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Sequential Phase 1 and Phase 2 randomized, controlled trials of the safety, immunogenicity and efficacy of combined pre-erythrocytic vaccine antigens RTS,S and TRAP formulated with AS02 Adjuvant System in healthy, malaria naïve adults

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ABSTRACT

In an attempt to improve the efficacy of the candidate malaria vaccine RTS,S/AS02, two studies were conducted in 1999 in healthy volunteers of RTS,S/AS02 in combination with recombinant *Plasmodium falciparum* thrombospondin-related anonymous protein (TRAP). In a Phase 1 safety and immunogenicity study, volunteers were randomized to receive TRAP/AS02 (N = 10), RTS,S/AS02 (N = 10), or RTS,S + TRAP/AS02 (N = 20) at 0, 1 and 6-months. In a Phase 2 challenge study, subjects were randomized to receive either RTS,S + TRAP/AS02 (N = 25) or TRAP/AS02 (N = 10) at 0 and 1-month, or to a challenge control group (N = 8). In both studies, the combination vaccine had an acceptable safety profile and was acceptably tolerated. Antigen-specific antibodies, lymphoproliferative responses, and IFN-γ production by ELISPOT assay elicited with the combination vaccine were qualitatively similar to those generated by the single component vaccines. However, post-dose 2 anti-CS antibodies in the RTS,S + TRAP/AS02 vaccine recipients were lower than in the RTS,S/AS02 vaccine recipients. After challenge, 10 of 11 RTS,S + TRAP/AS02 vaccinees, 5 of 5 TRAP/AS02 vaccinees, and 8 of 8 infectivity controls developed parasitemia, with median pre-patent periods of 13.0, 11.0, and 12.0 days, respectively. The absence of any prevention or delay of parasitemia by TRAP/AS02 suggests no apparent added value of TRAP/AS02 as a candidate vaccine. The absence of significant protection or delay of parasitemia in the 11 RTS,S + TRAP/AS02 vaccine recipients contrasts with previous 2 dose studies of RTS,S/AS02. The small sample size did not permit identifying statistically significant differences between the study arms. However, we speculate, within the constraints of the challenge study, that the presence of the TRAP antigen may have interfered with the vaccine efficacy previously observed with this regimen of RTS,S/AS02, and that any future TRAP-based vaccines should consider employing alternative vaccine platforms.

1. Introduction

Adjuvanted RTS,S (RTS,S/AS), a candidate malaria vaccine consisting of the recombinant protein RTS,S, which is comprised of sequences of the circumsporozoite protein (CSP) and hepatitis B surface antigen (HBsAg), is uniquely able to protect malaria-naïve adult subjects after experimental malaria challenge against infection [1–5], and African adults and children exposed to diverse strains against clinical and severe disease [6–11]. Other strategies have been concurrently explored to improve the efficacy of adjuvanted RTS,S, including formulation with more potent adjuvants [12–14], prime-boost regimens with alternative vaccine platforms expressing the CSP [15–18] and evaluation of other adjuvanted *Plasmodium falciparum* antigens [19–21] individually or in combination with RTS,S [22,23]. We report two clinical evaluations which aimed at improving adjuvanted RTS,S by combining it with the recombinant thrombospondin related anonymous protein (TRAP) of *P. falciparum*, PfTRAP [24]. PfTRAP is one of several adhesive proteins [25] naturally expressed in sporozoite [26] and hepatic stages [27]. The candidacy of PfTRAP as a vaccine antigen is supported by several considerations. First, PfTRAP, like CSP, binds specifically to sulfated glycoconjugates on hepatic cells [28], suggesting an essential role in sporozoite infectivity, confirmed using PfTRAP knockout parasites [29]. Second, immunization of rodents with PfTRAP analogs alone or in combination with CSP protects them against parasitemia after experimental challenge with infectious sporozoites [30,31]. Third, several Phase 2 trials of a viral-vectored PfTRAP-based multi-antigen vaccine have consistently delayed [32,33], and in some instances prevented [34], patent parasitemia after experimental challenge with mosquito-borne malaria.

We present the initial Phase 1 study conducted to assess the safety and immunogenicity of RTS,S/AS combined with PfTRAP, and the subsequent Phase 2 study in malaria naïve adults to assess safety, immunogenicity, and efficacy.

2. Methods

2.1. Study subjects and eligibility

The Phase 1 trial was conducted in males or females 18–50 years old at the Clinique Notre-Dame de Grâce, Gosselies, Belgium. The Phase 2 challenge trial, conducted at the Walter Reed Army Institute of Research (WRAIR), USA, enrolled male or females aged 18–45 years, with no history of malaria or previous administration of an investigational malaria vaccine. In both studies, subjects were eligible if healthy as established by medical history, clinical examination and laboratory screening, and were seronegative for HBsAg and hepatitis C. The Phase 1 study started in 1998 and was completed in 1999 and the Phase 2 study was conducted and completed in 1999 (see Supplementary Appendix).

2.2. Study design

Subjects in the Phase 1, open trial, were randomized to TRAP/AS02, RTS,S/AS02 or TRAP + RTS,S/AS02 groups (ratio 1:1:2) to receive 3 doses of vaccine administered at 0, 1, 6-months.

The Phase 2, double-blind, challenge trial was originally planned to recruit subjects to 2 cohorts; the first cohort to undergo sporozoite challenge after 2 doses and the second after 3 doses of study vaccine. Due to lack of protective efficacy of both vaccines in the first cohort, the second cohort was not enrolled. Subjects in cohort 1 were randomized to receive 2 doses of RTS,S + TRAP/AS02 or TRAP/AS02 (ratio 2.5:1) at 0, 1-months, with sporozoite-infected mosquito challenge planned for 7–30 days after Dose 2. A second randomization was conducted prior to challenge (for details, see Supplementary Appendix).

