Strain-specific *Plasmodium falciparum* multifunctional $CD4^+$ T cell cytokine expression in Malian children immunized with the FMP2.1/$AS02_A$ vaccine candidate

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Abstract

Based on *Plasmodium falciparum (Pf)* apical membrane antigen 1 (AMA1) from strain 3D7, the malaria vaccine candidate FMP2.1/AS02$_A$ showed strain-specific efficacy in a Phase 2 clinical trial in 400 Malian children randomized to 3 doses of the AMA1 vaccine candidate or control rabies vaccine on days 0, 30 and 60. A subset of 10 *Pf*(-) (i.e., no clinical malaria episodes) AMA1 recipients, 11 *Pf*(+) (clinical malaria episodes with parasites with 3D7 or Fab9-type AMA1 cluster 1 loop [c1L]) AMA1 recipients, and 10 controls were randomly chosen for analysis. Peripheral blood mononuclear cells (PBMCs) isolated on days 0, 90 and 150 were stimulated with full-length 3D7 AMA1 and c1L from strains 3D7 (c3D7) and Fab9 (cFab9). Production of IFN-γ, TNF-α, IL-2, and/or IL-17A was analyzed by flow cytometry. Among AMA1 recipients, 18/21 evaluable samples stimulated with AMA1 demonstrated increased IFN-γ, TNF-α, and IL-2 derived from $CD4^+$ T cells by day 150 compared to 0/10 in the control group ($P<0.0001$). Among AMA1 vaccinees, $CD4^+$ cells expressing both TNF-α and IL-2 were increased in *Pf*(-) children compared to *Pf*(+) children. When PBMCs were stimulated with c3D7 and cFab9 separately, 4/18 AMA1 recipients with an AMA1-specific $CD4^+$ response had a significant response to one or both c1L. This suggests that recognition of the AMA1 antigen is not dependent upon c1L alone. In summary, AMA1-specific T cell responses were notably increased in children immunized with an AMA1-based vaccine candidate. The role of $CD4^+TNF\text{-}\alpha^+IL\text{-}2^+$-expressing T cells in vaccine-induced strain-specific protection against clinical malaria requires further exploration. Clinicaltrials.gov Identifier: NCT00460525

Keywords: Malaria, Pediatric, T cell, Multifunctional, AMA1, FMP2.1/AS02$_A$

1. Introduction

The development of an effective vaccine against the malaria parasite *Plasmodium falciparum* (*Pf*) is a formidable challenge due to the complex life cycle, the multifaceted host immune response and the long evolutionary history between the parasite and the human host. Children are particularly at risk due to ineffective immune responses that fail to control illness, in contrast to semi-immune adults from the same endemic locations, who are still infected but do not become ill [1-3]. Centuries of co-evolution have enabled *Pf* to develop several means of immune evasion in the human host [4, 5]. Selective pressure driven by the host immune response results in polymorphism within parasite antigens exposed extracellularly on a host cell or on the surface of the parasite [6, 7]. The expression of polymorphic protein sequences is one of the key factors that has hindered development of a malaria vaccine that elicits a cross-protective immune response against multiple allelic variants.

One such polymorphic protein is the apical membrane antigen 1 (AMA1). This relatively conserved apicomplexan protein manifests numerous point mutations and is expressed on the apical surface of all plasmodium species. The role that AMA1 plays in cellular invasion is unclear; however, it is expressed at two stages in the malaria life cycle, the hepatic stage before and during sporozoite invasion of hepatocytes and the erythrocytic stage before merozoite invasion of erythrocytes [8-10]. Anti-AMA1 antibodies appear to inhibit reorientation of the apical end of the merozoite to the erythrocyte surface, erythrocyte binding, and initial formation of the parasite-erythrocyte tight junction *in vitro* [11-15]. AMA1 vaccination induced protective efficacy against death and reduced levels of parasitemia after challenge in animal models [16-20]. In light of *in vitro* and *in vivo* data, this molecule is a leading blood stage vaccine candidate against *Pf*.

In humans, vaccination with the AMA1 ectodomain from *Pf* stain 3D7 (FMP2.1) adjuvanted with GlaxoSmithKline Vaccines' (GSK) $AS02_A$ demonstrated strain-specific efficacy (64%) against clinical malaria in Malian children but did not demonstrated cross-strain protection [6, 21]. A likely explanation for the lack of cross-strain protection is the highly polymorphic nature of the AMA1 ectodomain, specifically eight highly polymorphic sites on a twelve amino acid sequence identified as the cluster 1 loop (c1L). A previous study in this population found that onset of clinical symptoms from *Pf* infection were more likely when subsequent infections with *Pf* strains demonstrated changes in the amino acid sequences of the ectodomain of AMA1 [22]. Further analysis revealed that this association was driven by the magnitude of amino acid changes within the c1L (amino acid positions 196 to 207). The dominant predictor of clinical malaria was the extent of polymorphic changes occurring at residue 197. These observations suggest that it might be possible to increase the efficacy of an AMA1 vaccine by selectively targeting the immune response to c1L alleles that are predominantly expressed by malaria strains present in a particular geographic location [6].

Following vaccination with FMP2.1/$AS02_A$, a robust increase in AMA1-specific antibody production is observed. This elevation in humoral immunity correlated with protection, but was not sufficient to fully protect against all clinical malaria episodes [21]. The extent to which an AMA1-specific T cell response protects against clinical malaria is largely unknown. Production of IL-5 and IFN-γ by AMA1-specific T cells following FMP2.1/$AS02_A$ vaccination has been measured in adult Phase I studies by ELISpot [23, 24]. In the present study, we assessed the 3D7 AMA1-specific T cell effector response utilizing a subset of peripheral blood mononuclear cells (PBMC) derived from a Phase 2 FMP2.1/$AS02_A$ vaccine candidate trial in Malian children aged 1-6 years [21]. Furthermore, we evaluated allele-specific $CD4^+$ and $CD8^+$

T cell responses to AMA1 c1L variants that differed at position 197 (strain 3D7-like c1L (c3D7) and strain Fab9-like c1L (cFab9) that differs only at position 197) as they relate to strain-specific clinical infections. We report on the cell-mediated immune responses induced by the FMP2.1/AS02$_A$ vaccine in Malian children compared to a rabies vaccine control cohort and characterize the multifunctional T cell response induced by vaccination.

2. Materials and Methods

2.1. Trial design and ethics

Bandiagara (pop. ~13,600) is located in Mali, West Africa and has intense seasonal transmission (July-December) of *P. falciparum* malaria. The entomologic inoculation rate is 20-60 infected bites per month during peak transmission, falling to near zero during the dry season. The malaria disease burden is high, with children aged < 14 years experiencing a mean of 1.7-3.3 clinical episodes of uncomplicated malaria a year between 2009-12 [25] and a 2.3% annual incidence of severe malaria among children aged < 6 years [26]. A Phase 2 controlled, double-blind trial testing the malaria vaccine candidate FMP2.1 antigen [The Walter Reed Army Institute of Research (WRAIR)] based upon *Pf* AMA1 from strain 3D7 and adjuvanted to AS02$_A$ (GSK), was conducted in 400 Malian children aged 1-6 years randomly assigned to receive 3 doses of the AMA1 vaccine candidate or a control rabies vaccine (RabAvert®, Chiron Vaccines) on days 0, 30 and 60 [21]. Enrollment, vaccination and collection of venous-blood samples were conducted at the Bandiagara Malaria Project research clinic from May 2007 to February 2008. The trial was conducted in compliance with the Declaration of Helsinki. Study protocols were reviewed and approved by the Institutional Review Boards (IRB) of the University of Bamako in Mali, the University of Maryland, WRAIR. and the U.S. Army Surgeon General. Village permission to conduct research was obtained from village chiefs, government officials and

traditional healers before study initiation. Individual written informed consent was obtained from the parent or legal guardian of each child before screening and enrollment. The primary end point was a clinical episode of malaria, defined as fever (axillary temperature of 37.5°C or higher) with an asexual *Pf* density of at least 2,500 parasites/mm^3 on thick blood film. Full study details and vaccine formulation have been described previously [21].

2.2. Plasmodium falciparum *infection genotyping*

Cross-protective efficacy of the FMP2.1/$AS02_A$ vaccine candidate against symptomatic Pf infections was determined by identifying which polymorphic sites were associated with increased number of clinical episodes [6]. The vaccine had a high protective efficacy against strains with identical amino acid residues at the 8 polymorphic sites within the cluster 1 loop (c1L) regardless of polymorphic changes elsewhere in AMA1. In particular, high vaccine efficacy was associated with absence of polymorphic changes at amino acid position 197 within the c1L. Strains were organized into genotypes based on the sequence of the c1L and selection of the vaccine genotype (3D7, D**E**RHFDKY) and a genotype with a polymorphic change at 197 only (with respect to the c1L) known as Fab9 (D**Q**RHFDKY) were chosen for this study based on these data. The distribution of strains expressing c3D7 or cFab9 has been found to be comparable in Mali [22].

