ROLE OF SICKLE-CELL TRAIT, MALARIA AND EPSTEIN BARR VIRUS CO-INFECTION ON EBNA-1-SPECIFIC T-CELL IMMUNITY IN THE ETIOLOGY OF ENDEMIC BURKITT LYMPHOMA IN CHILDREN AGED 4-9 YEARS FROM WESTERN KENYA

By

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ABSTRACT

Etiological mechanism underlying endemic Burkitt lymphoma (eBL), remain unknown despite the long-standing epidemiological link between *Plasmodium falciparum* and Epstein Barr Virus (EBV) co-infections. Although past studies have linked malaria and EBV in eBL etiology none has investigated the role of sickle cell trait (HbAS) and malaria transmission on the quality of T-cell memory phenotype and function. Furthermore, there is paucity of data on the role of common infectious agents in eBL etiology. Previous studies have shown an age-dependent deficiency in EBV-specific CD8+ T-cell-mediated IFN-γ immunosurveillance in children from malaria holoendemic regions. Further, deficiency in Epstein-Barr nuclear antigen-1 (EBNA-1)-specific IFN-γ responses has been associated with eBL diagnosis in children aged 4 to 9 years. EBNA-1 is the sole antigen expressed by EBV-infected B cells as well as EBV-associated tumours. This case-control study hypothesized that qualitative differences in EBNA-1- specific T-cell phenotype and function as well as serology existed in malaria-exposed children and, thus in part, explains their increased risk for eBL. Moreover, the study examined the relationship between HbAS and EBV titers and how it relates to anti-EBV immunity. Thus to test this hypothesis, healthy children from western Kenya with divergent malaria exposure histories: Kisumu (n=24), a malaria holoendemic region and Nandi (n=23), a hypoendemic region; were age-matched to eBL patients (n=31). T-cell memory phenotype and function was evaluated by multiparameter flow cytometry against T-cell lineage markers and functional phenotype based on IFN-γ, IL-10, IL-17 and PD-1 expression after *ex vivo* stimulation with EBNA-1 peptides. Multiplex ELISA was used to determine serological profiles. EBV titers were determined by qPCR, while PCR-based Restriction Fragment Length Polymorphism (RFLP) was used to determine HbAS genotype. Pairwise comparisons were done using Mann-Whitney U test while multiple comparisons were done using Kruskal-Wallis tests. Logistic regression analysis was used to determine the association between HbAS trait and viral titers while Chi square was used to test for differences in proportions of HbAS genotypes. Results show that EBNA-1-specific IFN-γ responses were significantly lower in eBL and Kisumu compared to Nandi children (p=0.01). IFN-γ was generated from $CD4^+$ effector memory T cells and a heterogeneous combination of $CD8^+$ T cells (p<0.0001). The frequencies of PD-1 expressing T cells were low in eBL patients and highest in Kisumu children than Nandi children for both CD4+ T cells (p=0.0237) and CD8+ T cells (p=0.0423). The expression of PD-1 and IFN-γ in response to EBNA-1 was mutually exclusive. Further, Kisumu and eBL children had higher cellular and plasma EBV titers compared to Nandi children (p=0.0001). Although HBAS genotype was more common in Kisumu children than other groups (χ^2= 23.42, df 2, p<0.0001), it was neither associated with EBV titers nor eBL (OR=0.5473, 95% CI, 0.2835-1.057, p=0.0996). This suggests that EBNA-1-specific deficiencies in eBL may be due to a deletion of EBNA-1-specific T-cell population as a consequence of T-cell exhaustion. These results show that HbAS trait does not offer protective advantage against eBL and hence all children in malaria holoendemic regions should be treated as vulnerable to eBL risk irrespective of HbAS genotype. Further, results show that EBV peptide such as EBNA-1 can be exploited further as a possible vaccine candidate against EBV associated tumours. This study recommends a paradigm shift from use of total EBV viral loads as a prognostic biomarker to use for cellular viral loads in clinical management of eBL. In addition, there is need for development of novel immunotherapeutic agents such as adoptive autologous EBNA-1 specific T cells as well as PD-1 mediated blockade in clinical management of eBL.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Although Endemic Burkitt lymphoma (eBL) is the most prevalent childhood cancer in equatorial Africa (Magrath, 1990a) its etiological mechanisms remain poorly understood. In these regions the annual eBL incidence varies between 1 to 10 in a 100,000 children (Mwanda *et al.,* 2004), however this rate is much higher in regions in malaria holoendemic region such as Kisumu County (Rainey *et al.*, 2007a). The peak age range for this cancer is between 4 and 9 years (Mwanda, 2004).

Despite the fact that eBL is associated with *Plasmodium falciparum* and Epstein Barr Virus (EBV) co-infection (Dalldorf *et al.*, 1964), the possible role of other common infectious agents has not been investigated. In areas of eBL incidence (Rainey *et al.*, 2007b), there are reports of high prevalence rates of other common infectious diseases such as measles, schistosomiasis and bacterial infections (Opisa *et al.*, 2011; Parodi *et al.*, 2013). To date, there are no studies that have investigated whether children who develop eBL have different serological profiles to these common infectious agents or not.

Previous studies in western Kenya have shown that chronic malaria exposure is associated with high EBV titers measured from whole blood and a younger age of primary EBV infection (Moormann *et al.*, 2005; Piriou *et al.*, 2012). However, none of these studies has explored differences in viral titers between different compartments of blood (cellular versus plasma).

Children aged 4-9 years residing in malaria holoendemic regions have been reported to have a deficiency of IFN-γ mediated EBV-specific CD8+ T-cell immunity (coinciding with the peak-age incidence of eBL) (Moormann *et al.*, 2007). However, whether this deficiency is specific to IFN-γ or to other cytokines such as IL-10 or IL-17 that have been associated with malaria exposure (de Kossodo and Grau, 1993), has not been investigated. Furthermore, there are no studies that have examined whether this recall response is universal to all EBV latently expressed antigens such as EBNA2 and EBNA3A or specific to certain EBV antigens.

Studies in western Kenya utilizing ELISPOT, which is a less sensitive test than flow cytometry have shown selective deficiency of EBNA-1 specific IFN-γ CD8+ T cells in children presenting with eBL (Moormann *et al.*, 2009). As a result, T memory architecture with respect to EBNA-1 specific IFN-γ T cells remain to be described.

Since *P. falciparum* is an important covariate in eBL etiology, and eBL occurs after a child has survived the critical age when malaria is severe (Moormann *et al.*, 2011), factors that protect the host against severe forms of the disease (Klein, 1978; Artavanis-Tsakonas *et al.*, 2003; Doolan *et al.*, 2009; López *et al.*, 2010; Vannberg *et al.*, 2011) need to be evaluated in context of shaping the resultant EBNA-1-specific immunity. One such well-described genetic marker frequent in western Kenya is sickle-cell trait (HbAS) (Moormann *et al.*, 2003a). The role of HbAS genotype has not been evaluated in the context of EBV infection and possibly eBL etiology. Thus, the relationship between HbAS/AA and EBV viral titers as a functional measure of viral control, and as well as a potential biomarker for eBL risk was investigated in the current study.

1.2 Problem Statement

Endemic Burkitt lymphoma has been described as a polymicrobial disease with multifaceted aetiologies (Rochford *et al.,* 2005). However, the possible role of underlying sero-profiles to other common infections in etiology of eBL remains unknown.

Studies in western Kenya have shown high EBV titers in children aged between 4 and 9 years as measured from whole blood (Moormann *et al.*, 2005). However, no study has evaluated the differences in the compartments of blood to distinguish between cellular and plasma viral burden that can be used as realistic biomarker.

Although malaria has been known to have immune modulatory effect on the human host (Theander, 1992), the effect of malaria exposure history in shaping the quality and function of EBV specific T-cell immunity as a factor in the etiology of eBL remains un-investigated. Further, it is not known whether this effect is global to all EBV latent antigens or specific to certain antigens. Moreover, the inhibitory effect of malaria infection on EBNA-1 specific T-cell immunity as exemplified by expression of molecules such as PD-1 has not been explored in the context of eBL.

The quality and function of T cells to an antigen is shaped by the cytokine milieu (Hunt and Grau, 2003). Although IFN-γ-producing T cells against EBV antigens have been correlated with protection against EBV-associated lymphoproliferative disorders (Mautner and Bornkamm, 2012; Rowe *et al.*, 2001), the impact of malaria on modulating host cytokine milieu such as IL-10 or IL-17 that may be permissive to eBL has not been studied. Although a myriad of factors have been implicated in shaping long-term T-cell responses (Kedzierska *et al.,* 2006; Saule *et al.,* 2006; Allen *et al.,* 2011; Turner *et al.,* 2006) and the development of

short-term effector memory T-cell subsets (Stemberger *et al.*, 2007) that could increase susceptibility to eBL, no study, to date, has examined the relationship between HbAS, which is a well-characterized human mutation in western Kenya that confers protection against severe forms of *P. falciparum* malaria and concomitant parasitemia and eBL or resultant EBV specific T cell immunity.

1.3 Objectives of the Study

1.3.1 General Objective

To investigate the role of sickle cell trait, holoendemic *Plasmodium falciparum* malaria and EBV co-infections on EBNA-1-specific T-cell immunity in the etiology of eBL.

1.3.2 Specific Objectives

1. To determine differences in serological profiles (total IgG levels) to EBV antigens (EA, EBNA-1, VCA, Zta), malaria antigens (AMA-1, MSP1, LSA1, TRAP, CelTos), measles and schistosomiasis, between children with eBL and healthy control children living in areas with divergent *P. falciparum* malaria transmission intensities.

2. To determine EBV viral loads measured from different compartments of blood between children presenting with eBL and healthy children living in areas with divergent *P. falciparum* transmission and exposure histories.

3. To examine differences in frequencies of EBNA-1-specific, cytokine-producing (i.e. IFN-γ, IL-10 and IL-17) CD4+ and CD8+ memory T cells between children presenting with eBL and healthy children living in areas with divergent *P. falciparum* transmission and exposure histories.

4. To compare frequencies of EBNA-1, EBNA2 and EBNA3A cytokine producing T cells between children presenting with eBL and healthy children living in areas with divergent *P. falciparum* transmission and exposure histories.

5. To determine effector-memory phenotypic characteristics of EBNA-1-specific, cytokine-producing CD4+ and CD8+ T cells between children presenting with eBL and healthy control children, living in areas with divergent *P. falciparum* transmission dynamics and exposure histories.

6. To determine frequencies of EBNA-1-specific, PD-1-expressing CD4+ and CD8+ T-cell subsets between children presenting with eBL and healthy children, living in areas with divergent *P. falciparum* transmission dynamics and exposure histories.

7. To determine differences in EBNA-1-specific immune architecture between age-matched healthy children controls living in areas with divergent *P. falciparum* malaria transmission intensities and children presenting with eBL stratified by beta-Haemoglobin genotype (HbAA/AS).

1.4 Research Questions

1. What are the differences in serological profiles to EBV antigens (EA, EBNA-1, VCA, Zta), malaria antigens (AMA-1, MSP1, LSA1, TRAP, CelTos), measles and schistosomiasis between children diagnosed with eBL to age-matched, healthy control children living in areas with divergent *P. falciparum* malaria transmission?

2. What are the differences in EBV loads measured from peripheral whole blood compartments (i.e. cellular versus cell-free virus) comparing children diagnosed with eBL to age-matched, healthy control children living in areas with divergent *P. falciparum* malaria transmission?

3. What are the differences in frequency of EBNA-1-specific cytokine (i.e. IFN-γ, IL-10 and IL-17)-producing CD4+ and CD8+ T cells, comparing children diagnosed with eBL to age-matched healthy control children living in areas with divergent *P. falciparum* malaria transmission?

4. What are the differences in the frequencies of EBNA-1, EBNA2 and EBNA3A cytokine producing T cells between children presenting with eBL and healthy children living in areas with divergent *P. falciparum* transmission and exposure histories.

5. What are the effector-memory phenotypic characteristics (i.e. CD4+, CD8+, CD4+5RA, CCR7, CD27) of EBNA-1-specific, cytokine-producing CD4+ and CD8+ T cells, comparing children diagnosed with eBL to age-matched, healthy control children living in areas with divergent *P. falciparum* malaria transmission?

6. What are the differences in frequencies of EBNA-1-specific, PD-1-expressing CD4+ and CD8+ T-cell subsets, comparing children diagnosed with eBL to age-matched, healthy control children living in areas with divergent *P. falciparum* malaria transmission?

7. What are the differences between HbAS genotypes (sickle-cell trait, HbAS, polymorphism) in EBV viral control and EBNA-1-specific immune architecture,

comparing eBL children to age-matched, healthy control children living in areas with divergent *P. falciparum* malaria transmission?

1.5 Significance of the Study

Although eBL was first described 50 years ago in Uganda, (Burkitt, 1958; Burkitt and O'Connor, 1961; Burkitt, 1962, 1983), the incidence still remains high in equatorial regions where *P. falciparum* transmission is high and resources are limited for cancer awareness or for obtaining prompt diagnosis and treatment.

Understanding how malaria modulates the host immunity such as through induction of PD-1 expression can be beneficial in the development of immunotherapies that can be used by populations at increased risk of eBL. Differences in frequencies of EBNA-1-specific T-cell capacity between healthy children and those with eBL will shed light on how these differences potentially lead to the genesis of eBL. Understanding such mechanisms will be pivotal in designing vaccine candidates that can elicit appropriate protective immunity. Understanding these differences in quality of T cell immunity in healthy children compared to eBL patients will lead to novel immunotherapies using EBNA-1 as a vaccine candidate.

Clinical management of lymphoproliferative disorders requires constant monitoring of viremia as a marker of prognosis (Bingler *et al.*, 2008; Stevens *et al.*, 2002). A reliable biomarker is desirable especially for a tumour such as eBL. Findings from this study shed light on the appropriate blood component to use to monitor prognosis in eBL patients or patients who may be at risk of tumorigenesis. Lastly, understanding host genetic factors that increase risk of eBL can lead to early diagnosis and clinical management of children that are at increased risk of the cancer.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology and etiology of endemic Burkitt lymphoma

Endemic Burkitt lymphoma was first clinically described in African children in 1958 by Dennis Burkitt (Burkitt, 1958; Davies *et al.,* 1964). The highest incidence of eBL is in equatorial Africa and Papua New Guinea (5-15 cases per 100,000 children) (Bishop *et al.*, 2000). This malignancy is a high-grade B-cell neoplasm with a high proliferative index and a incidence rate in males compared to females (Magrath, 1990b; Parkin *et al.*, 1989). Within equatorial Africa, there is an uneven geographic distribution of eBL, suggesting that malaria may be a risk factor (Dalldorf *et al.,* 1964). In fact, there is a strong correlation between holoendemic malaria and incidence of eBL (Dalldorf *et al.,* 1964; Mwanda *et al.,* 2004b).

This cancer characteristically manifests as a distortion of the facial skeleton, although other anatomical sites, such as the abdomen have been implicated (reviewed by Orem *et al.,* 2008) (Fig. 2.1). The cancer is more frequent around Lake Victoria and along the costal region of Kenya which are malaria holoendemic regions (Mwanda *et al.*, 2004; Rainey *et al.*, 2007a). The annual incidence rate of this cancer is quite high around Lake Victoria than other parts of the country(Rainey *et al.*, 2007b).

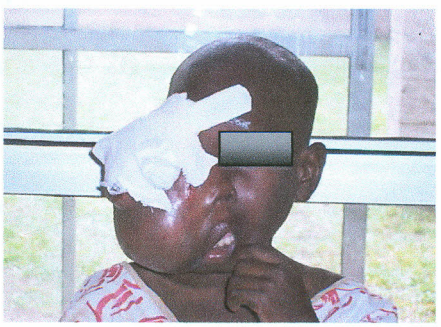


Fig. 2.1 A child presenting with facial form of endemic Burkitt Lymphoma

(Source: http://www.frtomskids.org/images/Burkitts1.jpg)

The peak age range for eBL occurrence is between 4 and 9 years (Mwanda, 2004), which curiously happens after the age when malaria exerts its most profound impact on morbidity and mortality in children (WHO, 2012) and years after primary EBV infection (de-The, 1970). This observation implies that cumulative malaria exposure coupled up with high EBV loads in children (Moormann *et al.,* 2005) sets the stage for subsequent events that increase the risk of eBL (Fig. 2.2).

