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Gamma Interferon Responses to *Plasmodium falciparum* Liver-Stage Antigen 1 and Thrombospondin-Related Adhesive Protein and Their Relationship to Age, Transmission Intensity, and Protection against Malaria

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Gamma interferon (IFN-γ) responses to the *Plasmodium falciparum* antigens liver-stage antigen 1 (LSA-1) and thrombospondin-related adhesive protein (TRAP) are thought to be important in protection against malaria. Optimal methods of testing and the effects of age and transmission intensity on these responses are unknown. IFN-γ responses to LSA-1 and TRAP peptides were assessed by the enzyme-linked immunospot assay (ELISPOT) and enzyme-linked immunosorbent assay (ELISA) in children and adults from areas of stable and unstable malaria transmission in Kenya. Adults in the areas of stable and unstable transmission had similar frequencies and levels of IFN-γ responses to LSA-1 and TRAP as determined by ELISPOT and ELISA. In contrast, IFN-γ responses to the LSA-1 T3 peptide (assessed by ELISPOT) and to any LSA-1 peptide (assessed by ELISA) were less frequent in children in the area of unstable transmission than in children in the area of stable transmission. IFN-γ responses to LSA-1 were more frequently detected by ELISA than by ELISPOT in the stable-transmission area. IFN-γ responses detected by ELISA and ELISPOT did not correlate with each other. In children in the stable-transmission area, IFN-γ responses to LSA-1 peptides assessed by ELISA, but not by ELISPOT, were associated with protection against clinical malaria and anemia. IFN-γ responses to LSA-1 appear to require repeated *P. falciparum* exposure and/or increased age and, as measured by ELISA, are associated with protection against clinical malaria and anemia.

A better understanding of the immunity correlates of protection against infection and disease due to *Plasmodium falciparum* malaria that evolve during the course of repeated natural infections would advance the development and testing of human malaria vaccines. Gamma interferon (IFN-γ) is believed to eliminate directly or represent a molecular correlate of T-cell toxicity against liver-stage *P. falciparum* (9, 30, 38). There has consequently been great interest in assessing the relationship between IFN-γ responses to the preerythrocytic antigens liver-stage antigen 1 (LSA-1) and thrombospondin-related adhesive protein (TRAP) and parasitologic and clinical endpoints. Studies of T-cell immunity to these antigens conducted in various populations in areas where malaria is endemic, however, have not been uniform in their results or conclusions. IFN-γ responses to LSA-1, for example, were reported to be associated with protection against subsequent infection in children with mild malaria in Gabon (27) but did not correlate with time to infection after drug-induced parasitologic cure of adults and children in Kenya (20, 25). Similarly, preexisting IFN-γ responses to TRAP correlated with higher hemoglobin levels in a prospective examination of Kenyan children (35), whereas there was not a significant relationship between IFN-γ response and attacks of clinical malaria in children and adults in another area of Kenya (11). The apparently conflicting conclusions of such studies may stem from technical, demographic, and epidemiologic factors. From a technical perspective, the method of assessing IFN-γ production may be important. Recent studies conducted in The Gambia of responses to TRAP and circumsporozoite protein (CSP) assessed the number of IFN-γ-secreting mononuclear cells by the enzyme-linked immunospot assay (ELISPOT) (10, 11, 37), while most studies of LSA-1 have measured IFN-γ production by enzyme-linked immunosorbent assay (ELISA) (20, 25, 27, 35). Interestingly, although an early report suggested an increased sensitivity of the ELISPOT compared to the ELISA for detecting T-cell IFN-γ responses in persons naturally exposed to malaria (23), there have been to our knowledge no subsequent comparative studies of the two methods for residents of areas where malaria is endemic. Furthermore, all published studies that have reported an association of LSA-1- or TRAP-induced IFN-γ responses with protection against malaria infection or disease have assessed IFN-γ production by ELISA (6, 27, 29, 35). With respect to demographic and epidemiologic variables, the seasonality, stability, and intensity of malaria transmission; the ages and genetic backgrounds of the study participants; and the specific clinical and parasitologic variables chosen as endpoints may influence the correlation

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with preexisting or concurrent cytokine responses. To gain a better understanding of these complexities, we evaluated LSA-1- and TRAP-driven IFN-γ responses by both ELISPOT and ELISA in children and adults living in two geographically proximate but epidemiologically distinct areas of western Kenya. One is a lowland area where malaria transmission is stable and intense, and the other is a highland area where transmission is episodic and unstable and where the pattern of endemicity has been described as epidemicity prone (21, 28).

MATERIALS AND METHODS

Study sites and populations. Participants were recruited from the villages of Kanyawegi and Kipsamoite in western Kenya. Kanyawegi is located in an area of Kisumu District where malaria is holoendemic, with stable, perennial high-level malaria transmission, though seasonal variations occur (25). The parasitemia prevalence in children under 5 years of age at the time of sample collection was 95%. Kipsamoite in Nandi District is located in an epidemic-prone highland area with unstable malaria transmission (14, 28). Testing was performed at a time of low transmission in Kipsamoite, when parasitemia prevalence in children under 5 years of age was 5.6%.