2.3. Sample collection and randomization

A Phase 2 study was conducted in 400 Malian children aged 1-6 years randomly assigned to receive 3 doses of the FMP2.1/$AS02_A$ (henceforth termed AMA1) vaccine candidate or a control rabies vaccine (RabAvert®, Chiron Vaccines) on Days 0 (D0), 30 and 60, with D0 occurring before the start of the malaria transmission season [21]. Pediatric volunteer whole blood (5-10 mL) was collected at the study clinic into sterile Eppendorf (sera) or EDTA tubes

(PBMCs) at appropriate study visits and immediately refrigerated. Sera collection was processed and frozen as described previously [27]. Blood was processed by density centrifugation, within 2-4 hours of acquisition, utilizing lymphocyte separation medium (ICN Biomedical Inc, Aurora, OH) following standard techniques [28]. PBMCs were resuspended in media and linear-rate frozen to -70°C in isopropyl alcohol containers (Nalgene, USA) at the field site before transfer to liquid nitrogen storage containers for shipment to the University of Maryland's Center for Vaccine Development (CVD). Samples for this experiment were randomly chosen by an unblinded investigator from the pool of samples that had at least 10.0 x 10^6 cells at Days 0 (D0), D90 and D150 and distributed between three groups; 1) AMA1 vaccinated children who remained free of clinical *Pf* malaria [*Pf*(-)] by Day 240 post vaccination 1 (n = 10); 2) AMA1 vaccinated children who sustained one or more episodes of clinical *Pf* malaria [*Pf* (+)] and were genotyped as parasites with 3D7 or Fab9-type AMA1 c1L (n = 11; and 3) Rabies control vaccine recipients (n = 10). Randomization was restricted to those AMA1 vaccine recipients that had a D90 anti-AMA1 titer within the 25th to 75th percentile as an indication of an immunologic response. AMA1 *Pf*(-) children were age and residence-matched (by neighborhood, a.k.a.. quartier) to rabies control children.

2.4. PBMC Stimulation

PBMC were thawed, washed and rested overnight at 36°C, 5% CO_2. A portion of the cells (2.0 x 10^6) was removed to serve as negative (media) and positive (stimulation with 10 μg/ml SEB; Sigma, St. Louis, MO) controls. The remaining PBMC were split into three aliquots (total ~ 3.0 x 10^6 cells) and stimulated with; 1) 5 μg/ml AMA1 ectodomain of the 3D7 strain [29] 2) 5 μg/ml 3D7-like c1L (GenBank: AAN35928.1, DEMRHFYKDNKY, 95% pure, 21st Century Biochemicals, Marlboro, MA); or 3) 5 μg/ml Fab9-like c1L (GenBank: ACB87787.1,

DQMRHFYKDNKY, 95% pure, 21st Century Biochemicals) separately. Each experiment consisted of a media negative control, a positive control with *Staphylococcus* enterotoxin B (SEB), and cell stimulation with three *Pf* stimulation conditions (AMA1, 3D7-like c1L, or Fab9-like c1L) and run concomitantly at each time point. Samples were excluded if the viability or the numbers of PBMC recovered after thawing were insufficient (n=1, i.e., D90 for subject 316). Using an optimized protocol, cells were stimulated for 2 hours before protein transport was blocked by adding 0.5 μl/tube GolgiPlug (BD Pharmingen) followed by overnight incubation [30]. The concentration for AMA1 stimulation was previously optimized [30] and c1L concentrations were matched for consistency.

2.5. Flow cytometry staining and analysis

PBMC surface molecules were stained with fluorochrome-labeled monoclonal mouse anti-human antibodies (mAbs) against CD3 (ECD, Clone UCHT1, Beckman Coulter, Indianapolis, IN), CD19 (Pacific Blue, Clone Sj25-c1, Invitrogen, Frederick, MD), CD14 (Pacific Blue, Clone Tük4, Invitrogen), CD8 (FITC, Clone Hit8a, BD Biosciences, San Jose, CA), CD45RA (QDot 655, Clone MEM-56, Invitrogen, Eugene, OR), CD62L (APC-eFluor780, Clone Dreg-56, eBioscience, San Diego, CA), and CD4 (Biotin, Clone Sk3, BD Biosciences followed by streptavidin (Pacific Orange, Invitrogen), followed by fixation/permeabilization (Medium A and B, Invitrogen) and intracellular staining with mAbs to CD69 (PE, Clone FN50, eBioscience), IFN-γ (APC, Clone B27, BD Biosciences), TNFα (Alexa Fluor 700, Clone MAb11BD, Biosciences), IL17A (PerCP-Cy5.5, Clone eBio64DEC17, eBioscience), and rat anti-human antibody against IL2 (PE-Cy7, Clone MQ1-17H12, BD Biosciences). An amine reactive dye (ViVid, Invitrogen, Oregon) was used as a dead cell discriminator. A total of 200,000-700,000 events (mean ~450,000) in the forward and side scatter (FS/SS) lymphocyte

gate were collected per sample. B lymphocytes ($CD19^+$) and innate immune cells ($CD14^+$) were excluded from analysis. Doublets/aggregates were subtracted from analysis and gate placement determined with the aid of Fluorescence Minus One (FMO) controls**.** Cells were resuspended in 1% paraformaldehyde fixation buffer and analyzed on an LSR II flow cytometer (BD Biosciences). List-mode data files were analyzed using WinList 7.1 (Verity Software House, Topsham, ME). Specimens were included in the analysis if; 1) the cell viability was >75% after thawing and 2) cells were shown to be functionally active as determined by the production of IFN-γ by at least 0.2% $CD3^+$ cells after stimulation with SEB. Live ($ViVid^-$) lymphocyte ($CD3^+CD19^-CD14^-$) populations were gated for analysis of cytokine expression and antigen-specific activation ($CD69^+$) of $CD4^+$ and $CD8^+$ T cells. Positive detection of $CD4^+$ and $CD8^+$ T cell cytokine expression for responses that were observed in less than 0.1% of the lymphocyte population was defined as a signal at least two times above background detected in the negative control. The average number of $CD4^+$ T cells gated per experiment was ~85,000 (range 38,000 – 174,000) lymphocytes. We performed hierarchical gating strategies for viable T cells ($ViVid^-$ $CD14^-CD19^-CD3^+$) gated to $CD4^+$ and $CD8^+$ T cell subsets. Analysis of the $CD4^+$ and $CD8^+$ memory subpopulations was completed using the T cell surface molecules CD45RA and CD62L which enables the characterization of T memory subsets as T central memory (T_{CM}; $CD45RA^-$ $CD62L^+$), T effector memory (T_{EM}; $CD45RA^-CD62L^-$), and terminally differentiated effector memory (T_{EMRA}; $CD45RA^+CD62L^-$) [32] (gating strategy depicted in Supplemental Fig. 1). The significance of the antigen-specific responses was determined by χ^2 analysis of the difference in net number of positive events in the media control versus the experimental AMA1 antigen and c1L subunits. Investigators were blinded and samples anonymized until the χ^2 analysis was completed.

206 *2.6. Enzyme-linked immunosorbent assays (ELISAs)*

207 ELISA assays to detect the presence of anti-AMA1 IgG were performed at WRAIR as
208 described previously [21]. Results were obtained for each randomized study participant. In order
209 to rule out concomitant Schistosoma infection that has been demonstrated to distort immunologic
210 findings [30, 31], *Schistosoma haematobium* ELISA assays to detect the presence of anti-soluble
211 egg antigen (SEA) and anti-soluble worm antigen protein (SWAP) antibodies, were performed as
212 described [31]. Briefly, sera collected from children randomized in this study were diluted to
213 1:250 and plated in duplicate. Plates were developed with 1:5000 HRP-conjugated goat anti-
214 human IgG (KPL Inc, Gaithersburg, MD) followed by TMB substrate solution (KPL, Inc.,
215 Gaithersburg, MD). Phosphoric acid 1M was used to stop the reaction and the optical density
216 (OD) was read at 450 nm after 20 minutes. Background ODs from uncoated wells were
217 subtracted from those of antigen-coated wells to adjust for nonspecific binding. Lower limits of
218 detection were set at dilutions of 1:50 for SEA and SWAP. Pooled sera from 10 local Malian
219 adults (adult prevalence of *S. haematobium* is >50%) were used as positive controls. Sera with a
220 result near the lower limit of detection for anti-SEA antibodies were repeated at 1:10 dilutions to
221 confirm the validity of negative results.

222 *2.7. Statistics*

223 Statistical evaluation of the data was completed using GraphPad Prism 5 (GraphPad
224 Software, Inc., San Diego, CA). Student t-test (two-tailed), Mann-Whitney U test, or Kruskal-
225 Wallis were used to compare continuous data and χ^2 analysis, using Mantel-Haenszel or Fisher
226 Exact (two-tailed) as appropriate, was performed for categorical data. Spearman's rank
227 correlation coefficient was calculated as a measure for statistical dependence between
228 nonparametric measures. A significance level of $P<0.05$ was considered statistically significant.

