Aus der

Medizinischen Universitätsklinik und Poliklinik Tübingen

Abteilung VII, Tropenmedizin

Guiding clinical malaria vaccine development using immune cell monitoring and controlled human malaria infection

Thesis submitted as requirement to fulfill the degree "Doctor of Philosophy" in *Experimental Medicine* (PhD)

at the

Faculty of Medicine

Eberhard Karls Universität

Tübingen

by

Ibanez Molina, Javier

2023

Dean: Professor Dr.B. Pichler

First reviewer: Professor Dr. B. Mordmüller
Second reviewer: Professor S. Wagner, PhD

Date of oral examination: 19.05.2023

TABLE OF CONTENTS

1 INTRODUCTION 4

1.1 Vaccines.. 4

1.1.1 Vaccine types.. 4

1.1.2 Vaccine-induced immunity... 5

1.1.3 Vaccine adjuvants 7

1.1.4 Vaccination practices... 8

1.2 The malaria vaccines technology roadmap.. 10

1.2.1 Plasmodium lifecycle... 11

1.2.2 Malaria vaccines in clinical development.. 13

1.2.3 Evaluation of Malaria vaccine candidates 16

1.2.4 Immune cell monitoring in vaccine trials 17

1.2.1 Immunity to malaria... 18

1.2.2 Correlates of Protection 18

1.3 Objectives.. 19

2 RESULTS AND MANUSCRIPTS ACCEPTED FOR PUBLICATION...21

2.1 Chapter 1: Systematic monitoring of the bona fide pro-inflammatory CD4+ T helper cells in a PfSPZ-CVac phase II clinical trial with CHMI. 21

2.2 Chapter 2: Cellular and antibody response in GMZ2-vaccinated African volunteers in a Controlled Human Malaria Infection trial.. 33

3 DISCUSSION..50

3.1 Influence of malaria life cycle stages on immune responses to the *Plasmodium falciparum* sporozoite chemoprophylaxis vaccine and parasite-specific T cell activation as biomarker for vaccine efficacy.. 51

3.2 Dissecting cellular responses in semi-immune Africans vaccinated with the GMZ2 malaria vaccine candidate in a Controlled Human Malaria Infection trial. 59

4 SUMMARY...65

5 ZUSAMMENFASSUNG.....67
6 BIBLIOGRAPHY.....70
7 DECLARATION OF CONTRIBUTIONS.....92
8 ACKNOWLEDGEMENTS.....94

1 INTRODUCTION

1.1 Vaccines

Genuinely, vaccines are biological inventions. They play a vital role in modern medicine to prevent infection and disease through the induction of immune memory against specific infectious agents. The World Health Organization (WHO) estimates that vaccinations prevent more than 3.5 million deaths annually[1].

Since Edward Jenner demonstrated the potential to prevent infection through "vaccination" in 1798[2], vaccines have had formidable impact on global health. Jenner made key contribution in vaccine development by advancing knowledge established by Benjamin Jetsy and others, who first demonstrated the principle of vaccination decades earlier. Jetsy demonstrated that milkmaids exposed to cowpox infections did not contract smallpox and inoculated members of his own family with cowpox to demonstrate that such procedure may induce protection against smallpox. Variolation, a similar procedure but using material from human infections, was introduced in Britain by Lady Mary Wortley Montagu in 1721 following prior observations about variolation during her time in Constantinople (1717)[3], and later advanced by Jesty. However, Jenner is recognized for publicizing the procedures and thereby introducing them on a large scale. Historical records place the origin of vaccination in Central Asia before the 10th century.

1.1.1 Vaccine types

There are numerous ways of classifying vaccines. One way to classify vaccines is according to the method of production[4], namely whole germ (live-attenuated or killed vaccines) and those that use a fragment or single molecules of the germ; so-called subunit vaccines. Live-attenuated vaccines are based on weakening a germ's pathogenicity until it is not able to cause disease anymore. Alternatively, live vaccines can be produced by using closely related non-pathogenic organisms to induce protection against pathogens (e.g., vaccinia virus, Bacillus Calmette-Guérin). Examples for live vaccines are those against *Variola major* and *Variola minor*, *Mycobacterium tuberculosis*, *Salmonella typhi,* yellow fever virus, poliovirus, measles, mumps, and rubella viruses, varicella-zoster virus, and rotavirus. Killed whole organism vaccines are also produced by chemical or physical inactivation of the fully infectious agent such as cholera, pertussis, polio (IPV), rabies, tick-borne encephalitis, and hepatitis A.

Sub-unit vaccines involve use of products, toxins, or fragments of a pathogen as a vaccine. Toxoid vaccines are produced by chemically inactivating toxins produced by the germ e.g., *Corynebacterium diphtheriae* (Diphtherietoxin), and *Clostridium tetani* (Tetanus neurotoxin; TeNT). Polysaccharide vaccines are also classified as sub-unit vaccines because they comprise sugars present on the surface of many bacteria such as *Streptococcus pneumonia*, *Neisseria meningitidis*, *Haemophilus influenzae* B. As immunity induced by polysaccharide vaccines is typically lower and short-lived, glycoconjugate vaccines have been developed. They constitute of polysaccharides that are chemically conjugated with a protein antigen to induce higher level of protection against pathogens e.g., *Haemophilus influenzae* B, *Streptococcus pneumonia* (7,10 and 13 valent), *Neisseria meningitidis* C and *Neisseria meningitidis* A.

Owing to advances in the field of genetic engineering since the 1970's, a new vaccine approach based on biotechnological techniques has increased the number of new vaccines in the global pipeline. For instance, genetic engineering enables production of proteins in heterologous organisms, known as *recombinant protein* vaccines. Here, the advantage is that organisms that are safe and highly efficient in production (e.g., *Escherichia coli* or yeast) can be used. They are easily maintained in the laboratory and production can be easily scaled up using established methods. Recombinant protein vaccines are on the market and confer protection against for example hepatitis B, meningococcal serotype B and human papillomavirus. Lately, the mRNA vaccine technology[5] has emerged to simplify the procedure by directly use the human host cell machinery to produce the antigen of interest. This technique proved to be particularly helpful and applicable in the recent development of vaccines against SARS-CoV-2. Similar to this, non-pathogenic and attenuated viruses that are utilized as vectors can have the genome of a targeted pathogen, such as the Ebola virus, modified. Examples are Adenoviral-, Vesicular Stomatitis- or Modified Vaccinia Ankara-based vectors, leading to vaccines known as recombinant vector vaccines. This strategy helps ensure that the genetic information is delivered successfully into the cellular cytoplasm.

1.1.2 Vaccine-induced immunity

Vaccines are made to stimulate the immune system and generate effector and memory responses against pathogens[6]. Once the vaccine is inoculated, immune cells find and decode the information contained in the vaccine to ultimately generate an effector response. The effector response differentiates two main arms, the so-called innate (not specific and short-term) and the adaptive one (antigen-specific and long term). Within the adaptive one, there are two main cell types, the B cells whose response is generally based on the creation of antibodies

(humoral immunity), and the T cells, more specialized in detecting and destroy infected cells (cellular immunity)[7]. Long-lasting immunization requires the differentiation of a certain number of antigen-specific B and T cells following the expansion-retraction cycle of inflammation after the first encounter with cognate antigens. Surviving antigen-specific cells support immunological memory conferring faster, more focused, and larger secondary responses as compared to priming responses. B cells differentiate into two principal memory cell types, the plasma cells, and the memory B cells. While memory B cells are specialized in rapidly responding against a new infection, plasma cells may migrate to the bone marrow producing antibodies to maintain a constant level in plasma over time[7].

Not all antibodies generated have the same capacity to act against infection. Differences in the number of binding sites covered by (Avidity), and the strength of these binding sites (Affinity), have a direct impact in the durability of the antibody response[8]. High-affinity antibodies are often made by the CD4+ T cell-assisted primed B cells in the lymphatic tissues such as the lymph nodes, the spleen, or the thymus. Within those tissues are the follicles, where ultimately specific structures known as germinal centers are generated to facilitate the generation of memory B and plasma cells. The emergence of both memory B and plasma cells occur through multiple interactions between B cells and a specialized subset of CD4+ T helper cell known as T-follicular helper cell (T_{FH}). From these iterations, affinity maturation of B cell receptors, and thereby antibodies are canonically accomplished. The process, known as somatic hypermutation, results from introducing random mutations in the Ig genes followed by the survival of those B cells producing high affinity antibodies. It happens during B cell proliferation that is regulated by the CD40 (B cell):CD40L (T_{FH}) interaction[8].

However, B cells might differentiate into antibody secreting-plasma cells also in a T cell-independent manner. This process occurs extrafollicularly before even the formation of the germinal centers, and it is equally responsible of the generation of a substantial number of memory B cells[9].

In a similar fashion, the strength of the T cell response is highly influenced by three main factors, the antigen presenting cell; APC (cell type, enabled activation pathways, level of activation), location, and the chemical milieu (interleukins: cytokines and chemokines) present in the body niche where the contact between cells occurs. T cells can be differentiated into two main types by the expression of the co-receptors CD8 and CD4. Both types are essential in T cell memory development. CD8+ T cells become cytotoxic. They detect and

kill infected cells via direct mechanisms, e.g., the release of enzymes (e.g., perforin, granzyme) or indirectly, through the release of cytokines (e.g., IFN-γ). In order to influence B and CD8+ T cells to develop into effectors and ultimately form memory, CD4+ T cells are crucial for controlling the adaptive immune response.

Human CD4 T cells can distinguish different subsets according to the expression of transcription factors and the secretion of cytokines[10]. T_{FH} are distinguished from the other major groups by the expression of the BCL6 transcription factor and interleukin 21 (IL21). T-helper 1 cells (Th1) express T-bet and produce IFN-γ, TNF-α, and IL2. T-helper 2 cells (Th2) are characterized by expressing GATA3, and secreting IL4, IL5, and IL13. T-helper 17 cells (Th17) are identified by the RORC2 transcription factor signature and produce IL17, IL22 and IL26. The T-regulatory cells (Treg) are characterized by FOXP3, and the secretion of TGF-β[10].

Altogether, vaccine-induced immunity relies on the combination of several factors including the environment, nutrition, genetic background, microbiome, sex, age, antigen nature, or vaccine type[11]. The combination of such factors, make the acquisition of a high quality and long-lasting protection a real puzzle[12]. Thus, the optimization of the prime and boost vaccine regimens is crucial to be explore as well as the use of approaches (e.g., adjuvants, drugs) aiming to modulate and ultimately enhance the type and durability of the vaccine-induced memory.

1.1.3 Vaccine adjuvants

Adjuvants are chemical substances that are combined with a vaccine antigen aiming to generate a formulation capable to induce stronger and/or longer immune responses in the host than using the antigen alone. The word adjuvant comes from the Latin *adjuvare*, which means "to help". The concept of vaccine adjuvancy was enunciated long ago by Ramon[13] and later popularized among others by Charles A. Janeway [14]. Adjuvants have been repeatedly, and critically discussed by a wider audience. E.g., the association between the increasing number of vaccines given during the first two years of age and diagnosed cases of autism in US (1995-2015)[15]. Skepticism about vaccines has seemingly divided the opinion of educated people since then[16].

Aluminum salts such as Alhydrogel (Alum) were among the first adjuvant tested for human use about one century ago[17]. Later, other adjuvants such as liposome-preparations (e.g. CAF 01), detoxified lipopolysaccharides (monophosphoryl lipid A), oligonucleotides (CpG), saponins or nanoparticles have increased the list of adjuvants available for human use [18–20] .

In addition to enhance the stimulation of the APCs by promoting vaccine-antigen uptake, Alum contribute to the recruitment of other innate cells such as neutrophils, monocytes and eosinophils[21]. Among the APCs, dendritic cells (DCs) are particularly efficient in immune cell recruiting, and antigen presentation to T cells through major histocompatibility complex (MHC) molecules[22]. Adjuvants not only enhances immunity at the dendritic cell level but also influences the type of response generated[23].

Finding alternative strategies to enhance the immune response to recombinant and synthetic antigens, which are frequently not immunogenic, has made significant progress, e.g., presenting antigens on virus-like particles (VLPs); nanoparticles that resemble virus but cannot replicate[24,25].

1.1.4 Vaccination practices

Before vaccines are licensed for marketing, they are tested in a series of clinical trials. The aims of those trials are to determine whether if a specific medical intervention is safe and effective. Clinical trials are typically conducted in three to four phases[26]. Every trial phase has a different main objective. Phases are connected by a development plan of the medical intervention under evaluation with the goal of market authorization. In phase I, the aim is to test for the first time, the safety level and tolerability of a new intervention in a small group of people. In phase II, the aim is to increase the number of subjects and determining both safety and efficacy data. Generally, phase II trials are designed to detect common adverse effects and potential risk factors. In phase III, the medicine is tested in an environment like its intended use when marketed, usually with high number of volunteers[26]. The goals there, are to assess efficacy in the group of patients in need of the intervention and it with placebo or other interventions when available. It is also the first-time assessment of safety is done in a group to detect infrequent adverse event. A phase IV trial may be performed after receiving the approval for the new intervention by authorized regulatory agency. The goals there, are reporting the effectiveness of a new intervention and collect more safety data associated with the implementation of the new intervention at population scale[26].

In vaccinology, an important decision to determine is the route for the vaccine administration. The criteria for selecting optimal vaccine administration route are influence by factors such as the composition, immunogenicity, immune mechanism, and site of immune activation. Established routes for vaccine administration are (i) intramuscular (i.m.) e.g., Diphtheria, tetanus toxoids, and acellular pertussis or “DTaP”, (ii) subcutaneous (s.c.) e.g., measles,

mumps, and rubella or “MMR”, (iii) intranasal (i.n.) e.g., live attenuated influenza vaccine or “LAIV”, (iv) oral (p.o.) e.g., rotavirus or “RV1-5”, (v) intradermal (i.d.) e.g., BCG. Attenuated *P. falciparum* sporozoites must be given intravenously (i.v.) to be efficacious[27]. This route may become more important in the future as other vaccines also show increased efficacy when given i.v.[28,29] . Despite so many possibilities, there is still space for development of novel vaccine administration strategies in an effort to develop less painful and invasive or more targeted delivery systems such as microneedle patches[30] or edible formulations[31].

Timing of vaccination is an important variable for successful immunization. There are a few basic concepts that are generally accepted but often not systematically investigated. Among them, the interval between doses, the age when being vaccinated or the sequence of concurrent and subsequent vaccinations. Essentially, it is assumed that vaccines administered too close to each other produce suboptimal immunization as well as increasing the likelihood to develop some adverse reaction (e.g., Tetanus-Diphtheria toxoid)[32]. Most vaccines are given within the first year of life. However, a growing body of evidence show that key physiological features of the human immunity are age-dependent and insufficient maturity may lead also to suboptimal immunizations. For instance, the anatomical areas within the immune follicles where the antibody maturation takes place (GC) are not fully developed until 18 months of age, the bone marrow niches- where blood cells are produced- are still in development before 12 months of age, or the antibody restriction by maternal antibodies is exerted until they totally wane after 4-6 months post-delivery[33].

Furthermore, vaccine immunogens can be combined as single product, and different combinations have been developed since 1948[34]. Combined vaccines (e.g., DTaP, MMR) generally are favored over single injections of the same immunogens. Although combined vaccines are more expensive than single ones, they offer different advantages related with the time required to get all vaccines, the number of shots needed and therefore the potential concern associated with the number of injections or adverse events particularly in children[32]. In line with the advantages, experience in travel medicine show that simultaneous vaccination is well tolerated and effective covering travelers[35] as much as individuals living in remote areas of the world where patient return is unlikely[36]. To license a combined vaccine, noninferiority clinical trials[37] should be conducted and their outcome must demonstrate that the new product is not worse than the standard (i.e. single vaccine) by a reasonable boundary

known as delta (Δ). Delta selection is based on the clinical significance which is often a matter of discussion with the regulatory agency.

To date, vaccines targeting for about twenty pathogens are licenced[38] with most of the vaccines developed against viral infections and just few for bacterial infections. Most vaccines are a unique class of pharmaceutical products considered "drugs" and a "biological product" at the same time. Finally, licensing is given by medical agencies or specific departments assigned by countries such as the US Food and Drug administration; FDA[39] or the European Medicines agency; EMA[40]. In addition, the WHO[41] also makes recommendations ("prequalification") which ultimately may be adopted internationally.

Despite their proven cost-effectiveness[42], vaccine acceptance relies on trust as the intervention is typically done in large groups of healthy individuals [43]. Because of this, vaccines still continue facing social criticism, and often strong opposition[44,45] . Unsupported beliefs, and misconceptions are commonly behind the mistrust argued against the use of vaccines. Among them, the understanding that vaccines are unsafe and directly lead to health problems (e.g., neurological diseases or infertility)[46–49]. A majority of the people arguing against vaccines belong to the self-recognized anti-vaccination movement, popularly known as "anti-vaxxers" [50]. In the recent times, this opposition have caused for instance the reappearance of measles in the numerous areas of US or the disturbances in the implementation of the mRNA vaccines against SARS-CoV-2[51] . A Strategic Advisory Group of Experts (SAGE) was established by the WHO in 2012 in response to these movements with the objective of describing and identifying the factors causing low vaccine uptake as well as developing methods to alleviate "vaccine hesitancy"[15].

1.2 The malaria vaccines technology roadmap

Malaria is a disease of poverty[52]. Although both curable and preventable, malaria continues to pose a serious threat to world health, with an estimated 241 million infections and 627, 000 fatalities expected in 2020. By far most malaria related deaths occur among children below five years old[54]. Despite substantial gain in reducing malaria transmission since 2000, attributed to scale-up of interventions and control programs, recent years have witnessed a stagnation of this trend with poverty, political instability, and withdrawal of health programs[53].

The use of interventions such as combination drug therapy, early diagnosis, and insecticide-treated bed nets have greatly reduced malaria mortality[55]. However, with increasing drug resistance, insecticide resistance and difficulties in implementation, these measures alone are

not sufficient to eradicate malaria globally. Therefore, health organizations and the scientific community have acknowledged the potential advantage of a highly effective malaria vaccine to accomplish control and elimination[56]. By 2030, the WHO and its collaborators established a strategic objective to create malaria vaccines that are effective against clinical malaria in at least 75% of patients and may be used in all malaria-endemic regions. These vaccines will target *P. falciparum* and *P. vivax*[57] .

However, due to the parasite's complexity and multistage lifecycle, developing a malaria vaccine is difficult. Plasmodium-specific immune responses are life cycle stage-, species-, and strain-specific[58].

1.2.1 Plasmodium lifecycle

Every *Plasmodium* species has a complicated life cycle that involves infecting both humans and mosquitoes [*Figure 1*]. In humans, *Plasmodium* naturally infects two types of tissues: hepatocytes in the liver and red blood cells. The infection of a human starts when an infected female mosquito from the *Anopheles* genus releases sporozoite stage parasites during a blood meal. Within a very short time (typically less than 30 minutes), the sporozoites invade hepatocytes and mature for 6-7 days until they become mature merozoites that are released into the blood. This stage of the infection occurs without symptoms. One sporozoite can produce up to 30.000 merozoites[59]. In the blood stream, the merozoites of *P. falciparum, P. ovale,* and *P. malariae* invade the mature red blood cells, whereas *P. vivax* merozoites infect preferentially young red blood cells (reticulocytes)[59]. During the blood stage, malaria parasite-infected red blood cells mature through three different phenotypic stages: ring, trophozoite and schizont. In the mature schizont, segmentation of the daughter merozoites is followed by parasite egress into the blood stream, where new red blood cells are immediately infected. Thereafter, infectious merozoites emerge in the blood and immediately infect new red blood cells. The duration of this cycle depends on the plasmodial species: 72 hours for *P. malariae*, 48 hours for *P. falciparum*, *P. vivax*, and *P. ovale*, and 24 hours for *P. knowlesi*. Most of the merozoites continue to cycle and lead to an exponential increase in parasite numbers whereas a small proportion to merozoites differentiate into dimorphic sexual forms called gametocytes during the blood stage and lead to mosquito infection when ingested by *Anopheles* female mosquitos.

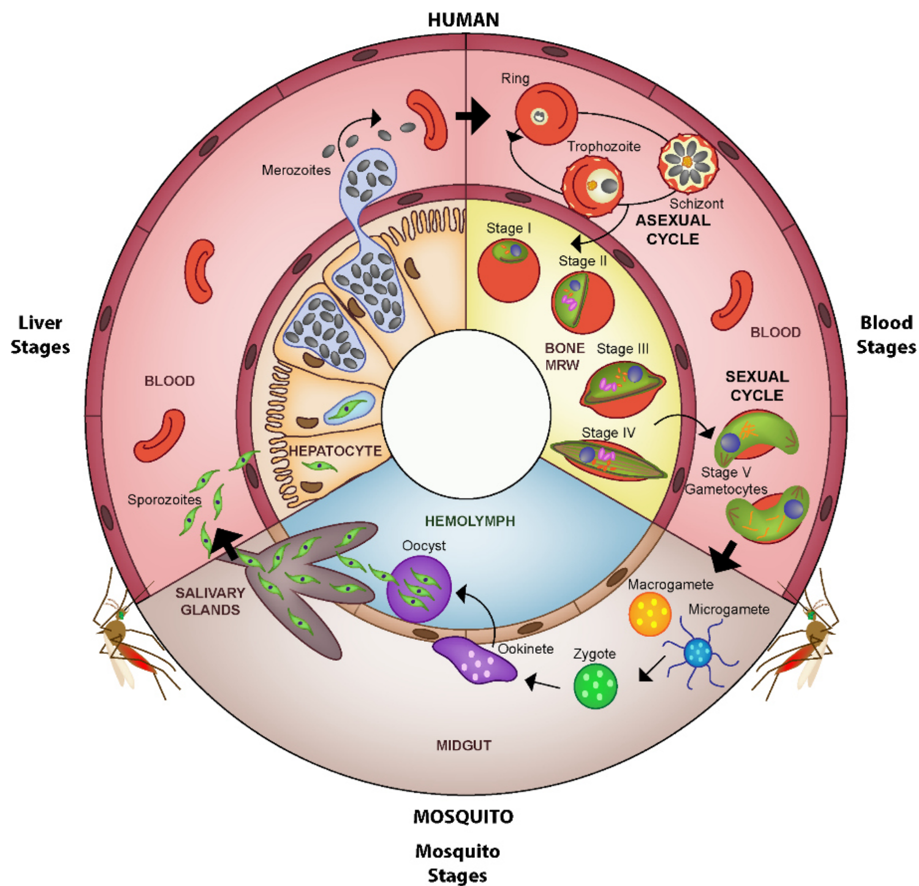


Figure 1. ***Malaria life cycle***. *In the human host, sporozoites released by malaria-infected female Anopheles mosquitoes reach liver hepatocytes, undergo liver schizogony and develop into 10^4 to 10^5 merozoites. Seven days after mosquito inoculation, merozoites enter in the blood circulation and infect red blood cells. Most of the merozoites maturate inside the red blood cells through different stages (Ring, Trophozoite, and Schizont), while a small fraction develops to germinal cells known as gametocytes. Mature gametocytes (both male microgametes, and female macrogametes) are taken up by the mosquito host. Sexual reproduction takes place in the mosquito midgut. Microgametes and macrogametes fuse to zygotes and mature into motile ookinetes. Ookinetes infiltrate the mosquito's midgut wall and become oocysts. The oocysts undergo sporogony and release sporozoites, which travel to the salivary glands of the mosquito and subsequently are injected into a fresh human host.*

Source : *Nilsson, SK et al. PLoS Pathog 11(6): e1004871 (2015).*

© 2015 Nilsson et al.

In the mosquito's midgut, the differentiation of micro- (male) and macro- (female) gametes occurs followed by sexual mating, and formation of a zygote. The zygote's matures into a gliding diploid form called ookinete. The ookinete subsequently transverse the midgut epithelium and develop into cysts called oocyst. The oocyst undergoes cytoplasmic subdivisions until clearly differentiated sporozoites are formed. Finally, sporozoites are discharged and localize to the salivary glands of the mosquito. Sporozoites are injected into the human host during a blood meal to end the cycle. This will spread another sickness among people.

1.2.2 Malaria vaccines in clinical development

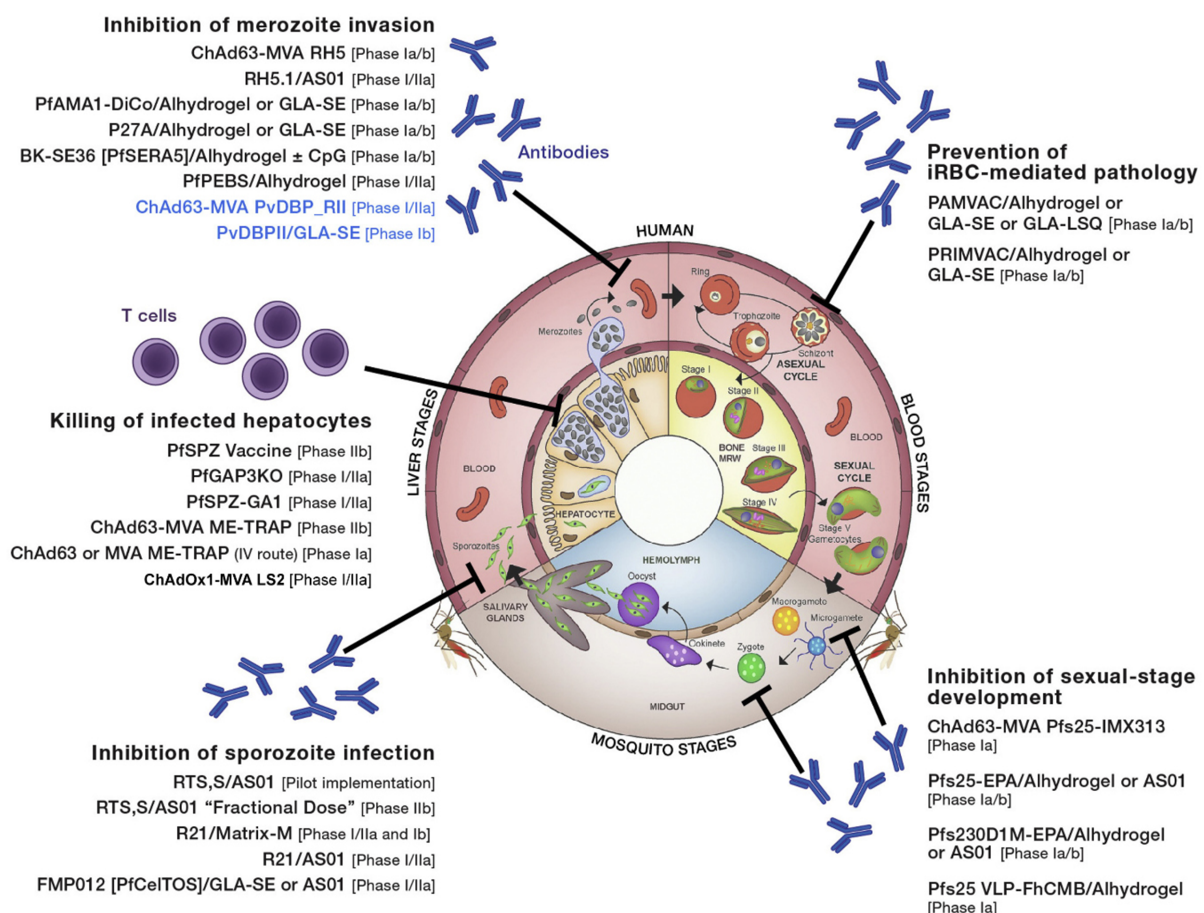
Several malaria vaccines candidates are in the development pipeline [*Figure 2*]. According to the stage of the malaria lifecycle that is being targeted, they can be categorized. For stages that occur within the human host, malaria vaccines are distinguished into two main types: pre-erythrocytic, and asexual blood-stage vaccines. Pre-erythrocytic vaccines are aimed to block infection before or during the liver stage. While some pre-erythrocytic vaccines are subunit vaccines designed against single antigens displayed by sporozoites such as the circumsporozoite protein (CSP) or thrombospondin-related adhesion protein (TRAP)[60], others are based on the attenuation of the whole sporozoite. Asexual blood-stage vaccines, on the other side, have the goal to reduce the merozoite invasion of blood cells or reduce pathology associated with adhesion of infected red blood cells[61], thus avoiding the clinical pathology of the disease.

A third group of malaria vaccines are focused on the transmission of malaria gametocytes from human hosts to the mosquito vectors. The so-called transmission-blocking vaccines are made to produce antibodies that the mosquito ingests while feeding on blood and uses to attack the zygote or sexual phases, thereby preventing infection of the mosquito. Here, antibodies do not act in the human host bit in the mosquito through blockade of plasmodial sexual reproduction[62].

All malaria subunit vaccine candidates are formulated with adjuvants (e.g., Alhydrogel, AS01, GLA-SE, Matrix-M) or require an immune-stimulatory component like expression in chimpanzee adenoviruses (ChAd), modified vaccinia Ankara virus (MVA), or presentation on a virus like particle (e.g., RTS,S) to induce sufficient levels of immune responses.

RTS, S/AS01, commercially known as Mosquirix, is the most advanced malaria vaccine and the only one recommended by WHO for widespread use in children[63]. It was finally approved following 21 years after of testing, including in the largest malaria vaccine trial in sub-Saharan Africa[64].

Mosquirix targets the sporozoite stage before liver infection through generation of antibodies binding to CSP. CSP is the major protein on the surface of the malaria sporozoite. Mosquirix vaccination requires four doses, and its efficacy is rather modest (~36%) with some variability between study populations[65]. Several studies have revealed that vaccine efficacy between populations vaccinated with RTS, S/A01 vary with age, location, or anemia status in African children [66].



*<u>Figure 2</u>. **Malaria Vaccines**. Vaccine candidates against malaria can be classified according to the malaria life cycle stage which is targeted (e.g., Sporozoites, Liver stage infection, Blood stage infection, Sexual stage development). Many of the malaria vaccine candidates are designed as subunit vaccines combining a dominant antigen and an adjuvant. Subunit vaccines are thought to generate antigen-specific antibodies capable of blocking parasite development or reducing malaria-related pathogenesis. Alternatively, whole sporozoite vaccines (WSV) based on the attenuation of fully infectious sporozoites have emerged as vaccine platform. WSV are conceived to stimulate the T-cell response in the liver stage besides antigen-specific antibodies. Other vaccine candidates designed to stimulate mostly the T-cell response in the liver, use not replicative viral vectors (e.g., Chimpanzee adenovirus 63; ChAd63 or Modified vaccinia virus; MVA) encoding malaria antigens (e.g., ME-TRAP or LS2).*

<u>Source</u> : Simon J. Draper et al. Cell Host & Microbe (2018).

© 2018 The Author(s). Published by Elsevier BV.

Besides Mosquirix, attenuated sporozoites have been tested as a pre-erythrocytic vaccine since the early 1970s[67,68]. Since then, it has been reproducibly shown that immunization with irradiated sporozoites can confer protection against challenge from a mosquito infected with non-attenuated sporozoites[68].

To date, technical advances that led to the production of pharmaceutical grade sporozoites according to good manufacturing practice (GMP), pioneered by the American company Sanaria Inc[69], has revived hope that a whole cell malaria vaccine is possible. Intravenously injectable suspensions of purified, metabolically active, radiation-attenuated *P. falciparum* (Pf) sporozoites (SPZ) where thus the first successful whole cell, pre-erythrocytic vaccine candidate (PfSPZ Vaccine)[70]. In addition to radiation-attenuated sporozoites[27,71] (RAS), genetically attenuated parasites[72] (GAP) and chemo-attenuated parasites are currently developed. Chemo-attenuation of sporozoites entails administration of viable sporozoites in combination with antimalarial drugs to attenuate the parasite *in vivo*. This approach is known as chemoprophylaxis with sporozoites (CPS)[73]. When PfSPZ are used, then the approach is referred to as PfSPZ Chemoprophylaxis Vaccine (PfSPZ-CVac)[74–76].

Although RTS, S/AS01 and the whole sporozoite vaccines (WSV) are both pre-erythrocytic vaccines, an important aspect which differentiates the two approaches is the type of immune response elicited. On one hand, Mosquirix immunogenicity generates mostly but not only[77], an anti-CSP humoral response[78,79] while WSV also stimulate cell-mediated responses leading to

generation of cytotoxic antigen-specific CD8+ T cells[80]. Furthermore, RTS,S/AS01 vaccine tends to induce rather short-term protection in malaria naïve volunteers[81] whereas for PfSPZ-based vaccines longer periods of protection reaching up to one-year protection after completing a vaccination schedule have been reported [81].

1.2.3 Evaluation of Malaria vaccine candidates

To develop preclinical malaria vaccines, candidates need to be evaluated in a series of human clinical trials. In Phase I, small numbers of healthy adults (e.g., 20-100 participants) are recruited to assess the effect of vaccine dose and formulation on safety, tolerability, and immunogenicity. A larger number of subjects are recruited in Phase II, where immunogenicity, safety and efficacy or surrogates of efficacy using different doses, schedules and formulations are tested[82]. Subsequently, a pivotal Phase III program is undertaken for in-depth characterization of the vaccine in conditions that are as close as possible to the intended use. In the best case, Phase III is followed by market authorization. Phase III normally involves the largest number of participants, usually in the thousands, and is conducted at multiple sites[83,84].

The best way to evaluate human vaccines early in development is through the exposure of human subjects to the vaccine formulation. Human volunteers are still intentionally infected under controlled circumstances to evaluate the effectiveness of a medication or vaccination. Thereby, the concept of controlled human infection model[85] (CHI), has progressed tremendously over the years accompanied by crucial ethical considerations and debates[86]. In the malaria field, for more than thirty years, controlled human malaria infection models (CHMI) have been used to generate critical efficacy data that inform the transition of clinical trials from Phase IIa (efficacy in malaria-naïve volunteers assessed by CHMI) to late stage development[87] .

Given the absence of reliable surrogates of vaccine efficacy for malaria vaccine candidates, CHMI trials offer an interesting tool to accelerate clinical vaccine development. Despite the higher cost, duration, and complexity, CHMI trials are efficient as they provide clear outcome measures and can prevent unnecessary further development of non-efficacious vaccines[85].

The challenge of finding adequate correlates of protection in malaria vaccine trials persists. As discussed by McCall et al[88], there are several reasons why identification of immunological correlates of protection against malaria is so difficult. Some relate to biological aspects such as the parasite complexity or its antigenic diversity while others are related with the degree of natural immunity present in endemic areas.

It may however, be possible to identify correlates of protection using robust methods and evaluate as many variables as possible using a controlled human infection model and validate them in studies in malaria-endemic regions[12,89].

By now, several potential correlates of protection have been explored in the framework of both the subunit and whole sporozoite malaria vaccines. For instance, a study involving RTS, S AS01, revealed that the antibody response against the NANP repeat of CSP are associated with protection[90]. On the other hand, although inconsistently, it has been demonstrated that CSP-specific CD4+ T cells that produce IFN- have been linked to protection in some trial responses[77]. Another example from malaria unexposed subjects, is the correlation between the elicited T cell response (LAMP-1+ IFN-γ+) and the reduction of the infected red blood cells following vaccination with some of the TRAP subunit vaccines after CHMI[91] .

Whole sporozoite-based vaccines elicit anti-CSP neutralizing antibodies with preferences for the NANP repeated region[92], whereas antibodies targeting both the N- and C-terminal parts are possibly better in fixing complement[93] and inducing cellular phagocytosis of the opsonized sporozoites[94]. The level of PfSPZ-based vaccine-induced anti-CSP responses do not correlate consistently with protection in CHMI. Vaccine-induced anti-CSP IgM can block the sporozoite invasion in *in vitro* assays, and anti-CSP monoclonal antibodies have been shown to be protective after passive immunization[95].

1.2.4 Immune cell monitoring in vaccine trials

Immune cell monitoring (ICM) can be used to track the study participant`s immune responses over the course of vaccination. Phenotyping of cells over time, e.g., using multiparameter flow cytometry (MFC), gives an opportunity to understand how vaccination influences certain cell type clusters. ICM applied during vaccination can contribute to a better understanding of vaccination outcome when partnering vaccine trials controlled human infection (e.g., CHMI).

To perform ICM, blood is regularly drawn from volunteers and analyzed using flow cytometry. However, high-costs and complicated equipment limit flow cytometry use in remote and small laboratories. This was demonstrated by limited use of ICM during malaria vaccine clinical trials compared to cancer research. From 2000 to 2021, only 15 malaria research articles in PubMed adopted ICM (search term: “Immune cell monitoring + malaria vaccine”) compared to 219 for cancer vaccine. Despite the current relative lack of data, ICM use has increased dramatically due to the in-depth analyses it offers[96,97].

For example, ICM is advancing the current understanding of the human immunity generated living in Africa which in turn is crucial to designing better malaria vaccines[98,99].

1.2.1 Immunity to malaria

Following repeated exposure, people living in malaria endemic areas develop mechanisms that reduce disease morbidity. This naturally acquired immunity (NAI), is not sterilizing, depends on age[66], and wanes in absence of restimulation[100]. Protective immunity is associated with increased levels of antibodies against different antigens expressed in infected erythrocytes (e.g., PfEMP1, GLURP, MSP1, PfEMP1 variant VAR2CSA)[101] but also by cellular responses e.g. by activated dendritic cells, polymorphonuclear leukocytes, B and T cells[102]. Anti-malaria antibodies work through invasion inhibition, blocking sequestration, parasite and infected red blood cell opsonization[103] that can lead to the activation of complement[104] and the phagocytosis mediated by neutrophils and monocytes[105].

1.2.2 Correlates of Protection

Many vaccines protect mainly due to the development of specific antibodies or other immune reactions that must reach a certain level to act and can be detected either directly or through their functional activity. However, antibody concentration and function are only part of a successful immune response and in some cases may not be dominant or correlate with observed protection. This is the case for some of the most challenging diseases such as tuberculosis[106], malaria[107], dengue[108], typhoid[109] , hepatitis A or influenza in elderly[110] where protection also depends on cellular responses.

The interest in finding efficacious vaccines for malaria or tuberculosis[111] has stimulated the exploration of other cell types beyond the traditional CD4 and CD8 αβ T cells such as Vγ9Vδ2 gamma delta T[112] or natural killer (NK) cells [113,114] due to their fast response, cell-independent priming, cytotoxicity, or presence of surface receptors for Fc antibody fragments.

Despite the presence of published guidance[115], terminology about correlates of protection is not well defined or consistent in the literature. For some authors, a "correlate of protection" (CoP) is defined as biomarker statistically associated with protection[116], while for others the same meaning is termed "surrogate"[117]. CoPs are classified in two main classes, the mechanistic correlates of protection (mCoP) when the mechanism orchestrated by the immune response is truly the cause of the observed protection, and the non-mechanistic"; nCoP, when a certain immune mechanism is associated with protection despite being not the true mechanism[118].

WHO differentiates between correlates and surrogates of protection. Thus, while correlates are all CoPs, only those CoPs which have a causal relationship between vaccine-induced immune marker and the predefined study endpoint fulfil the definition of a surrogate[115].

The European Agency for the evaluation of Medical Products, defines the term “serological surrogate” when a certain antibody concentration correlates with protection[115].

