EFFECT OF MATERNAL *PLASMODIUM FALCIPARUM* MALARIA INFECTION DURING PREGNANCY ON TRANS-PLACENTAL TRANSFER OF EPSTEIN BARR VIRUS-SPECIFIC IgG ANTIBODIES, FETAL LYMPHOCYTE HOMEOSTASIS AND CYTOKINES IN CHULAIMBO

BY

Ogolla Sidney Onyango

A Thesis Submitted in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy in Biomedical Science and Technology (Medical Immunology) of Maseno University.

DEPARTMENT OF BIOMEDICAL SCIENCE AND TECHNOLOGY

MASENO UNIVERSITY

DECLARATION

I declare that this thesis is my original work and has not been submitted to any other University. The work has been presented to Maseno University as a fulfillment for the award of Doctor of Philosophy in Biomedical Science and Technology (Medical Immunology). No part of this thesis may be reproduced without prior permission of the author and/or Maseno University.

Ogolla Sidney Onyango (PG/PHD/0145/2012)

Signature……..Date…….

This thesis has been submitted for examination with our approval as university supervisors.

SUPERVISORS

1. Prof. Collins Ouma, PhD

Department of Biomedical science and Technology

Maseno University,

Kisumu, Kenya.

Signature……..Date…….

2. Prof. Rosemary Rochford, PhD

Department of Microbiology and Immunology

SUNY Upstate Medical University,

750 East Adams St. Syracuse,

New York, USA.

Signature……..Date…….

ACKNOWLEDGEMENT

I would like to appreciate my University academic and research supervisor Prof. Collins Ouma of Maseno University for excellent mentorship and supervision during my research and thesis write up. Special gratitude goes to Prof. Rosemary Rochford, for the opportunities, strong scientific mentorship during my post-graduate studies, her kind indulgence and painstaking corrections during my research work are highly appreciated and to that, I owe a debt of gratitude. To my colleagues at the at the laboratory Dr. Odada Peter, Dr. Maxwell Majiwa, Dr. Kiprotich Chelimo, Dr. Ibrahim Daud, Eunice Toko, Emily Koech, Cliff Oduor you deserve hearty thanks for providing a conducive and stimulating scientific environment during my studies. Special thanks also goes to the staff at Prof. Rochford's lab at the Upstate Medical University especially Dr. Christina, Dr. Eric Worlford, Dr. Joel Wilmore, Julie Ritchie, Nancy Fiore, Chandrav De, Paul Bassarel and Katie for their hospitality during my visit. To the SUNY/CWRU/KEMRI projects staff, thank you for being there with me. This work would not be possible without the support from the National Cancer Institute at the National Institutes of Health grant no. CA102667 and D43 doctoral training grant (NCI 153707).

DEDICATION

To all the mothers and infants who took part in this study.

ABSTRACT

Co-infection with malaria and Epstein Barr virus (EBV) have been implicated in the etiology of endemic Burkitt's lymphoma (eBL) but the precise mechanism by which these two agents lead to the pathogenesis of eBL has been partially elucidated. In addition, living in a malaria holoendemic area is a risk factor to earlier primary EBV infection that results in higher viral loads and poor control of the virus, a risk factor in the etiology of eBL. Children from Chulaimbo, a malaria holoendemic region are infected with EBV early in life when typically maternal antibodies should protect them against the infection. This study investigated the effect of maternal malaria infection during pregnancy on the transfer of EBV-specific IgG antibodies and fetal lymphocyte homeostasis in pregnant women from Chulaimbo. The specific aims were: to determine the effect of malaria infection during pregnancy and maternal hypergammaglobulinemia on the transfer of EBV-specific antibodies; to determine the effect of *in utero* malaria exposure on cord blood cytokines and chemokines; to determine the effect of *in utero* malaria exposure on cord blood Treg cells and characterize B and T-cells in cord blood following *in utero* malaria exposure. In a longitudinal analysis, 70 pregnant women were analyzed from Chulaimbo (malaria holoendemic area); flow cytometric analysis of Treg cells, B and T-cell phenotypes were performed on cord blood mononuclear cells (CBMCs). Maternal total IgG was measured using Enzyme-linked Immunosorbent Assay (ELISA) while EBV serological analysis and cord blood cytokine levels was done by Luminex assays. Differences in antibody, cytokine, Treg, B and T-cells between malaria-exposed and -unexposed were compared using Mann-Whitney U test. Multivariate regression analysis assessed the effect of malaria exposure on the transfer of EBV-specific antibodies while controlling for a set of confounders. A higher proportion (63%) of the infants were exposed to malaria *in utero.* Maternal malaria infection during pregnancy resulted in significant reduction in the transfer of anti-VCA and anti-EBNA1 antibodies to the neonates by 13% and 22%, respectively. The levels of cytokines and chemokines between malaria-exposed and -unexposed cord blood were comparable. Significantly higher frequency of Treg cells was observed in malaria-exposed compared to -unexposed cord blood (p=0.0005). There were higher transitional B-cells and activated classical memory B-cells but lower naïve B-cells in malaria exposed cord blood compared to unexposed cord blood (p=0.0089, p=0.0257 and p=0.0053, respectively). However, T-cell homeostasis in cord blood was not affected by maternal malaria infection during pregnancy. Results suggest that malaria exposed neonates are susceptible to early primary EBV infection due to low antibody levels, reduced transfer of maternal antibodies, inhibition of EBV-specific immune responses by the increased Treg cells and altered B-cell homeostasis. Findings from this study will help to explore potential avenues for delaying the early primary EBV infection in children experiencing holoendemic malaria who are at risk of eBL.

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LIST OF ABBREVIATIONS AND ACRONYMS

APC	Antigen Presenting Cells
BCR	B cell Receptor
BSA	Bovine Serum Albumin
BL	Burkitt's lymphoma
CBMC	Cord Blood Mononuclear Cell
CD	Cluster of Differentiation Antigen
CMV	Cytomegalovirus
CTL	Cytotoxic T-cell
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
EBER	Epstein Barr Virus encoded small RNA
eBL	Endemic Burkitt's Lymphoma
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic Acid
EBNA	Epstein Barr Virus Nuclear Antigen
EAd	Early Antigens Diffuse
FOXP3	Forkhead box p3
GC	Germinal Center
HHV-8	Human Herpes Virus-8
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
HSV	Herpes Simplex Virus

Ig	Immunoglobulin
KEMRI	Kenya Medical Research Institute
LMP	Latent Membrane Proteins
NPC	Nasopharyngeal Carcinoma
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
Treg	T Regulatory Cells
TT	Tetanus Toxoid
UNICEF	United Nations Children's Fund
VCA	Viral Capsid Antigen
WHO	World Health Organization
Zta	Z Transactivator Antigen

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Endemic Burkitt's (eBL) lymphoma is a cancer affecting children aged between 2 and 15 years of age (Mwanda *et al.,* 2004), accounting for up to 74% of all childhood malignancies in tropical Africa (Mwanda *et al.,* 2004), and is the most prevalent pediatric cancer in Kenya (Makata *et al.,* 1996; Mwanda *et al.,* 2004). The geographic distribution of eBL incidence coincides within malaria holoendemic regions where *P. falciparum* malaria infections are often chronic or repeated early in life (Rainey *et al.,* 2007). Repeated malaria infections during infancy and early primary EBV infection have been implicated in the pathogenesis of eBL, however, the pathologic process leading to eBL is not well understood (Rochford *et al.,* 2005).

Maternal antibodies that are passively acquired transplacentally have been shown to be protective against various infections during infancy (de Moraes-Pinto and Hart 1997). However, factors such as maternal HIV infection, maternal hypergammaglobulinaemia, placental malaria, preterm birth, levels of total and specific IgG and nature of antigen affect the trans-placental transfer of maternal antibodies to the infant (de Moraes-Pinto *et al.,* 1998; Okoko *et al.,* 2002b; Okoko *et al.,* 2001a; Okoko *et al.,* 2001c; Scott *et al.,* 2005) and this may leave an infant vulnerable to infections early in life.

Evidence suggest that EBV-specific maternal antibodies are protective against EBV infection in the first few years of life (Biggar *et al.,* 1978). Therefore, the levels of EBV-specific maternal antibodies could be an important determinant of EBV infection

early in life as low levels would leave an infant susceptible to infection by 6 months (Biggar *et al.,* 1978; Chan *et al.,* 2001; Geser *et al.,* 1982). Indeed children from malaria holoendemic regions are infected by EBV by six months of age (Piriou *et al.,* 2012); age at which maternal antibodies should protect them from the infection. The susceptibility of children from malaria holoendemic area to early primary EBV infection could be linked to impaired transfer of EBV-specific antibodies. Chulaimbo Sub-county in western Kenya is a malaria holoendemic region and children resident in this region have an increased risk of eBL due to early primary EBV infection (Piriou *et al.,* 2012). Despite this, the effect of malaria infection during pregnancy and maternal hypergammaglobulinemia on the trans-placental transfer of the well-characterized antibodies against EBV lytic (VCA, Zta, EAd) and EBV latent (EBNA1) to neonates of mothers from Chulaimbo remains unknown. Maternal hypergammaglobulinemia, characterized by increased concentrations of total IgG is a common complication of pregnancy that result from infections such as malaria. Hypergammaglobulinemia inhibits the transfer of antibodies by saturating the FcRn receptor, so pathogen-specific antibodies cannot be transported (Palmeira *et al.,* 2012). It remains to be determined whether maternal hypergammaglobulinemia limits the transfer of the EBV-specific antibodies to the neonates. This study therefore determined the effect of maternal malaria infection during pregnancy on the transfer of EBV-specific antibodies to the neonates.

Cord blood cytokine and chemokine levels modify the risk of various infections during infancy. Cord blood cytokine levels have been shown to modify the risk to malaria during infancy (Adegnika *et al.,* 2008; Kabyemela *et al.,* 2013; Malhotra *et al.,* 2009; Malhotra *et al.,* 2005). Colonization of the placenta by malaria parasites can result

in the priming of neonatal immunity and shifting of the cytokine and chemokine balance. Chemotaxis deficiencies as well as shifts in cytokine profiles could potentially contribute to the susceptibility of children from malaria holoendemic region to early primary EBV infection. It remains to be determined whether the shift in cytokine balance increases the susceptibility to early primary EBV infection. Therefore, concentrations of cytokines and chemokines were determined in cord blood from mothers who were either exposed or unexposed to malaria during pregnancy to assess their impact on neonatal immunity to EBV.

One possible mechanism by which EBV evades the host immune system and establishes persistent infection is by induction of T regulatory (Treg) cells. Treg cells are a subclass of T-cells that are involved in suppression of immune functions. Once Treg cells are activated, they inhibit immune responses in a non-specific manner (Hu *et al.*, 2010; Thornton and Shevach 1998; Thornton and Shevach 2000). Infection with *P. falciparum* malaria was demonstrated to induce significant expansion of Treg cells (Goncalves *et al.,* 2010) and since Treg cells suppress immune responses in a non-specific manner, the increased Treg cells may potentially inhibit immune responses to EBV during primary infection in children. However, it is unknown if maternal malaria infection during pregnancy modulates the frequency of Treg cells or their phenotypes in cord blood. This study determined the effect of *in utero* malaria exposure on the frequency of Treg cells in cord blood.

Persistent antigenic challenge such as exposure to malaria *in utero* may have adverse effect to the fetal developing immune system. Malaria infection is known to perturb the distribution of lymphocytes in peripheral circulation in children (Asito *et al.*,

2008), as well as increased cell death of the lymphocytes (Toure-Balde *et al.,* 1996). This abrogation of lymphocyte subsets may in turn result in altered cellular and humoral immune responses to EBV as indicated by loss of T-cell mediated immunity (Moormann *et al.,* 2007). Malaria infection causes the perturbation in the distribution of lymphocytes in children but whether *in utero* exposure to malaria perturbs lymphocyte distribution in cord blood is yet to be determined. This study determined the effect of *in utero* exposure to malaria on the frequency and phenotypes of cord blood lymphocytes.

1.2 Problem Statement

Children from malaria holoendemic area are infected with EBV earlier in infancy, when typically maternal antibodies should protect them from EBV infection. This early exposure results in higher viral loads and poor control of the virus, a risk factor in the etiology of eBL. Current understanding posits that the levels of EBV-specific maternal antibodies are important determinant to the susceptibility of infants to EBV infection early in life, however, it remains to be determined whether the EBV-specific antibodies are transferred to the infant and whether maternal malaria infection and hypergammaglobulinemia affects the transfer of these antibodies.

Sequestration of infected erythrocytes within the placenta can result in the priming of neonatal immunity and shifting of the cytokine and chemokine balance. Levels of cord blood cytokines and chemokines increases the susceptibility of neonates to malaria and HIV infections during infancy. It has not been investigated whether shifts in cytokine balance and chemotactic deficiencies following maternal malaria infection modulates neonatal susceptibility to early primary EBV infection.

Viruses establish persistent infection by induction of Treg cells by suppressing immunity against the virus and aid in establishing persistent infection. In HSV infection, when Treg cells are present at the time of infection the level of protective immunity against the virus is impaired while depletion of Tregs prior to the infection resulted in robust HSV-specific responses. Whether maternal malaria infection during pregnancy induces Treg cells in cord blood and subsequently suppresses immune response to EBV during primary infection remains to be determined.

Malaria infection is known to perturb the distribution of lymphocytes in peripheral circulation as well as increased cell death in children acutely infected with malaria. This abrogation of lymphocyte subsets resulted in altered immune responses to EBV as indicated by loss of T-cell mediated immunity. Although extensive information is available on the types and frequencies of lymphocytes that populate the peripheral blood of children, it remains to be determined whether lymphopoiesis is altered in neonates exposed to *P. falciparum* malaria *in utero*.

1.3 Main Objective

To determine the effects of maternal malaria infection during pregnancy on trans-placental transfer of EBV-specific maternal antibodies and on fetal immunoregulatory cells.

1.3.1 Specific objectives

1. To determine the effect of malaria infection during pregnancy and maternal hypergammaglobulinemia on the trans-placental transfer of anti-VCA-p18, -EBNA1, -Zta, -EAd and –VCA-gp125-specific maternal antibodies to neonates of mothers from Chulaimbo.

2. To assess the effect of malaria exposure *in utero* on the profiles of cord blood cytokines and chemokines.
3. To establish the effect of *in utero* exposure to malaria on the frequency of T regulatory cells in cord blood.
4. To phenotypically characterize B and T-cells in cord blood following *in utero* malaria exposure.

1.3.2 Research questions

1. What is the effect of maternal malaria infection and hypergammaglobulinemia during pregnancy on the transfer of anti-VCA-p18, -EBNA1, -Zta, -EAd and –VCA-gp125-specific maternal antibodies?
2. What effects do *in utero* malaria exposure have on the profiles of cord blood cytokines?
3. What is the effect of *in utero* exposure to malaria on the frequency of T-regulatory cells in cord blood?
4. What are the phenotypes of cord blood B and T-cells following *in utero* malaria exposure?

1.4 Significance Of The Study

Maternal malaria infections have adverse effects on both the mother and her unborn infant. Findings from this study have unraveled avenues to explore as part of delaying or preventing primary EBV infection in children resident in *P. falciparum* holoendemic region of western Kenya who are at greater risk of eBL. Since maternally transferred antibodies are important to the neonate before they develop *de novo* antibodies, findings from this study further unraveled the potential role of chronic antigen

exposure (maternal malaria infection during pregnancy) on neonatal immunity to EBV and may help to explore potential avenues for delaying the early primary EBV infection in children experiencing holoendemic malaria who are at risk of eBL.

A possible mechanism by which EBV subverts the immune system to establish chronic infection could be by the induction of regulatory T cells (Tregs). The frequency and functional capacity of the regulatory cells may also affect the level of viral persistence and therefore elucidating the role of Treg cells before or during primary EBV infection is important because opportunities for immune manipulation may be exposed at this initial stage of infection thereby aiding in preventing the risk of early EBV infection and subsequent risk of developing eBL. Results from this study provides information that can be used in the manipulation of immune the system in averting the early exposure to EBV thereby preventing early primary EBV infection.

Lymphopoiesis and lymphocyte recirculation is a tightly regulated process. However, infections could abrogate the process and result in altered phenotypes. If *in utero* exposure to malaria alters B and T-cell phenotypes, then cellular and humoral immune responses to EBV during primary infection as well as immune responses to childhood vaccines may also be altered. This study have important implications that may help in designing vaccines to be used in children resident in malaria endemic regions.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epstein Barr Virus (EBV)

Epstein Barr virus (EBV) is a gamma-herpes virus that belongs to the sub-family of Gammaherpesvirinae (Babcock *et al.,* 1998). It is a human virus that has the ability to elicit the proliferation and immortalization of infected B-cells into lymphoblasts both *in vivo* and *in vitro* (Babcock *et al.,* 1998; Joseph *et al.,* 2000). EBV primarily infects resting B-cells and may also infect non-dividing epithelial cells (Babcock *et al.,* 1998; Joseph *et al.,* 2000).

2.1.1 Biology of EBV

The EBV genome is made up of a double stranded DNA molecule of about 175 kilobase pairs organized into a series of unique internal and terminal repeat domains (Baer *et al.,* 1984). The genome encodes about 80 different proteins that are involved in both the latent and lytic phases of its cycle and the entire genome persist in proliferating B-cells as linear covalently closed circular episomal DNA (Kieff and Liebowitz 1990).

2.1.2 Infections in humans, different populations and age groups

There are two strains of EBV that differ in the sequence of viral genes expressed during latent infection that can infect humans. These include type A (EBV-1) and type B (EBV-2) (Dambaugh *et al.,* 1984; Sample *et al.,* 1986). It is estimated that EBV infects over 95% of adult population worldwide (Crawford 2001). Primary infection occurs horizontally during childhood mainly through contact with maternal or caretakers' saliva (Yao *et al.,* 1985) and this coincides with the period at which maternal immunity diminish

(Biggar *et al.*, 1978). After primary infection, EBV establishes a lifelong latent infection and rarely causes disease unless the host-virus immune balance is upset (Donati *et al.*, 2006a). Initial EBV seroepidemiological studies showed that primary infection occurs by three years of age in developing countries (Biggar *et al.*, 1978) while in developed countries, the infection is delayed until adolescence where it causes a self limiting infection called infectious mononucleosis. However, recent evidence has emerged showing that EBV infects children by three months of age (Piriou *et al.*, 2012), age at which they should still be protected by maternal antibodies.

Some members of the herpes virus like cytomegalovirus (CMV), herpes simplex virus (HSV) are commonly transmitted *in utero* (Grant *et al.*, 1981). Primary infection with EBV during pregnancy and subsequent trans-placental transmission is rare but it has been demonstrated that it can cross the placenta. EBV infection of the human cervix has also been documented (Sixbey *et al.*, 1986) raising the possibility of neonatal infection. However, no work has been done on the outcome of fetal infection by EBV.

Studies have demonstrated increased serological markers of EBV reactivation during pregnancy (Costa *et al.*, 1985; Icart *et al.*, 1981; Meyohas *et al.*, 1996) but other studies (Meyohas *et al.*, 1996) suggested that pregnancy alone cannot induce any significant increase in infected B-cells. Two studies detected a higher proportion of antibodies to early antigen (EA) in pregnant compared to non-pregnant women (Costa *et al.*, 1985; Fleisher and Bolognese 1982), suggesting reactivation during pregnancy, however, these studies did not determine what effect this reactivation has on trans-placental transfer of the antibodies against the virus.

Previous studies have attempted to determine whether mother-to-child EBV transmission occurs. A study in 1970's detected EBV infected cells in 1 out of about 700 samples tested while another study identified EBV transformed cells in cord blood of 1 out of 40 samples. On the other hand, another study reported that no EBV transformed cells were isolated from blood of neonates (Joncas *et al.,* 1974), therefore it has not been conclusively proven whether EBV is transmitted *in utero*.

It is known that malaria causes reactivation of EBV thereby increasing the number of EBV infected B-cells and as such the effect of maternal malaria infection during pregnancy on the trans-placental transfer of EBV specific antibodies remains unknown. Maternal malaria infection often results in the colonization of the placenta by infected erythrocytes, a condition known as placental malaria (Bulmer *et al.,* 1993). This colonization often results in the damage of the placental architecture and may in turn interfere with the trans-placental transfer of materials. It has been demonstrated that EBV-specific maternal antibodies protect an infant for up to six months of age (Biggar *et al.,* 1978), but it is not known what effect maternal malaria infection or placental malaria exerts on the transfers of the EBV-specific maternal antibodies.

Transmission of EBV early in life can induce tolerance and therefore can limit immune response to the virus raising the possibility that this may favor the development of BL. It has been demonstrated that primary EBV infection in African children occurs early in life with nearly all the children being EBV seropositive by age of two years and seroconversion occurring as early as three months of age (de-The 1977). In addition, it has been demonstrated that living in a malaria endemic region is a predictor to the early EBV infection in infancy (Piriou *et al.,* 2012). This raises an interesting suggestion that

events early in an infant's life, such as maternal infections may results in EBV infection early in life. This study therefore determined the effect of maternal malaria infection during pregnancy on the transfer of EBV-specific antibodies to the neonates.

2.1.3 Transmission

Primary infection with EBV mainly occurs through contact with the virus in saliva in infants while in young adults, the virus is mainly transmitted orally and periodic shedding of the virus from the salivary tissue is a necessary feature of the virus (Straus *et al.,* 1993). It has also been suggested that sharing of food or pre-mastication of food for infants can transmit the virus. Alternatively, the virus can be transmitted indirectly through contact with contaminated utensils or toys. Other studies have shown that EBV can be transmitted by blood transfusion or bone marrow transplant, but these are rare cases (Shearer *et al.,* 1985). There is also evidence of viral particle presence in cervical epithelium and semen but sexual transmission has not been proven (Sixbey *et al.,* 1986).

2.1.4 Establishment and maintenance of infection

EBV passes through the salivary tract to infect epithelial cells of the oropharynx where they replicate and release progeny virions from the cells that in turn infect resting naïve B-cells. Once it infects the naïve resting B-cells, the virus switches on the latent proteins i.e. EBV nuclear antigens (EBNA 1-6) and Latent membrane proteins (LMP), (Thorley-Lawson and Allday 2008; Tomkinson *et al.,* 1993) that drive the resting naïve B-cells into an activated lymphoblast (Abbot *et al.,* 1990), which then traffic to the follicles where they initiate germinal center reactions by switching off the expression of EBNA-2 and switching on latency 2 (Thorley-Lawson and Mann 1985). During the

latency 2 program the virus switches on the expression of LMP1, LMP2A and LMP2B (Wang *et al.,* 1997). LMP1 drives class switch recombination while LMP2 has been implicated in driving somatic hyper-mutation (He and Chen 2003). After this, the cells exit germinal centers and enter the memory B-cell compartment where EBV switches on EBV latency 0 program (Thorley-Lawson and Allday 2008). These memory B-cells can re-enter the tonsils, where they undergo-plasma cell differentiation leading to lytic replication and release of EBV virions into saliva or re-infection of other B-cells (Laichak *et al.,* 2005).