3. Results

3.1. Determination of T cell sub-populations and cytokine production

Data was generated from all 31 children (Table 2). T cell data are presented in Tables 2 and 3 for a randomly chosen subset of AMA1 vaccinated children who experienced a malaria infection genotyped as 3D7-like or Fab9-like with respect to the eight polymorphic amino acids within the c1L (AMA1 *Pf*(+); n = 11) or who remained free of clinical malaria disease (AMA1 *Pf*(-); n = 10) in the post-vaccination follow-up period. A subset of children that received the rabies control vaccine (n = 10) were chosen to match the ages and quartier (residence location) of the *Pf*(-) AMA1 vaccinated children included in this analysis. Peripheral blood mononuclear cells (PBMC) obtained at three time points (D0, D90 and D150) were examined for vaccine antigen-specific responses (the full-length ectodomain of AMA1) and c1L-specific responses (amino acid positions 196 to 207) via multiparameter flow cytometry. Anti-AMA1 antibody titers determined by ELISA that were previously measured during the vaccine trial were correlated to CMI responses in the AMA1 *Pf*(-) and AMA1 *Pf*(+) children chosen for this study (discussed further in section 3.6.).

3.2. Intracellular cytokine expression to full-length AMA1 stimulation

Antigen recognition to AMA1 was measured after stimulation of study participant PBMC and quantification of intracellular cytokine production (TNF-α, IFN-γ, IL-2 and IL-17A) (Table 2). No significant antigen-specific cytokine response was noted in any child at D0 before vaccination. Among AMA1 recipients, 18/21 (86%) evaluable samples stimulated with full-length AMA1 ectodomain demonstrated significantly increased levels of a combination of IFN-γ,

TNF-α, or IL-2 derived from $CD4^+$ T cells by D150 compared to 0/10 in the rabies control group ($p<0.0001$, χ^2 Fisher exact analysis). Most AMA1 vaccinated children (15/18 (83%)) demonstrated AMA1-specific $CD4^+$ T cell cytokine production by D90 (i.e., 1 month post-3rd vaccination) and the remaining 3 non-responders subsequently developed antigen-specific $CD4^+$ T cell effector response by D150 (i.e., 3 months post-3rd vaccination). The geometric mean percentage of antigen-specific, cytokine-expressing (IFN-γ, TNF-α or IL2) $CD4^+$ T cells was statistically higher at Day 90 and Day 150 as compared to Day 0 (Mann-Whitney Rank Sum: D90: 0.01 vs. 0.28, $P < 0.0001$ and D150: 0.01 vs. 0.20, $P < 0.0001$; Kruskal-Wallis: D0 vs. D90 $P < 0.0001$ and D0 vs. D150 $P < 0.0001$)) but was not significantly different between D90 and D150 (Mann-Whitney Rank Sum: 0.28 vs. 0.20, $P = 0.17$) (Fig. 1A). Very little IL-17A was detected (Fig. 1B-D). Expression of TNF-α and IL-2 by AMA1-specific $CD4^+$ T cells was detected by D90 and persisted through D150. In contrast, IFN-γ derived from $CD4^+$ T cells was observed to peak by D90 and wane by D150. Although 5/10 rabies vaccine recipients experienced a clinical malaria episode during follow-up, all 10 failed to mount a detectable AMA1-specific $CD4^+$ T cell effector response. This observation supports the notion that protective immune responses are likely attributable to the AMA1 vaccine candidate rather than natural malaria exposure. Three AMA1 vaccinated children failed to mount detectable AMA1-specific $CD4^+$ T cell responses at any time point. Of note, these children experienced a 3D7-like clinical infection during follow-up.

We further stratified the results of AMA1 vaccinated children between those who remained free of clinical malarial disease during the course of study follow-up (AMA1 *Pf*(-)) and those who developed a clinical 3D7 or Fab9-like malaria infection (AMA1 *Pf*(+)) compared to the rabies control recipients (Fig. 1B-D). While the absolute number of $CD4^+$ T cells secreting

measurable cytokine was low, significantly greater numbers of both AMA1 *Pf*(+) and AMA1 *Pf*(-) children produced IFN-γ, TNF-α, and IL-2 at D90 and D150 compared to rabies control children (Table 2 and Fig. 1). Regarding differences within the AMA1 vaccinated children stratified by seasonal acquisition of malaria, AMA1 *Pf*(-) recipients produced significantly more IL-2 than AMA1 *Pf*(+) children by D150 (P = 0.024). Interestingly, a trend towards increased TNF-α production was also noted in AMA1 *Pf*(-) recipients (P = 0.10). Taken together, these results indicate that vaccination with FMP2.1/AS02$_A$ elicits AMA1-specific CD4$^+$ responses as indicated by measurable cytokine production.

3.3. Multifunctional cytokine expression to full-length AMA1 stimulation

We next evaluated the presence of multifunctional (simultaneous expression of 2 or more cytokines) CD4$^+$ and CD8$^+$ (described in following section) memory T cells in response to the AMA1 antigen in PBMC isolated from AMA1 *Pf*(-) and AMA1 *Pf*(+) children (Fig. 2). Over the length of follow-up, single cytokine expression (IFN-γ, TNF-α, and IL-2) in CD4$^+$ T cells accounted for a geometric mean of 33.8% of the total amount of cytokine-expressing cells in AMA1 *Pf*(-) children and 34.5% in AMA1 *Pf*(+) children at D90. IL-17A responses were not detected. The percentages of single positive cells remained comparable in AMA1 *Pf*(-) (21.2%) and AMA1 *Pf*(+) (27.8%) children at D150.

Dual cytokine expression (i.e., single cells expressing a combination of two cytokines simultaneously) accounted for 37.6% of all cytokine-expressing CD4$^+$ T cells in AMA1 *Pf*(-) recipients while double-positive cells represented 51.2% of the CD4$^+$ response in AMA1 *Pf*(+) recipients at D90. The proportion of dual cytokine expression substantially increased from baseline in AMA1 *Pf*(-) children by D150 (64.2%) whereas dual cytokine responses in AMA1 *Pf*(+) recipients remained steady (56.1%), however, this increase from D90 was not significant

297 (P = 0.41). Of the multifunctional responses measured, the combination of TNF-α^+IL-2^+-
298 expressing $CD4^+$ T cells was predominant, comprising a geometric mean of 13.7% of cytokine-
299 expressing cells at D90 and increasing to 20.9% by D150 in AMA1 *Pf*(-) children (p = 0.43).
300 Among AMA1 *Pf*(+) children, TNF-α^+IL-2^+-expressing $CD4^+$ T cells also dominated at D90
301 comprising 12.9% but did not increase over the course of the study (D150 = 12.8%).

302 Triple-cytokine expressing $CD4^+$ T cells among AMA1 *Pf*(-) volunteers accounted for
303 28.6% of cytokine-expressing cells at D90 (Fig. 2C), with the majority (15.9%) expressing
304 IFNγ^+TNFα^+IL2^+ at Day 90 but falling to 5.8% by Day 150 (Fig. 2A-B). This compares to a
305 value of 5.9% in AMA1 *Pf*(+) volunteers at Day 90; a value that remained unchanged at Day 150
306 (5.5%). While the accentuation of triple-positive excreting cells in the children who did not
307 acquire malaria was markedly higher than children who developed a malaria infection, this did
308 not meet statistical significance (p = 0.25) at D90. Minimal numbers of quadruple-positive (IFN-
309 γ^+TNF-α^+IL-2^+IL-$17A^+$) $CD4^+$ T cell responses were detected due to a lack of measurable IL-
310 17A cytokine (Fig. 1).

311 Memory subsets were also examined to determine which $CD4^+$ subpopulation was
312 predominantly expressing cytokines in an AMA1-specific manner. The majority of the AMA-
313 specific response originated from the CD45RA$^-$CD62L$^-$ effector memory T cells (T_{EM}, 42-89%)
314 in AMA1 recipients regardless of time point or study group (AMA1 *Pf*(-) versus AMA1 *Pf*(+)).
315 The AMA1-specific CD45RA$^-$CD62L$^+$ central memory (T_{CM}) $CD4^+$ T cell response ranged from
316 0-19% with minimal difference observed between study groups or time points. The AMA1-
317 specific response from terminally differentiated effector cells (T_{EMRA}) was marginal in all AMA1
318 recipient study groups (data not shown).

