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Interferon-gamma responses to *Plasmodium falciparum* liver-stage antigen-1 and merozoite-surface protein-1 increase with age in children in a malaria holoendemic area of western Kenya

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Abstract

Background: In areas of high-level, year-round malaria transmission, morbidity and mortality due to malaria decrease after the first two to three years of life. This reduction may be related to the development of cellular immunity to specific antigens expressed in the different life-cycle stages of *Plasmodium falciparum*.

Methods: A cross sectional study was conducted to evaluate T cell cytokine responses to the *P. falciparum* pre-erythrocytic antigen liver-stage antigen-1 (LSA-1) and the blood-stage antigen merozoite-surface protein-1 (MSP-1) in children under five years of age residing in a malaria holoendemic region of western Kenya. Interferon-γ (IFN-γ) and interleukin-10 (IL-10) responses to the LSA-1 T3 peptide (aa 1813–1835) and the MSP-1 aa20–39 peptide were tested in 48 children.

Results: The proportion of children producing IFN-γ to LSA-1 and to MSP-1 increased with age: in the 0–12, 13–24, 25–36 and 37–48 month age groups, zero, 11.1, 36.4 and 40% of children had IFN-γ responses to LSA-1 (p = 0.019), and 10, 10, 27.7 and 40% of children had IFN-γ responses to MSP-1 (p = 0.07), respectively. In contrast, the proportion of children producing IL-10 to LSA-1 and MSP-1 was similar in all age groups.

Conclusion: The data suggest that development of IFN-γ responses to LSA-1 and MSP-1 requires increased age and/or repeated exposure, whereas IL-10 responses to these antigens may occur at any age and with limited exposure. The data also demonstrate that by the age of 4 years, children in a malaria holoendemic area develop frequencies of IFN-γ responses to LSA-1 and MSP-1 similar to those seen in adults in the area.

Background

In malaria holoendemic areas, where malaria is stable and intense throughout the year, morbidity and mortality from *Plasmodium falciparum*malaria occur primarily in children aged 6–24 months and decrease significantly after 24 months of age [1]. Age-related protection from *P.*

falciparum infection and disease in these areas is thought to be related to acquisition of cellular and humoral immunity to pre-erythrocytic and blood-stage *P. falciparum* antigens [2].

T lymphocyte cytokine production to *P. falciparum* pre-erythrocytic and blood-stage antigens, particularly IFN-γ and IL-10 production, appears to be important in induction and maintenance of immunity to *P. falciparum* in naturally exposed populations [2]. The pre-erythrocytic antigen liver-stage antigen-1 (LSA-1) is recognized by cytotoxic T lymphocytes (CTL) in animal models and humans naturally exposed to malaria [3,4]. *In vitro* studies in areas of stable and unstable malaria transmission have shown that LSA-1 evokes strong IFN-γ and IL-10 responses, and these responses correlate with protection from infection [5–9]. Development of cellular immunity to blood-stage antigen merozoite-surface protein-1 (MSP-1) in experimental and human malaria involves mainly $CD4^+$ T cell IFN-γ responses [2,10–12]. In some rodent malaria models, TH1 cells producing IFN-γ and IL-2 are important for controlling infection in its early phases, while TH2 cells producing IL-4 and IL-10, together with antibodies, are important for parasite clearance in the later phases of infection [2].

Our prior studies and those of other groups have documented that IFN-γ and IL-10 responses to LSA-1 are present in older children (>5 years) and adults in malaria endemic areas [5–8]. However, the frequency of these responses in children aged 0–4 years is not well described. An understanding of the development of IFN-γ and IL-10 responses to LSA-1 and MSP-1 in children with natural exposure to malaria from birth to 4 years of age is important because this age group is the target group for malaria vaccine development.

