Circumsporozoite-Specific T Cell Responses in Children Vaccinated with RTS,S/AS01E and Protection against P falciparum Clinical Malaria

Ally Olotu1, Philippe Moris2, Jedidah Mwacharo1, Johan Vekemans3, Domitila Kimani1, Michel Janssens2, Oscar Kai1, Erik Jongert2, Marc Lievens2, Amanda Leach2, Tonya Villafana3,4, Barbara Savarese3, Kevin Marsh1,5, Joe Cohen2, Philip Bejon1,5

1 Kenya Medical Research Institute/ Wellcome Trust Programme, Centre for Geographic Medicine Research, Coast, Kilifi, Kenya, 2 GlaxoSmithKline Biologicals, Rixensart, Belgium, 3 PATH Malaria Vaccine Initiative (MVI), Bethesda, Maryland, United States of America, 4 MedImmune, LLC, Gaithersburg, Maryland, United States of America, 5 Centre for Clinical Vaccinology and Tropical Medicine, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom

Abstract

Background: RTS,S/AS01E is the lead candidate pre-erythrocytic malaria vaccine. In Phase IIb field trials the safety profile was acceptable and the efficacy was 53% (95%CI 31%–72%) for protecting children against clinical malaria caused by P. falciparum. We studied CS-specific T cell responses in order to identify correlates of protection.

Methods and Findings: We used intracellular cytokine staining (for IL2, IFNγ, and TNFα), ex-vivo ELISPOTs (IFNγ and IL2) and IFNγ cultured ELISPOT assays to characterize the CS-specific cellular responses in 407 children (5–17 months of age) in a phase IIb randomized controlled trial of RTS,S/AS01E (NCT00380393). RTS,S/AS01E vaccines had higher frequencies of CS-specific CD4+ T cells producing IFNγ, TNFα or IL2 compared to control vaccines. In a multivariable analysis TNFα+ CD4+ T cells were independently associated with a reduced risk for clinical malaria among RTS,S/AS01E vaccinees (HR = 0.64, 95%CI 0.49–0.86, p = 0.002). There was a non-significant tendency towards reduced risk among control vaccinees (HR = 0.80, 95%CI 0.62–1.03, p = 0.084), albeit with lower CS-specific T cell frequencies and higher rates of clinical malaria. When data from both RTS,S/AS01E vaccines and control vaccinees were combined (with adjusting for vaccination group), the HR was 0.74 (95%CI 0.62–0.89, p = 0.001). After a Bonferroni correction for multiple comparisons (n=18), the finding was still significant at p = 0.018. There was no significant correlation between cultured or ex vivo ELISPOT data and protection from clinical malaria. The combination of TNFα+ CD4+ T cells and anti-CS antibody statistically accounted for the protective effect of vaccination in a Cox regression model.

Conclusions: RTS,S/AS01E induces CS-specific Th1 T cell responses in young children living in a malaria endemic area. The combination of anti-CS antibody concentrations titers and CS-specific TNFα+ CD4+ T cells could account for the level of protection conferred by RTS,S/AS01E. The correlation between CS-specific TNFα+ CD4+ T cells and protection needs confirmation in other datasets.

Introduction

RTS,S is the lead candidate pre-erythrocytic malaria vaccine [1]. The vaccine antigen consists of 19 copies of the central tandem repeats and C-terminal region of the P. falciparum circumsporozoite protein (CS) fused to hepatitis B surface antigen (HBsAg), and co-expressed with unfused HBsAg in Saccharomyces cerevisiae cells. The two proteins spontaneously assemble in the yeast cells to form virus-like particles. The RTS,S antigen has been tested with two different alternative Adjuvant Systems: AS02 or...
AS01. Both Adjuvant Systems contain the immunostimulants monophosphoryl lipid A (MPL®) and QS21, formulated either with an oil-in-water emulsion (AS02) or with liposomes (AS01).

Formulated in either Adjuvant System, the RTS,S antigen induces high concentrations of anti-circumsporozoite protein (CS) antibodies [2,3,4,5,6,7]. Correlations between anti-CS concentrations and protection against infection were statistically significant on experimental challenge with *P. falciparum* in malaria naïve adults [7], of borderline significance on natural challenge of semi-immune adults [4], and significant on natural challenge of children in a malaria endemic area [8]. Anti-CS titers did not correlate with protection against clinical malaria episodes in children [4,9], but we recently identified a non-linear relationship between concurrent (rather than peak) anti-CS titers and protection from clinical malaria in children [10].

CD4+ T cell responses to pre-erythrocytic antigens prevent intra-hepatocytic parasites developing in both human and mouse studies [11,12]. Potential mechanisms include TNFα induced apoptosis [13] or inhibition of parasite growth [14] and IFNγ induced NO production [15]. RTS,S-activated T cell mediated immune responses have been assessed using proliferation assays, cytokine production on cell culture, intracellular cytokine staining and flow-cytometry, and *ex vivo* and cultured ELISPOT assays [16,17].

RTS,S/AS immunization induces a CD4+ T cell response but little or no detectable CD8+ T cell response [7,18,19,20,21]. Sun et al observed IFNγ-producing CD8+ T cells, but only after cells were stimulated for 10–14 days *in vitro* [22]. Barbosa et al reported CD8+ T cell responses after 42 hours *in vitro* stimulation on comparing RTS,S/AS02 vaccinees with control vaccinees at 10 weeks, but not at 4 weeks, post immunization [23].

The frequency of poly-functional CD4+ T cells identified by intracellular cytokine staining (ICS) correlated with protection from *P. falciparum* infection after experimental challenge in adults [7,24]. In a field study, Reece et al reported a correlation between protection against re-infection and cultured IFNγ ELISPOT assays using a single conserved T cell epitope from the CS protein [20]. However, this analysis was not adjusted for anti-CS titers, and did not include ICS studies. A borderline correlation between single cytokine ICS results and protection from *P. falciparum* infection was shown in a field study in infants [23].

