EFFECTS OF MALARIA ENDEMICITY ON THE DEVELOPMENT OF IMMUNITY IN KENYAN CHILDREN

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ABSTRACT

CYNTHIA JOY SNIDER: Effects of Malaria Endemicity on the Development of Immunity in Kenyan Children
(Under the direction of Steve Meshnick, MD, PhD)

The heterogeneity of *Plasmodium falciparum (Pf-)* malaria endemicity affords an opportunity to explore the differential effects of *Pf*-malaria infections on the development of immunity. Focusing on two areas in western Kenya with disparate *Pf*-malaria transmission intensities, this dissertation 1) examined how recurrent *Pf*-malaria infections affected Epstein-Barr virus (EBV) lytic and latent antigen CD8+ T-cell IFN-γ response among EBV co-infected infected children, and 2) described the differential patterns of *Pf*-malaria antibody responses and how they waned over time. We analyzed data collected over a two-year time period from children residing in Kisumu (high malaria transmission) and Nandi (low malaria transmission). We observed a 46% decrease in the prevalence of positive EBV lytic antigen IFN-γ response among children living in the Kisumu when compared to Nandi (PR: 0.54; 95% CI: 0.30-0.99). Further analysis revealed impairment of EBV lytic antigen IFN-γ responses among 5-9 year olds. We did not identify any differences in *Pf*-malaria exposure and EBV latent antigen IFN-γ response. Results suggest there may be a loss of immunological control of the EBV lytic cycle among children repeatedly infected with *Pf*-malaria. Our second analysis on *Pf*-malaria antibody responses revealed that proportions of positive IgG responses to select blood-stage antigens (apical membrane antigens-1 3D7 and FVO strains) and the pre-erythrocytic liver

stage antigen-1 antigen were higher in Kisumu than Nandi ($P < .05$). There was a clear trend in the increase of IgG responses with age in Nandi but not in Kisumu where even the youngest age group had a high proportion of antibody responses. Overall, IgG responses waned over a six-month period in both districts. However the magnitude of the median relative change in antibody responses was generally greater in Nandi than Kisumu particularly among children 0-4 year olds to the antigens AMA-1 3D7, AMA-1 FVO, AND MSP-1_{42} 3D7 ($P < .05$). These findings indicate patterns of naturally acquired immunity evolve, and wane, differently as a result of heterogeneous *Pf*-malaria transmission intensities and age.

To the children and families of the Kisumu/Nandi cohort for sharing their experiences in an effort to make eBL an obsolete form of childhood cancer.

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AIDS	Acquired Immunodeficiency Syndrome
AMA-1	Apical Membrane Antigen - 1
CI	Confidence Interval
CIDR1α	Cysteine-rich Interdomain Region 1α
CTL	Cytotoxic T-lymphocyte
eBL	endemic Burkitt's lymphoma
EBNA	Epstein-Barr Nuclear Antigen
EBV	Epstein-Barr Virus
EMM	Effect Measure Modifier
EIR	Entomological Inoculation Rates
ELISPOT	Enzyme-linked Immunospot Assay
GEE	Generalized Estimating Equations
GLM	Generalized Linear Model
Hb	Hemoglobin (AS, SS = sickle cell trail; AA = not sickle cell trait)
HIV	Human Immunodeficiency Virus
IgG	Immunoglobulin
IFN-γ	Interferon-gamma
IRB	Institutional Review Board
LSA-1	Liver Stage Antigen – 1
LLPC	Long-lived Plasma Cells
LCL	Lymphoblastoid Cell Line
MHC	Major Histocompatibility Complex

MTI	Malaria Transmission Intensity
MSP-1	Merozoite Surface Protein - 1
MBC	Memory B-cells
NAI	Naturally Acquired Immunity
OR	Odds Ratio
PfEMP1	*Plasmodium falciparum* Erythrocyte Membrane Protein 1
Pf-malaria	*Plasmodium falciparum* malaria
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PHA	Phytohemmagglutinin
PR	Prevalence Ratio
RBC	Red Blood Cells
SLPC	Short-lived Plasma Cells
SES	Socioeconomic Status
SFU	Spot-forming Units
VCA	Viral-capsid Antigen

CHAPTER ONE: SPECIFIC AIMS

Malaria is one of the leading causes of morbidity and mortality around the world, causing an estimated 225 million illnesses in 2009, resulting in approximately 781,000 deaths.[1] The global burden disproportionately affects those living in sub-Saharan Africa where 78% of illnesses and 91% of deaths were reported.[1] Furthermore, children <5 years of age carry the highest burden of morbidity and mortality; globally 85% of deaths were in this age groups.[1]

Yet the morbidity and mortality estimates do not accurately convey the true burden of malaria because they do not take into account the consequences of co-infections such as *Pf*-malaria and Epstein - Barr virus (EBV). These two pathogens have been implicated in the development of endemic Burkitt's lymphoma (eBL).[2] eBL is the most common cancer among children in equatorial Africa and has been estimated to account for over 70% of childhood cancers in that region.[3, 4] EBV infection has been hypothesized to be the first step in the multi-step carcinogenesis of eBL. EBV causes a life-long infection in B-lymphocytes.[5] The second step in eBL carcinogenesis is believed to be recurrent *Pf*-malaria; however, the mechanism of its influence on EBV persistence is unclear. Studies have focused on establishing the relationship of each of these co-factors to eBL yet few studies have examined the interaction between these two pathogens.

What is clear is that the global burden of *Pf*-malaria infections reaches beyond the numbers. The incidence of malaria is decreasing in many parts of the world, but a need remains for an effective vaccine to supplement traditional control measures such as indoor residual spraying, insecticide treated bednets, and chemotherapy if reductions in morbidity and mortality are to be sustained.[6] Differential patterns of naturally acquired immunity exist between areas with varying levels of malaria transmission and this may be partially explained by the short lifespan of antibody responses. Studies have attempted to identify immunological markers of *Pf*-malaria infections in areas of high malaria transmission, but few studies have been conducted in areas of low malaria transmission.

We exploited the heterogeneous malaria transmission intensities of two areas in western Kenya to examine the issues we have raised. First, we investigated the role of recurrent *Pf*-malaria exposure on EBV-specific cellular immunity. Building upon cross-sectional ecological studies that have examined the effect of district-level *Pf*-malaria exposure on EBV infection, we incorporated additional time points to elucidate the longitudinal association of these two pathogens. We then explored the patterns of naturally acquired immunity in these two settings, describing the relative change in antibodies over time.

<u>Specific Aim 1.</u> To assess the effect of recurrent *Pf*-malaria exposure on Epstein Barr Virus latent and lytic antigen CD8+ T-cell IFN-γ responses over time.

Hypothesis: Recurrent *Pf*-malaria infections deplete EBV-specific CD8+ T-cell IFN-γ responses.

Overview: We used data collected from a cohort of children 10 months to 15 years of age residing in two areas of disparate malaria transmission intensities to examine changes in EBV-specific CD8+ T-cell lytic and latent responses over a two-year time period. We used a district- and individual-level definition of recurrent *Pf*-malaria to explore if malaria transmission intensity (district-level definition) is an adequate surrogate for individual-level *Pf*-malaria exposure.

Specific Aim 2.

a. To describe malaria antibody responses among children in malaria holoendemic and hypoendemic areas, contrasting any differences between the areas.

Hypothesis: Children residing in holoendemic areas should have higher levels of antibodies to blood-stage antigens than children residing in hypoendemic areas. In addition, antibody responses to blood-stage antigens in the holoendemic area should show an age-trend whereas antibodies should be relatively evenly distributed across age groups in the hypoendemic area.

b. To describe the relative change in malaria antibodies over time in malaria holoendemic and hypoendemic areas, highlighting any differences between the areas.

Hypothesis: Antibodies to blood-stage antigens are reportedly short-lived therefore antibodies should decrease over time. Yet children in holoendemic areas should have developed protective immunity such that the magnitude of their decrease should be less than children residing in malaria hypoendemic areas.

Overview: We used data from a cohort of children 10 months to 15 years of age residing in two areas characterized by heterogeneous malaria transmission intensities to describe the prevalence of positive antibody responses to select blood-stage antigens. We calculated the relative change in antibody responses over a six-month period to characterize the change over time.

CHAPTER TWO: BACKGROUND AND SIGNFICANCE

Areas of heterogeneous malaria transmission intensity (MTI) provide a natural setting to investigate the influence of malaria infection on the development of immunity in children. The unique geographic distribution of eBL cases in high *Pf*-malaria transmission areas of sub-Saharan Africa and Papua New Guinea led investigators to conclude that holoendemic *Pf*-malaria exposure played a role in the pathogenesis of eBL.[7-9] Furthermore, differential patterns of naturally acquired immunity (NAI) to severe malaria between populations exposed to varying levels of malaria have been observed in a number of studies.[10, 11] In this chapter, the role of *Pf*-malaria infection in the development of eBL and *Pf*-malaria humoral immunity will be reviewed after a general introduction to malaria.

Malaria

Malaria is a parasitic vectorborne-disease that led to approximately 225 million infections in 2009 resulting in an estimated 781,000 deaths, 85% of deaths in children < 5 years of age.[1] The greatest burden of morbidity and mortality is in sub-Saharan Africa where 78% of illnesses and 91% of deaths were reported.[1] In Kenya, the incidence of malaria is highest among children <5 years old and is the most common cause of morbidity and mortality in children <2 years old.[12]

There are five species of the *Plasmodium* parasite that infect humans; we will focus on *Plasmodium falciparum* which is responsible for the majority of human infections, including severe manifestations such as cerebral malaria, severe anemia, respiratory distress, and kidney failure.[13] *Plasmodium* parasites are restricted in geography to the distribution of the *Anopheles* vector.[13] Generally, these are areas where the temperatures ranges from 16-33^{o}C and the altitude is less than 2,000 meters.[14] Approximately 50% of the world's population live in malarious areas of sub-Saharan Africa, Asia, Central and South America, and the Pacific Islands (Figure 2.1).[13, 15] Areas of high malaria transmission are found predominantly in sub-Saharan Africa and Southeast Asia.[15]

The MTI depends on temperature, rainfall, vector, and the availability of infected humans. Historically, classification has been according to the proportion of children 2-9 years old with parasitemia or splenomegaly.[14] Hypoendemic ($\leq$10%) and mesoendemic (11-50%) areas have seasonal or unstable malaria transmission whereas hyperendemic (50-75%) and holoendemic (>75%) areas have stable malaria transmission.[14] In recent years, the use of entomological inoculations rates (EIR) has also been utilized to characterize MTI. Areas with an EIR < 10 have been used to describe areas of unstable and low transmission whereas areas with EIR $\geq$ 10 typically have high and stable transmission.[16]

Recurrent *Pf*-malaria and EBV Co-infection

It has been theorized that repeated infections with *Pf*-malaria adversely affects the immunological control of EBV persistent infection, leading to the development of

eBL.[2] Yet the mechanism of interaction between the two pathogens remains unknown. To appreciate why this is, an understanding of eBL and EBV is necessary. Evidence linking these two diseases will be discussed as well as evidence that links *Pf*-malaria infections to eBL. Discussion about how *Pf*-malaria infection could affect EBV persistence will be followed by what is currently known about the interaction of the two pathogens as it relates to CD8+ T-cell response.

Endemic Burkitt's Lymphoma

eBL is an aggressive B-cell non-Hodgkin's lymphoma in which tumors double in size within 24-48 hours.[17-19] eBL is ultimately caused by a chromosomal translocation of the c-*myc* oncogene on chromosome 8 with one of the heavy or light immunoglobulin (Ig) chains.[17, 20, 21] The c-*myc* proto-oncogene regulates cell apoptosis, differentiation and proliferation and the translocation of this gene leads to deregulation of vital cellular control.[17, 22] Translocation is believed to be due to a very rare occurrence during gene rearrangement or class switching.[23, 24]

eBL was first characterized in 1958 by Denis Burkitt when he published his observations on 32 Ugandan children between the ages of 2-14 who presented with a sarcoma of the jaw.[20] Additional findings and reports determined eBL was commonly observed in equatorial Africa (referred to as "The Lymphoma Belt") but not in northern or southern Africa (Figure 2.2).[25-28]

eBL is the most common cancer among children in Equatorial Africa where it is estimated to account for over 70% of childhood cancers (Figure 2.3).[17, 22] In Kenya alone, it accounts for 37-43% of childhood cancers.[12] The incidence of eBL

in children <15 years in high malarious areas ranges from 5-15 per 100,000 per total population per year.[17, 22, 25, 27, 29] eBL is commonly reported in children between the ages of 2-12 years with no cases reported in children <1 and few cases reported in 1 year-old children.[20, 21, 25, 30] In general, the peak age range is 4-8 years, while in Kenya the peak age is 6 years.[8, 12, 31, 32] Males are more often diagnosed with eBL than females with a sex ratio ranging from 1.6-3.2 males for every female diagnosed with eBL.[12, 25, 27, 32, 33] This observation cannot be explained as there are no apparent differences in admissions rates between males and females.[25]

Although an aggressive cancer, eBL responds well to chemotherapy.[8, 18, 26] Chemotherapeutic drugs are able to target tumor cells effectively as a result of the rapid cell proliferation.[26] In addition, if a portion of the tumor can be removed, either by surgery or chemotherapy, there is indication the patient's own immune system can control and eliminate the rest.[26]

The pathogenesis of eBL is hypothesized to involve EBV as the initiator and recurrent *Pf*-malaria as the promoter of EBV-infected B-lymphocyte proliferation, increasing the opportunity for a c-*myc* translocation, the hallmark of eBL.[2] Due to the limited geographic distribution of eBL in equatorial Africa, Burkitt initially suggested the cause of eBL was an "arthropod vectored-virus."[26] However, Dalldorf suggested in 1962 that two factors were responsible for eBL: 1) an agent found globally, and 2) an environmental factor such as *Pf*-malaria.[8] It was just two years later when Epstein, Achong, and Barr identified a new virus in electron micrographs taken of cultured eBL cells, later named the Epstein-Barr Virus (EBV).[34] In 1982, Klein synthesized contemporary research into a cohesive explanation of the three

stages of eBL evolution that still prevails today.[2] The first stage entails primary EBV infection; dé The and colleagues identified that pre-eBL children had significantly higher antibody titers to the EBV viral capsid antigen (VCA) than matched controls.[2, 27] Immunological modulation by recurrent *Pf*-malaria exposure is the second step in the process. *Pf*-malaria has been proposed to impair EBV-specific T-cell immunity[19, 35] and/or lead to proliferation of EBV-infected B-lymphocytes.[2, 19] This impairment and proliferation increases the probability of the occurrence of a c-*myc* translocation which leads to stage 3, the development of eBL.[2, 19, 22]

Studies of eBL etiology that directly examine the relationship between recurrent *Pf*-malaria and EBV are challenging. The relatively low incidence of eBL cases, 5-15 per 100,000 per total population per year depending on location, makes longitudinal cohort studies of eBL difficult to conduct.[17, 22, 25, 27, 29] A prospective seroepidemiological study was conducted over a two year span collecting samples from 42,000 children less than 9 years old, of which only 14 eBL cases were available for inclusion in analyses.[27] Furthermore, establishing temporality of exposure-outcome is an important concern for eBL case-control studies because there is no clearly established biomarker for the development of eBL. Likewise, there is no clear biomarker for the accumulation of repeated *Pf*-malaria infections. Furthermore, it is possible that EBV biomarkers (e.g., antibody titers, EBV load) assessed as exposures may in fact be the result of eBL disease (i.e., reverse causality).[33]

The only two studies assessing the joint effects of *Pf*-malaria and EBV among eBL cases showed an important interaction.[33, 36] Both case-control studies found the

odds of high antibody levels to both EBV and *Pf*-malaria in eBL cases to be significantly higher than controls with low antibody levels to both EBV and *Pf*-malaria.[33, 36] In one study, the odds ratio (OR) for the joint effect was 13.2 (95% confidence interval [CI]: 3.8-46.6) while the OR for *Pf*-malaria alone was 1.4 (95% CI: 0.3-6.3) and EBV alone was 5.7 (95% CI: 1.6-20.7).[36] In the other study, the OR for the joint effect of EBV and *Pf*-malaria was 5.0 (95% CI: 2.8-8.9) while the OR for *Pf*-malaria alone was 1.1 (95% CI: 0.5-2.4) and EBV alone was 1.0 (95% CI: 0.5-2.2).[33] Although OR estimates are imprecise, the magnitude of association and the synergistic effect indicates that the interaction of the two pathogens play an important role in the pathogenesis of eBL.

There is an important interaction between recurrent *Pf*-malaria and EBV yet the mechanism behind the interaction is not well understood. In the absence of eBL longitudinal studies that could focus on the interplay between recurrent *Pf*-malaria and EBV, other types of studies can provide insights.

Epstein-Barr Virus

Epidemiology of EBV

EBV is a ubiquitous virus that is transmitted orally through saliva.[5, 13] Over 90% of adults carry antibodies to EBV and young children often contract the virus from sharing toys and consuming foods pre-chewed by parents.[3, 5, 13, 37] EBV is shed continuously in the saliva of healthy EBV seropositive individuals although very few viruses are infectious.[38]

In developing countries, 99% of primary infections occur in children by the age of 3 years.[22, 39] Maternal antibodies to EBV offers protection in young infants until 5-7 months.[39] Infection in young children is asymptomatic and serological testing is often the only evidence of past infection.[40-42] In contrast, the age of primary infection in developed countries is delayed until adolescence or young adulthood of which 35-50% presents as symptomatic infectious mononucleosis.[5, 39, 43] The difference in age of primary infection is attributed to improved living conditions and hygiene in developed countries. [41, 44] There has been some evidence that females have higher anti-VCA immunoglobulin (IgG) titers than males, although there does not appear to be any difference in the age of primary infection by sex.[45, 46]

Infection, Persistence, and Immune Response

EBV is a double stranded DNA virus of the gamma herpes family that is B-lymphotrophic.[41] The location of viral replication is still debated but evidence suggests EBV infects epithelium cells and naïve B-lymphocytes of the oropharyngeal epithelium.[5, 19, 38, 47, 48] Similar to other herpes viruses, EBV has two life cycles: lytic (productive) and latent (non-productive).[41, 49] During lytic infection, the virus replicates within the epithelial cells and naïve B-lymphocytes, leading to cell death when virions are released.[41] The control of EBV proliferation during this phase is brought about by T-cell response, especially cytotoxic CD8+ T-lymphocytes (CTL).[50]

EBV causes a life-long latent infection in resting memory B-lymphocytes.[5, 19] A proportion of EBV-infected naïve B-lymphocytes escape the host's immune system by migrating to lymph nodes where they pass through the follicle to form germinal center reactions, colonizing the resting memory B-lymphocyte pool.[5, 19, 47] These EBV-infected memory B-lymphocytes contain viral genetic material but do not produce virions.[5] Latently infected memory B-lymphocytes are capable of polyclonal proliferation or activation to the lytic cycle.[41, 48] This latter shift occurs when latently-infected memory B-lymphocytes differentiate into plasma cells.[47, 51]

EBV primary infection leads to a strong T cell-mediated response which also dominates immunosurveillance and control of EBV latency.[22, 35, 50, 52] Natural killer cells of the innate immune system are the first to respond during primary infection. [22] This is quickly followed by CTL response which targets both lytic and latent viral proteins.[19, 35, 50] In healthy EBV seropositive individuals, EBV-specific memory CTL cells represent up to 5% of all circulating CTL cells, indicating the pivotal role of T-cell immunosurveillance in controlling EBV latent infection.[19, 22, 50] EBV is a powerful B-lymphocyte mitogen that leads to continued proliferation in vivo and transformation to immortalized lymphoblastoid cell lines (LCL) in vitro.[22, 42, 50, 53, 54] Failure to control latent infection leads to uncontrolled proliferation of EBV-infected B lymphocytes.[22]

EBV-infected B-lymphocytes evade T-cell immunosurveillance through a number of mechanisms: down-regulating expression of almost 100 viral genes to just 12 thereby rendering it non-immunogenic, reducing the expression of major histocompatibility complex (MHC) class I receptors on the cell surface to prevent

CTL recognition, producing viral cytokines that enhance B-lymphocyte infectivity and counteract immune response, and inhibiting apoptosis.[17, 37, 55-57]

After primary infection, EBV load is dependent on immunosurveillance of latent EBV infection. EBV load in healthy seropositive individuals is stable over time with 1-50 latently infected cells per million circulating B-lymphocytes.[55, 58] Increases in EBV loads may be a consequence of reduced T-cell control, as observed in post-transplantation patients and AIDS cases.[49, 59] However no point prevalence correlation has been observed between EBV loads and EBV-specific CD8+ T-cell IFN-γ response.[35] In addition, EBV DNA detected in plasma and serum is a combination of encapsidated and naked DNA, signifying active EBV replication and apoptosis, respectively.[48, 58, 60] Individuals with a mixture of encapsidated and naked EBV DNA have the highest EBV loads.[58]

Maintenance of EBV persistence is not well understood but is believed to be a balance between latent infection, replication (i.e., lytic phase), and the immune response.[48, 57, 59, 61] EBV-infected resting memory B-lymphocytes are a reservoir for persistent infection and the shift from latent infection to replication occurs when a portion of these latently-infected lymphocytes differentiate into plasma cells.[51, 56] The signal that triggers plasma cell differentiation is unknown.[56] EBV replication leads to viral shedding in the saliva and infection of naïve B-lymphocytes that migrate to the lymph nodes where they seed additional pools of uninfected resting memory B-lymphocytes.[48, 56] Replication elicits an immune response which once again brings the virus under control.[56, 57]

Relationship between EBV and eBL

EBV is a necessary but not a sufficient cause of eBL.[24] It is recognized that chromosomal translocation of the c-*myc* oncogene initiates the development of eBL, yet there is evidence to suggest EBV has an active role in its pathogenesis. First, EBV was discovered in electron micrographs of cultured eBL cells.[34] Subsequent tumor studies found approximately 97-98% of eBL tumors were positive for EBV. [2, 22, 50, 62] Second, eBL tumors develop from a cell (or cells) already infected with EBV, suggesting EBV infection occurs in the early stages of the pathogenesis of eBL rather than after tumor development.[24, 63] Third, individuals diagnosed with eBL had high anti-VCA titers prior to development of eBL.[41, 44, 64, 65] As anti-VCA titers are stable over time, the high titers before and after eBL onset may indicate suppression of EBV-specific T-cell immunity. Fourth, EBV leads to the transformation of normal B-lymphocytes to immortalized LCL in vitro; in vivo, EBV leads to proliferation of EBV-infected B-lymphocytes.[22, 42, 50, 53, 54] This proliferation increases the risk for eBL to emerge. Finally, EBV is linked to other cancers, such as nasopharyngeal carcinoma, and has demonstrated neoplastic growth in immunocompromised individuals suffering from B-lymphocyte lymphoproliferative disease.[24, 27]

Pf-malaria and EBV Co-infection

Chronic *Pf*-malaria is immune-modulating.[26] Studies of acute *Pf*-malaria infection during vaccination programs have found that *Pf*-malaria alters the capacity of children's immune systems to mount a response.[66-68] Immune competence rapidly

returns with parasite clearance indicating children with asymptomatic infection may still experience a degree of modulated immune response.[22, 67-71]

Malaria is a powerful B-lymphocyte mitogen. In vitro, the cysteine-rich interdomain region 1α (CIDR1α) domain of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is able to activate proliferation of B-lymphocytes from individuals with no previous malaria exposure.[72] It targets activation of memory B-lymphocytes and also provides protection against apoptosis.[73] A recent study demonstrated a direct link between CIDRα and EBV infected B-lymphocytes suggesting CIDRα could trigger reactivation of EBV-infected B-lymphocytes during acute malaria infection.[61] In areas of high malaria transmission, asymptomatic parasitemia may result in persistent proliferation of memory B-lymphocytes.[73]

Relationship between *Pf*-malaria and eBL

Pf-malaria is a cofactor in the pathogenesis of eBL (necessary but not sufficient). It is not *Pf*-malaria infection itself that is believed to be a cofactor but rather the prolonged exposure to *Pf*-malaria infection results in elevated risk of eBL development.[2, 29, 31, 32] The highest incidence of eBL has been identified in malaria holoendemic areas where early investigations noted the similarity in the geographical distribution of eBL and holoendemic malaria in equatorial Africa and Papua New Guinea.[26, 27, 30] For example, of the 1,005 eBL cases identified from 1988-1997 in Kenya, only 7% were from malaria free areas.[12] In addition, the introduction of antimalarials in areas of high malaria transmission led to a decrease

in the number of eBL cases thereby suggesting a link.[7, 32] Furthermore in holoendemic areas where control programs were instituted, eBL is rarely detected.[7]

The coincidence of *Pf*-malaria infection and eBL suggests a temporal relationship. Symptomatic *Pf*-malaria peaks in children aged 2-3 years while eBL peaks in 4-8 years.[74] Two additional interesting observations are noted in Burkitt's work in Uganda. First, the average age of eBL patients in malarious areas was 8.1 years, while in low risk malarious areas it was 16.2 years.[25] In addition, immigrants from unstable malaria transmission areas of Uganda who moved to holoendemic areas presented with eBL in late adolescents and adulthood, almost 50% over the age of 15.[25] Morrow's work also found a similar pattern as most adult cases of eBL that he identified were born in hypoendemic areas.[32]

Furthermore, there is also evidence that children with sickle cell trait (HbAS), which protects from severe *Pf*-malaria infections, have lower incidence of eBL than children without the trait (HbAA).[75] Finally, eBL cells appear to have their origin in germinal-cells and chronic *Pf*-malaria infection induces germinal center hyperactivity.[22, 24, 63] Viral reactivation coupled with expanded germinal centers increases the risk for a random mutation to occur in an EBV-infected germinal center lymphocyte.[24, 63]

Current Knowledge of the Effect of Recurrent *Pf*-malaria Infections on EBV Infection

This section focuses on current understanding of recurrent *Pf*-malaria infection and EBV T-cell response. A summary of studies can be found in Table 2.1.