319 *3.4. $CD4^+$ T cell responses to the c1L subunit peptides*

In addition to full-length AMA1, PBMC were stimulated separately with the c1L from strains 3D7 (c3D7) and Fab9 (cFab9) to further assess the role of the c1L as compared to full-length ectodomain of AMA1, in overall immune response and to assess the effect of polymorphisms at amino acid 197 as a mechanism of evasion of cell-mediated immunity (Table 3). Of the evaluable samples, 3/21 AMA1 vaccine recipients had a significant $CD4^+$ T cell response to both strains of c1L ($p \leq 0.0001$, χ^2 analysis) and all 3 were AMA1 *Pf*(-) subjects. One of these 3 participants had a detectable response at D0, D90, and D150 suggesting that the responses preceded and possibly were boosted by AMA1 vaccination. The other two individuals developed a detectable response post-immunization (D90 and D150). An additional 2/21 AMA1 recipients responded to either c3D7 (D150) or cFab9 (D90). Both responders contracted a 3D7-like clinical malaria infection during the post-vaccination follow-up period. Of the rabies control recipients, 2/10 demonstrated peptide-specific responses. One individual responded to both c1L sequences and the other to the c3D7 only; however, these observed $CD4^+$ T cell responses were present at D0 for both. Interestingly, despite evidence of pre-study immunity, both of these children developed clinical malaria due to parasites with c1L heterologous to both c3D7 and cFab9. Responders to c3D7 demonstrated higher total IFNγ expression (geomean: D0 = 16.9%; D90 = 16.7%, D150 = 14.7%) than cFab9 responders (geomean: D0 = 7.2%; D90 = 7.4%, D150 = 8.8%) beginning at D0 and continuing through D150.

3.5. $CD8^+$ T cell responses to full-length AMA1 and c1L subunit peptides

Coupled with the analysis of the antigen-specific $CD4^+$ T cell response to AMA1 vaccination, we examined the $CD8^+$ T cell sub-population in response to stimulation with the ectodomain of AMA1 or to the c1L subunit peptides (not shown). As has been described in previous studies, [37] we, similarly, failed to detect appreciable numbers of $CD8^+$ T cell

responses to accurately interpret trends. Evidence of antigen-specific $CD8^+$ T cell cytokine expression after PBMC stimulation with AMA1 or either c1L was observed in a small number of children (i.e., 3/21 AMA1 vaccinated children, and 1/10 rabies vaccine recipients). The rabies recipient with antigen-specific $CD8^+$ T cell recognition responded to AMA1 and both c1L subunit peptides with an IFN-γ^+ response. Low-grade response was noted at D0 (IFN-γ^+: AMA-1 0.07%; c3D7 0.08%; cFab9 0.09%) and D90 (IFN-γ^+: AMA-1 0.03%; c3D7 0.07%; cFab9 0.02%) suggesting the presence of a pre-existent immune response that was not boosted by natural exposure during the malaria transmission season. Of note, this child did not develop malaria during the period of observation. Of the three AMA1 vaccinated children, two were in the AMA1 *Pf*(-) group and responded to the 3D7-specific c1L peptide. However, one had demonstrable IFN-γ^+ (0.23%) and IFN-γ^+TNF-α^+ (0.31%) response at D0, which waned through D150 (IFN-γ^+: 0.14%; IFN-γ^+TNF-α^+ 0.17%) suggestive of pre-existing immunity to the 3D7 strain of parasite and not likely vaccine-induced immune response. The second had detectable D150 $CD8^+$ T cell effector response to AMA1 characterized predominantly by a population of cells expressing IFN-γ alone (0.2%). This same child demonstrated antigen recognition of the Fab9 c1L subunit at D150 characterized by IFN-γ (0.13%) and TNF-α (0.21%) cytokine excretion but without developing a detectable clinical Fab9 infection. Of note, this is the only AMA1 vaccinated child with a detectable $CD8^+$ response to AMA1 at D150 (IFN-γ^+ 0.2%). The third child experienced a clinical Fab9-like infection at D103 and demonstrated peptide-specific recognition of the Fab9 c1L subunit characterized by IFN-γ cytokine expression at D90 (0.21%). The possible association between onset of a Fab9 clinical infection and an allele-specific $CD8^+$ response to the cFab9 is notable; however, it was observed in this subject alone. As indicated by the low number of AMA1 vaccine recipients with a significant $CD8^+$ response to AMA1, this

vaccination strategy induced a predominant if not complete $CD4^+$ response to the vaccine antigen in this clinical trial. This vaccine strategy did not induce an antigen-specific multifunctional $CD8^+$ response in any of the AMA1 recipients that demonstrated a positive $CD8^+$ T cell response.

3.6. Humoral response to AMA1

We reviewed concomitant humoral responses in the randomized children for whom flow cytometric analysis was conducted. The AMA1-specific antibody titer was statistically higher at Day 90 and Day 150 as compared to Day 0 (Kruskal-Wallis: D0 vs. D90 $P < 0.0001$ and D0 vs. D150 $P < 0.0001$). An increase in anti-AMA1 IgG was observed in AMA1 *Pf*(+) (p=0.001) and *Pf*(-) (p=0.002) recipients over D0 measurements, which peaked by D90 and remained elevated at D150 (Fig. 3A). Conversely, a delayed rise in antibody titers was noted in rabies control with a peak antibody level at D150. These data mirror the kinetics of our findings in CMI assays however the anti-AMA1 IgG responses in AMA1 *Pf*(-) and AMA1 *Pf*(+) children were similar (D90, p=0.85), suggesting a lack of correlation between 3D7-strain-specific antibody production and strain-transcendent protection against malaria [21]. The change in anti-AMA-1 IgG responses from D0 to D90 in *Pf*(-) and *Pf*(+) children that had a clinical infection before D90 were compared and the magnitude of the IgG responses were similar in both groups (Δ D90, p=0.75).

We next stratified the analysis of differential antibody responses between AMA1 *Pf*(-) and AMA1 *Pf*(+) children by examining children who contracted a 3D7-like clinical infection compared to a Fab9-like clinical infection (Fig. 3B). A significant elevation in antibody titer at D60 (p=0.052), D90 (p=0.009), and D150 (p=0.030) was noted in children who contracted a Fab9-like clinical malaria infection compared to children with a 3D7-like clinical malaria

infection. Moreover, AMA1 *Pf*(+) 3D7+ children had decreased anti-AMA1 IgG titers as compared to AMA1 *Pf*(-) children at D60 (p=0.019) and D90 (p=0.013). Conversely, an increase in anti-AMA1 IgG titer was observed when comparing AMA1 *Pf*(+) Fab9 to AMA1 *Pf*(-) subjects at D90 but did not achieve statistical significance (p = 0.073).

We examined the correlation between the fold-change in AMA1-specific $CD4^+$ T cell responses between Days 0 and 90 and Days 0 and 150 against the anti-AMA1 IgG fold-change in titer responses at the same time points to elucidate if measured cell-mediated immune (CMI) responses associated with concomitant humoral response. Whereas a significant increase in detectable CMI and IgG was measured between D0 and D90 as well as D0 and D150 (Fig. 1A and Fig. 3), we did not detect any correlation between the total AMA1-specific $CD4^+$ T cell response and the anti-AMA1 titer between individual volunteers (D0-D90: P = 0.67, Spearman ρ = 0.1521; D0-D150: P = 0.56, Spearman ρ = -0.1129).

3.7. Humoral response to Schistosoma haematobium *egg antigen (SEA)*

A pre-existing condition, such as a parasitic coinfection, could conceivably alter the malaria-specific $CD4^+$ response to the vaccine antigen in the children included in this study. Coinfection with the helminth parasite *S. haematobium* has been shown to provide a protective effect against the acquisition of malaria in Malian children and has been shown to change the peripheral cytokine profiles, alter circulating regulatory T cell populations and affect memory B cell antigen recognition in Malian children [30, 31]. Thus, the presence of *S. haematobium* anti-SEA titers was assessed by ELISA at D0 for each child in this study (Table 1). We defined positive antibody responders for this endemic region as individuals with an antibody titer >3 SE above the mean of age-matched Malian children with no urinary excretion of *S. haematobium* eggs (defined as titer > 2,880) [26]. One rabies control recipient was positive for anti-SEA

antibodies (titer of 3,379) and one was borderline elevated (titer 1,956) suggesting that *S. haematobium* coinfections were not a confounding variable in our immunologic findings.

