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## Stability of interferon-gamma and interleukin-10 responses to Plasmodium falciparum liver stage antigen-1 and thrombospondin-related adhesive protein in residents of a malaria holoendemic area


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## STABILITY OF INTERFERON- $\gamma$ AND INTERLEUKIN-10 RESPONSES TO *PLASMODIUM FALCIPARUM* LIVER STAGE ANTIGEN-1 AND THROMBOSPONDIN-RELATED ADHESIVE PROTEIN IN RESIDENTS OF A MALARIA HOLOENDEMIC AREA

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**Abstract.** The stability of anti-malarial immunity will influence the interpretation of immunologic endpoints during malaria vaccine trials conducted in endemic areas. Therefore, we evaluated cytokine responses to *Plasmodium falciparum* liver stage antigen-1 (LSA-1) and thrombospondin-related adhesive protein (TRAP) by Kenyans from a holoendemic area at a 9-month interval. The proportion of adults with interferon- $\gamma$  (IFN- $\gamma$ ) responses to 9-mer LSA-1 peptides was similar at both time-points, whereas responses from children decreased ( $P < 0.05$ ). Response to the longer, 23-mer LSA-1 peptide was variable, decreasing in adults and children over time ( $P < 0.02$  and  $P < 0.001$ , respectively). The proportion of children with IFN- $\gamma$  responses to either antigen at the second time-point was significantly lower than that of adults, yet more adults responded to 9-mer TRAP peptides ( $P < 0.02$ ). In contrast, the proportion of interleukin-10 responses to LSA-1 and TRAP was similar at both time-points for both age groups. Most noteworthy was that even when the repeat cross-sectional frequency of cytokine responses was the same, these responses were not generated by the same individuals. This suggests that cytokine responses to LSA-1 and TRAP are transient under natural exposure conditions.

### INTRODUCTION

A major achievement in the fight against malaria will be the development of a safe and efficacious vaccine that can be delivered to infants and children living in malaria endemic areas of the tropics. Evaluating clinical endpoints for vaccine efficacy in this setting will be costly, time consuming, and require large numbers of participants to achieve sufficient power to detect differences in malaria-related morbidity between vaccinated and control groups.<sup>1,2</sup> Identification and validation of reliable immunologic correlates of protection against infection and morbidity are therefore important goals of malaria vaccine research.

The ongoing search for immune correlates of protection against malaria includes characterizing T-cell responses to malaria antigens that would prevent or diminish the intensity and frequency of blood stage infection, the cause of clinical illness such as fever and anemia. In the case of pre-erythrocytic vaccine development, it has been shown that inoculation of malaria-naïve individuals with hundreds of irradiated sporozoites leads to complete protection against patent blood stage infection after challenge with mosquitoes containing infective sporozoites. This immunity is transient and wanes within several months.<sup>3</sup> Observations of mouse malaria models and of humans exposed to irradiated sporozoites indicate that T-cell cytokine responses, particularly interferon- $\gamma$  (IFN- $\gamma$ ), are important in effecting and modulating this immunity that is largely directed against liver stage parasites.<sup>3–7</sup> In contrast to the immunity elicited by large numbers of irradiated sporozoites, the strength of the immune response developed after repeated exposure to mosquito transmitted

infective sporozoites that characterizes natural immunity is variable and less clearly associated with protection against infection and morbidity. Complete protection against the progression of malaria parasites from the liver to blood stage does not develop in the latter situation, although there is evidence of an exposure- and age-related delay in time to re-infection.<sup>8</sup>

