ASSOCIATION OF INTERFERON-γ RESPONSES TO PRE-ERYTHROCYTIC STAGE VACCINE CANDIDATE ANTIGENS OF PLASMODIUM FALCIPARUM IN YOUNG KENYAN CHILDREN WITH IMPROVED HEMOGLOBIN LEVELS:

XV. ASEMBO BAY COHORT PROJECT

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Abstract. Previous studies in animal models have revealed an association between interferon-γ (IFN-γ), produced by CD8+ T cells and irradiated sporozoite-induced sterile immunity. To determine whether IFN-γ can serve as a marker of pre-erythrocytic protective immunity in individuals naturally exposed to malaria, we characterized IFN-γ and lymphocyte proliferative responses to previously defined CD8+ cytotoxic T lymphocyte (CTL) epitopes from six pre-erythrocytic stage antigens in 107 children six months to two years old from a community-based birth cohort in western Kenya. We found that IFN-γ positive responders had higher hemoglobin (Hb) levels and significantly reduced prevalence of severe malarial anemia one month after the test compared with IFN-γ non-responders, suggesting that IFN-γ immune responses to these pre-erythrocytic antigens were associated with protection against malarial anemia. Children who responded by lymphocyte proliferation had a significantly longer time to first documented malaria parasitemia after birth; however, there was no correlation between the presence of lymphocyte proliferative response and higher Hb levels. We propose that IFN-γ production could be used as a potential marker of protective immunity against malaria associated anemia in young children living in malaria holoendemic areas.

INTRODUCTION

Malaria imposes an intolerable disease burden on the 2.4 billion people living in tropical areas. Currently, there is an estimated 300–500 million clinical cases and 1.1–2.7 million deaths yearly. Sub-Saharan Africa accounts for 90% of these malaria-related deaths, which are mostly the result of infection with Plasmodium falciparum in young children and pregnant women.1

There is a consensus that an effective vaccine along with other malaria control measures is needed for an effective control and prevention of malaria. Among the malaria vaccine candidates presently being developed, pre-erythrocytic stage vaccine candidates and multicomponent vaccines that include determinants from pre-erythrocytic antigens are attractive. The protective effects of such a vaccine would prevent sporozoites from invading hepatocytes and developing to maturity. Consequently, the chance of successful development of blood stage parasitemia and clinical disease is reduced, as well as the development of gametocytes and the potential for transmission.2

Earlier studies have implicated CD8+ cytotoxic T lymphocytes (CTLs) in protection against pre-erythrocytic-stage malaria parasites. In vitro studies in animal models have shown that CD8+ CTLs can eliminate plasmodium-infected hepatocytes.3 Although there is no direct evidence of involvement of CTL responses in protection against pre-erythrocytic parasites in humans with natural infection, CTL responses have been demonstrated in individuals naturally exposed to P. falciparum4–7 and immunized with irradiated sporozoites.5 A number of studies have shown that the CTL responses are not only major histocompatibility complex (MHC)–restricted6,7–8 but also affected by variations within the CTL epitopes.6,9 Thus, parasite diversity plus human MHC polymorphisms pose an impediment to the development of single epitope-based pre-erythrocytic stage vaccines. Multivalent and multistage vaccines and HLA alleles with significant overlapping peptide-binding specificities (supertypes) are expected to overcome problems associated with genetic diversity of parasite antigens and MHC restriction.5,10

A substantial body of evidence has been accumulated that points to the pivotal role of pre-erythrocytic stage antigen-specific cytokine responses in protection against infection. Earlier studies in animal models reported that interferon-γ (IFN-γ) is active against infected hepatocytes and that IFN-γ produced by CD8+ CTLs induced the infected hepatocytes to produce L-arginine-derived nitrogen oxides toxic to the intracellular parasites.11–13 A recent study in BALB/c mice provided evidence that the immunity induced by immunization with irradiated sporozoites or DNA vaccines was initiated by parasite-specific CD8+ T cells and was dependent on the production of IFN-γ and interleukin-12 (IL-12).14 Another study in mice using multiple malaria CD8+ T cell epitope vaccine formulations also showed that protection correlated with high levels of IFN-γ-secreting cells.15 In addition, a study in humans reported a positive correlation between pre-erythrocytic stage specific IFN-γ secretion and CTL responses in volunteers immunized with irradiated sporozoites.5 More recently, several studies conducted in different malaria-endemic regions have highlighted an association between liver stage antigen-1 (LSA-1)–driven IFN-γ or IL-10 responses and P. falciparum infection/morbidity.16–19

