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Puzzle pieces from malaria vaccine clinical trials

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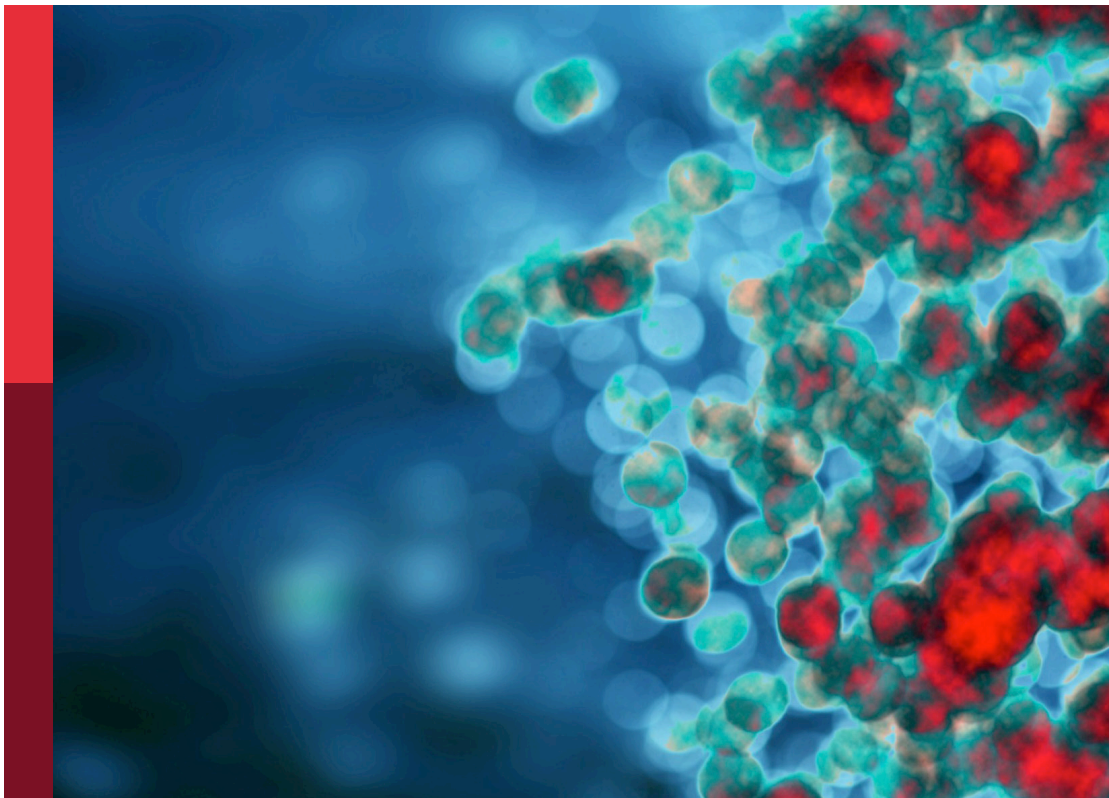
Published in
Frontiers in Immunology



ISSN 1664-8714
ISBN 978-2-83251-660-7
DOI 10.3389/978-2-83251-660-7

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Puzzle pieces from malaria vaccine clinical trials

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Citation

Palacpac, N. Q., Mordmüller, B., Tsuboi, T., Tiono, B. A., eds. (2023). *Puzzle pieces from malaria vaccine clinical trials*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-83251-660-7

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frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 30 January 2023
DOI 10.3389/fimmu.2023.1138763

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 06 January 2023
ACCEPTED 23 January 2023
PUBLISHED 30 January 2023

CITATION
Palacpac NMQ, Tiono AB, Mordmüller B and Tsuboi T (2023) Editorial: Puzzle pieces from malaria vaccine clinical trials. *Front. Immunol.* 14:1138763. doi: 10.3389/fimmu.2023.1138763

Editorial: Puzzle pieces from malaria vaccine clinical trials

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KEYWORDS

malaria, *Plasmodium*, vaccine, controlled human malaria infection, clinical trials

Editorial on the Research Topic
Puzzle pieces from malaria vaccine clinical trials

The recent endorsement of RTS,S/AS01 for broad use in children residing in regions with moderate to high malaria transmission is a key milestone in the fight against malaria (1, 2). However, there is still a lot more work to be done. Clinical trials can be challenging in terms of study design, ethics, costs, and field implementation (logistics, recruitment of human volunteers and retention, societal perceptions, etc.), but are critical for evidence generation, new tools and innovative technological approaches. Careful analysis and insight make each clinical trial an important puzzle piece providing clues to help us understand limitations/knowledge gaps that are stumbling blocks to safe, stable, easy-to-administer, cost-effective vaccines. This *Research Topic* also highlights an important key-ingredient of successful research: the extensive collaborations and multidisciplinary/transdisciplinary consortiums at the root of each activity.

The *Plasmodium falciparum* life cycle: peaks and troughs of vaccine development

The complexity of the parasite lifecycle; gaps in our understanding on the interactions of the parasite and host; and the amazing capacity of malaria-infected red blood cell for immune subversion and immunosuppression (3) are high hurdles for vaccine development. Ramjith et al., 2022 developed mathematical models, presented power analysis considerations, and made an online tool to allow data analysis and sample size estimation when conducting trials for transmission-blocking interventions. The authors describe what data are needed for either an assessment of transmission-blocking activity or transmission-reducing activity and where power can be increased while considering the many confounders involved. The models seek to maximize the informativeness of future transmission-blocking intervention trials, and allow pre- and post-intervention comparisons.

Nunes-Cabaço et al., 2022 traced the history of the clinical assessment of whole-sporozoite malaria vaccines from its earliest concept in 1967. Several milestones were discussed, including the achievements in PfSPZ (*Pf* sporozoite) vaccine production and controlled human malaria infection (CHMI) studies. To date, PfSPZ vaccines seem highly

protective in malaria-naïve adults but somewhat less active in African adults. First results from studies in infants and small children have been disappointing.

Sex has recently gathered attention as a variable that can influence immune response, vaccine efficacy and safety. Clinical trials of PfSPZ-based vaccines in the US, Germany, Kenya, Tanzania, Mali, Burkina Faso, and Equatorial Guinea showed that participants older than 11 years of age had sex-associated differences in vaccine-induced antibody response but no sex-related differences in protection (in CHMI or field clinical trials) (KC et al., 2022). Several trials also show that antibody levels against sporozoites were not predicting protection per se, and that prior malaria exposure significantly resulted in lower antibody responses, even in females with higher antibody levels than their male counterparts.

The perspective article by Owalla et al., 2022 emphasizes the need for highly sensitive parasite diagnostics in endemic settings. Indeed, low-density infections in malaria-endemic areas are common, often ignored and their influence in trial outcomes and end-point assessments remain unclear. The authors compared the current tools for determining infection status and suggest frequent dried blood spot sampling with pooled qRT-PCR as a cost-effective strategy to circumvent infection monitoring blind-spots in clinical trials and epidemiological studies.

The study by Xu et al., 2022 provides preliminary data for delivering a multi-antigen vaccine in a single vaccine formulation in the form of a multi-layer nanoparticle. The authors tested trimethyl chitosan-based layer-by-layer nano-assembly vaccine platform as a delivery vehicle for three antigens: CSP, AMA1, and MSP1. Biophysical characteristics of the delivery platform showed promise.

At the preclinical stage is another vaccine antigen, a fragment of *P. falciparum* Rh5-interacting protein (PfRipr5) (Takashima et al., 2022). PfRipr5 has been identified as a promising blood-stage vaccine candidate and is proceeding into clinical testing. The GMP-compliant recombinant protein was produced using the insect cells-baculovirus expression vector system and tested in pre-clinical model. Three human-acceptable adjuvant formulations tested head-to-head: Alhydrogel®, GLA-SE or CAF®01 showed comparable levels of anti-PfRipr5 antibodies. The highest functional activity by growth inhibition assay (GIA) was obtained in PfRipr5 with CAF®01.

Bougouma et al., 2022 reported the results of a phase Ib trial of the BK-SE36 vaccine candidate based on the serine repeat antigen-5 in 12- to 60-month-old children living in a malaria endemic area in Burkina Faso. The safety and immunogenicity of BK-SE36 were demonstrated in this age group for the first time. In general, the vaccine was safe and similarly immunogenic when given subcutaneously and intramuscularly; and as expected, subcutaneous vaccination led to more adverse events than the intramuscular route. The increase in IgG titers after vaccination was more pronounced in 12–24 months than in 25–60 month-old children, and a delayed third dose significantly boosted the immune response.

Looking for clues in a follow-up study after a Phase 2b multi-center clinical trial of the GMZ2 vaccine (4), in-depth anti-GMZ2 antibody responses were investigated in one of the sites where the highest incidence of malaria was observed (Dassah et al., 2022). The study showed the importance of naturally acquired immunity; the influence of age and parasite threshold at which fever is triggered; and the relatively high pre-existing anti-merozoite antibodies in Burkinabe children.

Needles in a haystack: CHMI challenges and clues

CHMI is increasingly becoming an important tool for the clinical evaluation of candidate drugs and vaccines as well as a model to dissect the heterogeneity in immune response to malaria (5). The study by de Jong et al., 2022 assessed antibody responses in two CHMI trials (with or without *P. falciparum* gametocyte exposure) to disentangle stage-specific signals and identify responses specific to sexual stage parasites *vs* asexual stage antibody response. The study provide insight into the humoral responses to two transmission-blocking vaccine candidates (Pfs48/45 and Pfs230) and identified new antigens that may be developed as markers for gametocyte exposure.

Salkeld et al., 2022 used CHMI in attempts to mimic the field observation of blood-stage malaria immunity acquired throughout several clinical episodes. After three homologous blood-stage CHMI, majority of the subjects did not show measurable functional anti-parasite immunity based on reduced parasite growth/multiplication rate but repeat infections did show boosting of antibody responses to MSP1 and AMA1. The work demonstrated the safety of repeated CHMI with no major differences in clinical symptoms or laboratory markers of infection across primary to tertiary challenges.

Last but not the least, while clinical trials against *P. falciparum* are making progress, the highs and lows in *P. vivax* had come in trickles (6, 7). *P. vivax* is the most dominant malaria parasite throughout Asia-Pacific and South America (with detection currently increasing in sub-Saharan Africa). The species is considered a key obstacle in malaria elimination (8) because of its unique biology and absence of a routine continuous *in vitro* cultivation method that have largely restricted research efforts to develop interventions. Roobsoong et al., 2022 present the challenges of using *P. vivax*-CHMI, particularly the stringent and safe preparation of the parasites to be used, the logistics and limitations of sporozoite- and blood-stage CHMI of *P. vivax*.

Conclusions

We hope that by highlighting progress, challenges and limitations in malaria vaccine clinical trials, this Research Topic will be useful in creating a shared vision that a malaria-free world needs concerted and evolving action.

Author contributions

NP, AT, BM, and TT are the four editors of the Research Topic, wrote this editorial and approved the final version.

Acknowledgments

We wish to thank all authors who responded to our call for contributions. Furthermore, we are also indebted to all the reviewers and editors who contributed their valuable time and energy to ensure robust, comprehensive, and open-minded reviews that went into

every single manuscript published on this Research Topic. Last but not least to the editorial office (including the submission and production teams) for all their support.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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ORIGINAL RESEARCH
published: 02 June 2022
doi: 10.3389/fimmu.2022.899223

GMZ2 Vaccine-Induced Antibody Responses, Naturally Acquired Immunity and the Incidence of Malaria in Burkinabe Children

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GMZ2 is a malaria vaccine candidate evaluated in a phase 2b multi-centre trial. Here we assessed antibody responses and the association of naturally acquired immunity with incidence of malaria in one of the trial sites, Banfora in Burkina Faso. The analysis included 453 (GMZ2 = 230, rabies = 223) children aged 12-60 months old. Children were followed-up for clinical malaria episodes for 12 months after final vaccine administration. Antibody levels against GMZ2 and eleven non-GMZ2 antigens were measured on days 0 and 84 (one month after final vaccine dose). Vaccine efficacy (VE) differed by age group (interaction, (12-35 months compared to 36-60 months), p = 0.0615). During the twelve months of follow-up, VE was 1% (95% confidence interval [CI] -17%, 17%) and 23% ([CI] 3%, 40%) in the 12 - 35 and 36 – 60 months old children, respectively. In the GMZ2 group, day 84 anti-GMZ2 IgG levels were associated with reduced incidence of febrile malaria during the follow up periods of 1-6 months (hazard ratio (HR) = 0.87, 95% CI = (0.77, 0.98)) and 7-12 months (HR = 0.84, 95%CI = (0.71, 0.98)) in the 36-60 months old but not in 12-35 months old children. Multivariate analysis involving day 84 IgG levels to eleven non-vaccine antigens, identified MSP3-K1 and GLURP-R2 to be associated with reduced incidence of malaria during the 12 months of follow up. The inclusion of these antigens might improve GMZ2 vaccine efficacy.

Keywords: GMZ2, MSP3-K1, GLURP-R2, *Plasmodium falciparum*, malaria vaccine, naturally acquired immunity

OPEN ACCESS

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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: *18 March 2022*
Accepted: *22 April 2022*
Published: *02 June 2022*

Citation:
Dassah S, Adu B, Tiendrebeogo RW, Singh SK, Arthur FKN, Sirima SB and Theisen M (2022) GMZ2 Vaccine-Induced Antibody Responses, Naturally Acquired Immunity and the Incidence of Malaria in Burkinabe Children. Front. Immunol. 13:899223. doi: 10.3389/fimmu.2022.899223

INTRODUCTION

GMZ2 is a *Plasmodium falciparum* candidate vaccine, and is designed with the aim to emulate naturally acquired anti-malarial immunity (1). It is composed of conserved domains of two asexual blood-stage antigens of *P. falciparum*, glutamate-rich protein (GLURP) and merozoite surface protein (MSP) 3 which are major epitopes for antibodies (2, 3). The rationale for including these

antigens is based on a series of immune epidemiological studies from diverse malaria endemic regions. Individuals living in malaria endemic areas gradually acquire immunity to clinical malaria (4, 5). This naturally acquired immunity (NAI) takes years of exposure to develop and is characterized by a low grade parasitemia in the presence of strong *P. falciparum*-specific immune responses (6). Immunoglobulin (Ig) G antibodies are thought to play a particularly important role in NAI (4, 7). Immuno-epidemiological studies of responses to GLURP and MSP3 have consistently demonstrated that high levels of specific antibodies are associated with protection against febrile malaria (8–16), in areas with different transmission intensity [ranging from >200 infective bites per person per year in a study in Senegal (16) to approximately 2 to 3 infective bites per person per year in a study in Sudan (15)], and with respect to their geographical locations, suggesting that GMZ2 might potentially confer immunity against clinical malaria in diverse endemic settings. Further, one of these studies suggested that antibodies against GLURP and MSP3 act in a complementary manner to control parasite multiplication (12). It is now generally accepted that protective immunity depends on a robust antibody response against multiple antigens (17–20), and it has been proposed that the magnitude and breadth of specific responses are critical in this respect (17).

While the exact immune mechanism(s) involved in NAI remains elusive, we and others have shown that monocyte mediated opsonic phagocytosis (OP) of *P. falciparum* blood-stage merozoites (8, 21, 22) and antibody-dependent cellular inhibition (ADCI) (23) are elicited during the acquisition of NAI. Recently, we further demonstrated that neutrophils may also help to eliminate circulating merozoites from blood during NAI (24).

Collectively, immuno-epidemiological studies together with pre-clinical studies in rodents and New World monkeys (25–27) led to the manufacturing and clinical testing of GMZ2 adjuvanted with alhydrogel® (alum). GMZ2/alum was well tolerated and immunogenic in three phase 1 studies (28–30) and a phase 2b multi-centre trial in African children 12-60 months old (31). Overall, the trial showed that GMZ2 had a modest efficacy in the target population (31). In a sub-analysis we found that VE was higher in children 3–4 years of age (20% (4%, 33%)] compared to children 1–2 years of age [6% (-8%, 18%). An interaction with age is consistent with the proposed mode of action of GMZ2, which aims to mimic, boost, and broaden the breadth of NAI.

Here, we present the detailed immunological evaluation of samples from the GMZ2/alum phase 2b study collected at the Banfora site in Burkina Faso. Antibodies against GMZ2 and established targets of NAI were measured and evaluated against the incidence of clinical malaria.

METHODS

Ethics Statement

Data for this study was obtained from the GMZ2/alum phase 2b clinical trial. The trial was monitored by the GMZ2 Scientific Coordinating Committee, local safety monitors, independent clinical monitors and an independent data safety monitoring committee (IDMC). The local Ethics Committees and regulatory authorities for each site and country approved the clinical trial protocol before the start of the trial. Signed informed consent was obtained from parent/guardian of children before their inclusion in the study. The protocol was registered with the Pan African clinical trial registry with registration number ATMR2010060002033537.

Study Site and Design

The study used 453 (GMZ2 = 230, rabies=223) children's specimen collected from Banfora, Burkina Faso in the GMZ2/alum phase 2b clinical trial. Malaria is endemic in Burkina Faso and occurs throughout the year, with seasonal peak between June and October, a period when rainfall is highest. *P. falciparum* is responsible for nearly 100% of all clinical malaria cases and children under five years and pregnant women are the populations at highest risk. Study design and details were previously described (31). Briefly, children were randomized to either receive three doses of GMZ2/alum or rabies vaccine on days 0, 28 and 56 and were passively followed in the ensuing months for febrile malaria episodes up to month 12 from the last vaccine dose. Any child reporting to the local health facility and/or to study team with fever or history of fever 48 hours prior to reporting at the health facility had peripheral blood taken for malaria parasitaemia determination by microscopy. Febrile malaria episode was defined as parasitaemia count of ≥ 5000 parasites/μl and fever or history of fever within the past 48 hours prior to reporting sick. Since age-dependent pyrogenic thresholds have not been determined in the present study, which is spanning multiple age groups and transmissions seasons, we have used a single parasite threshold throughout. Sera were collected at scheduled intervals between May, 2011 and February, 2012 and stored at -80°C until this analysis. To assess immune responses following the GMZ2/alum immunization and the risk of clinical malaria, baseline (Day 0) sera and sera collected one month (Day 84) after final vaccine dose were used.

Blood Smear for Malaria Parasite Detection

Thin and thick blood films were prepared from a finger prick. The thin film was fixed with methanol for a few seconds. Both blood films were then stained with 10% Giemsa stain for 15 minutes for malaria parasite identification and quantification. The stained blood smears were rinsed with running tap water for about 10 seconds and allowed to air dry. Malaria parasites were counted (trophozoites) against 200 white blood cells (WBCs) on the thick film by two independent experienced microscopist using a light microscope under oil immersion at 100x magnification. Negative result was assigned after examining 200 high power fields of the thick film at x100 magnification. Parasite counts were converted to parasites density/μL of blood assuming 8000 WBCs/μL of blood. Malaria species identification was done using thin blood smears.

Multiplex Luminex Assay for Antibody Quantification

IgG antibody levels were determined against a panel of 11 antigens (nMSP3-K1, MSPDBL2, GLURP-R2, MSP6, MSP3.3, MSP3.7,

MSP2-3D7, SERA5, *Pf*38, *Pf*12 and MSP1-19) and GMZ2 in a multiplex assay as described elsewhere (32). Briefly, recombinant proteins were coupled to $1.25x10^7$ microspheres beads per bead region. 100μL of the beads mixture containing 1250 beads per bead region were added to a pre-wetted 96 well microtiter plate. Serum samples diluted at 1:1000 were added and incubated for 2 hours. A secondary antibody, phycoerythrin (PE) -labelled goat antihuman IgG (Jackson Immuno Research) was added at 1:3500 for the detection of IgG bound antibodies and incubated for 1 hour. For the quantification of IgG subclasses, mouse antihuman IgG1 or IgG3 diluted 1:5000 were added followed by a PE-labelled goat antimouse IgG diluted 1:200. Between steps, plates were washed 3 times each with assay buffer E (ABE: PBS [pH = 7.4], 0.1% bovine serum albumin [BSA], 0.02%Tween 20 and 0.05% sodium azide). Mean fluorescent intensity (MFI) was measured with Luminex 200 Bio-Plex analyser (Bio-Rad Laboratories, Inc.).

Statistical Analysis

Data were analyzed using Stata version 15 (College Station, Texas) and GraphPad Prism version 8. Differences in geometric mean antibody levels were compared using a t-test after log transforming antibody data to base 10. Cox regression was used to estimate vaccine efficacy and to determine the association of antibody levels with incidence of clinical malaria, using a robust standard error to allow for repeated events in the same child. To compare effects by age group and time period, Wald tests were used to assess interactions. To standardise the antibody levels to the 12 antigens, levels were transformed to logarithms and the logged values, x, then transformed to z-scores $\frac{(x-\bar{x})}{s}$, where $\bar{x}$ is the mean and s the standard deviation of the logged values.

RESULTS

Baseline Characteristics

The phase 2b efficacy trial of GMZ2/alum was conducted at 5 sites in East- West- and Central-Africa (31). The present analysis include participants from one of the sites, Banfora, a village with high malaria transmission in Burkina Faso where 590 children were randomized. Of these, 547 received all three doses of the vaccine (272 in the GMZ2 group and 275 in the rabies vaccine group). Samples and data were available from 453 children (82.82% of the ATP population), 223 in the GMZ2 group and 230 in the rabies vaccine group. The distribution of gender, age, and bed net use were similar in the two groups (**Table 1**).

Febrile Malaria Episodes During Follow-Up

During the 12-months of follow-up (after dose 3 of vaccine was administered), 98.9% of the malaria episodes were *P. falciparum* mono-infections. The remaining episodes were mixed infections of *P. falciparum* and *P. ovale* or *P. malariae*. Children from the study cohort were stratified into younger (12-35 month) and older (36-60 month) age groups (based on age groups reported in the phase 2b trial) (31). The incidence of malaria episodes decreased per 1000 person years at risk with increasing age in both vaccine groups (**Figure 1A**) consistent with age-dependent acquisition of naturally acquired immunity (NAI) in the study population. We also plotted the geometric mean parasite density of each age group for all malaria cases (**Figure 1B**). The geometric mean parasite densities during febrile malaria cases decreased with increasing age.

TABLE 1 | Demographic characteristics of participants by trial arm, stratified by age and gender.

Variable		Rabies group n = 223 (%)	GMZ2 group n = 230 (%)
Age category	12 - 35 months	120 (53.8)	104 (45.2)
	36 – 60 months	103 (46.2)	126 (54.8)
Gender	Female	104 (46.6)	114 (49.6)
	Male	119 (53.4)	116 (50.4)
Bed net use	No	32 (14.3)	38 (16.5)
	Yes	191 (85.7)	192 (83.5)

n = the number of children in a group; % = percentage out of the group.

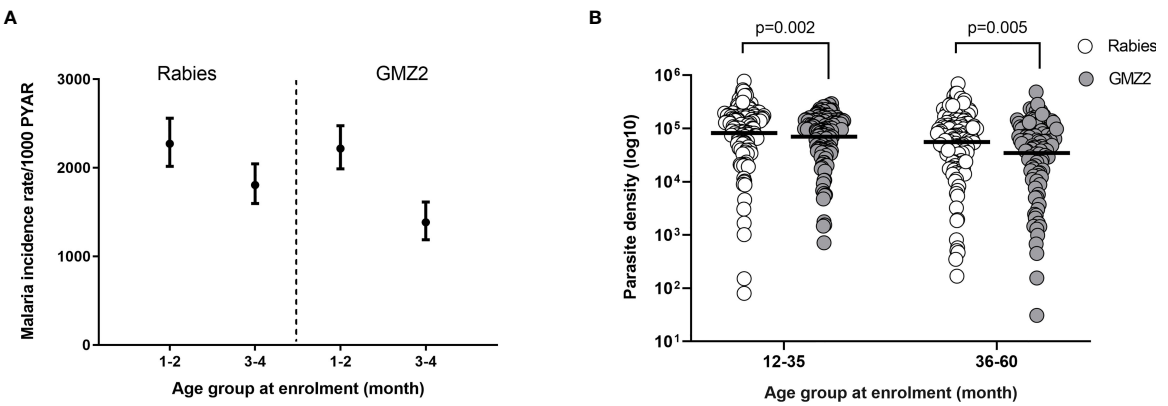

FIGURE 1 | Clinical malaria incidence rate per randomization arm and parasite density by age group. **(A)** Incidence rate of febrile malaria by vaccine group per 1000-person year at risk (PYAR). **(B)** Geometric mean *P. falciparum* density by age group in both vaccine groups. P values were determined by t-test after log (base 10) transforming parasite density. Error bars represent geometric mean with 95% confidence intervals.

Importantly, geometric mean parasite densities were significantly (t-test, $p \leq 0.005$) lower in the GMZ2 compared to the rabies groups in both age groups (**Figure 1B**).

Vaccine Efficacy Is Age-Dependent in the Study Population

We have previously observed a significant vaccine efficacy (VE) in the ATP analysis (31). In Banfora, we found that VE (using parasite density ≥ 5,000/uL and fever/history of fever) was higher in older children during the twelve months of follow-up (**Figure 2**). VE was 1% (95% confidence interval [CI] -17%, 17%) and 23% ([CI] 3%, 40%) in the younger and older children, respectively (**Figure 2**). To identify antibody specificities involved in VE, we investigated not only vaccine-induced antibodies but also naturally acquired antibodies against merozoite surface proteins not present in the vaccine because such antibodies may act in concert with GMZ2 antibodies.

GMZ2 IgG Increase With Vaccination and Correlates With Decreased Parasitaemia

GMZ2 IgG levels were first compared between vaccine groups at days 0 and 84 (one month after final vaccine dose), respectively. Levels of GMZ2 IgG were similar between the vaccine groups at day 0, however, at day 84, the GMZ2 group had significantly higher levels than the rabies group (t-test, $p < 0.001$) (**Figure 3A**). To assess whether antibody boosting depends on age, we compared GMZ2 antibody levels at days 0 and 84 for each age group. GMZ2 IgG levels were higher at day 84 compared to day 0 in both age groups (**Figure 3B**). The fold increase in GMZ2 specific IgG (i.e. day 84 GMZ2-IgG/day 0 GMZ2-IgG) was significantly higher in GMZ2 vaccinated children than those in the rabies vaccine group for each age group (**Figure 3C**). While the fold increase in the rabies group reflects natural exposure the increase in GMZ2 IgG levels in the GMZ2 vaccine group is a result of vaccination as well as natural exposure. Finally, we assessed the effect of a 10-fold increase in GMZ2 antibody level on parasite densities during febrile malaria in each vaccine group and in the overall cohort in separate multiple linear regression analysis adjusting for age of children. There was a significant decrease in parasitaemia associated with a 10-fold increase in GMZ2 specific antibodies in the overall study population and in the rabies group. However, although the same trend was observed in the GMZ2 group, the decrease in parasitaemia was not statistically significant [β = -0.23, 95%CI=(-0.56;0.11), p = 0.187], (**Figure 3D**). This suggests other non-GMZ2 IgG antibodies may have contributed to the decreased parasitaemia observed in the GMZ2 group (**Figure 1B**).

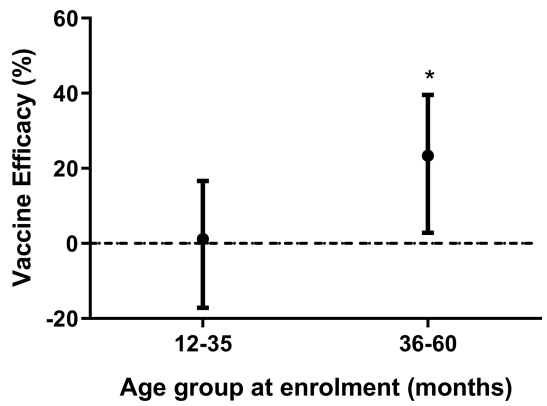

FIGURE 2 | Vaccine efficacy stratified by age. Vaccine efficacy (VE) (using parasite density ≥ 5,000/uL and fever/history of fever) after 12 months of follow up stratified by age group. Error bars represent 95% confidence intervals. Cox regression model was used to calculate hazard ratios, 95% confidence intervals and p values for each age group. VE was defined as 100 × (1-HR), where HR is the hazard ratio from the Cox regression. The horizontal dashed line indicates (VE = 0). Asterisks represent P values (*P < 0.05).

GMZ2 IgG Was Associated With Reduced Incidence of Febrile Malaria

The relationship between GMZ2 IgG levels on day 84, and the incidence of febrile malaria from that time point until 12 months post dose 3, was investigated separately in each vaccine group. The association differed by age group (interaction p-value 0.011). In the rabies group, there was no association between GMZ2 IgG levels and incidence of malaria in any of the age groups at any of the defined follow up periods (months 1-6 and 7-12, respectively) (**Figure 4A**). Similarly, in the GMZ2 group, there was no association between levels of GMZ2 IgG and malaria incidence in the younger children at any of the defined follow up periods. However, in the older children, GMZ2 IgG levels were significantly associated with reduced incidence of malaria during months 1-6 [hazard ratio (HR) = 0.87, 95%CI = (0.77, 0.98)] and 7-12 [HR = 0.84, 95%CI = (0.71, 0.98)] months of the follow-up period (**Figure 4B**).

GMZ2 Vaccine Induced Antibodies Promote Opsonic Phagocytosis

Recently we developed a bead-based phagocytosis assay (BPA) to measure the functional activity of antibodies against distinct merozoite surface antigens (20). The GMZ2 vaccine antigen was immobilized on the surface of internally dyed microsphere beads and BPA activities of vaccine-induced antibodies were quantified in samples collected at days 0 and 84 from all study participants. A wide range of phagocytic activities were observed (**Figure 5**). At day 0, samples from both vaccine groups showed similar functional activities (**Figure 5A**). At day 84, there was a significant difference in BPA activity between the GMZ2 and rabies groups, demonstrating that GMZ2/alum elicit functional antibodies. The increase in functional activities was significant (t-test, $p<0.0001$) in both age groups (**Figure 5B**).

Anti-Merozoite IgG Levels Increased in Both Vaccine Groups During Follow-Up

First, we used a flow cytometry-based immunofluorescence assay (FC-IFA) to quantify anti-merozoite antibodies (33). In each age group, day 84 levels of merozoite IgG were higher than those at baseline (day 0) irrespective of the vaccine group (**Figure 6A**).

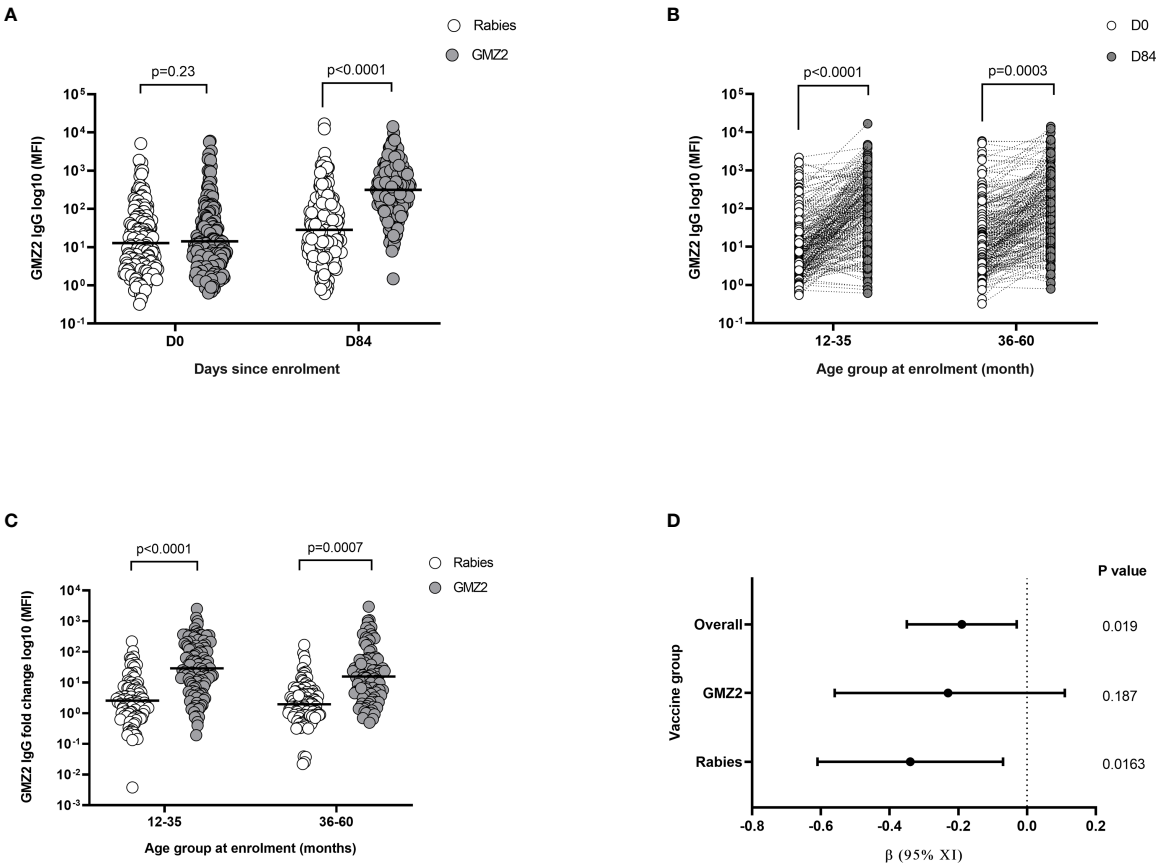

FIGURE 3 | Antibody responses after GMZ2 vaccination and association with parasite density. **(A)** Mean fluorescent intensity (MFI) representing GMZ2 specific IgG levels compared between the two vaccine groups at day 0 (D0) and day 84 (D84). **(B)** Mean fluorescent intensity (MFI) representing GMZ2 specific IgG levels compared between day 0 (D0) and day 84 (D84) for the two age groups. **(C)** Fold increase (day 84 GMZ2-IgG/day 0 GMZ2-IgG) in GMZ2-IgG levels between day 0 and day 84 compared between the vaccine groups for each age group. All P values in panels **(A–C)** respectively were determined by t-test after log (base 10) transforming data. Horizontal lines represent geometric means. **(D)** Association between GMZ2 specific IgG levels and parasite density during febrile malaria in the overall study population, the GMZ2 vaccine group alone and the rabies vaccine group alone. Beta (β) coefficients, confidence intervals and p values were calculated using separate multiple linear regressing models adjusting for age. Antibody and parasite density data were both log (base 10) transformed prior to use in the models. The vertical dotted line indicates no association with between GMZ2 IgG and parasite density (β = 0).

When the study population was categorized according to vaccine group, there was no difference between merozoite IgG levels in children who received GMZ2/alum and those who received the rabies vaccine at either day 0 or day 84 (**Figure 6B**). In contrast, levels of merozoite IgG did increase between days 0 and 84 in either vaccine group suggesting the contribution of naturally acquired antibody boosting. Next, we used the merozoite opsonic phagocytosis (OP) assay to assess the functional activity of anti-parasite IgG at day 0 and day 84 (8, 24). Irrespective of the vaccine group, day 84 merozoite OP values were higher than day 0 values in both younger and older children (**Figure 6C**). However, there were no differences when merozoite OP values were compared between the vaccine groups on day 0 and day 84, respectively (**Figure 6D**).

Collectively, these findings are consistent with the notion that anti-merozoite immunity develops in the present study population irrespective of vaccine group.

Dissecting Merozoite Specific IgG Responses Associated With Febrile Malaria in the Study Population

Having shown through the FC-IFA that natural exposure boosted anti-merozoite IgG responses in the study population, we next sought to delineate the potential specific merozoite antigens involved. Levels of merozoite specific antibodies were measured against a panel of 11 merozoite antigens not present in GMZ2 (**Supplementary Table S1**). There was a high variability in the IgG levels for the different antigens in the different vaccine groups. However, for each antigen, day 84 IgG levels appeared higher than day 0 IgG levels irrespective of the vaccine group suggesting a boosting through natural exposure (**Supplementary Table S1**). After transforming to logarithms (base=10) and calculating a z-score for the transformed variable, the association between incidence of malaria and day 84 antigen-specific antibody levels was assessed using Cox regression with a

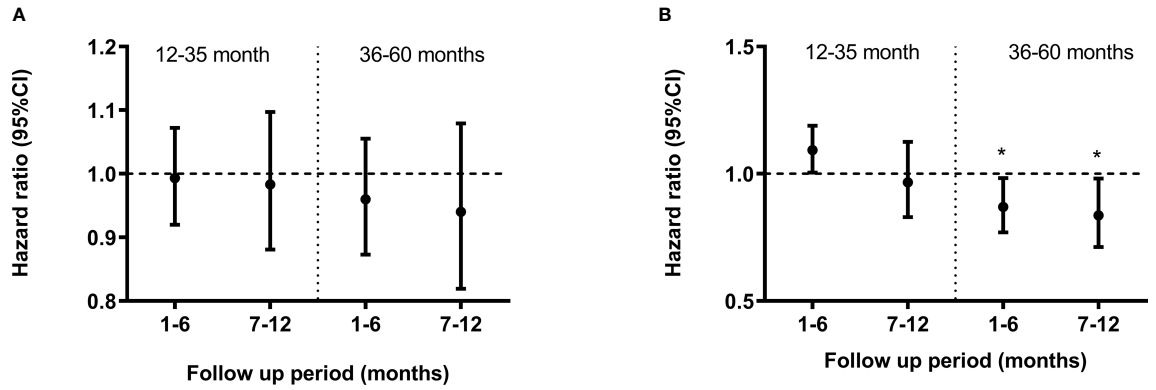

FIGURE 4 | Association between GMZ2 antibody levels and clinical malaria stratified by age group. Cox regression model was used to calculate hazard ratios, 95% confidence intervals and p values for antibody levels in the rabies vaccine group **(A)** and GMZ2 vaccine group **(B)** for each age group. Error bars represent 95% confidence intervals. The horizontal dashed line indicates no association with protection (HR = 1). Asterisks represent P values (*P < 0.05). Malaria episodes were collected over 12 months of follow up.

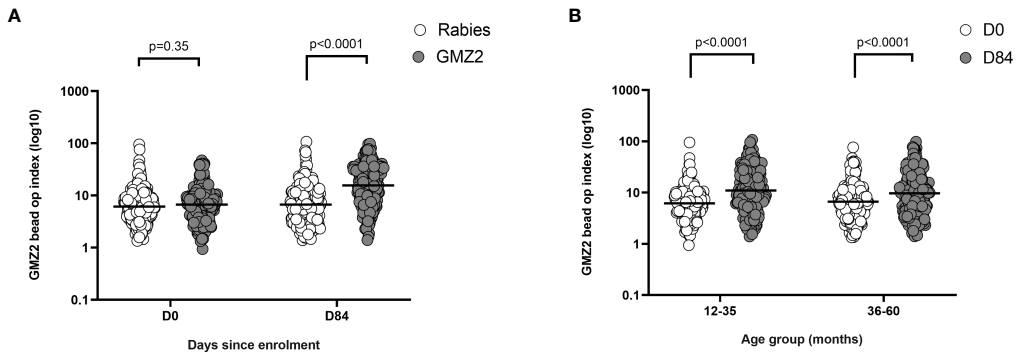

FIGURE 5 | GMZ2 bead OP index in relation to age and vaccine group. **(A)** GMZ2 coated bead OP compared between the vaccine groups at day 0 (D0) and day 84 (D84). **(B)**. GMZ2 coated bead opsonic phagocytosis (OP) levels compared between day 0 (D0) and day 84 (D84) for the different age groups. P values were determined by t-test after log (base 10) transforming data. Horizontal lines represent geometric means.

robust standard error to allow for repeated events within the same child and adjusted for age group and trial arm (GMZ2/alum or rabies vaccine) (**Table 2**). Antibodies against MSP3-K1 (HR = 0.87, 95% CI: 0.81 - 0.94 for a unit increase in z-score), MSPDBL2 (HR = 0.89, 95% CI: 0.83 - 0.97), GLURP-R2 (HR = 0.90, 95% CI: 0.84 - 0.97) and MSP3.7 (HR = 0.93, 95% CI: 0.87 – 1.00) were associated with reduced incidence of malaria in the study population. There was no evidence that the associations differed between the arms of the trial. Antibodies against all 11 antigens were then entered into the model to obtain estimates of independent association for each antibody adjusted for effects of the other antibodies. In the multivariate analysis, antibodies against only two antigens, MSP3-K1 (HR = 0.88, 95% CI: 0.80 – 0.97, p = 0.007) and GLURP-R2 (HR = 0.88, 95% CI: 0.80 – 0.98, p = 0.015), were independently associated with reduced incidence of malaria (**Table 2**). In the multivariate model, age remained strongly associated with malaria incidence indicating that the panel of immune responses measured only partially explained the reduction in incidence with age. A limitation is that we were not able to measure exposure to malaria, an important confounder, we may therefore have underestimated the strength of associations. Nonetheless, the data suggest that antibodies against MSP3-K1 and GLURP-R2 may have independently contributed to reducing malaria incidence in the study population during the clinical trial period and future GMZ2 designs could benefit from their incorporation.

DISCUSSION

In summary, we showed VE in Banfora increased with increasing age of the children at enrolment, and older children (36-60 months) benefitted most from GMZ2/alum vaccination in the 12 months of follow-up. We further showed that naturally acquired antibodies to MSP3-K1 and GLURP-R2 measured at day 84 (one

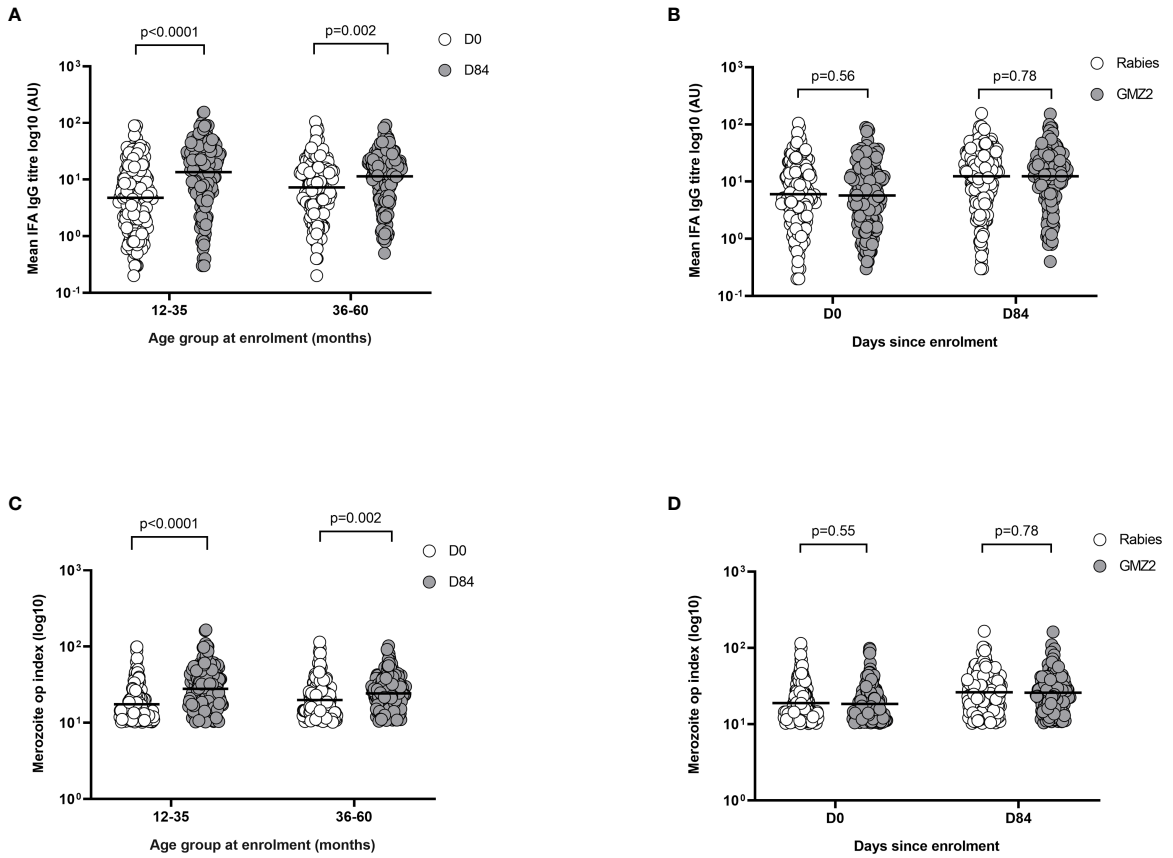

FIGURE 6 | Merozoite IFA levels in relation to age and febrile malaria. **(A)** Merozoite IFA levels compared between day 0 (D0) and day 84 (D84) for the different age and vaccine groups. **(B)** Merozoite IFA levels compared between the vaccine groups at day 0 (D0) and day 84 (D84). **(C)** Merozoite opsonic phagocytosis (OP) index compared between day 0 (D0) and day 84 (D84) for the different age groups. **(D)** Merozoite opsonic phagocytosis (OP) index compared between the vaccine groups at day 0 (D0) and day 84 (D84). P values were determined by t-test after log (base 10) transforming data. Error bars represent geometric mean and 95% confidence intervals. IFA, Immunofluorescence assay; AU, Antibody unit.

TABLE 2 | Association of non-GMZ2 antibodies with malaria incidence in the study population.

Variable	Hazard ratio [a] (95%CI)	P-value	AdjustedHazard ratio [b] (95%CI)	P-value
nMSP3-K1	0.87 (0.81,0.94)	<0.001	0.88 (0.80,0.97)	0.007
MSPDBL2	0.89 (0.83,0.97)	0.006	0.98 (0.88,1.09)	0.686
GLURP-R2	0.90 (0.84,0.97)	0.008	0.88 (0.80,0.98)	0.015
MSP6	0.92 (0.85,1.00)	0.054	1.01 (0.91,1.12)	0.901
MSP3.3	0.93 (0.86,1.00)	0.060	0.93 (0.86,1.01)	0.094
MSP3.7	0.93 (0.87,1.00)	0.048	0.96 (0.87,1.06)	0.375
SERA5	1.00 (0.93,1.07)	0.942	1.05 (0.96,1.15)	0.263
nMSP2-3D7	0.95 (0.88,1.02)	0.147	0.99 (0.90,1.10)	0.897
*Pf*38	1.00 (0.93,1.07)	0.935	1.01 (0.91,1.12)	0.867
*Pf*12	1.01 (0.94,1.09)	0.820	1.01 (0.91,1.11)	0.907
MSP1-19	1.04 (0.97,1.12)	0.283	1.10 (0.99,1.22)	0.092

[a]*Association of each variable with malaria incidence, adjusted for age and trial arm.*

[b]*Independent association for each variable adjusted for effects of all the other variables.*

95% confidence interval (95%CI). MSP, merozoite surface protein; MSPDBL2, merozoite surface protein duffy binding-like domain 2; GLURP-R2, glutamate rich protein region 2; SERA5, serine rich antigen 5; Pf, Plasmodium falciparum.

month after final vaccine dose), were associated with reduced malaria incidence in the study population during the trial period. Incorporation of these antigens in some form into future GMZ2 designs may help improve VE.

Considering all sites in the GMZ2 efficacy study, we observed that children in the GMZ2/alum group with high levels of GMZ2 IgG had a lower incidence of clinical malaria, after adjusting for age, compared with children with low levels (31). Here, we performed a series of association analyses in the GMZ2/alum vaccine group to further examine possible effects of GMZ2 IgG on protection against febrile malaria. Anti-GMZ2 antibody responses were investigated with respect to quantity and functional activity in the phagocytosis assay. Collectively, these assays demonstrated that GMZ2 vaccine elicited high levels of cytophilic IgG antibodies, which were capable of promoting phagocytosis of GMZ2-coated beads. Thus, supporting the notion that GMZ2 IgG may enhance merozoite-phagocytosis by blood leukocytes (24, 34). Contrary to expectations merozoite-phagocytosis was not stronger in the GMZ2 group compared to the rabies group suggesting that children in Banfora possesses relatively high levels of pre-existing anti-merozoite antibodies. We also, cannot rule out possibilities of antibodies against GMZ2 vaccine to mediate anti-malaria activity through other antibody-dependent mechanisms like Antibody Dependent Cellular Inhibition (ADCI) and inhibition of merozoite invasion,

We found that increasing anti-GMZ2 IgG levels were associated with reduced incidence of febrile malaria in older children 36-60 months of age during the first 12 months of follow-up. However, these analyses did not establish anti-GMZ2-IgG as the sole correlate of vaccine protection as they did not exclude potential confounders such as acquisition of antibodies to other blood stage antigens and age-dependent maturation of cell mediated immunity (35). Although IgG antibodies are thought to be the main effector molecule mediating protection against febrile malaria, cellular immune responses may also play a role through T-cell help for producing a robust antibody response or through multifunctional effector memory T cells producing IFN-γ, TNFα, and IL-2 (36). Whether GMZ2/alum enhance antigen-specific pluripotent lymphocytes remains to be investigated. Likewise, IgM antibodies may also play a role in malaria immunity. Recently, it was convincingly demonstrated that levels of specific IgM antibodies are associated with a reduced risk of clinical malaria in a longitudinal cohort study of children and that such antibodies may block merozoite invasion of red blood cells in a complement-dependent manner (37). Whether GMZ2-vaccine specific IgM antibodies play a similar role in the present cohort remains to be investigated.

We further observed that older children had lower parasite densities during febrile malaria attacks than the younger ones and that this difference was most pronounced in the GMZ2 vaccine group. This finding is consistent with observations that the parasite threshold at which fever is triggered depends on the age of the affected child. Older individuals were found to have a much lower pyrogenic threshold compared to younger ones (38, 39). When considering all study participants in Banfora, we further observed that increased levels of GMZ2 IgG were significantly associated with decreased parasitemias in these febrile attacks. This association was not observed in the GMZ2 group suggesting that these children have a lower pyrogenic threshold compared to children in the rabies vaccine group. It might be speculated that GMZ2 vaccination modulate the dynamics of parasitemia and the occurrence of fever. Pyrogenic cytokines Interlukin-1 IL1, IL6, and Tumor Necrosis Factor (TNF) are produced in response to malaria parasites (40). Of these, IL6, together with prostaglandin E2 (PGE2), is considered to be a major pyrogenic mediator of fever (reviewed in (41). Whether GMZ2 vaccination is affecting pyrogenic cytokine production and modulation of pyrogenic threshold triggered by malaria parasites through this inflammatory cytokines-neuronal body temperature regulatory axis mechanism remains to be determined.

It has previously been proposed that multiple anti-merozoite antibody specificities act in concert to provide protection against clinical malaria (17, 20) after a certain threshold has been reached (17). To determine whether several antibody specificities might also be involved in reducing clinical malaria incidence in Banfora, levels of distinct antibody specificities were assessed in the study population. We found, in a multivariate analysis involving eleven naturally acquired antibodies where the association of each antibody is adjusted for the effect of all the others, that levels of IgG against MSP3-K1 and GLURP-R2 were independently associated with reduced incidence of clinical malaria. Interestingly, both of these protein sequences are related to the GMZ2 constituent antigens, as they are derived from MSP3 and GLURP, respectively. The MSP3 antigen is a well-established target of naturally acquired immunity (42, 43) and analysis of sequences from most parasite isolates from malaria endemic populations show a distinct dimorphism belonging to either MSP3-3D7 or MSP3-K1 type alleles (44). In a Kenyan study, MSP3-K1 specific IgG was significantly associated with reduced risk of clinical malaria after adjusting for the effect of antibodies against other antigens such as AMA1 and MSP2 (45). Similarly, antibodies against GLURP-R2 have been associated with protection against malaria in several endemic populations including Burkina Faso, Ghana, and India (9, 14, 46). The finding that naturally acquired antibodies against variable regions of GLURP and MSP3 are associated with protection against febrile malaria in children from Banfora support the notion that antibodies against both conserved and variable domains are involved in protective mechanisms (16, 42, 47). While antibodies against the variable domains are thought to contribute to allele-specific immunity (42), antibodies against the conserved domains may provide protection against multiple parasite strains prevailing in the endemic population [reviewed in (48)]. Taken together, these findings suggest that future design of GMZ2 may benefit from the inclusion of variable epitopes from the MSP3 and GLURP antigens to improve efficacy. Whether such vaccine specific responses would be strain-specific remains to be investigated.

Other blood-stage malaria vaccines such as MSP-1 and AMA-1 intended to block or reduce the invasion of erythrocytes by malaria merozoites (4, 49, 50) have either shown no or little protection in

Phase 2b efficacy studies (51–54). The main reason for these failures might be related to difficulties associated with the production of recombinant antigens with native conformations. However, polymorphisms observed for several of these malaria antigens in different parasite strains may also explain the lack of protective efficacy. Allele-specific vaccine efficacy has been reported in multiple trials of malaria vaccines, such as AMA1 (55), RTS,S (56) as well as those containing attenuated whole sporozoites (57). Considering the worldwide dynamics of *P. falciparum* parasites with different distributions among different regions and the finding that parasites may evolve over time possibly as a result of immune selection [reviewed in (58)], polymorphisms in key malaria antigens is considered a major obstacle to vaccine development. Although, the GMZ2 constituent antigens are relatively conserved (11, 59), it is possible that the limited VE might be due to some degree of strain-specific immunity. Overall, allelic-specific protection analysis of the GMZ2 trial may provide critical insights into putative strain-specific responses resulting in the development of more efficacious vaccine.

In conclusion, GMZ2/Alhydrogel VE was more pronounced in older children, and this may reflect a synergistic interaction between vaccine-induced and naturally acquired immune responses. Interestingly, additional epitopes from the variable regions of GLURP and MSP3 were identified as potential candidates for inclusion in future GMZ2 designs for improved efficacy. The study contributes important insights that could be useful in developing more efficacious blood-stage malaria vaccines that will benefit from a positive influence of naturally acquired immunity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The local Ethics Committees and regulatory authorities for Burkina Faso, Gabon, Ghana and Uganda reviewed and approved the clinical trial protocol before the start of the trial. Signed informed consent was obtained from parent/guardian of children before their inclusion in the study. The protocol was registered with the Pan African clinical trial registry with registration number ATMR2010060002033537.

AUTHOR CONTRIBUTIONS

SD, RT, and SKS performed the experiments. MT and SBS designed the clinical study. BA and MT designed the experiments and analysis. BA and MT wrote the manuscript. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants from the European and Developing Countries Clinical Trials Partnership (grant IP.2007.31100.001), the German Federal Ministry of Education and Research (BMBF, grants 01KA0804 and 01KA1402) and Ministry of Foreign Affairs of Denmark (DFC file no.14-P01-GHA).

ACKNOWLEDGMENTS

We thank the children and their parents and guardians who volunteered to participate in the study, and without whose cooperation this study would have been impossible. We thank the Data and Safety Monitoring Board (Trudie Lang (CHAIR), Brian Faragher, Blaise Genton, Angelina Kakooza, Grégoire Adzoda, John E. Williams, and Dao Fousseni) for their recommendations throughout the study. We also thank Dr. Subhash Singh for his critical comments and helpful suggestions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.899223/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 03 June 2022
doi: 10.3389/fimmu.2022.899615

Quantifying Reductions in *Plasmodium falciparum* Infectivity to Mosquitos: A Sample Size Calculator to Inform Clinical Trials on Transmission-Reducing Interventions

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: 18 March 2022
Accepted: 10 May 2022
Published: 03 June 2022

Citation:
Ramjith J, Alkema M, Bradley J, Dicko A, Drakeley C, Stone W and Bousema T (2022) Quantifying Reductions in Plasmodium falciparum Infectivity to Mosquitos: A Sample Size Calculator to Inform Clinical Trials on Transmission-Reducing Interventions. Front. Immunol. 13:899615. doi: 10.3389/fimmu.2022.899615

Malaria transmission depends on the presence of mature *Plasmodium* transmission stages (gametocytes) that may render blood-feeding *Anopheles* mosquitos infectious. Transmission-blocking antimalarial drugs and vaccines can prevent transmission by reducing gametocyte densities or infectivity to mosquitos. Mosquito infection outcomes are thereby informative biological endpoints of clinical trials with transmission blocking interventions. Nevertheless, trials are often primarily designed to determine intervention safety; transmission blocking efficacy is difficult to incorporate in sample size considerations due to variation in infection outcomes and considerable inter-study variation. Here, we use clinical trial data from studies in malaria naive and naturally exposed study participants to present an online sample size calculator tool. This sample size calculator allows studies to be powered to detect reductions in the proportion of infected mosquitos or infection burden (oocyst density) in mosquitos. The utility of this online tool is illustrated using trial data with transmission blocking malaria drugs.

Keywords: malaria, transmission, gametocyte, anopheles, mosquito, elimination, trial, oocyst

INTRODUCTION

Despite considerable improvements in access to efficacious antimalarial treatment and increased uptake of preventive strategies such as insecticide treated bed nets and indoor residual spraying, malaria is still responsible for over 2 million infections and approximately 627.000 deaths each year (1). The spread of resistance against antimalarial drugs (1) further highlights the need for additional tools in the fight against malaria. Tools that reduce the efficient transmission of malaria are considered particularly useful (2). Malaria transmission to mosquitos is initiated in the human host,

when a small proportion of asexual parasites differentiate into gametocytes, the sexual reproductive forms of the parasite. When the human host is bitten by a female *Anopheles* mosquito and gametocytes are taken up with the bloodmeal, gametes are formed. Sexual reproduction starts when male gametes fertilize female gametes to form zygotes that transform into motile ookinetes that penetrate the mosquito midgut wall to form an oocyst. The presence of oocysts is typically used as evidence for successful transmission to mosquitos. After approximately 8-12 days, sporozoites are released from the oocyst and colonize the salivary glands of the mosquito, thereby rendering it infectious upon its next bite.

Transmission blocking drugs can clear or sterilize gametocytes (3–5); transmission blocking vaccines are typically designed to elicit antibodies against surface antigens of *Plasmodium* gamete [e.g. Pfs230, Pfs48/45 (6)], zygote or ookinete forms [Pfs25 (7)] or mosquito midgut antigens [AnAPN1 (8)] and thereby prevent parasite development in mosquitos. Recently, monoclonal antibodies against gamete antigens have also been proposed as transmission-reducing tools by passive immunization (9, 10).

The ultimate public health endpoint of these transmission blocking interventions is a reduction in the force of infection and thereby the incidence of malaria infection in a population (11). However, studies with these public health endpoints are complex in design, expensive and logistically challenging to implement, typically involving cluster-randomized or stepped wedge designs. Early phase testing of transmission blocking efficacy requires biological endpoints that more directly estimate of human-to-mosquito transmission. These early trials may involve naturally infected gametocyte carriers or individuals participating in controlled human malaria infection (CHMI) studies where gametocytes are induced (12–14). In both studies, mosquitos may be allowed to feed directly on the skin of parasite carriers or on a venous blood sample that is offered through a membrane (15); mosquitos can subsequently be assessed for infection status.

These functional assays allow samples from early phase clinical studies to be used for meaningful assessments of vaccine efficacy. Although there is considerable recent interest in the transition from oocyst to sporozoites, and whether this involves a developmental bottleneck (16–18), the majority of oocyst-infected mosquitos will become sporozoite-positive mosquitos (19) and until a minimum oocyst or sporozoite density is defined to render an infected mosquito infectious, the proportion of mosquitos that become infected is considered the most relevant measure of the transmissibility of naturally acquired infections. The transmission-blocking activity (TBA) of an intervention are defined as its ability to reduce the proportion of mosquitos that is infected. It is also possible that interventions do not completely prevent mosquito infection but reduce the infection burden in mosquitos (i.e. oocyst density). Transmission-reducing activity (TRA) is defined as the achieved reduction in oocyst density compared to controls (20). While studies in gametocyte carriers are typically designed to measure TBA, experiments that determine the ability of test samples to reduce the transmission of *in vitro* cultured gametocytes typically measure TRA. In standard membrane feeding assays (SMFA), high densities of cultured *P. falciparum* gametocytes are offered to mosquitos in the presence of test and control samples. SMFA are optimized to achieve high oocyst densities in control mosquitos to maximize precision and reproducibility (21). Because of this high infection intensity in control mosquitos, even highly potent samples may not prevent oocyst formation completely and TRA is the common readout of SMFA (20).

Sample size estimates for transmission blocking efficacy outcomes are challenging. When candidate vaccines, drugs or monoclonal antibodies enter clinical testing, they are typically primarily evaluated for safety in small first-in-human trials (7) that are powered on outcomes other than efficacy outcomes. Transmission assays are inherently noisy and considerable between-site variation exists in the performance of mosquito assays. Two additional complicating factors are the negative binomial distribution of oocysts (22–24), which is especially relevant when using oocyst density as an outcome measure, and the strong correlation between mosquito observations from the same individual in studies with naturally infected gametocyte carriers. Despite these challenges, mosquito feeding assays offer opportunities to maximize informativeness of trials with transmission-blocking interventions. In this context, we describe a negative binomial mixed effects model for TRA endpoints and a mixed effects logistic regression for TBA endpoints. Using these models, we designed a calculator tool that allows i) power analysis for transmission blocking intervention trials based on both TRA or TBA efficacy endpoints by means of mosquito feeding assays, ii) statistical analysis of data to either determine reference values for the power analysis or to quantify TRA and TBA as study outcomes. The practical application of the calculator is demonstrated with two clinical datasets.

MATERIALS AND METHODS

The Statistical Models

In order to calculate empirical power to detect reduction in oocyst prevalence/proportion of infected mosquitos, we simulated transmission data to estimate the effects of different levels of Transmission Blocking Activity (TBA). These data contain a binary outcome (a mosquito can either be infected or not infected), therefore a mixed effects logistic regression model was used. For power calculations for studies with reduction of oocyst density as an endpoint, the negative binomial distribution of oocysts has to be taken into account. This distribution is required as the majority of oocysts is found in a small proportion of all mosquitos (22–24). Therefore, to calculate the empirical power to estimate the effect of different levels of Transmission Reducing Activity (TRA), a mixed effects negative binomial regression model was used. For both models, we used mixed effects models, meaning we modelled both fixed effects of TBA and TRA as well as random intercepts. Both logistic regression and negative binomial regression models assume that the data are independent. Violations in this assumption leads to underestimated standard errors and thus an increased

likelihood of false positive findings. Including random intercepts in the models, allows for the correlation between outcomes for mosquito samples from the same participant to be accounted for; which we refer to as the intra-cluster correlation. The random intercepts are used to allow participant-level variation in pre-intervention transmissibility, i.e. a participant-specific baseline proportion of infected mosquitos (for TBA models) or participant-specific baseline geometric mean oocyst density (for TRA models).The mathematical details for the data simulation algorithm and the statistical models and tests are given in the **Supplementary Information**.

To estimate empirical power for TBA, the calculator relies on user specifications of i) baseline proportion infected mosquitos, ii) anticipated TBA and iii) the intra-cluster correlation that is used to directly estimate the variance of the random effects. The value that is entered for baseline proportion of infected mosquitos is ideally based on site-specific data from preceding (pilot) studies or, if unavailable, on best estimates from existing literature, taking into account variation in gametocyte density in the study population (**Figure 1B**). The percentage of transmission inhibition that we expect the studied intervention to achieve, the anticipated TBA, can be estimated based on pre-clinical data. The intra-cluster correlation reflects the correlation of infection between mosquitos fed on the same participant and is determined based on the variance of the intercepts in the pilot dataset. Thus likelihood of infection in mosquitos fed from one sample is highly correlated when the intra-cluster correlation is close to 1, and independent when the intra-cluster correlation is 0.

Similarly, to estimate empirical power for TRA, the calculator relies on four user-defined specifications of: i) baseline geometric mean oocyst density, ii) anticipated TRA, iii) variance of the random intercepts, and iv) the dispersion parameter. The baseline geometric mean oocyst density, variance of the random effects and dispersion parameter can be calculated in the data analysis tool preferably using site-specific individual mosquito level data from preceding (pilot) studies. The anticipated TRA is user-defined and

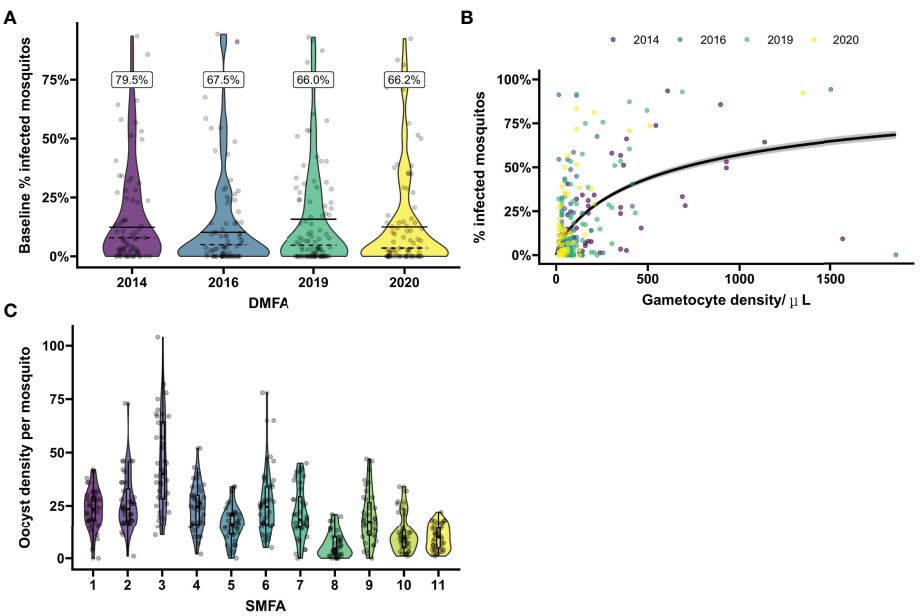

FIGURE 1 | Baseline mosquito infectivity in studies assessing transmission blocking or transmission reducing activity. **(A)** Variation in the proportion of infected mosquitos from natural gametocyte carriers. Violin plots show the percentage of infected mosquitos at baseline as determined by mosquito feeding assays on microscopically detected *P. falciparum* gametocyte carriers in Mali prior to intervention in four separate transmission blocking intervention trials performed in 2014, 2016, 2019 and 2020. On average 66.2 mosquitos were dissected per sample. Dashed lines indicate the mean baseline percentage of infected mosquitos when including all enrolled gametocyte carriers. The black lines indicate the mean baseline percentage of infected mosquitos including only samples that were infectious to mosquitos. The percentage above the plot gives the percentage of infectious gametocyte carries for each year. Dots show the values of individual baseline samples. **(B)** Microscopically estimated gametocyte density in relation to mosquito infection rates. The relationship between log gametocyte density by microscopy and probability of a mosquito being infected was modelled with logistic regression. The black line indicates the expected proportion of infected mosquitos across given gametocyte densities. Samples positive for gametocytes by microscopy at baseline were included from the four trials presented in **(A)**. To demonstrate the effect of entry criteria (i.e. the minimum gametocyte density required for participation) on pre-intervention infectiousness, the average slope over the total of 4 trials was presented. However, average gametocyte density as well as the modeled slopes differ between the different years, emphasizing the variability of baseline proportion of infected mosquitos and need of site-specific baseline parameters for sample size calculations. Dots show values per individual sample, colors correspond with the trials as presented in **(A)**. **(C)** Variation in oocyst density in experiments with *in vitro* cultured gametocytes. Violin plots show the oocyst density per mosquito for pooled malaria-naïve control sera in 11 separate mosquito membrane feeding experiments with cultured *P. falciparum* NF54 gametocytes. Sera were tested in duplicate with 20 mosquito dissections per sample. Boxes indicate IQR and median oocyst density per experiment, whiskers indicate full range. Dots indicate oocyst counts in individual mosquitos.

can for example be based on pre-clinical data. The dispersion parameter in the negative binomial regression model controls over-dispersion – which is the case when the empirical variation in the data is larger than that predicted from the model. The dispersion parameter and random effects variance together control the extent of inter-cluster correlation, for which an estimate is displayed in the input panel of the power calculator.

Finally, for both power calculations, a user-specified level of significance and a testing threshold should be specified. For the levels of significance, one of two choices is possible, 0.025 or 0.05, of which 0.025 is often preferred for one-sided tests/superiority trials and applicable for most envisaged use scenarios where TBA or TRA is anticipated to exceed a certain threshold value. This threshold can be zero, when merely testing whether an intervention reduces transmission compared to the pre-intervention control condition; often it is more informative to demonstrate that TBA/TRA is significantly larger than a higher threshold, for instance a minimum TRA of 80% has historically been proposed to identify potent interventions for further development. It is relevant to realize that this threshold TBA or TRA value is different from the anticipated TBA/TRA value, the value that we expect and is typically higher than the threshold level we aim to compare it to.

