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SHORT REPORT

Children with endemic Burkitt lymphoma are deficient in EBNA1-specific IFN-γ T cell responses

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Endemic Burkitt lymphoma (eBL) is the most common childhood cancer in equatorial Africa and is linked to Epstein–Barr virus (EBV) and *Plasmodium falciparum* coinfections early in life. Epstein–Barr nuclear antigen 1 (EBNA1) is the sole viral latent antigen expressed in BL tumors. Loss of EBNA1-specific immune surveillance could allow eBL emergence. Therefore, EBNA1-specific T cell responses were analyzed by IFN-γ ELISPOT in Kenyan children with eBL and compared to healthy children with divergent malaria exposure. Significantly fewer children with eBL, 16% (7/44) had EBNA1-specific IFN-γ responses in contrast to healthy children living in a malaria holoendemic area or in an area with sporadic malaria transmission, 67% (40/60) and 72% (43/60) responders, respectively ($p < 0.003$). Children with eBL maintained IgG_1 dominated antibody responses to EBNA1 similar to healthy children suggesting a selective loss of IFN-γ secreting EBNA1-specific T cells in the presence of intact humoral immunity. $CD8^+$ T cell responses to EBV lytic and latent antigens not expressed in the tumors were similarly robust in eBL patients compared to healthy children. In addition, $CD4^+$ T cell responses to a malaria protein, merozoite surface protein 1, were present in lymphoma patients. This study demonstrates a selective loss of EBNA1-specific T cell responses in children with eBL and suggests a potential immunotherapeutic target for this EBV-associated lymphoma.
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Epstein–Barr virus (EBV) is etiologically associated with a number of malignancies that occur in immune competent hosts including nasopharyngeal carcinoma, mixed-cellularity Hodgkin's disease, and endemic Burkitt lymphoma (eBL).[1] Although there are different patterns of viral gene expression in EBV-associated malignancies, all EBV tumors express the viral latent protein Epstein–Barr nuclear antigen 1 (EBNA1).[1] An important question is how these tumors escape the host immune response when viral antigens are present.

Endemic BL is the most common childhood malignancy in Equatorial Africa and occurs in children between the ages of 2 and 14 years with a peak incidence of 6 years of age.[2] Holoendemic malaria is thought to be an etiologic cofactor in this malignancy. Although the exact mechanisms are unknown, repeated malaria infections in young children are thought to drive B-cell expansion and suppress T cell immunity thus allowing for the emergence of a malignant clone. We recently reported that 5–9 year old children living in a holoendemic malaria region had depressed $CD8^+$ T cell responses against both EBV lytic and latent antigens,[3] supporting a model for malaria driven immune modulation as one potential mechanism. However, nothing is known of the EBV-specific T cell responses in children with eBL.

EBNA1 is the only latent protein expressed in eBL tumors. Escape from immune surveillance by eBL was originally thought to occur because BL cells have defects in MHC Class I antigen processing and the gly-ala repeat domain of the EBNA1 inhibits proteasomal processing and EBNA1 mRNA translation.[4–6] In contrast to this lack of $CD8^+$ T cell recognition of EBNA1 and eBL, recent studies have shown that EBNA1-specific $CD4^+$ T cells can be isolated from nearly all EBV-seropositive healthy adults,[7,8] and these $CD4^+$ T cells are cytolytic for constitutively HLA class II expressing BL cell lines which express EBNA1 protein.[9] However, if the host is able to mount a cytolytic $CD4^+$ T cell response to EBNA1, why do eBL tumors escape host immune control? In this study, we examined EBNA1-specific $CD4^+$ T cell responses in comparison to EBV-specific $CD8^+$ and malaria-specific $CD4^+$ T cell responses in Kenyan children with eBL, and compared these to healthy Kenyan children with divergent patterns of malaria exposure. This allowed us to address the question of whether children with eBL were able to mount an EBNA1-specific T cell response.