2.1.5 Epstein Barr Virus serology

After primary infection, IgM antibodies to VCA appear first, followed by IgG antibodies (de-The *et al.,* 1978). Primary EBV infection is usually determined by the detection of VCA IgM antibodies and the absence of EBNA antibodies. In healthy EBV carriers, EBNA1 IgG and VCA IgG antibodies persist for life, whereas the early antigen diffuse (EAd)-specific IgG falls rapidly after primary infection (Miller 1990). Detection of high levels of anti-EAd IgG in latent EBV infection has been associated with reactivation of EBV (Rahman *et al.*, 1991).

Recent data has demonstrated that the levels of VCA, EBNA1, Zta and EAd-specific IgG antibodies were significantly higher in children from malaria holoendemic area compared to children from malaria sporadic area (Piriou *et al.*, 2009), levels of these antibodies were significantly higher following reactivation of EBV by *P. falciparum* malaria. In addition, it was demonstrated that children from malaria holoendemic areas experience early primary EBV infection compared to children from malaria sporadic area. However, what predispose infants from malaria holoendemic area to EBV infection

early in life is unknown. As such, the current study measured the levels of anti-EBV-specific antibodies in the mothers and in their neonates to determine if malaria during pregnancy could potentially interfere with the trans-placental transfer of EBV-specific maternal antibodies.

2.1.6 EBV-associated malignancies

Epstein Barr Virus (EBV) is an oncogenic virus and infection is associated with the development of a number of malignancies (Young and Rickinson 2004). This virus only causes the malignancies and cancer under conditions of host immunosuppression or when viral proteins are unusually expressed, however, these by themselves are not sufficient cause. The most common associations are with endemic Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin's disease and some T-cell lymphomas. Others include EBV-associated hemophagocytic syndrome, natural killer (NK)-cell lymphoma, lymphoproliferative diseases in immunocompromised hosts, gastric carcinoma and smooth-muscle tumors (Crawford 2001; Kawa 2000).

2.1.7 Burkitt's lymphoma

Burkitt's lymphoma is a distinct form of non-Hodgkin's lymphoma and it is the most common pediatric cancer in equatorial Africa where it accounts for 74% of all childhood malignancies (Burkitt 1983). Burkitt's lymphoma can be classified as endemic, sporadic or HIV associated (Parkin *et al.*, 2003). Sporadic Burkitt's lymphoma accounts for 20-30% of non-Hodgkin's lymphoma in children in developed countries. It affects the abdominal region and can be detected at any age. Endemic Burkitt's lymphoma is almost exclusively found in Africa (Freedman and Friedberg, 2006), affects mainly the facial skeleton in children aged between 2 to 9 years and is the most common

pediatric cancer in equatorial Africa (Orem *et al.*, 2007). In Kenya, there is evidence of uneven geographical distribution in the incidences of eBL with most of the cases occurring within the malaria endemic region (Rainey *et al.,* 2007). In eBL, majority of tumors have EBV DNA in all malignant cells. In young adults, BL manifest as acute infectious mononucleosis (AIM) that is characterized by rapid expansion of virus specific CD8 T-cells in peripheral blood (Callan *et al.,* 1998). In children in developing countries such as Kenya, primary EBV infections occur within the first few years of life (de-The 1977; Moormann *et al.*, 2005) and are often asymptotic infections.

2.2 Role of Malaria In EBV Infection and Disease

Co-infection with *P. falciparum* malaria and EBV has been implicated in the genesis of eBL. Although the association between EBV and eBL has been established, the role of *P. falciparum* malaria has not been clearly elucidated. Malaria causes a complex pattern of immune modulation accompanied by polyclonal B lymphocyte activation leading to increased numbers of circulating EBV infected B-cells (Whittle *et al.,* 1984). The highest density parasitemia is observed in children aged between 6-11 months old and coincidentally it is at this age that primary EBV infection is likely to occur (Rochford *et al.,* 2005). It has been reported that acute malaria causes impairment of EBV-specific T-cell immunity (Gunapala *et al.,* 1990) and that this impaired EBV-specific T-cell responses is indicated by the loss of IFN-γ mediated killing of virus infected cells (Moss *et al.,* 1983). Rochford *et al*., (2005) proposed two possible mechanisms to explain the relationship between EBV and *P. falciparum* malaria in the etiology of eBL. The first theory suggests that *P. falciparum* malaria induces B-cell expansion and consequently EBV reactivation leading to the expansion of infected B-cell,

with a possibility of *c-myc* translocation that is the hallmark of eBL. This was supported by data demonstrating that children acutely infected with malaria or living in malaria endemic regions have altered distribution of B-cells (Asito *et al.,* 2008; Asito *et al.,* 2011). The other theory suggests that infection with *P. falciparum* malaria impairs EBV-specific T-cell immunity leading to poor control of the virus by the immune system. This theory is supported by observation that children living in malaria endemic areas have diminished EBV-specific T-cell immunosurveillance between the ages of 5 and 9 years, which coincides with the peak age incidence of eBL (Moormann *et al.,* 2007). Recent evidence has emerged demonstrating that children from malaria holoendemic region experience earlier primary EBV infection (Piriou *et al.,* 2012). This susceptibility may be linked to impaired transplacental transfer of EBV-specific antibodies to the neonates.

2.3 Malaria in Pregnancy

The WHO/UNICEF estimates that about 30 million pregnancies occur each year in Africa with majority residing in areas where malaria transmission is stable. In areas where malaria is endemic like in this study population, the frequency and severity of malaria infection is greater in primigravidae and secundigravidae than in multigravidae (Brabin 1991; Okoko *et al.,* 2002a; Tako *et al.,* 2005). The risk of malaria has been observed to increase two to four folds in primigravidae than in multigravidae (Brabin 1991; McGregor *et al.,* 1983). This susceptibility is associated with the ability of the malaria parasites to express pregnancy specific antigens such as variant surface antigens (VSA) and primigravidaes do not have adequate specific immunity to clear them, however, immunity is gained with successive pregnancies. A study by (Shulman and Dorman 2003) found out that pregnant women have a higher rate of parasitaemia and

parasite density compared to non-pregnant women and an outstanding feature of malaria infection during pregnancy is presentation of parasitized placenta at delivery. Erythrocytes infected with *P. falciparum* sequester in the maternal placental vascular space where they replicate, a condition referred to as placental malaria. The main features of placental malaria are the presence of parasites within the intervillous space, thickening of the trophoblastic membrane, presence of malaria pigment or fibrin deposits (Matteelli *et al.*, 1997). Placental parasitaemia are usually very high (over 50%) but in contrast, peripheral parasitaemia are usually very low or absent (Ibhanesebhor and Okolo 1992; Shulman and Dorman 2003).

Plasmodium falciparum infection of the placenta is a major medical challenge among pregnant women. Some of the adverse effects of placental malaria include maternal anemia, delivering a low birth weight (LBW) baby, intra-uterine growth retardation (IUGR), infant mortality, congenital infection and fetal parasite exposure. The pathologic alteration of the placenta by the sequestration of infected erythrocytes can impair the trans-placental transfer of antibodies, however, the exact mechanisms are not fully understood (Uneke 2007; Walter *et al.*, 1982).

2.4 Transplacental Transfer of Maternal Antibodies

Maternal antibodies transferred across the placenta or via breast milk are important in the defense against various pathogens during the first few months of an infant's life. With time, maternal antibodies are replaced by the infant's own antibodies produced either by natural infection with the pathogens or by immunization. The fact that most of the IgG in fetal blood is of maternal origin shows that they are transported across the placenta into fetal circulation (Simister 2003) and at birth, fetal IgG exceeds

maternal levels (Kohler and Farr 1966). The transport of IgG across the placenta is mediated by Fc receptor of IgG including FcγR and FcRn (Israel *et al.,* 1995; Simister and Story 1997; Story *et al.,* 1994) and involves an active process whereby neonatal FcRn binds IgG and crosses the syncytiotrophoblast into the endothelium of fetal capillaries. A number of studies have reported the selective transfer of IgG subclasses across the placenta with IgG1 and IgG4 being transferred more efficiently than IgG2 and IgG3 (Garty *et al.,* 1994).

A number of factors such as maternal HIV, maternal hypergammaglobulinemia, placental malaria, preterm birth and levels of specific antibodies can potentially lead to inadequate trans-placental transfer of some maternal antibodies and this may confer less protection of the infant (de Moraes-Pinto *et al.,* 1996; de Moraes-Pinto *et al.,* 1998; Scott *et al.,* 2005; Wesumperuma *et al.,* 1999). A study by (Cumberland *et al.,* 2007) in Kilifi where malaria transmission is perennial reported a reduced antibody levels in women with active or past placental malaria infection compared to placental malaria negative mothers.

A study by (de Moraes-Pinto *et al.,* 1996) found out that maternal HIV infection was associated with reduced trans-placental transfer of antibodies against Varricella Zoster Virus (VZV), tetanus, measles and pneumonia. This same study also demonstrated that the efficacy of trans-placental transfer of these antibodies decreased as the respective maternal antibodies increased. Another study in expectant women from Kilifi reported lower tetanus antibodies levels, reduced trans-placental transfer and a lower cord plasma levels of the tetanus antibodies in women with HIV infection compared to HIV negative mothers (Cumberland *et al.,* 2007).

The effect of placental malaria on trans-placental transfer of tetanus antibodies is controversial. A study in Papua New Guinea where malaria transmission is very high demonstrated that trans-placental antibody transfer of tetanus antibody is reduced in women with placental malaria compared to women without (Brair *et al.,* 1994). In contrast, other studies in The Gambia and Malawi have shown that there is no effect of placental malaria on trans-placental transfer of tetanus antibodies (de Moraes-Pinto *et al.,* 1996; Okoko *et al.,* 2001b). Further, the trans-placental transfer of antibodies to CMV is reported to be more efficient than the trans-placental transfer of antibodies specific to the Herpes group of viruses (Gotlieb-Stematsky *et al.,* 1983).

Maternal hypergammaglobulinemia (high levels of total IgG) is usually caused by non-specific stimulation of B-cells. These high levels of IgG can potentially inhibit the transfer of antibodies by saturating the FcRn receptors and thereby limiting the transfer of pathogen-specific antibodies. This observation can be supported by data that demonstrated significant negative correlation between maternal levels of total IgG and the transfer ratios IgG to measles (Goncalves *et al.,* 1999).

However, the effect of maternal malaria infection during pregnancy on the trans-placental transfer of EBV-specific antibodies remains unknown. Therefore this study investigated the effect of maternal malaria infection during pregnancy and maternal hypergammaglobulinaemia on the efficiency of trans-placental transfer of EBV-specific antibodies in mother child pairs from Chulaimbo in Western Kenya where malaria transmission is endemic.

2.5 Cytokines and Chemokines In Cord Blood/Neonates

Maternal malaria infection and as well as other factors within the intrauterine environment may alter the cytokine balance in the placenta (Fried *et al.,* 1998; Suguitan *et al.,* 2003) but it is unclear whether cord blood cytokines are transported from maternal circulation into fetal circulation or produced by cord blood cells. Cord blood cytokine levels have been shown to modify the risk to malaria during infancy (Adegnika *et al.,* 2008; Kabyemela *et al.,* 2013; Malhotra *et al.,* 2009; Malhotra *et al.,* 2005). For example studies in Tanzania reported an association between cord blood levels of pro inflammatory cytokine IL-1β and TNF and risk of severe malaria during infancy (Brickley *et al.,* 2015; Kabyemela *et al.,* 2013). In addition, maternal malaria infection can alter the expression of certain cytokines. This is supported by observations of lower cord blood levels of IFN-γ in neonates whose mothers were uninfected with malaria during pregnancy compared to the malaria infected mothers in an area of Sudan that experiences unstable malaria. On the other hand, there was no significant difference in cord blood levels of regulatory cytokines IL-4 and IL-10 in the neonates from the two groups of mothers (Bayoumi *et al.,* 2009). They also reported high levels of IL-12 in cord blood of infants born to malaria-infected mothers. This led them to suggest that the high level of this cytokine was responsible for the low prevalence of *P. falciparum* antigens in the infants (Bouyou-Akotet *et al.,* 2004). In utero exposure to HIV-1 altered the expression of chemokines and increased the susceptibility of the neonates to HIV-1 infection (Bunders *et al.,* 2014). These observations suggest that levels of cord blood cytokines influence the susceptibility to malaria and HIV-1 infection during infancy. However, the levels of cytokines and chemokines in cord blood following *in utero*

malaria exposure and subsequent susceptibility to EBV infection in infants remains to be determined.

2.6 Regulatory T-Cells and Their Role In Persistent Viral Infections

Regulatory T-cells (Tregs) are a distinct subpopulation of T-cells that arise from the thymus or periphery and play a central role in immunological self tolerance and maintenance of immune homeostasis (Sakaguchi 2004). Treg are divided into two groups i.e. natural Treg and induced Treg. Natural Treg develop from $CD4^{+}CD8^{+}CD3^{high}$ T-cells in the thymus (Cupedo *et al.,* 2005; Darrasse-Jeze *et al.,* 2005). They comprise about 5 - 10% of $CD4^{+}$ T-cells in adult human PBMC (Bacchetta *et al.,* 2005). Phenotypically, they are defined by constitutive expression of the high affinity IL-2 receptor α chain (CD25) and the transcription factor forkhead box p3 (FOXP3) (Sakaguchi 2000; Shevach 2002). Tregs can be isolated from the thymus and umbilical cord blood and these cells have suppressive abilities (Wing *et al*., 2002; Wing *et al*., 2005). In contrast, adaptive Treg arise in the periphery, expressing FOXP3 only when activated by certain stimulatory conditions.

To date, FOXP3 is the most definitive marker for Tregs (Sakaguchi, 2004). Therefore, unless otherwise stated, the phenotype of Tregs will be defined as $CD4^{+}CD25^{+}FOXP3^{+}$ Treg and $CD8^{+}CD25^{+}FOXP3^{+}$ Treg cells.

Immune response to viruses can be influenced by the activity of cell types that express regulatory functions (Rouse *et al.,* 2006). Studies have demonstrated that the first indication that a virus might subvert the immune response by inducing Treg activity was

with the identification of Treg as the cause of the suppression of $CD8^+$ T-cell response (Iwashiro *et al.,* 2001).

The mechanism of Tregs suppression is still contentious and not fully understood. There are various mechanisms that have been raised to explain Treg mode of action. These mechanisms include cell contact dependent mechanism, suppression by inhibitory cytokines, suppression by modulation of APC function and cytolysis including perforins and granzyme dependent killing mechanisms (Vignali *et al.,* 2008). Treg suppress proliferation and IFN-γ production of both $CD4^+$ and $CD8^+$ T-cells (Bacchetta *et al.,* 2005), further, Tregs are able to suppress the proliferation of antigen stimulated naïve T-cells *in vitro* (Thornton and Shevach 1998) although the precise mechanism by which this is achieved is yet to be elucidated. A study by (Zhao *et al.,* 2006) demonstrated that activated Tregs can kill B-cells and other APC's. It has been proposed that multiple mechanisms may operate in Treg suppression and that Tregs may express various molecules on their surface that might contribute to their suppressive functions (Sakaguchi *et al.,* 2009).

Various studies that investigated the roles of Treg in viral infections found differences in the frequency of these regulatory cells in the peripheral blood of infected compared to uninfected individuals. The frequency of $CD4^+CD25^+$Treg was elevated in chronic HCV infected individuals compared to uninfected individuals (Boettler *et al.,* 2005). Treg cells isolated from HCV patients suppressed virus-specific $CD8^+$ T-cell proliferation and IFN- γ production, and depletion of these cells resulted in increased IFN-γ production in response to HCV proteins (Boettler *et al.,* 2005; Cabrera *et al.,* 2004). In addition, the role of $CD8^+$ Tregs in HCV infection has been established

(Billerbeck *et al.,* 2007). In HIV infection, Tregs have been shown to limit HIV specific responses affecting both $CD4^+$ and $CD8^+$ T-cell functions (Aandahl *et al.,* 2004; Weiss *et al.,* 2004). Studies with HSV have demonstrated that at time of infection, when Tregs are present, the levels of protective immunity against HSV are impaired and that depletion of Treg prior to infection resulted in elevated HSV-specific CD4 T-cell responses (Suvas *et al.,* 2003; Tako *et al.,* 2005).

Studies have been done on the role of Treg cells in EBV infection whereby cells were cultured using EBNA-1 and an expanded pool of $CD4^+$T-cells with suppressive functions was reported (Voo *et al.,* 2005). These suppressive cells may affect the cytotoxic T-cell (CTL) response to EBV and thus the level of viral persistence and reactivation, potentially creating an environment conducive for the outgrowth of EBV-infected cells and therefore tumor development.

Treg frequency and functional capacity may affects the level of viral persistence and therefore elucidating their role could provide an understanding into the susceptibility of infants to early EBV infection and thus the development of EBV-related tumors (Marshall *et al.,* 2004). Despite this, the effect of *in utero* sensitization to malaria on the frequency and phenotypes of Treg cells in cord blood remains unknown.

2.7 B and T-Cell Homeostasis

Lymphocyte development usually starts with the pluripotent hematopoietic stem cells in the fetal liver. The pluripotent hematopoietic stem cells differentiate into committed progenitors of B and T-cell through a tightly regulated process. B-cells originate from pluripotent hematopoietic stem cells that differentiate into mature B-cells through different intermediary cell types that are defined by the expression of different

cell surface receptors (Blom and Spits, 2006). The B-cell lymphoid progenitor undergoes serial B-cell development after receiving growth promoting signals from bone marrow stromal cells, (Hardy *et al.*, 1991). The immature B-cells exit the bone marrow to become mature naive B-cells (Billips *et al.,* 1995; Sims *et al.,* 2006) and migrate via the peripheral blood to secondary lymphoid tissues (Klein *et al.,* 2003), where they interact with antigens and T-cell derived signals to give rise to memory B-cells (Tangye *et al.*, 2003). These memory B-cells undergo somatic hypermutations and immunoglobulin isotype switching, producing high affinity antigen specific antibodies in the germinal centers (Tangye *et al.*, 2003).

Based on the surface expression of the IgD, IgG, IgM, CD27, CD21 and CD10, B cells can be classified as; transitional B-cells ($CD19^{+}CD10^{+}IgD^{+}CD27^{-}$), naïve B cells ($CD19^{+}IgD^{+}CD27^{-}$), memory B-cells ($CD19^{+}IgD^{-}CD27^{+}$) (Bohnhorst *et al.,* 2001; Chang *et al.,* 2008; Sanz *et al.,* 2008; Sims *et al.,* 2005). The naïve B-cells then differentiate into memory B-cells upon encountering antigens (Lindsley *et al.,* 2007) that can be further classified as; marginal zone B-cells ($CD19^{+}CD27^{+}IgD^{+}C27^{+}$) or class-switched memory B-cells ($CD19^{+}CD27^{+}IgD^{-}IgM^{-}$).

T-cell precursors (pro T-cell) migrate from the bone marrow to the thymus (Bhandoola and Sambandam 2006). They are phenotypically characterized as $CD4^{-}CD8^{-}$ (double negative). The pro T-cell looses the stem cell marker (CD44) and starts the TCR-β gene re-arrangement to become a pre T-cell that are $CD4^{+}CD8^{+}$ (double positive) (Germain 2002). These cells begin to proliferate and undergo the TCR-α gene rearrangement. These then undergo both positive and negative selection within the thymus to become T-cells with a defined TCR and single expression of CD4 or CD8 on

their surface that are released into circulation (Goldrath and Bevan 1999; Jameson *et al.*, 1995). In the secondary lymphoid organs, once the naïve T-cells encounter antigens presented by the APC together with the appropriate costimulatory signal, they are activated and differentiate into blasts cells (Mempel *et al.,* 2004). Based on the surface expression of CD45 isoforms, T-cells can either be naïve i.e. have never encountered antigens before and phenotypically characterized as CD45RA or are memory i.e. cells derived from naïve cells after encountering antigens and are phenotypically characterized by expression of CD45RO (Suarez *et al.,* 2002). Although there are other markers that can be used in combination with CD45 isoforms to delineate naïve from memory T-cells, this study used the basic markers CD45RA/RO because of the limitation of the FACSCANTO to eight parameters.

Despite the fact that lymphocyte development is a tightly regulated process, abrogation in their differentiation has been reported (Bohnhorst *et al.,* 2001; Asito *et al.,* 2008; Weiss *et al*., 2009). Indeed defects potentially occur during the formation and revision of antigenic receptors (Shaffer *et al*., 2002). Alternatively, persistent antigenic challenge, such as continuous malaria exposure may result in the destruction of lymphoid organs and in turn affect the process of lymphopoiesis. For example, in a study at the coast of Kenya where parasitic infections are endemic, a study investigated the effect of maternal parasitic infections on the development of the infant's humoral immunity (King *et al.,* 1997). The results demonstrate that chronic exposure to parasites during pregnancy can stimulate the development of antigen specific responses as well as development of T-cell memory (King *et al.,* 1997). This study therefore determined

whether chronic antigenic challenge (*in utero* malaria exposure) perturbs the distribution of lymphocytes in cord blood.

CHAPTER THREE

METHODOLOGY

3.1 Study Area

This study was conducted at the antenatal clinic (ANC) and the maternity wing of Chulaimbo sub-district hospital in Kisumu West District in Nyanza Province in Western Kenya (Appendix 1). It is located about 25Km Northwest of Kisumu town, in the lowland areas of Lake Victoria at an altitude of 1328 meters above sea level. The hospital serves a predominantly rural population of about 70,800 individuals and assists over 200 deliveries each year. In this area, malaria transmission is holoendemic with two seasonal peaks; June to August and November to December (Juma *et al.,* 2008). The rainfall is bimodal with long rains from March to May and short rains from October to December (Juma *et al.,* 2008). Since there is persistent transmission of *P. falciparum*, there is high incidence of eBL in this study area (Rainey *et al.,* 2007).