Study Populations and Reference Values

In order to provide reference values to inform envisioned future transmission studies, two datasets from recent clinical trials with transmission endpoints were analyzed in the calculator. For oocyst prevalence data (i.e. the proportion of infected mosquitos), individual-level data from studies examining the impact of transmission-reducing antimalarial drugs were used (25). This exemplar dataset was selected to illustrate reductions in the proportion infected mosquitos (i.e. transmission blocking activity; TBA) following interventions. In this study, naturally infected gametocyte carriers were included and transmissibility to mosquitos was assessed before and after a drug intervention. Briefly, naturally infected gametocyte carriers with microscopically detectable *P. falciparum* gametocyte densities (>16 gametocytes/μL) were recruited and treated with conventional artemisinin-combination therapy (a 3-day regimen administered by weight of 320mg dihydroartemisinin and 40 mg piperaquine per tablet) alone or combined with low doses of gametocytocidal drugs (0.0625 mg/kg, 0.125mg/kg, 0.25 mg/kg 0.5 mg/kg of primaquine). Before and after initiation of treatment, venous blood was drawn and offered to locally reared mosquitos that were examined 7 days later for the presence of oocysts (binary outcome: absent/present) with on average 70.5 mosquitos dissected per blood sample. This key dataset was complemented with data of four independent trials (4, 25–27) that evaluated the transmission blocking efficacy of gametocytocidal drugs by means of direct membrane feeding assays in a single site in Ouelessebougou, Mali. These additional data were used to examine variation in the proportion of infected mosquitos at baseline, prior to administration of any transmission blocking drugs. The number of gametocytes determined by microscopy at screening was used to demonstrate how parasitological enrolment criteria such as gametocyte density influenced baseline infectivity and thereby the efficiency of TBA assessments.

For oocyst density data, we used standard membrane feeding assay (SMFA) results from a clinical trial NCT04238689 with a highly potent transmission blocking monoclonal antibody as reference dataset. The efficacy of the monoclonal antibody was studied in malaria-naïve study participants; their serum samples being offered to mosquitos in the presence of high densities of cultured *P. falciparum* NF54 gametocytes in the SMFA, rendering this reference appropriate to illustrate reductions in oocyst density as an outcome (i.e. transmission-reducing activity; TRA). This dataset included 20 subjects before administration of a transmission blocking monoclonal antibody and 10 subjects post administration who were selected to have partial TRA. On average 19.4 mosquitos were dissected with a median oocyst density of 42 in mosquitos fed on pre-intervention samples. Post-administration samples were selected where the mean TRA value was approximately 80% (median 77.1%, range 62.4-86.1%), a TRA value traditionally used as threshold to support further development of transmission-blocking interventions (28, 29). This dataset was complemented with 11 independent experiments performed for the abovementioned clinical trial (30) to examine variance in oocyst density for SMFA experiments conducted at the same site (Radboudumc, Nijmegen, the Netherlands). The only entry criterion for SMFA experiments was a proportion of infected mosquitos of >70%, a pre-defined quality control threshold (20).

All trials in Mali received ethics approval by the Ethics Committee of the Faculty of Medicine, Pharmacy, and Dentistry of the University of Science, Techniques, and Technologies of Bamako (Bamako, Mali), and the Research Ethics Committee of the London School of Hygiene & Tropical Medicine (London, UK). The trial in the Netherlands received approval from the Arnhem-Nijmegen Committee on Research Involving Humans.

Simulation Scenarios

To show a range of power calculations for TRA and TBA respectively, we allowed different combinations of parameter values for the data simulation and empirical power calculations. For both TBA/TRA we allowed sample sizes n = (10, 20, 30, 40) for participants and m = (20, 40, 60) for mosquitos, based on conventional group sizes (31–35). We used consensus thresholds of meaningful efficacy (24, 28, 29, 36) to define anticipated TBA/TRA = (70%, 80%, 95%) and thresholds for detecting TBA/TRA larger than τ = (50%, 80%, 90%). Further, for TRA we considered baseline geometric mean oocyst densities of μ_0 = (20, 30, 45) (37) and for TBA we considered baseline proportion infected mosquitos of p_0 = (10%, 15%, 25%) based on a meta-analysis of membrane feeding experiments (38). For intra-cluster correlation we considered values of 0 for independence and 0.5 or 0.35 for TBA or TRA analyses, respectively, as motivated by the reference data. For TRA, the intra-cluster correlation depends on both variance of the random effects and the dispersion parameter, so to keep the results comparable, we used a fixed dispersion parameter as estimated by the data analysis and varied the

variance of the random effects to determine the intra-cluster correlation (0, 0.35). We only considered a significance level of 0.025, being interested in one-tailed testing whether TRA or TBA were larger than a pre-defined threshold. A significance level of 0.025 for a one-tailed test is essentially the equivalent of a significance level of 0.05 for a two-tailed test; the empirical power for a significance level of 0.05 is always larger.

Software

All data analysis was conducted using R4.1.1 (39). in RStudio (40) making use of the mgcv package (41, 42) for the analysis. Rshiny was used to develop the app (43). The app is currently hosted on https://bousema-lab.shinyapps.io/transmission_sample_size/.

RESULTS

First, we evaluated the variance in baseline proportion of infected mosquitos from four previous transmission blocking intervention trials. Mosquito infection prevalence prior to the intervention differed per study and varied from 14.2% – 17.4% when including non-infectious participants, or from 21.1% - 24.0% when including only infectious participants (**Figure 1A**). The proportion of infected mosquitos at baseline was strongly correlated with concurrent gametocyte density (**Figure 1B**). Whilst this association has repeatedly been described (44–47) and the current analysis was not intended to improve on such estimates, the association is of immediate relevance in designing studies and selecting the study population. The proportion infected mosquitos at baseline is highly dependent on eligibility criteria: e.g. when using a threshold of >50 gametocytes/μL as selection criterion, the average proportion of infected mosquitos at baseline was 25%, increasing to 35% when using a threshold of >100 gametocytes/μL, and 44% for a threshold of >200 gametocytes/μL. The shape of the association between gametocyte density and mosquito infection rates may vary between study sites and years (48). To inform the size of pilot experiments to determine site-specific baseline data on the proportion of infected mosquitos, we explored how precision in estimates of baseline infectivity and intra-cluster correlation depends on the size of the study population. For this, we used one study population [2014 study in **Figure 1A** (25)] to randomly select subjects from. The true proportion of infected mosquitos for the average participant in the entire population (n=81) was 17.0% (95% CI: 13.0%, 21.6%); the true intra-cluster correlation in the entire population was 0.52 (95% CI: 0.42, 0.61). The distribution of estimates of the baseline infectivity and the intra-cluster correlation for 100 simulations with sampling sizes of 10 to 75 participants are presented in **Figures 2A, B**, respectively. When taking 100 random samples of 40 participants, 84% of the estimations of baseline proportion of infected mosquitos were within the 95% CI of the complete dataset of 81 participants. With this same sampling size of 40 participants, 93% of the estimations for intra-cluster correlation were within the 95% CI of the complete dataset. Forty participants in pilot experiments may thus provide reasonably precise estimates of baseline parameters. We next performed power calculations using an anticipated TBA = 90%, a threshold TBA of 80%, number of participants = 20 and number of dissected mosquitos per sample = 30 and a significance level of 0.025. The power was 73% using the true reference parameters of the full dataset (i.e. baseline proportion of infected mosquitos = 17.0% and intra-cluster correlation = 0.52). For a sample size of 40, approximately half of the estimates for proportion infected and intra-cluster correlation led to an estimated power within the acceptable power range with 5% margin of error,

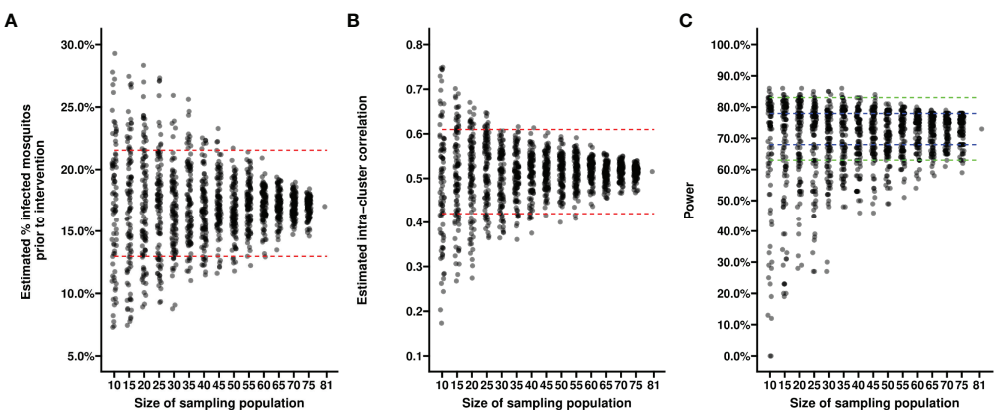

FIGURE 2 | Estimates of baseline proportion of infected mosquitos using different sample sizes. Each dot reflects the estimated average percentage of infected mosquitos (y-axis) **(A)** or the estimated intra-cluster correlation **(B)** in a random sample of participants from a transmission blocking intervention trial performed in Mali in 2014. Each dot in **(C)** reflects the power estimate derived from the estimates of average percentage of infected mosquitos and intra-cluster correlation from a random sample of participants, based on an anticipated TBA of 90%, a threshold TBA of 80%, a number of participants of 20, a number of dissected mosquitos of 30 and a significance level of 0.025. Sizes of the sampled populations range from 10 to 75 participants, with intervals of 5 participants (x-axis). For each sample size scenario, 100 random samples selected without replacement were taken from the trial dataset with a total of 81 participants. Red dashed lines indicate 95% confidence intervals of the averages in the total trial population. Blue dashed lines indicate a 5% margin of error, green dashed lines indicate a 10% margin of error from the power estimate based on the reference values derived from the complete dataset.

and approximately 80% were within the acceptable power range when the margin of error was increased to 10% (**Figure 2C**).

Similarly, variance in baseline oocyst density was evaluated using the SMFA outcomes of malaria-naïve control sera from 11 separate experiments. Despite experiments being conducted with the same parasite line (NF54) and mosquito species (*An. stephensi*) in the same laboratory, the mean oocyst density per mosquito was highly variable over the experiments and reached 6.5 – 44.6 oocysts/mosquito (**Figure 1C**). These findings illustrate the need for site-specific baseline parameters for sample size calculations and, for SMFA, adequate controls.

To demonstrate the utility of the tool for data analysis, data from a previous transmission blocking intervention trial using proportion of infected mosquitos by membrane feeding as the primary outcome measure (25) were analyzed in the mixed-effects logistic regression model. For a trial with natural gametocyte carriers, the reduction in the proportion of infected mosquitos (TBA) is the preferred outcome. TBA was estimated as a function of the baseline proportion of infected mosquitos and the estimated odds ratio from the model. These estimates included individuals who were not infectious to mosquitos. Prior to treatment, 18.5% (15/81) of the participants in this trial was non-infectious (25) and, including these individuals, the estimated proportion of infected mosquitos for the average person prior to treatment was 17.0% (95% CI: 13.0%, 21.6%) (2014 study in **Figure 1A**). Based on previous demonstrations of the potency of primaquine in preventing transmission (25, 49), we used the analysis tool to test whether transmission was reduced by at least 80% following primaquine treatment. In our exemplar dataset, the proportion of infected mosquitos was reduced to 2.0% (95% CI: 1.2%, 3.2%) (**Figure 3A**). TBA was estimated at 88% (95% CI: 82.2%, 91.9%), significantly larger than the threshold of 80% (p=0.0058). The intra-cluster correlation was estimated to be 0.52.

Subsequently, we used the data analysis tool to compare two out of five intervention arms from the same trial. In the study arm receiving 0.125mg/kg of primaquine, the estimated proportion of infected mosquitos for the average person was 11.0% prior treatment, which was reduced to 1.6% after intervention. The TBA is estimated to be 85.88% (95% CI: 76.93%, 91.36%), not significantly higher than the threshold of 80% (p=0.0824). For the study arm receiving 0.5 mg/kg of primaquine, the estimated 12.7% of mosquitos that was infected for the average person at baseline, was reduced to 0.8% post-intervention, resulting in a TBA of 94.0% (95% CI: 86.4%, 97.4%), significantly higher than the threshold of 80% (p=0.0021). TBA was not statistically significantly different between arms (p=0.0793). The intra-cluster correlation was estimated to be 0.47.

Informed by these reference values we used a baseline proportion of infected mosquitos of 15% and an intra-cluster correlation of 0.5 to perform power calculations for envisioned future trials with transmission reducing interventions.

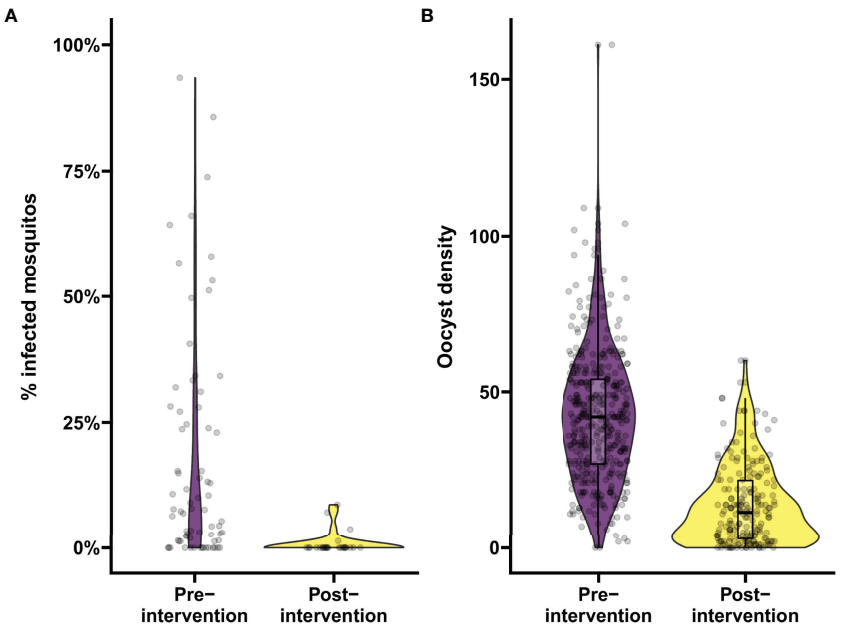

FIGURE 3 | Analyzing the proportion of infected mosquitos and oocyst burden in mosquitos using the app. Demonstration of the data analysis output from the app. **(A)** Analysis of changes in percentage of infected mosquitos. Violin plots show the percentage of infected mosquitos pre- and post-intervention as determined by mosquito feeding assays from natural gametocyte carriers in a transmission trial performed in Mali in 2014, in purple and yellow respectively. On average 70.5 mosquitos were dissected per sample. **(B)** Analysis of changes in infection burden (oocyst density). Violin plots show oocyst density pre- and post-intervention from samples selected from a trial evaluating a transmission blocking monoclonal antibody in malaria naïve subjects by means of mosquito feeding experiments with cultured gametocytes, in purple and yellow respectively. On average 19.4 mosquitos were dissected per sample. Boxes indicate median and IQR, whiskers indicate range, outliers are presented as dots and were defined as >1.5*IQR.

Alternatively, all trials with the same enrolment criteria (**Figure 1A**) could be used to inform reference values for an envisioned future trial; these values are provided in **Supplementary Table 1**. We used several different sample sizes of human participants (n=10, n=20, n=30, n=40), numbers of mosquitos dissected (m=20, m=40, m=60), anticipated TBA values (70%, 80%, 95%) that we expect the efficacy of our studied transmission blocking intervention will be, and threshold TBA values (>50%, >80%, >90%) that we wish to show the TBA of the studied intervention exceeds. One example of the output of the power calculator for such a power estimation is shown in **Figure 4**. We repeated simulations using a range of baseline proportion of infected mosquitos (10%, 15% and 25%), and an intra-cluster correlation of 0; results are shown in **Table 1**. Note that this table is based on parameters that are representative for the study site of the datasets analyzed in this paper, but different values for intra-cluster correlation and other baseline parameters may represent better the users' own study site and can be computed using the data analysis tool.

Similarly, we analyzed data of a recent trial using reductions in oocyst density (i.e. transmission reducing activity; TRA) by SMFA as the endpoint (30) in the mixed-effects negative binomial regression model. The number of 41.3 oocysts per mosquito for the average person at baseline was reduced to 10.5 after intervention (**Figure 3B**), resulting in a TRA of 74.5% (95% CI: 71.3, 77.3), significantly higher than the threshold of 70% (p=0.0035). The standard deviation of the random intercepts was 0.393 and the dispersion parameter was estimated as 3.316 which were used to estimate an ICC of 0.35.

Next, we compared two of the intervention arms from the same trial in the analysis tool. For the first arm the number of 52.7 oocysts per mosquito for the average person at baseline was reduced to 20.2 after intervention, resulting in a TRA of 61.6% (95% CI: 55.0, 67.3), not higher than the threshold of 70% is (p=0.9987). For the second arm, the number of 35.9 oocysts per mosquito for the average person at baseline was reduced to 5.3 oocysts per mosquito after intervention, resulting in a TRA of 85.3%, significantly higher than the threshold of 70% (p<0.001). Estimates of TRA were significantly different between arms (p<0.001). The standard deviation of the random intercepts was 0.36 and the dispersion parameter was estimated as 3.358 which were used to estimate an ICC = 0.31.

Power calculations for envisioned future studies were performed using a number of 20, 30 or 45 oocysts per mosquito for the average

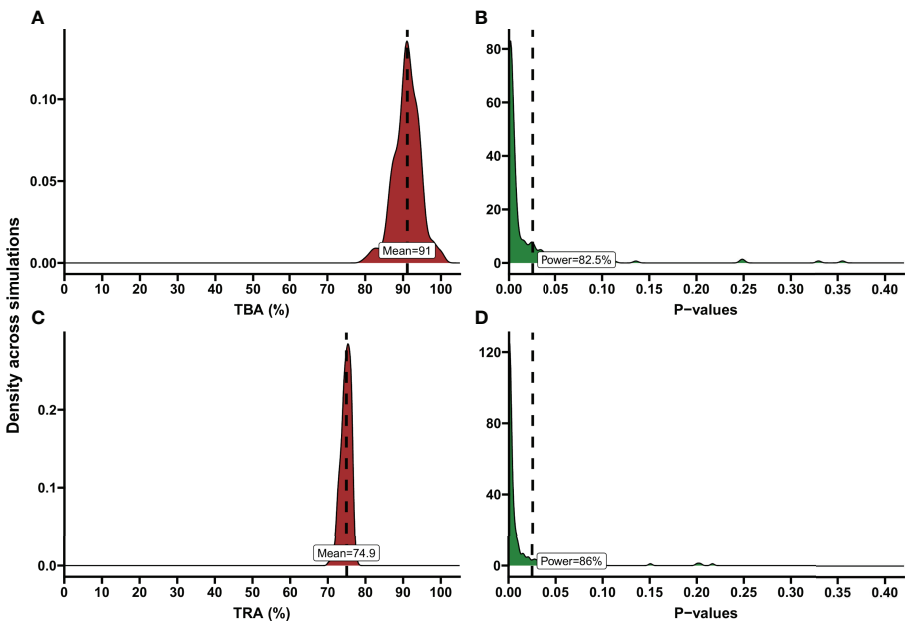

FIGURE 4 | Examples of TBA and TRA power estimations. Demonstration of power calculation output of the app. These are illustrated using density plots, where the y-axis indicates the kernel density estimates for the values on the x-axis, which is a smoothed version of the histogram. **(A)** Percentage of transmission blocking activity (TBA) across simulations. The dashed line shows the mean TBA across stimulations; the red shaded area shows the distribution of TBA estimates in simulations. As an example, variables based on the data analysis of a transmission blocking intervention trial performed in Mali, 2014 were entered in the power calculator. Number of simulations: 200, Anticipated TBA: 90%, Threshold: 80%, Number of subjects: 20, Number of dissections per subject: 40, Baseline proportion infected: 17.02%, Intra-cluster correlation: 0.5, Level of significance: 0.025. **(B)** P-values across simulations, using the variables as described in **(A)**. Dashed line shows level of significance (0.025); the green shaded area shows the distribution of p-values across simulations. Estimated power is presented in the text box. **(C)** Percentage of transmission reducing activity (TRA) across simulations. Dashed line shows the mean TRA across stimulations. As an example, variables based on the data analysis of a trial evaluating the TRA of a monoclonal antibody were entered in the power calculator. Number of simulations: 200, Anticipated TRA: 75%, Threshold: 70%, Number of subjects: 10, Number of dissections per subject: 20, Geometric mean number of oocysts for the average subject pre-treatment: 41.27, Anticipated standard deviation of the random intercepts: 0.393, Anticipated dispersion parameter 3.316, Intra-Cluster Correlation: 0.35, Level of significance: 0.025. **(D)** P-values across simulations, using the variables as described in **(C)**. Dashed line shows level of significance (0.025). Estimated power is presented in the text box.

TABLE 1 | Power for trials using reduction in proportion of infected mosquitos as functional outcome.

			ICC = 0									*ICC* = 0.5								
	n	m	Anticipated TBA=70			Anticipated TBA=80			Anticipated TBA=95			Anticipated TBA=70			Anticipated TBA=80			Anticipated TBA=95		
			τ =50	τ =80	τ =90	τ =50	τ =80	τ =90	τ =50	τ =80	τ >90	τ =50	τ =80	τ =90	τ =50	τ =80	τ =90	τ =50	τ =80	τ =90
p_0 = 10%	10	20	17.5	0	0	30	0	0	48.5	0	0	18.5	0	0	36.5	0	0	29	1.5	0
		40	40	0.5	0	84.5	2.5	0	81	27.5	0	49	0	0	78.5	0	0	53.5	17.5	0
		60	52	0	0	89.5	4.5	0	91.5	71	0.5	65.5	0	0	91.5	0.5	0	64.5	38	1
	20	20	32	0	0	70	1.5	0	91.5	27.5	0	53	0	0	82.5	0.5	0	53.5	17.5	0
		40	66	0	0	99	2	0	96	87	14.5	86	0	0	99	3	0	76.5	65.5	4
		60	82.5	0	0	100	3	0	100	100	35	95.5	0.5	0	99.5	8	0	84.5	81	20
	30	20	30	0	0	90	0	0	100	79	0	67.5	0	0	96.5	1.5	0	78.5	60	1
		40	74	0	0	100	0	0	100	100	40.5	95.5	0	0	100	6	0	94	92	19.5
		60	92	0	0	100	0	0	100	100	48	99	0	0	100	7.5	0	97	97	52
	40	20	51	0	0	100	0	0	100	99	2.5	83.5	0	0	100	1.5	0	87	84	2
		40	99.5	0	0	100	0	0	100	100	66.5	100	0	0	100	7.5	0	96	96	50.5
		60	98.5	0	0	100	0	0	100	100	79.5	100	0	0	100	10	0	99	99	78
p_0 = 15%	10	20	29	0	0	55	1	0	73.5	2.5	0	35	0	0	61	0.5	0	48	9	0
		40	58	0	0	92	5	0	94	63	0	65.5	0	0	90	1	0	72	46.5	1
		60	65	0	0	99.5	3.5	0	99	90	19.5	77.5	0	0	99	2.5	0	79.5	70	6.5
	20	20	42.5	0	0	89.5	7	0	96	61	0	73	0	0	95.5	3	0	70	55	3
		40	87.5	0	0	100	5	0	100	99	22	93.5	0	0	100	7	0	88	87	26
		60	92.5	0	0	100	6	0	100	100	40	99.5	0	0	100	10.5	0	93.5	93.5	53.5
	30	20	57.5	0	0	97.5	0	0	100	83	10.5	77	0	0	99.5	2	0	91	88.5	7.5
		40	98	0	0	100	1	0	100	100	37	100	0	0	100	9	0	97	97	56
		60	100	0	0	100	3.5	0	100	100	57	100	0	0	100	12.5	0	98	98	78
	40	20	71	0	0	100	0	0	100	96.5	23	91	0	0	100	2.5	0	96	95.5	21
		40	100	0	0	100	0	0	100	100	56.5	100	0	0	100	9.5	0	99	99	79
		60	100	0	0	100	0	0	100	100	82.5	100	0	0	100	14	0	100	100	92.5
p_0 = 25%	10	20	39	0	0	87	3.5	0	95	55	0	51.5	0	0	91	1	0	71	35.5	0.5
		40	78.5	0	0	100	2.5	0	100	96.5	26	81.5	0	0	99.5	4.5	0	88.5	84	12.5
		60	88.5	0	0	100	7	0	100	100	41	88.5	0	0	100	7	0	90	89	32
	20	20	59	0	0	100	6.5	0	100	94	11.5	84	0	0	100	5	0	91.5	89.5	14.5
		40	94	0	0	100	5.5	0	100	100	40.5	96	0	0	100	10	0	97.5	97.5	63
		60	100	0	0	100	11.5	0	100	100	70.5	99	0	0	100	12	0	98.5	98.5	76.5
	30	20	80	0	0	100	0	0	100	100	18	94	0	0	100	3.5	0	98	98	40.5
		40	100	0	0	100	0	0	100	100	80.5	100	0	0	100	8	0	100	100	81
		60	100	0	0	100	1.5	0	100	100	92.5	100	0	0	100	12.5	0	100	100	87.5
	40	20	92	0	0	100	0	0	100	100	19	98.5	0	0	100	3	0	100	100	55
		40	100	0	0	100	0	0	100	100	97	100	0	0	100	10.5	0	100	100	93.5
		60	100	0	0	100	0	0	100	100	99	100	0	0	100	16.5	0	100	100	97.5

ICC, intra-cluster correlation; TBA, transmission blocking activity; n, number of participants; m, number of mosquitos dissected; p_0 baseline proportion of infected mosquitos; τ, the threshold at which we wish to test that the anticipated TBA exceeds.

person at baseline and an intra-cluster correlation of 0 or 0.35 using several variations in trial characteristics (number of participants, number of dissections per sample, TRA threshold, anticipated TRA) (**Table 2**). An example of the power calculator output is shown in **Figure 4**. The following general patterns can be derived and are in agreement with the literature (21): i) for both TBA and TRA, empirical power is highly dependent on site specific parameters: oocyst density or proportion of infected mosquitos at baseline and intra-cluster correlation; ii) power can be increased substantially by using higher number of dissected mosquitos per sample.

DISCUSSION

We present a mathematical framework to calculate power and analyze data in transmission blocking intervention studies using either TBA or TRA as the efficacy outcome. These methods are made accessible in an online tool that allows users to perform their own analyses and power calculations in pre- and post-intervention comparisons as well as study designs that compare an intervention- to a control group.

After years of relative neglect, there is an increasing interest in the impact of novel antimalarial drugs on transmission (50–52) and transmission blocking vaccines (53). An important advantage of transmission-blocking interventions is that there are informative biological endpoints for efficacy (11) for which there have been efforts to qualify assays (54). However, the large number of variables in mosquito feeding assays complicates both analysis and power calculations for transmission blocking intervention studies. The anticipated transmission inhibition, number of participants, number of mosquitos, baseline gametocyte density, baseline proportion of infected mosquitos or oocyst density all affect the power in such trials. What adds to that complexity, is that there are site-specific conditions (38)

TABLE 2 | Power for trials using reduction in oocyst density as functional outcome.

			ICC= 0									*ICC*= 35								
	n	m	Anticipated TRA=50			Anticipated TRA=70			Anticipated TRA=80			Anticipated TRA=50			Anticipated TRA=70			Anticipated TRA=80		
			τ =35	τ =40	τ =45	τ =55	τ =60	τ =65	τ =65	τ =70	τ =75	τ =35	τ =40	τ =45	τ =55	τ =60	τ =65	τ =65	τ =70	τ =75
μ_0 = 20	5	10	60.5	32	13.5	89	59.5	24.5	99	85.5	43.5	55.5	32.5	14.5	87.5	60	21.5	99.5	86	42
		20	87.5	61	17.5	99.5	92	36.5	100	99	69	87	56.5	16	99.5	89.5	33	100	98	67.5
		30	97.5	78	24	100	98	58.5	100	100	89.5	97	77.5	25	100	97.5	59.5	100	100	91
	10	10	91.5	57	14.5	100	93	39.5	100	99.5	70	88.5	57.5	16.5	99.5	91	37.5	100	99	67
		20	100	83	41	100	100	71	100	100	93	99	86	36	100	100	71	100	100	91
		30	100	95.5	49	100	100	82.5	100	100	99.5	100	96	48	100	100	83.5	100	100	100
	20	10	100	94	26.5	100	100	70.5	100	100	96.5	100	92	31.5	100	100	73.5	100	100	94
		20	100	100	66	100	100	97	100	100	100	100	99.5	63.5	100	100	90	100	100	100
		30	100	100	74.5	100	100	97.5	100	100	100	100	100	72.5	100	100	99	100	100	100
μ_0 = 30	5	10	58.5	32	11.5	95	63	25.5	100	87.5	44.5	59	34	11	92	65.5	20	99	89	45.5
		20	90	57	15	99.5	93	41.5	100	100	74.5	89	55.5	16.5	100	91	40	100	99	71
		30	100	81	28.5	100	99.5	57	100	100	93	97.5	81.5	27.5	100	98.5	57.5	100	100	92
	10	10	91	59	17	100	95	45	100	100	76	92.5	58.5	14.5	100	93.5	39.5	100	99.5	76
		20	99.5	86.5	32.5	100	100	74	100	100	95	99.5	86	32.5	100	100	72	100	100	93
		30	100	97	52	100	100	84	100	100	100	100	97.5	50.5	100	100	85.5	100	100	99.5
	20	10	100	90.5	36	100	100	80	100	100	95.5	100	92.5	33	100	100	78	100	100	97.5
		20	100	98	56.5	100	100	93.5	100	100	100	100	100	63	100	100	93	100	100	100
		30	100	100	76	100	100	100	100	100	100	100	100	73.5	100	100	99	100	100	100
μ_0 = 45	5	10	61	38	10	95.5	68.5	29	100	95	45.5	59.5	34	10	95	67.5	28.5	100	93.5	43.5
		20	90	58	19	100	94.5	39.5	100	100	77	90.5	60.5	18.5	100	92	37.5	100	100	74.5
		30	98.5	81	27	100	99.5	65	100	100	93.5	98.5	83.5	24	100	99.5	63	100	100	94
	10	10	91	59.5	20.5	100	96.5	49	100	100	78.5	91	60.5	15.5	100	97	45.5	100	100	76
		20	100	87	36	100	100	71.5	100	100	94.5	99.5	89.5	37	100	100	73.5	100	100	95
		30	100	98.5	49.5	100	100	86	100	100	100	100	99	49	100	100	89.5	100	100	100
	20	10	100	93.5	38	100	100	82.5	100	100	100	100	91	33	100	100	80	100	100	99
		20	100	100	64.5	100	100	97.5	100	100	100	100	100	65.5	100	100	95	100	100	100
		30	100	100	76	100	100	100	100	100	100	100	100	79	100	100	99.5	100	100	100

ICC, intra-cluster correlation; TBA, transmission blocking activity; n, number of participants; m, number of mosquitos dissected; μ_0 baseline oocyst density; τ, the threshold at which we wish to test that the anticipated TBA exceeds.

such as feeding protocol (e.g. direct skin versus membrane feeding, duration of feeding, type of artificial membrane), donor characteristics (e.g. minimum parasite or gametocyte density) and mosquito characteristics (e.g. receptivity and survival rate), that lead to site-specific differences in baseline oocyst density, proportion of infected mosquitos or number of mosquitos available for dissection (38, 48). As illustrated in the current analysis, there can even be considerable variation between experiments conducted at the same study site with the same procedures and inclusion criteria (**Figure 1**) (48, 55). It is thus imperative to obtain site-specific baseline estimates of infectivity in pilot experiments prior to designing transmission-blocking intervention trials. For studies in natural gametocyte carriers, one approach is to determine in pilot experiments what percentage infected mosquitos can be achieved with the enrolment criteria of the envisaged clinical trial. We explored the number of gametocyte carriers that should be included in such pilot experiments by randomly selecting participants from our study population. Including a minimum of 40 donors allowed us to approximate the 'true' mosquito infection prevalence and intra-cluster correlation with sufficient precision to allow power calculations. An alternative or complementing approach would be to test whether the association between gametocyte density and mosquito infection rates in the envisaged study population follows that of a recent multi-site study (48) and subsequently decide what a minimum gametocyte density should be for study participants to be enrolled in the study. Using stringent eligibility criteria can increase the pre-intervention proportion of infected mosquitos, resulting in an increased efficiency of the trial. As an example, using the reference values of the transmission blocking intervention trial described above (i.e. intra-cluster correlation = 0.52, anticipated TBA = 88%, number of participants = 20, number of dissected mosquitos per experiment = 40, threshold value of TBA = 80%), by only including participants with a minimal gametocyte density of 100 gametocytes/μL, the baseline proportion of infected mosquitos can increase from 17.0% to 34.6%, resulting in a power increase from 82.5% to 92%. Whilst it may be challenging to recruit these rare high-density gametocyte carriers, it will increase study power.

Our analyses emphasize the value of a site-adaptable tool to analyze data and make power calculations for trials with transmission-blocking interventions. The high variability in baseline data highlights that use of site-specific baseline data is strongly recommended for obtaining reference values to enter in the power calculator, instead of using the preset reference values or power estimations as presented in **Tables 1**, **2**, that are based on the datasets described in this manuscript.

An important consideration to consider when using the app is that the power calculations are based on finding TRA or TBA above a certain threshold within study arms or within a total

study population, but not on the comparison of transmission blocking efficacy between two intervention arms. Although the app is not designed for this, the data analysis tool does offer the opportunity for some alternative analyses. If the number of infectious individuals (i.e. the number of individuals infecting at least one mosquito) is a preferred outcome instead of TBA or TRA, the analysis tool can be used by generating a dataset with only one fictive dissected mosquito per individual and entering 0 for non-infectious and 1 for infectious individuals in the column for number of infectious mosquitos. Additionally, the analysis tool could be used for other paired assessments of infectivity (for example the relative transmission of a primary vivax infection compared to a recrudescent infection).

In conclusion, we have developed a tool for analysis and power calculation of transmission blocking intervention trials that is accessible on https://bousema-lab.shinyapps.io/transmission_sample_size/. This supports the inclusion of functional mosquito feeding assays to assess intervention efficacy in early phase trials and thereby maximize their informativeness. This may accelerate the clinical development of transmission blocking interventions. At present, mosquito feeding assays remain a surrogate endpoint for public health impact that requires confirmation; predicting the association between intervention efficacy in terms of reductions in the proportion of infected mosquitos and the public health impact at population level is a high priority.

DATA AVAILABILITY STATEMENT

Datasets used to demonstrate the utility of the app are available upon request to the corresponding author. Requests to access these datasets should be directed to teun.bousema@radboudumc.nl.

ETHICS STATEMENT

All trials in Mali received ethics approval by the Ethics Committee of the Faculty of Medicine, Pharmacy, and Dentistry of the University of Science, Techniques, and Technologies of Bamako (Bamako, Mali), and the Research Ethics Committee of the London School of Hygiene & Tropical Medicine (London, UK). The trial in the Netherlands received approval from the Arnhem-Nijmegen Committee on Research Involving Humans. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JR and MA analyzed the data and drafted the first version of the manuscript; JB, AD, CD, and WS contributed data and assisted in data analysis and interpretation and in the drafting of the manuscript. TB conceived the study, contributed to data analysis, and drafted the first version of the manuscript. JR, MA and TB designed the app and JR programmed the app. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by the Gates Foundation (INV-002098). JB received support from the UK Medical Research Council (MRC) and the UK Department for International Development (DFID; MR/K012126/1) under the MRC–DFID Concordat agreement and as part of the EDCTP2 program supported by the EU. TB is supported by a European Research Council Consolidator Grant (ERC-CoG 864180; QUANTUM). TB and MA are further supported by an AMMODO Science Award (2019).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.899615/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 18 July 2022
DOI 10.3389/fimmu.2022.930956

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 28 April 2022
ACCEPTED 28 June 2022
PUBLISHED 18 July 2022

CITATION
de Jong RM, Alkema M, Oulton T, Dumont E, Teelen K, Nakajima R, de Assis RR, Press KWD, Ngotho P, Tetteh KKA, Felgner P, Marti M, Collins KA, Drakeley C, Bousema T and Stone WJR (2022) The acquisition of humoral immune responses targeting *Plasmodium falciparum* sexual stages in controlled human malaria infections. *Front. Immunol.* 13:930956. doi: 10.3389/fimmu.2022.930956

The acquisition of humoral immune responses targeting *Plasmodium falciparum* sexual stages in controlled human malaria infections

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Individuals infected with *P. falciparum* develop antibody responses to intra-erythrocytic gametocyte proteins and exported gametocyte proteins present on the surface of infected erythrocytes. However, there is currently limited knowledge on the immunogenicity of gametocyte antigens and the specificity of gametocyte-induced antibody responses. In this study, we assessed antibody responses in participants of two controlled human malaria infection (CHMI) studies by ELISA, multiplexed bead-based antibody assays and protein microarray. By comparing antibody responses in participants with and without gametocyte exposure, we aimed to disentangle the antibody response induced by asexual and sexual stage parasites. We showed that after a single malaria infection, a significant anti-sexual stage humoral response is induced in malaria-naïve individuals, even after exposure to relatively low gametocyte densities (up to ~1,600 gametocytes/mL). In contrast to antibody responses to well-characterised asexual blood stage antigens that were detectable by day 21 after infection, responses to sexual stage antigens (including transmission blocking vaccine candidates Pfs48/45 and Pfs230) were only apparent at 51 days after infection. We found antigens previously associated with early gametocyte or anti-gamete immunity were highly represented among responses linked with gametocyte exposure. Our data provide detailed insights on the induction and kinetics of antibody responses to gametocytes and identify novel antigens that elicit antibody responses exclusively in individuals with gametocyte exposure. Our findings provide target

10.3389/fimmu.2022.930956

identification for serological assays for surveillance of the malaria infectious reservoir, and support vaccine development by describing the antibody response to leading vaccine antigens after primary infection.

KEYWORDS

malaria, *Plasmodium falciparum*, sexual stage, gametocyte antigens, antibody responses, controlled human malaria infection (CHMI)

Introduction

Gametocytes are the only life stage of *Plasmodium falciparum* that can initiate successful infection in anopheline mosquitoes. The human infectious reservoir in malaria endemic areas is therefore defined by the presence of mature male and female gametocytes in the blood. Interventions to reduce this reservoir or prevent transmission by direct interference with sexual stage development inside mosquitoes could facilitate efforts to achieve malaria elimination (1, 2).

From the point of erythrocyte invasion by a sexually committed merozoite it takes 10 to 12 days for *P. falciparum* gametocytes to fully mature; during this time, they pass through five distinct developmental forms (stages I-V). Immature gametocytes sequester primarily in the bone marrow and spleen outside the peripheral circulation (3–5). They are released back into the circulation to fully mature, after which they can be transmitted to mosquitoes during a blood meal. In the mosquito midgut, gametocytes egress from the host cell, and differentiate into male and female gametes that rapidly undergo fertilisation. In humans, intact immature gametocytes produce proteins that are exported to the erythrocyte surface and elicit an immune response (6). Early reports suggest that naturally acquired antibodies can directly affect gametocyte morphology and maturation, and as a result these antibodies may be able to affect gametocyte numbers and time in circulation (7, 8). Recent evidence indicates that antibodies specific to putative immature gametocyte erythrocyte surface antigens may promote phagocytosis (6), but to what extent gametocytes are specifically targeted and killed in circulation remains unclear. In contrast, there is abundant evidence that immune responses to intra-erythrocytic gametocyte proteins can inhibit gamete fertilisation in the mosquito midgut, when gametes are exposed to the blood meal content after egress from the Red blood cell (9). These target antigens form the basis of advanced transmission blocking vaccines (10).

At present, little is known about antibodies specific for gametocyte proteins, besides the well-characterised gamete fertility proteins (Pfs48/45 and Pfs230). Studies indicate that these proteins may not be the sole contributors to natural transmission blocking immunity (11), so there is an imperative to investigate immune responses to a wider sexual stage protein catalogue. The use of gametocyte specific antibodies as biomarkers of gametocyte carriage and infectiousness may also help identify the infectious reservoir in population-wide surveillance. Prior studies that identified gametocyte-enriched or specific proteins used proteomic data, without reference to immunogenicity (11, 12). Studies assessing anti-gametocyte antibody responses have focused on naturally exposed populations and are thus complicated by lack of effective controls and inherent variance in prior parasite exposure. Controlled human malaria infection (CHMI) models (13) in which malaria naïve volunteers are deliberately infected with *P. falciparum* parasites, provide a powerful tool to study immune responses during a well-characterized primary infection. Classical CHMI does not allow for evaluation of interventions affecting transmission, as gametocytes arise approximately 10 days (14) after asexual parasitaemia peaks, by which time participants have received full curative treatment that does not allow for gametocyte development. Recently, the CHMI model has been adapted to allow safe induction of gametocytes in study participants (12, 15, 16). In these models, volunteers were infected with *P. falciparum* 3D7 parasites and sub-curative treatment of asexual parasites allowed the development of viable mature male and female gametocytes. Infection by injection of infected red blood cells appeared to induce higher gametocyte densities and a higher likelihood of infecting mosquitoes, compared to infection through mosquito bites (16) and resulted in lower inflammation (17).

Here, we assessed antibody responses to sexual stage antigens among participants of a CHMI transmission study (16) after a single induced infection. We examined the immunogenicity of gametocyte proteins, the acquisition of gametocyte-specific antibodies and their association with preceding gametocyte exposure. Bead-based antibody assays allowed us to assess antibody responses to sub-units of the transmission blocking vaccine antigens Pfs48/45 and Pfs230, with comparison to well characterised antibody biomarkers of blood stage infection. Using a protein microarray, we set out to identify novel antigens that are targeted by antibodies uniquely induced after gametocyte exposure,

which is of value in the context of serological assay development for gametocyte surveillance.

Materials and methods

Clinical trial samples

Samples were collected in a CHMI transmission trial conducted between May and November 2018 (16). Individuals were infected either by the bites of 5 P. *falciparum* 3D7 infected mosquitoes (SPZ Gct, n =12), or by intravenous injection with ~2,800 *P. falciparum* 3D7 infected human erythrocytes (BS Gct, n=12). Parasitaemia was monitored by 18s quantitative polymerase chain reaction (qPCR); after parasitaemia reached a prespecified treatment threshold, participants received a gametocyte permissive sub-curative dose of piperaquine (480 mg) (16). Serum and citrate plasma samples were collected on prespecified time points prior to and after challenge infection. Plasma samples were selected for analyses from blood samples taken prior to challenge (C-1), and at days C+7 (BS Gct), C+9 (SPZ Gct), C+21 and C+36 in BD Cell Preparation Tubes with sodium citrate. One serum sample was selected from day C+51, which was collected in BD SST™ II Advance tubes.

As a control for gametocyte exposure in our antibody assays, additional plasma samples were analysed from control participants in a CHMI study where no gametocyte exposure was anticipated. Although gametocyte exposure was deemed highly unlikely due to early curative treatment, the absence of gametocytaemia was not formally demonstrated. This study was conducted between April 2011 and March 2012 (18); volunteers were infected by mosquito bite (SPZ Control: 5 P. *falciparum* 3D7 infected mosquitoes, participants n=5) or an intravenous blood stage injection (BS Control: 1,962 *P. falciparum* 3D7 infected erythrocytes, participants n=5) and treated with a standard curative regimen of atovaquone/proguanil upon thick smear positivity. Here, we analysed antibody responses in 5 volunteers infected by mosquito bite (SPZ Control, acting as a control for SPZ Gct) and 5 infected with blood stages (BS Control, acting as a control for BS Gct). Plasma samples were collected prior to challenge (C-1), and at days C+7 (BS Control only), C+9 (SPZ Control only), C+21 and C+36 in BD Cell Preparation Tubes with sodium citrate. Plasma samples were collected in BD Cell Preparation Tubes with sodium citrate.

Asexual parasite densities were determined by 18S qPCR on prespecified timepoints as described previously (16, 18, 19). Gametocyte densities were determined using quantitative reverse-transcriptase polymerase chain reaction (qRTPCR) for *ccp4* (female) and *pfmget* (male) messenger RNA, with a limit of detection of 0.1 male or female gametocyte/µL (20).

Both trials were performed at the Radboud university medical centre (Nijmegen, the Netherlands) following approval by the central committee on research involving human subjects (CCMO) under NL34273.091.10 and NL63552.000.17. All study participants provided written informed consent and both trials were registered at clinicaltrials.gov under NCT01236612 and NCT03454048. Antibody responses were specified as an exploratory outcome measure in the CHMI transmission study; the current analyses are thereby ancillary to the main study evaluation that focused on safety, gametocyte density and infectivity.

Gametocyte and asexual ELISA

Gametocyte and asexual extracts were prepared as described previously (21). Nunc MaxiSorp™ 96-wells plates (ThermoFisher) were coated overnight at 4°C with 100 µl, equivalent to 75,000 gametocytes or 40,000 asexual parasites, per well. Plates were blocked with 5% skimmed milk in PBS and subsequently incubated with an 1:50 dilution of citrate plasma. Detection was done with 1:40,000 dilution Goat anti-Human IgG HRP (Invitrogen, Cat. No. **31412).** ELISAs were developed by adding 100 µL tetramethylbenzidine and stopped with 50 µL 0.2M H_2SO_4. Absorbances were read at 450nm on an iMark™ microplate absorbance reader (Bio-Rad).

ELISA analyses were performed using Auditable Data Analysis and Management System for ELISA (ADAMSEL FPL v1.1). We included serial diluted control serum from a Dutch missionary that experienced many malaria episodes as a standard curve. The standard curve was plotted on a logarithmic scale and fitted to a power trend line (R2> 0.99), optical density (OD) measurements for each test sample (average of duplicates that were no more than 25% different) were converted to arbitrary units (AU) relative to the control serum, where undiluted control serum was defined to contain 100 AU of IgG.

Bead-based antibody quantification

IgG antibodies against 21 antigens, one targeting pre-erythrocytic stages (Circumsporozoite protein [CSP] (22)), 15 targeting the asexual blood stage (Erythrocyte binding antigen [EBA140, EBA175 and EBA181] (23); Early transcribed membrane protein 5 [Etramp-5] (24); Glutamate rich protein 2 [GLURP-R2] (25); Heat shock protein 40 [HSP40] (24); Merozoite surface protein 1-19 [MSP1-19] (26), Merozoite surface protein 2 [MSP2-ch150/9 (3D7 family allele) (27), and MSP2-DD2 (FC27 family allele) (28); Schizont egress antigen 1 [SEA-1] (29); Skeleton binding protein 1 [SBP-1] (30); Apical membrane antigen 1 [AMA1] (31); and Reticulocyte binding protein homologue [RH2.2 (32), RH4.2 (33), RH5.1 (34)] and five (belonging to 2 proteins) targeting sexual stages [four fragments of Pfs48/45; Pfs230-CMB (35)] were quantified for all samples for each participant using a Luminex MAGPIX©

suspension bead array, as described previously (36). The proper conformation of Pfs48/45 recombinant proteins was validated using conformational dependent rat monoclonal antibodies (37). A complete list of antigens is provided in Table S1. Briefly, plasma/serum samples were assayed at a dilution of 1:200. Secondary antibody was an R-phycoerythrin conjugated goat anti-human IgG (Jackson Immuno Research, PA, USA; 109-116-098) diluted to 1:200. Data are presented as background adjusted median fluorescence intensities (MFI), or the same measure as a log2 ratio of each individual's adjusted MFI at baseline.

Protein microarray

De novo protein microarrays were designed and printed at the University of California, Irvine, to assess antibody responses to a panel of gametocyte enriched *P. falciparum* proteins. The backbone for protein selection was an analysis of specificity for the gametocyte stage, as scored by determining frequency of detection across 11 proteomic analyses. This analysis is described in detail elsewhere (12). In summary: Proteins were binned from low to high abundance and weighted according to the retrieval rates of proteins in two curated lists of 'gold standard' gametocyte and asexual genes, consisting of genes that are known to be specific for either asexual stages (n = 45) or gametocytes (n = 41). High expression of gametocyte gold standard proteins with concurrent absence of non-gametocyte gold standard proteins resulted in a high gametocyte score, calculated from the fraction of retrieved gametocyte genes over retrieved non-gametocyte genes. All scores were log-transformed and summed over all data sets.

Full criteria for inclusion on the array are presented in Table S2. In addition to gametocyte specificity, proteins were prioritized according to their likelihood of gamete surface expression (based on gene ontological terms, domain prediction, or empirical evidence), or association with gametocyte exposure (38), transmission blocking immunity (39) or antibody recognition of immature gametocyte infected red blood cell (giRBC) surfaces (6). In total, a selection of 600 unique *P. falciparum* genes were selected for cloning. Sequences encoding the proteins were obtained from a 3D7 strain reference genome, with sequences longer than 1000 amino acids split into multiple fragments (overlaps of at least 17 amino acids). PCR amplification and cloning were successful for 568 unique genes (making up 943 distinct sequences) all of which were expressed in an *in vitro* transcription and translation (IVTT) system (5 Prime, Gaithersburg, MD, USA) according to manufacturer instructions, and as described previously (39, 40). Arrays were printed onto 8-pad nitrocellulose-coated glass AVID slides (Grace Bio-Labs, Inc., Bend, OR, USA) using an Omni Grid Accent robotic microarray printer (Digilabs, Inc., Marlborough, MA, USA). Each array and subarray contained IgG positive controls, for quality control, and negative controls containing the products of the IVTT reaction without PCR vector, for data normalisation. Samples were processed as described previously, with small deviations. Samples were probed at a final dilution on of 1:200, with secondary antibody (Southern Biotech, Goat Anti-Human IgG-TXRD) at a concentration of 0.5 µg/mL (39). Arrays were scanned on a GenePix 4300A High-Resolution Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA). Local background was assessed for each protein target automatically, with foreground MFI determined using irregular threshold pixel density mapping. Correction for background was done for each spot using the 'backgroundCorrect' function of the *limma* package (41). Background corrected values were transformed using the base 2 logarithm and normalised to systematic effects by subtracting the median signal intensity of the negative IVTT controls (internally within four subarrays per sample). The final normalised data are a log2 MFI ratio relative to the background reactivity of each sample/sub-array: a value of 0 represents equality with the background, and a value of 1 indicates a signal twice as high.

Data analysis

Data analysis was performed using R (R foundation for statistical computing, Vienna, Austria; version 4.1.2) (42), STATA (StataCorp. 2021. College Station, TX: StataCorp LLC; Release 17), or Graphpad PRISM (Graphpad software, San Diego, CA, USA; version 8). Total parasite and gametocyte area under the curve (AUC) was computed using GraphPad prism software with the formula AUC = $(\Delta X)^*(Y1 + Y2)/2$, where X is the time in days and Y the parasite density at a given timepoint. Correlation between parasite and serological data were analysed by Spearman's rank order correlation. For the analysis of breadth in the purified antigen antibody assays, antigens were considered 'recognised' for a given timepoint and participant if an antibody response (log transformed background adjusted MFI) exceeded the mean plus 2*SD of all individuals (n=34) against the same antigen pre-challenge. For the analysis of breadth in the protein microarray antibody assays, antigen recognition was defined as any log2 MFI ratio value greater than 1 (double the signal with respect to the internal array control). Serological data were log transformed and compared between timepoints within cohorts by paired t-tests, and between cohorts by students t-tests. For recombinant protein assays, significance thresholds were adjusted by Bonferroni correction for comparisons of multiple antigens. Comparison between array responses between timepoints and cohorts were conducted with bayes moderated t-tests with adjustment for false discovery (43).

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Results

In the CHMI transmission trial, 12 participants were infected by mosquito bite (SPZ Gct) and 12 participants were infected by intravenous injection of asexual parasites (BS Gct) (16). All participants developed parasitaemia with median onset of qPCR detectable parasitaemia on day 7 (Interquartile range (IQR) day 7 – 9) in SPZ Gct and on day 5 (IQR day 5 – 5) in BS Gct (Figure S1). To clear asexual parasitaemia but permit gametocyte maturation, participants received treatment with low dose piperaquine (480mg) on day 12.25 (Median, Interquartile range (IQR) day 10.5 – 12.5) in SPZ Gct, and all BS Gct participants were treated on day 8. This resulted in qRT-PCR detectable gametocytes post treatment in 11/12 participants in SPZ Gct and 12/12 participants in BS Gct (16).

In the control CHMI cohorts, 5 participants were infected by mosquito bite (SPZ Control) and 5 participants were infected by intravenous injection of asexual parasites (BS Control) with the same *P. falciparum* 3D7 parasite clone as the CHMI transmission trial. In contrast to the CHMI Gct trial, participants from control cohorts received a full curative treatment with atovaquone/proguanil that does not permit gametocyte maturation, initiated on day 12.3 (Median, IQR day 9.8 – 12.3) in SPZ Control and on day 8 (IQR day 8 - 8) in BS Control. As such, these participants were included in our analyses as negative controls without gametocyte exposure (Table 1), although there was no formal demonstration of gametocyte negativity.

Neither peak total parasite density nor AUC were significantly different between SPZ Gct and SPZ Control (peak density, p=0.091; AUC, p=0.058), and between BS Gct and BS Control (peak density, p=0.673; AUC, p=0.206) or between SPZ Gct and BS Gct (peak density, p=0.478; AUC, p=0.977). However, significantly higher peak gametocyte densities were observed in BS Gct compared to SPZ Gct (p<0.001, Mann-Whitney U; Table 1) (16). Total parasite AUC and gametocyte specific AUC were positively associated for both SPZ Gct and BS Gct (Figure S2A); a tighter correlation was observed for BS Gct, reflecting the controlled timing of blood stage infection and subsequent schizogony in this cohort.

Antibody response to crude gametocyte extract does not reflect preceding gametocyte exposure

Extracts from mixed asexual stage and mature gametocytes from laboratory cultured *P. falciparum* NF54 were prepared to assess antibody responses in the CHMI participants to native parasite proteins (Figure 1). At C+35/36 after infection, a statistically significant increase in anti-asexual stage antibodies was found compared to baseline in both SPZ Gct and BS Gct (p<0.0001) and corresponding SPZ Control (p<0.0001) and BS Control (p=0.002). Anti-asexual stage IgG antibody levels post infection were not statistically different between infection routes SPZ Gct and BS Gct (p=0.22), and borderline significant between SPZ Control and BS Control (p=0.050).

IgG levels against gametocyte extract were also significantly higher post CHMI compared to baseline in the SPZ Gct and BS Gct groups (p<0.0001). There was no statistically significant difference in antibody responses to gametocyte extract between SPZ Gct and BS Gct cohorts (p=0.31). Although participants in control cohorts were not exposed to sexual stage parasites during their CHMI, some subjects infected by mosquito bites (SPZ Control) showed an increase in anti-sexual stage IgGs in response to infection. On average, antibody responses to gametocyte extract were higher after infection for both control cohorts (SPZ Control, p=0.034, BS Control, p=0.0017), though for SPZ Control this increase was disproportionally driven by a single volunteer (after exclusion, p=0.075).

There were no statistically significant correlations between total parasite AUC or gametocyte AUC and either asexual or gametocyte antibody responses by ELISA (Figures S2B,C).

Sexual stage-specific antibodies are induced after limited gametocyte exposure

We observed that antibody responses to crude gametocyte extracts cannot be used to discriminate between responses to asexual and sexual parasite stimuli. This indicates, perhaps unsurprisingly, an abundance of proteins of unknown stage-

TABLE 1 Parasitaemia and gametocytaemia for different cohort.

		Mosquito bite (sporozoite) infection		Asexual stage infection	
		SPZ Control	SPZ Gct	BS Control	BS Gct
Total parasites	Median peak density in parasites/µL (IQR)	7,413 (2,336 – 28,371)	32,807 (7,137 – 50,831)	44,668 (27,184 – 74,505)	27,700 (9,818 – 81,091)
	Median AUC in parasites/µL/day (IQR)	8,682 (2,782 – 29,890)	37,654 (15,430 – 71,484)	62,470 (39,606 – 103,950)	38,735 (11,366 – 75,145)
Gametocytes	Median peak density in gametocytes/µL (IQR)	ND	14 (10 – 64)	ND	1,304 (308 – 1,607)
	Median AUC in gametocytes/µL/day (IQR)	ND	1,574(596 – 3,018)	ND	11,043 (2,715 – 14,866)

AUC, Area under the curve; IQR, Interquartile range; ND, Not determined.

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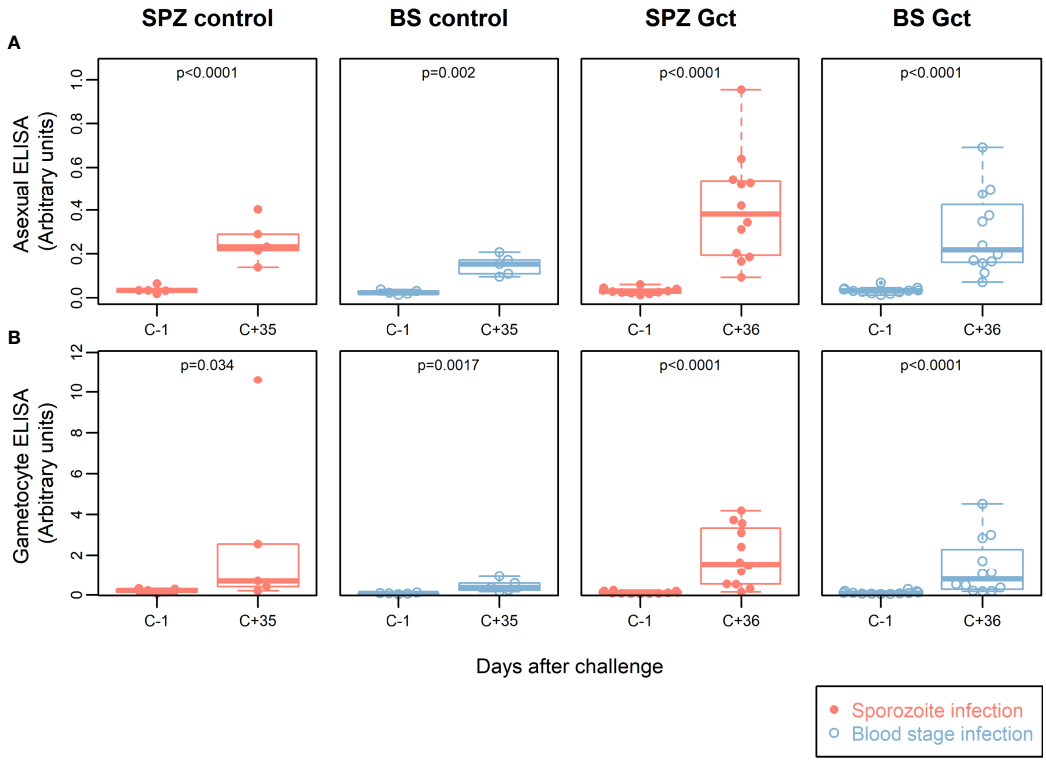

FIGURE 1
Participant parasite exposure and antibody response to crude parasite extracts. IgG antibody responses to crude asexual and gametocyte extracts. In all plots, red solid circles denote mosquito bite (sporozoite) infection cohorts (SPZ Control, SPZ Gct) and blue hollow circles denote asexual parasite infection (BS Control, BS Gct). **(A)** Anti-asexual antibody response pre- and post- (day 36) infection. **(B)** Anti-gametocyte antibody response pre- and post- (day 36) infection. p = p-values from paired t-tests on log-transformed data.

specificity in the gametocyte extract that formed the basis of this assay. We therefore analysed antibody responses to 21 well-characterized asexual stage, sporozoite, and sexual stage recombinant proteins, including TBV candidates Pfs48/45 and Pfs230, in a multiplexed bead-based antibody assay.

At baseline, the median number of antigens recognised per individual in SPZ Gct and BS Gct were 0.5 and 0, respectively (Figure S3). By C+36, all individuals were seropositive to at least one antigen, with a statistically significant increase in antibody breadth in SPZ Gct ($p = 0.025$), but not BS Gct ($p = 0.12$). At C+51, 21/21 antigens were recognised by at least one individual in both cohorts. Between C+36 and C+51, there was no significant increase in breadth of response in SPZ Gct ($p = 0.23$), but a significant increase in BS Gct ($p = 0.015$). Breadth of antigen recognition in the control cohorts was similar to the Gct groups at relative time points; the increase in breadth scores from baseline to C+35 was statistically significant in both SPZ Control ($p = 0.041$) and BS Control ($p = 0.0086$) and the change in median score was equal between control groups (SPZ Control, change in median = +2; BS Control, change in median = +2). In contrast, the increase in the median number of antigens recognized in the Gct groups over the same time period was nearly twice as great in SPZ Gct (change in median = +3.5) compared to BS Gct (change in median +2).

As observed in the cell extract assays, quantitative antibody responses to specific recombinant antigens increased significantly over the period of observation (Figure 2). In the Gct cohorts, the earliest independently significant increases in antibody response were observed at C+21 for a small number of non-sexual stage antigens (Table S3). However, after adjustment for multiple comparisons the only significant response observed at day 21 was against CSP in the SPZ Gct cohort ($p=0.001$). At C+35/36 a greater number of antigens showed increased responses, with those to PfMSP1-19 in SPZ Gct and BS Gct, and GLURP-R2 in SPZ Gct remaining significant after adjustment for false discovery. At C+51, several asexual antigens remained significantly elevated including ETRAMP5 and GLURP-R2 in SPZ Gct, EBA175, MSP2-DD2 and PfAMA-1 in BS Gct, and PfMSP1-19 in both cohorts. After adjustment for false discovery, antibody responses to the sexual stage antigens Pfs230, Pfs48/45 full length, and the 10C fragment of Pfs48/45 were all significantly higher at C+51 compared to baseline in BS

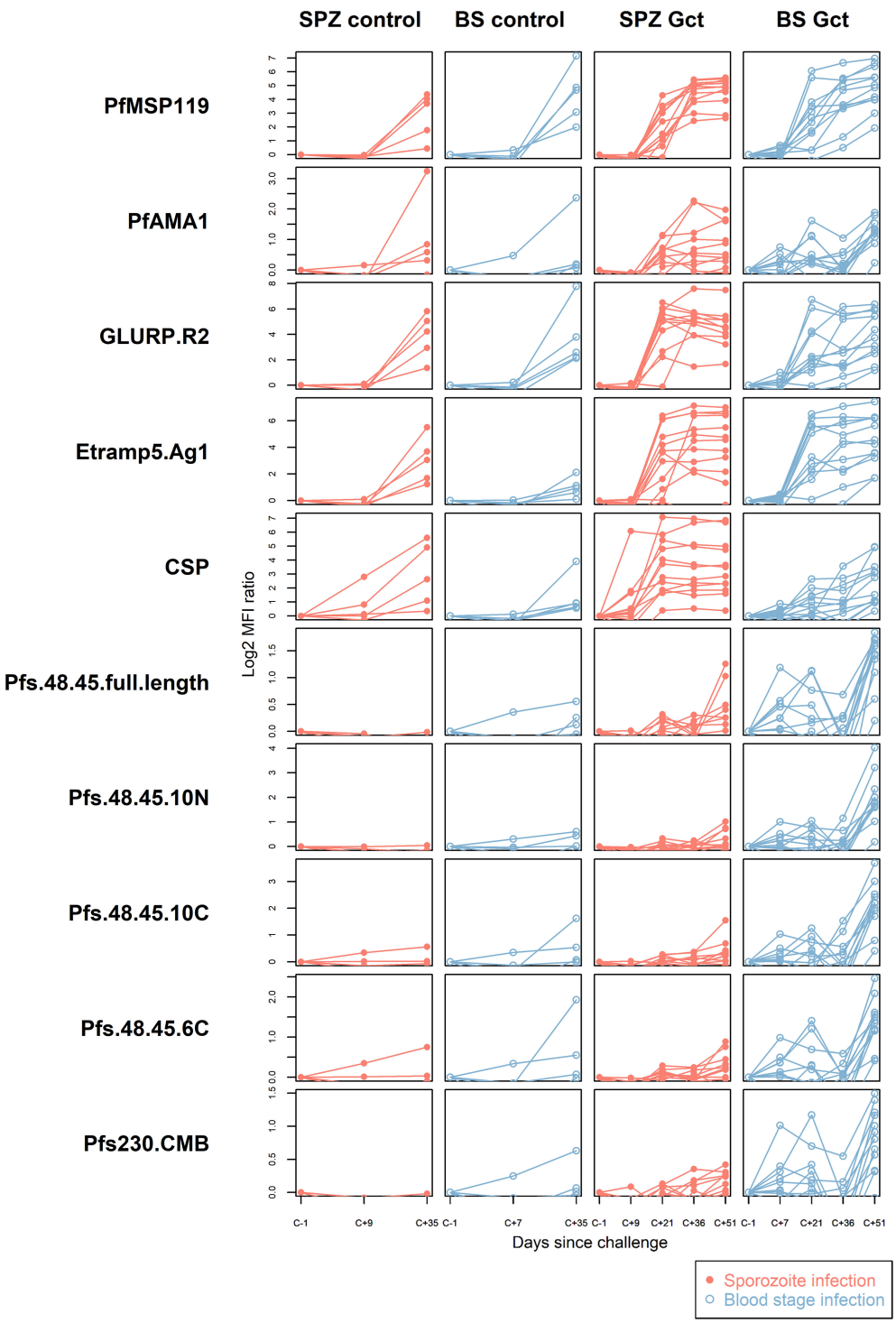

FIGURE 2
Antibody responses to purified recombinant proteins. IgG antibody responses to a selection of purified sporozoite, asexual and sexual stage antigens. Statistical analysis of response over time compared to baseline for all antigens is in Supplemental Table S3. In all plots, red solid circles denote mosquito bite (sporozoite) infection cohorts (SPZ Control, SPZ Gct) and blue hollow circles denote asexual parasite infection (BS Control, BS Gct). Data are presented as log2 median fluorescence intensity (MFI) ratios of response over baseline, each line representing a single individual.

Gct, but not SPZ Gct. No statistically significant antibody responses to gametocyte antigens were observed in SPZ Gct at any point, or in either cohort before C+51.

In the control cohorts, independently significant responses were observed for HSP40 in SPZ Control, and EBA140 and PfMSP1-19 in BS Control; these increases were not statistically significant after adjustment for false discovery. It should be noted that samples were only available until C+36 for these cohorts.

Responses to gametocyte antigens occur after responses to asexual antigens

To determine possible shifts in antibody responses during follow-up, supportive of responses specifically induced by gametocytes, we next compared antibody responses and their relative rankings between days C+35/36 and C+51. For this, antibody responses to all antigens were ranked by median magnitude of response in each cohort (Figure S4) and compared between C+35/36 and C+51 timepoints.

Within the CHMI Gct cohorts, median magnitude-ranked responses to sexual stage antigens were most increased between C+36 and C+51 in BS Gct, with Pfs48/45-10C, Pfs48/45-10N and Pfs48/45 full length moving up by 5, 4 and 5 positions, respectively (Table S4). In SPZ Gct, Pfs48/45-10C and Pfs48/45-10N moved up by 1 and 2 positions, though Pfs48/45 full length fell by 1 position. Little to no change was observed for Pfs48/45-6C or Pfs230-CMB in either cohort between timepoints. Sexual stage antigen rankings at C+35/36 between SPZ Gct and SPZ Control were similar, while median responses tended to rank higher in BS Control than in BS Gct at the equivalent timepoints.

Antibody response to gametocyte infected erythrocyte surface antigens are among those correlated with cumulative gametocyte exposure

The correlation between each recombinant protein biomarker and asexual or gametocyte exposure was assessed by analysing antigen-specific antibody data from the final timepoint of observation (C+51) in the CHMI Gct cohorts (Figure S5, Table S5) in relation to prior parasite biomass (i.e. area under the curve of density over time). In SPZ Gct, responses to Pfs230 and Pfs48/45-10N were independently correlated with asexual and gametocyte AUC, but these correlations were not statistically significant after adjustment for multiple comparisons. For BS Gct, several asexual and sexual stage responses were independently correlated with asexual and gametocyte AUC; after adjustment only PfMSP1-19 showed a statistically significant positive correlation with asexual AUC (R^2 = 0.64, p=0.0017), and only Pfs48/45-10C showed a statistically significant positive correlation with gametocyte AUC (R^2 = 0.64, p=0.0019).