Material and methods

Study population and design

From August 2005 to March 2006, we prospectively enrolled 44 Kenyan children admitted to Nyanza Provincial General Hospital (NPGH), Kisumu, Kenya. Confirmation of BL diagnosis was made by histology from a fine-needle aspirate stained with May-Grunewald Giemsa and reviewed by Dr. Margaret Oduor, the hospital's Clinical Pathologist. Blood samples were taken from children with eBL upon admission to the hospital and before administration of the first dose of cytotoxic chemotherapy.

Because eBL is the most prevalent pediatric cancer in this region enrolling enough children diagnosed with cancer other than eBL was not feasible. Therefore, we enrolled 120 healthy children from 2 regions of Kenya with divergent risk for BL and malaria exposure. The first region is Kisumu District where malaria is holoendemic, *i.e.* intense, perennial malaria transmission and the incidence of eBL is high.[2,10] The second region is the Nandi District in the highlands of western Kenya where malaria transmission is sporadic and unstable and the risk of eBL is very low.[2,10] In addition, adults from the Kisumu region were included as a referent group and as a basis of comparison for EBNA1-specific immunity in healthy US adults.[8] The mean ages of children enrolled in this study were 5.7, 6.2 and 6.9 years for Nandi, Kisumu and eBL

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children, respectively. The 3 groups of children were compared for hematological indices and found to have similar values within normal ranges for their age. The mean white blood cell counts were 8.4, 11.7 and 10×10^3 per μl and mean hemoglobin were 10.4, 11.3 and 13.7 gm/dl for Nandi, Kisumu and eBL children, respectively.

Ethical approval for the study was obtained from Institutional Review Boards at Case Western Reserve University (CWRU) and SUNY Upstate Medical University and the Ethical Review Committee at the Kenya Medical Research Institute (KEMRI).

Isolation of peripheral blood mononuclear cells

Peripheral blood was collected in sodium heparinized tubes and transported to the CWRU/KEMRI laboratory located at the Centre for Global Health 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, Invitrogen, Paisley, Scotland, UK) supplemented with 10% heat inactivated human AB serum, 50 μg/ml gentamicin, 10 mM Hepes and 2 mM glutamine for cytokine stimulation assays. Plasma and whole blood was frozen for later serologic and DNA analyses.

Assessment of EBV- and malaria-specific T cell responses by IFN-γ ELISPOT

IFN-γ ELISPOT assays and selection of HLA Class I peptides from EBV lytic (BRLF1, BZLF1, BMLF1) and EBV latent (EBNA 3A, 3B, 3C) antigens has been previously described.[3] Fifty-one overlapping EBNA1 peptides covering the C′ terminal domain (amino acids 400–641) as previously described[8] were used as a total pool (EATP) and 5 subpools consisting of 10–11 peptides in each subpool (EAI-V). IFN-γ responses to phytohemagglutinin (Sigma-Aldrich, St. Louis, MO) at 1 μg/ml and a recombinant blood stage malaria protein (merozoite surface protein-1, MSP1, 42 kDa, 3D7 strain)[11] were controls. The specificity of EBV- and malaria-antigen responses was tested on PBMCs from 20 malaria unexposed adult American volunteers. EBV seropositive American PBMC donors had robust cytokine responses to EBV peptides but lacked responses to malaria peptides (data not shown). PBS was added to 0.5×10^6 PBMCs per well as the negative control and represented the background level of IFN-γ produced by unstimulated cells. The number of spot forming units (SFU) per well was counted using ImmunoSpot Scanning & Imaging Software, Version 4.0 (Pharmingen).

Determination of EBNA1-specific IgG subclass

ELISA for IgG subclass titers were done as described by the manufacturer (Sigma/Diamedix; Miami, FL). Plasma samples were diluted at 1:20 and 1:10 and run in triplicate. EBNA1 and IgG subclass titers were assessed by evaluating plasma samples from patients and healthy volunteers at serial dilutions from 1:25 to 1:3200. After plotting absorption values, the point of inflection was calculated to determine the IgG titer for each subclass.

Determination of EBV viral load

EBV viral load was determined by whole peripheral blood DNA extraction as previously described with minor modifications.[12] For the real-time quantitative PCR, EBV and β-actin were amplified using previously described primer/probes[12] using BioRad MasterMix in the BioRad IQ3 cycler. To quantitate EBV viral load, serial dilutions of cellular DNA obtained form an EBV negative BL line (BL41) were used to generate a standard curve for total cellular DNA. EBV viral load was calculated using serial dilutions of an EBV plasmid containing the amplified EBV region. For statistical analysis, samples below the threshold of detection were changed to 0.5 before natural log (ln) transformation.

