Cellular and humoral immunity in malaria pre-exposed Tanzanian children and adults following vaccination with RTS,S, the most advanced malaria vaccine, and after whole sporozoite based controlled human malaria infections
Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von Prof. Dr. Marcel Tanner, Prof. Dr. Claudia Daubenberger, Prof. Dr. Steffen Borrmann


Prof. Dr. Jörg Schibler

Dekan
to my beloved wife, children, parents, brothers and sisters.
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<tbody>
<tr>
<td>ACT</td>
<td>Artemisinin-based Combination Therapy</td>
</tr>
<tr>
<td>AMA</td>
<td>Apical membrane antigen</td>
</tr>
<tr>
<td>CelTOS</td>
<td>Cell-traversal protein for ookinetes and sporozoites</td>
</tr>
<tr>
<td>CHMI</td>
<td>Controlled human malaria infection</td>
</tr>
<tr>
<td>CSA</td>
<td>Chondroitin sulfate A</td>
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<tr>
<td>CSP</td>
<td>Circumsporozoite Protein</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immuno-Sorbent Assay</td>
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<tr>
<td>ICAM1</td>
<td>Intracellular adhesion molecule1</td>
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<tr>
<td>IFN</td>
<td>Interferon gamma</td>
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<tr>
<td>IHI</td>
<td>Ifakara Health Institute</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRBCs</td>
<td>Infected Red Blood cells</td>
</tr>
<tr>
<td>ITNs</td>
<td>Insecticide-Treated Nets</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Spraying</td>
</tr>
<tr>
<td>ITTP</td>
<td>Intent to treat population</td>
</tr>
<tr>
<td>PPP</td>
<td>Per protocol population</td>
</tr>
<tr>
<td>LLITNs</td>
<td>Long Lasting Insecticide-Treated Nets</td>
</tr>
<tr>
<td>LSA</td>
<td>Liver stage antigen</td>
</tr>
<tr>
<td>MDGs</td>
<td>Millennium Development Goals</td>
</tr>
<tr>
<td>MVI</td>
<td>Malaria Vaccine Initiative</td>
</tr>
<tr>
<td>NIMR</td>
<td>National Institute for Medical Research</td>
</tr>
<tr>
<td>PATH</td>
<td>Program for Appropriate Technology in Health</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red Blood cells</td>
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<td>WHO</td>
<td>World Health Organization</td>
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## List of abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LAMP</td>
<td>Loop-mediated isothermal amplification</td>
</tr>
<tr>
<td>mRDT</td>
<td>Malaria Rapid Diagnostic Tests</td>
</tr>
<tr>
<td>MSP</td>
<td>Merozoite surface protein</td>
</tr>
<tr>
<td>PfEMP1</td>
<td>Plasmodium falciparum erythrocyte membrane protein1</td>
</tr>
<tr>
<td>PfHRP2</td>
<td>Plasmodium falciparum Histidine-Rich Protein2</td>
</tr>
<tr>
<td>PLDH</td>
<td>Plasmodium Lactate Dehydrogenase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>qtr-PCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>Swiss-TPH</td>
<td>Swiss Tropical and Public Health institute</td>
</tr>
<tr>
<td>SSP</td>
<td>Sporozoites surface protein</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrotic factor</td>
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Summary

Introduction

Malaria is caused by intracellular organisms that belong to the genus Plasmodium. In 2015, there were an estimated 438,000 deaths and 214 million clinical illnesses due to malaria infection, of which the majority were in sub-Saharan African children below five years of age. Amongst the five species that are known to infect humans, Plasmodium falciparum causes the most severe disease, mostly in children and pregnant women in sub-Saharan Africa. Despite malaria control programs being operational for many years, malaria elimination in most endemic regions is far from being achieved. Vaccination is considered the most cost effective method of preventing infectious diseases. To date, there are no effective vaccines available for parasitic infections, despite the existence of strong evidence of acquired immunity in most parasitic infections studied. It is therefore highly likely that the addition of an effective tool such as a vaccine to the current malaria control strategy would have a strong positive impact on our ability to control this disease. In the first part of this thesis, we aimed to investigate the vaccine efficacy as well as the cellular and humoral immunity of African paediatric volunteers vaccinated with the most clinically advanced malaria vaccine; the RTS, S/AS01.

Meanwhile, novel vaccination and testing approaches are being pursued to improve or replace the recombinant subunit malaria vaccine approach to meet the goals formulated in the Malaria Vaccine Roadmap of WHO (http://www.who.int/immunization/topics/malaria/vaccine_roadmap/en). These goals strategized that by 2030, licensed vaccines targeting Plasmodium falciparum and Plasmodium vivax should encompass the following two objectives, for use by the international public health community:

i) First, it should have a protective efficacy of at least 75 percent against clinical malaria and be suitable for administration to appropriate at-risk groups in malaria-
endemic areas.

ii) Secondly, it should reduce transmission of the parasite and thereby substantially reduce the incidence of human malaria infection; enable elimination in multiple settings and be suitable for administration in mass campaigns.

Currently, the most promising candidate seems to be the whole malaria sporozoite approach, which is formed of cryopreserved, purified whole live-attenuated (either by irradiation or genetic attenuation) sporozoites. One of the novel tools used to analyze induced vaccine efficacy in sub-Saharan Africa experimentally vaccinated volunteers is controlled human malaria infection (CHMI). Many CHMIs using infectious mosquito bites or purified sporozoites have been successfully conducted in the USA and Europe over many years, but this approach had not been employed in sub-Saharan Africa until 2012. The aim of the second part of this thesis was to describe the potential of using CHMI as a tool to accelerate malaria vaccine development in sub-Saharan Africa and to dissect malaria-specific immunity induced by CHMI based on our trial conducted in 2012 in Bagamoyo.

Methods and findings

In the first part of this thesis (Chapter 4), the aim was to investigate safety, efficacy, cellular and humoral immunity in RTS,S/AS01 vaccinated Tanzanian paediatric populations. Adverse events were used to determine the safety of the RTS,S/AS01 vaccine in this age group (paper I), ELISA to measure the vaccine-induced CS-specific antibodies and Luminex to measure vaccine-induced cytokine responses (paper II and III). Furthermore, flow cytometry was used to investigate vaccine-induced cellular immune responses (paper III). We also looked into the implications and practicalities of immunological sampling in the African paediatric population. We did community sensitization and collected blood samples from 400 children for immunological study (paper IV). We showed that in 6-12 week old infants, vaccine efficacy against clinical malaria 14 months after first vaccination was
30.1% (95% CI, 23.6 to 36.1) in the intention-to-treat (ITT) and 31.3% (97.5% CI, 23.6 to 38.3) in the per-protocol (PP) population. Furthermore, the vaccine efficacy against severe malaria was 26.0% (95% CI, −7.4 to 48.6) and 36.6% (95% CI, 4.6 to 57.7) in the ITT and PP populations, respectively. The safety of the vaccine in terms of serious adverse events showed similar trends in both study groups. We identified two main RTS, S/AS01 vaccine induced cellular immune mechanisms: (i) Th1-related responses such as CS-specific IFN-γ, GM-CSF and IL-15 are associated with protection and (ii) Th2-related responses mediated by CS-specific IL5 and RANTES are associated with increased odds of malaria. Moreover, antibody avidity alone did not predict protective efficacy in the current study. The induction of RTS, S/AS01 protective Th1 and pro-inflammatory responses was lower in infants compared to children; a scenario that might explain the lower efficacy observed in the infant cohort. Furthermore, we also showed that immunology studies in the paediatric population can feasibly be conducted in African research institutions.

In the second part of this thesis (Chapter 5), we conducted in 2012 the first CHMI using cryo-preserved purified non-attenuated sporozoites in Tanzanian adult volunteers with previous malaria exposure (paper V). In this study, the humoral and cellular immune responses elicited following CHMI were evaluated (paper VI and VII). We used adverse events to determine the safety of the CHMI model in malaria pre-exposed volunteers. We also used blood slide microscopy to define sporozoite infectivity rates, Luminex assays to examine the sporozoite-induced antibodies, B-cell Elispot analysis, single cell RNA sequencing, flow cytometry and cell sorting followed by in vitro stimulation assays to investigate and define the affected innate and adaptive immune responses following CHMI (paper VIII). Our studies showed that: (i) CHMI is safe, tolerable and infective when used in malaria endemic regions, (ii) a single dose of intradermal sporozoite (PfSPZ) challenge elicited long-lived merozoite-opsonizing antibodies and long-lasting innate and innate-like lymphocyte populations, (iii) When we compared Dutch (malaria naïve) and Tanzanian
Acknowledgement

(malaria exposed) subjects undergoing the same challenge study, Dutch subjects responded differently to PfSPZ challenge compared to Tanzanian (malaria pre-exposed) subjects.

Conclusion

Substantial investment in research and development is needed to develop a highly efficacious malaria vaccine. To date, the recombinant subunit vaccines are yet to give the desired levels of protection for malaria elimination but seem to prevent malaria disease in high transmission settings. Large scale manufacturing, storage and distribution of live whole malaria sporozoite-based vaccines for mass administration need further development. So far, data generated from the PfSPZ vaccine trials conducted in the USA, Europe and in African research institutions imply that malaria naive individuals respond better to malaria vaccines than malaria pre-exposed individuals. The question remains to be, “what exactly constitutes the reason for lack of durable protection against malaria infection in endemic areas?” The most important factor in accelerating future vaccine development is a better understanding of the biology and nature of acquired immunity, which will lead to improved vaccine design. We have established the foundation for using CHMI to assess efficacy of new interventions against malaria and to study the mechanisms of the lack of protection conferred by different malaria vaccines in endemic settings. This study has opened new doors in the field of malaria intervention, whereby malaria vaccine and drug efficacy can be easily tested using CHMI in the target population.
CHAPTER 1: Introduction
1.1. Malaria: overview

1.1.1. The parasites

Malaria infection is caused by intracellular organisms that belong to the genus *Plasmodium* in the Phylum *Apicomplexa* within the *Protista* kingdom. The Phylum *Apicomplexa* is estimated to consist of over 5,000 species, many of which are responsible for causing a wide range of serious diseases in humans, livestock, wild animals and invertebrates (Gubbels and Duraisingh, 2012; Levine, 1988). The genus *Plasmodium* consists of more than 100 species, of which four species have long been recognized to infect humans and an additional species, known to naturally infect macaques, has recently also been reported to infect humans (Knowles and Gupta; White, 2008). Malaria is transmitted to humans through the bite of infected female anopheline mosquitoes. Out of over 400 anopheline species that are known to date, only forty are considered to be dominant vector species (Sinka et al., 2012). The prevalence of malaria species of public health importance varies worldwide and is linked to the geographical and environmental preferences of the parasite (Figure 1) (Blanford et al., 2013).

![Figure 1: Global distribution of *P. falciparum* and *P. vivax* by countries. (Feachem et al., 2009)](image-url)
Below we highlight the characteristics of the five malaria species known to infect homo-sapiens.

### 1.1.1.1. *Plasmodium falciparum*

*P. falciparum* is found in tropical and subtropical areas of the world, and is known to be the principal cause of malaria-related morbidity and mortality. Pregnant women, children and immunocompromized individuals constitute the most vulnerable population, with over half a million deaths in Africa alone estimated to be caused by *P. falciparum* infection in children under five (WHO, 2015). The severity of *P. falciparum* infection is mainly due to its ability to multiply in the red blood cells (RBCs) every 48 hours during its asexual blood stage cycle. Destruction of RBCs leads to significant RBC loss and contributes to anaemia, which is often observed in chronically infected persons. *P. falciparum* infection of the RBC leads to novel, parasite-encoded protein expression on the surface of infected RBCs (iRBCs). These surface expressed proteins, such as the Var gene encoded proteins, enable *P. falciparum* to adhere to endothelial cells in small vasculature (sequestration), hence creating chances for physically obstructing small blood vessels in the brain and kidneys. The iRBCs rosetting in the brain may result in cerebral malaria, a complication that may lead to mental retardation or death (Cooke et al., 2000; Idro et al., 2010).

### 1.1.1.2. *P. vivax*

*P. vivax* is the most prevalent human malaria parasite outside of Africa, due to its adaptation to relatively cooler climate zones. Although *P. vivax* contributes to widespread morbidity in endemic areas, *P. vivax* infection is rarely severe or fatal (Howes et al., 2016; White., 2011a). *P. vivax* is well known for its ability to develop dormant stages in the liver that can be reactivated. Reactivated *P. vivax* can cause asexual blood stage infections several months or years after the primary infectious mosquito bite. Currently, only
Primaquine is known to be an effective treatment against \textit{P. vivax} dormant liver stage forms, with the disadvantage that it requires 14 days of consistent drug adherence with significant side effects in glucose-6-phosphate dehydrogenase (G6PD) patients (Ashley et al., 2014; John et al., 2012; White, 2011b).

\textit{P. vivax} control is challenging due to factors such as its ability to be transmitted by a broad range of anopheline vectors species, the early biting behavior of mosquitoes that transmit the parasite, the emergence of gametocytes stages before onset of illness and the difficulty of diagnosing asexual blood stage infection due to low parasitemia (Bassat et al., 2016).

\subsection*{1.1.1.3. \textit{P. ovale}}
\textit{P. ovale} is a relatively rare species, mostly found in West-Africa and the Pacific islands. \textit{P. ovale} exhibits Schüffner's dots and infected erythrocytes can be normal or slightly enlarged; a characteristic feature that is also shared by \textit{P. vivax}, making it difficult to distinguish the two species microscopically (Collins and Jeffery, 2005a). Similar to \textit{P.vivax}, \textit{P. ovale} can form a latent liver stage called hypnozoite, which makes the parasite capable of causing disease several months or years after primary infection (Collins and Jeffery, 2005b; Markus, 2011). Furthermore, \textit{P. ovale} tends to present a low parasite burden and mild clinical symptoms, making routine diagnosis challenging. Recently, two \textit{P. ovale} subspecies, classic (\textit{P. ovale curtis}) and variant (\textit{P. ovale wallikeri}) have been characterized (Sutherland et al., 2010). In Africa, the higher prevalence of \textit{P. ovale} relative to \textit{P. vivax} could be explained by the ability of \textit{P. ovale} to infect populations carrying the null Duffy blood group, which provides natural resistance to \textit{P. vivax} (Collins and Jeffery, 2005b).

\subsection*{1.1.1.4. \textit{P. malariae}}
\textit{P. malariae} is found in sub-Saharan Africa, Southeast Asia, Indonesia and in the Western Pacific Islands (Collins and Jeffery, 2005a). Compared to the other human malaria parasite
species, *P. malariae* has a distinct asexual blood stage life cycle that lasts for three days. Although *P. malariae* does not cause complicated malaria at the beginning, it is known that if untreated, *P. malariae* patients have higher risk of developing a life-long, chronic infection that may result in serious complications such as nephrotic syndrome (Badiane et al., 2014). In most cases *P. malariae* distributions have been found to coincide with that of *P. falciparum*. In malaria-endemic Africa, mixed infections of *P. malariae* and *P. falciparum* are common. However, to reveal the presence of low-parasitemia *P. malariae* within mixed infections, molecular techniques such as polymerase chain reaction (PCR) are required (Collins and Jeffery, 2007).

### 1.1.1.5. *P. knowlesi*

*P. knowlesi* has recently been reported to be a significant cause of zoonotic malaria, particularly in Malaysia. *P. knowlesi* is abundant in Southeast Asia as a natural pathogen of long-tailed and pig-tailed macaques. Despite the fact that *P. knowlesi* morphologically resembles *P. falciparum* and *P. malariae*, *P. knowlesi* has a short asexual blood stage cycle of approximately 24 hours, unlike *P. falciparum* and *P. malariae* which requires 48 hours to complete their asexual blood stage cycle. Importantly, *P. knowlesi* infections can progress rapidly from uncomplicated to severe presentations across age groups (Vythilingam et al., 2016; Wesolowski et al., 2015).

### 1.1.2. Epidemiology of *P. falciparum* malaria

*P. falciparum* is a major cause of malaria that impacts most heavily in children and pregnant women in sub-Saharan Africa, Southeast Asia and South America. By 2015, an estimate of 214 million (range: 149–303 million) cases of malaria have been reported to cause about 438 000 deaths (range: 236 000–635 000), predominantly in African children younger than five years old (WHO, 2015). For the past decades, *P. falciparum* malaria has become one
of the most studied diseases globally. *P. falciparum* was endemic in Southern Europe and North America until the beginning of the 20th century. Comprehensive surveillance and treatment programs, including the use of Dichlorodiphenyltrichloroethane (DDT) for vector control has since lead to the elimination of malaria in these regions.

To date, the importance of malaria remains prominent on a global scale, despite the recent decline in malaria cases (Berberian, 1948; Meshnick and Dobson, 2001). Although malaria mortality rates amongst children have declined by over 50% within the past decade, the WHO 2014 world malaria report indicated that *P. falciparum* malaria was still responsible for about 10% of Africa’s entire disease burden in 2013, with children (WHO, 2014) and in primigravidae (Menendez et al., 2000) being the most affected populations. Some of the ongoing hurdles in malaria control programs are caused by environmental changes such as heavy rains after drought and the associated focal expansion of vectors, socio-economic factors and civil wars in some part of Africa leading to mass population movement (White et al., 2014). Additionally, the availability of efficacious drugs is constantly under threat due to the development of drug resistance by the parasite and insecticide resistance of the vector (Jindal et al., 2014).

### 1.1.3. Life cycle of *P. falciparum*

The life cycle of *P. falciparum* alternates between the human host and the transmitting vector, Anopheles mosquito. During its development, the parasite undergoes a number of morphological changes that take place in a variety of tissues in both the mosquito and human vector (Bijker et al., 2013; Pradel and Frevert, 2001). Sporozoites, the infectious form of *P. falciparum*, are injected into the human dermis or capillaries during the blood meal of an infected female Anopheles mosquito. It is estimated that some of the
sporozoites may be contained at the site of injection for up to 6 hours before migrating to the liver (Yamauchi et al., 2007). After invading hepatocytes, it takes approximately 6-7 days for the parasite to multiply within hepatocytes (Pradel and Frevert, 2001). At the time of leaving the liver, several thousand of merozoites are contained within merosomes, which is most likely to avoid host cell defense mechanisms (Vaughan et al., 2012). Merozoites are finally released from the ruptured merosomes, infecting RBCs to start the asexual blood stage development (Cowman and Kappe, 2006). In the erythrocytic phase, merozoites undergo asexual multiplication, turning from a ring to a trophozoite stage and finally to a schizont stage. When the schizonts reach full maturation, the iRBCs burst; freeing the 16-20 newly developed merozoites to invade fresh, non-infected RBCs.

The asexual blood stage developmental cycle lasts for 48 hours and is the cause of the symptoms associated with malaria disease. During this stage, a proportion of merozoites will commit to differentiate into male and female gametocytes (Alano, 2007). After a female mosquito feeds on human blood containing gametocytes, the gametocytes are released from the iRBCs in the mosquito midgut and develop into gametes. The fertilized female macrogametes develop into zygotes and then into actively moving ookinetes that traverse the midgut epithelium and further develop to oocysts. Fully developed and matured oocysts release sporozoites that migrate through the body cavity to the salivary gland. The cycle of human infection begins again when the infected female mosquito feeds on the next human host (Arama and Troye-Blomberg, 2014; Baer et al., 2007; White et al., 2014) (summarized in Figure 2).
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Figure 2: Plasmodium species life cycle in both mosquito vector and human host

The malaria parasite life cycle involves two hosts. The female Anopheles mosquito and the human host (1). Sporozoites injected by mosquito migrate and infect liver cells (2) and mature into schizonts (3), which rupture and release merozoites (4). After this initial replication in the liver (exo-erythrocytic schizogony-A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony-B). Merozoites infect red blood cells (5). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites (6). Some of the merozoites differentiate into sexual erythrocytic stages (gametocytes) (7). The gametocytes, male and female, are ingested by an Anopheles mosquito during a blood meal (8). The parasites multiplication in the mosquito is known as the sporogonic cycle(C). While in the mosquito’s stomach, the microgametes penetrate the macrogametes generating zygotes (9). The zygotes in turn become motile and elongated (ookinetes) (10) which invade the midgut wall of the mosquito where they develop into oocysts (11). The oocysts grow, rupture, and release sporozoites (12), which make their way to the mosquito’s salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle (1) (CDC, 2013).

1.1.4. Pathogenesis of P. falciparum malaria

The P. falciparum liver stage in humans is clinically silent and therefore inherently difficult to study. Clearly, the liver stage does not cause obvious signs and symptoms of ongoing malaria infection. In contrast, the pathogenesis of P. falciparum malaria disease during the erythrocytic life cycle stage has been well studied. P. falciparum malaria pathogenesis is initiated when merozoites invade the RBC. The different pathophysiological outcomes of P.
falciparum asexual blood stage infection are summarized in Figure 3. After RBC invasion, a principal ligand known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) starts to be expressed on the surface, which is encoded by a highly polymorphic multigene family, also known as the var genes (with around 60 copies per genome) (Newbold et al., 1999). PfEMP1 has been reported to play a pivotal role in *P. falciparum* malaria pathogenesis by mediating the cytoadherence of iRBCs to endothelial surface receptors in the capillaries (Cooke et al., 2000). The sequestration of iRBCs is the result of the interaction between parasites-derived proteins present on surface of iRBCs (mainly PfEMP1) and several host molecules expressed on the surface of small blood vessel endothelial cells. Some key receptors for parasite adhesion are known, and include chondroitin sulfate A (CSA), the intercellular adhesion molecule 1 (ICAM1) and CD36. These parasite–host protein interactions have been found to be associated with pathology in the placenta (CSA), brain (ICAM-1) and in other organs (CD36) (Cooke et al., 2000).

Recently, the involvement of intravascular fluid depletion during severe malaria and impaired microcirculation leading to cardiac dysfunction has been reported. However, such findings warrant further detailed clinical evaluation (Mishra et al., 2013). A huge increase in parasite density and biomass during erythrocytic cycle leads to the systemic release of pro-inflammatory cytokines including tumor necrotic factors (TNF). It is thought that TNF production levels regulated by the promoter region contribute to pathophysiological processes such as anaemia and cerebral malaria (McGuire et al., 1999). Combining these processes of cytokine release, innate immune system hyperactivation, vascular obstruction, inflammation and damage leads to a multitude of organs to be affected including the kidney, brain, lung, heart and placenta (Figure 3) (Gazzinelli et al., 2014).
Figure 3: A series of pathogenesis that is suggested to occur during different stages of malaria parasite life cycle in human host.

(a) After a mosquito bite, sporozoites travel to the liver to infect hepatocytes and develop into merozoites that are released in the bloodstream. Repeated cycles of red blood cell (RBC) invasion, replication and merozoite release will result in the exponential growth of the parasite population and lead to disease. IRBCs will circulate containing ring-stage parasites, and a small proportion of merozoites will develop into male and female gametocytes that infect mosquitoes, completing the parasite life cycle. (b) The removal of IRBCs by splenic macrophages or the uptake of free haemozoin results in the activation of innate immune receptors and cytokines which cause paroxysms and induce the expression of adhesion molecules that helps mediate parasite sequestration. The sequestration of infected reticulocytes is less intense. Haemolysis of infected and bystander (uninfected) RBCs, uptake of altered RBCs by splenic macrophages and cytokine-induced impairment of erythropoiesis cause anaemia. Free haemoglobin catalyses oxidative damage, hypoxia and lactic acidosis, promoting metabolic acidosis, which is aggravated by the altered renal function that is observed in patients with malaria. (Adapted from: Nature Reviews Immunology) (Gazzinelli et al., 2014)

1.1.5. Malaria diagnosis

Early diagnosis and treatment of malaria can substantially reduce disease-related morbidity and mortality and further contributes to the reduction of malaria transmission. Malaria diagnosis involves identifying malaria parasites or antigens/products in a patient’s blood (Tangpukdee et al., 2009). Microscopy has been the gold standard for malaria diagnosis for many years both in research and in the field. This method detects parasites...
in the capillary blood of infected individuals via thick and thin blood smears. In many cases, reliable and accurate microscopy results can only be achieved if the test is performed by qualified and well trained microscopists. However, in the real-life situation of many resource constrained settings, lack of reliable electricity supply to operate microscopes, shortage of high quality reagents supply and the low level of training of the microscopists to perform routine blood slide reading constitute serious challenges to the utilization of microscopy for public health purposes (Michael, 2015).

Recently, a rapid, sensitive and easy to use malaria diagnostic test has been introduced widely, which relies on the detection of parasite specific antigens such as *P. falciparum* Histidine-Rich Protein 2 (*Pf*HRP2), *Plasmodium* lactate dehydrogenase (*PLDH*) or Aldolase (*PfAldolase*), (WHO, 2011). These rapid diagnostic tests (RDTs) do not require trained personnel or specialized equipment for malaria diagnosis and therefore they have been massively distributed into the public health system (T3 program: http://www.who.int/malaria/areas/test_treat_track/en/). However, RDTs are also associated with disadvantages; for example they are relatively expensive, do not quantify parasites, only have a sensitivity of around 100 parasites/microliter of blood and are sensitive to environmental storage temperature and humidity (WHO, 2011; Wongsrichanalai et al., 2007). In addition, *P. falciparum*-specific species identification is possible only with *Pf*HRP2 based kits, with other human malaria species indistinguishable and grouped under non-*P. falciparum*. RDTs based on pLDH can distinguish between *P. falciparum*, *P. vivax* and the other malaria species (pan-malaria), however they lack sensitivity compared to *Pf*HRP2-based RDTs (Wongsrichanalai et al., 2007).

Molecular biological techniques based on polymerase chain reaction (PCR) have shown higher sensitivity compared to RDT and are also able to distinguish not only between
different *Plasmodium* species but also between different *P. falciparum* strains (Gunawardena and Karunaweera, 2015; Shaukat et al., 2012). QPCR based detection of malaria infection is also suitable for accurate quantification of malaria parasites. However, qPCR for malaria diagnosis is not wide-spread since it is expensive, requires well-maintained equipment and higher level technical expertise, currently limiting its use to research purposes (Zimmerman and Howes, 2015).

### 1.1.6. Current malaria control strategies

Malaria control means reduction of disease incidence, prevalence, morbidity or mortality within a focal area to an acceptable level following proper intervention strategies. Elimination is defined as taking control one step further and aims at reducing incidence in a certain geographical region close to zero. While malaria eradication is defined as permanent global reduction of the incidence of infection to zero, extinction is reduction of a specific infectious agent to a point that it no longer exists in nature or in the laboratory (Andrews and Langmuir, 1963). The current malaria control strategy encompasses two major domains: (i) prevention; which works against the transmission of the parasite from mosquito vector to humans and (ii) case management; that focuses on the development of illness and severe disease. Global disease control efforts have successfully impacted on (i) control of many infectious diseases including malaria, (ii) elimination of diseases such poliomyelitis in many countries and (iii) eradication of diseases such as smallpox. Internationally, the call towards malaria elimination and eventual eradication has turned out challenging, in regards to translating it into reality in the near future (MMWR, 1999; WHO, 2014). Currently, 80 countries with ongoing malaria transmission are classified by WHO as being in the malaria elimination phase and 4 countries, namely United Arab Emirates, Morocco, Turkmenistan and Armenia, have been recently certified by WHO to have eliminated the disease (Cibulskis et al., 2016; Newby et al., 2016).
1.1.6.1. Malaria vector control

Vector control is considered an essential tool in malaria control strategies and is mainly based on targeting mosquitoes, the vector for transmission (Giardina et al., 2014). This has been possible through large scale implementation of indoor residual spraying of insecticides and insecticide treated bed nets as well as by draining bodies of stagnant water, which is the habitat of the larvae stage. These approaches successfully led to malaria elimination in Europe and the USA. In Africa, there have been undeniable advances in the fight against malaria in recent years, whereby we could see a trend of malaria incidence dropping by around 37% in most malaria endemic areas since 2000 (Cotter et al., 2013; Mharakurwa et al., 2013; Roucher et al., 2014). It is most likely that the use of insecticide treated bed nets, indoor residual spraying and rapid treatment of malaria cases have played an important role in getting closer to the malaria-specific target of the Millennium Development Goals (target C), which aims to reduce the incidence of malaria by 75% (WHO, 2015). However, some studies caution this optimism and have reported lack of evidence for significantly decreasing malaria incidence based on these interventions, questioning the universal trend across settings and transmission intensities (Okiro et al., 2011, 2013).

1.1.6.2. Malaria treatment

Malaria is curable and preventable if treated adequately and promptly. However, *P. falciparum* parasites have developed resistance against the cheapest and most widely used antimalarial including chloroquine and Sulfadoxine-Pyrimethamine (SP) (Schneider and Kim, 2013). WHO recommends artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated *P. falciparum* malaria. Amongst ACTs, Artemisinin-lumefantrine (ALU) is used as first line therapy for uncomplicated malaria according to
Tanzanian National Guidelines for Malaria Diagnosis and Treatment (Adjuik et al., 2004; WHO, 2016). The ongoing emergence of antimalarial drug resistant parasite strains in South-East-Asia and appearance of pyrethroid resistant vector strains throughout sub-Saharan Africa are a considerable threat to the current global malaria control programmes (Menard and Dondorp, 2017; Mulamba et al., 2014). Drug development of a single compound takes up to an average of 15 years through different stages of clinical development. In recent years, thousands of chemical structures have gone through pre-clinical testing and some even reached early clinical testing (Gamo et al., 2010). With no new drug class expected to reach the market for at least the next five years, there is a need to strengthening current malaria surveillance programs to ensure proper use of available antimalarial drugs and early resistance detection (Roses, 2008).

Learning from the regions that have successfully eliminated malaria in the past, it is obvious that malaria could be controlled and focally eliminated with a combination of complementary strategies put in place. As we embark on eliminating malaria in Africa, a combination of the current control strategies as well as novel approaches, which include simple, robust and sensitive diagnostic tools, better drugs as well as an effective vaccine will be required (WHO, 2015).

1.1.6.3. Malaria vaccines

To date, vaccination is the most cost effective method of preventing infectious diseases (Karunamoorthi, 2014; Loucq, 2013). The potential of vaccination programmes to control viral and bacterial diseases such as poliomyelitis, measles, diphtheria, tetanus, rabies and the eradication of smallpox in humans have been remarkable success stories of vaccines (Loucq, 2013). Development of an effective malaria vaccine has been a major goal of
malaria researchers for many decades. The revised malaria vaccine technology roadmap is targeting to have a vaccine that will achieve malaria elimination in multiple settings and at the same time being highly efficacious against clinical malaria by 2030 (WHO, 2013). The complexity of the Plasmodium life cycle, lack of clear understanding of important biological processes that lead to potent, long-lasting host immune responses and the high parasite diversity have severely hampered efforts to develop an effective malaria vaccine (Crompton et al., 2010). For many decades, scientists have explored several vaccine design approaches that target different stages of the parasite life cycle. These include pre-erythrocytic, blood-stage and transmission blocking vaccine approaches (Hill, 2011; Wang et al., 2009). So far RTS,S/AS01E is the only vaccine to show a partial protective effect against clinical malaria among young children in a phase 3 clinical trial and WHO has planned for an implementation programme (MVIP) in three countries beginning from 2018 (WER-WHO, 2016).

1.2. Immune responses against malaria

1.2.1. Humoral immunity against malaria

Antibodies have long been known to play a critical role in natural immunity against malaria. The most direct evidence that antibodies are important mediators of immunity to malaria comes from passive transfer studies, in which antibodies from malaria-immune adults were successfully used to treat patients with severe malaria. Studies in mice deficient in Fc-γ receptors further supported an important role for antibodies in protection (Cohen et al., 2010; Sabchareon et al., 1991). Antibodies may protect an individual against malaria by a variety of mechanisms, including inhibition of sporozoites or merozoite invasion of host cells. Furthermore, studies have demonstrated that antibodies in the plasma of malaria immune volunteers (Hill et al., 2013) and RTS,S/AS01E vaccine immunized volunteers
(Schwenk et al., 2003) were able to opsonize free merozoites and sporozoites, respectively, and mediate in-vitro phagocytosis by monocytes.

Although natural malaria infections fail to induce sterile protection - even after several years of repeated exposure to the parasite- a partially protective, short-lived antibody response could be observed (Kinyanjui et al., 2007). Studies have shown that children from malaria endemic regions build up partial immunity that protects against severe disease as they grow older - however a complete sterile immunity has very rarely been reported (Portugal et al., 2013). Moreover, the partially induced natural immunity has shown to wane as person moves away from malaria endemic regions into a region without malaria exposure. During the Plasmodium life cycle, the parasite expresses a wide range of highly polymorphic proteins (Doolan et al., 2009). The complex interplay of polymorphic parasite proteins with the host immune system is most likely the reason for partial protection observed under natural exposure. Therefore, naturally developing immunity against malaria is not a good guideline for developing an effective vaccine against the disease (Waters, 2006).

1.2.2. Cellular immunity against malaria

Studies have associated cellular immune responses, particularly CD8+ T cells secreting IFN-g, with pre-erythrocytic immunity (Epstein et al., 2011; Malik et al., 1991). Some evidence suggests that cellular immune responses induced by malaria infection may protect against both pre-erythrocytic and erythrocytic parasite stages (Schneider and Kim, 2013). The pre-erythrocytic stage parasite develops within the hepatocytes, thereby making infected hepatocytes targets for cytotoxic, major histocompatibility complex class I (MHC class I) restricted CD8 T cells (Epstein et al., 2011; Guebre-Xabier et al., 1999). It is
commonly thought that this kind of immunity is dependent on the expression of parasite antigens on the hepatocyte surface in conjunction with MHC class I (Villarino and Schmidt, 2013a). The MHC molecules are key players in regulating adaptive immune responses following invasion of foreign pathogens. Two major classes of MHC molecules, namely MHC class I and MHC class II, have been identified (Erickson, 1987; de Groot et al., 2016). MHC class I molecules are found on most cell types, and function by binding and presenting endogenously, intracellularly derived peptides to CD8 T cells. MHC class II molecules reside on the surface of professional antigen presenting cells and function by binding exogenously derived proteins that will be processed intracellularly to be presented to CD4 T cells. The importance of MHC molecules on influencing the outcome of a protective vaccine response has been demonstrated (O et al., 2014). Good peptide binding to the MHC groove is essential, but not the sole determinant of antigen presentation (Wieczorek et al., 2017). The development of a peptide-based malaria vaccine should also consider the influence of MHC genotypes in the immune responses of target population (Comber and Philip, 2014; Wieczorek et al., 2017). T cell mediated mechanisms that result in destruction of malaria infected hepatocytes in humans are not well elucidated. They may include cell death induced by contact between the lymphocyte effector and target cells, killing through release of cytokines such as interferon gamma (IFN-g) that induce hepatocytes to produce nitric oxide or destruction via antibody-dependent cellular cytotoxicity (Seguin et al., 1994; Trimnell et al., 2009; Villarino and Schmidt, 2013b). IRBCs do not express MHC class I or class II molecules. This renders erythrocytes not a direct target for T- cell recognition and effector mechanisms. However, the iRBCs are liable to binding of antibodies that target surface-exposed foreign antigens. This binding could result in opsonization, thereby making the parasitized erythrocytes more susceptible to clearance by phagocytic cells in the spleen and destruction by complement fixation (Doll and Harty, 2014; Urban et al., 1999).
1.3. **Malaria pre-existing responses and the magnitude of vaccine induced immunity**

Recent studies have revealed that pre-existing immunity may modulate the magnitude and specificity of immune responses induced by subsequent immunizations (Bergmann-Leitner et al., 2012; Kannanganat et al., 2010). Pre-existing immunity was demonstrated to impede the ability of human adenoviruses to induce strong innate and adaptive immune responses when used as a vaccine delivery system (Croyle et al., 2008; Kannanganat et al., 2010). Whether the observed slow development of protective immunity in populations living in malaria endemic areas reflects the down regulation of T cell specific immunity following natural parasite infection remains to be revealed. Malaria specific memory B cells (MBCs) can be maintained for many years, even after absence of repeated exposure (Migot et al., 1995; Ndungu et al., 2013). However, there is also evidence demonstrating that protective immunity gradually declines if a person moves away from a malaria endemic area for a longer time period (Färnert et al., 2015). The role of pre-existing immunity on the development of vaccine induced immunity needs further investigation. A better defined role of pre-existing immunity on vaccine-induced immunity in the endemic population would aid our understanding of how individuals previously exposed to malaria will respond to different immunization regimens.

1.4. **Malaria vaccines development**

Vaccines are tailored to induce protective immune responses against infectious diseases when applied prior to the contact with the pathogen itself (Riedel, 2005). For many years, malaria vaccine development has faced a number of challenges. Amongst those is the ability of the parasite to evade host immune responses by camouflaging itself through expression of various antigenic forms in both the mosquito vector (Ramphul et al., 2015)
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and human host (Stanisic et al., 2013; Wykes et al., 2014; Zheng et al., 2014). Immunological assays and system biology approaches have been used to predict the antigen targets that may serve as better candidates for today’s vaccine development programs (Hagan et al., 2015). For nearly half a century, different vaccine development approaches have been employed. However, knowledge gaps of protective immune mechanisms that develop under natural conditions severely hamper these programs.

Likewise, the lack of vaccine-induced protection observed thus far for most malaria vaccine candidates as they progress into human clinical trials makes malaria vaccine development highly empirical (Bouharoun-Tayoun et al., 1990; Nakaya and Pulendran, 2015; Plotkin, 2008; Weiss and Jiang, 2012). To date, many of the current malaria vaccine development strategies have focused on utilizing (i) multiple components that will be effective against more than one parasite life cycle stage, (ii) multiple epitopes to overcome genetic and antigenic variation and (iii) multiple components that will induce both humoral and cellular immune responses. The ideal vaccine does not only prevent clinical disease but also infection and blocks transmission (Doolan and Hoffman, 1997; Kumar et al., 2002; Lozano and Patarroyo, 2007; Shi et al., 1999).

Three stages of the parasite life cycle have been pursued as targets by different vaccines, namely the pre-erythrocytic stage vaccines, the asexual blood stage vaccines and a vaccine targeting the parasite within the vector thus preventing transmission. An overview of the vaccine approaches targeting the different life cycle stages are given below (Figure 4):
An infected female anopheles mosquito bite injects sporozoites into capillaries (1). The sporozoites pass quickly into the human liver (2). The sporozoites multiply asexually in the liver cells over the next 7 to 10 days, causing no symptoms (3). In an animal model, the parasites, in the form of merozoites, are released from the liver cells in vesicles, journey through the heart, and arrive in the lungs, where they settle within lung capillaries. The vesicles eventually disintegrate, freeing the merozoites to enter the blood phase of their development (4). In the bloodstream, the merozoites invade red blood cells (erythrocytes) and multiply again until the cells burst. Then they invade more erythrocytes. This cycle is repeated, causing fever each time parasites break free and invade blood cells (5). Some of the infected blood cells leave the cycle of asexual multiplication. Instead of replicating, the merozoites in these cells develop into sexual forms of the parasite, called gametocytes that circulate in the blood stream (6). When a mosquito bites an infected human, it ingests the gametocytes, which develop further into mature sex cells called gametes (7). The fertilized female gametes develop into actively moving oocinetes that burrow through the mosquito's midgut wall and form oocysts on the exterior surface (8). Inside the oocyst, thousands of active sporozoites develop. The oocyst eventually bursts, releasing sporozoites into the body cavity that travel to the mosquito’s salivary glands (9). The cycle of human infection begins again when the mosquito bites another person (10) (PATH- MVI, 2014).

1.4.1. Pre-erythrocytic malaria vaccine approaches

The pre-erythrocytic (PE) stage malaria vaccine development approach has recently attracted the attention of many scientists in the field, since it has shown the most promising outcomes when compared with vaccines aimed at targeting other malaria developmental stages. The PE vaccine approach aims at targeting the infective stage of *P. falciparum*, the sporozoite and the liver stage. Sporozoites can be targeted by
neutralizing antibodies that remove infective sporozoites from the circulation and prevent them from reaching the liver (Behet et al., 2014; Nahrendorf et al., 2014). If sporozoites escape from these antibodies and other innate immune mechanisms they will find their final destination – the liver hepatocytes. It is thought that liver stage parasites are recognized by CD8+ T cells that target and selectively kill these infected liver cells. In summary, these immune effector arms prevent merozoites being released to iRBC thereby preventing the development of clinical symptoms (Chakravarty et al., 2007; Reyes-Sandoval et al., 2011; Weiss and Jiang, 2012).

1.4.1.1. Recombinant PE vaccine approaches

The most researched PE vaccine stage protein is the *P. falciparum* circumsporozoite (CS) protein. This protein is expressed in abundance during sporogony, the sporozoite stage and the early hepatic stages of *P. falciparum* infection (Coppi et al., 2011). This protein is known to be an important mediator of sporozoite gliding motility and cell traversal in the midgut of the mosquito and within hepatocytes of the mammalian host (Coppi et al., 2011). Antibodies directed against CS protein have been shown to reduce the ability of the parasite to glide and traverse into the hepatocytes and hence are indirectly associated with reduced risk of clinical malaria (John et al., 2008). The critical roles of CSP as an immunodominant antigen that induce protection against PE stage of *P. falciparum* have been well characterized in pre-clinical studies in animals (Kumar et al., 2006). The NANP repeat domain is known to be a B-cell epitope and the C-terminal region is known to be a T-cell epitope (Cohen et al., 2011). Moreover, studies conducted in rodent models and humans have shown that antibodies directed to NANP CSP repeat region have consistently demonstrated the ability to neutralize sporozoites infectivity (Mehlhorn, 2011). Under natural conditions, sporozoites are known to travel rapidly to the liver after mosquito bite delivery hence creating limited time for the humoral immunity to act on them. The C-
terminal non-repetitive region of CSP can be processed by professional antigen presenting cells or infected hepatocytes and presented on the surface to initiate CD4 and CD8 T cell responses (Gordon et al., 1995; Singh et al., 2007). The discovery that a genetically engineered hepatitis B viral envelope can be used as a carrier matrix for different peptides lead to the use of this technology to design a CSP-based malaria vaccine that utilizes the hepatitis B surface antigen (HBsAg) in this manner (Rutgers et al., 1988).

The RTS, S malaria vaccine - developed by GlaxoSmithKline (GSK) Biologicals - is the most advanced malaria subunit vaccine. It consists of a hybrid molecule of CSP and HBsAg and free HBsAg recombinantly expressed by \textit{S. cerevisiae}. This hybrid molecule encompasses a part of the central repeat and the C-terminal region which are fused to the N-terminal region of HBsAg. The vaccine assembles itself into particles that also include unfused HBsAg (Kester et al., 2007). Furthermore, RTS, S is powered by a complex adjuvant (AS) system (Cohen et al., 2010), either AS02 (\textit{Quillaja saponiria} 21 (QS21), monophosphoryl lipid A (MPL) and an oil-in-water emulsion) or AS01 (QS21, MPL and liposomes) (Stoute et al., 1997) (Figure 5).
In its first clinical development phase conducted in malaria naïve volunteers, the RTS,S/AS01 vaccine has shown to be safe and immunogenic. In malaria semi-immune individuals, RTS,S/AS01 has been clearly shown to induce protective antibodies in infants in Mozambique (Aponte et al., 2007). When co-administered within the expanded program on immunization (EPI) scheme in Tanzanian infants (Abdulla et al., 2008) protection against clinical malaria was observed. In the phase 3 trial, three primary doses of vaccine was reported to reduce cases of clinical malaria by 28% and 18% in 5-17 months (children) and 6-12 weeks (infants) respectively (RTS,S Clinical Trials Partnership, 2015). Furthermore, analysis of pooled data from several phase 2 studies revealed that efficacy against clinical malaria was inversely related to transmission intensity (Bejon et al., 2013). In general, RTS,S/AS01 vaccine efficacy was shown to be about 36% in the first year; waning off to an efficacy of 2.5% in the fourth year, followed
by a partial rebound in clinical malaria cases during the fifth year in a high malaria exposed cohort (Olotu et al., 2016). The efficacy of 4.4% that was obtained during a 7 year follow-up compromised the benefits of the efficacy shown during the short term follow-up. In a phase III clinical trial conducted in 7 African countries in 11 trial centers with a wide range of malaria endemicity, RTS, S/AS01 was generally reported to have higher vaccine efficacy in children (5-17 months) than in infants (6-12 weeks) providing protection against clinical and severe malaria for up to 1.5 years following third vaccination (RTS,S Clinical Trials Partnership, 2014). These outcomes of the phase III RTS,S/AS01 vaccine trial have been somewhat disappointing (RTS,S Clinical Trials Partnership, 2014; RTS,S Clinical Trials Partnership et al., 2012)) but provided the proof that large phase III vaccine trials can be conducted in Sub-Saharan Africa. Although RTS,S/AS01 was approved by the European Medicines Agency for active immunization of children aged 6 weeks to 17 months against malaria, the WHO did not recommend the inclusion of RTS,S/AS01 in the Expanded Programme of Immunizations. The RTS,S/AS01 vaccine will be tested in a pilot study for larger scale implementation under real-life settings in Ghana, Kenya and Malawi starting in 2018 (http://www.path.org/news/press-room/809/).

1.4.1.2. Viral vectored PE vaccine approaches

Poxvirus vectors have been the most studied live viral system for delivering antigens in human and animal models, since their use in smallpox eradication in the 1970’s (Limbach and Paoletti, 1996; Moss, 2011). This virus has been demonstrated to be a useful vector for vaccination purposes, due to its ability to generate recombinant viruses that express a variety of foreign antigens, and which confer protection to immunized animals (Jacobs et al., 2009; Sánchez-Sampedro et al., 2015). An example of a PE subunit malaria vaccine with a multi-epitope was developed in the United Kingdom by a group from Oxford
University (Prieur et al., 2004). Since then, the Oxford group has been conducting a cascade of clinical trials with a prime boost approach aiming at provoking T-cell immune responses against liver stage parasites. In early 2002, a vaccine construct consisting of attenuated fowl pox virus strain 9 (FP9) with multi epitope fused to thrombospondin related adhesion protein (ME-TRAP) entered clinical trials in British and Gambian volunteers (Moorthy et al., 2004). TRAP is a PE protein that plays a major role in parasite gliding motility and infectivity of liver cells (Sultan et al., 1997). However, no protection was demonstrated when tested against febrile disease in children living in malaria endemic areas (Bejon et al., 2006).

Adenovirus vectors are amongst the few other viruses that attracted the attention of the vaccine research and development field. The replication-deficient adenovirus serotype 5 (Ad5) vector can be produced by having part of its genome removed and replaced by insertion of a foreign, non-viral target DNA (Bangari and Mittal, 2006). In the malaria vaccine field, the use of the Ad5 vectors remains challenging, as pre-existing neutralizing antibodies against the adenovirus vector were found to be associated with failure to induce appropriate immune responses in vaccinated individuals, both in the USA and Africa (Fausther-Bovendo and Kobinger, 2014; Nwanegbo et al., 2004; Saxena et al., 2013). Recombinant adenovirus serotype 5 (Ad5) vector expressing circumsporozoite protein (CSP) given either alone or together with apical membrane antigen 1 (AMA-1) expressing Ad5 virus was demonstrated to have increased IFN-γ specific CD8 T cell responses, albeit low antibodies responses. Additionally, the CHMI conducted with volunteers immunized using Ad5 with CSP and AMA-1 did not show protective efficacy (Sedegah et al., 2011).
1.4.2. DNA vaccine approaches

In the early 1990’s, advance in molecular biology technology lead to the finding that DNA or RNA inoculated directly into mice resulted in induction of cellular immune responses (Wolff et al., 1990). A vaccine trial conducted in the mouse model revealed that the injection of an influenza hemagglutinin glycoprotein-encoding plasmid via the skin or muscular route could induce protective immunity against a challenge with a lethal influenza virus (Fynan et al., 1993). The success of the DNA vaccine approach was also demonstrated in early stages of clinical trials (phase I) for different diseases (Khan et al., 2013). However, currently there is no single DNA based malaria vaccine that has moved to phase II clinical trials. This calls for a better design of DNA vaccine constructs and incorporating better promoters driving the gene expression in vivo, as well as novel delivery technologies that can enhance immune responses in humans (Ferraro et al., 2011).

1.4.3. The live attenuated whole parasite based vaccine approaches

The failure in clinical advancement for many subunit malaria vaccine approaches re-stimulated the concept of using whole sporozoites as a PE malaria vaccine. In the early 70’s, research revealed that immunization of mice and humans with radiation-attenuated sporozoites delivered by mosquitos could confer sterile protection against experimental malaria challenge (Clyde et al., 1973). Since then, the sporozoite-based approach has been considered as a gold standard for evaluation of malaria vaccine and drug efficacy in humans. CHMI studies that involve infecting healthy volunteers with live malaria sporozoites through infected mosquito bites was limited so far to few sites in Europe (Oxford and Nijmegen) and the USA (University of Maryland and NIH). In sub-Saharan Africa, the lack of phase I clinical trial facilities, trained medical and laboratory teams, together with the absence of high security mosquito rearing facilities has so far prohibited
testing in this important target population (Shekalaghe et al., 2014).

The recent technological advancements driven by the biotechnology company Sanaria (http://www.sanaria.com) to manufacture large quantities of aseptic, purified, cryopreserved *P. falciparum* sporozoites (*PfSPZ*) that meet stringent regulatory standards and which can be administered intravenously to volunteers using needles has opened up the possibility for testing PE vaccines at an early developmental stage in sub-Saharan countries (Hoffman et al., 2010). The whole sporozoite approach, either in the form of radiation-attenuated sporozoites (RAS) (Clyde et al., 1973; Nussenzweig et al., 1969), genetically attenuated parasites (GAPs) (Mueller et al., 2005) or wild type (WT) sporozoites under chloroquine prophylaxis (CPS) (Putrianti et al., 2009; Roestenberg et al., 2009), have been proven to confer protection against experimental sporozoite challenge in malaria naïve volunteers (Doolan and Hoffman, 2000; Epstein et al., 2011). Amongst these possible whole parasite vaccine approaches, RAS and GAP are seen by many scientists as the most promising candidates for vaccination of humans against malaria (Mac-Daniel et al., 2014). The recent study conducted in the USA has demonstrated that the irradiated sporozoites (irrSPZ)-based vaccines can provide 100% short-term protection against homologous CHMI, making it one of the major leaders in the malaria vaccine field (Seder et al., 2013).