To identify novel antibody biomarkers of gametocyte exposure, 943 protein targets (mapping to 568 gene IDs) were printed on microarrays, following selection for their enrichment in gametocyte stages based on transcriptomic and proteomic evidence (Table S2), or inclusion as known *Plasmodium* biomarkers. Antibody breadth increased significantly after infection (C+35/36) in all cohorts except for SPZ Control (Figure 3A). Mean magnitude of response to all antigens for each participant increased significantly in SPZ Gct and BS Gct, but not in the controls, while a significant increase in mean magnitude of response to each target protein was observed for all cohorts (Figures 3B, C). Correlation in mean response to each target between the two included post-infection timepoints (C+36 compared to C+51) was near perfect for SPZ Gct (R^2 = 0.99, p<0.0001) and BS Gct (R^2 = 0.98, p<0.0001).

At the level of individual targets, 216 of the 943 IVTT protein targets on the array displayed a significant increase in antibody response from pre-infection to either C+36 or C+51 in SPZ Gct, and 91 showed a significant increase in BS Gct. Four of the 91 targets with statistically significant responses in BS Gct were uniquely responsive in this cohort: PF3D7_0905300 (dynein heavy chain, putative; 1.65 fold increase compared to baseline), PF3D7_1302000 (EMP1-trafficking protein; 1.31 fold increase), and PF3D7_0721100 (conserved protein, unknown function; 1.32 fold increase), and PF3D7_1306500 (MORN repeat protein, putative, 1.15 fold increase) (Table S6). In the control cohorts, the only statistically significant increase in antibody response (C-1 to C+35) after adjustment for multiple comparisons was PF3D7_0206800 (MSP2) in SPZ Control.

To further distinguish antibody responses elicited by the principally asexual stimulus of the control CHMI studies from responses to the asexual and gametocyte stimulus of the Gct studies, the mean magnitude of response to each target post-infection was compared between control and Gct cohorts according to infection methodology. A threshold for negligible change in response between time points in the control cohorts was set arbitrarily as any percent difference in MFI of 7.5% or less, while a positive response cut-off for the Gct cohorts was set at 25%. 348 targets showed increases in magnitude (>25% absolute fold change) from pre- to post- (C+36 or C+51) infection in the Gct cohorts (Figure 4). Of these, 67 were responsive only in the SPZ Gct and/or BS Gct and not in their respective control cohorts; 53 were unique to SPZ Gct (median maximum percent change between cohorts at C+36 or C+51: 37.5% [IQR 30.7-47.4]), 8 were unique to BS Gct (35.0% [30.3-39.0]), and 6 were shared in both (SPZ Gct: 37.1% [32.9-41.6], BS Gct: 38.6 [29.0-44.1]) (Table S6). Nineteen targets were uniquely identified in the day 36 analysis, 6 in the day 51 analysis, and 42 were identified as responsive in both. A sub-selection of putative biomarkers of gametocyte exposure was

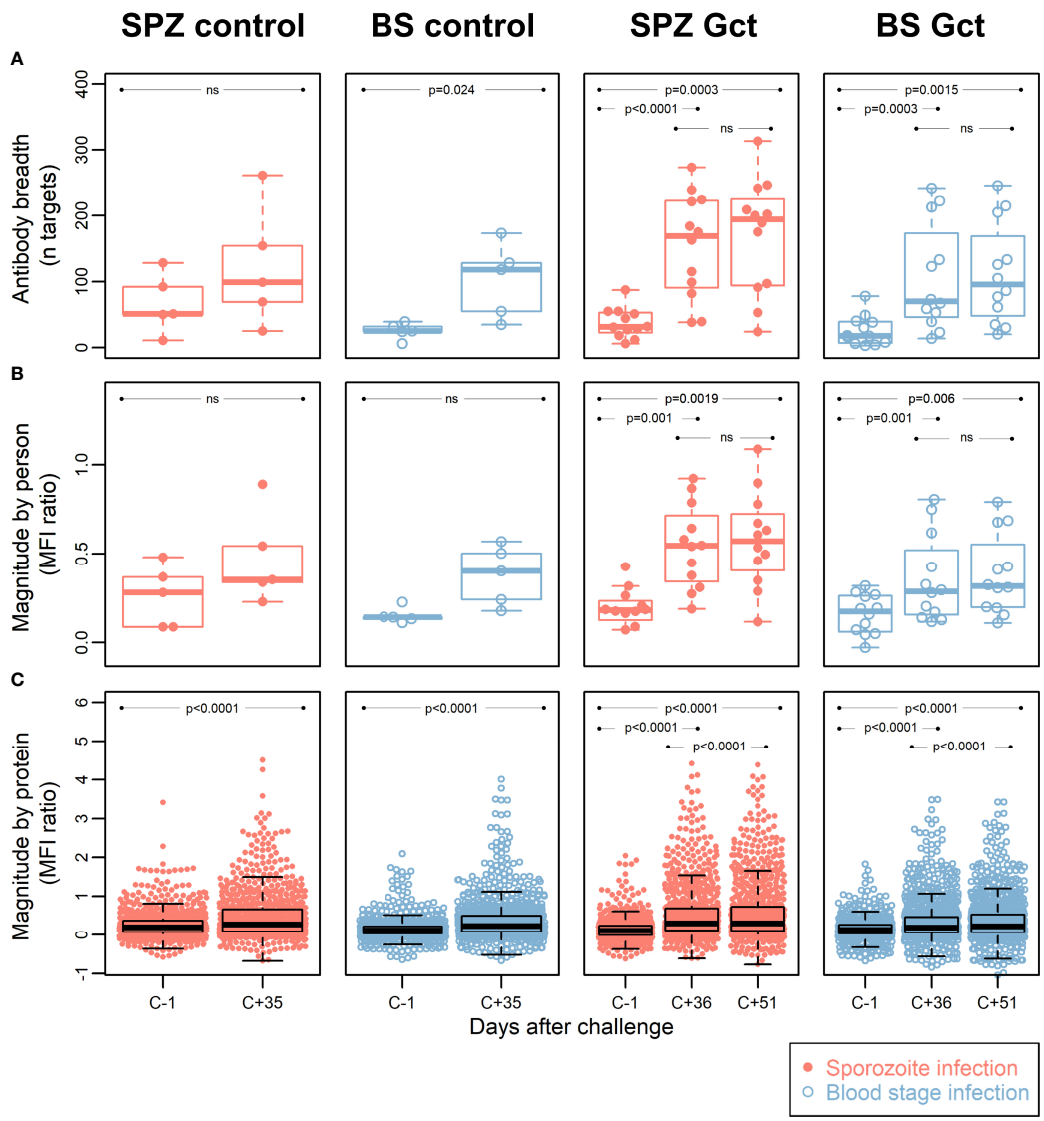

FIGURE 3
Antibody breadth and magnitude. Data points for each individual within each cohort group are represented with dots in a beeswarm pattern. Overlayed boxplots represent the median (thick line), interquartile range (box limits) and the 25th/75th percentiles plus 1.5*IQR (whiskers). **(A)** Antibody breadth is the number of protein targets (out of a total of 943) with responses above background, for each individual. For SPZ Gct, the mean MFI ratio of antibody responses to all proteins for one individual was -0.47 at C-1; this data point was not included in the plot, but the parameters of the relevant box plot were calculated from all data points. **(B)** Antibody magnitude is shown as the mean magnitude of response by each individual in a cohort group/timepoint to all protein targets. Magnitude of response is shown as a log2 MFI ratio, where 0 represent no change relative to background, and 1 represents doubling with respect to background. **(C)** Antibody magnitude is shown as the mean magnitude of response to each protein target by all individuals in a cohort group/timepoint, with units as in **(B)** P-values are from paired two-sided t-tests for difference between C-1 and C+35/36, C-1 and C+51, or C+36 and C+51, as indicated. MFI: median fluorescence intensity; IQR: Interquartile range. P = P-value from paired t-tests. Ns = Not significant at p = 0.05.

made and is shown in Table 2. This includes the four targets described above with significantly higher antibody responses (day C+36 and/or 51) compared to baseline in BS Gct but not SPZ Gct, six with responses in both SPZ Gct and BS Gct but not in their methodological control cohorts, and six with response in either SPZ Gct or BS Gct where a response was observed at C+51 but not at C+36 (two targets met more than one of these criteria). There was no significant correlation between antibody responses to these targets and total parasite AUC in either SPZ or BS Gct (Figure S6A), and no correlation between response and gametocyte AUC in SPZ Gct (Figure S6B). For BS Gct, 4 targets showed independently significant or borderline significant associations with gametocyte AUC: PF3D7_0721100 (R^2 = 0.33, p=0.051; conserved *Plasmodium* protein, unknown

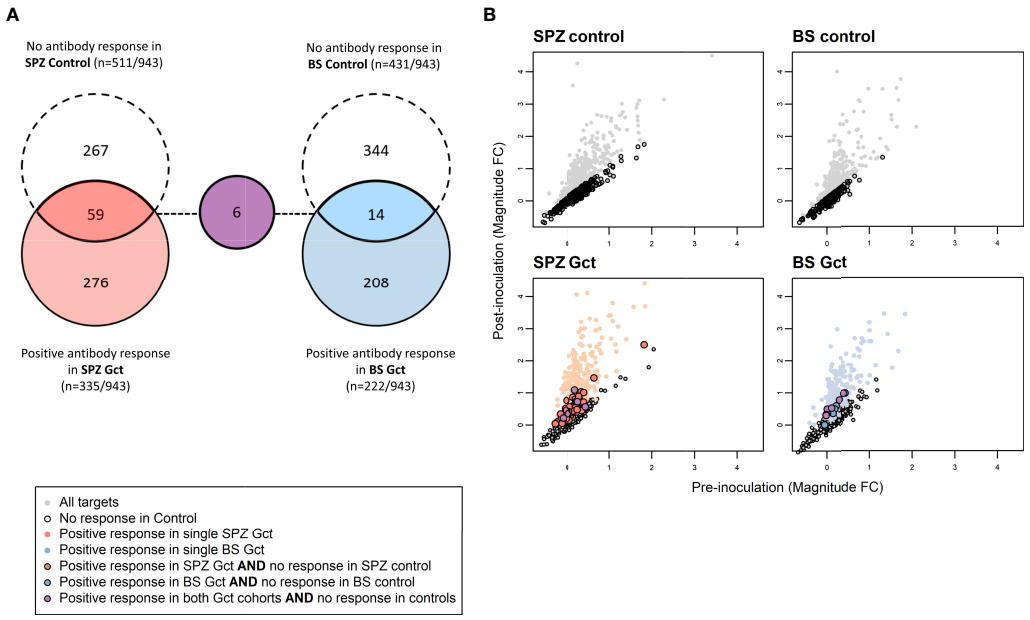

FIGURE 4
Differences in antibody response to microarray targets associated with gametocyte exposure. **(A)** Array targets demonstrating minimal response (<7.5% increase in the mean magnitude of response between pre- [day 0] and post- [day 35] inoculation timepoints) in the control CHMI cohorts are shown in red for SPZ Control (sporozoite inoculum) and blue for BS Control (asexual inoculum). Array targets demonstrating a positive response (>7.5% increase in the mean magnitude of response between pre- [day 0] and post- [day 36] inoculation timepoints) in the control CHMI cohorts are shown in dashed circles for each cohort, with overlap representing those targets with minimal response in control and positive response in Gct cohorts. **(B)** Axes show pre- and post-inoculation mean magnitude of response (log2 MFI ratio) to each target on the array. Mean magnitude for each target is represented by a single marker. Grey markers are those with increased responses after inoculation in the Control cohorts. Red and blue markers are as in **(A)** Targets with responses only in the CHMI Gct cohorts are outlined in black.

function), PF3D7_1302000 (R^2 = 0.47, p=0.014; EMP1-trafficking protein [PTP6]), PF3D7_0726400 (R^2 = 0.37, p=0.037; conserved *Plasmodium* membrane protein, unknown function), and PF3D7_1016300 (R^2 = 0.34, p=0.046; glycophorin binding protein [GBP]). None of these remained significant after adjustment for false discovery.

The 69 array targets meeting any of our criteria for further investigation (Table S6) mapped to 64 unique gene IDs; three IDs were represented by two peptides (PF3D7_1250100; osmiophilic body protein (G377), PF3D7_0212400; conserved *Plasmodium* membrane protein, unknown function, PF3D7_1328000; conserved *Plasmodium* protein, unknown function) and one was represented by three peptides (PF3D7_1038400; gametocyte specific protein Pf11-1). Association with cell membranes was highly represented (n/N=27/65) among predicted gene ontological terms (Table S6). Antibody responses to several well-characterised blood stage antigens were associated with gametocyte exposure in our analyses, including PF3D7_1228600 (merozoite surface protein 9, MSP9), PF3D7_0711700 (erythrocyte membrane protein 1, PfEMP1 [VAR]), and PF3D7_0102200 (ring-infected erythrocyte surface antigen, RESA). Well-characterised sexual stage antigens included PF3D7_1302100 (gamete antigen 27/25 [G27/25]), PF3D7_1102500 (parasite/early gametocyte exported protein PHISTB/GEXP02]), and PF3D7_1038400 (gametocyte-specific protein [Pf11-1]). Overall, 4/64 targets were specific to asexual stages, 42 were specific or enriched in gametocytes, 17 showed more evenly shared stage expression, and 1 was unclassifiable. Antibody responses to eleven of the 64 gene products identified here were noted for their association with naturally acquired transmission blocking immunity (TBI) in a previous analysis of individuals from Burkina Faso, Cameroon and Gambia (39). Nine of the 64 genes (three also linked with TBI) were identified as putative early gametocyte erythrocyte surface antigens in a previous analysis of rodent infections and sera from Malawi (6).

Discussion

To improve our understanding of naturally acquired immunity after gametocyte exposure, we assessed antibody responses to antigens in parasite and gametocyte extracts, selected recombinant *P. falciparum* proteins and a large panel of

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TABLE 2 Antibody targets putatively linked with higher gametocyte exposure as identified by protein microarray.

Gene ID	Name	Description	Criteria for selection	Note
PF3D7_0905300		dynein heavy chain, putative	*	
PF3D7_1306500		MORN repeat protein, putative	*	Naturally acquired transmission blocking immunity (mosquito stage) (39)
PF3D7_1351000		phosphatidylinositol transfer protein, putative	BS/51***	
PF3D7_1125200		ubiquitin-like domain-containing protein, putative	BS/51***	
PF3D7_0721100		conserved Plasmodium protein, unknown function	BS/51*/***	Early giRBC membrane antigen, putative (6)
PF3D7_1409400		conserved Plasmodium membrane protein, unknown function	SPZ/51***	
PF3D7_1135600	CAPD3	condensin-2 complex subunit D3, putative	SPZ/51***	
PF3D7_1016300	GBP130	glycophorin binding protein (GBP)	SPZ/51***	Early giRBC membrane antigen, putative (6)
PF3D7_1328000		conserved Plasmodium protein, unknown function	SPZ and BS/36 and 51**	
PF3D7_0307900		conserved Plasmodium protein, unknown function	SPZ and BS/36 and 51**	
PF3D7_0726400		conserved Plasmodium membrane protein, unknown function	SPZ and BS/36 and 51**	Early giRBC membrane antigen, putative (6) AND Naturally acquired transmission blocking immunity (mosquito stage) (39)
PF3D7_0201900	EMP3	erythrocyte membrane protein 3 (EMP3)	SPZ and BS/36 and 51**	Early giRBC membrane antigen, putative (6)
PF3D7_1320800		dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	SPZ and BS/36 and 51**	
PF3D7_1302000	PTP6	EMP1-trafficking protein (PTP6)	SPZ and BS/36 and 51*/**	Early giRBC membrane antigen, putative (39)

Summary table listing targets with significantly higher antibody responses (day C+36 and/or 51) compared to baseline in BS Gct but not SPZ Gct (*n=4), in both SPZ Gct and BS Gct compared to their methodological control cohorts (**n=6), and in either SPZ Gct or BS Gct where a response was observed at C+51 but not at C+36 (***n=6 [overlapping]). **BS** = Antibody response in blood stage infection cohort, SPZ = Antibody response in sporozoite infection cohort, 36 = Antibody response at C+36 compared to methodology matched control, 51 = Antibody response at C+51 compared to methodology matched control. Full details of these targets and all targets described in the text are given in Supplemental Table 6.

gametocyte-enriched proteins in volunteers from CHMI cohorts with different exposures to gametocytes. We showed that antibody responses to sexual stages are induced after a single exposure to relatively low gametocyte densities (peak densities up to ~1,600 gametocytes/mL). The antibody response to sexual stage-specific proteins was higher in participants exposed to higher gametocyte densities and was observed later than the response to well-characterised asexual antibody responses. Furthermore, we identified a list of known and new antigens that elicit antibodies that are associated with gametocyte exposure.

A handful of studies aimed to identify antibody responses to gametocyte-specific antigens in naturally exposed individuals. Several seroepidemiological studies, limited to the well-known gametocyte antigens Pfs48/45 and Pfs230 (44, 45), demonstrated rapid and short-lived gametocyte-specific antibody responses (46, 47), which do not necessarily increase with age as responses to some asexual antigens do (44, 48). Skinner et al. analysed antibody responses in the sera of Malian children to a large panel of putatively gametocyte-specific antigens based on the first published *P. falciparum* proteome. Comparing responses before and after the malaria season, they identified high seroprevalence of antibodies to the proteins Pfs16, PF3D7_1346400, PF3D7_1024800 and PfMDV1, indicating that these may be important biomarkers of gametocyte exposure. More recently, Muthui et al. set out to analyse naturally acquired antibody responses to seven antigens selected based on potential gametocyte surface expression, including Pfs230, PfMDV1 and five previously uncharacterized gametocyte antigens (49). They demonstrated antibody responses to all seven antigens, and suggested that PF3D7_0303900, PF3D7_1314500, and PF3D7_0208800 may have potential as markers of high gametocytaemia. Though undoubtably useful, these studies were not designed to assess the immune response to incident infection or accurately validate

serological biomarkers as indicators of prior gametocyte exposure. In field settings, such assessments are challenging and require longitudinal observations before and after infection with sensitive quantification of parasite and gametocyte exposure. Complementary to the studies in natural gametocyte exposure populations, CHMI provides a unique opportunity in which the absence of prior exposure is guaranteed, and parasitaemia and gametocytaemia are monitored with high precision to provide metrics for cumulative exposure to different parasite life stages. In our current study, all CHMI cohorts showed an overall increase in antibody responses after infection to antigens in asexual as well as gametocyte extracts. CHMI control cohorts were exposed to asexual parasites but probably not to mature gametocytes: while gametocyte commitment may have occurred (50) and, potentially, early-stage gametocytes may have developed, early treatment of volunteers with a curative regimen of atovaquone/proguanil upon thick smear positivity makes it very unlikely that gametocytes completed maturation. Nevertheless, responsiveness in our gametocyte ELISA in CHMI control cohorts is not unexpected since the majority of antigens are shared between parasite stages (12), and responses to crude gametocyte extract were previously shown to be a poor predictor of gametocyte carriage (51).

Importantly, responses against sexual stage specific antigens were highest in our cohort that was exposed to the highest gametocyte burden, with antibodies to Pfs48/45 and Pfs230 showing a statistically significant increase during follow-up in the BS Gct group. In this cohort, anti-Pfs48/45 antibody responses were also strongly associated with the preceding gametocyte biomass; this response was observed only at day 51 after CHMI, indicating a slight lag from peak gametocytaemia (c. day 20) to its commensurate response (not observed at day 36) compared to the peak in parasite density (day 8) and the observation of a response (from day 21). Given the relative sizes of the asexual and gametocyte biomass and their anticipated antigenic insults, these findings are broadly in line with expectations. It is noteworthy though that despite the relative scarcity of gametocytes, antibody responses to some sexual stage antigens (Pfs48/45-10C) were ranked higher than the majority of asexual antigens, indicating that the native Pfs48/45 protein is highly immunogenic. One unexpected finding was the antibody response to CSP after infection with blood stage parasites; we hypothesize that the abundance of low complexity, highly immunogenic repeat regions shared by CSP and some blood stage antigens may have resulted in a degree of cross-reactivity (52). Unexpected blood stage antigen reactivity has been observed previously after RTS'S vaccination (53).

Pfs48/45 and Pfs230 represent two well-described gametocyte antigens, but as there are hundreds of proteins predicted to be enriched or specifically expressed in gametocytes (12, 54), these two represent a tiny fraction of antigens that are likely to induce antibodies during gametocyte exposure. The ability to detect gametocyte-specific antibodies in the BS Gct cohort encouraged us to try to identify other gametocyte antigens that induced specific responses. We thus compared antibody responses to a large panel of gametocyte-enriched proteins on a protein microarray between the SPZ Gct and BS Gct cohorts. A long list of gene products (n=64) was identified for further analysis if they: 1) Showed significant antibody response to infection in the high (BS Gct) but not in the low (SPZ Gct) gametocyte cohort; or 2) Showed antibody responses in either the low or high Gct cohorts while having a negligible response in control CHMI cohorts. Encouragingly, we identified multiple antigens that are known to be gametocyte-specific, including Pfs16 (55), Pfg27 (55), Pf11-1 (56) and Pfg377 (57). Furthermore, there was considerable overlap with gametocyte proteins on the surface of infected red blood cells identified by Dantzler et al. (6), and with antigens that were identified as being associated with functional transmission reducing activity (39). Given that antibody responses in our CHMI cohort participants were low, as compared to individuals with rare functional anti-gametocyte immunity (39, 58), and as expected, based on a single relatively low infection burden, we did not expect or assess the functionality of responses but focused on the kinetics of antibody acquisition and the potential utility of responses as biomarkers of gametocyte exposure.

Our findings are based on detailed assessments of parasite exposure and antibody responses following a first encounter with *Plasmodium* parasites in a small number of volunteers. A limitation of this study is the relatively low gametocyte exposure with median peak densities of 1,304 (IQR 308 – 1,607) gametocytes/mL combined with a relatively short duration of exposure. Whilst these densities are similar to that observed in many asymptomatic infections in endemic settings, much higher densities and, in particular, much longer exposure to gametocytes can be observed in naturally infected individuals (59). As such, it is conceivable that our analysis did not identify all markers of epidemiologically relevant gametocyte carriage. A second limitation, as described above, is that we did not formally demonstrate absence of gametocyte exposure in control cohorts. If gametocytes had developed in control subjects, our comparisons between cohorts may have resulted in a conservative interpretation of unique gametocyte responses. A further limitation is the limited number of timepoints for immunological assessments. Control cohorts did not have a late timepoint (C+51) of sampling and we were unable to examine antibody longevity beyond day 51. Assessing the duration of detectable antibody titers, isotype and avidity beyond this timeframe, and whether re-infection will boost and/or change these responses will be valuable in the characterization of antibodies as biomarkers of infectivity and could have implications for the development of transmission blocking vaccines (TBV). Natural boosting of antibody

responses has been noted as an advantage for TBVs that target pre-fertilisation gametocyte antigens. We found that all BS Gct participants showed an increase in antibodies to TBV candidate antigens Pfs48/45 and Pfs230, including the Pfs48/45-6C and Pfs230CMB fragments that are similar to TBV targets currently in human trials (Ref (60). and clinicaltrials.gov: NCT04862416). Our findings suggest relevant natural boosting of Pfs48/45 and Pfs230 antibody responses; we further report on the first distinct analysis of responses against full length Pfs48/45, as well as three Pfs48/45 fragments: 10C, 10N and 6C.

In conclusion, we found increased humoral responses to *P. falciparum* sexual stages after exposure to a single CHMI, irrespective of gametocyte densities. The cohort with highest gametocyte exposure showed more sexual-stage specific responses compared to the cohort exposed to low gametocytes, while overall parasite responses were higher in SPZ Gct. Using a protein microarray we identified potential gametocyte specific antigens.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the central committee on research involving human subjects (CCMO). The patients/participants provided their written informed consent to participate in this study.

Author contributions

RJ, MA, WS, and TB conceived and designed the study. RJ, MA, WS, TO, ED, KT performed experiments and data analysis. WS and TB designed the microarray, with input from MM and KD. KKAT and RJ designed proteins used in the bead-based antibody assays. RN and RR constructed the protein microarray under supervision of PF. RJ, MA, TO and WS performed the data analysis, with contributions from MM, PN, KC and CD. RJ, MA, TO, WS and TB wrote the manuscript with input from all authors. All authors approved the final manuscript.

Funding

This work was supported by a Sir Henry Wellcome fellowship (number 218676/Z/19/Z) from the Wellcome Trust (UK) awarded to WS. TB, RMdJ and MA are supported by a fellowship from the European Research Council (ERC-CoG 864180; QUANTUM). CD and TO are supported by a project grant from the Bill and Melinda Gates foundation (INDIE OPP1173572), and KKAT is supported by the Intellectual Ventures Global Good Fund and a UKRI Connecting Capabilities fund/Bloomsbury SET (CCF-17-7779). MM and PN are supported by a Programme grant from the UKRI Medical Research Council (MR/T016272/1) and Wellcome Center award 104111.

Acknowledgments

We would like to thank James Beeson for provision of recombinant protein, and Marga van de Vegte-Bolmer, Geert-Jan van Gemert, and Kjerstin Lanke for their assistance with laboratory assays, and Jackie Cook and David Macleod for their data analytical assistance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.930956/full#supplementary-material

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frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 17 August 2022
doi: 10.3389/fimmu.2022.900080

Layer-by-Layer Delivery of Multiple Antigens Using Trimethyl Chitosan Nanoparticles as a Malaria Vaccine Candidate

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

Received: *19 March 2022*
Accepted: *15 June 2022*
Published: *17 August 2022*

Citation:
Xu Y, Zhou Z, Brooks B, Ferguson T, Obliosca J, Huang J, Kaneko I, Iwanaga S, Yuda M, Tsuji Y, Zhang H, Luo CC, Jiang X, Kong X-P, Tsuji M and Tison CK (2022) Layer-by-Layer Delivery of Multiple Antigens Using Trimethyl Chitosan Nanoparticles as a Malaria Vaccine Candidate. Front. Immunol. 13:900080. doi: 10.3389/fimmu.2022.900080

Developing a safe and effective malaria vaccine is critical to reducing the spread and resurgence of this deadly disease, especially in children. In recent years, vaccine technology has seen expanded development of subunit protein, peptide, and nucleic acid vaccines. This is due to their inherent safety, the ability to tailor their immune response, simple storage requirements, easier production, and lower expense compared to using attenuated and inactivated organism-based approaches. However, these new vaccine technologies generally have low efficacy. Subunit vaccines, due to their weak immunogenicity, often necessitate advanced delivery vectors and/or the use of adjuvants. A new area of vaccine development involves design of synthetic micro- and nano-particles and adjuvants that can stimulate immune cells directly through their physical and chemical properties. Further, the unique and complex life cycle of the *Plasmodium* organism, with multiple stages and varying epitopes/antigens presented by the parasite, is another challenge for malaria vaccine development. Targeting multistage antigens simultaneously is therefore critical for an effective malaria vaccine. Here, we rationally design a layer-by-layer (LbL) antigen delivery platform (we called LbL NP) specifically engineered for malaria vaccines. A biocompatible modified chitosan nanoparticle (trimethyl chitosan, TMC) was synthesized and utilized for LbL loading and release of multiple malaria antigens from pre-erythrocytic and erythrocytic stages. LbL NP served as antigen/protein delivery vehicles and were demonstrated to induce the highest *Plasmodium falciparum* Circumsporozoite Protein (PfCSP) specific T-cell responses in mice studies as compared to multiple controls. From immunogenicity studies, it was concluded that two doses of intramuscular injection with a longer interval (4 weeks) than traditional malaria vaccine candidate dosing would be the vaccination potential for LbL NP

vaccine candidates. Furthermore, in PfCSP/Py parasite challenge studies we demonstrated protective efficacy using LbL NP. These LbL NP provided a significant adjuvant effect since they may induce innate immune response that led to a potent adaptive immunity to mediate non-specific anti-malarial effect. Most importantly, the delivery of CSP full-length protein stimulated long-lasting protective immune responses even after the booster immunization 4 weeks later in mice.

Keywords: chitosan, layer-by-layer, releases, malaria vaccine, multiple antigens

1 INTRODUCTION

Malaria kills over 260,000 children under five years old in Africa every year. The first malaria vaccine RTS,S/AS01 (Mosquirix®), an advanced recombinant protein-based vaccine was approved in 2021 for children under 5 years old. However, compared with other vaccinations, RTS, S/AS01 has only modest efficacy preventing approximately 30% of severe malaria cases after a series of four injections (1). The recombinant protein is a pre-erythrocytic stage circumsporozoite protein (CSP). It targets parasites before they can infect the liver, but this is only relevant for one stage of the parasite's complex life cycle. One of the primary difficulties in malaria vaccination is the complexity of the multistage life cycle of *Plasmodium* and the intricate host-parasite interactions during the course of malaria infection. An optimal malaria vaccine would efficiently target multiple stages of the parasite life cycle (2). Further, vaccines offer another tool that could take pressure off continued use of combined malaria treatment drugs if one drug becomes resistant (2).

Due to the weak immunogenicity of investigational subunit vaccines, they often require advanced delivery vectors and/or the use of adjuvants (3). Design of novel adjuvant or nanoparticle delivery vectors that can stimulate immune cells and enhance vaccine efficacy has brought hope for future vaccine development. Improving the efficiency of vaccines by combination of adjuvants and advanced delivery systems based on controlled release technology is also one of the major priorities of the World Health Organization program for vaccine development (4). The goal is to develop a controlled release system to induce protective immune responses as soon as possible after the first immunization, while also providing prolonged immunity with negated or reduced administration of boosts.

To develop a more effective malaria vaccine with protective immune response and delivery of multiple life cycle stage antigens, we describe here a trimethyl chitosan-based layer-by-layer (LbL) nano-assembly vaccine platform (LbL NP) that enables the LBL delivery and release of multiple malaria antigens in a controllable manner. We have successfully constructed the LbL NP with efficient loading of different stages of antigens. It encapsulates a *Plasmodium falciparum* malaria parasite blood stage apical membrane antigen PfAMA-1 or merozoite surface antigen PfMSP-1 inside the core. The pre-erythrocytic stage antigen PfCSP (full length) is absorbed and stabilized on the shell layer of LbL construct. The size of the LbL NP vaccine candidates can be tuned from 200 nm to 400 nm, which are suitable for intramuscular injection (5). The highly positive charged surface of the trimethyl chitosan nanoparticles is beneficial for loading multiple antigens and confers greater solubility due to the trimethylation on the chitosan surface. A set of LbL NP was synthesized for encapsulation and loading with pre-erythrocytic and erythrocytic stage antigens at high efficiency of 70%-98%. The release of antigens was controlled between several days to months by tuning the charge and layer composition of the construct. The released antigens were characterized to verify that they maintained their stability and antigenicity. Most importantly, LbL NP served as antigen/protein delivery vehicles and induced the highest *Plasmodium falciparum* Circumsporozoite Protein (PfCSP) specific T-cell responses in mice, as compared to other adjuvants. Two doses of intramuscular injection with a longer interval (4 weeks) than other current vaccine candidates between them induced the high titer of humoral response against PfCSP. Furthermore, 5 of 6 mice were protected against a malaria challenge after receiving a booster of LbL NP delivery of full length of CSP as the vaccine candidate. Finally, general biosafety and dose tolerance studies demonstrated that LbL NP could be applied safely at less than 5 mg/kg with no significant adverse effects.

2 MATERIALS AND METHODS

2.1 Materials

Chitosan (75–85% deacetylated, mol wt. 50–190kDa), Sodium tripolyphosphate (TPP), Tween 80 (Cat.8221870500, Sigma-Aldrich); Poly sodium 4-styrenesulfornate (PSS, average Mw~70k, Cat. 243051, Sigma-Aldrich), Sodium Hyaluronate (HA, average Mw~60k, Cat. 9067-32-7, Glentham)

2.2 Ethics Statement

All animal experiments were carried out in strict accordance with the Policy on Humane Careand Use of Laboratory Animals of the United States Public Health Service. The protocol wasapproved by the Institutional Animal Care and Use Committee (IACUC) at The Columbia University (Animal Welfare Assurance no. D16-00003) and Michigan State University (Animal Welfare Assurance no. A3955-01). In addition, all components of the University are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC Unit #1047).

2.3 Animals

In the immunogenicity and efficacy studies, female BALB/c mice 8-10 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions. We only used female BALB/c to determine the infectivity of the transgenic PfCSP/Py parasites, as shown in previous published literature as a reference (6). In the safety studies, Sprague-Dawley rats were obtained from Charles River Laboratories and weighed 234-272 g at the time of use. Animals were individually housed, with free access to standard rodent chow and fresh water throughout the study. Animals were maintained on an automated 12/12-hour light/dark cycle with 7:00 am as the start of the light phase.

2.4 Parasites

We have previously generated the transgenic parasite, PfCSP/Py Spz by inserting a construct expressing the PfCSP at the locus of the *P. yoelii* CSP gene by double cross-over homologous recombination (7). The PfCSP/Py parasite, which is a useful tool to evaluate human malaria vaccine based on PfCSP in a mouse model was then shipped to Sanaria Inc., where the parasites were purified and cryopreserved. We focused on PfCSP/Py Spz challenge study to demonstrate the LbL NP delivery efficacy in this paper.

2.5 Expression and Purification of the Recombinant PfCSP, PfAMA-1 and PfMSP-1

The recombinant PfCSP was expressed in bacteria as reported by Zhang et al. (7). Briefly, the PfCSP plasmid (synthesized by Genscript) was transformed into the BL21(DE3) E. coli strain. The construct was subcloned into the *E. coli* pET-11a expression vector downstream of the T7 promoter using the NdeI and Bam HI restriction sites. The resulting transcribed gene incorporates additional amino acid sequence of HHHHHHHH at its 3' end and the PfCSP expression was induced using isopropyl 1-thio-β-galactopyranoside (IPTG, 1mM) at 20°C when the culture reached an optical density of 0.5 at 600 nm. Cells from the overnight culture were pelleted by centrifugation, resuspended in lysis buffer, and passed through a French press three times. The lysate was centrifuged for 30 min at 10,000 × *g* to pellet down the inclusion bodies and cellular debris. The PfCSP was purified using Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography. Nickel-captured CSP was then refolded in a refolding buffer and was subsequently dialyzed and concentrated. The gel image was captured in order to check the MW of produced CSP. The final yield was around 5 mg from a liter of LB culture with purity greater than 95%.

The recombinant AMA-1 and MSP-1 proteins were produced by mammalian cells. Briefly, the AMA-1 and MSP-1 genes were codon optimized for mammalian cell expression and synthesized by a commercial vendor (Genscript, NJ) with a secretion signal and a C-terminal 8x His tag. They were then cloned into the modified expression vector pVRC8400 (kindly provided by the Vaccine Research Center, National Institutes of Health). Plasmid DNAs encoding these proteins were transiently transfected into Freestyle 293-F cells, cultured in Erlenmeyer flasks using 25 to 30% of the nominal volume, and rotated at 120 rpm under standard humidified conditions (37°C and 5% CO2). Cells were allowed to secrete the malaria proteins for 4-5 days. Cell supernatants were filtered and loaded onto Ni-NTA beads, and proteins were eluted with 250 mM imidazole. Proteins were buffer exchanged into PBS by dialysis.

2.6 Statistical Analyses

Data was collected on an Excel spreadsheet. Graphic interpretation of the data was performed using GraphPad Prism 9.0.0. Statistical comparisons of vaccinated groups to vehicle control were made using Kruskal-Wallis one-way analysis of variance followed by post-hoc testing using Dunn's multiple comparisons test. In all cases, a $p<0.05$ was considered statistically significant.

2.7 TMC Synthesis and Characterization

Trimethyl chitosan (TMC) samples with different degrees of quaternization were synthesized according to a previous method (8), with some modifications of the reaction time. Briefly, chitosan was methylated by methyl iodide in a strong base (NaOH) solution at 60°C for a single time or multiple times to obtain TMC with different degrees of quaternization. The products were dissolved in NaCl solution and then purified by dialysis against the water and then lyophilized to be ready for next step encapsulation of antigens. The purified products were then analyzed by 1H NMR spectroscopy. The FTIR spectra of TMC and chitosan were measured using Nexus 6700 FTIR with Diamond ATR insert.

2.8 TMC-TPP Nanoparticle and TMC-TPP-Protein Nanoparticle Synthesis, Loading, and Release Tests

The TMC nanoparticles were prepared by ionic gelation of TMC with TPP anions. 10 mg of TMC was dissolved in 5 ml of water to make a 2 mg/ml solution. Subsequently, 2 ml of TPP solution at concentrations (2 mg/ml) was added drop-by-drop to the above solution under magnetic stirring at room temperature for 0-60 min. To optimize the synthesis parameters of LbL NP, we first chose BSA as a model protein for the load and release test since it has similar size of targeted malaria proteins. And then, we used targeted CSP, AMA-1 and MSP-1 proteins for the test. Protein was first dissolved in the TMC solution for stirring of 15 min before adding the TPP. The size and zeta potential of the TMC nanoparticles were measured with a Zetasizer (Nano ZS90, Malvern Panalytical). The particle-size distribution of the nanoparticles is reported as a polydispersity index (PDI). All measurements were performed in triplicate. The loading efficiency and capacity of the protein loaded TMC nanoparticles were determined by separating the nanoparticles from free proteins by centrifugation at 18,000×g for 15 min. The amount of free/unloaded protein in the supernatant was measured by micro-BCA protein assay. The supernatant of non-loaded TMC nanoparticle suspension was used as a blank. The loading efficiency and loading capacity of the nanoparticles

were calculated as follows and all measurements were performed in triplicate.

$$\text{LE}(\%) = (\text{total amount of protein} - \text{free protein})/\text{total amount of protein} \times 100\,\%$$

$$\text{LC}(\%) = (\text{total amount of protein} - \text{free protein})/\text{nanoparticles dry} \times 100\,\%$$

To identify and quantify loading and release of different proteins in LbL NP, we used fluorescence dye labelled technology for protein quantification. 2 mL of TMC solution was mixed with 1 mL and 0.5 mL of Texas-red 594-protein solution (Thermo Fisher, 1 mg/mL) in separated glass vials. Into each mixture solution, 50 μL of Tween 80 was added as non-ionic surfactant. After stirring for 10 minutes to fully mix TMC, BSA (or CSP, or AMA or MSP), and Tween 80, 2 mL of TPP solution (2 mg/mL) was slowly added under constant stirring. After reaction for 1 hour, the reaction solutions were purified by gradient centrifugation with 10 μL of glycerol three times. The samples were then redispersed into DI water, and the second layer of protein labelled by Alexa Fluor 488 (Thermo Fisher) was added with or without a protection layer of polystyrene sulfonate (PSS) or Hyaluronate (HA). The purified NPs were redispersed in PBS immediately for burst release testing at 37°C and the supernatant was also collected from the purification to calculate the encapsulation efficiency/burst release. NPs were kept in PBS at 37°C in an orbital shaker for up to 5 weeks to monitor the releases. During each period of data collection, old PBS buffer was exchanged with new buffer and the released protein was measured by fluorescence and UV-Vis spectrometry. Dynamic Light Scattering DLS and Zeta potential (Zetasizer Nano ZS90, Malvern Panalytical) were used for NP size distribution and surface charge at each step of formation of LbL NP or LbL NP-protein complexes.

Vaccine candidate preparation: After determining the composition of LbL NP using BSA as the model protein, the malaria protein PfCSP, PfAMA-1, and PfMSP-1 were encapsulated or absorbed on the LbL NP using these methods. Trimethyl chitosan (TMC) was dissolved in DI water at a concentration of 1 mg/mL, and tripolyphosphate (TPP) was dissolved in DI water at a concentration of 1 mg/mL. 1 mL of TMC solution was mixed with 250 μL of PfCSP solution at a concentration of 1 mg/mL. After TMC/PfCSP interaction for 30 minutes, 200 μL of TPP solution was added into the TMC/PfCSP mixture to form LbL NP CSP. After reacting for 1 hour, the reaction was stopped from stirring, and the solution was purified by centrifugal filtration. The final product was fully purified and then redispersed into 500 μL of sterile PBS.

For the three protein LbL formulations, TMC was dissolved in DI water at a concentration of 1 mg/mL, and tripolyphosphate (TPP) was dissolved in DI water at a concentration of 1 mg/mL. 1 mL of TMC solution was mixed with 91 μL of PfAMA-1 solution at a concentration of 1.64 mg/mL and 181 μL of PfMSP-1 solution at a concentration of 0.83 mg/mL. After mixing TMC and PfAMA-1/PfMSP for 30 minutes, 200 μL of TPP solution was added into the TMC/PfAMA-1/PfMSP mixture to form LbL NP AMA-1/MSP. After reacting for 1 hour, 150 μL of PfCSP solution at a concentration of 1 mg/mL was added to form LbL NP AMA-1/MSP-1/CSP formulation. After reacting for another 1 hour, the reaction was stopped, and the solution was purified by centrifugal filtration. The final product was redispersed into 300 μL of sterile PBS. The unloaded nanoparticles were synthesized using the same method which will be used as control. The final volume was concentrated to 500 μl.

Adjuvant Montanide formulation preparation: 250 μL of PfCSP solution at a concentration of 1 mg/mL was concentrated by using a protein concentrator (molecular weight cutoff 30K, Thermo Scientific™ Pierce™). Into the concentrated CSP solution (volume was ~20 μL), sterile PBS was added to achieve a final volume of 150 μL. Then, CSP solution was mixed with 350 μL of Montanide ISA 720 VG ST solution using two syringes for obtaining water in oil emulsion, for a final volume of 500 μL. A similar process was used for the three-malaria antigen formulations. 152 μL of AMA-1 at a concentration of 1.64 mg/ml, 250 μL of CSP at a concentration of 1 mg/ml, and 301 μL of MSP-1 at a concentration of 0.83 mg/ml were mixed and concentrated using the protein concentrator (Thermo Fisher, 10K, MWCO) by centrifugation. Into the concentrated protein solution (volume was ~20 μL), sterile PBS was added until the final volume was 150 μL. Then, 350 μL of ISA720 VG ST solution was emulsified with the protein solution. The final volume of solution was 500 μL and was transferred to an empty vial. As the control group, 150 μL of PBS was emulsified with adjuvant Montanide ISA 720 VG ST to obtain final volume of 500 μL.

Adjuvant 7DW8-5 formulation preparation: 250 μL of CSP solution at a concentration of 1 mg/mL was added of 225 μL of PBS which brought the total volume to 475 μL. Subsequently, 25 μL of 7DW8-5 solution at a concentration of 1 mg/mL was added into CSP solution. The final volume was 500 μL. Similar to CSP protein formulation, 152 μL of AMA-1 at a concentration of 1.64 mg/ml, 250 μL of CSP at a concentration of 1mg/ml, and 301 μL of MSP-1 at a concentration of 0.83 mg/ml were mixed and concentrated using a protein concentrator (molecular weight cutoff 30K). Into the concentrated protein solution (volume was ~20 μL), sterile PBS was added until the final volume was 475 μL. And then, 25 μL of 7DW8-5 solution at a concentration of 1 mg/mL was added into mixed three protein CSP/AMA-1/MSP-1solution. The final volume was 500 μL. As the control group, 475 μL of PBS was added with adjuvant 7DW8-5 to obtain final volume of 500 μL.

2.9 Antigenicity and Binding Kinetics Measurements of LbL NP CSP Complex Using nanoSPRi Platform

An antigenicity test was used for evaluation of released protein to identify its antigenicity. The method is an enzyme-linked immunosorbent assay (ELISA). Malaria antigens were prepared at concentrations of 20 μg/mL and 10 μg/mL in PBS, pH 7.4. 100 μL of each preparation were added in duplicate to wells of a 96-well ELISA plate and incubated at room temperature for 1 hour.

Plates were washed in PBS-Tween 5 x. 300 μL of blocking buffer was added to each well and incubated overnight at 4C. Plates were washed in PBS-Tween 5 x. 100 μL of 10 μg/mL primary antibody in PBS was added to each well (excluding no-antibody control wells) and incubated at room temperature for one hour. Plates were washed in PBS-Tween 5 x. 100 μL of a 1:2000 dilution of goat anti-human HRP IgG secondary antibody was added to each well (excluding no-antibody control wells) and incubated at room temperature for 1 hour. Plates were washed in PBS-Tween 5 x. 100 μL of TMB substrate was added to each well and color allowed to develop for ~ 25 minutes. 100 μL of 2 M sulfuric acid was added to each well and readings taken at OD450 nm.

The construction of nanoSPRi platform for malaria protein evaluation involves a sandwich assembly of malaria's antibody/antigen matched pair. In this design (details provided in Results and **Figure S1**), a capture antibody (CAb, e.g., anti-CSP) array was immobilized on the chip. The chip was prepared with 5 spots (repeats) for each sample (5-10 samples) to enable multiplex antigen detection. A sample containing malaria antigen (e.g., PfCSP) was then injected in the flow cell. The antigen was then specifically bound with the CAb array. The biotinylated detection antibody (e.g., biotinylated anti-CSP) was used as the detection system for bound antigen while streptavidin-coated nano enhancer quantum dots (QDs) were used as the signal amplification technique. When a QD is covalently bound to the detection antibody *via* biotin-streptavidin interaction, QD adds mass to the sandwich construct resulting in appreciable SPRi signal detection. The SPRi measurements were carried out as follows. First, the sensing chip was functionalized with thiolated protein A solution for 2 hrs in a humidity chamber (65-75% relative humidity). Thereafter, the chip was washed with deionized (DI) water, dried with a nitrogen stream, and allowed to form a self-assembly layer of protein A on the chip surface for at least 3 hrs prior to use. The functionalized chip was then be spotted with anti-malaria capture antibody (3C1 or 2A10) in 5 replicates for 4 different concentrations (125, 250, 500, and 1000 μg/mL). The resulting spots were then incubated inside a humidity chamber for at least two hours. The chip was then washed with DI water, air dried by gentle stream of nitrogen, mounted onto the SPRi instrument, and treated with Luna Labs' proprietary blocking agents. The instrument was (16) calibrated using a high salt concentration (25 mM NaCl) before analysis. Analysis was done by first injecting 1× PBS buffer to obtain a baseline signal. A single injection of 10 ng/mL of released PfCSP protein solution or control PfCSP original soultion was introduced in the flow cell at a running buffer flow rate of 20 μL/min to allow effective capture of the antigen by immobilized antibody spots on the sensing chip surface. The obtained sensorgrams were globally fit to a 1:1 biomolecular interaction model (software: SrubberGen, HORIBA Scientific) to calculate binding kinetic parameters: k_a, k_d and K_D.

2.10 Safety Studies

We evaluated the tolerability of a trimethylated chitosan nanoparticles administered intramuscularly to male Sprague-Dawley rats twice over 14 days. In total, sixteen male Sprague-Dawley rats were assigned to 4 groups (vehicle control or three dose levels of nanoparticle [n=4/group]) as shown in **Table S1**. Clinical observations were recorded up to once daily and body weights were assessed prior to dosing and at least twice weekly thereafter. The tissues/organs were also collected and weighed from all animals: heart, liver, kidney, and muscle tissues at the site of administration. Tissues/organs were processed using standard H&E staining. Microscopic evaluations of tissues/organs were conducted by a qualified veterinary pathologist.

2.11 Immunogenicity Studies

We formulated vaccine candidates by loading one pre-erythrocytic protective antigen PfCSP and two blood stage antigens PfAMA-1 and PfMSP-1 in the LbL NP structure as described in section 2.3. Three formulations were delivered for the animal studies for either two or three doses by intramuscular injections to compare the vaccine candidate performance (**Table 1**). They were LbL NP-CSP (TMC-TPP encapsulated PfCSP); LbL NP-AMA-1/CSP-1 (TMC-TPP encapsulated AMA-1 inside of core, and PfCSP in the outside layer); LbL NP-CSP/AMA-1/MSP-1 (TMC-TPP encapsulated MSP-1 with second layer of AMA-1 and the outside layer is CSP). Additionally, another two adjuvants Montanide ISA 720 [Seppic Inc. (9)], a natural metabolizable nonmineral oil and a highly refined emulsifier of mannite monooleate family, and 7DW8-5 (10), a recently identified novel analog of α-galactosylceramide (α-GalCer) that enhances the level of malaria-specific protective immune were used to compare and incorporated with these LbL NP formulations.

In total, 24 female BALB/c mice (n=4 per group) were immunized intramuscularly with each formulation twice or three times at a 3-week interval to determine the vaccine candidate potential dosing. For each animal, an injection containing 10 μg of protein was administered for each dose. Three weeks after the boost, mouse sera were collected for serology analysis of the antibody titers of PfCSP, PfMSP-1, and PfAMA-1 for each formulation and the numbers of IFN-γ-secreting T cells in spleens of mice immunized with antigens by intramuscular injection was measured by IFN-γ enzyme linked immunospot (ELISPOT) assay (10).

TABLE 1 | Vaccine candidate formulation compositions in Immunogenicity Study. 1-12 group animals were injected by two doses of vaccine candidates, but 13-24 group animals were injected by three doses.

Groups:	Immunization regimens
1, 13	LbL NP-CSP + ISA720
2, 14	LbL NP-AMA-1/CSP + ISA720
3, 15	LbL NP-CSP/AMA-1/MSP-1 + ISA720
4, 16	LbL NP + ISA720 only
5, 17	LbL NP-CSP + 7DW8-5
6, 18	LbL NP-AMA-1/CSP + 7DW8-5
7, 19	LbL NP-CSP/AMA-1/MSP-1 + 7DW8-5
8, 20	LbL NP + 7DW8-5 only
9, 21	LbL NP-CSP
10, 22	LbL NP-AMA-1/CSP
11, 23	LbL NP-CSP/AMA-1/MSP-1
12, 24	LbL NP only

2.12 Efficacy Studies

We prepared nine vaccine candidate samples for nine group of animals with two doses for each group of animals and one group using unloaded LbL NP as a control. These samples included two control adjuvant groups (Montanide ISA 720 VG ST and 7DW8-5). 10 groups (n=6 for each group, nine vaccine candidates and one control) of 8-10 weeks old female BALB/c mice were immunized intramuscularly with each formulation twice with a 4-week interval between two doses. For each animal, an injection containing 10 μg of protein was applied for each dose. Four weeks after the two doses, naïve as well as immunized mice were challenged with 1000 transgenic PfCSP/Py sporozoites intravenously. The infectivity of PfCSP/Py Spz was determined by the presence or absence of parasites (parasitemia) in the blood of the challenged mice. This was done by way of microscopic examination of Giemsa-stained thin blood smears made from one drop of blood extracted from the tail vein of the mice from day 4 to day 12 post-Spz challenge.

3 RESULTS

3.1 TMC Precursor Synthesis Optimization and Characterization

The critical component of the LBL vaccine delivery platform is trimethyl chitosan (TMC), which allows antigens to be properly loaded to form nanoparticles. We first synthesized TMC from chitosan which creates a positively charged molecule that is soluble in aqueous solutions. To accomplish this, we performed a methylation reaction two times to achieve trimethylation. The proton nuclear magnetic resonance (1H-NMR) spectrum of TMC characterization was shown in **Figure 1A**. According to the literature (11), the signal at 3.22 ppm corresponds to the methyl group at the N, N, N-trimethylated site, the signal at 2.72 ppm corresponds to the methyl group at the N, N-demethylated site, and the signals ranging from 4.8 to 5.4 ppm were attributed to the hydrogen atom bonded to the carbon 1 of the glycoside ring. The degree of quaternization (DQ) was calculated as about 50.5% using the following equation:

$$\%DQ(\%) = \left[\left(\frac{\int TM}{\int H}\right) \times \frac{1}{9}\right] \times 100$$

where $\int_{TM}$ was the integral of the trimethyl amino group (quaternary amino group) peak at 3.3 ppm and $\int_{H}$ was the integral of the 1H peaks from 4.7 to 5.7 ppm. The DQ was used for evaluation of trimethylation degree every time after trimethylation and also serves as the quality control factor for TMC production.

The FTIR band shown in the **Figure 1B** at 1,471 cm^{-1} was attributed to angular deformation of C-H bonds of methyl groups existing in higher proportion in TMC (8), as compared with chitosan only. The bands at 2,918 cm^{-1} that appear in the FTIR spectrum of TMC were attributed to characteristic stretching of C-H bonds. The FTIR spectrum further demonstrated that we successfully performed the trimethylation of chitosan.

3.2 Parameter Determination and Optimization for LbL NP-Antigen Formulation, Antigen Loading and Release

To form TMC LbL NPs, a cross linker is required. Tripolyphosphate (TPP) is a non-toxic polyanion that can crosslink with TMC to form uniform nanoparticles under certain condition. By using a cross linker, the encapsulation and loading of multiple antigens on the TMC nanoparticles can also be achieved. For making nanoparticles only, we mixed TMC and TPP solution as indicated in the Methods Section. 60 min of reaction time was selected for core-shell layer structural nanoparticle formation (**Figure 2**). After 15 min of reaction, the particles were starting to form. After 30 minutes of reaction time, the nanoparticles were formed at approximately 50 nm

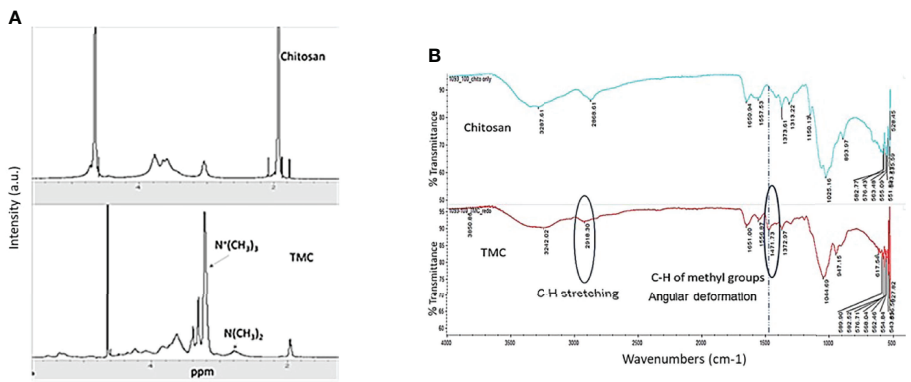

FIGURE 1 | **(A)** Characteristic 1H-NMR spectrum of Chitosan and N-trimethyl chitosan (TMC) in the D2O. **(B)** FTIR spectra of Chitosan and N-trimethyl chitosan (TMC).

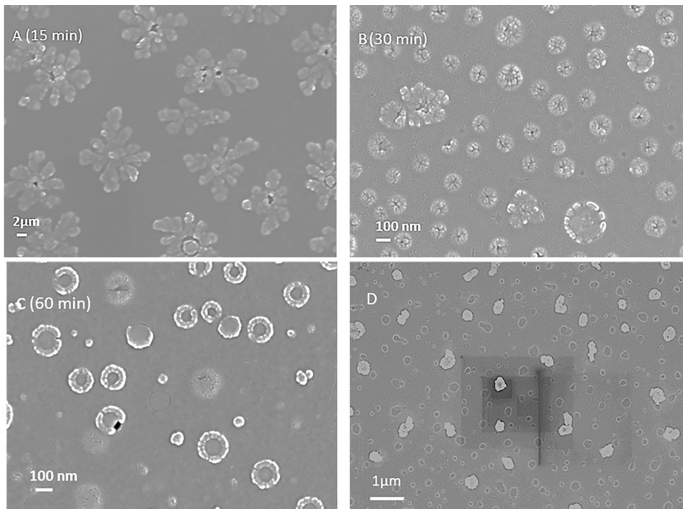

FIGURE 2 | **(A-C)** Scanning electron microscope images of LbL NP formations at different reaction time (15, 30 and 60 min). 60 min was found to be the optimized time for the core-shell structural nanoparticle formation. **(D)** SEM image of LbL NP encapsulated with protein.

diameter as a single nanoparticle, though they also self-assembled to start forming the core-shell layer structure nanoparticles at a size range of 200-300 nm (**Figure S2**). After 30-60 minutes, the nanoparticles were found to be stable in the solution. Zeta potential analysis was used paralleled with DLS for measuring surface charge of nanoparticles during the synthesis. TMC precursors had a highly positive charge of +40~+50 mV, and TMC-TPP nanoparticle formation lowered the surface charge to approximately +20~+30 mV due to the TPP crosslinking.

In addition to the TPP-crosslinked TMC nanoparticle "core," we also investigated the another negatively charged long chain polymer PSS as the crosslinker to compare with TPP. As shown in **Table S2**, the charge of NPs changed from negatively charged to positively charged when the ratio of TMC : PSS decreased from 0.91: 1 to 0.83: 1. As a result, we focused on those composition that has ratios higher than 1:0.83 for the protein loading since it requires a positively charged surface. At the same time, we investigated the charge changes when we included both TPP and PSS in the composition and tuned the ratio between TMC : TPP to PSS. As shown in **Table S3**, once we added PSS during NP formulation, the surface charge of the NP significantly decreased from highly positive charge + 23 mV to + 4 mV. Also, we observed the presence of white aggregates formed during the NP synthesis after adding higher amount of PSS (TMC : PSS ratio >10:1). These results indicated that adding the PSS could cause aggregates of NPs if the amount is high. As a result, we worked to optimize the amount of PSS used in the composition to form stable NPs without precipitation. Since PSS has shown stronger interaction with TMC. At the same time, another negatively charged polymer hyaluronate (HA, 60K MW), was selected for comparison with PSS as an alternative option for the outer-layer coating. Different amounts of HA were tested similarly to that performed for PSS. However, we did not see aggregates during the LbL NP formation which indicated that HA would be a good candidate for LbL NP formulation.

We next evaluated protein loading and encapsulation inside LbL NP. First, bovine serum albumin (BSA) was selected as the model protein for nanoparticle loading and release tests due to the size similarity to the malaria antigens targeted in this study to obtain preliminary data. We could encapsulate BSA in TMC-TPP nanoparticle with size of 320 ± 82 nm which is larger than the pure NPs (224 ± 20 nm) as indicated in **Figure S2**. Also, the empty core was encapsulated with protein which showed a fully solid nanoparticle in SEM image (**Figure 2D**).

We chose two different dyes for the labelling of BSAs to present different proteins in the LbL encapsulation and loading test. Once we determined the optimized composition of LbL NP using these model proteins, we worked on the malaria antigen proteins. One BSA was labelled with Texas Red dye (abs: 594 nm) for encapsulation in the core of the chitosan LbL NP, and a second BSA with Alexa Fluor dye (abs: 488 nm) dye labelling was immobilized on the NP surface without and with adding the outer protection layer (blue circle) polystyrene sulfonate (PSS) or hyaluronate (HA) as shown in the **Figure 3A**. The two dye-labelled BSA proteins can be identified separately using UV-Vis spectroscopy, as shown in **Figure 3A**. Results indicated that there was no interference between the emission peak of each dye when they were mixed in solution and measured simultaneously.

The loading efficiency (LE) of core protein encapsulation was calculated at approximately 85.2%. However, during the loading process for the second layer protein, the first layer of protein exhibited a burst release. The LE slightly decreased to 82.3% without a protection layer of PSS, while it further decreased to 74.2% with PSS coating. However, the loading efficiency of the second layer protein was approximately 91.2% without PSS and 84.5% with PSS. Also, from the release test we could see that the encapsulated protein was released significantly faster as expected,

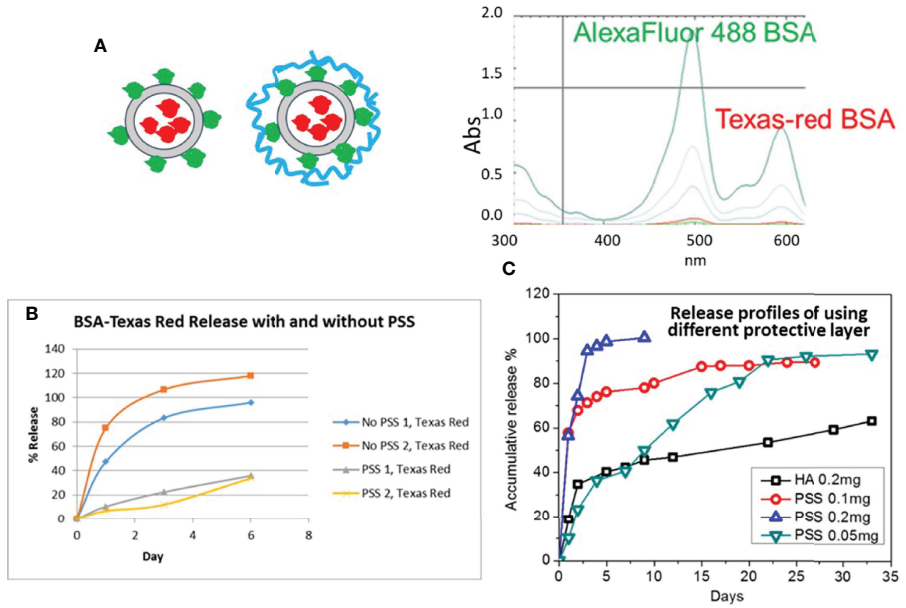

FIGURE 3 | **(A)** Schematic of dye Alexa Fluor 488 (green) and Texas-red labelled BSA LbL loading on NPs (red circle) by two approaches without and with second protection layer coating (Blue); UV-Vis spectra of two dye labelled protein mixture. **(B)** Release profile of NP formulation with and without PSS as the protective layer. **(C)** Release profile of LbL NP formulation with different mass amount of PSS or HA as the protective layer.

so the protective layer is necessary to obtain a long-term release profile (**Figure 3B**).

Secondly, to achieve the release profile for each protein layer, we optimized the composition of the second layer of PSS and TPP. We determined that the ratio of TMC : TPP:PSS should be kept below 1:0.2:0.5 for protein loading. The final formulation loading efficiency of protein can be reached as high as 91.2% or 97.5% when we applied TMC : TPP:PSS ratio of 1:0.2:0.2 or 1:0.2:0.1 respectively. Similar to PSS, HA used as the outer layer was also evaluated for effect on protein release. The test for the selected formulation release were monitored for more than a month, with results shown in **Figure 3C**. The burst release of the Texas Red BSA protein for these samples was 30-40% for using higher PSS contents (0.1 mg), but when we decreased the amount of PSS to 0.05 mg or for 0.2 mg of HA coating, the burst release decreased to 10% and 24%, respectively. Also, the encapsulated protein was released significantly faster with higher content of PSS layer, and the release rate decreased with a reduction in the amount of PSS used. For achieving a month interval release profile, we need to limit the coating amount between 0.05-0.1 mg per 1mg TMC. HA used as the coating for the outer layer provided even longer protein sustained release for if we need to achieve greater than one month release profile.

At the final optimization, we chose the loading and release of a second layer of BSA using 0.05 mg (High) and 0.01 mg (Low) of PSS or HA as the protective layer. When we used PSS, the encapsulation efficiency for BSA was as high as 98.5% and 93.4%, for the inside (red) and outside (green) layers, respectively (**Table 2**). Results also show that over the first 4 days, both protein layers have similar release profiles (**Figure 4**). However, after a week of release, the outer layer of protein ("2nd Protein") demonstrated an increased release rate as compared to the inner core layer of protein ("1st protein"). It was clear that when less protective polymer used (0.01 mg), there is a faster release rate. However, for HA coated LbL NP formulation, the inner layer (first protein) release reached 42% of total after 30 days for samples with lower amounts of HA protective layer, as compared to only 15% of release when we used higher amounts of protective coating of HA. However, the second protein was almost entirely released for higher HA samples. For the PSS protective coating samples, the release was higher than 80% at 30 days. However, the higher PSS coating, the first protein was released at a lower amount (40%) compared to low PSS coating with almost 80% released. These results indicated that we could control the protein release profile by tuning the protective layer of coating amount and compositions. Using BSA as the model protein, we have proven the concept using LbL NP for multiple antigen loading/encapsulation and delivery. We used this model to apply for malarial antigen candidates pre-erythrocytic protective antigen CSP and blood stage protective antigen AMA-1, MSP-1 with further investigation and animal studies. The loading efficiency for these three proteins were in the range of 70-98%.

The optimized ratio of TMC : CSP was determined at 10:2.5 if CSP is the only protein encapsulated in the core of the nanoparticles. The CSP has found relatively more negative charge in PBS solution than BSA protein which caused the decreased NP size with lower zeta potential compared with LbL NP-BSA formulation. However, for two- and three-protein loading, CSP was loaded in the outer layer of the formulation. The average size of TMC NPs loaded with CSP was much smaller

TABLE 2 | LbL NP composition and encapsulation and loading efficiency for each of the vaccine candidates.

Sample	TMC (mg)	TPP (mg)	PSS (mg)	HA (mg)	BSA (red, mg)	LE (%)	BSA (Green,mg)	LE (%)	Zeta potential (mV)
High PSS	1	0.2	0.05		0.25	98.5	0.25	93.4	5.0
Low PSS	1	0.2	0.01		0.25	74.2	0.25	64.3	12.1
High HA	1	0.2		0.05	0.25	97.0	0.25	97.3	3.0
Low HA	1	0.2		0.01	0.25	70.2	0.25	54.6	10.5

than AMA-1 due to the protein surface charge and sizes. As a result, we slightly modified the ratio of TMC to AMA-1 to 10:1.5 to achieve highly stable in PBS and uniform size distribution of NPs when we loaded both MSP-1 and CSP LbL in one formulation. As shown in the **Table 3**, loaded with multiple proteins, the average size was increased to 305.5 nm for two layers of protein loading and 339.1 nm for three-layer protein loading, however, which were each found to be stable at an acceptable size range for intramuscular injection. These three formulations that were used in the animal studies.

3.3 Evaluate the Antigenicity and Integrity of Antigen Loaded Chitosan NPs Complexes

LbL NP malaria antigens were prepared as shown in the Method section. To confirm that the antigenicity of each antigen on LbL NPs will not be altered following entrapment or loading on the chitosan surface. An enzyme-linked immunosorbent assay (ELISA) was used to evaluate the effect of the preparation process on released protein binding, and a nano-enhanced Surface Plasmon Resonance Image (nanoSPRi) method was used to monitor binding kinetics of release antigens. After each step of antigen loading on trimethyl chitosan nanoparticles, the antigens were released and collected for evaluation. From ELISA results shown in **Figures 5A–C**), we observed that released AMA-1, CSP, and MSP-1 showed similar ELISA profiles as control antigens without any processing to the antibody responses.

We next evaluated malaria antigens using nanoSPRi to confirm that the antigen has similar binding kinetics and affinity to the correlated antibodies before and after loading and released from the NPs. Binding strength/affinity of malaria protein to their respective ligand (i.e., antibody) is an important factor when determining the efficacy of the developed vaccine. It is related to a kinetic parameter KD, the ratio of dissociation to association constants (kd/ka) of the antigen/antibody interaction. The lower the KD value, the higher the affinity of the antibody to its antigen. For this test, we focused on PfCSP as the representative target. The binding kinetics of malaria antigen CSP with its antibody pair was determined using nanoSPRi platform. Here, we chose a specific monoclonal antibody (3C1) that recognize the central repeat and C-terminal regions of PfCSP and a gold standard monoclonal antibody 2A10 (7). As shown in **Figures 6A, B**, the sensorgram fit for CSP/3C1 (antibody) binding interaction was more pronounced when compared to that of CSP/2A10 (antibody). The calculated KD values for CSP/3C1 and CSP/2A10 antigen/antibody pairs were 3.1×10^{-9} M and 1.3×10^{-8} M, respectively. This indicates that the binding strength of CSP/3C1 pair was stronger than that of a CSP/2A10 set. These results demonstrated that both antibodies could be used to obtain a sandwich construct for malaria detection. We next evaluated the binding interactions of the released CSP (rCSP) from chitosan. Results are shown in **Figures 6C–F** of the sensorgrams for detection of CSP. No significant increase of antigen signal (delta value of 15) before

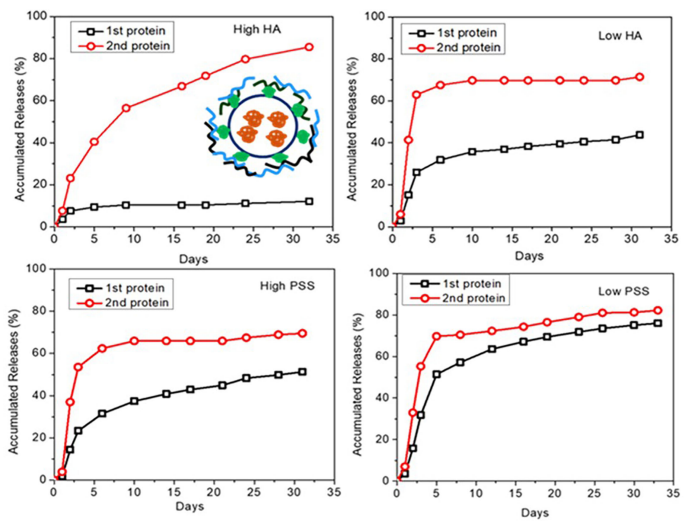

FIGURE 4 | Dye-labelled protein release profiles for different formulations of LbL NP by tuning of amount of outside of PSS or HA protective layer (insert is the schematic for two layers of loading of dye labelled proteins by coated with protective layers).