Studies of T cell cytokine responses in young children have been hampered by the difficulty in obtaining venipuncture blood samples from these children and in obtaining adequate numbers of mononuclear cells for testing of responses. Finger-prick blood sampling was successfully used in this study with lower cell concentrations to conduct a cross-sectional study of IFN-γ and IL-10 responses to LSA-1 and MSP-1 in children aged 1–48 months in a malaria holoendemic area of western Kenya.

Methods

Study population

Blood samples for cytokine testing were collected from a total of 48 children aged 1–48 months in the location of Kanyawegi, western Kenya, an area of intense, perennial malaria transmission. Written informed consent was obtained from the parents or guardians of all children who participated in the study. 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, Cleveland, OH.

Blood collection and transportation

Approximately 0.5–1 ml volume of blood was collected from each individual by finger-prick method. The finger to be pricked was cleaned with 70% alcohol and pricked using a sterile lancet. The first drop of blood was wiped using dry gauze and the finger was then squeezed gently to get 0.5–1 ml of blood in a microtainer containing the anticoagulant EDTA (BD Microtainer™, Becton Dickson, Franklin Lakes, USA). Blood samples were then transported to the Case Western Reserve University laboratory at the Center for Vector Biology and Control Research, KEMRI, Kisian, Kenya.

Microscopy

Thick and thin blood smears were stained using 5% Giemsa solution and examined for *Plasmodium* species by two microscopists. Parasite density/μl of blood was calculated by counting the number of parasites per 200 white blood cells and multiplying by 40, assuming an average white blood cell count of 8000/μl.

Isolation of peripheral blood mononuclear cells (PBMC) and cell culture

Peripheral blood mononuclear cells (PBMC) were separated from the whole blood by Ficoll-Hypaque density gradient centrifugation. The final pellet was suspended in 0.5 ml of RPMI 1640 medium (Gibco, Invitrogen, Paisley, Scotland, UK) supplemented with 5% heat inactivated human AB serum, 50 mg/ml gentamicin, 10 Mm Hepes and 2 mM glutamine. Cells were plated in duplicate at a final concentration of 1×10^6 cells/ml, 200 μl/well, on 96 well U-bottom microtiter plates (Microtest™, Becton Dickson). Cell culture supernatants were removed after 72 hours and tested for the presence of IFN-γ and IL-10.

Cytokine enzyme linked immunosorbent assay (ELISA)

Supernatant testing for IFN-γ and IL-10 was performed using two-site ELISA. 96 well ELISA microtiter plates (Immulon^R 4 HBX, Thermo Labsysytems, East Forge Parkway, Franklin, USA) were coated with 50 μl/well of primary antibody and incubated at 4 °C overnight. The plates were washed twice using 1x PBS with 0.05% Tween 20 (PBS-T), blocked with 50 μl /well of 3% BSA and incubated at 37 °C for 1 hour. Plates were then washed twice, and samples, standards and blanks added at 50 μl /well. Plates were incubated at 37 °C for 2 hours, washed twice and 50 μl/well of biotinylated secondary antibody was added. Plates were incubated at room temperature for 45 minutes, washed twice and 50 μl/well of streptavidin

alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA) at a 1:2000 dilution was added. Plates were incubated for 30 minutes at room temperature and washed six times. 50 μl /well of alkaline phosphatase substrate (Sigma Diagnostics, Inc. St. Louis, MO) was added. Optical density (O.D.) of the standards and samples were read at 405 nm when the highest standard reached an O.D. between 1.0 and 1.4 nm. Sample O.D. values were compared to standards with known concentrations of IFN-γ and IL-10 and sample concentrations extrapolated from a standard curve. Values of baseline (unstimulated) culture supernatants were subtracted from those of peptide/mitogen-stimulated culture supernatants.