### 1.4.4. Blood stage malaria vaccines approaches

Asexual blood stage malaria vaccine approaches aim at reducing the growth rate of blood stage infections which will in turn prevent or curb clinical disease severity. It has been suggested that people who have survived several encounters of malaria exposure develop natural immunity over time (Doolan et al., 2009). The goal of this type of a vaccine
approach would therefore be to accelerate development of blood stage immunity without suffering the consequences of infection. Several products that fall under this category have been developed or are under initial stages of development. Few candidates have entered phase 1b and 2b clinical trials (WHO, 2015). *P. falciparum* merozoite surface protein 3 (MSP3) has undergone extensive testing in clinical trials. This 48 kDa protein contains both conserved and divergent regions in a wide range of *P. falciparum* isolates. In the early eighties, Khusmith S et al. reported that a combination of serum from immune West African adults and monocytes from malaria non-exposed individuals was able to suppress *in vitro* the proliferation of *P. falciparum* parasites (Khusmith and Druilhe, 1983). Many research groups were engaged in identifying which component within these West African sera could be responsible for mediating this parasite growth inhibitory effect. Oeuvray et al., demonstrated that antibodies against MSP3 function in cooperation with blood monocytes to promote asexual blood stage killing in an antibody-dependent cellular inhibition assay (Oeuvray et al., 1994). Long term clinical protection from malaria disease was associated with the IgG3 isotype of antibodies binding to MSP3 (Roussilhon et al., 2007).

To date, several clinical trials have been conducted to test a multivalent MSP3 subunit malaria vaccine produced in *Escherichia coli*. This MSP3 subunit vaccine was proven to be safe and immunogenic in pre-clinical studies (Bang et al., 2011), phase 1a clinical trials in European volunteers (Audran et al., 2005) and several phase 1b clinical trials involving volunteers from malaria endemic countries (Lusingu et al., 2009; Nebie et al., 2009; Sirima et al., 2009). The improvement of the vaccine with a view to inducing highly protective immune responses is on-going. A phase 2b proof-of-concept clinical trial to assess the protective effect of the MSP3 long synthetic peptide vaccine against all clinical malaria episodes in children aged 12-48 months is on-going in West Africa (Clinical trial.gov, 2015).
The most advanced subunit blood stage vaccines is GMZ2, which has successfully reached phase 2 testing. The GMZ2 vaccine is composed of a fusion of the *P. falciparum* glutamate-rich protein (GLURP) and merozoite surface protein 3 (MSP3). This vaccine was demonstrated to be safe and immunogenic in a phase 1a study conducted in malaria naïve European volunteers (Esen et al., 2009) as well as when given intramuscularly in a phase 1b trial conducted in semi-immune African volunteers (Bélard et al., 2011). A recent study has shown that GMZ2 elicits high levels of antibodies in both non-exposed and malaria-exposed volunteers with broadly neutralizing activity in vitro against a variety of *P. falciparum* strains (Jepsen et al., 2013). However, a phase 2b randomized, controlled trial of the efficacy of three doses of GMZ2 performed in Burkinabe, Gabonese, Ghanaian and Ugandan children resulted in an efficacy of just 14% [95% CI: 3.6%, 23%] by ATP analysis, when the cohort was adjusted for age and site. Despite the low efficacy, the GMZ2 was shown to be well tolerated, immunogenic and protected children from developing clinical disease and reduced the incidence of malaria (Bélard et al., 2011; Sirima et al., 2016). From such findings, it’s obvious that improvement of the efficacy of GMZ2 vaccine is a critical requirement. Other asexual blood stage antigens that have undergone extensive testing as blood stage candidates are MSP 1 (Lin et al., 2014) and AMA1 (Harvey et al., 2014). Both of these antigens have provided limited protection against clinical disease, most likely due to the enormous sequence polymorphisms of these vaccine candidates that exist in field populations (Sheehy et al., 2012; Thera et al., 2011).

A whole iRBC approach has also been proposed, resembling the whole sporozoite approach in the sense that the complete organism is used as vaccine. The development of this approach faces challenges in terms of manner of attenuation, scaling up of production and GMP compliant production (Stanisic and Good, 2015).
1.4.5. **Malaria transmission blocking vaccines**

The growing interest in intervening with the parasite life cycle by generating antibodies that block transmission from the human host to the mosquito has led to advancements in developing transmission blocking vaccines (TBV). TBV are expected to complement other malaria vaccines and intervention strategies such as bed nets and to reduce the spread of drug resistant malaria parasite strains. The concept of developing TBVs has been around for many years, but few scientists choose to implement research in this area. Until recently, relatively few TBV candidates are in the early developmental phases. In a study performed in the mouse model, it was demonstrated that immunizing mice with Pfs25-CP VLPs (a Pfs25 recombinant protein fused to the Alfalfa mosaic virus coat protein) using either a two-dose or a single-dose vaccination regimen induced functional antibodies that exhibited parasite transmission activity through a six month study period (Jones et al., 2013). A phase 1a trial conducted at the John Hopkins Centre for Immunization Research, USA showed that significant antibody responses were detectable in volunteers who completed the lowest scheduled doses of Pfs25/ISA 51 vaccine but showed high reactogenicity (Wu et al., 2008). In addition to *P. falciparum* TBVs, the recent published data also showed that a DNA plasmid containing the *P. vivax* gametocyte genes Pvs48/45 and Pvs47 could induce antibodies that significantly reduced the proliferation of *P. vivax* oocysts in the mosquito mid gut (Tachibana et al., 2015). Recombinant protein from *P. vivax* ookinetes surface protein (*Pvs25*) was expressed in *S. cerevisiae*, purified and formulated in Alhydrogel. In a phase I study, this vaccine formulation induced transmission blocking immunity against *P. vivax* (Malkin et al., 2005).

1.5. **Controlled human malaria infection in malaria endemic countries**

CHMI are a critical component of research and development in malaria vaccine and drug
development. The availability of a standardized CHMI model to predict outcomes in clinical evaluation in the field will help evade the current problem of a lack of predictive assays or good correlates of protection (Moorthy et al., 2009). CHMI through infected mosquitoes is the most commonly practiced method but only few centers globally have the capacity to infect mosquitoes with a laboratory strain of malaria that is easily treatable by Chloroquine. In recent years, Sanaria Inc., USA has produced at GMP standard fully infectious, aseptic, purified \( P. falciparum \) sporozoites (\( PfSPZ \) challenge), which opened the possibility of conducting CHMI using a syringe and needle all over the world. A similar technique has led to the manufacturing of irradiation attenuated sporozoites, which could be used as a malaria vaccine candidate (Hoffman et al., 2010). The safety of CHMIs has been extensively demonstrated already over 80 years ago, when it was used as treatment of neuro-syphilis (O'LEARY PA, 1927). In 1986, the first CHMI study using infected mosquitoes that had been fed on cultured \( P. falciparum \) gametocytes was reported (Chulay et al., 1986). The potency and viability of \( PfSPZ \) challenge was further demonstrated in a clinical evaluation study conducted in Europe and in the US whereby to date, over 1000 volunteers have participated in CHMI studies (Chulay et al., 1986; Sauerwein et al., 2011; Spring et al., 2014). Results from these studies report reproducible and high infection rates in study subjects in combination with a good safety profile. CHMI have not been routinely performed in people living in malaria endemic Africa. Although it is possible to find volunteers with minimal malaria exposure in Africa, it is not clear whether the CHMI studies will have the same success rate and resulting immune responses when compared to malaria naïve adults from Europe and USA. These knowledge gaps, together with availability of sites in an endemic area with the infrastructure required for assessing participant safety and qualified medical staff creates the possibility of conducting CHMI studies in Tanzania. These studies will allow early evaluation of vaccine induced protection and novel drugs in one of the main target populations. It is hoped that performing CHMI
studies in malaria endemic countries will contribute to the acceleration of malaria vaccine and drug development by increasing the number of trials that can be conducted simultaneously (Chilengi, 2009).
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References


CHAPTER 2

Rationale and research questions
Chapter 2: Rationale and research question

2. Rationale and research questions addressed in this thesis

Development of a malaria vaccine that targets the pre-erythrocytic stages of *P. falciparum* has recently attracted the attention of many research groups. One of the most advanced malaria vaccine candidates to date is the recombinant subunit vaccine, the RTS,S/AS01. RTS,S/AS01 has been under extensive research since the 1980’s, and has demonstrated the ability to reduce the rate of acquisition of new blood stage infections and the multiplicity of infections in vaccinees. The broad evidence from challenge studies conducted in the USA and in African adult or paediatric field trials with varying transmission intensities, suggests a critical role for CSP-specific antibodies and/or CD4+ T cells in protection against infection. However, until lately, there has been no consensus on the immunological mechanisms that led to such findings and no clear correlate of protection for RTS,S/AS01 vaccine has been described. The first section of this thesis aims to address following research questions (Aim 1):

1. Is the RTS,S/AS01 vaccine safe, tolerable with improved or similar efficacy during large multicenter phase III trial when compared to outcome of phase II studies?

2. What will be the acceptability and feasibility of collecting PBMC samples from children aged 5 to 17 months at first bleeding for ancillary cellular immunology studies in Bagamoyo?

3. What are the markers of RTS,S/AS01 induced immune responses and do they correlate to vaccine induced protection in different age groups conducted in African children?
Most recombinant subunit malaria vaccines have failed to induce high level and long lasting protection. Novel approaches are being tested to improve vaccine induced protection. The most promising approach is the whole sporozoite based vaccine, which uses the whole organism either irradiation inactivated or in its live non-attenuated form under drug coverage.

CHMI has been successfully conducted in more than 1,500 volunteers in the US and Europe, but until 2012 it had not been employed in volunteers living in sub-Saharan Africa. The mechanisms of protective immunity after natural malaria infection or CHMI of malaria pre-exposed individuals remain subject of further investigation. We have conducted CHMI using cryo-preserved purified non-attenuated sporozoites in adult male Tanzanian volunteers with previous malaria exposure in order to address following research questions (Aim 2):

1. Is intradermal injection of PfSPZ challenge to malaria pre-exposed subjects safe and infective in comparison to malaria-naïve subjects?
2. How does the immune response of malaria pre-exposed compare to malaria-naïve subjects after exposure to similar doses of PfSPZ challenge?
3. What are the changes in humoral and cellular immune responses following PfSPZ challenge?
CHAPTER 3

Aim of the thesis
3. **Aims of my thesis**

3.1. **Aim 1:**

To evaluate the safety, immunogenicity and protective efficiency of the RTS,S/AS01 vaccine in Tanzanian infants and children during a phase III multicenter clinical trial.

To address Aim 1, we monitored adverse events to determine safety of RTS,S/AS01; we used ELISA to measure vaccine induced CSP-specific antibodies; Luminex to measure ex-vivo vaccine induced cytokine production as well as polychromatic flow cytometry to investigate quality and quantity of vaccine induced cellular immune responses.

3.2. **Aim 2:**

To evaluate safety, infectivity, humoral and cellular immune responses following intradermal CHMI in malaria pre-exposed Tanzanian adults.

To address aim 2, we determined the safety of CHMI in malaria pre-exposed volunteers by monitoring adverse events; we used blood slide microscopy to define sporozoites infectivity rate; Luminex to examine CHMI induced antibodies; B-cell Elispot to monitor MBC; single cell RNA sequencing, flow cytometry, cell sorting and stimulation assays to investigate innate immune responses following CHMI.
CHAPTER 4

Investigation of vaccine efficacy, and cellular and humoral immunity in RTS,S/AS01E vaccinated volunteers in Tanzania
PAPER I

A Phase 3 Trial of RTS,S/AS01 Malaria Vaccine in African Infants

A Phase 3 Trial of RTS,S/AS01 Malaria Vaccine in African Infants

The RTS,S Clinical Trials Partnership

ABSTRACT

BACKGROUND

The candidate malaria vaccine RTS,S/AS01 reduced episodes of both clinical and severe malaria in children 5 to 17 months of age by approximately 50% in an ongoing phase 3 trial. We studied infants 6 to 12 weeks of age recruited for the same trial.

METHODS

We administered RTS,S/AS01 or a comparator vaccine to 6537 infants who were 6 to 12 weeks of age at the time of the first vaccination in conjunction with Expanded Program on Immunization (EPI) vaccines in a three-dose monthly schedule. Vaccine efficacy against the first or only episode of clinical malaria during the 12 months after vaccination, a coprimary end point, was analyzed with the use of Cox regression. Vaccine efficacy against severe malaria, safety, and immunogenicity were also assessed.

RESULTS

The incidence of the first or only episode of clinical malaria in the intention-to-treat population during the 14 months after the first dose of vaccine was 0.31 per person-year in the RTS,S/AS01 group and 0.40 per person-year in the control group, for a vaccine efficacy of 30.1% (95% confidence interval [CI], 23.6 to 36.1). Vaccine efficacy in the per-protocol population was 31.3% (97.5% CI, 23.6 to 38.3). Vaccine efficacy against severe malaria was 26.0% (95% CI, −7.4 to 48.6) in the intention-to-treat population and 36.6% (95% CI, 4.6 to 57.7) in the per-protocol population. Serious adverse events occurred with a similar frequency in the two study groups. One month after administration of the third dose of RTS,S/AS01, 99.7% of children were positive for anti-circumsporozoite antibodies, with a geometric mean titer of 209 EU per milliliter (95% CI, 197 to 222).

CONCLUSIONS

The RTS,S/AS01 vaccine coadministered with EPI vaccines provided modest protection against both clinical and severe malaria in young infants. (Funded by GlaxoSmithKline Biologicals and the PATH Malaria Vaccine Initiative; RTS,S ClinicalTrials.gov number, NCT00866619.)
Considerable gains have been achieved in malaria control during the past decade. Nonetheless, malaria remains a major public health concern. In 2010, an estimated 216 million cases of malaria and 655,000 malaria-related deaths occurred, with the vast majority of deaths occurring in African children.

The RTS,S/AS01 candidate malaria vaccine targets the pre-erythrocytic stage of the Plasmodium falciparum parasite. It was developed to reduce clinical and severe malaria in African children. Ideally, it would be administered through the well-established Expanded Program on Immunization (EPI).

In 2011, we reported the results for the first coprimary end point from an ongoing phase 3 trial, which showed that during 12 months of follow-up, RTS,S/AS01 had an efficacy against clinical and severe malaria of 55.8% (97.5% confidence interval [CI], 50.6 to 60.4) and 47.3% (95% CI, 22.4 to 64.2), respectively, among children 5 to 17 months of age at enrollment (per-protocol analysis). Vaccine efficacy against severe malaria among children 6 to 12 weeks of age and those 5 to 17 months of age combined was 34.8% (95% CI, 16.2 to 49.2) during an average of 11 months of follow-up (range, 0 to 22). We now report on the second coprimary end point from the same trial: efficacy against clinical malaria during 12 months of follow-up among infants 6 to 12 weeks of age at enrollment, when RTS,S/AS01 was coadministered with EPI vaccines.

**METHODS**

**STUDY DESIGN**

Details of the study methods have been described previously and are provided in the Supplementary Appendix and the study protocol, both of which are available with the full text of this article at NEJM.org. This phase 3, randomized, controlled, double-blind trial is being conducted at 11 centers in 7 African countries with a range of malaria-transmission intensity (Fig. S1 in the Supplementary Appendix). The trial is designed to evaluate vaccine efficacy, safety, and immunogenicity for 32 months after the first dose of study vaccine in children 6 to 12 weeks of age or 5 to 17 months of age at enrollment. The trial includes three study groups in each age category: infants who received three doses of RTS,S/AS01 administered at 1-month intervals and a booster dose 18 months after the third dose, infants who received three doses of RTS,S/AS01 at 1-month intervals without a booster dose, and a control group of infants who received a non-malaria comparator vaccine. The analysis described in this report combines the first two groups (referred to as the RTS,S/AS01 group) and compares this group with the control group 14 months after the first dose of vaccine administered in children 6 to 12 weeks of age (Fig. S2 in the Supplementary Appendix). The trial protocol was approved by all relevant ethics review boards and national regulatory authorities (Tables S1A and S1B in the Supplementary Appendix). Written informed consent was obtained from the children's parents or guardians. The study was undertaken in accordance with Good Clinical Practice guidelines.

**STUDY OVERSIGHT**

The trial was sponsored by GlaxoSmithKline Biologicals (GSK), the vaccine developer and manufacturer, and funded by both GSK and the Program for Appropriate Technology in Health (PATH) Malaria Vaccine Initiative, which received a grant from the Bill and Melinda Gates Foundation. All study centers received study grants from the Malaria Vaccine Initiative, which also provided funding for authors' travel and accommodations related to this trial. All the authors reviewed all manuscript drafts, approved the final version of the manuscript, and made the decision to submit it for publication. No GSK authors were involved in the collection or analysis of the data; the analysis was performed by an independent statistician. The authors had full access to the results. The authors remain unaware of study-group assignments in this ongoing trial and do not have access to the raw data at this point. Details of the contributions of all the authors to the study are available in the Supplementary Appendix. The Clinical Trials Partnership Committee and Writing Group vouch for the completeness and accuracy of the data presented and for the fidelity of this report to the study protocol.

**RANDOMIZATION AND VACCINATION**

From December 2009 through January 2011, a total of 6537 infants 6 to 12 weeks of age were randomly assigned to one of the three study groups in a 1:1:1 ratio. Three doses of the RTS,S/AS01 or
The comparator vaccine, meningococcal serogroup C conjugate vaccine (Menjugate, Novartis), were coadministered with EPI vaccines according to the World Health Organization EPI schedule. EPI vaccines comprised a diphtheria–tetanus–whole-cell pertussis–hepatitis B–Haemophilus influenzae type b pentavalent vaccine (Tritanrix HepB Hib, GSK) and an oral poliovirus vaccine containing serotypes 1, 2, and 3 (Polio Sabin, GSK). The study and pentavalent vaccines were administered intramuscularly at different protocol-specified injection sites.

**SURVEILLANCE FOR CLINICAL AND SEVERE MALARIA**

Passive surveillance for malaria began at the time of the first vaccination. Parents or guardians of the study participants were encouraged to seek care at a health facility if the child had any signs of illness, and transportation was facilitated. All participants who presented to a study facility with reported or documented fever during the previous 24 hours were evaluated for malaria.

The primary efficacy end point for this analysis was the incidence of clinical malaria, defined as an illness in a child who was brought to a study facility with an axillary temperature of 37.5°C or higher and *P. falciparum* asexual parasitemia at a density of more than 5000 parasites per cubic millimeter or a case of malaria meeting the primary case definition of severe malaria (Table S2 in the Supplementary Appendix). Different parasite thresholds were used for secondary case definitions (Table 1). Participants who were hospitalized were evaluated for severe malaria on the basis of a protocol-defined algorithm (Table S3 in the Supplementary Appendix).

**SAFETY SURVEILLANCE**

Data regarding serious adverse events were recorded by means of passive surveillance beginning after the first dose of vaccine. Verbal autopsies were conducted for deaths that occurred outside study facilities. Information was collected on all unsolicited reports of adverse events that occurred within 30 days after vaccination and on reactogenicity (pain, swelling, redness at the injection site, drowsiness, fever, irritability or fussiness, or loss of appetite) within 7 days after vaccination among the first 200 participants enrolled at each center. Symptom intensity was assessed with the use of standardized methods (Table S4 in the Supplementary Appendix).

**IMMUNOGENICITY**

Anti-circumsporozoite antibodies were measured by means of enzyme-linked immunosorbent assay in the first 200 infants enrolled at each study center at screening and 1 month after dose-3. An antibody titer of 0.5 EU per milliliter or greater was considered to be positive.

**LABORATORY AND RADIOLOGIC PROCEDURES**

Laboratory and radiologic procedures have been reported previously and are described in the Supplementary Appendix.

**STATISTICAL ANALYSIS**

The statistical methods have been described in detail previously. We used Cox regression models (1 minus hazard ratio) to evaluate vaccine efficacy against the first or only episode of clinical malaria, using the study center as a stratification factor that allowed for differential baseline hazards. For the coprimary end point, vaccine efficacy against clinical malaria during 12 months of follow-up in the two age categories, 97.5% confidence intervals were used, ensuring an overall two-sided alpha level of 5%. The proportionality of hazards was evaluated by means of Schoenfeld residuals and models, including time-varying covariates. Secondary analyses, which included evaluations based on other case definitions and an analysis including multiple episodes of clinical malaria, were performed with the use of negative binomial regression. Vaccine efficacy against severe malaria was defined as 1 minus the risk ratio and is presented with 95% confidence intervals and Fisher’s exact P values.

Primary analyses of vaccine efficacy were based on the per-protocol population, which included all participants who received three doses
### Table 1. Efficacy of the RTS,S/AS01 Vaccine against Clinical and Severe Malaria in Infants Enrolled at 6 to 12 Weeks of Age.

<table>
<thead>
<tr>
<th>Variable</th>
<th>RTS,S/AS01 Vaccine</th>
<th>Control Vaccine</th>
<th>Protective Efficacy</th>
<th>Adjusted for Covariates*</th>
<th>Protective Efficacy</th>
<th>P Value</th>
<th>% (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Events</td>
<td>Person-Yr</td>
<td>Event Rate</td>
<td>No. of Events</td>
<td>Person-Yr</td>
<td>Event Rate</td>
<td>% (CI)†</td>
<td>P Value</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
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<td>------------</td>
<td>---------------</td>
<td>------------</td>
<td>------------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Clinical malaria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First or only episode</td>
<td>1161</td>
<td>3163</td>
<td>0.37</td>
<td>714</td>
<td>1474</td>
<td>0.48</td>
<td>31.3 (23.6–38.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>All episodes</td>
<td>1283</td>
<td>3073</td>
<td>0.42</td>
<td>770</td>
<td>1429</td>
<td>0.54</td>
<td>30.3 (23.7–36.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Intention-to-treat population</td>
<td>2301</td>
<td>3604</td>
<td>0.64</td>
<td>1626</td>
<td>1790</td>
<td>0.91</td>
<td>32.9 (26.3–38.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Severe malaria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All episodes</td>
<td>3995</td>
<td>4106</td>
<td>0.31</td>
<td>782</td>
<td>1949</td>
<td>0.40</td>
<td>30.1 (23.6–36.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Primary case definition</td>
<td>3995</td>
<td>4004</td>
<td>0.31</td>
<td>782</td>
<td>1949</td>
<td>0.40</td>
<td>30.1 (23.6–36.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Secondary case definition</td>
<td>4358</td>
<td>4376</td>
<td>0.30</td>
<td>792</td>
<td>1949</td>
<td>0.40</td>
<td>29.9 (23.4–36.1)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* In the adjusted analyses, data were stratified according to study site with adjustment for the distance to the nearest outpatient health facility.
† All end points are presented with 95% confidence intervals except for the coprimary end point, which is presented with 97.5% confidence intervals. The coprimary end point was defined as vaccine efficacy against a first or only episode of clinical malaria, according to the primary case definition.
‡ The primary case definition of clinical malaria was an illness in a child brought to a study facility with a temperature of ≥37.5°C and *Plasmodium falciparum* asexual parasitemia at a density of >5000 parasites per cubic millimeter or a case of malaria meeting the primary case definition of severe malaria.
§ The primary case definition of severe malaria was *P. falciparum* asexual parasitemia at a density of >5000 parasites per cubic millimeter with one or more markers of disease severity and without a diagnosis of a coexisting illness. The secondary case definition of severe malaria was present or could not be ruled out. Markers of severe disease were prostration, respiratory distress, a Blantyre coma score of ≤2 (on a scale of 0 to 5, with higher scores indicating a higher level of consciousness), two or more observed or reported seizures, hypoglycemia, acidosis, elevated lactate level, or hemoglobin level of <5 g per deciliter. Coexisting illnesses were defined as proven pneumonia, meningitis on analysis of cerebrospinal fluid, bacteremia, or gastroenteritis with severe dehydration.
of a study vaccine coadministered with EPI vaccines and who were included in efficacy surveillance, starting 14 days after the third dose of a study vaccine. The modified intention-to-treat population included all participants who received at least one dose of a study vaccine. In the adjusted analyses, vaccine efficacy was adjusted for study center and distance to the nearest outpatient facility (≤5 km vs. >5 km). Data were censored 14 months after the first dose of vaccine, or at the date of emigration, withdrawal of consent, or death.

Serious adverse events were coded from clinician-assigned diagnoses according to the preferred terms of the Medical Dictionary for Regulatory Activities and were based on available clinical and laboratory evidence.

The primary analysis of immunogenicity was based on the per-protocol population. Anti-circumsporozoite antibody titers were plotted and evaluated after the third dose of a study vaccine on the basis of seropositivity levels and geometric mean titers.

RESULTS

STUDY POPULATION

In total, 6537 infants 6 to 12 weeks of age were enrolled; 6003 (91.8%) were included in the per-protocol analysis (Fig. 1, and Fig. S3 in the Supplementary Appendix). Baseline demographic characteristics were similar in the two study groups (Table S5 in the Supplementary Appendix). The numbers of participants and malaria episodes according to study center are shown in Table S6 in the Supplementary Appendix. As expected, the majority of malaria episodes were reported by centers in areas with the highest transmission; 43.5% of all clinical malaria episodes were reported by two high-transmission sites in western Kenya. These two sites, combined with the site in Nanoro, Burkina Faso (where transmission is high but seasonal), accounted for 72.6% of clinical malaria episodes in this analysis. The rate of use of insecticide-treated nets was 85.8% overall and was similar in the two study groups. Indoor residual spraying was conducted as a public health intervention at four study centers; at those centers, spraying coverage was low (Table S7 in the Supplementary Appendix).

VACCINE EFFICACY AGAINST CLINICAL AND SEVERE MALARIA

In the per-protocol population, the incidence of a first or only episode of clinical malaria meeting the primary case definition during 12 months of follow-up was 0.37 per person-year in the RTS,S/AS01 group and 0.48 per person-year in the control group, for a vaccine efficacy of 31.3% (97.5% CI, 23.6 to 38.3). Kaplan–Meier curves are shown in Figures 2A and 2B. Vaccine efficacy was not constant over time (P<0.001 by Schoenfeld residuals), with efficacy higher at the beginning than at the end of the follow-up period (Table S8 in the Supplementary Appendix). Vaccine efficacy against all clinical malaria episodes was 32.9% (95% CI, 26.3 to 38.8). Estimates of efficacy against clinical malaria were consistent across all case definitions and in both adjusted and intention-to-treat analyses (Table 1).

At least one episode of severe malaria occurred in 58 of 3995 infants (1.5%) in the RTS,S/AS01 group and in 46 of 2008 infants (2.3%) in the control group, for a vaccine efficacy of 36.6% (95% CI, 4.6 to 57.7) in the per-protocol population. In the intention-to-treat population, at least one episode of severe malaria occurred in 77 of 4358 infants (1.8%) in the RTS,S/AS01 group and in 52 of 2179 infants (2.4%) in the control group, for a vaccine efficacy of 26.0% (95% CI, −7.4 to 48.6) (Table 1, and Tables S15 and S16 in the Supplementary Appendix).

SAFETY

Serious Adverse Events

Serious adverse events were reported in 17.9% (95% CI, 16.8 to 19.1) of recipients of the RTS,S/AS01 vaccine and in 19.2% (95% CI, 17.6 to 20.9) of recipients of the meningococcal vaccine (Table 2, and Table S9 in the Supplementary Appendix). A total of 94 infants died: 66 of 4358 infants (1.5%; 95% CI, 1.2 to 1.9) in the RTS,S/AS01 group and 28 of 2179 infants (1.3%; 95% CI, 0.9 to 1.9) in the control group. Causes of death were similar in the two groups; none of the deaths were thought to be related to vaccination (Table S10 in the Supplementary Appendix). Serious adverse events that were considered to be related to a study vaccine occurred in 7 infants: 4 of the 4358 infants in the RTS,S/AS01 group and 3 of the 2179 infants in the control group; 4 events (2 in each group)
7082 Infants were assessed for eligibility

545 Were not eligible
364 Did not meet criteria
84 Withdrew consent
65 Moved or were lost to follow-up
30 Had other reasons

6537 Underwent randomization and received study vaccine

4358 Received dose 1 of RTS,S/AS01 (ITT population)

2179 Received dose 1 of control vaccine (ITT population)

213 Did not complete vaccination
27 Died or withdrew for medical reasons
47 Had consent withdrawn or parent or guardian declined to participate
84 Moved or were lost to follow-up
55 Had other reasons

4235 Received dose 2

2134 Received dose 2

4145 Received dose 3

2090 Received dose 3

150 Were not included in the per-protocol analysis
7 Did not meet inclusion criteria
113 Were out of interval for dose regimen
16 Were lost to follow-up after dose 3
14 Had other reasons

3995 Were included in the per-protocol population

82 Were not included in the per-protocol analysis
5 Did not meet inclusion criteria
64 Were out of interval for dose regimen
3 Were lost to follow-up after dose 3
10 Had other reasons

260 Did not complete vaccination
21 Died
4 Had consent withdrawn
235 Moved or were lost to follow-up

1830 Attended visit 16 (12 mo after dose 3)

1642 Attended visit 16 (12 mo after dose 3)

503 Did not complete visit 16
37 Died
16 Had consent withdrawn
450 Moved or were lost to follow-up

89 Did not complete vaccination
9 Died or withdrew for medical reasons
20 Had consent withdrawn or parent or guardian declined to participate
38 Moved or were lost to follow-up
22 Had other reasons

113 Were out of interval for dose regimen
16 Were lost to follow-up after dose 3
14 Had other reasons

11139 Were included in the per-protocol population

2008 Were included in the per-protocol population

Figure 1. Enrollment of Infants 6 to 12 Weeks of Age.
ITT denotes intention to treat.
were episodes of fever for which infants were hospitalized for investigation. One infant (in the control group) had anaphylaxis, one infant (in the RTS,S/AS01 group) had a suspected injection-site infection related to the pentavalent vaccine, and one infant (in the RTS,S/AS01 group) had repeated febrile seizures associated with a respiratory infection. The frequency of seizures within 7 days after vaccination, reported previously, was similar in the two study groups.3

Meningitis of any cause was reported as a serious adverse event in 11 infants: 9 of the 4358 infants in the RTS,S/AS01 group and 2 of the 2179 infants in the control group (relative risk in the RTS,S/AS01 group, 2.3; 95% CI, 0.5 to 10.4). A pathogen was identified for 7 of the events (salmonella in 3 episodes of meningitis and pneumococcus in 4 episodes). The 4 remaining events, with no pathogen identified, were reported by a single study center (3 episodes of meningitis in the RTS,S/AS01 group and 1 episode in the control group). Of the 11 episodes of meningitis, 2 were new (1 due to pneumococcus and 1 due to salmonella); the 9 other episodes have been reported previously.3 Investigator-driven medical review of previously reported meningitis episodes led to reclassification of 1 episode as an episode of pneumonia and reclassification of 4 episodes without cause as 2 episodes of pneumococcal meningitis and 2 of salmonella meningitis. Four of the episodes of meningitis occurred within 30 days after vaccination.

**Adverse Events**

Unsolicited reports of adverse events within 30 days after vaccination were recorded with similar frequency in the RTS,S/AS01 group (79.4%; 95% CI, 77.2 to 81.5) and in the control group (81.3%; 95% CI, 78.3 to 84.1). No clinically important imbalances were observed (Table S11A in the Supplementary Appendix). Information on unsolicited reports of adverse events related to the vaccine or leading to withdrawal within 30 days after vaccination is shown in Table S11B in the Supplementary Appendix. The frequency of solicited reports of local symptoms was similar among infants who received the RTS,S/AS01 vaccine and among those who received the pentavalent vaccine (Table S13 in the Supplementary Appendix). Systemic reactogenicity was higher in the RTS,S/AS01 group than in the control group (Fig. 3, and Table S12 in the Supplementary Appendix). Postvaccination fever was reported after 30.6% of doses (95% CI, 29.2 to 32.0) in the RTS,S/AS01 group and after 21.1% of doses (95% CI, 19.4 to 22.8) in the control group. A temperature higher than 39°C was reported after less than 1% of doses. The incidence of mucocutaneous disease was similar in the two study groups (Table S14 in the Supplementary Appendix).
Before vaccination, 34.3% and 35.2% of infants in the RTS,S/AS01 and control groups, respectively, were positive for anti-circumsporozoite antibodies but at low titers (Fig. S4 in the Supplementary Appendix). One month after the third dose of the study vaccine, 99.7% of infants in the RTS,S/AS01 group were positive for anti-circumsporozoite antibodies, with a geometric mean titer of 209 EU per milliliter (95% CI, 197 to 222).

**Table 2. Serious Adverse Events in Infants 6 to 12 Weeks at Enrollment during 14 Months after the First Dose of Vaccine (Intention-to-Treat Population).**

<table>
<thead>
<tr>
<th>Variable</th>
<th>RTS,S/AS01 Vaccine (N = 4358)</th>
<th>Control Vaccine (N = 2179)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Infants</td>
<td>% (95% CI)</td>
</tr>
<tr>
<td><strong>Serious events in all infants</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥1 Serious adverse event</td>
<td>782</td>
<td>17.9 (16.8–19.1)</td>
</tr>
<tr>
<td>≥1 Serious adverse event, excluding malaria</td>
<td>760</td>
<td>17.4 (16.3–18.6)</td>
</tr>
<tr>
<td>≥1 Fatal serious adverse event†</td>
<td>66</td>
<td>1.5 (1.2–1.9)</td>
</tr>
<tr>
<td>≥1 Serious adverse event related to vaccine</td>
<td>4</td>
<td>0.1 (0.0–0.2)</td>
</tr>
<tr>
<td>≥1 Serious adverse event within 30 days after vaccination</td>
<td>192</td>
<td>4.4 (3.8–5.1)</td>
</tr>
<tr>
<td><strong>Events with an incidence ≥0.5%†</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonia</td>
<td>302</td>
<td>6.9 (6.2–7.7)</td>
</tr>
<tr>
<td>Gastroenteritis</td>
<td>260</td>
<td>6.0 (5.3–6.7)</td>
</tr>
<tr>
<td>Malaria</td>
<td>184</td>
<td>4.2 (3.6–4.9)</td>
</tr>
<tr>
<td>Anemia</td>
<td>90</td>
<td>2.1 (1.7–2.5)</td>
</tr>
<tr>
<td>Febrile convulsion</td>
<td>82</td>
<td>1.9 (1.5–2.3)</td>
</tr>
<tr>
<td>Bronchialitis</td>
<td>28</td>
<td>0.6 (0.4–0.9)</td>
</tr>
<tr>
<td>Convulsion</td>
<td>41</td>
<td>0.9 (0.7–1.1)</td>
</tr>
<tr>
<td>Bronchopneumonia</td>
<td>35</td>
<td>0.8 (0.6–1.1)</td>
</tr>
<tr>
<td>Upper respiratory tract infection</td>
<td>36</td>
<td>0.8 (0.6–1.1)</td>
</tr>
<tr>
<td>Salmonella sepsis</td>
<td>26</td>
<td>0.6 (0.4–0.9)</td>
</tr>
<tr>
<td>Malnutrition</td>
<td>29</td>
<td>0.7 (0.4–1.0)</td>
</tr>
<tr>
<td>Sepsis</td>
<td>26</td>
<td>0.6 (0.4–0.9)</td>
</tr>
<tr>
<td>HIV infection‡</td>
<td>27</td>
<td>0.6 (0.4–0.9)</td>
</tr>
<tr>
<td>Enteritis</td>
<td>11</td>
<td>0.3 (0.1–0.5)</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>16</td>
<td>0.4 (0.2–0.6)</td>
</tr>
<tr>
<td>Measles</td>
<td>20</td>
<td>0.5 (0.3–0.7)</td>
</tr>
<tr>
<td>Pyrexia</td>
<td>15</td>
<td>0.3 (0.2–0.6)</td>
</tr>
</tbody>
</table>

* More than one fatal serious adverse event could be attributed to a single infant if there was more than one underlying cause of death (e.g., meningitis and sepsis).
† Events are listed according to the preferred terms in the Medical Dictionary for Regulatory Activities.
‡ HIV denotes human immunodeficiency virus.

**IMMUNOGENICITY**

DISCUSSION

This phase 3 trial showed that in young infants, the RTS,S/AS01 candidate vaccine provided modest protection against malaria when coadministered with EPI vaccines. The efficacy of RTS,S/AS01 reported here is lower than that observed in a phase 2 trial involving infants at three of the phase 3 trial sites, in which RTS,S/AS01 was coadministered with EPI vaccines. In that trial, geo-
Metric mean titers of anti-circumsporozoite antibodies after vaccination were similar to those measured here, but vaccine efficacy against clinical malaria was 61.6% (95% CI, 35.6 to 77.1). Although we wish to avoid overinterpretation of the results of this previously reported small phase 2 trial with wide confidence intervals, it is notable that this higher estimate of efficacy comes from a study conducted at sites in areas with low-to-moderate malaria transmission. It is possible that the pooled estimate across the 11 centers in the phase 3 trial obscures differences in vaccine efficacy according to transmission intensity and that these two sets of results are compatible with each other.

The efficacy of the RTS,S/AS01 vaccine reported here is also lower than that reported previously among older children recruited for this trial at the same study centers.3 A likely explanation for the lower vaccine efficacy among infants is an age-dependent differential immune response to the vaccine. This concept is supported by the lower anti-circumsporozoite antibody titers observed in infants (geometric mean titer, 209 EU per milliliter; 95% CI, 197 to 222) as compared with titers in older children (621 EU per milliliter; 95% CI, 592 to 652), reported previously.3 Although the titer of anti-circumsporozoite antibodies is not an established correlate of the level of protection, an association with efficacy has been observed in several trials.17–21 Infants may have mounted a lower immune response than older children owing to coadministration of RTS,S/AS01 with routine EPI vaccines, an inhibitory effect of maternally derived anti-circumsporozoite antibodies, an absence of priming with hepatitis B vaccine or with P. falciparum infection, or the infant’s immature immune system.

Coadministration of RTS,S/AS01 with the pentavalent vaccine and the oral poliovirus vaccine might have resulted in immune interference and contributed to the lower anti-circumsporozoite antibody titers in the younger infants. Two phase 2 studies have explored the immunologic response to the related RTS,S/AS02 vaccine, either when coadministered with a diphtheria–tetanus–pertussis–hepatitis B vaccine or when given 2 weeks afterward. The geometric mean titer of anti-circumsporozoite antibodies was lower when vaccines were coadministered than when they were staggered (70 EU per milliliter [95% CI, 54 to 90] vs. 200 EU per milliliter [95% CI, 151 to 265]).20,21 However, vaccine efficacy against infection was similar in the two trials (65.2% [95% CI, 20.7 to 84.7] during 6 months after vaccination and 65.9% [95% CI, 42.6 to 79.8] during 3 months after vaccination, respectively).

An absence of priming with hepatitis B vaccine or with P. falciparum infection may also have contributed to the lower anti-circumsporozoite antibody titers. In this trial, infants simultaneously received a hepatitis B surface antigen (HBsAg)–containing combination vaccine and the RTS,S vaccine, which contains HBsAg fused as a carrier protein to the circumsporozoite protein. Immune interference on concurrent administration of similar protein components has been described.22 In contrast, in older children vaccinated against hepatitis B, memory T-cell reactivation may have enhanced the anti-circumsporozoite antibody response to RTS,S/AS01.22 One study showed a tendency toward higher anti-circumsporozoite antibody responses in children who had been vaccinated against hepatitis B than in children who had not previously received hepatitis B vaccine.23 Maternally derived antibodies can interfere with the immune response in young infants; such interference is common with live vaccines, such as the measles vaccine, but can also occur with some protein vaccines.24,25 Similarly, pas-
sively acquired antibodies to either HBsAg or the circumsporozoite components of the RTS,S/AS01 vaccine might have suppressed immune responses. Finally, although most protein vaccines and polysaccharide–protein conjugate vaccines are immunogenic in young infants, improved immunogenicity and efficacy have often been achieved when vaccination has extended beyond the first few months of life.22,26,27

As previously reported in older children,9 statistical models indicated nonproportionality of hazards over time. This could be due to waning vaccine efficacy, differential acquisition of natural immunity, or other factors that may influence the model,28 such as heterogeneity of exposure, the vaccine effect at the individual level, or both.29,30 If vaccine efficacy does wane, this might contribute to the lower observed efficacy among infants than among older children, especially because young infants may be less susceptible to malaria in the immediate postvaccination period owing to maternally acquired immunity, fetal hemoglobin, lower exposure, and other factors.31

The 11 sites of the phase 3 trial cover a wide range of malaria-transmission intensity. The inclusion of sites in high-transmission or seasonal-transmission areas and the large proportion of cases of severe and clinical malaria from these sites might have contributed to the lower vaccine efficacy among infants in this trial than in earlier trials involving infants. The implications of the large representation of malaria episodes from high-transmission areas may become apparent when site-specific data are analyzed at a later date, as specified by the protocol. Estimates of site-specific vaccine efficacy and the corresponding estimates of clinical or severe malaria episodes averted will help to determine what role this vaccine might have in malaria control. Exploration of factors that might affect vaccine efficacy, including the effect of maternal antibodies, the role of immune interference by EPI vaccines, the effect of the RTS,S/AS01 booster, and status with respect to previous exposure to *P. falciparum* parasites, will provide crucial information for the further development of this vaccine and for other malaria vaccines under development.32

Overall, fatal, or vaccine-related serious adverse events were balanced between the study groups. In the previous analysis, which included infants and older children, the incidence of meningitis was imbalanced between the RTS,S/AS01 and control groups.3 The imbalance remains, but we now have clarified that the majority of cases had a bacterial cause. We will continue to monitor the incidence of meningitis throughout the trial. The imbalance in the incidence of rash, observed in previous RTS,S studies,12,33 was not confirmed in this larger trial.

This phase 3 trial shows efficacy of the RTS,S/AS01 vaccine. Data from the remainder of this trial and additional studies in progress will contribute to the understanding of the complex interplay among the intensity of exposure to malaria, the immune response, and vaccine efficacy. Supported by GlaxoSmithKline Biologicals (GSK) and the PATH Malaria Vaccine Initiative, which received a grant from the Bill and Melinda Gates Foundation.

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REFERENCES


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PAPER II

The effect of immunization schedule with the malaria vaccine candidate RTS,S/AS01E on protective efficacy and anti-circumsporozoite protein antibody avidity in African infants

The effect of immunization schedule with the malaria vaccine candidate RTS,S/AS01E on protective efficacy and anti-circumsporozoite protein antibody avidity in African infants

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Abstract

Background: The malaria vaccine RTS,S induces antibodies against the Plasmodium falciparum circumsporozoite protein (CSP) and the concentration of Immunoglobulin G (IgG) against the repeat region of CSP following vaccination is associated with protection from P. falciparum malaria. So far, only the quantity of anti-CSP IgG has been measured and used to predict vaccination success, although quality (measured as avidity) of the antigen-antibody interaction shall be important since only a few sporozoites circulate for a short time after an infectious mosquito bite, likely requiring fast and strong binding.

Methods: Quantity and avidity of anti-CSP IgG in African infants who received RTS,S/AS01E in a 0-1-2-month or a 0-1-7-month schedule in a phase 2 clinical trial were measured by enzyme-linked immunosorbent assay. Antibody avidity was defined as the proportion of IgG able to bind in the presence of a chaotropic agent (avidity index). The effect of CSP-specific IgG concentration and avidity on protective efficacy was modelled using Cox proportional hazards.

Results: After the third dose, quantity and avidity were similar between the two vaccination schedules. IgG avidity after the last vaccine injection was not associated with protection, whereas the change in avidity following second and third RTS,S/AS01E injection was associated with a 54% risk reduction of getting malaria (hazard ratio: 0.46; 95% confidence interval (CI): 0.22-0.99) in those participants with a change in avidity above the median. The change in anti-CSP IgG concentration following second and third injection was associated with a 77% risk reduction of getting malaria (hazard ratio: 0.23, 95% CI: 0.11-0.51).

Conclusions: Change in IgG response between vaccine doses merits further evaluation as a surrogate marker for RTS,S efficacy.

Trial registration: ClinicalTrials.gov Identifier NCT00436007.

Keywords: Malaria, RTS,S, Vaccine, Plasmodium falciparum, Antibody, Avidity, Correlate of protection
Background
Malaria has an enormous public health impact and new preventive interventions are urgently needed. After more than 100 years of research on malaria vaccines, RTS,S was the first pre-erythrocytic vaccine candidate that entered phase III clinical development [1-3]. RTS,S contains hepatitis B surface antigen (HBsAg) together with a fusion protein of HBsAg and a carboxy-terminal fragment of Plasmodium falciparum circumsporozoite protein (CSP), co-expressed in yeast and formulated with a proprietary adjuvant (AS01). The exact mechanism of RTS,S-mediated protection is not known, although Immunoglobulin G antibodies (IgG) against the CSP repeat region are likely to play an important role since the concentration of anti-CSP IgG partly explains protection in most studies that assessed efficacy of RTS,S in African children [4-6]. In addition, passive transfer of anti-CSP IgG can protect animals from subsequent challenge [7,8]. Besides concentration, many other properties determine antibody function. Among them are availability of effector molecules, post-translational modification, isotype, subclass, affinity and avidity of antibodies. It is difficult to measure all these characteristics in one sample, particularly in the small sample volumes obtained during clinical trials in infants. Affinity, defined as the strength of interaction between an epitope and an antibody binding site, would be a particularly interesting variable to measure in the context of anti-CSP IgG-mediated immunity, since the time of interaction with the parasite is short (less than 30 minutes [9]), sporozoites are strongly diluted and few. In fact, only one successful hepatocyte infection is sufficient to initiate and maintain blood stage infection. Studies in mice have shown that high antibody affinity against a synthetic CSP immunogen is positively associated with protection [8,10] and most studies in humans indicate that anti-CSP IgG concentration explains only parts of the vaccine-mediated protection. Increase in antibody affinity after repeated antigen exposure is the result of affinity maturation due to somatic hypermutation. The rate and extent of maturation may be influenced by several factors, including nature, route and dose of the antigen, adjuvants and carriers as well as the immunization schedule. In the present study antibody avidity was measured. It is a representation of the strength of interaction between antibodies and antigens in a complex and besides antibody affinity, valences of antibodies and antigens as well as structural features of the complex are important determinants of avidity. For CSP, it has been shown that the use of some adjuvants can increase the avidity of anti-CSP IgG after vaccination of human volunteers [11]. In this study IgG avidity against the repeat region of CSP was measured after the second and third injection of RTS,S/AS01E in infants that received the vaccine as part of a phase IIb clinical trial to assess safety and efficacy of RTS,S/AS01E in the age-group targeted by the expanded programme on immunization (EPI) [5,12].

Methods
Clinical trial
The objective of the study was to explore the effect of anti-CSP IgG avidity on RTS,S vaccine efficacy in naturally exposed infants. Details of the clinical trial have been published previously [5,12]. Briefly, safety and efficacy of RTS, S/AS01E when given through the EPI was assessed in 511 children from Gabon, Ghana and Tanzania. Participants were randomly assigned to one of three intervention arms: 1) RTS,S/AS01E as three injections, one month apart (0, 1, 2 months schedule [012]; n = 170), 2) RTS,S/AS01E extended schedule (0, 1, 7 months schedule [017]; n = 170) or 3) control (EPI vaccines alone; n = 171). Malaria was defined as parasitaemia >500 parasites per μl and an axillary temperature >37°C. The efficacy of RTS,S against first malaria episodes, detected by passive case detection, was equivalent in the two schedules one year after the third injection. The study followed Good Clinical Practice guidelines, the Declaration of Helsinki (4th revision) and received approval from the appropriate local and national ethics committees of each site. In addition, ethical review by the ethics committees of the London School of Hygiene and Tropical Medicine Ethic Committee, the Swiss Tropical Institute Committee and the Western Institutional Review Board was sought. The trial is registered with ClinicalTrials.gov (NCT00436007).

Antibody measurements
Antibodies against CSP were measured by evaluating IgG responses against the CSP-repeat region, using a validated enzyme-linked immunosorbent assay (ELISA) with R32LR as the coating antigen [13]. An anti-CSP IgG titre of 0.5 ELISA units per millilitre (EU/mL) or greater was considered to be positive. For measurements of avidity of IgG against the repeat region of CSP, samples were evaluated as described [13], but in two different plates; one treated with a chaotropic agent and one untreated plate. As chaotropic agent a 1 M solution of ammonium thiocyanate (NH₄SCN) was added in the treatment plate while 0.05% Tween-20 in PBS was added in the untreated plate and both CSP ELISA plates were further washed and developed as described [13]. The avidity index (AI) was calculated as the ratio of the concentration of anti-CSP IgG (EU/ml) that remained bound to the coated antigen after treatment with NH₄SCN, divided by the concentration of IgG (EU/ml) that remained bound to the coated antigen in the untreated plate. Anti-CSP IgG quantification and avidity were measured at the Center for Vaccinology, Ghent University Hospital, Belgium.
For statistical modelling the logarithm of anti-CSP IgG concentration was used since previous data showed that log-transformation results in a better fit to the normal distribution. AI was analysed in the two RTS,S-vaccinated arms and after the second and third vaccination. Since the majority of infants before vaccination and those receiving control vaccine do not have measurable anti-CSP IgG, AI cannot be calculated. Delta AI (dAI) was defined as the difference in AI between the second and third vaccination. Similarly, delta CSP (dCSP) was defined as the difference in anti-CSP IgG concentration between the second and third vaccination.