Adults were defined as individuals ≥15 years old, and children were defined as those individuals ≤8 years old. To test for nonspecific IFN-γ responses to malaria antigen peptides, blood was obtained from 18 North American adults who had never traveled to areas where malaria is endemic.

Informed consent and ethical approval. Written informed consent was obtained from the study participants or the parents or guardians of individuals under 18 years of age. Ethical approval was obtained from the Ethical Review Committee at the Kenya Medical Research Institute and the Human Investigations Institutional Review Board at Case Western Reserve University and University Hospitals of Cleveland.

Blood sample collection, isolation of peripheral blood mononuclear cells, and cell culture. Ten- to 20- and 5- to 8-ml blood samples were collected by venipuncture from adults and children, respectively. Ten microliters of blood was used to determine hemoglobin concentration. Peripheral blood mononuclear cells (PBMC) were separated from whole blood by Ficoll-Hypaque density gradient centrifugation. PBMC were suspended in RPMI 1640 medium (Gibco, Invitrogen, Paisley, Scotland, United Kingdom) supplemented with 10% heat-inactivated human AB blood-type serum, 50 μg of gentamicin/μl, 10 mM HEPES, and 2 mM glutamine.

Antigens and mitogens for cytokine testing. Peptides that were likely to induce IFN-γ responses in the Kenyan populations studied were chosen for testing. The LSA-1 and TRAP 9-mer peptides chosen were predicted to bind to HLA supertypes common in western Kenya (8). The longer LSA-1 (T3) and TRAP (tr51) peptides chosen had elicited strong IFN-γ responses in other Kenyan populations (11, 20). LSA-1 peptides included three 9-mer predicted T-cell epitopes, ls84 (amino acids [aa] 84 to 92; LPMSNVKNV) (3), ls94 (aa 94 to 102; QTNFKSLLR) (15), and ls1881 (aa 1881 to 1889; LFHIFDGDN) (V. Brusic, personal communication), and the 23-mer T-cell epitope T3 (aa 1813 to 1835; NENLDDLDEGIEKSSEELSEEKI) (6, 20). TRAP peptides were the 9-mer tr539 (aa 539 to 547; TPYAGEPAP) (8) and 20-mer tr51 (aa 526 to 545; AGLAYKFVVPGAATPYAGEP) (11). Peptides were synthesized and purified by high-performance liquid chromatography to >95% purity (Sigma Genosys, St. Louis, Mo.) and used at a concentration of 10 μg/ml. Phytohemagglutinin (PHA) at 1 μg/ml was used as a positive mitogen control.

IFN-γ testing by ELISPOT and ELISA. Sterile ELISPOT 96-well microtiter plates (Millipore, Inc., Billerica, Mass.) were precoated at 4°C overnight with 5 μg of human anti-IFN-γ monoclonal antibodies (Endogen M-700A)/ml. The plates were washed with sterile phosphate-buffered saline (PBS) and blocked with 10% heat-inactivated fetal bovine serum in PBS. Isolated PBMC were plated at 0.5×10^6 cells in duplicate wells, and PHA and LSA-1 and TRAP peptides were added. PBS alone was used as the negative control and represented the background level of IFN-γ produced by nonstimulated cells. The cells were incubated at 37°C in 5% CO_2 for 5.5 days (~132 h). Plates were washed, and a second biotinylated anti-IFN-γ monoclonal antibody (Endogen M-701B) was applied (0.75 μg/ml) for 1.5 h at 37°C. This treatment was followed by washing, incubation with a 1:2,000 dilution of streptavidin-conjugated horseradish peroxidase (DAKO P0397) for 2 h at room temperature, washing, and color development by addition of 1% 3-amino-9-ethyl-carbazole in 0.1 M acetate buffer catalyzed by 0.015% hydrogen peroxide. The reaction was stopped after 10 to 20 min by washing with water. Plates were dried in the dark at room temperature. The number of spot-forming units (SFU) per well was counted by using ImmunoSpot scanning and imaging software (Pharmingen). Results are expressed as numbers of SFU per 10^6 PBMC. Responses to malaria peptides were not detected for PBMC from 18 malaria-nonexposed healthy adults. A pool of Epstein-Barr virus peptides covering multiple HLA types known to be present in both geographic areas was used as a positive 9-mer peptide control, and both malaria-exposed and malaria-naïve adults responded to it (data not shown). A 132-h incubation time was chosen because earlier experiments showed optimal peptide-specific responses in samples from the lowland population incubated for this amount of time compared to responses at 16 or 72 h (data not shown). Overnight (16-h) incubation produced no IFN-γ responses in cells from 30 adults from the area of low levels of transmission.

