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

INAUGURALDISSERTATION
zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von
Maxmillian Gideon Mpina
Aus Sumbawanga, Rukwa, United Republic of Tanzania
Basel, 2018

Originaldokument gespeichert auf Dokumentenserver der Universität Basel
edoc.unibas.ch

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von
Prof. Dr. Marcel Tanner, Prof. Dr. Claudia Daubenberger, Prof. Dr. Steffen Borrmann

Basel, den 13. December 2016

Prof. Dr. Jörg Schibler

Dekan

……..to my beloved wife, children, parents, brothers and sisters.

Table of Contents

CHAPTER 1: Introduction ... **1**

1.1. Malaria : Overview ... 1

1.1.1. The parasite ... 1

1.1.1.1. *Plasmodium falciparum* ... 2
1.1.1.2. *Plasmodium vivax* ... 2
1.1.1.3. *Plasmodium ovale* ... 3
1.1.1.4. *Plasmodium malariae* ... 3
1.1.1.5. *Plasmodium knowlesi* ... 4

1.1.2. Epidemiology of *Plasmodium falciparum* ... 4

1.1.3. Life cycle of *Plasmodium falciparum* malaria ... 5

1.1.4. Pathogenesis of *Plasmodium falciparum* malaria ... 7

1.1.5. Malaria diagnosis ... 9

1.1.6. Current malaria control strategies ... 11

1.1.6.1. Malaria vector control ... 12
1.1.6.2. Malaria treatment ... 12
1.1.6.3. Malaria vaccines ... 13

1.2. Immune responses against malaria ... 14

1.2.1. Humoral immunity against malaria ... 14

1.2.2. Cellular immunity against malaria ... 15

1.3. Malaria pre-existing responses and the magnitude of vaccine induced immunity ... 17

1.4. Malaria vaccines development ... 17

1.4.1. Pre-erythrocytic malaria vaccine approaches ... 19

1.4.1.1. Recombinant pre-erythrocytic vaccine approaches ... 20
1.4.1.2. Viral vectored pre-erythrocytic vaccine approaches ... 23

1.4.2. DNA vaccine approaches ... 25

1.4.3. The live attenuated whole parasite based vaccine approaches ... 25

1.4.4. Blood stage malaria vaccine approaches ... 26

1.4.5. Malaria transmission blocking vaccines (TBV) ... 29

1.5. Controlled human malaria infection in malaria endemic countries ... 29

CHAPTER 2: Rationale and research questions ... 47

CHAPTER 3: Aim of the thesis and approaches ... 50

CHAPTER 4: Investigation of vaccine efficacy and cellular and humoral immunity in RTS,S/AS01E vaccinated volunteers in Tanzania 52

4.1. Paper I: A phase 3 trial of RTS,S/AS01E malaria vaccine in African infants.. 53

4.2. 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 children 66

4.3. Paper III: Distinct helper T cell type 1 and 2 responses associated with malaria protection and risk in RTS,S/AS01E vaccines 73

4.4. 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.. 84

CHAPTER 5: Controlled human malaria infections as a tool to accelerate malaria vaccine development in Africa and to dissect malaria specific immunity 104

5.1. Paper V: Controlled human malaria infection of Tanzanians by intradermal injection of aseptic, purified, cryopreserved *Plasmodium falciparum* sporozoites105

5.2. Paper VI: Impact of malaria pre-exposure on anti-parasite cellular and humoral immune responses after controlled human malaria infection 117

5.3. Paper VII: Controlled human malaria infection boots pre-erythrocytic stage transcending and merozoite opsonizing antibody in malaria semi-immune Tanzanian adults.. 130

5.4. Paper VIII: Controlled human malaria infection leads to long-lasting changes in innate and innate-like lymphocyte populations... 155

CHAPTER 6: Discussion, conclusion and recommendations.......................... 172

6.1. General discussion.. 173

6.1.1. Investigation of safety, efficacy and immunogenicity of RTS,S/AS01 vaccine in Tanzania paediatric population.. 173

6.1.2. Controlled human malaria infection as a cornerstone to accelerate malaria vaccine development in Africa 181

6.2. Conclusion 186

6.3. Recommendations 187

6.4. Contribution of the current thesis 188

CHAPTER 7: PhD supplementary work and curriculum vitae.......................... 194

7.1. Appendices 195

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 196

7.1.2. Antiviral innate immune activation in HIV-infected adults negatively affects HIV/IC31- induced vaccine specific memory $CD4^+$ T cells 204

7.1.3. Maturation and MIP-1 beta production of cytomegalovirus-specific T cell responses in Tanzanian children, adolescents and adults: Impact by HIV and *Mycobacterium tuberculosis* co-infections... 213

Table of figures

Figure 1: Global distribution of *P. falciparum* and *P vivax* by countries.............................. 1
Figure 2: Plasmodium species life cycle in both mosquito vector and human host............7.
Figure 3: A series of pathogenesis that is suggested to occur during different stages of malaria parasite life cycle in human host 9
Figure 4: Stages in *P. falciparum* life cycle that are deemed potential target for vaccine development 19
Figure 5: Schematic representation of RTS, S vaccine particle assembly 22

List of abbreviations

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

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

Acknowledgement

First and foremost, I would like to thank almighty God, for his guidance throughout my PhD journey. It would have never been possible without his amazing grace and endless blessings.

I would also like to express my sincere gratitude to my supervisor Prof. Claudia Daubenberger for her superb academic and scientific support. Despite of residing far from Basel, you have always given me opportunities to develop as a scientist. For that I say Asante sana, Mungu akubariki.

I would like to express my great appreciation to Prof. Marcel Tanner, the former director of Swiss TPH for being my faculty representative and ensuring all my financial and academic matters are settled. You are one of the very rare people that have ever lived in this planet. Mungu akupe maisha marefu, uzidi kuona matunda ya kazi za mikono yako. Amina.

I would like to offer my special thanks to Dr Salim Abdullah, the former IHI director and Christine Mensch, the student administrator, Swiss TPH travel and accommodation office for your immense support during my study.

I wish to thank various people for their contribution to this project; Anneth-Mwasi Tumbo, Catherine M, Tedson L, Tobias S and Tobias R, Julian R, dada Isabelle Z, Damien P, Nicole L, Martina F, Glenda R, Julian S; from you I have learned the true meaning of team work.

Drs Rumisha SF, Masimba P , Mwingira F, Dilip A, Lutambi A, Mwanyika H, Kasasa S, Amek N, for always organizing the party and make my living in Europe felt like home. You were my true mates and your support will never be forgotten.

I wish to acknowledge the help provided by office mates; Urassa A, Mzee T, Mwangoka G, Kafuruki S, Juma O, Mihayo M, and Khadija S: Your smiles, jokes and encouragement during my writing process cannot be compensated. I am very grateful for your support.

Many thanks to my family for continuously caring for me and understand you had when I said; I was not going to visit you. My mom Owonyesiga, for your everlasting love, asking me to quit my PhD if I found it is difficult. Mama those words were like fuel for me to work harder. My dad Gido, for telling me that “hard work always pays” I thank you immensely. God bless you all.

My children Manolito and Innah for jumping on the computer and always messing up with my writing moments, but you always made me laugh, smile, and gave me energy to carry on. This PhD is dedicated to you sweethearts.

I am grateful to my beloved wife Lightness John Maya for your warm support and taking care of the family while I was away from home.

My special thanks are extended to all study participants, Bagamoyo and Swiss TPH team who supported me in one way or another.

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-g, 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 (*Pf*SPZ) 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

(malaria exposed) subjects undergoing the same challenge study, Dutch subjects responded differently to *Pf*SPZ 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 *Pf*SPZ 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).

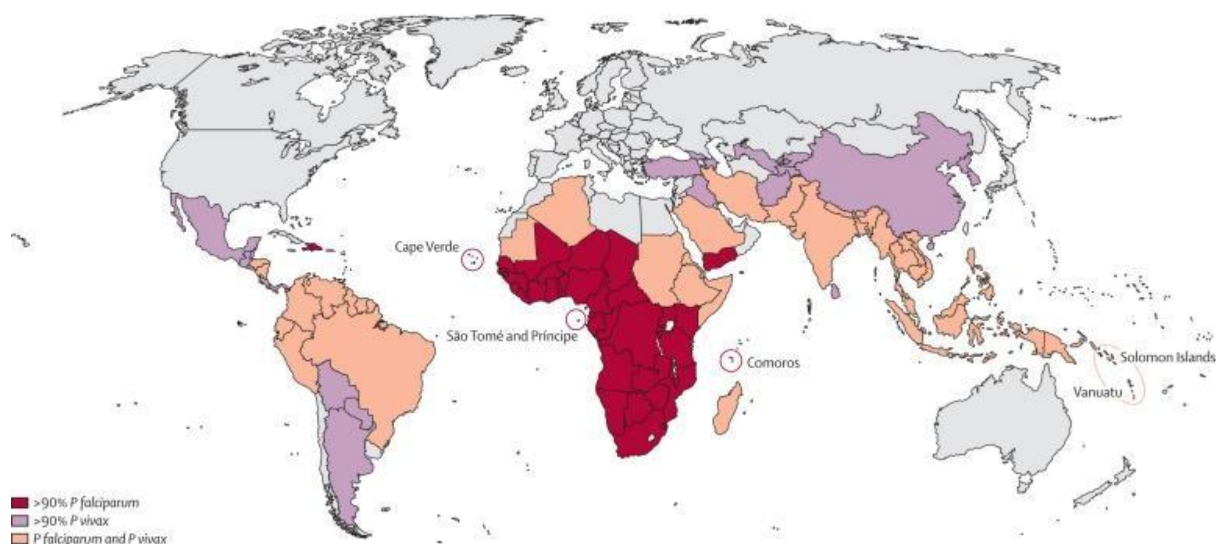


Figure 1: Global distribution of *P. falciparum* and *P. vivax* by countries. (Feachem et al., 2009)

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 *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).

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).

1.1.1.3. P. ovale

P. ovale is a relatively rare species, mostly found in West-Africa and the Pacific islands. *P. ovale* exhibits Schüffner's dots and infected erythrocytes can be normal or slightly enlarged; a characteristic feature that is also shared by *P. vivax,* making it difficult to distinguish the two species microscopically (Collins and Jeffery, 2005a). Similar to *P.vivax*, *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, *P. ovale* tends to present a low parasite burden and mild clinical symptoms, making routine diagnosis challenging. Recently, two *P. ovale* sub-species, classic (*P. ovale curtis*) and variant (*P.ovale wallikeri*) have been characterized (Sutherland et al., 2010). In Africa, the higher prevalence of *P. ovale* relative to *P. vivax* could be explained by the ability of *P. ovale* to infect populations carrying the null Duffy blood group, which provides natural resistance to *P. vivax* (Collins and Jeffery, 2005b).

1.1.1.4. P. malariae

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).

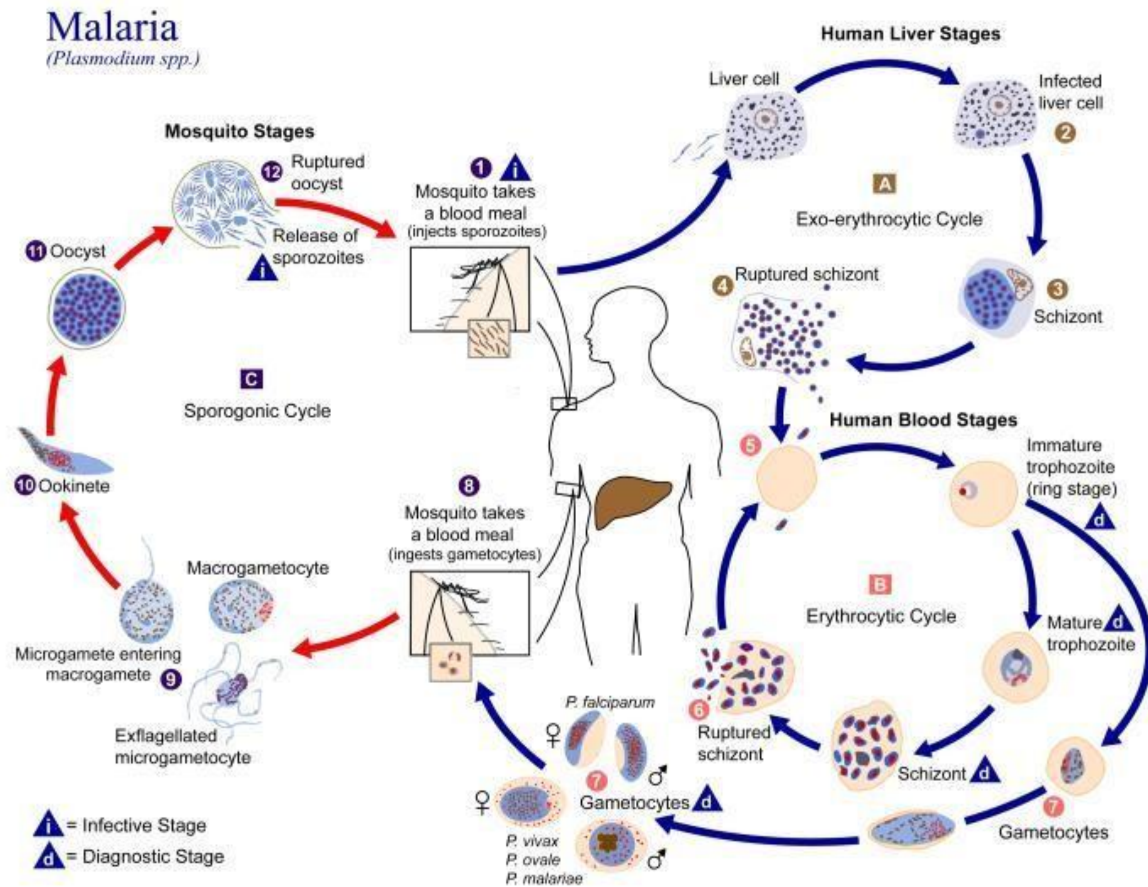


Figure 2: ***Plasmodium species*** **life cycle in both mosquito vector and human host**

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).

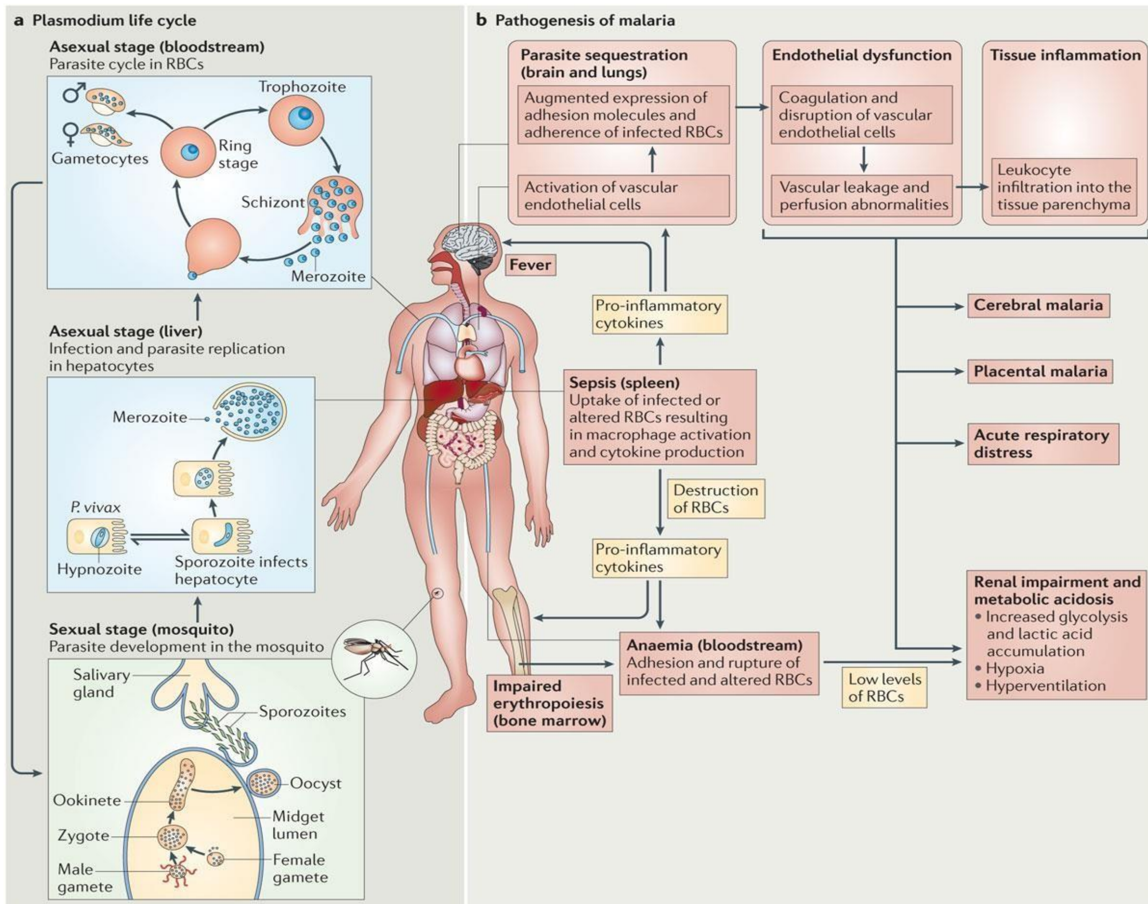


Figure 3: **A series of pathogenesis that is suggested to occur during different stages of malaria parasite life cycle in human host**

(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 iRBCs disrupts blood flow, promotes blood clots, injures endothelial cells and ruptures vascular walls, leading to the extravasation of vascular content and local tissue inflammation. These mechanisms contribute to acute respiratory distress, cerebral malaria or placental malaria. 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 (*P*LDH) 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)

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):

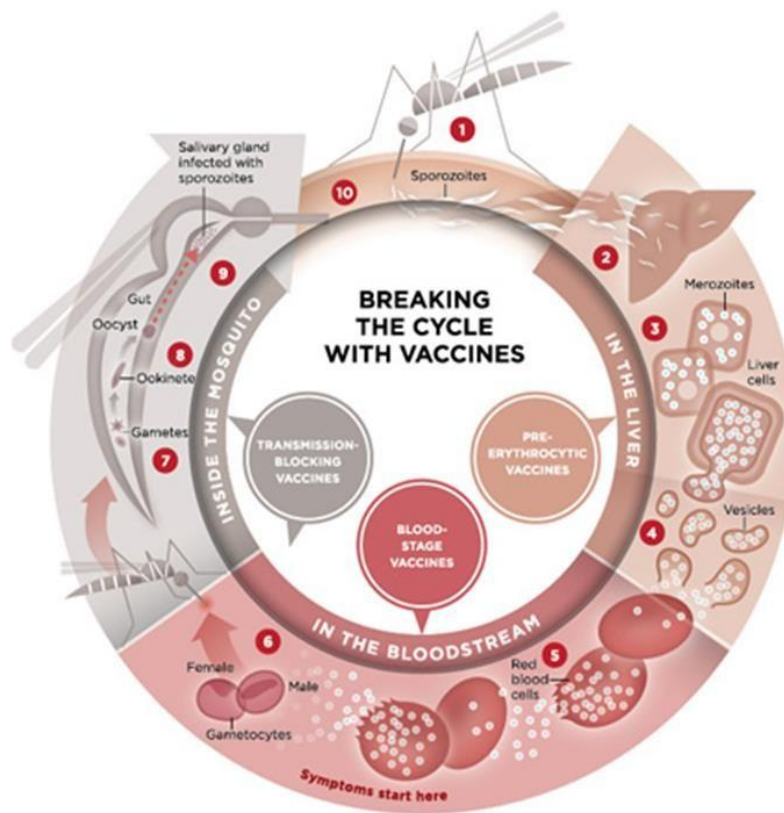


Figure 4: **Stages in *P. falciparum* life cycle that are deemed potential target for vaccine development**

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 ookinetes 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 *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 (*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).

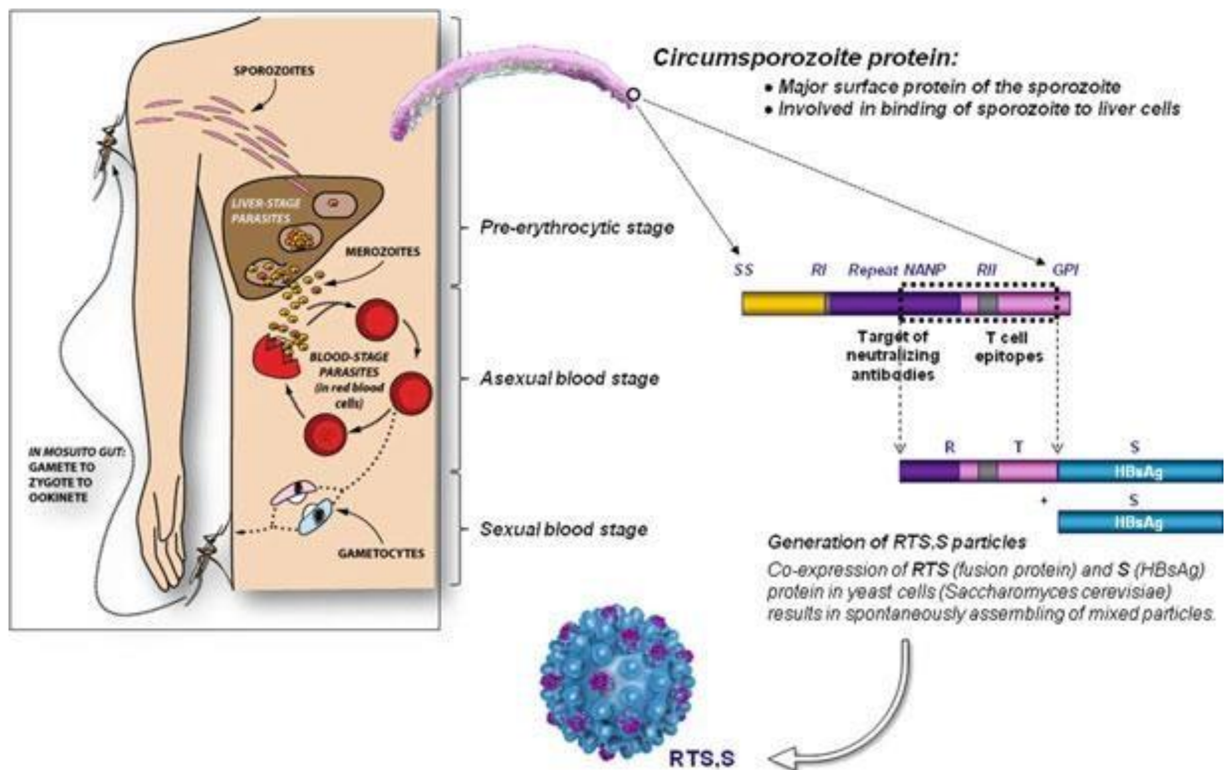


Figure 5: **Schematic representation of RTS,S vaccine particle assembly**

A central tandem repeat (B-cell epitope) and C-terminal region of the *P.falciparum* circumsporozoite protein (T-cell epitope) are fused to N-terminal of the S antigen of Hepatitis B virus (HBsAg) (B) and co-expressed together with unfused HBsAg S in *C.cereviciae* yeast to form a vaccine particle (Cohen et al., 2011).

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 (*Pf*SPZ) 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 (*Pv*s25) 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 (*Pf*SPZ 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 *Pf*SPZ 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).

References

Abdulla, S., Oberholzer, R., Juma, O., Kubhoja, S., Machera, F., Membi, C., Omari, S., Urassa, A., Mshinda, H., Jumanne, A., et al. (2008). Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants. N. Engl. J. Med. *359*, 2533–2544.

Adjuik, M., Babiker, A., Garner, P., Olliaro, P., Taylor, W., White, N., and International Artemisinin Study Group (2004). Artesunate combinations for treatment of malaria: meta-analysis. Lancet Lond. Engl. *363*, 9–17.

Alano, P. (2007). Plasmodium falciparum gametocytes: still many secrets of a hidden life. Mol. Microbiol. *66*, 291–302.

Andrews, J.M., and Langmuir, A.D. (1963). The Philosophy of Disease Eradication. Am. J. Public Health Nations Health *53*, 1–6.

Aponte, J.J., Aide, P., Renom, M., Mandomando, I., Bassat, Q., Sacarlal, J., Manaca, M.N., Lafuente, S., Barbosa, A., Leach, A., et al. (2007). Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet *370*, 1543–1551.

Arama, C., and Troye-Blomberg, M. (2014). The path of malaria vaccine development: challenges and perspectives. J. Intern. Med. *275*, 456–466.

Ashley, E.A., Recht, J., and White, N.J. (2014). Primaquine: the risks and the benefits. Malar. J. *13*, 418.

Audran, R., Cachat, M., Lurati, F., Soe, S., Leroy, O., Corradin, G., Druilhe, P., and Spertini, F. (2005). Phase I malaria vaccine trial with a long synthetic peptide derived from the merozoite surface protein 3 antigen. Infect. Immun. *73*, 8017–8026.

Badiane, A.S., Diongue, K., Diallo, S., Ndongo, A.A., Diedhiou, C.K., Deme, A.B., Ma, D., Ndiaye, M., Seck, M.C., Dieng, T., et al. (2014). Acute kidney injury associated with Plasmodium malariae infection. Malar. J. *13*, 226.

Baer, K., Klotz, C., Kappe, S.H.I., Schnieder, T., and Frevert, U. (2007). Release of hepatic Plasmodium yoelii merozoites into the pulmonary microvasculature. PLoS Pathog. *3*, e171.

Bang, G., Prieur, E., Roussilhon, C., and Druilhe, P. (2011). Pre-clinical assessment of novel multivalent MSP3 malaria vaccine constructs. PloS One *6*, e28165.

Bangari, D.S., and Mittal, S.K. (2006). Development of nonhuman adenoviruses as vaccine vectors. Vaccine *24*, 849–862.

Bassat, Q., Velarde, M., Mueller, I., Lin, J., Leslie, T., Wongsrichanalai, C., and Baird, J.K. (2016). Key Knowledge Gaps for Plasmodium vivax Control and Elimination. Am. J. Trop. Med. Hyg. 16–0180.

Behet, M.C., Foquet, L., van Gemert, G.-J., Bijker, E.M., Meuleman, P., Leroux-Roels, G., Hermsen, C.C., Scholzen, A., and Sauerwein, R.W. (2014). Sporozoite immunization of

human volunteers under chemoprophylaxis induces functional antibodies against pre-erythrocytic stages of Plasmodium falciparum. Malar. J. *13*, 136.

Bejon, P., Mwacharo, J., Kai, O., Mwangi, T., Milligan, P., Todryk, S., Keating, S., Lang, T., Lowe, B., Gikonyo, C., et al. (2006). A Phase 2b Randomised Trial of the Candidate Malaria Vaccines FP9 ME-TRAP and MVA ME-TRAP among Children in Kenya. PLoS Clin. Trials *1*.

Bejon, P., White, M.T., Olotu, A., Bojang, K., Lusingu, J.P., Salim, N., Otsyula, N.N., Agnandji, S.T., Asante, K.P., Owusu-Agyei, S., et al. (2013). Efficacy of RTS,S malaria vaccines: individual-participant pooled analysis of phase 2 data. Lancet Infect. Dis. *13*, 319–327.

Bélard, S., Issifou, S., Hounkpatin, A.B., Schaumburg, F., Ngoa, U.A., Esen, M., Fendel, R., de Salazar, P.M., Mürbeth, R.E., Milligan, P., et al. (2011). A Randomized Controlled Phase Ib Trial of the Malaria Vaccine Candidate GMZ2 in African Children. PLoS ONE *6*, e22525.

Berberian, D.A. (1948). The use of DDT residual spray in malaria control and its effect on general sanitation in rural districts. J. Palest. Arab Med. Assoc. *3*, 49–61.

Bergmann-Leitner, E.S., Duncan, E.H., Mease, R.M., and Angov, E. (2012). Impact of pre-existing MSP142-allele specific immunity on potency of an erythrocytic Plasmodium falciparum vaccine. Malar. J. *11*, 315.

Bijker, E.M., Bastiaens, G.J.H., Teirlinck, A.C., van Gemert, G.-J., Graumans, W., van de Vegte-Bolmer, M., Siebelink-Stoter, R., Arens, T., Teelen, K., Nahrendorf, W., et al. (2013). Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity. Proc. Natl. Acad. Sci. U. S. A. *110*, 7862–7867.

Blanford, J.I., Blanford, S., Crane, R.G., Mann, M.E., Paaijmans, K.P., Schreiber, K.V., and Thomas, M.B. (2013). Implications of temperature variation for malaria parasite development across Africa. Sci. Rep. *3*, 1300.

Bouharoun-Tayoun, H., Attanath, P., Sabchareon, A., Chongsuphajaisiddhi, T., and Druilhe, P. (1990). Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J. Exp. Med. *172*, 1633–1641.

Chakravarty, S., Cockburn, I.A., Kuk, S., Overstreet, M.G., Sacci, J.B., and Zavala, F. (2007). CD8+ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes. Nat. Med. *13*, 1035–1041.

Chilengi, R. (2009). Clinical development of malaria vaccines: should earlier trials be done in malaria endemic countries? Hum. Vaccin. *5*, 627–636.

Chulay, J.D., Schneider, I., Cosgriff, T.M., Hoffman, S.L., Ballou, W.R., Quakyi, I.A., Carter, R., Trosper, J.H., and Hockmeyer, W.T. (1986). Malaria transmitted to humans by

mosquitoes infected from cultured Plasmodium falciparum. Am. J. Trop. Med. Hyg. *35*, 66–68.

Cibulskis, R.E., Alonso, P., Aponte, J., Aregawi, M., Barrette, A., Bergeron, L., Fergus, C.A., Knox, T., Lynch, M., Patouillard, E., et al. (2016). Malaria: Global progress 2000 – 2015 and future challenges. Infect. Dis. Poverty *5*.

Clinical trial.gov (2015). Phase 2B Double Blind, Randomized, Controlled Trial to Evaluate the Safety, Immunogenicity and Protective Efficacy of MSP3-LSP Vaccine Candidate Adjuvanted in Aluminium Hydroxide (AIOH) Against Plasmodium Falciparum Clinical Malaria in Healthy Children Aged 12-48 Months in Mali.

Clyde, D.F., Most, H., McCarthy, V.C., and Vanderberg, J.P. (1973). Immunization of man against sporozite-induced falciparum malaria. Am. J. Med. Sci. *266*, 169–177.

Cohen, J., Nussenzweig, V., Nussenzweig, R., Vekemans, J., and Leach, A. (2010). From the circumsporozoite protein to the RTS, S/AS candidate vaccine. Hum. Vaccin. *6*, 90–96.

Cohen, J., Benns, S., Vekemans, J., Leach, A., and Schuerman, L. (2011). Development of the RTS,S/AS Vaccine Candidate from Concept to Phase III. In Progress in Parasitology, H. Mehlhorn, ed. (Springer Berlin Heidelberg), pp. 121–133.

Collins, W.E., and Jeffery, G.M. (2005a). Plasmodium ovale: Parasite and Disease. Clin. Microbiol. Rev. *18*, 570–581.

Collins, W.E., and Jeffery, G.M. (2005b). Plasmodium ovale: Parasite and Disease. Clin. Microbiol. Rev. *18*, 570–581.

Collins, W.E., and Jeffery, G.M. (2007). Plasmodium malariae: Parasite and Disease. Clin. Microbiol. Rev. *20*, 579–592.

Comber, J.D., and Philip, R. (2014). MHC class I antigen presentation and implications for developing a new generation of therapeutic vaccines. Ther. Adv. Vaccines *2*, 77–89.

Cooke, B., Coppel, R., and Wahlgren, M. (2000). Falciparum malaria: sticking up, standing out and out-standing. Parasitol. Today Pers. Ed *16*, 416–420.

Coppi, A., Natarajan, R., Pradel, G., Bennett, B.L., James, E.R., Roggero, M.A., Corradin, G., Persson, C., Tewari, R., and Sinnis, P. (2011). The malaria circumsporozoite protein has two functional domains, each with distinct roles as sporozoites journey from mosquito to mammalian host. J. Exp. Med. *208*, 341–356.

Cotter, C., Sturrock, H.J.W., Hsiang, M.S., Liu, J., Phillips, A.A., Hwang, J., Gueye, C.S., Fullman, N., Gosling, R.D., and Feachem, R.G.A. (2013). The changing epidemiology of malaria elimination: new strategies for new challenges. Lancet *382*, 900–911.

Cowman, A.F., and Kappe, S.H.I. (2006). Malaria's Stealth Shuttle. Science *313*, 1245–1246.

Crompton, P.D., Pierce, S.K., and Miller, L.H. (2010). Advances and challenges in malaria vaccine development. J. Clin. Invest. *120*, 4168–4178.

Croyle, M.A., Patel, A., Tran, K.N., Gray, M., Zhang, Y., Strong, J.E., Feldmann, H., and Kobinger, G.P. (2008). Nasal Delivery of an Adenovirus-Based Vaccine Bypasses Pre-Existing Immunity to the Vaccine Carrier and Improves the Immune Response in Mice. PLoS ONE *3*.

Doll, K.L., and Harty, J.T. (2014). Correlates of protective immunity following whole sporozoite vaccination against malaria. Immunol. Res. *59*, 166–176.

Doolan, D.L., and Hoffman, S.L. (1997). Multi-gene vaccination against malaria: A multistage, multi-immune response approach. Parasitol. Today Pers. Ed *13*, 171–178.

Doolan, D.L., and Hoffman, S.L. (2000). The complexity of protective immunity against liver-stage malaria. J. Immunol. Baltim. Md 1950 *165*, 1453–1462.

Doolan, D.L., Dobaño, C., and Baird, J.K. (2009). Acquired Immunity to Malaria. Clin. Microbiol. Rev. *22*, 13–36.

Epstein, J.E., Tewari, K., Lyke, K.E., Sim, B.K.L., Billingsley, P.F., Laurens, M.B., Gunasekera, A., Chakravarty, S., James, E.R., Sedegah, M., et al. (2011). Live attenuated malaria vaccine designed to protect through hepatic $CD8^+$ T cell immunity. Science *334*, 475–480.

Erickson, R.P. (1987). Natural history of the major histocompatibility complex. Am. J. Hum. Genet. *40*, 468–469.

Färnert, A., Wyss, K., Dashti, S., and Naucler, P. (2015). Duration of residency in a non-endemic area and risk of severe malaria in African immigrants. Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis. *21*, 494–501.

Fausther-Bovendo, H., and Kobinger, G.P. (2014). Pre-existing immunity against Ad vectors: humoral, cellular, and innate response, what's important? Hum. Vaccines Immunother. *10*, 2875–2884.

Feachem, R.G.A., Phillips, A.A., and Targett, G. rey A. (2009). Shrinking the Malaria Map: A Prospectus on Malaria Elimination. ResearchGate.

Ferraro, B., Morrow, M.P., Hutnick, N.A., Shin, T.H., Lucke, C.E., and Weiner, D.B. (2011). Clinical applications of DNA vaccines: current progress. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. *53*, 296–302.

Fynan, E.F., Webster, R.G., Fuller, D.H., Haynes, J.R., Santoro, J.C., and Robinson, H.L. (1993). DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. U. S. A. *90*, 11478–11482.

Gamo, F.-J., Sanz, L.M., Vidal, J., de Cozar, C., Alvarez, E., Lavandera, J.-L., Vanderwall, D.E., Green, D.V.S., Kumar, V., Hasan, S., et al. (2010). Thousands of

chemical starting points for antimalarial lead identification. Nature *465*, 305–310.

Gazzinelli, R.T., Kalantari, P., Fitzgerald, K.A., and Golenbock, D.T. (2014). Innate sensing of malaria parasites. Nat. Rev. Immunol. *14*, 744–757.

Giardina, F., Kasasa, S., Sié, A., Utzinger, J., Tanner, M., and Vounatsou, P. (2014). Effects of vector-control interventions on changes in risk of malaria parasitaemia in sub-Saharan Africa: a spatial and temporal analysis. Lancet Glob. Health *2*, e601-615.

Gordon, D.M., McGovern, T.W., Krzych, U., Cohen, J.C., Schneider, I., LaChance, R., Heppner, D.G., Yuan, G., Hollingdale, M., and Slaoui, M. (1995). Safety, immunogenicity, and efficacy of a recombinantly produced Plasmodium falciparum circumsporozoite protein-hepatitis B surface antigen subunit vaccine. J. Infect. Dis. *171*, 1576–1585.

de Groot, N.G., Bontrop, R.E., and Doxiadis, G.G.M. (2016). Major Histocompatibility Complex (MHC). In The International Encyclopedia of Primatology, (John Wiley & Sons, Inc.), p.

Gubbels, M.-J., and Duraisingh, M.T. (2012). Evolution of apicomplexan secretory organelles. Int. J. Parasitol. *42*, 1071–1081.

Guebre-Xabier, M., Schwenk, R., and Krzych, U. (1999). Memory phenotype CD8(+) T cells persist in livers of mice protected against malaria by immunization with attenuated Plasmodium berghei sporozoites. Eur. J. Immunol. *29*, 3978–3986.

Gunawardena, S., and Karunaweera, N.D. (2015). Advances in genetics and genomics: use and limitations in achieving malaria elimination goals. Pathog. Glob. Health *109*, 123–141.

Hagan, T., Nakaya, H.I., Subramaniam, S., and Pulendran, B. (2015). Systems vaccinology: Enabling rational vaccine design with systems biological approaches. Vaccine.

Harvey, K.L., Yap, A., Gilson, P.R., Cowman, A.F., and Crabb, B.S. (2014). Insights and controversies into the role of the key apicomplexan invasion ligand, Apical Membrane Antigen 1. Int. J. Parasitol. *44*, 853–857.

Hill, A.V.S. (2011). Vaccines against malaria. Philos. Trans. R. Soc. Lond. B Biol. Sci. *366*, 2806–2814.

Hill, D.L., Eriksson, E.M., Li Wai Suen, C.S.N., Chiu, C.Y., Ryg-Cornejo, V., Robinson, L.J., Siba, P.M., Mueller, I., Hansen, D.S., and Schofield, L. (2013). Opsonising Antibodies to P. falciparum Merozoites Associated with Immunity to Clinical Malaria. PLoS ONE *8*, e74627.

Hoffman, S.L., Billingsley, P.F., James, E., Richman, A., Loyevsky, M., Li, T., Chakravarty, S., Gunasekera, A., Chattopadhyay, R., Li, M., et al. (2010). Development of a metabolically active, non-replicating sporozoite vaccine to prevent Plasmodium falciparum malaria. Hum. Vaccin. *6*, 97–106.

Howes, R.E., Battle, K.E., Mendis, K.N., Smith, D.L., Cibulskis, R.E., Baird, J.K., and Hay, S.I. (2016). Global Epidemiology of Plasmodium vivax. Am. J. Trop. Med. Hyg. *95*, 15–34.

Idro, R., Marsh, K., John, C.C., and Newton, C.R. (2010). Cerebral Malaria; Mechanisms Of Brain Injury And Strategies For Improved Neuro-Cognitive Outcome. Pediatr. Res. *68*, 267–274.

Jacobs, B.L., Langland, J.O., Kibler, K.V., Denzler, K.L., White, S.D., Holechek, S.A., Wong, S., Huynh, T., and Baskin, C.R. (2009). Vaccinia Virus Vaccines: Past, Present and Future. Antiviral Res. *84*, 1–13.

Jepsen, M.P.G., Jogdand, P.S., Singh, S.K., Esen, M., Christiansen, M., Issifou, S., Hounkpatin, A.B., Ateba-Ngoa, U., Kremsner, P.G., Dziegiel, M.H., et al. (2013). The malaria vaccine candidate GMZ2 elicits functional antibodies in individuals from malaria endemic and non-endemic areas. J. Infect. Dis. *208*, 479–488.

Jindal, H., Bhatt, B., Malik, J.S., SK, S., and Mehta, B. (2014). Malaria vaccine. Hum. Vaccines Immunother. *10*, 1752–1754.

John, C.C., Tande, A.J., Moormann, A.M., Sumba, P.O., Lanar, D.E., Min, X.M., and Kazura, J.W. (2008). Antibodies to pre-erythrocytic Plasmodium falciparum antigens and risk of clinical malaria in Kenyan children. J. Infect. Dis. *197*, 519–526.

John, G.K., Douglas, N.M., von Seidlein, L., Nosten, F., Baird, J.K., White, N.J., and Price, R.N. (2012). Primaquine radical cure of Plasmodium vivax: a critical review of the literature. Malar. J. *11*, 280.

Jones, R.M., Chichester, J.A., Mett, V., Jaje, J., Tottey, S., Manceva, S., Casta, L.J., Gibbs, S.K., Musiychuk, K., Shamloul, M., et al. (2013). A Plant-Produced Pfs25 VLP Malaria Vaccine Candidate Induces Persistent Transmission Blocking Antibodies against Plasmodium falciparum in Immunized Mice. PLoS ONE *8*.

Kannanganat, S., Nigam, P., Velu, V., Earl, P.L., Lai, L., Chennareddi, L., Lawson, B., Wilson, R.L., Montefiori, D.C., Kozlowski, P.A., et al. (2010). Preexisting Vaccinia Virus Immunity Decreases SIV-Specific Cellular Immunity but does not diminish Humoral Immunity and Efficacy of a DNA/MVA Vaccine. J. Immunol. Baltim. Md 1950 *185*, 7262–7273.

Karunamoorthi, K. (2014). Malaria Vaccine: A Future Hope to Curtail the Global Malaria Burden. Int. J. Prev. Med. *5*, 529–538.

Kester, K.E., McKinney, D.A., Tornieporth, N., Ockenhouse, C.F., Heppner, D.G., Hall, T., Wellde, B.T., White, K., Sun, P., Schwenk, R., et al. (2007). A phase I/IIa safety, immunogenicity, and efficacy bridging randomized study of a two-dose regimen of liquid and lyophilized formulations of the candidate malaria vaccine RTS,S/AS02A in malaria-naïve adults. Vaccine *25*, 5359–5366.

Khan, S.M., Reece, S.E., Waters, A.P., Janse, C.J., and Kaczanowski, S. (2013). Why

are male malaria parasites in such a rush?: Sex-specific evolution and host-parasite interactions. Evol. Med. Public Health *2013*, 3–13.

Khusmith, S., and Druilhe, P. (1983). Cooperation between antibodies and monocytes that inhibit in vitro proliferation of Plasmodium falciparum. Infect. Immun. *41*, 219–223.

Kinyanjui, S.M., Conway, D.J., Lanar, D.E., and Marsh, K. (2007). IgG antibody responses to Plasmodium falciparum merozoite antigens in Kenyan children have a short half-life. Malar. J. *6*, 82.

Knowles, R., and Gupta, D. A study of monkey-malaria, and its experimental transmission to man. Ind Med *Gaz 67*, 301–320.

Kumar, K.A., Sano, G., Boscardin, S., Nussenzweig, R.S., Nussenzweig, M.C., Zavala, F., and Nussenzweig, V. (2006). The circumsporozoite protein is an immunodominant protective antigen in irradiated sporozoites. Nature *444*, 937–940.

Kumar, S., Epstein, J.E., Richie, T.L., Nkrumah, F.K., Soisson, L., Carucci, D.J., and Hoffman, S.L. (2002). A multilateral effort to develop DNA vaccines against falciparum malaria. Trends Parasitol. *18*, 129–135.

Levine, N.D. (1988). Progress in Taxonomy of the Apicomplexan Protozoa. J. Protozool. *35*, 518–520.

Limbach, K.J., and Paoletti, E. (1996). Non-replicating expression vectors: applications in vaccine development and gene therapy. Epidemiol. Infect. *116*, 241–256.

Lin, C.S., Uboldi, A.D., Marapana, D., Czabotar, P.E., Epp, C., Bujard, H., Taylor, N.L., Perugini, M.A., Hodder, A.N., and Cowman, A.F. (2014). The merozoite surface protein 1 complex is a platform for binding to human erythrocytes by Plasmodium falciparum. J. Biol. Chem. *289*, 25655–25669.

Loucq, C. (2013). Vaccines today, vaccines tomorrow: a perspective. Clin. Exp. Vaccine Res. *2*, 4–7.

Lozano, J.M., and Patarroyo, M.E. (2007). A rational strategy for a malarial vaccine development. Microbes Infect. Inst. Pasteur *9*, 751–760.

Lusingu, J.P.A., Gesase, S., Msham, S., Francis, F., Lemnge, M., Seth, M., Sembuche, S., Rutta, A., Minja, D., Segeja, M.D., et al. (2009). Satisfactory safety and immunogenicity of MSP3 malaria vaccine candidate in Tanzanian children aged 12-24 months. Malar. J. *8*, 163.

Mac-Daniel, L., Buckwalter, M.R., Berthet, M., Virk, Y., Yui, K., Albert, M.L., Gueirard, P., and Ménard, R. (2014). Local immune response to injection of Plasmodium sporozoites into the skin. J. Immunol. Baltim. Md 1950 *193*, 1246–1257.

Malik, A., Egan, J.E., Houghten, R.A., Sadoff, J.C., and Hoffman, S.L. (1991). Human cytotoxic T lymphocytes against the Plasmodium falciparum circumsporozoite protein.

Proc. Natl. Acad. Sci. U. S. A. *88*, 3300–3304.

Malkin, E.M., Durbin, A.P., Diemert, D.J., Sattabongkot, J., Wu, Y., Miura, K., Long, C.A., Lambert, L., Miles, A.P., Wang, J., et al. (2005). Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria. Vaccine *23*, 3131–3138.

Markus, M.B. (2011). Malaria: origin of the term “hypnozoite.” J. Hist. Biol. *44*, 781–786.

McGuire, W., Knight, J.C., Hill, A.V., Allsopp, C.E., Greenwood, B.M., and Kwiatkowski, D. (1999). Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles. J. Infect. Dis. *179*, 287–290.

Mehlhorn, H. (2011). Progress in Parasitology (Springer Science & Business Media).

Menard, D., and Dondorp, A. (2017). Antimalarial Drug Resistance: A Threat to Malaria Elimination. Cold Spring Harb. Perspect. Med.

Menendez, C., Ordi, J., Ismail, M.R., Ventura, P.J., Aponte, J.J., Kahigwa, E., Font, F., and Alonso, P.L. (2000). The impact of placental malaria on gestational age and birth weight. J. Infect. Dis. *181*, 1740–1745.

Meshnick, S.R., and Dobson, M.J. (2001). The History of Antimalarial Drugs. In Antimalarial Chemotherapy, P.J.R. MD, ed. (Humana Press), pp. 15–25.

Mharakurwa, S., Mutambu, S.L., Mberikunashe, J., Thuma, P.E., Moss, W.J., Mason, P.R., and Southern Africa ICEMR Team (2013). Changes in the burden of malaria following scale up of malaria control interventions in Mutasa District, Zimbabwe. Malar. J. *12*, 223.

Michael, O.S. (2015). Malaria RDT diagnosis: Magnifying a curious point. Travel Med. Infect. Dis. *13*, 267–268.

Migot, F., Chougnet, C., Henzel, D., Dubois, B., Jambou, R., Fievet, N., and Deloron, P. (1995). Anti-malaria antibody-producing B cell frequencies in adults after a Plasmodium falciparum outbreak in Madagascar. Clin. Exp. Immunol. *102*, 529–534.

Mishra, S.K., Behera, P.K., and Satpathi, S. (2013). Cardiac involvement in malaria: an overlooked important complication. J. Vector Borne Dis. *50*, 232–235.

MMWR, C.-C. for D.C. and (1999). The Principles of Disease Elimination and Eradication.

Moorthy, V.S., Imoukhuede, E.B., Milligan, P., Bojang, K., Keating, S., Kaye, P., Pinder, M., Gilbert, S.C., Walraven, G., Greenwood, B.M., et al. (2004). A Randomised, Double-Blind, Controlled Vaccine Efficacy Trial of DNA/MVA ME-TRAP Against Malaria Infection in Gambian Adults. PLoS Med *1*, e33.

Moorthy, V.S., Diggs, C., Ferro, S., Good, M.F., Herrera, S., Hill, A.V., Imoukhuede, E.B., Kumar, S., Loucq, C., Marsh, K., et al. (2009). Report of a consultation on the optimization of clinical challenge trials for evaluation of candidate blood stage malaria

vaccines, 18-19 March 2009, Bethesda, MD, USA. Vaccine *27*, 5719–5725.

Moss, B. (2011). Smallpox vaccines: targets of protective immunity. Immunol. Rev. *239*, 8–26.

Mueller, A.-K., Labaied, M., Kappe, S.H.I., and Matuschewski, K. (2005). Genetically modified Plasmodium parasites as a protective experimental malaria vaccine. Nature *433*, 164–167.

Mulamba, C., Riveron, J.M., Ibrahim, S.S., Irving, H., Barnes, K.G., Mukwaya, L.G., Birungi, J., and Wondji, C.S. (2014). Widespread Pyrethroid and DDT Resistance in the Major Malaria Vector Anopheles funestus in East Africa Is Driven by Metabolic Resistance Mechanisms. PLoS ONE *9*.

Nahrendorf, W., Scholzen, A., Bijker, E.M., Teirlinck, A.C., Bastiaens, G.J.H., Schats, R., Hermsen, C.C., Visser, L.G., Langhorne, J., and Sauerwein, R.W. (2014). Memory B-Cell and Antibody Responses Induced by Plasmodium falciparum Sporozoite Immunization. J. Infect. Dis. *210*, 1981–1990.

Nakaya, H.I., and Pulendran, B. (2015). Vaccinology in the era of high-throughput biology. Philos. Trans. R. Soc. Lond. B. Biol. Sci. *370*.

Ndungu, F.M., Lundblom, K., Rono, J., Illingworth, J., Eriksson, S., and Färnert, A. (2013). Long-lived Plasmodium falciparum specific memory B cells in naturally exposed Swedish travelers. Eur. J. Immunol. *43*, 2919–2929.

Nebie, I., Diarra, A., Ouedraogo, A., Tiono, A.B., Konate, A.T., Gansane, A., Soulama, I., Cousens, S., Leroy, O., and Sirima, S.B. (2009). Humoral and cell-mediated immunity to MSP3 peptides in adults immunized with MSP3 in malaria endemic area, Burkina Faso. Parasite Immunol. *31*, 474–480.

Newbold, C., Craig, A., Kyes, S., Rowe, A., Fernandez-Reyes, D., and Fagan, T. (1999). Cytoadherence, pathogenesis and the infected red cell surface in Plasmodium falciparum. Int. J. Parasitol. *29*, 927–937.

Newby, G., Bennett, A., Larson, E., Cotter, C., Shretta, R., Phillips, A.A., and Feachem, R.G.A. (2016). The path to eradication: a progress report on the malaria-eliminating countries. Lancet Lond. Engl. *387*, 1775–1784.

Nussenzweig, R., Vanderberg, J., and Most, H. (1969). Protective immunity produced by the injection of x-irradiated sporozoites of Plasmodium berghei. IV. Dose response, specificity and humoral immunity. Mil. Med. *134*, 1176–1182.

Nwanegbo, E., Vardas, E., Gao, W., Whittle, H., Sun, H., Rowe, D., Robbins, P.D., and Gambotto, A. (2004). Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States. Clin. Diagn. Lab. Immunol. *11*, 351–357.

O, E., Lee, Y.-T., Ko, E.-J., Kim, K.-H., Lee, Y.-N., Song, J.-M., Kwon, Y.-M., Kim, M.-C.,

Perez, D.R., and Kang, S.-M. (2014). Roles of major histocompatibility complex class II in inducing protective immune responses to influenza vaccination. J. Virol. *88*, 7764–7775.

Oeuvray, C., Bouharoun-Tayoun, H., Gras-Masse, H., Bottius, E., Kaidoh, T., Aikawa, M., Filgueira, M.C., Tartar, A., and Druilhe, P. (1994). Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes. Blood *84*, 1594–1602.

Okiro, E.A., Bitira, D., Mbabazi, G., Mpimbaza, A., Alegana, V.A., Talisuna, A.O., and Snow, R.W. (2011). Increasing malaria hospital admissions in Uganda between 1999 and 2009. BMC Med. *9*, 37.

Okiro, E.A., Kazembe, L.N., Kabaria, C.W., Ligomeka, J., Noor, A.M., Ali, D., and Snow, R.W. (2013). Childhood malaria admission rates to four hospitals in Malawi between 2000 and 2010. PloS One *8*, e62214.

O'LEARY PA (1927). Treatment of neurosyphilis by malaria: Report on the three years' observation of the first one hundred patients treated. J. Am. Med. Assoc. *89*, 95–100.

Olotu, A., Fegan, G., Wambua, J., Nyangweso, G., Leach, A., Lievens, M., Kaslow, D.C., Njuguna, P., Marsh, K., and Bejon, P. (2016). Seven-Year Efficacy of RTS,S/AS01 Malaria Vaccine among Young African Children. N. Engl. J. Med. *374*, 2519–2529.

PATH- MVI (2014). Life cycle of the malaria parasite | Malaria Vaccine Initiative.

Plotkin, S.A. (2008). Vaccines: correlates of vaccine-induced immunity. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. *47*, 401–409.

Portugal, S., Pierce, S.K., and Crompton, P.D. (2013). Young Lives Lost as B Cells Falter: What We're Learning about Antibody Responses in Malaria. J. Immunol. Baltim. Md 1950 *190*, 3039–3046.

Pradel, G., and Frevert, U. (2001). Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion. Hepatol. Baltim. Md *33*, 1154–1165.

Prieur, E., Gilbert, S.C., Schneider, J., Moore, A.C., Sheu, E.G., Goonetilleke, N., Robson, K.J.H., and Hill, A.V.S. (2004). A Plasmodium falciparum candidate vaccine based on a six-antigen polyprotein encoded by recombinant poxviruses. Proc. Natl. Acad. Sci. U. S. A. *101*, 290–295.

Putrianti, E.D., Silvie, O., Kordes, M., Borrmann, S., and Matuschewski, K. (2009). Vaccine-like immunity against malaria by repeated causal-prophylactic treatment of liver-stage Plasmodium parasites. J. Infect. Dis. *199*, 899–903.

Ramphul, U.N., Garver, L.S., Molina-Cruz, A., Canepa, G.E., and Barillas-Mury, C. (2015). Plasmodium falciparum evades mosquito immunity by disrupting JNK-mediated apoptosis of invaded midgut cells. Proc. Natl. Acad. Sci. U. S. A. *112*, 1273–1280.

Reyes-Sandoval, A., Wyllie, D.H., Bauza, K., Milicic, A., Forbes, E.K., Rollier, C.S., and

Hill, A.V.S. (2011). CD8+ T effector memory cells protect against liver-stage malaria. J. Immunol. Baltim. Md 1950 *187*, 1347–1357.

Riedel, S. (2005). Edward Jenner and the history of smallpox and vaccination. Proc. Bayl. Univ. Med. Cent. *18*, 21–25.

Roestenberg, M., McCall, M., Hopman, J., Wiersma, J., Luty, A.J.F., van Gemert, G.J., van de Vegte-Bolmer, M., van Schaijk, B., Teelen, K., Arens, T., et al. (2009). Protection against a malaria challenge by sporozoite inoculation. N. Engl. J. Med. *361*, 468–477.

Roses, A.D. (2008). Pharmacogenetics in drug discovery and development: a translational perspective. Nat. Rev. Drug Discov. *7*, 807–817.

Roucher, C., Rogier, C., Sokhna, C., Tall, A., and Trape, J.-F. (2014). A 20-Year Longitudinal Study of Plasmodium ovale and Plasmodium malariae Prevalence and Morbidity in a West African Population. PloS One *9*, e87169.

Roussilhon, C., Oeuvray, C., Müller-Graf, C., Tall, A., Rogier, C., Trape, J.-F., Theisen, M., Balde, A., Pérignon, J.-L., and Druilhe, P. (2007). Long-term clinical protection from falciparum malaria is strongly associated with IgG3 antibodies to merozoite surface protein 3. PLoS Med. *4*, e320.

RTS,S Clinical Trials Partnership (2014). Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. PLoS Med. *11*, e1001685.

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

RTS,S Clinical Trials Partnership, Agnandji, S.T., Lell, B., Fernandes, J.F., Abossolo, B.P., Methogo, B.G.N.O., Kabwende, A.L., Adegnika, A.A., Mordmüller, B., Issifou, S., et al. (2012). A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. N. Engl. J. Med. *367*, 2284–2295.

Rutgers, T., Gordon, D., Gatoye, A., Hollingdale, M., Hockmeyer, W., Rosenberg, M., and De Wilde, M. (1988). Hepatitis B surface antigen as carrier matrix for the repetitive epitope of the circum-sporozoite protein of Plasmodium falciparum. Biotechnology *6*, 1065–1070.

Sabchareon, A., Burnouf, T., Ouattara, D., Attanath, P., Bouharoun-Tayoun, H., Chantavanich, P., Foucault, C., Chongsuphajaisiddhi, T., and Druilhe, P. (1991). Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am. J. Trop. Med. Hyg. *45*, 297–308.

Sánchez-Sampedro, L., Perdiguero, B., Mejías-Pérez, E., García-Arriaza, J., Di Pilato, M., and Esteban, M. (2015). The Evolution of Poxvirus Vaccines. Viruses *7*, 1726–1803.

Sauerwein, R.W., Roestenberg, M., and Moorthy, V.S. (2011). Experimental human

challenge infections can accelerate clinical malaria vaccine development. Nat. Rev. Immunol. *11*, 57–64.

