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Exposure to Holoendemic Malaria Results in Suppression of Epstein-Barr Virus–Specific T Cell Immunosurveillance in Kenyan Children

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Background. Malaria and Epstein-Barr virus (EBV) infection are cofactors in the pathogenesis of endemic Burkitt lymphoma (eBL). The mechanisms by which these pathogens predispose to eBL are not known.

Methods. Healthy Kenyan children with divergent malaria exposure were measured for responses to EBV latent and lytic antigens by interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) assay and interleukin (IL)-10 ELISA. Phytohemagglutinin (PHA), purified protein derivative (PPD), and T cell epitope peptides derived from merozoite surface protein (MSP)-1, a malaria blood-stage antigen, were also evaluated.

Results. Children 5–9 years old living in an area holoendemic for malaria had significantly fewer EBV-specific IFN- γ responses than did children of the same age living in an area with unstable malaria transmission. This effect was not observed for children <5 years old or those >9 years old. In contrast, IFN- γ responses to PHA, PPD, and *Plasmodium falciparum* MSP-1 peptides did not significantly differ by age. IL-10 responses to EBV lytic antigens, PPD, and PHA correlated inversely with malaria exposure regardless of age.

Conclusions. Children living in malaria-holoendemic areas have diminished EBV-specific T cell immunosurveillance between the ages of 5 and 9 years, which coincides with the peak age incidence of eBL.

Burkitt lymphoma was first described in African children in 1958 [1]. The highest incidence of endemic Burkitt lymphoma (eBL) is in equatorial Africa and Papua New Guinea (5–15 cases/100,000 children) [2, 3]. Within Africa, there is an uneven geographic distribution of eBL, which led Dalldorf [4] in 1962 to suggest that malaria may be a risk factor for lympho-

magenesis. In fact, there is a strong correlation between residence in areas of intense, perennial malaria transmission (i.e., holoendemic malaria) and the incidence of eBL [3, 5].

eBL, the first human cancer discovered to have a viral etiology, is associated with Epstein-Barr virus (EBV) infection [6]. EBV is restricted to humans and infects the vast majority of the world's population [7]. In countries such as Kenya, EBV infection generally occurs by 2 years of age [8–11] and persists for life as a latent infection in memory B cells [12]. The lytic phase of viral replication is thought to occur after plasma cell differentiation [13–15]. The virus is periodically shed in the saliva, suggesting that there is ongoing lytic-cycle reactivation [16].

Immunosurveillance and control of EBV is dominated by CD8⁺ human leukocyte antigen (HLA) class I-restricted cytotoxic T lymphocyte (CTL) interferon (IFN)- γ responses to both latent and lytic viral epitopes [17, 18]. An EBV-specific CD8⁺ T cell subset that has reduced cytotoxicity and that secretes interleukin (IL)-10 has also been described [19]. Two possible but not

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mutually exclusive models have been proposed to explain how holoendemic malaria could affect EBV latency and immunity in children and thereby increase the risk of eBL: suppression of EBV-specific T cell immunity and/or expansion of the latently infected B cell pool (reviewed in [20, 21]). The few studies that have investigated the role played by malaria in T cell control of EBV-infected B cells in clinical samples have used an in vitro regression assay that measures the ability of T cells to control the outgrowth of EBV-transformed B lymphocytes [22–24]; regression of colonies is thought to be mediated by T cells that express CD8 and produce IFN- γ [25, 26]. Using this assay, Moss et al. [23] found that adults living in malaria-holoendemic regions of Papua New Guinea had impaired EBV-specific T cell responses. Later observations from The Gambia reported that children experiencing acute clinical malaria had impaired EBV-specific T cell immunity [22, 24]. However, conclusions from this earlier work were based on a relatively small number of children or on adults with mature T cell immunity, whose responses might differ from those of children. In addition, regression assays do not identify the effector T cells or cytokine mediators involved in the control of EBV-infected B cells, and no distinction was made between EBV-specific immunosuppression and generalized depression of T cell immunity.

The study described here investigated the effect of malaria on EBV immunity and assessed whether the intensity and duration of malaria exposure influences EBV-specific IFN- γ and IL-10 responses in healthy children from 2 epidemiologically distinct areas of western Kenya that differ markedly in the incidence of eBL [3, 5, 27, 28]. In addition, malaria-specific cytokine responses were examined concomitantly to address the question of whether there are global versus EBV-specific alterations in T cell immunity in children exposed to malaria.

PARTICIPANTS, MATERIALS, AND METHODS

Participants. Approval for this study was obtained from the Kenya Medical Research Institute (KEMRI) National Ethical Review Committee and the Institutional Review Board for Human Studies at the University Hospitals of Cleveland, Case Western Reserve University. Written, informed consent was obtained from the guardians or parents of the study participants.

Study participant recruitment and sample collection were conducted in July–August 2002 in 2 epidemiologically distinct areas of Kenya (Kisumu District and Nandi District), as described elsewhere [11]. The characteristics of the study population are summarized in table 1. Children from the holoendemic-malaria transmission area are referred to as “Kisumu children” ($n = 104$), and children from the sporadic, unstable malaria transmission area are referred to as “Nandi children” ($n = 127$). Children (1–14 years old) were enrolled if they had overall good health and were excluded from analysis if they were EBV seronegative [11]. Of note, 77% of the Kisumu children had asymptomatic *Plasmodium falciparum* infection detected by blood smear, which is typical for resident children from this area. In contrast, 16% of the Nandi children had positive blood smears after a recent malaria epidemic, demonstrating the dramatic difference in malaria exposure between the 2 populations.

Sample collection. Peripheral blood was collected in sodium-heparinized tubes and transported to the Case Western Reserve University laboratory located at KEMRI’s Center for Vector Biology and Control Research in Kisumu for processing the same day. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by ficoll-hypaque density gradient centrifugation and suspended in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated human AB serum, 50

Table 1. Study participant demographics.

