Plasmodium falciparum-Specific Cellular Immune Responses after Immunization with the RTS,S/AS02D Candidate Malaria Vaccine in Infants Living in an Area of High Endemicity in Mozambique[∇]

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Results from clinical trials in areas where malaria is endemic have shown that immunization with RTS,S/AS02A malaria vaccine candidate induces partial protection in adults and children and cellular effector and memory responses in adults. For the first time in a malaria vaccine trial, we sought to assess the cell-mediated immune responses to RTS,S antigen components in infants under 1 year of age participating in a clinical phase I/IIb trial of RTS,S/AS02D in Mozambique. Circumsporozoite protein (CSP)-specific responses were detected in approximately half of RTS,S-immunized infants and included gamma interferon (IFN- γ), interleukin-2 (IL-2), and combined IL-2/IL-4 responses. The median stimulation indices of cytokine-producing CD4⁺ and CD8⁺ cells were very low but significantly higher in RTS,S-immunized infants than in infants that received the comparator vaccine. Protection against subsequent malarial infection tended to be associated with a higher percentage of individuals with CSP-specific IL-2 in the supernatant ($P = 0.053$) and with higher CSP-specific IFN- γ -producing CD8⁺ T-cell responses ($P = 0.07$). These results report for the first time the detection of malaria-specific cellular immune responses after vaccination of infants less than 1 year of age and pave the way for future field studies of cellular immunity to malaria vaccine candidates.

Malaria remains one of the major world health problems affecting between 200 and 400 million people annually and causing 2 to 3 million deaths, mostly children and pregnant women living in sub-Saharan Africa (37). Infections by *Plasmodium falciparum*, one of the four species of plasmodia that affect humans, cause 80 to 90% of the malaria cases and are responsible for 95% of all malaria-associated deaths (14). Since most of the worldwide malaria burden is due to *P. falciparum*, efforts for prevention and eradication of malaria have focused on this parasite, and a *P. falciparum*-customized malaria vaccine is one of most promising tools (12, 25, 26).

The most abundant and immunogenic antigen on the surface of *Plasmodium* sporozoites is the circumsporozoite protein (CSP), which is a target for vaccine development (9, 10, 17, 27). In vaccines based on irradiated sporozoites and CSP in human and mouse models, antibodies to circulating sporozoites, followed by cell-mediated responses to the protein after invasion of hepatocytes, have been described as crucial for the generation of protective responses (7, 11, 13, 28, 29).

RTS,S is a subunit malaria vaccine candidate based on the CSP of *P. falciparum* that has been under study for many years.

The chimeric vaccine contains a portion of the NANP-repeats, all four NVDP-repeats, and the complete carboxyl-terminal region of CSP suggested to be targets for humoral and cellular immunity, along with the amino-terminal region of HbsAg (HBS) (16). The malaria vaccine candidate RTS,S (GlaxoSmithKline, Rixensart, Belgium) formulated with the adjuvant system AS01 or AS02 has proven to confer partial protective immunity against malaria infection in malaria-naïve adults (20, 21, 41), as well as in adults and infants in areas where malaria is endemic (2–6). Clinical safety, immunogenicity, and efficacy trials in infants and children have shown RTS,S/AS02 to be safe and protective and to induce high antibody titers (2, 4, 6, 34).

Although the induction of a CSP-specific humoral response after RTS,S vaccination has been well described, the generation of cellular immune responses has not yet been addressed in infants or young children immunized with the RTS,S vaccine candidate. In adults, protection conferred by the RTS,S vaccine has been associated with acquisition of strong antibody and cellular responses to the CSP fragment of RTS,S (20, 22). Malaria naïve volunteers immunized with RTS,S/AS02 frequently develop strong proliferative and IFN- γ -producing T-cell responses to peptides representing T-cell epitopes (Th2R and Th3R) present in the vaccine (22). A correlation between protection against experimental challenge and the CSP-specific production of IFN- γ by CD4⁺ and CD8⁺ T cells has been described in a limited number of individuals (42).