Saxena, M., Van, T.T.H., Baird, F.J., Coloe, P.J., and Smooker, P.M. (2013). Pre-existing immunity against vaccine vectors – friend or foe? Microbiology *159*, 1–11.

Schneider, K.A., and Kim, Y. (2013). Genetic hitchhiking under heterogeneous spatial selection pressures. PloS One *8*, e61742.

Schwenk, R., Asher, L.V., Chalom, I., Lanar, D., Sun, P., White, K., Keil, D., Kester, K.E., Stoute, J., Heppner, D.G., et al. (2003). Opsonization by antigen-specific antibodies as a mechanism of protective immunity induced by Plasmodium falciparum circumsporozoite protein-based vaccine. Parasite Immunol. *25*, 17–25.

Sedegah, M., Tamminga, C., McGrath, S., House, B., Ganeshan, H., Lejano, J., Abot, E., Banania, G.J., Sayo, R., Farooq, F., et al. (2011). Adenovirus 5-vectored P. falciparum vaccine expressing CSP and AMA1. Part A: safety and immunogenicity in seronegative adults. PloS One *6*, e24586.

Seder, R.A., Chang, L.-J., Enama, M.E., Zephir, K.L., Sarwar, U.N., Gordon, I.J., Holman, L.A., James, E.R., Billingsley, P.F., Gunasekera, A., et al. (2013). Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science *341*, 1359–1365.

Seguin, M.C., Klotz, F.W., Schneider, I., Weir, J.P., Goodbary, M., Slayter, M., Raney, J.J., Aniagolu, J.U., and Green, S.J. (1994). Induction of nitric oxide synthase protects against malaria in mice exposed to irradiated Plasmodium berghei infected mosquitoes: involvement of interferon gamma and CD8+ T cells. J. Exp. Med. *180*, 353–358.

Shaukat, A.M., Gilliams, E.A., Kenefic, L.J., Laurens, M.B., Dzinjalamala, F.K., Nyirenda, O.M., Thesing, P.C., Jacob, C.G., Molyneux, M.E., Taylor, T.E., et al. (2012). Clinical manifestations of new versus recrudescent malaria infections following anti-malarial drug treatment. Malar. J. *11*, 207.

Sheehy, S.H., Duncan, C.J., Elias, S.C., Choudhary, P., Biswas, S., Halstead, F.D., Collins, K.A., Edwards, N.J., Douglas, A.D., Anagnostou, N.A., et al. (2012). ChAd63-MVA–vectored Blood-stage Malaria Vaccines Targeting MSP1 and AMA1: Assessment of Efficacy Against Mosquito Bite Challenge in Humans. Mol. Ther. *20*, 2355–2368.

Shekalaghe, S., Rutaihwa, M., Billingsley, P.F., Chemba, M., Daubenberger, C.A., James, E.R., Mpina, M., Ali Juma, O., Schindler, T., Huber, E., et al. (2014). Controlled Human Malaria Infection of Tanzanians by Intradermal Injection of Aseptic, Purified, Cryopreserved Plasmodium falciparum Sporozoites. Am. J. Trop. Med. Hyg. *91*, 471–480.

Shi, Y.P., Hasnain, S.E., Sacci, J.B., Holloway, B.P., Fujioka, H., Kumar, N., Wohlhueter, R., Hoffman, S.L., Collins, W.E., and Lal, A.A. (1999). Immunogenicity and in vitro protective efficacy of a recombinant multistage Plasmodium falciparum candidate vaccine. Proc. Natl. Acad. Sci. U. S. A. *96*, 1615–1620.

Singh, A.P., Buscaglia, C.A., Wang, Q., Levay, A., Nussenzweig, D.R., Walker, J.R., Winzeler, E.A., Fujii, H., Fontoura, B.M.A., and Nussenzweig, V. (2007). Plasmodium circumsporozoite protein promotes the development of the liver stages of the parasite. Cell *131*, 492–504.

Sinka, M.E., Bangs, M.J., Manguin, S., Rubio-Palis, Y., Chareonviriyaphap, T., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P., Temperley, W.H., et al. (2012). A global map of dominant malaria vectors. Parasit. Vectors *5*, 69.

Sirima, S.B., Tiono, A.B., Ouédraogo, A., Diarra, A., Ouédraogo, A.L., Yaro, J.B., Ouédraogo, E., Gansané, A., Bougouma, E.C., Konaté, A.T., et al. (2009). Safety and immunogenicity of the malaria vaccine candidate MSP3 long synthetic peptide in 12-24 months-old Burkinabe children. PloS One *4*, e7549.

Sirima, S.B., Mordmüller, B., Milligan, P., Ngoa, U.A., Kironde, F., Atuguba, F., Tiono, A.B., Issifou, S., Kaddumukasa, M., Bangre, O., et al. (2016). A phase 2b randomized, controlled trial of the efficacy of the GMZ2 malaria vaccine in African children. Vaccine *34*, 4536–4542.

Spring, M., Polhemus, M., and Ockenhouse, C. (2014). Controlled human malaria infection. J. Infect. Dis. *209 Suppl 2*, S40-45.

Stanisic, D.I., and Good, M.F. (2015). Whole organism blood stage vaccines against malaria. Vaccine *33*, 7469–7475.

Stanisic, D.I., Barry, A.E., and Good, M.F. (2013). Escaping the immune system: How the malaria parasite makes vaccine development a challenge. Trends Parasitol. *29*, 612–622.

Stoute, J.A., Slaoui, M., Heppner, D.G., Momin, P., Kester, K.E., Desmons, P., Wellde, B.T., Garçon, N., Krzych, U., and Marchand, M. (1997). A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group. N. Engl. J. Med. *336*, 86–91.

Sultan, A.A., Thathy, V., Frevert, U., Robson, K.J., Crisanti, A., Nussenzweig, V., Nussenzweig, R.S., and Ménard, R. (1997). TRAP is necessary for gliding motility and infectivity of plasmodium sporozoites. Cell *90*, 511–522.

Sutherland, C.J., Tanomsing, N., Nolder, D., Oguike, M., Jennison, C., Pukrittayakamee, S., Dolecek, C., Hien, T.T., Rosário, D., E, V., et al. (2010). Two Nonrecombining Sympatric Forms of the Human Malaria Parasite Plasmodium ovale Occur Globally. J. Infect. Dis. *201*, 1544–1550.

Tachibana, M., Suwanabun, N., Kaneko, O., Iriko, H., Otsuki, H., Sattabongkot, J., Kaneko, A., Herrera, S., Torii, M., and Tsuboi, T. (2015). Plasmodium vivax gametocyte proteins, Pvs48/45 and Pvs47, induce transmission-reducing antibodies by DNA immunization. Vaccine *33*, 1901–1908.

Tangpukdee, N., Duangdee, C., Wilairatana, P., and Krudsood, S. (2009). Malaria Diagnosis: A Brief Review. Korean J. Parasitol. *47*, 93–102.

Thera, M.A., Doumbo, O.K., Coulibaly, D., Laurens, M.B., Ouattara, A., Kone, A.K., Guindo, A.B., Traore, K., Traore, I., Kouriba, B., et al. (2011). A field trial to assess a blood-stage malaria vaccine. N. Engl. J. Med. *365*, 1004–1013.

Trimnell, A., Takagi, A., Gupta, M., Richie, T.L., Kappe, S.H., and Wang, R. (2009). Genetically attenuated parasite vaccines induce contact-dependent CD8+ T cell killing of Plasmodium yoelii liver stage-infected hepatocytes. J. Immunol. Baltim. Md 1950 *183*, 5870–5878.

Urban, B.C., Ferguson, D.J., Pain, A., Willcox, N., Plebanski, M., Austyn, J.M., and Roberts, D.J. (1999). Plasmodium falciparum-infected erythrocytes modulate the maturation of dendritic cells. Nature *400*, 73–77.

Vaughan, A.M., Mikolajczak, S.A., Wilson, E.M., Grompe, M., Kaushansky, A., Camargo, N., Bial, J., Ploss, A., and Kappe, S.H.I. (2012). Complete Plasmodium falciparum liver-stage development in liver-chimeric mice. J. Clin. Invest. *122*, 3618–3628.

Villarino, N., and Schmidt, N.W. (2013a). CD8+ T Cell Responses to Plasmodium and Intracellular Parasites. Curr. Immunol. Rev. *9*, 169–178.

Villarino, N., and Schmidt, N.W. (2013b). CD8+ T Cell Responses to Plasmodium and Intracellular Parasites. Curr. Immunol. Rev. *9*, 169–178.

Vythilingam, I., Wong, M.L., and Wan-Yussof, W.S. (2016). Current status of Plasmodium knowlesi vectors: a public health concern? Parasitology 1–9.

Wang, R., Smith, J.D., and Kappe, S.H.I. (2009). Advances and challenges in malaria vaccine development. Expert Rev. Mol. Med. *11*, e39.

Waters, A. (2006). Malaria: new vaccines for old? Cell *124*, 689–693.

Weiss, W.R., and Jiang, C.G. (2012). Protective CD8+ T lymphocytes in primates immunized with malaria sporozoites. PloS One *7*, e31247.

WER-WHO (2016). WHO | The Weekly Epidemiological Record (WER).

Wesolowski, R., Wozniak, A., Mila-Kierzenkowska, C., and Szewczyk-Golec, K. (2015). Plasmodium knowlesi as a Threat to Global Public Health. Korean J. Parasitol. *53*, 575–581.

White, N.J. (2008). Plasmodium knowlesi: The Fifth Human Malaria Parasite. Clin. Infect. Dis. *46*, 172–173.

White, N.J. (2011a). Determinants of relapse periodicity in Plasmodium vivax malaria. Malar. J. *10*, 297.

White, N.J. (2011b). Determinants of relapse periodicity in Plasmodium vivax malaria. Malar. J. *10*, 297.

White, M.T., Bejon, P., Olotu, A., Griffin, J.T., Bojang, K., Lusingu, J., Salim, N., Abdulla, S., Otsyula, N., Agnandji, S.T., et al. (2014). A combined analysis of immunogenicity, antibody kinetics and vaccine efficacy from phase 2 trials of the RTS,S malaria vaccine. BMC Med. *12*, 117.

WHO (2013). WHO | Malaria vaccine technology roadmap.

WHO (2014). WHO | World Malaria Report 2014.

WHO (2016). WHO | Overview of malaria treatment.

WHO, W. (2011). WHO | Malaria rapid diagnostic test performance. Results of WHO product testing of malaria RDTs: Round 3 (2010-2011).

WHO, W. (2015). WHO | Fact Sheet: World Malaria Report.

Wieczorek, M., Abualrous, E.T., Sticht, J., Álvaro-Benito, M., Stolzenberg, S., Noé, F., and Freund, C. (2017). Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation. Front. Immunol. *8*.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P.L. (1990). Direct gene transfer into mouse muscle in vivo. Science *247*, 1465–1468.

Wongsrichanalai, C., Barcus, M.J., Muth, S., Sutamihardja, A., and Wernsdorfer, W.H. (2007). A Review of Malaria Diagnostic Tools: Microscopy and Rapid Diagnostic Test (RDT). Am. J. Trop. Med. Hyg. *77*, 119–127.

Wu, Y., Ellis, R.D., Shaffer, D., Fontes, E., Malkin, E.M., Mahanty, S., Fay, M.P., Narum, D., Rausch, K., Miles, A.P., et al. (2008). Phase 1 Trial of Malaria Transmission Blocking Vaccine Candidates Pfs25 and Pvs25 Formulated with Montanide ISA 51. PLoS ONE *3*.

Wykes, M.N., Horne-Debets, J.M., Leow, C.-Y., and Karunarathne, D.S. (2014). Malaria drives T cells to exhaustion. Front. Microbiol. *5*, 249.

Yamauchi, L.M., Coppi, A., Snounou, G., and Sinnis, P. (2007). Plasmodium sporozoites trickle out of the injection site. Cell. Microbiol. *9*, 1215–1222.

Zheng, H., Tan, Z., and Xu, W. (2014). Immune Evasion Strategies of Pre-Erythrocytic Malaria Parasites. Mediators Inflamm. *2014*.

Zimmerman, P.A., and Howes, R.E. (2015). Malaria diagnosis for malaria elimination. Curr. Opin. Infect. Dis.

CHAPTER 2

Rationale and research questions

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 *Pf*SPZ 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 *Pf*SPZ challenge?
3. What are the changes in humoral and cellular immune responses following *Pf*SPZ 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

The article has been published in the New England Journal of Medicine (2012). Doi: 10.1056/NEJMoa1208394.

ORIGINAL ARTICLE

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

The RTS,S Clinical Trials Partnership

The authors are listed in the Appendix. All the authors assume responsibility for the overall content and integrity of the article. Address reprint requests to Ms. Sara Mian-McCarthy at PATH Malaria Vaccine Initiative, Communications and Advocacy Unit, 455 Massachusetts Ave. NW, Suite 1000, Washington, DC 20001-2621, or at smian-mccarthy@path.org.

This article was published on November 9, 2012, at NEJM.org.

N Engl J Med 2012;367:2284-95.
DOI: 10.1056/NEJMoa1208394

Copyright © 2012 Massachusetts Medical Society.

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 all malaria episodes, 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.[1,2] 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.[1]

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).[3] 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[3-7] 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[6] 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.[8]

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.[9] EPI vaccines comprised a diphtheria–tetanus–whole-cell pertussis–hepatitis B–*Hemophilus 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).[4,10]

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.[11] 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). Information on related adverse events within 30 days after vaccination was collected for all participants. Study clinicians used clinical judgment to decide whether an adverse event was likely to be related to the vaccine. In an analysis of previous RTS,S studies, rash was observed more frequently in children vaccinated with RTS,S than in controls.[12] Rashes and mucocutaneous diseases occurring within 30 days after vaccination and seizures occurring within 7 days after vaccination were reported according to Brighton Collaboration guidelines[13,14] (see the Methods section in the Supplementary Appendix).

IMMUNOGENICITY

Anti-circumsporozoite antibodies were measured by means of enzyme-linked immunosorbent assay[15] 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 millimeter or greater was considered to be positive.

LABORATORY AND RADIOLOGIC PROCEDURES

Laboratory and radiologic procedures have been reported previously[5] and are described in the Supplementary Appendix.

STATISTICAL ANALYSIS

The statistical methods have been described in detail previously.[3,7] 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.

Variable	RTS,S/AS01 Vaccine			Control Vaccine			Protective Efficacy		Protective Efficacy Adjusted for Covariates*	
	No. of Events	Person-Yr	Event Rate	No. of Events	Person-Yr	Event Rate	% (CI)†	P Value	% (95% CI)	P Value
Clinical malaria‡										
Per-protocol population (12 mo after third dose of vaccine)										
First or only episode										
>5000 parasites/mm³ and temperature ≥37.5°C (coprimary end point)	1161	3163	0.37	714	1476	0.48	31.3 (23.6–38.3)	<0.001	31.5 (24.7–37.6)	<0.001
>0 parasites/mm³ and measured or reported fever	1475	2921	0.50	879	1328	0.66	32.4 (26.5–37.9)	<0.001	32.6 (26.7–38.0)	<0.001
>500 parasites/mm³ and temperature ≥37.5°C	1282	3073	0.42	770	1429	0.54	30.3 (23.7–36.2)	<0.001	30.4 (23.8–36.3)	<0.001
>20,000 parasites/mm³ and temperature ≥37.5°C	1005	3256	0.31	630	1535	0.41	31.4 (24.2–37.9)	<0.001	31.6 (24.4–38.1)	<0.001
All episodes, >5000 parasites/mm³ and temperature ≥37.5°C	2301	3604	0.64	1626	1790	0.91	32.9 (26.3–38.8)	<0.001	33.0 (26.4–38.9)	<0.001
Intention-to-treat population (14 mo after first dose of vaccine)										
First or only episode, >5000 parasites/mm³ and temperature ≥37.5°C	1283	4106	0.31	782	1949	0.40	30.1 (23.6–36.1)	<0.001		
All episodes, >5000 parasites/mm³ and temperature ≥37.5°C	2615	4688	0.56	1864	2345	0.79	32.9 (26.7–38.5)	<0.001		
	No. of Infants	No. Affected	Affected rate	No. of Infants	No. Affected	Affected rate	% (95% CI)	P Value		
			%			%				
Severe malaria§										
Per-protocol population (12 mo after third dose of vaccine)										
Primary case definition	3995	58	1.5	2008	46	2.3	36.6 (4.6–57.7)	0.02		
Secondary case definition	3995	63	1.6	2008	51	2.5	37.9 (8.3–57.8)	0.01		
Intention-to-treat population (14 mo after first dose of vaccine)										
Primary case definition	4358	77	1.8	2179	52	2.4	26.0 (−7.4–48.6)	0.09		
Secondary case definition	4358	83	1.9	2179	58	2.7	28.4 (−1.9–49.4)	0.06		

* 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 diagnosis of a coexisting illness. The secondary 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, including cases in which a coexisting illness 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 radiographically 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*[16] 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)

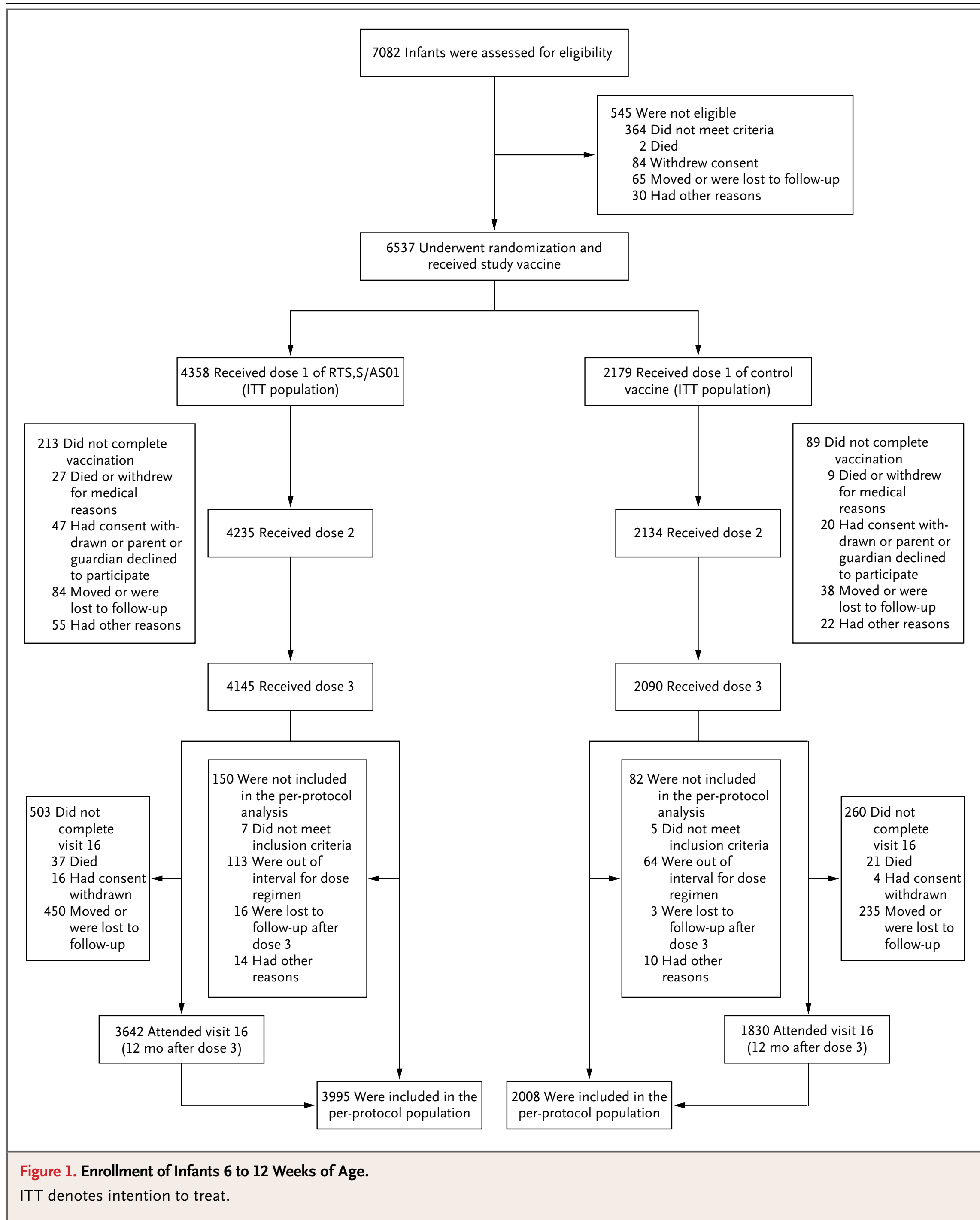


Figure 1. Enrollment of Infants 6 to 12 Weeks of Age.
ITT denotes intention to treat.

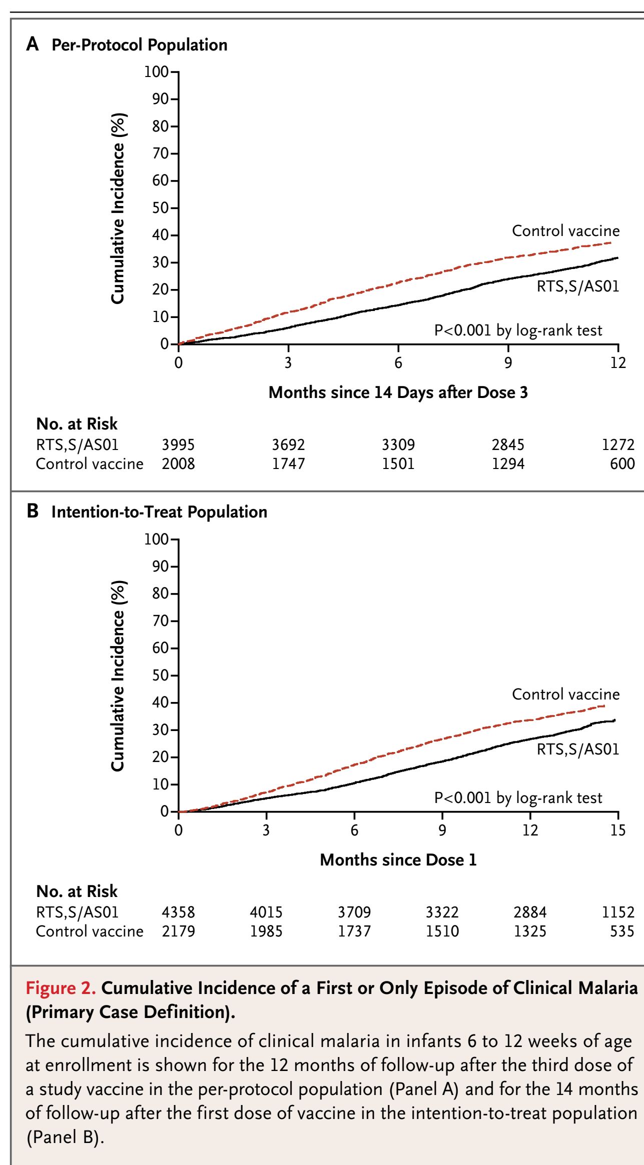


Figure 2. Cumulative Incidence of a First or Only Episode of Clinical Malaria (Primary Case Definition).

The cumulative incidence of clinical malaria in infants 6 to 12 weeks of age at enrollment is shown for the 12 months of follow-up after the third dose of a study vaccine in the per-protocol population (Panel A) and for the 14 months of follow-up after the first dose of vaccine in the intention-to-treat population (Panel B).

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 meningococcal vaccine and was lower than that observed with 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).

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

Variable	RTS,S/AS01 Vaccine (N=4358)		Control Vaccine (N=2179)	
	No. of Infants	% (95% CI)	No. of Infants	% (95% CI)
Serious events in all infants				
≥1 Serious adverse event	782	17.9 (16.8–19.1)	419	19.2 (17.6–20.9)
≥1 Serious adverse event, excluding malaria	760	17.4 (16.3–18.6)	407	18.7 (17.1–20.4)
≥1 Fatal serious adverse event*	66	1.5 (1.2–1.9)	28	1.3 (0.9–1.9)
≥1 Serious adverse event related to vaccine	4	0.1 (0.0–0.2)	3	0.1 (0.0–0.4)
≥1 Serious adverse event within 30 days after vaccination	192	4.4 (3.8–5.1)	96	4.4 (3.6–5.4)
Events with an incidence ≥0.5%†				
Pneumonia	302	6.9 (6.2–7.7)	152	7.0 (5.9–8.1)
Gastroenteritis	260	6.0 (5.3–6.7)	139	6.4 (5.4–7.5)
Malaria	184	4.2 (3.6–4.9)	115	5.3 (4.4–6.3)
Anemia	90	2.1 (1.7–2.5)	58	2.7 (2.0–3.4)
Febrile convulsion	82	1.9 (1.5–2.3)	46	2.1 (1.5–2.8)
Bronchiolitis	28	0.6 (0.4–0.9)	21	1.0 (0.6–1.5)
Convulsion	41	0.9 (0.7–1.3)	19	0.9 (0.5–1.4)
Bronchopneumonia	35	0.8 (0.6–1.1)	20	0.9 (0.6–1.4)
Upper respiratory tract infection	36	0.8 (0.6–1.1)	19	0.9 (0.5–1.4)
Salmonella sepsis	26	0.6 (0.4–0.9)	16	0.7 (0.4–1.2)
Malnutrition	29	0.7 (0.4–1.0)	7	0.3 (0.1–0.7)
Sepsis	26	0.6 (0.4–0.9)	10	0.5 (0.2–0.8)
HIV infection‡	27	0.6 (0.4–0.9)	9	0.4 (0.2–0.8)
Enteritis	11	0.3 (0.1–0.5)	12	0.6 (0.3–1.0)
Urinary tract infection	16	0.4 (0.2–0.6)	10	0.5 (0.2–0.8)
Measles	20	0.5 (0.3–0.7)	7	0.3 (0.1–0.7)
Pyrexia	15	0.3 (0.2–0.6)	11	0.5 (0.3–0.9)

* 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

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).

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-

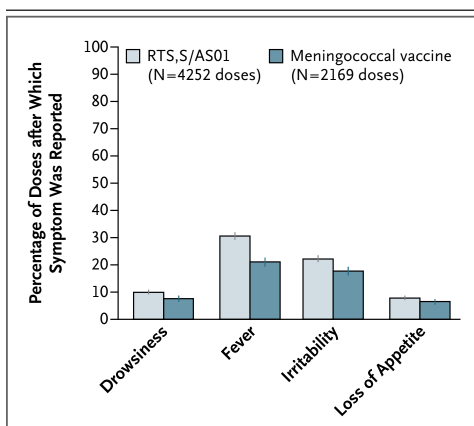


Figure 3. Incidence of Solicited Reports of Adverse Events during the 7-Day Postvaccination Period after Each Dose (Intention-to-Treat Population).

Solicited reports of adverse events during the 7-day postvaccination period were recorded and analyzed in the first 200 infants enrolled at each study site.

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).[17] 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,[3] 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.

Drs. Aide, Greenwood, and Woods report receiving grant support from GlaxoSmithKline through their institutions. Drs. Aponte and Sacarlal report receiving consulting fees from GlaxoSmithKline through their institutions. Drs. Jongert and Olivier report being employees of GlaxoSmithKline, and Drs. Ballou, Guerra, Lapierre, Leach, Ofori-Anyinam, and Vekemans and Mr. Lievens report being employees of and holding stock in GlaxoSmithKline. Dr. Rettig reports receiving travel support from the PATH Malaria Vaccine Initiative, and Mr. Bawa reports receiving travel support from the PATH Malaria Vaccine Initiative through his institution. Drs. Bejon, Mwambingu, and Olotu report receiving grant support from the PATH Malaria Vaccine Initiative through their institutions. Dr. Cohen reports receiving consulting fees from and holding stock in GlaxoSmithKline, being a former employee of GlaxoSmithKline, and being a named inventor on several patents and patent applications related to malaria-vaccine development, the rights to which have been assigned to GlaxoSmithKline. Dr. D'Alessandro reports receiving consulting fees and lecture fees from Sigma-Tau Pharmaceuticals through his institution and lecture fees from Novartis through his institution. Dr. Kaslow reports holding stock and stock options in Merck. Dr. Loucq reports holding stock in GlaxoSmithKline. Dr. Lusingu reports receiving grant support, payment for the development of education presentations, and travel support from the PATH Malaria Vaccine Initiative through his institution and grant support from GlaxoSmithKline through his institution. Dr. Marsh reports receiving travel support and payment for board membership from Novartis. Dr. Njuguna reports receiving consulting fees from GlaxoSmithKline and grant support from the PATH Malaria Vaccine Initiative through her institution. Dr. Schellenberg reports receiving consulting fees from the PATH Malaria Vaccine Initiative. Dr. Tanner reports receiving payment for board membership from the UBS Optimus Foundation, payment for board membership from Novartis through his institution, grant support and travel support from the PATH Malaria Vaccine Initiative through his institution, and travel support from Sanaria through his institution. No other potential conflict of interest relevant to this article was reported.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

APPENDIX

The authors are as follows: **Albert Schweitzer Hospital, Lambaréné, Gabon, and Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany:** Selidji Todagbe Agnandji, M.D., M.P.H., Bertrand Lell, M.D., José Francisco Fernandes, M.D., Béatrice Peggy Abossolo, M.D., Barbara Gaelle Nfono Ondo Methogo, M.D., Anita Lumeka Kabwende, M.D., Ayola Akim Adegnika, M.D., Ph.D., Benjamin Mordmüller, M.D., Saadou Issifou, M.D., Ph.D., Peter Gottfried Kremsner, M.D.; **Centro de Investigação em Saúde de Manhiça, Manhiça, Mozambique, and Barcelona Center for International Health Research (CRESIB)–Universitat de Barcelona, Hospital Clinic, Barcelona:** Jahit Sacarlal, M.D., M.P.H., Ph.D., Pedro Aide, M.D., Ph.D., Miguel Lanaspa, M.D., John J. Aponte, M.D., Ph.D., Sonia Machevo, M.D., Sozinho Acacio, M.D., Helder Bulo, D.V.M., Betuel Sigauque, M.D., Ph.D., Eusébio Macete, M.D., M.P.H., Ph.D., Pedro Alonso, M.D., Ph.D.; **Ifakara Health Institute, Bagamoyo, Tanzania, and Swiss Tropical and Public Health Institute, Basel, Switzerland:** Salim Abdulla, M.D., Ph.D., Nahya Salim, M.D., Rose Minja, C.O., Maxmillian Mpina, M.Sc., Saumu Ahmed, M.D., Ali Mohammed Ali, M.Sc., Ali Takadir Mtoro, M.D., Ali Said Hamad, M.D., Paul Mutani, M.D, Marcel Tanner, Ph.D.; **Institut de Recherche en Science de la Santé, Nanoro, Burkina Faso, and Institute of Tropical Medicine, Antwerp, Belgium:** Halidou Tinto, Pharm.D., Ph.D., Umberto D'Alessandro, M.D., Ph.D., Hermann Sorgho, Ph.D., Innocent Valea, Pharm.D., Biébo Bihoun, M.D., Issa Guiraud, M.D., Berenger Kaboré, M.D., Olivier Sombié, M.D., Robert Tinga Guiguemdé, M.D., Ph.D., Jean Bosco Ouédraogo, M.D., Ph.D.; **KEMRI/CDC Research and Public Health Collaboration, Kisumu, Kenya:** Mary J. Hamel, M.D., D.T.M.&H., Simon Kariuki, Ph.D., Martina Oneko, M.D., Chris Odero, Dip.Clin.Med., H.N.D.P.H., Kephas Otieno, H.N.D.M.L.T., Norbert Awino, B.Ed, P.Dip.P.M., Meredith McMorrow, M.D., M.P.H., Vincent Muturi-Kioi, M.B., Ch.B., Kayla F. Laserson, Sc.D., Laurence Slutsker, M.D., M.P.H.; **KEMRI–Walter Reed Project, Kombewa, Kenya:** Walter Otieno, M.D., M.Med., Ph.D., Lucas Otieno, M.D., M.P.H., Nekoye Otsyula, M.B., Ch.B., Stacey Gondi, M.C.H.D., Allan Otieno, M.B., Ch.B., M.Med., Victorine Owira, B.A., Esther Oguk, Dip.Clin.Med., George Odongo, B.Sc., Jon Ben Woods, M.D., Bernhards Ogutu, M.D., Ph.D.; **KEMRI–Wellcome Trust Research Program, Kilifi, Kenya:** Patricia Njuguna, M.B., Ch.B., Roma Chilengi, M.D., M.P.H., Pauline Akoo, M.B., Ch.B., Christine Kerubo, M.B., Ch.B., Charity Maingi, R.N., M.P.H., Trudie Lang, Ph.D., Ally Olotu, M.B., Ch.B., Philip Bejon, M.B., B.S., D.T.M.&H., Ph.D., Kevin Marsh, M.D., M.R.C.P., D.T.M.&H., Gabriel Mwambingu, H.N.D.M.L.T.; Kintampo Health Research Center, **Kintampo, Ghana, and London School of Hygiene and Tropical Medicine, London:** Seth Owusu-Agyei, Ph.D., Kwaku Poku Asante, M.D., M.P.H., Kingsley Osei-Kwakye, M.D., M.P.H., Owusu Boahen, M.P.H., David Dosoo, M.Sc., Isaac Asante, M.B.A., George Adjei, M.Sc., Evans Kwara, M.D., Daniel Chandramohan, M.D., Ph.D., Brian Greenwood, M.D.; **National Institute for Medical Research, Korogwe, Tanzania, University of Copenhagen, Copenhagen, and London School of Hygiene and Tropical Medicine, London:** John Lusingu, M.D., Ph.D., Samwel Gesase, M.D., Anangisye Malabeja, M.D., Omari Abdul, M.D., Coline Mahende, M.Sc., Edwin Liheluka, M.P.H., Lincoln Malle, Dip.M.L.T., Martha Lemnge, Ph.D., Thor G. Theander, M.D., D.Sc., Chris Drakeley, Ph.D.; **School of Medical Sciences, Kumasi, Ghana:** Daniel Ansong, M.B., F.W.A.C.P., Ch.B., Tsiri Agbenyega, M.B., Ch.B., Ph.D., Samuel Adjei, M.B., Ch.B., P.G.Dip., Harry Owusu Boateng, M.B., Ch.B., M.P.H., M.W.A.C.P., Theresa Rettig, M.D., John Bawa, M.B.A., Justice Sylverken, M.B., Ch.B., Grad.Dip., M.W.A.C.P., David Sambian, Dip.Lab.Tech., Anima Sarfo, M.B., Ch.B., Alex Agyekum, M.Phil.; **University of North Carolina Project, Lilongwe, Malawi:** Francis Martinson, M.B., Ch.B., M.P.H., Ph.D., Irving Hoffman, M.P.H., Tisungane Mvalo, M.B., B.S., Portia Kamthunzi, M.B., B.S., M.Trop.Paed., D.T.C.H., Rutendo Nkomo, M.B., Ch.B., Tapiwa Tembo, M.Sc., Gerald Tegha, Dip.Lab.Tech., M.Sc., Mercy Tsidya, Dip.Edu., Jane Kilembe, B.Sc., Chimwemwe Chawinga, Dip.Clin.Med.; **GlaxoSmithKline Vaccines, Wavre, Belgium (in alphabetical order):** W. Ripley Ballou, M.D., Joe Cohen, Ph.D., Yolanda Guerra, M.D., Erik Jongert, Ph.D., Didier Lapierre, M.D., Amanda Leach, M.R.C.P.C.H., Marc Lievens, M.Sc., Opokua Ofori-Anyinam, Ph.D., Aurélie Olivier, Ph.D., Johan Vekemans, M.D., Ph.D.; and **PATH Malaria Vaccine Initiative, Washington, DC (in alphabetical order):** Terrell Carter, M.H.S., David Kaslow, M.D., Didier Leboulleux, M.D., Christian Loucq, M.D., Afiya Radford, B.S., Barbara Savarese, R.N., David Schellenberg, M.D., Marla Sillman, M.S., Preeti Vansadia, M.H.S.

REFERENCES

1. World malaria report 2011. Geneva: World Health Organization, 2011.

2. Steketee RW, Campbell CC. Impact of national malaria control scale-up programmes in Africa: magnitude and attribution of effects. Malar J 2010;9:299.

3. The RTS,S Clinical Trials Partnership. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. N Engl J Med 2011;365:1863-75.

4. Vekemans J, Marsh K, Greenwood B, et al. Assessment of severe malaria in a multicenter, phase III, RTS,S/AS01 malaria candidate vaccine trial: case definition, standardization of data collection and patient care. Malar J 2011;10:221.

5. Swysen C, Vekemans J, Bruls M, et al. Development of standardized laboratory methods and quality processes for a phase III study of the RTS,S/AS01 candidate malaria vaccine. Malar J 2011;10:223.

6. Leach A, Vekemans J, Lievens M, et al. Design of a phase III multicenter trial to evaluate the efficacy of the RTS,S/AS01 malaria vaccine in children across diverse transmission settings in Africa. Malar J 2011;10:224.

7. Lievens M, Aponte JJ, Williamson J, et al. Statistical methodology for the evaluation of vaccine efficacy in a phase III multi-centre trial of the RTS,S/AS01 malaria vaccine in African children. Malar J 2011;10:222.

8. ICH harmonised tripartite guideline: guideline for Good Clinical Practice. International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (ICH). June 1996:30-3, 41-52 (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E6_R1/Step4/E6_R1__Guideline.pdf).

9. Immunization in practice module 2: EPI vaccines. Geneva: World Health Organization, 2004:26.

10. Bejon P, Berkley JA, Mwangi T, et al. Defining childhood severe falciparum malaria for intervention studies. PLoS Med 2007;4(8):e251.

11. Verbal autopsy standards: ascertaining and attributing cause of death. Geneva: World Health Organization, 2007.

12. Vekemans J, Guerra Y, Lievens M, et al. Pooled analysis of safety data from pediatric phase II RTS,S/AS malaria candidate vaccine trials. Hum Vaccin 2011;7:1309-16.

13. Beigel J, Kohl KS, Khuri-Bulos N. Rash including mucosal involvement: case definition and guidelines for collection, analysis, and presentation of immunization safety data. Vaccine 2007;25:5697-706.

14. Bonhoeffer J, Menkes J, Gold MS, et al. Generalized convulsive seizure as an adverse event following immunization: case definition and guidelines for data collection, analysis, and presentation. Vaccine 2004;22:557-62.

15. Clement F, Van Braeckel E, Desombere I, et al. Validation of an enzyme-linked immunosorbent assay for the quantification of human IgG directed against the repeat region of the circumsporozoite protein of the parasite Plasmodium falciparum. Malar J (in press).

16. MedDRA term selection: points to consider: ICH-endorsed guide for MedDRA users. Release 4.2 (http://www.meddramsso.com/files_acrobat/ptc/9491-1410_TermSelPTC_R4_2_sep2011.pdf).

17. Asante KP, Abdulla S, Agnandji S, et al. Safety and efficacy of the RTS,S/AS01E candidate malaria vaccine given with expanded-programme-on-immunisation vaccines: 19 month follow-up of a randomised, open-label, phase 2 trial. Lancet Infect Dis 2011;11:741-9. [Erratum, Lancet Infect Dis 2011;11:727.]
18. Kester KE, Cummings JF, Ofori-Anyinam O, et al. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J Infect Dis 2009;200:337-46.
19. Olotu A, Lusingu J, Leach A, et al. Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5-17 months in Kenya and Tanzania: a randomised controlled trial. Lancet Infect Dis 2011;11:102-9. [Erratum, Lancet Infect Dis 2011;11:159.]
20. Abdulla S, Oberholzer R, Juma O, et al. Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants. N Engl J Med 2008;359:2533-44.
21. Aponte JJ, Aide P, Renom M, et al. Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet 2007;370:1543-51.
22. Dagan R, Poolman J, Siegrist CA. Glycoconjugate vaccines and immune interference: a review. Vaccine 2010;28:5513-23.
23. Lell B, Agnandji S, von Glasenapp I. A randomized trial assessing the safety and immunogenicity of AS01 and AS02 adjuvanted RTS,S malaria vaccine candidates in children in Gabon. PLoS One 2009;4(10):e7611.
24. Hodgins DC, Shewen PE. Vaccination of neonates: problem and issues. Vaccine 2012;30:1541-59.
25. Siegrist CA. Mechanisms by which maternal antibodies influence infant vaccine responses: review of hypotheses and definition of main determinants. Vaccine 2003;21:3406-12.
26. Prymula R, Plisek S. Clinical experience with DTPw-HBV ad DTPw-HBV/Hib combination vaccines. Expert Opin Biol Ther 2008;8:503-13.
27. Insel RA. Potential alterations in immunogenicity by combining or simultaneously administering vaccine components. Ann N Y Acad Sci 1995;754:35-47.
28. Therneau TM, Grambsch PM. Modeling survival data: extending the Cox model. New York: Springer, 2000.
29. White MT, Griffin JT, Drakeley CJ, Ghani AC. Heterogeneity in malaria exposure and vaccine response: implications for the interpretation of vaccine efficacy trials. Malar J 2010;9:82.
30. Halloran ME, Longini IM Jr, Struchiner CJ. Design and analysis of vaccine studies. New York: Springer, 2010.
31. Brabin B. An analysis of malaria parasite rates in infants: 40 years after Macdonald. Trop Dis Bull 1990;87:R1-R21.
32. World Health Organization. Malaria vaccine “rainbow tables” (http://www.who.int/vaccine_research/links/Rainbow/en/index.html).
33. Agnandji ST, Asante KP, Lyimo J, et al. Evaluation of the safety and immunogenicity of the RTS,S/AS01E malaria candidate vaccine when integrated in the expanded program of immunization. J Infect Dis 2010;202:1076-87. [Erratum, J Infect Dis 2011;203:1344.]
Copyright © 2012 Massachusetts Medical Society.

ICMJE SEEKING TWO NEW MEMBER JOURNALS

The International Committee of Medical Journal Editors (ICMJE) is seeking two new member journals to be represented by their editors-in-chief. Information about the ICMJE is available at www.icmje.org. Candidate journals should meet the following criteria:

- be a peer-reviewed general medical journal that publishes original research involving humans
- have a governance structure that ensures editorial independence
- have an editor with experience in the position who expects to continue in the position for at least another 3 years
- be financially able to support the editor’s participation in ICMJE activities

In considering candidates, the ICMJE may seek to improve the balance of geographic areas and publishing models among its membership.

To apply, editors-in-chief of interested journals should submit the following materials to the ICMJE (at icmje@acponline.org):

- brief curriculum vitae
- cover letter describing the journal, including but not necessarily limited to details of the journal’s history, sponsor or publisher, governance structure, publishing model (e.g., subscription, author-pays open access), target audience, print circulation and online traffic, number of manuscript submissions per year, processes used to select material for publication, acceptance rate, databases where indexed, website address, and guidelines for authors
- statement on how the journal might benefit from ICMJE membership and how the ICMJE might benefit from the journal’s membership (should not exceed 1000 words)

The deadline for applications is January 31, 2013.

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 article has been published in Malaria Journal (2015). Doi: 10.1186/s12936-015-0605-7.

Ajua *et al. Malaria Journal* (2015) 14:72
DOI 10.1186/s12936-015-0605-7

MALARIA JOURNAL

RESEARCH Open Access

The effect of immunization schedule with the malaria vaccine candidate RTS,S/AS01$_E$ on protective efficacy and anti-circumsporozoite protein antibody avidity in African infants

Anthony Ajua[1], Bertrand Lell[1,2], Selidji Todagbe Agnandji[2], Kwaku Poku Asante[3], Seth Owusu-Agyei[3,4], Grace Mwangoka[5], Maxmilliam Mpina[5], Nahya Salim[5], Marcel Tanner[5,6], Salim Abdulla[5], Johan Vekemans[7], Erik Jongert[7], Marc Lievens[7], Pierre Cambron[7], Chris F Ockenhouse[8], Peter G Kremsner[1,2] and Benjamin Mordmüller[1,2*]

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/AS01$_E$ 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/AS01$_E$ 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

* Correspondence: benjamin.mordmueller@uni-tuebingen.de
[1]Eberhard Karls Universität Tübingen, Institut für Tropenmedizin, Wilhelmstraße 27, 72074 Tübingen, Germany
[2]Centre de Recherches Médicales de Lambaréné (CERMEL), BP118 Lambaréné, Gabon
Full list of author information is available at the end of the article

BioMed Central

© 2015 Ajua et al.; licensee BioMed Central. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

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/$AS01_E$ in infants that received the vaccine as part of a phase IIb clinical trial to assess safety and efficacy of RTS,S/$AS01_E$ 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/$AS01_E$ 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/$AS01_E$ as three injections, one month apart (0, 1, 2 months schedule [012]; n = 170), 2) RTS,S/$AS01_E$ 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 (NH_4SCN) 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 NH_4SCN, 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

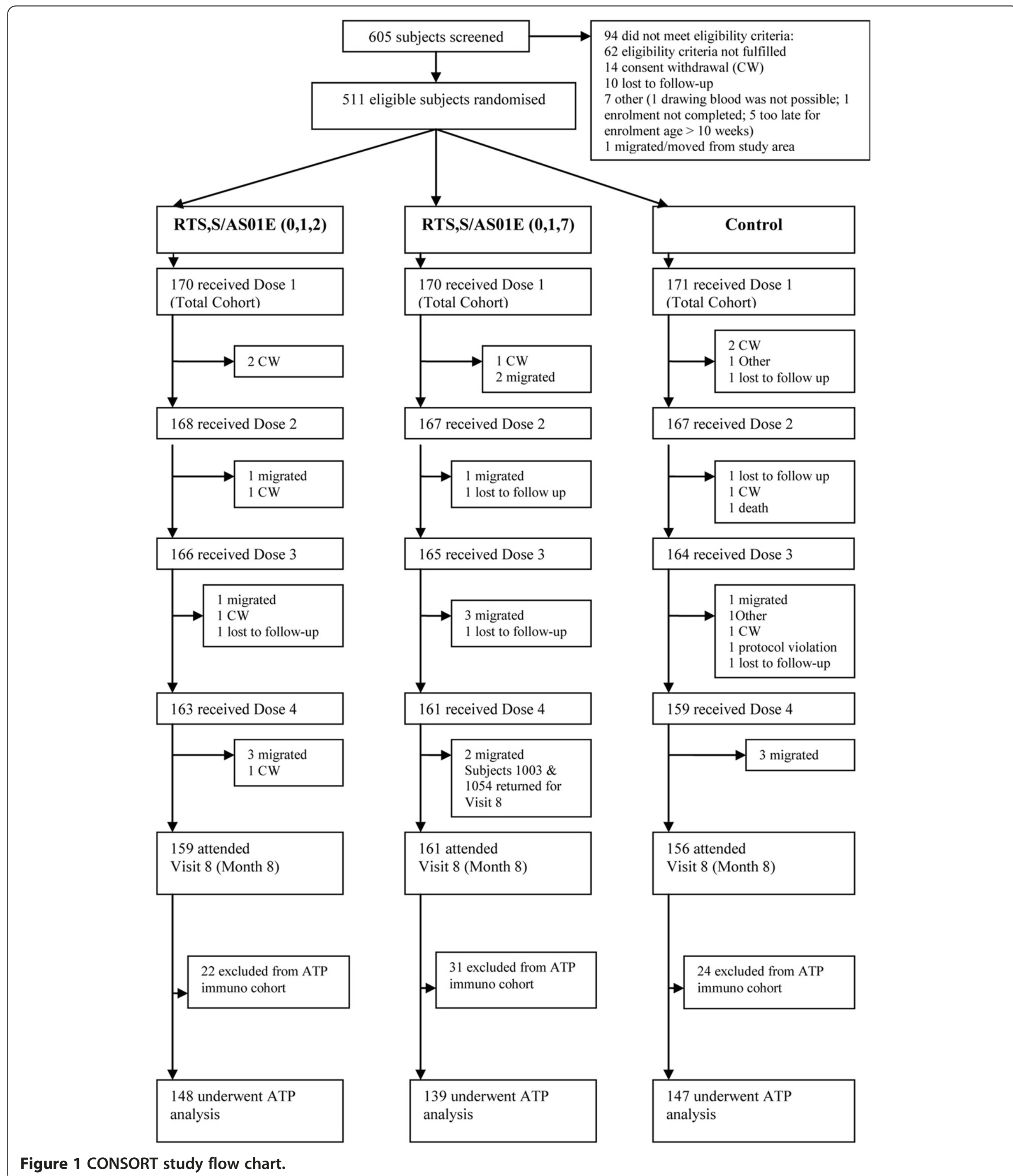


Figure 1 CONSORT study flow chart.

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

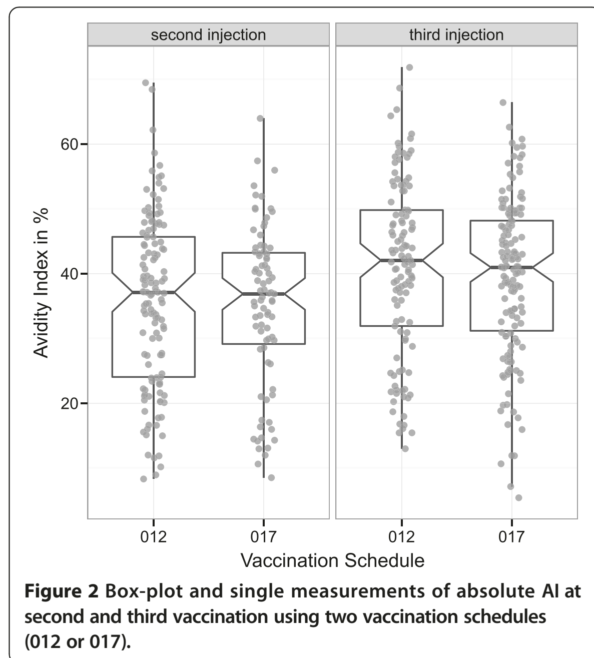


Figure 2 Box-plot and single measurements of absolute AI at second and third vaccination using two vaccination schedules (012 or 017).

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.

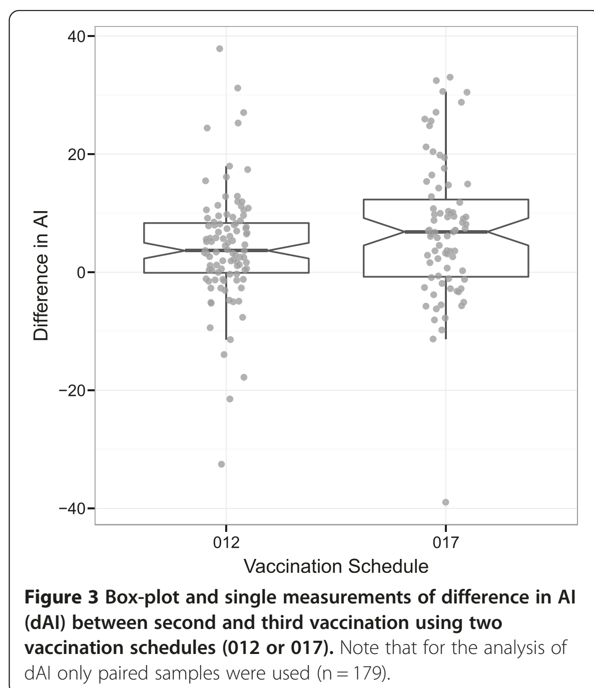


Figure 3 Box-plot and single measurements of difference in AI (dAI) between second and third vaccination using two vaccination schedules (012 or 017). Note that for the analysis of dAI only paired samples were used (n = 179).

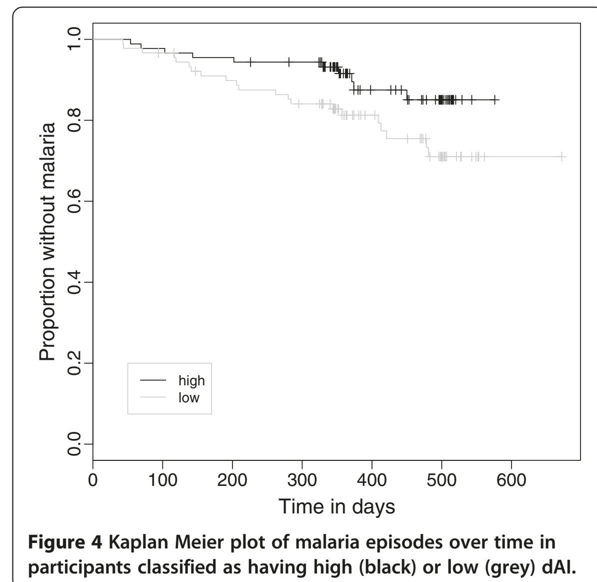


Figure 4 Kaplan Meier plot of malaria episodes over time in participants classified as having high (black) or low (grey) dAI.

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. Preclinical 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. JV, EJ, ML, and PC are employees of the GlaxoSmithKline group of companies. JV, EJ and ML receive GlaxoSmithKline stock and/or options. CFO is an employee of PATH-MVI. Other authors report no conflicts of interest other than study funding.

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, 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.

Acknowledgements

The authors thank the participants and their parents, the community members and the Chiefs in the traditional areas, and the management and staff of the local collaborating institutions (the Kintampo Municipal Hospital, Ghana Health Service, and the Kintampo North and South Health Directorates in Kintampo). We also thank Jarno Jansen (Keyrus Biopharma, on behalf of GSK Vaccines) for publication management and editorial assistance. The Deutsche Forschungsgemeinschaft and the Open Access Publishing Fund of the University of Tübingen supported publishing this manuscript under a Creative Commons Attribution License.

Author details

[1]Eberhard Karls Universität Tübingen, Institut für Tropenmedizin, Wilhelmstraße 27, 72074 Tübingen, Germany. [2]Centre de Recherches Médicales de Lambaréné (CERMEL), BP118 Lambaréné, Gabon. [3]Kintampo Health Research Centre, PO Box 200, Kintampo, Ghana. [4]Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK. [5]Bagamoyo Research and Training Centre of Ifakara Health Institute, Bagamoyo, 360 Kiko Avenue, Mikocheni, PO Box 78373, Dar es Salaam, Tanzania. [6]Swiss Tropical and Public Health Institute, Basel, Switzerland. [7]GlaxoSmithKline Biologicals, Rixensart, Belgium. [8]PATH Malaria Vaccine Initiative, 455 Massachusetts Avenue NW, Suite 1000, Washington, DC 20001, USA.

Received: 9 October 2014 Accepted: 2 February 2015
Published online: 13 February 2015

References

1. Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BGNO, Kabwende AL, et al. A phase 3 trial of RTS, S/AS01 malaria vaccine in African infants. N Engl J Med. 2012;367:2284–95.
2. Agnandji ST, Lell B, Soulanoudjingar SS, Fernandes JF, Abossolo BP, Conzelmann C, et al. First results of phase 3 trial of RTS, S/AS01 malaria vaccine in African children. N Engl J Med. 2011;365:1863–75.
3. RTS,S Clinical Trials Partnership. Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. PLoS Med. 2014;11:e1001685.
4. Olotu A, Lusingu J, Leach A, Lievens M, Vekemans J, Msham S, et al. Efficacy of RTS, S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5–17 months in Kenya and Tanzania: a randomised controlled trial. Lancet Infect Dis. 2011;11:102–9.
5. Asante KP, Abdulla S, Agnandji S, Lyimo J, Vekemans J, Soulanoudjingar S, et al. Safety and efficacy of the RTS, S/AS01E candidate malaria vaccine given with expanded-programme-on-immunisation vaccines: 19 month follow-up of a randomised, open-label, phase 2 trial. Lancet Infect Dis. 2011;11:741–9.
6. Aponte JJ, Aide P, Renom M, Mandomando I, Bassat Q, Sacarlal J, et al. Safety of the RTS, S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet. 2007;370:1543–51.
7. Egan JE, Weber JL, Ballou WR, Hollingdale MR, Majarian WR, Gordon DM, et al. Efficacy of murine malaria sporozoite vaccines: implications for human vaccine development. Science. 1987;236:453–6.
8. Porter MD, Nicki J, Pool CD, Debot M, Illam RM, Brando C, et al. Transgenic parasites stably expressing full-length *Plasmodium falciparum* circumsporozoite protein as a model for vaccine down-selection in mice using sterile protection as an endpoint. Clin Vaccine Immunol. 2013;20:803–10.
9. Fairley H. Chemotherapeutic suppression and prophylaxis in malaria. Trans R Soc Trop Med Hyg. 1945;38:311–55.
10. Reed RC, Louis-Wileman V, Wells RL, Verheul AF, Hunter RL, Lal AA. Re-investigation of the circumsporozoite protein-based induction of sterile immunity against *Plasmodium berghei* infection. Vaccine. 1996;14:828–36.
11. Rickman LS, Gordon DM, Wistar Jr R, Krzych U, Gross M, Hollingdale MR, et al. Use of adjuvant containing mycobacterial cell-wall skeleton, monophosphoryl lipid A, and squalane in malaria circumsporozoite protein vaccine. Lancet. 1991;337:998–1001.
12. Agnandji ST, Asante KP, Lyimo J, Vekemans J, Soulanoudjingar SS, Owusu R, et al. Evaluation of the safety and immunogenicity of the RTS, S/AS01E malaria candidate vaccine when integrated in the expanded program of immunization. J Infect Dis. 2010;202:1076–87.
13. Clement F, Dewar V, Van Braeckel E, Desombere I, Dewerchin M, Swysen C, et al. Validation of an enzyme-linked immunosorbent assay for the quantification of human IgG directed against the repeat region of the circumsporozoite protein of the parasite *Plasmodium falciparum*. Malar J. 2012;11:384.
14. Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, Chantavanich P, et al. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am J Trop Med Hyg. 1991;45:297–308.
15. Busse CE, Czogiel I, Braun P, Arndt PF, Wardemann H. Single-cell based high-throughput sequencing of full-length immunoglobulin heavy and light chain genes. Eur J Immunol. 2014;44:597–603.
16. Muellenbeck MF, Ueberheide B, Amulic B, Epp A, Fenyo D, Busse CE, et al. Atypical and classical memory B cells produce *Plasmodium falciparum* neutralizing antibodies. J Exp Med. 2013;210:389–99.
17. Bachmann MF, Kalinke U, Althage A, Freer G, Burkhart C, Roost H, et al. The role of antibody concentration and avidity in antiviral protection. Science. 1997;276:2024–7.
18. Stoute JA, Slaoui M, Heppner DG, Momin P, Kester KE, Desmons P, et al. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS, S Malaria Vaccine Evaluation Group. N Engl J Med. 1997;336:86–91.