In order to examine associations with protection against clinical malaria, we assessed the CS-specific cellular immune responses in 447 children using ICS, *ex vivo* IFNγ and IL2 ELISPOT, and cultured IFNγ ELISPOT assays in a phase II b randomized clinical trial of RTS,S/AS01E versus control, in which we observed 53% (95%CI 31%–72%) protection against clinical malaria [25]. The blood volumes sampled in children prevented us from using an ICS assay previously reported in adult studies [7], but a whole blood ICS assay requiring smaller blood volumes has been developed and used in two phase II trials in Ghana [26] and Gabon [27]. These studies showed that the vaccine induced CD4+ IL2, TNFα or IFNγ producing cells, but CD40L was not detectable using the whole blood assay for children in Sub-Saharan Africa. We therefore did not include CD40L staining in the assay for our study.

The qualification of correlates of immunity and surrogates of protection has been recently reviewed [28,29]. The Prentice criteria require that: a) vaccination predicts protection; b) vaccination predicts the potential surrogate; c) the surrogate predicts protection among vaccinees and d) that the surrogate accounts for all the effect of vaccination [30]. If vaccination is an independent predictor of outcome including the potential surrogate in the analysis, this suggests that other mechanisms are involved. On the other hand, if including the potential surrogate in analysis removes vaccination as a predictor, this is consistent with the effect of vaccination being mediated by the surrogate marker.

**Methods**

The study protocol and its amendments received ethical and scientific approval from Kenya Medical Research Institute National Ethics Committee, National Institute for Medical Research of Tanzania, the Oxford Tropical Research Ethics Committee, the London School of Hygiene and Tropical Medicine Ethics committee and the Western Institutional Review Board in Seattle. The study was conducted in accordance with the Helsinki Declaration of 1964 (revised 1996) and Good Clinical Practice guidelines and was overseen by an Independent data monitoring committee and local safety monitors. Written informed consent was obtained using approved Swahili or Giriama consent forms. Illiterate parents thumb printed the consent form which was countersigned by an independent, literate witness.

We conducted a randomized controlled trial to evaluate the efficacy and safety of RTS,S/AS01E against clinical malaria episodes due to *P. falciparum* infection in Kilifi, Kenya and Korogwe, Tanzania. There were 894 children between the two sites, of which the 447 children enrolled in Kilifi were assessed for vaccine induced cellular immunity using ICS and ELISPOT.

Details on randomization, immunization and surveillance have been published previously [25]. In Kilifi, 447 children 5–17 months old were randomized and received either RTS,S/AS01E or rabies vaccine in a 1:1 ratio according to 0, 1, 2 month schedule. Both vaccines were given intramuscularly in the left deltoid. The primary end point was clinical malaria, defined as the presence of fever (axillary temperature ≥37.5°C) and *P. falciparum* parasitaemia ≥2500/μL. Active and passive surveillance for malaria was conducted by field workers and study personnel at local dispensaries.

Children were vaccinated between March and August 2007. Blood was taken for immunological studies before vaccination, one month post dose 3, then on March 2008 irrespective of the time of recruitment (i.e. between 4 and 10 months post dose 3, mean 8 months), 12 months post dose 3 and in October 2008 irrespective of time of recruitment, (i.e. between 12 and 18 months post dose 3, mean 15 months). Peak malaria transmission was between May and August 2008.

**CS antibody measurement**

Antibodies to the *P. falciparum* circumsporozoite protein (CS) tandem repeat epitope were assessed by ELISA at the Center for Vaccinology, Ghent University Hospital, Belgium. Results were reported in EU/mL. Plates were adhered with the recombinant antigen R32LR that contained the sequence [NVDP]NANC15][LR] [31].

**Peptides**

A set of 32 15-mer, peptides were used, overlapping by 11 amino acids to cover the full length of the CS antigen used in the vaccine (3D7 strain). All these peptides were used in a single pool for the ICS studies, but they were divided into three pools for ELISPOT studies, namely; a) the conserved region including the NANC repeats, b) the variant TH2R region and c) the variant TH3R region and conserved CS.T3T region (Table 1).

**ELISPOT assays**

Peripheral blood mononuclear cells (PBMC) were separated and incubated in RPMI medium (Sigma-Aldrich) with 10% Human AB serum. We used Millipore MAIP S45 plates and MabTech antibodies for ELISPOT assays according to the
Table 1. Peptide pools.

| Peptide | Sequence |
|---------|----------|
| NaNP and conserved region peptides pool | NaNP and conserved region peptides pool |
| Pept 1 | MMAP DPNANPNANPN |
| Pept 2 | NaNP NaNPANPNANP |
| Pept 3 | DPNA NPANPNANPNQ |
| Pept 4 | NPNA NPNNQNGQNGQ |
| Pept 5 | NPNN QNGQNGQNGHNM |
| Pept 6 | NQNG NQGQNMMPNPD |
| Pept 7 | NGQG HNMMPNPNNV |
| Pept 8 | HNMP NDPNMRVYDNA |
| Pept 9 | NQPM RNVDENANANS |
| Pept 10 | RNVD ENANANSVKN |
| Pept 11 | ENAN ANSAVKNNNNE |
| TH2R region peptides pool | TH2R region peptides pool |
| Pept 12 | ANSA VKNNNNEEPSD |
| Pept 13 | VKNV NNEEPSDKHIK |
| Pept 14 | NNEE PSDHKEKYLEN |
| Pept 15 | PSDK HIKEYNKKNQ |
| Pept 16 | HIKE YLNQONLSLST |
| Pept 17 | YLNK IQNLSLEWSP |
| Pept 18 | IQNS LTEWSPCSCVT |
| Pept 19 | LSTE WSPCSEVTCGNG |
| TH3R/CS.T3T region peptides pool | TH3R/CS.T3T region peptides pool |
| Pept 20 | WSPC SVTGGNGQVQR |
| Pept 21 | SVTC GNGQVQKRPG |
| Pept 22 | GNGI QVRIKPGSANK |
| Pept 23 | QVRI KPGSANPKPD |
| Pept 24 | KPGS ANPKPDELDYA |
| Pept 25 | ANPK KDELDYANDIE |
| Pept 26 | KDEL DYANDIEKIC |
| Pept 27 | DYAN DYEKICKIMEK |
| Pept 28 | DIEK KMEKICSSV |
| Pept 29 | KICK MKCSSVFNV |
| Pept 30 | MECK SSFVNNVNSSI |
| Pept 31 | KCSS VFNNVNSSIGL |

All three peptide pools were combined for the ICS assay. The pools were used separately for the ex vivo and plating out of the cultured ELISPOT assay. doi:10.1371/journal.pone.0025786.t001

ELISPOT analysis

ELISPOT wells were assayed in duplicate, and the final result was the mean of two wells. The negative control well result was subtracted from each peptide well. ELISPOT’s failed quality control if the negative control well had more than 25 spots or the positive control had less than 30 spots. The results from the three peptide pools were added to calculate total responses. Results are presented as number of spots per million incubated PBMC.