Besides, the international conference on harmonization (ICH) uses the term “validated surrogate endpoint” for an endpoint where there is strong evidence that it predicts clinical outcome[115].

1.3 Objectives

The search for correlates of protection through the systematic monitoring of immune responses following vaccination, may identify biomarkers that facilitate the choice and optimization of vaccines. This may lead to the formulation of hypotheses on causal relationships and thereby have implications on antigen design, identification of significant interactions, and prediction of susceptibility of an individual or a population. If validated, data generated can be used as a surrogate to clinically develop a vaccine when measuring vaccine efficacy is not feasible, e.g., due to ethical issues[119].

This thesis aimed to investigate cellular immunity following vaccination. I investigated anti-malarial cellular immunity using systematic immune monitoring of circulating cell populations in two distinct, randomized, placebo-controlled, double-blinded vaccine trials using controlled human malaria infection (CHMI) to assess vaccine efficacy. One of the two malaria vaccine candidates (PfSPZ-CVac) was tested in European malaria-naïve volunteers, whereas the subunit GMZ2 vaccine was evaluated in healthy adult African participants.

Outcome of each of the CHMIs was associated with immune parameters and their relation was investigated to better understand protection from malaria. To structure this thesis, each study is confined in a separate chapter:

1. Characterization of proinflammatory CD4+ Th1-like cells following stimulation with malaria antigens as potential surrogate of protection in a trial comparing two PfSPZ-CVac immunization schedules.
2. Investigation of the effect of immunization with the malaria vaccine GMZ2 adjuvanted with Alhydrogel or CAF01 on cell-mediated vaccine immunogenicity through characterization of B and T cell phenotypes.

2 RESULTS AND MANUSCRIPTS ACCEPTED FOR PUBLICATION

2.1 Chapter 1: Systematic monitoring of the bona fide pro-inflammatory CD4+ T helper cells in a PfSPZ-CVac phase II clinical trial with CHMI.

Publication Nr.1

Efficacy, T cell activation and antibody responses in accelerated Plasmodium falciparum sporozoite chemoprophylaxis vaccine regimens

Ibanez, J., Fendel, R., Lorenz, FR. et al.

npj Vaccines 7, 59 (2022)
https://doi.org/10.1038/s41541-022-00473-1

---

npj | vaccines

www.nature.com/npjvaccines

ARTICLE OPEN

Efficacy, T cell activation and antibody responses in accelerated *Plasmodium falciparum* sporozoite chemoprophylaxis vaccine regimens

Javier Ibanez[1,2,8], Rolf Fendel[1,2,3,8], Freia-Raphaella Lorenz[1], Patricia Granados-Bayon[1], Sina Brückner[1], Meral Esen[1,2,3], Mihály Sulyok[1], Zita Sulyok[1], Steffen Borrmann[1,2,3], Petra Bacher[4,5], Alexander Scheffold[4], Stephen L. Hoffman[6], Peter G. Kremsner[1,2,3] and Benjamin Mordmüller[1,2,3,7]✉

Repeated direct venous inoculation of *Plasmodium falciparum* sporozoites (PfSPZ) together with antimalarial chemoprophylaxis (PfSPZ–CVac) is the most potent way to induce sterile immunity against *P. falciparum* infection in malaria-naive volunteers. However, established schedules are complex and long. Here, we tested two accelerated three-dose schedules (28- and 10-day regimen) assessing efficacy by controlled human malaria infection (CHMI) against placebo, comparing vaccine-specific T cell and antibody responses by antigen-reactive T cell enrichment (ARTE) and protein microarray, respectively. Both regimens were similarly efficacious (67 and 63% vaccine efficacy) but different in the induction of vaccine-specific T cells and antibodies. The 10-day regimen resulted in higher numbers of antigen-specific CD4+ effector memory pro-inflammatory T cells and a broader antibody response compared with the 28-day regimen. Usually in nature, *P. falciparum* liver stage lasts about 6.5 days. The short vaccination-interval of the 10-day regimen prolongs the time of continuous exposure to liver-stage parasites, which may explain the stronger response. Besides dose and number of vaccinations, duration of liver-stage exposure is a factor to optimize PfSPZ–CVac immunogenicity.

npj Vaccines (2022)7:59 ; https://doi.org/10.1038/s41541-022-00473-1

INTRODUCTION

The global burden of malaria declined in the first fifteen years of the XXI century, but since then, it has reached a plateau. The latest world malaria report published in 2021 by the World Health Organization even shows that the malaria cases and deaths increased in the last year to reach 241 million cases and 627 000 malaria deaths[1].

The COVID-19 pandemic has compromised the goals of the Global Technical Strategy to Combat Malaria 2016–2030[2] and put the progress made over the past decades at risk. Effective SARS-CoV-2 vaccines were developed within less than a year and have a major impact on the pandemic. Likewise, an effective vaccine against malaria[3] could be a game changer. Proof-of-concept studies for malaria vaccines in humans were first conducted about 50 years ago using irradiated *Plasmodium falciparum* (Pf)-infected mosquitoes[4,5]. Only very recently, the WHO endorsed the first malaria vaccine, RTS,S, for widespread use[6]. Even though this new vaccine might save thousands of lives per year, it is far from optimal as it protects children in endemic areas only for approximately 30%[7].

The recent development of methods to manufacture aseptic, purified, cryopreserved Pf sporozoites (PfSPZ) from mosquito salivary glands under GMP conditions for injection into humans has great potential to further improve the malaria vaccine landscape and makes attenuated whole-parasite vaccines a leading candidate in the field[8].

The most potent immunization strategy known so far uses replication-competent sporozoite (SPZ)-stage parasites that are attenuated in vivo. Here, attenuation is achieved using an antimalarial drug that allows progressing of infection from inoculation to maturation in the liver, the clinically silent phase of the infection, but is highly active against asexual blood-stage parasites (e.g., chloroquine). The concept of in vivo attenuation or "infection-treatment" is uncommon in human medicine, but successfully established in veterinary medicine and widely used in the prevention of East Coast Fever (ECF), a disease of cattle caused by *Theileira parva*. This veterinary vaccine shows high efficacy in preventing clinical ECF and mortality[9].

A second approach to immunize individuals using attenuated PfSPZ is the so-called PfSPZ Vaccine, in which sporozoites are rendered replication-defective by γ-irradiation. The superior potency of in vivo attenuation over approaches using replication-incompetent PfSPZ is likely due to the prolonged exposure to liver-stage parasites, a higher antigen load, and a broader immune response[10].

Previously and as part of the present study, we established an immunization regimen dubbed PfSPZ chemoprophylaxis vaccine (PfSPZ–CVac), with three inoculations of cryopreserved PfSPZ (PfSPZ Challenge), given every four weeks to volunteers under chloroquine chemoprophylaxis for 10 weeks[10]. Three direct venous inoculations (DVI) of 5.12×10^4 PfSPZ (on days 0, 28, and 56) protected all volunteers from infection, whereas lower doses dose-dependently led to partial protection[10].

The immunological mechanism behind PfSPZ-mediated protection is not known. We have previously shown that vaccine efficacy (VE) is associated with a higher number of circulating memory CD4+ T cells producing IFN-γ, IL-2, and/or TNF-α following in vitro stimulation with PfSPZ or infected red blood cells (iRBC)[10]. Antibody titers against Pf circumsporozoite protein (CSP), the

[1]Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany. [2]German Center for Infectious Diseases (DZIF), Partner Site Tübingen, Tübingen, Germany. [3]Centre de Recherches Médicales de Lambaréné (CERMEL), Lambaréné, Gabon. [4]Institute of Immunology Christian-Albrechts-University of Kiel, Kiel, Germany. [5]Institute of Clinical Molecular Biology, Christian-Albrechts-University of Kiel, Kiel, Germany. [6]Sanaria Inc, Rockville, MD 20850, USA. [7]Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands. [8]These authors contributed equally: Javier Ibanez, Rolf Fendel. ✉email: benjamin.mordmueller@radboudumc.nl

main surface protein of PfSPZ, increased with dose, but were not associated with protection[10]. However, the immunization scheme of our previous study was still very complex and lengthy. For the development toward a vaccine with better applicability in travel medicine as well as in endemic settings, the time span of the regimen needs to be significantly shortened. The present study reports the results of two more realistic vaccination regimens. The number of interventions was reduced and the schedule shortened to 28 and subsequently down to 10 days.

Regarding immunogenicity, we characterized humoral responses by ELISA and protein microarrays, as well as cellular immune responses. The antigen-reactive enrichment methodology[11] (ARTE) using Pf-infected red blood cells (iRBC) as proxy of Pf-infected hepatocytes was implemented to monitor ex vivo, the CD40L+CD4+ T cell specificity during immunization and before the day of challenge (C-1). ARTE has been successfully employed previously to explore antigen- specific CD4+ T cell subsets generated against various infections and immune pathologies[12–15] but not yet to characterize the immune response of any malaria vaccine candidate. The two-step technique, based on the magnetic isolation of fresh cells bearing the surface ligand CD40L (CD154) plus the intracellular detection of the same CD40L marker in combination with other pro-inflammatory cytokines such as TNF-α[14], aimed not only to detect the bona fide T-helper cells, but also allowed to distinguish cognate responses within the naive (CD45RO−) or memory (CD45RO+) T cell repertoire.

The longer PfSPZ–CVac regimen with PfSPZ inoculations on days 0, 14, and 28 using repeat inoculations with a timing that allows three separate liver-stage developments had a similar VE but surprisingly was less immunogenic than the shorter one with PfSPZ inoculations on days 0, 5, and 10. In the shorter vaccination schedule, the booster vaccinations are given when the parasites of the previous dose are still in the liver stage. This results in a total period of 16 days during which parasites are constantly present in the liver.

RESULTS

Participants, safety, and reactogenicity

Volunteers were allocated to a placebo group (normal saline, $n = 6$) or to a vaccine group with three direct venous inoculations (DVI) of 51,200 PfSPZ either 14 days ($n = 10$) or 5 days ($n = 9$) apart, for a total immunization regimen of 28 and 10 days, respectively. Basic demographic data are summarized in Supplementary Table 1 and the CONSORT diagram is shown in Supplementary Fig. 1. All volunteers received chemoprophylaxis with chloroquine. In addition, half of the volunteers ($n = 5$ verum, $n = 2$ placebo) of the 28-day regimen also received 2 g of extended-release azithromycin (Zithromax Uno) on the day of the first vaccination. The rationale to use azithromycin was based on studies in mouse models in which the drug allowed the plasmodial liver stage to undergo schizogony, but meanwhile blocked the biogenesis and inheritance of the apicoplast. The azithromycin-treated animals did not develop patent asexual blood-stage parasitemia and azithromycin-mediated attenuation led to pre-erythrocytic immunity comparable to chloroquine prophylaxis[16,17].

Twenty-five volunteers were enrolled in the study and received at least one immunization (intention-to-treat—ITT), 22 participants completed the trial as per protocol (PP). Two volunteers (1 placebo, 1 PfSPZ–CVac 10-day regimen) withdrew from the study following the third vaccination (98 and 74 days, respectively) and before controlled human malaria infection (CHMI) (Supplementary Fig. 2). In all but one volunteer (PfSPZ–CVac 28-day regimen without azithromycin), chloroquine treatment was fully effective in treating parasitemias in the first asexual blood cycle (Supplementary Fig. 2). The individual that unexpectedly developed the parasitemia (178 parasites/μL) during immunization was excluded from the study. Circulating parasites isolated from this participant were fully sensitive to chloroquine ex vivo (50% inhibitory concentration: 3 nM). Traces of chloroquine were detected in plasma, although concentration was below the level of quantification of the assay (<5 ng/mL). In contrast to this, three other randomly selected volunteers had chloroquine plasma concentrations of 98, 119, and 115 ng/mL, which is well above the inhibitory concentrations. Chloroquine was administered at the clinical trial site in the presence of a physician and a second team member, but swallowing was not monitored (e.g., by oral inspection). It is most likely that the participant who unexpectedly developed the parasitemia did not swallow the tablet but kept it in the oral cavity and discarded it when leaving the examination room. The volunteer was successfully treated with atovaquone-proguanil but disagreed with further pharmacokinetic investigations.

A total of 313 adverse events (AE) were reported in 25 randomized volunteers. No serious AE (SAE) occurred. In total, 194 AEs were reported during the immunization (Supplementary Fig. 3 and Supplementary Table 2) and 119 AEs during the challenge infection were solicited (Supplementary Table 3). The most frequent clinical AEs were headache (20 episodes), fatigue (20), and diarrhea (12). The most frequent laboratory AEs were increased alanine (7) and aspartate (5) aminotransferases (ALT and AST, respectively), elevated lactate dehydrogenase, LDH (8), and high monocyte count (9). In total, 276 AEs were mild (Grade 1), and 27 were moderate (Grade 2). Six severe Grade 3 AEs were reported, including increased ALT, lymphopenia, and thrombocytopenia associated with the case of malaria during immunization. Lymphopenia and two Grade 3 gastrointestinal bleedings were reported in the 10-day group. All Grade 3 AEs resolved uneventfully.

Parasitemia kinetics during vaccination and vaccine efficacy

Circulating asexual blood-stage parasites were detectable 7–9 days following each vaccination in the 28-day regimen. Peak parasitemia tended to decrease during sequential vaccinations. This trend was more pronounced in volunteers who were later protected against CHMI (Fig. 1a, Table 1). Parasitemia also peaked approximately 8 days after each vaccination in the 10-day regimen with decreasing peak parasitemia after the second and third DVIs. Here, the decrease in peak parasitemia was not associated with protection. All five placebo recipients developed parasitemia following inoculation of PfSPZ Challenge. VE was 67% (6/9, $p = 0.000045$) and 63% (5/8, $p = 0.012$) in subjects immunized at 14- and 5-day intervals, respectively, as we have reported before[10] (Fig. 1b).

Effect of azithromycin

We explored if a single dose of 2 g of extended-release azithromycin, given at the day of vaccination, can block development of (transient) asexual blood-stage parasitemia, as described above. Seven of the 14 volunteers (2 placebo and 5 vaccinees) of the 28-day regimen received azithromycin in addition to chloroquine prophylaxis at their first vaccination. All five who received PfSPZ developed transient asexual blood-stage parasitemia, although at a lower level, compared with chloroquine alone ($p = 0.027$, Kruskal–Wallis test on AUC) (Supplementary Fig. 2).

Vaccination induces parasite-specific CD40L+CD4+CD45RO+T-helper cells with a pro-inflammatory signature

To profile T cell reactivity following vaccination with high sensitivity, antigen-reactive T cell enrichment[11] (ARTE) was performed following in vitro iRBC or uRBC stimulation of PBMC. Thereby, the pro-inflammatory CD40L+TNF-α+IFN-γ+CD45RO+/CD4+T (Th1) memory helper-cell response and the stimulated

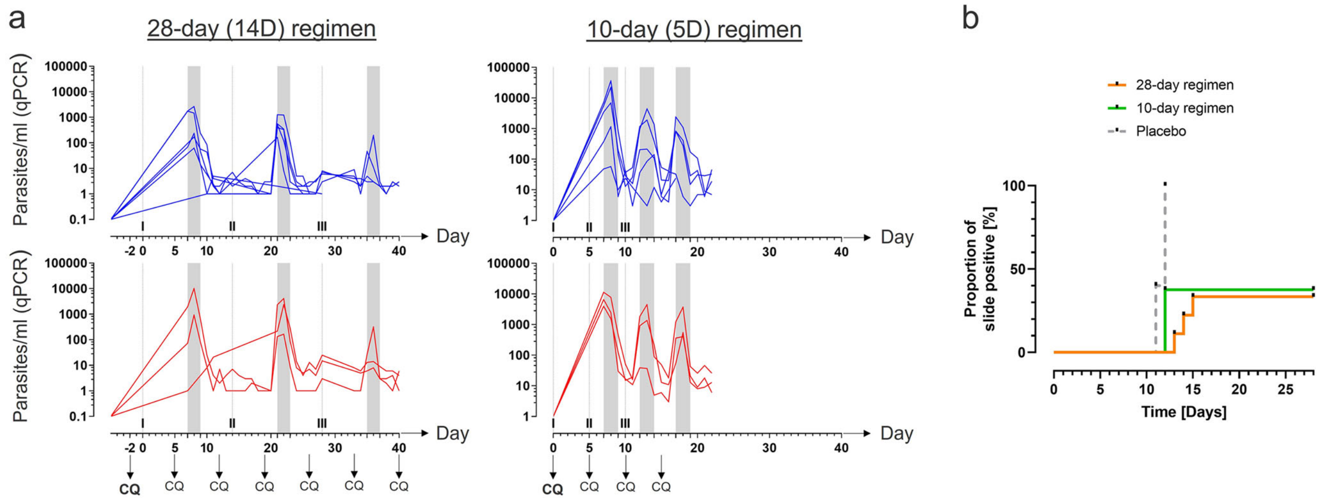


Fig. 1 Submicroscopic infected red blood cells (iRBC) are detectable during the vaccination period. **a** The kinetics of the parasites detectable by RT-qPCR in the peripheral blood during the vaccination period using PfSPZ–CVac is shown. The left panels represent the parasitemia during the 28-day regimen and the right panel represents the 10-day regimen. Individuals being protected against the subsequent CHMI are shown in blue, unprotected are shown in red. Gray vertical bars represent the expected 7–9-day prepatent period after direct venous inoculation (DVI). Arrows indicate the time points when participants received chloroquine (CQ) tablets. **b** Proportion of slide positivity after CHMI is depicted using a Kaplan–Meier plot.

Table 1. Kinetic of transient parasitemia during the vaccination period in the 28-day and 14-day regimen in protected and unprotected individuals.

Vaccine dose (PfSPZ)	Vaccination time points	CHMI outcome	Immunization	Median parasitemia (# PCR positive/# total participants)	Day peak parasitemia
51200	0,14,28	Protected	I	204 (5/6)	8 (+8)
51200	0,14,28	Unprotected	I	933 (2/3)	8 (+8)
51200	0,14,28	Protected	II	457 (6/6)	21 (+7)
51200	0,14,28	Unprotected	II	2378 (3/3)	22 (+8)
51200	0,14,28	Protected	III	3 (4/6)	36 (+8)
51200	0,14,28	Unprotected	III	14 (3/3)	36 (+8)
51200	0,5,10	Protected	I	6953 (5/5)	8 (+8)
51200	0,5,10	Unprotected	I	6556 (3/3)	7 (+7)
51200	0,5,10	Protected	II	215 (5/5)	13 (+8)
51200	0,5,10	Unprotected	II	1372 (3/3)	13 (+8)
51200	0,5,10	Protected	III	813 (5/5)	17 (+7)
51200	0,5,10	Unprotected	III	545 (3/3)	18 (+8)

naive T cell response (CD40L+TNF-α+IFN-γ+CD45RO−/CD4+) specific for Pf was monitored during the whole vaccination period. The frequencies of iRBC-reactive T cells were measured by flow cytometry. The gating strategy is summarized in Supplementary Figs. 3 and 4 and the calculation scheme to estimate the frequency of stimulated T cells is given in Supplementary Fig. 5.

At baseline, iRBC treatment did not stimulate any memory Th1 or naive T-helper cells (Fig. 2). At day 14 (II, 28-day regimen) and day 15 (III5, 10-day regimen) respectively, the memory Th1 frequency increased only in vaccinated subjects, but not in the placebo group (Fig. 2a). The naive T-helper cells did not react to the iRBC stimulation after vaccination (Fig. 2b). Interestingly, two individuals of the 28-day regimen group who did not develop any parasitemia during the first immunization, also had the lowest frequency of iRBC-specific memory Th1 cells in that group (0.007 and 0.006%, median 0.021%), which is in the range of placebo (median 0.005%) and the 10-day vaccinee group at five days after the first immunization (median 0.003%) (Fig. 2, black dots). This observation indicates that in these individuals, iRBC-specific reactivity could not be induced by a single dose of PfSPZ alone and exposure to late liver stage/asexual blood stage eventually might be required. Despite this observation that these two individuals showed very low reactivity, the frequency of memory Th1 cells did not directly correlate with the overall exposure to parasites in the blood, represented by the calculated area under the curve of the parasitemia during the first immunization (Supplementary Fig. 6).

PfSPZ–CVac induces iRBC-specific CD40L+CD4+pro-inflammatory Th1 cells one day before CHMI (C-1)

Both immunization regimens (28-day and 10-day) significantly increased the frequency of polyfunctional effector memory (TEM, CD40L+TNF-α+IFN-γ+CD45RO+CD197−/CD4+) parasite antigen-specific T cells by day C-1 (Fig. 3). The 28-day vaccine regimen tended to generate about two times lower frequencies of polyfunctional TEM cells than the 10-day regimen, but the difference was not statistically significant. Also, TNF-α monofunctional TEM was significantly elevated in both regimens (Fig. 3b),

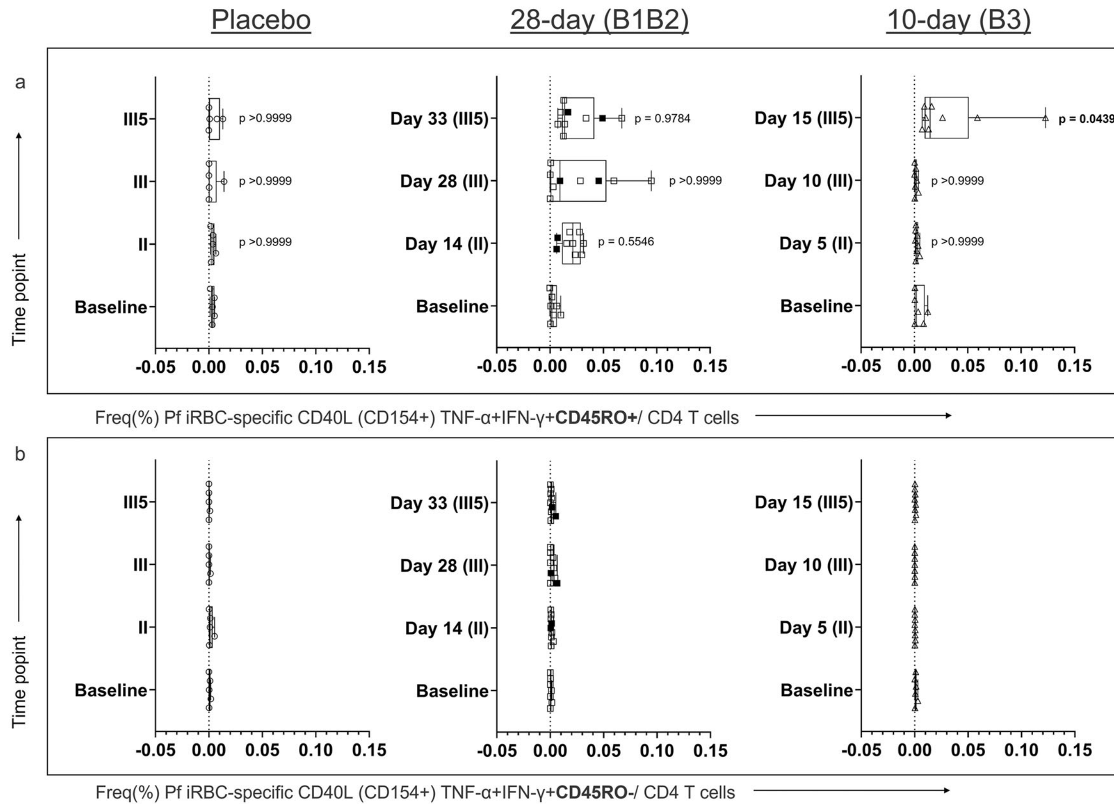


Fig. 2 Ex vivo level of iRBC-specific polyfunctional memory and naive helper T cells (Th1). PBMCs were stimulated with iRBCs. Frequency of (**a**) memory Th1 cells (CD40L+CD4+TNF-α+IFN-γ+CD45RO+) and (**b**) naive Th1 cells (CD40L+CD4+TNF-α+IFN-γ+CD45RO–) were quantified by flow cytometry. Black filled squares represent the frequency of Th1 iRBC specific for two participants who did not develop detectable parasitemia after receiving the first immunization dose. Kruskal–Wallis test was used to assess the difference in the frequency of stimulated T cell population during the vaccination period. Statistical significance was considered when $p < 0.05$.

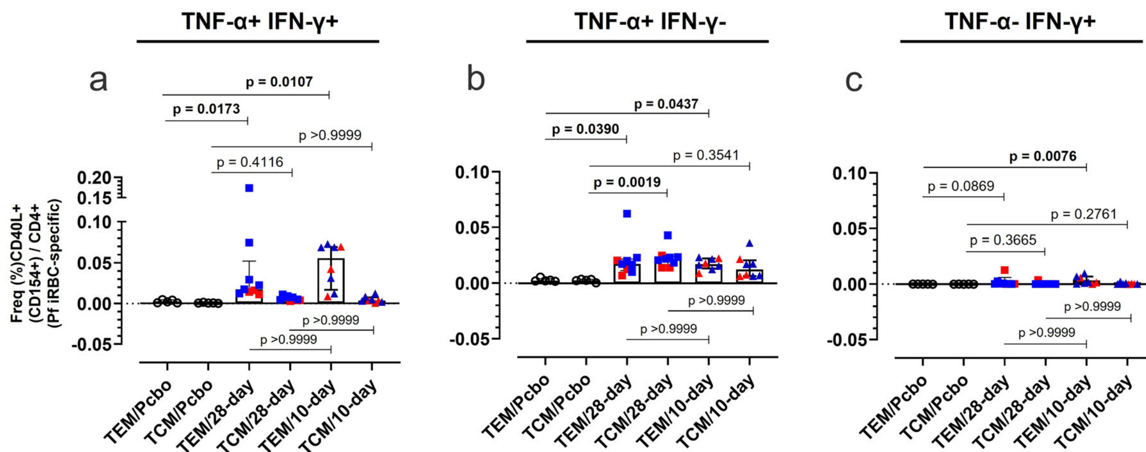
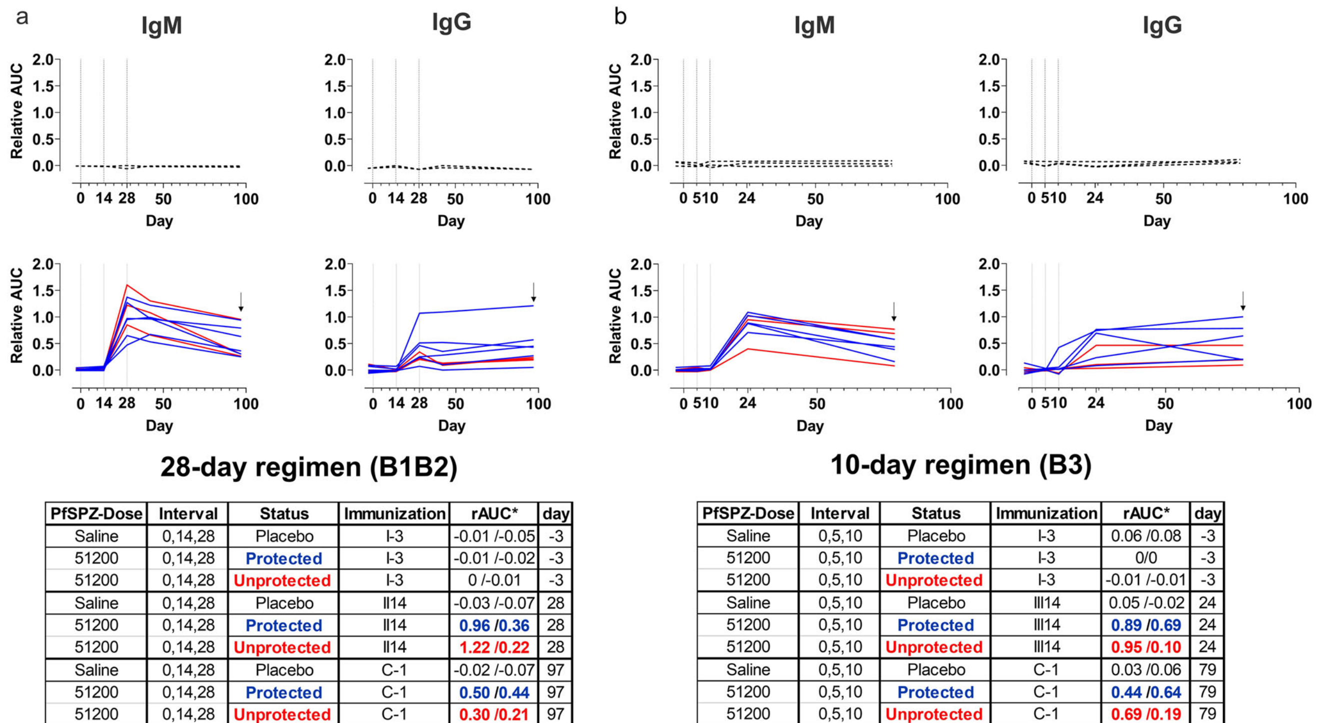


Fig. 3 Pf-iRBC-specific activated mono- (TNF-α+ or IFN-γ+) and polyfunctional (TNF-α+ and IFN-γ+) effector memory and central memory CD4+T cells one day before CHMI. iRBC-specific cell frequencies obtained following ARTE are shown, comparing the influence of each regimen in the acquisition of circulating effector (TEM) or central (TCM) memory cells. (**a**) Polyfunctional T cells, (**b**) TNF-α+IFN-γ– monofunctional T cells, and (**c**) TNF-α– IFN-γ+ monofunctional T cells. Squares: 28-day regimen vaccinees; Triangles: 10-day regimen vaccinees; open circles: placebo. Red: no protection after CHMI; blue: protection after CHMI. Bar plots represent median and error bars show the interquartile range (IQR). The nonparametric Kruskal–Wallis with Dunn's test is performed to analyze the differences between PfSPZ vaccinees (28-day, open squares and 10-day regimen, open triangles) and the nonimmunized volunteers (placebo, Pcbo). Differences between groups are considered statistically significant (bold) if $p < 0.05$.

and IFN-γ monofunctional TEM was only significantly elevated in the 10-day regimen (Fig. 3c).

Polyfunctional central memory cells (TCM, CD40L+TNF-α+IFN-γ+CD45RO+CD197+/CD4+) were similarly elevated in both the 10-day and the 28-day regimen after immunization as compared with the respective placebo controls (Fig. 3a). Only in the 28-day regimen, TNF-α monofunctional TCM was significantly elevated, all other estimated monofunctional TCM populations were elevated compared with the placebo control, although not statistically significant (Fig. 3b, c).



PfSPZ-Dose	Interval	Status	Immunization	rAUC*	day
Saline	0,14,28	Placebo	I-3	-0.01 /-0.05	-3
51200	0,14,28	Protected	I-3	-0.01 /-0.02	-3
51200	0,14,28	Unprotected	I-3	0 /-0.01	-3
Saline	0,14,28	Placebo	II14	-0.03 /-0.07	28
51200	0,14,28	Protected	II14	0.96 /0.36	28
51200	0,14,28	Unprotected	II14	1.22 /0.22	28
Saline	0,14,28	Placebo	C-1	-0.02 /-0.07	97
51200	0,14,28	Protected	C-1	0.50 /0.44	97
51200	0,14,28	Unprotected	C-1	0.30 /0.21	97

PfSPZ-Dose	Interval	Status	Immunization	rAUC*	day
Saline	0,5,10	Placebo	I-3	0.06 /0.08	-3
51200	0,5,10	Protected	I-3	0/0	-3
51200	0,5,10	Unprotected	I-3	-0.01 /-0.01	-3
Saline	0,5,10	Placebo	III14	0.05 /-0.02	24
51200	0,5,10	Protected	III14	0.89 /0.69	24
51200	0,5,10	Unprotected	III14	0.95 /0.10	24
Saline	0,5,10	Placebo	C-1	0.03 /0.06	79
51200	0,5,10	Protected	C-1	0.44 /0.64	79
51200	0,5,10	Unprotected	C-1	0.69 /0.19	79

Fig. 4 IgM and IgG ELISA titers against the malaria circumsporozoite protein (CSP). Analysis of IgM and IgG relative area under the curve (rAUC) against the CSP protein is represented for each vaccine regimen assessed. Sera were collected to perform ELISA (28-day = I-3, I14, II14, III14, and III69. 10-day = I-3, I5, II5, III14, and III69). B1B2 participants following 28-day regimen (**a**) and volunteers in B3 following the 10-day regimen (**b**). Blue lines describe IgM or IgG anti-CSP when vaccinees resulted protected after CHMI. Red lines by contrast, represent unprotected vaccinees and black dashed lines those who received placebo control (Pcbo). Vertical dotted lines indicate immunization days (0, 14, and 28 at 14-D interval and 0, 5, and 10 at 5-D interval). Arrows indicate the antibody levels against the CSP protein one day before challenge (C-1). Bottom tables summarize rAUC titers against CSP protein for each regimen, immunoglobulin isotype, status obtained after PfSPZ challenge (CHMI), and day of the study. (*) rAUC median per subgroup and immunoglobulin isotype backward slash separated (IgM (left)/IgG (right)).

ELISA shows similar IgG kinetics against the *P. falciparum* circumsporozoite protein (CSP)

IgG and IgM antibodies specific for the main surface protein of PfSPZ, CSP, were investigated in all volunteers by ELISA on each vaccination day, 14 days after the final boost and one day before CHMI. Both, CSP-specific IgG and IgM were negative at baseline in all participants. Participants following the 28-day regimen reached peak levels about 4 weeks after receiving the first vaccination (II14), and remained at similar levels until C-1 (Fig. 4a). This was unexpected since the third vaccination was expected to further increase the anti-CSP antibody levels. In the 10-day regimen group, the CSP-specific antibodies were not detectable before the last immunization on day 10, in 7 out of 8 individuals. In one individual, CSP-specific IgG could be detected just before the third immunization. In this group, antibody levels peaked by day 24 after the first vaccination (Fig. 4b). In parallel, the CSP-specific IgM response peaked after receiving the second or the third immunization dose following the 28- or 10-day regimen, respectively (Fig. 4). The IgM relative area under the curve was on average higher than the IgG response by II14 (28-day regimen, Fig. 4a) or III14 (10-day regimen, Fig. 4b), but declined between III14 and C-1. By contrast, the IgG levels remained at similar levels from III until C-1. Furthermore, differences between protected and unprotected vaccinees were not statistically significant at C-1 for any of the explored antibody isotypes. However, the anti-CSP IgG level was higher in protected (median relative area under the curve (rAUC) = 0.45) than in unprotected volunteers (median rAUC = 0.2) (Student's *t*-test, two-sided, $p = 0.073$).

Protein microarray

The overall IgG antibody response against Pf was estimated by protein microarray in PfSPZ-exposed vaccinees ($n = 9$, 28-day regimen and $n = 8$, 10-day regimen), as well as in the placebo controls ($n = 5$) one day before CHMI (Fig. 5). The array comprises 262 Pf protein fragments representing 228 unique proteins as described before[18].

IgG antibodies recognizing pre-erythrocytic malaria proteins such as the liver-stage antigen 1 (LSA1) and the liver-specific protein 2 (LISP2) were strongly induced in PfSPZ–CVac vaccinees. In addition, antigens associated with late liver stage and blood stage, like MSA180 and MSP10, were significantly elevated in the vaccinated group (Fig. 5a). Interestingly, the only antigen associated with protection in this study is MSP5, and antibodies specific for the liver-stage antigen LISP2 are even more elevated in the unprotected individuals (Fig. 5b).

Strikingly, when both vaccination regimens were compared, the parasite antigen-specific IgG antibody levels following the 10-day regimen were significantly higher than in the 28-day regimen (Fig. 5c). These antibodies were specific to several late liver-stage/early blood-stage antigens (PHISTb; Pf helical interspersed subtelomeric b, ApiAP2; Pf Apicomplexa plant-like transcription factor proteins, and MSP10; Pf merozoite surface protein 10).

DISCUSSION

Exposure to whole pre-erythrocytic-stage parasites has been one of the first successful vaccination approaches to malaria and at the moment seems still to be the most efficacious method to

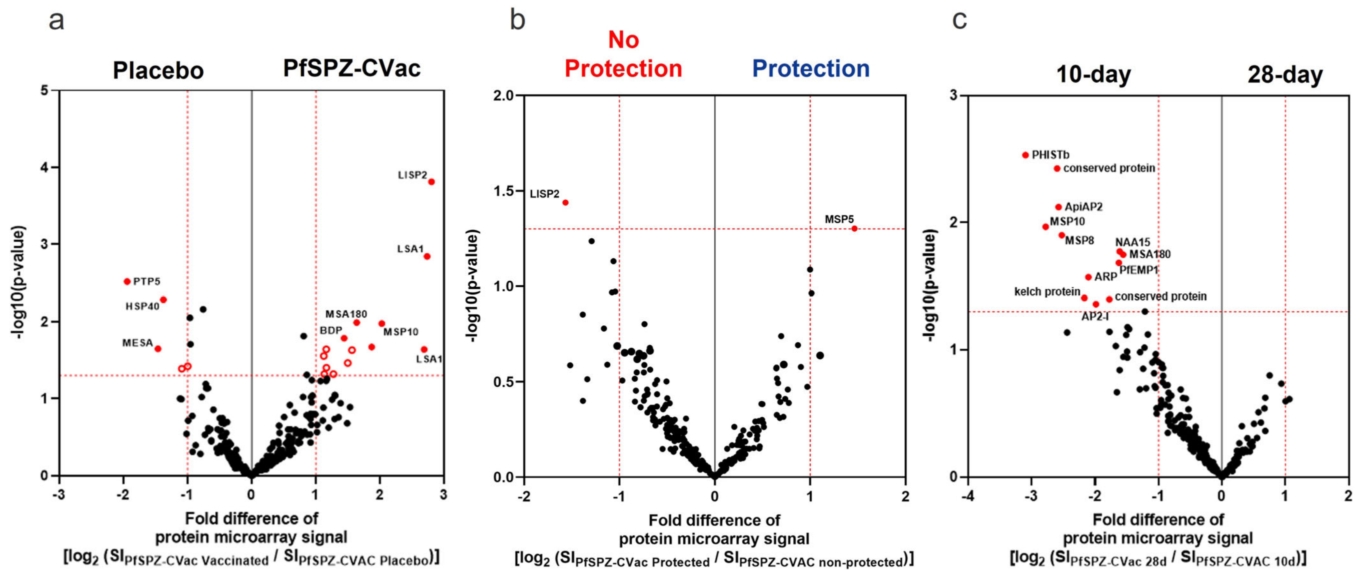


Fig. 5 Screening of elicited humoral immune response using protein microarrays. Sera from all volunteers collected one day before CHMI (C-1) were assessed on protein microarrays containing 262 Pf proteins representing 228 unique antigens. Analysis was performed on C-1 data after subtraction of the individual background reactivity. Volcano plots represent the protein microarray data analyzed by the groups of the (**a**) antigen reactivity in vaccinated donors against placebo controls (immunogenicity), (**b**) reactivity of protected individuals against unprotected individuals, and (**c**) reactivity in the 28-day regimen vs. the 10-day regimen. Differentially recognized antigens (*p*-value <0.05 and fold change >2) are depicted in red. P- values are estimated using the two-sided Welch-corrected Student's *t*-test.

immunize against malaria in vertebrate hosts[19]. Seminal studies on human hosts receiving x-irradiated sporozoites by mosquito bite performed in the 1970s, showed that vaccination with attenuated sporozoites is safe and generates high antibody titers against the main surface protein of the sporozoite, the CSP protein[4]. Since then, the concept was reproduced with different modes of attenuation[20,21] but it was only about twenty years ago that it was turned into an approach to develop a vaccine rather than being a proof-of-concept to develop other, mostly recombinant vaccines. The technical challenges of manufacturing and preserving billions of pharmaceutical-grade sporozoites for human use were overcome by the company Sanaria Inc.[22] and clinical development was led by an international effort, including institutions from America, Europe, Asia, and Africa. In this framework, clinical trials have used whole sporozoites that were physically (PfSPZ Vaccine)[23–27], chemically (PfSPZ–CVac)[10,18,28,29] or genetically (PfSPZ–GAP1)[30] attenuated in both malaria-naive and lifelong malaria-exposed human subjects.