An ELISPOT to determine IFN-γ levels was considered positive if the frequency of SFU in the stimulated well was significantly greater than that in the unstimulated background well ($P < 0.05$). The significance test was a chi-square comparison of two proportions, adjusted for small sample size and assuming Poisson distribution.

For ELISA IFN-γ measurements, PBMC were plated at a final concentration of 10^6 PBMC/ml in 96-well (200 μl/well) U-bottom microtiter plates (Microtest; Becton Dickinson). Cell culture supernatants were removed after ~120 h and tested for the presence of IFN-γ by ELISA as previously described (5). Values for baseline (unstimulated) culture supernatants were subtracted from those for peptide- or mitogen-stimulated culture supernatants. The cutoff value for a positive IFN-γ response by ELISA was defined as greater than the mean plus 2 standard deviations of the values from 18 North American control subjects. If no response greater than that of unstimulated PBMC for a specific peptide was observed, a cutoff value of 20 pg/ml was used. Cutoff values for the peptides were the following: for T3, 20 pg/ml; for ls84, 85 pg/ml; for ls94, 43 pg/ml; for ls1881, 51 pg/ml; and for tr539, 20 pg/ml. Responses to tr51 were not evaluated by ELISA.

Hemoglobin level and hemoglobin S. Hemoglobin levels were assessed by beta-hemoglobin photometry (Hemocue Corp., Lake Forest, Calif.). Results were expressed as grams of hemoglobin per deciliter of blood. The hemoglobin S mutation was detected by PCR amplification followed by restriction enzyme Bsu361 digestion as previously described (19).

Microscopic detection of blood-stage malaria. Malaria infection was diagnosed by microscopic inspection of thick and thin blood smears. Blood smears were stained with Giemsa stain, and slides were examined by two experienced microscopists. The microscopists were blind to the study protocol and read each slide twice. A smear was deemed negative when no parasites were observed after counting microscopic fields that included at least 200 leukocytes. Density of parasitemia was expressed as the number of asexual *P. falciparum* organisms per microliter of blood, assuming a leukocyte count of 8,000 per μl.

Active surveillance for clinical malaria. Children and adults who contributed PBMC for evaluation of IFN-γ responses were asked to participate in a clinical follow-up study. Those who consented were monitored weekly over a 36-week period for development of clinical malaria, defined as the presence of self-reported fever, chills, headache, or severe malaise, as well as *P. falciparum* infection on a blood smear. A second endpoint, clinical malaria with high-density parasitemia, was defined as the presence of malaria symptoms and a *P. falciparum* density of ≥4,000 parasites/μl. This density was previously found to have the best overall sensitivity and specificity for measured fever in a large epidemiologic study of malaria morbidity in children aged 1 to 14 years in this area of western Kenya (1). Individuals in the active-surveillance cohort contacted the clinical officer if they had the symptoms listed above and were treated with sulfadoxine-pyrimethamine unless they were allergic to this medication, in which case they were given amodiaquine or quinine. At the end of clinical surveillance, hemoglobin testing was repeated. Only children from the lowland stable-transmission area had sufficient episodes of clinical malaria and anemia to allow for comparison of these episodes to IFN-γ responses.

Statistical analysis. Individuals who had IFN-γ responses to PHA, which included all individuals tested by ELISPOT and all but two individuals tested by ELISA, were assessed for peptide-induced IFN-γ responses. Differences in the frequencies of positive cytokine responders were compared by using the χ^2 test. Quantitative differences in the levels of cytokine production were evaluated by a *t* test of log-transformed values. For the purposes of log transformation, cytokine values of 0 were set at 1 pg/ml. Similar *P* values were obtained when cytokine levels were compared by using the nonparametric Mann-Whitney U test. Geometric mean levels rather than median levels are presented because the median cytokine level for all peptides was 0 (since the percentage of responders to individual peptides was always <50%). The correlation between levels of IFN-γ measured by ELISPOT and ELISA was assessed by the Spearman rank test. Frequencies of anemia and prevalences of clinical malaria in children with or

TABLE 1. ELISPOT IFN-γ responses to LSA-1 and TRAP peptides in adults and children in areas of stable and unstable transmission

Subjects (age [yr])	Peptide(s)	Area of stable transmission		Area of unstable transmission	
		No. positive/ total no. tested (%)	Geometric mean SFU (range)	No. positive/ total no. tested (%)	Geometric mean SFU (range)
Adults (≥15)	LSA-1 T3	24/86 (27.9)	7.8 (0–102)	29/87 (33.3)	7.8 (0–306)
	LSA-1 9-mer	32/86 (37.2)	11.6 (0–204)	40/87 (46.0)	12.3 (0–340)
	Any LSA-1	38/86 (44.2)	17.0 (0–204)	46/87 (52.9)	15.3 (0–340)
	Any TRAP	21/83 (25.3)	9.0 (0–177)	20/82 (24.4)	7.7 (0–204)
Children (≤8)	LSA-1 T3	15/57 (26.3)	5.8 (0–66)	3/33 (8.3)[a]	3.8 (0–52)[b]
	LSA-1 9-mer	21/56 (37.5)	7.6 (0–246)	10/36 (27.8)	11.0 (0–180)
	Any LSA-1	23/57 (40.3)	10.1 (0–245)	10/36 (27.8)	12.0 (0–180)
	Any TRAP	11/46 (23.9)	5.1 (0–243)	10/31 (32.3)	5.6 (0–87)