Pre-erythrocytic *Plasmodium falciparum* vaccine candidate antigens now or soon to undergo clinical trials in malaria endemic areas include circumsporozoite antigen (CSP),<sup>9</sup> liver stage antigen-1 (LSA-1),<sup>10</sup> and thrombospondin-related adhesive protein (TRAP).<sup>11</sup> LSA-1 is the only *P. falciparum* antigen known to be expressed exclusively in hepatocytes. CSP and TRAP are present on the surface of infective sporozoites and are involved in the invasion of liver cells. T-cell immunity to LSA-1 and TRAP is commonly observed in residents of malaria endemic areas, but the value and reproducibility of specific cytokine responses as correlates of protective immunity in this setting are not well established. For example, some reports conclude that pre-existing IFN- $\gamma$  responses to LSA-1 are predictive of delayed time to re-infection or a lower rate of blood stage infection, whereas others show no correlation.<sup>12–15</sup> Similarly, LSA-1-driven IL-10 responses have been suggested to be predictive of resistance to re-infection.<sup>16</sup> Flanagan and others<sup>17</sup> reported that the overall frequency of *ex vivo* IFN- $\gamma$  responses to 14 overlapping TRAP peptides was 40% of 217 donors living in Kilifi, Kenya. The response rates diminished from 70% to 36% in a subset of 69 individuals selected for re-testing 1 year after the initial examination. Factors contributing to the apparent variability of T-cell cytokine responses include the age of the individuals examined, recent or current exposure to sporozoites, the presence of blood stage infection, treatment with anti-malarial drugs, the specific cytokines selected for study, and the stability of immune responses over time. To address these gaps in knowledge, we conducted a study of

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adults and children living in a malaria holoendemic area of western Kenya during two consecutive seasons of differing transmission. LSA-1- and TRAP-driven IFN- $\gamma$  and interleukin-10 (IL-10) responses were evaluated for population prevalence and concordance over the 9-month period.

## MATERIALS AND METHODS

**Ethical review and informed consent.** This study was approved by the Ethical Review Committee of the Kenya Medical Research Institute and the Institutional Review Board for Human Studies at University Hospitals of Cleveland, Case Western Reserve University. Written informed consent was obtained from all participants  $\geq 18$  years old and from guardians of participants  $< 18$  years old.

**Study site and repeated cross-sectional study design.** Participants were recruited from the Kanyawegi sub-location in Kisumu District, Nyanza Province, Kenya. Malaria transmission in this area is high (annual entomological inoculation rates can exceed 300 infectious bites per person) with seasonal variation.<sup>18</sup> Immunologic measurements reported here were performed two times over a 9-month interval. The first measurement was in November 2000 when the monthly entomological inoculation rate was estimated to be three infectious bites per person. The second immunologic measurement was performed in August 2001 when the monthly entomological inoculation rate was estimated to be 3- to 4-fold higher (Kim Lindblade, personal communication).

**Blood sample collection and processing.** Adults ( $\geq 18$  years) donated 12–16 mL blood and children (2–9 years) donated 5–8 mL blood collected in sodium heparin tubes. Samples were transported at room temperature to the laboratory for processing. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density centrifugation and resuspended in complete RPMI containing 10% human AB serum, 10 mmol/L L-glutamine, and 10  $\mu\text{g}/\text{mL}$  gentamicin. Typical yields were  $2 \times 10^6$  PBMC/mL blood from children and  $1 \times 10^6$  PBMC/mL blood from adults. Sufficient PBMCs for cytokine analyses were obtained from 93 adults and 66 children during the first collection and 114 adults and 119 children during the second collection. Fifty-four adults and 59 children donated blood at both time-points. The study population was 48% female. Exclusion criteria included pregnancy and self-reported treatment of malaria 2 weeks before blood collection.

**LSA-1 and TRAP peptides.** LSA-1 and TRAP peptides used for the study are listed in Table 1. These peptides have been shown to elicit T-cell responses in Africans naturally exposed to malaria<sup>17,19,20</sup> and were based on the major histocompatibility complex (MHC) Class 1 types present in our study population.<sup>21</sup> Long and short peptides from LSA-1 and TRAP were synthesized and purified to  $>95\%$  by high-performance liquid chromatography (Sigma Genosys, Gainesville, TX).

