Reduced transplacental transfer of a subset of Epstein-Barr virus specific antibodies to neonates of mothers infected with *Plasmodium falciparum* malaria during pregnancy

Sidney Ogolla\(^a,b\), Ibrahim I. Daud\(^a,c\), Amolo S. Asito\(^a,d\), Odada P. Sumba\(^a\), Collins Ouma\(^b\), John Vulule\(^a\), Jaap M. Middeldorp\(^b\), Arlene E. Dent\(^f\), Saurabh Mehta\(^d\), Rosemary Rochford\(^h\)#*

\(^a\)Center for Global Health Research, Kenya Medical Research Institute, Kisumu, Kenya; \(^b\)Maseno University, Kisumu, Kenya; \(^c\)Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya; \(^d\)School of Physical and Biological Sciences, Jaramogi Oginga Odinga University of Science and Technology Bondo, Kenya; \(^e\)Department of Pathology, VU University Medical School, Amsterdam, The Netherlands, \(^f\)Case Western Reserve University, OH, USA, \(^g\)Division of Nutritional Sciences, Cornell University, Ithaca, NY, USA, \(^h\)Department of Microbiology and Immunology, SUNY Upstate Medical University, Syracuse, NY, USA

Running title: Reduced transplacental transfer of EBV-specific antibodies

#Corresponding author:  Prof. Rosemary Rochford, Department of Immunology and Microbiology, University of Colorado, Denver, 12800 E. 19\(^{th}\) Ave, Aurora, CO 80045-2537. Phone: 303-724-9960; e-mail: rosemary.rochford@ucdenver.edu
Current address: Health Challenges and Systems, African Population and Health Research Center, Nairobi, Kenya

Current address: Department of Immunology and Microbiology, University of Colorado, Denver, Aurora, CO, USA
Abstract

Over 35% of children from a malaria endemic region are infected with Epstein-Barr virus (EBV) by six months of age. This susceptibility may be linked to impaired transplacental transfer of antibodies. In this study, we determined the effect of malaria exposure during pregnancy on the transfer of EBV-specific maternal antibodies in a region of western Kenya that experiences endemic malaria. Pregnant mothers were recruited and followed up until delivery to determine neonatal malaria exposure. EBV lytic (VCA, Zta, EAd), EBV latent (EBNA1) and tetanus-specific IgG antibodies were measured in 70 paired maternal and cord blood samples using a Luminex-bead based assay. A high proportion (63%) of the infants were exposed to malaria in utero. Levels of EBV and tetanus-specific antibodies were similar in malaria-infected mothers and in mothers who had no detectable malaria infection. Malaria exposed neonates had significantly lower levels of anti-EBNA1, anti-Zta and anti-EAd antibodies as compared to their mothers. In utero malaria exposure resulted in significant reduction in transplacental transfer of anti-VCA-p18 and -EBNA1 antibodies by 13% and 22% respectively. Neonates received significantly low levels of anti-Zta and –EAd antibodies irrespective of malaria exposure. In multivariate analysis, in utero malaria exposure was associated with a significant reduction in the transfer of anti-VCA-p18 and -EBNA1 antibodies to the neonates (p=0.0234 and p=0.0017, respectively). Malaria during pregnancy results in differential transfer of EBV-specific antibodies from the mother to the fetus. The impaired
transplacental transfer of some antibodies may lead to the malaria-exposed neonates being susceptible to early EBV infection.
INTRODUCTION

Endemic Burkitt’s lymphoma (eBL) is a distinct form of non-Hodgkin’s lymphoma and is the most common pediatric malignancy in malaria endemic regions of sub-Saharan Africa (1). Both infection with Epstein Barr virus (EBV) and repeated episodes of *P. falciparum* malaria are known risk factors for eBL (2) but the mechanism(s) by which these two agents interact to promote the emergence of malignant B cell clones has not been elucidated. Recently, we found that infants from a malaria endemic region of Western Kenya were infected with EBV by 6 months of age (3). Living in malaria endemic regions was a predictor of this early age of primary infection. This aberrant primary EBV infection may set the stage for lymphoma development as previously hypothesized (4-6).