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FIG. 1. Timeline of immunizations and blood sample collection. Timing for the blood samples used for cellular immunity studies are indicated: BS0, preimmune; BS1, 4 weeks postimmunization; and BS2, 10.5 weeks postimmunization.

Current efforts are under way to proceed to phase III clinical trials with the RTS,S vaccine, despite no currently identified immune correlates of protection for vaccination with RTS,S in infants or young children. The present study was integrated into a phase I/IIb clinical trial of the RTS,S/AS02D candidate vaccine in infants in a rural area of malaria endemicity in Mozambique (4). We sought here to examine the cellular responses in infants vaccinated with RTS,S/AS02D and further the development of assays for use in malaria vaccine trials in infants and young children, the population most vulnerable to severe malaria.

MATERIALS AND METHODS

Study population. This study was integrated into a randomized placebo-controlled phase I/IIb clinical trial of the RTS,S/AS02D malaria candidate vaccine in infants living in a rural area of Mozambique (4) (registry URL, clinicaltrials.gov; trial registration no., NCT00197028). Briefly, mothers in their last trimester of pregnancy living in the Ilha Josina and Tanginga communities of Manhica district in Maputo, Mozambique, were invited to enroll their newborns after delivery in the clinical trial. After informed consent was obtained, the mothers were counseled regarding sexually transmitted diseases and tested for human immunodeficiency virus and hepatitis B virus infection (UniGold HIV [Trinity Biotech]; Determine HIV1-2 and HBsAg [Abbott Laboratories]). Mothers with a positive result for either serological test were excluded from the study and referred to the Manhica District Hospital for clinical management according to national guidelines.

Vaccines. The pediatric version of RTS,S/AS02A contains 25 μ g of RTS,S antigen and 0.125 ml of AS02D adjuvant as described by Macete et al. in 2007 (23, 24). The control vaccine Engerix-B is from GlaxoSmithKline, Belgium. TETRActHib (Adventis Pasteur, France), the vaccine used in the Expanded Programme on Immunization (EPI) program, is a lyophilized vaccine combining diphtheria and tetanus toxoid-pertussis vaccine (DTP) with *Haemophilus influenzae* type b conjugate vaccine (polyribosyl ribitol phosphate conjugated to tetanus protein).

Immunizations. All enrolled children received the standard polio (Chiron/Novartis, San Francisco, CA) and DTPw/Hib (DTP plus *H. influenzae* type b) vaccines as their EPI vaccinations at 8, 12, and 16 weeks of age at their local community health post. At the first EPI vaccination visit, they were randomly distributed into two immunization groups to receive either the RTS,S/AS02D or the hepatitis B Engerix-B vaccines. These intramuscular immunizations were given in a staggered manner blindly 2 weeks after the EPI vaccines at weeks 10, 14, and 18. A diagram of the immunization schedule is shown in the Fig. 1.

Blood samples. Approximately 2.0 ml of blood was drawn from infants prior to study vaccination (blood sample BS0) and then 4 and 10.5 weeks (blood samples BS1 and BS2) after the third immunization with RTS,S/AS02D or hepatitis B vaccine by heel prick, finger prick, or venipuncture for later visits. Blood was collected into heparin tubes at an ambient temperature and processed within 3 h for cellular immunology and biochemistry assays.

Malaria case detection. Malaria infections caused by *P. falciparum* were assessed by active detection and by passive case detection at health facilities in the study area as described previously (4). Active detection occurred at predefined intervals in which a blood slide for parasitemia determination was collected, and the axillary temperature was recorded irrespective of symptoms. Passive case detection was done through monitoring of all attendances at health facilities and ascertainment of episodes of clinical malaria, including blood smear for infants with documented fever (37.5°C or higher) or history of fever in the preceding 24 h, as described in detail elsewhere (24). Infants with any parasitemia > 0 were considered cases and used in the current analysis. After vaccination, parasitemia-positive infants were not included in the risk period for malaria for 28 days after receiving treatment. Clinical management was provided according to standard national guidelines.