An important criterion in establishing disease causality is that exposure occurs before disease. Recalling the chronology of the three stages of eBL progression – EBV infection, recurrent *Pf*-malaria, and eBL (i.e., c-*myc* translocation) – it is clear that there is the potential for chronic *Pf*-malaria to affect EBV latent infection, giving rise to eBL (Figure 2.4). As previously noted, 99% of primary EBV infections occur in children by the age of 3 years in developing countries.[22, 39] In holoendemic areas, symptomatic *Pf*-malaria infections peak in children aged 2-3 years, although infections continue into the teen years.[74] This would indicate children are already EBV seropositive when they experience repeated *Pf*-malaria infections in their early childhood. Recall that eBL peaks in children 4-8 years, soon after the peak age-related incidence for *Pf*-malaria infection.[74]

There are two prevailing, though not mutually exclusive, theories on the role of recurrent *Pf*-malaria on EBV infection and immunity. The first hypothesizes impaired EBV-specific T-cell immunity.[19] EBV-specific T-cell immunity is responsible for immunosurveillance and control of EBV latent and lytic proteins; however, chronic *Pf*-malaria may impair EBV-specific T-cell immunity, resulting in inadequate immunosurveillance of EBV. [19, 35] The second theory hypothesizes that EBV-infected B-lymphocytes proliferate as an individual is repeatedly exposed to *Pf*-malaria.[19] The increased number of EBV-infected B-lymphocytes thereby increases the risk for the emergence of an EBV-infected B-lymphocyte with the malignant mutation, leading to the development of eBL.[19]

Acute *Pf*-malaria infection leads to impaired T-cell control of persistent EBV infection. Studies comparing children's T-cell response during and 3-4 weeks after

Pf-malaria infection found a significant increase in the number of EBV-infected B-lymphocytes and high regression indices, both supporting the conclusion of a loss of CTL function.[76-78] By nature of these studies, it is clear that acute *Pf*-malaria infections have a transient effect on T-cell response. Furthermore, findings from case-control studies comparing acutely infected *Pf*-malaria individuals with healthy adults came to the same conclusion.[69, 79]

The cumulative effect of *Pf*-malaria infections on T-cell control of persistent EBV infection has not been well researched. Two ecological studies comparing holoendemic areas to areas of unstable malaria transmission have been conducted yielding different conclusions. One study found a significant difference in T-cell function between the two areas, with individuals living in holoendemic areas showing a loss of T-cell response.[80] However this study was conducted among adults who have mature immune systems as compared to children. A more recent study among children found no overall difference between the two areas; however, among children residing in the holoendemic area, a significant decrease in T-cell response was observed in children 5-9 years old compared to other age groups.[35] Although using malaria transmission as a surrogate for cumulative *Pf*-malaria infections is not ideal, these findings provide the only understanding we currently have on the cumulative effect of *Pf*-malaria exposure on latent EBV infection.

Naturally Acquired Immunity to *Pf*-malaria Infection

Unlike other infectious diseases, such as measles, life-long protective immunity (i.e., sterilizing immunity) never develops to *Pf*-malaria and individuals remain vulnerable

to re-infection throughout life.[16, 81] Yet partial immunity is developed over time whereby individuals progress from symptomatic illness with high parasite densities to asymptomatic illness and low density parasitemia.[16]

To understand the complex weave of interactions between the parasite and immunity in the human host, an understanding of the parasite life cycle and the critical role of humoral immunity is necessary. This will set the foundation for the description of how NAI evolves and how the longevity of antibodies affects NAI. Finally, we will review how NAI is influenced by heterogeneous patterns of transmission and age and the implications this has for protection from symptomatic, or clinical, disease.

Life Cycle of *Pf*-malaria

Pf-malaria parasites require two hosts as part of their life cycle, female *Anopheles* mosquitoes and humans, but our focus will be on the lifecycle in the human host (Figure 2.5).[82, 83] When an infected female mosquito bites a human host, she releases parasites called sporozoites into the bloodstream where they rapidly invade hepatocytes, or liver cells.[82, 83] These parasites mature in liver cells during this pre-erythrocytic, or liver stage.[82, 83] Upon rupture, merozoites (extracellular form of the parasite) are released and invade erythrocytes, or red blood cells (RBCs). Invasion of the RBC begins with a reversible attachment between the merozoite and RBC (Figure 2.6).[82, 84, 85] The merozoite then reorients its apical end to bring it in contact with the RBC where an irreversible, tight attachment is formed.[82, 84, 85] It enters the RBC surrounded by a protective vacuole that seals once the merozoite is fully

integrated into the RBC[82, 85] where they undergo asexual replication (referred to as erythrocytic or blood stage).[83] Most parasites in the blood stage continue in asexual replication, infecting more RBCs when merozoites are released.[82, 83] A small proportion of parasites do not return to asexual replication but enter the sexual stage where they differentiate into male or female gametocytes.[82, 83] Gametocytes are ingested by mosquitoes to continue their lifecycle in mosquitos.[83, 86]

Symptomatic infection in the human host is restricted to the asexual blood-stage when merozoites replicate in RBCs and are released into the bloodstream.[86, 87] This release of merozoites leads to inflammatory responses that cause the hallmark malaria symptoms of fever, rigor, and nausea.[86, 87] Parasites of the liver stage and gametocytes do not cause symptomatic illness and this has been hypothesized to be due to their poor immunogenic capacity.[6]

Humoral Immunity to *Pf*-malaria Infection

Studies in the early 1960's demonstrated that gamma globulin from healthy malaria-immune adults were effective in reducing the parasite density and clinical symptoms of malaria in children hospitalized with malaria.[88-90] These studies established the critical role of antibodies to blood-stage antigens as a significant component in NAI to malaria infections. The mechanisms by which antibodies can control *Pf*-malaria infection include direct elimination of parasites, prevention of merozoite invasion of RBCs, and aiding in the detection and clearance of infected RBCs.[82, 91-93]

Blood-stage antigens of particular interest are the apical membrane antigen (AMA)-1 and merozoite surface protein (MSP)-1 and both have been the focus of vaccine development. AMA-1 is a highly polymorphic antigen[82] and is highly immunogenic.[94, 95] Antibodies to AMA-1 antibodies have been shown to prevent reorientation of the merozoite on its apical end thereby preventing the critical attachment necessary for invasion.[85] Recently, AMA-1 was also found to be expressed by sporozoites of the pre-erythrocytic stage. Once again, AMA-1 appears to play a vital role in cell invasion because antibodies to AMA-1 prevented invasion of hepatocytes.[96] Like AMA-1, MSP-1 is a highly polymorphic antigen but it is not as immunogenic as AMA-1.[95 97] MSP-1 coats the surface of merozoites but its precise function of remains unknown.[84] It is processed into a number of fragments including MSP-$1_{42.}$ and during invasion of the RBC, MSP-$1_{42.}$ is further processed into MSP-1_{19} and MSP-1_{33}.[91] Antibodies to MSP-1 have been shown to prevent merozoite invasion of RBCs.[82, 98] In addition, animal studies demonstrated that antibodies to MSP-1 protected primates from infection.[97]

Sporozoites of the pre-erythrocytic stage are reportedly poorly immunogenic because they have less antigenic polymorphisms, indicating less pressure from the host's immune system.[6] Yet antibodies to sporozoites have been detected suggesting a potentially contributory role in NAI.[6] The suggested mechanisms of protection are similar to those proposed for blood stage antibodies - direct elimination of sporozoites, prevention of sporozoite invasion of hepatocytes, and clearance of infected hepatocytes.[93] Levels of pre-erythrocytic antibodies, such as the liver stage antigen (LSA)-1, have been reported to appear only after many years

of *Pf*-malaria exposure,[6, 93] although one study reported LSA-1 IgG levels in infants.[99] However, IgG responses in children are reportedly lower than adults.[100] Even though IgG responses to pre-erythrocytic antigens have been observed to be consistently lower than levels of blood-stage antibodies,[6, 93] comparisons between *Pf*-malaria immune and naïve individuals suggest that antibodies confer a degree of immunity against parasites.[6] Furthermore, significantly higher levels of LSA-1 antibodies were detected in high malaria transmission areas as compared to low areas indicating they may be involved in some level of protection.[101]

Evolution of NAI

Susceptibility to malaria is universal barring specific genetic traits that offer partial resistance to *Pf*-malaria infection.[13, 87] Individuals with heterozygous sickle cell trait (HbAS) have partial resistance to *Pf*-malaria, leading to reduced parasite density and lower risks for severe forms of malaria whereas individuals homozygous for the trait (HbSS) appear to be at greater risk for severe disease.[13, 87] Glucose-6-phosphate dehydrogenase deficiency is another genetic trait that protects individuals from severe manifestations of *Pf*-malaria infection.[102]

The development of NAI is a complex process that occurs between the *Pf*-malaria parasites and human host. Parasites incorporate various techniques to evade, and even suppress, the human host's immune response. The life cycle of parasites encompasses a diverse array of expressed antigens. Furthermore, parasites have developed polymorphic regions on antigens believed to be the result of frequent targeting by the host's immune response.[6, 82, 91, 93, 103] Allelic

polymorphisms have led to different genotypes, or strains, within a single species, such as 3D7 and FVO.[16] The predilection of parasites for liver and RBCs with limited or no major histocompatibility complex molecules means an efficient T-cell response cannot be mounted.[6, 82, 92] Immune suppression strategies include malaria-specific B- and T-cell apoptosis (cell-death) and the prevention of dendritic cells from maturing thereby preventing antigen recognition and presentation to T-cells.[6, 103, 104]

In this web of interactions with parasites, humans have evolved a complex immune response that manages to transform symptomatic illness to asymptomatic control of parasite density.[16] This response has been described in three phases (Figure 2.7). During the first phase, immunity to severe, life threatening infections is acquired after just a few infections despite an increasing prevalence of parasites.[81, 93, 105] The next phase brings about clinical, or anti-disease, immunity whereby individuals no longer suffer from symptomatic illness in spite of relatively high parasite densities.[16, 81] Acquisition of partial immunity to parasite immunity (anti-parasite) is the final phase, where parasitemia density remains low throughout infection.[16, 81]

The mechanisms responsible for this slow evolution of immunity are not well understood and should not be assumed to be similar for all phases.[93] It has been reasoned that maturation of immunity requires years in order to be exposed to variants of each antigen, although there is evidence of partial immunity to heterologous strains in the form of lower parasite densities and shortened courses of symptomatic illness.[16, 93] Symptomatic illness has been purported to be the result of

an individual being exposed to a novel variant in which there was no prior exposure.[6, 106] As individuals experience repeated *Pf*-malaria infections, they accumulate a sufficient antibody repertoire such that they no longer suffer from symptomatic illness but continue to carry parasites.[16, 93] Described in the 1900's by Koch as "premunition," low levels of parasites was believed to be necessary for maintaining immunity; interruption led to a loss in immune response.[16, 81]

Longevity of Antibodies

The loss of immunity has been attributed to the short lifespan of serum antibodies to *Pf*-malaria antigens[107, 108] but this may not be universal nor this simple. Naïve B-cells that encounter merozoites differentiate into short-lived plasma cells (SLPCs), secreting over 5,000 IgG molecules per second.[109] SLPCs have been estimated to survive between 8 hours to as long as 10 days.[109] Meanwhile some naïve B-cells make their way into germinal centers of follicles in the lymph nodes where they develop into high affinity antibodies, either differentiated B-cells called long-lived plasma cells (LLPCs) or memory B-cells (MBCs).[109-112] An estimated 80-90% of LLPCs are sequestered in the bone marrow where they continuously secrete antibodies, maintaining serum antibody levels even in the absence of infection; their lifespan in humans is unknown.[109-113] SLPCs and LLPCs are vital for the initial control in infection.[109-111] MBCs are critical for rapid secondary responses, having a low threshold for activation but peaking 6-8 days after re-exposure to their cognate antigen.[109, 111]

A hallmark of the immune response is the ability to recognize antigens that have already encountered. A lack of recognition therefore implies an absence of memory. The immune response to *Pf*-malaria infections suggests an absence of memory because people become repeatedly infected and ill. Therefore attempts have been made to delineate the contribution of LLPCs and MBCs to serum antibody levels but conclusions have been conflicting.[92] One study concluded there was a defect in MBCs to specific *Pf*-malaria antigens,[98] but another study reported slow and steady acquisition of MBCs to the same *Pf*-malaria antigens investigated in the first study.[111] Once again, the hurdle in developing long-term memory may be the challenges created by the *Pf*-malaria parasite whereby appropriate, high affinity antibody responses cannot be developed due to an ever changing repertoire of antigens on the merozoites.[109, 111] Although the activation of MBCs is typically antigen-dependent, there is evidence of antigen-independent activation (referred to as bystander activation) of *Pf*-malaria-specific MBCs antibodies.[109-111, 114]

Evidence arguing for the short lifespan of serum antibodies is numerous. First, high levels of antibody responses have been associated with parasitemia,[97, 98, 109, 115, 116] but antibody responses declined after symptomatic infection ceased[97] or once effective treatment commenced.[6, 97] In areas that experience seasonal transmission of *Pf*-malaria, studies have found that antibodies wane over time from the high to low season.[6, 100, 107, 117, 118] Furthermore, a commonly cited situation is when adults return to malaria endemic areas and suffer from symptomatic infection despite repeated *Pf*-malaria infection during their childhood.[92] Studies have reported that antibody responses to MSP-1 and other antigens are short-lived in infants[119, 120]

and children compared to adults.[92, 109, 117] Yet these differences may be due to the maturity of immune systems in adults compared to infants and children.

Studies that have estimated the half-lives of antibodies have been limited and inconsistent. IgG1 responses were reported to have a half-life of 21 days.[108] However, another study that examined IgG1 responses specific to Pf-malaria IgG responses (combination of blood-stage antigens including MSP-1 and AMA-1) reported IgG1 responses had a half-life of 10 days.[107] Still another study determined the half-life of MSP-1_{19} responses was 49 years.[94] The contradictory findings may be attributed to different antigens tested but nevertheless, the half-life of antibodies remains an open area for research and discussion.

What is likely a more accurate description about the longevity of antibody responses is that there is a careful and coordinated balance between both short- and long-lived responses whereby clinical immunity develops rather than parasite immunity.[109] The short life-span of antibodies is not indicative of a lack of immunological memory especially because rapid proliferation of antibody responses have been observed after exposure.[6] In addition, regardless of the MTI, only a few *Pf*-malaria infections may be necessary to protect from severe, non-cerebral malaria.[105] Reexamining the example of adults returning to malaria endemic areas, what is often overlooked is that their course of illness is often shorter in duration and their risk of severe forms are less than immune naïve individuals.[6] For example, in Madagascar after the unraveling of intense malaria control efforts from previous decades, a 1987 malaria outbreak left young adults and children with high proportions of symptomatic illness whereas older adults who were exposed to

repeated *Pf*-malaria infections as children suffered from less disease.[121] Hence antibodies do persist in the absence of antigens that stimulate an immune response.[109]

Malaria Transmission Intensity and Age Effect on the Evolution of Immunity

The patterns of NAI in individuals vary by malaria transmission intensity and age with the exception of infection in infants. Maternal antibodies appear to provide protection among infants from fever, high density parasitemia, and severe illness until they are about 6 months old when protection begins to wane.[16, 87, 99, 102, 122, 123]

In areas of stable and high malaria transmission, symptomatic *Pf*-malaria infection, including severe forms, disproportionately affect children ≤ 3 years old with monthly parasite prevalence among this age group reported to be as high as 90%.[10, 11, 87, 119, 122-125] At 6 months of age, infants become susceptible to infection.[16, 87, 102, 122, 123] Parasite densities were observed to peak in infants 6-11 months followed by a rapid decline with age.[122, 126] There is a subsequent decline in symptomatic illness and eventual decrease in parasite density with age, which is attributed to the accumulation of antibodies in response to repeated infection.[10, 122] This same pattern of acquired immunity is also observed for adults who move from low to high malaria endemicity areas.[10, 16]

Immunity in individuals residing in areas of low malaria transmission is not well as developed as demonstrated by the burden of symptomatic *Pf*-malaria infections being distributed across all age ranges while severe *Pf*-malaria infections

typically is reported in 1-5 year olds.[10, 11, 14, 16, 123, 125, 126] High febrile illnesses among all ages have been reported during periodic outbreaks.[6]

Studies of antibody responses have generally found IgG responses to AMA-1,[94, 95, 101] MSP-1_{19},[94, 95, 101, 127, 128] and LSA-1 [94, 101, 129] to be greater in high transmission areas as compared to low areas. In high transmission areas, they have also shown that the prevalence of AMA-1[95, 101, 119, 130] including AMA-1 FVO[131], MSP-1_{19}[95, 120, 128, 132, 133], MSP-1_{42}[130], and LSA-1[99-101, 119] antibodies increase with age but not always for MSP-1_{19}[134, 135] or LSA-1[130, 136]. Limited studies have been conducted in areas of low malaria transmission and these have found that AMA-1[95, 101] and MSP-1_{19} [95, 101, 128] increased with age but not consistently for AMA-1 FVO[131] or MSP-1_{19}.[134, 137] LSA-1 responses were significantly higher in adults in one study[118] but another study detected no age trend.[101] An interesting observation was that symptomatic illness was not found to be correlated with parasite density in areas of high malaria transmission whereas in areas of low transmission, there was a clear correlation.[16]

There is evidence that age may be independently related to the NAI although teasing out its independent effect in areas of high malaria transmission has been problematic. Adults who move into high malaria transmission areas suffer from symptomatic illness yet they develop the ability to prevent fever and control parasitemia faster than children.[16] Furthermore, adults are at greater risk for severe illness as compared to children as MTI declines.[16, 138] The risk of cerebral malaria is low in all children < 5 years old regardless of MTI, but in areas of low- or moderate-transmission, there is a J-shaped association with increasing age, whereas in areas

of high transmission, risk of cerebral malaria remains low.[138] The initial decrease in the J-shape was hypothesized to be due to the acquisition of immunity to severe malaria among young infants.[105, 138]

Evidence of Clinical Protection from *Pf*-malaria Infection

Efforts have been underway to develop of an effective vaccine for *Pf*-malaria infection. There is growing evidence that a multivalent vaccine that can elicit an immune response to a number of antigens will be necessary.[82, 93, 103, 119] As previously mentioned, the vaccine will have to overcome the vast array of evasive strategies utilized by parasites. To compound this problem, a further challenge has been identifying antibodies that are immunological markers of protection.[92] Few studies have examined the protective effect of antibodies to multiple antigens.[136, 139] What complicates research further is that less than <1% of known antigens have been studied thus far.[92] Prioritizing antigens for investigation has been hindered by the lack of knowledge on the precise function of a majority of the known antigens.[82] Finally, most studies have been conducted in areas of stable malaria transmission but patterns of NAI vary across MTI. Therefore it is unclear if the mechanism driving NAI in these areas are relevant to individuals infrequently exposed to *Pf*-malaria.

Studies of protection from symptomatic *Pf*-malaria infection in sub-Saharan Africa have generally found little evidence of protection associated with AMA-1. Only one study found that AMA-1 antibodies were protective[140] and another determined a combination of AMA-1 and MSP-2 antibodies reduced the risk of symptomatic *Pf*-malaria infection.[141] Still the majority of studies indicated no association.[127, 132, 136,

[142] Evidence of a protective effect of MSP-1_{19} antibodies is inconsistent. Although positive associations have been detected[127, 128, 133, 137, 141] there have also been reports of no association.[132, 134, 135, 140, 142] Limited studies on 3D7 and FVO strains have generally suggested antibodies to AMA-1 3D7,[130, 131] AMA-1 FVO[131], and MSP-1 3D7[130] are protective of symptomatic *Pf*-malaria infection, but antibodies to MSP-1 FVO are not.[130] Finally, the MSP-1_{19} block 2 has been linked with protection from symptomatic illness.[143-145]

A recent meta-analysis of population-based longitudinal studies generally corroborated these findings between AMA-1 and MSP-1 and protection from symptomatic *Pf*-malaria infection.[91] Antibodies to the 3D7 strain of AMA-1 reduced the risk of symptomatic illness whereas results for the FVO strain of AMA-1 were not as conclusive.[91] Antibodies to MSP-1_{19} led to an 18% decrease in the risk of symptomatic illness among individuals with detectable IgG responses as compared to non-responders.[91] Further analysis revealed that there was a 15% decline in the risk of symptomatic illness for every doubling of antibody responses (log base 2).[91] Studies of MSP-1_{42} were not reported.

LSA-1 antibodies independent of other pre-erythrocytic antigens does not appear to confer clinical immunity.[129] However, in conjunction with other pre-erythrocytic antigens, LSA-1 antibodies have been reported to be protective of symptomatic illness.[136, 139]

Summary

The burden of *Pf*-malaria infections is not independent of other infectious diseases circulating in a community. The repeated interaction of *Pf*-malaria parasites with persistent EBV infections can lead to eBL but the precise mechanism of interaction is shrouded in uncertainty. Two critical components to improve our understanding of the interaction between these two pathogens are 1) the use of an individual-level definition of *Pf*-malaria that accounts for recurrent *Pf*-malaria rather than acute, and 2) a study time period that allows for an examination of EBV persistence and immunity in response to recurrent *Pf*-malaria exposure. This dissertation aimed to incorporate these two missing components to gain further insight on the interplay of these two co-factors of eBL.

The disproportionate burden of *Pf*-malaria infections in sub-Saharan Africa, particularly among children, justified on-going research and treatment to ensure a continuing reduction in the rate of illness. Vaccine trials are underway in the hopes of creating an effective addition to current malaria control strategies. Yet could differences in antibody responses between areas of disparate malaria transmission intensities affect the effectiveness of vaccines? Furthermore the reported short lifespan of antibodies presents challenges in the development of long-lasting immunity. This dissertation aimed to improve the understanding of the differential patterns of immunity development between areas of heterogeneous malaria transmission intensities, including the decay of immune responses over time.

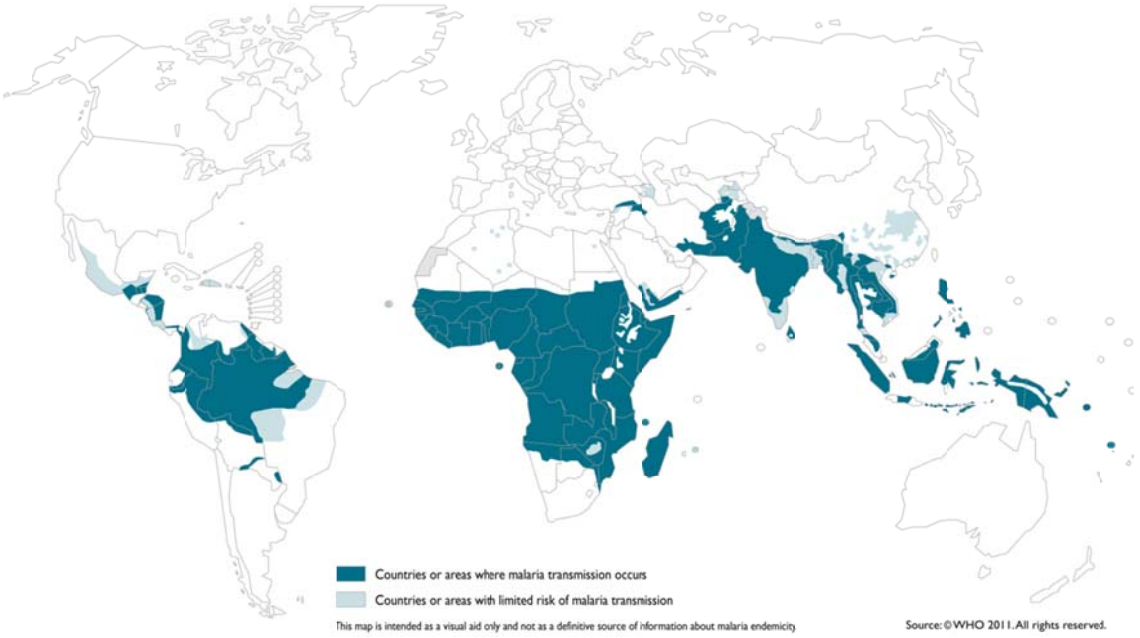


FIGURE 2.1. The geographic distribution of malaria risk, 2010.[146] Reproduced from the World Health Organization.