Whole blood ICS assay

Whole blood was stimulated in Kilifi within 2 hours of being drawn. 350 μl of whole blood plus 100 μl of phosphate buffered saline (PBS) was incubated in three different 15 ml Falcon tube, with 1 μg/ml of anti-CD28 anti-CD49d monoclonal antibodies (supplied by BD). After 2 hours, Brefeldin A was added to a final concentration of 1 μg/ml and incubation was continued overnight at 37 °C ± 1- CO2 5 to 7%. EDTA was then added to a final concentration at 5 mM, and after 15 minutes 1 ml FACS lysing solution (BD). The positive control was stimulated using staphylococcal enterotoxin B (SEB) and negative control was PBS without peptides. Circumsporozoite antigen peptides were added to the third tube to a final concentration of 1 μg/ml (see Table 1). The cells were then washed in PBS and re-suspended in PBS with 10% DMSO and stored at −70°C for transport to GSK in Rixensart. In GSK, cells were thawed, washed and stained with alexa-fluor 700 conjugated anti-CD3 (Pharmingen), peridinin-chlorophyll (PerCP)-conjugated anti-CD4 (BD Biosciences), and allophycocyanin (APC)-H7 conjugated anti-CD8 antibodies (BD Biosciences). Cells were fixed and permeabilized using the Cytofix/Cytoperm buffer kit (Pharmingen), and stained with APC conjugated anti-IL-2 (Pharmingen), fluorescein-isothiocyanate (FITC)-conjugated anti-IFN-γ (Pharmingen) and phycoerythrin (PE) cyanin–7 (Cy7)-conjugated anti-TNFα (Pharmingen). Cells were washed, re-suspended in fetal-calf-serum (FCS)-containing phosphate buffered saline (PBS) and analyzed on a BD™ LSR II flow cytometer (BD Biosciences). Events were counted using the automatic gating on the FACSDiva software (BD Biosciences). Conventional rules were used to gate on single cells, then the lymphocyte subset based on forward and side scatter, CD3 and CD4/CD8 positive cells and then cytokine expression was classified into positive/negative using FACSDiva software. An example of the output with gating shown is given in Supporting Information S1. We required at least 10,000 CD4+ events and 5,000 CD8+ events had been acquired, and we acquired more than 50,000 CD4+ events for the majority of samples (>90%). Results from antigen-stimulated cultures were not excluded from analysis on the basis of positive/negative control results, in the absence of established criteria. Data are represented as background subtracted GS-specific events per million CD4+ or CD8+ T cells.

Assays were conducted according to sample availability, since blood samples were limited to 3 mls. In order of priority, the assays conducted were; ICS, IFNγ ex vivo ELISPOT, IL2 ex vivo ELISPOT and cultured ELISPOT. Samples were processed within 3 hours of being taken. ICS samples were stored for 3 to 4 months at −70°C before staining. The samples were processed during the double-blind phase of the study.

Statistical analysis

Geometric mean responses are calculated and a Student’s T test was performed on log-transformed values to compare between vaccination groups. A paired T test on log-transformed values was used to compare time-courses, and correlations between assays were examined using Pearson’s product moment calculation on
log-transformed values. Cox regression for the primary endpoint (clinical malaria with \textit{P. falciparum} density $\geq 2500/\mu L$) was adjusted for age at first vaccination, village, distance from the health facility, bed net use and anti-circumsporozoite (CS) antibody levels by dichotomizing concurrent anti-CS titers at 40 EU/mL [10]. Cellular responses were analyzed as time-varying covariates, applying the result from the time of the most recent clinic visit. A Bonferroni correction was subsequently calculated for the independently significant explanatory variables. Responses were log transformed to produce normal distributions before inclusion in the Cox regression models. Analysis was conducted on the According To Protocol vaccinees. STATA version 10 was used.

Results

Blood samples were processed from 407 children. Data were acquired from 1,066 ICS assays (from three different clinic visits), 660 cultured ELISPOTs (from four different clinic visits), 780 \textit{ex vivo} ELISPOTs for IFN$\gamma$ (from three clinic visits) and 453 \textit{ex vivo} ELISPOTs for IL2 production (from three clinic visits). 56 (8%), 12 (2%) and 21 (5%) assays failed quality control criteria for positive and negative controls for cultured, and \textit{ex vivo} IFN$\gamma$ and \textit{ex vivo} IL2 ELISPOTs, respectively. For ICS assay, the results from the positive control were at least 100 cells per million above the negative control for 1045 (98.0%), 1057 (99.1%) and 1055 (99.0%) for IFN$\gamma$, IL2 and TNF$\alpha$, respectively.

The geometric mean responses to the negative control were 75, 165 and 159 cells per million for IFN$\gamma$, IL2 and TNF$\alpha$ ICS results respectively, and average responses to positive control were 3,768, 10,895 and 3,454 cells per million. The mean responses to CS antigen vary by timepoint and by vaccination group, but the ranges were 11 to 25, 10 to 681 and 8 to 426 for IFN$\gamma$, IL2 and TNF$\alpha$, respectively. There was no variation in responses to control by time point ($p = 0.15$, $p = 0.15$, $p = 0.6$) or by vaccination group at the first timepoint post vaccination ($p = 0.4$, $p = 0.36$ and $p = 0.39$). An example of the flow cytometry analysis is shown in Figure 1.

