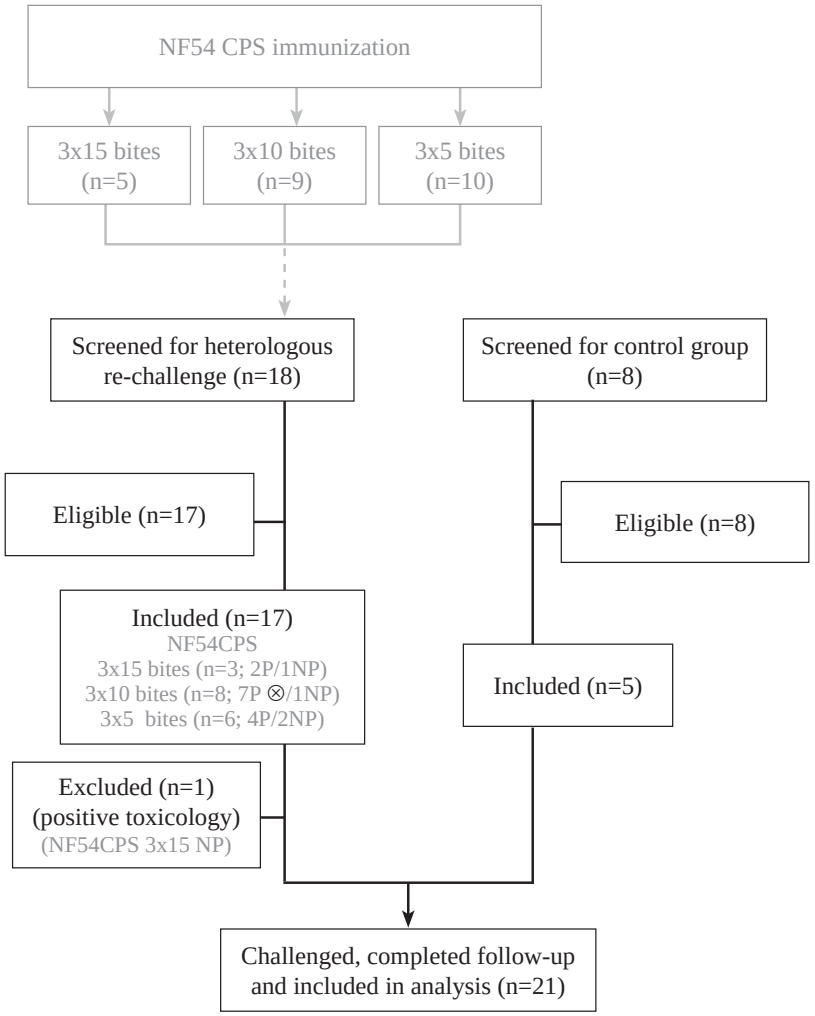

Figure 1 Study flow diagram. The previous NF54 CPS immunization study is shown in grey. P=NF54 protected, NP=NF54 unprotected. ⊗=Volunteer presumptively treated on day 10.5 after NF54 challenge and considered NF54 protected

Seventeen NF54 CPS-immunized volunteers and five controls were included. One volunteer had to be excluded because of a positive urine toxicology test for cannabis and was

treated with atovaquone/proguanil two days after challenge. Two of the remaining included volunteers had previously received the highest dose of NF54 CPS (3x15 bites), 8 a medium dose (3x10 bites) and 6 the lowest dose (3x5 bites). Thirteen were NF54 protected, of which one volunteer was presumptively treated because of a non-malaria related SAE on day 10,5 after NF54 challenge, but considered NF54 protected (9).

None of the female volunteers were pregnant or lactating. Serology for HIV, hepatitis B and hepatitis C was negative in all volunteers. Plasma samples tested by Enzyme-Linked ImmunoSorbent Assay (ELISA) against crude NF54 asexual blood stages were negative in all control volunteers. None of the volunteers had travelled to a malaria-endemic area within 6 months prior to the start of the study. All volunteers provided written informed consent before screening.

Study procedures

All volunteers were challenged simultaneously by exposure to five bites of *Anopheles stephensi* mosquitoes infected with the NF135.C10 *P. falciparum* clone (8) in August 2012. This heterologous challenge was performed 14 months after the last NF54 CPS immunization and 9.5 months after NF54 challenge. Volunteers were followed-up on an outpatient basis once daily on days 5-6 after challenge, twice daily between days 7-15 and once daily between days 16-21. During each visit, blood was drawn for parasite detection by thick smear. Volunteers were treated with 1000 mg atovaquone and 400 mg proguanil once daily for three days according to Dutch national malaria guidelines as soon as parasites were detected by thick smear, or on day 21 after challenge if they had remained thick smear negative. The last visit for volunteers was conducted in February 2013.

Safety parameters were determined daily: platelet counts were determined in EDTA blood with the Sysmex XE-2100 (Sysmex Europe GmbH. Norderstedt. Germany). D-dimer concentrations were assessed in citrate plasma by STA-R Evolution (Roche Diagnostics, Almere, The Netherlands; upper limit of detection 5000 ng/ml), Highly sensitive (Hs) Troponine T and Lactate Dehydrogenase (LDH) were determined in serum by Modular E170 (Roche Diagnostics, Almere, The Netherlands).

Endpoints

The primary endpoint was time to parasitemia after challenge infection as assessed by thick smear. Blood was screened by microscopy for parasites as described before, and the thick smear was considered positive if two unambiguous parasites were detected in 0.5µL of blood, confirmed by a second independent reader. Volunteers were considered protected when thick smears remained negative up until 21 days after challenge.

Secondary endpoints were the kinetics of parasitemia and frequency of signs and symptoms. Parasitemia was retrospectively quantified by qPCR on samples collected up

to twice daily from day 5 until day 21 after challenge as described previously (10) with some modifications. Briefly, 5µL Zap-oglobin II Lytic Reagent (Beckman Coulter) was added to 0.5ml of EDTA blood, after which the samples were mixed and stored at -80°C. After thawing, samples were spiked with the extraction control Phocine Herpes Virus (PhHV) and DNA was extracted with a MagnaPure LC isolation station. Isolated DNA was resuspended in 50µl H_2O and 5µl was used as template. For the detection of *P. falciparum*, the primers as described earlier (10) and the TaqMan MGB FAM-labelled probe 5'-AACAATTGGAGGGCAAG-3' were used. For quantification of PhHV the primers 5'-GGGCGAATCACAGATTGAATC-3', 5'-GCGGTTCCAAACGTACCAA-3' and the probe Cy5-5'-TTTTTATGTGTCCGCCACCATCTGGATC-3' were used.

Adverse events (AEs) reported by volunteers or observed by the investigator were recorded according to the following scale: mild (grade 1; easily tolerated), moderate (grade 2; interferes with normal activity) or severe (grade 3; prevents normal activity). Fever was recorded as grade 1 (37.5-38.0°C), grade 2 (38.0-39.0°C) or grade 3 (>39.0°C).

Statistical analysis

All possibly and probably (both solicited and unsolicited) related AEs were tabulated, grouped and analyzed by calculating the average number of mild, moderate or severe AEs per volunteer in each group. Statistical analyses were performed using GraphPad Prism 6.02. Differences in prepatent period and parasitemia at time of treatment between two groups (NF54 protected and controls) were tested by Mann Whitney test, and between the three dose groups by Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. A p value of <0.05 was considered statistically significant.

Results

Heterologous protection induced by CPS immunization

Sterile heterologous protection against NF135.C10 was complete in 15% (2/13) of NF54 protected volunteers (Figure 2A). Patency was significantly delayed in the other 11 volunteers, indicative of partial protection [median prepatent period determined by thick smear was 10.5 days (range 9.0-15.5) versus 8.5 days (range 8.5-8.5) in controls; p=0.0005 (Table 1, Figure 2B)]. Seven out of 11 partially protected subjects showed a delay in patency by qPCR of at least 48 hours, and thus more than one *P. falciparum* multiplication cycle.

The 3 volunteers previously not protected against NF54 were neither protected against NF135.C10 (Table 1, Figure 1). The prepatent period by thick smear did not differ significantly between NF54 CPS immunization dose groups (Figure 2C/2D). Parasitemia at time of treatment was higher in controls compared to CPS-immunized (p=0.047; Figure 3).

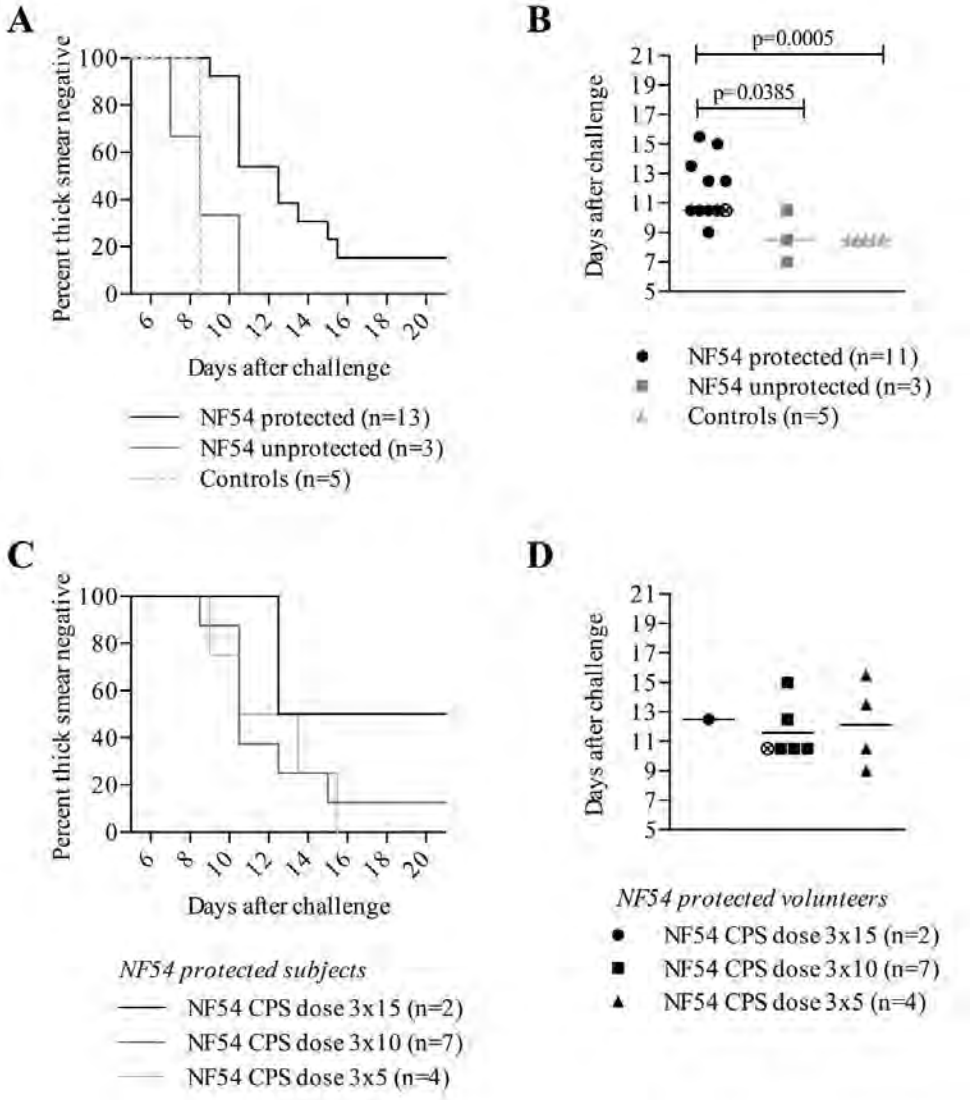

Figure 2 Protection and prepatent period after heterologous NF135.C10 challenge. Left panels: Kaplan-Meier curves showing percentage of thick smear negative volunteers after NF135.C10 challenge according to previous NF54 protection status (A) and NF54 CPS immunization dose for NF54-protected subjects (C). Right panels: The corresponding distribution of prepatent period of thick smear positive volunteers is shown in dot plots according to NF54 protection status (B) and NF54 CPS immunization dose (D). Lines represent medians. ⊗=Volunteer presumptively treated after NF54 challenge and considered NF54 protected.

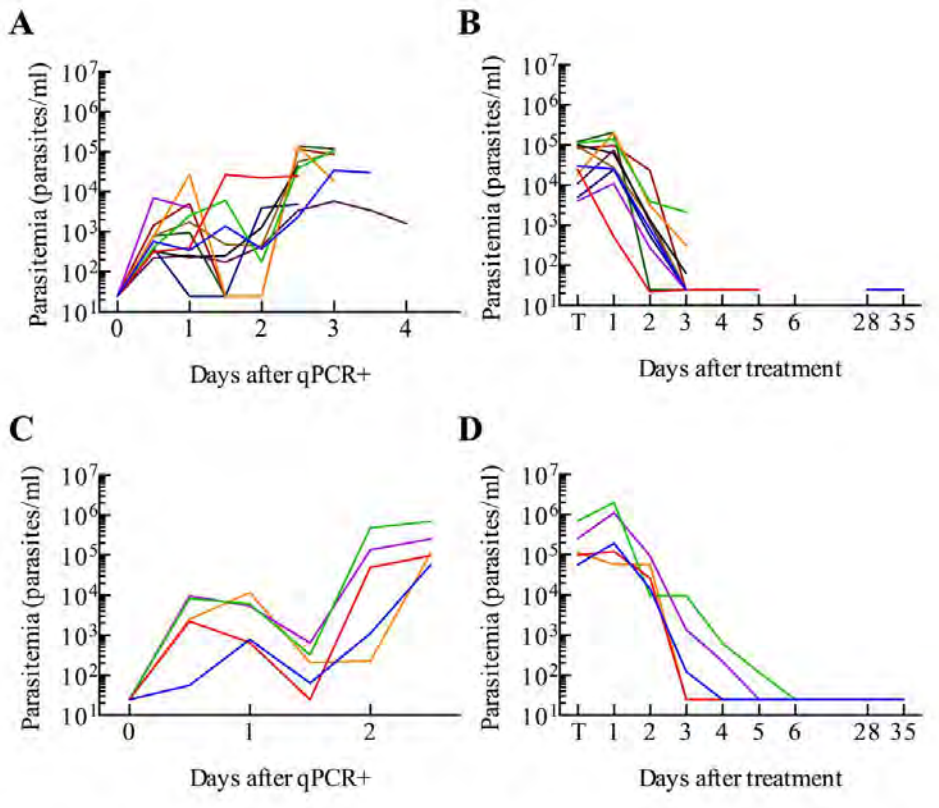

Figure 3 Parasitemia before and after treatment. Parasitemia measured by qPCR up until initiation of treatment (A and C) and from treatment onwards (B and D) in previously NF54 protected volunteers (A and B) and controls (C and D). Each line represents an individual subject with the same colour before and after treatment. Values shown as 25 Pf/ml were negative (i.e. half the detection limit of the qPCR: 50 parasites/ml).

Adverse events

All volunteers reported possibly or probably related AEs after challenge. Partially protected volunteers and controls showed a peak of AEs on the first day after start of treatment (Figure 4). Fourteen volunteers experienced related grade 3 AEs, which were more frequently reported in partially protected than in control volunteers (8/10 versus 2/5 respectively). There were no serious AEs. In partially protected volunteers, delayed patency concurred with earlier onset of AEs relative to the time of parasite-detection by thick smear. While control volunteers did not experience any AEs up until one day before parasite-detection by thick smear, partially protected volunteers experienced AEs as early as three days before thick smear positivity.

Table 1 Protection against NF135.C10 challenge after NF54 CPS immunization

	NF135.C10 Protected (n)	**NF135.C10 TS+ (n)**	**Prepatent period [a]**
Previously NF54 protected			
3x15	1	1	12.5
3x10	1	6	10.5 (10.5-15.0)
3x5	0	4	12.0 (9.0-15.5)
all	2	11	10.5 (9.0-15.5)***
NF54 unprotected			
3x15	0	0	
3x10	0	1	8.5
3x5	0	2	8.8 (7.0-10.5)
all	0	3	8.5 (7.0-10.5)
Malaria-naive controls	0	5	8.5 (8.5-8.5)

Sixteen previously CPS-immunized and challenged with *P. falciparum* NF54 volunteers in a CPS dose de-escalation and challenge trial were re-challenged with clone NF135.C10.

[a] in days, median (range). TS: Thick smear

***: p=0.0005 compared to controls

All controls and one partially protected volunteer showed persisting fever (maximum 39.0 °C) and/or mild to moderate complaints in the evening of day 3 after start of treatment. Resolution of the AEs took longer (up to 7 days) in controls compared to partially protected volunteers, and to historical controls (11). Additional thick smears performed in these volunteers on day 4, 5 and 6 after start of treatment were negative. All volunteers recovered fully without requiring additional antimalarial treatment.

Safety parameters

Hs troponin T concentrations remained within normal range (<0.03 µg/L) in all volunteers. LDH was elevated in ten volunteers after initiation of treatment (median maximum value 242 U/L, range 182-718 U/L) and returned within normal range (0-248 U/L) during follow-up. D-dimer levels were elevated in all volunteers (median maximum value 1748 ng/ml, range 524 – <5000 ng/ml) and returned within normal range (0-220 ng/ml) during follow-up. The number of platelets decreased below lower reference value ($150x10^9$/L) in 13 volunteers (median lowest value $127x10^9$/L, range 51-$275x10^9$/L) without apparent clinical manifestations of bleeding or thrombotic complications. Safety parameters returned within normal range in all volunteers after treatment.

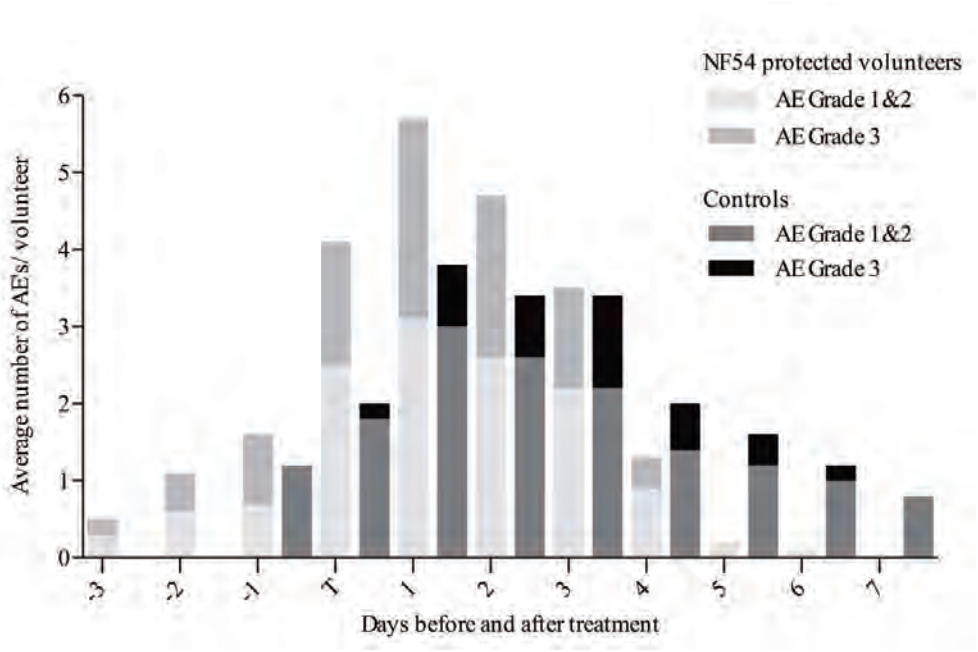

Figure 4 Adverse events before and after initiation of treatment. Average number of possibly and probably related (both solicited and unsolicited) AEs per previously NF54 protected or control volunteer in relation to the time of positive thick smear (day of treatment). Time points are plotted towards day of treatment, depicted as 'T', from 3 days before until 7 days after start of treatment.

Discussion

Our principle finding is that protection against a heterologous challenge infection with NF135.C10 is present in NF54 CPS-immunized and protected volunteers challenged more than one year before. Heterologous protection against NF135.C10 was complete in 15% (2/13) of volunteers while there was a delayed patency of more than 48 hours in 54% (7/13) of subjects. Taking into account a mean multiplication factor of 11.1 (11) and the presumed absence of functional blood stage immunity at this low parasitemia (3), this delay indicates that liver parasite load was reduced by approximately 91%. In three out of these seven volunteers a delay of more than two or three cycles was observed, indicating an estimated reduction of >99%. Three volunteers with no protection in the earlier homologous NF54 challenge study were also fully susceptible to NF135.C10.

Previous CPS studies showed that protection is mediated by immunity against pre-erythrocytic stages rather than asexual blood stages (3). NF135.C10 originates from Cambodia, while NF54, isolated near Schiphol Amsterdam airport, likely originates from West Africa

(8). Both isolates show distinct differences in genes encoding three well-established antigens (MSP-1, MSP-2 and GLURP) as well as in the rif repetitive elements (8). The target antigens of CPS-mediated protection remain to be elucidated in further studies including possible differences in antigen-specific responses to NF54 and NF135.C10.

Heterologous protection was incomplete in the majority of NF135.C10 re-challenged volunteers demonstrated by a delayed patency compared to controls. Apart from the genetic/antigenic variation between NF135.C10 and NF54, and thus insufficient breadth of the induced immune response, this incomplete heterologous protection may relate to a number of alternative explanations: i) Waning immunity: the heterologous challenge was performed at 14 months, rather than the usual 2 to 5 months post CPS immunization; ii) Suboptimal sporozoite immunization dose received by the majority (14/16) of volunteers, indicating an antigen threshold for complete protection (9). The minimally required immunization dose may increase for longevity of homologous protection and may be even higher for (long-lasting) heterologous protection. This trial was not powered to detect any dose-response relationships, but the two fully protected volunteers had indeed been immunized with the medium and high dose; iii) A possible difference between NF54 and NF135C.10 in sporozoite infectivity for liver cell invasion and/or maturation. This is supported by the higher first peak of NF135.C10 parasitemia compared to historical NF54 controls (2871 *Pf*/ml versus 456 *Pf*/ml respectively (11).

In partially protected volunteers, delayed patency concurred with earlier onset of AEs in relation to parasite-detection by thick smear. This might be due to the longer time frame before parasitemia reaches the thick smear detection limit. Alternatively, early immune recognition of blood stage parasites by the host may result in an increased inflammatory response and subsequent increase in AEs. A comparable effect was observed in a previous trial, where CPS-immunized subjects who received a blood-stage challenge developed inflammatory markers and fever earlier than naïve controls (3).

Compared to partially protected volunteers, control volunteers showed prolonged AEs after treatment. This continuation of AEs until day 7 after treatment has not been observed in previous CHMI trials with either strain NF54 or NF135.C10, neither in the CPS studies nor in RAS studies (5). Whether this represents an incidental finding or strain-specific characteristics needs to be investigated in future trials.

In conclusion, NF54 CPS immunization induces heterologous protection against the geographically and genetically distinct *P. falciparum* NF135.C10 clone. Increasing the immunization dose, altering the immunizing strain, or even immunization with a combination of strains may further improve protection. These results and further optimization of CPS immunization regimens will prove highly valuable for the clinical development of whole sporozoite vaccines.

Acknowledgements

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CHAPTER 10

Sporozoite immunization of human volunteers under mefloquine prophylaxis is safe, immunogenic and protective: a double-blind randomized controlled clinical trial

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PLoS One. 2014 Nov 14;9(11):e112910.

Abstract

Immunization of healthy volunteers with chloroquine ChemoProphylaxis and Sporozoites (CPS-CQ) efficiently and reproducibly induces dose-dependent and long-lasting protection against homologous *Plasmodium falciparum* challenge. Here, we studied whether chloroquine can be replaced by mefloquine, which is the only other licensed anti-malarial chemoprophylactic drug that does not affect pre-erythrocytic stages, exposure to which is considered essential for induction of protection by CPS immunization. In a double blind randomized controlled clinical trial, volunteers under either chloroquine prophylaxis (CPS-CQ, n = 5) or mefloquine prophylaxis (CPS-MQ, n = 10) received three sub-optimal CPS immunizations by bites from eight *P. falciparum* infected mosquitoes each, at monthly intervals. Four control volunteers received mefloquine prophylaxis and bites from uninfected mosquitoes. CPS-MQ immunization is safe and equally potent compared to CPS-CQ inducing protection in 7/10 (70%) versus 3/5 (60%) volunteers, respectively. Furthermore, specific antibody levels and cellular immune memory responses were comparable between both groups. We therefore conclude that mefloquine and chloroquine are equally effective in CPS-induced immune responses and protection.

Introduction

Malaria remains one of the most important infectious diseases worldwide and still causes approximately 207 million cases and 627,000 deaths every year (1). Anti-disease immunity against malaria is not easily induced: in endemic areas this takes many years of repeated exposure to develop (2), and sterile protection against infection does not seem to be induced at all (3). Also candidate vaccines have shown only limited protective efficacy so far (4, 5). Novel vaccines and drugs can be tested for efficacy at an early stage of clinical development in Controlled Human Malaria Infection (CHMI) studies, exposing a small number of healthy volunteers to *Plasmodium falciparum* by bites from infected *Anopheles* mosquitoes. Immunization of healthy volunteers under chloroquine ChemoProphylaxis with Sporozoites (CPS-CQ immunization) efficiently, reproducibly and dose-dependently induces protection against homologous CHMI (6, 7), shown in a subset of volunteers to last for more than 2 years (8). CPS-CQ immunization requires exposure to bites from only a total of 30-45 *P. falciparum* infected mosquitoes to induce 89–95% protection (6, 7, 9). In contrast, protection by immunization with radiation-attenuated sporozoites (RAS) requires a minimum of 1000 infected mosquito bites (10), or intravenous injection of five times 135,000 cryopreserved sporozoites (11).

The unprecedented efficiency of the CPS immunization regime may relate to its design: in contrast to RAS, CPS immunization allows full liver stage development and exposure to early blood-stages. Moreover, chloroquine is known for its immunomodulatory capacities (12-14) that may play a role in induction of protection, which is mediated by pre-erythrocytic immunity (9) including antibodies directed against sporozoites (15-17), and likely T cells targeting liver-stages (7). Next to chloroquine, mefloquine (MQ) is the only licensed drug for chemoprophylaxis that does not affect pre-erythrocytic stage development (18). We therefore aimed to assess whether chloroquine could be replaced by mefloquine for CPS immunization. In a double blind randomized controlled clinical trial we assessed safety, immunogenicity and protection against challenge for CPS-MQ compared to CPS-CQ.

