The Association between the Comprehensive Epstein–Barr Virus Serologic Profile and Endemic Burkitt Lymphoma

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ABSTRACT

Background: The discovery of Epstein–Barr virus (EBV) in Burkitt lymphoma tumors represented the first link between a virus and cancer in humans, but the underlying role of this virus in endemic Burkitt lymphoma remains unclear. Nearly all children in Burkitt lymphoma–endemic areas are seropositive for EBV, but only a small percentage develop disease. Variation in EBV-directed immunity could be an explanatory cofactor.

Methods: We examined serum from 150 Burkitt lymphoma cases and 150 controls using a protein microarray that measured IgG and IgA antibodies against 202 sequences across the entire EBV proteome. Variation in the EBV-directed antibody repertoire between Burkitt lymphoma cases and controls was assessed using unpaired *t* tests. ORs quantifying the association between anti-EBV IgG response tertiles and Burkitt lymphoma status were adjusted for age, sex, and study year.

Results: Thirty-three anti-EBV IgG responses were elevated in Burkitt lymphoma cases compared with controls ($P \leq 0.0003$). Burkitt lymphoma–associated IgG elevations were strongest for EBV proteins involved in viral replication and antiapoptotic signaling. Specifically, we observed ORs $\geq$4 for BMRF1 (early antigen), BBLF1 (tegument protein), BHRF1 (Bcl-2 homolog), BZLF1 (Zebra), BILF2 (glycoprotein), BLRF2 [viral capsid antigen (VCA)p23], BDLF4, and BFRF3 (VCAp18). Adjustment for malaria exposure and inheritance of the sickle cell variant did not alter associations.

Conclusions: Our data suggest that the anti-EBV serologic profile in patients with Burkitt lymphoma is altered, with strong elevations in 33 of the measured anti-EBV IgG antibodies relative to disease-free children.

Impact: The Burkitt lymphoma–specific signature included EBV-based markers relevant for viral replication and antiapoptotic activity, providing clues for future Burkitt lymphoma pathogenesis research.

Introduction

The suspicion of Dennis Burkitt that a pathogen was responsible for the rapid-onset pediatric tumors he observed in Ugandan children led to the 1964 discovery of the first virus linked to a human cancer, Epstein–Barr virus (EBV; ref. 1). In addition to Burkitt lymphoma, EBV has been linked over the past approximately 50 years to additional lymphomas, including a subset of Hodgkin and non-Hodgkin lymphoma, as well as epithelial carcinomas of the stomach and nasopharynx (2, 3). Despite progress toward understanding the extent to which this oncogenic virus contributes to the global cancer burden, the specific role of EBV in the pathogenesis of Burkitt lymphoma, the first identified EBV-related tumor, remains enigmatic. Whereas previous research supported limited EBV protein expression in Burkitt lymphoma tumors (4, 5), recent work in Burkitt lymphoma cell lines provides evidence of a broader EBV proteome associated with this disease (6).

Early reports following the discovery of EBV demonstrated that Ugandan children with higher levels of antibody against the viral capsid antigen (VCA IgG titers) were more likely to develop Burkitt lymphoma (7). These data were used as supportive evidence of a causal role of EBV in Burkitt lymphoma tumors. However, antibody-based work to identify comprehensive serologic patterns that associate with this pediatric tumor have not been conducted, with research to date focusing on immune responses to less than five of the nearly 100 EBV transcripts (8–10). Recently developed protein microarray technology capable of measuring antibodies targeting the full EBV proteome provides a unique tool to fill this knowledge gap (11, 12). We utilized this multiplex technology, targeting antibody responses to 202 peptide sequences representing 86 EBV proteins, to probe serum from 300 Ghanaian children, including 150 with endemic Burkitt lymphoma.

Materials and Methods

The sera were collected during a previously described study conducted in Ghana by the NCI (13, 14) between 1965 and 1994. Relevant to the project described here, we selected 150 children diagnosed with histologically or cytologically confirmed Burkitt lymphoma (age range, 0–17 years) and 150 apparently healthy control children, frequency-

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matched to cases on sex, age (5-year intervals), and enrollment period (10-year intervals). Cases and controls were enrolled into the original study after obtaining permission from a parent or guardian; children at least eight years of age also provided individual assent. All laboratory testing was conducted under a protocol approved by the QIMR Berghofer Medical Research Institute Human Research Ethics Committee and James Cook University (Cairns, Australia).

EBV protein microarray

Sera were evaluated using a protein microarray targeting both IgG and IgA antibodies against 199 EBV protein sequences representing nonredundant open-reading frames and predicted splice variants in 86 proteins from five EBV strains (AG876, Akata, B95-8, Mutu, and Raji; refs. 11, 12). We also included three synthetic EBV peptides for which circulating antibodies are putative cancer biomarkers [ref. 15; VCAp18, Epstein–Barr nuclear antigen (EBNA)-1, and early antigen (EAd) p47], bringing the total number of anti-EBV probes measured on the array to 202. Details of each sequence printed on the array have been published and are available upon request (16).

