

AID expression in peripheral blood of children living in a malaria holoendemic region is associated with changes in B cell subsets and Epstein-Barr virus

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The development of endemic Burkitt's lymphoma (eBL) is closely associated with Epstein-Barr virus (EBV) infection and holoendemic malaria infections. The role of EBV in the development of malignancy has been studied in depth, but there is still little known about the mechanisms by which malaria affects Burkitt's lymphomagenesis. Activation induced cytidine deaminase (AID) expression is necessary for the introduction of c-myc translocations that are characteristic of BL, but a link between AID and EBV or malaria is unclear. To determine whether frequency of malaria exposure leads to increased AID expression in peripheral blood mononuclear cells (PBMC) we examined two cohorts of children in western Kenya with endemic and sporadic malaria transmission dynamics. High frequency of malaria exposure led to increased expression of AID, which coincided with decreases in the IgM⁺ memory B cells. In the children from the malaria endemic region, the presence of a detectable EBV viral load was associated with higher AID expression compared to children with undetectable EBV, but this effect was not seen in children with sporadic exposure to malaria. This study demonstrates that intensity of malaria transmission correlates with AID expression levels in the presence of EBV suggesting that malaria and EBV infection have a synergistic effect on the development of c-myc translocations and BL.

Endemic Burkitt's lymphoma (eBL) is a rapidly dividing B cell malignancy that is fatal if untreated and it is found primarily in children in sub-Saharan Africa.¹ The etiology of eBL is closely linked to infection with Epstein-Barr virus (EBV) and holoendemic exposure to malaria.^{2,3} EBV is a ubiquitous virus that is found in >90% of people worldwide, and is present in

nearly all cases of endemic African BL.⁴ A common feature of eBL is the translocation of the oncogene c-myc to the control of the immunoglobulin promoter leading to constitutive expression of c-myc.^{5,6} Translocations of c-myc in the presence of EBV are sufficient to produce transformed cells,⁶ but the etiology of the c-myc translocations and whether malaria plays a role in inducing these translocations is unknown.

The induction of c-myc translocations is likely an early event in the development of eBL.⁷ Studies in humans and mice have shown that there is a direct link between the activity and expression of activation induced cytidine deaminase (AID) and c-myc translocations.⁸ AID is a necessary enzyme for somatic hypermutation (SHM) and class-switch recombination (CSR).⁹ AID expression is generally restricted to germinal center B cells within the spleen and lymph nodes.⁹ AID is rarely expressed in the peripheral blood of healthy individuals, however it has been shown that people infected with HIV have AID expression in circulating lymphocytes.¹⁰ In addition, HIV infected patients were more likely to have high levels of AID expression in their blood during the years preceding diagnosis with non-Hodgkin's lymphoma than patients that did not develop lymphoma.¹⁰ HIV-associated BL is similar to African endemic BL because both diseases are initiated by chronic infection, and both have c-myc translocations.

Key words: malaria, plasmodium, Epstein-Barr Virus, Burkitt's lymphoma, activation induced cytidine deaminase

Abbreviations: AID: activation induced cytidine deaminase; CSR: class-switch recombination; eBL: endemic Burkitt's lymphoma; EBNA: Epstein-Barr nuclear antigen; EBV: Epstein-Barr virus; GPI: glycosylphosphatidylinositol; LMP: latent membrane protein; MBC: memory B cell subsets; PBMC: peripheral blood mononuclear cell; SHM: somatic hypermutation; TLR: Toll-like receptor

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What's new?

Activation-induced cytidine deaminase (AID) expression has been linked to translocations in the c-myc transcription factor that can cause Burkitt's lymphoma, a tumor closely associated with Epstein Barr Virus (EBV) and malaria infections. Here the authors demonstrate a first link between AID expression and exposure to malaria in the peripheral blood of children. They further show that AID expression is linked to detectable EBV in children living in a region of high malaria transmission. These findings shed new light on how malaria and EBV co-infections can increase the risk of developing Burkitt's lymphoma.

EBV has several reported effects on AID expression in B cells by different mechanisms. For example, the EBV latent membrane protein (LMP)-1 is capable of acting in place of CD40 to activate infected B cells and lead to AID mediated CSR.¹¹ In contrast, another EBV latent protein, Epstein-Barr nuclear antigen (EBNA)-2, was shown to inhibit AID expression during proliferation of infected B cells. In addition to direct effects on AID expression, infection with EBV could lead to the rescue of cells from apoptosis that are over-expressing c-myc.⁶ High EBV viral loads such as those observed in children from malaria holoendemic regions,¹² could increase the likelihood of a B cell with a c-myc translocation getting rescued from cell death.³

Plasmodium falciparum malaria has several components that act as B cell activators during the course of infection. *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) is a polyclonal B cell activator leading to increased cytokine and Ig secretion and activation marker expression.¹³ *P. falciparum* is also capable of activating B cells through Toll-like receptors (TLR). The hemoglobin metabolism breakdown product, hemozoin, bound to DNA of *Plasmodium* parasites is capable of stimulating B cells through TLR9.¹⁴ Of note, AID expression and activity can be induced through TLR9 activation.¹⁵ In addition, *Plasmodium* glycosylphosphatidylinositol (GPI) anchors are capable of stimulating TLR2.¹⁶ The effects of these B cell activators could be even greater in a population that is chronically exposed to malaria.