2.3. Vaccines

The two recombinantly produced vaccine antigens, RTS,S and TRAP, were manufactured by GlaxoSmithKline (GSK) Biologicals (Rixensart, Belgium). The RTS,S vaccine antigen has been described [12]. The TRAP antigen is a recombinant protein produced in, and purified from, the culture supernatant of insect cells (*Spodoptera frugiperda* Sf9 cell line) infected with a recombinant baculovirus (AcMNPV). The baculovirus expresses a truncated form of the TRAP gene derived from *P. falciparum* strain NF54 (clone 3D7). The final purified antigen consists of a 493 amino acid long polypeptide comprising amino acids 26 (arginine/R) to 511 (lysine/K) of the authentic TRAP protein, extended at its carboxy terminal end by the addition of 7 histidine residues. The antigens (RTS,S/TRAP or TRAP) were presented as lyophilized pellets in single dose vials. Just before administration, each pellet was reconstituted with liquid AS02 Adjuvant System [12]. Subjects received 50 μg RTS,S or 25 μg TRAP or both 50 μg of RTS,S and 25 μg of TRAP together with 50 μg MPL, and 50 μg QS21 in an oil/water emulsion as a 0.5 mL dose, by intramuscular injection.

2.4. Safety assessments

Local and systemic adverse events (AEs) were systematically assessed using standardized criteria as previously reported [2] (see Supplementary Appendix). All unsolicited reports of AEs occurring within 30 days, and of reactogenicity within 4 days, of vaccination were recorded. Serious AEs (SAEs) were collected throughout the study. Hematological and biochemical tests for safety evaluation were performed and any clinically significant values noted.

2.5. Assessment of humoral immune response

Antibodies (IgG) against the CS central repeat tetrapeptide epitopes were measured using ELISA with recombinant R32LR as the capture antigen as described previously [35,36]. Antibodies against TRAP were measured by ELISA using the vaccine antigen as the capture antigen, and expressed as titers.

2.6. Assessment of CMI response

2.6.1. Peripheral blood mononuclear cell collection

For both studies, the peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood on a density gradient and stored in liquid nitrogen as described previously [37].

2.6.2. Lymphoproliferative assay

Lymphoproliferative (LP) results were expressed as stimulation indices (SI*) which are the ratio between the quantities of 3*H*-thymidine incorporated by the cells in the presence of a specific antigen and the ones incorporated by the cells cultured in medium alone (for assay methodologies, see the Supplementary Appendix).

2.6.3. Antigen-stimulated IFN-γ and IL-5 secretion by PBMC

IFN-γ and IL-5 secretion by whole PBMC was measured in supernatant harvested from antigen-stimulated PBMC after 120 h by commercial ELISA kit (respectively IFN-γ EASIA®; Medgenix, Fleurus, Belgium or Biosource International, Camarillo, CA). Further detail is provided in the Supplementary Appendix.

2.6.4. Ex vivo IFN-γ and IL-4 ELISPOT assays

ELISPOT assays were conducted as previously described (see Supplementary Appendix) [5,38].

2.7. Efficacy assessment

Immunized volunteers and infectivity controls underwent standardized malaria challenge (day of challenge [DOC]) over 2 consecutive days, ranging from 16 to 27 days, after the second immunization. Parasitemia was detected through daily blood films starting 7 days post challenge; volunteers were censored at 30 days post challenge if no parasitemia was detected. Volunteers who developed parasitemia were treated with a standard oral course of chloroquine (total 1500 mg base given in divided doses: 600 mg initially followed by 300 mg at 6, 24 and 48 h) under direct supervision.

2.8. Statistical analyses

2.8.1. Study cohorts

For the Phase 1 trial all analyses are presented for the intention to treat (ITT) population which included all subjects who received at least 1 dose of study vaccine. For the Phase 2 trial, safety data are presented for the ITT population and immunogenicity and efficacy data for a modified ITT population, excluding volunteers receiving vaccine subject to temperature deviations (see Section 3.1).

2.8.2. Analysis of reactogenicity and safety

Summaries were calculated for the incidence, intensity, and relationship of solicited and unsolicited AEs (see Supplementary Appendix).

2.8.3. Analysis of humoral immune responses

The percentage of subjects with seropositive levels of anti-CS antibodies ($\geq$1 μg/mL) was determined. Antibody titers were summarized by GMT with 95% CI. GMT calculations were performed by taking the anti-log of the mean of the log titer transformations. Anti-CS antibody titers of <1 μg/mL were assigned a value of 0.5 μg/mL for the purpose of GMT calculation. For each vaccine group, anti-TRAP antibody titers were described and GMTs with 95% CI were calculated; no 0 values were found.

2.8.4. Analysis of cell mediated immune responses

Descriptive analyses in terms of LP response, expressed as stimulation indices (SI*), and measurements of IFN-γ and IL-5 secretion in the culture supernatant of the stimulated cells, are shown for the Phase 1 study. Results for ELISPOT assays were described as spot forming cells per million for the Phase 2 study.

2.8.5. Sample size

Both studies were designed to assess the safety, immunogenicity and efficacy (Phase 2 study only) of each individual vaccination regimen, and not for the support of inter-group comparisons. Only descriptive analysis was planned and the sample size was not statistically computed.