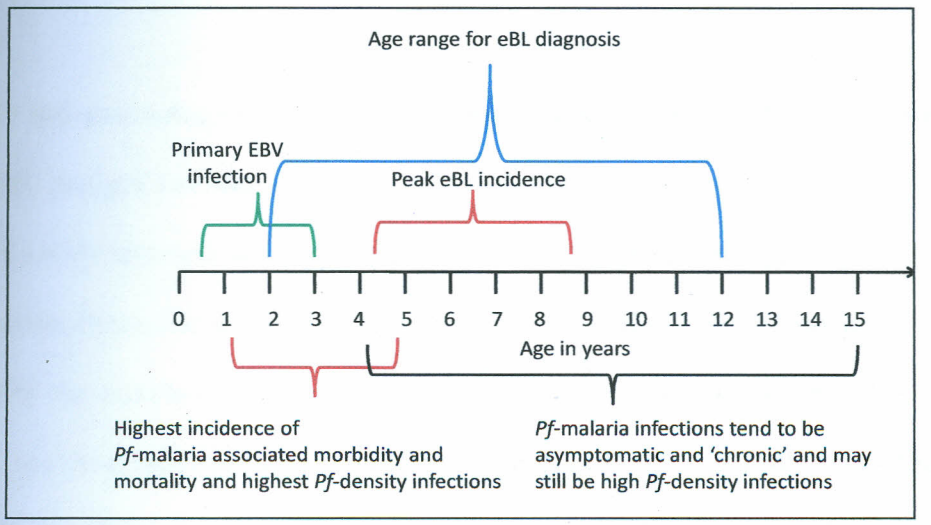


Fig. 2.2 Chronology of P. falciparum malaria and EBV infections in relation to eBL incidence
(*Courtesy* Moormann *et al.*, 2011)

African children are infected with EBV earlier in life than Americans and Europeans: 44% of African children are seropositive by the age of 12 months, 81% by 2 years and nearly 100% by 3 years (Biggar *et al.*, 1978; Piriou *et al.*, 2012). Historical studies sought to investigate the impact of malaria on EBV control using a rather crude *in vitro* regression assay (Gunapala *et al.*, 1990; Moss *et al.*, 1983). These studies suggested that holoendemic malaria exposure (Moss *et al.*, 1983) and acute episodes of clinical malaria (Gunapala *et al.*, 1990; Whittle *et al.*, 1984) impaired EBV-specific T-cell immunity. The CD4+ to CD8+ T-cell ratio was inverted due to an absolute rise in CD8+ T cells during acute malaria attacks (Gunapala *et al.*, 1990). These previous studies though seminal, were limited by examination

of small numbers of study participants and the nature of the populations examined. In addition, regression assays did not identify the effector cells or cytokine mediators involved in the control of EBV-infected B cells. Finally, these experiments neither made distinctions between EBV-specific immunosuppression and an overall transient depression of T-cell responses associated with malaria, nor did they include examination of responses in children diagnosed with eBL.

2.2 EBV Immunobiology and the Role of *c-myc* translocation in eBL lymphomagenesis

2.2.1 EBV Antigen Profiles

There is a wide spectrum and polymorphic array of gene products expressed by EBV during its life cycles (lysogenic and lytic stages) (Rickinson and Kief, 2001). The latent life cycle is marked by the expression of nine proteins, six associated nuclear antigens EBNA-1, 2, 3A, 3B, 3C, and three latent membrane proteins (LMP-1, LMP-2A and LMP-2B). Other proteins expressed by EBV during latency include untranslated RNA, the EBV encoded mRNA (EBERs) and other family of BamH1A transcripts (Rowe *et al.*, 2009; 1992, Bornkamm, 2009). During the latency transcription program, EBV genes are under strict control of the master transcription factor EBNA2 (Roughan and Thorley-Lawson, 2009; Rickinson and Kief, 2001). This latency program is characterized by limited viral protein expression that safeguards the maintenance of the viral reservoir in the face of strong immune responses (Masucci and Ernberg, 1994). Thus immune responses targeting latent antigens would be desirable in controlling chronic infections such as eBL.

The lytic stage is the productive cycle in which viral proteins are produced (Allen, 2005). The lytic phase from latency is marked by expression of EBV immediate early lytic transcript BZLF1 which encodes for Zta protein (Allen, 2005; Rickinson and Kief, 2001). This involves

the induction of latent membrane protein 1 (LMP-1), LMP-2A and LMP-2B as early lytic cycle antigens (Rowe *et al.*, 2008). Other viral antigens include early antigen (EAd), Leader proteins (LP), viral capsid antigen (VCA) and EBV-induced membrane antigen (MA) (Allen, 2005; Rickinson and Kief, 2001). Normally, there is low-grade lytic cycle reactivation in immunocompetent individuals leading to occasional shedding of the virus in the saliva (Yao *et al.*, 1989) possibly for self-perpetuation (Walling *et al.*, 2001). Viral reactivation can also occur due to host internal physiological and biochemical changes as well as extrinsic stimulation due to *P. falciparum* malaria (Chene *et al.*, 2009; Donati *et al.*, 2004) or exposure to plant extracts such as *Euphorbia tirucalli* (Mannucci *et al.*, 2012). Serological titers can be used to distinguish primary infection from remote infection (Gulley and Tang, 2008). During primary EBV infection, high IgG and IgM anti-VCA titers accompany symptoms of acute infectious mononucleosis (AIM) (Elgh and Linderholm, 1996; Gulley and Tang, 2008) (Fig. 2.3). To date very few studies have investigated how antibody titers to both latent and lytic antigen in children living malaria regions can act as a risk factor in the aetiology of eBL.

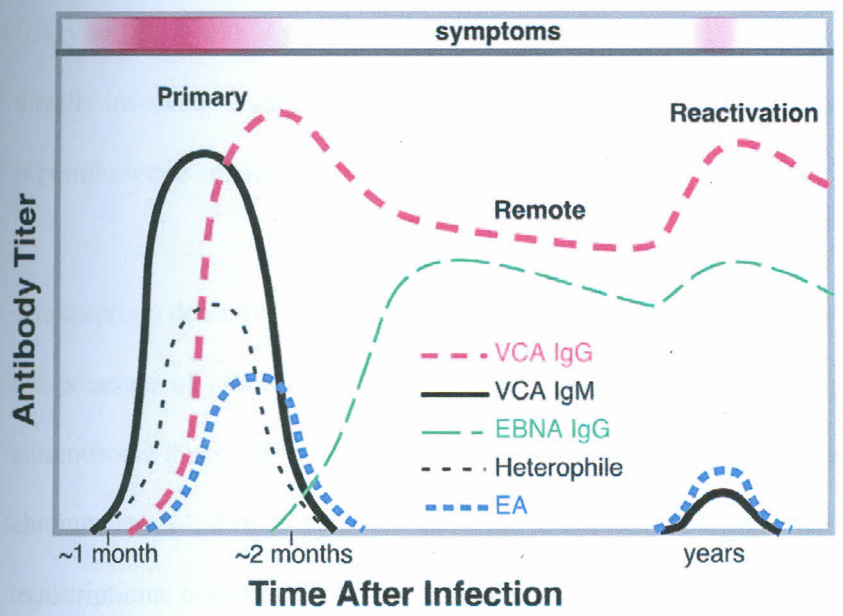


Fig. 2.3 EBV Serological Titers Change With Time
(*Courtesy of* Gulley and Tang, 2008)

2.2.2 The Role of *c-myc* Genetic Lesion in eBL etiology

EBV as a causal factor in the etiology of eBL has not been without controversy (Bornkamm, 2009); however, compelling evidence supports its role. EBV has the ability to make tumour cells resistant to apoptosis once the *c-myc* translocation has occurred (Thorley and Allday, 2008). As a result, it has been postulated that EBV should not be seen as a *conditio sine qua non* for the development of eBL but rather a facilitative factor in its development once the *c-myc* gene translocation has occurred. This is because there are a minority of cases that have been reported where BL occurs in the absence of EBV infection (Bornkamm, 2009). It is, therefore, be evident that genetic lesion from whatever origin is the defining moment in the catalytic events that lead to eBL emergence when other contributing risk factors, such as age, immune status, sex and environment, are in place (Magrath, 2012). The presence of EBV, therefore, has two roles that are not mutually exclusive: first, heightening the molecular events (complementing the activity of *c-myc* gene) and secondly, increasing the likelihood of genetic lesions giving rise to translocation, thus setting the stage for eBL lymphomagenesis (Nagy *et al.*, 2009). Apart from the oncogenic property, EBV load has been suggested to directly involved in shaping the T-cell differentiation pathway augmenting a phenotype that is permissive to enhanced viral survival and persistence (Hou *et al.*, 2009; Wang *et al.*, 2012).

The common denominator in eBL and other EBV-associated lymphoproliferative disorders is the presence of chromosomal translocation affecting the *c-myc* gene on the long arm of chromosome 8 and one of the immunoglobulin heavy or kappa and lambda light chain loci on chromosome 14, 2 or 22 respectively. This translocation brings the *c-myc* oncogene under the transcriptional control of the immunoglobulin locus (Klein, 1978; Nagy *et al.*, 2009). It is this cytogenetic abnormality that drives unregulated cell growth and proliferation that is the hallmark of eBL (Thorley-Lawson and Allday, 2008). The *c-myc* oncogene is a regulator

gene that codes for a transcription factor. The expression of this gene is tightly regulated but when it is dysregulated as happens in eBL, it results in uncontrolled growth and proliferation of cells. These cells posses immortal properties which is a defining signature of many cancers (Klein *et al.*, 1976; Thorley-Lawson and Allday, 2008).

2.3 Endemic Burkitt Lymphoma as a Polymicrobial Disease

Although there are several non-mutually exclusive hypotheses to support a causal role for the interaction between *P. falciparum* and EBV in the aetiology of eBL (Rochford *et al.*, 2005; Chene *et al.*, 2009), this does not explain why only a few children living in malaria holoendemic regions develop eBL. All such hypotheses are premised on the classical observation that a strong geographical and epidemiological overlap between malaria endemicity and incidence of eBL exist (Burkitt, 1962; Rainey *et al.*, 2007a). However the role of other possible mechanism in the etiology of eBL remain to be described. Although past studies have alluded to eBL as having multifactorial etiology, there has been no study to date that has investigated the possible association of common infections such as schistosomiasis, measles, bacterial infections as well as other viral infections in the etiology of eBL. These diseases are found at high prevalence rates in the regions that report high eBL incidence (Parodi *et al.*, 2013).

Published reports have shown that regions around Lake Victoria experience high prevalence rates of schistosomiasis (Opisa *et al.*, 2011; Sher *et al.*, 1989). This infection has immune modulating properties that may have a possible role in the etiology of eBL, but whether children who develop eBL have a different schistosomiasis exposure remains to be studied since coincidentally regions that have high prevalence of schistosomiasis also report high incidence of eBL (Orem *et al.*, 2007).

Measles is another childhood diseases that have been associated with cancer (Parodi *et al.*, 2013; Tennant *et al.*, 2013) as well as immune modulation and quite common in regions around Lake Victoria (Ohuma *et al.*, 2009). The possible effect of this disease has not been investigated in the context of eBL. Comparing the serological status of children diagnosed with eBL and healthy ones from same region may shed more light on whether children who develop eBL have a different exposure history that puts them at increased risk of tumorigenesis.

2.4 EBV viremia as a biomarker and risk of Cancer

The monitoring of EBV viral loads have been used as both a risk factor in the etiology of eBL and other EBV associated lymphoproliferative disorders (de-Thé, 1985) as well as a prognostic marker after initiating chemotherapy (Bingler *et al.*, 2008; Slyker *et al.*, 2013; Stevens *et al.*, 2002). Past studies in western Kenya have measured EBV viral loads from whole blood without differentiating whether it was of plasma or of cellular origin (Moormann *et al.*, 2005; Piriou *et al.*, 2012, 2009). A previous study in western Kenya showed that children less than 5 years old, living in malaria holoendemic regions, had high EBV loads as compared to those living in a region of hypoendemic *P. falciparum* transmission (Moormann *et al.*, 2005). A reliable measure of viremia that can be used for monitoring for eBL risk and other lymphoproliferative disorder is therefore urgently needed. Furthermore, the impact of the high EBV loads, compounded with often-asymptomatic *P. falciparum* infections on EBV-specific T-cell differentiation, is poorly understood.

2.5 Modulatory Effects of Malaria on Host Cytokine Response

Malaria, a clinical condition due to infection by *Plasmodium* species, is a leading cause of morbidity and mortality globally (WHO, 2012). Protective immunity to malaria is a product

of excruciating cumulative exposure history and is dependent on intensity and burden of infection. However when such immunity develops, it fails to induce sterile immunity (Snow *et al.,* 1997; Marsh and Kinyanjui, 2006). The clinical manifestations of malaria include anaemia, cerebral malaria, paroxysms, periodic fever, vomiting, anorexia, joint pains and body aches (Kwiatkowski and Greenwood, 1989). Although malaria may modulate the host immune system, (Hviid *et al.,* 1992; Theander, 1992; Peyron *et al.,* 1994), the exact mechanisms that contribute to increased risk eBL genesis have not been well delineated.

The fact that the malaria parasite (*Plasmodium* species) has developed strategies to evade the host immune response is one of the defining markers of its successful parasitism (Theander, 1992; Ramasamy, 1998; Hogh, 1996). It has been shown that the parasite can have immune inhibitory effects on the host's CD4+ and CD8+ T cells (Artavanis-Tsakonas *et al.*, 2003; Marsh and Kinyanjui, 2006; Theander, 1992). Further, it has been shown that, the parasite can modulate the host defenses (Marsh and Kinyanjui, 2006; Moormann *et al.*, 2011; Niikura *et al.*, 2011; Ramasamy, 1998). Understanding the impact of this modulation on the development and maintenance of T-cell immunity to other secondary infections such as EBV that would increase the risk for eBL, would guide intervention strategies and diagnostic management of this pediatric cancer.

One hallmark of clinical pathology due to *P. falciparum* malaria infection is the uncontrolled pro-inflammatory immune responses (Ramasamy, 1998). This immuno-pathological disorder is the cytokine storm that is associated with clinical malaria (de Kossodo and Grau, 1993; Hunt and Grau, 2003). Understanding the differences in cytokine patterns between populations at risk of eBL would help in proper management of populations at risk of this cancer. To date, no data exists that has examined how malaria modulates the immune system

via cytokine milieu as a risk factor in eBL etiology. There is need therefore to study how malaria polarizes the host immunity either towards type 1 (IFN-γ) or type 2 cytokine (IL-10) (de Kossodo and Grau, 1993) production that could be a risk factor in eBL tumorigenesis. Besides the two well-defined cytokines involved in malaria pathology, IL-17 has recently been described as another independent pathway involved in malaria clinic-pathology (Bueno *et al.*, 2012). IL-17 has also been also shown to enhance viral persistence as well down-regulate Th2 responses (Awasthi and Kuchroo, 2009; Bueno *et al.*, 2012; Hou *et al.*, 2009). The roles of these cytokines have not been well described in the etiology of eBL.

2.6 The Effect of Malaria Transmission Pressure On EBV-specific T-Cell Immunity

Studies involving EBV-related malignancies have suggested a pre-existing immuno-modulation model for emergence of these cancers. This shows that selective loss of EBV-specific immunity predisposes to disease (Heller *et al.*, 2008). Such immune modulation can be due to chemotherapy as happens in post transplant lymphoproliferative disorders (Rowe *et al.*, 2001) or HIV-associated BL (Slyker *et al.*, 2013) or malaria in the case of eBL (Dalldorf *et al.*, 1964). Once infected, EBV resides in memory B cells. It is interesting to note that *P. falciparum* preferentially activates memory B cells (Donati *et al.*, 2004, 2006; Chêne *et al.*, 2007a). Long-lasting antigenic stimulation has long been suggested to be risks factor in the development of several B-cell malignancies such eBL and multiple myeloma (Brown *et al.*, 2008). However, the mechanism by which malaria perturbs T cell compartment leading to eBL remains to be investigated. . BL is a relatively rare cancer in light of ubiquitous malaria and EBV infections, suggesting a more complicated synergist model.

Studies have shown that chronic or acute clinical malaria infections not only impairs EBV-specific T-cell immunity and causes polyclonal activation of EBV-infected B cells, but also

induces shifts in B-cell homeostasis (Marsh *et al.,* 1989; Rasti *et al.,* 2005). Healthy EBV-infected individuals have efficient EBV-specific immunosurveillance against both lytic and latent EBV antigens (Khanna and Burrows, 2000; Rickinson and Moss, 1997). However, children presenting with eBL have been shown to be selectively deficient in EBNA-1-specific IFN-γ CD8+ T cells (Moormann *et al.*, 2009; Snider *et al.*, 2012). Whether this deficiency is confined to the CD8+ compartment of T cells or transcends to the CD4+ compartment is not known. Furthermore, it is not clear whether this deficiency is specific to EBNA1 or to other EBV latent antigens such as EBNA2 or EBNA3A.