**IFN- $\gamma$  ELISPOT and IL-10 ELISA.** For IFN- $\gamma$  ELISPOT assays, PBMCs were plated at  $0.5 \times 10^6$  cells/well in Millipore MAIP ELISPOT 96-well microtiter plates (Millipore Corp., Billerica, MA) pre-coated at 4°C overnight with 5  $\mu\text{g}/\text{mL}$  human anti-IFN- $\gamma$  monoclonal antibodies (Endogen M-700A, Rockford, IL), washed with sterile phosphate-buffered saline (PBS), pH 7.4, and blocked with 10% heat-inactivated fetal

TABLE 1  
LSA-1 and TRAP peptides used for this study

Malaria antigen (amino acid position*)	Amino acid sequence	Abbreviation
LSA-1 (84–92)	LPM SNV KNV	ls84
LSA-1 (94–102)	QTN FKS LLR	ls94
LSA-1 (1881–1889)	LFH IFD GDN	ls1881
LSA-1 (1813–1835)	NEN LDD LDE GIE KSS EEL SEE KI	T3
TRAP (51–59)	LLM DCS GSI	tr51
TRAP (539–547)	TPY AGE PAP	tr539
TRAP (51–70)	LLM DCS GSI RRH NWV NHA VP	tp6

\* Numbers represent amino acid position based on NCBI GenBank *Entrez* accession no. CAA39663 for LSA-1 and A46283 for TRAP.

bovine serum in PBS. PBMC were incubated at 37°C in 5% CO<sub>2</sub> for 5 days in culture medium alone (negative control), 10  $\mu\text{g}/\text{mL}$  malaria peptide, and 1  $\mu\text{g}/\text{mL}$  phytohemagglutinin (PHA; positive control). Details of subsequent steps and enumeration of spot forming units (SFUs)/ $10^6$  PBMCs are as described.<sup>13</sup> For IL-10 assays, PBMC were plated at  $0.2 \times 10^6$  cells/well in sterile U-bottom polystyrene microtiter plates (Falcon 35-3077; Becton Dickinson & Co, Franklin Lakes, NJ) and incubated with malaria peptides, PHA, or PBS alone for 5 days as described for the IFN- $\gamma$  ELISPOT. Supernatants were kept frozen at  $-20^\circ\text{C}$  until IL-10 ELISAs were performed.<sup>14</sup>

Malaria peptides were tested under the same incubation conditions using PBMCs obtained from 20 healthy malaria-naïve controls from Cleveland, OH. A pool of EBV peptides was used as positive 9-mer peptide controls. Positive responses to the EBV peptides were observed in the both the malaria exposed and malaria-naïve donors (data not shown). Malaria-naïve volunteers had no detectable IFN- $\gamma$  ELISPOT responses to any of the malaria peptides. A positive IL-10 response by a study participant was scored if the value was greater than 2 SD above the mean of the 20 control malaria-naïve blood donors. IL-10 cut-off values for the various peptides were as follows: 20 pg/mL for T3, 85 pg/mL for ls84, 43 pg/mL for ls94, 51 pg/mL for ls1881, and 20 pg/mL for tr539, tr51, and tr6.

**Malaria infection and surveillance for clinical malaria.** Malaria infection was determined by microscopic inspection of blood smears. Parasitemia was quantified by counting the number of asexual parasites observed after visualizing at least 200 leukocytes. Densities were calculated assuming a leukocyte count of 8,000/ $\mu\text{L}$  blood. Beginning in January 2001, study participants enrolled in immunologic studies in November 2000 were visited weekly by trained field assistants and queried about signs and symptoms of malaria.<sup>13</sup> Participants contacted their village field assistant if they had fever, chills, headache, or severe malaise. Clinical malaria was defined as the presence of any of these symptoms with  $\geq 4,000$  parasites/ $\mu\text{L}$  blood. Individuals with clinical malaria were treated with sulfadoxine-pyrimethamine (the drug recommended by the Kenya Ministry of Health for treatment of uncomplicated malaria at the time) unless allergic to this medication, in which case they were given amodiaquine or quinine.