TABLE 3 | Parameters for the formulations for the efficacy studies.

Formulations(Main components)	Ratio of each component (weight ratio)	DLS (nm)	Zeta potential
LbL NP	10	129.1± 45.6	28.1 ± 5.8
LbL NP-CSP	10:2.5	236.6 ± 97.6	14.2 ± 9.3
LbL NP-AMA-CSP	10:1.5:1.5	305.5 ± 92.6	13.2 ± 5.6
LbL NP-AMA-MSP-CSP	10:1.5:1.5:1.5	339.1 ± 121.4	11.2 ± 4.9

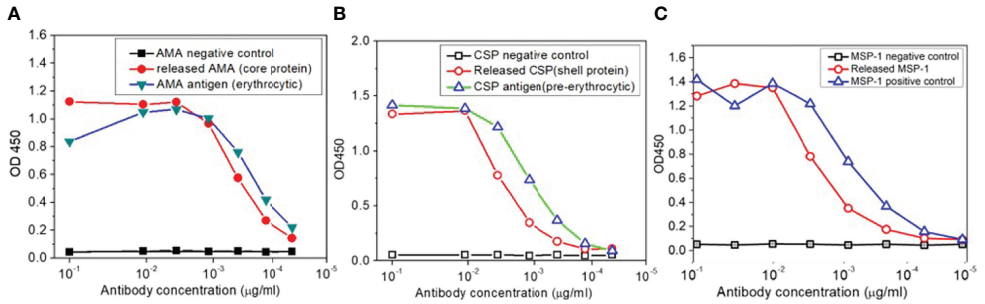

FIGURE 5 | **(A)** ELISA antigenicity test for comparison of released AMA, **(B)** CSP and **(C)** MSP-1 antigens from TMC nanoparticles with corresponding malaria antigens.

and after the CSP release was observed. These results indicate that the released CSP maintained its binding function and was very similar to the original CSP.

3.4 Perform Animal Safety Studies

There were no significant concerns, or any nanoparticle related clinical observations found during the safety studies using Sprague-Dawley rats after administration of 0-25 mg/kg dose of nanoparticles. Only we found several animals exhibited bruising or scabbing of the tail as a result of tail vein blood collections. There were also no statistically significant differences in mean body weights between groups on Day 1, 15, and 17 (**Figure 7**). In addition, there were no differences in heart, liver, or kidney weight between groups. We also evaluated the hematology, clinical chemistry, and histopathology parameters after administration of different doses of nanoparticles. While not statistically significant, white blood cell counts appeared elevated in nanoparticle treated animals compared with vehicle

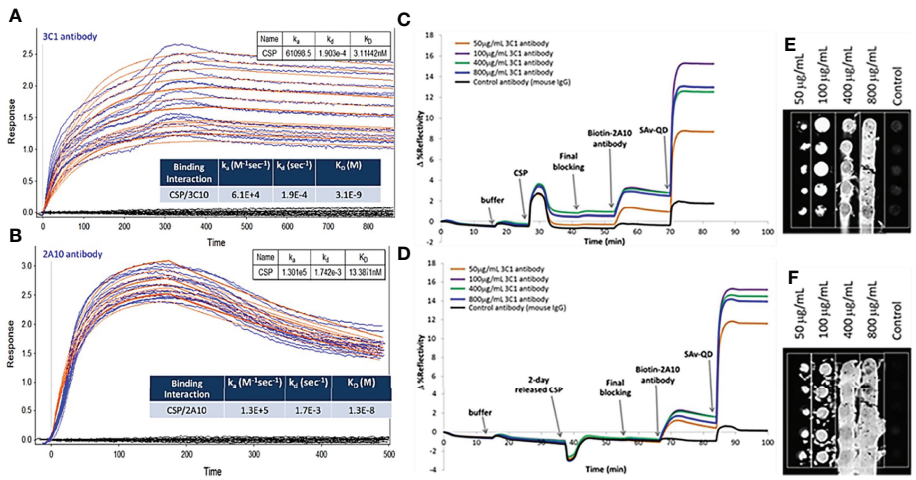

FIGURE 6 | Sensorgram fits and corresponding images of **(A, B)** CSP/3C1 and CSP/2A10 malaria antigen/antibody pairs using a 1:1 biomolecular interaction model to obtain kinetic parameters ka, kd and KD as shown in the insert Table. Anti-CSP antibody (3C1 or 2A10) was spotted as capture antibody (CAb) in 4 different concentrations. CSP protein (10 μg/mL) was then injected in the flow cell and allowed to interact with the immobilized CAb on the chip surface to determine association and dissociation of protein. **(C, E)** Sensorgrams for the detection of CSP and **(D, F)** 2-day released CSP from chitosan nanoparticles. Anti-CSP 3C1 as a capture antibody while anti-CSP 2A10 as detection antibody.

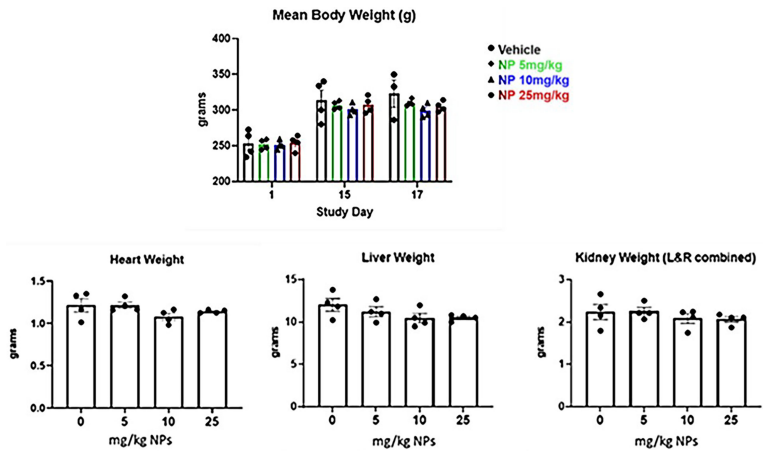

FIGURE 7 | Mean body weight and organ weight changes over time after IM injection of two doses of LbL NP at concentration of 0-25mg/kg in Sprague-Dawley rats.

controls. Albumin decreased in a dose-dependent manner while globulin increased in treated animals. These alterations likely resulted from an inflammatory process. Na/K ratios increased in a dose-dependent manner, possibly due to transcellular shifts or increased renal excretion following induction of initial hyperkalemia upon skeletal muscle damage. Remaining parameters (total proteins, hemoglobin, MCV, MCH, platelets, MPV, neutrophils, lymphocytes, monocytes, etc.) were comparable across groups in this short-term study. While vehicle control skeletal muscle was normal, nanoparticle-treated animals had similarly affected hind limb lesions at the site of injection. The muscle bundles were dissected by edema and inflammation. Heart, kidney, and liver were normal in all study animals. This result provides safety dose range for immunogenicity and efficacy studies at below 5 mg/kg. We used 2.2 mg/kg dose for nanoparticle delivery antigens which is far below the safety dose ranges in the immunogenicity and efficacy study.

3.5 Mouse Immunogenicity Studies of Chitosan Loaded Multiple Stage Antigen Releases

We formulated vaccine candidates by loading pre-erythrocytic protective antigen CSP and blood stage protective antigen AMA-

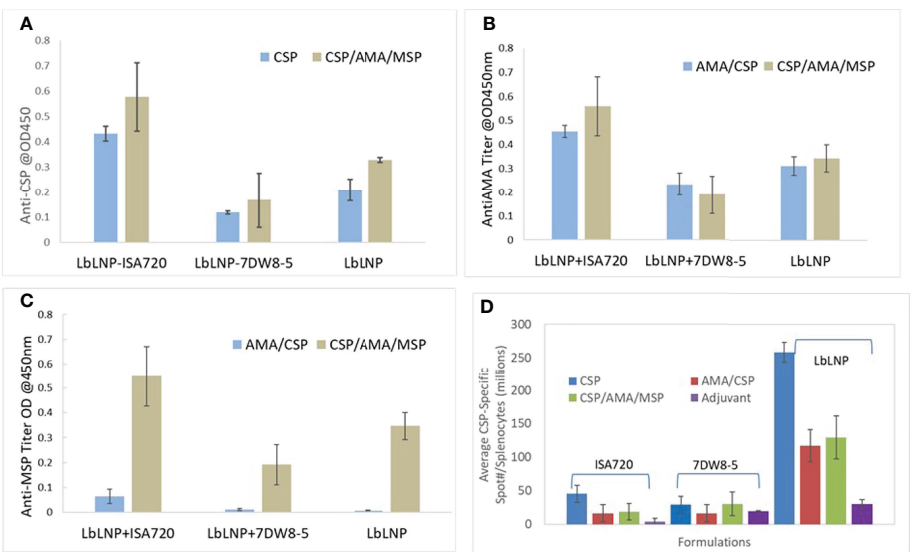

FIGURE 8 | ELISA of anti-CSP **(A)**, anti-AMA-1 **(B)** and anti-MSP-1 **(C)** induced by 2 doses of immunization of different vaccine candidates incorporated with and without adjuvant ISA 720 and 7DW8-5. Each group has four mice, and the data were averaged from these 4 mouse sera at 20,000 dilutions. **(D)** PfCSP of P. falciparum -specific T cell responses (IFN-γ ELISPOT) induced by immunization with LbL NP formulations and compared with two other adjuvants ISA 720 and 7DW8-5 using in the vaccine formulations for BALB/c mice study by 2-dose intramuscular injection.

1, MSP-1 in the LbL NP (**Table 1** and Section 3.2 for details). After dosing, mouse sera were collected for serology analysis of the antibody titers using ELISA of CSP, MSP1 and AMA1 for each formulation and the numbers of IFN-γ-secreting T cells in spleens of mice immunized with antigens by intramuscular injection were measured by IFN- γ enzyme linked immunospot (ELISPOT) assay.

From the cellular response and ELISA results shown in **Figures 8A–C**), it was demonstrated that LbL NP possessed an adjuvant effect. Especially in the group with adjuvant ISA 720 (see **Table 1** for details), it produced the highest humoral responses after administrated two doses of vaccine candidates in sera samples (20,000 dilutions). Also, we found out that in the 2-dose administration, this should be enough dosing to induce sufficient response compared to 3-doses for all the antigens. In the LbL NP group, we found the three-antigen loaded formulation with 2-doses demonstrated the highest humoral responses in sera samples but not for other combination adjuvant groups. However, injection of unloaded LbL NP alone as the negative control also resulted in a very low responses (<0.05) of antibody against PfCSP. Results from the ELISPOT CD4 T-cell response study demonstrated that LbL NP group formulations showed greater responses in CSP-specific CD4 T-cells than the other two adjuvant groups (ISA72 and 7DW8-5, **Figure 8**). In the 3-dose results, these responses continued to increase for the NP-CSP formulations (data not shown here). The NP vaccine candidate group alone induced the highest PfCSP specific T-cell response. We inferred that chitosan nanoparticles have a sugar like structure which is similar to the PfCSP sugar structure and is likely to induce higher cellular responses. This statement of potential adjuvant effect will be further investigated in future studies. The LbL NP vaccine candidate is such a potent immunogen that it may induce antibodies that have reactivity to CSP, AMA-1 and MSP. So far, we have concluded that the LbL NP is an extremely potent vaccine vector. 2-doses of immunization with a longer interval between them, likely 4 weeks, has potentially shown to induce the highest humoral response against CSP. We worked on this dosing plan in the efficacy studies.

3.6 Perform Protective Efficacy Studies by Parasite Challenge in Mouse Model

We next investigated the efficacy of LbL NP-antigen vaccine candidates to protect against sporozoite challenge in a mouse model, as analyzed by the presence or absence of parasitemia in thin blood smears. Intramuscular injection was used as the administration route and Malaria proteins were formulated using LbL NP and other two adjuvants for comparison. For the efficacy studies, both immunized and naïve mice were challenged intravenously with a recombinant rodent malaria strain, *Plasmodium yoelii* parasite that bears a full-length PfCSP, called PfCSP/Py sporozoite (Spz) (6), kindly provided by Sanaria Inc.

First, we injected 3 groups (N=4 per group) of female BALB/c mice intravenously with 375, 750, and 1500 Spz of the PfCSP/Py parasites. The infectivity of PfCSP/Py Spz was determined by the presence or absence of parasites (parasitemia) in the blood of the challenged mice. All the mice that received 1500, as well as 750 spz, developed parasitemia by Day 7 post challenge. However, three out of four mice that received 375 spz failed to develop parasitemia. Therefore, we challenged vaccinated mice with 1000 PfCSP/Py Spz in the experiments to investigate efficacy.

Single (CSP) and three-protein (AMA-1/MSP-1/CSP) LbL NP formulations were prepared by ionic gelation using the developed method (Method in section 2). The optimized ratio of TMC : CSP was determined at 10:2.5 if CSP is the only protein encapsulated in the core of the nanoparticles. For three-protein loading, CSP was loaded in the outer layer of the formulation. For three protein formulations, the ratio of TMC to CSP, AMA-1 and MSP-1 was 10:1.5:1.5:1.5 which were described in Section 3.2 and **Table 3**. As can be seen in **Table 4** efficacy results, all naïve mice were successfully infected with transgenic malaria parasites. Five out of six mice immunized with adjuvanted CSP (Groups 2, 5, 8) were protected (100-83.3% protection) compared to the naïve mouse group, indicating that the single antigen CSP vaccine displayed good efficacy. Immunization with CSP/AMA1/MSP1 with 7DW8-5 (Group 6) and NP-CSP/AMA1/MSP1 (Group 9) also induced a moderate protection (66.7%) compared to the naïve mouse group, resulting in protection for four out of six mice. When we compared these vaccinated groups with internal control groups (adjuvant alone Groups 7 and 10), CSP + 7DW8-5 (Group 5), and NP-CSP (Group 8) still demonstrate statistically significant efficacy ($p<0.05$, Fisher's test). However, immunization with CSP/AMA1/MSP1 with ISA720 (Group 3) was able to protect only two out of six mice (33.3%), which was identical to that seen in mice immunized with ISA720 alone (Group 4). The reason why CSP alone seems more potent is likely because when more than one protein is combined, the presentation of a single antigen could be slightly diminished due to competition at the level of antigen-presentation, as multiple proteins will compete for MHC class I and class II-mediated presentation.

4 DISCUSSION

It is important that the delivery vehicle used for antigen delivery is highly stable and uniformly dispersed in the human blood system. The limited solubility of chitosan and chitosan-based materials therefore hinders its use and application for a wide range of biological environments. The reductive methylation of chitosan for obtaining N, N, N-trimethyl chitosan (TMC) is a good strategy for overcoming such limitations because TMC can be soluble in distilled water, in PBS solution, and in alkaline or acidic aqueous solutions. The solubility of TMC across the range of pH is due to the shifting in charge density originated by methylation of primary amino groups on chitosan. Also, the methylation of chitosan results in a high positive charge on the surface of TMC which is beneficial for loading of negatively charged biological molecules. FTIR and NMR analysis of our synthesis indicates that the TMC was successfully prepared and trimethylation was successful.

The ionic gelation method is considered the most suitable method for protein loading on TMC NPs. The presence of cross linker TPP and surface coating chemistries of PSS or HA have been evaluated to select the optimized composition for development of malaria vaccine candidates. The use of TPP as a crosslinker was found necessary for successful protein encapsulation within the core of the nanoparticle. PSS as a surface protection coating was found to moderately decrease the loading amount of core and second layer proteins. However, this was determined acceptable since it is used as a protective layer to prevent outer layer proteins from immediate release and degradation. PSS has a very strong affinity to TMC as compared to HA during the formation of NPs. As a result, if we apply PSS as the protective layer, the amount of PSS needs to be limited to less than 0.05 mg per mg of TMC to avoid large precipitations. Release testing also confirmed that undesired burst release was lower if we decreased the protect layer PSS amount. HA performed similar function as PSS, but it provided more benefits that it reduced precipitation of nanoparticle when we used the same amount of coating as PSS. And it also helped in prolonged the release of loaded protein compared with using PSS as the protected layer. HA could be the most beneficial for the formation of LbL NP vaccine candidate. Combined, we concluded that the protective layer is necessary and required for achieving long-term release profiles.

Three multiple stage malaria life cycle antigens were successfully encapsulated and loaded on TMC nanoparticles. These antigens are pre-erythrocytic stage antigens (i) circumsporozoite protein (CSP; the major antigen on the sporozoite surface and its fragments have been included in the most clinically advanced malaria vaccine RTS, S) (12). However, RTS, S does not include the N-terminal region of CSP. Adoptive transfer of a monoclonal antibody specific for the N-terminus of the P. falciparum CSP, strongly inhibits the infection of rodent malaria sporozoites expressing the N-terminus of P. falciparum CSP (13). The erythrocytic stage antigens (ii) apical membrane antigen 1 (AMA1; involved in merozoite invasion of red blood cells and essential to the proliferation and survival of the malarial parasite, and its antibodies have shown to be protective (14), and (iii) merozoite surface protein 1 (MSP1; highly immunogenic in humans and numerous studies suggest it is an effective target for a protective immune response (15). We worked with GenScript for synthesis of the plasmid to produce full size of CSP protein. As widely known, a wide variety of factors regulate and influence gene expression levels, and GenScript used OptimumGene™ algorithm to consider as many of these factors as possible, producing the single gene that can reach the highest possible level of expression. In this case, the native gene employs tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery. They increased the codon usage bias in *E. coli* by upgrading the Codon Adaptation Index (CAI) to 0.74. CAI of 1.0 is considered to be perfect in the desired expression organism.

ELISA results demonstrated that both free and entrapped protein after release from NPs possessed similar responses to their antibodies. Binding strength/affinity of released proteins to their respective receptors is an important factor when determining the efficacy of the developed vaccine. Binding, specificity, affinity, kinetics, and active binding concentration were determined from the shape of produced surface plasmon resonance imaging sensorgrams. These observations demonstrated that released PfCSP maintained binding properties to corresponding antibodies 2A10 and 3C1 following loading and release in the LbL NPs.

Many candidate vaccines evaluated to date fail to achieve protection against certain human pathogens, such as malaria, and this is primarily due to their poor cellular immunogenicity (16). As a result, it is important that newly developed adjuvant LbL NP may add value when it is used as a stand-alone manner or in combination with existing adjuvants such as ISA 720 (9) and 7DW8-5 (16). Here, we found *in vivo* immunogenicity tests using LbL NPs as antigen/protein delivery vehicles demonstrated immunoadjuvant properties. The LbL NP formulation groups showed the greatest PfCSP specific T-cell responses in mice and also strong titers of humoral responses. Specific IgG was detected in all mice receiving vaccine formulation with sera dilutions between 4,000 and 20,000 after 2 doses. Finally, we challenged with *P. yoelli* parasites that express only PfCSP, and therefore, we saw the protective immune response targeted against PfCSP only. When we determined the level of protection by the presence or absence of parasitemia in thin blood smears, we found that 5 of 6 mice

TABLE 4 | Protection of mice immunized with vaccine candidates against transgenic PfCSP/Py sporozoites administrated intravenously.

Vaccine formulations	Protected/Challenged	p Value
Group 1: Naive	0/6	
Group 2: CSP+ISA720	6/6	0.061[a]
Group 3: CSP/AMA1/MSP1+ISA720	2/6	1[a]; 0.24[b]
Group 4: ISA720 only	2/6	
Group 5: CSP+7DW8-5	5/6	0.008[a]*; 0.015[b]*
Group 6: CSP/AMA1/MSP1+7DW8-5	4/6	0.24[a]; 0.061[b]
Group 7: 7DW8-5 only	1/6	
Group 8: CSP+LbL NPs	5/6	0. 008[a]*; 0.015[b]*
Group 9: CSP/AMA1/MSP1+LbL NPs	4/6	0.24[a]; 0.061[b]
Group 10: LbL NPs only	1/6	

[a]*The p value between vaccine candidate group to each adjuvant group.*

[b]*The p value between vaccine candidate group to naïve group.*

**p<0.05 significant.*

were protected against malaria challenge after boost of LbL NP delivery of full length of CSP as the vaccine candidate. Thus, we systematically demonstrated that intramuscular injection of LbL NP leads to a more potent adjuvant effect than commercial ISA720 in the efficacy studies. However, if we establish PfCSP/PfAMA-1/PfMSP-1 triple transgenic parasites and challenge them, the LbL NP expressing the three proteins may exert a better efficacy compared to a single protein-expressing LbL NP vaccine. Although the protective immunity induced by PfCSP (one antigen) may be weaker, a combined protective immunity induced by all 3 proteins may be more potent due to additive or synergistic effect. Also, it is rare to see protection lasting for more than 4 weeks after a booster dose. To the best of our knowledge, there have been no other malaria vaccines found that can sustain sterile protection for more than 2 weeks.

Also, we observed that LbL NPs are potentially a good adjuvant candidate for vaccine delivery in order to obtain long-lasting protection. The LbL NP found may elicit innate immune response that was potent to mediating non-specific anti-malarial effect. In the safety studies, we found LbL NP at a dose of less than 5 mg/ml were also determined biocompatible and safe in male Sprague-Dawley rats. While these studies suggest a protective response using LBL NP as the delivery vector, additional studies are necessary to fully understand the potential of the nanoparticle approach due to the smaller number of mice per group in this study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at The Columbia University (Animal Welfare Assurance no. D16-00003) and Michigan State University (Animal Welfare Assurance no. A3955-01).

AUTHOR CONTRIBUTIONS

Conceptualization by YX. Methodology was done by ZZ, BB, TF. JO, HZ, XJ, CL prepared the immunogens and evaluated the antigen functionality. IK, SI, MY have made the transgenic PfCSP/Py Spz. JH, YT and MT have conducted the immunogenicity and efficacy studies in mice. YX drafted the manuscript, and CT, X-PK and MT participated in reviewing and editing the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This material is based upon work supported by the US Army Medical Research and Materiel Command under Contract No W81XWH-18-C-0073. And also it is supported by the Army/DHA SBIR Program/US Army Medical Research and Development Command (USAMRDC)/Walter Reed Army Institute of Research (WRAIR), Military Infectious Diseases Research Program (MIDRP)/JPC-2 under Contract No. W81XWH21C0057.

ACKNOWLEDGMENTS

We thank Dr. Matthew Bernard and Dr. Krieger-Burke Teresa at the Michigan State University *In Vivo* Facility for nanoparticle safety and tolerance studies. We thank Sanaria, Inc. for kindly provide us with cryopreserved Pf/Py Spz.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.900080/full#supplementary-material

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Conflict of Interest: Authors YX, ZZ, BB, TF, JO and CT were employed by company Luna Labs USA.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Immunology

TYPE Clinical Trial
PUBLISHED 22 August 2022
DOI 10.3389/fimmu.2022.984323

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 01 July 2022
ACCEPTED 01 August 2022
PUBLISHED 22 August 2022

CITATION
Salkeld J, Themistocleous Y, Barrett JR, Mitton CH, Rawlinson TA, Payne RO, Hou MM, Khozoee B, Edwards NJ, Nielsen CM, Sandoval DM, Bach FA, Nahrendorf W, Ramon RL, Baker M, Ramos-Lopez F, Folegatti PM, Quinkert D, Ellis KJ, Poulton ID, Lawrie AM, Cho J-S, Nugent FL, Spence PJ, Silk SE, Draper SJ and Minassian AM (2022) Repeat controlled human malaria infection of healthy UK adults with blood-stage *Plasmodium falciparum*: Safety and parasite growth dynamics. *Front. Immunol.* 13:984323. doi: 10.3389/fimmu.2022.984323

Repeat controlled human malaria infection of healthy UK adults with blood-stage *Plasmodium falciparum*: Safety and parasite growth dynamics

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In endemic settings it is known that natural malaria immunity is gradually acquired following repeated exposures. Here we sought to assess whether similar acquisition of blood-stage malaria immunity would occur following repeated parasite exposure by controlled human malaria infection (CHMI). We report the findings of repeat homologous blood-stage *Plasmodium falciparum* (3D7 clone) CHMI studies VAC063C (ClinicalTrials.gov NCT03906474) and VAC063 (ClinicalTrials.gov NCT02927145). In total, 24 healthy, unvaccinated, malaria-naïve UK adult participants underwent primary CHMI followed by drug treatment. Ten of these then underwent secondary CHMI in the same manner, and then six of these underwent a final tertiary CHMI. As with primary CHMI, malaria symptoms were common following secondary and tertiary infection, however, most resolved within a few days of treatment and there were no long term sequelae or serious adverse events related to CHMI. Despite detectable induction and boosting of anti-merozoite serum IgG antibody responses following each round of CHMI, there was no clear evidence of anti-parasite immunity (manifest as reduced parasite growth *in vivo*) conferred by repeated challenge with the homologous parasite in the majority of volunteers. However, three volunteers showed some variation in parasite growth dynamics *in vivo* following repeat CHMI that were either modest or short-lived. We also observed no major differences in clinical symptoms or laboratory

markers of infection across the primary, secondary and tertiary challenges. However, there was a trend to more severe pyrexia after primary CHMI and the absence of a detectable transaminitis post-treatment following secondary and tertiary infection. We hypothesize that this could represent the initial induction of clinical immunity. Repeat homologous blood-stage CHMI is thus safe and provides a model with the potential to further the understanding of naturally acquired immunity to blood-stage infection in a highly controlled setting.

Clinical Trial Registration: ClinicalTrials.gov, identifier NCT03906474, NCT02927145.

KEYWORDS

malaria, *Plasmodium falciparum*, controlled human malaria infection (CHMI), malaria immunology, human challenge model

Introduction

Controlled human malaria infection (CHMI) is the most developed experimental human microbial infection model. It has been critical to advancing new therapeutic drugs and vaccines, including the most advanced anti-sporozoite vaccine for *Plasmodium falciparum*, RTS,S/AS01, and has provided key insights into parasite immuno-biology and pathogenesis of disease (1). CHMI was pioneered in the fascinating era of malariotherapy, in which deliberate malaria infection, delivered either *via* mosquito bite or by blood transfusion, was administered as treatment for neurosyphilis prior to the availability of penicillin in the mid-1940s (2). Since it was the induction of fever that led to improvement of neurosyphilitic symptoms, patients were routinely re-challenged with both homologous and heterologous strains (with *P. vivax* and to a lesser extent with *P. falciparum*) and this became the standard treatment of neurosyphilis. Retrospective examinations of reinfection data in patients using homologous *P. vivax* or homologous and/or heterologous *P. falciparum* demonstrated a reduction in fever episodes ("clinical immunity") during the secondary infection as well as reductions in parasitaemia ("anti-parasite immunity"), suggesting some partial immunity can be conferred from one previous parasite exposure (3, 4). However, this phenomenon has not been re-explored by CHMI in the "modern era".

In the natural exposure setting (i.e. in malaria-endemic countries), infection with *P. falciparum* gradually allows older children and adults to develop asymptomatic infections whereby they are able to tolerate high levels of parasitaemia without symptoms or clinical signs of malaria (5). This naturally-acquired form of clinical immunity is widely understood to involve a complex interplay of cellular and humoral immune mechanisms, and to develop over time, following repeated exposures to multiple parasite strains.

Repeat homologous blood-stage CHMI (with malaria caused by Plasmodium parasites of any species) has the potential to further the understanding of the impact of prior malaria exposure on a subsequent infection in a highly controlled and experimental setting. However, prior to recent studies in Oxford, only one previous study had administered a second intravenous *P. falciparum* blood-stage challenge in humans (6). Five malaria-naïve volunteers were repeatedly inoculated with infected erythrocytes at low doses, followed by administration of anti-malarial drugs before development of clinical infection. Three of the four volunteers who completed the study were protected from infection after three rounds of low dose blood-stage challenge and cure, with no parasite DNA detected by quantitative PCR (qPCR) after the fourth inoculation. However, with very small numbers, lack of a control group and detection of residual atovaquone, which may have confounded the observed outcome, clear interpretation of these results is difficult (6, 7). In contrast, many healthy adult volunteers, in other malaria vaccine clinical trials worldwide, have been re-challenged with *P. falciparum* malaria delivered by mosquito bite (8–11) or as injected cryopreserved sporozoites (12–14). However, in most cases these were vaccinees who had shown evidence of vaccine-induced sterilizing or partial immunity following their primary CHMI. These studies also aimed to assess the durability of vaccine-induced pre-erythrocytic immunity as opposed to any impact on the subsequent blood-stage of infection.

More recently, as part of the VAC063 RH5.1/AS01$_B$ blood-stage vaccine efficacy trial (15), we administered a secondary

homologous *P. falciparum* (3D7 clone) blood-stage challenge to a subset of unvaccinated infectivity control volunteers with unexpected results. Upon primary CHMI in the part of the study called "VAC063A", all fifteen infectivity controls were diagnosed at ~10,000 parasites per mL of blood within 8-12 days as routinely seen in this CHMI model in malaria-naïve adults. Subsequently, following secondary homologous CHMI approximately 4 months later (called "VAC063B"), six out of eight volunteers showed identical blood-stage parasitaemia to the primary infection, however, two out of eight participants demonstrated a reduction in their parasite multiplication rate (PMR) as compared to primary infection and the remainder of the secondary CHMI cohort. Indeed, one of these two volunteers showed undetectable blood-stage infection out to 19 days.

Given anti-parasite immunity is slow to acquire, these findings after a single acute primary exposure were not anticipated. However, they did suggest that repeat homologous blood-stage CHMI could provide a model to further interrogate the immunological mechanisms that underlie the acquisition of anti-parasite immunity, which could then guide development of new vaccination strategies. Here, we therefore sought to further develop this model by inviting these previous participants back to take part in a final follow-on challenge study (called "VAC063C"), to enable the assessment of safety and parasite growth dynamics following tertiary, secondary and primary homologous blood-stage CHMI.

Results

Participant flow and demographics

Twenty-five volunteers were screened for the VAC063C study. Sixteen of these were malaria-naïve healthy adult volunteers, and the other nine volunteers were screened following invitation to participate, after their previous enrolment and receipt of blood-stage *P. falciparum* CHMI as unvaccinated infectivity controls in the VAC063 study (A and B CHMIs, ClinicalTrials.gov NCT02927145) (15) (Figure 1A). Eleven volunteers were subsequently enrolled, comprising six volunteers who had previously undergone two CHMIs in the VAC063 study (Group 1), two volunteers who had received a single prior CHMI (Group 2) and a further three malaria-naïve volunteers to act as primary infectivity controls (Group 3). Over the entire study period, only a single participant withdrew prior to completion (after 45 days of follow-up post-CHMI) due to personal reasons. Table S1 compares the demographics of those undergoing primary, secondary or tertiary CHMI in the VAC063C study as well as the pooled demographic data across all three CHMI studies (VAC063A, B and C). Age, gender, ethnicity and body mass index (BMI) were comparable across all participants undergoing primary, secondary and tertiary CHMI.

In total, across the three studies (VAC063 A, B and C), 24 participants underwent one CHMI only, 10 volunteers received two homologous CHMIs and 6 received three homologous CHMIs. The intervals between the CHMI periods were approximately 4 months (VAC063A to B) and 8 months (VAC063B to C) (Figure 1B).

Period of controlled human malaria infection

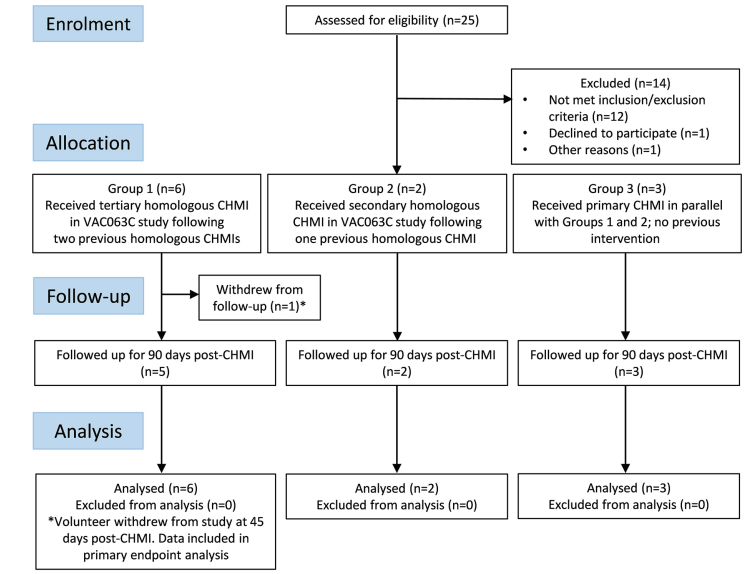
Blood-stage CHMI, with the *P. falciparum* 3D7 clone, was initiated for the VAC063C study on 6th November 2018; this was approximately 8 months after completion of the preceding VAC063B CHMI (Figure 1B). Exactly as for VAC063A and VAC063B, the CHMI was initiated by administration of the inoculum to each participant *via* an intravenous injection. This contained approximately 777 parasitised erythrocytes in 5 mL of normal saline, as estimated by a limiting dilution assay of the inoculum (executed as soon as the final participant had received it). This was highly comparable to the dose of approximately 452 and 857 parasitised erythrocytes administered to participants during the VAC063A and B CHMIs, respectively (15). All eleven participants subsequently developed patent blood-stage parasitaemia and were diagnosed and treated at pre-defined parasitaemia/clinical thresholds. All participants completed the course of antimalarials as prescribed and all follow-up visits were completed by 14th February 2019.

Safety of repeat homologous *P. falciparum* CHMI

There were no serious adverse events (SAEs) or unexpected reactions deemed possibly, probably or definitely related to CHMI, blood draws or study drugs during the course of the VAC063C study and no participants withdrew due to study-related adverse events (AEs). Otherwise, only a single SAE deemed unrelated to study interventions occurred during VAC063C; abdominal pain secondary to suspected renal calculus requiring overnight hospital admission. We next proceeded to analyse the safety data from the VAC063C study alone, as well as data from all participants combined across the three VAC063 blood-stage CHMI trials.

The maximum severity reported and relative frequencies of CHMI-related solicited AEs for all participants receiving primary, secondary and tertiary CHMI across the three CHMI studies are shown in Figures 2A, C, with unsolicited AEs shown in Table S2. Data on solicited AEs from the VAC063C study only are shown in Figure 2B. There were no major differences in the number or type of AE reported across the primary, secondary and tertiary CHMI groups, although severe AEs were limited to those undergoing primary or secondary CHMI (with the caveat

A



B

	Primary CHMI	Secondary CHMI	Tertiary CHMI	VAC063C study only
Total number of participants undergoing CHMI	24 15 VAC063A (Group 6) 6 VAC063B (Group 9) 3 VAC063C (Group 3)	10 8 VAC063B (Group 8) 2 VAC063C (Group 2)	6 VAC063C (Group 1)	6 primary 2 secondary 3 tertiary
Interval between CHMIs		Post primary 4 months(A->B) 8 months(B->C)	Post secondary 8 months (B->C)	

FIGURE 1
VAC063C flow chart of study design and volunteer recruitment. **(A)** Enrolment into the VAC063C study began in October 2018. Twenty five volunteers were screened for eligibility. Eleven eligible volunteers were identified, six of whom were VAC063 participants returning for a tertiary controlled human malaria infection (CHMI) (Group 1), two of whom were VAC063 participants returning for a secondary CHMI (Group 2) and three of whom were newly enrolled participants for primary CHMI (Group 3). Clinical follow-up continued until 90 days after challenge (C+90) and was completed by 14th January 2019. Volunteer demographics are summarized in Table S1. **(B)** In total (across three CHMI studies, VAC063A, B and C) 24 participants underwent primary CHMI, 10 of whom subsequently underwent secondary CHMI and six of those participants returned for a tertiary CHMI. The first CHMI (VAC063A) was on 14th November 2017, the second (VAC063B) was on 6th March 2018 and the third (VAC063C) was on 6th November 2018.

that a smaller number of participants underwent tertiary CHMI). The most commonly reported AEs were headache, fatigue, malaise, followed by feverishness, chills, sweats, myalgia, nausea and arthralgia. A minority of volunteers reported diarrhoea or vomiting. Maximum severity of AEs peaked between 24 and 48 hours post-diagnosis (after starting antimalarial drug treatment) and most AEs had resolved within a few days of CHMI or completing treatment with no long-term sequelae (Figure 3). A minority of participants also reported AEs possibly related to taking antimalarial treatment (artemether/lumefantrine or atavoquone/proguanil). These occurred in the 24-48 hour period after initiating treatment and resolved quickly (Figure S1).

We next analysed the frequency and severity of objective clinical observations and laboratory AEs (Figure 4; Table S3). We observed no differences in haemoglobin or platelet counts across the three cohorts undergoing repeat *P. falciparum* infection. However, lymphocytopaenia was more frequent following secondary and tertiary CHMI (affecting >60% participants) compared to <30% following primary CHMI. There was also a trend toward less severe pyrexia with each successive CHMI (Figures 4A, C). In nearly all cases, these

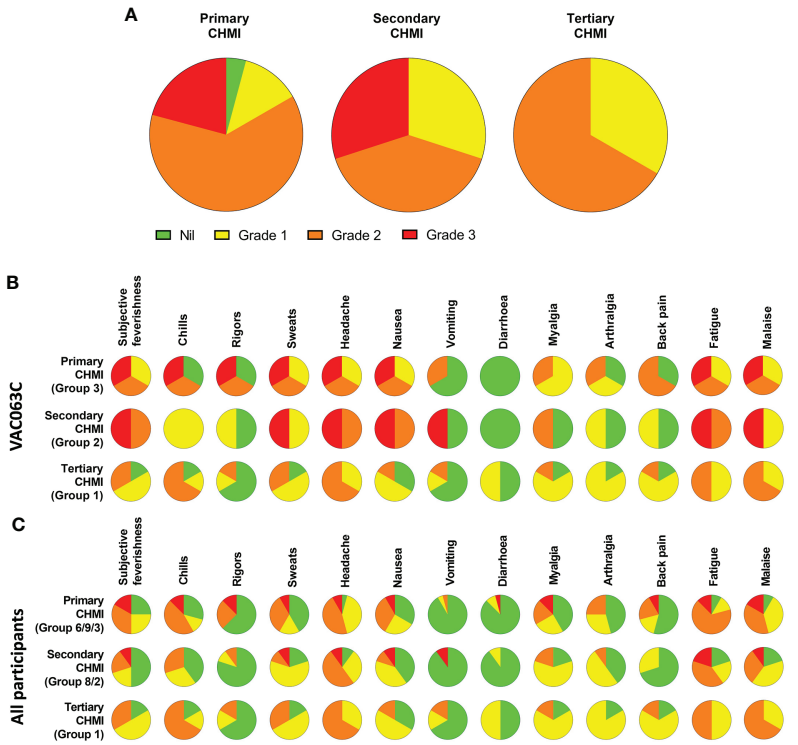


FIGURE 2
Safety of repeated *P. falciparum* blood-stage CHMI. Participants were asked about the presence of 13 solicited systemic adverse events (AE) at each study visit following CHMI. **(A)** The maximum severity of any solicited AE reported by each participant in the 48 hours before and after diagnosis during primary (n=24), secondary (n=10) and tertiary (n=6) CHMI is shown as a proportion of the total number of participants. **(B)** VAC063C: The solicited AEs recorded during the CHMI period are shown as the maximum severity reported by each participant and as a proportion of the participants reporting each individual AE for primary (n=3), secondary (n=2) and tertiary (n=6) CHMI. AE data was collected until 90 days after challenge. Colour coding as per panel **(A, C)** The solicited AEs recorded during the CHMI period are shown as the maximum severity reported by each participant and as a proportion of the participants reporting each individual AE for primary (n=24), secondary (n=10) and tertiary (n=6) CHMI for all participants across the three CHMI studies. Groups refer to specific study group numbers in VAC063A, B and C. Colour coding as per panel **(A)**.

clinical changes were transient and coincided with malaria diagnosis. Pyrexia resolved within the next 48 hours and lymphocytes normalised by 6 days post-treatment (T+6) in VAC063C or by 28 days post-CHMI (C+28) in VAC063A and B (noting T+6 was not assessed in these earlier studies).

During VAC063C we also noted a transaminitis occurring in Group 3 participants (those undergoing primary CHMI), as measured by significantly higher peak alanine aminotransferase (ALT) compared to secondary and tertiary participants ($P = 0.03$ by Kruskal-Wallis test). This was seen at 6 days post-diagnosis and treatment (T+6) for two out of three primary CHMI participants (Figure 4B) but was not apparent in any participant undergoing secondary or tertiary CHMI. We potentially failed to observe this degree of transaminitis in the VAC063A and VAC063B cohorts because these studies did not include the T+6 assessment (Figure S2), although four milder elevations of ALT were observed. Further examination of the two volunteers with transaminitis in VAC063C revealed similar increases in aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT), and that the participants had neither signs nor symptoms of hepatitis. One participant reported mild loss of appetite and, upon examination, mild epigastric and suprapubic tenderness were present but without right upper quadrant pain or hepatosplenomegaly. Their AST normalised after 3 days and ALT after 12 days, although GGT did not fully normalise until the last study visit (day C+90). The second participant remained completely asymptomatic and all transaminases (ALT, AST and GGT) normalised after 28 days. In both participants bilirubin, alkaline phosphatase (ALP) and markers of synthetic liver function (albumin and coagulation screen) remained normal. All participants were screened for blood-borne infections (hepatitis B, hepatitis C, human immunodeficiency virus (HIV), cytomegalovirus (CMV), Epstein-Barr virus (EBV)) prior to enrolment; both participants had been EBV positive prior to CHMI. However, in order to rule out new blood-borne infections as the cause of liver derangement, both participants were re-screened post-CHMI, approximately six weeks after their screening bloods. Both were

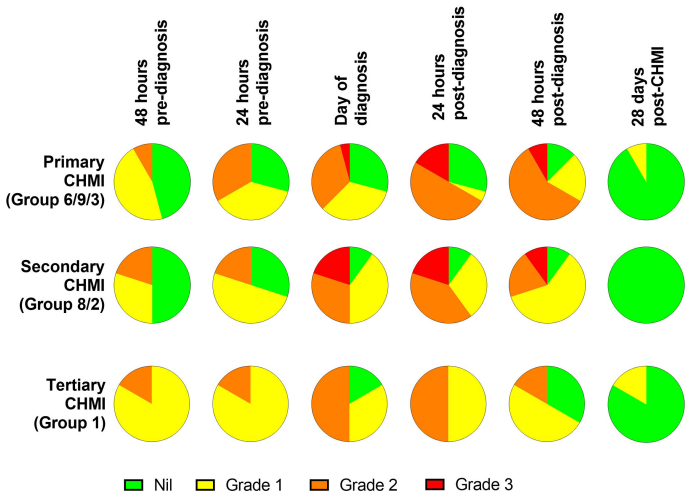


FIGURE 3
Symptom evolution during repeated *P. falciparum* blood-stage CHMI. The maximum severity of any solicited systemic adverse events (AE) recorded at the indicated time points during the CHMI period for each participant for primary (n=24), secondary (n=10) and tertiary (n=6) CHMI.

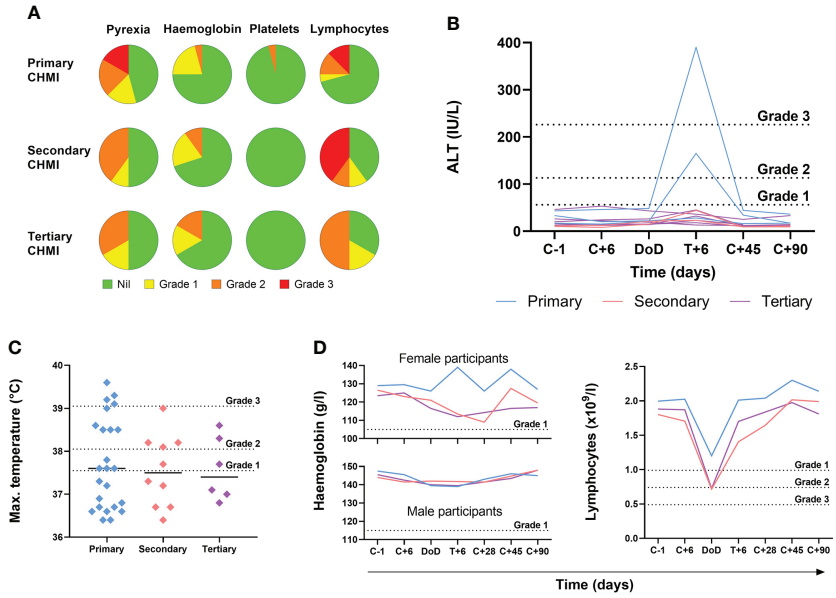


FIGURE 4
Objective measurements and laboratory AEs during repeated *P. falciparum* blood-stage CHMI. **(A)** Frequency and severity of pyrexia, anaemia, thrombocytopaenia and lymphopaenia during primary (n=24), secondary (n=10) and tertiary (n=6) CHMI from screening until 90 days post-CHMI. **(B)** Alanine aminotransferase (ALT) for each participant in VAC063C at each timepoint for primary (n=3), secondary (n=2) and tertiary (n=6) CHMI showing significant transaminitis after treatment of the primary infection (P = 0.03 as calculated by Kruskal-Wallis test of peak ALT values). Dashed lines show the local cut off for Grade 1, 2 and 3 abnormalities. Time is number of days post-CHMI, except for day of diagnosis (DoD) and day 6 post-treatment (T+6) which vary by participant. The same information for VAC063 A and B (which did not include a T+6 assessment) is presented in Figure S2. **(C)** Maximum recorded temperature during primary (n=24), secondary (n=10) and tertiary (n=6) CHMI. Individual data points and the median are shown. Dashed lines show the local cut off for Grade 1, 2 and 3 abnormalities. Kruskal-Wallis test showed no significant difference between primary, secondary and tertiary CHMI (P = 0.31). **(D)** Median haemoglobin concentration and lymphocyte count for all participants across VAC063A, B and C over time for primary (n=24), secondary (n=10) and tertiary (n=6) CHMI (of which n=18 were female and n=22 male). Time points are the same as in panel **(B)** Kruskal-Wallis test showed no significant difference between primary, secondary and tertiary CHMI (minimum haemoglobin concentration P = 0.93; minimum lymphocyte count P = 0.19).

retested for CMV, hepatitis B, hepatitis C, human immuno deficiency virus (HIV) and screened for hepatitis A and hepatitis E infection. All serology was negative with the exception of a positive hepatitis A virus IgG in one of the participants however, they were also positive on retro spective testing of their screening bloods, consistent with prior vaccination.

Parasite growth dynamics following repeat homologous *P. falciparum* CHMI

Across the three VAC063 blood-stage CHMI studies, all participants developed detectable blood-stage parasitaemia and were diagnosed according to clinical and parasitological criteria defined in the trial protocols. Individual parasitaemias were measured over time by qPCR, and are shown for all volunteers undergoing primary (n=24), secondary (n=10) and tertiary (n=6) CHMI (Figure 5A; Table S4). Analysis of the median qPCR result showed highly comparable acute blood-stage parasite growth across each repeat infection cohort (Figure 5B). We confirmed that volunteers were diagnosed at similar levels of parasitaemia across each cohort (approximately 10,000 parasites per mL [p/mL] blood) with no significant differences observed; $P = 0.79$, Kruskal-Wallis test (Figure 5C).

Overall, there was no evidence that prior blood-stage *P. falciparum* infection affected subsequent homologous parasite growth, as evidenced by comparable time-to-diagnosis (Figure 5D) across the three cohorts; $P = 0.16$, log-rank test. The median time-to-diagnosis was 9 days post-primary CHMI

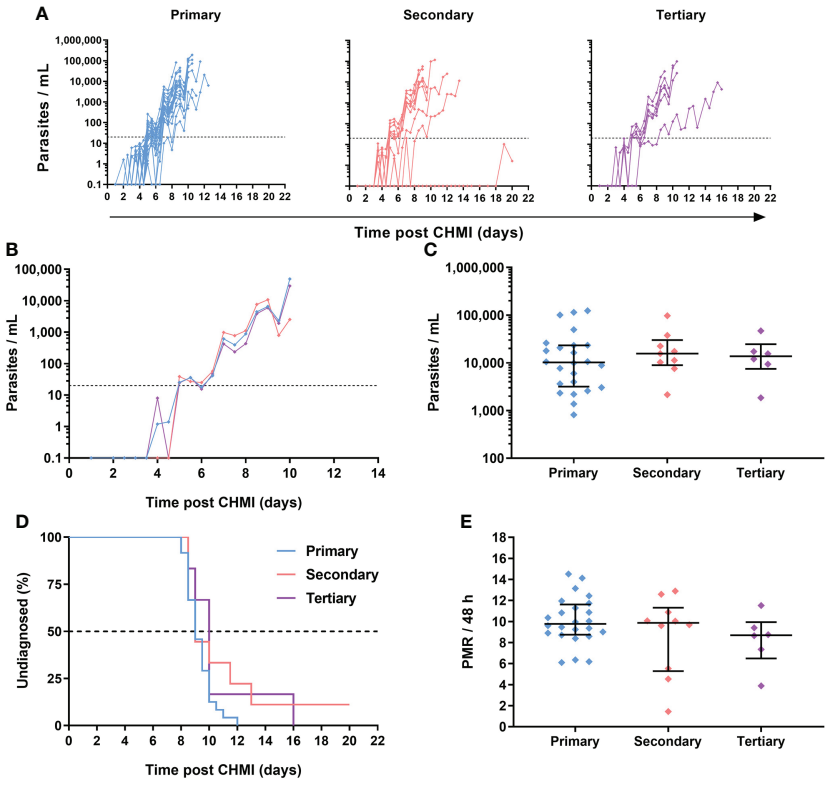


FIGURE 5
Parasite growth dynamics following repeated *P. falciparum* blood-stage CHMI. **(A)** qPCR data for primary (n=24), secondary (n=10) and tertiary (n=6) CHMI across the VAC063A, B and C studies. Parasitaemia measured by qPCR over time in parasites/mL (p/mL) blood is shown for each volunteer. CHMI was initiated by intravenous inoculation of *P. falciparum* infected erythrocytes on day 0. The lower limit of quantification is indicated by the dotted line at 20 p/mL; values below this level are plotted for information only. **(B)** Median qPCR data are shown for primary, secondary and tertiary CHMI. Colours as in panel A. **(C)** Parasitaemia at the time of diagnosis. Individual data points and the median ± inter-quartile range for primary (n=24), secondary (n=9) and tertiary (n=6) CHMI are shown. Note one participant requested antimalarial treatment at day C+20 during secondary CHMI without reaching the diagnostic criteria so no data point is shown for this participant. *P* value as calculated by Kruskal-Wallis test showed no significant difference between groups. **(D)** Kaplan-Meier plot of time to diagnosis in days. Note one secondary CHMI participant requested treatment and was censored at day C+20. Log-rank (Mantel-Cox) test showed no significant difference in time to diagnosis between primary, secondary and tertiary CHMI. **(E)** The parasite multiplication rate (PMR) per 48 hours was modelled from the qPCR data up until the time point of diagnosis. Individual data points and median ± inter-quartile range for primary, secondary and tertiary CHMI PMR are shown. *P* value as calculated by Kruskal-Wallis test showed no significant difference.

(n =24), 9 days post-secondary CHMI (n=10), and 10 days post-tertiary CHMI (n=6). Similar results were observed for the VAC063C protocol pre-specified primary analysis of parasite growth dynamics by comparison of the parasite multiplication rate (PMR) with no significant difference in the PMRs between the three groups. The median PMR per 48 h for primary CHMI was 9.8, 9.9 for secondary and 8.7 for tertiary, $P = 0.3$, Kruskal-Wallis test (Figure 5E).

Nevertheless, in contrast to the majority (7/10) of volunteers undergoing repeat CHMI who showed near identical blood-stage parasitaemia across all infections (Figure 6A), we noted a small subset of three volunteers where some change in the phenotype of parasite growth occurred (Figures 6B–D). Two participants demonstrated slower blood-stage parasite growth following successive CHMIs. The first participant had a relatively low PMR following primary CHMI and then a consistent but modest reduction in PMR during subsequent parasite exposures (4-month then 8-month interval) with PMR per 48 h falling from 6.1 to 4.5 and then to 3.9 after the secondary and tertiary CHMIs, respectively (Figure 6B). The second volunteer had a 1.7-fold drop in their PMR per 48 h (9.4 to 5.5) between primary and secondary CHMI (administered 8 months apart), but did not go on to receive a tertiary challenge (Figure 6C). Finally, one participant completely suppressed parasitaemia post-secondary CHMI (which occurred 4 months post-primary CHMI) until day C+19 (Figure 6D). At this point, blood parasitaemia was detected by qPCR for the first time but remained below the lower limit of quantification at 20 p/mL. A similar result was obtained on day C+20, however at this point the participant requested antimalarial treatment so was drug-treated at low-level parasitaemia and never met criteria for diagnosis. A pre-treatment blood sample was also cultured in the laboratory and outgrowth of *P. falciparum* parasites was subsequently confirmed *in vitro* (data not shown). However, upon tertiary CHMI during the VAC063C study (8 months later), this same volunteer developed blood-stage parasitaemia with essentially identical growth to that observed following their primary CHMI. Further clinical investigation yielded no obvious reason for suppressed parasite growth in the blood following this volunteer's secondary CHMI, and a subsequent screen for the presence of antimalarial drugs in plasma proved negative. Indeed, all antimalarial compounds tested were below the limit of detection in all plasma samples tested, with the exception of lumefantrine. In total, three volunteers were tested, two volunteers displaying reduced parasite growth and one volunteer displaying consistent parasite growth. Lumefantrine gave a low-level signal across all samples from the three volunteers, with almost identical within-individual results pre- and post-CHMI (samples taken 2-3 weeks apart). The significance of these very low levels of detectable lumefantrine in all samples remain unexplained but, as it was present both in volunteers with altered and consistent parasite growth across challenges, cannot explain the observed differences in parasite growth rates.

Antibody responses following repeat homologous CHMI

Finally, we assessed serum IgG antibody responses by ELISA to three commonly-studied merozoite antigens – the 19 kDa C-terminus of merozoite surface protein 1 ($MSP1_{19}$), apical

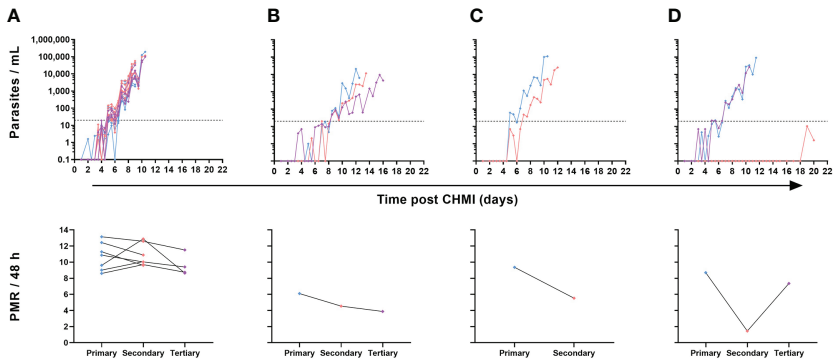


FIGURE 6
Individual variation in parasite growth dynamics of repeated *P. falciparum* blood-stage CHMI. qPCR data for primary (blue), secondary (pink) and tertiary (purple) CHMI. Parasitaemia is shown over time for each volunteer who underwent more than one CHMI (total n=10). The PMR per 48 hours was modelled from the qPCR data up until the time point of diagnosis for each participant for each CHMI and is shown below the relevant parasite growth graph. **(A)** Data from participants who showed minimal variation in parasite growth dynamics between each CHMI (n=7 for primary and secondary CHMI, of whom n=4 also underwent tertiary CHMI). **(B)** One participant showed consistently slower parasite growth with each subsequent CHMI. **(C)** One participant showed slower parasite growth during secondary CHMI compared to primary but did not undergo a tertiary CHMI. **(D)** One participant showed completely suppressed parasite growth on secondary CHMI until day C+19 but no change compared to primary upon tertiary CHMI.

membrane antigen 1 (AMA1) and reticulocyte-binding protein homologue 5 (RH5). $MSP1_{19}$-specific serum IgG responses were detectable at day C+28 in >90% volunteers following initial CHMI. These were subsequently boosted by each successive CHMI and were relatively well-maintained between parasite exposures (Figure 7A). In contrast, serum IgG responses to AMA1 were less dominant after the primary CHMI (with responses only detected in a minority of volunteers). These then boosted following repeat CHMI in a similar manner to $MSP1_{19}$, however, these responses dropped back to baseline between each successive *P. falciparum* exposure (Figure 7B). Responses to RH5 were undetectable in all volunteers at all time-points tested (data not shown).

We also screened serum samples from all participants undergoing more than one CHMI for the presence of red cell alloantibodies, which could have been raised by the repeated exposure to the blood-stage inoculum. Samples were tested both at baseline (before primary CHMI) and at final follow-up (day C+90) after the final (whether secondary or tertiary) CHMI. All tested samples were negative for alloantibodies (data not shown).

Discussion

This is the first assessment of repeat homologous *P. falciparum* blood-stage CHMI in the modern era, where volunteers have reached diagnostic criteria before initiation of anti-malarial therapy. Although a relatively small number of volunteers (ten in total) underwent repeated CHMI and the interval between repeat infections varied between four and eight months, overall we have demonstrated that repeated exposure to homologous blood-stage parasites by CHMI is safe in healthy adults. However, it does not lead to the development of any significant anti-parasitic blood-stage immunity in the vast majority of re-challengees.

The frequencies of CHMI-associated solicited AEs were within expected ranges in all participants undergoing a primary CHMI, consistent with previously reported studies of primary CHMI using both blood-stage and sporozoite infection models (15–18). Inference about relative frequency of solicited AEs in re-challenged participants is limited by relatively small sample size, selection bias of returning participants and inter-subject variability in reporting of AEs. However, our findings suggest that there are no major differences in the frequencies of AEs following homologous re-challenge, or any increased risk, as compared to primary CHMI. The majority of AEs also resolve within a few days of treatment.

There were also no statistically significant differences in the frequencies of objective clinical or laboratory markers of malaria infection across the repeat CHMIs, although we noted the highest levels of pyrexia occurred in a subset of volunteers undergoing primary CHMI. This is not inconsistent with the retrospective analyses of the old neurosyphilis treatment data (4), although the key difference here is that modern CHMIs only allow acute blood-stage infection prior to treatment in contrast to uncontrolled parasitaemia which almost certainly led to more effective acquisition of anti-malarial clinical immunity. We also noted the average lymphocyte counts were lower following secondary and tertiary infections. Lymphocytopaenia following primary CHMI is well-documented (19), although further migration of cells from the periphery in repeat CHMI may reflect an immunological recall response upon secondary and tertiary infection, similar to our previous observations on antigen-specific B cells induced by subunit blood-stage vaccination prior to CHMI (20).

We also detected a derangement in liver transaminases following primary CHMI in approximately 25% of the participants (two in VAC063C and four in VAC063A and B), with the two in VAC063C of greatest severity and noticeably occurring at six days post-treatment initiation. This has not been

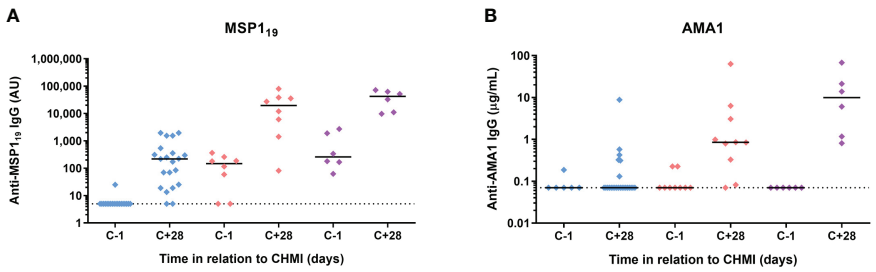


FIGURE 7
Induction of serum antibody responses to merozoite antigens during CHMI. **(A)** Serum anti-$MSP1_{19}$ total IgG ELISA was conducted on samples from participants for primary (n=19 at C-1; n=21 at C+28), secondary (n=8) and tertiary (n=6) CHMI. Sera from the pre-CHMI (C-1) and 28 days post-CHMI (C+28) time points were tested. **(B)** Serum anti-AMA1 total IgG as measured by standardized ELISA on samples from participants for primary (n=6 at C-1; n=24 at C+28), secondary (n=9 at C-1; n=10 at C+28) and tertiary (n=6) CHMI. Samples colour coded as per previous figures. Individual data points and median are shown.

previously observed in our centre, potentially due to lack of routine testing at the T+6 time point. Given that the abnormalities rapidly improved, these AEs would not have been detected at later follow-up visits, raising the possibility that frequency of liver function test (LFT) abnormalities post-primary CHMI may have been previously underestimated. LFT abnormalities are known to occur in both naturally acquired and experimental infection. In blood-stage primary CHMI studies performed at QIMR, Australia, moderate and severe elevations of transaminase elevations enzymes have been reported, peaking at 4-12 days after antimalarial treatment, with ALT/AST ratio >1 and normal bilirubin in both *P. falciparum* and *P. vivax* infection models (21). Our findings are consistent with this pattern and given timing of onset and resolution, together with the observation that abnormal ALT levels normalise after treatment in severe malaria, suggest the transaminitis is likely to relate directly to CHMI, with infection and inflammation driving increased ALT rather than antimalarial drugs. This suggestion is supported by a larger dataset from the Netherlands where CHMI participants with transaminitis had a significantly higher parasite burden over the course of the CHMI than those without such abnormalities (22). However, since the transaminitis affected only a small subset of the primary CHMI cohort, individual host factors may also have contributed. Importantly, all ALT abnormalities were transient in nature, resolved spontaneously and were not associated with clinical symptoms or any derangement of synthetic liver function, so these findings do not raise clinical concern or preclude further testing using this repeat CHMI model, however, monitoring over similar time-points in future studies is warranted. Moreover, we also saw no derangement in LFTs after secondary or tertiary CHMI. We hypothesise that this could represent a degree of clinical immunity, given transaminitis is a collateral marker of tissue damage. This is consistent with modified T cell responses to limit cytotoxicity after a single drug-treated infection, which has been demonstrated in our complementary study of the host response to repeat blood-stage infection in the VAC063 trial (23).

The blood-type of the donor of the red blood cells infected with 3D7 clone *P. falciparum* was Group O Rhesus negative, and the volume administered for CHMI is extremely small, equivalent to only a few microlitres of blood. As such, the risk of development of red cell alloantibodies following blood-stage CHMI was deemed to be very low. Nevertheless, as an exploratory measure, participants who were re-challenged in the VAC063A, B and C studies, and therefore re-exposed to the same inoculum, were retrospectively screened for IgG alloantibodies to red cells pre-CHMI and post their final follow-up. All samples tested were negative for red cell alloantibodies, consistent with single CHMI data from other groups using the same challenge inoculum (James McCarthy, personal communication). These data also suggest that an antibody response against the challenge inoculum itself was not a confounding factor in these re-challenge studies, which is also consistent with the very similar parasite growth kinetics observed in the majority of re-challengees.

Across the repeat CHMIs, all participants developed patent parasitaemia and reached diagnostic criteria, with the exception of the one participant who failed to reach diagnosis after secondary CHMI. By and large, our data suggest minimal to no acquisition of effective anti-parasitic blood-stage immunity occurs following one or two drug-treated and acute exposures to *P. falciparum*, even when using the homologous parasite clone. This is not inconsistent with observations from natural infection in the field, albeit here exposure to heterologous parasite strains will occur, repeat infections may be more frequent and blood-stage parasitaemias are likely to reach higher levels prior to treatment. This is also in stark contrast to the highly effective pre-erythrocytic immunity afforded by repeat exposure to sporozoite CHMI under drug cover (24, 25). We nonetheless were able to observe the priming and boosting of antibody responses to the well-studied merozoite antigens $MSP1_{19}$ and AMA1, but not RH5. This hierarchy of immuno-dominance of *de novo* antibody induction is consistent with our previous observations in other primary CHMI trials (26, 27), and relatively short-lived responses boosted by each successive malaria exposure is consistent with observations from field studies spanning the malaria season (28). Notably our repeat CHMI data would strongly suggest these responses, as measured, remain well below a threshold that could contribute to reduced blood-stage parasite growth *in vivo*.

Nonetheless, we did note three participants of interest whose PMR varied across the repeat CHMIs. In one volunteer parasites grew consistently slowly, in another a modest reduction in PMR was seen upon secondary CHMI, and finally one volunteer showed a dramatic suppression of growth following secondary CHMI that was not repeated following tertiary CHMI. We could identify no demographic or obvious host factors to explain these observations, and these three volunteers had no history of prior malaria exposure. The latter participant's only abnormality on blood tests was the development of a moderate anaemia by 28 days post-secondary CHMI, and their haemoglobin immediately preceding CHMI had been borderline low (111 g/dL). The relationship between iron status and malaria is complex (29) but it is unlikely that a short-term moderate anaemia would have caused such significant suppression of parasite growth. This is also supported by the fact that when a tertiary infection was administered the participant's haemoglobin dropped again to similar levels but parasite growth conversely thrived. Also no systemic supplemental iron was known to be taken at the time of CHMI that could explain the increased growth rate. Instead, the highly anomalous PMR seen after secondary infection might be explained by one or more of the following: i) the

induction and then waning of an effective immune response, or the induction of a highly specific immune response against a variant surface antigen on the infected red blood cell that was only expressed by the parasite during the secondary CHMI; ii) surreptitious or accidental self-treatment with a drug with an antimalarial effect; or iii) an operator error in the administration of the secondary challenge inoculum. The latter would seem unlikely, because even if inoculated with a single parasite, with 10-fold blood-stage growth every 48 hours, this would not lead to such prolonged qPCR negativity in a non-immune subject. Regarding drugs, we could identify no such intervention, and performed an anti-malarial drug screen that was negative. Further studies would thus be needed to investigate if this was indeed a highly effective, but variant antigen-specific, immune response.

Overall, our data support the safety of repeat CHMI with blood-stage *P. falciparum* but suggest this approach will unlikely provide a model to study effective anti-parasitic immunity. Ongoing work, including with the recently established *P. vivax* blood-stage CHMI model in Oxford (30), will instead focus on using the unique opportunities afforded by CHMI to interrogate mechanisms of clinical immunity with the goal of informing next-generation interventions specifically designed to offer protection against disease.

Methods

Study design and participants

VAC063C was a non-blinded blood-stage *P. falciparum* controlled human malaria infection (CHMI) study to evaluate the safety and parasite growth dynamics of primary, secondary and tertiary blood-stage *P. falciparum* CHMI of healthy malaria-naïve UK adults. Healthy, malaria-naïve and non-pregnant adults aged 18-50 were invited to participate in the study. Volunteers were recruited and challenged at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM), part of the Oxford Vaccine Centre (OVC) at the University of Oxford. Eleven volunteers were enrolled in total. A full list of inclusion and exclusion criteria is reported in Supplementary Methods. Allocation to study group (Groups 1 and 2) was based on previous involvement and CHMI exposure in the VAC063 trial (ClinicalTrials.gov NCT02927145) (15), with new malaria-naïve volunteers comprising Group 3. The primary endpoint of the study was safety of repeat homologous CHMI (as measured by active and passive collection of clinical and laboratory AEs after each CHMI) and qPCR-derived parasite multiplication rate (PMR) was the primary endpoint for the assessment of parasite growth dynamics.

Data were pooled for analysis from participants who were enrolled in both VAC063C (November 2018) and in two previous CHMI studies under the preceding VAC063 protocol – VAC063A (November 2017) and VAC063B (March 2018). Briefly, the VAC063 protocol encompassed VAC063A and VAC063B, and was an open label, non-randomised Phase I/IIa clinical trial evaluating vaccine efficacy of the recombinant blood stage malaria protein RH5.1 in $AS01_B$ adjuvant (15). The VAC063C trial was conducted to investigate the durability of any protective anti-parasite immunity measured in control (non-vaccinated) participants upon homologous re-challenge for the second or third time.