Vaccine induced anti-CS T cell responses: ICS assays

CD4$^+$ and CD8$^+$ anti-CS T cell responses were detected in both vaccination groups using ICS. There were no significant differences between the groups pre-vaccination. Vaccination with RTS,S/AS01E induced CD4$^+$ but no CD8$^+$ anti-CS T cell responses. The strongest responses were seen for IL2 producing CD4$^+$ T cells at one month post vaccination (a mean of 681 cells per million, 95\%CI 585–792), followed by TNF$\alpha$ (426 cells per million, 95\%CI 362–502), and weak IFN$\gamma$ responses (25 cells per million, 95\%CI 18–34) (Table 2, Figure 2). These levels

Figure 1. An example plot of FACS data acquired following intra-cellular cytokine staining is shown for negative control (medium only), positive control (i.e. staphylococcal enterotoxin B, SEB) and CS peptides.

doi:10.1371/journal.pone.0025786.g001
### Table 2. Geometric means of CMI assays by clinic visit and by vaccination group.

| Visit | Rabies | RTS,S/AS01e |
|-------|--------|-------------|
|       | Mean (95%CI) | N | Mean (95%CI) | N | p |
|       | ICS: CD4+ve cells: IFNg | Screen | 118 (93–145) | 172 | 119 (96–145) | 182 | 0.73 |
|       | ICS: CD4+ve cells: IL2 | Screen | 103 (82–127) | 173 | 95 (76–117) | 182 | 0.52 |
|       | ICS: CD8+ve cells: TNFa | Screen | 86 (71–108) | 172 | 81 (64–102) | 182 | 0.69 |
|       | ICS: CD8+ve cells: IL2 | Screen | 156 (121–191) | 171 | 190 (155–233) | 174 | 0.79 |
|       | IFNg cultured ELISPOT: NANP and conserved region | Screen | 27 (23–31) | 149 | 33 (30–36) | 160 | 0.8 \\* |
|       | IFNg cultured ELISPOT: TH2R region | Screen | 33 (26–40) | 148 | 32 (29–34) | 160 | 0.8 \\* |
|       | IFNg cultured ELISPOT: TH3R/CS.T3T region | Screen | 31 (24–38) | 149 | 30 (27–33) | 160 | 0.8 \\* |

### Table 2. Cont.

| Visit | Rabies | RTS,S/AS01e |
|-------|--------|-------------|
|       | Mean (95%CI) | N | Mean (95%CI) | N | p |
|       | IFNg ex vivo ELISPOT: TH2R region | Screen | 15 (13–17) | 152 | 14 (12–16) | 163 | 0.56 |
|       | IFNg ex vivo ELISPOT: TH3R/CS.T3T region | Screen | 18 (15–20) | 152 | 17 (15–20) | 163 | 0.79 |
|       | IL2 ex vivo ELISPOT: All CS peptides summed | Screen | 44 (33–57) | 153 | 42 (33–53) | 163 | 0.71 |

*for p < 0.05 or ** for p < 0.005 where Mean for Rabies group < Mean for RTS,S/AS01e group. # for p < 0.05 or ## for p < 0.005 where Mean for Rabies group > Mean for RTS,S/AS01e group.

ELISPOT assays

Three different peptide pools were used for the ELISPOT assays, allowing a more detailed analysis of immunogenicity. Cultured ELISPOT results were higher among RTS,S/AS01e vaccines than among rabies vaccines at 1 month and 6.5 months post vaccination, but not at 12 months. IFNg ex vivo ELISPOT results did not vary by vaccination group at any timepoint. IL2 ex vivo ELISPOT responses were significantly higher in RTS,S/AS01e vaccines at 1 month post vaccination, but not at 6.5 months (Table 2) compared with rabies vaccines.

For both the cultured IFNg ELISPOT and ex vivo IL2 ELISPOT, the vaccine induced cellular responses were limited to two peptide pools (i.e. TH2R and TH3R/CS.T3T pools, Table 1). No responses were detected to the third peptide pool (NANP and conserved region peptides; Figure 3).
Table 3. Inter-assay Correlation coefficients of CMI assays at 1 month post vaccination with RTS,S/AS01E.

|                | CD4+ IFNγ | CD4+ IL2 | CD4+ TNFα | Antibody (CS) | Cultured IFNγ | IFNγ ELISPOT | IL2 ELISPOT |
|----------------|-----------|----------|-----------|---------------|---------------|--------------|-------------|
| CD4+ IFNγ      | 1         |          |           |               |               |              |             |
| CD4+ IL2       | 0.38***   | 1        |           |               |               |              |             |
| CD4+ TNFα      | 0.32***   | 0.66***  | 1         |               |               |              |             |
| Antibody (CS)  | 0.14***   | 0.35***  | 0.26***   | 1             |               |              |             |
| Cultured IFNγ  | 0.14**    | 0.15**   | 0.18***   | 0.22***       | 1             |              |             |
| IFNγ ELISPOT   | −0.05     | −0.05    | 0.02      | 0.03          | −0.02         | 1            |             |
| IL2 ELISPOT    | 0.01      | 0.15*    | 0.11*     | −0.06         | 0.31***       | 1            |             |

* = p<0.05, ** = p<0.01, *** = p<0.001.

doi:10.1371/journal.pone.0025786.t003

Time course of responses (ICS assays)

The frequencies of IL2, TNFα and IFNγ producing CD4+ T cells by ICS was significantly higher at one month after the final vaccination with RTS,S/AS01E compared with pre-vaccination levels (p<0.0001, p<0.0001, p = 0.0006, respectively). There was then a fall in responses between 1 month and 12 months post vaccination, falling to pre-vaccination levels for IL2 (p<0.0001) and TNFα (p<0.0001). IFNγ producing CD4+ T cells remained above pre-vaccination levels, albeit at low frequency throughout. However, there was an even more pronounced fall in CD4+ T cell responses among control vaccinees (Table 2), and so RTS,S/AS01E vaccinated children had substantially higher CD4+ T cell responses than control vaccinees at 12 months post vaccination (p<0.0001 for TNFα and IL2, p = 0.009 for IFNγ).