Site, characteristic	Age group			All
	1–4 years	5–9 years	10–14 years	
Kisumu				
Study participants enrolled, no.	32	36	36	104
Hemoglobin level, mean, g/dL	9.7	12.2	12.5	11.6
Children with malaria-positive smear, ^a %	77	72	83	77
Body temperature, mean, °C	36.9	36.7	37.0	36.9
Nandi				
Study participants enrolled, no.	36	48	43	127
Hemoglobin level, mean, g/dL	12.0	12.9	13.5	12.8
Children with malaria-positive smear, ^a %	8	14	26	16
Body temperature, mean, °C	37.2	37.3	37.0	37.2

^a *Plasmodium falciparum* prevalence differed significantly between study sites ($P < .0001$).

$\mu\text{g/mL}$ gentamicin, 10 mmol/L HEPES, and 2 mmol/L glutamine for the cytokine-stimulation assays.

Peptide selection. Molecular HLA class I genotyping was performed previously on a random, unrelated subset of residents from each study population [29]. The frequency of HLA class I alleles was similarly heterogeneous in Kisumu and Nandi but not significantly different from each other. Therefore, our selection of HLA class I-restricted epitope peptides did not create a response bias between study populations that would result from differences in HLA genotype [29]. Previously described HLA class I-restricted EBV epitope peptides from immunodominant lytic and latent antigens [17, 18] were selected and pooled. Peptides from EBV lytic (BRLF1, BZLF1, and BMLF1) and EBV latent (Epstein-Barr nuclear antigen [EBNA] 3A, EBNA3B, and EBNA3C) antigens were selected on the basis of their predicted binding affinities to prevalent HLA alleles in our study population (table 2). Peptides were synthesized and purified to >95% by high-performance liquid chromatography and were lyophilized for stability (Sigma Genosys). Peptides were reconstituted in 30% (wt/vol) dimethyl sulfoxide and diluted in sterile PBS (GIBCO) to a concentration of 0.1 mg/mL. EBV peptides were pooled so that each peptide was used at a final concentration of 10 $\mu\text{g/mL}$.

Malaria-specific cytokine responses were assessed using previously described T cell epitopes of merozoite surface protein (MSP)-1, a protein expressed during the erythrocytic stage of the parasite life cycle. A C-terminal peptide referred to as M1 (VTHEYQELVKKLEALEDAV; residues 20–39) [30] and an N-terminal peptide referred to as M2 (GISYYEKVLAKYKDDLE; residues 1467–1483) [31] were used for ex vivo cytokine-stimulation assays. The specificity of malaria-peptide responses was tested on PBMCs from 20 malaria-unexposed adult US volunteers. EBV-seropositive US PBMC donors had robust cyto-

kine responses to the EBV peptides but lacked responses to the malaria peptides (data not shown). Two positive controls were used to stimulate 1×10^5 PBMCs/well: the mitogen phytohemagglutinin (PHA; Sigma-Aldrich) at 1 $\mu\text{g/mL}$ and purified protein derivative (PPD) at 10 $\mu\text{g/mL}$ (Tubersol 5 TU, Amerisource Bergen). PBS was added to 0.5×10^6 PBMCs/well as the negative control and represented the background level of IFN- γ produced by unstimulated cells.

Cytokine assays. IFN- γ enzyme-linked immunospot (ELISPOT) assays were performed using sterile Millipore MAIP ELISPOT 96-well microtiter plates precoated at 4°C overnight with 5 $\mu\text{g/mL}$ human anti-IFN- γ monoclonal antibody (Endogen M-700A). The plates were washed with sterile PBS and blocked with 10% heat-inactivated fetal bovine serum. PBMCs plated at 0.5×10^6 cells/well plus stimulant were incubated at 37°C in 5% CO₂ for 72 h. Plates were washed and a second biotinylated anti-IFN- γ monoclonal antibody (Endogen M-701B) was applied (0.75 $\mu\text{g/mL}$) for 1.5 h at 37°C, followed by washing, incubation with a 1:2000 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 mol/L acetate buffer catalyzed by 0.015% hydrogen peroxide. The reaction was stopped after 10–20 min by washing with distilled water. Plates were dried in the dark at room temperature. The number of spot-forming units per well was counted using ImmunoSpot scanning and imaging software (version 4; Pharmingen). Results are expressed as spot-forming units per 1×10^6 PBMCs.

IL-10 ELISAs were performed after PBMC stimulation under conditions similar to those of the IFN- γ ELISPOT assay but with a final concentration of 0.2×10^6 cells/well in 200 μL of complete RPMI in U-bottom microtiter plates (Microtest; BD). Cell culture supernatants were removed after 72 h and tested

Table 2. Epstein-Barr virus (EBV) lytic and latent peptides.

EBV protein	Cycle	Peptide pool	Amino acid		HLA restriction
			Sequence	Residues	
BRLF1	Lytic	EP1	DYC NVL NKE F	EBV 28–37	A24
BRLF1	Lytic	EP1	RVR AYT YSK	EBV 148–156	A3
BZLF1	Lytic	EP1	RAK FKQ LL	EBV 190–197	B8
BMLF1	Lytic	EP1	GLC TLV AML	EBV 280–288	A2
EBNA3A	Latent	EP2	FLR GRA YGL	EBV 325–333	B8
EBNA3A	Latent	EP2	RPP IFI RRL	EBV 379–387	B7
EBNA3A	Latent	EP2	SVR DRL ARL	EBV 596–604	A2
EBNA3A	Latent	EP2	RLR AEA QVK	EBV 603–611	A3
EBNA3B	Latent	EP2	TYS AGI VQI	EBV 217–225	A24
EBNA3B	Latent	EP2	VEI TPY KPT W	EBV 657–666	B44
EBNA3C	Latent	EP2	RRI YDL IEL	EBV 258–266	B27

NOTE. EBNA, Epstein-Barr nuclear antigen; HLA, human leukocyte antigen.

Table 3. Aggregate cytokine responses, by children (1–14 years old) with divergent malaria exposure.