PAPER III

Distinct helper T cell type1 and 2 responses associated with malaria protection and risk in RTS,S/AS01E vaccinees

The article has been published in the Journal of Clinical Infectious Diseases (2017).
Doi: 10.1093/cid/cix429.

Clinical Infectious Diseases

MAJOR ARTICLE

IDSA Infectious Diseases Society of America   hivma hiv medicine association   OXFORD

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

Gemma Moncunill,[1,2] Maxmillian Mpina,[3,a] Augusto J. Nhabomba,[2,a] Ruth Aguilar,[1,b] Aintzane Ayestaran,[1,b] Héctor Sanz,[1,b] Joseph J. Campo,[1,2] Chenjerai Jairoce,[2] Diana Barrios,[1] Yan Dong,[4] Núria Díez-Padrisa,[1] José F. Fernandes,[5] Salim Abdulla,[6] Jahit Sacarlal,[2,7] Nana A. Williams,[1] Jaroslaw Harezlak,[4] Benjamin Mordmüller,[5,8] Selidji T. Agnandji,[5] John J. Aponte,[1,2] Claudia Daubenberger,[9,10] Clarissa Valim,[11,12,c] and Carlota Dobaño[1,2,c]

[1]ISGlobal, Barcelona Centre for International Health Research, Hospital Clínic–Universitat de Barcelona, Catalonia, Spain; [2]Centro de Investigação em Saúde de Manhiça, Maputo, Mozambique; [3]Ifakara Health Institute, Bagamoyo Research and Training Centre, Tanzania; [4]Department of Epidemiology and Biostatistics, School of Public Health, Indiana University, Bloomington; [5]Centre de Recherches Médicales de Lambaréné, Albert Schweitzer Hospital, Gabon; [6]Ifakara Health Institute, Dar es Salaam, Tanzania; [7] Faculdade de Medicina da Universidade Eduardo Mondlane, Maputo, Mozambique; [8]Institute of Tropical Medicine, German Center for Infection Research, University of Tübingen, Germany; [9]Swiss Tropical and Public Health Institute, Basel, and [10]University of Basel, Switzerland; [11]Department of Osteopathic Medical Specialties, Michigan State University, East Lansing; and [12]Department of Immunology and Infectious Diseases, Harvard T. H. Chan School of Public Health, Boston, Massachusetts

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 $P \le .01$). 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 (T_H1)–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 T_H1-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 T_H1 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

Received 6 February 2017; editorial decision 18 April 2017; accepted 4 May 2017; published online May 13, 2017.

[a]M.M. and A.J.N. contributed equally.

[b]R.A., A.A., and H.S. contributed equally.

[c]C.V. and C.Do. contributed equally.

Correspondence: G. Moncunill, ISGlobal, Barcelona Centre for International Health Research (CRESIB), Hospital Clínic–Universitat de Barcelona, Carrer Rosselló 153 (CEK Bldg), E-08036 Barcelona, Catalonia, Spain (gemma.moncunill@isglobal.org).

Clinical Infectious Diseases® 2017;65(5):746–55

© The Author 2017. Published by Oxford University Press for the Infectious Diseases Society of America. All rights reserved. For permissions, e-mail: journals.permissions@oup.com.

DOI: 10.1093/cid/cix429

(T_H1) 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 recruitment. 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

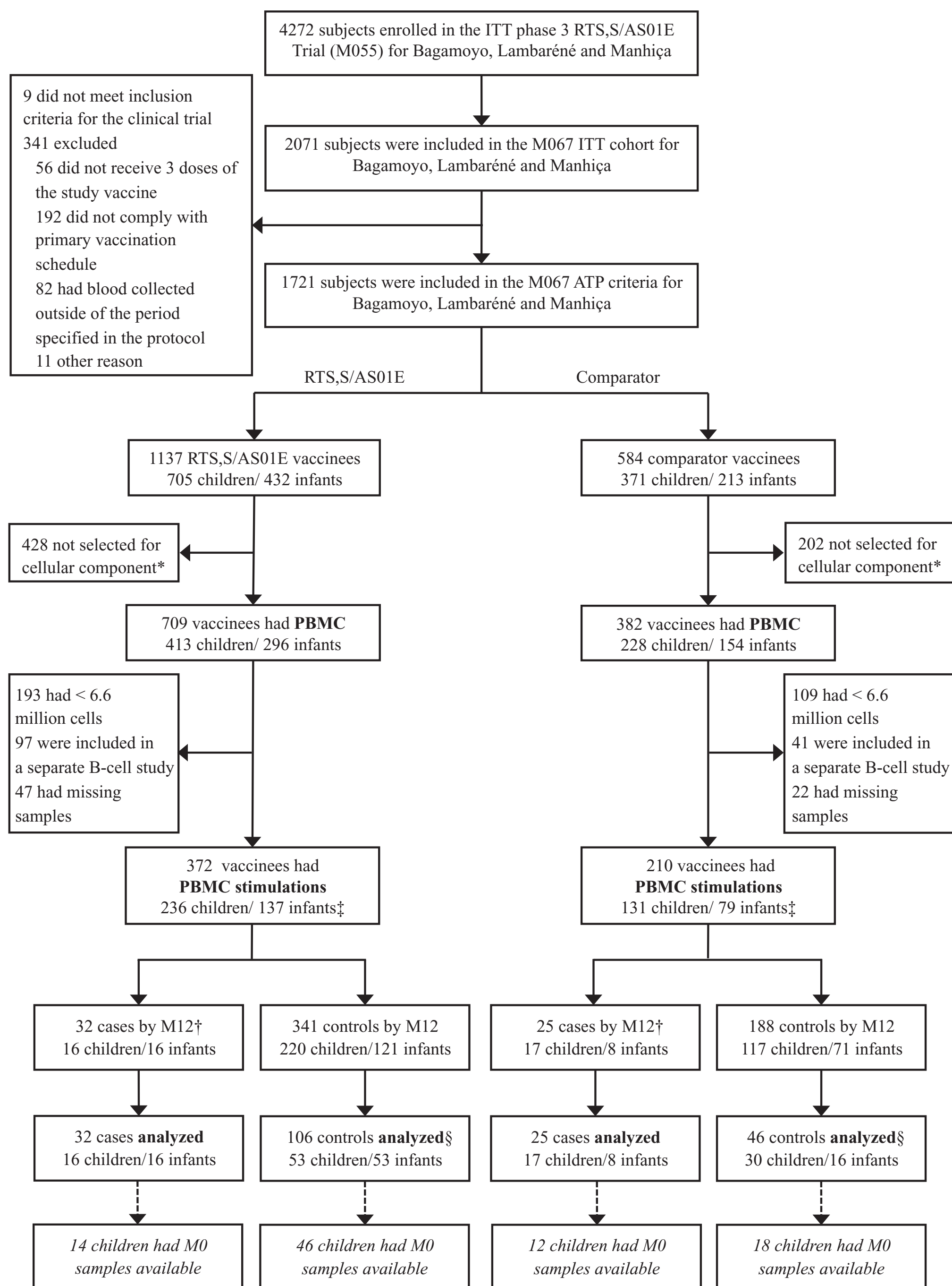


Figure 1. Study profile. *Based on the study protocol, only a subset of vaccinees were enrolled for the cellular component of the immunology study MAL067 (M067), ancillary to the RTS,S/AS01E phase 3 trial MAL055 (M055). ‡Per study protocol, peripheral blood mononuclear cells (PBMCs) were supposed to 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 vaccinees, and 1:2 case:controls were selected among comparator vaccinees. 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.

package [13]. Secondary analysis and details are described in the Supplementary Methods.

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

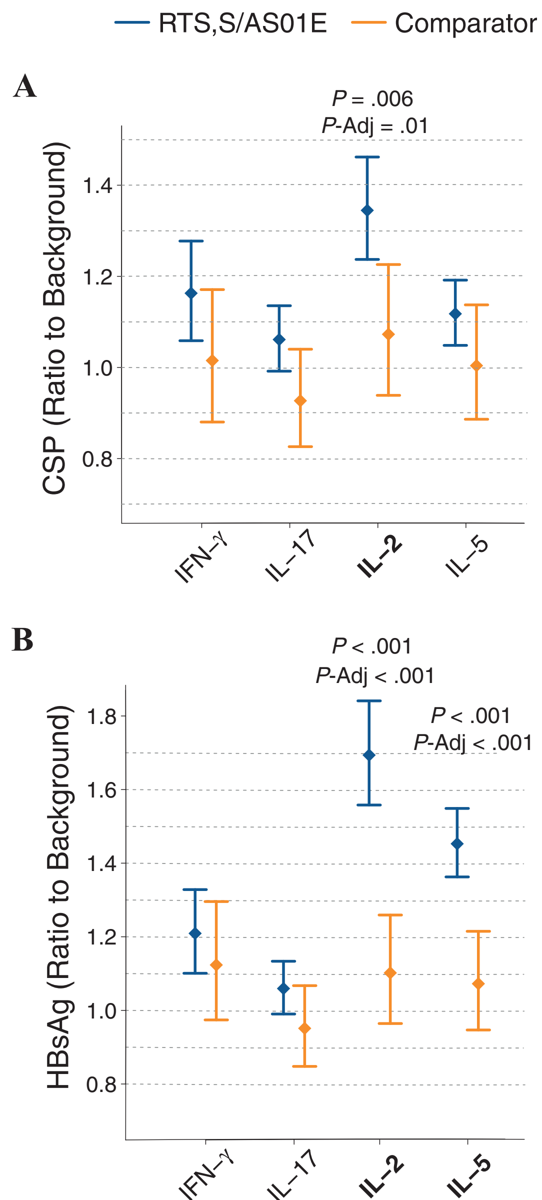


Figure 2. Circumsporozoite protein (CSP; *A*) or hepatitis B surface antigen (HBsAg; *B*) primary marker ratios 1 month postimmunization with RTS,S/AS01E or a comparator vaccine. Diamonds show geometric means of CSP or HBsAg ratios to controls and bars show the 95% confidence intervals. *P* values were 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.

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

	Children			Infants			Difference Age Groups	
Marker	RTS,S/AS01E (n = 69)	Comparator (n = 47)	*P* Value[a]	RTS,S/AS01E (n = 69)	Comparator (n = 24)	*P* Value[a]	*P* Value[b]	Adjusted *P* Value[b]
IFN-γ	36 (24–54)	15 (10–25)	.008	11 (7–16)	10 (4–22)	.88	.002	.007
G-CSF	1314 (1081–1598)	885 (658–1190)	.03	629 (511–774)	693 (452–1062)	.69	<.001	<.001
GM-CSF	345 (265–451)	159 (98–258)	.007	145 (104–201)	149 (71–313)	.95	<.001	<.001
IL-10	337 (260–437)	180 (112–289)	.03	128 (98–166)	164 (93–290)	.44	<.001	<.001
IL-12	2090 (1650–2647)	1094 (739–1619)	.007	841 (687–1029)	811 (533–1232)	.88	<.001	<.001
IL-15	162 (137–191)	104 (81–134)	.005	110 (96–127)	110 (73–168)	1	.01	.03
IL-1β	2491 (2142–2898)	1285 (833–1984)	.007	1210 (1019–1438)	1145 (792–1656)	.79	<.001	<.001
IL-1RA	3422 (2763–4238)	2166 (1605–2924)	.02	1653 (1400–1952)	1505 (913–2479)	.73	<.001	<.001
IP-10	32 (27–40)	26 (22–30)	.07	23 (20–25)	22 (17–28)	.85	.003	.009
TNF	1297 (1035–1625)	521 (303–898)	.004	287 (193–425)	316 (168–594)	.8	<.001	<.001

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

Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon gamma; IL, interleukin; IP, interferon-gamma-induced protein; TNF, tumor necrosis factor.

[a] *P* values for the comparison of RTS,S and comparator vaccinees were computed based on *t* tests of $\log_{10}$-transformed values.

[b] *P* values for assessing differences between age cohorts (interaction with age) were computed through linear regressions and were adjusted for multiple testing using a Holm approach for primary markers and a Benjamini-Hochberg approach for secondary markers.

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 5), and most associations were weak and different from associations with CSP ratios. In contrast, no clear associations were detected in comparator vaccinees.

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 vaccinees (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

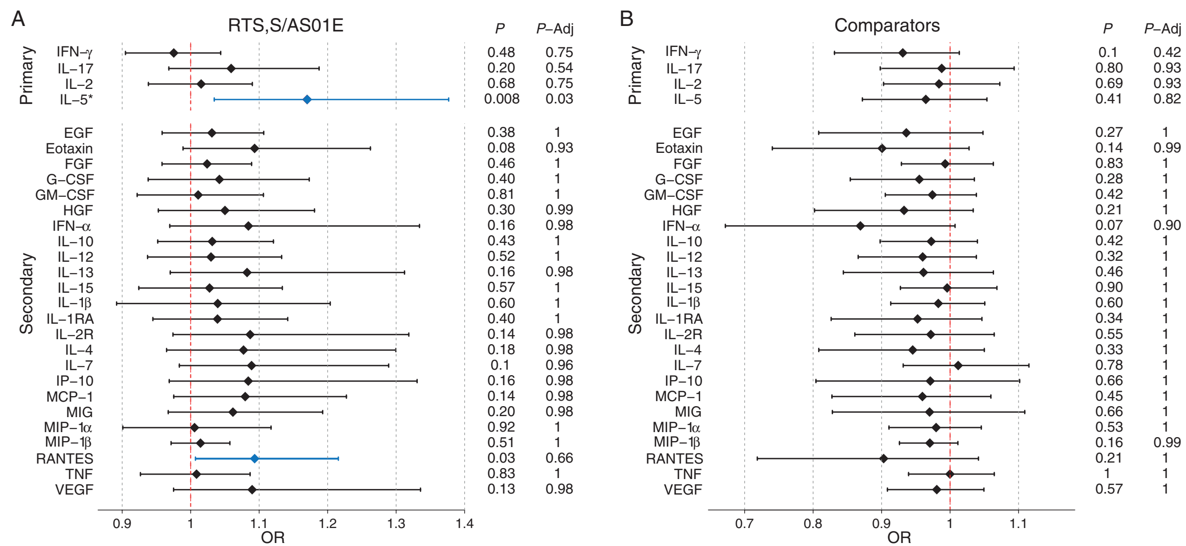


Figure 3. Correlation between clinical malaria and circumsporozoite protein (CSP) marker ratios in RTS,S/AS01E vaccinees (*A*) and comparator vaccinees (*B*). Odds ratios and 95% confidence intervals per 10% increase in primary and secondary marker ratios (CSP ratio to control) 1 month postimmunization. Blue indicates markers that were significantly associated (without adjustment for multiple testing) with clinical malaria in logistic mixed-effects models. *P* values were adjusted (*P*-Adj) for multiple testing through a permutation approach. N = 137 RTS,S/AS01E vaccinees and 70 comparator vaccinees. *The association between clinical malaria and the interleukin 5 ratios was different between RTS,S/AS01E and comparator vaccinees, with *P* value for interaction test adjusted for multiple testing = .049. Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IFN, interferon; IL, interleukin; IP, interferon-gamma-induced protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; OR, odds ratio; RANTES, regulated on activation normal T-cell expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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).

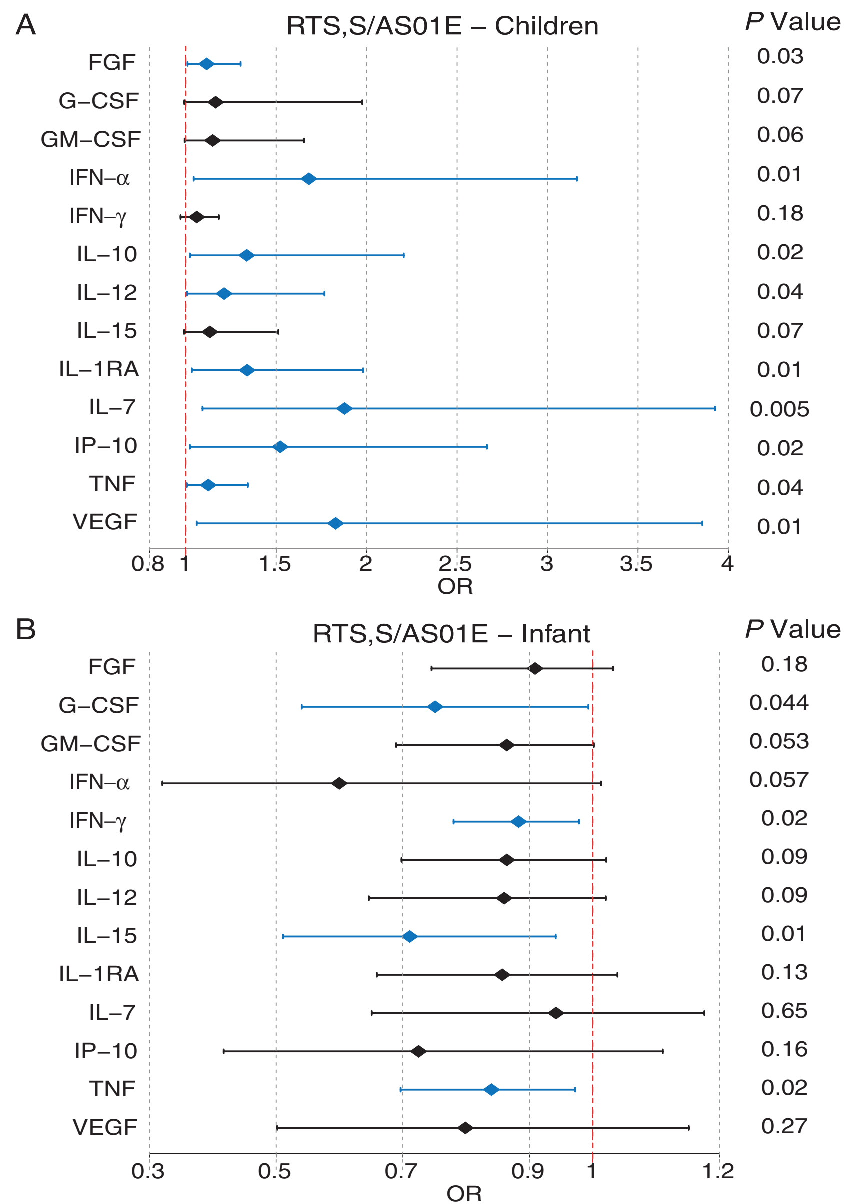


Figure 4. Correlation between clinical malaria and circumsporozoite protein (CSP) marker ratios in RTS,S/AS01E-vaccinated children (*A*) and infants (*B*). Odds ratios and 95% confidence intervals per 10% increase in marker ratios (CSP ratio to control) 1 month postimmunization. Only markers that had 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 69 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 T_H1 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.

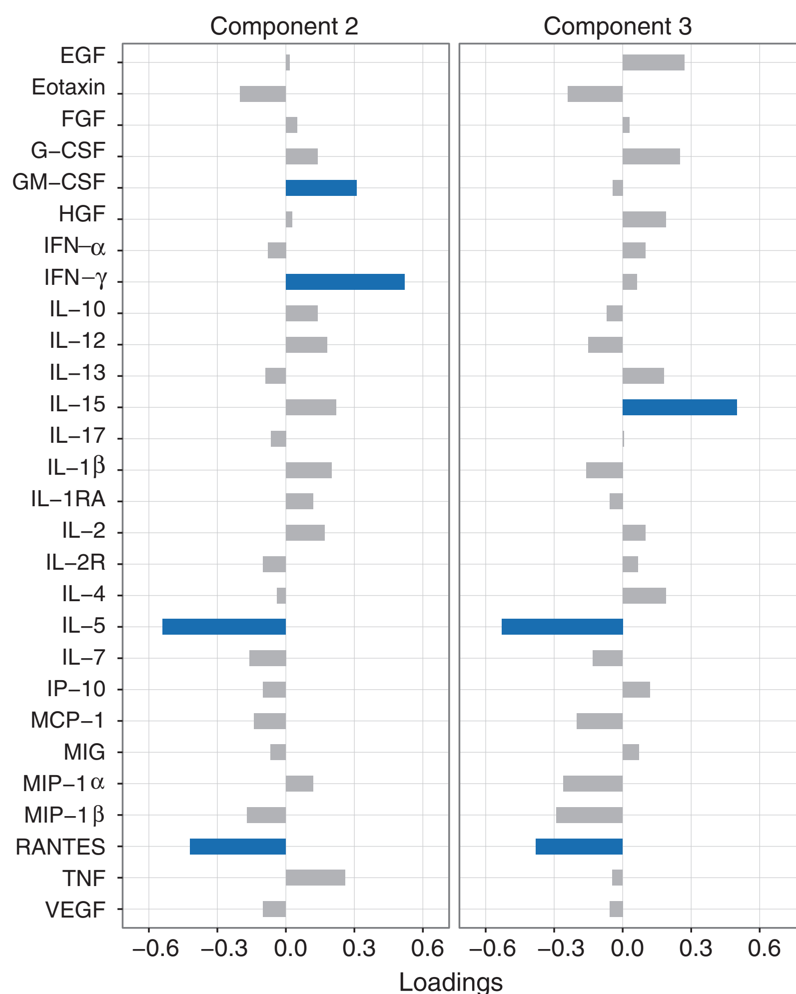
DISCUSSION

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 T_H1-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 T_H2 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 T_H2 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 T_H1 and T_H2 responses could partially explain the association of IL-5 with malaria in RTS,S/AS01E vaccinees. IL-5$^+$ T_H2 cells probably constrain protective T_H1 responses and inhibit several macrophage functions [18]. Exploratory analyses of IFN-γ/IL-5 ratios indicate that a skewed response to T_H2 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. T_H1 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, including NK T cells and CD8$^+$ T cells, which may be involved in RTS,S/AS01E-induced cellular responses to liver-stage parasites. GM-CSF, produced by T_H1 and T_H17, NK, and B cells and macrophages, induces effector functions in granulocytes, monocytes, macrophages, and eosinophils, is critical for dendritic cells, and increases IFN-γ secretion [25]. Therefore, IL-15 and GM-CSF could contribute to induction or enhancement of T_H1-mediated protective responses.

Detected age differences in immunogenicity are in line with immune ontogeny. Newborns have a biased cellular response toward a T_H2 profile, due to diminished T_H1 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



	Component 2	Component 3
Univariate models		
OR (95% CI)	0.36 (0.2 - 0.64)	0.54 (0.32 - 0.92)
AUC	0.714	0.675
Multivariate model		
OR (95% CI)	0.35 (0.2 - 0.62)	0.46 (0.25 - 0.86)
AUC	0.749	

Figure 5. Association of combinations of circumsporozoite protein (CSP) marker ratios with clinical malaria with the combinations obtained through partial least squares (PLS) regression in RTS,S/AS01E vaccinees. Bars quantify the importance (loadings) of each marker (using CSP to control ratios) for specific marker combinations (PLS component) that were significantly associated with clinical malaria (component 1 association with clinical malaria was not statistically significant, $P = .2$). Markers that substantially contributed for the component (loadings <-0.3 or >0.3) are highlighted in blue. Odds ratio and predictive accuracy (area under the receiver operating characteristic curve) from univariate and multivariate models with the selected combination of markers as variables are shown. N = 137 RTS,S/AS01E vaccinees. Abbreviations: AUC, area under the receiver operating characteristic curve; CI, confidence interval; EGF, epidermal growth factor; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IFN, interferon; IL, interleukin; IP, interferon-gamma-induced protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; OR, odds ratio; RANTES, regulated on activation normal T-cell expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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_H1-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 *P. 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 Manhiça, 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 isotypes 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

Author contributions. G. M., C. Do., and C. Da. conceived and designed the cellular immunology experiments. M. M., C. Da., J. F. F., C. Do., A. J. N., C. J., J. J. C., and G. M. processed the samples and performed the stimulations. M. M., A. N., D. B., and R. A. performed the Luminex assays. C. V., J. J. A., J. H., C. Do., and G. M. led development of the experimental and analytical plans. A. A., H. S., and C. V. analyzed the study data and interpreted results with G. M.. J. H. performed the Luminex plate design and provided key intellectual contribution to the analysis of the pilot and the main case-control study. J. J. A., J. H., H. S., Y. D., and C. V. analyzed the pilot study. N. A. W. and N. D.-P. managed and coordinated the study. S. A., S. T. A., J. S., and P. L. A. were site principal investigators (PIs) of the RTS,S/AS01E phase 3 clinical trial. B. M., S. T. A., C. Da., and C. Do. were site PIs of the immunology study. G. M., A. A., C. V., and C. Do. wrote the first drafts of the manuscript. B. M., A. S. T., J. J. C., C. Da., R. A., J. J. C., A. J. N., J. J. A., and H. S. contributed to the writing of the manuscript. All authors agree with manuscript results and conclusions.

Acknowledgments. We thank all the participants of the study and their families. We thank the MAL055 clinical team; the field and laboratory personnel from CERMEL, CISM, ISGlobal, and IHI-BRTC, in particular Laura Puyol, Bendita Zavale, Lázaro Quimice, Nelito Ernesto José, and Helder Bulo; and Carole Amroune and Claudia Hernández for their support. We are also grateful to the members of the MAL067 Vaccine Immunology Workgroup and its Working Groups (Statistical and Cellular). We also thank GSK Vaccines and PATH Malaria Vaccine Initiative for their support in the conduct of the MAL067 Study. We thank Marc Lievens for his help in the selection of samples. ISGlobal is a member of the CERCA Programme, Generalitat de Catalunya.

Financial support. This work was supported by the PATH Malaria Vaccine Initiative; the National Institute of Allergy and Infectious Diseases, National Institutes of Health (grant number R01AI095789); the Ministerio de Economía y Competitividad (Instituto de Salud Carlos III) (award number PI11/00423); and the Agència de Gestió d'Ajuts Universitaris i de Recerca AGAUR (award number 2014SGR991). C. Do. was recipient of a Ramon y Cajal Contract from the Ministerio de Economía y Competitividad (award number RYC-2008–02631). G. M. was recipient of a Sara Borrell–Instituto de Salud Carlos III fellowship (award number CD010/00156).

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.

References

1. RTS,S Clinical Trials Partnership, Agnandji ST, Lell B, et al. A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. N Engl J Med **2012**; 367:2284–95.
2. Agnandji ST, Lell B, Soulanoudjingar SS, et al. First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. N Engl J Med **2011**; 365:1863–75.
3. RTS,S Clinical Trials Partnership. Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. Lancet **2015**; 386:31–45.
4. Barbosa A, Naniche D, Aponte JJ, et al. *Plasmodium falciparum*-specific cellular immune responses after immunization with the RTS,S/AS02D candidate malaria vaccine in infants living in an area of high endemicity in Mozambique. Infect Immun **2009**; 77:4502–9.

5. Agnandji ST, Fendel R, Mestré M, et al. Induction of *Plasmodium falciparum*-specific CD4+ T cells and memory B cells in Gabonese children vaccinated with RTS,S/AS01(E) and RTS,S/AS02(D). PLoS One **2011**; 6:e18559.
6. Ansong D, Asante KP, Vekemans J, et al. T cell responses to the RTS,S/AS01(E) and RTS,S/AS02(D) malaria candidate vaccines administered according to different schedules to Ghanaian children. PLoS One **2011**; 6:e18891.
7. Olotu A, Moris P, Mwacharo J, et al. Circumsporozoite-specific T cell responses in children vaccinated with RTS,S/AS01E and protection against *P falciparum* clinical malaria. PLoS One **2011**; 6:e25786.
8. Ndungu FM, Mwacharo J, Kimani D, et al. A statistical interaction between circumsporozoite protein-specific T cell and antibody responses and risk of clinical malaria episodes following vaccination with RTS,S/AS01E. PLoS One **2012**; 7:e52870.
9. Horowitz A, Hafalla JC, King E, et al. Antigen-specific IL-2 secretion correlates with NK cell responses after immunization of Tanzanian children with the RTS,S/AS01 malaria vaccine. J Immunol **2012**; 188:5054–62.
10. Kester KE, Cummings JF, Ofori-Anyinam O, et al; RTS,S Vaccine Evaluation Group. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J Infect Dis **2009**; 200:337–46.
11. RTS,S Clinical Trials Partnership. Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. PLoS Med **2014**; 11:e1001685.
12. White MT, Verity R, Griffin JT, et al. Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a phase 3 randomised controlled trial. Lancet Infect Dis **2015**; 3099:1–9.
13. R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, **2015**.
14. Neafsey DE, Juraska M, Bedford T, et al. Genetic diversity and protective efficacy of the RTS,S/AS01 malaria vaccine. N Engl J Med **2015**; 373:2025–37.
15. Rivino L, Messi M, Jarrossay D, Lanzavecchia A, Sallusto F, Geginat J. Chemokine receptor expression identifies pre-T helper (Th)1, pre-Th2, and nonpolarized cells among human CD4+ central memory T cells. J Exp Med **2004**; 200:725–35.
16. Upadhyaya B, Yin Y, Hill BJ, Douek DC, Prussin C. Hierarchical IL-5 expression defines a subpopulation of highly differentiated human Th2 cells. J Immunol **2011**; 187:3111–20.
17. Stewart VA, McGrath SM, Walsh DS, et al. Pre-clinical evaluation of new adjuvant formulations to improve the immunogenicity of the malaria vaccine RTS,S/AS02A. Vaccine **2006**; 24:6483–92.
18. Male D, Brostoff J, Roth D, Roitt I. Immunology. 7th edition. Elsevier Health Sciences; **2006**.
19. Jacobsen EA, Helmers RA, Lee JJ, Lee NA. The expanding role(s) of eosinophils in health and disease. Blood **2012**; 120:3882–90.
20. Swanson BJ, Murakami M, Mitchell TC, Kappler J, Marrack P. RANTES production by memory phenotype T cells is controlled by a posttranscriptional, TCR-dependent process. Immunity **2002**; 17:605–15.
21. Crompton PD, Moebius J, Portugal S, et al. Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. Annu Rev Immunol **2014**; 32:157–87.
22. Sun P, Schwenk R, White K, et al. Protective immunity induced with malaria vaccine, RTS,S, is linked to *Plasmodium falciparum* circumsporozoite protein-specific CD4+ and CD8+ T cells producing IFN-gamma. J Immunol **2003**; 171:6961–7.
23. Schwenk R, Lumsden JM, Rein LE, et al. Immunization with the RTS,S/AS malaria vaccine induces IFN-γ(+)CD4 T cells that recognize only discrete regions of the circumsporozoite protein and these specificities are maintained following booster immunizations and challenge. Vaccine **2011**; 29:8847–54.
24. Lumsden JM, Schwenk RJ, Rein LE, et al. Protective immunity induced with the RTS,S/AS vaccine is associated with IL-2 and TNF-α producing effector and central memory CD4 T cells. PLoS One **2011**; 6:e20775.
25. Shi Y, Liu CH, Roberts AI, et al. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know. Cell Res **2006**; 16:126–33.
26. Goenka A, Kollmann TR. Development of immunity in early life. J Infect **2015**; 71(suppl 1):S112–20.
27. Rowe J, Macaubas C, Monger T, et al. Heterogeneity in diphtheria-tetanus-acellular pertussis vaccine-specific cellular immunity during infancy: relationship to variations in the kinetics of postnatal maturation of systemic Th1 function. J Infect Dis **2001**; 184:80–8.
28. Gessner BD, Knobel DL, Conan A, Finn A. Could the RTS,S/AS01 meningitis safety signal really be a protective effect of rabies vaccine? Vaccine **2017**; 35:716–21.
29. Shann F. Nonspecific effects of vaccines and the reduction of mortality in children. Clin Ther **2013**; 35:109–14.
30. Kleinnijenhuis J, Quintin J, Preijers F, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A **2012**; 109:17537–42.
31. Kremsner PG, Winkler S, Brandts C, et al. Prediction of accelerated cure in *Plasmodium falciparum* malaria by the elevated capacity of tumor necrosis factor production. Am J Trop Med Hyg **1995**; 53:532–8.
32. Luty AJ, Perkins DJ, Lell B, et al. Low interleukin-12 activity in severe *Plasmodium falciparum* malaria. Infect Immun **2000**; 68:3909–15.
33. Warimwe GM, Fletcher HA, Olotu A, et al. Peripheral blood monocyte-to-lymphocyte ratio at study enrollment predicts efficacy of the RTS,S malaria vaccine: analysis of pooled phase II clinical trial data. BMC Med **2013**; 11:184.
34. Tsang JS, Schwartzberg PL, Kotliarov Y, et al; Baylor HIPC Center; CHI Consortium. Global analyses of human immune variation reveal baseline predictors of postvaccination responses. Cell **2014**; 157:499–513.
35. Fourati S, Cristescu R, Loboda A, et al. Pre-vaccination inflammation and B-cell signalling predict age-related hyporesponse to hepatitis B vaccination. Nat Commun **2016**; 7:10369.
36. Nakaya HI, Hagan T, Duraisingham SS, et al. Systems analysis of immunity to influenza vaccination across multiple years and in diverse populations reveals shared molecular signatures. Immunity **2015**; 43:1186–98.

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

Mpina M[1,2,3] , [3] Lukindo T[1], Tumbo A[1,2,3], Rutishauser T[2,] , Tanner M[2,3], Abdulla S[1], Daubenberger C[2,3]

1. Ifakara Health Institute, Bagamoyo Branch, Bagamoyo, Tanzania
2. Swiss Tropical and Public Health Institute, Basel, Switzerland
3. University of Basel, Basel Switzerland

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-g responses assessed by ex-vivo ELISpot were higher in protected than infected individual (12). In addition, analysis of IL2, IFN-g 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-g, 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/AS0 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-g, 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 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 *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). The

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).**

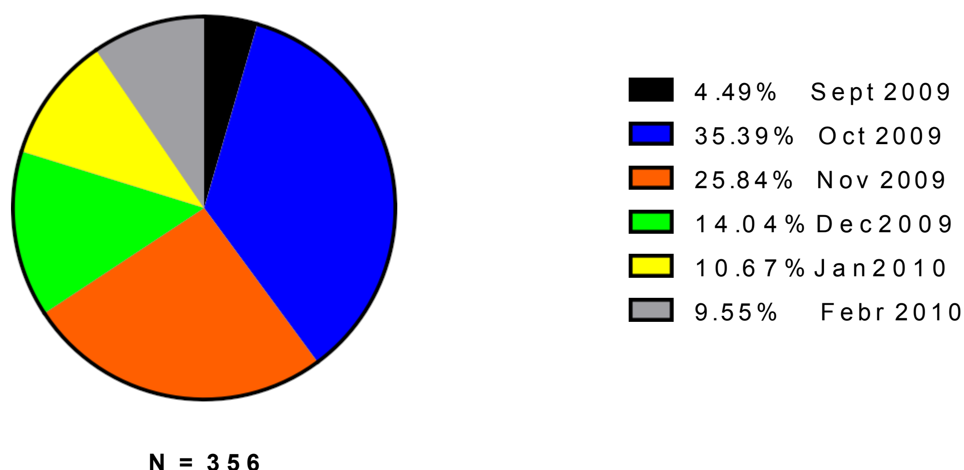


Figure 6: Number of children enrolled in Bagamoyo study site by months during recruitment period

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.

Sample type	Baseline	Month 3	Month 20	Month 21	Month 32
PBMC	356 (100%)	353 (99.2%)	0	322 (90.5%)	318 (89.3%)
Plasma	356	353	0	322	318
Filter paper	0	0	336	0	0

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.

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

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.

Frequency of children recruited at Bagamoyo study area by village

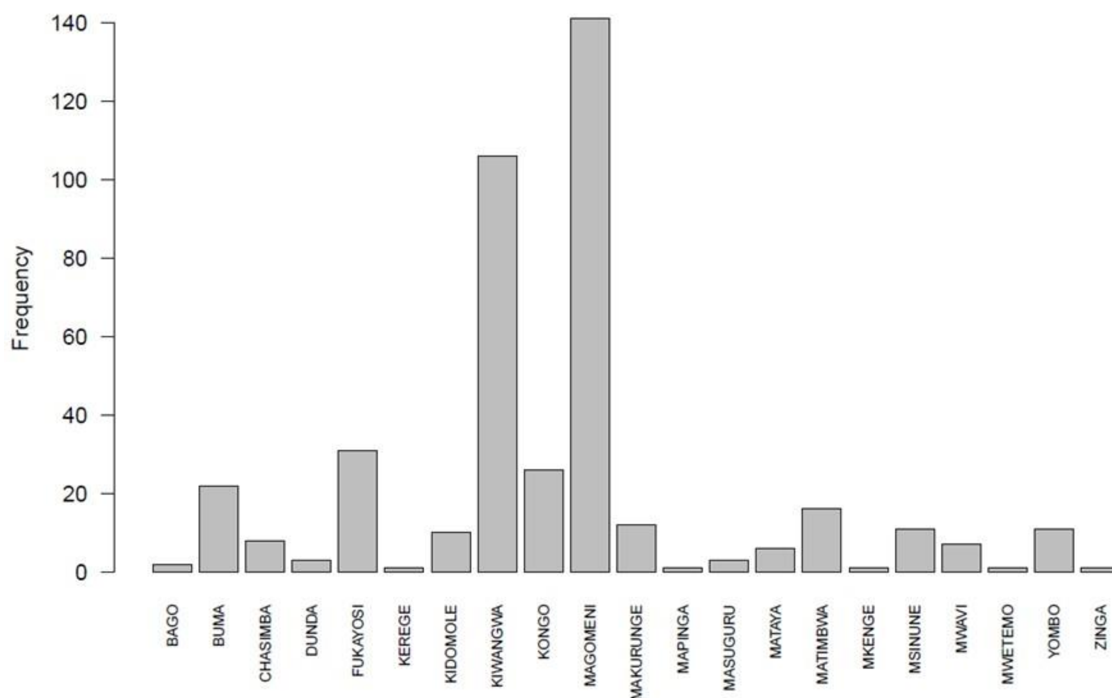


Figure 2: Frequencies of children recruited per village within Bagamoyo clinical trial study area

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

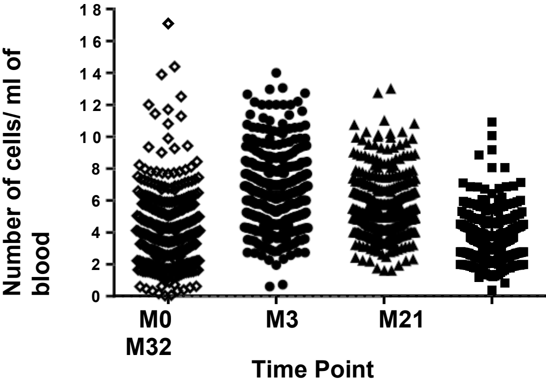


Figure 3: Shown are sampling time points M0, M3, M23 and M34 of the Bagamoyo cohort

Percentage PBMCs viability of sample collected on different time point during

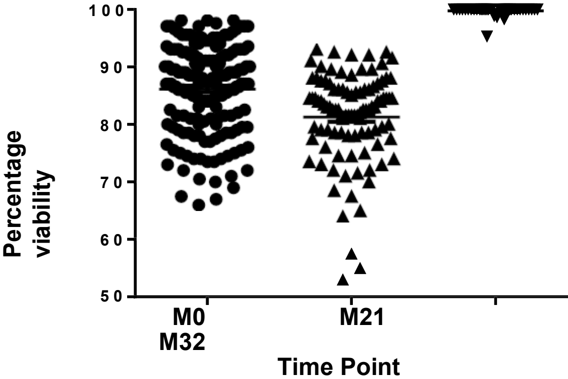


Figure 4: Total viable PBMC counted upon day of thawing (day1-yield) and after over-night rest in culture media (day2- recovery). Differences analysed by Wilcoxon non-parametric non-paired test (N=115)

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

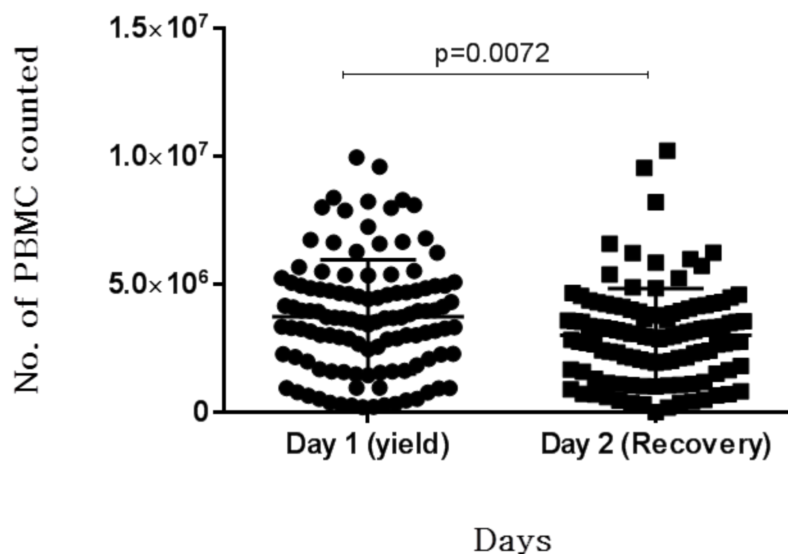


Figure 5: Total viable PBMC counted upon day of thawing (day1-yield) and after over-night rest in culture media (day2- recovery). Differences analysed by Wilcoxon non-parametric non-paired test (N=115)

Percentage viability upon thawing and after overnight rest in

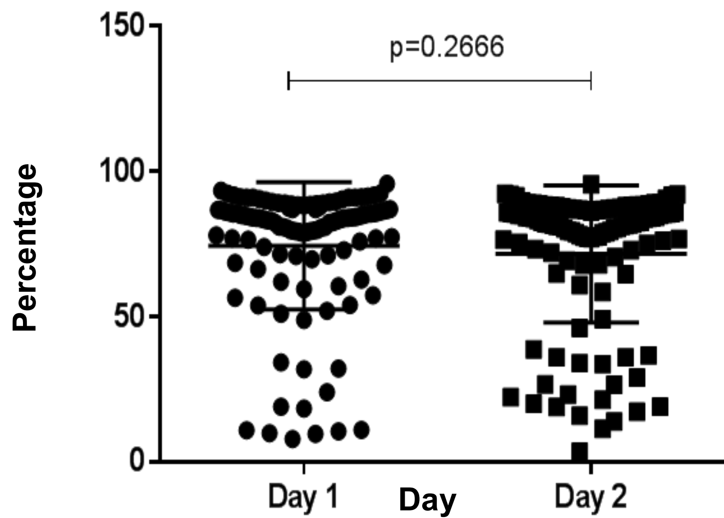


Figure 6: Percentage PBMC viability upon day of thawing (day1) and after over-nigh rest in culture media (day2). Differences analysed by Wilcoxon non-parametric non-paired test (N=115)

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.

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

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

Flow cytometer gating strategies

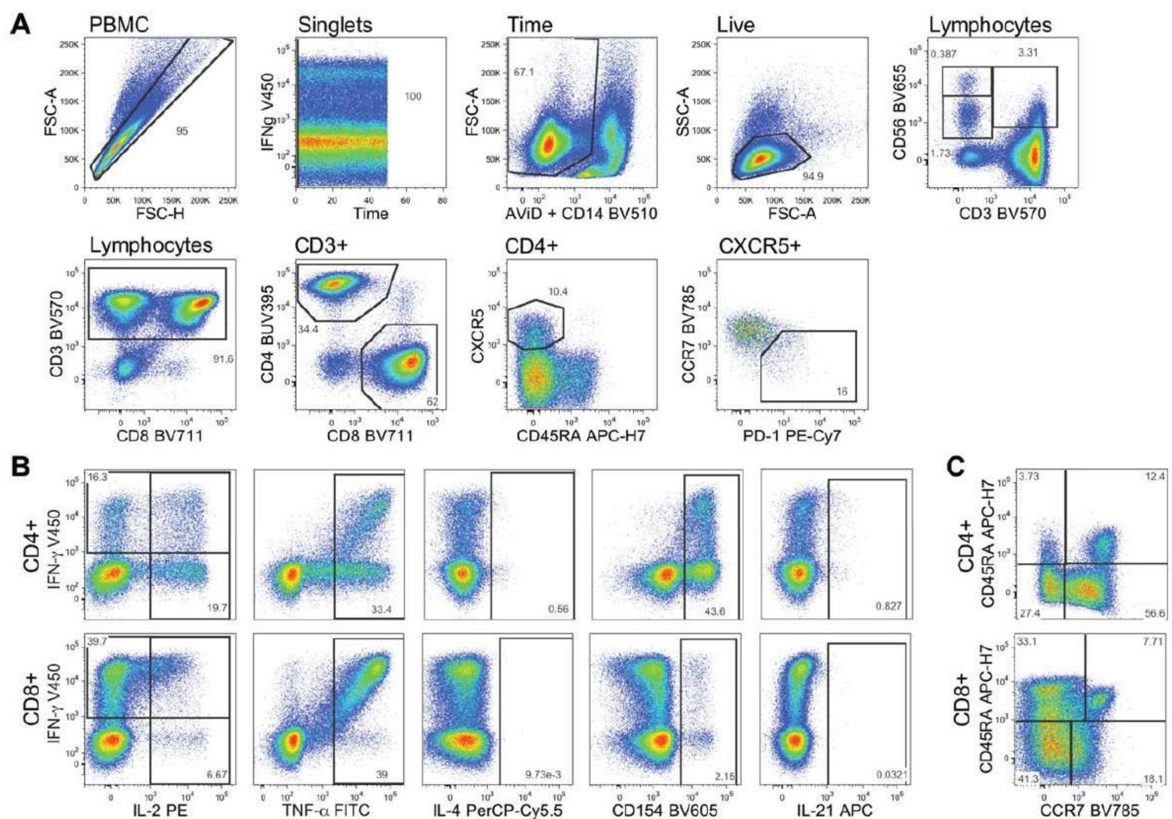


Figure 7: Example of the staining and gating strategy for PBMC stimulated with Staphylococcal enterotoxin B (SEB). All gates for non-functional markers were defined using fluorescence minus one (FMO) controls whereas gates for functional markers were defined using the unstimulated samples. **A:** Gating 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 secretes IFN-g and IL-2 after stimulation with HBsAg and CSP antigen invitro. In line with our finding, the IFN-g 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 lager 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.

Acknowledgement

We thank all parents/Guardians, children and staff of the Ifakara Health Institute, Bagamoyo Research and Training Centre and Swiss Tropical and Public Health Institute who contributed so much to this project

Author details

1-Ifakara Health Institute, Bagamoyo branch, 360 Kiko Avenue, Mikocheni, PO Box 78373, Dar es Salaam, Tanzania. 2-Swiss Tropical and Public Health Institute, Basel, Switzerland.
3-University of Basel, Basel Switzerland

References

1. Plowe CV, Alonso P, Hoffman SL. The Potential Role of Vaccines in the Elimination of Falciparum Malaria and the Eventual Eradication of Malaria. J Infect Dis. 2009 Dec 1;200(11):1646–9.

2. Cohen J, Nussenzweig V, Nussenzweig R, Vekemans J, Leach A. From the circumsporozoite protein to the RTS, S/AS candidate vaccine. Hum Vaccin. 2010 Jan;6(1):90–6.

3. Stoute JA, Slaoui M, Heppner DG, Momin P, Kester KE, Desmons P, et al. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group. N Engl J Med. 1997 Jan 9;336(2):86–91.

4. Bojang KA, Milligan PJ, Pinder M, Vigneron L, Alloueche A, Kester KE, et al. Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. Lancet. 2001 Dec 8;358(9297):1927–34.

5. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, Milman J, et al. Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. Lancet Lond Engl. 2004 Oct 16;364(9443):1411–20.

6. Bejon P, Lusingu J, Olotu A, Leach A, Lievens M, Vekemans J, et al. Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. N Engl J Med. 2008 Dec 11;359(24):2521–32.

7. Aponte JJ, Aide P, Renom M, Mandomando I, Bassat Q, Sacarlal J, et al. Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet. 2007 Nov 3;370(9598):1543–51.

8. Abdulla S, Oberholzer R, Juma O, Kubhoja S, Machera F, Membi C, et al. Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants. N Engl J Med. 2008 Dec 11;359(24):2533–44.

9. Casares S, Brumeanu T-D, Richie TL. The RTS,S malaria vaccine. Vaccine. 2010 Jul 12;28(31):4880–94.

10. Sacarlal J, Aide P, Aponte JJ, Renom M, Leach A, Mandomando I, et al. Long-term safety and efficacy of the RTS,S/AS02A malaria vaccine in Mozambican children. J Infect Dis. 2009 Aug 1;200(3):329–36.

11. Lalvani A, Moris P, Voss G, Pathan AA, Kester KE, Brookes R, et al. Potent Induction of Focused Th1-Type Cellular and Humoral Immune Responses by RTS,S/SBAS2, a Recombinant Plasmodium falciparum Malaria Vaccine. J Infect Dis. 1999 Nov 1;180(5):1656–64.

12. Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, Moris P, et al. Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J Infect Dis. 2009 Aug 1;200(3):337–46.

13. Sun P, Schwenk R, White K, Stoute JA, Cohen J, Ballou WR, et al. Protective immunity induced with malaria vaccine, RTS,S, is linked to Plasmodium falciparum circumsporozoite protein-specific CD4+ and CD8+ T cells producing IFN-gamma. J Immunol Baltim Md 1950. 2003 Dec 15;171(12):6961–7.

14. Pinder M, Reece WHH, Plebanski M, Akinwunmi P, Flanagan KL, Lee E a. M, et al. Cellular immunity induced by the recombinant Plasmodium falciparum malaria vaccine, RTS,S/AS02, in semi-immune adults in The Gambia. Clin Exp Immunol. 2004 Feb;135(2):286–93.

15. Wang R, Epstein J, Charoenvit Y, Baraceros FM, Rahardjo N, Gay T, et al. Induction in

humans of CD8+ and CD4+ T cell and antibody responses by sequential immunization with malaria DNA and recombinant protein. J Immunol Baltim Md 1950. 2004 May 1;172(9):5561–9.

16. Barbosa A, Naniche D, Aponte JJ, Manaca MN, Mandomando I, Aide P, et al. Plasmodium falciparum-Specific Cellular Immune Responses after Immunization with the RTS,S/AS02D Candidate Malaria Vaccine in Infants Living in an Area of High Endemicity in Mozambique. Infect Immun. 2009 Oct;77(10):4502–9.

17. Agnandji ST, Fendel R, Mestré M, Janssens M, Vekemans J, Held J, et al. Induction of Plasmodium falciparum-specific CD4+ T cells and memory B cells in Gabonese children vaccinated with RTS,S/AS01(E) and RTS,S/AS02(D). PloS One. 2011;6(4):e18559.

18. Ansong D, Asante KP, Vekemans J, Owusu SK, Owusu R, Brobby NAW, et al. T cell responses to the RTS,S/AS01(E) and RTS,S/AS02(D) malaria candidate vaccines administered according to different schedules to Ghanaian children. PloS One. 2011;6(4):e18891.

19. Olotu A, Moris P, Mwacharo J, Vekemans J, Kimani D, Janssens M, et al. Circumsporozoite-Specific T Cell Responses in Children Vaccinated with RTS,S/AS01E and Protection against P falciparum Clinical Malaria. PLoS ONE [Internet]. 2011 Oct 6 [cited 2015 Aug 4];6(10). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3188575/

20. Lumsden JM, Schwenk RJ, Rein LE, Moris P, Janssens M, Ofori-Anyinam O, et al. Protective immunity induced with the RTS,S/AS vaccine is associated with IL-2 and TNF-α producing effector and central memory CD4 T cells. PloS One. 2011;6(7):e20775.

21. Horowitz A, Hafalla JCR, King E, Lusingu J, Dekker D, Leach A, et al. Antigen-specific IL-2 secretion correlates with NK cell responses after immunization of Tanzanian children with the RTS,S/AS01 malaria vaccine. J Immunol Baltim Md 1950. 2012 May 15;188(10):5054–62.

22. Vahey MT, Wang Z, Kester KE, Cummings J, Heppner DG, Nau ME, et al. Expression of genes associated with immunoproteasome processing of major histocompatibility complex peptides is indicative of protection with adjuvanted RTS,S malaria vaccine. J Infect Dis. 2010 Feb 15;201(4):580–9.

23. Daubenberger CA. Gene-expression analysis for prediction of RTS,S-induced protection in humans. Expert Rev Vaccines. 2010 May;9(5):465–9.

24. Vahey MT, Wang Z, Kester KE, Cummings J, Heppner DG, Nau ME, et al. Expression of genes associated with immunoproteasome processing of major histocompatibility complex peptides is indicative of protection with adjuvanted RTS,S malaria vaccine. J Infect Dis. 2010 Feb 15;201(4):580–9.

25. Pichyangkul S, Kum-Arb U, Yongvanitchit K, Limsalakpetch A, Gettayacamin M, Lanar DE, et al. Preclinical Evaluation of the Safety and Immunogenicity of a Vaccine Consisting of Plasmodium falciparum Liver-Stage Antigen 1 with Adjuvant AS01B Administered Alone or Concurrently with the RTS,S/AS01B Vaccine in Rhesus Primates. Infect Immun. 2008 Jan;76(1):229–38.

26. Moncunill G, Dobaño C, McElrath MJ, De Rosa SC. OMIP-025: evaluation of human T- and NK-cell responses including memory and follicular helper phenotype by intracellular cytokine staining. Cytom Part J Int Soc Anal Cytol. 2015 Apr;87(4):289–92.

27. Howie SR. Blood sample volumes in child health research: review of safe limits. Bull World Health Organ. 2011 Jan 1;89(1):46–53.

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

The article has been published in the American Journal of Tropical Medicine and Hygiene (2014). Doi: 10.4269/ajtmh.14-0119.

Am. J. Trop. Med. Hyg., 91(3), 2014, pp. 471–480
doi:10.4269/ajtmh.14-0119
Copyright © 2014 by The American Society of Tropical Medicine and Hygiene

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

Seif Shekalaghe, Mastidia Rutaihwa, Peter F. Billingsley, Mwajuma Chemba, Claudia A. Daubenberger, Eric R. James, Maximillian Mpina, Omar Ali Juma, Tobias Schindler, Eric Huber, Anusha Gunasekera, Anita Manoj, Beatus Simon, Elizabeth Saverino, L. W. Preston Church, Cornelus C. Hermsen, Robert W. Sauerwein, Christopher Plowe, Meera Venkatesan, Philip Sasi, Omar Lweno, Paul Mutani, Ali Hamad, Ali Mohammed, Alwisa Urassa, Tutu Mzee, Debbie Padilla, Adam Ruben, B. Kim Lee Sim, Marcel Tanner,† Salim Abdulla,† and Stephen L. Hoffman*†

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, PfSPZ Challenge, were used to infect Dutch volunteers by intradermal injection. We conducted a double-blind, placebo-controlled trial to assess safety and infectivity of PfSPZ Challenge in adult male Tanzanians. Volunteers were injected intradermally with 10,000 ($N = 12$) or 25,000 ($N = 12$) PfSPZ or normal saline ($N = 6$). PfSPZ Challenge was well tolerated and safe. Eleven of 12 and 10 of 11 subjects, who received 10,000 and 25,000 PfSPZ respectively, developed parasitemia. In 10,000 versus 25,000 PfSPZ 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. PfSPZ 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 syphilis[1,2] 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,[4–6] 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 (PfSPZ) that are highly infectious, a product called PfSPZ Challenge.[11–13] When young adult Dutch volunteers were injected intradermally (ID) with doses of 2,500, 10,000, or 25,000 PfSPZ (divided into two 50 μL injections), five of six volunteers developed parasitemia in all three groups, and the time from injection of PfSPZ Challenge to detection of parasites 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 PfSPZ Challenge in Africans, we conducted a double-blind, placebo-controlled trial to assess the safety and infectivity of ID-administered PfSPZ 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 PfSPZ in 50 μL were injected ID into the deltoid area of both upper arms for a total of 10,000 PfSPZ.[12] In a second group we increased the dose to 25,000 PfSPZ, and based on findings in murine model systems, which suggested ways to improve the efficiency of ID injections,[14] we divided the total dosage into four injections of 6,250 PfSPZ, each in 10 μL.