Memory B cells were originally thought be delineated by expression of CD27 and the absence of IgD. More recently, however, multi-color flow cytometric analysis has identified several memory B cell subsets (MBC).^{17,18} Each MBC subset is thought to have a different source or function. Classical MBCs are generally germinal center derived and have undergone CSR.¹⁷ However, it is possible to have classical MBCs that maintain their IgM expression despite evidence of SHM in this population. MZ-like MBCs have been described as being enriched for B cell receptors that are more autoreactive than classical MBCs.¹⁹ The atypical MBC subset is unique in that it is considered to have an exhausted phenotype.^{18,20} In addition, MBCs that express FcRL4 have a distinct phenotype, which stems from the inhibitory domains associated with the FcRL4 protein.²¹ Several studies have reported changes in MBCs associated with diseases such as HIV,²⁰ malaria,¹⁸ and Hepatitis C virus.¹⁹ Further study into the changes in MBC subset distribution could lead to insight into infection-associated cancers such as eBL.

Several studies have examined the effects of *P. falciparum* infection on B cell homeostasis. Kassa *et al.* found that adults with acute malaria infections had decreased percentages of CD19+ B cells.²² Similarly, we found that children in Western Kenya with acute malaria had decreased CD19+ B cells and also decreased classical MBC compared to a 4-week recovery sample.²³ While examining the B cell responses to acute infection is important,²⁴ it is also necessary to understand how repeated infections and transmission intensity affect B cell homeostasis. We characterized the peripheral B cell subsets in a longitudinal cohort of infants enrolled from two regions of Western Kenya that experience different patterns of malaria transmission intensity throughout the year: Kisumu district, where malaria transmission and eBL is high, and Nandi district, where transmission and risk for eBL is low and episodic.²⁵ No differences were observed between the infants from the two sites in frequencies of naive B cells or classical MBC. However, the levels of non-class switched IgD+CD27+ MBC were significantly lower in Kisumu infants relative to Nandi at 12, 18 and 24 months of age. These data suggest that even early in life, malaria transmission is altering the development of MBC subsets.

Further evidence for effects of malaria on alterations in B cell homeostasis is the increase in atypical MBC expanded in children and adults in Mali, which has high malaria transmission.^{18,20} They were defined as "exhausted" B cells based on their poor proliferative response in vitro and it was proposed that they may contribute to poor antibody responses.²⁰ As eBL is thought to derive from MBC,²⁶ understanding whether there are alterations in MBC subsets in children living in areas with high malaria transmission and eBL risk is important.

In this study, we analyzed peripheral blood mononuclear cells (PBMC) isolated from children at 36 months of age from Kisumu and Nandi regions of Kenya and performed a detailed B cell phenotype analysis. The B cell subsets were then correlated with AID expression and EBV viral load.

Material and Methods**Study Population**

Two cohorts of children were established in predominantly rural areas of western Kenya that have previously been described.^{25,27,28} The cohorts were from populations with divergent levels of malaria transmission due to their geographical location. The cohort with holoendemic/high malaria transmission is in Kisumu District ($n = 54$) within Nyanza Province and sporadic/low malaria transmission is

characteristic of the cohort from Nandi District ($n = 34$) of the Rift Valley Province. The cohorts are part of a larger study and have been previously described.^{29,30} Infants were enrolled at ~1 month of age during a 3-month period between April 2006 and June 2006. All children were born to HIV-seronegative mothers and their health was closely monitored and treated for illness as per Kenya Ministry of Health guidelines. Infants were followed from 1 month of age through 36 months. The present study is focused on the blood drawn at 36 months of age. Temperature and hemoglobin levels were assessed at time of sample collection to eliminate any children experiencing clinical malaria. The Kenya Medical Research Institute Ethical Review Committee and the Institutional Review Board at State University of New York Upstate Medical University gave ethical approval for this study.

Blood Collection

Study participants were examined and children that were not febrile had 1–3 ml of blood drawn by venipuncture into heparinized vacutainer tubes. Within 4 hr of collection, PBMC were isolated by ficoll density gradient centrifugation. The cells were frozen in freezing media (RPMI, 20% Fetal Bovine Serum, 10% DMSO) and stored in liquid nitrogen until analysis.

Flow Cytometry

PBMC were thawed and stained with the LIVE/DEAD Aqua (Life Technologies, Grand Island, NY) viability dye according to the manufacturer protocol. Before fixation, cells were incubated with Human Fc Receptor Binding Inhibitor (eBioscience, San Diego, CA) then stained with the following monoclonal antibodies: CD38-PE-TexasRed (Life Technologies), IgD-FITC (BD Biosciences, San Jose, CA), IgM-Brilliant Violet 421, CD307d (FcRL4)-PE, CD3-Alexa Fluor 700, CD10-PE/Cy7, Streptavidin-APC/Cy7, CD19-PerCP/C5.5 (BioLegend, San Diego, CA), CD24-Biotin, CD21-APC, and CD27-650NC (eBioscience). Cells were then fixed in 2% formaldehyde and analyzed within 1–2 hr on an LSR Fortessa (BD Biosciences). An average of 8×10^5 events were collected (range of 2×10^5 to 2×10^6 events), which resulted in an average of 6.7×10^4 total B cells analyzed (range of 5×10^3 to 2×10^5 B cells). All flow cytometry data was processed using FlowJo Software (Tree Star, San Carlos, CA). Percentages presented for total CD19+ B cell population (CD19+, CD3–) are derived from the percentage of cells within the live gate based on LIVE/DEAD Aqua staining that is within the lymphocyte gate. All B cell subset percentages are the frequency of the total B cell gate (CD19+, CD3–) described above, with the exception of the IgM+ MBC, which is presented as frequency of CD19+, CD3–, CD10–, CD27+ and IgD–.