4. Discussion

We demonstrate that immunization with the FMP2.1/AS02$_A$ vaccine elicits a CD4$^+$ T cell response in the subset of AMA1 vaccinated children examined. Among AMA1 vaccine recipients, 18 of 21 children demonstrated significantly increased AMA1 antigen-specific levels of IFN-γ, TNF-α, and IL-2 derived from CD4$^+$ T cells by D150 compared to 0/10 who received the control rabies vaccine over the course of a malaria transmission season. These findings are in contrast to data collected from a Malian adult population via ELISpot after the same FMP2.1/AS02$_A$ vaccine regimen in which all participants, including control rabies vaccine recipients, demonstrated an AMA1-specific IFN-γ production [24]. The high baseline of cell-mediated IFN-γ production in Malian adults versus the pediatric population may be the result of a natural exposure to malaria inducing a lymphocyte response not yet acquired in children. While the cytokine expression profiles for AMA1 vaccinated children who developed malaria (either 3D7-like or Fab9-like strains) and those who remained free of malaria were similar, multifunctional CD4$^+$ cells expressing both TNF-α and IL-2 were increased in AMA1 *Pf*(-) children compared to AMA1 *Pf*(+) children. Thus, as a group, AMA1 vaccinated children who remained free of malaria during the malaria transmission season appeared to have an enhanced immune response compared to vaccinated children who developed malaria during the same time period. However, the observed AMA1-specific CD4$^+$ responses in individual vaccine recipients did not correlate with the level of anti-AMA1 antibody titers in *Pf*(-) or *Pf*(+) children, illustrating the lack of understanding of immune correlates of protection against *P. falciparum*

[33-35]. The increased level of anti-AMA1 antibody titers measured in children with a Fab-9 like clinical infection compared to children with a 3D7-like infection suggests that some degree of strain-specific humoral immunity was observed in this study. Contrastingly, the overall increase in multifunctional $CD4^+$ cells expressing both TNF-α and IL-2 cell-mediated immune response in the AMA1 *Pf*(-) children suggests evidence of strain-transcending cellular immunity following vaccination with an AMA1 vaccine based on the 3D7 ectodomain.

A temporal shift in triple-positive (IFN-γ^+TNF-α^+IL-2^+) $CD4^+$ cytokine-expressing cells was also noted in AMA1 *Pf*(-) children compared to AMA1 *Pf*(+) children, and while it did not reach statistical significance with the numbers in our analysis, this observation suggests a possible role in protection against clinical malaria infection. The "quality" or multifunctionality of T cells has been shown to be a barometer of vaccine efficacy [36]. Moreover, the vaccine antigen-specific $CD4^+$ T cell response appears largely to originate from the $CD45RA^-CD62L^-$ effector memory subset. Children vaccinated with FMP2.1/$AS02_A$ demonstrated very little detectable AMA1-specific $CD8^+$ T cell response with the exception of one child with a positive IFN-γ response at D150 in support of the notion that FMP2.1/$AS02_A$ predominantly induced a $CD4^+$ memory response in this vaccine trial. However, peripheral detection of immune response does not necessarily reflect local hepatic immunity where $CD8^+$ T cell-derived IFN-γ appears to correlate with protection against malaria [37] and may represent a central weakness in our ability to interpret immunological results in humans.

We hypothesized that an association may exist between the onset of clinical symptoms from *Pf* infection and changes in the amino acid of the ectodomain of AMA1. Previous studies from this site in Mali suggest that a dominant predictor of clinical malaria was the extent of polymorphic changes occurring at residue 197 [6]. We therefore examined the cellular immune

response after PBMC stimulation with peptides corresponding to the c1L of the 3D7 (c3D7) and Fab9 (cFab9) haplotypes, which differ from each other only at amino acid position 197. However, only a small fraction of the AMA1 recipients demonstrated a positive response to either c1L suggesting that either c1L is not likely the primary epitope recognized by AMA1-specific $CD4^+$ T cells or that the immunologic assays utilized for detection do not provide a full measure for understanding the intricacies of the human immune response to malaria. A limited number of samples were available for this clinical trial in which a small percentage of the children experienced a clinical 3D7-like or Fab-9 like infection. Nevertheless, a measurable $CD4^+$ response was detected with the vaccine antigen (AMA1), while this was not the case for the c1L subunit peptides, adding credibility to the notion that there was no detectable T cell response to the c1L subunit peptides rather than lack of adequate response detection.

It is also possible that the adjuvant $AS02_A$ may have influenced the phenotype of the resulting AMA1-specific $CD4^+$ T cell response. It is known that certain components of an adjuvant can sway the T cell response toward a predominant Th1 or Th2 response. The $AS02_A$ adjuvant was found to induce a predominantly Th1 response in antigen-specific T cells [38-40]. The protein subunit vaccine RTS,S is a vaccine that targets the hepatic stage of the *Pf* life cycle rather than the erythrocytic stage as in AMA1 [40]. Lumsden et al. examined the effect of RTS,S adjuvanted to $AS02_A$ and observed IL-2^+ and TNF-α^+IL-2^+CD4^+ antigen-specific T cell responses [41] comparable to the $CD4^+$ responses observed in this study. Furthermore, a study to assess the immunogenicity of the Mtb72F/$AS02_A$ vaccine, a candidate vaccine against the intracellular pathogen *Mycobacterium tuberculosis*, reported an antigen-specific $CD40L^+$TNF-α^+IL-2^+ and a $CD40L^+$IFN-γ^+TNF-α^+IL-2^+ $CD4^+$ T cell response [42]. Leroux-Roels et al. observed these T cells responses at time points that coincide with those reported in this study.

While the primary vaccine antigen was different and the study population did not include children from the developing world such as in our results, the possibility that the adjuvant may influence immunologic similarities cannot be discounted.

5. Conclusion

Vaccines that target blood stage antigens are intended to reduce morbidity and mortality and may be important components of future multi-antigen vaccines. We demonstrate a vaccine-specific $CD4^+$ T cell response in the AMA1 vaccine that has demonstrated strain-specific protection in field studies [6, 21]. While we did not detect strain-specific immunologic variation at the level of c1L, a multifunctional T cell response as well as antibody titer was elevated in vaccinated children protected against malaria. The possibility that a particular subset of multifunctional T cells within this Th1 response is essential for protection against intracellular pathogens, such as *Pf*, warrants further exploration.

References

1. Chan JA, Howell KB, Reiling L, Ataide R, Mackintosh CL, Fowkes FJ, et al. Targets of antibodies against *Plasmodium falciparum*-infected erythrocytes in malaria immunity. J Clin Invest 2012;122:3227-3238.
2. Chelimo K, Embury PB, Sumba PO, Vulule J, Ofulla AV, Long C, et al. Age-related differences in naturally acquired T cell memory to *Plasmodium falciparum* merozoite surface protein 1. PLoS One 2011;6:e24852.
3. Tran TM, Li S, Doumbo S, Doumtabe D, Huang CY, Dia S, et al. An intensive longitudinal cohort study of Malian children and adults reveals no evidence of acquired immunity to *Plasmodium falciparum* infection. Clin Infect Dis 2013;57:40-47.
4. Pacheco MA, Battistuzzi FU, Junge RE, Cornejo OE, Williams CV, Landau I, et al. Timing the origin of human malarias: the lemur puzzle. BMC Evol Biol 2011;11:299.
5. Takala SL, Plowe CV. Genetic diversity and malaria vaccine design, testing and efficacy: preventing and overcoming 'vaccine resistant malaria'. Parasite Immunol 2009;31:560-573.
6. Ouattara A, Takala-Harrison S, Thera MA, Coulibaly D, Niangaly A, Saye R, et al. Molecular basis of allele-specific efficacy of a blood-stage malaria vaccine: vaccine development implications. J Infect Dis 2013;207:511-519.
7. Tanabe K, Mita T, Palacpac NM, Arisue N, Tougan T, Kawai S, et al. Within-population genetic diversity of *Plasmodium falciparum* vaccine candidate antigens reveals geographic distance from a Central sub-Saharan African origin. Vaccine 2013;31:1334-1339.

8. Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, et al. A proteomic view of the *Plasmodium falciparum* life cycle. Nature 2002;419:520-526.

9. Howell SA, Well I, Fleck SL, Kettleborough C, Collins CR, Blackman MJ. A single malaria merozoite serine protease mediates shedding of multiple surface proteins by juxtamembrane cleavage. J Biol Chem 2003;278:23890-23898.

10. Silvie O, Franetich JF, Charrin S, Mueller MS, Siau A, Bodescot M, et al. A role for apical membrane antigen 1 during invasion of hepatocytes by *Plasmodium falciparum* sporozoites. J Biol Chem 2004;279:9490-9496.

11. Thomas AW, Deans JA, Mitchell GH, Alderson T, Cohen S. The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit *Plasmodium knowlesi* invasion of erythrocytes. Mol Biochem Parasitol 1984;13:187-199.

12. Hodder AN, Crewther PE, Anders RF. Specificity of the protective antibody response to apical membrane antigen 1. Infect Immun 2001;69:3286-3294.

13. Mitchell GH, Thomas AW, Margos G, Dluzewski AR, Bannister LH. Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells. Infect Immun 2004;72:154-158.

14. Dutta S, Lee SY, Batchelor AH, Lanar DE. Structural basis of antigenic escape of a malaria vaccine candidate. Proc Natl Acad Sci U S A 2007;104:12488-12493.

15. Riglar DT, Richard D, Wilson DW, Boyle MJ, Dekiwadia C, Turnbull L, et al. Super-resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte. Cell Host Microbe 2011;9:9-20.