The method and timepoint of attenuation of the parasites is important for achieving successful immunization, with killed sporozoites being not protective[31] and strategies that allow full parasite development in the liver being the most effective[32]. One elegant way of achieving such late attenuation is the use of chemoprophylaxis based on a drug that kills asexual blood-stage but not liver-stage parasites. Proof-of-concept in mice[33] and humans[34] showed that this approach is highly effective.

Previously, we have translated this approach into a highly efficacious vaccine candidate using cryopreserved PfSPZ[10]. As the current immunization schedule is complex and therefore difficult to implement as a vaccine to be used in endemic regions and for travelers, we aimed at shortening and simplifying the regimen using CHMI as a measure of vaccine efficacy.

Healthy, malaria-naïve European young adults immunized with two regimen of three inocculations with fully infectious Pf sporozoites under chloroquine prophylaxis (PfSPZ–CVac) showing similar VE levels (28-day, 67% and 10-day regimen, 63%)[10]. Both regimens were safe and well tolerated in line with the previous 56-day regimen[10] using the same PfSPZ dose. Recently, the 10-day regimen was assessed in US subjects and showed high VE (75%) using double number of PfSPZ per dose[35]. However, both 10-day regimens followed the same pattern regarding number and grade of AEs reporting more AEs after receiving the first vaccination shot at the time of peaking parasitemia. A subsequent trial conducted in Tübingen, allowed us to study a PfSPZ–CVac regimen partially inspired by the 10-day regimen with three immunizations of 1.1×10^5 PfSPZ given at days 1, 6, and 29 under similar chloroquine dosage. Of interest, we achieved a high degree of heterologous protection (77% VE) challenging the vaccinees immunized using parasite strain PfSPZ NF54 of African origin with the genetically distant Pf 7G8 strain, which was isolated in South America[18].

Volunteers receiving 5.12×10^4 PfSPZ per dose developed adverse events related to the blood and lymphatic-system, including thrombocytopenia, lymphopenia, or leukopenia. General symptoms such as chills or fatigue were present in ~80% of all subjects. Mild nervous-system disorders such as dizziness or headache were similarly frequent. The use of twice the amount of PfSPZ per dose given at days 1, 6, and 29 further increased the likelihood of dizziness and headache[18]. In addition, the present study found an increase in both alanine and aspartate aminotransferase liver enzymes, which is consistent with the study published by Murphy et al.[35] but not consistent with the study using the 56-day regimen[10].

One participant allocated to chloroquine in the 28-day regimen became thick blood-smear positive following the first vaccination. At this time, he had no malaria symptoms but became symptomatic during treatment (Supplementary Fig. 2b). It is most likely that he did not swallow the chloroquine tablets as post hoc-measured plasma levels detected only traces of the drug (in agreement with a short exposure while keeping the tablets in the mouth). This shows that despite observed intake, parasite attenuation with an orally given drug has nonnegligible risks, which could lead to severe consequences when such an approach is used outside a tightly controlled clinical trial. Coformulation with PfSPZ or, at least, parenteral administration of the drug, would circumvent this problem. Chloroquine could have been given by intramuscular injection, but is not well tolerated and

would require large volumes[36]. Intravenous injection is life-threatening, particularly when given as a bolus[37].

The exploratory use of azithromycin in one of the two 28-day groups was done to assess if it can be used as an alternative to chloroquine but with a better adverse-event profile because the asexual blood stage could be omitted[16] and its general good safety profile. Unfortunately, all participants developed asexual blood-stage parasitemia, albeit at lower levels (Supplementary Fig. 8). Thus, chemoprophylaxis in the two 28-day regimen groups (B1 and B2) was based on chloroquine alone, as per-protocol[10]. Nevertheless, the effect of azithromycin on parasitemia permits to hypothesize that a higher dose given either over a longer period or later during liver stage, could prevent blood-stage parasitemia. In a similar fashion, the use of pyrimethamine has recently shown that full protection is possible when the drug is given late during liver stage, preventing the asexual blood-stage development[38].

In agreement with other PfSPZ–CVac studies involving malaria-naive PfSPZ vaccinees[10,18,34,35], parasite-specific activated Th1 memory cells were equally expanded at C-1, irrespective of the regimen adopted, although the time between the third immunization and C-1 was key for the 10-day regimen vaccinees to catch up similar frequencies. Furthermore, we could associate the condensed regimen of 10 days with an elevated frequency of parasite-specific pro-inflammatory TEM cells, even though TEM cell number was not associated with protection against CHMI (Fig. 3).

Analysis of the IgM and IgG titers against the CSP protein, showed similar antibody anti-CSP profiles for both protected and unprotected participants, regardless of the vaccine regimen during the immunization phase (Fig. 4). Similarly, a higher number of PfSPZ per dose assessed in malaria-naive subjects following both the 56-day regimen[10,21] and a shorter 10-day regimen[35] did not change the pattern. Moreover, not only the dose, but also the use of pyrimethamine as PfSPZ–CVac partner drug instead of CQ has lately demonstrated a similar pattern[38]. The fact that in a 56-day regimen with lower PfSPZ number per dose (1.28×10^4), there was no difference between protected ($n = 6$) and unprotected ($n = 3$)[10] individuals, but in a study using higher dose of PfSPZ per dose (1.1×10^5), a significant difference could be detected[18], which may suggest a threshold for the anti-CSP IgG level that correlates with protection.

Furthermore, the antibody-response analysis performed after vaccination but before challenge (C-1) showed to be stronger following the regimen of 10 days compared with the 28 days (Fig. 5c). Consistent with the previous 56-day regimen performed in Tübingen using 5.12×10^4 PfSPZ[10], or the 28-day regimen (with immunizations at days 0, 5, and 28) using 1.1×10^5 PfSPZ[18] per dose, the antibody profile revealed an increased IgG reactivity against liver-stage markers such as LSA1 and LISP2 in all PfSPZ–CVac vaccinees (Fig. 5a). Moreover, vaccinees following the 10-day regimen showed 4–6 times higher IgG-reaction intensity towards Pf proteins associated with the liver-to-blood interface such as PHISTb, ApiAP2, or MSP10 than the 28-day regimen (Fig. 5c). This strong difference is surprising, but might be explained by the extended exposure of the immune system to liver-stage parasites of approximately 16 consecutive days, whereas the exposure to liver-stage parasites in the 28-day regimen is approximately 3 times 6.5 days with intermittent periods of blood-stage parasites, and a few days without any exposure to the parasite (Supplementary Fig. 9). Interestingly, the results presented here also show that MSP5 is strongly associated with protection after homologous PfSPZ Challenge. This protein is expressed in sporozoites, late liver stage, as well as the blood stage, and has been found to be immunogenic[10]. Likewise, it has been shown to be highly immunogenic in other vaccine trials using PfSPZ Vaccine before (Tumbo et al., in preparation). In agreement with it, the 28-day regimen (with vaccination at days 0, 5, and 28) using a higher number of PfSPZ per dose also revealed that protection against PfSPZ Challenge is strongly associated with another protein anchored in the merozoite surface protein 2 (MSP2). In the same way, MSP2 is associated with later stages of Pf infection in the liver[39]. Thereby, it is reasonable to believe that generating protective antibodies against two immunogenic antigens covering both initial and later phases of the pre-erythrocytic malaria such as CSP and MSP5, should offer a superior degree of protection against Pf.

Despite the interesting findings, the present work has some limitations affecting the extent of their conclusions. The sample size of the study is small. While this allows determination of clinically significant VE, it is difficult to determine immune parameters responsible for protection. Further studies should investigate, in addition to other parasite-specific CD4+ T cell subtypes, the contribution of the innate cell types (e.g., NK, NKT, and γ9δ2-T cells) to control parasite growth. In addition, immune response to whole sporozoites or to specific highly relevant antigens such as CSP might have revealed the role of single antigens in the overall immune response. Overlapping peptides might have been used to investigate specific CD4+ and CD8+ T cell response. For the future vaccine development, it also will be indispensable to extend the follow-up period to investigate the durability of protection.

In summary, study evidence shown here in addition to the findings by Murphy et al.[35] highlights that it is safe to compress the PfSPZ–CVac regimen to ten days maintaining yet a high VE, which might be even further improved by increasing the number of PfSPZ number per dose.

METHODS

TÜCHMI-002 Stage-B study design

This single-center, randomized, placebo-controlled, double-blinded phase-I/-II study was conducted from April 2015 to December 2015 at the Institute of Tropical Medicine in Tübingen, Germany. The PfSPZ formulation team was unblinded and thus was not involved in clinical or diagnostic activities.

Approval was obtained from the Ethics Committee of the Eberhard Karls University and University Clinics (EudraCT number 2013-003900-38, National Clinical Trial number (NCT): NCT02115516). The study was performed in accordance with Good Clinical Practice/International Conference on Harmonization guidelines. The sample size was calculated using the function nBinomial of the R package gsDesign[40] which estimates the sample size required to detect a difference between two rates. According to study protocol, treatment allocation was random and blinded with a ratio of 2:1 (PfSPZ: placebo). A dedicated member of the formulation team, who was not involved in volunteer management or diagnostic activities, was responsible for keeping the randomization envelopes and dosing schedule, while a third party outside the study team and sponsor, generated and distributed the randomization list.

The primary efficacy endpoint was assigned to the proportion of volunteers parasitemic within 21 days after CHMI, while the secondary efficacy endpoint considered the time to detect parasitemia (prepatent period). The primary safety endpoint was set as the occurrence of related Grade-3 adverse events (AEs) from the first chemoprophylactic dose uptake (I-2) and PfSPZ challenge administration (I) until the end of the study. The secondary safety point focused on the appearance of any related AE from the time of the first administration of an immunizing regimen (PfSPZ–CVac) until the end of the study.

We recruited healthy, nonpregnant, malaria-naive volunteers aged between 18 and 45 years using the university email list.

Since the study was performed ambulatory, special safety precautions were applied to minimize risks of Pf malaria. To check the volunteers' understanding of the trial, after providing informed consent, participants had to correctly fill out a questionnaire of ten questions about risks and obligations. If they failed their first attempt, volunteers had the opportunity to discuss the study and retry once. A second failed test led to study exclusion. Moreover, volunteers had to provide full contact details and name two contact persons, of which at least one had to live nearby and know about the participants' whereabouts during the trial.

Out of the 46 volunteers screened, 21 were not eligible and 25 were enrolled and randomized to receive either 5.12×10^4 PfSPZ Challenge of the NF54 strain or placebo (Supplementary Fig. 1).

Early conceptual design counted with three study groups, including two placebo controls each and randomization, was as follows: 5:2 participants for Group B1 (5.12×10^4 PfSPZ: placebo), 5:2 participants for Group B2 (5.12×10^4 PfSPZ NF54: placebo), and 9:2 participants for Group B3 (5.12×10^4 PfSPZ NF54: placebo).

In Group B1, participants received 51,200 PfSPZ NF54 two weeks apart, using a chemoprophylactic regimen with CQ (10 mg/kg CQ base-loading dose, followed by weekly dosing with 5 mg/kg CQ base) for 6 doses.

Volunteers of Group B2 received the same PfSPZ dose two weeks apart and CQ chemoprophylaxis, but 2 g of extended–released azithromycin (ER-AZ) upon the first PfSPZ Challenge inoculation was also administered.

Volunteers of Group B3 received the most condensed immunization regimen with simultaneous administration of CQ and PfSPZ on days 0, 5, and 10 followed by an additional CQ dose on day 15.

Ten weeks after the last immunization, a challenge via CHMI was performed in all groups and individuals. CHMI was done by DVI of 3200 PfSPZ of the NF54 strain. Active follow-up of the participants was conducted 140 days after CHMI.

TÜCHMI-002 Stage-B study-design modifications

Twenty-two healthy malaria-naive young adult volunteers out of 46 assessed for eligibility were chosen following a randomized double-blinded placebo-controlled immunization trial to explore and compare immunogenicity. In brief, volunteers were randomly distributed into two exploratory groups. The first one was the result of fusing previous B1 with B2 groups after azithromycin failure confirmed by qPCR data after the first PfSPZ DVI (I). Therefore, subjects receiving thereafter remaining two immunizations (II and III) fourteen days apart, remained as the 28-day regimen group ($n = 14$). There, 9/12 participants received PfSPZ Challenge under chloroquine (PfSPZ–CVac), while 3/12 were administered with placebo (NaCl 0.9%). The original B3 group remained as 10-day regimen group ($n = 11$, 9/11 receiving PfSPZ–CVac and 2/11 receiving placebo).

Safety assessment

Safety was assessed through capture of adverse events (AE) (Supplementary Tables 2 and 3). The allocation ratio of 2:1 between immunized and placebo-treated volunteers was used to maximize the amount of safety/tolerability data related to PfSPZ Challenge with chemoprophylaxis. AE (and as part of these adverse reactions [AR]) was graded according to a predefined scale, whereas a study physician did the grading of unexpected AEs. Clinical AEs were elicited by spontaneous reporting and a defined set of open questions to improve discriminative capacity. Laboratory abnormalities were reported as AE when outside the reference range. The clinical team judged small deviations from the reference range as "non clinically significant". Nevertheless, from day three after the "first immunization" (I-3) on, all laboratory abnormalities were regarded as AE. Laboratory-safety analyses were done before inoculation of the vaccine or administration of the loading dose (I-3, II-3, and III-3), one day before CHMI (C-1), on the day of the first parasitemia after CHMI for those who get positive, twenty-eight days after challenge (C28), and at any time the clinical team decided that a laboratory analysis was required. Due to the condensed immunization schedule of Group B3, the second and third laboratory-safety analyses were done on the day of PfSPZ Challenge inoculation.

PfSPZ Challenge product

Cryopreserved NF54 PfSPZ was produced by Sanaria Inc. (Rockville, US). The parasites were isolated from infected mosquitoes bred under sterile conditions. Sporozoites were stored and transported in liquid nitrogen vapor phase at −140 to −196 °C. Formulation and reconstitution were made in Tübingen on the day of infection. Volunteers were inoculated within 20 min after thawing of PfSPZ.

Parasitemia monitoring

qPCR and thick blood smears (TBS) were performed daily from 6 to 21 days after DVI according to the methods published elsewhere[10]. TBS were reviewed once daily by at least two delegated and trained members of the study team. Positivity was accepted in the case of concordant results. Antimalarial treatment (atovaquone/proguanil or artemether lumefantrin) was initiated in the case of TBS positivity.

Sample collection

From all volunteers, heparinized whole blood was collected three days before the first immunization (I-3), before the second and the third immunizations (II and III), 5 days after the third immunization (III5), and one day before challenge (C-1). Sera were collected at days I-3, II, III, III14, and C-1 from all participants.

Statistical analysis

Efficacy analyses were performed on the per-protocol (PP) population. Primary efficacy was calculated using the unconditional exact Boschloo test, vaccine efficacy was expressed as (1 minus relative risk) x100%. Survival analysis was performed by using the log-rank test and plotted with inverse Kaplan–Meier curves. Malaria positivity was accepted when qPCR detected ≥100 parasites per milliliter of blood. A p-value < 0.05 deemed to indicate statistical significance. Statistical analyses were performed under R version 3.1.2.1 with exact2x2 and rms packages. All statistical tests were performed two-sided.

Antigen-reactive T cell enrichment (ARTE)

Peripheral blood mononuclear cells (PBMCs) were obtained by ficoll-paque plus density gradient centrifugation (GE Healthcare Life Science, ref# 17-1440) from the heparinized whole blood. All assays were performed using fresh PBMC samples.

In brief, PBMCs from each participant were placed in RPMI 1640 medium (Sigma-Aldrich), supplemented with 5% (v/v) AB serum (Lonza) and 2 mM L-glutamine (PAA Laboratories) for 18 h at 37 °C at 5% CO2. Hence, in order to enrich the Ag-specific T cells, plate wells containing 1×10^7 PBMCs were stimulated for 5 h with the following stimulants: (a) negative control; 500 µl of thawed uninfected red blood cells (RBC) from a culture collection aliquoted and stored at −80 °C, (b) 500 µl of a thawed aliquot containing infected red blood cells (iRBCs, Pf3D7 laboratory strain; 90% schizonts by microscopy), and (c) 5 µg/ml of Staphylococcus endotoxin B (SEB, Sigma-Aldrich; ref# S4881) as a positive control. All samples reached a final volume of 1500 µl/well.

In addition to the stimulants cited above, all samples were incubated in the presence of 1 µg/ml anti-CD40 (#Cat 130-094-133) and 1 µg/ml anti-CD28 (#Cat 130-093-375) functional-grade pure antibody (Ab) (both Miltenyi Biotec).

Brefeldin A (Sigma, #Cat B7151) was employed at 1 µg/ml for 2 h to stop cytokine release. As a negative control, nonautologous uninfected RBCs (uRBCs, type O+) obtained from the blood- donation center (Zentrum für Klinische Transfusionmedizin Tübingen gemeinnützige GmbH) were used for each participant.

After incubation, a small fraction of 30 µl from the original sample (ORI) was taken to assess the original phenotyping before magnetic separation by direct staining with 65 µl of the original fraction staining mix. The leftover sample was centrifuged at $300 \times g$ for 5 min. The pelleted cells were labeled with 10 µl of anti-CD40L-Biotin (Miltenyi Biotec, #Cat 130-092-658) for 10 min at 4 °C. Samples were subsequently labeled using the Anti-Biotin MicroBeads (#Cat 130-090-485) and washed two times with PEB buffer (1xPBS, 2 mM EDTA, and 0.5% BSA) before being loaded into the magnetic columns to perform the anti-biotin magnetic sorting separation (MACS) (Miltenyi Biotec, #Cat 130-042-201).

Once the columns had retained the coated cells, 60 µl of the staining mix for the surface staining was added to each column. Following, 1xPBS (Gibco, Life Technologies) was used two times before eluting the sample with 500 µl of PEB. The eluted cells were collected in 1.5-ml tubes (Eppendorf) to be fixed with 200 µl of Fixable Live/Dead (Inside stain kit, Miltenyi Biotec, 1:100).

A second round of magnetic separation was performed over the fixed suspension using a new set of columns. Previously, a rising step with 500 µl of PEB was done to prepare the new columns.

Once the fixed positive fraction was retained again, the columns were washed out with 500 µl of PEB before adding 200 µl of Inside Perm buffer (Inside stain kit, Miltenyi Biotec, #Cat 130-090-477) into the column. Then, 60 µl of the intracellular staining cocktail was added to every column. After 15 min of incubation at room temperature, the columns were washed out with 200 µl of Inside Perm buffer. Eluted cells with 1000 µl of PEB buffer were collected into 1.5 ml of Eppendorf tubes to be centrifuged at 300 g for 5 min. The pellet was resuspended in 200 µl of PEB buffer to flow

cytometry analysis. If not mentioned otherwise, the monoclonal antibodies were purchased from Miltenyi Biotech.

Original (ORI) staining. About 5 µl of the following monoclonal antibodies plus 35 µl of PEB were used per sample. Monoclonal antibodies: anti-CD8-PerCP (#Cat 130-008-057, 1:13), anti-CD14-PerCP (#Cat 130-098-072, 1:13), anti-CD20-PerCP (#Cat 130-098-097, 1:13), anti-CD4-VioBlue (#Cat 130-099-683, 1:13), anti-CD45RO-PE-Vio 770 (#Cat 130-099-692, 1:13), and anti-CCR7-FITC (UCHL-1, Becton Dickinson Biosciences, 1:13).

Positive-selection (PS) staining. About 6 µl of the following monoclonal antibodies plus 20 µl of 1xPBS were used per sample to stain the surface of the CD40L+−T cells: anti-CD8-VioGreen (#Cat 130-096-902, 1:10), anti-CD14-VioGreen (#Cat 130-096-875, 1:10), anti-CD20-VioGreen (#Cat 130-096-094, 1:10), anti-CD4-APC-Vio770 (#Cat 130-100-457, 1:10), anti-CD45RO-FITC (#Cat 130-095-462, 1:10), and anti-CCR7-PE (#Cat 130-099-361, 1:10). Permeabilized samples were stained with 6 µl of anti-CD40L-VioBlue (#Cat 130-096-217, 1:10), in combination with markers for the following cytokines: anti-TNF-α-PE-Vio 770 (6 µl) (#Cat 130-096-755, 1:10), and anti-IFN-γ-PerCP 5.5 (0.6 µl) (Biolegend, #Cat 502526, 1:100)

T cell monitoring data analysis

After PBMC isolation (step 0), 10^7 cells were placed in three separate wells. Each well was stimulated with the uninfected red blood cells (uRBC), the Pf-infected red blood cells (iRBCs, 90% Schizont), or the Staphylococcal enterotoxin B (SEB) at each timepoint. In step 1, 30 µl of each stimulated sample (original sample, ORI) is used to calculate the number of CD4+ T cells stimulated with uRBCs, iRBCs, or SEB (Supplementary Fig. 5). The remaining sample of each well is magnetically isolated to enrich the CD40L + population stimulated with uRBC, iRBC, or SEB (step 2). The resulting positive samples (PS) are analyzed by flow cytometry for the intracellular staining of TNF-α and IFN-γ. Moreover, each subpopulation of mono- or polyfunctional T cells was classified into effector memory T cells (TEM), central memory T cells (TCM), and naive T cells (TN) using the antibodies anti-CD45RO and anti-CCR7 (Supplementary Figs. 3 and 4).

In step 3, the net percentage of iRBC-specific CD40L+CD4+ T cells is calculated according to the formula (Equation 1, Supplementary Fig. 6).

Equation 1: Calculation of net percentage of iRBC-specific CD40L+CD4+ T cells using the ARTE

$$\text{net fraction iRBC specific TEM} = \frac{\text{iRBC stimulated TEM events} - \text{uRBC stimulated TEM events}}{\text{fraction of iRBC stimulated Th cells} \times 10^7 \text{ Total stimulated PBMCs}} \times 100\%$$

Cells were measured using the BD FACS Canto II. FlowJo V10 was used to analyze flow cytometry data. Both statistical analysis and figures were generated using GraphPad Prism 8.1.2 (GraphPad Software, San Diego, CA) and FACSDiva software (BD Biosciences).

ELISA

IgM and IgG antibodies directed against recombinant PfCSP were measured via ELISA, as described previously[41] although including some modifications.

High-binding 96-well plates (Corning Costar 96-Well EIA/RIA Plates) were coated overnight at 4 °C with 20 ng/well of PfCSP diluted in 100 µl/well of 0.1 M sodium bicarbonate buffer (pH 9.6). Plates were washed three times using 0.1% Tween 20 in 1 X PBS buffer and blocked with 200 µl/well of blocking buffer containing 5% bovine serum albumin, 0.1% Tween 20, and 0.5 mM EDTA in 1 X PBS buffer. Following 3 h of incubation at room temperature on an orbital shaker, the plates were washed three times. Samples were added as unique in twofold serial dilutions of plasma in 100 µl/well of blocking buffer covering a dilution range from 1:400 down to 1:51,200.

On every plate, positive- and negative-control sera were included and equally diluted. Plasma of a naive European donor served as negative control. Pooled plasma derived from vaccinees of a different PfSPZ immunization study (MAVACHE day III14, verification phase, NCT02704533) was used as positive control.

Plates were incubated for 1 h at room temperature on the shaker before being washed three times. Secondary antibodies (Peroxidase-conjugated AffiniPure Goat anti-human IgG Fc fragment, Jackson ImmunoResearch) were added in a concentration of 0.088 µg/ml in 100 µl/well of blocking buffer and plates were incubated for 1 h at room temperature on the shaker. Following three washes, 100 µl/well of TMB peroxidase substrate was added and plates were incubated for approximately 10 min in the dark. The reaction was stopped by adding 50 µl/well of 1 M hydrochloric acid. Optical densities (OD) were determined in the CLARIOstar microplate reader (BMG LABTECH) at 450 nm and 620 nm.

ELISA data analysis

Data analysis was done in R version 3.6.2 using the packages "drc" (Ritz, Baty et al., 2015), "DescTools" (Signorell et al., 2018), "tidyr" (Wickham, Lionel, 2018), and "ggplot2" (Wickham, 2016). Signals were corrected for their backgrounds by subtracting the ODs measured at 620 nm from the ODs measured at 450 nm. For every control and sample, a four-parameter logistic curve was fitted using the common logarithm of the dilution factor (400–5.12×10^4) as x- and the measured ODs as y-values. The area under the curve (AUC) was calculated and related to the AUC of positive and negative control on the corresponding plate that were set to 1.0 or 0.0, respectively (Equation 2).

Equation 2: Calculation of relative relative area under the curve (AUC) for measurement of ELISA

$$AUC_{\text{relative (rAUC)}} = \frac{AUC_{\text{test sample}} - AUC_{\text{negative control}}}{AUC_{\text{positive control}} - AUC_{\text{negative control}}}$$

Microarray assay

Protein microarrays to assess antibody reactivity against Pf antigens have been performed according to optimized procedures[42–44]. Microarrays were produced as described previously at the University of California Irvine, Irvine, California, USA[45]. In total, 251 Pf proteins were expressed using an *Escherichia coli* lysate in vitro expression system and spotted on a 16-pad ONCYTE AVID slide, representing 212 important Pf antigens. All antigens spotted on the array have been published before[18]. Secondary antibodies (goat anti-human IgG Qdot®800) were obtained from Grace Bio-Labs Inc. (Bend, OR).

Plasma was withdrawn from all study participants one day before challenge (C-1) by phlebotomy and stored at −80 °C. Plasma samples were diluted 1:100 in 0.05X Super G Blocking Buffer (Grace Bio-Labs Inc.) containing 10% *E. coli* lysate (GenScript, Piscataway, NJ) and incubated for 30 min on a shaker at room temperature (RT). Meanwhile, microarray slides were rehydrated using 0.05X Super G Blocking buffer at RT. Subsequently, rehydration buffer was removed, and samples added onto the slides. Samples were incubated for 2 h at RT on a shaker (180 rpm). Afterward, diluted plasma samples were removed, and microarrays washed using 1X TBST buffer (Grace Bio-Labs, Inc.). Secondary antibodies were applied at a dilution of 1:250 and incubated for 2 h. After a final washing step, slides were dried by centrifugation at 500 g for 10 min. Slide images were taken in a ArrayCAM® Imaging System using the ArrayCAM 400-S Microarray Imager Software (Grace Bio-Labs Inc.).

Microarray data analysis

Microarray data were analyzed using the R statistical software package version 3.6.2. All images were manually checked for any noise signal. Image quality was very high, but rare blurry spots were removed from further analysis. Spot signals were corrected for local background reactivity by applying a normal–exponential convolution model[46] using a saddle-point approximation for initial parameter estimation[47] (available in the limma package[48] v3.28.14). Data were then log2-transformed to approach a normal distribution of spot signals. Interarray normalization reducing the effect of sample-specific background reactivity to *E. coli* antigens contained in the spots' matrix was performed by subtracting the median signal intensity of mock expression spots on a particular array from the actual spot signals measured on the same array. Differential recognition of antibodies in the different study outcome-groups was analyzed by Student's t-test, and the respective *p*-value and fold-change differences of antibody-level means were given. Heatmaps, boxplots, and volcano plots were generated using the gplots, ggplot, and PAA packages, respectively.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

All data supporting the findings of this study are available upon reasonable request from the corresponding author.

CODE AVAILABILITY

The code used for data analysis is available upon reasonable request from the corresponding author and Rolf Fendel (rolf.fendel@uni-tuebingen.de). For statistical analyses, R statistical software package version 3.6.2 and GraphPad Prism 8.1.2 (GraphPad Software, San Diego, CA) was used.

Received: 6 October 2021; Accepted: 18 March 2022;
Published online: 31 May 2022

REFERENCES

1. World malaria report 2021. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2021 (2021).
2. World Health Organization & Global Malaria Programme. *Global technical strategy for malaria, 2016–2030*. (2015).
3. Duffy, P. E. & Patrick Gorres, J. Malaria vaccines since 2000: progress, priorities, products. *npj Vaccines* **5**, 1–9 (2020).
4. Clyde, D. F., Most, H., McCarthy, V. C. & Vanderberg, J. P. Immunization of man against sporozite-induced falciparum malaria. *Am. J. Med. Sci.* **266**, 169–177 (1973).
5. Rieckmann, K. H., Carson, P. E., Beaudoin, R. L., Cassells, J. S. & Sell, K. W. Letter: sporozoite induced immunity in man against an Ethiopian strain of Plasmodium falciparum. *Trans. R. Soc. Trop. Med. Hyg.* **68**, 258–259 (1974).
6. Maxmen, A. Scientists hail historic malaria vaccine approval — but point to challenges ahead. *Nature* https://doi.org/10.1038/d41586-021-02755-5 (2021).
7. Hogan, A. B., Winskill, P. & Ghani, A. C. Estimated impact of RTS,S/AS01 malaria vaccine allocation strategies in sub-Saharan Africa: A modelling study. *PLoS Med.* **17**, e1003377 (2020).
8. Richie, T. L. et al. Progress with Plasmodium falciparum sporozoite (PfSPZ)-based malaria vaccines. *Vaccine* **33**, 7452–7461 (2015).
9. Lynen, G. et al. East Coast fever immunisation field trial in crossbred dairy cattle in Hanang and Handeni districts in northern Tanzania. *Trop. Anim. Health Prod.* **44**, 567–572 (2012).
10. Mordmüller, B. et al. Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. *Nature* **542**, 445–449 (2017).
11. Bacher, P. et al. Antigen-reactive T cell enrichment for direct, high-resolution analysis of the human naive and memory Th cell repertoire. *J. Immunol.* **190**, 3967–3976 (2013).
12. Scheffold, A., Schwarz, C. & Bacher, P. Fungus-specific CD4 T cells as specific sensors for identification of pulmonary fungal infections. *Mycopathologia* **183**, 213–226 (2018).
13. Bacher, P. et al. Human anti-fungal Th17 immunity and pathology rely on cross-reactivity against Candida albicans. *Cell* **176**, 1340–1355.e15 (2019).
14. Bacher, P. et al. Low-avidity CD4+ T cell responses to SARS-CoV-2 in unexposed individuals and humans with severe COVID-19. *Immunity* **53**, 1258–1271.e5 (2020).
15. Bacher, P. et al. Regulatory T cell specificity directs tolerance versus allergy against aeroantigens in humans. *Cell* **167**, 1067–1078.e16 (2016).
16. Friesen, J. et al. Natural immunization against malaria: causal prophylaxis with antibiotics. *Sci. Transl. Med.* **2**, 40ra49–40ra49 (2010).
17. Andersen, S. L. et al. Efficacy of azithromycin as a causal prophylactic agent against murine malaria. *Antimicrob. Agents Chemother.* **38**, 1862–1863 (1994).
18. Sulyok, Z. et al. Heterologous protection against malaria by a simple chemoattenuated PfSPZ vaccine regimen in a randomized trial. *Nat. Commun.* **12**, 2518 (2021).
19. Clyde, D. F., McCarthy, V. C., Miller, R. M. & Hornick, R. B. Specificity of protection of man immunized against sporozoite-induced falciparum malaria. *Am. J. Med. Sci.* **266**, 398–403 (1973).
20. Itsara, L. S. et al. The development of whole sporozoite vaccines for Plasmodium falciparum malaria. *Front. Immunol.* **9**, 2748 (2018).
21. Goh, Y. S., McGuire, D. & Rénia, L. Vaccination with sporozoites: models and correlates of protection. *Front Immunol.* **10**, 1227 (2019).
22. Hoffman, S. L. et al. Development of a metabolically active, non-replicating sporozoite vaccine to prevent Plasmodium falciparum malaria. *Hum. Vaccin.* **6**, 97–106 (2010).
23. Epstein, J. E. et al. Protection against Plasmodium falciparum malaria by PfSPZ Vaccine. *JCI Insight* **2**, e89154 (2017).
24. Lyke, K. E. et al. Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection. *Proc. Natl Acad. Sci. USA* **114**, 2711–2716 (2017).
25. Olotu, A. et al. Advancing global health through development and clinical trials partnerships: a randomized, placebo-controlled, double-blind assessment of safety, tolerability, and immunogenicity of PfSPZ vaccine for malaria in healthy Equatoguinean men. *Am. J. Trop. Med. Hyg.* **98**, 308–318 (2018).
26. Jongo, S. A. et al. Safety and differential antibody and T-cell responses to the Plasmodium falciparum sporozoite malaria vaccine, PfSPZ vaccine, by age in Tanzanian adults, adolescents, children, and infants. *Am. J. Trop. Med. Hyg.* **100**, 1433–1444 (2019).
27. Jongo, S. A. et al. Increase of dose associated with decrease in protection against controlled human malaria infection by PfSPZ vaccine in Tanzanian adults. *Clin. Infect. Dis.* **71**, 2849–2857 (2020).
28. Healy, S. A. et al. Chemoprophylaxis vaccination: phase I study to explore stage-specific immunity to Plasmodium falciparum in US adults. *Clin. Infect. Dis.* **71**, 1481–1490 (2020).
29. Jongo, S. A. et al. Immunogenicity and protective efficacy of radiation-attenuated and chemo-attenuated PfSPZ vaccines in equatoguinean adults. *Am. J. Trop. Med. Hyg.* **104**, 283–293 (2021).
30. Roestenberg, M. et al. A double-blind, placebo-controlled phase 1/2a trial of the genetically attenuated malaria vaccine PfSPZ-GA1. *Sci. Transl. Med.* **12**, eaaz5629 (2020).
31. Freund, J., Thomson, K. J., Sommer, H. E., Walter, A. W. & Schenkein, E. L. Immunization of rhesus monkeys against malarial infection (p. knowlesi) with killed parasites and adjuvants. *Science* **102**, 202–204 (1945).
32. Belnoue, E. et al. Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. *J. Immunol.* **172**, 2487–2495 (2004).
33. Beaudoin, R. L., Strome, C. P., Mitchell, F. & Tubergen, T. A. Plasmodium berghei: immunization of mice against the ANKA strain using the unaltered sporozoite as an antigen. *Exp. Parasitol.* **42**, 1–5 (1977).
34. Roestenberg, M. et al. Protection against a malaria challenge by sporozoite inoculation. *N. Engl. J. Med.* **361**, 468–477 (2009).
35. Murphy, S. C. et al. PfSPZ-CVac efficacy against malaria increases from 0% to 75% when administered in the absence of erythrocyte stage parasitemia: a randomized, placebo-controlled trial with controlled human malaria infection. *PLoS Pathog.* **17**, e1009594 (2021).
36. Salako, L. A., Aderounmu, A. F. & Walker, O. Influence of route of administration on the pharmacokinetics of chloroquine and desethylchloroquine. *Bull. World Health Organ.* **65**, 47–50 (1987).
37. Looareesuwan, S. et al. Cardiovascular toxicity and distribution kinetics of intravenous chloroquine. *Br. J. Clin. Pharm.* **22**, 31–36 (1986).
38. Mwakingwe-Omari, A. et al. Two chemoattenuated PfSPZ malaria vaccines induce sterile hepatic immunity. *Nature* https://doi.org/10.1038/s41586-021-03684-z (2021).
39. Eacret, J. S., Gonzales, D. M., Franks, R. G. & Burns, J. M. Immunization with merozoite surface protein 2 fused to a Plasmodium-specific carrier protein elicits strain-specific and strain-transcending, opsonizing antibody. *Sci. Rep.* **9**, 9022 (2019).
40. Hermans, L. et al. At equipotent doses, isradipine is better tolerated than amlodipine in patients with mild-to-moderate hypertension: a double-blind, randomized, parallel-group study. *Br. J. Clin. Pharm* **38**, 335–340 (1994).
41. Maskus, D. J. et al. Characterization of a novel inhibitory human monoclonal antibody directed against Plasmodium falciparum apical membrane antigen 1. *Sci. Rep.* **6**, 39462 (2016).
42. Taghavian, O. et al. Antibody profiling by proteome microarray with multiplex isotype detection reveals overlap between human and Aotus nancymaae controlled malaria infections. *Proteomics*, https://doi.org/10.1002/pmic.201700277 (2018).
43. Kobayashi, T. et al. Distinct antibody signatures associated with different malaria transmission intensities in Zambia and Zimbabwe. *mSphere* **4**, e00061-19 (2019).
44. Obiero, J. M. et al. Antibody biomarkers associated with sterile protection induced by controlled human malaria infection under chloroquine prophylaxis. *mSphere*, https://doi.org/10.1002/pmic.201700277 (2019).
45. Doolan, D. L. et al. Profiling humoral immune responses to P. falciparum infection with protein microarrays. *Proteomics* **8**, 4680–4694 (2008).
46. Ritchie, M. E. et al. A comparison of background correction methods for two-colour microarrays. *Bioinformatics* **23**, 2700–2707 (2007).
47. Silver, J. D., Ritchie, M. E. & Smyth, G. K. Microarray background correction: maximum likelihood estimation for the normal-exponential convolution. *Biostatistics* **10**, 352–363 (2009).
48. Ritchie, M. E. et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* **43**, e47 (2015).

ACKNOWLEDGEMENTS

We want to thank all study participants and the study team at the Institute of Tropical Medicine. The clinical trial was funded by the German Federal Ministry of Education and Research (BMBF) through the German Center for Infection Research (DZIF). Manufacture of investigational product by Sanaria was supported in part by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under SBIR award numbers 5R44AI058375 and 5R44AI055229.

AUTHOR CONTRIBUTIONS

J.I. and R.F. contributed equally to the paper. Conceived and designed the experiments: J.I. and R.F. Conceived and designed the clinical trial: B.M., P.G.K., and S.L.H. Performed the experiments: J.I., S.Br., P.G.B., F.R.L., and R.F. Analyzed the data: J.I., F.R.L., R.F., M.S., Z.S., and B.M. Contributed reagents/materials: B.M., P.G.K., and S.L.H. Drafted the first paper: J.I., R.F., and B.M. Revision of the paper for important intellectual content: all authors. Read and approved the final version of the paper: all authors.

FUNDING

Open Access funding enabled and organized by Projekt DEAL.

COMPETING INTERESTS

BM and PGK received funding from the DZIF. SLH, associated with Sanaria Inc., has a potential conflict of interest. All other authors declare no conflict of interest.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41541-022-00473-1.

Correspondence and requests for materials should be addressed to Benjamin Mordmüller.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022

1.1 Chapter 2: Cellular and antibody response in GMZ2-vaccinated African volunteers in a Controlled Human Malaria Infection trial.

Publication Nr.2

Cellular and antibody response in GMZ2-vaccinated Gabonese volunteers in a controlled human malaria infection trial

Nouatin, O., Ibáñez, J., Fendel, R. et al.