The prevalence of malaria is estimated to be 28% (Jenkins *et al.,* 2015) and *P. falciparum* is responsible for approximately 97% of the malarial infections with residents in this area receiving 100-300 infective mosquito bites per annum (Bloland *et al.,* 1999). Malaria in children under five is estimated to be 17% (Division of Malaria Control 2010). The prevalence of HIV in men and women was estimated to be 14.2% and 20.5%, respectively (Ministry of Health 2014).

There are 24 health facilities within Maseno Division but Chulaimbo sub-district hospital was preferred due to the fact that it is within the malaria holoendemic area, the facility's ability to offer curative and preventative services including ANC, inpatient,

maternity, prevention of mother-to-child (PMTC) services as well as HIV research and training by Academic Model Providing Access to Healthcare (AMPATH).

3.2 Study Population

This study was conducted among pregnant mothers attending antenatal clinic (ANC) at the Chulaimbo sub-district hospital. Antenatal clinic attendance at the hospital is relatively high (Daud *et al.,* 2014b). The prevalence of maternal malaria infections during pregnancy within the larger Kisumu district was estimated at 18% (Ouma *et al.,* 2007). HIV prevalence among women in the larger Kisumu County is 20% (Division of Malaria Control 2010).

3.3 Study Design

This was a longitudinal prospective study where the pregnant mothers were recruited and followed up during the routine antenatal clinic (ANC) visits (up to 4 ANC visits per mother) until they delivered.

3.3.1. Sample size determination

Taking the prevalence of malaria in pregnancy at 18% (Ouma *et al.,* 2007), 95% confidence interval and 10% as the margin of error, the sample size for this study was calculated using the formula below (Cochran 1977);

$$\mathbf{n} = \frac{\mathbf{Z^2 x (p) x (1-p)}}{\mathbf{c^2}}$$

Where:
n = required sample size
Z = value corresponding to the confidence level (standard value of 1.96 for a 95% confidence level)

p = estimated prevalence of maternal malaria in the study area
c = margin of error at (standard value of 0.10)

Calculation:

$$\mathbf{n} = \frac{\mathbf{1.96^2} \times \mathbf{0.18} \times \mathbf{(1-0.18)}}{\mathbf{0.1^2}}$$

n=56

Between June to December 2008, 835 pregnant women attended the ANC at Chulaimbo sub District hospital. However, only 334 (40% of the 835) of these mothers delivered at the hospital. To cater for the loss to follow-up, the number of pregnant women that were recruited into the study in the first instance was calculated using the formula below (Whitley and Ball 2002);

$$\mathbf{N} = \frac{\mathbf{n}}{\mathbf{(1-q)}}$$

Where n = Final sample size (56)

q = % loss to follow-up (60% based on those lost to follow-up; 835-334=(501/835)×100)

$$\mathbf{N} = \frac{\mathbf{56}}{\mathbf{(1-0.6)}}$$

N= 140

As such, this study required to enroll a total of 140 pregnant mothers during the first ANC visit who were then followed up for the subsequent ANC visits until they delivered (and considering the loss to follow-up) to achieve the minimum sample size of 56.

3.3.2 Inclusion criteria

Pregnant women living within a radius of ten kilometers from the hospital, singleton pregnancy, uncomplicated vaginal delivery, willing to return to the hospital for routine clinical visits and delivery and signed the written informed consent form.

3.3.3 Exclusion criteria

HIV positive pregnant women, delivery at home, failure to give consent, twin pregnancy, delivery by caesarian section, blood transfusion <24 hours before delivery, complications during pregnancy, significant anti-partum hemorrhage or miscarriage and other infectious diseases besides malaria during pregnancy.

3.4 Methods of Data Collection

3.4.1 Collection of demographic data

A detailed questionnaire was administered to the pregnant women who consented to the study. The information collected included; age, origin, gravidity, number of children, bed net usage, intermittent preventative treatment in pregnancy (IPTp) and tetanus vaccination.

3.4.2 Sample collection

During enrolment and subsequent ANC visits, 200-500µL of maternal venous blood was collected by venipuncture or finger prick into EDTA microtainers (BD, Franklin Lakes, NJ). Within twelve hours of delivery, 5ml of blood was drawn by venipunture into heparinized vacutainers^{TM}. Immediately after delivery, but after the umbilical blood has stopped pulsing, the umbilical cord was clamped and cut. The whole placenta was sprayed with 70% alcohol, wiped with 2% iodine tinture followed by 1% iodine tinture

and alcohol swab. Then using sterile surgical gauze, the whole length of the cord was wiped to remove any blood clots. To reduce the possibility of cross-contamination with maternal lymphocytes, 50mL of blood was collected from the umbilical vein at the distal side of the placenta with the needle pointing to the uterus directly into a heparinised tube and a 500µL EDTA tube. Two thick and thin blood smears were also prepared immediately.

The placenta was then placed on a raised sterile wire mesh stand, with the chorionic plate (fetal side) facing down to promote blood accumulation and intra-villous blood (IVB) space accessibility. A 14-gauge needle attached to a syringe was directed approximately 0.5cm deep through the wire mesh into the intervillous space, denoted as dark-purple regions, while carefully avoiding puncture of the surrounding fetal vessels on the surface of the chorionic plate. The syringe was gently pulled to create a vacuum initiating blood flow, followed by withdrawal to allow for dripping blood, transfer about 50ml, into a heparin tube and 500µL into EDTA tube, then thick and thin blood smears were also made. All samples were transported within two hours to the SUNY/KEMRI laboratory located at Centre for Global Health Research (CGHR) in Kisumu and processed on the same day. Plasma from cord blood of North American mothers (malaria naive), who had never travelled to any malaria endemic regions of the world were also included as controls.

3.4.3 Microscopic investigation of *P. falciparum* parasites

Parasitemia was determined at time of blood collection by performing a thick and thin blood smear and staining with 5% Giemsa solution to quantify parasites. Two microscopists examined the slides and a third microscopist resolved any discrepancies in

the slide reading. Parasite density was expressed as the number of asexual *P. falciparum* per μL of blood and the pregnant women with detectable parasites in the smear were treated according to the Kenya Ministry of Health guidelines.

3.4.4 CBMC isolation and cryopreservation

Cord blood mononuclear cells (CBMC)'s were separated from sodium heparin anti-coagulated whole blood by standard Ficoll-Hypaque density gradient centrifugation. In this procedure, the anti-coagulated blood was layered carefully onto equal amount of Ficoll-paque (GE Healthcare, Sweden) in a 50mL tube and then centrifuged at 450×g for 30 minutes. Plasma was transferred into Sarstedt tubes (Sarstedt, Germany) and stored at -80^{0}C while the CBMCs was collected using a sterile 10mL pipette and transferred into another 50mL tube. The cells were washed by adding sterile 1×PBS, pH 7.2, without calcium or magnesium followed by centrifugation for 15 minutes at 350×g at room temperature. The supernatant was aspirated off, the pellet broken by gentle flicking of the tube and then washed again as described above and centrifuged for 10 minutes at 350×g. The supernatant was aspirated, the pellets broken by gently flicking the tubes and the cells resuspended in 1mL of sterile 1 × PBS, pH 7.2. 10µL of 0.4% Trypan blue solution was used to dilute the cells in a 1:1 ratio to aid in visualizing the cells under a microscope and using a Haemocytometer to calculate the yield using the formula [cell count in 1ml = (# cells counted in 4 squares)×5×2×10^4]. The calculations of the cell count were carried out in Microsoft Excel sheets. Freezing media was added based on the number of cells and then transferred to -80^{0}C freezer and then to liquid nitrogen after overnight chilling.

3.4.5 EBV viral load quantification and parasite genotyping in maternal and cord blood

DNA was extracted from 200µl EDTA blood using QIAamp DNA mini kit (Qiagen, Maryland, USA) according to the manufacturers' protocol. DNA was eluted off the column in an equivalent volume of water and stored at -20^{o}C.

The following conditions were used for Q-PCR: an initial denaturation at 50^{0}C for 2 min, 95^{0}C for 10 min, and 45 cycles of 95^{0}C for 15 seconds and 60^{0}C for 1 minutes. EBV DNA levels were determined using primers and probes designed to detect a 70bp region of the EBV BALF5 gene (Kimura *et al.,* 1999; Moormann *et al.,* 2005), Appendix 2. To generate a standard curve, EBV positive plasmid generated from the PCR product was used. The viral loads were normalized to the β-actin copy number, log transformed and then calculated based on copies of EBV genome/ml. *P. falciparum* DNA was detected using PCR primer and probes designed to detect the *P. falciparum* 18s gene, Appendix 3.

3.4.6 Total IgG ELISA

Plates were coated with anti-human IgG monoclonal antibody and incubated overnight at 4^{0}C. The plates were then washed twice with wash buffer (PBS, 0.05% Tween-20) and blocked using PBS with 3% BSA then incubated at 37^{0}C for one hour. After four wash steps, plasma samples and standards diluted 1:100 in sample/conjugate dilution buffer (PBS, 0.05% Tween 20, 1% Triton X-100, 3 % BSA) were added and incubated for one hour at 37 ºC. Again, the plates were washed four times with wash buffer and HRP conjugated anti-human IgG monoclonal antibody added and incubated for one hour at 37^{0}C.

After the incubation period, the plates were washed four times and 5,5,3,3, tetramethylbenzidine (TBM) solution added and incubated in the dark for 30 minutes at room temperature. The reaction was stopped by addition of 1M H_2SO_4 stop solution and the plates were read on an automated V_{max} Kinetic microplate reader (Molecular devices, CA, USA) at 405nm using Molecular Devices Softmax Pro-software version 4.7.15 (Molecular devices, CA, USA). Total IgG concentrations were determined by extrapolation from the standard curve.

3.4.7 Assessment of EBV and TT-specific antibodies in maternal and cord blood

In order to detect anti-EBV and TT-specific IgG antibodies against a panel of antigens, a previously described protocol was used (Middeldorp and Meloen 1988; Piriou *et al.*, 2009). Briefly, different amounts of antigens were coupled with $1x10^6$ pre-activated carboxylated microspheres (Luminex, Austin TE, USA) in 500µl of 100mM MES pH 6.0 buffer. The beads were then washed and stored in PBS, 0.1% BSA, 0.002% Tween-20, 0.05% Sodium Azide, pH 7.4 at 4^0C until use. A 1.2 µm 96 well Millipore filter plate was pre-wetted with 100µL/well of PBS, 0.05% Tween-20 (PBT) and aspirated using a vacuum manifold. Using a multichannel pipettor 50µL of the 2X concentrated working microsphere mixture was aliquoted into the appropriate wells of the filter plate, then plasma samples were added to the appropriate wells. This reaction was gently mixed using a multichannel pipettor and the filter plate covered and incubated for 30 minutes at room temperature in a plate shaker. The supernatants was aspirated using a vacuum manifold and the plates washed twice with 100µL/well of PBT and then 100µL of goat anti-human IgG R-PE-conjugated detection antibody added and the microspheres resuspended by pippeting up and down a number of times. A further, 30

minute incubation at room temperature in a plate shaker followed then the microspheres were re-suspended in 100µL of PBT and immediately 75 beads per set were acquired and analyzed on a Bio-Plex system (Luminex Corporation, USA). EBV-seronegative and -seropositive controls were used in each plate. Since VCA, EBNA1, Zta and EAd standards were not commercially available, results of the assay were expressed as Mean Fluorescent Intensity (MFI) of at least 75 beads for each EBV and TT-specific antigen tested.

3.4.8 Cytokine Testing by Multiplex Bead Assay System

Cytokine levels in cord blood were determined using Invitrogen 25-plex human cytokine kit (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. The multi screen filter plates were pre-wetted and 25µl of human cytokine magnetic antibody bead solution added to each well. These were washed twice using 200µl of PBT (PBS, 1% BSA, 0.05 Tween 20) and then 50µl of incubation buffer was added to each well. Then, 100 µl/well of standards (human 25-plex cytokines) and plasma samples (Kenyans and North Americans) were added to the appropriate wells, covered in aluminum foil and incubated at room temperature for two hours in an orbital shaker. After incubation, the plates were washed twice, 100µl/well of biotynilated detector antibody was added and incubated at room temperature for one hour on an orbital shaker. After a two step wash, 100µl of diluted streptavidin-RPE was added to each well and incubated at room temperature for 30 minutes on an orbital shaker. Excess streptavidin-RPE was washed off and 125µl of working wash solution was added to each well and data was acquired immediately on Luminex 100 using Bioplex manager V4.0.

Concentrations of the cytokines in plasma in pg/ml were extrapolated from the individual cytokine and chemokine standard curves.

3.4.9 Thawing of cells

The cryovials were removed from liquid nitrogen and thawed in a 37^0C water bath and transferred to a conical tube with pre-warmed thaw media (10% FBS in RPMI). These were spun at 300×g for 12 minutes, then the supernatant aspirated and washed with pre-warmed thaw media and spun again. The supernatant was aspirated and the tube gently flicked, then the cells re-suspended in 1.5ml of warm thaw media and incubated at 37^0C, 5% CO_2 overnight. The cells were then harvested, washed, supernatant aspirated, cells re-suspended in 1ml of complete media and cell viability checked using 0.4% trypan blue solution.

3.4.10 Staining for flow cytometry

Half a million CBMCs were aliquoted into labelled polystyrene tubes (Becton Dickinson, USA), 1ml of flow buffer (PBS, 0.5% BSA) was added to each tube then spun at 450×g for 5 minutes at 4^0C and decanted. To inhibit non-specific binding to the fc receptors, human FcγR-binding inhibitor was added to each tube, incubated for 10 minutes at 4^0C then 1ml of flow buffer added, spun at 450×g for 5 minutes at 4^0C and decanted. Appropriate monoclonal antibodies specific for different cell surface markers were added, Appendix 4. The tubes were vortexed and incubated on ice for 30 minutes in the dark. After incubation, 2mL of cold flow buffer was added to each tube and vortexed gently and then spun at 450×g for 5 minutes at 4^0C. The supernatant was aspirated off and 0.5mL of 1% paraformaldehyde added to all the tubes, vortexed gently and incubated in the dark for 20 minutes at room temperature. A one wash step with 1ml flow buffer follows then the

cell resuspended in 100µL of flow buffer added. This was followed by permibialization of the cells by addition of 50µL of fix/perm buffer (BD Pharmigen, San Diego, CA, USA) and incubated for 15 minutes. Anti-FOXP3 PE monoclonal antibody was added and incubated for 30 minutes in the dark. The tubes were then washed once with 1ml of flow buffer, spun, decanted, resuspended in 300µL of flow buffer and data acquired immediately.

3.4.11 Flow cytometry acquisition and analysis

Data was acquired immediately using FACSDiva software on FACSCanto II flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). The fluorescent intensity measurement was done using logarithmic amplifiers whereas the forward scatter and side scatter measurement was made using linear amplifiers. Flowjo software v9.7 (Tree star Inc, USA) was applied to collect data for compensation and processing of data before statistical analysis.

3.5 Statistical Analysis

The ratio of EBV and TT-specific antibody levels in cord blood to that in respective maternal blood [cord: maternal (CMR)] was used as a measure of trans-placental antibody transfer (Okoko *et al.,* 2001a). Comparison was made between malaria infected and uninfected mothers or malaria-exposed and -unexposed neonates. Multiple linear regression was used to assess the effect of *in utero* malaria exposure on the trans-placental transfer of EBV and TT-specific maternal antibodies while accounting for a set of covariates such as maternal hypergammaglobulinemia, maternal age, parity, gestational age and gestational age at first malaria exposure. Differences in the median levels of antibodies in maternal venous blood to that in their paired neonate were

determined using Wilcoxon Matched pair test. The differences in antibody levels and cytokine levels between malaria-exposed and -unexposed infants were compared using Mann-Whitney U test. Correlation between the levels of maternal EBV and TT-specific antibodies in relation to those in cord blood was calculated using Spearman correlation test.

The frequency of Treg cells in malaria exposed cord blood relative to malaria unexposed cord blood was compared using Mann-Whitney U test. The differences in frequency of B and T-cell phenotypes in CBMC's were compared in neonates of malaria infected and uninfected mothers using Mann-Whitney U test. 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.

3.6 Ethical Considerations

Approvals for this study were obtained from the Kenya Medical Research Institute (KEMRI) Ethical Review Committee and Ethical Review Boards of SUNY Upstate Medical University Hospital, USA (Appendix 5). Written informed consent (Appendix 6) was obtained from all the mothers. Blood collection through venipuncture or finger prick can cause temporary discomfort, bruises and pain and to reduce these risks, only trained and qualified phlebotomist from the Kenya Ministry of Health performed these procedures. In addition, sterile disposable lancets were used and all sharps were stored in the appropriate biohazard sharps' containers before disposal. The participation in the study was voluntary, the participants were free to withdraw at any time during the study, and access to health care was not dependent on participation in this

study. All the samples collected were coded for identification and only the investigator and the supervisors had access to the data.

CHAPTER FOUR

RESULTS

4.1 Characteristics of the Study Population

The demographic and clinical characteristics of the study population are shown in table 4.1. Mean maternal age in the malaria-exposed group was 23.4 [SD ±6.1] years while that in the non -exposed group was 21.8 [SD ±6.8] years.

Based on the malaria microscopy and QPCR results, a pregnant mother was infected with malaria when malaria parasites were detected in any of the ANC visits, or at delivery. Subsequently, an infant was exposed *in utero* when their mother had detectable parasites during pregnancy. Using this criterion, 63% (44) of the infants were exposed to malaria *in utero* while 37% (26) were unexposed to malaria in utero.

Maternal EBV infection was defined by the presence of anti-VCA antibodies as determined by VCA-p18 ELISA; all the women in this study were EBV seropositive. EBV DNA and *P. falciparum* DNA was not detected in any cord blood. 95% of the expectant mothers in malaria-exposed group and 92% in the malaria non-exposed group had reported receiving tetanus vaccine during the current pregnancy. Primigravid mothers in malaria-exposed and -unexposed group were 36% and 30%, respectively. The mean birth weight of infants born to mothers who had malaria during pregnancy was 3171g compared to 3146g for the infants born to mothers who had no detectable malaria during pregnancy.

Table 4.1: General characteristics of the mothers and their infants.

Factor	Malaria exposed	Malaria unexposed	*p*-value
Maternal characteristics			
n	44 (63)	26 (37)	0.041
Maternal age (mean) [±SD]	22.36 [6.10]	21.77 [6.79]	0.566
<20	18 (41)	12 (46)	0.790
>20	26 (59)	14 (54)	0.763
Gestational age			
<37 weeks	8 (18)	4 (15)	0.900
>37 weeks	33 (75)	18 (69)	0.648
Gravida			
Primigravida	16 (36)	7 (30)	0.784
Secundigravida	12 (28)	12 (40)	0.548
Multigravida	16 (36)	7 (30)	0.784
TT vaccine (current pregnancy)	42 (95)	24 (92)	0.627
Bed net use	32 (73)	20 (77)	0.750
EBV seroprevalence (%)	44(100)	26(100)	1.000
Mean Total IgG [±SD] mg/ml	29.15 [10.12]	25.29[8.93]	0.112
Neonatal characteristics			
Mean Birth weight (grams) [±SD]	3171 [444]	3146 [420]	0.923
Time of malaria exposure			
Early	31 (70)		
Late	13 (30)		

Values represent n(%) unless otherwise stated. Early exposure to malaria was defined as <26 weeks gestation while late exposure was defined as >26 weeks gestation. Differences between the two groups were compared using *t* test. N- number, SD- standard deviation, EBV – Epstein Barr Virus, TT – Tetanus toxoid. Seven women reported gestational ages that were unreliable and not used in this analysis. Four women did not have their ANC books, so TT vaccination could not be confirmed.

4.2 Effect of *In Utero* Malaria Exposure on Transfer of EBV-Specific Antibodies

To determine the effect of *in utero* malaria exposure on transplacental transfer of anti-EBV-specific IgG antibodies, the levels of the antibodies in maternal venous blood at delivery and in respective cord blood were first determined, then the levels of the IgG antibodies in maternal venous blood were correlated to that in cord blood.

4.2.1 Anti-EBV and TT-specific antibody levels in mothers and their neonates

To determine the levels of anti-EBV and TT-specific antibodies in mother venous blood at delivery and their corresponding cord blood, plasma IgG antibodies were tested in a 6-plex Luminex assay. The maternal mean fluorescent intensity (MFI) values for EBV-specific IgG to VCA-p18, EBNA1, Zta, EAd, VCA-gp125 and TT were 13407, 6697, 1268, 3728, 5510 and 16633, respectively, while those in cord blood were 12645, 3985, 165.5, 331, 4893 and 15903. This study reports significantly higher levels of anti-EBNA1, -Zta and -EAd IgG antibodies in maternal blood compared to that in cord blood (all $p<0.0001$). The levels of anti-VCA-p18, anti-VCA-gp125 and anti-TT IgG antibodies were comparable between the mothers and their neonates (Figure 4.1).

There were comparable levels of antibodies against VCA-p18, EBNA1, Zta, EAd, VCA-gp125 and TT in both the malaria infected and uninfected mothers. Although there were higher levels of antibodies against VCA-p18, EBNA1, EAd, VCA-gp125 and TT in neonates whose mother did not have malaria during pregnancy compared to neonates whose mothers had malaria during pregnancy, and these levels were comparable (Table 4.2). Furthermore, the levels of total IgG in the mothers or the infants in the malaria exposed vs. the unexposed groups, were comparable (Table 4.2).

This study then compared the levels of EBV and TT-specific antibody in maternal blood and in respective cord blood. Irrespective of malaria exposure status, there was no significant difference in the levels of antibodies against VCA-p18, VCA-gp125 and TT in the mothers and their neonates (figure 4.2). The levels of anti-EBNA1 antibodies in the mothers who never had malaria were comparable to those in their neonates (p=0.069). However, the levels of anti-EBNA1 antibodies were significantly lower in malaria exposed neonates compared to the unexposed neonates (p=0.0002). Irrespective of malaria exposure status, the levels of anti-Zta and -EAd antibodies were significantly lower in the neonates compared to their mothers (all p<0.0001) (Figure 4.2).