Study oversight

The VAC063C study was designed and conducted in the UK at the CCVTM, University of Oxford. The study was registered on ClinicalTrials.gov (NCT03906474) and received ethical approval from the UK National Health Service Research Ethics Services (South Central – Oxford A reference 18/SC/0521). All participants provided written informed consent and consent was verified before each CHMI. The study was conducted according to the principles of the current revision of the Declaration of Helsinki 2008 and in full conformity with the ICH guidelines for Good Clinical Practice (GCP). GCP compliance was independently and externally monitored by the Clinical Trials and Research Governance (CTRG) Team at the University of Oxford. Details of the previous VAC063 study approvals and oversight (from which primary and secondary CHMI data were used for a pooled analysis) are as previously described (15).

Controlled human malaria infection

The blood samples used as infectious inocula in this study were produced by Drs Gregor Lawrence, Allan Saul and colleagues at QIMR in Brisbane, Australia in 1994 and consist of aliquots of *P. falciparum* (clone 3D7) infected erythrocytes taken from a single donor (31). Blood was collected at the Australian Red Cross Blood Bank in an aseptic manner using standard blood bank equipment. The leukocytes were removed with a leukocytic filter. The thawing and washing of the cells reduced the amount of serum transferred with the red cells by a factor of 1000, compared to injecting the same volume of blood. The red cells were cryopreserved using a protocol from the American Association of Blood Banks Technical Manual that is normally employed for freezing blood from patients and donors with rare blood groups. The blood group of the donor was group O and Rhesus negative, Kell antigen negative. To date, CHMI of malaria-naïve individuals using this inoculum has always resulted in parasitaemia as detected by qPCR and/or microscopy (16, 18, 31, 32).

Between 1994 and 2003 the cryopreserved samples to be used in this trial were stored in dedicated liquid nitrogen cylinders in a secure facility at QIMR. In 2003 the samples

were transferred to Biotec Distribution Ltd., Bridgend, UK and then to Thermo Fisher Bishop's Stortford, Hertfordshire, UK, in 2007 where they have been stored on behalf of the University of Oxford in temperature-monitored liquid nitrogen. Participants in VAC063C were each infected by direct intravenous inoculation of *P. falciparum* (clone 3D7) blood-stage parasites in the same way as for the VAC063A and B CHMI studies. The target inoculum dose was 1000 parasitised erythrocytes per participant. The inoculum was thawed and prepared under strict aseptic conditions as previously described (15) and volunteers received infected red cells in a total volume of 5 mL normal saline, followed by a saline flush. Subjects were observed for 1 h post-CHMI before discharge. The order in which volunteers from different groups were inoculated was interspersed in case of time effects on viability of the parasites. Following CHMI, blood samples were taken once on day one post-challenge (day C+1) and twice daily from day two (day C+2) for qPCR (target gene = 18S ribosomal RNA), to measure parasite density in real time.

Diagnosis of malaria was made on the basis of presence of symptoms in-keeping with malaria infection together with qPCR ≥5,000 parasites/mL (p/mL) or any available qPCR ≥10,000 p/mL, even if asymptomatic. Note in the VAC063A study, thick blood films were also evaluated at each time-point by experienced microscopists and diagnosis required volunteers to fulfil two out of three criteria: a positive thick blood film (one viable parasite in 200 fields) and/or qPCR ≥5,000 parasites/mL and/or symptoms consistent with malaria. In VAC063B and C microscopy was removed as a diagnostic tool to reduce the risk of participants being diagnosed prematurely, without any impact on participant safety. Participants were treated with a course of artemether/lumefantrine (Riamet) at diagnosis (n=9), or where contraindicated, with a course of atovaquone/proguanil (Malarone) (n=2). Half of the Riamet doses and all Malarone doses were directly observed by study investigators. After completion of treatment, follow-up visits were conducted at 6 days post-diagnosis/treatment (T+6), as well as at 28, 45 and 90 days post-CHMI (C+28, C+45 and C+90). This was similar though not identical to the follow-up schedule for VAC063A and B CHMIs where a T+6 visit was not included.

Safety analysis

Participants were reviewed once on day 1 post-challenge and twice-daily from day 2. At each visit they were asked a list of symptoms commonly associated with malaria infection ('solicited' symptoms including feverishness, malaise, fatigue, arthralgia, back pain, headache, myalgia, chills, rigors, sweats, headache, nausea, vomiting and diarrhoea). The severity of any reported symptom was then graded by the participant from 1 (mild) to 3 (severe), using the severity grading criteria shown in Supplementary Material. These were all recorded by the investigator as solicited AEs if they occurred during the 28 day period post-CHMI (or until completion of antimalarial treatment). Pyrexia was scored as follows: absent (≤ 37.5°C), mild (37.6 - 38.2°C), moderate (38.3 - 38.9°C) and severe (≥ 39° C) and participants were also asked to measure and record their temperature in a diary card if they experienced feverishness outside of their clinic visit. Blood samples for full blood count and biochemistry were obtained prior to CHMI (C-1) and then at C+6, diagnosis, T+6, C+45 and C+90. These were evaluated at Oxford University Hospitals NHS Trust providing 5-part differential white cell counts and quantification of electrolytes, urea, creatinine, bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (ALP) and albumin. Blood was also tested serologically for evidence of Hepatitis B, Hepatitis C, HIV, EBV and CMV infection prior to CHMI. Blood tests were carried out at other time-points if clinically indicated.

All other (unsolicited) AEs were collected until day 90 post-CHMI and their likely causality in relation to CHMI or antimalarial drugs assessed and assigned a MedDRA code as described in the protocol. All AEs considered possibly, probably or definitely related to CHMI/antimalarial drugs were reported (Table S2), and all laboratory AEs at least possibly related to study interventions are also reported (Table S3).

qPCR and PMR modelling

Quantitative PCR was performed as previously reported for the VAC063A and B studies (15), and these data were used to model the PMR. The arithmetic mean of the three replicate qPCR results obtained for each individual at each timepoint was used for model-fitting. Negative individual replicates and data points which, based upon the mean of the three replicates, were negative or below the lower limit of quantification (20 parasites/mL), were handled as specified in the laboratory qPCR standard operating procedure. PMR was calculated using a linear model fitted to $\log_{10}$-transformed qPCR data (33). As previously, fitted lines were constrained to pass through the known starting parasitaemia, calculated from the results of a limiting-dilution-based assay of the number of viable parasites in the inoculum (34) and a weight-based estimate of each volunteer's blood volume (70mL/kg) (35).

Total IgG ELISAs

ELISAs to $MSP1_{19}$, AMA1 and RH5 (all 3D7 sequence) were performed on serum samples using standardised methodology, all as previously described (15, 17).

Red cell alloantibody measurement

Serum samples were retrospectively screened for IgG alloantibodies to red cells (performed by Oxford University Hospitals NHS Foundation Trust using Capture-R® Ready-Screen® solid phase system) at C-1 (pre-CHMI) and C+90 (final post-CHMI follow-up).

Antimalarial drug screen

Plasma taken both pre-CHMI and at day of diagnosis was sent for antimalarial compound testing at the Walter Reed Army Institute of Research (WRAIR) in the USA. The antimalarial compounds tested included amodiaquine, artemisinin, atovaquone, chloroquine, clindamycin, doxycycline, lumefantrine, mefloquine, proguanil, pyrimethamine, quinine, and sulfadoxine. The compounds were separated using a Waters Acquity UPLC using a Cortecs C18 2.7μM, 2.1 X 50mm column with a 5.5 minute gradient using 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Detection was performed on a Waters TQ-S mass spectrometer using electrospray ionization in positive ion mode with the exception of Atovaquone which was analysed in negative ion mode. Parent ions were isolated, fragmented and one product mass monitored.

Samples were sent from two participants who demonstrated reduced parasite growth after repeat CHMI and from one participant who had the same growth rate across all three CHMIs for comparison.

Statistical analysis

Data were analysed using GraphPad Prism version 9.3.1 for Windows (GraphPad Software Inc., California, USA). Statistical tests used are reported in the Results text, and included two-tailed Kruskal-Wallis test with Dunn's multiple comparison post-test, and log-rank analysis (Mantel Cox) of the Kaplan Meier curves. A value of $P < 0.05$ was considered significant.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by UK National Health Service Research Ethics Services (South Central – Oxford A reference 18/SC/0521). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceived and performed the experiments: YT, JB, CM, TR, RP, MH, BK, NE, CN, DM, FB, WN, RL, MB, FR-L, PF, DQ, KE, IP, PS, SS, SD, and AM. Analysed the data: JS, YT, SS, JB, NE, SD, and AM. Project Management: J-SC and FN. Wrote the paper: JS, YT, SS, SD, and AM. All authors contributed to the article and approved the submitted version.

Funding

The VAC063 clinical trial was funded by the Office of Infectious Diseases, Bureau for Global Health, USAID, under the terms of Malaria Vaccine Development Program (MVDP) contract AID-OAA-C-15-00071, for which Leidos, Inc. was the prime contractor. The opinions expressed here are those of the authors and do not necessarily reflect the views of the USAID. This work was also supported in part by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC). The views expressed are those of the authors and not necessarily those of the NIHR or the Department of Health and Social Care. TAR held a Wellcome Trust Research Training Fellowship [108734/Z/15/Z] and CMN held a Wellcome Trust Sir Henry Wellcome Postdoctoral Fellowship [209200/Z/17/Z]. SJD is a Jenner Investigator and held a Wellcome Trust Senior Fellowship [106917/Z/15/Z].

Acknowledgments

The authors are grateful for the assistance of Natalie Lella, Lola Matthews, Yara Neves Silva and Nicola Turner for recruitment activities and clinical trials administration, Daniel Marshall Searson for database management, Julie Furze for laboratory assistance, and Richard Morter, Amy Flaxman and Duncan Bellamy for qPCR support (Jenner Institute, University of Oxford); Amy Duckett and Carly Banner for arranging contracts (University of Oxford); James McCarthy for providing the 3D7 inoculum (QIMR, Australia); Amy Noe (Leidos Life Sciences) and Lorraine Soisson (U.S. Agency for International Development, USAID) for useful discussions; William Dennis and Chau Vuong (WRAIR, USA) for assisting with the antimalarial drug testing; and all the study volunteers.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.984323/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 25 August 2022
DOI 10.3389/fimmu.2022.978591

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 26 June 2022
ACCEPTED 08 August 2022
PUBLISHED 25 August 2022

Safety and immunogenicity of BK-SE36 in a blinded, randomized, controlled, age de-escalating phase Ib clinical trial in Burkinabe children

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Background: A blood-stage vaccine targeting the erythrocytic-stages of the malaria parasite *Plasmodium falciparum* could play a role to protect against clinical disease. Antibodies against the *P. falciparum* serine repeat antigen 5 (SE47 and SE36 domains) correlate well with the absence of clinical symptoms in sero-epidemiological studies. A previous phase Ib trial of the recombinant SE36 antigen formulated with aluminum hydroxyl gel (BK-SE36) was promising. This is the first time the vaccine candidate was evaluated in young children below 5 years using two vaccination routes.

Methods: Safety and immunogenicity of BK-SE36 was assessed in a double-blind, randomized, controlled, age de-escalating phase Ib trial. Fifty-four Burkinabe children in each age cohort, 25–60 or 12–24 months, were randomized in a 1:1:1 ratio to receive three doses of BK-SE36 either by intramuscular (BK IM) or subcutaneous (BK SC) route on Day 0, Week 4, and 26; or the control vaccine, Synflorix® *via* IM route on Day 0, Week 26 (and physiological saline on Week 4). Safety data and samples for immunogenicity analyses were collected at various time-points.

Results: Of 108 subjects, 104 subjects (96.3%) (Cohort 1: 94.4%; Cohort 2: 98.1%) received all three scheduled vaccine doses. Local reactions, mostly mild or of moderate severity, occurred in 99 subjects (91.7%). The proportion of

subjects that received three doses without experiencing Grade 3 adverse events was similar across BK-SE36 vaccines and control arms (Cohort 1: 100%, 89%, and 89%; and Cohort 2: 83%, 82%, and 83% for BK IM, BK SC, and control, respectively). BK-SE36 vaccine was immunogenic, inducing more than 2-fold change in antibody titers from pre-vaccination, with no difference between the two vaccination routes. Titers waned before the third dose but in both cohorts titers were boosted 6 months after the first vaccination. The younger cohort had 2-fold and 4-fold higher geometric mean titers compared to the 25- to 60-month-old cohort after 2 and 3 doses of BK-SE36, respectively.

Conclusion: BK-SE36 was well tolerated and immunogenic using either intramuscular or subcutaneous routes, with higher immune response in the younger cohort.

Clinical Trial Registration: https://pactr.samrc.ac.za/TrialDisplay.aspx?TrialID=934, identifier PACTR201411000934120.

KEYWORDS

SE36, malaria blood-stage vaccine, serine repeat antigen, SERA5, *Plasmodium falciparum*, safety, immunogenicity

Introduction

Malaria is a huge public health problem. The significant decline in global morbidity and mortality rates achieved from 2000 to 2015 have largely stalled in recent years (1, 2). 95% of the estimated 241 million malaria cases in 2020 occurred in children living in sub-Saharan Africa (2). The Strategic Advisory Group on Malaria Eradication recommended the continuous development of improved vaccines to contribute to existing control strategies as well as future sustainable elimination (3).

Following the 6 October WHO recommendation for the first malaria vaccine, RTS,S/AS01, expanded use for children in moderate-to-high transmission settings, on 2 December 2021, Global Alliance for Vaccines and Immunizations (GAVI) approved the vaccine program for endemic countries across Africa (4, 5). RTS,S/AS01, the anti-sporozoite vaccine based on the *P. falciparum* circumsporozoite protein, is to be provided in a four-dose schedule to children from 5 months of age (5). In the multi-site phase III trial of this pre-erythrocytic vaccine, vaccine efficacy was 36.3% (95% CI 31.8–40.5) in 5–17 month old children who received 3 doses at 0, 1, and 2 months, plus a booster at 20 months (6). The vaccine does not confer sterile immunity and clinical malaria developed in the vaccinated population (6, 7). A vaccine that can control morbidity and possibly limit the next stages of human-to-mosquito transmission would be a valuable tool.

The *P. falciparum* serine repeat antigen 5 (SERA5) is an abundant essential blood-stage antigen secreted in large amounts into the lumen of the parasitophorous vacuole (8). Recent studies highlight its various roles from parasite egress to immune evasion. Conditional knockout of SERA5 caused a defect in the regulation of the lag phase that controls RBC membrane disruption, "explosive" rupture, and merozoite disposal (9). Interaction with calcium dependent protein kinase 1 (PfCDPK1) led to enhanced cytosolic Ca2+ levels that served as a trigger for merozoite egress (10). In addition, the N-terminal 47kDa fragment is bound to host vitronectin which in turn bound other host proteins camouflaging the merozoites against the host immune system (11).

A recombinant form of SERA5 N-terminal domain (SE36) was selected for clinical development based on: (a) epidemiological studies showing high antibody titers that inversely correlate with malaria symptoms and severe disease (12, 13); (b) *in vitro* studies demonstrating induction of antibodies that are inhibitors of parasite growth, exert antibody-dependent complement-mediated lysis of schizonts, or antibody-dependent monocyte-mediated parasite growth inhibition (12, 13); and (c) non-human primate challenge studies demonstrating protection against *P. falciparum* challenge infection (14). SE36 was prepared under Good Manufacturing Practice (GMP) standards, formulated with aluminum hydroxide gel (AHG) to yield BK-SE36. Phase I

safety and immunogenicity trials of BK-SE36 were conducted in healthy, malaria naive Japanese adults (13), and in malaria exposed Ugandan volunteers aged 6- to 32-year-old (15). The encouraging results from the phase I trial in Uganda justified the conduct of a trial in younger cohorts, which we report here. The primary endpoints were the safety and reactogenicity of BK-SE36 administered subcutaneously or intramuscularly in healthy 12- to 60-month-old Burkinabe children. Secondary endpoints were humoral and cellular immune responses. This phase Ib trial provides safety and immunogenicity data with regards to two administration routes and the utility of a third dose at Week 26 (6 months after the first dose) for BK-SE36 in 1- to 5-year-old.

Methods

Trial site and population

The study was conducted at the Banfora trial centre of the Centre National de Recherche et de Formation sur le Paludisme (CNRFP). The Unité de Recherche Clinique de Banfora (URC-B), located about 400 km from Ouagadougou, Burkina Faso, is situated within the complex of the regional hospital. The trial participants were drawn from the Banfora Health Demographic Surveillance System (DSS) which covers a total population of 30,000 inhabitants. Bed net coverage in the area was around 80% (16) but indoor residual spraying (IRS) was considered inadequate or nil (17, 18). *P. falciparum* is responsible for 93% of malaria cases, with the rest attributed to monoinfections due to *P. malariae* (2%) and mixed infections of *P. falciparum* + *P. malariae* (5%) (19). The common vectors are *Anopheles gambiae*, *A. coluzzi* and *A. arabiensis* (16–18). Children under five years of age are the population subgroup of highest risk. Although transmission occurs throughout the year, about 60% of clinical cases are reported during June–September coinciding with the rainy months of May–November (19).

Study design and objectives

This double-blind, randomized, controlled, age de-escalating, phase Ib clinical trial with a single-blind follow-up phase (Clinical trial registry PACTR201411000934120) enrolled 108 healthy, malaria-exposed African children. Children in both Cohort 1 (aged 25–60 months, n = 54) and Cohort 2 (aged 12–24 months, n = 54) were randomized into 3 treatment arms in a 1:1:1 ratio receiving: (a) 3 full doses of BK-SE36 by the subcutaneous route (BK SC), (b) 3 full doses of BK-SE36 by the intramuscular route (BK IM), and (c) 2 doses of the licensed *Pneumococcal* polysaccharide conjugate decavalent Synflorix® vaccine, alternate with 1 dose of physiological saline by the IM route (control arm). The primary objective was to assess safety and reactogenicity; the secondary objective was to assess the immune response. For age de-escalation, Cohort 2 vaccination started after the recommendation of an independent safety monitoring committee (ISMC) who reviewed safety data from Cohort 1 up to and including 7 days of active follow-up post Dose 2.

The inclusion of a third dose at Week 26 (W26; Day 182) was intended to increase the immune response and evaluate the effect of a booster dose. The safety and immunogenicity of two doses was demonstrated in a previous trial in Uganda (15); while a three-dose regimen had been tested in malaria naïve Japanese adults (13). The common vaccination route in Japan, where the BK-SE36 vaccine was developed, is the subcutaneous route (SC). As the intramuscular route (IM) is the standard route of administration in the national Expanded Programme on Immunization, it was deemed important to add this treatment arm. Moreover, in some vaccines, IM administration is associated with a better immune response and fewer injection site reactions (20). The dosing interval of 28-days was similar to the minimum interval in vaccine doses according to the guidelines from the Advisory Committee on Immunization Practices (21). The sample size was calculated based on the safety objective. A group size of 18 subjects gives a minimum power of 85% to detect 1 or more SAEs that occur with a frequency of at least 10%. Allowing for losses to follow-up, a group size of 15 would still provide ≥79% power to detect 1 or more SAEs that occur with a frequency of at least 10%.

Screening, enrolment, randomization, and blinding

Community consent was obtained from local village leaders and community members. Based on data from the DSS in the study area, all children aged 12- to 60-month-old and their parents/guardians were invited to local community meetings to explain the study. Those interested were invited to participate in a public lottery to randomly select participants for a screening visit. When the infant's/child's name was called, the parent/guardian randomly selected a sealed envelope containing "YES" or "NO". "YES" would mean that the infant/child was invited for a screening visit. At the trial site, informed consent was sought and parent(s)/guardian(s) were asked to sign/thumbprint consent forms prior to performing any study related procedure. A literate, impartial witness was present for illiterate parent(s)/guardian(s).

Participants meeting the eligibility criteria (Supplementary Material) were assigned to treatment arms using a computer-generated randomization list. Randomization used permuted random block sizes of 6 and 9. For each cohort, allocation to a treatment number was based on the order that the subject presented for vaccination. The trial pharmacist opened sequentially numbered opaque sealed envelopes after ensuring that the child/infant before him met the eligibility criteria and

had been given a study ID number. An independent vaccinator performed vaccine administration. All other study staff were blinded to treatment assignment. The trial remained double-blind until one month after the booster dose.

Intervention, storage, and masking

BK-SE36 is produced by expressing recombinant SE36 based on amino acid residues 17–192 and 226–382 of *P. falciparum* (Honduras-1) SERA5 in *E. coli* BL21(DE3) pLysS (13). The purified protein was mixed with aluminum hydroxide gel in PBS. GMP-grade BK-SE36 vaccine was supplied by the Research Foundation for Microbial Diseases of Osaka University in single-dose vials as a lyophilized white powder that was reconstituted prior to vaccination (Lot number SER04B). When reconstituted with 1.3 mL of the supplied diluent (Japanese Pharmacopoeia water, Lot number D13T04), the opaque/opalescent liquid suspension contained 100 µg/mL of SE36 protein and 1 mg/mL aluminum. One mL was used for administration.

The control vaccine was Synflorix® (GlaxoSmithKline Biologicals s.a.), a 10-valent adsorbed pneumococcal polysaccharide conjugate vaccine purchased locally in mono-dose, prefilled glass syringe (Lot numbers ASPNA361AA and ASPNA765AE). As per manufacturer recommendation, based on the participant age group, vaccinations (0.5 mL dose) were delivered with an interval of at least 2 months between the 2 primary doses: Synflorix® was administered on Day 0 (D0) and Week 26 (W26); and physiological saline on Week 4 (W4). Physiological saline (Otsuka Pharmaceutical Factory, Inc) was supplied in twist-off type multi-dose plastic ampoules (Lot number K4J78).

All study vaccines were securely stored at 5 ± 3°C with limited access. To preserve blinding, masked, similar type syringes were used for vaccination (including for Synflorix® and saline).

Trial visits and safety assessments

Vaccinations were conducted on D0, W4/D28 and W26/D182. Following each vaccination, subjects were observed in the clinic for at least 60 minutes for any immediate local or systemic adverse events (AEs). Safety outcomes were also evaluated daily at home for the next 6 days, and during clinic visits on days 7 and 28 post vaccination. Daily contact visits were also done at D240–330 prior to clinic visit at D365. Monthly visits were conducted on D395–D455 by a field worker to check the participants' status and refer them to the trial center, if necessary. The final clinic visit was at D477. During clinic visits, hematology safety tests included hemoglobin (Hb) and red cell indices (MCV, MCH, MCHC), white blood cell count (WBC) with differential absolute neutrophil count (ANC), RBC, and platelet count. Alanine aminotransferase (ALT), aspartate amino transferase (AST), total bilirubin, and creatinine were also assessed. The number and percentage of participants with AEs, serious adverse events (SAEs), AEs leading to withdrawal, and clinically significant hematological and biochemical abnormalities were recorded. The severity of AEs was assessed and evaluated based on a 3-grade scale (Grade 1 = mild, Grade 2 = moderate, or Grade 3 = severe) by the investigators (Supplementary Material). Fever, as presented here, was determined by measurement rather than reported history. A rapid diagnostic test and thick- and thin- blood smears were prepared whenever a subject presented with an axillary temperature of ≥ 37.5°C or a history of fever within the past 24 hours.

Malaria diagnosis was done by light microscopy in the Parasitology Unit at URC-B. Two thick and thin blood smears were prepared for each subject for samples obtained at D0, vaccination days (D28, D182), 28 days post vaccination (D56 and D210), D365, D477, and whenever clinical malaria was suspected at any unscheduled visit.

Immunogenicity assessment

Anti-SE36 IgG antibody titers before vaccination (D0, D182), 4 weeks after each vaccination (D28, D56, D210), and at D365 and D477 were measured by ELISA. ELISA measurements, outsourced to a GLP certified testing facility (CMIC Pharma Science Co., Ltd., Japan), were performed using standardized methodology and expressed in titers calculated using an equilibrium line assay (13, 15). When clinical malaria was diagnosed during passive surveillance, samples for IgG analyses were obtained whenever possible for the initial unscheduled visit and one week after treatment. At each arm and each visit, the number and proportion of individuals with detectable SE36 IgG were reported. For serum samples with anti-SE36 IgG antibody levels below the limit of detection, a value of 8 was assigned for statistical analyses. Geometric mean titers were calculated at each time point. IgG1 and IgG3 subclasses were determined (22) for those with detectable anti-SE36 antibody titers 4 weeks after the second (W8/D56) and third (W30/D210) vaccinations.

T cell cytokine (IL-5, IL-13, and IFNγ) measurements for samples obtained before vaccination, 4 weeks post second (W8/D56) and third (W30/D210) vaccinations, and at D365 and D477 were done at the Immunology and Parasitology Laboratory at CNRFP using Human Th1/Th2 Cytokine ELISA Kit (Abcam, UK) according to manufacturer's instructions. In brief, antibodies specific for IFNγ, IL-5 and 1L-13 were precoated respectively onto corresponding microtiter plates and samples, including standards of known concentrations, were incubated at room temperature for 2 h 30 min. The wells were then washed 3 times with PBS and antibody cocktails from

the ELISA kits were added into wells, incubated at room temperature for 1h, washed and horseradish peroxidase conjugated streptavidin were added for 45 min. After removal of non-bound HRP conjugate, TMB substrate was added and incubated for 30 min in the dark at room temperature. Following incubation with stop solution, absorbance at 450 nm was measured with the microplate reader Biotek ELx808 (Winooski, Vermont 05404-0998 USA).

The mapping of protective epitopes in the SE36 antigen was done by ELISA (14) using serum samples obtained at W8/D56 and W30/D210 at the Department of Molecular Protozoology, Research Institute for Microbial Diseases (RIMD), Osaka University.

Statistical methods

Statistical analyses were performed at the London School of Hygiene and Tropical Medicine using Stata ver 15 (Statacorp, College Station, TX, USA, www.stata.com). All safety analyses were descriptive in nature and presented as frequency distributions by vaccination group. For continuous variables, box-whisker plots, medians, inter-quartile ranges, and ranges were used. Antibody titers are presented in terms of the geometric mean for each time point and treatment arm. Two separate analyses were done: an analysis that included all subjects who received at least one vaccine dose and a separate analysis per protocol that included all subjects who received all three doses at the correct time interval. As similar results were obtained with both datasets, safety and immunogenicity data shown here are for all subjects who received at least 1 vaccination. Exact Binomial Proportion was used to estimate the proportion of subjects that received 3 doses without experiencing Grade 3 adverse events.

For comparison of anti-SE36 antibody titers, and fold-change in antibody titers in BK and SC arms, statistical tests (t-tests of log(titer)) were performed at 2 time points (D182, prior to Dose 3; D210, 4 weeks post Dose 3).

Ethical and regulatory approval

The study was conducted according to the principles of the Declaration of Helsinki (2013), the ICH guidelines for GCP (CPMP/ICH/135/95) July 1996 (and its Revision 2 dated 9 November 2016), and in full conformity with relevant country regulations. Ethical reviews were conducted in Burkina Faso: Comité d'Éthique pour la Recherche en Santé du Burkina Faso (Ref: 2014-12-144) and Comité Institutionnel de Bioéthique du CNRFP (Ref: n°2014/071/MS/SG/CNRFP/CIB, N°2016/000008/MS/SG/CNRFP/CIB); Japan: Scientific Committee/Institutional Review Committee of the Research Institute for Microbial Diseases (Ref: 26-6), Osaka University (Ref: 574); and United Kingdom: London School of Hygiene and Tropical Medicine Research Ethics Committee (Ref: 9175). Approval for the clinical trial (N°2015:658/MS/CAB) and importation permit (N° 20150016/MS/SG/DGPML/DRLP/SHPS/KKG) for the Investigational Products (IP) were obtained from Agence Nationale de Régulation Pharmaceutique (ARPN, previous name: Direction Générale de la Pharmacie, du Médicament et des Laboratoires (DGPML).

Results

Participant distribution, recruitment and demographic data

Seventy-seven children were screened for inclusion in Cohort 1 (25- to 60-month-old), of whom 54 were enrolled and randomized to three study arms each with 18 subjects (BK SC, BK IM, and control) (Figure 1A). Those who were excluded (n = 23) did not meet inclusion criteria (n = 12), declined to participate (n = 1), or were not enrolled because the sample size had already been achieved (n = 10). Seven days after Dose 2 of Cohort 1, the ISMC assessed the safety data prior to the Go decision to start the vaccination in Cohort 2 (12- to 24-month-old). For Cohort 2, of 94 subjects screened, 40 were excluded (n = 33, did not meet inclusion criteria; n = 2, declined to participate; n = 5, not enrolled because the sample size had already been achieved) (Figure 1B).

The vaccination of Cohort 1 started on July 4, 2015 (Dose 1) and the last vaccination (Dose 3) was completed by January 11, 2016. Follow-up was completed on Oct 28, 2016. Thirty-three children (92%) received all 3 doses of BK-SE36 and 18 (100%) received 2 doses of Synflorix (and physiological saline at Dose 2). Three subjects, all from the BK IM arm did not receive Dose 3 (n = 1, withdrew consent; n = 2, withdrawn by the investigator due to participation in another trial, and another due to erythema).

Vaccination of Cohort 2 began on October 12, 2015 (Dose 1) and was completed by April 18, 2016 (Dose 3). Follow-up was completed on February 7, 2017. Thirty-five children (97%) received all 3 doses of BK-SE36 and 18 (100%) received 2 doses of Synflorix (and physiological saline at Dose 2). One subject (BK SC) withdrew consent before Dose 3.

Demographic characteristics at enrolment among arms in each cohort were broadly similar (Table 1).

BK-SE36 safety and reactogenicity

In terms of immediate reactogenicity, within the first 60 minutes post-vaccination, abnormal pulse rates were reported in all vaccination arms of Cohort 1 and Cohort 2 after each vaccination (Supplementary Table S1). Grade 1 pain/limitation of limb movement was reported for one BK SC subject of Cohort

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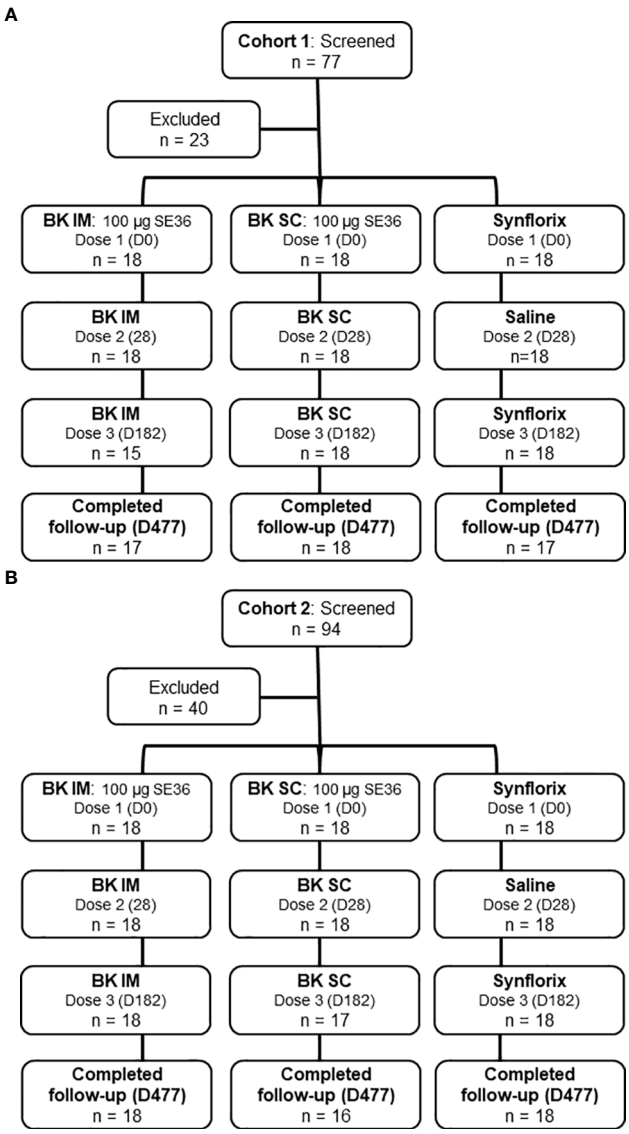

FIGURE 1
Trial profile. **(A)** Cohort 1 (25-60-month-old). The reasons for exclusion are: did not meet inclusion criteria (n=12), declined to participate (n=1) and sample size reached (n=10). **(B)** Cohort 2 (12-24-month-old). The reasons for exclusion are: did not meet inclusion criteria (n=33), declined to participate (n=2) and sample size reached (n=5). Results presented comes from all subjects who received at least 1 vaccination.

1 within 60 min of Dose 1. No other solicited local or systemic reactions were reported within the hour after each vaccination.

Local adverse events observed during the trial included pain at the injection site, swelling, erythema/redness, and induration (Table 2). Overall (Cohort 1 and 2 combined), the most commonly reported local events were induration (67%, 60%, and 53% for Dose 1, Dose 2, and Dose 3, respectively in BK-SE36 arms *vs* 53%, 17%, 22% for control arm) and pain (64%, 61%, and 26% for Dose 1, Dose 2, and Dose 3, respectively in BK-SE36 arms *vs* 67%, 22%, 14% in the control arm) (Supplementary Table S2). Table 2 shows that, between arms, BK SC reported more AEs than BK IM (*e.g.* In Cohort 1: induration: 67%–89% for BK SC *vs* 0–44% for BK IM; pain: 67%–83% for BK SC *vs* 13–61% for BK IM; in Cohort 2: induration: 82%–100% for BK SC *vs* 17–56% for BK IM; pain: 12%–89% for BK SC *vs* 11%–44% for BK IM). Local events were either mild or moderate; and most resolved within 1–2 weeks without treatment. The longest induration resolved 44 days post Dose 1 for a BK SC subject in Cohort 2; the longest induration in the control arm resolved 27 days post Dose 1. Both were Grade 1 AEs. The longest recorded redness lasted for 158 days for 1 BK SC subject in Cohort 1 (Grade 1, observed after Dose 2) *vs* 2 days for control.

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TABLE 1 Baseline characteristics of the study participants at enrollment within each vaccine arm.

Study cohorts		Cohort 1: 25-60 months				Cohort 2: 12 –24 months			
Arm		**BK-SE36**		**Control (Synflorix)**	**Total**	**BK-SE36**		**Control (Synflorix)**	**Total**
Route		**SC**	**IM**	**IM**	**SC**	**IM**	**IM**		
n		**18**	**18**	**18**	**54**	**18**	**18**	**18**	**54**
Gender	Male	7 (39%)	7 (39%)	6 (33%)	20 (37%)	8 (44%)	6 (33%)	8 (44%)	22 (41%)
	Female	11 (61%)	11 (61%)	12 (67%)	34 (63%)	10 (56%)	12 (67%)	10 (56%)	32 (59%)
Age (months)	(mean ± SD)	43.7 ± 11.3	46.1 ± 9.6	47.8 ± 9.0	45.9 ± 10	18.5 ± 3.7	18.2 ± 3.1	19.3 ± 3.0	18.7 ± 3.3
Height (cms)	(mean ± SD)	93 ± 7	96 ± 6	96 ± 7	95 ± 7	78 ± 4	77 ± 3	79 ± 4	78 ± 3
Weight (kgs)	(mean ± SD)	13.5 ± 1.8	14.8 ± 1.8	14.8 ± 2.2	14.4 ± 2.0	9.4 ± 1.0	9.0 ± 1.1	9.5 ± 1.4	9.3 ± 1.20
Body mass index (BMI)	(mean ± SD)	15.6 ± 1.1	16.0 ± 1.4	15.9 ± 1.4	15.8 ± 1.3	15.5 ± 1.6	15.0 ± 1.3	15.2 ± 1.2	15.2 ± 1.4

SC, subcutaneous route; IM, intramuscular route; n, no. of subjects.

For systemic events, there were 3 Grade 3 fever events: from a control subject in Cohort 1 after Dose 1 (not related), a control subject in Cohort 2 after Dose 1 (possibly related) and a BK IM subject in Cohort 2 after Dose 2 (not related). Other AEs (loss of appetite, irritability and drowsiness) were less common (Table 2), generally mild and resolved within 3 days.

The most frequently reported AEs at any time during the trial period were respiratory tract infections (bronchitis, rhinitis, and cough) with Cohort 2 (younger cohort) having more events than Cohort 1. Gastrointestinal disorders (enteritis and gastroenteritis) were also common in Cohort 2 in all treatment arms (Table 3). Most of the AEs were due to common childhood illnesses. Only one related AE (Table 2), urticaria in a BK IM subject, was reported in Cohort 1. The AE occurred 2 days post Dose 2, was moderate in severity, and resulted in the discontinuation of the third dose (as per the investigator's decision). The event resolved 5 days after onset. In Cohort 2, there were 8 mild to moderate AEs judged related to vaccination with no consequence on study continuation. Diarrhea was reported in four subjects (1 in BK IM, 2 in BK SC and 1 in control arm; one subject in addition to diarrhea reported vomiting), in all cases occurring within a day of vaccination and resolving ≤ 2 days after onset. Another BK SC subject had pruritus 4 days post Dose 1 which resolved 2 days after onset. One subject in the control arm had fever 2 days post Dose 2 which resolved (without treatment) 2 days after onset. High transaminasemia occurred 7 days post Dose 2 in one BK SC subject, of Grade 2 severity and resolved (without treatment) by 22 days after onset.

No serious adverse events (SAEs) were judged related to vaccination. Four (4) SAEs were reported in Cohort 1 (BK SC, n = 2 and control arm, n = 2) (Supplementary Table S3). Seven (7) SAEs were reported in Cohort 2 (BK IM, n = 3; BK SC, n = 2; and control, n = 2). All SAEs were due to severe malaria with most cases resolving within a week (longest around 10 days). Aside from SAEs, a Grade 3 event (high transaminasemia) occurred in Cohort 2 (BK SC) 160 days after Dose 3. The event was judged unrelated to vaccination and had resolved (without treatment) by 22 days after onset. The proportions of subjects that received three doses without experiencing Grade 3 adverse events were similar in all vaccination arms for both cohorts (Cohort 1: 100%, 89%, and 89% for BK IM, BK SC, and control, respectively; Cohort 2: 83%, 82%, and 83% for BK IM, BK SC, and control, respectively) (Supplementary Table S3). None of the SAEs or Grade 3 events resulted in the discontinuation of vaccination.

With regards to laboratory AEs, large variations, above or below the reference range, were observed in hematology and biochemistry parameters but most were considered to be clinically not significant and the child was well. In both cohorts, most out-of-range values in hematology were observed in platelets, MCV, MCH and ESR (additionally Cohort 2 has out-of-range values also in RBC and MCHC) but no strong evidence or pattern was repeated across treatment arms in both cohorts which could be interpreted as vaccine related. Some laboratory fluctuations led to or were correlated with AEs. In Cohort 1, fluctuations in Hb and platelet (n = 3) were linked to anemia (BK IM, n = 1; BK SC, n = 1; control, n = 2). Some elevated liver enzyme fluctuations were linked to high transaminasemia (BK SC, n = 2). Abnormal ALT, AST, and bilirubin values were also linked to hepatitis A (BK SC, n = 2; BK IM, n = 1). In Cohort 2, an abnormal Hb value was linked to anemia in the control group. Elevated liver enzymes were linked to high transaminasemia (BK SC, n=2; BK IM, n=1; control, n=1). In addition, elevated liver enzymes (ALT and AST) in 4 children in the control arm were also linked to hepatitis A.

Humoral and cellular response to BK-SE36 vaccination

Geometric means titers (GMT) with 95% confidence intervals by vaccine arm and visit are shown in Table 4. Notably, detectable (> 8, the assigned value for statistical

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TABLE 2 Summary of local and systemic adverse events (full analysis set).

	Cohort 1 (25-60 months)								
	Dose 1			Dose 2			Dose 3		
n	BK IM 18	BK SC 18	Control 18	BK IM 18	BK SC 18	Control 18	BK IM 15	BK SC 18	Control 18
Local									
Pain	9 (50%)*	13 (72%)	13 (72%)	11 (61%)	15 (83%)	6 (33%)	2 (13%)	12 (67%)	4 (22%)
Swelling	4 (22%)	2 (11%)	5 (28%)	6 (33%)	5 (28%)	1 (6%)	3 (20%)	1 (6%)	3 (17%)
Redness/Erythema	0	0	0	3 (17%)	5 (28%)	2 (11%)	0	5 (28%)	0
Induration	8 (44%)	12 (67%)	11 (61%)	7 (39%)	16 (89%)	2 (11%)	0	16 (89%)	2 (11%)
Systemic									
Fever	0	1 (6%)	2 (11%)	1 (6%)	1 (6%)	1 (6%)	0	0	0
Loss of appetite	0	0	0	1 (6%)	0	1 (6%)	0	0	0
Irritability/fussiness	0	0	0	0	0	0	0	0	0
Drowsiness	0	0	1 (6%)	1 (6%)	1 (6%)	1 (6%)	0	0	0
Other AEs suspected to be related to study vaccine									
Urticaria				1 (6%)					
	Cohort 2 (12-24 months)								
n	**18**	**18**	**18**	**18**	**18**	**18**	**18**	**17**	**18**
Local									
Pain	8 (44%)	16 (89%)	11 (61%)	8 (44%)	10 (56%)	2 (11%)	2 (11%)	2 (12%)	1 (6%)
Swelling	5 (28%)	4 (22%)	3 (17%)	6 (33%)	4 (22%)	0	3 (17%)	5 (29%)	4 (22%)
Redness/Erythema	0	7 (39%)	2 (11%)	0	4 (22%)	0	1 (6%)	3 (18%)	1 (6%)
Induration	10 (56%)	18 (100%)	8 (44%)	3 (17%)	17 (94%)	4 (22%)	6 (33%)	14 (82%)	6 (33%)
Systemic									
Fever	2 (11%)	0	2 (11%)	1 (6%)	2 (11%)	0	0	0	0
Loss of appetite	1 (6%)	1 (6%)	1 (6%)	1 (6%)	0	0	0	1 (6%)	0
Irritability/fussiness	0	0	0	0	0	0	0	1 (6%)	0
Drowsiness	0	0	1 (6%)	0	2 (11%)	0	0	0	0
Other AEs suspected to be related to study vaccine									
Pruritus	0	1 (6%)	0	0	0	0	0	0	0
Pyrexia	0	0	0	0	0	1 (6%)	0	0	0
Diarrhea	0	1 (6%)	0	0	1 (6%)	0	1 (6%)	0	1 (6%)
Vomiting	0	0	0	0	1 (6%)	0	0	0	0
Increased transaminase	0	0	0	0	1 (6%)	0	0	0	0

no. of children experiencing an event = n (% of children); BK IM = BK-SE36 via intramuscular route; BK SC = BK-SE36 via subcutaneous route

analyses), pre-vaccination anti-SE36 IgG antibodies were present in all arms at D0, prior to any vaccination (Cohort 1: 5/36 in BK arms (GMT for 5 subjects with detectable titers: 139.5, CI 50.2-387.6), 4/18 in the control arm (n = 4, GMT 216.4, CI 6.7-7012); Cohort 2: 17/36 in BK arms (n = 17, GMT 58.7, CI 37.4-92.2), 9/18 in the control arm (n = 9, GMT 52.8, CI 26.7-104.7)).

In Cohort 1, 4 weeks after Dose 1 (D28) only a small rise in titers in both BK-SE36 arms was observed (Table 4). Four weeks after Dose 2 (D56), anti-SE36 IgG antibodies increased substantially in both BK-SE36 arms (10.4-fold change for BK IM, 7.3-fold change for BK SC) while the titers remained at a similar level in the control arm (1.7-fold change) (Supplementary Table S4). Titers waned at D182, before Dose 3 (6 months after Dose 1). Four weeks after Dose 3 (D210), the anti-SE36 IgG titers reached peak levels (16.5-fold change for BK IM, 11.2-fold change for BK SC). At D365 (26 weeks after Dose 3) and D477 (42 weeks after Dose 3), titers waned again to levels similar to D28 (28 days after Dose 1). There was no evidence of a difference when comparing GMTs between BK IM and BK SC using t-tests performed at 2-time points (before Dose 3 (D182): $p = 0.50$; 4 weeks post Dose 3 (D210): $p = 0.83$ for comparison between BK IM and BK SC) (Table 4). Antibody titers were relatively stable from D0 to D477 in the control group.

In the younger cohort, on D28, high anti-SE36 IgG antibody titers were seen in both BK-SE36 arms, indicating a good

TABLE 3 Frequently reported adverse events.

	BK-SE36 IM		BK-SE36 SC		Control	
	Cohort 1 n = 18	Cohort 2 n = 18	Cohort 1 n = 18	Cohort 2 n = 18	Cohort 1 n = 18	Cohort 2 n = 18
Bronchitis	11 (61%)* [19]	13 (72%) [39]	14 (78%) [26]	15 (83%) [41]	10 (56%) [20]	13 (72%) [29]
Rhinitis	11 (61%) [15]	13 (72%) [39]	10 (56%) [18]	16 (89%) [39]	11 (61%) [20]	14 (78%) [33]
Cough	4 (22%) [6]	0	3 (17%) [4]	3 (17%) [3]	3 (17%) [4]	0
Enteritis	0	9 (50%) [14]	0	7 (39%) [13]	2 (11%) [2]	7 (39%) [11]
Gastroenteritis	0	2 (11%) [2]	0	3 (17%) [3]	0	4 (22%) [4]

*no. of children experiencing an event (% of children), [total no. of events]

response immediately after Dose 1 (compared to Cohort 1) (Table 4). Fold-change after Dose 2 (D56) was comparable to the level of fold-change obtained from Cohort 1 after Dose 3 (D210) (Supplementary Table S4). Four weeks after Dose 3 (D210), the anti-SE36 IgG titers reached peak levels (37.6-fold for BK IM and 22.3-fold for BK SC). At D365 and D477 titers waned to levels slightly higher than D28 (28 days after Dose 1), except for the BK SC arm at D477 (Table 4). Again, there was no evidence of a difference when comparing GMT between vaccination routes using t-tests on log titers performed at 2-time points (before Dose 3 (D128): $p = 0.48$; 4 weeks after Dose 3 (D210): $p = 0.99$ for comparison between BK IM and BK SC). GMT and fold change in antibody titers in the control group remained relatively stable from D0–D477.

For serum samples with detectable anti-SE36 IgG antibody levels, IgG1 and IgG3 concentrations were measured 4 weeks after Doses 2 and 3 (Table 5). For both cohorts, it appears that BK-SE36 induced a more pronounced IgG1 subclass dominant

TABLE 4 Total anti-SE36 IgG antibody.

	BK-SE36 Intramuscular		BK-SE36 Subcutaneous		Control (Synflorix® + saline) Intramuscular	
	n	GMT (95% CI)	n	GMT (95% CI)	n	GMT (95% CI)
			Cohort 1			
Day 0	18	9.4 (6.7, 13.0)	18	15.1 (8.0, 28.8)	18	16.6 (7.2, 38.5)
Day 28	18	18.2 (10.0, 33.1)	18	29.7 (15.7, 55.9)	18	29.7 (13.8, 63.9)
Day 56	18	97.2 (47.0, 200.9)	18	110.6 (63.6, 192.4)	18	28.5 (13.7, 59.3)
Day 182	17	33.1 (19.1, 57.3)	18	43.4 (23.5, 80.1)	18	28.5 (13.8, 58.9)
Day 210	17	155.3 (79.2, 304.6)	18	169.5 (92.6, 310.2)	18	24.0 (11.4, 50.5)
Day 365	17	27.9 (12.9, 60.4)	18	37.7 (20.5, 69.2)	17	18.0 (8.8, 37.0)
Day 477	17	38.3 (16.2, 90.7)	18	56.0 (27.5, 113.8)	17	43.4 (19.3, 98.0)
			Cohort 2			
Day 0	18	16.9 (9.9, 28.6)	18	24.9 (13.3, 46.9)	18	20.6 (11.6, 36.4)
Day 28	18	65.6 (38.0, 113.4)	18	63.7 (37.6, 107.8)	18	27.5 (15.2, 49.9)
Day 56	17	271.7 (144.5, 510.9)	16	304.0 (148.0, 624.6)	18	16.6 (10.4, 26.5)
Day 182	18	21.8 (10.7, 44.7)	17	29.4 (18.2, 47.6)	18	8.7 (7.6, 9.8)
Day 210	18	634.6 (284.3, 1416)	16	640.2 (374.8, 1093)	18	8.4 (7.6, 9.4)
Day 365	18	109.0 (50.3, 235.9)	16	93.9 (39.8, 221.2)	18	22.2 (13.4, 36.8)
Day 477	18	98.9 (39.7, 245.9)	16	38.6 (20.5, 72.9)	17	12.8 (8.9, 18.5)

Subjects were vaccinated at Day 0, 28 and 182; Day 28, 56 and 210 = 4 weeks after Dose1, 2, and 3, respectively; GMT = geometric mean titre (95% confidence interval); n = number of subjects;
Cohort 1: $p = 0.50$ for comparison of BK-SE36 arms at Day 182 (prior to Dose 3) and $p = 0.83$ for comparison of BK-SE36 arms at Day 210 (4 weeks post Dose 3).
Cohort 2: $p = 0.48$ for comparison of BK-SE36 arms at Day 182 (prior to Dose 3) and $p = 0.99$ for comparison of BK-SE36 arms at Day 210 (4 weeks post Dose 3).

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TABLE 5 Concentration of anti-SE36 IgG1 and IgG3 subclasses.

		Cohort 1					
		BK-SE36 Intramuscular		BK-SE36 Subcutaneous		Control (Synflorix® + saline) Intramuscular	
		n	GMC (95% CI)	n	GMC (95% CI)	n	GMC (95% CI)
IgG1	Day 56	16	13.6 (7.2, 25.4)	17	14.0 (8.9, 22.0)	10	5.2 (1.5, 18.2)
	Day 210	16	16.1 (8.5, 30.4)	17	19.8 (11.9, 33.0)	8	4.7 (0.9, 23.3)
IgG3	Day 56	16	2.2 (0.5, 8.9)	17	1.0 (0.3, 3.7)	10	1.2 (0.2, 8.9)
	Day 210	16	1.9 (0.6, 6.7)	17	0.4 (0.1, 1.2)	8	4.7 (0.5, 46.9)
		Cohort 2					
IgG1	Day 56	17	28.7 (16.6, 49.9)	16	25.5 (12.7, 51.4)	8	4.0 (1.1, 15.1)
	Day 210	17	66.2 (37.9, 115.7)	16	57.5 (38.6, 85.6)	1	3.2
IgG3	Day 56	17	8.5 (2.9, 24.4)	16	9.7 (2.6, 36.9)	8	0.6 (0.1, 4.3)
	Day 210	17	3.5 (0.9, 13.1)	16	8.0 (2.9, 22.3)	1	0.1

Day 56, 4 weeks after Dose 2; Day 210, 4 weeks after Dose 3; GMC, geometric mean concentration (95% confidence interval); n, number of subjects.

response. Changes in IgG3 were not as marked as for the IgG1 subclass, although higher geometric mean concentrations were observed in Cohort 2.

Considering T-cell cytokines IL-5, IL-13, and IFNγ, several subjects in Cohort 1 did not show detectable levels at visits D56, D210, D365 and D477 (Supplementary Table S5). In Cohort 2, more subjects had detectable IL-5 and higher levels of IL-13, although these levels were highly variable and observed in all vaccine arms including the control.

Reactivity of serum to peptides covering the whole sequence of the SE36 protein showed that in both cohorts at D56 and D210, sera from all BK-SE36 vaccinees reacted most strongly with synthetic peptides 7, 8, 9, and 15 (Supplementary Figure S1). Control sera reacted most strongly to peptides 1, 7, 8, and 15.

Discussion

The primary objective of the study was to assess the safety and reactogenicity of 3 full doses of BK-SE36 (100 µg SE36 protein with AHG as an adjuvant) when administered on D0, D28, and D182, by either subcutaneous or intramuscular route, in healthy African children naturally exposed to the parasite *P. falciparum*. The sample size was small, the immunological analysis descriptive in nature, and the study did not include functional assays or a comprehensive assessment of the cell-mediated immune response. However, this was the first study to assess safety and immunogenicity in an age group (12–60 months) that has not been included in previous vaccine trials of BK-SE36 and, likewise, to compare the immune response to vaccination using IM and SC routes for this vaccine candidate. Traditionally, aluminum adjuvanted vaccines are recommended for IM and live attenuated virus vaccines for the SC route (20). However, the common vaccination route in Japan, where BK-SE36 was developed, is SC. IM vaccinations are easier to perform, and this route remains the standard worldwide, with injections generally well tolerated. This trial shows that similar to Havrix® (hepatitis A vaccine, inactivated) and Priorix-Tetra™ (measles–mumps–rubella–varicella [MMRV] combination vaccine), both routes are immunogenic (23, 24).

One hundred and four of 108 participants completed three vaccinations, and overall 68/72 (94%) children received all 3 vaccinations of BK-SE36. BK-SE36 showed an acceptable safety profile in this population of Burkinabe children. There were no serious adverse events, unexpected reactions or safety concerns considered to be related to BK-SE36 during the course of the trial. All SAEs (n = 11) reported were hospitalizations due to severe malaria judged not related to vaccination and most resolved in less than two weeks. The proportion of children that received three doses without experiencing Grade 3 adverse events were similar across vaccination arms in both cohorts.

Reactogenicity was similar to that seen in the Japanese phase Ia (13) and Ugandan phase Ib (15) clinical trials. The most frequently reported solicited local AEs were induration and pain, mostly mild to moderate in terms of severity. Although more (and longer) cases of local reactogenicity were reported in the BK SC arm than in the BK IM or the control arms, there was no apparent increase in AEs at Week 26 (Dose 3). No distinct differences were seen between the age groups. Other AEs related to vaccination (urticaria, pruritus, diarrhea, vomiting and high transaminasemia) were mild to moderate in severity. Overall, the safety profile was comparable to that of the control pneumococcal polysaccharide conjugate vaccine Synflorix®.

BK-SE36 induced a clear humoral immune response. Total anti-SE36 IgG antibody titers increased 4 weeks after Dose 2 and

Dose 3 in the BK-SE36 arms in both cohorts. Overall, mean anti-SE36 protein antibody titer values were higher at these visits in the BK-SE36 arms compared to the control group and only the BK IM and BK SC arms showed >2.5-fold change in antibody titers after each vaccination. Cohort 2 (12–24 months) had 2-fold and 4-fold higher antibody titers than Cohort 1 after Dose 2 and Dose 3 of BK-SE36, respectively. The control arm, in both cohorts, had titers that remained low from Day 0–Day 577.

Some subjects (in Cohort 1, 5 children randomized in BK arms and 4 in control; Cohort 2, 17 children in BK arms and 9 in control) had anti-SE36 IgG antibodies pre-vaccination suggesting presence of residual maternal antibodies or exposure to *P. falciparum* infections early in life (25–27). In a surveillance study looking at antibody titers to merozoite antigens in children residing in both high (Banfora, Burkina Faso) and low (Keur Soce, Senegal) malaria transmission areas consistently low antibody titers were also observed (28). Although antibody titers were found to decline in the first few months, presumably due to the loss of maternal antibodies, the rate of waning of maternally derived and the degree of naturally acquired anti-SE36 IgG antibody titers (including seropositivity) has not been thoroughly studied in this age group; although age-related acquisition as a result of natural infection has been noted in previous studies (13, 27). Indeed, the first two doses for both age cohorts in this trial were administered during the rainy season (where malaria transmission is high), but high titers are not expected because of low immunogenicity and the clear age-dependency for IgG specific to SE36 (13). In the present study, in the absence of robust baseline data, it is not clear if existing pre-vaccination anti-SE36 IgG antibodies are due to natural exposure or are residual maternal antibodies.

SE36 was observed to tightly bind to host prote*in vitro*nectin that can act as a molecular camouflage (11) and thus repeated infections and presence of these vitronectin-bound SE36 complex could inevitably result to immune tolerance against SE36 molecule. In the Ugandan adult cohort, no significant increase in antibody titers were observed after 2 vaccinations of BK-SE36, in contrast to 6–10 year old Ugandan children where the proportion of subjects with >2-fold increase in antibody titers was 73% (15). In this trial, the proportion of subjects with >2-fold increase in antibody titers after Dose 2 was 83% for Cohort 1 and 79% for Cohort 2.

In BK-SE36 vaccinees, a booster vaccination (Dose 3) resulted in higher immune responses. This is in contrast to an earlier trial in Japanese adults that showed no significant difference in antibody titer post Dose 3 when compared to values obtained post Dose 2 (13). Differences in vaccination schedule may have contributed to the improved immunogenicity. In Japanese adults the three vaccinations were in 21-days interval, whereas, in both Burkinabe cohorts Dose 3 was delayed to Week 26 (182 days from Dose 1 or 154 days after Dose 2). Antibody levels dropped to near pre-vaccination titers 5 months after Dose 2, but 28 days post Dose 3, antibodies were boosted, sometimes to levels higher than those induced 28 days after two vaccinations. Following Dose 3, the proportion of subjects with >2-fold increase in antibody titers was 89% for Cohort 1 and 97% for Cohort 2. In trials of two other vaccine candidates, improved immunogenicity was also observed for a delayed dose schedule (29, 30).

After D210, GMT values drop at D365 (26 weeks after Dose 3; fold change in antibody titer compared to baseline was only 2.7-fold in Cohort 1 and 4.9-fold in Cohort 2) and D444 (42 weeks after Dose 3; Cohort 1 = 3.9-fold change, Cohort 2 = 3.1-fold change); but not to levels similar to the control arm (Cohort 1, D365 = 1.0-fold change, D444 = 2.5-fold change; Cohort 2, D365 = 1.1-fold change, D444 = 0.6-fold change). Notably, the high transmission season was 6 months after Dose 3 for Cohort 1 and 2 months after Dose 3 for Cohort 2. Improvement in vaccination schedules (timing of third dose) should also be explored. It is envisioned that protection can be obtained when high anti-SE36 antibody titers induced by vaccination (4 weeks after Dose 3) coincide with the time of greater risk of contracting malaria (*i.e.*, during the rainy season when transmission is highest).

No marked difference was observed when BK-SE36 was administered *via* the intramuscular or subcutaneous route of the vaccination. As previously reported, the vaccine induced response was composed mostly of IgG1 (22). With regards to epitope mapping, binding was observed in all arms to peptide 15, the binding site of the host protein, vitronectin. This binding property has been found to be more or less conserved in global *P. falciparum* isolates (11). Control sera reacted also to peptide 1. Synthetic peptide 1 lies in the intrinsically unstructured octamer repeat region at the N-terminal domain of SE36. All BK-SE36 arms reacted most strongly with peptides 7, 8, and 9 which corresponds to domains in the middle of the SE36 molecule proximal to the serine repeat region (14). Binding to peptide regions that lie in characteristically disordered or intrinsically unstructured regions (*e.g.* peptides 1, 7, 8, 9) further implies the absence of a strict conformational requirement for SE36 to be able to elicit an immune response (14).

The contribution of SE36 antigen-specific helper T cells remains unclear as cytokine secretion levels were low overall for IL-5, IL-13 (used as Th2 response markers) and IFNγ (as a Th1 response marker). The proportion of subjects with cytokine responses was higher in the younger cohort (Cohort 2), but no marked associations were found in relation to vaccine arm. Aside from small sample size, the wide variability seen may be attributed to an immature immune system, short-lived responses or very low response levels that fall below the threshold of detection in peripheral blood sampling. Only three T cell response markers were used and further studies are needed to cover other cytokines in the cellular immune response repertoire. Studies investigating protective efficacy against malaria infection and clinical

disease are needed for more robust conclusions. So far, previous trials (13, 15, 22) and sero-epidemiological studies (12–14) suggest some subtle differences in antibody IgG subclass profile, fine epitope specificity, and potential differences in T-helper cell responses between immune response observed in vaccinees and immune response observed as a result of natural infection. These differences need to be further explored. Nevertheless, in congruence with prior trials, BK-SE36 is a promising blood-stage malaria vaccine candidate.

Conclusions

BK-SE36 malaria vaccine appears to be well-tolerated when given to healthy semi-immune 12-60 month old children in Burkina Faso, at the dose of 100 µg, subcutaneously or intramuscularly on Days 0, 28, and 182. Although, BK-SE36 was immunogenic in both cohorts, whichever administration route was used, the IM route appears to have a lower risk of adverse reactions at the site of vaccination than the SC route. Moreover, younger children (12–24 months old) showed better immune response. The third dose at Week 26 boosted the humoral response to BK-SE36 in both age cohorts. This study supports the design and conduct of a phase IIb double-blind study in children under 5 years.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Comité d'Éthique pour la Recherche en Santé du Burkina Faso (Ref: 2014-12-144) and Comité Institutionnel de Bioéthique du CNRFP (Ref: n°2014/071/MS/SG/CNRFP/CIB, N°2016/000008/MS/SG/CNRFP/CIB) (Burkina Faso); Scientific Committee/Institutional Review Committee of the Research Institute for Microbial Diseases (Ref: 26-6), Osaka University (Ref: 574) (Japan); and London School of Hygiene and Tropical Medicine Research Ethics Committee (Ref: 9175) (United Kingdom). Approval for the clinical trial (N°2015:658/MS/CAB) and importation permit (N°20150016/MS/SG/DGPML/DRLP/SHPS/KKG) for the Investigational Products (IP) were obtained from Agence Nationale de Régulation Pharmaceutique (ARPN, previous name: Direction Générale de la Pharmacie, du Médicament et des Laboratoires (DGPML). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

SS, EB, AT, IN, OL, SH, FD, NP, and TH contributed to the conception and design of the study. EB, AT, IN, AO, SaC, AD, IS, JY, and SS were responsible for study implementation at the study site. AZO organized the database. SiC performed the statistical analysis. TH, NP, SH, and SS analyzed the data. MY, NA, TT, and NP for materials/reagents/assay development and analysis tools. EB and NP wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This work was supported by the Global Health Innovative Technology Fund (G2014-109) awarded to the Consortium; MEXT KAKENHI Grant Numbers 15651988, 24249024, Funds for Integrated Promotion of Social System Reform and Research and Development (38201103-01), Grant for Translational Research Network Program (JP20lm0203135, AMED) (C-13) awarded to TH; Grant for Cyclic Innovation for Clinical Empowerment by AMED (17nk0101206j0003) to Nobelpharma Co., Ltd (NPC, Tokyo, Japan); and Irish Aid to EVI. An additional in-kind contribution was also obtained from NPC. The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

Acknowledgments

We thank study volunteers and their families for participation in the study and acknowledge the support of the URCB Centre (Unite de Recherche Clinique de Banfora), the Local Safety Monitor and Independent Safety Monitoring Committee for their support (Ken J. Ishii, Yoshio Hirota, Meta Roestenberg, Kwaku Poku Asante and Marceline T. Yaméogo). Guidance of the regulatory authorities and ethics committees in Burkina Faso, UK, Japan; and the Independent Scientific Advisory Committee (Carole Long, Mahamadou Thera, Takafumi Tsuboi and Chetan Chitnis) during the trial and follow up study are much appreciated. We also gratefully acknowledge the clinical trial assistance of Lydia Dabré (GRAS), the quality assurance assistance of Nathalie Imbault (EVI), laboratory assistance of Hideko Yoshikawa, Sawako Itagaki and Yuko Oishi (RIMD); the administration and logistical support of Nadège Kinda (GRAS), Sten Larsen

Finnsson and Thorsten Kohaut (EVI), and Kumiko Tai (RIMD). Vaccine manufacture and supply was supported and undertaken by The Research Foundation for Microbial Diseases of Osaka University (BIKEN), Japan. The trial was sponsored by NPC; logistics, support and all other activities are duly acknowledged from Masanori Osakabe, Takako Aburada, Taka Sato, Satoru Murayama, and Motomichi Kouno.

Conflict of interest

TH is the inventor of BK-SE36 and all rights have now been turned over to NPC. NP served as contract researcher for NPC, Apr - Sept 2017. SH, FD and OL received support from NPC for salaries, travel and CRO cost for clinical monitoring. EB, SaC, AD, AZO, JY also received support from NPC for salaries during the long term follow-up.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.978591/full#supplementary-material

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Bougouma EC, Palacpac NMQ, Tiono AB, Nebie I, Ouédraogo A, Houard S, Yagi M, Coulibaly SA, Diarra A, Tougan T, Ouedraogo AZ, Soulama I, Arisue N, Yaro JB, D'Alessio F, Leroy O, Cousens S, Horii T and Sirima SB (2022) Safety and immunogenicity of BK-SE36 in a blinded, randomized, controlled, age de-escalating phase Ib clinical trial in Burkinabe children.
Front. Immunol. 13:978591.
doi: 10.3389/fimmu.2022.978591

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 08 September 2022
DOI 10.3389/fimmu.2022.977472

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to
Parasite Immunology,
a section of the journal
Frontiers in Immunology

RECEIVED 24 June 2022
ACCEPTED 17 August 2022
PUBLISHED 08 September 2022

CITATION
Nunes-Cabaço H, Moita D and
Prudêncio M (2022) Five decades of
clinical assessment of whole-
sporozoite malaria vaccines.
Front. Immunol. 13:977472.
doi: 10.3389/fimmu.2022.977472

Five decades of clinical assessment of whole-sporozoite malaria vaccines

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In 1967, pioneering work by Ruth Nussenzweig demonstrated for the first time that irradiated sporozoites of the rodent malaria parasite *Plasmodium berghei* protected mice against a challenge with infectious parasites of the same species. This remarkable finding opened up entirely new prospects of effective vaccination against malaria using attenuated sporozoites as immunization agents. The potential for whole-sporozoite-based immunization in humans was established in a clinical study in 1973, when a volunteer exposed to X-irradiated *P. falciparum* sporozoites was found to be protected against malaria following challenge with a homologous strain of this parasite. Nearly five decades later, much has been achieved in the field of whole-sporozoite malaria vaccination, and multiple reports on the clinical evaluation of such candidates have emerged. However, this process has known different paces before and after the turn of the century. While only a few clinical studies were published in the 1970's, 1980's and 1990's, remarkable progress was made in the 2000's and beyond. This article reviews the history of the clinical assessment of whole-sporozoite malaria vaccines over the last forty-nine years, highlighting the impressive achievements made over the last few years, and discussing some of the challenges ahead.

KEYWORDS

vaccine, plasmodium, sporozoite, clinical trial, protective efficacy, immunogenicity

Introduction

The 6 October 2021 will be forever engraved in the history of the fight against malaria as the date when RTS,S, the first vaccine against this devastating disease, was recommended by the World Health Organization (WHO) to be given to children living in regions with moderate-to-high transmission of *Plasmodium falciparum* (Pf) malaria. RTS,S, a subunit vaccine based on the Pf circumsporozoite protein (CSP), was initially developed by the Walter Reed Army Institute of Research (WRAIR) and GlaxoSmithKline (GSK), in 1987. A long path followed, during which the vaccine was evaluated in multiple clinical trials in malaria-endemic regions, leading to its eventual

endorsement. Immunogenicity studies have indicated that RTS,S exerts its protective effect through antibodies against PfCSP and through $CD4^+$ T cell responses, but no clear immune correlates of protection have been identified (1, 2). Results from a large Phase III clinical study have shown that 4 doses of the vaccine present relatively modest and rapidly waning 25.9% and 17.3% effectiveness against clinical and severe malaria, respectively, in newborns aged 6–12 weeks, and 36.3% and 32.2% efficiency against clinical and severe malaria, respectively, in children aged 5–17 months [(3) and reviewed in (4)]. A post-approval plan comprising 4 complementary Phase IV studies that will evaluate safety, effectiveness and impact of RTS,S in the context of its real-life implementation will support the ongoing evaluation of the vaccine's benefit-risk and inform decision-making for its potential wider implementation across sub-Saharan Africa (5). Moreover, RTS,S is not expected to protect against the other human malaria parasites, namely *P. vivax* (Pv), *P. ovale*, *P. malariae*, and the zoonotic *P. knowlesi* (6). Thus, in spite of this landmark achievement, the licensing of RTS,S should not be viewed as the end of the road in the quest for a malaria vaccine. Rather, it should be seen as a stepping stone towards the WHO's ambitious goals of, by 2030, licensing vaccines targeting Pf and Pv with protective efficacy of at least 75 percent against clinical malaria and that substantially reduce the incidence of human malaria infection (7).