Quantitative PCR

EBV viral load was determined by extracting DNA from up to 200 μ l of blood using the Qiagen DNeasy kit (Qiagen,

Valencia, CA) according to the manufacturer protocol. DNA was eluted in water and stored at -20°C until analysis. The detection of the EBV BALF5, *P. falciparum* and human β -actin genes was performed using primers and probes previously described.^{12,31,32} RNA was isolated from PBMC that were not stained for flow cytometric analysis using the Qiagen RNeasy kit (Qiagen) according to the manufacturer protocol. The volume of RNA put into the reverse transcription reaction was based upon the cell number and was performed using the High Capacity cDNA Reverse Transcription kit (Life Technologies) according to the manufacturer protocol. AID mRNA levels were determined by using the TaqMan Primers and Probe set Hs00757808_m1 (Life Technologies) and the HPRT gene was used as a control for the presence of cDNA (Integrated DNA Technologies, Coralville, IA). AID qPCR was performed with a standard curve generated by inserting the PCR fragment into a TOPO vector (Life Technologies) according to the manufacturer protocol.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA). Differences between the two cohorts were compared using Mann-Whitney U-test. Correlation analysis was performed with a Spearman rank correlation test. Statistical analysis was considered significant if p values were ≤ 0.05 .

Results

Study Population and Clinical Characteristics

Two cohorts of children were recruited and followed from 1 month of age from two rural study sites in western Kenya. These cohorts have been the focus of several previously published studies on the effects of malaria transmission on EBV infection.^{28–30} The first study site was in Nyanza Province of western Kenya in the Kisumu District near Lake Victoria. This area has holoendemic malaria transmission and an increased risk of eBL, and will be referred to as Kisumu.²⁷ The second study site was in the Rift Valley Province within the Nandi District. This site has a low malaria transmission rate with only sporadic outbreaks and will be referred to as Nandi.²⁷ Each cohort was monitored until 3 years of age at regularly scheduled intervals. There were a significantly higher number of children in Kisumu with detectable *P. falciparum* parasitemia by blood smear or qPCR analysis at nearly every time-point tested compared to Nandi, confirming differences in malaria transmission between these two sites.²⁹ For the current study, we analyzed samples obtained at 3 years of age. Although none of the study participants had evidence of fever (temperature $>37.5^\circ\text{C}$) or anemia at time of sample collection, we also assessed whether there were any detectable *P. falciparum* parasites by Q-PCR. Only three children from Kisumu and none from Nandi were positive for *P. falciparum* by qPCR. The absence of fever or anemia suggested an asymptomatic infection in these children and they were included in subsequent analysis.