Statistics
Analysis of the effect of IgG avidity on protective efficacy was exploratory and not detailed in the statistical analysis plan of the original study. IgG responses between the groups were analysed by descriptive statistics and represented as boxplots together with the individual measurements. The effect of anti-CSP IgG concentration and AI on risk of malaria was calculated using the according-to-protocol (ATP) dataset with a Cox proportional hazards model in R v2.15.2. For statistical modelling antibody concentrations were log-transformed. To calculate the effect of dAI and dCSP on the occurrence of malaria episodes with a Cox proportional hazards model, values were dichotomized on the median dAI or dCSP and labelled as 'high' and 'low', respectively. All models included the covariates schedule and site. If appropriate, other covariates were added as reported in the results section. A p-value below 0.05 was considered significant and 95% confidence intervals (95% CI) are given where appropriate.

Results
After screening 605 participants, 170 received RTS,S in the standard (012) and 170 in the extended (017) schedule, as depicted on the CONSORT flowchart of the primary study (Figure 1). Samples from 315 (300 ATP) participants were available for immunological analysis (012: n = 154 [148]; 017: n = 161 [152]). Paired immunological samples to calculate dAI were available from 187 (179 ATP) participants (012: n = 103 [100]; 017: n = 84 [79]).

As reported earlier [5], high anti-CSP IgG titres after three vaccine injections were associated with a reduction in subsequent incidence of clinical malaria: the hazard ratio of a ten-fold increase in anti-CSP IgG was 0.52 (95% CI: 0.34–0.81), which corresponds to a 48% risk reduction.

Absolute AI after two (012: 35.9, 017: 34.9; t-test p = 0.57) and three (012: 41.2, 017: 39.3; t-test p = 0.22) RTS,S injections were similar between the two vaccination schedules (Figure 2). As expected, an increase in AI between the second and third vaccination was present (Figure 3). Increase in delta AI (dAI) was slightly, albeit not statistically significant, higher in the 017 (7.1) group compared to the 012 (4.2) group (delta: 3.0; 95% CI: −0.3–6.1; t-test p = 0.08).

To explore the effect of AI, dAI and dCSP on malaria risk, three Cox proportional hazard models were defined and tested. AI after the third injection, corrected for site, schedule and anti-CSP IgG concentration, did not explain a significant reduction in risk of clinical malaria (Model 1; hazard ratio: 0.99, 95% CI: 0.97–1.02). Participants were then divided on the median in dCSP and dAI ‘high’ and ‘low’ responders and included as categorical variable in the model. Classification as ‘high-dCSP’ was associated with a significant risk reduction (77%) compared to the ‘low dCSP’ group in a model corrected for site and schedule (Model 2; hazard ratio: 0.23, 95% CI: 0.11–0.51). When dAI, corrected for site, schedule and dCSP was analysed, the hazard ratio between high and low responders separated by the median, was 0.46 (Model 3; 95% CI: 0.22–0.99; Wald test p = 0.049), hence classification as ‘high dAI’ group member is associated with a 54% risk reduction (Figure 4).

Discussion
The complex interplay of vaccine-primed immune mediators that define a successful response upon pathogen encounter is not well understood. Cellular and humoral components have important roles, although in various compositions, depending on the pathogen and the host. Antibodies are the prototypic vaccine-induced immune mediators and play an important role in anti-malarial immunity during the pre-erythrocytic [8,10] as well as the erythrocytic stage [14] of the disease, as shown by passive transfer experiments in mice and man. The sheer concentration of antigen-specific antibodies is normally used to measure immunization success and serves as a surrogate to estimate protective efficacy. The clinical development of RTS,S is a unique opportunity to investigate the effect of further variables such as antibody avidity, isotype or subclass on vaccine efficacy, since clinical (true) efficacy is known [5], being 57% (95% CI: 33–73) with the 012 schedule and 32% (95% CI: 16–45) following the 017 schedule.

Here, anti-CSP IgG avidity was measured to assess if it predicts vaccine efficacy in a phase II clinical trial of RTS,S independent of anti-CSP IgG concentration [5,12]. Regardless of the vaccination scheme and site, avidity did not improve prediction over anti-CSP IgG concentration alone. This may mean that: i) the assay is not sensitive enough to reflect avidity; ii) collinearity between antibody concentration and avidity blurs the effect of avidity; or, iii) that avidity is not an important determinant of vaccine efficacy. In this study IgG concentration and avidity was measured after the second and third
vaccine injection. This approach is valid to assess if the immune system reacted to vaccination successfully. Since kinetics of IgG vary over time and the study was performed under natural exposure to malaria parasites, the time of encounter with the parasite becomes an important variable. This is in contrast to controlled human malaria infection (CHMI) studies, where the time of infection is defined. Hypothetically, the difference in IgG concentration (and avidity) between second and third vaccination could be a better predictor of effective antibody-mediated protection than concentration after the third vaccine injection, because it better reflects the
further evolution of antibody responses until next parasite encounter. The present data argue for the use of this approach since it was shown that a high dCSP predicts protective efficacy and dAI explains part of the protection in the RTS,S vaccinated children (Model 3). How AI evolves over time and if it is a useful predictor of vaccine efficacy remains to be validated with further, independent and confirmatory studies.

Nevertheless, this observation adds a new component to the search of correlates of protection and the understanding of the immune responses elicited by pre-erythrocytic malaria vaccine candidates such as RTS,S. Since adjuvants also have a profound effect on the speed of avidity maturation [11], the effect of avidity on vaccine efficacy could even be analysed with interventional studies that assess the effect of timing between immunizations (as in this study) and different adjuvants on protective efficacy while direct measures of maturation of the immune system such as single-cell based sequencing of IgG genes of anti-CSP memory B-cells [15,16] are performed. This may be particularly interesting for antigens such as CSP that are not highly immunogenic per se, because highly immunogenic antigens often induce antibodies with strong avidity over a short period of time and a threshold antibody concentration is appropriate to predict their efficacy [17]. Other studies in the development of RTS,S (e.g., challenge experiments [18] and the recently completed phase III trial [1-3]) will certainly provide additional information and may establish the measurement of avidity as one biomarker for vaccine efficacy. Additionally, such knowledge may guide the design of next generation vaccines and administration schemes.

Conclusions
So far, the most robust correlate of protection for the malaria vaccine candidate RTS,S is anti-circumsporozoite (CSP) IgG concentration following immunization. Pre-clinical data and theoretical considerations suggest that avidity may have an additional impact on protective
efficacy. It is shown that an increase in anti-CSP IgG concentration and avidity between second and third vaccine injection is associated with a strong risk-reduction for malaria after immunization. This finding shall influence the way of analysis of immunological correlates of protection since using change in antibody concentration and avidity rather than single measurements enables improved modelling of immune-effector function at the time of pathogen encounter and hence more powerful prediction of vaccine efficacy.

Consent
Written informed consent was obtained from each child’s parent(s). Illiterate parents were informed about the study in the presence of an impartial and literate witness and informed consent was documented by thumbprint of the parent and signature of the witness.

Abbreviations
AI: Avidity index; dAI: delta AI; CSP: Plasmodium falciparum circumsporozoite protein; dCSP: Delta CSP; ATP: according to protocol; IgG: Immunoglobulin G.

Competing interests
This study was funded by PATH-MVI and GlaxoSmithKline Biologicals SA. GM and MT report receiving funding for study-related travels. MT reports receiving financial compensation for activities outside the submitted work for board membership of the Optimus Foundation and the Novartis Institute for Tropical Diseases, having grants pending from both PATH-MVI and the Bill and Melinda Gates Foundation, and receiving travel reimbursements from PATH-MVI and Sanaria Corp. IV, EJ, ML, and PC are employees of the GlaxoSmithKline group of companies. JV, VI, and ML receive GlaxoSmithKline stock and/or options. CFO conceived and supervised the study. All authors contributed to writing and review of the manuscript. All authors read and approved the final manuscript.

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Authors’ contributions
AA and BM drafted the manuscript and performed the statistical analysis. BL, STA, KPA, SO-A, GM, MM, and NS collected the data and performed analyses. MT, SA, JV, EJ, ML, and PC, CFO, and PGK conceived and supervised the study. All authors contributed to writing and review of the manuscript. All authors read and approved the final manuscript.

References
PAPER III

Distinct helper T cell type 1 and 2 responses associated with malaria protection and risk in RTS,S/AS01E vaccinees

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Distinct Helper T Cell Type 1 and 2 Responses Associated With Malaria Protection and Risk in RTS,S/AS01E Vaccinees

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Background. The RTS,S/AS01E malaria vaccine has moderate efficacy, lower in infants than children. Current efforts to enhance RTS,S/AS01E efficacy would benefit from learning about the vaccine-induced immunity and identifying correlates of malaria protection, which could, for instance, inform the choice of adjuvants. Here, we sought cellular immunity-based correlates of malaria protection and risk associated with RTS,S/AS01E vaccination.

Methods. We performed a matched case-control study nested within the multicenter African RTS,S/AS01E phase 3 trial. Children and infant samples from 57 clinical malaria cases (32 RTS,S/25 comparator vaccinees) and 152 controls without malaria (106 RTS,S/46 comparator vaccinees) were analyzed. We measured 30 markers by Luminex following RTS,S/AS01E antigen stimulation of cells 1 month postimmunization. Crude concentrations and ratios of antigen to background control were analyzed.

Results. Interleukin (IL) 2 and IL-5 ratios were associated with RTS,S/AS01E vaccination (adjusted P ≤ .01). IL-5 circumsporozoite protein (CSP) ratios, a helper T cell type 2 cytokine, correlated with higher odds of malaria in RTS,S/AS01E vaccinees (odds ratio, 1.17 per 10% increases of CSP ratios; P value adjusted for multiple testing = .03). In multimarker analysis, the helper T cell type 1 (Th1)–related markers interferon-γ, IL-15, and granulocyte-macrophage colony-stimulating factor protected from subsequent malaria, in contrast to IL-5 and RANTES, which increased the odds of malaria.

Conclusions. RTS,S/AS01E-induced IL-5 may be a surrogate of lack of protection, whereas Th1-related responses may be involved in protective mechanisms. Efforts to develop second-generation vaccine candidates may concentrate on adjuvants that modulate the immune system to support enhanced Th1 responses and decreased IL-5 responses.

Keywords. malaria; vaccine; immunity; cellular immune responses; cytokines.

RTS,S/AS01E is the most advanced malaria vaccine candidate in development, having completed a phase 3 trial in Africa, in which the 1-year vaccine efficacy (VE) against clinical malaria was 56% in children aged 5–17 months and 31% in infants aged 6–12 weeks [1–3]. The mechanisms of RTS,S/AS01E-induced protection and the reasons for the moderate efficacy and the lower protection in infants than children remain unclear. Identifying immune correlates of protection can shed light on these questions, help improve RTS,S/AS01E, and rationally design the next generation of malaria vaccines to control and eliminate this disease.

RTS,S is a subunit vaccine targeting the pre-erythrocytic stage of Plasmodium falciparum infection and is based on the circumsporozoite protein (CSP). The vaccine consists of a recombinant protein containing part of the CSP fused to and coexpressed with the hepatitis B surface antigen (HBsAg). RTS,S in the phase 3 trial was formulated with the AS01 adjuvant that consists of 3-O-desacyl-4′-monophosphoryl lipid A (MPL), QS21, and liposomes. In previous trials, RTS,S induced high titers of anti-CSP immunoglobulin G (IgG) [4–10], which recently were shown to correlate with the magnitude and duration of VE in children and infants [11, 12]. Unlike antibodies, cellular responses in endemic areas are low to moderate and mainly based on helper T cell type 1

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(T_h)1 responses, specifically CD4+ T cells expressing, interleukin (IL) 2, tumor necrosis factor (TNF), and interferon (IFN)-γ [4-9]. No investigated cellular response has predicted RTS,S-induced protection consistently in pediatric phase 2 trials [7, 8].

RTS,S/AS01E-induced cellular immunity is probably complex and involves different cell types and immune mediators. Most studies performed to date measured a limited number of parameters, restricted by the reduced amounts of blood obtained in children. Multiplex bead arrays and the Luminex platform allow multiparameterization of numerous markers in small volumes. Use of this technology in 2 studies including a limited number of markers suggested that RTS,S vaccination elicited secretion of the cytokines IL-2 and IL-4 [4, 9]. Herein, we used a multiplex immunoassay to measure cellular responses following ex vivo stimulations with vaccine antigens in cells freshly isolated at pre- and postimmunization in children and infants from 3 African sites of the phase 3 trial.

METHODS
Study Design
Written informed consent was obtained from children’s parents/guardians before enrollment. The study protocol was approved by all relevant ethics review boards and national regulatory authorities (Supplementary Methods). We performed a matched case-control study nested within the RTS,S/AS01E phase 3 trial, described elsewhere [3]. In brief, the trial enrolled infants (6–12 weeks) and children (5–17 months) who were vaccinated with either RTS,S/AS01E or a comparator vaccine—that is, rabies vaccine (children) or meningococcal C conjugate vaccine (infants), administered at study months zero, 1, and 2. Peripheral blood mononuclear cells (PBMCs) were collected and stimulated ex vivo at month 0 before vaccination and 30 days after the third dose of vaccine (M3) from 368 children in 3 trial sites: Bagamoyo (Tanzania), Lambaréné (Gabon), and Manhiça (Mozambique); and from 219 infants in Manhiça and Lambaréné (only M3). This study included all malaria cases detected 1–12 months postvaccination from the according-to-protocol immunology cohort whose samples were available (Figure 1, Supplementary Table 1). Malaria cases were defined as subjects who sought care at a health facility and had any P. falciparum asexual parasitemia by blood smear. Controls were matched to cases based on site, age group, and time of vaccination and follow-up. Up to 1:4 case:controls (average 1:3) were selected among RTS,S/AS01E vaccinees, and 1:2 case:controls were selected among comparator vaccinees. Investigators conducted assays blinded to vaccination and protection status.

Peripheral Blood Mononuclear Cell Stimulations and Multiplex Bead Array Assay
PBMCs were stimulated fresh with peptide pools covering the 2 vaccine antigens: CSP (31 peptides) and HBsAg (53 peptides) [7]. Dimethyl sulfoxide, the solvent for peptide pools, was used alone as a background control (henceforth “background” or “mock stimulation”). Thirty cytokines, chemokines, and growth factors were quantitated in cell supernatants using the Cytokine Human Magnetic Panel from Life Technologies (Supplementary Methods).

Selection of Markers and Metrics for Primary Analysis in a Pilot Study
Four cytokines (IL-2, IL-5, IL-17, and IFN-γ) were selected as primary markers for primary and secondary analyses in a pilot study that included 153 children who were not in the main matched case-control study. Markers were selected based on immunogenicity (Supplementary Table 2), precision, reliability, accuracy, uniqueness, and biological relevance. The primary outcome for antigen-specific responses was defined in the pilot study as the ratio between the crude concentration after antigen stimulations (CSP or HBsAg) and after mock stimulations (Supplementary Methods).

Statistical Analysis
Primary analysis of immunogenicity and correlates of protection was based on ratios of primary markers at M3 and focused on RTS,S/AS01E vaccinees and CSP responses. To distinguish correlates of RTS,S/AS01E-induced immunity from correlates of naturally acquired immunity, results of RTS,S/AS01E and comparator vaccinees were contrasted through interaction tests with vaccination group. Marker responses between RTS,S/AS01E and comparator vaccinees were contrasted through linear models and t tests. Impact of baseline ratios on postvaccination ratios was assessed through linear regression models, with baseline ratios as predictors. Analyses of correlates of protection were based on comparisons of cases and controls for CSP ratios in logistic regression models with a random intercept to account for matching strata. Odds ratios (ORs) were scaled to represent a 10% increase in ratios or in concentrations. Additionally, for interpretability, relevant ORs were scaled based on standard deviations. To identify groups of markers associated with malaria, marker ratios were analyzed in combination and selected through regression with elastic net and through partial least squares discriminant analysis (PLS-DA).

All tests were 2-sided and considered statistically significant to a .05 α-level. Analyses were adjusted for multiple testing through permutation (maximum T). P values for the 4 primary markers were adjusted (P adj) separately from the 24 secondary markers. P values for CSP and HBsAg stimulations were adjusted separately. Adjustments for multiple testing when assessing interactions with vaccination status, age group, or sex were done through Holm (primary markers) and Benjamini-Hochberg (secondary markers) approaches. When age interactions were statistically significant, the age-specific association was reported. Analyses were conducted using the R software.
9 did not meet inclusion criteria for the clinical trial
341 excluded
56 did not receive 3 doses of the study vaccine
192 did not comply with primary vaccination schedule
82 had blood collected outside of the period specified in the protocol
11 other reason

4272 subjects enrolled in the ITT phase 3 RTS,S/AS01E Trial (M055) for Bagamoyo, Lambaréné and Manhiça
2071 subjects were included in the M067 ITT cohort for Bagamoyo, Lambaréné and Manhiça
1721 subjects were included in the M067 ATP criteria for Bagamoyo, Lambaréné and Manhiça

1137 RTS,S/AS01E vaccinees 705 children/ 432 infants
584 comparator vaccinees 371 children/ 213 infants

2103 vaccinees had PBMC 1413 children/ 870 infants
1219 vaccinees had PBMC 853 children/ 496 infants

372 vaccinees had PBMC stimulations 236 children/ 137 infants‡
382 vaccinees had PBMC 228 children/ 154 infants

109 had < 6.6 million cells 61 were included in a separate B-cell study 22 had missing samples

193 had < 6.6 million cells 97 were included in a separate B-cell study 47 had missing samples
428 not selected for cellular component*

32 cases by M12‡ 16 children/16 infants
341 controls by M12 220 children/121 infants
25 cases by M12‡ 17 children/8 infants
188 controls by M12 117 children/71 infants

14 children had M0 samples available 46 children had M0 samples available 12 children had M0 samples available 18 children had M0 samples available

25 cases by M12 17 children/8 infants
188 controls by M12 117 children/71 infants

12 cases analyzed 16 children/16 infants
106 controls analyzed‡ 53 children/53 infants
25 cases analyzed 17 children/8 infants
46 controls analyzed‡ 30 children/16 infants

32 cases analyzed 16 children/16 infants
106 controls analyzed‡ 53 children/53 infants
25 cases analyzed 17 children/8 infants
46 controls analyzed‡ 30 children/16 infants

32 cases analyzed 16 children/16 infants
106 controls analyzed‡ 53 children/53 infants
25 cases analyzed 17 children/8 infants
46 controls analyzed‡ 30 children/16 infants

25 cases by M12 17 children/8 infants
188 controls by M12 117 children/71 infants

14 children had M0 samples available 46 children had M0 samples available 12 children had M0 samples available 18 children had M0 samples available

Figure 1. Study profile. *Based on the study protocol, only a subset of vaccinates were enrolled for the cellular component of the immunology study MAL067 (M067), ancillary to the RTS,S/AS01E phase 3 trial MAL055 (M055). ‡Per study protocol, peripheral blood mononuclear cells (PBMCs) were supposed to be freshly stimulated and have supernatant collected whenever >6.6 million cells were harvested. The priority was to cryopreserve 5 million PBMCs. Stimulations were performed when additional PBMCs were available for at least circumsporozoite protein and mock stimulations (0.8 million each). In the Manhiça site, after we collected samples from 292 subjects in each age cohort, all subsequent samples were assigned to a different study involving B cells and no fresh stimulations were performed. †Four subjects (1 RTS,S/AS01E- and 3 comparator-vaccinated children) who were not originally in the according-to-protocol cohort were inadvertently included in the analysis. The subjects had the blood sample collected more than the 30 days after the primary vaccination defined in the protocol. ‡Up to 1:4 case:controls (average 1:3) were selected among RTS,S/AS01E vaccinates, and 1:2 case:controls were selected among comparator vaccinates. Controls that were not matched to any case were not analyzed. Abbreviations: ATP, according-to-protocol; ITT, intention-to-treat; M0, study month 0; M12, study month 12; PBMC, peripheral blood mononuclear cell.
RESULTS

Study Population and Markers at Preimmunization

Samples from 209 subjects were analyzed (Figure 1). Among RTS,S/AS01E vaccinees, 15 cases and 51 controls were from Bagamoyo, 1 case and 2 controls from Lambaréné, and 16 cases and 53 controls from Manhiça. Vaccinees in Bagamoyo and Lambaréné were all children, and in Manhiça nearly all were infants (95%). Preimmunization CSP ratios in RTS,S/AS01E- and comparator-vaccinated children (baseline samples were not collected in infants) were comparable, except for IFN-γ, which was statistically significantly higher in RTS,S/AS01E vaccinees (Supplementary Table 3).

Immunogenicity Markers

When comparing CSP ratios between RTS,S/AS01E and comparator vaccinees at M3, only IL-2 was significantly higher in RTS,S/AS01E than in comparator vaccinees (Figure 2A, Supplementary Figure 1A). For HBsAg ratios, IL-2 and the helper T cell type 2 (T\(_{h2}\)) cytokine IL-5 were significantly higher in RTS,S/AS01E vaccinees (Figure 2B, Supplementary Figure 1B). The effect of vaccination on ratios was comparable in children and infants and in both sexes (\(P\)-adj > .10 for interaction with age and with sex).

Concentrations of primary markers IL-2 and IL-5 after CSP and HBsAg stimulations were significantly higher in RTS,S/AS01E than in comparator vaccinees (Supplementary Figure 2). Unlike analysis with ratios, concentrations after CSP, HBsAg, and mock stimulations were different between age groups for several markers (\(P\)-adj for interaction with age <.05; Supplementary Table 4). In children, CSP and background responses of most of these markers were higher in RTS,S/AS01E vaccinees than in comparator vaccinees, including the T\(_{h1}\)-related cytokines IFN-γ, IL-12, IL-15, and the proinflammatory markers TNF and IL-1β (Table 1; Supplementary Table 4). In contrast, no differences in marker concentrations between vaccination groups were detected in infants. No differences in immunogenicity by sex were found (\(P\)-adj > .10 for interaction with sex).

Impact of Preimmunization Markers on RTS,S/AS01E Immunogenicity

Baseline responses to CSP due to previous immunity or to HBsAg due to hepatitis B vaccination (included in the expanded program of immunization) could affect immunogenicity of RTS,S/AS01E in children. Therefore, we analyzed the impact of prevaccination ratios of primary markers on the postvaccination ratio of the same or any other marker (Supplementary Table 5). For CSP ratios in RTS,S/AS01E-vaccinated children, baseline IFN-γ was consistently and positively correlated with postvaccination ratios of some markers, including the immunogenicity marker IL-2, the T\(_{h1}\)-related cytokine IL-15, and the homeostatic cytokine IL-7 (Supplementary Figure 3). For HBsAg ratios, baseline IFN-γ was negatively correlated with some postvaccination CSP ratios, including IFN-α, IFN-γ, and vascular endothelial growth factor (VEGF). Baseline IL-2

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Figure 2. Circumsporozoite protein (CSP; A) or hepatitis B surface antigen (HBsAg; B) primary marker ratios 1 month postimmunization with RTS,S/AS01E or a comparator vaccine. Diamonds show geometric means of CSP or HBsAg ratios to controls and bars show the 95% confidence intervals. \(P\) values were computed through t tests of the log\(_{10}\) transformed ratios and were adjusted for multiple testing (\(P\)-Adj) through a permutation approach. Only statistically significant \(P\) values are shown. There were 137 RTS,S/AS01E and 70 comparator vaccinees for CSP (A) and 133 RTS,S/AS01E and 63 comparator vaccinees for HBsAg (B). Abbreviations: CSP, circumsporozoite protein; HBsAg, hepatitis B surface antigen; IFN-γ, interferon gamma; IL, interleukin.
HBsAg ratios correlated with higher levels of some postvaccination CSP ratios (eg, IFN-α). Few baseline HBsAg ratios were associated with postvaccination HBsAg ratios in RTS,S/AS01E vaccinees (Supplementary Table S5), and most associations were weak and different from associations with CSP ratios. In contrast, no clear associations were detected in comparator vaccinees.

### Table 1. Comparisons of Marker Concentrations After Circumsporozoite Protein Stimulations in RTS,S/AS01E and Comparator Vaccinees by Age Cohort When Significant Age Interactions Were Detected

<table>
<thead>
<tr>
<th>Marker</th>
<th>Children</th>
<th>Infants</th>
<th>Difference Age Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RTS,S/AS01E (n = 69)</td>
<td>Comparator (n = 47)</td>
<td>P value*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>36 (24–54)</td>
<td>15 (10–25)</td>
<td>.009</td>
</tr>
<tr>
<td>G-CSF</td>
<td>1314 (1081–1598)</td>
<td>885 (658–1190)</td>
<td>.03</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>245 (265–461)</td>
<td>159 (98–256)</td>
<td>.07</td>
</tr>
<tr>
<td>IL-10</td>
<td>237 (260–437)</td>
<td>180 (112–289)</td>
<td>.03</td>
</tr>
<tr>
<td>IL-12</td>
<td>2090 (1650–2647)</td>
<td>1094 (739–1619)</td>
<td>.07</td>
</tr>
<tr>
<td>IL-15</td>
<td>162 (137–191)</td>
<td>104 (81–134)</td>
<td>.005</td>
</tr>
<tr>
<td>IL-17α</td>
<td>2491 (2142–2898)</td>
<td>1285 (833–1984)</td>
<td>.007</td>
</tr>
<tr>
<td>IL-1β</td>
<td>3422 (2763–4238)</td>
<td>2166 (1605–2924)</td>
<td>.02</td>
</tr>
<tr>
<td>IP-10</td>
<td>32 (27–40)</td>
<td>26 (22–30)</td>
<td>.008</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>36 (24–54)</td>
<td>15 (10–25)</td>
<td>.009</td>
</tr>
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<td>GM-CSF</td>
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<td>IP-10</td>
<td>32 (27–40)</td>
<td>26 (22–30)</td>
<td>.008</td>
</tr>
</tbody>
</table>

Data are presented as geometric mean, pg/mL (95% confidence interval) unless otherwise indicated.

### Figure 3.

#### Primary/Secondary Marker Analysis

*Correlates of Clinical Malaria: Single-Marker Analysis*

Figure 3 shows the ORs for 10% increases in postvaccination CSP ratios of primary and secondary markers and malaria in RTS,S/AS01E (Figure 3A) and comparator vaccines (Figure 3B). Only IL-5 was significantly associated with the odds of malaria in RTS,S/AS01E vaccinees (OR, 1.17 per 10% increases of IL-5 CSP ratios [95% confidence interval, 1.03–1.38]). Although the

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chemokine RANTES (regulated on activation normal T-cell expressed and secreted) was also associated with increased odds of malaria in RTS,S/AS01E vaccinees (OR, 1.09 [confidence interval, 1.01–1.22]), the association was not significant after adjusting for multiple testing. The ORs of IL-5 and RANTES, scaled by changes in standard deviation units of the ratio, were 2.29 and 1.61, respectively. Furthermore, the association of IL-5 with malaria was specific to RTS,S/AS01E vaccinees (P(adj) = .049 for vaccination interaction; Supplementary Table 6). No marker was associated with malaria in comparator vaccinees. Based on these results, we explored the association of IFN-γ/IL-5 ratio with malaria, and a higher ratio was found to be protective against malaria (P = .004; Supplementary Figure 4).

Figure 4. Correlation between clinical malaria and circumsporozoite protein (CSP) marker ratios in RTS,S/AS01E-vaccinated children (A) and infants (B). Odds ratios and 95% confidence intervals per 10% increase in marker ratios (CSP ratio to control) 1 month postimmunization. Only markers that had statistically significant interactions with age were analyzed. Blue indicates markers that were significantly associated with clinical malaria in logistic mixed-effect models. N = 68 RTS,S/AS01E-vaccinated children and 89 RTS,S/AS01E-vaccinated infants. Abbreviations: FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP, interferon-gamma-induced protein; OR, odds ratio; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
The associations of nearly half of the markers with malaria varied between children and infants, including IFN-γ (Figure 4; Supplementary Table 6). In children, increases in CSP ratios of several markers significantly increased the odds of malaria. On the contrary, increases in CSP ratios of TH1 and proinflammatory cytokines IFN-γ, IL-15, and TNF and the granulocyte colony-stimulating factor (G-CSF) in infants protected from malaria. Sex of vaccinees did not confound associations between ratios and malaria—that is, neither impacted ORs nor P values (data not shown).

In secondary analyses (data not shown), we found that marker concentrations were not correlated with malaria.

Correlates of Clinical Malaria: Multiple-Marker Analysis

When analyzing combinations of marker ratios in models selected by elastic net, IFN-γ (OR, 0.90), granulocyte-macrophage colony-stimulating factor (GM-CSF) (OR, 0.94), IL-15 (OR, 0.93), IL-5 (OR, 1.38), and RANTES (OR, 1.15) were predictive of malaria in RTS,S/AS01E vaccinees (Supplementary Figure 5). Two components identified by PLS-DA were independently protective against malaria in RTS,S/AS01E vaccinees (Figure 5). In line with previous results, the markers that contributed more (loadings > −0.3 or >0.3) to 1 component were GM-CSF, IFN-γ, IL-5, and RANTES and to the other component were IL-15, IL-5, and RANTES.

DISCUSSION

We found that IL-5 CSP ratios at postvaccination increased the odds of malaria in RTS,S/AS01E-vaccinated children and infants, a result that could explain the lack of protection in numerous vaccinees through a different mechanism than the previously reported strain-specific VE in children [14]. In multimarker analysis, IL-5 and RANTES increased the odds of malaria in RTS,S/AS01E vaccinees, whereas the TH1-related markers IFN-γ, IL-15, and GM-CSF were correlated with RTS,S/AS01E-induced protection. IFN-γ and IL-15 were also associated with protection in single-marker analysis in infants, as well as TNF and G-CSF. Although associations of marker ratios with vaccination were comparable between age groups, associations with concentrations were significantly different for several markers. Proinflammatory and the above-reported protective markers were significantly higher in RTS,S/AS01E-vaccinated than in comparator-vaccinated children, but these differences were not observed in infants. These results suggest that RTS,S/AS01E is less immunogenic in infants, and may explain the lower VE in this age group [12]. Of note, RTS,S/AS01 responses were higher for HBsAg than CSP. This may be due to a higher immunogenicity of HBsAg than CSP, to the higher proportion of HBsAg in the RTS,S vaccine, or to hepatitis B vaccination.

IL-5 is mainly produced by activated Th2 cells and restricted to effector memory T cells that are differentiated after recurrent antigenic exposure [15, 16]. IL-5 has never been examined in RTS,S trials, although IL-4, another Th2 cytokine, was previously found to be elevated in RTS,S-vaccinated infants [4]. In a preclinical study, the AS01B and AS02A adjuvants induced IL-5 in addition to IFN-γ responses [17]. To our knowledge, there is no epidemiological evidence of IL-5 association with occurrence of clinical malaria. Functional polarity between Th1 and Th2 responses could partially explain the association of IL-5 with malaria in RTS,S/AS01E vaccinees. IL-5+ Th2 cells probably constrain protective Th1 responses and inhibit several macrophage functions [18]. Exploratory analyses of IFN-γ/IL-5 ratios indicate that a skewed response to Th2 increases the odds of malaria. Additionally, the effect of IL-5 in promoting eosinophil responses [18] could contribute to the increased odds of malaria. Eosinophils have important regulatory functions and may restrict inflammation and increase plasma cell responses [19]. RANTES, the other marker that increased the odds of malaria, is produced by memory T cells and macrophages in PBMCs [18, 20] and is involved in chemoattraction of T cells and, together with IL-5, of eosinophils [18]. We speculate that IL-5 and RANTES in RTS,S/AS01E vaccinees could be acting together on eosinophils to regulate antibody responses through plasma cells, jeopardizing protective responses.

IFN-γ, mainly produced by T cells and natural killer (NK) cells, seems to be involved in the protection mediated by pre-erythrocytic vaccines [21]. CSP-specific IFN-γ T-cell and NK cell responses were elicited by RTS,S vaccination in adult challenge studies [22–24] and in clinical trials from endemic areas [4, 6, 7, 9], but their effect on protection remains unclear. Th1 responses are involved in protection against intracellular pathogens through cell-mediated immunity, and IFN-γ is crucial for parasite killing through the induction of nitric oxide [18, 21]. IL-15, a cytokine produced by dendritic cells and monocytes in PBMCs, is important for activation of NK and T cells, and increase around 12 months of age [26, 27]. Therefore, IL-15 and GM-CSF could contribute to induction or enhancement of Th1-mediated protective responses.

Detected age differences in immunogenicity are in line with immune ontogeny. Newborns have a biased cellular response toward a Th2 profile, due to diminished Th1 and proinflammatory responses that persist during the first months of life and increase around 12 months of age [26, 27]. Therefore, the age differences in RTS,S immunogenicity may be explained by diminished responsiveness in infants that may impact any vaccine [26, 27] depending on the adjuvant and antigen immunogenicity. Curiously, we detected similar marker differences in the mock stimulations, reflecting nonspecific responses upon RTS,S/AS01E vaccination. A nonspecific effect of RTS,S has not
been observed, but the nonspecific effect of other vaccines in early life has been described [28–30]. Surprisingly, despite low immunogenicity in infants, more markers were correlated with protection in infants than in children, including the T$_{h}$1-related cytokines IFN-γ, IL-15, and TNF. On the contrary, in children, some markers including TNF were associated with increased...
odds of malaria. Despite this apparent paradox, different levels of cytokines may have different effects. For instance, moderate levels of cytokines like TNF have been described to control *Plasmodium falciparum* infection [31], but exacerbated levels are biomarkers of proinflammatory responses involved in pathogenesis of malaria [32]. Also, differences may be given by diverse cell origin as monocytes, macrophages, NK cells, and also effector memory and central memory T cells may produce it.

Several baseline marker ratios had an impact on postimmunization ratios in children. Responses affected included the immunogenicity marker IL-2, the protective cytokines IFN-γ and IL-15, and IL-7, IFN-α, and VEGF, which were associated with higher odds of malaria in children. This suggests that immune status and previous responses to malaria and hepatitis B vaccine may influence RTS,S/AS01E immunogenicity. If malaria baseline immunity alters vaccine responses, it could further explain the lower VE in infants. Indeed, IgG data against CSP suggest that baseline levels have an impact on immunogenicity [12]. Also, baseline cell composition and inflammation have previously been associated with RTS,S efficacy [33] and postvaccination responses in other vaccine studies [34–36]. Future work assessing cell phenotypes and activation, cytokine-expressing CD4⁺ T cells, and antibody responses to CSP and HBsAg at baseline may help confirm this hypothesis.

Imbalances in age distribution across sites and the nonavailability of baseline samples in infants may limit the strength of some of our conclusions. Most children were from Bagamoyo and infants from Maniça, but because malaria transmission was similar in both sites, it is unlikely that age differences were confounded by study site. Finally, our study did not include samples from areas of high endemicity, nor had information about other factors, for example, coinfections that could impact vaccine responses.

In summary, we identified 2 possible different and antagonistic cellular immune mechanisms induced by RTS,S/AS01E vaccination: IL-5 (and RANTES) T⁺H₂ responses associated with increased odds of malaria; and IFN-γ and other T⁺H₁-related responses (GM-CSF, IL-15) associated with protection. Moreover, we detected lower induction of protective T⁺H₁ and proinflammatory responses by RTS,S/AS01E in infants than children, whereas T⁺H₂ responses were similar, which could contribute to the decreased VE in infants. Further analysis of cytokine-expressing cells together with isotopes and specificities of antibody responses to RTS,S/AS01E will clarify the role of these identified correlates in protection or lack thereof. Our findings may transcend antigen specificity and underscore the need to understand the impact of baseline immune status and factors that may modulate any pediatric vaccine responses in Africa. Our results reveal undesirable vaccine responses that may abrogate the protection of RTS,S/AS01E and other vaccines, but that might be overcome by improved formulations. Adjuvants that modulate the immune system to support a potent T⁺H₁ response during the first months of life and avoid counteracting responses may be required.

**Supplementary Data**

Supplementary materials are available at Clinical Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

**Notes**


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**Potential conflicts of interest.** J. J. A. received funds from GSK to participate in a data and safety monitoring board for a new pneumococcal vaccine. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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PAPER IV

Mal067 ancillary immunology study of the phase 3 RTS,S/AS01E vaccine trial in Bagamoyo: The implication for immunological sampling of African paediatrics

Working paper
Mal067 ancillary immunology study of the phase 3 RTS,S/AS01E vaccine trial in Bagamoyo: The implication for immunological sampling of African paediatrics

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Introduction

Malaria remained a major public health concern for many decades. Highly efficacious malaria vaccines are considered to be the cornerstone for successful malaria eradication (1). The RTS, S malaria vaccine; a GlaxoSmithKline (GSK) Biologicals design, is a mixture of RTS, the chimeric recombinant protein containing polypeptide region of circumsporozoite protein (CSP), the hepatitis B virus surface antigen (HBsAg), and S, the recombinant protein containing HBsAg alone (2). RTS, S adjuvated with AS01 has been shown to be safe and to induce significant protection against Plasmodium falciparum (P. falciparum) infection and or clinical malaria in naïve adult (3), semi-immune adults (4) semi-immune children (5,6) and infants (7,8). In previous studies, the RTS, S vaccination have proved to induce high anti-CSP IgG titre and modest cellular immune responses (9,10). However the role of cell-mediated-immunity in RTS, S-induced protection is as yet unresolved. There remained inconclusive results to the induction of cytotoxic T cells by TRS, S, although significant Th1 cytokines-mediated responses have been demonstrated (11–19). In naïve adults there are indication that cellular immunity to CSP may be important. IL-2 and IFN-γ responses assessed by ex-vivo ELISpot were higher in protected than infected individual (12). In addition, analysis of IL2, IFN-γ TNF and CD40L by intracellular cytokines staining (ICS) and flow cytometry suggested that higher CD4+ Th1 cell responses might be associated with protection. A recent study showed that frequencies of TNF and IL2 producing T effector and T effector memory cells restimulated with CSP peptide were higher in protected non-immune vaccinees (20). In African children IL2, IFN-γ, and TNF cytokine responses are produced among vaccinated individuals after stimulation of fresh whole blood with CSP and HBs peptide; albeit in low magnitude with heterogeneity in the intensity of responses. In Gabonese children, IL2 CD4+ T cell responses could be detected upon CSP antigen re-stimulation after RTS, S/AS01 vaccination (17). In Tanzania, CD69 expressing T cells were present in the higher numbers in vaccinated than unvaccinated children after stimulation of peripheral blood mononuclear cells with CSP and HBs peptide (21). In Mozambican infants, increased levels of secreted IFN-γ, IL2, and IL4 to both
vaccine antigen components were observed in supernatants as well as IFN- and ILs by ICS up to ten weeks post dose 3 (16). Secreted IL-2 was the strongest and most frequent CSP-specific responses and was detected in more than 25% of the RTS, S/AS01 immunized infants at ten weeks post dose 3. The induction of CD8+ T cells remained inconclusive and has been detected in only this trial in Mozambique (16). However, the key question regarding the mode of action of RTS, S and the duration of protection elicited by the vaccine remain open. There is only few studies that tried to identify markers associated with RTS,S induced protection against malaria using gene expression microarrays (22,23). A study carried out in naïve USA adult volunteers vaccinated with RTS, S/AS01 or RTS, S/AS02 and experimental challenged with *P. falciparum* showed different gene expression in four components of immunoproteasome. Those genes are responsible for processing of peptide for MHC presentation distinguished protected and no-protected individuals before the parasites challenge (24). It remains to be established whether similar patterns would apply to children from malaria endemic area naturally exposed to *P. falciparum* and other infections. Here we report an analysis of cellular immune responses of children enrolled, between the 5-17 months of age, in a Phase IIIb trial of RTS, S/AS01 in Bagamoyo Tanzania.

**Materials and methods**

**Ethical consideration**

The protocol was approved by institutional review boards (IRBs) of the Ifakara Health Institute (IHI/IRB/No.A59) and National Institute for Medical Research Tanzania (NIMR/ HQ/R.8a/Vol. IX/792), and the Ethikkommission beider Basel (EKBB), Basel, Switzerland (EKBB 319/11). The protocol was also approved by TFDA (Ref. No. CE.57/180/04/41), and the trial was registered at ClinicalTrials.gov (NCT00866619). The study was conducted in accordance with Good Clinical Practice guidelines. Before enrolment into the study, the parents or guardians of a child participants were given an inform consent form to read and upon understanding it, they were allowed to give the
will for their child to participate in trial. No child was recruited in the study without having informed consent process completed.

**Study site and design**

This ancillary immunology arm of the phase 3, randomized, controlled, double-blind trial was conducted at 11 centers in 7 African countries with a range of malaria-transmission intensity. The Ifakara health institute, Bagamoyo branch, Tanzania was part of 11 sites in seven African countries that participated in the whole study. The main trial was designed to evaluate vaccine efficacy, safety, and immunogenicity during an average period of 49 months (range: 41–55 months) after the first dose of study vaccine in children and an average period of 41 months (range: 32–48 months) after the first dose of study vaccine in young infants. Children with a moderate or severe illness, a major congenital defect, malnutrition requiring hospitalization, a haemoglobin concentration 5.0 g/dl, or 8.0 g/dl with clinical signs of decompensation, a history of atypical febrile seizures, a neurological disorder, or WHO stage III or stage IV HIV disease at the time of recruitment were considered not eligible for participating in trial. Insecticides treated nets were provided or made available to any child who presented for screening.

**Blood sample collection and processing**

A total of 400 children aged between 5 -17 months were recruited out of 800 children enrolled for the main study. 3.8 mL of whole blood were collected into cell preparation tubes (CPT Vacutainer BD), from each child at baseline, 3, 21 and 32 months after first vaccination. Additionally, at months 20, approximately 0.2 mL blood was collected into a filter paper blood spots for the parasite genotyping. Blood were transported at room temperature to the laboratory for processing within 1 hour after collection, as this was critical for the quality and sensitivity of cell mediated immune assays.

In the lab, blood were centrifuged and processed according to manufacture instructions. Plasma was separated and aliquoted into 2 mL cryovials and frozen at -80ºC. PBMCs were collected from the
tubes, washed, counted using Countess automated cell counter from Invitrogen and stored at -80ºC for 18-24 hrs before transferred into liquid nitrogen (LN2) for longer storage. At least 5 million PBMCs per volunteer per bleeding time point were frozen down in LN2 and the left over PBMC were used for fresh stimulations onsite with Dimethyl sulfoxide (DMSO), CSP, Phytohemagglutinin (PHA), Apical membrane antigen 1 (AMA1) and Hepatitis B antigen (HBsAg). The erythrocyte pellets form the CPT tubes were carefully collected and stored for retrospective determination of \textit{P. falciparum} infections.

**Recovery and viability of PBMCs from Bagamoyo after thawing the cryopreserved cells**

To assess whether the stored PBMC are viable and the vials have the sufficient number of cells for the purpose of our analysis, subjects with more than one vials stored (N=115) were selected for participating in this pilot study. Only the baseline and month 3 PBMCs were included in this experiment. Briefly PBMC sample were thawed from the liquid nitrogen, washed, re-suspended into culture medium (RPMI 1640, 10% FBS) and then counted using GUAVA technologies (Germany). The numbers counted and the viabilities obtained from this count were recorded as day 1. The cells were then left incubated overnight with culture medium (RPMI 1640, 10% FBS) and then washed, re-suspended into culture medium and counted to obtain the recovery count. The number counted and viabilities obtained were recorded as day 2.

**Intracellular cytokine staining (ICS)**

To test the functionality of cryopreserved PBMCs, the rate of IFN-g/IL-2 secretion was determined using PBMCs from 115 children. The PBMC were stimulated by the CSP and HBsAg antigen which are the components of the RTS, S vaccine. Intracellular cytokine staining assays were performed and IL-2 and IFN-g responses quantified by flow cytometry. The IL-2 and IFN-g were selected as marker of functionality based of the previous studied outcomes that showed IFN-g/IL-2 responses following vaccination with RTS, S (25). ICS was performed according to the published method by Moncunill et al (26). Briefly, PBMC samples from 115 RTS, S vaccinated children were stimulated with CSP, and HBsAg, and stained with commercial available reagents (26).
primary measurement in this study was the cytokine response for CD4+ and CD8+ T-cell subsets producing IFN-γ and/or IL-2 to CSP and HBsAg. Data was acquire by flow cytometry directly from the plates using the high throughput sampler (HTS) until reaching dead volume of at least 50 μL.

**Statistical analysis**

Descriptive statistics are used to describe participant’s recruitment patterns with relative to the villages of their origin. The Fisher’s exact Test were used to calculate the IL-2/IFN-g responses rates and the difference between day 1 and day 2 viability and recovery were tested by using Mann Whitney test by using a GraphPad Prism 6 (USA). The p<0.05 were considered significant.

**Results**

**Recruitment outcome and study sampling adherence**

Recruitment for this study took place between mid-September 2009 and February 2010. A total of 400 children were recruited at the baseline. However, only 356 met according to protocol (ATP) criteria and their data were used in this analysis. The number of recruitment for each month varied substantially with the lowest number observed at the beginning of the study recruitment, September 2009. However, the recruitment was improved on October and November, before falling again in December to February, albeit remained above the original number (Figure 1).
Figure 6: Number of children enrolled in Bagamoyo study site by months during recruitment period

Furthermore, despite that the study follow up persisted intensively for nearly 3 years, our data demonstrate that, on the subsequent visit, adherence remained higher on month 3 (99.2%), month 21 (90.5%) and on month 32 (90.5%). The overview of sample collected during the course of this study is depicted in Table 1.

Table 1: Overview of the number of subjects recruited in Bagamoyo site at baseline and those adhering to subsequent sample collection visit (3, 20, 21, 32 months) after vaccination.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Baseline</th>
<th>Month 3</th>
<th>Month 20</th>
<th>Month 21</th>
<th>Month 32</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td>356 (100%)</td>
<td>353 (99.2%)</td>
<td>0</td>
<td>322 (90.5%)</td>
<td>318 (89.3%)</td>
</tr>
<tr>
<td>Plasma</td>
<td>356</td>
<td>353</td>
<td>0</td>
<td>322</td>
<td>318</td>
</tr>
<tr>
<td>Filter paper</td>
<td>0</td>
<td>0</td>
<td>336</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Additionally, we investigated amongst the sample collected, the number of subjects who had their PBMCs successfully stimulated on site. Our data suggest that over ninety percent of children had the correct amount of blood collected hence demonstrate the efficient of our phlebotomist. (Table 2)

**Table 2:** Overview of the total number of PBMCs collected during all time points (3, 20, 21, 32 months) and those stimulated onsite. In bracket is the percentage of total number within respective time point.

<table>
<thead>
<tr>
<th>Time points</th>
<th>Total PBMC collected (%)</th>
<th>Freshly stimulated (%)</th>
<th>Not stimulated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>356 (100%)</td>
<td>316 (88.8%)</td>
<td>40 (11.2%)</td>
</tr>
<tr>
<td>Month 3</td>
<td>353 (99.2%)</td>
<td>338 (95.8%)</td>
<td>15 (4.3%)</td>
</tr>
<tr>
<td>Month 21</td>
<td>322 (90.5%)</td>
<td>314 (97.5%)</td>
<td>8 (2.5%)</td>
</tr>
<tr>
<td>Month 32</td>
<td>318 (89.3%)</td>
<td>288 (90.6%)</td>
<td>30 (9.4%)</td>
</tr>
</tbody>
</table>

**Community awareness influences the number of participant recruited across the study area**

To investigate the influence of community responses amongst villages, we stratified the subjects groups according to their village of origin. A total of 21 villages were included in the study area. This includes Bago, Chasimba, Dunda, Fukayosi, Kerege, Kidomole, Kiwangwa, Kongo, Magomeni, Makurunge, Mapinga, Masuguru, Mataya, Matimbwa, Mkenge, Msinune, Mwavi, Mwetemo, Yombo and Zinga. Together, our data demonstrated that, Magomeni and Kiwangwa had the highest frequency of subject participation compared to the other villages (Figure 2). Several clinical trials studies have been previously conducted in Kiwangwa and Magomeni, this could explanation the higher frequency seen in the two villages as community is aware of the clinical trials therefore it was not questionable to agree to participate.
Recovery and viability of PBMCs from children at point of collection varies between time points

The recovery of PBMCs isolated from all the bleeding time points were measured at baseline (mean ± SME) (4.54±0.43 million cells/ml of blood), month 3 (6.47±0.12 million cells/ml of blood), month 23 (5.58 ± 0.11 million cells/ml of blood) and Month 32 (4.39 ± 0.84 million cells/ml of blood) (Figure 3). Additionally, the overall percentage viability for the isolated PBMC at different time points was (86.1% ± 0.57 million cells/ml of blood) at baseline, (81.3 ± 0.79 million cells/ml of blood) at month 23, (99.7 ± 0.14 million cells/ml of blood) at month 32. Viability data for the month 3 samples was not obtained (Figure 4).
Number of peripheral blood mononuclear cells (PBMCs) recovered from the samples collected in Bagamoyo

![Graph showing number of PBMCs recovered from samples collected at different time points M0, M3, M21, and M32.]

**Figure 3:** Shown are sampling time points M0, M3, M23 and M34 of the Bagamoyo cohort.

Percentage PBMCs viability of sample collected on different time point during

![Graph showing percentage viability of PBMCs at different time points M0, M3, M21.]

**Figure 4:** Total viable PBMC counted upon day of thawing (day1-yield) and after over-night rest in culture media (day2- recovery). Differences analysed by Wilcoxon non-parametric non-paired test (N=115)
Recovery and viability of PBMCs from Bagamoyo after thawing the cryopreserved cells

To assess the quality of stored PBMC in terms of viability and quantity (numbers), subjects with more than one vials stored (N=115) were selected for participating in this pilot study. The data suggest an evidence of significant difference in number of PBMC recovery between thawing and resting time points, \(p = 0.0072\) (Figure 5). However, our data do not show any evidence of differences in PBMCs viabilities between the day of thawing (day1) and after over-night resting in culture (day2) \(p = 0.2666\) (Figure 6)

![Total PBMC recovered after thawing and over-night rest in culture media](image)

**Figure 5:** Total viable PBMC counted upon day of thawing (day1-yield) and after over-night rest in culture media (day2-recovery). Differences analysed by Wilcoxon non-parametric non-paired test (N=115)
Percentage viability upon thawing and after overnight rest in culture media (day2). Differences analysed by Wilcoxon non-parametric non-paired test (N=115)

**Figure 6**: Percentage PBMC viability upon day of thawing (day1) and after overnight rest in culture media (day2). Differences analysed by Wilcoxon non-parametric non-paired test (N=115)

The CD4+ T cells but not CD8 T cells from cryopreserved PBMCs from Tanzanian children secretes IFN-g and IL-2 cytokines following stimulation with HBsAg and CSP antigens

To test the functionality of cryopreserved PBMCs, the rate of IFN-g/IL-2 was determined using PBMCs from 115 children. Our data shows that the stored PBMC remained functional after 5 years of storage in LN2. Albeit in low magnitude, the CD4-T cells and not CD8 T cell were found to secret IL-2 and IFN-g cytokines (Table 3). The flow cytometer gating strategies is shown in figure 7
Table 3: T cell responses analysed base on their IFN-g and IL-2 secretion rate following stimulation with CSP and HBsAg of cryopreserved PBMCs from Tanzania children.