Each child's serum was tested on a single microarray that included laboratory controls and four "no DNA" (no translated protein) spots to assess person-specific background. After testing, slides were scanned on an Axon GenePix 4300A (Molecular Devices); raw fluorescence intensities were corrected for spot-specific background; corrected data were transformed using variance stabilizing normalization (vsn) in Gmine; and output was standardized to person-specific background (mean +1.5 SD of four "no DNA" spots). Positivity was defined as a standardized signal intensity $\geq$1.0. The standardized signal intensity for each spot was further grouped into tertiles (three categories), with cutoffs for the categories defined using equal thirds of the antibody distribution among the 150 controls.

Twenty-five samples were tested in duplicate, blinded to laboratory personnel, to assess assay reproducibility specific to this study population. The average coefficient of variation (CV) across the 202 EBV sequences was 17% [interquartile range (IQR), 14%–20%] for IgG and 15% (IQR, 8%–16%) for IgA.

Malaria antibody testing and sickle cell carriage

We utilized available data on malaria antibody status from a subset of samples included in earlier studies (17). In brief, IgG antibody data were available from ELISA targeting two blood-stage vaccine candidates (SE36 and MSP1), one blood-stage diagnostic antigen (HRPII), and the cutaneous-stage diagnostic antigen 6NANP. Malaria antibody output was categorized using previously published cutoffs; samples with ELISA arbitrary unit (AU) readings less than the assay limit of quantification were classified as negative, and elevated versus low categories were defined using the AU distribution among nondiseased control children (17).

Because malaria resistance genes can also influence risk of infection and parasite load, we leveraged information from 173 children that detailed inheritance of the hemoglobin variant associated with sickle cell disease—rs334 in the *HBB* gene. A glutamic acid to valine change at amino acid seven (Glu7Val) results in the production of hemoglobin S, as opposed to hemoglobin A, and is associated with malaria symptom severity. Laboratory details on Sanger sequencing done on genomic DNA extracted from 400 μL of blood with Qia MinElute Virus Spin kit (Qiagen) have been described previously (18).

Statistical approach

We filtered out antibody responses that did not meet a CV threshold of 20%, excluding 47 IgG and 19 IgA antibodies. For the remaining 338 antibodies (155 IgG and 183 IgA), differences in the mean standardized signal intensity between children with Burkitt lymphoma and disease-free controls were assessed using an unpaired Student *t* test. Case–control differences were considered statistically significant at the $P \leq 0.0003$ threshold (equivalent to Bonferroni-corrected $P \leq 0.05$). ORs quantifying the association between the three-level categorical variable for each antibody and Burkitt lymphoma status were estimated using logistic regression models adjusted for sex, age, and study year. P_{trend} values were calculated from a model with each three-level antibody marker treated as an ordinal variable. To determine whether EBV–Burkitt lymphoma associations were confounded by other potential Burkitt lymphoma risk factors, we included the malaria antibodies and sickle cell variant described above as covariates in logistic regression models. In addition, we included interaction terms (i.e., the cross product of anti-EBV and antimalaria antibodies) in regression models to determine whether the anti-EBV IgG response was differentially associated with Burkitt lymphoma based on malaria exposure.

Prediction analytics were used to ascertain which of the 33 anti-EBV IgG antibodies with case–control differences meeting the $P \leq 0.0003$ threshold were most informative for classifying children as having Burkitt lymphoma or being disease-free. The dataset of 300 children was split into the training (60% of data) and validation (40% of data) sets. Random forest models were executed using R software in the training set to identify which IgG antibodies had the highest mean decrease accuracy, a metric reflecting each variable's importance in classifying Burkitt lymphoma status. This process was repeated 100 times in the training set, and the list was narrowed down to the four anti-EBV IgG antibodies determined to be the most important classifiers in at least 50% of the 100 iterations. We then used a support vector machine (SVM) in the validation set (40% of the original data; $N = 120$) to test how well this parsimonious immune signature discriminated Burkitt lymphoma status. Specifically, the SVM was trained using 30 randomly selected samples from the validation set, and the signature's accuracy for classifying Burkitt lymphoma status was assessed in the remaining 90 samples. This process was repeated 40 times, and the overall performance of the immune signature was summarized by averaging the area under the receiver operating curve (AUC) across these repetitions.

Among controls, we evaluated whether a history of malaria exposure or inheritance of the sickle cell disease variant was associated with the level of anti-EBV antibody response using log-linear models (SAS PROC GENMOD, log link). Restriction of these analyses to controls allowed us to examine the correlations in the absence of potential disease effects (i.e., influence of disease on the IgG response).

Results

This study characterized the EBV antibody profile in sera from 150 Ghanaian children with endemic Burkitt lymphoma and 150 disease-free children who were frequency matched to cases on age, sex, and year of blood draw (**Table 1**). Approximately two thirds of the children included in this study were males, and the average age at blood draw (age at Burkitt lymphoma diagnosis for cases) was 8.3 years (IQR, 6–10 years). The vast majority of children mounted a detectable IgG response to the synthetic EBNA-1 peptide (controls = 83%; cases = 77%), reflecting ubiquitous early-life exposure to EBV in this setting.

