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

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

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

Results. Interleukin (IL) 2 and IL-5 ratios were associated with RTS,S/AS01E vaccination (adjusted $P \leq .01$). IL-5 circumsporozoite protein (CSP) ratios, a helper T cell type 2 cytokine, correlated with higher odds of malaria in RTS,S/AS01E vaccinees (odds ratio, 1.17 per 10% increases of CSP ratios; *P* value adjusted for multiple testing = .03). In multimarker analysis, the helper T cell type 1 (T_H1)–related markers interferon-γ, IL-15, and granulocyte-macrophage colony-stimulating factor protected from subsequent malaria, in contrast to IL-5 and RANTES, which increased the odds of malaria.

Conclusions. RTS,S/AS01E-induced IL-5 may be a surrogate of lack of protection, whereas T_H1-related responses may be involved in protective mechanisms. Efforts to develop second-generation vaccine candidates may concentrate on adjuvants that modulate the immune system to support enhanced T_H1 responses and decreased IL-5 responses.

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

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

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

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(T_H1) responses, specifically $CD4^+$ T cells expressing, interleukin (IL) 2, tumor necrosis factor (TNF), and interferon (IFN)–γ [4–9]. No investigated cellular response has predicted RTS,S-induced protection consistently in pediatric phase 2 trials [7, 8].

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

METHODS

Study Design

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

Peripheral Blood Mononuclear Cell Stimulations and Multiplex Bead Array Assay

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

Selection of Markers and Metrics for Primary Analysis in a Pilot Study

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

Statistical Analysis

Primary analysis of immunogenicity and correlates of protection was based on ratios of primary markers at M3 and focused on RTS,S/AS01E vaccinees and CSP responses. To distinguish correlates of RTS,S/AS01E-induced immunity from correlates of naturally acquired immunity, results of RTS,S/AS01E and comparator vaccinees were contrasted through interaction tests with vaccination group.

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

All tests were 2-sided and considered statistically significant to a .05 α-level. Analyses were adjusted for multiple testing through permutation (maximum T). *P* values for the 4 primary markers were adjusted (*P*-adj) separately from the 24 secondary markers. *P* values for CSP and HBsAg stimulations were adjusted separately. Adjustments for multiple testing when assessing interactions with vaccination status, age group, or sex were done through Holm (primary markers) and Benjamini-Hochberg (secondary markers) approaches. When age interactions were statistically significant, the age-specific association was reported. Analyses were conducted using the R software