<table>
<thead>
<tr>
<th>T cell</th>
<th>Antigen</th>
<th>N</th>
<th>Filtered</th>
<th>IFN-g/IL-2</th>
<th>95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>CSP</td>
<td>184</td>
<td>26</td>
<td>8/158 (5.1%)</td>
<td>(2.8%, 9.7%)</td>
</tr>
<tr>
<td></td>
<td>HBsAg</td>
<td>114</td>
<td>40</td>
<td>12/65 (18.5%)</td>
<td>(10.9%, 29.6%)</td>
</tr>
<tr>
<td>CD8+</td>
<td>CSP</td>
<td>184</td>
<td>35</td>
<td>0/149 (0.0%)</td>
<td>(0.0%, 2.5%)</td>
</tr>
<tr>
<td></td>
<td>HBsAg</td>
<td>114</td>
<td>60</td>
<td>0/54 (0.0%)</td>
<td>(0.0%, 6.6%)</td>
</tr>
</tbody>
</table>

The Fisher’s exact test were used to determine the proportional of IL2 and IFN-g producing T cell

Flow cytometer gating strategies

Figure 7: Example of the staining and gating strategy for PBMC stimulated with Staphylococcal enterotoxin B (SEB). All gates for non-functional markers were defined using fluorescence minus one (FMO) controls whereas gates for functional markers were defined using the unstimulated samples. A: Gating hierarchy to identify NK cells, NKT-like cells, CD4+ and CD8+ T cells, and TFH-like cells. Initial gating is done on FSC-H and FSC-A to discriminate singlets, followed by the exclusion of events collected during a period of time early in collection when fluctuations may occur. In this example, there were no problems of fluctuations and the time gate was minimized to avoid
exclusion of any events. Dead cells and monocytes are excluded by an amine reactive dye and the CD14 marker in the same dump channel. Lymphocytes are gated using FSC-A and SSC-A. Subsequent gating discriminates two subsets of NK cells by CD56 and CD3 expression (CD56dimCD3- and CD56hiCD3-) and NKT-like cells (CD56+CD3+). Within the gate of lymphocytes, CD3+ cells are identified, followed by identification of CD4+ and CD8+ T cells. Of note, NKT-like cells are not excluded from classical T cells and therefore are overlapping populations. Finally, TFH-like cells are identified as CXCR5+ CD45RA- CD4+ T cells that have a low expression of CCR7 and arePD-1+. 

**B:** Functional markers for CD4+ and CD8+ T cells. A gate is applied for each cytokine, not taking into account the coexpression of other markers. Boolean gates are then created based on these gates to identify cells expressing different combinations of markers. 

**C:** The expression level of CCR7 and CD45RA is examined within CD4+ and CD8+ T-cell subsets to later provide insight into the memory phenotype of the antigen specific cells.

### Discussion

We found that the CD4 T cells but not CD8 T cells in PBMCs collected from Tanzanian children secrete IFN-γ and IL-2 after stimulation with HBsAg and CSP antigen invitro. In line with our finding, the IFN-γ and IL-2 secreting CD4 T cell were demonstrated in several RTS, S studies previously conducted in rhesus primate (25), naive adult individuals (20), and in children and infants living in malaria endemic regions (18). This finding implies that the quality of PBMC collected in Bagamoyo site during the course of RTS, S study was well maintained even after five years of storage in LN2. The result of this pilot study highlights the need for further analysis to evaluate in larger sample size the cellular and humoral immune responses induced by RTS, S malaria vaccine in paediatric population in natural transmission settings. Efficient recovery, higher percentage of viability and functionality of PBMCs are essential for reliable investigation of the ways in which the immune system responds to a certain interventions. However, blood sampling in paediatric population for the purpose of assessment of their immune responses has been very challenging amongst the parents, researchers and ethics committees (27). This is because the children become restless during blood draw and the mother gets nervous witnessed blood is taken from her child. In the current study we managed to collect 3.8 ml of blood from each child and isolate PBMC. From this we showed that, an average of 4.5 million lymphocytes per millilitre of blood could be
recovered from children aged between 5 and 17 months old. To date, data indicative of how many PBMCs can be obtained in a millilitre of blood collected from African children population are scarce. This study took advantage of samples collected during the RTS, S/AS01 clinical trial conducted in Bagamoyo Tanzania to establish the average number of PBMCs, their viability and functionality. This information may be helpful in planning future sample repositories or as a comparison for other immunological studies involving African paediatric population.

At the beginning of the study we thought to recruit more that 90% of children within 3 months of recruitment period. However this was not achieved and instead the recruitment rate of approximately 5- 35% was attained. The possible explanation for this was that, parents needed time to digest information given during sensitization meetings and decide whether they need their child to be included in the study of not. We could show that the recruitment rate went up on the October 2009, implying that parents were content with the study information hence became willing to let their children participate. On the other hand, it could be that sensitization meetings were not sufficient enough to convince parent to participate, hence parents needed time to see what happened to those who were convinced at the first place. However, despite slow rate of recruitment, adherence was well maintained. Approximately 99% of all children recruited at baseline attended the month 3 visit and blood was drawn from them for different laboratory tests. On the subsequent visit, adherence slightly went down up to 89% by the end of the study on month 32 of follow up. Such adherence after three years of follow up suggests that the follow up system was well set and village health care workers were performing the duty of trucking the participants efficiently. The elevated frequencies of participation observed in Kiwangwa and Magomeni area remains to be revealed as the two area represent different geographical backgrounds (rural and urban respectively). Furthermore, this finding suggests that the parents were satisfied with the services they received in the context of RTS, S clinical trial benefits, the reason why they opt to continue participating for such long time. Our data also shows that an average of 93% (range 88.8% to 97.5%) of all samples collected in respective time point, the PBMCs were stimulated onsite. The ability to obtain enough number of cells for onsite stimulation reflects the availability of sufficient amount of blood from the collection clinic.
We further demonstrated that the average recovery of freshly isolated PBMCs from all the bleeding time points were 5.25 million cells/ml of blood (range 4.39 to 6.47 million cells/ml of blood). Additionally, the overall percentage viability for the isolated PBMC at different time points was 89.0% (range from 81.3% to 99.7%). The quality (viability) of PBMC stored in LN2 and quantity (numbers) is critical for generation of credible immune response data. Our data suggest an evidence of significant difference in number of PBMC recovery between thawing and resting time points. However, we could not see any evidence of differences in PBMCs viabilities between the day of thawing (day1) and after over-night resting of PBMCs in culture (day2). The difference in numbers observed between time of thawing and after resting in culture is not uncommon, as some PBMCs are lost during the washing steps of thawing procedures while the remaining PBMC showed similar viability. The major limitation for this was CPT tube that resulted in occurrence of some red blood cell contamination in the isolated PBMC hence creating difficulties in counting. The volume of blood collected from children was also variable as it was difficult to obtain the exactly stipulated blood volume form these children. Overall, we found that sufficient PBMC could be obtained from 3.8 mls of blood in African paediatric population and hence more studies in how immune responses in children responds to various interventions could possibly be conducted in African settings.

**Conclusion**

The study of cellular immune responses amongst African paediatric population remained to be challenging due to difficulties in obtaining enough blood samples. However, children are most vulnerable population for many infectious diseases. Securing adequate information on how their immune functions responds against different infections is critical for development of interventions such as drugs and vaccine for children population. Vaccine studies including malaria vaccines are conducted in paediatric population residing in malaria endemic areas, and it is hoped that results
from these studies will highlight the possibilities of involving children as the target population on validating the information obtained in previous clinical trial phases conducted in adults regarding Ag-specific CD4 and CD8 T cells responses.

**Author Contributions**
MM drafted the manuscript and performed the statistical analysis. AT, TL, GM and TR collected the data and performed analyses. CD conceived and supervised the study. All authors contributed to writing and review of the manuscript. All authors read and approved the final manuscript.

**Abbreviations**
CSP: Plasmodium falciparum circumsporozoite protein; ATP: according to protocol; IgG: Immunoglobulin G; LN: Liquid nitrogen; IFN-g: Interferon gamma; IL2: Interleukin 2; CD: Cluster of differentiation; PBMC: Peripheral blood mononuclear cells

**Competing interests**
This study was funded by PATH-MVI and GlaxoSmithKline Biologicals SA. Authors report no conflicts of interest other than study funding.

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References


CHAPTER 5

Controlled Human malaria infections as a tool to accelerate malaria vaccine development in Africa and to dissect malaria specific immunity
PAPER V

Controlled Human Malaria Infection of Tanzanians by Intradermal Injection of Aseptic, Purified, Cryopreserved *Plasmodium falciparum* Sporozoites

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Controlled Human Malaria Infection of Tanzanians by Intradermal Injection of Aseptic, Purified, Cryopreserved *Plasmodium falciparum* Sporozoites


Ifakara Health Institute, Bagamoyo Research and Training Centre, Bagamoyo, Tanzania; Sanaria Inc., Rockville, Maryland; Protein Potential LLC, Rockville, Maryland; Swiss Tropical and Public Health Institute, Basel, Switzerland; University of Basel, Switzerland; Radboud University Nijmegen Medical Center, Department of Medical Microbiology, Nijmegen, The Netherlands; Howard Hughes Medical Institute and Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland; Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania

Abstract. Controlled human malaria infection (CHMI) by mosquito bite has been used to assess anti-malaria interventions in > 1,500 volunteers since development of methods for infecting mosquitoes by feeding on *Plasmodium falciparum* (Pf) gametocyte cultures. Such CHMIs have never been used in Africa. Aseptic, purified, cryopreserved Pf sporozoites, PISPZ Challenge, were used to infect Dutch volunteers by intradermal injection. We conducted a double-blind, placebo-controlled trial to assess safety and infectivity of PISPZ Challenge in adult male Tanzanians. Volunteers were injected intradermally with 10,000 (N = 12) or 25,000 (N = 12) PISPZ or normal saline (N = 6). PISPZ Challenge was well tolerated and safe. Eleven of 12 and 10 of 11 subjects, who received 10,000 and 25,000 PISPZ respectively, developed parasitemia. In 10,000 versus 25,000 PISPZ groups geometric mean days from injection to Pf positivity by thick blood film was 15.4 versus 13.5 (P = 0.023). Alpha-thalassemia heterozygosity had no apparent effect on infectivity. PISPZ Challenge was safe, well tolerated, and infectious.

INTRODUCTION

Controlled human malaria infection (CHMI), intentional infection of subjects with malaria parasites, has been used for treating patients with syphilis1,2 and in research for nearly a century.3 Since the development in the 1980s of methods for infecting mosquitoes by feeding on *Plasmodium falciparum* (Pf) gametocyte cultures.4,4 CHMI has been used repeatedly and successfully in more than 1,500 volunteers in the United States and Europe.6–10 Africa suffers the most morbidity and mortality from malaria, and thus could benefit significantly by using CHMI to facilitate development of new vaccines, drugs, and diagnostics for malaria, and for understanding innate and acquired resistance to the parasites that cause malaria. However, until now such CHMIs had never been used in Africa.

There are a number of reasons why CHMI has not been established in Africa. One is that the phase 1 clinical trial facilities and teams necessary to safely and professionally carry out such trials have not been available until recently for such studies. A second reason is that from 1985 to 2010, all CHMI studies in which volunteers were infected with Pf sporozoites were conducted by exposure to the bites of Pf-infected *Anopheles* mosquitoes not native to Africa, and produced in high security facilities difficult to establish, run, and maintain in Africa. To address the first limitation, we established a phase 1 clinical trial center at the Ifakara Health Institute (IHI), Bagamoyo, Tanzania. At the same time it became possible to manufacture aseptic, purified, cryopreserved Pf sporozoites (PISPZ) that are highly infectious, a product called PISPZ Challenge.11–13 When young adult Dutch volunteers were injected intradermally (ID) with doses of 2,500, 10,000, or 25,000 PISPZ (divided into two 50 µL injections), five of six volunteers developed parasitemia in all three groups, and the time from injection to Pf positivity by thick blood smear was ~13 days in all three groups. Thus, there was infection, but no dose response, presumably because increasing the dose did not increase the numbers of sporozoites that exited the skin, entered the circulation, and invaded hepatocytes.12

To begin the process of understanding how to use PISPZ Challenge in Africans, we conducted a double-blind, placebo-controlled trial to assess the safety and infectivity of ID-administered PISPZ Challenge in 30 male, highly educated, Tanzanian residents of Dar es Salaam, Tanzania, who had had minimal exposure to Pf malaria during the previous 5 years. As a bridge to the Dutch study, one group received the regimen used in one of the Dutch groups: 5,000 PISPZ in 50 µL were injected ID into the deltoid area of both upper arms for a total of 10,000 PISPZ.12 In a second group we increased the dose to 25,000 PISPZ, and based on findings in murine model systems, which suggested ways to improve the efficiency of ID injections,17 we divided the total dosage into four injections of 6,250 PISPZ, each in 10 µL.

As in Dutch12 and British13 subjects, PISPZ Challenge was safe, well tolerated, and infectious in young adult Tanzanian males. In the bridging group the infection rate, but not the pre-patent period, was comparable to that observed for the same dose in young adult Dutch subjects. These findings provide the foundation for using CHMI with PISPZ Challenge to assess the protective efficacy of antimalarial vaccines and drugs in Africa.

MATERIALS AND METHODS

Study design and population. This single center, double-blind, randomized, controlled trial was conducted in Bagamoyo,
Tanzania between February and August 2012. Thirty healthy male volunteers 20 to 35 years of age were recruited from higher learning institutions in Dar es Salaam. Screening for eligibility took place at the newly established Bagamoyo Clinical Trial Unit (BCTU) of the Ifakara Health Institute (IHI). Volunteers were screened using predetermined inclusion and exclusion criteria based on clinical examinations and laboratory tests. These included medical history and physical examinations, and standard hematology, biochemistry, malaria, human immunodeficiency virus, hepatitis B and C, and sickle cell tests. In addition subjects were screened for α-thalassemia. In the initial screening α-thalassemia trait was an exclusion criterion because of a theoretical concern that these individuals might be less susceptible to Pf infection. As screening progressed it became clear that a substantial proportion of the local population was heterozygous for α-thalassemia so it would be important to include this population in the volunteer pool to understand if the heterozygous condition was more resistant to malaria infection by experimental challenge and pose an obstacle that would need to be addressed in future vaccine studies. Volunteers who indicated that they had not had an episode of documented malaria in the past 5 years were included. They also had malaria thick smears, and any subject who was positive was excluded.

Screening for α° and αα° α-thalassemia caused by deletions. One milliliter (1 mL) of venous blood was collected in EDTA tubes and stored at −80 °C. The DNA was extracted from 100 µL whole blood with ZR Genomic DNA-Tissue MiniPrep (ZymoResearch, Irvine, CA) according to manufacturer’s recommendations. We used primers to amplify the hem α2 globin gene as a control and the 3.7 kb, 4.2 kb, and 20.5 kb deletion junction fragments of the α-thalassemia variants that could be easily identified by size as described. The multiplex polymerase chain reaction (PCR) primers used were a 2/3del F, 3.7del/20.5del R, a2 R, 4.2del F, 4.2del R, and 20.5del F. The PCR reaction contained additionally 1× Q-solution 2.5 U HotStarTag DNA polymerase in supplied reaction buffer (Qiagen, Valencia, CA) and 100 ng of genomic DNA. Reactions were carried out on a thermal cycler (Gene Amp 2700, Applied Biosystems), with an initial 15-minute denaturation at 95°C, 38 cycles of 95°C for 20 seconds, 60°C for 20 seconds, 72°C for 90 seconds, and a final extension at 72°C for 5 minutes. Following amplification, 5 µL of product was electrophoresed through a 1.5% agarose gel with 0.6 µg/mL ethidium bromide in 1× TBE buffer at 7 volts/cm for 1 hour. The gel was visualized on an UV transilluminator. Resulting PCR products were purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced on both strands with the Big Dye Terminator v3.1 sequencing kit on an ABI 3130XL sequencer (Applied Biosystems). The Sequence reads were aligned against reference genes for hemoglobin alpha 1 and 2 (NCBI Gene ID: 3039 and 3040, GRCh37.p10) and polymorphisms were identified according to human dbSNP (Build 137). Sequence analysis was done by the Geneious 6.1.5 software package.

All volunteers gave written informed consent before screening and being enrolled in the study. The trial was performed in accordance with Good Clinical Practices, an Investigational New Drug (IND) application filed with the U.S. Food and Drug Administration (US FDA) (IND 14267), and an Investigational Medical Product Dossier (IMPD) filed with the Tanzanian Food and Drug Administration (TFDA). The protocol was approved by institutional review boards (IRBs) of the Ifakara Health Institute (IHI/IRB/No25) and National Institute for Medical Research Tanzania (NIMR/ HQ/R.8a/VolDX/1217), and the Ethikkommission beider Basel (EKBB), Basel, Switzerland (EKBB 319/11). The protocol was also approved by TFDA (Ref. No. CE.57/1804/A/50), and the trial was registered at ClinicalTrials.gov (NCT01540903).

Intervention and randomization. The intervention material was aseptic, purified, cryopreserved PISPZ (PISPZ Challenge) isolated from salivary glands of asexually reared mosquitoes. Details regarding the production, cryopreservation, quality control, potency, and CHMI have been described. The lot of PISPZ Challenge used in this study had been manufactured and then cryopreserved in liquid nitrogen vapor phase (LNVP) 12 months before administration. It was a different lot than the lot used in the Netherlands; however, it was the same lot that was used in Oxford in which 5 of 6 volunteers who received 2,500 PISPZ ID and 4 of 6 volunteers who received 7,500 PISPZ IM developed malaria. The results of the sporozoite membrane integrity (viability) and 6-day hepatocyte potency assay (potency) were similar to those for the previous trials (Supplemental Table 1). Thirty eligible volunteers were randomly allocated to the experimental (PISPZ Challenge) or control (normal saline) groups. Twenty-four volunteers received PISPZ Challenge and six received normal saline (BD Medical/Surgical, BD Pre-filled Normal Saline Flash Syringe, Columbus, Nebraska). The volunteers and clinicians were blinded as to whether the volunteers received PISPZ Challenge or normal saline.

CHMI. Administration of PISPZ Challenge and immediate follow-up. PISPZ Challenge was administered ID in two different dose groups. Twelve volunteers were inoculated with 10,000 PISPZ ID in two injection sites, one 50 µL injection in each deltoid, each injection containing 5,000 PISPZ. Twelve volunteers were inoculated with 25,000 PISPZ ID in four injection sites, two 10 µL injections in each deltoid, each injection containing 6,250 PISPZ. Three control volunteers were assigned to each of the two dose
Inoculation of PISPZ Challenge. Results were graded according to a predetermined table (Supplemental Table 2 adapted from FDA guidelines, http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm009177.pdf). Adverse events were divided into those that occurred during the first 5 days after inoculation of PISPZ Challenge, and were attributed to the administration of the study product, and those that occurred from Day 6 onward, and were attributed to Pf infection (malaria).

The possibility of cardiac damage was assessed, because a cardiac-related serious adverse event (SAE) had been reported in 2007 in the Netherlands in a volunteer who was immunized with an experimental Pf subunit vaccine, underwent CHMI by exposure to the bites of five Pf-infected mosquitoes, developed malaria, and was treated with an anti-malarial. In case symptoms or signs that could be related to a cardiac event developed, the event was reported, and those in that experimental group.

The volunteers were observed in the ward for 24 hours after administration of PISPZ Challenge and discharged to home. They were given diaries and thermometers for recording of adverse events and temperature. Results were graded according to a predetermined table (Supplemental Table 2 adapted from FDA guidelines, http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm009177.pdf). Adverse events were divided into those that occurred during the first 5 days after inoculation of PISPZ Challenge, and were attributed to the administration of the study product, and those that occurred from Day 6 onward, and were attributed to Pf infection (malaria).

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25,000 PfSPZ dose (N = 12), or control (N = 6) groups (Figure 1 and Table 1). In groups 1, 2, and the control group, 7, 1, and 2 volunteers were heterozygous for the α-thalassemia trait, respectively.

**Parasitemia. Thick blood smear.** Eleven volunteers who received 10,000 PfSPZ (Table 2A) and 10 volunteers who received 25,000 PfSPZ (Table 2B) developed parasitemia detected by thick blood smear and confirmed by qPCR. One of the other two subjects in the 25,000 PfSPZ group was treated for a false positive thick blood smear on Day 11 (qPCR was negative), and was eliminated from the analysis. The second other volunteer in the 25,000 PfSPZ group was treated for a false positive thick blood smear on Day 19; qPCR was negative throughout for this volunteer, and this volunteer was considered to have not developed parasitemia as no subject in the study developed parasitemia by qPCR after Day 16, and this volunteer was followed by qPCR through Day 19. Thus, 11 of 12 volunteers in the 10,000 PfSPZ group and 10 of 11 evaluable volunteers in the 25,000 PfSPZ group developed Pf parasitemia after ID injection of PfSPZ Challenge. Volunteers in the 10,000 PfSPZ group had a significantly different pre-patent period than in the 25,000 PfSPZ group (geometric mean [GM] of 15.4 and 13.5 days, Wilcoxon, \( P = 0.023 \)). The GM parasite densities were 8.9 and 7.0 parasites/mL blood, respectively, at the time of first thick smear positivity.

**qPCR.** Parasitemia was determined by qPCR on all samples collected after CHMI. The qPCR was performed retrospectively, after volunteers had been diagnosed and treated. The sensitivity of qPCR was considered to be 20 parasites/mL of blood. The qPCRs were first positive 9.0 to 16.0 days after inoculation of PfSPZ Challenge (Table 2). Consistent with thick blood smear results, the volunteers in the 10,000 PfSPZ group had a longer time to positive qPCR than those in the 25,000 PfSPZ group (GM of 12.6 and 11.1 days, respectively), however the difference did not reach the level of statistical significance (Wilcoxon, \( P = 0.076 \)). The GM parasite densities were 0.11 and 0.16 parasites/mL blood, respectively, at the time of first qPCR positivity.

Parasite growth was cyclical and similar in both dose groups (Figure 2). Using a previously described method,\(^25\) the parasite multiplication rate in the bloodstream could not be determined with confidence because of the high variability of the amplification dynamics among individual subjects. However, there did not appear to

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**Table 1.** Volunteer characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Control (normal saline)</th>
<th>Group 1 (10,000 PfSPZ)</th>
<th>Group 2 (25,000 PfSPZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td>N = 6</td>
<td>N = 12</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Age at screening (years)</td>
<td></td>
<td>Mean ± SD</td>
<td>25.7 ± 3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>25.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Min, max</td>
<td>21.5, 30.9</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td></td>
<td>Mean ± SD</td>
<td>20.7 ± 2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>19.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Min, max</td>
<td>19, 24</td>
</tr>
<tr>
<td>Height (cm)</td>
<td></td>
<td>Mean ± SD</td>
<td>168 ± 4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Min, max</td>
<td>164, 174</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td></td>
<td>Mean ± SD</td>
<td>58.4 ± 6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>58.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Min, max</td>
<td>51, 66</td>
</tr>
</tbody>
</table>

*BMI = body mass index.*
be a significant difference between the replication rate in the Tanzanians and the replication rate in the Dutch subjects.\(^2\)

Parasite genotypes. Parasites from the positive volunteers were genotyped at six polymorphic microsatellite loci to confirm that they were from PfSPZ Challenge (Pi NF54) and not acquired by natural exposure to infected mosquitoes in the Bagamoyo area. Parasites from all 21 volunteers had identical microsatellite signatures to Pf NF54 (Table 3), indicating that they were infected with the challenge strain and not locally transmitted Pf parasites. Later, on Days 67 and 89 after CHMI, two volunteers developed Pf malaria. These infections were also genotyped and both carried non-Pf NF54 alleles at all six microsatellite loci (Table 3), indicating that they were infected with naturally acquired, locally transmitted Pf parasites.

Effect of \(\alpha\)-thalassemia heterozygosity on parasitemia. The GM parasite density for volunteers who were heterozygous for \(\alpha\)-thalassemia and those who were non-\(\alpha\)-thalassemic in the 10,000 PfSPZ were not significantly different at the time of detection of parasites by microscopy, 11.1 versus 7.4 parasites/µL (GM) in non-\(\alpha\)-thalassemia volunteers. The GM time to blood slide positivity in the single \(\alpha\)-thalassemia heterozygous subject was no different from GM time to blood slide positivity in the non-\(\alpha\)-thalassemia subjects, 12.7 versus 13.6 days (Table 4).

Adverse events (AEs). Clinical adverse events. A summary of the number of volunteers in each group with AEs during the 28 days after injection of normal saline or PfSPZ Challenge is shown in Table 5. The occurrence of AEs was similar in all groups. Most AEs recorded after administration of PfSPZ were consistent with symptoms and/or signs associated with clinical malaria. Table 6 provides the total number of AEs that occurred during Days 0–28 broken down by Grade (1–3); 75% were Grade 1. Table 7 delineates the specific solicited and unsolicited AEs that occurred during Days 0–28 after injection of normal saline or PfSPZ Challenge. There were 63 AEs in groups 2 (10,000 PfSPZ) and 3 (25,000 PfSPZ) and only nine of the 63 AEs (14%) occurred during the first 5 days after injection of PfSPZ Challenge. There were no acute systemic allergic reactions after the injection. The most common study-related events were headache, malaise, fatigue, and arthralgia, all symptoms consistent with malaria. Surprisingly, saline control subjects who did not have malaria had these symptoms

### Table 2

<table>
<thead>
<tr>
<th>Volunteer code</th>
<th>Thick smear</th>
<th>qPCR</th>
<th>Parasite density at first day positive (Pf/µL)</th>
<th>Parasite density by qPCR at time of diagnosis by thick smear (Pf/µL)</th>
<th>Parasite density at diagnosis (Pf/µL)</th>
<th>Parasite density positive (day)</th>
<th>Pre-patent period (day)</th>
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<td>50041-20</td>
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<td>4.0</td>
<td>13.0</td>
<td>0.36</td>
<td>3.00</td>
<td>12.8</td>
<td>12.8</td>
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<tr>
<td>60008-20</td>
<td>12.8</td>
<td>4.0</td>
<td>10.5</td>
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<td>8.00</td>
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<td>12.7</td>
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<tr>
<td>60026-20</td>
<td>12.7</td>
<td>11.0</td>
<td>11.5</td>
<td>0.21</td>
<td>9.00</td>
<td>17.6</td>
<td>17.6</td>
</tr>
<tr>
<td>70001-20</td>
<td>14.2</td>
<td>80.0</td>
<td>11.0</td>
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<td>23.00</td>
<td>14.2</td>
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<tr>
<td>70014-20</td>
<td>15.8</td>
<td>21.0</td>
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<td>83.00</td>
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<td>70031-20</td>
<td>14.2</td>
<td>11.0</td>
<td>11.0</td>
<td>0.05</td>
<td>17.00</td>
<td>14.2</td>
<td>14.2</td>
</tr>
<tr>
<td>70044-20</td>
<td>17.6</td>
<td>6.0</td>
<td>15.0</td>
<td>0.07</td>
<td>6.00</td>
<td>17.6</td>
<td>17.6</td>
</tr>
<tr>
<td>90047-20</td>
<td>13.7</td>
<td>4.0</td>
<td>11.0</td>
<td>0.09</td>
<td>0.10</td>
<td>13.7</td>
<td>13.7</td>
</tr>
<tr>
<td>Geom. mean</td>
<td>15.4</td>
<td>8.9</td>
<td>12.6</td>
<td>0.11</td>
<td>4.10</td>
<td>15.4</td>
<td>15.4</td>
</tr>
<tr>
<td>No. of positives</td>
<td>11/12</td>
<td>11/12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{*}\)This volunteer was treated on Day 11 as a result of reporting of a positive thick blood smear. Review of this thick blood smear indicated it was negative, and all qPCR results on this volunteer were negative. Thus, this volunteer was considered as negative for Plasmodium falciparum infection for subsequent analyses, because no volunteers first became qPCR positive on Day 19 or later.

\(^{†}\)This volunteer was treated on Day 11 as a result of reporting of a positive thick blood smear. Review of this thick blood smear indicated it was negative, and all qPCR results on this volunteer were negative. Thus, it is not known if this volunteer would have developed Plasmodium falciparum infection, and this volunteer has been excluded from the analysis. Thus, 11 volunteers were considered to be in this group, and 10 were documented to have developed Plasmodium falciparum parasitemia.
at a frequency similar to that of subjects who received PfSPZ Challenge.

Clinical serious adverse events (SAEs)—all unrelated. There were two subjects who had unrelated SAEs in this study. The first SAE was in a 26-year-old volunteer who was diagnosed as having gastroenteritis/dysentery of grade 3 severity at the time of hospitalization, which was 33 days post administration of PfSPZ Challenge. The second SAE occurred in a 22-year-old volunteer who was admitted to the hospital and diagnosed with severe malaria (axillary temperature > 40°C, altered mental status, and smear positive) on day 89 after administration of PfSPZ Challenge (the volunteer was previously treated and successfully cleared of parasites on Day 14 post inoculation of PfSPZ Challenge). Parasites taken from this volunteer at the time of diagnosis of severe malaria were genotyped using microsatellite markers, and these parasites were shown not to be the Pf NF54 parasites in PfSPZ Challenge (see above, and Table 3). Both volunteers with SAEs recovered uneventfully.

Laboratory test abnormalities. Serum chemistries (glucose, creatinine, aspartate aminotransferase [AST], alanine aminotransferase [ALT], and bilirubin) and hematological parameters (e.g., hemoglobin, white blood cell count, platelet count) were assessed beginning on Day 5 after inoculation of normal
when assessed on Day 5 after inoculation of normal saline and PfSPZ Challenge, laboratory abnormalities were infrequent and self-limited and similar in incidence rate among all three groups (Supplemental Table 3). From Day 6 to Day 28 post inoculation the incidence rate of abnormalities was slightly increased, but the incidence rate of abnormalities was similar in normal saline controls and those who received PfSPZ Challenge (Supplemental Table 3).

**DISCUSSION**

In this study, we showed for the first time that inoculation of healthy, young adult, African males with aseptic, purified, cryopreserved *P. falciparum* sporozoites, a product called PfSPZ Challenge, was safe, well tolerated, and infective. In fact, the infection rates in the Tanzanians were as good, if not better than the infection rates in young adult Dutch with no previous exposure to malaria.12

Eleven of 12 subjects who received 10,000 PfSPZ ID in two divided 50 μL doses and 10 of 11 who received 25,000 PfSPZ ID in four divided 10 μL doses became parasitemic. In the Netherlands, 5 of 6 who received 10,000 and 5 of 6 who received 25,000 PfSPZ ID in two divided 50 μL doses became parasitemic.

However, the GM pre-patent period for the Tanzanians who received the 10,000 PfSPZ dosage regimen was 15.4 days, and the GM pre-patent period for the Dutch was 12.6 days ($P = 0.0192$, Wilcoxon 2-tailed). There are multiple possible explanations for the prolonged pre-patent period in the Tanzanians. One is that naturally acquired immunity reduced the number of parasites that invaded or fully developed in the liver, thereby reducing the numbers of parasites that were released from the liver and prolonging the pre-patent period. Another possibility is that the replication rate in the blood was reduced as a result of naturally acquired immunity or innate resistance, and that this reduced replication prolonged the pre-patent period. Unfortunately, the variability in the qPCR results did not allow for definitively determining if this was the case, but it appeared

**Table 3**

Microsatellite genotypes of Pf infections in volunteers after PfSPZ Challenge

<table>
<thead>
<tr>
<th>Strain or sample ID</th>
<th>Microsatellite Loci*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA controls</td>
<td>HB3</td>
</tr>
<tr>
<td></td>
<td>TA81</td>
</tr>
<tr>
<td></td>
<td>Poly a</td>
</tr>
<tr>
<td></td>
<td>PK2</td>
</tr>
<tr>
<td></td>
<td>TA40</td>
</tr>
<tr>
<td></td>
<td>ARA2</td>
</tr>
<tr>
<td></td>
<td>TA87</td>
</tr>
<tr>
<td>NSFP4</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>166</td>
</tr>
<tr>
<td></td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>94</td>
</tr>
</tbody>
</table>

**Table 4**

Pre-patent periods and parasite densities by microscopy by α-thalassemia status

<table>
<thead>
<tr>
<th>Group 1*</th>
<th>Pre-patent period (Days)</th>
<th>Parasite density at diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volunteer ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90047-20</td>
<td>13.74</td>
<td>4.0</td>
</tr>
<tr>
<td>70001-20</td>
<td>14.11</td>
<td>80.0</td>
</tr>
<tr>
<td>60026-20</td>
<td>12.71</td>
<td>11.0</td>
</tr>
<tr>
<td>70014-20</td>
<td>15.76</td>
<td>21.0</td>
</tr>
<tr>
<td>10002-20</td>
<td>18.64</td>
<td>5.0</td>
</tr>
<tr>
<td>30035-20</td>
<td>18.66</td>
<td>5.0</td>
</tr>
<tr>
<td>Geom. mean</td>
<td>15.43</td>
<td>11.1</td>
</tr>
<tr>
<td>No. positive</td>
<td>6/7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2*</th>
<th>Pre-patent period (Days)</th>
<th>Parasite density at diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volunteer ID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60051-20</td>
<td>12.72</td>
<td>4.0</td>
</tr>
<tr>
<td>30053-20</td>
<td>13.72</td>
<td>4.0</td>
</tr>
<tr>
<td>80058-20</td>
<td>13.68</td>
<td>15.0</td>
</tr>
<tr>
<td>20056-20</td>
<td>18.67</td>
<td>5.0</td>
</tr>
<tr>
<td>40068-20</td>
<td>13.44</td>
<td>7.0</td>
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<tr>
<td>30060-20</td>
<td>13.47</td>
<td>7.0</td>
</tr>
<tr>
<td>50050-20</td>
<td>12.72</td>
<td>7.0</td>
</tr>
<tr>
<td>60072-20</td>
<td>14.04</td>
<td>7.0</td>
</tr>
<tr>
<td>20070-20</td>
<td>12.62</td>
<td>13.0</td>
</tr>
<tr>
<td>20064-20</td>
<td>11.05</td>
<td>7.0</td>
</tr>
<tr>
<td>Geom. mean</td>
<td>12.72</td>
<td>13.59</td>
</tr>
<tr>
<td>No. positive</td>
<td>1/1</td>
<td>9/10</td>
</tr>
</tbody>
</table>

* The proportion of volunteers who were heterozygous for α-thalassemia trait was higher in the 10,000 PfSPZ group than in the 25,000 PfSPZ group (chi-squared, 2-tailed, $P = 0.001$). There were no statistically significant differences in infection rates, parasitemia, or parasite/mL blood between subjects heterozygous for α-thalassemia trait, and those who were not.
that the replication rate was similar to the 11.5-fold replication rate every 48 hours seen in the Dutch volunteers.1 Thus, we do not know why the pre-patent period was longer in the Tanzanians. However, because the replication rates seemed to be similar in the Dutch and Tanzanian subjects, we think that there may have been fewer merozoites released from the livers of the Tanzanians.

Interestingly, the GM pre-patent period in the group that received 25,000 PfSPZ was significantly different (13.5 days) than was the pre-patent period in the group that received 10,000 PfSPZ (15.4 days) \( (P = 0.023) \). This 2.5-day difference can only partially be accounted for by increasing the numbers of PfSPZ by 2.5-fold. It is likely that more efficient administration of the PISPZ contributed to the delay by increasing the number of sites of administration from two to four and decreasing the volume of the injections from 50 to 10 \( \mu \)L, as we have seen in mice.14

Hemoglobinopathies, disorders of hemoglobin structure, and production, are one of the most common monogenic disorders in humans. \( \alpha \)-thalassemia is a hemoglobinopathy resulting from deletion of one \( (\alpha-) \) or both \( (\alpha-) \) \( \alpha \) genes from chromosome 16. Its wide distribution in populations living in places like Tanzania, where malaria has been or still is present, has been hypothesized to result from protection against severe or lethal malaria.20 This study was not designed (i.e. powered) to be able to assess the differences in infection rates, pre-patent periods, and parasite densities between individuals with and without \( \alpha \)-thalassemia trait. Nonetheless, there was no indication of any differences between those carrying this trait and those who did not have it. This finding is consistent with a number of field studies that showed that individuals who were heterozygous or homozygous for \( \alpha \)-thalassemia had similar rates of asymptomatic parasitemia and similar manifestations of uncomplicated malaria as non-thalassemic individuals.17

Administration of PISPZ Challenge was extremely well tolerated. There were no serious adverse events. In fact, the incidence rate of AEs was the same in the control group that received normal saline as it was in the groups that received PISPZ Challenge (Tables 5–7). Very few of the AEs occurred during the first 5 days after inoculation of PISPZ Challenge, and thus very few were attributed to the injection of study product or malaria. Numbers in parenthesis occurred within the first 5 days after injection of study product.

## Table 5

<table>
<thead>
<tr>
<th>Number of volunteers with clinical adverse events, Days 0–28 post injection*</th>
<th>Saline control (N = 6)</th>
<th>Group 1 (10,000 PfSPZ) (N = 12)</th>
<th>Group 2 (25,000 PfSPZ) (N = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adverse events</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>(no. of volunteers, %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any adverse event</td>
<td>6 (100)</td>
<td>9 (75)</td>
<td>9 (75)</td>
</tr>
<tr>
<td>Serious adverse event</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Solicited adverse event</td>
<td>6 (100)</td>
<td>9 (75)</td>
<td>9 (75)</td>
</tr>
<tr>
<td>Unsolicited adverse event</td>
<td>2 (33)</td>
<td>6 (50)</td>
<td>2 (17)</td>
</tr>
</tbody>
</table>

* A volunteer was counted at most once within each event type.

## Table 6

<table>
<thead>
<tr>
<th>Number of clinical adverse events, Days 0–28 post-injection</th>
<th>Saline control</th>
<th>Group 1 (10,000 PfSPZ)</th>
<th>Group 2 (25,000 PfSPZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of AEs</td>
<td>22</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>Total solicited AEs</td>
<td>14</td>
<td>30</td>
<td>34</td>
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<tr>
<td>Grade 1</td>
<td>14</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td>Grade 2</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Grade 3</td>
<td>2*</td>
<td>1†</td>
<td>0</td>
</tr>
<tr>
<td>Total unsolicited AEs</td>
<td>9</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Grade 1</td>
<td>9</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Grade 2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

** An adverse effect (AE) was recorded more than once if it resolved and subsequently reappeared during the 28-day interval.

† Severe rash and pruritis (each grade 3) in a volunteer beginning 10 days post-injection.

Studies that showed the incidence of AEs was the same in the control group that received normal saline as it was in the groups that received PISPZ Challenge (Tables 5–7). Very few of the AEs occurred during the first 5 days after inoculation of PISPZ Challenge, and thus very few were attributed to the injection of PISPZ Challenge (Table 7). Most occurred during the period when the volunteers were being diagnosed with Pl parasitemia and being treated. However, even during this period the incidence rate was similar in the control and experimental groups. One of the striking findings was how few symptoms and signs attributable to Pl parasitemia were experienced by the subjects who developed malaria. In the Dutch study 9 of 18 volunteers reported fever, whereas only 2 of 24 (\( P = 0.004 \), Fisher’s exact test, 2-tailed) reported fever in this study. In the Netherlands 3 of 18 had chills, whereas in this study only 1 of 24 had chills (\( P = 0.068 \)). We assume this low level of symptoms and signs was attributable to naturally acquired or innate immunity, which ameliorated clinical manifestations. A panel of six polymorphic microsatellite loci was successfully used to identify challenge strain (Pl NF54) infections and differentiate them from naturally acquired \( P. falciparum \) parasites circulating in Bagamoyo. This technique has been previously shown to be highly effective at differentiating genetic variants of \( P. falciparum \) within countries and across
larger geographic regions. Here, we show that the assay is also sensitive enough to generate reliable multi-locus genotypes from infected blood with parasite densities as low as five parasites/L blood. Although microsatellites are useful in identifying the infecting strain, they do not provide information on genetic regions of vaccine-induced selection or escape following heterologous challenge. Future studies will include genome-wide characterization of breakthrough infections in PISPZ Vaccine trials using DNA microarrays and genome sequencing to assess cross-strain protection and identify loci under vaccine-induced immune selection. This approach may help to pinpoint the genetic regions encoding the key antigens responsible for driving strain-specific immune responses and inform the development of next-generation multivalent whole organism vaccines in the event that efficacy is found to be strain-specific.

In conclusion, we have established the foundation for using CHMI with PISPZ Challenge in Africa, where malaria is endemic. Improvements in administration, such as direct venous inoculation (DVI) (Mordmueller B, submitted), will soon be used in Bagamoyo in CHMI studies to assess the protective efficacy of the PISPZ Vaccine in Tanzanians.

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REFERENCES

**Supplemental Table 1**

Results of potency and sporozoite membrane integrity assays (SMIA) on the lot of PfSPZ Challenge used in the clinical trial in Bagamoyo, Tanzania.*

<table>
<thead>
<tr>
<th>Time point</th>
<th>Potency (no. of parasites expressing PfMSP-1/well)</th>
<th>% Viability (sporozoite membrane integrity assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>32.7 ± 1.5</td>
<td>98.2%</td>
</tr>
<tr>
<td>Release</td>
<td>29.3 ± 3.1</td>
<td>87.4% ± 5.9%</td>
</tr>
<tr>
<td>3 Month</td>
<td>27.3 ± 0.6</td>
<td>84.6% ± 1.9%</td>
</tr>
<tr>
<td>6 Month</td>
<td>26.7 ± 1.5</td>
<td>83.6% ± 5.5%</td>
</tr>
<tr>
<td>Post-last clinical dose–Oxford†</td>
<td>26.3 ± 2.5</td>
<td>86.3% ± 6.5%</td>
</tr>
<tr>
<td>12 Month</td>
<td>27.3 ± 0.6</td>
<td>86.2% ± 1.3%</td>
</tr>
<tr>
<td>Post-last clinical dose–Tanzania (18 month)</td>
<td>24.0 ± 1.7</td>
<td>81.7% ± 2.6%</td>
</tr>
</tbody>
</table>

*Fresh PfSPZ used for the lot of PfSPZ Challenge used in this clinical trial produced 10% more PfMSP-1-expressing parasites in this assay than did PfSPZ that had been cryopreserved for several days (Release). At 18 months, several weeks after inoculation of the last volunteers in Bagamoyo, the PfSPZ had a 17% reduction in the SMIA as compared with fresh PfSPZ. There was an 11% reduction in the results of the sporozoite membrane integrity of cryopreserved PfSPZ at the time of Release, as compared with fresh PfSPZ. At 18 months, several weeks after inoculation of the last volunteers in Bagamoyo, the PfSPZ had a 17% reduction in the SMIA as compared with fresh PfSPZ.

**Supplemental Table 2**

Normal ranges at BRTC Laboratory and corresponding modified FDA Guidelines for toxicity grading*.

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal Range</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (meq/l)</td>
<td>136–146</td>
<td>N/A</td>
<td>146–147</td>
<td>148–150</td>
<td>&gt; 150</td>
</tr>
<tr>
<td>Sodium (meq/l)</td>
<td>132–135</td>
<td>130–131</td>
<td>125–129</td>
<td>&lt; 125</td>
<td></td>
</tr>
<tr>
<td>Potassium (meq/l)</td>
<td>3.5–5</td>
<td>5.3–5.4</td>
<td>5.5–5.6</td>
<td>&lt; 5.6</td>
<td></td>
</tr>
<tr>
<td>Potassium (meq/l)</td>
<td>N/A</td>
<td>3.3–3.4</td>
<td>3.1–3.2</td>
<td>&lt; 3.1</td>
<td></td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>53–106</td>
<td>107–150</td>
<td>151–177</td>
<td>178–221</td>
<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>3.89–6.0</td>
<td>6.01–6.94</td>
<td>6.95–11.1</td>
<td>&gt; 11.1</td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>5–34</td>
<td>1.1–2.5 x ULN</td>
<td>2.6–5 x ULN</td>
<td>5.1–10 x ULN</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>0–55</td>
<td>1.1–1.5 x ULN</td>
<td>1.6–2.0 x ULN</td>
<td>3.0–3.0 x ULN</td>
<td></td>
</tr>
<tr>
<td>Bilirubin (μmol/L)</td>
<td>0–6.1</td>
<td>1.1–1.5 x ULN</td>
<td>1.6–2.0 x ULN</td>
<td>3.0–3.0 x ULN</td>
<td></td>
</tr>
<tr>
<td>Total white blood count x 1,000/μL</td>
<td>3.2–11.7</td>
<td>2.5–3.1</td>
<td>1.5–2.5</td>
<td>1.0–1.5</td>
<td></td>
</tr>
<tr>
<td>Neutrophil count x 1,000/μL</td>
<td>2.0–6.9</td>
<td>1.5–1.999</td>
<td>1.0–1.499</td>
<td>0.500–0.999</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte count x 1,000/μL</td>
<td>0.87–3.19</td>
<td>0.750–0.869</td>
<td>0.500–0.749</td>
<td>0.250–0.499</td>
<td></td>
</tr>
<tr>
<td>Eosinophil count x 1,000/μL</td>
<td>0.47</td>
<td>0.701–1.5</td>
<td>1.501–5.0</td>
<td>&gt; 5.0</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>12.9–18.3</td>
<td>12.5–13.5</td>
<td>10.5–12.4</td>
<td>8.5–10.4</td>
<td></td>
</tr>
<tr>
<td>Platelets x 1,000/μL</td>
<td>146–345</td>
<td>125–145</td>
<td>100–124</td>
<td>25–99</td>
<td></td>
</tr>
</tbody>
</table>


**Supplemental Table 3**

Laboratory abnormalities that developed after administration of normal saline or PfSPZ Challenge from Day 5 through Day 28 post inoculation.

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal saline controls (N = 30)</th>
<th>10,000 PfSPZ (N = 12)</th>
<th>25,000 PfSPZ (N = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased AST</td>
<td>6 (2), 14 (2)</td>
<td>9 (1), 9 (2), 12 (1), 12 (2)</td>
<td>5 (3), 14 (3), 15 (1), 18 (2)</td>
</tr>
<tr>
<td>Increased ALT</td>
<td>14 (3), 15 (1)</td>
<td>9 (2), 12 (1)</td>
<td>5 (2), 14 (2), 15 (1)</td>
</tr>
<tr>
<td>Increased Bilirubin</td>
<td>18 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
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*Each entry represents a specific, different laboratory abnormality.
1° For those designated “†”, the abnormality occurred, resolved, and recurred.
AE = adverse event; AST = aspartate aminotransferase; ALT = alanine aminotransferase.

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PAPER VI

Impact of malaria pre-exposure on anti-parasite cellular and humoral immune responses after controlled human malaria infection

Impact of Malaria Preexposure on Antiparasite Cellular and Humoral Immune Responses after Controlled Human Malaria Infection

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To understand the effect of previous malaria exposure on antiparasite immune responses is important for developing successful immunization strategies. Controlled human malaria infections (CHMIs) using cryopreserved Plasmodium falciparum sporozoites provide a unique opportunity to study differences in acquisition or recall of antimalaria immune responses in individuals from different transmission settings and genetic backgrounds. In this study, we compared antiparasite humoral and cellular immune responses in two cohorts of malaria-naive Dutch volunteers and Tanzanians from an area of low malarial endemicity, who were subjected to the identical CHMI protocol by intradermal injection of P. falciparum sporozoites. Samples from both trials were analyzed in parallel in a single center to ensure direct comparability of immunological outcomes. Within the Tanzanian cohort, we distinguished one group with moderate levels of preexisting antibodies to asexual P. falciparum lysate and another that, based on P. falciparum serology, resembled the malaria-naive Dutch cohort. Positive P. falciparum serology at baseline was associated with a lower parasite density at first detection by quantitative PCR (qPCR) after CHMI than that for Tanzanian volunteers with negative serology. Post-CHMI, both Tanzanian groups showed a stronger increase in anti-P. falciparum antibody titers than Dutch volunteers, indicating similar levels of B-cell memory independent of serology. In contrast to the Dutch, Tanzanians failed to increase P. falciparum-specific in vitro recall gamma interferon (IFN-γ) production after CHMI, and innate IFN-γ responses were lower in P. falciparum lysate-seropositive individuals than in seronegative individuals. In conclusion, positive P. falciparum lysate serology can be used to identify individuals with better parasite control but weaker IFN-γ responses in circulating lymphocytes, which may help to stratify volunteers in future CHMI trials in areas where malaria is endemic.

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tionary volunteers, resulting in similar infection rates (24). As a follow-up, we here present results of the malaria-specific humoral and cellular immune responses in Tanzanian and Dutch volunteers who were inoculated intradermally with the same number of live P. falciparum PFSPZs during these CHMI studies.

MATERIALS AND METHODS

Human ethics statement. The Dutch trial (23) was approved by the Central Committee for Research Involving Human Subjects of The Netherlands (NL31858.091.10) and registered at Clinicaltrials.gov, identifier NCT 01086917. The Tanzanian trial (24) was approved by institutional review boards of the Ikaraka Health Institute (IHI/IRR/No25), the National Institute for Medical Research Tanzania (NIMR/HQ/R.Ra/Vol.IX/1217), the Ethikkommission beider Basel (EKKB), Basel, Switzerland (EKBB 319/11), and the Tanzanian Food and Drug Administration (reference no. CE.57/180/04A(150)) and registered at Clinicaltrials.gov, identifier NCT 01540903. All study teams complied with the Declaration of Helsinki and good clinical practice, including monitoring of data, and all volunteers gave written informed consent.