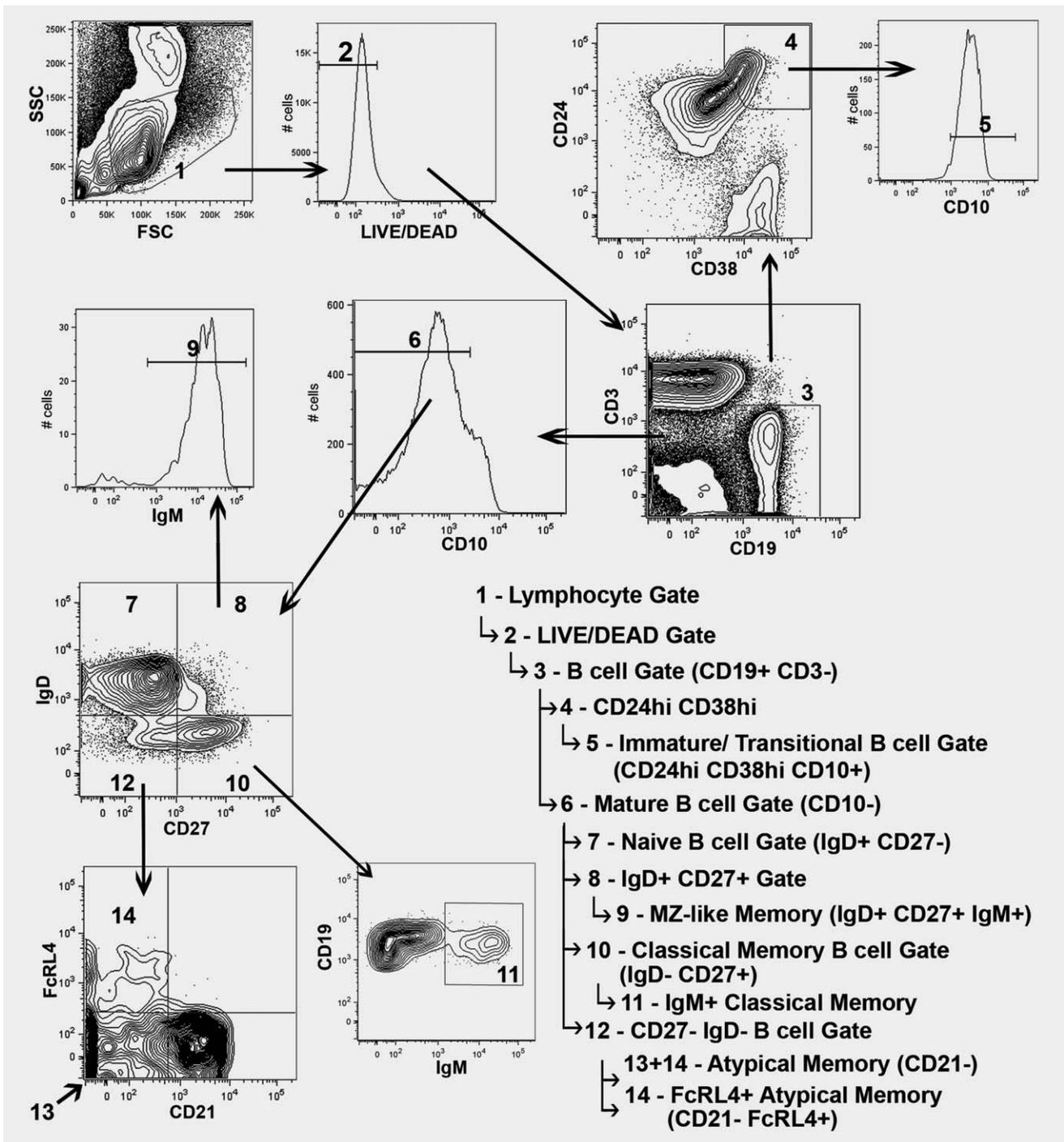


Figure 1. Representative flow cytometry demonstrating the gating strategy used for analysis. Flow cytometry images shown are representative of study participants from Kisumu, which suffer a high frequency of malaria transmission. Beginning with the upper left panel, samples were gated as shown, with parent gating denoted by indented arrows in the legend.

Children From Areas With High Malaria Transmission Have Differences in Memory B Cell Subsets Compared to Children From a Low Malaria Transmission Region

To determine the impact of repeated malaria infection as occurs in Kisumu on the distribution of peripheral B cell subsets in 3-year-old children, we performed a comprehensive

11-color flow cytometric analysis on PBMCs isolated from children at each study site. Samples were analyzed for the presence of CD19, CD3, CD10, CD24, CD38, CD27, CD21, IgM, IgD, FcRL4 and binding to an amine reactive viability dye. The staining strategy allowed us to perform detailed analysis on B cell subsets, as demonstrated in

Table 1. Summary of B cell phenotyping in Nandi and Kisumu children

	Kisumu mean	Kisumu range	Nandi mean	Nandi range	<i>p</i> value
Total B cells	10.98	3.23–21.1	12.26	3.7–23.4	0.1548
Immature/Transitional	9.39	2.49–22.6	10.90	3.99–19.7	0.0436
Naïve	51.34	20.6–69.1	47.52	24.4–65.6	0.1062
Classical memory	13.52	4.75–37.1	14.72	4.08–35.5	0.4769
MZ-like memory	5.22	1.51–10.2	6.89	3.64–11.2	0.001
IgM+ Classical	11.06	3.11–28.5	18.41	6.97–46.2	<0.0001
Atypical memory	3.82	0.786–11.6	2.40	0.995–6.36	0.0008
FcRL4+ atypical memory	0.46	0.071–1.96	0.32	0.081–0.91	0.0374
CD27– IgD– B cells	8.67	3.35–18.6	6.40	2.14–20.5	0.0007

Mean percentage and range are shown for each B cell subset as shown in Figure 2.

Figure 1. We analyzed the total CD19+ B cell population, as well as the immature/transitional, naïve and MBCs (summarized in Table 1). The MBCs included classical (CD27+ IgD–), atypical (CD27– IgD– CD21–) and MZ-like (CD27+ IgD+ IgM+) MBCs. There was no statistically significant difference in the total CD19+ B cell percentages between the two study cohorts (Fig. 2a). The percentage of immature B cells of the total CD19+ B cell population was significantly decreased in Kisumu compared to Nandi (Fig. 2b).

The distributions of MBCs are altered in adults from malaria endemic regions.¹⁸ To determine whether a high frequency of malaria transmission affects children by altering the balance between B cell memory subsets we analyzed the cell surface expression of CD27 and IgD on mature (CD19+, CD10–) B cells. There was no statistical difference in the percentages naïve B cells (CD19+, CD10–, CD27–, IgD+) (Fig. 2c) or of classical memory B cells (CD19+, CD10–, CD27+, IgD–) between both sites (Fig. 2d). This suggests the development and maintenance of the naïve and classical MBCs is not affected by differential exposure to malaria transmission consistent with our earlier observations using a more limited panel of cell surface markers.³⁰ However, there was a significant decrease in the frequency of IgM+ B cells (CD19+, CD10–, CD27–, IgD–, IgM+) in the classical memory pool of children in Kisumu compared to Nandi ($p < 0.0001$).

Previous studies of the Kisumu and Nandi cohorts at 1–2 years of age have shown an increase in atypical memory B cells in children from Kisumu, but were unable to determine whether these populations included immature B cells, due to technical limitations.³⁰ Our panel allowed us to define the atypical memory B phenotype, by the excluding CD10+ cells. The percentage of CD27– IgD– B cells was significantly higher in children from Kisumu, compared to Nandi, at 3 years of age (Fig. 2i). The increase in CD27–IgD– B cells with more malaria exposure was due to an increase in atypical memory B cells (CD19+, CD10–, CD27–, IgD–, CD21–) that were significantly higher in Kisumu, compared to Nandi (Fig. 2g). The atypical memory B cell pool consists

predominantly of IgM– cells (range of 75–98% IgM–) (data not shown). Further characterization of the atypical memory B cells revealed that the Kisumu cohort exhibited higher expression of FcRL4+ than Nandi (Fig. 2h). The higher levels of atypical memory B cells in children from Kisumu coincide with lower levels of MZ-like memory B cells (CD19+, CD10–, CD27+, IgD+, IgM+) (Fig. 2e).