Inter assay correlations

There were strong correlations between the different cytokines detected by ICS, and also between IL2/IFNγ ELISPOT results one month after vaccination with RTS,S/AS01E (Table 3). Cultured ELISPOT results were significantly associated with ICS results, but not with ex vivo ELISPOT results (IFNγ or IL2). Antibody titres were associated with all the cellular assays except ex vivo IFNγ ELISPOT results (Table 3).

Correlates of immunity

After vaccination with RTS,S/AS01E, an increasing frequency of TNFα producing, CS-specific CD4+ cells detected using ICS was associated with a reduced risk of clinical malaria (HR = 0.64 for each 10 fold increase in the frequency of CD4+ TNFα + T cells, 95%CI 0.49–0.86, p = 0.002). On ICS, IFNγ production by CS-specific CD4+ T cells was associated with a reduced risk of clinical malaria of borderline significance (p = 0.07, Table 4). TNFα and IFNγ producing CS-specific CD4+ T cells were at much lower frequencies among control vaccinees, but nevertheless were associated with reduced risks of clinical malaria of borderline significance. When data from both RTS,S/AS01E vaccinated and control vaccinees were combined (with adjusting for vaccination group), the overall hazard ratios were 0.74 (95%CI 0.62–0.89, p = 0.001) and 0.79 (95%CI 0.67–0.94, p = 0.007) for TNFα and IFNγ, respectively. On Bonferroni adjustment, these p values were 0.018 and 0.13, respectively. Similar results were observed when adjusting for anti-CS antibody titres as a continuous variable (HR = 0.75, 95%CI 0.62–0.91, p = 0.003 with p = 0.054 after Bonferroni adjustment, and HR = 0.81, 95%CI 0.68–0.95, p = 0.01 with p = 0.13 after Bonferroni adjustment for TNFα producing CD4+ T cells and IFNγ producing CD4+ T cells, respectively).

In order to display the effect graphically, the cellular responses were split by tertile (Figure 4). The middle tertiles for TNFα are at intermediate risk, suggesting a continuous change in risk as the frequency of TNFα cells increases rather than a threshold effect.

When the frequencies of TNFα producing CD4+ T cells and IFNγ producing CD4+ T cells were combined in the same model, TNFα producing CD4+ T cell frequency was the independent factor (i.e. HR = 0.76, 95%CI 0.64–0.89, p = 0.001 compared with HR = 0.84, 95%CI 0.71–1.00, p = 0.050 for the frequency of IFNγ producing CD4+ T cells). An interaction term generated by multiplying the frequency of TNFα producing CD4+ T cells by antibody concentrations was not significant in determining risk (HR = 0.79, 95%CI 0.51–1.2, p = 0.29).

On applying the fourth of the Prentice criteria, we found that vaccination group was still an independent predictor of clinical malaria risk in a multivariable model including CD4+ TNFα + T cells (HR = 0.69, 95%CI 0.48–0.97, p = 0.036). In other words, only 42% of the effect of vaccination could be accounted for by CD4+ TNFα + T cells. However, when anti-CS titers were added to the model the effect of vaccine became non-significant (HR = 0.93, 95%CI 0.62–1.42, p = 0.76, i.e. 87% of the effect of vaccination was accounted for).

Hence, while neither CD4+ TNFα + T cells nor anti-CS antibodies alone accounted for all of the effect of vaccination with RTS,S/AS01E on clinical malaria risk, the combination of CD4+ TNFα + T cells and anti-CS antibodies together could account for all of the statistical effect of vaccination.

Discussion

Vaccination with RTS,S/AS01E induced circumsporozoite protein (CS) specific T cell responses in 5–17 month-old children living in a malaria endemic area. The frequency of CD4+ TNFα + T cells on ICS was associated with protection from clinical malaria. Although the use of 15-mer peptides may have been sub-optimal to demonstrate CD8+ T cell responses, we did in fact identify both CD4+ and CD8+ responses above negative control conditions for both RTS,S/AS01E and control vaccinees. However, the CD8+ responses were apparently not induced by vaccination, and presumably are the result of exposure to malaria parasites [32].

If TNFα producing CD4+ T cells are causally related to protection, they should be associated with protection whether they are acquired by vaccination or by natural exposure to malaria
Figure 2. The time course of anti-CS CD4+ ICS responses and summed ELISPOT responses is shown per time point for RTS,S/AS01E and control vaccination groups. * indicates $p<0.05$ and ** indicates $p<0.005$.

Figure 3. ELISPOT responses are shown for the individual stimulating peptide pools at 1 month post vaccination with RTS,S/AS01E.

doi:10.1371/journal.pone.0025786.g002

doi:10.1371/journal.pone.0025786.g003
parasites. Indeed, a borderline statistical correlation between TNF-α CD4+ T cells and a reduced risk of clinical malaria was observed among control vaccinees. However, the overall level of protection afforded by T cells among RTS,S/AS01E vaccinees was greater, since the vaccinated children had 2–3 fold more TNF-α at 1 month and 8–10 fold more at 12 months post vaccination. The frequency of TNF-α CD4+ T cells at 12 months post vaccination was below screening (i.e. pre-vaccination) levels, and may reflect short-term changes in exposure [35]. Taking together the antibody data, the consistent pattern in ICS and ELISPOT studies, the stability of positive and negative control responses, and the accounting for background reactivity by subtracting negative control responses from antigen-specific responses, show no significant variation over time for positive and negative controls, we conclude that the T cell responses are raised by exposure to malaria during the transmission season, but are short-lived and therefore not sustained once exposure falls. We did not identify an association between protection and T cell responses detected by the cultured IFN-γ assay, as previously reported [20]. However, we tested pools of peptides rather than individual peptides, and the previously reported association was specific to the CS.T3T peptide. The CS.T3T peptide was contained in a pool of TH3R/CS.T3T peptides. In other studies, the CS.T3T peptide accounted for more than half of the overall response seen in the TH3R/CS.T3T peptide pool [36]. Furthermore, we identified very little IFN-γ production in our study. Previous studies showing marked IFN-γ production have been done in adults [18], and IFN-γ production may be suppressed in children in malaria endemic areas [37].