Clinical trial design. Samples for immunological analysis were obtained from two CHMI trials (23, 24). The first trial, performed at Radboud University Medical Center, Nijmegen, The Netherlands, was composed of 18 healthy Dutch subjects between the ages of 19 and 30 years with no history of malaria. Any volunteer who was positive for P. falciparum serology or had resided in an area where malaria is endemic within the previous 6 months was excluded from the trial. Three groups (n = 6 per group) were infected by intradermal injections of 2,500, 10,000, or 25,000 cryopreserved PfSPZs (NF54 strain). By day 21, 15/18 volunteers had developed parasites detectable by positive blood thick smear (TS); 5/6 in each group (23). There were no differences in parasite densities at diagnosis between the three dose groups (23). For immunological analysis, nine P. falciparum-positive volunteers of the 10,000 (n = 4) and 25,000 (n = 5) PfSPZ dose groups were selected based on availability of plasma and peripheral blood mononuclear cells (PBMCs).

The second trial was carried out in Bagamoyo, Tanzania, with volunteers residing in Dar es Salaam (an area where malaria is hypendemic). Twenty-four males between 20 to 35 years of age were enrolled and confirmed to be free of parasites by real-time quantitative PCR (qPCR). Subjects with a self-reported history of clinical malaria in the previous 5 years were excluded. The volunteers were divided into two groups with 12 volunteers per group and infected by intradermal injections of either 10,000 or 25,000 cryopreserved PfSPZs (NF54 strain). A total of 21/24 became both qPCR and blood smear positive by day 21 after infection (24). The three P. falciparum-negative volunteers were excluded from analysis in the present study.

PBMCs, citrate anticoagulated plasma samples from Dutch volunteers, and serum samples from Tanzanian volunteers were collected and cryopreserved 1 day before challenge (pre-CHMI) and after treatment (post-CHMI; day 35 and day 28 after infection).

DNA extraction and qPCR analysis. A total of 5 μl Zap-Ogolobin II lytic reagent (Beckman Coulter) was added to 500 μl of EDTA blood, after which the samples were mixed and stored at −80°C.

DNA extraction and quantification of parasitemia by qPCR in the Dutch CHMI trial were performed in Nijmegen as described previously (25), with slight modifications. Briefly, after thawing, samples were spiked with murine white blood cells as an extraction control, and DNA was extracted with a MagnaPure LC isolation station. For detection of the extraction control and P. falciparum, primers for the murine albumin gene and P. falciparum 18R rRNA were used as described previously (25).

Additionally, the P. falciparum 18R rRNA TaqMan MGB probe AAG AAT TGG AGG GCA AGG−6-carboxyfluorescein (FAM) was used.

DNA extraction and qPCR in the Tanzanian trial were carried out at the Leiden University Medical Center, Leiden, The Netherlands, as described previously (26). Plasmodium hericius 1 (PhHV-1) was added to the isolation lysis buffer to serve as an internal control. For quantification of PhHV, the primers GCGCGAATTCGAGTTGATCGT and GCCGTTGCAAACGTACCAA and the probe Cy5–TTTTATGTGTCCGCCACCATC TTGATC were used.

P. falciparum (NF54 strain) standard curves for both qPCR assays were prepared in Nijmegen by titration of ring-stage-infected red blood cells (RBC) in uninfected human blood. The two qPCR assays in both sites were confirmed to yield the same results when quantifying the P. falciparum content in sequential samples from four CHMI volunteers.

Parasite material for immunological analysis. The P. falciparum NF54 strain used in both CHMI trials is the parental strain of the 3D7 clone (27). P. falciparum (NF54 strain) blood-stage parasites were cultured in RPMI 1640 containing 10% human A+ serum and a 5% hematocrit erythrocyte suspension in a semiautomated culture system and regularly screened for mycoplasma contamination. For in vitro stimulation assays, asynchronous parasites harvested at a parasitemia of approximately 10 to 20% were purified by centrifugation on a 63% Percoll density gradient to obtain mature asexual stages. This resulted in concentrations of parasitemia levels of about 80 to 90%, consisting of more than 95% schizonts/mature trophozoites. P. falciparum-infected RBC (PRBC) were washed twice in RPMI, cryopreserved in glycerol-containing freeze medium, and used upon thawing in stimulation assays. Mock-cultured uninfected erythrocytes (uRBC) were obtained similarly and served as the control.

P. falciparum lysates for enzyme-linked immunosorbent assay (ELISA) were prepared by extracting purified schizonts/mature trophozoites with 1% sodium deoxycholate and 2.5 μl phenylmethylsulfonyl fluoride protease inhibitor for 15 min at room temperature (RT).

Recombinant and synthetic proteins. Recombinant proteins of circumsporozoite protein (CSP) and liver-stage antigen 1 (LSA-1) were used to probe humoral responses toward preerythrocytic stages, while crude P. falciparum lysates were used to assess antibody reactivity toward blood stages. Apical membrane protein 1 (AMA-1) and exported protein 1 (EXP-1) are expressed in both preerythrocytic and asexual stages.

Full-length P. falciparum NF54 CSP with repeats was produced in Escherichia coli by Gennova Biopharmaceuticals Ltd., Pune, India. A recombinant LSA-1 construct, LSA-NRC, was expressed in E. coli, incorporating the N- and C-terminal regions of the protein and two of the centrally placed 17-aminocidic repeats for the 3D7 LSA-1 sequence (PlasmoDB–PF3D7_1036400) (28). Both the N- and C-terminal regions as well as the repeats are highly conserved between NF54 and 3D7. The major difference is the greater number of repeats, which are the primary target of anti-LSA-1 antibodies (29), in the Nf54 sequence than in the 3D7 sequence (30). Amino acids 25 to 545 of codon-optimized AMA-1 of the P. falciparum FVO strain were expressed in the methylotrophic yeast Pichia pastoris (31, 32). A peptide covering the C-terminal amino acids 73 to 162 of the integral parasitophorous vacuolar membrane protein EXP-1 (Swiss-Prot database primary accession number P04926) was chemically synthesized using solid-phase 9-fluorenylmethoxy carbonyl (Fmoc) chemistry and differs from the 3D7 sequence only by a single amino acid in position 160 (33).

ELISA to assess antibody reactivity. Ninety-six-well PolyStyrene flat-bottom plates (Nunc MaxiSorp; Thermo Scientific) were coated with 2 μg/ml of CSP, EXP-1, and AMA-1, 0.25 μg/ml of LSA-1, or P. falciparum lysate at the equivalent of 20,000 PRBC/well in phosphate-buffered saline (PBS) and incubated overnight at 4°C. Plates were blocked with 5% milk in PBS. All of the following washing steps were carried out with PBS–0.05% Tween (PBST). Using 1% milk in PBST, plasma or serum samples were serially diluted in duplicate starting at 1:50 to 1:800 for protein antigen and 1:250 to 1:4,000 for P. falciparum lysate and incubated for 3 h at room temperature. Bound IgG was detected using horseradish peroxidase (HRP)–conjugated anti-human IgG (Thermo Scientific; diluted 1:50,000 in sample buffer). Plates were developed using tetramethylbenzidine (TMB) peroxidase substrate (tebu-bio). The reaction was stopped using an equal volume of 0.2 M H2SO4, and absorbance was measured with a spectrophotometer plate reader at 450 nm (Anthos 2001 ELISA plate reader).
A serial dilution of a pool of sera from 100 hyperimmune Tanzanian individuals (HIT) (20) individuals living in an area where malaria is highly endemic was used as a reference standard and was included on each plate. The reactivity for each antigen in undiluted HIT serum was defined as 100 arbitrary units (AU). Optical density (OD) values were converted into AUs by using the four-parameter logistic curve fit using the Auditable Data Analysis and Management System for ELISA (ADAMSV-111, http://www.malarialresearch.eu/content/software).

For each antigen, all time points of an individual volunteer were assayed on the same plate. To determine whether Tanzanians had a positive P. falciparum serology (by recognition of P. falciparum lysate), the mean (+ 2 standard deviations [SD]) baseline antibody titer against P. falciparum lysate of the Dutch volunteers was used as the cutoff for positivity.

In vitro PBMC stimulation assay to assess cellular responses. Venous whole blood was collected into citrated Vacutainer CPT cell preparation tubes (Becton Dickinson). PBMCs were obtained by density gradient centrifugation, washed three times in cold PBS, counted, frozen at 10^7 cells/ml in fetal calf serum (FCS) with 10% dimethyl sulfoxide, and stored in vapor-phase nitrogen. After being thawed, PBMCs were counted and cultured at a concentration of 500,000 cells/well in a 96-well round-bottom plate and stimulated in duplicate at a ratio of 1:2 with 10^6 P. falciparum NF54-infected RBC or uRBC for either 24 h or 6 days in a total volume of 200 μl.

Flow cytometry. Cells were stained and analyzed by flow cytometry either directly ex vivo or after 24 h or 6 days in vitro stimulation. Cells were stained first for viability with LIVE/DEAD fixable Aquia dead cell stain (Invitrogen) or fixable viability dye eFluor 780 (eBioscience) and later with three different staining panels for surface markers for the 24-h stain, CD3 PerCP (UCH1; BioLegend), CD56-phycocerythrin (PE) (HCDC56; Biolegend), anti-T-cell receptor (TCR) Pan γδ/PE (IMMU150; Beckman Coulter), CD4 Pacific Blue (OKT4; Beckman Coulter), CD8 allophycocyanin (APC)-H7 (SK1; BD Pharmingen), CD45RO energy coupled dye (ECD) (UCH1; Beckman Coulter), and CD62L PeCy7 (DREG56; eBioscience); for the ex vivo stain, the only changes from the previous stain were CD3 V500 (clone SP342; BD Horizon) and CD8 PerCP (RPA-T8; BioLegend); and for the third-panel 6-day stain, CD3 PeCy7 (OKT3; BioLegend) and CD8 PerCP (RPA-T8; BioLegend). For intracellular staining, cells were incubated with different monoclonal antibodies (MAbs) depending on the staining panels. After 30 min of incubation at RT, cells were washed and permeabilized with FixxX fix/perm buffer (eBioscience) for 30 min on ice and stained in permeabilization buffer (eBioscience) with IFN-γ fluoroscein isothiocyanate (FITC) (4S.3B; eBioscience; 24-h stain) and FixxX eFluor660 (PCH101; eBioscience; ex vivo stain) or Ke67 FITC (R56; BD Pharmingen) and FixxX eFluor660 (PCH101; eBioscience; 6-day stain). Cells were collected on a CyAn ADP 9-color flow cytometer (Dako/Beckman Coulter) and analyzed using FlowJo software (Tree Star, Inc.) version 9.6. All assays were conducted with the same batch of PBIRBC and uRBC, with all time points of one volunteer assayed in one experiment to prevent interassay variations. Natural killer T cells (NKT) and gamma delta T cells (γδT) were analyzed in the same gate and henceforth are referred to as NKT-γδT cells.

Statistical analysis. Statistical analysis was performed in GraphPad Prism 5. Differences within the cohorts and between time points were analyzed per volunteer by a Wilcoxon matched-pairs signed-rank test and those between groups were analyzed by a Mann-Whitney U test. The relationship between baseline antibody titers and the increase in antibody titers was analyzed by Spearman correlation, and P values of <0.05 were considered statistically significant. Cellular responses were corrected for the background by subtracting responses to uRBC from responses to PBIRBC for each sample; resulting negative values were set to zero.

RESULTS

Tanzanian volunteers have higher baseline antibody titers than Dutch subjects. Pre-CHMI antibody titers were significantly higher in Tanzanian than in the malaria-naive Dutch volunteers for crude P. falciparum lysate (P < 0.03), with 12/21 Tanzanians having titers higher than the mean (+2 SD) titers of Dutch volunteers. This was also true for pre-CHMI antibodies to the individual parasite antigens AMA-1 (P < 0.015; 13/21) and CSP (P < 0.04; 15/21), and the same trend was found for EXP-1 (P < 0.06; 8/21) (Fig. 1A). Elevated LSA-1 antibody titers were found in 5/21 Tanzanians, but there was no significant difference between Dutch and Tanzanian groups at the group level (P < 0.16). Within the Tanzania cohort, there was a wide range of pre-CHMI antibody responses, with some volunteers showing only low responses, comparable to those of the Dutch cohort. To address whether there was a general division into high and low responders to malaria antigens, we stratified Tanzanian individuals based on their reactivity to P. falciparum lysate (containing a large number of late-liver- and blood-stage antigens) (Fig. 1B). Compared to their P. falciparum lysate-seronegative counterparts (n = 9), seropositive Tanzanians (n = 12) had significantly higher antibody titers against the cross-stage antigens AMA-1 (P = 0.005; 7.6-fold higher median titer) and EXP-1 (P = 0.04; 5.4-fold higher). The same trend was found for the sporozoite antigen CSP (P = 0.09; 2.4-fold higher) and the liver-stage antigen LSA-1 (P = 0.06; 1.7-fold) (Fig. 1C).

P. falciparum lysate seropositivity prior to CHMI is associated with reduced initial blood-stage parasitemia. We next assessed whether preexisting humoral responses might be associated with the control of parasites in Tanzanian volunteers. In line with stronger humoral responses in the Tanzanian cohort than in the Dutch cohort at baseline, Tanzanian volunteers became qPCR positive significantly later than the Dutch volunteers (Fig. 2A), with a median prepatent period of 11.0 days (interquartile range [IQR], 11.0 to 13.5) in Tanzanians and 10.0 days (9.5 to 11.0) in Dutch volunteers (P = 0.009). Similarly, prepatency by TS was also longer in Tanzanians (median [IQR], 13.7 days [12.75 to 16.7]) than in the Dutch (12.6 days [12.3 to 14]) (P = 0.035). The time between detection by qPCR and by TS was comparable for both cohorts (median [IQR] for Tanzanians, 2.6 days [2.2 to 3.15]; Dutch, 3.0 days [2.0 to 3.15]; P = 0.88), but Dutch volunteers had a significantly higher peak parasite density (median number of parasites/milliliter [IQR] for Tanzanians, 12,000 [6,800 to 15,000]; Dutch, 74,000 [26,000 to 190,000]; P = 0.02), possibly due to slight differences in the thick smear protocol between the two sites. Within the Tanzanian cohort, there was no significant difference in time to qPCR-detectable parasitemia between volunteers who were either P. falciparum lysate seropositive or seronegative at baseline (P = 0.16) (Fig. 2B), nor was there a difference in prepatency by TS (P = 0.41) or time between detection by qPCR and TS (P = 0.15). However, seropositive Tanzanian volunteers had a significantly lower parasite load at the time of first qPCR-detectable parasitemia than their seronegative counterparts (P = 0.033) (Fig. 2C). This difference remained evident, but became smaller, by the time the first peak in parasite load was reached (P = 0.05; Fig. 2D). Across all Tanzanian volunteers, pre-CHMI antibody titers for CSP (Pearson r = 0.45, P = 0.04), but no other antigens, correlated significantly with prepatency by qPCR (see Fig. S1 in the supplemental material).

P. falciparum-specific antibody responses are more efficiently increased in Tanzanians than in Dutch volunteers after CHMI. Post-CHMI, antibody responses increased significantly in the Tanzanian volunteers for P. falciparum lysate (P < 0.001; 15/21 with a >3-fold increase in titers), AMA-1 (P = 0.002; 6/21), EXP-1 (P < 0.0001; 14/21), LSA-1 (P = 0.001; 5/21), and CSP...
In contrast, the Dutch volunteers showed significant increased titers only against EXP-1 ($P = \text{0.001}$; 7/9), CSP ($P = \text{0.008}$; 6/9), and *P. falciparum* lysate ($P = \text{0.008}$; 3/9), while responses to LSA-1 and AMA-1 remained low and unaltered (Fig. 3B). Compared to the Dutch, Tanzanians showed a trend for stronger induction or boosting of responses based on the overall fold increase in titers to *P. falciparum* lysate, AMA-1, and LSA-1 (Fig. 3C) and significantly higher absolute increases in titers for these antigens (Fig. 3D). Volunteers in both cohorts were subjected to CHMI with either 10,000 or 25,000 PfSPZs. However, the only significant difference in antibody responses between the two dose groups was a slightly higher response in the 25,000 than in the 10,000 dose group for *P. falciparum* lysate in Dutch volunteers ($P = \text{0.04}$) and a similar trend for the Tanzanians for CSP ($P = \text{0.07}$) (see Fig. S2 in the supplemental material). Post-CHMI, *P. falciparum* lysate-seronegative Tanzanians had a significantly stronger fold increase in antibody titers against AMA-1 ($P = \text{0.02}$) and EXP-1 ($P = \text{0.04}$) than seropositive individuals, with a similar trend for *P. falciparum* lysate ($P = \text{0.082}$) and CSP ($P = \text{0.095}$), but no difference in LSA-1 responses ($P = \text{0.80}$) (Fig. 3E). For the entire cohort, the fold increase of *P. falciparum* lysate ($P = \text{0.01}$) and AMA-1 ($P = \text{0.001}$) responses upon CHMI correlated negatively with the baseline response. The absolute increase in titers, however, was not different for any of the antigens between the two

FIG 1 Baseline malaria-specific antibody titers indicate previous exposure in Tanzanian volunteers. Antibody reactivity against crude *P. falciparum* lysate and the *P. falciparum* antigens AMA-1, EXP-1, LSA-1, and CSP was tested prior to malaria infection. A pool of sera from 100 hyperimmune Tanzanians (HIT) was used as a reference. Reactivity for each antigen in undiluted HIT serum was set at 100 arbitrary units (AUs). (A) Responses between Dutch (D; circles; $n = \text{9}$) and Tanzanian (T; squares; $n = \text{21}$) cohorts were compared using a Mann-Whitney U test. (B) Tanzanian volunteers were stratified based on *P. falciparum* lysate recognition at baseline as Sero + ($n = \text{12}$; black box plots) or Sero − ($n = \text{9}$; white box plots), using the mean + 2 SD of Dutch volunteers (gray circles) as a cutoff for positivity. (C) Pre-CHMI responses of seropositive and seronegative Tanzanians were analyzed by ELISA for individual *P. falciparum* antigens and compared by Mann-Whitney U test. Scatter plots show individual data points, horizontal lines indicate the median of the group, and error bars indicate the interquartile range (IQR). Dashed lines indicate the mean + 2 SD of antibody titers in Dutch volunteers for each respective antigen.

FIG 2 Preexposure to malaria is associated with difference in prepatency and parasitemia after CHMI. Parasitemia after CHMI was determined by qPCR. The day of first parasite detection by qPCR after PfSPZ injection is shown for Dutch (D; gray circles; $n = \text{9}$) compared to Tanzanians (T; gray squares; $n = \text{21}$) (A) and for *P. falciparum*-seropositive ($n = \text{12}$; black squares) and -seronegative ($n = \text{9}$; white squares) Tanzanian volunteers (B). The parasite load (number of *P. falciparum* parasites per milliliter of blood) at the first day of qPCR-detectable parasitemia (C) and the time of first peak parasite density (D) are shown for *P. falciparum*-seropositive ($n = \text{12}$; black squares) and -seronegative ($n = \text{9}$; white squares) Tanzanian volunteers. Data are shown as median ± IQR. Groups were compared by a Mann-Whitney U test.
groups (Fig. 3F). Notably, *P. falciparum* lysate-seronegative Tanzanians thereby also showed a greater absolute increase in antibody titers than malaria-naïve Dutch volunteers.

Dutch but not Tanzanian volunteers show cellular recall responses induced by CHMI. To analyze the acquisition of cellular responses after CHMI, we investigated *in vitro* IFN-γ responses upon incubation with PfRBC for 24 h (Fig. 4A and B). At baseline, *P. falciparum*-specific IFN-γ responses were comparable between Dutch and Tanzanians for all cell subsets except for NKT-γδT cells, which showed slightly higher responses in the Dutch (*P* =...
Dutch but not Tanzanian volunteers show increased *P. falciparum*-specific *in vitro* IFN-γ production after CHMI. PBMCs collected pre- and post-CHMI from Dutch (D; circles; n = 9; post = 35 days after CHMI) and Tanzanian (T; squares; n = 21; post = 28 days after CHMI) volunteers were stimulated with PfRBC for 24 h. (A) After stimulation, cells were stained for surface expression of CD3, CD4, γδTCR, CD8, and CD56 to gate lymphocyte subsets. NKT and γδT were gated as a combined population. (B) Intracellular IFN-γ is shown for total lymphocytes after 24 h of uRBC or PfRBC stimulation. Graphs show the proportions of cells with *P. falciparum*-specific IFN-γ production, comparing Dutch and Tanzanian volunteers pre-CHMI (C) and post-CHMI (D), and comparing pre- and
FIG 5  P. falciparum-specific in vitro IFN-γ production by innate lymphocytes from Tanzanian volunteers is dependent on baseline serological status. PBMCs collected from Tanzanian volunteers who either had a positive P. falciparum (Sero+; n = 12; black squares) or negative P. falciparum (Sero--; n = 9; white squares) serology prior to challenge (baseline) were stimulated with PfRBC for 24 h and stained for intracellular IFN-γ. Panels show IFN-γ production by lymphocyte subsets: (A) CD4+ T cells; (B) CD8+ T cells; (C) NKT-γδT cells; (D) CD56dim; (E) CD56bright NK cells. Data are shown as median ± IQR of responses corrected for the background by subtracting responses to uRBC. Differences in responses between the seropositive and seronegative groups were analyzed by a Mann-Whitney U test. P. falciparum-specific IFN-γ responses of pre-CHMI PBMCs from malaria-naive Dutch volunteers (D; n = 9; gray circles), who had negative P. falciparum serology, are shown as a comparator.

0.077) (Fig. 4C). Post-CHMI, previously malaria-naive Dutch volunteers showed significant increases in P. falciparum-specific IFN-γ production by all lymphocyte subsets analyzed (CD4+, P = 0.004; CD8+, P = 0.009; CD56dim, P = 0.004; CD56bright NK, P = 0.008; NKT-γδT, P = 0.004) (Fig. 4E to I). In contrast, Tanzanian volunteers showed little or no increase in IFN-γ-producing cells (CD4+, P = 0.74; CD8+, P = 0.065; CD56dim, P = 0.49; CD56bright NK, P = 0.75; NKT-γδT, P = 0.92). In both cohorts and at both time points, CD56bright NK cells showed significantly higher IFN-γ responses than CD56dim NK cells (Dutch pre/post-CHMI, P = 0.004; Tanzanian pre/post-CHMI, P = 0.0001). Significantly increased IFN-γ responses were found in both the effector (CD4+, P = 0.004; CD8+, P = 0.008) and central (CD4+, P = 0.004; CD8+, P = 0.012) memory T-cell compartments in the Dutch cohort, while the Tanzanians showed no such increase (see Fig. S3 in the supplemental material). As a result, post-CHMI IFN-γ responses in the Dutch were significantly higher for all cell subsets than in the Tanzanian cohort (Fig. 4D). Within the Tanzanian cohort, there was an overall trend for higher P. falciparum-specific pre-CHMI IFN-γ responses in those volunteers that had particularly short prepatency by qPCR (see Fig. S1 in the supplemental material). CD8+ T cells from Dutch (P = 0.04) but not Tanzanian (P = 0.47) volunteers showed increased proliferative responses post-CHMI (see Fig. S4A in the supplemental material). The proliferation of CD4+ T cells in response to PfRBC was not significantly altered post-CHMI in either cohort (P = 0.25 in Dutch and P = 0.11 in Tanzanians) (see Fig. S4B).

Analyzing the lymphocyte subset compositions in both cohorts, we found that they were largely stable between pre- and post-CHMI time points, with the exception of a prominent and significant post-CHMI increase of the NKT-γδT proportion in the Dutch volunteers and a clear decrease of CD56dim NK proportions in both cohorts. There was further no significant difference in the proportions of CD4+ CD45RO Foxp3+ T cells between the two cohorts, which could have explained differences in responsiveness (see Table S1 in the supplemental material). For the Tanzanian cohort, we observed a significant reduction of Foxp3+ T cells post-CHMI (P = 0.008), while for the Dutch volunteers there was no change (P = 0.44). Stimulation with the phorbol myristate acetate (PMA) and ionomycin mitogens showed that lymphocytes of both Dutch and Tanzanian volunteers were fully functional and able to produce IFN-γ at comparable levels both pre- and post-CHMI (see Fig. S4C).

Reduced innate IFN-γ responses in Tanzanian volunteers are associated with preexisting humoral immunity and thus potential preexposure. Finally, we analyzed whether the degree of preexposure reflected by differences in malaria-specific humoral immunity might affect cellular recall responses to PfRBC. Cells from P. falciparum lysate-seronegative Tanzanian volunteers had significantly higher responses to PfRBC (pre-CHMI) than those from seropositive Tanzanian volunteers (NKT-γδT, P = 0.05; CD56dim, P = 0.01; CD56bright NK, P = 0.04) and were of a magnitude comparable to those observed in the Dutch volunteers (Fig. 5). CD4+ and CD8+ T cells showed a similar pattern, although the difference between seropositive and seronegative Tanzanians did not reach statistical significance (Fig. 5A and B). Post-CHMI, these differences remained, and there was no increase in these responses in either of the two groups.

DISCUSSION

In the present study, we performed a side-by-side comparison of antimalarial immune responses initiated or recalled by CHMI in two cohorts of young adults, from The Netherlands and Tanzania, with different malaria exposure histories and genetic backgrounds. Recruitment of Tanzanian volunteers took place in Dar es Salaam, an urban area where malaria is hypoendemic. Inclusion criteria into this CHMI trial were tailored to ensure minimal recent exposure to P. falciparum; Tanzanian volunteers had not experienced a clinical episode of malaria for 5 years, as self-reported, and were confirmed free of parasites by qPCR before CHMI took place. Nevertheless, more than 50% of the 21 Tanzanian volunteers had a positive P. falciparum lysate serology before CHMI.
based on *P. falciparum* lysate ELISA, which is a standard exclusion criterion in Dutch CHMI trials. In line with this, increased baseline antibody titers for the *P. falciparum* antigens CSP, LSA-1, EXP-1, and AMA-1 were found in the Tanzania cohort and particularly in those with positive serology for *P. falciparum* lysate. This clearly indicates previous exposure to the malaria parasite in this cohort. Asymptomatic infections, which can often occur in people living in areas with low malarial endemicity (34), in the 5 years of no self-reported clinical malaria preceding CHMI might have led to a maintenance of higher antibody responses.

As might be expected, preexisting antibodies to *P. falciparum* antigens appeared to have an effect on the outcome of CHMI. Tanzanians had a significantly longer prepatency based on qPCR detection than the previously malaria-naive Dutch. Furthermore, *P. falciparum* lysate-seropositive Tanzanians had a lower parasite load at the time of first detection by qPCR and at the time of the first peak of parasite load than seronegative Tanzanians. The first peak of blood-stage parasitemia can be used as a proxy for parasite load at the time of first detection than the previously malaria-naive Dutch. Furthermore, *P. falciparum* lysate-seropositive Tanzanians had a lower parasite load at the time of first detection by qPCR and at the time of the first peak of parasite load than seronegative Tanzanians. The first peak of blood-stage parasitemia can be used as a proxy for parasite liver load (16, 35). Our results might therefore indicate the emergence of fewer parasites from the liver and hence better control of liver load (35). Our results might therefore indicate the emergence of fewer parasites from the liver and hence better control of liver load (16, 35).

Within the Tanzanian cohort, *P. falciparum* lysate-seropositive Tanzanians showed a similar absolute and accordingly much greater fold increase in antibody titers than their seropositive counterparts. At baseline, these volunteers had significantly lower responses to most parasite antigens than their seropositive counterparts and largely resembled the malaria-naive Dutch cohort. The fact that this group showed a greater increase in antibody titers than the Dutch strongly suggests that despite largely negative *P. falciparum* serology, these individuals have a stable *P. falciparum*-specific memory B-cell repertoire (40, 41). Given the same increase in absolute antibody titers, their memory B-cell repertoire appears to be of a magnitude similar to that of the *P. falciparum* lysate-seropositive Tanzanians, despite lower circulating plasma antibody levels.

Of note, humoral responses were assessed using a number of recombinant or synthetic proteins, which are not fully identical to the sequences of these proteins expressed by the NF54 strain used in both CHMI trials. While most antigens used in this study were of the 3D7 sequence and thus closely resemble the NF54 sequence, AMA-1 responses were assessed using the FVO strain sequence. AMA-1 is known for its extensive antigenic polymorphism and to cause strain-specific immunity (42, 43). Nevertheless, there is a significant antigenic overlap between AMA-1 alleles allowing for cross-reactivity across different strains, both in terms of functional activity and recognition, which affects mainly the magnitude of the response (42, 44-46). Based on these data, we consider it unlikely that assessment of AMA-1 responses using the NF54 or 3D7 sequence would have yielded different qualitative results. However, absolute titers would likely have been higher when using the AMA-1 sequence homologous to the CHMI strain.

Another potential confounder is the fact that the *P. falciparum* strains that Tanzanian volunteers were naturally exposed to prior to CHMI with *P. falciparum* NF54 are unknown. As any study examining preexposed individuals, analysis of antigen-specific responses against polymorphic antigens has therefore the additional limitation that it is difficult to match this unknown exposure history. This has to especially be taken into account when investigating CHMI-induced boosting of potential preexisting responses in this cohort, which might be masked by using antigens of different strain origins. However, despite the fact that Tanzanians likely experienced exposure to a variety of *P. falciparum* strains prior to CHMI, there was (i) a clear division into seropositive and seronegative individuals not just by total *P. falciparum* NF54 lysate but also by all individual antigens analyzed and (ii) a clearly stronger increase in antibody titers in Tanzanians than in malaria-naive Dutch volunteers to several antigens, including AMA-1. We cannot exclude, however, that this might have even been more pronounced when using antigens of different strain origins for analysis.

Although the Tanzanian volunteers in the present study showed evidence of humoral immune memory, parasite-specific IFN-γ production by adaptive T-cell subsets was not higher than in malaria-naive Dutch volunteers at baseline and remained unchanged after CHMI. In contrast, previously naive Dutch volunteers showed a significant increase in IFN-γ production by CD4 and CD8 T-cell subsets after a primary infection, consistent with
what has been shown previously (47, 48). The lack of increased proliferative and Th1 responses 1 month after CHMI in Tanzanian volunteers could be partially due to immunosuppression following exposure to blood-stage parasites during CHMI. Such T-cell immunosuppression is well described for malaria (49–58) and, although usually resolved within 2 weeks (52, 53), can persist for more than 4 weeks (50). That Dutch volunteers are not equally affected by this might be due to the fact that such immunosuppressive effects appear to be more pronounced in immune than in nonimmune donors, as shown elsewhere (59). T regulatory cells (Tregs) are one potential mediator of suppressed IFN-γ production by adaptive cells. Increased Treg numbers during or after malaria are a well-reported phenomenon (60), and malaria parasites can enhance the suppressive activity of Tregs (61). While Dutch and Tanzanian volunteers had similar Treg proportions, we cannot exclude that Tregs in Tanzanian volunteers might be functionally more active, potentially due to past priming in malaria infections. That this apparent lack of a Th1 immune response in the Tanzanian cohort is malaria specific is supported by the fact that Dutch and Tanzanian volunteers showed similar Th1 responses to mitogen stimulation both pre- and post-CHMI. Nevertheless, an additional influence of genetic background, which may explain differential P. falciparum-specific responses described in settings where malaria is endemic, cannot be excluded (62, 63).

NK cells are rapidly activated by malaria parasites, contribute to the early IFN-γ response during blood-stage infection (64), and can eliminate infected erythrocytes in vivo in a contact-dependent manner (65). Importantly, there is a functional dichotomy: the rarer CD56bright cells are more prominent in lymphatic tissues and are superior cytokine producers, while the CD56dim subset harbors a stronger cytotoxic potential (66–68). However, NK cells have usually been examined as one population, and the exact contributions of CD56dim and CD56bright NK cell subsets to malaria immunity thus remain to be established. An in vitro study on PBMCs from malaria-naive donors found greater IFN-γ production by CD56bright than by CD56dim NK cells only in response to cytokines but not upon PIRBC stimulation (69). However, consistent with their generally reported greater cytotoxic potential, only CD56dim cells showed degranulation upon PIRBC stimulation (69). Our findings that CD56bright NK cells show a greater IFN-γ response than CD56dim NK cells to PIRBC both before and after a primary malaria infection, as well as a memory-like effect of this response, are in line with findings from a previous CHMI trial (70). Tanzanian volunteers showed the same functional difference between the two NK cell subsets but no memory effect in either subset after CHMI. It was previously shown that depletion of αβ T cells abrogates memory-like IFN-γ responses of innate cells otherwise observed after CHMI (47, 70). Therefore, the absence of increased PIRBC-specific αβ T-cell responses post-CHMI in Tanzania volunteers might be one reason why P. falciparum-specific IFN-γ production by NK cells and other innate lymphocytes (NKT and γδ T cells) was also not increased post-CHMI. The fact that P. falciparum lysate-seronegative Tanzanians had higher innate IFN-γ responses than did seropositive Tanzanians already at baseline is a further indication that reduced Th1 responses are likely linked to the degree of previous malaria exposure. Of note, the proportion of CD56bright cells remained unaltered after CHMI, while the CD56dim subset had a smaller contribution to the PBMC compartment post-CHMI in both cohorts. It remains to be established whether this means that, in contrast to memory-like IFN-γ production, other NK cell functions, such as migration out of the circulation and engagement in antimalaria immune responses at other sites, such as the spleen, are unaffected and fully functional in preexposed individuals.

Within the Tanzanian cohort, there was no association of pre-existing P. falciparum-specific IFN-γ responses by T-cell subsets or innate lymphocytes with the parasitological outcome of CHMI, i.e., prepatency or parasite load at first detection by qPCR. If at all, those with higher IFN-γ responses to blood-stage PRBC had a shorter prepatency. This does not, however, exclude that cellular responses play a role in the prolonged prepatency of Tanzanian volunteers and specifically those with positive P. falciparum serology. One possible reason for the lack of such an association is that PRBC were chosen as a stimulus for P. falciparum-specific responses. This was done due to the relatively large antigenic overlap between blood-stage and (late) liver-stage parasites (71, 72). However, responses to sporozoite and early-liver-stage antigens, which may be more relevant when assessing responses responsible for reducing the parasite load by targeting the earlier stages of infection, are likely to be missed using PIRBC as a stimulus. Moreover, our analysis was restricted to P. falciparum-specific IFN-γ production, and it is likely that other responses, for instance, degranulation as a proxy for cytotoxicity, may be more relevant readouts (71). Finally, the only accessible compartment for analysis of cellular responses in these human trials was peripheral blood. P. falciparum-specific responses, and particularly those responsible for reducing liver infection, might, however, be enriched or primarily located in other sites, for instance, in tissue-resident memory cells in the liver (73, 74). This might be even more pronounced in preexposed individuals, where such a tissue-resident memory population might have already been established.

Noteworthy, the differential immune responses described herein may explain why Tanzanian volunteers reported fewer clinical symptoms, than their Dutch counterparts during CHMI after PISPZ injection (24). On the one hand, pre-existing antibody responses may reduce the initial parasite load and mediate antidisease immunity. Additionally, the relatively lower P. falciparum-specific cellular Th1 responses observed in the Tanzanian cohort than in the Dutch cohort might also be beneficial. Dutch volunteers preexposed to infected mosquito bites under chloroquine prophylaxis exhibited stronger IFN-γ production and earlier clinical symptoms when reexposed to blood-stage parasites than malaria-naive volunteers during their first infection (15). Thus, a shift away from Th1 responses in preexposed volunteers may make them less vulnerable to fever and other inflammation-induced symptoms.

In conclusion, our data show that previous malaria exposure is associated with some degree of parasite control during liver-stage or early-blood-stage infection after CHMI, humoral immune memory, and reduced antiparasite Th1 responses in the circulating lymphocyte compartment. Positive P. falciparum lyse serology can be used to identify individuals with better parasite control but weaker peripheral blood Th1 responses, which may help to stratify volunteers in future CHMI trials in areas where malaria is endemic. However, assessment of memory B-cell responses might be explored as a potentially better tool than serology to define preexposure per se. Important questions to be addressed in future studies include (i) which readouts other than Th1 responses should be used to determine immunization-induced cellular immunity and (ii) how differences in preexisting malaria-specific
immune responses affects the outcome of whole parasite immunity and vaccination approaches in areas where malaria is endemic.

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REFERENCES


PAPER VII:

Controlled human malaria infection boosts pre-erythrocytic stage transcending and merozoite opsonizing antibody responses in malaria semi-immune Tanzanian adults

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Controlled human malaria infection boosts pre-erythrocytic, stage transcending merozoite opsonizing antibody responses in malaria semi-immune Tanzanian adults

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Abstract

Background:
Controlled human malaria infections (CHMI) provide a unique platform for malaria research. Data describing CHMI-induced immune responses have been largely confined to malaria naive populations. Here, we assessed changes in malaria-specific humoral immune response in malaria pre-exposed healthy Tanzanian volunteers after a single intradermal application of purified, metabolically active *P. falciparum* sporozoites (*Pf*SPZ challenge).

Methods:
We compared side by side changes in serum antibody levels using malaria antigen specific multiplex bead arrays, antibody secreting memory B cell (MBC) responses and functional merozoite opsonization activity in each volunteer. We analyzed the results from pairs of visits using the Wilcoxon signed ranks test.

Results:
When compared to baseline, peak antibody titers binding to recombinant MSP-1 (alleles 3D7 and FVO), AMA-1, CSP and LSA-1 were observed on day 28 after *Pf*SPZ challenge. Antibodies binding to CelTOS, EBA-175 and SSP2 did not alter. MSP-1 and AMA-1 specific antigen secreting cells (ASCs) measured in peripheral blood where highest on day 28 after *Pf*SPZ challenge, but rapidly declined to near baseline levels on day 168. Merozoite opsonizing antibody responses increased following *Pf*SPZ challenge in some but not all individuals. When volunteers were stratified according to baseline opsonizing activity, low baseline responders showed significantly increase of merozoite opsonizing activity on days 28, 56 and 168 after *Pf*SPZ challenge, while no change occurred in the group with high opsonization at baseline. Outcomes of multiplex bead arrays, B-cell ELISpot analysis and merozoite opsonization assays in each volunteer followed similar trends after *Pf*SPZ challenge.
challenge, suggesting that boosting of pre-erythrocytic and asexual blood stage humoral immune responses can be assessed reliably by three assessment methods.

**Conclusion:**

*PfSPZ* challenge induces a significant albeit short lived boost in pre-erythrocytic and stage transcending specific antibody levels and ASCs. The changes in merozoite opsonizing antibodies were strongly influenced by pre-existing immunity, as only volunteers with a low level of opsonizing antibodies at baseline showed a consistent increase 6 months following the CHMI. CHMI studies in malaria pre-exposure volunteers paves the way towards a greater understanding of the immunological conditions that favor the development of lasting protective humoral malaria immunity in vaccine target population.

**Clinical trials**

Gov.identifier: NCT01540903

**Keywords**

*Plasmodium falciparum*, controlled human malaria infection, multiplex bead array, antibody secreting cells, merozoite opsonization, malaria semi-immune individuals
Background

Malaria is a preventable and treatable vector borne disease caused by parasite of the genus *Plasmodium*, with *P. falciparum* responsible for the majority of malaria morbidity and mortality in Sub-Saharan Africa [1]. The *P. falciparum* life cycle alternates between the female mosquito and the human host. In humans, after sporozoite inoculation, *P. falciparum* undergoes pre-erythrocytic liver stage development followed by the asexual blood stages, with parasites replicating in the red blood cells[1]. Asexual blood stage parasitemia is then followed by sexual stage development in the human host. After feeding on parasite-carrying humans, sexual replication occurs within the mosquito and is followed by sporozoite development in the salivary gland, and thus completes the cycle. The critical role of antibodies in protection against severe and clinical malaria in humans has been unequivocally established in passive transfer experiments[2][3]. Naturally acquired humoral immune responses in malaria have been described in many immuno-epidemiological studies [4][5]. Repeated malaria infections may result in clinical immunity that is hardly ever completely protective against infection. This phenomenon is multifactorial, due to both extensive *P. falciparum* antigen diversity and a failure to mount appropriately long-lived humoral responses[6,7]. Memory B cells (MBC), together with bone marrow resident long lived plasma cells, are thought to be the foundation of humoral immune memory after pathogen or vaccine exposure [8]. These B cells are responsible for the rapid, high titer, high affinity secondary antibody responses elicited upon re-exposure to the same infection or vaccine[9,10]. In malaria, several studies have demonstrated that MBC are present at low frequency following natural exposure, and that ‘atypical’ MBC that have been linked to impaired humoral immunity in HIV can be detected as well in malaria exposed volunteers[11,12].
CHMI has become the cornerstone of early clinical testing of potential malaria vaccine candidates, including the current most advanced malaria vaccine, RTS,S[13]. Until recently, CHMI studies were limited to few research institutions in the USA and Europe due to the requirement for rearing *P. falciparum* infected mosquitoes in the laboratory[14]. CHMI studies present an ideal setting to analyze cellular and humoral immunity to *P. falciparum* antigens since the infection occurs under highly defined conditions with well-known dosages and genotypes [14]. Recently, accessibility to cryo-preserved, metabolically active *P. falciparum* sporozoites has enabled the first CHMI studies to occur in malaria endemic countries [15]. To date, CHMI has been widely deployed to study immune responses against the pre-erythrocytic stage in both naïve and semi-immune population[16–21]. However, there remains no consensus on how immune responses against *P. falciparum* vary between diverse ethnic populations, and the impact of transient, low-density blood stage infections on malaria immunity has not been thoroughly investigated [22,23]. Questions like the impact of previous malaria exposure on infection dynamics, consequences of haemoglobinopathies on asexual blood stage development, infection dosing and application routes can now be addressed in malaria endemic settings through CHMI studies [22]. Here we describe the dynamics of humoral immune responses targeting pre-erythrocytic, stage transcending (MSP-1 and AMA-1) and asexual blood stage parasites using three complementary approaches after a single intradermal inoculation of purified, metabolically active *P. falciparum* sporozoites [21].
Material and methods

Ethics statement
The study was approved by the ethics committee of the National Institute of Medical Research, Tanzania (NIMR/HQ/R.8a/Vol.IX/1217), the ethics committee of Basel, Switzerland (EKBB 319/11) and the institutional review board of the Ifakara Health Institute (IHI) (IHI/IRB /No25). The study protocol was approved by the Tanzania Food and Drug Authority (TFDA) (Ref. No. CE.57/180/04A/50). The trial was registered at ClinicalTrials.gov (NCT01540903). Written informed consent was obtained from all participants. All the trial procedures were conducted by following the ICH-GCP guidelines for protection of human well-being.

Study participants
This study took place between February and August 2012 at the Ifakara Health Institute, Bagamoyo Research and Training Centre, located about 70 miles north from Dar-es-Salaam, Tanzania. Thirty participants, aged between 20 and 35 years old, were recruited from Dar-es-Salaam Institute of Technology (DIT), College of Business Education (CBE) and Institute of Finance management (IFM). These colleges enroll students from all over Tanzania. Participant inclusion and exclusion criteria and dose assignment for the groups have been explained elsewhere [21]. Volunteers who indicated to have no documented evidence of malaria episodes in the past 5 years were included. At enrollment, volunteers were tested for malaria infection using thick blood smear and only microscopy blood smear negative volunteers were enrolled in the trial [21].
Peripheral Blood Mononuclear Cell collection

Blood samples were drawn by venipuncture into sodium citrate-containing cell preparation tubes (BD, Vacutainer CPT) on days 0, 28, 56 and 168 after sporozoite inoculation. PBMC were isolated according to the manufacturer’s instructions, frozen in fetal bovine serum (FBS) (PAA laboratory GmbH) containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), and stored in liquid nitrogen (-191°C). PBMC samples collected at the different time points from one distinct subject were always thawed and assayed in parallel.

Serum and plasma sample collection

Serum was collected from 4 mL of whole blood in blood collection tubes with clot activators (Becton Dickinson, 369032) and allowed to stand at room temperature for 1 hour to facilitate clot formation. Blood collection tubes were then centrifuged at 2000 x g for 10 minutes at 22 °C, and aliquots of serum stored at -80°C. Plasma was obtained from BD Vacutainer CPT immediately prior to collection of PBMCs, aliquoted and stored at -80°C.

Antibody Luminex analysis

Nine recombinant proteins were coupled to Magplex magnetic microspheres (Luminex Corp., Austin, Texas) whose fluorescent signature was detected in ten different regions using xMAP® technology: Region 33: anti-Human IgG F(ab’2)(Sigma, Tres Cantos, Spain); Region 35: Bovine Serum Albumin (BSA); Region 36: Apical membrane antigen 1, 3D7 strain (AMA-1 3D7); Region 38: 42 kDa merozoite surface protein 1, 3D7 strain (MSP-1423D7); Region 42: 42 kDa merozoite surface protein 1, FVO strain (MSP-142FVO); Region 43: fragment II of region II of the 175 kDa erythrocyte binding protein (EBA-175, or Pf F2);
Region 44: Cell traversal protein for ookinetes and sporozoites (CelTOS); Region 45: liver-stage antigen 1 (LSA-1); Region 46: sporozoite surface protein 2 (SSP2); Region 52: circumsporozoite surface protein (CSP); AMA-1 3D7 strain and EBA-175 were produced at the International Centre for Genetic Engineering and Biotechnology, New Delhi, India (ICGEB). MSP-142 3D7 and FVO strains were produced at the Walter Reed Army Institute of Research, (WRAIR) Silver Spring, MD, USA. The pre-erythrocytic antigens CelTOS, LSA-1, SSP2 and CSP were produced at Protein Potential, LLC in Rockville, Maryland, USA.

The multiplex suspension array technology (SAT) panel was prepared as previously reported [23,24], with the following alteration: super-paramagnetic polystyrene 6.5 µm COOH-microspheres were coupled to corresponding antigens. Briefly, microspheres were washed and activated with Bead Activation Buffer (100mM monobasic sodium phosphate, pH 6.2) and simultaneous addition of Sulfo-NHS (N-hydroxysulfosuccinimide) and EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) at 50 mg/ml in a total volume of 100µL for 20 min. Microspheres were coupled overnight at 4ºC with optimized concentrations of corresponding protein in 500 µL PBS or MES reactions, then blocked with 500 µL PBS-BN (PBS with 1% BSA and 0.05% sodium azide), quantified and stored in multiplex aliquots at 1,000 microspheres/µL/region (or single-plex for region 33 anti-human IgG F(ab’)2 at 2,000 microspheres/µL) in PBS-BN, protected from light at 4ºC. An 11-point standard curve was used in each 96-well flat bottom mylar plate (Greiner One). A 2-fold serial dilution starting at 150 ng/mL ChromPure purified human IgG (Jackson Immuno Research Laboratories) was mixed with region 33 (anti-Human IgG) microspheres in single plex at 2,000 microspheres per well and placed for 1 h at room temperature at the same time that serum samples for the study were incubated with the multiplex microsphere mix at 1,000 microspheres per region per well. Study samples, positive and negative controls were tested
in duplicates at dilutions 1:500 and 1:20000. The plate was washed three times with 200 µL/well of PBS with 0.05% Tween20 (PBST) on a magnetic 96-well plate block. Anti-human IgG-biotin (Sigma, Tres Cantos, Spain) diluted 1:1000 in PBS-BN was added at 100 µL per well and incubated at room temperature with agitation and protection from light for 45 min. Plates were washed as before, and streptavidin-R-phycoerythrin diluted 1:1000 in PBS-BN was added at 100 µL per well and incubated for 25 min as before. Plates were washed as before and microspheres were resuspended in 100 µL of PBS-BN per well and read using a Luminex 200 instrument with xPONENT version 3.1 (Luminex Corp, Texas). At least 50 beads per analyte were acquired per sample. Crude median fluorescence intensity (MFI) and background fluorescence from blank wells were exported. A 5-parameter logistic regression with inverse variance weighting (1/MFI^2) was selected for the standard curve fitting due to superior fit with antibody data:

\[ y = A_{\text{max}} + \frac{A_{\text{min}} - A_{\text{max}}}{(1 + \left(\frac{x}{EC50}\right)^\beta)^\epsilon} \]

Where \( A_{\text{min}} \) is the lower asymptote, \( A_{\text{max}} \) is the upper asymptote, \( \beta \) is the slope at the inflection point of the sigmoidal curve, \( EC50 \) is the concentration at the inflection point, and \( \epsilon \) is a factor of asymmetry. The parameters of the standard curve in each plate were used to estimate the unit concentration (units relative to ng/mL IgG) for each analyte. The standard curve for each Luminex assay plate was used to normalize the estimated concentration of antibodies to each antigen, reported as antibody units (AU, or Units) per mL, through interpolation. Measurements above and below the limits of quantification were given arbitrary high and low Unit values beyond the dynamic range, respectively.
Memory B cell ELISpot analysis

*P. falciparum* antigen-specific ASCs were quantified by a modified version of the recently developed method [25]. Briefly, PBMCs were thawed and cultured with stimulation media consisting of 10%FBS in RPMI 1640, plus a cocktail of polyclonal activators: 20 ng/ml β-mercaptoethanol, 2.5 µg/ml CpG oligonucleotide ODN-2006 (Eurofins MWG/Operon, Huntsville, AL), 20ng/ml Protein A from *Staphylococcus aureus* Cowan (SAC) (Sigma-Aldrich, St. Louis, MO), 50ng/ml pokeweed mitogen (Sigma-Aldrich), and 50 ng/ml recombinant IL-10 (BD Biosciences) at 37 °C in 5% CO₂ atmosphere for six days. Afterwards, cells were collected, washed and distributed in 96-well ELISpot plates (Millipore Multiscreen HTS IP Sterile plate 0.45 µm, hydrophobic, high-protein binding). To detect ASCs, ELISpot plates (Millipore) were prepared by coating with either a 10 µg/ml solution of polyclonal goat antibodies specific for human IgG (Caltag H17000) to detect all IgG-secreting cells, a 1% solution of bovine serum albumin (BSA) as a non-specific protein control, a 5 µg/ml solution of MSP1 (1:1 mixture of FVO and 3D7 strains) or a 5 µg/ml solution of AMA-1 (3D7 strain only). Sources of these recombinant antigens are described in the Luminex section above. All Plates were then blocked using 1 % bovine serum albumin. Stimulated PBMCs were washed and tested in duplicate over five serial dilutions starting at 5x10⁵ cells/well and six serial dilutions starting at 4 x 10⁴ cells/well for the detection of antigen-specific ASCs and total IgG ASCs, respectively. After a six-hour incubation, plates were washed in PBS and 0.05% PBS-Tween20 (PBST), and incubated at 4°C overnight with a 1:1000 dilution of alkaline phosphatase-conjugated goat antibodies specific for human IgG [(gamma)-Invitrogen] in PBST. Plates were washed, developed with BCIP/NBT, thoroughly washed again with ddH2O and dried in the dark. ELISpot plates were quantified using an AID Elispot reader (Start V-spot reader spectrum) and reported as frequencies of ASCs per 10⁶ PBMCs. The limit of detection of the ASCs ELISpot assay for this analysis was five
ASCs per $10^6$ PBMC based on the mean of ASCs in the 1% BSA control. Assay failure was defined as fewer than 1000 IgG+ ASCs per $10^6$ PBMCs after the six-day culture.