Antigens and mitogens for cytokine tests

The frequencies and levels of cytokine responses to LSA-1 and MSP-1 were tested using the previously described MSP-1 peptide aa20 to 39, amino acid (aa) sequence VTHESYQELVKKLEALEDAV [11] LSA-1 peptide T3 (aa 1813 to 1835), aa sequence NENLDDLDEGIEKSSEELSEEKI [5]. These peptides have been demonstrated to elicit T cell cytokine responses from individuals in malaria endemic populations [5,9,11]. Peptides were synthesized and purified by high-performance liquid chromatography (HPLC) to > 95% purity (Sigma Genosys, St. Louis, MO) and used at a concentration of 10 μg/ml. Phytohaemagglutinin (PHA) at 5 μg/ml and phorbol-12-meristate-13-acetate plus Ionomycin (PMA-I) at 20 ng/ml and 1 μg/ml, respectively, were used as positive mitogen controls. PHA and PMA-I responses were similar. Only PHA responses are presented in this paper.

Statistical Analysis

Only individuals with cytokine concentrations of ≥ 20 pg/ml to PHA or PMA-I were analysed for peptide-induced cytokine responses. Individuals with peptide-induced cytokine concentrations of ≥ 20 pg/ml were considered positive responders for that peptide while those with cytokine concentrations ≤ 20 pg/ml were considered non-responders to that peptide. Frequencies of cytokine responses were compared across age groups (0–12, 13–24, 25–36 and 37–48 months old) by chi-square analysis for trend. Log-transformed cytokine levels were compared across age groups by analysis of variance (ANOVA). For the purpose of log transformation, all stimulated supernatants with no production of cytokine above media were given a value of 1 pg/μl. Stata 7.0 (Stata Corporation, Texas, USA) was used for all statistical analysis.

Results

Frequency and density of P. falciparum *parasitaemia across age groups*

Frequency and density of parasitemia did not differ significantly across age groups (Table 1). Presence of parasitemia did not correlate with IFN-γ or IL-10 responses or levels to either LSA-1 or MSP-1 (Tables 2 and 3), and levels of parasitemia did not correlate with cytokine levels to any peptide (Spearman's rho 0.04 – 0.15, all p values >0.1).

Table 1: Frequencies and density of *P. falciparum* parasitemia, by age.

Age (months)	No. of positives/Total (%)	Geo. mean density, parasites/μl (range)[a]
0–12	6/11 (54.55)	3992.73 (0–29880)
13–24	11/12 (91.67)	10466.67 (0–38360)
25–36	10/13 (76.92)	5873.85 (0–29680)
37–48	9/12 (75.00)	10083.33 (0–47120)
P	NS[b]	NS[c]

[a] Geometric mean *P. falciparum* density in each age group. [b] NS = not significant. $P < 0.05$ considered significant, as determined by χ^2 test for homogeneity across age groups. [c] NS = not significant. $P < 0.05$ considered significant, as determined by analysis of variance (ANOVA) of log-transformed *P. falciparum* density across age groups.

Frequency and levels of IFN-g and IL-10 cytokines across age groups

Frequencies and levels of IFN-γ and IL-10 responses to PHA were similar across age groups (Tables 4 and 5). However, frequencies of IFN-γ responses to LSA-1 increased significantly with age ($P = 0.019$) and a similar trend was seen in frequencies of IFN-γ responses to MSP-1 ($P = 0.07$) (Table 4). Geometric mean cytokine levels of IFN-γ responses to LSA-1 and MSP-1 also increased with age, although these increases did not achieve statistical significance (Table 4). In contrast, frequencies and levels of IL-10 responses were similar across age groups (Table 5). IL-10 levels were generally much lower than IFN-γ levels.