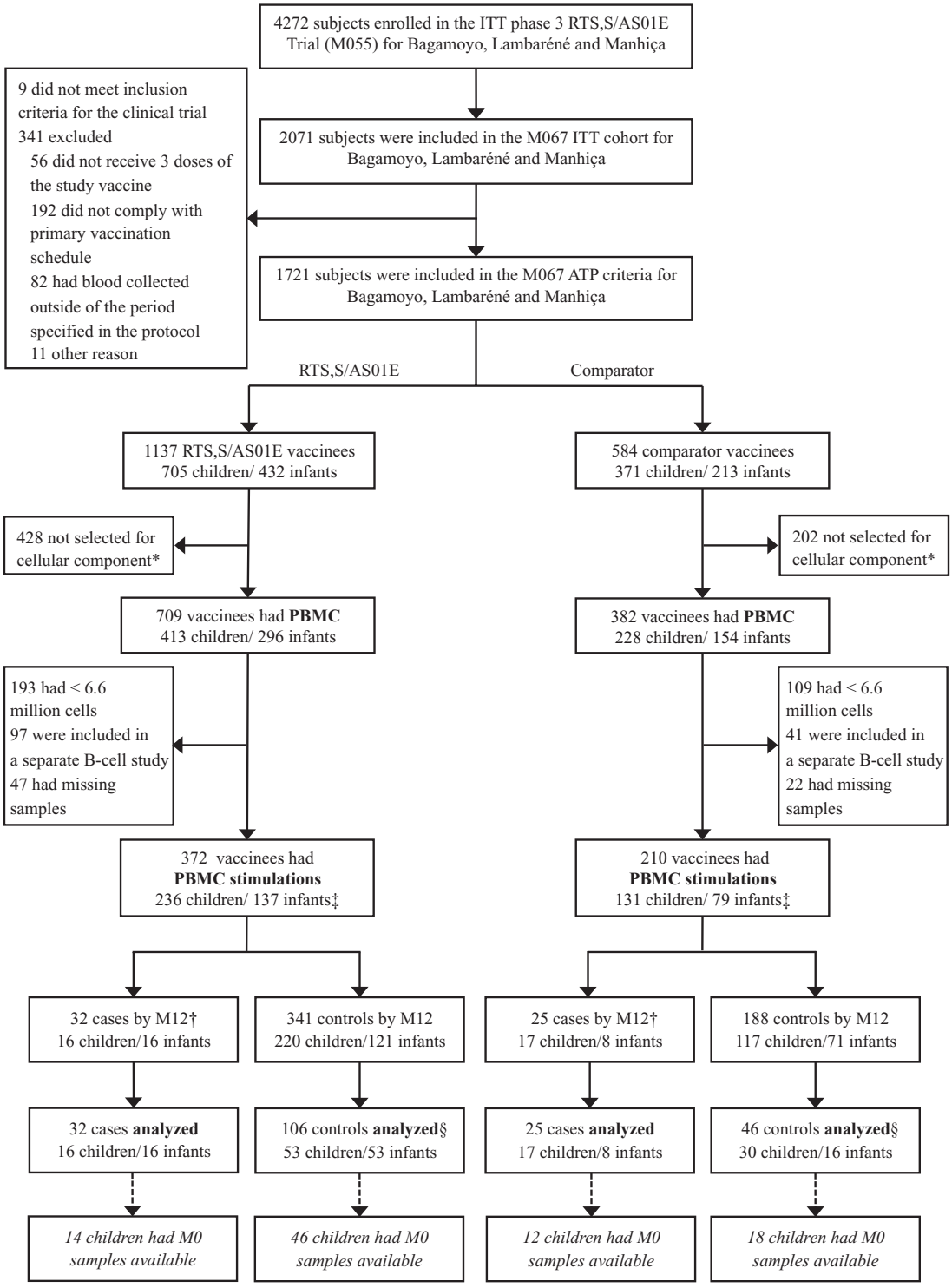


Figure 1. Study profile. *Based on the study protocol, only a subset of vaccinees were enrolled for the cellular component of the immunology study MAL067 (M067), ancillary to the RTS,S/AS01E phase 3 trial MAL055 (M055). ‡Per study protocol, peripheral blood mononuclear cells (PBMCs) were supposed to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

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

RESULTS

Study Population and Markers at Preimmunization

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

Immunogenicity Markers

When comparing CSP ratios between RTS,S/AS01E and comparator vaccinees at M3, only IL-2 was significantly higher in RTS,S/AS01E than in comparator vaccinees (Figure 2A, Supplementary Figure 1A). For HBsAg ratios, IL-2 and the helper T cell type 2 (T_H2) cytokine IL-5 were significantly higher in RTS,S/AS01E vaccinees (Figure 2B, Supplementary Figure 1B). The effect of vaccination on ratios was comparable in children and infants and in both sexes (*P*-adj > .10 for interaction with age and with sex).

Concentrations of primary markers IL-2 and IL-5 after CSP and HBsAg stimulations were significantly higher in RTS,S/AS01E than in comparator vaccinees (Supplementary Figure 2). Unlike analysis with ratios, concentrations after CSP, HBsAg, and mock stimulations were different between age groups for several markers (*P*-adj for interaction with age <.05; Supplementary Table 4). In children, CSP and background responses of most of these markers were higher in RTS,S/AS01E vaccinees than in comparator vaccinees, including the T_H1-related cytokines IFN-γ, IL-12, IL-15, and the proinflammatory markers TNF and IL-1β (Table 1; Supplementary Table 4). In contrast, no differences in marker concentrations between vaccination groups were detected in infants. No differences in immunogenicity by sex were found (*P*-adj > .10 for interaction with sex).