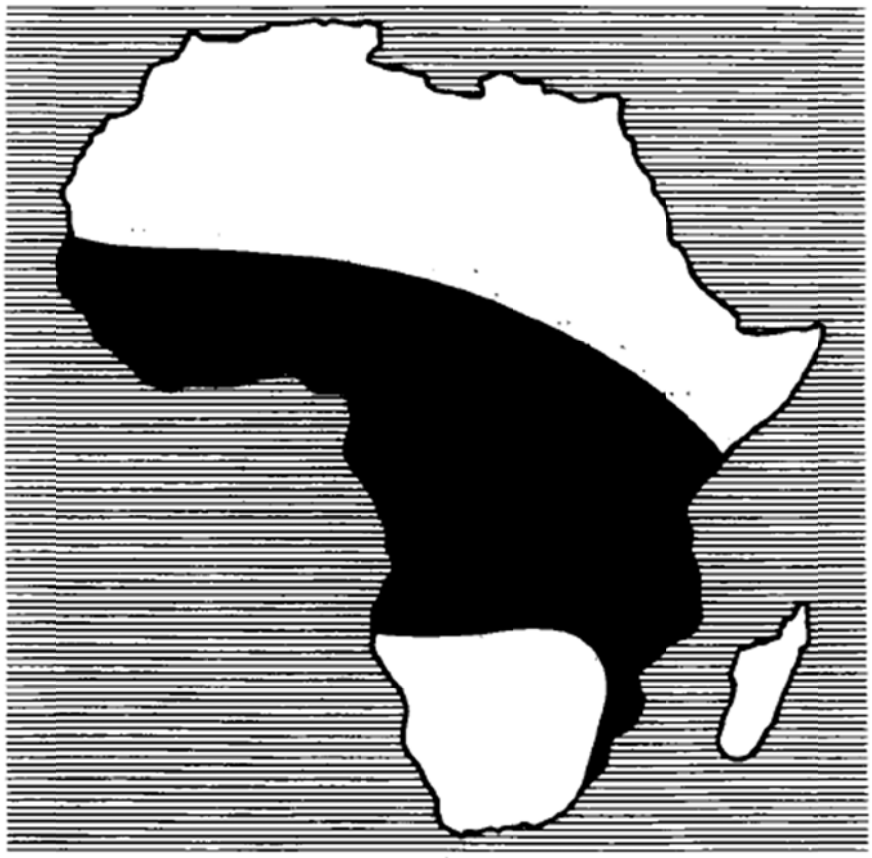


FIGURE 2.2. The lymphoma belt of Africa showing the approximate distribution of tumors. Adapted by permission from John Wiley and Sons: Cancer 51(10):1777-1786 ©1983.

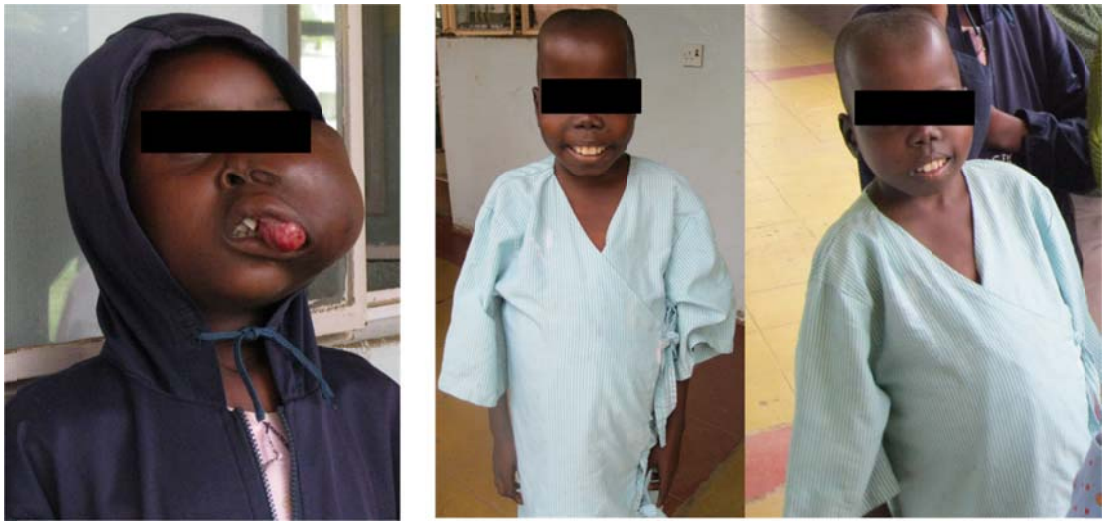


FIGURE 2.3. Images of a girl and boy with eBL tumor manifestations in different areas of the body. Photo on the left is a girl with a mandibular tumor. Photo in the center and right are of the same boy with an abdominal tumor.

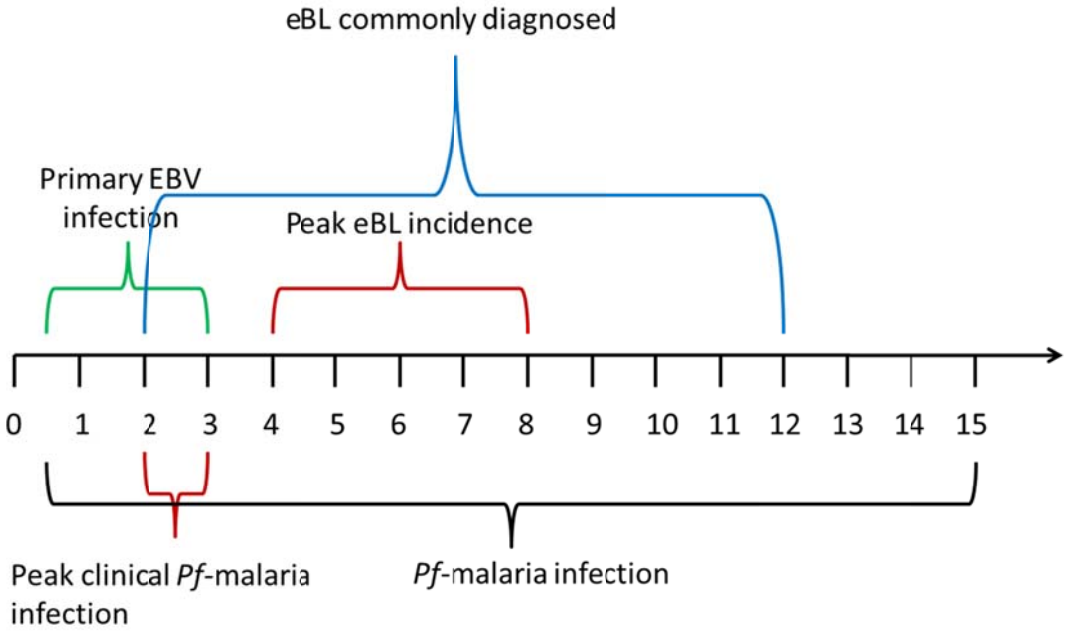


FIGURE 2.4. Chronology of proposed events in the carcinogenesis of eBL.

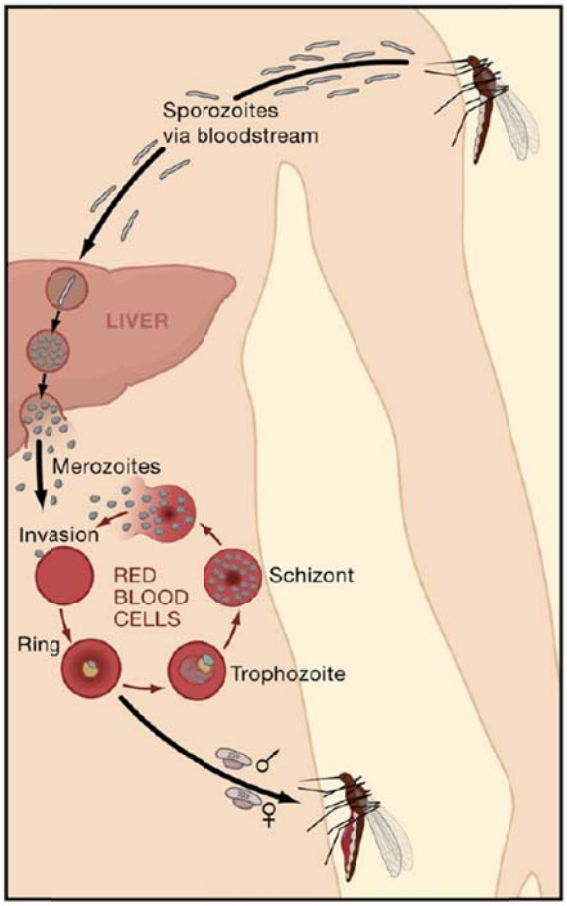


FIGURE 2.5. The lifecycle of the malaria parasite. Reprinted from Cell, 124(4), Cowman AF, Crabb BS, Invasion of red blood cells by malaria parasites, 755-766, ©2006, with permission from Elsevier.

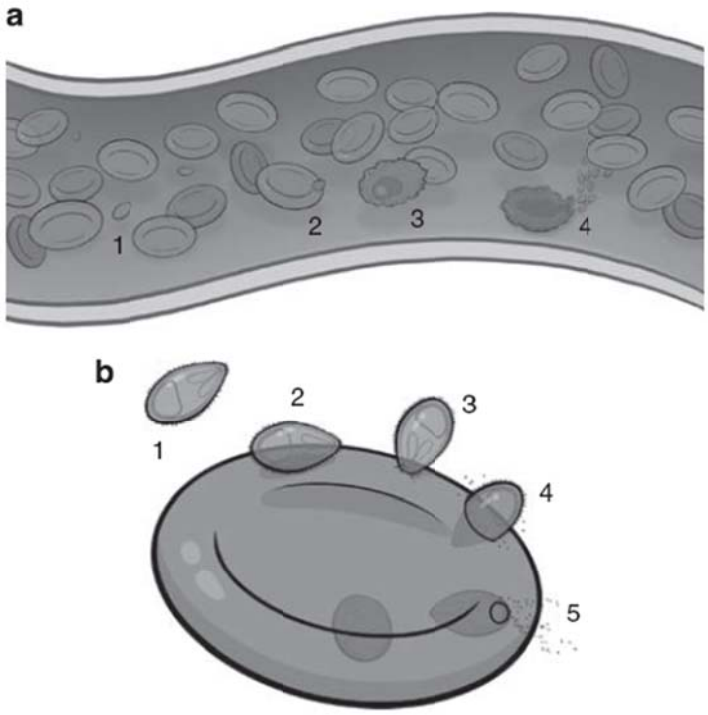


FIGURE 2.6. The blood-stage lifecycle of Plasmodium. (a) Depiction of the lifecycle. (b) Merozoite invasion of a red blood cell. Adapted by permission from Macmillan Publishers Ltd: Immunology and Cell Biology 87(5):377-390 ©2009

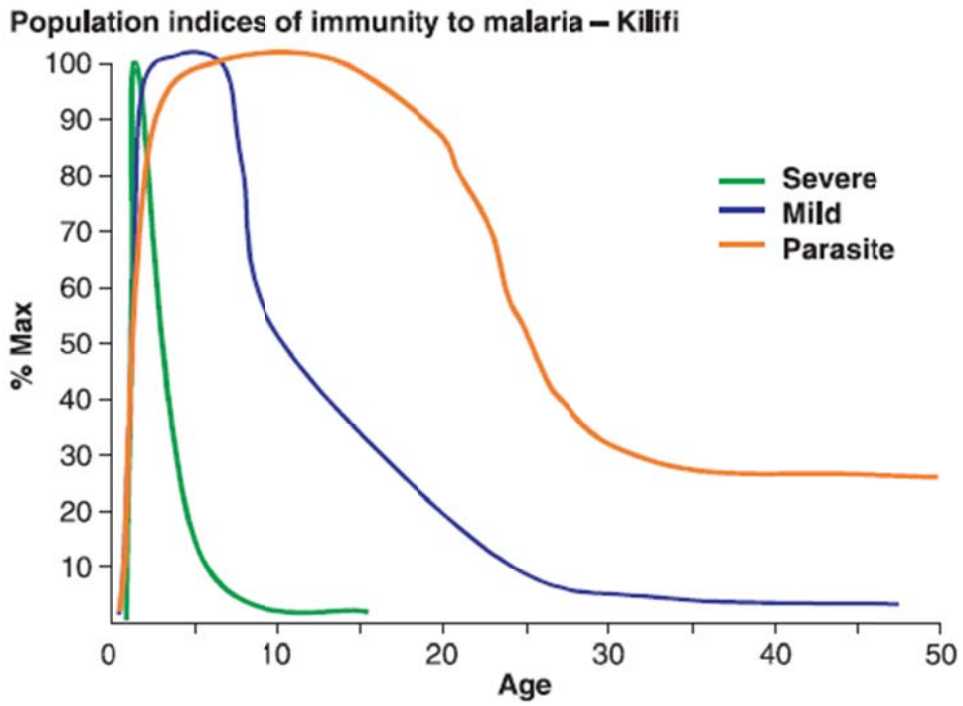


FIGURE 2.7. Population indices of immunity to malaria in Kilifi, Kenya. Age patterns for asymptomatic parasite prevalence as well as mild and severe malaria are shown. The y-axis shows the maximum prevalences recorded in Kilifi. Adapted by permission from John Wiley and Sons: Parasite Immunology 28(1-2):51-60 ©2006

TABLE 2.1. Summary of *Pf*-malaria and EBV research related to T-cell response.

Authors	Study Design	Results
Moss[80], 1983 Papua New Guinea	Ecological; adults from holoendemic and sporadic areas as well as and Caucasian controls	1. Higher regression endpoints for individuals in holoendemic area compared to control groups. No difference between two control groups. 2. No differences among three groups to anti-EBNA or anti-VCA. 3. Spontaneous transformation of uninfected cells in individuals from holoendemic area but not control groups.
Kataaha[53], 1984 UK	Laboratory; adults with no history of *Pf*-malaria infection	1. EBV can induce normal lymphocytes into LCL in vitro. 2. *P. falciparum* products can induce normal lymphocytes into LCL in vitro.
Whittle[78], 1984 Gambia	Case-control; During/post *P. falciparum* infection in children 5-18 years old.	1. High regression indices during acute *P. falciparum* infection. 2. During acute *P. falciparum* infection, number and proportion of T-helper cells was reduced. 3. Proportion of B-lymphocytes increased during acute *P. falciparum* infection.
Gunapala[69], 1990 UK	Case-control; acute *P. falciparum* infection in UK residents aged 11-51 years, healthy EBV seropositive UK adults	1. A larger proportion of cases had higher regression endpoints than controls. 2. Cases had higher numbers of B-lymphocytes and CD8+ T-cells; ratio of CD4:CD8 reduced compared to controls. 3. The proportion of lymphocytes transformed to LCL in cases was significantly higher than controls. 4. Cases had higher anti-VCA titers than controls.
Moormann[35], 2007 Kenya	Ecological; Children 1-14 yo from holoendemic and sporadic areas	1. No difference in cytotoxic T-cell response between two areas but in holoendemic area observed a loss of T-cell response in children 5-9 years old compared to younger and older children. 2. Children in holoendemic area had significantly less Interleukin 10 responses to EBV lytic peptides than those in the sporadic area.

Authors	Study Design	Results
Njie[79], 2009 Gambia	Case-control; Children 3-14 years old with symptomatic *P. falciparum*, age-matched children with no parasitemia, Gambian adults not parasitemic, UK adults with no parasitemia, all seropositive for EBV	1. Gambian adults had significantly higher viral loads than UK adults. 2. Gambian children with acute *Pf*-malaria had viral loads 5-6 times higher than control children ($p<0.001$). 3. EBV loads higher in children 3-5 years old than older children although not statistically significant. 4. No significant difference in EBV loads in children with acute *Pf*-malaria 4-6 weeks after infection. 5. Gambian children had weak T-cell responses during acute *Pf*-malaria infection compared to adult controls; recovery levels were similar to adult controls (no significance testing due to small sample size).

CHAPTER THREE: DESCRIPTION OF DATA SOURCES

Study Setting

The Kisumu/Nandi Cohort Study was established in western Kenya, an area located within the eBL "Lymphoma Belt" (Figure 2. 2). Two sites with disparate MTI levels were selected (Figure 3.1). The first site was located in Kisumu District in Nyanza Province, located in the lowlands on the banks of Lake Victoria. Malaria transmission in this area has been described as high and perennial, i.e. holoendemic. The other site was in Nandi District in the Rift Valley Province, located in the highlands 150 km northeast of Kisumu where malaria transmission has been characterized as low, unstable and prone to epidemics, i.e. hypoendemic. These areas were referred to by their district names, Kisumu (holoendemic) and Nandi (hypoendemic).

Study Population

In Kisumu district, Kanyawegi sublocation was selected for participation based on interest in the study and willingness of children (with parental permission) to provide venous blood samples. An estimated 3,000 individuals reside in the 6-7 villages in this sublocation. Fishing was the predominant occupation. The Kipsamoite sublocation in Nandi District was also selected and similar to Kanyawegi, there were 6-7 villages with an estimated population of 3,500 residents. Subsistence farming

was the main occupation in this area. In both locations, homes were built using locally available materials.

Study Sampling

Local meetings were held to introduce and explain the purpose of the study to community members. Study staff waited to enroll children for a few months after the introductory meetings to provide parents an opportunity to consider the study.

Each household and household member had been assigned a unique study identification number in 1999 for a demographic study. This information was used to randomly sample households for inclusion in the study. The number of participants enrolled from each village was set to ensure proportional geographic representation of each study site. Written informed consent was obtained from parents or guardians of study participants.

Investigators enrolled a total of 236 children. Based on the age-incidence of eBL in western Kenya, investigators made an effort to enroll approximately the same number of children within the following age groups: 2-4 years, 5-9 years, and 10-14 years (Table 3.1). It should be noted during the enrollment process, 13 children aged 5 months - 23 months and 2 teenagers aged 15 year olds were enrolled. Investigators also enrolled approximately the same ratio of children by sex.

Selection Criteria

The following inclusion criteria were used for enrollment in the study:

- Permanent residency in the study site as defined by sleeping in the home at least 10 months of the year;
- "Healthy" appearance as assessed by the clinical officer (i.e., no signs of chronic illness or malnutrition);
- Parental consent for blood sample collection.

The following criteria were used to exclude children from the study:

- Clinical officer observed signs of severe *Pf*-malaria infection;
- HIV infection was known or suspected.

Data Collection

Three surveys were conducted between July, 2002 and August, 2004. The timing of the surveys and corresponding number of participants are summarized in Table 3.2.

During each survey, a standardized form was used to collect general demographic information (e.g., date of birth, sex), and malaria related information (e.g. bednet use). A copy of the form can be found in Appendix A. In addition, blood was collected for *Pf*-malaria testing (thick and thin blood smears), *Pf*-malaria serology testing (AMA-1 and MSP-1_{42} to 3D7 and FVO strains, and LSA-1) and EBV testing (EBV-specific CD8+ T cell IFN-γ).

Human Subjects Research

The Office of Human Research Ethics at the University of North Carolina at Chapel Hill determined the proposed research (Study # 08-1117) did not require IRB

approval as it did not constitute human subjects research. The original study was under the auspices of a National Institute of Health K08 Award (Principal Investigator – Dr. Moormann), AI51565, and approved by the University Hospitals of Cleveland, Case Western Reserve University IRB and the Ethical Review Committee for the Kenya Medical Research Institute. Letters from these organizations can be made available upon request to Dr. Moormann (by phone at (508) 856-8826 or by email at ann.moormann@umassmed.edu).

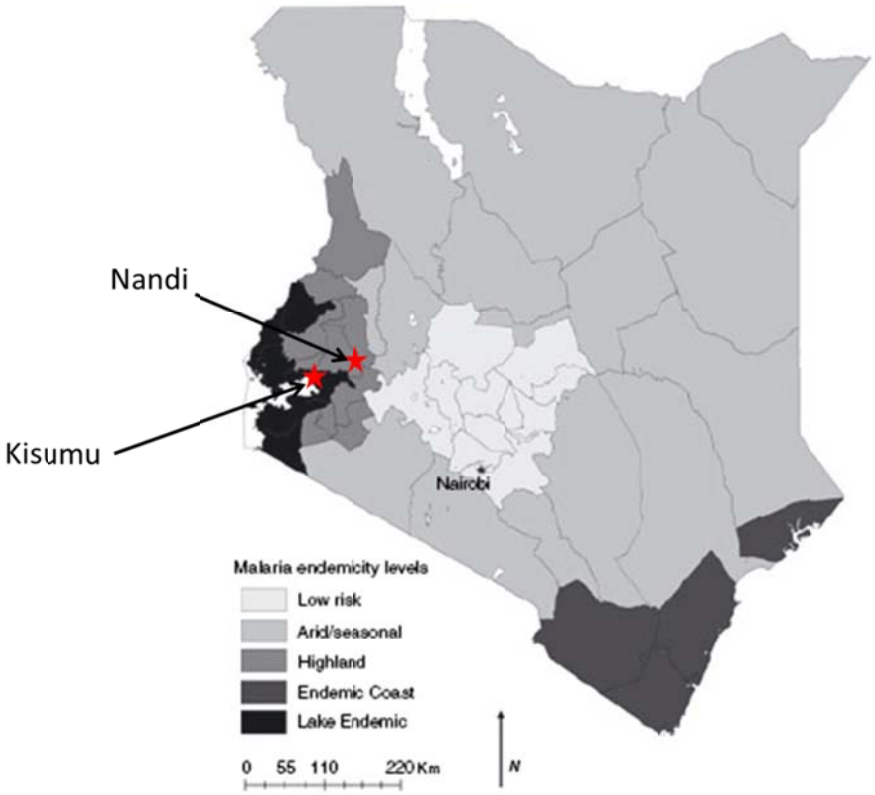


FIGURE 3.1. Map of Kisumu and Nandi districts by levels of malaria endemicity, Kenya. Adapted by permission from John Wiley and Sons: Tropical Medicine and International Health 12(8):936-943 ©2007.

TABLE 3.1. Summary of children enrolled in the Kisumu/Nandi Cohort Study

	Kisumu District n (%)	Nandi District n (%)
Total Enrolled	106 (45)	130 (55)
Age		
<2	9 (8.5)	4 (3)
2-4	26 (24.5)	39 (30)
5-9	39 (36.8)	46 (35.5)
10-14	32 (30.2)	39 (30)
>14	0	2 (1.5)
Male	62 (58.5)	64 (50.8)

TABLE 3.2. Summary of survey periods and corresponding number of participants.

Study Period	Number of Participants
July– August 2002 (baseline)	236
February–March 2003 (6-month follow up)	210
July–August 2004 (2-year follow up)	174

CHAPTER FOUR: METHODS

Specific Aim 1

To assess the effect of recurrent *Pf*-malaria exposure on Epstein Barr Virus latent and lytic antigen CD8+ T-cell IFN-γ responses over time.

Study Design Overview

Using the Kisumu/Nandi Cohort, we assessed the cumulative effect of repeated *Pf*-malaria infections on EBV latent and lytic antigen CD8+ T-cell IFN-γ responses over time. We used data from all three surveys from this cohort because the setting (i.e., holoendemic and hypoendemic) permitted exploration of district- and individual-level definition of recurrent *Pf*-malaria infections. Furthermore, EBV and *Pf*-malaria test results at three time points over a two-year period were available to address our objective.

Selection Criteria

Inclusion criteria: All cohort members who were seropositive for EBV at baseline.

Exclusion criteria: Children with uninterpretable EBV latent and lytic antigen CD8+ T-cell IFN-γ responses.

Measurements

Outcome: EBV latent and lytic antigen CD8+ T-cell IFN-γ response from each of the three survey periods was a dichotomous variable. Originally measured as a continuous variable by the IFN-γ enzyme-linked immunospot (ELISPOT) assay, the X^2 Fisher's Exact Test was used to classify responses (1=positive response, 0=no response).

Exposure: Two definitions of *Pf*-malaria were used to explore the impact of *Pf*-malaria at the district and individual level.