**Statistical analysis.** An IFN- $\gamma$  ELISPOT was considered positive if the proportion of SFUs in the stimulated well was significantly greater compared with the unstimulated background well ( $P < 0.05$ ). The significance test was a  $\chi^2$  com-

parison of two proportions adjusting for small sample size and assuming a Poisson distribution. An IL-10 ELISA response was considered positive if it was greater than the cut-off value based on the malaria-naïve negative control studies as described above. A  $\chi^2$  test of homogeneity was used to determine whether the proportion of children with IFN- $\gamma$  and IL-10 responses were significantly different than that of the adults. A two-sided Wilcoxon (Mann-Whitney) rank sum test was used to determine whether one group had a lower IFN- $\gamma$  precursor frequency or lower mean IL-10 response than another group. The correlation between responses to both cytokines was assessed by Spearman rank test. A  $\chi^2$  test of homogeneity was used to determine whether the proportion of individuals with cytokine responses was significantly different between time-points. All analyses were conducted in SAS version 8.2 (SAS, Cary, NC).

## RESULTS

**Proportion of IFN- $\gamma$  and IL-10 responders comparing children to adults at two times.** Eighty-six adults and 59 children from the first collection in November 2000 and 93 adults and 96 children from the second collection in August 2001 had interpretable IFN- $\gamma$  ELISPOT results. Data from the first collection showed no significant differences for the proportion of responders when children were compared with adults for any of the peptides assayed (e.g., 16–29% in each age group responded to one or more of the LSA-1 peptides and 14–22% to the TRAP peptides; Table 2). Nine months later, however, fewer children than adults responded. This was true for all of the peptides tested at both time-points (e.g., PBMCs from 7 of 96 [7%] children versus 38 of 92 [41%] adults made IFN- $\gamma$  when stimulated with the tr6 peptide;  $P = 0.001$ ).

The pattern of IL-10 responses differed from IFN- $\gamma$ . First, the proportion of individuals responding to any of the peptides was lower regardless of age or the time of blood collection (i.e., the range of IL-10 response rates for children was 0–19% [ $N = 27$ –74] and 9–32% [ $N = 39$ –74] for adults; Table 3). Second, with the exception of one TRAP peptide (tr539), the frequency of IL-10 responses was not significantly different for children compared with adults at either time-point. There was no correlation between IFN- $\gamma$  and IL-10 responses for any of the peptides (results not shown).

To determine if there was a difference in the magnitude of cytokine responses between adults and children over the 9-month study period, data for all persons with detectable cytokine responses were compared. The range for IFN- $\gamma$  responses detected by ELISPOT for any malaria peptide was 30–200 SFU/10<sup>6</sup> PBMCs with no significant difference ( $P > 0.1$ ) among the seven peptides tested. The magnitude of response did not significantly differ by age or time of collection (data not shown). Similarly for IL-10, the amount of cytokine produced did not differ significantly according to malaria peptide, age, or time of collection (data not shown). The range of IL-10 responses to the various malaria peptides was 90–200 pg/mL.

**Stability of cytokine responses for individuals examined at both time-points.** Samples from the same individuals were obtained at both collection periods from 54 adults and 59 children. Comparison of cytokine responses was determined using IFN- $\gamma$  ELISPOT results for adults only because the repeat

TABLE 2  
Repeat cross-sectional proportions of children and adults with IFN- $\gamma$  ELISPOT responses to LSA-1 and TRAP