Whole-blood cultures and peptide stimulation. To stimulate and culture whole blood, 250 μ l of heparinized blood was dispensed into three aliquots supplemented with 150 μ l of RPMI 1640 (incomplete medium; Invitrogen/Gibco-BRL), containing 20 mM HEPES, 100 U of penicillin ml^{-1} , 100 μ g of streptomycin ml^{-1} , 2 mM L-glutamine, and mouse anti-human CD28 and anti-human CD49d antibodies (BD Biosciences, San Jose, CA) at final concentrations of 1.2 μ g ml^{-1} each. The cultures were stimulated as follows with (i) CSP peptides encompassing sequences present in RTS,S; (ii) HBS peptides present in RTS,S; and (iii), as a negative control, the peptide diluent alone, dimethyl sulfoxide at a 1/1,000 dilution. CSP and HBS peptides were 15-mer long, overlapping peptides by 11 amino acids used at 1.25 μ g ml^{-1} as described previously (39). Due to small volumes of blood, positive control stimulation with mitogen (phytohemagglutinin) was not possible for most of the donors and was performed in ca. 10% of the samples in parallel with acridine orange-ethidium bromide staining to ensure viability of >95%. Peptide stimulation incubation was performed at 37°C in 5% CO₂ for ~42 h, after which 100 μ l of the supernatant was collected and stored at -80°C until use in cytokine detection assays. An approximate 42- to 48-h stimulation yielded the best signal-to-noise ratio compared to 24- and 72-h incubations (data not shown). Brefeldin A was added to the culture (Golgi Plug; BD Pharmingen, San Diego, CA), and cells were kept in incubation for another 6 h prior to flow cytometry.

Intracellular cytokine staining (ICS). After an approximately 42-h peptide stimulation, erythrocytes were lysed by adding fluorescence-activated cell sorting lysing solution (BD Biosciences). Cells were then stained with fluorochrome-conjugated antibodies to the cell surface markers anti-CD8/APC, anti-CD4/fluorescein isothiocyanate, and anti-CD3/PerCP (BD Pharmingen); fixed and

permeabilized with a solution containing paraformaldehyde and saponin (Cytofix/Cytoperm; BD Biosciences), and stained with phycoerythrin-conjugated anti-IFN- γ or anti-IL-2 antibodies (FastImmune; BD Biosciences). A minimum of 50,000 gated lymphocyte events were collected on a BD FACSCalibur cytometer, and the unlabeled clones for anti-CD3, anti-CD4, anti-CD8, anti-IFN- γ , and anti-IL-2 immunoglobulin G antibodies and Calibrite beads (BD Biosciences) were used for compensation settings. To reduce interassay variations, we used the control stimulated cells as a reference for specific stimulation. Plots of CD3-CD4 and CD3-CD8 versus each cytokine were generated for control stimulated cells, and a threshold gating was placed to objectively exclude at least 99.5% cytokine-phycoerythrin-negative control cells from the cytokine-positive quadrant. The same threshold was placed on the cells stimulated with CSP and HBS peptides. Thus, antigen-specific cells were defined as the CD3⁺ CD4⁺ or CD3⁺ CD8⁺ cells in the quadrant above the threshold. Stimulation indexes (SIs) were calculated as ratios between the proportions of peptide-specific cells over the proportion of control stimulated cells for each individual in order to account for donor variation in background signal.