As in Dutch[12] and British[13] subjects, PfSPZ 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 PfSPZ 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,

*Address correspondence to Stephen L. Hoffman, Sanaria Inc., 9800 Medical Center Drive, Rockville, MD 20850. E-mail: slhoffman@sanaria.com
†These authors contributed equally to this study.

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 and 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 a+ and a^0 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 of whole blood with ZR Genomic DNA-Tissue MiniPrep (ZymoResearch, Irvine, CA) according to manufacturer's recommendations. We used primers to amplify the alpha 2 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.[15] The multiplex polymerase chain reaction (PCR) primers used were a 2/3.7del 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 HotStarTaq 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, Foster City, CA), with an initial 15-minute denaturation at 96°C, 30 cycles of 98°C for 45 seconds, 60°C for 90 seconds, 72°C for 135 seconds, and a final extension at 72°C for 5 minutes. Following amplification, 10 μL of the product were electrophoresed through a 1.5% agarose gel with 0.6 μg/mL ethidium bromide in 1× TBE buffer first at 7 volts/cm for 1 hour followed by 3 volts/cm for an additional 2 hours. The gel was visualized on an UV transilluminator. The wild-type aa/aa loci yielded a PCR product of 1,800 base pair (bp), whereas the 3.7 kb deletion, 4.2 kb deletion and 20.5 kb deletion resulted in PCR products of 2,029 bp, 1,628 bp, and 1,007 bp, respectively.

Screening for a+ and a^0 α-thalassemia caused by non-deletion mutations. The three exons of each alpha 2 and alpha 1 gene were amplified and sequenced to determine all non-deletional mutations that cause a^+ and a^0 α-thalassemia. Each 25 uL reaction contained 0.5 μM of each primer hem alpha F, hem alpha 1 R, hem alpha 2 R, and also 1× Q-solution 2.5 U HotStarTaq DNA polymerase in supplied reaction buffer (Qiagen) 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/Vol.IX/1217), and the Ethikkommission beider Basel (EKBB), Basel, Switzerland (EKBB 319/11). The protocol was also approved by TFDA (Ref. No. CE.57/180/04A/50), and the trial was registered at ClinicalTrials.gov (NCT01540903).

Intervention and randomization. The intervention material was aseptic, purified, cryopreserved PfSPZ (PfSPZ Challenge) isolated from salivary glands of aseptically reared mosquitoes.[16,17] Details regarding the production, cryopreservation quality control, potency, and CHMI have been described.[11–13,18,19] The lot of PfSPZ 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[12]; however, it was the same lot that was used in Oxford in which 5 of 6 volunteers who received 2,500 PfSPZ ID and 6 of 6 volunteers who received 25,000 PfSPZ IM developed malaria.[13] 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).[12,13]

Thirty eligible volunteers were randomly allocated to the experimental (PfSPZ Challenge) or control (normal saline) groups. Twenty-four volunteers received PfSPZ Challenge and six received normal saline (BD Medical/Surgical, BD Pre-filled Normal Saline Flush Syringe, Columbus, Nebraska). The volunteers and clinicians were blinded as to whether the volunteers received PfSPZ Challenge or normal saline.

CHMI. *Administration of PfSPZ Challenge and immediate follow-up.* PfSPZ Challenge was administered ID in two different dose groups. Twelve volunteers were inoculated with 10,000 PfSPZ ID in two injection sites, one 50 μL injection in each deltoid, each injection containing 5,000 PfSPZ. Twelve volunteers were inoculated with 25,000 PfSPZ ID in four injection sites, two 10 μL injections in each deltoid, each injection containing 6,250 PfSPZ. Three control volunteers were assigned to each of the two dose

groups and were inoculated with normal saline in the same way as those in that experimental group.

Immediately before use, a vial of PfSPZ Challenge was thawed and diluted with phosphate buffered saline containing human serum albumin in an aseptic environment. It was then injected by a blinded nurse within 30 minutes of thawing. After injection, volunteers were observed in the injection room for at least 5 minutes and thereafter were escorted by a nurse to the ward.

Diagnosis of malaria. Thick blood smears were obtained every 12 hours on Days 5 through 14 after injection with saline or PfSPZ Challenge and daily on Days 15 through 21 until positive or until Day 21. After initiation of treatment of a positive thick smear, thick smears were assessed until three consecutive daily smears were negative after treatment. Thick smears were also assessed on Day 28 after CHMI.

Slide preparation and reading was performed following a standard procedure. In brief, 10 μL of blood was placed uniformly on a 10 mm × 20 mm area of the slide, air dried, and stained with Giemsa, pH 7.2. Microscopes were calibrated and the number of passes/fields required to read 0.5 μL blood was determined. This amount of blood was assessed for the primary reads to determine if the slide was negative. If the volunteer was symptomatic, double this amount of blood was read. A blood smear was declared positive when one reader saw two parasites in 0.5 μL of blood and the presence of parasites was independently confirmed by a second reader. The pre-patent period was defined as the period between inoculation of PfSPZ Challenge and appearance of the first positive blood smear. Retrospectively, parasitemias were also determined by quantitative polymerase chain reaction (qPCR) performed on all samples collected after CHMI, as previously described.[20]

Treatment of malaria. Those who became smear positive were treated with a standard 3-day regimen of artemether/lumefantrine (Coartem), and were discharged after three consecutive negative smear results. Those who did not become positive during the first 21 days after CHMI were discharged and returned on Day 28. On this day, the study was unblinded and those who had received PfSPZ Challenge and had not developed infection were treated with artemether/lumefantrine (Coartem) irrespective of the blood smear results. All volunteers were seen on Days 56 and 168.

Assessment of adverse events. The volunteers were observed in the ward for 24 hours after administration of PfSPZ Challenge and discharged to home. They were given diaries and thermometers for recording of adverse events and temperatures. Volunteers returned on Day 5 after administration of PfSPZ Challenge for admission to the ward for assessment of safety and diagnosis and treatment of malaria.

During the period of follow-up all symptoms and signs (solicited and unsolicited) were recorded and graded by the attending physician as follows: mild (easily tolerated), moderate (interferes with normal activity), or severe (prevents normal activity). Axillary temperature was recorded as grade 1 (> 37.5–38.0°C), grade 2 (> 38.0–39.0°C), or grade 3 (> 39.0°C). Hematological and biochemical parameters were assessed minimally on Days 5, 9, 12, 28, 56, and 168 after inoculation of PfSPZ Challenge, and on the day of parasite positivity (day of initiating treatment). For those individuals who did not become positive by thick blood smear, these assays were also conducted on Days 15, 18, and 21 after inoculation of PfSPZ 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/ucm091977.pdf). Adverse events were divided into those that occurred during the 5 days after inoculation of PfSPZ 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 antimalarial.[21] In case symptoms or signs that could be related to a cardiac event developed during the study, blood was collected at baseline to be able to determine if there was any difference in the results of assays used to assess cardiac damage (e.g., troponins) before the trial began and at the time of such an event. We note that after our clinical trial in Tanzania, another cardiac event occurred in a volunteer in a PfSPZ-CVac vaccine trial in the Netherlands after CHMI by mosquito bite, diagnosis of malaria, and initiation of treatment.[22]

Genotyping of parasites. At the time of diagnosis and before treatment 4 mL of blood were collected for genotyping by microsatellite analysis to determine if the parasites were derived from PfSPZ Challenge (Pf NF54) or from a naturally acquired infection. The DNA was isolated from blood specimens using the QIAamp DNA Blood Midi Kit (Qiagen). Microsatellite markers Poly alpha, PfPK2, TA81, ARA2, TA87, and TA40 were amplified using hemi-nested PCR.[23,24] Capillary electrophoresis was performed using an Applied Biosystems 3730XL 96-capillary DNA sequencer and software. Capillary electrophoresis output files were analyzed using Genemapper 4.0 (Applied Biosystems). Genomic control strains 3D7 and HB3 (ATCC-MR4, Manassas VA) were included to determine characteristic peak morphology for each microsatellite locus and control for slight variations among runs. Microsatellite peaks above the threshold of 100 relative fluorescent units exhibiting characteristic morphology were scored. Peak sizes were determined by manual inspection of each electropherogram and then normalized against the Pf 3D7 control. Normalized peak sizes were compared with those observed in Pf NF54.

Statistical analysis. Data analysis was performed using SAS software version 9.3 (SAS Institute Inc., Cary, NC). Descriptive statistics were assessed, specifically the geometric mean, for the parasitemia results. Thick blood smear and qPCR results were compared between the 10,000 and 25,000 PfSPZ groups using a non-parametric test (Wilcoxon rank-sum test, two-tailed). The effects of α-thalassemia heterozygosity on parasitemia were compared within each group by non-parametric tests (Wilcoxon rank-sum test, two-tailed). Proportions were compared using χ^2 test (two-tailed).

RESULTS

Volunteers. In total 323 volunteers were recruited from higher learning institutions during the first information meeting. Out of these, 30 met eligibility criteria and were enrolled and randomized to the 10,000 PfSPZ dose (N = 12),

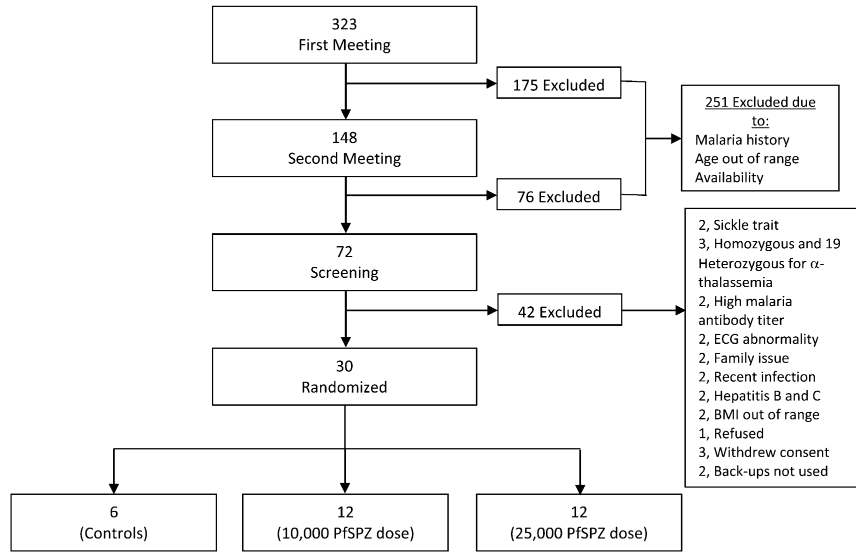


FIGURE 1. Flow chart of recruitment and study design.

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/μL 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/μL blood (110 and 160 parasites/mL blood), respectively, at the time of first qPCR positivity. The GM parasite densities by qPCR at the time of thick smear diagnosis in the two groups were 4.1 and 1.6 parasites/μL, respectively. qPCR was negative throughout the 21-day follow-up for the slide-negative volunteer who received 10,000 PfSPZ, and through Day 19 for the subject who received 25,000 PfSPZ and was treated on Day 19. It was also negative in all normal saline controls, except for one specimen on Day 19, which was determined to be caused by mislabeling of a specimen from a PfSPZ Challenge subject. 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

TABLE 1
Volunteer characteristics*

	Control (normal saline)	Group 1 (10,000 PfSPZ)	Group 2 (25,000 PfSPZ)
	N = 6	N = 12	N = 12
Sex			
Male	6	12	12
Age at screening (years)			
Mean ± SD	25.7 ± 3.0	25.9 ± 1.6	24.6 ± 2.3
Median	25.2	25.5	24.5
Min, max	21.5, 30.9	24.3, 30.5	20.7, 27.2
BMI (kg/m^2)			
Mean ± SD	20.7 ± 2.4	21.7 ± 3	20.5 ± 2.3
Median	19.3	20.7	19.7
Min, max	19, 24	18.4, 27.9	17.3, 25.4
Height (cm)			
Mean ± SD	168 ± 4.3	168.5 ± 8.2	171.7 ± 5.6
Median	166	169	172
Min, max	164, 174	158, 182	162, 179
Weight (kg)			
Mean ± SD	58.4 ± 6.3	61.7 ± 9.8	60.5 ± 8.8
Median	58.3	60.8	60.5
Min, max	51, 66	47, 79	45.5, 81.5

*BMI = body mass index.

TABLE 2
A. Thick smear and qPCR results, group 1 (10,000 PfSPZ)

	Thick smear		qPCR		
Volunteer code	Pre-patent period (day)	Parasite density at diagnosis (Pf/μL)	qPCR positive (day)	Parasite density at first day positive (Pf/μL)	Parasite density by qPCR at time of diagnosis by thick smear (Pf/μL)
10002-20	18.6	5.0	16.0	0.24	0.01
10023-20	18.7	15.0	15.0	0.12	13.00
30035-20	18.7	5.0	14.0	0.04	11.00
40010-20	N/A	N/A	N/A	N/A	N/A
50041-20	14.6	4.0	13.0	0.36	3.00
60008-20	12.8	4.0	10.5	0.11	8.00
60026-20	12.7	11.0	11.5	0.21	9.00
70001-20	14.2	80.0	11.0	0.21	23.00
70014-20	15.8	21.0	12.5	0.03	83.00
70031-20	14.2	11.0	11.0	0.05	17.00
70044-20	17.6	6.0	15.0	0.07	6.00
90047-20	13.7	4.0	11.0	0.09	0.10
Geom. mean	15.4	8.9	12.6	0.11	4.10
No. of positives	11/12		11/12		

B. Thick smear and qPCR results, group 2 (25,000 PfSPZ)

Volunteer code	Pre-patent period (day)	Parasite density at diagnosis (Pf/μL)	qPCR positive (day)	Parasite density at first day positive (Pf/μL)	Parasite density by qPCR at time of diagnosis by thick smear (Pf/μL)
20056-20	18.7	5	16.0	0.13	14.00
20064-20	11.1	7	9.0	0.07	5.00
20070-20	12.6	13	9.5	0.07	5.00
30053-20	13.7	4	12.0	0.17	0.17
30060-20	13.5	7	11.0	1.00	0.40
40055-20	N/A	N/A*	N/A	N/A	N/A
40068-20	13.4	7	11.0	0.26	2.00
50050-20	12.7	7	10.0	0.04	10.00
50057-20	N/A	N/A†	N/A	N/A	N/A
60051-20	12.7	4	11.0	0.13	1.00
60072-20	14.0	7	11.5	0.17	1.00
80058-20	13.7	15	11.0	0.36	0.12
Geom. mean	13.5	7	11.1	0.16	1.60
No. of positives	10/11		10/11		

*This volunteer was treated on Day 19 as a result of reporting of a positive thick blood smear. Review of this thick blood smear indicated that 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 *P. falciparum* parasitemia.

be a significant difference between the replication rate in the Tanzanians and the replication rate in the Dutch subjects.[12]

Parasite genotypes. Parasites from the positive volunteers were genotyped at six polymorphic microsatellite loci to confirm that they were from PfSPZ Challenge (Pf 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 α-thalassemia heterozygosity on parasitemia. The GM parasite density for volunteers who were heterozygous for α-thalassemia and those who were non-α-thalassemic in the 10,000 PfSPZ were not significantly different at the time of detection of parasites by microscopy, 11.1 versus 6.9 Pf/μL blood, respectively (Wilcoxon, $P = 0.5498$) (Table 4). The GM time to blood smear positivity (pre-patent period) was also not significantly different between α-thalassemia heterozygous and non- α-thalassemia volunteers, 15.4 and 15.4 days, respectively, in the 10,000 PfSPZ group (Wilcoxon, $P = 0.7922$). In the 25,000 PfSPZ group, the parasite densities at the time of detection of parasites by microscopy was 4.0 parasites/μL blood in the one α-thalassemia heterozygous volunteer and 7.4 parasites/μL blood (GM) in non-α-thalassemia volunteers. The time to blood slide positivity in the single α-thalassemia heterozygous subject was no different from GM time to blood slide positivity in the non-α-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

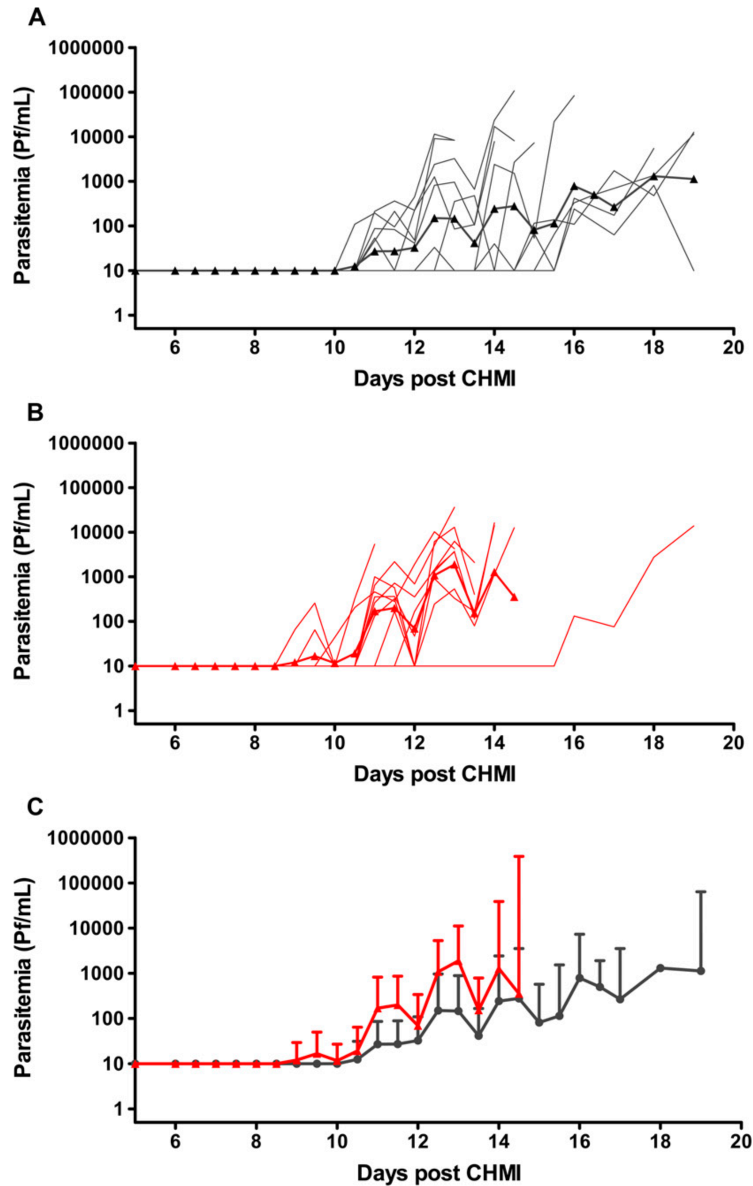


FIGURE 2. Parasite density as measured by qPCR in the 10,000 (**A**) and 25,000 (**B**) PfSPZ dose groups. Panels A (N = 11) and B (N = 10) show individual and geometric mean parasite density (parasites/mL) of positive volunteers from day of inoculation through last day of positivity after initiation of treatment. Panel C shows an overlay of geometric mean parasite densities with confidence intervals (95%) of positive volunteers in each group (Black line = Group 1, 10,000 PfSPZ; Grey line = Group 2, 25,000 PfSPZ).* *For Panel B, the geometric mean was calculated excluding the single volunteer who first became positive on Day 16.

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

TABLE 3
Microsatellite genotypes of Pf infections in volunteers after PfSPZ Challenge

	Strain or sample ID	Microsatellite Loci*					
		TA81	Poly a	PfPK2	TA40	ARA2	TA87
DNA controls	HB3	131	178	190	205	61	103
	3D7	122	148	166	220	64	94
	NF54	**122**	**148**	**166**	**220**	**64**	**94**
Post-CHMI infections	7059065	122	148	166	220	64	94
	8059066	122	148	166	220	64	94
	9059067	122	148	166	220	64	94
	2058376	122	148	166	220	64	94
	3059070	122	148	166	220	64	94
	4059071	122	148	166	220	64	94
	5059072	122	148	166	220	64	–†
	6059073	122	148	166	220	64	94
	7059074	122	148	166	220	64	94
	8059075	122	148	166	220	64	94
	9059076	122	148	166	220	64	94
	1059077	122	148	166	220	64	94
	2059078	122	148	166	220	64	94
	3059079	122	148	166	220	64	94
	4059080	122	148	166	220	64	94
	5059081	122	148	166	220	64	94
	3059088	122	148	166	220	64	94
	7059083	122	148	166	220	64	94
	8059084	122	148	166	220	64	94
	9059085	122	148	166	220	64	94
	6059082	122	148	166	220	64	94
Subsequent infections	4155794	118	155	157	228	59	103
	7060514	118	155	163	217	87	88

Gray shading indicates microsatellite alleles shared with NF54.
*Numbers represent microsatellite sizes in nucleotide base pairs, as determined by capillary electrophoresis.
†Did not amplify.

saline or PfSPZ Challenge. 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 4
Pre-patent periods and parasite densities by microscopy by α-thalassemia status

Heterozygous			Normal		
Volunteer ID	Pre-patent period (Days)	Parasite density at diagnosis	Volunteer ID	Pre-patent period (Days)	Parasite density at diagnosis
Group 1*					
90047-20	13.74	4.0	50041-20	14.64	4.0
70001-20	14.11	80.0	60008-20	12.75	4.0
60026-20	12.71	11.0	70044-20	17.63	6.0
70014-20	15.76	21.0	70031-20	14.16	11.0
10002-20	18.64	5.0	10023-20	18.73	15.0
30035-20	18.66	5.0			
Geom. mean	15.43	11.1		15.42	6.9
No. positive	6/7			5/5	
Group 2*					
60051-20	12.72	4.0	30053-20	13.72	4.0
			80058-20	13.68	15.0
			20056-20	18.67	5.0
			40068-20	13.44	7.0
			30060-20	13.47	7.0
			50050-20	12.72	7.0
			60072-20	14.04	7.0
			20070-20	12.62	13.0
			20064-20	11.05	7.0
Geom. mean	12.72	4.0		13.59	7.4
No. positive	1/1			9/10	

*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 = 0.011). There were no statistically significant differences in infection rates, parasitemia, or parasites/μL blood between subjects heterozygous for α-thalassemia trait, and those who were not.

TABLE 5
Number of volunteers with clinical adverse events, Days 0–28 post injection*

	Saline control (N = 6)	Group 1 (10,000 PfSPZ) (N = 12)	Group 2 (25,000 PfSPZ) (N = 12)
Adverse events (no. of volunteers, %)	n (%)	n (%)	n (%)
Any adverse event	6 (100)	9 (75)	9 (75)
Serious adverse event	0	0	0
Solicited adverse event	6 (100)	9 (75)	9 (75)
Unsolicited adverse event	2 (33)	6 (50)	2 (17)

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

that the replication rate was similar to the 11.5-fold replication rate every 48 hours seen in the Dutch volunteers.[12] 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-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 PfSPZ 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 μL, as we have seen in mice.[14]

Hemoglobinopathies, disorders of hemoglobin structure, and production, are one of the most common monogenic disorders in humans. α-thalassemia is a hemoglobinopathy resulting from deletion of one (–α) or both (–) α 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.[26] 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 α-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

TABLE 6
Number of clinical adverse events, Days 0–28 post-injection

	Saline control (N = 6)	Group 1 (10,000 PfSPZ) (N = 12)	Group 2 (25,000 PfSPZ) (N = 12)
Total number of AEs	22	42	42
Total solicited AEs			
Grade 1	14	30	34
Grade 2	4	2	6
Grade 3	2*	1†	0
Total unsolicited AEs			
Grade 1	2	8	1
Grade 2	0	0	1
Grade 3	0	1‡	0
Total SAEs	0	0	0

An adverse event (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.
†Severe headache attributable to controlled human malaria infection (CHMI).
‡Pharyngitis, etiology unclear, resolved after 3 days.
SAE = serious adverse event.

TABLE 7
Specific solicited and unsolicited adverse events, Days 0–28 post–injection*

Adverse event	Saline control N = 6	Group 1 10,000 PfSPZ N = 12	Group 2 25,000 PfSPZ N = 12
Total AEs	21 (2)	30 (3)	34 (6)
Fever	0	0	2
Headache	6 (1)	7	7 (1)
Malaise	1	5 (1)	4
Fatigue	3 (1)	5	6 (2)
Myalgia	3	2	1
Arthralgia	3	2	4
Nausea and/or vomiting	0	0	0
Chills	0	0	1
Diarrhea	0	0	0
Constipation	0	1	0
Abdominal pain	1	0	1 (1)
Chest pain/discomfort	0	1	0
Palpitations	0	0	0
Shortness of breath	0	0	0
Dizziness	1	0	0
Erythema, swelling, or pruritis at injection site	0	0	3 (2)
Rash or pruritus remote to injection site	2	0	0
Itching throat	0	0	1
Loss of appetite	0	3 (1)	1
Pain on swallowing/pharyngitis	1	2	0
Back pain	0	1 (1)	0
Neck pain	0	1	0
Elevated axillary temp.	0	0	3

*A volunteer was counted at most once within each event type, even if the AE cleared and reappeared. All AEs were considered either possibly or probably related to the injection of study product or malaria. Numbers in parentheses occurred within the first 5 days after injection of study product.

studies that showed that individuals who were heterozygous or homozygous for α-thalassemia had similar rates of asymptomatic parasitemia and similar manifestations of uncomplicated malaria as non-thalassemic individuals.[27]

Administration of PfSPZ 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 PfSPZ Challenge (Tables 5–7). Very few of the AEs occurred during the first 5 days after inoculation of PfSPZ Challenge, and thus very few were attributed to the injection of PfSPZ Challenge (Table 7). Most occurred during the period when the volunteers were being diagnosed with Pf 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 Pf 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 5 of 18 had chills, whereas in this study only 1 of 24 had chills ($P = 0.068$).[12] 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 (Pf 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.[28–32] 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/μL 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 PfSPZ Vaccine[11,18,19] 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 PfSPZ Challenge in Africans to establish the efficacy of new interventions against malaria and to study the mechanisms of protection conferred by hemoglobinopathies, glucose 6 phosphate dehydrogenase deficiencies, and innate and acquired immunity to malaria in settings 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 PfSPZ Vaccine[19] in Tanzanians.

Received February 26, 2014. Accepted for publication May 25, 2014.

Published online July 28, 2014.

Note: Supplemental tables appear at www.ajtmh.org.

Acknowledgments: We thank all volunteers and staff of the Ifakara Health Institute, Bagamoyo Research and Training Centre, Sanaria Inc., and Swiss Tropical and Public Health Institute who contributed so much to this project. Special thanks to Yonas Abebe, Alexander Hoffman, Thomas L. Richie, and Robert C. Thompson of Sanaria for editorial, legal, and administrative support, and the Sanaria manufacturing, quality control, regulatory, and clinical teams. We thank Rose Minja, Hery Mwandolela, and Ally Mtoro for safety review of volunteers during the trial and pharmacist Abel Mdemu for verification of formulation. We appreciate the advice of Barney Graham, David Diemert, and Zulfiqarali Premji, members of the Safety Monitoring Committee.

Financial support: The Tanzanian Commission on Science and Technology (COSTECH), the Ifakara Health Institute, and the Swiss Tropical Public Health Institute provided funding for this clinical trial. The development, manufacturing, and quality control release and stability studies of PfSPZ Challenge were supported in part by an NIAID Small Business Innovation Research grant, 5R44AI058375.

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

Authors' addresses: Seif Shekalaghe, Mastidia Rutaihwa, Mwajuma Chemba, Maximillian Mpina, Omar Ali Juma, Beatus Simon, Omar Lweno, Paul Mutani, Ali Hamad, Ali Mohammed, Alwisa Urassa, Tutu Mzee, and Salim Abdulla, Ifakara Health Institute, Bagamoyo Research and Training Centre, Bagamoyo, Tanzania, E-mails: sshekalaghe@ihi.or.tz, mrutaihwa@ihi.or.tz, mchemba@ihi.or.tz, Ojuma@ihi.or.tz, bbongole@ihi.or.tz, mmpina@ihi.or.tz, olweno@ihi.or.tz, pmutani@ihi.or.tz, ahamad@ihi.or.tz, amohammed@ihi.or.tz, aurassa@ihi.or.tz, tmzee@ihi.or.tz, and sabdulla@ihi.or.tz. Peter F. Billingsley, Eric R. James, Anusha Gunasekera, Anita Manoj, Elizabeth Saverino, Debbie Padilla, Adam Ruben, and Stephen L. Hoffman, Sanaria Inc., Rockville, MD, E-mails: pbillingsley@sanaria.com, ejames@sanaria.com, agunasekera@sanaria.com, amanoj@sanaria.com, esaverino@sanaria.com, dpadilla@sanaria.com, aruben@sanaria.com, and slhoffman@sanaria.com. Claudia A. Daubenberger, Tobias Schindler, and Maximillian Mpina, Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Basel, Switzerland, and University of Basel, Basel, Switzerland, E-mails: Claudia.Daubenberger@unibas.ch, Tobias.Schindler@stud.unibas.ch, and Max.Mpina@hotmail.com. Eric Huber, Department of Medicines Research, Swiss Tropical and Public Health Institute, Basel, Switzerland, E-mail: Eric.Huber@unibas.ch. L. W. Preston Church, Hospital Epidemiologist and Head Division of Infectious Diseases VA Hospital, Charleston, SC, E-mail: lwpchurch@sanaria.com. Cornelus C. Hermsen and Robert W. Sauerwein, Radboud University Nijmegen Medical Center, Department of Medical Microbiology, Nijmegen, The Netherlands, E-mails: R.Hermsen@ncmls.ru.nl and R.Sauerwein@mmb.umcn.nl. Christopher Plowe and Meera Venkatesan, Howard Hughes Medical Institute and University of Maryland Center for Vaccine Development, Baltimore, MD, E-mails: cplowe@medicine.umaryland.edu and mvenkate@medicine.umaryland.edu. Philip Sasi, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania, E-mail: philip.sasi@gmail.com. B. Kim Lee Sim, Sanaria Inc., and Protein Potential LLC, Rockville, MD, E-mail: ksim@protpot.com. Marcel Tanner, Department of Epidemiology and Public Health, Swiss Tropical and Public Health Institute, Basel, Switzerland, and University of Basel, Basel, Switzerland, E-mail: Marcel.Tanner@unibas.ch.

This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

REFERENCES

1. Mayne B, 1933. The injection of mosquito sporozoites in malaria therapy. *Public Health Rep 48:* 909–913.
2. Mayne B, Young M, 1941. The technique of induced malaria as used in the South Carolina State Hospital. *J Vener Dis Inf 22:* 271–276.
3. Boyd MF, 1949. *Malariology: A Comprehensive Survey of All Aspects of This Group of Diseases from a Global Standpoint.* Philadelphia, PA: W. B. Saunders.
4. Ifediba T, Vanderberg JP, 1981. Complete *in vitro* maturation of *Plasmodium falciparum* gametocytes. *Nature 294:* 364–366.
5. Campbell CC, Collins WE, Nguyen Dinh P, Barber A, Broderson JR, 1982. *Plasmodium falciparum* gametocytes from culture *in vitro* develop to sporozoites that are infectious to primates. *Science 217:* 1048–1050.
6. Chulay JD, Schneider I, Cosgriff TM, Hoffman SL, Ballou WR, Quakyi IA, Carter R, Trosper JH, Hockmeyer WT, 1986. Malaria transmitted to humans by mosquitoes infected from cultured *Plasmodium falciparum*. *Am J Trop Med Hyg 35:* 66–68.
7. Church LW, Le TP, Bryan JP, Gordon DM, Edelman R, Fries L, Davis JR, Herrington DA, Clyde DF, Shmuklarsky MJ, Schneider I, McGovern TW, Chulay JD, Ballou WR, Hoffman SL, 1997. Clinical manifestations of *Plasmodium falciparum* malaria experimentally induced by mosquito challenge. *J Infect Dis 175:* 915–920.
8. Epstein JE, Rao S, Williams F, Freilich D, Luke T, Sedegah M, de la Vega P, Sacci J, Richie TL, Hoffman SL, 2007. Safety and clinical outcome of experimental challenge of human volunteers with *Plasmodium falciparum*-infected mosquitoes: an update. *J Infect Dis 196:* 145–154.
9. Roestenberg M, O'Hara GA, Duncan CJ, Epstein JE, Edwards NJ, Scholzen A, van der Ven AJ, Hermsen CC, Hill AV, Sauerwein RW, 2012. Comparison of clinical and parasitological data from controlled human malaria infection trials. *PLoS ONE 7:* e38434.
10. Laurens MB, Duncan CJ, Epstein JE, Hill AV, Komisar JL, Lyke KE, Ockenhouse CF, Richie TL, Roestenberg M, Sauerwein RW, Spring MD, Talley AK, Moorthy VS, 2012. A consultation on the optimization of controlled human malaria infection by mosquito bite for evaluation of candidate malaria vaccines. *Vaccine 30:* 5302–5304.
11. Hoffman SL, Billingsley P, James E, Richman A, Loyevsky M, Li T, Charkravarty S, Gunasekera A, Chattopadhyay R, Li

M, Stafford R, Ahumada A, Epstein JE, Sedegah M, Reyes S, Richie TL, Lyke KE, Edelman R, Laurens MB, Plowe CV, Sim BKL, 2010. Development of a metabolically active, non-replicating sporozoite vaccine to prevent *Plasmodium falciparum* malaria. *Hum Vaccin 6:* 97–106.

12. Roestenberg M, Bijker EM, Sim BK, Billingsley PF, James ER, Bastiaens GJ, Teirlinck AC, Scholzen A, Teelen K, Arens T, van der Ven AJ, Gunasekera A, Chakravarty S, Velmurugan S, Hermsen CC, Sauerwein RW, Hoffman SL, 2013. Controlled human malaria infections by intradermal injection of cryopreserved *Plasmodium falciparum* sporozoites. *Am J Trop Med Hyg 88:* 5–13.
13. Sheehy SH, Spencer AJ, Douglas AD, Sim BK, Longley RJ, Edwards NJ, Poulton ID, Kimani D, Williams AR, Anagnostou NA, Roberts R, Kerridge S, Voysey M, James ER, Billingsley PF, Gunasekera A, Lawrie AM, Hoffman SL, Hill AV, 2013. Optimizing controlled human malaria infection studies using cryopreserved parasites administered by needle and syringe. *PLoS ONE 8:* e65960.
14. Ploemen IH, Chakravarty S, van Gemert GJ, Annoura T, Khan SM, Janse CJ, Hermsen CC, Hoffman SL, Sauerwein RW, 2013. *Plasmodium* liver load following parenteral sporozoite administration in rodents. *Vaccine 31:* 3410–3416.
15. Tan AS, Quah TC, Low PS, Chong SS, 2001. A rapid and reliable 7-deletion multiplex polymerase chain reaction assay for alpha-thalassemia. *Blood 98:* 250–251.
16. Lyke KE, Laurens M, Adams M, Billingsley PF, Richman A, Loyevsky M, Chakravarty S, Plowe CV, Sim BK, Edelman R, Hoffman SL, 2010. *Plasmodium falciparum* malaria challenge by the bite of aseptic *Anopheles stephensi* mosquitoes: results of a randomized infectivity trial. *PLoS ONE 5:* e13490.
17. Laurens MB, Billingsley P, Richman A, Eappen AG, Adams M, Li T, Chakravarty S, Gunasekera A, Jacob CG, Sim BK, Edelman R, Plowe CV, Hoffman SL, Lyke KE, 2013. Successful human infection with *P. falciparum* using three aseptic *Anopheles stephensi* mosquitoes: a new model for controlled human malaria infection. *PLoS ONE 8:* e68969.
18. Epstein JE, Tewari K, Lyke KE, Sim BK, Billingsley PF, Laurens MB, Gunasekera A, Chakravarty S, James ER, Sedegah M, Richman A, Velmurugan S, Reyes S, Li M, Tucker K, Ahumada A, Ruben AJ, Li T, Stafford R, Eappen AG, Tamminga C, Bennett JW, Ockenhouse CF, Murphy JR, Komisar J, Thomas N, Loyevsky M, Birkett A, Plowe CV, Loucq C, Edelman R, Richie TL, Seder RA, Hoffman SL, 2011. Live attenuated malaria vaccine designed to protect through hepatic CD8+T cell immunity. *Science 334:* 475–480.
19. Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, Holman LA, James ER, Billingsley PF, Gunasekera A, Richman A, Chakravarty S, Manoj A, Velmurugan S, Li M, Ruben AJ, Li T, Eappen AG, Stafford RE, Plummer SH, Hendel CS, Novik L, Costner PJ, Mendoza FH, Saunders JG, Nason MC, Richardson JH, Murphy J, Davidson SA, Richie TL, Sedegah M, Sutamihardja A, Fahle GA, Lyke KE, Laurens MB, Roederer M, Tewari K, Epstein JE, Sim BK, Ledgerwood JE, Graham BS, Hoffman SL, for the VRC 312 Study Team, 2013. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. *Science 341:* 1359–1365.
20. Adegnika AA, Verweij JJ, Agnandji ST, Chai SK, Breitling LP, Ramharter M, Frolich M, Issifou S, Kremsner PG, Yazdanbakhsh M, 2006. Microscopic and sub-microscopic *Plasmodium falciparum* infection, but not inflammation caused by infection, is associated with low birth weight. *Am J Trop Med Hyg 75:* 798–803.
21. Nieman AE, de Mast Q, Roestenberg M, Wiersma J, Pop G, Stalenhoef A, Druilhe P, Sauerwein R, van der Ven A, 2009. Cardiac complication after experimental human malaria infection: a case report. *Malar J 8:* 277.
22. van Meer MP, Bastiaens GJ, Boulaksil M, de Mast Q, Gunasekera A, Hoffman SL, Pop G, van der Ven AJ, Sauerwein RW, 2014. Idiopathic acute myocarditis during treatment for controlled human malaria infection: a case report. *Malar J 13:* 38.
23. Anderson TJ, Su XZ, Bockarie M, Lagog M, Day KP, 1999. Twelve microsatellite markers for characterization of *Plasmodium falciparum* from finger-prick blood samples. *Parasitology 119:* 113–125.
24. Shaukat AM, Gilliams EA, Kenefic LJ, Laurens MB, Dzinjalamala FK, Nyirenda OM, Thesing PC, Jacob CG, Molyneux ME, Taylor TE, Plowe CV, Laufer MK, 2012. Clinical manifestations of new versus recrudescent malaria infections following anti-malarial drug treatment. *Malar J 11:* 207.
25. Roestenberg M, de Vlas SJ, Nieman AE, Sauerwein RW, Hermsen CC, 2012. Efficacy of preerythrocytic and blood-stage malaria vaccines can be assessed in small sporozoite challenge trials in human volunteers. *J Infect Dis 206:* 319–323.
26. Harteveld CL, Higgs DR, 2010. Alpha-thalassaemia. *Orphanet J Rare Dis 5:* 13.
27. Taylor SM, Parobek CM, Fairhurst RM, 2012. Hemoglobinopathies and the clinical epidemiology of malaria: a systematic review and meta-analysis. *Lancet Infect Dis 12:* 457–468.
28. Baliraine FN, Afrane YA, Amenya DA, Bonizzoni M, Vardo-Zalik AM, Menge DM, Githeko AK, Yan G, 2010. A cohort study of *Plasmodium falciparum* infection dynamics in Western Kenya Highlands. *BMC Infect Dis 10:* 283.
29. Schultz L, Wapling J, Mueller I, Ntsuke PO, Senn N, Nale J, Kiniboro B, Buckee CO, Tavul L, Siba PM, Reeder JC, Barry AE, 2010. Multilocus haplotypes reveal variable levels of diversity and population structure of *Plasmodium falciparum* in Papua New Guinea, a region of intense perennial transmission. *Malar J 9:* 336.
30. Griffing SM, Mixson-Hayden T, Sridaran S, Alam MT, McCollum AM, Cabezas C, Marquino Quezada W, Barnwell JW, De Oliveira AM, Lucas C, Arrospide N, Escalante AA, Bacon DJ, Udhayakumar V, 2011. South American *Plasmodium falciparum* after the malaria eradication era: clonal population expansion and survival of the fittest hybrids. *PLoS ONE 6:* e23486.
31. Mobegi VA, Loua KM, Ahouidi AD, Satoguina J, Nwakanma DC, Amambua-Ngwa A, Conway DJ, 2012. Population genetic structure of *Plasmodium falciparum* across a region of diverse endemicity in West Africa. *Malar J 11:* 223.
32. Yalcindag E, Elguero E, Arnathau C, Durand P, Akiana J, Anderson TJ, Aubouy A, Balloux F, Besnard P, Bogreau H, Carnevale P, D'Alessandro U, Fontenille D, Gamboa D, Jombart T, Le Mire J, Leroy E, Maestre A, Mayxay M, Menard D, Musset L, Newton PN, Nkoghe D, Noya O, Ollomo B, Rogier C, Veron V, Wide A, Zakeri S, Carme B, Legrand E, Chevillon C, Ayala FJ, Renaud F, Prugnolle F, 2012. Multiple independent introductions of *Plasmodium falciparum* in South America. *Proc Natl Acad Sci USA 109:* 511–516.

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*

Time point	Potency (no. of parasites expressing PfMSP-1/well)	% Viability (sporozoite membrane integrity assay)
Fresh	32.7 ± 1.5	98.2%
Release	29.3 ± 3.1	87.4% ± 5.9%
3 Month	27.3 ± 0.6	84.6% ± 1.9%
6 Month	26.7 ± 1.5	83.6% ± 5.5%
Post-last clinical dose–Oxford[13] 9 Month	26.3 ± 2.5	86.3% ± 6.5%
12 Month	27.3 ± 0.6	86.2% ± 1.3%
Post-last clinical dose–Tanzania (18 month)	24.0 ± 1.7	81.7% ± 2.6%

*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 27% reduction in potency by this assay 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*

Test	Normal Range	Grade 1	Grade 2	Grade 3	Grade 4
Sodium (meq/l)–hypernatremia	136–146	N/A	146–147	148–150	> 150
Sodium (meq/l)– hyponatremia		132–135	130–131	125–129	< 125
Potassium (meq/l)– hyperkalemia	3.5–5	5.1–5.2	5.3–5.4	5.5–5.6	> 5.6
Potassium (meq/l)– hypokalemia		N/A	3.3–3.4	3.1–3.2	< 3.1
Creatinine (μmol/L)	53–106	107–150	151–177	178–221	> 221 or requires dialysis
Glucose (mmol/L)–hyperglycemia	3.89–6.0	6.01–6.94	6.95–11.1	> 11.1	
Glucose (mmol/L)– hypoglycemia		3.61–3.88	3.05–3.60	2.50–3.04	< 2.50
AST (U/L)	5–34	1.1–2.5 × ULN	2.6–5 × ULN	5.1–10 × ULN	>10 × ULN
ALT (U/L)	0–55	1.1–2.5 × ULN	2.6–5 × ULN	5.1–10 × ULN	>10 × ULN
Bilirubin (μmol/L)	0–6.1	1.1–1.5 × ULN	1.6–2.0 × ULN	2.0–3.0 × ULN	> 3.0 × ULN
Total white blood count × 1,000/μL	3.2–11.7	2.5–3.1	1.5–2.5	1.0–1.5	< 1.0
Neutrophil count × 1,000/μL	2.0–6.9	1.5–1.999	1.0–1.499	0.500–0.999	< 0.500
Lymphocyte count × 1,000/μL	0.87–3.19	0.750–0.869	0.500–0.749	0.250–0.499	< 0.250
Eosinophil count × 1,000/ μL	0–0.7	0.701–1.5	1.501–5.0	> 5.0	
Hemoglobin (g/L)	12.9–18.3	12.5–13.5	10.5–12.4	8.5–10.4	< 8.5
Platelets × 1,000/μL	146–345	125–145	100–124	25–99	< 25

*Guidance for Industry - Toxicity Grading Scale for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials – 2007 (http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm091977.pdf). AST = aspartate aminotransferase; ALT = alanine aminotransferase; ULN = upper limit of normal.

Supplemental Table 3

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

	Normal saline controls (N = 6) 1st day AE noted* (maximum Grade)†	10,000 PfSPZ (N = 12) 1st day AE noted (maximum Grade)*	25,000 PfSPZ (N = 12) 1st day AE noted (maximum Grade)*
Increased AST	6 (2), 14 (2)	9 (1), 9 (2), 12 (1), 12 (2)	5 (3), 14 (3), 15 (1), 18 (2)
Increased ALT	14 (3), 15 (1)	9 (2), 12 (1)	5 (2), 14 (2), 15 (1)
Increased Bilirubin		9 (2)	
Increased Creatinine	18 (1)		
Hypoglycemia	5 (2), 28 (1)	15 (1) and 19 (1), 15 (1), 15 (1)	5 (2) and 12 (3)
Hyperglycemia	5 (2)	5 (2), 15 (1)	5 (1), 5 (2), 11 (1), 13 (2), 13 (2), 19 (2)
Leukopenia		18 (1)	
Leukocytosis	14 (1)		
Neutropenia	5 (1) and 18 (1), 6 (1) and 28 (2), 9 (2), 27 (1)	5 (2), 5 (2), 9 (1), 12 (1) and 18 (1), 12 (1), 27 (1), 28 (1)	5 (2), 12 (1), 27 (2)
Lymphopenia	21 (1)	15 (1), 18 (2)	14 (1), 14 (3), 15 (1)
Eosinophilia		5 (1)	
Anemia		28 (1), 28 (1)	11 (1), 15 (2)
Thrombocytopenia	6 (1)	5 (2) and 12 (2), 9 (2), 13 (3), 18 (1)	15 (1)
Proteinuria		5 (3), 5 (2), 12 (2), 12 (2), 28 (3)	5 (2), 9 (2)
Hematuria		28 (2)	

*Each entry represents a specific, different laboratory abnormality.
†For those designated "and", the abnormality occurred, resolved, and recurred.
AE = adverse event; AST = aspartate aminotransferase; ALT = alanine aminotransferase.

PAPER VI

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

The article has been published in the Journal of Infection and Immunity (2015). Doi: 10.1128/IAI.03069-14.

IAI Journals.ASM.org

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

Joshua M. Obiero,[a]* Seif Shekalaghe,[b] Cornelus C. Hermsen,[a] Maxmillian Mpina,[b] Else M. Bijker,[a] Meta Roestenberg,[a]* Karina Teelen,[a] Peter F. Billingsley,[c] B. Kim Lee Sim,[c] Eric R. James,[c] Claudia A. Daubenberger,[d,e] Stephen L. Hoffman,[c] Salim Abdulla,[b] Robert W. Sauerwein,[a] Anja Scholzen[a]

Radboud University Medical Center, Department of Medical Microbiology, Nijmegen, The Netherlands[a]; Ifakara Health Institute, Bagamoyo Research and Training Center, Bagamoyo, Tanzania[b]; Sanaria Inc., Rockville, Maryland, USA[c]; Swiss Tropical and Public Health Institute, Department of Medical Parasitology and Infection Biology, Basel, Switzerland[d]; University of Basel, Basel, Switzerland[e]

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.

In 2012, *Plasmodium falciparum* malaria caused an estimated 207 million cases and 627,000 deaths, of which 90% occurred in children under 5 years of age and in pregnant women in sub-Saharan Africa (1). Major control efforts have been implemented with some success (2, 3), but malaria eradication will likely require a safe and highly protective vaccine. Subunit vaccines have thus far shown moderate efficacy at best. RTS,S is the only vaccine candidate in phase 3 trials but, despite averting substantial numbers of malaria cases (4), shows only 30 to 50% reduction in clinical disease after 12 months depending on both age and malaria endemicity and even less after 18 months (5–7). These results stress the need for more effective second-generation vaccines. Key requirements are not only the identification of novel immunogens but also a better understanding of protection-related immune responses. This includes the effect of previous malaria exposure on immune responses upon reexposure or vaccination (8, 9).

During the past 3 decades, controlled human malaria infection (CHMI) trials have become an indispensable tool not only in assessing the efficacy of candidate vaccines (10, 11) but also in evaluating immune responses induced by exposure to the malaria parasite (12–15). CHMI trials have so far been performed in countries were malaria is not endemic in previously unexposed individuals (11, 16–19). A logical next step is to study the potential differences in the acquisition, maintenance, or recall of immune responses in individuals from different transmission settings and genetic backgrounds (20, 21). The availability of aseptic, purified, cryopreserved, live *P. falciparum* sporozoites (PfSPZs; PfSPZ Challenge) (22) opens up opportunities to carry out CHMI trials in countries where malaria is endemic, since it bypasses the need for infecting local *Anopheles* mosquitoes with *P. falciparum* or importing *P. falciparum*-infected mosquitoes to the trial site. The first PfSPZ Challenge trial in malaria-naive Dutch volunteers demonstrated an infectivity rate of 83% after intradermal injections, independent of the dose given (23). Recently, PfSPZ Challenge was used for the first time during a CHMI trial in healthy adult male Tan-

Received 10 December 2014 Returned for modification 26 January 2015 Accepted 9 March 2015

Accepted manuscript posted online 16 March 2015

Citation Obiero JM, Shekalaghe S, Hermsen CC, Mpina M, Bijker EM, Roestenberg M, Teelen K, Billingsley PF, Sim BKL, James ER, Daubenberger CA, Hoffman SL, Abdulla S, Sauerwein RW, Scholzen A. 2015. Impact of malaria preexposure on antiparasite cellular and humoral immune responses after controlled human malaria infection. Infect Immun 83:2185–2196. doi:10.1128/IAI.03069-14.

Editor: J. H. Adams

Address correspondence to Robert W. Sauerwein, robert.sauerwein@radboudumc.nl, or Anja Scholzen, anja.scholzen@radboudumc.nl.

* Present address: Joshua M. Obiero, University of California Irvine, Department of Medicine, Division of Infectious Diseases, Irvine, California, USA; Meta Roestenberg, Leiden University Medical Center, Department of Infectious Diseases, Leiden, The Netherlands.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.03069-14.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/IAI.03069-14

zanian 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 Tanzanians and Dutch volunteers who were inoculated intradermally with the same number of live 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 Ifakara Health Institute (IHI/IRB/No25), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/1217), the Ethikkommission beider Basel (EKBB), Basel, Switzerland (EKBB 319/11), and the Tanzanian Food and Drug Administration (reference no. CE.57/180/04A/50) 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 hypoendemic). 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 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-Oglobin 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 AAC AAT TGG AGG GCA AG–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). Phocine herpesvirus 1 (PhHV-1) was added to the isolation lysis buffer to serve as an internal control. For quantification of PhHV, the primers GGGCGAATCACAGATTGAATC and GCGGTTCCAAACGTACCAA and the probe Cy5-TTTTTATGTGTCCGCCACCATCTGGATC 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 (PfRBC) 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 lysate for enzyme-linked immunosorbent assay (ELISA) was prepared by extracting purified schizonts/mature trophozoites with 1% sodium desoxycholate and 2.5 μl phenylmethanesulfonyl 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-amino-acid 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 PfRBC/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 1:60,000 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 H_2SO_4, 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 (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 (AUs). 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 (ADAMSEL-v1.1; http://www.malariaresearch.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 of *in vitro* stimulation. Cells were stained first for viability with LIVE/DEAD fixable Aqua 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-phycoerythrin (PE) (HCD56; Biolegend), anti-T-cell receptor (TCR) Pan γ/δ-PE (IMMU510; 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 Foxp3 fix/perm buffer (eBioscience) for 30 min on ice and stained in permeabilization buffer (eBioscience) with IFN-γ fluorescein isothiocyanate (FITC) (4S.B3; eBioscience; 24-h stain) and Foxp3 eF660 (PCH101; eBioscience; *ex vivo* stain) or Ki67 FITC (B56; BD Pharmingen) and Foxp3 eF660 (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 batches of PfRBC 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 PfRBC 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 Tanzanians 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 prepatancy 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

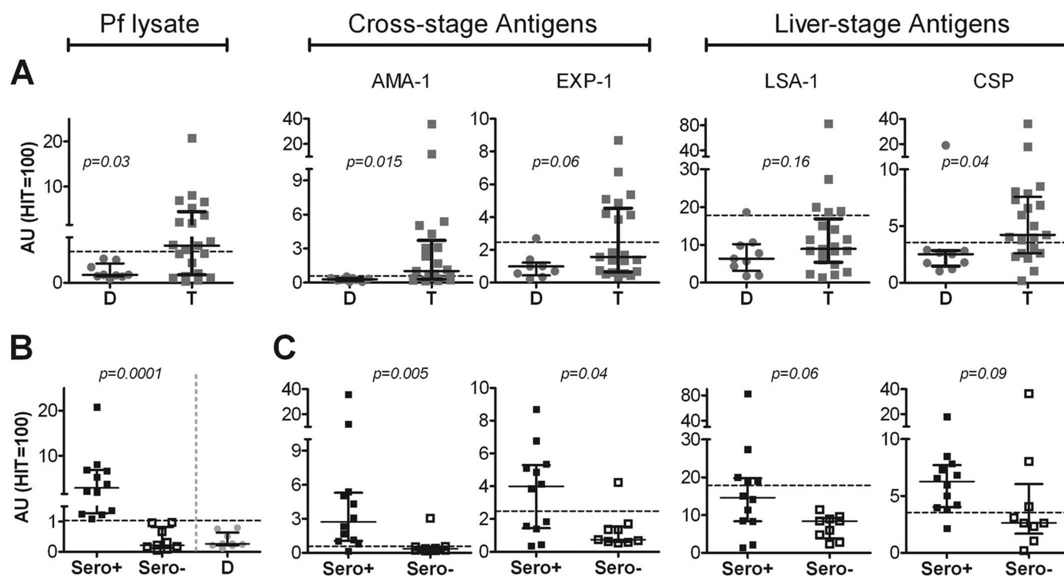


FIG 1 Baseline malaria-specific antibody titers indicate previous exposure in Tanzanian volunteers. Antibody reactivity against crude *P. falciparum* lysate and the *P. falciparum* antigens AMA-1, EXP-1, LSA-1, and CSP was tested prior to malaria infection. A pool of sera from 100 hyperimmune Tanzanians (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; $n = 9$) and Tanzanian (T; squares; $n = 21$) cohorts were compared using a Mann-Whitney U test. (B) Tanzanian volunteers were stratified based on *P. falciparum* lysate recognition at baseline as Sero+ ($n = 12$; black box plots) or Sero− ($n = 9$; 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.

($P < 0.001$; 11/21) (Fig. 3A). In contrast, the Dutch volunteers showed significant increased titers only against EXP-1 ($P = 0.004$; 7/9), CSP ($P = 0.008$; 6/9), and *P. falciparum* lysate ($P = 0.008$; 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 ($P = 0.04$) and a similar trend for the Tanzanians for CSP ($P = 0.07$) (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 ($P = 0.02$) and EXP-1 ($P = 0.04$) than seropositive individuals, with a similar trend for *P. falciparum* lysate ($P = 0.082$) and CSP ($P = 0.095$), but no difference in LSA-1 responses ($P = 0.80$) (Fig. 3E). For the entire cohort, the fold increase of *P. falciparum* lysate ($P = 0.01$) and AMA-1 ($P = 0.001$) 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

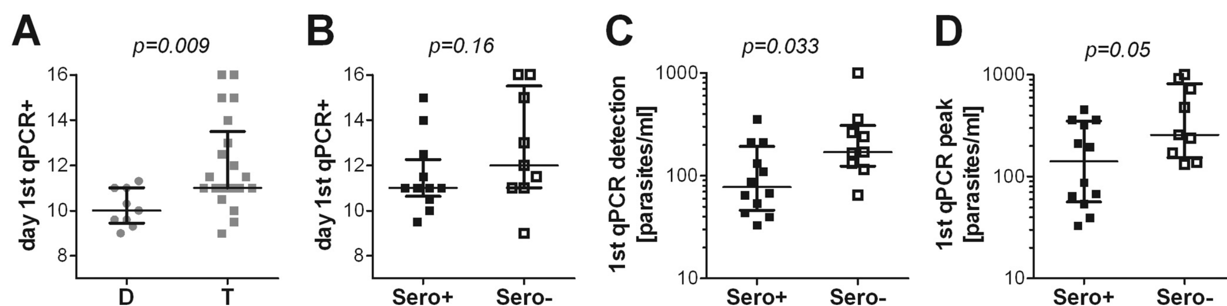


FIG 2 Preexposure to malaria is associated with difference in prepatency and parasitemia after CHMI. Parasitemia after CHMI was determined by qPCR. The day of first parasite detection by qPCR after PfSPZ injection is shown for Dutch (D; gray circles; $n = 9$) compared to Tanzanians (T; gray squares; $n = 21$) (A) and for *P. falciparum*-seropositive ($n = 12$; black squares) and -seronegative ($n = 9$; 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 ($n = 12$; black squares) and -seronegative ($n = 9$; white squares) Tanzanian volunteers. Data are shown as median ± IQR. Groups were compared by a Mann-Whitney U test.