2.8.6. Analysis of efficacy

Efficacy was assessed by comparison of malaria incidence and time to onset of parasitemia. Fisher's Exact test was used for the comparison of malaria incidence between the control and each treated group. A Kaplan–Meier analysis was performed on time to onset of parasitemia, testing between the control and the two treatment groups using the log-rank statistic.

3. Results

3.1. Subject cohort

The study flow for both trials is provided in Fig. 1.

In the Phase 1 study, 40 subjects were enrolled and randomized (RTS,S/AS02 *N* = 10, TRAP/AS02 *N* = 10, RTS,S + TRAP/AS02 *N* = 20). The mean age of subjects was 34.3 years (range: 19–48 years), 60% were males and all were Caucasian.

In the Phase 2 study, 43 subjects were enrolled (RTS,S + TRAP/AS02 *N* = 25, TRAP/AS02 *N* = 10, control *N* = 8). Thirteen recipients of Dose 2 of RTS,S + TRAP/AS02 and 5 recipients of Dose 2 of TRAP/AS02 inadvertently received vaccine that may have been transiently stored at subzero temperatures. As temporary freezing might have reduced the potency of the vaccine, these subjects were excluded from participating in the malaria challenge. Of the 43 subjects enrolled, the mean age was 34.2 years (range: 20–45 years), 61% were males and the majority were Caucasian (49%) or African–American (40%).

3.2. Safety outcomes

Transient pain at the injection site was the most frequently reported solicited local AE across vaccine groups in both studies, occurring with a similar incidence in each vaccine group (after 87–100% of doses) (Table 1). The frequency of Grade 3 pain was similar after vaccination across vaccine groups and studies (after 17–35% of doses). Grade 3 redness and swelling occurred after <7% of doses in any vaccine group. All Grade 3 AEs resolved within the initial 72-h follow-up period after each vaccination, with the majority of symptoms resolved within the first 24 h.

The most frequently reported solicited general symptom in the Phase 1 study was myalgia (after 47–63% of doses across groups) and in the Phase 2 study fatigue (after 30–32% of doses across groups). Grade 3 general AEs occurred after <7% of doses in any vaccine group. In the Phase 1 study all Grade 3 symptoms were considered to have a 'probable'/'suspected' (PB/SU) relationship to vaccination and in the Phase 2 study, one report of Grade 3 malaise in a recipient of RTS,S + TRAP/AS02 was judged to have a PB/SU relationship to vaccination.

Unsolicited AEs with a PB/SU relationship to vaccination were infrequent: influenza-like symptoms in 7 subjects (2 TRAP/AS02, 1 RTS,S/AS02, 4 RTS,S + TRAP/AS02), rigors in 1 subject (RTS,S + TRAP/AS02) and hypesthesia (numbness of arm lasting 2 days) in 1 subject (RTS,S + TRAP/AS02) in the Phase 1 study; flu-like symptoms in 1 subject (RTS,S + TRAP/AS02) and upper respiratory tract infection in 1 subject (RTS,S + TRAP/AS02) in the Phase 2 study. No unsolicited AE with a PB/SU relationship to vaccination was of Grade 3 intensity.

In both studies, no SAE was reported and no subject was withdrawn because of an AE. No clinically significant hematological, biochemical, or urine abnormalities were observed.

3.3. Humoral immunogenicity outcomes

3.3.1. Anti-CS response

In both studies, prior to vaccination, no volunteer had anti-CS antibodies (Table 2). In the Phase 1 study, the post immunization anti-CS GMTs at each timepoint were higher, but not statistically so, after administration of RTS,S/AS02 compared to RTS,S + TRAP/AS02. Post Dose 2, the anti-CS GMT in the RTS,S/AS02 group (85 μg/mL [95% CI: 53, 138]) tended to be higher than the RTS,S + TRAP/AS02 group (56 μg/mL [95% CI: 31, 100]) and higher than that of the corresponding Phase 2 post Dose 2 anti-CS GMT in the RTS,S + TRAP/AS02 group (35 μg/mL [95% CI: 20, 62]).

A. Phase 1 Safety and Immunogenicity Study
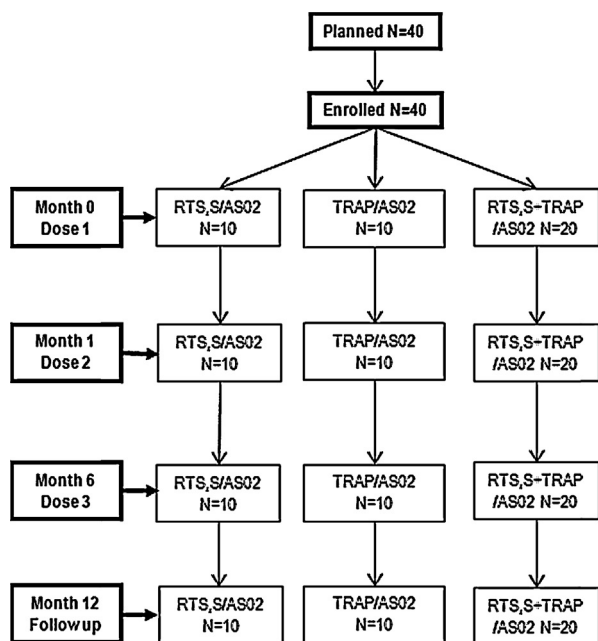
B. Phase 2 Challenge Study

Fig. 1. Study flow diagrams.