Malar J 21, 191 (2022)

https://doi.org/10.1186/s12936-022-04169-8

Nouatin *et al. Malaria Journal* (2022) 21:191
https://doi.org/10.1186/s12936-022-04169-8

Malaria Journal

RESEARCH **Open Access**

Cellular and antibody response in GMZ2-vaccinated Gabonese volunteers in a controlled human malaria infection trial

Odilon Nouatin[1,2,3,4*†], Javier Ibáñez[2,3†], Rolf Fendel[1,2,3*†], Ulysse A. Ngoa[1], Freia-Raphaella Lorenz[2], Jean-Claude Dejon-Agobé[1,5], Jean Ronald Edoa[1,2], Judith Flügge[2,3], Sina Brückner[2], Meral Esen[1,2,3,16], Michael Theisen[6,7,8], Stephen L. Hoffman[9], Kabirou Moutairou[4], Adrian J. F. Luty[10,11], Bertrand Lell[1,12], Peter G. Kremsner[1,2,3], Ayola A. Adegnika[1,2,3,13,14†] and Benjamin Mordmüller[1,2,3,15†]

Abstract

Background: Antibody and cellular memory responses following vaccination are important measures of immunogenicity. These immune markers were quantified in the framework of a vaccine trial investigating the malaria vaccine candidate GMZ2.

Methods: Fifty Gabonese adults were vaccinated with two formulations (aluminum Alhydrogel and CAF01) of GMZ2 or a control vaccine (Verorab). Vaccine efficacy was assessed using controlled human malaria infection (CHMI) by direct venous inoculation of 3200 live *Plasmodium falciparum* sporozoites (PfSPZ Challenge). GMZ2-stimulated T and specific B-cell responses were estimated by flow cytometry before and after vaccination. Additionally, the antibody response against 212 *P. falciparum* antigens was estimated before CHMI by protein microarray.

Results: Frequencies of pro- and anti-inflammatory $CD4^+$ T cells stimulated with the vaccine antigen GMZ2 as well as B cell profiles did not change after vaccination. IL-10-producing $CD4^+$ T cells and $CD20^+$ IgG^+ B cells were increased post-vaccination regardless of the intervention, thus could not be specifically attributed to any malaria vaccine regimen. In contrast, GMZ2-specific antibody response increased after the vaccination, but was not correlated to protection. Antibody responses to several *P. falciparum* blood and liver stage antigens (MSP1, MSP4, MSP8, PfEMP1, STARP) as well as the breadth of the malaria-specific antibody response were significantly higher in protected study participants.

Conclusions: In lifelong malaria exposed adults, the main marker of protection against CHMI is a broad antibody pattern recognizing multiple stages of the plasmodial life cycle. Despite vaccination with GMZ2 using a novel formulation, expansion of the GMZ2-stimulated T cells or the GMZ2-specific B cell response was limited, and the vaccine response could not be identified as a marker of protection against malaria.

†Odilon Nouatin, Javier Ibáñez and Rolf Fendel contributed equally to the work and share first authorship

†Ayola A. Adegnika and Benjamin Mordmüller share the last authorship and contributed equally to this work

*Correspondence: paterneodilon@gmail.com; rolf.fendel@uni-tuebingen.de

[1] Centre de Recherches Médicales de Lambaréné, BP : 242, Lambaréné, Gabon
[2] Institute of Tropical Medicine, University Tübingen, Wilhelmstr. 27, 72074 Tübingen, Germany
Full list of author information is available at the end of the article

© The Author(s) 2022. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

Trial registration PACTR; PACTR201503001038304; Registered 17 February 2015; https://pactr.samrc.ac.za/TrialDisplay.aspx?TrialID=1038

Keywords: GMZ2, Cytokine, Memory B cells, P. falciparum, CHMI, Microarray

Background

Malaria remains one of the leading causes of maternal and infant mortality in the world [1]. The tools currently available for malaria control include vector control, chemoprophylaxis, prompt diagnosis and use of effective anti-malarial drugs [1]. In addition to existing tools, an effective malaria vaccine would be a game changer for elimination and eradication programmes [2]. Many malaria vaccine candidates have been tested, including GMZ2, a recombinant protein vaccine candidate that targets the asexual blood stages of *Plasmodium falciparum*. GMZ2 comprises a combination of Glutamate-Rich Protein (GLURP) and Merozoite Surface Protein 3 (MSP3) expressed in *Lactococcus lactis* [3]. It has been tested in several studies and has proven to be immunogenic in terms of vaccine-specific IgG production and specific memory B cell generation when using aluminum hydroxide as adjuvant [4–6]. A multicentre Phase II randomized, controlled trial in malaria endemic regions showed significant but low efficacy of the GMZ2-Alhydrogel formulation, ranging from 3.6 to 23% [7]. The latter result raised the question of whether the choice of Alhydrogel as adjuvant is optimal. Particularly, inducing pro-inflammatory T cell-mediated responses could be advantageous. The cationic adjuvant formulation (CAF01) is an adjuvant that has already been used in clinical trials to induce $CD8^+$ cytotoxic T lymphocytes against HIV-1 (human immunodeficiency virus-1) peptides [8], and to promote long-lived *Mycobacterium tuberculosis*-specific $CD4^+$ T-cell responses [9].

Cytokine producing $CD4^+$ T cells have been shown to play an important role in protection against *P. falciparum* infection following immunization with the malaria vaccine RTS, S [10–14]. Moreover, polyfunctional T cells have been associated with higher protective efficacy after vaccination [15–18]. Thus, CAF01 was chosen as a novel adjuvant partner for GMZ2. One of the goals of using CAF01 as an adjuvant for the GMZ2 vaccine candidate was also to enhance the memory response. Memory B cells (MBCs) produce antibodies of switched isotypes with higher affinity [19], and their development and maintenance is modulated by *P. falciparum* infection [20, 21].

In the current study, two regimens of GMZ2 (30 µg and 100 µg), adjuvanted with CAF01 and one regimen (100 µg) of GMZ2 adjuvanted with Alhydrogel were used to investigate in a randomized, controlled, double-blind, phase 1 clinical trial the safety, tolerability, immunogenicity and vaccine efficacy [22]. To estimate the protective efficacy, the study population was challenged using viable cryopreserved *P. falciparum* sporozoites in a controlled human malaria infection (CHMI) by direct venous inoculation (DVI). As reported previously, none of the vaccination regimens could improve protection against malaria infection, and the elicited humoral immune response was not predictive for protection. Nevertheless, surprisingly, the level of antibody levels against the antigen GMZ2 before the vaccination could predict protection against CHMI [22].

Here, the frequencies of cytokine-producing $CD4^+$ T cells, the circulating GMZ2-specific B cells following immunization, and the antibody response to a range of over 200 *P. falciparum* antigens were evaluated. The potential of these biomarkers to predict protection against infection or clinical symptoms after CHMI was assessed.

Methods

Study design and population

Assessment of intracellular cytokine producing $CD4^+$ T cells and B cell responses after vaccine antigen GMZ2 stimulation was nested within a Phase 1 trial aiming to assess the safety, immunogenicity, and efficacy of GMZ2 adjuvanted with CAF01 in fifty Gabonese adults with lifelong exposure to malaria. As part of the inclusion criteria, all participants were tested negative for hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency viruses (HIV) as well as for malaria parasites at baseline. Detailed information concerning the study design is given elsewhere [22]. Standardized CHMI using the parasite strain *P. falciparum* NF54 was conducted by direct venous inoculation of PfSPZ Challenge (Sanaria Inc.), as described previously [23–25]. Vaccine efficacy was defined as protection from clinical symptoms of malaria or protection from a parasitaemia above 1000 parasites per µl. Peripheral blood mononuclear cells (PBMC) were isolated from venous blood collected at inclusion (D0), and again 28 days after the third vaccination (D84) and were used for immunological assays. Plasma isolated from blood collected at C-1 (one day before the CHMI) was used to quantify the antibody titre against several *P. falciparum* antigens, and to measure the antibody breadth.

Blood collection, PBMC isolation and stimulation

The different steps of PBMC isolation after sample collection, and of PBMC stimulation are described in detail elsewhere [26]. Briefly, after counting, cells were suspended in the culture medium and stimulated for 18 h with either the vaccine antigen (GMZ2, 4 µg/ml, Henogen S.A. Belgium) or staphylococcal enterotoxin B (SEB, 1 µg/ml, Sigma-Aldrich) as positive control. Unstimulated cells as negative control were incubated in culture medium alone. The culture was done in the presence of anti-CD28/CD49d antibodies (BD Biosciences). Two hours after PBMC stimulation, 1 µg/ml of Golgi Plug (BD Biosciences) was added. Each plate used for stimulation included PBMC isolated both on D0 and on D84 from the same participant. The working conditions remained stable for all samples.

Intracellular cytokine staining (ICS)

Isolated PBMCs were stained first with Aqua live/dead (Life Technologies) and incubated at room temperature in the dark for 30 min. After washing, they were suspended in 50 µl of filtered Flow Cytometry Buffer FCB (1X PBS (Gibco), 0.5% BSA (Sigma-Aldrich) 2 mM EDTA (Thermo Fischer Scientific)) and 50 µl of Staining Buffer (FCB 2% Beriglobin (CSL Behring)) and stained with anti CD3-PercP Cy5.5 (eBioscience), anti CD4-FITC, (BD Biosciences) followed by an incubation at 4 °C in the dark for 30 min. Anti-IL-10-PE, anti-IL-13-BV711, anti-IL-2-BV785, anti-IFNγ-BV421, anti-TNFα-BV605 and anti-IL4-PE CF594 (all Biolegend) intracellular staining was done according to manufacturer's recommendations (BD Biosciences). Cell acquisition was performed using a Spectral Cell Analyzer cytometer SP6800 (Sony Biotechnology) 15-color cytometer, with 100,000 cells per tube as the total number of cells acquired. Polyfunctional $CD4^+$ T cells were defined as $CD4^+$ T cells expressing any combination of IFN-γ, IL-2 or TNF. IL-10, IL-4 or IL-13 producing CD4+ T cells are defined as CD3+CD4+IL-10+, CD3+CD4+IL-10−IL-4+ and CD3+CD4+IL-10−IL-13+, respectively.

GMZ2 labelling and circulating GMZ2-specific memory B cell staining

Molecular Probes' Alexa Fluor® 647 Protein Labeling Kit was used to label GMZ2 vaccine antigen with the Alexa Fluor 647 dye as described by the manufacturer. Briefly, the GMZ2 solution was added to the vial of reactive dye containing a magnetic stir bar. The solution was mixed to fully dissolve the dye, and the reaction mixture was stirred for 1 h at room temperature. Then, the provided purification resin was stirred thoroughly to ensure a homogeneous suspension, and the resin was pipetted into the column allowing excess buffer to drain away into a beaker. The reaction mixture was then loaded onto the column. The reaction was rinsed with elution buffer. The elution buffer was added slowly to elute the labelled protein. Two colored bands were observed representing the labelled protein and the unincorporated dye respectively. The labelled protein was collected into a provided collection tube. The reliability of the achieved labelling was tested by spectroscopy before storing at 4 °C for further use.

After thawing, PBMCs were rested overnight at 37 °C and 5% CO_2 in a cell incubator. Then, cells were stained with Aqua live/dead (Life Technologies) at room temperature in the dark for 30 min. Additionally, circulating GMZ2-specific memory B cells were stained using anti-CD20-BV570 (Biolegend), anti-CD27-BV421 (BD Biosciences), anti-IgG-PE (Biolegend), and the recombinant protein GMZ2 coupled to AF647 (AF647 from Life Technologies) for 30 min at 4 °C in the dark, washed twice and resuspended in 300 µl of flow cytometry buffer to be acquired by the Spectral cell analyzer SP6800. In total, 300,000 cells were acquired per sample and GMZ2-specific B cells were further characterized within the $CD20^+IgG^+CD27^-$ and $CD20^+IgG^+CD27^+$ populations.

Cell count estimation and assay optimization

Blood counts were obtained from Centre de Recherches Médicales de Lambaréné (CERMEL). Total lymphocyte counts were used to calculate the estimated cell counts per phenotype according to their frequencies following both T and B gating strategies (Additional file 1: Fig. S1a and S1b). For both T cell and B cell assays, all time points per volunteer were measured in a single experiment after several optimization tests, namely the calibration of the flow cytometer, the titration of the monoclonal antibodies, the titration of the stimulating antigen, determination of the optimal stimulation period and the assay was repeated with a positive control several times to ensure assay stability.

GMZ2-specific IgG concentration measurement

The anti-GMZ2 total IgG was measured by enzyme-linked immunosorbent assay (ELISA) on isolated plasma collected at D0 and at D84 as described by Esen et al. [5] with minor modifications. These modifications consisted of the dilution of the plasma sample in PBS, 3% non-fat milk, 0.1% Tween 20, and the use of peroxidase conjugated goat anti-human IgG (Invitrogen) at a 1:65,000 dilution [22]. As reference for the assay, European malaria-naive pooled sera was taken as negative control whereas the pooled sera from Gabonese adults was used as positive control.

Microarray assay

Protein microarray-based assessments of antibody reactivity against *P. falciparum* antigens were performed as described before with some modifications [27]. Microarrays were produced as described previously at the University of California Irvine, Irvine, CA [28]. In total, 251 *P. falciparum* proteins were expressed using an *Escherichia coli* lysate in vitro expression system and spotted on a 16-pad ONCYTE AVID slide, representing 212 *P. falciparum* antigens. The antigens spotted on the array are summarized in the Additional file 2: Table S2. Secondary antibodies (goat anti-human IgG QDot®800) were obtained from Grace Bio-Labs, Inc., (Bend, OR).

The plasma samples from the Gabonese donors were taken one day before challenge by phlebotomy and stored at − 80 °C. In addition, plasma samples from European donors were obtained from malaria-naïve study participants in CHMI trials performed in Tübingen, Germany. For use on the microarray, plasma samples were diluted 1:100 in 0.05X Super G Blocking Buffer (Grace Bio-Labs Inc.) containing 10% *E. coli* lysate (GenScript, Piscataway, NJ) and incubated for 30 min on a shaker at room temperature (RT). Meanwhile, microarray slides were rehydrated using 0.05X Super G Blocking buffer at RT. Subsequently, rehydration buffer was removed, and samples added on the slides. Samples were incubated for 2 h at RT on a shaker (180 rpm). Afterwards, diluted plasma samples were removed, and microarrays washed using 1X TBST buffer (Grace Bio-Labs, Inc.). Subsequently, secondary antibodies (anti-human IgG Q800, Grace Biolabs, #110,635), were applied at a dilution of 1:250 and incubated for 2 h. After a final washing step, slides were dried by centrifugation at 500*g* for 10 min. Slide images were taken using an ArrayCAM® Imaging System (Grace Bio-Labs Inc.) using the ArrayCAM 400-S Microarray Imager Software.

Statistical analysis

Microarray data analysis

Microarray data were exported from the Imaging software and further analysed using the R statistical software package version 3.6.2. All images were manually checked for any noise signal. Image quality was very high, but rare blurry spots were removed from further analysis. Background correction was performed according to the maximum likelihood estimation for the normal-exponential convolution model [29] using the saddle-point approximation (available in the limma package v3.28.14). Subsequently, data was normalized by log2-transformation. Finally, median normalization was performed to normalize the different assays for background activity of antibodies binding to *E. coli* lysate using mock expression spots. Analysis of plasma antibody levels in the groups with different study outcomes were analysed by Student's t-test, and the respective p value and fold-change differences of antigen-specific antibody level means were given. Antibody breadth was estimated by comparing the antibody levels of the vaccinated subjects to a cohort of malaria naïve volunteers. For each of the subjects, the number of antibody levels with higher than four-fold the reactivity of the mean of the respective reactivity of the malaria-naïve population was enumerated and defined as the individual antibody breadth.

In addition, the vaccine-specific antibody response at baseline was tested for correlation with the individual antigens spotted on the protein microarrays using Student's t-test. Heatmaps, box plots and volcano plots were generated using the gplots, ggplot and PAA packages, respectively.

Flow cytometry data analysis

Flow cytometer data was analysed using FlowJo Version 10, graphs and statistical analysis were done using GraphPad Prism Version 6, and R (R Core Team (2017), R Foundation for Statistical Computing, Vienna, Austria).

Proportion of activated CD4+ T cells were gated as CD3+ CD4+ cytokine+. Data are expressed as substraction of the gated unstimulated from the antigen-specifically stimulated sample, including subsequent normalization using the average of the positive control stimulation. Flow cytometry data showing erratic staining or samples with viability (Live/dead staining) below 70% were excluded from the analysis for both ICS and B-cell staining.

Results

Baseline characteristics of the study population

The timeline of the study design is depicted in Fig. 1. Baseline characteristics were similar between the groups (Table 1). From the 50 participants included in this study, sufficient PBMC samples for all analyses were available from 44 participants. Blood samples collected before the first immunization (D0), and one month after the last immunization (D84) were used for this study. Among these participants whose PBMC samples were available, six volunteers received a control vaccine (Verorab), 11 received 100 µg GMZ2-Alhydrogel, 7 received 30 µg GMZ2-CAF01 and 20 received 100 µg GMZ2-CAF01. Results of the clinical trial have been published before [22]. The vaccine efficacy (VE) for the treatment arms is

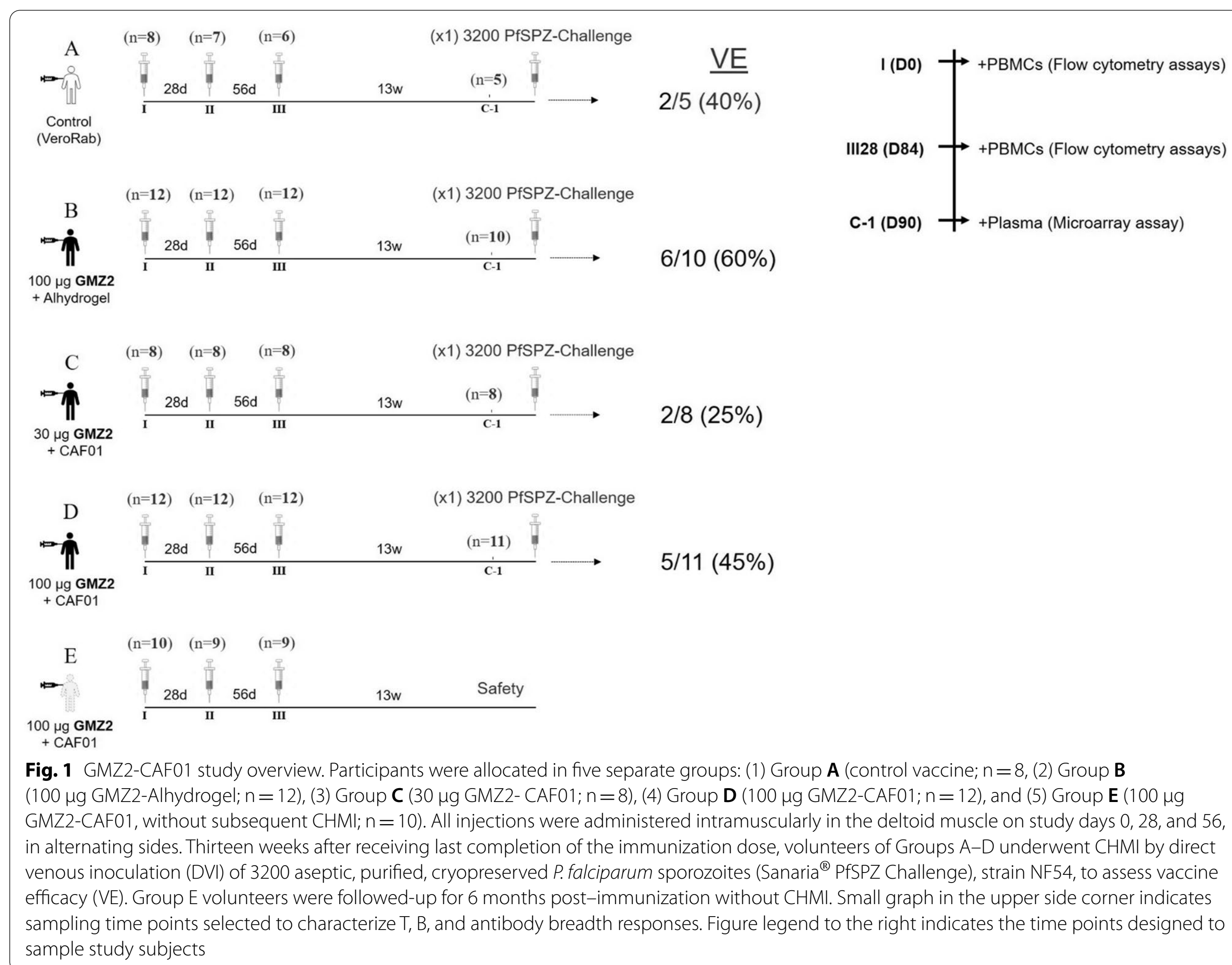


Fig. 1 GMZ2-CAF01 study overview. Participants were allocated in five separate groups: (1) Group **A** (control vaccine; n = 8, (2) Group **B** (100 µg GMZ2-Alhydrogel; n = 12), (3) Group **C** (30 µg GMZ2- CAF01; n = 8), (4) Group **D** (100 µg GMZ2-CAF01; n = 12), and (5) Group **E** (100 µg GMZ2-CAF01, without subsequent CHMI; n = 10). All injections were administered intramuscularly in the deltoid muscle on study days 0, 28, and 56, in alternating sides. Thirteen weeks after receiving last completion of the immunization dose, volunteers of Groups A–D underwent CHMI by direct venous inoculation (DVI) of 3200 aseptic, purified, cryopreserved *P. falciparum* sporozoites (Sanaria® PfSPZ Challenge), strain NF54, to assess vaccine efficacy (VE). Group E volunteers were followed-up for 6 months post–immunization without CHMI. Small graph in the upper side corner indicates sampling time points selected to characterize T, B, and antibody breadth responses. Figure legend to the right indicates the time points designed to sample study subjects

Table 1 Baseline characteristics of the study population

	Rabies	100 µg GMZ2 Alum	30 µg GMZ2 CAF01	100 µg GMZ2 CAF01	p value
Number	N = 6	N = 11	N = 07	N = 20	
Age (years) *	23 [22–33.75]	25 [23–26]	22 [20–25]	22 [19–33]	0.451
Body mass index (kg/m2) #	21.90 (2.74)	23.02 (2.82)	21.78 (1.43)	22.15 (2.04)	0.635
Hemoglobin (g/dl) #	13.73 (1.35)	14.63 (1.31)	13.51 (1.68)	13.84 (1.29)	0.309
White blood cells (cells/µl) *	5.70 [5.3–6.25]	4.90 [4.2–7]	5.30 [4.3–6.7]	5.10 [4.1–6.9]	0.726
Lymphocytes (cells/µl) *	2.17 [1.9–2.3]	2.32 [1.8–2.6]	2.59 [1.8–2.8]	1.74 [1.5–2.5]	0.396
Monocytes (cells/µl) *	0.54 [0.4–0.6]	0.44[0.3–0.6]	0.49 [0.3–0.5]	0.47 [0.3–0.6]	0.507
Neutrophils (cells/µl) *	2.49 [2–2.9]	2.32 [1.2–2.6]	1.82 [1.3–2.5]	1.84 [1.2–2.5]	0.420
Eosinophils (cells/µl) *	0.27 [0.16–0.86]	0.17 [0.11–0.94]	0.61 [0.12–0.91]	0.34 [0.24–0.94]	0.821
Basophils (cells/µl) *	0.03 [0.02–0.08]	0.06 [0.03–0.11]	0.05 [0.02–0.07]	0.05 [0.02–0.06]	

N number of subjects

* Median and [Interquartile range], Kruskal–Wallis test

Mean (Standard Deviation), ANOVA

Table 2 Cox proportional analysis pre-immunization

	Risk to be treated after CHMI					
	Cell frequency (Pre-immunization)			Estimated cell number (Pre-immunization)		
	HR	95%CI	p value	HR	95%CI	p value
$IFN_{\gamma}^{+}IL2^{+}TNF\alpha^{+}$ $CD4^{+}$ T	1.49	0.92–2.40	0.10	1.02	0.99–1.04	0.13
$IFN_{\gamma}^{+}IL2^{+}$ $CD4^{+}$ T	0.92	0.52–1.64	0.78	0.98	0.79–1.20	0.86
$IFN_{\gamma}^{+}TNF\alpha^{+}$ $CD4^{+}$ T	1.28	0.46–3.55	0.63	0.99	0.95–1.03	0.85
$IL2^{+}TNF\alpha^{+}$ $CD4^{+}$ T	1.15	0.58–2.27	0.68	1.00	0.99–1.01	0.56
IgG positive CD20 + B cell	0.85	0.56–1.10	0.16	1.00	0.99–1.00	0.59
GMZ2-specific B cells	1.01	0.94–1.1	0.64	1.00	0.99–1.00	0.07
CD27 negative GMZ2-specific B cells	1.01	0.91–1.12	0.74	1.00	0.99–1.00	0.21
CD27 positive GMZ2-specific B cells	1.39	0.98–1.96	0.06	1.01	0.99–1.02	0.07

Treatment after CHMI was administered to those who developed malaria or to those whose parasitaemia was more than 1000 parasites per µL.

p value was significant when less than 0.05

CI confidence interval, *HR* hazard ratio.

summarized in Fig. 1, none of the experimental vaccinations improved protection against the challenge in comparison to the rabies control vaccine significantly.

GMZ2 stimulated $CD4^{+}$ T cells response

Cytokine-producing $CD4^{+}$ T cells were gated on viability and surface markers as well as intracellular cytokine pattern (Additional file 1: Fig. S1a).

Cytokine (IFN-γ, TNF, IL-2, IL-10, IL-13, and IL-4) production of $CD4^{+}$ T cells was assessed by measuring the fraction of single and multiple cytokine-positive cells following in vitro stimulation with medium alone (mock stimulation), GMZ2 antigen, or with the positive control (SEB). Stimulation with positive control SEB reached stimulation levels of 2–4%, which has been reported similarly before [30, 31]. As expected, most participants circulating pro-inflammatory $CD4^{+}$ T cells reacted to the stimulation with the vaccine antigen GMZ2 at baseline (Fig. 2a), most likely due to their lifelong natural exposure to malaria parasites. All $CD4^{+}$ T pro-inflammatory cytokine combinations had similar values at D0 and D84, regardless of the intervention (i.e., GMZ2 or control vaccine). To increase statistical power during analyses, all groups of those who received the GMZ2 vaccine candidate were pooled. Nevertheless, no difference between the GMZ2 vaccinated groups and the control group on D84 was found.

The GMZ2 antigen stimulated IL-4^{+} and IL-13^{+} $CD4^{+}$ T cell frequencies were similar in the Rabies vaccinated as well as in GMZ2 vaccinated groups before and after vaccination, whereas the proportion of total IL-10 producing $CD4^{+}$ T cells increased significantly in the GMZ2 vaccinated group (45-fold, 95% CI = 20 – 71), as well as in the control group (20-fold, 95% CI 5–35) following vaccination (Fig. 2b). Besides, the proportion of IL-10^{+} $CD4^{+}$ cells were not statistically different between GMZ2- and control-vaccinated groups at D84. Interestingly, additional analyses using estimated blood counts showed an increase in the number of double (IFN-γ^{+}/TNF-α^{+} or $IL2^{+}$/TNF- α^{+}) and triple positive proinflammatory cytokine (IFN-γ^{+}/$IL2^{+}$/TNF-α^{+}) producing $CD4^{+}$ T cells after stimulation with GMZ2. In addition, also total number of $IL4^{+}$ and $IL10^{+}$ anti-inflammatory T-cells, but not $IL13^{+}$ T-cells increased in cell number after vaccination (Additional file 1: Fig. S2).

(See figure on next page.)

Fig. 2 $CD4^{+}$ T cell frequencies following immunization. Isolated PBMCs were stimulated with either medium alone, the vaccine antigen GMZ2, or Staphylococcal enterotoxin B (SEB) as positive control. Thereafter, intracellular cytokine staining was performed, and the cells measured by flow cytometry. Data are expressed after subtraction of unstimulated cell frequencies from that of stimulated with the positive control (SEB), and with GMZ2, and normalization with the average of positive control values. The comparison of the pro-inflammatory cytokine producing $CD4^{+}$ T cells (Fig. 2a), and the anti-inflammatory cytokine producing $CD4^{+}$ T cells (Fig. 2b) between D0 and D84 was performed in those receiving the control vaccine and in those vaccinated with GMZ2 (including those vaccinated with 100 µg GMZ2-Alhydrogel (opened dots), 30 µg GMZ2-CAF01 (grey dots), 100 µg GMZ2-CAF01 (black dots) using Wilcoxon test with Bonferroni correction for multiple comparisons. p value less than 0.05 is considered as statistically significant. All time points per volunteer were measured in a single experiment after several optimization tests, and individual volunteers were measured in separate experiments. Symbols represent individual samples. Red lines represent the median values with interquartile range.

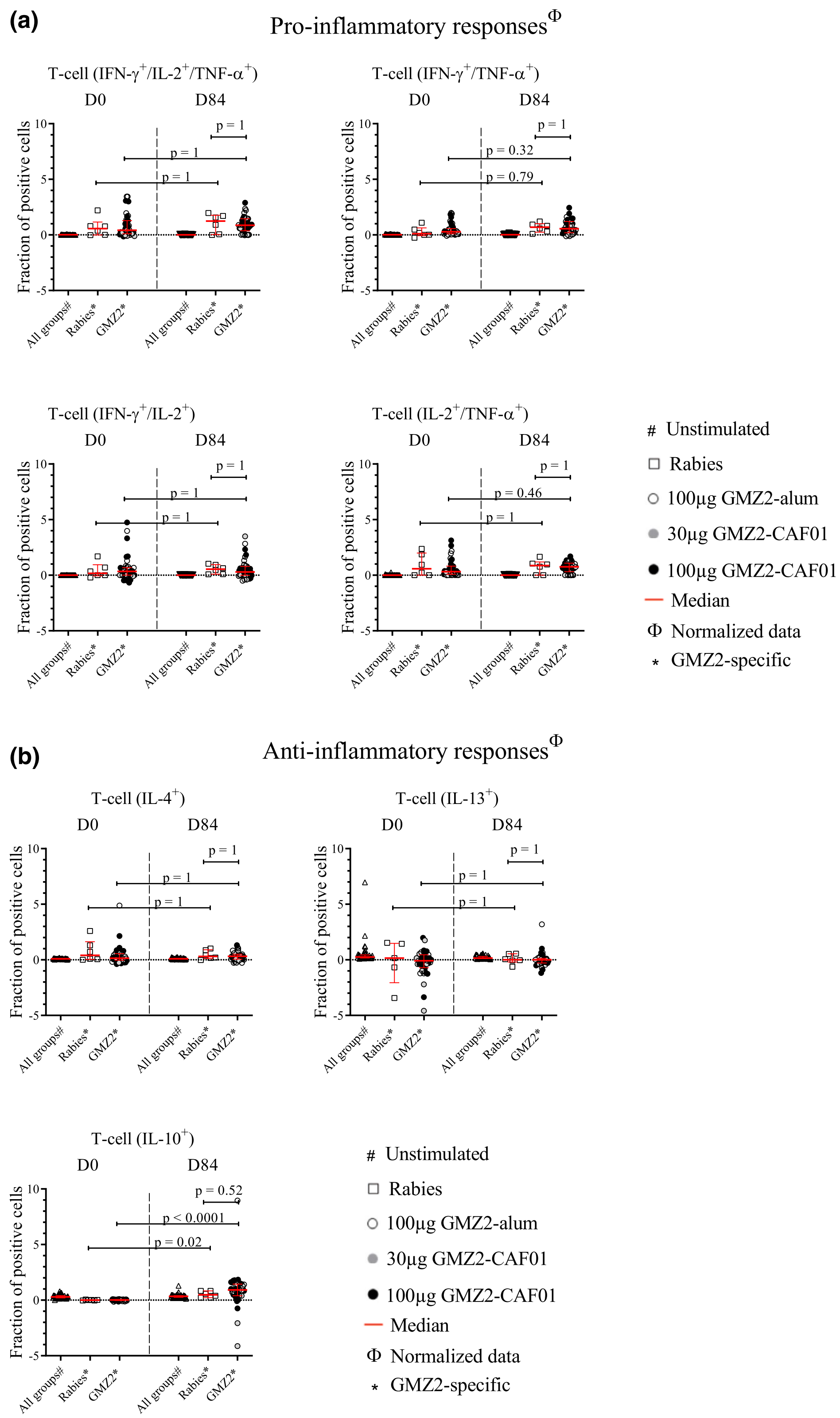


Fig. 2 (See legend on previous page.)

Circulating B cell response to GMZ2

GMZ2-specific B cells were gated on viability and surface markers as well as reactivity to GMZ2 (Additional file 1: Fig. S1b). Here, the generation of circulating GMZ2-specific memory B cells after vaccination was determined by flow-cytometric estimation of antigen-specific $CD20^+$ IgG^+ B cells. Data showed no increment in the $CD20^+IgG^+$ cell frequency in all GMZ2 vaccinated individuals at D84. Similarly, $CD20^+IgG^+$ cell frequency in response to the vaccine antigen GMZ2 remained without changes and neither $CD27^+$ nor $CD27^-$ GMZ2-specific cell frequencies were associated with any increment post vaccination (Fig. 3). Interestingly, estimated cell counts of the total lymphocyte count numbers showed slightly higher $CD20^+IgG^+$ counts at D84 ($p < 0.05$). However, GMZ2-specific $CD20^+IgG^+$ cells did not increase after immunization (Additional file 1: Fig. S3).

T and B cells stimulated post-immunization with the vaccine antigen GMZ2 are not associated with protection against clinical malaria in CHMI

Exploratory analyses of data collected at D0 and D84 were done in a subgroup of participants who underwent CHMI to assess whether pre- or post-immunization cellular patterns were associated with protection from experimentally induced malaria [22]. Thereby, higher frequencies of $CD20^+IgG^+$ B cells were found to be associated with protection from parasitaemia after CHMI (Fig. 4). Surprisingly, neither T-stimulated nor GMZ2-specific B cells (Fig. 4). No significant association between the pre-immunization (D0) cell frequencies (Fig. 5a) or the estimated cell counts (Fig. 5b) and the time to malaria treatment after CHMI was observed. In the same way, no significant association between the baseline cytokine producing $CD4^+$ T cells and the outcome in CHMI was observed (Table 2).

At D84, protected volunteers had a higher number of circulating IgG positive $CD20^+$ B cells compared to those

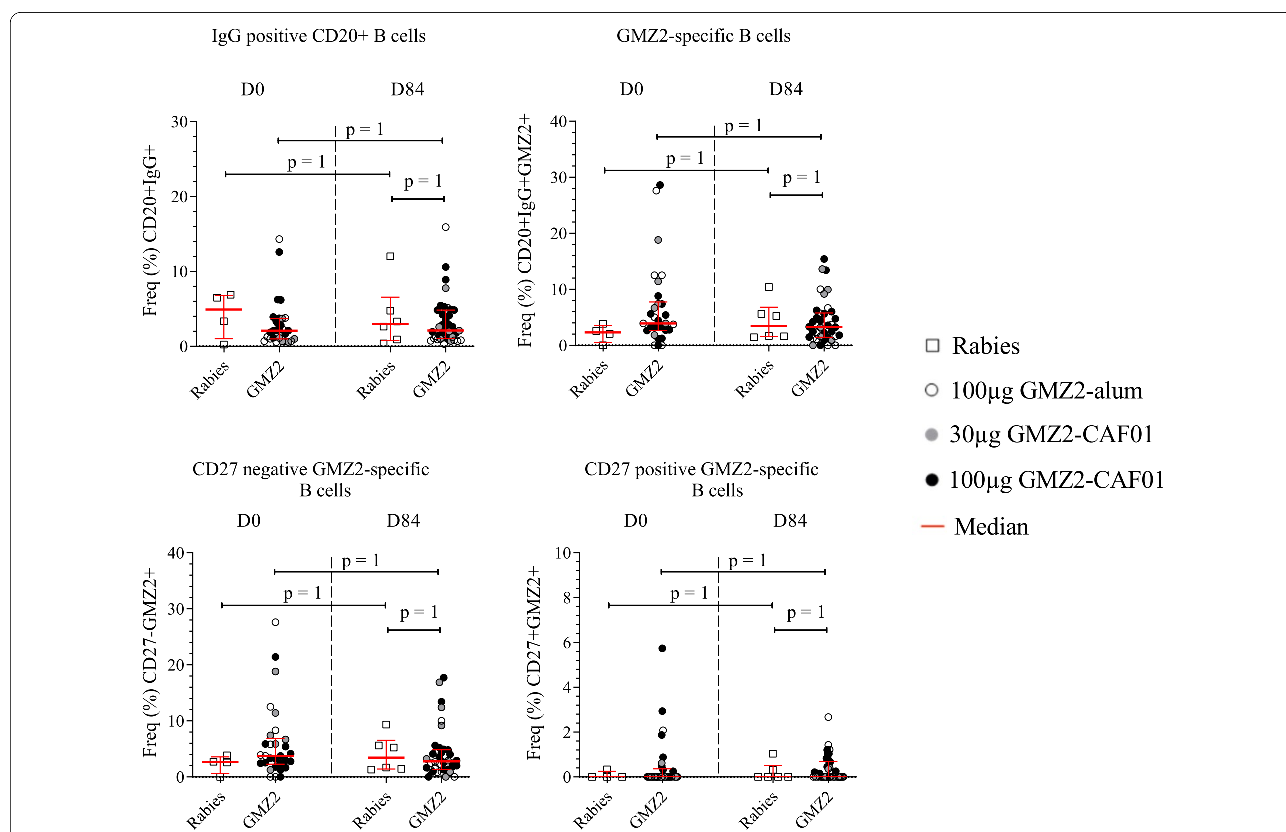


Fig. 3 GMZ2-specific B cell frequencies following immunization. B cells were estimated using cryopreserved PBMCs without additional stimulation. The comparison of the GMZ2-specific memory B cells frequencies between D0 and D84 was performed in those receiving the control vaccine and in those vaccinated with GMZ2 (including those vaccinated with 100 µg GMZ2-Alhydrogel (opened dots), 30 µg GMZ2-CAF01 (grey dots), 100 µg GMZ2-CAF01 (black dots) using Wilcoxon test with Bonferroni correction for multiple comparisons. p value less than 0.05 is considered as statistically significant. All time points per volunteer were measured in a single experiment after several optimization tests, and individual volunteers were measured in separate experiments. Symbols represent individual samples. Red lines represent median values with interquartile range

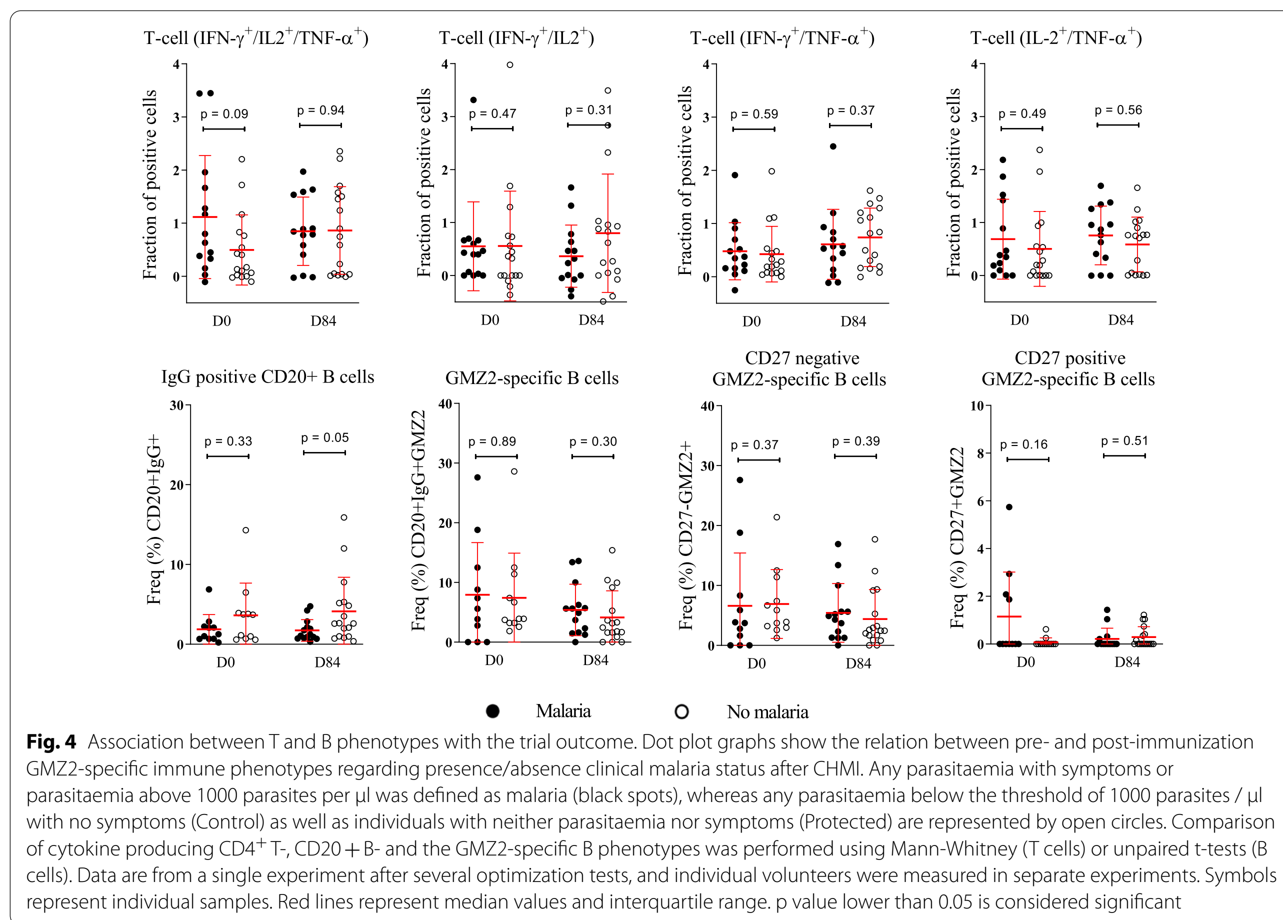


Fig. 4 Association between T and B phenotypes with the trial outcome. Dot plot graphs show the relation between pre- and post-immunization GMZ2-specific immune phenotypes regarding presence/absence clinical malaria status after CHMI. Any parasitaemia with symptoms or parasitaemia above 1000 parasites per µl was defined as malaria (black spots), whereas any parasitaemia below the threshold of 1000 parasites / µl with no symptoms (Control) as well as individuals with neither parasitaemia nor symptoms (Protected) are represented by open circles. Comparison of cytokine producing $CD4^+$ T-, CD20 + B- and the GMZ2-specific B phenotypes was performed using Mann-Whitney (T cells) or unpaired t-tests (B cells). Data are from a single experiment after several optimization tests, and individual volunteers were measured in separate experiments. Symbols represent individual samples. Red lines represent median values and interquartile range. p value lower than 0.05 is considered significant

having malaria although this difference did not reach statistical significance (p = 0.06) (Additional file 1: Fig. S4). Furthermore, an association between higher IgG positive $CD20^+$ B frequency and the time to malaria treatment after CHMI (D84) was observed (Additional file 1: Fig. S5a) whereas post-immunization estimated counts (Additional file 1: Fig. S5b) did not. Additionally, Cox regression using post-immunization data showed that the hazard of malaria in CHMI was not significantly associated with the proportion of cells at D84 (Additional file 1: Table S1).