Assay, stimulant	Proportion (%) positive		<i>P</i>
	Kisumu	Nandi	
IFN-γ ELISPOT assay			
EP1	36/104 (34.6)	52/117 (44.4)	.14
EP2	30/104 (28.8)	41/117 (35.0)	.32
M1	17/103 (16.5)	26/113 (23.0)	.23
M2	25/104 (24.0)	31/113 (27.4)	.57
PPD	90/103 (87.4)	103/115 (89.6)	.61
PHA	92/104 (88.5)	109/117 (93.2)	.22
IL-10 ELISA			
EP1	2/104 (1.9)	22/126 (17.5)	.0001
EP2	1/104 (1.0)	6/126 (4.8)	.095
M1	5/104 (4.8)	14/126 (11.1)	.084
M2	7/104 (6.7)	18/126 (14.3)	.067
PPD	30/102 (29.4)	76/123 (61.8)	.00001
PHA	68/104 (65.4)	103/127 (81.1)	.0067

NOTE. Not all children had a sufficient peripheral blood mononuclear cell yield to test every stimulation condition, as reflected by the denominators. Boldface type indicates a significant difference ($P < .05$) between Kisumu and Nandi children for that stimulant. ELISPOT, enzyme-linked immunospot; EP1, Epstein-Barr virus (EBV) lytic peptide pool; EP2, EBV latent peptide pool; M1 and M2, malaria peptides; PHA, phytohemagglutinin; PPD, purified protein derivative.

for IL-10 by ELISA as described elsewhere [27]. Values for baseline (unstimulated) culture supernatants were subtracted from those for the peptide/mitogen-stimulated culture supernatants. The concentration of cytokine secreted was determined against a standard curve by use of recombinant IL-10 with a sensitivity of 10 pg/mL.

Data analyses. Analyses were conducted using SAS (version 8.2; SAS Institute). An IFN- γ ELISPOT response was considered to be positive if the proportion of spot-forming units in the stimulated well was significantly greater than that in the unstimulated background well by a χ^2 Fisher's exact test ($P < .05$). Proportions of responders were compared across study sites and age categories by use of a χ^2 Fisher's exact test. An IL-10 ELISA response to the malaria peptides was considered to be positive if it was >2 SDs above the mean response for the malaria-naïve negative controls; the cutoff value for MSP-1 M1 and M2 peptides was 40 pg/mL. Responses to EBV peptides were found only for PBMCs from EBV-seropositive donors (data not shown). A χ^2 test for homogeneity was used to determine whether the proportion of children with IFN- γ and IL-10 responses was significantly different from that of the adults. Continuous values of IFN- γ precursor frequency or IL-10 ELISA values among positive responders were compared across study sites by use of a 2-sided Wilcoxon (Mann-Whitney *U*) rank sum test. The correlation between an individual's re-

sponse to both cytokines was assessed by use of the McNemar test.

RESULTS

IFN- γ responses in children with differing exposure to malaria.

Aggregate IFN- γ ELISPOT responses in 1–14-year-old children

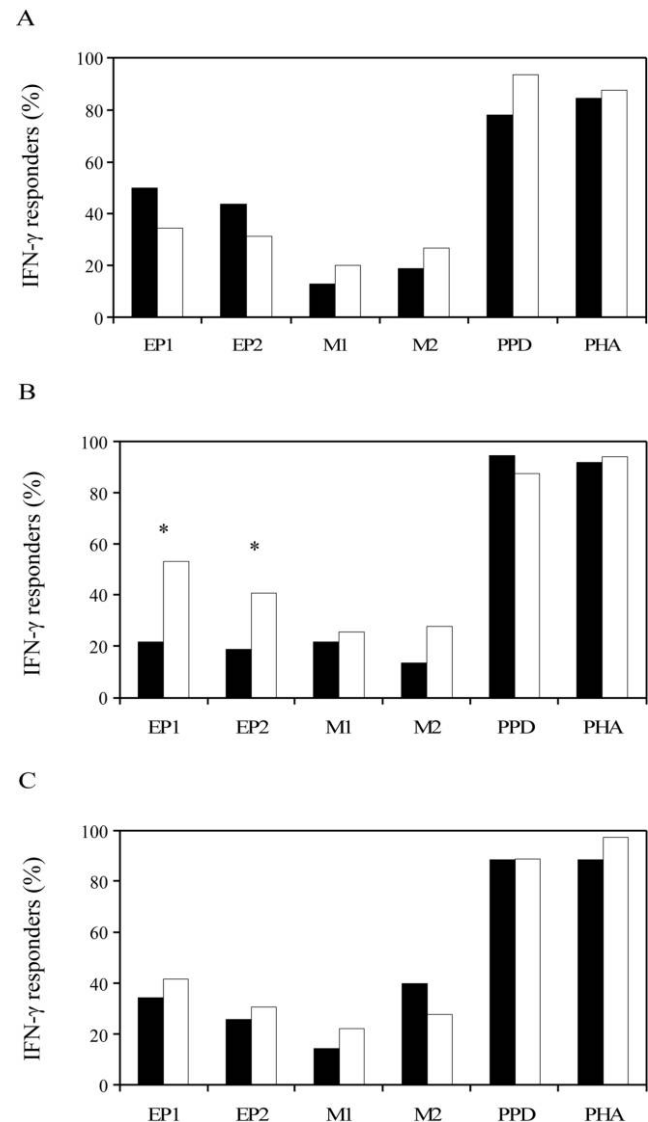
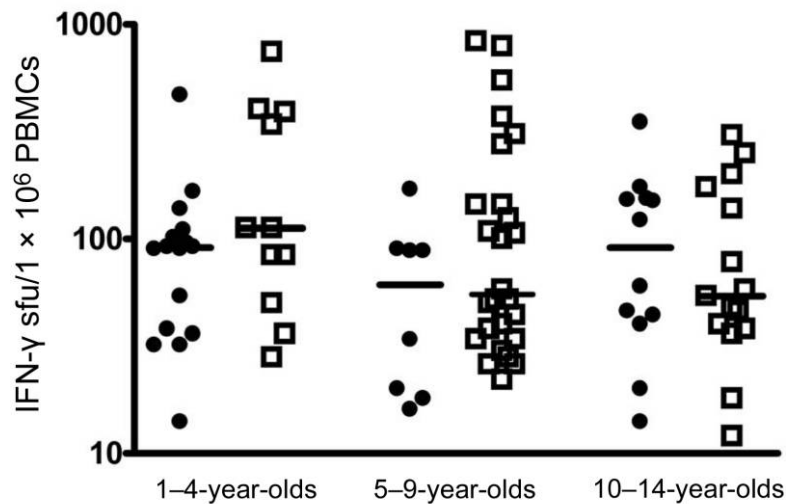