Methods

Study subjects

Healthy subjects between 18 and 35 years old with no history of malaria were screened for eligibility based on medical and family history, physical examination and standard hematological and biochemical measurements. Urine toxicology screening was negative in all included subjects; none of the subjects were pregnant or lactating. Serological analysis for HIV, hepatitis B, hepatitis C and *P. falciparum* asexual blood-stages was negative in all subjects. All subjects had an estimated 10-year risk smaller than 5% of developing a

cardiac event as estimated by the Systematic Coronary Evaluation System adjusted for the Dutch population (19). None of the subjects had travelled to a malaria-endemic area during or within 6 months prior to the start of the study. All subjects provided written informed consent before screening. The Central Committee for Research Involving Human Subjects of The Netherlands approved the study (NL 37563.058.11). Investigators complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. This trial is registered at ClinicalTrials.gov, identifier NCT01422954.

Study design and procedures

This single center, double blind randomized controlled trial was conducted at Leiden University Medical Center (Leiden, the Netherlands) from April 2012 until April 2013

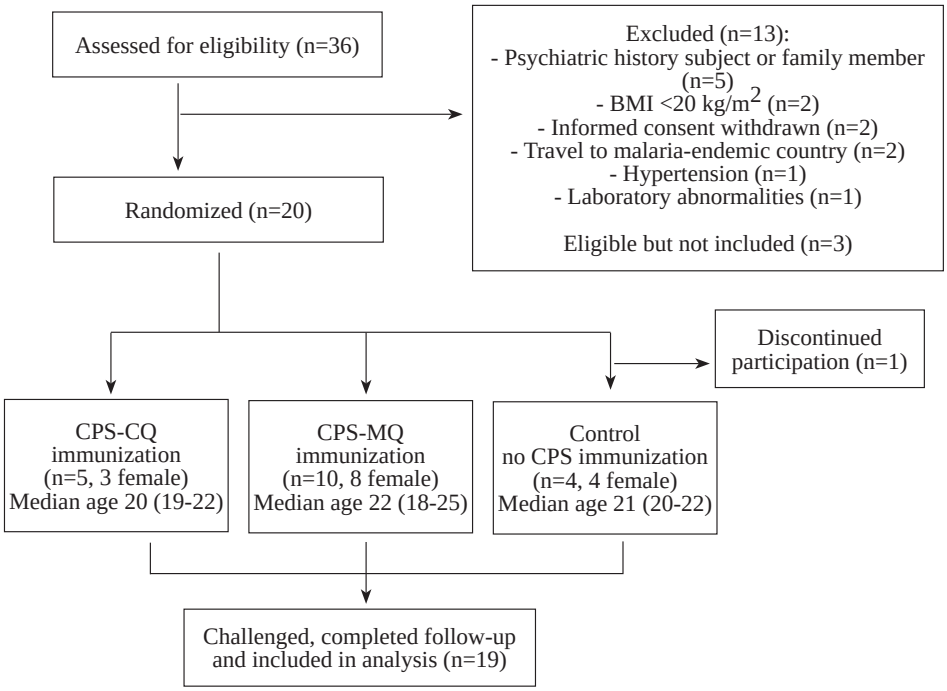

Figure 1 Study flow diagram. Thirty-six subjects were screened for eligibility, of whom twenty were included in the trial and randomized over three groups. One control subject was excluded after initiation of chemoprophylaxis but before the first immunization because of an unexpected visit to a malaria-endemic area during the study period. In a double-blind fashion, fifteen subjects received either CPS-CQ or CPS-MQ immunization and four control subjects received bites from uninfected mosquitoes and mefloquine prophylaxis. Subjects received a challenge infection by bites of five infected mosquitoes sixteen weeks after discontinuation of prophylaxis.

(Figure 1). Twenty subjects were randomly divided into three groups by an independent investigator using a computer-generated random-number table. Subjects, investigators and primary outcome assessors were blinded to the allocation. Subjects in the CPS-CQ group (n = 5) received a standard prophylactic regimen of chloroquine consisting of a loading dose of 300 mg on the first and fourth day and subsequently 300 mg once a week for 12 weeks. Subjects in the CPS-MQ group (n = 10) and the control group (n = 5) received mefloquine prophylaxis starting with a loading split dose regimen to limit potential side-effects: 125 mg twice per week for a duration of 3 weeks and subsequently 250 mg once a week for 12 weeks. Chloroquine and mefloquine were administered as capsules, indistinguishable from each other. During this period all subjects were exposed to the bites of 8 *Anopheles* mosquitoes three times at monthly intervals, starting 22 days after start of mefloquine prophylaxis and 8 days after start of chloroquine prophylaxis.

Volunteers in the CPS-CQ and CPS-MQ groups received bites from mosquitoes infected with the *P. falciparum* NF54 strain, control subjects received bites from uninfected mosquitoes. The immunization dose was based on our previous dose-de-escalation trial (7) and aimed to establish partial protection in the CPS-CQ group in order to enable detection of either improved or reduced protection in the CPS-MQ group. Sample sizes were calculated based on the expected difference of 4 days in prepatent period between the CPS-CQ and CPS-MQ groups, a standard deviation of 1.6 and 2.3 days respectively, an α of 5% and a power of 0.90. This calculation resulted in a CPS-CQ group of 4 and a CPS-MQ group of 8 subjects. To account for possible dropouts based on (perceived) side effects we included one and two extra volunteers in the CPS-CQ and CPS-MQ groups respectively. The control group was included as infectivity control for the challenge infection.

On days 6 to 10 after each immunization by mosquito exposure, all subjects were followed on an outpatient basis and peripheral blood was drawn for blood smears, standard hematological measurements, cardiovascular markers and retrospective qPCR.

Twenty weeks after the last immunization, sixteen weeks after discontinuation of prophylaxis, all subjects were challenged by the bites of five mosquitoes infected with the homologous NF54 *P. falciparum* strain, according to previous protocols (20). After this challenge-infection, all subjects were checked twice daily on an outpatient basis from day 5 up until day 15 and once daily from day 16 up until day 21 for symptoms and signs of malaria. Thick blood smears for parasite detection were made during each of these visits after challenge, hematological and cardiovascular markers were assessed daily. As soon as parasites were detected by thick smear, subjects were treated with a standard curative regimen of 1000 mg atovaquone and 400 mg proguanil once daily for three days according to Dutch national malaria treatment guidelines. If subjects remained thick smear negative, they were presumptively treated with the same curative regimen on day 21 after challenge infection. All subjects were followed closely for 3 days after initiation of treatment and

complete cure was confirmed by two negative blood smears after the last treatment dose. Chloroquine and mefloquine levels were measured retrospectively in citrate-plasma from the day before challenge by liquid chromatography (detection limit for both chloroquine and mefloquine: 5 µg/L) (21).

Anopheles stephensi mosquitoes for immunizations and challenge-infection were reared according to standard procedures at the insectary of the Radboud university medical center. Infected mosquitoes were obtained by feeding on NF54 gametocytes, a chloroquine- and mefloquine-sensitive *P. falciparum* strain, as described previously (22). After exposure of volunteers, all blood-engorged mosquitoes were dissected to confirm the presence of sporozoites. If necessary, feeding sessions were repeated until the predefined number of infected or uninfected mosquitoes had fed.

Endpoints

The primary endpoint was prepatent period, defined as the time between challenge and first positive thick blood smear. Secondary endpoints were parasitemia and kinetics of parasitemia as measured by qPCR, adverse events and immune responses.

Detection of parasites by thick smear

Blood was sampled twice daily from day 5 until day 15 and once daily from day 16 up until day 21 after challenge and thick smears were prepared and read as described previously (9). In short, approximately 0.5 µl of blood were assessed by microscopy and the smear was considered positive if two unambiguous parasites were seen.

Quantification of parasitemia by qPCR

Retrospectively, parasitemia was quantified by real-time quantitative PCR (qPCR) on samples from day 6 until day 10 after each immunization and from day 5 until day 21 after challenge as described previously (23), with some modifications. Briefly, 5 µl Zap-Oglobin II Lytic Reagent (Beckman Coulter) was added to 0.5 ml of EDTA blood, after which the samples were mixed and stored at –80°C. After thawing, samples were spiked with the extraction control Phocine Herpes Virus (PhHV) and DNA was extracted with a MagnaPure LC isolation instrument. Isolated DNA was resuspended in 50 µl H_2O, and 5 µl was used as template. For the detection of *P. falciparum*, the primers as described earlier (23) and the TaqMan MGB probe AAC AAT TGG AGG GCA AG-FAM were used. For quantification of PhHV the primers GGGCGAATCACAGATTGAATC, GCGGTTCCAAACGTACCAA and the probe Cy5-TTTTTATGTGTCCGCCACCATCTGGATC were used. The sensitivity of qPCR was 35 parasites/ml of whole blood.

Adverse events and safety lab

Adverse events (AEs) were recorded as following: mild events (easily tolerated), moderate events (interfering with normal activity), or severe events (preventing normal activity). Fever was recorded as grade 1 (>37×5°C–38×0°C), grade 2 (>38×0°C–39×0°C) or grade 3 (>39×0°C). Platelet and lymphocyte counts were determined in EDTA-anti-coagulated blood with the Sysmex XE-2100 (Sysmex Europe GmbH, Norderstedt, Germany). D-dimer concentrations were assessed in citrate plasma by STA-R Evolution (Roche Diagnostics, Almere, The Netherlands).

Immunological analyses

In order to assess cellular immune memory responses, peripheral blood mononuclear cell (PBMC) re-stimulation assays were performed as described previously (7). PBMCs were collected, frozen in fetal calf serum containing 10% dimethylsulfoxide, and stored in vapor phase nitrogen before initiation of prophylaxis (baseline; B) and one day before the challenge infection (C-1).

After thawing, PBMCs were re-exposed *in vitro* to *P. falciparum*-infected red blood cells (*Pf*RBC) and incubated for 24 hours at 37°C in the presence of a fluorochrome-labeled antibody against CD107a. Uninfected red blood cells (uRBCs) were used as a negative control. During the last 4 hours of incubation, 10 µg/ml Brefeldin A and 2 µM Monensin were added, allowing cytokines to accumulate within the cells. As a positive control, 50 ng/ml PMA and 1 mg/ml ionomycin were added for the last four hours of incubation. After 24h stimulation, cells were further stained with a viability marker and fluorochrome-labeled antibodies against CD3, CD4, CD8, CD56, γδ-T cell receptor, IFNγ and granzyme B (Table S1 (7)). For each volunteer, cells from all time points were tested in a single experiment: thawed and stimulated on the same day and stained the following day. Samples were acquired on a 9-color Cyan ADP (Beckman Coulter) and data analysis was performed using FlowJo software (version 9.6.4; Tree Star). A representative example showing the full gating strategy is shown in Figure S1. Gating of cytokine-positive cells was performed in a standardized way by multiplying a fixed factor with the 75 percentile of the geometric Mean Fluorescent Intensity (MFI) of cytokine negative PBMCs for each volunteer, time point and stimulus. Responses to uRBC were subtracted from the response to *Pf*RBC for each volunteer on every time point.

Plasma for the assessment of malaria-specific antibodies was collected and stored at baseline (B), 27 days after the first immunization (I1; one day before the second immunization), 27 days after the second immunization (I2; one day before the third immunization), and one day before the challenge infection (C-1). Antibody titers were assessed as described previously (17). In summary, serially diluted citrate plasma was used to perform standardized enzyme-linked immunosorbent assay (ELISA) in NUNC™ Maxisorp plates (Thermo

Scientific) coated with 1 µg/ml circumsporozoite protein (CSP), liver-stage antigen-1 (LSA-1) or merozoite surface protein-1 (MSP-1) antigen, diluted in PBS. Bound IgG was detected using horseradish peroxidase (HRP) conjugated anti-human IgG) (Thermo Scientific, 1/60000) and Tetramethylbenzidine (all Mabtech). Spectrophotometrical absorbance was measured at 450 nm. OD values were converted into AUs by four-parameter logistic curve fit using Auditable Data Analysis and Management System for ELISA (ADAMSEL-v1.1, http://www.malariaresearch.eu/content/software; accessed 27 October 2014). Levels of antibodies were calculated in relation to a pool of 100 sera from adults living in a highly endemic area in Tanzania (HIT serum (24)), which was defined to contain 100 arbitrary units (AU) of IgG directed against each antigen.

Statistical analyses

The proportion of protected subjects in the CPS-CQ versus CPS-MQ group was tested with the Fisher's exact test using Graphpad Quickcalcs online and the 95% confidence interval (CI) of protection for each group was calculated by modified Wald Method (25). Further statistical analyses were performed with GraphPad Prism 5. Differences in prepatent period and time from qPCR positivity until thick smear positivity were tested by Mann Whitney test. Antibody levels are shown as individual titers with medians and differences between time points were analyzed by Friedman test with Dunn's multiple comparison post-hoc test. Induction of cellular immune responses was tested for CPS-CQ and CPS-MQ groups separately by Wilcoxon matched-pairs signed rank test (B versus C-1). A p-value of <0.05 was considered statistically significant. Analyses of parasitemia were performed on log transformed data, the geometric mean peak parasitemia after each immunization was calculated using the maximum parasitemia for each subject.

Results

Safety of CPS-CQ and CPS-MQ immunization

Twenty out of 36 screened subjects (median age 21 years; range 18–25) were included in the study (Figure 1). One control subject was excluded between start of prophylaxis and the first immunization because of an unexpected intermittent visit to a malaria-endemic area. Thick blood smears performed from day 6 up until day 10 after each immunization remained negative in all volunteers. As determined retrospectively by qPCR, 2/5 subjects in the CPS-CQ group and 7/10 subjects in the CPS-MQ group showed sub-microscopic parasitemia after the first immunization (geometric mean peak parasitemia for positive subjects: 948 parasites/ml [range 228–3938] and 256 parasites/ml [range 48–1559] respectively, Figure 2). After the second immunization, four CPS-MQ subjects showed sub-microscopic parasitemia (geometric mean peak parasitemia for positive subjects 104 parasites/ml [range 48–223]),

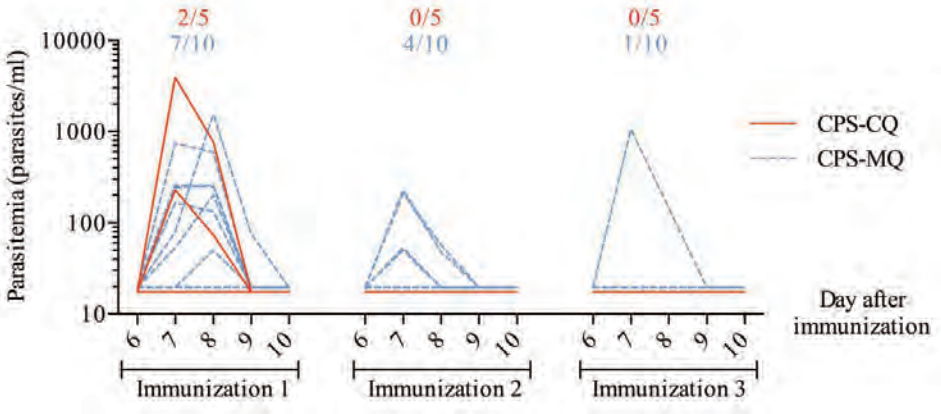

Figure 2 Parasitemia during CPS immunization. Parasitemia was determined retrospectively, once daily from day 6 until day 10 after each immunization, by real-time quantitative PCR (qPCR). Each line represents an individual subject from the CPS-MQ (dashed blue lines) or CPS-CQ group (red lines). The number of subjects with a positive qPCR/total number of volunteers in the CPS-MQ (blue) and CPS-CQ (red) groups after each immunization are shown above the graph. Values shown as 17.5 on the log-scale were negative (i.e. half the detection limit of the qPCR: 35 parasites/ml).

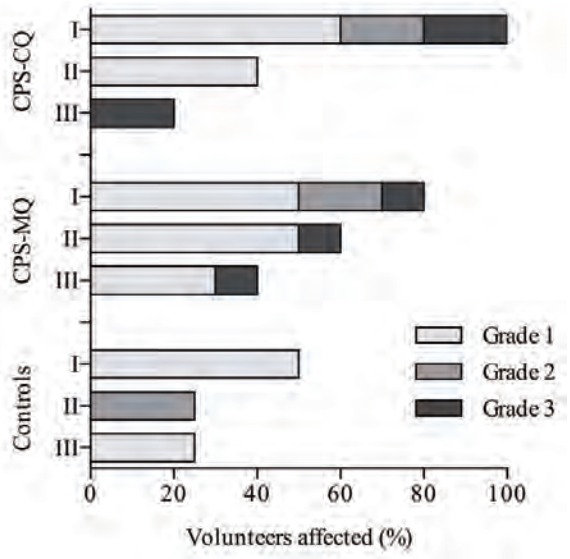

Figure 3 Adverse events during CPS immunization. Percentage of volunteers in each group experiencing possibly or probably related AE after the first (I), second (II) and third (III) immunization. AEs were evaluated at each visit and graded for severity as described in the methods paragraph: mild (light grey), moderate (dark grey) and severe (black). Only the highest intensity per subject is listed. No Serious Adverse Events occurred.

while none of the CPS-CQ subjects showed parasitemia. After the third immunization, only one CPS-MQ subject showed parasitemia by qPCR (peak parasitemia 1059 Pf/ml).

After the first immunization, all subjects (5/5) in the CPS-CQ group and almost all CPS-MQ subjects (8/9) experienced possibly or probably related AEs. One subject in each group had a grade 3 AE (headache and vomiting, respectively). Two control volunteers reported mild AEs (Figure 3 and Table S2). After the second immunization, two CPS-CQ volunteers and six volunteers in the CPS-MQ group had mild AEs. Two control subjects experienced moderate and severe headache, respectively. After the third immunization, one volunteer in the CPS-CQ group and four CPS-MQ volunteers had AEs; one control subject experienced mild AEs (Figure 3 and Table S2). One CPS-CQ subject reported moderate sleeping problems while taking chloroquine prophylaxis. One control subject had moderate problems with initiation of sleep and another control subject experienced vivid dreams under mefloquine prophylaxis. Other than mild to moderate dizziness and sleep related AEs, which all resolved after chemoprophylaxis was stopped, no neuropsychiatric AEs occurred. No serious adverse events occurred.

During immunization, one subject each in the CPS-CQ, CPS-MQ and control groups showed platelet counts below the lower limit of normal ($150x10^9$/L); lowest values $105x10^9$/L, $116x10^9$/L and $131x10^9$/L, respectively. Three, five and two subjects from the

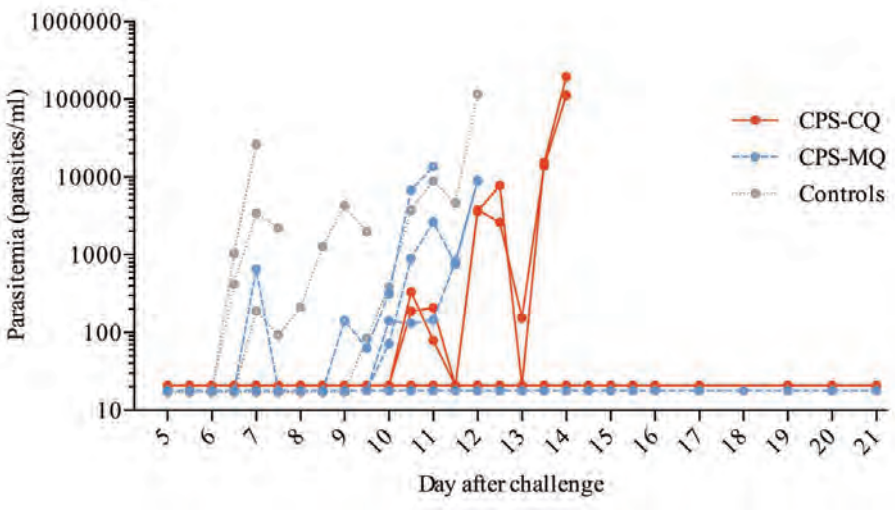

Figure 4 Parasitemia after challenge infection. Parasitemia was assessed retrospectively by real-time quantitative PCR (qPCR) twice daily from day 5 until day 15 and once daily up until day 21 after challenge. Each line represents an individual subject. Red lines represent CPS-CQ immunized volunteers (n = 5), dashed blue lines CPS-MQ immunized subjects (n = 10) and dotted grey lines malaria-naive control subjects (n = 4). Values shown as 17.5 on the log-scale were negative (i.e. half the detection limit of the qPCR: 35 parasites/ml).

CPS-CQ, CPS-MQ and control groups respectively, showed leukocyte counts below the lower limit of normal ($4x10^9$/L); mean lowest value during immunization period: $3.8x10^9$/L [SD 1.2], $4.0x10^9$/L [SD 1.1] and $4.2x10^9$/L [SD 0.7] respectively. No subject developed leukocyte counts lower than $2.0x10^9$/L. One volunteer in each group showed leukocyte counts above the upper limit of normal ($10x10^9$/L; highest values $10.8x10^9$/L, $13.8x10^9$/L and $10.1x10^9$/L respectively). After the first immunization, 3/5 CPS-CQ subjects, 7/10 in the CPS-MQ group and none in the control group developed elevated d-dimer levels (>500 ng/ml). After the second immunization, six CPS-MQ subjects but none in the CPS-CQ or control groups showed elevated d-dimer levels. After the third immunization, three CPS-MQ subjects showed elevated d-dimer levels, while none of the subjects in the other groups did.

Protection against challenge infection

In the CPS-CQ group 3/5 subjects and in the CPS-MQ group 7/10 volunteers were protected against challenge infection (Fisher's exact test p = 1.0). All control subjects became thick smear positive (median day 8.5, range 7–12, p = 0.03 versus CPS-immunized subjects; Table 1). None of the protected subjects showed parasitemia by qPCR at any time point during follow-up (Figure 4). The median prepatent period was not significantly different between the CPS-CQ and CPS-MQ groups, neither when protected subjects were arbitrarily set at a prepatent period of 21 days (p = 1.00), nor when comparing unprotected subjects only (p = 0.1). The median chloroquine plasma concentration on the day before challenge infection was 9 µg/L (range 7–10) in the CPS-CQ group, and the median mefloquine concentration was 24 µg/L (range 5–116) in the mefloquine groups.

Table 1 Protection against challenge infection after CPS-CQ and CPS-MQ immunization.

Group	Protection			Unprotected volunteers					
				Day of positivity after challenge[c]					
	n	%	p	Thick smear	p	qPCR	p	ΔTS+qPCR+[c]	p
CPS-CQ	3/5	60 (23-88)		14.0 (14.0-14.0)		11.3 (10.5-12.0)		2.8 (2.0-3.5)	
CPS-MQ	7/10	70 (39-90)	1.0[d]	12.0 (11.0-12.0)	0.10[f]	10.0 (9.0-10.0)	0.10[f]	2.0 (2.0-2.0)	0.40[f]
Control	0/4	0% (0-55)	0.03[e]	8.5 (7.0-12.0)	0.048[g]	6.3 (5.0-9.5)	0.056[g]	2.5 (1.5-2.5)	0.70[g]

[a] Presented as protected/total number of subjects

[b] Presented as % protected (95% CI by modified Wald Method)

[c] Presented as median (range) days.

[d,e] p-value calculated by Fisher's exact test comparing [d]CPS-MQ versus CPS-CQ or [e]control versus all CPS-immunized subjects

[f,g] p-value calculated by Mann Whitney test comparing [f]CPS-MQ versus CPS-CQ or [g]control versus all CPS-immunized subjects (both excluding protected subjects)

Immunogenicity of CPS-CQ and CPS-MQ

Antibodies against the pre-erythrocytic antigens CSP and LSA-1 and the cross-stage antigen MSP-1 were assessed by ELISA. Antibodies against CSP were induced in both CPS-CQ and CPS-MQ immunized volunteers ($p<0.05$ and $p<0.01$ respectively, on C-1; Figure 5A and 5B), but not significantly higher in protected compared to unprotected subjects ($p = 0.88$ and $p = 0.48$ respectively). Antibodies against LSA-1 were only significantly induced in CPS-MQ immunized volunteers on I2 ($p<0.001$; Figure 5C and 5D), although not higher in protected subjects ($p = 0.39$). Anti-MSP-1 antibodies by CPS immunization were not statistically significant increased in either group (Figure 5E and 5F).

IFNγ production by both adaptive and innate cell subsets in response to *in vitro P. falciparum* re-stimulation was induced by both CPS-CQ and CPS-MQ (Figure S2), without a clear quantitative or qualitative difference between the study groups. Next, CD107a expression by CD4 T cells and granzyme B production by CD8 T cells, both associated with protection in a previous CPS-CQ trial (7), were assessed by flow cytometry. Four out of 5 CPS-CQ and 8/10 CPS-MQ immunized subjects showed induction of CD107a expression by CD4 T cells upon *in vitro* re-stimulation after immunization (Figure 6A and 6B). Although volunteer numbers were too low to reach statistical significance, the magnitude of this response appeared to be associated with protection for CPS-CQ (Figure 6A), while for CPS-MQ it was not (Figure 6B). Granzyme B production by CD8 T cells was not significantly induced in either CPS-CQ or CPS-MQ group, nor was it associated with protection (Figure 6C and 6D).

After challenge, MSP-1 specific antibodies were boosted in all unprotected volunteers (fold change median 20.4 (range 7.1–33.6), 76.0 (5.7–06.3) and 7.7 (2.9–15.3) for CPS-CQ, CPS-MQ and control groups respectively). None of the protected subjects showed an increase in MSP-1 antibody levels on C+35 compared to C-1 (median fold change 1.0 (range 1.0–1.3) and 1.0 (0.6–2.4) for CPS-CQ and CPS-MQ groups, respectively).

Discussion

Immunization of healthy volunteers with *P. falciparum* sporozoites while taking mefloquine prophylaxis is safe, induces both humoral and cellular immune responses and protects against homologous malaria challenge.