AID mRNA Expression is Higher in Children From Kisumu and AID Expression Correlates With a Decrease in MZ-Like Memory

To determine the effects of malaria transmission on the expression of AID in PBMC, RNA was isolated from PBMC, and quantified by RT-qPCR on a per cell basis. All children in our study had detectable AID expression in their PBMC, albeit at low copy number per cell. Children living in Kisumu did not have a significantly ($p = 0.2211$) higher number of AID copies per cell than children in Nandi (Fig. 3). Because we had already observed differences in the percentages of B cell subsets between Kisumu and Nandi cohorts, we next wanted to know if any B cell subset correlated with levels of AID expression independent of frequency of malaria exposure by comparing the phenotype data to AID expression from both high and low malaria transmission sites combined. Higher levels of AID expression significantly correlated with a decrease in the MZ-like memory B cell population (Fig. 4). There was no significant correlation between AID levels and percentages of other B cell subsets (data not shown).

Children That Had a Detectable EBV Viral Load Had Higher AID Expression in Kisumu, But Not in Nandi

As EBV latent proteins have been shown to both activate and repress AID expression in B cell culture systems,^{33,34} we next determined whether elevated EBV load was associated with AID expression. DNA extracted from whole blood was analyzed by qPCR for EBV viral load as an indicator of cell associated virus. Although all children in this cohort are EBV seropositive,²⁹ no difference in the percentage of children

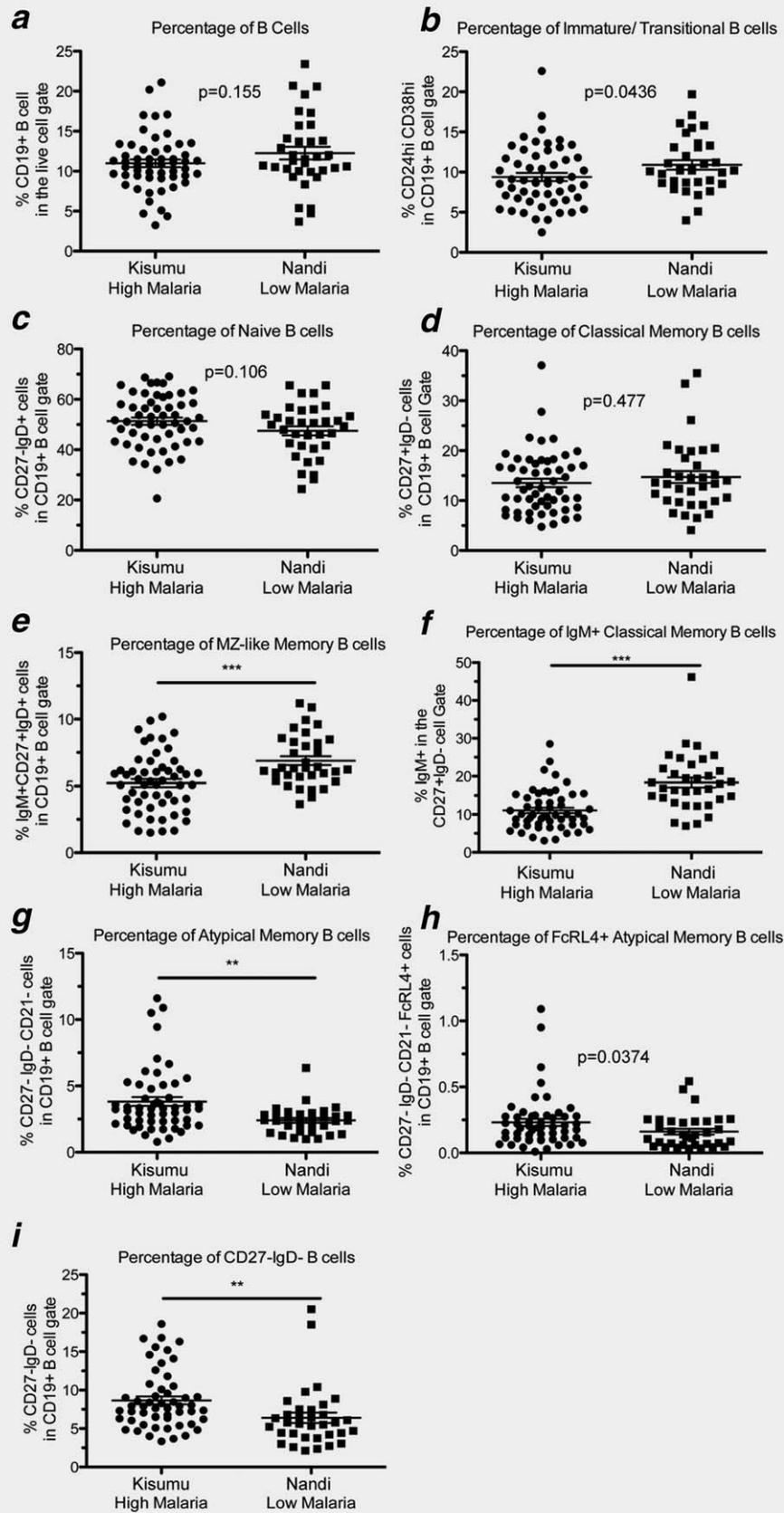


Figure 2. Atypical, FcRL4+ and IgM Memory B cell subsets were significantly different in children living in the presence of high malaria transmission regions. Comparison of the different B cell populations in children from high and low malaria transmission regions based on the gating strategy shown in Figure 1. The numbers in parentheses below the line number in the flow chart from Figure 1 demonstrating the hierarchy of populations. (a) Percentage of B cells in the live cell gate (3). (b) Transitional/Immature B cells (4), (c) Naive B cells (7), (d) Classical Memory B cells (10), (e) MZ-like Memory B cells (9), (f) IgM+ Classical memory B cells (11), (g) Atypical Memory B cells (13), (h) FcRL4+ Atypical Memory B cells (13 + 14) and (i) CD27- IgD- B cells (12). Kisumu $n = 54$, Nandi $n = 34$. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$. p -value determined by a Mann-Whitney U-test.