- At the district level, recurrent *Pf*-malaria **exposure** was a time-invariant exposure defined according to the level of malaria transmission (1=Kisumu - holoendemic, 0=Nandi - sporadic).
- At the individual level, recurrent *Pf*-malaria **infection** was a time-varying exposure defined as the cumulative average of *Pf*-malaria infections (ranges from 0 to 1 at each survey period, where 1= parasitemia detected at present and previous survey periods and 0=no parasitemia detected at present or previous survey periods.).

Additional covariates:

At the individual level, age, district of residence, HIV status, nutritional status, schistosomiasis infection, sex, and socioeconomic status (SES) have been identified as potential confounders of the exposure-outcome relationship.

- HIV testing was not conducted as part of study activities; unmeasured potential confounder.

- Nutritional status was not assessed; unmeasured potential confounder.
- Schistosomiasis testing was not conducted; unmeasured potential confounder.
- Information on SES was not collected; unmeasured potential confounder.

Age and sex will be included in our analysis as covariates.

- Age was modeled as a time-varying covariate using dummy indicators. The categories were selected for comparison with previous work (0-4 years, 5-9 years, ≥10 years). The ≥10 year age group was used as the referent group.
- District of residence (site) was a time-invariant dichotomous variable (1=Kisumu, 0=Nandi).
- Sex was a time-invariant dichotomous variable (1=Male, 0=Female).
- Survey time period was modeled as a time-invariant covariate using dummy indicators. The baseline survey period was used as the referent group.

Data Analysis

We used weighted log-binomial regression models with generalized estimate equations (GEE) and robust variance estimators to characterize variation of EBV latent and lytic antigen CD8+ T-cell IFN-γ responses over time with 1) recurrent *Pf*-malaria exposure (district) as an exposure, and 2) recurrent *Pf*-malaria infection (individual) as an exposure.

The GEE approach was used account for repeated outcome measures.[147, 148] Random effects models could have been used, however the GEE was preferred for our analyses because we were interested in estimating the population-averaged change in the outcome for a unit change in the exposure whereas random effects models estimate the change in the outcome for each cluster in the population.[149] A limitation of the GEE approach is that to estimate robust standard errors, large numbers of clusters with few repeated measures are needed. [149] Fortunately the Kisumu/Nandi cohort fulfilled this criteria as it was composed of 230 clusters (i.e., subjects) with three repeated measures.[149]

GEEs use a "quasi-likelihood" approach, an extension of the maximum likelihood estimation.[147, 148] Estimates are interpreted similarly to linear or logistic regression models.[150, 151]

The GEE takes the same form as the Generalized Linear Model (GLM)

$$g(\mu) = \beta_0 + \sum_{h=1}^{p} \beta_h X_h$$

where μ is the mean response *E(Y)*, $g(\mu)$ is a function of the mean, β_0 is the baseline mean when all predictor variables equal 0, *p* are independent variables, X_h represents the vector of predictor variables, and β_h is the mean change when X_h =1.[150]

The difference between GLM and GEE is the underlying assumptions and approach to estimating the parameters and variances.[150, 151] Both models require

specification of the link function and variance function; however, GEEs require specification of the working correlation matrix.[150, 151] The inclusion of the correlation parameters allows GEE to model within-subject correlation.[150, 151]

In our analyses we used the independent as the "best guess" for the working correlation matrix; the independent working correlation matrix assumes there is no correlation within a subject when calculating the point estimate.[150, 151] We also used robust standard errors which provided unbiased estimates even if the working correlation matrix was inaccurate. We used the generalized Wald Test to build the final multivariable model as a quasi-likelihood analysis does not generate the Likelihood Ratio test commonly used for model comparisons.[150]

An important assumptions when using GEE is that data are missing completely at random; missingness of data is independent of observed and unobserved data.[150] We had missing observations in the two subsequent follow-up surveys. Upon examination of the pattern of missing observations, we concluded missingness was related to age therefore our data were not missing completely at random. We weighted the final model using inverse probability weights. A summary of this approach is provided in Appendix B.

Descriptive Analysis: Frequencies and proportions were calculated for all measurements and summarized in tables or figures. Continuous outcomes were not normally distributed therefore data were described using medians and non-parametric testing was used to identify any differences between levels of a variable.

Bivariable Analysis: The crude relationship between the exposure-outcome was explored using the log-binomial regression with GEE. Covariate-outcome relationships were also explored. Findings from the analyses were summarized in tables and figures.

Assessment of Effect Measure Modification: Covariates were initially examined as potential effect measure modifiers (EMM) of the exposure-outcome relationship. One at a time, the covariate (main effect) and an exposure-covariate interaction term were added to the crude model (interaction model). The generalized Wald test was used to compare the crude and interaction models to test the significance of the interaction term. Any covariates significant at an alpha-level of 0.20 or less were classified as EMM and included in the final model. All models were stratified by district of residence.

Assessment of Confounding: Covariates not identified as EMM were assessed as potential confounders. Causal diagrams for the relationship between *Pf*-malaria exposure (district) and repeated infection (individual) are presented in Figure 4.1. District level –No additional covariates were included in the model. Individual level – Age has continuously been reported as a confounder of the exposure-outcome relationship therefore it was adjusted for in multivariate models. Sex and survey period were assessed as potential confounders; they did not lead to a >10% change in the unadjusted PR but were included in the final model.[149]

Multivariable Analysis: We began our analysis with a fully adjusted weighted log-binomial model including the exposure, EMMs, and confounders. We used the independent working correlation matrix with robust variance estimators. All measured covariates were included in the model as EMM or confounder therefore we did not our fully adjusted model was or finalized model.

Power

Our study population was fixed; we simulated data to calculate power estimates using the GEE approach with three repeated measures examining the effect of *Pf*-malaria exposure (district) at a 0.05 two-sided significance level. The power estimates for recurrent *Pf*-malaria exposure (district) for both lytic and latent pools of EBV-specific CD8+ T-cell IFN-γ response are summarized in Figure 4.2. We had 80% power to detect a PR of 1.93 in EBV-specific CD8+ T-cell IFN-γ response (lytic) as a result of *Pf*-malaria exposure. We had over 80% power to detect a PR of 1.96 in specific CD8+ T-cell IFN-γ response (latent) as a result of *Pf*-malaria exposure.

Limitations

There were several potential limitations of Specific Aim 1.

- Interpreting findings from models using a district-level definition of recurrent *Pf*-malaria exposure are subject to the ecological fallacy whereby population-based estimates are attributed to the individual. We took caution when making interpretations of our results, highlighting this potential limitation.

- The individual-level definition of recurrent *Pf*-malaria infection may not accurately reflect the true experience of participants. Between baseline and subsequent follow-up surveys, participants may have had *Pf*-malaria infections which were not captured by this study design, especially in the 18 month gap between the last two follow-up surveys.
- Testing to confirm HIV status was not conducted; children were deemed healthy by clinician judgment. Although HIV is a potential confounder in the association of *Pf*-malaria infection and EBV, it does not appear HIV infection was present in this cohort at the time of the study. At the time of the study, HIV treatment was not readily available in Kenya and as a result, HIV infected individuals often died within two years of HIV-related symptom onset. Subsequent follow up of these children, as recent as 2009, found no deaths among the children.
- Although Schistosomiasis infection was not conducted, an examination of the causal diagram between the exposure-outcome identified age and sex as measured antecedents of schistosomiasis infection (schistosomiasis was identified as a collider); adjusting for a collider may bias our estimate. Hence the lack of schistosomiasis testing for children is unlikely to bias our results.
- Nutritional status was not measured; children enrolled in the study were deemed healthy by clinician judgment with no obvious signs of malnourishment. Hence, nutritional status was unlikely to be a potential confounder at the time of the study.

- SES was an unmeasured potential confounder. Families living in villages at both districts were likely to be classified as low SES as the main occupation was fishing (Kisumu) and farming (Nandi), with homes constructed of locally available materials. Given the SES composition of the villages, it appears SES was not a potential confounder at the time of the study.

Specific Aim 2

a. To describe malaria antibody responses among children in malaria holoendemic and hypoendemic areas, contrasting any differences between the areas.

b. To describe the relative change in malaria antibodies over time in malaria holoendemic and hypoendemic areas, highlighting any differences between the areas.

Study Design Overview

We used data collected in July-August 2002 (baseline) and February-March 2003 (six-month follow) from the Kisumu/Nandi Cohort to describe *Pf*-malaria antibody responses. This cohort was amenable for our objective because data were available from two districts characterized by different malaria transmission intensities. In addition, *Pf*-malaria-specific antibody test result were available.

Selection Criteria

Inclusion criteria: All cohort members.

Exclusion criteria: Children with uninterruptable antibody results.

Measurements and Analysis Plan – Specific Aim 2a

Outcome: Dichotomous IgG responses to AMA-1 3D7, AMA-1 FVO, MSP-1_{42} 3D7, MSP-1_{42} FVO, and LSA-1 where 1=positive, 0=negative. Originally measured in mean fluorescence intensity (MFI), we standardized results into arbitrary units (AU) to account for plate-to-plate variability. Standardization was done by dividing the each participant's MFI antibody response by the negative controls' mean + three standard deviations. AU>1 were classified as a positive IgG response.

Exposure: Our analysis was descriptive therefore age, parasitemia status, and sex were treated as exposures.

- Age was a categorical variable: 0-4 years, 5-9 years, ≥10 years. Age was defined as the child's age at the time of the survey.
- Parasitemia status was a dichotomous variable (1=parasitemic, 0=not parasitemic).
- Sex was a dichotomous variable (1=Male, 0=Female).

Descriptive Analysis: Frequencies and proportions were calculated and summarized in tables and figures. Median AU values were calculated for each IgG response and summarized in a table. Medians were used because the distribution of the

continuous outcome was not normally distributed. Data were stratified by district of residence.

Bivariable Analysis: All analyses were stratified by district of residence. The Chi-square test and Cochran Armitage trend test were used to assess any significant differences among the levels of each exposure as well as to compare responses between the two districts. Spearman's rank correlation coefficients were used to examine any correlation between outcomes.

Measurements and Data Analysis Plan – Specific Aim 2b

Outcome: Continuous AU values of AMA-1 3D7, AMA-1 FVO, MSP-1_{42} 3D7, MSP-1_{42} FVO, and LSA-1 antibodies measured as relative change over six-months.

Relative change was calculated using the following formula:

$$= \frac{\text{(IgG response at six-month follow-up – IgG response at baseline)}}{\text{IgG response at baseline.}}$$

Exposure: Our analysis was descriptive therefore age group, parasitemia status, and sex were treated as exposures.

- Age was a categorical variable: 0-4 years, 5-9 years, ≥10 years. Age was defined as the child's age at the baseline survey.
- Parasitemia status was a categorical variable:
    - Parasitemia at both times

    - Parasitemia at baseline survey only
    - Parasitemia at six-month follow-up survey only
    - Aparasitemic at both surveys.
- Sex was a dichotomous variable (1=Male, 0=Female).

Descriptive Analysis: The distribution of the continuous outcome was examined and was not normally distributed. Therefore we used medians to describe the data. Data were stratified by district of residence and summarized using boxplots and tables.

Bivariable Analysis: All analyses were stratified by district of residence. Non-parametric tests were used to identify significant differences between levels of each exposure as well as to compare responses between the two districts. Exact tests were used for small samples sizes. An extension of the Wilcoxon rank-sum test was used to assess for trends of ordinal exposures. Spearman's rank correlation coefficients were used to assess any correlation in the relative change of antibody responses between outcomes.

Missing observations: Surveys were conducted on 210 of the original 236 children at six-month follow up. This represented an 11% loss to follow-up. We did not identify any significant differences by age group or sex between children who did and did not participate in the six-month follow-up survey.

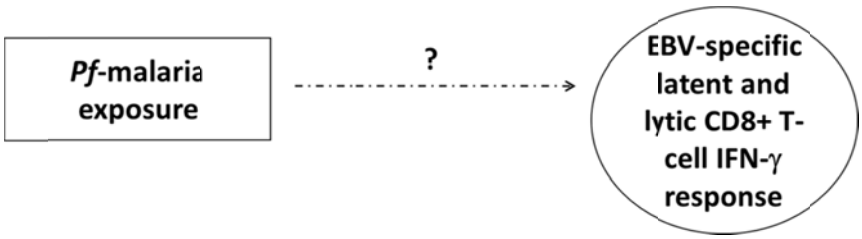
Limitations – Specific Aim 2

There were several potential limitations of Specific Aim 2.

- Cross-sectional studies measure exposure and outcome at one point in time. It was possible that children with elevated antibody responses cleared their parasitemia just before the survey. Therefore we may not have captured accurately the relationship between parasitemia status and antibody response. However, this would have predominantly been an issue in Kisumu where children experienced *Pf*-malaria infection more often than Nandi children. Given the large proportion of children who had parasitemia detected in Kisumu (>76%) at both surveys, this issue was likely to be minimal.
- The use of AU permitted standardization of antibody responses to account for plate-to-plate variability. However there is no intrinsic meaning of AU values. In addition, cutoffs for AU values differ across studies therefore and they cannot be directly compared across studies although directionality of responses are comparable.
- Caution must be taken when interpreting the relative change in antibody responses. A 200% increase in antibody response in a child originally classified as a negative responder does not imply the child has become a positive responder.
- We cannot say if observed differences in antibody responses reflect functional differences.
- Due to limited power, we were unable to detect small yet meaningful differences. For example, we had few children in Kisumu (n=6) who were aparasitemic at both surveys and few children in Nandi (n=5) who were

parasitemic at both surveys. Hence we were unable to detect differences in the median relative change in IgG response by parasitemia status for most antibodies.

A.



B.

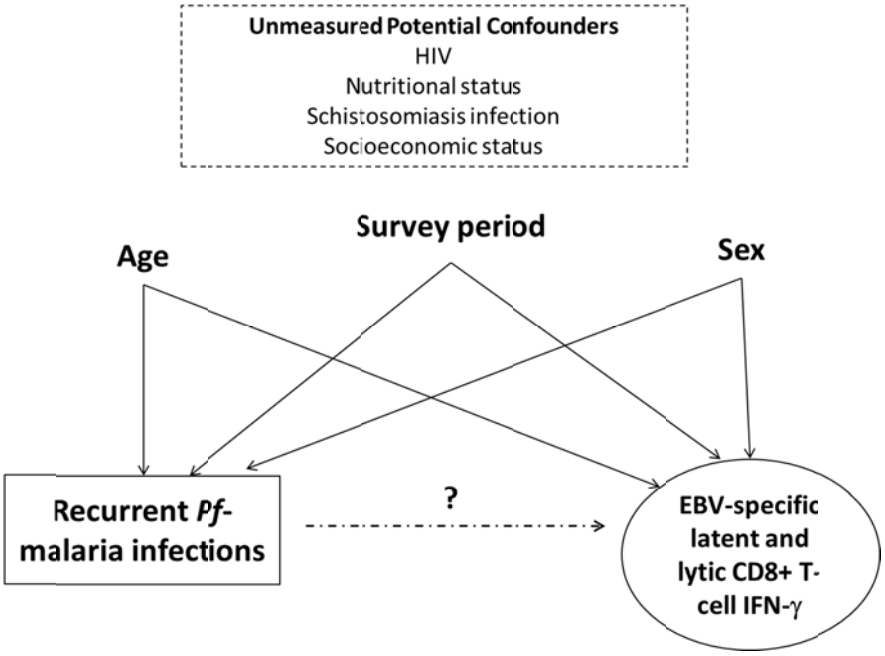
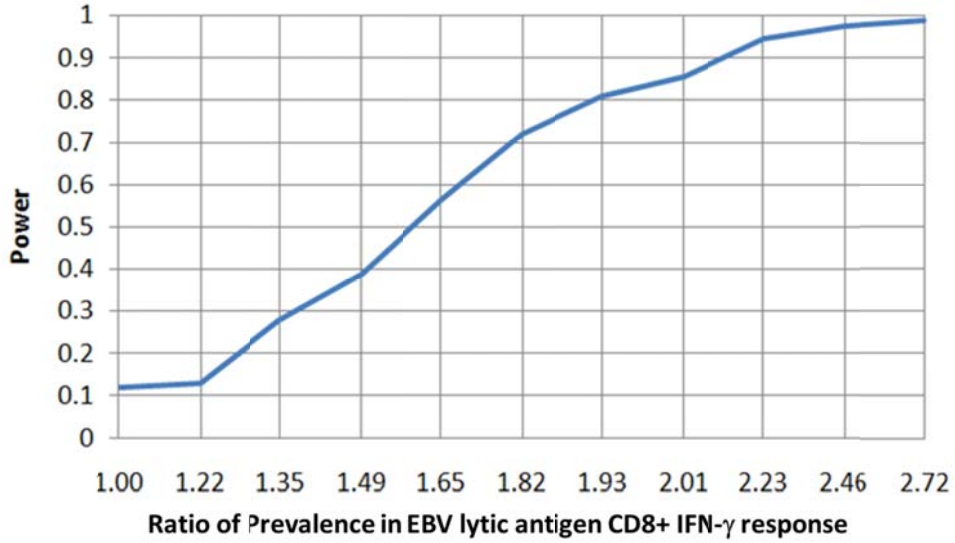


FIGURE 4.1. Causal diagrams depicting the relationship between Pf-malaria and EBV-specific latent and lytic CD8+ T-cell IFN-γ response. Graph A illustrates the district-level relationship while Graph B depicts the individual-level relationship.

A.



B.

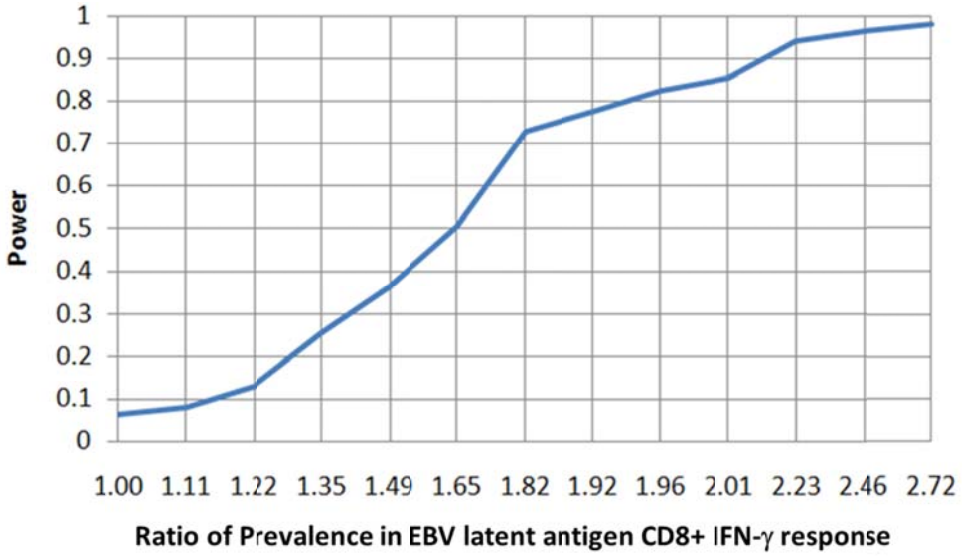


FIGURE 4.2. Power estimates for log-binomial regression using GEE to account for three repeated measures. Graph A (top) shows the power estimates for *Pf*-malaria exposure (district) for a lytic pool of EBV-specific CD8+ IFN-γ response. Graph B (bottom) shows the power estimates for *Pf*-malaria exposure (district) for a latent pool of EBV-specific CD8+ IFN-γ response .

CHAPTER FIVE: Recurrent *Plasmodium falciparum* Malaria Infections in Kenyan Children Diminish T-cell Immunity to Epstein Barr Virus Lytic but not Latent Antigens

ABSTRACT

Background. *Plasmodium falciparum* malaria (*Pf*-malaria) and Epstein Barr Virus (EBV) infections coexist in children at risk for endemic Burkitt's lymphoma (eBL); yet studies have only glimpsed the cumulative effect of *Pf*-malaria on EBV-specific immunity.

***Methods*.** Using pooled EBV lytic and latent CD8+ T-cell epitope-peptides, IFN-γ ELISPOT responses were surveyed three times among children (10 months to 15 years) in Kenya from 2002-2004. Prevalence ratios (PR) and 95% confidence intervals (CI) were estimated in association with *Pf*-malaria exposure, defined at the district-level (Kisumu: holoendemic; Nandi: hypoendemic) and the individual-level.

Results. We observed a 46% decrease in positive EBV lytic antigen IFN-γ responses among 5-9 year olds residing in Kisumu compared to Nandi (PR: 0.54; 95% CI: 0.30-0.99). Individual-level analysis in Kisumu revealed further impairment of EBV lytic antigen responses among 5-9 year olds consistently infected with *Pf*-malaria compared to those never infected. There were no observed district- or individual-level differences between *Pf*-malaria exposure and EBV latent antigen IFN-γ response.

Conclusions. The gradual decrease of EBV lytic antigen but not latent antigen IFN-γ responses after primary infection, suggests a specific loss in immunological control over the lytic cycle in children residing in malaria holoendemic areas; further refining our understanding of eBL etiology.

INTRODUCTION

Plasmodium falciparum (*Pf*) malaria and Epstein Barr Virus (EBV) have been identified as co-factors in the pathogenesis of endemic Burkitt's lymphoma (eBL) [2] which is estimated to account for 70% of cancers among children in equatorial Africa [3, 4]. In areas with intense perennial malaria transmission (holoendemic), the highest incidence of eBL is in children aged 4-8 years [17, 22, 25, 27, 29, 74], in contrast to areas with low malaria transmission (hypoendemic) where eBL is rarely reported [7, 25, 30].

It has been hypothesized that *Pf*-malaria infections promote eBL in two mutually-compatible ways. In developing countries, most children experience primary EBV infection by 3 years of age, followed by life-long infection in memory B-lymphocytes [5, 19]. *P. falciparum* induces polyclonal B-cell expansion and lytic EBV reactivation [61], thus increasing the number of latently-infected B-cells. In otherwise healthy individuals, interferon-gamma (IFN-γ) secreting cytotoxic CD8+ T-cells mediate immunosurveillance of EBV [19, 22, 35, 50, 52, 152]. Repeated *Pf*-malaria infections could hence lead to exhaustion or hypo-responsiveness of EBV latent or lytic antigen CD8+ T-cells, thus increasing the chance for this EBV-associated malignancy to arise.

Limited evidence supports an impaired EBV-specific T-cell response in association with *Pf*-malaria. Using an in vitro regression assay as a measure of cytotoxicity, children with acute *Pf*-malaria demonstrated a transient loss of control over B-cell outgrowth [76-78]. Furthermore, case-control studies comparing acutely *Pf*-malaria infected individuals with healthy adults came to the same conclusion [69, 79]. However, the cumulative effect of repeated often asymptomatic *Pf*-malaria infections on EBV persistence has not been thoroughly studied. [2, 29, 31, 32] Two ecological studies provide the minimum understanding we have on the relationship. A study among adults found a loss of EBV-specific T-cell control among those exposed to holoendemic compared to hypoendemic malaria [80]. A second study only found significantly lower EBV latent and lytic antigen IFN-γ responses in children 5-9 years old residing in the holoendemic area compared to other age groups and children from a hypoendemic area [35].

The objective of this study was to examine the influence of cumulative *Pf*-malaria on EBV latent and lytic antigen CD8+ T-cell IFN-γ ELISPOT responses in children over a two-year period.

METHODS

The Kisumu/Nandi cohort has been previously described [59]. In brief, the cohort consists of 236 children, randomly selected and between 10 months and 15 years at enrollment, from two districts in western Kenya with disparate *Pf*-malaria transmission intensities: Kisumu is characterized as holoendemic and Nandi as hypoendemic. Due to the age-related incidence of eBL, an equal distribution of

children by age and sex were enrolled from each area: children 0-4 years have an elevated risk of eBL whereas 5-9 year olds are at highest risk and ≥10 years old have the lowest risk. Data were collected from 2002-2004 using a standardized survey. Three face-to-face interviews were conducted at baseline (July-August 2002), six month follow-up (February-March 2003), and two-year follow-up (July-August 2004). Blood was also collected for malaria and EBV testing.

Pf-malaria infection was confirmed on thick and thin blood smears by microscopy. Testing of EBV-specific T-cell response by IFN-γ ELISPOT has been previously described [35]. Lytic (BRLF1, BZLF1, and BMLF1) and latent (Epstein-Barr nuclear antigen [EBNA] 3A, EBNA 3B, and EBNA3C) antigens were selected and pooled for testing. One positive control (mitogen phytohemmagglutinin [PHA]) was used to stimulate wells and a negative control (phosphate buffer saline [PBS]) was used to measure background IFN-γ response in unstimulated wells. Assays were condensed into a three-week period using the same reagents and personnel to minimize inter-assay variability. Cytotoxic T-lymphocyte (CTL) ImmunoSpot scanning and imaging software (version 4; Cellular Technology Ltd, Shaker Heights, OH) was used to count the number of spot-forming units (SFU) per well; results were expressed as SFU per million peripheral blood mononuclear cells (PBMC). Using a two-sided Fisher's exact test ($P < .05$), EBV lytic and latent epitope-peptide CD8+ T-cell IFN-γ responses were categorized as positive or negative. A positive response was recorded if the proportion of SFUs in the stimulated well was significantly different from the proportion of SFU in the unstimulated well. The magnitude of response was calculated by subtracting the SFU in PBS wells (negative control)

from the SFU in the stimulated wells. The median value for the negative control wells was 4 SFU per million PBMCs (range 0 to 772 SFU/million PBMC). Median values were calculated among positive responders only.