[a] $P = 0.05$ in a χ^2 analysis comparing frequencies of positive responses in areas of stable and unstable transmission.
[b] $P = 0.03$ in a t test comparing log-transformed IFN-γ levels in areas of stable and unstable transmission.

without IFN-γ responses to malaria peptides were compared by using the χ^2 test. Logistic regression was used to estimate the risk of anemia and clinical malaria after adjusting for age, initial infection, and the presence of hemoglobin S.

RESULTS

IFN-γ responses measured by ELISPOT. IFN-γ responses to LSA-1 and TRAP peptides in 86 adults and 57 children from the lowland stable-transmission area and in 87 adults and 36 children from the highland unstable-transmission area were assessed by ELISPOT. Background ELISPOT IFN-γ responses in both areas were similar, with geometric means of 26.4 and 11.8 SFU per 10^6 PBMC in the stable-transmission area among adults and children, respectively, and geometric means of 34.6 and 16.6 SFU per 10^6 PBMC in the unstable-transmission area among adults and children, respectively. For adults in both areas, proportions of responses to the LSA-1 and TRAP peptides ranged from 24.4 to 52.9%, and geometric mean numbers above background of SFU to the LSA-1 and TRAP peptides ranged from 7.8 to 28.3 SFU per 10^6 PBMC (Table 1). There was no difference according to area of residence. For children, there were also no significant differences in the proportions of those with responses to the 9-mer LSA-1 or TRAP peptides (frequencies ranged from 23.9 to 37.5%). In contrast, the frequency and level of IFN-γ responses to the LSA-1 T3 peptide were lower for children living in the unstable-transmission area than for children from the stable-transmission site (frequency, 8.3% versus 26.3%, $P = 0.05$; geometric mean number of SFU per 10^6 PBMC, 3.8 versus 5.8, $P = 0.03$) (Table 1). HLA class I alleles were similarly distributed in highland and lowland populations (4), and IFN-γ responses detected by ELISPOT or ELISA to the 9-mer LSA-1 and TRAP peptides were not HLA class I restricted (A. M. Moormann, unpublished data). IFN-γ ELISPOT results did not correlate with the presence or level of parasitemia for adults or children from either study site (data not shown).

IFN-γ responses measured by ELISA. IFN-γ responses to LSA-1 and TRAP peptides in 60 adults and 43 children from the stable-transmission area and 66 adults and 24 children from the unstable-transmission area were assessed by ELISA. Frequencies and levels of IFN-γ responses to LSA-1 and TRAP peptides were similar for adults from both areas. In contrast, the frequencies and levels of IFN-γ responses to one or more of the LSA-1 peptides were lower for children in the unstable-transmission area (frequency, 25.0 versus 58.1%, $P = 0.05$; geometric mean level, 11 versus 30 pg/ml, $P = 0.04$) (Table 2). IFN-γ ELISA results did not correlate with the presence or level of parasitemia for adults or children from either study site (data not shown).

Comparison of IFN-γ responses measured by ELISPOT and ELISA. The decision to categorize an individual as a responder

TABLE 2. ELISA IFN-γ responses to LSA-1 and TRAP peptides in adults and children in areas of stable and unstable transmission

Subjects (age [yr])	Peptide(s)	Area of stable transmission		Area of unstable transmission	
		No. positive/ total no. tested (%)	Geometric mean (pg/ml) (range)	No. positive/ total no. tested (%)	Geometric mean (pg/ml) (range)
Adults (≥15)	LSA-1 T3	29/60 (48.3)	18 (0–3,874)	27/66 (40.9)	15 (0–5,362)
	LSA-1 9-mer	30/60 (50.0)	27 (0–4,115)	26/62 (41.9)	19 (0–3,598)
	Any LSA-1	39/60 (65.0)	60 (0–4,115)	33/66 (50.0)	36 (0–5,362)
	TRAP tr539	20/55 (36.4)	7 (0–1,429)	17/45 (37.8)	10 (0–4,783)
Children (≤8)	LSA-1 T3	18/43 (41.9)	9 (0–2,209)	6/24 (25.0)	6 (0–6,480)
	LSA-1 9-mer	19/38 (50.0)	20 (0–1,996)	5/22 (22.7)[a]	6 (0–2,714)
	Any LSA-1	25/43 (58.1)	30 (0–2,209)	8/24 (25.0)[a]	11 (0–6,480)[b]
	TRAP tr539	7/31 (22.6)	3 (0–1,930)	0/9 (0.0)	2 (0–19)

[a] $P \leq 0.05$ in a χ^2 analysis of frequencies of positive responses in areas of stable and unstable transmission.
[b] $P = 0.04$ in a t test of log-transformed IFN-γ levels in areas of stable and unstable transmission.