16. Deans JA, Knight AM, Jean WC, Waters AP, Cohen S, Mitchell GH. Vaccination trials in rhesus monkeys with a minor, invariant, *Plasmodium knowlesi* 66 kD merozoite antigen. Parasite Immunol 1988;10:535-552.

17. Collins WE, Pye D, Crewther PE, Vandenberg KL, Galland GG, Sulzer AJ, et al. Protective immunity induced in squirrel monkeys with recombinant apical membrane antigen-1 of *Plasmodium fragile*. Am J Trop Med Hyg 1994;51:711-719.

18. Anders RF, Crewther PE, Edwards S, Margetts M, Matthew ML, Pollock B, et al. Immunisation with recombinant AMA-1 protects mice against infection with *Plasmodium chabaudi*. Vaccine 1998;16:240-247.

19. Stowers AW, Kennedy MC, Keegan BP, Saul A, Long CA, Miller LH. Vaccination of monkeys with recombinant *Plasmodium falciparum* apical membrane antigen 1 confers protection against blood-stage malaria. Infect Immun 2002;70:6961-6967.

20. Xu H, Hodder AN, Yan H, Crewther PE, Anders RF, Good MF. $CD4^+$ T cells acting independently of antibody contribute to protective immunity to *Plasmodium chabaudi* infection after apical membrane antigen 1 immunization. J Immunol 2000;165:389-396.

21. Thera MA, Doumbo OK, Coulibaly D, Laurens MB, Ouattara A, Kone AK, et al. A field trial to assess a blood-stage malaria vaccine. N Engl J Med 2011;365:1004-1013.

22. Takala SL, Coulibaly D, Thera MA, Batchelor AH, Cummings MP, Escalante AA, et al. Extreme polymorphism in a vaccine antigen and risk of clinical malaria: implications for vaccine development. Sci Transl Med 2009;1:2ra5.

23. Polhemus ME, Magill AJ, Cummings JF, Kester KE, Ockenhouse CF, Lanar DE, et al. Phase I dose escalation safety and immunogenicity trial of *Plasmodium falciparum* apical

559 membrane protein (AMA-1) FMP2.1, adjuvanted with AS02A, in malaria-naive adults at
560 the Walter Reed Army Institute of Research. Vaccine 2007;25:4203-4212.
561 24. Lyke KE, Daou M, Diarra I, Kone A, Kouriba B, Thera MA, et al. Cell-mediated
562 immunity elicited by the blood stage malaria vaccine apical membrane antigen 1 in
563 Malian adults: results of a Phase I randomized trial. Vaccine 2009;27:2171-2176.
564 25. Coulibaly D, Travassos MA, Kone AK, Tolo Y, Laurens MB, Traore K, et al. Stable
565 malaria incidence despite scaling up control strategies in a malaria vaccine-testing site in
566 Mali. Malar J 2014;13:374.
567 26. Lyke KE, Dicko A, Kone A, Coulibaly D, Guindo A, Cissoko Y, et al. Incidence of
568 severe *Plasmodium falciparum* malaria as a primary endpoint for vaccine efficacy trials
569 in Bandiagara, Mali. Vaccine 2004;22:3169-3174.
570 27. Lyke KE, Dabo A, Sangare L, Arama C, Daou M, Diarra I, et al. Effects of concomitant
571 *Schistosoma haematobium* infection on the serum cytokine levels elicited by acute
572 *Plasmodium falciparum* malaria infection in Malian children. Infect Immun
573 2006;74:5718-5724.
574 28. Lyke KE, Burges RB, Cissoko Y, Sangare L, Kone A, Dao M, et al. HLA-A2 supertype-
575 restricted cell-mediated immunity by peripheral blood mononuclear cells derived from
576 Malian children with severe or uncomplicated *Plasmodium falciparum* malaria and
577 healthy controls. Infect Immun 2005;73:5799-5808.
578 29. Dutta S, Lalitha PV, Ware LA, Barbosa A, Moch JK, Vassell MA, et al. Purification,
579 characterization, and immunogenicity of the refolded ectodomain of the *Plasmodium*
580 *falciparum* apical membrane antigen 1 expressed in *Escherichia coli*. Infect Immun
581 2002;70:3101-3110.

30. Lyke KE, Dabo A, Arama C, Daou M, Diarra I, Wang A, et al. Reduced T regulatory cell response during acute *Plasmodium falciparum* infection in Malian children co-infected with *Schistosoma haematobium*. PLoS One 2012;**7**:e31647.

31. Lyke KE, Wang A, Dabo A, Arama C, Daou M, Diarra I, et al. Antigen-specific B memory cell responses to *Plasmodium falciparum* malaria antigens and *Schistosoma haematobium* antigens in co-infected Malian children. PLoS One 2012;**7**:e37868.

32. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 2004;22:745-763.

33. Moormann AM, Sumba PO, Chelimo K, Fang H, Tisch DJ, Dent AE, et al. Humoral and cellular immunity to *Plasmodium falciparum* merozoite surface protein 1 and protection from infection with blood-stage parasites. J Infect Dis 2013;208:149-158.

34. Malaguarnera L, Musumeci S. The immune response to *Plasmodium falciparum* malaria. Lancet Infect Dis 2002;2:472-478.

35. Beck HP, Felger I, Genton B, Alexander N, al-Yaman F, Anders RF, et al. Humoral and cell-mediated immunity to the *Plasmodium falciparum* ring-infected erythrocyte surface antigen in an adult population exposed to highly endemic malaria. Infect Immun 1995;63:596-600.

36. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 2008;8:247-258.

37. Epstein JE, Tewari K, Lyke KE, Sim BK, Billingsley PF, Laurens MB, et al. Live attenuated malaria vaccine designed to protect through hepatic CD8(+) T cell immunity. Science 2011;334:475-480.

38. Garcon N, Chomez P, Van Mechelen M. GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. Expert Rev Vaccines 2007;6:723-739.

39. Pashine A, Valiante NM, Ulmer JB. Targeting the innate immune response with improved vaccine adjuvants. Nat Med 2005;11:S63-68.

40. Garcon N, Heppner DG, Cohen J. Development of RTS,S/AS02: a purified subunit-based malaria vaccine candidate formulated with a novel adjuvant. Expert Rev Vaccines 2003;2:231-238.

41. Lumsden JM, Schwenk RJ, Rein LE, Moris P, Janssens M, Ofori-Anyinam O, et al. Protective immunity induced with the RTS,S/AS vaccine is associated with IL-2 and TNF-alpha producing effector and central memory CD4 T cells. PLoS One 2011;6:e20775.

42. Leroux-Roels I, Leroux-Roels G, Ofori-Anyinam O, Moris P, De Kock E, Clement F, et al. Evaluation of the safety and immunogenicity of two antigen concentrations of the Mtb72F/AS02(A) candidate tuberculosis vaccine in purified protein derivative-negative adults. Clin Vaccine Immunol 2010;17:1763-1771.

Acknowledgments:

We would like to thank the people of Bandiagara, Mali for their participation and support of this vaccine study. The Walter Reed Army Institute of Research (WRAIR) kindly provided the purified recombinant AMA1-derived protein FMP2.1 used for cellular stimulation and formed the basis of the FMP2.1/AS02A vaccine candidate for this study. Additionally, we would like to thank GlaxoSmithKline Vaccines for providing the $AS02_A$ adjuvant. The conduct of the trial was

supported by a contract N01AI85346 and a cooperative agreement U19AI065683 from the National Institute of Allergy and Infectious Diseases, grant D43TW001589 from the Fogarty International Center, National Institutes of Health and contract W81XWH-06-1-0427 from the United States Department of Defense and the United States Agency for International Development. Vaccine production was supported by the United States Agency for International Development, Washington, D.C. and by the Military Infectious Diseases Research Program, Fort Detrick, MD. The immunologic assays and SG were funded by contract U01AI065683-06 from the International Collaboration on Infectious Diseases Research. CVP and MBL are supported by the Howard Hughes Medical Institute. This abstract was presented in part to the American Society of Tropical Medicine and Hygiene, Washington, D.C., November 2013, abstract #1443.

Figure Legends:

Figure 1 A-D. Vaccination with AMA1 elicits an antigen-specific $CD4^+$ cytokine response. Depicted is the net percentage of total $CD4^+$ antigen-specific intracellular expression of IFN-γ, TNF-α, IL-2, and IL-17A at Days 0, 90 and 150 after vaccination (**A**) as measured by flow cytometry. The significance of the differences between the results from AMA1-vaccinated children at D0 as compared to D90 and D150 was determined using a nonparametric two-tailed Mann-Whitney U test as described below for panels B-D. Additionally, results were stratified between in rabies control recipients (Rb), AMA1 vaccine recipients that remained free of clinical *Pf* infection [*Pf*(-)], and AMA1 recipients that contracted a clinical *Pf* infection [*Pf*(+)] by time point and depicted as percentage of net positive $CD4^+$ cytokine expressing cells (**B-D**). The geometric mean of positive responders is indicated by the horizontal bars. The significance of the differences between the results from AMA1-vaccinated children as compared to the rabies control cohort was examined (unless specifically indicated) using a nonparametric two-tailed Mann-Whitney U test with statistically significant results depicted by asterisks (*$p<0.05$, **$p<0.005$).