Whole-sporozoite (WSp) vaccines (Figure 1) have emerged as a possible strategy to immunize against malaria since the demonstration that X-irradiated sporozoites of *P. berghei* (Pb) could induce protective immune responses against an intravenous challenge with fully infective Pb parasites (8). Interest in WSp vaccination increased following the initial demonstration by Clyde *et al.* that radiation-attenuated Pf sporozoites could also afford protective immunity against homologous Pf malaria (9). However, for a long time, WSp vaccination was considered impractical, and the barriers to the development of WSp vaccines seemed all but insurmountable (10). Nevertheless, research into this area gained momentum in the early 2000's and, one by one, many of these barriers were overcome, through the efforts of several laboratories around the world and, pivotally, by the remarkable technological and scientific progress made by Stephen L. Hoffman's team at Sanaria, Inc. and its network of collaborators.

Nearly five decades have elapsed since the first clinical assessment of a WSp vaccine by Clyde *et al.*, in 1973 (9). Whereas throughout the first 3 decades of this period such trials involved a total of only about two dozen human subjects (10, 11), this number has risen exponentially since then, generating an impressive amount of data on the immunogenicity and protective efficacy of WSp vaccines in humans (Figure 2). Here, we review the knowledge accumulated through these clinical studies, at a time when the prospect of WSp vaccines becoming a reality in a not-so-distant future seems more realistic than ever.

Clinical evaluation of whole-sporozoite vaccines

Controlled human malaria infection

The widely used term Controlled Human Malaria Infection (CHMI) is technically incorrect, since, as McFadden eloquently states, "malaria is a disease, not an organism" (12). As such, describing infection by *Plasmodium* parasites as "malaria infections" is no more right than referring to HIV infections as "AIDS infections" or to SARS-CoV-2 infections as "COVID-19 infections". However, the term CHMI appears to have been adopted by the community and, since it seems unlikely that it will be replaced by the more accurate "Controlled Human Infection by Malaria Parasites" (CHIMP) or "Controlled

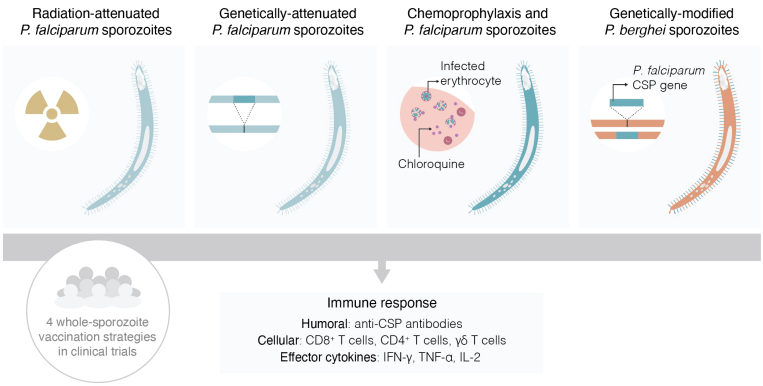

FIGURE 1
Schematic representation of the four types of whole-sporozoite vaccines against malaria assessed in clinical trials.

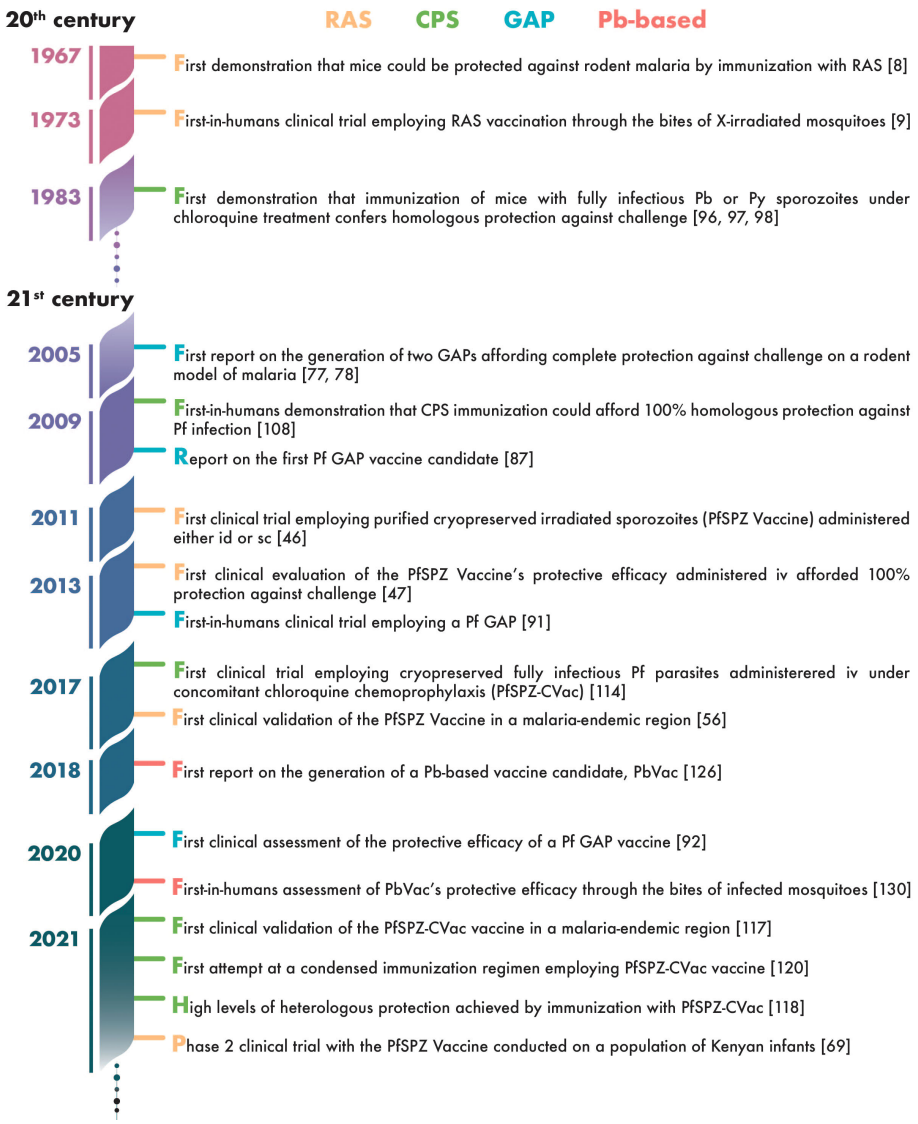

FIGURE 2
Timeline of landmark achievements in the development of whole-sporozoite vaccines against malaria.

Human *Plasmodium* Infection" (CHPI), will be employed throughout this review.

CHMI is of paramount importance in the context of malaria vaccinology, as amply discussed in several reviews (13–20). Both early and recent studies aimed at assessing WSp vaccine candidates in the clinic have resorted to CHMI, employing the strictly controlled exposure of trial participants to the bites of laboratory-reared, *Plasmodium*-infected mosquitoes (21). CHMI by the bites of five mosquitoes consistently infects all malaria-naïve volunteers (22), although exposure to the bites of 3 aseptically-raised Pf-infected mosquitoes has also been proposed as a safe, effective procedure for CHMI in malaria-naïve adults (23). While the NF54 strain of Pf is most commonly employed for CHMI by mosquito bite, the 7G8, NF135.C10 and NF166.C8 Pf strains have also been reported as eligible for use in such studies (24). An alternative to mosquito bite-based CHMI lies in the use of Sanaria, Inc.'s PfSPZ Challenge, consisting of infectious, aseptic, purified, vialed, cryopreserved Pf sporozoites, which can be administered by needle and syringe (25). Dose-finding trials have shown that intravenous (iv) injection of 3200 PfSPZ Challenge leads to a geometric mean pre-patent period similar to that observed following the bites of 5 Pf-infected mosquitoes (26). Whether CHMI by mosquito bite is preferable to the iv route, or vice-versa, remains a matter of some controversy. While the former is the more natural route of infection, it does not allow the exact estimation of the number of

inoculated sporozoites. Nevertheless, efforts have been made to standardize mosquito-administered CHMI (27), reducing the impact of this biological variability. On the other hand, PfSPZ Challenge enhances access to CHMI, including in malaria-endemic regions [(28, 29) and recently reviewed in (30)], which otherwise would be limited to the few research facilities with the capability to carry out Pf infections of mosquitoes for experimental purposes (25).

Radiation-attenuated sporozoites

An appropriate dose of ionizing radiation (UV, X-ray and γ) can prevent replication of a pathogenic organism, while preserving metabolic activity (31). Radiation-attenuated *Plasmodium* sporozoites (RAS) retain their ability to infect liver cells but are unable to replicate and progress to form erythrocyte-infectious merozoites, likely as a result of extensive DNA damage, accompanied by downregulation of DNA repair genes (32). In 1967, Ruth Nussenzweig and colleagues reported for the first time that mice could be protected against rodent malaria by immunization with RAS (8). Publication of this report created hope that humans could be completely protected against malaria, inspiring others to explore the prospect of WSp immunization in the clinic (33). To this day, RAS remain the gold-standard of whole-organism vaccination against human malaria.

Early studies of RAS immunization in humans

Inspired by Ruth Nussenzweig's pioneering report, in 1973, a team at the University of Maryland School of Medicine commenced trials to vaccinate human volunteers with Pf RAS, delivered by the bites of X-irradiated mosquitoes. In the first report of these studies, one of three volunteers fed on by 379 mosquitoes over the course of 84 days did not develop malaria following an infective challenge with sporozoites delivered by non-irradiated mosquitoes 15 days after the last immunization (9). This volunteer then underwent an additional 5 immunization sessions, during which he was exposed to a total of 819 irradiated mosquitoes, and remained protected against a second infectious sporozoite challenge 12 days after the last immunization (9). These observations constitute the first demonstration of the protective efficacy of RAS vaccination in the clinic. Interestingly, having remained malaria-free for 2 months after the second sporozoite challenge, the same volunteer was challenged by intravenous injection of Pf trophozoites, and developed parasitemia and clinical symptoms 5 days later (9). Although the authors may not have fully realized this at the time, this was also the first indication that the protection afforded by WSp vaccination is purely restricted to the pre-erythrocytic stage of *Plasmodium* infection. A subsequent report describes the first RAS immunization against both Pf and Pv through the bites of irradiated mosquitoes infected with either of these parasites. A single volunteer received immunizing doses of Pf or Pv on different days and at different intervals, and was subsequently challenged with infectious parasites of either species delivered by non-irradiated mosquitoes. The experimental setup employed might be considered less than appropriate nowadays, particularly considering the small number of study participants, the irregular immunization schedules, and the concomitant use of both human parasites. The subject underwent immunization with Pf sporozoites delivered by a total of 1806 irradiated mosquitoes, which protected him against a Pf but not a Pv challenge. Subsequent immunization by exposure to a total of 739 Pv-infected, irradiated mosquitoes conferred protection against Pv challenges for up to six months (34). Another volunteer immunized by the bites of 728 irradiated Pv-infected mosquitoes was reported to be unprotected against a Pv challenge one week after the last immunization, but was protected one week after the last inoculation of an additional series of 1251 bites (35). Finally, three volunteers immunized by the bites of 440-987 irradiated, Pf-infected mosquitoes were protected for 8 weeks against an infectious Pf challenge, but no protection was observed in volunteers exposed to 200 or fewer irradiated mosquitoes (36). Overall, of 11 volunteers who were immunized in the 1970s by the bites of irradiated, Pf-infected mosquitoes, five displayed species-specific (37) protection against a subsequent exposure to infective sporozoites of different Pf strains.

It would be more than a decade until the next clinical studies of WSp vaccines took place. In 1991, two groups of volunteers were vaccinated by repeated exposure to the bites of Pf-infected, X-irradiated mosquitoes. While two volunteers in group 1, exposed to 625 and 715 irradiated mosquitoes, were unprotected against an infectious Pf challenge delivered by mosquito bite, all three volunteers in group 2, who were exposed to a total of 1563-1681 immunizing bites, were fully protected against a Pf challenge three weeks after the last immunization (38). One of these subjects received a series of booster immunization bites approximately three months after that first challenge and was re-challenged nine months after that, remaining immune to virulent sporozoites (39). Between 1989 and 1999, another eleven volunteers were immunized at the Naval Medical Research Center and the Walter Reed Army Institute for Research. The results of these trials are summarized in a publication by Hoffman *et al.* in 2002, and show that ten of eleven volunteers immunized by the bites of 1001-2927 irradiated mosquitoes infected with Pf strain NF54 were protected against a homologous challenge two to nine weeks after the last immunization (11). Furthermore, four out of five protected subjects were also protected against a Pf re-challenge 23-42 weeks after a secondary immunization, and two volunteers

were protected when re-challenged with the heterologous 7G8 strain of Pf (11). This report constitutes a landmark in WSp malaria vaccination, demonstrating not only that protective immunity elicited by Pf RAS is strain-transcendent, but also that it may persist for at least 10 months. These findings created a renewed interest in WSp vaccination against human malaria and paved the way for an entirely new era of research in this field.

WSp vaccination by injection: purification and cryopreservation of Pf sporozoites

The enthusiasm generated by the observations outlined above was curbed by the generally accepted conviction that a vaccine whose administration required the bites of more than 1000 mosquitoes was clinically impractical [reviewed in (40, 41)]. However, and contrary to what had successfully been done in rodent models, the injection of infected mosquito salivary gland material into humans posed unacceptable medical risks (41). This realization entailed several immediate concerns, arising from the (i) practical limitations in infecting mosquitoes with Pf, which depended on feeding on volunteers with circulating Pf gametocytes; (ii) relatively small numbers of sporozoites in the salivary glands of Pf-infected mosquitoes; and (iii) absolute necessity for adequate purification and preservation of Pf sporozoites intended for immunization. The first of these concerns had been overcome by the development of methods for *in vitro* culturing of Pf parasites in 1976 (42), and for gametocyte production from these cultures in 1982 (43). The challenges imposed by the other two concerns meant that, for the best part of the first decade of the 21st century, clinical trials employing RAS remained scarce (44). This situation changed dramatically thanks to the persistence of Stephen L. Hoffman and his team at Sanaria, Inc., who set out to develop methods to increase sporozoite yields in infected mosquitoes, as well as to purify and preserve these parasites (40). Their efforts culminated in the successful manufacture of the PfSPZ Vaccine, consisting of aseptically purified, metabolically active, non-replicating (irradiated), cryopreserved Pf sporozoites of the NF54 strain, suitable for clinical use (45) and GMP-compliant (31). This remarkable achievement completely changed the prospects for WSp vaccination, and prompted a surge of clinical trials to assess and optimize the immunogenicity and efficacy of RAS-based immunization.

Establishing the proof-of-principle of PfSPZ vaccination

In the first attempt at human vaccination with PfSPZ Vaccine, the vaccine was administered either by intradermal (id) or subcutaneous (sc) injection to a total of 80 volunteers, 44 of whom subsequently underwent homologous CHMI by mosquito bite, alongside 18 non-immunized controls. The results were nothing less than disappointing, with only two of the challenged vaccinees protected against infection, and none of the others displaying even a delay in time to detectable parasitemia (46). Unfazed by these results, the authors employed several animal models to dissect the immune responses elicited by injection of the vaccine through different routes. Their results provided unequivocal evidence that intravenous (iv) injection of PfSPZ Vaccine elicited significantly more potent immune responses than id and sc administration of the vaccine (46). These observations paved the way for the clinical evaluation of PfSPZ Vaccine's protective efficacy when administered by iv injection and, in 2013, the Sanaria team reported for the first time that five doses of 1.35 x 10^5 iv-injected PfSPZ Vaccine (strain NF54) conferred 100% protection against an infectious challenge with PfNF54 parasites delivered by mosquito bite 3 weeks after the last immunization (47). This landmark study constituted the first demonstration that a WSp vaccine delivered by needle and syringe could confer high levels of protection against human malaria. Aided by the subsequent demonstration that PfSPZ vaccination could confer long-term protection against malaria (48), these findings laid the foundations for an ambitious plan to further the clinical development of PfSPZ Vaccine and other related products (49).

Protection against heterologous challenge

The demonstration that five doses of the PfSPZ Vaccine could induce high levels of protection against homologous challenge in trials conducted in the USA raised several questions, including whether it would be possible to reduce the number of vaccine doses employed, and if such protection would hold upon a heterologous challenge and/or in malaria-endemic regions. The issues of dose reduction and heterologous protection were addressed in several clinical trials reported from 2017 onwards. Heterologous protection studies commonly employ the South American Pf isolate 7G8 (50, 51), which is genetically diverse from the PfSPZ Vaccine's PfNF54 strain (52). In fact, a recent analysis of the genome, proteome and $CD8^+$ T cell epitopes of various Pf strains has shown that Pf7G8 is more distant from PfNF54 than any one of more than 700 African isolates investigated, suggesting that Pf7G8 constitutes a stringent surrogate for the vaccine's field efficacy in Africa (53). In a report from 2017, 5 doses of 2.7×10^5 PfSPZ were shown to confer 92.3% and 80.0% protection against homologous (Pf3D7, a clone of PfNF54) and heterologous (Pf7G8) CHMI delivered by mosquito bite three weeks after the last immunization, respectively, but efficacy against the latter dropped dramatically to 10% twenty-four weeks after the final

immunization (52). The same study also showed that a 3-dose regimen of 4.5 × 10^5 PfSPZ conferred 86.7% and 57.1% protection against homologous CHMI by mosquito bite three and twenty-four weeks after the last immunization, respectively (52). These results indicate that heterologous protection may be less pronounced and less durable than homologous protection, raising concerns about the vaccine's efficacy in the field. Nevertheless, another study revealed 64% protection against homologous challenge 19 weeks after the last of three immunizations with 9.0 × 10^5 PfSPZ at 8-week intervals, and 83% of the protected subjects who underwent a repeat heterologous challenge with Pf7G8 parasites 33 weeks after the final immunization remained protected (54). Very recently, vaccination with 9 × 10^5 PfSPZ on days 1, 8, and 29 was found to be similarly protective against homologous (PfNF54, 77% overall efficacy) and heterologous (Pf7G8, 79% overall efficacy) CHMI delivered iv at 3 or 9-10 weeks after immunization (55).

Protective efficacy in malaria-endemic regions

The first clinical evaluation of the PfSPZ vaccine in a malaria-endemic region was conducted in healthy Malian adults, naturally exposed to malaria. Trial participants were exposed to five doses of iv-delivered 2.7 × 10^5 PfSPZ at days 0, 28, 56, 84, and 140 during the dry season, and were actively followed up for 24 weeks during the transmission season. The results of this trial were reported in 2017 and indicated a vaccine efficacy of 51.7% (56), which is markedly lower than observed in a previous CHMI trial in the USA with a similar vaccine dose and administration schedule (52). Shortly afterwards, an identical vaccination regimen was employed to administer PfSPZ to Tanzanian adults. Challenge by homologous iv CHMI three weeks after the last immunization revealed only 20% protection, and all protected individuals remained uninfected after a re-challenge at 24 weeks (57). Interestingly, antibody responses to PfCSP in these studies, as in a PfSPZ Vaccine immunogenicity study carried out in Equatorial Guinea (58), were lower than in the volunteers in the USA (57). These observations indicate that malaria-naïve individuals in the USA respond better to the vaccine than malaria-exposed individuals in Africa. This may result from the immune modulation caused by repeated exposure to malaria, and suggests that enhancing the vaccine's immunogenicity and achieving sterile protection in endemic regions might require increasing the dose of PfSPZ and changing the interval between immunizations (49). In an attempt to increase vaccine efficacy in Tanzania, another trial was conducted where the PfSPZ dose was increased to 9 × 10^5 or 1.8 × 10^6, and the number of doses was reduced to 3, at 8-week intervals. Interestingly, and perhaps somewhat surprisingly, this study revealed an association between an increase in the dose and a decrease in vaccine efficacy. In fact, while 100% of the participants who received the 9 × 10^5 dose were protected against homologous (PfNF54) iv CHMI at 3 or 11 weeks, only 33% of those who received the 1.8 × 10^6 dose were protected against homologous (PfNF54) iv CHMI at 7.4 weeks (59). More recently, three doses of 1.8 × 10^6 PfSPZ at 1-, 13- and 19-week intervals afforded 51% efficacy against natural Pf transmission in Mali (60).

Multi-dose priming

The ability to elicit effective heterologous protection is an absolute requirement for a malaria vaccine to be deployed in the field, where multiple Pf strains likely coexist. Sanaria has therefore concentrated a large part of their recent efforts on improving PfSPZ's heterologous protection. Hypothesizing that induction of liver-resident $CD8^+$ T cells, which are pivotal for vaccine efficacy (61), could be enhanced by repeated priming with low PfSPZ vaccine doses, two multi-dose priming studies followed by CHMI were recently undertaken. In a clinical trial in the USA, 5 doses of 4.5 x 10^5 PfSPZ vaccine administered iv on days 1, 3, 5 and 7, and week 16 (referred to as multi-dose priming and delayed boosting) protected 40% of the subjects against heterologous challenge with Pf7G8 delivered by mosquito bite 12 weeks after the last immunization (62). Relevantly, in the same study, three immunizations with 9.0 × 10^5 PfSPZ at 8-week intervals (standard dose) afforded only 20% protection against heterologous Pf7G8 challenge by mosquito bite at 12 weeks, and three 8-weekly administration of 1.8 x 10^6 PfSPZ (escalated dose) afforded only 23% protection against heterologous Pf7G8 CHMI by mosquito bite at 24 weeks (62). More recently, the efficacy of multi-dose priming regimens of PfSPZ Vaccine against homologous (PfNF54) CHMI administered iv 6-7 weeks after the final immunization was evaluated in a clinical trial in Equatorial Guinea. In this study, four multi-dose priming regimens, with or without delayed boosting, were evaluated, all of which using doses of 9 x 10^5 PfSPZ delivered iv: days 1, 3, 5, 7 and 113; days 1, 3, 5 and 7; days 1, 3, 5, 7 and 29; and days 1, 8, and 29. A significant 51.3% protection was only observed for the regimen in which the vaccine was administered on a 4-week schedule, on days 1, 8, and 29 (63). The delayed boosting immunization schedule yielded a protective efficacy of ~40%, which is similar to that observed in the USA trial (62), but was not statistically significant (63). Perhaps surprisingly, protection afforded by the 2-dose multi-prime regimen (days 1, 8, and 29; 51.3%) was higher than that afforded by 4-dose multi-prime (days 1, 3, 5, 7 and 29; 10.7%), clearly a matter that demands additional investigation.

Vaccination of children and infants

Malaria exerts its heavier mortality burden on children and infants, with 77% of total malaria deaths in 2020 occurring under

the age of 5 years-old (64). With this in mind, Sanaria, Inc. initiated an assessment of the safety and feasibility of iv administration of the PfSPZ Vaccine, aiming to conduct an efficacy trial on this age group. These assessments took place in Tanzania (65) and Kenya (66, 67), and were accompanied by a careful analysis of caregiver and community perceptions and experiences regarding participation in these studies (68). These efforts culminated in a recently reported phase 2 trial conducted in western Kenya on a population of 336 infants aged 5-12 months, naturally exposed to malaria. The vaccine was administered in three iv doses of 4.5×10^5, 9.0×10^5 or 1.8×10^6 PfSPZ spaced by 8 weeks, with a 12-month follow-up period. Although vaccine efficacy against clinical malaria was estimated at 45.8% in the highest-dose group at the study's 3-months exploratory endpoint, significant protection against Pf infection was not observed in any dose group at the 6 months primary endpoint (69). These disappointing findings indicate that immune responses to the PfSPZ Vaccine are age-dependent, and may be explained by major differences between infants and older children and adults in the priming of PfSPZ-specific T cell responses (65, 69), and/or by the presence of low-level Pf parasitemia at the time of administration of the first vaccine dose (69, 70). In any case, these results clearly do not support the use of the PfSPZ Vaccine in infant populations, whose immune systems are immature, particularly for T-cell responses (71).

Genetically-attenuated parasites

Plasmodium parasites express several genes encoding pre-erythrocytic stage-specific proteins, some of which may be essential for the parasite's intra-hepatic development (72). Genetically-attenuated parasites (GAP) have been engineered to abrogate the expression of one or more genes essential for completion of their developmental process in the liver. Targeted deletion of these genes results in parasites that are able to infect hepatocytes but arrest their liver-stage development at defined points, remaining unable to establish a symptomatic blood-stage infection *in vivo* (73). A potential advantage of GAP- over RAS-based immunization is that the former constitute a homogeneous population of parasites with defined genetic identity and attenuation phenotype, which may be designed to induce optimal protective immunity (74). It is usually accepted that the immunity induced by parasites whose liver development arrests later is superior to that induced by early-arresting parasites (75, 76). Therefore, the development of a late-arresting PfGAP that can elicit effective protective immunity against malaria remains an attractive objective to which much attention has been devoted.

GAPs: From mice to humans

Effective vaccination employing genetically attenuated *Plasmodium* parasites was first demonstrated in rodent models of malaria in the mid-2000's. In 2005, Mueller *at al.* and van Dijk *et al.* showed that immunization of mice with Pb sporozoites deficient in the upregulated in infective sporozoites gene 3 (*uis3*) or in the *p36p* gene, respectively conferred complete protection against a challenge with infectious Pb sporozoites (77, 78). Over the next few years following these landmark studies, several reports emerged showing that highly effective protective immunity could be elicited by immunization by iv injection of other rodent parasite mutants, including *p52-/p36*-deficient *P. yoelii* (Py) (79), *uis3-/uis4*-deficient Pb (80) and Py (81), and purine nucleoside phosphorylase (*pnp*)-deficient Py (82), multidrug resistance-associated protein 2 (*mrp2*)-deficient Pb (83), and *b9-/slarp*-deficient Pb, followed by an iv challenge employing fully infective sporozoites of the same species (84).

Naturally, the success for GAP-based vaccination in rodents sparked an interest in the use of this approach to create vaccine candidates against human malaria. The genetic design of replication-competent vaccine strains holds the promise for a potent, broadly protective malaria vaccine (85). The development of appropriate genetic manipulation methods enabled the targeted deletion of genes in order to create Pf GAPs that arrest during hepatic development and that lack drug-resistance markers (86–88). Subsequent technical advances in genetic manipulation enhanced the efficiency and pace for generation of transgenic *Plasmodium* parasites (85). The first Pf GAP was reported in 2009 and consisted of a Pf parasite lacking the *p52* and *p36* genes, whose liver arrest was confirmed *in vitro* and in a liver-humanized mouse model (87). Since then, several other Pf GAPs have been reported in the literature, including Pf$b9^-$/$slarp^-$ (84), Pf$p52^-$/$p36^-$/$sap1^-$ (89) and Pf$mei2^-$ (90). Several of these candidates have been, are currently, or will likely undergo evaluation in a clinical setting.

Clinical evaluation of Pf GAP vaccines

The number of Pf GAP candidates tested in humans is currently limited. The first report of such a clinical study dates from 2013, when Pf$p52^-$/$p36^-$ sporozoites (87) were administered to six malaria-naïve volunteers by the bites of infected female *Anopheles stephensi* mosquitoes. Subjects were initially exposed to 5 bites/volunteer, which was followed by exposure to ~200 bites/volunteer one month later. Although all volunteers remained blood stage-negative after the low dose exposure, one volunteer developed parasitemia after exposure to 263 bites, activating a Stopping Rule in the study (91). Genotyping analysis confirmed that the parasite in the peripheral circulation of this volunteer was Pf$p52^-$/$p36^-$, showing that a breakthrough infection, rather than a reversion to wild-type Pf, had occurred (91). This observation highlights the need to identify gene deletions, or a combination thereof, that ensure the parasite's complete arrest in the liver of the immunized subjects. In an attempt to achieve this, an additional

deletion was included to generate the Pf*p52*$^{-}$/*p36*$^{-}$/*sap1*$^{-}$ parasite, termed PfGAP3KO (89). To confirm immunization safety, PfGAP3KO was administered to 10 subjects by a single exposure to the bites of 150 to 200 bites per volunteer. All participants in this study remained blood stage-negative, indicating complete attenuation of PfGAP3KO in humans, and paving the way for the evaluation of its protective efficacy in the clinic (89).

The first Pf GAP to have undergone an evaluation of its protective efficacy in humans is Pf*b9*$^{-}$/*slarp*$^{-}$ (84). Aseptic, purified, and cryopreserved Pf*b9*$^{-}$/*slarp*$^{-}$ sporozoites were manufactured by Sanaria, Inc., creating the PfSPZ-GA1 Vaccine. No breakthrough infections were observed following the iv administration of three doses of 4.5×10^5 or 9.0×10^5 PfSPZ-GA1 Vaccine at 8-week intervals (92). Subjects were then challenged by mosquito bite CHMI with PfNF54 parasites 3 weeks after the last immunization. Although all vaccine groups showed a significant increase in pre-patency time, only 1 of 12 volunteers in the 4.5×10^5 PfSPZ-GA1 group and 2 of 13 volunteers in the 9.0×10^5 PfSPZ-GA1 group were sterily protected (92). Even though these results may appear somewhat disappointing, this is a landmark trial in that it constitutes the first clinical assessment of the protective efficacy of a Pf GAP vaccine. Furthermore, it should be noted that all volunteers from a Pf RAS control group, immunized with three doses of 4.5×10^5 PfSPZ Vaccine, developed parasitemia (92), which may reflect a particularly high stringency of the PfNF54 mosquito bite challenge employed in this study.

The clinical evaluation of PfGAP3KO's immunogenicity and protective efficacy was reported very recently. In this trial, the vaccine was delivered by three (with 4 weeks between the first and second vaccinations and the 8 weeks between the second and third vaccinations) or five (with 4 weeks between the first four vaccinations and 8 weeks between the fourth and fifth vaccinations) immunizations, with ~200 PfGAP3KO-infected mosquito bites per immunization. CHMI was carried out by the bites of PfNF54-infected mosquitoes either 4 weeks after the last immunization of the 6 volunteers in each of study arms 1 and 2, or 26 weeks after the first CHMI for study participants in both study arms who did not have any detectable Pf infection after the first CHMI. The vaccine protected 50% of the volunteers in either study arm after the first CHMI, and protected 1 of the 6 volunteers who undertook the second CHMI (93).

The road ahead for Pf GAP vaccination remains wide open, with efforts ongoing towards the identification of late-arresting replication-competent Pf parasites that are completely attenuated and highly immunogenic. Moreover, existing mutants, such as Pf*mei2*$^{-}$, are already undergoing clinical evaluation, and several others are likely to follow. Finally, the possibility of iteratively improving these parasites through the expression of additional antigens or immunomodulatory elements offers the prospect of a rationale for the creation of increasingly efficacious and versatile Pf GAP candidates (85).

Chemoprophylaxis and sporozoites

Depending on their molecular target and mode of action, antiplasmodial drugs may act either on multiple or only on specific stages of the parasite's life cycle. Immunization by ChemoProphylaxis and Sporozoites (CPS) relies on the ability of an antiplasmodial compound to provide a prophylactic cover against the symptomatic stage of *Plasmodium* infection following the administration of non-attenuated sporozoites. Thus, the inoculated, replication-competent, parasites are able to infect, develop and egress from hepatic cells unencumbered, but are eliminated prior to egress or following merozoite release into the blood stream, during the first wave of invasion of red blood cells (94). Liver infection elicits potent pre-erythrocytic immune responses, while the appearance of disease symptoms is prevented by the presence of the circulating drug. Unrestricted liver stage growth expands parasite biomass and antigenic repertoire to a greater extent than what occurs with RAS and GAP, potentially enhancing immunogenicity and decreasing the dose of immunizing parasites required for protection. In addition, the presence of an abortive blood-stage infection may elicit humoral immune responses against blood-stage *Plasmodium* antigens (95). Early pre-clinical studies showed that immunization of mice with fully infectious Pb (96, 97) or Py (98) sporozoites under chloroquine treatment, a drug that specifically targets blood stage parasites (99), conferred significant protection against a sporozoite challenge with the same parasite species. Since then, similar results have been obtained employing other antiplasmodial drugs, such as primaquine (100), mefloquine (101), pyrimethamine (102), piperaquine (103), artesunate (104), clindamycin (105), azithromycin (105) and arteether (106). More recently, CPS employing *P. knowlesi* (Pk) sporozoites and chloroquine was also shown to confer significant protection against Pk infection in a non-human primate model (107). Collectively, these pre-clinical observations paved the way to a wide array of studies aimed at assessing the potential of CPS immunization for vaccination against human malaria.

CPS immunization by mosquito bite

The first-in-humans demonstration that CPS immunization could afford high levels of sterile protection against Pf infection was provided by a landmark study in 2009, carried out at Nijmegen's Radboud University Medical Centre. In this seminal study, ten volunteers were exposed to the bites of 12 to 15 PfNF54-infected mosquitoes in three immunization sessions at 1-month intervals, whilst under the cover of a prophylactic chloroquine regimen. Five control subjects received an equivalent number of non-infected mosquito bites and were subjected to a similar chloroquine regimen. Both

groups of volunteers were challenged by homologous CHMI delivered by mosquito bite 8 weeks after the last immunization dose (4 weeks after the discontinuation of chloroquine prophylaxis). Whereas all control subjects developed PfNF54 parasitemia, all immunized volunteers were protected against infection, indicating a striking 100% homologous protective efficacy of this immunization method (108). Importantly, a homologous re-challenge of six protected volunteers 2.5 years after the original study revealed that four of them remained sterilely protected, while the remaining two showed prolonged prepatent periods (109).

In a trial aimed at discerning the contributions of pre-erythrocytic and erythrocytic immunity for the protection afforded by Pf CPS vaccination, 4 out of 5 subjects (80%) taking chloroquine prophylaxis and immunized by 3 exposures to the bites of 15 PfNF54-infected mosquitoes at 1-month intervals were fully protected against a homologous CHMI by mosquito bite (110). In another group of 9 similarly immunized volunteers, none was protected against a blood-stage challenge by iv administration of asexual PfNF54 parasites, showing that protection against malaria CPS immunization is entirely mediated by pre-erythrocytic immunity (110). In a subsequent trial, 60 and 70% homologous protection was observed for volunteers under either chloroquine or mefloquine prophylaxis, respectively, who were exposed 3 times to 8 PfNF54-infected mosquitoes at monthly intervals (111).

The enthusiasm generated by the high protective efficacy observed in these homologous CHMI trials led to the assessment of the protection conferred by CPS immunization against heterologous parasite strains. Thus, in a follow-up study, 16 volunteers previously immunized by CPS employing PfNF54 parasites delivered by mosquito bite and homologously challenged with the same parasite strain were re-challenged 14 months after the last immunization with Pf strain NF135.C10. Only 2 out of 13 volunteers that were previously fully protected against PfNF54 were also fully protected against Pf NF135.C10, while the remaining 11 displayed an increased pre-patent period (112). These somewhat disappointing results were made even more so by the results of a subsequent clinical trial. There, CPS immunization with PfNF54 protected 5 out of 5 volunteers against a PfNF54 challenge 14 weeks after the last immunization, but sterilely protected only 2 out of 10 and 1 out of 9 volunteers against CHMI with Pf strains NF135.C10 and NF166.C8, respectively, all delivered by mosquito bite (113). These findings raise important questions regarding the potency of the immune responses required for effective heterologous protection following CPS immunization and the optimization thereof. This may involve the use of an immunizing Pf strain with intrinsically higher liver stage infectivity, an increase in the immunization dose or an alteration of the immunization regimen (113). Some of these challenges can at least be partially addressed by resorting to iv administration of the immunizing parasites, as discussed below.

Enter Sanaria's PfSPZ-CVac

In view of the promising results of early CPS vaccine trials in the clinic, the team at Sanaria, Inc. and its collaborators posited that PfSPZ Challenge could serve as a replacement for mosquito bite delivery of immunizing Pf parasites, hence giving rise to a CPS vaccine approach termed PfSPZ-Chemoprophylaxis Vaccine (PfSPZ-CVac) (49). In the first clinical trial with PfSPZ-CVac, 3-4 id administrations of 7.5 x 10^4 PfSPZ employing chloroquine as the drug partner induced no sterile protection against homologous CHMI with PfSPZ Challenge (114). With the benefit of hindsight, it is now clear that this is not a surprising result, given the poor immunogenicity of vaccine administration by the id route, as observed in PfSPZ vaccine studies ongoing at the time (46, 47). Thus, in a subsequent landmark trial carried out at the University of Tübingen, PfSPZ-CVac was administered iv, with chloroquine as the partner drug. A dose-dependent protective effect of the vaccine was observed, with 100% of the volunteers immunized by three doses of 5.12×10^4 sporozoites at 28-day intervals being protected against homologous iv CHMI with PfSPZ Challenge (PfNF54) 10 weeks after the last immunization (115). Remarkably, not only was this the first time that complete sterile immunity by PfSPZ-CVac was observed in the clinic, but also this was achieved with sporozoite doses 1-2 orders of magnitude lower than those required by RAS immunization with PfSPZ Vaccine, as outlined above. These results confirmed the high immunogenicity of the PfSPZ-CVac immunization approach, opening the door for further optimization of the immunization regimen and its assessment against heterologous CHMI or in the field (116).

The first assessment of PfSPZ-CVac in a malaria-endemic region took place in Equatorial Guinea and was reported in 2021. In this clinical trial, 3 doses of 2.7×10^6 PfSPZ Vaccine or 1.0×10^5 PfSPZ-CVac were administered at 8- or 4-week intervals, respectively, to different groups of volunteers. Immunized subjects underwent homologous CHMI by iv administration of PfSPZ Challenge (PfNF54) at a median of 14 weeks after the last immunization. Vaccine efficacies were 27 and 55% for PfSPZ Vaccine and PfSPZ-CVac, respectively, and were not statistically different from each other (117). Pre-patency as assessed by thick blood smear was significantly longer for PfSPZ Vaccine, but not for PfSPZ-CVac recipients, than controls (117). This trial constitutes the first head-to-head comparison of PfSPZ Vaccine and PfSPZ-CVac efficacies. It should be noted that the efficacy of both immunizations was lower than that observed in homologous CHMI studies in malaria-naïve volunteers employing lower vaccination doses (54, 115), once again indicating that immunization regimens in the field require further optimization.

Heterologous protection by PfSPZ-CVac vaccination

The issue of vaccination dose began to be assessed in a trial reported in 2021, where PfSPZ-CVac was used in combination with either chloroquine or pyrimethamine at a dose of 2×10^5 sporozoites, a 4-fold increase relative to that employed in the Mordmuller *et al.* study (115). In this study, subjects received 3 monthly immunizations with either partner drug, and underwent CHMI by iv administration of PfSPZ Challenge 3 months after the last immunization. The data revealed 100% heterologous (Pf7G8) protection in the chloroquine group, whereas 87.5 and ~78% protective efficacy was observed against homologous (PfNF54) and heterologous (Pf7G8) challenge, respectively, in the pyrimethamine group (118). These remarkable results constitute the first demonstration that high levels of heterologous protection can be achieved for at least 3 months through PfSPZ-CVac vaccination, which is significantly higher than what had been observed for RAS immunization with 9×10^5 PfSPZ Vaccine (62). However, in a very recent study in Mali, 3 doses of 2×10^5 PfSPZ-CVac (chloroquine) administered at 0, 4 and 8 weeks afforded only an estimated, non-statistically significant, protective efficacy of ~33% against naturally transmitted Pf infection over a 48-week surveillance period spanning wet and dry seasons (119).

Condensed PfSPZ-CVac immunization regimens

Also in 2021, a condensed immunization regimen employing PfSPZ-CVac and chloroquine was attempted for the first time. Inoculation of 1.1×10^5 sporozoites, twice the dose employed in the Mordmuller *et al.* study (115), on days 1, 6 and 29, yielded 77% protection against heterologous (Pf7G8) iv CHMI with PfSPZ Challenge 12 weeks after the last immunization (120). The importance of this study lies not only on the high protective efficacy observed, but also on the fact that in the immunization regimen employed chloroquine was administered only on the days of vaccine inoculation, limiting to three the number of visits to complete vaccination (120). In yet another study from 2021, two condensed regimens of three administrations of $5.12x10^4$ PfSPZ-CVac seven days apart and of $1.024x10^5$ PfSPZ-CVac five days apart, using chloroquine as the partner drug, were assessed in the clinic. The two regimens gave very different protections against homologous CHMI with PfSPZ Challenge (PfNF54), with the 7-day group showing 0% protective efficacy, and the higher-dose, 5-day group displaying 75% protective efficacy (121). It should be noted that vaccine administration to the former group coincided with patent parasitemia, suggesting that this may be associated with the observed lack of sterile immunity (121). Finally, in a very recent assessment of accelerated PfSPZ-CVac vaccination regimens, volunteers underwent three-dose immunization regimens at days 0/14/28 or at days 0/5/10, employing 5.12×10^4 sporozoites/dose and chloroquine as the partner drug. Homologous CHMI was performed by iv administration of PfSPZ Challenge (PfNF54) 10 weeks after the last immunization. The two immunization regimens yielded similar protective efficacies of 67 and 63% for 28- and 10-day vaccination schedules, respectively, but the latter resulted in more pronounced cellular and humoral immune responses than the former (122). Collectively, these results pave the way for further development of an effective condensed regimen of PfSPZ-CVac immunization, capable of eliciting protective immunity in the field.

P. berghei-based vaccination against human malaria

Rodent *Plasmodium* parasites are the most widely employed models of malaria research, particularly in what concerns the investigation of the pre-erythrocytic stages of infection (123). In recent years, rodent malaria parasites have also emerged as potential candidates for WSp immunization against human malaria. The idea draws from the origins of vaccination, when Edward Jenner unknowingly established the notion of cross-species protective immunity, by successfully using cowpox to vaccinate humans against smallpox (124). The notion that a similar principle may apply to Pb and human malaria parasites is supported by the presence of cross-species epitopes in different malaria parasites (125), and is strengthened by the high percentage of predicted T cell epitopes shared between the former and the latter (126). Besides, Pb's high amenability to genetic modification, solidified by years of experience in this area, enables the insertion of selected human *Plasmodium* antigens into neutral loci of its genome, effectively turning the rodent parasite into a unique platform for expression of heterologous *Plasmodium* antigens (127). Immunization with such chimeric Pb sporozoites is therefore expected to elicit not only cross-species immune responses, but also targeted immunity against human malaria parasites arising from those heterologous immunogens (128).

Pre-clinical validation of Pb-based WSp vaccination

The concept of Pb-based WSp vaccination was validated in 2018 through the generation of PbVac, a Pb parasite that expresses PfCSP under the control of the strictly pre-erythrocytic Pb*uis4* promoter (126). Pre-clinical characterization of PbVac showed that it expresses both the endogenous PbCSP and the heterologous PfCSP at the

surface of sporozoites and liver stages, and that it displays wild-type Pb-like mosquito and hepatic infectivity levels (126). Employing liver- and blood-humanized mouse models, PbVac was also shown to invade and develop inside human hepatocytes and to be unable to replicate inside human erythrocytes. Moreover, and crucially, PbVac was found to infect human primary hepatocytes with significantly higher efficacy than Pf, which may potentially entail high levels of human liver infectivity (126). Immunization of rabbits by the bites of PbVac-infected mosquitoes elicited cross-species cellular immune responses, as well as PfCSP-specific antibody responses that functionally inhibit infection of human hepatocytes by Pf, both *in vitro* and in liver-humanized mice (126). Collectively, these data unequivocally demonstrated PbVac's potential for immunization against Pf malaria, warranting its evaluation in the clinic. However, this posed a significant challenge, not only because there was no previous history of experimental administration of rodent malaria parasites to humans, but also due to the fact that PbVac is a genetically modified organism, and that sporozoites of this parasite can only be generated in mosquitoes infected by feeding on the blood of infected mice. Thus, several additional studies were performed to ensure the safety of PbVac for human use, including the creation of a Master Cell Bank, whole-genome sequencing of the transgenic parasite, a complete set of microbiological analyses, and tissue distribution and drug-sensitivity studies (129). The complete set of pre-clinical data gathered in these studies (126, 129) paved the way for its assessment in humans.

Clinical assessment of PbVac

The first-in-humans assessment of PbVac was reported in 2020 and consisted of a phase 1/2a clinical trial, in which PbVac sporozoites were administered to volunteers by the bites of infected female *A. stephensi* mosquitoes. Safety was assessed in a phase 1 dose-escalation study, in which groups of volunteers were exposed to the bites of 5, 25 and 75 PbVac-infected mosquitoes, with no breakthrough infections or serious adverse events recorded (130). In phase 2a of the study, 12 volunteers were immunized by four exposures to the bites of 75 PbVac-infected mosquitoes, spaced by 4 (between the first and second and between the second and third immunizations) or 8 (between the third and fourth immunizations) weeks, and were challenged 3 weeks after the last immunization by PfNF54-infected mosquito bites. A significant delay in blood stage patency and a significantly lower parasite density at first detection in the blood was observed in immunized volunteers, corresponding to an estimated 95% decrease in PfNF54 liver load for vaccinated subjects relative to non-immunized controls (130). It should be noted that the 4 x 75 PbVac-infected mosquito bites employed in this study corresponds to a much lower vaccine dose than that delivered by the more than 1000 mosquito bites previously used for immunization with Pf RAS (11). Thus, although no sterile protection was observed in the PbVac study, the marked reduction in liver parasite load triggered by immunization with the clearly sub-optimal dose employed, alongside the dose-dependent humoral and cellular immune responses observed (130), support further exploration of Pb-based vaccination against malaria. To this end, the production of aseptically purified, vialed, cryopreserved PbVac sporozoites that can be administered by parenteral injection at defined doses is currently ongoing in collaboration with Sanaria, Inc. Furthermore, the possibility of inserting multiple heterologous genes in the Pb genome (131) under the control of suitable pre-erythrocytic promoters (132) enables the generation of transgenic Pb parasites that express genes from different human *Plasmodium* parasites and from different stages of their life cycle. This possibility is particularly appealing in the case of Pv, for which an *in vitro* culture system is yet to be achieved (133), which severely limits the development of a WSp vaccine. Thus, transgenic Pb parasites expressing suitable Pv antigens may serve as unique surrogates for WSp vaccination against this human malaria parasite.

Immune responses elicited by whole-sporozoite vaccination

WSp vaccines primarily aim at boosting the host's immunity through the generation of effective and long-lasting immune responses that control and/or eliminate the parasite during the pre-erythrocytic stage of its life cycle. The investigation of these immunological mechanisms and their correlation with protection have been the focus of multiple studies that led to a thorough, yet still incomplete, picture of the immunity that ensues following vaccination, as recently reviewed (134–136). Although several studies have suggested a relation between some immune parameters and protection, a definitive immune correlate of protective efficacy of WSp vaccination remains to be clearly identified. Studies in mice and non-human primates have provided robust evidence that a large part of the pre-erythrocytic immune response that leads to protection is cell-mediated in the liver [reviewed in (61, 137)]. However, the fact that, in humans, immune parameters can only be analyzed in the peripheral circulation constitutes a limitation to the assessment of the global WSp-associated immunological landscape in the clinic. Moreover, it is likely that different WSp vaccines may produce distinct humoral and cellular response signatures that define protective immunity. In this chapter we will outline the main humoral and cellular immune responses identified during the clinical assessment of a variety of WSp vaccine candidates.

Antibody-mediated responses

Vaccines commonly act by inducing an antibody-mediated response against specific microorganisms or their constituents. The humoral responses induced by WSp vaccines are largely

directed at pre-erythrocytic antigens, with CSP, the most abundant protein on the surface of sporozoites, representing the hallmark parasite target [reviewed in (134, 136, 138, 139)]. Sporozoite- or CSP-specific antibodies are consistently induced in response to WSp vaccination of malaria-naïve individuals, and some studies have reported a correlation of antibody titers with RAS (47, 69), CPS (115), GAP (92) or PbVac (130) immunization doses, or with PfSPZ-induced protection (48, 92). Importantly, pre-exposure has been identified as a limiting factor for the magnitude of the humoral responses elicited by RAS (57, 58, 140) and PfSPZ-CVac (117) immunizations. Whether a similar effect is observed following immunization with other types of WSp vaccines remains to be addressed.

In addition to the magnitude of the humoral response, it is also important to assess the functionality of the antibodies generated by vaccination. Antibodies against sporozoites or their antigens may limit the infection in several ways, including by decreasing their motility (141), inhibiting hepatocyte invasion and parasite development (142), or mediating their destruction through mechanisms such as antibody-dependent cytotoxicity or phagocytosis upon opsonization (143, 144). The functionality of the circulating antibodies induced by WSp vaccination can be assessed by a variety of *in vitro* assays or *in vivo* studies, as recently reviewed (145). An important role for antibodies in pre-erythrocytic immunity was initially established through the observation that patency following administration of Pb sporozoites to naïve mice was delayed by passive transfer of serum from RAS-immunized mice (146). A functional role for antibodies elicited by PfSPZ (48), CPS (147) and Pf GAP3KO (89) immunization has been demonstrated *in vivo* using liver-humanized mouse models.

Different WSp vaccine approaches lead to distinct extents of parasite development in the liver, hence differing in the breadth of *Plasmodium* antigens presented to the host. Accordingly, antibodies to asexual and sexual erythrocytic antigens were low to undetectable following PfSPZ Vaccine (47) and early-arresting GAP (91) immunizations, while humoral responses against both pre-erythrocytic and cross-stage *Plasmodium* antigens are induced by CPS vaccination (148). Functional antibodies against the immunodominant CSP, which is common across WSp vaccine strategies, are prevalent in all WSp immunization approaches [reviewed in (134); see also (92, 130, 149–151)]. Nonetheless, antibodies against non-CSP proteins from CPS-immunized volunteers were shown to block Pf parasite development in hepatocytes *in vitro* and *in vivo* (152). In fact, several other antigens besides CSP currently constitute promising vaccine candidates, including thrombospondin-related adhesion protein (TRAP) (153) and cell-traversal protein for *Plasmodium* ookinetes and sporozoites (CelTOS) (154). Excitingly, Pb-based WSp vaccination (126, 130) offers a platform that may be used as a backbone for insertion of multiple genes, to elicit tailored humoral immune responses that enhance and/or synergize with those induced against CSP. This strategy may trigger humoral immunity against multiple Pf strains, as well as against other *Plasmodium* species, such as Pv, to overcome current limitations of the existing WSp vaccination approaches.

Cellular immunity

Cellular immunity is critical for the protection elicited by RAS immunization in rodent and non-human primate models, and most pre-clinical data indicate a central role for $CD8^+$ T cells and interferon-γ (IFN-γ) in protection by this vaccination approach (46, 155, 156). In addition, other cell populations, including $CD4^+$ T cells, γδ T cells and natural killer (NK) cells, can also play a role in mediating protection [reviewed in (135, 137)].

$CD8^+$ T cells recognize pathogen-derived peptides bound to MHC class I molecules on the surface of antigen presenting cells or infected cells, and can eliminate liver stage *Plasmodium* parasites either directly, such as through perforin-mediated lysis (157), or indirectly, through cytokine (e.g. IFN-γ, TNF-α) production [reviewed in (135, 137)]. Sterile immunity induced by RAS vaccination in mice is abolished upon depletion of $CD8^+$ T cells or IFN-γ (155, 158), and IFN-γ directly impairs *Plasmodium* development in human hepatocytes in culture (159). In general, immunizations of humans by RAS (48, 65, 69) and CPS (92, 115) do not consistently nor robustly induce Pf-specific $CD8^+$ T cells in the blood of vaccinated subjects. Nevertheless, some studies reported the detection and dose-dependent increase in the frequency of those cells after vaccination by RAS (47), GAP (91, 92) or PbVac (130), although this did not correlate with protection or patency. Moreover, increased granzyme B expression by $CD8^+$ T cells was associated with protection following CPS vaccination (111). The overall suboptimal detection of parasite-specific $CD8^+$ T cells in the blood is likely associated with their predominant tissue residency. Indeed, Pf-specific IFN-γ-producing $CD8^+$ T cells produced upon RAS immunization of non-human primates are mainly localized to the liver, where they can be present at up to 100 times higher frequencies than in the blood (46, 48). These studies have highlighted the importance of vaccine administration route (iv>>id or sc), dose and schedule on the formation of tissue-resident $CD8^+$ T cell responses, which likely extends to the other WSp immunization strategies.

$CD4^+$ T cells can have a multiplicity of roles in mediating protective immunity in malaria, including aiding the survival and differentiation of $CD8^+$ T cells (160, 161), the development of efficient B cell responses (162, 163), or by acting directly through pro-inflammatory cytokine (eg. IFN-γ, TNF-α, IL-2)

production (reviewed in (135, 137, 164). Many studies have reported the presence of Pf-specific $CD4^+$ T cells, and particularly of polyfunctional memory Th1 cells (producing IFN-γ, TNF-α and/or IL-2), in the blood of volunteers immunized with RAS (47, 48, 54, 57, 65), CPS (92, 115), GAP (91, 92) and PbVac (130), but they were only associated with protective immunity following CPS vaccination (108, 109, 115). In addition, the increased expression of the degranulation marker CD107a on $CD4^+$ T cells has also been associated with protection against homologous (111) but not heterologous (113) challenge following CPS immunization. Importantly, Pf-specific polyfunctional memory $CD4^+$ T cell responses were low to undetectable in PfSPZ-vaccinated infants in Tanzania (65) and Western Kenya (69), raising concerns regarding the implementation of the PfSPZ vaccination strategy in this immunologically immature population.

γδ T cells, which represent 2-5% of total T cells in humans, are unconventional T cells that are not restricted by classical MHC-mediated antigen presentation. The major subset of γδ T cells in the blood, $Vγ9^+Vδ2^+$, recognizes stress or pathogen-related phosphoantigens that specifically and robustly activate them to proliferate, secrete cytokines (such as IFN-γ and TNF-α), and display cytotoxic behavior [reviewed in (165, 166)]. Accordingly, human γδ T cells are innate responders to *Plasmodium* parasites *in vitro* (167) and are able to directly kill merozoites (168). Data from animal studies have provided evidence that γδ T cells can inhibit *Plasmodium* hepatic development (169), and are necessary for the generation of protective $CD8^+$ T cell responses and for sterile protection following RAS vaccination (170), among other functions (reviewed in (135, 137, 171). In RAS vaccine clinical trials, γδ T cells expanded upon immunization of malaria-naïve and pre-exposed volunteers (47, 48, 54, 170), and the frequency of $Vδ2^+$ γδ T cells was found to be predictive of protection, both at baseline and prior to CHMI (48). $Vδ2^+$ γδ T cell expansion was further reported in some studies following CPS (115, 118) and PbVac (130) immunization. Hence, γδ T cells, and specifically the $Vδ2^+$ subset, represent a potential correlate of protection that warrants further exploration (136).

NK and NKT cells are important innate and innate-like effector cells that are abundant in the liver, and have been implicated in cell-mediated immunity to liver stage *Plasmodium* infection [reviewed in (172, 173)]. Although not extensively analyzed in the context of WSp vaccination, NK and NKT cells were shown to contribute to the increase in IFNγ production by lymphocytes responding to Pf following CHMI (174), and NK cells were found to upregulate activation and proliferation markers during CPS immunization (94). Importantly, an increase in NK and NKT cell frequencies was found following PbVac immunization, which, for the latter population, correlated the prepatent period of vaccinated individuals (130). Future vaccine development studies should further investigate in depth these and other innate and innate-like populations, as well as related pathways, in light of recent data on their involvement in immune signatures that potentially correlate with protection (175).

Final remarks: Lessons from the past, challenges for the future

Looking back to the history of research on WSp vaccines against malaria, it is clear that much has been achieved, particularly during this last decade. While until the early 2010's progress was relatively slow, and only a handful of clinical trials had been performed, this number has risen dramatically since then. During this period, Sanaria Inc.'s achievements have revolutionized the field, transforming an attractive, yet unpractical, immunization strategy into a family of injectable products suitable for vaccination and CHMI. The PfSPZ Vaccine alone has now been administered to more than 1700 volunteers in over 20 clinical trials, PfSPZ-CVac has been assessed in a large array of clinical studies and immunization regimens, and PfSPZ Challenge has been used for CHMI of several dozen subjects (31). It was also during this period that Pf GAP vaccination was first evaluated in the clinic, as was a novel WSp immunization strategy based on the use of genetically modified Pb parasites. We presently understand the elicitation of immunity by WSp vaccines better than ever before, and major technical hurdles that once seemed unsurmountable have now been overcome. And yet, the road travelled so far was not without pitfalls, and many important challenges still lay on the path ahead. Despite progress in the automation of mosquito dissections (176), an effective system for *in vitro* production of Pf sporozoites remains unavailable. Nevertheless, Sanaria, Inc. have publicly announced that major achievements have been made in this regard, and it is very likely that these findings will be published in the near future. Although much has been learned from the immunological analyses of clinical samples from participants in multiple trials (145), immune correlates of malaria vaccine efficacy remain largely undefined (136). On the other hand, the disappointing results of the only clinical trial of a WSp vaccine in infants raises justified concerns about the effectiveness of this immunization approach in that age group (69). Moreover, the higher protective efficacy in malaria naïve volunteers when compared to malaria pre-exposed volunteers (47, 52, 56, 59), as well as the variable levels of protection afforded by different regimens of PfSPZ-CVac vaccination (115, 118, 120, 121), suggest that additional optimization of immunization regimens with these vaccines is

required. Finally, the relatively low clinical efficacy of the PfSPZ-GA1 and PbVac candidates (92, 130) demands additional development of these promising, yet still suboptimal, vaccination approaches. Several of these issues will more than likely be addressed in future clinical trials, either planned or ongoing. According to clinicaltrials.gov, there are currently several active, recruiting, or not yet recruiting trials of WSp malaria vaccines, including studies aimed at assessing PfSPZ Vaccine efficacy in Malian women of childbearing age (NCT03989102) and in Malian children (NCT04940130), as well as against heterologous CHMI in malaria-naïve USA adults (NCT04966871), and a head-to-head comparison between an early-arresting [GA1: P*fb9*$^{-}$/*slarp*$^{-}$ (84)] and a late-arresting [GA2: P*fmei2*$^{-}$ (90)] GAP is currently ongoing at the Leiden University Medical Center (NCT04577066). Plans are also being made for the clinical evaluation of the safety and protective efficacy of parentally injected PbVac. Moving forward, these and other studies will continue to compound our accumulated knowledge on human immunization with WSp malaria vaccines, bringing their use for preventing disease and, ultimately, contributing to its elimination, ever closer to reality.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

MP acknowledges the "la Caixa" Foundation for Grant HR21-848, the GSK OpenLab Foundation for grant TC269, and Fundação para a Ciência e Tecnologia (FCT) for grant PTDC-SAU-INF-29550-2017. HN-C acknowledges funding from FCT (reference DL57/2016/CP1451/CT0011).

Acknowledgments

Helena Pinheiro is gratefully acknowledged for designing Figure 1 of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Perspective
PUBLISHED 20 September 2022
DOI 10.3389/fimmu.2022.1003452

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 26 July 2022
ACCEPTED 22 August 2022
PUBLISHED 20 September 2022

CITATION
Owalla TJ, Hergott DEB, Seilie AM, Staubus W, Chavtur C, Chang M, Kublin JG, Egwang TG and Murphy SC (2022) Rethinking detection of pre-existing and intervening *Plasmodium* infections in malaria clinical trials. *Front. Immunol.* 13:1003452. doi: 10.3389/fimmu.2022.1003452

Rethinking detection of pre-existing and intervening *Plasmodium* infections in malaria clinical trials

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Pre-existing and intervening low-density *Plasmodium* infections complicate the conduct of malaria clinical trials. These infections confound infection detection endpoints, and their immunological effects may detract from intended vaccine-induced immune responses. Historically, these infections were often unrecognized since infrequent and often analytically insensitive parasitological testing was performed before and during trials. Molecular diagnostics now permits their detection, but investigators must weigh the cost, complexity, and personnel demands on the study and the laboratory when scheduling such tests. This paper discusses the effect of pre-existing and intervening, low-density *Plasmodium* infections on malaria vaccine trial endpoints and the current methods employed for their infection detection. We review detection techniques, that until recently, provided a dearth of cost-effective strategies for detecting low density infections. A recently deployed, field-tested, simple, and cost-effective molecular diagnostic strategy for detecting pre-existing and intervening *Plasmodium* infections from dried blood spots (DBS) in malaria-endemic settings is discussed to inform new clinical trial designs. Strategies that combine sensitive molecular diagnostic techniques with convenient DBS collections and cost-effective pooling strategies may enable more thorough and informative infection monitoring in upcoming malaria clinical trials and epidemiological studies.

KEYWORDS

***Plasmodium falciparum*, pre-existing infection, intervening infection, clinical trial, at-home DBS, 18S rRNA**

Introduction

Clinical trials are critical for evaluating candidate malaria vaccines and drugs. Such trials are routinely conducted in malaria-endemic sites as field efficacy trials (1–5) and in both endemic and non-endemic sites as controlled human malaria infection (CHMI) studies (6–9). In all cases, it is generally accepted that the *Plasmodium* infection status of the participants is established at the time of trial enrollment. In malaria-endemic regions, participants may have been recently exposed to *Plasmodium* parasites, so it is possible that participants may be actively infected at the time of trial eligibility and enrollment assessments. Consequently, many studies are designed to start with anti-malarial drug treatment of some or all participants to eliminate any pre-existing *Plasmodium* parasites at the outset of the trial (4, 6, 7, 10, 11). In CHMI studies in non-endemic regions (8, 9) and field efficacy trials involving children 5-17 months in endemic settings (1–3, 5, 12), pre-treatment is not usually considered because participants are usually assumed not to have pre-existing *Plasmodium* infections. However, at least one pre-existing, low-density *Plasmodium falciparum* infection was encountered during screening and eligibility procedures at a U.S.-based non-endemic CHMI study site (S. Murphy, J Kublin, pers. comm.), which highlights the need for pre-enrollment testing worldwide. Pre-enrollment testing is a requirement for any non-endemic CHMI study intending to use a recently qualified *Plasmodium* 18S rRNA biomarker in lieu of thick blood smears (TBS) for detecting infections in such studies (13). Pre-enrollment testing has also been used in one CHMI study in an endemic region (14). On the other hand, following vaccination, most field studies in malaria endemic settings rely on passive case detection for endpoint efficacy assessments such as time to first infection or to first episode of clinical malaria (1, 5). Studies employing active case detection through weekly or monthly visits usually only collect a thick blood smear (TBS) if a participant reports a temperature of ≥37·5°C or history of fever and other malaria-related symptoms within the last 24 hours (2–4). Examples of the current field practices employed by investigators during the pre-enrollment, follow-up sampling and efficacy endpoint assessment are as shown in Table 1. It is clear that reliance on symptoms as well as weekly or monthly sampling and low sensitivity techniques may miss out on pre-existing and emerging *de novo* low density infections, which may confound vaccination efforts and ultimately affect efficacy estimates.

Abbreviations: DBS, Dried blood spot; TBS, Thick blood smear; PCR; polymerase chain reaction; qRT-PCR, Quantitative real-time polymerase chain reaction; NAAT; nucleic acid amplification test; ACD; active case detection; PCD; passive case detection.

What are the consequences of low-density pre-existing and intervening infections on measurement of parasitological efficacy endpoints in vaccine studies?

The presence or absence of *Plasmodium* parasites or of a parasite-derived biomarker are often used in studies designed to assess time to first infection or time to first clinical episode as efficacy endpoints. Such assessments depend on accurate identification of pre-existing *Plasmodium* infections at enrollment and during follow-up. However, definitive determination of the infected *vs.* uninfected baseline status of a participant can be difficult because a significant proportion of *Plasmodium* infections in endemic regions exist at low densities (15–17), which are often below the limit of detection (LoD) of standard field diagnostic tools such as TBS and rapid diagnostic tests (RDTs) (18, 19). Even in studies that use molecular tests, low-density infections may be missed because of the highly dynamic nature of the parasite – densities may be too low to be detected at the time of sampling. The inability to rule out pre-existing, low-density infections prior to vaccination and to detect their emergence during vaccination or in the subsequent efficacy assessment period may potentially confound trial outcomes and endpoint assessments. For example, undetected low-density infections could progress to higher density, detectable infections soon after enrollment – such infections would not be expected to be abrogated by vaccination with pre-erythrocytic vaccines and yet such pre-existing but undetectable infections could end up being counted as new infections in the study data, which could falsely reduce the calculated efficacy of a candidate vaccine product. Similarly, the inability to detect the emergence of low-density *de novo*/intervening infections after vaccination will extend the parasite detection time and has the potential to falsely amplify the calculated efficacy of the vaccine. Despite the likely influence of pre-existing and intervening low-density infections on vaccine efficacy, the significance and magnitude of such impacts is still poorly understood. For some vaccines, it is likely that vaccine efficacy is reduced when vaccinations are given concurrent with erythrocyte stage parasitemia, which was shown, for example, to reduce sporozoite-based vaccine efficacy in a CHMI model (9). However, there could be circumstances where the timing of an infection potentially enhance efficacy. For example, a study of the ChAd63/MVA ME-TRAP vaccine in Kenya resulted in 67% efficacy against field-acquired infections (20), which was higher than that observed in Senegal (21). *Post-hoc* analysis showed that the rate of *Plasmodium* infections during the vaccination period were much higher at the Kenyan sites than at the Senegalese sites (21), which could have modulated either anti-erythrocyte or liver-stage immunity. The complex effects of low *vs.* high parasitemias and other parasite, host, and environmental factors on the immune system must be evaluated in the future to develop and safeguard malaria vaccines.

TABLE 1 Examples of clinical trial strategies for pre-vaccination treatment, follow-up sampling, and efficacy endpoint assessments.

Vaccine candidate	Clinical trial design	Pre-vaccination treatment? (if any)	Infection detection endpoint?	Follow-up during efficacy and infection detection?	Reference
RTS,S	Field trial at 11 African sites in children	None (enrolled infants and children 5-17 months)	Clinical malaria; severe malaria (TBS)	PCD for >18 months	(1)
R21	Field trial in Burkina Faso in children 5-17 months	None. Participants tested for malaria if fever ≥37·5°C.	Clinical malaria (TBS)	ACD monthly for 6 months plus PCD. TBS obtained if temperature ≥37·5°C or history of fever within the last 24 h.	(2)
SPf66	Field trial in The Gambia in children 5-11 months	Antimalarial treatment before first and third vaccination (SP)	Clinical malaria (TBS)	ACD twice weekly for 4.5 months plus PCD. TBS obtained if temperature ≥37·5°C or history of fever within the last 24 h.	(3)
DNA/MVA ME-TRAP	Field trial in The Gambia in children and adults	Antimalarial treatment prior to 3rd dose of vaccination (SP)	Infection by TBS	ACD and weekly TBS for 11 weeks.	(4)
ChAd63 MVA ME-TRAP	Field trial in Burkina Faso in 5-17 months	None (enrolled infants and children 5-17 months)	First clinical malaria episode (RDT & TBS)	PCD and TBS if temperature ≥37·5°C or history of fever within the last 24 h	(5)
GMZ2	CHMI in adults in an endemic region (Gabon)	Antimalarial treatment prior to vaccination (clindamycin)	Infection by TBS & qRT-PCR	ACD for 6-35 days	(7)
PfSPZ	Phase 2 field trial in Kenya in children	None (enrolled children 5-12 months)	Clinical malaria and infection (TBS)	ACD (RDT) and PCD (TBS/qPCR) every two weeks for 12 months	(12)
PfSPZ CHMI	CHMI in adults in an endemic region (Kenya)	None prior to CHMI; tested for existing infection	Clinical malaria & qPCR (treated at ≥500 parasites/μL)	ACD (blood drawn twice per day from days 8-15 and once from days 16-22 post-CHMI)	(14)

ACD, active case detection; PCD, passive case detection; SP, sulfadoxine-pyrimethamine.

What are the consequences of pre-existing infections on measurement of immunological efficacy endpoints in vaccine studies?