Ex vivo ELISPOT studies did not correlate with ICS studies for the same cytokines, even though both use overnight stimulations, and ICS results were 10-fold higher than ELISPOT results. This may be partially explained by measuring ELISPOT assays per million PBMC, whereas ICS is measured per million CD4+ T cells. However, ex vivo and cultured ELISPOTs identify different

### Table 4. The hazard ratio from Cox regression models (with 95% CI) for the outcome clinical malaria by CMI assays.

| Assay       | Both datasets | Rabies Vaccines | RTS,S/AS01E Vaccines |
|-------------|--------------|-----------------|----------------------|
|             | HR (95%CI)   | p               | HR (95%CI)           | p               |
| ICS: CD4 cells |
| IFN-γ       | 0.79(0.67–0.94) | 0.007           | 0.81(0.66–1.01)     | 0.058          |
| IL2         | 0.90(0.76–1.07) | 0.23             | 0.97(0.78–1.22)    | 0.81           |
| TNF-α       | 0.74(0.62–0.89) | 0.001           | 0.80(0.62–1.03)    | 0.084          |
| ICS: CD8 cells |
| IFN-γ       | 1.07(0.91–1.25) | 0.43             | 1.13(0.93–1.37)    | 0.21           |
| IL2         | 0.85(0.69–1.05) | 0.13             | 0.92(0.70–1.21)    | 0.56           |
| TNF-α       | 0.87(0.72–1.04) | 0.12             | 0.83(0.66–1.05)    | 0.11           |
| IFN-γ cultured ELISPOT |
| NANP        | 1.01(0.65–1.57) | 0.95             | 0.90(0.54–1.52)    | 0.7            |
| TH2R        | 0.76(0.51–1.14) | 0.18             | 0.67(0.30–1.52)    | 0.34           |
| TH3R        | 0.94(0.67–1.32) | 0.72             | 0.99(0.61–1.61)    | 0.97           |
| Sum         | 0.95(0.67–1.34) | 0.77             | 0.92(0.54–1.57)    | 0.77           |
| IFN-γ ex vivo ELISPOT |
| NANP        | 1.61(1.01–2.55) | 0.044            | 1.54(0.87–2.72)    | 0.14           |
| TH2R        | 1.0(0.59–1.69)  | 1                | 0.9(0.44–1.85)     | 0.78           |
| TH3R        | 1.62(1.04–2.52) | 0.032            | 1.57(0.82–2.99)    | 0.17           |
| Sum         | 1.35(0.86–2.12) | 0.2              | 1.32(0.73–2.4)     | 0.35           |
| IL2 ex vivo ELISPOT |
| NANP        | 1.18(0.56–2.51) | 0.67             | 1.02(0.29–3.39)    | 0.99           |
| TH2R        | 0.57(0.23–1.45) | 0.24             | 0.49(0.13–1.79)    | 0.28           |
| TH3R        | 0.94(0.45–1.97) | 0.87             | 0.73(0.21–2.49)    | 0.61           |
| Sum         | 0.83(0.38–1.83) | 0.65             | 0.81(0.25–2.56)    | 0.72           |

HR = Hazard Ratio for each log (i.e. ten-fold) increase in frequency of T cells. Confidence intervals are 5–95%. HRs are adjusted by anti-CS antibody titre (in 2 groups), age, area of residence, ITN use and distance from the dispensary. NANP = NANP and conserved region peptides pool, TH2R = TH2R region peptides pool, TH3R = TH3R and CS.T3T region peptide pool, Sum = all three peptide pools summed.

doi:10.1371/journal.pone.0025786.t004

---

parasites. Indeed, a borderline statistical correlation between TNF-α CD4+ T cells and a reduced risk of clinical malaria was observed among control vaccinees. However, the overall level of protection afforded by T cells among RTS,S/AS01E vaccinees was greater, since the vaccinated children had 2–3 fold more TNF-α CD4+ T cells at 1 month and 8–10 fold more at 12 months post vaccination. The frequency of TNF-α CD4+ T cells at 12 months post vaccination was below screening (i.e. pre-vaccination) levels, and may reflect short-term changes in exposure [35]. Taking together the antibody data, the consistent pattern in ICS and ELISPOT studies, the stability of positive and negative control responses, and the accounting for background reactivity by subtracting negative control responses from antigen-specific responses, show no significant variation over time for positive and negative controls, we conclude that the T cell responses are raised by exposure to malaria during the transmission season, but are short-lived and therefore not sustained once exposure falls. We did not identify an association between protection and T cell responses detected by the cultured IFN-γ assay, as previously reported [20]. However, we tested pools of peptides rather than individual peptides, and the previously reported association was specific to the CS.T3T peptide. The CS.T3T peptide was contained in a pool of TH3R/CS.T3T peptides. In other studies, the CS.T3T peptide accounted for more than half of the overall response seen in the TH3R/CS.T3T peptide pool [36]. Furthermore, we identified very little IFN-γ production in our study. Previous studies showing marked IFN-γ production have been done in adults [18], and IFN-γ production may be suppressed in children in malaria endemic areas [37].

Ex vivo ELISPOT studies did not correlate with ICS studies for the same cytokines, even though both use overnight stimulations, and ICS results were 10-fold higher than ELISPOT results. This may be partially explained by measuring ELISPOT assays per million PBMC, whereas ICS is measured per million CD4+ T cells. However, ex vivo and cultured ELISPOTs identify different
cell populations, the latter more closely reflecting a central memory phenotype [38,39,40]. Hence it is possible that ICS and cultured ELISPOT identify central memory cells, but ex vivo ELISPOT responses to TH3R/CS.T3T peptides pool (lower row). Where more than one third of responses were at the lower limit of detection, the lower two tertiles are combined (and hence only 2 tertiles are displayed on some plots). For CD4+ TNFα responses, the tertiles were 1 to 154 (lower), 155 to 407 (middle) and 408 to 28,840 (upper) cells per million for control vaccinees, and 1 to 26 (lower), 27 to 165 (middle) and 166 to 10,000 (upper) cells per million for RTS,S/AS01E vaccinees. For CD4+ IFNγ responses the tertiles were 1 to 12 (lower), 13 to 66 (middle) and 67 to 8,320 (upper) cells per million for RTS,S/AS01E vaccinees, and 1 to 40 (lower) and 41 to 5,980 (upper) cells per million for control vaccinees. The time point “0 months” refers to the time of a blood draw. Cellular responses were analyzed as time-varying covariates, where the effect of cellular responses from all available blood draws was related to clinical malaria episodes during the period of monitoring after each measurement. Therefore, each RTS,S vaccinee could contribute to 2 periods of monitoring. These three assays were selected for the figure because significant associations on Cox regression were seen (Table 4).