Although most children harbored evidence of some exposure to the virus, an examination of immune profiles against the full complement

Table 1. Characteristics of children in the study population, by Burkitt lymphoma (BL) status.

Participant characteristics	Total ($N = 300$)	BL cases ($n = 150$)	Controls ($n = 150$)
Sex			
Male	190 (63.3%)	95 (63.3%)	95 (63.3%)
Female	110 (36.7%)	55 (36.7%)	55 (36.7%)
Age in years			
0–5	63 (21.0%)	31 (20.7%)	32 (21.3%)
6–7	73 (24.3%)	38 (25.3%)	35 (23.3%)
8–9	76 (25.3%)	37 (24.7%)	39 (26.0%)
10–17	88 (29.3%)	44 (29.3%)	44 (29.3%)
Year of diagnosis			
1968–1973	60 (20.0%)	31 (20.7%)	29 (19.3%)
1974–1981	142 (47.3%)	72 (48.0%)	70 (46.7%)
1982–1992	98 (32.7%)	47 (31.3%)	51 (34.0%)
Elevated malaria antibodies[a]			
SE36	131 (43.7%)	56 (37.3%)	75 (50.0%)
MSP1 (SE0203)	171 (57.0%)	84 (56.0%)	87 (58.0%)
HRPII	131 (43.7%)	64 (42.7%)	67 (44.7%)
6NANP	159 (53.0%)	70 (46.7%)	89 (59.3%)
Sickle cell disease variant, rs334			
AA genotype	152 (87.9%)	97 (89.8%)	55 (84.6%)
AS genotype[b]	21 (12.1%)	11 (10.2%)	10 (15.4%)

[a]SE36 and MSP1 are vaccine candidate antibodies with previously reported inverse associations with Burkitt lymphoma; HRPII is a marker of blood-stage malaria infection; 6NANP is a cutaneous-stage diagnostic antigen.
[b]The S allele corresponds to sickle cell–associated variant rs334 in the *HBB* (hemoglobin) gene, with data available from 173 children in this study.

of viral proteins demonstrated marked differences in the degree of EBV exposure between children with versus without Burkitt lymphoma. Case–control comparisons of the mean standardized signal intensity for each of the 202 anti-EBV antibodies on the array revealed nominal ($P < 0.05$) elevations in 91 IgG and 3 IgA antibodies. Thirty-three of the anti-EBV IgG antibodies remained significantly elevated in endemic Burkitt lymphoma cases after adjustment for multiple testing ($P \leq 0.0003$). **Figure 1A** illustrates this pattern of anti-EBV IgG elevation specific to Burkitt lymphoma cases and highlights the three antibodies with the most pronounced P values, including anti-BHRF1 (Bcl-2 homolog) IgG and anti-BMRF1 (EA) IgG from both the *ag876* and *mutu* EBV strains.

The Burkitt lymphoma–associated IgG signature included immune responses to transcripts across the full viral proteome, including significant ($P \leq 0.0003$) responses against five distinct phases of the viral life cycle (**Fig. 1B**). After adjustment for the child's age, sex, and year of blood draw, the strongest associations between the anti-EBV IgG response and Burkitt lymphoma ($OR_{\text{highest vs. lowest tertile}}$ 4.5–7.0) were observed for antibodies targeting proteins involved in viral replication and virion production [BMRF1 (EA) and BBLF1(tegument protein)], antiapoptotic functions [BHRF1(Bcl-2 homolog)], and the switch from latent phase to active, lytic replication [BZLF1 (Zta); **Table 2**]. In addition, elevated anti-EBV IgG targeting a glycoprotein (BILF2), a late lytic-cycle regulatory protein (BDLF4), and two components of the viral capsid [BLRF2(VCAp23) and BFRF3 (VCAp18)] were associated with at least a 4-fold increase in Burkitt lymphoma ($OR_{\text{highest vs. lowest tertile}} \geq 4.0$).

We separated our data into training and validation sets to identify four of the 33 $P \leq 0.0003$ EBV targets that were most informative for classifying Burkitt lymphoma status: BHRF1 (Bcl-2 homolog), BMRF1 (EAp47), BBLF1 (tegument protein), and BZLF1 (ZTA). The average performance [area under the receiver operating curve (AUC)] of this four-marker IgG immune signature for classifying a child as being a Burkitt lymphoma cases versus a disease-free control was 0.76 (range, 0.6–0.9).

Notably, the elevated anti-EBV IgG profile in Burkitt lymphoma cases persisted after accounting for malaria metrics, with no appreciable change in the OR trends after inclusion of antimalaria IgG antibody or inheritance of the sickle cell genetic variant in regression models (Supplementary Figs. S1 and S2). However, we did observe