Age group comparisons			
LSA-1 peptides	% children ( <i>N</i> )	% adults ( <i>N</i> )	<i>P</i> value
ls84	Nov. 2000	24% (13/55)	23% (20/86)
	Aug. 2001	4% (3/84)	18% (17/93)
	<i>P</i> value	0.001*	0.411
ls94	Nov. 2000	16% (9/55)	26% (22/86)
	Aug. 2001	6% (6/96)	27% (25/92)
	<i>P</i> value	0.048	0.810
ls881	Nov. 2000	24% (12/49)	24% (21/86)
	Aug. 2001	ND	37% (31/83)
	<i>P</i> value	ND	0.069
T3	Nov. 2000	25% (15/59)	29% (26/86)
	Aug. 2001	5% (5/96)	15% (14/93)
	<i>P</i> value	0.001*	0.023*
TRAP peptides	Nov. 2000	20% (9/44)	22% (18/83)
	Aug. 2001	ND	38% (30/79)
	<i>P</i> value	ND	0.023*
tr51	Nov. 2000	14% (6/42)	16% (13/83)
	Aug. 2001	8% (8/96)	39% (36/92)
	<i>P</i> value	0.292	0.001*
tp6	Nov. 2000	ND	ND
	Aug. 2001	7% (7/96)	41% (38/92)
	<i>P</i> value	ND	0.001*

\* Significant *P* value < 0.05.  
ND, not done.

cross-sectional frequency of responders was the same, whereas responses from the children showed an overall decrease from November 2000 to August 2001 (Table 2). There were too few individuals who had IL-10 responses to make meaningful comparisons (Table 3). For this analysis, an individual's peptide-specific immune response was categorized as "stable" if IFN- $\gamma$  was detected at both time-points, "lost" if positive at the first and negative at the second collection, "gained" if negative at the first and positive at the second collection, and "non-responsive" if negative at both time-points. These data are summarized in Table 4. First, we observed that the proportion of persons with "stable" responses was low regardless of the peptide tested. The peptide that most consistently stimulated an IFN- $\gamma$  response was ls1881, but this was found in only 6 of 43 (14%) individuals. Second, "loss" or "gain" of responses between the first and second time of PBMC collection was observed in a greater proportion of individuals (the range for persons in these categories was 7–36%). None of the LSA-1 or TRAP peptides stood out as being associated with either category, possibly with exception of responses to LSA-1 T3, which were "lost" by 15 of 47 (32%) participants and tr539 and tr51, which were "gained" by 16 of 44 (36%) individuals. The most common category was "non-responders," which varied from 42% of participants for ls1881 to 62% of participants for ls84.

**Relationship of *P. falciparum* parasitemia, symptomatic malaria, and anti-malarial drugs to cytokine responses.** The



TABLE 3

Repeat cross-sectional proportions of children and adults with IL-10 ELISA responses to LSA-1 and TRAP

Age group comparisons			
	% children ( <i>N</i> )	% adults ( <i>N</i> )	<i>P</i> value
<b>LSA-1 peptides</b>			
ls84			
Nov. 2000	6% (2/36)	16% (7/48)	0.135
Aug. 2001	10% (5/48)	25% (11/44)	0.065
<i>P</i> value	0.425	0.315	
ls94			
Nov. 2000	6% (2/34)	17% (7/42)	0.148
Aug. 2001	19% (14/73)	13% (6/46)	0.383
<i>P</i> value	0.073	0.632	
ls881			
Nov. 2000	3% (1/31)	10% (4/41)	0.280
Aug. 2001	ND	16% (5/31)	
<i>P</i> value		0.418	
T3			
Nov. 2000	6% (2/35)	9% (4/43)	0.554
Aug. 2001	19% (14/74)	21% (10/47)	0.751
<i>P</i> value	0.069	0.117	
<b>TRAP peptides</b>			
tr539			
Nov. 2000	0% (0/27)	28% (11/39)	0.003*
Aug. 2001	ND	32% (10/31)	
<i>P</i> value		0.713	
tr51			
Nov. 2000	ND	ND	
Aug. 2001	14% (10/73)	19% (9/47)	0.425
<i>P</i> value			
tp6			
Nov. 2000	ND	ND	
Aug. 2001	10% (7/69)	15% (7/47)	0.441
<i>P</i> value			