Determination of concentration of cytokines in supernatant. Detection of human IFN- γ , IL-2, and IL-4 in 25 μ l of each supernatant, diluted 1:2, was performed by using a four-bead CBA-Flex customized kit and analyzed using the FCAP array software (v.1.0.1; BD Biosciences). To assess the antigen-specific production of cytokines, concentration values from the control-stimulated culture supernatants were subtracted from the concentration values from peptide-stimulated supernatants for the same blood sample. Threshold values for evaluating positive responses in supernatant were placed above the 98th percentile for all preimmune cytokine levels using reverse cumulative distribution plots. The cytokine cutoffs were therefore set as follows: IFN- γ at 20 pg ml⁻¹, IL-2 at 100 pg ml⁻¹, and IL-4 at 5 pg ml⁻¹.

Statistical methods. The according-to-protocol (ATP) cohort included subject samples that met all eligibility criteria and complying with all procedures defined in protocol. Criteria for analysis of cellular immunity results included children who correctly completed the protocol of three immunizations that were followed up for 10 weeks and had an available sample (referred to as the cell-mediated immunity [CMI] cohort) (Fig. 1).

Reverse cumulative distribution plots were used for rapid visual assessment of the distributions (32). Differences between both vaccine groups in intracellular median SIs and cytokine median concentrations in the supernatant were assessed by using the Wilcoxon rank-sum test and comparison of proportions was performed by using the Fisher exact test. McNemar chi-square and Wilcoxon rank-sum tests were used to compare preimmune and postimmune proportions and the distribution of positive responses in supernatant responders. Analyses were performed using Stata 9 software (StataCorp LP).

RESULTS

Study population and samples included for analysis of cellular immunity. From August 2005 to September 2006, 214 infants were enrolled in the phase I/IIb double-blind randomized placebo-controlled trial of RTS,S/AS02D in Mozambique as described previously (4). Whole-blood cell cultures for cell immunogenicity measures were performed from 206 blood samples taken before immunization at BS0, from 186 samples taken 4 weeks after the third immunization (BS1), and from 188 samples taken 10.5 weeks after the third immunization (BS2) (Fig. 1). Detection of secreted (supernatant) and non-secreted (intracellular) cytokines were measured in the same whole-blood cell cultures. The data were available for the assessment of secreted cytokines at BS1 and BS2 for 62 and 65% of the samples, respectively, whereas for ICS from BS1 and BS2 the data were available from 80 and 70% of the samples, respectively. Missing data were equally distributed between the immunization groups.

Basal levels of secreted cytokines. Prior to assessing postimmunization cytokine secretion, basal preimmune cytokine production was analyzed. Preimmune specific levels of IFN- γ , IL-2, and IL-4 were low (apart from one outlier) ranging from mostly undetectable up to 45.2, 51.6, and 5.3 pg ml⁻¹, respec-

tively. Median white blood cell counts were similar between the RTS,S/AS02D and hepatitis B immunization groups at BS0 (9.7, with an interquartile range [IQR] of 8.3 to 11.8, and 9.9, with an IQR of 8.7 to 11.6), as well as at other time points (data not shown).

CSP-specific cytokine responses in supernatants after immunization. CSP-specific cytokine concentrations were assessed after immunization at 4 weeks (BS1) and 10.5 weeks (BS2) postimmunization. The median concentration of CSP-specific IL-2 was significantly higher in the RTS,S/AS02D vaccine group compared to the hepatitis B vaccine group both at BS1 and BS2 (Fig. 2). The median CSP-specific IL-2 concentrations at BS1 for the RTS,S/AS02D group and for the hepatitis B group were 24.5 (IQR = 1.0 to 43.6) and 0.0 (IQR = 1.0 to 22.6), respectively, and at BS2 the medians were 25.3 (IQR = 1.0 to 95.5) and 0.0 (IQR = 1.0 to 20.8), respectively. A higher proportion of infants produced IFN- γ , IL-2, and IL-4 after immunization with RTS,S/AS02D compared to the preimmune group (Fig. 3). In particular, the proportion of infants with an IL-2 response was significantly higher in the RTS,S/AS02D group than in the hepatitis B group at both 4 weeks (BS1, $P < 0.01$ [Fisher exact test]) and 10.5 weeks (BS2, $P < 0.001$ [Fisher exact test]) after immunization (Fig. 3B). Interestingly, as shown in Fig. 3, a significantly higher proportion of positive responders was more often observed at BS2 after a longer (10.5-week) postimmunization interval.