The lytic and latent phases of EBV life cycle induces distinct antibodies in response to specific lytic and latent antigens. Anti-EBV nuclear antigen (EBNA)-1 antibodies are produced against EBNA-1, the only antigen expressed in latently infected memory B cells and in eBL tumors (7). Anti-viral capsid antigen (VCA), early antigen (EA), immediate early protein (Zta) antibodies are produced against their respective target lytic antigens (8). Clinically, elevated levels of anti-VCA and EBNA-1 IgG antibodies have been used as evidence of past infection (9), while the presence of IgG antibodies to the EBV early antigens (EAd and Zta) generally reflect recent or reactivated infections (10, 11). Although EBV-specific antibody patterns reflect dynamics of EBV activity in adults, few studies have addressed this issue in infants and children (3, 12-15) or in newborns (16, 17).
More importantly, no comparison to maternal antibody levels has been made nor has there been an analysis of how maternal malaria infections impact transplacental transfer of EBV-specific antibodies.

Mothers transfer pathogen specific antibodies to their infants during pregnancy. These passive antibodies from the mother provide protection to the infant before they develop de novo antibodies (18). These antibodies are mainly acquired through transplacental transfer. The transport of maternally derived IgG across the placenta is mediated by Fc receptor of IgG including FcγR and FcRn (19-21). It is an active and selective process whereby neonatal FcRn binds IgG and crosses the syncytiotrophoblast and releases IgG into the endothelium of fetal capillaries.

Maternal factors such as placental malaria, HIV infection, maternal hypergammaglobulinemia, and preterm birth, have been shown to inhibit the efficient mother-to-child transfer of pathogen specific antibodies (22-27). For example, in a study in the rural coastal area of Kenya, placental malaria infection as well as HIV infection was associated with a significant reduction in the transfer of anti-tetanus IgG antibodies to the neonates (26). In a rural Gambian population, placental malaria infection was associated with significant reduction in transplacental transfer of antibodies against herpes simplex virus (HSV), varicella-zoster virus (VZV) and respiratory syncytial virus (RSV) (27). Maternal HIV infection was associated with reduced transplacental transfer of IgG.
antibodies against tetanus toxoid, measles virus and varicella-zoster virus (22). Together, these studies suggest that maternal infections during pregnancy can interfere with the efficient vertical transfer of pathogen specific antibodies potentially leaving infant susceptible to infections early in life. Transplacental transfer of EBV specific maternal antibodies to their infants in the context of maternal malaria infection has not been investigated. 

Given that over 35% of infants from a malaria endemic region can be infected with EBV during infancy (3) when typically maternally acquired antibodies should protect them against EBV infection, we tested the hypothesis that maternal malaria infection reduced the transplacental transfer of EBV-specific maternal antibodies to the neonates. In addition, the above-mentioned studies looked at antibody transfer in the context of placental malaria infection as determined by the presence of malaria parasites during delivery. The long-term effect of maternal malaria infection (e.g. infection with malaria at any time during pregnancy) on antibody transfer remains poorly understood. This study investigated the effect of maternal malaria infection during pregnancy on the efficiency of transplacental transfer of well characterized EBV-specific antibodies (e.g. VCA, EBNA1, EAd, and Zta) and tetanus toxoid-specific antibodies in mother-child pairs from a region of Western Kenya where malaria transmission is high.

MATERIALS AND METHODS
**Study population.** This study was conducted at the antenatal clinic (ANC) and maternity ward of Chulaimbo Sub-District hospital in Kisumu West District. This hospital mainly serves a rural population that experiences holoendemic malaria with two seasonal peaks; June to August and November to December (28). The inclusion criteria consisted of pregnant women of any gravidity, <24 weeks gestation, having a normal full blood count, HIV negative, residency within a 10km distance of the hospital and willing to return to the hospital for delivery, follow up clinical procedures and laboratory testing. Gestational age was evaluated by measurement of fundal height and history of the last menstrual period. All pregnant women were enrolled in a six-month period from June to November 2011. The demographics of this study population have been previously described (29). Briefly, at enrollment, demographic and antenatal care data was captured. A total of 200 pregnant women were enrolled. Of these, 25 were HIV-positive and were excluded from these analyses. The remaining 175 pregnant women who were HIV-1 negative were followed up monthly through antenatal visits (up to 4 follow-up visits per mother) until delivery. Of these, 93 pregnant women delivered at the hospital and 70 of them had complete mother-child pair of plasma samples that were analyzed in the current study.