\* Significant *P* value < 0.05.  
ND, not done.

prevalence of blood stage infection was 61% (57/93) in adults and 91% (60/66) in children when the first immunologic study was performed in November 2000. Mean parasite densities were 636 and 6615 parasites per microliter for adults and children, respectively. At the time of the second collection in August 2001, 51% (58/114) of adults (mean parasite density, 410/ $\mu$ L) and 78% (93/119) of children (mean parasite density, 4,969/ $\mu$ L) had blood stage infection. The difference in parasite prevalence between time-points was significant for children ( $P = 0.028$ ) but not for adults ( $P = 0.13$ ). Gametocyte rates were lower ( $P = 0.01$ ) in children over time, decreasing from 29% (19/66) to 13% (16/119). Five adults met the diagnostic criteria for clinical malaria at the time of blood collection and received anti-malarial treatment. Children were more likely to be symptomatic with *P. falciparum* parasitemia at the two cross-sectional surveys, 37% (22/59) and 28% (27/96) in November 2000 and August 2001, respectively, and were treated with sulfadoxine-pyrimethamine. Active surveillance for clinical malaria began immediately after the first immunologic measurement was performed. Forty-nine percent (58/119) of children had a symptomatic malaria episode during the 9-month study period and were treated with anti-malarial drugs. IFN- $\gamma$  or IL-10 responses were not associated with prior or concurrent parasitemia (both children and adults) or prior and concurrent treatment of symptomatic malaria (children only).

## DISCUSSION

Studies of malaria-naïve humans and rodents immunized with pre-erythrocytic malaria vaccine antigens have shown that malaria-specific T-cell IFN- $\gamma$  responses are closely linked with protection against challenge infection.<sup>3,5-7</sup> In contrast, T-cell responses to the same antigens by persons with immunity resulting from repeated exposures to mosquito-borne sporozoites differ substantially. This is evident by less vigorous and more complex responses that include IFN- $\gamma$ , IL-10, and tumor necrosis factor- $\alpha$  and a weak association with protection when measured as time to re-infection.<sup>14,22</sup> Appreciation of this variability in background T-cell responses will be important for malaria vaccine development in endemic populations where immune correlates may serve as primary endpoints for immunogenicity and secondary endpoints for parasitological or clinical efficacy. To understand better the stability of T-cell cytokine responses to LSA-1 and TRAP in target populations for malaria vaccine testing, we assessed IFN- $\gamma$  and IL-10 responses to LSA-1 and TRAP peptides at two time-points over a 9-month interval in children and adults living in a malaria holoendemic area of western Kenya. We found that the proportion of children and adults with IFN- $\gamma$  responses far exceeded those with IL-10. Additionally, IFN- $\gamma$  and IL-10 responses were not concordant, and IFN- $\gamma$  responses by adults were not stable over time. Temporal changes in population and individual T-cell responses did not correlate with the presence of blood stage infection, transmission as estimated by entomologic inoculation rate, symptomatic malaria, or treatment with anti-malaria drugs.

The impact of malaria transmission on the acquisition and maintenance of T-cell responses to the antigens studied here has to date not been well characterized. An earlier study we conducted in the highlands of western Kenya suggested that IL-10 responses to LSA-1 correlated with protection against infection that was evident between the rainy and dry seasons, whereas IFN- $\gamma$  responses were unchanged.<sup>23</sup> In contrast to the high endemicity in the region where this study was done, malaria transmission in the highlands of western Kenya is sporadic, with long intervening periods of low or absent blood stage infection. Taken together, these data suggest that the pattern of exposure to sporozoites qualitatively influences immunity to pre-erythrocytic antigens, such that during periods of low transmission, maintenance of IL-10 responses is favored, whereas high levels of exposure shift the response toward IFN- $\gamma$ . This hypothesis is supported by observations comparing children with severe versus mild malaria in Gabon. IL-10 responses were associated with lower parasite densities in mild malaria cases and correlated inversely with severe malaria and high-density parasitemia.<sup>15</sup>