The development of field-useable tools for assessing protective immunologic end points of vaccines is one of the critical needs in malaria vaccine development and testing. Over the last three decades, the conventional 51Cr-release assay has been the technique for measuring CTL reactivity in the immune response to pre-erythrocytic parasites. However, this method is technically demanding. Therefore, there is a need for the development of simple and reproducible surrogate
methods to measure malaria-specific CTL reactivity. The culture supernatant enzyme-linked immunosorbent assay (ELISA), which measures cytokine secretion by cultured lymphocytes, the ELISPOT, which enumerates cytokine-secreting T cells in response to stimulation by T cell epitopes, and tetramer analysis, which detects peptide-specific CD8+ T cells, are three alternative assays. These three methods are better than the conventional 51Cr-release assay. Both the culture supernatant ELISA and ELISPOT methods are simple and can be used in many field laboratory settings, but the use of the tetramer technique requires special equipment that may limit its use in the field. We conducted this study in Kenyan children six months to two years old to determine 1) the correlation between IFN-γ response/lymphocyte proliferation and malaria infection/malaria-related disease, and 2) whether these immunologic readouts can serve as markers of protection against malaria.

MATERIALS AND METHODS

Study area. This study was carried out as part of the Asembo Bay Cohort Project (ABCP) at Asembo Bay area in Nyanza province of western Kenya, recently described in detail elsewhere. In this area, malaria transmission occurs throughout the year, with two peak transmission seasons from March to May and October to December. The annual entomologic inoculation rates have been reported to range between 100 and 300 per year, and P. falciparum accounts for more than 90% of the malaria cases in this area. Children less than two years of age experience the greatest morbidity and mortality from malaria, with severe anemia as the major cause of malaria-related mortality in this area. In the ABCP, routine blood samples from infants were collected monthly from birth onwards for the detection of malaria parasites and the determination of hemoglobin (Hb) concentrations. Children who were febrile (axillary temperature ≥ 37.5°C) with any density of parasitemia or afebrile with parasitemia more than 5,000/µL of blood were treated with standard doses of sulfadoxine/pyrimethamine (SP). Written informed consent was obtained from the parents or guardians of participating infants/children in the ABCP. The Institutional Review Board of the Centers for Disease Control and Prevention (CDC) and the National Ethical Review Committee of the Kenya Medical Research Institute approved the ABCP.

Study subjects and design. Because young children are the population at the greatest risk for malaria infection, we chose children six months to two years old participating in the ABCP for the immunologic study. One hundred seven children six months to two years old participating in the ABCP for the immunologic study. One hundred seven children six months to two years old participating in the ABCP for the immunologic study. One hundred seven children six months to two years old participating in the ABCP for the immunologic study. One hundred seven children six months to two years old participating in the ABCP for the immunologic study. One hundred seven children six months to two years old participating in the ABCP for the immunologic study.

Blood collection, sample processing, and laboratory procedures. Fingerprick blood samples (750 µL) were collected into sterile microvette tubes containing EDTA (Sarstedt, Nümbrecht, Germany) and transported to the laboratory at 4°C within six hours. Thick and thin smears were prepared and examined by light microscopy for malaria parasites. Hemoglobin levels were determined using the Coulter counter (Coulter A+ T 10; Coulter Corporation, Miami, FL). Electrophoresis was used for detection of the sickle cell trait. The blood was diluted with 2 mL of sterile phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation through Ficoll-Hypaque. The PBMCs were then spin-washed twice in PBS and re-suspended in RPMI 1640 medium (BRL/Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% heat-inactivated human AB+ serum (donors not previously exposed to malaria), 5% heat-inactivated fetal bovine serum, 2 mM glutamine, streptomycin (100 µg/ml), and penicillin (100 units/ml). The viability of the cell preparation was determined using the trypan blue dye exclusion method. The cell preparations were generally found to be more than 95% viable.