Impact of Preimmunization Markers on RTS,S/AS01E Immunogenicity

Baseline responses to CSP due to previous immunity or to HBsAg due to hepatitis B vaccination (included in the expanded program of immunization) could affect immunogenicity of RTS,S/AS01E in children. Therefore, we analyzed the impact of prevaccination ratios of primary markers on the postvaccination ratio of the same or any other marker (Supplementary Table 5). For CSP ratios in RTS,S/AS01E-vaccinated children, baseline IFN-γ was consistently and positively correlated with postvaccination ratios of some markers, including the immunogenicity marker IL-2, the T_H1-related cytokine IL-15, and the homeostatic cytokine IL-7 (Supplementary Figure 3). For HBsAg ratios, baseline IFN-γ was negatively correlated with some postvaccination CSP ratios, including IFN-α, IFN-γ, and vascular endothelial growth factor (VEGF). Baseline IL-2

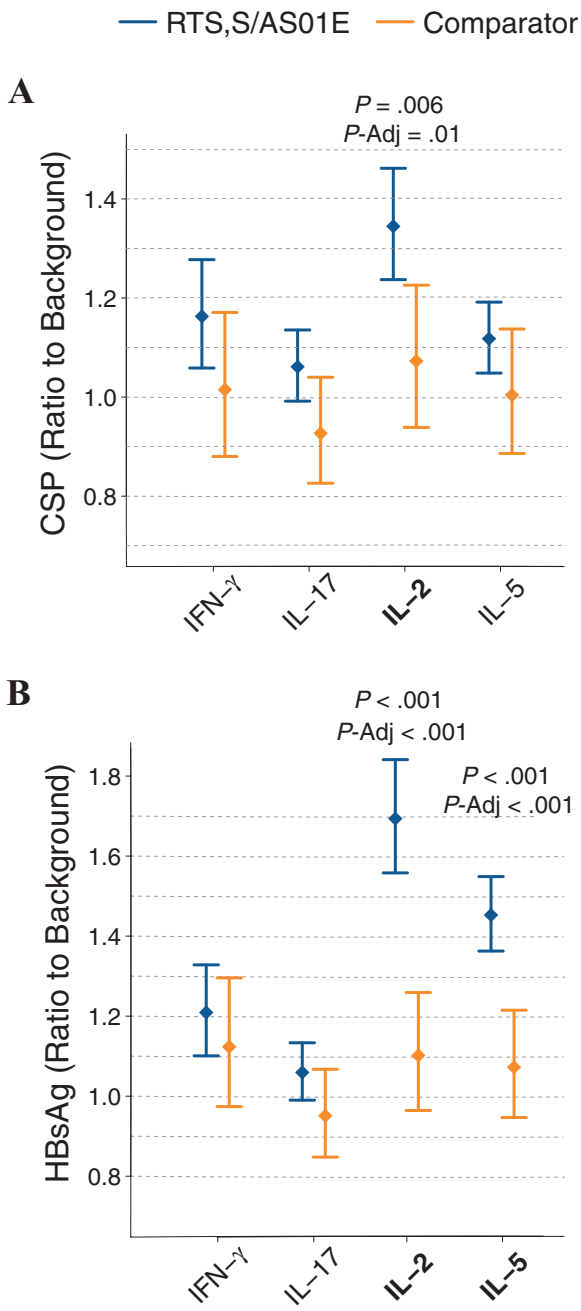


Figure 2. Circumsporozoite protein (CSP; *A*) or hepatitis B surface antigen (HBsAg; *B*) primary marker ratios 1 month postimmunization with RTS,S/AS01E or a comparator vaccine. Diamonds show geometric means of CSP or HBsAg ratios to controls and bars show the 95% confidence intervals. *P* values were computed through *t* tests of the $\log_{10}$-transformed ratios and were adjusted for multiple testing (*P*-Adj) through a permutation approach. Only statistically significant *P* values are shown. There were 137 RTS,S/AS01E and 70 comparator vaccinees for CSP (*A*) and 133 RTS,S/AS01E and 63 comparator vaccinees for HBsAg (*B*). Abbreviations: CSP, circumsporozoite protein; HBsAg, hepatitis B surface antigen; IFN-γ, interferon gamma; IL, interleukin.

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

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

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

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

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

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

HBsAg ratios correlated with higher levels of some postvaccination CSP ratios (eg, IFN-α). Few baseline HBsAg ratios were associated with postvaccination HBsAg ratios in RTS,S/AS01E vaccinees (Supplementary Table 5), and most associations were weak and different from associations with CSP ratios. In contrast, no clear associations were detected in comparator vaccinees.