**Merozoite Phagocytosis Assay**

Merozoite phagocytosis assays were performed as described previously [26,27], with minor alterations. Briefly, magnetically-purified schizonts were treated with E64 for 8 hours [28], and then passed through a 1.2-μm filter to release merozoites. Haemozoin was removed by magnetic separation, and purified merozoites were then stained with Ethidium Bromide (EtBr). Merozoites were incubated with the study participant’s plasma at 1:4000 dilutions and then combined with the THP-1 cells at a ratio 6:1. THP-1 mediated phagocytosis was arrested after 40 minutes. The percentage of EtBr fluorescent THP-1 cells was determined by flow cytometry (FacsCalibur, BD Sciences). Plasma pools from Australian and malaria hyper-immune adults collected in Papua New Guinea were used as non-exposed and hyper-immune controls, respectively.

**Statistical methods**

We displayed the log-transformed response variables using dot plots and scatter plots. We summarized the skewed outcomes using the median and 90% central range, and compared the results from pairs of visits using the Wilcoxon signed ranks test. We defined high and low responders using the median value as a cut-off. The analyses were carried out in R version 3.1.0.[29]
Results

Participants and study details

Thirty healthy male volunteers were recruited into this CHMI study. Two groups of 12 volunteers received 1x10^4 and 2x10^4 of live, purified, non-attenuated *P. falciparum* sporozoites (*Pf*SPZ challenge) intra-dermally, respectively. As a third group, six volunteers served as double-blinded placebo controls and were treated essentially the same except that they received saline instead of *Pf*SPZ challenge. The participants demographic information such as age, weight, height and BMI have been described[21]. Three participants that received *Pf*SPZ were excluded from the analysis presented here because they did not develop asexual blood stage parasitemia[21]. Remaining 27 participants were followed up for all scheduled visits at days 0, 9, 28, 56 and 168 except for another three participants for which one volunteer missed day 168 visit and two volunteers were excluded due to suspected clinical malaria episodes between study days 56 and 168.

Serum antibody levels binding to pre-erythrocytic and asexual blood stage antigens during CHMI

We assessed IgG antibody levels binding to known malaria antigens in serum samples collected at days 0, 9, 28, 56 and 168 using multiplex bead arrays. Antigens tested included liver stage antigen 1 (LSA-1), circumsporozoite protein (CSP), Cell-traversal protein for ookinetes and sporozoites (CeITOS), sporozoite surface protein 2 (SSP2), merozoite surface protein 1 (MSP-1 allele 3D7), MSP-1 (allele FVO), erythrocyte binding antigen 175 (EBA-175), and the apical membrane antigen 1 (AMA-1). At baseline, all participants had detectable antibodies against the CSP, MSP-1 and AMA-1. Four (14.8%) volunteers lacked detectable antibody responses against LSA-1 and CeITOS and three volunteers (11.1%) were negative for SSP2 (Figure 1).
Figure 1:

Multiplex-bead array based analysis of pre-erythrocytic, stage-transcending and asexual blood stage malaria antigen-specific serum antibody levels in *P.falciparum* sporozoites challenged (left column) and placebo controls (right column). Mean log 10 antibody unit (AU) after CHMI binding to (a) MSP-1, (allele 3D7), (b) MSP-1,(allele FVO), (c) AMA-1, (allele 3D7), (d) CSP, (e) LSA-1. Error bars indicate 95% confidence intervals of the mean. Solid circles: Volunteers infected with *P. falciparum* sporozoites, empty circles: saline control volunteers.
IgG antibody levels against MSP-1 (allele 3D7) (a), MSP-1 (allele FVO) (b), AMA-1 (allele 3D7) (c), CSP (d), LSA-1 (e) increased significantly, with the highest responses measured on day 28 after PfSPZ challenge (Figure 1, left column). Little variation in all malaria antigen specific humoral immune responses were measured over time in the placebo controls (Figure 1, right column). Antibody levels binding to EBA-175, CelTOS and SSP2 did not differ over the study period (Supplementary Figure 1). Although the inoculated sporozoites expressed the 3D7 allele of MSP-1, IgG responses to both MSP-1 alleles (3D7 and FVO) increased significantly (Table 1).

Relative changes of MSP-1 (alleles 3D7 and FVO), AMA-1, CSP and LSA-1 specific antibody levels from each individual’s baseline results are given in Table 1. Significant increases in relation to baseline were observed on days 28 and 56. On day 168, antibody levels targeting MSP-1 and CSP only remained significantly higher when compared to baseline, which was more pronounced for the 3D7 allelic form of MSP-1.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Day 28 vs 0</th>
<th>p-value</th>
<th>Day 56 vs 0</th>
<th>p-value</th>
<th>Day 168 vs 0</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP-1 (3D7)</td>
<td>32065 (-1407,1990947)</td>
<td>&lt;0.001</td>
<td>14039 (-794, 450948)</td>
<td>&lt;0.001</td>
<td>2372 (160994)</td>
<td>0.008</td>
</tr>
<tr>
<td>MSP-1 (FVO)</td>
<td>26743 (-1216,2059911)</td>
<td>&lt;0.001</td>
<td>13219 (-621,567900)</td>
<td>&lt;0.001</td>
<td>860 (9882,335375)</td>
<td>0.03</td>
</tr>
<tr>
<td>AMA-1</td>
<td>4523 (-15264,100269)</td>
<td>0.046</td>
<td>5244 (-11788, 70530)</td>
<td>0.03</td>
<td>31431,36785</td>
<td>0.70</td>
</tr>
<tr>
<td>CSP</td>
<td>475 (36,4400)</td>
<td>&lt;0.001</td>
<td>270 (-168,4759)</td>
<td>&lt;0.001</td>
<td>126 (-289,2890)</td>
<td>0.04</td>
</tr>
<tr>
<td>LSA-1</td>
<td>44 (-30,2001)</td>
<td>0.01</td>
<td>22 (-186,1543)</td>
<td>0.05</td>
<td>0 (-231,828)</td>
<td>0.94</td>
</tr>
</tbody>
</table>
Table 1:
Summary of differences in IgG responses (shown as AU) between time points days 28, 56 and 168 in relation to baseline of CHMI. The 90% central range is given in brackets. The p-values shown are obtained from the Wilcoxon matched-pairs signed-rank test.

**MSP1 and AMA1 specific memory B cell responses are boosted after intradermal PfSPZ challenge**

Next we used the B cell EliSpot analysis to follow frequencies of ASCs in peripheral blood [30]. At baseline, 10 out of 27 (37 %) and 12 out of 27(44%) participants had ASC against MSP-1 (both alleles were tested in combination) and AMA-1, respectively (range MSP-1: 1-60 spots/million PBMC and range AMA-1: 1-20 spots/million PBMC). The number of MSP-1 and AMA-1 specific ASCs cells was determined relative to the total number of IgG-producing cells measured in the same experiment (baseline range of total IgG-producing cells 1,250-25,988) as described in [30]. The number of MSP-1 and AMA-1 specific ASCs increased following CHMI and reached an observed peak at day 28, with a rapid decline on days 56 and 168 (Figures 2A and 2C). The number of MSP-1 and AMA-1 specific ASCs in the saline inoculated group remained largely unchanged over the time course followed (Figure 2B and 2D). Importantly, the total number of IgG-producing cells remained unchanged during the CHMI (Supplementary Figure 2).
Figure 2: MSP-1 and AMA-1 specific ASCs increase after intradermal PfSPZ challenge

The log of the proportion of MSP-1 specific ASCs(a) and AMA-1 specific ASCs(b) in PfSPZ challenge inoculated volunteers (closed circles) are shown. In Figure 2B and 2D, respective results obtained from placebo control volunteers are depicted (open circles).

The relative increases of MSP-1 and AMA-1 specific ASCs in comparison to baseline values are given in Table 2. Between baseline, days 28 and 56, statistically significant increases were observed for both antigens. On day 168, ASCs have returned to baseline levels, most likely reflecting a rapid decline of circulating ASCs frequencies.
Merozoite opsonizing antibodies increase following *Pf*SPZ challenge

Merozoite opsonizing antibodies have been demonstrated to be a robust correlate of natural acquired immunity to malaria[31]. Therefore, we assessed whether the increase of IgG antibodies binding to MSP-1 and AMA-1 following CHMI (measured by multiplex bead array and B cell EliSpot analysis) corresponded to an increase in functional merozoite opsonizing antibodies. Using a validated *in vitro* phagocytosis assay [26,27] (Supplementary Figure 3), merozoite opsonizing antibodies were observed in all individuals prior to CHMI - strongly suggesting prior malaria exposure (Figure 3). In the *Pf*SPZ challenged group, an increase in opsonizing antibodies were observed (Figure 3A) while the saline controls did not change over time (Figure 3B).

<table>
<thead>
<tr>
<th>Antibody secreting cells</th>
<th>Day 28 versus 0 p value</th>
<th>Day 56 versus 0 p value</th>
<th>Day 168 versus 0 p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log % MSP-1specific ASCs/total ASCs</td>
<td>0.18 (-0.003, 6.86)</td>
<td>0.068 (-0.01, 2.43)</td>
<td>0.001 (-0.13, 0.89)</td>
</tr>
<tr>
<td>Log % AMA-1specific ASCs/total ASCs</td>
<td>0.028 (-0.05, 0.35)</td>
<td>0.007 (-0.05, 0.16)</td>
<td>0.029 (-0.05, 0.16)</td>
</tr>
</tbody>
</table>

Table 2:

Relative increase in MSP-1 and AMA-1specific ASCs presented as the median of the differences between day 28, 56 and 168 and baseline. The 90% central range is given in brackets. The p-values are obtained from the Wilcoxon matched-pairs signed-rank test.
Figure 3: Opsonization of merozoites increases after intradermal PfSPZ challenge.
Increase in opsonizing antibody responses in (a) all *Pf*SPZ challenged volunteers post CHMI, (b) placebo controls (c) in *Pf*SPZ challenged volunteers with low baseline opsonizing activity (d) in *Pf*SPZ challenged volunteers with high baseline opsonizing activity.

Baseline levels of merozoite opsonizing antibodies were diverse in our volunteers. We investigated whether the baseline activity of opsonizing antibodies influenced subsequent responses to CHMI. Participants were grouped into high and low responders using the median value of the baseline as cut-off value. Participants with lower levels of opsonization activity at baseline showed strong induction of opsonization at 28, 56 and 168 days following sporozoite inoculation (Figure 3C). In contrast, individuals displaying high baseline opsonization activity showed no increase following CHMI (Figure 3D). To directly test whether the volunteers with low and high responses at baseline had different responses after CHMI, we used an interaction test. The increase in merozoite opsonization after CHMI was significantly greater for the baseline low versus high responders at day 28 (p=0.04) and day 56 (p=0.01). We found no correlation between baseline opsonization activity and parasite pre-patent period.
<table>
<thead>
<tr>
<th></th>
<th>Median difference (90% central range)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Volunteers combined</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 9 vs day 0</td>
<td>-1.7 (-7.4, 4.8)</td>
<td>0.12</td>
</tr>
<tr>
<td>Day 28 vs day 0</td>
<td>0.07 (-10.7, 40.5)</td>
<td>0.26</td>
</tr>
<tr>
<td>Day 56 vs day 0</td>
<td>0.5 (-6.5, 38.1)</td>
<td>0.12</td>
</tr>
<tr>
<td>Day 168 vs day 0</td>
<td>3.0 (-8.2, 32.6)</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Low baseline responders</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 9 vs day 0</td>
<td>-1.46 (-5.96, 5.96)</td>
<td>0.49</td>
</tr>
<tr>
<td>Day 28 vs day 0</td>
<td>17.04 (-1.5, 45.31)</td>
<td>0.01</td>
</tr>
<tr>
<td>Day 56 vs day 0</td>
<td>18.91 (-1.33, 40.18)</td>
<td>0.01</td>
</tr>
<tr>
<td>Day 168 vs day 0</td>
<td>12.05 (-0.93, 31.37)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>High baseline responders</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 9 vs day 0</td>
<td>-2.08 (-7.85, 4.36)</td>
<td>0.28</td>
</tr>
<tr>
<td>Day 28 vs day 0</td>
<td>0.07 (-12.89, 18.23)</td>
<td>0.58</td>
</tr>
<tr>
<td>Day 56 vs day 0</td>
<td>0.53 (-7.11, 19.36)</td>
<td>0.70</td>
</tr>
<tr>
<td>Day 168 vs day 0</td>
<td>3.46 (-9.73, 27.13)</td>
<td>0.70</td>
</tr>
</tbody>
</table>

**Table 3:**
Relative increase in merozoite opsonization activity presented as the median of the differences between day 28, 56 and 168 and baseline. The 90% central range is given in brackets. The p-values are obtained from the Wilcoxon matched-pairs signed-rank test.

**Comparison of malaria specific humoral immunity measurements after CHMI in individual volunteers**
The serum and PBMC samples collected after a defined challenge of a single *P. falciparum* strain enabled us to compare consequences for malaria specific humoral immunity using three different methods, namely multiplex bead array, B cell ELISpot analysis and in vitro merozoite opsonization assay. Interestingly, a high degree of concordance was observed between MSP-1(allele 3D7)ASCs, MSP-1(allele3D7)bead array and merozoite opsonization assays (Figure 4). For example, volunteer 10023 showed significant increases while volunteer 20064 remained low in all three measurement methods (Figure 4). AMA-1 specific responses were comparable to the MSP-1 specific humoral immune responses (Supplementary Figure 4A). Humoral immune responses in the control group showed little variation throughout the follow up period confirming that the alterations in antibody measures were induced by the *PfSPZ* challenge (Supplementary Figure 4B).
**Figure 4:**

Comparison of malaria antigen specific humoral immune responses upon *Pf*SPZ challenge in Tanzanian adults. Results from each volunteer are depicted. Red lines: Bead array-based detection of MSP-1 specific serum antibodies; Blue lines: B cell ELISpot analysis; Green lines: merozoite opsonization assay.
Discussion

We demonstrate here that a single intradermal inoculation with aseptic, purified, cryo-preserved non-attenuated sporozoites in semi-immune adult Tanzanian volunteers boosted pre-erythrocytic and stage-transcending malaria-specific humoral immunity. Volunteers participating in the CHMI were recruited from higher education institutions living in Dar es Salaam for reduced likelihood of heavy malaria pre-exposure. At enrollment, study participants with lowest CSP and EXP-1 specific serum antibodies antibody levels were selected from a larger pool of eligible volunteers. However, since anti-malarial antibodies were detected in baseline samples across the three monitoring techniques used here, these volunteers are unlikely to be malaria-naive. Those volunteers who became blood smear positive after CHMI were treated with a 3-day regimen of Artemether/lumefantrine (Coartem), and were discharged from the research hospital after three consecutive negative blood smear results. Retrospective analyses of blood samples collected during CHMI by the more sensitive quantitative PCR showed that subclinical, asexual blood-stage parasitemia was present for 2-3 days prior to drug treatment [21]. Therefore, the low-density asexual blood-stage infection experienced by CHMI participants likely explains the boost in stage-transcending and asexual blood stage immunity.

Here, we tested for antibodies to recombinant CSP and LSA-1 by multiplex bead array. Both antigens are exclusively expressed by sporozoites and liver-stage parasites. IgG antibodies to these antigens increased rapidly following CHMI, suggesting that inoculated sporozoites and developing liver stage parasites resulted in recall of pre-existing immunity. The increase in CSP and LSA-1 specific antibodies after CHMI are in line with results published by Obiero et al.[32], however, we included in our study a longer follow up period after PfSPZ challenge
PAPER VIII

Controlled human malaria infection leads to long-lasting changes in innate and innate-like lymphocyte populations

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Controlled Human Malaria Infection Leads to Long-Lasting Changes in Innate and Innate-like Lymphocyte Populations

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Animal model studies highlight the role of innate-like lymphocyte populations in the early inflammatory response and subsequent parasite control following Plasmodium infection. IFN-γ production by these lymphocytes likely plays a key role in the early control of the parasite and disease severity. Analyzing human innate-like T cell and NK cell responses following infection with Plasmodium has been challenging because the early stages of infection are clinically silent. To overcome this limitation, we examined blood samples from a controlled human malaria infection (CHMI) study in a Tanzanian cohort, in which volunteers underwent CHMI with a low or high dose of Plasmodium falciparum sporozoites. The CHMI differentially affected NK, NKT (invariant NKT), and mucosal-associated invariant T cell populations in a dose-dependent manner, resulting in an altered composition of this innate-like lymphocyte compartment. Although these innate-like responses are typically thought of as short-lived, we found that changes persisted for months after the infection was cleared, leading to significantly increased frequencies of mucosal-associated invariant T cells 6 mo postinfection. We used single-cell RNA sequencing and TCR αβ-chain usage analysis to define potential mechanisms for this expansion. These single-cell data suggest that this increase was mediated by homeostatic expansion-like mechanisms. Together, these data demonstrate that CHMI leads to previously unappreciated long-lasting alterations in the human innate-like lymphocyte compartment. We discuss the consequences of these changes for recurrent parasite infection and infection-associated pathologies and highlight the importance of considering host immunity and infection history for vaccine design.

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Infection with the apicomplexan parasite Plasmodium falciparum causes malaria and resulted in an estimated 214 million clinical cases of disease and 438,000 deaths globally in 2015 (1). Children are particularly vulnerable until the age of 5 y, when they become more resistant to severe forms of malaria. P. falciparum sporozoites (PfSPZs) are transmitted to the human host during feeding of infected female Anopheles mosquitoes. These sporozoites travel through blood vessels to the liver where they develop into the symptomatically silent pre-erythrocytic life cycle stage. Immunization with high numbers of attenuated PfSPZs can induce protection against malaria infection (2–7). Importantly, resistance to disease is rather short-lived due to the development of short-lived immunological memory (8). Over time, repeated exposure eventually leads to decreased disease symptoms; however, resistance against subsequent infections is limited. Pre-erythrocytic stage–specific protective immunity requires a combination of liver-resident adaptive cellular immune mechanisms, including cytotoxic CD8 T cells and CD4 T cells that secrete IFN-γ and provide help to sporozoite-neutralizing Ab-producing B cells (8, 9), but other immune processes may be of importance as well. Innate-like lymphocyte responses precede adaptive immune responses, and animal model data suggest that these early immune responses are critical for the outcome of subsequent adaptive immune responses (8).

Innate-like lymphocytes have been studied in a variety of infections because of their ability to rapidly secrete IFN-γ and TNF-α and other effector molecules that control early inflammatory responses and pathogen load (10, 11). These innate and innate-like
subsets include NK cells, NKT (invariant NKT [iNKT]) cells, and mucosal-associated invariant T (MAIT) cells. NK cells (CD3-CD56-) are abundant in human blood (~13% of lymphocytes) and liver (~31% of lymphocytes) (12). NK cell function is controlled by a balance of inhibitory (recognizing MHC class I) and activating signals, such as stress-induced ligands from the ULBP family (13). MAIT cells (CD3+ CD161hi Vα24+) make up 1–8% of T cells in blood and mucosal tissues and 20–45% of T cells in the liver (14, 15). The MAIT cell TCR consists of an invariant TCRα-chain (Vα27.2) and a limited range of possible β-chains (16, 17), but MAIT cells have recently been shown to have greater TCR heterogeneity than initially expected (18–20). The MAIT TCR recognizes Ag in the context of the MHC-related protein 1, which includes bacterial metabolites of the riboflavin synthesis pathway (21). We demonstrated that inflammatory and TCR signals synergize to induce MAIT cell effector function (22), although inflammatory signals can be sufficient to activate MAIT cells (23, 24). iNKT cell (CD5+ Vα24Jα18) abundance is substantially lower in humans compared with the mouse model system. iNKT cells typically account for ~0.02% of T cells in blood and ~0.5% of T cells in the liver (25). The iNKT cell TCR consists of an invariant TCRα chain (Vα24Jα18) and a limited range of possible β-chains (typically Vβ 11), and it recognizes lipids presented by the MHC class I-like protein CD1d (26).

A shared feature of these cell populations is their ability to quickly respond to inflammatory signals, such as IL-12, IL-15, and IL-18 (26, 27). A consequence of this inflammation-mediated activation is the acquisition of effector function, including expression of IFN-γ and granyme B (grzB) (23). IFN-γ is of particular interest because it seems critical in mediating immunity against the parasite (28–35). Importantly, although inflammation appears limited during the liver stage of malaria, the proinflammatory response is pronounced during the asexual blood stage, with an increase in IL-12 and IL-18 serum concentrations (36–39). Thus, the initial inflammatory environment elicited by the infection may be sufficient to activate these cell populations, leading to secretion of effector molecules, such as IFN-γ and TNF-α. Importantly, animal model data suggest that the early inflammatory environment, and particularly IFN-γ, seems to play an important role in the ensuing parasite control, as well as disease progression, including the development of cerebral malaria (10, 40, 41). It is unknown how accurately the mouse model mimics these innate-like lymphocyte responses in human infection, because to our knowledge, the function and fate of NK and innate-like T cells following Plasmodium infection of humans have not been elucidated in detail.

To address this question, we specifically examined changes in the NK cell, NKT cell, and MAIT cell populations using samples from a controlled human malaria infection (CHMI) study in a Tanzanian cohort of young adult males (age 20–35 y) exposed to aseptic P. falciparum (PfSPZ) challenge (42). We found that CHMI significantly affected the MAIT cell population frequencies and that changes in the MAIT cell population persisted for ≥168 d postinfection. To further interrogate these changes in MAIT cell frequency, we used a novel method to reconstruct TCR αβ-chain usage from single-cell RNA-sequencing (RNAseq) data. Analysis of this data suggests that the MAIT cell population rebound after the blood-stage parasitemia is driven by homeostatic expansion mechanisms. Together, these data demonstrate that CHMI induces long-lasting changes in the composition of the innate-like lymphocyte compartment, with the most pronounced changes occurring in the MAIT cell population. We discuss the implications of these data in the context of recurrent infection, pathology, and vaccine development.

Materials and Methods

Ethics statement

PBMCs analyzed in this study were collected during the CHMI trial conducted in Tanzania and described in Shekalaghe et al. (42). This CHMI was conducted as a single-center, randomized, double-blind controlled trial enrolling volunteers following predefined inclusion and exclusion criteria. All volunteers gave written informed consent before screening and being enrolled in the study, and the trial was performed in accordance with Good Clinical Practices, an investigational new drug application filed with the U.S. Food and Drug Administration (14267), and an Investigational Medical Product Dossier filed with the Tanzanian Food and Drug Administration. The protocol was approved by institutional review boards (IRBs) of the Ifakara Health Institute (IHI/IRB/No25), the National Institute for Medical Research, Tanzania (NIMR/HQ/R.8a/VI.12/171), and the Ethikkommission Beider Basel (Basel, Switzerland; EKBB 319/11). The protocol was also approved by the Tanzanian Food and Drug Administration (Reference Number CE.57/180/04/A/50), and the trial was registered at https://clinicaltrials.gov/ (NCT01549093).

Samples analyzed

Cryopreserved samples including only the participant identifier (ID; no personal or identifiable information) from this study were sent from the Ifakara Health Institute to the Fred Hutchinson Cancer Research Center for analysis with approval by the IRB (number 7349). In the 2.5 × 10^7 (25K) PfSPZ group, one volunteer had to be excluded from the analysis as a result of premature malaria treatment, and only volunteers who developed asexual blood-stage parasites after administration of Sanaria PfSPZ challenge were included in the analysis. Venous blood was drawn prior to the intradermal injection of PfSPZ challenge (day 0) and at subsequent time points (days 9, 28, 56, and 168) (42). Volunteers were monitored for onset of the asexual blood-stage parasitemia by examination of thick blood smears by microscopy and were treated with a curative dose of Coartem following national guidelines. A very limited number of samples were also available from blood drawn at the time of microscopy-based diagnosis of asexual blood-stage parasitemia. Importantly, although these volunteers were not malaria naive, they had not had a documented malaria episode in the past 5 y. PBMC samples were stored and shipped to the Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center for analysis by flow cytometry or RNAseq or were used for ex vivo–stimulation assays. Lymphocyte counts of volunteers participating in this CHMI were collected using a Sysmex XS-800i machine.

Flow cytometry

For the phenotypic identification of lymphocyte populations, bulk PBMCs or sorted MAIT cells were stained with Aqua Live/Dead Fixable Dead Cell Stain (Invitrogen). Our gating strategy and representative FACS plots are shown in Supplemental Fig. 2, and our four FACS analysis panels are shown in Supplemental Fig. 3.

Cell sorting

PBMCs were stained with the described sort panel and sorted on a FACSAria (BD) into complete RP10. Populations sorted were CD8+ MAIT (CD3+CD8+CD161hiVα7.2+). All sorts were performed on a FACSAria II.

Single-cell RNAseq processing, sequencing, and alignment

After flow sorting, single cells were captured on the Fluidigm C1 Single-Cell Auto Prep System (C1), lysed on a chip, and subjected to reverse transcription and cDNA amplification using the SMARTer Ultra Low Input RNA Kit for the Fluidigm C1 System (Clontech, Mountain View, CA). Sequencing libraries were prepared using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA), according to C1 protocols (Fluidigm). Barcoded libraries were pooled and quantified using a Qubit Fluorometer (Thermo Scientific, Life Technologies, Grand Island, NY). Single-read sequencing of the pooled libraries was carried out on a HiScanSQ or an HSeq2500 sequencer (Illumina) with 100-base reads, using Truseq v3 Cluster and SBS kits (Illumina) with a target depth ≥ 2.5 M reads. Sequences were aligned to the UCSC Human Genome Assembly version 38, and gene expression levels were quantified using RSEM and TPM values loaded into GSEA analyses. We used the MAST analysis platform (23) for subsequent single-cell analysis steps. Quality-control parameters for the single-cell RNAseq data included expression of ≥500 genes, alignment rate > 80%, library size > 10,000, and exon rate > 30% (all four criteria have to be met for a single cell to be included). MAIT cells were...
analyzed directly after sorting and were not stimulated prior to single-cell RNAseq processing.

**MAIT cell–stimulation assay**

A total of 2000–20,000 CD8+ MAIT cells was left untreated or were treated with IL-12 (eBioscience), IL-15 (eBioscience), and IL-18 (MBL) at 100 ng/ml or with a combination of the cytokines and anti-CD3/CD28-coupled beads (Invitrogen). At 20 h, GolgiPlug (BD) was added at a 1:1000 final dilution. For intracellular staining, cells were fixed with Cytofix/Cytoperm and permeabilized with Perm/Wash (both from BD), per the manufacturer’s instructions, and stained for intracellular cytokines. Samples were run on an LSR II (BD) and analyzed using FlowJo software (TreeStar).

**Statistical analysis**

Statistical significance comparing control and treated subjects was assessed using a Type II ANOVA for a linear mixed-effect model with patient-level random intercepts, with α level of 5%. R functions lmer and Anova in lme4 and car packages were used for the calculations. Comparison of time points with each other was done using a paired t test with FDR correction.

**Results**

*Innate-like lymphocyte frequencies change in a PfSPZ infection dose–dependent manner*

To study the innate-like lymphocyte response to PfSPZ infection, we analyzed samples from a trial conducted in African volunteers pre-exposed to malaria with intradermal application of $1 \times 10^4$ (10K) or 25K purified, cryopreserved nonattenuated PfSPZs ($n = 12$ each group). Eleven of twelve volunteers in the 10K PfSPZ group and 10 of 11 evaluable volunteers in the 25K PfSPZ group developed asexual blood-stage parasitemia after intradermal injection of PfSPZs (42). Blood was drawn prior to the intradermal challenge (day 0) and at subsequent time points (days 9, 28, 56, and 168) (42). Volunteers were monitored for the onset of the blood parasitemia stage by blood smear and were treated with Coartem following a positive blood smear to clear the infection. A very limited number of samples were also available from blood drawn at the time of blood parasitemia diagnosis. Importantly, although these volunteers were not malaria naive, they had not had a documented malaria episode in the past 5 y. Thus, the design of this study allowed us to determine how NK, iNKT, and MAIT cell function and frequencies change early (day 9) following CHMI and to longitudinally examine the duration of these changes in a cohort that is highly relevant for malaria vaccine efforts.

We initially determined whether an infection with 10K or 25K PfSPZs affects the composition of the innate-like lymphocyte compartment in the venous blood by flow cytometry. We examined the frequency of MAIT cells (CD3+Vα7.2+CD161hi), NK cells (CD3-CD56+), and iNKT cells (CD3+Vα24Jα18+) on days 0 and 9 postinfection and then after the Coartem treatment on days 28, 56, and 168. The gating strategies used and representative flow cytometry plots are shown in Supplemental Fig. 2.

We observed a significant increase in the frequency of MAIT cells within the CD8+ T cell population at 168 d postinfection.
(p = 0.0174) in the 25K-dose challenge group. Significant changes in the frequency of MAIT cells within the CD8+ T cell population following infection with the 10K dose were not observed (Fig. 1A, left versus right panels), indicating that MAIT cells respond in an infection dose–dependent manner. This was recapitulated when calculating the absolute number of CD8+ MAIT cells in the blood (Supplemental Fig. 1). Importantly, the absolute number of CD3+ T cells (cells per microliter blood) did not change significantly over time, indicating that this population serves as a suitable reference point to display T cell subset frequencies (Supplemental Fig. 1). We next examined the frequencies of NK cells and iNKT cells (Fig. 1B, 1C). NK cell frequencies in the lymphocyte population were reduced significantly on day 28 (p = 0.0005) and on day 56 (p = 0.0083) postinfection, particularly in the high challenge group (Fig. 1B). NK cell frequencies are shown as the percentage of the CD3+ lymphocyte population, but the same result is seen when analyzing NK cell numbers per microliter of blood (Supplemental Fig. 1C). In contrast to NK and MAIT cells, iNKT cell frequencies did not change significantly following the infection, regardless of the PISPZ dose (Fig. 1C). Given the changes in the MAIT cell population following CHMI in the 25K challenge group and considering their functional potential (IFN-γ production) and abundance in the liver (up to 40% of all T cells), we primarily focused on the more abundant CD8+ MAIT cell subset for the rest of the study to determine the consequences of CHMI on the MAIT cell population.

**MAIT cell frequencies change following PISPZ infection**

We examined the frequency of MAIT cells (CD3+Vα7.2+CD161hi; CD8+ and CD8− subsets) on days 0, 9, 28, 56, and 168 during the blood parasitemia stage and then after the Coartem treatment on days 28, 56, and 168. To better dissect the changes in the MAIT cell population, we looked at the frequency of all (CD8+ MAIT cells)

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**FIGURE 2.** MAIT cell frequency is altered following intradermal injection with PISPZs. Shown are changes in the frequency of MAIT cells over time for each individual volunteer. Significant changes are indicated with an asterisk (*), and p values were determined using a t test. **(A)** Percentage of MAIT cells in the CD3+ population in the blood in the 25K PISPZ group. Significant changes compared with day-0 baseline were observed at the blood parasitemia stage (p = 0.0302), on day 56 (p = 0.0022), and on day 168 (p = 0.0072). **(B)** Frequency of CD8+ MAIT cells and **(C)** within the CD3+ cell population. Statistical significance comparing control and treated subjects was done using a Type II ANOVA for a linear mixed-effect model. Comparison of time points with each other was done using a paired t test with FDR. Significant changes compared with day-0 baseline were observed for CD8+ MAIT cells at the blood parasitemia stage (p = 0.0278) and on day 168 (p = 0.0239). Significant changes were detected for CD8− MAIT cells on day 28 (p = 0.0003), day 56 (p = 0.0008), and day 168 (p = 0.0002). The yellow line in each graph represents the median. Volunteer IDs are listed, with each volunteer depicted in a distinct color.
and CD8\(^+\) MAIT cells within the T cell (CD3\(^+\)) compartment (Fig. 2A) and then subdivided the population into the CD8\(^+\) (Fig. 2B) and CD8\(^-\) (Fig. 2C) subsets. We found that the frequency of MAIT cells within the CD3\(^+\) population decreased significantly during the blood parasitemia stage \((p = 0.0302)\) and rebounded thereafter, with MAIT cell frequencies on day 168 exceeding the initial day-0 baseline level \((p = 0.0072, \text{Fig. } 2A)\). This pattern was also observed when the MAIT cell population was divided into CD8\(^-\) and CD8\(^+\) cell subsets (Fig. 2B, 2C). Importantly, availability of the blood-stage parasitemia samples was limited to this MAIT cell analysis experiment; thus, these samples could not be included in any subsequent experiments.

**MAIT cell activation occurs in a limited manner during the course of the infection**

Exposure to proinflammatory cytokines is sufficient to induce MAIT cell activation \((23, 24)\). Release of parasites from the liver into the bloodstream is associated with a proinflammatory immune response, which could activate MAIT cells. Thus, we first addressed whether there are signs of MAIT cell activation on day 9 postinfection. CD69 is an early marker of T cell activation and although an increase in CD69 surface expression is often used as an indicator of TCR signaling, inflammatory signals are also sufficient to induce expression \((43)\). We examined surface expression levels of CD69 and found no significant changes on day 9 or later (Fig. 3A). Similarly, the frequency of CD25\(^+\)- and CD40L\(^+\)-expressing MAIT cells did not increase significantly on days 9 and 28 following challenge with PfSPZs (Fig. 3B, 3C). CD25\(^+\) MAIT cells were significantly decreased on day 56 \((p = 0.0011)\) (Fig. 3B). Finally, NKG2D expression did not change significantly postinfection (Fig. 3D). Together, these flow cytometry data suggest that ex vivo MAIT cell activation is minimal at the interrogated time points.

**MAIT cell effector function is limited on day 9 postinfection**

To determine whether there is early (day 9) acquisition of effector function, we examined the ex vivo expression of TNF-\(\alpha\), IL-17, IFN-\(\gamma\), and grzB by intracellular cytokine staining and flow cytometry (Fig. 4). We found no evidence of significant ex vivo MAIT cell effector function on days 9, 28, 56, and 168 postinfection. We considered two possibilities for the lack of ex vivo effector function: a lack of activating inflammatory signals and parasite-induced impairment of function. To distinguish between these two possibilities, we next tested how MAIT cells isolated before and after blood-stage parasitemia respond to TCR and cytokine stimulation ex vivo.

**MAIT cells remain functional following infection with PfSPZs**

We isolated MAIT cells from five donors by FACS and examined whether their ability to respond to ex vivo stimulation was changed on days 9 and 28 postinfection compared with day 0. We measured the ability of MAIT cells to express IFN-\(\gamma\), grzB, and IL-17A/F.
following stimulation with proinflammatory cytokines (IL-12/15/18) or a combination of TCR signals delivered by anti-CD3/CD28–coated beads and IL-12/15/18. Regardless of the time point postinfection, MAIT cells responded to both stimulation conditions and almost uniformly expressed grzB (Fig. 5A) and IFN-γ (Fig. 5B). A much smaller fraction expressed IL-17A or IL-17F at all three time points (Fig. 5C, 5D). Together, these data suggest that MAIT cells remain responsive to TCR and cytokine–mediated stimulation on day 9 or 28 postinfection. To determine whether we can identify MAIT cell activation early postinfection, we next examined MAIT cells from one donor using single-cell RNA-seq.

Single-cell RNA-seq analysis of MAIT cells
We isolated MAIT cells by FACS from donor 20064 and used the C1 Fluidigm system to interrogate changes in gene-expression signatures on a single-cell level by comparing data from days 0, 9, and 28 after CHMI. Using an analysis method that we developed previously (23), we found that some, but not all, MAIT cells express grzB or IFN-γ transcripts on day 9 postinfection, but not on days 0 and 28 (Fig. 6A), suggesting that some MAIT cells are indeed partially activated on day 9 postinfection.

To acquire a more global understanding of the MAIT cell changes, we used the single-cell data to compare the transcriptome of these single cells at days 0, 9, and 28 postinfection. We found that the MAIT cell population displayed a distinct RNA-expression profile on each day (Fig. 6B), suggesting that they do respond to the PISPZ challenge by day 9 and do not return to baseline transcriptional status by day 28. To better understand the nature of these changes, we next reconstructed the TCRα and TCRβ sequences from the transcriptome and analyzed the CDR3 region to identify clonal diversity (Fig. 6C, Supplemental Fig. 4). We did not observe significant changes in the relative distribution of MAIT cell clones by day 28, suggesting that the expansion of MAIT cells is not dominated by a distinct TCR-bearing clonal population.

We did not observe significant changes in the expression of IL-7Rα and the cγ-chain (needed for signal transduction and shared with other cytokine receptors) on days 9 and 28 (Fig. 6D). Although IL-7 is typically associated with promoting homeostatic proliferation (44), recent studies suggest that IL-7 can induce MAIT cell cytotoxic function (45, 46). The increase in mRNA encoding for the cγ-chain on MAIT cells was confirmed at the protein level by flow cytometry staining (Fig. 6E). Given this potential change in responsiveness to cytokine signals, we next wanted to determine whether the expanded MAIT cell population is distinct, in terms of trafficking properties, from the preinfection MAIT cell population.

Traffic properties in MAIT cells
To determine whether the tissue-trafficking properties of MAIT cells change postinfection, we used FACS to examine chemokine receptor expression following CHMI. To specifically determine the liver- and tissue-homing potential of MAIT cells during CHMI, we analyzed the expression of CCR6, CXCR6, and CXCR3. Briefly, CCR6 is a key receptor to enable trafficking to inflamed tissues, and CXCR6 plays an essential role for trafficking
to the liver (47–49). The majority of MAIT cells expressed CCR6 and CXCR6 on their surface prior to CHMI. Overall, we found that expression of CCR6 and CXCR6 was fairly stable over time (Fig. 7A, 7B). Only a small fraction of MAIT cells expressed CXCR3, which has recently been shown to orchestrate migration of Ag-specific T cells (50). We observed an initial significant decrease (day 0 versus day 28, \( p = 0.0078 \)) in the frequency of CXCR3+ CD8+ MAIT cells that was followed by an increase over time that approached the initial expression frequency (Fig. 7C).

**Discussion**

CHMI is a powerful approach to study immune responses following defined exposure to *P. falciparum* in different human populations (51). We report that a single intradermal infection with aseptic, cryopreserved PfSPZs has profound long-lasting effects on the innate-like lymphocyte compartment in the peripheral blood of adult Tanzanian volunteers pre-exposed to malaria. To our knowledge, MAIT cells in malaria-naive volunteers undergoing similar CHMI approaches have not been studied (51). We observed a significant decrease in the frequency of blood-circulating MAIT cells following intradermal infection with 25K PfSPZs during early blood-stage parasitemia (days 11–18 post-infection). This was followed by a rebound in the frequency of MAIT cells that exceeded the initial baseline frequency and was sustained for months after the infection (Figs. 1, 2, Supplemental Fig. 1). The long-lasting (168-d) increase in MAIT cell frequency that we observed is remarkable, because an acute infection or vaccine application typically changes the innate immune system only temporarily before it returns to baseline. In contrast, long-lasting changes are seen in the adaptive immune system due to Ag-specific adaptive memory immune responses. This was elegantly demonstrated by Tsang and colleagues (52) on a systems biology level in the context of the influenza vaccine. Our data suggest that an acute infection with PfSPZ did not just result in a temporary immune perturbation but instead had a long-lasting impact on MAIT cells. Importantly, these changes occurred in an infection dose–dependent manner in volunteers infected with 25K, but not 10K, PfSPZs (Fig. 1). These two doses resulted in

![FIGURE 5](image-url)
*P. falciparum* infections with different geometric mean asexual erythrocytic stage parasitemia prepatent periods of 15.4 d (10K) and 13.5 d (25K) by microscopy (p = 0.023) and 12.2 d (10K) and 11.1 d (25K) by quantitative PCR (p = 0.076) (42). This kinetic difference is presumably due to the release of higher numbers of merozoites from the liver in the 25K PfSPZ group that resulted in a shorter prepatent period in comparison with the 10K PfSPZ group. Malaria treatment decision was based on the detection of asexual blood-stage parasites in the blood by microscopy. Given the difference in detection sensitivity between quantitative PCR and microscopy, the immune system had likely been exposed to asexual blood-stage parasites for ∼2.5–3 d in both groups before initiation of Coartem treatment. Based on this similarity in the duration of the blood-stage phase of infection in the two dosage groups, the infection dose–dependent differences that we observed (Fig. 1) are likely due to an increased inflammatory response in the liver following infection with 25K PfSPZ as opposed to a prolonged infection during the blood stage.

Interestingly, early after 25K PfSPZ infection, the NK cell frequency decreased significantly, similarly to MAIT cells (Fig. 1B), but then slowly (by day 168) returned to baseline levels. This decrease contrasts with data from an animal model demonstrating that NK cells in the blood expand robustly and early postinfection (40). Support for the notion that NK cells contribute to the antiparasite immune response comes from a recent study showing that human NK cell responses. Finally, iNKT cell frequencies were stable, regardless of the challenge dose (Fig. 1C). We argue that this is unexpected given the many similarities between iNKT and MAIT cells in terms of proinflammatory cytokine responsiveness (26). Future studies will need to address why MAIT cells, but not iNKT cells, are affected by CHMI.

To examine whether MAIT cell function is altered in addition to frequency, we next examined MAIT cells for expression of markers that indicate recent activation (CD69, CD25), as well as expression of cytokines (IL-17, TNF-α, IFN-γ, grzB). MAIT cells did not show a strong increase in expression of biomarkers that indicate activation at early or later time points (Fig. 3) or that displayed ex vivo effector function on day 9 or later postinfection.
Importantly, MAIT cells remained fully responsive to activating signals ex vivo (Fig. 5). These data demonstrate that MAIT cell function is not lost and argue against the existence of inhibitory signals that impair MAIT cell function following PfSPZ infection. This is in contrast to reports that suggest parasite-specific adaptive immune responses are impaired by the presence of asexual blood-stage parasites (11). Determining that MAIT cells remain functional is highly relevant, because mechanisms that activate MAIT cells in the liver could be of interest as a potential therapeutic strategy against the liver-stage disease. Because of their high abundance in the liver (up to 45% of T cells), MAIT cells may be involved in maintaining liver homeostasis during malaria parasite infections and might provide early antiparasite effector mediators (e.g., IFN-γ production) before the adaptive immune response is recruited and fully developed. The size of the MAIT cell population in the liver likely exceeds the size of the adaptive Ag-specific T cell population that could be induced by vaccination. In this context, it is also important to consider that MAIT cells have high expression levels of CCR6 and CXCR6, allowing them to traffic to the liver. Importantly, we found evidence of partial activation by single-cell RNAseq (Fig. 6A), suggesting that there are some activating, but yet unknown, stimuli early postinfection. It is noteworthy that, despite minimal initial activation, the MAIT cell transcriptome did not return to baseline in the time frame tested, because we still found transcriptional differences on day 28 postinfection (Fig. 6B). Future studies will need to examine the MAIT cell transcriptome at later time points postinfection to determine how long this functional alteration lasts.

Next, we used these single-cell RNAseq MAIT cell transcriptome data to reconstruct TCRα- and β-chain usage in the MAIT cell population on days 0, 9, and 28 from the same donor. Although MAIT cells express an invariant α-chain, there are differences in the CDR3 junctions that allowed us to track distinct MAIT cell clones (Fig. 6C). Given that we observed an increase in MAIT cell frequency starting by day 28 in an infection dose-dependent manner, we asked how the TCR αβ distribution changed at these three time points. We wanted to distinguish whether the increase on day 28 is due to a relocation of the same MAIT cell population back into circulation after its sharp decline on the day of parasitemia or whether it is indicative of replenishment of the MAIT cell population via homeostatic mechanisms or de novo generation from the thymus. Our single-cell RNAseq data did not reveal significant changes in the TCR repertoire among days 0, 9, and 28, indicative of an overall MAIT cell expansion rather than a clonotype-driven response (Fig. 6C). Interestingly, a previous study reported a sustained expansion of γδ T cells following a primary CHMI in naive volunteers, but it is
still unclear whether this expansion is polyclonal or clonally driven (51, 54). Importantly, in the context of the increased IL-7Rα and γc coexpression on day 28, we argue that the increase in the MAIT cell population is triggered by homeostatic expansion. Homeostatic expansion occurs in response to available growth factors and is typically driven by cytokines (e.g., IL-7 and IL-15) whose receptor uses the common γ-chain (55, 56). Increased responsiveness to IL-7 through enhanced IL-7Rα expression could be of specific interest because it also positively affects MAIT cell effector function (45, 46).

Given the temporary decrease in MAIT cell frequency in the blood of the 25K PfSPZ group, we considered that MAIT cells could migrate to other tissues or, alternatively, undergo apoptosis. A decrease in blood MAIT cell frequencies following infection is not unusual and has been reported in several other instances, including active tuberculosis (57) and HIV (58–60), although the fate of the MAIT cells in these situations has not been fully resolved. In an effort to determine whether migration may play a role in these MAIT cell changes, we examined the expression patterns of three highly relevant chemokine receptors for homing to the liver and other tissues: CCR6, CXCR6, and CXCR3 (Fig. 7). We did not find any significant differences in the expression pattern of CCR6 and CXCR6, with only a temporary decrease in CXCR3 expression early postinfection. This shows that MAIT cells remain responsive to chemotactic signals but does not provide insight into the relationship between blood and tissue MAIT cells. Ultimately, it would be necessary to examine human liver tissue to understand the dynamics of the MAIT cell response.

MAIT cells are rare in mice; however, a recently developed mouse model with MAIT cell frequencies that approximate those in humans opens new possibilities for studying MAIT cells in an animal model (61).

How biologically relevant are these changes in the MAIT cell and NK cell populations? NK cells play an important and non-redundant role in host defense against viruses, particularly herpesviruses. Although the frequency of NK cells eventually normalized again, it raises the question whether the PfSPZ-induced NK cell decrease may have a temporary general negative impact on host immunity, given the critical role of NK cells for protection against herpesvirus infections (62). This is particularly interesting in the context of EBV infections, because coinfection is a major risk factor for developing Burkitt lymphoma, and exposure to EBV may also exacerbate well-characterized pathologies that have been associated with excessive inflammatory responses. It is important to keep in mind that we studied the immune response following a defined single malaria infection event initiated by purified, cryopreserved sporozoites of a defined genotype, NF54, and treatment occurred upon detection of the blood stage of infection. Under natural conditions, humans are exposed to multiple infectious mosquito bites carrying a range of parasite genotypes, and treatment is typically not immediate. Thus, the well-defined CHMI approach allows us to study the immune response in the context of the liver stage and the early blood stage of infection, which will help to interpret data from more complex natural infection studies.

Changes in the initial immune response affect the ensuing adaptive immune response, which is of relevance in the context of vaccine efficacy. Our data demonstrate changes in the NK and MAIT cell compartment months after CHMI. Additional studies are now required to address how these changes alter the outcome of repeated malaria infections (delivered by CHMI or natural infections) that could be beneficial or detrimental for malaria-induced pathology, viral or bacterial coinfections, and vaccine take and responsiveness in individuals with recent history of malaria exposure, particularly infants and children (66).

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Disclosures

S.L.H. is associated with Sanaria Inc., who manufactured the PfSPZ challenge, and thus has potential conflicts of interest. The other authors have no financial conflicts of interest.

References


S Fig 1. CD3⁺, CD8⁺ MAIT and NK cell counts per microliter blood. The lymphocyte counts per microliter blood were determined and subsets counts were calculated based on frequency established by FACS. (A) CD3⁺ cell counts did not change significantly over time, while (B) CD8⁺ MAIT cell counts increased significantly by day 168 (p < 0.05) going from a mean value 44 (d 0) cells to 70 (day 168) cells per microliter blood. (C) NK cell numbers decreased significantly on days 28 and 56.
**S Fig 2. Gating strategy and representative FACS plots.** Our gating scheme to identify CD4, CD8, iNKT, CD8+ and all MAIT and NK cells is illustrated and representative FACS plots are shown.
### Supplemental Figure 3

**Chemotaxis Panel**

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<tr>
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<td>BV421</td>
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<td>BD</td>
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<tr>
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<td>GB11</td>
<td>BD</td>
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<td>G3H6</td>
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**NK Panel**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Fluor</th>
<th>Clone</th>
<th>Provider</th>
</tr>
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<tbody>
<tr>
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<td>V510</td>
<td>N/A</td>
<td>Invitrogen</td>
</tr>
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<td>BV786</td>
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<td>α-IFNγ</td>
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S Fig 3. FACS Panels. Shown are the FACS panels used to examine function ex vivo and iNKT, NK and MAIT cell frequencies, chemotaxis and activation characteristics.