Immunologic assays. In this study, we examined IFN-γ and lymphocyte proliferative responses to the previously defined 46 CD8+ CTL epitopes representing six pre-erythrocytic stage antigens and malarial anemia at the time of testing and one month after testing for the immune responses.

Peptides. The previously defined 46 CD8+ CTL epitopes representing six pre-erythrocytic stage antigens were used in this study. The peptide sequences and the HLA binding specificities of the peptides are shown in Table 1. These peptides were synthesized using Fmoc chemistry at the Bio-engineering Core Facility, National Center for Infectious Diseases, CDC (Atlanta, GA). Peptides were more than 90% pure as determined by reverse-phase high-performance liquid chromatography. These peptides are recognized in the context of HLA-A2, A3, and B7 supertypes and HLA-B35-, B53-, and B58-restricted epitopes. We chose these peptides based on previous studies, which had shown that the Kenyan population in this study area who were HLA typed, 1) 39% expressed HLA-A2, 23.4% expressed HLA-A3, and 34.5% expressed HLA-B7, and 2) 7% expressed B35, 18% expressed B53, and 20% expressed B58 (Udhayakumar V et al., unpublished data). These peptides were recognized by 84.8% of the immune adults from this area. Peptides from the same antigen, some with significant overlapping HLA-binding specificities (supertypes) and some MHC-restricted, were pooled for use in this study to minimize limitations associated with parasite polymorphism and MHC restriction. These were referred to as single antigen pools. In addition, we pooled peptides from different antigens (referred to as grouped antigens), previously reported to induce only MHC-restricted CTL responses, to compare with the single antigen pools. Although we are not aware of any antagonism that has been reported for the peptides used in this study, earlier studies showed that the use of pooled peptides did not interfere with the responses to the individual peptides.

Lymphocyte proliferation assay. Lymphocyte proliferation
TABLE 1

Peptide sequences for CTL epitopes from different pre-erythrocytic stage antigens of *Plasmodium falciparum*.

<table>
<thead>
<tr>
<th>Code</th>
<th>AA position</th>
<th>Sequence</th>
<th>HLA</th>
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<tr>
<td>L6</td>
<td>1786–1794</td>
<td>KPIVQYDNF</td>
<td>B53</td>
<td>8</td>
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<tr>
<td>LS8</td>
<td>1850–1857</td>
<td>KPNKSLY</td>
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<td>LS5</td>
<td>1854–1861</td>
<td>KSLYDEHI</td>
<td>B58</td>
<td>4, 22</td>
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<td>CP26</td>
<td>368–375</td>
<td>KPKDELDP</td>
<td>B35</td>
<td>6, 8</td>
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<td>EX23</td>
<td>77–84</td>
<td>ATSVLAGL</td>
<td>B58</td>
<td>7</td>
</tr>
<tr>
<td>TR26</td>
<td>3–11</td>
<td>HLGNVKLYL</td>
<td>A2</td>
<td>4</td>
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<tr>
<td>TR29</td>
<td>51–59</td>
<td>LLMDCGSI</td>
<td>A2</td>
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<tr>
<td>LA72</td>
<td>1260–1266</td>
<td>MELKKELEK</td>
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<td>LA90</td>
<td>1524–1532</td>
<td>EPKDEIVPE</td>
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*LSA-1 peptides*

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<tr>
<td>PL 740</td>
<td>94–102</td>
<td>QTNFKSSLR</td>
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<td>105–113</td>
<td>GVSENIFLK</td>
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<td>RINEEKEKH</td>
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<td>PL 750</td>
<td>1855–1863</td>
<td>SLYDEHIK</td>
<td>A3*</td>
<td>5</td>
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<tr>
<td>PL 751</td>
<td>60–68</td>
<td>VLSHNSYK</td>
<td>A3*</td>
<td>5</td>
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<tr>
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<td>16–24</td>
<td>LLIHFINGK</td>
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<tr>
<td>PL 764</td>
<td>1851–1862</td>
<td>KSLYDEHIK</td>
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<td>1851–1862</td>
<td>KSLYDEHIK</td>
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*TRAP peptides*

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<td>PL 735</td>
<td>394–402</td>
<td>GLIMVLSFL</td>
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<td>PL 737</td>
<td>7–16</td>
<td>ILSVSSFLFV</td>
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*CSP peptides*

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<td>344–353</td>
<td>VTCGNGOVR</td>
<td>A3*</td>
<td>5</td>
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<tr>
<td>PL 755</td>
<td>85–93</td>
<td>KLRKPKHKK</td>
<td>A3</td>
<td>5</td>
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*†* Grouped antigens comprised peptides from the following pre-erythrocytic stage antigens: LSA-1 (ls6, ls8, ls53); CSP (cp26); EXP-1 (Ex23); TRAP (Tr26, Tr29); and LSA-3 (La72, La90).