Correlates of Clinical Malaria: Single-Marker Analysis

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

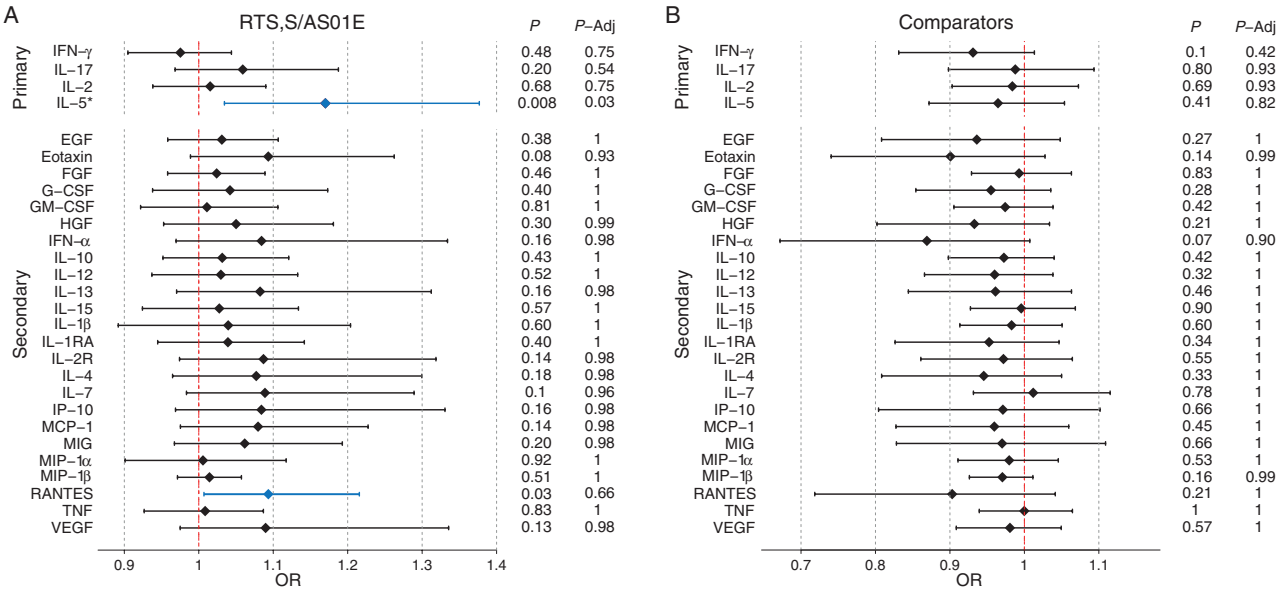


Figure 3. Correlation between clinical malaria and circumsporozoite protein (CSP) marker ratios in RTS,S/AS01E vaccinees (*A*) and comparator vaccinees (*B*). Odds ratios and 95% confidence intervals per 10% increase in primary and secondary marker ratios (CSP ratio to control) 1 month postimmunization.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