GMZ2-specific IgG concentration and B cells baseline

GMZ-specific antibodies were specifically elicited by GMZ2 vaccination regimen (Fig. 6a–c). The association between B cells and the concentration of anti-GMZ2 IgG was analysed. This was done as anti-GMZ2 IgG concentration at baseline, but not after vaccination was shown to be predictive for CHMI outcome, as was already previously published [22].

GMZ2-specific IgG concentration was not associated with the frequencies of neither the $CD20^+IgG^+$ B or the $CD20^+IgG^+$ GMZ2 + B cells (Fig. 7). Likewise, the estimated B-cell counts circulating in the blood were not associated with the antigen-specific IgG concentration (Additional file 1: Fig. S6).

Pre-existing antibody response in lifelong malaria-exposed volunteers predicts protection from malaria in CHMI

To quantify the immune response against a large set of malaria antigens, protein microarrays were performed in the semi-immune study population as well as a European malaria-naïve control population (Fig. 8a). Mean antibody responses to 86 of the 251 expressed *P. falciparum* antigens were at least two-fold higher and significantly different in the African population compared to the European population. Further analysis within the Gabonese study population revealed that antibody responses were significantly higher in the population protected from clinical malaria (no symptoms and either submicroscopic parasitaemia or no parasitaemia during the 35 days of follow up post-CHMI) than in the population that developed symptoms in CHMI (Fig. 8b). Elevated

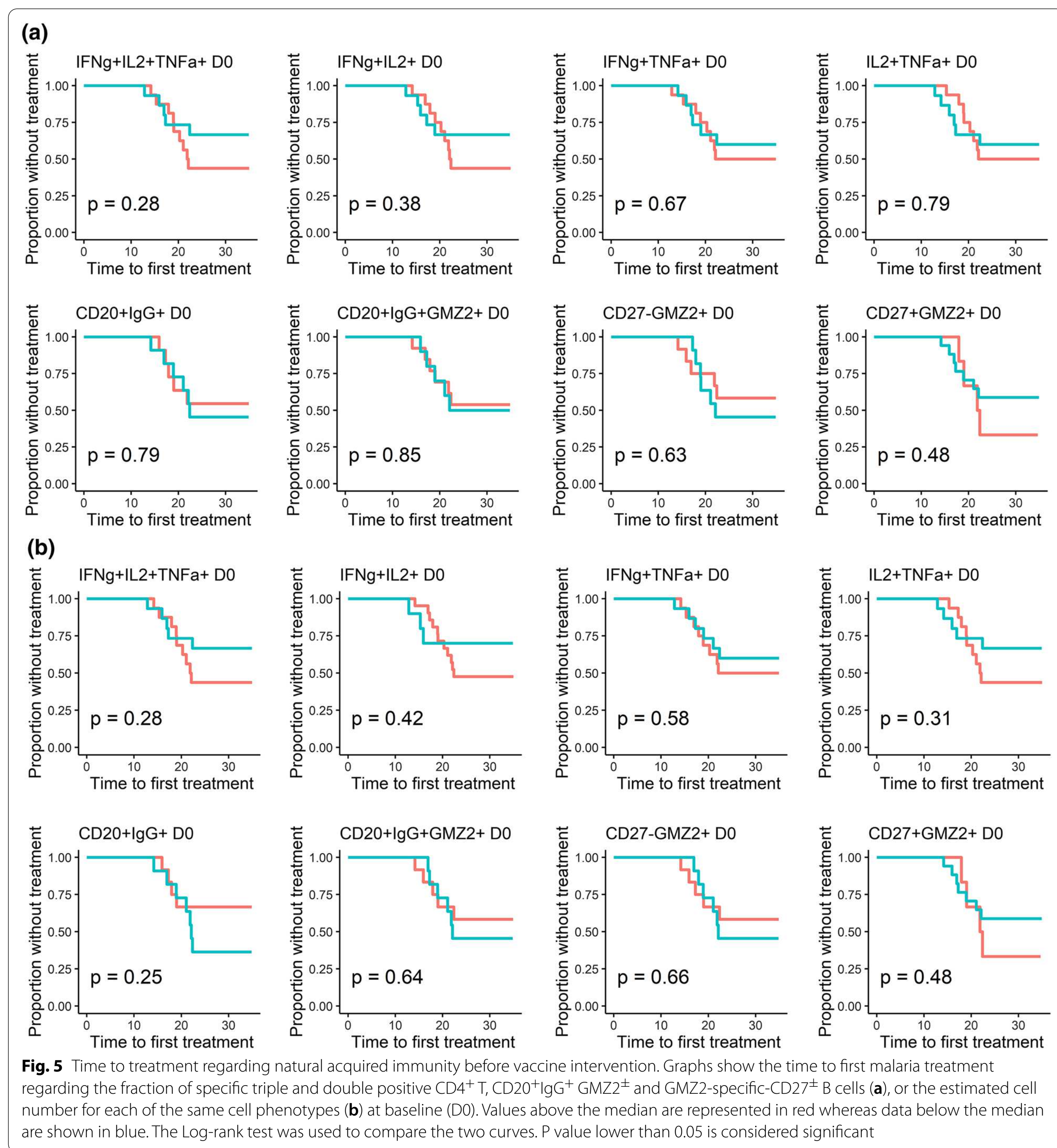


Fig. 5 Time to treatment regarding natural acquired immunity before vaccine intervention. Graphs show the time to first malaria treatment regarding the fraction of specific triple and double positive CD4^{+} T, CD20^{+}IgG^{+} GMZ2$^{\pm}$ and GMZ2-specific-CD27$^{\pm}$ B cells (**a**), or the estimated cell number for each of the same cell phenotypes (**b**) at baseline (D0). Values above the median are represented in red whereas data below the median are shown in blue. The Log-rank test was used to compare the two curves. P value lower than 0.05 is considered significant

antibody levels were directed against a range of antigens. The pattern included antigens expressed at various stages of the life cycle and localized at different sites (merozoite surface, erythrocyte membrane, intracellular, sporozoite protein) of the parasite (Additional file 2: Table S2). In total, 23 spots, representing 21 different antigens, were significantly and at least twofold higher recognized by specific antibodies in the protected group vs. the unprotected group. These included well characterized, highly immunogenic antigens that are described as markers for long-term exposure. Some have been investigated as vaccine candidates, such as merozoite surface protein 1 (MSP1), MSP4, MSP8, erythrocyte binding antigen 181 (EBA-181), sporozoite threonine and asparagine-rich

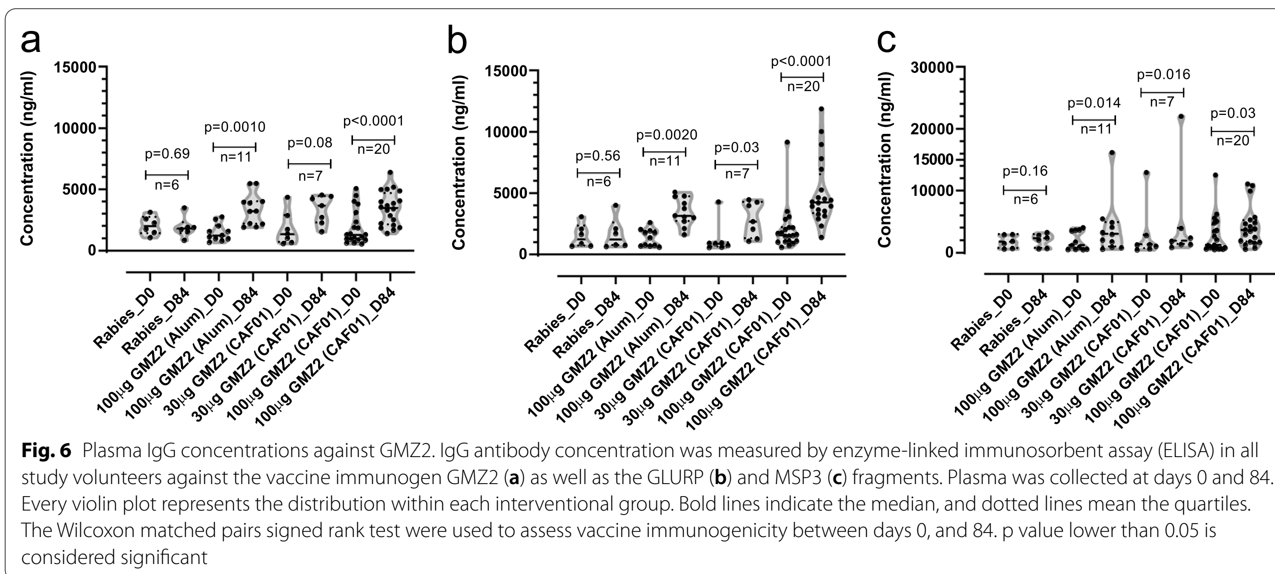


Fig. 6 Plasma IgG concentrations against GMZ2. IgG antibody concentration was measured by enzyme-linked immunosorbent assay (ELISA) in all study volunteers against the vaccine immunogen GMZ2 (**a**) as well as the GLURP (**b**) and MSP3 (**c**) fragments. Plasma was collected at days 0 and 84. Every violin plot represents the distribution within each interventional group. Bold lines indicate the median, and dotted lines mean the quartiles. The Wilcoxon matched pairs signed rank test were used to assess vaccine immunogenicity between days 0, and 84. p value lower than 0.05 is considered significant

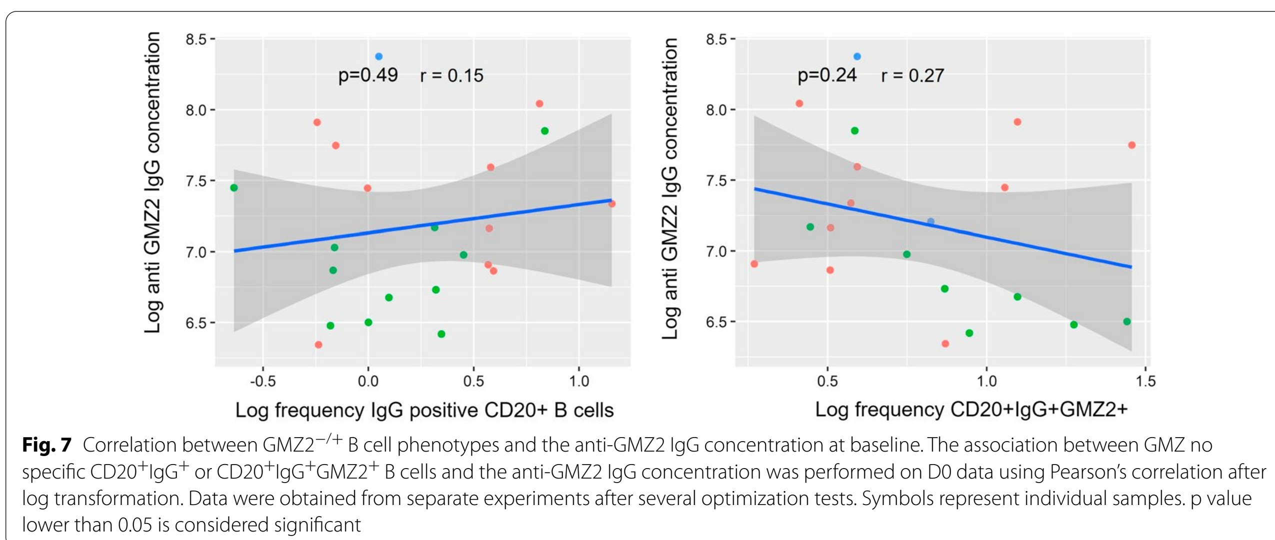


Fig. 7 Correlation between GMZ2$^{-/+}$ B cell phenotypes and the anti-GMZ2 IgG concentration at baseline. The association between GMZ no specific CD20^{+}IgG^{+} or CD20^{+}IgG^{+}GMZ2^{+} B cells and the anti-GMZ2 IgG concentration was performed on D0 data using Pearson's correlation after log transformation. Data were obtained from separate experiments after several optimization tests. Symbols represent individual samples. p value lower than 0.05 is considered significant

(See figure on next page.)

Fig. 8 Protein microarray using plasma from volunteers undergoing CHMI. Figure shows the heatmap from protein microarrays in the semi-immune study population as well as a European malaria-naïve control population (**a**). The intensity of antibody responses in the population being protected from clinical malaria and those who developed malaria were compared and shown as volcano plot. The red circles are antigens being at least two-fold higher and significantly upregulated in the respective group (**b**). **c** shows the heatmap in participants having at least two-fold higher antibody response in the protected group vs. the unprotected group, thus showing the raw data of the red dots in Fig. 8b. Gene ID according to PlasmoDB are given. **d** shows the breadth of the antibody response in those who develop malaria (n = 15), those who control the parasitaemia (n = 12), those having full protection (n = 7), in the negative control (1 European malaria-naïve sample, measured in 5 technical replicates) and in European naïve controls (n = 13). Data are from a single experiment after several optimization tests, and individual volunteers were measured in separate experiments. Differential antibody recognition in the different allocated study outcomes was analysed by Student's t-test. p value less than 0.05 is considered as statistically significant. *NC* Negative control, *N* malaria-naïve subjects, *M* subjects having clinical symptoms of malaria after CHMI, *C* subjects controlling parasitaemia, *P* subjects fully protected after CHMI

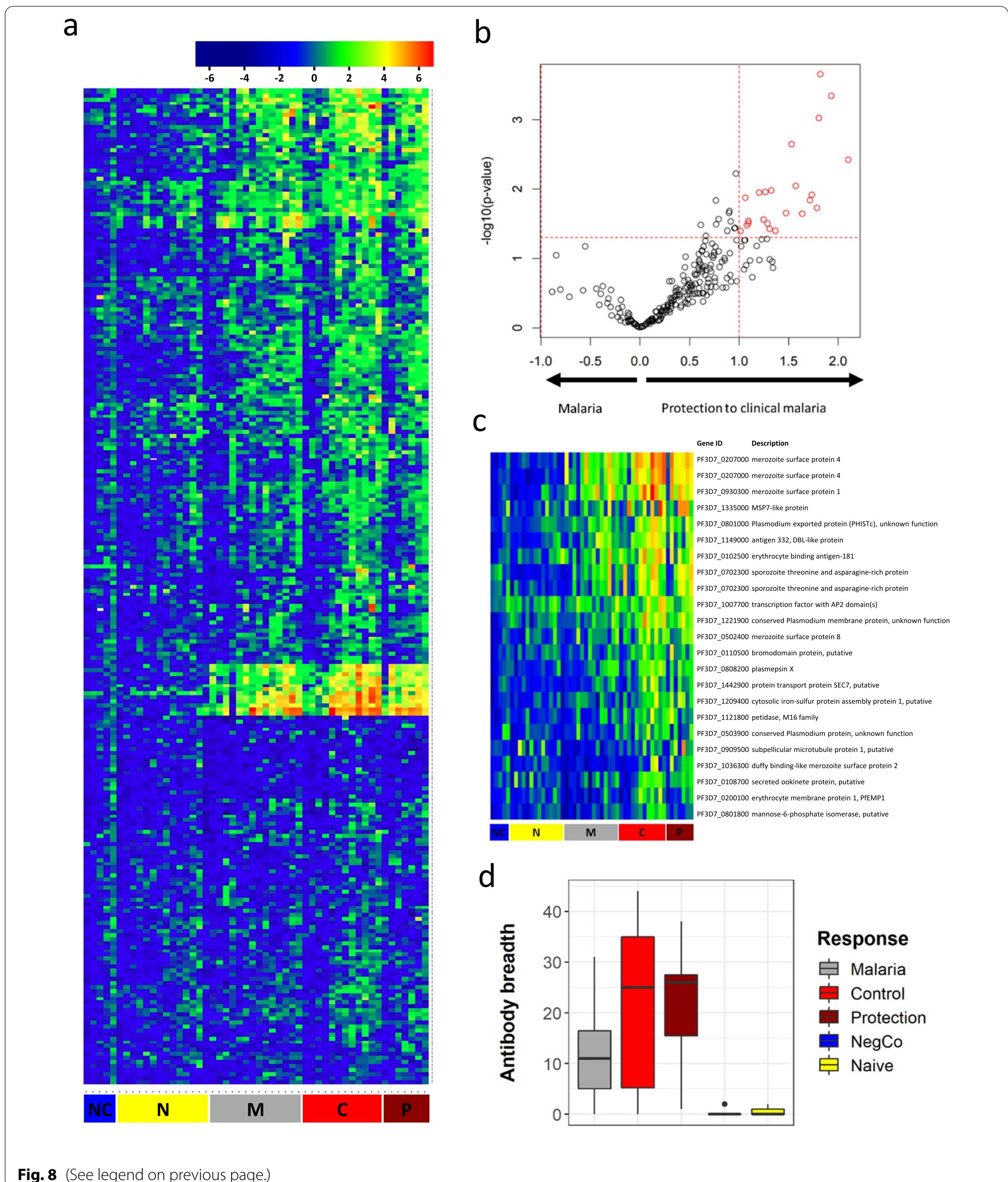


Fig. 8 (See legend on previous page.)

protein (STARP). Individual results for the antigens associated with protection against clinical malaria are depicted in Fig. 8c.

Neither GLURP nor MSP3 were contained on the microarray and therefore could not be part of this specific analysis, but these antigens have thoroughly been

analysed in previous work by ELISA (Fig. 6) [22]. In addition, the correlation of the antibody response against the vaccine antigens with any of the antigens represented on the protein microarray was investigated (Additional file 3: Table S3). Interestingly, the immune response to MSP3 correlated to several other members of the MSP-family (MSP5, MSP10, MSP11). Also, it is noticeable that the response measured to GMZ2, GLURP and MSP3 also correlated with the antibody response against several PfEMP1 proteins.

Immunity to clinical malaria develops after years of repetitive exposure to the parasite, potentially increasing the breadth of the antigens recognized by the immune system. As expected, volunteers who developed clinical symptoms had a significantly lower breadth of highly recognized antigens than those who controlled parasitaemia. Regardless, the antibody response in this population was still much higher than in European naïve controls (Fig. 8d).

Discussion

In this exploratory study, the cellular and humoral immune responses to the GMZ2 malaria vaccine candidate adjuvanted with either Alhydrogel or CAF01 was assessed. Likewise, it was hypothesized that vaccine specific $CD4^+$ T cell and $CD20^+$ IgG^+ B cell responses are induced and that they are associated with protection against CHMI.

The observation that immunization with GMZ2 adjuvanted with either Alhydrogel or CAF01 did not significantly augment the $CD4^+$ T cell proportion was unexpected, however, an increase in the estimated number of GMZ2-stimulated $CD4^+$ T cells was detectable (Additional file 1: Fig. 2). The mechanism of action of these two adjuvants is well described and responses should be measurable in healthy adult volunteers [32] although previous studies have been only conducted in animal models [33, 34] and this study is the first one to directly compare both adjuvants in humans. On one hand, it is possible that stoichiometry between adjuvant and vaccine antigen plays a role besides the chemical nature of the adjuvant, since both vaccine formulations have been optimized in animal models before; and an increase in GMZ2-specific antibodies could be observed. On the other hand, another explanation relying on the marked $CD4^+$ T cell activity at baseline may provide a scenario where the saturated activation of T cells and/or previous activation of dendritic cells (DCs) before vaccination may reflect the influence of the frequent natural exposure to *P. falciparum* [35]. It has also been shown that elevated levels of $CD161^+CD4^+$ T cells and malaria-specific IFN-γ-production predicted protection against CHMI [36].

The elevated levels of GMZ2-induced IL-10 following vaccination in both GMZ2 and Rabies vaccinees suggest, that immunoregulatory mechanisms were induced by the inflammatory stimulus of vaccination to help dampening proinflammatory responses [37]. Concerning the explored immunophenotypes, it was surprising not to see higher frequencies of circulating GMZ2-specific $CD20^+$ IgG^+ B cells following immunization. In contrast, GMZ2-specific B cells were increased after vaccination in malaria-naïve adults using Alhydrogel-adjuvanted GMZ2 at D84 [5]. Moreover, GMZ2 vaccine seemed to not stimulate higher frequencies of neither the $CD27^-$ cluster, theoretically involving both putative atypical ($CD21^-CD27^-$) and activated ($CD21^+CD27^-$) memory B cells, nor the $CD27^+$ B cells (putative classical memory B cells). Natural exposure to malaria parasites through repeated infections can induce protective antibodies [38], whilst simultaneously expanding the population of atypical memory B cells [39]. In future clinical trials investigating vaccines in malaria-endemic countries, this should be investigated in more detail.

Conversely, it was found that after immunization, higher frequency of $CD20^+$ IgG^+ B cells was related to the absence of clinical malaria after CHMI. However, a link between the level of GMZ2- specific memory B cells and the concentration of anti-GMZ2 antibodies was not observed; something that has been seen previously with GMZ2 [5] and other vaccines [40, 41]. Likewise, it was not possible to assign any antigen specific immune phenotype with the prevention of clinical malaria.

On the protein microarray, a set of anti-plasmodial antibodies detected before CHMI were associated with protection. The targeted antigens included several biomarkers of exposure [42] and well-described malaria vaccine candidates. Associations between antibody breadth and protection, as well as responses to specific malaria antigens and protection, have been described in malaria-naïve volunteers immunized with sporozoites under chemoprophylaxis [36, 43]. There, either patterns or specific antigens were associated with protection. So far, highly specific biomarkers predicting protection at a general level are still not found, these could be patterns of overall exposure or immune response to specific antigens or domains. Interestingly enough, the pre-existing antibody response against the vaccination antigens at baseline correlated with several markers of long-term exposure to malaria parasites displayed on the microarray. Antigens of the array used in this study were down-selected from previous studies [28, 43–47] and may be further simplified for an assay that predicts protection following natural or artificial exposure to parasites.

Interestingly, a higher breadth of antibody responses was also associated with protection. Certainly, this fact reinforces the hypothesis that protection is mediated by individual patterns of *P. falciparum* antigen-recognition rather than a single antigen, although some antigens are more dominant than others. Both antigens contained in GMZ2 – MSP3 and GLURP – are among those dominant ones since baseline activity against them predicts protection against CHMI, as it has been already shown [22].

This study presents some limitations, most importantly small number of volunteers per group, which only allows the detection of large effects. In addition, specific T cell stimulation was done using the vaccine antigen and not peptides or multimers. Despite these limitations, the study provides important insights on the difficulties to develop a malaria vaccine for malaria-endemic regions. Further exploration of the immunological aspects of naturally acquired immunity in endemic regions are a necessary step in the design and clinical development of any future malaria vaccine with an impact where it is most needed [36].

Immunization with GMZ2 formulated with CAF01 or Alhydrogel did not successfully induce robust increases in $CD4^+$ T or $CD20^+$ IgG^+ B cell responses. Disappointingly, data concerning the expansion of estimated $CD20^+IgG^+$ B cell counts highlighted that GMZ2-specific cells did not contribute to reduce the risk of clinical malaria in CHMI.

Conclusion

The GMZ2-reactive T and B cell patterns examined here show the dominant role of naturally acquired immunity in controlling malaria clinical episodes in the high endemic area of Gabon. Therefore, the differences observed in clinical trials in endemic settings compared to malaria-naïve volunteers stress even more the point that inclusion of populations form malaria-endemic areas early in the clinical development is important.

Abbreviations

Alhydrogel: Alhydrogel hydroxide; CAF: Cationic adjuvanted formulation; CD: Cluster of differentiation; CHMI: Controlled human malaria infection; HBSS: Hank's balanced salt solution; IgG: Immunoglobulin G; IL: Interleukin; IFN-γ: Interferon gamma; PBMC: Peripheral blood mononuclear cells; RPMI: Roswell Park memorial institute medium; Th: T helper; TNF: Tumour necrosis factor; VE: Vaccine efficacy.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-022-04169-8.

Additional file 1: Figure S1a. Gating strategies for cytokine producing $CD4^+$ T cell identification. **Figure S1b.** Gating strategy for GMZ2-reactive memory B cell identification. **Figure S2.** Estimated number of $CD4^+$ T cells producing cytokines on unstimulated, vaccine antigen GMZ2 and Staphylococcal endoterotoxin B (SEB) stimulated cells following immunization. Symbols represent individual samples in unstimulated, GMZ2 stimulated and SEB-stimulated conditions. All time points per volunteer were measured in a single experiment after several optimization tests, and individual volunteers were measured in separate experiments. Red lines represent median values with interquartile range. p value lower than 0.05 is considered significant. **Figure S3.** Estimated number of B cells with or without GMZ2-reactivity following immunization. Symbols represent individual samples. All time points per volunteer were measured in a single experiment after several optimization tests, and individual volunteers were measured in separate experiments. Red lines represent the median values with interquartile range. p value lower than 0.05 is considered significant. **Figure S4.** Association between pre/post-immunization GMZ2-specific immune cells and trial outcome. Dot plot graphs show the relation between the estimated number of pre/post-immunization GMZ2 stimulated cytokine producing $CD4^+$ T cells (upper side), the number of and B cells subsets (bottom side) regarding clinical malaria status after CHMI. Monotone increase of parasitemia with symptoms (Malaria) is represented by black spots. Low oscillating parasitemia with no symptoms (Control) plus individuals with neither parasitemia nor symptoms (Protected) are represented by open circles. Comparison of the cell number of GMZ2 stimulated $CD4^+$ T cells, of CD20+ B cells and the GMZ2-specific B subsets was performed using Mann-Whitney (for T cells) or unpaired t-tests (for B cells). Data are from a single experiment after several optimization tests, and individual volunteers were measured in separate experiments. Symbols represent individual samples. **Figure S5.** Post-immunization cell frequencies and the time to treatment after CHMI. Graphs show the time to first malaria treatment regarding the fraction of specific triple and double positive $CD4^+$ T cells, total B cells and the $CD27^{+/-}$ cluster subsets of GMZ2-specific within $CD20^+IgG^+$ B cells (a), or the number of GMZ2-stimulated triple and double positive $CD4^+$ T cells, and the number of total B cells and different $GMZ2^+$B cells (b) at D84. Values above the median are represented in red whereas data below the median are shown in blue. The Log-rank test was used to compare the two curves. p value lower than 0.05 is considered significant. **Figure S6.** Correlation between the estimated number of B cell phenotypes and the anti-GMZ2 IgG concentration at baseline. The association between the estimated number of CD20+IgG+ B cells, the estimated number of GMZ2-specific B cells and the anti-GMZ2 IgG concentration, was performed on D0 data using Pearson's correlation after log transformation. Data are from a single experiment after several optimization tests, and individual volunteers were measured in separate experiments. Symbols represent individual samples. A p-value less than 0.05 is considered as statistically significant. **Figure S7.** B cell phenotypes frequency following immunization regarding vaccine intervention. Frequencies of $CD20^+$IgG positive, $CD20^+IgG^+$GMZ2-specific and GMZ2-specific $CD27^{+/-}$ B cells between D0 and D84 are compared for all volunteers regarding vaccine intervention. Vaccinated subjects with Rabies control vaccine are represented with opened squares. GMZ2 vaccinated discriminate vaccinees receiving 100µg GMZ2-Alhydrogel (opened dots), 30µg GMZ2-CAF01 (grey dots), and 100µg GMZ2-CAF01 (black dots). Wilcoxon test following by Bonferroni correction for multiple comparison is performed to test statistical significance. p value below 0.05 is considered statistically significant. Data are from a single experiment after several optimization tests, and individual volunteers were measured in separate experiments. Symbols represent individual samples. Red lines represent the median values with interquartile range. p value lower than 0.05 is considered significant. **Table S1**. Cox proportional analysis post-immunization

Additional file 2: Table S2. Complete list of antigens spotted on the protein microarray.

Additional file 3: **Table S3.** The antibody response against the vaccine antigen (GMZ2) or any of the main components (MSP3, GLURP), estimated before vaccination, was tested for correlation with any of the antibody responses, as estimated by the protein microarray before CHMI. The log-transformed ELISA data and the protein microarray data were tested usind Student's t-test for correlation. Antigens with correlations resulting in p-values < 0.05 are shown.

Acknowledgements
We are grateful to the study participants, and the nurses and fieldworkers involved in this clinical study. We particularly thank those who provided CAF01 and the PfSPZ Challenge (Sanaria Inc.) as well as the immunology teams of CERMEL and the Institute of Tropical Medicine.

Author contributions
Conceived and designed the experiments: ON, JI, RF, AAA, BM, ME. Performed the experiments: ON, JI, RF. Generated the figures: ON, JI, RF. Analysed the data: ON, JI, RF, FRL. Interpreted the data analysis: ON, JI, RF, BM. Contributed reagents/materials/protocols: UAN, AAA, BM, JF, SB, KM, AJL, ME, BL, PGK, MT, SLH. Wrote Clinical Trial protocol: UAN, AAA, BM. Performed the clinical trial: UAN, JCDA, JRE, BL. Wrote the paper: ON, JI, RF, BM. Provided manuscript editorial contributions: RF, BM, UAN, AJL. All authors read and approved the final manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL. This work is carried out on the GMZ2-CAF01 project which is supported by the German Center for Infection Research (TTU03.801, TTU 03.702, TTU 03.703).

Availability of data and materials
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The original study was approved by the Comité National d'Ethique de la Recherche (CNER) of Gabon, under the reference N°004/2015/SG/P. The trial was performed according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use Good Clinical Practice guidelines and the Declaration of Helsinki and is registered with the Pan-African Clinical Trials Registry (trial number PACTR201503001038304). Written informed consent was received from all study participants before inclusion into the clinical trial.

Consent for publication
Not applicable.

Competing interests
As CEO of Sanaria Inc., SLH has a potential conflict of interest. All other authors declared that they have no competing interests.

Author details
[1]Centre de Recherches Médicales de Lambaréné, BP : 242, Lambaréné, Gabon. [2]Institute of Tropical Medicine, University Tübingen, Wilhelmstr. 27, 72074 Tübingen, Germany. [3]German Centre for Infection Research (DZIF), Partner Site, Tübingen, Germany. [4]Département de Biochimie Et de Biologie Cellulaire, Faculté des Sciences et Techniques, Université d'Abomey-Calavi, Cotonou, Bénin. [5]Center of Tropical Medicine and Travel Medicine, Department of Infectious Diseases, Amsterdam University Medical Centers, Amsterdam, The Netherlands. [6]Department for Congenital Disorders, Statens Serum Institut, Copenhagen, Denmark. [7]Centre for Medical Parasitology at Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark. [8]Department of Infectious Diseases, Copenhagen University Hospital, Rigshospitalet, Denmark. [9]Sanaria Inc., Rockville, MD 20850, USA. [10]Centre d'Etude et de Recherche sur le Paludisme Associé à la Grossesse et a l'Enfance, Calavi, Bénin. [11]MERIT, Université de Paris, Paris, IRD, France. [12]Department of Medicine I, Division of Infectious Diseases and Tropical Medicine, Medical University of Vienna, Vienna, Austria. [13]Department of Parasitology, Leiden University Medical Centre (LUMC), 2333 ZA Leiden, The Netherlands. [14]Fondation pour la Recherche Scientifique, 72 BP45 Cotonou, Bénin. [15]Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands. [16]Cluster of Excellence: EXC 2124: Controlling Microbes to Fight Infection, Tübingen, Germany.

Received: 25 October 2021 Accepted: 23 April 2022
Published online: 17 June 2022

References

1. WHO. World malaria report 2021. Geneva, World Health Organization, 2021. https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2021. Accessed 25 Feb 2022.
2. White MT, Verity R, Churcher TS, Ghani AC. Vaccine approaches to malaria control and elimination: insights from mathematical models. Vaccine. 2015;33:7544–50.
3. Theisen M, Soe S, Brunstedt K, Follmann F, Bredmose L, Israelsen H, et al. A *Plasmodium falciparum* GLURP-MSP3 chimeric protein; expression in *Lactococcus lactis,* immunogenicity and induction of biologically active antibodies. Vaccine. 2004;22:1188–98.
4. Belard S, Issifou S, Hounkpatin AB, Schaumburg F, Ngoa UA, Esen M, et al. A randomized controlled phase Ib trial of the malaria vaccine candidate GMZ2 in African children. PLoS ONE. 2011;6:e22525.
5. Esen M, Kremsner PG, Schleucher R, Gassler M, Imoukhuede EB, Imbault N, et al. Safety and immunogenicity of GMZ2 - a MSP3-GLURP fusion protein malaria vaccine candidate. Vaccine. 2009;27:6862–8.
6. Mordmuller B, Szywon K, Greutelaers B, Esen M, Mewono L, Treut C, et al. Safety and immunogenicity of the malaria vaccine candidate GMZ2 in malaria-exposed, adult individuals from Lambarene. Gabon Vaccine. 2010;28:6698–703.
7. Sirima SB, Mordmuller B, Milligan P, Ngoa UA, Kironde F, Atuguba F, et al. A phase 2b randomized, controlled trial of the efficacy of the GMZ2 malaria vaccine in African children. Vaccine. 2016;34:4536–42.
8. Gram GJ, Karlsson I, Agger EM, Andersen P, Fomsgaard A. A novel liposome-based adjuvant CAF01 for induction of CD8(+) cytotoxic T-lymphocytes (CTL) to HIV-1 minimal CTL peptides in HLA-A*0201 transgenic mice. PLoS ONE. 2009;4:e6950.
9. van Dissel JT, Joosten SA, Hoff ST, Soonawala D, Prins C, Hokey DA, et al. A novel liposomal adjuvant system, CAF01, promotes long-lived *Mycobacterium tuberculosis*-specific T-cell responses in human. Vaccine. 2014;32:7098–107.
10. Sun P, Schwenk R, White K, Stoute JA, Cohen J, Ballou WR, et al. Protective immunity induced with malaria vaccine, RTS, S, is linked to *Plasmodium falciparum* circumsporozoite protein-specific CD4+ and CD8+ T cells producing IFN-gamma. J Immunol. 2003;171:6961–7.
11. Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, et al. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS, S/AS01B and RTS, S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J Infect Dis. 2009;200:337–46.
12. Lumsden JM, Schwenk RJ, Rein LE, Moris P, Janssens M, Ofori-Anyinam O, et al. Protective immunity induced with the RTS, S/AS vaccine is associated with IL-2 and TNF-alpha producing effector and central memory CD4 T cells. PLoS ONE. 2011;6:e20775.
13. Moncunill G, Mpina M, Nhabomba AJ, Aguilar R, Ayestaran A, Sanz H, et al. Distinct helper T cell type 1 and 2 responses associated with malaria protection and risk in RTS, S/AS01E vaccinees. Clin Infect Dis. 2017;65:746–55.
14. Agnandji ST, Fendel R, Mestré M, Janssens M, Vekemans J, Held J, et al. Induction of *Plasmodium falciparum*-Specific CD4+ T cells and memory B cells in Gabonese children vaccinated with RTS, S/AS01E and RTS, S/AS02D. PLoS ONE. 2011;6:e18556.
15. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. Nat Med. 2007;13:843–50.