Many studies have found that malaria vaccine efficacy is reduced in studies in endemic regions compared to efficacy against CHMI in non-endemic sites [discussed in (22)]. An extensive review of the contributing immunological, parasitological, vectorial, and environmental factors is beyond the scope of this paper. Instead, the following section highlights several recent clinical trial outcomes that demonstrate the consequences of pre-existing infections on vaccine study outcomes. First, a recent CHMI study at a non-endemic U.S. site showed that the administration of the second and third doses of *P. falciparum* sporozoite-based vaccine at 7-day intervals, concurrent with the emergence of low-density blood stage *Plasmodium* parasites (<20 estimated parasites/μL; TBS-negative) completely eliminated the otherwise high efficacy achieved when blood stage parasites were absent during vaccination with a two-fold higher dose of the same vaccine given at 5-day intervals (9). Second, field clinical trials of the recently WHO-approved RTS,S vaccine and other candidates reveal that immunity induced by candidate malaria vaccines is dependent on specific antibodies and requires an active response involving B cells and CD4$^+$ T cells (1, 23, 24). However, active TBS-positive *Plasmodium* infections induced altered phenotypes and functionalities of dendritic cells (25), B cells (26) and T cells (27), causing a disruption in host immune responses to antigenic epitopes. Furthermore, natural exposure to persistent *P. falciparum* infections is known to increase the frequency of atypical memory B cell and CD4$^+$ T cells expressing phenotypic markers of exhaustion (28). Therefore, pre-existing infections may alter immune reactivity by down-regulating vaccine-induced immune responses, providing a probable reason as to why promising results of experimental malaria vaccine candidates in non-endemic regions have often not been replicated in malaria-endemic areas (29). Whether low density infections are as impactful as higher density infections is currently unknown. Consequently, detection of pre-existing infections is imperative to control the confounding effects of such infections and to facilitate reliable and consistent interpretation of clinical trial results in different cohorts at different clinical sites under different transmission pressures.

Low density *Plasmodium* infections – a frequent complicating factor worldwide

To our knowledge, there are no widely-accepted, standardized approaches for detection of pre-existing *Plasmodium* infections in malaria clinical trials in endemic regions. Nonetheless, the emerging literature suggests that

these infections are common and therefore overlooked. A recent DBS study in a hyperendemic region of Uganda enrolled asymptomatic, RDT-negative persons to better understand the natural history of asymptomatic low-density infections (17). Amongst adults and children, 58% (76/130) of RDT-negative individuals had *Plasmodium* 18S rRNA detectable by quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) at some point during the 28-day collection period. This study is notable because DBS samples were self-collected using daily finger prick sampling to observe the dynamic behavior of these asymptomatic low-density infections. This study is discussed in a later section as well to provide a roadmap for improving infection detection in future endemic site clinical trials. Prevalent, dynamic asymptomatic low-density *Plasmodium* infections have also been reported by other investigators in different regions of malaria endemicity (15, 16, 30). In Mozambique, analysis of parasite densities collected at seven time points over 28 days in a cohort of asymptomatic men revealed that 81% were cumulatively parasite PCR positive by day 28 and that parasite densities continued to vary in individuals over that 28-day period (16). Similarly, a study in a low transmission setting in Vietnam also showed 32% of samples were PCR positive and that parasite densities in asymptomatic carriers oscillated over time (30). These low-density parasitemias would be considered to be pre-existing infections in malaria vaccine clinical trials. However, if testing is not planned throughout the study or if the testing modality is insufficiently sensitive, then such infections would go undetected and the consequence to the efficacy estimates of the experimental vaccine would be largely unknown.

Diagnostic methods for detecting low-density infections in malaria clinical trials

Since 2010, WHO advised that all diagnoses of malaria febrile illnesses be accompanied by a confirmatory parasitological test (31), which could be microscopy, RDTs, or molecular testing. Such methods may detect low-density *Plasmodium* infections, albeit with different degrees of success. TBS, RDTs, and molecular tests can be conducted on capillary (fingerstick) blood or peripheral whole blood, which can be collected and stored as liquid blood or preserved as DBS. In clinical trials, Phase 1-2 studies typically schedule more frequent testing and use more analytically sensitive tests compared to Phase 3 studies. Test selection considerations also include the study population (infants, children, adults), the clinical and laboratory capabilities of the site, and assay costs. It should be noted that some malaria clinical trials have used clinical signs and symptoms of malaria as an eligibility criterion for enrollment (8) or as a trigger for diagnostic testing (2), but such approaches completely ignore the larger pool of pre-existing, low-density infections and *de novo* emerging intervening infections.

TBS microscopy

Microscopic examination of TBS remains a standard method for the diagnosis of *Plasmodium* infections and for estimating parasite densities in most field studies. Briefly, preparation of a TBS involves spreading a drop of blood obtained *via* finger stick or venipuncture onto a clean, dry microscope slide. The TBS is allowed to dry, then erythrocytes are lysed and nuclei are stained with Giemsa stain for 10-30 minutes depending on the specific method. Parasite detection is performed under an oil-immersion light microscope at a total magnification of ~1000-fold. TBS microscopy allows for definitive identification of *Plasmodium* species by well-trained microscopists. The advantages and disadvantages of TBS have been extensively reviewed (32, 33). For our purposes, we will focus on three key factors: quality, scalability, and proximity. First, high-quality microscopy (like all high-quality laboratory testing) requires ongoing proficiency testing and quality control, which can be difficult since microscopy is more operator-dependent than the other testing methods. Even with high quality microscopy, the field-use LoD of TBS is relatively high at ~50-100 parasites/μL (34, 35) – this LoD would miss many asymptomatic and intervening low-density infections. The inability to detect low-density infections means that infected persons may be erroneously enrolled in studies or the subsequent emergence of such an infection can be delayed or missed during or after vaccination. Second, TBS microscopy is laborious and does not scale easily with increasing numbers of clinical trial samples. TBS may be required at frequent defined study time points, but also must be available on-demand for clinically-significant cases. The turnaround time for a small number of TBS is such that clinically-actionable data can be obtained within hours, but as the number of slides increases, it becomes harder to provide timely reporting. CHMI studies do not enroll extremely large numbers of participants, but daily TBS is at least usually required during periods when patent parasitemia is anticipated (8, 23, 24, 36). In contrast, field efficacy studies have less frequent sampling but usually enroll larger cohorts of participants (2, 7, 8, 10, 23, 37). Thus, in both studies, delivery of timely, high quality TBS results can be difficult. Nonetheless, TBS can be performed at or near the clinic. While proximity to the clinical site is critical for symptomatic case management, such proximity may be less important when monitoring and following up on low-density infections.

RDTs

RDTs are lateral flow immunochromatographic tests that detect *Plasmodium* antigens in whole blood (usually histidine rich protein-2 (HRP2) for *P. falciparum* and lactate dehydrogenase (LDH) for all species). They have the advantages of ease of use, rapid turnaround time suitable for point-of-care or near point-of-care use, and therefore, deployability. However, the LoD for most marketed

RDTs is ~200 parasites/μL (38), which would miss many pre-existing and intervening low-density infections. Newer 'ultrasensitive' RDTs (uRDT) have LoDs about 10-fold better than standard RDTs (39, 40), but these are not yet widely available. In addition to the high LoD, *P. falciparum* parasites with deletions in the HRP2-coding gene can lead to false negative RDT results (41, 42), which limits their use. RDTs may also remain positive following parasite clearance due to persistent antigenemia even in appropriately treated persons such that RDTs are not considered to be a test of cure. Finally, RDTs do not provide any quantitative assessment of parasite density, which is useful for modeling parasite growth and estimating the impact of partially protective vaccines through measures such as estimated liver burden. RDTs have not been widely used as an efficacy endpoint in malaria vaccine clinical trials, though some groups are beginning to assess RDT diagnostic performance against TBS and qPCR in some CHMI trials (43).

Nucleic acid amplification tests

Over the past forty years, a wide variety of nucleic acid amplification tests (NAATs) have been developed for many infectious diseases including malaria. In simplest terms, NAATs generally involve a nucleic acid extraction step followed by an amplification/detection step using oligonucleotide-specific reagents. Methods include PCR, qRT-PCR, and nucleic acid-based sequence amplification, which have been reviewed previously (32). Methods vary with respect to the amount of blood sampled, the amount of extracted nucleic acid carried into the amplification step, the strategy for detection, the target gene(s) or RNA sequence(s), the choice of oligonucleotide-specific reagents, and the scale of testing. For the purposes of detecting low-density infections in clinical trials, we recommend that NAATs should only be considered for use if they can reliably detect infections at densities <1 parasite/μL. Some NAATs achieve even more sensitive LoDs in the 0.001-0.02 parasite/μL range. Such assays generally sample 0.05-1 mL of blood, a much greater volume than can be examined by TBS. Sensitive NAATs can detect blood stage infections 1-4 days before TBS [reviewed in (13)]. NAATs are also less operator dependent than TBS and more scalable than TBS or RDTs for monitoring low density infections. Because of their superiority over TBS and RDTs, a variety of NAATs have been used in CHMI trials at both non-endemic sites (8, 9, 36, 44) and endemic sites (14, 23). To provide proficiency testing across different malaria NAAT platforms, the World Health Organization has established a formal external quality assurance scheme for malaria NAAT laboratories (45, 46). One drawback to NAATs is the requirement for sophisticated instrumentation and staff training, which often leads to NAATs being performed at only centralized/reference laboratories. A potential technical drawback is that some NAATs can also detect gametocytes and produce positive results at low densities that cannot be adjudicated by microscopy. Detection of gametocytes may lead to exclusion at enrollment, and detection of potentially pre-existing gametocytes following vaccination may confound parasitological efficacy endpoints, especially if more convenient, less sensitive testing were used at enrollment. The influence of gametocytes on molecular diagnostic tests used for malaria vaccine efficacy endpoints requires additional study and consideration as these tests become more widely adopted. As the field advances, these considerations will need to be balanced to implement NAAT strategies that speed turnaround times, simplify clinical site scheduling and sampling, provide clear and actionable data, and save on human resources and financial costs without sacrificing quality.

A strategy for more frequent, cost-effective testing to avoid clinical trial blind-spots

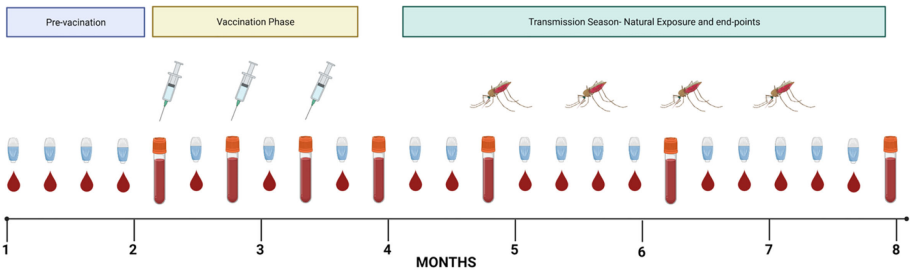
DBS collection is a convenient, minimally invasive blood collection technique that does not require a clinic or phlebotomist. DBS remain stable over a wide range of temperature and storage conditions, and thus allow retrospective analyses without sacrificing sample integrity. A recent meta-analysis determined that DBS were non-inferior to venous blood samples for qualitative detection of *Plasmodium* parasites across a variety of settings (47). However, DBS continue to be mainly used for sample collection in clinic and field settings by trained healthcare professionals. Nevertheless, DBS have been used successfully for self-collection of samples for a variety disease conditions such as HIV (48, 49), hepatitis (50), and diabetes (51).

Recently, an alternative and cost-effective sampling approach based on at-home DBS collection combined with pooled *Plasmodium* 18S rRNA qRT-PCR was determined to be feasible, well tolerated, cost-effective, analytically sensitive, and convenient for detecting low density infections in asymptomatic adults and children in an endemic area (17). This feasibility study of daily at-home DBS collection was conducted in 130 (100 adults and 30 children) community members in a rural, malaria-endemic setting in Uganda (17). In this study, participants were minimally trained in DBS collection by study staff at enrollment and were supplied with DBS collection packages for at-home use for the subsequent six days. DBS were returned by participants to the clinic on the seventh day and retraining was conducted if necessary. Thereafter, each week, participants were provided with all materials to collect daily at-home DBS until the following week and this was repeated until day 28. Compliance with at-home DBS collection was extremely high, with 85% of participants collecting all DBS over the 28-day period. Only five (4%) participants withdrew from the study early due to pain or inconvenience of the collection procedures - details about the study are recently published (17). Accuracy of the at-home collected DBS as a parasite detection tool for low-density infections was also assessed using a recently adapted pooled qRT-PCR strategy (52). The method involved

conducting initial DBS runs using within-participant pools of up to 10 samples per pool (equal to 10 daily DBS collections per pool). If the pool was negative, all samples were reported as negative. If the pool was positive, samples were deconvoluted and re-run individually. DBS pooling reduced costs associated with testing individual qRT-PCR negative samples, and qRT-PCR provided highly sensitive detection of parasite 18S rRNA biomarker. The feasibility of at-home, self-collected DBS in rural settings could improve the ability to conduct surveillance studies and trial follow-up. Additional data from this study will soon be forthcoming to share the prevalence and complexity of the asymptomatic infections seen in these participants (D. Hergott, S. Murphy, pers. comm.).

Malaria vaccine clinical trials in endemic regions usually involve periodic follow-up for months during and after vaccination. Study designs are intended to be long enough to capture a sufficient number of infections in the community to render a verdict regarding vaccine efficacy between two or more groups, and sampling is intended to be frequent enough so as to not miss an infection that could come and go between visits. However, participants are usually not sampled more than once a week and sometimes only once a month depending on the number of participants and the study (Table 1). Furthermore, in many studies, there is little or no infection detection monitoring during the vaccination period. These untested periods leave blind-spots in the study data that could potentially help to explain trial outcomes. Low-cost, at-home DBS collections with pooled qRT-PCR testing provide a way to comprehensively assess infection status before and during a study to avoid such blind-spots (Figure 1). This strategy reduces the number of clinic visits per participant, and pooled sample analysis also reduces the number of qRT-PCR runs per participant, thereby reducing cost. DBS can be collected before and throughout a study and delivered to the clinic site on a convenient basis once a week. The frequency of DBS collection, delivery, and testing for a given trial site would be informed by existing knowledge of the site's seasonality and intensity of transmission. Home-collected samples could even potentially be mailed to a coordinating laboratory or picked up by village health workers or other Ministry of Health networks with access to the community. This approach would save time, human resources, and money associated with large scale and frequent sample collection and analysis. While this study was conducted using qRT-PCR, it is possible that other NAAT methods could also be similarly used with this overall strategy, provided that the assay LoD is sufficiently sensitive to detect pre-existing and intervening low-density infections at the pooled sample step.

The DBS testing described herein would not need to be done immediately after collection because this approach would be restricted to monitoring of low-density asymptomatic persons. Clinical trial sites would need to continue to provide TBS or RDTs to manage acutely-ill participants and initiate treatment as needed. From an ethical perspective, there is no current WHO mandate to treat asymptomatically-low density infected persons despite the known frequency of this type of infection throughout malaria-endemic parts of the world. If there was a long interval between collection and testing, the results may not be actionable for



Per participant samples	Traditional Design	Added DBS spots
Samples collected	7	25
Minimum RT-PCR runs	1	3
Maximum RT-PCR runs	7	25

FIGURE 1
Proposed testing strategy for more frequent DBS collections with pooled qRT-PCR. In this theoretical vaccine clinical trial scenario, enrollment and vaccination take place over the first four months of the study followed by a four-month efficacy phase during the transmission season as shown. In addition to the typical whole venous blood collections (test tube icons), more comprehensive testing can be achieved by adding repeated DBS collections during the intervening time periods (small drop of blood icons). These DBS collections could be at-home or in the clinic as needed. The number of collections could be adjusted to a daily frequency or to less frequent collection as needed. The inset table shows the number of samples collected if only venous blood was specified ('Traditional Design') or if venous blood and DBS were collected ('Added DBS spots') and then calculates the minimum and maximum number of qRT-PCR tests that would need to be tested to determine each participant's infection status; this calculation assumes a first qRT-PCR pool size of n = 10. The minimum number of runs would occur if all three pools were negative, whereas the maximum number of runs would occur if all three pools were positive and required deconvolution.

an individual participant. If the testing was conducted with a shorter turnaround time, it may be possible to relay actionable information back to clinic sites to inform treatment of participants. In addition to providing clear and comprehensive vaccine efficacy data, this infection status data could also help to better understand local prevalence and transmission characteristics. This sort of testing strategy could also be employed in large scale surveillance and longitudinal cohort studies over an even wider range.

Conclusions

Malaria clinical trials that incorporate at-home DBS sample collection coupled with pooled qRT-PCR sample analysis may be better able to conveniently and cost-effectively detect pre-existing and intervening low-density *Plasmodium* infections in study participants. This rich information could provide valuable insights that will help us better understand why vaccines are efficacious in some participants and settings but not others, which could accelerate the development of new and improved malaria vaccines for the world.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics Statement

The perspective presented here is based in part on our recently completed study that was approved by the National HIV/AIDS Research Committee of the Uganda National Council for Science and Technology (UNCST) (Approval #: ARC 228) as well as the University of Washington Institutional Review Board (STUDY00009434). No potentially identifiable human data is presented in this article.

Author contributions

TO, SM, JK, and TE conceived the idea. TO drafted the paper. DH, AS, WS, CC, and MC reviewed the first draft. All authors contributed to the article and approved the submitted version.

Funding

OPP1212966/INV-009313 (SM), NIH R21AI146763 (SM).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 25 October 2022
DOI 10.3389/fimmu.2022.1006716

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 29 July 2022
ACCEPTED 30 September 2022
PUBLISHED 25 October 2022

CITATION
KC N, Church LWP, Riyahi P, Chakravarty S, Seder RA, Epstein JE, Lyke KE, Mordmüller B, Kremsner PG, Sissoko MS, Healy S, Duffy PE, Jongo SA, Nchama VUNN, Abdulla S, Mpina M, Sirima SB, Laurens MB, Steinhardt LC, Oneko M, Li M, Murshedkar T, Billingsley PF, Sim BKL, Richie TL and Hoffman SL (2022) Increased levels of anti-PfCSP antibodies in post-pubertal females versus males immunized with PfSPZ Vaccine does not translate into increased protective efficacy. *Front. Immunol.* 13:1006716. doi: 10.3389/fimmu.2022.1006716

Increased levels of anti-PfCSP antibodies in post-pubertal females versus males immunized with PfSPZ Vaccine does not translate into increased protective efficacy

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Background: While prior research has shown differences in the risk of malaria infection and sickness between males and females, little is known about sex differences in vaccine-induced immunity to malaria. Identifying such differences could elucidate important aspects of malaria biology and facilitate development of improved approaches to malaria vaccination.

Methods: Using a standardized enzyme-linked immunosorbent assay, IgG antibodies to the major surface protein on *Plasmodium falciparum* (Pf)

sporozoites (SPZ), the Pf circumsporozoite protein (PfCSP), were measured before and two weeks after administration of a PfSPZ-based malaria vaccine (PfSPZ Vaccine) to 5-month to 61-year-olds in 11 clinical trials in Germany, the US and five countries in Africa, to determine if there were differences in vaccine elicited antibody response between males and females and if these differences were associated with differential protection against naturally transmitted Pf malaria (Africa) or controlled human malaria infection (Germany, the US and Africa).

Results: Females ≥ 11 years of age made significantly higher levels of antibodies to PfCSP than did males in most trials, while there was no indication of such differences in infants or children. Although adult females had higher levels of antibodies, there was no evidence of improved protection compared to males. In 2 of the 7 trials with sufficient data, protected males had significantly higher levels of antibodies than unprotected males, and in 3 other trials protected females had higher levels of antibodies than did unprotected females.

Conclusion: Immunization with PfSPZ Vaccine induced higher levels of antibodies in post-pubertal females but showed equivalent protection in males and females. We conclude that the increased antibody levels in post-pubertal females did not contribute substantially to improved protection. We hypothesize that while antibodies to PfCSP (and PfSPZ) may potentially contribute directly to protection, they primarily correlate with other, potentially protective immune mechanisms, such as antibody dependent and antibody independent cellular responses in the liver.

KEYWORDS

PfSPZ Vaccine, malaria vaccine, *Plasmodium falciparum*, PfCSP, antibodies, humoral immunity, sex, gender

Introduction

In 2020, malaria caused 241 million clinical episodes and 627,000 deaths (1), the highest number of deaths since 2012. The worsening situation has occurred despite an annual investment of >$3 billion in intensive control measures, indicating a saturation of capacity to achieve further impact (2, 3). The WHO estimated that there were more deaths in Africa from malaria than from COVID-19 in 2020 (4), resulting in 40-fold more disability life years (DALYS) lost from malaria in Africa in 2020 than from COVID-19 from February 2020 to March 2021 (5).

Despite the global malaria control efforts, progress has slowed in recent years and there is an urgent need for highly effective malaria vaccines. A malaria vaccine, RTS,S/AS01, has been recently recommended for implementation in young African children by the World Health Organization based on the results of a pilot implementation program in Ghana, Malawi, and Kenya in 920,000 infants in which the vaccine reduced malaria hospitalizations by 21% and severe malaria by 30% (6). Our long-term goal is the development of a much more effective malaria vaccine that can be used to eliminate malaria because it prevents infection with Pf. We use whole *Plasmodium falciparum* (Pf) sporozoites (SPZ), the entire parasite, as the immunogen in our vaccines (7).

Our first-generation malaria vaccine is Sanaria® PfSPZ Vaccine, which is made up of radiation-attenuated, aseptic, purified, cryopreserved PfSPZ. It has been tested in 21 clinical trials in the United States (US), Europe, and six African countries (8–29). A meta-analysis of 13 double-blind, placebo-controlled trials of PfSPZ Vaccine, 11 of which were conducted in Africa, revealed no significant difference in adverse event patterns between vaccinees and controls who received normal saline (NS) (16–21, 25–29). Vaccine efficacy (VE) reached 100 percent against homologous (same Pf strain as the vaccine, NF54) controlled human malaria infection (CHMI) at 3-7 weeks after the last dose of vaccine (17, 27, 30), and 78 percent against heterologous (Pf7G8 strain) CHMI at 3 and 9-

10 weeks (14, 28), and lasted for at least 14 months against homologous (13) and 8 months against heterologous CHMI (15). VE against Pf infection has been demonstrated in field trials in African adults to last at least 18 months and vary from 47 to 85 percent depending on the trial, dosage regimen and population assessed (29). This protection is seen despite antibody and cellular immune responses that are many-fold lower than in malaria-naive adults in Germany or the US.

Vaccination-induced protective immunity is mediated by a complex combination of innate, humoral, and cell-mediated immune responses (31–36). The influence of biological sex on immunity has gathered attention in recent years, and a growing body of data suggests that sex-specific effects may result in variable immunological and efficacy outcomes after vaccination (32). Females tend to have greater antibody responses than males, higher basal immunoglobulin levels and higher B cell numbers (32, 33, 35–37).

In all our clinical trials we have assessed, in the same laboratory, the IgG antibody responses to the major protein on the surface of PfSPZ, the Pf circumsporozoite protein (CSP), prior to immunization and 2 weeks after the last immunizing dose. In a number of the trials, especially the field trials, anti-PfCSP antibody levels were higher in vaccinees who were protected as compared to those who were not protected (16, 25, 27). In this paper we report our analysis of the comparative anti-PfCSP antibody responses and protective efficacy between male and female vaccinees in 11 clinical trials in the US, Germany, Kenya, Tanzania, Mali, Burkina Faso, and Equatorial Guinea.

Methods

Selection of clinical trials

All clinical trials of PfSPZ Vaccine were considered for inclusion. Trials were included if they met the following criteria: 1) PfSPZ Vaccine was administered by direct venous inoculation (DVI); 2) The trial included female participants; 3) Datasets including participant demographics, net OD 1.0 (see ELISA methods for definition of Net OD 1.0) anti-PfCSP levels by ELISA and vaccine efficacy outcomes (when assessed) were available for analysis. Because participants were not assessed for biological sex, the data collected on sex are represented by self-identified or parent-identified gender. To assess differences in potential effects of changes in the hormonal milieu associated with puberty, the data were divided into study participants < 11 years of age and ≥ 11 years of age as part of the analysis.

IgG antibodies to PfCSP by ELISA

IgG antibodies to the Pf circumsporozoite protein (CSP) were assessed by ELISA as previously described (38). Briefly, 96-well plates (Nunc MaxiSorp Immuno Plate) were coated overnight at 4°C with 2 μg/mL of a nearly full length recombinant PfCSP protein [described in (38)] in 50 μL per well in coating buffer (Coating Solution Concentrate Kit, KPL, Catalog# 5150-0014). Plates were washed three times with 2 mM imidazole, 160 mM NaCl, 0.02% Tween 20, 0.5 mM EDTA and blocked with 1% Bovine Serum Albumin (BSA) blocking buffer (10% BSA Diluent/Blocking Solution, KPL, Catalog# 5140-0006) containing 1% non-fat dry Milk for 1 h at 37°C. Plates were washed three times and serially diluted serum samples (in triplicates) were added and incubated at 37°C for 1 h. After three washes, peroxidase labelled goat anti-human IgG (Anti-Human IgG (H+L) Antibody, Peroxidase-Labeled, KPL, Catalog #5220-0330) was added at a dilution of 0.1 μg/ml and incubated at 37°C for 1 h. Plates were washed three times, ABTS peroxidase substrate was added for plate development, and the plates were incubated for 75 min at room temperature. The plates were read with a Spectramax Plus 384 microplate reader (Molecular Devices) at 405 nm. The data were collected using SoftMax Pro GXP v5 and fit to a 4-parameter logistic curve, to calculate the serum dilution yielding an optical density reading of 1.0 (OD 1.0). A negative control (pooled sera from non-immune individuals from a malaria free area) was included in all assays. Serum from an individual with anti-PfCSP antibodies was used as a positive control. The same negative and positive controls were used in all assays. The assay was conducted on sera obtained prior to immunization and 2 weeks after the last immunization. Samples were considered positive if the difference between the post-immunization OD 1.0 and the pre-immunization OD 1.0 (net OD 1.0) was ≥50 and the ratio of the post-immunization OD 1.0 to pre-immunization OD 1.0 (ratio) was ≥3.0.

Statistical and meta-analysis methods

The Net OD 1.0 ELISA anti-PfCSP levels were calculated for each participant in a trial to compare the antibody levels between female and male participants. First, the net OD 1.0 ELISA anti-PfCSP levels were obtained by calculating the difference between pre-immunization and two weeks post last immunization levels measured for each participant. Then, the negative net antibodies were replaced with a value of 1 for the logarithmic presentation of data. Finally, the net antibody levels between female and male participants were compared using the Kruskal-Wallis test (SAS 9.4). The non-parametric Wilcoxon-Mann-Whitney test was used to determine statistical significance for fold change values of antibody levels.

The protection risk ratio (RR) between male and female vaccine participants was compared to evaluate the vaccine efficacy in males and females. The RR as a parameter does not depend on aspects of study design, which vary between studies. This feature supported comparing multiple clinical trial outcomes obtained from different populations, population sizes, and vaccine doses. The RR was obtained from (39).

10.3389/fimmu.2022.1006716

$$RR = \frac{a/n_1}{c/n_2} \quad (1)$$

Where a was the number of protected male participants in a trial vaccine group, n_1 was the total number of males in that trial's vaccine group. The c and n_2 were the number of protected females and total female participants in the vaccine group. In a random-effects meta-analysis, $Ln(RR)$ was each trial's study effect (r_i). In the random-effects meta-analysis, the 95% confidence interval (CI) for RR was calculated using (2):

$$CI = \hat{r}_i \pm 1.96 \times \hat{\sigma} \quad (2)$$

Where σ for was the each trial estimated standard error obtained from (3):

$$\hat{\sigma}_i^2 = 1/a - 1/n_1 + 1/c - 1/n_2 \quad (3)$$

The z-statistic value for each trial was then estimated by (4):

$$z_i = \hat{r}_i/\hat{\sigma}_i \quad (4)$$

We obtain the two-tailed p-value for a trial by p=2[1−$\Phi(z)$], where $\Phi(z)$ was the standard normal cumulative distribution (39). Lastly, the overall RR of all trials was calculated using the random-effects modified inverse variance method for trial weights. The modified weight was calculated by (5):

$$w_i = \tilde{w}_{inv} + \ln(n_1 + n_2) \quad (5)$$

The inverse variance estimates study weight and can be presented by $\tilde{w}_{inv} = 1/\hat{\sigma}_i$. The logarithmic summation modifies the study weights (modified variance) to overcome the possible small study size problem due to numerous small sample size trials (40, 41). Finally, the Q-statistics and I^2 values for the random-effect analysis were measured to report the heterogeneity of the meta-analysis on male and female vaccine efficacy.

Results

Clinical trials

Data from 11 clinical trials were available for analysis (Table 1). These included 8 adult (age ≥ 18 years) trials with immunology and efficacy data; 1 trial with infants (ages 5 to 12 months) and children (ages 1 to 9 years) with immunology for all ages and efficacy for the infant cohort; and 2 trials with infants (ages 6-12 months), children (ages 1-17 years) and adults (age ≥ 18 years) with immunology data for all 3 age groups but efficacy data only for adults. Trials conducted in the US and Germany enrolled malaria naïve adults; efficacy was assessed using controlled human malaria infection (CHMI). Trials conducted in sub-Saharan Africa (Tanzania, Equatorial Guinea, Burkina Faso, Kenya, Mali) enrolled participants with varying degrees of prior exposure to Pf; efficacy, when evaluated, was assessed against either naturally acquired infection (Kenya, Mali, Burkina Faso) or CHMI (Tanzania, Equatorial Guinea).

Antibodies to PfCSP by ELISA by sex

In all trials, antibody levels against PfCSP were assessed prior to the first dose of vaccine and 2 weeks after the final vaccine dose. The antibody level was the serum dilution at which the optical density (OD) was 1.0. The net OD 1.0, the difference between the post- and pre-vaccination OD 1.0 levels, is reported. As reported in prior studies, antibody levels for males and females combined were substantially higher in adult study participants from sites where malaria is not endemic compared with malaria endemic areas (16, 17, 19) [Jongo, unpublished]. Antibody levels at sites located in Tanzania and Equatorial Guinea where infants to adults were assessed, correlated inversely with age (19) [Jongo, unpublished]. Net OD 1.0 PfCSP antibody levels were higher in female study participants compared with male participants in 10 of 12 trials (Figure S1); in 5 trials (EGSPZV2 (Equatoria Guinea, 2016), EGSPZV3 (Equatoria Guinea, 2018), MLSPZV2 (Mali 2) (Mali, 2014), WRAIR 2080 (US, 2014) and MAVACHE (Germany, 2016)) this difference was statistically significant. Net PfCSP antibody levels were higher in males in three trials – BSPZV2 (Tanzania, 2015) and KSPZV1 (Kenya, 2016). All 3 trials included children, and all participants in the Kenya trials were less than 9 years of age. When the trial data were segregated according to age ≥ or < 11 years old, all studies showed higher net PfCSP antibody levels in females age ≥ 11years compared with males, with the difference significant in 5 trials (Figure 1). In the 4 clinical trials with infants and children, the net PfCSP antibody levels in participants under age 11 years were not significantly different, but levels were higher in males in 3 of the 4 trials (Figure 2).

Vaccine efficacy by sex

In the adult trials, vaccine efficacy was determined by CHMI at predetermined time points after the final vaccine dose or by natural exposure over a 24-week period after the final vaccine dose (Table 1). A meta-analysis of vaccine efficacy by sex (Figure 3) was done for 9 of the 10 trials in which protective efficacy was assessed in adults. The EGSPZV2 (Equatorial Guinea, 2016) had only 1 female who participated in CHMI (and was protected) and was not included in the analysis. In one trial, the MLSPZV2 (Mali 2) (Mali, 2016) trial, meta-analysis demonstrated a trend towards greater vaccine efficacy in females (RR 0.53, CI 0.28 - 1.01, p=0.057, chi-squared). However, the

10.3389/fimmu.2022.1006716

TABLE 1 Characteristics of the individual trials included in this analysis.

Study (Country, year trial started)	Vaccinees Male/Female	Dose	Dosing Interval (days)	Efficacy Assessment
US and German Adults age _18 years				
VRC 312 (USA, 2011) (9) *(NCT01441167)*	5/9	1.35x10^5 PfSPZ	Group 4a – 1, 29, 113, 141 and 189 Group 4b – 1, 29, 57, 85 and 134 Group 4c – 1, 29, 57 and 106	CHMI (NF54) 3 weeks post final dose
WRAIR 2080 (USA, 2014)) (14) *(NCT02215707)*	20/14	2.7x10^5 PfSPZ	1, 29, 57, 85 and 141	CHMI (NF54, 7G8) 3 weeks post final dose
		4.5x10^5 PfSPZ	1, 57 and 113	
Warfighter 2 (USA, 2016) (23) *(NCT02601716)*	36/21	4.5x10^5 PfSPZ	1, 3, 5, 7 and 113	CHMI 12 weeks post final dose
		9.0x10^5 PfSPZ	1, 57 and 113	
		1.8x10^6 PfSPZ	1, 57 and 113	CHMI 24 weeks post final dose
		2.7x10^6 PfSPZ (1st dose) then 9.0x10^5 PfSPZ	1, 57 and 113	
MAVACHE (Germany, 2016) (28) *(NCT02704533)*	8/4	9.0x10^5 PfSPZ	1, 8 and 29	CHMI 3 weeks post final dose
African children and adults age ≥ 11 years				
MLSPZV1 (Mali, 2014) (16) *(NCT01988636)*	35/7	2.7x10^5 PfSPZ	1, 29, 57, 85 and 141	Naturally acquired infection 4 to 24 weeks post final dose
BSPZV2 (Tanzania, 2015) (19, 22) *(NCT02613520)*	13/12	9.0x10^5 or 1.8x10^6 PfSPZ	1, 57 and 113	CHMI (age ≥ 18 years) 3-11 weeks post final dose
MLSPZV2 (Mali, 2016) (27) *(NCT02627456)*	41/15	1.8x10^6 PfSPZ	1, 57 and 113	Naturally acquired infection 0 to 24 weeks post final dose
BFSPZV1 (Burkina Faso, 2016) (29) *(NCT02663700)*	21/18	2.7x10^6 PfSPZ	1, 57 and 113	Naturally acquired infection 0 to 24 weeks post final dose
EGSPZV2 (Equatorial Guinea, 2016) [(24), Jongo et al., manuscript in preparation] *(NCT02859350)*	23/4	2.7x10^6 PfSPZ	1, 57 and 113	CHMI (age ≥ 18 years) 14-33 weeks post final dose
EGSPZV3 (Equatorial Guinea, 2018) [(26) Jongo et al., AJTMH 2022]	64/13	9.0x10^5 PfSPZ	Group 1 - 1, 3, 5, 7 and 113 Group 2 - 1, 3, 5 and 7 Group 3 - 1, 3, 5, 7 and 29 Group 4 - 1, 8 and 29	CHMI 3 weeks post final dose
African infants and children age 5 months – 11 years				
BSPZV2 (Tanzania, 2015) (19) *(NCT02613520)*	14/20	4.5x10^5, 9.0x10^5 or 1.8x10^6 PfSPZ	1, 57 and 113 days	N/A
KSPZV1 (part 1) (Kenya, 2016) (42) *(NCT02687373)*	20/25	1.35x10^5, 2.7x10^5 or 4.5x10^5	1 dose	N/A
		9.0x10^5 or 1.8x10^6 PfSPZ	1 and 57 days	
KSPZV2 (part 2) (Kenya, 2016) (20) *(NCT02687373)*	109/88	4.5x10^5, 9.0x10^5 or 1.8x10^6 PfSPZ	1, 57 and 113 days	Naturally acquired infection 2 to 52 weeks post final dose
EGSPZV2 (Equatorial Guinea, 2016) (Jongo et al., manuscript in preparation) *(NCT02859350)*	18/15	1.8x10^6 PfSPZ	1, 57 and 113 days	N/A

overall results of the meta-analysis demonstrated no difference in protective efficacy by sex (RR 1.02, CI 0.21-5.05, p=0.96, chi-squared). In the only pediatric trial to assess vaccine efficacy, KSPZV1 (part 2) (Kenya, 2016), there was no difference in efficacy by sex (RR 1.06, CI 0.76-1.39, p=0.76, chi-squared).

Antibodies by sex and protection status

Net OD 1.0 PfCSP antibody levels by sex and protection status within each of the 7 individual trials of adults in which sample sizes were adequate to make comparisons (MLSPZV1

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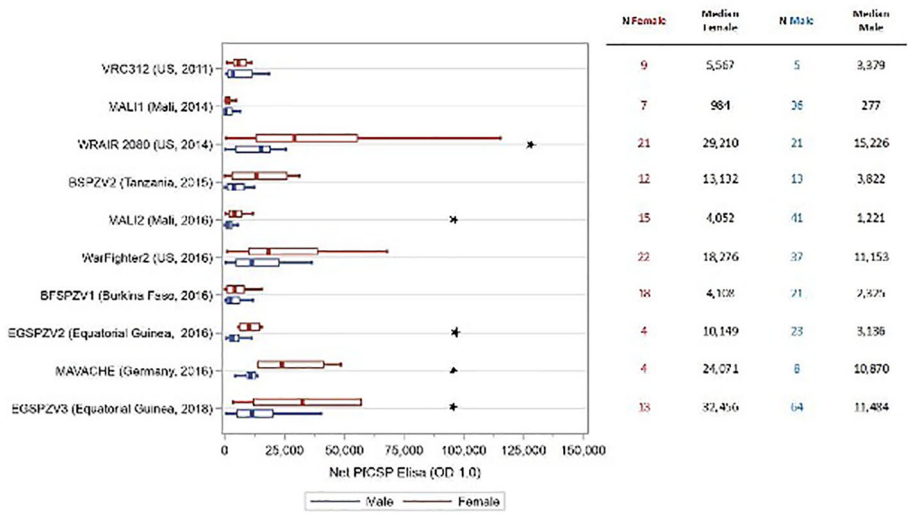

FIGURE 1
Net OD 1.0 PfCSP antibody level by sex in study participants age ≥ 11 years receiving PfSPZ Vaccine. Study name and time of study start are shown in the left of the figure, with the number of participants of each sex and the median net OD 1.0 PfCSP antibody levels for each sex in the right-hand columns. Box plots display the median, interquartile range and minimum/maximum for each trial with female participants represented in red and male participants in blue. The difference in net PfCSP antibody responses between females and males was statistically significant ($p<0.05$, Kruskal-Wallis test) in 5 of the trials (★).

(Mali 1) (Mali, 2014), MLSPZV2 (Mali 2) (Mali, 2016), WRAIR 2080 (US, 2014), Warfighter 2 (US, 2016), BFSPZV1 (Burkina Faso, 2016), MAVACHE (Germany, 2016), EGSPZV3 (Equatorial Guinea, 2018)) did not yield a consistent relationship among antibody level, protection and sex (Figure S2). Among participants who were protected, antibody levels were higher in females than in males in 6 of 7 trials and the differences were statistically significant in 3 (WRAIR 2080 (US,

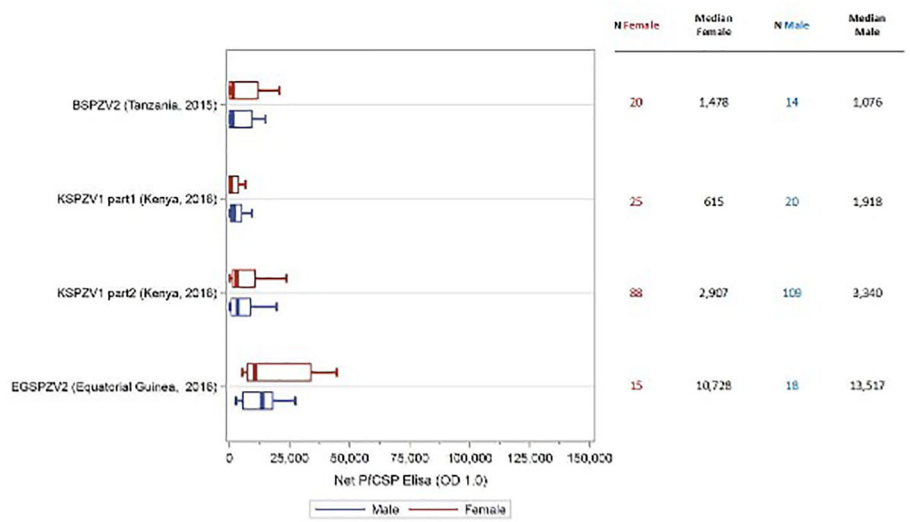

FIGURE 2
Net OD 1.0 PfCSP antibody level by sex in study participants age < 11 years receiving PfSPZ Vaccine. Study name and time of study start are shown in the left of the figure, with the number of participants of each sex and the median net PfCSP antibody levels for each sex in the right-hand columns. Box plots display the median, interquartile range and minimum/maximum for each trial with female participants represented in red and male participants in blue. There was no difference in the net PfCSP antibody responses between females and males in this age group.

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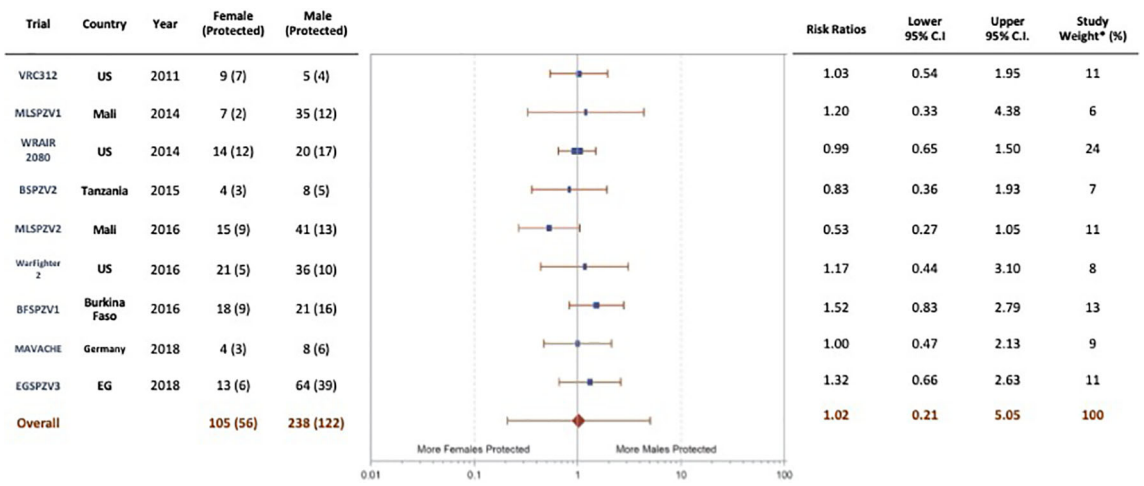

FIGURE 3
Protection status by sex in adult vaccinees (age ≥ 18 years) in trials of PfSPZ Vaccine, random-effects model. Although one trial (MLSPZV2) showed a trend towards a significant difference in vaccine efficacy favoring females, the conclusion of the meta-analysis was no difference in the efficacy of PfSPZ Vaccine in males compared with females (weighting – modified variance; I^2-11.7% (-66%, 53%); p=0.97, chi-squared).

2014), MAVACHE (Germany, 2016), EGSPZV3 (Equatorial Guinea, 2018)) of the 6 trials. In 3 trials (WRAIR 2080 (US, 2014), Warfighter 2(US, 2016), and BFSPZV1 (Burkina Faso, 2016)), antibody levels were significantly higher in protected vs unprotected females. In 2 trials (MLSPZV1 (Mali 1) (Mali, 2014) and MLSPZV2 (Mali 2) (Mali, 2016)), antibody levels were significantly higher in protected vs unprotected males and in one additional trial (EGSPZV3 (Equatorial Guinea, 2018)) the difference was borderline significant (p=0.059).

Discussion

In 100% of ten clinical trials in Mali, Tanzania, Burkina Faso, Equatorial Guinea, the US, and Germany females ≥11 years of age (Figure 1) made higher levels of antibodies to PfCSP than did males, and these differences were significant in five of the ten studies. In contrast, in four studies in participants <11 years old in Africa, there were no significant differences in levels of antibodies to PfCSP between females and males, and in three of the four studies, males had higher levels of antibodies (Figure 2). These findings are consistent with prior reports on sex differences in vaccine induced antibody responses. Adult females, for example, have shown stronger antibody responses to immunizations for influenza, hepatitis B, herpes virus, yellow fever, rabies, and smallpox virus than males (7, 31, 33, 36, 43). Sex differences in humoral immunity exist throughout life in some cases, while in others, such as appears to be the case with PfSPZ Vaccine, differences are found only after puberty, implying that genes and hormones are both likely involved (31).

Although females have shown higher vaccine-induced antibodies in many studies, it has not been consistently linked to increased vaccination effectiveness in females (44, 45). In our case, the differences in antibody responses between males and females were not mirrored by differences in efficacy; protection against CHMI or against transmission in the field appeared not to be influenced by sex (Figure 3). This suggests that other immune mechanisms, such as antibody-dependent or antibody-independent cell-mediated responses, are the major determinants of protection. However, we have not systematically assessed the functional capacity of antibodies in the sera of females vs. males to inhibit PfSPZ invasion of hepatocytes, which has been significantly associated with protection in some clinical trials, even when anti-PfCSP antibody level was not significantly associated (9). It is generally believed that PfSPZ-based vaccination protects against malaria infection through CD8 T cell responses that home to the liver, although other mechanisms may be involved as well (8). We surmise that antibody responses may correlate with other responses more mechanistically involved in protection, as suggested in prior publications on PfSPZ Vaccine (8, 9) and thereby act as a biomarker. This is consistent with the finding that, depending on the trial, antibody responses in non-protected individuals in one trial may be higher than antibody responses in protected individuals in another trial. For example, antibody responses in non-protected individuals in EGSPZV3 (Equatorial Guinea, 2018) (Figure S2G) were higher in both males and females than responses in protected individuals in MLSPZV1 (Mali 1) (Mali, 2014) (Figure S2A), MLSPZV2 (Mali 2) (Mali, 2016) (Figure S2B) or BFSPZV1 (Burkina Faso, 2016) (Figure S2E). If antibody levels were the primary determinants of protection, this would not be the case.

A consistent finding from our studies is that individuals with prior malaria exposure, such as African adults, have significantly lower antibody responses to PfCSP than malaria naïve adults (Figure 1, Figure S1) (14, 16, 17, 19, 23, 27). We think this is primarily due to immune dysregulation due to lifelong exposure to malaria parasites, but elimination of the PfSPZ for immunization by naturally acquired adaptive immune responses and immunosuppression due to concomitant helminth and other infections may also contribute (46). Interestingly, in trials including African infants and children there is a negative correlation between age and antibodies to PfCSP with the highest levels in infants and young children (19). Antibody levels in these children approach the responses seen with malaria-naive adults (19). Regardless, in participants ≥ 11 years of age, antibody levels were higher in females than their male counterparts despite the degree of prior malaria exposure (Figure 1).

An effect of dose and dosing interval was not specifically examined in this analysis. Antibody levels appear to increase with increasing total vaccine dose in groups with similar degrees of prior exposure to Pf in both males and females (Figure 1). Regardless of the dose and dosing interval used, all trials evaluating children and adults ≥ 11 years of age, antibody levels were higher in females compared with males (Figure 1).

In this study, the interplay between sex, antibody levels and protection was not straightforward. In three of seven trials with sufficient data for evaluation (one in Burkina Faso, two in the US), protected females showed statistically significantly higher antibody responses than non-protected females and males did not (Figures S2C–E) while in two different trials (both in Mali), protected males showed statistically significantly higher antibody responses than non-protected males and females did not (Figures S2A, B). In two trials (Germany and Equatorial Guinea), there were no significant differences between protected and unprotected males or females (Figures S2F, G). At this point, we are not able to explain these differences.

The finding that sex-related differences in protection were not revealed in this study has important practical implications. For example, there is no need to consider varying immunization regimens between males and females. Nevertheless, it will be important to continue monitoring for sex-related differences as the clinical development program for PfSPZ-based vaccines moves forward.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study we present for publication is an analysis of data from multiple contributing studies. For every study included in this analysis: 1. Each study was individually IRB/ethics committee approved – in some cases this was one or more institutional IRB with or without a national IRB. 2. All studies required informed consent. For the studies enrolling participants under the age of 18, parental or guardian consent was a requirement for study participation. The host country definition for consent requirements was used for each trial. 3. The analysis of antibody responses and the collection of demographics, including sex, was explicitly included in each study protocol from which the data presented in this manuscript was derived. 4. The full list of ethical review committees, by trial: 1. VRC 312 - US National Institute of Allergy and Infectious Diseases (NIAID; National Institutes of Health [NIH]) IRB 2. Mali 1 - Faculté de Médecine de Pharmacie et d'OdontoStomatologie [FMPOS] IRB (Bamako, Mali); US National Institute of Allergy and Infectious Diseases (NIAID; National Institutes of Health [NIH]) IRB 3. WRAIR 2080 – Walter Reed Army Institute of Research IRB 4. BSPZV2 – Ifakara Health Institute IRB; National Institute for Medical Research (Tanzania) IRB 5. Mali 2 - Faculté de Médecine de Pharmacie et d'OdontoStomatologie [FMPOS] IRB (Bamako, Mali); US National Institute of Allergy and Infectious Diseases (NIAID; National Institutes of Health [NIH]) IRB 6. Warfighter 2 – Naval Medical Research Center IRB; University of Maryland IRB 7. BFSPZV1 – Burkina Faso Ethics Committee for Health Research (Burkina Faso); University of Maryland IRB 8. KSPZV1 – Kenya Medical Research Institute (KMRI) IRB; US Centers for Disease Control and Prevention (CDC) IRB 9. EGSPZV2 - Ifakara Health Institute IRB; Comité Ético Nacional de Guinea Ecuatorial (CENGE) 10. MAVACHE - Ethikkommission der Medizinischen Fakultat und am Universitatsklinikum Tübingen (Tübingen, Germany) 11. EGSPZV3 - Ifakara Health Institute IRB; Comité Ético Nacional de Guinea Ecuatorial (CENGE). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

SH, TR, LC, SC, and NK conceived, designed, and defined the analysis plan, and interpreted results. NK performed and analyzed the antibody assays. PR developed algorithms, gathered and analyzed the data. SH, TR, LC, and NK drafted the manuscript. RS, JE, KL, BM, PK, MS, SH, PD, SJ, VN, SA, SS, MLa, LS, and MO conducted the clinical trials, TM provided regulatory support, PB provided training and laboratory support, BS provided the

investigational product, and TR and LC supervised the clinical trials for the sponsor. All authors read, commented on, and approved the final version of the manuscript.

Funding

The clinical trials were funded in whole or in part with funds from the following: US National Institute of Allergy and Infectious Diseases, National Institutes of Health, including the Division of Microbiology and Infectious Diseases, the Laboratory of Malaria Immunology and Vaccinology, and the Vaccine Research Center, The Department of Defense Joint Warfighter Medical Research Program, The US Army Medical Research and Development Command, Military Infectious Diseases Research Program, The Naval Medical Research Center Advanced Medical Development Program, The Ministry of Health and Social Welfare and the Ministry of Mines and Hydrocarbons, Equatorial Guinea, Marathon EG Production Limited, Noble Energy, Atlantic Methanol Production Company, and EG Liquified Natural Gas, and German Federal Ministry of Education and Research (BMBF) through the German Center for Infection Research (DZIF). Sanaria, Inc. funded the data analysis. Marathon EG Production Limited, Noble Energy, Atlantic Methanol Production Company, and EG Liquified Natural Gas were not involved in the study design, the collection, analysis or interpretation of data, the writing of this article, or the decision to submit it for publication.

Acknowledgments

We wish to acknowledge the invaluable support of the Sanaria Manufacturing, Quality, Regulatory and Clinical Teams and Protein Potential Assays Team, Rockville MD, USA: especially Yonas Abebe, Anusha Gunasekera, Elizabeth Saverino, Mei-Chun Chen, Abraham Eappen, Tao Li, Anita Manoj, Richard Stafford, Adam Richman, Adam Ruben, Yun Wu, Aderonke Awe, Asha Patil, LiXin Gao, Faith Beams, Virak Pich, Keith Nelson, Yingda Wen, Bing Jiang, Maria Orozco, Rui Xu, James Overby, Steve Matheny, Yeab Getachew, Enni Fomumbod, Mary King, Michelle Laskowski, Patricia De La Vega, Tint Wai, Jonathan Jackson, Meghan Marquette, Henry Huang, and Debbie Padilla. We also warmly thank the many institutions, investigators and their teams who were or are involved in the conduct of completed, ongoing and planned trials of PfSPZ products, for their collaboration.

Conflict of interest

NK, LC, PR, SC, TM, PB, BS, TR and SH are salaried employees of Sanaria Inc., the developer and owner of PfSPZ Vaccine and sponsor of the clinical trials. In addition, BS and SH have a financial interest in Sanaria Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare this study received funding from Sanaria Inc.

The funder had the following involvement in the study: proposal of the study hypothesis, conduct of the ELISAs and data analysis.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1006716/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 27 October 2022
DOI 10.3389/fimmu.2022.1002430

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 25 July 2022
ACCEPTED 17 October 2022
PUBLISHED 27 October 2022

CITATION
Takashima E, Nagaoka H, Correia R, Alves PM, Roldão A, Christensen D, Guderian JA, Fukushima A, Viebig NK, Depraetere H and Tsuboi T (2022) A novel asexual blood-stage malaria vaccine candidate: PfRipr5 formulated with human-use adjuvants induces potent growth inhibitory antibodies. *Front. Immunol.* 13:1002430. doi: 10.3389/fimmu.2022.1002430

A novel asexual blood-stage malaria vaccine candidate: PfRipr5 formulated with human-use adjuvants induces potent growth inhibitory antibodies

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PfRipr is a highly conserved asexual-blood stage malaria vaccine candidate against *Plasmodium falciparum*. PfRipr5, a protein fragment of PfRipr inducing the most potent inhibitory antibodies, is a promising candidate for the development of next-generation malaria vaccines, requiring validation of its potential when formulated with adjuvants already approved for human use. In this study, PfRipr5 antigen was efficiently produced in a tank bioreactor using insect High Five cells and the baculovirus expression vector system; purified PfRipr5 was thermally stable in its monomeric form, had high purity and binding capacity to functional monoclonal anti-PfRipr antibody. The formulation of purified PfRipr5 with Alhydrogel®, GLA-SE or CAF®01 adjuvants accepted for human use showed acceptable compatibility. Rabbits immunized with these formulations induced comparable levels of anti-PfRipr5 antibodies, and significantly higher than the control group immunized with PfRipr5 alone. To investigate the efficacy of the antibodies, we used an *in vitro* parasite growth inhibition assay (GIA). The highest average GIA activity amongst all groups was attained with antibodies induced by immunization with PfRipr5 formulated with CAF®01. Overall, this study validates the potential of adjuvanted PfRipr5 as an asexual blood-stage malaria vaccine candidate, with PfRipr5/CAF®01 being a promising formulation for subsequent pre-clinical and clinical development.

KEYWORDS

asexual blood-stage malaria vaccine, PfRipr5, *Plasmodium falciparum*, adjuvant, Alhydrogel, GLA-SE, CAF01

10.3389/fimmu.2022.1002430

Introduction

Three main malaria vaccine types have been considered to target the different life cycle stages of *Plasmodium falciparum*, namely, pre-erythrocytic vaccines, asexual blood-stage vaccines, and transmission-blocking vaccines. Last year, the World Health Organization (WHO) for the first time recommended the use of a pre-erythrocytic vaccine based on circumsporozoite protein (CSP), RTS,S/AS01, for the prevention of *P. falciparum* malaria in children living in regions with moderate to high transmission (1). However, in the RTS,S/AS01 phase 3 trial (2) the vaccine efficacy is modest, and efficacy against clinical malaria wanes more rapidly than efficacy against infection (especially in high transmission settings) due to higher levels of naturally acquired immunity by the blood-stage infection in the control cohort than the vaccine cohort (3). Therefore, to maintain blood-stage immunity an asexual blood-stage vaccine is considered an important addition to a pre-erythrocytic vaccine (4).

High polymorphism levels in *P. falciparum* asexual blood-stage malaria vaccine antigens often result in strain-specific immunity that hampers vaccine efficacy in the clinical trials (5). Thus, developing vaccines based on conserved antigens across multiple strains could be a more straightforward approach to attain high protective efficacy in the field (6). The PfRipr/PfCyRPA/Rh5 protein complex is considered to play one of the central roles in the sequential molecular events leading to *P. falciparum* merozoite invasion (7). Since all three subunit proteins are highly conserved and naturally acquired antibody responses in humans against each of them are associated with clinical protection against malaria (8–10), the PfRipr/PfCyRPA/Rh5 protein complex components are considered as promising asexual blood-stage vaccine candidates. A phase I/IIa trial of RH5.1, a recombinant protein-based antigen derived from Rh5, formulated with AS01B adjuvant, showed induction of antibodies in humans that can significantly reduce the growth of asexual blood-stage parasites *in vivo* following controlled human malaria infection (CHMI) using blood-stage parasites (11, 12), thus making Rh5 the leading asexual blood-stage vaccine candidate from the PfRipr/PfCyRPA/Rh5 complex. However, the RH5.1 vaccine-induced protection was modest and only led to a 1- to 2-day delay in time to diagnosis without sterile protection (11).

To develop an improved next-generation asexual blood-stage malaria vaccine targeting the PfRipr/PfCyRPA/Rh5 complex, PfRipr is one of the promising antigen targets because of the following accumulated evidence. Specific antibodies raised against recombinant PfRipr protein exhibited strain-transcending inhibition of *P. falciparum in vitro* growth (7). We have successfully expressed the cysteine-rich region of PfRipr (K279-D995 amino acids (aa)) using the wheat germ cell-free system (WGCFS) (13). Next to investigating the protective efficacy of the antibodies, we used an *in vitro* parasite growth inhibition assay (GIA), and demonstrated the significant GIA activity of anti-PfRipr antibodies using both homologous *P. falciparum* 3D7 and heterologous FVO strains *in vitro* (14). However, using PfRipr as vaccine target is challenging due to its large size (126-kDa in full length) and highly cysteine-rich nature (87 cysteine residues). To overcome this barrier, we expressed 11 truncated protein fragments derived from PfRipr spanning 152-aa to 215-aa each. We then immunized animals using each PfRipr fragment to generate fragment specific polyclonal antibodies. By GIA measurement of the antibodies, we identified PfRipr5 (aa C720-D934) as a protein fragment inducing the most potent growth inhibitory antibodies as comparable level to the antibodies against full-length PfRipr (15). In agreement with this finding, an independent report showed that an anti-PfRipr monoclonal antibody (mAb) with GIA activity recognized aa N816-L860, a part of PfRipr5 (16). Although a direct comparison of GIA with polyclonal anti-RH5.1 and anti-PfRipr5 antibodies has not been conducted yet, this previous study suggested that rabbit polyclonal antibodies raised against recombinant PfRipr proteins with Freund adjuvant would have a comparable or greater GIA activity than anti-Rh5 antibodies in four laboratory strains of *P. falciparum* (16). In addition, only one non-synonymous single-nucleotide polymorphisms with minor allele frequency 9.13% is found in PfRipr5 (A755G), as opposed to those found in RH5.1 (H148D, Y147H, S197Y, C203Y, and I140M); thus PfRipr5 is more conserved than RH5.1 (7). Therefore, PfRipr5 is regarded as a promising asexual blood-stage vaccine candidate antigen for next-generation asexual blood-stage and combination vaccines against *P. falciparum*.

Adjuvants play a key role to enhance the efficacy of weakly-immunogenic antigens and/or to induce appropriate immune responses (17). Since most of the subunit malaria vaccine antigens considered to date are weak immunogens, the choice of adjuvant is a critical component for malaria vaccine development (18). Aluminum-based adjuvants are considered the gold standard among the human applicable adjuvants thanks to their safety and track-record (19), but novel adjuvants might be a better choice for a malaria vaccine. Formulation of PfAMA1-DiCo [an asexual blood-stage vaccine candidate based on the three recombinant variants of *P. falciparum* apical membrane antigen 1 (AMA1)] and PRIMVAC and PAMVAC (two placental malaria vaccines based on the VAR2CSA protein) with a non-aluminum-based adjuvant, glucopyranosyl lipid adjuvant–stable emulsion (GLA-SE) were shown to be safe and well-tolerated, and induced higher levels of functional antibodies compared to aluminum-based adjuvant, Alhydrogel® (20–22). GLA-SE is a TLR4 agonist with potential to enhance the Th1 cell-mediated cytotoxic T lymphocyte (CTL) response and shown to be safe and well tolerated in human subjects in multiple phase I clinical trials (18, 23). In addition to GLA-SE, CAF®01 is a novel two-component liposomal adjuvant system composed of a cationic liposome vehicle [dimethyldioctadecylammonium (DDA)] stabilized with trehalose 6,6-dibehenate (TDB), a synthetic

variant of mycobacterial glycolipid cord factor which is recognized by the C-type lectin receptor MINCLE and has been shown to be safe in human trials (24, 25). The CAF®01 adjuvant has recently been tested in a phase 1/2a GMZ2 asexual blood-stage malaria vaccine clinical trial for the first time and the GMZ2/CAF®01 vaccine was well tolerated and immunogenic in humans (26). This CAF®01's unique mode of action makes it an attractive candidate adjuvant for a future malaria vaccine.

In this study, we produced PfRipr5 antigen using insect cells and a baculovirus expression vector system, and performed a head-to-head comparison of its antigenicity when formulated with adjuvants for human use, specifically Alhydrogel®, GLA-SE, and CAF®01, as well as functional activity of the rabbit antibodies, to further advance the development of a PfRipr5-based malaria vaccine candidate.

Materials and methods

Production of PfRipr5

PfRipr5 recombinant protein was produced in a 50 L stirred-tank bioreactor (Sartorius, Göttingen, Germany) by infecting insect High Five cells (Invitrogen, Carlsbad, CA) at 2 $\times 10^6$ cell/mL with a recombinant baculovirus encoding *pfripr5* nucleotide sequence and His_6-tag for purification, using a multiplicity of infection of 0.1 virus per cell, as described elsewhere (27). Cells were expanded by sub-culturing at 0.3-0.5 $\times 10^6$ cell/mL every 2-3 days when cell density reached 2-3 $\times 10^6$ cell/mL in Insect-XPRESS™ (Sartorius) and at 27°C, using shake-flasks (Corning, Corning, NY) of 500 mL (N-4 stage) and 2000 mL (N-3 stage), and stirred tank-bioreactors (Sartorius) of 2 L (N-2 stage), 10 L (N-1 stage) and 50 L (production stage, N). For shake-flask cultures, cells were maintained in a shaking incubator (Inova 44R – Eppendorf, Hamburg, Germany) set to 100 rotations per minute (rpm) and with 2.54 cm shaking diameter. For bioreactor cultures, pO_2 was set to 30% of air saturation and was maintained by varying the agitation rate from 60 to 270 rpm and the percentage of O_2 in the gas mixture from 0 to 100%, the gas flow rate was set to 0.01 volume per volume per minute (vvm).

Purification of PfRipr5

Purification of secreted PfRipr5 was carried out on ÄKTA Explorer 100 systems (Cytiva, Tokyo, Japan) as described elsewhere (27). In brief, cell culture bulk was clarified using a Sartopore 2 30" 0.45 µm + 0.2 µm filter (Sartorius), loaded on a Histrap HP column (Cytiva), and protein was eluted with a linear Imidazole gradient. The eluate was concentrated using a Vivaflow 200 Hydrosart 10 kDa (Sartorius) and loaded into a Superdex 75 prep grade XK50/100 gel size-exclusion chromatography column (SEC) (Cytiva), from which fractions corresponding to monomeric PfRipr5 were collected. The collected fractions were loaded in a HiPrep desalting 26/10 column (Cytiva), the eluate was concentrated as mentioned above, and then sterile-filtered (0.2 µm). The final sample was formulated in 16 mM sodium phosphate buffer, 250 mM NaCl, at pH 8.0, aliquoted and stored at -80°C.

Cell concentration and viability

Cell concentration and viability were assessed using a Cedex HiRes Analyzer (Roche, Basel, Switzerland).

SDS-PAGE and western blot

SDS-PAGE and Western blot analyses were performed as described elsewhere (28). Reduced (R) samples were treated with NuPAGE Sample Reducing Agent 1× for 10 minutes at 70°C, whereas for non-reduced samples (NR) water was mixed instead. Then, both samples were run in the same gel (4-12% Bis_Tris, NuPAGE). For PfRipr5 identification by Western blot, anti-PfRipr5 antiserum previously generated in rabbits (15) was used (dilution 1:1000), and an anti-rabbit IgG antibody conjugated with alkaline phosphatase was used as secondary antibody (dilution 1:5000). Protein band detection was performed with NBT/BCIP 1-Step (Thermo Fisher Scientific, Waltham, MA). Densitometry analysis of SDS-PAGE gels was performed using Fiji software (29).

Protein concentration

Protein concentration was determined by spectrophotometry at 280 nm using the mySPEC equipment (VWR, Radnor, PA).

Dynamic light scattering

The size distribution of the purified PfRipr5 was analyzed by dynamic light scattering (DLS) on a Spectro Light 600 (Xtal Concepts, Hamburg, Germany).

High performance liquid chromatography-Size-exclusion chromatography

Purified PfRipr5 protein was analyzed in a HPLC system equipped with Photodiode Array Detector (Waters, Milford, MA). Purified sample was loaded in a XBridge BEH 125 Å SEC 3.5 µm HPLC column (Waters), equilibrated in buffer

containing 16 mM sodium phosphate, 250 mM NaCl, at pH 8.0, at a flow rate of 0.86 mL/min. Twenty micrograms of PfRipr5 was injected, and the eluted proteins were detected at 280 nm.

Thermal shift assay

Purified PfRipr5 was mixed with a thermal shift dye (Thermo Fisher Scientific) in a MicroAmp™ EnduraPlate™ Optical 96-Well Fast Clear Reaction Plate with Barcode (Thermo Fisher Scientific) to a final volume of 20 µL (n = 2 measurements). Thermal shift assay was performed in a QuantStudio 7 Flex RealTime PCR System (Thermo Fisher Scientific), with excitation and emission wavelengths of 580 and 623 nm, respectively. Plates were heated from 25°C to 90° C (rate of 0.016°C per second) and fluorescence was measured. Results were analyzed using the Protein Thermal Shift™ Software V1.3.

Surface plasmon resonance

Surface plasmon resonance (SPR) was carried out in Biacore X100 (Cytiva) as we previously described (27).

Adjuvant and PfRipr5 formulation

The PfRipr5 antigen was formulated with three adjuvants compatible for human use. Alhydrogel® was provided from Croda Denmark (Frederikssund, Denmark), CAF®01 was provided from Statens Serum Institut (SSI; Copenhagen, Denmark), and GLA-SE was provided from Access to Advanced Health Institute (AAHI; Seattle, WA). SSI and AAHI performed the compatibility studies using their routine assays to evaluate suitability of the adjuvants for formulation with PfRipr5. The PfRipr5 was diluted in 10 mM Tris buffer with 2% glycerol (pH=7.0) to the target concentration in each vaccine formulation. Five vaccine formulations (500 µL/dose) were devised, namely (i) Alhydrogel® vaccine formulations containing Alhydrogel® (5 mg/mL) and either 100 µg/mL (low dose) or 400 µg/mL (high dose) of PfRipr5, (ii) CAF®01 vaccine formulations containing CAF®01 (1250 µg/mL DDA and 250 µg/mL TDB), and either 100 µg/mL (low dose) or 400 µg/mL (high dose) of PfRipr5, and (iii) GLA-SE vaccine formulation containing GLA-SE (50 µg/mL) and 400 µg/mL (high dose) of PfRipr5. In human clinical trials, often 100 µg will be set as the highest dose. To evaluate a dose range, we used half (50 µg/dose) as low dose and twice (200 µg/dose) as high dose in this rabbit study. In the case of GLA-SE formulation study, if the higher antigen concentration formulation is compatible, we will be able to expect compatibility with the lower antigen concentration. Therefore, we only tested the high dose in the case of the GLA-SE formulation.

The antigen-adjuvant compatibility of all formulations was assessed *via* visual inspection and pH measurement at room temperature (RT) to mimic the on-site preparation for Alhydrogel® or CAF®01 formulations one hour post formulation and for GLA-SE formulations at 0-, 4-, and 24-hour post formulation at RT and 5˚C. In addition, the interaction of the PfRipr5 antigen with Alhydrogel® or CAF®01 was further evaluated by mixing the adjuvant with 100 µg/mL or 400 µg/mL of the PfRipr5 protein, centrifuging at 14,000 ×*g* for 15 minutes (Alhydrogel® formulations) or 137,400 ×*g* for 30 minutes (CAF®01 formulations), and measuring non-adsorbed protein in the supernatant using bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). In the case of GLA-SE formulation, antigen stability was assessed by sandwich-ELISA established in our recent study using functional mouse anti-PfRipr mAb (clone 29B11) as capture antibody (27). Because we have previously reported the binding of PfRipr5 with mouse anti-PfRipr5 mAb 29B11, shown to have a potent GIA activity (27), thereby being used as proxy for predicting its biological activity.