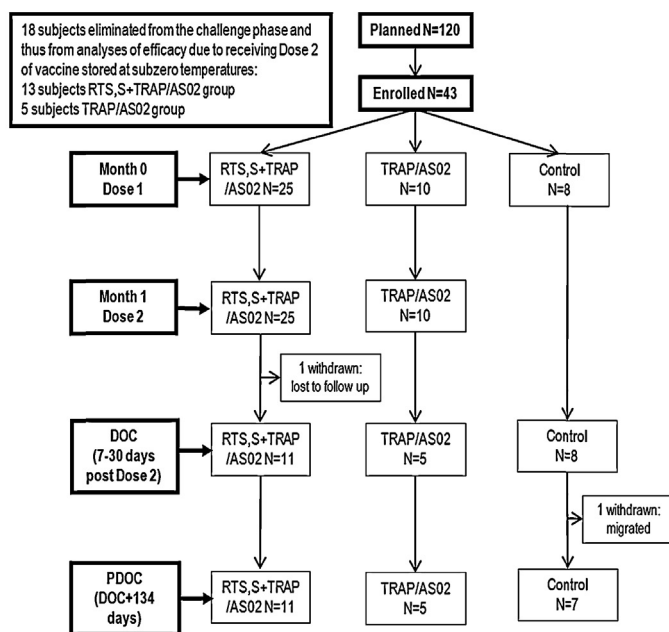In the Phase 1 study, all 40 subjects received all 3 doses of study vaccine. In the Phase 2 study, all 35 volunteers who received study vaccine received both doses; 13 recipients of Dose 2 of RTS,S + TRAP/AS02 and 5 recipients of Dose 2 of TRAP/AS02 received vaccine that may have been transiently stored at subzero temperatures and were excluded from malaria challenge due to the possibility of reduced vaccine potency. Replacement vaccine for the second dose was administered to the remaining 12 RTS,S + TRAP/AS02 subjects and 5 TRAP/AS02 subjects. DOC—day of challenge; PDOC—post day of challenge.

3.3.2. Anti-TRAP response

In the Phase 1 study, an increase in anti-TRAP GMTs was observed after subsequent doses of TRAP/AS02 and RTS,S + TRAP/AS02 (Table 3); GMTs were similar in both groups. Six months post Dose 3 (day 360), anti-TRAP GMTs had decreased to post Dose 2 GMT levels in both the TRAP/AS02 and RTS,S + TRAP/AS02 groups.

In the Phase 2 study, the highest anti-TRAP GMTs were observed post Dose 2 (DOC) in both the TRAP/AS02 and RTS,S + TRAP/AS02 groups; GMTs were similar in both groups. At 134 days post DOC,

Table 1

Incidence of solicited symptoms reported after all doses (total vaccinated cohort).

| | | Phase 1 safety and immunogenicity study | | | | | | Phase 2 challenge study | | | |
| | | TRAP/AS02 N = 30 | | RTS,S/AS02 N = 30 | | RTS,S + TRAP/AS02 N = 60 | | RTS,S + TRAP/AS02 N = 50 | | TRAP/AS02 N = 20 | |
Local symptoms		N	%	N	%	N	%	N	%	N	%
Pain	Total	26	87	26	87	50	83	46	92	20	100
	Grade 3	5	17	6	20	10	17	14	28	7	35
Redness	Total	4	13	6	20	20	33	13	26	7	35
	>50 mm	1	3	2	7	1	2	3	6	0	0
Swelling	Total	6	20	11	37	14	23	7	14	2	10
	>50 mm	0	0	0	0	2	3	0	0	0	0
General symptoms											
Arthralgia/joint pain	Total	10	33	7	23	20	33	4	8	0	0
	Grade 3	0	0	0	0	1	2	3	6	0	0
Fatigue	Total	9	30	11	37	31	52	16	32	6	30
	Grade 3	0	0	0	0	3	5	0	0	0	0
Fever	Total	4	13	5	17	20	33	5	10	0	0
	>39 °C	1	3	2	7	2	3	0	0	0	0
Gastrointestinal	Total	2	7	4	13	9	15	5	10	0	0
Headache	Total	8	27	6	20	16	27	10	20	5	25
Malaise	Total	0	0	1	3	3	5	12	24	2	10
	Grade 3	0	0	0	0	0	0	1	2	0	0
Myalgia	Total	17	57	14	47	38	63	10	20	4	20
	Grade 3	0	0	1	3	0	0	0	0	0	0

N—number of administered doses.

N/%—number/percentage of doses followed by at least one type of symptom.

Grade 3 pain—spontaneously painful when moved.

Grade 3 general symptoms—adverse event which prevented normal, everyday activities.

All Grade 3 symptoms resolved during the 72-h follow-up period following each vaccination.

Table 2
Seropositivity rates and GMTs for anti-CS antibodies.

Phase 1 study	TRAP/AS02			RTS,S/AS02			RTS,S + TRAP/AS02		
Timing	N	S+ N (%)	GMT [95% CI] (μg/mL)	N	S+ N (%)	GMT [95% CI] (μg/mL)	N	S+ N (%)	GMT [95% CI] (μg/mL)
Prevaccination	10	0 (0.0)	<0.5	10	0 (0.0)	0.1 [0.0, 0.2]	20	0 (0.0)	<0.5
14 Days PI (day 14)	10	0 (0.0)	<0.5	10	10 (100)	14.9 [6.6, 33.8]	20	18 (90)	9.2 [3.9, 21.6]
14 Days PII (day 42)	10	0 (0.0)	<0.5	10	10 (100)	85.4 [52.9, 137.6]	20	19 (95)	55.8 [31.0, 100.4]
14 Days PIII (day 194)	10	0 (0.0)	<0.5	10	10 (100)	150.0 [95.2, 236.1]	20	19 (95)	59.8 [27.7, 129.1]
6 Months PIII (day 360)	10	0 (0.0)	<0.5	10	10 (100)	72.4 [39.5, 132.7]	20	18 (90)	29.0 [11.1, 75.6]