HBS-specific production of cytokines in supernatant after immunization. Since HBsAg was present in both the RTS,S/AS02D and the control hepatitis B vaccines, HBS stimulation was expected to generate cytokine production in both immunized groups. Children immunized with the RTS,S formulation showed slightly higher HBS-specific IFN- γ and IL-2 responses compared to the hepatitis B vaccine group (data not shown). Compared to preimmune cytokine production, the proportion of children with a HBS-specific IFN- γ and IL-2 production increased significantly for both vaccine groups at both 4 weeks (BS1) and 10.5 weeks (BS2) (Fig. 3C and D). However, there was a greater proportion of infants producing HBS-specific IL-2 in the RTS,S group than in the hepatitis B vaccine group (Fig. 3, $P_{IL-2} = 0.05$ [Fisher exact test]).

Assessment of CSP- and HBS-specific intracellular cytokine production. To describe the role of CD4⁺ and CD8⁺ T cells in IFN- γ and IL-2 responses to RTS,S, intracellular IFN- γ and IL-2 cytokine staining was performed in the same cell culture after supernatant collection. Averages of $21,155 \pm 9,763$ CD4⁺ T cells and $8,532 \pm 4,521$ CD8⁺ T cells were analyzed for IFN- γ intracellular staining, and averages of $17,100 \pm 7,542$ CD4⁺ T cells and $6,917 \pm 3,773$ CD8⁺ T cells were analyzed for IL-2 intracellular staining. The percentage of antigen-specific cytokine-expressing cells was generally very low and, after subtraction of control-stimulated values, the maximum ranged from 3.5% to 6% depending on the stimulating antigen. Median SIs for both CD4⁺ and CD8⁺ T cells ranged from 0.81 to 1.34 in both immunization groups (Table 1). When CSP-specific responses were assessed, it was observed that children immunized with RTS,S had a higher median SI of CSP-specific IFN- γ -producing CD8⁺ T cells (Table 1, $P_{IFN-\gamma} = 0.029$ [Wilcoxon rank-sum test]) and CSP-specific IL-2-producing CD4⁺ T cells than did the hepatitis B vaccination group (Table 1, $P_{IL-2} = 0.043$ [Wilcoxon rank-sum test]). Evaluation of HBS-

FIG. 2. Reverse cumulative distribution of cytokine concentration in supernatants from CSP-stimulated cell cultures. Plots for preimmune (a to c) and for 4-week (d to f) and 10.5-week (g to i) postimmunization samples are shown in the top, middle, and bottom rows, respectively. Immunization groups are represented by boldface (RTS,S/AS02D) and dotted (hepatitis B) lines. Arrowheads show the concentration cutoffs for positive responses: 20 pg ml⁻¹ for IFN- γ , 100 pg ml⁻¹ for IL-2, and 5 pg/ml-1 for IL-4. The Wilcoxon rank-sum test was used to compare cytokine concentrations between both immunization groups. Significant differences ($P < 0.01$) are indicated by asterisks (**).

FIG. 3. Frequency of CSP- and HBS-specific cytokine responses in supernatants of children immunized with RTS,S/AS02D and hepatitis B (Engerix-B) vaccines before immunization (BS0, □), 4 weeks postimmunization (BS1, ▨), and 10.5 weeks postimmunization (BS2, ■). Differences in the proportions of positive responders before and after immunization (brackets) and between immunization groups (arrows) were assessed by using the McNemar chi-square and Fisher exact tests, respectively. Significant differences: *, $P < 0.05$; **, $P < 0.001$.

specific responses showed that the median SI of HBS-specific IL-2 and IFN- γ -producing CD8⁺ T cells was also higher in the RTS,S group than in the hepatitis B vaccine group (Table 1, $P_{\text{IFN-}\gamma} = 0.015$ and $P_{\text{IL-2}} = 0.030$ [Wilcoxon rank-sum test]).