There is paucity of data on the breadth and magnitude of EBNA-1-specific immunity in children living in malaria-holoendemic regions. Understanding the immune landscape of the healthy controls and comparing it to age-matched eBL patients would be critical in deciphering some of mechanisms that may mediate the etiology of eBL. EBNA-1 is the only expressed protein in all EBV-associated malignancies, EBV-positive proliferating B cells in EBV-seropositive, healthy carriers and during latency stages of the virus infection (Allen, 2005; Rickinson and Kief, 2001; Rowe *et al.,* 2009). Further, seminal studies in Kenya have shown that it is only EBNA-1 that shows immune stratification based on malaria exposure (Moormann *et al.*, 2007).

The expression of EBNA-1 in all EBV-associated malignancies elevates it to a special status as a viral gene product that can be exploited for vaccine construction, immunotherapeutic as well as diagnostic biomarker (Lee *et al.*, 2004). It has long been known that EBNA-1 is not processed by the classical endogenous MHC Class I pathway but, instead, by autophagy (Münz *et al.*, 2000; Schmid and Münz, 2007; Taylor and Rickinson, 2007). This is because EBNA-1, a 60-80 kDa polypeptide, contains Gly/Ala repeat domains that were initially

believed to mediate inhibition of antigen processing by the proteosome, the main catalytic machinery for generation of MHC class I ligands and therefore CD8+ T-cell epitopes. However, it was later shown that this protein could be recognized by CD8+ T-cells (reviewed by Münz *et al.*, 2000). Previous studies have shown that endogenously expressed EBNA-1 protein is accessible to the MHC-1 pathway, despite the Gla/Ala-repeats (Lee *et al.*, 2004, Tellam *et al.*, 2004). Several MHC-restricted epitopes from this protein have since been characterized and shown to be immunogenic (Lee *et al.*, 2004, Tellam *et al.*, 2004), thus making it possible to study T cell response to them.

The role of CD4+ T-cells as principal mediators of anti-viral immunity and malignancy has gained popularity (Brown, 2010). The CD4+ T cells have long been recognized as crucial for effective antiviral immunity: they are essential for induction and maintenance of cytotoxic T-cell responses, can exert cytolytic activity on their own as well as provide help for humoral immune responses (Ressing *et al.*, 2008). The CD4+ T-cells routinely respond to a large pool of EBV antigens drawn from both the latent and lytic life cycle (Brown, 2010; Münz *et al.*, 2000; Nazaruk et al., 1998; Ning *et al.*, 2011; Paludan and Münz, 2003; Wilson and Morgan, 2002). CD4+ T cells can access EBNA-1-derived peptides through autophagy, a process where antigens bypass the normal exogenous pathway in which the antigen is taken up from the extracellular milieu before being processed through the endo-lysosomal compartment (Cavignac and Esclatine, 2010; Lee *et al.*, 2009; Taylor and Rickinson, 2007). Thus, EBNA-1, is nonetheless potentially an important immunological target (Lee *et al.*, 2004; Leight and Sugden, 2000). Thus both CD4+ and CD8+ T cells are important artillery in immunosurveillance of latent EBV infection. It is for this reason that both CD4+ and CD8+ EBNA-1 specific T cell memory phenotypes and function need to be described.

Seminal studies from western Kenya that have reported diminished CD8+ T-cell responses against latent and lytic EBV antigens, strengthen the view that recurrent and intense *P. falciparum* infection could be responsible for the observed T-cell immune deficiencies (Moormann *et al.*, 2007). The reported loss of EBNA-1-specific immune surveillance can lead to escape of EBV transformed-B cell malignant clones that allow for eBL emergence. Therefore, the mechanisms of this loss need to be investigated to design novel clinical and immunotherapeutic interventions against eBL.

2.7 *P. falciparum*, PD-1 mediated T cell Exhaustion as a factor in the etiology of eBL

There is increasing evidence of pronounced *P. falciparum* induced immune-modulatory effects on EBV-immunosurveillance in children who have not yet 'learned' how to properly control the viral bursts associated with repeated malaria infections (Moormann *et al.*, 2005; Njie *et al.*, 2009). One of the possible mechanism through which malaria could be shaping the immune landscape is through the induction of immune inhibitory molecules such as PD-1 (Butler *et al.*, 2012).

The expression of Programmed death (PD-1) molecule and its associated ligands (PD-L1 and PD-L2) have been reported in many studies as either a marker of exhaustion or activation (Yamamoto *et al.*, 2008). The effect of these molecules on immune system impairment has been reported more frequently for T cells than B cells and for immune evasion strategies by tumours (Yamamoto *et al.*, 2008). PD-1 has been implicated in reduced T-cell proliferation and reduced CD8+ cytotoxicity (Francisco *et al.*, 2010). However, whether the same mechanism exists in the EBV-malaria nexus in the etiology of eBL is unknown. A detailed understanding of the immune architecture using T cell lineage functional and phenotypic

markers would expand our understanding of eBL aetiology and may be useful biomarkers in staging of this tumour.

2.9 Characterization of T cell memory phenotypes and possible Role in eBL Etiology

To date, no study has characterized the quality and phenotype of EBV-specific T-cell profiles in malaria-endemic populations, including children diagnosed with eBL. As such, it would be important to create an understanding of childhood immunity enlisted to control early-age infections with EBV within the setting of malaria co-infections and, thus, lend important clues to the etiology of eBL.

To characterize T cell memory phenotypes, a pan lymphocyte marker, CD45 that consists of several isoforms: CD45RA and CD45RO is used (Sallusto *et al.*, 2004; Saule *et al.*, 2006). Whereas RO is a marker of memory or recall, RA is a marker associated with naïve T-cell lymphocytes (Sallusto *et al.*, 2004; Saule *et al.*, 2006). Another T cell lineage marker, CCR7, a C-C chemokine receptor 7 (CD197) that functions as a homing receptor is used with together with CD45 to classify T cells into four phenotypes: naïve T cells (T_N), central memory T cells (T_{CM}), effector memory T cells (T_{EM}) and effector memory expressing CD45RA (T_{EMRA}) (Sallusto *et al.*, 2004; Saule *et al.*, 2006). Central memory T cells, characterized by the expression of CD45RO+, CCR7 and CD62L provide reactive memory which home to T-cell areas of secondary lymphoid organs with little or no effector function, but readily proliferate and differentiate into effector cells upon antigenic challenge. The effector memory T cells on the other hand are CD45RO+, have lost CCR7 expression, are heterogeneous for the expression of CD62L and show immunological features characteristic for homing to inflamed tissues with accelerated effector functions (Sallusto *et al.*, 2004).

Each T-cell phenotype has distinct pattern of cytokine responses. Although CD8+ T_{EM} produce a lot of perforin upon activation both CD4+ and CD8+ T_{EM} secrete large amounts of IL-2, IFN-γ or IL-15 (Rufer *et al.*, 2003; Sallusto *et al.*, 2004). T_{CM} on the other hand secrete high quantities of IL-2, IFN-γ or IL-4. The pool of circulating subsets of peripheral T cells is influenced by the nature and magnitude of an infection as well as the age of the host (Saule *et al.*, 2006; Hislop *et al.*, 2007). There is precedent for an acute infection to manifest a different pattern of T-cell lineage evolution as opposed to a chronic infection or cancer as observed using viral infection in murine models (Angelosanto *et al.*, 2012). This change is equally marked by a hierarchical succession in T-cell function (Davenport *et al.*, 2002a; Rufer *et al.*, 2003; Sallusto *et al.*, 2004; Saule *et al.*, 2006) which is modelled by environmental and host genetic factors (Arese, 2006; Cao *et al.*, 2004; Hill, 2001). This characteristic pattern of lineage changes will either confer protection or susceptibility (Angelosanto *et al.*, 2012). It is for this reason that the quality of T-cell memory subsets needs to be evaluated in the context of malaria and EBV with regard to eBL genesis.

Earlier studies in western Kenya investigated T cell responses to EBV by ELISPOT assay (Moormann *et al.*, 2009, 2007). ELISPOT is a less sensitive assay that utilizes large quantities of cells and does not distinguish the phenotype of antigen specific cells. To address these limitations, there is need to compare proportions of EBNA-1-specific CD4+ and CD8+ T-cell memory subsets by flow cytometry, which a is high throughput assay. It is hypothesized that children from a malaria hypoendemic region will present with different T-cell phenotypes compared to those from holoendemic regions. These differences may provide clues about eBL etiological mechanisms.

2.10 Sickle-Cell Trait and eBL: The unresolved question for 45 years

Host genetic factors have been implicated in either susceptibility or resistance to many infectious and non-infectious diseases. Although *P. falciparum* has been linked in the etiology of eBL, the rarity of this malignancy (with an annual incidence 2 in 100,000 children (Morrow, 1985) relative to the prevalence of malaria and EBV infections within pediatric populations malaria holoendemic regions suggests additional risk factors. Several host genetic factors that offer protective advantage against several infectious diseases such as malaria (Hill, 2001). Some of the well characterized host genetic factors in western Kenya include Glucose-6-phosphate dehydrogenase deficiency (G6PDH) and sickle cell trait (HbAS) (Moormann *et al.*, 2003a). The frequencies of these traits are high which coincidentally have high eBL incidence (Rainey *et al.*, 2007a).

It has been 45 years on since it was proposed that HbAS trait could play a role in decreasing incidence of eBL (Nkrumah and Perkins, 1976; Williams *et al.*, 2005b). Homozygous individuals (HbSS) suffer from sickle-cell disease, whereas heterozygosity (HbAS) confers protection against clinical malaria, 60% protection against high parasitemia (Allison, 1954a, 1954b; Williams, 2006) and 70-90% protection against severe disease (Aidoo *et al.*, 2002a; Gilles *et al.*, 1967; Hill *et al.*, 1991). If malaria disease severity and parasitemia play a causal role in eBL etiology, then the incidence of eBL would be lower in those with HbAS trait (Nkrumah and Perkins, 1976; Pike *et al.*, 1970; Williams, 1966).

The frequency of HbAS genotype is high in western Kenya and differs according to malaria exposure (Aidoo *et al.*, 2002a; Aluoch and Aluoch, 1993; Moormann *et al.*, 2003b; Williams *et al.*, 2005). It is necessary to investigate whether HbAS has any impact on EBV burden, which in turn affect host immune architecture to EBV as a mechanism towards eBL

aetiology. The relationship between HbAA/HbAS genotype and EBV titers as a functional measure of viral control and a potential biomarker of disease risk need to be explored. Furthermore, the role of HbAS has not been fully appreciated in terms of shaping immune landscape permissible for eBL lymphomagenesis.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Sites

The current study was carried out in western Kenya in areas that have different malaria transmission dynamics. Kenya strides across the equator on latitude 4°N and 4°S and longitude 34° and 41°E. The country measures 580,367 square kilometres with varied geographical regions. Due to varied topography and climatic conditions, malaria transmission is high at the coast and western part of the country while the middle part of the country has mountainous terrain and experience hypoendemic malaria transmission. Kenya has an estimated population of 42 million (as per the Kenya National Bureau of Statistics (http://www.knbs.or.ke) and World Bank 2012 census estimates (http://data.worldbank.org/country/Kenya)).

The age-matched healthy control children to the case eBL group were enrolled from three sites in western Kenya utilizing infrastructure of two sub-district hospitals (Chulaimbo-Kisumu and Mosoriot-Nandi as well Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH)) in regions differing in malaria endemicity (Ernst *et al.*, 2006; Ndenga *et al.*, 2006). Chulaimbo Rural Health Training Centre (-0°2'15'N, 34°38'17E or -0.037972, 34.638299) is the regional sub-district hospital that serves rural residents of Kisumu County while Mosoriot Rural Health Centre serves rural residents of Nandi County. These sites further differ in their incidence of eBL (Rainey *et al.*, 2007a). Mosoriot (highlands) (0°19'19N, 35°10'24'E or 0.322078, 35.172429) found in Nandi County is in a region of hypoendemic (low and unstable) malaria and located at an altitude of over 3000m above sea level. High rainfall and humidity, but moderately low temperatures, characterize this region.

This region also has a low incidence of eBL (Rainey *et al.*, 2007a). JOOTRH (0°5'19'S, 34°46'19E or -0.088697, 34.772016) is the regional referral centre for all pediatric cancers in western Kenya. Chulaimbo (lowlands), located near the shores of Lake Victoria in Kisumu County is a region that experiences holoendemic malaria (chronic and intense transmission patterns) (Bloland *et al.*, 1999) and has a high incidence of eBL (Rainey *et al.*, 2007a; Rainey *et al.*, 2007b). This area is located at an altitude of about 1133m above sea level and is characterized by high humidity, rainfall and temperatures throughout the year. Healthy controls were enrolled after medical evaluation by a clinical officer attached to this study.

Annual entomologic inoculation rates (EIR) in Kisumu may exceed 300 infectious bites per person (John *et al.*, 2005) even though this area has more recently experienced lower malaria transmission due to the success of malaria control programs (O'Meara *et al.*, 2011). The eBL group typically reside in malaria holoendemic areas of western Kenya (Rainey *et al.*, 2007a, 2007b). (See attached Appendix 1 and 2 showing malaria endemicity in Kenya and eBL prevalence rates in Nyanza province, respectively, Appendix 3 shows the geographical location of the study sites).

3.2 Study Populations

Pediatric patients diagnosed with eBL and admitted at Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) formerly Nyanza Provincial General Hospital (NPGH), the regional referral centre for cancer treatment were enrolled in this study after obtaining written informed consent. The eBL diagnosis was made based on cytological and histological finds of fine needle stained with May-Grunewald Giemsa stain. A clinical cytologist and pathologist independently confirmed this assessment. Findings from this group were

compared to healthy age-matched controls (aged below 10 years) drawn from western Kenya from regions differing in malaria endemicity and incidence of eBL.

3.3 Study Design

This was a case-control study design. In this study, eBL children were matched by age and malaria exposure with healthy children from the same locality (geographical and malaria exposure) as well as intentionally collecting age-matched controls from a malaria hypoendemic region to serve as the 'gold standard' comparison for a healthy immune response associated with viral control (defined as no detectable or low EBV levels by qPCR in EBV seropositive individuals).

3.4 Inclusion and Exclusion Criteria

3.4.1 Inclusion Criteria

Before a healthy child was enrolled to the study, the following conditions had to be fulfilled:

i. Aged between 4 and 9 years, which is the peak incidence age for eBL.
ii. Physically healthy as determined by a Clinical officer after physical examination (see Appendix 9: Healthy Children Enrolment Form).
iii. Auxiliary temperatures of 37.5°C.
iv. Permanent residents of Kisumu or Nandi Counties.
v. Written informed parental or guardian consent for blood to be drawn.
vi. For children presenting with eBL, enrolment occurred prior to induction of chemotherapy and after admission to the JOOTRH irrespective of the district of origin.

3.4.2 Exclusion Criteria

Exclusion criteria included:

i. For healthy controls: presence of diseases that compromise immune responses such as HIV infection and/or malnutrition.

ii. Evidence of fever due to lower respiratory infection, bacterial or viral infection.

iii. Anaemic - (haemoglobin (Hb) less than 5.0g/dL).

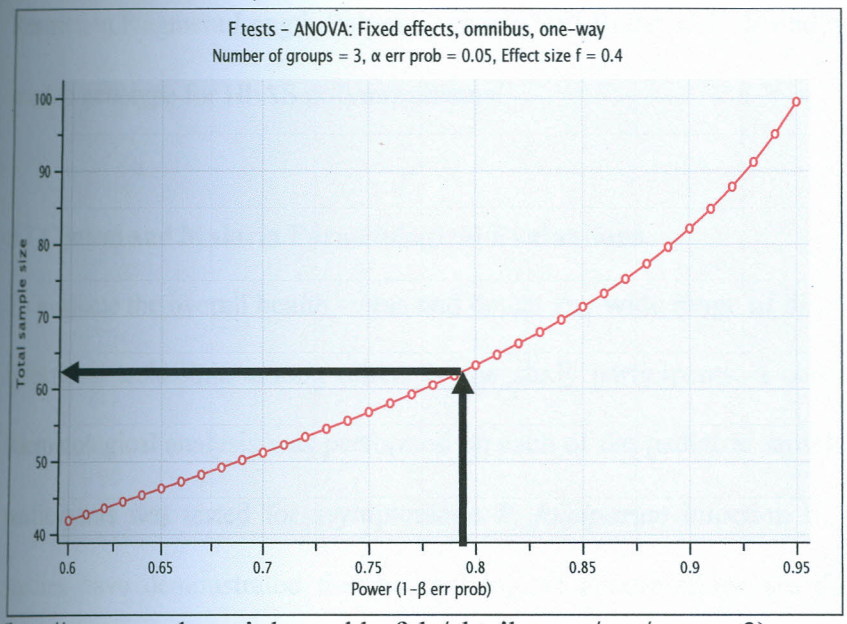
iv. Refusal to consent.

v. Non-permanent residents of Kisumu and Nandi Counties.

vi. Aged below 4 years or above 9 years, which are ages outside the peak incidence ages for eBL.