Analyses were restricted to EBV seropositive children at baseline [59]. We used two definitions of cumulative *Pf*-malaria. First, *Pf*-malaria exposure was defined according to the malaria transmission intensity of the district (district-level definition): Kisumu (holoendemic) or Nandi (hypoendemic). Next, *Pf*-malaria infection was defined as the cumulative average of *P. falciparum* infection (parasitemia) in a participant over the three survey periods (individual-level definition). The value ranged from 0 (never infected) to 1 (always infected); results and discussion focus on children who were always infected (referred to as recurrent) and never infected. With the individual-level definition, we also included the covariates age group, district, sex, and when the survey was conducted (referred to as survey period) in the analysis. We first examined covariates as potential effect measure modifiers using an a priori cutoff of $P = .20$. In the absence of evidence of effect measure modification, we included covariates in the model as potential confounders.

For descriptive analyses, we used the χ^2 statistic to measure associations between categorical exposures and outcomes. We used the two-sided Wilcoxon rank sum (Mann-Whitney *U*)/Kruskal Wallis test for continuous outcomes. For multivariable analyses, we used weighted log-binomial regression with robust variances to estimate the prevalence ratios (PR) and corresponding 95% confidence intervals (CI). We used generalized estimating equations (GEE) with robust

variance estimators to account for correlation due to repeated measurements. A weighted model with inverse probability weights was used to address missing observations due to children not participating in all surveys. An explanation of our approach can be found in Appendix B. We also conducted complete case analyses and found no differences in the PR or 95% CI; therefore we report results from the weighted analyses. Data were analyzed in SAS 9.1.3 (Cary, NC).

Written informed consent was obtained from a parent or guardian of the participant. This study was approved by the Institutional Review Boards at the University Hospitals of Cleveland, Case Western Reserve University where Dr. Moormann was affiliated at the time this study was done and also obtained from the Ethical Review Committee for the Kenya Medical Research Institute. It was deemed exempt by the Institutional Review Board at the University of North Carolina at Chapel Hill.

RESULTS

Participant Summary

Of the 236 children enrolled, 230 (97.5%) were seropositive for EBV [59]. Our weighted analysis included 149 children who participated in all surveys and had interpretable EBV-specific T-cell responses (Table 5.1). The age and sex distribution between the districts were not significantly different ($P = .11$ and $P = .30$, respectively). Children in Kisumu experienced more *Pf*-malaria infections than children in Nandi ($P < .001$); only 3% of Kisumu children were never infected

compared to 78% in Nandi. This was despite a classically defined malaria outbreak in Nandi during the survey periods (Figure 5.1).

The Magnitude of EBV-specific IFN-γ Responses Did Not Differ Significantly by Malaria Endemicity

The proportion of positive IFN-γ responses to PHA (positive control) demonstrates that children from both districts were equally able to elicit an IFN-γ response indicating no global signs of immune dysfunction (Table 5.2). There were no significant differences in median values of EBV lytic or latent CD8+ T-cell IFN-γ responses between children of similar age groups across districts. Therefore, *Pf*-malaria exposure does not appear to influence the magnitude of EBV-specific IFN-γ responses.

***Pf*-malaria Exposure (District-level) and EBV-specific T-cell IFN-γ Responses**

EBV lytic antigen CD8+ T-cell IFN-γ responses. We observed a few intriguing patterns in the prevalence of positive EBV lytic antigen CD8+ T-cell IFN-γ response when children were stratified into age groups by their baseline age (age group cohorts) (Figures 5.2A and 5.2C). In Kisumu, the prevalence of positive responses in the 0-4 year and 5-9 year cohorts decreased from baseline to first follow-up, but remained unchanged in the ≥10 year cohort. By the second follow-up, responses increased among the 0-4 and 5-9 year cohorts while responses decreased in ≥10 year cohort. However, children in the 5-9 year cohort had the lowest prevalence at each survey period. In Nandi, responses declined in all age group cohorts from

baseline to first follow-up and remained almost unchanged in the 5-9 year and ≥10 year cohorts by the second follow-up. In the 0-4 year cohort, however, responses increased. The patterns and prevalence of responses among the age group cohorts were similar at all survey periods, varying <10%.

Using the district-level definition of *Pf*-malaria and the weighted model described earlier, we estimated the prevalence of positive responses in Kisumu was 0.70 (95% CI: 0.45-1.08) times the prevalence in Nandi although this 30% difference was not significant. In Kisumu, there were no significant differences in positive responses in children 0-4 years (PR: 1.39, 95% CI: 0.60-3.20) and 5-9 years (PR: 0.74, 95% CI: 0.37-1.48) when compared to children ≥10 years (Figure 5.3A). Likewise in Nandi, the prevalence of positive responses in children 0-4 years (PR: 1.10, 95% CI: 0.60-2.02) and 5-9 years (PR: 1.04, 95% CI: 0.61-1.76) did not differ significantly from children ≥10 years. When similar age groups were compared between districts, we detected a significant difference in children 5-9 years where the prevalence of positive responses in Kisumu was 0.54 (95% CI: 0.30-0.99) that of children in Nandi (Figure 3A). No other differences by age group were found.

EBV latent antigen CD8+ T-cell IFN-γ responses. Examining the patterns in the prevalence of positive EBV latent antigen CD8+ T-cell IFN-γ response by age group cohorts, there was variation within and between districts (Figures 5.2B and 5.2D). In Kisumu, the prevalence at baseline was highest among the 0-4 year cohort but then decreased to nearly the same prevalence as the other age group cohorts. In Nandi, there was a decreasing trend from baseline to second follow-up for the 0-4 year and 5-9 year cohorts. However, the ≥10 years cohort had the highest prevalence of

response at baseline that decreased by the first follow-up but rebounded by the second follow-up.

From our weighted model, we observed the prevalence of positive responses in Kisumu was 0.80 (95% CI: 0.51-1.25) times the prevalence in Nandi, although not significant. In Kisumu, the prevalence of positive responses in children 0-4 years (PR: 1.93, 95% CI: 0.91-4.13) and 5-9 years (PR: 1.22, 95% CI: 0.61-2.45) was not significantly different from children ≥10 years, although there was a decrease in prevalence with increasing age group (Figure 5.3B). Similarly in Nandi, responses among children 0-4 years (PR: 0.72, 95% CI: 0.35-1.48) and 5-9 years (PR: 0.84, 95% CI: 0.47-1.49) did not differ significantly from children ≥10 years old, although there was a slight increase in response with increasing age. Despite these interesting trends, there were no significant differences in the prevalence of positive responses when similar age groups were compared between districts.

Pf-malaria Infection (Individual-level) and EBV-specific T-cell IFN-γ Responses

EBV lytic antigen CD8+ T-cell IFN-γ responses. Using the individual-level definition of *Pf*-malaria and weighted model described earlier, we found the association between recurrent *Pf*-malaria infections and EBV lytic antigen CD8+ T-cell IFN-γ response varied by age group and survey period. We therefore used two models. In the first model, we stratified results by age group, while adjusting for sex and survey period. Similarly in the second model, we stratified by survey period while adjusting for sex and age group.

We noted three observations from our analysis. First, the PR of recurrent *Pf*-malaria infections and positive IFN-γ responses among Kisumu children were consistently lower than Nandi children for all age groups and survey periods (Table 5.3). In general, there is a two-fold difference in the PR between Kisumu and Nandi although not significant (P = .32). Secondly, the association between recurrent *Pf*-malaria infections and IFN-γ responses varied by age group. In both Kisumu and Nandi, the prevalence of positive responses among children 0-4 years with recurrent *Pf*-malaria infections was higher than that of similarly aged children never infected. In Nandi, the difference was statistically significant. Finally, the PR of recurrent *Pf*-malaria infections and IFN-γ responses to EBV lytic antigens varied by survey period in both districts. At baseline, for both districts, the PR of positive responses among children with recurrent *Pf*-malaria infections was greater compared to children never infected; this result was statistically significant in Nandi, but not Kisumu. However, the PR decreased at subsequent study periods; the prevalence of positive responses among children with recurrent *Pf*-malaria infection diminished over time compared to children never infected. This could reflect functional diminishment of responsive EBV lytic antigen T-cells under continuous pressure from *Pf*-malaria.

EBV latent antigen CD8+ T-cell IFN-γ responses. Using our weighted model, we did not observe any variation by age group (Table 5.4) or survey period (data not shown). In Kisumu, for all age groups, the adjusted prevalence of positive EBV latent antigen CD8+ T-cell IFN-γ response was higher among children with recurrent *Pf*-malaria infections compared to those never infected (Table 5.4). There was a two-fold difference in the PR for children 0-4 years and >10 years with recurrent *Pf*-

malaria infections than children 5-9 years. In Nandi, children 0-4 years with recurrent *Pf*-malaria infections had fewer positive responses than children never infected, and a PR that was three-fold lower than older children. However, children in older age groups with recurrent *Pf*-malaria infections had higher positive responses than similarly aged children never infected. Despite estimates for Kisumu and Nandi being imprecise and not statistically significant, the observations suggest that children 5-9 years in Kisumu are unable to mount the type of T-cell response as younger and older children. Meanwhile, in Nandi, the increasing PR with age may reflect how a maturing immune system, not continuously exposed to *Pf*-malaria, is able to induce a T-cell response to latent antigens even when co-infected with *Pf*-malaria.

DISCUSSION

Our study demonstrates that the prevalence of positive EBV lytic- but not latent-antigen CD8+ T-cell IFN-γ responses decreases in a malaria holoendemic area and not a hypoendemic area. This suggests that children repeatedly infected with *Pf*-malaria eventually lose functional IFN-γ producing CD8+ T-cells in response to EBV lytic antigens. In an effort to control viral replication induced by recurrent *Pf*-malaria infections [61], we hypothesize that EBV lytic antigen CD8+ T-cells have become exhausted and unable to produce IFN-γ or alternatively these cells were culled through apoptosis. As a result of the loss of responsive EBV lytic antigen CD8+ T-cells, more B-lymphocytes could become latently infected by EBV, and thus gradually increasing the risk of eBL. These findings are consistent with previous

studies of this cohort, which detected significantly higher median EBV viral load and EBV-specific IgG antibodies to EBV lytic and latent antigens in the holoendemic compared to hypoendemic area [59, 153].

Furthermore, the association between *Pf*-malaria infections and positive EBV lytic antigen CD8+ T-cell IFN-γ responses varied by age group. The EBV lytic antigen deficiency was most pronounced among children 5-9 years old in the malaria holoendemic area and was further potentiated in those recurrently infected with *Pf*-malaria. In our individual-level analysis, these children had the lowest PR of positive responses while this same age group in the hypoendemic area appeared to be affected little. Additionally, the patterns observed in the age group cohorts clearly showed that the 5-9 year cohort in Kisumu had the lowest prevalence of positive responses among all age group cohorts, in both districts, at each survey period. The sustained inability to produce an effective EBV lytic antigen CD8+ T-cell IFN-γ response among 5-9 year olds may be an etiologically relevant event in eBL development because eBL is most often diagnosed in this age group. Finally, the inconsistency of patterns between age group cohorts within a district suggests there is an age-dependent interaction between *Pf*-malaria and EBV-specific T-cell response. Studies of immune mechanisms that induce exhaustion or deletion are needed to understand maintenance of EBV-specific T-cell immunity in children.

This study is an important early step to understanding the cumulative effect of *Pf*-malaria infections on EBV-specific T-cell immunity over time. Availability of data over two-years permitted identification of potentially important biological and environmental mechanisms that only become apparent over time. For example, the

association between *Pf*-malaria infection and positive EBV lytic antigen CD8+ T-cell IFN-γ responses varied by age group and survey period. The variation noted with age group is expected because there is an age-dependent increase in T-cell immunity as children develop protection against *Pf*-malaria after repeated infections [154]. Children in malaria holoendemic areas acquire immunity to *Pf*-malaria and EBV during the first years of life, and ongoing studies will compare the development of *Pf*-malaria to EBV-specific T-cell memory.

Using data, collected during a two-year period, also allowed us to use an individual-level definition for *Pf*-malaria infections. Unlike other studies, our definition accounted for the cumulative effect of *Pf*-malaria infection which has been hypothesized to be critical in the pathogenesis of eBL, rather than the transient effect typically observed with acute *Pf*-malaria infection [2]. However, our definition was vulnerable to misclassification because *Pf*-malaria infection was assessed only twice during the two-year follow-up. Therefore, we may not have captured participants' malaria histories accurately. This misclassification was likely to be differential because children in the holoendemic area were exposed to *Pf*-malaria parasites at a higher frequency, averaging two malaria infections per year, than children in the hypoendemic area [122]. Therefore, we may have underestimated or overestimated the PR for *Pf*-malaria and EBV-specific T-cell responses in the holoendemic area.

A strength of our study was the use of two definitions for *Pf*-malaria: 1) district-level according to malaria transmission intensity, and 2) individual-level based on measured *Pf*-malaria infection. Although our findings of EBV lytic antigen

CD8+ T-cell IFN-γ responses were consistent with both definitions, our findings of EBV latent antigen CD8+ T-cell IFN-γ responses were inconsistent. This may have been due to the limited power or an underestimation of the influence of *Pf*-malaria infections in hypoendemic areas. However, it also highlights the potential pitfall in attributing district-level results to the individual, also known as the ecological fallacy. The inconsistency may have been due to other factors that differed between the districts and unrelated to malaria transmission intensities. Therefore, we conclude that the use of malaria transmission intensity as a surrogate for malaria infection has been informative yet future studies should endeavor to prospectively collect *Pf*-malaria and EBV co-infections information from individuals to more accurately describe this complex relationship.

There were several potential confounders that were not captured in our study, specifically HIV status, nutritional status, schistosomiasis infection, and socioeconomic status. However, we do not believe the absence of these confounders materially affected our findings. When data were collected in western Kenya from 2002-2004, HIV testing in infants was conducted only when medically warranted. All children were examined by a clinician and had no obvious signs of illness or malnourishment, and no deaths have been reported as of 2009. Schistosomiasis infection was unmeasured yet an examination of the *Pf*-malaria and EBV response relationship indicated adjusting for schistosomiasis infection would have biased our analysis. If measured, participants and their families would likely have been classified as low socioeconomic status because the main occupation was

fishing (Kisumu) and farming (Nandi) in both rural study areas with homes constructed of locally available materials.

Our findings on EBV lytic antigen CD8+ T-cell IFN-γ responses were consistent with the studies that have used residence area (malaria transmission intensity) to explore the cumulative effect of *Pf*-malaria infections on EBV-specific T-cell response. We observed fewer positive EBV lytic antigen CD8+ T-cell IFN-γ responses among 5-9 year old than older children [35]. We also identified a reduction in EBV-specific T-cell response among children living in a holoendemic compared a hypoendemic area [80]. The consistency of our findings with previous studies is important given our limited sample size and precision. Meanwhile, our analysis of EBV lytic antigen CD8+ T-cell IFN-γ response at the individual-level supports findings from previous studies that used residence area as a surrogate for malaria infection.

However, we did not detect the same statistically significant district-level difference in positive EBV latent antigen CD8+ T-cell IFN-γ responses among 5-9 year olds as a previous study [35]. This discrepancy may be due to the limited power of our study. Furthermore, the difference between our individual-level analysis and the previous study may also be due to the use of a surrogate definition of *Pf*-malaria.

This study design marks a step toward examining the individual-level association of *Pf*-malaria infections and EBV-specific T-cell IFN-γ responses and identifies a potential difference between children recurrently infected with *Pf*-malaria compared to children never infected. To adequately quantify this effect, a longitudinal study should be considered which could accurately measure *Pf*-malaria

infection and changes in *Pf*-malaria and EBV-specific T-cell immunity over time. The temporal aspects of future studies will be vital to elucidating the precise mechanism by which repeated *Pf*-malaria infections affect EBV persistence and immunity.

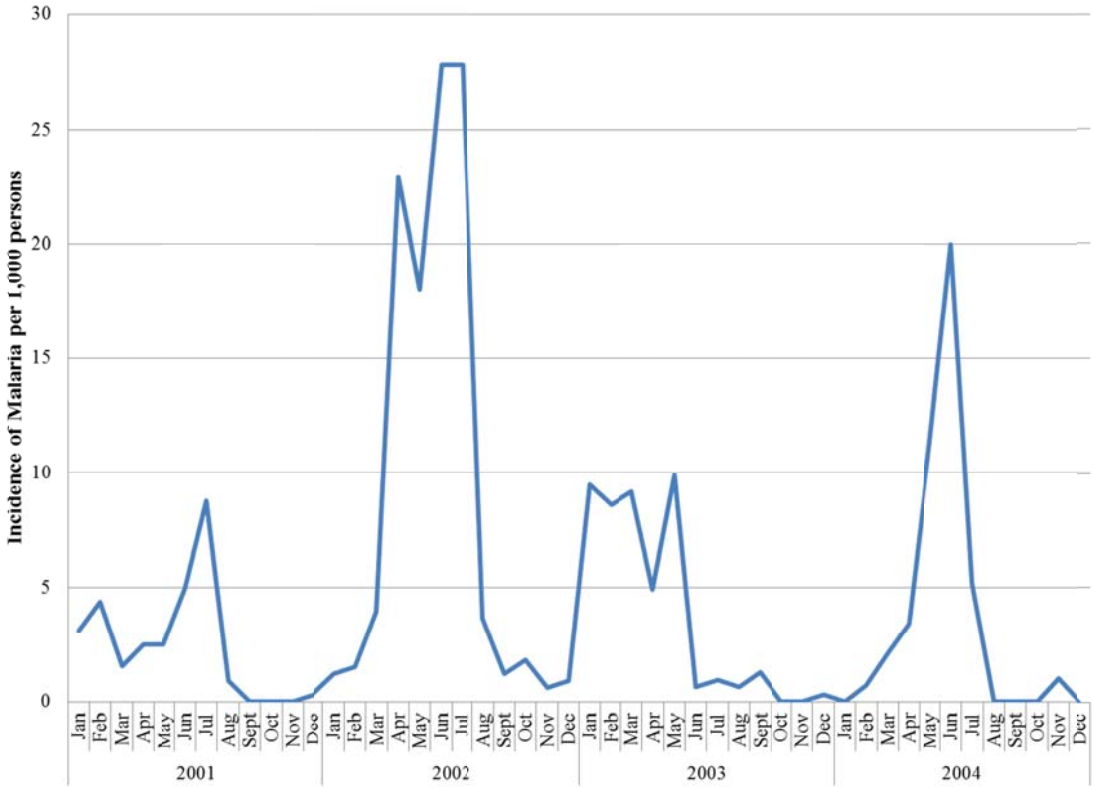
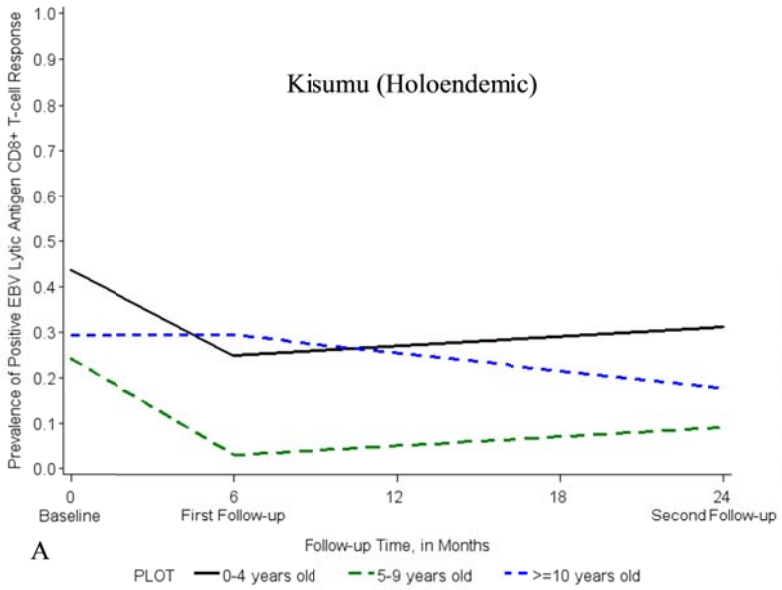
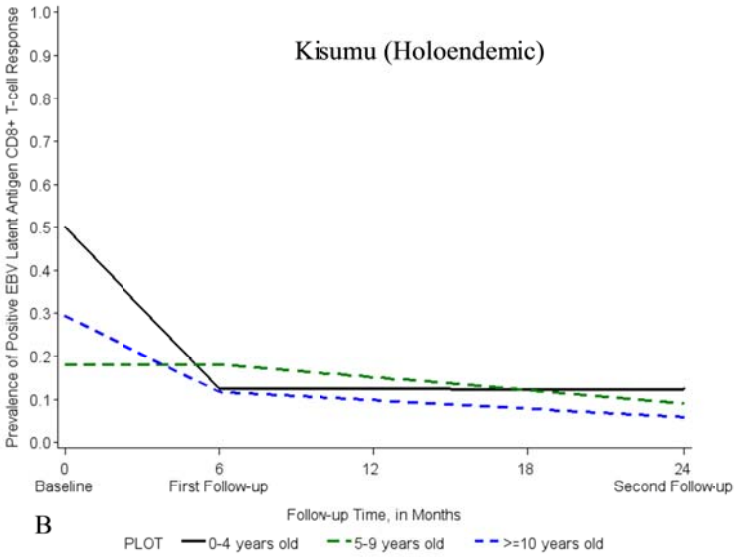
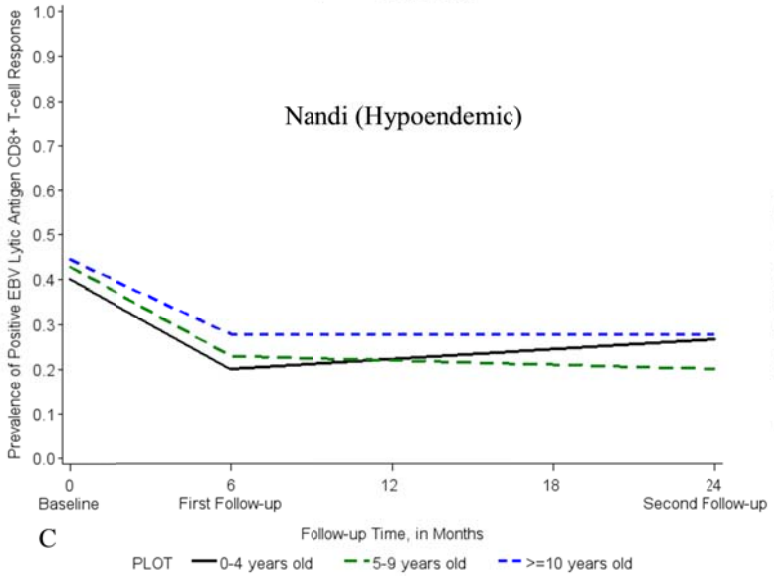


FIGURE 5.1. Malaria incidence in the highland area of Kipsamoite, 2001-2004.