All the women included in the study were tested for HIV as part of Maternal-to-Child-Transmission (MCTC) of HIV programs in accordance with the Kenya Ministry of Health national guidelines. All the mothers received an average of two doses of sulphadoxine-pyrimethamine (SP) that was administered by directly
observed therapy at the ANC visits as part of the recommendation of Kenya Ministry of Health for malaria prophylaxis. Approvals for this study were obtained from the Kenya Medical Research Institute (KEMRI) Ethical Review Committee and Ethical Review Board of SUNY Upstate Medical University Hospital, USA. Written informed consent was obtained from all the mothers.

**Sample collection and preservation.** During the ANC visits and within 12 hours of delivery, 200-500µL of maternal venous blood was collected by venipuncture or finger prick into EDTA microtainers (BD, Franklin Lakes, NJ). After delivery, ~500µL of cord blood, the representative pool of blood circulating in the neonates, was collected from the umbilical vein into EDTA microtainers (BD, Franklin Lakes, NJ). All samples were immediately transported to the SUNY Upstate University laboratory at KEMRI’s Centre for Global Health Research and plasma was separated from whole blood and stored at -80°C until antibody assays were performed.

**Malaria diagnosis.** Malaria parasite load and plasmodium species was determined in maternal and cord blood samples as previously described (29). An infant was considered exposed to malaria *in utero* if any of the blood smears and/or quantitative (Q)-PCR results at any of the ANC visits, maternal blood at delivery, or in the cord blood were positive for *P. falciparum*. An infant was considered not malaria exposed if the blood smears and/or RTQ-PCR results at
any of the ANC visits, maternal blood at delivery or in the cord blood were negative.

**EBV peptide antigens and Luminex-based suspension beads assays.** EBV-specific antibodies were detected using 5 synthetic peptides covering immunodominant epitopes of the viral capsid antigen VCA-p18 subunit, VCA gp125 subunit, EBV nuclear antigen 1 (EBNA1), early diffuse antigen complex (EAd) and the immediate early protein, Z transcriptional activator (Zta) (13, 30, 31). The EBV gp125 is another major capsid immunogen of VCA, independent from p18. Tetanus toxoid (TT) antigen (Calbiochem, Darmstadt, Germany) was used as a control since the mothers are routinely immunized against tetanus during pregnancy. The choice of the above mentioned antigens were based on their well characterized serological profiles as previously described (13, 32, 33).

To detect EBV and TT-specific IgG against this panel of EBV peptides and TT, we used a Luminex-bead based suspension assay using a previously described protocol (13). Plasma was diluted 1:100 prior to testing. At least 75 beads per region were acquired on Bioplex reader (Bio Rad, Hercules, CA) and results expressed as mean fluorescence intensity (MFI).

**VCA- and EBNA1-specific IgG subclass ELISA.** EBV specific IgG subclass antibody distribution was analyzed in the mother-child pairs of plasma samples using two synthetic peptides covering the immunodominant epitopes of VCA-
p18 and EBNA1 by ELISA as previously described (13, 32) with modifications. Peroxidase conjugated sheep antibodies specific to the different human IgG subclasses (Invitrogen, Camarillo, CA) was added and the plates developed using tetramethylbenzidine (TMB) substrate. Plasma samples were diluted 1:100. The optical densities were measured at 490nm on BioRad microplate reader using microplate manager version 6 (BioRad, Hercules, CA). Antibody levels for IgG subclass responses were expressed in arbitrary units (AU), which were calculated by dividing the test sample ODs by the mean OD+2 standard deviations (SDs) from EBV negative control sera.

Total IgG ELISA. Determination of total IgG in maternal plasma was done using human IgG total ELISA kit from eBioscience (San Diego, CA) following manufacturer’s instruction. The plates were read at 450nm (Molecular Devices, Sunnyvale, CA) and the concentration of total IgG (mg/ml) in plasma was extrapolated from the standard curve.

Statistical analysis. The levels of EBV and TT-specific IgG antibodies between malaria-exposed and non-exposed neonates as well as malaria-infected and uninfected mothers were compared using Mann Whitney U test. Placental transfer was measured as the ratio of the level of specific antibody in cord blood to that in respective maternal blood, i.e. the cord to maternal ratio (CMR), (26, 27). Correlation between the levels of maternal EBV and TT-specific antibodies in relation to those in cord blood was calculated using Spearman correlation test.
Linear regression analysis was used to assess the effect of a number of variables such as maternal age, parity, birth weight, and gestational age at first malaria exposure on the transplacental transfer of EBV-specific antibodies. In multivariate analysis, we adjusted for maternal age, hypergammaglobulinemia, and gestational age at exposure, as potential confounders as they are known or suspected risk factors for the outcome. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad software) and Stata statistical software version 11 (StataCorp). For all statistical analyses, a two-sided p-value of ≤0.05 was considered significant.