Discussion

This study documents that IFN-γ responses to peptides encoding T cell epitopes for the pre-erythrocytic antigen LSA-1 and the blood-stage antigen MSP-1 are infrequent in the first year of life but increase with age through the age of 4 years in children in a malaria holoendemic area. By the age of 4 years, frequency and level of IFN-γ responses to the single LSA-1 peptide T3 exceeded those of adults in an area of highly seasonal malaria transmission [5], and approached those of adults in a malaria holoendemic area (John CC, unpublished data). Frequencies of IFN-γ responses to the MSP-1 peptide, aa 20–39, were also similar by the age of 4 years to those reported in other MSP-1 peptides in adults in malaria endemic areas [13,14]. In contrast, frequencies of IL-10 responses to LSA-1 and MSP-1 peptides did not differ with age, and levels and frequencies of IL-10 responses were much lower than IFN-γ responses. These findings suggest that age and/or

Table 2: Frequencies and geometric mean levels of IFN-γ responses to PHA, LSA-1 and MSP-1 peptides by presence of *P. falciparum* parasitemia

Condition	PHA		MSP-1 (aa 20–39)		LSA-1, T3	
Blood smear	No. pos./Tot. (%)[a]	Geo. mean (range)[b]	No. pos./Tot (%)	Geo. mean (range)	No. pos./Tot (%)	Geo. mean (range)
P. falciparum positive	36/36 (100)	2545.4 (164.8–6891.6)	7/32 (21.9)	145.8 (0–1245.9)	7/31 (22.6)	105.4 (0–254.3)
P. falciparum negative	12/12 (100)	1908 (231.1–4254.1)	2/9 (22.2)	28.4 (0–150.3)	2/8 (25.0)	39.0 (0–983.5)
P	NS[c]	NS[d]	NS	NS	NS	NS

[a] A positive response was defined as any response ≥ 20 pg/ml above that of unstimulated cell culture supernatants and had a positive or negative blood smear. [b] Geometric mean cytokine levels in pg/ml. [c] NS = not significant. $P < 0.05$ considered significant, as determined by χ^2 test for trend across age groups. [d] $P < 0.05$ was considered significant as determined by analysis of variance of log-transformed values across age groups.

Table 3: Frequencies and geometric mean levels of IL-10 responses to PHA, LSA-1 and MSP-1 peptides by presence of *P. falciparum* parasitemia

Condition	PHA		MSP-1 (aa 20–39)		LSA-1, T3	
Blood smear	No. pos./Tot. (%)[a]	Geo. mean (range)[b]	No. pos./Tot (%)	Geo. mean (range)	No. pos./Tot (%)	Geo. mean (range)
P. falciparum positive	25/36 (69.4)	175.5 (0–847.4)	5/32 (15.6)	23.7 (0–445.9)	2/31 (6.5)	6.9 (0–125.2)
P. falciparum negative	11/12 (91.7)	330.1 (8.4–1247.4)	2/9 (22.2)	14.7 (0–67.7)	1/8 (12.5)	16.6 (0–125.2)
P	NS[c]	NS[d]	NS	NS	NS	NS

[a] A positive response was defined as any response ≥ 20 pg/ml above that of unstimulated cell culture supernatants and had a positive or negative blood smear. [b] Geometric mean cytokine levels in pg/ml. [c] NS= not significant. $P < 0.05$ considered significant, as determined by χ^2 test for trend across age groups. [d] $P < 0.05$ was considered significant as determined by analysis of variance of log-transformed values across age groups.

Table 4: Frequencies and geometric mean levels of IFN-γ responses to PHA, LSA-1 and MSP-1 peptides by age.

Condition	PHA		MSP-1 (aa 20–39)		LSA-1, T3	
Age (months)	No. pos./Tot. (%)[a]	Geo. mean (range)[b]	No. pos./Tot (%)	Geo. mean (range)	No. pos./Tot (%)	Geo. mean (range)
0–12	11/11 (100)	2481.8 (164.9–6206.5)	1/10 (10.0)	12.2 (0–105.5)	0/9 (0.0)	0 (0–0.0)
13–24	12/12 (100)	1859.7 (95.1–3635.4)	1/10 (10.0)	124.6 (0–1245.9)	1/9 (11.1)	71.7 (0–645.2)
25–36	13/13 (100)	2875.7 (547.1–6891.6)	3/11 (27.3)	137.7 (0–1003.4)	4/11 (36.4)	68.6 (0–443.2)
37–48	12/12 (100)	2294.1 (99.8–4254.1)	4/10 (40.0)	203.7 (0–1097)	4/10 (40.0)	217.9 (0–983.5)
P	NS[c]	NS[d]	0.070	NS	0.019	NS