TABLE 3. ELISPOT IFN-γ responses to LSA-1 peptides and clinical outcomes for children in an area of stable, high transmission[a]

Clinical outcome[b]	LSA-1 T3 peptide		LSA-1 9-mer peptides		Any LSA-1 peptide	
	% Responder (n = 13)	% Nonresponder (n = 36)	% Responder (n = 18)	% Nonresponder (n = 30)	% Responder (n = 20)	% Nonresponder (n = 29)
Clinical malaria	69.2	50.0	61.1	50.0	65.0	48.3
Clinical malaria with high-density parasitemia	53.9	27.8	38.9	30.0	45.0	27.6
Anemia (at time of sample collection)	30.8	27.8	33.3	23.3	30.0	27.6
Anemia (at 36-week follow-up)	38.5	30.6	38.8	26.6	35.0	31.0

[a] P values for the frequencies of responders compared to those of nonresponders by χ^2 analysis were >0.05 for all conditions.
[b] High-density parasitemia is defined as >4,000 *P. falciparum* parasites/μl of blood. Anemia is defined as <11 g of hemoglobin/dl of blood.

differed according to whether ELISA or ELISPOT was used. For example, in the unstable-transmission area, where 56 adults were tested for IFN-γ responses to LSA-1 by both methods, 29 individuals were positive by ELISPOT and 31 individuals were positive by ELISA, but only 15 individuals were positive by both methods. Thus, approximately 50% of those who tested positive by ELISA did not test positive by ELISPOT and vice versa. There was also no correlation between the number of IFN-γ SFU measured by ELISPOT and IFN-γ cytokine levels in response to LSA-1 peptides measured by ELISA (Spearman's rho values, <0.01-0.11; all P values were >0.05), and there was a weak correlation between the strengths of ELISA and ELISPOT responses to TRAP peptide tr539 (Spearman's rho = 0.21; P = 0.017).

The proportion of individuals with malaria peptide-driven IFN-γ responses to one or more LSA-1 peptide was higher when measured by ELISA than by ELISPOT for both adults and children in the stable-transmission area (for adults, 65.0 versus 44.2% [P = 0.013] for ELISA and ELISPOT, respectively; for children, 58.1 versus 40.3% [P = 0.08] for ELISA and ELISPOT, respectively). In this area, the proportion of individuals with IFN-γ responses was higher when tested by ELISA than by ELISPOT even if we used a less stringent definition of a positive response, e.g., >5 SFU per 10^6 PBMC. Proportions of IFN-γ responses determined by ELISA and ELISPOT were nearly the same for both age categories for residents of the unstable-transmission area (for adults, the value obtained by ELISA was 50.0% versus an ELISPOT value of 52.9%, with a P value that was not significant; for children, the value obtained by ELISA was 25.0% versus an ELISPOT value of 27.8%, with a P value that was not significant) (Tables 1 and 2).

ELISPOT and ELISA IFN-γ responses to LSA-1 and TRAP peptides and their relationship with clinical malaria in children in the stable-transmission area. Fifty-six children in the area of stable transmission were monitored for the 36 weeks following IFN-γ testing for the development of clinical malaria, as defined in Materials and Methods. Forty-nine of these children's IFN-γ responses to LSA-1 and TRAP peptides were assessed by ELISPOT, the responses of 38 children were assessed by ELISA, and the responses of 31 children were assessed by both methods. IFN-γ production in response to individual malaria peptides and LSA-1 or TRAP peptides in aggregate was assessed by ELISPOT or ELISA for correlation with the following conditions: (i) anemia (<11 g of hemoglobin/dl of blood) at the time of IFN-γ testing; (ii) anemia 36 weeks after IFN-γ testing; (iii) clinical malaria during the 36-week period of observation; and (iv) clinical malaria with high-density parasitemia (≥4,000 parasites/μl) during the 36-week period of observation.