Figure 1. Proportion of interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) responders, by age group. The proportion of IFN- γ ELISPOT responders was compared between study sites according to the following age groups: 1–4-year-olds (A), 5–9-year-olds (B), and 10–14-year-olds (C). Black bars show positive responders from Kisumu, and white bars show positive responders from Nandi. Stimulation conditions included the following: an Epstein-Barr virus (EBV) lytic peptide pool (EP1); an EBV latent peptide pool (EP2); malaria peptides (M1 and M2); purified protein derivative (PPD); and phytohemagglutinin (PHA). Asterisks indicate that significantly fewer 5–9-year-olds from Kisumu responded to the EBV lytic ($P = .003$) and EBV latent ($P = .03$) peptide pools than did Nandi children of the same age (χ^2 Fisher's exact test).

A. EBV lytic responses



B. EBV latent responses

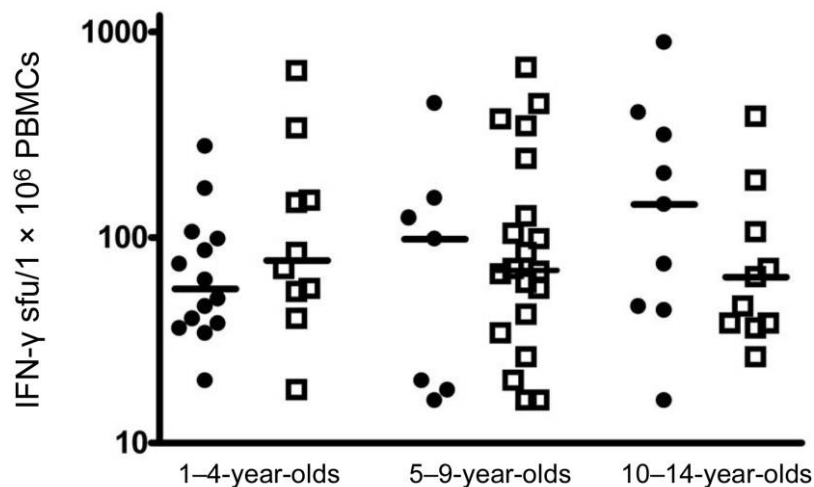


Figure 2. Magnitude of interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) responses, by each age group. The magnitude of Epstein-Barr virus (EBV) lytic (A) and latent (B) IFN- γ responses was compared by age group and by residence (Kisumu vs. Nandi) for positive responders only. IFN- γ responses are expressed as spot-forming units per 1×10^6 peripheral blood mononuclear cells (PBMCs). The no. of spot-forming units for each responder is represented as black circles for Kisumu children and as white squares for Nandi children, and a median bar is shown for each age group.

from the malaria-holoendemic area in Kisumu District and the malaria epidemic-prone area in Nandi District are shown in table 3. No significant differences in IFN- γ ELISPOT responses to the EBV lytic peptide pool (EP1: BRLF1, BZLF1, and BMLF1), the EBV latent peptide pool (EP2: EBNA3A, EBNA3B, and EBNA3C), MSP-1 T cell epitopes (M1 and M2), PPD, or PHA were observed when children of all ages with different cumulative malaria exposure were compared.

Because malarial infection and clinical morbidity are highest in children <5 years old and because protection against malaria

is acquired with age and duration of exposure to malaria [32, 33], we categorized the study participants into age groups as follows: (1) 1–4 years old, when malaria susceptibility is the greatest and the peak incidence of eBL has not yet been reached; (2) 5–9 years old, when malaria susceptibility is declining and the incidence of eBL is greatest; and (3) 10–14 years old, when antimalaria immunity has developed and the incidence of eBL has declined. IFN- γ ELISPOT responses were analyzed according to age group and compared between the malaria-endemic study sites. Although the proportion of EBV-specific responders

was similar for Kisumu and Nandi children aged 1–4 and 10–14 years (figure 1A and 1C), fewer 5–9-year-old children from Kisumu than from Nandi produced IFN- γ to both the EBV lytic (EP1, 21.6% vs. 53.1%; $P = .003$) and the EBV latent (EP2, 18.9% vs. 40.8%; $P = .03$) peptides (figure 1B). In contrast, the proportion of IFN- γ responders to the MSP-1 peptides, PHA, and PPD did not significantly differ by age group or malaria exposure. Interestingly, when the proportion of EBV responders was compared between age groups among the Kisumu children, the lowest proportion was found in the 5–9-year-olds (18.9%) compared with the 1–4-year-olds (43.8%) and the 10–14-year-olds (25.7%) ($P < .001$). This age-associated loss of EBV-specific IFN- γ response was not observed for the Nandi children.

We next compared the magnitude of IFN- γ responses among the Kisumu and Nandi children with positive ELISPOT responses. The range of positive IFN- γ responses was highly variable: 12–836 sfu/ 1×10^6 PBMCs for EP1 and 16–1322 sfu/ 1×10^6 PBMCs for EP2. Median responses were not significantly different between age groups and study sites for either of the EBV peptide pools (figure 2).

IL-10 responses by children with differing exposure to malaria. Aggregate IL-10 ELISA responses in 1–14-year-old children from the malaria-holoendemic area in Kisumu and malaria epidemic-prone area in Nandi are shown in table 3. We observed significantly fewer IL-10 responders to the EBV lytic peptide pool ($P = .0001$), PPD ($P = .00001$), and PHA ($P = .0067$) in children from Kisumu than from Nandi. In contrast, the proportion of IL-10 responders to the EBV latent peptide pool and the malaria peptides was similar between study sites.