3.5 Sample Size and Power Calculation

The sample size for this study was calculated using the G*power software (Faul *et al.*, 2007). Utilizing this software, certain parameters are either from a pilot study or published work is necessary. To achieve a statistical power of 80% ($\beta \geq 0.8$ is acceptable for biological studies) and a critical limit of $\alpha = 0.05$ for significance level with a large effect size, using mean EBV titers for the three study groups as one of the outcome parameters (mean log EBV copies per microgram of human DNA=3.0 or 3000 copies of EBV per microgram of DNA) (Moormann *et al.*, 2005), a total of 63 children (a minimum of 21 from each from the three study groups) were required to be enrolled. (See the Fig. 3.1).



(http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3).

Fig. 3.1: Sample Size and Power Calculations

To establish the quality of assays for this study, 22 adults were enrolled. These controls were Kenyan adult samples (n=11) from the same study sites and a further 11 North American adults with no prior malaria exposure. The inclusion of these adult controls was specifically to serve as quality controls as well as for optimization of the assays performed and not for analyses.

3.6 Data Collection Methods

For objective 1, the method of data collection was Multiplex ELISA for measuring total IgG levels to selected common infectious agent's antigens (EBV, *P. falciparum*, measles and schistosomiasis). For objective two, Quantitative PCR (qPCR) was used to measure EBV viral titers after DNA extraction either from blood pellet or from plasma. Multiparameter Flow cytometry was used to obtain data for objective 3,4 5 and 6. The quality of EBNA-1-specific T-cell phenotypes was investigated by gating on cytokine positive cells after *ex vivo* stimulation with a pool of 50, 15-mer (overlapping by 14) EBNA-1 peptides. These peptides were drawn from the C-terminal end of the EBNA-1 protein. Lastly for objective 7,

Restriction Fragment Length Polymorphisms (RFLP) based-PCR and gel electrophoresis was used to genotype for HbAS polymorphisms.

3.7 Clinical and Malaria Parasitological Evaluations

To evaluate the overall health status and detect any wide range of disorders such as anaemia, infections, leukaemia among others in the study participants, a complete blood count and haematological analysis was performed on each of the pediatric samples. Further, each study participants was tested for asymptomatic *P. falciparum* infection by microscopy. Several studies have demonstrated that haematological abnormalities are the most common side effects of lymphomas and chronic infections(Kirchhoff *et al.*, 2008; Teuffel *et al.*, 2008).

3.7.1 Demographic Characteristics

During enrolment, characteristics such as ethnicity, gender and age were collected before a sample was drawn to ensure matching. For clarity of reference, these groups of children were referred to as **Kisumu** (stable/high malaria transmission region-holoendemic malaria, high risk of eBL), **Nandi** (unstable/low malaria transmission-hypoendemic malaria, low risk of eBL) and **BL** (children with confirmed diagnosis of eBL). Kisumu and BL children were ethnically matched and were of Luo ethnic group. The Nandi group had to be of the Kalenjin ethnicity and specifically of the Nandi sub-ethnic group.

3.7.2 Malaria Parasitological Examination

Malaria diagnosis was determined by microscopic examination of Giemsa-stained thick and thin smears at 1000× under oil immersion. Parasitemia was determined per microlitre (µl) of blood by counting asexual parasites per 200 white blood cells, assuming an average 8000 white blood cells per µl of blood as per the WHO standard criteria (Dowling and Shute,

1966). A smear was considered negative when no parasites were observed after counting microscopic fields that included at least 200 leukocytes. Any study participant testing positive for *P. falciparum* was treated by qualified medical staff attached to the project following the Kenya Ministry of Health guidelines for treatment of uncomplicated malaria.

3.7.3 Complete Blood Count (CBC)

An automated coulter counter (Beckman Coulter, Boulevard, California, USA) was used to quantify the full hemogram (blood profile) of all the subjects.

3.8 Laboratory Procedures

3.8.1 Choice and Selection of EBV Peptide Pools

Overlapping 8-10-mer EBNA1 peptide pools from the C-terminal region of the protein was used to stimulate the cells as it contains both CD4+ and CD8+ T-cell epitopes (see Appendix 4 for list of EBNA1 peptides). EBNA2 and EBNA3A, representing other EBV latent antigens were used in the study as control peptides to investigate the selective specificity of EBNA-1 response.

These commercial overlapping peptides (EBNA-1, -2 and -3A) were synthesized and purified to greater than 70% purity by high performance-liquid chromatography (HPLC) and were lyophilized for stability (21st Century Biochemical, Marlboro, Massachusetts, USA). Peptides were reconstituted in 5 % (wt/vol) dimethyl sulfoxide and diluted in sterile 1× PBS (GIBCO) to a stock concentration of 0.2 mg/ml. The EBV peptides were pooled so that each peptide was used at a concentration of 2 µg/ml (see Appendix 4 for List of EBNA-1 peptides used in the study).

3.8.2 Study Assays Optimization

All assays and result presented in this thesis were obtained after a series of optimization experiments using EBV seropositive healthy US adults as well as Kenyan adults (with no malaria exposure) ensuring quality assurance/quality control and reproducibility before screening actual samples.

3.8.3 Blood Sample Collection and Processing

About 2-5 ml of venous blood samples was collected by venepuncture from each study participant by a qualified KEMRI phlebotomist. The blood was collected in heparinized green top BD Vacutainer tubes® (BD, UK) for PBMC isolation and purple top EDTA microtainer tubes® (BD, UK) for EBV titre determination. The sample was transported to UMMS/KEMRI laboratories at the Centre for Global Health Research, Kisumu and processed within 3 hours. The peripheral blood mononuclear cells (PBMCs) were separated from whole blood by Ficoll-hypaque (GE Healthcare, Sweden) density gradient centrifugation method. The isolated buffy coat of PBMCs was washed twice in 1×PBS, counted and cryo-preserved until used for flow cytometric analyses.

3.8.3.1 Cryopreservation of PBMCs

The isolated PBMCs were suspended in complete culture media (RPMI 1640 supplemented with 10% heat inactivated human serum type AB). The cells were enumerated by use of an improved Neubauer chamber haemocytometer (Thermo Scientific, Swedesboro, USA) and the cells re-suspended in freezing media (10% DMSO and 90% Foetal Bovine Serum (FBS) (Invitrogen, New York, USA) at a concentration of 6×10^6 cells/ml. Sterile conditions were maintained during the whole procedure. The cells were aliquoted in appropriately labelled and pre-chilled 1.8 ml cryotubes (Nalgene, Thermo Scientific, Swedesboro, USA). The cells

to be cryopreserved were chilled in FBS on ice for 30 min to ensure maximum cell viability after freezing. The cryotubes with the cells were then placed in a freezing unit containing Isopropyl alcohol and stored at -80 °C overnight before being transferred to vapour phase liquid nitrogen the next day where they were stored until ready for use.

3.8.3.2 Protocol for Thawing of PBMC

The cryopreserved cell vials were removed from liquid nitrogen tank and transported on dry ice to the laboratory. The vials were quickly thawed in a 37^0C water bath. When there was just a small frozen piece (pea size) in the vial, the cell suspension was quickly transferred into a 15 ml conical tube containing 10 ml of pre-warmed thaw media with 10 IU/ml of DNAse in a Biosafety laminar flow hood. The cell suspension was gently allowed to mix with the thaw media. The empty cryovial was rinsed with approximately 1ml of thaw media and this added to the cell suspension. The tube was then spun at 250× g for 7 min at room temperature (RT). The supernatant was discarded, the pellet re-suspended gently using a pipette ensuring that no bubble were formed, before washing the cells once more in warm thaw media. The supernatant was then discarded, the cells re-suspended in 5 ml of complete culture media (10% heat inactivated Human type AB serum in RPMI 1640 supplemented with 0.05% L-glutamine and 0.05% Penstrep) and cell population determined by counting the cells in 0.4% Trypan Blue/Turk's solution to determine cell viability.

3.8.3.3 PBMC Culture Conditions

The counted PBMCs left overnight at 37°C in a 5% CO_2 humidified incubator were washed, re-counted and plated at a concentration of 5×10^6 cells per well. The cells were cultured in complete RPMI (10% human serum AB, 2 mM L-glutamine, 20 mM HEPES, and pen/strep). The cultures were then stimulated using the following conditions with the indicated final

concentrations in parenthesis: overlapping EBNA-1 peptides (10 µg/ml), EBNA2 (10 µg/ml), EBNA3A (10 µg/ml) for 24 hours. Positive control wells were stimulated with 2µg /ml of SEB while the negative control wells had 1x PBS. After 18 hours of incubation, the cells were plugged (blocked) by addition of both 6µg/mL Golgi Stop™ (BD Biosciences, San Jose, California, USA) and 10 µg/mL Golgi Plug™ (BD Biosciences) due to differences in their mode of action to trap the intracellular cytokine for the last six hours of culture. Whereas Golgi Plug™ contains Brefeldin A (BFA), Golgi StopTM contains monensin both of which are protein transport inhibitors. Monensin is an inhibitor of trans-Golgi function where it prevents acidification of endocytic vesicles, while BFA inhibits protein transport between endoplasmic reticulum and the Golgi by preventing the exocytosis of cytokine containing vesicle thus allowing for visualization of cytokine production following stimulation. Both Golgi Stop™ and Golgi Plug™ blocks intracellular protein transport process resulting in accumulation of cytokines or protein of interest in the Golgi complex enhancing the detectability of cytokine producing cells. The cells were then harvested and stained with a panel of fluorochrome-conjugated antibodies for flow cytometry analysis (See Appendix 5 for cell surface and intracellular staining protocol for Flow Cytometry).

3.8.4 T-cell Immunophenotyping

Peripheral blood T-cell immunophenotyping was analysed by standard flow cytometric technique. The screening of different T-cell phenotypes was carried out using a panel of monoclonal antibodies to T-cell lineage markers conjugated to Quantum dot 605 (Qdot), phycoerythrin-Texas Red (PE-TR), BD™Horizon V500 (V500), Allophycocyanin Fluorscent 700 (APC Fluor 700), Peridinin-Chlorophyll Protein-Cyanide 5.5 (Percp-Cy 5.5), Alexa eFluorscein 780 (Alexa eFlour 780), and PE-cyanin 7 (PE-Cy 7) against the lineage-associated T-cell markers CD3, CD4+, CD8+, CD4+5RA, PD-1, CCR7 and CD27

respectively; and a dump channel consisting of monoclonal antibodies conjugated Pacific Blue (PB) against CD14 and CD19 lymphocyte markers. The Live/Dead® vivid violet molecular probe was used as a live marker. For the functional immunophenotyping, the cells were permeabilized and then monoclonal antibodies conjugated to phycoerythrin (PE), Allophycocyanin (APC) and Fluorescein isothiocyanate (FITC) against IL-17, IL-10 and IFN-γ, respectively were used. Appropriate Fluorescent Minus One tubes (FMOs) were used alongside the no antigen stimuli in all setups. The flow data was acquired on a LSRII flow cytometer (BD Biosciences, New Jearsey, USA) after gating and acquiring 100,000 events in the lymphocyte gate on a forward and side scatter dot plot using the FACSDiva ver. 6.1 software program (Becton Dickinson Immunocytometry systems, San Jose, USA).

3.8.5 T cell Memory Immunophenotyping

The phenotype of EBNA-1-specific IFN-γ-effector cells were characterized based on two T cell lineage markers described earlier by Sallusto and others (Sallusto *et al* 2004) (Fig. 3.2).

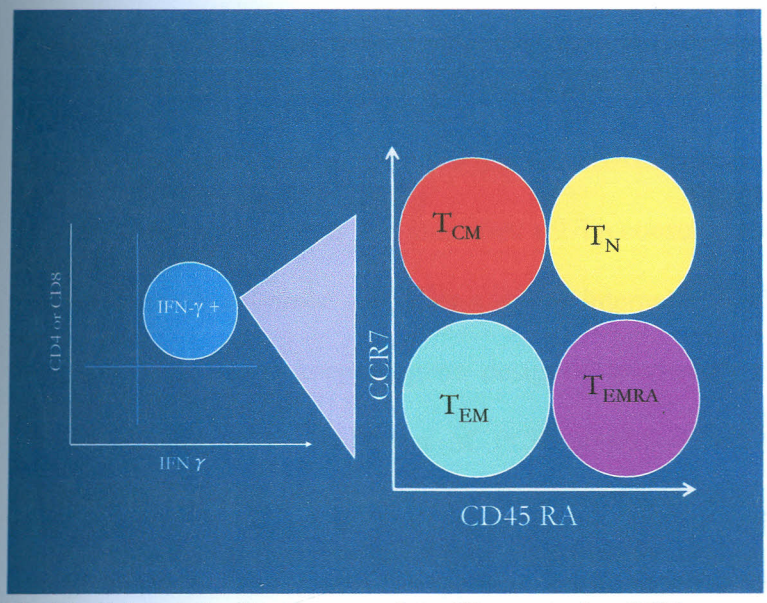


Fig 3.2: Schematic diagram used to Characterize IFN-γ-specific effector-T-cell memory phenotype

3.8.6 Quantitative Polymerase Chain Reaction for EBV Titers

The EDTA anti-coagulated blood was centrifuged at 1000 rpm (8000×g) (Eppendorf microcentrifuge 5415R, Hoffman-LaRoche Inc., USA) for 10 min to separate plasma from the blood pellet. An equivalent vol/vol of sterile 1× PBS was used to replace the aliquoted plasma. The ensuing blood pellet was stored at -80°C until DNA extraction while the plasma was stored at -20°C to be used for serology. EBV viral load was determined according to a previous protocol (Hayden *et al.*, 2008)) and later modified by Moormann and others (Moormann *et al.*, 2005). Briefly, EBV DNA was extracted from 200µl aliquot of either sample (Blood pellet or plasma) using QIAamp DNA Blood mini kit (Qiagen, Inc., Valencia, CA) with elution into 100 µl of 10 mM Tris-Cl-0.5 mM EDTA buffer (pH 9.0) according to the manufacturer's instructions. The eluted DNA was appropriately labelled and stored at -80°C until use. For the plasma, EBV titers were evaluated both with and without DNAse at 37°C for 30min treatment to investigate the quantity of lytic infective free viruses.

For EBV qPCR, two PCR primers and Taqman ® probes (Applied Bio system, Foster City, CA, USA) (that detect a 70bp region of the EBV BALF5 gene) combinations were used. The first probe specifically targets and amplifies a 97bp conserved sequence in the EBV EBNA-1 gene while the second probe amplifies a spiked internal control (IC- human beta actin gene) which acts as a house keeping gene designed to prevent false negative results due to inefficient extraction or inhibition of PCR amplification. Previously designed primers amplifying a conserved sequence of viral EBV DNA polymerase (BALF5) gene and a fluorogenic probe for this area (70bp) have been described by (Kimura *et al.*, 1999). The quantitative (q)-PCR cycle was as follows: 2 min at 50 °C, 10 min at 95 °C, 42 cycles of 15 sec at 95 °C and 1 min at 60 °C using a thermal cycler model I Cycler TM optical module (BioRad laboratories, Hercules, CA). IQ Supermix (BioRad laboratories, Hercules, CA) was

used for all reactions. To generate a standard curve, two commercial lots of fluorimetrically quantified EBV B95-8 DNA (Advanced Biotechnologies Inc., Columbia, MA) were used to create a six point tenfold serial dilution curve ranging from 2.65 copies/µl to 2.62×10^6 copies/µl. All the samples were assayed in duplicate, of which the mean value was taken as the DNA copy number. The EBV viral copy was calculated by extrapolation to a standard curve consisting of serially diluted EBV DNA standards. The lowest detection limit for this assay was 1 copy of EBV genome per µg of human beta-actin DNA multiplexed in the assay (Piriou *et al.*, 2012), while plasma viremia was normalized to EBV copies per µl of plasma. Figure 3.3 illustrates the standard curve that was used to extrapolation values for each samples run. The BL41 lymphoma cell line DNA was used as no template control to check against non-specific genomic amplification as it is EBV negative (Farrell *et al.*, 1991).