ELISPOT IFN-γ responses to LSA-1 peptides did not correlate with any of the four outcomes (Table 3). These data contrasted with the correlations found for responses measured by ELISA (Table 4). Children with ELISA IFN-γ responses to the LSA-1 T3 peptide were significantly less likely to develop clinical malaria (P = 0.005) or clinical malaria with high-density parasitemia (P = 0.004) than those without such responses. In addition, children with responses to one or more of the LSA-1 peptides were less likely than those without such responses to be anemic during follow-up (P = 0.005). After adjustment for (i) age, (ii) the presence of blood-stage infection at the time PBMC were collected, and (iii) hemoglobin S levels, individuals with IFN-γ responses to LSA-1 T3 remained less likely to develop clinical malaria (odds ratio [OR], 0.15;

TABLE 4. ELISA IFN-γ responses to LSA-1 peptides and clinical outcomes for children in an area of stable, high transmission

Clinical outcome[a]	LSA-1 T3 peptide		LSA-1 9-mer peptides		Any LSA-1 peptide	
	% Responder (n = 16)	% Nonresponder (n = 22)	% Responder (n = 16)	% Nonresponder (n = 17)	% Responder (n = 22)	% Nonresponder (n = 16)
Clinical malaria	37.5	81.8[b]	56.2	64.7	50.0	81.3
Clinical malaria with high-density parasitemia	12.5	59.1[c]	37.5	29.4	27.3	56.3
Anemia (at time of sample collection)	25.0	45.5	29.3	43.7	31.8	43.8
Anemia (at 36-week follow-up)	12.5	36.4	12.5	35.3	9.1	50.0[b]

[a] High-density parasitemia is defined as >4,000 *P. falciparum* parasites/μl of blood. Anemia is defined as <11 g of hemoglobin/dl of blood.
[b] The P value for the frequency of responders compared to that of nonresponders, by χ^2 analysis, was 0.005.
[c] The P value for the frequency of responders compared to that of nonresponders, by χ^2 analysis, was 0.004.

TABLE 5. ELISA IFN-γ responses to LSA-1 peptides and risk of adverse clinical outcome, adjusted for age, hemoglobin S level, and prior infection[a]

Clinical outcome[b]	LSA-1 T3 peptide		LSA-1 9-mer peptides		Any LSA-1 peptide	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Clinical malaria	0.15 (0.03–0.77)	0.02	0.56 (0.11–2.86)	NS	0.23 (0.08–1.25)	NS
Clinical malaria with high-density parasitemia	0.10 (0.01–0.68)	0.02	1.37 (0.28–6.71)	NS	0.30 (0.06–1.40)	NS
Anemia (at time of sample collection)	0.54 (0.08–3.71)	NS	1.62 (0.22–11.72)	NS	0.51 (0.07–3.84)	NS
Anemia (at 36-week follow-up)	0.49 (0.07–3.57)	NS	0.13 (0.01–1.36)	NS	0.08 (0.01–0.77)	0.03

[a] All ORs and *P* values were calculated by logistic regression analysis. NS, not significant.
[b] High-density parasitemia is defined as >4,000 *P. falciparum* parasites/μl of blood. Anemia is defined as <11 g of hemoglobin/dl of blood.

95% confidence interval [CI], 0.03 to 0.77) or clinical malaria with high-density parasitemia (OR, 0.10; 95% CI, 0.01 to 0.68). Moreover, children with IFN-γ responses to one or more LSA-1 peptide remained less likely to be anemic (OR, 0.08; 95% CI, 0.01 to 0.77) than those without these responses (Table 5). Hemoglobin levels were significantly higher in individuals who responded to one or more LSA-1 peptides than in individuals who did not respond (12.1 versus 11.0 g/dl; $P = 0.03$). ELISA- and ELISPOT-determined IFN-γ responses to the TRAP peptides were too infrequent to allow for comparison of clinical outcomes in individuals with and without these responses.

DISCUSSION

IFN-γ responses to preerythrocytic malaria antigenic peptides have been shown with murine models to mediate directly or indirectly (through nitric oxide production) resistance to liver-stage parasites induced by immunization with radiation-attenuated sporozoites (7). These responses may, by inference, also be important in naïve adult volunteers protected against *P. falciparum* sporozoite challenge by prior exposure to the bites of irradiated infective mosquitoes (18). Given the progress of the development and testing of malaria vaccines directed against preerythrocytic stages of *P. falciparum* (2, 16, 33, 34, 40–44), a marker such as T-cell IFN-γ responsiveness would be a valuable surrogate to evaluate vaccine immunogenicity and efficacy in populations living in areas where malaria is endemic. This issue has been addressed in field-based studies conducted in several areas of the world where malaria is endemic, including sub-Saharan Africa, Papua New Guinea, and Latin America. These reports in general show that: (i) peptide-stimulated IFN-γ may be produced by both $CD4^+$ and $CD8^+$ T cells (3, 6, 11, 12, 20); (ii) responses to LSA-1 and TRAP peptides, as measured by various methods and with various numbers of peptides, are demonstrable in ~10 to 40% of individuals, with studies of LSA-1 (3, 6, 20) but not TRAP (11) demonstrating an age-related increase in the rate of responses; (iii) the instability in ELISPOT IFN-γ responses exists at the individual level (11); and (iv) an inconsistent relationship exists between these responses and parasitologic and clinical endpoints such as time to reinfection and malaria morbidity (11, 20, 25, 27, 35).