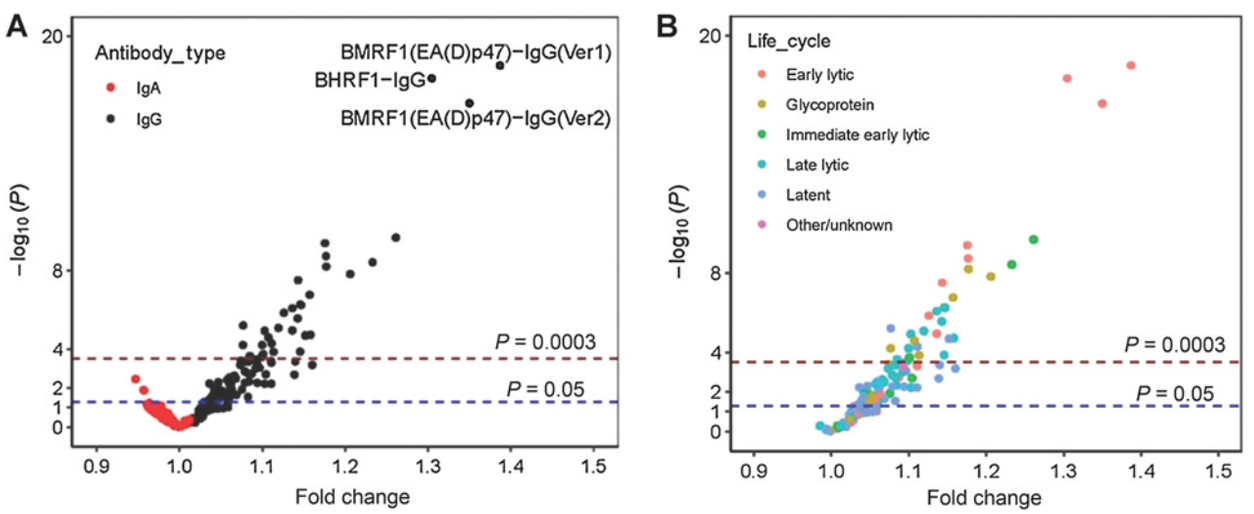

Figure 1.
A, The *x*-axis displays the fold change (case vs. control ratio of standardized signal intensity) for all antibodies with CV ≤ 20%. The *y*-axis illustrates the *P* value corresponding to the case versus control mean difference *t* test. A total of 33 IgG markers were elevated in children with Burkitt lymphoma relative to controls at the $P \leq 0.0003$ (Bonferroni $P < 0.05$) threshold. The probes with the three lowest *P* values are highlighted. Responses are restricted to IgG antibody in **B** and are color coded to indicate the phase of the viral life cycle represented by each array probe/EBV protein. Ver1: ag876 strain; Ver2: mutu strain.

Table 2. OR and 95% CI for the association between anti-EBV antibody level and Burkitt lymphoma (BL) in Ghanaian children.