[a] A positive response was defined as any response ≥ 20 pg/ml above that of unstimulated cell culture supernatants. [b] Geometric mean cytokine levels in pg/ml. [c] NS= not significant. $P < 0.05$ considered significant, as determined by χ^2 test for trend across age groups. [d] $P < 0.05$ was considered significant as determined by analysis of variance of log-transformed values across age groups.

repeated exposure are necessary for development of IFN-γ but not IL-10 responses to LSA-1. The low frequencies and levels of IL-10 responses may reflect truly decreased IL-10 responses to LSA-1 and MSP-1 peptides in this population, but they may also reflect the effects of the finger-prick sampling method used, the relatively small number of mononuclear cells tested (2.0×10^5 cells), and testing for responses to single LSA-1 and MSP-1 peptides.

LSA-1 is a vaccine candidate antigen expressed exclusively in the liver-stage of *P. falciparum* infection [15]. Studies in malaria endemic areas of Africa have documented that IFN-γ and IL-10 responses to LSA-1 correlate with protection from infection [5–9]. In addition, a recent study in children from a malaria holoendemic area of western Kenya documented that IFN-γ responses to a pooled group of pre-erythrocytic antigens including LSA-1 were associated with protection from *anaemia* [16]. These findings suggest that IFN-γ and IL-10 responses to LSA-1 will

Table 5: Frequencies and geometric mean levels of IL-10 responses to PHA, LSA-1 and MSP-1 peptides by age.

Condition	PHA		MSP-1 (aa 20–39)		LSA-1, T3	
Age (months)	No. pos./Tot. (%)[a]	Geo. mean (range)[b]	No. pos./Tot (%)	Geo. mean (range)	No. pos./Tot (%)	Geo. mean (range)
0–12	7/11 (63.6)	241.4 (0–1247.0)	2/10 (20.0)	8.6 (0–48.93)	0/9 (0.0	1.3 (0–11.6)
13–24	9/12 (75.0)	155.6 (2.26–847.5)	3/10 (30.0)	19.4 (0–67.70)	1/9 (11.1)	17.5 (0–125.2)
25–36	10/13 (76.9)	195.8 (0–800.0)	0/11 (0.0)	2.7 (0–11.88)	0/11 (0.0)	1.1 (0–7.8)
37–48	10/12 (83.3)	267.7 (0.02–806.2)	2/10 (20.0)	57.9 (0–44.95)	2/10 (20.0)	16.6 (0–118.4)
P	NS[c]	NS[d]	NS	NS	NS	NS

[a] A positive response was defined as any response ≥ 20 pg/ml above that of unstimulated cell culture supernatants. [b] Geometric mean cytokine levels in pg/ml. [c] NS= not significant. $P < 0.05$ considered significant, as determined by χ^2 test for trend across age groups. [d] $P < 0.05$ was considered significant as determined by analysis of variance of log-transformed values across age groups.

be important markers of immunogenicity in LSA-1-containing vaccines. IFN-γ responses to LSA-1 are thought to mediate protection from infection by eliminating infected hepatocytes through induction of the nitric oxide pathway [17]. The mechanism for IL-10-associated protection from infection is less well defined, but may involve chemoattraction of CD8+ T cells [18] or augmented antibody-dependent cellular inhibition activity [6]. IFN-γ and IL-10 responses to MSP-1, a blood-stage vaccine candidate antigen, have also been documented in adults in malaria endemic areas [12–14,19], and rodent vaccination models suggest that IFN-γ responses to MSP-1 are important in protection from infection [20,21]. Further human studies are required to document whether IFN-γ or IL-10 responses to MSP-1 are protective in human populations.