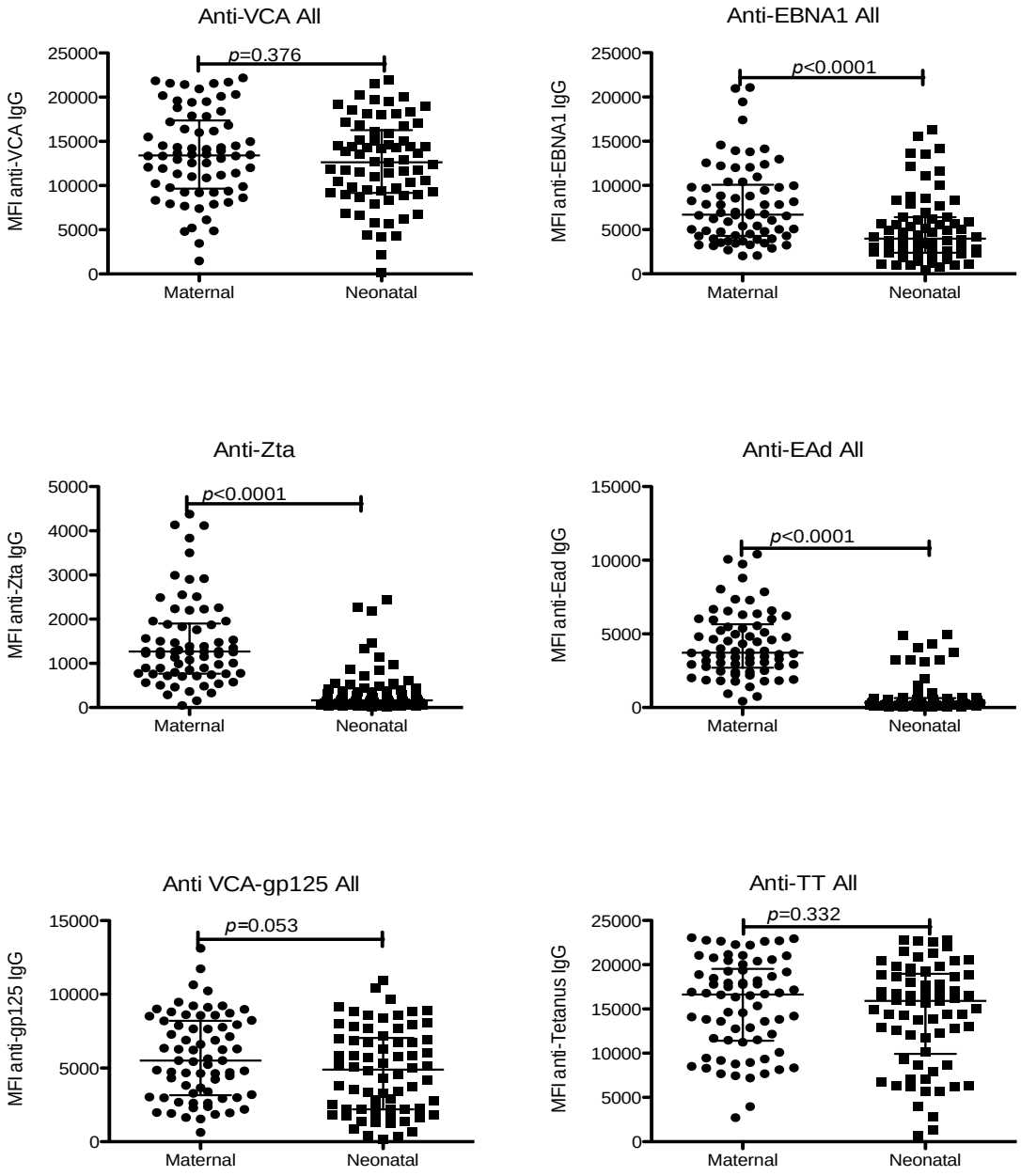

Figure 4.1: Levels of anti-EBV and TT-specific antibodies in all mothers' venous blood compared to the levels in respective cord blood regardless of malaria status. The MFI of at least 75 Luminex beads acquired for each of the antigen tested is indicated on the y-axis. $p \leq 0.05$ were considered significant as determined by Wilcoxon Matched pair test. Horizontal bars represent median values for each. VCA-Viral capsid antigen, EBNA1-EBV nuclear antigen, EAd-Early antigen diffuse, Zta-Z transactivation antigen, TT-tetanus toxoid

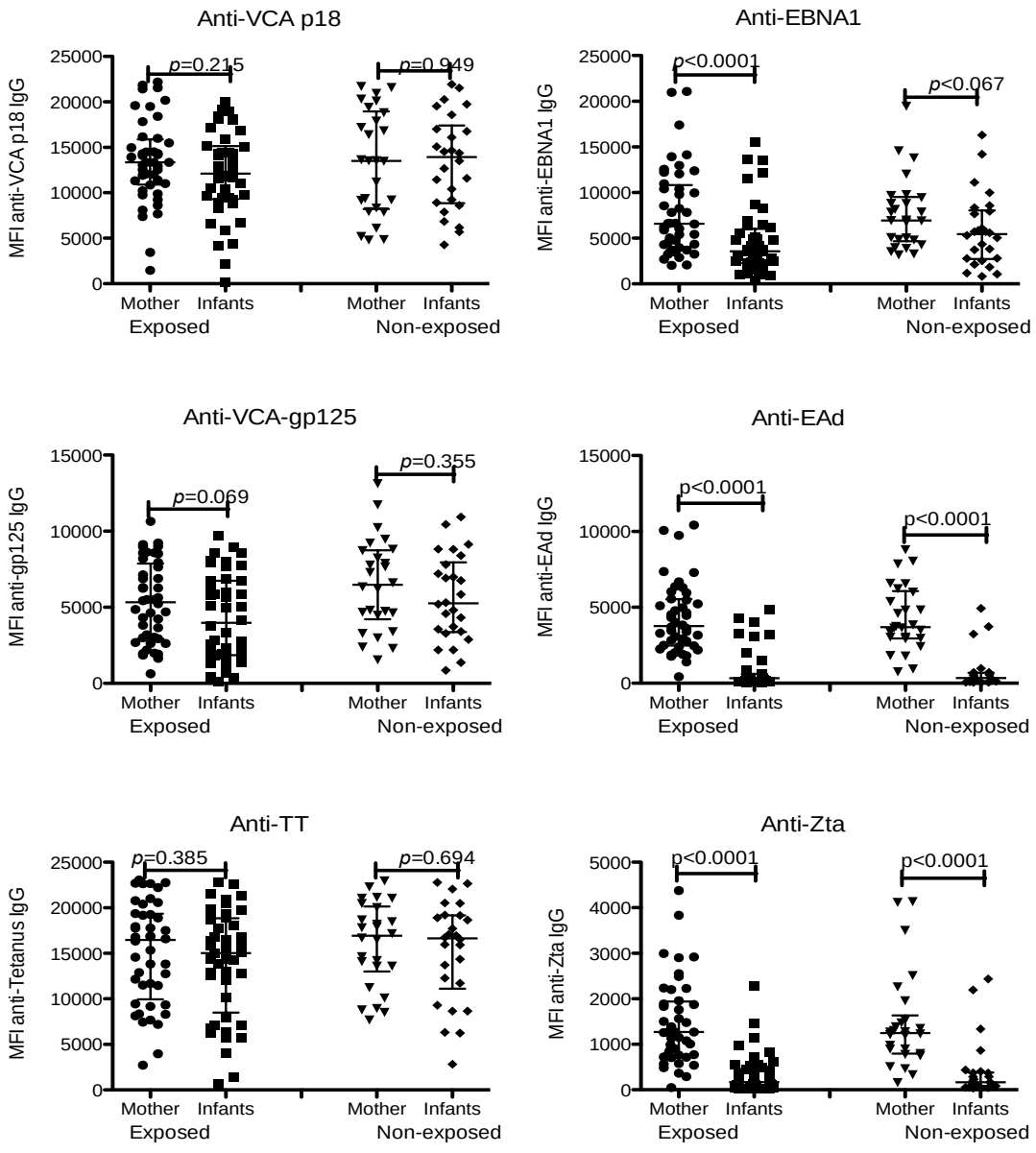

Figure 4.2: Levels of anti-EBV and TT-specific antibodies in mothers and their corresponding infants based on *P. falciparum* malaria status. The MFI of at least 75 Luminex beads acquired for each of the antigen tested is indicated on the y-axis. $p \leq 0.05$ was considered significant as determined by Wilcoxon Matched pair test.
VCA-Viral capsid antigen, EBNA1-EBV nuclear antigen, EAd-Early antigen diffuse, Zta-Z transactivation antigen, TT-tetanus toxoid

Table 4.2: Levels of EBV and tetanus specific antibodies in mothers and their neonates

	Mothers			**Neonates**		
	Malaria infected	**Malaria Uninfected**	***p-value***	**Malaria Unexposed**	**Malaria exposed**	***p-value***
Anti-VCA-p18	13337 (10898-15868)	13520 (8229-18957)	0.947	13933 (8855-17404)	12113 (9312-15135)	0.378
Anti-EBNA1	6590 (4043-10824)	6956 (4669-9510)	0.803	5445 (2712-8047)	3577 (2278-6024)	0.140
Anti-Zta	1268 (761.8-1939)	1249 (791.8-1637)	0.903	165 (86.25-381)	168 (91-478.5)	0.976
Anti-EAd	3770 (2482-5540)	3699 (2953-6064)	0.832	347.5 (143.8-691.5)	331 (190.8-620.8)	0.757
Anti-VCA-gp125	6475 (4201-8738)	5334 (2942-7868)	0.140	5256 (3370-7951)	3982 (1853-6741)	0.067
Anti-TT	16466 (9952-19338)	16918(13001-20138)	0.516	16639 (11120-19175)	15022 (8491-18846)	0.385
Total IgG (pg/ml)	26.71(21.27-39.08)	24.13 (17.96-31.63)	0.112	26.80 (22.04-31.78)	27.26 (22.54-38.19)	0.373

The data are medians of at least 75 beads of anti-EBV and TT-specific IgG antibodies. 25^{th} and 75^{th} quartiles are in parenthesis. Statistical differences of $p \leq 0.05$ are considered significant as determined by Mann-Whitney U test. VCA-Viral capsid antigen, EBNA1-EBV nuclear antigen, EAd-Early antigen diffuse, Zta-Z transactivation antigen, TT-tetanus toxoid.
Hypergammaglobulinemia was defined as having total IgG concentration of greater that 30pg/ml.

4.2.2 Association between maternal and neonatal anti-EBV and TT antibodies

It has been documented that levels of antibodies in cord blood correlate to that in respective maternal blood. To determine if antibody levels in the mother influence the levels of antibodies in the neonate, this study correlated the levels of anti-EBV and TT-specific maternal antibodies to that in the respective cord blood. In the malaria unexposed group, there was a strong positive correlation between maternal and neonatal anti-VCA antibodies (r=0.908, $p<0.0001$). This was similar to the other antibodies tested i.e. anti-EBNA1, -Zta, -gp125 and -TT (r=0.907, $p<0.0001$, r=0.793, $p<0.0001$, r=0.927, $p<0.0001$, r=0.888, $p<0.0001$), respectively. However, there was no correlation between maternal and neonatal anti-EAd antibodies (r=0.191, $p=0.350$) (Figure 4.3).

In the malaria-exposed group, there was a positive correlation between the levels of anti-VCA-p18, -EBNA1, -Zta, -VCA-gp125, -TT in the mothers to their respective neonate (r=0.642, r=0.746, r=0.773, r=0.842 and r=903, respectively), (Figure 4.3). This study observed a weak correlation between maternal and neonatal anti-EAd antibodies (r=0.277, $p=0.065$) (Figure 4.3).

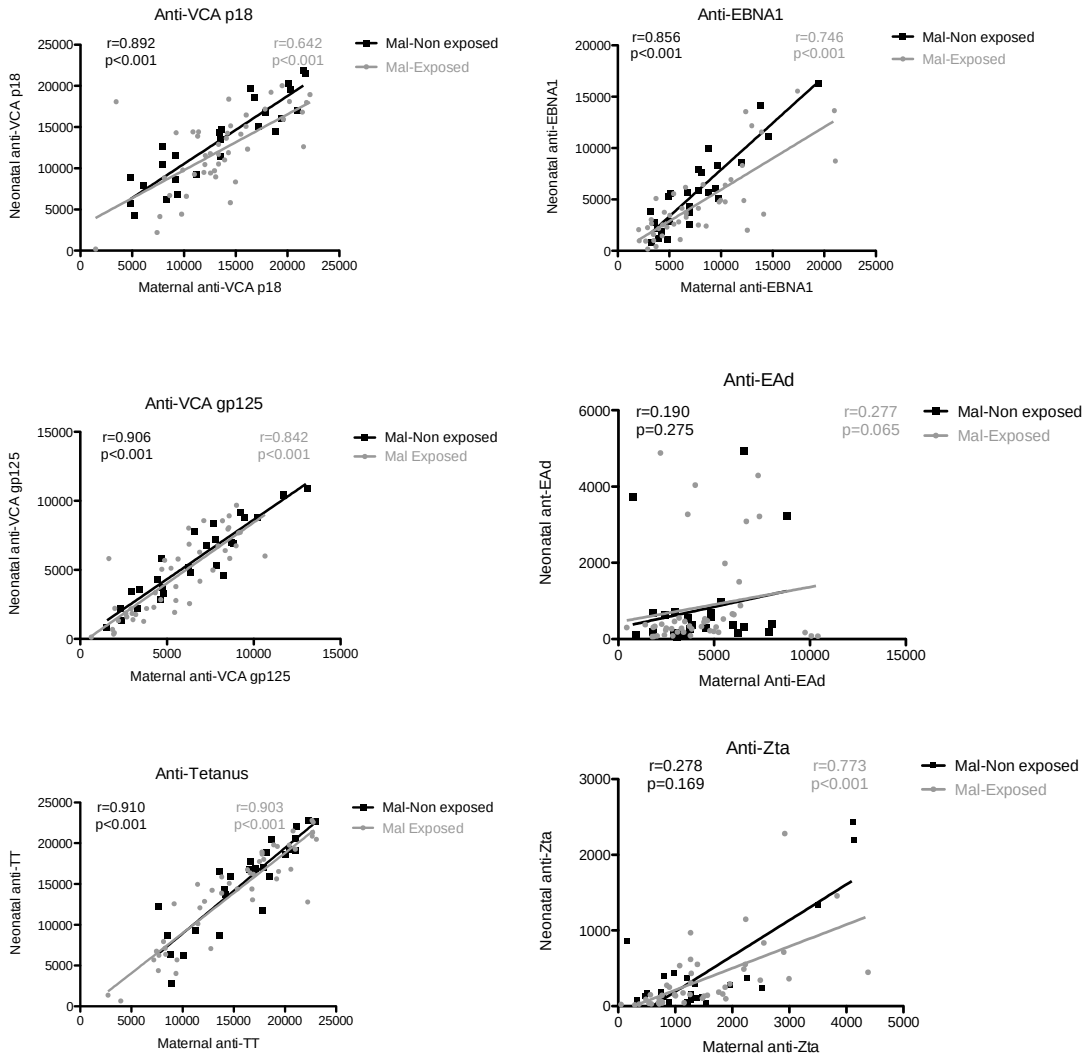

Figure 4.3: Correlation between maternal and neonatal anti-EBV and anti-TT specific antibodies in malaria exposed and non-exposed groups. Spearman Correlation test assessed correlation between maternal and infant antibody levels where $p \leq 0.05$ were considered significant. VCA-Viral capsid antigen, EBNA1-EBV nuclear antigen, EAd-Early antigen diffuse, Zta-Z transactivation antigen, TT-tetanus toxoid

4.2.3 Transplacental transfer of maternal anti-EBV and TT-specific antibodies

This study then determined the amount of anti-EBV and TT-specific antibodies that were transferred to the neonates irrespective of malaria exposure. As illustrated in

Table 4.3, the amount of anti-VCA-p18, EBNA1, Zta, EAd, VCA-gp125, and TT-specific IgG antibodies that were transferred to the neonate were 94%, 60%, 13%, 9%, 89% and 96%, respectively.

Table 4.3: Transfer of EBV and Tetanus specific antibodies from the mothers to their neonates.

	Maternal MFI	**Neonatal MFI**	**% Transfer**
Anti-VCA-p18	13407	12645	94
Anti-EBNA1	6697	3985	60
Anti-Zta	1268	165.5	13
Anti-EAd	3728	331	9
Anti-VCA-gp125	5510	4893	89
Anti-TT	16633	15903	96

Data are median of MFI of at least 75 beads set acquired.
% of antibodies transferred was determined by the formula; (level of antibody in neonates/levels of antibodies in mothers) × 100.

4.2.4 Reduction in anti-EBV-specific antibody transfer due to *in utero* malaria exposure

The transplacental transfer of antibodies from the mother to her neonate is an active and selective process whose efficacy is dependent on a number of factors. The level of specific antibody in cord blood to that in their respective maternal blood; cord to maternal ratio (CMR), was used to determine the efficacy of transplacental transfer of antibodies. In addition, the proportional reduction in transplacental transfer of antibodies due to *in utero* malaria exposure or maternal hypergammaglobulinemia was determined. *In utero* exposure to malaria resulted in significant reduction in the transfer of anti-VCA-p18 and anti-EBNA1 antibodies by 13.4% and 21.65%, respectively (p=0.023 and

p=0.002, respectively). No significant reduction in transfer of anti-EAd, anti-Zta, anti-VCA-gp125 and anti-TT antibodies were observed (Table 4.4).

Maternal hypergammaglobulinemia resulted in 5.08%, 17.64%, 18.18%, 12.66% and 8.79% reduction in the transfer of anti-EBNA1, -Zta, -EAd, -gp125 and tetanus specific antibodies, respectively. However, normogammaglobulinemic mothers transferred more anti-VCA-p18 IgG antibodies to their neonates compared to the hypergammaglobulinemic mothers (Table 4.5).

In multivariate analysis, after adjusting for potential confounding factors such as maternal hypergammaglobulinemia, maternal age and gestational age at first malaria exposure, *in utero* malaria exposure was associated with significant reduction in the transfer of anti-VCA-p18 and anti-EBNA1 antibodies from the mothers to the neonates (*p*=0.009 and *p*=0.042 respectively). No significant reductions were observed for anti-Zta, -EAd, -VCA-gp125-specific or TT-specific IgG antibodies (Table 4.6).

Table 4.4: Effect of malaria exposure on the efficiency of transplacental transfer of EBV specific maternal antibodies.

	CMR Malaria exposed	**CMR Malaria non-exposed**	**% reduction**	***p*-value**
Anti-VCA-p18	0.862 (0.754 - 1.001)	0.9960 (0.8285 - 1.182)	13.4	**0.023**
Anti-EBNA1	0.546 (0.426 - 0.809)	0.7620 (0.5580 - 1.012)	21.65	**0.002**
Anti-Zta	0.151 (0.080 - 0.313)	0.1530 (0.0735 - 0.3195)	0.25	0.950
Anti-EAd	0.102 (0.048 - 0.176)	0.0680 (0.0350 - 0.1655)	-3.4	0.349
Anti-VCA-gp125	0.697 (0.552 - 0.979)	0.8495 (0.6800 - 0.9723)	15.25	0.064
Anti-TT	0.949 (0.816 - 0.103)	0.9810 (0.8540 - 1.040)	3.2	0.458

Data are median CMR. The 25^{th} and 75^{th} quartiles are in parenthesis. Maternal and cord blood plasma samples were tested for the presence of anti-EBV and TT specific IgG antibodies by Luminex bead based assay. Placental transfer was determined as CMR i.e. ratio of antibody in cord to that in respective maternal blood. Percentage reduction due to malaria was determined by the formula; (CMR of non-exposed – CMR of exposed)*100. Statistical differences of $p \leq 0.05$ are considered significant as determined by Mann-Whitney test.

Table 4.5: Effect of maternal gammaglobulinemia on efficiency of transplacental transfer of EBV specific maternal antibodies.

	Normogammaglobulinemia	**Hypergammaglobulinemia**	***% reduction***
Anti-VCA-p18	1.01	0.86	-17.44
Anti-EBNA1	0.56	0.59	5.08
Anti-Zta	0.14	0.17	17.64
Anti-EAd	0.09	0.11	18.18
Anti-VCA-gp125	0.69	0.76	12.66
Anti-TT	0.83	0.91	8.79

Data are median cord to maternal ratio of specific antibodies (CMR). Maternal and cord blood plasma samples were tested for the presence of anti-EBV and TT specific IgG antibodies by Luminex bead based assay. Placental transfer was determined as CMR i.e. ratio of antibody in cord to that in respective maternal blood. Percentage reduction due to malaria was determined by the formula; (CMR of non-exposed – CMR of exposed)*100. Statistical differences of $p \leq 0.05$ are considered significant as determined by Mann-Whitney test.

Table 4.6: Multivariate linear regression analysis of the transplacental transfer of EBV and TT-specific antibodies

Outcome	Intercept	SE	*p*-value
Log Anti-VCA-p18	0.01	0.33	**0.009**
Log Anti-EBNA1	0.70	0.40	**0.042**
Log Anti-Zta	-2.06	0.75	0.185
Log Anti-EAd	-2.68	1.00	0.469
Log Anti-VCA-gp125	0.03	0.37	0.365
Log Anti-TT	0.21	0.27	0.410

The values were adjusted for neonatal values plus maternal age, hypergammaglobulinemia, and gestational age at first malaria exposure. SE=Standard error of the regression, *p*-values were determined from the regression analysis. VCA-Viral capsid antigen, EBNA1-EBV nuclear antigen, EAd-Early antigen diffuse, Zta-Z transactivation antigen, TT-tetanus toxoid

In summary, maternal malaria infection during pregnancy results in the differential transplacental transfer of EBV-specific antibodies to the neonates. Transplacental transfer of anti-TT specific antibodies was not affected by maternal malaria infection during pregnancy.

4.3 Effect of *In Utero* Malaria Exposure on Cord Blood Cytokines Levels

It was hypothesized that maternal malaria infection during pregnancy would result in increased levels of pro inflammatory cytokines in cord blood. The cytokines that were tested using a multiplex bead based assay included; IL-1beta, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, GM-CSF, IFN-alpha, IFN-gamma, IP-10, MCP-1, MIG, MIP-1alpha, MIP-1beta, RANTES and TNF-alpha. Five (5) cytokines (i.e. IL-5, IL-10, IL-13, IL-15 and IL-17) were undetectable in cord blood plasma regardless of malaria exposure. Since the Kenyan mothers may have been

infected with malaria at some point in their life, cytokine levels in cord blood from North American mothers with no history of malaria exposure were also analyzed as controls since they have never been infected with malaria. This study did not observe any IL-13, IL-15, IL-17, IFN-alpha, IL-7 and MIG in these North American cord blood samples. Table 4.7 shows the mean concentration of the cytokines and chemokines in the cord blood samples.