EBV protein and array sequence[a]	*t* test *P*	BL mean (SD)	Control mean (SD)	BL positivity	Control positivity	OR tertile 2 (95% CI)[b]	OR tertile 3 (95% CI)	*P*-trend
BMRF1 [EA(D)_p47] YP_001129454.1-67745-68959	<0.0001	1.27 (0.39)	0.92 (0.21)	69.3%	32.0%	1.80 (0.85–3.82)	7.03 (3.58–13.8)	<0.0001
BMRF1 [EA(D)_p47] AFY97929.1-67486-68700	<0.0001	1.26 (0.39)	0.93 (0.20)	68.7%	33.3%	2.03 (0.98–4.19)	6.79 (3.49–13.2)	<0.0001
BBLF1 (Tegument protein) YP_001129480.1-109516-109289	<0.0001	1.31 (0.29)	1.12 (0.23)	87.3%	70.7%	1.36 (0.67–2.76)	5.64 (2.98–10.7)	<0.0001
BHRF1 (Bcl-2 homolog) YP_001129442.1-42204-42779	<0.0001	1.18 (0.31)	0.90 (0.18)	68.7%	24.0%	0.70 (0.32–1.49)	4.96 (2.71–9.08)	<0.0001
BBLF1 (Tegument protein) AFY97956.1-108555-108328	<0.0001	1.27 (0.29)	1.08 (0.24)	85.3%	64.7%	1.76 (0.90–3.47)	4.79 (2.54–9.01)	<0.0001
BZLF1 [Zebra (Zta)] CAA24861.1-102338-102210	<0.0001	1.09 (0.36)	0.87 (0.21)	44.0%	15.3%	1.86 (0.94–3.68)	4.46 (2.41–8.28)	<0.0001
BILF2 (glycoprotein) YP_001129503.1-139063-138317	<0.0001	1.16 (0.28)	0.98 (0.22)	72.7%	46.0%	1.94 (1.00–3.77)	4.44 (2.37–8.31)	<0.0001
BLRF2 [viral capsid antigen (VCA) p23] YP_001129461.1-76771-77259	<0.0001	1.48 (0.33)	1.31 (0.28)	92.0%	86.0%	1.80 (0.92–3.53)	4.27 (2.32–7.89)	<0.0001
BDLF4 (late lytic-cycle regulator) YP_001129488.1-117560-116883	<0.0001	1.24 (0.28)	1.10 (0.20)	84.7%	65.3%	1.86 (0.96–3.61)	4.13 (2.23–7.63)	<0.0001
BFRF3 (VCAp18) CAA24838.1-61507-62037	<0.0001	1.54 (0.31)	1.39 (0.25)	92.7%	94.0%	1.04 (0.53–2.05)	4.01 (2.21–7.28)	<0.0001
BZLF2 [glycoprotein (gp) 42] YP_001129466.1-90630-89959	<0.0001	1.36 (0.23)	1.26 (0.19)	96.0%	94.7%	1.83 (0.95–3.50)	3.66 (2.00–6.72)	<0.0001
BDLF3 (gp150) AFY97964.1-118644-117940	<0.0001	1.26 (0.29)	1.09 (0.26)	80.7%	60.7%	1.87 (0.99–3.55)	3.50 (1.91–6.41)	<0.0001
BBRF3 (gpM) YP_001129479.1-107679-108896	<0.0001	1.08 (0.28)	0.94 (0.18)	60.0%	36.7%	1.69 (0.89–3.22)	3.42 (1.88–6.22)	<0.0001
BDLF3 (gp150) YP_001129490.1-119605-118901	<0.0001	1.20 (0.34)	0.99 (0.26)	72.7%	52.7%	1.30 (0.68–2.48)	3.35 (1.86–6.04)	<0.0001
BZLF1 [Zebra (Zta)] YP_001129467.1-90855-90724	<0.0001	1.11 (0.36)	0.90 (0.21)	48.0%	20.0%	1.24 (0.66–2.33)	2.77 (1.55–4.93)	0.0003
BALF2 [EA(D)_p138][c] YP_001129510.1-165796-162410-1	<0.0001	0.95 (0.21)	0.83 (0.15)	38.7%	11.3%	1.09 (0.58–2.05)	2.69 (1.52–4.76)	0.0003
BPFL1[d] CAA24839.1-71527-62078-2	<0.0001	1.25 (0.34)	1.10 (0.21)	78.0%	66.0%	1.09 (0.58–2.03)	2.66 (1.50–4.72)	0.0004
BPFL1 YP_001129449.1-59370-49906-3	<0.0001	0.97 (0.21)	0.88 (0.16)	33.3%	19.3%	1.50 (0.81–2.77)	2.66 (1.48–4.75)	0.0008
Latent membrane protein (LMP) 1 AFY97906.1-168167-168081	<0.0001	1.16 (0.17)	1.08 (0.15)	84.0%	70.0%	1.88 (1.01–3.47)	2.63 (1.45–4.77)	0.0017
BPFL1 YP_001129449.1-59370-49906-2	<0.0001	1.04 (0.24)	0.92 (0.18)	46.7%	31.3%	1.73 (0.94–3.19)	2.57 (1.42–4.64)	0.0019
Epstein–Barr nuclear antigen (EBNA) 3B YP_001129464.1-83074-83430	<0.0001	1.01 (0.29)	0.87 (0.24)	44.7%	23.3%	1.03 (0.55–1.92)	2.51 (1.42–4.45)	0.0007
BVRF2 (VCAp40) YP_001129501.1-136465-138282	<0.0001	1.01 (0.26)	0.88 (0.17)	42.7%	20.0%	1.18 (0.63–2.19)	2.41 (1.37–4.24)	0.0014
BDLF2 (glycoprotein that binds BMRF2) YP_001129491.1-120928-119666	<0.0001	1.09 (0.25)	0.98 (0.17)	57.3%	40.0%	1.01 (0.55–1.86)	2.34 (1.33–4.12)	0.0019
BcLF1 (VCAp160) CAA24794.1-137466-133321-1	<0.0001	0.88 (0.26)	0.77 (0.19)	23.3%	12.7%	1.29 (0.71–2.36)	2.24 (1.26–3.97)	0.0046
BcLF1 (VCAp160) AFY97965.1-125044-120899-1	<0.0001	0.88 (0.28)	0.76 (0.20)	30.7%	9.3%	1.26 (0.69–2.29)	2.17 (1.23–3.82)	0.0061
BLLF1 (gp350) YP_001129462.1-79936-77276	<0.0001	1.02 (0.25)	0.91 (0.21)	45.3%	30.0%	1.17 (0.64–2.17)	2.09 (1.19–3.68)	0.0076
EBNA3A AFY97915.1-80252-82747	<0.0001	1.21 (0.30)	1.08 (0.20)	76.0%	69.3%	1.13 (0.62–2.06)	1.99 (1.13–3.49)	0.0139
BALF2 [EA(D)_p138] YP_001129510.1-165796-162410-2	<0.0001	1.02 (0.29)	0.90 (0.16)	39.3%	22.0%	1.17 (0.64–2.13)	1.92 (1.09–3.35)	0.0188
BdRF1 (VCAp40) AFY97974.1-136284-137321	<0.0001	0.82 (0.29)	0.72 (0.16)	15.3%	4.7%	0.80 (0.44–1.46)	1.67 (0.97–2.89)	0.049
BFRF3 (VCAp18) YP_001129448.1-49335-49865	0.0002	1.56 (0.31)	1.44 (0.25)	92.7%	94.6%	0.88 (0.45–1.75)	3.71 (2.07–6.67)	<0.0001
BFRF1 (viral egress protein) YP_001129446.1-46719-47729	0.0002	0.88 (0.23)	0.79 (0.15)	14.0%	6.7%	1.45 (0.79–2.69)	2.51 (1.41–4.47)	0.0014
BSLF2/BMLF1 (lytic switch protein) YP_001129456.1-71967-70589	0.0002	0.88 (0.21)	0.80 (0.15)	20.0%	8.0%	1.51 (0.83–2.74)	2.14 (1.20–3.82)	0.0101
BRRF2 (promotes viral progeny) AFY97943.1-93884-95497	0.0003	1.24 (0.28)	1.13 (0.25)	82.0%	72.7%	1.31 (0.71–2.41)	2.41 (1.36–4.39)	0.002