Although most volunteers experienced AEs after the first immunization, their frequency declined after subsequent immunizations in line with a reducing number of volunteers developing parasitemia. The majority of AEs was mild, with only 10–20% of subjects experiencing a grade 3 AEs after each immunization. In general, the reported neurologic and psychiatric side effects of mefloquine are a major concern limiting its acceptability and clinical application. In this study, mild to moderate dizziness and sleep-related complaints

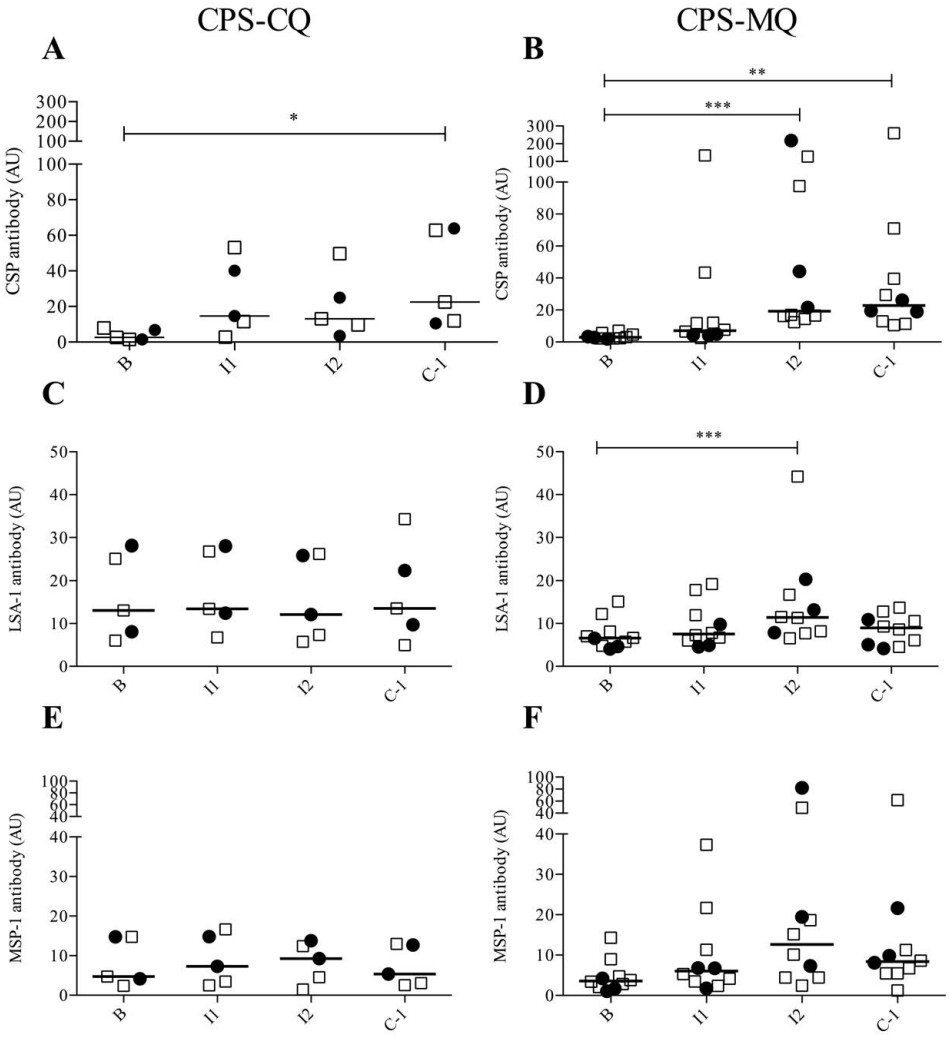

Figure 5 Antibody responses induced by CPS-CQ and CPS-MQ immunization. Antibodies against CSP (A and B; in AU), LSA-1 (C and D), and MSP-1 (E and F) were analyzed at baseline (B), 28 days after the first (I1) and second (I2) immunization and one day before challenge (C-1; 20 weeks after the last immunization) for all CPS-CQ (A, C and E, n = 5) and CPS-MQ (B, D and F, n = 10) immunized volunteers. Data are shown as individual titers with medians. Open squares indicate protected subjects, filled circles indicate unprotected subjects. Differences between the time points were analyzed by Friedman test with Dunn's multiple comparison post-hoc test. Significant differences are indicated by asterices with * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

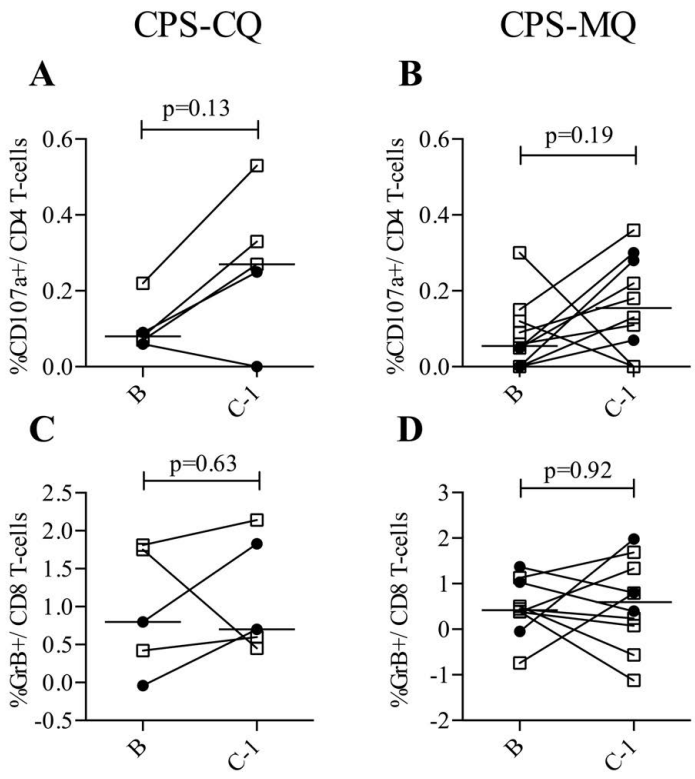

Figure 6 Cellular immune responses: CD107a expression by CD4 T cells and granzyme B production by CD8 T cells. CD107a expression by CD4 T cells after *Pf*RBC re-stimulation, corrected for uRBC background in CPS-CQ (A) and CPS-MQ (B) groups; granzyme B production by CD8 T cells after *Pf*RBC re-stimulation, corrected for uRBC background in CPS-CQ (C) and CPS-MQ (D) groups. Symbols and lines represent individual subjects before immunization (B) and one day before challenge (C-1). Open squares indicate protected subjects, filled circles indicate unprotected subjects. Differences between B and C-1 for all subjects were tested by Wilcoxon matched-pairs signed rank test.

occurred in a small number of subjects in both chloroquine and mefloquine groups. Although this study was not powered to detect differences in AEs, frequency of neuropsychiatric AEs did not appear to differ between both drugs. This is in line with most reports in literature comparing AEs of mefloquine or chloroquine (with or without proguanil) for chemoprophylactic use (26-29) although one study found more neuropsychiatric AEs in subjects taking mefloquine by retrospective questionnaire (30). Taking the small sample size into consideration, both CPS-CQ and CPS-MQ immunization regimens appear to be reasonably well tolerated and safe. In 2013, however, after completion of this study, the U.S. Food and Drug Administration (FDA) issued a boxed warning for mefloquine, stating that neurologic

side effects might be permanent. This might lead to adjustment of prophylaxis guidelines and limitation of mefloquine use where alternatives are available, as for now it remains a recommended antimalarial prophylactic for several target groups (31).

In previous studies we showed that 19/20 subjects (95%) were protected after bites from 45 infected mosquitoes, 8/9 (89%) after bites from 30 and 5/10 (50%) after bites from 15 infected mosquitoes during chloroquine prophylaxis (6, 7, 9). The 60–70% protection observed in the current CPS-CQ and CPQ-MQ groups, immunized with bites from 24 mosquitoes, demonstrates the reproducibility of CPS immunization and indicates a linear relationship between immunization dose and protection. This confirms the consistency of the CPS approach and is remarkable, given the assumed variation in the number of sporozoites injected by mosquitoes (32). This study further establishes CPS immunization as a worthwhile immunization protocol to relatively easily induce protection and create differentially protected cohorts to study target antigens and correlates of protection, both of which would be highly valuable tools in the search for *P. falciparum* vaccines and biomarkers of protection (33).

Although the study was not powered to detect these differences, there are hints suggestive of more efficient induction of protection by CPS-CQ compared to CPS-MQ: i) the two unprotected CPS-CQ volunteers showed a longer prepatent period than the CPS-MQ subjects (14 versus 12 days, Mann-Whitney test $p = 0.13$); ii) induction of immunity required less immunizations in the CPS-CQ group i.e. none of these subjects showed blood-stage parasites after the second immunization while subjects in the CPS-MQ group still developed parasitemia after the second and third immunization. If there is a difference between CPS-CQ and CPS-MQ in protective efficacy, it is small, but possibly detectable in larger cohorts or when the immunization dose is further reduced.

Induction of anti-circumsporozoite antibodies by CPS-CQ is consistent with previous work, but neither anti-LSA-1, nor MSP-1 antibodies were induced by CPS-CQ in the current study (17). Antibodies against the latter antigens are dose-dependently induced (17), and the current immunization regime using bites from 3×8 *P. falciparum*-infected mosquitoes might have been insufficient (7). The induction of cellular *P. falciparum*-specific memory responses, as reflected by IFNγ production, is in line with previous CPS-CQ studies, even though limited sample size hampered statistical significance for some cell types. Interestingly, CD107a expression by CD4 T cells upon *in vitro* re-stimulation, associated with protection in a previous CPS-CQ study (7), appeared again to be associated with protection in the CPS-CQ group, but not the CPS-MQ group. Granzyme B production by CD8 T cells upon *in vitro* re-stimulation did not appear to be a reproducible marker of protection in this second CPS study (7). Whether this might be related to immunization dose remains to be investigated in future CPS trials.

The striking efficiency of CPS immunization might at least be partly due to the

established immune modulating properties of the 4-amino-quinoline chloroquine (12), possibly reflected by the more efficient induction of degranulating CD4 T cells. Chloroquine has been shown to increase cross-presentation in hepatitis B vaccination and influenza (13, 14), and thus may enhance cellular immune responses considered essential for protection against liver-stages (12). For mefloquine, a 4-methanolquinoline, this immune-modulating property has, to our knowledge, not been reported. A possible strategy to assess whether chloroquine and/or mefloquine indeed have immune enhancing effects on whole sporozoite immunization would be to compare immunization with RAS in the presence or absence of these drugs.

Mefloquine or chloroquine plasma concentrations were still detectable in all volunteers one day before the challenge infection. Possible contributing effects of these remaining drug levels to the protective efficacy outcome were considered in several ways; i) The interval between first qPCR and thick smear positivity, as proxy for parasite multiplication, was 2.8 in the CPS-CQ group, 2.0 in the CPS-MQ group and 2.5 in the control group. This interval is similar to previous CHMI studies with the NF54 *P. falciparum* strain in the absence of prophylactic drug levels (7, 34); ii) the two volunteers with the highest mefloquine levels (116 and 77 µg/L) were control subjects who became thick smear positive with only a minimal delay in patency within the time-frame of historical controls (35); iii) plasma chloroquine and mefloquine levels at C-1 were in all volunteers well below the minimum therapeutic concentration (CQ: 30 µg/L (36)) or the concentration at which breakthrough infections are observed in non-immune people (MQ <406 – 603 µg/L (37)). iv) We cannot rule out that protected subjects experienced transient parasitemia after challenge, which was cleared in the first blood-stage cycle by remaining drug levels. But because parasitemia was not detected by qPCR in any of the protected subjects at any time point after challenge potential parasitemia must have been below the qPCR detection limit of 35 parasites/ml, indicating a reduction of at least 92% in liver load, given a geometric mean height of the first peak or parasitemia in non-immune historical controls of 456 parasites/ml (35); v) None of the protected subjects showed a boost in anti-MSP-1 antibodies after challenge while all unprotected subjects did, suggesting that protected subjects did not experience blood-stage parasitemia after challenge. (9). From these combined data we believe that remaining drug concentrations are unlikely to have contributed to the observed protection, although this cannot be formally excluded.

A review of rodent studies using different attenuation methods for whole sporozoite immunization shows that increased development of the parasite in the liver, but absence of blood-stage parasitemia during immunization is associated with the highest protective efficacy (38). It would therefore be interesting to investigate CPS immunization with alternative antimalarials with varying targets in the parasite life cycle. CPS immunization with causal prophylactic drugs affecting liver-stages, e.g. primaquine, will likely results in a reduction of AEs because of reduced or absent blood-stage exposure. Whether antigen-

exposure is sufficient to induce protection when the liver-stage is abrogated, remains to be answered.

In conclusion, we show that immunization of healthy volunteers under mefloquine prophylaxis with *P. falciparum* sporozoites is safe, immunogenic and protective. These findings could have important implications for malaria vaccine development and further development of CPS approaches.

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Supporting Information

Table S1 Antibodies used for flow cytometry.

Target	Fluorochrome	Clone	Supplier
Fixable viability	Aqua	N/A	eBioscience
CD3	PerCP	UCHT-1	Biolegend
CD4	ECD	SFCI12T4D11	BeckmanCoulter
CD8	APC-H7	SK1	BD Biosciences
γδTcell receptor	PE	IMMU510	BeckmanCoulter
CD56	Biotin	HCD56	Biolegend
Streptavidin	eF660	N/A	eBioscience
IFNγ	PeCy7	4S.B3	Biolegend
CD107a	Pacific Blue	H4A3	Biolegend
Granzyme B	FITC	GB11	Biolegend

Table S2 Possibly and probably related adverse events during CPS immunization*# (next page).
Number of volunteers (%)*; for each subject, only the highest intensity is listed.
#CQ, MQ: after start of respective chemoprophylaxis up until first immunization; I, II, III: after the first, second and third immunization respectively.

		CPS-CQ				CPS-MQ					Control		
		CQ	**I**	**II**	**III**	**MQ**	**I**	**II**	**III**	**MQ**	**I**	**II**	**III**
Abdominal pain	None	5 (100)	4 (80)	5 (100)	5 (100)	10 (100)	9 (90)	10 (100)	10 (100)	2 (50)	3 (75)	4 (100)	4 (100)
	Mild	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	1 (25)	1 (25)	0 (0)	0 (0)
	Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Arthralgia	None	5 (100)	5 (100)	5 (100)	5 (100)	10 (100)	10 (100)	10 (100)	10 (100)	4 (100)	4 (100)	4 (100)	4 (100)
	Mild	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Chest pain	None	5 (100)	5 (100)	5 (100)	5 (100)	10 (100)	10 (100)	10 (100)	10 (100)	4 (100)	4 (100)	4 (100)	4 (100)
	Mild	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Chills	None	5 (100)	5 (100)	5 (100)	5 (100)	10 (100)	10 (100)	10 (100)	10 (100)	4 (100)	4 (100)	4 (100)	4 (100)
	Mild	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Diarrhea	None	5 (100)	5 (100)	4 (80)	5 (100)	10 (100)	9 (90)	10 (100)	10 (100)	4 (100)	4 (100)	4 (100)	4 (100)
	Mild	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Dizziness	None	4 (80)	5 (100)	5 (100)	5 (100)	10 (100)	9 (90)	9 (90)	9 (90)	3 (75)	4 (100)	4 (100)	4 (100)
	Mild	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)
Fatigue	None	5 (100)	5 (100)	5 (100)	5 (100)	10 (100)	9 (90)	10 (100)	10 (100)	4 (100)	4 (100)	4 (100)	4 (100)
	Mild	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fever	None	5 (100)	4 (80)	5 (100)	5 (100)	10 (100)	7 (70)	10 (100)	8 (80)	4 (100)	4 (100)	4 (100)	4 (100)
	Mild	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (30)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Headache	None	3 (60)	0 (0)	4 (80)	4 (80)	7 (70)	4 (40)	5 (50)	7 (70)	4 (100)	4 (100)	3 (75)	3 (75)
	Mild	2 (40)	3 (60)	1 (20)	0 (0)	3 (30)	4 (40)	4 (40)	3 (30)	0 (0)	0 (0)	0 (0)	1 (25)
	Moderate	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	2 (20)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)
	Severe	0 (0)	1 (20)	0 (0)	1 (20)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Malaise	None	5 (100)	4 (80)	5 (100)	5 (100)	10 (100)	9 (90)	10 (100)	9 (90)	4 (100)	4 (100)	4 (100)	4 (100)
	Mild	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Myalgia	None	5 (100)	4 (80)	5 (100)	5 (100)	10 (100)	10 (100)	9 (90)	9 (90)	4 (100)	4 (100)	4 (100)	4 (100)
	Mild	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Nausea	None	4 (80)	4 (80)	5 (100)	5 (100)	10 (100)	7 (70)	10 (100)	10 (100)	2 (50)	4 (100)	4 (100)	4 (100)
	Mild	1 (20)	1 (20)	0 (0)	0 (0)	0 (0)	2 (20)	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Vomiting	None	5 (100)	4 (80)	5 (100)	5 (100)	10 (100)	9 (90)	10 (100)	10 (100)	4 (100)	4 (100)	4 (100)	4 (100)
	Mild	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Moderate	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Vivid dreams or other sleeping problems	None	4 (80)	5 (100)	5 (100)	5 (100)	10 (100)	10 (100)	10 (100)	10 (100)	2 (50)	3 (75)	4 (100)	4 (100)
	Mild	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25)	1 (25)	0 (0)	0 (0)
	Moderate	1 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Other	None	4 (80)	4 (80)	5 (100)	5 (100)	10 (100)	4 (40)	8 (80)	8 (80)	3 (75)	4 (100)	4 (100)	3 (75)
	Mild	1 (20)	1 (20)	0 (0)	0 (0)	0 (0)	4 (40)	2 (20)	2 (20)	0 (0)	0 (0)	0 (0)	1 (25)
	Moderate	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25)	0 (0)	0 (0)	0 (0)

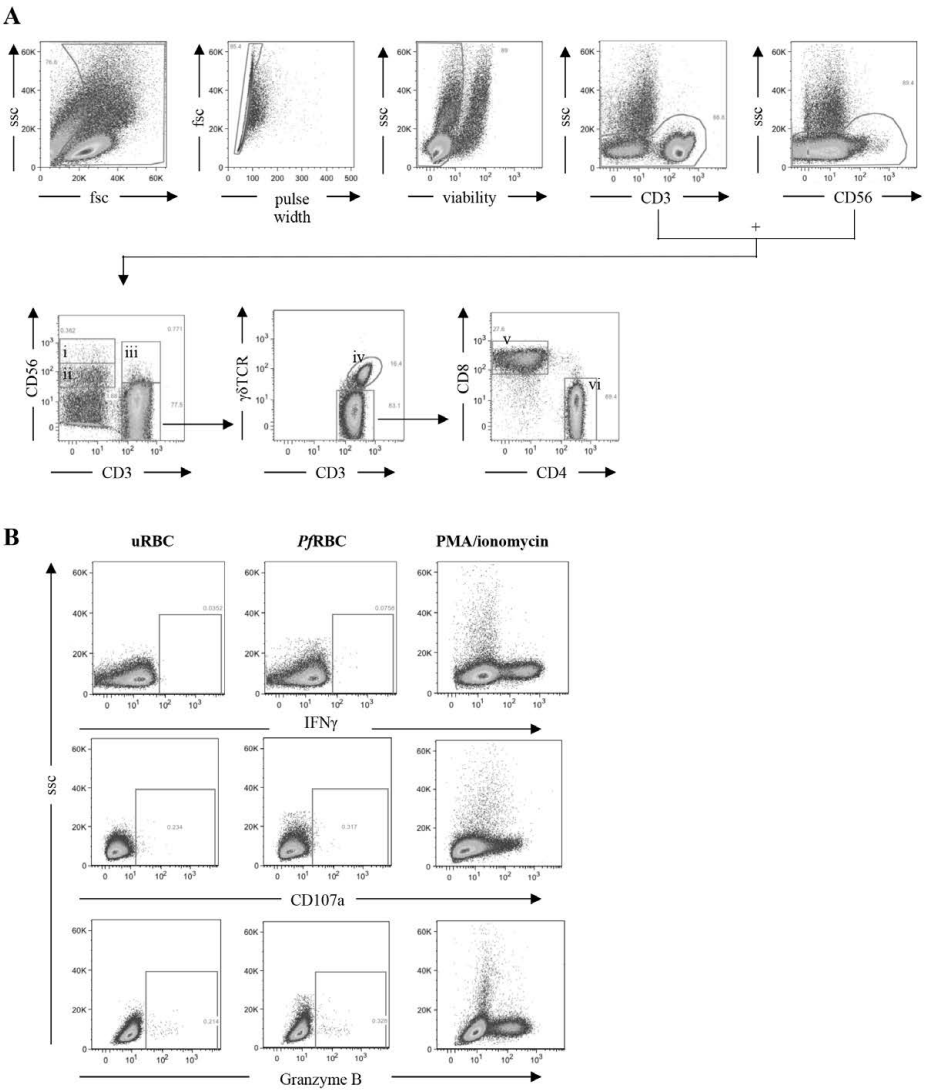

Figure S1 Gating strategy (A) Representative flow cytometry plots for a uRBC stimulated sample from one volunteer at baseline (before immunization). Singlet viable PBMCs were subdivided into (i) CD56hi NK cells, (ii) CD56dim NK cells, (iii) NKT cells, (iv) γδT cells, (v) CD8 T cells, (vi) CD4 T cells.

(B) Gating of IFNγ, CD107a and granzyme B positive cells for uRBC, *Pf*RBC and PMA/ionomycin re-stimulated cells at baseline. For uRBC and *Pf*RBC stimulation CD4 T cells are shown, for PMA/ionomycin total viable PBMCs. Within each sample, gating of cytokine-positive cells was performed in a standardized way by multiplying a fixed factor with the 75 percentile of the geometric Mean Fluorescent Intensity (MFI) of cytokine negative PBMCs

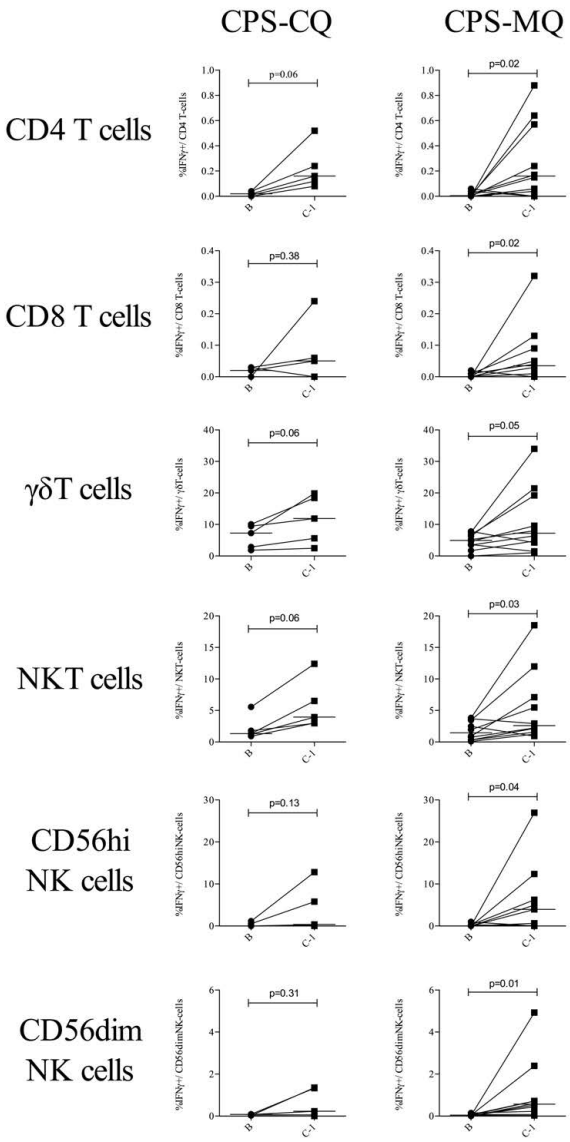

Figure S2 Cellular immune responses: IFNγ production. IFNγ production by different cell subtypes in response to *in vitro* re-stimulation with *Pf*RBC (corrected for uRBC background), before immunization (B) and one day before challenge (C-1). Differences between B and C-1 were tested by Wilcoxon matched-pairs signed rank test.

CHAPTER 11

Controlled human malaria infections by intradermal injection of cryopreserved Plasmodium falciparum sporozoites

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Abstract

Controlled human malaria infection with sporozoites is a standardized and powerful tool for evaluation of malaria vaccine and drug efficacy but so far only applied by exposure to bites of *Plasmodium falciparum* (Pf)-infected mosquitoes. We assessed in an open label Phase 1 trial, infection after intradermal (ID) injection of respectively 2,500, 10,000, or 25,000 aseptic, purified, vialed, cryopreserved Pf sporozoites (PfSPZ) in 3 groups (n = 6/group) of healthy Dutch volunteers. Infection was safe and parasitemia developed in 15 of 18 volunteers (84%), 5 of 6 volunteers in each group. There were no differences between groups in time until parasitemia by microscopy or qPCR, parasite kinetics, clinical symptoms or laboratory values. This is the first successful infection by needle and syringe with PfSPZ manufactured in compliance with regulatory standards. After further optimization, the use of such PfSPZ may facilitate and accelerate clinical development of novel malaria drugs and vaccines.

Introduction

Malaria caused by *Plasmodium falciparum* (Pf) causes approximately one million deaths and 250 million clinical cases annually (1, 2). Implementation of insecticide impregnated bed nets, residual insecticide spraying, and combinations of antimalarial drugs, has reduced malaria-associated morbidity and mortality in many areas (1). Questions related to sustainability of this effort, however, have led to a recent delineation of requirements for new tools (3, 4). A safe, long-acting anti-malarial drug and a highly effective malaria vaccine would be powerful tools for control and elimination of Pf malaria.

Progress has been facilitated by the capacity to infect volunteers under controlled conditions in order to test new vaccines and drugs. Infection of volunteers by exposure to laboratory-reared *Anopheles* spp. mosquitoes transmitting Pf sporozoites (SPZ) (5) was first introduced for treatment of neurosyphilis in the 1920s (6). The development of drugs such as chloroquine (7), primaquine (8). and atovaquone (9) were facilitated by these controlled human malaria infections (CHMIs). The ability to culture Pf gametocytes (10-12) enhanced the capacity to produce infected mosquitoes for CHMI studies. Although potentially serious, Pf malaria can be radically cured at the earliest stages of blood infection when risks are virtually absent. CHMIs are restricted to a few specialized centers that can produce PfSPZ-infected mosquitoes, where more than 1,300 volunteers have been safely infected by the bites of PfSPZ-infected mosquitoes since 1986, primarily for clinical trials of drugs (9) and malaria vaccines (5, 13-21), but also for trials of diagnostic tests (22), and studying human immune responses to Pf (23).

In addition to the use of CHMIs for testing vaccines and drugs, controlled infections can also be used to immunize against malaria. For example, immunization with radiation-attenuated PfSPZ by bites of mosquitoes protects > 90% of volunteers according to the published literature (24-26), and recently 100% protection against CHMI was achieved by immunization of volunteers taking a prophylactic regimen of chloroquine, with PfSPZ administered by mosquito bites (27, 28).