The plasma level of pro-inflammatory cytokine IL-2R were 64.42pg/ml in North America cord blood, were significantly higher (both $p<0.0001$) in malaria-exposed 290.2 pg/ml and malaria-unexposed (233.29pg/ml) and with no difference between malaria-exposed and -unexposed cord blood ($p=0.24$). The plasma levels of pro-inflammatory cytokines IL-2, GM-CSF and IFN-γ were significantly high in North American cord blood (1.88pg/ml, 5.1pg/ml and 30.26pg/ml, respectively) compared to either malaria-exposed or -unexposed Kenyan cord blood (Table 7). There were comparable levels of other pro-inflammatory cytokines IL-1β, IL-12 and TNF-α between the three groups (Table 4.7).

Plasma levels of anti-inflammatory cytokine IL-4 was significantly higher (both $p<0.0001$) in North American cord blood (45.3pg/ml) compared to malaria exposed (7.01pg/ml) or malaria unexposed cord blood (14.22pg/ml). The levels of anti-inflamatory cytokines IL-1RA and IFN-α were comparable (Table 4.7).

The plasma level of RANTES was 2491.81pg/ml in North American cord blood and was significantly enhanced (both $p<0.0001$) in malaria-exposed (32340.pg/ml) and malaria-unexposed cord blood (34355pg/ml) compared to North American cord blood. Similarly, the plasma levels of MIP-1β were significantly low (both $p<0.0001$) in North

American cord blood (24.6pg/ml) compared to either malaria-exposed (49.28pg/ml) or malaria-unexposed cord blood (54.82pg/ml), with no significant difference in the malaria-exposed and -unexposed cord blood (p=0.958). The levels of other chemokines; Eotaxin, IL-8, IP-10, MIP-1α and MCP-1 between the three groups were comparable. Similarly, malaria-exposed and -unexposed cord blood had comparable levels of MIG in plasma (14.63pg/ml vs. 17.85pg/ml, respectively).

Table 4.7: Cord blood cytokine and chemokine levels

	Malaria-exposed		**Malaria-unexposed**			**Malaria naïve**			
	Mean	SD	Mean	SD	***p*-value-[a]**	Mean	SD	***p*-value-[b]**	***p*-value-[c]**
IL-1beta	7.67	4.95	9.21	7.72	1.00	14.82	14.06	1.00	1.00
IL-2	1.23	1.08	1.00	0.82	1.00	1.88	1.64	**0.001**	**0.006**
IL-2R	290.2	164.68	233.29	171.76	0.24	64.42	63.46	**<0.0001**	**<0.0001**
IL-6	11.87	30	49.48	273	1.00	14.4	7.51	0.134	**0.024**
IL-12	119.06	48.88	114.07	37.12	1.00	130.6	53.78	0.521	0.964
GM-CSF	0.27	0.158	0.34	0.265	0.162	5.10	0.23	**<0.0001**	**<0.0001**
IFN-gamma	21.13	7.17	21.36	6.08	1.00	30.26	17.38	**0.001**	**0.001**
TNF-alpha	3.11	2.35	2.65	2.26	1.00	3.59	4.62	0.705	1.00
IL-1RA	1257.98	209.19	2203	5890	0.891	1188	753.86	0.318	1.00
IL-4	7.01	0.00	14.22	0.00	1.00	45.3	4.49	**<0.0001**	**<0.0001**
IFN-alpha	30.75	6.88	37.3	19.48	1.00				
Eotaxin	2.38	2.13	26.85	147.09	1.00	15.56	6.16	1.00	0.455
IL-8	41.57	46.16	598.09	2570	1.00	18.29	15.47	0.687	1.00
IP-10	20.34	12.25	30.5	55.32	0.677	28.41	13.48	1.00	0.755
MIP-1alpha	92.69	192.16	114.41	237.31	1.00	38.05	13.24	0.248	0.092
MIP-1beta	49.28	32.48	54.82	62.45	0.958	24.6	16.05	**<0.0001**	**<0.0001**
MIG	14.63	7.72	17.85	20.73	1.00				
MCP-1	87.67	190.25	68.82	144.58	1.00	111.32	57.64	0.717	1.00
RANTES	32340.82	15688.45	34355	15436	1.00	2491.81	1369.96	**<0.0001**	**<0.0001**

Values presented are the concentration of the cytokines and chemokines in pg/ml. [a]Comparison between Malaria-unexposed and -exposed infants; [b]Comparison between Malaria unexposed and Naïve infants; [c]Comparison between Malaria-exposed and Naïve infants. *p*-values ≤0.05 are considered statistically significant.

Next, this study determined if the concentrations of pro-inflammatory cytokines and chemokines in cord blood was influenced by a common process i.e. exposure to malaria *in utero*. The levels of pro-inflammatory cytokine and chemokines in cord blood were correlated and the results are summarized in Tables 4.8 and 4.9. IL-8 had significant positive correlation to IL-6, GM-CSF and TNF- α (r=0.446, $p<0.0001$, r=456, $p<0.0001$ and r=423, $p=0.0001$, respectively) but significant negative correlation to IFN-γ (r=-0.382, $p=0.0005$). TNF-α correlated positively with GM-CSF (r=0.491, $p<0.0001$) but negatively to IFN-γ (r=-0.403, $p=0.0002$). GM-CSF positively correlated with IL-12 (r=0.259, $p=0.0210$) but negatively with IFN-γ (r=-0.645, $p<0.0001$), Table 4.8.

MIP-1α had positive correlation to MIP-1β, IP-10 and MIG (r=0.570, $p<0.0001$, r=0.234, $p=0.038$ and r=0.239, $p=0.034$, respectively). RANTES positively correlated with Eotaxin (r=0.233, $p=0.040$) but negatively with MCP-1 (r=-0.271, $p=0.017$). Eotaxin had positive correlation with MIP-1β (r=0.367, $p=0.008$), Table 4.9.

Table 4.8: Correlation between cord blood pro-inflammatory cytokines

	IL-1beta	IL-6	IL-12	IL-17	GM-CSF	IFN-gamma	TNF-alpha	IL-8
IL-1beta		0.119	-0.127	-0.035	0.092	-0.031	0.213	0.141
IL-6			-0.108	0.209	0.066	-0.117	0.143	**0.446**
IL-12				-0.068	**0.259**	-0.056	0.154	0.066
IL-17					-0.035	0.004	-0.069	0.174
GM-CSF						**-0.645**	**0.491**	**0.456**
IFN-gamma							**-0.403**	**-0.382**
TNF-alpha								**0.423**
IL-8								

Values are correlation coefficients. Values in bold have significant correlation coefficients of $p \leq 0.05$ determined by Spearman correlation test. IL-Interleukin, IFN-Interferon, TNF-Tumor necrosis factor, GM-CSF-Granulocyte monocyte colony stimulating factor

Table 4.9: Correlation between cord blood pro-inflammatory chemokines

	RANTES	Eotaxin	MIP-1alpha	MIP-1beta	MCP-1	IP-10	MIG
RANTES		**0.233**	0.048	0.079	**-0.270**	-0.003	0.051
Eotaxin			0.158	**0.369**	0.170	-0.075	0.034
MIP-1alpha				**0.570**	0.215	**0.234**	**0.239**
MIP-1beta					0.206	0.209	0.049
MCP-1						0.013	0.010
IP-10							0.220
MIG							

Values are correlation coefficients. Values in bold have significant correlation coefficients of $p \leq 0.05$ as determine by Spearman correlation test.

This study then determined if time of exposure to malaria *in utero* determines the concentration of cytokines in cord blood. The infants whose mothers who had detectable malaria during the last trimester were considered to be exposed late while those infants whose mothers had detectable malaria during the first or second trimesters were considered to be exposed early. This study reports that there was no significant difference in the concentration of cytokines or chemokines between the two groups (Table 10). In IL-6, the infants who are exposed late had significantly higher concentrations (19.43pg/ml) compared to the infants who were exposed early (9.982pg/ml) $p=0.035$ (Table 4.10).

Table 4.10: Effect of time of maternal malaria infection on the concentrations of cord blood cytokines and chemokines

	Early		**Late**		
	Mean	SD	Mean	SD	*p*-value
IL-1beta	4.5	3.2	4.7	3.0	0.735
IL-2	1.6	0.9	1.8	1.1	0.895
IL-2R	266.1	140.3	372.2	218.5	0.188
IL-6	10.0	27.6	19.4	38.3	**0.035**
IL-12	120.5	50.9	114.1	43.6	0.780
GM-CSF	1.3	0.7	1.5	0.7	0.705
IFN-gamma	21.8	7.4	19.0	6.0	0.348
TNF-alpha	3.5	2.2	3.4	1.8	0.989
IL-1RA	1278.0	187.9	1188.0	269.5	0.207
IL-4	1.4	1.0	1.3	0.0	0.393
IFN-alpha	17.78	13.1	18.96	12.11	0.732
Eotaxin	2.381	1.894	2.578	2.831	0.779
IL-8	33.73	45	43.87	46.89	0.664
IP-10	20.67	13.67	15.55	6.909	0.475
MIP-1alpha	24.06	60.89	86.23	248.1	0.932
MIP-1beta	47	33.4	57.04	29.38	0.141
MIG	10.79	7.589	9.661	6.201	0.545
MCP-1	53.34	52.01	82.64	63.62	0.096
RANTES	19933	10168	18224	8950	0.975

Cytokine and chemokine levels were measured in maternal venous blood and cord blood at delivery. Values presented are the concentration of the cytokines and chemokines in pg/ml. *p*-values ≤0.05 are considered statistically significant.

In summary, *in utero* exposure to malaria does not modify the levels of pro-inflammatory and anti-inflammatory cytokines and chemokines in cord blood.

4.4 Effect of Maternal Malaria Infection During Pregnancy on Frequency of $CD4^+$ and $CD8^+$Treg Cells In Cord Blood

To evaluate the impact of malaria exposure during pregnancy on the frequency of *ex vivo* Treg cells in cord blood, CBMC's were isolated, stained using anti-CD3, anti-CD4, anti-CD8, anti-CD25 and anti-FOXP3 monoclonal antibodies and analyzed using eight color flow cytometer to identify $CD4^+$ T-cells that co-express CD25 and FOXP3. An infant was considered exposed to malaria *in utero* if *P. falciparum* was detected in any of the maternal ANC visit sample, maternal venous, placental blood or cord blood sample at delivery by either blood smear of QPCR. Figure 4.4 represents the gating strategy used to identify Treg cells in cord blood. Representative flow cytometry dot plot of the gated $CD4^+$Treg cells in malaria exposed (a) and unexposed (b) infants are shown in Figure 4.5.

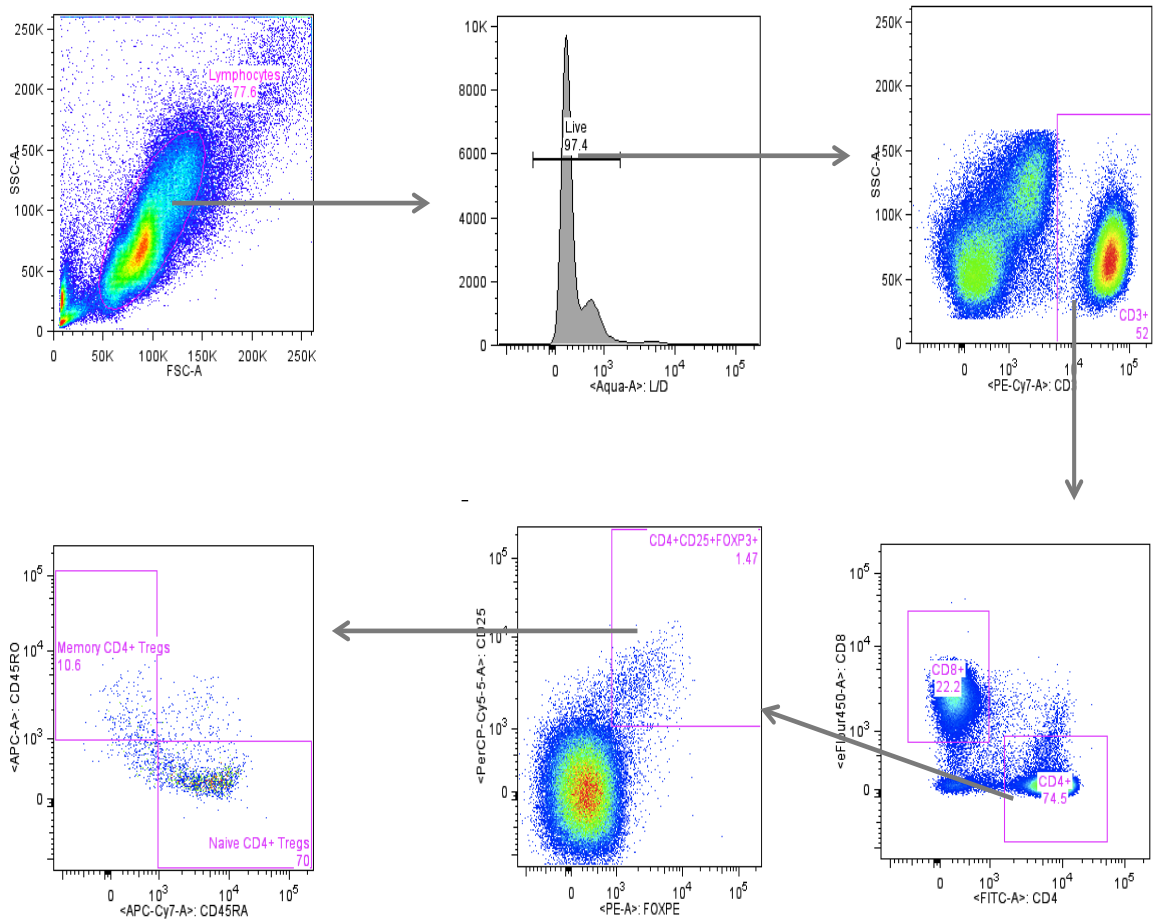

Figure 4.4: Representative flow cytometric gating strategy used to identify Treg cells. Based on the side scatter and forward scatter characteristics, the cells were first gated on the lymphocytes, to exclude dead cells, a live/dead discrimination dye was used, and then the cells were gated on $CD3^{+}$ and $CD4^{+}$. Fluorescence minus one (FMO) controls were used to derive the gates.

This study reports that in malaria exposed neonates, the median *ex vivo* $CD4^+$ T-cells that expressed the Treg phenotype ($CD25^+FOXP3^+$) was 2.23% (IQR 0.738-6.865) relative to malaria unexposed neonate 0.653% (IQR 0.063-1.263), Figure 4.6. These results suggest that *in utero* exposure to malaria results in significant expansion of Treg cells in cord blood.

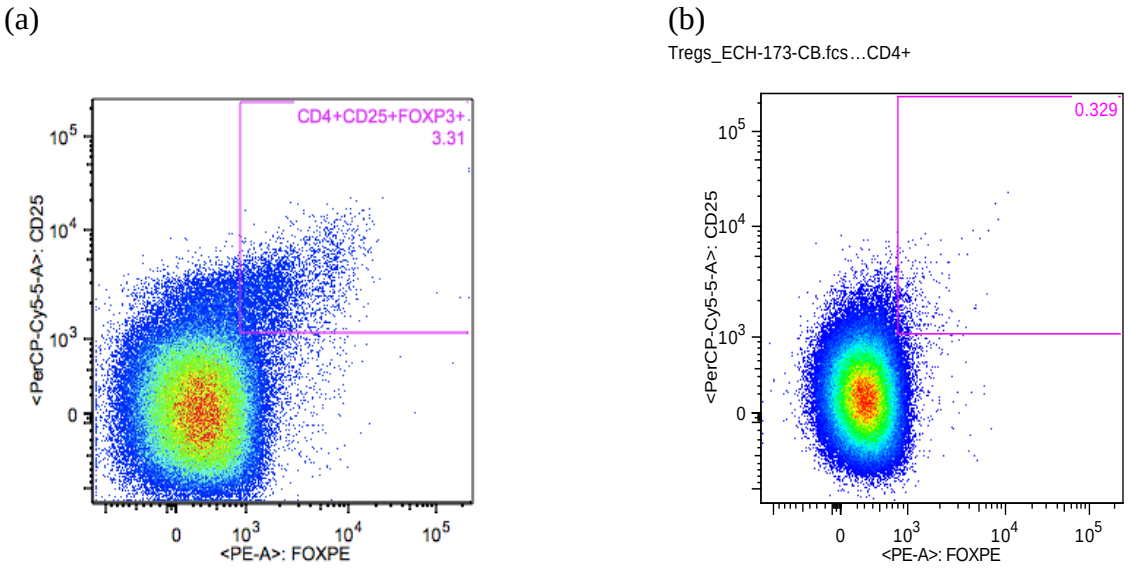

Figure 4.5: Representative dot plots showing the frequency of Treg cells in one representative sample from the malaria exposed (a) and unexposed (b) cord blood. Fluorescence minus one (FMO) controls were used in deriving the gates.

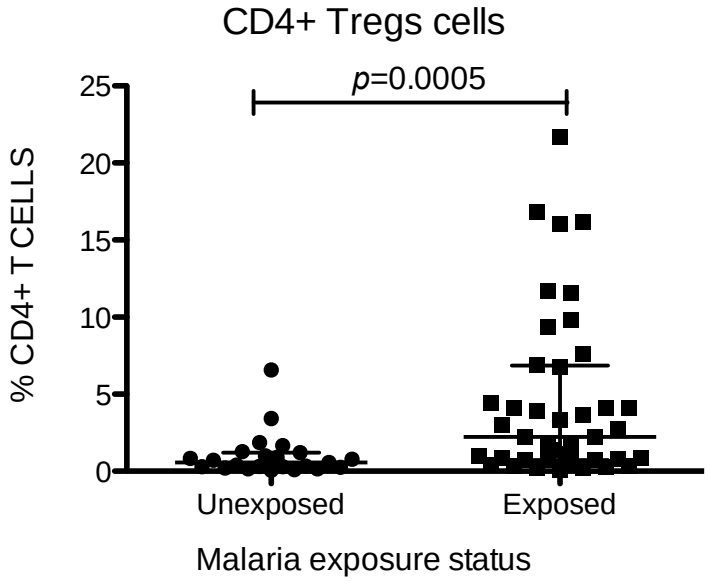

Figure 4.6: *Ex vivo* $CD4^+$Treg cells frequencies in cord blood are significantly increased in malaria exposed cord blood. CBMC's were isolated from cord blood and analyzed by flow cytometry. The $CD25^+FOXP3^+$ Treg cells are expressed as a percentage of $CD4^+$ T-cells. Groups were compared using two-tailed Mann–Whitney tests where $p \leq 0.05$ was considered significant.

To determine if infants exposed to malaria *in utero* have altered frequencies of $CD8^+$ Treg cells, this study quantified the percentage of $CD8^+$ T-cells that co-express CD25 and FOXP3 in cord blood from mothers with or without malaria during pregnancy. As shown in Figure 4.7, the results demonstrate that compared to the unexposed neonates, the median frequencies of *ex vivo* $CD8^+$ Treg cells in cord blood of malaria exposed were thrice those of the unexposed (0.108% and 0.039% respectively). This difference was however not significant, p=0.436, suggesting that *in utero* exposure to malaria did not significantly alter the frequencies of $CD8^+$ Tregs in cord blood.

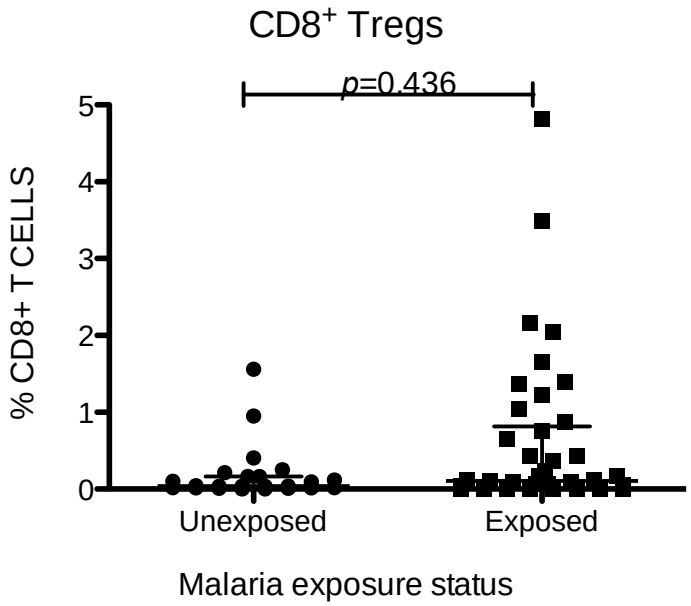

Figure 4.7: *Ex vivo* CD8$^+$Treg cells in cord blood. *In utero* exposure to malaria did not significantly alter the frequency of CD8$^+$Treg cells.

4.4.1 Treg cell phenotypes in cord blood

To determine if *in utero* exposure to malaria leads to perturbation of the distribution of the Treg cell phenotypes, further gating based on the surface expression of CD45RA and CD45RO was done on the CD4$^+$ and CD8$^+$Treg cells. As shown in table 4.11, the median frequency of CD4$^+$Treg cells expressing the naïve marker (CD45RA) were comparable in cord blood of malaria-exposed and -unexposed neonates (53.6% vs. 59.3% respectively, p=0.140). Similarly, the frequency of memory CD4$^+$Treg cells in cord blood of malaria-unexposed infants was similar to that in cord blood of malaria exposed infants (16.7% and 9.6% respectively, p=0.252).

There was similar frequency of naive CD8$^+$Treg cells in cord blood of malaria-exposed and -unexposed infants, (74.5% and 62.65% respectively, p=0.822). Likewise,

the median frequency of memory $CD8^+$Treg cells was similar in cord blood of malaria unexposed and exposed infants, *p*=0.464.

Table 4.11: Phenotypes of $CD4^+$ and $CD8^+$Treg cells in cord blood.

		Malaria Unexposed	Malaria Exposed	*p*-value
$CD4^+$ Treg cells	Naive	53.6 (29.35-69.78)	59.3 (40.5-81.4)	0.140
	Memory	16.7 (4.7-50.9)	9.6 (2.6-30.2)	0.252
$CD8^+$ Treg cells	Naïve	62.65 (50-95)	74.5 (0.0-99.9)	0.822
	Memory	0.0 (0.0-1.8)	0.0 (0.0 -0.0)	0.464

The values are median frequencies. In parenthesis are the 25^{th} and 75^{th} percentiles. Significant difference was determined by Mann-Whitney U test, where $p<0.05$ was considered significant.