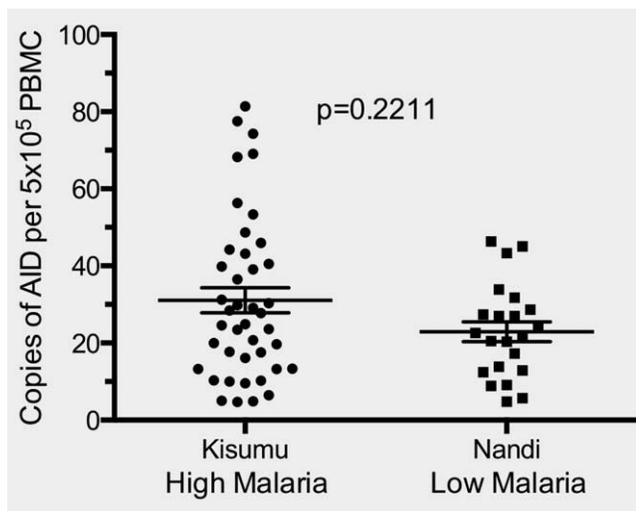


Figure 3. AID mRNA expression is trending but not significantly increased in PBMC of children in a high malaria transmission region. AID mRNA copies per 5×10^5 PBMC from children in high and low malaria transmission regions. Kisumu $n = 42$, Nandi $n = 22$. p -value determined by a Mann-Whitney U-test.

with detectable EBV load at 36 months of age was observed (52.4% in Kisumu vs. 50% in Nandi). The mean viral load for children from Kisumu was 7.66 copies/ μ g and from Nandi, 9.23 copies/ μ g and not significantly different between the two sites. To determine whether there was detectable viral latency gene expression in the PBMC, we performed qPCR for EBV nuclear antigen (EBNA)-1. All of the samples were negative for EBNA-1 expression aside from three children (data not shown). The three children with EBNA-1 expression did not have a distinct phenotype for any parameter as compared to the rest of the children in their cohort and were therefore not excluded from analysis.

We next grouped the study participants based on whether they had measurable EBV viral load at the time of blood draw (3-year old) or no detectible EBV. To determine whether the presence of detectable levels of EBV correlated with a change in the levels of AID expression, we compared EBV viral load-positive and-negative children within Kisumu or Nandi. There was an increase in AID expression in the EBV+ children from Kisumu compared to the EBV- children in the same site (Fig. 5a). Children from Nandi had similar levels of AID expression, regardless of whether they were EBV viral load-positive or -negative (Fig. 5b). When comparing only the EBV+ children between Kisumu and Nandi there was a significantly higher level of AID expression in the blood of the children exposed to higher levels of malaria transmission in Kisumu (Fig. 5c). These data suggest that there is relationship between malaria transmission and EBV infection that leads to increased AID expression.

Discussion

A key step to the development of eBL is the translocation of c-myc oncogene to the control of the immunoglobulin

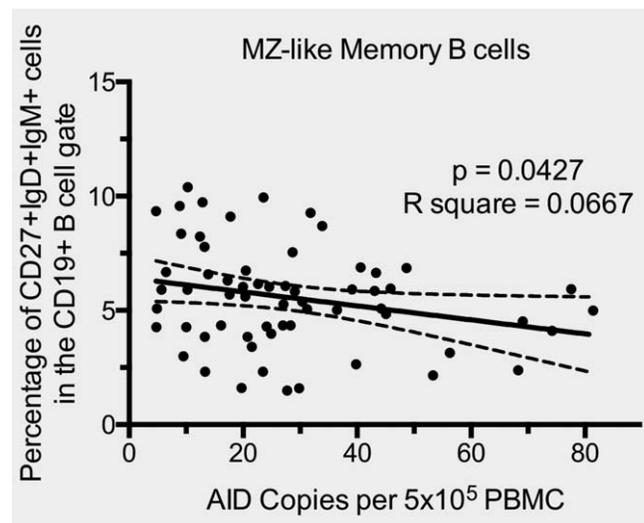


Figure 4. Increased AID expression correlates with a decrease in the MZ-like MBC. The copies of AID per 5×10^5 PBMC (x-axis) compared to the percentage of IgM Memory B cells (y-axis) for each child from both the high and low malaria transmission regions. p values were determined by correlation analysis. The solid line is a linear regression line and dotted lines represent the 95% confidence interval. $n = 64$.

promoter. This event is thought to require the activity of AID,^{35,36} which is tightly regulated under normal circumstances.³⁷ It has been suggested that chronic activation of AID expression by *P. falciparum* or HIV could be the link to the increased risk for B cell malignancies in the infected populations.¹ However, there have been no studies to date demonstrating an increase in AID expression correlated with malaria infection. Studies of HIV associated non-Hodgkin's lymphoma show that AID expression is detectable in peripheral blood before diagnosis with lymphoma, but the B cell subset that expresses AID is unknown.¹⁰ Several groups have demonstrated the effects of *Plasmodium* infection on peripheral blood B cells,^{18,30,38} with a particular emphasis on memory B cells, due to the lack of long-lived immunity to malaria. MBCs are also important in the context of BL development, because phenotyping of malignant cells has shown a significant amount of SHM, suggesting BL is derived from a post-germinal center B cell.³⁹ Our study is unique because we enrolled children from geographically proximate regions in western Kenya that were at a high or low risk for eBL due to differences in malaria transmission between the sites. While we cannot rule out the possibility of unknown environmental factors contributing to the phenotypic characteristics found in the two cohorts of children, the malaria burden stands out as the most significant difference between the two sites. In our high-risk cohort from the malaria endemic region of Kisumu, we observed that the children with a detectible EBV viral load in Kisumu had significantly higher expression of AID than children with detectible EBV in Nandi. Memory B cell populations in our cohorts were significantly altered by high frequency malaria transmission. Children from Kisumu