* Denotes intracellularly-detected markers
* Denotes reagents used only in low dose CHMI samples
* Denotes reagents used only in high dose CHMI samples
Supplemental Figure 4

S Fig 4. (A) single-cell RNAseq was also performed on a 2nd donor, but RNAseq data quality (transcript number and length) was substantially lower compared to the donor shown in Fig 6. Although not used for whole transcriptome analysis, we used this donor’s data to reconstruct TCR sequence information as outlined in Fig. 6 (B) Showing combined data from both subjects. E.cell = the number of cells with expanded TCRs (>4); NE. cell = the number of cells with non-expanded TCRs (<=4); E.int = E. cells shared between donors. There was not significant difference in the distribution of cell with expanded vs. non-expanded TCRs over time (p-value = 0.6364).
CHAPTER 6

General discussion, conclusion and recommendations
6.1. General discussion

This chapter discusses the outcomes of malaria vaccine and challenge studies performed in the context of my PhD thesis. We evaluated the most advanced pre-erythrocytic malaria vaccine, RTS,S/AS01 in Bagamoyo, Tanzania – a setting where malaria is endemic and where the target population for this vaccine lives. We provide novel insights on the immunological evaluation of RTS,S/AS01 induced immunity and its relation to measured vaccine efficacy against *P. falciparum* malaria in African children and infants (Chapter 4). In addition we discuss the potential of using CHMI in malaria endemic countries as a tool for accelerating malaria vaccine and drug development in the relevant target population (Chapter 5). We further give recommendations for possible improvement of the current vaccine development approaches and outline the contribution of the current PhD work in the field of malaria vaccine development.

6.1.1. Investigation of safety, efficacy and immunogenicity of RTS,S/AS01 vaccine in Tanzania paediatric population

Volunteer safety while receiving a novel, non-registered investigational product is top priority in clinical trials, particularly when targeting healthy populations in vaccine development. The RTS,S/AS01 malaria vaccine candidate, tested in a large phase III trial, is the most advanced vaccine so far in the malaria vaccine development pipeline. Despite the large-scale testing of RTS,S/AS01 in eleven African centers and the long follow up period of 32 months, important knowledge gaps remain. Mode of action, immune correlates of protection and factors driving the reduced vaccine efficacy in different malaria endemicities remain unclear. The
question of why the RTS,S/AS01 vaccine shows lower efficacy in infants when compared to children of 5-17 months and how the different outcomes of the vaccine trials are affected by pre-existing malaria endemicity is far from clear.

Moreover, the modest increase of IgG antibody titers to the NANP repeat region post-booster dose at month 20 compared to the peak response post-dose three is remarkable (JOINT TECHNICAL EXPERT GROUP., 2015). In addition, the question of whether children vaccinated with RTS,S/AS01 are prone to a malaria rebound when living in higher malaria transmission regions is of serious concern (Olotu et al., 2016). Surprisingly, little is known about relevant antibody subclasses and the avidity and functionality of protective antibodies required for conferring protection. Similarly, the quality, kinetics and quantity of vaccine-specific cellular immune response and their relation to long term protection remain obscure.

In Bagamoyo, safety and efficacy was assessed during this phase 3 randomized, controlled, double- blinded trial, in infants aged 6-12 weeks at time of enrolment (chapter 4; paper I). In this trial, infants were randomly assigned to receive three doses of the RTS,S/AS01 vaccine or a comparator vaccine, administered intramuscularly at 1-month intervals, with or without a fourth dose given 20 months after the first dose and participants were followed up through passive surveillance. In this study, we showed that the RTS,S/AS01 vaccine generally demonstrated an excellent safety profile. A comparable safety profile was also reported in early phases of RTS,S/AS01 when trials were conducted in malaria naïve population in the US and Europe (Schwartz et al., 2012).
However, several safety signals have become apparent during recent data analysis such as the mortality increase in girls (Klein et al., 2016). The Global Advisory Committee on Vaccine Safety (GACVS) reported a 2-fold increase mortality in girls than in boys vaccinated with RTS,S/AS01; when a combined additional safety signal analysis was performed by gender and study arm (WER-WHO, 2016). While girls are more at risk of being neglected by their families in certain cultures, there is also clear evidence that sex-based differences in susceptibility to infectious diseases and immunity against vaccines exist (Klein et al., 2015, 2015; Ruggieri et al., 2016). So far, unexplained serious adverse events such as cerebral malaria, meningitis and death are slightly higher in the RTS,S/AS01 vaccinees when compared to the control group. Such observations could be incidental findings, as some of the cases were realized later without any obvious timely relationship to vaccination. These safety signals make further investigation of the safety of RTS,S/AS01 when deployed under “real life conditions” a must before approval for routine use is granted.

We further evaluated the efficacy of the RTS,S/AS01 malaria vaccine in a phase 3 study that included 6,537 infants expecting that the outcome would be similar or even better when compared to what has been observed earlier in phase I and phase II studies. However, the efficacy achieved in this phase III trial was lower when compared to the efficacy demonstrated in the early studies conducted in a comparable environment and age group (Asante et al., 2011). In striking contrast to a moderate efficacy observed in the current RTS,S/AS01 vaccine trial in infants; the previous trials of RTS,S/AS01 demonstrated superior efficacy when tested in malaria naïve subjects (Kester et al., 2007, 2009; Leroux-Roels et al., 2014), semi-
immune African adults volunteers (Polhemus et al., 2009) and fairly encouraging efficacy in older children (5-17 months) (Agnandji et al., 2011). In order to address the question as to why the RTS,S/AS01 vaccine appears to be more efficacious in naive and semi-immune children than in infants living in Africa, we have started to analyze the blood samples collected from the children cohort during our trial in Bagamoyo and the analysis is on ongoing.

At the moment, we could hypothesize that the level of efficacy observed in the current study is a clear-cut finding, making the effort for designing a second generation vaccine crucial. However, age-dependent immune response could be another possible explanation. It is well established that resistance to severe malaria increases with increase in age and exposure (Doolan et al., 2009; Griffin et al., 2015; Olotu et al., 2011). Infant immune systems are not well developed and have limited exposure to antigens in utero. Therefore protection against infections in this population is thought to be heavily dependent on their provision of protective maternal antibodies and well-functioning innate immune systems (PrabhuDas et al., 2011). This is also supported by higher anti-CSP antibodies detected in older children compared to infants, albeit antibodies have not been well established as marker of protective vaccine induced immunity (Kester et al., 2009; Olotu et al., 2011).

An alternative explanation could be that the RTS,S/AS01 vaccine protects individuals from developing blood stage infection, leading to a slower acquisition of immunity to blood-stage parasites. Such scenario will result in a rebound of clinical malaria cases at a later life stage, as recently demonstrated by Olotu and
colleagues (Olotu et al., 2016). It could also be true that other factors such as heterogeneity of natural exposure and individual based vaccine intake and responses played a role in the observed lower efficacy in the infant group. Co-administration of RTS,S/AS01 with other EPI vaccines could be another cause of inferior efficacy shown in the current trial.

Cellular immune interference has been shown to have an impact when two vaccines containing similar protein components are administered in parallel (Dagan et al., 1998). The hepatitis B vaccine that was co-administered with RTS,S/AS01 has a fusion protein that also incorporates the HBsAg. This could also be a reason for the disappointing vaccine efficacy observed in the infant cohort. However, this tendency warrants further detailed analysis of the impact of co-administering the vaccine with EPI vaccines. Presence of maternal antibodies, fetal haemoglobin and low exposure in infants has been shown to play critical roles in efficacy of other vaccines in infants (Billig et al., 2012; Edwards, 2015). However, despite that in this particular study these parameters were not tested, a slightly higher efficacy that was reported in children cohort suggests pre-existing hemoglobinopathies might not underline lower efficacy in the infant cohort.

Cellular and humoral immune responses are both important in protection against malaria infection (Good and Doolan, 1999; Schofield and Mueller, 2006) and are likely to be affected by the level of malaria exposure. African field trials of malaria vaccines have mostly generated evidences of humoral immune responses, while data regarding cellular immunity remains under explored (White et al., 2015). The most challenging hurdles in conducting the cellular immune assays in infants and
children are a limited blood volume that can be obtained from this population, getting an acceptable frequency of repeat sample collection and gaining ethical and parental approval for the enrolment of usually healthy children in research studies (MOORMANN, 2009). The quality and quantity of the PBMC isolation in any cellular immune response assays is also critical. In the current study we managed to collect PBMCs and plasma samples at different time points and cryo-preserved them in liquid nitrogen vapor phase and -80°C, respectively for future investigation of vaccine-induced cellular and humoral immune responses. We showed that the collected PBMCs were still good quality in terms of viability and quantity even after 5 years of storage (chapter 4; paper IV).

In this thesis work, we also demonstrated that Th1 responses could be the best immunological markers for RTS,S/AS01 vaccine induced immunity while Th2 responses, in particular IL5 and RANTES, might represent markers for lack of protective immune responses. We detected a lower induction of protective Th1 (IFN-g, IL15 and GM-CSF) responses by RTS,S/AS01 in infants compared to children while Th2 (IL4 and IL13) and the pro-inflammatory markers (TNF and IL-1β) responses remained similar in both cohorts (chapter 4; paper III). These findings provide further supporting evidence as to why infants in the RTS,S/AS01 phase III study had lower vaccine efficacy compared to the children cohort.

Previous studies of RTS,S/AS01 also reported similar trends of increased responses in children rather than in infants when they compared anti-CSP antibodies amongst children and infant cohorts (White et al., 2015). The accumulating evidence from multiple previous clinical challenge trials the USA,
adult or paediatric field trials in different age groups and across the distinct transmission settings of The Gambia, Kenya, Mozambique and Tanzania suggest that the protective mechanism of the RTS,S/AS01 is strongly supported by antibodies against the CSP repeat region (Moorthy and Ballou, 2009). Since maternal antibodies have been reported to interfere with antibody generation during vaccination (Edwards, 2015; Niewiesk, 2014); we hypothesize that the poor efficacy observed in the RTS,S/AS01 infant cohort could also be due to effect of maternal antibodies. We argue that vaccine efficacy in young children was much better since maternal antibodies persist for a short period in early life and during the age of 5-17 months most of the maternal antibodies have disappeared.

We further evaluated the quality of RTS,S/AS01 induced anti-CSP antibodies in a phase 2 study aiming to investigate the effect of RTS,S/AS01 vaccination schedule on protective efficacy and avidity of anti-CSP antibodies in 6-12 weeks African infants (chapter 4; paper II). We showed that the magnitude of increase of anti-CSP IgG concentration and avidity between second and third vaccine injection and not the anti-CSP IgG concentration and avidity alone is associated with a strong risk reduction for malaria after immunization. This is in line with the findings of recent studies showing that, despite malaria exposed children having a higher antibody avidity compared to children with less malaria exposure; the observed avidity alone did not predict protection (Olotu et al., 2014). Several explanations for lack of correlation between efficacy and avidity of RTS,S/AS01 induced antibodies in children could be: First, the co-linearity between antibody concentration and avidity might have blurred the effect of avidity in this study.
On the other hand, the finding may also suggest that avidity might not be an important determinant of vaccine efficacy or that our assay was not sufficient to reflect the avidity. Second, our assay was designed to detect antibodies against the CSP-repeat region, which was shown to be an immunodominant epitope. Neglecting to measure antibodies against the C-terminal flanking region might potentially exclude some relevant antibody responses that could explain the effectiveness of the vaccine (Watt et al., 2013). The use of new techniques such as Surface Plasmon Resonance (SPR) could be an alternative and more sensitive method in future to investigate antibody avidity induced by vaccination compared to the thiocyanate elusion method (Reddy et al., 2012).

In Conclusion, acquired immunity to malaria develops with increasing age and repeated infections. Understanding immune correlates underlying protection against malaria in early life will facilitate vaccine development. Our results highlight the need for revisiting and designing a vaccination schedule and formulation that will modulate the immune system towards a potent Th1 response during the first months of life and avoid counteracting responses such as IL-5 and maternal antibodies. A complete understanding of the mechanism underlying the acquisition of protective immunity in malaria exposed individuals is crucial for outlining the strategies for a better malaria vaccine development.
6.1.2. Controlled human malaria infections as a cornerstone to accelerate malaria vaccine development in Africa

Controlled human malaria infections (CHMI) have provided a landscape for malaria researchers to test malaria vaccine efficacy in well-controlled setting. The call for urgency in the development of an efficacious malaria vaccine has led to increased demand for conducting early stage CHMI-based efficacy studies in malaria endemic regions (Laurens et al., 2012). The experimental \textit{P. falciparum} challenge model in human using a syringe and needle was first introduced into African settings in 2012. CHMI has demonstrated the greatest ability to predict efficacy of malaria vaccines in malaria-naive adults (Spring et al., 2014), and here for the first time in the history of malaria vaccine development, we evaluated whether CHMI can reproduce such results in pre-exposed population. In this study (chapter 5; paper V), we show for the first time that inoculation of healthy, young adult, Tanzanian males with aseptic, purified, cryopreserved \textit{P. falciparum} sporozoites (\textit{PfSPZ} challenge), was safe, well tolerated and was as infective as in malaria naïve volunteers. We used samples collected during this CHMI study to explore adaptive cellular immune activation following different doses of \textit{PfSPZ} challenge (chapter 5; paper VI), examined the memory B cells, antibodies and their opsonising activities before and after CHMI (chapter 5; paper VII), and the impact on innate-like lymphocytes such as MAIT cell, iNK T cell and NK cells (chapter 5; paper VIII).

To understand the effect of malaria pre-exposure on anti-parasite immunity during CHMI, we compared the role of pre-existing antibodies in a CHMI study conducted
in malaria naïve Dutch adults and malaria semi-immune Tanzanian adults who received essentially the same dose of \( \text{PfspZ} \) challenge delivered intradermally (chapter 5; paper VI). Here we show that pre-existing antibodies to \( P. falciparum \) antigens had a significant effect on the outcome of the CHMI as demonstrated by the longer prepatency period and lower parasite load at the time of first detection as assessed by qPCR in the Tanzanian versus the Dutch cohort. Antibody responses in naïve Dutch subjects versus malaria semi-immune Tanzanian subjects who received same dose of \( \text{PfspZ} \) challenge were also compared. The higher fold increase in anti-malaria antibodies observed in Tanzania subjects but not in Dutch subjects after CHMI suggests the presence and boosting of pre-existing humoral immunity (chapter 5; paper VI). However, it remains to be answered in the future studies whether and which proportion of these boosted humoral responses resulted from naturally acquired immunity; or have been elicited by the longer and transient asexual blood stage parasitaemia exposed to the immune system before malaria treatment initiation. Our results indicate that the immune response against malaria in pre-exposed individuals might differ from that of malaria-naïve individuals during CHMI.

In chapter 5, paper VII; we present findings from a merozoite opsonization assay that was used as a measure of functionality of antibodies detected before, during and after CHMI. We showed that the antibodies produced had a peak level of opsonization function one month after CHMI, and these responses remained significantly higher after 6 months compared to baseline. Of note, the boosting of opsonising antibody responses was evident in volunteers who - at the time of intradermal sporozoite injection - showed lower functional antibody levels but not in
volunteers with higher levels. These differences in boosting the level of opsonizing antibodies observed in lower and higher responders could be explained by the differences in malaria pre-exposure status amongst the two groups of naïve and pre-exposed adults. Whether, the observed increase in percentage phagocytosis reflects the maximum threshold that people in malaria endemic regions could achieve remains to be tested in future sporozoite vaccine trials. However, our study was not designed to evaluate the impact of increased opsonization activity on protective efficacy. In summary, these findings highlight the need for detailed investigation to identify and validate the best antibody functional assays that will predict and correlate with malaria or vaccine induced humoral immune responses in malaria pre-exposed volunteers.

Our findings also show that malaria specific cellular responses, as measured by IFN-g secreting CD4 and CD8 T cells subsets, were more pronounced in malaria naïve volunteers than in semi-immune volunteers, despite the two cohorts having similar baseline responses before CHMI (chapter 5; paper VI). We hypothesize that malaria naïve volunteers mount immune responses that are more biased towards T cell mediated immune responses, which are likely to be directed toward the liver stage parasites (anti-infection), while malaria pre-exposed volunteers mount immune response that are more biased towards B cell immune responses directed towards blood stage (anti-disease). However, this hypothesis remains to be further investigated and validated in *P. falciparum* vaccine trials comparing malaria naïve and pre-exposed volunteers.
We further showed that a single dose of PfSPZ challenge boosted MBC against MSP-1 and AMA-1 with the maximum peak observed one month after challenge, which returned to baseline after 6 months (chapter 5; paper VII). Based on these findings, we hypothesize that in malaria semi-immune volunteers the MBC responses following CHMI resulted from a secondary response but had short-lived plasma cell expansion as we observed peak responses around 2 weeks after asexual blood stage detection. The observed decline of MBC after CHMI could also be explained by antigen-specific plasma cells that migrate to the bone marrow, the predominant site of antibody production (Crotty et al., 2003; Slifka et al., 1995), resulting in fewer MBCs recirculating in the peripheral blood. We further showed that the quantity of antibodies and opsonising functions after PfSPZ challenge followed a similar trend to that observed for MBCs in each individual. We are left with an open question as to whether the slow development of natural acquired immunity observed in people living in malaria endemic areas is a result of immune dysregulation and how should this be overcome during malaria vaccine development.

The question as to how the human innate-like lymphocyte compartment is involved in malaria infections is not well characterized. In the current study, we showed for the first time that CHMI affects innate-like lymphocytes - in particular the MAIT cell populations - in a PfSPZ challenge dose-dependent manner. Furthermore, while these innate-like responses are perceived to be short-lived, we found that changes in the MAIT cell population persisted even 6 months after the infection was cleared. It is yet to be investigated if these changes in innate-like lymphocyte composition are beneficial or detrimental for immune responses – particularly in populations that
live in malaria endemic regions. As the MAIT cells likely play essential roles in the timely initiation of adaptive immune responses during infection, the mechanisms that activate MAIT cells in the liver could be of interest as a potential therapeutic strategy against liver-stage malaria infection. The single-cell RNA-seq analysis data also suggests that CHMI leads to a clonally driven increase of innate-like lymphocytes that may be mediated by homeostatic expansion-like mechanisms through enhanced expression of IL-7 receptors.

Together, our data demonstrates that CHMI studies using PfSPZ challenge can be safely performed in malaria endemic African countries where individuals with varying degree of pre-exposure reside. These studies will generate essential information required for future malaria vaccine design.
6.2. Conclusion

Substantial investment in research and development is needed to develop an effective malaria vaccine. The information gained from the RTS,S/AS01 vaccine development approach highlights the possible ways to overcome malaria vaccine development obstacles. It is obvious that, although recombinant subunit vaccines do not yet give the desired levels of protection, these types of vaccine approaches can be easily manufactured and feasibly delivered in disease endemic setting. Manufacturing of live sporozoite vaccines for mass administration and the requirements for the storage remain to be clearly resolved. Studies conducted in the US, European and African individuals when testing the whole sporozoite approach have shown that malaria-naïve individuals respond better than malaria-exposed individuals (Jongo et al., 2018; in press). The question remained, “what exactly constitutes the reason for lack of durable protection against malaria infection in endemic areas?” The most probable way of accelerating future vaccine development is a better understanding of the host-pathogen interaction and the nature of acquired immunity that will eventually be translated into better vaccine design. Conduct of CHMIs in malaria exposed volunteers has opened new doors in the malaria vaccinology field. It is now likely that malaria vaccines and drugs could be easily tested for their efficacy in the target population. We have established the foundation for using CHMI to assess the efficacy of new interventions against malaria and to study the mechanisms of protection conferred by different malaria vaccines in endemic settings. Improvements such as direct venous inoculation (DVI) of irradiation attenuated sporozoites or live sporozoites under chloroquine prophylaxis will provide more information on the protective efficacy of the PfSPZ vaccines approach in endemic populations.
6.3. Recommendations

Several recommendations to improve the current vaccine approaches could be delineated from our work. These include the use of prime-boost strategies whereby a combination of different antigen delivery systems encoding the same epitopes or antigen, delivered at an interval of a few weeks apart is applied. Furthermore, efficient protection against malaria is likely the result of the combined effect of multiple, additive and low levels of a variety of immune responses targeting a broad range of antigens resulting in individual protective signatures. Therefore, an addition of more PE and/or asexual blood stage antigens to the current RTS,S formulation that covers a wide range of parasites could be one option for improvement. This should also include antigens directed against sexual stages or ookinetes as they will add the essential transmission blocking component. An alternative strategy would be to start from scratch with the vaccine development efforts, by identifying novel critical antigens amongst the thousands of antigens expressed by the parasite and select those which are strongly provoking the immune system and provide solid protection.

To circumvent the hurdles of generating successful recombinant subunit vaccines, whole parasites that are attenuated either by irradiation, drugs or genetic modifications could be the better option for achieving higher efficacy. However, much work has to be done in this model as we would need to identify the right dose and immunization schedule that will induce long-lasting immunity in malaria pre-exposed populations.

To date, it is unclear which assay is the best for studying relevant immune functions in malaria immunity. Therefore, identifying and validating the best
functional assays for characterization of both cellular and humoral immune responses will help to identify relevant antigens. Analyses of the host and parasite transcriptome, proteome, expression cloning and new imaging technology could make our goal for developing a second generation malaria vaccine that protects more than 80% against clinical malaria possible.

The achievement of RTS, S vaccine clinical development has been accelerated by collaboration from many international and local organizations working together to support a series of clinical trials in Africa. Strengthening this cooperation will further build or enable sharing of high technology laboratory capacities that are available today to Scientists from economically stronger countries. Human resources and expertise for designing better clinical trials in Sub-Saharan Africa is a must. By doing so, we will all together to support and thrive, in order to achieve detailed investigations on how malaria immunity works in naturally exposed individuals and use this platform for designing a better and effective second generation malaria vaccine. Additionally, a longer-term follow-up is needed for full assessment of the vaccine risks and benefits. A thorough evaluation of the risk-benefit, feasibility and cost-effectiveness to determine the overall impact of RTS,S/AS01 in the community is highly needed, for the goal of implementation of RTS,S across the continent to be unequivocally recommended.
6.4. Contribution of the current thesis

This PhD thesis has made several contributions to the field of malaria vaccines trials. We have reported for the first time through this work that vaccine trials involving paediatric populations can feasibly be incorporated into EPI programmes and conducted in large populations in the African setting. This information can be adapted for testing of other drug and vaccine products in similar settings. Furthermore, we have demonstrated that individuals living in different disease endemicity might respond differently to the vaccine candidate. This work also showed that \( P.\) \( SPZ \) challenge response from the malaria naïve population was more biased toward cellular immunity while malaria pre-exposed population responded toward humoral immunity. Such novel knowledge will contribute to the better design of a second generation malaria vaccine that will be more potent in the malaria endemic population. Through this PhD work we have also for the first time contributed to knowledge that CHMI could safely be conducted in pre-exposed malaria population with a similar infectivity outcome. This information led to a series of malaria vaccine clinical trials that involved CHMI in large number of African research institutes.
References


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CHAPTER 7

Appendices: PhD supplementary work and Curriculum Vitae
7.1. Appendices

During the course of this PhD work, several contributions were made to the research work published in Lancet infectious disease, Clinical and vaccine immunology and PLOS One. The first study (published in Lancet infectious disease) aimed to assess the performance of the novel T-cell activation marker–tuberculosis (TAM-TB) assay for the diagnosis of active tuberculosis in children with symptoms that suggest tuberculosis. The second study (published in Clinical and vaccine immunology) aimed to analyze mRNA expression levels before and shortly after H1/IC31-Tuberculosis vaccination and correlate these with vaccine-specific central (TCM) and effector memory CD4 T cell (TEM) responses. The third study (published in PLOs One), aimed to investigate the expression of Mip-1β and the T cell maturation marker CD27 within CMVpp65-specific CD4+ and CD8+ T cells in relation to age, HIV and active Tuberculosis (TB) co-infection in a cohort of Tanzanian volunteers. Despite that these studies are not directly linked to the topic of my thesis, my contribution in laboratory analysis of the samples and the review of manuscripts worth it recognitions.
7.1.1 Assessment of the novel T-cell activation marker–tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study

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Summary

Background The diagnosis of paediatric tuberculosis is complicated by non-specific symptoms, difficult specimen collection, and the paucibacillary nature of the disease. We assessed the accuracy of a novel immunodiagnostic T-cell activation marker–tuberculosis (TAM-TB) assay in a proof-of-concept study to identify children with active tuberculosis.

Methods Children with symptoms that suggested tuberculosis were prospectively recruited at the NIMR-Mbeya Medical Research Center in Mbeya, and the Ifakara Health Institute in Bagamoyo, Tanzania, between May 10, 2011, and Sept 4, 2012. Sputum and peripheral blood mononuclear cells were obtained for Mycobacterium tuberculosis culture and performance assessment of the TAM-TB assay. The children were assigned to standardised clinical case classifications based on microbiological and clinical findings.

Findings Among 210 children screened, we selected a subgroup of 130 to ensure testing of at least 20 with culture-confirmed tuberculosis. 17 of 130 children were excluded because of inconclusive TAM-TB assay results. The TAM-TB assay enabled detection of 15 of 18 culture-confirmed cases (sensitivity 85.3–91.5%, CI 0.5–96.6–96.4). Specificity was 66.6–89.6% (CI 80.8–0.9%–80.9%) in the cases that were classified as not tuberculosis (n=63), with little effect from latent tuberculosis infection. The TAM-TB assay identified five additional patients with highly probable or probable tuberculosis, in whom M tuberculosis was not isolated. The median time to diagnosis was 14–45 for culture.

Interpretation The sputum-independent TAM-TB assay is a rapid and accurate blood test that has the potential to improve the diagnosis of active tuberculosis in children.

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Introduction

Tuberculosis in children is a serious public health problem. Recent estimates of the tuberculosis disease burden in children, based on the results of a systematic literature review and mathematical modelling, suggest that about 1 million children developed tuberculosis worldwide in 2010, including 280,000 incident cases in the African region. Surveillance data for children remain imprecise because paediatric tuberculosis is often either underdiagnosed or overdiagnosed in high-burden countries. The lack of accurate and rapid diagnostic methods contributes to tuberculosis morbidity and mortality in children and hampers the assessment of new drugs and vaccines in paediatric populations.

Diagnosis of active tuberculosis in children poses a major challenge. Clinical symptoms of tuberculosis in children are often non-specific and resemble those of common paediatric illnesses, including pneumonia and malnutrition. Adequate respiratory specimens are difficult to obtain for bacterial confirmation, particularly in very young children who are unable to expectorate and in whom diagnostic yields are poor because of the paucibacillary nature of the disease. Hence, the diagnosis is routinely made on the basis of a combination of clinical features, contact history, chest radiography, and tuberculin skin test, and often with scoring charts that have poor diagnostic accuracy. The Xpert MTB/RIF assay enables timely, sensitive, and specific molecular detection of pulmonary tuberculosis and rifampicin resistance in adults, but its value in young children is greatly reduced. In a recent meta-analysis, the calculated pooled sensitivity was 66.6% for Xpert MTB/RIF against culture in expectorated or induced sputa, or gastric lavage specimens from children with suspected tuberculosis. WHO strongly recommends Xpert MTB/RIF as the initial diagnostic test in children suspected of having multidrug-resistant or HIV-associated tuberculosis, and only conditionally in all children suspected of having tuberculosis.

Immunodiagnostic tests, such as the tuberculin skin test and interferon-γ release assays—ie, QuantiFERON-TB Gold and T-SPOT.TB—do not depend on the presence of Mycobacterium tuberculosis in collected samples. Although these tests have use in screening...
special risk populations for latent tuberculosis infection, they cannot enable the crucial distinction between active tuberculosis disease and latent tuberculosis infection. Failure to accurately identify cases with active tuberculosis restricts the clinical application of these tests in endemic regions where latent tuberculosis infection is ubiquitous.

Results of previous studies in adults showed that flow-cytometric analysis of the CD27 on circulating M tuberculosis-specific T cells can discriminate active tuberculosis from latent tuberculosis infection. Loss of CD27 expression on M tuberculosis-specific CD4 T cells is a marker of active tuberculosis due to persistent antigenic stimulation and probably relates to increased cellular homing to the site of disease. In a proof-of-concept study, we assessed the performance of the new T-cell activation marker–tuberculosis (TAM-TB) assay for the diagnosis of active tuberculosis in children with symptoms that suggest tuberculosis.

Methods

Study population

This prospective diagnostic assessment was done at two Tanzanian research sites—the NIMR-Mbeya Medical Research Center, Mbeya, and the Ifakara Health Institute, Bagamoyo. Children older than 6 months and younger than 16 years with signs or symptoms that suggested tuberculosis were enrolled from May 10, 2011, until Sept 4, 2012, and followed up for a minimum of 5 months. At least one of the following eligibility criteria had to be met: persistent, non-remitting cough for more than 14 days that did not respond to antibiotics; repeated episodes of fever within the past 14 days that did not respond to antibiotics; malaria had been excluded; weight loss or failure to thrive during the previous 3 months; and signs and symptoms that suggested extrapulmonary tuberculosis. Children who received tuberculosis treatment in the past 12 months were excluded. The children were referred from peripheral health facilities and local hospitals. Because of cost restrictions, a subgroup of 130 children was selected from a larger cohort of 290 children to ensure testing of at least 20 children with culture-confirmed tuberculosis after prevalence estimation at the two sites. All other clinical information was masked from the investigators doing the selection with a list of patient identification numbers with corresponding disease classifications.

The Institutional Review Board of the Ifakara Health Institute, the Mbeya Medical Research and Ethics Committee, and the Medical Research Coordinating Committee of Tanzania approved the study protocol. We obtained written informed consent from a literate parent or legal guardian. In cases of illiteracy, informed oral consent was attained by an independent witness. Children older than 7 years provided assent for participation.

Classification and reference standard

Classification of children was based on the results of the clinical and microbiological assessment as culture-confirmed tuberculosis (culture-positive for M tuberculosis), highly probable tuberculosis (chest radiograph consistent with tuberculosis confirmed by two independent reviewers, histology or cytology typical for tuberculosis, or fluorescent or acid-fast bacilli on microscopy), probable tuberculosis (clinically suspected tuberculosis without objective findings as above), not tuberculosis (alternative diagnosis established and clinical resolution without antituberculosis treatment), or indeterminate (any other combination; appendix). Culture-confirmed tuberculosis was used as the reference standard for sensitivity analysis and not tuberculosis as the reference standard for specificity assessment.

Clinical and laboratory procedures

Clinical procedures at enrolment comprised medical history, physical examination, HIV testing, CD4 T-cell count, interferon-γ release assay (QuantiFERON-TB Gold, Cellestis, Melbourne, VIC, Australia), and chest radiography. Chest radiographs were classified as strongly indicating, uncertain, or not tuberculosis by two independent experts from whom all clinical and diagnostic information was masked. Malnutrition was assessed on the basis of weight-for-age Z score in children aged up to 10 years (less than –2) and body-mass index in those older than 10 years (<2 SD below normal). If feasible, at least three induced or two expectorated respiratory specimens were obtained on consecutive days. Induced sputum was obtained in accordance with a standard protocol. Fine-needle aspiration biopsies of enlarged lymph nodes were done when clinically indicated, as per standard protocol.

After N-acetyl-L-cysteine–sodium hydroxide decontamination, each sputum sample pellet was assessed with microscopy by use of Ziehl-Neelsen staining. At least one sample was inoculated on both liquid (BACTEC...
MGIT 960, Becton Dickinson, Franklin Lakes, NJ, USA) and solid media (Loewenstein-Jensen culture). Positive cultures were confirmed by use of microscopy, and subsequent MPT64 antigen or molecular tests (Genotype MTBDC or CM, Hain Lifescience, Nehren, Germany) or both. GenoType MTBDRplus (Hain Lifescience) or phenotypic drug-susceptibility testing (BACTEC MGIT 960 SIRE kit, Becton Dickinson) was used for resistance testing. Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) was done in at least one sputum sample according to the manufacturer’s instructions. All tests, including TAM-TB assay, were done by trained laboratory technical staff masked to clinical information and radiological results.

Results from established diagnostic procedures were made available to support clinical management in accordance with national and international guidelines. Results of the experimental TAM-TB assay were not released.

**TAM-TB assay**

Details of the underlying biological principle of the TAM-TB assay, definition of cutoff values in an optimisation cohort of adults (n=87), and additional background information are provided in the appendix.

Briefly, the TAM-TB assay is used to measure the CD27 phenotype of CD4 T cells producing interferon-γ in response to M tuberculosis antigens according to a standard intracellular cytokine staining procedure. 

M tuberculosis-specific CD4 T-cell responses were judged positive when at least five CD4 T-cell interferon-γ-producing events were detected, and the proportion of interferon-γ-producing CD4 T cells after antigen stimulation was greater than 0·05% and was at least twice the frequency of that in the negative controls.

Well characterised samples from adults (appendix) were used to identify the CD27 median fluorescence intensity (MFI) ratio with optimum discriminatory power between latent tuberculosis infection and active tuberculosis. Cryopreserved peripheral blood mononuclear cells obtained at the baseline visit were stimulated for 12–16 h with a set of overlapping ESAT-6/CFP-10 peptides (Elephants and Peptides, Potsdam, Germany) and purified protein derivative (Statens Serum Institut, Copenhagen, Denmark) before staining with fluorochrome-labeled antibodies. We compared the MFI of CD27 staining on interferon-γ-positive M tuberculosis-specific CD4 T cells to the CD27 MFI value for all CD4 T cells to define the CD27 MFI ratio for a sample (appendix). CD27 MFI ratio results were

**Table 1: Demographics and clinical characteristics of study participants by classification group**

<table>
<thead>
<tr>
<th>Classification</th>
<th>All patients (n=113)</th>
<th>Culture-confirmed tuberculosis (n=18)</th>
<th>Highly probable tuberculosis (n=8)</th>
<th>Probable tuberculosis (n=12)</th>
<th>Not tuberculosis (n=63)</th>
<th>Indeterminate (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>6 (1·2–10·9)</td>
<td>4 (1·5–12·7)</td>
<td>8 (1·2–11·9)</td>
<td>6 (1·8–10·5)</td>
<td>6 (1·2–9·8)</td>
<td>5·4 (2·2–10·5)</td>
</tr>
<tr>
<td>Female sex</td>
<td>52 (46%)</td>
<td>8 (44%)</td>
<td>5 (63%)</td>
<td>6 (50%)</td>
<td>27 (43%)</td>
<td>6 (50%)</td>
</tr>
<tr>
<td>Symptoms at enrolment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cough</td>
<td>108 (96%)</td>
<td>16 (89%)</td>
<td>6 (75%)</td>
<td>12 (100%)</td>
<td>62 (98%)</td>
<td>12 (100%)</td>
</tr>
<tr>
<td>Fatigue or lethargy</td>
<td>28 (25%)</td>
<td>8 (44%)</td>
<td>2 (25%)</td>
<td>2 (17%)</td>
<td>16 (25%)</td>
<td>0</td>
</tr>
<tr>
<td>Wheezing</td>
<td>16 (14%)</td>
<td>1 (6%)</td>
<td>1 (13%)</td>
<td>3 (25%)</td>
<td>9 (14%)</td>
<td>2 (17%)</td>
</tr>
<tr>
<td>Breathing difficulties</td>
<td>47 (42%)</td>
<td>10 (56%)</td>
<td>4 (50%)</td>
<td>8 (67%)</td>
<td>22 (35%)</td>
<td>2 (25%)</td>
</tr>
<tr>
<td>Fever</td>
<td>82 (73%)</td>
<td>16 (89%)</td>
<td>6 (75%)</td>
<td>9 (75%)</td>
<td>45 (71%)</td>
<td>6 (50%)</td>
</tr>
<tr>
<td>Chest pain</td>
<td>23 (20%)</td>
<td>1 (6%)</td>
<td>0 (1%)</td>
<td>1 (8%)</td>
<td>18 (29%)</td>
<td>3 (25%)</td>
</tr>
<tr>
<td>Haemoptysis</td>
<td>4 (4%)</td>
<td>1 (6%)</td>
<td>0 (1%)</td>
<td>1 (8%)</td>
<td>1 (2%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>Enlarged lymph nodes</td>
<td>12 (11%)</td>
<td>5 (28%)</td>
<td>1 (13%)</td>
<td>1 (8%)</td>
<td>5 (8%)</td>
<td>0</td>
</tr>
<tr>
<td>Weight</td>
<td>55 (49%)</td>
<td>13 (72%)</td>
<td>3 (38%)</td>
<td>5 (42%)</td>
<td>32 (51%)</td>
<td>2 (17%)</td>
</tr>
<tr>
<td>Abdominal pains</td>
<td>20 (18%)</td>
<td>5 (28%)</td>
<td>2 (25%)</td>
<td>3 (25%)</td>
<td>9 (14%)</td>
<td>8 (67%)</td>
</tr>
<tr>
<td>Malnutrition</td>
<td>60 (53%)</td>
<td>12 (67%)</td>
<td>4 (50%)</td>
<td>5 (42%)</td>
<td>24 (38%)</td>
<td>5 (42%)</td>
</tr>
<tr>
<td>HIV infection</td>
<td>33 (29%)</td>
<td>4 (22%)</td>
<td>3 (38%)</td>
<td>7 (58%)*</td>
<td>15 (24%)</td>
<td>4 (33%)</td>
</tr>
<tr>
<td>WHO immunological staging</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not clinically significant</td>
<td>7/33 (21%)</td>
<td>0/4 (0%)</td>
<td>0/1 (0%)</td>
<td>2/7 (29%)</td>
<td>4/15 (27%)</td>
<td>1/4 (25%)</td>
</tr>
<tr>
<td>Mild</td>
<td>6/33 (18%)</td>
<td>1/4 (25%)</td>
<td>1/1 (33%)</td>
<td>2/7 (29%)</td>
<td>2/10 (13%)</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td>Advanced</td>
<td>1/33 (3%)</td>
<td>1/4 (25%)</td>
<td>0/3 (0%)</td>
<td>0/7 (0%)</td>
<td>0/15 (0%)</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td>Severe</td>
<td>19/33 (58%)</td>
<td>2/4 (50%)</td>
<td>2/1 (67%)</td>
<td>3/7 (43%)</td>
<td>9/15 (60%)</td>
<td>1/4 (25%)</td>
</tr>
<tr>
<td>On antiretroviral therapy at enrolment</td>
<td>13/33 (39%)</td>
<td>2/4 (50%)</td>
<td>1/1 (33%)</td>
<td>2/7 (29%)</td>
<td>7/15 (47%)</td>
<td>1/4 (25%)</td>
</tr>
<tr>
<td>Positive tuberculin skin test</td>
<td>31/103 (30%)</td>
<td>13/17 (76%)</td>
<td>3/8 (38%)</td>
<td>2/11 (18%)</td>
<td>9/57 (16%)</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>Positive interferon-γ release assay</td>
<td>27/110 (25%)</td>
<td>13/18 (72%)</td>
<td>1/7 (14%)</td>
<td>3/12 (25%)</td>
<td>8/63 (13%)</td>
<td>4/12 (33%)</td>
</tr>
<tr>
<td>Positive tuberculin skin test interferon-γ release assay</td>
<td>47/112 (42%)</td>
<td>17/18 (94%)</td>
<td>3/8 (38%)</td>
<td>4/12 (33%)</td>
<td>15/63 (24%)</td>
<td>4/11 (36%)</td>
</tr>
</tbody>
</table>

Data are median (IQR), number (%), or n/N (%). *p=0·033 compared with not tuberculosis distribution (Fisher’s exact test).
consistent in 17 independent quality control assessments of two batches of peripheral blood mononuclear cells (appendix).

Adult patients with tuberculosis had significantly higher CD27 MFI ratios than did control patients after ESAT-6/CFP-10 or purified protein derivative stimulation; area under operating characteristic curves were 0.931% and 0.881%, respectively (appendix). We set CD27 MFI ratio thresholds at greater than 5 for ESAT-6/CFP-10 and greater than 13 for purified protein derivative responses to achieve a balanced TAM-TB assay sensitivity of 83.3% (95% CI 68.6–93.0) and specificity of 83.7% (69.3–93.2) in this optimisation cohort. Once these cutoff values were ascertained, samples from paediatric tuberculosis suspects were tested (validation cohort). TAM-TB assays were done at two independent laboratories according to identical standard operating procedures.

**Statistical analysis**
Calculation of medians, IQR, test accuracy measures (sensitivity and specificity), Kruskall-Wallis ANOVA with post-test correction (Dunn’s), and Mann-Whitney and Fisher’s exact tests were done with GraphPad Prism software (version 4.03).

**Role of the funding source**
The study was funded by the European and Developing Countries Clinical Trials Partnership, which had no role in study design, data gathering, analysis, and interpretation, or writing of the report. The corresponding author had full access to all the data and final responsibility for the decision to submit for publication.

**Results**
After assay optimisation in adults (optimisation cohort; appendix), the diagnostic performance of the TAM-TB assay was assessed in 130 children suspected to have tuberculosis (validation cohort); samples from 113 (87%) children were eligible for analysis. Figure 1 shows the study profile; 17 (13%) children were excluded from the study because of inconclusive results. 18 (16%) of 113 eligible children had culture-confirmed tuberculosis, eight (7%) highly probable tuberculosis, 12 (11%) probable tuberculosis, 63 (56%) not tuberculosis, and 12 (11%) indeterminate cases (figure 1). A fine-needle aspiration biopsy of enlarged lymph nodes was done in five children; the final classifications were culture confirmed tuberculosis (n=3), probable tuberculosis (n=1), and not tuberculosis (n=1). All culture-confirmed tuberculosis cases were positive on assessment of a respiratory specimen, including one with additional cytology that suggested tuberculous lymphadenitis. Nine (50%) of 18 children with culture-confirmed tuberculosis were sputum smear-positive. Six of eight patients with highly probable tuberculosis had chest radiographs that strongly suggested tuberculosis; one had chronic granulomatous lymphadenitis, and one was sputum smear-positive. Patients with probable tuberculosis had symptoms that suggested infection, which resolved completely on tuberculosis treatment, but radiographic signs and laboratory investigations were non-conclusive.

Table 1 shows the demographic and clinical characteristics of the children. The median age of the 113 children included in the analysis was 6-1 years (table 1). 33 of 113 children had HIV infection (table 1). According to the WHO immunological classification for established HIV infection, 19 of the HIV-infected children had severe immunodeficiency (table 1). 37 of 38 children with culture-confirmed, highly probable, or probable tuberculosis received tuberculosis treatment.

![Figure 2: Venn diagram of positive Mycobacterium tuberculosis culture, Xpert MTB/RIF, and TAM-TB assay results](image)

**TAM-TB=T-cell activation marker—tuberculosis.**

<table>
<thead>
<tr>
<th>Culture-confirmed tuberculos (n=18)</th>
<th>Highly probable tuberculosis (n=8)</th>
<th>Probable tuberculosis (n=12)</th>
<th>Not tuberculosis (n=63)</th>
<th>Indeterminate (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay-positive cases</strong></td>
<td>15 (85%)</td>
<td>3 (38%)</td>
<td>2 (17%)</td>
<td>2 (3%)</td>
</tr>
<tr>
<td><strong>Assay-negative cases</strong></td>
<td>3 (17%)</td>
<td>5 (63%)</td>
<td>10 (83%)</td>
<td>61 (97%)</td>
</tr>
</tbody>
</table>

Table 2: T-cell activation marker—tuberculosis assay results by classification groups

<table>
<thead>
<tr>
<th>Culture-confirmed tuberculosis (n=18)</th>
<th>Highly probable tuberculosis (n=8)</th>
<th>Probable tuberculosis (n=12)</th>
<th>Not tuberculosis (n=63)</th>
<th>Indeterminate (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity (95% CI)</strong></td>
<td>83.3% (35.9–99.6)</td>
<td>83.3% (51.6–97.9)</td>
<td>83.3% (58.6–96.4)</td>
<td>83.3% (51.6–96.4)</td>
</tr>
<tr>
<td><strong>Positive/total</strong></td>
<td>5/6</td>
<td>10/12</td>
<td>22/25</td>
<td>25/30</td>
</tr>
<tr>
<td><strong>Specificity (95% CI)</strong></td>
<td>95.6% (84.9–99.5)</td>
<td>100% (81.5–100)</td>
<td>96.8% (89.0–99.6)</td>
<td>96.8% (89.0–99.6)</td>
</tr>
<tr>
<td><strong>Negative/total</strong></td>
<td>43/45</td>
<td>18/18</td>
<td>61/63</td>
<td>61/63</td>
</tr>
<tr>
<td><strong>Disease prevalence</strong></td>
<td>7/115 (6%)</td>
<td>22/175 (13%)</td>
<td>29/290 (10%)</td>
<td></td>
</tr>
<tr>
<td><strong>Positive predictive value</strong></td>
<td>54.9%</td>
<td>100%</td>
<td>74.5%</td>
<td></td>
</tr>
<tr>
<td><strong>Negative predictive value</strong></td>
<td>98.9%</td>
<td>97.7%</td>
<td>98.1%</td>
<td></td>
</tr>
</tbody>
</table>

Data are n/N (%) unless otherwise indicated. Culture-confirmed tuberculosis and not tuberculosis were the reference standards for sensitivity and specificity. Calculation of predictive values was based on disease prevalence in the paediatric cohort (n=296).

Table 2: Sensitivity, specificity, and predictive values of the T-cell activation marker—tuberculosis assay

---

**Table 3:**

<table>
<thead>
<tr>
<th>Culture-confirmed tuberculosis (n=18)</th>
<th>Highly probable tuberculosis (n=8)</th>
<th>Probable tuberculosis (n=12)</th>
<th>Not tuberculosis (n=63)</th>
<th>Indeterminate (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sensitivity (95% CI)</strong></td>
<td>83.3% (35.9–99.6)</td>
<td>83.3% (51.6–97.9)</td>
<td>83.3% (58.6–96.4)</td>
<td>83.3% (51.6–96.4)</td>
</tr>
<tr>
<td><strong>Positive/total</strong></td>
<td>5/6</td>
<td>10/12</td>
<td>22/25</td>
<td>25/30</td>
</tr>
<tr>
<td><strong>Specificity (95% CI)</strong></td>
<td>95.6% (84.9–99.5)</td>
<td>100% (81.5–100)</td>
<td>96.8% (89.0–99.6)</td>
<td>96.8% (89.0–99.6)</td>
</tr>
<tr>
<td><strong>Negative/total</strong></td>
<td>43/45</td>
<td>18/18</td>
<td>61/63</td>
<td>61/63</td>
</tr>
<tr>
<td><strong>Disease prevalence</strong></td>
<td>7/115 (6%)</td>
<td>22/175 (13%)</td>
<td>29/290 (10%)</td>
<td></td>
</tr>
<tr>
<td><strong>Positive predictive value</strong></td>
<td>54.9%</td>
<td>100%</td>
<td>74.5%</td>
<td></td>
</tr>
<tr>
<td><strong>Negative predictive value</strong></td>
<td>98.9%</td>
<td>97.7%</td>
<td>98.1%</td>
<td></td>
</tr>
</tbody>
</table>

Data are n/N (%) unless otherwise indicated. Culture-confirmed tuberculosis and not tuberculosis were the reference standards for sensitivity and specificity. Calculation of predictive values was based on disease prevalence in the paediatric cohort (n=296).

Table 2: Sensitivity, specificity, and predictive values of the T-cell activation marker—tuberculosis assay
### Table 4: Cases with discrepant culture and TAM-TB assay results: clinical characteristics at enrolment and follow-up visits by age

<table>
<thead>
<tr>
<th>Clinical case classification and reason for classification</th>
<th>Tuberculosis skin test or QuantiFERON</th>
<th>HIV status</th>
<th>First-visit clinical diagnosis, treatment, and response</th>
<th>Follow-up clinical diagnosis, treatment, and outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture-negative and TAM-TB assay-positive cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 years</td>
<td>Positive</td>
<td>Negative</td>
<td>Pneumonia Amoxycillin</td>
<td>Pulmonary tuberculosis later confirmed by culture and Xpert MTB/RIF</td>
</tr>
<tr>
<td>7 years</td>
<td>Positive</td>
<td>Negative</td>
<td>Pneumonia Amoxycillin</td>
<td>Pulmonary tuberculosis</td>
</tr>
<tr>
<td>9 years</td>
<td>Positive</td>
<td>Positive</td>
<td>Lymphadenitis Cafraxone</td>
<td>Tuberculosis lymphadenitis</td>
</tr>
<tr>
<td>11 years</td>
<td>Positive</td>
<td>Positive</td>
<td>Pneumonia Cafraxone</td>
<td>Pulmonary tuberculosis</td>
</tr>
<tr>
<td>5 years</td>
<td>Positive</td>
<td>Positive</td>
<td>Chest infection Cafraxone</td>
<td>Pulmonary tuberculosis</td>
</tr>
<tr>
<td>7 years</td>
<td>Positive</td>
<td>Positive</td>
<td>Pneumocystis pneumonia plus Mycobacterium intracellum Co-trimoxazole</td>
<td>Recovered from initial diagnosis</td>
</tr>
<tr>
<td>10 years</td>
<td>Positive</td>
<td>Positive</td>
<td>Bronchectasis Metronidazole</td>
<td>Bronchectasis, malaria</td>
</tr>
<tr>
<td>Culture-positive and TAM-TB assay-negative cases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 years</td>
<td>Positive</td>
<td>Negative</td>
<td>Pulmonary tuberculosis, malnutrition 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin</td>
<td>Pulmonary tuberculosis</td>
</tr>
<tr>
<td>8 years</td>
<td>Positive</td>
<td>Negative</td>
<td>Nephrotic syndrome Fuosuricide, prednisolone Deterioration, hospital admission</td>
<td>Pulmonary tuberculosis (only retrospectively confirmed)</td>
</tr>
<tr>
<td>2 years</td>
<td>Negative</td>
<td>Negative</td>
<td>Pulmonary tuberculosis, malnutrition 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin</td>
<td>Pulmonary tuberculosis</td>
</tr>
</tbody>
</table>

Positive tuberculin skin test suggested by a lesion of at least 10 mm in HIV-uninfected or at least 5 mm in HIV-infected children. TAM-TB=T-cell activation marker tuberculosis.
and one child died before the positive *M tuberculosis* culture result became available and treatment could be started. 33 of 37 children reported symptom resolution on treatment, one child with highly probable tuberculosis died, and three who were classified as culture confirmed, highly probable, or probable tuberculosis were lost to follow-up. No drug resistance was detected in the culture-confirmed cases.