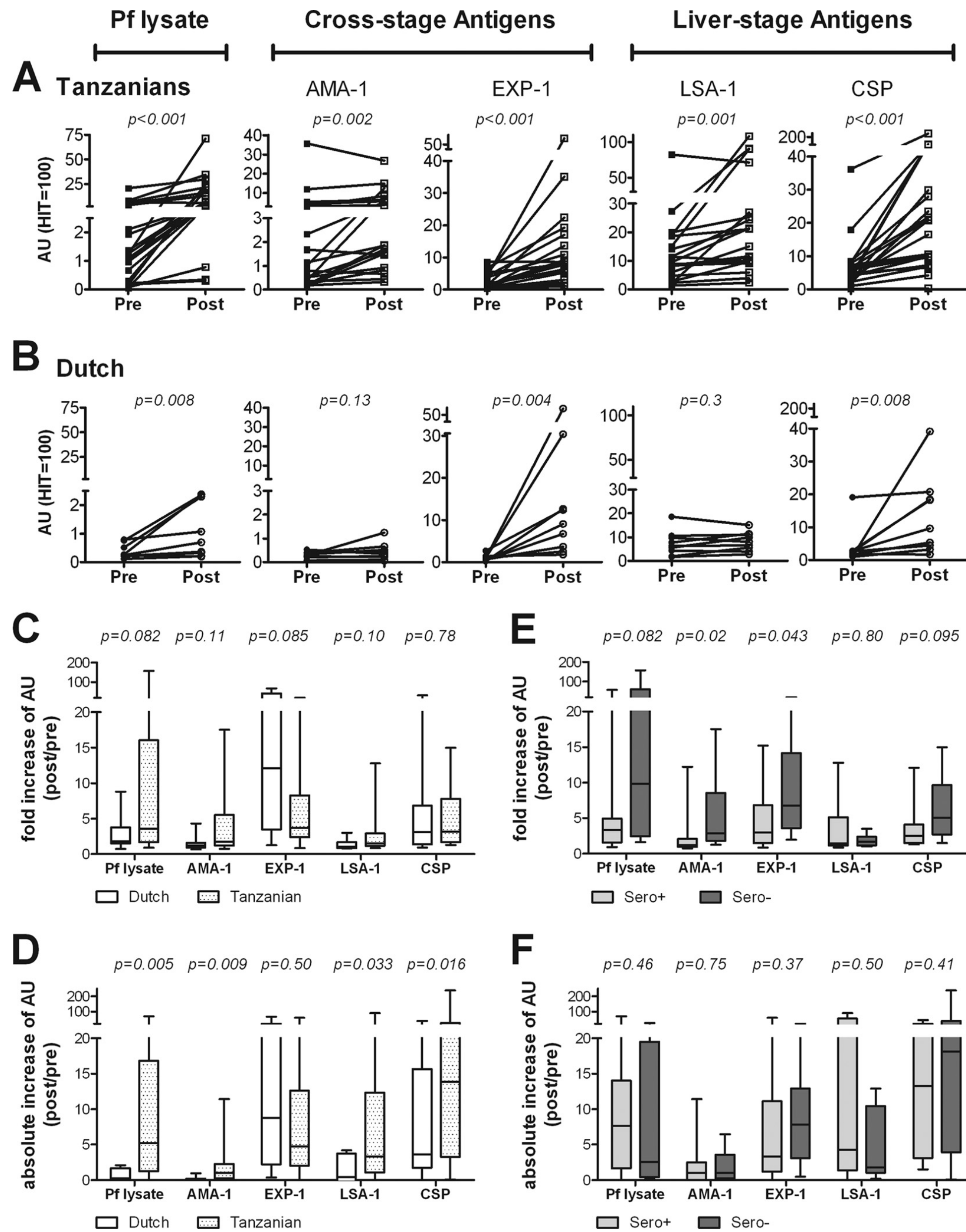


FIG 3 Increased humoral responses in Dutch and Tanzanians after CHMI. Antibody titers were measured 4 weeks (Tanzanians; squares; $n = 21$) (A) and 5 weeks (Dutch; circles; $n = 9$) (B) after intradermal infection with PfSPZs. Graphs show the antibody reactivity (AUs) pre-CHMI (black) and post-CHMI (white) against crude *P. falciparum* lysate, AMA-1, EXP-1, LSA-1, and CSP. Plots show individual data points, with lines connecting the two time points for each volunteer. Responses in the two cohorts pre- and post-CHMI were compared by using a Wilcoxon matched-pairs signed-rank test. The fold increase (ratio of AU post-CHMI/AU pre-CHMI) (C, E) and absolute increase in antibody reactivity (AU post-CHMI minus AU pre-CHMI) (D, F) were calculated for Dutch versus Tanzanian volunteers (C, D) and in Tanzanian volunteers classified as *P. falciparum* lysate seropositive or seronegative at baseline (E, F). Data are shown as whisker plots, with boxes indicating the median and IQR, and whiskers indicating the minimum/maximum values. Responses were compared using 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-naive 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 ($P =$

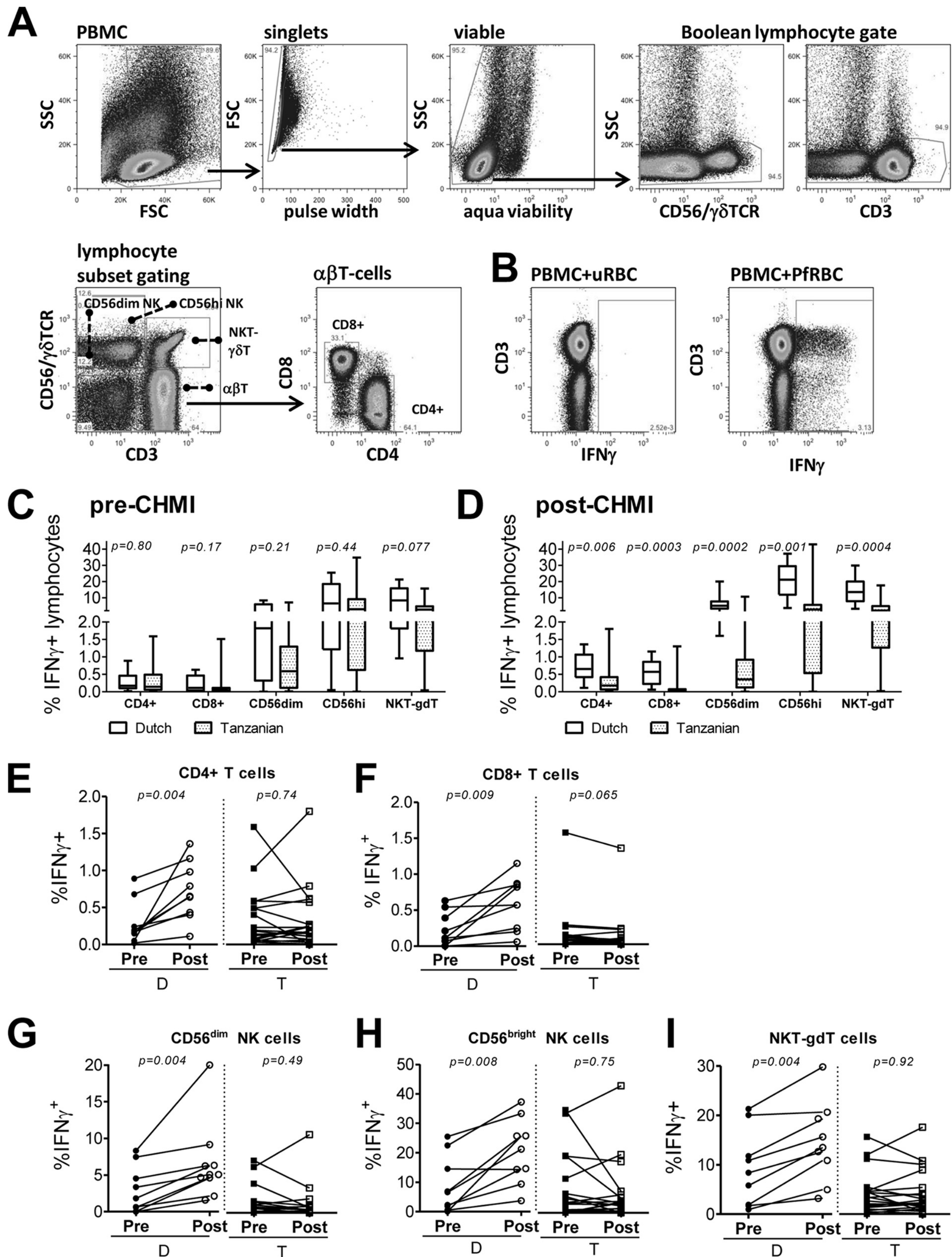


FIG 4 Dutch but not Tanzanian volunteers show increased *P. falciparum*-specific *in vitro* IFN-γ production after CHMI. PBMCs collected pre- and post-CHMI from Dutch (D; circles; $n = 9$; post = 35 days after CHMI) and Tanzanian (T; squares; $n = 21$; post = 28 days after CHMI) volunteers were stimulated 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

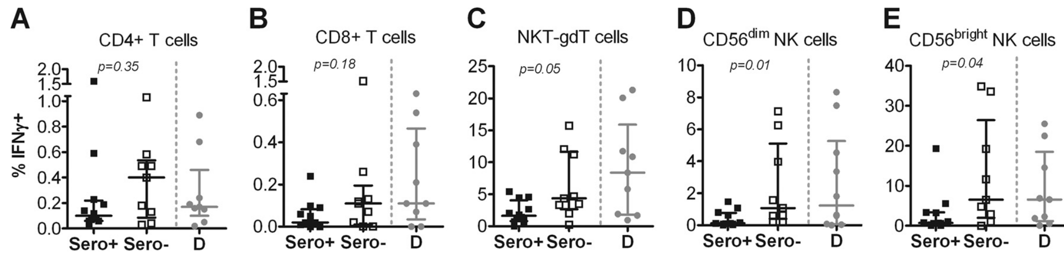


FIG 5 *P. falciparum*-specific *in vitro* IFN-γ production by innate lymphocytes from Tanzanian volunteers is dependent on baseline serological status. PBMCs collected from Tanzanian volunteers who either had a positive *P. falciparum* (Sero+; $n = 12$; black squares) or negative *P. falciparum* (Sero−; $n = 9$; 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, $P \leq 0.0001$). 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

post-CHMI responses within each cohort for $CD4^+$ T cells (E), $CD8^+$ T cells (F), CD56dim NK cells (G), CD56bright NK cells (H), and NKT-γδT cells (I). For each volunteer, all time points were measured in a single experiment. Data are shown as whisker plots with boxes indicating the median with IQR and whiskers the minimum/maximum values (C and D) or individual data points with lines connecting the two time points for each volunteer (E to I). All responses were corrected for the background by subtracting responses to uRBC. Responses pre-CHMI and post-CHMI were compared using a Wilcoxon matched-pairs signed-rank test.

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 liver load (16, 35). Our results might therefore indicate the emergence of fewer parasites from the liver and hence better control of either initiation or progression of liver-stage infection in preexposed individuals within the Tanzanian cohort and in Tanzanians than in the Dutch cohort. In line with such an effect of preexisting antisporozoite immunity would be the observation that those Tanzanian volunteers with a longer prepatancy by qPCR also had higher baseline antibody titers against the sporozoite antigen CSP. However, the first detection by qPCR in both the Dutch and Tanzanians after intradermal PfSPZ injection was uncharacteristically late compared to that for infection by mosquito bite routinely conducted in The Netherlands and elsewhere (11, 16, 18, 19, 23, 24, 35). This is likely due to less efficient liver-stage infection by this route and hence a low initial blood-stage load that reaches the qPCR detection limit only later. A lower first detectable parasitemia in seropositive Tanzanians than in seronegative Tanzanians might therefore additionally be attributed to the control of blood-stage replication prior to qPCR detection. Antibodies directed against AMA-1, for instance, are known to interfere with blood-stage multiplication (36) but can also confer protection against liver-stage infection (37). Since recognition of both cross-stage and liver-stage antigens was stronger in the Tanzanian cohort than in the Dutch, and particularly in the seropositive individuals, both possibilities remain open. Our data, however, support antibody control of blood-stage replication only during the initial phase of sub-qPCR-detectable parasitemia: while parasite multiplication based on PCR data could not be directly compared due to the high variability of the amplification dynamics (24), there was no difference between prepatency by TS and qPCR in the two Tanzanian groups.

Upon CHMI, Dutch volunteers showed a slight but significant induction of humoral responses against CSP, *P. falciparum* lysate, and EXP-1, while Tanzanians boosted responses to all antigens examined. Reactivity to the CSP sporozoite antigen is expected after PfSPZ inoculation and consistent with previous findings (38, 39). Increased antibody responses against *P. falciparum* lysate and EXP-1 likely reflect cross-stage reactivity between late-liver-stage and blood-stage merozoites, while exposure to developing liver stages (LSA-1) in naive volunteers appears insufficient to induce a detectable antibody response. Similarly, limited exposure to blood-stage-expressed AMA-1, due to early curative treatment, appears to prevent induction of detectable titers in naive volunteers, which is consistent with results even after multiple CHMIs (39). Consistent with preexposure, Tanzanians showed on the group level a greater increase in titers for *P. falciparum* lysate, AMA-1, and LSA-1 than the previously malaria-naive Dutch. Within the Tanzanian cohort, *P. falciparum* lysate-seronegative 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 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 PfRBC stimulation (69). However, consistent with their generally reported greater cytotoxic potential, only CD56dim cells showed degranulation upon PfRBC stimulation (69). Our findings that CD56bright NK cells show a greater IFN-γ response than CD56dim NK cells to PfRBC 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 PfRBC-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 preexisting *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 PfRBC 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 PfRBC 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 PfRBC 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, such as fever, than their Dutch counterparts during CHMI after PfSPZ injection (24). On the one hand, preexisting 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* lysate 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 affect the outcome of whole parasite immunization and vaccination approaches in areas where malaria is endemic.

ACKNOWLEDGMENTS

We thank all the trial volunteers and the staff from the Radboud University Medical Center, the Ifakara Health Institute/Bagamoyo Research and Training Centre, Sanaria Inc., and the Swiss Tropical and Public Health Institute, all of whom made this study possible. We thank S. Singh (Gennova Biopharmaceuticals), D. Lanar, and G. Corradin for providing recombinant and synthetic CSP, LSA-1, and EXP-1 proteins, respectively. We thank M. van de Vegte-Bolmer, R. Siebelink-Stoter, and W. Graumans for culture and isolation of PfRBC.

S.L.H. is chief executive and scientific officer at Sanaria Inc. B.K.L.S., P.F.B., and E.R.J. are employees of Sanaria Inc., which manufactured and provided PfSPZ Challenge, and thus do have a potential conflict of interest. There are no other conflicts of interest for all other authors.

This work was supported by Top Institute (TI) Pharma (grant number T4-102), the FP7-founded European Virtual Institute of Malaria Research (EVIMalaR; grant agreement number 242095), the Tanzanian Commission on Science and Technology (COSTECH), the Ifakara Health Institute, and the Swiss Tropical Public Health Institute. A.S. received a long-term postdoctoral fellowship from the European Molecular Biology Organization (EMBO). The development and manufacturing of cryopreserved *P. falciparum* sporozoites (PfSPZ Challenge) were further supported by Small Business Innovation Research (SBIR) (grants R44AI058375-03, -04, -05, -05S1) from the National Institute of Allergy and Infectious Diseases at the National Institutes of Health (NIAID/NIH), USA, and through agreement number 07984 from the Program for Appropriate Technology in Health (PATH) Malaria Vaccine Initiative (with funds from the Bill and Melinda Gates Foundation). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

J.M.O., S.S., C.C.H., E.M.B., M.R, C.A.D., S.L.H., S.A., R.W.S., and A.S. designed the studies and experiments. S.S., E.M.B., and M.R. performed the clinical studies and collected clinical data. J.M.O., C.C.H., K.T., and A.S. conducted experiments. J.M.O., C.C.H., and A.S. analyzed the data. M.M., P.F.B., B.K.L.S., E.R.J., S.L.H., and A.S. collected/prepared/contributed vital reagents. J.M.O., C.C.H., R.W.S., and A.S. interpreted the data. J.M.O., C.C.H., R.W.S., and A.S. wrote the manuscript. All authors read and approved the final manuscript.

REFERENCES

1. **WHO.** 2013. World malaria report. WHO, Geneva, Switzerland.
2. **Feachem RG, Phillips AA, Hwang J, Cotter C, Wielgosz B, Greenwood BM, Sabot O, Rodriguez MH, Abeyasinghe RR, Ghebreyesus TA, Snow RW.** 2010. Shrinking the malaria map: progress and prospects. Lancet **376:**1566–1578. http://dx.doi.org/10.1016/S0140-6736(10)61270-6.
3. **Mendis K, Rietveld A, Warsame M, Bosman A, Greenwood B, Wernsdorfer WH.** 2009. From malaria control to eradication: the WHO perspective. Trop Med Int Health **14:**802–809. http://dx.doi.org/10.1111/j.1365-3156.2009.02287.x.
4. **RTS,S Clinical Trials Partnership.** 2014. Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. PLoS Med **11:**e1001685. http://dx.doi.org/10.1371/journal.pmed.1001685.
5. **RTS,S Clinical Trials Partnership, Agnandji ST, Lell B, Fernandes JF, Abossolo BP, Methogo BG, Kabwende AL, Adegnika AA, Mordmuller B, Issifou S, Kremsner PG, Sacarlal J, Aide P, Lanaspa M, Aponte JJ, Machevo S, Acacio S, Bulo H, Sigauque B, Macete E, Alonso P, Abdulla S, Salim N, Minja R, Mpina M, Ahmed S, Ali AM, Mtoro AT, Hamad AS, Mutani P, Tanner M, Tinto H, D'Alessandro U, Sorgho H, Valea I, Bihoun B, Guiraud I, Kabore B, Sombie O, Guiguemde RT, Ouedraogo JB, Hamel MJ, Kariuki S, Oneko M, Odero C, Otieno K, Awino N, McMorrow M, Muturi-Kioi V, Laserson KF, Slutsker L, et al.** 2012. A phase 3 trial of RTS,S/AS01 malaria vaccine in African infants. N Engl J Med **367:**2284–2295. http://dx.doi.org/10.1056/NEJMoa1208394.
6. **Bejon P, White MT, Olotu A, Bojang K, Lusingu JPA, Salim N, Otsyula NN, Agnandji ST, Asante KP, Owusu-Agyei S, Abdulla S, Ghani AC.** 2013. Efficacy of RTS,S malaria vaccines: individual-participant pooled analysis of phase 2 data. Lancet Infect Dis **13:**319–327. http://dx.doi.org/10.1016/S1473-3099(13)70005-7.
7. **Bojang KA, Milligan PJM, Pinder M, Vigneron L, Alloueche A, Kester KE, Ballou WR, Conway DJ, Reece WHH, Gothard P, Yamuah L, Delchambre M, Voss G, Greenwood BM, Hill A, McAdam KPWJ, Tornieporth N, Cohen JD, Doherty T.** 2001. Efficacy of RTS,S/AS02 malaria vaccine against *Plasmodium falciparum* infection in semiimmune adult men in The Gambia: a randomised trial. Lancet **358:**1927–1934. http://dx.doi.org/10.1016/S0140-6736(01)06957-4.
8. **Struik SS, Riley EM.** 2004. Does malaria suffer from lack of memory? Immunol Rev **201:**268–290. http://dx.doi.org/10.1111/j.0105-2896.2004.00181.x.
9. **Doolan D, Dobaño C, Baird J.** 2009. Acquired immunity to malaria. Clin Microbiol Rev **22:**13. http://dx.doi.org/10.1128/CMR.00025-08.
10. **Sauerwein R, Roestenberg M, Moorthy V.** 2011. Experimental human challenge infections can accelerate clinical malaria vaccine development. Nat Rev Immunol **11:**57–64. http://dx.doi.org/10.1038/nri2902.
11. **Stoute JA, Slaoui M, Heppner DG, Momin P, Kester KE, Desmons P, Wellde BT, Garcon N, Krzych U, Marchand M.** 1997. A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. RTS,S Malaria Vaccine Evaluation Group. N Engl J Med **336:**86–91.
12. **Clyde D.** 1975. Immunization of man against *falciparum* and *vivax* malaria by use of attenuated sporozoites. Am J Trop Med Hyg **24:**397–401.
13. **Hoffman S, Goh L, Luke T, Schneider I, Le T, Doolan D, Sacci J, de la Vega P, Dowler M, Paul C, Gordon D, Stoute J, Church L, Sedegah M, Heppner D, Ballou W, Richie T.** 2002. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. J Infect Dis **185:**1155–1164. http://dx.doi.org/10.1086/339409.
14. **Kester K, McKinney D, Tornieporth N, Ockenhouse C, Heppner D, Hall T, Krzych U, Delchambre M, Voss G, Dowler M, Palensky J, Wittes J, Cohen J, Ballou W, RTS,S Malaria Vaccine Evaluation Group.** 2001. Efficacy of recombinant circumsporozoite protein vaccine regimens against experimental *Plasmodium falciparum* malaria. J Infect Dis **183:**640–647. http://dx.doi.org/10.1086/318534.
15. **Bijker E, Bastiaens G, Teirlinck A, van Gemert GJ, Graumans W, van de Vegte-Bolmer M, Siebelink-Stoter R, Arens T, Teelen K, Nahrendorf W, Remarque E, Roeffen W, Jansens A, Zimmerman D, Vos M, van Schaijk B, Wiersma J, van der Ven AJ, de Mast Q, van Lieshout L, Verweij J, Hermsen C, Scholzen A, Sauerwein RW.** 2013. Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity. Proc Natl Acad Sci U S A **110:**7862–7867. http://dx.doi.org/10.1073/pnas.1220360110.
16. **Roestenberg M, O'Hara GA, Duncan CJA, Epstein JE, Edwards NJ, Scholzen A, van der Ven AJAM, Hermsen CC, Hill AVS, Sauerwein RW.** 2012. Comparison of clinical and parasitological data from controlled human malaria infection trials. PLoS One **7:**e38434. http://dx.doi.org/10.1371/journal.pone.0038434.
17. **Engwerda CR, Minigo G, Amante FH, McCarthy JS.** 2012. Experimentally induced blood stage malaria infection as a tool for clinical research. Trends Parasitol **28:**515–521. http://dx.doi.org/10.1016/j.pt.2012.09.001.
18. **Lyke KE, Laurens M, Adams M, Billingsley PF, Richman A, Loyevsky M, Chakravarty S, Plowe CV, Sim BK, Edelman R, Hoffman SL.** 2010. *Plasmodium falciparum* malaria challenge by the bite of aseptic *Anopheles stephensi* mosquitoes: results of a randomized infectivity trial. PLoS One **5:**e13490. http://dx.doi.org/10.1371/journal.pone.0013490.
19. **Hodgson SH, Ewer KJ, Bliss CM, Edwards NJ, Rampling T, Anagnostou NA, de Barra E, Havelock T, Bowyer G, Poulton ID, de Cassan S, Illingworth JJ, Douglas AD, Mange PB, Collins KA, Roberts R, Gerry S, Berrie E, Moyle S, Colloca S, Cortese R, Sinden RE, Gilbert SC, Bejon P, Lawrie AM, Nicosia A, Faust SN, Hill AV.** 2015. Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing CS & ME-TRAP against controlled human malaria infection in malaria naive individuals. J Infect Dis **211:**1076–1086. http://dx.doi.org/10.1093/infdis/jiu579.
20. **Roestenberg M, McCall M, Hopman J, Wiersma J, Luty A, van Gemert G, van de Vegte-Bolmer M, van Schaijk B, Teelen K, Arens T, Spaarman L, de Mast Q, Roeffen W, Snounou G, Rénia L, van der Ven A, Hermsen C, Sauerwein R.** 2009. Protection against a malaria challenge by

sporozoite inoculation. N Engl J Med **361:**468–477. http://dx.doi.org/10.1056/NEJMoa0805832.

21. **Seder RA, Chang LJ, Enama ME, Zephir KL, Sarwar UN, Gordon IJ, Holman LA, James ER, Billingsley PF, Gunasekera A, Richman A, Chakravarty S, Manoj A, Velmurugan S, Li M, Ruben AJ, Li T, Eappen AG, Stafford RE, Plummer SH, Hendel CS, Novik L, Costner PJ, Mendoza FH, Saunders JG, Nason MC, Richardson JH, Murphy J, Davidson SA, Richie TL, Sedegah M, Sutamihardja A, Fahle GA, Lyke KE, Laurens MB, Roederer M, Tewari K, Epstein JE, Sim BK, Ledgerwood JE, Graham BS, Hoffman SL, VRC 312 Study Team.** 2013. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. Science **341:**1359–1365. http://dx.doi.org/10.1126/science.1241800.
22. **Hoffman SL, Billingsley PF, James E, Richman A, Loyevsky M, Li T, Chakravarty S, Gunasekera A, Chattopadhyay R, Li M, Stafford R, Ahumada A, Epstein JE, Sedegah M, Reyes S, Richie TL, Lyke KE, Edelman R, Laurens MB, Plowe CV, Sim BK.** 2010. Development of a metabolically active, non-replicating sporozoite vaccine to prevent *Plasmodium falciparum* malaria. Hum Vaccin **6:**97–106. http://dx.doi.org/10.4161/hv.6.1.10396.
23. **Roestenberg M, Bijker E, Sim B, Billingsley P, James E, Bastiaens G, Teirlinck A, Scholzen A, Teelen K, Arens T, van der Ven A, Gunasekera A, Chakravarty S, Velmurugan S, Hermsen C, Sauerwein R, Hoffman S.** 2013. Controlled human malaria infections by intradermal injection of cryopreserved *Plasmodium falciparum* sporozoites. Am J Trop Med Hyg **88:**5–13. http://dx.doi.org/10.4269/ajtmh.2012.12-0613.
24. **Shekalaghe S, Rutaihwa M, Billingsley PF, Chemba M, Daubenberger CA, James E, Mpina M, Ali Juma O, Schindler T, Huber E, Gunasekera A, Manoj A, Simon B, Savarino E, Church LW, Hermsen CC, Sauerwein RW, Plowe CV, Venkatesan M, Sasi P, Lweno O, Mutani P, Hamad A, Mohammed A, Urassa A, Mzee T, Padilla D, Ruben A, Lee Sim BK, Tanner M, Abdullah S, Hoffman SL.** 2014. Controlled human malaria infection of Tanzanians by intradermal injection of aseptic, purified, cryopreserved *Plasmodium falciparum* sporozoites. Am J Trop Med Hyg **91:**471–480. http://dx.doi.org/10.4269/ajtmh.14-0119.
25. **Hermsen CC, Telgt DS, Linders EH, van de Locht LA, Eling WM, Mensink EJ, Sauerwein RW.** 2001. Detection of *Plasmodium falciparum* malaria parasites *in vivo* by real-time quantitative PCR. Mol Biochem Parasitol **118:**247–251. http://dx.doi.org/10.1016/S0166-6851(01)00379-6.
26. **Adegnika AA, Verweij JJ, Agnandji ST, Chai SK, Breitling LP, Ramharter M, Frolich M, Issifou S, Kremsner PG, Yazdanbakhsh M.** 2006. Microscopic and sub-microscopic *Plasmodium falciparum* infection, but not inflammation caused by infection, is associated with low birth weight. Am J Trop Med Hyg **75:**798–803.
27. **Walliker D, Quakyi IA, Wellems TE, McCutchan TF, Szarfman A, London WT, Corcoran LM, Burkot TR, Carter R.** 1987. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science **236:**1661–1666. http://dx.doi.org/10.1126/science.3299700.
28. **Hillier CJ, Ware LA, Barbosa A, Angov E, Lyon JA, Heppner DG, Lanar DE.** 2005. Process development and analysis of liver-stage antigen 1, a preerythrocyte-stage protein-based vaccine for *Plasmodium falciparum*. Infect Immun **73:**2109–2115. http://dx.doi.org/10.1128/IAI.73.4.2109-2115.2005.
29. **Fidock DA, Gras-Masse H, Lepers JP, Brahimi K, Benmohamed L, Mellouk S, Guerin-Marchand C, Londono A, Raharimalala L, Meis JF, Langsley G, Roussilhon C, Tartar A, Druilhe P.** 1994. *Plasmodium falciparum* liver stage antigen-1 is well conserved and contains potent B and T cell determinants. J Immunol **153:**190–204.
30. **Zhu J, Hollingdale MR.** 1991. Structure of *Plasmodium falciparum* liver stage antigen-1. Mol Biochem Parasitol **48:**223–226. http://dx.doi.org/10.1016/0166-6851(91)90117-O.
31. **Kocken C, Withers-Martinez C, Dubbeld M, van der Wel A, Hackett F, Valderrama A, Blackman M, Thomas A.** 2002. High-level expression of the malaria blood-stage vaccine candidate *Plasmodium falciparum* apical membrane antigen 1 and induction of antibodies that inhibit erythrocyte invasion. Infect Immun **70:**4471–4476. http://dx.doi.org/10.1128/IAI.70.8.4471-4476.2002.
32. **Faber B, Remarque E, Kocken C, Cheront P, Cingolani D, Xhonneux F, Jurado M, Haumont M, Jepsen S, Leroy O, Thomas A.** 2008. Production, quality control, stability and pharmacotoxicity of cGMP-produced *Plasmodium falciparum* AMA1 FVO strain ectodomain expressed in *Pichia pastoris*. Vaccine **26:**6143–6150. http://dx.doi.org/10.1016/j.vaccine.2008.08.055.
33. **Meraldi V, Nebié I, Moret R, Cuzin-Ouattara N, Thiocone A, Doumbo O, Esposito F, Traoré A, Corradin G, Terenzi S.** 2002. Recognition of synthetic polypeptides corresponding to the N- and C-terminal fragments of *Plasmodium falciparum* Exp-1 by T-cells and plasma from human donors from African endemic areas. Parasite Immunol **24:**141–150. http://dx.doi.org/10.1046/j.1365-3024.2002.00447.x.
34. **Bottius E, Guanzirolli A, Trape J-F, Rogier C, Konate L, Druilhe P.** 1996. Malaria: even more chronic in nature than previously thought; evidence for subpatent parasitaemia detectable by the polymerase chain reaction. Trans R Soc Trop Med Hyg **90:**15–19. http://dx.doi.org/10.1016/S0035-9203(96)90463-0.
35. **Roestenberg M, de Vlas SJ, Nieman AE, Sauerwein RW, Hermsen CC.** 2012. Efficacy of preerythrocytic and blood-stage malaria vaccines can be assessed in small sporozoite challenge trials in human volunteers. J Infect Dis **206:**319–323. http://dx.doi.org/10.1093/infdis/jis355.
36. **Arnot DE, Cavanagh DR, Remarque EJ, Creasey AM, Sowa MP, Morgan WD, Holder AA, Longacre S, Thomas AW.** 2008. Comparative testing of six antigen-based malaria vaccine candidates directed toward merozoite-stage *Plasmodium falciparum*. Clin Vaccine Immunol **15:**1345–1355. http://dx.doi.org/10.1128/CVI.00172-08.
37. **Schussek S, Trieu A, Apte SH, Sidney J, Sette A, Doolan DL.** 2013. Immunization with apical membrane antigen 1 confers sterile infection-blocking immunity against *Plasmodium* sporozoite challenge in a rodent model. Infect Immun **81:**3586–3599. http://dx.doi.org/10.1128/IAI.00544-13.
38. **Felgner PL, Roestenberg M, Liang L, Hung C, Jain A, Pablo J, Nakajima-Sasaki R, Molina D, Teelen K, Hermsen CC, Sauerwein R.** 2013. Pre-erythrocytic antibody profiles induced by controlled human malaria infections in healthy volunteers under chloroquine prophylaxis. Sci Rep **3:**3549. http://dx.doi.org/10.1038/srep03549.
39. **Nahrendorf W, Scholzen A, Bijker EM, Teirlinck AC, Bastiaens GJ, Schats R, Hermsen CC, Visser LG, Langhorne J, Sauerwein RW.** 2014. Memory B-cell and antibody responses induced by *Plasmodium falciparum* sporozoite immunization. J Infect Dis **210:**1981–1990. http://dx.doi.org/10.1093/infdis/jiu354.
40. **Ndungu FM, Lundblom K, Rono J, Illingworth J, Eriksson S, Farnert A.** 2013. Long-lived *Plasmodium falciparum* specific memory B cells in naturally exposed Swedish travelers. Eur J Immunol **43:**2919–2929. http://dx.doi.org/10.1002/eji.201343630.
41. **Ndungu FM, Olotu A, Mwacharo J, Nyonda M, Apfeld J, Mramba LK, Fegan GW, Bejon P, Marsh K.** 2012. Memory B cells are a more reliable archive for historical antimalarial responses than plasma antibodies in no-longer exposed children. Proc Natl Acad Sci U S A **109:**8247–8252. http://dx.doi.org/10.1073/pnas.1200472109.
42. **Kennedy MC, Wang J, Zhang Y, Miles AP, Chitsaz F, Saul A, Long CA, Miller LH, Stowers AW.** 2002. *In vitro* studies with recombinant *Plasmodium falciparum* apical membrane antigen 1 (AMA1): production and activity of an AMA1 vaccine and generation of a multiallelic response. Infect Immun **70:**6948–6960. http://dx.doi.org/10.1128/IAI.70.12.6948-6960.2002.
43. **Remarque EJ, Faber BW, Kocken CH, Thomas AW.** 2008. Apical membrane antigen 1: a malaria vaccine candidate in review. Trends Parasitol **24:**74–84. http://dx.doi.org/10.1016/j.pt.2007.12.002.
44. **Drew DR, Hodder AN, Wilson DW, Foley M, Mueller I, Siba PM, Dent AE, Cowman AF, Beeson JG.** 2012. Defining the antigenic diversity of *Plasmodium falciparum* apical membrane antigen 1 and the requirements for a multi-allele vaccine against malaria. PLoS One **7:**e51023. http://dx.doi.org/10.1371/journal.pone.0051023.
45. **Terheggen U, Drew DR, Hodder AN, Cross NJ, Mugyenyi CK, Barry AE, Anders RF, Dutta S, Osier F, Elliott SR, Senn N, Stanisic DI, Marsh K, Siba PM, Mueller I, Richards JS, Beeson JG.** 2014. Limited antigenic diversity of *Plasmodium falciparum* apical membrane antigen 1 supports the development of effective multi-allele vaccines. BMC Med **12:**183. http://dx.doi.org/10.1186/s12916-014-0183-5.
46. **Remarque EJ, Faber BW, Kocken CH, Thomas AW.** 2008. A diversity-covering approach to immunization with *Plasmodium falciparum* apical membrane antigen 1 induces broader allelic recognition and growth inhibition responses in rabbits. Infect Immun **76:**2660–2670. http://dx.doi.org/10.1128/IAI.00170-08.
47. **Teirlinck A, McCall MB, Roestenberg M, Scholzen A, Woestenenk R, de Mast Q, van der Ven AJ, Hermsen C, Luty A, Sauerwein R.** 2011.

Longevity and composition of cellular immune responses following experimental *Plasmodium falciparum* malaria infection in humans. PLoS Pathog 7:e1002389. http://dx.doi.org/10.1371/journal.ppat.1002389.
48. **Teirlinck A, Roestenberg M, van de Vegte-Bolmer M, Scholzen A, Heinrichs M, Siebelink-Stoter R, Graumans W, van Gemert GJ, Teelen K, Vos M, Nganou-Makamdop K, Borrmann S, Rozier Y, Erkens MA, Luty A, Hermsen C, Sim B, van Lieshout L, Hoffman S, Visser L, Sauerwein R.** 2013. NF135.C10: a new *Plasmodium falciparum* clone for controlled human malaria infections. J Infect Dis **207**:656–660. http://dx.doi.org/10.1093/infdis/jis725.
49. **Riley EM, Andersson G, Otoo LN, Jepsen S, Greenwood BM.** 1988. Cellular immune responses to *Plasmodium falciparum* antigens in Gambian children during and after an acute attack of falciparum malaria. Clin Exp Immunol **73**:17–22.
50. **Ho M, Webster HK, Looareesuwan S, Supanaranond W, Phillips RE, Chanthavanich P, Warrell DA.** 1986. Antigen-specific immunosuppression in human malaria due to *Plasmodium falciparum*. J Infect Dis **153**:763–771. http://dx.doi.org/10.1093/infdis/153.4.763.
51. **Chemtai AK, Okelo GB.** 1989. Suppression of T-cell proliferative response in *Plasmodium falciparum* malaria patients—preliminary results. East Afr Med J **66**:787–791.
52. **Bygbjerg IC, Jepsen S, Theander TG.** 1986. Lymphocyte response to purified *Plasmodium falciparum* antigens during and after malaria. Acta Trop **43**:55–62.
53. **Theander TG, Bygbjerg IC, Andersen BJ, Jepsen S, Kharazmi A, Odum N.** 1986. Suppression of parasite-specific response in *Plasmodium falciparum* malaria. A longitudinal study of blood mononuclear cell proliferation and subset composition. Scand J Immunol **24**:73–81.
54. **Williamson WA, Greenwood BM.** 1978. Impairment of the immune response to vaccination after acute malaria. Lancet **i**:1328–1329.
55. **Mabey DC, Brown A, Greenwood BM.** 1987. *Plasmodium falciparum* malaria and *Salmonella* infections in Gambian children. J Infect Dis **155**:1319–1321. http://dx.doi.org/10.1093/infdis/155.6.1319.
56. **Gunapala DE, Facer CA, Davidson R, Weir WR.** 1990. *In vitro* analysis of Epstein-Barr virus: host balance in patients with acute *Plasmodium falciparum* malaria. I. Defective T-cell control. Parasitol Res **76**:531–535.
57. **Greenwood BM, Bradley-Moore AM, Bryceson AD, Palit A.** 1972. Immunosuppression in children with malaria. Lancet **i**:169–172.
58. **Cook IF.** 1985. Herpes zoster in children following malaria. J Trop Med Hyg **88**:261–264.
59. **Riley EM, Jobe O, Blackman M, Whittle HC, Greenwood BM.** 1989. *Plasmodium falciparum* schizont sonic extracts suppress lymphoproliferative responses to mitogens and antigens in malaria-immune adults. Infect Immun **57**:3181–3188.
60. **Scholzen A, Minigo G, Plebanski M.** 2010. Heroes or villains? T regulatory cells in malaria infection. Trends Parasitol **26**:16–25. http://dx.doi.org/10.1016/j.pt.2009.10.004.
61. **Minigo G, Woodberry T, Piera KA, Salwati E, Tjitra E, Kenangalem E, Price RN, Engwerda CR, Anstey NM, Plebanski M.** 2009. Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria. PLoS Pathog **5**:e1000402. http://dx.doi.org/10.1371/journal.ppat.1000402.
62. **McCall MB, Hopman J, Daou M, Maiga B, Dara V, Ploemen I, Nganou-Makamdop K, Niangaly A, Tolo Y, Arama C, Bousema JT, van der Meer JW, van der Ven AJ, Troye-Blomberg M, Dolo A, Doumbo OK, Sauerwein RW.** 2010. Early interferon-gamma response against *Plasmodium falciparum* correlates with interethnic differences in susceptibility to parasitemia between sympatric Fulani and Dogon in Mali. J Infect Dis **201**:142–152. http://dx.doi.org/10.1086/648596.
63. **Torcia MG, Santarlasci V, Cosmi L, Clemente A, Maggi L, Mangano VD, Verra F, Bancone G, Nebie I, Sirima BS, Liotta F, Frosali F, Angeli R, Severini C, Sannella AR, Bonini P, Lucibello M, Maggi E, Garaci E, Coluzzi M, Cozzolino F, Annunziato F, Romagnani S, Modiano D.** 2008. Functional deficit of T regulatory cells in Fulani, an ethnic group with low susceptibility to *Plasmodium falciparum* malaria. Proc Natl Acad Sci U S A **105**:646–651. http://dx.doi.org/10.1073/pnas.0709969105.
64. **Inoue S, Niikura M, Mineo S, Kobayashi F.** 2013. Roles of IFN-gamma and gammadelta T cells in protective immunity against blood-stage malaria. Front Immunol **4**:258. http://dx.doi.org/10.3389/fimmu.2013.00258.
65. **Chen Q, Amaladoss A, Ye W, Liu M, Dummler S, Kong F, Wong LH, Loo HL, Loh E, Tan SQ, Tan TC, Chang KT, Dao M, Suresh S, Preiser PR, Chen J.** 2014. Human natural killer cells control *Plasmodium falciparum* infection by eliminating infected red blood cells. Proc Natl Acad Sci U S A **111**:1479–1484. http://dx.doi.org/10.1073/pnas.1323318111.
66. **Jacobs R, Hintzen G, Kemper A, Beul K, Kempf S, Behrens G, Sykora KW, Schmidt RE.** 2001. CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells. Eur J Immunol **31**:3121–3127. http://dx.doi.org/10.1002/1521-4141(2001010)31:10<3121::AID-IMMU3121>3.0.CO;2-4.
67. **Cooper MA, Fehniger TA, Caligiuri MA.** 2001. The biology of human natural killer-cell subsets. Trends Immunol **22**:633–640. http://dx.doi.org/10.1016/S1471-4906(01)02060-9.
68. **Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J.** 2009. CD56bright natural killer (NK) cells: an important NK cell subset. Immunology **126**:458–465. http://dx.doi.org/10.1111/j.1365-2567.2008.03027.x.
69. **Korbel DS, Newman KC, Almeida CR, Davis DM, Riley EM.** 2005. Heterogeneous human NK cell responses to *Plasmodium falciparum*-infected erythrocytes. J Immunol **175**:7466–7473. http://dx.doi.org/10.4049/jimmunol.175.11.7466.
70. **McCall MB, Roestenberg M, Ploemen I, Teirlinck A, Hopman J, de Mast Q, Dolo A, Doumbo O, Luty A, van der Ven A, Hermsen C, Sauerwein R.** 2010. Memory-like IFN-γ response by NK cells following malaria infection reveals the crucial role of T cells in NK cell activation by *P. falciparum*. Eur J Immunol **40**:3472–3477. http://dx.doi.org/10.1002/eji.201040587.
71. **Bijker EM, Teirlinck AC, Schats R, van Gemert GJ, van de Vegte-Bolmer M, van Lieshout L, IntHout J, Hermsen CC, Scholzen A, Visser LG, Sauerwein RW.** 2014. Cytotoxic markers associate with protection against malaria in human volunteers immunized with *Plasmodium falciparum* sporozoites. J Infect Dis **210**:1605–1615. http://dx.doi.org/10.1093/infdis/jiu293.
72. **Tarun AS, Peng X, Dumpit RF, Ogata Y, Silva-Rivera H, Camargo N, Daly TM, Bergman LW, Kappe SH.** 2008. A combined transcriptome and proteome survey of malaria parasite liver stages. Proc Natl Acad Sci U S A **105**:305–310. http://dx.doi.org/10.1073/pnas.0710780104.
73. **Nganou-Makamdop K, van Gemert GJ, Arens T, Hermsen CC, Sauerwein RW.** 2012. Long term protection after immunization with *P. berghei* sporozoites correlates with sustained IFNgamma responses of hepatic CD8+ memory T cells. PLoS One **7**:e36508. http://dx.doi.org/10.1371/journal.pone.0036508.
74. **Tse SW, Cockburn IA, Zhang H, Scott AL, Zavala F.** 2013. Unique transcriptional profile of liver-resident memory CD8+ T cells induced by immunization with malaria sporozoites. Genes Immun **14**:302–309. http://dx.doi.org/10.1038/gene.2013.20.

PAPER VII:

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

To be submitted to Malaria Journal

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

Maxmillian Mpina[1, 2, 3, §],Danika L. Hill[6,7,8],Joseph Campo[4], Amanda Ross[2, 3], Anneth Tumbo [1, 2, 3] Louis Schofield[6,7], Carlota Dobaño[4] , Seif Shekalaghe[1] , Peter F. Billingsley[5], Kim Lee Sim [5], Stephen L. Hoffman [5], Salim Abdulla [1], Marcel Tanner [2, 3],Claudia A. Daubenberger[2, 3, §]

1 Ifakara Health Institute, Bagamoyo Research and Training Centre, P.O. Box 74, Bagamoyo, Tanzania

2 Swiss Tropical and Public Health Institute, Socinstrasse 57, CH 4002 Basel, Switzerland

3 University of Basel, Petersplatz 1, Basel, 4051, Switzerland

4 ISGlobal, Hospital Clinic-Universitat de Barcelona, Carrer Rosselló 132, Barcelona, Spain.

5 Sanaria Inc., 9800 Medical Center Dr., Suite A209, Rockville, MD 20850, Maryland, USA

6 The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville VIC 3052, Victoria Australia

7 Department of Medical Biology, University of Melbourne, Parkville VIC, 3010

8 Babraham Institute, Babraham Research Campus, Cambridge CB22 3AT, United Kingdom

§ Corresponding authors

Maxmillian Mpina (mmpina@ihi.or.tz)

Danika L. Hill (danikaleahill@gmail.com)

Joseph Campo (joseph jcampo@antigendiscovery.com)

Amanda Ross (Amanda.Ross@unibas.ch)

Anneth Tumbo (atumbo@ihi.or.tz)

Louis Schofield (schofield@wehi.edu.au)

Carlota Dobaño (carlota.dobano@isglobal.org)

Seif Shekalaghe (seif sshekalaghe@ihi.or.tz)

Peter F. Billingsley (pbillingsley@sanaria.com)

Kim Lee Sim (ksim@sanaria.com, ksim@protpot.com)

Stephen L. Hoffman (slhoffman@sanaria.com)

Salim Abdulla (sabdulla@ihi.or.tz)

Marcel Tanner (marcel.tanner@unibas.ch)

Claudia A. Daubenberger (Claudia.Daubenberger@unibas.ch)

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 SSP2did 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, suggesting that boosting of pre-erythrocytic and asexual blood stage humoral immune responses can be assessed reliably by three assessment methods.

Conclusion:

*Pf*SPZ 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 (-191ºC). 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 ºC, 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-1_{42}3D7); Region 42: 42 kDa merozoite surface protein 1, FVO strain (MSP-1_{42}FVO)¸ Region 43: fragment II of region II of the 175 kDa erythrocyte binding protein (EBA-175, or PfF2);

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-1_{42} 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:

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

Where A_{min} is the lower asymptote, $A_{\max}$ is the upper asymptote, β is the slope at the inflection point of the sigmoidal curve, $EC50$ is the concentration at the inflection point, and ϵ 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 ºC in 5% CO_2 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 $5x10^5$ cells/well and six serial dilutions starting at 4×10^4 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 10^6 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, 56and 168 except for another three participants for which one volunteer missed day 168visit 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 (CelTOS), 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 CelTOS and three volunteers (11.1%)were negative for SSP2 (Figure 1).

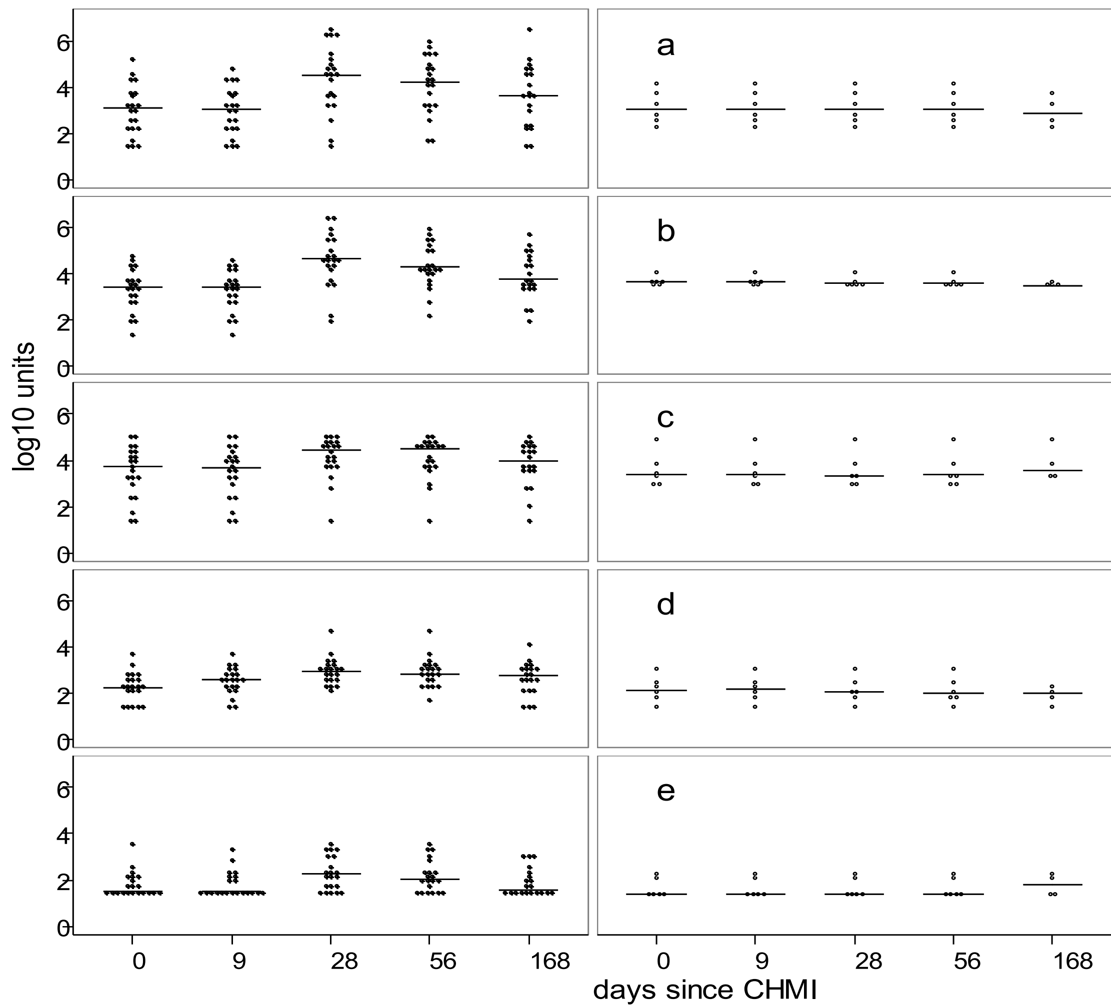


Figure 1:

Multiplex-bead array based analysis of pre-erythrocytic, stage-transcending and asexual blood stage malaria antigen-specific serum antibody levels in *Pf*SPZ 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 *Pf*SPZ 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.

Antigen	Day 28 vs 0	p-value	Day 56 vs 0	p-value	Day 168 vs 0	p-value
MSP-1 (3D7)	32065 (-1407,1990947)	<0.001	14039 (-794, 450948)	<0.001	2372 (-4068, 160994)	0.008
MSP-1 (FVO)	26743 (-1216,2059911)	<0.001	13219 (-621,567900)	<0.001	860 (-9882,335375)	0.03
AMA-1	4523 (-15264,100269)	0.046	5244 (-11788, 70530)	0.03	506 (-31431,36785)	0.70
CSP	475 (36,4400)	<0.001	270 (-168,4759)	<0.001	126 (-289,2890)	0.04
LSA-1	44 (-30,2001)	0.01	22 (-186,1543)	0.05	0 (-231,828)	0.94

Table 1:

Summary of differences in IgG responses (shown as AU) between time points days 28, 56 and 168in 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 *Pf*SPZ 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-1specific 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).

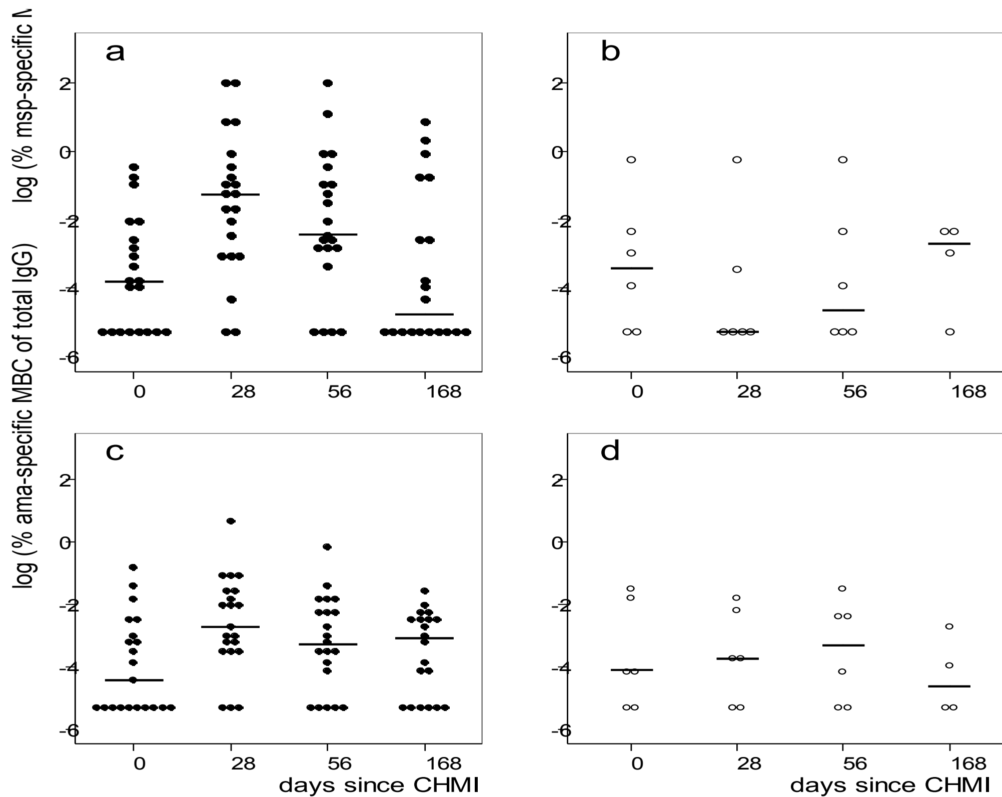


Figure 2: MSP-1 and AMA-1 specific ASCs increase after intradermal *Pf*SPZ challenge

The log of the proportion of MSP-1specific ASCs(a) and AMA-1 specific ASCs(b) in *Pf*SPZ 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.

Antibody secreting cells	Day 28 versus 0	p value	Day 56 versus 0	p value	Day 168 versus 0	p value
Log % MSP-1specific ASCs/total ASCs	0.18 (-0.003, 6.86)		0.068 (-0.01, 2.43)	0.001	0.0 (-0.13, 0.89)	0.26
Log % AMA-1specific ASCs/total ASCs	0.028 (-0.05, 0.35)	0.007	0.029 (-0.05, 0.16)	0.05	0.005 (-0.215, 0.074)	0.55

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.

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).

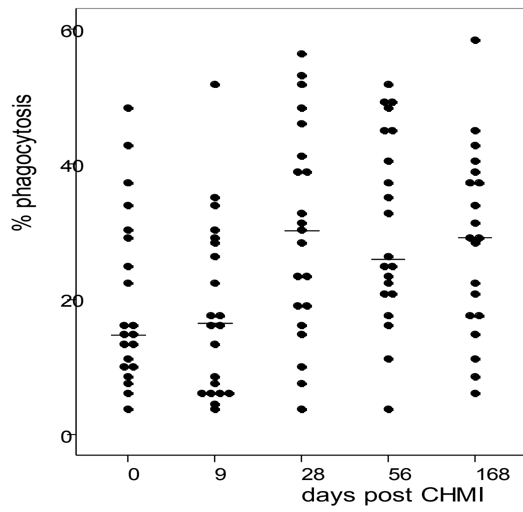


Figure 3A

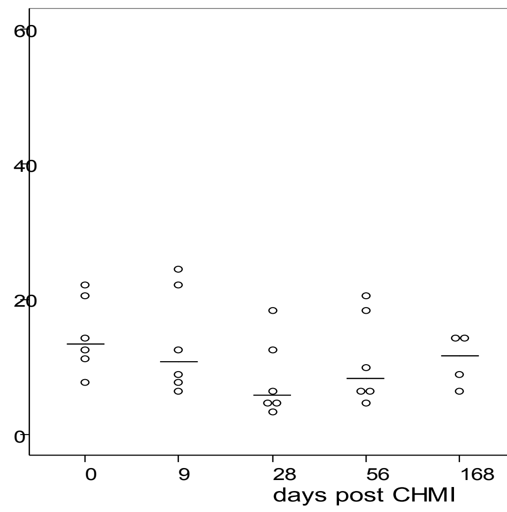


Figure 3B

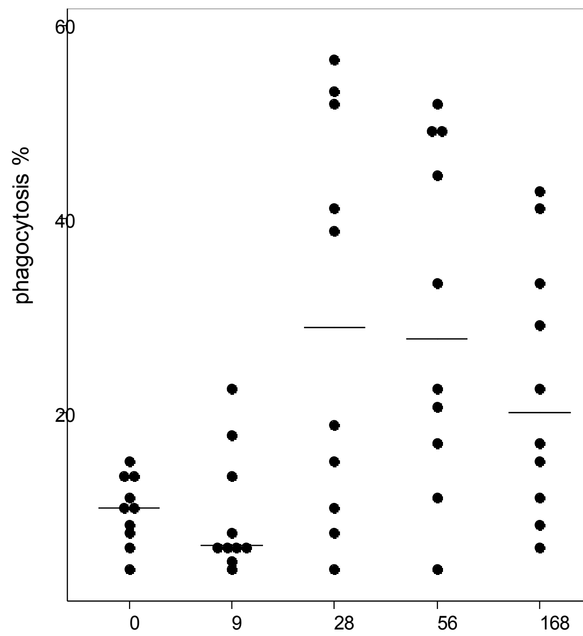


Figure 3C

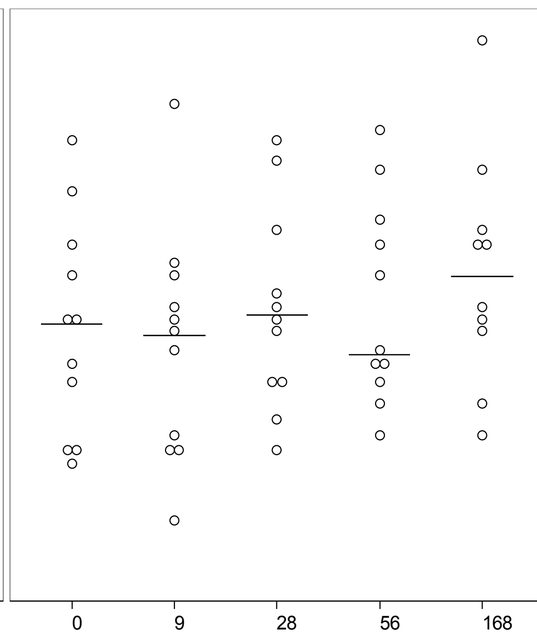


Figure 3D

Figure 3:Opsonization of merozoites increases after intradermal *Pf*SPZ 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, 56and 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.