Kisumu (Holoendemic)
Prevalence of Positive EBV Lytic Antigen CD8+ T-cell Response
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
6
12
18
24
Baseline
First Follow-up
Second Follow-up
A
Follow-up Time, in Months
PLOT
0-4 years old
5-9 years old
>=10 years old


Kisumu (Holoendemic)
Prevalence of Positive EBV Latent Antigen CD8+ T-cell Response
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
6
12
18
24
Baseline
First Follow-up
Second Follow-up
B
Follow-up Time, in Months
PLOT
0-4 years old
5-9 years old
>=10 years old


Nandi (Hypoendemic)
Prevalence of Positive EBV Lytic Antigen CD8+ T-cell Response
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
6
12
18
24
Baseline
First Follow-up
Second Follow-up
C
Follow-up Time, in Months
PLOT
0-4 years old
5-9 years old
>=10 years old

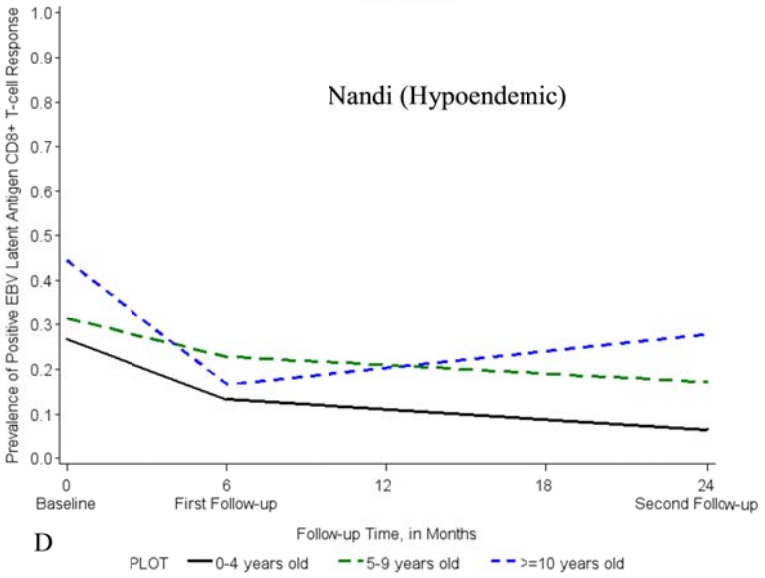

Nandi (Hypoendemic)
Prevalence of Positive EBV Latent Antigen CD8+ T-cell Response
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
6
12
18
24
Baseline
First Follow-up
Second Follow-up
D
Follow-up Time, in Months
PLOT
0-4 years old
5-9 years old
>=10 years old

FIGURE 5.2. Change in the prevalence of positive EBV lytic (A and C) and latent (B and D) antigen CD8+ T-cell IFN-γ response by age group at baseline, Kenya 2002-2004. Age group at each survey period is based on age at baseline. In Kisumu: 16 (0-4 years), 33 (5-9 years) and 17 ($\geq$10 years). In Nandi: 30 (0-4 years), 35 (5-9 years) and 18 ($\geq$10 years).

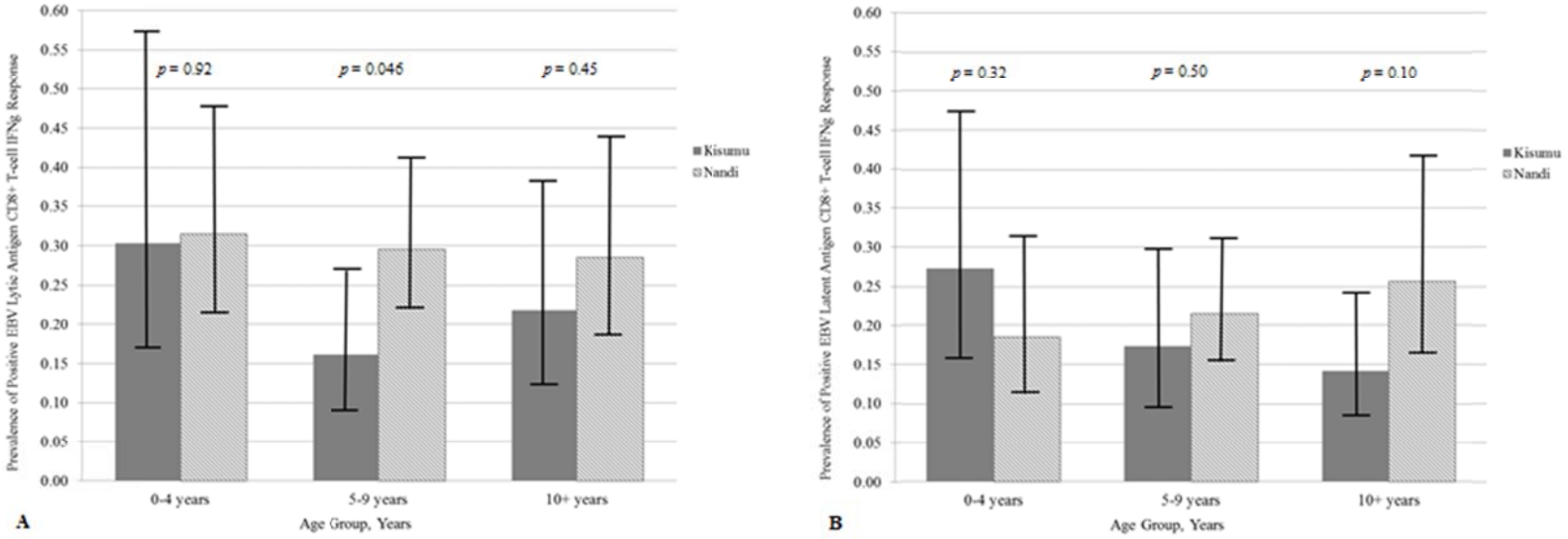


FIGURE 5.3. Prevalence of positive EBV lytic (A) and latent (B) antigen CD8+ T-cell IFN-γ response by age group and district of residence, Kenya 2002-2004. Age group was classified as a time-varying factor. For both graphs, the number of observations for children in each age group in Kisumu was: 33 (0-4 years), 87 (5-9 years) and 78 (≥10 years). The number of observations for children in each age group in Nandi was: 54 (0-4 years), 125 (5-9 years) and 70 (≥10 years). *P*-values for differences between areas of residence by age group are indicated.

TABLE 5.1. Summary of participants in the Kisumu/Nandi Cohort, Kenya 2002-2004[a]

	Site				Total
	Kisumu (holoendemic)		Nandi (hypoendemic)		
	n	%	n	%	n
Sex					
Male	39	59.1	38	45.8	77
Female	27	40.9	45	54.2	72
Age (in years)					
0-4	16	24.2	30	36.1	46
5-9	33	50.0	35	42.2	68
≥10	17	25.8	18	21.7	35
Malaria infections					
All surveys	38	57.6	0	0	38
Two surveys	20	30.3	4	4.8	24
One survey	6	9.1	14	16.9	20
Never	2	3.0	65	78.3	67
Total	66		83		149

NOTE. n, number; %, percentage.

[a] Data in the table are weighted according to the 149 children who participated in all surveys and had interpretable Epstein-Barr virus (EBV) specific CD8+ T-cell IFN-γ response.

TABLE 5.2. Prevalence and magnitude of EBV-specific CD8+ T-cell IFN-γ response by site of residence and age group, Kenya 2002-2004[a]

	EBV lytic antigens			EBV latent antigens			PHA[b]	
	n	%	Median[c] (range)	n	%	Median[c] (range)	n	%
Baseline (July-August 2002)								
Kisumu								
0-4 years	7/16	43.8	96 (14-166)	8/16	50.0	43 (20-98)	13/16	81.3
5-9 years	8/33	24.2	67 (18-170)	6/33	18.2	47 (16-448)	31/33	93.9
≥10 years	5/17	29.4	150 (20-350)	5/17	29.4	46 (16-404)	15/17	88.2
Nandi								
0-4 years	12/30	34.3	98 (28-836)	8/30	26.7	70 (18-146)	28/30	93.3
5-9 years	15/35	42.9	50 (22-792)	11/35	31.4	84 (42-668)	33/35	94.3
≥10 years	8/18	22.9	53 (36-304)	8/18	44.4	88 (26-1322)	18/18	100
First follow-up (February-March 2003)								
Kisumu								
0-4 years	4/16	25.0	46 (40-128)	2/16	12.5	55 (32-78)	16/16	100
5-9 years	1/33	3.0	20 (20)	6/33	18.2	15 (14-132)	31/33	93.9
≥10 years	5/17	29.4	30 (18-162)	2/17	11.8	23 (18-28)	16/17	94.1
Nandi								
0-4 years	6/30	20.0	98 (24-744)	4/30	13.3	77 (32-128)	26/30	86.7
5-9 years	8/35	22.9	82 (16-1742)	8/35	22.9	58 (20-248)	34/35	97.1
≥10 years	5/18	27.8	54 (32-382)	3/18	16.7	54 (14-354)	18/18	100
Second follow-up (July-August 2004)								
Kisumu								
0-4 years	5/16	31.3	76 (30-84)	2/16	12.5	106 (64-148)	15/16	93.8
5-9 years	3/33	9.1	60 (56-150)	3/33	9.1	42 (24-74)	33/33	100
≥10 years	3/17	17.7	250 (40-288)	1/17	5.9	16 (16)	17/17	100
Nandi								
0-4 years	8/30	26.7	50 (14-384)	2/30	6.7	69 (58-80)	25/30	83.3
5-9 years	7/35	20.0	76 (14-278)	6/35	17.1	59 (14-214)	31/35	88.6
≥10 years	5/18	27.8	26 (14-130)	5/18	27.8	56 (22-122)	18/18	100

NOTE. n, number; %, percentage; EBV, Epstein-Barr Virus; PHA, Phytohemagglutinin.

[a] Data in the table are weighted according to the 149 children who participated in all surveys and had interpretable Epstein-Barr Virus (EBV) specific CD8+ T-cell IFN-γ response.

[b] Phytohemagglutinin (PHA) was used as a positive control.

[c] Median EBV-specific CD8+ T-cell IFN-γ responses were calculated among children with positive responses and is expressed as spot forming units (SFU) per 1 x 10^6 peripheral blood mononuclear cells (PBMC).

TABLE 5.3. Unadjusted and adjusted prevalence ratio (PR) and 95% confidence interval (CI) for *Pf*-malaria infection and positive EBV lytic antigen CD8+ T-cell IFN-γ response by age group and survey period, Kenya 2002-2004

	Kisumu Constant *Pf*-malaria infection versus no infection		Nandi Constant *Pf*-malaria infection versus no infection	
	PR	95% CI	PR	95% CI
Unadjusted	0.64	0.23-1.77	1.43	0.73-2.81
Age groups [a]				
0-4 years	1.31	0.28-6.18	3.00	1.72-5.23
5-9 years	0.53	0.15-1.88	1.16	0.39-3.45
≥10 years	0.78	0.16-3.53	0.98	0.33-2.95
Survey periods [b]				
Baseline	1.24	0.49-3.11	1.76	1.07-2.91
Six months	0.29	0.05-1.62	0.73	0.17-3.22
Two years	0.21	0.05-0.92	0.22	0.02-3.23

NOTE. *Pf*-malaria, *Plasmodium falciparum* malaria; EBV, Epstein-Barr virus; PR, prevalence ratio; CI, confidence interval; Ref, referent group.

[a] Adjusted for sex and survey period. Unstratified estimate for constant *Pf*-malaria compared to never infected in Kisumu ($P = 0.72$) and Nandi ($P = 0.97$) were not significant. Specific details on the number and prevalence of positive responses for each age group are included in Table 2.

[b] Adjusted for sex and age group. Unstratified estimate for constant *Pf*-malaria compared to never infected was not significant infected in Kisumu ($P = 0.65$) but significant in Nandi ($P = 0.03$). The number of children in Kisumu for each survey period was 66 and the number of children in Nandi was 83.

TABLE 5.4. Unadjusted and adjusted prevalence ratio (PR) and 95% confidence interval (CI) for *Pf*-malaria infection and positive EBV latent antigen CD8+ T-cell IFN-γ response, Kenya 2002-2004

	Kisumu Constant *Pf*-malaria infection versus no infection		Nandi Constant *Pf*-malaria infection versus no infection	
	PR	95% CI	PR	95% CI
Unadjusted	1.60	0.37-6.92	1.54	0.71-3.35
Age groups [a]				
0-4 years	2.10	0.22-19.65	0.51	0.08-3.37
5-9 years	1.14	0.26-4.99	1.47	0.58-3.63
≥10 years	2.68	0.38-18.73	1.82	0.83-3.99

NOTE. *Pf*-malaria, *Plasmodium falciparum* malaria; EBV, Epstein - Barr virus; PR, prevalence ratio; CI, confidence interval.

[a] Adjusted for sex and survey period. Unstratified estimate for constant *Pf*-malaria compared to never infected in Kisumu ($P = 0.32$) and Nandi ($P = 0.13$) were not significant. Specific details on the number and prevalence of positive responses for each age group are included in Table 2.

CHAPTER SIX: Children's Antibody Responses to Select Malaria Antigens Differentially Develop and Wane by Malaria Transmission Intensity in Kenya

ABSTRACT

Background. The development of malarial antibodies that mediate protective immunity to *Plasmodium falciparum (Pf)* infection depend on malaria transmission intensity. However, more information is needed on the heterogeneity and kinetics of this multi-antigen response, particularly in areas of low malaria transmission.

Methods. A cohort of 236 children aged 10 months to 15 years, living in areas of holoendemic (Kisumu) and hypoendemic (Nandi) *Pf*-malaria transmission in Kenya, were surveyed at baseline and six-months. Determinants of IgG responses to five *P. falciparum* antigens (AMA-1 3D7, AMA-1 FVO, MSP-1_{42} 3D7, MSP-1_{42} FVO, and LSA-1) were contrasted between the two areas. We also examined the relative change of antibody responses between the two surveys (six-months).

Results. The proportion of positive IgG responses for all age groups was higher in Kisumu than Nandi; these were significant ($P < .05$) for AMA-1 3D7, AMA-1 FVO, and LSA-1 for both surveys. Antibody responses increased with age in Nandi but not in Kisumu. The magnitude of the decrease in the relative change in IgG responses to AMA-1 3D7, AMA-1 FVO and MSP-1_{42} 3D7 over a six-month period was two-fold

greater ($P < .05$) among children 0-4 years old in Nandi compared to similarly aged children in Kisumu. Antibody responses to AMA-1 3D7, AMA-1 FVO, $MSP1_{42}$-3D7, and LSA-1 among aparasitemic children were higher ($P < .05$) in Kisumu than Nandi. There were differences ($P < .05$) in antibody responses by parasitemia status in Nandi but few in Kisumu. Males in Kisumu had higher ($P < .05$) antibody responses to AMA-1 3D7, AMA-1 FVO, $MSP1_{42}$-3D7, and LSA-1 than those in Nandi. All measured antibodies correlated strongly with one another in Nandi ($P < .001$) but few correlated in Kisumu. In general, antibodies waned over the six-month period by age, parasitemia status, and sex in both districts. The magnitude of the relative change in antibody responses was often more pronounced in Nandi than Kisumu. The correlation in the median relative change in antibodies responses bore similar patterns to those observed in other cross-sectional studies.

***Conclusion*.** Important differences in the pattern of naturally acquired immunity to *P. falciparum* exist by age, parasitemia status, and sex between areas of holoendemic and hypoendemic malaria transmission. These findings highlight the need to consider these factors when considering which antigens to target for vaccine development.

INTRODUCTION

Across the globe, an estimated 225 million individuals experienced malaria infections in 2009, resulting in an estimated 780,000 deaths [1]. Efforts have been underway to create an effective vaccine that can further reduce the global burden of

malaria-related morbidity and mortality, which are greatest on the African continent where 78% of infections and 91% of deaths occurred, the majority in children <5 years [1].

Almost 50 years ago, studies of passive transfer of serum gamma-globulin from *Plasmodium falciparum* (*Pf*-) malaria immune adults to symptomatic children demonstrated that antibodies played a key role in controlling *Pf*-malaria infection [88-90, 155]. Yet the identification of an effective vaccine has been hindered by our limited understanding of how immunologic memory to *Pf*-malaria is developed and sustained in humans as well as the selection of immunogenic malaria antigens as vaccine candidates [82]. Naturally acquired immunity to *Pf*-malaria does not lead to sterile immunity; adults residing in *Pf*-malaria endemic areas develop partial immunity that leads to asymptomatic infection in the presence of parasites. Rather, the critical role for antibodies appears to be their ability to establish protective immunity to clinical *Pf*-malaria infections by reducing the density of blood-stage parasitemia [122] after repeated exposures to *Pf*-malaria, thereby preventing severe manifestations of *Pf*-malaria infection (reviewed in [93]). Antibodies to blood-stage antigens have been shown to prevent merozoites from attaching and invading red-blood cells (reviewed in [91, 93]). However, it is unclear which antibody, or combination of antibodies, confer protection from clinical *Pf*-malaria infections.

A recent meta-analysis of population-based cohort studies found that individuals with IgG responses to the merozoite surface protein (MSP)-1_{19} were at less risk for clinical *Pf*-malaria infection compared to those without IgG responses [91]. A similar conclusion was made for the 3D7 variant of the apical membrane antigen

(AMA)-1 but for AMA-1 FVO, findings were inconsistent [91]. However, most studies included in the meta-analysis were conducted in areas of stable perennial and seasonal malaria transmission. Malaria endemicity influences the development of protective immunity [11, 124] and studies examining IgG responses in hypoendemic (low and unstable) malaria areas are limited. One potential explanation for this differential pattern may be the reportedly short half-life of IgG1 responses. IgG1 responses were reported to have a half-life of 21 days [108] but another more recent study of a combination of blood-stage antigens (including MSP-1 and AMA-1) reported IgG1 responses had a half-life of 10 days [107]. The implications would be that individuals infrequently exposed to *Pf*-malaria would not be able to sustain necessary antibody levels for protection from clinical *Pf*-malaria infection.

To gain further insight into the transmission-intensity dependent heterogeneity of malaria antibody responses, we described IgG responses of children residing a *Pf*-malaria holoendemic area and contrasted them to a hypoendemic area for the blood-stage antigens AMA-1 3D7, AMA-1 FVO, MSP-1_{42} 3D7, and MSP-1_{42} FVO. We also included the pre-erythrocytic liver stage antigen (LSA)-1. Moreover, we calculated the relative change in IgG responses over a six-month period to examine and contrast the pattern of change between the two districts.

METHODS

Data from the Kisumu/Nandi cohort was used and has been previously described [59]. The cohort consists of 236 children aged 10 months to 15 years from Kisumu and Nandi districts in western Kenya. *Pf*-malaria transmission in Kisumu is described as

holoendemic (perennial and stable) while in Nandi transmission is hypoendemic (low and unstable). Data were collected in July-August 2002 (baseline survey) and February-March 2003 (six-month follow up survey).

Although *Pf*-malaria is holoendemic in Kisumu, there are relative peaks in transmission intensity after the long rains (March-May) and short rains (October-December) [156]. Therefore the baseline survey was conducted after the long rains and the six-month follow-up survey was conducted before the long rains in following year. Nandi experienced a classically defined malaria outbreak during the baseline survey and a peak in transmission six months later with an intervening period of low malaria incidence (C John, unpublished data).

Equal distributions of children were enrolled by age and sex. During the two face-to-face interviews, standardized forms were used to collect data and blood was collected for *Pf*-malaria parasitemia and antibody testing. Parents or guardians of participants provided written informed consent. The Institutional Review Board at the University Hospitals of Cleveland, Case Western Reserve University (AM's affiliation at the time of the study) and the Ethical Review Committee for the Kenya Medical Research Institute approved the original study. The study was exempted by the Institutional Review Board at the University of North Carolina at Chapel Hill.

Confirmation of *Pf*-malaria infection was done by microscopy of thick and thin blood smears. Testing for *Pf*-malaria antibody used the same approach as previously described [153]. Immunoglobulin G (IgG) specific for the AMA-1 3D7, AMA-1 FVO, MSP-1_{42} 3D7, MSP-1_{42} FVO, and LSA-1 were detected using a bioplex bead-based assay. One-thousand beads of each malaria antigen were placed in

wells with plasma from participants and diluted to 1:5000. Included on each plate were negative controls (US residents with no history of malaria) and positive controls (pooled samples from Kisumu residents). Preparation of beads and testing were conducted on all samples at the same time on the same machine to reduce potential variation due to differences in bead preparation and assay. Although antibody results were calculated as mean fluorescence intensity (MFI), slight plate-to-plate variation led to a need to standardize results. Therefore results are expressed in arbitrary units (AU). For each plate, the participant's AU values were calculated by dividing each participant's MFI antibody response by the negative controls' mean MFI plus three standard deviations. AU values greater than 1.0 indicated a positive IgG response.

We analyzed cross-sectional data collected at baseline and six-month follow-up stratified by district. Outcomes were dichotomous positive/negative IgG responses to all five *Pf*-malaria antigens. Exposures of interest were age group (0-4 years, 5-9 years, and $\geq$10 years), parasitemia status (positive/negative), and sex. The Chi-square test and Cochran Armitage trend test were used to assess any significant differences among the levels of each exposure as well as to compare responses between the two districts. Spearman's rank correlation coefficient was used to assess correlation among the different *Pf*-malaria malaria antibodies measured as continuous AU values.

To describe the changes in *Pf*-malaria antibody response over time, we assessed the relative change in IgG response to the five *Pf*-malaria antigens over the six-month period between the two survey periods, stratified by district. However,

the number of children who participated in the latter survey decreased from 236 to 210 children, representing an 11% loss to follow-up. We did not identify any significant differences between the population of children who participated in the six-month follow-up survey and those who did not. Continuous AU values were used to calculate the relative change in IgG response with the formula [(IgG response at baseline – IgG response at six-month follow-up)/IgG response at baseline]. Exposures included age group at baseline, parasitemia status (parasitemia at both survey periods, parasitemia at first survey period only, parasitemia at second survey period only, and never parasitemic), and sex. Levels of our exposure parasitemia were created based on previous findings that the presence of parasitemia was associated with higher levels of blood-stage antibodies [115, 116]. IgG responses to the five *Pf*-malaria antigens were not normally distributed therefore we used the nonparametric two-sided Wilcoxon rank sum (Mann-Whitney *U*)/Kruskal Wallis test to examine any differences among the exposure levels and between districts. The Exact Wilcoxon/Kruskal-Wallis test was used for small sample sizes. An extension of the Wilcoxon rank-sum test was used to test the trend of ordinal variables. Spearman's rank correlation coefficients were calculated to assess correlation in the relative change between the different malaria antibodies. All analyses were conducted in SAS 9.2 (Cary, NC).

RESULTS

Study participants. Of the 236 children in the cohort, interpretable results were available for 229 (97%) children at baseline and 207 (88%) children at six-month

follow-up (Table 6.1). There were no differences by age group and sex between the districts. A significant difference by parasitemia status was observed between Kisumu and Nandi children ($P < .001$) at both survey periods; an estimated >76% of children in Kisumu were parasitemic at both survey periods compared to <16% in Nandi. The median antibody response to all antigens except MSP-1_{42} FVO was significantly higher in Kisumu compared to Nandi (Table 6.2).

The proportion of positive IgG responses at baseline and six-month follow-up was higher in Kisumu than Nandi. In general, the proportion of positive IgG responses was higher in Kisumu than Nandi by age group, parasitemia status, and sex; many of these differences reached statistical significance.

When antibody responses were examined by age group, we found that in Kisumu (Figure 6.1A) there were a high proportion (>80%) of positive IgG responses to all *Pf*-malaria antigens among all age groups at baseline except MSP-1_{42} FVO; yet there was an increasing trend in positive IgG response to MSP-1_{42} FVO with increasing age from 60-83% ($P = .045$). There remained a high proportion of positive IgG responses to the *Pf*-malaria antigens at the six-month follow-up (Figure 6.1B) with the exception of MSP-1_{42} FVO; responses increased from 48%-68% with age but this was not significant ($P = .15$). In contrast, the proportion of positive IgG responses to *Pf*-malaria antigens was relatively low in Nandi (<77%) compared to Kisumu at both survey periods. At baseline, this difference in positive responses was significant for all age groups for all *Pf*-malaria antigens except MSP-1_{42} FVO where the proportion of positive responders was similar between the districts (Figures 6.1A and 6.1C). At six-month follow-up, significant differences by age groups between the

districts remained for AMA-1 3D7, AMA-1 FVO, MSP-1_{42} 3D7, and LSA-1 for all age groups except among the $\geq$10 year old responders to MSP-1_{42} 3D7 where responses were similar between districts (Figures 6.1B and 6.1D). Furthermore in Nandi, a trend in increasing positive IgG responses with age group was observed for all *Pf*-malaria antigens; however, this was not statistically significant (Figure 6.1C). At six-month follow-up this increase in IgG response with age group was statistically significant for all *Pf*-malaria antigens except LSA-1 (Figure 6.1D). Taken together, these results indicate the proportion of positive IgG responses among all age groups is higher in Kisumu relative to Nandi for all antigens except MSP-1_{42} FVO; however, age trends with IgG responses were primarily detected in Nandi and not Kisumu.

In our assessment of parasitemia status and antibody response, we found that IgG responses to AMA-1 3D7, AMA-1 FVO, and MSP-1_{42} FVO did not differ by parasitemia status at baseline or six-month follow-up in Kisumu; however, significant differences were noted for LSA-1 at both survey periods and MSP-1_{42} 3D7 at six-month follow-up (Figures 6.2A and 6.2B). In Nandi, however, significant differences in IgG responses to all *Pf*-malaria antigens by parasitemia status were observed at baseline. Differences in Nandi were also observed at six-month follow-up for AMA-1 3D7, AMA-1 FVO, and LSA-1. When the districts were contrasted, we observed similar proportions of positive IgG responses among parasitemic children at baseline and six-month follow-up (except for AMA-1 3D7 and AMA-1 FVO during the latter survey). However, aparasitemic children in Kisumu had elevated proportions of positive IgG responses than aparasitemic children in Nandi at both survey periods for all antigens except MSP1_{42}-FVO (Figures 6.2A and 6.2B). This would suggest

that aparasitemic children in Kisumu were able to maintain antibodies to pre-erythrocytic and blood-stage antigens in the absence of stimulation from parasites. Meanwhile, children who were parasitemic were able to elicit an immune response regardless of their residence.