4.4.2 Effect of time of exposure on the frequency of $CD4^+$ and $CD8^+$ Treg cells

In this study, it was hypothesized that timing of malaria infection during pregnancy would lead to perturbation of Treg cells and Treg cell phenotypes. To test this hypothesis, the mothers were classified as *late* if malaria was detected during the last trimester while they were classified as *early* if malaria was detected during either the first or second trimester of pregnancy, then the frequencies of Treg cells as well as Treg cell phenotypes were compared between the two groups. As shown in table 4.12, there was a trend towards higher frequency of $CD4^+$Treg cells in cord blood from mothers who had malaria early in pregnancy compared to mothers who had malaria late in pregnancy, this difference was however not significant (2.91% and 1.41%, *p*=0.4619). The frequency of naive $CD4^+$Treg cells was comparable between the infants who were exposed early and late (59.35% and 56.2%, *p*=0.670). On the other hand, infants who were exposed late in pregnancy had thrice the frequency of memory $CD4^+$Treg cells compared to infants who

were exposed early in pregnancy, though this difference was not significant (21.4% and 7.99% respectively, p=0.200).

Infants who were exposed early during pregnancy had a significantly higher frequency of $CD8^+$Treg cells compared to infants who were exposed during the third trimester (0.17% and 0.006% respectively, p=0.007). However, majority of $CD8^+$Treg cells in both early and late groups expressed the naive phenotype (94% and 90.6%, respectively, p=0.317). On the other hand, few of the $CD8^+$Treg cells expressed the memory T-cell marker, Table 4.12.

Table 4.12: Frequencies of Treg cells and their phenotypes in cord blood.

	Early Exposure	**Late Exposure**	***p*-value**
$CD4^+$Treg cells	2.91 (0.74-7.10)	1.41 (0.11-4.4)	0.462
Naïve $CD4^+$Tregs	59.35 (40.9-83.3)	56.2 (38.7-80.6)	0.667
Memory $CD4^+$Tregs	7.99 (1.48-30.0)	21.4 (6.25-42.9)	0.200
$CD8^+$Treg cells	0.17 (0.07-1.25)	0.006 (0-0.04)	**0.007**
Naïve $CD8^+$Treg cells	94.0 (87.9-96.3)	90.6 (87.5-93.3)	0.317
Memory $CD8^+$Treg cells	0.35 (0-2.97)	3.21 (0-5.050)	0.218

The values are median frequencies. In parenthesis are the 25^{th} and 75^{th} percentiles. Significant difference was determined by Mann-Whitney U test, where $p<0.05$ was considered significant.

In summary, *in utero* exposure to malaria results in the expansion of CD4+Treg cells in cord blood. However, the frequency of CD8+Treg cells in cord blood was not altered by this exposure.

4.5 Effect of *In Utero* Malaria Exposure on Immunophenotypic Characteristics of B and T-Cells In Cord Blood

To determine the phenotypic characteristics of lymphocytes in cord blood following *in utero* malaria exposure, this study first quantified the percentages of B and T-cells in cord blood then phenotypically characterized the B and T-cells based on the cell surface expression of naïve and memory markers.

4.5.1 Analysis of T-cell lymphocytes in cord blood

In order to determine shifts in lymphocyte subsets in cord blood following *in utero* malaria exposure, this study quantified the $CD3^+$ lymphocytes in cord blood from mothers with or without malaria during pregnancy. As shown in Figure 4.8, the results demonstrated that although there was a trend towards higher percentage of total $CD3^+$ cells in the malaria-exposed group compared to the unexposed, this difference was not significant (p=0.206). The median percentage of $CD4^+$ and $CD8^+$ T-cells were also comparable in the malaria-exposed and -unexposed groups (p=0.634 and p=0.343, respectively). These results suggest that *in utero* exposure to malaria does not alter the frequency of T-cell lymphocytes or T-cell subsets in cord blood.

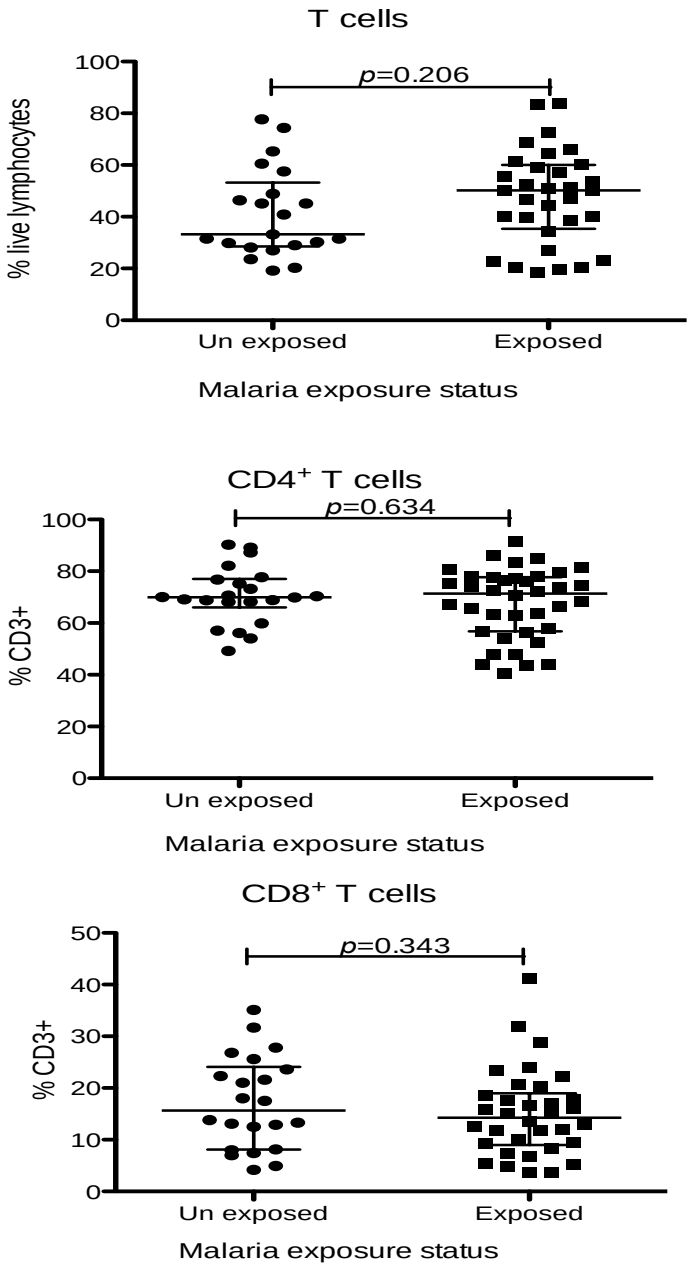

Figure 4.8: T-cell lymphocyte subsets in cord blood based on *P. falciparum* malaria infection of the mother. (A) Total T-cells, (B) CD4+ T-cells and (C) CD8+ T-cells. Differences in the frequencies of total T-cells, CD4 and CD8 T-cells between the two groups were determined using Mann-Whitney test.

4.5.2 Immunophenotypic characteristics of T-cells in cord blood

This study then quantified the phenotypes of $CD4^+$ and $CD8^+$ T-cells to determine whether exposure to malaria *in utero* alters these T-cell phenotypes. Naive and memory $CD4^+$ and $CD8^+$ T-cell were defined based on the surface expression of either CD45RA or CD45RO. Figure 9 shows a representative flow cytometry dot plots for the phenotypes. The naive phenotype was the most predominant in both $CD4^+$ and $CD8^+$ T-cell compartments. There was no significant difference in either naive or memory $CD4^+$ and $CD8^+$ T-cell in CBMC's of malaria-exposed and -unexposed infants (Table 4.13), suggesting that exposure to *P. falciparum* malaria *in utero* did not alter the distribution of naive or memory $CD4^+$ and $CD8^+$ T-cells.

Table 4.13: Distribution of T-cell subsets in cord blood

		Malaria Exposed	**Malaria Unexposed**	***p*-value**
$CD4^+$ T-cells	Naïve	87.4 (83.8-91.65)	88.0 (78.38-91.48)	0.791
	Memory	2.110 (0.507-5.220)	3.375 (0.709-10.09)	0.178
$CD8^+$ T-cells	Naïve	92.70 (87.5-95.7)	91.80 (89.13-95.65)	0.658
	Memory	0.441 (0-4.32)	2.895 (0.108-7.78)	0.077

CBMC's were isolated from cord blood and analyzed by flow cytometry. Values represented are median percentages of either $CD4^+$ or $CD8^+$ T-cells. The 25^{th} and 75^{th} percentiles are in parenthesis. Differences between the two groups were determined using Mann-Whitney U test.

4.5.3 Effect of time of exposure on the frequency of cord blood T-cell phenotypes

Previous studies have shown that malaria during early or late in pregnancy has severe consequences to the infant. In this study, the effect of time of exposure to malaria *in utero* on the T-cell phenotypes was determined. Infants were classified as *late* if their mothers had detectable malaria during the last trimester of pregnancy while they were classified as *early* if their mothers had malaria during the first or second trimester of pregnancy. Data demonstrate that there was no significant difference in the frequencies of cord blood naive $CD4^+$ and $CD8^+$ T-cells as well as the frequencies of cord blood memory $CD4^+$ and $CD8^+$ T-cells between infants in the *early* and *late* exposure groups (Table 4.14).

Table 4.14: Effect of time of exposure on the frequency of Treg cells and T-cell phenotypes in cord blood.

	Early	**Late**	***p-value***
Naïve $CD4^+$ T-cells	88.85 (86.05-91.93)	84.30 (61.3-91.5)	0.257
Memory $CD4^+$ T-cells	1.685 (0.488-4.653)	3.210 (1.150-6.840)	0.222
Naïve $CD8^+$ T-cells	94.0 (87.9-96.3)	90.6 (87.5-93.3)	0.317
Memory $CD8^+$ T-cells	0.346 (0-2.970)	3.210 (0-5.050)	0.218

Values are median percentages. Interquartile ranges are in parenthesis. Statistical differences between the two groups were determined using Mann-Whitney U test.

4.5.4 Frequency of B-cell lymphocytes in cord blood

In order to determine shifts in B-cell lymphocytes in cord blood following *in utero* malaria exposure, this study quantified the percentages of $CD19^+$ lymphocytes in cord blood from mothers with or without malaria during pregnancy. As shown in figure 4.9, the results demonstrate that the percentage of total $CD19^+$ cells was similar in both the malaria-exposed and -unexposed cord blood (p=0.916).

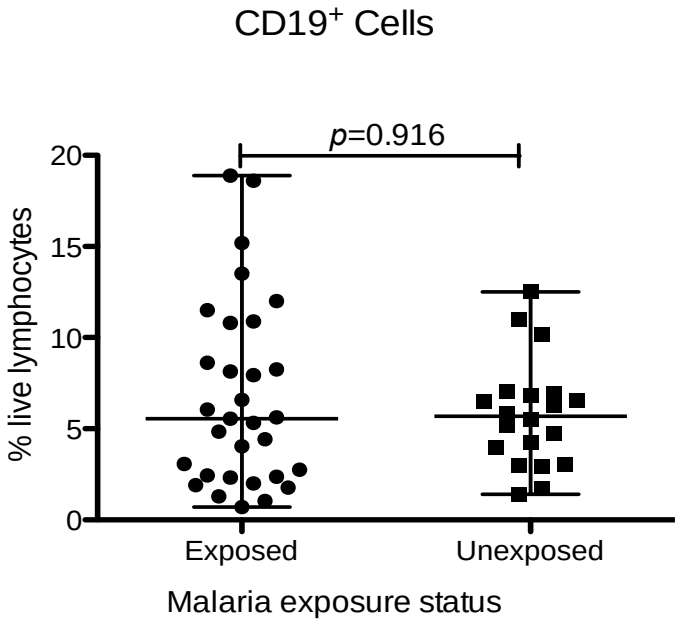

Figure 4.9: The percentage of B-cells was comparable between malaria-exposed and -unexposed cord blood. Mann-Whitney U test was used to test for differences between the two groups.

4.5.5 Alteration of the frequency of activated classical, naïve and transitional B-cells following *in utero* malaria exposure

Since *P. falciparum* malaria infection is associated with the perturbation in the distribution of lymphocyte subsets in children (Asito *et al.*, 2008), this study hypothesized that maternal malaria infection during pregnancy may also lead to altered lymphocyte subsets in cord blood. To better understand the differences in B-cell subset distribution in cord blood following *in utero P. falciparum* exposure, the B-cells were further gated based on the expression of IgD, IgG, IgM CD21 and CD27, to subdivide B cell populations into distinct B cell subsets

The frequency of transitional B-cells were significantly higher in malaria exposed cord blood compared to unexposed cord blood; 21.5% (range 8.55 – 31.1) *vs*. 8.545% (range 3.445 – 16.53), p=0.009 (Table 4.15). On the other hand, naïve B-cells were significantly higher in malaria unexposed cord blood relative to the malaria exposed cord blood; 77.4% (range 53.23 – 90.9) vs. 39.7% (range 16.3 – 64.1), p=0.005 (Table 15). There was no significant difference in the frequency of $CD27^{+}IgD^{-}$ (classical memory B-cells) between malaria-exposed and -unexposed cord blood. However, activated classical memory B-cells were significantly higher in malaria exposed cord blood relative to unexposed cord blood; 18.6% (8.830 – 48.4) vs. 7.94% (4.42 – 18.75), p=0.026. The frequency of resting classical memory B-cells was comparable between malaria-exposed and -unexposed cord blood (p=0.952), Table 4.15.

The frequency of marginal zone memory B-cells were comparable in malaria-exposed and –unexposed cord blood. Similarly, the frequency of atypical memory B-cells as well as non-class switched B-cells were comparable between malaria-exposed and -unexposed cord blood, Table 4.15.

Table 4.15: Median percentage of B-cell lymphocyte subsets in cord blood

	Medians (25^{th} and 75^{th} percentiles)		
B-cell population	**Malaria Exposed**	**Malaria Unexposed**	***p-value***
Atypicals	11.70 (6.92 - 53.10)	13.35 (9.49 - 29.05)	0.487
Atypical memory B-cells	10.60 (4.11 - 21.60)	9.50 (6.49 - 18.23)	0.899
Classical memory	0.82 (0.24 - 1.52)	1.03 (0.41 - 3.08)	0.237
Activated Classical memory	18.60 (8.83 - 48.4)	7.94 (4.42 - 18.75)	**0.026**
Resting Classical memory	0.0 (0 - 0.62)	0 (0 - 0.68)	0.952
Non class switched	1.07 (0.29 - 2.71)	0.57 (0.34 - 1.26)	0.149
Marginal zone memory	7.38 (0.67 - 48.43)	1.93 (0.12 - 8.54)	0.404
Naive B-cells	39.70 (16.30 - 64.10)	77.4 (53.23 - 90.90)	**0.005**
Transitional B-cells	21.50 (8.55 - 31.10)	8.55 (3.45 - 16.53)	**0.009**

CBMCs were analyzed by flow cytometric staining with various combinations of monoclonal antibodies. For each population, the percentages given are the medians of the proportions of B cells subsets within the live $CD19^{+}$ B cell gate. The 25^{th} and 75^{th} percentiles are in parenthesis. Statistical differences between the two groups were determined using Mann-Whitney U test. Statistically significant $p \leq 0.05$ are in bold.

In summary, this study reports that malaria exposure during the prenatal period results in the perturbation in B-cell compartment. However, the T-cell compartment was not significantly affected by maternal malaria exposure during pregnancy.

CHAPTER FIVE

DISCUSSION

5.1 The Effect of *In Utero* Malaria Exposure on Transplacental Transfer of EBV-Specific Antibodies

Continuous exposure to *Plasmodium falciparum* during infancy and early age of Epstein Barr virus (EBV) infection are risk factors in eBL pathogenesis, but how these two etiological factors interact with host immune system to promote development of malignant B-cell clones is still not clear. Data from a previous study demonstrated that children from malaria holoendemic areas are infected by EBV earlier in life, resulting in poor control of the virus (Piriou *et al.*, 2012); it was postulated in the current study that maternal infection with *P. falciparum* malaria during pregnancy may interfere with the transfer of EBV-specific antibodies from the mother to the neonates thereby leaving the infants susceptible to early primary EBV infection. The data presented in this study certainly demonstrates that maternal malaria infection during pregnancy results in significant reduction in the transfer of anti-VCA-p18 and EBNA1 antibodies. Furthermore data presented in this study shows that regardless of *in utero* malaria exposure, the levels of anti-Zta and -EAd antibodies are significantly low in the neonates.

Irrespective of maternal *P. falciparum* malaria infection status during pregnancy, the levels of anti-EBV and TT-specific antibodies in the mothers were comparable. This is in contrast to a previous study that found elevated antibody levels in children who are chronically exposed to malaria (Piriou *et al.*, 2009). These two studies may not be comparable because while this study compared anti-EBV-specific antibody levels in adults, the previous study compared anti-EBV-specific antibody levels in children.

However, the difference could be attributed to the fact that pregnancy reactivates EBV (Haeri *et al.,* 2010) such that the levels of EBV-specific antibodies would be similar in both groups of women. Nevertheless, *in utero*, malaria exposure resulted in significantly less anti-VCA-p18 and anti-EBNA1 IgG antibodies being transferred from the malaria infected mothers to the malaria-exposed neonates. This difference remained significant even after adjusting for other confounding variables that are known to interfere with the placental transport of antibodies.

The reduced transplacental transfer of anti-VCA-p18 and anti-EBNA1 antibodies due to *in utero* malaria exposure could have resulted from the fact that maternal malaria infection during pregnancy causes placental abnormalities such as thickening in the placental tissues (Bulmer *et al.,* 1993; Rogerson and Beeson 1999), resulting in the reduced transfer of specific antibodies to the infant. In addition, *P. falciparum* infestation of the placenta may have caused damage or alteration to the Fc receptors that are responsible for transporting antibodies across the placenta, resulting in reduced transfer of these antibodies.

Since there is a preferential transplacental transfer of IgG subclasses, it was also hypothesized that the EBV antigens may be eliciting IgG2 response that is transported across the placenta less efficiently that either IgG1 or IgG3, with a consequence of reduced transplacental transfer. However, what remains to be established is why anti-VCA antibodies that are also produced during the lytic phase are efficiently transported across the placenta yet anti-Zta and EAd antibodies are not. In this study, it is thought that maternal malaria infection could have caused isotype switch such that the dominant

IgG that is presented is not efficiently transported across the placenta into fetal circulation.

One surprising finding from this study is that anti-Zta and -EAd antibodies are not efficiently transported across the placenta into fetal circulation, resulting in low levels of these antibodies in the neonates. Though there is evidence of viral reactivation during pregnancy in this cohort (Daud *et al.,* 2014a), it was expected that maternal levels of antibodies against Zta and EAd would be high resulting in more antibodies being transferred to the neonates. However, these results show that the levels of antibodies against Zta and EAd in the neonates are significantly low compared to the levels in the mothers. Furthermore, malaria-exposed neonate had higher CMR compared to the unexposed, suggesting that exposure to malaria resulted in more antibodies being transferred.

A number of factors can explain the low levels of anti-Zta and EAd antibodies as well as the inefficient transfer of these antibodies to the neonate. First, relative to the levels of anti-VCA or anti-TT antibodies in the mothers, the levels of anti-Zta and -EAd were low in the mothers and since the levels of antibodies in the mother determines the levels in the neonates, it is expected that the neonates would have low levels of antibodies. Irrespective of the levels of other antibodies, when comparing the levels of anti-Zta and -EAd antibodies in the mothers and their neonates, the levels of antibodies transferred were significantly very low and very little of these antibodies were transferred to the neonates. Secondly, the EBV antibodies that were studied represent different phases of the virus life cycle. EBNA1 is mainly produced during latent infections while anti-VCA, -Zta and -EAd antibodies are mainly produced during lytic phase of the virus

life cycle. Detection of anti-Zta and -EAd IgG antibodies at high levels has been associated with viral reactivation (Donati *et al.,* 2006a; Rahman *et al.,* 1991). In healthy EBV carriers, IgG antibodies against both EBNA1 and VCA persist for life (Miller 1990), as the virus is continuously being shed so the levels of these antibodies should be high in the mothers as reported in this study.

Thirdly, 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 capillaries. High levels of anti-VCA and –TT antibodies as well as other 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 -EAd antibodies to bind to finite number of FcRn receptors, thereby limiting the transfer of anti-Zta and EAd antibodies to the neonates. However, this observation warrants further studies to investigate the mechanism.

Another plausible explanation to the low levels of anti-EAd antibodies in the mother could be the fact that the levels of anti-EAd antibodies falls relatively fast after infection (Miller 1990), and since majority of IgG are transported across the placenta during the last trimester of pregnancy (Saji *et al.,* 1999), the rapid decay of these antibodies may result in very little antibodies being transferred to the neonates as reported in this study. This is in contrast to the high IgG antibodies against both VCA and EBNA1 that persist for life in healthy carriers (Miller 1990). This factor may also explain the lack of correlation between maternal and neonatal anti-EAd antibodies observed in this study.

The high levels of anti-TT antibodies in the mothers observed in this study could be attributed to the tetanus toxoid vaccine that the mothers receive during pregnancy as part of the focused antenatal care (FANC). Consequently, the result of the high levels of anti-TT antibodies in the neonates could be attributed to the high anti-TT antibodies in the mothers, as the levels of antibodies in the mother is a determinant. In this study, the transplacental transfer of antibodies against tetanus was more efficient and was not affected by maternal malaria infection. The results for the transfer of tetanus antibodies may be attributed to the active transport of IgG1 and IgG3 subclass of IgG that is mainly elicited by protein antigens such as tetanus toxoid (Palmeira *et al.,* 2012).

The findings from this study also demonstrate that malaria exposure does not affect the transplacental transfer of tetanus-specific antibodies, an observation consistent with previous studies that demonstrated that placental malaria does not have an effect on the transplacental transfer of tetanus antibodies (de Moraes-Pinto *et al.,* 1996; Okoko *et al.,* 2001b). However, these results are in contrast to other studies that demonstrated that placental malaria results in reduced transplacental transfer of tetanus antibodies (Brair *et al.,* 1994; Cumberland *et al.,* 2007). Data from this is of particular interest since in contrast to previous studies that looked at the effect of placental malaria as determined by the presence of malaria parasites in the mother at birth on antibody transfer, this study followed up the expectant mother through their pregnancy to determine time of exposure of the neonates *in utero*.