Note: Bold text is used to highlight the canonical EBV protein name. The remaining (non-bolded) text describes the sequence details of the array probe.
Abbreviation: CI, confidence interval.
[a]The table is ordered by (i) *t* test *P* value (lowest to highest) and (ii) OR tertile 3 (highest to lowest).
[b]The odds of being a Burkitt lymphoma case were calculated from a regression model that included age, sex, year of blood draw, and a three-level variable (tertiles) for anti-EBV antibody level. The tertiles were calculated using the underlying antibody distribution among disease-free controls. All ORs are expressed relative to the referent group of tertile 1 (lowest third of antibody distribution).
[c]Single-stranded (ss) DNA-binding protein.
[d]Lytic cycle protein involved in immune evasion.

evidence of an interaction between EBV and malaria. The associations between Burkitt lymphoma and four of our top 10 anti-EBV IgG markers were more pronounced in children with elevated levels of the diagnostic malaria antigen HRPII ($P_{interaction} < 0.05$; Supplementary Table S1). For example, the overall OR between Burkitt lymphoma and IgG targeting VCAp23 was 4.27 [95% confidence intervals (CI), 2.32–7.89]. Intriguingly, this association was more muted in children with negative or low (AU < 107.24) anti-HRPII IgG (OR = 2.48; 95% CI, 1.13–5.46) and much stronger among those with elevated (AU $\geq$107.24) anti-HRPII IgG (OR = 9.05; 95% CI, 3.25–25.2; $P_{interaction} = 0.02$). We observed no evidence of interaction between the top 10 anti-EBV IgG markers and inheritance of the sickle cell variant (data not shown).

Among control children, neither inheritance of the sickle cell disease variant nor metrics of malaria exposure significantly ($P \leq 0.0003$) influenced anti-EBV antibody patterns (data not shown).

Discussion

This study represents the most comprehensive evaluation to date of EBV-directed immunity in children diagnosed with endemic Burkitt lymphoma. Our results suggest that the anti-EBV serologic profile is different in Burkitt lymphoma cases, with strong elevations in 33 of the measured IgG antibodies relative to disease-free children of the same age and sex. These Burkitt lymphoma–associated antibody elevations were unique to IgG rather than IgA and were independent of malaria. Notably, the strongest Burkitt lymphoma–EBV associations reflected exposure to proteins involved in viral replication as well as the antiapoptotic Bcl-2 homolog.

In agreement with earlier research conducted in Uganda shortly after the discovery of EBV (7), we confirm associations of Burkitt lymphoma with IgG antibodies against peptides comprising the EBV viral capsid, including VCAp18, -p23, -p40, and -p160, as well as the switch protein Zta (10). Our current data further suggest that IgG antibodies against a multitude of other EBV proteins are also associated with Burkitt lymphoma. In addition to IgG, we examined anti-EBV IgA antibodies in the context of Burkitt lymphoma. IgA reflects recent exposure along mucosal surfaces such as the oral epithelium and has proven to be an informative biomarker for EBV-associated epithelial tumors (e.g., nasopharyngeal carcinoma; refs. 19–21). Children in this study mounted IgA responses to approximately 35% of the examined EBV proteins on average (Supplementary Fig. S3), consistent with prior work reporting ongoing EBV reactivation and shedding in the saliva of Ugandan mothers and children (22). However, IgA responses did not differ between cases and controls. It appears that IgG, which reflects systemic exposure to EBV's infection of circulating B cells, is a more relevant disease marker for this pediatric B-cell tumor.

The most pronounced Burkitt lymphoma–related IgG differences included lytic-phase targets essential for viral replication [BMRF1 (EA) and BZLF1 (Zta)] and virion production [BBLF1 (tegument protein); ref. 23]. Interestingly, these EBV proteins were also part of the four-marker immune signature that most accurately classified Burkitt lymphoma status. Ongoing EBV replication likely reflects an impaired T-cell response that allows the virus to continue expressing the proteins necessary for replication and spread in the B-cell compartment. It is plausible that such ongoing viral replication increases the number of infected B cells and provides more opportunities for a Burkitt lymphoma–initiating event. However, our cross-sectional study design could not determine whether the observed lytic protein expression occurred before or after disease onset. Lytic viral activity in tumor cells or the surrounding microenvironment after disease onset could also feasibly play a role in pathogenesis (24–26). Although antibodies do not directly reflect tumor gene expression, our findings are notable in light of reports of lytic-phase transcripts in Burkitt lymphoma–derived cell lines and African Burkitt lymphoma tumor biopsies (6, 27), as well as the recent identification of a viral BZLF1 polymorphism in Burkitt lymphoma tumors that can enhance EBV lytic replication (28).