Rabbit immunization

All rabbit immunizations were subcontracted to Kitayama Labes Co. Ltd (Ina, Japan), and the antisera were provided by the company. In brief, Japanese white rabbits (n=6 per group) were subcutaneously immunized with the PfRipr5 protein alone (50 µg/shot) or with PfRipr5 antigen (0, 50, and 200 µg/shot) formulated with the aforementioned adjuvants at the specific concentrations in 500 µL injection, twice at three-week intervals (Day 0 and Day 21). Antisera were collected two weeks after the last immunization (Day 35).

Enzyme-linked immunosorbent assay

ELISA was conducted to measure anti-PfRipr5 rabbit antibody titer. The following buffers were used: (i) coating buffer, containing 50 mM sodium carbonate buffer pH 9.5, (ii) blocking buffer, containing 1% (w/v) bovine serum albumin (BSA) (nacalai tesque, Kyoto, Japan) in phosphate buffered saline (PBS), (iii) dilution buffer, containing 0.1% BSA in PBS, and (iv) stopping buffer, containing 1 M sulfuric acid (FUJIFILM Wako Pure Chemical, Osaka, Japan). Briefly, flat-bottom 96-well ELISA plates (Corning) were coated with 100 ng per well of PfRipr5 diluted with coating buffer. Plates were blocked with 300 µL/well of blocking buffer for 1 hour at 37˚ C. Five-times serial dilutions of each test rabbit serum starting from 1000-times dilution were prepared in dilution buffer. Diluted sera were added to antigen-coated wells in triplicate (50 µL/well) and

incubated for 1 hour at 37°C. Plates were washed with washing buffer using a plate washer, and incubated with 100 µL/well of the goat anti-rabbit IgG conjugated with horseradish peroxidase (GE Healthcare, Chicago, IL) for 1 hour at 37°C . After washing, the substrate (0.1 mg/well of o-phenylenediamine, FUJIFILM Wako) diluted with 5 mM citric acid buffer pH 5.0 was added, and the plates were incubated at 37°C for 15 minutes. Reactions were stopped by adding 100 µL/well of stopping buffer. Absorbance was promptly measured at 492 nm using a Spectramax M3 microplate reader (Molecular Devices, Sunnyvale, CA). Reciprocal serum dilutions that gave a mean absorbance value of 0.5 at 492 nm were determined as the endpoint titers.

Culturing *P. falciparum* and growth inhibition assay

Based on the highly conserved nature of PfRipr5 (16), we only used the *P. falciparum* 3D7 strain for the GIA to determine the functional activity of anti-PfRipr5 IgG. *P. falciparum* 3D7 strain was kindly provided by the National Institute of Allergy and Infectious Diseases (NIAID), and asexual stage of the parasite was cultured as described elsewhere (30). Total rabbit IgGs for GIA were purified from individual immune rabbit antisera with HiTrap protein G-Sepharose columns (GE Healthcare, Camarillo, CA) according to the manufacturer's protocol. The GIA activity of the total IgGs from rabbit antisera against the PfRipr5 proteins was determined at 20 mg/mL final concentration over one cycle of *P. falciparum* 3D7 parasite replication. Parasitemia was determined by flow cytometry as described previously (15). Briefly, the parasite cultures were synchronized the day before the start of the GIA, so that the majority of parasites were at the late trophozoite-to-schizont stage at the start of the GIA. Twenty microliters of parasite-infected erythrocyte (pRBC) suspension (0.3% parasitemia and 2% hematocrit) and 20 µl of IgGs were added per well of half-area flat-bottom 96-well cell culture microplates (Corning) and gently mixed. For a control, 20 µl of culture medium was added to the pRBC suspension. Cultures were incubated at 37°C in humidified, gassed (90% N_2, 5% O_2, and 5% CO_2), airtight boxes. After 25 hours of incubation, when most of the invading parasites had developed to the early trophozoite stage, the pRBC were pelleted by brief centrifugation (1,300 ×*g* for 5 min) and washed once in 100 µl PBS. The cells were then incubated with 50 µl of diluted (1:1,000 in PBS) SYBR green I (Invitrogen) for 10 min at RT and washed once in PBS. Parasitemia was measured by flow cytometry with a FACSCanto II (BD Biosciences, San Jose, CA) by the acquisition of 50,000 events per sample. Data were analyzed with FlowJo 9.1 software (Tree Star, Ashley, OR) by first gating for intact erythrocytes by side scatter and forward scatter parameters and subsequently determining the proportion of SYBR green I-positive cells. Rabbit IgGs obtained after immunization with Freund adjuvant formulated hexa-histidine-tagged glutathione S-transferase (His-GST) and region 3 to 5 of erythrocyte binding antigen 175 of *P. falciparum* (PfEBA175) (15) were used as a negative and positive controls, respectively. For each GIA, four independent experiments were carried out in triplicate to confirm the reproducibility and average GIA activities among four replicates obtained from each rabbit IgG were used for analyses.

Statistical analysis

All statistical analyses were performed with GraphPad Prism (ver. 9.4.0) (GraphPad Software, San Diego, CA). Difference of the mean antibody titers and GIA activities among groups was tested by one-way ANOVA with Tukey's multiple comparisons test. Pearson's correlation coefficient between ELISA titers and GIA activities was calculated. P-values less than 0.05 were considered as statistically significant.

Results

PfRipr5 production

PfRipr5 was produced using insect High Five cells and the baculovirus expression vector system (IC-BEVS) at 50 L scale, and the quality of purified product was assessed by SDS-PAGE, western blot, Dynamic light scattering (DLS), High performance liquid chromatography-Size-exclusion chromatography (HPLC-SEC), Thermal shift assay (TSA) and Surface plasmon resonance (SPR).

Baculovirus infection kinetics followed a typical profile of a low MOI production process, i.e. High Five cell growth from 0-24 hours post-infection (hpi), onset of cell viability drop at 48 hpi, and culture harvest at 72 hpi (when cell viability reaches approx. 80%) (Figure 1A). Overall production yield was 0.8 mg/L, similar to previous reports (27). Bands corresponding to the expected molecular weight (Mw) size of monomeric PfRipr5 were identified by SDS-PADE and Western blot in the purified material (Figure 1B), with purity >85% (Table 1). HPLC and DLS data shows a single peak within the expected Mw (25-30 kDa) and radius (~10 nm), suggesting that purified PfRipr5 was mostly in monomeric form (Figures 1C, D). In addition, the thermal stability of purified PfRipr5 (as assessed by thermal shift assay, melting temperature = 54 ± 2°C) as well as its ability to bind to the anti-PfRipr mAb 29B11 (as assessed by SPR, KD = $1.73 \pm 1.6 \times 10^{-9}$ M) were confirmed (Table 1).

These data demonstrates that the purified PfRipr5 antigen herein produced in insect cells had high quality and thus was suitable for further formulation with adjuvants and animal immunizations.

10.3389/fimmu.2022.1002430

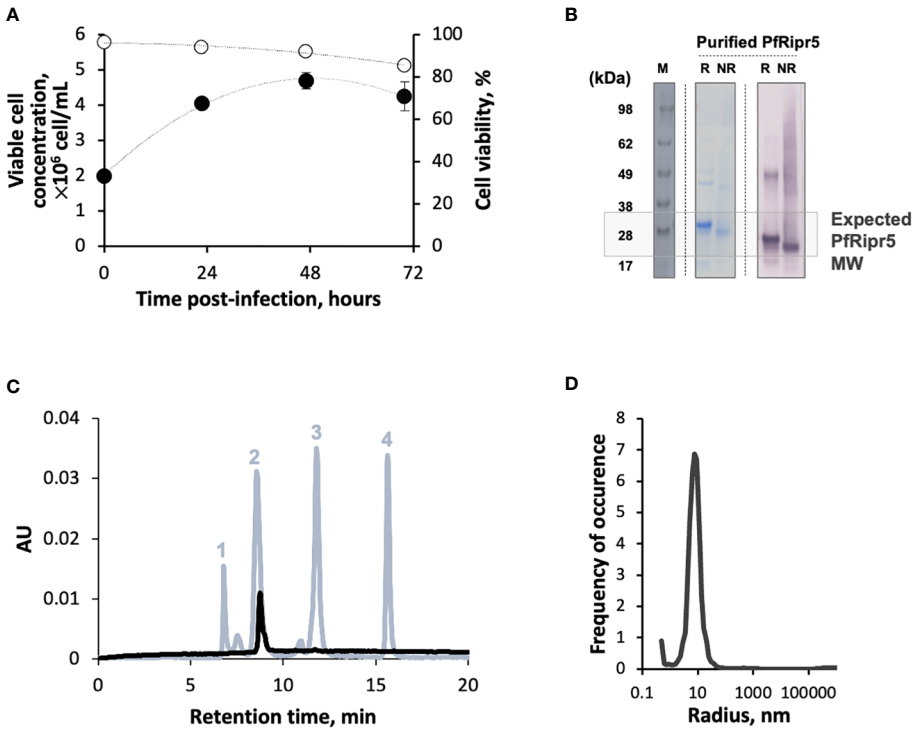

FIGURE 1
Production and characterization of PfRipr5. **(A)** Viable cell concentration (filled circles) and cell viability (open circles) throughout production process. **(B)** Identification of PfRipr5 by SDS-PAGE (middle panel) and Western blot (right panel) in purified sample. M denotes pre-stained protein standard SeeBlue® Plus2, R denotes reduced sample, NR denotes non-reduced sample. **(C)** HPLC-SEC analysis of purified PfRipr5 protein (black line). The protein standard mix (grey line) used for estimation of PfRipr5 molecular weight was composed by (1) thyroglobulin (660 kDa), (2) ovalbumin (44.2 kDa), (3) ribonuclease A (13.7 kDa), and (4) uracil (112 Da). AU denotes absorbance unit (280 nm). **(D)** Size distribution profile of purified PfRipr5 assessed by dynamic light scattering (average of three measurements). A representative data of one biological replicate (n = 1) was shown.

PfRipr5 formulation

The PfRipr5 antigen was formulated with three different adjuvants, Alhydrogel®, CAF®01, and GLA-SE, and the PfRipr5-adjuvant compatibility was confirmed following analyses optimized for each adjuvant.

Visual inspection of the PfRipr5 formulated with Alhydrogel® or CAF®01 showed no changes as compared to Alhydrogel® or CAF®01 alone, respectively. Likewise, the pH of the PfRipr5/Alhydrogel® (7.2) and the PfRipr5/CAF®01 (7.0) was similar to that of each adjuvant alone, thus suggesting their chemical stability (Table 2). Quantification of PfRipr5 protein in the supernatant following centrifugation of these formulations was performed using BCA protein assay and showed that the concentration of non-adsorbed PfRipr5 in all the Alhydrogel® and CAF®01 formulations was below the 25 µg/mL detection limit even for the high-dose PfRipr5 formulations, suggesting that all PfRipr5 antigen was adsorbed to both Alhydrogel® and CAF®01 (Table 2). Visual inspection of PfRipr5 formulated with GLA-SE and GLA-SE alone was performed at 0, 4, and 24 hours post-formulation at 5°C and RT through assessment of color, opacity, and phase (Table 3); no visual variations of the 3 parameters were observed for any of the groups (PfRipr5/GLA-SE formulation and GLA-SE alone) or time points. Similarly, no major changes in pH were observed. Finally, sandwich-ELISA data suggests no apparent loss in binding affinity of the PfRipr5/GLA-SE formulation to the functional anti-PfRipr5 mAb 29B11 when compared to PfRipr5 antigen control, indicating that the desired conformation of a functional epitope in PfRipr5 recognized by the anti-PfRipr5 mAb 29B11 is

TABLE 1 Characterization of purified PfRipr5.

Production yield, mg/L	Purity, %*	Melting temperature, °C**	KD, M*** (mAb 29B11)
0.8	> 85	54 ± 2	$1.73 \pm 1.6 \times 10^{-9}$

*Purity assessed by densitometry analysis of SDS-PAGE.
**Melting temperature assessed by thermal shift assay.
***KD: equilibrium dissociation constant between PfRipr5 and mAb 29B11 assessed by surface plasmon resonance.

TABLE 2 Formulation of PfRipr5 with Alhydrogel® and CAF®01.

	Alhydrogel®	100 µg/mL PfRipr5+ Alhydrogel®	400 µg/mL PfRipr5+ Alhydrogel®	CAF®01	100 µg/mL PfRipr5 +CAF®01	400 µg/mL PfRipr5 +CAF®01
Appearance*	Opaque suspension	Opaque suspension	Opaque suspension	Opaque suspension	Opaque suspension	Opaque suspension
pH**	7.2	6.5	7.3	7.0	6.5	7.2
Non-adsorbed PfRipr5***	–	Below detection level of 25 µg/ml	Below detection level of 25 µg/ml	–	Below detection level of 25 µg/ml	Below detection level of 25 µg/ml

*Appearance was determined by visual inspection.
**pH of the final vaccine formulation was measured, as this may be an indicator of chemical stability.
***Non-adsorbed PfRipr5 protein concentration in the supernatant after the centrifugation was determined by BCA.

TABLE 3 Formulation of PfRipr5 with GLA-SE.

	GLA-SE					GLA-SE + PfRipr5				
		5°C		RT			5°C		RT	
	T=0	T=4h	T=24h	T=4h	T=24h	T=0	T=4h	T=24h	T=4h	T=24h
Appearance*	White Opaque One Phase	No change	No change	No change	No change	White Opaque One Phase	No change	No change	No change	No change
pH**	6.36	6.36	6.34	6.35	6.30	6.91	6.87	6.79	6.84	6.84
Sandwich-ELISA***	No apparent loss of formulated PfRipr5 binding to mAb 29B11 in all the assay conditions in comparison with PfRipr5 antigen control (T=0)									

*Appearance was determined by visual inspection and was recorded at each time point according to three parameters: 1) color, 2) opacity, and 3) phase.
**pH of the final vaccine formulation was measured.
***Sandwich-ELISA was used to investigate the desired conformation of PfRipr5 is maintained when mixed with GLA-SE or not. Because we have previously reported the binding of PfRipr5 with mouse anti-PfRipr5 mAb 29B11, shown to have a potent GIA activity (27), thereby being used as proxy for predicting its biological activity.
RT, Room temperature.

maintained when mixed with GLA-SE throughout the assessed time (Table 3).

These results indicate the acceptable compatibility of the PfRipr5 with all the tested adjuvants, and thus rabbit immunization using these vaccine formulations were performed.

Immunogenicity of adjuvanted PfRipr5

Rabbits (n=6 per group) were subcutaneously immunized with the above-mentioned formulations. PfRipr5 alone (50 µg) induced significantly higher anti-PrRipr5 antibodies (Mean ELISA titer = 3.4 $\times 10^4$) than all the adjuvant alone groups (Alhydrogel®(Alum), GLA-SE (GLA), and CAF®01 (CAF) with 0 µg PfRipr5, $P<0.05$) suggesting that the PfRipr5 protein itself is immunogenic in rabbits (Figure 2A). Formulation of PfRipr5 with Alhydrogel®, GLA-SE, and CAF®01 induced statistically significant higher levels of antibodies in most low dose (50 µg) (Mean ELISA titers: Alum = 1.0 $\times 10^5$ ($P<0.01$); CAF = 1.0 $\times 10^5$ ($P<0.01$)) and in all high dose groups (200 µg)[Mean ELISA titers: Alum = 8.8 $\times 10^4$ ($P<0.05$); GLA = 1.2 $\times 10^5$ ($P<0.001$); CAF = 1.1 $\times 10^5$ ($P<0.001$)] than the PfRipr5 alone group, the exception being the low dose (50 µg) formulation with GLA-SE (Mean ELISA titer = 8.0 $\times 10^4$); no statistically significant difference was attained for the anti-PfRipr5 antibody titers between high and low dose within the adjuvant groups and across the adjuvant groups.

GIA activity of the antibodies induced upon rabbit vaccination with adjuvanted PfRipr5

To address the functional activity of the rabbit antibodies induced upon rabbit immunization, *in vitro* GIA was conducted. The GIA activities of IgG induced by PfRipr5 GLA-SE and CAF®01 formulations were higher in the high dose (200 µg) (Mean %GIA: GLA = 36.2%; CAF = 49.4%) than in the low dose (50 µg) (Mean %GIA: GLA = 10.9%; CAF = 38%) groups, respectively (Figure 2B). In contrast, the GIA activity of IgG induced by PfRipr5 Alhydrogel® formulation was higher in the low dose (50 µg) (Mean %GIA = 37%) than in the high dose (200 µg) (Mean %GIA = 19.9%) groups. In Figure 2B, the GIA activities of IgG induced by low dose (50 µg) PfRipr5/Alhydrogel® formulation ($P<0.01$), high dose (200 µg) PfRipr5/GLA-SE ($P<0.01$), and low (50 µg)($P<0.01$) and high dose (200 µg)($P<0.0001$) PfRipr5/CAF®01 groups showed statistically significant levels of GIA activities compared to the

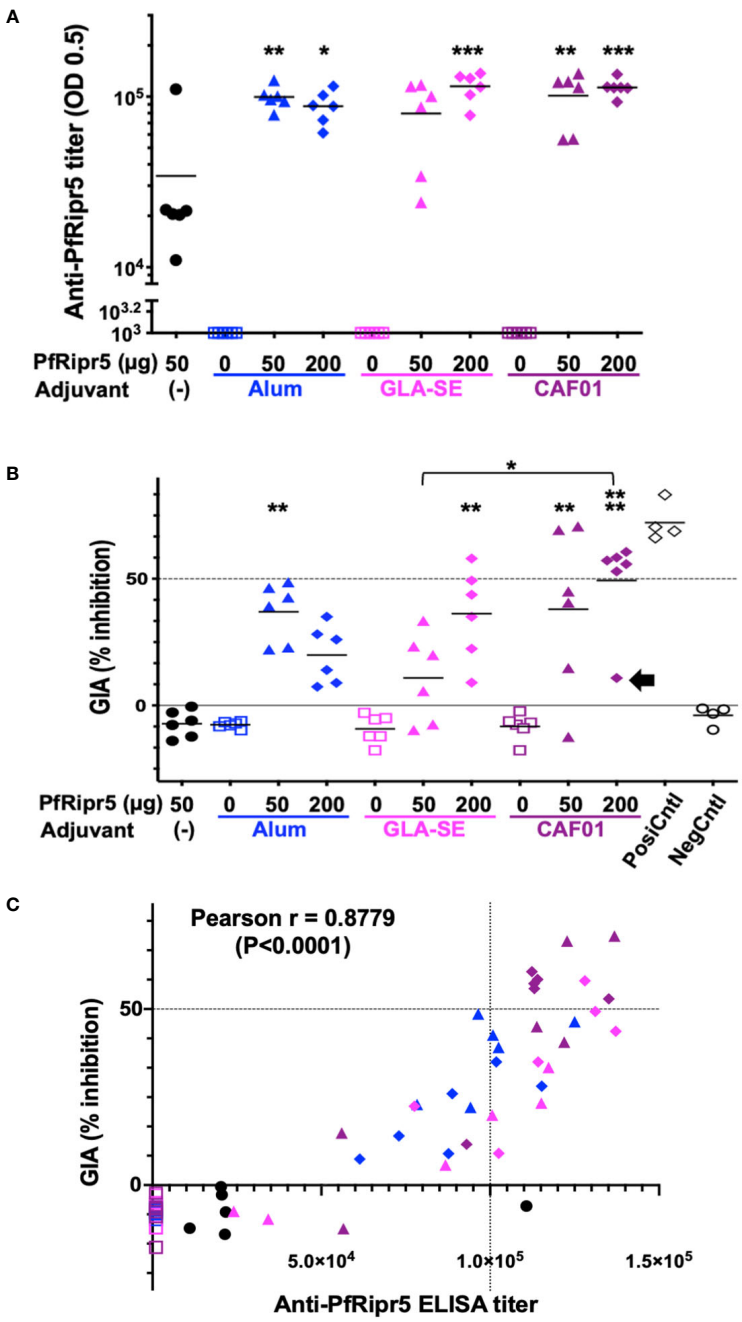

FIGURE 2
Immunogenicity of adjuvanted PfRipr5 and GIA activity of anti-PfRipr5 rabbit antibodies. PfRipr5 antigen only [(-); black filled circles], Adjuvant only (open squares), 50 μg dose of PfRipr5 (filled triangles), 200 μg dose of PfRipr5 (filled diamonds), formulation with Alhydrogel® (Alum; blue), GLA-SE (GLA; magenta), and CAF®01 (CAF; purple). Difference of the mean antibody titers and GIA activities among groups was tested by one-way ANOVA with Tukey's multiple comparisons test. P values less than 0.05 was considered as statistically significant difference and all the asterisks represent the significant difference against the PfRipr5 without adjuvant group unless otherwise indicated (*P<0.05; **P<0.01; ***P<0.001; ****: P<0.0001). **(A)** ELISA titer of anti-PfRipr5 antibodies. Reciprocal serum dilutions that gave a mean absorbance at 492 nm of 0.5 were determined as the endpoint titers. OD, optical density. **(B)** GIA activities of the total IgG purified from anti-PfRipr5 antibodies. For each GIA, four independent experiments were carried out in triplicate to confirm reproducibility and each data point represents average GIA of each rabbit IgG samples in four independent experiments. Rabbit IgGs immunized with Freund adjuvant formulated PfEBA175_region 3 to 5 (15) (PosiCntl; black open diamonds) and His-GST (NegCntl; black open circles) were used as a positive and negative controls, respectively. Black arrow indicates one outlier rabbit GIA data in the 200 μg PfRipr5/CAF®01 group. **(C)** Correlation between anti-PfRipr5 ELISA titers and GIA activities (n = 60). Pearson's correlation coefficient was calculated as r = 0.8779 (P<0.0001).

PfRipr5 (50 μg)/no-adjuvant group. In addition, significantly higher GIA activity was also observed in the high dose (200 μg) PfRipr5/CAF®01 than low dose (50 μg) PfRipr5/GLA-SE groups. No statistically significant difference was attained for the other GIA activities between high and low dose within the adjuvant groups and across the adjuvant groups. However, importantly, eight rabbit IgG samples out of 60 samples demonstrated ≧50% GIA activities, belonging to the PfRipr5/CAF®01 groups (high dose: five rabbits; and low dose: two rabbits) and high dose PfRipr5/GLA-SE (one rabbit). Notably, if one outlier rabbit in the high dose (200 μg) PfRipr5/CAF®01 group was excluded from the analysis (Figure 2B, highlighted with black arrow), the mean GIA activity in this group (57.0%) would be comparable to that of the positive control group (Mean %GIA = 72.2%). Although PfRipr5 alone group induced anti-PfRipr5 antibody titers (Figure 2A), no GIA activity was observed (Figure 2B), thus demonstrating that the antigen-adjuvant formulations here devised are essential to obtain enough levels of anti-PfRipr5 antibodies for functional activity.

Overall, the CAF®01 adjuvanted PfRipr5 induced antibodies with the highest GIA activity thus being the most suitable formulation for subsequent pre-clinical studies.

Correlation between ELISA titers and GIA activities

Anti-PfRipr5 antibody titers and GIA activities from 60 rabbits showed statistically significant positive correlation (Pearson's correlation coefficient, r = 0.8779; P<0.0001) (Figure 2C). Especially, a group of IgG samples with higher GIA activities (≧50%) and higher antibody titers were mainly from rabbits immunized with CAF®01 formulations. In Supplement Figure S1, higher mean GIA activity was observed in the high dose (200 μg) PfRipr5/CAF®01 than high dose (200 μg) PfRipr5/GLA-SE group with similar levels of anti-PfRipr5 antibody titers, although statistical difference has not been reached significant. This observation indicates that higher IgG titer correlates with higher GIA activity and suggests that further efforts should focus on how to increase the anti-PfRipr5 specific antibody titers for the development of a potent PfRipr5-based vaccine formulation.

Discussion

In this study, we tested head-to-head the adjuvanticity of PfRipr5 protein formulated with three adjuvants for human use (Alhydrogel®, GLA-SE, and CAF®01), to prioritize a PfRipr5-based malaria vaccine candidate for further advance its development. The PfRipr5 antigen herein produced was shown to be thermally stable in its monomeric form, having high purity and binding capacity to functional monoclonal anti-PfRipr antibody, thus assuring its quality for the subsequent use in animal experiments.

Polyclonal antibodies generated against PfRipr, PfCyRPA, or Rh5, were shown to inhibit merozoite invasion of *P. falciparum in vitro* (31–34), and thus the PfRipr/PfCyRPA/Rh5 complex components have been considered as promising asexual blood-stage vaccine candidates against *P. falciparum* malaria (35). Recently, Healer et al. (16) showed that rabbit antibodies raised against PfRipr resulted in significantly higher levels of GIA than those raised against either PfCyRPA or Rh5, whereas antibodies against the PfRipr/PfCyRPA/Rh5 complex showed the lowest level of inhibition, suggesting that the epitopes of some neutralizing antibodies were buried in the PfRipr/PfCyRPA/Rh5 ternary complex. These observations suggest that antibodies raised against PfRipr are more inhibitory than those raised against PfCyRPA, Rh5, or the PfRipr/PfCyRPA/Rh5 complex, and that vaccination with PfRipr alone might be sufficient to attain the desired parasite growth inhibition. In line with these findings, we have also previously shown strong growth inhibitory capacity of anti-PfRipr5 antibodies which also block PfRipr/Rh5 interaction, as well as that between PfRipr and its erythrocyte-surface receptor, SEMA7A (15). In the current study, we showed that all adjuvanted PfRipr5 formulations were stable during the period needed for vaccine administration. Furthermore, the observed immunogenicity, GIA activity of rabbit antibodies, and statistically significant positive correlation between antibody titers and GIA activities induced by PfRipr5 formulations suggest that PfRipr5 based vaccine development is feasible.

Recently, the first phase1/2a trial of Rh5 based blood-stage vaccine candidate, RH5.1, was conducted (11). In that study, the RH5.1/AS01B significantly reduced the *in vivo* parasite growth rate after blood-stage CHMI with *P. falciparum*. They also showed that *in vitro* GIA activity using purified human IgG significantly correlated with *in vivo* parasite growth rate. While the GIA measures a neutralization activity of purified IgG, the system lacks immune cells, complement, and other vaccine-induced antibody isotypes/subclasses. Therefore, in addition to the GIA activities induced by anti-PfRipr5 antibodies, it will still be worthwhile to investigate other immune pathways induced by PfRipr5 in first-in-human studies to evaluate the full potential of the PfRipr5 vaccine and its ability to induce *in vivo* efficacy and the capacity of natural infection to boost vaccine induced immune responses.

Some *P. falciparum* antigens are known to be highly immunogenic during natural infection (36, 37). In contrast, native PfRipr and Rh5 are weakly immunogenic antigens during natural infection (6, 10). Consistently, there was no evidence of natural boosting of anti-Rh5 antibodies in the primary CHMI using blood-stage *P. falciparum* challenge (11). Importantly, the current study shows that PfRipr5 antigen alone was immunogenic to rabbits without any adjuvant, although the generated antibodies could not induce significant GIA activities.

Investigating whether anti-PfRipr5 antibody titers can be boosted when vaccinated humans receive multiple natural infections or CHMIs could be performed in future studies to understand the possibility of natural boosting of the vaccine-induced anti-PfRipr antibody titers.

Protein-based subunit malaria vaccine candidates that have been developed to date have poor immunogenicity. Therefore, targeted delivery of subunit vaccines *via* systems possessing adjuvant properties is of paramount importance (18) to ensure effective delivery and ability to increase protective immunity (38); the latter requires neutralizing antibodies (39, 40) and optimal Th1-mediated immunity (41). Several new-generation adjuvants in vaccine formulations have been approved for human use (42). A number of adjuvants, Alhydrogel®, CpG ODN, Montanide ISA, GLA-SE, GLA-LSQ, Adjuvant Systems, Matrix-M, and CAF®01 have been used for clinical trials assessing subunit malaria vaccines (18, 26, 43), and selection of the proper adjuvant needs to be tested antigen by antigen manner. In this study, we have explored adjuvanticity of the PfRipr5 antigen formulated with Alhydrogel®, GLA-SE, or CAF®01 because Alhydrogel® is considered as the gold standard (19), GLA-SE showed better immunogenicity than Alhydrogel® in some malaria vaccine clinical trials (20–22), and CAF®01 was previously used as novel adjuvant for a malaria vaccine candidate (26). In this study, the GIA activities of IgG induced by low dose Alhydrogel® formulation, high dose GLA-SE, and low and high dose CAF®01 formulations showed statistically significant levels of GIA activities compared to the PfRipr5 no-adjuvant group. The high dose CAF®01 formulation showed the highest significance ($P<0.0001$) (Figure 2B). Furthermore, the number of rabbits with high GIA activities (≧50%) was highest in the PfRipr5/CAF®01 groups (high dose: 5/6 rabbits; and low dose: 2/6 rabbits). Finally, higher mean GIA activity was also observed in the high dose CAF®01 formulation than high dose GLA-SE group even with similar levels of anti-PfRipr5 antibody titers (Supplementary Figure S1). Although these results will be further strengthened in the future using larger number of animals to increase the statistical power, the PfRipr5/CAF®01 formulation was identified as the most promising vaccine candidate for further development because of its higher immunogenicity and induction of functional antibodies in rabbits. In fact, for the same antibody titers, the GIA activity of rabbit IgG induced by PfRipr5/CAF®01 formulation is higher than those induced by PfRipr5/GLA-SE formulations. These findings might be explained by the difference of antibody quality, such as epitope repertories and avidity. Additionally, the low dose of PfRipr5/Alhydrogel® formulation showed higher antibody titers than high dose of Alhydrogel® formulation. The immune mechanism underlining these results needs to be investigated further.

In general, it should be noted that it is difficult to predict adjuvanticity in humans from animal experiments. For instance, the use of a CAF®01-based formulation of GMZ2, one of the blood-stage malaria vaccine candidates, was superior to Alhydrogel® in preclinical studies but not in human trials (26). In contrast, a chlamydia vaccine candidate CTH522 adjuvanted with CAF®01 had a better immunogenicity than Alhydrogel® formulation in humans (25). Thus, adjuvanticity in humans is considered to be vaccine antigen dependent. Nonetheless, the fact that CAF®01 has the potential to induce potent inhibitory antibodies in rabbits supports further pre-clinical and clinical studies with this formulation.

In conclusion, we have identified that the GIA activity of rabbit IgG from PfRipr5/CAF®01 (200 μg) group was the highest among all the groups (approximately 50% inhibition), which is similar to the GIA activity of antibodies elicited against PfRipr5 with non-human applicable Freund's adjuvant formulation (15). Based on the promising GIA results, the PfRipr5/CAF®01 formulation is suggested as the most suitable for subsequent pre-clinical and clinical development.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Kitayama Labes Co., Ltd. (Ina, Japan). All animal immunizations were commercially conducted at Kitayama Labes Co., Ltd. (Ina, Japan).

Author contributions

ET, AR, AF, NV, HD and TT conceived and designed experiments. ET, HN, RC, DC, JG and AR conducted experiments. ET, AF, PA, AR and TT analyzed the data. ET, AF, NV, RC, HD and TT wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by the Japan-based Global Health Innovative Technology (GHIT) Fund (grant ID: T2018-151). This work was also supported in-part by JSPS KAKENHI (grant numbers: JP18H02651, JP20H03481, JP21H02724, JP21K06990, and JP21KK0138) and by a research fund from Sumitomo Pharma Co., Ltd. iNOVA4Health – UIDB/04462/2020 and UIDP/04462/2020, a program financially supported by Fundação para a Ciência e Tecnologia (FCT)/Ministério da

Ciência, Tecnologia e Ensino Superior, through national funds is acknowledged. Funding from INTERFACE Programme, through the Innovation, Technology and Circular Economy Fund (FITEC), is gratefully acknowledged. This work was supported by FCT through the initiatives "Investigador FCT" Program (IF/01704/2014), Exploratory Research and Development Project (IF/01704/2014/CP1229/CT0001), and PhD fellowship (Ricardo Correia - SFRH/BD/134107/2017). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

The Alhydrogel® was a kind gift from Dr. Erik B. Lindblad, R&D Unit, Croda, Denmark. We thank the Japanese Red Cross Society for providing human erythrocytes and plasma for culturing *P. falciparum* parasites. We also thank Miwa Ochi, Yuiko Ogasawara, Ai Yanase, and Yusuke Hara for their technical assistance, and Flavia D'Alessio, Ezenwa James Onyemata, Robert Adamu Shey and Masauso Moses Phiri for project management support.

Conflict of interest

AF is employed by Sumitomo Pharma Co., Ltd. and TT and ET are supported by a research fund from Sumitomo Pharma Co., Ltd. AF, TT, ET, and HN are inventors on patent PCT/JP 2017/040532 on a malaria vaccine antigen, PfRipr5. DC is co-inventor on patents on the cationic adjuvant formulations CAF. All rights have been turned over to SSI, which is a non-profit government research facility. JAG works for AAHI which has patent rights in the GLA-SE adjuvant formulation. These involvements did not influence the design of the study, the collection, analysis, access to, and interpretation of data, or the writing of the manuscript.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1002430/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Correlation between mean anti-PfRipr5 ELISA titers and mean GIA activities (n = 6 rabbits/group). PfRipr5 antigen only (black filled circle), Adjuvant only (open squares), 50 µg dose of PfRipr5 (filled triangles), 200 µg dose of PfRipr5 (filled diamonds), formulation with Alhydrogel® (Alum; blue), GLA-SE (GLA; magenta), and CAF®01 (CAF; purple). Pearson's correlation coefficient was calculated as r = 0.9438 (P<0.0001).

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frontiers | Frontiers in Immunology

TYPE Perspective
PUBLISHED 05 January 2023
DOI 10.3389/fimmu.2022.1006954

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasite Immunology, a section of the journal Frontiers in Immunology

RECEIVED 29 July 2022
ACCEPTED 09 December 2022
PUBLISHED 05 January 2023

CITATION
Roobsoong W, Yadava A, Draper SJ, Minassian AM and Sattabongkot J (2023) The challenges of *Plasmodium vivax* human malaria infection models for vaccine development. *Front. Immunol.* 13:1006954. doi: 10.3389/fimmu.2022.1006954

The challenges of *Plasmodium vivax* human malaria infection models for vaccine development

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Controlled Human Malaria Infection models (CHMI) have been critical to advancing new vaccines for malaria. Stringent and safe preparation of a challenge agent is key to the success of any CHMI. Difficulty producing the *Plasmodium vivax* parasite *in vitro* has limited production of qualified parasites for CHMI as well as the functional assays required to screen and down-select candidate vaccines for this globally distributed parasite. This and other challenges to *P. vivax* CHMI (*Pv*CHMI), including scientific, logistical, and ethical obstacles, are common to *P. vivax* research conducted in both non-endemic and endemic countries, with additional hurdles unique to each. The challenges of using CHMI for *P. vivax* vaccine development and evaluation, lessons learned from previous and ongoing clinical trials, and the way forward to effectively perform *Pv*CHMI to support vaccine development, are discussed.

KEYWORDS

***Plasmodium vivax in vitro* culture, controlled human malaria infection, CHMI, malaria human challenge study, *P. vivax* vaccine, Malaria Infection Study Thailand, MIST**

Introduction

Inducing human challenge by inoculation with malaria-infected blood was first used as a treatment (malariotherapy) for neurosyphilis in Europe and the United States in the early 1900s (1, 2).

More recently, Controlled Human Malaria Infection (CHMI) has been applied to the fields of malaria vaccine and drug development. The advent of *in vitro* culture methods for *P. falciparum* in the mid-1970s exponentially expedited studies on several aspects of *in vitro* research, one of which is the use of these cultured parasites in human infection studies (3, 4). By 1986, investigators at WRAIR published the first report on *Pf*CHMI

using mosquitoes infected with gametocytes from *in vitro* cultured parasites (5). Worldwide, thousands of healthy trial participants have been infected with *P. falciparum* sporozoites (6, 7) and more than 500 with blood-stage parasites (8–13). *Pf*CHMI is now well established in both non-endemic countries and numerous African trial sites and is an important tool in the rapid assessment and down-selection of candidate antimalarial drugs and vaccines. The re-establishment of mosquito-bite induced *Pv*CHMI under current ethical and regulatory guidelines was initiated in the mid-2000s. As opposed to *Pf*CHMI, where using laboratory-cultured gametocytes is feasible, the source of *P. vivax* gametocytes for infecting mosquitoes is naturally-infected humans. Until 2018 there had only been a handful of published studies in three areas of the world, Colombia, USA and Australia (14–22), and no experience in Europe until a group in Oxford (UK) was the second globally to produce a cryopreserved bank of a *P. vivax*-infected blood suitable for *Pv*CHMI (23). McCarthy and team were the first to use this technique to evaluate new drugs for *P. vivax* in healthy volunteers in Australia (14). This article summarizes the challenges and progress with continuous culture of *P. vivax*, the different challenge protocols (mosquito bite & blood stage infection), and the ethical and logistical issues in setting up *Pv*CHMI models for *P. vivax* vaccine development.

Continuous culture of *P. vivax* – An update

P. vivax has raised the bar when it comes to difficulties in conducting robust CHMI studies, particularly due to the lack of a continuous parasite culture method. Finding culture conditions that could support asexual propagation while maintaining productive gametocyte production would impact hugely on the time required to develop effective vaccines and drugs. The lack of a continuous *in vitro* culture system has thus long-hampered an in-depth understanding of this parasite's biology. Together, these obstacles have challenged the development of functional assays with which to screen and down-select candidate vaccines and drugs. This includes *in vitro* assays of growth inhibition activity (GIA) using cultured blood-stage parasites, widely used in the *P. falciparum* field to screen for functional antibody responses. Consequently, this has delayed the identification of optimal combinations of blood-stage antigens that could be targeted to successfully inhibit *P. vivax* blood-stage growth by vaccination. Several groups have recently succeeded in establishing short-term *P. vivax* culture for invasion inhibition assays using enriched reticulocytes from cord blood (24), but such methods are still dependent on access to fresh *P. vivax* isolates from patients, limiting the routine use of such assays to endemic regions. Moreover, in the absence of a *P. vivax* blood-stage culture system that can also yield gametocytes, the production of infected mosquitoes for sporozoite- and/or transmission-stage studies (25) also requires access to blood samples from *P. vivax* patients (14, 23). Filling this gap will be key to spear-heading *P. vivax* research and vaccine development.

Since the success of *P. vivax in vitro* culture using the Chesson strain adapted from non-human primate to human blood (26), several attempts to grow the *P. vivax* parasite exponentially *in vitro* have relied on two key factors: the culture micro-environments (26–30) and host reticulocytes (26–34). Reticulocytes derived from hematopoietic stem cells and immortalized erythroid progenitors have been shown to support *P. vivax* maturation (29, 32, 34–36); however, the production cost is still high and only small-scale production has been achieved. The culture microenvironments have direct impacts not only on parasite development but also stabilize the healthiness of the reticulocyte. Among different culture conditions and different sources of reticulocyte that have been tried (26, 28–30, 32–34, 37), none of these two-dimensional systems could lead to exponential growth nor reliable infective gametocyte production. The transcending progression from two-dimensional (2D) to three-dimensional (3D) culture systems, which mimic the microenvironment of the desired functional organ (38–40), could fuel the progression of the continuous culture of *P. vivax* blood stage. The 3D human bone marrow, which exhibits the structural features of human bone marrow while supporting the maintenance of hematopoietic stem cells (39), can be further utilized for *P. vivax* culture. On the other hand, progress has been made on vivax research using humanized mouse models. Two humanized mice models have been used to propagate *P. vivax* erythrocytic stage successfully (41–43). In the human liver-chimeric mouse model (huHep mouse), the mouse liver has been repopulated with human hepatocytes, and has been shown to support the complete exo-erythrocytic stage development of *Plasmodium spp* (42, 44). This huHep mouse model has been further utilized for *P. vivax* by infusing the human reticulocytes, allowing the exo-erythrocytic merozoites to invade and develop to erythrocytic stage, including gametocytes (43). The recently developed Human Immune System Human Erythrocyte mouse model (HIS-HEry), repopulated with human erythropoietic progenitors in mouse bone marrow, provides robust circulating human reticulocytes which support the *in vivo* propagation of *P. vivax* erythrocytic stage and importantly infective gametocytes (41). The passive transfer of *P. vivax* infected blood from the donor HIS-HEry infected mouse to the recipient uninfected mouse allows the continuous *in vivo* propagation of this parasite. These advances in 3D culture systems and humanized mouse models have enlightened *P. vivax* research, and drug and vaccine development.

How to challenge volunteers using CHMI: Mosquito-bite or blood-stage challenge model?

The type of *P. vivax* CHMI model (initiated by mosquito-bite delivered sporozoites or direct blood-stage inoculation) chosen for a particular clinical study will depend on the aspect of immunity that is being interrogated, and/or the intervention being tested (drug or vaccine) and/or the lifecycle stage against which these are active. An overview of challenge agent production for mosquito-bite and blood-stage *Pv*CHMI is summarized in Figure 1.

One advantage of mosquito-bite challenge is that it mimics the route of natural infection. However, it requires access to infected patients to initiate the production of infected mosquitoes and the constant production of mosquitoes, as well as relevant entomological expertise. There are also substantial logistics associated with the shipment of mosquitoes, safety testing of the donor blood sample(s) and timing with carefully pre-planned vaccination studies usually occurring in other locations or countries. It also inevitably leads to the use of genetically variable parasite isolates, and the issue of hepatic dormancy and potential relapse has to be accounted for when planning any clinical trial involving human participants (18).

In contrast, a blood-stage *P. vivax* CHMI model involves intravenous injection of blood-stage *P. vivax* parasites. Infected participants are monitored for the development of symptoms and blood-stage infection by daily qPCR in real-time. The parasite multiplication rate (PMR) is modeled from the qPCR data and the impact on PMR is usually the primary endpoint measure of vaccine "efficacy" for blood-stage vaccine candidates. A blood-stage CHMI model has been the preferred model to test blood-stage vaccines for *P. falciparum* (9, 45). This also offers a more feasible approach for *Pv*CHMI studies in a non-endemic setting, and aspects of these studies can be more easily standardized. In particular, this approach can allow the delivery of multiple studies with the same challenge strain of parasite, and the same inoculum size can be administered to each participant (9, 12). Because this route of infection bypasses the liver stage, there is no (known) risk of *P. vivax* relapse. However, *P. vivax* blood-stage inocula are not widely available.

Establishment of infected mosquitoes for *Pv*CHMI

Regulatory, logistical and ethical concerns

Regulatory requirements for conducting CHMI are stringent and vary according to the country in which they are being performed. Because there are no cultured lots of *P. vivax*,

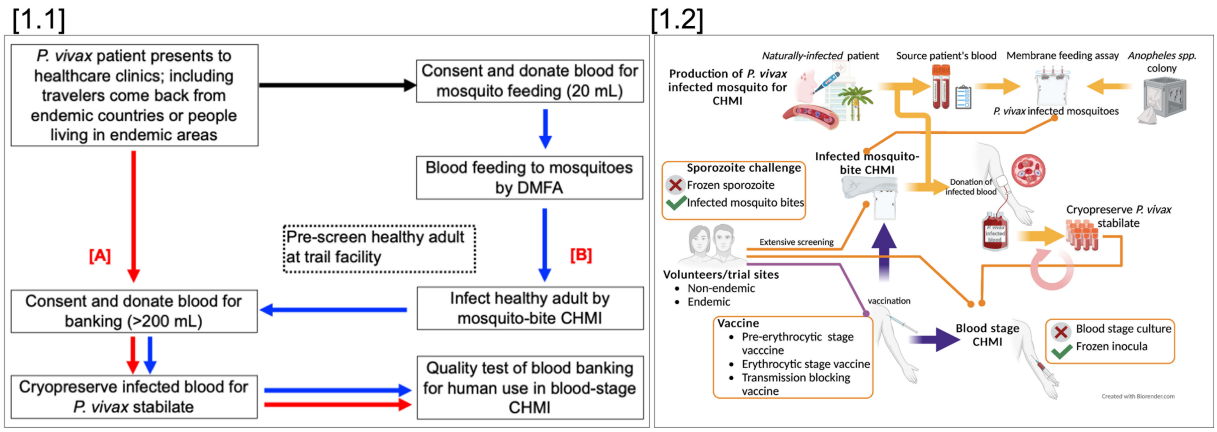

FIGURE 1
Overview of *Pv*CHMI to support vaccine development; [1.1] Strategies to produce a *P. vivax* infected blood bank. *P. vivax* infected blood can be obtained from *P. vivax* infected travelers returning from endemic areas or from patients seeking treatment at health facilities in endemic areas. Collected blood, after the informed consent process, is used to prepare blood banking directly (>200 mL) **(A)**, or fed to laboratory-reared mosquitoes through a direct membrane feeding assay (DMFA) to produce *P. vivax* infected-mosquitoes (less blood volume required for membrane DMFA). In parallel, healthy volunteers must be identified, pre-consented for mosquito-bite CHMI and a blood donation. Shipment of infected mosquitoes from the field site must then be critically timed to infect the donor volunteer(s) *via* mosquito-bite CHMI **(B)**. These volunteers are then monitored closely for blood donation to produce a *P. vivax* stabilate. [1.2] Utilization and challenges of different *Pv*CHMIs. Sporozoite-induced CHMI can be done through mosquito bite-challenge but not injection of sporozoites. Mosquito bite-CHMI can be used to evaluate all stages of vaccines including pre-erythrocytic stage vaccines, blood-stage vaccines, and transmission-blocking vaccines. Blood-stage induced CHMI can be used to evaluate blood-stage and transmission-blocking vaccines.

10.3389/fimmu.2022.1006954

parasites need to be obtained from infected human donors. Therefore, there are additional regulatory, logistical and ethical considerations in conducting *Pv*CHMI. Among the more important considerations are the potential for inadvertent transmission of adventitious agents from the donor to the recipient and the potential of relapse due to inadequate treatment of dormant hypnozoites. To ameliorate these concerns, the donor and recipient inclusion and exclusion criteria for *Pv*CHMI differ from those of *Pf*CHMI and are discussed below.

Plasmodium vivax-infected patient (donor) blood screening

After the bleeding of patients for mosquito feeding, a blood sample needs to be screened for blood- and vector-borne infections, due to the potential risks associated with the inadvertent transmission of such infections to subsequent healthy volunteers. All centers therefore screen the donors for blood borne diseases as per national blood and transplant guidelines. The main screening for blood-borne pathogens is similar in all trials. The different screening is usually related to mosquito-borne diseases that vary between regions/countries. For example, there is no local transmission of Chagas disease in Thailand, so that blood screening for this disease is not required while it transmits locally in Colombia; thus, the study will require screening for this disease in blood donors. Knowledge of infections with the potential for transmission by *Anopheles* spp. mosquitoes in these settings is an important consideration. Although *Anopheles* spp. mosquitoes are the primary vector for the transmission of malaria, some are known to transmit lymphatic filariasis and may serve as vectors for certain endemic viral infections (46). The mosquitoes for CHMI are derived from qualified laboratory-reared colonies, in order to minimize the transmission risk of other infections from the patient's blood (on which the mosquito has fed), region-specific vector-borne testing has been undertaken by all centers, including, but not limited to, Filaria, Chikungunya, Japanese encephalitis, Dengue, Zika and West Nile viruses. The list of mosquito-borne diseases for each country is available from the Ministry of Public Health and local CDC. The safety of the volunteers from the other mosquito-borne diseases after challenge by mosquito bite is the major concern for local IRBs in all countries conducting the trial. The highly qualified mosquito is an important key for success of sporozoite-*Pv*CHMI and can be evaluated from blood feeding rate and mortality rate, besides they must be free of other mosquito borne diseases. The high-quality vector colony usually does not depend on the number of years the colony has been established but rather on the staff experiences.

Healthy volunteer inclusion/exclusion criteria

Inclusion and exclusion criteria for volunteers in CHMI studies are usually rigorous, but for mosquito-bite delivered *P. vivax* malaria there are a few added complexities. The susceptibility of the mosquito-bite recipients to *P. vivax* infection needs to be confirmed, and this requires the Duffy antigen receptor for chemokines (DARC) (now called the atypical chemokine receptor 1, ACKR1) (33) to be present on the surface of their red cells. This requirement for DARC positivity also applies to *P. vivax* blood-stage CHMI. It is also important to ensure volunteers in mosquito bite-induced *Pv*CHMI do not have an adverse reaction to primaquine (PQ), as this is standard radical cure for dormant hypnozoites. PQ causes hemolysis in individuals deficient in glucose 6-phosphate dehydrogenase (G6PD) (47, 48); therefore, only subjects defined as having normal G6PD phenotype are recruited. In one *Pv*CHMI study (15), failure of radical cure with PQ was observed in two subjects resulting in multiple relapses. Investigations revealed that these individuals had either a non-functioning or reduced-functioning cytochrome P450 isoenzyme 2D6 (CYP2D6) genotype ("poor metabolizers" and "intermediate metabolizers" of PQ, respectively) and so were at greater risk for relapsing *P. vivax* malaria compared with those with a fully functioning CYP2D6 ("extensive metabolizers") (49). This was a note of warning that drug failure can be difficult to predict; Oxford were subsequently able to mitigate against this by screening their volunteers for CYP2D6 genotype prior to mosquito-bite *Pv*CHMI. As a final test, they monitored participants' sera for satisfactory clearance of PQ over 24 hours after administration of a test dose. These parameters are also of major relevance to the field as it is estimated that the combination of G6PDH deficiency and reduced functioning CYP2D6 account for nearly 40% of the population at risk of *P. vivax* infection ineligible for PQ therapy (50). For blood-stage CHMI in which hypnozoite formation does not occur, screening for G6PD and CYP2D6 can be omitted, as PQ treatment is not indicated.

Challenge to obtain *P. vivax* blood-stage parasites to set up a blood-stage *Pv*CHMI

Infected mosquitoes are required to produce a new cryopreserved stabilate bank of *P. vivax*-infected blood. To get infected mosquitoes, a blood donation from an infected patient is required. There are two possible strategies to achieve this goal (14, 23). The first is waiting for a returning traveler from an

endemic area with febrile *P. vivax* illness (Figure 1.1). This is the approach that the McCarthy group used to start parasite banking (14). This method is unpredictable in terms of timing and location, giving minimal notice and bringing logistical challenges for the clinical and laboratory teams, also giving no choice of patient or isolate. The second method is to produce *P. vivax*-infected mosquitoes from an endemic setting and to use the infected mosquitoes to bite volunteers (mosquito challenge-sporozoite *Pv*CHMI) to produce a blood-parasite bank for further blood-stage challenge studies. The latter allows for a more controlled and largely predictable process; however, it brings the added complication of conducting a small sporozoite *Pv*CHMI trial in carefully pre-screened "donor" individuals in order to obtain infected blood. An advantage is that different batches of mosquitoes can be selected to maximize the chance of obtaining a clonal isolate. Moreover, healthy volunteers can be carefully screened and selected in the desired (often non-endemic) country for blood group and other safety considerations so that they meet the criteria to become a safe "universal blood donor". Lastly, the timing of production of infected mosquitoes and subsequent *Pv*CHMI can be planned in advance, so that both the clinical and laboratory teams are fully prepared. Establishment of a good insectary for malaria transmission is not that simple. Choosing the right species of vector is important and parasite-vector competency is key to ensuring a good batch of *P. vivax*-infected mosquitoes for sporozoite-induced *Pv*CHMI. The ethics committees in Thailand only allow the university to establish colonies of local vectors, but not imported species. The logistics challenge to deliver infected mosquitoes from endemic countries to the trial site in the countries, or to non-endemic countries, are much different. Ground or air transport within the country will require less complicated arrangements, requires only short-time prior notification with less documentation. The delivery of the mosquitoes to the trial sites at non-endemic countries must follow International Air Transport Association (IATA) guidelines, which have specific requirements for documents related to the infected mosquitoes, and specific packing and labeling to ensure that safety precautions are implemented. The import permit to ship the mosquitoes to institutes located in different countries will differ. In the US, the recipient is required to obtain an import permit from the US-CDC, while a letter from the recipient's institute is required to receive the infected mosquitoes. Not all airlines will allow hand-carried infected mosquitoes into the passenger cabin and this needs to be arranged in advance. A possible alternative is to ship the infected mosquitoes *via* a commercial courier by packing them in a temperature-controlled box. This route would involve a longer transportation time from packing at the original site until arrival at the trial sites after customs clearance. In some cases, this took more than 72 h and only healthy infected mosquitoes could survive this mode of shipment. The logistical issue related to mosquito delivery will be a major concern for any trial being conducted in a non-endemic country.

P. vivax-infected healthy volunteers donating blood for future blood-stage *Pv*CHMI

A further complication arises when blood from *P. vivax*-infected volunteers is used to initiate future blood-stage *Pv*CHMI studies by intravenous administration to other volunteers. Apart from passing an extensive blood-borne infection screen, eligible volunteers need to be universal blood donors (Blood Group O, Rhesus D negative, RH-). This is required to minimize the risk of any transfusion reactions occurring with future administration of their parasitized red cells (i.e., the final *P. vivax* "challenge" inoculum). Testing the blood donor's red cells for the Kell antigen is also important if this is to be administered to female volunteers, due to the potential risk in pregnancy of developing hemolytic disease of the newborn in relation to Kell antigen incompatibility.

Learning from recent *Pv*CHMI studies

Until early 2022, mosquito-bite induced *Pv*CHMI had been conducted in just four countries – two endemic countries, Colombia and Thailand, and two non-endemic countries, the USA and UK. Blood-stage inoculation to induce *Pv*CHMI was first established by McCarthy and team, where *P.vivax*-infected blood was banked from infected patients directly (14, 51). The group at Oxford has also established a blood stage model to induce *Pv*CHMI, but instead used a controlled parasite banking method where healthy volunteers were carefully screened and selected for mosquito-bite infection with *P. vivax* before donating infected blood for banking. The specific studies to support vaccine development are briefly described here. Information generated from these studies have help research teams to design the better trials that suit to the local research environment (local IRB, logistics and regulatory).

Colombia

*Pv*CHMI, under modern guidelines, delivered *via* the bite of laboratory-reared, membrane-fed mosquitoes was established in the 2000s with yeoman's work done by Herrera and colleagues in Colombia. An insectary was established to ensure access to mosquitoes prior to the first study in Cali, Colombia. After infecting mosquitoes with blood from donors in Buenaventura,

they transported their mosquitoes to Cali- a distance of about 72 miles - to conduct *Pv*CHMI. In the first study (19) they performed bite-titration and established that 3 ± 1 bites resulted in a 100% infection success rate. This included the establishment of an insectary followed by establishing a reproducible infection in humans. Once established, the challenge model has been used to assess vaccine efficacy (20, 22). This is the first study to conduct the trial with less than 5 mosquito-bites. The following studies have used the standard 5 bites due to the different mosquito species—*An. albimanus* is the vector for Colombian studies, while *An. dirus* is the vector for WRAIR, Oxford and Thai studies. Arévalo-Herrera, et al, demonstrated that immunization of volunteers with *P. vivax* radiation-attenuated sporozoites (*Pv*RAS) was safe, immunogenic, and induced sterile immunity in 42% of the duffy positive volunteers in Colombia. This trial used significant numbers of volunteers for *Pv*RAS immunization compared with the trial in 1974 (52). The findings from this study confirm that immunization with *Pv*RAS is safe, immunogenic and induces sterile immunity in 42% of volunteers. This is the first study to confirm that inducing sterile protection with *Pv*RAS, as seen with *Pf*RAS, is possible. The study also identified some key immune determinants of sterile protection against *P. vivax*, which can guide the development of an effective vaccine against *P. vivax*. The detailed protocol used in this study is also published as a supplement.

WRAIR, USA

WRAIR, in collaboration with NMRC and AFRIMS in Bangkok, began by establishing a *Pv*CHMI model in the US in 2009 to assess the efficacy of a pre-erythrocytic stage vaccine. In the US, all human clinical studies are regulated by the US-FDA, in addition to the IRB. Following extensive review and additional guidance, *Anopheles dirus* mosquitoes were fed with blood collected from infected donors in northwestern Thailand. These mosquitoes were transported to Bangkok, a distance of about 300 miles, by road. An aliquot of donor blood was also shipped to the US for blood- and vector-borne testing. Batches of mosquitoes were hand-carried to the US in secure containers following approval obtained from the CDC for the import of infectious biological agents and vectors in accordance with 42 CFR section 71.54. and approvals from the US Department of Agriculture, the US Department of Transportation, Transport Security Administration, International Air Transport Association as well as the commercial airline. Following arrival, the mosquitoes were maintained in the WRAIR insectary before the conduct of *Pv*CHMI. Only United Airlines allowed hand-carriage of the infected mosquitoes onto the plane. Recently, United Airlines stopped flying between Bangkok and the US, so that mosquito shipment by this route is no longer available. Courier shipment has been used to ship mosquitoes from Thailand to the collaborators after the trials at WRAIR in 2009. The overall duration required from packing the mosquitoes to arrival at the destination insectary was usually about 60 h, but in certain cases with delayed flight and custom clearance, this may be up to 72 h. The key success factor is the quality of the mosquitoes, as longer shipment times affect the survival rate of the mosquitoes and impact on the sporozoite development required for sporozoite-*Pv*CHM.

Two separate lots of mosquitoes infected with *P. vivax*, genotyped as Type 1(VK210) based on the CSP sequence (53), were successfully transported and used to challenge a total of 12 subjects, 6 per study (54). Following this, a third lot was used to assess the efficacy of a CSP-based vaccine (15). The last study required more coordination as the planning and immunization schedule began approximately 4 months prior to the challenge. An unexpected challenge was faced following the third study, where two subjects experienced relapses despite treatment with PQ, as previously described (15, 49). Subsequent sporozoite-induced *Pv*CHMI studies have since excluded anyone who does not have an extensive metabolizer CYP2D6 phenotype.

Australia

The research team has established a method to prepare a *P. vivax*-infected blood bank for further intervention studies (14). This has accelerated the *Pv*CHMI, as the established protocol has shown a safe and reproducible clinical model in malaria-naive individuals. Collins et al. (51) demonstrated the safe, reproducible, and efficient transmission of *P. vivax* gametocytes from humans to mosquitoes, and established an experimental model that will accelerate the development of interventions targeting multiple stages of the *P. vivax* life cycle. More detailed protocols for steps to conduct the trial in this study were published as supplement to the paper. This provides a useful reference for other researchers who want to establish *Pv*CHMI, especially in non-endemic countries, starting with the *P. vivax* patient as blood donor. The advantage of preparing blood banking directly from *P. vivax* patients is high parasitemia. However, the large blood volume collected from symptomatic patients may raise concerns among IRBs in endemic countries for safety and feasibility to prepare blood samples for further use, as endemic populations usually stay in more remote areas with limited infrastructure and access to hospitals or public-health centers.

Oxford, UK: How to make the parasite bank and test it for human use?

To produce a cryopreserved stabilate of infected blood for *Pv*CHMI trials at Oxford, *P. vivax*-infected mosquitoes were obtained through a collaboration with the Mahidol Vivax

Research Unit in Bangkok, Thailand. The whole process from identifying cases in the field and feeding mosquitoes to extensive safety, viability and clonality testing in two different countries, shipping mosquitoes to the UK and finally infecting healthy pre-screened participants by mosquito-bite *Pv*CHMI, required the alignment of multiple stars, but was completed within just 14 days, as summarized in Figure 2. The preparation of infected mosquitoes for Oxford and the following Mahidol studies was similar to the WRAIR study.

Following *Pv*CHMI, initiated by five infectious mosquito bites, volunteers were monitored closely for parasitemia and symptoms. On day 14 post-CHMI, both volunteers, with parasites and symptoms, were admitted to the Oxford clinical trials unit, and parasitized erythrocytes collected *via* a 250 mL blood sample (23). The challenge agent is produced stringently and safely (55, 56) following guidance on the minimum requirements for human challenge agents manufactured outside a GMP facility, and is based on principles that can be applied across high-, middle- and low-income countries (55, 57). Neither IRB in the UK or Thailand had question the manufacture of the challenge agent outside of a strict GMP setting. The UK regulators did not request to review the blood bank development protocol outside of the context of a vaccine (CTIMP) study. However, the blood banking at Oxford was manufactured in-house under "GMP-like" conditions, with full QA/QP oversight, sterile conditions and full audit trail. Thailand also does not have specific regulation for challenging agents.

Following cryopreservation of the infected blood, the process of stringent quality control testing for a number of parameters began, including sterility, mycoplasma and endotoxin. In parallel, an extensive safety screen for blood-borne infections was performed on the plasma, and an *in vitro* short-term culture viability assay was set up. Parasite DNA was then isolated and sequenced (Sanger Institute, Cambridge), to allow the analysis of leading vaccine candidate antigens and multigene families, including the vivax interspersed repeat (VIR) genes. This high-quality genome was named PvW1 and its analysis is expected to guide the future assessment of candidate vaccines and drugs, as well as experimental medicine studies (23). Only the parasite CSP gene was identified from the *P. vivax* strain used in the WRAIR trials.

Thirty-seven healthy volunteers have to-date been infected by blood-stage *Pv*CHMI with the Thai PvW1 clone with no safety concerns (Hou MM et al., unpublished data). In addition, the inoculum has been used to test the only two available clinical-stage blood-stage *P. vivax* vaccine candidates; viral-vectors ChAd63 and MVA expressing *P. vivax* Duffy-binding protein region II (PvDBPII) and protein-in-adjuvant PvDBPII in Matrix-M™ adjuvant (58, 59). As a direct result of this work, the first ever efficacy result has been obtained for a *P. vivax* blood-stage vaccine (60). The next steps include efficacy testing of this leading vaccine candidate in both naïve and exposed populations in endemic Thailand.

Malaria infection study Thailand (MIST) Mahidol University, Thailand

The Malaria Infection Study Thailand (MIST) is underway. It commenced in 2018, and is the first in Asia.

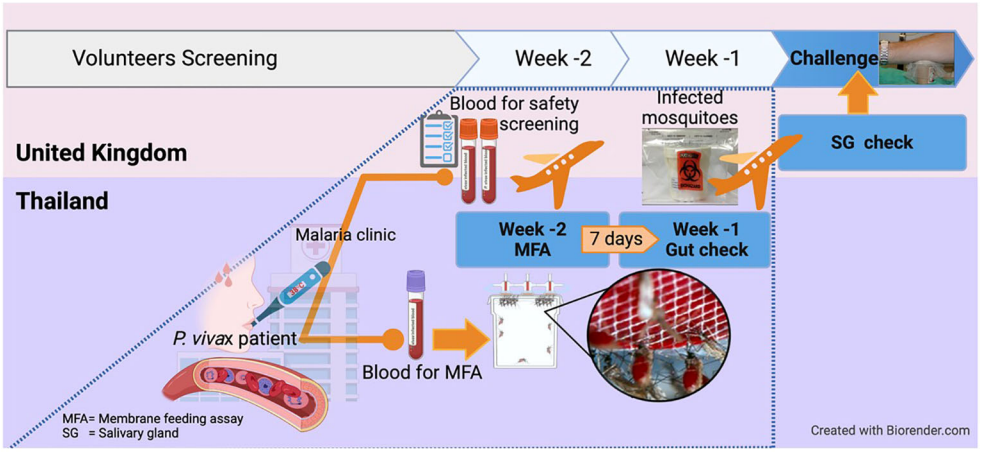

FIGURE 2
Excellent (time) match maker, the 2 weeks-notice! For mosquito-bite CHMI conducted in the UK, mosquitoes in Thailand were infected by *Plasmodium vivax*-infected patients and then shipped to the UK within 2 weeks. This is because *Plasmodium vivax* takes about 14 days for sporozoites to develop and reach the salivary glands, ready for CHMI. This also allowed time for completion of all safety laboratory tests required on the infected blood donor. Serum and whole blood samples were shipped to Oxford in real-time for extensive safety screening, including testing for blood-borne and vector-borne infections. Molecular speciation of the *P. vivax* isolate was also confirmed. The overall process required pre-organizing of all logistics to ship the patient blood and obtain the results within a week after collection, before mosquitoes were shipped on a pre-determined schedule.

The study protocols for mosquito-bite *Pv*CHMI (MIST-1), blood injection *Pv*CHMI (MIST-2) and blood stage vaccine evaluation (MIST-3) have been adapted from the Oxford studies with some modification to meet the requirements of the local populations and Institutional Review Board concerns. The first challenge study (MIST-1) was conducted in 2019, in two volunteers, to allow production of a *P. vivax*-infected blood stabilate (Sattabongkot, unpublished data). Several rounds of consultation with local IRBs were required before submitting the protocols to both Mahidol and Oxford and obtaining IRB approval. Since Thai IRBs had rejected a similar study proposed in the late 1990s due to concerns regarding relapse in volunteers, the MIST-1 protocol included information on the relapse pattern of *P. vivax* Thai isolates and the efficacy of PQ treatment in a Thai population (61), ensuring volunteer safety and follow-up. The MIST-2 protocol was revised many times due to the uncertain impact of the Covid-19 situation on the screening of healthy volunteers before admission to the trial ward. A limitation for blood banking is that only RH+ O+ volunteers could be recruited for MIST-1 as RH- status is rare in the Thai population. With stringent inclusion/exclusion criteria required by the local IRB and the safety concerns (including allergy to insect bites, other hematological tests and CYP450 status), the likelihood of eligibility was as low as 1:6 for MIST-1. Blood-stage vaccine evaluation (MIST-3) using blood-stage challenge is dependent on completion of the MIST-2 trial, as parasite development in Thai volunteers will be used to design the MIST-3 trial.

Discussion

Collaborative international efforts have led to the successful establishment of both sporozoite- and blood-stage *Pv*CHMI studies (14–21, 23). Despite the late start, and the complexities associated with a mosquito-bite induced *Pv*CHMI, studies in three non-endemic (UK, USA, and Australia), and two endemic countries (Colombia and Thailand) have been performed within less than 15 years, with one or both of these models (14–16, 23). The approach of producing a *P. vivax*-infected blood stabilate from healthy donors instead of patients facilitates the carefully planned and stringent production of the agent and the subsequent rigorous comparison of different vaccines or drugs by using the same *P. vivax* strain(s) across studies in different locations and populations. It also allows for the most relevant strain(s) for a particular region/population to be used. However, infection of the blood donor first requires a mosquito-bite induced *Pv*CHMI, which necessitates collaboration with teams working in endemic areas with an insectary of local vectors. Knowledge of disease epidemiology and parasite biology in different areas will help research teams plan for appropriate patient testing and optimize future *Pv*CHMI trial designs until parasite *in vitro* culture is better established. For vaccine efficacy trials that require sporozoite stage *Pv*CHMI, producing qualified *P. vivax* infected mosquitoes in endemic countries is still required until continuous culture of *P. vivax* producing infective gametocytes is established. This is the most crucial step to minimize all potential challenges to conduct *Pv*CHMI and accelerate the vaccine and drug development against *P. vivax*.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by VAC068 (ref 20, creation of Oxford blood bank): NCT03377296; Ethics: Approved by NRES Oxfordshire A Research Ethics Committee, ref 17/SC/0389; VAC069 (ref 20 – testing of blood bank in malaria-naïve recipients): NCT03797989; Approved by NRES South Central Hampshire A Research Ethics Committee, ref 18/SC/0577; VAC071 (ref 42 – viral-vectored DBP vaccine): NCT04009096; Approved by Oxfordshire Research Ethics Committee, ref 19/SC/0193; EudraCT (regulatory) ref 2019-000643-27; VAC079 (ref 42 – DBP protein in Matrix M vaccine): NCT04201431; Approved by Oxfordshire Research Ethics Committee A, ref 19/SC/0330; EudraCT (regulatory) ref 2019-002872-14; ForMIST1: •FTMEC: MUTM 2020-038-03, •OxTREC: 43-19, •Clinical trial registration number: NCT4083508; For MIST2: •FTMEC: MUTM 2021-047-02, •OxTREC: 15-21, •Clinical trial registration number: NCT05071079

In the review of published work from other groups, the EC approval statements can be reviewed from original publications. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Funding

The *Pv*CHMI work in Oxford by AMM and SJD was funded, in part, by the European Union's Horizon 2020 research and innovation program under grant agreement 733073 for MultiViVax; the United Kingdom Medical Research Council (MRC) Confidence in Concept Scheme at the University of Oxford (MC_PC_16056); and by the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC). The views expressed are those of the authors and not necessarily those of the NIHR or the Department of Health and Social Care. Further *Pv*CHMI work in Oxford and Thailand by WR, AM, SD and JS was funded by the Wellcome Trust Malaria Infection Study in Thailand (MIST) program [212336/Z/18/Z].

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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