doi:10.1371/journal.pone.0025786.g004

Figure 4. Survival plots with time to first episode of clinical malaria plotted for RTS,S/AS01E (left columns) and control vaccinees (left and right columns) according to tertile of CD4+ TNFα responses (top row), CD4+ IFNγ responses (middle row) and IFNγ ex vivo ELISPOT responses to TH3R/CS.T3T peptides pool (lower row). Where more than one third of responses were at the lower limit of detection, the lower two tertiles are combined (and hence only 2 tertiles are displayed on some plots). For CD4+ TNFα responses, the tertiles were 1 to 154 (lower), 155 to 407 (middle) and 408 to 28,840 (upper) cells per million for RTS,S/AS01E vaccinees, and 1 to 26 (lower), 27 to 165 (middle) and 166 to 10,000 (upper) cells per million for control vaccinees. For CD4+ IFNγ responses the tertiles were 1 to 12 (lower), 13 to 66 (middle) and 67 to 8,320 (upper) cells per million for RTS,S/AS01E vaccinees, and 1 to 40 (lower) and 41 to 5,980 (upper) cells per million for control vaccinees. The time point “0 months” refers to the time of a blood draw. Cellular responses were analyzed as time-varying covariates, where the effect of cellular responses from all available blood draws was related to clinical malaria episodes during the period of monitoring after each measurement. Therefore, each RTS,S vaccinee could contribute to 2 periods of monitoring. These three assays were selected for the figure because significant associations on Cox regression were seen (Table 4).

doi:10.1371/journal.pone.0025786.g004

The CD4+ T cell response associated with protection in our analysis (i.e. TNFα production) were at a low frequency (mean 426 cells per million CD4 cells at peak). Higher frequency responses have been required to achieve protection in sporozoite challenge studies [7]. However, the antibody concentrations associated with protection are also higher in sporozoite challenge studies [7,10]. These differences in outcome may be explained by the greater sporozoite inoculum used in challenge studies compared with exposure in the field [41]. The IFNγ response that was apparently associated with protection in our study was very low frequency and barely above the limit of detection (25 cells per million), and the apparent association is likely to reflect the association between IFNγ and TNFα rather than an independent effect. We were unable to assess polyfunctionality in the present study, because the high number of samples (n = 1066, with three conditions per sample) required an automated gating strategy using FACSDiva software (BD Biosciences). The FACSDiva analysis used for this study did not include Boolean gates in order to obtain polyfunctional T cell data. Further analysis to determine polyfunctionality in this large dataset is ongoing.

Multiple comparisons (i.e. 18) have been undertaken to identify the association between TNFα producing CD4+ T cells and protection from clinical malaria. However, the association was highly significant (p = 0.001) and remains significant after a Bonferroni correction (p = 0.018).

The Prentice criteria have been proposed as a way of qualifying surrogate endpoints [42,43] and include four criteria [30]. We found that the combination of anti-CS titers and TNFα producing CD4+ T cells met all the criteria (i.e. vaccination was associated with protection; anti-CS titers and CD4+ TNFα+ T cells were both independently associated with vaccination; were both
independently associated with protection; and the combination of anti-CS titers and CD4+ TNFα+ T cells, but not either alone, could account for the effect of vaccination in multi-variable Cox regression analysis). We found no significant interaction between anti-CS and TNFα+ T cells.

Microheterogeneity of malaria exposure has been observed in Kilifi [44], and may confound the association between antibodies to blood stage malaria antigens and the risk of malaria [33]. However, this is unlikely to explain the association between CD4+ TNFα+ T cells and protection from clinical malaria for two reasons: The direction of confounding was in the opposite direction in this cohort (i.e. microheterogeneity led to a confounded association between increasing antibody levels and increasing risk of malaria rather than protection), and the association with protection is more marked in RTS,S/AS01E vaccines rather than control vaccines.

There is strong evidence that anti-CS antibodies inhibit sporozoite invasion [45], supporting a causal relationship, and TNFα may reduce the parasite’s intrahepatic development [13,14]. However, it is possible that the frequency of CD4+ TNFα+ T cells is associated with another causal mediator of immunity (for instance better quality antibody responses, enhanced T cell memory or polyfunctionality). These further characterizations of the immune response should now be a priority, since establishing an immunological surrogate endpoint will accelerate the development of candidate malaria vaccines, inform monitoring the persistence of immune responses and inform the timing of a booster dose.

Supporting Information

Supporting Information S1

Acknowledgments

We are grateful to the parents of participants and village and district authorities for their cooperation; the data and safety monitoring board, chaired by Prof. Malcolm Molyneux, and the local safety monitor, Dr. Jay Berkley in Kilifi; Edna Ogada, Juliana Wambua (site coordinator), and Dorothy Mwachiro (community liaison officer) for providing support in Kilifi; and the staff of the Malaria Project Team at GlaxoSmithKline — in particular, Mickael Mestre (technical assistance), Olivier Jauniaux (technical assistance), Robbert Van-der-Most and Lode Schuurman for pre-submission reviews of the manuscript, Nathalie Annez, Sarah Bennis (professional writer), Srilakshmi Pranesh, Marie-Sylvie Remacle (professional writer) and Laurence Viguier.

Author Contributions

Conceived and designed the experiments: PM JMV JM DK MJ OK EJ PB. Analyzed the data: AO ML PB. Wrote the paper: AO JV AL JC PB.