Respiratory specimens could be collected from each child; 103 (91%) of 113 children provided at least three induced or two expectorated sputum samples. Figure 2 shows the overlap between children with a positive *M tuberculosis* culture, Xpert MTB/RIF, or TAM-TB assay, and table 2 shows the TAM-TB assay results. 15 of 18 children with culture-confirmed tuberculosis had a positive TAM-TB assay result (sensitivity 83·3%, 95% CI 58·6–96·4; table 3). Of the 63 cases classified as not tuberculosis, 61 had a negative TAM-TB assay (specificity 96·8%, 89·0–99·6; table 3). Findings were similar at the two independent sites Bagamoyo and Mbeya (table 3). The TAM-TB assay was positive in two (13%) of these 15 children. Xpert MTB/RIF enabled detection of 12 (67%) of 18 culture-confirmed tuberculosis cases, giving a sensitivity of 66·7% (95% CI 41·1–85·6) when two or more sputum samples obtained on consecutive days were analysed. The sensitivity was 55·6% (31·3–77·6) when only the first sputum sample was analysed. The specificity of Xpert MTB/RIF was 99·6% (94·2–100; data were missing for one person). Xpert MTB/RIF was positive in two of three children with culture-confirmed tuberculosis who were TAM-TB assay negative (figure 2). The sensitivity achieved with the combination of TAM-TB assay and Xpert MTB/RIF was 94·4% (72·6–99·1). The median time to detection by use of culture, defined as the period between enrolment and first *M tuberculosis* confirmation, was 19·5 days (IQR 14·0–45·0), whereas TAM-TB assay results were obtained within 24 h after recovery of peripheral blood mononuclear cells.

**Discussion**

The TAM-TB assay showed good sensitivity and excellent specificity with *M tuberculosis* culture as a reference standard. Specific detection of active tuberculosis in children was based on cutoff values set from the optimisation study in adults. Retrospectively lowering CD27 ratio thresholds to 2 for ESAT-6/CFP-10 and 7 for purified protein derivative stimulation, would further improve assay sensitivity from 83·3% to 88·9% (95% CI 88·0–99·4%) without affecting specificity with additional detection of highly probable (n=1), probable tuberculosis (n=1), and indeterminate (n=1) cases. Hence, cutoff values specifically optimised for children might further improve TAM-TB accuracy. Contrary to molecular-based assays like Xpert MTB/RIF and microbiological tests, the TAM-TB assay can be done on a readily available peripheral blood sample and is not limited by the paucibacillary nature of active tuberculosis in children. Consistent test performance at both study sites suggests the assay is robust and repeatable. Of greatest clinical relevance is that the TAM-TB assay provides an answer within a day of blood collection, which is important because early treatment initiation can be crucial in young children at high risk of disseminated tuberculosis disease.22

Of the children with discrepant results, five of eight children who were *M tuberculosis* culture-negative and TAM-TB assay positive had highly probable or probable tuberculosis. The clinical classification used might have included some overdiagnosis.1 International consensus definitions of intrathoracic tuberculosis were not available when the study was designed.23 However, detailed case assessment suggested that these children probably did have active tuberculosis, suggesting that the TAM-TB assay is probably at least as sensitive as *M tuberculosis* culture.

Despite access to state-of-the-art tuberculosis diagnostics, most children enrolled in our study were
treated for tuberculosis based on a combination of epidemiological and clinical findings. This shows the poor diagnostic usefulness of *M tuberculosis* culture in clinical practice, related to difficult specimen collection, suboptimum sensitivity in children with paucibacillary disease, and long turnaround times. Suboptimum sensitivity undermines the suitability of the test as a reference standard and complicates optimal assessment of diagnostic accuracy. Consistent with previous paediatric studies, Xpert MTB/RIF enabled the detection of culture-confirmed paediatric cases from expectorated or induced sputa with a sensitivity of 68·7% and a specificity of 100%. Compared with *M tuberculosis* culture, the combination of Xpert MTB/RIF and TAM-TB, two assays with a turnaround time of less than 24 h, had a sensitivity of 94%, missing one case. This child had severe nephrotic syndrome and culture-confirmation was only achieved after the child had passed away. The combination of TAM-TB assay and Xpert MTB/RIF enabled the detection of more tuberculosis cases than did culture when both culture-confirmed and highly probable tuberculosis cases were included in the reference standard.

The TAM-TB assay was highly accurate in identifying children without tuberculosis, including 13 children with a positive interferon-γ release assay or the result of the tuberculin skin test. Hence, the assay has a high specificity for tuberculosis disease in children despite immunological evidence of previous *M tuberculosis* exposure, consistent with data from the adult optimisation cohort and a previous study in adults. Only two not tuberculosis cases were incorrectly assigned. Both children were infected with HIV, one had simultaneous infection with non-tuberculous mycobacteria and both had latent tuberculosis infection. These false-negative TAM-TB results were caused by a predominance of mycobacteria-specific effector memory T-cell responses in peripheral blood. In view of a positive CFP-10/ESAT-6 response in the child without non-tuberculous mycobacteria infection, the positive TAM-TB assay result might suggest recent *M tuberculosis* infection that did not progress to active disease during the period of observation. In the other case, the non-tuberculous mycobacteria (*M interjectum*) identified in the sputum might have elicited the positive TAM-TB assay result. Implementation of additional highly immunogenic *M tuberculosis*-specific antigens in the next generation TAM-TB assay could further improve specificity and sensitivity.

The current version of the TAM-TB assay has several limitations, mainly related to cost and technical complexity. It requires advanced blood processing procedures, antigenic stimulations, flow cytometry equipment, and well-trained staff. Refinement and simplification are in progress to optimise diagnostic performance and make it compatible with cytometers that are in widespread use for measurement of CD4 T-cell counts in HIV/AIDS-affected countries. The TAM-TB assay did not generate valid test results in 13% of the samples of peripheral blood mononuclear cells, but this might be attributed to reduced T-cell viability and decreased cytokine production after cryopreservation. Ideally, the TAM-TB assay should be done on fresh whole blood samples—eg, in antigen precoated tubes similar to the commercial QuantiFERON In-Tube system (Cellexis). Low CD4 T-cell counts due to severe HIV infection might remain a problem even when fresh blood samples are used. The small sample size of this proof-of-concept study resulted in very wide confidence intervals and did not allow systematic assessment of test performance in very young, malnourished, or HIV-infected children who have recently been started on antiretroviral therapy. These factors might negatively affect the accuracy of the TAM-TB assay. Additional studies need to be done to specifically address test performance in these patient groups. The assessment of the TAM-TB assay in a study population enriched for culture-confirmed tuberculosis cases might have been a source of bias, particularly for the analysis including the under-represented classification groups. Although the TAM-TB assay cannot provide information about drug susceptibility, use of concomitant Xpert MTB/RIF testing can address this limitation.
To our knowledge, this study is the first to assess the diagnostic performance of the novel TAM-TB assay in children (panel). Importantly, it was done in a region with a high tuberculosis incidence and recruited children with symptoms that suggested tuberculosis who represent the real-life diagnostic challenge in these settings. Despite a need for further refinement and testing in other regions with high burden of tuberculosis, our results suggest that the spu-tum-independent TAM-TB assay is a major advance for the rapid and accurate diagnosis of tuberculosis in children.

Contributors

KR, MH, and GG designed the study. GG designed the TAM-TB assay. PC, ES, AR, NEN, EM, KS, FH, FL, LI, MH, and KR oversee enrolment, patient care, or standard laboratory work. DF, FM, AB, MC, MM, CG, and CD were responsible for the flow-cytometry analysis. DF, FM, ES, KR, and GG did the data management and analysis. DF, KR, GG, CD, and BM wrote the draft of the report. BJM provided expert advice. All authors contributed to data gathering and interpretation, and revision of the report.

Declaration of interests

We declare no competing interests.

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References

7.1.2 Antiviral Innate Immune Activation in HIV-Infected Adults Negatively Affects H1/IC31-Induced Vaccine-Specific Memory CD4+ T Cells

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Tuberculosis (TB) remains a global health problem, with vaccination being a necessary strategy for disease containment and elimination. A TB vaccine should be safe and immunogenic as well as efficacious in all affected populations, including HIV-infected individuals. We investigated the induction and maintenance of vaccine-induced memory CD4+ T cells following vaccination with the subunit vaccine H1/IC31. H1/IC31 was inoculated twice on study days 0 and 28 among HIV-infected adults with CD4+ lymphocyte counts of >350 cells/mm^3. Whole venous blood stimulation was conducted with the H1 protein, and memory CD4+ T cells were analyzed using intracellular cytokine staining and polychromatic flow cytometry. We identified high responders, intermediate responders, and nonresponders based on detection of interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) expressing central (TCM) and effector memory CD4+ T cells (TEM) 182 days after the first immunization. Amplicon-based transcript quantification using next-generation sequencing was performed to identify differentially expressed genes that correlated with vaccine-induced immune responses. Genes implicated in resolution of inflammation discriminated the responders from the nonresponders 3 days after the first inoculation. The volunteers with higher expression levels of genes involved in antiviral innate immunity at baseline showed impaired H1-specific TCM and TEM maintenance 6 months after vaccination. Our study showed that in HIV-infected volunteers, expression levels of genes involved in the antiviral innate immune response affected long-term maintenance of H1/IC31 vaccine-induced cellular immunity. (The clinical trial was registered in the Pan African Clinical Trials Registry [PACTR] with the identifier PACTR201105000289276.)

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production of abundant quantities of type I interferon (IFN) (6, 7).

In HIV-infected individuals, vaccine take and durability of the immune system are negatively affected upon manifestation of progressed HIV disease (8). The HIV is detected by the innate immune system, primarily by recognition of viral nucleic acids. Endosomal TLR-7 and -8 and cytosolic RIG-1-like receptors sense single-stranded viral RNA (ssRNA), which leads to secretion of IFN-α/β. DCs and monocytes express TLR-7 and -8 and cytosolic RIG-1-like receptors sense IC31 in BCG-vaccinated, HIV-infected adults with a CD4+ T cell count of 350 cells/mm³ (10). The principal finding was that the vaccine was safe and immunogenic. CD4+ lymphocyte counts and viral loads remained stable during the entire study period. The aim of this study was to analyze mRNA expression levels and T cell responses to boost vaccination in HIV-infected individuals, since the downstream signaling cascades of TLR-7, TLR-8, and TLR-9 are shared (6, 7).

We conducted a phase II, double-blind, randomized, placebo-controlled trial to evaluate the safety and immunogenicity of H1/IC31 in BCG-vaccinated, HIV-infected adults with a CD4+ lymphocyte count of >350 cells/mm³ (10). The principal finding was that the vaccine was safe and immunogenic. CD4+ lymphocyte counts and viral loads remained stable during the entire study period. The aim of this study was to analyze mRNA expression levels and T cell responses to boost vaccination in HIV-infected individuals, since the downstream signaling cascades of TLR-7, TLR-8, and TLR-9 are shared (6, 7).

For ICS, samples from each phlebotomy date of one particular participant were thawed simultaneously in a water bath at 37°C. Cells were transferred to tubes containing phosphate-buffered saline (PBS) (BioWhittaker), washed, and permeabilized using Perm/Wash solution (BD Biosciences). The flow cytometry staining was completed with the following anti-human antibody panel: CD3-BV421 (MOPC-21; BD Biosciences), CD4-BV505 (653; Invitrogen), CD8-peridinin chlorophyll protein (PerCP)-Cy5.5 (SK1; BD Biosciences), CCR7-phycocerythrin (PE) (120503; eBioscience), IFN-γ-APC (5607; BD Biosciences), tumor necrosis factor alpha (TNF-α)-Cy7-PE (M111; eBioscience), interleukin-2 (IL-2)-fluorescein isothiocyanate (FITC) (5344.111; BD Biosciences), IL-17A-647 (SCPL1362; eBioscience), and CD45RA-BV570 (H1001; eBioscience) (see Table 2.1 in the MyFlowCyt Reporting Standard in the supplemental material). For each fluorochrome, a single-stained mouse IgG1 control was included. The samples were acquired on an LSR II flow cytometer (BD Biosciences, San Jose CA) equipped with green (532 nm), red (640 nm), and blue lasers (488 nm) (see Table 3.1 in the MyFlowCyt Reporting Standard in the supplemental material). On average, 900,000 events per sample were recorded. Data analysis was performed using FlowJo software version 10.0.7 (Tree Star). The following gating strategy was used (Fig. 1): CD3-positive cells were gated by plotting CD3-BV421 versus side scatter area (SSC-A). The singlets were selected by plotting forward scatter area (FSC-A) versus forward scatter height (FSC-H), and the lymphocytes were gated with FSC-A versus SSC-A. Time versus IL-2-FITC was gated to exclude bubbles and various fluidics pressures. The IFN-γ-Alexa 700 versus CD8-PerCP-Cy5.5 plot was used to gate the CD8-negative cells. The CD4-positive cells were selected by plotting IFN-γ-Alexa 700 versus CD4-PE 605. The memory subsets were defined using the CD45RA-BV570 versus CCR7-PE plot. To identify cytokine-positive cells from all 4 memory subsets, each cytokine was plotted against CD4-PE 605. FlowJo version 10.0.7 was used for the analysis. This project focused on the detection of the cytokines IL-2, IFN-γ, and TNF-α. The production of IL-17 by CD4+ T cells was not significantly different after booster vaccination (10).

A detailed description following the MyFlowCyt Reporting Standard for flow cytometry experiments (13) can be accessed in the supplemental material.

Extraction of total RNA. Total RNA was directly extracted from PaxGene blood RNA tubes using the PaxGene blood RNA kit (PreAnalytiX; Hombrechtikon, Switzerland) according to the manufacturer’s protocol.

The concentration of total RNA and the RNA integrity number were determined by an Agilent RNA 6000 nano kit (Agilent Technologies, Waldbronn, Germany). RNA samples were stored at −80°C.

AmplicSeq panels. We used two custom-made AmplicSeq primer panels to quantify gene expression. The Pathway Reporter Panel covered 917 genes, providing a general snapshot of the whole human transcriptome. The rationale and design of this panel are described elsewhere (35).

The second AmplicSeq primer panel, the Immune Response Panel, was developed based on a literature-driven collection of 526 genes implicated in innate and adaptive immune responses. Functional enrichment analysis with the biological process (BP) terms of Gene Ontology suggests that the prioritized genes are highly enriched in innate and adaptive immune response pathways. The selected genes were submitted via a web interface
for primer design and synthesis using proprietary algorithms (AmpliSeq; Life Technologies).

**AmpliSeq technology**

The amplification and quantification of mRNA for subsequent analysis was performed using a capillary-based system, specifically the Ion PicoArray, with the Ion PicoArray HD Kit and the Ion Torrent PGM (Life Technologies, Carlsbad, CA, USA). Libraries were made from 5-20 ng of total RNA, according to the manufacturer’s recommendations. The amplified libraries were then used for sequencing on the Ion PicoArray HD System, and the generated reads were aligned to the reference genome (10). The statistical analysis was performed using the MiSeq software (14). After correction for the T cell response obtained from unstimulated controls, the absolute counts of cytokine- (IFN-\(\gamma\) and/or IL-2 and/or TNF-\(\alpha\))-expressing T~CM~ or T~EM~ and counts of T~CM~ or T~EM~ negative for cytokine expression were compared between day 0 and each follow-up (day 14, day 56, day 70, and day 182) (see Table 2). Final responders were defined based on a comparison of day 0 to day 182 and with a false discovery rate (FDR) of \(>0.0001\) for a non-responder, an FDR of \(>10^{-15}\) for an intermediate responder, and an FDR of \(<10^{-15}\) for a high responder.

**RESULTS**

**H1/IC31 phase II clinical trial design.** We conducted a phase II, double-blind, randomized, placebo-controlled trial in Bagamoyo,

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**FIG 1 Gating strategy used for flow cytometric analysis of H1-induced memory CD4\(^+\) T cell cytokine expression.** The plots show the sequential gating hierarchy of one representative sample: CD3\(^+\) T cells; single cells; lymphocytes; time; and CD8\(^+\) T cells, which were further gated for CD4\(^+\) cells and split into all memory subsets on the basis of CCR7 and CD45RA surface markers. The bottom row comprises the plots with the cytokine expression gates for H1 fusion protein-stimulated T~EM~ at day 182. IL-2, TNF-\(\alpha\), and IFN-\(\gamma\) expression levels were measured. T~CM~, central memory CD4\(^+\) T cells; T~EM~, effector memory CD4\(^+\) T cells; H1, hybrid virus.
TABLE I H1/IC31 clinical trial overview and results

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Placebo group (n = 4)</th>
<th>H1/IC31 vaccinated group (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median [IQR] yr)</td>
<td>35 (26–49)</td>
<td>39 (34–45)</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>3/1</td>
<td>9/10</td>
</tr>
<tr>
<td>No. (%) with prior BCG vaccination (self-report)</td>
<td>4 (100)</td>
<td>19 (100)</td>
</tr>
<tr>
<td>CD4 count (median [IQR] cells/mm³)</td>
<td>575 (425–785)</td>
<td>19 (100)</td>
</tr>
<tr>
<td>Viral load (median [IQR] copies/mL)</td>
<td>33,802 (514–49,875)</td>
<td>37,932 (8,754–50,950)</td>
</tr>
</tbody>
</table>

*IQR, interquartile range; F, female; M, male.

Table 1: Clinical trial overview and results.

Placebo group and H1/IC31 vaccinated group were compared for various parameters such as age, gender, CD4 count, and viral load.

Tanzania (10). Twenty-four HIV-positive volunteers were enrolled and vaccinated twice with H1/IC31 (n = 20) or placebo (n = 4) at baseline (day 0) and day 56. One H1/IC31-vaccinated volunteer dropped out due to pregnancy. Whole-blood samples for transcriptome analysis were taken on study days 0, 3, and 59 after each vaccination. Whole-blood samples for immunogenicity assays were taken on study days 0, 14, 56, 70, and 182 for long-term follow-up. All volunteers were BCG vaccinated prior to the study and were antiretroviral therapy (ART) naive with CD4 counts of >350 cells/mm³ (Table 1). Generally, the H1/IC31 vaccine was safe and well tolerated with no significant impact on the CD4⁺ lymphocyte count and HIV load after vaccination.

Cytokine-producing memory CD4⁺ T cell subsets. Based on chemokine receptor CCR7 and surface marker CD45RA expression, two subsets of CD4⁺ memory T cells, Tcm and TEM, were identified (Fig. 1) (18). We measured the development and magnitude of the H1/IC31-induced memory CD4⁺ T cell subsets expressing IL-2, IFN-γ, and TNF-α following in vitro stimulation with the H1 protein. At day 182, TCM and TEM were significantly elevated compared to levels in placebo controls (see Fig. S1 in the supplemental material). To measure the quality of the immune response, we discriminated the memory CD4⁺ T cell subsets according to their polyfunctional cytokine expression profile. Figure 2A displays the mean percentages of cytokine expression of all memory subsets according to their polyfunctionality. In agreement with Reither et al. (10), the H1/IC31-specific TCM and TEM…

FIG 2: Cytokine expression profile of memory CD4⁺ T cell subsets following H1 fusion protein stimulation. Volunteers receiving H1/IC31 at days 0 and 56 are included. (A) Mean percentages of all memory CD4⁺ T cell subsets on the basis of all possible combinations of IL-2, IFN-γ, and TNF-α expression are given. Radial and not area of each segment represent the percentages of CD4⁺ T cell subsets (CM and TEM) expressing all combinations of IL-2, IFN-γ, and TNF-α expression following stimulation with H1. P values correspond to significance testing comparing each study day to study day 0 (a = 0.05, b = 0.0125, c = 0.001; **, P < 0.001). A two-sided Wilcoxon signed-rank test with the Bonferroni correction was applied. TCM, central memory CD4⁺ T cells; TEM, effector memory CD4⁺ T cells; H1, H1 vaccine; 1, 1 vaccination days.
were predominantly trifunctional or bifunctional, expressing IL-2 and TNF-α.

In Fig. 2B and C, TCM and TEM are displayed separately. In a comparison of day 0 to day 182, statistically significant increases of IL-2 and TRAIL in TCM were observed. This was also observed in bifunctional TCM expressing IL-2 with TNF-α (P < 0.05) after 182 days. The expression levels of IL-2 and TRAIL were significantly increased in TEM compared to baseline. The expression levels of IL-2 and TRAIL in TEM were significantly increased in TEM compared to baseline.

Definition of vaccine responder groups. To investigate potential innate immune mechanisms that yield differential vaccine immunogenicity in our study participants, we divided the volunteers according to their cytokine responses (IFN-γ, IL-2, and/or TNF-α), comparing H1-specific TCM and TEM.

An overview of potential responder groups comparing each study day to the baseline at day 0 is given in Table 2. In a comparison of day 70 to day 182, several volunteers shifted from responders to nonresponders, indicating discriminative memory maintenance.

No significant difference in relation to the viral load and CD4+ lymphocyte counts between these groups was observed (Table 3).

Grouping the volunteers based on a comparison of day 0 to day 182 allowed us to identify the innate immune mechanisms that potentially lead to improved TCM and TEM maintenance.

Gene expression data. On average, 4,686,802 reads (95% confidence interval [CI], 4,712,807 reads) per sample were generated and mapped to either the 826 genes of the AmpliSeq Immune Response Panel or the 917 genes of the Pathway Reporter Panel. A dynamic range of 5 orders of magnitude was observed, ranging from genes with no detectable expression like IFN-γ to genes expressing more than 160,000 reads like lysozyme and CD74. Both AmpliSeq panels shared 355 genes, whose transcript abundance measurements closely correlated (R² = 0.9829).

The expression levels of nine endogenous control genes did not differ significantly between the 24 volunteers included here across all time points, supporting the robustness of the AmpliSeq-based targeted transcriptome measurement approach (Fig. 3A).

Vaccine-induced differential gene expression. Next we addressed if early changes in the whole-blood gene expression levels of the targeted 1,388 genes might distinguish the three responder groups of each memory subset. The results are shown in Fig. 3B, and more detailed information is provided in Table S1 in the supplemental material. TCM and TEM intermediate and high responders showed upregulated gene expression levels 3 days postvaccination for TCM responders, 5 genes, namely, COL1A1, ELN, CTFG, SERPINE1, and POSTN, were identified as upregulated. TEM responders had an additional 5 genes, including COL3A1, COL1A2, FN1, ID1, and DCN, with increased expression levels.

Most of the 10 differentially expressed genes are either annotated in BTMs involving integrin interactions or extracellular matrix remodeling (ELN or SERPINE1). ID1 is involved in leukocyte differentiation. HSPA1A is the only gene which was upregulated in a TEM nonresponder. This gene encodes a heat shock protein and is involved in the stress response. No differential gene expression was observed in the placebo controls.

Differential immune activation at baseline. Next we addressed if gene expression levels at baseline before the first vaccination could distinguish the responder groups. Table 4 shows an overview of all genes that differed significantly at baseline between the vaccine responder groups based either on TCM or TEM. Compared to levels in nonresponders, higher gene expression levels were observed for ITGB4, CLEC1C, and CXCL6 for TCM high responders and for PRKDC, CD19, DNAJB5, MARCO, and IRS2 for TEM high responders. TGM2, OAS1, and IFI27 showed the lowest expression levels among TCM high responders, whereas AICDA, NFATC2, IFI27, IFIT3, IFIT1, and CXCL10 were expressed at lower levels in TEM high responders. Tissue transglutaminase (TGM) has a role in HIV pathogenesis and enhanced levels of its degradation product, α(ε-glutamyl)lysine, are found in the blood of patients with progressed HIV disease (19). AICDA and NFATC2 take part in the antiviral immune response with the

### Table 2: Overview of responder groups based on either TCM or TEM

<table>
<thead>
<tr>
<th>T cell type</th>
<th>Day 14</th>
<th>Day 56</th>
<th>Day 70</th>
<th>Day 182</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM</td>
<td>6/6</td>
<td>11/35</td>
<td>5/68</td>
<td>8/56</td>
</tr>
<tr>
<td>TEM</td>
<td>3/5/11</td>
<td>4/5/10</td>
<td>2/25</td>
<td>4/69</td>
</tr>
</tbody>
</table>

### Table 3: Demographics including information about HIV infection status of final responder groups based on the comparison of day 0 to day 182.

<table>
<thead>
<tr>
<th>Day 182 responder group</th>
<th>No. of volunteers</th>
<th>Age (yr)</th>
<th>No. F/No. M</th>
<th>Viral load (log10)</th>
<th>CD4+ count (cells/mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>4</td>
<td>38 ± 13</td>
<td>3/1</td>
<td>11.5 ± 5.3</td>
<td>575 ± 250</td>
</tr>
<tr>
<td>TCM</td>
<td>High responder</td>
<td>6</td>
<td>35 ± 5</td>
<td>2/4</td>
<td>12.1 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>Intermediate responder</td>
<td>5</td>
<td>46 ± 5</td>
<td>2/3</td>
<td>14.0 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>Nonresponder</td>
<td>8</td>
<td>38 ± 6</td>
<td>6/2</td>
<td>14.3 ± 2.7</td>
</tr>
<tr>
<td>TEM</td>
<td>High responder</td>
<td>9</td>
<td>38 ± 7</td>
<td>4/5</td>
<td>12.3 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>Intermediate responder</td>
<td>6</td>
<td>40 ± 9</td>
<td>2/4</td>
<td>14.4 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>Nonresponder</td>
<td>4</td>
<td>39 ± 5</td>
<td>4/0</td>
<td>14.0 ± 1.2</td>
</tr>
</tbody>
</table>

*Data are means ± SD.

F: female; M: male.
Antiviral Immune Activation and Vaccination Outcome

FIG 3 (A) Gene expression of endogenous control genes. Variations in expression levels of nine control genes between subjects and over time and log 2-fold changes between baseline and the two visits after each vaccination for all 24 volunteers are shown. (B) Heat map showing vaccine-induced differential gene expression levels between TCM and TEM responder groups. Log2-fold changes in gene expression levels 3 or 59 days after vaccination compared to prevaccination levels are shown. Differentially expressed genes were identified using edgeR with an FDR of <0.1 (**, significant for TCM and TEM; *, significant for TEM only). Only changes in gene expression 3 days after vaccination were significant.

roles of affinity maturation and class switching of antibodies and inhibition of viral replication (19, 20). According to annotation with BTMs, the lower expressed genes indicated in bold above are involved in innate antiviral immunity. We analyzed the expression levels of all genes available in both AmpliSeq panels that belonged to these BTMs (Fig. 4A). Clearly, at baseline both nonresponder groups showed higher transcription levels of members of BTM M150 (innate antiviral response), M165 (enriched in activated dendritic cells), M75 (antiviral IFN signature), M127 (type I interferon response), M13 (innate activation by cytosolic DNA sensing), and M68 (RIG-1-like receptor signaling).

The expression levels of genes involved in innate immunity to viruses (IRF7, IFI27, IFIT3, EIF2AK2, DHX58, IFIT1, CCL8, and OAS1) showed an association with HIV-1 viral loads (Fig. 4B). Toll-like receptor 8 variant. The AmpliSeq approach allows for nucleotide sequencing of the amplified fragments using the Ion Torrent approach. We are aware that this study was not designed (e.g., powered) to assess the association of polymorphic genes with vaccine-induced immune responses. One single nucleotide polymorphism (SNP) in TLR-8 (rs3764880) was previously reported to result in slower disease progression in HIV-infected individuals and reduced activation of the NF-κB pathway (21). The functional variant of TLR-8 showed the A1G polymorphism (rs3764880) that alters the start ATG codon of TLR-8 into a GTG triplet. The resulting truncated TLR-8 (1,038 amino acids [aa]) exhibits a shorter signal peptide. Seven volunteers expressed the TLR-8 A1G; of these, three volunteers harbored the homozygous TLR-8 A1G. Interestingly, none of the volunteers with the TLR-8 A1G belonged to the TEM nonresponder group, and all three subjects expressing the homozygous TLR-8 A1G were TCM high responders. Importantly, subjects with the homozygous TLR-8 A1G have a reduced expression of antiviral gene signatures at baseline (Fig. 5A). Independent of the responder grouping, at day 182 volunteers with the homozygous TLR-8 A1G have on average the highest percentages of trifunctional and bifunctional (IL-2 and TNF-α) TCM and TEM (Fig. 5B and C).

DISCUSSION

The study presented here provides the first insights into the possible link between chronic immune activation in HIV infection and lack of maintenance of vaccine-induced TCM and TEM responses. Data on the impact of chronic, untreated HIV infection on experimental and routine vaccination outcomes are scarce (22). The identification of immune-related genes that are expressed before vaccination or induced shortly after vaccination influencing cellular immune responses in HIV-positive volunteers will be of great value (23).

Determinants of induction and long-term maintenance of memory CD4+ T cell responses generally in humans are only partially known (24). The H1/IC31 vaccine has been tested in several phase I clinical trials, including HIV-negative and BCG-unvaccinated and BCG-vaccinated and M. tuberculosis-exposed European volunteers (4, 25). In the BCG- and M. tuberculosis-naive European volunteers, IFN-γ-producing H1-specific T cells were detectable by ELISPOT analysis until 131 weeks after the first vaccination. Only limited retraction of IFN-γ production in cell culture

TABLE 4 Genes which are differentially expressed between the different vaccine responder groups for TCM and TEM

<table>
<thead>
<tr>
<th>T cell type</th>
<th>Genes with higher expression in responders</th>
<th>Lower than those in nonresponders</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCM</td>
<td>ITGB4, CLEC1A, CXCL6</td>
<td>TGM2, OAS1, IFI27</td>
</tr>
<tr>
<td>TEM</td>
<td>PRKDC, CD19, DNAJB5, MARCO, IRS2</td>
<td>AICDA, NFATC2, IFIT3, IFIT1, CXCL10</td>
</tr>
</tbody>
</table>

aDifferentially expressed genes were identified using edgeR with an FDR of <0.1 and are grouped as having higher or lower expression levels in the high-responder group than in the nonresponder group.

bGenes in bold are involved in innate antiviral immunity.
supernatants from H1-stimulated peripheral blood mononuclear cells (PBMCs) was measured between study weeks 14 and 131 (4). In the current study, we observed an expansion of H1-specific TCM and TEM in all volunteers at study day 70, which is 2 weeks after the booster vaccination. However, when followed up until day 182, a retraction of H1-specific TCM and TEM was observed. Interestingly, this retraction differed greatly between volunteers, which allowed us to group them based on the comparison of H1/IC31-induced TCM and TEM responses on day 182 in relation to those on day 0.

Compared with microarray analyses, the AmpliSeq approach has a higher sensitivity and dynamic range, and its results compare precisely with RNA sequencing-derived whole-transcriptome data (11). Results obtained with both gene panels strongly supported the idea that the AmpliSeq-based transcriptome monitoring approach is very robust and results in highly reproducible data sets suitable for high-throughput analysis of clinical samples. Here, to our knowledge, we have employed this targeted transcriptome monitoring tool in an experimental vaccine trial for the first time.

Higher expression levels of genes involved in the extracellular matrix and integrin interactions and cell adhesion were observed in volunteers who maintained H1-specific TCM and TEM responses until day 182. Searching the literature, we found that cells involved in wound healing like alternatively activated macrophages (26), μ6T cells (27), monocyte-derived multipotential cells (28), fibrocytes (17), and endothelial progenitor cells (29) have all been described to express these particular genes.

Muyanja et al. showed that the cellular and humoral immune responses to the highly efficacious live yellow fever vaccine YF-17D in East African individuals were substantially lower than those of European volunteers treated identically (30). Prior to vaccination, the East African volunteers presented an increased activated immune microenvironment with higher frequencies of exhausted and activated natural killer cells, differentiated B and T cells, and proinflammatory monocytes (30). We therefore compared the gene expression profiles measured at baseline between our responder groups. Significantly higher expression levels of genes involved in innate antiviral immune responses like type I IFN signaling and viral sensing through cytosolic RIG-I-like receptors were observed in the nonresponders. A close correlation between HIV loads and expression levels of this group of genes indicates that the chronic HIV infection is driving the higher expression levels of these antiviral responses. In line with these re-
The activation of RIG-1-like receptors resulted in selective suppression of TLR signaling and thus supports our findings (33). The HIV was found to specifically infect activated memory CD4+ T cells with potential consequences for the maintenance of vaccine-specific memory responses (34). In conclusion, higher activation of the components of the innate immunity due to HIV stimulation at baseline results in impaired H1-specific TCM and TEM maintenance.

In addition to access to the expression level, the AmpliSeq approach also provides access to the nucleotide sequence of the respective amplified gene fragment. This enabled us to identify volunteers carrying the previously known TLR-8 A1G single nucleotide sequence variant. Volunteers expressing this TLR-8 A1G gene on both chromosomes had lower gene expression levels of the innate antiviral immune response genes. This was linked to longer maintenance of vaccine-specific TEM responses until day 182 compared to that for the wild-type TLR-8 carriers.

The inverse relationship between chronic innate antiviral immune activation by HIV and sustained H1/IC31-induced vaccine-specific cellular immune responses has high relevance for future vaccine development and monitoring programs. Patients receiving ART potentially present with lower activation of innate antiviral immunity, and thus the effect of treating patients with ART prior to vaccination has to be evaluated. The presence of the TLR-8 A1G variant in HIV-infected individuals might provide an immune genetic background, supporting a more desired vaccination outcome. This observation warrants future studies using larger numbers of HIV-infected and -noninfected volunteers undergoing experimental or routine vaccination.

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Maturation and Mip-1β Production of Cytomegalovirus-Specific T Cell Responses in Tanzanian Children, Adolescents and Adults: Impact by HIV and Mycobacterium tuberculosis Co-Infections

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Abstract

It is well accepted that aging and HIV infection are associated with quantitative and functional changes of CMV-specific T cell responses. We studied here the expression of Mip-1β and the T cell maturation marker CD27 within CMVpp65-specific CD4+ and CD8+ T cells in relation to age, HIV and active Tuberculosis (TB) co-infection in a cohort of Tanzanian volunteers (16 years of age, n = 108 and >18 years, n = 79). Independent of HIV co-infection, IFNγ+ CMVpp65-specific CD4+ T cell frequencies increased with age. In adults, HIV co-infection further increased the frequencies of these cells. A high capacity for Mip-1β production together with a CD27low phenotype was characteristic for these cells in children and adults. Interestingly, in addition to HIV co-infection active TB disease was linked to further down regulation of CD27 and increased capacity of Mip-1β production in CMVpp65-specific CD4+ T cells. These phenotypic and functional changes of CMVpp65-specific CD4 T cells observed during HIV infection and active TB could be associated with increased CMV reactivation rates.

Introduction

Primary cytomegalovirus (CMV) infection occurs often at birth or during adolescence with prolonged viral shedding into the urine and saliva that can persist for years. In adults, primary CMV infection is typically controlled within 6 months [11]. Thereafter, CMV persists lifelong in
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A state of clinical latency [2,3]. The CMV-specific adaptive immune response efficiently suppresses overt viral replication and prevents viral shedding for decades. Aging is notably associated with accumulation of high frequencies of oligoclonal CMV-specific CD8+ T cells [3]. In late-stage AIDS patients (CD4 counts <50/μl), CMV infection can cause disseminated or localized end-organ disease [3]. CMV-specific CD4+ T cell responses appear to be crucial for CMV control in murine models and in humans [6,43]. In adults, these CMV-specific CD4+ T cells are characterized by: (i) highly differentiated CD27-/CCR7 effector memory phenotype; (ii) propensity to secrete the CCR5 ligand Mip-1β and (iii) high cytolytic activity against CMV infected cells and these characteristics differ from HIV- or Vaccinia virus-specific CD4+ T cells [7].

CMV-specific CD4+ T cells are relatively resistant to HIV infection and HIV mediated depletion [8], [9], [10], and this has been linked to their propensity to secrete Mip-1β. Mip-1β has also been connected to the expression of the immune senescence marker CD57 [11]. However, the underlying immunological mechanisms that “imprint” this particular phenotype and high capacity for Mip-1β production are unclear. Recent or on-going antigen exposure causes differentiation of pathogen-specific CD4+ T cells into CD27+ effector memory phenotype and can inflict functional changes [7,10,12–14]. One factor contributing to an increased capacity to produce Mip-1β could be recurrent or transient, subclinical reactivation of CMV throughout life. To address this hypothesis, we studied cell frequencies, Mip-1β production and CD27 expression of CMV-specific CD4+ and CD8+ T cells in children, adolescence and adults in relation to age, HIV and active tuberculosis infection in individuals from Tanzania, where CMV infection prevalence reaches up to 100% in adults [15].

Results and Discussion

Detection of CMV-specific T cell responses using polychromatic flow cytometry after in vitro stimulation is a reliable marker for determining the CMV infection status in children [16]. CMVpp65-specific CD4+ and CD8+ T cell responses were characterized in a Tanzanian cohort (n = 187) encompassing children (<10 years of age, n = 79), adolescents (10–16 years of age, n = 29) and adults (>18 years of age, n = 79). This Tanzanian cohort is described in greater detail [17]. The gating strategy and representative flow cytometry plots are shown in Fig 1. Study subjects were stratified according to age and their HIV and TB co-infection status. In the absence of a reliable test for definite childhood TB classification, HIV- paediatric TB suspects included culture-confirmed TB (n = 14) and uncertain TB classification (n = 16). HIV+ paediatric TB suspects included culture confirmed TB (n = 4), and 13 subjects with uncertain TB classification (n = 13). In 46 HIV+ and 13 HIV+ children an alternative diagnosis could be established after 5 months of clinical follow-up excluding active TB [17].

As expected, the prevalence of CMVpp65-specific T cell responses increased with age (Fig 2A). In the HIV+ group, CMVpp65-specific T cell responses were detectable in 48.5% (16 of 33) of children <5 years of age, in 77.3% (17 of 22, Fig 2B) of children between 5 and 10 years, in 81% (17 of 21) of adolescents (10–16 years) and 96% (48 of 50) of adults (Fig 2A, P<0.0001). In the HIV+/AIDS group, CMVpp65-specific T cell responses were detectable in 70% (7 of 10) of children <5 years of age, in 92.8% (13 of 14) of children (5–10 years), in 87.5% (7 of 8) of adolescents (10–16 years) and 89.7% (26 of 29) of adults. Interestingly, in children <10 years of age, HIV infection was associated with an increased prevalence of CMVpp65-specific T cell responses (p = 0.067). These data suggest that within the studied Tanzanian population, CMV infections are mostly acquired within the first 10 years of life with HIV infection being a potential risk factor for early acquisition of CMV infection. When compared to West-African populations, CMV infection seems to be acquired at an older age where by the age of
Fig 1. Gating strategies and CMV responses evaluation. A) Morphological and CD marker expression gating strategy of CD4 and CD8 T cell. B) Representative CD4 and CD8 T cell IFNγ response from a significant responder. C) Gating strategy for the CD27 MFI Fold Change calculation of IFNγ producing cells compared to all CD4 or CD8 T cells. D) Gating strategy for the determination of IFNγ producing cells that were also positive for Mip1β production.

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Fig 2. CMVpp65-specific T cell responses in relation to age and HIV infection status. (A) Frequency of subjects with or without CMVpp65-specific T cell responses (CD4+ or CD8+) is shown stratified by age group and HIV status. B) The frequency of subjects with CMVpp65-specific T cell responses distributed within the CD4+ and/or CD8+ T cell compartment is shown after stratification by HIV status. The number of subjects in each group is indicated within brackets. CMVpp65-specific T cell responses were considered positive if the frequency of IFNγ+ T cells was 2-fold above the negative control and at least 0.05% of the parent population. Statistical analysis was performed using the chi-square test for trend (A) and chi-square test (B).

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12 months close to 100% of children showed serological evidence of CMV infection or had released CMV DNA in urine [18,19].

CD4 T cell responses targeting purified protein derivatives (PPD) followed a similar trend (data not shown), indicating that detection of Mycobacteria-specific T cells in adolescents and adults are not a consequence of BCG vaccination during early infancy (BCG vaccination coverage for patients <18 years was 97.7% (43 of 44, unknown status n = 1). These findings are consistent with the disappearance of circulating Mycobacteria-specific CD4 T cell responses upon BCG vaccination within one year following vaccination [20].

Next, we compared the contribution of CD4+ or CD8+ T cells to the CMV-specific T cell responses in children (<10 years) and adults (>18 years). A high number of children (36.4% for HIV- and 45% for HIV+) mounted CMV responses exclusively within the CD8+ T cell subset irrespective of their HIV status. In HIV+ individuals, the difference between children and adults reached statistical significance with more individuals having both CD4+ and CD8+ CMV-specific T cell responses in the adult HIV+ group (Fig 2B, P = 0.037).

Adults showed increased frequencies of CMVpp65-specific CD4+ T cells compared to children <10 years regardless of their HIV infection status (Fig 3A, HIV-: median 0.15% vs. 0.06%, P = 0.0004; HIV+: median 0.26 vs. 0.07%, P = 0.002). A higher frequency of CMVpp65-specific CD8+ T cell responses in adults was also observed, particularly within the context of HIV
infection (Fig 3C, median 0.15% vs. 0.37%, P = 0.017) [4,21]. Absolute counts of CMVpp65-specific CD4+ T cells no longer increased with age and were significantly affected by HIV co-infection in children and adults (Fig 3B, P = 0.045 and P = 0.0276 respectively). In line with previous reports showing age related differences in the proportion of IFNγ+ T cells in response to SEB stimulation [22–24], we also observed significantly reduced frequencies and absolute counts of IFNγ+CD4+ T cell responses after SEB stimulation in children (Fig 4). This could reflect the higher proportion of naïve T cells in children, that cannot express IFNγ [25] and might also be linked to IFNγ promoter hyper methylation of naïve T cells in infants [26]. Importantly, the proportion of responders with CMVpp65-specific CD4+ T cells was similar between HIV+ (12 of 19, 63.1%) and HIV− (11 of 20, 55%) children (P = 0.53). This demonstrates that CMV-specific CD4+ T cells persisted in children <10 years of age during concurrent HIV infection as described previously for adults [10,27], even though absolute numbers of CMVpp65-specific CD4+ T cells were reduced in HIV+ adults and children (Fig 3B). Relative resistance of CMV-specific CD4+ T cells to HIV-induced depletion has been linked to their high capacity for Mip-1B secretion [7] together with their mature phenotype, characterized by a high proportion of cells expressing the senescence marker CD57 [28]. Expression of CD57 on
CMV-specific CD4⁺ T cells correlates with an increased capacity to produce Mip-1β [10]. We thus wanted to determine whether the capacity for Mip-1β production is associated with aging in our cohort. There was no linear relationship between age and capacity for Mip-1β production in IFNγ⁺ CMV-specific CD4⁺ T cells in HIV subjects (data not shown). Both HIV- adults and children had IFNγ⁺ CMV-specific CD4⁺ T cells characterized by a CD27⁻⁺ phenotype (P = 0.332) and a similar capacity to co-express Mip-1β (median 32.4% v 50%, P = 0.372, Fig 5 and Fig 1C and 1D for gating strategy details). A previous study reported a predominance of undifferentiated CD27⁺ CMV-specific CD4⁺ T cells in infants [29] and an unusual immature CD27 phenotype predominance in adults contrasting with several other reports [10],[30],[31]. Differences in study population or methodology could account for this discrepancy. Interestingly, down regulation of CD27 and increased frequencies of Mip-1β co-producing CD4⁺ T cells occurred in adults with active TB (P = 0.036 and 0.0596) and particularly in those co-infected with HIV (P = 0.008 and 0.002). This result demonstrates to our knowledge for the first time that active TB infection alone is associated with phenotypic and functional changes in CMV-specific CD4⁺ T cell response in adults. A significant impact of HIV and active
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tuberculosis on the CD27 phenotype of CMV-specific CD8⁺ T cells was not observed (Fig 6A, and 6B). Based on these results, we hypothesize that the CMV virus might reactivate during active TB, particularly so in HIV+ subjects—driving further antigen-driven CMV-specific cellular maturation and increased Mip-1β production [9,10]. This is supported by the known hematopoietic niche for latent CMV viruses which reactivate upon differentiation of myeloid precursor into tissue macrophages [32]. Cellular recruitment including monocyte-derived macrophages is essential for the granuloma formation that typifies tuberculosis pathogenesis [33]. Similar changes were not detected in HIV+ children. However, the number of children included in the analyses and the fact that all enrolled children were TB suspects limits the validity of this finding.

Conclusion

We show that CMVpp65-specific T cell responses are detectable in the peripheral blood of most Tanzanian children indicating that acquisition of CMV infection typically occurs during infancy and early childhood with HIV being a risk factor. In comparison to adults, there was an increased proportion of CD8⁺ T cells participating in the response and this was particularly apparent in HIV+ children. In children, CMVpp65-specific CD4⁺ T cells had a mature CD27⁺ effector memory phenotype and a high capacity for Mip-1β production similar to those detected in adults, and there was no apparent effect of HIV co-infection. Despite some limitations of the current study, such as the hospital based inclusion of children, our results suggest that the high capacity for Mip-1β production of CMV-specific CD4⁺ T cells is independent of age. Importantly, we observed for the first time that active TB impacts on the CMV-specific CD4⁺ T cell phenotype. This may reflect recent CMV reactivation and would indicate a previously unknown interplay between CMV virus infection and active tuberculosis pathogenesis.

Materials and Methods

Ethics Statement

The study was conducted at two Tanzanian research sites—the NIMR-Mbeya Medical Research Center, Mbeya, and the Ifakara Health Institute, Bagamoyo. The Institutional Review Board of the Ifakara Health Institute, the Mbeya Medical Research and Ethics Committee, and the Medical Research Coordinating Committee of Tanzania approved the study protocols. We obtained written informed consents from adults or for children from a literate parent or legal guardian who participated in the TB child study [17] or the Worm HIV Interaction study (adults only) [34].

Study subjects and blood sampling

Children older than 7 years provided assent for participation. Children and adolescents (Ç16 years old) were enrolled in the context of clinical consultation for illnesses that resemble active tuberculosis. HIV/TB healthy controls and MTB culture-confirmed TB cases were enrolled into the adult group (Ç18 years old). Blood was collected into Vacuette CPDA tubes and PBMC isolation performed using Ficoll gradient centrifugation (Leucosep, Greiner Bio-One GmbH) before cryopreservation in fetal calf serum containing 10% DMSO within 6h of phlebotomy.

Reagents

Complete Medium (CM) (RPMI 1640 W/Glutamax and Penicillin-Streptomycin (Gibco, Invitrogen), 100μg/ml, 10% heat-inactivated FCS (FBS Gold A11-151, PAA Germany). Anti-
CD4-PerCP Cy5.5 (Oct4) and HLA-DR Pe-Cy7 (LN3) were purchased from eBioscience. Anti-CD8 Horizon V500 (RPA-T8); anti-CD27-APC H7 (M-T271); anti-CD3-Pac Blue (UCHT-1); anti-IFNγ-FITC (B27); anti-CD154-APC (TRAP1); anti-Mip-1β-PE (D21-1351); CST and compensation beads were obtained from BD Pharmingen.

**Intracellular cytokine staining**

PBMC were thawed and washed twice in CM containing benzonase (Novagen, 25KU, 1/5000 V/V). Viability was assessed using trypan blue exclusion after a resting period of 2–6 hours at 37°C in complete medium. Cells were stimulated for 12h to 16h in complete medium containing Brefeldin A (Sigma-Aldrich, 5ug/ml) and CD28/CD49d antibodies (Pharmingen, 1ug/ml) with CMVpp65 peptide set (JPT Peptide Technologies, 2ug/ml), Staphylococcal Enterotoxin B (Sigma-Aldrich, 0.8ug/ml) or nothing as a negative control. Intracellular cytokine staining was performed using a standard staining protocol [10]. Acquisition of cells was performed on BD FACS Canto2 (NIMR-MMRC, Mbeya) or BD Fortessa (IHI-BRTC, Bagamoyo). Instruments were calibrated before each run using BD Cytometer Setup and Tracking Beads according to manufacturer’s recommendations. Data were analysed using FlowJo 9.X (Tree Star). T cell responses were considered positive, if the frequency of IFNγ+ cells was above 0.05% of the parent population and >2-fold the background frequency in the unstimulated control. GraphPad Prism (San Diego, CA, USA), version 4.03, was used for statistical analysis. Anonymized patient characteristics and the respective flow cytometry data statistics are provided as S1 Table.

**Supporting Information**

S1 Table. Participant level data; anonymized patient characteristics and respective flow cytometry data statistics. Column legends: A) Tuberculosis classification (see [17]); B) Age (years); C) HIV status (1: positive, 2: negative); D) CD4 cell counts per μl of blood; E) CD4 T cell percentages observed after PBMC stimulation; F) Signif: CD4 T cell response to CMVpp65 stimulation passed threshold for significance (see methods). NS: Not significant; G to I) Percentages of CD4 T cell producing one or both of the indicated cytokine(s) observed after PBMC stimulation with CMVpp65 (γ: IFN-γ, MIP: Mip-1β); J) Proportion of CD4 T cell producing Mip-1β that also produced IFN-γ; K) CD27 MFI Fold Change of IFNγ producing cells compared to all CD4 T cells (see Fig 1 for gating strategy); L to Q) as for F to K) but for CD8 T cell responses to CMVpp65 PBMC stimulation.

(XLSX)

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**Author Contributions**

Conceived and designed the experiments: CG KR MH. Performed the experiments: DP FM AB MM MC PM. Analyzed the data: DP CD KR CG. Involved in study participant’s recruitment: PC FH ES NN. Analysed TB diagnostic results: AR.

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