RESULTS

Characteristics of the study population. Plasma from 70 mother-child pairs was analyzed in this study. A neonate was considered exposed to malaria if *P. falciparum* malaria was detected in maternal venous blood at any ANC visit, at delivery in the placenta or venous blood, or in cord blood. A neonate was considered non-exposed if *P. falciparum* malaria was not detected in the maternal venous blood at any ANC visit, at delivery, or in cord blood. It may be possible that we did not capture all malaria infections in the mother, but for the purpose of this comparison, we labeled this group non-exposed. Using this criterion, 63% (44/70) of the neonates in this study were exposed to malaria *in utero* (Table 1).
The demographic and clinical characteristics of this study population are shown in Table 1. Mean maternal age in the malaria-exposed group was 22.36 years (SD±6.1) while that in the non-exposed group was 21.77 years (SD±6.79). To determine if the mothers were infected with EBV, anti-VCA-p18 IgG antibodies were measured in the enrollment ANC plasma samples by ELISA (13, 32). All the women in this study were EBV seropositive. Ninety-five percent of the pregnant mothers in malaria-exposed group and 92% in the malaria non-exposed group had received tetanus toxoid (TT) vaccine during the current pregnancy. Primigravid mothers in malaria exposed and non-exposed group were 36% and 30%, respectively. Only four neonates weighed less than 2.5 kg, which is defined as low birth weight (LBW). There were 2 infants (5%) with LBW in the malaria exposed and 2 (8%) in the malaria non-exposed group. Hypergammaglobulinemia was defined as levels of total IgG greater than 30 mg/ml. Seven mothers (27%) in the malaria un-infected group and sixteen (36%) mothers in the malaria infected group had hypergammaglobulinemia.

**Comparison of levels of EBV and TT-specific antibodies in mothers and their neonates.** We first compared the levels of EBV and TT-specific antibodies in mothers and their paired neonates to determine if malaria exposure during pregnancy affected the levels of antibodies in the mothers or in the cord blood of the infants. To do this, we used a Luminex-bead based assay that allowed us to measure antibodies to EBV lytic (e.g. VCA-p18, VCA-gp125, EAd, Zta,) and latent (e.g. EBNA1) antigens as well as TT antigen (13). We included the TT...
antigen as a reference antigen, as there are numerous studies evaluating the transplacental transfer of anti-TT antibody (22, 26, 34, 35). The levels of EBV specific anti-VCA-p18, VCA-gp125 and anti-TT antibodies were comparable in the mothers and their neonates irrespective of malaria exposure (Fig. 1). In contrast, both the malaria-exposed and malaria non-exposed neonates had significantly lower levels of anti-Zta and anti-EAd antibodies in the cord blood as compared to their mothers (both \( p < 0.001 \)). Interestingly, malaria exposed neonates had significantly lower anti-EBNA1 antibodies compared to their mothers (\( p < 0.001 \)), while there was no significant difference in the level of anti-EBNA1 antibodies between the mothers and their neonates that were non-exposed to malaria (Fig. 1). Because the pregnant mothers were infected with malaria at different time points during pregnancy, we compared the levels of anti-EBV and TT antibodies in neonates who were exposed to malaria early (<26 weeks gestation) vs. late (>26 weeks gestation) in pregnancy and found no significant difference in the levels of anti-VCA-p18, -EBNA1, -Zta, -EAd, -VCA-gp125 and anti-TT between the two groups of neonates (data not shown).