Few other studies have addressed the stability of T-cell cytokine responses to pre-erythrocytic malaria antigens. A repeat cross-sectional study by Kurtis and others<sup>24</sup> of adult Kenyans living in a malaria holoendemic area near the study site described here supports the notion that LSA-1-driven IFN- $\gamma$  responses are unstable. In contrast with our results, these workers observed that the frequency of responses to LSA-1 was greater rather than less during the period of high than low transmission. The apparent lack of stability of IFN- $\gamma$  responses reported here may be explained by several possible mechanisms. First, if T-cell responses to LSA-1 and TRAP are short-lived and depend on recent exposure to liver stage

TABLE 4  
Stability of IFN- $\gamma$  ELISPOT responses by adults at two time-points over a 9-month interval

Category	Percent in each category for each peptide (N/total)					
	ls84	ls94	ls1881	T3	tp539	tp51
Stable response	4% (2/47)	9% (4/47)	14% (6/43)	0% (0/47)	13% (5/39)	5% (2/44)
Lost response	17% (8/47)	17% (8/47)	14% (6/43)	32% (15/47)	10% (4/39)	7% (3/44)
Gained response	17% (9/47)	15% (7/47)	30% (13/43)	19% (9/47)	31% (12/39)	36% (16/44)
Non-responders	62% (29/47)	60% (28/47)	42% (18/43)	49% (23/47)	46% (18/39)	52% (23/44)

*P. falciparum*, it is possible that antigenic polymorphism may contribute to the gain, loss, or lack of responses during the first and second collection periods. Polymorphisms in the region of the *LSA-1* gene corresponding to ls84 have been observed, whereas the gene sequences corresponding to the TRAP peptides we used for our study are believed to be relatively conserved.<sup>17,25,26</sup> Second, co-existing blood stage infection could suppress pre-erythrocytic immunity through inhibition of dendritic cell functions such as antigen processing or expression of costimulatory molecules.<sup>27–29</sup> We have not examined whether this mechanism is operative but note the lack of correlation of IFN- $\gamma$  responses with concurrent blood stage infection and parasite density. Third, sampling of T-cell populations with specificity for liver stage malaria antigens may be limited by the ability to detect such cells in the peripheral circulation, especially in persons who are naturally infected and presumably have few viable liver stage *P. falciparum* present at one time. In this context, mouse malaria models suggest that T cells with specificity for hepatic stage malaria may be preferentially localized in the liver.<sup>30</sup> It is noteworthy that T-cell cytokine responses to vaccine candidates that include the TRAP peptides studied here are readily detectable in the peripheral blood of malaria-naïve humans after administration of experimental vaccines by prime boost strategies such as plasmid DNA followed by recombinant non-replicating poxviruses.<sup>31,32</sup>

Continuing study of the persistence and stability of effector and memory T cells with specificity for pre-erythrocytic antigens is warranted if the goal of defining reproducible immune correlates of protection is to be achieved. We believe that such studies should include comparison of the levels of total and antigen-specific CD4 and CD8 T cell subsets responses over shorter periods of time (e.g., at 1- or 2-week intervals), so that limitations of interpretation related to temporal stability can be limited by excluding changes in transmission intensity and other variables such as exposure to other infectious pathogens. Such work will ultimately be useful in understanding whether the apparent lack of long-term memory in human falciparum malaria is related to T-cell attrition and apoptosis.<sup>33–35</sup> A better understanding of whether and how T-cell memory to malaria develops in humans will hopefully lead to rational strategies for boosting and maintaining the immunity elicited by a malaria vaccine.

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