Phase 2 study	RTS,S + TRAP/AS02			TRAP/AS02		
Timing	N	S+ N (%)	GMT [95% CI] (μg/mL)	N	S+ N (%)	GMT [95% CI] (μg/mL)
Prevaccination	12	0 (0)	–	5	0 (0)	–
14 Days PI (day 14)	12	10 (83)	7.9 [1.7, 36.2]	5	1 (20)	<0.5
28 Days PI (day 28)	12	10 (83)	8.5 [2.3, 31.5]	5	0 (0)	<0.5
16–27 Days PII (DOC)	11	11 (100)	35.1 [19.7, 62.2]	5	0 (0)	<0.5
DOC + 134 days	10	9 (90)	6.2 [2.6, 14.8]	5	0 (0)	<0.5

S+—seropositivity defined as ≥1.0 μg/mL of anti-CS antibody. Anti-CS antibody titers of <1 μg/mL were assigned a value of 0.5 μg/mL for the purpose of GMT calculation.
N—number of subjects tested (Phase 1 study, ITT cohort; Phase 2 study, subjects receiving normal vaccine at Dose 2 in modified ITT immunogenicity cohort).
PI, PII and PIII—post Dose 1, post Dose 2 and post Dose 3.
DOC—day of challenge.

anti-TRAP GMTs had decreased but were still above post Dose 1 values in both vaccine groups.

3.3.3. Cell mediated immunogenicity outcomes

In the Phase 1 study, antigen specific proliferative responses to RTS,S in recipients of RTS,S/AS02 or RTS,S + TRAP/AS02 and to TRAP in recipients of TRAP/AS02 or RTS,S + TRAP/AS02 were markedly elevated over baseline values. Proliferation to RTS,S was similar in both the RTS,S/AS02 and RTS,S + TRAP/AS02 groups and to TRAP in both the TRAP/AS02 and RTS,S + TRAP/AS02 groups (see Supplementary Appendix). Cellular responses were boosted by the third vaccination and responses persisted at day 360.

Measurements of IFN-γ and IL-5 in culture supernatant in response to antigen-specific stimulation showed substantial induction post second vaccination; no meaningful increase was observed post third vaccination. No real differences in RTS,S stimulated responses were observed between RTS,S and RTS,S/TRAP vaccinated groups (see Supplementary Appendix).

In the Phase 2 study, RTS,S stimulated IFN-γ responses in PBMC cultures derived from subjects vaccinated with RTS,S + TRAP/AS02 greatly exceeded baseline responses (Fig. 2). RTS,S did not elicit IFN-γ responses in PBMC cultures from subjects vaccinated with TRAP/AS02. TRAP-specific IFN-γ responses were observed in PBMC cultures from RTS,S + TRAP as well as TRAP vaccinated subjects, but not in pre-vaccination PBMC cultures.

Analysis of IL-4 responses in parallel cultures of PBMC from pre- and post-vaccinated subjects showed a similar pattern of reactivity (Fig. 3). Pre-immune PBMC showed no notable responses to either RTS,S or to TRAP. Post vaccination IL-4 responses elicited with RTS,S and TRAP were antigen-specific in that TRAP recalled responses in TRAP and RTS,S + TRAP recipients, whereas RTS,S recalled responses only in RTS,S + TRAP vaccinees. Of note, while PBMC from RTS,S + TRAP recipients showed higher IFN-γ responses to RTS,S than TRAP, results for IL-4 responses to both antigens were similar.

3.4. Vaccine efficacy

Of the 24 volunteers who underwent challenge, patent parasitemia developed in 10 of 11 RTS,S + TRAP/AS02 vaccinees, all 5 TRAP/AS02 vaccinees, and all 8 infectivity controls (Fig. 4). Fisher's exact tests of the proportion of subjects infected indicated that neither vaccinated group differed from control (p = 1.0). The median pre-patent period from challenge to infection was 13.0, 11.0 and

Table 3
GMTs for anti-TRAP antibodies.

Phase 1 study[a]	TRAP/AS02		RTS,S/AS02		RTS,S + TRAP/AS02	
Timing	N	GMT [95% CI] (EL.U/mL)	N	GMT [95% CI] (EL.U/mL)	N	GMT [95% CI] (EL.U/mL)
14 days PI (Day 14)	8	304 [129,721]	–	–	16	341 [236,494]
14 days PII (Day 42)	10	2251 [1050,4828]	–	–	20	2178 [1308,3627]
14 days PIII (Day 194)	10	4410 [3136,6201]	2	235 [189,292]	20	3152 [2212,4491]
6 months PIII (Day 360)	10	1879 [1472,2397]	6	231 [121,440]	20	1411 [886,2247]

Phase 2 study	RTS,S + TRAP/AS02		TRAP/AS02	
Timing	N	GMT [95% CI] (EL.U/mL)	N	GMT [95% CI] (EL.U/mL)
14 Days PI (day 14)	12	79 [16,389]	5	7 [0.1, 547]
28 Days PI (day 28)	12	22 [3,187]	5	20 [0.3, 1362]
16–27 Days PII (DOC)	11	2237 [1052,4759]	5	1877 [1468,2399]
DOC + 134 days	11	178 [23,1367]	5	628 [328,1204]