Malaria infection causes polyclonal stimulation of B-cells, and this may result in increased levels of specific or non-specific IgG in blood. In this study, maternal hypergammaglobulinemia caused the reduction of antibodies transferred to the neonate

by between 5 – 18%. This may have caused the saturation of the FcRn receptors such that they cannot transfer antibodies to the neonates.

5.2 Cytokines and Chemokines In Cord Blood

The sequestration of infected erythrocytes in the placenta may result in the sensitization of cord blood mononuclear cells to malaria antigens and induce the production of either pro-inflammatory and/or anti-inflammatory cytokines and chemokines (Malhotra *et al.,* (2005). The levels of some cord blood cytokines might in turn influence the susceptibility to diseases during infancy (Adegnika *et al.,* 2008; Brickley *et al.,* 2015; Kabyemela *et al.,* 2013; Malhotra *et al.,* 2009; Malhotra *et al.,* 2005). It was hypothesized in the current study that maternal malaria infection would result in the increased levels of pro-inflammatory cytokines and chemokines in cord blood. However, results reported here show that both the malaria-exposed and -unexposed cord blood had comparable levels of cytokines. Although this was unexpected, it could be attributed to the influence of endemicity of malaria or other parasitic infections as that are common in this area and are known to influence the production of cytokines. Studies on cytokines in cord blood have shown that they vary greatly. A previous study reported that the levels of some cytokines were higher in cord blood and persisted into childhood and these levels were implicated in the protection against malaria infection (Kabyemela *et al.,* 2013). In concert with a study in Tanzania that compared cytokine levels in cord blood of infants born to mothers with or without placental malaria (Kabyemela *et al.,* 2013), this study also reports comparable levels of cord blood of pro inflammatory cytokines IL-1β and TNF-α when malaria exposed, malaria unexposed and malaria naive cord blood were compared. In contrast, a study in

Sudan reported that infants born to mothers without malaria had significantly lower levels of IFN-γ compared to infants of mothers with malaria (Bayoumi *et al.,* 2009). In this study however, the levels of pro inflammatory cytokines IL-2, GM-CSF and IFN-γ, and anti-inflammatory cytokine IL-4 were significantly higher in malaria naive cord blood plasma. These increased levels were surprising but they may be attributed to other prenatal factors such as maternal stress, other infections that were not considered or dietary patterns that are known to influence cytokine production. This conclusion can be supported by data from experimental animal models that demonstrated that plasma levels of pro inflammatory cytokines in offspring were associated with either increased maternal levels of glucocorticoids, maternal infections or their diet during pregnancy (Lillycrop *et al.,* 2005; Wyrwoll *et al.,* 2008).

Perturbations in the concentrations of chemokines may lead to chemotactic deficiency as well as susceptibility to infections during infancy. Since previous reports have associated low levels of RANTES to increased disease severity and mortality in children as well as suppression of erythropoiesis, (Bostrom *et al.,* (2012); (John *et al.,* 2006; Ochiel *et al.,* 2005; Were *et al.,* 2006), it was speculated that malaria-exposed infants would have low levels of RANTES and this could lead to poor control of EBV infection during childhood. The RANTES results presented in this study shows that malaria exposure *in utero* does not influence the levels of RANTES, as both the malaria-exposed and -unexposed neonates had comparable levels of RANTES. On the other hand, malaria naive infants had significantly lower levels compared to either malaria exposed or unexposed infants. These results contrasts a previous one carried out in Mali in which it was reported that levels of RANTES were higher in malaria un-infected

Dogon children compared to the infected children (Bostrom *et al.,* 2012). The increased levels of RANTES in Kenyan pregnant women may protect their neonates from early EBV infection. This can be supported by observations that RANTES plays an important role in inhibiting the replication of HIV (Coffey *et al.,* 1997). In addition, low levels of RANTES limited the ability of mice to control chronic viral infection and resulted in higher viral loads (Crawford *et al.,* 2011)

A previous study documented increased concentration of IL-8 in adults with severe malaria (Friedland *et al.,* 1993) and since IL-8 is a chemo-attractant cytokine involved in the recruitment of neutrophils to sites of inflammation, this study expected to observe increased levels of IL-8 in cord blood from malaria infected women. This may be further supported by the observations that levels of IL-8 correlate to malaria parasite density (Ayimba *et al.,* 2011; Burgmann *et al.,* 1995) suggesting that exposure to malaria *in utero* may have induced these responses. However, the levels of IL-8 in all the three groups in this study were comparable probably suggesting a common intrinsic factor that controls the expression of IL-8 among pregnant women.

The observations from these previous studies led them to suggest that the cord blood levels of these cytokines are influenced by the inflammation in the placenta. However, this study reports that some but not all the cytokines correlate, which might suggest that not all the pro inflammatory cytokines are influenced malaria exposure *in utero,* but some other factors such as other maternal parasitic infections, individual differences in expression could also have played a role.

The consequences of maternal malaria infection have been well documented, however, sentinel studies have looked into the effect of length or period of exposure on

the levels of cord blood cytokines. In order to evaluate the relationship between timing of exposure and levels of cytokines, malaria infection of the mothers was classified as either early or late. This study reported that apart from IL-6, there was no significant difference in levels of cytokines in cord blood when the two groups were compared, suggesting that the cord blood cytokine levels seen in this study are not influenced by the time of exposure but rather by some other factors.

5.3 Expansion of CD4+ Treg Cells Due To *In Utero* Malaria Exposure

In malaria endemic regions of Africa, about 25% of the pregnant women are infected by malaria (Desai *et al.,* 2007). Fetal complications that arise as a result of maternal malaria infection include intra-uterine growth retardation, stillbirth, premature delivery and low birth weight babies (Steketee 2003; Steketee *et al.,* 2001; Sullivan *et al.,* 1999).

Maternal malaria during pregnancy results in the sequestration of infected erythrocytes in the placenta and as a result, the unborn infant may be exposed to the infected erythrocytes or their soluble products. The exposure of the unborn infant to the infected erythrocytes or soluble products may induce tolerance or sensitize the infants immune system thereby influencing their susceptibility to malaria itself as well as other infections during infancy (Broen *et al.,* 2007; Malhotra *et al.,* 2009; Malhotra *et al.,* 2006).

Previous studies looking into the effect of fetal exposure to malaria mainly focused on placental malaria i.e. the infants were considered to be exposed if the mother had detectable parasites at parturition, but these studies neither described the timing nor

how frequent was the malaria infection. A study in the coastal region of Kenya where malaria is endemic classified the infants as exposed or unexposed depending on the maternal malaria infection at birth or production of specific cytokines by CBMC's and reported that exposed infants have increased susceptibility to malaria infection and anemia during childhood (Malhotra *et al.,* 2009). In contrast to the previous studies, this study followed up a group of expectant women during all their routine ANC visits until delivery to determine the effect of maternal *P. falciparum* infection at any time during pregnancy on the frequency of *ex vivo* Treg cells and phenotypes of the Treg cells in cord blood. This study also compared the frequency of *ex vivo* Treg cells in mothers with or without placental malaria, but since the numbers of placental malaria positive women were few, it would be more plausible to follow up such a study in a large cohort to draw conclusions. In addition, it is unknown whether persistent exposure of the infant to maternal malaria infection leads to perturbation of Treg cell numbers. The main finding in the current study is that maternal malaria infection during pregnancy may result in the exposure of the infant to malaria antigens (Malhotra *et al.,* 2006), which in turn results in the induction and expansion of *ex vivo* Treg cells in the infants' cord blood.

The expanded pool of Treg cells observed in this study was in response to exposure to *P. falciparum* antigens *in utero*. This observation is in concert with data from a previous study that demonstrated that malaria specific Treg cells are generated during the prenatal period due to *in utero* exposure to *P. falciparum* malaria infection (Brustoski *et al.,* 2006). This is further supported by data from a previous study that showed that Treg cell numbers increased in malaria infected individuals compared to the uninfected individuals (Minigo *et al.,* 2009), suggesting that the expanded pool of Treg

cells was in response to malaria infection. However, a study in the Gambia looking into the effect of placental malaria on fetal immunity reported that this exposure results in expansion of malaria-specific Treg cells in cord blood but not *ex vivo* Treg cells (Flanagan *et al.,* 2010). These observations together with the data presented in the current study demonstrate that maternal malaria infection during pregnancy results in the expansion of Treg cells in cord blood. A possible explanation to this could be that exposure to the infected erythrocytes or their soluble product results in the activation and/or expansion of either the thymus derived Tregs or the induction of Treg cells from naive T-cells in the periphery, though the mechanism of induction of Treg cells by malaria parasites warrants further studies. However, *in vitro* models have proposed that Treg cells may be induced by a strong TCR signal, contact with the APC as well as external signals from cytokines. Indeed, external signals such as cytokines could induce the conversion of $CD4^{+}CD25^{-}FOXP3^{-}$ T-cells to potent $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cells.

Exposure to *P. falciparum* malaria *in utero* results in the suppression of protective immunity to malaria with observation that Treg cells negatively affect naturally-acquired immunity to malaria (Todryk *et al.,* 2008) and once activated, Treg cells suppress immune functions non specifically (Jonuleit *et al.,* 2001; Shevach *et al.,* 2006). Thus, Tregs cells in cord blood might contribute to the suppression of immunity to EBV infection as well. Several studies have observed that infants born to mothers with placental malaria were more susceptible to *P. falciparum* infection during infancy (Le Hesran *et al.,* 1997; Malhotra *et al.,* 2009; Schwarz *et al.,* 2008). As demonstrated by another study in malaria endemic region of Kenya, the other effect of the increased frequency of Treg cells may be limiting the effector T-cell responses, which in turn leads

to the failure of the infants' immune system to mount functional antibody responses to malaria antigens (Dent *et al.,* 2006) and further suggested that maternal infection with other parasitic infections could also result in immune tolerance when the infants are naturally infected. The precise mechanism is contentious; however, it is thought that Treg cells negatively affect IFN-γ production and proliferation of T-cells.

Another effect of the increased pool of Treg cells is the inhibition of immune responses resulting in ineffective clearance of pathogens and consequently the establishment of chronic infections. For example, in viral infections, higher numbers of Treg cells were negatively associated with increased viral load. In HIV and HCV infections, Treg cells silence the anti-HIV or anti-HCV immune responses thereby contributing to the persistence of the infection (Boettler *et al.,* 2005; Weiss *et al.,* 2004) and the decrease in the numbers of Treg cells after antiviral therapy (Peng *et al.,* 2008) further gives support to this idea. Additionally, increase in the frequency of Treg cells has been associated with less efficient control of malaria parasites in children and adults (Todryk *et al.,* 2008; Walther *et al.,* 2005). Consequently, the increased frequency of cord blood CD4+ Treg cells observed in this study may inhibit/dampen an infant's immune response to EBV resulting in early primary infection.

Increased Treg frequency may have a major role in secondary infections, the magnitude of memory as well as debilitate the efficacy of vaccines. In individuals that received a trial vaccine against listeria, the $CD8^+$ T-cell responses were inhibited by Treg cells (Kursar *et al.,* 2002), while in a malaria mouse model, depletion of Treg cells before vaccination resulted in robust T-cell responses and better parasite control (Moore *et al.,* 2005), suggesting that Treg negatively affect the acquisition of immunity to malaria

infections. It is surprising that *in utero* exposure to malaria does not significantly alter the frequency of $CD8^+$ Treg cells as it does to $CD4^+$ Treg cells. Although there was a three-fold increase in the frequency of $CD8^+$ Treg cells in cord blood of exposed infants compared to the unexposed infants, this difference was not significant. Further studies with a larger sample size are needed to confirm these results.

Result from this study also shows that *in utero* exposure of the infant to malaria did not significantly alter the frequency of naive and memory Treg cells. Majority of the $CD4^+$ and $CD8^+$ Treg cells expressed the $CD45RA^+$ naive phenotype. These results are consistent with those from a previous study that reported that majority of the $CD4^+$ Treg cells were naive, but the corresponding maternal $CD4^+$ Treg cells mainly expressed the $CD45RO^+$ memory phenotype (Flanagan *et al.,* 2010). In addition, although findings from another study defined Treg cells as $CD4^+CD25^+$ T-cells, they showed that cord blood contains Treg cells that are functionally mature cells but are phenotypically naive (Takahata *et al.,* 2004).

The frequency of memory phenotype in cord blood Treg cells is low possibly due to either scarcity of antigens within the cord blood or the threshold of activation by the antigens had not been reached. Majority of these Treg cells were still naïve possibly because they had not encountered their TCR ligand that could have initiated the process of upregulating the activation markers and homing to lymph nodes to start expansion (Tang *et al.,* 2004). Alternative explanation to this scenario would be that since the amount of APC is critical to the induction of Treg cells and the fact that mature DC are more efficient in inducing the proliferation of Treg cells, cord blood has more immature DC (Yamazaki *et al.,* 2003) that could not prime the proliferation of these cells. Lastly,

these Treg cells may still need to undergo additional maturation steps in order for them to become responsive to antigens (Wing *et al.,* 2003).

It had been initially proposed that the duration and intensity of placental malaria infection was associated with Treg cells activity as antigen specific induction of Treg cells activity requires chronic low levels of antigen exposure (Brustoski *et al.,* 2006). To determine if time of exposure *in utero* influences Treg cells, this study classified the mothers as either infected early or late during pregnancy. Infants who were exposed early during prenatal period had significantly higher $CD8^{+}$Treg cells compared to infants who were exposed late during the prenatal period. This suggest that chronic exposure to *Pf* antigens allowed for optimal stimulation and exposure to the cytokines milieu that resulted in the generation of Treg cells or the induction of Treg cells from naïve T-cells.

5.4 Immunophenotypic Characteristics of B and T-cells In Cord Blood

Malaria infection during pregnancy is characterized by infestation of the placenta by infected erythrocytes and this may result in the placental transfer of the malaria parasites or their soluble products that may in turn prime the infant immune system to these antigens. Several studies have demonstrated that infants born to mothers with malaria during pregnancy are more susceptible to malaria and anemia during infancy (Cornet *et al.,* 1998; Le Hesran *et al.,* 1997; Schwarz *et al.,* 2008) as well as other infections. This susceptibility may be linked to the perturbed distribution of B and T-cells caused by maternal malaria infection during pregnancy. Indeed several studies have associated clinical malaria with the perturbation of lymphocyte subsets (Asito *et al.,* 2008; Kassa *et al.,* 2006; Worku *et al.,* 1997). In addition, malaria-specific B and T-cells have been isolated from cord blood of neonates born to mothers who had malaria during

pregnancy suggesting *in utero* exposure to malaria antigens (King *et al.,* 2002; Metenou *et al.,* 2007). Based on these observations, this study hypothesized that maternal malaria infection during pregnancy would result in the perturbation in the distribution of B and T-cell lymphocytes in cord blood. Data presented in this study demonstrate that *in utero* exposure to malaria antigens alters the distribution of some B-cell lymphocyte subsets while it has no effect on T-cell lymphocyte subsets in cord blood.

To identify the T-cells phenotypes, several studies have relied on the surface expression of the CD45 isoforms (RA/RO) to delineate between the naïve and memory T-cells. There are other markers that can be used in combination with CD45 isoforms to delineate naïve from memory T-cells, however this study used the basic markers CD45RA/RO because of the limitation of the FACSCANTO flow cytometer to eight parameters. During fetal development, CD45RA is the most common T-cell subset in the immunologically naïve neonates. As they develop and encounter antigens, they upregulate the expression of CD45RO to become memory T-cells. The data presented in this study demonstrate that *in utero* malaria exposure does not alter the *ex vivo* T-cell phenotypes in cord blood. In addition, the time of exposure to malaria *in utero* did not alter these phenotypes. These observations were unexpected since maternal malaria infection during pregnancy often results in the exposure of the neonate to the infected erythrocytes or their soluble products (Malhotra *et al.,* 2006; May *et al.,* 2009) that may in turn prime the neonatal cellular immune system *in utero*. In concert with data presented in this study, recent data from a malaria perennial region of Benin have demonstrated that placental infestation with *P. falciparum* malaria does not alter the frequency of naïve and memory $CD4^+$ T-cells in cord blood (Soulard *et al.,* 2011). In

addition, they found no association between the levels of placental parasitaemia and the frequencies of cord blood naïve and memory $CD4^+$ T-cells. Taken together, these observations suggest that maternal malaria infection alone is not sufficient to alter the T-cell homeostasis, but some other factors together with exposure to malaria *in utero* may play a role. Indeed, as suggested in a previous study (Soulard *et al.,* 2011), the neonatal immune responses to parasite-specific antigens may be determined by the *P. falciparum* placental immune responses rather than just the presence of the parasite within the placenta. Another plausible explanation would be that cord blood contains more immature APC (Sorg *et al.,* 1999), thereby not able to present antigens to the T-cells. Alternatively, the T-cells may be receiving insufficient co-stimulatory signal for them to start proliferating. This observation can be supported by data that compared adult naïve $CD4^+$ T-cells to naïve $CD4^+$ from cord blood and showed functional unresponsiveness of the cord blood naïve $CD4^+$ T-cells when stimulated by similar conditions (Hassan and Reen 1997). Although not investigated, this study considers that due to exposure to malaria *in utero,* the inhibitory molecules such as PD-1 and CTLA-4 may have been upregulated in cord blood thereby limiting primary T-cell activation and expansion. Since the inhibitory molecule PD-1 is a negative regulator of T-cell activation (Parry *et al.,* 2005) and the fact that PD-1 was upregulated in T-cells as well as in plasma from children and adults from malaria endemic region it may be possible that the activation and expansion of cord blood T-cells was inhibited due to upregulation of the inhibitory molecules (Wherry *et al.,* 2004).

This study also observed that *in utero* malaria exposure is associated with increased frequency of activated classical MBC in cord blood. Similarly, another study

in a malaria endemic region of Mali also demonstrated increased frequency of activated MBC compared to controls. By analogy, as previously suggested by another study (Donati *et al.,* 2006b), the increased activated classical MBC in cord blood could have been as a result of the preferential activation of memory B-cells by malaria antigens *in utero*.

One potential mechanism for the decline in the frequency of naïve B-cells observed in malaria exposed cord blood is that continual exposure of the CBMC's to malaria antigens resulted in the increased B-cell activation and potential differentiation of the naïve B-cells into other B-cell subsets. This observation can be supported by data from a previous study that demonstrated a decrease in the number of naïve B-cells in children following an episode of acute malaria (Asito *et al.,* 2008). Furthermore, the results from an earlier study that demonstrated that antigen-driven B-cell activation and differentiation results in the loss of IgD expression (Ni *et al.,* 2003). These observations may suggest that neonatal B-cells from malaria exposed cord blood are highly differentiated from the naïve B-cells compared to the B-cells in malaria unexposed cord blood.

The observation from this study that *in utero* malaria exposure does not lead to the expansion of atypical MBC contrast the observation from other studies that reported that persistent malaria exposure was associated with expansion of atypical MBC in children (Illingworth *et al.,* 2013; Weiss *et al.,* 2009) and associated with severity of the infection (Jacobi *et al.,* 2008). In line with this, a previous study demonstrated an increased frequency of atypical MBC in infants from a malaria endemic region (Asito *et al.,* 2011). However, these studies may not be comparable to the current study because as

the previous studies analyzed B-cell subsets in children with divergent malaria transmission, this study analyzed B-cell subsets in cord blood.

Previous studies have shown that acute clinical *P. falciparum* infection interferes with either B-cell homeostasis or trafficking in children from malaria endemic regions resulting in increased frequencies of transitional B-cells in peripheral blood (Asito *et al.*, 2008; Weiss *et al.,* 2009). Consistent with this observation, this study classified transitional B-cells as $CD19^{+}CD10^{+}IgD^{+}CD27^{+}IgM^{+}$ and report the increased frequency of transitional B-cells in malaria exposed cord blood probably suggesting that increased transitional B-cells may be due to increased trafficking due to *in utero* malaria exposure. Marginal zone memory B-cells, phenotypically identified as $CD19^{+}CD10^{-}$ $CD27^{+}IgD^{+}CD21^{+}IgM^{+}$ are a distinct population of mature B-cells that are domiciled in the spleen and are essential in the production of high affinity IgM in early phases of adaptive immune responses. This study observed a three fold increase in the frequency of marginal zone B-cells in malaria exposed cord blood compared to the unexposed cord blood. It is probable that the increased germinal center reactions as a result of the malaria exposure may have caused this increase.

In conclusion, data from this study demonstrate that *in utero* malaria exposure results in the differential perturbation in the B-cell compartment. However, functional studies on these CBMC's are warranted to better understand the effect of this altered phenotype on infant immune responses to EBV.

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATIONS

6.1 Summary Of Findings

The data presented in this study demonstrated that maternal malaria infection during pregnancy is associated with significant reduction in the transplacental transfer of anti-VCA and anti-EBNA1 antibodies to the neonate. Since in the current study other factors known to affect the transplacental transfer of antibodies were controlled for, the reduced transfer was due to *in utero* malaria exposure. Furthermore, regardless of malaria exposure, anti-Zta and anti-EAd antibodies are not efficiently transferred across the placenta, resulting in low levels in the neonates. This suggests that the malaria-exposed neonates may be susceptible to earlier primary EBV infection due to the altered transplacental transport of antibodies. Although this study postulated that *in utero* malaria exposure would result in increased levels of pro-inflammatory cytokines in cord blood, this study reports similar levels of pro-inflammatory cytokines in malaria-exposed and -unexposed cord blood. In addition this study observed increased concentration of inflammatory cytokines in cord blood from mothers who have never been exposed to malaria suggesting that other maternal factors such as stress and diet that were not taken into account in this study could be modulating the cytokine responses. This study also demonstrated that maternal malaria infection during pregnancy results in increased frequencies of Treg cells in cord blood. Since Treg inhibit immune responses non-specifically, it is plausible that these Tregs may not only dampen immune responses to EBV but to other infections as well. Data from this study have demonstrated that alteration of some B-cell phenotypes in cord blood following in utero malaria exposure.

In addition, cord blood B-cells from malaria exposed neonates are more differentiated. On the other hand, majority of both $CD4^+$ and $CD8^+$ T-cells were naïve. Finally, the altered transplacental transfer of EBV-specific IgG antibodies, and the abnormalities in B-cell development and trafficking due to maternal malaria infection during pregnancy may be a possible explanation to the earlier primary EBV infection in children from malaria holoendemic regions.