Statistical analysis

Analyses were conducted using SAS Version 8.2 (Cary, NC). An IFN-γ ELISPOT was considered positive if the proportion of SFU in the stimulated well was significantly greater compared to the unstimulated background well using a chi-square Fisher's exact test ($p < 0.05$); a more stringent criteria than merely counting a positive response as more than 5 SFC above background or as 2-fold above the background. Proportions of responders were compared across groups (eBL, Kisumu, Nandi) using Logistic Regression Analysis, with Odds Ratios and significance values calculated comparing the percent of eBL responders relative to each the other groups. *p*-Values less than 0.05 were taken to indicate significance whereas *p*-values between 0.06 and 0.08 were taken to be indicative of a trend. Responses to EBV peptides were found only from PBMC of EBV seropositive donors (data not shown for EBV seronegative donors). The magnitude of IFN-γ responses was generated using Analysis of Variance to compare outcomes by group, with *post-hoc* tests set to compare all pairs. Tukey's *post-hoc* were used for pairwise comparisons where we met homogeneity of variance assumption, otherwise Games-Howell was used (which adjusts for heterogeneity of variance).

Results

Children diagnosed with eBL are deficient in EBNA1-specific, IFN-γ producing T cell responses

To evaluate EBV-specific T cell immunity in children with eBL, 3 groups of Kenyan children were enrolled in this study: children diagnosed with eBL ($n = 44$), healthy children living in the holoendemic malaria region of Kisumu District (herein referred to as Kisumu) ($n = 60$), and healthy children living in the epidemic-prone malaria region of Nandi District (herein referred to as Nandi) ($n = 60$). We first asked whether there were differences in frequency of EBNA1-specific, IFN-γ producing T cell responses in the children with eBL compared to healthy children regardless of malaria exposure. To do this, freshly isolated PBMC from each of the groups were stimulated with an overlapping peptide library of the C-terminal domain of EBNA1 (aa400-641), which contains all but one described $CD4^+$ T cell epitopes of this antigen. This peptide library was divided in 5 EBNA1 peptide subpools consisting of 10 or 11 peptides each (EAI, EAII, EAIII, EAIV and EAV), or used as a total pool containing all 51 peptides (EATP). The peptide pools were previously shown to elicit IFN-γ from EBNA1-specific $CD4^+$ T cells in the majority of healthy adult EBV carriers.[8] Responses to the EBNA1 peptides were measured by IFN-γ ELISPOT assays. The proportion of positive responders in each group was compared and is presented in Table I. Children with eBL had significantly fewer IFN-γ responders to the total pool of EBNA1 peptide (7 of 44) compared to healthy Nandi (43 of 60) and Kisumu (40 of 60) children. When we examined responses to the EBNA1 peptide sub-pools (*i.e.* EAI-V), we also observed fewer responders in the eBL patients compared to the healthy children from both Nandi and Kisumu for all pools although this achieved statistical significance for only 1 of the 5 pools for Kisumu and 3 of 5 pools for Nandi children (Table I). Overall, the odds of having an IFN-γ response to EBNA1 are significantly greater in healthy children compared to children with eBL (Table I).

When PBMCs isolated from the eBL patients were stimulated with PHA, a general immune stimulant, 95% responded, similar to percent response observed in Kisumu (97%) and Nandi (92%) children, suggesting that children with eBL were not globally immune deficient. As a second test of immune competency, the proportion of eBL children responding to the malaria antigen MSP-1-primarily recognized by $CD4^+$ T cells[11]—was similar to the proportion of responders observed in both Kisumu children (36.4% and 25.0%, respectively) who reside in a malaria holoendemic area (Table I). The proportion of responders from Nandi was lower with only 13.8% responders as would be expected