[a] RTS,S/AS02 group: anti-TRAP antibody assay results for any subject at 14 days post Dose 1 or 14 days post Dose 2 and for many subjects at other timepoints were not available.
N—number of subjects tested (Phase 1 study, ITT cohort; Phase 2 study, subjects receiving normal vaccine at Dose 2 in modified ITT immunogenicity cohort).
PI, PII and PIII—post Dose 1, post Dose 2 and post Dose 3.
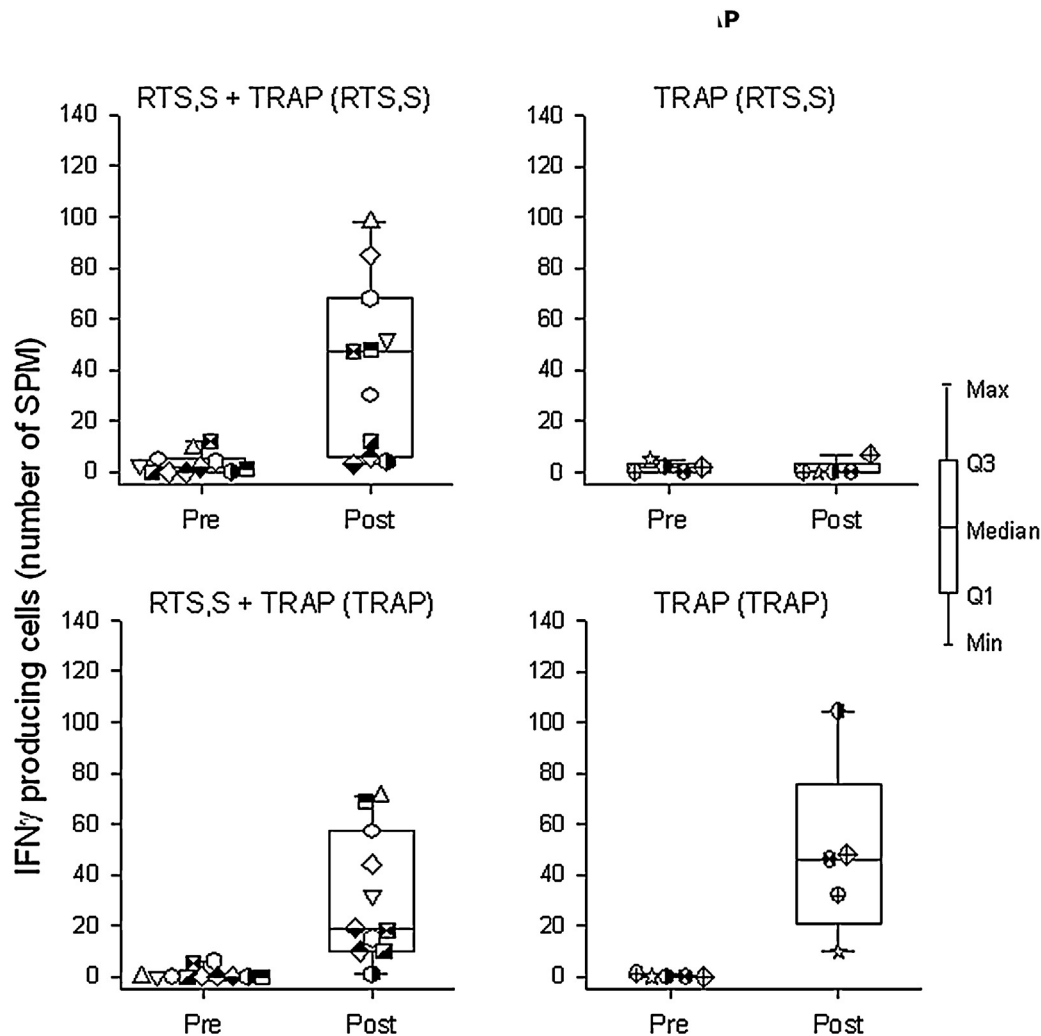DOC—day of challenge.

Fig. 2. *IFN-γ ELISPOT activity recalled with RTS,S or TRAP.*

IFN-γ producing cells measured by Elispots after *in vitro* stimulation of PBMCs with RTS,S or TRAP before vaccination (Pre) and at 2 weeks post second vaccination (Post) from subjects vaccinated with RTS,S + TRAP/AS02 (RTS,S + TRAP) vs with TRAP/AS02 (TRAP). The individual Elispot responses, expressed as the number of IFN-γ⁺ cells per 10⁶ PBMCs, are shown as dots/triangles and overall the minimum—Quartile1–median—Quartile-3 and maximum values are represented as well. Each individual subject is represented by a unique symbol and this symbol is used for all graphs.

12.0 days for the RTS,S + TRAP/AS02, TRAP/AS02 and infectivity control groups, respectively (log rank test: $p = 0.096$ RTS,S + TRAP/AS02 vs control, $p = 0.661$ TRAP/AS02 vs control).