	Median difference (90% central range)	p-value
All Volunteers combined		
Day 9 vs day 0	-1.7 (-7.4, 4.8)	0.12
Day 28 vs day 0	0.07 (-10.7, 40.5)	0.26
Day 56 vs day 0	0.5 (-6.5, 38.1)	0.12
Day 168 vs day 0	3.0 (-8.2, 32.6)	0.11
Low baseline responders		
Day 9 vs day 0	-1.46 (-5.96, 5.96)	0.49
Day 28 vs day 0	17.04 (-1.5, 45.31)	0.01
Day 56 vs day 0	18.91 (-1.33, 40.18)	0.01
Day 168 vs day 0	12.05 (-0.93, 31.37)	0.001
High baseline responders		
Day 9 vs day 0	-2.08 (-7.85, 4.36)	0.28
Day 28 vs day 0	0.07 (-12.89, 18.23)	0.58
Day 56 vs day 0	0.53 (-7.11, 19.36)	0.70
Day 168 vs day 0	3.46 (-9.73, 27.13)	0.70

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-1specific responses were comparable to the MSP-1specific 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 *Pf*SPZ challenge (Supplementary Figure4B).

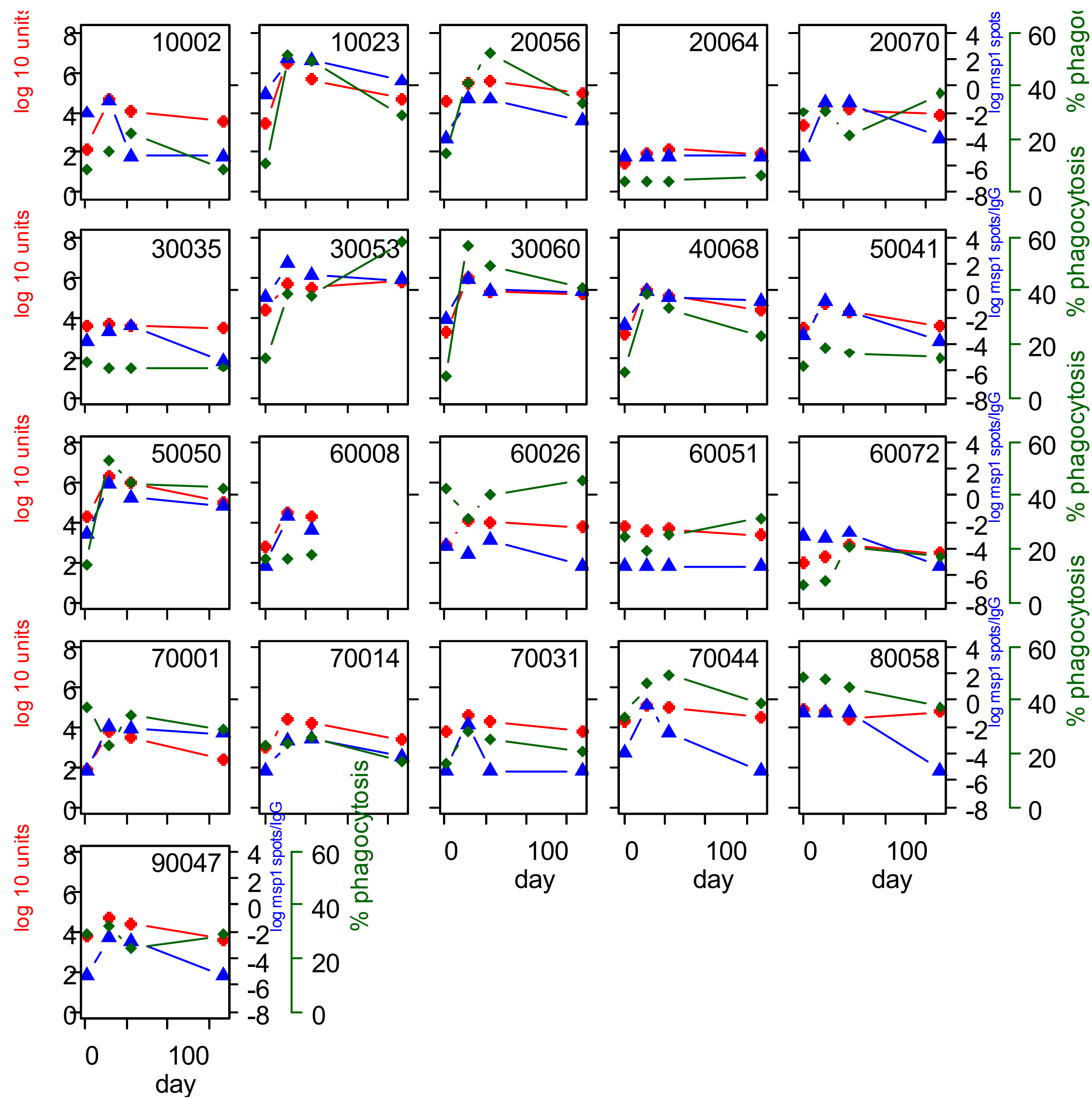


Figure 4:

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 *Pf*SPZ challenge

PAPER VIII

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

The article has been published in the Journal of Immunology (2017). Doi: 10.1056/NEJMoa1208394

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

Maxmillian Mpina,*,†,1 Nicholas J. Maurice,‡,1 Masanao Yajima,‡,§,1 Chloe K. Slichter,‡,¶,1 Hannah W. Miller,‡,1 Mukta Dutta,‡ M. Juliana McElrath,‡,¶ Kenneth D. Stuart,‖ Stephen C. De Rosa,‡ John P. McNevin,‡ Peter S. Linsley,# Salim Abdulla, Marcel Tanner,*,† Stephen L. Hoffman,†† Raphael Gottardo,‡ Claudia A. Daubenberger,*,† and Martin Prlic‡,¶**

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.
***The Journal of Immunology*, 2017, 199: 000–000.**

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

*Clinical Immunology Unit, Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, 4002 Basel, Switzerland; †University of Basel, Basel, 4001 Switzerland; ‡Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109; §Department of Mathematics and Statistics, Boston University, Boston, MA 02215; ¶Department of Global Health, University of Washington, Seattle, WA 98195; ‖Center for Infectious Disease Research, Seattle, WA 98109; #Benaroya Research Institute, Seattle, WA 98101; **Ifakara Health Institute, Bagamoyo Research and Training Centre, Bagamoyo, Tanzania; and ††Sanaria Inc., Rockville, MD 20850

[1]M.M., N.J.M., M.Y., C.K.S., and H.W.M. contributed equally to this work.

ORCIDs: 0000-0002-9300-6430 (M.M.); 0000-0002-2667-472X (N.J.M.); 0000-0002-9893-9256 (J.P.M.); 0000-0002-8960-4307 (P.S.L.); 0000-0002-0700-9505 (S.L.H.); 0000-0001-7136-0642 (C.A.D.); 0000-0002-0685-9321 (M.P.).

Received for publication November 23, 2016. Accepted for publication May 1, 2017.

This work was supported by National Institutes of Health Grants DP2 DE023321 (to M.P.), 2T32AI007509-16 (to C.K.S.), 1U19AI089986 (to K.D.S.), and U01 AI089859-05 (Pilot Grant to M.P.) and by the Swiss Tropical and Public Health Institute (to C.A.D.). The development, manufacturing, and quality-control release and stability studies of the Sanaria *Plasmodium falciparum* sporozoite challenge were supported in part by a National Institute of Allergy and Infectious Diseases Small Business Innovation Research Grant (5R44AI058375).

Address correspondence and reprint requests to Dr. Claudia A. Daubenberger or Dr. Martin Prlic, Swiss Tropical and Public Health Institute, Clinical Immunology, Socinstrasse 57, CH-4002 Basel, Switzerland (C.A.D.) or Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N, E5-110, P.O. Box 19024, Seattle, WA 98109 (M.P.). E-mail addresses: Claudia.Daubenberger@unibas.ch (C.A.D.) or mprlic@fhcrc.org (M.P.)

The online version of this article contains supplemental material.

Abbreviations used in this article: CHMI, controlled human malaria infection; FDR, false discovery rate; grzB, granzyme B; ID, identifier; iNKT, invariant NKT; IRB, institutional review board; 10K, 1×10^4; 25K, 2.5×10^4; MAIT, mucosal-associated invariant T; PfSPZ, *P. falciparum* sporozoite; RNAseq, RNA-sequencing.

Copyright © 2017 by The American Association of Immunologists, Inc. 0022-1767/17/$30.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1601989

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 ($CD3^+$ $CD161^{hi}$ $V\alpha7.2^+$) 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 (Vα7.2) 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 ($CD3^+$ $V\alpha24J\alpha18^+$) 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 V β 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 granzyme 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 PfSPZs (PfSPZ challenge) (42). We found that CHMI significantly affected the NK and 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 these 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/Vol.IX/1217), 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.57/180/04A/50), and the trial was registered at https://clinicaltrials.gov/ (NCT01540903).

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 2.5×10^4 (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 in liquid nitrogen and then 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 ($CD3^+CD8^+CD161^{hi}V\alpha7.2^+$) cells. 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 a HiSeq2500 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 R for 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 to 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 false discovery rate (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 1×10^4 (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 ($CD3^+V\alpha7.2^+CD161^{hi}$), NK cells ($CD3^-CD56^+$), and iNKT cells ($CD3^+V\alpha24J\alpha18^+$) 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

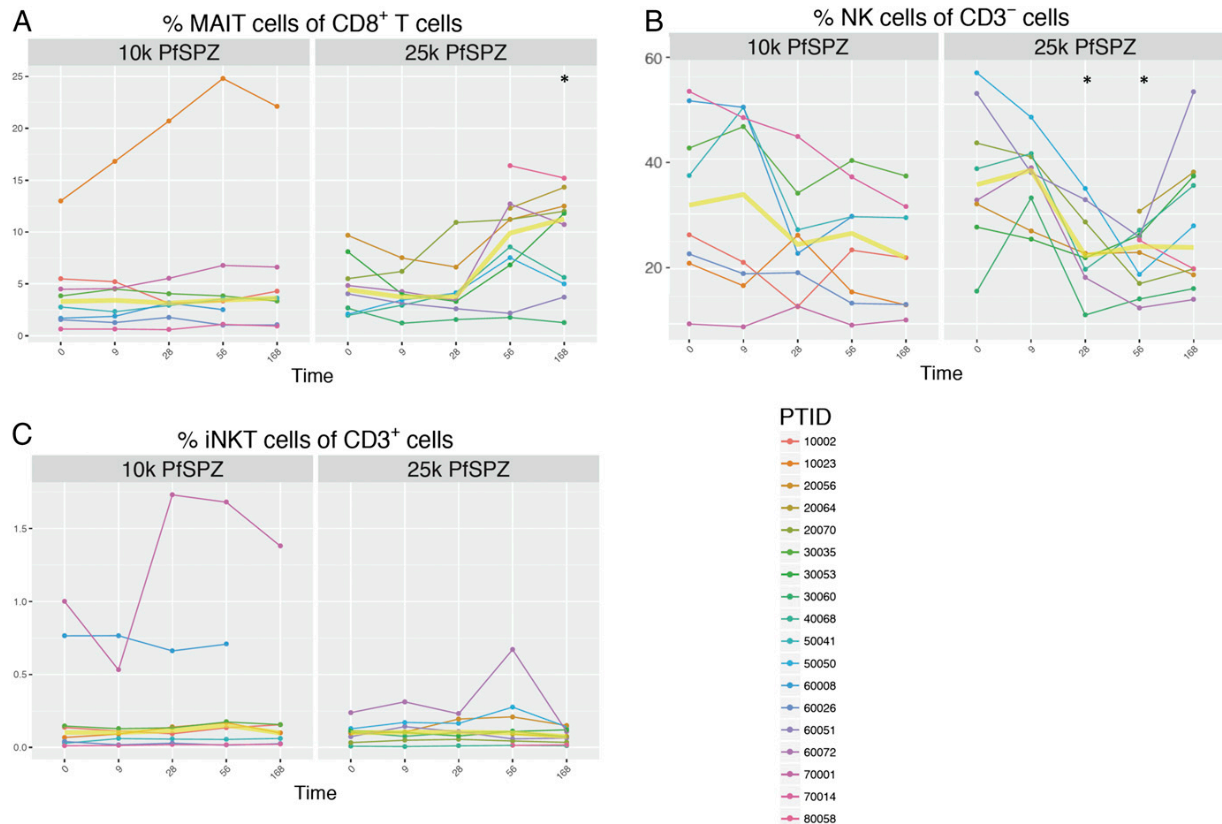


FIGURE 1. Changes in the innate-like T lymphocyte and NK cell compartment following intradermal injection of PfSPZs. Changes in the frequency of MAIT cells within the $CD8^+$ T cell population (**A**), the NK cell population (shown as frequency in $CD3^-$ lymphocyte population) (**B**), and the iNKT cell population (**C**) are shown for the low-dose challenge group (10k PfSPZ, left panels) and the high-dose challenge group (25k PfSPZ, right panels). The yellow line in each graph represents the median. Volunteer IDs are listed, with each volunteer depicted in a distinct color. Significant changes at time points following PfSPZ injection (compared with day 0) are indicated with an asterisk (*). Statistical significance comparing control and treated subjects was done using 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.

($p = 0.0174$) 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 PfSPZ 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 PfSPZ infection

We examined the frequency of MAIT cells ($CD3^+V\alpha7.2^+CD161^{hi}$; $CD8^+$ and $CD8^-$ subsets) on days 0 and 9 postinfection, 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 ($CD8^+$

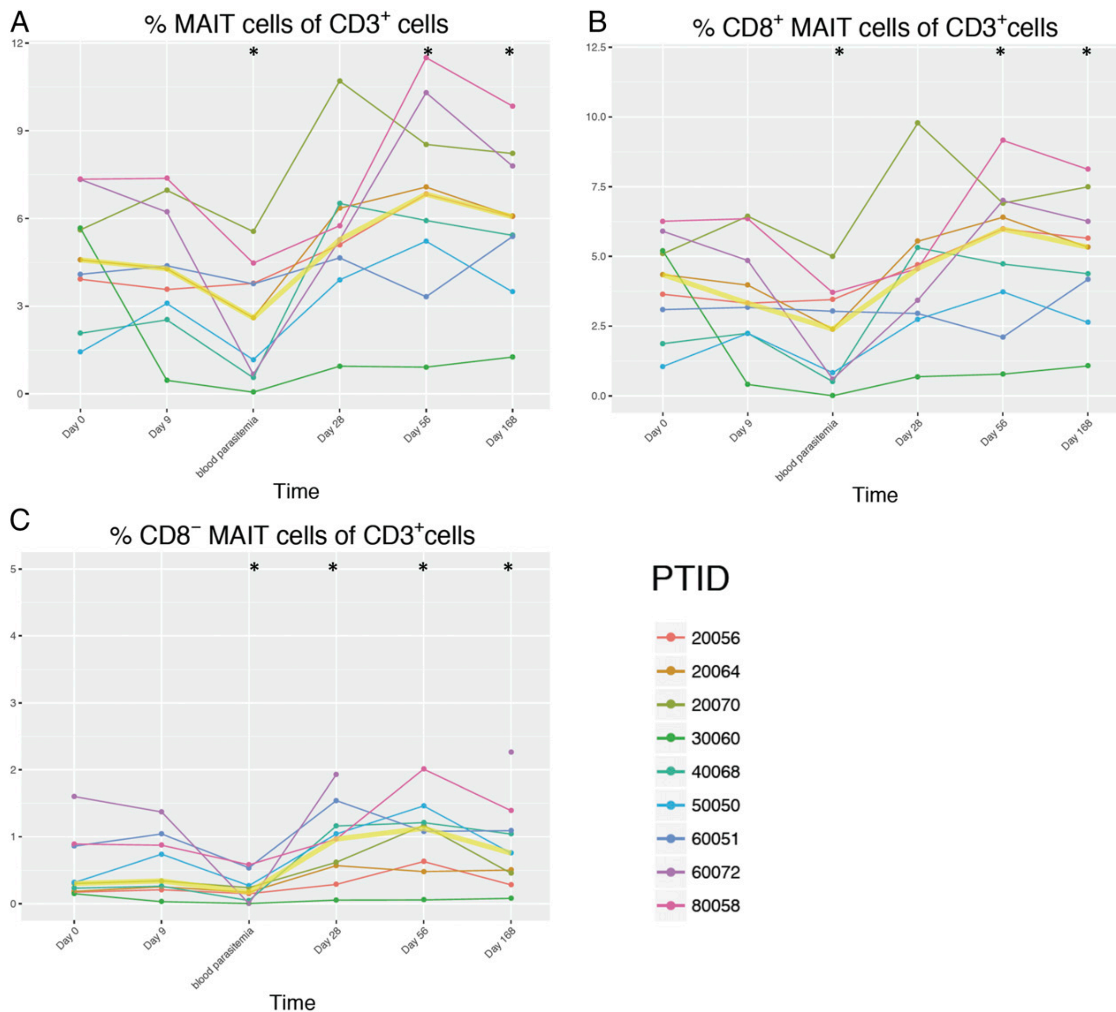


FIGURE 2. MAIT cell frequency is altered following intradermal injection with PfSPZs. Shown are changes in the frequency of MAIT cells over time for each individual volunteer. Significant changes are indicated with an asterisk (*), and p values were determined using a t test. (**A**) Percentage of MAIT cells in the $CD3^+$ population in the blood in the 25K PfSPZ 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 ($p = 0.0072$). Frequency of $CD8^+$ MAIT cells (**B**) and $CD8^-$ MAIT cells (**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, Fig. 2A). This pattern was also observed when the MAIT 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-α, IL-17, IFN-γ, 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-γ, grzB, and IL-17A/F

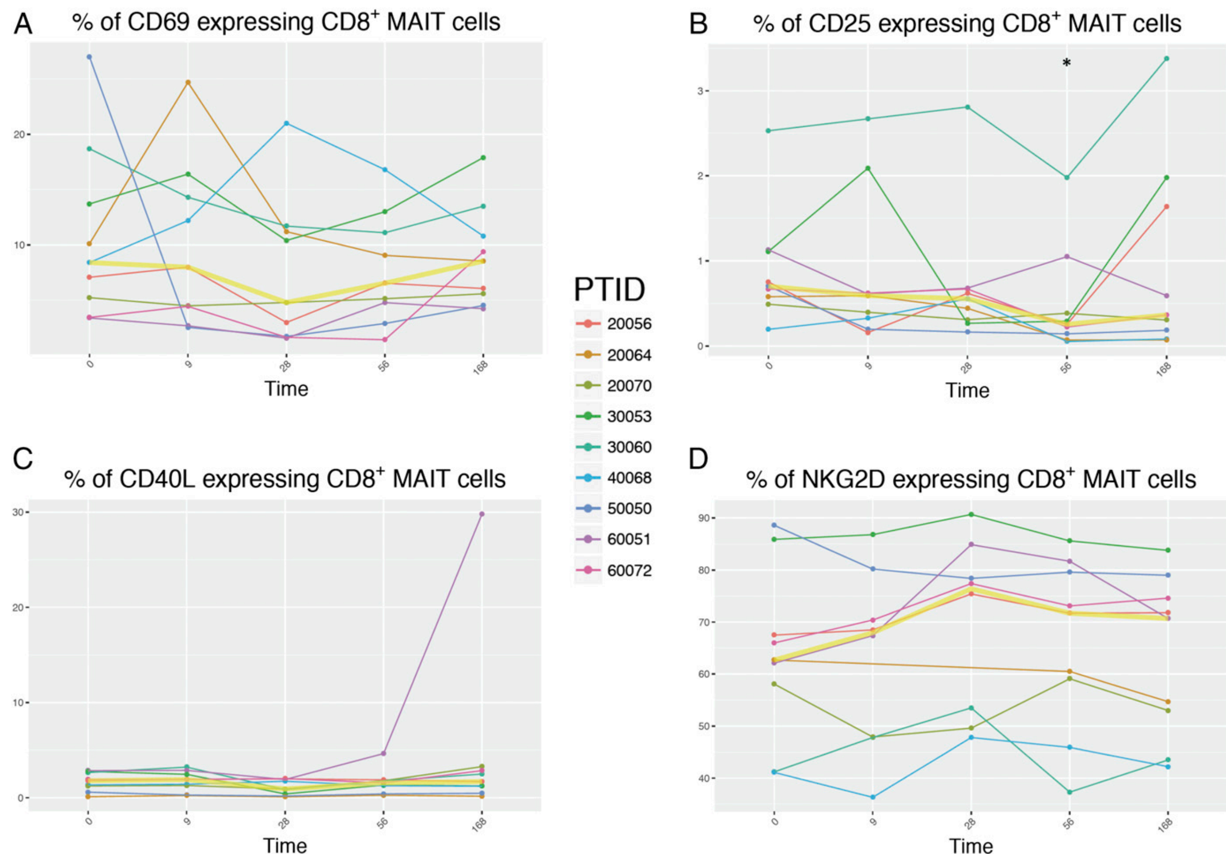


FIGURE 3. Limited MAIT cell activation early postinfection. Expression frequency of CD69 (**A**), CD25 (**B**), CD40L (**C**), and NKG2D (**D**) is shown for CD8$^+$ MAIT cells in the high-dose (25K PfSPZ) group. Significant changes compared with day 0 are indicated with an asterisk (*). Statistical 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. The yellow line in each graph represents the median. Volunteer IDs are listed, with each volunteer depicted in a distinct color.

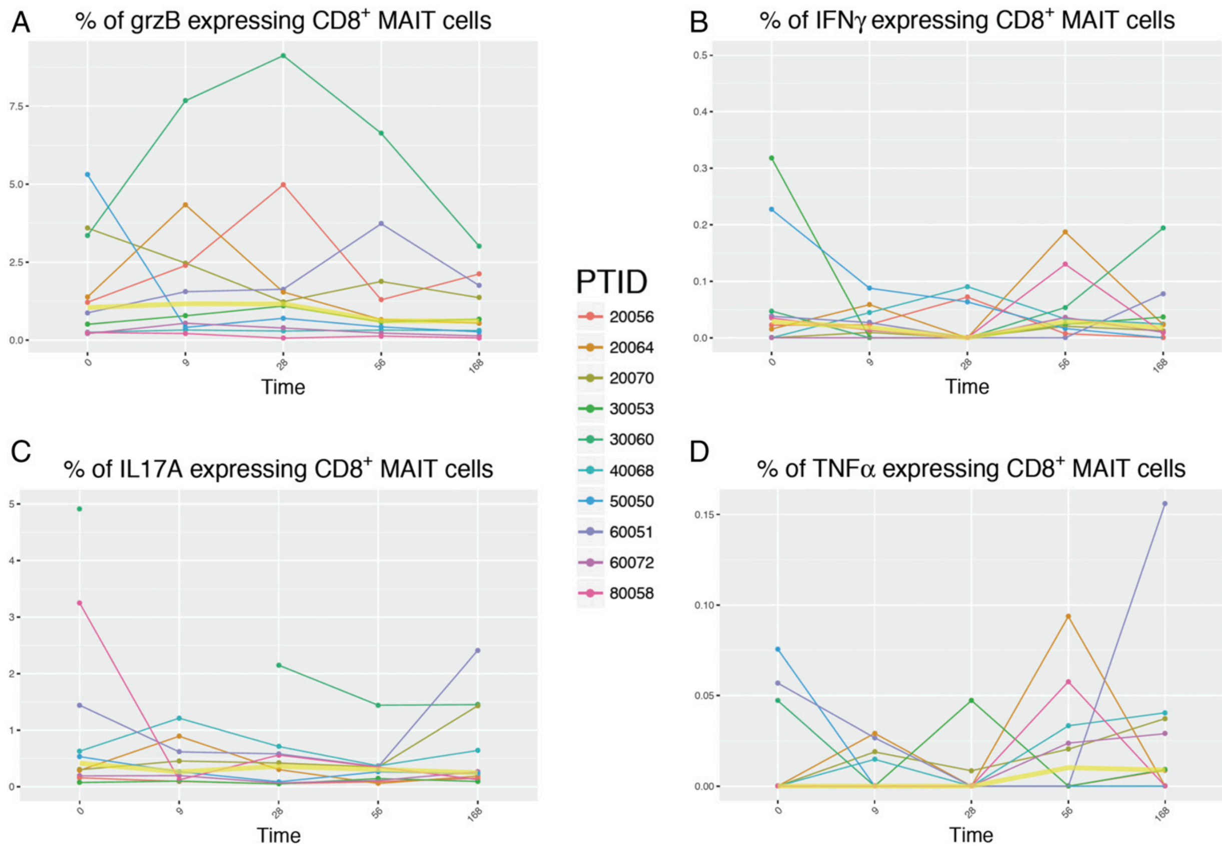


FIGURE 4. Minimal MAIT cell effector function early postinfection. Expression of grzB (**A**), IFN-γ (**B**), IL-17A (**C**), and TNF-α (**D**) on both MAIT cell subsets was measured in the 25K PfSPZ cohort. The yellow line in each graph represents the median. Statistical significance comparing control 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, but no significant changes were detected. Volunteer IDs are listed, with each volunteer depicted in a distinct color.

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 RNAseq.

Single-cell RNAseq 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 PfSPZ 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 found an increase in the coexpression 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.

Trafficking 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

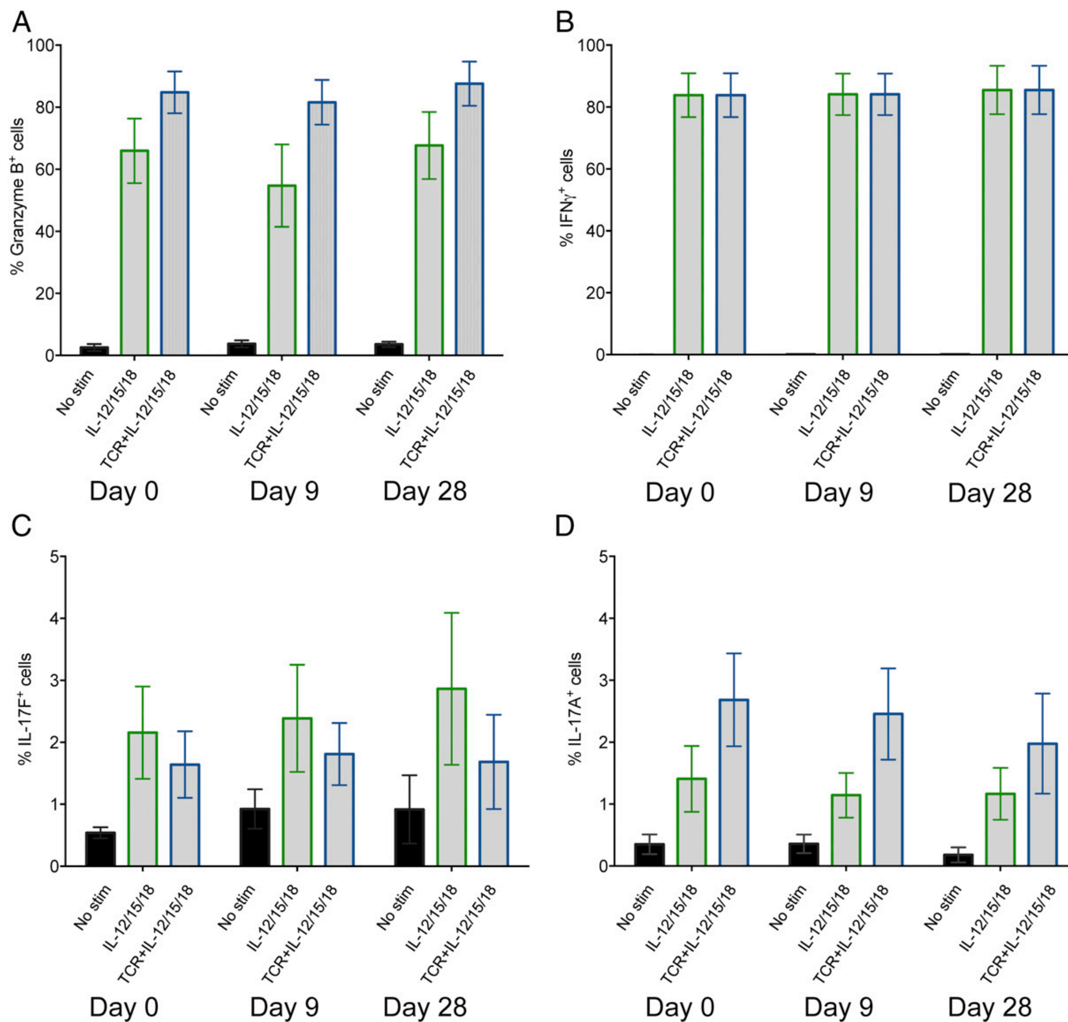


FIGURE 5. MAIT cells remain functional and responsive to stimulation ex vivo. FACS-purified CD8$^+$CD161^{hi}Vα7.2$^+$ cells were stimulated with cytokines (IL-12/15/18) or a combination of a TCR signal plus cytokines (TCR+IL-12/15/18), or were left unstimulated (No stim), for 24 h. Expression of grzB (**A**), IFN-γ (**B**), IL-17F (**C**), and IL-17A (**D**) was measured in five randomly chosen donors on days 0, 9, and 28. MAIT cells responded to each stimulation condition, but we did not detect significant changes when comparing MAIT cell responses to cytokine IL-12/15/18 (green bars) or TCR+IL-12/15/18 (blue bars) stimulation between the different time points (days 0, 9, and 28).

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, purified, 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

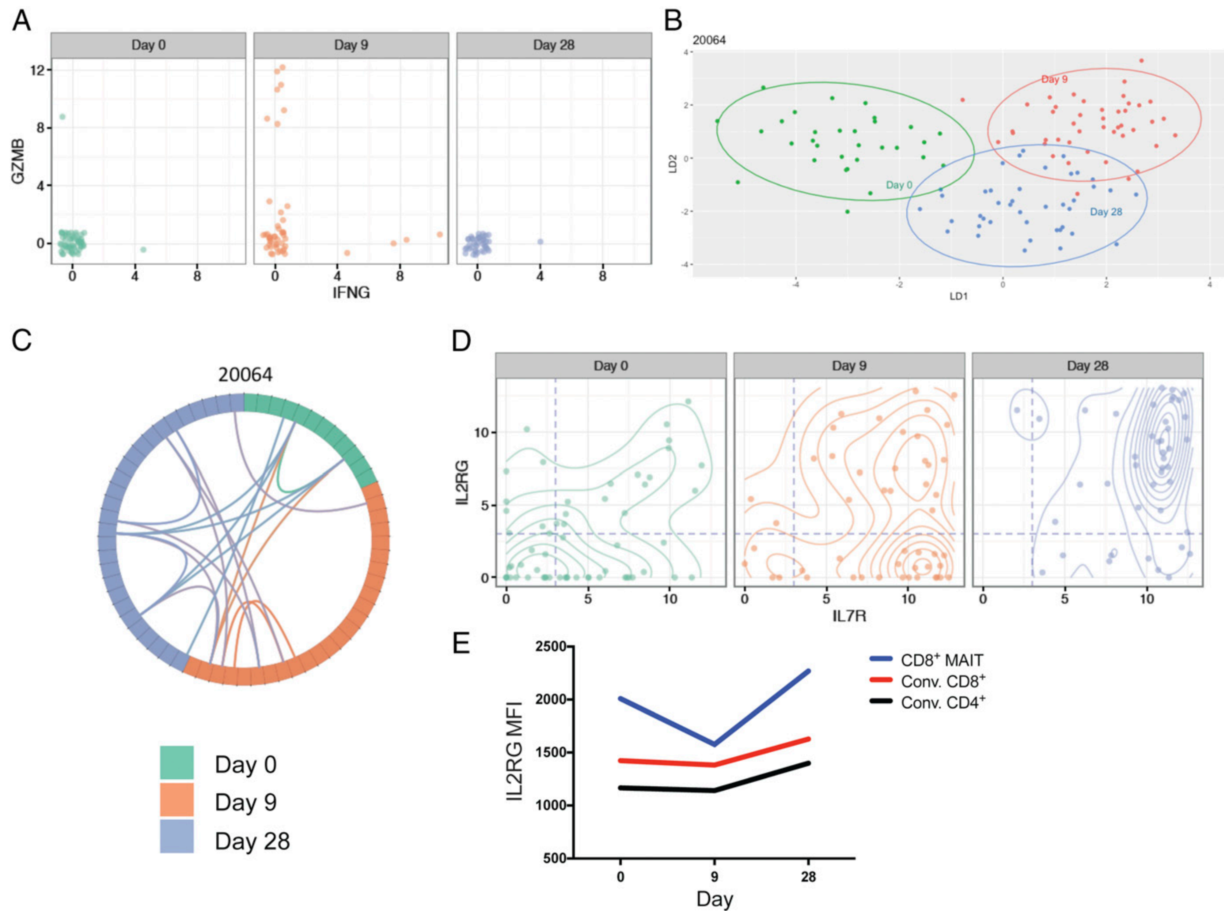


FIGURE 6. Single-cell RNAseq analysis of MAIT cells reveals partial activation and changes in the composition of the MAIT cell population. $CD8^+$ MAIT cells were sorted by FACS for single-cell RNAseq expression analysis by the Fluidigm C1 system. Cells sorted from donor 20064 on days 0, 9, and 28 are depicted in green, orange, and blue, respectively. (**A**) IFN-γ and grzB message are detected in some MAIT cells on day 9 postinfection but not on days 0 and 28. (**B**) Linear discriminant analysis of single MAIT cells sorted on days 0, 9, and 28 postsporozoite challenge. (**C**) Clonality in the MAIT cell population was examined by analyzing CDR3 usage from single cells by reconstructing their TCRα- and TCRβ-chains. Segments in the circle represent individual cells yielding a TCR sequence. Arcs connect cells sharing TCR clonotypes (TRA and/or TRB V gene, J gene, and CDR3 junction). (**D**) Changes in mRNA coexpression of IL-7Rα and cγ-chain (CD132) are shown in a contour plot. (**E**) The surface expression of CD132, measured by flow cytometry, increases on day 28, validating the single-cell RNAseq data [donor 20064 was used for (A–E)].

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 cells bind to and eliminate infected RBCs using cell contact–dependent mechanisms (53). Additional human studies will be required to determine which aspects of the mouse model system can be used to mimic 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 ex vivo 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

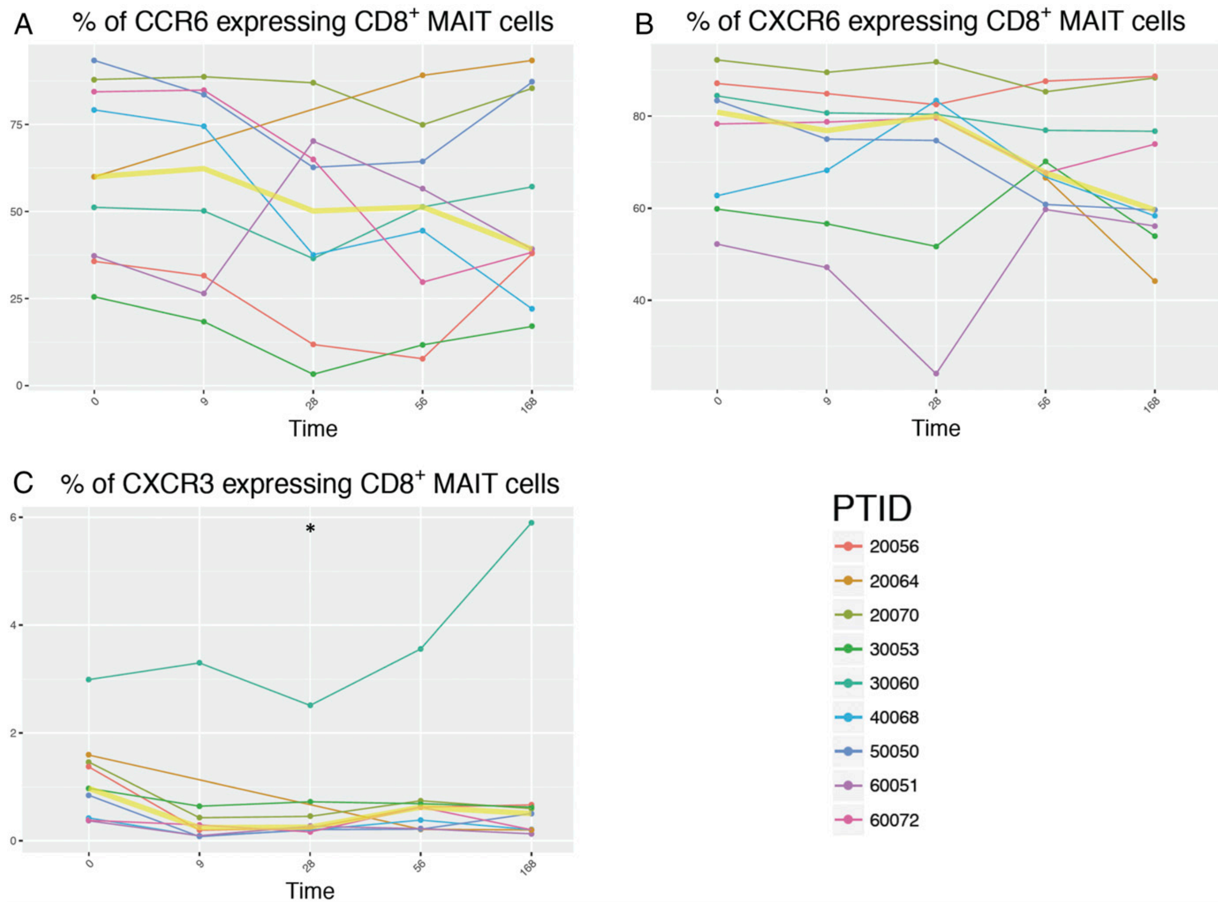


FIGURE 7. Changes in chemokine receptor expression months after the infection. The frequency of CCR6-expressing (**A**), CXCR6-expressing (**B**), and CXCR3-expressing (**C**) $CD8^+$ MAIT cells was determined by FACS in the 25K PfSPZ cohort. Statistical significance comparing control and treated subjects was done using Type II ANOVA for a linear mixed-effect model. Additional comparison of time points with each other was done using a paired *t* test with FDR. Significant changes are indicated with an asterisk (*), and *p* values were determined using a *t* test. The yellow line in each graph represents the median.

(Fig. 4). 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 cγ 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 nonredundant 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 malaria can affect immune responses against EBV (63–65). Finally, MAIT cells have been suggested to play a role in antibacterial immunity through sensing of MHC-related protein 1–bound microbial products, but they appear quite sensitive to inflammatory cues in the absence of Ag. Thus, MAIT cells may act as an amplifier of early proinflammatory signals, and an increase in MAIT cells may lead to a more profound subsequent proinflammatory response. In the context of malaria, these changes in the early immune response to the parasite may be beneficial and contribute to clearance, and they may also exacerbate 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).

Acknowledgments

We thank the James B. Pendleton Charitable Trust for their generous equipment donation. We thank Daryl Morris for a preliminary statistical analysis of the data and Gabriela Diaz for assistance with sample preparation. We thank Vivian Gersuk, Kimberly O'Brien, and Quynh-Anh Nguyen for excellent technical support for the single-cell RNAseq experiments. We thank all volunteers and staff of the Ifakara Health Institute, Bagamoyo Research and Training Centre, Sanaria Inc., and Swiss Tropical and Public Health Institute who contributed so much to the successful conduct of the CHMI.

Disclosures

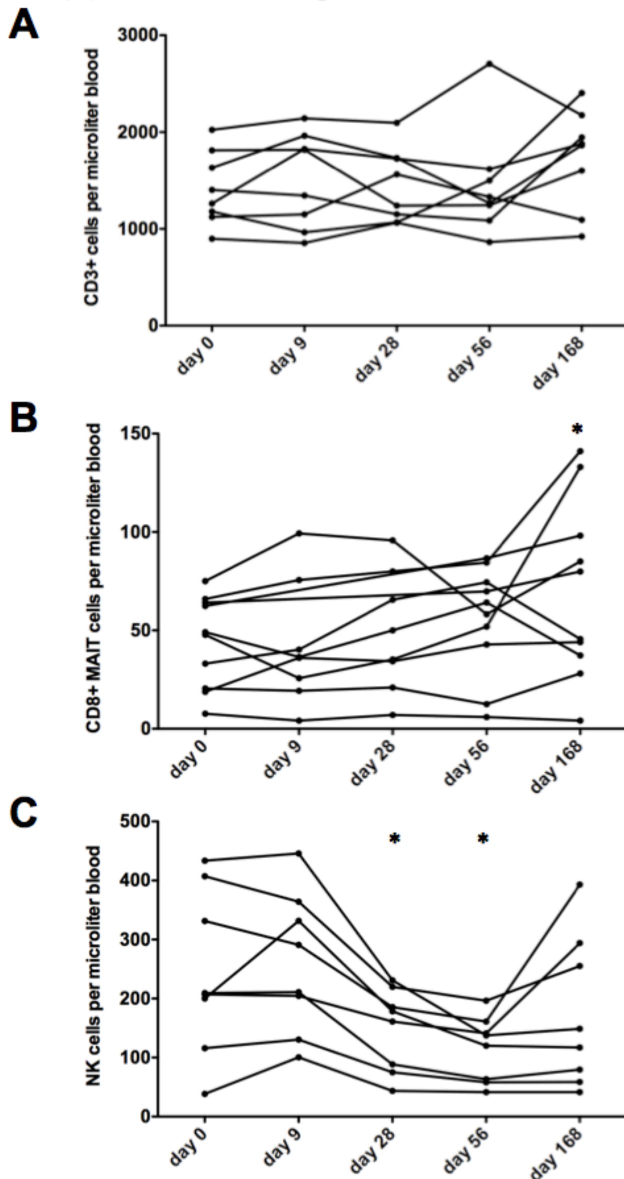
S.L.H. is associated with Sanaria Inc., who manufactured the PfSPZ challenge, and thus has potential conflicts of interest. The other authors have no financial conflicts of interest.

References

1. World Health Organization. 2015. *World Malaria Report 2015*. WHO Press, Geneva, Switzerland.
2. Epstein, J. E., K. M. Paolino, T. L. Richie, M. Sedegah, A. Singer, A. J. Ruben, S. Chakravarty, A. Stafford, R. C. Ruck, A. G. Eappen, et al. 2017. Protection against *Plasmodium falciparum* malaria by PfSPZ Vaccine. *JCI Insight* 2: e89154.
3. Ishizuka, A. S., K. E. Lyke, A. DeZure, A. A. Berry, T. L. Richie, F. H. Mendoza, M. E. Enama, I. J. Gordon, L. J. Chang, U. N. Sarwar, et al. 2016. Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. *Nat. Med.* 22: 614–623.
4. Lyke, K. E., A. S. Ishizuka, A. A. Berry, S. Chakravarty, A. DeZure, M. E. Enama, E. R. James, P. F. Billingsley, A. Gunasekera, A. Manoj, et al. 2017. Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection. *Proc. Natl. Acad. Sci. USA* 114: 2711–2716.
5. Mordmüller, B., G. Surat, H. Lagler, S. Chakravarty, A. S. Ishizuka, A. Lalremruata, M. Gmeiner, J. J. Campo, M. Esen, A. J. Ruben, et al. 2017. Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. *Nature* 542: 445–449.
6. Sissoko, M. S., S. A. Healy, A. Katile, F. Omaswa, I. Zaidi, E. E. Gabriel, B. Kamate, Y. Samake, M. A. Guindo, A. Dolo, et al. 2017. Safety and efficacy of PfSPZ Vaccine against *Plasmodium falciparum* via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial. *Lancet Infect. Dis.* 17: 498–509.
7. Seder, R. A., L. J. Chang, M. E. Enama, K. L. Zephir, U. N. Sarwar, I. J. Gordon, L. A. Holman, E. R. James, P. F. Billingsley, A. Gunasekera, et al; VRC 312 Study Team. 2013. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. *Science* 341: 1359–1365.
8. Crompton, P. D., J. Moebius, S. Portugal, M. Waisberg, G. Hart, L. S. Garver, L. H. Miller, C. Barillas-Mury, and S. K. Pierce. 2014. Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annu. Rev. Immunol.* 32: 157–187.
9. Doolan, D. L., and S. L. Hoffman. 2000. The complexity of protective immunity against liver-stage malaria. *J. Immunol.* 165: 1453–1462.
10. Hansen, D. S., M. C. D'Ombrain, and L. Schofield. 2007. The role of leukocytes bearing natural killer complex receptors and killer immunoglobulin-like receptors in the immunology of malaria. *Curr. Opin. Immunol.* 19: 416–423.
11. Stevenson, M. M., and E. M. Riley. 2004. Innate immunity to malaria. *Nat. Rev. Immunol.* 4: 169–180.
12. Doherty, D. G., and C. O'Farrelly. 2000. Innate and adaptive lymphoid cells in the human liver. *Immunol. Rev.* 174: 5–20.
13. Orr, M. T., and L. L. Lanier. 2010. Natural killer cell education and tolerance. *Cell* 142: 847–856.
14. Dusseaux, M., E. Martin, N. Serriari, I. Péguillet, V. Premel, D. Louis, M. Milder, L. Le Bourhis, C. Soudais, E. Treiner, and O. Lantz. 2011. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood* 117: 1250–1259.
15. Jo, J., A. T. Tan, J. E. Ussher, E. Sandalova, X. Z. Tang, A. Tan-Garcia, N. To, M. Hong, A. Chia, U. S. Gill, et al. 2014. Toll-like receptor 8 agonist and

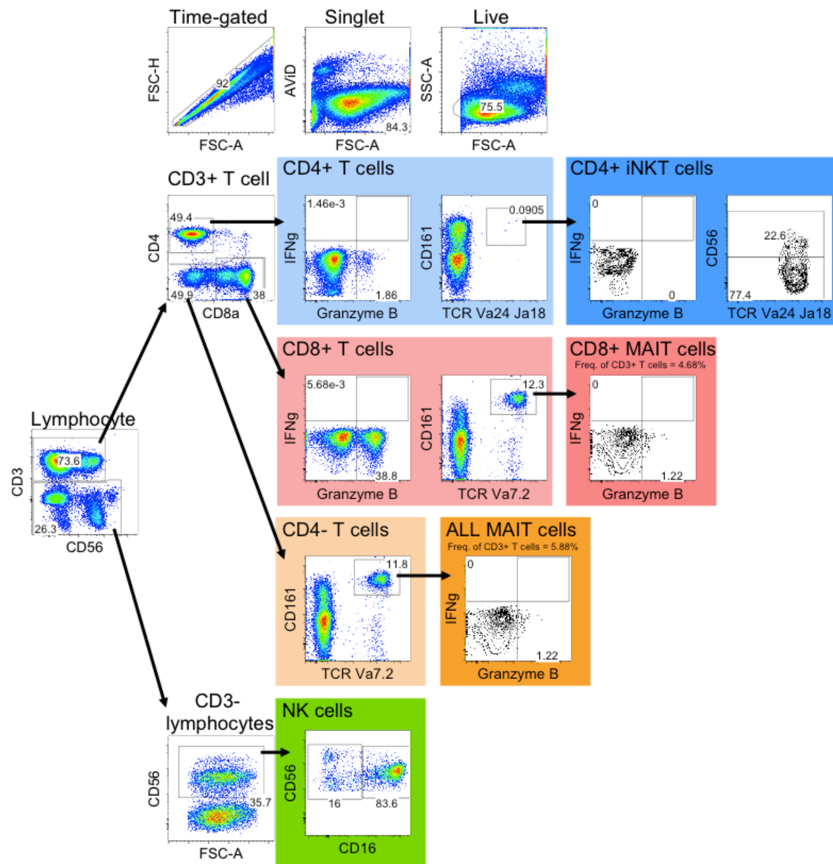
bacteria trigger potent activation of innate immune cells in human liver. *PLoS Pathog.* 10: e1004210.
16. Porcelli, S., C. E. Yockey, M. B. Brenner, and S. P. Balk. 1993. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J. Exp. Med.* 178: 1–16.
17. Treiner, E., L. Duban, S. Bahram, M. Radosavljevic, V. Wanner, F. Tilloy, P. Affaticati, S. Gilfillan, and O. Lantz. 2003. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* 422: 164–169.
18. Gold, M. C., J. E. McLaren, J. A. Reistetter, S. Smyk-Pearson, K. Ladell, G. M. Swarbrick, Y. Y. Yu, T. H. Hansen, O. Lund, M. Nielsen, et al. 2014. MR1-restricted MAIT cells display ligand discrimination and pathogen selectivity through distinct T cell receptor usage. *J. Exp. Med.* 211: 1601–1610.
19. Eckle, S. B., R. W. Birkinshaw, L. Kostenko, A. J. Corbett, H. E. McWilliam, R. Reantragoon, Z. Chen, N. A. Gherardin, T. Beddoe, L. Liu, et al. 2014. A molecular basis underpinning the T cell receptor heterogeneity of mucosal-associated invariant T cells. *J. Exp. Med.* 211: 1585–1600.
20. Lepore, M., A. Kalinichenko, A. Colone, B. Paleja, A. Singhal, A. Tschumi, B. Lee, M. Poidinger, F. Zolezzi, L. Quagliata, et al. 2014. Parallel T-cell cloning and deep sequencing of human MAIT cells reveal stable oligoclonal TCRβ repertoire. [Published erratum appears in 2014 *Nat. Commun.* 5: 4493.] *Nat. Commun.* 5: 3866.
21. Kjer-Nielsen, L., O. Patel, A. J. Corbett, J. Le Nours, B. Meehan, L. Liu, M. Bhati, Z. Chen, L. Kostenko, R. Reantragoon, et al. 2012. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* 491: 717–723.
22. Slichter, C. K., A. McDavid, H. W. Miller, G. Finak, B. J. Seymour, J. P. McNevin, G. Diaz, J. L. Czartoski, M. J. McElrath, R. Gottardo, et al. 2016. Distinct activation thresholds of human conventional and innate-like memory T cells. *JCI Insight* 1: e86292.
23. Finak, G., A. McDavid, M. Yajima, J. Deng, V. Gersuk, A. K. Shalek, C. K. Slichter, H. W. Miller, M. J. McElrath, M. Prlic, et al. 2015. MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data. *Genome Biol.* 16: 278.
24. Ussher, J. E., M. Bilton, E. Attwod, J. Shadwell, R. Richardson, C. de Lara, E. Mettke, A. Kurioka, T. H. Hansen, P. Klenerman, and C. B. Willberg. 2014. CD161++ CD8+ T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner. *Eur. J. Immunol.* 44: 195–203.
25. Kenna, T., L. Golden-Mason, S. A. Porcelli, Y. Koezuka, J. E. Hegarty, C. O'Farrelly, and D. G. Doherty. 2003. NKT cells from normal and tumor-bearing human livers are phenotypically and functionally distinct from murine NKT cells. [Published erratum appears in 2003 *J. Immunol.* 171: 5631.] *J. Immunol.* 171: 1775–1779.
26. Chandra, S., and M. Kronenberg. 2015. Activation and function of iNKT and MAIT cells. *Adv. Immunol.* 127: 145–201.
27. Prlic, M., and T. M. Hohl. 2011. iNKTs foil fungi. *Cell Host Microbe* 10: 421–422.
28. Schofield, L., J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, and V. Nussenzweig. 1987. Gamma interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites. *Nature* 330: 664–666.
29. McCall, M. B., and R. W. Sauerwein. 2010. Interferon-γ–central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. *J. Leukoc. Biol.* 88: 1131–1143.
30. Pombo, D. J., G. Lawrence, C. Hirunpetcharat, C. Rzepczyk, M. Bryden, N. Cloonan, K. Anderson, Y. Mahakunkijcharoen, L. B. Martin, D. Wilson, et al. 2002. Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum. Lancet* 360: 610–617.
31. Roestenberg, M., A. C. Teirlinck, M. B. McCall, K. Teelen, K. N. Makamdop, J. Wiersma, T. Arens, P. Beckers, G. van Gemert, M. van de Vegte-Bolmer, et al. 2011. Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. *Lancet* 377: 1770–1776.
32. Roestenberg, M., M. McCall, J. Hopman, J. Wiersma, A. J. Luty, G. J. van Gemert, M. van de Vegte-Bolmer, B. van Schaijk, K. Teelen, T. Arens, et al. 2009. Protection against a malaria challenge by sporozoite inoculation. *N. Engl. J. Med.* 361: 468–477.
33. Dodoo, D., F. M. Omer, J. Todd, B. D. Akanmori, K. A. Koram, and E. M. Riley. 2002. Absolute levels and ratios of proinflammatory and anti-inflammatory cytokine production in vitro predict clinical immunity to *Plasmodium falciparum* malaria. *J. Infect. Dis.* 185: 971–979.
34. D'Ombrain, M. C., L. J. Robinson, D. I. Stanisic, J. Taraika, N. Bernard, P. Michon, I. Mueller, and L. Schofield. 2008. Association of early interferon-gamma production with immunity to clinical malaria: a longitudinal study among Papua New Guinean children. *Clin. Infect. Dis.* 47: 1380–1387.
35. Luty, A. J., B. Lell, R. Schmidt-Ott, L. G. Lehman, D. Luckner, B. Greve, P. Matousek, K. Herbich, D. Schmid, F. Migot-Nabias, et al. 1999. Interferon-gamma responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. *J. Infect. Dis.* 179: 980–988.
36. Malaguarnera, L., S. Pignatelli, M. Musumeci, J. Simporè, and S. Musumeci. 2002. Plasma levels of interleukin-18 and interleukin-12 in *Plasmodium falciparum* malaria. *Parasite Immunol.* 24: 489–492.
37. Day, N. P., T. T. Hien, T. Schollaardt, P. P. Loc, L. V. Chuong, T. T. Chau, N. T. Mai, N. H. Phu, D. X. Sinh, N. J. White, and M. Ho. 1999. The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. *J. Infect. Dis.* 180: 1288–1297.
38. Lyke, K. E., R. Burges, Y. Cissoko, L. Sangare, M. Dao, I. Diarra, A. Kone, R. Harley, C. V. Plowe, O. K. Doumbo, and M. B. Sztein. 2004. Serum levels of the proinflammatory cytokines interleukin-1 beta (IL-1beta), IL-6, IL-8, IL-10, tumor necrosis factor alpha, and IL-12(p70) in Malian children with severe *Plasmodium falciparum* malaria and matched uncomplicated malaria or healthy controls. *Infect. Immun.* 72: 5630–5637.
39. Walther, M., J. Woodruff, F. Edele, D. Jeffries, J. E. Tongren, E. King, L. Andrews, P. Bejon, S. C. Gilbert, J. B. De Souza, et al. 2006. Innate immune responses to human malaria: heterogeneous cytokine responses to blood-stage *Plasmodium falciparum* correlate with parasitological and clinical outcomes. *J. Immunol.* 177: 5736–5745.
40. Kim, C. C., S. Parikh, J. C. Sun, A. Myrick, L. L. Lanier, P. J. Rosenthal, and J. L. DeRisi. 2008. Experimental malaria infection triggers early expansion of natural killer cells. *Infect. Immun.* 76: 5873–5882.
41. Hansen, D. S., N. J. Bernard, C. Q. Nie, and L. Schofield. 2007. NK cells stimulate recruitment of CXCR3+ T cells to the brain during *Plasmodium berghei*-mediated cerebral malaria. *J. Immunol.* 178: 5779–5788.
42. Shekalaghe, S., M. Rutaihwa, P. F. Billingsley, M. Chemba, C. A. Daubenberger, E. R. James, M. Mpina, O. Ali Juma, T. Schindler, E. Huber, et al. 2014. Controlled human malaria infection of Tanzanians by intradermal injection of aseptic, purified, cryopreserved *Plasmodium falciparum* sporozoites. *Am. J. Trop. Med. Hyg.* 91: 471–480.
43. Jiang, J., L. L. Lau, and H. Shen. 2003. Selective depletion of nonspecific T cells during the early stage of immune responses to infection. *J. Immunol.* 171: 4352–4358.
44. Prlic, M., L. Lefrancois, and S. C. Jameson. 2002. Multiple choices: regulation of memory CD8 T cell generation and homeostasis by interleukin (IL)-7 and IL-15. *J. Exp. Med.* 195: F49–F52.
45. Leeansyah, E., J. Svärd, J. Dias, M. Buggert, J. Nyström, M. F. Quigley, M. Moll, A. Sönnerborg, P. Nowak, and J. K. Sandberg. 2015. Arming of MAIT cell cytolytic antimicrobial activity is induced by IL-7 and defective in HIV-1 infection. *PLoS Pathog.* 11: e1005072.
46. Tang, X. Z., J. Jo, A. T. Tan, E. Sandalova, A. Chia, K. C. Tan, K. H. Lee, A. J. Gehring, G. De Libero, and A. Bertoletti. 2013. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J. Immunol.* 190: 3142–3152.
47. Germanov, E., L. Veinotte, R. Cullen, E. Chamberlain, E. C. Butcher, and B. Johnston. 2008. Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells. *J. Immunol.* 181: 81–91.
48. Jeffery, H. C., B. van Wilgenburg, A. Kurioka, K. Parekh, K. Stirling, S. Roberts, E. E. Dutton, S. Hunter, D. Geh, M. K. Braitch, et al. 2016. Biliary epithelium and liver B cells exposed to bacteria activate intrahepatic MAIT cells through MR1. *J. Hepatol.* 64: 1118–1127.
49. Yamazaki, T., X. O. Yang, Y. Chung, A. Fukunaga, R. Nurieva, B. Pappu, N. Martin-Orozco, H. S. Kang, L. Ma, A. D. Panopoulos, et al. 2008. CCR6 regulates the migration of inflammatory and regulatory T cells. *J. Immunol.* 181: 8391–8401.
50. Hickman, H. D., G. V. Reynoso, B. F. Ngudiankama, S. S. Cush, J. Gibbs, J. R. Bennink, and J. W. Yewdell. 2015. CXCR3 chemokine receptor enables local CD8(+) T cell migration for the destruction of virus-infected cells. *Immunity* 42: 524–537.
51. Scholzen, A., and R. W. Sauerwein. 2016. Immune activation and induction of memory: lessons learned from controlled human malaria infection with *Plasmodium falciparum. Parasitology* 143: 224–235.
52. Tsang, J. S., P. L. Schwartzberg, Y. Kotliarov, A. Biancotto, Z. Xie, R. N. Germain, E. Wang, M. J. Olnes, M. Narayanan, H. Golding, et al; Baylor HIPC Center; CHI Consortium. 2014. Global analyses of human immune variation reveal baseline predictors of postvaccination responses. [Published erratum appears in 2014 *Cell* 158: 226.] *Cell* 157: 499–513.
53. Chen, Q., A. Amaladoss, W. Ye, M. Liu, S. Dummler, F. Kong, L. H. Wong, H. L. Loo, E. Loh, S. Q. Tan, et al. 2014. Human natural killer cells control *Plasmodium falciparum* infection by eliminating infected red blood cells. *Proc. Natl. Acad. Sci. USA* 111: 1479–1484.
54. Teirlinck, A. C., M. B. McCall, M. Roestenberg, A. Scholzen, R. Woestenenk, Q. de Mast, A. J. van der Ven, C. C. Hermsen, A. J. Luty, and R. W. Sauerwein. 2011. Longevity and composition of cellular immune responses following experimental *Plasmodium falciparum* malaria infection in humans. *PLoS Pathog.* 7: e1002389.
55. Jameson, S. C. 2002. Maintaining the norm: T-cell homeostasis. *Nat. Rev. Immunol.* 2: 547–556.
56. Prlic, M., D. Kamimura, and M. J. Bevan. 2007. Rapid generation of a functional NK-cell compartment. *Blood* 110: 2024–2026.
57. Gold, M. C., R. J. Napier, and D. M. Lewinsohn. 2015. MR1-restricted mucosal associated invariant T (MAIT) cells in the immune response to *Mycobacterium tuberculosis. Immunol. Rev.* 264: 154–166.
58. Cosgrove, C., J. E. Ussher, A. Rauch, K. Gärtner, A. Kurioka, M. H. Hühn, K. Adelmann, Y. H. Kang, J. R. Fergusson, P. Simmonds, et al. 2013. Early and nonreversible decrease of CD161++ /MAIT cells in HIV infection. *Blood* 121: 951–961.
59. Wong, E. B., N. A. Akilimali, P. Govender, Z. A. Sullivan, C. Cosgrove, M. Pillay, D. M. Lewinsohn, W. R. Bishai, B. D. Walker, T. Ndung'u, et al. 2013. Low levels of peripheral CD161++CD8+ mucosal associated invariant T (MAIT) cells are found in HIV and HIV/TB co-infection. [Published erratum appears in 2014 *PLoS One* 9: e95115.] *PLoS One* 8: e83474.
60. Fernandez, C. S., T. Amarasena, A. D. Kelleher, J. Rossjohn, J. McCluskey, D. I. Godfrey, and S. J. Kent. 2015. MAIT cells are depleted early but retain functional cytokine expression in HIV infection. *Immunol. Cell Biol.* 93: 177–188.
61. Cui, Y., K. Franciszkiewicz, Y. K. Mburu, S. Mondot, L. Le Bourhis, V. Premel, E. Martin, A. Kachaner, L. Duban, M. A. Ingersoll, et al. 2015. Mucosal-

associated invariant T cell-rich congenic mouse strain allows functional evaluation. *J. Clin. Invest.* 125: 4171–4185.
62. Orange, J. S. 2013. Natural killer cell deficiency. *J. Allergy Clin. Immunol.* 132: 515–525, quiz 526.
63. Matar, C. G., N. T. Jacobs, S. H. Speck, T. J. Lamb, and A. M. Moormann. 2015. Does EBV alter the pathogenesis of malaria? *Parasite Immunol.* 37: 433–445.
64. Chattopadhyay, P. K., K. Chelimo, P. B. Embury, D. H. Mulama, P. O. Sumba, E. Gostick, K. Ladell, T. M. Brodie, J. Vulule, M. Roederer, et al. 2013. Holoendemic malaria exposure is associated with altered Epstein-Barr virus-specific CD8(+) T-cell differentiation. *J. Virol.* 87: 1779–1788.
65. Moormann, A. M., C. J. Snider, and K. Chelimo. 2011. The company malaria keeps: how co-infection with Epstein-Barr virus leads to endemic Burkitt lymphoma. *Curr. Opin. Infect. Dis.* 24: 435–441.
66. Moormann, A. M. 2009. How might infant and paediatric immune responses influence malaria vaccine efficacy? *Parasite Immunol.* 31: 547–559.