Since we observed similar levels of anti VCA-p18, -VCA-gp125 and -TT in the mothers and their neonates, we then correlated the levels of anti-EBV and TT specific antibodies in maternal venous blood to that in their neonates to determine if maternal antibody level is a predictor to the neonatal antibody levels. We observed a significant positive correlation between maternal and neonatal anti-VCA-p18, EBNA1, gp125 and TT antibodies regardless of malaria exposure.
Neonates born to mothers with malaria during pregnancy have reduced transplacental transfer of anti-VCA-p18 and anti-EBNA1 antibodies. We next determined if there was a reduction in EBV-specific antibody transfer due to exposure to malaria in utero. Placental transfer was measured as the ratio of the level of antibody in cord blood to that in respective maternal venous blood at delivery, i.e. the cord to maternal ratio (CMR). Transplacental transfer of antibodies to VCA-p18 and EBNA1 were significantly reduced from malaria infected mothers to their exposed neonates (by 13.40% and 21.65% respectively) (Table 2). There was no significant reduction in transplacental transfer of anti-Zta, anti-EAd, and anti-TT from the mothers to the neonates due to malaria exposure in utero ($p=0.950$, $p=0.349$, and $p=0.458$ respectively) although there was a trend to more significant reduction of anti-VCA gp125 ($p=0.064$) (Table 2).

When we performed a multivariate analysis to determine the effect of maternal malaria infection on transplacental transfer of anti-EBV and -TT antibodies while adjusting for potential confounding factors such as maternal age, hypergammaglobulinemia, and gestational age at first malaria exposure, we observed a significantly lower transfer of anti-VCA-p18 and anti-EBNA1 antibodies to the neonates from mothers who had malaria infection during...
pregnancy, compared to those who did not have malaria during pregnancy (p=0.009 and p=0.042, respectively) (Table 3). No significant reductions were observed for the other EBV-specific antibodies or for TT-specific antibodies.

**VCA-p18 and EBNA1 specific IgG subclass distribution in maternal venous and cord blood.** One possible explanation for the reduced transplacental transfer of anti-VCA and anti-EBNA antibodies could be differences in the IgG subclass distribution of these antibodies during pregnancy potentially due to viral reactivation. To test this possibility, we assessed the IgG subclass reactivity to EBNA-1 and VCAp18 antigens in the maternal venous blood and in the infant cord blood. Anti VCA-p18 IgG1 and IgG4 were detected in all the maternal and cord blood samples. Forty two percent (42%) and 40% of the cord blood samples had detectable anti VCA-p18 IgG2 and IgG3 antibodies respectively. EBNA1 IgG1 was detected in 98% and 88% of the maternal and cord blood samples respectively. Eighty three percent (83%) of maternal and cord blood samples had detectable EBNA1 IgG2 while EBNA1 IgG3 was detected in 71% and 59% of the maternal and cord blood samples. Thirty five percent and 36% of maternal and cord blood had detectable EBNA1 IgG4 respectively.

Next, we evaluated the levels of anti-VCAp18 and –EBNA-1 IgG sub-class levels in maternal venous and cord blood samples. Levels of VCA-p18 and EBNA1 specific IgG1 were significantly lower in cord blood compared to maternal venous blood (p=0.001 and 0.012 respectively). There were comparable levels of VCA-
specific IgG2, IgG3 and IgG4 in maternal venous and cord blood. Similarly, we did not observe any significant difference in the levels of EBNA1 specific IgG2, IgG3 and IgG4 between maternal venous and cord blood (all \( p>0.05 \))(Figure3). There were higher overall levels of anti-EBNA1 IgG2 antibodies as compared to the other IgG isotypes.
This study tested the hypothesis that exposure to *P. falciparum* malaria *in utero* would interfere with the transplacental transfer of EBV-specific antibodies from the mother to the neonate. Indeed, the data presented in this study demonstrate that the transfer of IgG antibodies to the EBV lytic VCA-p18 antigen and latent EBNA1 antigen from the mother to the neonate was significantly reduced following malaria exposure *in utero* and this effect remained significant even after adjusting for other confounding factors. We also observed that regardless of malaria exposure during pregnancy, neonates had significantly less EBV lytic anti-Zta and -EAd IgG antibodies.