4. Discussion

Both studies demonstrated the combination vaccine RTS,S + TRAP/AS02 had an acceptable safety profile and was generally well tolerated. Although not designed as non-inferiority trials and thus significantly underpowered to permit a formal conclusion, the anti-CS IgG responses in subjects who received both RTS,S + TRAP were substantially lower – at every time point measured – than in those who received RTS,S alone. While there may be alternative explanations, immune interference between TRAP and RTS,S must be considered as a leading explanation for the failure to see protection in the RTS,S/TRAP group. We have no real understanding as to how the anti-TRAP antibodies that were induced impacted on the anti-CS responses. While a specific correlate of protection for RTS,S has not been identified, analyses of potential correlates of protection consistently emphasize the association between protection and high levels of CS antibodies at the time of sporozoite exposure [2–5]. In the Phase II study

reported here, peak IgG responses to CS in the RTS,S/TRAP group were approximately 50% of what would have been typically observed in individuals receiving RTS,S alone. In contrast to CS, TRAP appears to be inherently more immunogenic, and in both the Phase 1 and Phase 2 studies, similar anti-TRAP humoral responses were observed with the combination and the component vaccines.

Immunological interference between antigens in combination vaccines is a well-known although highly unpredictable phenomenon that can occur even in the presence of a potent adjuvant. In the Phase 1 study, low levels of cross-reactive anti-TRAP antibody responses observed in the RTS,S/AS02 group may be due to antibodies directed against the thrombospondin-like type 1 sequence in the C terminus of CS [39,40,25]. At this point, there is no way of knowing conclusively as to whether or not measured or unmeasured immune responses to TRAP impacted on other aspects of the immune response induced by RTS,S.

In the Phase 1 study, the RTS,S- and TRAP-specific responses evaluated by proliferative responses, and IFN-γ and IL-5 secretion in the culture supernatant, were similar for vaccinees who received the combination RTS,S + TRAP/AS02 and for vaccinees who received either RTS,S/AS02 or TRAP/AS02. At the time of evaluation in 1999, assays were not in place to measure CS-specific cellular responses.
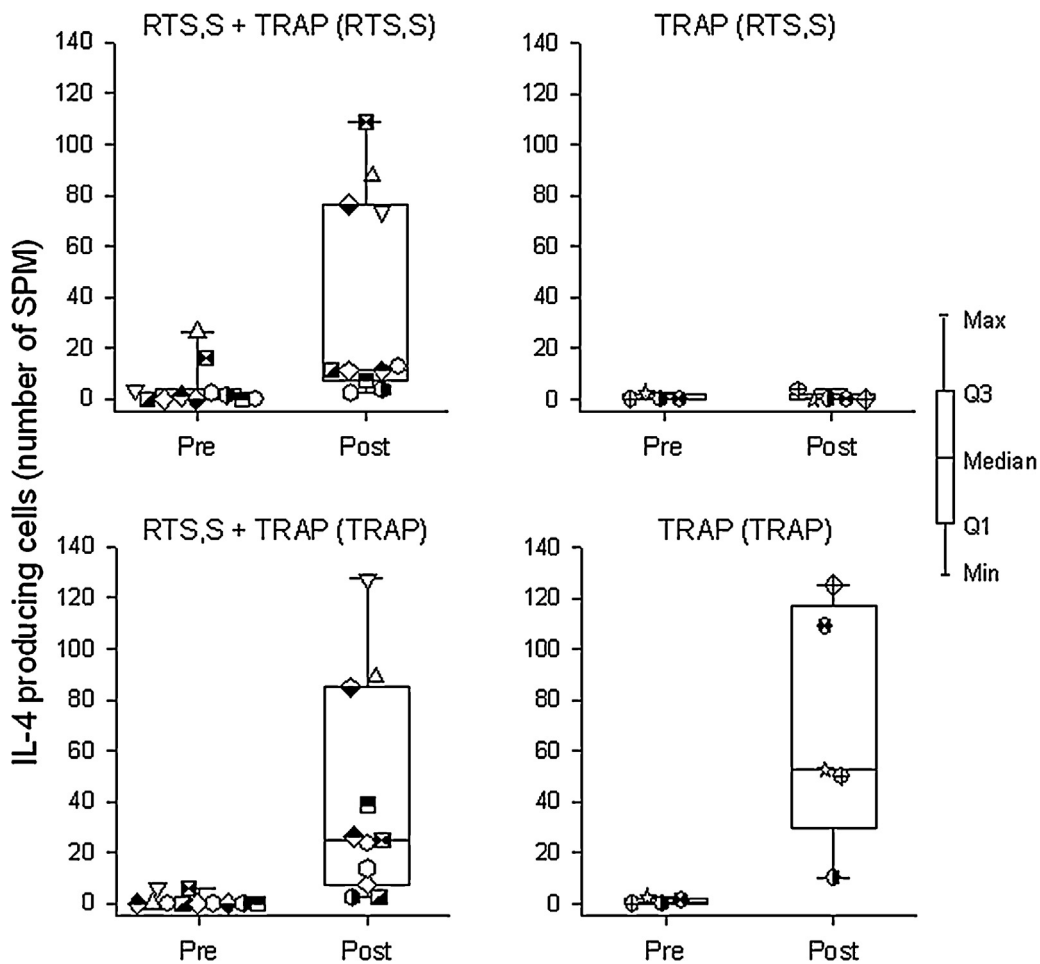
Fig. 3. *IL-4 ELISPOT activity recalled with RTS,S or TRAP.*
IL-4 producing cells measured by Elispots after *in vitro* stimulation of PBMCs with RTS,S or TRAP before vaccination (Pre) and at 2 weeks post second vaccination (Post) from subjects vaccinated with RTS,S + TRAP/AS02 (RTS,S + TRAP) vs with TRAP/AS02 (TRAP). The individual Elispot responses, expressed as the number of IFN-γ+ cells per 10^6 PBMCs, are shown as dots/triangles and overall the minimum—Quartile1–median—Quartile-3 and maximum values are represented as well. Each individual subject is represented by a unique symbol and this symbol is used for all graphs.