*‡* Refers to previously published peptide labels or our own peptide labels during this study.

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* was assayed as previously described, with modifications. Briefly, PBMCs were cultured in triplicate (5 × 10⁴ cells/well) in 96-well U-bottomed microtiter plates (Costar, Cambridge, MA) in a final volume of 200 µL of complete RPMI 1640 medium. The cells were stimulated with phytohemagglutinin (PHA) (Sigma, St. Louis, MO) at a concentration of 4 µg/ml and pooled peptides at concentrations of 10⁻⁵ µg/ml for each peptide. Cell culture plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂. On day five of incubation, pooled supernatants from triplicate wells were harvested and stored at -70°C until cytokines were assayed. Supernatants were replaced with an equal volume of RPMI 1640 complete medium and ³H-thymidine (1 µCi/well) (specific activity = 2 Ci [740.0 GBq]/mmol; DuPont New England Nuclear Research Products, Boston, MA) was added. The cultures were incubated for 16–18 hours. Cells were harvested onto filter mats using a cell harvester (LKB Instruments, Rockville, MD), and radioactive uptake was measured using a scintillation counter (LKB Instruments). Proliferative responses were expressed as stimulation indices (SIs) calculated using the formula: counts per minute (cpm) of triplicate stimulated cells/counts per minute (cpm) of triplicate un-stimulated control cells).

Cytokine assays. The concentration of IFN-γ in supernatants of the cell cultures were measured using a standard capture and detection sandwich ELISA, as per the recommendations of the manufacturer. Briefly, ELISA plates (Immulon 2; Dynatech Laboratories, McLean, VA) were coated overnight at 4°C with 1 µg/ml of primary antibody to IFN-γ (R&D Systems, Inc., Minneapolis, MN). The plates were then washed two times with wash buffer (PBS + 0.05% Tween 20) and blocked for two hours at room temperature with blocking buffer (wash buffer + 5% non-fat milk). The plates were washed three times, samples and cytokine standards were added, and the plates were incubated overnight at 4°C. The plates were then washed four times and secondary biotinylated anti-human IFN-γ monoclonal antibody (Endogen, Cambridge, MA) was added (0.5 µg/ml). The plates were incubated at room temperature for 45 minutes and then washed six times with wash buffer. A peroxidase-conjugated avidin reagent (ExtrAvidin; Sigma, St. Louis, MO) was added before stopping the reaction by adding 1 M H₃PO₄. The plates were read on an automated Vmax microplate reader at an absorbance of 450 nm (Molecular Devices Corporation). Cytokine concentrations in the supernatants were determined by extrapolation from standard curves. The detection limit of the assay was 3 pg/ml based on the standard curves.

Definitions. A child was considered to have a malaria infection if any asexual blood stage parasites were seen on a thick blood smear. Placental malaria was defined as any asexual blood stage parasites and pigment seen on a thick smear of placental blood. A child with an Hb level < 11 g/dL plus any density parasitemia was defined as having malaria-associated anemia, while those with Hb levels < 8 g/dL plus...
any density parasitemia were defined as having severe malarial anemia\(^{25}\) (Kuwile F. et al., unpublished data). A child was defined as having the sickle cell trait if both HbS and HbA were present and HbA predominated. We defined proliferative responders as children whose PBMCs had SIs \(\geq 2\) in response to one or more antigen pools. The children with IFN-\(\gamma\) production (\(\geq 3\) pg/ml) in response to one or more antigen pools were classified as IFN-\(\gamma\) positive responders as previously described.\(^{16}\) The non-responders were children whose PBMCs failed to proliferate (SIs < 2) or produce IFN-\(\gamma\) (< 3 pg/ml) in response to any antigen pool.