These highly protective immunization strategies could not be translated into an implementable vaccine, because they depended on inoculation of SPZ by mosquito bites. Inoculation of SPZ by injection would be a more feasible method and was performed through the early 1950s. The SPZ preparations used, however, were heavily contaminated with bacteria and mosquito material, and rates of infection with frozen and thawed SPZ were highly variable (29-33). A contemporary approach to production of SPZ for infection or vaccination requires generating aseptic SPZ-infected mosquitoes, purifying SPZ from mosquito tissues, vialing, preserving, and administering the SPZ by needle and syringe. Sanaria has met these requirements to produce infectious aseptic, purified, vialed, cryopreserved PfSPZ (PfSPZ Challenge), and produced and tested the world's first vaccine composed of these sporozoites (34, 35). Here, we report infection of volunteers with PfSPZ Challenge administered

intradermally (ID) by needle and syringe.

Materials and methods

Study population and study design

This open label, Phase 1 clinical trial was performed at Radboud University Nijmegen Medical Center, the Netherlands, from October 2010 to July 2011. Volunteers aged 18–35 years of age were screened for eligibility by medical history, physical examination, and laboratory tests of blood, serum, and urine, including standard hematological, biochemical, and pregnancy tests, and malaria, HIV, hepatitis B and hepatitis C serology. The main exclusion criteria were pregnancy, residence in a malaria-endemic area within the previous six months, positive Pf serology, symptoms, physical signs or laboratory test results suggestive of systemic disorders, and history of drug or alcohol abuse interfering with normal social function. All volunteers gave written informed consent.

Eighteen healthy malaria-naïve volunteers were included in this trial. Groups of six volunteers were injected intradermally (ID) with 2,500, 10,000, or 25,000 PfSPZ Challenge. The sample size of six per group had a power of 75% to show a difference between 2 of 6 volunteers infected in the 2,500 PfSPZ group and 6 of 6 volunteers infected in the 25,000 PfSPZ group. Dose escalation was done at a minimum interval of 3.5 weeks.

The trial was performed in accordance with Good Clinical Practice and an Investigational New Drug application filed with the U.S. Food and Drug Administration, and approved by the Central Committee for Research Involving Human Subjects of The Netherlands (CCMO NL31858.091.10). Clinicaltrials.gov identifier: NCT 01086917.

Study intervention (PfSPZ Challenge)

PfSPZ Challenge contains aseptic, purified, cryopreserved PfSPZ isolated from salivary glands of aseptically reared mosquitoes (34, 35). *Anopheles stephensi* mosquitoes were raised under aseptic conditions, then fed on cultured Stage V gametocytes of the NF54 strain of Pf (36). Approximately two weeks later, mosquito salivary glands containing PfSPZ were dissected, and PfSPZ were purified, formulated, vialed (15,000 PfSPZ per vial), and cryopreserved in liquid nitrogen vapor phase at –140°C to –196°C (35). PfSPZ Challenge released for clinical use met quality control specifications including sterility (USP 71 compendial assay), purity (Supplemental Figure 1), and potency (Table 1).

Potency was assessed as previously described (34, 35) by quantification of late liver stage parasites expressing Pf merozoite surface protein 1 (PfMSP-1) (37) in cultured human hepatocytes (HC-04 cells) (38) six days after addition of PfSPZ (Table 1, Supplemental Figure 2). For this 6-day hepatocyte potency assay, 4.0×10^4 HC-04 (1F9) cells/well in triplicate were infected with 5.0×10^4 PfSPZ and incubated for 6 days with daily media

change. Late liver stage parasites expressing PfMSP-1 were counted by staining the slides with an anti-PfMSP-1 mAb and fluorescently labeled secondary antibody. As previously described (34, 35) the membrane integrity of PfSPZ was used to assess cell viability (Table 1). For the sporozoite membrane integrity assay, propidium iodide and SYBR Green were added to 15,000 PfSPZ. PfSPZ were applied to a hemocytometer and incubated in a dark humidity chamber for 20 minutes, at which point the red PfSPZ (those with compromised membranes) and green PfSPZ (those with intact membranes) were counted under a fluorescent microscope. Those with intact membranes were considered viable, and viability is expressed as the percentage of total green PfSPZ over the total number of PfSPZ. Sporozoites were assessed before cryopreservation, for release of the lot, and to assess stability at defined time points after cryopreservation.

Table 1 Results of potency and sporozoite membrane integrity assays on the lot of PfSPZ Challenge used in this clinical trial

Time point	Potency (# of parasites expressing PfMSP-1/well)	% Viability (sporozoite membrane integrity assay)
Fresh	27 ± 4.6	ND
Release	20 ± 1.7	83.3% ± 6.5%
6 Month	18 ± 2.1	86.6% ± 1.9%
9 Month	20 ± 2.1	83.7% ± 8.4%
12 Month	21 ± 1.5	84.8% ± 3.0%
18 Month	20 ± 0.6	83.7% ± 4.2%
24 Month	18 ± 1.0	86.0% ± 1.5%
Pre-1st clinical dose (26 Month)	17 ± 0.6	79.4% ± 6.5%
Post-last clinical dose (30 Month)	16 ± 2.6	87.4% ± 1.9%

Fresh PfSPZ used for the lot of PfSPZ Challenge used in this clinical trial produced 26% more PfMSP-1-expressing parasites in this assay than did PfSPZ that had been cryopreserved for several days (Release); at 30 months, several weeks after inoculation of the last volunteers the PfSPZ had a 40.7% reduction in potency by this assay as compared to fresh PfSPZ. There was no reduction in the results of the sporozoite membrane integrity of cryopreserved PfSPZ during 30 months of storage.

The sporozoite membrane integrity assay was not done on fresh PfSPZ for this particular lot. In our most recent three production campaigns for PfSPZ Challenge, fresh viability was 97.8%, 99.0% and 98.2% while after cryopreservation viability was reduced to 90.9%, 91.5%, and 87.4% respectively, a mean reduction of 8.5%.

ND = not done.

The lot of PfSPZ Challenge used in this study had been cryopreserved in liquid nitrogen vapor phase for 27 (dose of 2,500 PfSPZ) to 30 months (dose of 25,000 PfSPZ) before administration. Immediately before use, a vial of PfSPZ Challenge was thawed and diluted

with phosphate buffered saline containing human serum albumin (HSA). Volunteers were injected within 30 minutes of thawing.

Controlled human malaria infection

Three groups of six volunteers each were injected ID with PfSPZ Challenge over the deltoid muscle, one injection in each upper arm. Each injection of 50 μL contained half the total dose. After injection, volunteers were observed for at least 60 minutes. Inoculations of volunteers were spaced 60 minutes apart. In each dose group, two volunteers were inoculated three days before the remaining four volunteers.

Volunteers made at least one daily outpatient clinical visit beginning five days after inoculation of PfSPZ Challenge. All symptoms and signs (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); fever was recorded as grade 1 (> 37.5°C–38.0°C), grade 2 (> 38.0°C–39.0°C) or grade 3 (> 39.0°C). Hematological and biochemical parameters were monitored daily. Because of a previous cardiac related serious adverse event (SAE) following CHMI with Pf infection (39), markers of cardiac damage and coagulation were assessed. Troponin, lactate dehydrogenase (LDH), platelets, and D-dimer were assessed daily during the period when blood stage parasitemia was expected, and for three days after initiating curative treatment with atovaquone/proguanil. If D-dimer or LDH were abnormal, blood samples were tested for fragmentocytes and von Willebrand cleaving protease activity, as markers for vascular endothelial cell activation (40). Final follow-up visits were on Days 35 and 140 after infection.

As soon as parasites were detected by microscopic examination of blood smears, volunteers were treated with atovaquone/proguanil (1,000/400 mg) administered orally once daily for three days. Complete cure was confirmed in all volunteers by two consecutive parasite-negative blood-slides after treatment, at least 4 days apart. Volunteers who did not develop parasitemia by Day 21 after challenge were presumptively treated with the same regimen.

Outcomes

The primary outcome was occurrence of Pf parasitemia detected by microscopic examination of blood smears. Sampling was done twice daily on Days 5 and 6 post-inoculation, thrice daily on Days 7–11, twice daily on Days 12–15, once daily on Days 16–21, and for two days after initiation of treatment for positive smears. To make thick blood smears, 15 μL of EDTA-anti-coagulated blood was spread on each well of a 3-well glass slide (CEL-LINE Diagnostic Microscope Slides, 30-12A-black-CE24; Braunschweig, Germany). After drying, wells were stained with Giemsa for 45 minutes, and examined at 1,000 × magnification to assess 0.5 μL of blood. The smear was scored as positive if two unambiguous parasites were found. Thus,

volunteers could be diagnosed with as few as 4 parasites/μL of blood. The pre-patent period was defined as the period between inoculation of PfSPZ Challenge and appearance of first positive blood smear.

Retrospectively, parasitemias were determined by real-time quantitative polymerase chain reaction (qPCR), performed on all samples collected after challenge, as previously described (41). The sensitivity of qPCR was 20 parasites/mL of blood.

Statistical analysis

Data analysis was performed using SPSS software version 16.0. Q-PCR results were assessed by analysis of variance (ANOVA) on log-transformed data.

Results

Parasitemia after injection of PfSPZ Challenge

Thirty-six healthy, malaria-naïve volunteers were screened and eighteen were included. All volunteers completed follow-up (Supplemental Figure 3). After ID injection of PfSPZ Challenge, 15 of the 18 volunteers developed a positive blood smear for Pf, five of six volunteers from each group (Table 2). The slide-negative volunteers in each group were presumptively treated with atovaquone/proguanil at 21 days post-infection.

Blood slides were first positive 11 to 14.3 days after administration of PfSPZ Challenge. The geometric mean (GM) pre-patent period was similar for all groups, i.e., 13.0, 12.7, and 13.0 days for the groups receiving 2,500, 10,000, and 25,000 PfSPZ Challenge, respectively (ANOVA $p = 0.92$). The GM parasite densities by microscopy at the time of diagnosis were 12.4, 11.2, and 23.4 parasites/μL blood (ANOVA $p = 0.69$ on log-transformed data) (Table 2).

Quantitative PCRs (qPCRs) were first positive 9.0 to 12.0 days after challenge (Table 2). Volunteers in the 2,500, 10,000, and 25,000 PfSPZ Challenge groups had similar GM times to first detection of parasites by qPCR of 10.6, 10.3, and 9.9 days (ANOVA $p = 0.486$) at a GM parasite density of 0.07, 0.2 and 0.2 parasites/μL blood (ANOVA $p = 0.24$), respectively. The GM parasite densities by PCR at the time of thick smear diagnosis were 35, 5, and 132 parasites/μL (ANOVA $p = 0.23$). qPCR was negative throughout the 21-day follow-up for the three slide-negative volunteers. Parasite growth was cyclical, and was similar in all dose groups (Figure 1), and the parasite replication rate in the bloodstream was comparable to that seen after CHMI by exposure to the bite of PfSPZ-infected mosquitoes, ~11.5-fold every 48 hours (42).

Table 2 Parasitemia data by thick blood smear and quantitative polymerase chain reaction (qPCR)

	Thick Smear		qPCR		
Volunteer code	Pre-patent period (day)	Parasite density at diagnosis (Pf/µL)	qPCR positive (day)	Parasite density at first day positive (Pf/µL)	Parasite density by qPCR at time of diagnosis by thick smear (Pf/µL)
Group 1 - 2,500 PfSPZ					
696-18	12.3	4	9.6	0.08	5
711-08	14.0	16	12	0.16	71
795-06	N/A	N/A	N/A	N/A	N/A
935-01	14.0	124	10.6	0.03	89
937-20	12.3	6	10.6	0.12	43
940-14	12.3	6	10.3	0.06	35
Geom. mean	13.0	12	10.59	0.1	35
# of positives	5/6		5/6		
Group 2 - 10,000 PfSPZ					
119-03	12.6	24	9.6	0.68	6
603-11	13.0	8	11	0.17	2
736-04	11.0	6	9.6	0.04	3
783-25	13.3	6	10.6	0.03	15
788-21	14.0	26	11	1.12	6
925-26	N/A	N/A	N/A	N/A	N/A
Geom. mean	12.7	11	10.34	0.2	5
# of positives	5/6		5/6		
Group 3 - 25,000 PfSPZ					
647-30	14.0	512	9.3	0.32	759
720-13	12.3	6	10.3	0.32	162
789-15	N/A	N/A	N/A	N/A	N/A
806-09	12.3	8	9	0.25	48
909-29	14.3	48	11.3	0.13	102
926-24	12.3	6	10	0.19	68
Geom. mean	13.0	23	9.95	0.2	132
# of positives	5/6		5/6		

N/A: not applicable; thick-smear negative volunteers were presumptively treated on day 21 after infection.

Safety

Local reactogenicity was not observed after ID administration of PfSPZ in any of the volunteers. All volunteers, including the three volunteers who did not develop parasitemia, reported solicited adverse events (AEs) considered possibly, probably, or definitely related to the trial procedures (clinical malaria) (Table 3). Headache was the most frequently reported AE, and occurred in all volunteers including the three who did not develop parasitemia. There were no significant differences among the groups in solicited AEs, which were most frequently reported between Days 12 and 18 post-injection. The percentage of volunteers with related grade 3 AEs was comparable to historical data from subjects subjected to CHMIs

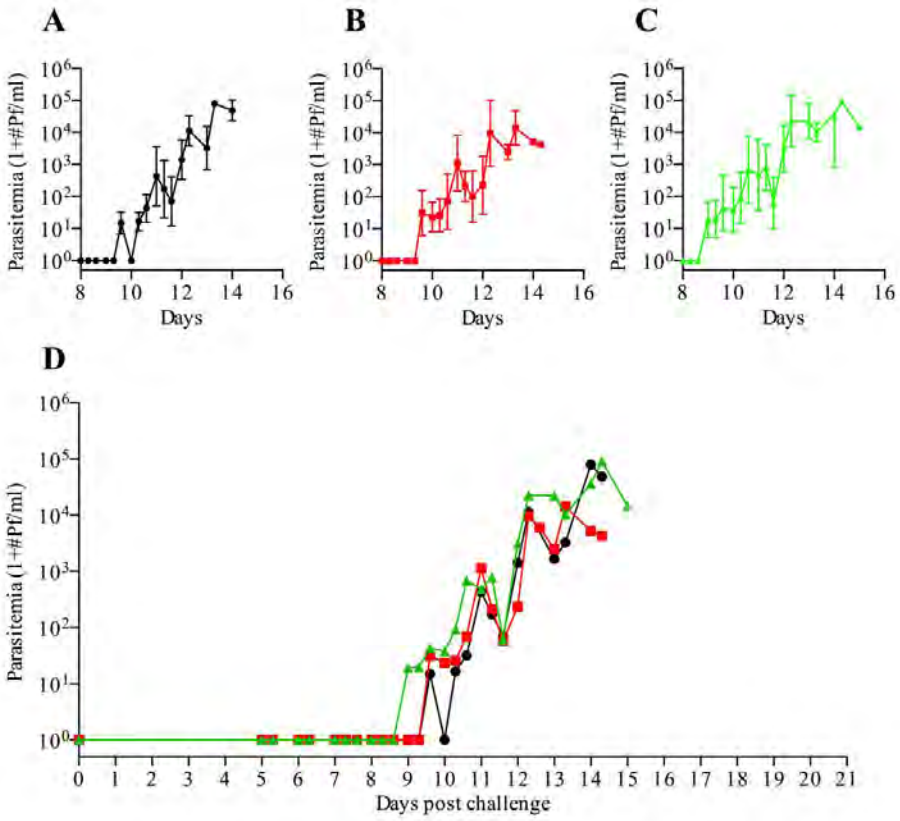

Figure 1 Parasite density as measured by qPCR in the 2,500 (A), 10,000 (B), and 25,000 (C) PfSPZ Challenge dose groups. Panels A, B, and C show geometric mean parasite density of positive volunteers per group with confidence intervals (N = 5 for all groups) from day of inoculation through last day of positivity after initiation of treatment. Panel D shows an overlay of geometric mean parasite densities of positive volunteers in each group.

by mosquito-bites (44% versus 49%, respectively) (42). The total number of solicited and unsolicited AEs reported over time is shown in Figure 2. There were few AEs before Day 7; PfSPZ Challenge inoculations were well tolerated.

Routine daily laboratory tests showed no clinically significant abnormalities before initiation of anti-malarial treatment. Three or four days after receiving the first dose of atovaquone/proguanil, four volunteers had thrombocyte levels in the range 78–95 × 10^9/L, which was below the lower limit of normal (120 × 10^9/L). Leukocyte counts decreased after initiation of treatment in all thick smear positive volunteers (minimum 2.89 × 10^9/L compared with 5.46 × 10^9/L at baseline). In thirteen volunteers, D-dimers were > 500 ng/mL, the upper limit of normal (ULN), at one or two days after initiation of anti-malarial treatment (range of

peaks: 540–10,200 ng/mL). D-dimer increases most likely reflect non-specific inflammatory responses to parasite-derived material released after initiation of treatment. In all volunteers, D-dimer concentrations normalized without complications. One volunteer had abnormal liver function tests at Day 2 post atovaquone/proguanil initiation. Maximum values were

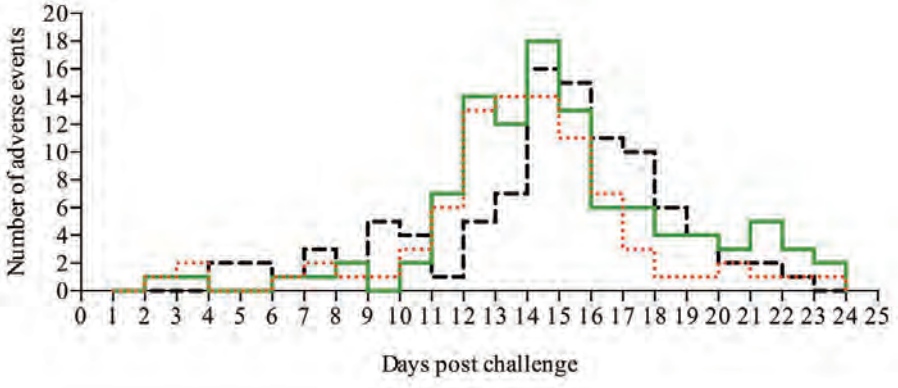

Figure 2 Number of possibly, probably, or definitely related solicited and unsolicited adverse events reported over time in the 2,500 (black dashed), 10,000 (red dotted) and 25,000 PfSPZ Challenge dose (green straight) groups.

526 units/L ASAT (ULN 40 units/L), 745 units/L ALAT (ULN 45 units/L), 777 units/L LDH (ULN 450 units/L), and 74 units/L γGT (ULN 50 units/L). Bilirubin and alkaline phosphatase were normal. Abnormal values had returned to baseline levels at Day 100 after infection.

One SAE occurred in a volunteer who reported chest pain one day after the first dose of atovaquone/proguanil. Based on medical history, the chest pain was initially considered possibly consistent with angina pectoris. Pain resolved within one hour without treatment. The volunteer was admitted to the cardiac care unit for monitoring for 6.5 hours. The first electrocardiogram (ECG) had a negative T-wave in V2, which was absent at the time of study initiation. All subsequent ECGs, beginning 2.5 hours after the first ECG, were comparable to baseline, with a negative T in V1 only. Troponin T levels were normal at the time of chest pain, 6 and 17 hours later, daily for the next three days and at trial Days 28 and 35. As per protocol, the trial was put on hold, and the event was reported to the Safety Monitoring Committee (SMC) and regulatory authorities. The SMC concurred with the principalinvestigator's attribution of the chest pain as "possibly related" to participation in the trial. The SMC concluded that although the cause of chest pain was not clear, the clinical data suggested that the SAE was not a serious cardiac event, and recommended resumption of the trial within three days of the event. The regulatory authorities concurred.

Table 3 Numbers of volunteers reporting solicited adverse events possibly, probably, or definitely related to administration of PfSPZ Challenge, with mean duration of events.*

	2,500 PfSPZ (n=6)		**10,000 PfSPZ (n=6)**		**25,000 PfSPZ (n=6)**	
Any adverse event	**No. of volunteers**	**Mean duration ± SD (days)**	**No. of volunteers**	**Mean duration ± SD (days)**	**No. of volunteers**	**Mean duration ± SD (days)**
Abdominal pain	1	2.9	1	0.04	2	0.3±0.1
Arthralgia	0	N/A	0	N/A	0	N/A
Chest pain	1	0.04	0	N/A	0	N/A
Chills	1	2.0	2	0.3±0.2	2	0.9±0.6
Diarrhea	0	N/A	0	N/A	1	0.8
Fatigue	5	2.9±3.3	3	2.5±1.7	5	3.0±3.9
Fever	3	1.6±1.5	2	1.8±0.6	4	0.8±0.4
Headache	6	1.1±1.1	6	1.5±1.6	6	1.4±2.6
Malaise	2	2.2±2.4	5	1.8±1.4	1	0.7
Myalgia	2	3.7±3.2	2	1.3±0.5	2	0.8±0.1
Nausea	3	1.7±1.3	5	0.9±0.9	3	1.0±0.9
Vomiting	0	N/A	2	0.01±0.0	0	N/A
Any	**6**	**2.0±1.4**	**6**	**1.1±0.8**	**6**	**1.1±1.0**
Grade 3 adverse event						
Fatigue	0	N/A	0	N/A	1	2.2
Fever	0	N/A	1	1.2	0	N/A
Headache	2	3.0±0.4	0	N/A	0	N/A
Malaise	1	4.8	0	N/A	1	0.1
Vomiting	0	N/A	2	0.01±0.0	0	N/A
Any	**2**	**3.9±0.2**	**3**	**0.6±0.0**	**2**	**1.2±0.0**

*****There were few AEs before day 7 (Figure 2). Thus, administration of PfSPZ Challenge was well-tolerated. The AEs were expected and attributed to malaria.

N/A: not applicable

Discussion

We report for the first time that healthy, malaria-naïve volunteers can be infected with *P. falciparum* malaria by injection of aseptic, purified, cryopreserved PfSPZ manufactured in compliance with regulatory standards. Five of six volunteers became infected when 2,500, 10,000 or 25,000 PfSPZ were inoculated ID. The AEs were comparable with those in mosquito bite challenge trials (17, 19, 42, 43). Virtually all related AEs were attributed to malaria, not to the inoculations with PfSPZ Challenge.

The capacity to infect volunteers with PfSPZ Challenge is dependent on the efficiency

of administration and the infectiousness/fitness of the cryopreserved PfSPZ. It can be expressed by the success rate of infection in the exposed individuals and/or the pre-patent period, i.e., the time from inoculation until first detected parasitemia. Since 1986 CHMIs have been performed by exposing volunteers to bites of laboratory-reared mosquitoes infected by feeding on Pf gametocyte-infected erythrocytes grown in culture (12). Essentially all volunteers challenged by bites of five PfSPZ-infected mosquitoes develop Pf parasitemia (5, 12, 17, 19). When numbers are reduced to one or two mosquitoes, success rates drop to 50% or less (43-45). The ID inoculation of the lowest dose of 2,500 cryopreserved PfSPZ Challenge, which resulted in infection of 5 of 6 volunteers in the current study, was thus at least as effective in achieving blood stage infection as the bites of 1–2 infected mosquitoes.

In regard to the pre-patent period the results were not straightforward. The pre-patent period in the 2,500 PfSPZ group was longer than was observed after 1–2 bites of PfSPZ (NF54)-infected mosquitoes at RUNMC (43) but shorter than after 1–2 bites of PfSPZ (3D7)-infected mosquitoes at the Naval Medical Research Center (44). The longer pre-patent period in our study compared with the pre-patent period after exposure to NF54-infected mosquitoes may have been caused by fewer developing liver stage schizonts after inoculation than after exposure to the bites of 1–2 PfSPZ-infected mosquitoes. Alternatively, replication in the liver stage could have been of lower magnitude or slower with the aseptic, purified, cryopreserved PfSPZ as compared with the fresh PfSPZ delivered by mosquito bite. Finally, the findings may just reflect expected biologic variability, because the study with 1–2 3D7 infected mosquitoes showed a longer pre-patent period than after PfSPZ Challenge (44).

The asexual erythrocytic stage parasites in our study replicated ~11.5-fold every ~48 hours. Thus, with a 10-fold increase in PfSPZ, the theoretical time until parasitemia by microscopic examination (pre-patent period) should have been 2 days less in the 25,000 PfSPZ group as compared with the 2,500 PfSPZ group. However, this was not the case as pre-patent periods of 13.0 and 13.0 days by microscopy and 10.59 and 9.95 days by qPCR were obtained in the 2,500 PfSPZ and 25,000 PfSPZ groups respectively. Thus, increasing the dose of PfSPZ Challenge 10-fold from 2,500 PfSPZ to 25,000 PfSPZ administered ID did not increase the percentage of infected volunteers or reduce the pre-patent period. Apparently, increasing the dose administered in two 50 μL injections did not result in higher numbers of PfSPZ getting from the skin to the circulation, invading and maturing in hepatocytes, eventually resulting in merozoites that invaded and multiplied in erythrocytes. Understanding this lack of dose response will be important for optimization of administration of PfSPZ Challenge. A possible explanation for this lack of dose response may be trapping of PfSPZ at the inoculation site. The use of five mosquitoes that probe in multiple sites must result in distribution of PfSPZ in the dermis and subcutaneous tissue in at least five different sites, and probably considerably more. Therefore, increase in the number of inoculation sites and injection of much smaller volumes (< 0.5 μL) may result in better infections. Such strategies

may also be useful for improving the efficiency of administration of the irradiated PfSPZ in the PfSPZ Vaccine. Although not as profound, there was a lack of a linear dose response in the first trial of the PfSPZ Vaccine in which irradiated PfSPZ were administered in 120 uL ID or SC (35).

To determine the minimal numbers of PfSPZ required to achieve 100% infection rates, and a pre-patent period similar to five PfSPZ-infected mosquitoes, it would be most useful to assess intravenous (IV) administration of PfSPZ Challenge. Data from studies in mice show that administration of purified cryopreserved *Plasmodium yoelii* (Py) SPZ required ~23 times more PySPZ administered ID than IV to achieve 80% infection rates (ID80) (Table 4). Similar differences in liver load *in vivo* between IV and ID routes of administration were demonstrated using luciferase-labeled, bioluminescent fresh *Plasmodium berghei* (Pb) SPZ (46). Thus, we will conduct studies to investigate the minimal IV-dose and to optimize non-IV administration by modifying the route of administration (e.g., ID, subcutaneous, intramuscular), inoculation volume, numbers of inoculations, and sites of injection.