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

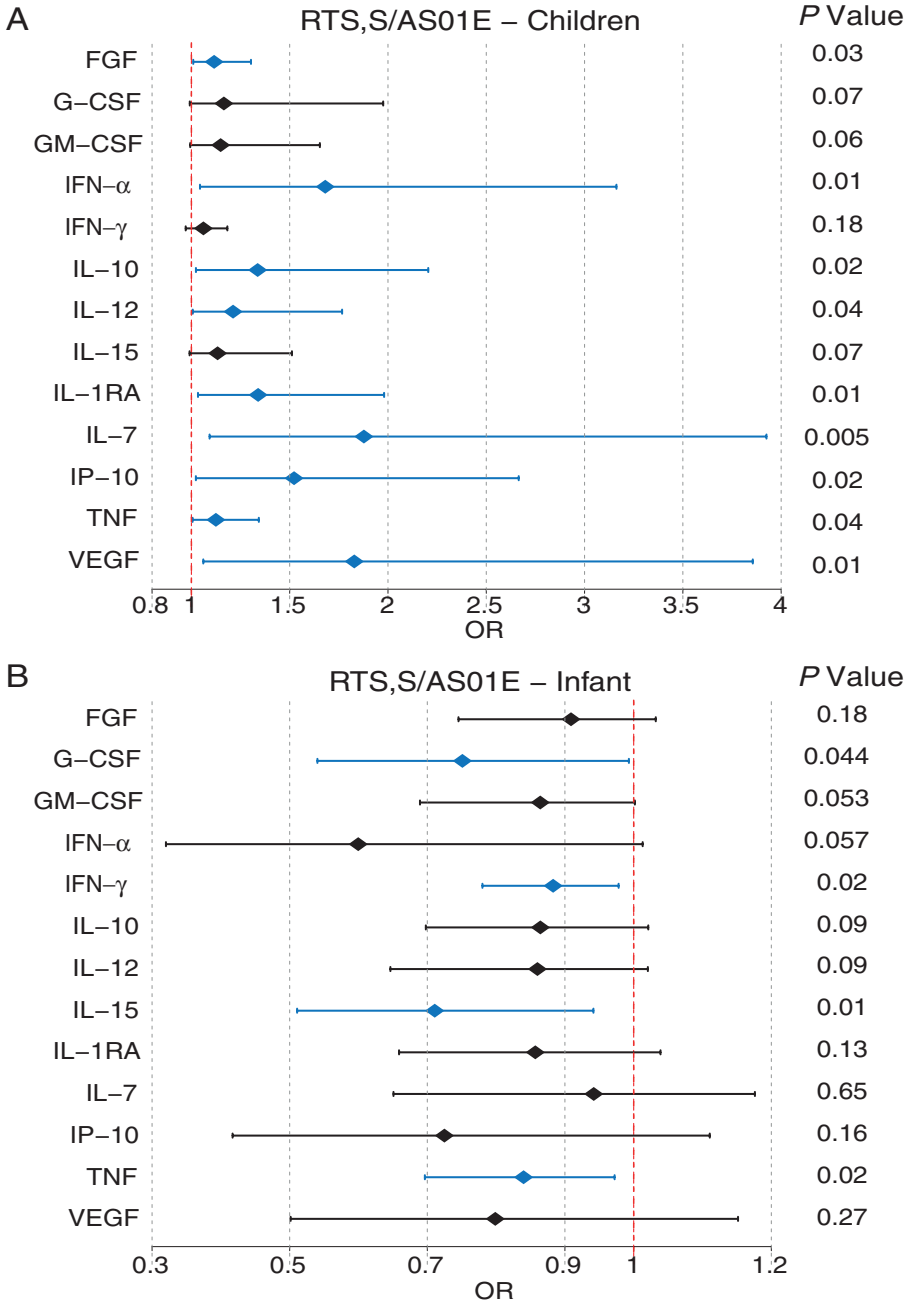


Figure 4. Correlation between clinical malaria and circumsporozoite protein (CSP) marker ratios in RTS,S/AS01E-vaccinated children (*A*) and infants (*B*). Odds ratios and 95% confidence intervals per 10% increase in marker ratios (CSP ratio to control) 1 month postimmunization. Only markers that had statistically significant interactions with age were analyzed. Blue indicates markers that were significantly associated with clinical malaria in logistic mixed-effect models. N = 68 RTS,S/AS01E-vaccinated children and 69 RTS,S/AS01E-vaccinated infants. Abbreviations: FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; IP, interferon-gamma-induced protein; OR, odds ratio; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

The associations of nearly half of the markers with malaria varied between children and infants, including IFN-γ (Figure 4; Supplementary Table 6). In children, increases in CSP ratios of several markers significantly increased the odds of malaria. On the contrary, increases in CSP ratios of T_H1 and proinflammatory cytokines IFN-γ, IL-15, and TNF and the granulocyte colony-stimulating factor (G-CSF) in infants protected from malaria. Sex of vaccinees did not confound associations between ratios and malaria—that is, neither impacted ORs nor *P* values (data not shown).