TABLE I – PERCENT IFN-γ RESPONDERS FOLLOWING STIMULATION OF PBMC WITH EBNA1 PEPTIDE POOLS (*i.e.*, EATP, EAI, EAII, EAIII, EAIV, EAV), MALARIA RECOMBINANT PROTEIN MSP1 (3D7), OR EBV-POOLED PEPTIDES (EP1/EP2)

eBL[1]		Kisumu			Nandi		
Antigens	% Positive (total $n = 44$)	% Positive (total $n = 60$)	Odds ratio	p-Value	% Positive (total $n = 60$)	Odds ratio	p-Value
EATP	16.1	66.7	10.57	0.00	71.7	13.37	0.00
EAI	4.5	11.7	2.77	0.22	23.7	6.53	0.02
EAII	13.6	30.0	2.71	0.06	25.4	2.16	0.15
EAIII	13.6	41.7	4.52	0.00	44.1	4.99	0.00
EAIV	6.8	15.0	2.41	0.21	20.3	3.49	0.07
EAV	4.5	15.0	3.71	0.11	22.0	5.94	0.02
MSP1 (3D7)	36.4	25.0	0.58	0.21	13.8	0.28	0.01
EP1/EP2	43.2	30.0	0.56	0.17	50.8	1.36	0.44

[1]Reference group for comparison to Kisumu and Nandi groups.

from children living in an area with unstable malaria transmission ($p = 0.01$).

We next compared the capacity of children with eBL and healthy children to respond to known EBV-specific $CD8^+$ T cell epitopes using peptide pools (EP1/EP2) that were previously described and contain peptides for both EBV lytic (BRLF1, BZLF1, BMLF1) and latent (EBNA3A, 3B and 3C) proteins.[3] No differences in the frequencies of IFN-γ responses to EBV-specific $CD8^+$ T cell epitopes in children with eBL compared to Kisumu or Nandi children were observed (Table I) suggesting that EBV $CD8^+$ T cell responses are not deficient in the children with eBL. Our previous results showed fewer IFN-γ responses to EBV-specific $CD8^+$ T cell epitopes restricted to 5–9 year old Kisumu children compare to Nandi children.[3] The healthy children examined here spanned the same age range (*i.e*. 2–14 years) as the eBL children. Therefore the age-specific (5–9 year olds) deficiency in IFN-γ responses to EBV-specific $CD8^+$ T cell epitopes was balanced by responses from the older and younger Kisumu children included in this analysis. When we examined IFN-γ EBV-specific $CD8^+$ T cell responses only from 5 to 9 year old eBL children, they were comparable to the healthy non-malaria exposed healthy 5–9 year olds from Nandi (data not shown).

Although the frequency of EBNA1-specific IFN-γ responders was lower in eBL children, we wanted to determine if the magnitude of this response in the 7 eBL children who did respond was similar to children from Kisumu or Nandi. Comparisons between eBL, Kisumu and Nandi children in the magnitude of their IFN-γ producing T cell responses to the individual EBNA1 peptide pools, the EBNA total pool (EATP) and to the malaria recombinant protein, MSP1 are shown in Figure 1. We found magnitude of IFN-γ responses to EATP to be significantly lower in eBL children compared to Nandi ($p < 0.001$) and Kisumu ($p < 0.001$) children, whereas Kisumu and Nandi children did not differ from each other ($p = 0.562$). When examining each EBNA1 peptide subpool, we found a significant difference overall between the 3 groups for EAI ($p < 0.003$) but the *post-hoc* comparisons revealed that Nandi children had significantly higher IFN-γ levels compared to eBL ($p < 0.012$) and Kisumu ($p < 0.02$) children, while the eBL and Kisumu children were not significantly different from one another ($p = 0.701$). For the EAIII pool there was a significant difference overall ($p < 0.002$), with *post-hoc* comparisons revealing that responses in eBL children were significantly lower than in Nandi ($p < 0.003$) and Kisumu ($p < 0.011$) children, whereas the level of responses between the Kisumu and Nandi children did not differ ($p = 0.591$). This was also true for EAV, with a significant difference overall ($p < 0.007$) and *post-hoc* comparisons showing the level of IFN-γ responses from eBL children significantly lower than from Nandi ($p < 0.007$) and Kisumu ($p < 0.029$) children, but healthy children with divergent malaria exposure did not differ from one another ($p = 0.836$). There were no significant differences in IFN-γ responses to EAII and EAIV among the 3 groups.