Figure 2. Magnitude and proportions of AMA1-specific multifunctional T cell responses in children stratified by presence or absence of clinical malaria (**A-C**). (A) Depicted are multifunctional subsets of $CD4^+$ T cells that were statistically significant after AMA1 stimulation of PBMC acquired from *Pf*(-) children and *Pf*(+) AMA1 vaccinated children. The geometric mean of responders is depicted by the horizontal black bars. (**B**) Shown are bar graphs demonstrating multifunctional cytokine subsets and magnitude of expression within $CD4^+$ T cells in *Pf*(-) children (white) compared to *Pf*(+) children (red) at Days 90 and 150. (**C**) Pie chart

depiction of proportions of single, double, and triple cytokine expression at Days 90 or 150 at the single cell level.

Figure 3. AMA1 vaccination elicits an AMA1-specific humoral response (**A-B**). Depicted are titers of anti-AMA1 IgG in vaccinated *Pf*(-) (blue square) and *Pf*(+) children (red triangle) versus rabies control (black circle) recipients with the level of significance compared to baseline values depicted for each time point (Days 0-150) (**A**). The level of significance for *Pf*(-) and *Pf*(+) as compared to Rb is depicted by asterisks as calculated by double-tailed Mann-Whitney U tests (*$p<0.05$, **$p<0.01$, ***$p<0.005$). (**B**) Anti-AMA1 IgG titers for AMA1 vaccinated children [*Pf*(-) children, *Pf*(+) children that contracted a Fab9-like clinical infection, and *Pf*(+) children that contracted a 3D7-like clinical infection]. Black horizontal bars signify the geometric mean and the levels of significance are indicated by asterisks as above (Mann Whitney Rank Sum Analysis).

Tables

Table 1: Baseline characteristics of randomized Malian children and features of study results stratified by study group.

Characteristic	AMA-1 *Pf*(+) (range) n = 11	AMA-1 *Pf*(-) (range) n = 10	P value[1]	Rabies Control (range) n = 10	P value[2]
Age (mean years ± SD)	4 ± 1.4	3.3 ± 1.8	ns	3.33.3 ± 1.8	ns
Female (%)	73	40	0.20	60	1.00
Clinical Malaria Episodes (by D240)	11/11	0/10	-	5/10	-
Parasite Density (Geometric mean/mm^3, range)	39,710 (7,564 - 98,359)	-----	-	37,199 (5,800 - 385,352)	ns
AMA-1 ELISA (mean titer D90)	211,698 (24,139-596,800)	176,295 (108,534-242,660)	0.85	39,205 (25-365,030)	≤ 0.001
Sh[3] SEA ELISA (number positive)	0/11	0/10	ns	1/10	ns

[1] P value of apical membrane antigen 1 (AMA1) vaccinated *Pf* (+) vs. *Pf* (-) study participants
[2] P value of AMA1 vaccinated (*Pf*(+) and *Pf*(-)) vs. Rabies control study participants
[3] ELISA to *S. haematobium* soluble egg antigen (SEA) (positive defined by titer > 2,880 measured at O.D. 450 nm)

Table 2: Results of $CD4^+$ intracellular cytokine expression after apical membrane antigen 1 (AMA1) antigenic stimulation of PBMC acquired from age-matched children randomized to AMA1 vaccination or Rabies control vaccination and stratified by acquisition of malaria *P. falciparum* (*Pf*) [*Pf* (-) vs. *Pf* (+)]. The Overall column represents the total number of children that demonstrated detectable cytokine expression in each group. Detectable results are further characterized by mean, geometric mean and range.

Group		**IFNγ**			**TNFα**			**IL2**			**IL17A**			**Overall**
		D0	**D90**	**D150**	**D0**	**D90**	**D150**	**D0**	**D90**	**D150**	**D0**	**D90**	**D150**	**Total**
Rabies n = 10		0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
AMA1 *Pf* (-) n = 10		0/10	5/10	3/10	0/10	8/10	8/10	0/10	7/10	7/10	0/10	0/10	0/10	10/10
	Mean	0.00	0.25	0.14	0.00	0.18	0.18	0.00	0.14	0.21	0.00	0	0	
	Geometric Mean	0.00	0.20	0.13	0.00	0.16	0.16	0.00	0.12	0.18	0	0	0	
	Range		0.09 - 0.48	0.08 - 0.2		0.09 - 0.4	0.06 - 0.31		0.06 - 0.37	0.06 - 0.43	-	-	-	
AMA1 *Pf* (+) n = 11		0/11	5/11	2/11	0/11	6/11	6/11	0/11	5/11	7/11	0/11	0/11	0/11	8/11
	Mean	0	0.14	0.13		0.18	0.10		0.17	0.09	0	0	0	
	Geometric Mean	0	0.12	0.11		0.14	0.09		0.15	0.08	0	0	0	
	Range		0.04 - 0.29	0.06 - 0.2		0.06 - 0.45	0.07 - 0.19		0.05 - 0.24	0.05 - 0.19	-	-	-	

Table 3: Results of $CD4^+$ and $CD8^+$ T cell intracellular cytokine expression after c3D7 and cFab9 antigenic stimulation of PBMC acquired from age-matched children randomized to apical membrane antigen 1 (AMA1) vaccination or Rabies control vaccination and stratified by acquisition of *P. falciparum* (*Pf*) malaria [*Pf* (-) vs. *Pf* (+)]. The Overall columns represents the total number of children that demonstrated detectable cytokine expression in each group.

	CD4+										**CD8+**									
Group	**c3D7**			**cFab9**			**Both**			**Overall**	**c3D7**			**cFab9**			**Both**			**Overall**
	D0	**D90**	**D150**	**D0**	**D90**	**D150**	**D0**	**D90**	**D150**	**Total**	**D0**	**D90**	**D150**	**D0**	**D90**	**D150**	**D0**	**D90**	**D150**	**Total**
Rabies n = 10	1/10	2/10	1/10	0/10	0/10	0/10	1/10	0/10	0/10	2/10	0/10	1/10	0/10	0/10	0/10	0/10	1/10	0/10	0/10	1/10
AMA1 *Pf* (-) n = 10	0/10	0/10	0/10	0/10	0/10	2/10	1/10	3/10	1/10	3/10	1/10	1/10	1/10	0/10	0/10	0/10	0/10	0/10	1/10	2/10
AMA1 *Pf* (+) n = 11	0/11	0/11	1/11	0/11	1/11	0/11	0/11	0/11	0/11	2/11	0/11	0/11	0/11	0/11	1/11	0/11	0/11	0/11	0/11	1/11