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

Correlates of Clinical Malaria: Multiple-Marker Analysis

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

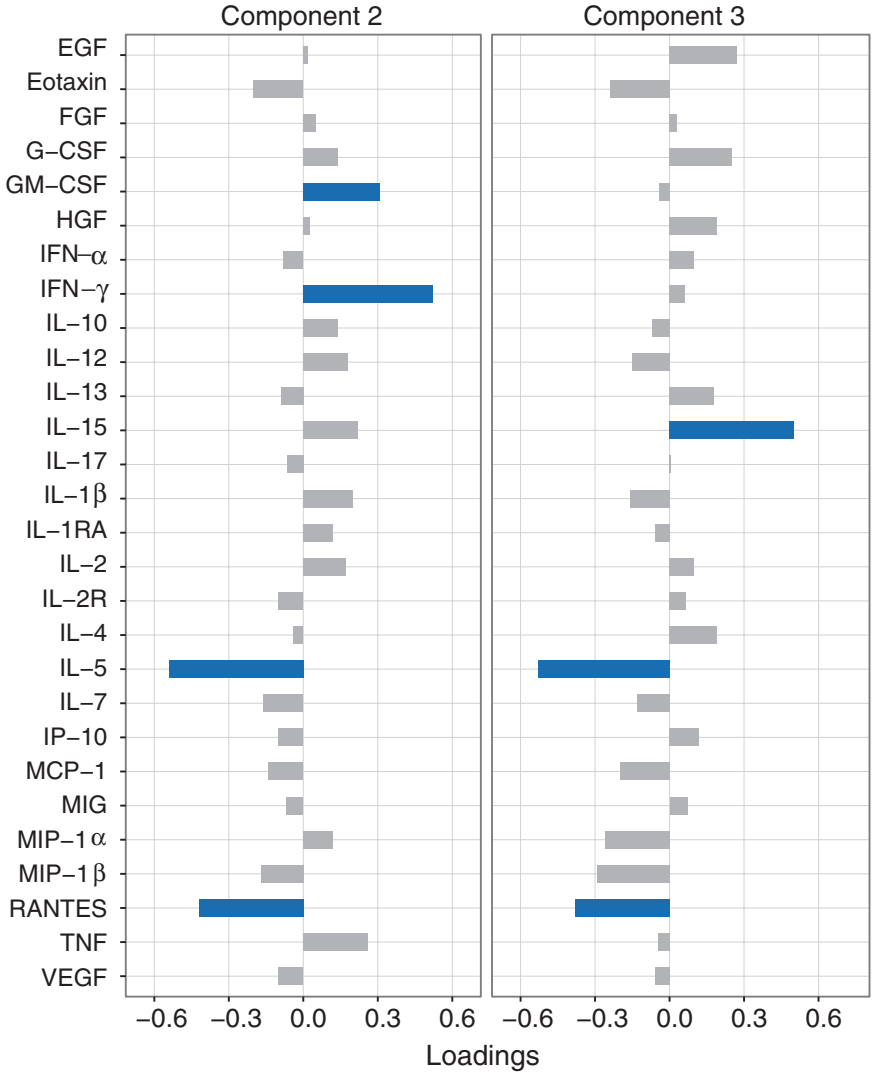
DISCUSSION

We found that IL-5 CSP ratios at postvaccination increased the odds of malaria in RTS,S/AS01E-vaccinated children and infants, a result that could explain the lack of protection in numerous vaccinees through a different mechanism than the previously reported strain-specific VE in children [14]. In multimarker analysis, IL-5 and RANTES increased the odds of malaria in RTS,S/AS01E vaccinees, whereas the T_H1-related markers IFN-γ, IL-15, and GM-CSF were correlated with RTS,S/AS01E-induced protection. IFN-γ and IL-15 were also associated with protection in single-marker analysis in infants, as well as TNF and G-CSF. Although associations of marker ratios with vaccination were comparable between age groups, associations with concentrations were significantly different for several markers. Proinflammatory and the above-reported protective markers were significantly higher in RTS,S/AS01E-vaccinated than in comparator-vaccinated children, but these differences were not observed in infants. These results suggest that RTS,S/AS01E is less immunogenic in infants, and may explain the lower VE in this age group [12]. Of note, RTS,S/AS01 responses were higher for HBsAg than CSP. This may be due to a higher immunogenicity of HBsAg than CSP, to the higher proportion of HBsAg in the RTS,S vaccine, or to hepatitis B vaccination.