When we compared the magnitude of IFN-γ expressed to MSP1, children with eBL had a significantly higher mean cytokine level compared to Kisumu and Nandi children ($p < 0.05$) as shown in Figure 1, demonstrating that children with eBL were not deficient in other $CD4^+$ T cell mediated responses.

Children with eBL have similar humoral immune response to EBNA1 as healthy children

One possible explanation for the inability of eBL patients to respond to EBNA1 peptides is that the T cell mediated IFN-γ response to EBNA1 was absent before BL tumorigenesis. If this was the case, then we would predict an absence of EBNA1 specific IgG_1 antibodies, because Th1 $CD4^+$ T cells assist B cells to make IgG_1 antibodies. To evaluate this possibility, IgG isotype specific ELISAs were done to determine if children with eBL had an EBNA1 antibody response and if so, what the dominant IgG isotype of this response was. Children with eBL produced IgG_1 subclass antibodies to EBNA1 similar to healthy children and adults (Fig. 2). This also argues against Th2 polarization by means of alternate cytokines, such as IL-10, being produced in response to EBNA1. Interestingly, the levels of anti-EBNA1 IgG1 were higher in the eBL patients than in the other groups. IgG_4 was detected in a small percentage of children with eBL but not IgG_2 or IgG_3. IgG_2 was only detected in one of the Nandi children (Fig. 2).

EBV viral load tends to be higher in children without EBNA1-specific IFN-γ responses

A recent study suggested that control of EBV correlates with $CD4^+$ T cell rather than $CD8^+$ T cell responses.[13] To test whether there was a link between EBNA1-specific, IFN-γ producing T cell responses and EBV viral load, we extracted DNA from whole blood and measured EBV viral load by RTQ-PCR.[12] The mean EBV viral load was then compared within each group of children stratified by EBNA1-specific IFN-γ response (Fig. 3). EBV viral load data were positively skewed, with a few extremely high values, and several very low or undetectable levels. As such, we analyzed natural log transformed EBV viral load using distribution-free nonparametric statistics. We employed the Mann–Whitney nonparametric statistic to compare IFN-γ producing EBNA1 (*i.e.* EATP) responders to nonresponders within our 3 groups (Nandi, Kisumu, eBL), with Fischer's exact tests of significance. A near-significant trend emerged for eBL and Kisumu children, showing that EBV viral load tended to be higher in children without EBNA1-specific IFN-γ responses in these 2 groups.

Discussion

Children in Equatorial Africa are at high risk for eBL, and this is linked to both holoendemic malaria exposure and early-age of EBV infection.[14] A long-standing hypothesis is that the repeated malaria infections experienced by children living in malaria