Hence, the RTS,S-specific responses recorded were the combined responses specific to both the HBs and CS antigen components of the RTS,S vaccine. In the Phase 2 trial, the vaccination regimens elicited low RTS,S- and TRAP-specific T cell responses, measured

Fig. 4. *Protection and time to parasitemia.* Log rank test: $p = 0.096$ RTS,S + TRAP/AS02 vs control, $p = 0.661$ TRAP/AS02 vs control.

by IFN-γ ELISPOT assay, and were notably lower when compared to other studies using the same methodology [5,38].

After challenge, all infectivity controls, 5 of 5 TRAP/AS02 vaccinees and 10 of 11 RTS,S + TRAP/AS02 vaccinees developed parasitemia. There was no evidence of any prevention or delay of parasitemia by TRAP/AS02. The absence of a significant delay or prevention of parasitemia by RTS,S + TRAP/AS02 contrasts sharply with the demonstration of prevention of parasitemia by published Phase 2 studies of 1 or 2 doses of RTS,S/AS02 in malaria-naïve adults following malaria challenge. Specifically, a single dose of RTS,S/AS02 protected 3 of 10 subjects, and 2 doses of RTS,S/AS02 protected 7 of 14 subjects in one trial against experimental malaria challenge [2] and in another trial protected 8 of 19 subjects [3].

In the challenge model [1–5] and in field studies in adults [6] and children [8,10,41–44] vaccinated with the candidate RTS,S/AS vaccine, an association between anti-CSP central repeat region antibody and protection was observed. Although two pediatric field trials reported a lack of association, the very high titers achieved in these children and the relatively short period of follow-up may have limited the ability to discriminate on the basis of differential CS responses [7,9]. In the challenge model, protected compared to non-protected recipients of RTS,S/AS have also demonstrated higher CS-specific CD4+ T cell and IFN-γ ELISPOT responses [5,38] and in a field trial in children, higher CS-specific TNFα CD4+ T cells [44].
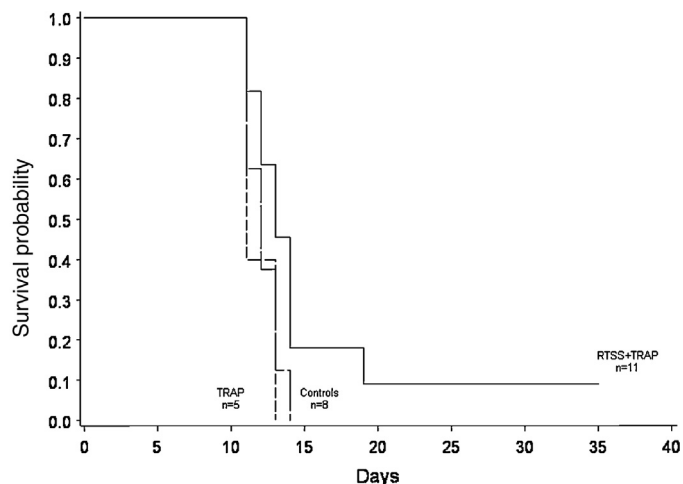
Other investigators have clearly established that TRAP is a valid a malaria vaccine candidate, although its ability to confer protection is entirely dependent on the way the antigen is delivered [45]. It is clear from this trial that antibodies and CD4+ T cell responses are insufficient, but when TRAP is delivered using heterologous prime boost such that potent CD8+ T cell responses are generated, compelling protection has been reported [46]. Based on these observations we are currently exploring whether the combination of RTS,S/AS01 plus ChAd63/MVA ME-TRAP will lead to enhanced levels of protection against experimental malaria challenge.

We recognize that there are a number of limitations associated with the challenge study, most notably a small sample size, which was further impacted by the exclusion of 18 subjects from the challenge phase. Further, the lack of an RTS,S/AS02 comparator does prevent direct, within-study efficacy comparisons between RTS,S, RTS,S/TRAP, and TRAP formulations. We conclude, within the constraints of the small sample size, that the presence of TRAP antigen may have interfered with vaccine efficacy previously observed with this regimen of RTS,S/AS02, and that future TRAP-based vaccines should consider employing alternative vaccine platforms.

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Conflict of interest statement

K.E. Kester, D.G. Heppner, C.F. Ockenhouse, R. Gasser, W.R. Ballou, D. Gordon, P. Duffy, G. Wortmann, and R. Miller were at the time of the study, officers of the US federal government, assigned at the Walter Reed Army Institute of Research. U. Krzych and C. Holland are employees at the Walter Reed Army Institute of Research. B. Wellde and G. Richmond were, at the time of the study, employees at the Walter Reed Army Institute of Research. P. Moris, O. Ofori-Anyinam, N. Tornieporth, M. Delchambre, G. Voss, W.R. Ballou, J. Cohen, and L. Vigneron are, or were at the time the study was planned and conducted, employees of the GlaxoSmithKline group of companies. P. Moris, O. Ofori-Anyinam, N. Tornieporth, M. Delchambre, G. Voss, W.R. Ballou, and J. Cohen own stock or stock options. W.R. Ballou, and D.G. Heppner are listed as inventors on patents or have patent applications covering various malaria vaccine candidates. J. Cohen is listed as an inventor on patents or patent applications related to RTS,S, TRAP and other malaria vaccine candidates, all assigned to GSK. D.G. Heppner declares receiving speaker fees from the National Defense University.

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The opinions expressed in this article are personal and are not to be construed as official positions of the United States Departments of the Army or Defense.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.06.033.

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