**Statistical analysis.** The data were analyzed using the SPSS statistical package (SPSS version 9.0; SPSS, Inc., Chicago, IL). The characteristics and the differences in the parasitologic/hematologic end points between the immune responders and non-responders were tested by the Student’s t-test and chi-square test. The correlation between the IFN-\(\gamma\) and proliferative responses was determined using Spearman’s rho correlation coefficient. Parasite densities (expressed as number of parasites per microliter of blood) were first transformed to natural logarithms before being used in the analyses. \(P\) values \(\leq 0.05\) (two-sided) were considered statistically significant.

**RESULTS**

**Frequency of the proliferative and IFN-\(\gamma\) responses.** The characteristics of the pre-erythrocytic stage antigen-specific IFN-\(\gamma\) and proliferative responders and non-responders are shown in Table 2. The responders and non-responders for both lymphocyte proliferative and IFN-\(\gamma\) responses were comparable in age, gender, prevalence of the sickle cell trait, proportion of children born to mothers with placental malaria, and treatment with SP.

The prevalence of the proliferative and IFN-\(\gamma\) responses is summarized in Figure 1. The proliferative responses to the mitogen PHA were more frequent than IFN-\(\gamma\) responses to the same mitogen. However, antigen-specific proliferative responses were generally low, with only 29% of the individuals tested responding to at least one antigen pool (overall responses), compared with IFN-\(\gamma\) responses in which 40% of the children produced IFN-\(\gamma\) in response to at least one antigen pool. Similarly, when the responses were stratified by antigen, the children who responded by proliferation were fewer than those who responded by producing IFN-\(\gamma\) (Figure 1). There was no correlation between the presence of lymphocyte proliferation and higher levels of IFN-\(\gamma\) responses to pre-erythrocytic antigens (\(r = 0.062, P = 0.526\)).

**Comparison of the IFN-\(\gamma\) and proliferative responses with the parasitologic and hematologic outcomes.** The parasitologic and the hematologic outcomes between the responders and non-responders for both proliferative and IFN-\(\gamma\) responses are compared in Table 3. The responders by proliferation had a significantly longer time to the first documented infection after birth than the non-responders (\(P = 0.007\), by Student’s t-test). The responders and non-responders by proliferation also differed in the prevalence of high-density parasitemia at the time of testing and parasitemia density at one month after testing for the immunologic parameters, with the responders having significantly higher prevalence/higher density parasitemia than the non-responders (\(P = 0.008\), by chi-square test and \(P = 0.045\), by Student’s t-test). There was no difference in the mean Hb levels and frequencies of malaria-associated anemia and severe malarial anemia at the time of testing and one month after testing between the responders and non-responders by proliferation. We did not find statistically significant differences in the time to the first documented infection between the IFN-\(\gamma\) responders and the non-

### Table 2

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Responders (n = 43)</th>
<th>Non-responders (n = 64)</th>
<th>(P^1)</th>
<th>Responders (n = 24)</th>
<th>Non-responders (n = 83)</th>
<th>(P^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (days)†</td>
<td>410 (365–457)</td>
<td>404 (368–439)</td>
<td>0.810</td>
<td>412 (371–472)</td>
<td>402 (369–435)</td>
<td>0.565</td>
</tr>
<tr>
<td>Gender ratio (M:F)</td>
<td>0.87</td>
<td>0.71</td>
<td>0.847</td>
<td>0.94</td>
<td>0.98</td>
<td>0.509</td>
</tr>
<tr>
<td>Proportion with sickle cell trait</td>
<td>0.28</td>
<td>0.19</td>
<td>0.270</td>
<td>0.25</td>
<td>0.22</td>
<td>0.735</td>
</tr>
<tr>
<td>Proportion born to mothers with placental malaria</td>
<td>0.22</td>
<td>0.14</td>
<td>0.375</td>
<td>0.1</td>
<td>0.2</td>
<td>0.851</td>
</tr>
<tr>
<td>Percentage of SP treatment at one month before testing</td>
<td>0.15</td>
<td>0.16</td>
<td>0.928</td>
<td>0.29</td>
<td>0.12</td>
<td>0.069</td>
</tr>
<tr>
<td>Percentage of SP treatment at the time of testing</td>
<td>0.10</td>
<td>0.22</td>
<td>0.108</td>
<td>0.14</td>
<td>0.18</td>
<td>0.634</td>
</tr>
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</table>

* The responders were defined as children whose peripheral blood mononuclear cells (PBMCs) secreted interferon-\(\gamma\) (IFN-\(\gamma\)) or had a stimulation index (SI) \(\geq 2\) in response to stimulation by at least one malarial antigen pool while the non-responders were children whose PBMCs neither produced IFN-\(\gamma\) nor proliferated (SI < 2) in response to stimulation by any of the malarial antigen pools. SP = sulfadoxine-pyrimethamine.