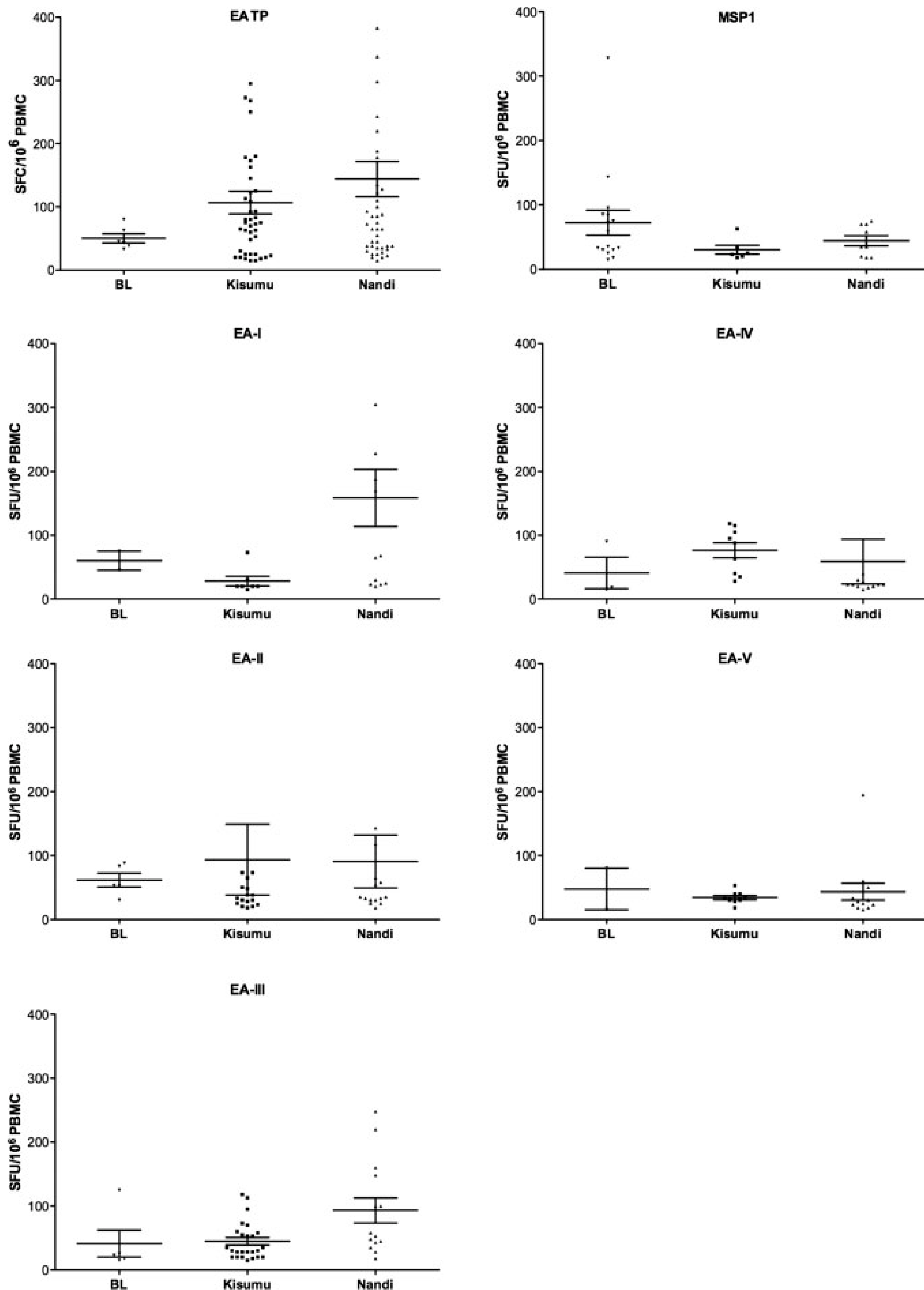
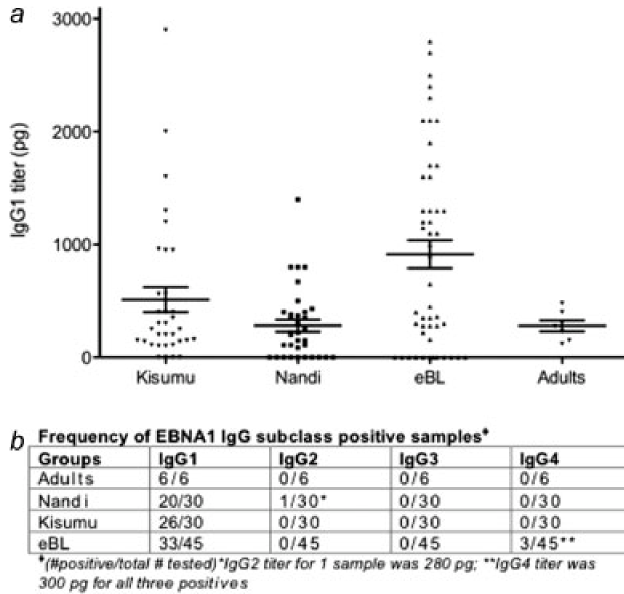