A number of conditions are known to affect the maternal-fetal transfer of IgG antibodies including HIV infection, placental malaria, and maternal hypergammaglobulinemia (23, 26, 27, 34). In this study, only HIV-negative mothers were enrolled and in our multivariate analysis we adjusted for hypergammaglobulinemia indicating that the effect of reduced transfer of anti-VCA-p18 and -EBNA1 antibodies was likely due to maternal malaria infection during pregnancy. It is not clear why this effect is selective for only two of the EBV antigens tested. However, we have previously shown that malaria infection during pregnancy results in EBV reactivation (29). It is possible that there was a shift in antibody isotype responses during EBV reactivation in the mothers such that a different isotype of IgG was dominant and was less efficiently transferred to the neonates. It is known that antigens that mainly elicit IgG1 or IgG3
responses are transported across the placenta more efficiently than either IgG2 or IgG4 (36, 37) with a consequence of reduced transplacental transfer of antibodies of these specificities. A previous study found that the IgG subclass distribution to VCA in acutely infected individuals or during viral reactivation was predominantly IgG1, no IgG2 was detected and the presence of IgG3 was indicative of viral reactivation (12). Consistent with this study, we found that IgG1 was the dominant sub-class of IgG in anti-VCA and anti-EBNA antibodies detected in maternal venous and infant cord blood. Thus, the reduction in transplacental transfer was likely due to decreased IgG1 transfer and not because of a shift in IgG sub-classes.

Antibodies to the EBV EAd lytic antigen have long been used clinically as a marker of EBV reactivation as they are short-lived (38). More recently, studies with another EBV lytic antigen, Zta, have also shown utility in indicating viral reactivation (10, 13). All the mothers in this study had both anti-Zta and –EAd antibodies at the time of delivery indicative of viral reactivation. This is consistent with studies that have demonstrated pregnancy induced viral reactivation (39, 40). One surprising finding from this study is that irrespective of malaria exposure, antibodies against Zta and EAd are not efficiently transported across the placenta into fetal circulation, resulting in low levels of anti-Zta and -EAd antibodies in the neonates. One possible explanation is the low maternal levels of anti-Zta and anti-EAd antibodies. This observation is supported by data from a previous study that demonstrated that levels of maternal antibodies to varicella
Zoster virus were the most important predictor of the neonatal antibody levels (41). The low maternal levels of anti-EAd antibodies could be due to the fact that the levels of anti-EAd antibodies fall relatively fast after infection (42). Since the majority of IgG are transported across the placenta during the last trimester of pregnancy (43), the rapid decay of anti-EAd antibodies may result in very little antibodies being transferred to the neonates as reported. This is in contrast to the high levels of IgG antibodies against both VCA-p18 and EBNA1 we observed and that persist for life in healthy carriers (42). This could also explain the lack of correlation between maternal and neonatal anti-EAd antibodies observed in the current study. Since some neonates in both malaria exposed and non-exposed groups had elevated anti-EAd IgG levels suggestive of active neonatal infection, EBV DNA was assessed in all the cord blood samples but were found to be negative (data not shown).

Another plausible explanation to the low levels of anti-Zta and anti-EAd IgG antibodies in cord blood would be the competitive binding of antibodies to the neonatal Fc receptors. The transfer of antibodies across the placenta is a selective process that is dependent on neonatal Fc receptors (FcRn). For antibodies to be transferred across the placenta, they have to bind to the FcRn, transported across the syncytiotrophoblast and into the fetal circulation (20, 43). Antibodies that are expressed at high levels in the mother may be competing with the antibodies that are expressed at low levels such as anti-Zta and anti-EAd for binding to limited number of FcRn receptors, thereby reducing the transfer of
anti-Zta and anti-EAd antibodies to the infants. However, this observation warrants further studies to investigate the mechanism. Lastly, the low levels of cord blood anti-Zta and -EAd antibodies could be explained by the fact that these antibodies may have been bound to the specific antigen and trapped within the placenta as immune complexes (44) and as a consequence, not transported into fetal circulation.

The finding from this study that maternal malaria infection during pregnancy did not affect the transplacental transfer of TT-specific antibodies is consistent with previous studies that demonstrated that placental malaria does not have an effect on the transplacental transfer of anti-TT antibodies (22, 35). However, these results are in contrast to other studies that demonstrated that placental malaria results in reduced transplacental transfer of anti-TT antibodies (26, 34). However, all of these previous studies focused on placental malaria as determined by the presence of malaria parasites in the mother at birth. In contrast, we assessed malaria infection at any time during pregnancy; thus, our results are not directly comparable with the results from previous studies.