† Values are the mean (95% confidence interval).

‡ Differences between groups were assessed using the Student’s t-test or chi-square test.
responders. The parasitemia levels at the different time points did not differ significantly between the IFN-γ responders and non-responders. There was also no difference at the time of testing in the Hb levels and frequencies of malaria-associated anemia and severe malarial anemia between the IFN-γ responders and non-responders. However, one month after testing for IFN-γ responses, the mean Hb levels of the responders were significantly higher than those of the non-responders ($P = 0.002$, by Student’s t-test). The results in Table 3 also show that the prevalence of severe malarial anemia was higher in the IFN-γ non-responders compared with the IFN-γ responders ($P = 0.010$, by chi-square test) one month after testing, although there was no difference in the prevalence of malaria-associated anemia between IFN-γ non-responders and non-responders at this time point.

**Relationship between IFN-γ production in response to different peptide pools and the Hb levels.** Having observed that the IFN-γ responders had significantly higher mean Hb levels one month after testing, we further analyzed this relationship when stratified by antigen. The results in Table 4A show that Hb levels for the responders remained high for all the antigens with statistically significant differences for responses to thrombospondin-related adhesive protein (TRAP) and grouped antigens (TRAP: $P = 0.014$ and grouped: $P = 0.009$, both by Student’s t-test). Since there was a general increase in Hb levels for the IFN-γ responders between the time of testing and one month after testing, we further compared the changes in mean Hb levels between the responders and non-responders stratified by antigen (Table 4B). We observed that there were increases in the mean Hb levels in the responders compared with the non-responders, and that the increases in mean Hb levels were statistically significant for the grouped antigens ($P = 0.003$, by Student’s t-test).

Due to the limited number of children who responded by proliferation to the individual antigen pools (1–6 children per antigen pool, except for grouped antigens, to which 11 children responded), we were unable to compare differences in the time to the first documented infection, the prevalence of parasitemia at the time of testing, and the parasitemia density one month after testing between the responders and non-responders stratified by antigen.

**DISCUSSION**

Epidemiologic studies suggest that the natural immunity in young children in areas endemic for malaria appears to be acquired in two stages. Children first develop an ability to resist the clinical effects of malaria infection (clinical or anti-disease immunity), followed by mechanisms that control parasitemia (anti-parasite immunity). Several immunologic studies have been undertaken to delineate the characteristics of naturally acquired immunity. This line of investigations will aid vaccine development as well as testing of vaccines in endemic areas. However, no immunologic markers for the clini-
Relationship between interferon-γ (IFN-γ) production and the hemoglobin levels (g/dL)

**Table 4**

| Peptides | Responders | Non-responders | P  
|----------|------------|----------------|---
| LSA-1    | 10.7 (9.7–11.6) (12) | 10.2 (9.6–10.7) (57) | 0.419  
| TRAP     | 11.4 (10.5–12.2) (11) | 9.8 (9.3–10.3) (54) | 0.014  
| CSP      | 10.7 (9.9–11.6) (14) | 10.0 (9.5–10.5) (47) | 0.171  
| EXP-1    | 11.0 (10.4–11.6) (18) | 10.2 (9.6–10.8) (50) | 0.132  
| PFS16    | 11.1 (10.5–11.7) (14) | 10.0 (9.5–10.6) (54) | 0.070  
| Grouped  | 11.5 (10.3–12.3) (17) | 9.8 (9.4–10.4) (72) | 0.009  
| Overall responses | 11.0 (10.4–11.4) (43) | 9.6 (9.1–10.2) (64) | 0.002  

*A The hemoglobin levels were measured one month after testing for the IFN-γ production. For definitions of abbreviations, see Table 1.