There were no differences in IgG responses between males and females in either district, during either survey (data not shown). Yet at baseline, males and females in Kisumu had a significantly higher proportion ($P < .05$) of positive responses when compared to their counterparts in Nandi to AMA-1 3D7 (>97% vs <64%), AMA-1 FVO (>95% vs <68%), MSP-1_{42} 3D7 (>95% vs <66%), and LSA-1 (>90% vs <56%). This significant difference was also present at six-month follow-up, where higher IgG responses to MSP-1_{42} FVO was found among females in Kisumu compared to Nandi (71% vs 46%). Adjusting for parasitemia status accounted for differences between females but significant differences remained among males.

Correlations between antibodies differed by malaria endemicity. Examination of the correlation between antibodies highlighted a few interesting observations (Tables 6.3A-6.3D). In Kisumu, correlations between allelic variants of the same antigen (AMA-1 3D7 and AMA-1 FVO; MSP-1_{42} 3D7 and MSP-1_{42} FVO) were high (Spearman's rho range: 0.755-0.953) at both survey periods (Tables 6.3A and 6.3B). Furthermore, there were relatively strong correlations (Spearman's rho range: 0.490 - 0.575) between LSA-1 and MSP-1_{42} antigens at both survey periods. At six-month follow-up, antibodies to LSA-1 were also slightly correlated to the AMA-1 antigens (Spearman's rho range: 0.268 - 0.284) but not at baseline. There was slight correlation between AMA-1 FVO and MSP-1_{42} 3D7 (Spearman's rho: 0.226) at

baseline but this was not observed at six-month follow-up. However, in Nandi, there were significantly strong correlations between all the antibodies (Spearman's rho range: 0.563-0.967) (Tables 6.3C and 6.3D). The pattern of correlation among antibodies suggest that in holoendemic areas such as Kisumu, specific antibodies are produced in response to *Pf*-malaria infection while in hypoendemic area such as Nandi, a broad array of antibodies are produced.

The relative change in antibody responses to malaria antigens suggests waning of antibodies over time. Overall, the median relative change in antibody response for all antigens decreased over the six-month period in Kisumu and Nandi by age group, parasitemia status, and sex (Tables 6.4 and 6.5). This suggests that antibody responses wane over time and therefore a proportion of antibodies are inferred to be short-lived, even in holoendemic areas.

In Kisumu, there were no significant differences in the median relative change by age group for any of the *Pf*-malaria antigens (Table 6.4). In addition, there was no observed trend in the relative change of antibody responses with age group. In Nandi, there were no differences among the age groups; however, there was a pattern observed for all antigens (except MSP-1_{42} 3D7) in which the magnitude of the relative change became smaller with increasing age (Table 6.5). Hence the children in the youngest age groups experienced the largest decline in antibody responses compared to children in older age groups. When the median relative change in antibody responses was compared between the districts, the magnitude of the decrease in antibody response was larger in Nandi (40-52%) compared to Kisumu (12-22%). These 2-3 fold differences were significant for AMA-1 3D7, AMA-

1 FVO, and MSP-1_{42} 3D7 among children 0-4 years of age and AMA-1 FVO, and MSP-1_{42} FVO among children 5-9 years old (Tables 6.4 and 6.5).

We observed a number of intriguing patterns in the relative change of antibody responses in relation to parasitemia status. There was a significant difference in the median relative change in antibody responses to MSP-1_{42} 3D7 by parasitemia status in Kisumu (Table 6.4); however, there were no significant findings in Nandi (Table 6.5). As previously noted, the median relative change in antibody responses over the six-month period decreased regardless of parasitemia status; however, the degree of the median relative change in antibody response varied by parasitemia status and district (Tables 6.4 and 6.5). In Kisumu, the largest median relative change was among children who were aparasitemic at both survey periods, ranging from 38% (MSP-1_{42} 3D7) to 69% (LSA-1). This indicates that in holoendemic areas, the lack of immune stimulation from infection results in a substantial loss of antibodies compared to those infected at least once during the surveys. In Nandi, the pattern is not as clear. The greatest decrease in the median relative change in antibody responses to MSP-1_{42} 3D7 (60%), MSP-1_{42} FVO (48%), and LSA-1 (59%) were among children who were parasitemic at baseline only. These results are plausible because we would expect children with limited exposure to *Pf*-malaria infection to have a sudden increase in antibody responses while parasitemic, resulting in a sizable decrease once parasitemia was cleared. However, for AMA-1 3D7, the largest change (40%) was among children who were parasitemic at six-month follow-up only while for AMA-1 FVO (42%) it was among children who were aparasitemic at both survey periods. The finding for AMA-1 3D7 is counterintuitive

because we would expect the largest increase in the median relative change to be among those who later became parasitemic, not the largest decrease. Meanwhile, the finding for AMA-1 FVO is less puzzling but still unexpected. We did not expect children in Nandi who were aparasitemic at both survey periods to have elevated antibody levels. However, a closer examination of aparasitemic children showed that the median relative change in antibody responses to all antigens ranged from a 33-45% decrease, once again suggesting that antibodies wane without parasite stimulation. As for the smallest amount of median relative change in antibody responses, patterns were similar in Kisumu and Nandi. Children parasitemic at both survey periods had the smallest median relative change for AMA-1 3D7 and AMA-1 FVO whereas the smallest median relative change for MSP-1_{42} 3D7, MSP-1_{42} FVO and LSA-1 were among children who were parasitemic only at six-month follow-up. Both of these findings are feasible because we would expect few changes in antibody responses in children who were parasitemic at both survey periods, or whose antibody responses would have increased as a result of parasitemia during the latter survey.

We also noted interesting patterns in parasitemia status when we drew comparisons between Kisumu and Nandi (Tables 6.4 and 6.5). First, among children who were parasitemic at both survey periods, the magnitude of the median relative change was greater in Kisumu than Nandi for all antigens except MSP-1_{42} FVO. This pattern of a greater magnitude in median relative change in Kisumu compared to Nandi was also observed among children who were aparasitemic at both survey periods. Both observations suggest that antibody responses wane more so in

Kisumu than Nandi and are inconsistent with the idea that children in holoendemic areas should have less of a decline in antibody responses over time than those in hypoendemic areas. However, upon further examination, there were few children (n=5) in Nandi who were parasitemic and few children (n=6) in Kisumu who were aparasitemic at both survey periods. Data from these limited observations could explain the inconsistency between our expectations and our results. Second, the magnitude in the median relative change was greater in Nandi than Kisumu among children who were parasitemic only at baseline. This is in accordance with our expectation that children repeatedly infected with *Pf*-malaria would be able to maintain antibodies better than children with limited exposure. Finally, among children who were parasitemic only at six-month follow-up, the magnitude of the median relative change was once again greater in Nandi than Kisumu. Likewise this observation is plausible because children repeatedly infected with *Pf*-malaria should theoretically have antibody levels greater than children rarely infected.

In general, the median relative change in antibody response was greater in females than males in Kisumu but these were not significant (Table 6.4). Males in Nandi had higher median relative change in antibody responses than females but this also did not reach statistical significance (Table 6.5). Significant differences in the median relative change were identified between males in Kisumu and Nandi for AMA-1 3D7, AMA-1 FVO, and MSP-1_{42} FVO whereas only a significant difference among females between the two districts was noted for AMA-1 FVO. Once again, these differences are linked to heterogeneity of malaria transmission intensities at the districts.

Antibodies do not decrease independently of one another over time. We detected significant correlations in the relative change in antibody responses to all antigens in Kisumu and Nandi (Table 6.6) and these correlations mimicked patterns previously observed in the cross-sectional analyses. In Kisumu, the strongest correlations were between genotypes of the same antigen (Spearman's rho range: 0.816-0.914) while moderately strong correlations were observed between LSA-1 and the MSP-1_{42} genotypes (Spearman's rho range: 0.623-0.682). The correlations between the AMA-1 genotypes to MSP-1_{42} genotypes and LSA-1 genotypes were modestly correlated (Spearman's rho range: 0.374-0.387) whereas in the cross-sectional analyses, these were not correlated. Once again in Nandi, all relative changes in antibody responses were strongly correlated to one another (Spearman's rho range: 0.504-0.927) whereas the correlation between MSP-1_{42} 3D7 and LSA-1 (Spearman's rho: 0.289) was modestly correlated, but still significant. These findings suggest that antibody responses do not decrease independently of one another but rather, there is a general waning of antibody responses over time, the pattern of which varies with the transmission intensity of the location.

DISCUSSION

As expected, we observed a higher proportion of positive IgG responses to select *Pf*-malaria antigens by age, parasitemia status, and sex in a *Pf*-malaria holoendemic area compared to a hypoendemic area. We also detected significant differences in the median relative change in antibody responses over a six-month period by age group and sex between the districts; intriguing patterns were observed between

districts in the relative change in antibody responses by parasitemia status. Within each district, however, there were few differences by age and parasitemia status in Kisumu whereas a number of important differences were detected in Nandi; there were no observed differences by sex in either district. We also did not observe any differences in the median relative change in antibody responses over six-months within each district by age, parasitemia status, or sex.

Our findings of positive IgG response in relation to age group between the districts highlight the disparate immunological experiences of children living in *Pf*-malaria holoendemic areas compared to hypoendemic areas. Children in areas like Kisumu experience repeated *Pf*-malaria infections shortly after birth, building a repertoire of antibodies that help them develop immunity to clinical malaria infection by 2-3 years and decreased parasite density between ages 10-14 [122]. However, the development of antibody responses is delayed in areas such as Nandi where children and adults of all ages are still susceptible to clinical malaria infection [10, 124, 157]. Hence it is not surprising that we detected significant differences in the proportion of positive IgG responses to the blood-stage antigens between the two districts. We only detected a few patterns of increasing antibody response (MSP-1_{42} FVO and LSA-1) with age in Kisumu, although not always significant. The lack of an age trend among AMA-1 responders is not unexpected as AMA-1 (3D7 and FVO) is highly immunogenic [94], as evidenced by the near saturation of responses among all age groups at both time points. Furthermore, young children often have the highest levels of parasites, which are associated with elevated antibody responses. Yet what is of interest is the apparent age trend observed in Nandi, which we would have

expected in Kisumu. Of interest is that Nandi experienced a classically defined malaria outbreak during the first survey and a peak in transmission during the second survey. Thus the pattern we observed in Nandi likely exemplifies the age trend often used to characterize the development of immunity in stable areas of malaria transmission. Furthermore, the magnitude of the median relative change differed between the two districts by age groups. Although antibodies appeared to wane over time in both districts, the magnitude of the median relative change in IgG response to AMA-1 3D7, AMA-1 FVO, and MSP-1_{42} 3D7 among Nandi children aged 0-4 years was a 2-3 fold difference compared to similarly aged children in Kisumu ($P < .05$). Furthermore, there was a general pattern in Nandi in which the magnitude of the relative change decreased from the youngest age group to the oldest age group. Once again, this reinforces the theory that young children in hypoendemic malaria transmission areas have lower antibody levels than children in holoendemic areas. Our findings of a lack of trend in Kisumu between age group and antibody response reiterates Drakeley's caution that using age-specific seroprevalence data to estimate malaria transmission intensities are highly dependent on the immunogenicity of antigens that are used as well as the geographic variability of the parasitic strain [94].

There was a striking difference in IgG responses for AMA-1 3D7, AMA-1 FVO, MSP-1_{42} 3D7, and LSA-1 between the districts among aparasitemic children. Antibodies to MSP-1 and AMA-1 are reportedly short-lived [107], therefore we would not expect to see high levels of positive IgG responses in aparasitemic individuals. However, immunity in those repeatedly infected with *Pf*-malaria reflects past and

present infections. Therefore we would hypothesize that antibodies to *Pf*-malaria infection would be produced from a combination of short-lived plasma cells (SLPC) and long-lived plasma cells (LLPC), of which LLPCs would accumulate over time in individuals repeatedly infected with *Pf*-malaria [111]. This is further supported by our observation of the magnitude of the relative change in antibody response by parasitemia status. Although there was a general waning of antibody responses over time suggesting that SLPCs comprise a substantial proportion of antibodies, the magnitude of the median relative change in antibody responses was smaller in Kisumu compared to Nandi among children parasitemic only at baseline or six-month follow-up. This suggests that LLPCs persist in Kisumu children whereas the increase in antibodies in Nandi children was due to SLPCs. However, we are cautious in our interpretations because parasitemia status and antibody responses were measured at the same time. Antibodies can persist after parasite clearance hence children who were aparasitemic but had elevated antibody responses may have cleared parasites shortly before the surveys, particularly in children living in Kisumu. Future studies that are able to accurately assess the temporal relationship between parasitemia and antibody response should be conducted to corroborate our findings.

The differences in positive IgG responses detected by sex between Kisumu and Nandi were unexpected. Heterogeneity in bednet usage could not explain this phenomenon because only 4% of participants in Kisumu and <1% in Nandi reported using bednets at the time the surveys were conducted0. After adjusting for parasitemia status, there was still a significant difference among males between the

districts. We can only conclude that there must be other factors unmeasured in our study that differed between the two groups.

Our observed patterns of correlation in antibody responses in the districts were consistent with another study in this area [101]. In Nandi there was strong correlation between all the antibodies suggesting that children periodically infected with malaria can elicit an immune response to a repertoire of antibodies. However in Kisumu, strong correlation in responses was only observed between the different AMA-1 genotypes, MSP-1 genotypes, and MSP-1 genotypes to LSA-1. The lack of evidence for correlation among the other antibodies (for example, AMA-1 3D7 and MSP-1 3D7) may be due to the high proportion of positive antibody responses, which provides limited statistical ability to detect significant correlations. This observation should serve as a cautionary note to researchers trying to identify immunological determinants of protection. Strong correlation among antibodies in hypoendemic *Pf*-malaria areas does not equate to protective immunity nor does limited correlation among antibody responses indicate the absence of protective immunity in holoendemic *Pf*-malaria areas. Future studies should examine correlations among additional pre-erythrocytic and blood stage-antigens as well as study areas with varying *Pf*-malaria transmission intensities to further explore this intriguing paradox.

There are limitations with our study. Like other studies of malaria antibody response, we are unable to address whether differences in IgG responses between the districts, or relative changes in antibody levels within an individual, reflect functional modifications in immune response. We may have also encountered

situations in which children were no longer parasitemic at the time of the survey but their antibody responses were still elevated. This was likely to occur predominantly in Kisumu where children experience numerous infections. However, we were able to detect parasitemia in >76% of Kisumu children at both surveys. Therefore this issue was likely to be minimal and did not affect our findings. Furthermore, although the use of AUs provides a means to standardize results within a study, AU values are not directly comparable across different studies. Hence we could only draw comparisons about directionality of findings. Another limitation is the nature of the relative change calculation and interpretation. A 200% increase may indicate change in IgG response from 0.2 AU to 0.6 AU yet this child remains a negative IgG responder. Finally, due to the limited power of our study, we were unable to examine more complex relationships in our analyses. For example, our analysis of parasitemia and relative change in antibodies levels over time was limited by the number of children who were aparasitemic at both survey periods in Kisumu (n=6) and parasitemic at both survey periods in Nandi (n=5). As a result, we did not have power to detect small yet important differences by parasitemia status.

Our study findings are generally consistent with previous studies of IgG responses in relation to malaria transmission intensity. We found a higher proportion of antibody responders with increasing malaria transmission intensity [94, 95, 101]. We detected significant differences in IgG responses by age group between the two districts and similar age trends within the districts [95, 101]. We also noted the saturation of AMA-1 (3D7, FVO) IgG responses across all age groups in Kisumu but not Nandi [94]. The increase in the proportion of antibody responses with age in Nandi may have

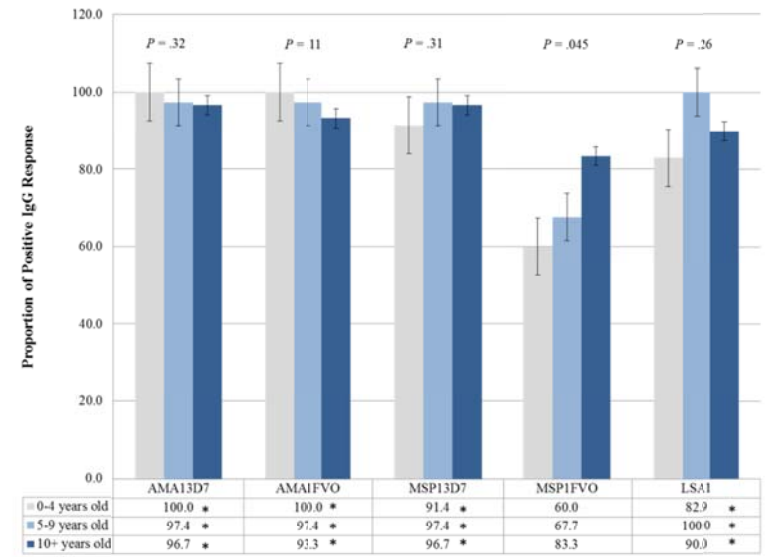
been due to the *Pf*-malaria infections from an on-ongoing outbreak yet our findings are similar to other studies in low malaria transmission areas where an age trend for AMA-1 and MSP-1 antigens was observed [94, 95, 101]. Patterns of correlation between antibody responses were consistent with a previous study by Noland and colleagues, including MSP-1 antibody patterns despite the use of different target antigens ($MSP\text{-}1_{42}$ in this study, $MSP\text{-}1_{19}$ in the other study) [101]. However, correlations differed from another study by Chelimo and colleagues that found a strong correlation between AMA-1 and MSP-1 antibodies, as well as a weak but significant correlation between LSA-1 and AMA-1 antibodies [119]. However the study led by Chelimo was conducted among infants and young children who have immunological capacities different from older children. We detected a difference in LSA-1 responses by parasitemia status in the malaria holoendemic area unlike Noland's study, despite both studies having been conducted in the same study area though during different time periods [101]. Differences may be attributed to assay and the use of AUs which varied between the studies. Our detection in an area of low malaria transmission of differences in IgG responses to AMA-1 and LSA-1 by parasite status was consistent with a study by Bull and colleagues that found similar differences during a low malaria-transmission season [115]. However, John and colleagues did not detect differences in LSA-1 antibody responses by parasitemia status [118]. Finally, our findings that antibodies wane over time regardless of malaria transmission intensity are also consistent with previous studies [100, 107].

Our findings reveal important differences in antibody responses between areas of high and low malaria transmission that could be used to inform future research on

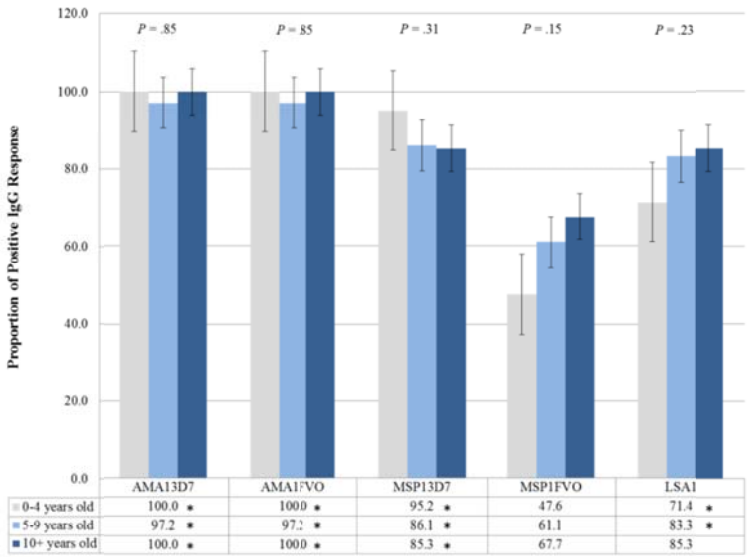
immunological markers of *Pf*-malaria protective immunity and vaccine development. First, we did not observe an age-trend in antibody responses in Kisumu because the proportion of positive responders in all groups was >83%, except to MSP-1_{42} FVO. This suggests that children <5 years of age are able to mount an immune response similar to older children. Could these two antigens induce an immune response that protects from clinical *Pf*-malaria infections? Studies of clinical *Pf*-malaria infections have often examined antigens independently. However it is likely that a multivalent vaccine will be necessary therefore longitudinal studies that examine multiple antibody responses in unison are needed. Furthermore, we identified that antibody responses waned over time, regardless of the malaria transmission intensity. However, the magnitude of change varied by antigens and was more pronounced among young children in the hypoendemic area. Many questions remain about B-cell memory to the array of *Pf*-malaria antigens. Are SLPCs the predominant source of antibodies in children infrequently infected with *Pf*-malaria, or only in young children? Does the loss in response represent a functional loss in immune response? Are the half-lives of IgG1 antibodies the same for all *Pf*-malaria antigens? There is a clear need for further research into the heterogeneity in function and longevity of *Pf*-malaria specific antibodies. Finally, as we pointed out earlier, patterns of antibody correlations differed between areas of holoendemic and hypoendemic malaria transmission. Caution should be taken when making interpretations because strong correlations among antibodies do not necessarily indicate protective immunity. Likewise, limited correlation among antibodies does not suggest the absence of protective immunity.

CONCLUSION

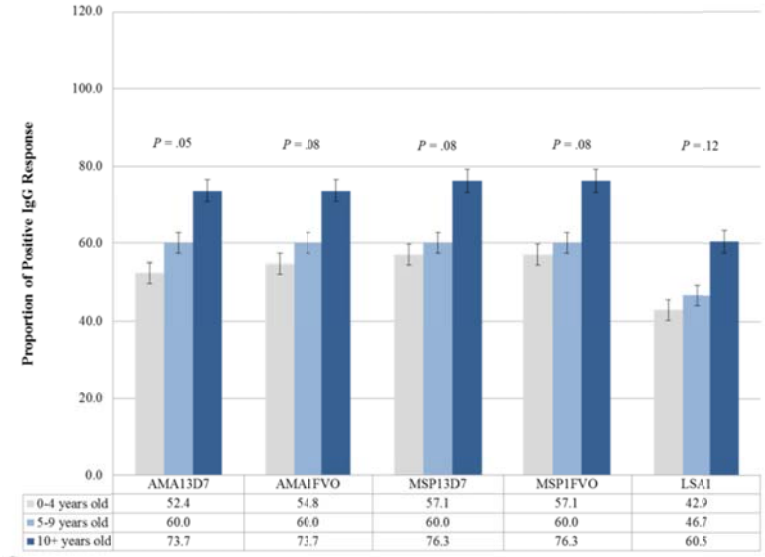
This study identified disparities in the proportion of positive IgG responses to select *Pf*-malaria antigens between a holoendemic and hypoendemic area of *Pf*-malaria transmission by age, parasitemia status, and sex. An overall waning of antibody responses was observed over six-months by age and parasitemia status; however, differences between the study populations in the magnitude of median relative change in antibody response by age was detected. These findings highlight the important differences in naturally acquired immunity between areas of disparate malaria transmission intensities and have important implications when assessing the effectiveness of vaccines across populations.