In an attempt to define more clearly the technical and epidemiological variables that determine IFN-γ responses in populations from areas where malaria is endemic, we evaluated LSA-1- and TRAP-driven responses in children and adults in two areas of western Kenya that differ markedly in transmission conditions. One is a highland area of Nandi District where transmission is episodic and outbreaks of severe malaria occasionally appear (14, 28), and the other is a lowland area of Kisumu District where transmission is stable and uncomplicated and where severe malaria predominates during infancy (39). The present study addresses how age, area of residence, and the method of detecting IFN-γ influence LSA-1- and TRAP-driven responses. The study findings suggest that IFN-γ responses to LSA-1 and TRAP develop in response to age and/or repeated exposure, as would be experienced by adults from either study site and by the children living in the area with perennial malaria transmission. A further novel finding is that IFN-γ responses to LSA-1 as measured by ELISA but not ELISPOT correlate with protection against uncomplicated malaria morbidity.

Frequencies of IFN-γ responses to LSA-1 and TRAP (by ELISA and ELISPOT) were similar in adults in the areas of unstable and stable transmission. In contrast, frequencies of IFN-γ responses to the LSA-1 T3 peptide (by ELISPOT) and to any LSA-1 peptide (by ELISA) were significantly lower in children from the area of unstable transmission than in children from the area of stable transmission. We previously documented that children under 2 years of age in this area of stable transmission have low frequencies of IFN-γ responses to LSA-1, despite frequent malaria exposure (5). Taken together, these findings suggest that the development of IFN-γ responses to LSA-1 and TRAP requires repeated, prolonged exposure in children but may require much less intense exposure in adults. Thus, both age and transmission intensity appear to play roles in the development of IFN-γ responses to LSA-1.

ELISPOT of IFN-γ responses to HLA class I-restricted cytotoxic T lymphocyte epitopes has been proposed as a less cumbersome way of identifying putatively protective $CD8^+$ cytotoxic T lymphocytes than the traditional chromium-51 release assay (30). Numerous recent animal (24, 38) and human (10, 11, 13, 37, 44) studies have used ELISPOT testing for IFN-γ as their primary measure of immunity. However, in human populations it has not been established whether the level of IFN-γ production (as measured by ELISA) or the number of mononuclear cells producing IFN-γ (as measured by ELISPOT) is a better correlate of protection against infection and disease. In the present study, frequencies of IFN-γ responses to LSA-1 were greater by ELISA than by ELISPOT in the lowland, stable-transmission area. The PBMC incubation period for IFN-γ testing by ELISA and ELISPOT used in

the present study was similar to that used in prior studies documenting an association of protection from infection or disease with ELISA IFN-γ responses to preerythrocytic antigens (27, 35) but longer than that used in studies assessing ex vivo ELISPOT IFN-γ responses to TRAP and CSP (11, 37). The longer incubation period was used for ELISPOT because ELISPOT IFN-γ responses were not found in this study population after the shorter 16-h incubation period. The frequencies of ELISPOT IFN-γ responses to the two TRAP peptides described here are similar to those reported by other investigators (11), so it is unlikely that the lower frequencies of IFN-γ responses to LSA-1 peptides as detected by ELISPOT than by ELISA are the result of technical differences in laboratory testing. It is also unlikely that ELISA IFN-γ responses to LSA-1 were nonspecific, given the significantly decreased frequency of these responses to LSA-1 in children in the unstable-transmission area and the lack of high-level responses in malaria-naïve subjects. The study findings therefore suggest that ELISA is a more sensitive assay than ELISPOT for the assessment of IFN-γ responses in areas of stable malaria transmission. The nature of these assays requires a different definition of a positive response for each assay, as ELISA measures a concentration and ELISPOT measures a proportion, but results did not differ even if a comparison to results for malaria-naïve controls was used as a positive ELISPOT response (e.g., >5 SFU per 10^6 PBMC above background).

The fairly low frequencies and levels of ELISPOT IFN-γ responses to individual TRAP and LSA-1 peptides observed in the present study, and to TRAP (10, 11) and CSP peptides (37) in other studies, suggest that in naturally exposed populations, preerythrocytic-stage malaria peptides elicit relatively low frequencies of IFN-γ-secreting cells. However, these cells may produce large amounts of IFN-γ, as measured by ELISA. The lack of correlation between IFN-γ ELISA and ELISPOT results in the present study is consistent with this notion, and the decreased risk of clinical malaria and anemia associated with LSA-1-induced IFN-γ responses as measured by ELISA but not by ELISPOT supports the premise that the amount of IFN-γ produced is an important correlate of protective immunity against preerythrocytic stages of *P. falciparum*. Indeed, to date all studies that have documented an association between IFN-γ responses to preerythrocytic antigens and protection against infection (6, 27) or disease (29, 35) in populations from areas where malaria is endemic have measured IFN-γ production by ELISA. Strong ELISPOT IFN-γ responses to malarial antigens have been documented in malaria-naïve volunteers given preerythrocytic *P. falciparum* DNA vaccines or RTS,S (42, 44), but the findings presented here suggest that for vaccine studies of naturally exposed populations, IFN-γ responses should initially be assessed by both methods.