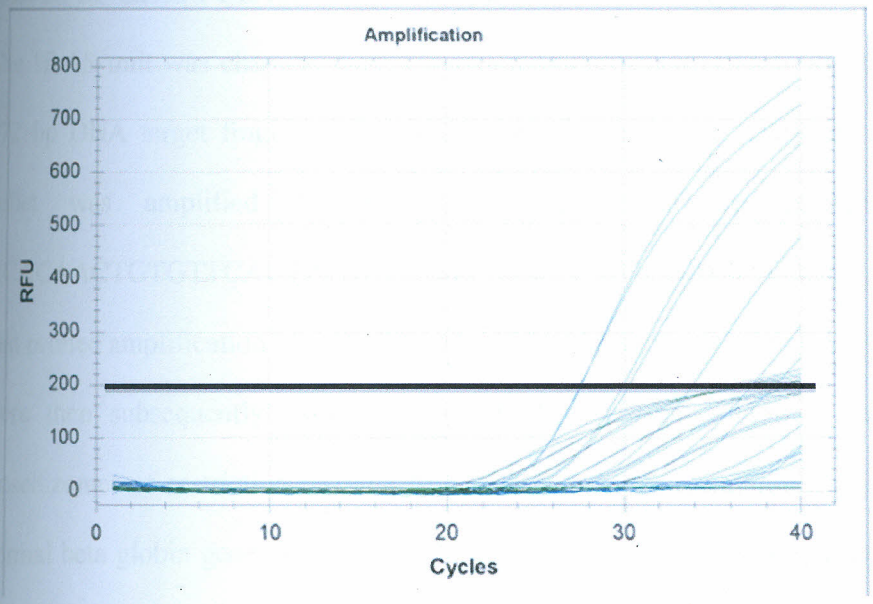


Fig.3.3 (A) RT-QPCR amplification CT values and cycles

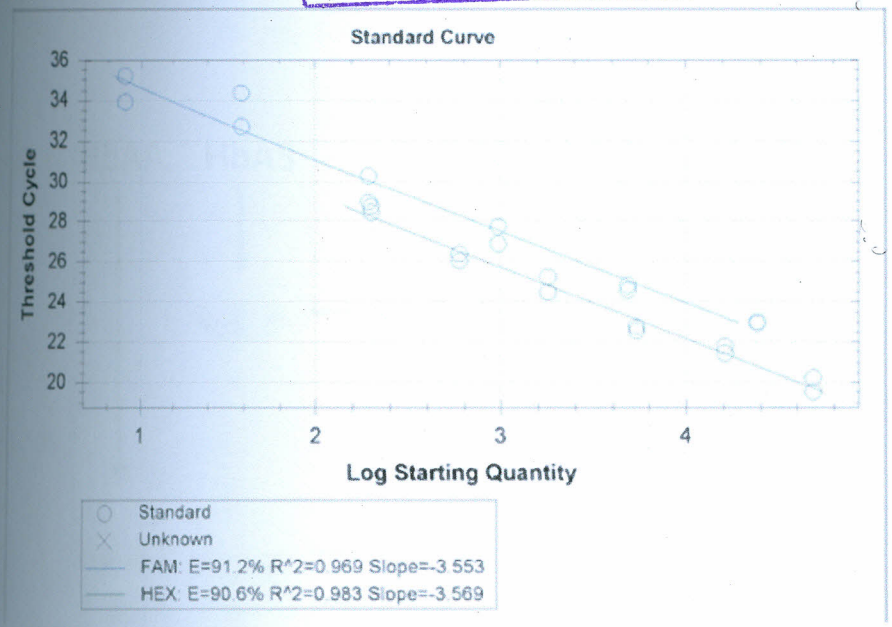


Fig.33 (B) EBV and Beta actin standard Curves

3.8.7 RFLP-based PCR for Sickle-Cell trait (HbAS) Genotyping

The HbAS trait was characterized as previously published (Husain *et al.*, 1995). Briefly, a 772 bp DNA target fragment from human beta-globin gene, extracted from whole blood pellet was amplified by PCR using the following primers: beta 1 (5'-ACACAACTGTGTTCACTAGC-3') and beta 2 (5'-CAACTTCATCCACGTTCACC-3'), that primed amplification of an 110-base-pair (bp) segment of beta globin gene. The products were then subsequently subjected to restriction enzyme digestion using Bsu 361, an isoschizomer of restriction enzyme Mst II, which has a recognition site at codon 6 in the normal beta globin gene, and cleaves the normal amplified beta globin DNA into fragments of 254, 228 and 202bp for HbAA and 430, 254, 228 and 202bp for HbAS. The digested products were then separated by gel electrophoresis (1.5% agarose gel) (Fig. 3.3).

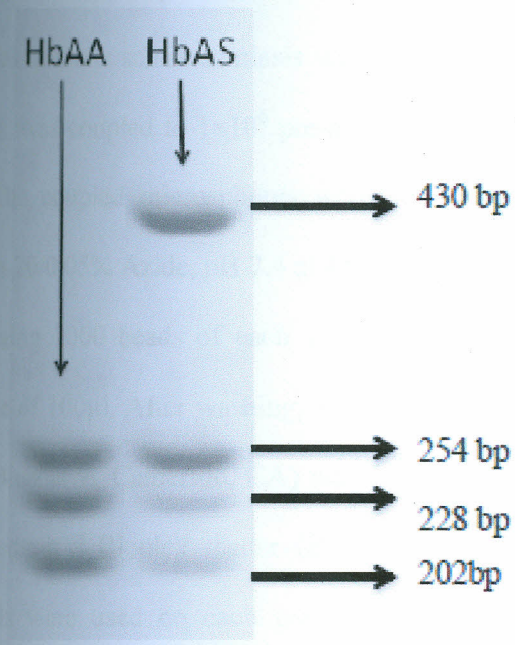


Fig. 3.4: A photomicrograph of gel electrophoresis showing beta haemoglobin genotypes after Bsu361 restriction enzyme digestion.

HbAA is digested into 3 fragments of 254, 228 and 202bp while HbAS is digested into 4 gene fragments of 430,254, 228 and 202bp.

3.8.8 Luminex Serology Assay

EBV-specific IgG antibody levels were determined using a microsphere suspension assay as previous described (Piriou *et al.*, 2009) on a CFX96TM Real Time System C1000TM Thermal Cycler (BioRad laboratories, Hercules, CA, USA). Briefly, synthetic peptides representing the immunodominant epitopes for the viral capsid antigen P18 (VCA), Epstein-Barr nuclear antigen 1 (EBNA-1), diffuse early antigen complex (EAd), and immediate early protein (Zta) antigens of EBV aka ZEBRA were coupled to carboxylated microspheres (Luminex, Austin, TE) following the manufacturer's protocol with some slight modifications. Other antigens used in the assay were derived from *P. falciparum* representing pre and

erythrocytic life cycles: Liver Stage antigen 1 (LSA-1), merozoite surface protein -1 (MSP-1), CelTos, circumsporozoite protein (CSP) and apical membrane antigen 1 (AMA-1), measles as wells schistosomiasis worm antigen protein 1 (SWAP). To start, 20 µg of each peptide was coupled to 1×10^6 pre-activated microspheres in a 500 µl of 100 mM MES pH 6.0. The coupled microspheres were washed and resuspended in PBS/0.1%BSA/0.02% Tween-20/0.05% Azide, pH 7.4 at 4°C until use. Antigen-specific IgG levels was detected by incubating 1000 beads of each antigen per well with plasma diluted at 1:100, in a final volume of 100µl. After washing, a 1:200 dilution of PE-conjugated Goat $F(ab)_2$ anti-human IgG (Biosource, Camarillo, CA) was added. At least 75 beads of each region/antigen were acquired on a Bioplex reader (Bio-Rad, Hercules, CA). EBV seronegative and positive controls were used on each plate. The results of the assay (levels of antibodies) were expressed as the mean fluorescent intensity (MFI) of at least 75 beads for each EBV antigen. Data from this assay has previously been validated in previous studies (Fachiroh *et al.*, 2006; Piriou *et al.*, 2009) and found to have very high correlations for all antigens when performed against the convectional ELISA.

3.8.9 Soluble PD-1 ELISA

Circulating human soluble PD-1 (sPD-1) from the plasma was determined using DuoSet ELISA Development Kit (R&D System Inc., Minneapolis, Minnesota, USA) according to the manufacturer's instruction. Briefly, 100µl /well of standards, samples (diluted 1:100 reagent diluent), and blanks were added to 96-well microtiter plates (Costar EIA plate R&D Systems Inc., Minneapolis, MN, USA) that had been pre- coated overnight at 4°C with goat anti-human PD-1 capture (primary) antibody (R&D System Inc., Minneapolis, Minnesota, USA). The plates were then incubated at room temperature for two hours before biotinylated detection (secondary) goat anti-human PD-1 antibody (R&D System Inc., Minneapolis, Minnesota, USA) was added. After washing and addition of streptavidin HRP conjugate and

alkaline phosphatase substrate (R&D System Inc., Minneapolis, Minnesota, USA), the quantity of sPD-1 was then determined colorimetrically. Optical density (O.D.) of the standards and samples were read at 450 nm wavelengths. The correction wavelength was set at 540 to take care of readings that maybe off scale and subtracted from 450nm readings. Sample O.D. values were compared to provided standards with known concentrations of PD-1 and sample concentrations extrapolated from a standard curve (R&D System Inc., Minneapolis, Minnesota, USA). Values of baseline (blanks) were subtracted from those of samples.

3.8.10 PD-1 Blocking Experiments

To investigate whether blockade of PD-1 pathway could lead to reversal of the IFN-γ response, PBMCs cultures were set-up in parallel with and without anti-human CD274 antibody (eBioscience- B7 HI) at a concentration of 1μg/ml before multiparameter flow cytometry. CD274 is a ligand for PD-1 and the interaction of PD-1 and CD274 (PD1-L1) leads to negative regulation of T-cell activation (Freeman *et al.*, 2000). Background response (PBS or unstimulated PBMCs) was subtracted from the each stimulation condition to obtain the net EBNA-1 specific T-cell response. See the gating strategy used below (Fig. 3. 5).

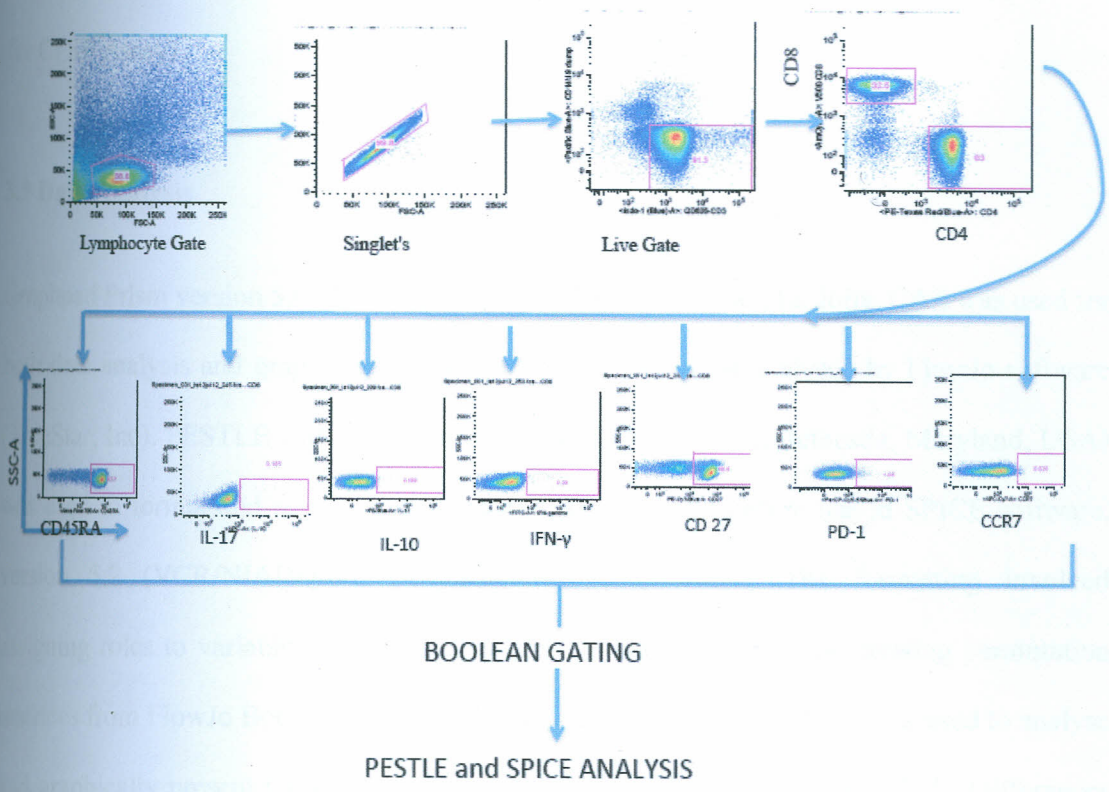


Fig. 3.5 T-cell Functional and Immunophenotyping Gating Strategy

3.8.11 CFSE Proliferation Assay for EBNA-1-Specific T cells

To investigate whether there were differences in the rate of EBNA-1-specific T-cell proliferation, Carboxy Fluorescein diacetate Succinimidyl Ester dye (CFSE) was incorporated in the culture media to tag proliferating cells. The assays works on the principle that covalently bound CFSE is divided equally between daughter cells allowing discrimination of successive cycles of proliferation (Hawkins *et al.*, 2007). Briefly thawed and rested cells were stained with CFSE at a concentration of 1×10^6 cells per mL for 15 min at 37°C in the dark. The cells were then washed twice with cold culture media, enumerated before stimulation and cultured for 5 days. On the fifth day, they were harvested and stained

as in described in section 3.5.6.4 above for CD3, CD4+ and CD8+ T-cells (See Appendix 5 for CFSE Assay).

3.9 Data Analysis

Graphpad Prism version 5.01 Software (Graphpad Software, Inc., La Jolla, CA.) was used for both data analysis and graph presentation. Flow data was first analysed by FlowJo software (TreeStar, Inc). PESTLE software version 1.7 (VCR/NIAD/NIH, Bethesda, Maryland, USA) was used to format and recode data generated from FlowJo before use in SPICE software, version 5.2 (VCR/NIAD/NIH, Bethesda, Maryland, USA). The formatting involved assigning roles to variables (values or categories), merging databases, creating permutation matrices from FlowJo Boolean gates and background subtraction. SPICE was used to analyse and graphically present the multiparametric FlowJo data formatted in PESTLE. Differences between measurement variables (viral loads, cytokine responses and IgG levels) were computed using Kruskal-Wallis and Wilcoxon signed rank tests while post-hoc Dunn's test and Mann-Whitney U test was used to evaluate differences between all pairs of data from the study sites. Correlation between continuous variables was done using non-parametric Spearman rank correlation test. The differences in HBAS genotypes frequencies were determined by Chi-square while the association between HbAS genotypes and EBV viral titers or EBNA1 specific T cells was by logistic regression analysis. All tests were two-tailed with p-value of 0.05 taken as the level of significance.

3.10 Ethical Considerations and Approval

The School of Graduate Studies provided scientific approval. Ethical approval for the study was obtained from the Ethical Review Committee at the Kenya Medical Research Institute (KEMRI) and the Committee for Protection of Human Subjects in Research of the

Institutional Review Board at the University of Massachusetts Medical School (UMMS), USA. Written informed consent, translated into three languages (Dholuo, Kiswahili and English) was obtained from parents or guardians of study participants (See Appendix 8).

All the samples collected were stripped of personal identity and coded using unique study identification numbers to protect study participant's privacy and only the Principal Investigator had access to personal details of the study participants, which was kept under key and lock.

Blood collection through venepuncture or finger prick can cause temporary discomfort, bruises and pain. To minimize risks, a qualified phlebotomist from the Ministry of Health carried out venepuncture. Furthermore, sterile butterfly blood collection needles were used and all sharps were stored in appropriate biohazard sharps' containers before disposal at KEMRI. Participation in the study was voluntary, the participants had a right to withdraw at any time during the study, and access to health care was not dependent on participation.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic Characteristics of the Study Participants

A total of 78 children were included in this study: Kisumu (n=24), Nandi (n=23) and BL (n=31), that was above the calculated minimum sample size of 63 study participants. The reason for this deviation was to take into consideration study participants whose PBMCs yields are poor as well as those whose cells may not be viable after cryopreservation and thawing process. The highest mean parasitemia density was observed among the Kisumu children with 3700 parasites per microliter of blood, consistent with children residing in a malaria holoendemic region (Snow *et al.*, 1998), followed by Nandi and eBL with 86.89 and 68.45 parasites per microliter, respectively (p=0.017) (Table 4.1).

4.2 Haematological Indices of the Pediatric Study Participants

Numerous studies have reported haematological abnormalities as the most common side effects of lymphomas and chronic infections (Kirchhoff *et al.*, 2008; Teuffel *et al.*, 2008) as happens in populations living in malaria holoendemic regions as well as children presenting with eBL. To eliminate possibilities of confounding factors that could otherwise skew the results, all children were tested for haematological indices. The measured haematological indices were within normal ranges based on reference values published on populations residing in the same geographical region (Zeh *et al.*, 2011). The Nandi children had higher levels of haemoglobin followed by Kisumu and lastly BL study group (p=0.025) consistent with residing at high altitude (3000m and 1133m above sea level respectively). There were no differences between the study populations in terms of peripheral white blood cell (WBC) counts (Table 4.2).