In previous studies in a highland area of Kenya with highly seasonal malaria transmission, it has been documented that IFN-γ responses to LSA-1 were less frequent in children than adults but were relatively stable across seasons, even in the absence of high-level transmission [5]. The frequency and level of IFN-γ responses to the LSA-1 T3 peptide tested in the present study were lower in adults in the highland area than in children aged 3–4 years in the holoendemic area of the present study. This suggests that repeated exposure may be the most important factor in development of IFN-γ responses to LSA-1. However, it is unclear whether the low frequency of IFN-γ responses to LSA-1 in children under 2 years of age is due to immaturity of the immune system, a need for sustained or cumulative exposure or both factors. Children under 2 years of age are at the highest risk for severe malarial anaemia. In light of the protection from *anaemia* associated with IFN-γ responses to pre-erythocytic antigens, as documented by Ong'echa et al [16], it will be important to determine whether high frequencies of IFN-γ responses to LSA-1 can be induced with LSA-1-containing vaccines in children under 2 years of age. Interestingly, frequencies of IFN-γ responses to LSA-1 in children aged 6 to 24 months were similar in the present study and that of Ong'echa et al, but PHA responses were significantly less frequent in children in the study of Ong'echa et al (~50% vs. >90% in the present study). The authors speculated that the low frequency of PHA-stimulated IFN-γ responses might be due to down-regulation of these responses by acute *P. falciparum* infection. The high frequencies of PHA-stimulated IFN-γ responses in the children in this study, most of whom were infected with *P. falciparum*, suggest that *P. falciparum* infection does not down-regulate mitogen stimulation of T cells, even in young children. Previous studies [5,6] and the present study have not documented any difference in frequency or levels of IFN-γ responses to LSA-1 in individuals with or without parasitemia, demonstrating that *P. falciparum* infection also does not suppress these responses in young children.

In previous studies in a highland area with highly seasonal malaria, it has been documented that IL-10 responses to LSA-1 were similar in children and adults and decreased dramatically in the absence of high transmission [5]. A recent study in a malaria endemic area of Gabon also documented similar IL-10 responses to LSA-1 in children and adults [22]. Taken together, these findings suggest that IL-10 responses to LSA-1 are not dependent on age or chronic, repeated exposure but may be transient and related to recent infection, among other factors. The IL-10 responses in this population of children were low-level and infrequent, but these responses were from samples taken at a single time point and may have been low for sampling or testing reasons. Given the protection from infection seen with IL-10 responses to LSA-1 in adults and older children in malaria endemic populations [5,6], it will be important to assess whether IL-10 responses in young children in this area are truly low, whether strong IL-10 responses can be induced by vaccines with high concentrations of antigen or specific adjuvants, and whether these responses are protective in this younger age group. The similar frequencies and levels of IL-10 responses to LSA-1 in children with and without parasitemia suggest

that active *P. falciparum* infection does not suppress or increase these responses.

IFN-γ responses to the blood-stage antigen MSP-1 showed the same age-related pattern as those to LSA-1. If the protection from malaria infection and disease associated with IFN-γ responses to MSP-1 in rodent malaria models is confirmed in human populations, future studies will need to investigate whether age, repeated exposure or both are necessary for induction of these responses.

Conclusion

The present study establishes that frequencies and levels of IFN-γ responses to LSA-1 and MSP-1 are low in the first 2 years of life but increase to close to adult levels by the age of 4 years, while IL-10 responses to these antigens appear unrelated to age, in children in a malaria holoendemic area of Kenya. Prospective cohort studies of children from birth to age 4 years in malaria endemic areas are required to determine how IFN-γ and IL-10 responses to LSA-1 and MSP-1 relate to prior infection, the longevity of these responses, and their relation to protection from infection and disease.

Author contributions

CK performed all sample testing and data analysis and wrote the original draft manuscript. POS and JWK contributed to study design. AVO contributed to sample testing and interpretation of results. CCJ contributed to study design, data analysis and interpretation of results. All authors had a part in revision of the final manuscript and approved the final manuscript.

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