Evaluation of association between CSP-specific cytokine responses and malaria infection. As described by Aponte et al. (4), “first or only” (FO) cases of *P. falciparum* infection postimmunization, as assessed by both active and passive detection of parasitemia, were documented during the follow-up period in 22 infants from the RTS,S/AS02D group and in 46 infants from the hepatitis B control group, with a resulting adjusted vaccine efficacy of 65.9% for malaria infection. The percentages of infants having had a FO malaria case were similar between the efficacy ATP cohort and the CMI cohort (24.4 and 24.2%, respectively). We sought to assess whether there was any difference in CSP-specific cytokine responses in the RTS,S/AS02D group between infants with reported malaria infection and those without. Table 2 shows the median CSP-specific intracellular CD4⁺ and CD8⁺ SIs for IL-2 and IFN- γ responses and the proportion of infants with positive IL-2 supernatant responses, according to FO cases of malaria parasitemia.

Among infants immunized with RTS,S, there was a trend toward a higher proportion of individuals who did not have an episode of malaria infection with a positive CSP-specific IL-2 response in supernatants (>100 pg ml⁻¹) compared to infants who suffered an episode of malarial infection ($P = 0.053$ [Fisher exact chi-square test], Table 2). Likewise, the median SI for IFN- γ -producing CD8⁺ T cells was higher in infants with no FO malarial infection than in those with FO malaria, although this difference did not reach significance ($P = 0.07$, Table 2). There was no difference in cytokine production be-

TABLE 1. Comparison of CSP- and HBS-specific IFN- γ and IL-2 CD4⁺ and CD8⁺ responses between RTS,S/AS02D and control hepatitis B immunization groups^a

T-cell stimulus	Time (wk) after third dose	Immunization group						<i>P</i> ^b
		Hepatitis B			RTS,S/AS02D			
		<i>n</i>	SI		<i>n</i>	SI		
			Median	IQR		Median	IQR	
IFN-γ								
CSP CD4 ⁺	4	75	1.20	0.77–1.61	75	1.16	0.85–1.75	0.611
	10.5	69	1.09	0.85–1.47	63	1.18	0.90–1.59	0.213
CSP CD8 ⁺	4	75	1.04	0.67–1.57	75	1.04	0.66–1.57	0.891
	10.5	69	1.05	0.67–1.41	63	1.25	0.84–1.85	0.029*
HBS CD4 ⁺	4	75	1.17	0.71–1.75	75	1.24	0.76–1.67	0.533
	10.5	69	1.03	0.74–1.47	63	1.08	0.77–1.59	0.769
HBS CD8 ⁺	4	75	1.08	0.63–1.71	75	1.06	0.74–1.70	0.423
	10.5	69	0.81	0.58–1.21	63	1.08	0.68–1.49	0.015*
IL-2								
CSP CD4 ⁺	4	69	1.05	0.71–1.65	70	1.32	0.95–2.00	0.052
	10.5	68	1.00	0.66–1.44	59	1.25	0.92–1.74	0.043*
CSP CD8 ⁺	4	69	1.04	0.66–1.86	70	1.14	0.78–1.86	0.626
	10.5	68	0.86	0.54–1.38	59	1.04	0.76–1.61	0.086
HBS CD4 ⁺	4	69	1.19	0.69–1.79	70	1.34	0.95–1.79	0.185
	10.5	68	1.04	0.72–1.37	59	1.18	0.81–1.88	0.065
HBS CD8 ⁺	4	69	1.14	0.62–1.67	70	1.16	0.83–1.65	0.451
	10.5	68	0.89	0.52–1.26	59	1.28	0.63–1.65	0.030*

^a Results are from ICS assays and are expressed as SIs as described in Materials and Methods. IQR = quartile 25–75. *n*, Number of subjects.^b As determined by the Wilcoxon rank-sum test. *, *P* < 0.05 (significance).

tween infants who suffered malaria infection in the hepatitis B vaccine group (*P* = 1.00 [data not shown]).