Figure 1 – Comparison of the magnitude of IFN-γ ELISPOT responses in children with eBL and healthy children. The magnitude of IFN-γ ELISPOT responses was compared between children with eBL ($n = 44$) and healthy children from Kisumu ($n = 60$) and Nandi ($n = 60$) for EBNA1 total peptide pool (EATP) and EBNA1 sub-pools, (*i.e*., EAI, EAII, EAIII, EAIV, EAV) and for malaria antigen MSP1 (3D7). IFN-γ spot forming units (SFU) minus PBS control SFU per 1×10^6 PBMCs are displayed (range of SFU in PBS wells was 0–12 SFU/10^6 PBMCs). Only responses above background are shown. Each dot represents an individual sample. Lines indicate the mean and SEM for each pool tested. There were significantly lower EATP levels in eBL children compared to Nandi ($p < 0.001$) and Kisumu ($p < 0.001$) children, and Kisumu and Nandi children did not differ from each other ($p = 0.562$). Significantly lower responses for children with eBL were also observed for EBNA1 sub-pools EAI, EAIII and EAV whereas there were no differences in responses for EAII and EAIV. For MSP1, children with eBL had significantly higher IFN-γ responses compared to Kisumu and Nandi children ($p < 0.05$).



Frequency of EBNA1 IgG subclass positive samples*

Groups	IgG1	IgG2	IgG3	IgG4
Adults	6/6	0/6	0/6	0/6
Nandi	20/30	1/30*	0/30	0/30
Kisumu	26/30	0/30	0/30	0/30
eBL	33/45	0/45	0/45	3/45**

*(#positive/total # tested) *IgG2 titer for 1 sample was 280 pg; **IgG4 titer was 300 pg for all three positives

FIGURE 2 – Mean EBNA1 IgG1 subclass levels in different populations. (*a*) Mean EBNA1 IgG1 levels in plasma were determined by ELISA for healthy Kisumu and Nandi children, children with eBL and healthy adults. Scatter plot show individual points for IgG1 titer and SEM is indicated for each group. (*b*) Frequency of EBNA1 IgG subclass positive samples. The frequency of response is shown for each EBNA1 IgG isotype tested.

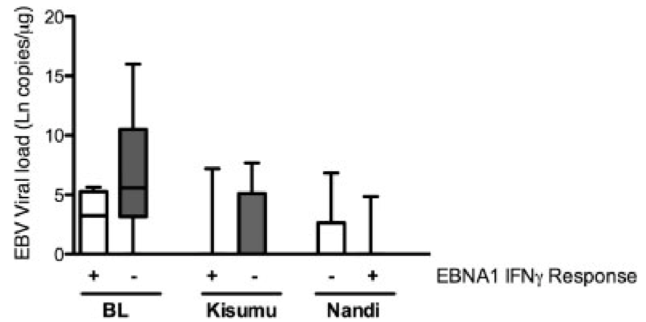


FIGURE 3 – Mean EBV viral load stratified by EBNA1-specific IFN-γ ELISPOT response among eBL patients and healthy children with divergent malaria exposure histories. EBV viral load was natural log transformed and then the mean EBV viral load was determined. The mean EBV viral load was compared within each group of children (eBL, Kisumu and Nandi) stratified by EBNA1-specific total pool (EATP) IFN-γ responders and non-responders (indicated as EBNA1 IFN-γ response). A near-significant trend emerged for eBL and Kisumu (but not the Nandi children). EBV viral load tended to be higher in children without IFN-γ responses to EBNA1 in these 2 groups.