Marked IgG differences in children with Burkitt lymphoma were also observed for the EBV homolog of Bcl-2 (BHRF1), an important piece of host antiapoptotic cellular signaling. As hypothesized previously, EBV's role in Burkitt lymphoma pathogenesis may be to keep transformed B cells alive (29), a function often attributed to EBNA3 (4, 5). The elevated serologic response to BHRF1 observed in these Ghanaian cases may also be attributable in part to the fact that BHRF1 transcription is a feature of "Wp latency," a pattern of viral protein expression observed in approximately 15% of endemic Burkitt lymphoma (5).

Previous studies have nicely demonstrated links between residence in high-malaria regions and early-life levels of plasma EBV and EBV-directed T-cell responses (30–32). We did not observe clear evidence that malaria exposure affected anti-EBV serologic immunity in disease-free children, in agreement with earlier VCA-based work from Ghana (33). More importantly, the main EBV–Burkitt lymphoma associations we report here persisted after adjustment for either malaria antibody level or the inheritance of the sickle cell rs334 variant. We do acknowledge evidence of an interaction between our strongest anti-EBV IgG antibody results and the malaria diagnostic antigen HRPII, supporting a synergistic model wherein the association between EBV and Burkitt lymphoma is more pronounced in children highly exposed to both pathogens. However, this requires independent, external validation.

The cross-sectional nature of these data did not allow us to elucidate whether children experienced these anti-EBV antibody differences prior to disease rather than at the time of Burkitt lymphoma diagnosis. The difficulty of conducting an adequately powered prospective study of this rare disease in younger children makes it unlikely that this limitation will be easily addressed in the future, but validation of the IgG markers identified here in subsequent case–control series is warranted. Of note, we did not observe uniform elevations for IgG in children with Burkitt lymphoma, arguing against a nonspecific disease effect—the presence of a tumor did not result in higher levels for all anti-EBV IgG antibodies.