Table 4 Infectivity in mice of purified, cryopreserved PySPZ administered IV or ID.

Intravenous (IV)				Intradermal (ID)			
No. of PySPZ Injected	**Number of mice**		**Proportion infected**	**No. of PySPZ Injected**	**Number of mice**		**Proportion infected**
	Infected	**Injected**			**Infected**	**Injected**	
33	2	5	40%	200	2	5	40%
100	1	5	20%	600	3	5	60%
300	5	5	100%	1800	3	5	60%
900	5	5	100%	5400	4	5	80%
80% infectious dose = 257 PySPZ 100% infectious dose = 528 PySPZ				80% infectious dose = 5871 PySPZ 100% infectious dose = N/A			

Purified, cryopreserved PySPZ were injected IV in the tail vein or ID at the base of the tail of 6-8 week old BALB/c. Infection was determined by examination of blood smears on days 7 and 14 after inoculation. The 80% and 100% infectious doses were calculated using CurveExpert ver. 1.4.

Next to route of administration, our manufacturing/cryopreservation process may also be responsible for reduced infectivity. *In vitro* assays of potency and viability estimate a maximum difference of 25–30% between fresh and cryopreserved PfSPZ Challenge (Table 1). Rodent model *in vivo* data, however, suggest that a ~7-fold loss in infectivity caused by cryopreservation is more likely (Table 5). Therefore, we will continue to concentrate our efforts on improvement of infectivity of PfSPZ Challenge. Interestingly, once the merozoites are released from the liver into the bloodstream they are as fit as non-cryopreserved, mosquito-administered parasites, as their replication rates are similar.

Table 5 Effect of cryopreservation on sporozoite membrane integrity and infectivity in mice inoculated intravenously with the same lot of P. yoelii sporozoites (PySPZ). Infectivity was the number of PySPZ required to infect 50% of BALB/c mice.

Date	Status of PySPZ	Viability (SMIA)	Number of PySPZ Inoculated (IV)	ID_{50} (Number of PySPZ)
Oct 2009	fresh	96.3%	24-12-6-3	8.9
Dec 2009	cryopreserved	72.7%	200-100-50-25	33.1
Dec 2009	cryopreserved	68.3%	200-100-50-25	62.1
Jan 2010	cryopreserved	67.7%	400-200-100-50-25	103.8
Feb 2010	cryopreserved	67.1%	400-200-100-50-25	55.2
Feb 2010	cryopreserved	71.6%	400-200-100-50-25	107
Feb 2010	cryopreserved	73.9%	400-200-100-50-25	34.5
Mean	cryopreserved	70.2%		66.0
Difference between fresh and cryopreserved PySPZ		26.1%		7.4-fold

Successful development and application of PfSPZ Challenge will increase the global capacity to conduct CHMIs, including in Africa where a CHMI consortium has been established with representative institutes from seven countries. This expansion of clinical sites conducting CHMIs will facilitate the clinical development of malaria vaccine candidates and anti-malarial drugs (3, 47). Another advantage of CHMI by PfSPZ Challenge may be a better-defined number of injected PfSPZ compared with the numbers administered by mosquito bites. This may decrease the large inter-individual variation in the estimated number of infected hepatocytes (48). Furthermore, using needle administration of defined quantities of PfSPZ Challenge from the same lot, will allow for comparisons of parallel and sequential clinical trials at multiple sites, including malaria-endemic areas. Finally, needle and syringe administration of cryopreserved PfSPZ is critical for potential development of whole PfSPZ vaccines where parasite development is arrested by radiation, anti-malarial drugs or genetic modification.

Freshly dissected, purified *P. yoelii* sporozoites (PySPZ) were assessed by the sporozoite membrane integrity assay (SMIA) as a measure of viability, and administered to BALB/c mice by intravenous (IV) injection. The remaining PySPZ from the same lot were cryopreserved, thawed at six different time points, assessed for viability by SMIA and administered IV to mice. To provide data for calculatiosn of the number of PySPZ that infected 50% of mice (ID_{50} calculated using an exponential association model $y=a(1-e^{-bx})$) (CurveExpert version 1×4) with fresh and cryopreserved PySPZ, groups of five mice each received PySPZ in de-escalating doses as indicated, and their infection status was determined by assessing Giemsa-stained blood smears 7-14 days after inoculation. The viability by SMIA

of purified, cryopreserved PySPZ was reduced 26.1% as compared to fresh, purified PySPZ. The cryopreserved PySPZ were 7.4-fold less infective than fresh PySPZ as it took 7.4 times more cryopreserved PySPZ to achieve 50% infection of mice.

In summary, we show that aseptic, purified, vialed, cryopreserved PfSPZ (PfSPZ Challenge) are infectious to humans for at least 2.5 years after cryopreservation. These data provide the rationale and foundation for a clinical trials program aimed at establishing a dose and route of PfSPZ that consistently achieves 100% infection rates. This will allow for the global expansion of sites that can conduct CHMIs for assessment of malaria vaccines and new drugs, and the potential to develop whole parasite vaccines based on cryopreserved PfSPZ.

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Disclosure

Sanaria Inc. manufactured PfSPZ Challenge, and Protein Potential LLC is affiliated with Sanaria. Thus, all authors associated with Sanaria or Protein Potential have potential conflicts of interest. There are no other conflicts of interest.

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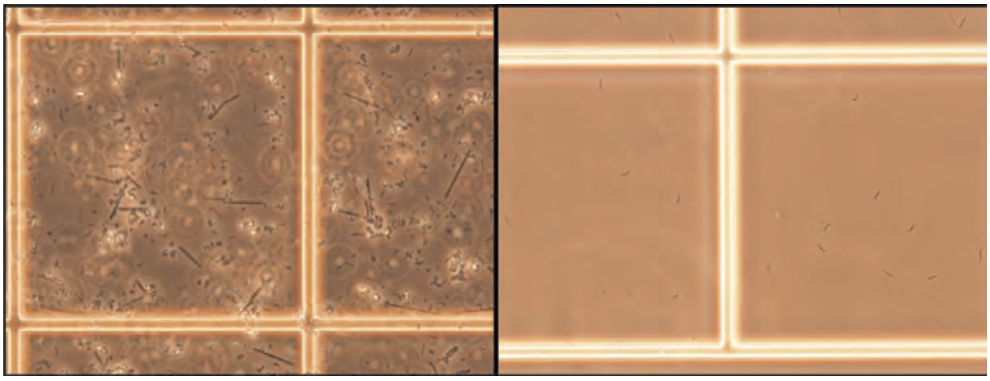
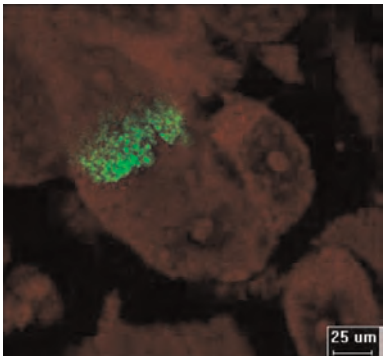
Supplemental Figures

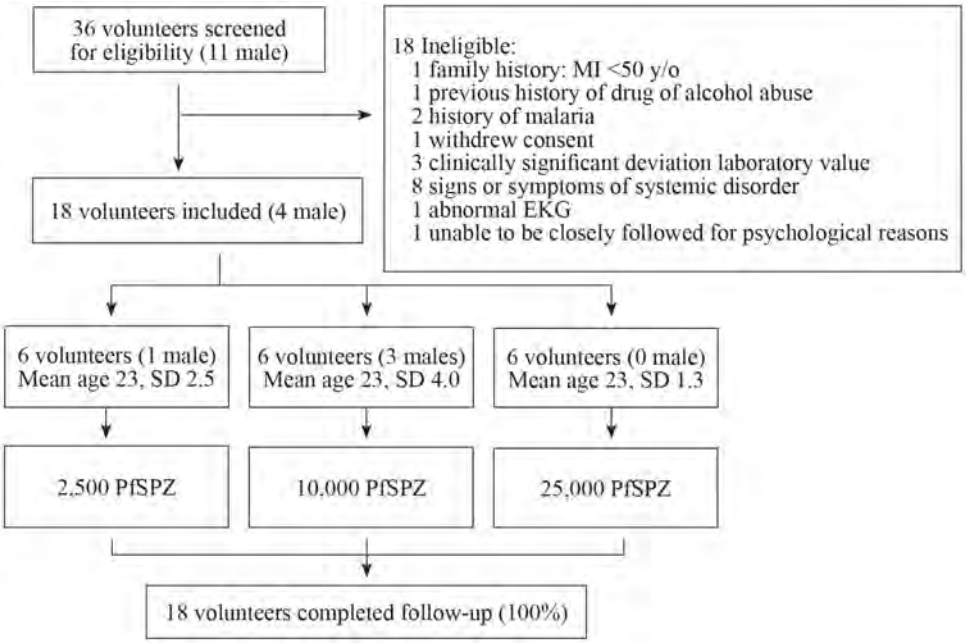


Before Purification (14 × 10^6 PfSPZ/ml) *After Purification (1.6 × 10^6 PfSPZ/ml)*

Supplemental Figure 1 PfSPZ before and after purification. Photomicrograph (200 ×) of the PfSPZ in the lot of PfSPZ Challenge used in the clinical trial prior to and after purification. The purification process reduced the amount of salivary gland material in the PfSPZ samples by greater than 99.9%.



Supplemental Figure 2 Liver-stage Pf parasite expressing PfMSP-1 after six days in culture. PfSPZ from the lot of PfSPZ Challenge used in this clinical trial were used to infect a human hepatocyte line *in vitro*. Six days later the parasites were stained with an antibody to PfMSP-1. PfMSP-1 is the major protein on the surface of merozoites, and is required for merozoite invasion of erythrocytes. It is only expressed in mature liver stage parasites and blood stage parasites, and is required for the survival of blood stage parasites. Potency of lots of PfSPZ Challenge is assessed by expression of PfMSP-1 *in vitro* (see Table 1). Image captured with a Zeiss LSM510 META laser scanning confocal microscope.



Supplemental Figure 3 Clinical trial profile.

CHAPTER 12

Construction of personalized health curves in disease space for human malaria infections

Else M. Bijker, Brenda Y. Torres, David S. Schneider, Robert W. Sauerwein

Manuscript in preparation

Abstract

Novel frameworks are needed to unravel complex host-pathogen interactions. A framework derived from population biology has previously been applied in fruit flies and mice to visualize infections in health-by-microbe disease space. By plotting the health of an individual against microbe load at several time points over the course of an infection, looping health curves were generated that traced the infection course through disease space. Such analyses have provided novel insights in tolerance and resistance to pathogens.

Here, we investigated such an approach for the first time with longitudinal data from humans, acquired using the controlled human malaria infection (CHMI) model. Retrospective longitudinal data from 91 subjects participating in seven CHMI studies were used to generate health curves of individual subjects from before the start of a malaria infection up until complete cure and recovery. Visualization in disease space enabled us to distinguish different levels of tolerance against *Plasmodium falciparum* malaria. These data illustrate the potential of these personalized health curves for rational monitoring and treatment of infections such as malaria on an individual basis.

Introduction

Interactions between hosts and microbes exist in many different forms, with varying kinetics and outcomes that can be difficult to disentangle. A theoretical framework derived from population biology has been proposed to improve our understanding of host-microbe interactions on an individual level (1). In this framework, a disease space is created in which the health of an individual is plotted against microbe load at several time points over the course of an infection. This produces a looping health curve that traces the infection course through disease space, and visualizes the relationship between health and pathogen load across the infection (1). Describing infections using disease space may provide a new framework to study parts and characteristics of infections that are difficult to visualize or quantify otherwise, such as the recovery period (1).

Studies in fruit flies and mice have shown that these animals indeed trace looping paths through disease space and that these loops can reveal 'safe' and 'dangerous' areas, providing novel insights in resistance and tolerance to pathogens *(B. Torres unpublished data)*. An important next step will be to show that infections in humans can also be described by personalized health curves in disease space. In order to create personalized health curves, it is very helpful if pathogen load can be quantified accurately and frequently over the course of the infection. *Plasmodium falciparum* infection will be a good candidate because of the availability of a sensitive quantitative real-time polymerase chain reaction (qPCR) for determination of parasite load in the circulation (2).

Analysis of cross-sectional data from children in a malaria-endemic area suggests that also humans trace curves through disease space, but limited information is available from the recovery period and longitudinal data are needed to confirm this finding *(B. Torres unpublished data)*. Controlled human malaria infection (CHMI) trials offer the possibility to collect such longitudinal datasets to assess whether paths in disease space can be constructed for humans. In these CHMI studies, healthy adult volunteers are exposed to *Plasmodium* parasites by exposure to infected *Anopheles* mosquitoes, intravenous injection of asexual blood stage parasites or injection of cryopreserved sporozoites. Participants are immediately treated for safety reasons when parasites are detected in their blood (3). CHMI trials are typically used to study the protective effect of novel vaccines or drugs, but can also be exploited to study basic characteristics of parasite-host interaction and pathophysiology of early malaria infection.

The aim of this study is to assess whether personalized health curves in disease space can be applied to study human *P. falciparum* infections, using retrospective longitudinal data from CHMI studies.

Methods

We retrospectively analyzed data from a total of 91 subjects from seven CHMI studies (4-10). Details of the studies are included in Table 1. All trials were approved by the Central Committee on Research involving Human Subjects (The Hague, The Netherlands), all subjects gave informed consent and investigators complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. Subjects were infected with *P. falciparum* malaria by either mosquito bites, injection of cryopreserved sporozoites or intravenous administration of infected erythrocytes. All subjects were treated with a curative regimen of atovaquone/proguanil when parasites were detected by thick smear. Subjects who remained thick smear negative were not included in this study. Some of the included subjects (n=37) had received chemoprophylaxis and sporozoites (CPS) immunization (4-6, 8), and were thus not malaria-naïve at the time of CHMI, while the other subjects (n=54) were (Table 1).

Table 1 Study subjects.

Clinicaltrials.gov identifier	*P. falciparum* strain	Challenge method	CPS-immunized	Number of subjects
NCT00757887 (8)	NF54	Mosquito bite	Yes	2
	NF54	Mosquito bite	No	5
NCT01218893 (6)	NF54	Mosquito bite	Yes	7
	NF54	Mosquito bite	No	5
NCT01422954 (5)	NF54	Mosquito bite	Yes	5
	NF54	Mosquito bite	No	4
NCT01236612 (4)	3D7	Mosquito bite	Yes	9
	3D7	Blood stage	No	5
	3D7	Blood stage	No	5
NCT01002833 (9)	NF54	Mosquito bite	No	7
	NF135.C10	Mosquito bite	No	3
NCT01660854 (10)	NF135.C10	Mosquito bite	Yes	14
	NF135.C10	Mosquito bite	No	5
NCT01086917 (7)	NF54	Intradermal injection of cryopreserved sporozoites	No	15
Total				91

Parasitemia and hematological parameters were assessed on samples collected one day before infection and from one day after infection onwards, up until 3 days after treatment and 28 or 35 days after infection when fully recovered. Parasitemia was quantified by qPCR as

described previously (2, 4-10). Samples for parasitemia were obtained once, twice or thrice daily (for details see Table S1). Platelet and lymphocyte counts and D-dimer concentrations were measured once daily on all these days as described previously (2, 4-10). IFNγ plasma levels were measured daily in the 7 subjects from the EHMI8B study (8) and the 14 blood-stage challenged subjects from the EHMI9 study (4) by ELISA. For time points where parasitemia was quantified but hematological parameters were not assessed (evening and night visits), these values were interpolated using LOWESS smoothing curve function in GraphPad Prism 6. Adverse events (AEs) were recorded during each follow-up visit as following: grade 1 (easily tolerated), grade 2 (interfering with normal activity), or grade 3 (preventing normal activity). Fever was recorded as grade 1 (>37.5°C – 38.0°C), grade 2 (>38.0°C – 39.0°C) or grade 3 (>39.0°C).

Individual health curves (Figures 1 and 4) were made using Tableau software (version 8.2). Figure 2 and Figure 5 were generated by performing Topological Data Analysis on the combined data set of parasite load, platelet counts, lymphocyte counts, D-dimer and IFNγ levels from all 91 subjects with the Ayasdi Iris software platform (ayasdi.com). Nodes in the network represent clusters of samples and edges connect nodes that contain samples in common (11, 12).

Cumulative duration of AEs was calculated for each subject by adding up the duration of all AEs per individual. The association between parasite load at time of thick smear positivity and cumulative duration of AEs was tested by Pearson correlation in GraphPad Prism (version 5). In order to quantify the tolerance status of each individual, the angle between parasitemia at time of treatment and the cumulative duration of AEs was calculated using the inverse tangent function in Microsoft Excel. Areas enclosed by the IFNγ by parasite load curves were calculated using the polygonloops function using Matlab (13). The difference between surface areas was tested by Mann-Whitney test in GraphPad Prism.

Results and discussion

Humans trace looping curves through disease space during malaria infection

We first used adverse events (AEs) as most direct and relevant measure of the subjects' health, and plotted these against parasite load over the course of a CHMI for each individual volunteer. This created looping curves of which representative examples are shown in Figure 1A. For most subjects parasitemia increased first and AEs occurred subsequently. When treatment was initiated, parasitemia reduced to zero and subjects recovered, thus closing the loop. The shape of these health curves was helpful to visualize differences between individuals, for example with regard to the recovery period: while for some subjects AEs decreased simultaneously with parasitemia, resulting in a collinear graph (e.g. subjects four and five), in others parasitemia declined first and AEs resolved subsequently (e.g. subjects

eleven and twelve). For all volunteers the curves ended where they started, without parasites in the blood and without symptoms or complaints, showing their resilience against this infection. Next to AEs, platelet numbers, lymphocyte counts and D-dimer concentrations created curves in disease space when plotted against parasite load over time (Figure 1B, C and D).

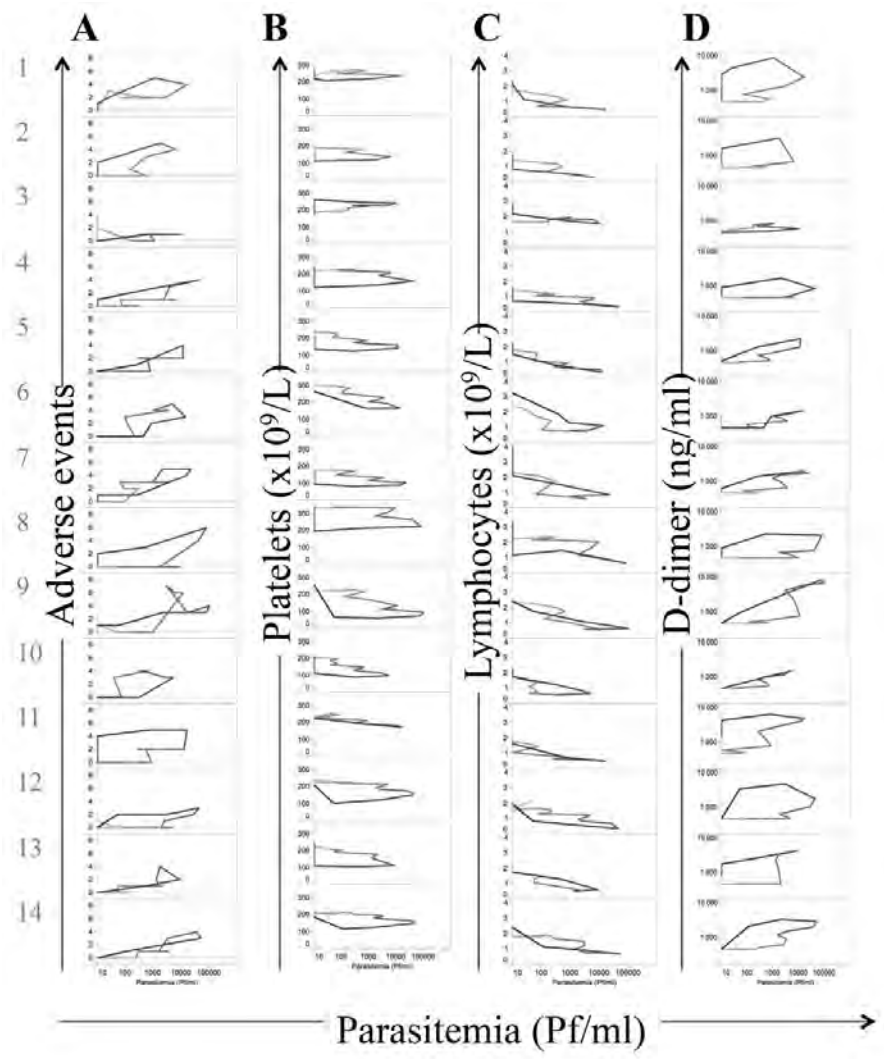


Figure 1 Loops in disease space from individual malaria infected human subjects. Loops were constructed in a disease space of parasitemia on the x-axis by (A) number of AEs, (B) platelets, (C) lymphocytes or (D) D-dimer on the y-axis. Each horizontal row of 4 graphs represents an individual i.v. blood-stage challenged subject (1-14) (4). Variables were

measured one day before challenge, and once to thrice daily from the day after challenge onwards. The line-color from light grey to black indicates increasing number of days after infection.

An alternative topological data analysis method (ayasdi.com) allowed us to visualize the course of the infection by combining all available data including parasite levels, platelet counts, lymphocyte counts, D-dimer levels and IFNγ levels from all 91 subjects. This analysis resulted in a ring-shaped structure that clearly represented the course of infection: time points

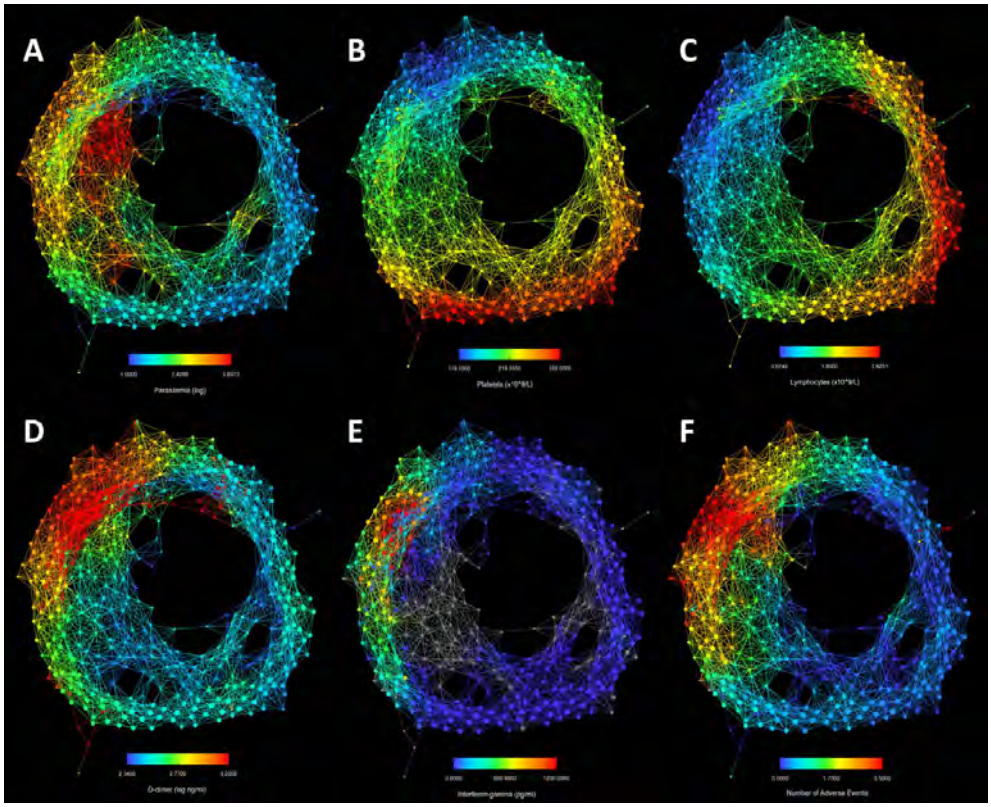


Figure 2 Topological data analysis of longitudinal data from controlled human malaria infections. Topological Data Analysis was performed on the combined data set of parasite load, platelet counts, lymphocyte counts, D-dimer and IFNγ levels from all 91 subjects with the Ayasdi Iris software platform (ayasdi.com). Nodes in the network represent clusters of samples and edges connect nodes that contain samples in common. *Norm correlation* was used as metric, and the lenses PCA coord 1 and PCA coord 2 were applied. Nodes are colored by the average value of their samples from dark blue (low) to red (high) for (A) parasite load, (B) platelet count, (C) lymphocyte count, (D) D-dimer levels, (E) IFNγ levels, and (F) adverse events.

showing high parasite load, IFNγ levels and D-dimer concentrations and low platelet and lymphocyte counts clustered together (Figure 2A-E). This topological data analysis based on parasite load and laboratory values also accurately co-located AEs (Figure 2F). The combined data clearly show that malaria infections in humans can be described by health curves in disease space.

Varying levels of tolerance to P. falciparum malaria can be visualized using disease space

There are three different ways for an organism to defend itself against infections: i) resistance, defined as the ability to control pathogen load, ii) tolerance i.e. the ability to limit severity of disease at a given pathogen load or iii) avoidance. Often, the first two mechanisms go hand in hand, and the balance between them determines the clinical course and outcome of an infection (14, 15). Also in malaria the distinction between tolerance and resistance has been recognized for a long time (16).

The number and severity of AEs experienced by volunteers during CHMI trials showed a significant variation between participants (Table 2). Although a small part may be explained by differences in reporting and/or use of escape medication, we believe that these data likely reflect true differences in disease severity after CHMI.

Table 2 Severity and duration of Adverse Events (AEs) after CHMI.

	Number of AEs per volunteer	**Cumulative duration of AEs per volunteer**
	Median (range)*	**Median (range)***
Grade 1	3 (1-11)	4.2 (0.1-30.5)
Grade 2	1 (0-8)	1.9 (0-20.4)
Grade 3	1 (0-9)	1.2 (0-17.8)
All AEs	7 (1-13)	9.6 (0.8-31.1)

* days

Severity of clinical disease, expressed as cumulative duration of AEs, and parasite load at time of detection by thick smear were significantly correlated (Figure 3A; Pearson $r=0.44$, $p<0.0001$), indicating that clinical presentation depends on parasite density, which is in line with data from endemic areas (17). The spread of data points around the correlation line, however, illustrates that subjects with the same parasite density can have quite a different cumulative duration of AEs. This suggests the existence of different tolerance levels against *P. falciparum* in this malaria-naïve population.