IL-5 is mainly produced by activated T_H2 cells and restricted to effector memory T cells that are differentiated after recurrent antigenic exposure [15, 16]. IL-5 has never been examined in RTS,S trials, although IL-4, another T_H2 cytokine, was previously found to be elevated in RTS,S-vaccinated infants [4]. In a preclinical study, the AS01B and AS02A adjuvants induced IL-5 in addition to IFN-γ responses [17]. To our knowledge, there is no epidemiological evidence of IL-5 association with occurrence of clinical malaria. Functional polarity between T_H1 and T_H2 responses could partially explain the association of IL-5 with malaria in RTS,S/AS01E vaccinees. IL-5^+ T_H2 cells probably constrain protective T_H1 responses and inhibit several macrophage functions [18]. Exploratory analyses of IFN-γ/IL-5 ratios indicate that a skewed response to T_H2 increases the odds of malaria. Additionally, the effect of IL-5 in promoting eosinophil responses [18] could contribute to the increased odds of malaria. Eosinophils have important regulatory functions and may restrict inflammation and increase plasma cell responses [19]. RANTES, the other marker that increased the odds of malaria, is produced by memory T cells and macrophages in PBMCs [18, 20] and is involved in chemoattraction of T cells and, together with IL-5, of eosinophils [18]. We speculate that IL-5 and RANTES in RTS,S/AS01E vaccinees could be acting together on eosinophils to regulate antibody responses through plasma cells, jeopardizing protective responses.

IFN-γ, mainly produced by T cells and natural killer (NK) cells, seems to be involved in the protection mediated by pre-erythrocytic vaccines [21]. CSP-specific IFN-γ T-cell and NK cell responses were elicited by RTS,S vaccination in adult challenge studies [22–24] and in clinical trials from endemic areas [4, 6, 7, 9], but their effect on protection remains unclear. T_H1 responses are involved in protection against intracellular pathogens through cell-mediated immunity, and IFN-γ is crucial for parasite killing through the induction of nitric oxide [18, 21]. IL-15, a cytokine produced by dendritic cells and monocytes in PBMCs, is important for activation of NK and T cells, including NK T cells and $CD8^+$ T cells, which may be involved in RTS,S/AS01E-induced cellular responses to liver-stage parasites. GM-CSF, produced by T_H1 and T_H17, NK, and B cells and macrophages, induces effector functions in granulocytes, monocytes, macrophages, and eosinophils, is critical for dendritic cells, and increases IFN-γ secretion [25]. Therefore, IL-15 and GM-CSF could contribute to induction or enhancement of T_H1-mediated protective responses.

Detected age differences in immunogenicity are in line with immune ontogeny. Newborns have a biased cellular response toward a T_H2 profile, due to diminished T_H1 and proinflammatory responses that persist during the first months of life and increase around 12 months of age [26, 27]. Therefore, the age differences in RTS,S immunogenicity may be explained by diminished responsiveness in infants that may impact any vaccine [26, 27] depending on the adjuvant and antigen immunogenicity. Curiously, we detected similar marker differences in the mock stimulations, reflecting nonspecific responses upon RTS,S/AS01E vaccination. A nonspecific effect of RTS,S has not



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

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

been observed, but the nonspecific effect of other vaccines in early life has been described [28–30]. Surprisingly, despite low immunogenicity in infants, more markers were correlated with protection in infants than in children, including the T_H1-related cytokines IFN-γ, IL-15, and TNF. On the contrary, in children, some markers including TNF were associated with increased

odds of malaria. Despite this apparent paradox, different levels of cytokines may have different effects. For instance, moderate levels of cytokines like TNF have been described to control *P. falciparum* infection [31], but exacerbated levels are biomarkers of proinflammatory responses involved in pathogenesis of malaria [32]. Also, differences may be given by diverse cell origin as monocytes, macrophages, NK cells, and also effector memory and central memory T cells may produce it.

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

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

In summary, we identified 2 possible different and antagonistic cellular immune mechanisms induced by RTS,S/AS01E vaccination: IL-5 (and RANTES) T_H2 responses associated with increased odds of malaria; and IFN-γ and other T_H1-related responses (GM-CSF, IL-15) associated with protection. Moreover, we detected lower induction of protective T_H1 and proinflammatory responses by RTS,S/AS01E in infants than children, whereas T_H2 responses were similar, which could contribute to the decreased VE in infants. Further analysis of cytokine-expressing cells together with isotypes and specificities of antibody responses to RTS,S/AS01E will clarify the role of these identified correlates in protection or lack thereof. Our findings may transcend antigen specificity and underscore the need to understand the impact of baseline immune status and factors that may modulate any pediatric vaccine responses in Africa. Our results reveal undesirable vaccine responses that may abrogate the protection of RTS,S/AS01E and other vaccines, but that might be overcome by improved formulations. Adjuvants that modulate the immune system to support a potent T_H1 response during the first months of life and avoid counteracting responses may be required.

Supplementary Data

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

Notes

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

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References

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

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