Figure 1

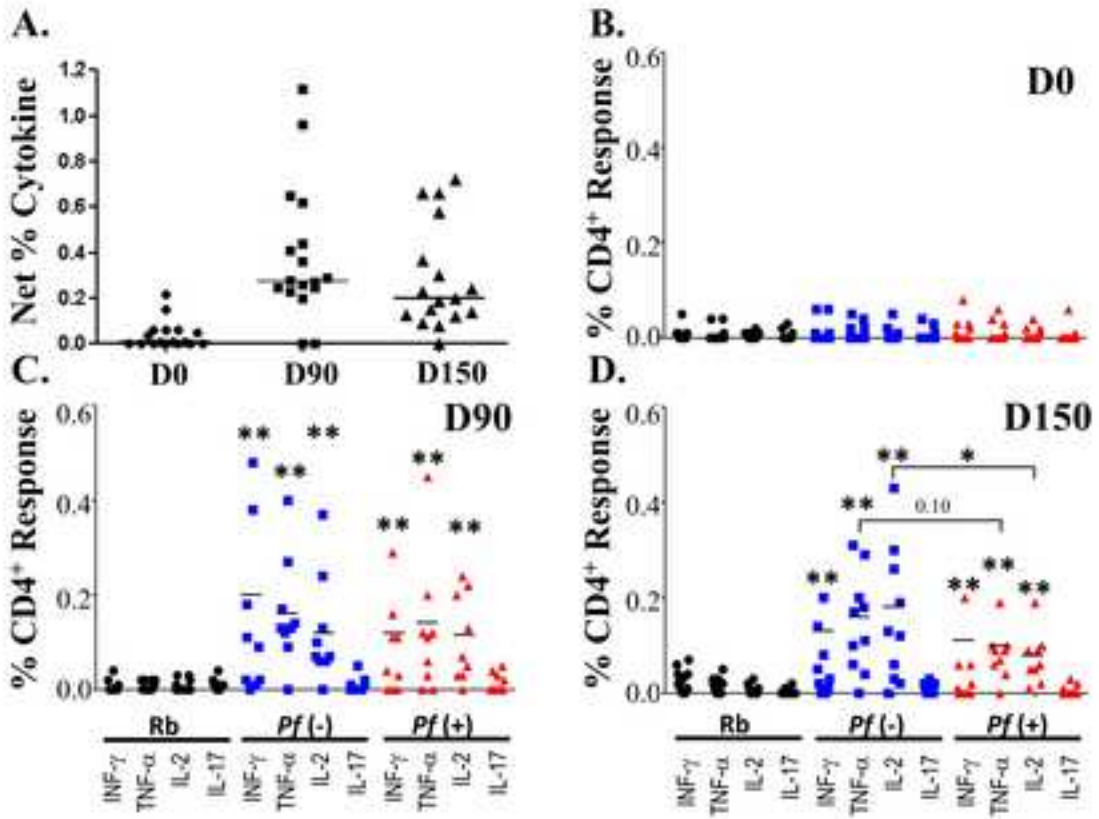

A.
Net % Cytokine
1.2
1.0
0.8
0.6
0.4
0.2
0.0
D0
D90
D150
B.
D0
% CD4+ Response
0.6
0.4
0.2
0.0
C.
D90
% CD4+ Response
**
Rb
Pf (-)
Pf (+)
INF-γ
TNF-α
IL-2
IL-17
D.
D150
*
0.10

Figure 2

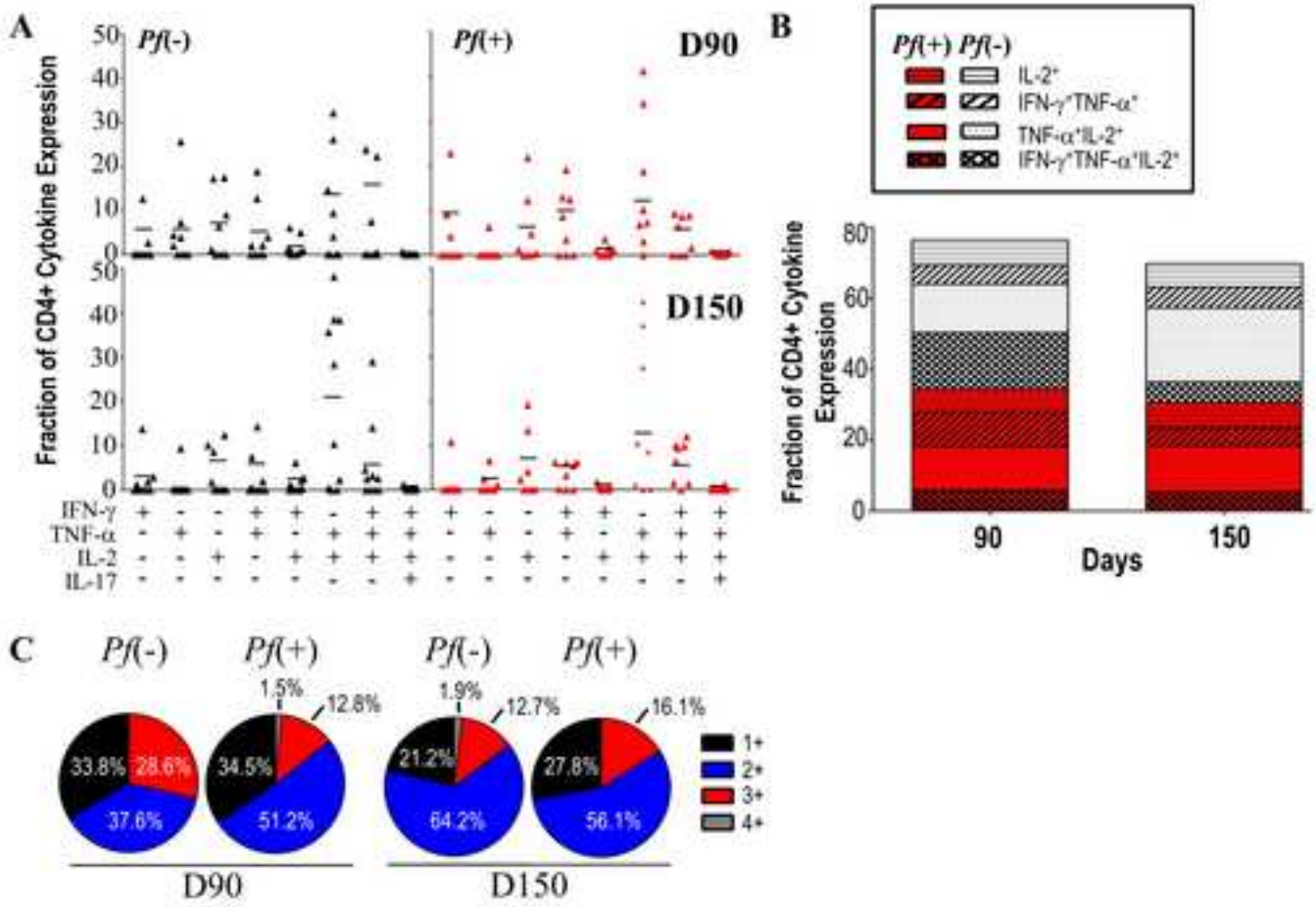
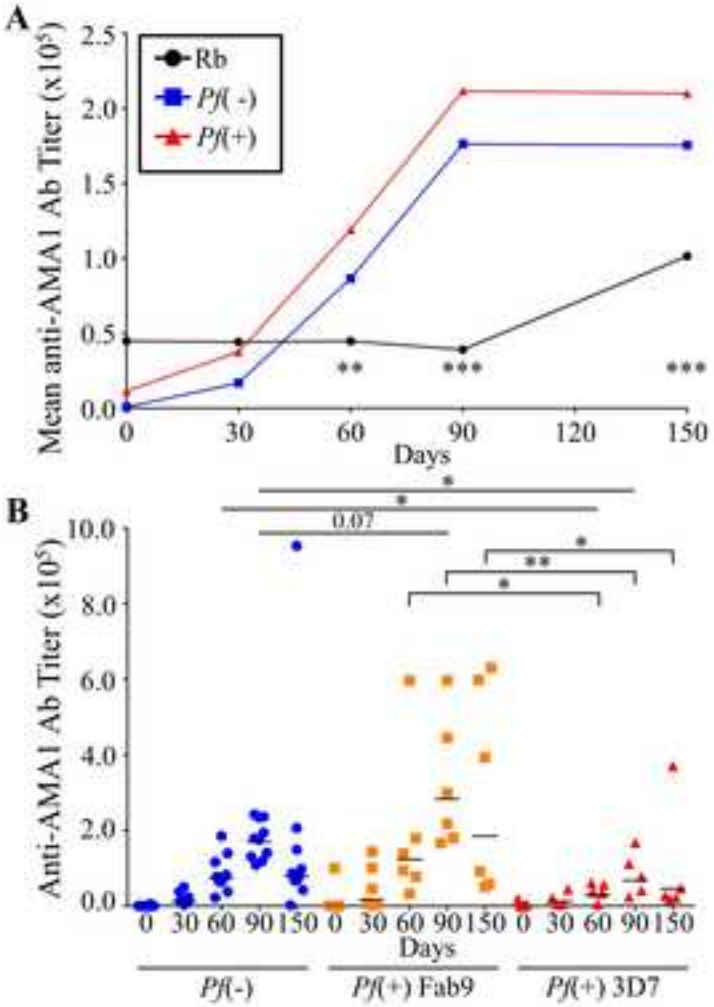

A
Pf(-)
Pf(+)
D90
D150
Fraction of CD4+ Cytokine Expression
IFN-γ
TNF-α
IL-2
IL-17
B
Pf(+) Pf(-)
IL-2+
IFN-γ+TNF-α+
TNF-α+IL-2+
IFN-γ+TNF-α+IL-2+
Fraction of CD4+ Cytokine Expression
90
150
Days
C
Pf(-)
Pf(+)
Pf(-)
Pf(+)
33.8%
28.6%
37.6%
1.5%
12.8%
34.5%
51.2%
1.9%
12.7%
21.2%
64.2%
16.1%
27.8%
56.1%
1+
2+
3+
4+
D90
D150

Figure 3


A
Mean anti-AMA1 Ab Titer (x10^5)
2.5
2.0
1.5
1.0
0.5
0.0
Rb
Pf(-)
Pf(+)
**
***
***
0
30
60
90
120
150
Days
B
Anti-AMA1 Ab Titer (x10^5)
10.0
8.0
6.0
4.0
2.0
0.0
0.07
*
*
*
**
*
0 30 60 90 150 0 30 60 90 150 0 30 60 90 150
Days
Pf(-)
Pf(+) Fab9
Pf(+) 3D7