Children were grouped by age as described above, and the proportion of IL-10 responders to each stimulus was then compared by study site (figure 3). Analysis of age-specific IL-10 responses to the EBV lytic peptides (EP1) demonstrated that 5–9-year-old Kisumu children had significantly fewer responses ($P = .009$) than did Nandi children of the same age. There were also fewer EP1 responders in the 10–14-year-old group of Kisumu children compared with Nandi children of the same age ($P = .051$). There were not enough children younger than 5 years with IL-10 responses to EP1 to show significant differences; however, no Kisumu children produced IL-10 in response to EP1, compared with 11% of Nandi children. The proportion of IL-10 responders to the EBV latent peptides (EP2) was too low to show any significant differences within age groups between the Kisumu and Nandi children. PPD- and PHA-driven IL-10 responses were significantly less frequent in 1–4-year-olds ($P < .001$), and PPD-driven IL-10 responses were also less frequent in 5–9 year olds from Kisumu than from Nandi ($P < .001$). The proportion of IL-10 responders to PPD and PHA was similar for older Kisumu and Nandi children.

IL-10 responses to the MSP-1 peptides were either absent or infrequent in children 1–4 years old regardless of study site and appeared to be similarly infrequent in the older age groups.

The magnitude of IL-10 responses was compared between age groups (figure 4). The median levels of IL-10 expressed by the Nandi children in response to the EBV lytic peptides were

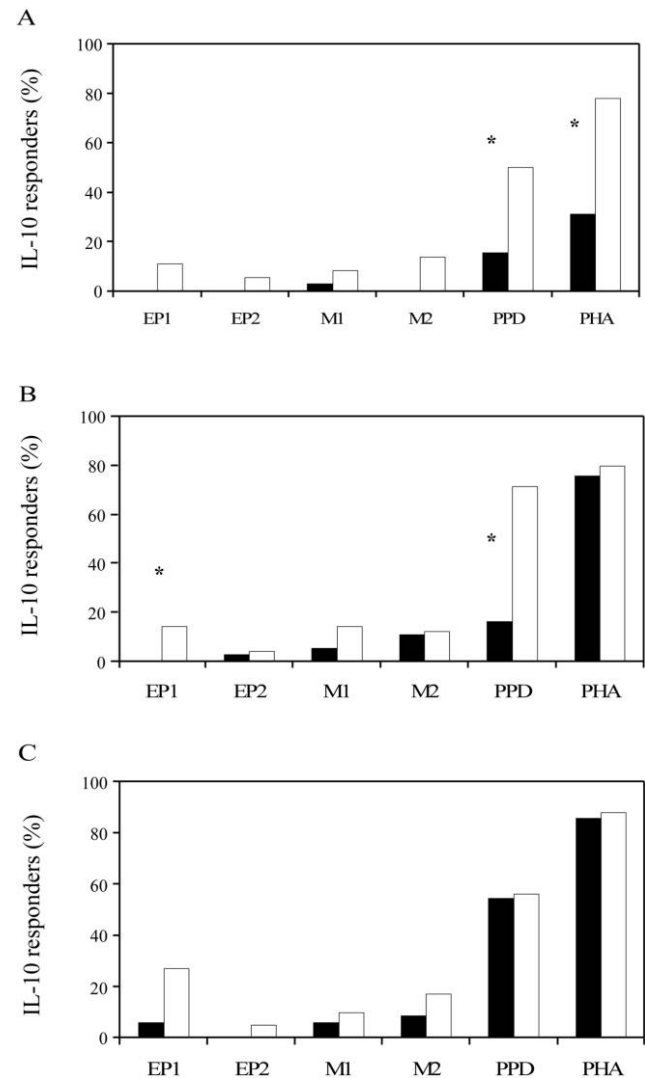


Figure 3. Proportion of interleukin (IL)-10 enzyme-linked immunospot (ELISPOT) responders, by age group. The proportion of IL-10 ELISA responders was compared between study sites according to the following age groups: 1–4-year-olds (A), 5–9-year-olds (B), and 10–14-year-olds (C). Black bars show positive responders from Kisumu, and white bars show positive responders from Nandi. Stimulation conditions included the following: an Epstein-Barr virus (EBV) lytic peptide pool (EP1); an EBV latent peptide pool (EP2); malaria peptides (M1 and M2); purified protein derivative (PPD); and phytohemagglutinin (PHA). Asterisks indicate that significant differences between Kisumu and Nandi children were observed among 5–9-year-olds for EP1 ($P = .009$) and PPD ($P < .001$) and among 1–4-year-olds for PPD and PHA ($P < .001$, for both). Among children 10–14 years old, the P value for the difference between Kisumu and Nandi children for EP1 was .051.