Table 4.1: Summary of Study Participants and General Characteristics

Study Group	*P.f.* exposure	Sample size (n)	Gender		Median age (yrs)	Mean vL (EBV copies /hDNA)	Mean *P.f.* parasitemia density
			Male (n)	Female (n)			
BL	Holoendemic	31	22(71.0%)	9 (29.0%)	8.5	5381.000	68.450
Kisumu	Holoendemic	30	14(46.7%)	16(53.3%)	8.2	504.600	3700.330
Nandi	Hypoendemic	28	15(53.6%)	13 (46.7%)	8.3	26.400	86.890
***p* value**						**0.026**	**0.017**

Data presented as means of tested variables.

Kruskal-Wallis test was used to analyse for differences between groups. Dunn's post hoc test was used to analyse for differences between all pairs of columns. Significant values ($p<0.005$) are in bold. (*P.f. –Plasmodium falciparum,* vL- EBV viral loads).

Table 4.2 Mean Haematological Indices across Study Populations

Study Population	Mean Haematological Indices							
	WBC ($10^3/\mu L$)	Hb (gm/L)	MCV (f/L)	MCH (pg)	MCHC	RBC ($10^6/\mu L$)	PCV/Hct (%)	Platelet count
BL	7.5	9.920	67.690	33.880	41.880	4.400	29.590	336
Kisumu	7.4	10.420	80.390	35.660	39.420	4.640	37.100	223
Nandi	7.4	11.420	80.710	37.700	42.080	3.790	39.290	295.6
p value	0.3679	**0.0250**	**<0.0001**	0.5838	**<0.0001**	0.5372	**<0.0001**	**0.0105**
Reference values ranges	(3.3-9.3)	(9.5-16.0)	(60-93)	(27-40)	(30-43)	(3.3-6.3)	(29.4-49.3)	(103-439)

Reference heamatological values for populations living in geographical same area, *courtesy* Kibaya *et al.,* 2008; Zeh *et al.,* 2011

Key: WBC- White Blood Cells; Hb- haemoglobin; MCV-Mean Cell Volume; MCH-Mean Cell Haemoglobin; MCHC-Mean Corpuscular Haemoglobin Concentration; RBC- Red Blood Cells; PCV- Packed Cell Volume. Differences in haematological indices were analysed using Kruskall-Wallis test. Significant values ($p < 0.05$) are in bold. Although there are some values which are statistically significant from analysis, they are not clinical significant as they are within the normal ranges as those obtained from populations living in the same area.

4.3 Serology Profiles for Common Infectious Agents across Study Populations

All study participants were tested for antibody titers against some of common infectious agents in the study sites (Petney and Andrews, 1998). The infectious agents tested included EBV, *P. falciparum* malaria, measles virus and schistosomiasis.

4.3.1 EBV Antigens Serology Profiles across Study Populations

It was generally observed that eBL and Kisumu children recorded high total IgG levels against the EBV antigens assayed as compared to the Nandi group. However there were no differences between the study groups in the levels of anti-EBNA-1 antibodies (p=0.0959).For VCA, eBL group posted significantly higher total IgG titers (median 25422 MFI; range: 21271-258210 MFI) followed by Kisumu (28942 MFI; range: 2400-186463 MFI) and lastly the Nandi study groups (median 23194 MFI; range: 114-143669) (p=0.0074). Further analysis showed that as much as eBL and Kisumu groups were comparable (p=0.0935), the Nandi group had very low VCA antibody titre compared to eBL group (p=0.0009). Kisumu and Nandi children had comparable anti-VCA titers (p=0.1043). For the Ead antigen, the eBL and Kisumu children recorded the highest levels of anti-EAd total IgG levels compared to Nandi children (p< 0.0001). These two antigens are from lytic EBV cycle and are an indicator of EBV reactivation. BL median titre level of 5871(756-45775) was comparable to that of Kisumu at 3423 (262-41805) (p=0.0891) but significantly higher than that of Nandi group of 429 (73.5-5437) (p> 0.0001).

There were significant differences between study groups in the levels of anti-Zebra total IgG levels (p<0.0001). The highest levels were observed in the BL group (11751, range 1328-133233), followed by Kisumu at 2016 (417-21698) while the least was observed among the

Nandi with a mean of 1162 (147-39309) (p=0.001). These levels were comparable between the Kisumu and BL children (p=0.2571) (Table 4.3).

4.3.2: Malaria Antigen Sero-Profiles across Study Populations

It was observed that populations residing in malaria holoendemic regions (BL and Kisumu) had higher total IgG levels to malaria antigens compared to those from malaria hypoendemic region (Nandi). It was observed that children from a malaria holoendemic region had the high levels of anti- AMA-1 levels compared to Nandi groups (p<0.0001). Kisumu children had high antibody titers of 48799 (range 4116-541208), followed by eBL group at 27103 (range 15083-275180) while the least levels were recorded in the Nandi group with a median of 870 (range 78-24536). For MSP-1, highest levels were observed among malaria holoendemic children (Kisumu and BL). Anti-MSP-1 levels were comparable between BL and Kisumu (p=0.4149) but significantly higher than the Nandi (p < 0.0001). Kisumu children had median titre levels of 60407 (3577-564361), BL group 31134 (10528-642848) while Nandi had the least levels at 4459 (433-469730.

Kisumu and BL children recorded the highest total IgG levels to CelTOS compared to Nandi (p<0.0001). Kisumu children had a mean of 5768 (51-59286), followed by BL group at 2581 (281-32431), which were comparable (p =0.6520), while the Nandi had the lowest levels at 919 (153-7215). Similar to the other malaria antigens tested, BL and Kisumu children had significantly higher titers to CSP compared to Nandi children (p < 0.0001). BL had the highest median level of 7196 (1337-165878), followed by Kisumu 6027 (687-24868) and Nandi 424 (114-8630) (p<0.0001). The same observation was made in regard to LSA seroprofiles. It was observed that living in a malaria holoendemic region was associated with high LSA titers. BL and Kisumu children had significantly high anti-LSA titers as compared

to Nandi children (p<0.0001). The eBL had the highest median titre level of 16127 (2785-159855) followed by Kisumu with 11668 (978-231735) and lastly Nandi with a median level of 1648 (95.5-6370) (Table 4.3).

4.3.3 Measles Sero-Profiles across Study Populations

There is some emerging data that common infectious agents may be associated with cancer participants (Parodi *et al.*, 2013; Tennant *et al.*, 2013). For measles exposure, it was observed that there were significant differences in the levels of anti-measles antibodies across study groups (p=0.0017). BL and Kisumu children had comparable median levels of anti-measles IgG titers (at 1241 (165-47291) and 1247 (508-5648), respectively) (p=0.8487) but which were significantly higher than the Nandi group with a median of 753 (178-2928) (Table 4.3).

4.3.4 Serology Profiles against Schistosomiasis SWAP Antigen

Schistosomiasis is a common helminthic infection along lake Victoria in Kisumu County(Opisa *et al.*, 2011) and many of the rural pediatric populations are equally at risk of developing eBL or schistosomiasis or both. Nandi children recorded the lowest median levels of anti-SWAP IgG titers at 473 (149-3191), followed by Kisumu at 2054 (399-9583) while the highest titers were in eBL with a median level of 4559 (203-37530) (p<0.0001). However BL and Kisumu children had comparable levels of anti-SWAP IgG levels (p=0.9033) (Table 4.3).

Table 4.3 Serological Profiles For Common Infectious Agents Across Study Group

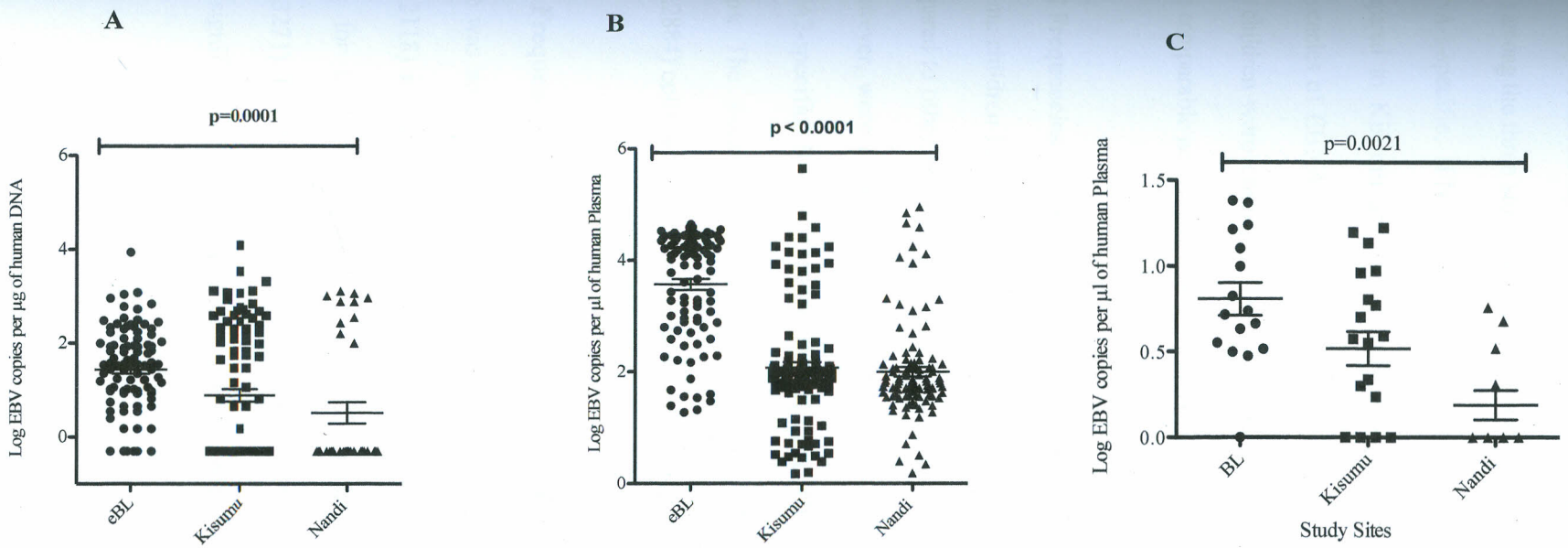
Study	EBV Antigens				Malaria Antigens					Measles	Schistosomiasis
Group	EBNA-1	VCA	Ead	Zebra	AMA-1	MSP-1	CelTOS	CSP	LSA	Measles	SWAP Antigen
BL	15631 (838-26322)	5871 (756-45775)	5871 (756-45775)	11751 (1328-133233)	27103 (15083-275180)	31134 (10528-642848)	2581 (281-32431)	7196 (1337-165878)	16127 (2785-159855)	1241 (178-2928)	4559 (203-37530)
Kisumu	14796 (722.5-26255)	3423 (262-41805)	3423 (262-41805)	2016 (417-21698)	48799 (4116-541208)	60407 (3577-564361)	5768 (51-59286)	6027 (687-24868)	11668 (978-231735)	1247 (508-5648)	2054 (399-9583)
Nandi	13867 (92-25965)	429 (73.5-5437)	429 (73.5-5437)	1162 (147-39309)	870 (78-24536)	4459 (433-46973)	919 (153-7215)	424 (114-8630)	1648 (95.5-6370)	753 (178-2928)	473 (149-3191)
p value	0.8256	**0.0074**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0017**	**<0.0001**

Key: EBNA-1- EBV Nuclear Antigen; VCA- Viral Capsid Antigen; Ead Early EBV antigen; Zebra-EBV zta antigen; MSP-1- Merozoite Surface Protein 1; AMA-1- Apical Membrane Antigen 1; CelTOS- cell-traversal protein for ookinetes and sporozoites antigen; CSP- CircumSporozoite Protein; LSA Liver Stage Antigen; SWAP- soluble worm antigen protein. Data represent means of total IgG levels as measured by multiplex ELISA. Differences in mean inflorescence intensity was analysed using Kruskall-Wallis test. Significant values are in bolt. In parenthesis are the ranges for each assayed antigen.

4.4 EBV Load Variation across Study Populations

There is controversy as to which compartment is appropriate or reliable for monitoring EBV viral load (whole blood, plasma or isolated PBMCs). While there is growing consensus that whole blood provides a sensitive and reliable estimate (Stevens *et al.*, 2001), the clinical utility of this measurement has not been determined for eBL patients or children at risk of developing eBL.

Therefore, EBV titers in both the cellular and plasma compartments were evaluated. For the blood compartment, the BL children had significantly higher median cellular EBV titers compared to Kisumu controls (14,810 versus 35.7 EBV copies per µg human DNA, respectively, $p<0.0001$) and significant differences existed between Kisumu and Nandi children (35.7, and 0.0 EBV copies per µg human DNA, respectively, $p=0.0003$). When EBV levels in the plasma (Fig. 4.1B) was compared, it was observed that BL group had significantly higher viremia levels (median 11,117 EBV copies per µl of plasma) compared to healthy children from malaria holoendemic region ($p<0.0001$). The median EBV viremia levels did not significantly differ between Kisumu and Nandi (5.4 and 4.1 EBV copies per µl of plasma, respectively) before DNAse treatment ($p=0.5127$) (Fig. 4.1A and B). However, when the plasma was treated with DNAse I before DNA extraction and EBV levels determined, it was observed that the levels of EBV tended towards zero suggesting presence of less lytic, encapsulated virus in the healthy children. However, it was noticed that still BL and Kisumu children had high viral loads than the Nandi ($p=0.0021$). There was no difference between the BL and in the quantity of encapsulated viruses even after DNAse 1 treatment (Fig. 4.1C).



A) Cell Associated EBV Titers **B) Total Plasma EBV Titers** **C) Encapsulated Plasma EBV DNA Titers**

Fig. 4.1: EBV Titers across Study Participants

The EBV titers were determined either from whole blood pellet of from plasma. This was to investigate differences in viral loads between the cell and plasma component. The viral titers were log transformed before statistical analysis since they did not obey Gaussian distribution. Panel A shows cell associated EBV titers while Panel B shows total plasma EBV titers before DNAse-1 treatment-Panel C shows samples after DNAse-1 treatment. Samples that used for DNAse 1 treatment parallel assay were those that had very high EBV titers in the plasma and were to investigate whether the high DNA in plasma was free or encapsulated. BL study group had significantly higher cell associated EBV titers compared to Kisumu children (p=0.0001.

4.5 T-cell Immunophenotyping Across study Populations

4.5.1 Frequencies of EBNA-1-Specific Cytokine producing T cells across study groups

This study observed significant differences in the frequencies of EBNA-1 specific IFN-γ- T cells among the three study populations. Nandi children had significantly higher frequencies of EBNA-1-specific, IFN-γ CD4+ T cells (p=0.0272) as well as CD8+ T cells (p=0.0087) compared to Kisumu and eBL study groups. However, no significant differences in the frequencies of EBNA-1- specific, IFN-γ CD4+ and CD8+ T cells between the Kisumu and eBL children were observed. The frequencies of EBNA-1-specific, IL-10 and IL-17 T cells were comparable across the study groups (Fig. 4.2).

4.5.2 Frequencies of EBNA2-Specific Cytokine Producing T cells across study groups

Kisumu children had marginally higher frequency of EBNA2-specific, IL-17 CD4+ T cells, compared to other groups (p=0.045). The frequencies of EBNA2-specific cells IL-17 CD8+ T however, were comparable (p=0.1365). There were no differences in the frequencies of EBNA2-specific, IL-10 CD4+ (p=0.6261) and CD8+ T (p=0.2715) cells across the study groups. The frequencies of EBNA2-specific, IFN-γ- CD4+ (p=0.5409) and CD8+ T (p=0.2884) cells were comparable across the study groups (Fig 4. 3).

4.5.3 Frequencies of EBNA3A-Specific Cytokine Producing T cells across study groups

There was no significant differences in the frequencies of EBNA3A-specific, IFN-γ CD4+ (p=0.2118) and CD8+ (p=0.4000) T-cells across study groups. The same observation was made for the frequencies of EBNA3A-specific IL-10 CD4+ (p=0.3376) and CD8+ (p=0.3271) T-cells. However, frequencies of CD4+ EBNA3A-specific, IL-17 CD4+ T cells were significantly higher in Kisumu children compared to other groups (p=0.0106 (Fig. 4. 4).