References

1. Ballou WR (2009) The development of the RTS,S malaria vaccine candidate: challenges and lessons. Parasite Immunol 31: 492–500.
2. Lel B, Agyare S, von Gleserapp I, Haerde S, Oyakhiromen S, et al. (2009) A randomized trial assessing the safety and immunogenicity of AS01 and AS02-adjuvanted RTS,S malaria vaccine candidate in children in Gabon. PLoS One 4: e7611.
3. Bojang KA, Olodude F, Pinder M, Otolorin-Oyinbo O, Viguier G, et al. (2005) Safety and immunogenicity of RTS,S/AS02A candidate malaria vaccine in Gambian children. Vaccine 23: 4148–4157.
4. Bojang KA, Milligan PJ, Pinder M, Viguier G, Alloureche A, et al. (2009) Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. Lancet 359: 1927–1934.
5. Doherty JF, Pinder M, Tornoeirth N, Carton C, Viguier L, et al. (1999) A phase I safety and immunogenicity trial with the candidate malaria vaccine RTS,S/AS02 in semi-immune adults in The Gambia. Am J Trop Med Hyg 61: 865–868.
6. Strum JA, Slaoaoi H, Heppner DG, Momin P, Kester KE, et al. (1997) A preliminary evaluation of a recombinant circumsporozoite protein vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation Group. N Engl J Med 336: 86–91.
7. Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, et al. (2004) Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by liposomal delivery. Vaccine 22: 4800–4894.
8. Moorthy VS, Ballou WR (2009) Immunological mechanisms underlying protection mediated by RTS,S; a review of the available data. Malar J 8: 312.
9. Ballou WR (2009) The development of the RTS,S malaria vaccine candidate: preliminary evaluation of a recombinant circumsporozoite protein vaccine. J Infect Dis 199: 1139–1144.
10. Olotu AI, Lusingu J, Leach A, Lievens M, Vekemans J, et al. (2011) Efficacy of RTS,S/AS02 malaria vaccine against Plasmodium falciparum malaria. RTS,S Malaria Vaccine Evaluation. J Infect Dis 204: 1411–1420.
in Gabonese children vaccinated with RTS,S/AS01(E) and RTS,S/AS02(D).
PloS ONE 6: e18559.
28. Plotkin SA (2008) Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis 47: 401–409.
29. Qin I, Gilbert PB, Corey I, McElreath MJ, Self SG (2007) A framework for assessing immunological correlates of protection in vaccine trials. J Infect Dis 196: 1304–1312.
30. Prentice RL (1989) Surrogate endpoints in clinical trials: definition and operational criteria. Stat Med 8: 431–440.
31. Macete EV, Sacarlal J, Aponge J, Leach A, Navia MM, et al. (2007) Evaluation of two formulations of adjuvanted RTS, S malaria vaccine in children aged 3 to 5 years living in a malaria-endemic region of Mozambique: a Phase 1/IIb randomized double-blind bridging trial. Trials 8: 11.
32. Flanagan KL, Lee EA, Gravenor MB, Reece WH, Urban BC, et al. (2001) Unique T cell effector functions elicited by Plasmodium falciparum epitopes in malaria-exposed Africans tested by three T cell assays. J Immunol 167: 4729–4737.
33. Bejon P, Cook J, Bergmann-Leitner E, Olotu A, Lusingu J, et al. (2011) Effect of the Pre-erythrocytic Candidate Malaria Vaccine RTS,S/AS01E on Blood Stage Immunity in Young Children. J Infect Dis 204: 9–18.
34. Kinyanjui SM, Bull P, Newbold CI, Marsh K (2003) Kinetics of antibody responses to Plasmodium falciparum-infected erythrocyte variant surface antigens, J Infect Dis 187: 667–674.
35. Bejon P, Turner L, Lavstsen T, Cham G, Olotu A, et al. (2011) Serological Evidence of Discrete Spatial Clusters of Plasmodium falciparum Parasites. PLoS ONE 6: e21711.
36. Dunachie SJ, Walther M, Vaola JM, Webster DP, Keating SM, et al. (2006) A clinical trial of prime-boost immunization with the candidate malaria vaccines RTS,S/AS02A and MVA-CS. Vaccine 24: 2050–2059.
37. Bejon P, Msacharo J, Kai O, Todryk S, Keating S, et al. (2007) The induction and persistence of T cell IFN-gamma responses after vaccination or natural exposure is suppressed by Plasmodium falciparum. J Immunol 179: 4193–4201.
38. Godkin AJ, Thomas HC, Openshaw PJ (2002) Evolution of epitope-specific memory CD8+ T cells after clearance of hepatitis C virus. J Immunol 169: 2214–2217.
39. Keating SM, Bejon P, Berthoud T, Vaola JM, Todryk S, et al. (2005) Durable Human Memory T Cells Quantifiable by Cultured Enzyme-Linked Immunospot Assays Are Induced by Heterologous Prime Boost Immunization and Correlate with Protection against Malaria. J Immunol 175: 5675–5680.
40. Todryk SM, Pathan AA, Keating S, Porter DW, Berthoud T, et al. (2009) The relationship between human effector and memory T cells measured by ex vivo and cultured ELISPOT following recent and distal priming. Immunology 128: 83–91.
41. Bejon P, Andrews L, Andersen RF, Dunachie S, Webster D, et al. (2005) Calculation of liver-to-blood inocula, parasite growth rates, and preerythrocytic vaccine efficacy, from serial quantitative polymerase chain reaction studies of volunteers challenged with malaria sporozoites. J Infect Dis 191: 619–626.
42. Kohberger RC, Jemido D, Noriega F (2008) Prediction of pertussis vaccine efficacy using a correlates of protection model. Vaccine 26: 3516–3521.
43. Ray ME, Bar K, Hussain MI, Hanks GE, Shipley WU, et al. (2009) Potential surrogate endpoints for prostate cancer survival: analysis of a phase III randomized trial. J Natl Cancer Inst 101: 228–236.
44. Bejon P, Williams TN, Liljander A, Noor AM, Wambua J, et al. (2010) Stable and unstable malaria hotspots in longitudinal cohort studies in Kenya. PLoS Med 7: e1000304.
45. Hollingdale MR, Appiah A, Leland P, do Rosario VE, Mazier D, et al. (1990) Activity of human volunteer sera to candidate Plasmodium falciparum circumsporozoite protein vaccines in the inhibition of sporozoite invasion assay of human hepatoma cells and hepatocytes. Trans R Soc Trop Med Hyg 84: 325–329.