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 chance 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.

Supplemental Figure 2



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

Reagent	Fluor	Clone	Provider
LIVE/DEAD	V510	N/A	Invitrogen
α-CD3	BV786	SK7	BD
α-CD4	Ax700	RPA-T4	BD
α-CD8α	PerCP-Cy5.5	SK1	BD
α-CD45RO	BV650	UCHL1	BD
α-CD52	APC-H7	4C8	BD
α-CD95 (Fas)	PE-Cy7	DX2	BD
α-CD161	BV605	HP-3G10	BioLegend
α-CD178 (FasL)[°]	APC	NOK-1	BD
α-CD183 (CXCR3)	BUV395	1C6/CXCR3	BD
α-CD186 (CXCR6)[•]	Alexa Fluor 647	K041E5	BioLegend
α-CD196 (CCR6)	BV421	11A9	BD
α-CD314 (NKG2D)	BB515	1D11	BD
α-TCR Vα7.2	PE	3C10	BioLegend

Activation Panel

Reagent	Fluor	Clone	Provider
LIVE/DEAD	V510	N/A	Invitrogen
α-CD3	BV786	SK7	BD
α-CD4	Ax700	RPA-T4	BD
α-CD8α	PerCP-Cy5.5	SK1	BD
α-CD25 (IL2Rα)	BV421	M-A251	BD
α-CD45RO	BV650	UCHL1	BD
α-CD69	BUV395	FN50	BD
α-CD132 (γ_c)[°]	PE-CF594	TUGh4	BD
α-CD152 (CTLA4)[‡•]	PE-CF594	BNI3	BD
α-CD154 (CD40L)	APC	89-76	BD
α-CD161	BV605	HP-3G10	BioLegend
α-CD223 (Lag3)	FITC	3DS223H	eBioscience
α-CD279 (PD1)	PE-Cy7	EH12.1	BD
α-TCR Vα7.2	PE	3C10	BioLegend

ICS Panel

Reagent	Fluor	Clone	Provider
LIVE/DEAD	V510	N/A	Invitrogen
α-CD3	BV786	SK7	BD
α-CD4	APC-eFluor 780	RPA-T4	eBioscience
α-CD8α	PerCP-Cy5.5	SK1	BD
α-CD45RO	BV650	UCHL1	BD
α-CD161	BV605	HP-3G10	BioLegend
α-TCR Vα7.2	PE	3C10	BioLegend
α-IFNγ	BUV395	B27	BD
α-IL-2	BV421	MQ1-17H12	BD
α-IL-17A	PE-Cy7	BL168	BioLegend
α-IL-17F[‡•]	PE-Cy7	SHLR17	eBioscience
α-TNFα	FITC	MAb11	eBioscience
α-Granzyme B	Alexa Fluor 700	GB11	BD
α-Granzyme K	eFluor 660	G3H69	eBioscience

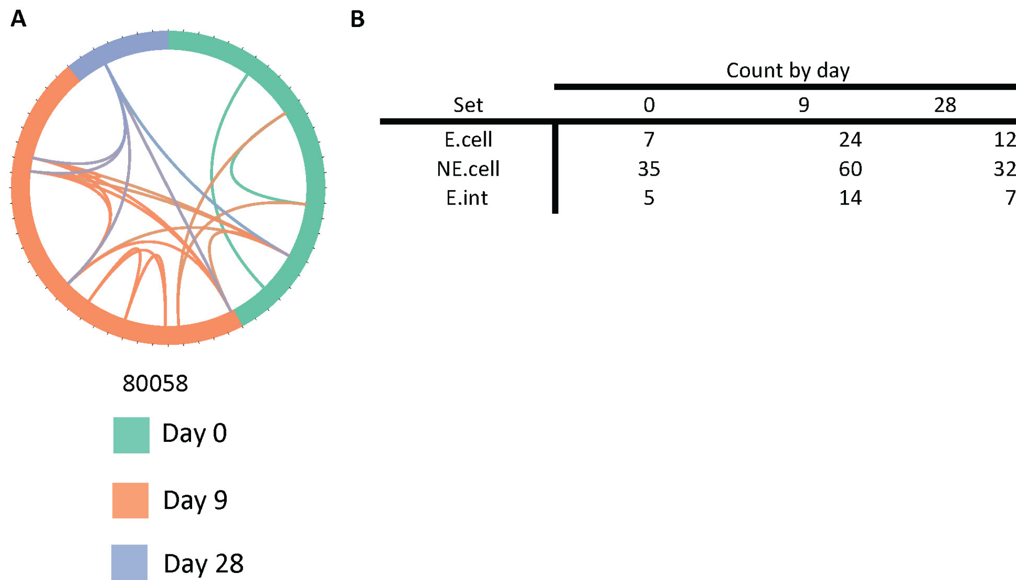
iNKT Panel

Reagent	Fluor	Clone	Provider
LIVE/DEAD	V510	N/A	Invitrogen
α-CD3	BV786	SK7	BD
α-CD4	FITC	L120	BD
α-CD8α	PerCP-Cy5.5	SK1	BD
α-CD16	PE-Cy7	3G8	BD
α-CD56	BV421	NCAM16.2	BD
α-CD161	BV605	HP-3G10	BioLegend
α-TCR Vα7.2	PE	3C10	BioLegend
α-TCR Vα24Jα18	APC	6B11	BioLegend
α-IFNγ	BUV395	B27	BD
α-Granzyme B	Alexa Fluor 700	GB11	BD

[‡] Denotes intracellularly-detected markers
[°] Denotes reagents used only in low dose CHMI samples
[•] Denotes reagents used only in high dose CHMI samples

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.

Supplemental Figure 4



Set	Count by day		
	0	9	28
E.cell	7	24	12
NE.cell	35	60	32
E.int	5	14	7

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 anaylsis 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 *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 *P. falciparum* sporozoites (*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 *Pf*SPZ 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 *Pf*SPZ 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 *Pf*SPZ 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 *Pf*SPZ vaccine trials comparing malaria naïve and pre-exposed volunteers.

We further showed that a single dose of *Pf*SPZ 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 *Pf*SPZ 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 *Pf*SPZ 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 *Pf*SPZ 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 *Pf*SPZ 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 *Pf*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

Agnandji, S.T., Lell, B., Soulanoudjingar, S.S., Fernandes, J.F., Abossolo, B.P., Conzelmann, C., Methogo, B.G.N.O., Doucka, Y., Flamen, A., Mordmüller, B., et al. (2011). First results of phase 3 trial of RTS,S/AS01 malaria vaccine in African children. N. Engl. J. Med. 365, 1863–1875.

Asante, K.P., Abdulla, S., Agnandji, S., Lyimo, J., Vekemans, J., Soulanoudjingar, S., Owusu, R., Shomari, M., Leach, A., Jongert, E., et al. (2011). Safety and efficacy of the RTS,S/AS01E candidate malaria vaccine given with expanded-programme-on-immunisation vaccines: 19 month follow-up of a randomised, open-label, phase 2 trial. Lancet Infect. Dis. 11, 741–749.

Billig, E.M., McQueen, P.G., and McKenzie, F.E. (2012). Foetal haemoglobin and the dynamics of paediatric malaria. Malar. J. 11, 396.

Crotty, S., Kersh, E.N., Cannons, J., Schwartzberg, P.L., and Ahmed, R. (2003). SAP is required for generating long-term humoral immunity. Nature 421, 282–287.

Dagan, R., Eskola, J., Leclerc, C., and Leroy, O. (1998). Reduced Response to Multiple Vaccines Sharing Common Protein Epitopes That Are Administered Simultaneously to Infants. Infect. Immun. 66, 2093–2098.

Doolan, D.L., Dobaño, C., and Baird, J.K. (2009). Acquired Immunity to Malaria. Clin. Microbiol. Rev. 22, 13–36.

Edwards, K.M. (2015). Maternal antibodies and infant immune responses to vaccines. Vaccine 33, 6469–6472.

Good, M.F., and Doolan, D.L. (1999). Immune effector mechanisms in malaria. Curr. Opin. Immunol. 11, 412–419.

Griffin, J.T., Hollingsworth, T.D., Reyburn, H., Drakeley, C.J., Riley, E.M., and Ghani, A.C. (2015). Gradual acquisition of immunity to severe malaria with increasing exposure. Proc. R. Soc. Lond. B Biol. Sci. 282, 20142657.

JOINT TECHNICAL EXPERT GROUP, MALARIA VACCINES (JTEG), and WHO (2015). Malaria Vaccine.

Kester, K.E., McKinney, D.A., Tornieporth, N., Ockenhouse, C.F., Heppner, D.G., Hall, T., Wellde, B.T., White, K., Sun, P., Schwenk, R., et al. (2007). A phase I/IIa safety, immunogenicity, and efficacy bridging randomized study of a two-dose regimen of liquid and lyophilized formulations of the candidate

malaria vaccine RTS,S/AS02A in malaria-naïve adults. Vaccine 25, 5359–5366.

Kester, K.E., Cummings, J.F., Ofori-Anyinam, O., Ockenhouse, C.F., Krzych, U., Moris, P., Schwenk, R., Nielsen, R.A., Debebe, Z., Pinelis, E., et al. (2009). Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J. Infect. Dis. 200, 337–346.

Klein, S.L., Marriott, I., and Fish, E.N. (2015). Sex-based differences in immune function and responses to vaccination. Trans. R. Soc. Trop. Med. Hyg. 109, 9–15.

Klein, S.L., Shann, F., Moss, W.J., Benn, C.S., and Aaby, P. (2016). RTS,S Malaria Vaccine and Increased Mortality in Girls. mBio 7, e00514–e00516.

Laurens, M.B., Duncan, C.J., Epstein, J.E., Hill, A.V., Komisar, J.L., Lyke, K.E., Ockenhouse, C.F., Richie, T.L., Roestenberg, M., Sauerwein, R.W., et al. (2012). A consultation on the optimization of controlled human malaria infection by mosquito bite for evaluation of candidate malaria vaccines. Vaccine 30, 5302–5304.

Leroux-Roels, G., Leroux-Roels, I., Clement, F., Ofori-Anyinam, O., Lievens, M., Jongert, E., Moris, P., Ballou, W.R., and Cohen, J. (2014). Evaluation of the immune response to RTS,S/AS01 and RTS,S/AS02 adjuvanted vaccines: randomized, double-blind study in malaria-naïve adults. Hum. Vaccines Immunother. 10, 2211–2219.

MOORMANN, A.M. (2009). How might infant and paediatric immune responses influence malaria vaccine efficacy? Parasite Immunol. 31, 547–559.

Moorthy, V.S., and Ballou, W.R. (2009). Immunological mechanisms underlying protection mediated by RTS,S: a review of the available data. Malar. J. 8, 312.

Niewiesk, S. (2014). Maternal Antibodies: Clinical Significance, Mechanism of Interference with Immune Responses, and Possible Vaccination Strategies. Front. Immunol. 5.

Olotu, A., Lusingu, J., Leach, A., Lievens, M., Vekemans, J., Msham, S., Lang, T., Gould, J., Dubois, M.-C., Jongert, E., et al. (2011). Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5-17 months in Kenya and

Tanzania: a randomised controlled trial. Lancet Infect. Dis. 11, 102–109.

Olotu, A., Clement, F., Jongert, E., Vekemans, J., Njuguna, P., Ndungu, F.M., Marsh, K., Leroux-Roels, G., and Bejon, P. (2014). Avidity of anti-circumsporozoite antibodies following vaccination with RTS,S/AS01E in young children. PloS One 9, e115126.

Olotu, A., Fegan, G., Wambua, J., Nyangweso, G., Leach, A., Lievens, M., Kaslow, D.C., Njuguna, P., Marsh, K., and Bejon, P. (2016). Seven-Year Efficacy of RTS,S/AS01 Malaria Vaccine among Young African Children. N. Engl. J. Med. 374, 2519–2529.

Polhemus, M.E., Remich, S.A., Ogutu, B.R., Waitumbi, J.N., Otieno, L., Apollo, S., Cummings, J.F., Kester, K.E., Ockenhouse, C.F., Stewart, A., et al. (2009). Evaluation of RTS,S/AS02A and RTS,S/AS01B in adults in a high malaria transmission area. PloS One 4, e6465.

PrabhuDas, M., Adkins, B., Gans, H., King, C., Levy, O., Ramilo, O., and Siegrist, C.-A. (2011). Challenges in infant immunity: implications for responses to infection and vaccines. Nat. Immunol. 12, 189–194.

Reddy, S.B., Anders, R.F., Beeson, J.G., Färnert, A., Kironde, F., Berenzon, S.K., Wahlgren, M., Linse, S., and Persson, K.E.M. (2012). High Affinity Antibodies to Plasmodium falciparum Merozoite Antigens Are Associated with Protection from Malaria. PLoS ONE 7, e32242.

Ruggieri, A., Anticoli, S., D'Ambrosio, A., Giordani, L., and Viora, M. (2016). The influence of sex and gender on immunity, infection and vaccination. Ann. Ist. Super. Sanita 52, 198–204.

Schofield, L., and Mueller, I. (2006). Clinical immunity to malaria. Curr. Mol. Med. 6, 205–221.

Schwartz, L., Brown, G.V., Genton, B., and Moorthy, V.S. (2012). A review of malaria vaccine clinical projects based on the WHO rainbow table. Malar. J. 11, 11.

Slifka, M.K., Matloubian, M., and Ahmed, R. (1995). Bone marrow is a major site of long-term antibody production after acute viral infection. J. Virol. 69, 1895–1902.

Spring, M., Polhemus, M., and Ockenhouse, C. (2014). Controlled human malaria infection. J. Infect. Dis. 209 Suppl 2, S40–S45.

Watt, G., Kantipong, P., Burnouf, T., Shikuma, C., and Philpott, S. (2013). Natural Scrub Typhus Antibody Suppresses HIV CXCR4(X4) Viruses. Infect. Dis. Rep. 5, e8.

WER-WHO (2016). WHO | The Weekly Epidemiological Record (WER).

White, M.T., Verity, R., Griffin, J.T., Asante, K.P., Owusu-Agyei, S., Greenwood, B., Drakeley, C., Gesase, S., Lusingu, J., Ansong, D., et al. (2015). Immunogenicity of the RTS,S/AS01 malaria vaccine and implications for duration of vaccine efficacy: secondary analysis of data from a phase 3 randomised controlled trial. Lancet Infect. Dis. 15, 1450–1458.

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

Damien Portevin, Felicien Moukambi, Petra Clowes, Asli Bauer, Mkunde Chachage, Nyanda E Ntinginya, Elirehema Mfinanga, Khadija Said, Frederick Haraka, Andrea Rachow, Elmar Saathoff, Maximilian Mpina, Levan Jugheli, Fred Lwilla, Ben J Marais, Michael Hoelscher, Claudia Daubenberger, Klaus Reither, Christof Geldmacher**

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 2ft0 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 83·3%, ft5% CI 58·6–ft6·4). Specificity was ft6·8% (ft5% CI 8ft·0–ftft·6) 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 1ft·5 days (IQR 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.**

Funding **European and Developing Countries Clinical Trials Partnership, German Federal Ministry of Education and Research, and Swiss National Science Foundation.**

Lancet Infect Dis 2014; 14: 931–38

Published **Online** September 1, 2014 http://dx.doi.org/10.1016/S1473-3099(14)70884-9

See **Comment** page 900

*Contributed equally

Swiss Tropical and Public Health Institute, Basel, Switzerland (D Portevin PhD, L Jugheli PhD, Prof C Daubenberger DVM, K Reither MD); **University of Basel, Basel, Switzerland** (D Portevin, L Jugheli, C Daubenberger, K Reither); **NIMR-Mbeya Medical Research Center, Mbeya, Tanzania** (F Moukambi MSc, P Clowes MD, A Bauer PhD, M Chachage PhD, N E Ntinginya MD); **Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich, Germany** (F Moukambi, P Clowes, A Bauer, A Rachow MD, E Saathoff PhD, Prof M Hoelscher FRCP, C Geldmacher PhD); **German Centre for Infection Research, Partner Site Munich, Germany** (A Rachow, E Saathoff, M Hoelscher, C Geldmacher); **Ifakara Health Institute, Bagamoyo, Tanzania** (E Mfinanga MD, K Said MD, F Haraka MD, M Mpina MSc, L Jugheli, F Lwilla PhD, K Reither); **and Children's Hospital at Westmead, Sydney Medical School, University of Sydney, Sydney, NSW, Australia** (Prof B J Marais FCPaed)

Correspondence to: Dr Klaus Reither, Swiss Tropical and Public Health Institute, 4051 Basel, Switzerland **klaus.reither@unibas.ch**

or

Dr Christof Geldmacher, Division of Infectious Diseases and Tropical Medicine, Medical Center of the University of Munich, 80802 Munich, Germany **geldmacher@lrz.uni-muenchen.de**

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.[1] Surveillance data for children remain imprecise because paediatric tuberculosis is often either underdiagnosed or overdiagnosed in high-burden countries.[2–4] 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.[4]

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.[2,5] 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.[6,7]

The Xpert MTB/RIF assay enables timely, sensitive, and specific molecular detection of pulmonary tuberculosis and rifampicin resistance in adults,[8] but its value in young children is greatly reduced. In a recent meta-analysis, the calculated pooled sensitivity was 66% for Xpert MTB/RIF against culture in expectorated or induced sputa, or gastric lavage specimens from children with suspected tuberculosis.[9] 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.[9]

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.[10] 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.[11–15] Loss of CD27 expression on *M tuberculosis*-specific CD4 T cells is a marker of active tuberculosis due to persistent antigenic stimulation[16] and probably relates to increased cellular homing to the site of disease.[17,18] 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, after 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

See Online for appendix

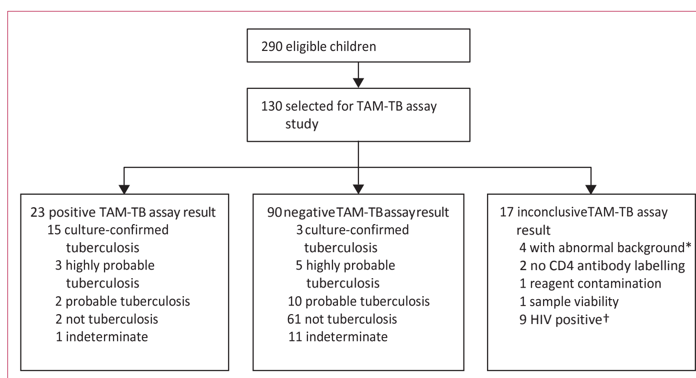


Figure 1: **Study profile**
TAM-TB=T-cell activation marker–tuberculosis. *Frequency of interferon-γ-positive CD4 T cells in the negative control greater than the median frequency plus 3 SD of the tested sample. †CD4 T cell count of less than 10 000 per μL and interferon-γ CD4 T cells less than 0·05%.

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 attested 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.[19] Fine-needle aspiration biopsies of enlarged lymph nodes were done when clinically indicated, as per standard protocol.[20]

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

	All patients (n=113)	Culture-confirmed tuberculosis (n=18)	Highly probable tuberculosis (n=8)	Probable tuberculosis (n=12)	Not tuberculosis (n=63)	Indeterminate (n=12)
Age (years)	6·1 (2·1–10·3)	4·6 (1·5–12·7)	8·1 (5·2–11·9)	6 (1·8–10·5)	6·1 (2·0–9·8)	5·4 (2·2–10·5)
Female sex	52 (46%)	8 (44%)	5 (63%)	6 (50%)	27 (43%)	6 (50%)
Symptoms at enrolment						
Cough	108 (96%)	16 (89%)	6 (75%)	12 (100%)	62 (98%)	12 (100%)
Fatigue or lethargy	28 (25%)	8 (44%)	2 (25%)	2 (17%)	16 (25%)	0
Wheezing	16 (14%)	1 (6%)	1 (13%)	3 (25%)	9 (14%)	2 (17%)
Breathing difficulties	47 (42%)	10 (56%)	4 (50%)	8 (67%)	22 (35%)	3 (25%)
Fever	82 (73%)	16 (89%)	6 (75%)	9 (75%)	45 (71%)	6 (50%)
Chest pain	23 (20%)	1 (6%)	0	1 (8%)	18 (29%)	3 (25%)
Haemoptysis	4 (4%)	1 (6%)	0	1 (8%)	1 (2%)	1 (8%)
Enlarged lymph nodes	12 (11%)	5 (28%)	1 (13%)	1 (8%)	5 (8%)	0
Weight loss	55 (49%)	13 (72%)	3 (38%)	5 (42%)	32 (51%)	2 (17%)
Abdominal pains	20 (18%)	5 (28%)	2 (25%)	3 (25%)	9 (14%)	1 (8%)
Malnutrition	60 (53%)	12 (67%)	4 (50%)	5 (42%)	24 (38%)	5 (42%)
HIV infection	33 (29%)	4 (22%)	3 (38%)	7 (58%)*	15 (24%)	4 (33%)
WHO immunological staging						
Not clinically significant	7/33 (21%)	0/4 (0%)	0/3 (0%)	2/7 (29%)	4/15 (27%)	1/4 (25%)
Mild	6/33 (18%)	1/4 (25%)	1/3 (33%)	2/7 (29%)	2/15 (13%)	0/4 (0%)
Advanced	1/33 (3%)	1/4 (25%)	0/3 (0%)	0/7 (0%)	0/15 (0%)	0/4 (0%)
Severe	19/33 (58%)	2/4 (50%)	2/3 (67%)	3/7 (43%)	9/15 (60%)	3/4 (75%)
On antiretroviral therapy at enrolment	13/33 (39%)	2/4 (50%)	1/3 (33%)	2/7 (29%)	7/15 (47%)	1/4(25%)
Positive tuberculin skin test	31/103 (30%)	13/17 (76%)	3/8 (38%)	2/11 (18%)	9/57 (16%)	4/10 (40%)
Positive interferon-γ release assay	27/110 (25%)	13/18 (72%)	1/7 (14%)	3/12 (25%)	8/61 (13%)	4/12 (33%)
Positive tuberculin skin test or interferon-γ release assay	47/112 (42%)	17/18 (94%)	3/8 (38%)	4/12 (33%)	15/63 (24%)	4/11 (36%)

Data are median (IQR), number (%), or n/N (%). *p=0·033 compared with not tuberculosis distribution (Fisher's exact test).

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

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 MTBC 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.[14] *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[21] (Elephants and Peptides, Potsdam, Germany) and purified protein derivative (Statens Serum Institut, Copenhagen, Denmark) before staining with fluorochrome-labelled 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

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

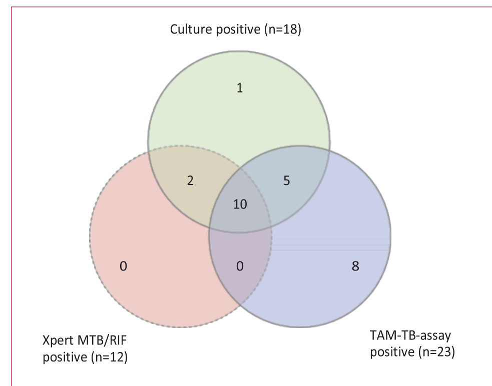


Figure 2: **Venn diagram of positive *Mycobacterium tuberculosis* culture, Xpert MTB/RIF, and TAM-TB-assay results**
TAM-TB=T-cell activation marker–tuberculosis.

	Culture-confirmed tuberculosis (n=18)	Highly probable tuberculosis (n=8)	Probable tuberculosis (n=12)	Not tuberculosis (n=63)	Indeterminate (n=12)
Assay-positive cases	15 (83%)	3 (38%)	2 (17%)	2 (3%)	1 (8%)
Assay-negative cases	3 (17%)	5 (63%)	10 (83%)	61 (97%)	11 (92%)

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

	Bagamoyo (n=63)	Mbeya (n=50)	All patients (n=113)
Sensitivity (95% CI)	83·3% (35·9–99·6)	83·3% (51·6–97·9)	83·3% (58·6–96·4)
Positive/total	5/6	10/12	15/18
Specificity (95% CI)	95·6% (84·9–99·5)	100% (81·5–100)	96·8% (89·0–99·6)
Negative/total	43/45	18/18	61/63
Disease prevalence	7/115 (6%)	22/175 (13%)	29/290 (10%)
Positive predictive value	54·9%	100%	74·5%
Negative predictive value	98·9%	97·7%	98·1%

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 main paediatric cohort (n=290).

Table 3: **Sensitivity, specificity, and predictive values of the T-cell activation marker–tuberculosis assay**

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;[22] 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 cytomorphology 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,[23] 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,

	Clinical case classification and reason for classification	Tuberculin skin test or QuantiFERON	HIV status	First-visit clinical diagnosis, treatment, and response	Follow-up clinical diagnosis, treatment, and outcome
Culture-negative and TAM-TB assay-positive cases					
15 years	Highly probable tuberculosis by radiograph	Positive	Negative	Pneumonia Amoxicillin No	Pulmonary tuberculosis later confirmed by culture and Xpert MTB/RIF 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
7 years	Highly probable tuberculosis by sputum smear	Positive	Negative	Pneumonia Amoxicillin No	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
9 years	Highly probable tuberculosis by lymph node cytology	Positive	Positive	Lymphadenitis Ceftriaxone No	Tuberculosis lymphadenitis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
11 years	Probable tuberculosis by symptoms, resolution after treatment	Negative	Positive	Pneumonia Ceftriaxone No	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
1 year	Probable tuberculosis by symptoms, resolution after treatment	Positive	Positive	Chest infection Cefalexin No	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
5 years	Not tuberculosis, no treatment, and healthy after 5 months	Positive	Positive	Pneumocystis pneumonia plus *Mycobacterium interjectum* Co-trimoxazole Resolved	Recovered from initial diagnosis
7 years	Not tuberculosis, no treatment, and healthy after 5 months	Positive	Positive	Pneumonia, urinary tract infection Antibiotics Resolved	Intestinal helminths Albendazole Resolved, new dry cough 6–9 months after recruitment
10 years	Indeterminate	Positive	Positive	Bronchiectasis Metronidazole Moderate	Bronchiectasis, malaria Antimalarial Persisting cough, mediastinal lymphadenopathy
Culture-positive and TAM-TB assay-negative cases					
14 years	Culture-confirmed tuberculosis with *Mycobacterium tuberculosis* in sputum	Positive	Negative	Pulmonary tuberculosis, malnutrition 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Unchanged	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved
8 years	Culture confirmed tuberculosis with *Mycobacterium tuberculosis* in sputum	Positive	Negative	Nephrotic syndrome Furosemide, prednisolone Deterioration, hospital admission	Pulmonary tuberculosis (only retrospectively confirmed) No tuberculosis treatment Death
2 years	Culture confirmed tuberculosis with *Mycobacterium tuberculosis* in sputum	Negative	Negative	Pulmonary tuberculosis, malnutrition 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Unchanged	Pulmonary tuberculosis 2 months of isoniazid, rifampicin, pyrazinamide, and ethambutol, and 4 months of isoniazid and rifampicin Resolved

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.

Table 4: **Cases with discrepant culture and TAM-TB assay results: clinical characteristics at enrolment and follow-up visits by age**

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 sensitivity of the TAM-TB assay was 69·2% (48·2–85·6) when both culture-confirmed and highly probable tuberculosis cases were included in the reference standard.

Table 4 summarises the clinical characteristics, tuberculin skin test and QuantiFERON-TB Gold results, diagnosis at enrolment, response to antibiotic treatment, and clinical follow-up of all children with discrepant TAM-TB assay and *M tuberculosis* culture results. Of the eight children who were TAM-TB assay positive but culture-negative (figure 2), three cases were classified as highly probable tuberculosis, two as probable tuberculosis, two as not tuberculosis, and one as indeterminate; six were HIV infected (table 4). Of the three children with highly probable tuberculosis, one was sputum smear-positive at enrolment, one was culture-confirmed during follow up, and one had cytological signs (chronic granulomatous lymphadenitis) that suggested tuberculosis. In the two probable tuberculosis cases, broad-spectrum antibiotics had no effect, but symptoms resolved on tuberculosis treatment (table 4). The TAM-TB assay was positive in two HIV-infected patients who were classified as not tuberculosis (table 4). *Mycobacterium interjectum* was identified in one case with an induration of 10 mm on the tuberculin skin test and an indeterminate QuantiFERON-TB Gold result. The other patient was QuantiFERON-TB Gold-positive. One HIV-infected child who was classified as indeterminate, had a complex clinical picture with various disease episodes that never fully resolved. Of the three children who were *M tuberculosis* culture-positive, but TAM-TB assay negative, two were malnourished (table 4). The third child had a nephrotic syndrome and died before culture results were available (table 4).

Of the 63 children classified as not tuberculosis, 15 (24%) had a positive tuberculin skin test or QuantiFERON-TB Gold test at enrolment, suggesting probable latent tuberculosis infection. 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 100% (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.[2,24]

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.[4] International consensus definitions of intrathoracic tuberculosis were not available when the study was designed.[25] 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 66·7% and a specificity of 100%.[26–28] 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.[15] 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-positive 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 needs 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 cryo-preservation.[29] 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 (Cellestis).[30] 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.[15,31] 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.

***Panel:* Research in context**

Systematic review

We searched PubMed up to June 10, 2014, using the terms "CD27", "diagnosis", "human", and "tuberculosis". Of the 33 results returned, seven were clinical studies from five independent laboratories and their results showed the potential of monitoring CD27 expression on *Mycobacterium tuberculosis*-specific CD4 T cells to diagnose active tuberculosis disease in adults. We did not find a report of the assessment of the use of *M tuberculosis*-specific T-cell phenotype for the diagnosis of active childhood tuberculosis.

Interpretation

Our report is the first to assess the accuracy of using CD27-expression analysis for the diagnosis of paediatric tuberculosis. Immunological data were generated and analysed by investigators from whom clinical information was masked. The T-cell activation marker–tuberculosis assay showed excellent sensitivity (83·3%) and specificity (96·8%) when *M tuberculosis* culture positivity was used as a reference standard, and enabled the detection of additional cases in culture-negative children who were clinically suspected of having tuberculosis. Despite a fairly small sample size and potential bias originating from cohort enrichment in culture-confirmed tuberculosis cases, the results of this proof-of-concept study show that immunodiagnostic tests that incorporate phenotypic characteristics of *M tuberculosis*-specific T cells have the potential to improve rapid detection of active tuberculosis in children.

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 sputum-independent TAM-TB assay is a major advance for the rapid and accurate diagnosis of tuberculosis in children.

Contributors

KR, MH, and CG designed the study. CG designed the TAM-TB assay. PC, ES, AR, NEN, EM, KS, FH, FL, LJ, MH, and KR oversaw enrolment, patient care, or standard laboratory work. DP, FM, AB, MC, MM, CG, and CD were responsible for the flow-cytometry analysis. DP, FM, ES, KR, and CG did the data management and analysis. DP, KR, CG, CD, and BJM 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.

Acknowledgments

We gratefully acknowledge funding from the European and Developing Countries Clinical Trials Partnership for this study as part of the TB CHILD project (IP.2009.32040.007). Cofunding was provided by the German Federal Ministry of Education and Research (grant 01KA1006), and the Swiss National Science Foundation (32EC30_131192/1). CG's salary is funded by the German Center for Infection Research. We thank all the children who participated in the study and their carers, and Philipp Metzger for his support in the immunology laboratory.

References

1 Jenkins HE, Tolman AW, Yuen CM, et al. Incidence of multidrug-resistant tuberculosis disease in children: systematic review and global estimates. *Lancet* 2014; **383:** 1572–79.

2 Perez-Velez CM, Marais BJ. Tuberculosis in children. *N Engl J Med* 2012; **367:** 348–61.

3 Marais BJ, Graham SM, Maeurer M, Zumla A. Progress and challenges in childhood tuberculosis. *Lancet Infect Dis* 2013; **13:** 287–89.

4 Cuevas LE, Petrucci R, Swaminathan S. Tuberculosis diagnostics for children in high-burden countries: what is available and what is needed. *Paediatr Int Child Heal* 2012; **32** (suppl 2)**:** S30–37.

5 Marais BJ, Pai M. New approaches and emerging technologies in the diagnosis of childhood tuberculosis. *Paediatr Respir Rev* 2007; **8:** 124–33.

6 Zar HJ, Connell TG, Nicol M. Diagnosis of pulmonary tuberculosis in children: new advances. *Expert Rev Anti Infect Ther* 2010; **8:** 277–88.

7 Graham SM. The use of diagnostic systems for tuberculosis in children. *Indian J Pediatr* 2011; **78:** 334–39.

8 Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane Database Syst Rev* 2014; published online Jan 21. DOI:10.1002/14651858.CD009593.pub3.

9 WHO. Xpert MTB/RIF: WHO policy update and implementation manual. http://www.who.int/tb/laboratory/xpert_launchupdate/en/ (accessed May 7, 2014).

10 Pai M, Denkinger CM, Kik SV, et al. Gamma interferon release assays for detection of *Mycobacterium tuberculosis* infection. *Clin Microbiol Rev* 2014; **27:** 3–20.

11 Streitz M, Tesfa L, Yildirim V, et al. Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis. *PLoS One* 2007; **2:** e735.

12 Jiang J, Wang X, Wang X, et al. Reduced CD27 expression on antigen-specific CD4+ T cells correlates with persistent active tuberculosis. *J Clin Immunol* 2010; **30:** 566–73.

13 Nikitina IY, Kondratuk NA, Kosmiadi GA, et al. Mtb-specific CD27low CD4 T cells as markers of lung tissue destruction during pulmonary tuberculosis in humans. *PLoS One* 2012; **7:** e43733.

14 Geldmacher C, Ngwenyama N, Schuetz A, et al. Preferential infection and depletion of *Mycobacterium tuberculosis*-specific CD4 T cells after HIV-1 infection. *J Exp Med* 2010; **207:** 2869–81.

15 Schuetz A, Haule A, Reither K, et al. Monitoring CD27 expression to evaluate *Mycobacterium tuberculosis* activity in HIV-1 infected individuals in vivo. *PLoS One* 2011; **6:** e27284.

16 De Jong R, Brouwer M, Hooibrink B, Van der Pouw-Kraan T, Miedema F, Van Lier RA. The CD27- subset of peripheral blood memory CD4+ lymphocytes contains functionally differentiated T lymphocytes that develop by persistent antigenic stimulation in vivo. *Eur J Immunol* 1992; **22:** 993–99.

17 Kapina MA, Shepelkova GS, Mischenko VV, et al. CD27^{low} CD4 T lymphocytes that accumulate in the mouse lungs during mycobacterial infection differentiate from CD27^{high} precursors in situ, produce IFN-gamma, and protect the host against tuberculosis infection. *J Immunol* 2007; **178:** 976–85.

18 Li L, Qiao D, Li Q, Zhang X, Lao S, Wu C. Distinct polyfunctional CD4+ T cell responses to BCG, ESAT-6 and CFP-10 in tuberculous pleurisy. *Tuberc Edinb Scotl* 2012; **ft2:** 63–71.

19 Zar HJ, Tannenbaum E, Apolles P, Roux P, Hanslo D, Hussey G. Sputum induction for the diagnosis of pulmonary tuberculosis in infants and young children in an urban setting in South Africa. *Arch Dis Child* 2000; **82:** 305–08.

20 Wright CA, Hesseling AC, Bamford C, Burgess SM, Warren R, Marais BJ. Fine-needle aspiration biopsy: a first-line diagnostic procedure in paediatric tuberculosis suspects with peripheral lymphadenopathy? *Int J Tuberc Lung Dis Off* 2009; **13:** 1373–79.

21 Geldmacher C, Schuetz A, Ngwenyama N, et al. Early depletion of *Mycobacterium tuberculosis*-specific T helper 1 cell responses after HIV-1 infection. *J Infect Dis* 2008; **1ft8:** 1590–98.

22 Bossuyt PM, Reitsma JB, Bruns DE, et al. The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. *Clin Chem* 2003; **4ft:** 7–18.

23 WHO. WHO case definitions of HIV for surveillance and revised clinical staging and immunological classification of HIV-related disease in adults and children. WHO. http://www.who.int/hiv/pub/guidelines/hivstaging/en/ (accessed June 20, 2014).

24 Newton SM, Brent AJ, Anderson S, Whittaker E, Kampmann B. Paediatric tuberculosis. *Lancet Infect Dis* 2008; **8:** 498–510.

25 Graham SM, Ahmed T, Amanullah F, et al. Evaluation of tuberculosis diagnostics in children: 1. Proposed clinical case definitions for classification of intrathoracic tuberculosis disease. Consensus from an expert panel. *J Infect Dis* 2012; **205** (suppl 2)**:** S199–208.

26 Nicol MP, Workman L, Isaacs W, et al. Accuracy of the Xpert MTB/RIF test for the diagnosis of pulmonary tuberculosis in children admitted to hospital in Cape Town, South Africa: a descriptive study. *Lancet Infect Dis* 2011; **11:** 819–24.

27 Rachow A, Clowes P, Saathoff E, et al. Increased and expedited case detection by Xpert MTB/RIF assay in childhood tuberculosis: a prospective cohort study. *Clin Infect Dis Off Publ Infect Dis Soc Am* 2012. DOI:10.1093/cid/cis190.

28 Zar HJ, Workman L, Isaacs W, et al. Rapid molecular diagnosis of pulmonary tuberculosis in children using nasopharyngeal specimens. *Clin Infect Dis Off Publ Infect Dis Soc Am* 2012; **55:** 1088–95.

29 Bull M, Lee D, Stucky J, et al. Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trials. *J Immunol Methods* 2007; **322:** 57–69.

30 Ferrara G, Losi M, D'Amico R, et al. Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: a prospective study. *Lancet* 2006; **367:** 1328–34.

31 Thomas TA, Mondal D, Noor Z, et al. Malnutrition and helminth infection affect performance of an interferon gamma-release assay. *Pediatrics* 2010; **126:** e1522–29.

CVI Journals.ASM.org

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

Nicole Lenz,[a,b] Tobias Schindler,[a,b,c] Benjamin M. Kagina,[d,e] Jitao David Zhang,[c] Tedson Lukindo,[e] Maxmillian Mpina,[a,b,f] Peter Bang,[g] Ingrid Kromann,[g] Søren T. Hoff,[g] Peter Andersen,[g] Klaus Reither,[a,b,f] Gavin J. Churchyard,[h,i,j] Ulrich Certa,[c] Claudia A. Daubenberger[a,b]

Swiss Tropical and Public Health Institute, Basel, Switzerland[a]; University of Basel, Basel, Switzerland[b]; Roche Pharmaceutical Research and Early Development, Department of Pharmaceutical Sciences/Translational Technologies and Bioinformatics, Roche Innovation Center Basel, Basel, Switzerland[c]; South African Tuberculosis Vaccine Initiative (SATVI), Institute of Infectious Disease and Molecular Medicine (IDM) and School of Child and Adolescent Health, University of Cape Town, South Africa[d]; Vaccines for Africa Initiative, Institute of Infectious Disease and Molecular Medicine (IDM), University of Cape Town, South Africa[e]; Bagamoyo Research and Training Centre, Ifakara Health Institute, Bagamoyo, United Republic of Tanzania[f]; Statens Serum Institute, Copenhagen, Denmark[g]; Aurum Institute, Johannesburg, South Africa[h]; London School of Hygiene and Tropical Medicine, London, United Kingdom[i]; School of Public Health, University of Witwatersrand, Johannesburg, South Africa[j]

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 56 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 (T_{CM}) and effector memory CD4$^+$ T cells (T_{EM}) 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 T_{CM} and T_{EM} 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.)

Vaccination represents one of the most successful health interventions for disease containment, elimination, and eventual eradication (1). Despite more than 50 years of widespread vaccination with *Mycobacterium bovis* bacille Calmette-Guérin (BCG), tuberculosis (TB) remains one of the world's most serious infectious diseases. In 2012, the World Health Organization (WHO) estimated that there were 9 million new clinical TB cases and 1 million people died from TB (2). Infection with HIV impairs host resistance, leading to a faster and higher rate of progression from latent to clinical TB (3). Consequently, there is an urgent need for development of improved TB vaccines that are more efficacious, safer, and more immunogenic than BCG in all populations, including HIV-infected individuals. H1/IC31 is a protein subunit vaccine against *Mycobacterium tuberculosis*, consisting of a fusion protein of the mycobacterial antigen 85B (Ag85B) and early secretory antigenic target 6 (ESAT-6) called hybrid 1 (H1) in combination with the adjuvant IC31. IC31 is composed of the cationic polyamino acid KLK and a single-stranded oligodeoxynucleotide with alternating sequences of the nucleic acids inosine and cytidine (ODN1a) (4). KLK enables simultaneous uptake of H1 and ODN1a in antigen-presenting cells and provides a platform for hyperefficient Toll-like receptor 9 (TLR-9) ligand recognition of ODN1a. Upon triggering of TLR-9, conventional dendritic cells and monocytes activate the MyD88-NF-κB dependent signaling cascade (5). This leads to secretion of proinflammatory cytokines and finally induction of adaptive immune responses. In addition to the MyD88-NF-κB-dependent pathway, plasmacytoid dendritic cells (pDC) have the unique ability to signal in a MyD88-interferon regulatory factor (IRF7)-dependent way. This yields

Received 13 February 2015 Returned for modification 9 March 2015
Accepted 19 April 2015
Accepted manuscript posted online 29 April 2015
Citation Lenz N, Schindler T, Kagina BM, Zhang JD, Lukindo T, Mpina M, Bang P, Kromann I, Hoff ST, Andersen P, Reither K, Churchyard GJ, Certa U, Daubenberger CA. 2015. Antiviral innate immune activation in HIV-infected adults negatively affects H1/IC31-induced vaccine-specific memory CD4$^+$ T cells. Clin Vaccine Immunol 22:688 – 696. doi:10.1128/CVI.00092-15.
Editor: H. F. Staats
Address correspondence to Claudia A. Daubenberger, claudia.daubenberger@unibas.ch.
N.L. and T.S. contributed equally to this work.
U.C. and C.A.D. contributed equally to this work.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/CVI.00092-15.
Copyright © 2015, American Society for Microbiology. All Rights Reserved.
doi:10.1128/CVI.00092-15

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

In HIV-infected individuals, vaccine take and durability of the immunity 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 proinflammatory cytokines and type I IFNs (9). Use of a TLR-9 agonist as an adjuvant needs to be tested and analyzed carefully 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/mm^3 (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 before and shortly after H1/IC31 vaccination and correlate these with vaccine-specific central (T_{CM}) and effector memory $CD4^+$ T cell (T_{EM}) responses. A whole venous blood stimulation assay (10) was used to monitor the vaccine-induced cellular immune response over a follow-up period of 182 days. With use of AmpliSeq and next-generation sequencing the expression changes of 1,388 genes in peripheral blood were determined (11). We describe for the first time that higher expression levels of genes involved in antiviral innate immunity at baseline reduce long-term maintenance of vaccine responses in HIV-infected volunteers.

MATERIALS AND METHODS

Ethics statement. This study was conducted according to good clinical practice guidelines and the Declaration of Helsinki. Informed consent was obtained from all participants. Detailed descriptions of the ethical approvals can be obtained from Reither et al. (10). The clinical trial was registered in the Pan African Clinical Trials Registry (PACTR) with the identifier PACTR201105000289276.

Participant enrollment and blood collection. A total of 24 HIV-positive, BCG-vaccinated volunteers with $CD4^+$ T cell counts of >350 cells/mm^3 from Bagamoyo, United Republic of Tanzania, were enrolled. Participants were randomly allocated in a ratio of 5:1 to receive placebo or H1/IC31 vaccine. One H1/IC31 vaccine recipient dropped out before the booster vaccination due to pregnancy. Injections of 0.5 ml containing 50 μg Ag85B-ESAT-6 and 500 nmol KLK plus 20 nmol ODN1a (adjuvant) or 0.4 ml Tris buffer (placebo) were administered intramuscularly on study days 0 and 56. For transcriptomics analysis, 2.5-ml samples of peripheral blood were collected via sterile venipuncture in RNA PaxGene Vacutainer tubes (PreAnalytiX) on days 0, 3, and 59. Samples of 9 ml of peripheral blood were collected in sodium heparin tubes and directly processed in whole-blood stimulation assays on days 0, 14, 56, 70, and 182. An extended description of the study design can be obtained from Reither et al. (10).

ICS and analysis by flow cytometry. The intercellular cytokine staining (ICS) and flow cytometry analyses were described in Reither et al. (10). The present project focused on the analysis of cytokine-producing memory $CD4^+$ T cell subsets. For whole-blood stimulation and ICS, the protocol of Hanekom et al. was followed (12). Briefly, *ex vivo* whole blood was directly stimulated with either the H1 fusion protein (5 μg/ml; Statens Serum Institute [SSI]) or phytohemagglutinin (5 μg/ml; BioWeb) or left unstimulated. The costimulatory antibodies anti-CD28 and anti-CD49d (0.5 μg/ml; Becton Dickinson [BD] Biosciences) were included in all assay conditions. Whole-blood samples were incubated for 7 h at 37°C and 5% CO_2 in an incubator and then treated with brefeldin A (10 μg/ml; Sigma-Aldrich). Subsequently, the whole-blood samples were transferred to a water bath at 37°C and incubated for an additional 5 h. Afterward, the water bath was switched off, and the water and contents were allowed to slowly reach room temperature. At 10 h after the water bath was switched off, the samples were harvested, EDTA (1.8 mM; Sigma-Aldrich) was added, and red blood cells were lysed in fluorescence-activated cell sorter (FACS) lysing solution (BD Biosciences). The remaining white blood cells were preserved in cryo solution containing 50% RPMI 1640 (Lonza), 40% fetal calf serum (BioWest), and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

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) (Bio-Whittaker), 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-QDot 605 (S3.5; Invitrogen), CD8-peridinin chlorophyll protein (PerCP)-Cy5.5 (SK1; BD Biosciences), CCR7-phycoerythrin (PE) (150503; eBioscience), IFN-μ-Alexa 700 (B27; BD Biosciences), tumor necrosis factor alpha (TNF-a)-Cy7-PE (Mb11; eBioscience), interleukin-2 (IL-2)-fluorescein isothiocyanate (FITC) (5344.111; BD Biosciences), IL-17-Alexa 647 (SCPL1362; eBioscience), and CD45RA-BV570 (HI100; eBioscience) (see Table 2.1 in the MyFlowCyt Reporting Standard in the supplemental material). For each fluorochrome, a single-stained mouse n CompBead (BD Biosciences) 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), violet (405 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-μ-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-μ-Alexa 700 versus CD4-QDot 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-QDot 605. FlowJo version 10.0.7 was used for the analysis. This project focused on the detection of the cytokines IL-2, IFN-μ, and TNF-a. The production of IL-17 by $CD4^+$ T cells was not significantly different after booster vaccination (10).

A detailed description following the MIFlowCyt 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.

AmpliSeq panels. We used two custom-made AmpliSeq 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 AmpliSeq primer panel, the Immune Response Panel, was developed based on a literature-driven collection of 826 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

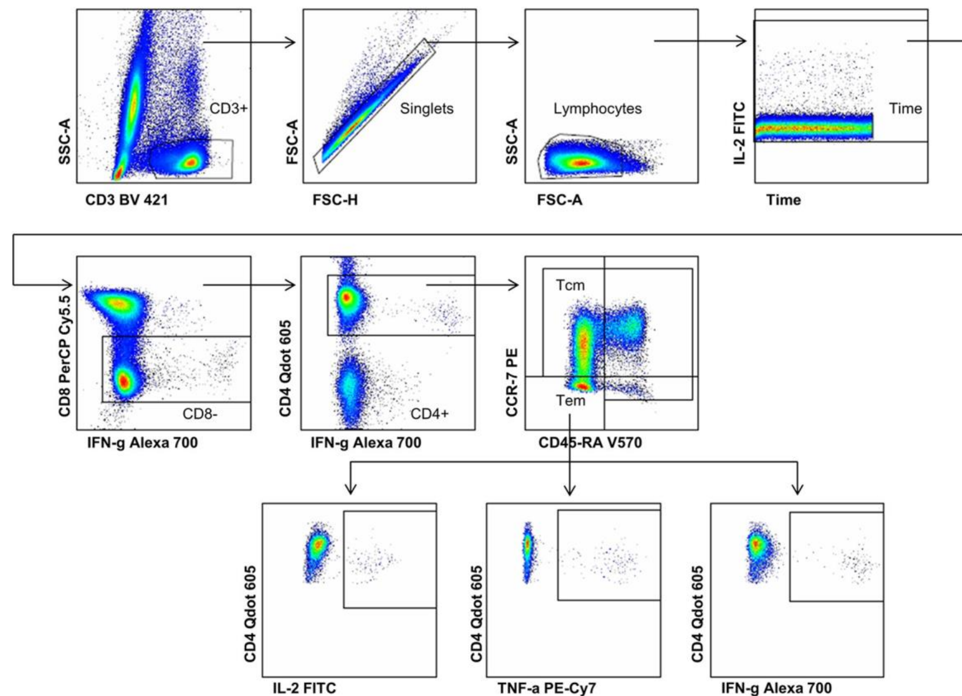


FIG 1 Gating strategy used for flow cytometric analysis of H1-induced memory CD4+ T cell cytokine expression. The plots show the sequential gating hierarchy of one representative sample: CD3+ T cells; single cells; lymphocytes; time; and CD8+ T cells, which were further gated for CD4+ cells and split 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-a, and IFN-μ expression levels were measured. T_{CM}, central memory CD4+ T cells; T_{EM}, effector memory CD4+ T cells; H1, hybrid 1.

for primer design and synthesis using proprietary algorithms (Ampliseq; Life Technologies).

Amplicon-based transcript quantification by semiconductor sequencing. The amplicon-based transcript quantification was performed using an ion proton semiconductor sequencer. This methodology was previously described by Zhang et al. (11) and strictly followed. Briefly, AmpliSeq libraries were prepared using 30 ng total RNA according to the protocol supplied with the ion AmpliSeq RNA library kit (catalog no. 4472335; Life Technologies, Carlsbad, CA, USA). The amplified and purified libraries were stored at —20°C. Library size distribution and concentration were measured using an Agilent high-sensitivity DNA kit (Agilent Technologies) according to the manufacturer's recommendation. Following library preparation, barcoded samples were pooled and processed together. The multiplexed library (total 8 pM) was linked to ion sphere particles and clonally amplified by emulsion PCR using the Ion PI template OT2 200 kit v3 with the Ion OneTouch 2 instrument according to the manufacturer's protocol (Life Technologies). Sequencing was performed using the Ion PI sequencing 200 kit v3 and the Ion Proton chip I, following the manufacturer's instructions, on the Ion Proton sequencer (Life Technologies). The generated reads were aligned to the *Homo sapiens* RNA canonical transcript reference hg19 and mapped to the genes of the corresponding AmpliSeq panel using the Torrent Mapping Alignment Program. Simultaneously, single nucleotide polymorphisms (SNPs) within the amplicons were identified during this process using the Ion Torrent Variant Caller.

Statistical analysis. (i) Analysis of memory CD4+ T cells. A two-sided Wilcoxon signed-rank test was performed to test for the significance of the polyfunctional memory CD4+ T cell subset between study days. The participants' responses to the vaccine were calculated using the Mimosa package within R software (14, 15). After correction for the T cell response obtained from unstimulated controls, absolute counts of cytokine (IFN-μ and/or IL-2 and/or TNF-a)-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 nonresponder, an FDR of $>10^{15}$ for an intermediate responder, and an FDR of $<10^{15}$ for a high responder.