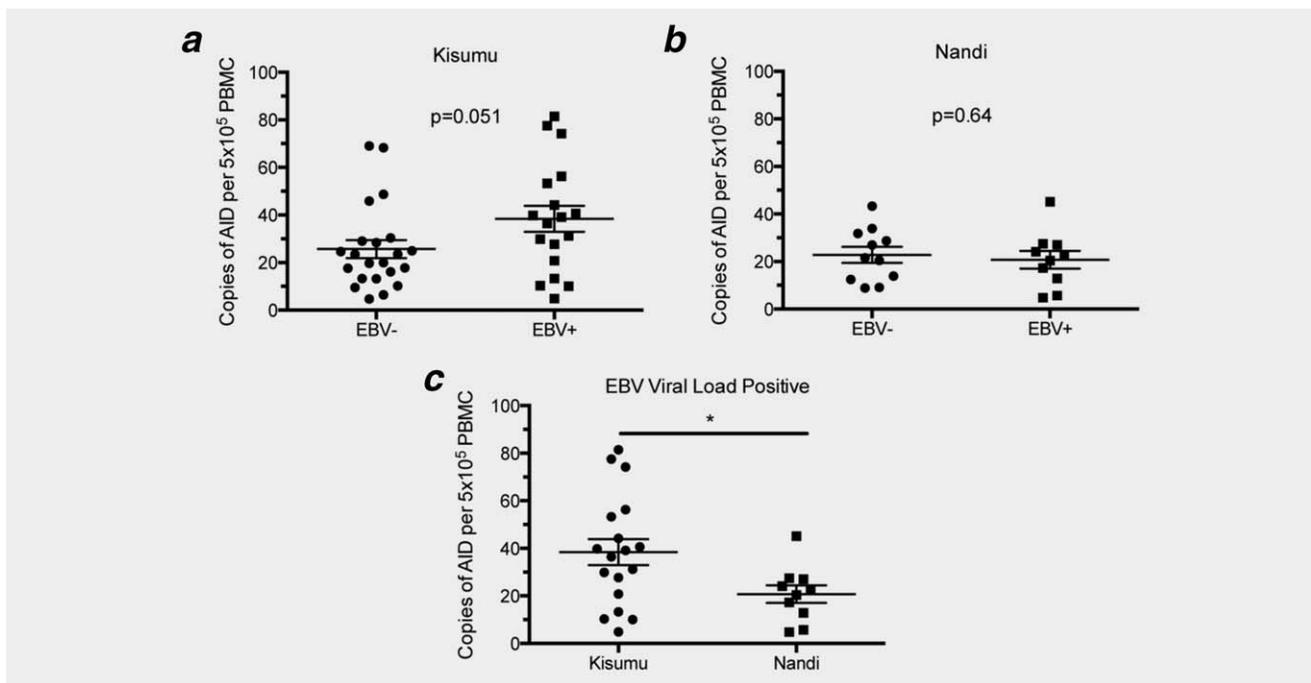


Figure 5. AID expression is increased in children with PCR-detectable EBV viral load in the high malaria transmission, but not the low malaria transmission regions. (a) AID mRNA expression levels from EBV+ and EBV- children from Kisumu (EBV- $n = 22$, EBV+ $n = 18$) or (b) Nandi (EBV- $n = 11$, EBV+ $n = 10$). (c) Comparison of EBV+ children from Kisumu versus EBV+ children from Nandi. p -value determined by Mann-Whitney U-test.

had lower levels of immature B cells and MZ-like memory, but higher percentages of atypical memory B cells. High percentages of atypical memory, and low levels of MZ-like MBCs, correlated with increased AID expression when analyzing the combined data from both sites. Together these data suggest that elevated AID expression is associated with a high frequency of malaria transmission, and B cell subset changes that are consistent with chronic stimulation.

AID expression in B cells is required for normal humoral immune functions, such as SHM and CSR, but those processes are normally restricted to germinal centers in secondary lymphoid organs.⁹ The detection of AID in B cells of children that do not have non-Hodgkin's lymphoma is important because it suggests that the role of malaria in the etiology of BL could be due to the ability of *Plasmodium* to induce AID expression and result in *c-myc* translocation. Another hypothesis for the etiology of BL has been that malaria leads to the loss of T cell control over EBV-infected cells.³¹ Our data does not rule out this hypothesis, but suggests an additional role for malaria within that model. An alternative hypothesis of malaria-induced lymphomagenesis is that the repeated exposure to malaria leads to continual reactivation of memory B cells in germinal centers, leading to an accumulation of somatic mutations.⁴⁰ Recent evidence has shown that AID expression is not necessarily limited to germinal centers and malaria could be non-specifically activating B cells in the peripheral blood.⁴¹ In addition, we measured higher AID expression in children from Kisumu with a

positive EBV viral load compared to the children with no or undetectable virus in their peripheral blood, but EBV viral load-positive children in Nandi had similar levels of AID compared to EBV negative children. These data suggest that EBV alone does not contribute to AID expression and that increased malaria exposure results in higher AID expression only in the presence of EBV. Malaria may be playing a role in upregulating AID activity resulting in increased EBV viral load due to the preference of EBV to infect B cells with a mutated immunoglobulin.⁴² Therefore, there is a possible synergy occurring between malaria and EBV that could lead to increased AID expression.