We next explored whether different levels of tolerance could be visualized using disease space. As shown in Figure 3B we defined subjects below the line as relatively tolerant and those above the line as relatively intolerant; subsequently the 'tolerance-level' of each volunteer

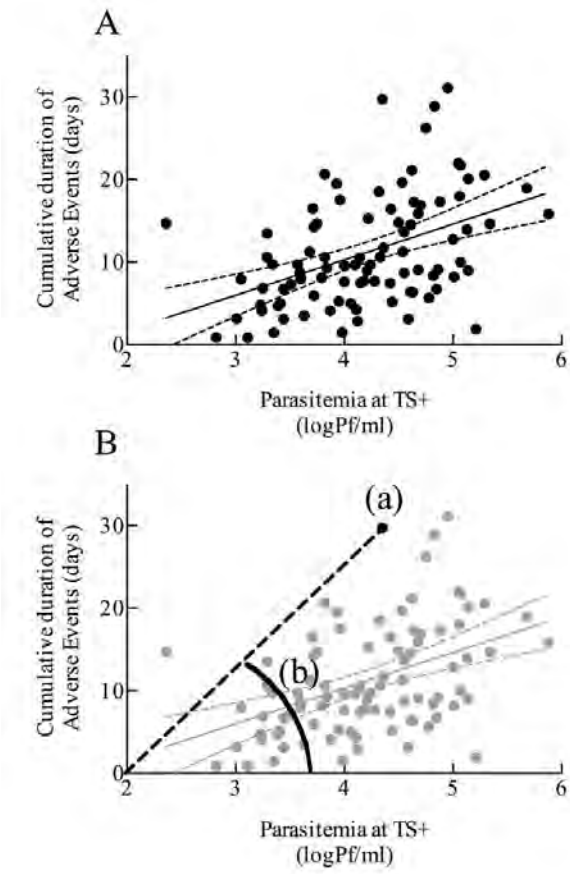


Figure 3 Association between parasitemia and disease severity in CHMI. For all 91 subjects, parasitemia at time of detection by thick smear (TS+) is plotted on the x-axis against the cumulative duration of AEs on the y-axis (A). The tolerance level of an individual (a) can be quantified by calculating the angle (b) between both parameters (B).

was quantified by calculating the angle between parasite load and cumulative duration of AEs. By plotting IFNγ plasma levels available from 21 volunteers against parasitemia, these different levels of tolerance could be distinguished in disease space (Figure 4). Within one trial (n=14 (4)), the surface area of these plots was significantly larger in intolerant compared to tolerant subjects (p=0.018).

We finally created a second topological graph using 'norm angle' as an alternative data metric. This metric calculates the angle between data points, and is used to assess whether two data rows are collinear rather than the absolute magnitude of their distance (ayasdi.com; Figure 5A). Using platelet counts, lymphocyte counts, D-dimer levels, parasitemia and IFNγ levels, subjects with different levels of tolerance were differentially located in disease space. While tolerant subjects mainly stayed in the central area of the graph, intolerant subjects occupied the outer region (Figure 5B).

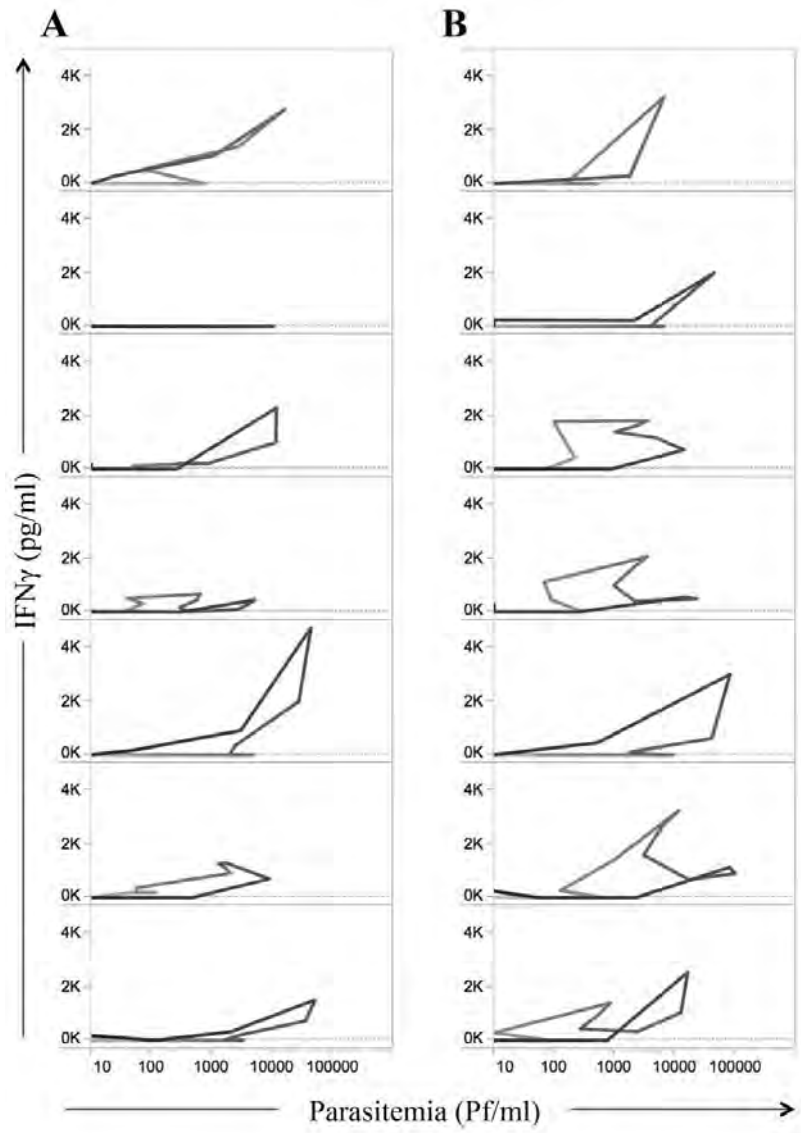


Figure 4 Loops in disease space distinguish tolerance phenotypes. Loops were constructed in disease space of parasitemia on the x-axis and IFNγ-level on the y-axis for all 14 blood-stage challenged subjects (4). These subjects were divided in 2 groups based on their tolerance level: (A) shows the 7 most tolerant subjects and (B) the 7 least tolerant subjects.

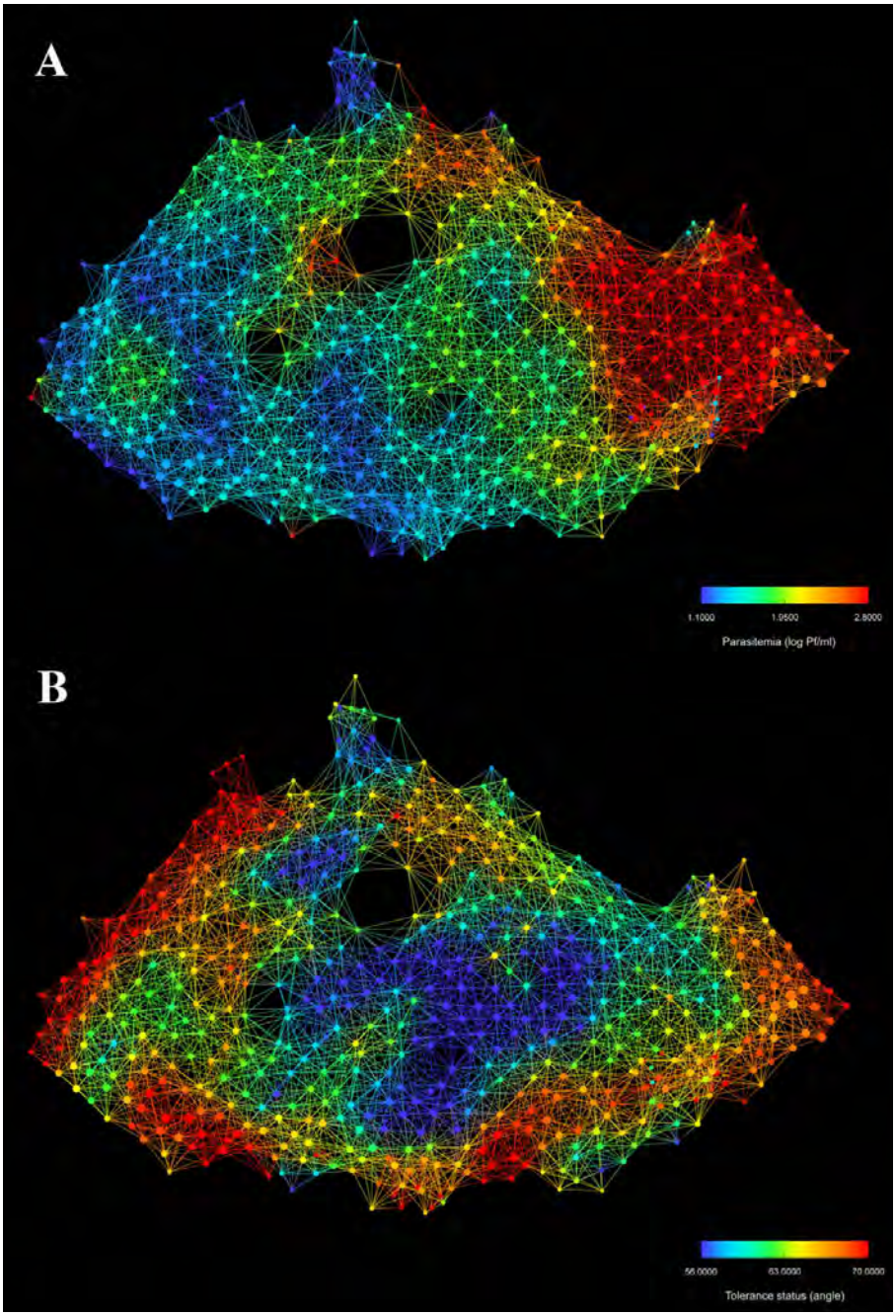


Figure 5 Tolerance level of CHMI volunteers determines their location in disease space. Topological Data Analysis was performed on the combined data set of parasite load, platelet counts, lymphocyte counts, D-dimer and IFNγ levels from all 91 subjects with the

Ayasdi Iris software platform (ayasdi.com) using norm angle as metric, and neighborhood lens 1 and neighborhood lens 2 as lenses. Nodes are colored by the average value of their samples for (A) parasite load or (B) tolerance level (angle), from dark blue (low) to red (high).

This study shows for the first time that human infections analyzed in disease space can provide important information by, for example, identifying and visualizing different levels of tolerance. For uncomplicated infections that respond sufficiently to antimalarial treatment, this analysis will not have clinical consequences. However, patients that are less tolerant or poorly responding to therapy, these curves may provide guidance for clinical care and treatment. For patients with severe malaria, or for example sepsis, where the balance between pro-inflammatory and anti-inflammatory cytokines is crucial for the outcome (18), the position and movement in disease space using biomarkers may direct personalized therapy (19). Since baseline samples will not be available from these patients, it will be impossible to construct a full health curve. Therefore, a next step will be to assess whether a limited number of longitudinal samples, constituting a vector in disease space, will be sufficient to provide useful information in such cases.

Future studies on malaria infections in endemic areas using disease space might elucidate patterns on tolerance and resistance after natural infection. In longitudinal cohort studies children can be treated with antimalarials before the start of the transmission season with intensive follow-up and regular blood-draws during the malaria season until development of parasitemia. Since only a proportion of these children will develop symptoms, analysis of immune markers and parasite kinetics might reveal pathways involved in tolerance and resistance in this partially immune population.

Conclusions

Data from this exploratory retrospective study show for the first time that disease space can be used to describe infections in humans. Personalized health curves have the potential to provide important information for clinical monitoring and treatment as well as insight in mechanisms and pathways involved in tolerance and resistance to malaria and other infectious diseases.

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Table S1 Daily frequency of blood sampling for quantification of parasitemia.

Study		Day -1	Day 1-4	Day 5-6	Day 7-11	Day 12-15	Day 16-21	Treatment	Day 1-3 post treatment	Day 28/35
NCT00757887 (8)		1	-	2	3	2	1	1	1	1
NCT01218893 (6)		1	-	2	2	2	2	1	1	1
NCT01422954 (5)		1	-	2	2	2	1	1	1	1
NCT 01236612 (4)	Mosquito bite	1	-	2	3	2	1	1	1	1
	Blood stage	1	1	2	3	2	1	1	1	1
NCT01002833 (9)		1	-	2	3	2	1	1	1	1
NCT01660854 (10)		1		1	2	2	1	1	1	1
NCT01086917 (7)		1	-	2	3	2	1	1	1	1

CHAPTER 13

General discussion

Malaria continues to cause significant morbidity and mortality worldwide, and an effective vaccine will be crucial to fight this disease. However, rational design and development of such a vaccine has been hampered by our limited knowledge on what constitutes protective immune responses. This thesis aims to improve our understanding of protection and immune responses against malaria using the chemoprophylaxis and sporozoites (CPS) immunization model and Controlled Human Malaria Infections (CHMIs).

Following the seminal proof-of-principle study (1), the CPS trials described in this thesis demonstrate a remarkable reproducibility in protective efficacy. CPS immunization of volunteers with bites from 3 times 12-15 mosquitoes infected with NF54 sporozoites consistently induces protection (19 of 20 subjects) against a standard NF54 challenge

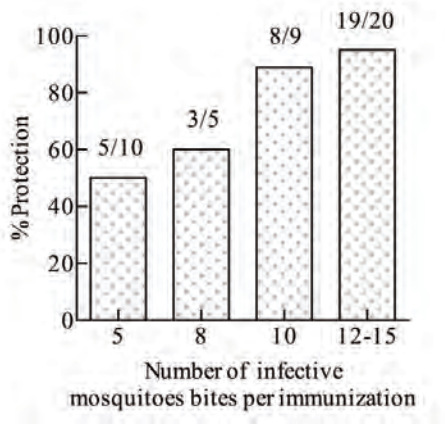

Figure 1 Protection after 3 CPS immunizations at monthly intervals with different numbers of bites.

infection (95%; chapters 6 and 7). Reduction of the number of infected mosquitoes results in gradually decreased protection to 5/10 subjects (50%) after immunization by three times 5 mosquitoes (Figure 1, chapters 7 and 10). These results reconfirm the impressive potency of CPS: the immunization dose is 20-30 times lower than for RAS requiring a minimum of 1000 bites and known as the only other method to induce complete sterile protection in the CHMI model (2). Its efficacy, potency, reproducibility and dose-dependency make the CPS protocol a unique methodology for immunological and vaccination studies in healthy volunteers.

The CPS immunization model can be exploited for two main purposes: the development of a whole parasite vaccine, and the investigation of protective immune responses. In this final chapter, I will discuss these two applications in the context of the existing body of knowledge, the novel insights gained from this thesis and recent developments. An important step towards application of a whole sporozoite vaccine will be to replace mosquito bites by injection of cryopreserved sporozoites (chapter 11) and I shall explore the implications and possibilities of this trajectory. A cornerstone in this thesis is the CHMI, and I will conclude this final chapter with some reflections on how our analysis of trust and control (chapter 2) may be used to improve the design and execution of clinical trials.

Whole sporozoite immunization: the road towards an applicable vaccine[1]

An ideal malaria vaccine is safe, practical, affordable, and induces a robust and long lasting protective immune response in all target populations including young children in Africa. The experience with RTS,S shows that it is possible to protect children in Africa against malaria by means of vaccination (3). This is encouraging, but the limited efficacy also demonstrates the need for a more effective "second-generation" vaccine. Immunization with whole sporozoites is highly efficacious when administered by mosquito bites or by intravenous administration (1, 4) (chapters 6, 7 and 10). The observed high protective efficacy strongly supports further exploration of their possible clinical development as a vaccine.

Chemoprophylaxis and sporozoites

In the CPS immunization protocol as originally described by Roestenberg et al (1) and applied in this thesis, subjects are exposed to sporozoites by bites from infected *Anopheles* mosquitoes. This limits the use of this protocol to a few specialized centers with parasite-culture facilities and well-equipped insectaries. Replacement of the mosquito bites by sporozoite injection with needle and syringe would greatly increase the possibilities for clinical application of CPS.

In this thesis, we show for the first time that volunteers can be infected with *P. falciparum* malaria by intradermal injection of aseptic, purified, cryopreserved sporozoites, a product developed by Sanaria Inc (PfSPZ Challenge; chapter 11). This opens the possibility to immunize subjects using chloroquine prophylaxis by injection of sporozoites with needle and syringe.

However, a first trial in which volunteers under chloroquine prophylaxis were immunized three or four times by intradermal administration of 75,000 cryopreserved sporozoites, showed no protection (5). CS-specific antibodies were induced with titers comparable to mosquito immunization, but LSA-1 antibodies and *P. falciparum* specific cellular memory responses were absent. Retrospective qPCR-analysis showed absence of parasitemia after all immunizations in all subjects, indicating that the parasite load achieved was much lower compared to immunization via mosquitoes, and apparently not sufficient to induce immunity. Protection after CPS immunization is dose-dependent (chapter 7), and this first PfSPZ-CVAC trial suggests that development of sufficient numbers of parasites developing in the liver may be more crucial than just exposure to high numbers of sporozoites. This hypothesis is in line

1 Parts of this paragraph were previously published in 1) Bijker EM, Sauerwein RW. Malariavaccins: de huidige stand van zaken. [Malariavaccines: an update. In Dutch]. *Tijdschrift voor Infectieziekten*, 2014 and 2) Bijker EM, Sauerwein RW. *Plasmodium falciparum* whole-parasite malaria vaccines. In: *Malaria Vaccine Development: Over 40 Years of Trials and Tribulations*. Corradin G, Engers H (Eds). Future Science Group, London, UK, 149–162

with historical animal studies showing that immunization with heat-inactivated sporozoites did not induce protection (6, 7) and a more recent review describing the importance of liver-stage development for protection in rodents (8). Therefore, it will be important to optimize the parasite liver load after administration of cryopreserved sporozoites.

After the first PfSPZ Challenge study at Radboudumc (chapter 11), a number of trials have been performed by Sanaria and collaborators to investigate alternative administration regimens, including an increased number of intradermal injections with fewer sporozoites per injection, intramuscular administration and intravenous injection. With the latter approach, 100% infection can be induced with a prepatent period approximating CHMI by mosquito bites (9, 10). Apparently, efficient migration of the sporozoites from the skin to the liver was a crucial hurdle after intradermal injection and could be circumvented by intravenous administration. A logical next step would thus be to perform CPS immunization with intravenous injection of sporozoites. This trial has indeed been performed and the results are expected soon (Clinicaltrials.gov NCT02115516).

In chapter 3 we hypothesized that the striking efficiency of CPS compared to RAS might be partially due to immune modulatory effects of chloroquine. Chloroquine was shown to enhance cross-presentation *in vitro* and in rodents, resulting in improved cytolytic responses (11, 12). In humans, co-administration of chloroquine with a hepatitis B vaccine booster significantly increased the number of virus-specific IFNγ-producing $CD8^+$ T cells (13). However, our results from rodent studies (chapter 5) and a clinical trial (chapter 10) do not support this concept so far: additional administration of chloroquine to mice that receive RAS immunization does not improve protection or immune responses, and replacement of chloroquine by mefloquine for CPS does not reduce immunogenicity or protective efficacy. Although we cannot formally rule out that chloroquine has a small direct immune-enhancing effect in CPS immunization, our work indicates that this effect is not substantive enough to warrant further investigation.

CPS with mefloquine is as immunogenic and protective as CPS with chloroquine (chapter 10). This observation that CPS immunization does not depend on chloroquine opens opportunities to investigate CPS with other drugs with killing effects at different points of the *Plasmodium* life cycle. A number of antimalarials have been shown to induce protection in rodent models. Primaquine and pyrimethamine affect parasites within the hepatocyte (14, 15) and azithromycin causes delayed death of intrahepatic parasites and release of noninfectious merozoites (16). The advantage of these drugs may be a potential reduction in adverse events as a result of the limited occurrence of blood-stage parasites. In contrast, chloroquine and mefloquine only affect blood-stage parasites, allowing the brief occurrence of parasitemia after immunization resulting in symptoms and complaints in the majority of subjects, including grade 3 adverse events (chapters 6, 7 and 10). Prevention of parasitemia will likely increase tolerability of the immunization regimen, but possibly at the expense of the efficacy

because of earlier arrest and thus diminished antigenic load and repertoire (8). Moreover, it remains to be investigated whether these drugs provide sufficient attenuation to prevent breakthrough infection, requiring additional administration of another compound such as chloroquine. These pros and cons of different attenuation methods comprise the classical balance between safety and immunogenicity of whole organism vaccination.

CPS immunization requires a much smaller number of mosquito bites to induce protection compared to RAS, but CPS vaccination by needle and syringe will require separate administration of chloroquine with the currently registered formulations, to prevent breakthrough infections. Realistically, clinical development of CPS as a vaccine for military personnel and selected traveler populations is worthwhile to explore. Large-scale implementation in malaria-endemic regions remains unlikely as long as a secure dosing schedule of CQ, currently requiring rigorous compliance from vaccinees, has not been established concomitant and limited to vaccination sessions.

Development and implementation of whole sporozoite vaccines

Sanaria Inc. has developed and tested the first GMP-produced whole sporozoite (RAS) vaccine: PfSPZ Vaccine. Sterile protection in 6/6 subjects has been obtained after intravenous, but not subcutaneous administration (4, 17). This vaccine has been taken forward for initial testing in Mali and Tanzania (Clinicaltrials.gov NCT01988636 and NCT02132299). Alternatively, genetically attenuated parasite (GAP) vaccines are still at early stages of clinical development, but may provide great potential. Compared to RAS, GAP vaccines provide the advantages of safer production and homogeneity of the product (18, 19). A number of crucial technical, economical and clinical hurdles need to be taken before application of whole sporozoite vaccines can be considered. For public health implementation, costs should be reasonable and the vaccine has to be suitable for administration to appropriate risk groups in malaria-endemic areas.

Currently, Sanaria's product PfSPZ Vaccine needs to be stored in liquid nitrogen vapor phase (LNVP) below -140°C and would have to be distributed through a LNVP cold chain, while the preferred storage condition as currently defined by the WHO is ambient, minimally 2-8°C. If the vaccine remains dependent on this storage method, its implementation will require large investments in storage and transport facilities at different levels in endemic countries (20). While high protective efficacy would probably legitimize such an investment, optimization of cryopreservation to increase infectivity of sporozoites, even when stored at higher temperatures, should be a focus of investigation.

Other points to be addressed include optimal vaccine dose, number of doses, interval between doses, site and route of administration. A new vaccine should ideally be administered along with the current vaccines in the Expanded Programme on Immunization (EPI) and therefore show no interference with other vaccines. The route of immunization should be

implementable on a large scale without the need for extensive healthcare workers' training. Currently, PfSPZ Vaccine only confers protection when administered intravenously, a route that has not been used for the administration of preventive vaccines before. While the intravenous approach may be effective and feasible for adults, it entails an enormous challenge for implementation in children in Africa. Although a very high vaccine-efficacy might prompt the community to invest in this unprecedented administration route, further research will be required to investigate the technical, ethical and practical feasibility in young African children. Ideally, immunization dose and regimen of PfSPZ Vaccine will be optimized for more conventional routes of delivery or administration with novel devices such as microneedles.

Induction of sustained protection is the hallmark of an effective vaccine. Longevity of protection in the absence of parasite exposure, but also the potential boosting of immune responses by repeated exposure to sporozoites in the field should be assessed. If vaccine-induced pre-erythrocytic immunity wanes, serious health consequences can be expected for individuals in endemic countries in the absence of clinical immunity (21). Furthermore, the possibility exists that antibodies in malaria-exposed individuals could capture the vaccine-sporozoites and therefore interfere with induction of protective immunity. This question remains open since protective efficacy of whole sporozoite immunization approaches has only been investigated in malaria-naïve volunteers until now, but should be addressed in the near future.

In summary, whole parasite immunization approaches hold great promise to play a role in the combat against malaria, once various technical, clinical and economic hurdles have been overcome. Furthermore, CPS has the potential to play a unique and pivotal role in the investigation of protective immune responses, which will inform vaccine development and could thereby have important implications.

CPS immunization as a model to investigate protective immune responses against P. falciparum malaria

One of the major findings from this thesis is that protective immunity induced by CPS immunization is directed against pre-erythrocytic stages (chapter 6). Although it is possible that a blood stage component would have contributed if we had not treated at time of thick smear positivity, it is clear that pre-erythrocytic immunity is the main component responsible for protection, legitimizing a focus of further research towards sporozoite- and liver-stages. Here, we will discuss our findings in relation to humoral, cellular and innate immunity in pre-erythrocytic protection, and discuss four important lines of future research: target antigens of protective immunity, immune signatures of protection, comparing naturally acquired versus experimentally induced protection and heterologous protection.

Humoral immunity in pre-erythrocytic protection

Antibodies can play a role in pre-erythrocytic protection through a number of possible mechanisms: i) Preventing sporozoites from exiting the proboscis at the biting site by forming immune complexes with soluble sporozoite proteins in the mosquito saliva: mosquitoes were shown to inject less sporozoites into RAS immunized mice or mice that were passively immunized with anti CS-protein monoclonal antibodies (22); ii) Inhibition of sporozoite motility. Sporozoites glide around and traverse the endothelium of the skin capillaries before entering the circulation (23); iii) Sporozoite clearance in circulation by opsonization; iv) Inhibition of hepatocyte traversal and/or invasion (24, 25); v) blockage of intrahepatic delevopment and/or elimination of liver stages, although the underlying mechanism of such an antibody-mediated effect remains unclear so far (26, 27).

There is ample evidence from animal studies that humoral immunity contributes to pre-erythrocytic protection. Naïve mice are protected against sporozoite challenge after passive transfer of serum or IgG from RAS-immunized mice (28-30). The circumsporozoite (CS) protein is a major target of these antibodies, and also monoclonal antibodies against the CS protein confer protection (31-35). Moreover, monkeys can be protected by a monoclonal antibody against the *P. vivax* CS protein repeat (36) or *P. knowlesi* sporozoites (37).

In vivo studies of pre-erythrocytic immunity in humans are hampered by the inaccessibility of the liver. As a substitute, a number of *in vitro* tests is available to investigate the effect of antibodies on sporozoite gliding (38, 39), traversal (40, 41), hepatocyte invasion and intrahepatic development (42-44). More recently, humanized mouse models, i.e. mice with functional human hepatocytes, have been generated, allowing the assessment of *in vivo* development of *P. falciparum* liver stages (42-45). From these assays, there is clear evidence that specific antibodies from semi-immune individuals and RAS, GAP or RTS,S immunized subjects can inhibit hepatocyte invasion of *P. falciparum* and/or *P. vivax* (42, 43, 45-50).