A recent study by Reece and colleagues of adults from an area of The Gambia where malaria is endemic, some of whom were vaccinated with the RTS,S vaccine, adds further fuel to the debate on optimal methods of testing IFN-γ responses in malaria-exposed populations. In that study, IFN-γ responses to CSP peptide 22 assessed by culture but not by ex vivo ELISPOT correlated strongly with protection from both infection and disease due to *P. falciparum* (36). The ELISAs and ELISPOTs performed in the present study, in which cells were incubated for 5 days, are essentially a cross between the traditional ex vivo assays and the longer 14-day cultured ELISPOT employed by Reece et al. At 5 days, the cells tested may be a mixture of central and effector memory cells. As noted in Materials and Methods, initial testing by the ex vivo method yielded no IFN-γ responses to the selected malaria peptides in 30 individuals, whereas a longer incubation time of 5 days resulted in the detection of responses. After 10 days of incubation, IFN-γ responses detected by ELISPOT to malaria peptides were seen in malaria-unexposed volunteers, though the levels of response were lower than those found by Reece et al. (unpublished data). Reece et al. do not describe whether responses to peptides with a 14-day incubation were tested for malaria-unexposed volunteers. Other differences between the two studies include the presence of CSP-vaccinated individuals, who had stronger ELISPOT IFN-γ responses, and the assessment of responses to only 15-mer CSP peptides, which were primarily $CD4^+$-T-cell mediated, in the study by Reece et al. The present study tested both 9-mer and longer LSA-1 and TRAP peptides and therefore likely detected both $CD8^+$- and $CD4^+$-T-cell-mediated responses. Reece et al. did not test IFN-γ responses to CSP by ELISA. The comparison of IFN-γ responses to CSP as measured by ELISA to ELISPOT IFN-γ responses in this malaria-exposed population, particularly in vaccinated individuals, would be of great interest.

IFN-γ responses to LSA-1 are thought to mediate protection against infection by elimination of developing parasites within infected hepatocytes through induction of the nitric oxide pathway (17). The protection against clinical malaria associated with IFN-γ responses to LSA-1 may thus operate through relative protection against high-density parasitemia; i.e., in semi-immune populations, the reduction in the development of liver-stage parasites and release of a smaller parasite inoculum from the liver may lead to blood-stage infection that is more efficiently eliminated or controlled, thereby limiting the risk of high-density parasitemia and morbidity. This possibility is consistent with the observation reported here that IFN-γ responses to LSA-1 were protective against high-density parasitemia and might explain why IFN-γ responses to LSA-1 were not associated with protection against infection in other studies that compared cytokine responses to the presence of any level of infection (20, 25). Protection against anemia may relate to decreased hemolysis and bone marrow suppression associated with episodes of clinical malaria (22, 26) or to IFN-γ-mediated upregulation of interleukin 12 production, which has been shown to increase bone marrow and splenic erythropoiesis in murine malaria (31, 32). Multiple factors likely contributed to the anemia observed 36 weeks after IFN-γ testing in the children in this study. However, there are several reasons to suggest that the association between ELISA IFN-γ responses to LSA-1 and protection against anemia is not a chance association or a marker of other effects. These reasons include the strength of the association, the similar trend for a protective association observed for LSA-1 T3 and 9-mer peptides, the persistence of a significant association after control for potential confounding factors, and the lack of an association between ELISPOT IFN-γ responses and protection against anemia. Cell depletion studies were not performed during this sample collection. Previous studies by our group in Papua New Guinea (4, 7) and highland Kenya (21) suggest that $CD8^+$ cells are the primary source of the IFN-γ induced by

both the 9-mer and T3 peptides, but the possibility of a significant contribution by $CD4^+$ cells in the present study populations cannot be excluded. The association of IFN-γ responses to the single T3 peptide with protection from malaria disease supports the idea that the peptide encodes a clinically important T-cell epitope of LSA-1.

Further study is required to determine whether IFN-γ production in response to these LSA-1 peptides is $CD4^+$- or $CD8^+$-T-cell dependent, to assess the role of other cytokines in the suppression or promotion of IFN-γ responses in populations in which malaria is endemic, and to determine if there is a threshold age at which these responses can be acquired without repeated, frequent malaria exposure. In addition, vaccine studies are necessary to determine whether strong IFN-γ responses to LSA-1 can be induced in children under 2 years of age in areas of stable, high transmission. The findings of the present study argue that LSA-1-induced IFN-γ responses should be assessed by ELISA as well as ELISPOT in future LSA-1 vaccine studies in areas where malaria is endemic.

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