DISCUSSION

This study is the first description of cellular immune responses induced by a candidate malaria vaccine (RTS,S/AS02D) in infants less than 1 year of age. Evaluation of cell-mediated responses to both the CSP and HBsAg components of RTS,S was performed in infants participating in a phase I/IIb clinical trial (4). Our data show that the RTS,S/AS02D vaccine was immunogenic in infants, eliciting detectable cellular immune responses to both CSP and HBS antigens after immunization.

Secreted IL-2 was the strongest and most frequent CSP-specific response observed and was detected in about a quarter (25.3%) of the RTS,S/AS02D-immunized infants at 10.5 weeks after immunization. In addition, HBS-specific IFN- γ and IL-2 responses were more frequently induced by the RTS,S/AS02D vaccine than by the control hepatitis B vaccine, possibly due to a stronger Th1 adjuvant effect of AS02D compared to alum present in the Engerix-B hepatitis B vaccine.

Although RTS,S/AS02D immunization induced statistically significant CSP-specific cellular immune responses detectable by ICS compared to the hepatitis B group or preimmune responses, the percentage of positive cells was very low, as shown by the SIs close to 1.0, and the biological significance of this is

TABLE 2. Associations between CSP-specific cytokine responses and malaria infection in RTS,S/AS02D-immunized infants

Variable ^a	Time (wk) after third dose	Malaria cases ^b						<i>P</i> ^c
		No			Yes			
		<i>n</i>	Median SI (IQR)	% Responders (no.)	<i>n</i>	Median SI (IQR)	% Responders (no.)	
SI								
IFN-γ (CD8 ⁺ T cells)	4	57	1.030 (0.650–1.571)		18	1.119 (0.852–1.455)		0.669
	10.5	46	1.398 (0.960–2.061)		17	1.121 (0.636–1.484)		0.074
IL-2 (CD4 ⁺ T cells)	4	56	1.355 (0.915–2.000)		14	1.294 (1.045–1.667)		0.860
	10.5	44	1.271 (0.943–1.794)		15	1.222 (0.903–1.594)		0.741
Cytokines in supernatant								
IFN-γ	10.5	54		9.3 (5)	17		0 (0)	0.328
IL-2	10.5	54		31.5 (17)	17		5.9 (1)	0.053
IL-4	10.5	54		13.0 (7)	17		0 (0)	0.188

^a CSP-specific cytokine responses are expressed as either SI values from ICS assays or as a percentage of infants with cytokines in supernatant (lower panel). *n*, Number of subjects.^b ATP cohort, for first or only episode of malaria infection from 2.5 to 6 months of follow-up.^c As determined by the Wilcoxon rank-sum test for the ICS SI values and as determined by the Fisher exact chi-square test for the proportion with supernatant cytokine response.

unknown. When significant differences were observed between immunization groups, median SIs for CSP-induced responses were ~0.25 higher in the RTS,S/AS02D group than those in the hepatitis B vaccine group. This translates into approximate 0.08 and 0.09% increments, respectively, in the CSP-specific CD4⁺ and CD8⁺ T-cell populations in children immunized with RTS,S. When comparing ICS to supernatant cytokines, no correlation was observed between secreted and intracellular cytokine levels (data not shown). This has often been described for different assays and may be due to the duration of in vitro stimulation and assay conditions favoring secretion and not having reached the peak of intracellular cytokine accumulation (1).