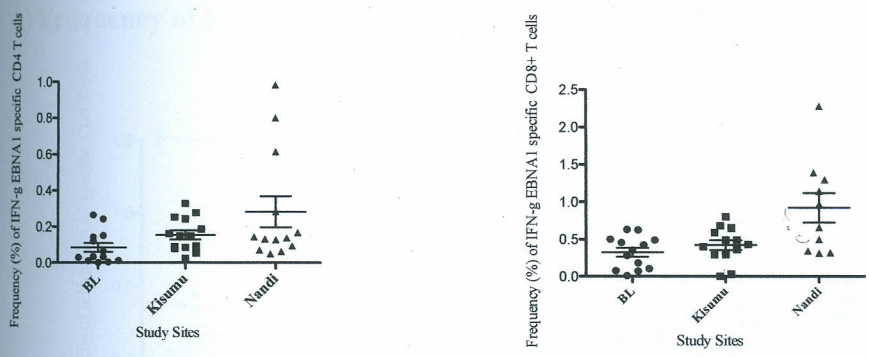


Fig. 4. 2: A) Frequency of EBNA-1-Specific, IFN-γ -Producing CD4+ and CD8+ T cells Across Study Populations

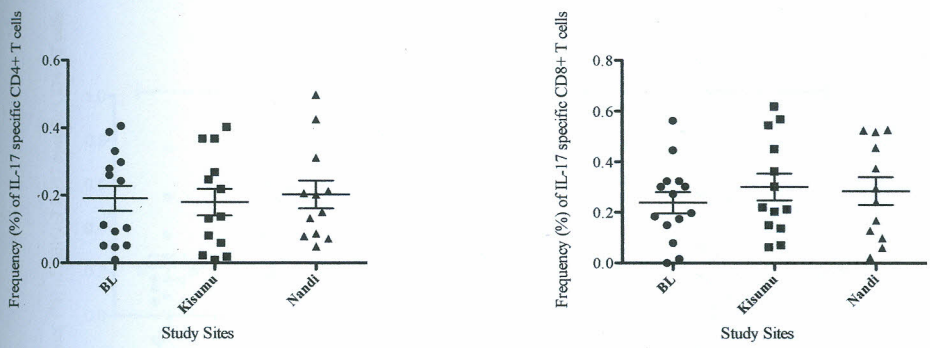


Fig. 4. 2: B) Frequency of EBNA-1-Specific, IL-17-Producing CD4+ and CD8+ T cells across Study Populations

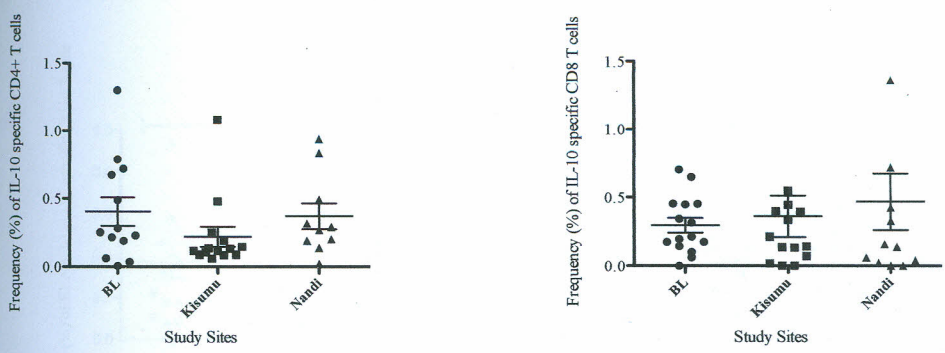
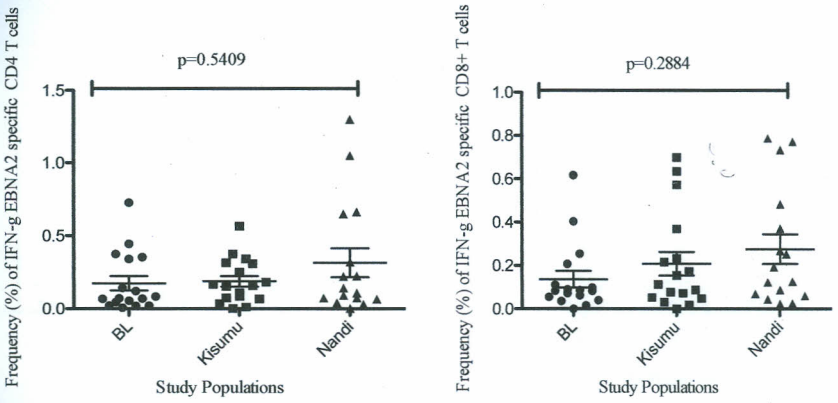


Fig. 4. 2: C) Frequency of EBNA-1-Specific, IL-10-Producing CD4+ and CD8+ T cells across the Groups

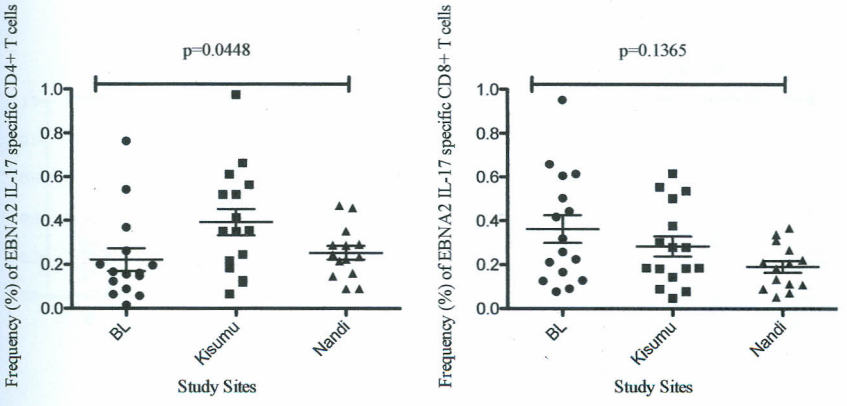
Frequencies of EBNA-1-cytokine-specific T cells after both surface and intracellular staining and acquisition. Presented data represents frequencies of EBNA-1-specific T cells for different cytokines after background subtraction. The lowest frequency of EBNA-1 specific IFN-γ CD4+ (p=0.0272) and CD8+ (p=0.0087) T was observed in BL children compared to other groups.

A) Frequency of EBNA-2-Specific, IFN-γ-Producing CD4+ and CD8+ T



cells

8. B) Frequency of EBNA-2-Specific IL-17-Producing CD4+ and CD8+ T cells



C) **Frequency of EBNA-2-Specific, IL-10-Producing CD4+ and CD8+ T cells**

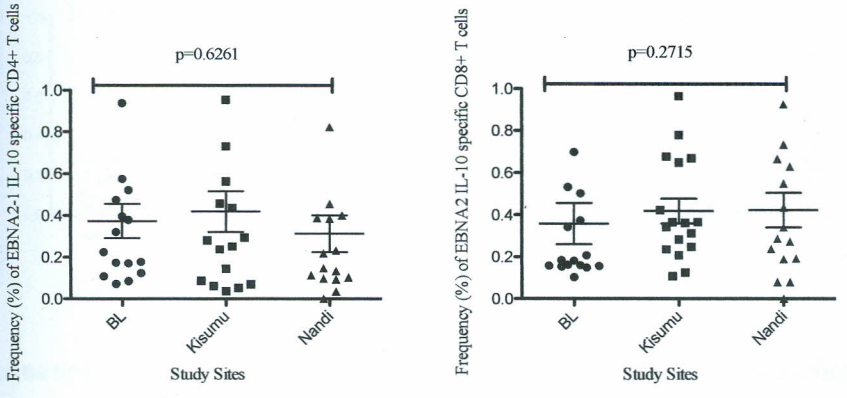
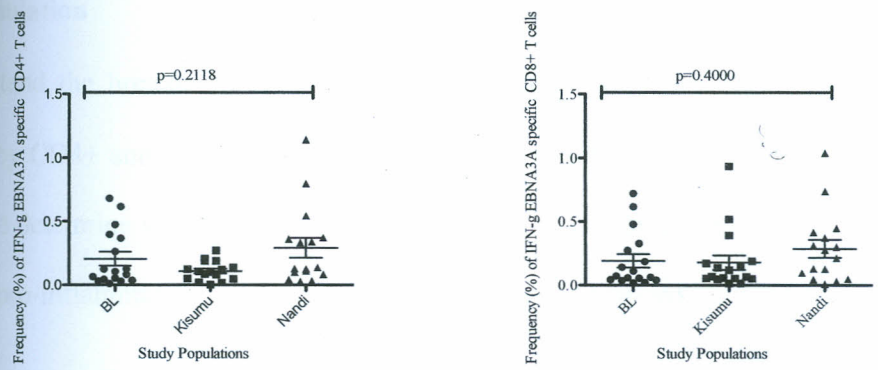


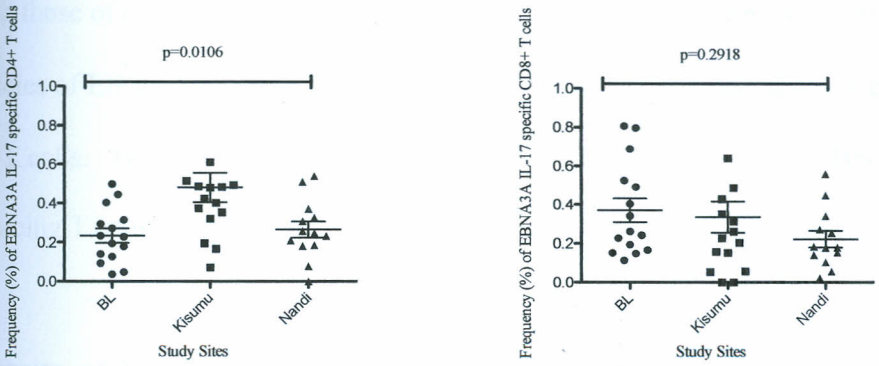
Fig. 4. 3: Frequency of EBNA-2-Specific, IFN-γ, IL-10 and IL-17-Producing CD4+ and CD8+ T cells across Study Populations

Frequencies of EBNA-2-specific T cells after both surface and intracellular staining and acquisition. Plotted data represent the net frequency response after subtratcion of background. The frequencies of EBNA-2 IFN-γ specific CD4+ T cells (p=0.5409 and CD8+ T (p=0.2884) we comparable between study groups.

A) Frequency of EBNA3A-Specific, IFN-γ-Producing CD4+ and CD8+ T cells



B) Frequency of EBNA3A-Specific, IL-17-Producing CD4+ and CD8+ T cells



C) Frequencies of EBNA3A-Specific, IL-10-Producing CD4+ and CD8+ T cells

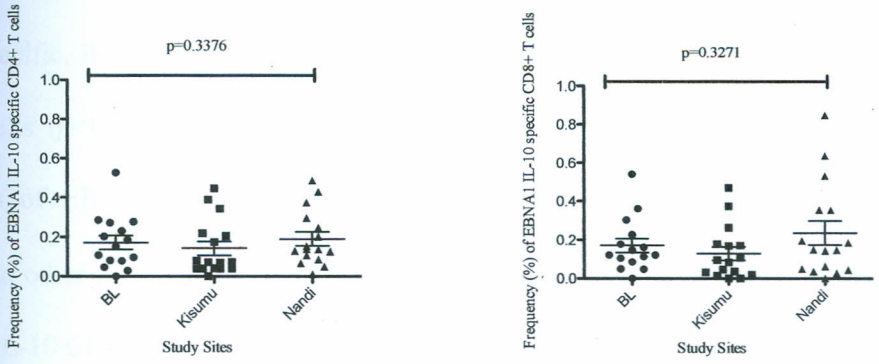


Fig. 4.4: Frequency of EBNA3A-Specific, IFN-γ-, IL-10- and IL-17-producing CD4+ and CD8+ T cells across Groups

Presented data represents frequencies of EBNA3A-specific T cells for different cytokines after background compensation. Frequencies of EBNA3A-IFN-γ-specific CD4+ T (p=0.2118) and CD8+ T cells (p=0.4000) were comparable across study groups. Same observation was made for the frequencies of EBNA3A IL-10 and IL-17 specific CD4+ and CD+ T cells

4.5.4 Comparative Analysis of Frequencies of Cytokine-Specific T cells within Each Study Population

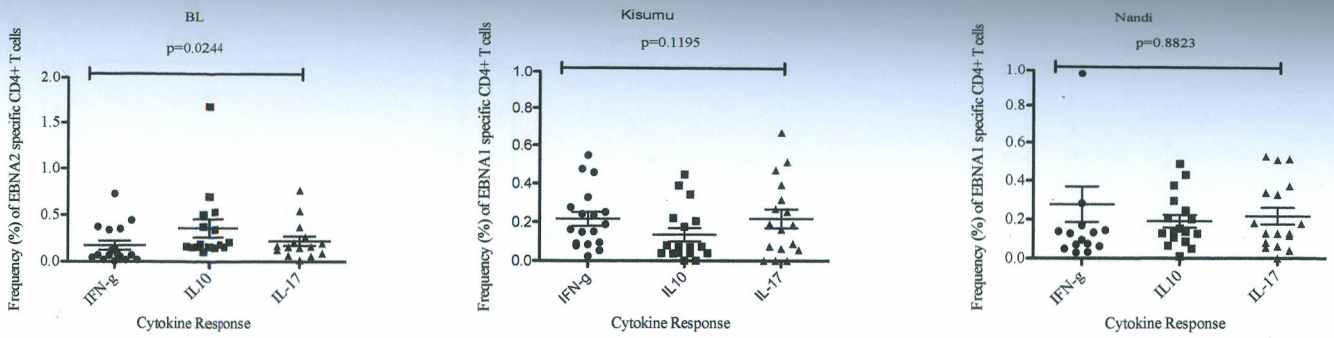
To understand the breathe of cytokine responses, frequencies of IFN-γ, IL-10 and IL-17 produced by CD4+ and CD8+ T cells in response to various EBV antigens were compared. This was to determine whether there was preferential production of a particular cytokine over the other (pro-inflammatory versus anti-inflammatory polarization).

It was observed that the frequencies of EBNA-1-specific, IFN-γ CD4+ T cells were lower compared to those of other cytokines (p=0.001) in BL children. In Kisumu and Nandi groups, the frequencies of EBNA-1-specific IFN-γ, IL-10 and IL-17 were comparable for both CD4+ and CD8+ T cells. The BL children had significantly higher frequencies of EBNA-1 CD8+ IL-17-producing T cells compared to other cytokines (p=0.0138) (Fig. 4.5).

The frequencies of EBNA2-specific IFN-γ T cells were contacted in Kisumu and BL compared to the Nandi group. Kisumu children had significantly lower frequencies of EBNA2-specific, IFN-γ-producing T cells relative to other cytokines for both the CD4+ and CD8+ T-cells (p=0.0041 and p=0.057, respectively). Similarly, BL study group had low frequencies of EBNA2-specific IFN-γ CD4+ (p=0.0244) and CD8+ (p=0.004) T cells compared to other cytokines. In the Nandi group, the frequencies of EBNA2-specific IFN-γ, IL-17 and IL-10 CD4+(p=0.8823) and CD8+ (p=0.2968) T cells were comparable (Fig. 4.6).