Our studies clearly show that functional antibodies and memory B cell responses against pre-erythrocytic stages are also generated by CPS immunization (51, 52). However, in this setting the magnitude of responses against CS protein serves as a marker of parasites exposure, rather than protection from challenge infection (52). Although CPS-induced antibodies functionally inhibit hepatocyte traversal *in vitro* and reduce liver-stage infection in humanized mice (51), their exact specificities in relation to CPS-induced protection remain to be investigated.

Induction of T cell responses against pre-erythrocytic stages

T cell deficient mice cannot be protected by sporozoite immunization, which suggests that T cells play a critical role in protection (53). T-cell activation is initiated and orchestrated by dendritic cells (DCs) that process and present antigens from pre-erythrocytic *Plasmodium* stages (54, 55). As such DCs in skin draining lymph nodes can process antigens from

sporozoites (56) while liver DCs can present antigens from either live (57) or apoptotic (58) infected hepatocytes (59, 60). Alternatively, proteins egress from the cell and are taken up, processed and presented by antigen presenting cells such as Kupffer cells. In a subsequent infection, memory CD8 T cells can recognize antigens presented on the surface of infected hepatocytes and kill these cells. When sporozoites reach the liver, they traverse through several hepatocytes before they finally settle to multiply and mature (61). Rodent studies indicate that both traversed and infected hepatocytes present CS protein-derived peptides on their surface (62). CS protein and other parasite antigens are probably transported to the cytoplasm of the hepatocyte (63-65) after which they are processed and presented to T cells on the cell surface (62).

Evidence for cellular immunity in pre-erythrocytic protection

Because of practical limitations in human studies, most of our knowledge on cellular immune responses against pre-erythrocytic stages is based on animal models. Protective immune mechanisms differ depending on for example the mouse strain and the *Plasmodium* species used (66), and we should emphasize the need to be careful in translating these findings to humans. However, evidence for an important role of both CD4 and CD8 T cells in pre-erythrocytic protection from animal models is substantial and consistent. CD8 T cell depletion of mice abrogates protection by GAP (67, 68), RAS (29, 30, 69-71) and CPS (72) immunization. And also in non-human primates CD8 depletion diminishes RAS-induced protection (73). CD4 T cell depletion reduces protection in some studies (30, 72), but not in others (29, 67, 69-71). In any case, CD4 T cells appear to provide important help during immunization for the induction of an appropriate CD8 response (74). In addition to these depletion studies, T cells from RAS-immunized animals were shown to have high cytolytic activity, to eliminate infected hepatocytes *in vitro* and to confer protection against sporozoite challenge upon passive transfer (29, 75-79). CD4 and CD8 T cells isolated form mice immunized with peptides from the *P. yoelii* CS protein inhibit liver-stage development *in vitro* (80), as do lymphocytes stimulated with CS peptides (81). The importance of antigen-presentation in the context of MHC-I was further confirmed by studies showing that β2 microglobulin knockout mice cannot be protected by RAS (82) or GAP (83) immunization. Together, these data provide strong evidence for the crucial role of CD8 T cells and the supportive role of CD4 T cells in pre-erythrocytic protection.

Consistent with this evidence from animal studies, T cell responses are induced in humans by both sporozoite immunization and natural exposure (84-88), with specificities for pre-erythrocytic antigens such as CS protein (85, 86, 88, 89), Liver Stage Antigen-1 (LSA-1) (90) and thrombospondin-related adhesion protein (TRAP) (91). Data from subunit vaccination studies indicate that T cells are important mediators of protection in humans. RTS,S for instance induces CS protein-specific CD4 T cells that produce IFN-γ

upon restimulation with peptides (49), with a higher frequency in protected compared to unprotected volunteers (49). Moreover, vaccination with the pre-erythrocytic multiple epitope –TRAP (ME-TRAP) using a chimpanzee adenovirus vector and a modified vaccinia virus Ankara booster induces specific IFN-γ producing CD8 T cells that correlate with sterile protection and prolonged prepatency (92).

The production of inflammatory cytokines such as IFNγ and TNFα (93, 94), is probably an important effector function of T cells against liver-stages (84, 85, 88), However, CD8-mediated protection after sporozoite immunization can occur independent of IFNγ in rodents (68, 95, 96), and cytotoxic mechanisms such as granzyme B or perforin-mediated killing are likely to contribute. Indeed, this thesis shows that granzyme B expression by CD8 T cells and degranulation of CD4 T cells were associated with protection against challenge infection (chapter 7). This emphasizes that it is crucial to assess a variety of markers when reviewing T cell responses, including markers of cytotoxicity, rather than cytokine production alone (97).

Innate immune responses in pre-erythrocytic protection

Vaccines are designed to induce adaptive memory responses, which have therefore rightly received the majority of attention. However, the innate immune system is crucial for the induction and orchestration of effective adaptive memory responses (98, 99), and there are even strong indications for a memory component in the innate immune compartment (100).

There is some evidence for a role of NK cells and γδ T cells in addition to CD8 and CD4 T cells in pre-erythrocytic protection. NK cell depletion significantly reduced protection by RAS immunization in several mouse strains (66). One study demonstrated inhibition of liver-stage development after sporozoite challenge by passive transfer of a γδ T cell clone isolated from an αβ deficient RAS immunized mouse (101).

This thesis provides additional clues that innate immune responses might contribute to CPS-induced protection. The frequency of γδT cells for instance increases dramatically upon CPS immunization (chapter 8), and these cells contribute significantly to IFNγ production upon restimulation (chapter 7, (102)). The origin, target antigens and functional role of these γδT cells remain unclear and warrant further investigation (103). For example, sequencing of γδT cell receptors before and after CPS immunization could clarify whether the expansion is antigen specific, i.e. creating clonal lines, or an aspecific bystander effect ((104), M. Mamedov, M. Davis lab, Stanford University School of Medicine, USA; personal communication).

Furthermore, a total of 11 out of 54 volunteers in three CPS-immunization studies remained qPCR negative during immunization, all except one of whom were protected against challenge. Since all volunteers included in these trials are malaria-naïve and thus lack adaptive memory responses, the difference between these volunteers and others with confirmed parasitemia during immunization might be explained by differences in innate

responses, although we cannot exclude that sub-qPCR parasitemia occurred. From rodent models, there is evidence that IL-6 can significantly inhibit liver schizont development (105) and that *Plasmodium* triggers a functional type 1 IFN response that reduces liver parasite burden (106). Variation in these or other innate pathways could thus account for the difference seen in CPS-immunized subjects and deserves further study.

Target antigens of pre-erythrocytic immunity

Although their efficacy appears to be modest as best so far, subunit vaccines may have substantial manufacturing advantages over whole sporozoite vaccine approaches. However, clinical development of malaria subunit vaccines has proven to be slow and difficult, mainly suffering from insufficient efficacy. RTS,S is most advanced showing 30-50% efficacy in endemic areas with waning and eventually complete loss of protection after three years (3, 107, 108). Clinical development might be accelerated by improved rational target antigen selection and vaccine design, and could greatly benefit from increased understanding of protective immunity.

The CS protein, the malaria target in RTS,S, has been a major focus of vaccine development for a number of good reasons: it is an immunodominant antigen, present on the surface of both the sporozoite and the infected hepatocyte (109) and there is plenty of evidence for a role in protective immunity (110) as described above. But an immune response against the CS protein is not absolutely required for the induction of protective immunity, since CS transgenic mice tolerant for the CS T-cell epitope can still be protected by RAS immunization, although less efficient than wild type mice (111). This findings, in addition to the limited efficacy of RTS,S and other subunit vaccines compared to whole sporozoite immunization, indicate that immune responses directed against multiple antigens are required for full sterile protection.

Identification of target antigens of protection will furthermore be important for a methodological reason. Assessment of cellular immune responses induced by CPS immunization have until now been investigated by *in vitro* restimulation with whole blood-stage parasites (chapters 7 and 10). While we found markers associated with protection using this approach, *in vitro* stimulation with peptides derived from relevant target antigens or *ex vivo* staining with tetramers might reduce the 'noise' in the response and allow for a clearer distinction between protective and unprotective responses. Identification of novel target antigens will thus be important for both the development of an effective second generation subunit vaccine and the study of immune mechanisms of protection.

Importantly, rather than being the result of a response against a single key antigen, sterile pre-erythrocytic immunity appears to be based on broad responses to numerous antigens (112, 113). The availability of the *P. falciparum* genome sequence (114) has facilitated the analysis of expression and transcription of the more than 5000 *Plasmodium* genes in its

different life cycle stages (115-117) and the identification of protein target antigens (112, 118, 119). A number of studies indeed indicate the importance of novel pre-erythrocytic antigens in protective immunity induced by sporozoite immunization. Screening of PBMCs from RAS-immunized subjects revealed 16 *P. falciparum* protein antigens that had previously not been associated with protection, out of which 2 were preferentially recognized by protected volunteers, while the 'traditional' antigens CSP, SSP2, LSA1 and EXP2 were detected mainly in nonprotected volunteers (112). Moreover, screening of antibodies from RAS immunized donors revealed a number of novel pre-erythrocytic antigens (119). These data support a strategy for further research aimed at the identification of combinations of (novel) antigens against which protective immune responses are directed.

For the induction of sterile pre-erythrocytic immunity, expression of *Plasmodium* antigens by infected hepatocytes appears important. In rodent studies, protection after RAS or GAP immunization can be abrogated by primaquine treatment, indicating that liver-stage antigen persistence is required (120, 121). Moreover, lower sporozoite immunization doses are sufficient to protect mice in protocols with longer liver stage development (8), and the superiority of CPS compared to RAS in humans might also be partially attributed to the increased breadth of liver stage antigens expressed during immunization. Taken together, the infected hepatocyte seems to be an attractive vaccine target. However, studying *Plasmodium* gene expression in liver stages is problematic because of the low efficiency and reproducibility of hepatocyte invasion by sporozoites *in vitro*. One approach to overcome this problem might be to isolate liver stage schizonts from uninfected hepatocytes by microdissection; laser capture microdissection of 1500 *P. yoelii* schizonts was shown to be sufficient for generating a cDNA liary from 1 μg of total RNA (117). An alternative option might be the use of parasites transfected with green fluorescent protein (GFP) allowing for FACS sorting and subsequent analysis of infect hepatocytes.

Of all *in vitro* hepatocyte models available, sporozoite invasion is most efficient in fresh primary hepatocytes, but their availability is limited and irregular. Investment in the generation of hepatocyte cell lines, optimization of hepatocyte-cryopreservation and development of novel platforms are therefore important. For example, a microscale human liver platform has been developed that is comprised of primary human hepatocytes and nonparenchymal cells in which full liver stage development of *P. falciparum* and *P. vivax* was established (122). Furthermore, application of hypoxic conditions might result in improved infections in hepatocyte cell lines (123), although superior results are still achieved with fresh hepatocytes.

Future work will exploit the reproducibility and efficiency of CPS immunization to identify novel target antigens of sterile pre-erythrocytic immunity for both cellular and humoral immunity (clinicaltrials.gov NCT02080026). Significant efforts have been made to identify target antigens in RAS immunization (112, 119, 124, 125), but the presence of late

liver stage development and early blood-stages in CPS probably allows for expression of a number of additional antigens that might be important for its unprecedented efficiency. For T cell targets, *in vitro* stimulation with synthetically produced, overlapping peptides might reveal novel targets. Alternatively, *Plasmodium* proteins can be expressed in *Escherichia coli* and then (cross-) presented by monocyte-derived DCs to which autologous T cells are added. Measurement of responses by these T cells, such as IFNγ production or degranulation, can be used in order to identify proteins that are recognized by CPS-immunized protected subjects (126). The advantage of this latter approach is that the processing of proteins is included in the assay, which might thus be a good representation of the *in vivo* processes. For the discovery of novel antibody targets, B cell receptor sequencing of plasmablasts after CPS immunization can be performed, in order to generate monoclonal antibodies that can be tested for specificity on protein microarrays (127) and for functional capacity *in vitro* or in humanized mice (128).

Immune signatures of protection

We identified degranulating, CD107a positive CD4 T cells and granzyme B producing CD8 T cells to be associated with protection after CPS immunization (chapter 7). The role of Th1 and cytotoxic T cells in CPS immunization was further supported by the increased expression of T-bet in both CD4 and CD8 T cells directly after immunization (chapter 8). While these observations aid to advance our understanding of protective immune responses, it remains a challenge to identify more accurate correlates of protection. Moreover, mechanistic studies will be required to reveal whether and how T cells actually contribute to protection.

Protective antimalarial immune responses are constituted of the two aspects that were discussed above: the human host, i.e. the quality and quantity of immune responses, and the *Plasmodium* parasite, i.e. the target antigens. It is unlikely that targeting a single antigen will result in a fully protective immune response (129). Similarly, it will probably be necessary to induce a multifaceted immune response including appropriate stimulation of the innate immune system and induction of both cellular and humoral memory responses. In order to fully understand protection, and to utilize this knowledge to design more effective vaccines, the next step will be to comprehensively analyze both target antigens and immune responses in conjunction, rather than separately, in order to identify immune signatures of protection.

Although the full breadth and diversity of the protective immune responses could not yet be revealed by the techniques and assays available for use in this thesis, we indeed found an indication supporting the immune signature hypothesis. We performed a lasso analysis on a panel of diverse cellular immune readouts after CPS immunization, including Th1, Th2 and Th17 cytokines, chemokines and cytotoxic markers. By performing this lasso analysis, we searched for a combination of markers, rather than a single marker, that predicted protection optimally. A combination of 11 makers indeed resulted in an improved area under the receiver

operator characteristic (ROC) curve compared to CD107a expression by CD4 T cells alone (0.87; 95%CI 0.73-1.0 versus 0.73; 95%CI 0.48-0.98). This supports the hypothesis that it is important to assess immune responses in relation to each other, as a signature of protection, rather than each single marker separately.

The next step will be to analyze target antigens of protective immunity, and combine these data with different approaches to assess the induced immune responses. Techniques for the identification of target antigens were discussed above. Assessment of immune responses could include gene-expression data *ex vivo* shortly after immunization or after *in vitro* restimulation of PBMCs using microarray or RNA sequencing (130), peripheral blood cytokine profiles and antigen specific memory responses. Humoral responses can be screened using protein microarray and confirmed by ELISA and functional assays. Cellular responses can be assessed *ex vivo* (chapter 8), or by flow cytometry or ELISpot after *in vitro* restimulation with whole parasites (chapters 7 and 10) or overlapping peptide pools. Flow cytometry technique used in this thesis allowed for the simultaneous assessment of 9 markers. Currently, the number of markers that can be assessed by flow cytometry using fluorochromes has increased to 20, and integration of mass spectrometry using rare-earth-metal isotopes overcomes the problem of spectral overlap of fluorescent dyes, extending the number of makers measured simultaneously to more than 35 (131). Once crucial target antigens are identified, this will also enable us to investigate the kinetics and characteristics of antigen-specific cells by tetramer-staining (132, 133) and perform functional cellular experiments, such as cytotoxic assays to assess lysis of peptide pulsed autologous target cells by T cells (78).

Combining data from such a comprehensive analysis of the induced immune responses on the one hand and target antigens on the other hand will create a multidimensional data set, which will be the next step towards identifying early predictive markers of vaccine efficacy and unraveling immune mechanisms of protection. The huge quantity of data generated will require intelligent bio-informatics approaches. The next biggest challenge will be to go from observational data to actual understanding; in order to make sense of the data, and assess the results for their clinical and immunological relevance, it will be necessary to build new collaborations between clinicians, immunologists, epidemiologists, statisticians and bio-informaticians working closely together in multidisciplinary teams (134). If such collaborations can be set up effectively, and novel bio-informatics approaches can be used in conjunction with classical immunology and vaccinology concepts and methods, important steps will be made to further advance our understanding of protective immunity against malaria. This knowledge may then enable us to trigger similar immune mechanisms with a vaccine.

Naturally acquired immunity versus experimentally induced protection

An alternative strategy to advance our understanding of target antigens and protective immune responses will be to compare naturally acquired immunity to experimentally induced protection. Residents of malaria-endemic areas develop immunity against *Plasmodium*, but this takes many years of repeated exposure. In areas of high and stable transmission, morbidity and mortality occur mainly during early childhood, while most malaria infections during adulthood are asymptomatic (135). This huge infectious reservoir of asymptomatic infected individuals is an important hurdle in elimination efforts. Sterilizing immunity, as induced by experimental RAS and CPS immunization, does not appear to occur in the field (136).

Comparative analysis of immune responses from CPS or RAS immunized subjects and clinically immune individuals from endemic areas might reveal important differences in antigen recognition and immune mechanisms of protection. Indeed, such an analysis of antibody response by protein microarray showed that antibodies from semi-immune and CPS immunized individuals recognized 202 and 192 antigens, respectively. While CPS immunized subjects recognized mainly pre-erythrocytic antigens, semi-immune individuals reacted mainly against blood stage proteins, and only 60 antigens were recognized by both groups (127).

This kind of information can be used to inform choices in vaccine design: should one aim for sterile pre-erythrocytic protection, as obtained in whole sporozoite immunization, with the risk of severe disease in the absence of a blood-stage component (chapter 6, (21))? Or should one focus on a vaccine directed against erythrocytic stages, in order to reduce clinical disease, although these vaccines have shown disappointing results until now? A logical step might be to focus on pre-erythrocytic stage vaccines in order to prevent infection, and thus transmission, as much as possible, but to invest in an erythrocytic component in parallel to prevent problematic reductions in blood-stage immunity (21). An interesting alternative in this context might be a vaccine aimed to reduce tolerance rather than resistance to the parasite. As proof-of-principle of such an approach, mice immunized with synthetic glycosylphosphatidylinositol (GPI), a *Plasmodium* toxin, were protected against severe malaria and fatality (137).

Heterologous protection

In chapter 9 we show that CPS immunization can induce protection against a heterologous *P. falciparum* strain. Although protection was partial in most subjects, this could possibly be explained by the suboptimal immunization dose, waning immunity and/or relatively high stringency of the NF135.C10 heterologous challenge. Substantial heterologous protection can hopefully be obtained in the near future by using an optimal immunization dose (clinicaltrials.gov NCT02098590). One way to further optimize the CPS protocol could be

to raise the immunization dose, since increasing antigen exposure might be required for the induction of protection in the following protection scenarios: homologous < long-lasting homologous < heterologous < long-lasting heterologous protection. Alternatively, using a mix of different *P. falciparum* strains for immunization could improve the breadth of the immune response and thereby heterologous protection.

Next to the importance of heterologous protection for the use of whole sporozoite immunization as an implementable vaccine, the lack of heterologous protection in the first study (chapter 9) can also be used to gain knowledge about protective target antigens. Once candidate antigens are identified in the context of homologous protection by CPS, these can be narrowed down by assessing recognition of their heterologous equivalents and association with protection against heterologous challenge in cell samples from this cohort. It will therefore be very important to annotate the sequences of the NF135.C10 and NF166.C8 strains, and compare sequences of candidate target antigens.

Challenge in a vial

In chapter 11 we show that healthy adult volunteers can be infected with *P. falciparum* malaria by intradermal injection of aseptic, purified, cryopreserved sporozoites (PfSPZ Challenge). This will enable centers worldwide to perform CHMI, while previously such trials were limited to centers with specialized insectaries. One limitation of our study was the absence of a dose that resulted in parasitemia in all subjects. Moreover, the prepatent period was longer than typically obtained in CHMI by mosquito bites (9). Optimization of viability, dose and administration route were thus required in order to improve infectivity. Following our study, a number of clinical trials has been performed at other sites to optimize administration of PfSPZ Challenge for CHMI.

Rodent studies investigated the effect of administration route on the infectivity of sporozoites administered by needle and syringe. While intramuscular administration resulted in significantly higher liver load compared to intradermal or subcutaneous injection, intravenous administration was even more efficient (138). Consequently, intramuscular administration was assessed in humans with 50% (3/6) and 100% (6/6) infection after injection of 2,500 and 25,000 sporozoites, respectively. In this trial, 5/6 subjects became infected after intradermal injection of 2,500 sporozoites with a prepatent period of 13.2 days, as in our study (chapter 11), demonstrating the reproducibility of PfSPZ Challenge (139). The prepatent period, however, was still longer than after mosquito bites. Next, intravenous administration of PfSPZ was assessed, resulting in 100% infection and a shorter prepatent period of approximately 11 days by injection of 3,200 PfSPZ (10), resembling inoculation by mosquito bites.

However, one should be cautious in replacing the natural inoculation route of mosquito

bite by intravenous administration and thereby circumventing the skin. The skin is a crucial organ in the immune system, and *Plasmodium* sporozoites are thought to modulate systemic immune responses through mechanisms in the skin (140). Interestingly, rodent models showed that bites from uninfected *Anopheles* mosquitoes conferred protection against *P. berghei* challenge (141). Similarly, mice exposed to bites from uninfected sand flies are protected against Leishmania major challenge (142, 143). The work in this thesis also indicates an effect of mosquito bites, independent of the parasite: control subjects that were receiving bites from uninfected mosquitoes while taking chloroquine prophylaxis showed increased proliferation and activation of mainly CD4 T cells (chapter 8).

Since intravenous and intramuscular administration of sporozoites bypass the skin and lack mosquito saliva-components, there is a risk of skewing study outcomes compared to the natural inoculation route. It will therefore be important to perform a direct comparison of both challenge methods, for example after CPS immunization, to assess the effect of circumventing the skin stage on immune responses and protection. Furthermore, the skin is an interesting organ to further explore, for example with a skin blister model. In this model, 10-15 mm suction blisters are created using a vacuum pump, separating the epidermis from the dermis. Analysis of cells in the blister fluid can then be used to investigate cellular immune responses in the skin. This model has been used to study the effect of aging on recall responses (144), and could now be used to assess the effect of mosquito saliva and sporozoites on immune responses in the skin.

Another possibility that is created by the availability of PfSPZ Challenge, is to perform CHMI studies in malaria-endemic areas. A first study was conducted in Tanzania in which 11 of 12 subjects who received 10,000 sporozoites intradermally developed parasitemia. The prepatent period was 15.4 days, compared to 12.6 in Dutch subjects (chapter 11). Whether this difference could be explained by acquired immunity or innate resistance affecting either pre-erythrocytic or blood-stages remained unclear (145), but this shows that PfSPZ Challenge might be useful to assess pre-existing immunity in endemic areas *(Obiero et al. submitted)*.

Looking at CHMIs through different eyes: interdisciplinary collaboration between biomedical and social sciences to improve clinical trial practice

CHMIs are a cornerstone in the work described in this thesis. Every type of clinical trial is a complex ensemble of scientific knowledge and instrumentation, of methodology and theory, of protocols and creativity. A clinical trial, like any scientific experiment, can thus be seen as a complex machinery that produces scientific knowledge. It is worth analyzing such machinery to better understand its functioning and then using those insights to adapt and improve the design and execution of clinical trials. In chapter 2 we presented CHMIs

as a 'strategic research site' for such research into the production of clinical trial knowledge in general, and for the role of trust and control therein in particular, because deliberately infecting healthy volunteers with malaria seems to require extra levels of safety—and thus of trust and control.

The scientific field that investigates how scientific knowledge is constructed is called "Science, Technology and Society studies" or "Science & Technology Studies" (STS) (146, 147). Much of this research uses qualitative methods, such as historical methods to investigate the origins of western scientific knowledge (148, 149), anthropological methods to study the practices in laboratories (150, 151), and sociological methods to investigate the development of scientific controversies (152, 153). In our study of the CHMI machinery, we used participant-observatory methods from the anthropology of science. Social-science research not only implies specific methodologies of data production and analysis, but also requires a different style of scholarly writing than is used in the natural sciences—the researcher-author needs to be more visible in the text (154, 155), resulting in the use of active rather than passive phrases and the use of 'I' rather than 'we'.[2] To signal this shift in style of research from natural science to anthropology of science, from discussing the results obtained with CHMIs to discussing the practice of doing CHMIs, the last part of this concluding chapter will be written in a social-science style that makes me more visible as author-researcher.

Doing a PhD means being trained to focus and specialize. This happens by immersion of the PhD student in research on a specific topic, and by socialization into a limited scientific community. Historians, philosophers and sociologists of science have argued that this could not be otherwise: only through many years of socialization will researchers learn the required spectrum of knowledge, skills, methods, instrumentation and literature (151, 152, 157). Under normal circumstances, this process results in a PhD student getting more and more versed in the paradigm (Kuhn's term) of that particular scientific specialty, during a process that is so gradual that many things are learnt without the student even noticing, and indeed forgetting what the world looked like before entering the field. Hence we call that knowledge 'tacit', since it is not explicated, not spoken about, not written down. It often is only when one meets an 'outsider', who is surprised by what the 'insider' finds self-evident and normal, that the 'insider' also realizes how much has been learnt during the socialization process that the PhD training is. For example, I was confronted with surprise and incomprehension from outsiders—including other biomedical scientists—when I mentioned that I infected healthy volunteers with malaria in my research. That made me realize: it is indeed quite remarkable what we are doing, how is it possible, what makes the CHMI machinery work?

2 Sometimes natural scientists erroneously interpret this stylistic difference as an indication that social-science knowledge is less 'scientific' and 'objective' than natural-science knowledge. However, social-science research makes the same objectivity claim as natural-science research does: its findings will be replicated if another anthropologist would do the same research again (156)

I realized that there was more to answering this question than the regular "well, we have figured out a way to do this". I was still enough of an outsider to recognize some of the surprise about what is going on in a CHMI trial; but also enough of an insider to recognize that much of the answer is implicit in the clinical and laboratory practices, and nowhere spelled out explicitly—it is, indeed, 'tacit knowledge'. Would it be possible to use my still ambiguous status of half-socialized in/outsider to reflect on these questions? Could I take a step back—or rather: step outside my socialization process—and reflect on my own research practices and explicate the various types of tacit knowledge, in order to learn about the broader issues of carrying out clinical trial research and the construction of scientific knowledge?

And that is what I started out to do in the research that resulted in chapter 2 of this thesis. Scientific knowledge does not exist only in articles, books and protocols. Rather, knowledge is produced, carried and sustained by people. Chapter 2 of this thesis maps all who make the CHMI trials possible: the sponsors, PIs, clinical investigators, but also the nurses, the technicians, the thick smear readers and the experts in parasite culture and mosquito breeding. The complex sociotechnical machinery of the trial runs on their knowledge, expertise and engagement. To describe and analyze their work and tacit knowledge thus also testifies of the crucial role they play in carrying out CHMIs—a nice addition to thanking them in the acknowledgment section of this thesis.