† Values are the mean (95% confidence interval) (individuals in the group).

‡ Differences between groups were assessed using the Student’s t-test.

**B. Changes in the hemoglobin levels between the time of testing and at one month later**

| Peptides | Responders | Non-responders | P  
|----------|------------|----------------|---
| LSA-1    | 0.93 (0.22–1.63) (12) | 0.84 (0.38–1.31) (57) | 0.878  
| TRAP     | 1.54 (0.38–2.69) (11) | 0.51 (0.06–0.95) (54) | 0.074  
| CSP      | 0.73 (0.12–1.34) (14) | 0.35 (0.16–0.80) (47) | 0.444  
| EXP-1    | 1.04 (0.21–1.88) (18) | 0.63 (0.18–1.08) (50) | 0.356  
| PFS16    | 1.09 (0.32–1.86) (14) | 0.63 (0.13–1.12) (54) | 0.374  
| Grouped  | 2.26 (0.96–3.56) (17) | 0.55 (0.11–1.00) (72) | 0.003  
| Overall responses | 1.44 (0.81–2.08) (43) | 0.19 (–0.29–0.66) (64) | 0.002  

*A The hemoglobin levels at the time of sampling were subtracted from those one month later and the mean of the increase in hemoglobin level for each antigen determined was then compared between the responders and the non-responders.

† Values are the mean hemoglobin increase (95% confidence interval) (individuals in the group).

‡ Differences between groups were assessed using the Student’s t-tests. Bold numbers indicate statistical significance.

cal immunity have been identified, especially among young children. Our previous epidemiologic studies conducted in the Asembo Bay area have shown that 6–24-month-old children experience the greatest morbidity and mortality from malaria, with severe anemia as the major cause of malaria-related mortality in this area.22 In the present study, we assessed the cellular immunologic responses to pre-erythrocytic stage vaccine candidate antigens in this age group. We used the approach of linking cross-sectional immunologic findings to parasitemia and Hb parameters at different time points, which enabled us to better understand the relationship between the immune responses and malaria infection or malaria-associated disease.

We have found that the IFN-γ responses to the pre-erythrocytic CTL epitopes are associated with higher Hb levels one month after testing, and have also shown that this response is involved in reducing the prevalence of severe malaria anemia in young Kenyan children (Table 3). The results obtained in one recent immuneepidemiologic study conducted in Gabon showed that IFN-γ responses to pre-erythrocytic stage LSA-1 were associated with resistance to re-infection with *P. falciparum* in young children; however, our study did not find a significant association between IFN-γ response and any parasitologic parameters. The lack of an association between IFN-γ response and parasitologic parameters observed in this study is consistent with the findings in other studies conducted in Kenya, which also reported lack of an association between IFN-γ production and parasitologic outcomes.17,18 Although the mechanisms involved in the association between IFN-γ response and protection against malaria anemia, but not with parasitologic outcomes, are unclear, the results of this study support earlier findings, which suggested the role of CTL-mediated responses against the liver stages of the malaria parasites in protection against severe malaria anemia.30 A recent study in a mouse model showed that IL-12 up-regulated by IFN-γ is one of key immune components in the protective immunity induced by immunization with irradiated sporozoites or DNA vaccines.14 Another study with a murine malaria model reported that IL-12 plays an important role in the protection against severe anemia via IL-12 up-regulation of the bone marrow and splenic erythropoiesis.31 Recent human studies also showed low plasma levels of IL-12 in Gabonese children with severe *P. falciparum* anemia, supporting the role of IL-12 in the up-regulation of Hb levels.32 Although we did not measure IL-12 production, we speculate that the association between IFN-γ responses to CTL epitopes of pre-erythrocytic stage antigens and increased Hb levels might be due to the production of IL-12 modulated by IFN-γ. Further studies will be needed to elucidate the association between pre-erythrocytic stage-specific IFN-γ production and protection against severe malaria anemia observed in this study population. Nevertheless, the findings in this study are important and suggest that the IFN-γ response to pre-erythrocytic stage CTL epitopes could play a role in clinical (anti-disease) immunity in young children who experience the greatest morbidity and mortality from malaria in this area.