(ii) AmpliSeq data analysis. AmpliSeq data were analyzed based on negative binomial distribution using the Bioconductor edgeR package (16). For each vaccine responder group, we performed differential expression analysis through the time course, comparing the two postvaccination time points to the prevaccination time point. Furthermore, gene expression was compared between the vaccine responder groups in order to identify values that differed from baseline. Standard settings were used, and only those genes with expression higher than 5 counts per million reads in at least 3 samples and an FDR of <0.1 were considered differentially expressed. Annotation of differentially expressed genes was performed using the previously published blood transcription modules (BTMs) (17). Statistical analysis was performed and plots were made using R version 3.1.0 (15).

RESULTS

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

TABLE 1 H1/IC31 clinical trial overview and results

Characteristic[a]	Value for: Placebo group (n = 4)	H1/IC31 vaccinated group (n = 19)
Age (median [IQR]) (yr)	38 (28—49)	39 (34—45)
Gender (F/M)	3/1	9/10
No. (%) with prior BCG vaccination (self-report)	4 (100)	19 (100)
No. (%) ART naive	4 (100)	19 (100)
CD4 count (median [IQR]) from baseline (cells/mm^3)	575.3 (425 – 617)[b]	656.9 (532—745)[b]
Viral load (median [IQR]) from baseline (copies/ml)	33,802 (514 – 49,875)[b]	37,932 (8,754—50,950)[b]

[a] IQR, interquartile range; F, female; M, male.
[b] No significant difference over the 182-day follow-up.

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/mm^3 (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, T_{CM} and T_{EM}, 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-μ, and TNF-a following *in vitro* stimulation with the H1 protein. At day 182, T_{CM} and T_{EM} 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 T_{CM} and T_{EM}

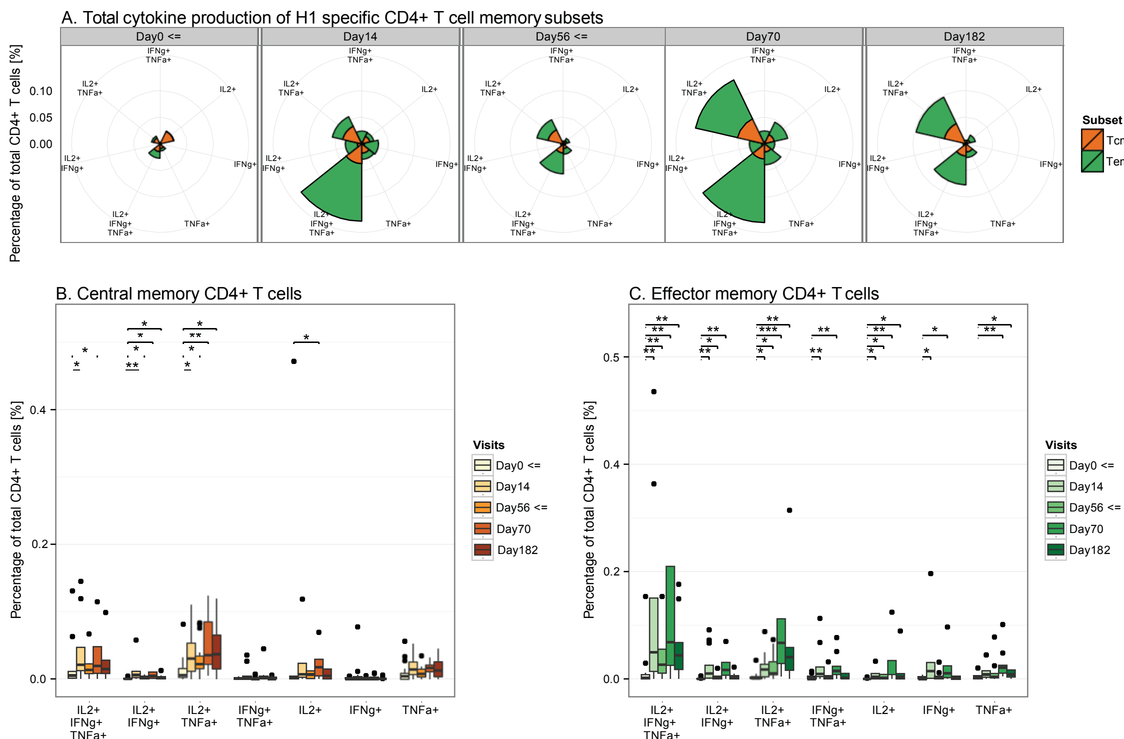


FIG 2 Cytokine expression profile of memory CD4$^+$ T cell subsets following H1 fusion protein stimulation. Volunteers receiving H1/IC31 at days 0 and 56 are included. (A) Mean percentages of all memory CD4$^+$ T cell subsets on the basis of all possible combinations of IL-2, IFN-μ, and TNF-a expression are given. Radii and not area of each segment represent the percentages of CD4$^+$ T cells. T_{CM} (B) and T_{EM} (C) expressing all combinations of IL-2, IFN-μ, and TNF-a expression following stimulation with H1. P values correspond to significance testing comparing each study day to study day 0 (*, P < 0.0125; **, P < 0.001; ***, P < 0.0001). A two-sided Wilcoxon signed-rank test with the Bonferroni correction was applied. T_{CM}, central memory CD4$^+$ T cells; T_{EM}, effector memory CD4$^+$ T cells; H1, hybrid 1; ☾, vaccination days.

TABLE 2 Overview of responder groups based on either T_{CM} or T_{EM}

T cell type	No. of nonresponders/intermediate responders/high responders at:			
	Day 14	Day 56	Day 70	Day 182
T_{CM}	6/6/7	11/3/5	5/6/8	8/5/6
T_{EM}	3/5/11	4/5/10	2/2/5	4/6/9

were predominantly trifunctional or bifunctional, expressing IL-2 and TNF-a.

In Fig. 2B and C, T_{CM} and T_{EM} are displayed separately. In a comparison of day 0 to day 182, statistically significant increases of bifunctional T_{CM} expressing IL-2 with TNF-a ($P < 0.05$) were observed. This was also observed in bifunctional T_{CM} expressing IL-2 with IFN-μ, although at extremely low percentages.

Trifunctional and bifunctional (IL-2 and TNF-a) T_{EM} were most significantly elevated in the comparison of day 182 to day 0 ($P < 0.01$), followed by monofunctional (IL-2 or TNF-a) T_{EM} ($P < 0.05$).

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-μ, IL-2, and/or TNF-a), comparing H1-specific T_{CM} and T_{EM}.

An overview of potential responder groupings 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 T_{CM} and T_{EM} maintenance.

Gene expression data. On average, 4,686,802 reads (95% confidence interval [CI], ±712,607 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-n to genes expressing more than 160,000 reads like lysozyme and CD74. Both AmpliSeq panels shared 355 genes, whose transcript abundance measurements closely correlated ($R^2 = 0.9829$).

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. T_{CM} and T_{EM} intermediate and high responders showed upregulated gene expression levels 3 days postvaccination. For T_{CM} responders, 5 genes, namely, COL1A1, ELN, CTGF, SERPINE1, and POSTN, were identified as upregulated. T_{EM} 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 involved in integrin interactions or extracellular matrix and cell adhesion (COL1A1, COL1A2, COL3A1, CTGF, FN1, and POSTN) or known to be part of extracellular matrix remodeling (ELN or SERPINE1). ID1 is involved in leukocyte differentiation. HSPA1A is the only gene which was upregulated in a T_{EM} 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 T_{CM} or T_{EM}. Compared to levels in nonresponders, higher gene expression levels were observed for ITGB4, CLEC1C, and CXCL6 for T_{CM} high responders and for PRKDC, CD19, DNAJB5, MARCO, and IRS2 for T_{EM} high responders. TGM2, **OAS1**, and **IFI27** showed the lowest expression levels among T_{CM} high responders, whereas AICDA, NFATC2, **IFI27**, **IFIT3**, **IFIT1**, and **CXCL10** were expressed at lower levels in T_{EM} high responders. Tissue transglutaminase (TGM) has a role in HIV pathogenesis and enhanced levels of its degradation product, ε(μ-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 3 Demographics including information about HIV infection status of final responder groups based on the comparison of day 0 to day 182

Day 182 responder group	No. of volunteers	Age (yr)[a]	No. F/no. M[b]	Viral load (log_2)[a]	$CD4^+$ count ($cells/mm^3$)[a]
Placebo	4	38 ± 13	3/1	11.5 ± 5.3	575 ± 250
T_{CM}					
High responder	6	35 ± 5	2/4	12.1 ± 3.8	823 ± 266
Intermediate responder	5	46 ± 5	2/3	14.0 ± 3.1	529 ± 64
Nonresponder	8	38 ± 6	6/2	14.3 ± 2.7	618 ± 181
T_{EM}					
High responder	9	38 ± 7	4/5	12.3 ± 3.4	698 ± 284
Intermediate responder	6	40 ± 9	2/4	14.4 ± 3.4	604 ± 90
Nonresponder	4	39 ± 5	4/0	14.9 ± 1.2	644 ± 226

[a] Data are means ± SD.
[b] F, female; M, male.

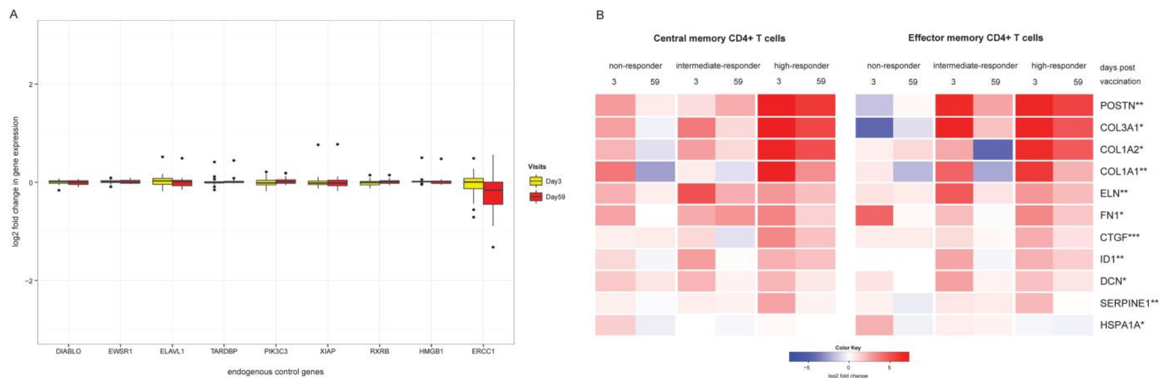


FIG 3 (A) Gene expression of endogenous control genes. Variations in expression levels of nine control genes between subjects and over time and $\log_2$-fold changes between baseline and the two visits after each vaccination for all 24 volunteers are shown. (B) Heat map showing vaccine-induced differential gene expression levels between T_{CM} and T_{EM} responder groups. Log_2-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 T_{CM} and T_{EM}; *, significant for T_{EM} 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-nB 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 T_{EM} nonresponder group, and all three subjects expressing the homozygous TLR-8 A1G were T_{EM} 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-a) T_{CM} and T_{EM} (Fig. 5B and C).

TABLE 4 Genes which are differentially expressed between the different vaccine responder groups for T_{CM} and T_{EM}

	Genes with expression levels:[a]	
T cell type	Higher than those in nonresponders	Lower than those in nonresponders[b]
T_{CM}	ITGB4	TGM2
	CLEC1A	**OAS1**
	CXCL6	**IFI27**
T_{EM}	PRKDC	AICDA
	CD19	NFATC2
	DNAJB5	**IFI27**
	MARCO	**IFIT3**
	IRS2	**IFIT1**
		CXCL10

[a] Differentially 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.
[b] Genes in bold are involved in innate antiviral immunity.

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 T_{CM} and T_{EM} 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-μ-producing H1-specific T cells were detectable by ELISpot analysis until 131 weeks after the first vaccination. Only limited retraction of IFN-μ production in cell culture

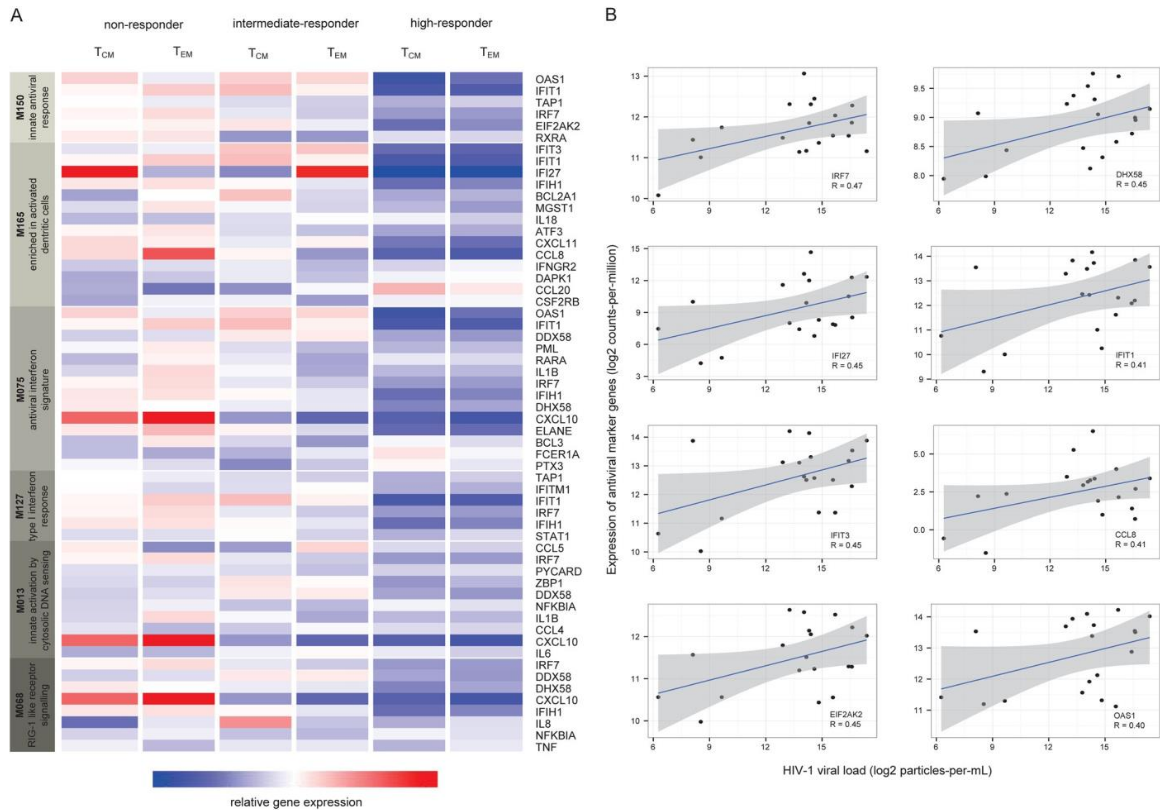


FIG 4 Differential gene expression at baseline prior to vaccination. (A) Innate antiviral signature at baseline. The expression levels of the five genes involved in innate antiviral immunity with their corresponding members of BTMs are shown for T_{CM} and T_{EM} responder groups. (B) Correlation of innate antiviral markers and viral load. Pearson correlation coefficients and 95% confidence intervals are shown. Only genes with Pearson correlation coefficients of >0.4 were considered. T_{CM}, central memory CD4^{+} T cells; T_{EM}, effector memory CD4^{+} T cells.

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 T_{CM} and T_{EM} 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 T_{CM} and T_{EM} was observed. Interestingly, this retraction differed greatly between volunteers, which allowed us to group them based on the comparison of H1/IC31-induced T_{CM} and T_{EM} 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 T_{CM} and T_{EM} responses until day 182. Searching the literature, we found that cells involved in wound healing like alternatively activated macrophages (26), μδ-T 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-1-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-

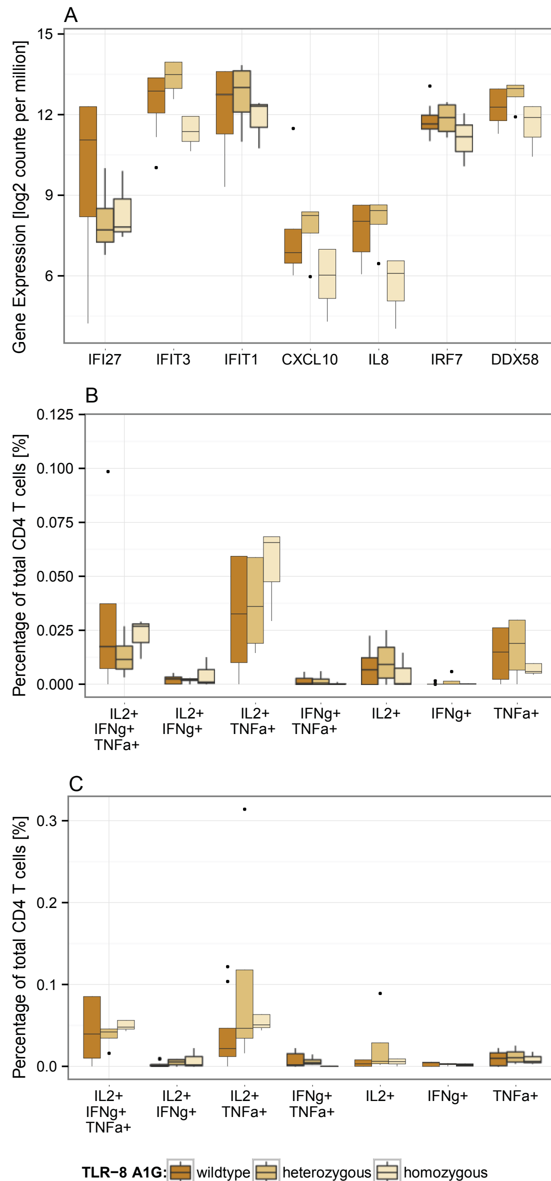


FIG 5 (A) Baseline expression of genes implicated in innate antiviral immunity comparing volunteers with either wild-type, heterozygous, or homozygous TLR-8 A1G. (B and C) Cytokine expression profiles of T_{CM} and T_{EM} following H1 fusion protein stimulation at day 182. The differences between volunteers with wild-type, heterozygous, and homozygous TLR-8 A1G are presented. Due to low numbers of volunteers with homozygous TLR-8 A1G, no statistical test was applied.

sults, HIV disease progressors are known to display a type I IFN chronic exposure signature when contrasted to HIV disease elite controllers (31, 32). The study by Negishi et al. reported that 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 T_{CM} and T_{EM} 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 T_{EM} 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.

ACKNOWLEDGMENTS

This work was supported by the European Developing Countries Clinical Trials Partnership (EDCTP) (grant IP.2009.32080.002) and the State Secretariat for Education and Research. The AmpliSeq analysis was funded by the Roche Innovation Center Basel.

We are grateful to all study participants. We also thank the sponsor, the Statens Serum Institut, and the members of the Data Safety Monitoring Board: Anthony Hawkridge, Jaap van Dissel, Clive Gray, Eyasu Makonnen, and Gibson Kibiki. We appreciate the support of Biolytix AG (www.biolytix.ch), who provided their RNA extraction platform.

REFERENCES

1. **Omer SB, Orenstein WA, Koplan JP.** 2013. Go big and go fast—vaccine refusal and disease eradication. N Engl J Med **368:**1374–1376. http://dx.doi.org/10.1056/NEJMp1300765.
2. **World Health Organization.** 2013. Global tuberculosis report. http://www.who.int/tb/publications/global_report/en/.
3. **Daley CL, Small PM, Schecter GF, Schoolnik GK, McAdam RA, Jacobs WR, Jr, Hopewell PC.** 1992. An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus. N Engl J Med **326:**231–235. http://dx.doi.org/10.1056/NEJM199201233260404.
4. **van Dissel JT, Soonawala D, Joosten SA, Prins C, Arend SM, Bang P, Tingskov PN, Lingnau K, Nouta J, Hoff ST, Rosenkrands I, Kromann I, Ottenhoff TH, Doherty TM, Andersen P.** 2010. Ag85B-ESAT-6 adjuvanted with IC31 promotes strong and long-lived *Mycobacterium tuberculosis* specific T cell responses in naive human volunteers. Vaccine **28:**3571–3581. http://dx.doi.org/10.1016/j.vaccine.2010.02.094.
5. **Aichinger MC, Ginzler M, Weghuber J, Zimmermann L, Riedl K, Schütz G, Nagy E, von Gabain A, Schweyen R, Henics T.** 2011. Adjuvating the adjuvant: facilitated delivery of an immunomodulatory oligonucleotide toTLR9 by a cationic antimicrobial peptide in dendritic cells. Vaccine **29:**426–436. http://dx.doi.org/10.1016/j.vaccine.2010.11.003.
6. **Blasius AL, Beutler B.** 2010. Intracellular Toll-like receptors. Immunity **32:**305–315. http://dx.doi.org/10.1016/j.immuni.2010.03.012.

7. **Kawasaki T, Kawai T.** 2014. Toll-like receptor signaling pathways. Front Immunol **5:**461. http://dx.doi.org/10.3389/fimmu.2014.00461.
8. **Okulicz JF, Mesner O, Ganesan A, O'Bryan TA, Deiss RG, Agan BK.** 2014. Hepatitis B vaccine responsiveness and clinical outcomes in HIV controllers. PLoS One **9:**e105591. http://dx.doi.org/10.1371/journal.pone.0105591.
9. **Iwasaki A.** 2012. Innate immune recognition of HIV-1. Immunity **37:** 389 – 398. http://dx.doi.org/10.1016/j.immuni.2012.08.011.
10. **Reither K, Katsoulis L, Beattie T, Gardiner N, Lenz N, Said K, Mfinanga E, Pohl C, Fielding KL, Jeffrey H, Kagina BM, Hughes EJ, Scriba TJ, Hanekom WA, Hoff ST, Bang P, Kromann I, Daubenberger C, Andersen P, Churchyard GJ.** 2014. Safety and immunogenicity of H1/IC31, an adjuvanted TB subunit vaccine, in HIV-infected adults with CD4$^+$ lymphocyte counts greater than 350 cells/mm^3: a phase II, multi-centre, double-blind, randomized, placebo-controlled trial. PLoS One **9:**e114602. http://dx.doi.org/10.1371/journal.pone.0114602.
11. **Zhang J, Schindler T, Küng E, Ebeling M, Certa U.** 2014. Highly sensitive amplicon-based transcript quantification by semiconductor sequencing. BMC Genomics **15:**565. http://dx.doi.org/10.1186/1471-2164-15-565.
12. **Hanekom WA, Hughes J, Mavinkurve M, Mendillo M, Watkins M, Gamieldien H, Gelderbloem SJ, Sidibana M, Mansoor N, Davids V, Murray RA, Hawkridge A, Haslett PA, Ress S, Hussey GD, Kaplan G.** 2004. Novel application of a whole blood intracellular cytokine detection assay to quantitate specific T-cell frequency in field studies. J Immunol Methods **291:**185 – 195. http://dx.doi.org/10.1016/j.jim.2004.06.010.
13. **Lee JA, Spidlen J, Boyce K, Cai J, Crosbie N, Dalphin M, Furlong J, Gasparetto M, Goldberg M, Goralczyk EM, Hyun B, Jansen K, Kollmann T, Kong M, Leif R, McWeeney S, Moloshok TD, Moore W, Nolan G, Nolan J, Nikolich-Zugich J, Parrish D, Purcell B, Qian Y, Selvaraj B, Smith C, Tchuvatkina O, Wertheimer A, Wilkinson P, Wilson C, Wood J, Zigon R, International Society for Advancement of Cytometry Data Standards Task Force, Scheuermann RH, Brinkman RR.** 2008. MIFlow-Cyt: the minimum information about a flow cytometry experiment. Cytometry A **73A:**926 – 930. http://dx.doi.org/10.1002/cyto.a.20623.
14. **Finak G, McDavid A, Chattopadhyay P, Dominguez M, De Rosa S, Roederer M, Gottardo R.** 2014. Mixture models for single-cell assays with applications to vaccine studies. Biostatistics **15:**87—101. http://dx.doi.org/10.1093/biostatistics/kxt024.
15. **R Core Team.** 2014. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
16. **Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP.** 2006. GenePattern 2.0. Nat Genet **38:**500 – 501. http://dx.doi.org/10.1038/ng0506-500.
17. **Li G, Cheng M, Nunoya J, Cheng L, Guo H, Yu H, Liu YJ, Su L, Zhang L.** 2014. Plasmacytoid dendritic cells suppress HIV-1 replication but contribute to HIV-1 induced immunopathogenesis in humanized mice. PLoS Pathog **10:**e1004291. http://dx.doi.org/10.1371/journal.ppat.1004291.
18. **Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A.** 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature **401:**708—712. http://dx.doi.org/10.1038/44385.
19. **Farrow MA, Kim EY, Wolinsky SM, Sheehy AM.** 2011. NFAT and IRF proteins regulate transcription of the anti-HIV gene, APOBEC3G. J Biol Chem **286:**2567 – 2577. http://dx.doi.org/10.1074/jbc.M110.154377.
20. **Xu W, Santini PA, Sullivan JS, He B, Shan M, Ball SC, Dyer WB, Ketas TJ, Chadburn A, Cohen-Gould L, Knowles DM, Chiu A, Sanders RW, Chen K, Cerutti A.** 2009. HIV-1 evades virus-specific IgG2 and IgA class switching by targeting systemic and intestinal B cells via long-range intercellular conduits. Nat Immunol **10:**1008 – 1017. http://dx.doi.org/10.1038/ni.1753.
21. **Oh DY, Taube S, Hamouda O, Kücherer C, Poggensee G, Jessen H, Eckert JK, Neumann K, Storek A, Pouliot M, Borgeat P, Oh N, Schreier E, Pruss A, Hattermann K, Schumann RR.** 2008. A functional Toll-like receptor 8 variant is associated with HIV disease restriction. J Infect Dis **198:**701 – 709. http://dx.doi.org/10.1086/590431.
22. **Kernéis S, Launay O, Turbelin C, Batteux F, Hanslik T, Boëlle PY.** 2014. Long-term immune responses to vaccination in HIV-infected patients: a systematic review and meta-analysis. Clin Infect Dis **58:**1130 – 1139. http://dx.doi.org/10.1093/cid/cit937.
23. **Seder RA, Hill AV.** 2000. Vaccines against intracellular infections requiring cellular immunity. Nature **406:**793 – 798. http://dx.doi.org/10.1038/35021239.
24. **Farber DL, Yudanin NA, Restifo NP.** 2014. Human memory T cells: generation, compartmentalization and homeostasis. Nat Rev Immunol **14:**24 – 35. http://dx.doi.org/10.1038/nri3567.
25. **van Dissel JT, Soonawala D, Joosten SA, Prins C, Arend SM, Bang P, Tingskov PN, Lingnau K, Nouta J, Hoff ST, Rosenkrands I, Kromann I, Ottenhoff TH, Doherty TM, Andersen P.** 2011. Ag85B-ESAT-6 adjuvanted with IC31 promotes strong and long-lived *Mycobacterium tuberculosis* specific T cell responses in volunteers with previous BCG vaccination or tuberculosis infection. Vaccine **29:**2100 – 2109. http://dx.doi.org/10.1016/j.vaccine.2010.12.135.
26. **Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M.** 2013. Macrophage plasticity and polarization in tissue repair and remodelling. J Pathol **229:**176 – 185. http://dx.doi.org/10.1002/path.4133.
27. **Rani M, Zhang Q, Schwacha MG.** 2014. Gamma delta T cells regulate wound myeloid CELL activity after burn. Shock **42:**133 – 141. http://dx.doi.org/10.1097/SHK.0000000000000176.
28. **Seta N, Kuwana M.** 2010. Derivation of multipotent progenitors from human circulating CD14$^+$ monocytes. Exp Hematol **38:**557 – 563. http://dx.doi.org/10.1016/j.exphem.2010.03.015.
29. **Gremmels H, Fledderus JO, van Balkom BW, Verhaar MC.** 2011. Transcriptome analysis in endothelial progenitor cell biology. Antioxid Redox Signal **15:**1092 – 1042. http://dx.doi.org/10.1089/ars.2010.3594.
30. **Muyanja E, Ssemaganda A, Ngauv P, Cubas R, Perrin H, Srinivasan D, Canderan G, Lawson B, Kopycinski J, Graham AS, Rowe DK, Smith MJ, Isern S, Michael S, Silvestri G, Vanderford TH, Castro E, Pantaleo G, Singer J, Gillmour J, Kiwanuka N, Nanvubya A, Schmidt C, Birungi J, Cox J, Haddad EK, Kaleebu P, Fast P, Sekaly RP, Trautmann L, Gaucher D.** 2014. Immune activation alters cellular and humoral responses to yellow fever 17D vaccine. J Clin Invest **124:**3147 – 3158. http://dx.doi.org/10.1172/JCI75429.
31. **Hyrcza MD, Kovacs C, Loutfy M, Halpenny R, Heisler L, Yang S, Wilkins O, Ostrowski M, Der SD.** 2007. Distinct transcriptional profiles in *ex vivo* CD4$^+$ and CD8$^+$ T cells are established early in human immunodeficiency virus type 1 infection and are characterized by a chronic interferon response as well as extensive transcriptional changes in CD8$^+$ T cells. J Virol **81:**3477 – 3486. http://dx.doi.org/10.1128/JVI.01552-06.
32. **Rotger M, Dalmau J, Rauch A, McLaren P, Bosinger SE, Martinez R, Sandler NG, Roque A, Liebner J, Battegay M, Bernasconi E, Descombes P, Erkizia I, Fellay J, Hirschel B, Miró JM, Palou E, Hoffmann M, Massanella M, Blanco J, Woods M, Günthard HF, de Bakker P, Douek DC, Silvestri G, Martinez-Picado J, Telenti A.** 2011. Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque. J Clin Invest **121:**2391 – 2400. http://dx.doi.org/10.1172/JCI45235.
33. **Negishi H, Yanai H, Nakajima A, Koshiba R, Atarashi K, Matsuda A, Matsuki K, Miki S, Doi T, Aderem A, Nishio J, Smale ST, Honda K, Taniguchi T.** 2012. Cross-interference of RLR and TLR signaling pathways modulates antibacterial T cell responses. Nat Immunol **13:**659 – 666. http://dx.doi.org/10.1038/ni.2307.
34. **Okoye AA, Picker LJ.** 2013. CD4$^+$ T-cell depletion in HIV infection: mechanisms of immunological failure. Immunol Rev **254:**54 – 64. http://dx.doi.org/10.1111/imr.12066.
35. **Zhang JD, Küng E, Boess F, Certa U, Ebeling M.** 2015. Pathway reporter genes define molecular phenotypes of human cells. BMC Genomics **16:** 342. http://dx.doi.org/10.1186/s12864-015-1532-2.

PLOS | ONE

RESEARCH ARTICLE

7.1.3

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

Damien Portevin[1,2]*, Félicien Moukambi[3,4], Maxmillian Mpina[5], Asli Bauer[3,4], Frederick Haraka[5], Mkunde Chachage[3], Philipp Metzger[1], Elmar Saathoff[4,6], Petra Clowes[3,4], Nyanda E. Ntinginya[3], Andrea Rachow[4,6], Michael Hoelscher[4,6], Klaus Reither[1,2,5], Claudia A. Daubenberger[1,2], Christof Geldmacher[4,6]*

1 Swiss Tropical and Public Health Institute, Basel, Switzerland, 2 University of Basel, Basel, Switzerland, 3 NIMR-Mbeya Medical Research Centre, Mbeya, Tanzania, 4 Department of Infectious Diseases and Tropical Medicine, Medical Centre of the University of Munich, Munich, Germany, 5 Ifakara Health Institute, Bagamoyo, Tanzania, 6 German Centre for Infection Research (DZIF), partner site Munich, Munich, Germany

* geldmacher@lrz.uni-muenchen.de (CG); damien.portevin@unibas.ch (DP)

OPEN ACCESS

Citation: Portevin D, Moukambi F, Mpina M, Bauer A, Haraka F, Chachage M, et al. (2015) 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. PLoS ONE 10(5): e0126716. doi:10.1371/journal.pone.0126716

Academic Editor: William A Paxton, Institute of Infection and Global Health, UNITED KINGDOM

Received: January 1, 2015

Accepted: April 7, 2015

Published: May 14, 2015

Copyright: © 2015 Portevin et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authors gratefully acknowledge funding from the European and Developing Countries Clinical Trials Partnership (EDCTP) for this study as part of the TB CHILD project (IP.2009.32040.007). Co-funding was provided by the German Federal Ministry of Education and Research (BMBF Grant 01KA1006), the Swiss National Science Foundation (32EC30_131192/1) and the German Research Foundation (DFG, grant SA 1878/1-1). Christof

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 (Ç16 years of age, n = 108 and Ç18 years, n = 79). Independent of HIV co-infection, $IFN\gamma^+$ 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 $CD27^{low}$ 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 [1]. Thereafter, CMV persists lifelong in

Geldmacher`s salary is funded by the German Center for Infection Research (DZIF). Damien Portevin's salary was funded by the Basel University (Förderung exzellenter junger Forschender). Philipp Metzger was supported by the German Academic Exchange Service (DAAD) with a PROMOS scholarship.

Competing Interests: The authors have declared that no competing interests exist.

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 [4]. In late-stage AIDS patients (CD4 counts <50/μl), CMV infection can cause disseminated or localized end-organ disease [5]. CMV-specific $CD4^+$ T cell responses appear to be crucial for CMV control in murine models and in humans [6],[4]. 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-1βproduction 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 (n = 31) 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

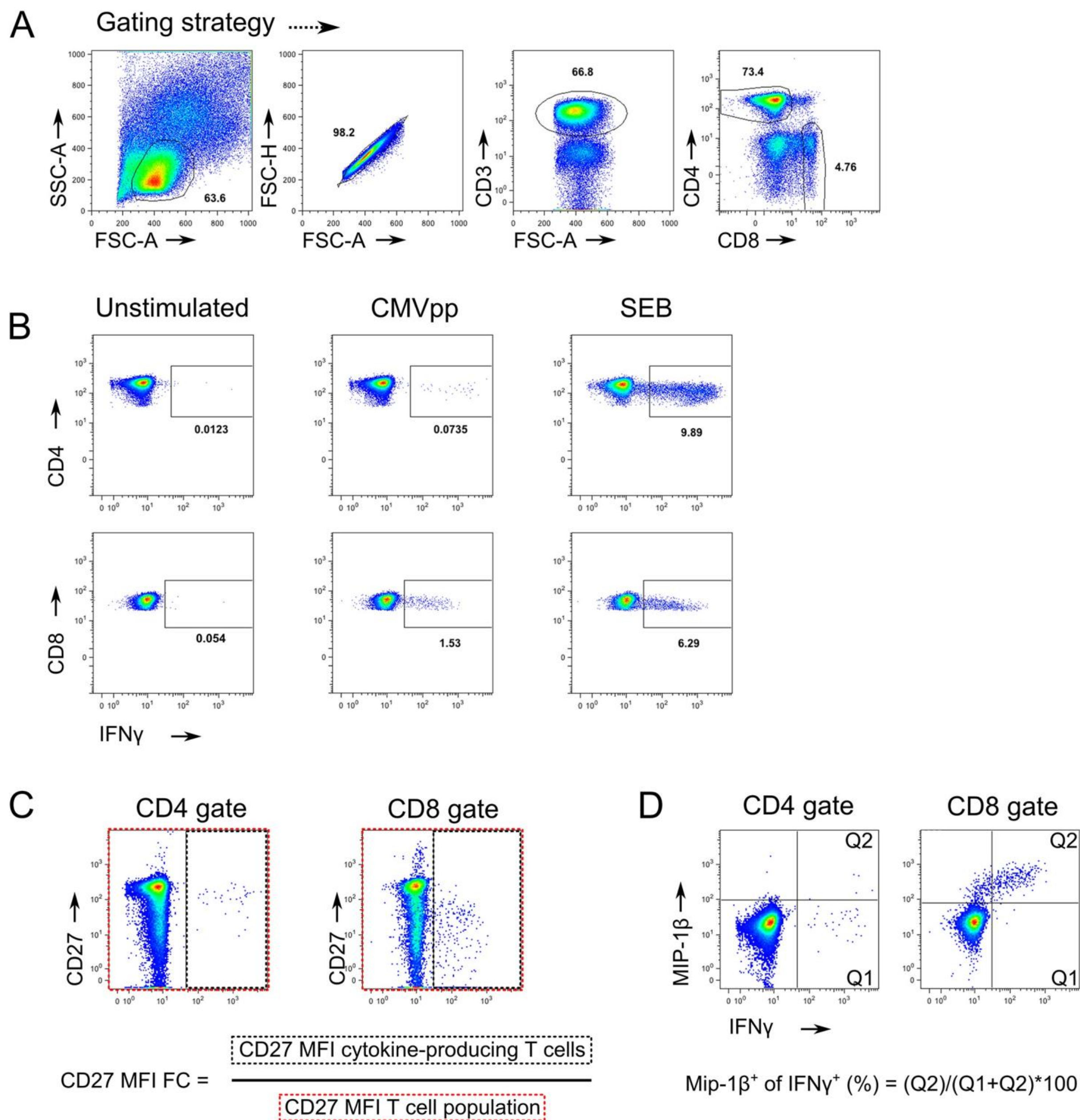


Fig 1. Gating strategies and CMV responses evaluation. A) Morphological and CD marker expression gating strategy of CD4 and CD8 T cell. B) Representative CD4 and CD8 T cell IFNγ response from a significant responder. C) Gating strategy for the CD27 MFI Fold Change calculation of IFNγ producing cells 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.

doi:10.1371/journal.pone.0126716.g001

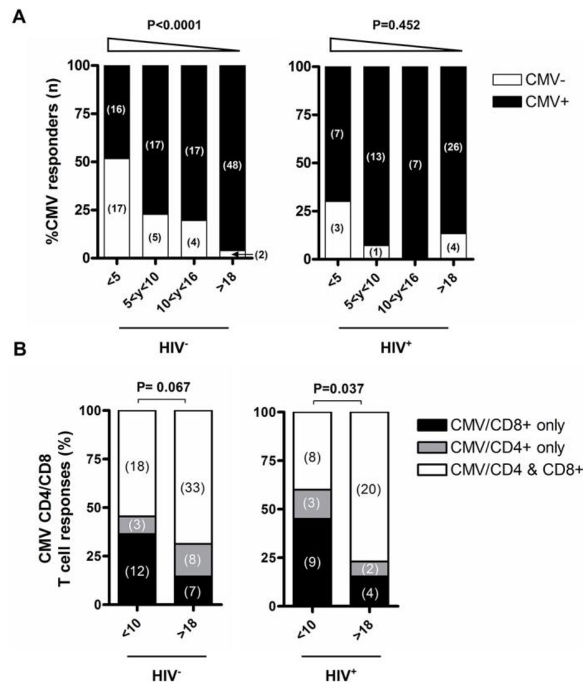


Fig 2. CMVpp65-specific T cell responses in relation to age and HIV infection status. (A) Frequency of subjects with or without CMVpp65-specific T cell responses ($CD4^+$ or $CD8^+$) is shown stratified by age group and HIV status. B) The frequency of subjects with CMVpp65-specific T cell responses distributed 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 $IFN\gamma^+$ 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).

doi:10.1371/journal.pone.0126716.g002

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

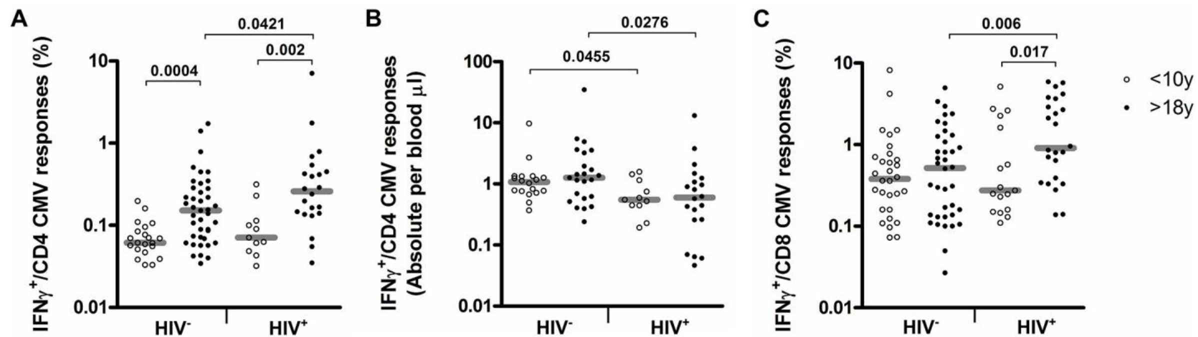


Fig 3. Frequencies and absolute counts of IFNγ+-CMVpp65–specific CD4+ or CD8+ T cell in children below 10 years of age and adults. Scatter-plots depicting A) the frequencies or absolute counts of CMV specific IFNγ+-CD4+ T cells s and C) frequencies of CMV specific IFNγ+-CD8+ T cells in the two-age groups and according to HIV status.

doi:10.1371/journal.pone.0126716.g003

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-1β secretion [7] together with their mature phenotype, characterized by a high proportion of cells expressing the senescence marker CD57 [28]. Expression of CD57 on

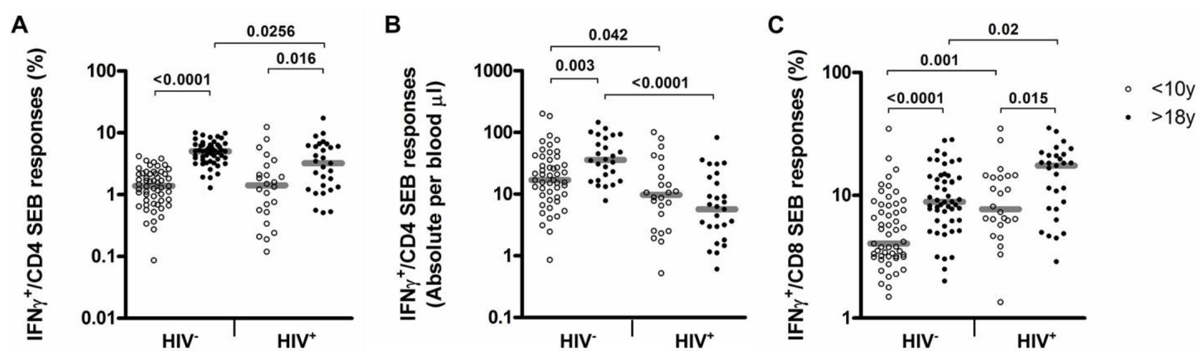


Fig 4. Frequencies and absolute counts of IFNγ+-SEB–specific CD4+ or CD8+ T cell in children below 10 years of age and adults. Scatter-plots depicting A) the frequencies or B) absolute counts of SEB specific IFNγ+-CD4+ T cells s and C) frequencies of SEB specific IFNγ+-CD8+ T cells in the two-age groups and according to HIV status.

doi:10.1371/journal.pone.0126716.g004

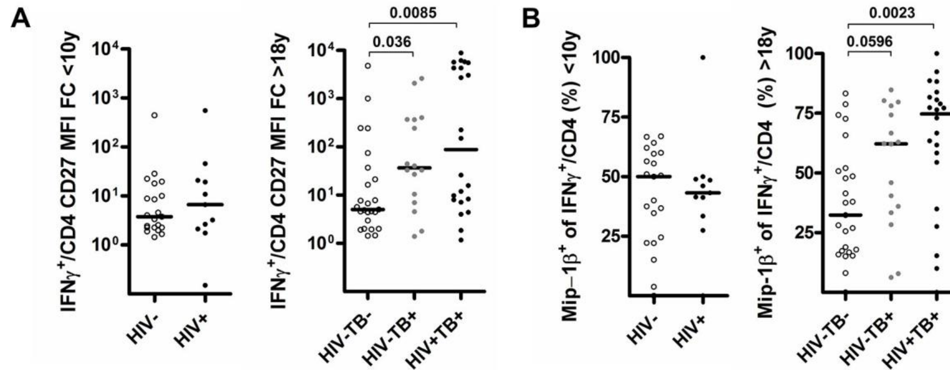


Fig 5. CD27 and Mip-1β expression of CMV specific IFNγ producing CD4 T cell responses are influenced by HIV as well as tuberculosis co-infection in adults. Scatter-plots illustrating (A) the CD27 MFI fold-change to all $CD4^+$ T cells and B) the proportion of cells producing Mip-1β of CMV specific $IFN\gamma^+$-$CD4^+$ T cell in patients under 10 or over 18 years old and according to HIV and TB infection status.

doi:10.1371/journal.pone.0126716.g005

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-1βproduction in $IFN\gamma^+$ CMV-specific $CD4^+$ T cells in HIV^- subjects (data not shown). Both HIV^- adults and children had $IFN\gamma^+$ CMV-specific $CD4^+$ T cells characterized by a $CD27^{low}$ 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

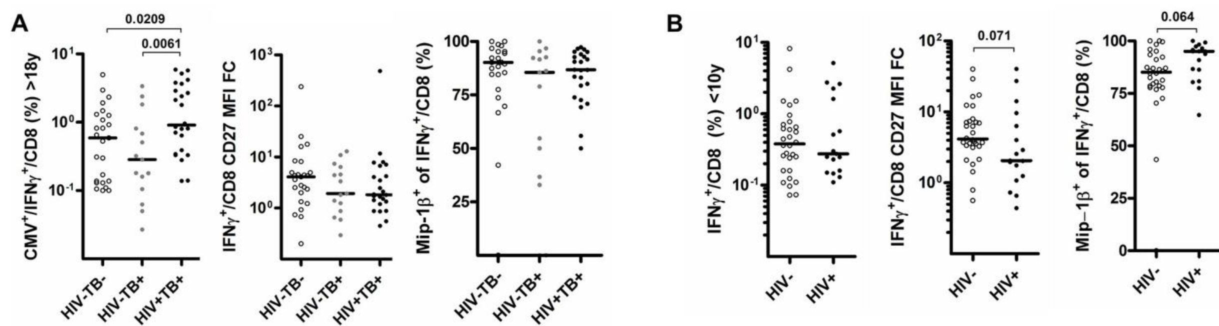


Fig 6. CD27 and Mip-1β expression of CMV specific IFNγ producing CD8 T cell responses are not significantly influenced by HIV as well as tuberculosis co-infection in adults. Scatter-plots illustrating (A) the CD27 MFI fold-change to all $CD8^+$ T cells and B) the proportion of cells producing Mip-1β of CMV specific $IFN\gamma^+$-$CD8^+$ T cell in patients under 10 or over 18 years old and according to HIV and TB infection status.

doi:10.1371/journal.pone.0126716.g006

tuberculosis on the CD27 phenotype of CMV-specific CD8$^+$ T cells was not observed (Fig 6A and 6B). Based on these results, we hypothesis that 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^{low} 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-1βproduction 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)

Acknowledgments

We thank all the children who participated in the study and their care takers and the adult study volunteers.

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. Wrote the paper: DP CD KR CG. Involved in study participant's recruitment: PC FH ES NN. Analysed TB diagnostic results: AR.

References

1. Cannon MJ, Hyde TB, Schmid DS. Review of cytomegalovirus shedding in bodily fluids and relevance to congenital cytomegalovirus infection. Rev. Med. Virol. 2011; 21:240-255. doi: 10.1002/rmv.695 PMID: 21674676

2. Adler SP. Cytomegalovirus and Child Day Care. N. Engl. J. Med. 1989; 321:1290-1296. doi: 10.1056/NEJM198911093211903 PMID: 2552316
3. Stagno S, Cloud GA. Working parents: the impact of day care and breast-feeding on cytomegalovirus infections in offspring. Proc. Natl. Acad. Sci. U. S. A. 1994; 91:2384-2389. PMID: 8146127
4. Waller ECP, Day E, Sissons JGP, Wills MR. Dynamics of T cell memory in human cytomegalovirus infection. Med. Microbiol. Immunol. (Berl.). 2008; 197:83-96. doi: 10.1007/s00430-008-0082-5 PMID: 18301918
5. Henry Masur JEK. Guidelines for Prevention and Treatment of Opportunistic Infections in HIV-Infected Adults and Adolescents; Recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America. Available: http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5804a1.htm [Accessed 2014 Sep. 22].
6. Bronke C, Palmer NM, Jansen CA, Westerlaken GHA, Polstra AM, Reiss P, et al. Dynamics of Cytomegalovirus (CMV)-Specific T Cells in HIV-1-Infected Individuals Progressing to AIDS with CMV End-Organ Disease. J. Infect. Dis. 2005; 191:873-880. doi: 10.1086/427828 PMID: 15717261
7. Casazza JP, Betts MR, Price DA, Precopio ML, Ruff LE, Brenchley JM, et al. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. J. Exp. Med. 2006; 203:2865-2877. doi: 10.1084/jem.20052246 PMID: 17158960
8. Waldrop SL, Pitcher CJ, Peterson DM, Maino VC, Picker LJ. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. J. Clin. Invest. 1997; 99:1739-1750. doi: 10.1172/JCI119338 PMID: 9120019
9. Casazza JP, Brenchley JM, Hill BJ, Ayana R, Ambrozak D, Roederer M, et al. Autocrine production of beta-chemokines protects CMV-Specific CD4 T cells from HIV infection. PLoS Pathog. 2009; 5: e1000646. doi: 10.1371/journal.ppat.1000646
10. Geldmacher C, Ngwenyama N, Schuetz A, Petrovas C, Reither K, Heeregrave EJ, et al. Preferential infection and depletion of Mycobacterium tuberculosis-specific CD4 T cells after HIV-1 infection. J. Exp. Med. 2010; 207:2869-2881. doi: 10.1084/jem.20100090 PMID: 21115690
11. Brenchley JM, Ruff LE, Casazza JP, Koup RA, Price DA, Douek DC. Preferential infection shortens the life span of human immunodeficiency virus-specific CD4+ T cells in vivo. J. Virol. 2006; 80:6801-6809. doi: 10.1128/JVI.00070-06 PMID: 16809286
12. Harari A, Vallelian F, Meylan PR, Pantaleo G. Functional heterogeneity of memory CD4 T cell responses in different conditions of antigen exposure and persistence. J. Immunol. Baltim. Md 1950. 2005; 174:1037-1045.
13. Streitz M, Tesfa L, Yildirim V, Yahyazadeh A, Ulrichs T, Lenkei R, et al. Loss of receptor on tuberculin-reactive T-cells marks active pulmonary tuberculosis. PloS One. 2007; 2:e735. doi: 10.1371/journal.pone.0000735 PMID: 17710135
14. Schuetz A, Haule A, Reither K, Ngwenyama N, Rachow A, Meyerhans A, et al. Monitoring CD27 expression to evaluate Mycobacterium tuberculosis activity in HIV-1 infected individuals in vivo. PloS One. 2011; 6:e27284. doi: 10.1371/journal.pone.0027284 PMID: 22087280
15. Brantsæter AB, Johannessen A, Holberg-Petersen M, Sandvik L, Naman E, Kivuyo SL, et al. Cytomegalovirus viremia in dried blood spots is associated with an increased risk of death in HIV-infected patients: a cohort study from rural Tanzania. Int. J. Infect. Dis. IJID Off. Publ. Int. Soc. Infect. Dis. 2012; 16:e879-885. doi: 10.1016/j.ijid.2012.08.003 PMID: 23031419
16. Ritter M, Schmidt T, Dirks J, Hennes P, Juhasz-Böss I, Solomayer EF, et al. Cytomegalovirus-specific T cells are detectable in early childhood and allow assignment of the infection status in children with passive maternal antibodies: Clinical immunology. Eur. J. Immunol. 2013; 43:1099-1108. doi: 10.1002/eji.201243100 PMID: 23280326
17. Portevin D, Moukambi F, Clowes P, Bauer A, Chachage M, Ntinginya NE, *et al*. Assessment of the novel T-cell activation marker-tuberculosis assay for diagnosis of active tuberculosis in children: a prospective proof-of-concept study. *Lancet Infect. Dis*. 2014. Available: http://linkinghub.elsevier.com/retrieve/pii/S1473309914708849 [Accessed 2014 Sep 18]. doi: 10.1016/S1473-3099(14)70884-9
18. Bello C, Whittle H. Cytomegalovirus infection in Gambian mothers and their babies. J. Clin. Pathol. 1991; 44:366-369. doi: 10.1136/jcp.44.5.366
19. Kaye S, Miles D, Antoine P, Burny W, Ojuola B, Kaye P, et al. Virological and Immunological Correlates of Mother-to-Child Transmission of Cytomegalovirus in The Gambia. J. Infect. Dis. 2008; 197:1307-1314. doi: 10.1086/586715 PMID: 18422443
20. Soares AP, Kwong Chung CKC, Choice T, Hughes EJ, Jacobs G, van Rensburg EJ, et al. Longitudinal Changes in CD4+ T-Cell Memory Responses Induced by BCG Vaccination of Newborns. J. Infect. Dis. 2013; 207:1084-1094. doi: 10.1093/infdis/jis941 PMID: 23293360

21. Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C, Ruchti F, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J. Exp. Med. 2005; 202:673-685. doi: 10.1084/jem.20050882

22. Hanna-Wakim R, Yasukawa LL, Sung P, Fang M, Sullivan B, Rinki M, et al. Age-Related Increase in the Frequency of CD4 + T Cells That Produce Interferon-γ in Response to Staphylococcal Enterotoxin B during Childhood. J. Infect. Dis. 2009; 200:1921-1927. doi: 10.1086/648375

23. Faist B, Fleischer B, Jacobsen M. Cytomegalovirus Infection- and Age-Dependent Changes in Human CD8+ T-Cell Cytokine Expression Patterns. Clin. Vaccine Immunol. 2010; 17:986-992. doi: 10.1128/CVI.00455-09 PMID: 20427631

24. Pera A, Campos C, Corona A, Sanchez-Correa B, Tarazona R, Larbi A, CMV Latent Infection Improves CD8+ T Response to SEB Due to Expansion of Polyfunctional CD57+ Cells in Young Individuals Björkström NK, ed. PLoS ONE. 2014; 9:e88538. doi: 10.1371/journal.pone.0088538 PMID: 24533103

25. Wilson CB, Westall J, Johnston L, Lewis DB, Dower SK, Alpert AR. Decreased production of interferon-gamma by human neonatal cells. Intrinsic and regulatory deficiencies. J. Clin. Invest. 1986; 77:860-867. doi: 10.1172/JCI112383

26. White GP, Watt PM, Holt BJ, Holt PG. Differential patterns of methylation of the IFN-gamma promoter at CpG and non-CpG sites underlie differences in IFN-gamma gene expression between human neonatal and adult CD45RO- T cells. J. Immunol. Baltim. Md 1950. 2002; 168:2820-2827.

27. Geldmacher C, Schuetz A, Ngwenyama N, Casazza JP, Sanga E, Saathoff E, et al. Early depletion of Mycobacterium tuberculosis-specific T helper 1 cell responses after HIV-1 infection. J. Infect. Dis. 2008; 198:1590-1598. doi: 10.1086/593017 PMID: 19000013

28. Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, Casazza JP, et al. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. Blood. 2003; 101:2711-2720. doi: 10.1182/blood-2002-07-2103 PMID: 12433688

29. Miles DJC, Sande M van der, Kaye S, Crozier S, Ojuola O, Palmero MS, et al. CD4 + T Cell Responses to Cytomegalovirus in Early Life: A Prospective Birth Cohort Study. J. Infect. Dis. 2008; 197:658-662. doi: 10.1086/527418

30. Libri V, Azevedo RI, Jackson SE, Di Mitri D, Lachmann R, Fuhrmann S, et al. Cytomegalovirus infection induces the accumulation of short-lived, multifunctional CD4+ CD45RA+ CD27 T cells: the potential involvement of interleukin-7 in this process: IL-7 induces re-expression of CD45RA in CD4+ T cells. Immunology. 2011; 132:326-339. doi: 10.1111/j.1365-2567.2010.03386.x PMID: 21214539

31. Edwards ES, Smith C, Khanna R. Phenotypic and transcriptional profile correlates with functional plasticity of antigen-specific CD4+ T cells. Immunol. Cell Biol. 2014; 92:181-190. doi: 10.1038/icb.2013.88 PMID: 24296812

32. Sinclair J, Reeves M. The intimate relationship between human cytomegalovirus and the dendritic cell lineage. Front. Microbiol. 2014; 5. Available: http://journal.frontiersin.org/Journal/10.3389/fmicb.2014.00389/full [Accessed 2014 Nov 25]. doi: 10.3389/fmicb.2014.00389

33. Lugo-Villarino G, Hudrisier D, Benard A, Neyrolles O. Emerging Trends in the Formation and Function of Tuberculosis Granulomas. Front. Immunol. 2013; 3. Available: http://journal.frontiersin.org/Journal/10.3389/fimmu.2012.00405/full [Accessed 2014 Dec 16]. doi: 10.3389/fimmu.2012.00405

34. Chachage M, Podola L, Clowes P, Nsojo A, Bauer A, Mgaya O, et al. Helminth-Associated Systemic Immune Activation and HIV Co-receptor Expression: Response to Albendazole/Praziquantel Treatment Nutman TB, ed. PLoS Negl. Trop. Dis. 2014; 8:e2755. doi: 10.1371/journal.pntd.0002755 PMID: 24675895