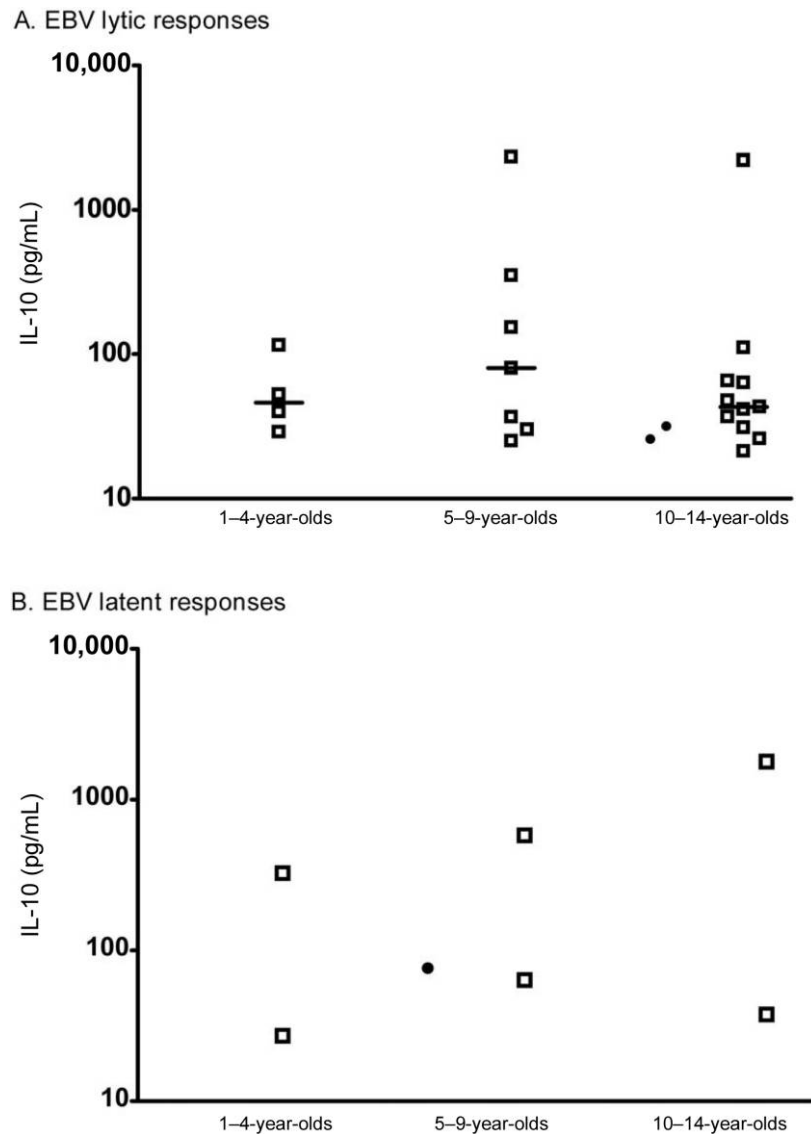


Figure 4. Magnitude of interleukin (IL)-10 enzyme-linked immunospot (ELISPOT) responses, by age group. The magnitude of Epstein-Barr virus (EBV) lytic (A) and latent (B) IL-10 responses was compared by age group and by residence (Kisumu vs. Nandi) for positive responders only. IL-10 responses are expressed as picograms per milliliter. The level of IL-10 for each responder is represented as black circles for Kisumu children and as white squares for Nandi children, and a median bar is shown for each age group.

similar between age groups. There were few or absent IL-10 responses to the EBV lytic and latent peptide pools in the Kisumu children. The Nandi children had a similar paucity of IL-10 responses to the EBV latent peptides.

Concordance of EBV-specific IFN- γ and IL-10 responses.

The concordance of cytokine responses was examined to determine whether IFN- γ nonresponders instead expressed IL-10. We found that the children were able to express both IFN- γ and IL-10 in response to PHA and PPD but that they tended to express either IFN- γ or IL-10 in response to EBV peptides. As shown in figure 3, too few children produced IL-10 in response to the EBV lytic or latent peptides to reach statistical significance, yet only 8 of the 88 children with IFN- γ responses

to the EBV lytic peptides had concomitant IL-10 responses regardless of malaria exposure. Similar findings were observed for responses to the EBV latent peptides. Only 4 of the 71 children with IFN- γ responses also produced IL-10.

DISCUSSION

Malaria has been identified as a cofactor in the etiology of eBL on the basis of the geographic overlap between the prevalence of this childhood cancer and residency in areas of high malaria endemicity. Although earlier work using in vitro regression assays of EBV outgrowth in peripheral blood suggests that malarial infection suppresses antiviral immunity and thereby fa-

vors the emergence of neoplastic B cells, the immune basis of this suppression has not been characterized in detail [22–24]. By comparing IFN- γ and IL-10 responses to EBV epitopes in a cross-section of healthy 1–14-year-old children living in 2 geographically proximate areas of western Kenya in which malaria endemicity ranges from holoendemic (Kisumu) to unstable and epidemic prone (Nandi), we made several observations that add to our knowledge of how malaria alters EBV immunity. First, there was an age-related loss of T cell IFN- γ responses to EBV lytic and latent HLA class I-restricted epitopes in 5–9-year-old children resident in the malaria-holoendemic area relative to younger and older children from the same area as well as children in all age groups from the epidemic-prone area. Second, the magnitude of IFN- γ responses (when a response was present) was not dependent on age or past malaria exposure. Third, IL-10 responses to EBV lytic peptides, PPD, and PHA were significantly less frequent in Kisumu children than in Nandi children, but this difference was resolved in children >9 years old. Considered in the context that the peak age incidence of eBL is 5–9 years [34], these data lend further credence to the notion that intense malaria exposure during the first 9 years after birth suppresses CTL IFN- γ activity and immunosurveillance against EBV-infected B cells at a time when neoplastic B cells are likely to emerge.

A secondary goal of our study was to determine whether the diminished childhood IFN- γ -mediated immunity to EBV associated with holoendemic malaria is antigen specific. On the basis of the observation that the frequency and strength of IFN- γ production in response to MSP-1 peptides, PPD, and PHA were similar for children in all age groups from both Kisumu and Nandi, it appears that the age-related loss of immunity is specific for EBV. These results do not formally exclude the possibility that reduced T cell IFN- γ responses extends to other microorganisms that commonly infect children in sub-Saharan Africa. In this regard, ongoing studies aim to determine whether CD8⁺ T cell immunity to cytomegalovirus, a persistent herpesvirus that is acquired during childhood in rural Africa, is modified in a manner that is distinct from or similar to that of EBV.

The cellular and molecular basis of the age-related changes in EBV immunity reported here, and why in particular T cell IFN- γ responses in 5–9-year-old children are depressed, remain to be determined. Several mechanisms may be considered. First, maturation of dendritic cells in 5–9-year-olds may be impaired as a consequence of chronic malaria exposure, as has been reported for in vitro studies of dendritic cells exposed to blood-stage *P. falciparum* [35]. Second, chronic malaria exposure may alter antigen processing by dendritic cells and other antigen-presenting cells through Toll-like receptor antagonism [36–38]. Third, chronic malaria exposure may lead to increased numbers of regulatory T cells, which decreases IFN- γ production by T

cells. A key feature that these potential immune-evasion strategies do not explain is the “recovery” of effector T cell immunity in 10–14-year-old children. To our knowledge, no studies have investigated the maturation of T cell immunity in children with chronic malarial infection.