The association of IFN-γ production with increased Hb levels in young Kenyan children was demonstrated by the responses to grouped antigens and the overall responses in this study. When the IFN-γ responses were further stratified by antigens, we observed that Hb levels for the IFN-γ responders remained high for all antigens, but showed statistical significance only for responses to TRAP and grouped antigens (Table 4A). These results suggest that the peptide pool from single antigen might induce weaker immune responses compared with that from multiple antigens, which lends support to the approach of including several well-characterized peptides from different antigens in a multivalent, multistage vaccines for optimum responses.10,15

Because of the limited cell numbers in the small volumes of blood collected from children, we were unable to conduct a T cell subset depletion assay to determine the cellular source of IFN-γ in this study. It is known that most of pre-erythrocytic stage T cell epitopes used in this study are degenerate cytoxic CD8+ T cell epitopes restricted by multiple HLA-A and HLA-B class I supertype alleles.3 More recently, it has been reported that some of these pre-erythrocytic stage antigens also contain HLA-DR CD4+ T cell epitopes restricted by multiple HLA class II supertype alleles.35 Interestingly, a high degree of overlap has been noted between the promiscuous class II CD4+ T cell epitopes reported recently and the degenerate class I CD8+ epitopes identified previously.5,33 Therefore, we speculate that both CD8+ and CD4+ T cells could respond to some of pre-erythrocytic stage antigens to produce the IFN-γ observed in this study.

In this study, we found that the children who responded by lymphocyte proliferation had a significantly longer time to the first documented malaria parasitemia after birth. It has been reported that multiple factors, such as maternal malaria infection during pregnancy, trans-placental malaria-specific an-
tibodies/soluble factors and host genetic factors in the child contribute to the susceptibility to malaria infection/disease and the capacity to mount immune responses to the acquired malaria parasitemia in the first few months of life.34–37 Although this study was not able to identify the major factor that leads to the susceptibility to the first malaria infection, the relationship between longer time to the first malaria infection and subsequent lymphocyte proliferation we observed may reflect the overall good status of cellular immunity.

Unlike IFN-γ responses, the lymphocyte proliferation to CTL peptides did not seem to have a protective effect against either anemia or parasitemia. In fact, among the proliferation responders, there was a trend to higher prevalence and density of infection than in the non-responders, but this reached statistical significance only at the time of and one month after immunologic testing (Table 3). It is not clear why the proliferative responder group had a higher prevalence and density of infection, particularly at one month post-immunologic testing. It is likely, in the case of one month before and at the time of testing, that malaria exposure/infection boosts/maintains the immune memory response in the form of continually expanding malaria-specific T cells. This speculation is supported by the previous finding and the view that continuous/persistence antigenic stimulation correlates with maintenance of T cell memory.38,39

A lower frequency of T cell proliferative and IFN-γ responses to both PHA and malaria pre-erythrocytic antigens was observed in this study when compared with other studies conducted in different malaria transmission areas or in different age groups of the same area.16–19 This could reflect the general immaturity of the immune system in children compared with adults. An alternative explanation could be that T cell responses were suppressed in population of children who are frequently exposed to malaria in this holoendemic area. In addition, our study showed that there was no association between the lymphocyte proliferative and IFN-γ responses to the antigens tested. This is consistent with other previous studies conducted in adult residents of Papua New Guinea19 and the Gambia,15 which also reported that IFN-γ responses to LSA-1 or CS peptides were dissociated from T-cell proliferative responses. Although the precise relationship (or line age) between proliferative T cells and IFN-γ-producing T cells to the pre-erythrocytic antigens and their differentiation is not clear, the results from this study suggest that these two immune responses play different roles in malaria infection. These results also indicate that understanding the potential relationship between memory and effector immune responses and their roles in conferring protection against disease will be critical in the development and testing of vaccines.

In summary, our results show that production of IFN-γ in response to pre-erythrocytic stage antigens is associated with increased Hb levels and reduced frequency of severe malarial anemia in young Kenyan children, suggesting that the immunity mediated by IFN-γ plays an important role in clinical protection against malarial anemia. However, the presence of a lymphocyte proliferative response was not associated with either increased Hb levels or protection against parasitemia. Thus, IFN-γ responses might be a suitable clinical protective immune marker in young children living in this holoendemic area for malaria when pre-erythrocytic stage vaccines are being evaluated/tested.

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REFERENCES