6.2 Conclusions

1. Maternal malaria infection during pregnancy results in significant reduction in the transplacental transfer of anti-VCA and anti-EBNA1 antibodies to the neonate. Regardless of malaria exposure, anti-Zta and anti-EAd antibodies are not efficiently transferred across the placenta, resulting in low levels in the neonates. This may result in increased susceptibility of malaria-exposed neonates to early primary EBV infection.
2. Maternal malaria infection during pregnancy does not significantly alter the levels of pro-inflammatory and anti-inflammatory cytokines and chemokines in cord blood.
3. Maternal malaria infection during pregnancy results in increased frequencies of $CD4^+$Treg cells in cord blood. However, this study demonstrated no significant differences in the frequency of $CD8^+$ Treg cells in cord blood.
4. *In utero* exposure to malaria results in higher frequency of transitional B-cells and activated classical memory in malaria-exposed cord blood compared to malaria unexposed cord blood. Malaria unexposed cord blood had higher naïve B cells

compared to malaria-exposed cord blood. Cord blood T-cell homeostasis in is not affected by maternal malaria infection during pregnancy.

6.3 Recommendations From This Study

1. Since malaria exposed neonates may be at risk of early primary EBV infection due to reduced transfer of EBV-specific antibodies, efforts to control malaria in pregnancy should be up scaled in order to prevent damage to the placenta. Alternatively, although not yet commercially available, the neonates could be immunized against EBV.
2. Levels of pro-inflammatory cytokines may not be responsible for earlier primary EBV infection. Cytokine and chemokine signaling pathways that may be involved in EBV infection should be investigated.
3. Since *in utero* malaria exposure results in increased frequency of Treg cells in cord blood and the fact that Treg cells dampen the immune system non specifically, it should be determined if these infants are also susceptible to other childhood infections.
4. The demonstration of altered distribution of B-cells in cord blood indicate the need to carry out extensive research on B-cell subset distribution in children who are exposed to malaria *in utero* to better understand the generation of humoral immunity following immunization.

6.4 Recommendations For Future Studies

1. This study focused on the effect of maternal malaria infection on the transfer of total IgG against EBV and TT-specific antibodies, future studies on transplacental

transfer of these antibodies should focus on IgG sub-class, to determine which sub-classes are efficiently transported across the placenta.

2. Apart from maternal malaria infection during pregnancy, other maternal characteristics, the intra-uterine environment and individual genetic polymorphism that may influence cytokine expression should also be investigated.
3. Future studies should elucidate if the increased Treg frequencies in malaria exposed cord blood results in the poor control of EBV during primary infection. Moreover, it should be determined whether the increased Treg cells frequencies also alter the infant's immune responses to other infections.
4. Future prospective studies with larger sample size should be carried out to determine the effect of the altered B-cell phenotype on the development of infant immunity to EBV as well as other childhood infections. In addition, the functional significance of the altered phenotypes should also be investigated.

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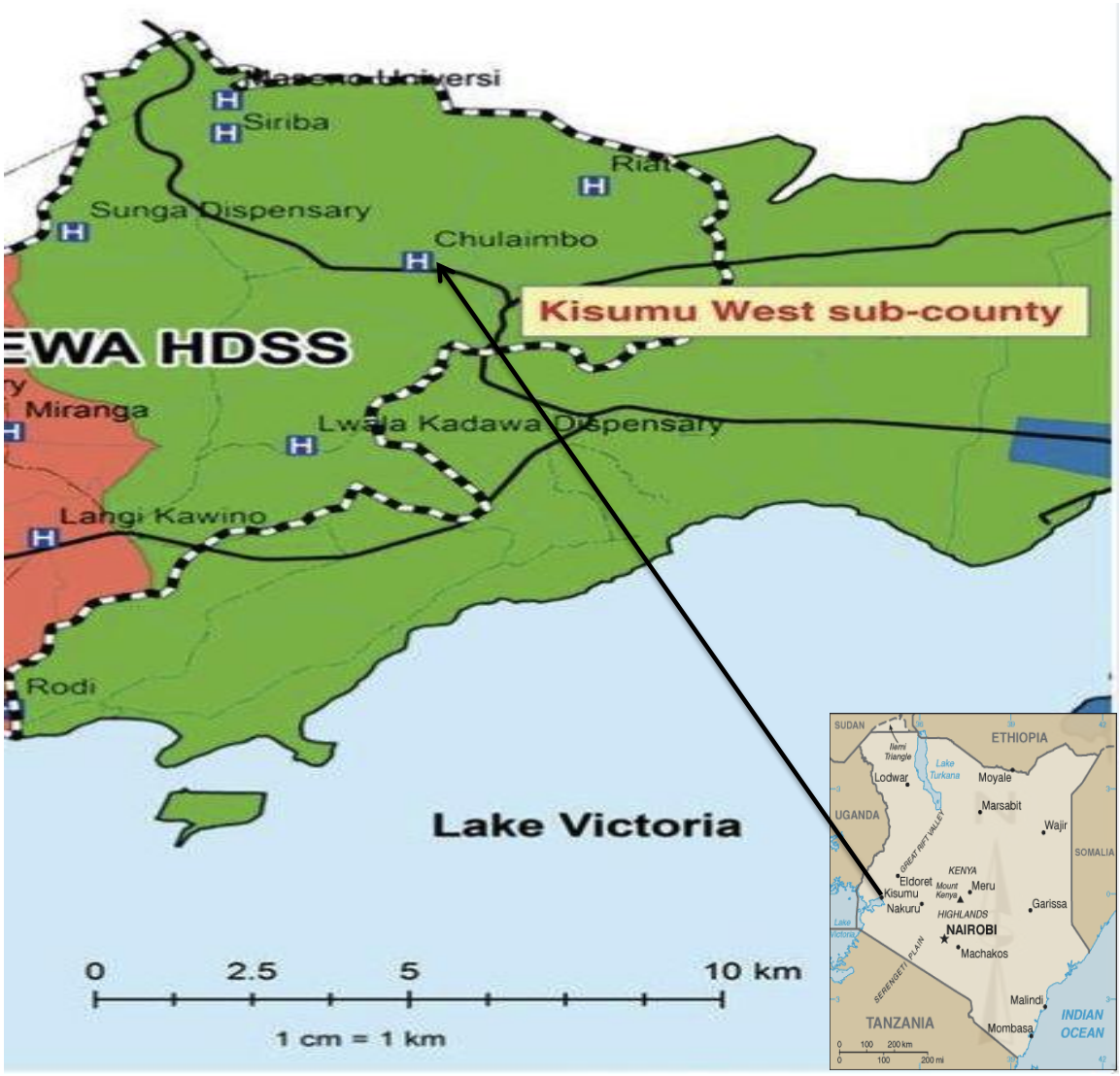
APPENDICES

Appendix 1: Map of Kenya showing the study area



Appendix 2: Primer sequence to detect a 70bp region of the EBV BALF5 gene

Forward 5′-CGGAAGCCCTCTGGACTTC-3′
Reverse 5′-CCCTGTTTATCCGATGGAATG-3′,

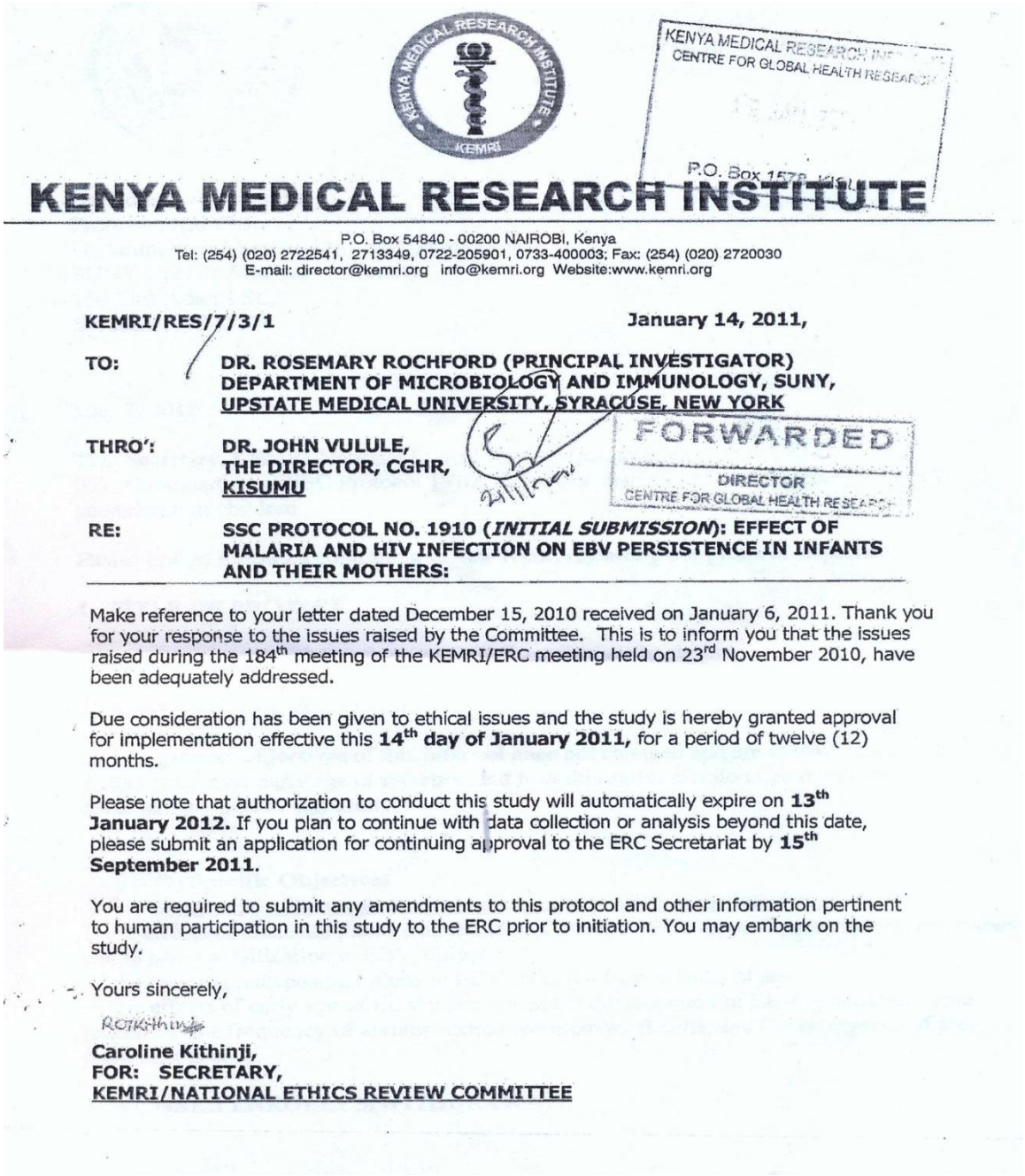
Appendix 3: Primer sequence to detect the *P. falciparum* 18s gene

Forward 5′- GTAATTGGAATGATAGGAATTTACAAGGT -3′
Reverse 5′-TCAACTACGAACGTTTTAACTGCAAC-3′,

Appendix 4: Staining panels used in the study

Panel 1(T-cell)	**Panel 2 (B-cell)**
CD3 PE-Cy7	CD10 Percp-Cy5.5
CD4 FITC	CD19 APC-Cy7
CD8 V500	CD21 FITC
CD25 Percp-Cy5.5	CD27 Pac Blue
Foxp3 PE	IgD PE-Cy7
CD45RA APC-Cy7	IgG PE
CD45RO APC	IgM APC
Live/Dead Aqua	Live/Dead Aqua

Appendix 5: Study approvals



KENYA MEDICAL RESEARCH INSTITUTE

KENYA MEDICAL RESEARCH INSTITUTE
CENTRE FOR GLOBAL HEALTH RESEARCH
P.O. Box 1578

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **January 14, 2011,**

TO: **DR. ROSEMARY ROCHFORD (PRINCIPAL INVESTIGATOR)**
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY, SUNY,
UPSTATE MEDICAL UNIVERSITY, SYRACUSE, NEW YORK

THRO': **DR. JOHN VULULE,**
THE DIRECTOR, CGHR,
KISUMU

FORWARDED
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

RE: **SSC PROTOCOL NO. 1910 (*INITIAL SUBMISSION*): EFFECT OF MALARIA AND HIV INFECTION ON EBV PERSISTENCE IN INFANTS AND THEIR MOTHERS:**

Make reference to your letter dated December 15, 2010 received on January 6, 2011. Thank you for your response to the issues raised by the Committee. This is to inform you that the issues raised during the 184th meeting of the KEMRI/ERC meeting held on 23rd November 2010, have been adequately addressed.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **14th day of January 2011,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **13th January 2012.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **15th September 2011.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

RCKithinji

Caroline Kithinji,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

Appendix 6: Consent form used in the study

SUNY Upstate Medical University Kenya Medical Research Institute Consent for Human Investigational Studies

Effects of malaria and HIV on EBV persistence in infants and their mothers

Study Participant Number: _____.

Background and Purpose:
Burkitt's lymphoma is a cancer that affects children and is thought to be caused by the presence of malaria and a virus called the Epstein Barr Virus (EBV). We know that children who live in malaria areas get EBV earlier in life than children that live in areas without malaria. These children are also likely to carry high levels of the virus. In this study we will look at the reasons why this is so.
Women who live in areas with high malaria such as Chulaimbo, when they become pregnant can get malaria parasites in their placenta. The presence of the parasite in the placenta has been known to cause many complications in babies after they are born. We think that mothers who suffer from malaria when they are pregnant may cause the higher levels of virus in their babies. The purpose of this study is to see if the presence of parasites in the placenta may result in babies getting high levels of EBV earlier in life and if this is also the reason that some children suffer from Burkitt's Lymphoma.
Dr. Rosemary Rochford from SUNY Upstate Medical University (SUNY Upstate) in Syracuse, USA and Dr Peter Odada Sumba at of the Kenya Medical Research Institute (KEMRI) are inviting you and your newborn child to participate in this research study.
We are approaching you at the antenatal clinic so that you may agree to participate in the study before your baby is born. If you agree to be in the study we will ask you to fill a form that will give us information about your pregnancy, where you live and when you are expected to deliver. When you come back to the hospital to deliver we will identify you as a study participant so that we may pay for your delivery costs.
We will ask for consent from you for your participation in the study. We will also ask you for consent for your newborn child to participate in this study.
Permission will be required from you for both you and your child. We are hoping to enroll 400 women and 400 infants into this study.

Study Procedures
1. Sample Collection:
In order to understand how strongly your child's body responds to malaria and to EBV, we have to know if you had malaria during your pregnancy and if your placenta has malaria parasites in it. We also want to know when your child is first infected with malaria and how many infections he/she will get and when he/she is infected with EBV. We will collect a small sample of blood from your finger today to test for malaria and also for some studies that will be carried out in our laboratories in Kisian. Each time that you come to the antenatal clinic, we will collect a small sample of blood from you. We will collect your placenta at delivery and will look for malaria parasites in it. We are also requesting for a teaspoon of blood from you once you are rested after your baby's

delivery, we will use a needle attached to a syringe to obtain the blood from your arm. We will also request a small blood sample from your child at delivery, which we will obtain from the umbilical cord.
After the birth of your baby, we will set up appointments for you to return to the clinic with your baby at 6, 10, 14, and 18 weeks of age. We will request to take blood samples from your baby at these appointments. A small amount of blood (a few drops), will be taken from your child's heel or finger. We will also schedule return visits to the clinic for when your baby is 6 months old, and at every 3-month interval to 24 months (2 yrs of age). Starting at the 6-month visit, we will request to take from your child a larger blood sample, equal to about 2 teaspoons. This sample will be drawn from a vein in your child's arm using a needle attached to a blood collection tube. It is important if you agree to be in this study that you will be around at least for the next year.
We will also collect from you breast milk and saliva samples to see if the EBV virus is in the milk and saliva and if this is how your baby gets infected. Breast milk and saliva will be collected at 6, 10, and 14 week visits. We will require about two teaspoons of breast milk and a teaspoon of saliva.
All samples will be transported to the SUNY Upstate/KEMRI laboratory in Kisumu. Tests done in the laboratory will tell us if you had malaria during pregnancy and when your child is infected with malaria and EBV and how your child's body protects him/her against malaria and against EBV. We will also carry out tests that will inform us of your child's immune system. We will monitor your child's growth every time we visit by measuring his/her height and weight.

2. Genetic studies:
We also would like to use part of your child's blood at the SUNY Upstate/KEMRI laboratory to do genetic studies. Genes are composed of the genetic material called
DNA. DNA is the part of the cell that is responsible for providing hereditary characteristics (such as eye color) and is used to build proteins. We would like to test his/her blood for genes including sickle cell trait, G6PD deficiency and HLA type that may protect against malaria. We may also test for other genes that are known to affect malaria and EBV. If we get any results from the lab studies that may affect your child's status with respect to malaria, we will inform you. We will also look at genes belonging to the malaria parasite and to EBV. This is important to see if your child has built defenses against different types of malaria parasites or EBV and similar viruses. Since the significance of the tests for changes in the malaria parasite or EBV is not known to you, we will not release the results of any genetic tests associated with the EBV and malaria parasite testing.
Do you accept for yours and your child's samples to be used for Genetic Studies?
YES_____ NO_____

3. Sample Storage:
Samples collected will be stored in the SUNY-KEMRI laboratories in Kisumu for the full duration of the study and the period required to analyze results. We may save some of your samples for many years to further study Burkitt's Lymphoma in the SUNY/KEMRI Labs in Kisian or in the labs of Dr Rochford at the SUNY Upstate Medical University. If at any time you wish to withdraw your agreement for us to save your and your child's

samples, please contact Dr. Peter Odada Sumba, the Research Project Manager, at KEMRI Kisumu Tel. 254-57-2022989 or 254-733-746854/254-720-766550 and we will destroy the samples.
Do you accept for yours and your child's samples to be stored and when necessary to be shipped to SUNY Upstate Medical University in the US for further investigations?
YES_____ NO_____

Risks:
There are few risks in having your blood taken from the placenta or your arm after delivery. There are also few risks from taking your breast milk and saliva. Minimal risks are associated with collecting your child's blood. The blood drawings include a little bleeding, pain, bruising and, rarely, infection. All of these are uncommon but may occur in very few people. We have never experienced these problems in our previous studies.
Answering questions during the antenatal enrolment interview will not cause any risk to you or your child.

Benefits:
We will be testing for malaria in your placenta, which is not normally done and is therefore a benefit. It is important to know if you had malaria in your placenta because it will tell us how your baby will fight off malaria and EBV later in life. Also as a benefit to your child, we will be making a visit every month to your home to collect samples at which time if your child is sick we will treat your child for fever, malaria, diarrhea and anemia according to the Kenya Ministry of Health Guidelines. Also, the information that we get from this study will be important for prevention programs for Burkitt's Lymphoma.

Alternatives:
You do not need to participate in this study to receive medical care for you and your baby. If you choose not to participate, you may still obtain normal delivery care at the hospital and medical care as provided by the Kenya Ministry of Health, including free testing and treatment for malaria.

Voluntary Participation:
Your participation in this research study is entirely voluntary. Refusing to participate will not alter your usual health care or involve any penalty or loss of benefits. If you decide to join the study, you may withdraw you and or your child at any time and for any reason.

Costs/ Payments:
We will pay for your delivery costs if you participate in this study. We will meet both bed and delivery fees**.** There are no additional costs to you or your child for participating in this study.
During your participation in the study, you and your child will receive free medical care and attention from the study assigned Clinical Officer for the duration of the study. If you withdraw from the study before you deliver, the study will not pay for your delivery costs. You will however be able to obtain normal delivery care at the hospital but the costs will be your responsibility. If you withdraw from the study after delivery, you will

not receive the free medical care and medication for study participants but you can still attend the clinic and receive services provided by the Ministry of Health. If you withdraw at any OTHER time, after our 1st home visit, your child will continue to receive free medical care from the study appointed clinical officer for the duration of the study period. Neither you nor your child will be paid for participating in this study.

Questions:
If you have any questions about this study you may speak to Dr. Peter Odada Sumba, the Research Program Manager, at the Center for Global Health Research, Kenya Medical Research Institute, PO Box 1578, Kisumu at 254-57-2022989 / 254-733-746854 / 254-720-766550 or to The Director of CGHR, KEMRI in Kisumu at 254-57-2022924. Queries pertaining to research subjects' rights may be made to the KEMRI/National Ethical Review Committee (ERC), PO Box 54840, Nairobi at 254- 2-02722541 or The Director of KEMRI, PO Box 54840, Nairobi at 254- 2-02722541.

In Case of Injury:
In the event of illness or physical injury resulting from taking part in this research study, please contact The Director of the Center for Global Health and Research (CGHR) at KEMRI in Kisumu at PO Box 1578, Kisumu 40100. Tel: 254-57-2022924 or Dr. Peter Odada Sumba at 254-57-2022989/ 254-733746854/254-720-766550
SUNY Upstate Medical University has no plans to give you money if you are injured. You have not waived any of your legal rights by signing this form.

Confidentiality of Records and Authorization to use/share protected health information for research: If you agree to participate in the study and if you allow your child to participate in this research, yours and your child's health information will be kept confidential. We will assign a number to you and your child that will appear on all the samples that we collect. Your names WILL NOT appear on any of these samples. This is being done to protect your and your child's medical information. There will be a few people that will have your name and numbers. These will be Dr. Rochford, Dr Odada Sumba and the study assistants. If for any reason your samples need to be shipped to the US for analysis, these samples will have only your study number and no names will be included. When we publish or present any of the findings from this study, your names will never be revealed. All information that we collect from you will be will be stored in locked cabinets at CGHR, KEMRI with only a few people in the Research Project, having access to these cabinets. This is also to ensure privacy and protection of your and your child's medical information.

Consent to participate in Research:
The nature and the purpose of the above research study have been explained to me. Signing below indicates that I have been informed about the research study in which I voluntarily agree to my participation and my child's participation. I have asked questions about the study and the information given to me has permitted me to make a fully informed and free decision about my and my child's participation in the study. By signing this consent form, I do not waive any legal rights, and the investigators are not relieved of any liability they may have. I can withdraw from this study at any time. A

copy of this consent form will be provided to me.
Signature of Parent_____ Date_____

Signature of Person Obtaining Consent/Authorization_____ Date _____

Signature of Witness_____ Date_____