16. Lindenstrom T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, Seder RA, et al. Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. J Immunol. 2009;182:8047–55.
17. Lin L, Finak G, Ushey K, Seshadri C, Hawn TR, Frahm N, et al. COMPASS identifies T-cell subsets correlated with clinical outcomes. Nat Biotechnol. 2015;33:610–6.
18. Mordmüller B, Surat G, Lagler H, Chakravarty S, Ishizuka AS, Lalremruata A, et al. Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. Nature. 2017;542:445–9.
19. Ahmed R, Gray D. Immunological memory and protective immunity: understanding their relation. Science. 1996;272:54–60.
20. Donati D, Zhang LP, Chene A, Chen Q, Flick K, Nystrom M, et al. Identification of a polyclonal B-cell activator in *Plasmodium falciparum*. Infect Immun. 2004;72:5412–8.
21. Ly A, Hansen DS. Development of B cell memory in malaria. Front Immunol. 2019;10:559.
22. Dejon-Agobe JC, Ateba-Ngoa U, Lalremruata A, Homoet A, Engelhorn J, Nouatin OP, et al. Controlled human malaria infection of healthy adults with lifelong malaria exposure to assess safety, immunogenicity, and efficacy of the asexual blood stage malaria vaccine candidate GMZ2. Clin Infect Dis. 2019;69:1377–84.
23. Mordmüller B, Supan C, Sim KL, Gómez-Pérez GP, Ospina Salazar CL, Held J, et al. Direct venous inoculation of *Plasmodium falciparum* sporozoites for controlled human malaria infection: a dose-finding trial in two centres. Malar J. 2015;14:117.
24. Gomez-Perez GP, Legarda A, Munoz J, Sim BK, Ballester MR, Dobano C, et al. Controlled human malaria infection by intramuscular and direct venous inoculation of cryopreserved *Plasmodium falciparum* sporozoites in malaria-naive volunteers: effect of injection volume and dose on infectivity rates. Malar J. 2015;14:306.
25. Lell B, Mordmüller B, Dejon Agobe JC, Honkpehedji J, Zinsou J, Mengue JB, et al. Impact of sickle cell trait and naturally acquired immunity on uncomplicated malaria after controlled human malaria infection in adults in Gabon. Am J Trop Med Hyg. 2018;98:508–15.
26. Nouatin O, Ateba Ngoa U, Ibanez J, Dejon-Agobe JC, Mordmüller B, Edoa JR, et al. Effect of immune regulatory pathways after immunization with GMZ2 malaria vaccine candidate in healthy lifelong malaria-exposed adults. Vaccine. 2020;38:4263–72.
27. Sulyok Z, Fendel R, Eder B, Lorenz F-R, Kc N, Karnahl M, et al. Heterologous protection against malaria by a simple chemoattenuated PfSPZ vaccine regimen in a randomized trial. Nat Commun. 2021;12:2518.
28. Doolan DL, Mu Y, Unal B, Sundaresh S, Hirst S, Valdez C, et al. Profiling humoral immune responses to *P. falciparum* infection with protein microarrays. Proteomics. 2008;8:4680–94.
29. Silver JD, Ritchie ME, Smyth GK. Microarray background correction: maximum likelihood estimation for the normal–exponential convolution. Biostatistics. 2009;10:352–63.
30. Pera A, Campos C, Corona A, Sanchez-Correa B, Tarazona R, Larbi A, et al. CMV latent infection improves CD8+ T response to SEB due to expansion of polyfunctional CD57+ cells in young individuals. PLoS ONE. 2014;9:e88538.
31. Mandala W, Harawa V, Munyenyembe A, Soko M, Longwe H. Optimization of stimulation and staining conditions for intracellular cytokine staining (ICS) for determination of cytokine-producing T cells and monocytes. Curr Res Immunol. 2021;2:184–93.
32. Awate S, Babiuk LA, Mutwiri G. Mechanisms of action of adjuvants. Front Immunol. 2013;4:114.
33. Lousada-Dietrich S, Jogdand PS, Jepsen S, Pinto VV, Ditlev SB, Christiansen M, et al. A synthetic TLR4 agonist formulated in an emulsion enhances humoral and Type 1 cellular immune responses against GMZ2–a GLURP-MSP3 fusion protein malaria vaccine candidate. Vaccine. 2011;29:3284–92.
34. Knudsen NP, Olsen A, Buonsanti C, Follmann F, Zhang Y, Coler RN, et al. Different human vaccine adjuvants promote distinct antigen-independent immunological signatures tailored to different pathogens. Sci Rep. 2016;6:19570.
35. Millington OR, Di Lorenzo C, Phillips RS, Garside P, Brewer JM. Suppression of adaptive immunity to heterologous antigens during *Plasmodium* infection through hemozoin-induced failure of dendritic cell function. J Biol. 2006;5:5.
36. de Jong SE, van Unen V, Manurung MD, Stam KA, Goeman JJ, Jochems SP, et al. Systems analysis and controlled malaria infection in Europeans and Africans elucidate naturally acquired immunity. Nat Immunol. 2021;22:654–65.
37. Jankovic D, Kugler DG, Sher A. IL-10 production by CD4+ effector T cells: a mechanism for self-regulation. Mucosal Immunol. 2010;3:239–46.
38. Triller G, Scally SW, Costa G, Pissarev M, Kreschel C, Bosch A, et al. Natural parasite exposure induces protective human anti-malarial antibodies. Immunity. 2017;47(1197–209):e10.
39. Weiss GE, Crompton PD, Li S, Walsh LA, Moir S, Traore B, et al. Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. J Immunol. 2009;183:2176–82.
40. Amanna IJ, Carlson NE, Slifka MK. Duration of humoral immunity to common viral and vaccine antigens. N Engl J Med. 2007;357:1903–15.
41. Mamani-Matsuda M, Cosma A, Weller S, Faili A, Staib C, Garçon L, et al. The human spleen is a major reservoir for long-lived vaccinia virus–specific memory B cells. Blood. 2008;111:4653–9.
42. Helb DA, Tetteh KK, Felgner PL, Skinner J, Hubbard A, Arinaitwe E, Mayanja-Kizza H, Ssewanyana I, Kamya MR, Beeson JG, Tappero J, Smith DL, Crompton PD, Rosenthal PJ, Dorsey G, Drakeley CJ, Greenhouse B. Novel serologic biomarkers provide accurate estimates of recent Plasmodium falciparum exposure for individuals and communities. Proc Natl Acad Sci U S A. 2015;112(32):E4438–47. https://doi.org/10.1073/pnas.1501705112.
43. Obiero JM, Campo JJ, Scholzen A, Randall A, Bijker EM, Roestenberg M, et al. Antibody biomarkers associated with sterile protection induced by controlled human malaria infection under chloroquine prophylaxis. mSphere. 2019;4:e00027-19.
44. Dent AE, Nakajima R, Liang L, Baum E, Moormann AM, Sumba PO, et al. *Plasmodium falciparum* protein microarray antibody profiles correlate with protection from symptomatic malaria in Kenya. J Infect Dis. 2015;212:1429–38.
45. Felgner PL, Roestenberg M, Liang L, Hung C, Jain A, Pablo J, et al. Pre-erythrocytic antibody profiles induced by controlled human malaria infections in healthy volunteers under chloroquine prophylaxis. Sci Rep. 2013;3:3549.
46. King CL, Davies DH, Felgner P, Baum E, Jain A, Randall A, et al. Biosignatures of exposure/transmission and immunity. Am J Trop Med Hyg. 2015;93:16–27.
47. Kobayashi T, Jain A, Liang L, Obiero JM, Hamapumbu H, Stevenson JC, et al. Distinct antibody signatures associated with different malaria transmission intensities in Zambia and Zimbabwe. mSphere. 2019;4:e00061-19.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

3 DISCUSSION

Since multiparameter flow cytometry (MFC) was developed in the 70's of last century[120], this tool has been instrumental in understanding the cellular compartment of the immune system in human health and disease. To date, the use of this technology makes it possible to analyze subsets of cells with distinctive phenotypes and functions. While phenotyping usually refers to the staining of cell-surface proteins, functional state is assessed by methods such as intracellular cytokine staining assay (ICS)[121], which serve as a marker for the quality of the T-cell response[122]. The ability of exploring vaccine-induced cellular heterogeneity is of particular interest for the assessment and improvement of vaccines. In HIV for instance, the use of MFC to monitor the T cell response has allowed to discriminate surrogates of HIV progression based on HIV-specific CD4+ and/or CD8+ T phenotypes[123]. Indeed, the study of the cellular responses against Tuberculosis has contributed to generate a diagnostic method based on the *in vitro* stimulation of PBMCs and the release of IFN-γ (IGRA)[124]. Moreover, different phenotypes identified by MFC potentially may serve as biomarkers for the next generation of diagnostics[125]. Likewise, in *Leishmania major* MFC showed that vaccine-induced CD4+ T cell producing Th1 cytokines (IFN-γ, IL2 and TNF-α) is a valuable correlate of protection in mice[126].

In addition, novel technologies combining flow cytometry with either three-dimensional imaging[127], mass spectrometry[99], single-cell transcriptomics[128], or single-cell metabolic analysis[129], are extending the understanding of the circulating cellular compartments. Furthermore, the combination of these new technologies provides highly dimensional data that may transform the understanding of vaccine-induced immunity, enabling for example, the prediction of the vaccine-induced immunogenicity through the early epigenetic reprogramming induced in myeloid cells[128,130], or extending our knowledge about the innate immune memory[131]. These technologies will progressively expand available data exponentially and will be combined further with other "-omics" platforms. From this rich data, approaches such as "Network Vaccinology" may lead to the creation of new and improved diagnostics, therapies, and vaccinations utilizing data from data repositories and knowledge of genetics, metabolism, system structure, and function[132]. Of note, those technologies are available and used mostly in high income economies nowadays, and a more global access is currently not evident.

What is likely though, is that much more laboratories, including those in malaria-endemic regions, will further investigate the in-depth immune profiling advanced by the single-cell analysis by mass spectrometry and more routine laboratory techniques including MFC.

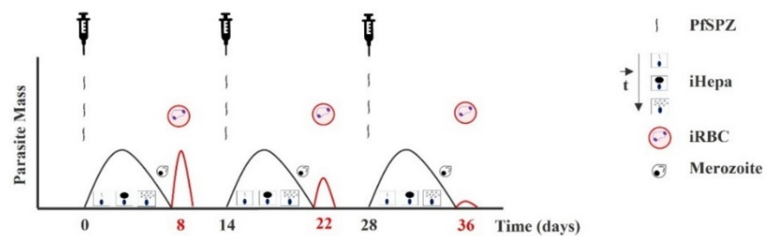
3.1 Influence of malaria life cycle stages on immune responses to the *Plasmodium falciparum* sporozoite chemoprophylaxis vaccine and parasite-specific T cell activation as biomarker for vaccine efficacy

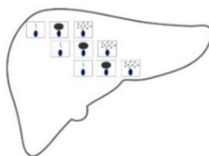
To date, most of the prophylactic vaccines available are optimized to lead to the production of neutralizing antibodies[133], and boosting doses are normally required to maintain a sufficient concentration in the serum. Besides antibodies, both natural infections and vaccines induce cellular memory responses such as cytotoxic CD8+ T cells and CD4+ T helper cells.

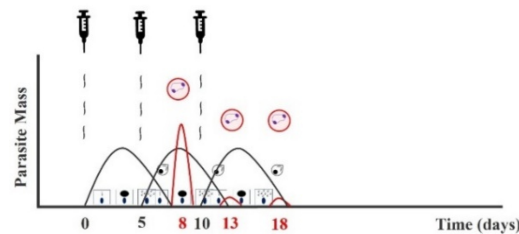
In the protection against malaria induced by malaria sporozoites, CD3+ T cell responses against multiple antigens[80] seem to play a significant role. Evidence for this comes from studies in mice[134] and nonhuman primates[71] intravenously immunized with sporozoites. In such studies, protection against infection with malaria parasites could be attained by inducing significant numbers of sporozoite-specific CD8+ T liver-resident cells. However, tissue-resident T cells are unlikely to leave the tissue where they belong[135], and therefore it is rather difficult to find them in peripheral blood, and thereby using them as reliable correlates or surrogates of protection. Several clinical trials have shown that CD4+ T cells producing inflammatory cytokines such as IFN-γ, IL2 and TNF-α (Th1), are consistently expanded in the periphery of malaria-naïve human subjects immunized with sporozoites, although only few of these studies have shown that such expansion correlates with protection[136]. A trial performed at the Institut für Tropenmedizin, Reisemedizin und Humanparasitologie (Tübingen) (TÜCHMI-002 trial, NCT02115516), showed that the generation of sterile protection against *P. falciparum* sporozoites (PfSPZ) was associated with higher frequencies of Pf-specific memory CD4+ Th1 cells. In this trial volunteers were immunized with fully infectious PfSPZ under chemoprophylaxis (PfSPZ-CVac). To achieve a 100% protection (9/9), three doses of 51,200 PfSPZ delivered intravenously at 4 weeks interval under chloroquine prophylaxis to avoid the Pf blood infection were required, whereas lower doses led to partial protection. The study found that expansion of reactive CD4+ T cells was dose-dependent and protection was associated with high numbers of polyfunctional CD4+ T cells (producing IFN-γ, IL-2 and TNF-α), whereas development of malaria was associated with CD4+ T cells producing TNF-α only [75].

Importantly, an exploratory analysis demonstrated that sterile protection was potentially distinguishable observing the expansion of CD4+ Th1 cells stimulated with malaria infected erythrocytes (iRBCs) shortly before CHMI (~10 weeks after the third vaccination).

In the first chapter, I hypothesized that systematic monitoring of pro-inflammatory malaria specific Th1 cell (CD40L+CD4+ T) circulating during or following immunization by MFC predicts protection against CHMI. To test this hypothesis, I obtained samples from participants enrolled in the second part of the TÜCHMI-002 trial that was designed to assess shortened immunization regimens with the optimal dose of 51,200 PfSPZ. Here, two groups of healthy malaria naive European young adults were immunized three times with 51,200 PfSPZ while receiving chemoprophylaxis with chloroquine (PfSPZ-CVac). The first group received three vaccinations 14 days apart (28-day regimen) and the second group received three doses every 5 days (10-day regimen). Thereby, this part of TÜCHMI-002 trial enabled us to analyse the influence of the time in between vaccinations in the generation of protective immune responses. The 28-day-regimen led to three distinct liver infection events of 6.5 days, each. The 10-day-regimen results in 16 consecutive days of liver infection (10-day regimen) [*Figure 3*].







<u>Figure 3</u>. ***TÜCHMI002 second arm overview****. The second arm of the PfSPZ-CVac clinical trial evaluated two condensed immunization schedules that either included three different instances of six days under malaria infection (28 days) or sixteen consecutive days of continued exposure to malaria antigens (10 days) (NCT02115516). Cartoon myth: Plasmodium falciparum sporozoites (PfSPZ). Hepatocytes infected over time (iHepa). Infected red blood cells (iRBC). Immunizations were carried out while receiving chemoprophylaxis with chloroquine (CQ). To sustain the treatment regimen against malaria blood stages, each participant got a CQ loading dose of 10 mg/kg two days prior to their first immunization and an additional CQ pill of 5 mg/kg every week. The bottom axis displays the anticipated waves of infected red blood cells (iRBCs) over the course of the 7-9-day prepatent liver phase cycle.*

Source : Ibanez, J., Fendel, R., Lorenz, FR. et al. npj Vaccines 7, 59 (2022).

© 2022 The Author(s). Published by Nature in partnership with the Sealy Institute for Vaccine Sciences.

The antigen reactive enrichment methodology (ARTE[137]) was used to monitor antigen-specific T cells during immunization and the day before the challenge (C-1). This method uses magnetic cell separation to enrich reactive (CD40L+) cells to be able to type antigen specific CD4+ T cells which occur at low numbers ($10^{-7/-4}$ in the naïve population, and $10^{-5/-2}$ in the memory population). By this method of enrichment, antigen-specific cells in both naive and memory CD4+ T cell compartments can be detected and characterized e.g., by measuring intracellular cytokine profiles of activated cells. A major advantage compared with methods using tetramers is that HLA typing and *a priori* knowledge about the recognized antigen is not required.

A previous experiment without prior enrichment showed that the amount of antigen-specific cells producing TNF-α and IFN-γ can distinguish between protected and unprotected vaccinees before CHMI[75]. I hypothesized that stimulation with iRBC also leads to expansion of CD40L+CD4+ T cells with other phenotypes, producing different cytokines, though at a lower number. For instance, in yet another study iRBC stimulation showed and association between the production of IL4 and protection following immunization with irradiated sporozoites[138].

In similar fashion, iRBC-induced IL17+ malaria-specific cells were reported in Malians[139], although it is unknown whether this cell type can be detected in PfSPZ vaccination of malaria-naïve subjects. If IL-17+ T cells play a role in protection in malaria is also not known.

As a result of monitoring *P. falciparum* specific CD40L+ enriched CD4+ T cells, I made three main observations; 1) Condensing the vaccination regimen from 28 to 10 days did not influence the frequency of pro-inflammatory memory (CD45RO+) cells during the immunization phase. Interestingly, similar frequencies of T cells producing IFN-γ and TNF-α were found at 14 (28-day regimen) or 15 days (10-day regimen) after first immunization, regardless of the three times higher cumulative number of PfSPZ in the 10-day regimen at this timepoint (51,200 infective sporozoites in the 28-day and 153,600 in the 10-day regimen). 2) The proinflammatory CD40L+CD4+ T frequency at C-1 was different: The 28-day regimen tended to promote less TNF-α+ IFN-γ+ effector memory T cells (TEM, CD45RO+CCR7-) compared to the condensed 10-day regimen but higher frequency of single TNF-α+ central memory T cells (TCM, CD45RO+CCR7+). In addition, the 10-day regimen resulted in higher frequencies of IFN-γ+ single TEM than the 28-day regimen. 3) During the immunization phase, the proinflammatory IL17+ CD40L+CD4+ T cell frequency detected by ARTE was about $10\text{-}10^2$ times lower than single TNF-α+, IFN-γ+ or TNF-α+ IFN-γ + CD40L+CD4+ T cell frequencies.

A side-by-side comparison between trials is difficult given the differences regarding trial design, methods and MFC antibody staining panels used to characterize the vaccine-induced T cell compartment. The implementation of highly standardised CHMI in the last years has favoured the exploration of different regimens of PfSPZ-CVac and other vaccines and made it possible to validate biomarkers such as T cell activation patterns. Interestingly, one study conducted in US (NCT02773979)[140] based on the TÜCHMI-002 results, compared two PfSPZ-CVac regimens of twenty-one days (3 doses of 51,200 or $1.024\text{x}10^5$ PfSPZ. One dose/week) and ten days (3 doses of $1.024\text{x}10^5$ PfSPZ. One dose/each 5 days). They found no correlation between the frequency of memory CD4+ T producing TNF-α, IFN-γ, or IL2 and vaccine efficacy (VE). Similar to my findings in the TÜCHMI-002 trial, the Th1 cell frequency induced by the 10-day regimen in the US study also tended to be higher in protected vaccinees.

Intriguingly, VE was absent following the 21-day regimen whereas it reached a 75% following the 10-day regimen. We therefore assume that emerging blood stages malaria parasites have a negative effect on VE when they coincide in time with the PfSPZ administration.

In the 28-day or longer regimens, blood stage is cleared at the time of second and third vaccination, whereas in the 10-day regimen parasites are not yet emerged when the second vaccination is done and are at a nadir during the third vaccination. All those regimens show high-level vaccine efficacy when the PfSPZ dose is ≥ $5x10^4$ sporozoites (67% for 28-day and 63% for 10-day).

Once differentiated, memory TCM and TEM cells are distributed towards different tissue sites across the human body. The CCR7 surface marker expressed in the TCM subset, is a homing receptor for lymph nodes, which allow the circulation through the lymphoid tissues, whereas TEM cells are rather migrating to peripheral tissue areas[141]. Different models have been developed to explain the lineage relationship between TCM, TEM and naïve T cells. Studies in mice support that the strength of the antigen exposure influences the polarization from TCM to TEM[142]. Thereby, the antigen strength, which can translate to the amount of antigen (vaccine dose) and the extension in time length of the antigen exposure, may explain the observed differences between regimens. Strikingly, the antibody profile assessed by protein microarray showed that prolonged continuous antigen exposure, induced by the TÜCHMI-002 10-day regimen also led to a distinct humoral response pattern. Levels of IgG antibodies targeting malaria proteins expressed at late liver stage (e.g., PHIS Tb, ApiAP2 or MSP10) were about 4-6 times higher compared to the 28-day regimen. In addition to the initial part of TÜCHMI-002 arm where the increment of the PfSPZ dose was behind the generation of sterile immunity [75], another example illustrating the importance of the antigen strength was given by the cited US study (NCT02773979). There, a two-fold increase in the PfSPZ dose used to immunize malaria-naïve volunteers with the same 10-day regimen as in TÜCHMI-002, led to higher VE (63% vs 75%). As the number of volunteers was low in both studies, this finding will need confirmation in other studies. Interestingly, when both dose and exposure factors were combined in a novel PfSPZ-CVac regimen VE was even higher (77%)[143]. In this study, immunization consisted in three vaccinations of $1.1x10^5$ PfSPZ intravenously inoculated at days 1,6, and 28. Chloroquine was the chemoprophylactic drug of choice and it was administered orally at same time that volunteers received vaccination. Following that schedule, for instance the IgG antibodies targeting malaria proteins expressed at liver stage (e.g., CSP, LSA-1 or MSA 180) were about 6-8 times higher compared to placebos[143].

Whether the PfSPZ-CVac-induced TEM cells detected by ARTE traffic to the liver to become liver-resident T cells remains unknown as human liver samples are difficult to obtain in the framework of such a study.

Based on data from mice and nonhuman primates immunized with irradiated PfSPZ, human CD40LCD4+ T cells are thought to primarily traffic to the spleen to "license" DCs enabling the CD8+T cell activation through direct DC-CD8+ T cell interaction through the "immunological synapse" and/or CD4+ T-DC-CD8+ T cell cross presentation[136]. Intriguingly, it is not well investigated the CD40LCD4+ T memory cell dynamics after subsequent exposure with the same malaria cognate antigens through PfSPZ vaccination.

Regarding the immune trafficking during vaccination phase, results provided by ARTE may be incomplete by the fact that migration through the lymphatic vessels could not be monitored. This could be important since the CCR7 ligand is expressed in lymphatic vessels[144], and potentially could be an alternative track for the PfSPZ-CVac-induced TCM cells to reach the infected tissues or draining lymph nodes. Thus, the underestimation of the lymph vessel trafficking is one of the possible explanations for the lack of correlation between VE and data about iRBC-specific CD4+ T frequency before CHMI. Studies in mice usually focused on CD8+ T cells and do not provide evidence for malaria specific TCM cells[145]. Although the mouse strain seems to influence the frequency of CD8+TEM and TCM cells when radio-attenuated experiments are performed using *P. berghei* or *P. yoelii*[146]. TEM but not TCM cells are related with protection against sporozoite infection[146]. For instance, another study in mice immunized with radio-attenuated *P. berghei* sporozoites as immunogen showed that most malaria-specific CD8+ T cells found in the liver after immunization responding quickly against sporozoite challenge (~96%) were TEMs and not TCMs[147].

Therefore, liver-resident CD8+ T cells induced by PfSPZ vaccination are likely a good candidate for a surrogate marker for protection against CHMI. PfSPZ-CVac-induced Th1-TEM cells could also have an indirect role in protection through activation of infected hepatocytes[148] as well as other immune cells residing in the liver such as NK- and NKT cells[149]. Of note, in PfSPZ-CVac the number of blood stage parasites usually decreases after second and third vaccinations[75,143,150]. This decrease may already be an outcome of the built up of the immune response and has been proposed as a biomarker of protection. As other attenuation methods (e.g., irradiation and most other genetic attenuation strategies) do not allow for blood stage parasites to occur, this candidate surrogate endpoint is of limited use, even in the field of whole sporozoite vaccine approaches.

Although the mechanisms controlling human memory T cell homeostasis are not fully elucidated, experiments assessing the half-life of human T cells show that human CD4+ live on average shorter than CD8+ T cells and TEM shorter than TCM (few months to one year, respectively)[151]. In that respect, a bias towards TEM or TCM through vaccination may have a direct implication for developing the best immunization approach. In particular, longevity of protection could be influenced. For instance, one study demonstrated about one year (59 weeks) of protection against CHMI in individuals intravenously immunized with 4 doses of 2.7×10^5 PfSPZ Vaccine [81]. Unfortunately, it was not investigated which kind of T cell memory was primarily present in those who were protected. Malaria-specific CD4+ T cells producing TNF-α, IFN-γ, and/or IL2 reached the highest frequency in the periphery one month after the first immunization and subsequent immunization rounds did not increase this peak anymore. Further characterization using the intracellular proliferation marker Ki-67, demonstrated that these cells followed an intense cycle of replication about one week after immunization. Interestingly, it was shown that proliferation of malaria-specific memory CD4+ T cells was associated with the concentration of anti-malarial antibodies after CHMI: When anti-malarial antibodies concentration was still high shortly after first CHMI, the frequency of malaria-specific memory Ki-67+ CD4+ T cells in the periphery was low. Contrastingly, higher frequency of malaria-specific memory Ki-67+ CD4+ T cells was detected at the time that anti-malarial antibodies concentration was lower[81]. In this context, one study using the live-attenuated YF-17D vaccine against yellow fever also showed that the frequency of the circulating YF-17D-specific Ki-67+ CD8+ T cells not only depends on the initial dose but also that such response saturated with increasing virus dose[152]. In the same study, it was also reported that activation of genes distinctive for TEM correlated with viral load.

Another study showed that the sterile immunity generated following an intravenous three-dose regimen (9×10^5 PfSPZ Vaccine) given each 8 weeks, is durable for about 33 weeks[153] at least. Durability has been proven also in one CPS study. There, protected subjects immunized by the exposure to malaria-infected mosquitos under chloroquine prophylaxis, remained malaria negative following another CHMI 28 months after initial immunization[154].

IFN-γ+ CD4+ and CD8+ T cell kinetics following vaccination with a live-attenuated smallpox vaccine [155] suggest shared patterns for CD4+ and CD8+ T cell responses induced by several live-attenuated vaccines.

However, only few vaccines induce very long protection (> 25 years). One example is YF-17D, where this phenotype is neither associated with TEM nor with TCM but with another cell type – “stem memory T cells” (TSCM)[156]. Generation of such long-term responses may be the goal of later generation of PfSPZ-based vaccines.

In the TÜCHMI-002 study, the accelerated 10-day regimen demonstrated an equivalent vaccine efficacy level than the 28-day (~63% vs ~67%) in malaria naïve subjects challenged 10 week after last immunization. Although no further monitoring of the PfSPZ-specific memory Th1 frequencies following CHMI, protection was related with IgG antibodies targeting proteins expressed in later stages of the hepatic malaria parasite such as the merozoite surface protein 5 (MSP5). Given that the 10-day regimen showed 4 times higher levels of IgG antibodies targeting other proteins of the MSP family such -8 or -10 is therefore expected that durability will be longer than the one offered by the 28-day regimen.

The short 10-day regimen has advantages over other PfSPZ-CVac regimens. It is more realistic in terms of daily practice at the clinic while reducing chloroquine intake from weeks or months to ten days. This reduces adverse reactions against chloroquine and simplifies the schedule for the vaccinee. However, based on immunological considerations, it would be possible that PfSPZ-CVac-induced TEM cell mediated protection acquired following the 10-day regimen could decline faster than following longer PfSPZ-CVac regimens. This clearly needs more investigation. In particular, when children and people from endemic areas are immunized.

In conclusion, the frequency of circulating proinflammatory parasite-specific TNF-α+ IFN-γ+ memory CD40L+CD4+ T cells did not show to be a reproducible predictor of vaccine efficacy in PfSPZ-CVac. On the other hand, condensed vaccination schedules are novel in the optimization of PfSPZ-CVac and have contributed to guide further clinical trials [143,157,158]. Together with CHMI as a tool of validation, the understanding about the human immunity against malaria is expected to increase in the future facilitating not only the optimization but also the design of better malaria vaccines.

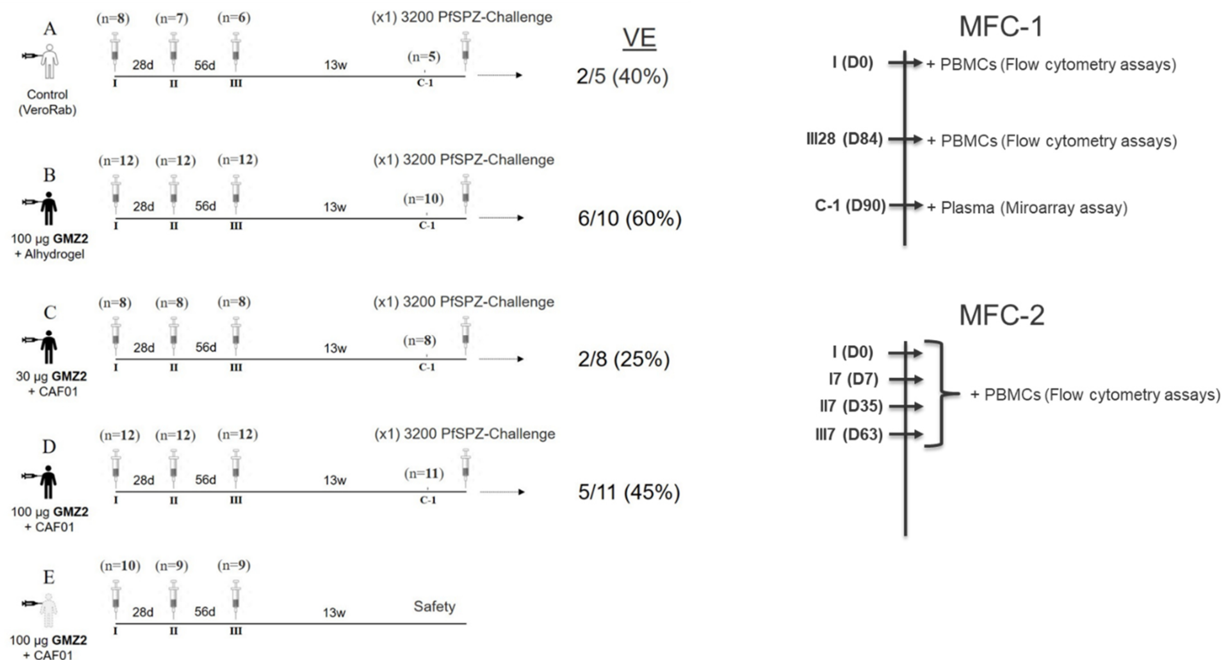
3.2 Dissecting cellular responses in semi-immune Africans vaccinated with the GMZ2 malaria vaccine candidate in a Controlled Human Malaria Infection trial.

People living in sub-Saharan Africa are frequently exposed to malaria parasites. Repeated infections during the first years pose a significant risk of severe disease but also contribute to the development of protective immune mechanisms which reduce morbidity and mortality later in life. Natural acquired immunity (NAI) against malaria is still not fully understood. Repeated exposure is important to maintain NAI at a high level[159]. Interestingly, chronic malaria exposure has been linked to elevated numbers of atypical memory B cells capable of secreting polyreactive anti-malarial neutralizing antibodies[160] and autoantibodies[161].

NAI is mediated by both antibody[162] and cellular[163] responses to several malaria proteins. Two of these proteins—merozoite surface protein 3 (MSP3) and glutamate rich protein (GLURP), which are both significantly produced in the blood stage of malaria—have been utilized as immunogens for potential malaria vaccines[164]. A fusion of two fragments of these proteins ($GLURP_{27-500}$ and $MSP3_{212-380}$) constitutes the backbone of the GMZ2 malaria vaccine candidate. GMZ2 adjuvanted with Alhydrogel adjuvant (Alum) has been shown to be immunogenic in malaria-naïve German and Gabonese vaccinees[165,166].

The goal of this chapter was to investigate potential associations between B and T cell phenotypes which may contribute to explain the outcome of the GMZ2-CAF01 clinical trial (PACTR201503001038304)[167]. Four different panels for MFC were designed to cover a variety of circulating CD19+ (B) and CD4+ (T) cell phenotypes. The goal of the GMZ2-CAF01 clinical trial, which was undertaken in Lambaréné (Gabon), was to assess the safety, tolerability, immunogenicity, and effectiveness of CAF01 and alum as adjuvants for the malaria vaccine candidate GMZ2. The study involved healthy adult African volunteers. CAF01 is a novel adjuvant made out of two molecules: A liposome (dimethyldioctadecyl-ammonium; DDA) and a synthetic version of the cord factor found in the cell walls of *Mycobacterium tuberculosis* (trehalose 6,6-dibehenate; TDB). Preclinical studies using GMZ2-CAF01 demonstrated superior immunogenicity that GMZ2-Alum[168]. Furthermore, CAF01 polarized the CD4+ T cell response towards Th1 and Th17[168]. The trial was motivated by the findings of a phase II trial, where children reaching high antibody response (Anti-GMZ2 IgG concentration) showed high vaccine efficacy levels[169].

Volunteers were allocated to one of 5 groups. 4/5 underwent CHMI about 13 weeks after last immunization. Group 1 received the rabies vaccine as control, group 2; 100µg GMZ2 + Alum, group 3; 30µg GMZ2 + CAF01, group 4 and group 5; 100µg GMZ2 + CAF01. Group 5 did not undergo CHMI to be able to identify late reactions to the vaccine. All vaccinations were administered intramuscularly, at time 0, and days 28 and 56. The study showed that GMZ2 was immunogenic, and antibody responses were comparable between groups immunized with formulations irrespective of the adjuvant (Alum or CAF01).



<u>Figure 4.</u> ***GMZ2-CAF01 workflow overview****. Left side figure illustrates the summary of the groups allocated within the clinical trial (Pan-African Clinical Trials: PACTR201503001038304) including group allocation, immunization schedule, and vaccine efficacy (VE) following controlled human malaria infection (CHMI). Two independent immunological analyses were conducted using study samples (Frozen PBMCs). In both investigations, multiparameter flow cytometry (MFC) was used to characterized T- and B-cell immune responses. In MFC-1, resting PBMCs were stimulated in vitro with the vaccine immunogen GMZ2 whereas in MFC-2 they were not. In MFC-1, the following time points were analyzed: Day of the first immunization (I, day 0), and twenty-eight days after third immunization (III28, day 84 of the study trial). Additionally, plasma samples were taken one day before challenge (C-1) to measure the antibody breadth response by protein-microarray. MFC-2 was focused on tracing T- and B-phenotype kinetics during immunization at baseline (I, day 0), and seven days after vaccination (days 7, 35 and 63 of the study trial).*

Source : Nouatin, O., Ibáñez, J., Fendel, R. et al. Malar J 21, 191 (2022).

© 2022 The Author(s). Published by Malaria Journal

Surprisingly, the first set of experiments conducted using frozen PBMCs comparing baseline cell frequencies with those acquired one month after the last immunization [MFC-2, *Figure 4*], showed that immunization with GMZ2 did not induce the expansion of pro-inflammatory CD4+ T cell or B cell responses in any of the interventional groups. Interestingly, further analyses pooling down all GMZ2 immunized study volunteers, revealed the increased frequency of anti-inflammatory CD4+T cells producing IL10 after vaccination (day 84) while a separated investigation on the same study trial showed increased concentrations of the soluble immune suppressor molecule HLA-G acquired during the immunization period[170].

A malaria protein microarray performed at baseline demonstrated that parasite-specific immunoglobulin G (IgG) acquired breadth predicted protection during CHMI better than the vaccine-induced anti-GMZ2 IgG titer in serum following vaccination[171].

In a second round of experiments also conducted on frozen samples [MFC-2, *Figure 4*, in preparation], my main interest was to investigate the role of circulating follicular T helper (cT_{FH}), and the B memory subsets during the immunization phase and if they play a role in the low level of vaccine efficacy of the two GMZ2 vaccine formulations. The kinetics of both circulating T and B phenotypes were monitored by MFC at baseline, and every 7 days after each immunization.

Follicular T Helper cells (T_{FH}) are known to play a crucial role in helping B cells through the formation of germinal centres within secondary lymph organs (e.g., spleen, lymph nodes). Important aspects of the humoral immune response such as the isotype class switching, antibody affinity maturation[172], or the production of both B cell memory cells (MBC) and plasma cells (PC) [173], depend on the T_{FH} -B cross-talk. Interestingly, CD4+ T cells committed to follicular helper lineage recirculate in the blood after B cell cross-talk as circulating T_{FH} (cT_{FH})[174]. The number of activated cT_{FH}, which are mobilized into the blood after immunization, has been proposed as correlate of vaccine protection[175].

The two tested GMZ2 vaccine formulations using two adjuvants can lead to different immune polarization (Th2 for Alum and Th1/Th17 for CAF01)[176]. I hypothesized that monitoring the cT_{FH} may show differences between the two adjuvants and show if CAF01 can modulated the immune response distinctly from alum.

The results extended previous observations but differences between immune responses between to the two vaccine formulations could not be detected.

At the cellular level, the first two immunizations were characterized by increased numbers of the cT_{FH}-2 effector memory cells (CXCR3-CCR6-), plasma cells (CD24-CD38hi), and double negative memory B cells (IgD-CD27-; DN) irrespective of any vaccine intervention. Likewise, expansion of activated (ICOS+/PD-1+), CXCR5+ CXCR3+CCR6+ T_{FH} (cT_{FH}-17.1) cell subset was also induced by vaccination although only after vaccine priming dose. Intriguingly, further analyses looking at the cT_{FH}-17.1 median fold change demonstrated an association between the strong expansion of effector memory the cT_{FH}-17.1 cells and the clinical onset of malaria after CHMI. This finding was unexpected.

Recent investigations aiming to understand the natural acquired immunity displayed by Gabonese adults identified a CD4+ T cell phenotype (CD161+) in response to the intravenous exposure to PfSPZ. Since CD161+CD4+ T cells can produce both IL17[177] and IFN-γ[99], I speculate with the idea that potentially could be the same cT_{FH}-17.1 here described [99].

Beyond, high numbers of CD161+CD4+ T cells were associated with the natural ability of controlling malaria infection[99]. Circulating effector memory cT_{FH}-1 subset (IFN-γ+) has been found to be associated with acute malaria episodes in Malian children. This finding suggests a negative role for Th1-like responses and may indicate a detrimental effect over the antibody response[178]. Following experiments using PBMCs from Malian children stimulated with iRBC demonstrated that CXCR3- cT_{FH} cells (cT_{FH}-2 and cT_{FH}-17) are better in providing help to B cells[178]. Thus, the moderate rise of the cT_{FH}-2 effector memory cell frequency may be a consequence of the immune modulation provided earlier by cT_{FH}-17.1.

Little is known about the cT_{FH}-17.1 subset. High frequencies of non-circulating T_{FH}-17.1 cells producing IFN-γ found in bronchoalveolar lavages of pulmonary sarcoidosis patients, have been linked to the plasticity of T_{FH} cells to transition from T_{FH}-17 to T_{FH}-1 under intense inflammation [179]. Furthermore, high frequencies of cT_{FH}-17.1 cells have been described to play a role in multiple sclerosis (MS) pathogenesis. In one study on the first line treatment for relapsing–remitting MS (RRMS) it was shown that untreated patients had higher frequencies of cT_{FH}-17.1 cells in peripheral blood compared to treated patients[180]. In the same study, patients treated for 6-12 months treatment showed a gradual increase the frequency of cT_{FH}-2 while decreasing for the cT_{FH}-17.1 and cT_{FH}-1 subsets.