A strength of this study is the longitudinal study design that allowed us to actively follow pregnant mothers through pregnancy up to delivery while other studies have focused mainly on placental malaria due to their cross-sectional study design. This allowed us to identify peripheral malaria infections of the mother during pregnancy rather than only malaria infection at delivery. In addition, we
used a more sensitive Q-PCR method (as compared to blood smears) to determine malaria infection status of the pregnant mothers at enrollment, subsequent follow-up, at delivery or in cord blood. However, whether the mothers had single or repetitive infections with malaria was not analyzed and is a limitation of the study. We did not analyze mothers with placental malaria separately from those with malaria infection at any time during pregnancy because there were so few mothers with placental malaria in this cohort. An additional limitation of our study was the modest number of mother-child pairs that was included in these analyses. Finally, while anti-VCA and –EBNA-1, -EAd and Zta antibodies are well described during infection with EBV, they are not known to be neutralizing antibodies. In contrast, antibodies against the EBV gp350 protein are neutralizing but have not been well characterized in human populations. Future studies will need to be done to analyze the levels of anti-gp350 antibodies in this cohort.

In summary, malaria infection during pregnancy results in impaired transplacental transfer of a subset of EBV specific antibodies. Inadequate transfer of anti-EBV antibodies from mothers to the neonates may predispose the infants to early EBV infection. Infection with EBV in infancy leads to poor control of the virus and has been hypothesized to be a risk factor for Burkitt's lymphoma. It remains to be determined whether this reduced transplacental transfer of anti-EBV antibodies predisposes the neonates to EBV infection in early infancy. This study
demonstrates the need for improved measures to prevent maternal malaria infections during pregnancy.

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FIGURE LEGENDS

FIG 1. Relative levels of EBV and TT-specific IgG antibodies in malaria exposed and non-exposed mothers and infants. Plasma was diluted at 1:100 and tested using a Luminex bead based assay. The mean fluorescence intensity (MFI) of 75 Luminex beads for each of the antigen tested is indicated on the y-axis. Significant p-values of paired t tests are indicated in the figures. Horizontal bars represent median values for each.

FIG 2. Correlation between maternal and infant anti-EBV and anti-TT specific antibodies in malaria exposed and non-exposed groups. Correlation between maternal and infant antibody levels was assessed by Spearman Correlation where p<0.05 was considered significant and only significant p-values are shown in the figure.

FIG 3. Levels of EBV specific IgG subclass in maternal venous blood and in their neonates. (A) Anti-VCA-p18 and (B) anti-EBNA1 specific IgG sub-class antibodies were measured by ELISA in the cord blood (CB) and maternal venous blood (VB) at delivery. Arbitrary units were calculated by dividing the test sample ODs by the mean OD+2 standard deviations (SDs) of the negative control sera. Horizontal bars represent the means and only significant p-values are shown in the figure.
Table 1. General characteristics of the mothers and their infants.

<table>
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<th>Malaria unexposed</th>
<th>p-value</th>
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<tr>
<td>n</td>
<td>44 (63)</td>
<td>26 (37)</td>
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<td>Maternal age (mean) [±SD]</td>
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<td>26 (59)</td>
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<td>24 (92)</td>
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<td>26(100)</td>
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<td>Mean Birth weight (grams) [±SD]</td>
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<td>3146 [420]</td>
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</tr>
<tr>
<td>Time of malaria exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>31 (70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late</td>
<td>13 (30)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as n (%) unless otherwise stated. Differences in proportions between the groups were determined by Fisher exact test. Early exposure to malaria was defined as <26 weeks gestation while late exposure was defined as >26 weeks gestation.
Table 2. Transplacental transfer of EBV and TT specific antibodies from the mother to the neonate

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CMR Malaria exposed (Median, IQR)</th>
<th>CMR Malaria non-exposed (Median, IQR)</th>
<th>% reduction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-VCA-p18</td>
<td>0.862 (0.754 - 1.001)</td>
<td>0.996 (0.829 - 1.182)</td>
<td>13.40</td>
<td>0.023</td>
</tr>
<tr>
<td>Anti-EBNA1</td>
<td>0.546 (0.426 - 0.809)</td>
<td>0.762 (0.558 - 1.012)</td>
<td>21.65</td>
<td>0.002</td>
</tr>
<tr>
<td>Anti-Zta</td>
<td>0.151 (0.080 - 0.313)</td>
<td>0.153 (0.074 - 0.320)</td>
<td>0.25</td>
<td>0.950</td>
</tr>
<tr>
<td>Anti-EAd</td>
<td>0.102 (0.048 - 0.176)</td>
<td>0.068 (0.035 - 0.166)</td>
<td>-3.40</td>
<td>0.349</td>
</tr>
<tr>
<td>Anti-VCA-gp125</td>
<td>0.697 (0.552 - 0.979)</td>
<td>0.850 (0.680 - 0.972)</td>
<td>15.25</td>
<td>0.064</td>
</tr>
<tr>
<td>Anti-TT</td>
<td>0.949 (0.816 - 1.013)</td>
<td>0.981 (0.854 - 1.040)</td>
<td>3.20</td>
<td>0.458</td>
</tr>
</tbody>
</table>