Understanding the role of the tacit knowledge that people use while doing research has been one result from STS research. Indeed this is so well established a finding, that it needs no further research or publications. Therefore, however insightful the explication of tacit knowledge was for myself as a medical PhD student, this research would not yield results that merit a publication in an STS journal. So, why do this STS research on CHMIs, present our findings at STS conferences, and submit it to an STS journal?

CHMI trials are, we have argued, a particularly strategic research site to study the role of trust and control in the construction of scientific knowledge. This is, as we have reviewed in chapter 2 of this thesis, a generally recognized but still under-researched insight in STS. For the sociotechnical machinery of the CHMI to work, we found that well-balanced combinations of control and trust need to be in place. In fact, we argue that it is tandems of trust and control that make the trials work. This analysis helps us to better understand why CHMI trials can be carried out successfully and how they produce valuable scientific knowledge. Moreover, we believe that our analysis can help to improve clinical trial research practices.

Here, I would like to briefly reflect on two issues that arose from this chapter. First, I will make a plea for interdisciplinary collaboration, of which many chapters in this thesis are examples, albeit in very different forms. Next, I will discuss some ways in which the insights from the STS chapter could become useful for clinical trial practice.

Interdisciplinary collaboration

Science is becoming increasingly interdisciplinary and I strongly believe that this is vital for the development of innovative concepts and ideas. In interdisciplinary collaboration constructs, ideas, viewpoints and methodologies are shared across fields. In this way, medical doctors like me can learn about protective immune responses against malaria from immunologists (chapters 7 and 8), use methodologies developed by fruit fly biologists to study tolerance (chapter 12) and assess big data together with bioinformaticians (this chapter). With each partner bringing knowledge and experience from their own domain, insights can be mutually inspiring and further developed. Also the collaboration between myself as a medical researcher and my father as an STS scholar was fruitful for both sides. For me, it helped to look at my own research practice and find ways to improve this; for him, it was an opportunity to study in more detail the role that trust and control play in the construction of scientific knowledge: a question that has been on the STS agenda for a long time, but could now be studied in this 'strategic research site'.

In the early years of STS, during the 1970's and 1980's, research was mainly about asking fundamental questions about the production of scientific knowledge and the role of knowledge in society. Questions about implications for practice, policy or politics were hardly raised. Although some argue that STS has *"always been intervening in one way or another - by offering different perspectives, by taking part in discussions, or merely by being at the sites"* (158), it is only since about a decade that frequent calls for a more engaged and interventionist STS can be heard (159, 160). In order to increase STS' impact on the practices of the research it studies, new collaborative relationships between STS-scholars and scientists need to be built (161). Or in Zuiderent-Jerak's words: it is needed to *"experiment with the co-construction of (medical) work practices (…) and critical STS"* (162). My collaboration as a medical researcher with an STS scholar, which led to the first chapter of this thesis, is an example of such an experiment.

For a constructive discussion on interventionist STS, it is important to explicate "what *STS researchers will bring to the table,* how *their contributions will enter joint processes of development, and* what *results will emerge"* (158). In our case, we presented and discussed the STS work described in this thesis during a work-in-progress meeting of the Radboudumc malaria research group. We presented and used some concepts from STS — such as tacit knowledge, social construction and interpretative flexibility — to analyze the practices of our CHMI research. These concepts are not part of the standard training of medical PhD students, nor of lab technicians and nurses. While senior researchers in the group implicitly knew from their experience what these concepts tried to capture and describe, researchers at the beginning of their career found it illuminating and helpful to look into the STS mirror that we were holding up to them. It helped to critically review the research practices of the group. For example, it explicated why protocols are not sufficient to transfer knowledge

about how to execute a procedure. This is something that everyone knew from experience, but often it was thought to be a 'failure' to properly execute the protocol. With the help of STS concepts, it now could be understood as an effect of not enough socialization, not valuing tacit knowledge, and not recognizing interpretative flexibility. For me personally, the collaboration with a senior STS researcher made me aware of the huge disciplinary differences between medical sciences and humanities and social science — in problem definition, data analysis, explanatory strategies, writing, publication, and even footnoting. I do not think it has made me a lesser medical researcher, though there is some risk in tampering with one's process of socialization. Looking at our CHMI practice from an STS perspective, for a while, has made me see some of its accomplishments and strengths in a clearer light — and this perhaps even made me a better medical researcher.

What, then, has STS brought to the table? First, it is a set of concepts to better describe and understand our scientific practice. Using those concepts is like looking into a mirror: we see who we are and what we do, but in a slightly different guise. No mirror is neutral and innocent, nor is the STS mirror — it will highlight certain aspects of our scientific practice more than others (163). Second, STS will also offer an engagement with our research practice that possibly will lead to changes in that practice, perhaps some adaptations in protocols or changes in the set-up of CHMIs. The next section will discuss one possible example: how reconsidering the role of trust and control in clinical trials was helpful to improve our clinical trial practices.

Using the analysis of tandems of trust and control to improve research practices

A suggestion that was raised in the chapter but we did not further elaborate on, was to perform a 'sensitivity analysis' of trust and control in clinical trials — an idea that in fact came up during the meeting with the Radboudumc malaria group. Although we will not use traditional methodologies of performing such an analysis, we believe it could be useful to attempt this as a thought experiment to improve research practices: what are the optimal location and degree of both trust and control in a specific situation? What is the effect of increasing or reducing either of them? I will try this, using the example of an independent audit of the malaria unit that was performed in spring 2014. An audit typically is a systematic evaluation of processes and records involved in a production line, in this case of infectious mosquitoes for CHMI. During this audit, it became clear that it was indeed useful to be conscious of the location and degrees of control and trust.

In any laboratory there is a lot of equipment, and it is important that this is controlled, i.e. calibrated and monitored. Let me take the example of freezers, in which samples and reagents are kept. In order to assure the quality of materials that are stored, it is important that freezer temperature is maintained within a pre-determined range around -80°C. One possible way to control the freezers is a monitoring system that can set off an alarm if the temperature

is out of range. This would allow the responsible person to take necessary action, for example move the content of the freezer to another freezer and call for technical assistance to repair the mal-functioning one. When we prepared for the audit of our malaria unit, not all freezers had such a monitoring system installed: we now had to choose whether to buy such a system, and decide on the degree of control we wanted to implement.

However, it is nowhere prescribed how freezers for this particular use should be monitored, nor is it self-evident what the right degree of control would be. The strictest form of control would be to install a constant monitoring system on all freezers with automated alarm to be set off as soon as the temperature gets out of a narrow predetermined range. At the other extreme, an option would be to only measure the temperature manually, do that weekly, and do it only in the most crucial freezers. While in the second example the technology of the freezers is trusted almost completely, in the first example trust has been shifted to the monitoring system. The obvious disadvantage of the second, very permissive option is, that if the temperature of the freezer goes temporarily out of range (between two measurements), it might compromise the frozen materials without anyone being aware. The risk of the first, very strict option, however, is that one relies exclusively on the monitoring and alarm system, which itself is a technology with the potential to fail. Moreover, the setting of a narrow range might set the alarm off very frequently, annoying the personnel in charge, and eventually stopping them from adequately responding to the alarm.

Therefore, it would probably be useful to consider other options somewhere midway in the control- and trust spectra. For example, one could decide to expand the temperature range beyond which the alarm would go off; one could choose not to monitor all freezers but only the ones that were crucial for the clinical trial processes; or one could consider installing a monitoring system without an alarm. In the latter option, the temperature course can be checked retrospectively by expert personnel, to assess whether the temperature has been out of range and whether materials may have been compromised. To make this choice of a midway option, it is crucial to what extent one relies on and trusts the expert judgment of the personnel that works with the freezer. They have to decide on the control measures, as they have the experience and (often tacit) knowledge to assess when the temperature of which fridge is crucial. It was interesting to see that the experienced auditor, in contrast to what some of us had expected, also recognized this. Instead of going to the extreme control side of the spectrum and obliging us to install continuous and strict monitoring on all fridges, the audit report took a midway position: *"The necessity to implement a daily recording of the temperatures of the fridges should be assessed."*

This example shows that it is useful to be conscious of and explicit about possible locations and degrees of control, where to invest in trust and the importance of trust for exercising control. More control is not always better, and trust in the technicians' expertise is required to find an optimal solution. The alternative of insisting on control by outsiders

or technical monitoring systems may be more risky than trusting your own technicians. In other words, the outcome of this thought experiment in sensitivity analysis of the freezer monitoring, may be to invest in trust in the technicians' expertise about control measurements, and thereby in fact using trust to make control work.

Conclusions and future perspectives

The work in this thesis contributes to the unraveling of protective immune responses against malaria. We show that sterile protection can be established by pre-erythrocytic immune responses, and find indications that cytotoxic T cells are important herein. Moreover, we find that CPS immunization can be performed with different antimalarial drugs and that CPS can induce heterologous protection. These studies have established CPS as a pivotal model to delineate protective immune responses, and the possibility to replace mosquito bites with injection by needle and syringe for sporozoite inoculation opens avenues to further develop whole sporozoite vaccines. The findings from this thesis will be important to focus future research on the identification of pre-erythrocytic target antigens and immune signatures of protection, in which interdisciplinary collaboration-platforms will play a central role. Together, these research lines will be instrumental in the development of an effective vaccine, which will be a crucial tool to combat malaria.

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SUMMARY

SAMENVATTING

Summary

Malaria is an important infectious disease with more than 3 billion people at risk, resulting in more than 200 million clinical cases and almost 600,000 deaths annually. The disease is caused by *Plasmodium* parasites and transmitted by bites from *Anopheline* mosquitoes. Implementation of effective interventions such as insecticide-treated bed nets, indoor insecticide spraying and targeted diagnosis and treatment with artemisinin-based combination therapy has led to a significant decrease in the burden of malaria in the past decades. However, these interventions are threatened by emerging drug resistance of parasites and resistance of mosquitoes to insecticides. Therefore, novel strategies are needed to effectively control malaria. A potent vaccine would be a crucial component of such a strategy, and understanding protective immune responses will be of great help to design and develop such a vaccine.

In this thesis we use models in healthy volunteers to investigate protective immunity against *P. falciparum* malaria. In Controlled Human Malaria Infections (CHMIs), healthy subjects are infected with malaria, either through bites from mosquitoes infected with *P. falciparum* sporozoites, or by venous inoculation of blood-stage forms of the parasite. In the chemoprophylaxis and sporozoites (CPS) immunization model, volunteers are exposed to live sporozoites while receiving chloroquine prophylaxis. The CPS immunization regime has previously been shown to induce very high levels of protection against challenge infection in a controlled setting. We use these models to advance our understanding of anti-malarial immunity induced by whole sporozoite immunization and thereby aim to augment clinical development.

The thesis starts with an interdisciplinary study, using the heuristics from Science Technology and Society studies (STS), to investigate the social construction of scientific knowledge in CHMI trials ***(chapter 2)***. Specifically, we argue that tandems of trust and control play a central role in the successful execution of CHMI trials and scientific experiments in general. We especially elaborate on the distinction between trust in and control of *persons, machines,* and *institutions* and argue that links between these different domains are crucial for the safe and effective execution of clinical trials. We also argue that trust and control are not just alternatives, which substitute for each other when one does not suffice, but that they actually work in conjunction and coproduce each other, to make scientific research work.

In the review in ***chapter 3*** we raise the hypothesis that chloroquine might be important for the efficient induction of immunity by CPS immunization through its known immune modulating capacities. In ***chapter 4*** we use the rodent malaria parasite *P. berghei* to investigate this hypothesis, but do not find supportive evidence in this model. In ***chapter 5***, we explore the option of translating chemoprophylaxis and parasite exposure into a field intervention with the aim of inducing protective immune responses. We propose that this could be evaluated in an area with a short but intensive transmission season, allowing for high exposure to pre-erythrocytic antigens while blood-stage infections are controlled by the

drug, and the possibility to evaluate induced protection in the next season.

Next, we use the CPS model to address two important questions in malaria immunity. We first investigate, in ***chapter 6***, towards which stages of the parasite lifecycle the protective immune responses are directed**.** The clinical trial described in this chapter shows that CPS-immunized subjects are not protected against a challenge with blood-stage parasites. Thus, CPS-induced protection is, at least primarily, mediated by immune responses against the pre-erythrocytic (sporozoite and liver) stages. We do, however, find evidence that immune recognition of blood-stages does occur after CPS immunization, although apparently not sufficient to protect against these parasite densities.

The second question is which immune responses are induced and whether these are associated with protection. Both cellular responses and antibodies appear to play a role in protection induced by live sporozoite based immunization; in this thesis we focused on cellular immune responses**.** In ***chapter 7*** we perform a dose de-escalation CPS trial, generating a differentially protected cohort: protection is dose-dependently induced, with almost 100% protection when immunized with bites from three times 15 mosquitoes, reducing to 50% protection when immunized with bites from three times five mosquitoes. This study design allows us to investigate immune correlates of protection. In vitro restimulation experiments reveal two cell types that are associated with protection: CD4 T cells expressing the degranulation marker CD107a and CD8 T cells producing granzyme B. In ***chapter 8*** we conduct an alternative analysis of CPS-induced immune responses by ex vivo lymphocyte phenotyping during immunization. These experiments indicate a role for Th1 responses and cytotoxic T cells in CPS-induced immunity, which is in line with the results from ***chapter 7.***

All CPS studies up until now have been performed with the same (homologous) NF54 strain for both immunization and challenge. It is, however, of critical importance to show protection against heterologous strains, given the huge diversity of strains in the field. ***Chapter 9*** describes a clinical trial in which NF54 CPS-immunized and challenged subjects were re-challenged with a different strain (NF135.C10). Although less efficient, NF54-induced CPS immunity also protects against NF135.C10 when tested after a period of more than a year. In ***chapter 10*** we show that CPS immunization in humans does not specifically depend on the choice of chloroquine for prophylaxis, as CPS with mefloquine is equally safe and protective. In addition, induced cellular and humoral immune responses do not differ significantly between both drug groups.

For clinical development of whole sporozoite based vaccines, it is obviously necessary to replace sporozoite inoculation through mosquito bites by needle and syringe. We show in ***chapter 11*** that CHMI can be performed by intradermal injection of aseptic, purified, vialed and cryopreserved *P. falciparum* sporozoites. These results will facilitate application of CHMI and accelerate malaria vaccine development, including whole sporozoite approaches.

Finally, in ***chapter 12***, we use the CHMI model for an alternative application by

exploring a novel framework derived from population biology. Our objective is to investigate tolerance against malaria by using so called "disease maps" to describe the relationship between the level of parasitemia and severity of disease. Visualization of the course of infection in disease space enables us to distinguish different levels of tolerance against *P. falciparum* malaria. The presented data illustrate the potential of this framework for rational monitoring and treatment of malaria on an individual basis.

In conclusion, this thesis sheds light on several aspects of protective immunity induced by whole sporozoite immunization: i) pre-erythrocytic stages as the target of immunity, ii) the dose-response relationship between immunization dose and protection, iii) the generation of heterologous protection, iv) the induction of cellular immune responses, v) the option to use different drugs for CPS and vi) the possibility to use cryopreserved sporozoites instead of bites by infected mosquitoes. We hereby aim to set an agenda for future work on the clinical development of whole sporozoite vaccines. This will be supported by the reproducibility and efficiency of CPS immunization and will include identification of novel target antigens and delineation of mechanisms of sterile pre-erythrocytic immunity. Rather than single immunological markers or parasitic antigens, we expect the focus of such studies to be on identifying immune signatures of protection. Altogether, CHMI and CPS immunization have proven to be highly valuable models in malaria vaccine research, and will likely continue to do so in future.

Samenvatting

Malaria is een belangrijke infectieziekte waaraan meer dan 3 miljard mensen worden blootgesteld, met meer dan 200 miljoen ziektegevallen en bijna 600.000 doden per jaar. De ziekte wordt veroorzaakt door *Plasmodium* parasieten die worden overgebracht door vrouwelijke *Anopheles* muggen. Implementatie van effectieve maatregelen zoals geïmpregneerde klamboes, het binnenshuis spuiten van insecticiden en gerichte diagnostiek en behandeling met artemisinine-combinatie therapie, heeft geleid tot een afname in het aantal malariagevallen in de afgelopen jaren. Deze interventies worden echter bedreigd door de opkomst van resistentie van parasieten en muggen. Nieuwe strategieën zijn dus nodig om malaria te bestrijden, en een effectief vaccin zou een belangrijk onderdeel zijn van een dergelijke strategie. Goed begrip van de beschermende afweerreacties is cruciaal om een vaccin te ontwerpen en ontwikkelen.

In dit proefschrift gebruiken wij modellen met gezonde vrijwilligers om beschermende immuniteit tegen *P. falciparum* malaria te onderzoeken. In *gecontroleerde humane malaria infecties* (CHMIs) worden gezonde vrijwilligers geïnfecteerd met malaria door hen bloot te stellen aan beten van geïnfecteerde muggen, of door intraveneuze toediening van bloedstadium parasieten. In het *chemoprofylaxe en sporozoïten* (CPS) immunisatie model worden vrijwilligers blootgesteld aan sporozoïten terwijl ze chloroquine profylaxe nemen. CPS immunisatie is zeer effectief gebleken in het opwekken van bescherming tegen een malaria infectie onder gecontroleerde omstandigheden. Wij gebruiken deze modellen om ons begrip van immuniteit tegen malaria te vergroten en op deze manier klinische ontwikkeling van sporozoïten-vaccins te bevorderen.

Dit proefschrift begint met een interdisciplinaire studie waarin we gebruik maken van de methodologie en het gedachtegoed van Wetenschap en Technologie studies om de sociale constructie van wetenschappelijke kennis in CHMI studies te onderzoeken ***(hoofdstuk 2)***. We betogen dat tandems van vertrouwen en controle een cruciale rol spelen in de succesvolle uitvoering van CHMI studies en wetenschappelijke experimenten in het algemeen. We maken onderscheid tussen vertrouwen in en controle van *personen, machines* en *instellingen* en betogen dat verbindingen tussen deze verschillende domeinen cruciaal zijn voor de veilige en effectieve uitvoering van klinische studies. We betogen ook dat vertrouwen en controle niet simpele alternatieven zijn die elkaar vervangen als een van beide niet voldoet, maar dat ze samen werken en elkaar coproduceren, om wetenschappelijk werk succesvol te laten zijn.

In de literatuurbespreking in ***hoofdstuk 3*** opperen we de hypothese dat chloroquine op basis van haar immuun-modulerende eigenschappen belangrijk is voor de efficiënte wijze waarop immuniteit door CPS immunisatie wordt opgewekt. In ***hoofdstuk 4*** gebruiken we de muizen-malariaparasiet *P. berghei* om deze hypothese te onderzoeken, maar vinden geen bewijs hiervoor in dit model. In ***hoofdstuk 5*** verkennen we de mogelijkheid om CPS te vertalen naar een interventie voor endemische gebieden, met als doel het opwekken van een

beschermende immuunreactie. We stellen voor om dit te onderzoeken in een gebied waar sprake is van een kort maar intensief transmissie-seizoen. Op deze manier vindt blootstelling aan een relatieve hoge dosis pre-erytrocytaire antigenen plaats, terwijl bloedstadium infecties worden gecontroleerd met hulp van de medicatie, en is er de mogelijkheid om in een opeenvolgend seizoen de opgewekte bescherming te evalueren.

Vervolgens gebruiken we het CPS model om twee belangrijke vragen te beantwoorden over malaria immuniteit. Ten eerste onderzoeken we, in ***hoofdstuk 6***, tegen welke stadia van de parasiet-levenscyclus de beschermende immuunrespons is gericht. De klinische studie in dit hoofdstuk laat zien dat CPS-geïmmuniseerde personen niet beschermd zijn tegen een infectie met bloedstadium parasieten. Bescherming door CPS is dus primair gebaseerd op een afweerreactie tegen pre-erytrocytaire stadia (sporozoïten en leverstadia). We vinden wel aanwijzingen voor herkenning van bloedstadium parasieten door het immuunsysteem, maar dit is dus niet voldoende om een beschermend effect te bewerkstelligen bij de lage parasietenconcentraties waarvan in het CHMI model sprake is.

De tweede vraag is welke immuunresponsen worden opgewekt, en of deze geassocieerd zijn met bescherming. Zowel cellulaire immuniteit als antistoffen lijken een rol te spelen in bescherming; in dit proefschrift concentreren we ons op de cellulaire responsen. In ***hoofdstuk 7*** beschrijven we een dosis de-escalatie CPS studie, waarin een gedifferentieerd cohort is gecreëerd, met bijna 100% bescherming na immunisatie met beten van driemaal 15 muggen, afnemend tot 50% bescherming na immunisatie met beten van driemaal vijf muggen. Deze studie stelt ons in de gelegenheid om op zoek te gaan naar cellulaire immuunresponsen die geassocieerd zijn met bescherming. Uit in vitro restimulatie experimenten komen twee celtypes naar voren die geassocieerd blijken te zijn met bescherming: CD4 T cellen die CD107a tot expressie brengen (een teken van degranulatie) en CD8 T cellen die granzyme B produceren. In ***hoofdstuk 8*** hebben we een alternatieve analyse van immuunresponsen uitgevoerd door middel van ex vivo fenotypering van lymfocyten tijdens CPS immunisatie. Deze experimenten wijzen op een rol voor Th1 responsen en cytotoxische T cellen, in lijn met de resultaten van ***hoofdstuk 7***.

Tot op heden zijn alle CPS studies uitgevoerd met dezelfde (homologe) NF54 stam voor zowel immunisatie als *challenge* infectie. Het is echter van cruciaal belang dat ook bescherming tegen een heterologe stam wordt aangetoond, gezien de enorme variatie tussen stammen in endemische gebieden. ***Hoofdstuk 9*** beschrijft een klinische studie waarin vrijwilligers die CPS immunisatie en een *challenge* infectie met NF54 hebben ondergaan, worden blootgesteld aan een *challenge* met de heterologe stam NF135.C10. Uit deze studie blijkt dat CPS immunisatie ook bescherming opwekt tegen een heterologe stam, alhoewel minder effectief dan homoloog. In ***hoofdstuk 10*** tonen we aan dat de werking van CPS immunisatie in mensen niet afhankelijk is van de keuze voor chloroquine als profylaxe: CPS immunisatie met mefloquine blijkt even veilig en effectief te zijn. Bovendien zijn ook

cellulaire en humorale immuunresponsen niet verschillend tussen beide groepen.

Voor de klinische ontwikkeling van sporozoïten-vaccins is het natuurlijk noodzakelijk dat het toedienen van sporozoïten door middel van muggenbeten wordt vervangen door een injectie met spuit en naald. We laten in ***hoofdstuk 11*** zien dat dit mogelijk is: we voeren CHMIs uit door intradermale injectie van aseptische, gezuiverde en bevroren *P. falciparum* sporozoïten. Deze resultaten zullen wereldwijde toepassing van CHMI faciliteren en de ontwikkeling van malaria vaccins, inclusief sporozoïten-vaccins, versnellen.

Tot slot gebruiken we in ***hoofdstuk 12*** het CHMI model voor een alternatieve toepassing door een nieuw raamwerk, afgeleid van de populatie biologie, te onderzoeken. Ons doel is om tolerantie tegen malaria te onderzoeken door de relatie tussen de hoogte van de parasitemie en de ernst van de ziekte te beschrijven in zogenaamde *"disease maps"*. Visualisatie van het beloop van de infectie in *"disease space"* maakt het mogelijk om verschillende niveaus van tolerantie tegen *P. falciparum* te onderscheiden. Deze data illustreren het potentieel van dit raamwerk voor rationele monitoring en behandeling van malaria op een individuele basis.

Concluderend werpt dit proefschrift licht op een aantal aspecten van immuniteit tegen malaria, opgewekt door immunisatie met sporozoïten: i) de pre-erytrocytaire stadia als aangrijpingspunt voor immuunresponsen, ii) de dosis-respons relatie tussen immunisatie dosis en bescherming, iii) het opwekken van heterologe bescherming, iv) de opgewekte cellulaire responsen, v) de mogelijkheid om andere medicatie voor CPS te gebruiken en vi) de mogelijkheid om bevroren parasieten te gebruiken in plaats van beten van geïnfecteerde muggen. Hiermee hopen wij bij te dragen aan het ontwikkelingsproces van sporozoïten-vaccins, waarin CPS immunisatie gezien de reproduceerbaarheid en efficiëntie een belangrijke rol zal kunnen spelen. Toekomstige studies zullen onder andere gericht zijn op het identificeren van nieuwe antigenen en het uitpluizen van de immunologische mechanismen van pre-erytrocytaire bescherming. In plaats van een enkele marker, zullen deze studies zich waarschijnlijk richten op het identificeren van een "immunologische handtekening" voor bescherming. CHMI en CPS immunisatie hebben bewezen zeer waardevolle modellen te zijn voor malaria vaccin onderzoek, en zullen deze rol hoogstwaarschijnlijk ook in de toekomst blijven vervullen.

DANKWOORD

CURRICULUM VITAE

LIST OF PUBLICATIONS

Dankwoord

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Curriculum vitae

Else Margreet Bijker (1984) was born in Delft, the Netherlands. She went to the Rudolf Steiner elementary school in Maastricht and completed Gymnasium at Jeanne d'Arc College cum laude. She studied medicine at Maastricht University, where she obtained her master degree cum laude and her medical degree in 2009. During her studies she performed research on various topics, and particularly on meningococcal sepsis in children in the departments of General Surgery and Pediatrics. She did an extra-curricular pediatrics internship in India, which inspired her to continue her career in research on tropical infectious diseases. In 2009 she started her PhD on malaria at the department of Medical Microbiology of the Radboud university medical center. During her PhD studies she published a number of papers, most of which are included in this thesis, and presented her work at several national and international conferences and seminars. She also actively participated in the writing of research proposals of which a number were granted, for example by The Netherlands Organization for Health Research and Development (ZonMW) and the Bill and Melinda Gates foundation. She won several conference prizes: the poster prize at the Top Institute Pharma Spring Meeting in 2012, the Elsevier Clinical Research Award at The American Society of Tropical Medicine and Hygiene Annual Meeting in 2012 and the price for best oral presentation at the Radboudumc Clinical PhD retreat in 2013. Else received a Frye stipendium "for excellent female PhD researchers" from the Radboud University in 2013. She was interviewed about her research on local and national radio and presented her work for a lay audience at the public event Kijk Live. In January 2015, she started her training in pediatrics at the Canisius Wilhelmina Hospital and the Radboud university medical center, Nijmegen.

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