DN B cells have been characterized in malaria, although mainly in autoimmune disorders like the systemic lupus erythematosus (SLE)[181]. In SLE patients, the DN B cells have been shown to react as autoreactive antigen secreting cells in response to TLR7 agonists[182]. Interestingly, one domain of the malaria Pf Erythrocyte Membrane Protein 1 (PfEMP1) has been characterized for driving polyclonal B cell activation through the increased expression of TLR7 and 10 in PBMCs isolated from European volunteers[183]. Within the CD27- B cells, the CD21- atypical memory B cell subset (Aty MBC) has been earlier detected in Gabonese volunteers[160]. Aty MBCs subset has been postulated as a biomarker of malaria exposure given that adults tend to accumulate more of these cells than children[184]. In here, although the CD27 marker was not included, the rapid increment in circulating DN B cells detected at day 7 after first immunization, was sustained over time as much as seven days after the third immunization (day 63), suggesting memory traits. In contrast to plasma cells, DN B cell frequency increment did not show to be associated with malaria outcome, and both protected, and no protected volunteers showed similar cell kinetics. Interestingly, studies in SLE have shown that DN B cells differentiated from an activated B cell precursor can further developed into extra follicular plasma cells. Moreover, investigations in healthy volunteers receiving vaccinations against influenza or thick borne encephalitis virus have shown coincidental increments of circulating DN B cells and plasma cells after immunization (day 7) in agreement with the kinetics studied here[181]. Thus, it seems that chronic exposure to malaria infections occurring in hyperendemic areas of the globe as Gabon, naturally tend to accumulate these cell subsets as an imperfect mechanism to reduce morbidity in adults, thus benefiting faster over quality responses.

An important explanation for the strong polarization towards anti-inflammatory immune responses and the nested modest results seen for the GMZ2 vaccine, may also be given by the frequent helminth coinfection occurring in Africa.[185] In that respect, hookworm infections have been associated with high levels of IL10[185,186] and may explain the IL10+CD4+ T cell results reported here. Confusingly, not all helminth species modulate equally the immune response. For instance, prior studies showed contrasting humoral responses against *Plasmodium falciparum* gametocytes when *Ascaris lumbricoides* or *Trichuris trichura* monoinfection was carried by Gabonese volunteers[187]. Furthermore, infection with another type of helminth, the *Schistosoma haematobium* trematode, has been associated with a positive effect over malaria infections[188].

Importantly, the analysis of the influence of helminth infections over study outcome, suggested that infection with either *Schistosoma haematobium* or the hookworm, *Strongyloides stercolaris* negatively affected the absence of clinical malaria after CHMI.

Collectively, this chapter underlined the difficulties to find a good cellular immune correlate of GMZ2-induced protection among the CD4+T, and B cell clusters, as much as developing a malaria vaccine for malaria-endemic regions. Moreover, cellular features potentially linking individuals highly exposed to malaria with autoimmune diseases brings back old reminiscences of the hygiene hypothesis[189] permitting to highlight the importance of investing in malaria research as a pathway to break down not only malaria but also other undesirable diseases.

In conclusion, although it is expected that the CHMI tool as well as other cutting-edge technologies will accelerate the next generation of malaria vaccines, only clinical trials conducted in endemic countries will truly show the potential of vaccine candidates to become licensed as well as impacting the fight against malaria in the world.

4 SUMMARY

Despite being treatable and preventable, malaria kills about half a million people each year, mostly in Africa. Notwithstanding many attempts to develop a malaria vaccine, only the vaccine RTS,S/AS01 (Mosquirix), has been fully developed and was recommended by the World Health Organization for use in children in malaria endemic areas on October 6th in 2021. RTS,S is a subunit vaccine and provides only partial protection. Manufacturing of pharmaceutical grade Plasmodium falciparum (Pf) sporozoites (SPZ) has been achieved in the past decade, making the development of plasmodial whole cell vaccines possible. This may be a novel and better platform to design malaria vaccines. In addition, the availability of cryopreserved infectious sporozoites has boosted possibilities to assess vaccine efficacy by using controlled human malaria infection (CHMI) trials. The most effective vaccination strategy to date is to inoculate PfSPZ while using chemoprophylactic antimalarials such as chloroquine – the PfSPZ Chemoprophylaxis Vaccine (PfSPZ-CVac). Although CHMI can test vaccine efficacy (VE), the immune mechanisms underneath are not fully understood. Particularly, the cellular immune response is poorly characterised compared to the humoral response (i.e. antibody titers). Thus, the systematic monitoring of cellular immune responses during immunization in combination with CHMI might be a valuable step to guide the clinical development of malaria vaccine candidates and identify immune mechanisms causally related to protection.

In Chapter 1, I investigated the influence of two accelerated regimens of PfSPZ-CVac on the generation of pro-inflammatory Pf-specific CD4+ T helper cells and their suitability as a surrogate of protection. The trial was conducted in healthy, malaria-naïve adults in Tübingen. To detect antigen-specific cells in peripheral blood with increased sensitivity, Plasmodium-specific T cells were enriched by magnetic-activated cell sorting followed by staining and multiparameter flow cytometry (MFC). Measurements were done using the flow cytometer FACS Canto II and stimulations were done with infected red blood cells (iRBCs). Uninfected red blood cells and Staphylococcal enterotoxin B (SEB) were also as negative and positive controls, respectively. Unexpectedly, I found that a condensed vaccination schedule, where the three vaccinations were given within 10 days induced higher frequencies of Plasmodium-specific CD40L+CD4+ TNF-α+/IFN-γ+ cells than a 28-day regimen. The response was also qualitatively different. The shorter regimen led to a polarized Th1 response with more

CD45RO+CCR7- effector memory T cells. This may lead to higher numbers of memory cells in the liver.

A tendency towards higher frequencies of specific CD40L+CD4+ TNF-α+/IFN-γ+ effector memory T cells was present in protected volunteers but no reliable correlate of protection could be identified. Future research will be needed to identify effector and regulatory responses that predict vaccine efficacy. It will be particularly important to include the characterization of Plasmodium-specific cytotoxic T cells.

More than 94% of the estimated malaria cases globally occur in Africa. Therefore, every malaria vaccine must be tested in malaria exposed volunteers to be sure that it has a significant public health impact. Unfortunately, for many vaccines, VE is lower in Africa. This effect is particularly strong in malaria vaccines. The GMZ2 vaccine was developed to prevent malaria and its complications by mimicking naturally acquired immunity. The vaccine antigen consists of a fusion protein between fragments of the merozoite surface protein-3 and the glutamate-rich protein. In Chapter 2, I investigated the immunogenicity of GMZ2 adjuvanted with two different immune modulators: Alhydrogel or CAF01. The study was performed in healthy, adult, lifelong malaria-exposed volunteers from Lambaréné, Gabon. MFC was used to systematically measure the T and B cell response, and to compare immune response patterns before and after immunization. Peripheral blood mononuclear cells were cryopreserved and measured upon completion of the trial using a Sony SP6800 Spectral Analyzer. Monitoring of GMZ2-stimulated CD4+ T, and CD20+ B cells showed that GMZ2 did not induce significant immune responses beyond the baseline. The low response to vaccination was unexpected as was the similar performance of the two adjuvants. VE in CHMI was similar between the groups, hence these findings may be expected. Notwithstanding the negative result, this study will help guiding the development of the next generation of blood stage malaria vaccines in malaria-exposed volunteers, where baseline responses play a major role in generating successful immune responses following vaccination.

In summary, the work presented as part of the thesis shows that systematic monitoring of the cellular immune responses by MFC, in combination with CHMI studies is a valid, and stringent approach to measure VE and identify correlates of protection, surrogate markers, and the effect of schedule and pre-existing immunity on vaccine responses. More work will be required to replace CHMI with immunological surrogate endpoints and understand vaccine-induced antimalarial immunity.

5 ZUSAMMENFASSUNG

An Malaria sterben jedes Jahr etwa eine halbe Million Menschen, vor allem in Afrika, obwohl die Erkrankung behandelbar und vermeidbar ist. Trotz zahlreicher Versuche, einen Malaria-Impfstoff zu entwickeln, wurde nur der Impfstoff RTS, S/AS01 (Mosquirix) von der Weltgesundheitsorganisation am 6. Oktober 2021 für die Anwendung bei Kindern in Malaria-Endemiegebieten empfohlen. RTS,S ist ein Proteinimpfstoff und hat eine Wirksamkeit vom etwa 30%. Ein weiterer Ansatz ist die Impfung mit Plasmodium falciparum (Pf) Sporozoiten (SPZ). In der letzten Dekade gelang es erstmals PfSPZ in pharmazeutischer Qualität herzustellen und tiefgefroren aufzubewahren. Dies macht die Anwendung von PfSPZ als hochwirksame Impfstoffe möglich. Darüber hinaus hat die Verfügbarkeit kryokonservierter infektiöser Sporozoiten die Möglichkeiten zur Bestimmung der Impfstoffwirksamkeit durch kontrollierte Malariainfektion des Menschen (controlled human malaria infection – CHMI) verbessert. Die bisher wirksamste Impfstrategie besteht darin, PfSPZ zu inokulieren und gleichzeitig chemoprophylaktisch wirksame Medikamente wie Chloroquin zu verwenden. Dieser Ansatz wird PfSPZ Chemoprophylaxis Vaccine (PfSPZ-CVac) genannt. Mittels CHMI kann die Wirksamkeit eines Impfstoffs getestet werden, die zugrundeliegenden Immunmechanismen sind jedoch nicht vollständig geklärt. Insbesondere die zelluläre Immunantwort ist nicht gut charakterisiert. Die humorale Antwort (Antikörpertiter) ist dagegen detailliert untersucht. Daher könnte eine systematische Messung der zellulären Immunantwort während der Immunisierung in Kombination mit einer CHMI ein wertvoller Schritt sein, um die Entwicklung von Malaria-Impfstoffkandidaten zu optimieren und kausale Zusammenhänge zwischen Immunantwort und Impfschutz zu identifizieren.

In Kapitel 1 untersuche ich den Einfluss von zwei verkürzten Impfschemata von PfSPZ-CVac auf die Bildung von pro-inflammatorischen Pf-spezifischen CD4+ T-Helferzellen und deren Eignung als Surrogat für den Schutz. Die Studie wurde an gesunden, Malaria-naiven Erwachsenen in Tübingen durchgeführt. Um antigenspezifische Zellen im peripheren Blut mit erhöhter Sensitivität nachzuweisen, wurden Plasmodium-spezifische T-Zellen durch magnetische Zellsortierung (magnetic-activated cell sorting – MACS) angereichert, anschließend gefärbt und mit Multiparameter-Durchflusszytometrie (multiparameter flow cytometry – MFC) gemessen. Die Messungen wurden mit dem Durchflusszytometer FACS

Canto II durchgeführt, die Stimulationen erfolgten mit infizierten roten Blutkörperchen (iRBCs). Als Negativ- bzw. Positivkontrollen wurden nicht infizierte rote Blutkörperchen und Staphylokokken-Enterotoxin B (SEB) verwendet.

Überraschenderweise stellte ich fest, dass ein verkürztes Impfschema, bei dem drei Impfungen innerhalb von 10 Tagen verabreicht wurden, Plasmodium-spezifische CD40L+CD4+ TNF-α+/IFN-γ+ Zellen stärker induzierte als ein 28-Tage-Schema. Die Reaktion war auch qualitativ unterschiedlich. Das kürzere Schema führte zu einer polarisierten Th1-Antwort mit mehr CD45RO+CCR7- Effektor-Gedächtnis-T-Zellen. Dies könnte zu einer höheren Anzahl von Gedächtniszellen in der Leber führen. Spezifische CD40L+CD4+ TNF-α+/IFN-γ+ Effektor-Gedächtnis-T-Zellen waren bei geschützten Probanden leicht erhöht, doch konnte kein zuverlässiges Korrelat des Schutzes ermittelt werden. Weitere Untersuchungen werden erforderlich sein, um Effektor- und regulatorische Reaktionen zu identifizieren, die die Wirksamkeit des Impfstoffs verlässlich vorhersagen. Besonders wichtig wird dabei die Charakterisierung der Plasmodium-spezifischen zytotoxischen T-Zellen sein.

Mehr als 94 % der geschätzten weltweiten Malariafälle treten in Afrika auf. Daher muss jeder Malariaimpfstoff auch bei Freiwilligen aus malariaendemischen Gebieten getestet werden. Dies stellt sicher, dass er eine signifikante Wirkung im gesundheitspolitisch wichtigsten Kontext hat. Leider ist die Wirksamkeit vieler Impfstoffe in Afrika geringer. Dieser Effekt ist bei Malariaimpfstoffen besonders ausgeprägt. Der GMZ2-Impfstoff wurde entwickelt, um Malaria und malaria-assoziierte Komplikationen zu verhindern, indem er die natürlich erworbene Immunität nachahmt. Das Impfstoffantigen besteht aus einem Fusionsprotein von Fragmenten des Merozoiten-Oberflächenproteins-3 und dem glutamatreichen Protein. In Kapitel 2 habe ich die Immunogenität von GMZ2 untersucht, das mit zwei verschiedenen Immunmodulatoren adjuvantiert wurde: Alhydrogel oder CAF01. Die Studie wurde an gesunden, erwachsenen, lebenslang malariaexponierten Freiwilligen aus Lambaréné, Gabun, durchgeführt. MFC wurde zur systematischen Messung der T- und B-Zell-Reaktion und zum Vergleich der Immunantwort vor und nach der Immunisierung eingesetzt. Die mononukleären Zellen des peripheren Blutes wurden eingefroren und nach Abschluss der Studie mit einem Sony SP6800 Spectral Analyzer gemessen. Die Analyse von GMZ2-stimulierten CD4+ T- und CD20+ B-Zellen zeigte, dass GMZ2 keine signifikanten Immunreaktionen über die Ausgangswerte vor Impfung hinaus auslöste. Die geringe Reaktion auf die Impfung war ebenso unerwartet wie die Beobachtung, dass die beiden Adjuvantien keine messbaren Unterschiede in der Aktivierung der Immunantwort auslösten. Die mittels CHMI bestimmte Wirksamkeit

war zwischen den Gruppen ebenfalls vergleichbar, so dass es nicht verwundert, dass keine großen Unterschiede in der malariaspezifischen Immunantwort zu finden waren.

Trotz der negativen Resultate, können die Ergebnisse bei der Entwicklung der nächsten Generation von Malariaimpfstoffen für die Verwendung in malariaendemischen Regionen hilfreich sein. Hier ist vor allem die natürlich erworbene, oft stark ausgeprägte Immunantwort vor Impfung zu beachten.

Zusammenfassend zeigen die im Rahmen dieser Dissertation vorgestellten Ergebnisse, dass die systematische Untersuchung der zellulären Immunreaktionen durch MFC in Kombination mit CHMI-Studien ein valider und stringenter Ansatz zur Messung der Wirksamkeit und zur Identifizierung von mit Schutz korrelierten Biomarkern, Surrogatmarkern ist. Außerdem kann die Auswirkung des Impfschemas und einer vorbestehenden Immunität auf die Impfantwort bestimmt werden. Weitere Arbeiten werden erforderlich sein, um CHMI durch immunologische Surrogatendpunkte ersetzen zu können und die impfstoffinduzierte Immunität bei Malaria zu verstehen.

6 BIBLIOGRAPHY

1. WHO [Online] (2022). Vaccines and immunization. Available: https://www.who.int/health-topics/vaccines-and-immunization.

2. Winkelstein, W. (1992). Not just a country doctor: Edward Jenner, scientist. *Epidemiol Rev* **14**, 1–15 (1992).

3. Flemming, A. (2020). The origins of vaccination. Nature Research .

4. Delany, I., Rappuoli R., De Gregorio E. (2014). Vaccines for the 21st century. EMBO Mol Med 6, 708–720.

5. Pardi, N., Hogan, M., Porter, F. (2018). mRNA vaccines - a new era in vaccinology. *Nat Rev Drug Discov* **17**, 261–279 (2018).

6. Plotkin, S. A., Orenstein, W. A., Offit, P. A. & Edwards, K. M. (2018). Plotkin's vaccines, Vaccine Immunology, pages 16-34, Elsevier, Inc.

7. Sallusto F., Lanzavecchia A., Araki K., Ahmed R. (2010). From Vaccines to Memory and Back. Immunity 33, 451–463.

8. Murphy, K., Travers, Paul, Walport, M. & Janeway, C (2012). Janeway's immunobiology. Garland Science.

9. Elsner, R. A. and Shlomchik, M. J (2020). Germinal Center and Extrafollicular B Cell Responses in Vaccination, Immunity, and Autoimmunity. Immunity 53, 1136–1150.

10. Geginat, J., Paroni, M., Maglie, S., Alfen, JS, Kastirr, I., Gruarin, P., De Simone, M., Pagani, M., Abrignani, S. (2014). Plasticity of Human CD4 T Cell Subsets. Front. Immunol. 5.

11. Poland, GA., Ovsyannikova, IG., Kennedy, RB. (2017). Personalized vaccinology: A review. Vaccine.

12. Pulendran, B., Li, S., Nakaya, HI. (2010). Systems vaccinology. Immunity 33, 516–529.

13. Rappuoli, R. (1994). Toxin inactivation and antigen stabilization: two different uses of formaldehyde. Vaccine 12, 579–581.

14. Janeway, CA Jr. (1989). Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 54 Pt 1, 1–13.

15. Salmon, DA., Dudley, MZ., Glanz, JM., Omer, SB. (2015). Vaccine Hesitancy: Causes, Consequences, and a Call to Action. Am J Prev Med 49, S391-398.

16. Browne, M. (2018). Epistemic divides and ontological confusions: The psychology of vaccine scepticism. Hum Vaccin Immunother 14, 2540–2542.

17. Matsiko, A. (2020). Alum adjuvant discovery and potency. Nature Research.

18. Panilaitis, B., Johri, A., Blank, W., Kaplan, D., Fuhrman, J. (2002). Adjuvant Activity of Emulsan, a Secreted Lipopolysaccharide from Acinetobacter calcoaceticus. *Clin Diagn Lab Immunol* **9**, 1240–1247.

19. Reimer, JM., Karlsson, KH., Lövgren-Bengtsson, K., Magnusson, SE., Fuentes, A., Stertman, L. (2012). Matrix-M^{TM} adjuvant induces local recruitment, activation and maturation of central immune cells in absence of antigen. *PLoS One* **7**, e41451.

20. Powell, TJ., Palath, N., DeRome, ME., Tang, J., Jacobs, A., Boyd, JG. (2011). Synthetic nanoparticle vaccines produced by layer-by-layer assembly of artificial biofilms induce potent protective T-cell and antibody responses in vivo. Vaccine 29, 558–569.

21. Marrack, P., McKee, AS., Munks, MW. (2009). Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol* **9**, 287–293.

22. Awate, S., Babiuk, LA., Mutwiri, G. (2013). Mechanisms of Action of Adjuvants. *Frontiers in Immunology* **4**, 114.

23. Nabel, G. J. (2013). Designing Tomorrow's Vaccines. N Engl J Med 368, 551–560.

24. Zeltins, A. (2013). Construction and characterization of virus-like particles: a review. *Mol Biotechnol* **53**, 92–107.

25. Zepeda-Cervantes, J., Ramírez-Jarquín, JO., Vaca, L. (2020). Interaction Between Virus-Like Particles (VLPs) and Pattern Recognition Receptors (PRRs) From Dendritic Cells (DCs): Toward Better Engineering of VLPs. Front Immunol 11, 1100.

26. Brody, T (2016). Clinical Trials: Study Design, Endpoints and Biomarkers, Drug Safety, and FDA and ICH Guidelines. Elsevier Science.

27. Seder, RA., Chang, LJ., Enama, ME., Zephir, KL., Sarwar, UN., Gordon, IJ., Holman, LA., James, ER., Billingsley, PF., Gunasekera, A., Richman, A., Chakravarty, S., Manoj, A., Velmurugan, S., Li, M., Ruben, AJ., Li, T., Eappen, AG., Stafford, RE., Plummer, SH., Hendel, CS., Novik, L., Costner, PJ., Mendoza, FH., Saunders, JG., Nason, MC., Richardson, JH., Murphy, J., Davidson, SA., Richie, TL., Sedegah, M., Sutamihardja, A., Fahle, GA., Lyke, KE., Laurens, MB., Roederer, M., Tewari, K., Epstein, JE., Sim, BK., Ledgerwood, JE., Graham, BS., Hoffman, SL.; VRC 312 Study Team. (2013). Protection Against Malaria by Intravenous Immunization with a Nonreplicating Sporozoite Vaccine. Science.

28. Irvine, EB., O'Neil, A., Darrah, PA., Shin, S., Choudhary, A., Li, W., Honnen, W., Mehra, S., Kaushal, D., Gideon, HP., Flynn, JL., Roederer, M., Seder, RA., Pinter, A., Fortune, S., Alter, G. (2021). Robust IgM responses following intravenous vaccination with Bacille Calmette–Guérin associate with prevention of Mycobacterium tuberculosis infection in macaques. Nat Immunol 22, 1515–1523.

29. Flickinger, JC Jr., Staudt, RE., Singh, J., Carlson, RD., Barton, JR., Baybutt, TR., Rappaport, JA., Zalewski, A., Pattison, A., Waldman, SA., Snook, AE. (2022). Chimeric adenoviral (Ad5.F35) and listeria vector prime-boost immunization is safe and effective for cancer immunotherapy. npj Vaccines 7, 1–10 (2022).

30. Li, J., Zeng, M., Shan, H., Tong, C. (2017). Microneedle Patches as Drug and Vaccine Delivery Platform. Curr Med Chem 24, 2413–2422.

31. Criscuolo, E., Caputo, V., Diotti, RA., Sautto, GA., Kirchenbaum, GA., Clementi, N. (2019). Alternative Methods of Vaccine Delivery: An Overview of Edible and Intradermal Vaccines. J Immunol Res.

32. National Center for Immunization and Respiratory Diseases (2011). General recommendations on immunization --- recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 60, 1–64.

33. Pollard, AJ., Bijker, EM. (2021). A guide to vaccinology: from basic principles to new developments. Nat Rev Immunol 21, 83–100.

34. Edwards, K. M., Karzon, D. T. (1990). Pertussis Vaccines. Pediatric Clinics of North America 37, 549–566.

35. Börner, N., Mühlberger, N., Jelinek, T. (2003). Tolerability of multiple vaccinations in travel medicine. J Travel Med 10, 112–116.

36. Schelling, E., Bechir, M., Ahmed, MA., Wyss, K., Randolph, TF., Zinsstag, J. (2007). Human and Animal Vaccination Delivery to Remote Nomadic Families, Chad. Emerg Infect Dis 13, 373–379.

37. Hahn, S (2012). Understanding noninferiority trials. Korean J Pediatr 55, 403–407.

38. WHO [Online] (2022). Immunization, Vaccines and Biologicals. Available: https://www.who.int/teams/immunization-vaccines-and-biologicals/diseases.

39. Commissioner, O. of the. U.S. Food and Drug Administration [Online] (2021). FDA. Available: https://www.fda.gov/home.

40. European Medicines Agency [Online] (2022). European Medicines Agency. Available: https://www.ema.europa.eu/en.

41. WHO [Online] (2022). Overview — History & Mission. WHO - Prequalification of Medical Products (IVDs, Medicines, Vaccines and Immunization Devices, Vector Control). Available: https://extranet.who.int/pqweb/vaccines.

42. Ultsch, B., Damm, O., Beutels, P., Bilcke, J., Brüggenjürgen, B., Gerber-Grote, A., Greiner, W., Hanquet, G., Hutubessy, R., Jit, M., Knol, M., von Kries, R., Kuhlmann, A., Levy-Bruhl, D., Perleth, M., Postma, M., Salo, H., Siebert, U., Wasem, J., Wichmann, O. (2016). Methods for Health Economic Evaluation of Vaccines and Immunization Decision Frameworks: A Consensus Framework from a European Vaccine Economics Community. Pharmacoeconomics 34, 227–244.

43. Larson, HJ., Clarke, RM., Jarrett, C., Eckersberger, E., Levine, Z., Schulz, WS., Paterson, P. (2018). Measuring trust in vaccination: A systematic review. Hum Vaccin Immunother 14, 1599–1609.

44. Gualano, MR., Olivero, E., Voglino, G., Corezzi, M., Rossello, P., Vicentini, C., Bert, F., Siliquini, R. (2019). Knowledge, attitudes and beliefs towards compulsory vaccination: a systematic review. Hum Vaccin Immunother 15, 918–931.

45. WHO [Online] (2022). Vaccines and immunization: Myths and misconceptions. Available: https://www.who.int/news-room/.

46. Principi, N., Esposito, S. (2020). Do Vaccines Have a Role as a Cause of Autoimmune Neurological Syndromes? Frontiers in Public Health.

47. DeStefano, F., Verstraeten, T., Jackson, LA., Okoro, CA., Benson, P., Black, SB., Shinefield, HR., Mullooly, JP., Likosky, W., Chen, RT.; Vaccine Safety Datalink Research Group, National Immunization Program, Centers for Disease Control and Prevention. Vaccinations and risk of central nervous system demyelinating diseases in adults. (2003). Vaccinations and Risk of Central Nervous System Demyelinating Diseases in Adults. Archives of Neurology 60, 504–509.

48. Gasparini, R., Panatto, D., Lai, PL., Amicizia, D. (2015). The ‘urban myth’ of the association between neurological disorders and vaccinations. J Prev Med Hyg 56, E1–E8.

49. Gonzalez, DC., Nassau, DE., Khodamoradi, K., Ibrahim, E., Blachman-Braun, R., Ory, J., Ramasamy, R. (2021). Sperm Parameters Before and After COVID-19 mRNA Vaccination. JAMA 326, 273–274.

50. Stolle, LB., Nalamasu, R., Pergolizzi, JV Jr., Varrassi, G., Magnusson, P., LeQuang, J., Breve, F.; NEMA Research Group. (2020). Fact vs Fallacy: The Anti-Vaccine Discussion Reloaded. Adv Ther 37, 4481–4490.

51. Burki, T (2020). The online anti-vaccine movement in the age of COVID-19. Lancet Digit Health 2, e504–e505.

52. Gallup, JL., Sachs, JD. (2001). The economic burden of malaria. Am J Trop Med Hyg 64, 85–96.

53. WHO [Online] (2021). World malaria report. Available: https://www.who.int.

54. WHO [Online] (2020). World malaria report: 20 years of global progress and challenges. Available: https://www.who.int.

55. Cohen, JM., Okumu, F., Moonen, B. (2022). The fight against malaria: Diminishing gains and growing challenges. Science Translational Medicine 14.

56. Alonso, P. L (2021). Malaria: a problem to be solved and a time to be bold. Nat Med 27, 1506–1509.

57. WHO [Online] (2013). Malaria Vaccine Technology Roadmap. Available: https://www.who.int.

58. Cooper, MM., Loiseau, C., McCarthy, JS., Doolan, DL. (2019). Human challenge models: tools to accelerate the development of malaria vaccines. Expert Rev Vaccines 18, 241–251.

59. Tan, MSY., Blackman, MJ. (2021). Malaria parasite egress at a glance. J Cell Sci 134.

60. Lu, C., Song, G., Beale, K., Yan, J., Garst, E., Feng, J., Lund, E., Catteruccia, F., Springer, TA. (2020). Design and assessment of TRAP-CSP fusion antigens as effective malaria vaccines. *PLoS One* **15**.

61. Mordmüller, B., Sulyok, M., Egger-Adam, D., Resende, M., de Jongh, WA., Jensen, MH., Smedegaard, HH., Ditlev, SB., Soegaard, M., Poulsen, L., Dyring, C., Calle, CL., Knoblich, A., Ibáñez, J., Esen, M., Deloron, P., Ndam, N., Issifou, S., Houard, S., Howard, RF., Reed, SG., Leroy, O., Luty, AJF., Theander, TG., Kremsner, PG., Salanti, A., Nielsen, MA. (2019). First-in-human, randomized, double-blind clinical trial of differentially adjuvanted PAMVAC, a vaccine candidate to prevent pregnancy-associated malaria. Clin. Infect. Dis.

62. Takashima, E., Tachibana, M., Morita, M., Nagaoka, H., Kanoi, BN., Tsuboi, T. (2021). Identification of Novel Malaria Transmission-Blocking Vaccine Candidates. Frontiers in Cellular and Infection Microbiology 11.

63. WHO [Online] (2021). WHO recommends groundbreaking malaria vaccine for children at risk. Available: https://www.who.int.

64. WHO [Online] (2021). The Weekly Epidemiological Record (WER). Available: https://www.who.int.

65. RTS,S Clinical Trials Partnership et al (2015). Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. The Lancet 386, 31–45.

66. Hill, DL., Carr, EJ., Rutishauser, T., Moncunill, G., Campo, JJ., Innocentin, S., Mpina, M., Nhabomba, A., Tumbo, A., Jairoce, C., Moll, HA., van Zelm, MC., Dobaño, C., Daubenberger, C., Linterman, MA. (2020). Immune system development varies according to age, location, and anemia in African children. Sci Transl Med 12.

67. Clyde, DF., Most, H., McCarthy, VC., Vanderberg, JP. (1973). Immunization of man against sporozite-induced falciparum malaria. Am. J. Med. Sci. 266, 169–177.

68. Itsara, LS., Zhou, Y., Do, J., Grieser, AM., Vaughan, AM., Ghosh, AK. (2018). The Development of Whole Sporozoite Vaccines for Plasmodium falciparum Malaria. Front Immunol 9.

69. Hoffman, SL., Vekemans, J., Richie, TL., Duffy, PE. (2015). The March Toward Malaria Vaccines. Am J Prev Med 49, S319-333.

70. Hollingdale, MR., Sedegah, M. (2017). Development of whole sporozoite malaria vaccines. Expert Rev Vaccines 16, 45–54.

71. Epstein, JE., Tewari, K., Lyke, KE., Sim, BK., Billingsley, PF., Laurens, MB., Gunasekera, A., Chakravarty, S., James, ER., Sedegah, M., Richman, A., Velmurugan, S., Reyes, S., Li, M., Tucker, K., Ahumada, A., Ruben, AJ., Li, T., Stafford, R., Eappen, AG., Tamminga, C., Bennett, JW., Ockenhouse, CF., Murphy, JR., Komisar, J., Thomas, N., Loyevsky, M., Birkett, A., Plowe, CV., Loucq, C., Edelman, R., Richie, TL., Seder, RA., Hoffman, SL. (2011). Live Attenuated Malaria Vaccine Designed to Protect Through Hepatic CD8+ T Cell Immunity. Science 334, 475–480.

72. Goswami, D., Minkah, NK., Kappe, SHI. (2019). Designer Parasites: Genetically Engineered Plasmodium as Vaccines To Prevent Malaria Infection. The Journal of Immunology 202, 20–28.

73. Bijker, EM., Bastiaens, GJ., Teirlinck, AC., van Gemert, GJ., Graumans, W., van de Vegte-Bolmer, M., Siebelink-Stoter, R., Arens, T., Teelen, K., Nahrendorf, W., Remarque, EJ., Roeffen, W., Jansens, A., Zimmerman, D., Vos, M., van Schaijk, BC., Wiersma, J., van der Ven, AJ., de Mast, Q., van Lieshout, L., Verweij, JJ., Hermsen, CC., Scholzen, A., Sauerwein, RW. (2013). Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity. PNAS 110, 7862–7867.

74. Roestenberg, M., McCall, M., Hopman, J., Wiersma, J., Luty, AJ., van Gemert, GJ., van de Vegte-Bolmer, M., van Schaijk, B., Teelen, K,. Arens, T., Spaarman, L., de Mast, Q., Roeffen, W., Snounou, G., Rénia, L., van der Ven, A., Hermsen, CC., Sauerwein, R. (2009). Protection against a malaria challenge by sporozoite inoculation. N Engl J Med 361, 468–477.

75. Mordmüller, B., Surat, G., Lagler, H., Chakravarty, S., Ishizuka, AS., Lalremruata, A., Gmeiner, M., Campo, JJ., Esen, M., Ruben, AJ., Held, J., Calle, CL., Mengue, JB., Gebru, T., Ibáñez, J., Sulyok, M., James, ER., Billingsley, PF., Natasha, KC., Manoj, A., Murshedkar, T., Gunasekera, A., Eappen, AG., Li, T., Stafford, RE., Li, M., Felgner, PL., Seder, RA., Richie,

TL., Sim, BK., Hoffman, SL., Kremsner, PG. (2017). Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. Nature 542, 445–449.

76. Roestenberg, M., Bijker, EM., Sim, BKL., Billingsley, PF., James, ER., Bastiaens, GJH., Teirlinck, AC., Scholzen, A., Teelen, K., Arens, T., van der Ven, AJAM., Gunasekera, A., Chakravarty, S., Velmurugan, S., Hermsen, CC., Sauerwein, RW., Hoffman, SL. Controlled Human Malaria Infections by Intradermal Injection of Cryopreserved Plasmodium falciparum Sporozoites. Am J Trop Med Hyg 88, 5–13.

77. Moris, P., Jongert, E., van der Most, RG. (2018). Characterization of T-cell immune responses in clinical trials of the candidate RTS,S malaria vaccine. *Hum Vaccin Immunother* **14**, 17–27.

78. White, MT., Verity, R., Griffin, JT., Asante, KP., Owusu-Agyei, S., Greenwood, B., Drakeley, C., Gesase, S., Lusingu, J., Ansong, D., Adjei, S., Agbenyega, T., Ogutu, B., Otieno, L., Otieno, W., Agnandji, ST., Lell, B., Kremsner, P., Hoffman, I., Martinson, F., Kamthunzu, P., Tinto, H., Valea, I., Sorgho, H., Oneko, M., Otieno, K., Hamel, MJ., Salim, N., Mtoro, A., Abdulla, S., Aide, P., Sacarlal, J., Aponte, JJ., Njuguna, P., Marsh, K., Bejon, P., Riley, EM., Ghani, AC. (2015). Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a phase 3 randomised controlled trial. The Lancet Infectious Diseases 15, 1450–1458.

79. Otieno, L., Guerra Mendoza, Y., Adjei, S., Agbenyega, T., Agnandji, ST., Aide, P., Akoo, P., Ansong, D., Asante, KP., Berkley, JA., Gesase, S., Hamel, MJ., Hoffman, I., Kaali, S., Kamthunzi, P., Kariuki, S., Kremsner, P., Lanaspa, M., Lell, B., Lievens, M., Lusingu, J., Malabeja, A., Masoud, NS., Mtoro, AT., Njuguna, P., Ofori-Anyinam, O., Otieno, GA., Otieno, W., Owusu-Agyei, S., Schuerman, L., Sorgho, H., Tanner, M., Tinto, H., Valea, I., Vandoolaeghe, P., Sacarlal, J., Oneko, M. (2020). Safety and immunogenicity of the RTS,S/AS01 malaria vaccine in infants and children identified as HIV-infected during a randomized trial in sub-Saharan Africa. Vaccine 38, 897–906.

80. Cockburn, IA., Seder, RA. (2018). Malaria prevention: from immunological concepts to effective vaccines and protective antibodies. Nat Immunol 19, 1199–1211.

81. Ishizuka, AS., Lyke, KE., DeZure, A., Berry, AA., Richie, TL., Mendoza, FH., Enama, ME., Gordon, IJ., Chang, LJ., Sarwar, UN., Zephir, KL., Holman, LA., James, ER., Billingsley, PF., Gunasekera, A., Chakravarty, S., Manoj, A., Li, M., Ruben, AJ., Li, T., Eappen, AG.,

Stafford, RE., K C N, Murshedkar, T., DeCederfelt, H., Plummer, SH., Hendel, CS., Novik, L., Costner, PJ., Saunders, JG., Laurens, MB., Plowe, CV., Flynn, B., Whalen, WR., Todd, JP., Noor, J., Rao, S., Sierra-Davidson, K., Lynn, GM., Epstein, JE., Kemp, MA., Fahle, GA., Mikolajczak, SA., Fishbaugher, M., Sack, BK., Kappe, SH., Davidson, SA., Garver, LS., Björkström, NK., Nason, MC., Graham, BS., Roederer, M., Sim, BK., Hoffman, SL., Ledgerwood, JE., Seder, RA. (2016). Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. *Nat Med* **22**, 614–623.

82. Artaud, C., Kara, L., Launay, O. (2019). Vaccine Development: From Preclinical Studies to Phase 1/2 Clinical Trials. Methods Mol Biol 2013, 165–176.

83. RTS,S Clinical Trials Partnership et al (2012). A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. N Engl J Med 367, 2284–2295.

84. Guerra Mendoza, Y., Garric, E., Leach, A., Lievens, M., Ofori-Anyinam, O., Pirçon, JY., Stegmann, JU., Vandoolaeghe, P., Otieno, L., Otieno, W., Owusu-Agyei, S., Sacarlal, J., Masoud, NS., Sorgho, H., Tanner, M., Tinto, H., Valea, I., Mtoro, AT., Njuguna, P., Oneko, M., Otieno, GA., Otieno, K., Gesase, S., Hamel, MJ., Hoffman, I., Kaali, S., Kamthunzi, P., Kremsner, P., Lanaspa, M., Lell, B., Lusingu, J., Malabeja, A., Aide, P., Akoo, P., Ansong, D., Asante, KP., Berkley, JA., Adjei, S., Agbenyega, T., Agnandji, ST., Schuerman, L. (2019). Safety profile of the RTS,S/AS01 malaria vaccine in infants and children: additional data from a phase III randomized controlled trial in sub-Saharan Africa. Hum Vaccin Immunother 15, 2386–2398.

85. Roestenberg, M., Kamerling, IMC., de Visser, SJ. (2018). Controlled Human Infections As a Tool to Reduce Uncertainty in Clinical Vaccine Development. Front Med (Lausanne) 5, 297.

86. Roestenberg, M., Mo, A., Kremsner, PG., Yazdanbakhsh, M. (2017). Controlled human infections: A report from the controlled human infection models workshop, Leiden University Medical Centre 4-6 May 2016. Vaccine 35, 7070–7076.

87. Matuschewski, K., Borrmann, S. (2019). Controlled Human Malaria Infection (CHMI) Studies: Over 100 Years of Experience with Parasite Injections. Methods Mol Biol 2013, 91–101.

88. McCall, MBB., Kremsner, PG., Mordmüller, B. (2018). Correlating efficacy and immunogenicity in malaria vaccine trials. Seminars in Immunology 39, 52–64.

89. Raeven, RHM., van Riet, E., Meiring, HD., Metz, B., Kersten, GFA. (2019). Systems vaccinology and big data in the vaccine development chain. Immunology 156, 33–46.

90. Young, WC., Carpp, LN., Chaudhury, S., Regules, JA., Bergmann-Leitner, ES., Ockenhouse, C., Wille-Reece, U., deCamp, AC., Hughes, E., Mahoney, C., Pallikkuth, S., Pahwa, S., Dennison, SM., Mudrak, SV., Alam, SM., Seaton, KE., Spreng, RL., Fallon, J., Michell, A., Ulloa-Montoya, F., Coccia, M., Jongert, E., Alter, G., Tomaras, GD., Gottardo, R. (2021). Comprehensive Data Integration Approach to Assess Immune Responses and Correlates of RTS,S/AS01-Mediated Protection From Malaria Infection in Controlled Human Malaria Infection Trials. Frontiers in Big Data 4.