Proportion of Positive IgG Response
120.0
100.0
80.0
60.0
40.0
20.0
0.0
P = .32
P = 11
P = .31
P = .045
P = .26
AMA13D7
AMA1FVO
MSP13D7
MSP1FVO
LSA1
0-4 years old 100.0 * 100.0 * 91.4 * 60.0 82.9 *
5-9 years old 97.4 * 97.4 * 97.4 * 67.7 100.0 *
10+ years old 96.7 * 93.3 * 96.7 * 83.3 90.0 *

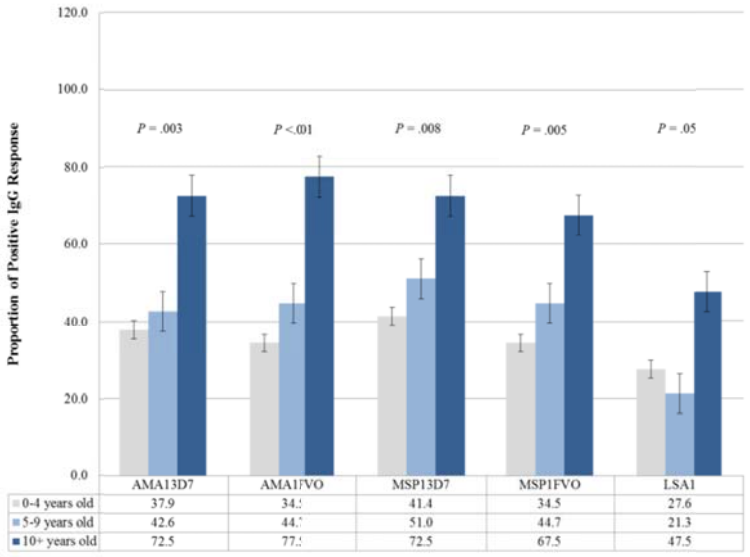
A


Proportion of Positive IgG Response
120.0
100.0
80.0
60.0
40.0
20.0
0.0
P = .85
P = .85
P = .31
P = .15
P = .23
AMA13D7
AMA1FVO
MSP13D7
MSP1FVO
LSA1
0-4 years old 100.0 * 100.0 * 95.2 * 47.6 71.4 *
5-9 years old 97.2 * 97.2 * 86.1 * 61.1 83.3 *
10+ years old 100.0 * 100.0 * 85.3 * 67.7 85.3

B


Proportion of Positive IgG Response
120.0
100.0
80.0
60.0
40.0
20.0
0.0
P = .05
P = .08
P = .08
P = .08
P = .12
AMA13D7
AMA1FVO
MSP13D7
MSP1FVO
LSA1
0-4 years old 52.4 54.8 57.1 57.1 42.9
5-9 years old 60.0 60.0 60.0 60.0 46.7
10+ years old 73.7 73.7 76.3 76.3 60.5

C


Proportion of Positive IgG Response
120.0
100.0
80.0
60.0
40.0
20.0
0.0
P = .003
P < .01
P = .008
P = .005
P = .05
AMA13D7
AMA1FVO
MSP13D7
MSP1FVO
LSA1
0-4 years old 37.9 34.2 41.4 34.5 27.6
5-9 years old 42.6 44.7 51.0 44.7 21.3
10+ years old 72.5 77.2 72.5 67.5 47.5

D

FIGURE 6.1. Proportion of IgG positive malaria antibody responses (>1 arbitrary units) by age group at baseline and six-month follow-up in Kisumu (A and B) and Nandi (C and D) in Kenya, 2002-2003. *P*-values in the graph represent the Cochran-Armitage Trend Test for differences in the proportion of positive antibody responses among age groups within a district. Asterisks represent statistically significant differences ($P < .01$) in the proportion of positive antibody responses between districts within the same age group. Bar represent error bars. At baseline, the number of children who were 0-4 years, 5-9 years, and $\geq$10 years in Kisumu was 35, 39, and 30, respectively while in Nandi, it was 42, 45, and 38, respectively. At the six-month follow-up, the number of children who were 0-4 years, 5-9 years, and $\geq$10 years in Kisumu was 21, 36, and 34, respectively and in Nandi, it was 29, 47, and 40, respectively.

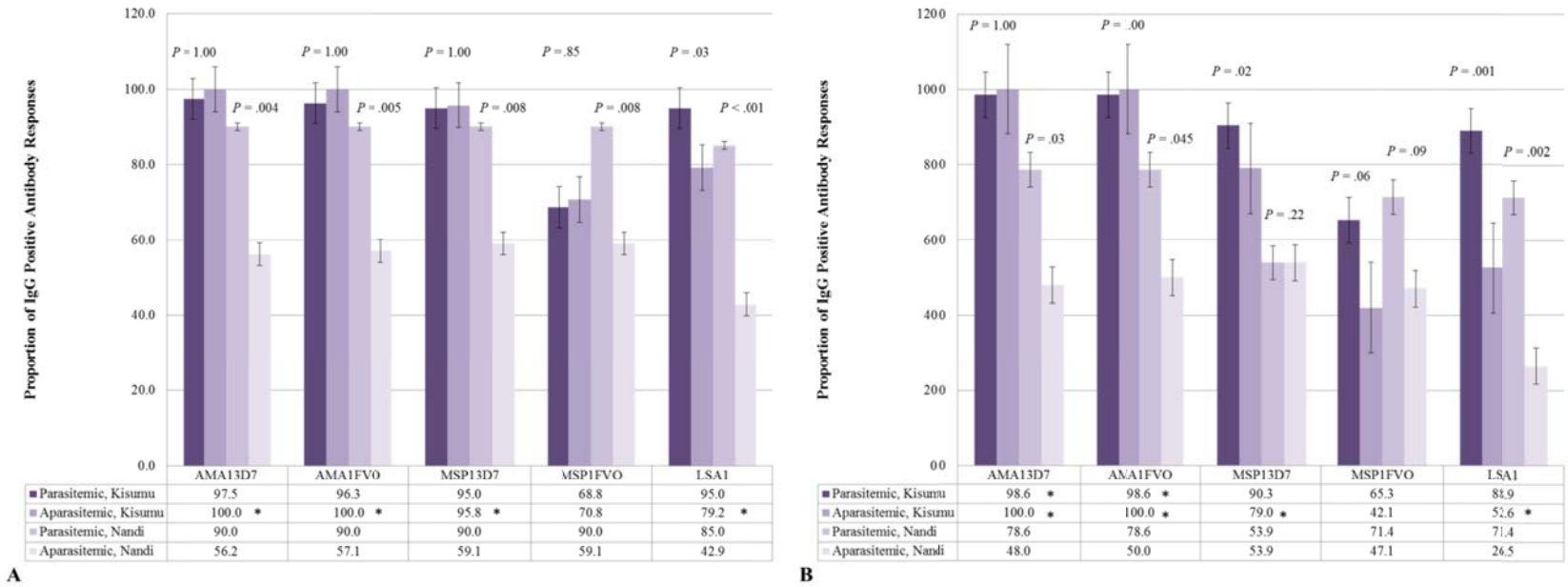


FIGURE 6.2. Proportion of IgG positive malaria antibody responses (>1 arbitrary units) by parasitemia in Kisumu and Nandi at baseline (A) and six-month follow-up (B) in Kenya, 2002-2003. *P*-values in the graph represent the Chi-square Test for differences in the proportion of positive antibody responses between parasitemia status within a district. Asterisks represent statistically significant differences ($P < .05$) in the proportion of positive antibody responses between districts within the same parasitemia status. Bar represent error bars. At baseline, the number of children who were parasitemic and aparasitemic in Kisumu was 80 and 24 while in Nandi it was 20 and 105. At six-month follow-up, the number of children who were parasitemic and aparasitemic was 72 and 19 while in Nandi it was 14 and 102, respectively.

TABLE 6.1. Summary of participants in the Kisumu/Nandi Cohort, Kenya 2002-2003

	Baseline				Six-month Follow-up			
	District				District			
	Kisumu (holoendemic)		Nandi (hypoendemic)		Kisumu (holoendemic)		Nandi (hypoendemic)	
	n	%	*n*	%	*n*	%	*n*	%
Sex								
Male	61	58.7	61	48.8	53	58.2	59	50.9
Female	43	41.3	64	51.2	38	41.8	57	49.1
Age group (in years)								
0-4	35	33.7	43	33.6	21	23.1	29	25.0
5-9	39	37.5	45	36.0	36	39.6	47	40.5
≥10	30	28.9	38	30.4	34	37.4	40	34.5
Parasitemia status								
Parasitemic	80	76.9	20	16.0	72	79.1	14	12.1
Aparasitemic	24	23.1	105	84.0	19	20.9	102	87.9
Total	104		125		91		116	

NOTE. n, number; %, percentage

TABLE 6.2. Median antibody responses for select malaria antigens at baseline by district, Kenya 2002

	Kisumu		**Nandi**		
	Median	Range	Median	Range	*P*-value*
AMA-1 3D7	4.53	0.17-7.24	2.04	0.05-7.68	<.001
AMA-1 FVO	4.57	0.16-6.77	2.18	0.04-10.83	<.001
MSP-1 3D7	5.19	0.22-12.66	3.14	0.05-15.37	.008
MSP-1 FVO	2.33	0.11-6.96	2.12	0.03-12.37	.63
LSA-1	3.71	0.39-10.88	0.99	0.18-9.17	<.001

**P*-values in table represent the Wilcoxon-Mann-Whitney U Test for differences in median antibody responses between Kisumu and Nandi. The number of participants in Kisumu and Nandi was 104 and 125, respectively. Antibody responses are measured in arbitrary units. See methods for explanation of arbitrary units calculation.

TABLE 6.3. Correlation* between malaria antibody responses at baseline and six-month follow-up in Kisumu (A and B) and Nandi (C and D), Kenya 2002-2003.

	AMA13D7	AMA1FVO	MSP13D7	MSP1FVO	LSA1
AMA13D7	1.0	0.846 <0.001	0.181 0.067	0.131 0.185	0.056 0.572
AMA1FVO		1.0	0.226 0.021	0.190 0.054	0.110 0.266
MSP13D7			1.0	0.824 <0.001	0.513 <0.001
MSP1FVO				1.0	0.542 <0.001
LSA1					1.0

A

	AMA13D7	AMA1FVO	MSP13D7	MSP1FVO	LSA1
AMA13D7	1.0	0.953 <0.001	0.161 0.129	0.140 0.187	0.268 0.010
AMA1FVO		1.0	0.187 0.076	0.177 0.092	0.284 0.006
MSP13D7			1.0	0.755 <0.001	0.575 <0.001
MSP1FVO				1.0	0.490 <0.001
LSA1					1.0

B

	AMA13D7	AMA1FVO	MSP13D7	MSP1FVO	LSA1
AMA13D7	1.0	0.951 <0.001	0.810 <0.001	0.769 <0.001	0.645 <0.001
AMA1FVO		1.0	0.836 <0.001	0.804 <0.001	0.667 <0.001
MSP13D7			1.0	0.963 <0.001	0.642 <0.001
MSP1FVO				1.0	0.601 <0.001
LSA1					1.0

C

	AMA13D7	AMA1FVO	MSP13D7	MSP1FVO	LSA1
AMA13D7	1.0	0.967 <0.001	0.816 <0.001	0.776 <0.001	0.638 <0.001
AMA1FVO		1.0	0.824 <0.001	0.778 <0.001	0.650 <0.001
MSP13D7			1.0	0.967 <0.001	0.610 <0.001
MSP1FVO				1.0	0.563 <0.001
LSA1					1.0

D

*Spearman's Rank Correlation Coefficients are presented on the first row and corresponding *P*-values are presented below.

TABLE 6.4. Relative change in malaria antibody responses (>1 arbitrary units) over six-months in Kisumu, Kenya 2002-2003.

		AMA-1 3D7			AMA-1 FVO			MSP-1 3D7			MSP-1 FVO			LSA-1		
	n	Median	Range	*P*-value	Median	Range	*P*-value	Median	Range	*P*-value	Median	Range	*P*-value	Median	Range	*P*-value
Age Group																
0-4 years	29	-0.20*	(-.56-1.14)	.47	-0.12*	(-.58-1.21)	.53	-0.17*	(-.88-2.45)	.61	-0.32	(-.93-1.15)	.59	-0.28	(-.90-1.72)	.40
5-9 years	34	-0.27	(-.57-.33)		-0.22*	(-.65-1.01)		-0.19	(-.97-.96)		-0.19*	(-.96-2.57)		-0.29	(-.77-2.03)	
10+ years	27	-0.23	(-.59-25.71)		-0.21	(-.67-26.94)		-0.27	(-.87-20.73)		-0.30	(-.86-18.82)		-0.38	(-.84-2.04)	
Parasitemia																
Both Surveys	57	-0.20	(-.58-25.71)	.11	-0.18	(-.63-26.94)	.15	-0.26	(-.88-20.73)	.04	-0.29	(-.93-18.82)	.07	-0.36	(-.90-2.04)	.17
Baseline Only	13	-0.27	(-.55-.31)		-0.19	(-.56-.15)		-0.42	(-.68-2.45)		-0.22	(-.88-1.12)		-0.46	(-.65-1.72)	
Six-month Only	14	-0.22	(-.46-.19)		-0.20	(-.58-.03)		0.02	(-.37-.96)		-0.05	(-.65-1.15)		-0.24	(-.57-1.31)	
Never	6	-0.50	(-.59-.22)		-0.54	(-.67- -.07)		-0.38	(-.97-.27)		-0.57	(-.96-.91)		-0.69	(-.84-.21)	
Sex																
Male	52	-0.25*	(-.59-.89)	.71	-0.19*	(-.67-1.21)	.96	-0.20	(-.97-2.45)	.59	-0.22*	(-.96-1.98)	.29	-0.29	(-.89-2.03)	.87
Female	38	-0.21	(-.56-25.71)		-0.20*	(-.63-26.94)		-0.21	(-.88-20.73)		-0.26	(-.88-18.82)		-0.35	(-.77-2.04)	

P-values in the table represent the Wilcoxon-Mann-Whitney U Test (sex) and Kruskal-Wallis Test (age group and parasitemia) for differences in the median relative change in antibody responses. Asterisks represent statistically significant differences ($P < .05$) in the median relative change in antibody responses between Kisumu and Nandi.

TABLE 6.5. Relative change in malaria antibody responses (≥1 arbitrary units) over six-months in Nandi, Kenya 2002-2003.

		AMA-1 3D7			AMA-1 FVO			MSP-1 3D7			MSP-1 FVO			LSA-1		
	n	Median	Range	*P*-value	Median	Range	*P*-value	Median	Range	*P*-value	Median	Range	*P*-value	Median	Range	*P*-value
Age Group																
0-4 years	37	-0.49	(-.96-5.80)	0.34	-0.49	(-.96-2.50)	0.19	-0.52	(-.95-2.98)	0.65	-0.48	(-.97-3.00)	0.75	-0.45	(-.93-2.26)	0.74
5-9 years	40	-0.35	(-.94-7.81)		-0.40	(-.94-4.19)		-0.30	(-.98-18.40)		-0.44	(-.96-11.58)		-0.38	(-.95-1.20)	
10+ years	34	-0.30	(-.86-4.61)		-0.33	(-.85-5.12)		-0.35	(-.96-9.69)		-0.38	(-.90-7.43)		-0.32	(-.96-7.73)	
Parasitemia																
Both Surveys	5	-0.07	(-.27-.13)	0.41	0.00	(-.34-.06)	0.12	-0.21	(-.53-.21)	0.13	-0.33	(-.65-.09)	0.83	0.20	(-.65-1.23)	0.08
Baseline Only	14	-0.35	(-.86-.067)		-0.20	(-.85-.24)		-0.60	(-.81-.003)		-0.48	(-.76-.96)		-0.59	(-.96-.14)	
Six-month Only	8	-0.40	(-.60-4.27)		-0.40	(-.66-4.35)		-0.16	(-.93-9.69)		-0.28	(-.90-7.43)		-0.16	(-.71-7.73)	
Never	84	-0.37	(-.96-7.81)		-0.42	(-.96-5.12)		-0.33	(-.98-18.4)		-0.45	(-.97-11.58)		-0.36	(-.95-4.28)	
Sex																
Male	56	-0.35	(-.92-7.81)	0.86	-0.35	(-.94-4.35)	0.33	-0.35	(-.95-18.4)	0.96	-0.45	(-.95-11.58)	0.76	-0.40	(-.94-7.73)	0.84
Female	55	-0.35	(-.96-4.61)		-0.39	(-.96-5.12)		-0.31	(-.98-2.98)		-0.39	(-.97-3.00)		-0.34	(-.96-2.53)	

P-values in the table represent the Wilcoxon-Mann-Whitney U Test (sex) and Kruskal-Wallis Test (age group and parasitemia) for differences in the median relative change in antibody responses.

TABLE 6.6. Correlation* between the relative change in malaria antibody responses in Kisumu (A) and Nandi (B) over six-months, Kenya 2002-2003.

	AMA13D7	AMA1FVO	MSP13D7	MSP1FVO	LSA1
AMA13D7	1.0	0.914 <0.001	0.387 <0.001	0.382 <0.001	0.380 <0.001
AMA1FVO		1.0	0.374 <0.001	0.382 <0.001	0.382 <0.001
MSP13D7			1.0	0.816 <0.001	0.682 <0.001
MSP1FVO				1.0	0.623 <0.001
LSA1					1.0

A

	AMA13D7	AMA1FVO	MSP13D7	MSP1FVO	LSA1
AMA13D7	1.0	0.927 <0.001	0.712 <0.001	0.724 <0.001	0.551 <0.001
AMA1FVO		1.0	0.688 <0.001	0.726 <0.001	0.504 <0.001
MSP13D7			1.0	0.904 <0.001	0.289 <0.001
MSP1FVO				1.0	0.554 <0.001
LSA1					1.0

B

* Spearman's Rank Correlation Coefficients are presented on the first row and corresponding *P*-values are presented below.

CHAPTER SEVEN: DISCUSSION

It has been over 50 years since Burkitt first published his description of eBL[20] and Cohen and McGregor demonstrated the protective effect of gamma-globulins.[88, 90] In the years since these important milestones in the history of *Pf*-malaria infection, advances have been made in our understanding the pathogenesis but there is still more work to be done to further reduce the global burden of diseases that are directly, and in-directly, attributable to *Pf*-malaria infection.

Summary of Findings

We have described a number of important findings in this dissertation that should guide future research on cumulative effect of *Pf*-malaria infections on EBV persistence and potential immunological markers of Pf-malaria immunity to symptomatic illness. In our first specific aim, we found that repeated *Pf*-malaria infections were associated with a loss in CD8+ T-cell production of IFN-γ against EBV lytic antigens but not latent antigens. This loss in CD8+ T-cell functionality was most pronounced among the age group in which eBL has been commonly reported. The loss of immunological control over EBV lytic replication may lead to more EBV latently infected B-lymphocytes thereby increasing the risk for developing eBL.

In our second specific aim, we found that antibody responses, and the proportion of positive responders, were higher in a malaria holoendemic areas when

compared to a hypoendemic area. Although antibody responses have been reported to increase with age in holoendemic areas, we observed a high proportion of positive responders across all age groups because even the youngest children had already experienced repeated *Pf*-malaria infections to have accumulated antibody responses. Interestingly, we did observe an age trend in the hypoendemic area, where they were experiencing an outbreak. We also found that antibody responses waned over time regardless of the malaria transmission intensities. However, the greatest magnitude of change was among children <5 years old in the hypoendemic area whereas it varied in the holoendemic area. These findings support the differential acquisition of antibodies to *Pf*-malaria infections by malaria transmission intensity and age.

Public Health Significance and Future Directions

Specific Aim 1: Findings suggest that children repeatedly exposed to *Pf*-malaria infections lose immunological control over latent EBV infection. Specifically, the ability of their CD8+ T-cell to produce IFN-γ to lytic antigens has diminished leading to reactivation of lytic replication. Longitudinal studies that can accurately measure EBV CD8+ T-cells IFN-γ and *Pf*-malaria infections are needed to corroborate our findings.

What is interesting about the finding of reduced EBV lytic antigen CD8+ T-cells response is that it coincides with the time frame in which children are beginning to develop protection from symptomatic illnesses to *Pf*-malaria infections and are able to better control parasite density. Therefore as immunity to *Pf*-malaria develops,

so does the risk for eBL. Though this is circumstantial evidence, it points to a critical time frame that should be further investigated as these two events are unlikely to be occurring independently of one another. Immune responses are a combination of innate, cellular, and humoral responses that work in unison to control infection, including multiple infections. Hence a more comprehensive approach should be considered when researching the interaction between *Pf*-malaria and EBV co-infection. It is understandable to reduce complex systems into simplified relationships and important information can be gleaned from studies like ours where we investigated one aspect of the immunologic response. Yet research should strive to build upon these simplified relationships to obtain a more accurate understanding of how the immune system orchestrates responses to *Pf*-malaria and EBV during co-infection. Furthermore, future research should be conducted to ascertain whether our observed decrease in CD8+ lytic antigen T-cell IFN-γ response among 5-9 year olds in a holoendemic malaria area contributes to the development eBL. Due to the difficulties in conducting a cohort study with eBL as an outcome, novel approaches will need to be considered.

Finally, our analysis examined the cumulative effect of *Pf*-malaria infections on latent EBV infection at the individual-level. The use of a surrogate for *Pf*-malaria infection at the district-level has been informative but does not, and cannot, take into account differences in individual-level risks for the if we are to This is a crucial step if we are to decipher the immunological relationship between *Pf*-malaria and EBV co-infections. Efforts should be made to build upon our attempt, using data from a

longitudinal study with shorter follow-up intervals so an accurate measure of cumulative *Pf*-malaria can be made.

Specific Aim 2: Our results highlight important differences in antibody responses to *Pf*-malaria antigens among children living in areas with different malaria transmission intensities. The lack of an age trend in the high malaria transmission area to select blood-stage antigens was due to a high proportion of positive responders in each age group. This suggests that the immune system of young children can mount an immune response similar to older children. More importantly, the lack of an age-trend for a number of antigens emphasizes that antibodies work in unison to eliminate an infection. Studies have typically examined antibodies independently of one another but it is unlikely a vaccine will target one antigen for a complex organism such as *Plasmodium falciparum*. To facilitate the development of a multivalent vaccine, it is important for research to begin focusing on how multiple antibody responses work together to overcome infection and illness.

Furthermore, we detected a decline in antibody responses over time, regardless of the malaria transmission intensity. But the magnitude of change was not the same for all antigens and was more pronounced for the youngest age group in the malaria low transmission area. This observation raises numerous questions. Are SLPCs responsible for fighting infection in children infrequently infected with *Pf*-malaria? Does the loss of antibody response represent a functional loss in immunity? Are the half-lives of IgG1 antibodies the same for all *Pf*-malaria antigens?

There is a clear need for further research into the function and longevity of antibodies to *Pf*-malaria.

Conclusion

The quest for an effective vaccine against *Pf*-malaria infections offers great hope for the future but we are still years, even decades away from developing a vaccine for use. Furthermore it is likely that the first generation of vaccine will be designed to protect from symptomatic and severe illness rather than infection. Although protection from symptomatic illness is vital, it may still leave children vulnerable to eBL. Therefore, we must continue in our pursuits to understand the multi-faceted pathogen that is *Pf*-malaria to gain knowledge on its natural history and interaction with other pathogens to aid in identifying novel approaches and strategies to reduce its impact on the lives of infants, children, and adults around the world.

APPENDIX A: CWRU/KEMRI Blood Sample Collection and Questionnaire Form 1

Study Title: Immunologic Studies of Endemic Burkitt's Lymphoma
Sample collection form 1: Blood sample collection for investigational studies

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Patient study number (STID) __ - __ - __ __ __ __ - __ __ - BL - __
(site) (village) (household) (individual) (round)

Date of sample collection __ __/ __ __ / __ __ **(dd/mm/yy)**

First name _____

Middle name _____

Last name _____

Date of birth __ __/ __ __ / __ __ **(dd/mm/yy)**

Sex ☐ **male (1)** ☐ **female (2)**

Has this child taken any anti-malarial medication for malaria within the past 2 weeks?

☐ **Yes (1)** ☐ **No (2)**

If yes, which medication _____

Has the child taken any traditional medicines?

☐ **Yes (1)** ☐ **No (2)**

If yes, which one(s) _____

Does this child sleep under a bed net? ☐ **Yes (1)** ☐ **No (2)**

If yes, is this net treated? ☐ **Yes (1)** ☐ **No (2)**

Has the house this child sleeps in been sprayed with insecticide by the Ministry of Health?

☐ **Yes (1)** ☐ **No (2)**

Ethnic group _____ ☐

List Samples collected:

- ❑ Blood smear _____(yes or no)
- ❑ Hemoglobin _____(gm/dl)
- ❑ Purple top (~ 500 μl) _____(yes or no)
- ❑ Green top (2-5 ml) _____(indicate amount taken)
- ❑ Other _____(specify type of specimen)

APPENDIX B: Calculation of Inverse Probability Weights

An assumption when using generalized estimating equations (GEE) is that missing data, or observations, are missing completely at random); missingness of data is independent of observed and unobserved data [158]. When data do not appear to satisfy this assumption, a less restrictive assumption on the missingness of data is needed.

Inverse probability weights (IPWs) are one method that can be used to address missing data, or observations, and is less restrictive about the missingness of data than GEE. When using IPW, observations are assumed to be missing at random; missing data are dependent on observed data but independent of unobserved data. [158] To calculate IPWs, the probability of participation is modeled using available predictor variables. The inverse of the predicted probabilities are calculated and assigned to each individual with complete data. Individuals with complete data are weighted to represent individuals with similar characteristics who have missing data.

To calculate the predicted probabilities for our study, we used logistic regression with first order interaction terms using the following equation:

$$Log[p_i/1-p_i] = b_0, + b_1(sex) + b_2(age\ group\ 0\text{-}4\ years) + b_3(age\ group\ 5\text{-}9\ years) + b_4(site\ of\ residence) + b_5(sex*age\ group\ 0\text{-}4\ years) + b_6(sex*age\ group\ 5\text{-}9\ years) + b_7(sex*site\ of\ residence) + b_8(age\ group\ 0\text{-}4\ years*site\ of\ residence) + b_9(age\ group\ 5\text{-}9\ years*site\ of\ residence).$$

Where p_i is the probability of child *i* (*i* =1,2,3…n) participating in all surveys. Taking the inverse of the predicted probabilities, the mean IPW was 1.44 and ranged from 1.14-1.92. Children who participated in all three surveys were assigned the mean IPW value whereas children with missing observations were assigned an IPW of 0.

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