A limitation of our ICS assay was that, due to the small volumes of blood available, whole blood was used instead of peripheral blood mononuclear cells (PBMC). ICS assays have been extensively optimized in PBMC, particularly from adults living in North America or Europe. Whole-blood assays may give more background in ICS than PBMC, potentially weakening the signal-to-noise ratio for antigen-specific responses and complicating compensation settings. The true differences between the immunization groups may thus be greater than detected in the present study. Furthermore, several studies have observed that the background levels in cells from African subjects may be higher due to more chronic activation and inflammation (18, 33, 43). The whole-blood ICS assay will thus require further optimization for use in African infants for malaria trials, similar to what has been done in the context of tuberculosis studies (15).

There are concerns about the adverse effect of maternal immunity and the immaturity of infants' immune systems on the induction of adequate antibody and effector T-cell IFN- γ responses (Th1) by malaria vaccines in neonates and infants living in areas of endemicity (35, 36). In this safety/efficacy trial in infants, RTS,S/AS02D immunization induced high titers of anti-CSP antibodies, suggesting that the presence of maternally transferred antibodies at a young immunization age did not significantly modify the antibody immune response (4). It has been suggested that IFN- γ T-cell responses to natural malaria exposure are infrequent in children (8, 38), and it is hypothesized that repeated exposure and a mature immune system may be required. In the present study, although weak, RTS,S immunization clearly induced CSP-specific T-cell responses, thus demonstrating that CSP-specific T-cell immunity can be elicited in infants less than 1 year of age.

In the main efficacy study, the RTS,S/AS02D immunization, along with EPI vaccines, was shown to be safe, to induce a strong antibody response to both CSP and HBS components, and to have a calculated efficacy of 65.9% against malaria infection (4). Since it is known that humoral immunity is not sufficient for protection against malaria infection, the role of cytokine-producing T cells has been under intense study in the effort to identify correlates of malaria vaccine efficacy. In past studies in adults, the RTS,S vaccine candidate has been shown by various techniques to elicit cellular immune responses (5, 20, 22, 30, 40–42). The use of different assays to measure antigen-specific T-cell activity, including both ex vivo and cultured lymphoproliferation, IFN- γ enzyme-linked immunospot, and ICS methods, complicates interpretations. In malaria-naïve adults, a trend has been observed toward a higher proportion of CSP-specific IFN- γ CD4⁺ and CD8⁺ responses in a small

number of protected individuals (19, 42). In adults in an area of malaria endemicity, cultured enzyme-linked immunospot assay revealed an association between IFN- γ -producing CD4⁺ cells and protection after RTS,S vaccination (31). To date, no trials of RTS,S vaccination in areas of malaria endemicity have reported detectable CD8⁺ responses, and published data in infants and young children are limited to antibody immunogenicity.

We found a suggestive association between not suffering an infection episode and higher levels of CSP-specific IL-2 in supernatant at 10.5 weeks postimmunization. In addition, there was a trend toward an association between not having a malaria infection episode and CSP-specific CD8⁺ IFN- γ responses, although these associations did not reach statistical significance, possibly due to the lack of power to make these associations with the given sample size. Surprisingly, stronger cytokine responses were found after longer postimmunization periods. The parallel increase of HBS-specific cytokine responses allows speculation that natural boosting by *P. falciparum* during the 6-week interval between BS1 and BS2 was not solely responsible for the increase. This suggests that a certain time period may be important to allow the development of RTS,S-induced protective T-cell immunity, as suggested in a previous RTS,S trial in adults (19). This interval may be more relevant in the development of infant immunity.

As an initial description of CSP-specific T-cell immune responses elicited in infants immunized with the RTS,S vaccine, the present study provides crucial background data for conducting further malaria immunology studies in infants and in identifying targets for immune correlates of protection. The methods and results reported here pave the way for future investigation both in optimizing assays adapted for detection of cell-mediated immunity in young African infants and in incorporating other parameters of T-cell function and phenotype in order to identify correlates of RTS,S vaccine efficacy.

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