Multi-parameter B cell phenotyping of children from regions of endemic and sporadic malaria transmission has not been performed with the breadth of our 11-color panel. Previous studies performed by our group on the Kisumu and Nandi cohorts were unable to definitively distinguish MBCs.³⁰ In addition, previously published studies from malaria endemic regions have primarily been performed on samples from adults. The cohort of children in our study is unique because we have followed them clinically from 1-month to 36-month old. Trends in B cell subset percentages are seen throughout the sampling period for these children and they remain steady through our analysis at 3 years of age. We previously demonstrated that there is a decrease in non-class switched (CD27+IgD+) and an increase in CD27-IgD- MBCs in the Kisumu cohort.³⁰ Our current findings confirm these trends, but we were able to better

define these populations as MZ-like memory B cells (CD27+ IgD+ IgM+) and atypical memory B cells (CD27- IgD- CD21-). The focus on MBCs is due to the presence of high levels of SHM in eBL, suggesting that the etiology is a post-germinal center B cell.³⁹ However, our data and data from other groups suggests that AID expression can be induced outside of the germinal center⁴¹ and therefore it is important to look at all B cell subsets. Analysis of the non-MBCs revealed a decrease in immature/transitional B cells in children living in a high malaria transmission area, but this decrease did not correlate to AID expression and represented only a minor change in the B cell pool in the blood.

With the goal of understanding the lack of long-lived immunity, several groups have reported the effects of malaria on B cell subsets.^{18,38} There are several changes in memory B cell populations, including an increase in atypical memory B cells in people living in malaria endemic regions.¹⁸ Atypical memory B cells have been described as “exhausted” memory B cells because they are functionally inactive.²⁰ There are some groups that refer to these as anergic B cells, and it has been suggested that they could function as B regulatory cells rather than memory B cells.⁴³ In our study, we show an increase in atypical memory B cells in children, consistent with what is seen in adults. However, the expansion of atypical memory B cells in the children from Kisumu is not as great as that demonstrated by other groups, and the percentage FcRL4+ atypical memory B cells of total B cells is less than 1%.¹⁸ There is some inconsistency in gating strategy for MBCs among other groups and we attempted to define the memory subsets taking into account the various methods used before.⁴⁴ We defined atypical B cells by their cell surface phenotype that was CD19+, CD10-, CD24-, CD27-, IgD- and CD21-. The addition of gating on IgD-negative B cells could account for the lower levels of atypical memory B cells in our study. In order for a B cell to be “memory” it must have experienced antigen, and therefore should be IgD-negative.⁴⁵ The chronic stimulation of B cells through the BCR can lead to the anergic phenotype of the cells in the atypical B cell pool, and therefore the increase in atypical B cells could be a result of repeated activation and have no functional role in malaria immunity.

IgM memory B cells have been described as marginal zone-like B cells, but recent evidence suggests that they are more similar to class-switched memory B cells than innate-like B cells.⁴⁶ MZ-like memory B cells are a lower percentage of total B cells in children from Kisumu compared to Nandi as shown in previous studies by our group at 12, 18 and 24 months of age.³⁰ In our study, we see that this trend continues through 36 months of age. In addition, there was a significant correlation of higher AID expression with a decrease in MZ-like memory B cells. The negative correlation of AID and MZ-like memory suggests that MZ-like memory B cells are not contributing to the expression of AID in PBMC. MZ-like memory B cells may be reduced in

malaria endemic regions due to chronic BCR stimulation resulting in a down-regulation of IgM on B cells. The decrease in AID expression associated with higher levels of MZ-like memory B cells could be due to the fact that higher AID levels would lead to increased class switching and therefore lower IgM expression. Our study was unable to definitively determine the B cell subset expressing AID by using a single cell approach because the limited amount of blood we could acquire from children and the technical difficulties associated with small cell numbers. Further studies are necessary to identify the cell type(s) responsible for increased AID expression.

The frequency of BL is estimated to be around five cases per 100,000 children living in equatorial Africa.^{25,27,47} Therefore, despite our detection of increased AID expression in children from a region with a high malaria frequency the likelihood of a child in our cohort developing BL is small, because our study started with less than 300 participants. Previous studies by Epeldegui *et al* have shown that AID is expressed in the peripheral blood of people infected with HIV that went on to develop non-Hodgkin’s lymphoma.¹⁰ The patient population of the Epeldegui *et al* study has several key differences compared to the current study of cohorts from Kisumu and Nandi. The use of the Multicenter AIDS Cohort Study patient population, which consisted of 6,972 patients followed longitudinally from the mid 1980’s to present, had advantages such as the length of patient exposure to HIV (5 years or more), the age of sampling (adulthood) and most importantly, the ability to select a group that had already developed lymphoma.¹⁰ We hypothesize that if we were able to look at the expression of AID before the development of BL we would detect similarly significant increases in AID expression, as shown in the study by Epeldegui *et al*. Unfortunately, the low frequency of BL and the limited resources available, did not allow for such a study in children in western Kenya.

The results of this study demonstrate that elevated AID expression and shifts towards higher atypical MBC frequency could be a consequence of a high frequency of malaria exposure in 3-year-old children from a region associated with a high risk for BL. High levels of AID were found to be primarily in children with detectable levels of EBV in the Kisumu District, suggesting that there was a synergistic effect of malaria and EBV. These data further provide evidence of altered MBCs in children with high malaria exposure leading to increased levels of atypical memory B cells and decreased MZ-like memory. In addition, there was a significant correlation with AID expression and a decrease in MZ-like memory regardless of the level of malaria exposure. Further research is necessary to determine how EBV and malaria act on these cells to increase AID expression and lead to malignancy.

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