We previously quantified EBV loads in 1–14-year-old children from Kisumu and Nandi and found that the highest mean levels were in the youngest age group (1–4 years old) from Kisumu, where malaria transmission is intense and stable [11]. The EBV-specific IFN- γ responses in the youngest age group could thus be generated by an effector T cell population that may not adequately contain viral replication and/or latency. This notion is supported by the lower magnitude of IFN- γ responses to both lytic and latent EBV peptides in the youngest children. In this context, we determined in the study participants described here whether high EBV loads were inversely proportional to EBV-specific IFN- γ responses. No correlation was observed, a finding similar to that reported in studies of the impact of HIV infection on EBV immunosurveillance [39]. Future work will determine whether this lack of correlation is related to transient incompetence or immunosuppression of EBV-specific CD8⁺ T cells—for example, failure to produce IFN- γ in response to cognate antigen or the presence of immunoregulatory T cells that actively suppress cytokine production.

The role played by IL-10 in the pathogenesis of eBL remains, at this point, speculative. We have previously found an inverse relationship between cytotoxicity and IL-10 expression in EBV-specific CD8⁺ T cell clones [19], and others have reported that IL-10 potentiates EBV-mediated B cell transformation by inhibiting memory T cells [40]. With regard to *P. falciparum*, elevated plasma IL-10 levels correlate with greater parasite densities and less-effective parasite clearance in children 1–4 years old [41]. There are also data suggesting that natural malarial infections bias toward Th2-like immunity [42, 43]. We therefore hypothesized that children with chronic malarial infection would have prominent EBV-specific IL-10 responses. Thus, the overall suppression of IL-10 responses in the younger children from the malaria-holoendemic area was unexpected. This suppression could be indicative of a global pattern of T cell exhaustion as a consequence of repeated malaria exposure. The inverse relationship between EBV-specific IFN- γ and IL-10 responses is consistent with a model for dominant T cell responses being either Th1 (IFN- γ) or Th2 (IL-10). However, because there were so few IL-10 responders, the significance of this finding is unclear at this time. A caveat of this interpretation is that we measured only human IL-10 and not the viral IL-10 homologue, BCRF1 [44].

Given that the incidence of eBL is ~2 cases/100,000 children [3], it is not feasible to evaluate directly immune risk factors that precede eBL tumorigenesis. Elucidation of how chronic malaria exposure contributes to the pathogenesis of eBL will

therefore require comparison with age-matched children presenting with eBL and prospective study of EBV immunity in healthy children with differing malaria exposure. This approach will not only advance our understanding of the role played by T cell immunosurveillance in controlling EBV latency but also contribute to understanding how malaria-related immunosuppression affects the development of immunity to other common pathogens and childhood vaccines.