This study characterized EBV-directed antibody responses to 202 peptide sequences representing 86 viral proteins in 300 Ghanaian children. The anti-EBV serologic profile was significantly different in children diagnosed with endemic Burkitt lymphoma, with elevations in 33 IgG markers compared with disease-free children. The association between anti-EBV IgG elevations and Burkitt lymphoma persisted after adjustment for malaria exposure and inheritance of the sickle cell genetic variant. This Burkitt lymphoma–specific signature included pronounced differences in the immune response against viral proteins involved in replication and antiapoptotic activity, providing clues for future research into EBV-mediated Burkitt lymphoma pathogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet 1964;1:702–3.
2. Cohen JI. Epstein-Barr virus infection. N Engl J Med 2000;343:481–92.
3. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer 2004;4:757–68.
4. Thorley-Lawson DA, Allday MJ. The curious case of the tumour virus: 50 years of Burkitt's lymphoma. Nat Rev Microbiol 2008;6:913–24.
5. Rowe M, Kelly GL, Bell AI, Rickinson AB. Burkitt's lymphoma: the Rosetta Stone deciphering Epstein-Barr virus biology. Semin Cancer Biol 2009;19:377–88.
6. Tierney RJ, Shannon-Lowe CD, Fitzsimmons L, Bell AI, Rowe M. Unexpected patterns of Epstein-Barr virus transcription revealed by a high throughput PCR array for absolute quantification of viral mRNA. Virology 2015;474:117–30.
7. Geser A, de The G, Lenoir G, Day NE, Williams EH. Final case reporting from the Ugandan prospective study of the relationship between EBV and Burkitt's lymphoma. Int J Cancer 1982;29:397–400.
8. Carpenter LM, Newton R, Casabonne D, Ziegler J, Mbulaiteye S, Mbidde E, et al. Antibodies against malaria and Epstein-Barr virus in childhood Burkitt lymphoma: a case-control study in Uganda. Int J Cancer 2008;122:1319–23.
9. Coghill AE, Hildesheim A. Epstein-Barr virus antibodies and the risk of associated malignancies: review of the literature. Am J Epidemiol 2014;180:687–95.
10. Asito AS, Piriou E, Odada PS, Fiore N, Middeldorp JM, Long C, et al. Elevated anti-Zta IgG levels and EBV viral load are associated with site of tumor presentation in endemic Burkitt's lymphoma patients: a case control study. Infect Agent Cancer 2010;5:13.
11. Liu Z, Coghill AE, Pfeiffer RM, Proietti C, Hsu WL, Chien YC, et al. Patterns of interindividual variability in the antibody repertoire targeting proteins across the Epstein-Barr virus proteome. J Infect Dis 2018;217:1923–31.
12. Coghill AE, Pfeiffer RM, Proietti C, Hsu WL, Chien YC, Lekieffre L, et al. Identification of a novel, EBV-based antibody risk stratification signature for early detection of nasopharyngeal carcinoma in Taiwan. Clin Cancer Res 2018;24:1305–14.
13. Nkrumah FK, Olweny CL. Clinical features of Burkitt's lymphoma: the African experience. IARC Sci Publ 1985;60:87–95.
14. Nkrumah FK, Perkins IV. Sickle cell trait, hemoglobin C trait, and Burkitt's lymphoma. Am J Trop Med Hyg 1976;25:633–6.
15. Chang C, Middeldorp J, Yu KJ, Juwana H, Hsu WL, Lou PJ, et al. Characterization of ELISA detection of broad-spectrum anti-Epstein-Barr virus antibodies associated with nasopharyngeal carcinoma. J Med Virol 2013;85:524–9.
16. Liu Z, Jarrett RF, Hjalgrim H, Proietti C, Chang ET, Smedby KE, et al. Evaluation of the antibody response to the EBV proteome in EBV-associated classic Hodgkin lymphoma. Int J Cancer 2019 Oct 16 [Epub ahead of print].
17. Aka P, Vila MC, Jariwala A, Nkrumah F, Emmanuel B, Yagi M, et al. Endemic Burkitt lymphoma is associated with strength and diversity of Plasmodium falciparum malaria stage-specific antigen antibody response. Blood 2013;122:629–35.
18. Legason ID, Pfeiffer RM, Udquim KI, Bergen AW, Gouveia MH, Kirimunda S, et al. Evaluating the causal link between malaria infection and endemic Burkitt lymphoma in northern Uganda: a Mendelian randomization study. EBioMedicine 2017;25:58–65.
19. Coghill AE, Hsu WL, Pfeiffer RM, Juwana H, Yu KJ, Lou PJ, et al. Epstein-Barr virus serology as a potential screening marker for nasopharyngeal carcinoma among high-risk individuals from multiplex families in Taiwan. Cancer Epidemiol Biomarkers Prev 2014;23:1213–9.
20. Henle G, Henle W. Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. Int J Cancer 1976;17:1–7.
21. Chien YC, Chen JY, Liu MY, Yang HI, Hsu MM, Chen CJ, et al. Serologic markers of Epstein-Barr virus infection and nasopharyngeal carcinoma in Taiwanese men. N Engl J Med 2001;345:1877–82.
22. Mbulaiteye SM, Walters M, Engels EA, Bakaki PM, Ndugwa CM, Owor AM, et al. High levels of Epstein-Barr virus DNA in saliva and peripheral blood from Ugandan mother-child pairs. J Infect Dis 2006;193:422–6.
23. Chiu YF, Sugden B, Chang PJ, Chen LW, Lin YJ, Lan YC, et al. Characterization and intracellular trafficking of Epstein-Barr virus BBLF1, a protein involved in virion maturation. J Virol 2012;86:9647–55.
24. Dutton A, Woodman CB, Chukwuma MB, Last JI, Wei W, Vockerodt M, et al. Bmi-1 is induced by the Epstein-Barr virus oncogene LMP1 and regulates the expression of viral target genes in Hodgkin lymphoma cells. Blood 2007;109:2597–603.
25. Tarbouriech N, Ruggiero F, de Turenne-Tessier M, Ooka T, Burmeister WP. Structure of the Epstein-Barr virus oncogene BARF1. J Mol Biol 2006;359:667–78.
26. Wang D, Liebowitz D, Kieff E. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell 1985;43(3 Pt 2):831–40.
27. Xue SA, Labrecque LG, Lu QL, Ong SK, Lampert IA, Kazembe P, et al. Promiscuous expression of Epstein-Barr virus genes in Burkitt's lymphoma from the central African country Malawi. Int J Cancer 2002;99:635–43.
28. Bristol JA, Djavadian R, Albright ER, Coleman CB, Ohashi M, Hayes M, et al. A cancer-associated Epstein-Barr virus BZLF1 promoter variant enhances lytic infection. PLoS Pathog 2018;14:e1007179.
29. Fitzsimmons L, Kelly GL. EBV and apoptosis: the viral master regulator of cell fate? Viruses 2017;9. doi: 10.3390/v9110339.
30. Piriou E, Kimmel R, Chelimo K, Middeldorp JM, Odada PS, Ploutz-Snyder R, et al. Serological evidence for long-term Epstein-Barr virus reactivation in children living in a holoendemic malaria region of Kenya. J Med Virol 2009;81:1088–93.
31. Snider CJ, Cole SR, Chelimo K, Sumba PO, Macdonald PD, John CC, et al. Recurrent Plasmodium falciparum malaria infections in Kenyan children diminish T-cell immunity to Epstein Barr virus lytic but not latent antigens. PLoS One 2012;7:e31753.
32. Piriou E, Asito AS, Sumba PO, Fiore N, Middeldorp JM, Moormann AM, et al. Early age at time of primary Epstein-Barr virus infection results in poorly controlled viral infection in infants from Western Kenya: clues to the etiology of endemic Burkitt lymphoma. J Infect Dis 2012;205:906–13.
33. Biggar RJ, Gardiner C, Lennette ET, Collins WE, Nkrumah FK, Henle W. Malaria, sex, and place of residence as factors in antibody response to Epstein-Barr virus in Ghana, West Africa. Lancet 1981;2:115–8.