holoendemic regions suppresses EBV specific T-cell immunity. Our earlier studies have supported this malaria-induced immune modulation model where we showed that 5–9 year old children, living in malaria holoendemic regions have diminished EBV-specific CD8$^+$ T cell responses relative to children living in malaria epidemic regions. What was not known was whether this diminished response also extended to children with eBL. In addition, although the EBV-specific CD8$^+$ T cell responses are targeted towards several of the EBV latent and lytic proteins, only the EBNA1 protein is expressed by eBL tumors. We show here that when children with eBL have a comparable EBV-specific CD8+ T cell response to healthy children, the eBL patients show a selective loss of the EBNA1-specific, IFN-γ producing T cells. EBNA1-specific CD4$^+$ T cells suppress tumor growth in a BL mouse model,[15] suggesting that the absence of IFN-γ responses to EBNA1 in eBL patients might allow the emergence of the malignant clone. When the loss of the EBNA1-specific IFN-γ response occurred cannot be determined from a cross-sectional study such as this. However, the presence of EBNA1 IgG in most of the eBL patients is suggestive that these children had an EBNA1 IFN-γ response at some point before the emergence of the tumor. The key question will be to understand why that loss occurred. One possibility is that EBV viral loads CD4$^+$ T cell responses may be exquisitely sensitive to the immunoregulatory influence of *P. falciparum*, particularly with respect to dendritic cell function[16] and that children chronically or repeated infected with malaria have impaired antigen presentation whereby T cell responses to EBV antigens are impaired due to a bystander effect. Alternatively, the high levels of EBNA-1 protein expressed by BL tumors could downregulate the IFN-γ T cell response due to T cell exhaustion.

In this study we compared T responses in children from Kisumu region (both eBL patients and healthy children) and children from the Nandi region. Although it was not possible to HLA type the study participants, previous work by our group has shown that there is no significant skewing of the HLA types between these 2 populations.[17] In addition, the EBNA-1 total pool of peptides span the entire C-terminal region of the protein encompassing all possible epitopes with the potential to be presented by any HLA type. Thus, the lack of EBNA1 IFN-γ responders in eBL patients is unlikely to be due to variance in HLA types between populations. We also examined whether there were clinical differences (*e.g.*, site of tumor location, response to treatment) between the 7 of 44 children that did respond and those that did not however, no differences were noted between the 2 groups (data not shown).

We found that children with elevated EBV viral load tended to lack EBNA-1 specific IFN-γ responses. Because EBNA1 is the only antigen expressed in latently infected memory B cells, this would suggest that those children with lower EBNA1 CD4$^+$ T cell responses might have a more difficult time controlling EBV persistence. Clearly more data is necessary but the trend we have observed is novel and intriguing. Ongoing longitudinal studies in our laboratories will allow us to more clearly define the temporal association between elevated EBNA1 antibody levels, EBV-specific T cell responses and EBV viral loads.

The EBNA-1 protein has gly-ala repeats that prevent processing and proper antigen presentation via MHC Class I.[4–6] However, there could be cross-reactive epitopes that are exogenously presented to CD8$^+$ T cells as has been described in mouse models.[18] So we cannot exclude a loss of EBNA1 to CD8 epitopes in our eBL patients. Of note, however, is that the eBL patients were able to respond to EBV peptide pools that contain epitopes specific for well described EBV CD8$^+$ T cell antigens (*e.g.* BRLF1, BZLF1, BMLF1, EBNA-3A, -3B, -3C) thus suggesting that the lack of EBNA1-specific IFN-γ responses in the eBL patients are likely to be CD4$^+$ T mediated. In addition, the eBL patients mounted robust IFN-γ responses to the malaria antigen, which is mediated by CD4$^+$ T cells.[19]

Consistent with our result in eBL, the report by Heller et al. show that there is loss of EBNA1-specific CD4$^+$ T cell responses in patients with EBV-positive,[20] but not EBV-negative Hodgkin lymphoma. Together, these studies argue that selective loss of EBNA1-specific IFN-γ response may be critical to the emergence of EBV-associated lymphomas. It is tempting to speculate that the absence of EBNA1- specific CD4$^+$ T cells predisposes EBV carriers to lymphoma development, even though the mechanisms leading to this select dysfunction most likely differ between eBL and Hodgkin lymphoma. Longitudinal studies of eBL patients will determine if restoration of EBNA1-specific T cell responses is associated with long-term survival. A universal loss in EBNA1-specific immune control could be exploited for immunotherapeutic approaches that induce cytolytic responses to EBNA1 in patients with EBV-associated malignancies.

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Author contributions: AMM designed experiments and wrote the manuscript; KNH performed EBNA1 ELISAs; KC performed ELISPOTS, PE performed ELISPOTS, RP-S performed biostatistical analysis; JO recruited patients for study and provided clinical input; MO performed histopathology; CM designed experiments; and RR designed experiments and wrote the manuscript.

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