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References

- Burkitt D. A sarcoma involving the jaws in African children. *Br J Surg* **1958**; 46:218–23.
- Bishop PC, Rao VK, Wilson WH. Burkitt's lymphoma: molecular pathogenesis and treatment. *Cancer Invest* **2000**; 18:574–83.
- Rainey JJ, Omenah D, Sumba PO, Moormann AM, Rochford R, Wilson ML. Spatial clustering of endemic Burkitt's lymphoma in high-risk regions of Kenya. *Int J Cancer* **2007**; 120:121–7.
- Dalldorf G. Lymphomas of African children with different forms or environmental influences. *JAMA* **1962**; 181:1026–8.
- Mwanda OW, Rochford R, Moormann AM, Macneil A, Whalen C, Wilson ML. Burkitt's lymphoma in Kenya: geographical, age, gender and ethnic distribution. *East Afr Med J* **2004**; (Suppl 8):S68–77.
- de-The G. The Epstein-Barr virus (EBV): a Rosetta stone for understanding the role of viruses in immunopathological disorders and in human carcinogenesis. *Biomed Pharmacother* **1985**; 39:49–51.
- Fleming AF. The epidemiology of lymphomas and leukaemias in Africa—an overview. *Leuk Res* **1985**; 9:735–40.
- Biggar RJ, Henle G, Bocker J, Lennette ET, Fleisher G, Henle W. Primary Epstein-Barr virus infections in African infants. II. Clinical and serological observations during seroconversion. *Int J Cancer* **1978**; 22: 244–50.
- Henle G, Henle W. Observations on childhood infections with the Epstein-Barr virus. *J Infect Dis* **1970**; 121:303–10.
- Henle W, Henle G. The relation between the Epstein-Barr virus and infectious mononucleosis, Burkitt's lymphoma and cancer of the post-nasal space. *East Afr Med J* **1969**; 46:402–6.
- Moormann AM, Chelimo K, Sumba OP, et al. Exposure to holoendemic malaria results in elevated Epstein-Barr virus loads in children. *J Infect Dis* **2005**; 191:1233–8.
- Souza TA, Stollar BD, Sullivan JL, Luzuriaga K, Thorley-Lawson DA. Peripheral B cells latently infected with Epstein-Barr virus display molecular hallmarks of classical antigen-selected memory B cells. *Proc Natl Acad Sci USA* **2005**; 102:18093–8.
- Rochford R, Hobbs MV, Garnier JL, Cooper NR, Cannon MJ. Plasmacytoid differentiation of Epstein-Barr virus-transformed B cells in vivo is associated with reduced expression of viral latent genes. *Proc Natl Acad Sci USA* **1993**; 90:352–6.
- Crawford DH, Iliescu V, Edwards AJ, Beverley PC. Characterisation of Epstein-Barr virus-specific memory T cells from the peripheral blood of seropositive individuals. *Br J Cancer* **1983**; 47:681–6.
- Thorley-Lawson DA. EBV the prototypical human tumor virus—just how bad is it? *J Allergy Clin Immunol* **2005**; 116:251–61, quiz 262.
- Yao QY, Ogan P, Rowe M, Wood M, Rickinson AB. Epstein-Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carriers. *Int J Cancer* **1989**; 43:67–71.
- Khanna R, Burrows SR. Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases. *Annu Rev Microbiol* **2000**; 54:19–48.
- Rickinson AB, Moss DJ. Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. *Annu Rev Immunol* **1997**; 15:405–31.
- Nazaruk RA, Rochford R, Hobbs MV, Cannon MJ. Functional diversity of the CD8⁺ T-cell response to Epstein-Barr virus (EBV): implications for the pathogenesis of EBV-associated lymphoproliferative disorders. *Blood* **1998**; 91:3875–83.
- Rochford R, Cannon MJ, Moormann AM. Endemic Burkitt's lymphoma: a polymicrobial disease? *Nat Rev Microbiol* **2005**; 3:182–7.
- Rickinson AB, Young LS, Rowe M. Influence of the Epstein-Barr virus nuclear antigen EBNA 2 on the growth phenotype of virus-transformed B cells. *J Virol* **1987**; 61:1310–7.
- Gunapala DE, Facer CA, Davidson R, Weir WR. In vitro analysis of Epstein-Barr virus: host balance in patients with acute *Plasmodium falciparum* malaria. I. Defective T-cell control. *Parasitol Res* **1990**; 76: 531–5.
- Moss DJ, Burrows SR, Castelino DJ, et al. A comparison of Epstein-Barr virus-specific T-cell immunity in malaria-endemic and -nonendemic regions of Papua New Guinea. *Int J Cancer* **1983**; 31:727–32.
- Whittle HC, Brown J, Marsh K, et al. T-cell control of Epstein-Barr virus-infected B cells is lost during *P. falciparum* malaria. *Nature* **1984**; 312:449–50.
- Moss DJ, Rickinson AB, Pope JH. Long-term T-cell-mediated immunity to Epstein-Barr virus in man. I. Complete regression of virus-induced transformation in cultures of seropositive donor leukocytes. *Int J Cancer* **1978**; 22:662–8.
- Gudgeon NH, Taylor GS, Long HM, Haigh TA, Rickinson AB. Regression of Epstein-Barr virus-induced B-cell transformation in vitro involves virus-specific CD8⁺ T cells as the principal effectors and a novel CD4⁺ T-cell reactivity. *J Virol* **2005**; 79:5477–88.
- John CC, Moormann AM, Sumba PO, Ofulla AV, Pregibon DC, Kazura JW. 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. *Infect Immun* **2004**; 72:5135–42.
- Moormann AM, John CC, Sumba PO, Tisch D, Embury P, Kazura JW. Stability of interferon-gamma and interleukin-10 responses to *Plasmodium falciparum* liver stage antigen-1 and thrombospondin-related adhesive protein in residents of a malaria holoendemic area. *Am J Trop Med Hyg* **2006**; 74:585–90.
- Cao K, Moormann AM, Lyke KE, et al. Differentiation between African populations is evidenced by the diversity of alleles and haplotypes of HLA class I loci. *Tissue Antigens* **2004**; 63:293–325.
- Parra M, Hui G, Johnson AH, et al. Characterization of conserved T- and B-cell epitopes in *Plasmodium falciparum* major merozoite surface protein 1. *Infect Immun* **2000**; 68:2685–91.
- Udhayakumar V, Anyona D, Kariuki S, et al. Identification of T and B cell epitopes recognized by humans in the C-terminal 42-kDa domain of the *Plasmodium falciparum* merozoite surface protein (MSP)-1. *J Immunol* **1995**; 154:6022–30.
- Bloland PB, Boriga DA, Ruebush TK, et al. Longitudinal cohort study of the epidemiology of malaria infections in an area of intense malaria transmission. II. Descriptive epidemiology of malaria infection and disease among children. *Am J Trop Med Hyg* **1999**; 60:641–8.
- Snow RW, Omumbo JA, Lowe B, et al. Relation between severe malaria morbidity in children and level of *Plasmodium falciparum* transmission in Africa. *Lancet* **1997**; 349:1650–4.
- Mwanda OW. Burkitt's lymphoma: the initial defining characteristics. *East Afr Med J* **2004**; (Suppl 8):S63–7.
- Urban BC, Ferguson DJ, Pain A, et al. *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature* **1999**; 400:73–7.
- Ocana-Morgner C, Mota MM, Rodriguez A. Malaria blood stage suppression of liver stage immunity by dendritic cells. *J Exp Med* **2003**; 197:143–51.
- Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N.

- Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* **2001**;193:233–8.
38. Wilson NS, Behrens GM, Lundie RJ, et al. Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nat Immunol* **2006**;7:165–72.
39. Piriou E, Jansen CA, Dort K, et al. Reconstitution of EBV latent but not lytic antigen-specific CD4+ and CD8+ T cells after HIV treatment with highly active antiretroviral therapy. *J Immunol* **2005**;175:2010–7.
40. Bejarano MT, Masucci MG. Interleukin-10 abrogates the inhibition of Epstein-Barr virus-induced B-cell transformation by memory T-cell responses. *Blood* **1998**;92:4256–62.
41. Hugosson E, Montgomery SM, Premji Z, Troye-Blomberg M, Bjorkman A. Higher IL-10 levels are associated with less effective clearance of *Plasmodium falciparum* parasites. *Parasite Immunol* **2004**;26:111–7.
42. Lee EA, Palmer DR, Flanagan KL, et al. Induction of T helper type 1 and 2 responses to 19-kilodalton merozoite surface protein 1 in vaccinated healthy volunteers and adults naturally exposed to malaria. *Infect Immun* **2002**;70:1417–21.
43. Reece WH, Plebanski M, Akinwunmi P, et al. Naturally exposed populations differ in their T1 and T2 responses to the circumsporozoite protein of *Plasmodium falciparum*. *Infect Immun* **2002**;70:1468–74.
44. Moore KW, Vieira P, Fiorentino DE, Trounstein ML, Khan TA, Mosmann TR. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRF1. *Science* **1990**;248:1230–4.