91. Stanisic, DI., McCall, MBB. (2021). Correlates of malaria vaccine efficacy. Expert Rev Vaccines.

92. Wang, LT., Pereira, LS., Flores-Garcia, Y., O'Connor, J., Flynn, BJ., Schön, A., Hurlburt, NK., Dillon, M., Yang, ASP., Fabra-García, A., Idris, AH., Mayer, BT., Gerber, MW., Gottardo, R., Mason, RD., Cavett, N., Ballard, RB., Kisalu, NK., Molina-Cruz, A., Nelson, J., Vistein, R., Barillas-Mury, C., Amino, R., Baker, D., King, NP., Sauerwein, RW., Pancera, M., Cockburn, IA., Zavala, F., Francica, JR., Seder, RA. (2020). A Potent Anti-Malarial Human Monoclonal Antibody Targets Circumsporozoite Protein Minor Repeats and Neutralizes Sporozoites in the Liver. Immunity 53, 733-744.e8.

93. Kurtovic, L., Behet, MC., Feng, G., Reiling, L., Chelimo, K., Dent, AE., Mueller, I., Kazura, JW., Sauerwein, RW., Fowkes, FJI., Beeson, JG. (2018). Human antibodies activate complement against Plasmodium falciparum sporozoites, and are associated with protection against malaria in children. BMC Med 16.

94. Feng, G., Wines, B.D., Kurtovic, L. (2021). Mechanisms and targets of Fcγ-receptor mediated immunity to malaria sporozoites. Nat Commun 12.

95. Wang, LT., Pereira, LS., Kiyuka, PK., Schön, A., Kisalu, NK., Vistein, R., Dillon, M., Bonilla, BG., Molina-Cruz, A., Barillas-Mury, C., Tan, J., Idris, AH., Francica, JR., Seder, RA. (2021). Protective effects of combining monoclonal antibodies and vaccines against the Plasmodium falciparum circumsporozoite protein. PLoS Pathog 17.

96. Hartmann, FJ., Babdor, J., Gherardini, PF., Amir, ED., Jones, K., Sahaf, B., Marquez, DM., Krutzik, P., O'Donnell, E., Sigal, N., Maecker, HT., Meyer, E., Spitzer, MH., Bendall,

SC. (2019). Comprehensive Immune Monitoring of Clinical Trials to Advance Human Immunotherapy. Cell Reports 28, 819-831.

97. Chattopadhyay, PK., Gierahn, TM., Roederer, M., Love, JC. (2014). Single-cell technologies for monitoring immune systems. Nat Immunol 15, 128–135.

98. Kazmin, D., Nakaya, HI., Lee, EK., Johnson, MJ., van der Most, R., van den Berg, RA., Ballou, WR., Jongert, E., Wille-Reece, U., Ockenhouse, C., Aderem, A., Zak, DE., Sadoff, J., Hendriks, J., Wrammert, J., Ahmed, R., Pulendran, B. (2017). Systems analysis of protective immune responses to RTS,S malaria vaccination in humans. PNAS 114, 2425–2430.

99. de Jong, SE., van Unen, V., Manurung, MD., Stam, KA., Goeman, JJ., Jochems, SP., Höllt, T., Pezzotti, N., Mouwenda, YD., Betouke Ongwe, ME., Lorenz, FR., Kruize, YCM., Azimi, S., König, MH., Vilanova, A., Eisemann, E., Lelieveldt, BPF., Roestenberg, M., Sim, BKL., Reinders, MJT., Fendel, R., Hoffman, SL., Kremsner, PG., Koning, F., Mordmüller, B., Lell, B., Yazdanbakhsh, M. (2021). Systems analysis and controlled malaria infection in Europeans and Africans elucidate naturally acquired immunity. Nat Immunol 22, 654–665.

100. Doolan, DL., Dobaño, C., Baird, JK. (2009). Acquired Immunity to Malaria. Clin Microbiol Rev 22, 13–36.

101. Gonzales, SJ., Reyes, RA., Braddom, AE., Batugedara, G., Bol, S., Bunnik, EM. (2020). Naturally Acquired Humoral Immunity Against Plasmodium falciparum Malaria. *Front. Immunol.* **0**.

102. Barry, A., Hansen, D. (2016). Naturally acquired immunity to malaria. *Parasitology* **143**, 125–128.

103. Tijani, MK., Lugaajju, A., Persson, KEM. (2021). Naturally Acquired Antibodies against Plasmodium falciparum: Friend or Foe? Pathogens 10.

104. Boyle, MJ., Reiling, L., Feng, G., Langer, C., Osier, FH., Aspeling-Jones, H., Cheng, YS., Stubbs, J., Tetteh, KK., Conway, DJ., McCarthy, JS., Muller, I., Marsh, K., Anders, RF., Beeson, JG. (2015). Human Antibodies Fix Complement to Inhibit Plasmodium falciparum Invasion of Erythrocytes and Are Associated with Protection against Malaria. Immunity 42, 580–590.

105. Healer, J., Chiu, CY., Hansen, DS. (2018). Mechanisms of naturally acquired immunity to P. falciparum and approaches to identify merozoite antigen targets. Parasitology 145, 839–847.

106. Bhatt, K., Verma, S., Ellner, JJ., Salgame, P. (2015). Quest for Correlates of Protection against Tuberculosis. Clin Vaccine Immunol 22, 258–266.

107. White, MT., Bejon, P., Olotu, A., Griffin, JT., Riley, EM., Kester, KE., Ockenhouse, CF., Ghani, AC. (2013). The Relationship between RTS,S Vaccine-Induced Antibodies, CD4+ T Cell Responses and Protection against Plasmodium falciparum Infection. PLoS One 8.

108. Friberg, H., Martinez, LJ., Lin, L., Blaylock, JM., De La Barrera, RA., Rothman, AL., Putnak, JR., Eckels, KH., Thomas, SJ., Jarman, RG., Currier, JR. (2020). Cell-Mediated Immunity Generated in Response to a Purified Inactivated Vaccine for Dengue Virus Type 1. mSphere 5.

109. Sztein, MB., Salerno-Goncalves, R., McArthur, MA. (2014). Complex Adaptive Immunity to Enteric Fevers in Humans: Lessons Learned and the Path Forward. Frontiers in Immunology 5.

110. Kumar, A., McElhaney, JE., Walrond, L., Cyr, TD., Merani, S., Kollmann, TR., Halperin, SA., Scheifele, DW. (2017). Cellular immune responses of older adults to four influenza vaccines: Results of a randomized, controlled comparison. Hum Vaccin Immunother 13, 2048–2057.

111. MacLennan, CA., Saul, A. (2014). Vaccines against poverty. PNAS 111, 12307–12312.

112. Junqueira, C., Polidoro, RB., Castro, G., Absalon, S., Liang, Z., Sen Santara, S., Crespo, Â., Pereira, DB., Gazzinelli, RT., Dvorin, JD., Lieberman, J. (2021). γδ T cells suppress Plasmodium falciparum blood-stage infection by direct killing and phagocytosis. Nat Immunol 22, 347–357.

113. Hart, GT., Tran, TM., Theorell, J., Schlums, H., Arora, G., Rajagopalan, S., Sangala, ADJ., Welsh, KJ., Traore, B., Pierce, SK., Crompton, PD., Bryceson, YT., Long, EO. (2019). Adaptive NK cells in people exposed to Plasmodium falciparum correlate with protection from malaria. J Exp Med 216, 1280–1290.

114. Burrack, K.S., Hart, G.T., Hamilton, S.E. (2019). Contributions of natural killer cells to the immune response against Plasmodium. Malar J 18.

115. WHO [Online] (2013). Correlates of vaccine-induced protection: methods and implications. Available: https://apps.who.int/iris/handle/10665/84288.

116. Plotkin, SA., Gilbert, PB. (2012). Nomenclature for immune correlates of protection after vaccination. Clin Infect Dis 54, 1615–1617.

117. Qin, L., Gilbert, PB., Corey, L., McElrath, MJ., Self, SG. (2007). A framework for assessing immunological correlates of protection in vaccine trials. J Infect Dis 196, 1304–1312.

118. Plotkin, SA. (2010). Correlates of protection induced by vaccination. Clin Vaccine Immunol 17, 1055–1065.

119. Human Vaccines Project [Online] (2021). Dr. Stanley Plotkin: Correlates of Protection by Vaccines. Available: https://www.youtube.com/watch?v=yuR8tSuvISY&t=1425s.

120. Shapiro, H. M (1983). Multistation multiparameter flow cytometry: a critical review and rationale. Cytometry 3, 227–243.

121. De Rosa, S. C. (2012). Vaccine applications of flow cytometry. Methods 57, 383–391.

122. Seder, RA., Darrah, PA., Roederer, M. (2008). T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 8, 247–258.

123. Maecker, H. T. (2009). Multiparameter Flow Cytometry Monitoring of T Cell Responses. Methods Mol Biol 485, 375–391.

124. Goletti, D., Delogu, G., Matteelli, A., Migliori, GB. (2022). The role of IGRA in the diagnosis of tuberculosis infection, differentiating from active tuberculosis, and decision making for initiating treatment or preventive therapy of tuberculosis infection. Int J Infect Dis.

125. Estévez, O., Anibarro, L., Garet, E., Martínez, A., Pena, A., Barcia, L., Peleteiro, M., González-Fernández, Á. (2020). Multi-parameter flow cytometry immunophenotyping distinguishes different stages of tuberculosis infection. Journal of Infection 81, 57–71.

126. Darrah, PA., Patel, DT., De Luca, PM., Lindsay, RW., Davey, DF., Flynn, BJ., Hoff, ST., Andersen, P., Reed, SG., Morris, SL., Roederer, M., Seder, RA. (2007). Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med 13, 843–850.

127. Duckworth, BC., Lafouresse, F., Wimmer, VC., Broomfield, BJ., Dalit, L., Alexandre, YO., Sheikh, AA., Qin, RZ., Alvarado, C., Mielke, LA., Pellegrini, M., Mueller, SN., Boudier, T., Rogers, KL., Groom, JR. (2021). Effector and stem-like memory cell fates are imprinted in distinct lymph node niches directed by CXCR3 ligands. Nat Immunol 22, 434–448.

128. Arunachalam, PS., Scott, MKD., Hagan, T., Li, C., Feng, Y., Wimmers, F., Grigoryan, L., Trisal, M., Edara, VV., Lai, L., Chang, SE., Feng, A., Dhingra, S., Shah, M., Lee, AS., Chinthrajah, S., Sindher, SB., Mallajosyula, V., Gao, F., Sigal, N., Kowli, S., Gupta, S., Pellegrini, K., Tharp, G., Maysel-Auslender, S., Hamilton, S., Aoued, H., Hrusovsky, K., Roskey, M., Bosinger, SE., Maecker, HT., Boyd, SD., Davis, MM., Utz, PJ., Suthar, MS., Khatri, P., Nadeau, KC., Pulendran, B. (2021). Systems vaccinology of the BNT162b2 mRNA vaccine in humans. Nature 596, 410–416 (2021).

129. Ahl, PJ., Hopkins, RA., Xiang, WW., Au. B., Kaliaperumal, N., Fairhurst, AM., Connolly, JE. (2020). Met-Flow, a strategy for single-cell metabolic analysis highlights dynamic changes in immune subpopulations. Commun Biol 3, 305.

130. Wimmers, F., Donato, M., Kuo, A., Ashuach, T., Gupta, S., Li, C., Dvorak, M., Foecke, MH., Chang, SE., Hagan, T., De Jong, SE., Maecker, HT., van der Most, R., Cheung, P., Cortese, M., Bosinger, SE., Davis, M., Rouphael, N., Subramaniam, S., Yosef, N., Utz, PJ., Khatri, P., Pulendran, B. (2021). The single-cell epigenomic and transcriptional landscape of immunity to influenza vaccination. Cell 184.

131. Netea, MG., Domínguez-Andrés, J., Barreiro, LB., Chavakis, T., Divangahi, M., Fuchs, E., Joosten, LAB., van der Meer, JWM., Mhlanga, MM., Mulder, WJM., Riksen, NP., Schlitzer, A., Schultze, JL., Stabell, Benn C., Sun, JC., Xavier, RJ., Latz, E. (2020). Defining trained immunity and its role in health and disease. Nat Rev Immunol 20, 375–388.

132. Creighton, R., Schuch, V., Urbanski, AH., Giddaluru, J., Costa-Martins, AG., Nakaya, HI. (2020). Network vaccinology. Semin Immunol 50.

133. Panagioti, E., Klenerman, P., Lee, LN., van der Burg, SH., Arens, R. (2018). Features of Effective T Cell-Inducing Vaccines against Chronic Viral Infections. Frontiers in Immunology 9.

134. Fernandez-Ruiz, D., Ng, WY., Holz, LE., Ma, JZ., Zaid, A., Wong, YC., Lau, LS., Mollard, V., Cozijnsen, A., Collins, N., Li, J., Davey, GM., Kato, Y., Devi, S., Skandari, R., Pauley, M., Manton, JH., Godfrey, DI., Braun, A., Tay, SS., Tan, PS., Bowen, DG., Koch-

Nolte, F., Rissiek, B., Carbone, FR., Crabb, BS., Lahoud, M., Cockburn, IA., Mueller, SN., Bertolino, P., McFadden, GI., Caminschi, I., Heath, WR. (2016). Liver-Resident Memory CD8(+) T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection. Immunity 45, 889–902.

135. McNamara, HA., Cai, Y., Wagle, MV., Sontani, Y., Roots, CM., Miosge, LA., O'Connor, JH., Sutton, HJ., Ganusov, VV., Heath, WR., Bertolino, P., Goodnow, CG., Parish, IA., Enders, A., Cockburn, IA. (2017). Up-regulation of LFA-1 allows liver-resident memory T cells to patrol and remain in the hepatic sinusoids. Science Immunology.

136. Cockburn, IA., Seder, RA. (2018). Malaria prevention: from immunological concepts to effective vaccines and protective antibodies. Nat. Immunol. 19, 1199–1211.

137. Bacher, P., Schink, C., Teutschbein, J., Kniemeyer, O., Assenmacher, M., Brakhage, AA., Scheffold, A. (2013). Antigen-reactive T cell enrichment for direct, high-resolution analysis of the human naive and memory Th cell repertoire. J Immunol 190, 3967–3976.

138. Bergmann, ES., Ballou, RW., Krzych, U. (1997). Detection of CD4+CD45RO+ T lymphocytes producing IL-4 in response to antigens on Plasmodium falciparum erythrocytes: an in vitro correlate of protective immunity induced with attenuated Plasmodium falciparum sporozoites. Cell Immunol 180, 143–152.

139. Metenou, S., Dembele, B., Konate, S., Dolo, H., Coulibaly, Y. I., Diallo, A. A., Soumaoro, L., Coulibaly, M. E., Coulibaly, S. Y., Sanogo, D., Doumbia, S. S., Traoré, S. F., Mahanty, S., Klion, A., & Nutman, T. B. (2011). Filarial infection suppresses malaria-specific multifunctional Th1 and Th17 responses in malaria and filarial coinfections. J Immunol 186, 4725–4733.

140. Murphy, SC., Deye, GA., Sim, BKL., Galbiati, S., Kennedy, JK., Cohen, KW., Chakravarty, S., Kc, N., Abebe, Y., James, ER., Kublin, JG., Hoffman, SL., Richie, TL., Jackson, LA. (2021). PfSPZ-CVac efficacy against malaria increases from 0% to 75% when administered in the absence of erythrocyte stage parasitemia: A randomized, placebo-controlled trial with controlled human malaria infection. PLoS Pathog 17.

141. Sallusto, F., Geginat, J., Lanzavecchia, A. (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu. Rev. Immunol. 22, 745–763.

142. Stemberger, C., Huster, KM., Koffler, M., Anderl, F., Schiemann, M., Wagner, H., Busch, DH. (2007). A Single Naive CD8+ T Cell Precursor Can Develop into Diverse Effector and Memory Subsets. Immunity 27, 985–997.

143. Sulyok, Z., Fendel, R., Eder, B., Lorenz, FR., Kc, N., Karnahl, M., Lalremruata, A., Nguyen, TT., Held, J., Adjadi, FAC., Klockenbring, T., Flügge, J., Woldearegai, TG., Lamsfus Calle, C., Ibáñez, J., Rodi, M., Egger-Adam, D., Kreidenweiss, A., Köhler, C., Esen, M., Sulyok, M., Manoj, A., Richie, TL., Sim, BKL., Hoffman, SL., Mordmüller, B., Kremsner, PG. (2021). Heterologous protection against malaria by a simple chemoattenuated PfSPZ vaccine regimen in a randomized trial. Nat Commun 12.

144. Hampton, HR., Chtanova, T. (2019). Lymphatic Migration of Immune Cells. Frontiers in Immunology 10.

145. Holz, LE., Fernandez-Ruiz, D., Heath, WR. (2016). Protective immunity to liver-stage malaria. Clin Transl Immunology 5.

146. Schmidt, NW., Butler, NS., Badovinac, VP., Harty, JT. (2010). Extreme CD8 T cell requirements for anti-malarial liver-stage immunity following immunization with radiation attenuated sporozoites. PLoS Pathog 6.

147. Berenzon, D., Schwenk, RJ., Letellier, L., Guebre-Xabier, M., Williams, J., Krzych, U. (2003). Protracted protection to Plasmodium berghei malaria is linked to functionally and phenotypically heterogeneous liver memory CD8+ T cells. J Immunol 171, 2024–2034.

148. Kurup, SP., Butler, NS., Harty, JT. (2019). T cell-mediated immunity to malaria. Nature Reviews Immunology 1.

149. Abuga, KM., Jones-Warner, W., Hafalla, JCR. (2021). Immune responses to malaria pre-erythrocytic stages: Implications for vaccine development. Parasite Immunology 43.

150. Ibanez, J., Fendel, R., Lorenz, FR. et al. (2022). Efficacy, T cell activation and antibody responses in accelerated Plasmodium falciparum sporozoite chemoprophylaxis vaccine regimens. npj Vaccines 7, 1–11.

151. Farber, DL., Yudanin, NA., Restifo, NP. (2014). Human memory T cells: generation, compartmentalization and homeostasis. Nat Rev Immunol 14, 24–35.

152. Akondy, RS., Johnson, PL., Nakaya, HI., Edupuganti, S., Mulligan, MJ., Lawson, B., Miller, JD., Pulendran, B., Antia, R., Ahmed, R. (2015). Initial viral load determines the

magnitude of the human CD8 T cell response to yellow fever vaccination. Proc. Natl. Acad. Sci. U.S.A. 112, 3050–3055.

153. Lyke, KE., Ishizuka, AS., Berry, AA., Chakravarty, S., DeZure, A., Enama, ME., James, ER., Billingsley, PF., Gunasekera, A., Manoj, A., Li, M., Ruben, AJ., Li, T., Eappen, AG., Stafford, RE., Kc, N., Murshedkar, T., Mendoza, FH., Gordon, IJ., Zephir, KL., Holman, LA., Plummer, SH., Hendel, CS., Novik, L., Costner, PJ., Saunders, JG., Berkowitz, NM., Flynn, BJ., Nason, MC., Garver, LS., Laurens, MB., Plowe, CV., Richie, TL., Graham, BS., Roederer, M., Sim, BK., Ledgerwood, JE., Hoffman, SL., Seder, RA. (2017). Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection. Proc. Natl. Acad. Sci. U.S.A. 114, 2711–2716.

154. Roestenberg, M., Teirlinck, AC., McCall, MB., Teelen, K., Makamdop, KN., Wiersma, J., Arens, T., Beckers, P., van Gemert, G., van de Vegte-Bolmer, M., van der Ven, AJ., Luty, AJ., Hermsen, CC., Sauerwein, RW. (2011). Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. The Lancet 377, 1770–1776.

155. Miller, JD., van der Most, RG., Akondy, RS., Glidewell, JT., Albott, S., Masopust, D., Murali-Krishna, K., Mahar, PL., Edupuganti, S., Lalor, S., Germon, S., Del Rio, C., Mulligan, MJ., Staprans, SI., Altman, JD., Feinberg, MB., Ahmed, R. (2008). Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. Immunity 28, 710–722.

156. Fuertes Marraco, SA., Soneson, C., Cagnon, L., Gannon, PO., Allard, M., Abed Maillard, S., Montandon, N., Rufer, N., Waldvogel, S., Delorenzi, M., Speiser, DE. (2015). Long-lasting stem cell-like memory CD8+ T cells with a naïve-like profile upon yellow fever vaccination. Sci Transl Med 7.

157. Jongo, SA., Urbano, V., Church, LWP., Olotu, A., Manock, SR., Schindler, T., Mtoro, A., Kc, N., Hamad, A., Nyakarungu, E., Mpina, M., Deal, A., Bijeri, JR., Ondo Mangue, ME., Ntutumu Pasialo, BE., Nguema, GN., Owono, SN., Rivas, MR., Chemba, M., Kassim, KR., James, ER., Stabler, TC., Abebe, Y., Saverino, E., Sax, J., Hosch, S., Tumbo, AM., Gondwe, L., Segura, JL., Falla, CC., Phiri, WP., Hergott, DEB., García, GA., Schwabe, C., Maas, CD., Murshedkar, T., Billingsley, PF., Tanner, M., Ayekaba, MO., Sim, BKL., Daubenberger, C., Richie, TL., Abdulla, S., Hoffman, SL. (2021). Immunogenicity and Protective Efficacy of

Radiation-Attenuated and Chemo-Attenuated PfSPZ Vaccines in Equatoguinean Adults. Am J Trop Med Hyg 104, 283–293.

158. Murphy, SC., Deye, GA., Sim, BKL., Galbiati, S., Kennedy, JK., Cohen, KW., Chakravarty, S., Kc, N., Abebe, Y., James, ER., Kublin, JG., Hoffman, SL., Richie, TL., Jackson, LA. (2021). PfSPZ-CVac efficacy against malaria increases from 0% to 75% when administered in the absence of erythrocyte stage parasitemia: A randomized, placebo-controlled trial with controlled human malaria infection. PLoS Pathog 17.

159. Langhorne, J., Ndungu, FM., Sponaas, AM., Marsh, K. (2008). Immunity to malaria: more questions than answers. Nat Immunol 9, 725–732.

160. Muellenbeck, MF., Ueberheide, B., Amulic, B., Epp, A., Fenyo, D., Busse, CE., Esen, M., Theisen, M., Mordmüller, B., Wardemann, H. (2013). Atypical and classical memory B cells produce Plasmodium falciparum neutralizing antibodies. J Exp Med 210, 389–399.

161. Rivera-Correa, J., Mackroth, M. S., Jacobs, T., Schulze Zur Wiesch, J., Rolling, T., & Rodriguez, A. (2019). Atypical memory B-cells are associated with Plasmodium falciparum anemia through anti-phosphatidylserine antibodies. eLife 8.

162. Hviid, L. (2005). Naturally acquired immunity to Plasmodium falciparum malaria in Africa. Acta Trop 95, 270–275.

163. Dinga, JN., Kimbung Mbandi, S., Cho-Ngwa, F., Fon, NP., Moliki, J., Efeti, RM., Nyasa, BR., Anong, DN., Jojic, N., Heckerman, D., Wang, R., Titanji, VP. (2014). Differential T-cell responses of semi-immune and susceptible malaria subjects to in silico predicted and synthetic peptides of Plasmodium falciparum. Acta Trop 135, 104–121.

164. Esen, M., Kremsner, PG., Schleucher, R., Gässler, M., Imoukhuede, EB., Imbault, N., Leroy, O., Jepsen, S., Knudsen, BW., Schumm, M., Knobloch, J., Theisen, M., Mordmüller, B. (2009). Safety and immunogenicity of GMZ2 - a MSP3-GLURP fusion protein malaria vaccine candidate. Vaccine 27, 6862–6868.

165. Mordmüller, B., Szywon, K., Greutelaers, B., Esen, M., Mewono, L., Treut, C., Mürbeth, RE., Chilengi, R., Noor, R., Kilama, WL., Imoukhuede, EB., Imbault, N., Leroy, O., Theisen, M., Jepsen, S., Milligan, P., Fendel, R., Kremsner, PG., Issifou, S. (2010). Safety and immunogenicity of the malaria vaccine candidate GMZ2 in malaria-exposed, adult individuals from Lambaréné, Gabon. Vaccine 28, 6698–6703.

166. Bélard, S., Issifou, S., Hounkpatin, AB., Schaumburg, F., Ngoa, UA., Esen, M., Fendel, R., de Salazar, PM., Mürbeth, RE., Milligan, P., Imbault, N., Imoukhuede, EB., Theisen, M., Jepsen, S., Noor, RA., Okech, B., Kremsner, PG., Mordmüller, B. (2011). A randomized controlled phase Ib trial of the malaria vaccine candidate GMZ2 in African children. PLoS One 6.

167. Dejon-Agobe, JC., Ateba-Ngoa, U., Lalremruata, A., Homoet, A., Engelhorn, J., Nouatin, OP., Edoa, JR., Fernandes, JF., Esen, M., Mouwenda, YD., Betouke Ongwe, EM., Massinga-Loembe, M., Hoffman, SL., Sim, BKL., Theisen, M., Kremsner, PG., Adegnika, AA., Lell, B., Mordmüller, B. (2019). Controlled Human Malaria Infection of Healthy Adults With Lifelong Malaria Exposure to Assess Safety, Immunogenicity, and Efficacy of the Asexual Blood Stage Malaria Vaccine Candidate GMZ2. Clin Infect Dis 69, 1377–1384.

168. van Dissel,, JT., Joosten, SA., Hoff, ST., Soonawala, D., Prins, C., Hokey, DA., O'Dee, DM., Graves, A., Thierry-Carstensen, B., Andreasen, LV., Ruhwald, M., de Visser, AW., Agger, EM., Ottenhoff, TH., Kromann, I., Andersen, P. (2014). A novel liposomal adjuvant system, CAF01, promotes long-lived Mycobacterium tuberculosis-specific T-cell responses in human. *Vaccine* **32**, 7098–7107.

169. Sirima, SB., Mordmüller, B., Milligan, P., Ngoa, UA., Kironde, F., Atuguba, F., Tiono, AB., Issifou, S., Kaddumukasa, M., Bangre, O., Flach, C., Christiansen, M., Bang, P., Chilengi, R., Jepsen, S., Kremsner, PG., Theisen, M.; GMZ2 Trial Study Group. (2016). A phase 2b randomized, controlled trial of the efficacy of the GMZ2 malaria vaccine in African children. *Vaccine* **34**, 4536–4542.

170. Nouatin, O., Ateba, Ngoa U., Ibáñez, J., Dejon-Agobe, JC., Mordmüller, B., Edoa, JR., Mougeni, F., Brückner, S., Bouyoukou Hounkpatin, A., Esen, M., Theisen, M., Moutairou, K., Hoffman, SL., Issifou, S., Luty, AJF., Loembe, MM., Agnandji, ST., Lell, B., Kremsner, PG., Adegnika, AA. (2020). Effect of immune regulatory pathways after immunization with GMZ2 malaria vaccine candidate in healthy lifelong malaria-exposed adults. *Vaccine* **38**, 4263–4272.

171. Nouatin, O., Ibáñez, J., Fendel, R., Ngoa, UA., Lorenz, FR., Dejon-Agobé, JC., Edoa, JR., Flügge, J., Brückner, S., Esen, M., Theisen, M., Hoffman, SL., Moutairou, K., Luty, AJF., Lell, B., Kremsner, PG., Adegnika, AA., Mordmüller, B. (2022). Cellular and antibody response in GMZ2-vaccinated Gabonese volunteers in a controlled human malaria infection trial. *Malaria Journal* **21**.

172. Vella, LA., Buggert, M., Manne, S., Herati, RS., Sayin, I., Kuri-Cervantes, L., Bukh Brody, I., O'Boyle, KC., Kaprielian, H., Giles, JR., Nguyen, S., Muselman, A., Antel, JP., Bar-Or, A., Johnson, ME., Canaday, DH., Naji, A., Ganusov, VV., Laufer, TM., Wells, AD., Dori, Y., Itkin, MG., Betts, MR., Wherry, EJ. (2019). T follicular helper cells in human efferent lymph retain lymphoid characteristics. *J Clin Invest* **129**, 3185–3200.

173. Morita, R., Schmitt, N., Bentebibel, SE., Ranganathan, R., Bourdery, L., Zurawski, G., Foucat, E., Dullaers, M., Oh, S., Sabzghabaei, N., Lavecchio, EM., Punaro, M., Pascual, V., Banchereau, J., Ueno, H. (2011). Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity 34, 108–121.

174. Tan, L., Nie, M., Song, L., Yu, S., Lan, G., Xie, X. (2021). The role of circulating T follicular helper cells in kidney transplantation. *Transplant Immunology* **69**.

175. Cárdeno, A., Magnusson, M.K., Quiding-Järbrink, M. et al. (2018). Activated T follicular helper-like cells are released into blood after oral vaccination and correlate with vaccine specific mucosal B-cell memory. Sci Rep 8.

176. Knudsen, NP., Olsen, A., Buonsanti, C., Follmann, F., Zhang, Y., Coler, RN., Fox, CB., Meinke, A., D'Oro, U., Casini, D., Bonci, A., Billeskov, R., De Gregorio, E., Rappuoli, R., Harandi, AM., Andersen, P., Agger, EM. (2016). Different human vaccine adjuvants promote distinct antigen-independent immunological signatures tailored to different pathogens. Sci Rep 6.

177. Fergusson, JR., Smith, KE., Fleming, VM., Rajoriya, N., Newell, EW., Simmons, R., Marchi, E., Björkander, S., Kang, YH., Swadling, L., Kurioka, A., Sahgal, N., Lockstone, H., Baban, D., Freeman, GJ., Sverremark-Ekström, E., Davis, MM., Davenport, MP., Venturi, V., Ussher, JE., Willberg, CB., Klenerman, P. (2014). CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. Cell Rep 9, 1075–1088.

178. Obeng-Adjei, N., Portugal, S., Tran, TM., Yazew, TB., Skinner, J., Li, S., Jain, A., Felgner, PL., Doumbo, OK., Kayentao, K., Ongoiba, A., Traore, B., Crompton, PD. (2015). Circulating Th1-Cell-type Tfh Cells that Exhibit Impaired B Cell Help Are Preferentially Activated during Acute Malaria in Children. Cell Reports 13, 425–439.

179. Ramstein, J., Broos, CE., Simpson, LJ., Ansel, KM., Sun, SA., Ho, ME., Woodruff, PG., Bhakta, NR., Christian, L., Nguyen, CP., Antalek, BJ., Benn, BS., Hendriks, RW., van

den Blink, B., Kool, M., Koth ,LL. (2015). IFN-γ–Producing T-Helper 17.1 Cells Are Increased in Sarcoidosis and Are More Prevalent than T-Helper Type 1 Cells. Am J Respir Crit Care Med 193, 1281–1291.

180. Cunill, V., Massot, M., Clemente, A., Calles, C., Andreu, V., Núñez, V., López-Gómez, A., Díaz, RM., Jiménez, MLR., Pons, J., Vives-Bauzà, C., Ferrer, JM. (2018). Relapsing–Remitting Multiple Sclerosis Is Characterized by a T Follicular Cell Pro-Inflammatory Shift, Reverted by Dimethyl Fumarate Treatment. Frontiers in Immunology 9.

181. Ruschil, C., Gabernet, G., Lepennetier, G., Heumos, S., Kaminski, M., Hracsko, Z., Irmler, M., Beckers, J., Ziemann, U., Nahnsen, S., Owens, GP., Bennett, JL., Hemmer, B., Kowarik, MC. (2020). Specific Induction of Double Negative B Cells During Protective and Pathogenic Immune Responses. Frontiers in Immunology 11.

182. Jenks, SA., Cashman, KS., Zumaquero, E., Marigorta, UM., Patel, AV., Wang, X., Tomar, D., Woodruff, MC., Simon, Z., Bugrovsky, R., Blalock, EL., Scharer, CD., Tipton, CM., Wei, C., Lim, SS., Petri, M., Niewold, TB., Anolik, JH., Gibson, G., Lee, FE., Boss, JM., Lund, FE., Sanz, I. (2018). Distinct Effector B Cells Induced by Unregulated Toll-like Receptor 7 Contribute to Pathogenic Responses in Systemic Lupus Erythematosus. Immunity 49, 725-739.

183. Simone, O., Bejarano, MT., Pierce, SK., Antonaci, S., Wahlgren, M., Troye-Blomberg, M., Donati, D. (2011). TLRs innate immunereceptors and Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) CIDR1α-driven human polyclonal B-cell activation. Acta Tropica 119, 144–150.

184. Ly A, Hansen DS. (2019). Development of B Cell Memory in Malaria. Front Immunol 10.

185. Amoani, B., Gyan, B., Sakyi, S.A. et al. (2021). Effect of hookworm infection and anthelmintic treatment on naturally acquired antibody responses against the GMZ2 malaria vaccine candidate and constituent antigens. BMC Infect Dis 21.

186. Hotez, PJ., Brooker, S., Bethony, JM., Bottazzi, ME., Loukas, A., Xiao, S. (2004). Hookworm infection. N Engl J Med. 19;351(8):799-807.

187. Gebru, T., Ajua, A., Theisen, M. et al. (2017). Recognition of Plasmodium falciparum mature gametocyte-infected erythrocytes by antibodies of semi-immune adults and malaria-exposed children from Gabon. Malar J 16.

188. Nouatin, O., Mengue, JB., Dejon-Agobé, JC., Fendel, R., Ibáñez, J., Ngoa, UA., Edoa, JR., Adégbité, BR., Honkpéhédji, YJ., Zinsou, JF., Hounkpatin, AB., Moutairou, K., Homoet, A., Esen, M., Kreidenweiss, A., Hoffman, SL., Theisen, M., Luty, AJF., Lell, B., Agnandji, ST., Mombo-Ngoma, G., Ramharter, M., Kremsner, P., Mordmüller, B., Adegnika, AA. (2021). Exploratory analysis of the effect of helminth infection on the immunogenicity and efficacy of the asexual blood-stage malaria vaccine candidate GMZ2. PLoS Negl Trop Dis 15.

189. Bach JF. (2002). The Effect of Infections on Susceptibility to Autoimmune and Allergic Diseases. N Engl J Med 347, 911–920.

7 DECLARATION OF CONTRIBUTIONS

The doctoral dissertation entitled "Guiding clinical malaria vaccine development using immune cell monitoring and controlled human malaria infection". is now submitted to the members of the PhD Board at the Faculty of Medicine, University of Tübingen. This is a record of an original work by Mr. Javier Ibanez and by co-authors from the Institut für Tropenmedizin, Reisemedizin und Humanparasitologie (Tübingen), Institute of Immunology (University of Schleswig-Holstein, Kiel), Interfaculty Institute for Cell Biology, Centre de Recherches Médicales de Lambaréné (Gabon), Center of Tropical Medicine and Travel Medicine, Department of Infectious Diseases, Amsterdam University Medical Center (Netherlands), Département de Biochimie et de Biologie Cellulaire, Faculté des Sciences et Techniques, Université d'Abomey-Calavi (Cotonou, Bénin), Department for Congenital Disorders, Statens Serum Institut (Copenhagen, Denmark), and Sanaria Inc (Rockville, USA). Two accepted publications (Publication Nr.1: npj vaccines. 2022 May 31;7(1):59. doi: 10.1038/s41541-022-00473-1; and publication Nr.2: Malaria Journal. 2022 June 17. 21, 191. doi: 10.1038/s41541-022-00473-1) are accomplished by Mr. Ibanez as a first author and remains together with a third side investigation currently as manuscript in preparation, the epicenter of this dissertation. We declare that Mr. Javier Ibanez contributed to all manuscripts regarding the study design, experimental design, data analyses and writing of the manuscripts.

Contributions of the PhD candidate and coauthors:

Chapter 1: I implemented, performed, and analyzed the multiparameter ARTE methodology for the monitoring of the proinflammatory CD40LCD4 T cells and drafted the first manuscript. Petra Bacher and Alexander Scheffold are the authors of the ARTE methodology and subsequently trainers of Javier Ibanez, Sina Brückner and Meral Esen. Patricia Granados-Bayon and Sina Brückner contributed to perform ARTE. Freia-Raphaella Lorenz performed the enzyme-linked immunosorbent assays and contributed to the analysis of the microarray data. Mihály and Zita Sulyok were clinicians in the study team. Steffen Borrmann, Meral Esen, and Peter G. Kremsner contributed to design the study trial. Meral Esen recruited the patients and facilitated the acquisition of the biochemical data. Rolf Fendel designed and analyzed the microarray data and contributed to write the manuscript. Benjamin Mordmüller designed the study trial, supervised the data analysis, and contributed to write the manuscript. Stephen L. Hoffman is Chief Executive and Scientific Officer at Sanaria Inc. All authors approved the final manuscript.

Chapter 2: I designed and performed multiparameter flow cytometry experiments, did the data analysis, and drafted the first manuscript. Odilon Nouatin shared first authorship and contributed performing laboratory experiments. Sina Brückner contributed to perform the enzyme-linked immunosorbent assays. Rolf Fendel designed and analyzed the microarray data and contributed to write the manuscript. Ulysse A. Ngoa and Ayola A. Agdenika recruited the patients. Ayola A. Adegnika contributed selecting the methods, acquiring biochemical data, and interpreting results. Benjamin Mordmüller contributed to design the study trial, supervised the data analysis, and contributed to write the manuscript. All authors approved the final manuscript.

8 ACKNOWLEDGEMENTS

I want to express my sincere gratitude to Prof. Dr. Benjamin Mordmüller and Prof. Dr. Peter G. Kremsner for giving me the opportunity to chase my doctoral studies at the Institute of Tropical Medicine, University of Tübingen. I thank Prof. Dr. Benjamin Mordmüller for his supervision, encouragement, and support. I am also deeply grateful to the Faculty of Medicine, University of Tübingen and my doctoral committee, (Prof. Dr. Benjamin Mordmüller, Prof. Samuel Wagner, Ph.D., and PD. Dr. Andreas Nüssler). Special thanks also to Dr. Rolf Fendel for taking time to discuss and improve the quality of my manuscripts. I also want to express my deepest thanks to Ph.D. Albert Lalremruata, Ph.D, MD. José Fernandes, Ph.D. Tamirat Gebru and Ph.D. David Nderu for encouraging me during my time as PhD student. I am grateful to the Institute of Tropical Medicine, who will be forever part of me. Endless thanks to Anette Knoblich, Jutta Kun, and our sadly missed Velia Grummes, for your loving help during my time in Tübingen.

Finalmente, quisiera agradecer a toda mi familia en España; en especial a Pepa, Pepe, y mis tres princesas, por haber sabido entender las motivaciones que me han llevado a alcanzar este momento. Igualmente, quisiera expresar mi sincero agradecimiento a mi brisa marinera del sur, por insuflar aire en mis velas tantas veces como las fuerzas para remar a destino vencieron al hastío.

I wanted to conclude giving voice to all persons not mentioned here, for contributing in many ways to this achievement. Thank you so much.

Javier Ibanez Molina