Data are median CMR. Interquartile ranges are in parenthesis. Maternal and cord blood plasma samples were tested for the presence of anti-EBV and TT specific antibodies by Luminex bead based assay. Cord to maternal ratio (CMR) i.e. ratio of specific antibody in cord blood to that in respective maternal blood was used to determine placental transfer of antibodies. Percentage reduction due to malaria was determined by the formula: (CMR of non-exposed – CMR of exposed)*100 Statistical differences of p≤0.05 are considered significant as determined by Mann-Whitney test.
Table 3. Multivariate linear regression analysis of the transplacental transfer of EBV and TT-specific antibodies

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Intercept</th>
<th>SE</th>
<th>Mean difference</th>
<th>SE</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Anti-VCA-p18</td>
<td>0.014</td>
<td>0.330</td>
<td>-0.573</td>
<td>0.212</td>
<td>0.009</td>
</tr>
<tr>
<td>Log Anti-EBNA1</td>
<td>0.703</td>
<td>0.395</td>
<td>-0.526</td>
<td>0.253</td>
<td>0.042</td>
</tr>
<tr>
<td>Log Anti-Zta</td>
<td>-2.065</td>
<td>0.749</td>
<td>-0.644</td>
<td>0.480</td>
<td>0.185</td>
</tr>
<tr>
<td>Log Anti-EAd</td>
<td>-2.681</td>
<td>0.998</td>
<td>-0.466</td>
<td>0.640</td>
<td>0.469</td>
</tr>
<tr>
<td>Log Anti-VCA-gp125</td>
<td>0.029</td>
<td>0.367</td>
<td>-0.215</td>
<td>0.235</td>
<td>0.365</td>
</tr>
<tr>
<td>Log Anti-TT</td>
<td>0.213</td>
<td>0.270</td>
<td>-0.143</td>
<td>0.173</td>
<td>0.410</td>
</tr>
</tbody>
</table>

The CMR were log transformed then values were adjusted for neonatal values plus maternal age, hypergammaglobulinemia, and gestational age at exposure.
Figure 1

Anti-VCA p18

Anti-EAd

Anti-EBNA1

Anti-TT

Anti-Zta

Anti-VCA-gp125

Anti-EAd

Anti-EBNA1

Anti-TT

Anti-Zta

Figure 1
<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Exposed</th>
<th>Non-Exposed</th>
<th>Correlation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-VCA p18</td>
<td>r=0.892</td>
<td></td>
<td></td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Anti-Zta</td>
<td>r=0.278</td>
<td></td>
<td></td>
<td>p=0.169</td>
</tr>
<tr>
<td>Anti-VCA gp125</td>
<td>r=0.906</td>
<td></td>
<td></td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Anti-EBNA1</td>
<td>r=0.856</td>
<td></td>
<td></td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Anti-EAd</td>
<td>r=0.190</td>
<td></td>
<td></td>
<td>p=0.275</td>
</tr>
<tr>
<td>Anti-Tetanus</td>
<td>r=0.910</td>
<td></td>
<td></td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Maternal Anti-VCA p18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal Anti-VCA p18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal Anti-Zta</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal Anti-Zta</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Maternal Anti-VCA gp125</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal Anti-VCA gp125</td>
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<td></td>
</tr>
<tr>
<td>Maternal Anti-EBNA1</td>
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<td></td>
<td></td>
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<tr>
<td>Neonatal Anti-EBNA1</td>
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<tr>
<td>Maternal Anti-EAd</td>
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<td></td>
</tr>
<tr>
<td>Neonatal Anti-EAd</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Maternal Anti-Tetanus</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal Anti-Tetanus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2
Figure 3

A

Anti-VCA-p18 antibody levels (AU)

B

Anti-EBNA1 antibody levels (AU)

p = 0.001

p = 0.012