The same trend was observed with respect to EBNA3A. Both BL and Kisumu study groups had significantly lower frequencies of EBNA3A-specific, IFN-γ-producing CD4+ T cells (p=0.0121 and p<0.0001, respectively) as well as CD8+ T cells (p=0.0016 and p=0.0013, respectively) compared to other cytokines (Fig. 4.7).

a) CD4+ T cells



b) CD8+ T cells

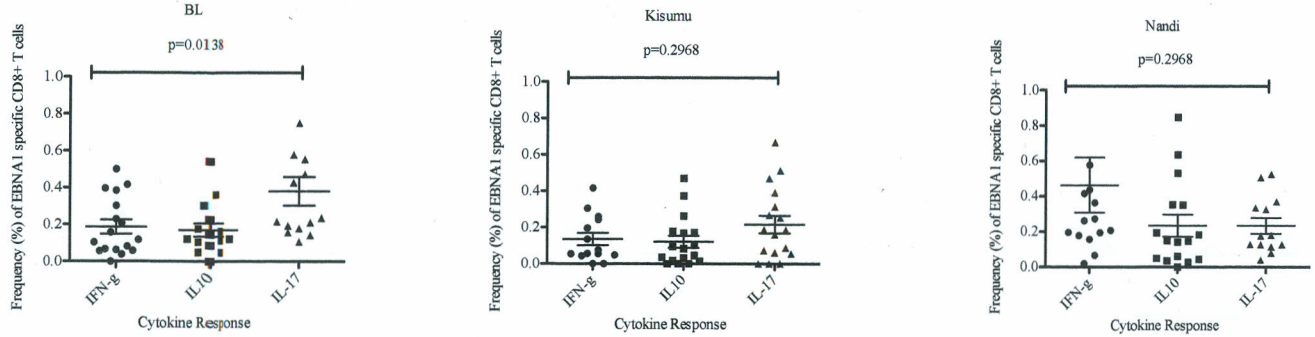
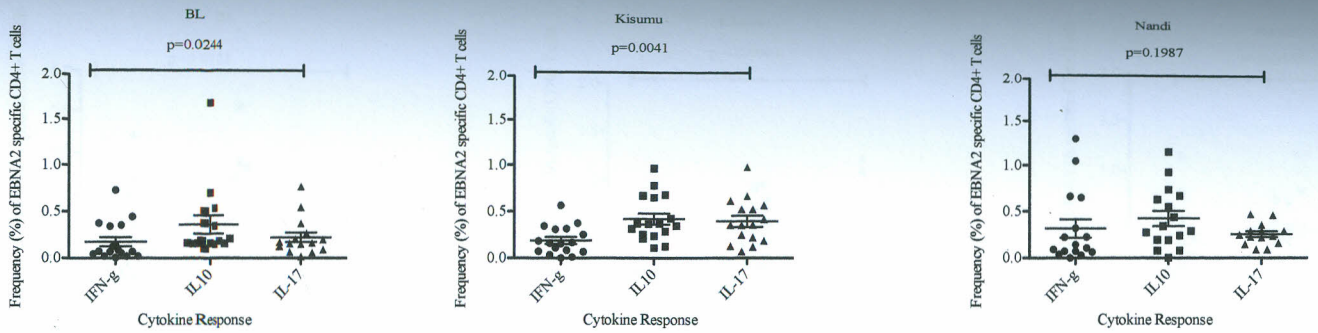


Fig 4. 5: Comparative Analysis of EBNA1-Specific, Cytokine-Producing T cells within Each Study Population

Frequency of EBNA-1-specific, cytokine-producing T cells after surface and intracellular staining. Presented data is the net response after background subtraction for every cytokine. Comparison made within the group to investigate for polarization of cytokine response. It is observed that IFN-γ T cells are the least in BL study group: BL CD4+ T cells (p=0.0244), CD8+ T cells (p=0.0138); Kisumu CD4+ (p=0.1195), CD8+ T cells (p=0. 2968); Nandi CD4+ T cells (p=0.8823), CD8+ T cells (p=0.2968).

a) CD4+ T cells



b) CD8+ T cells

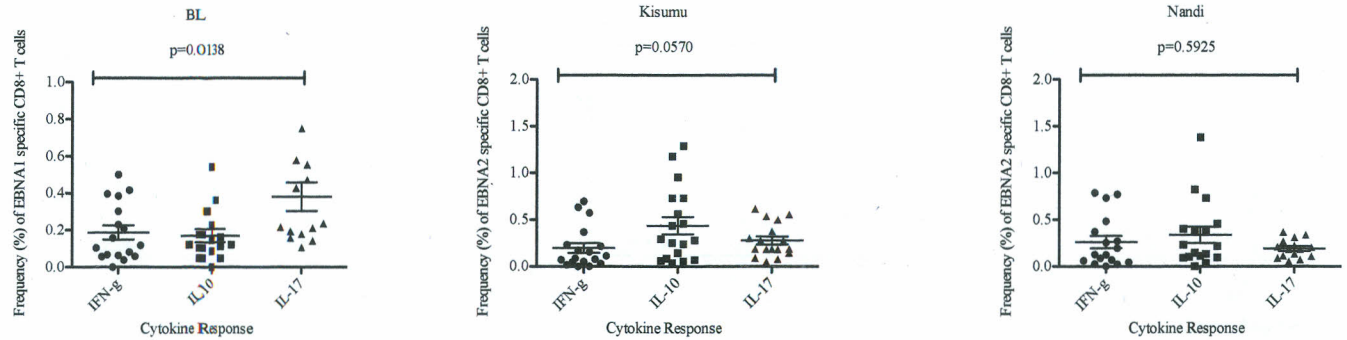
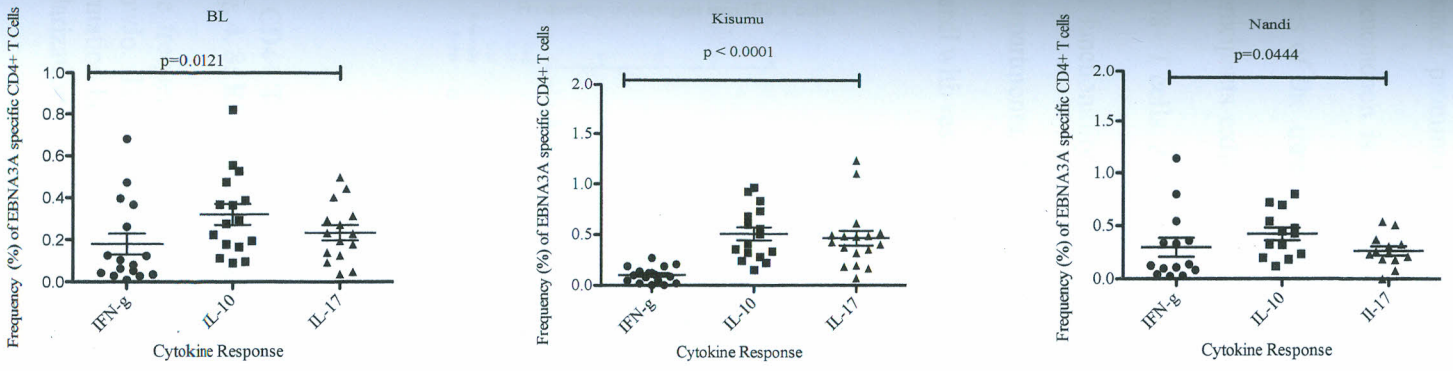


Fig 4. 6: Comparative Analysis of EBNA2-Specific, Cytokine-Producing T cells within Each Study Population

Frequency of EBNA-1-specific, cytokine-producing T cells after surface and intracellular staining. Presented data is the net response after background subtraction for every cytokine. Comparison made within the group to investigate for polarization of cytokine response. It is observed that IFN-γ T cells are the least in all study groups: BL CD4+ T cells (p=0.0244), CD8+ T cells (p=0.0138); Kisumu CD4+ (p=0.0041), CD8+ T cells (p=0.0570); Nandi CD4+ T cells (p=0.1987), CD8+ T cells (p=0.5925).

A) CD4+ T cells



B) CD8+ T cells

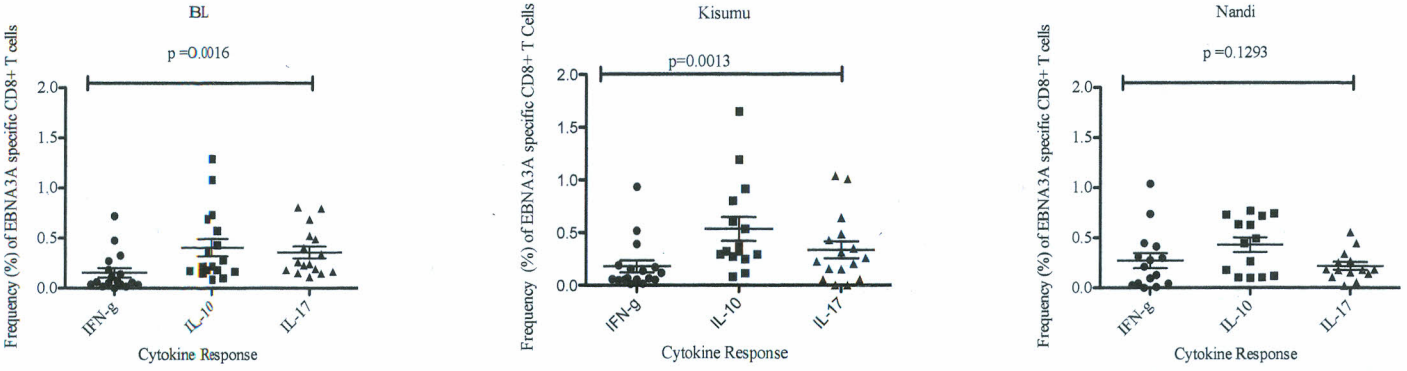
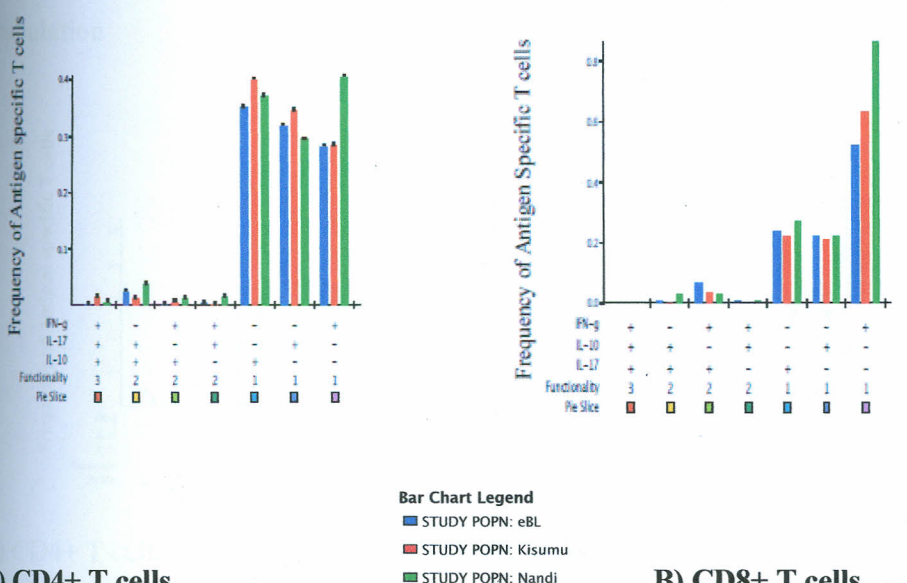


Fig. 4. 7: Comparative Analysis of EBNA3A-Specific, Cytokine-Producing CD4+ and CD8+ T cells Within Each Study Population

Represented are net T-cell frequencies of EBNA3A cytokine-specific T cells after gating on cytokine positive events and background compensated for in each analysis. In all study groups, the frequencies of IL-10-specific T cells were higher compared to other cytokines. However there was a reduction in the frequency of EBNA3A IFN-γ specific T cells in all study groups: BL CD4+ T cells (p=0.0121), CD8+ T cells (p=0.0016); Kisumu CD4+ T cells (p<0.0001), CD8+ T cells (p=0.0013); Nandi CD4+ T cells (p=0.0444), CD8+ T cells (p=0.1293).

4.5.5 Polarization and Polyfunctionality of Cytokine-Specific CD4+ and CD8+ T cells Across Study Populations

To further characterize the immune architecture of the study groups based on cytokine production, the responsive cells were analysed for possible multiple cytokine response. The phenomenon where T cells produce multiple cytokines against an infectious agents has gained prominence as a correlate of protective immunity (Ning *et al.*, 2011). Such phenomenon is known as polyfunctionality. Cytokine polyfunctionality was scored on the basis of the three cytokines with +3 as the highest score and +1 as the least. All study participants exhibited a polyfunctionality score of +3 to SEB in CD4+ T cells as well as CD8+ T cells. All the children studied exhibited single-cell one-cytokine (monofunctional or +1 functionality) response to all the three tested antigens in both CD4+ or CD8+ T cells compartments. There were no differences in polyfunctionality between BL, Kisumu and Nandi with respect to EBNA-1 (Fig 4.8).

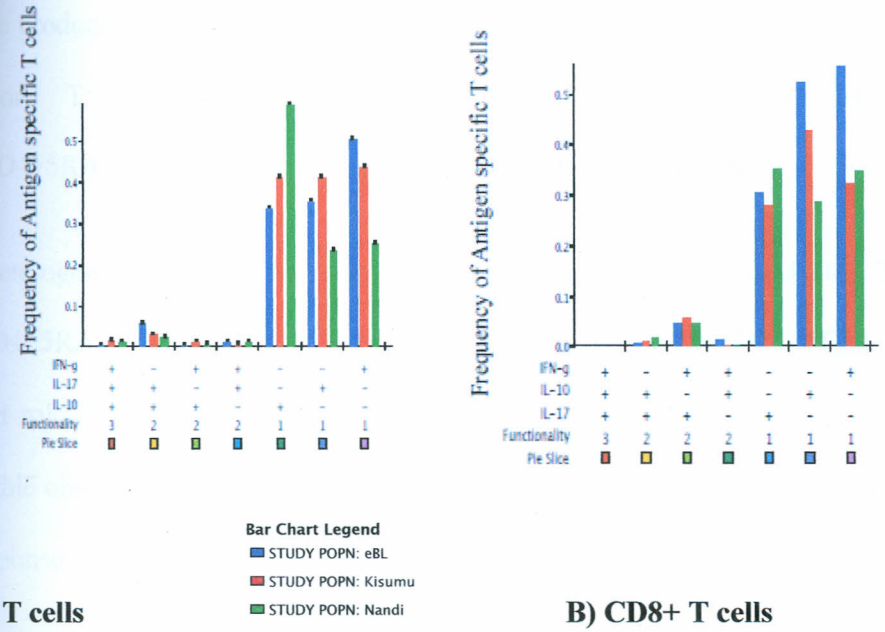


A) CD4+ T cells **B) CD8+ T cells**

Fig. 4. 8: EBNA-1 Cytokine-Producing Monofunctional cells across Study Populations

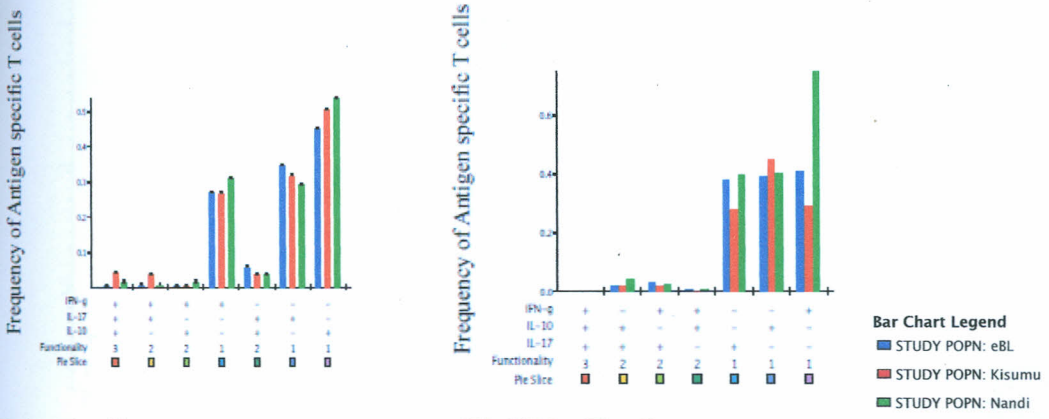
The frequencies of all cytokine specific T cells combined in a Boolean gating strategy in FlowJo. The Boolean gated events were then transferred to Pestle® software for matrix formation before analysis using SPICE®. Presented data shows that there polyfunctionality or polarization was not evident in both CD4+ and CD8+ T cells with respect to EBNA-1.

Similar to EBNA-1, cytokine response to EBNA2 and EBNA3A in all the three study populations was predominantly monofunctional with all monofunctionality pies being comparable. There was barely any evidence of polyfunctional or polarized cells to either EBNA2 or EBNA3A (Figs. 4.9 and 4.10).



A) CD4+ T cells **B) CD8+ T cells**

Fig. 4.9: EBNA-2 –Specific, Cytokine-Producing Monofunctional Cells across Study Population



A) CD4+ T cells B) CD8+ T cells

Fig. 4.10: EBNA-3A-Specific, Cytokine-Producing Monofunctional Cells across Study Populations

Antigen-specific T cells were analysed for either polyfunctionality or polarization to either EBNA2 or EBNA3A antigens by use of PESTLE® and SPICE® software programs. T cells exhibited monofunctionality to all antigens tested.

4.5.6 Characterization of EBNA-1-specific, IFN-γ-Effector T-cell Memory Subsets across Study Populations

Memory phenotypes of EBNA-1-specific IFN-γ-producing T cells were comparable across the three study populations. For the CD4+ T cells in all study populations, the main memory phenotype producing IFN-γ were CD4+5RA-CCR7- or T_{EM} ($p< 0.0001$). Other proportions of memory T cells: CCR7+CD4+5RA- (T_{CM}), CCR7-CD4+5RA+ (T_{EMRA}) and CCR7+CD4+5RA+ (T_N) were not very prominent in cytokine response.

Greater heterogeneity in T cell memory phenotypes was observed in all CD8+ T-cells with CCR7-CD4+5RA- or T_{EM}, CCR7+CD4+5RA+(T_N) and CCR7-CD4+5RA+(T_{EMRA}) playing a significant role in IFN-γ production to all the three EBV antigens tested ($p<0.0001$). The most notable observation was the presence CD8+ naïve-like phenotype that had capability of IFN-γ response (Fig. 4.11 A).

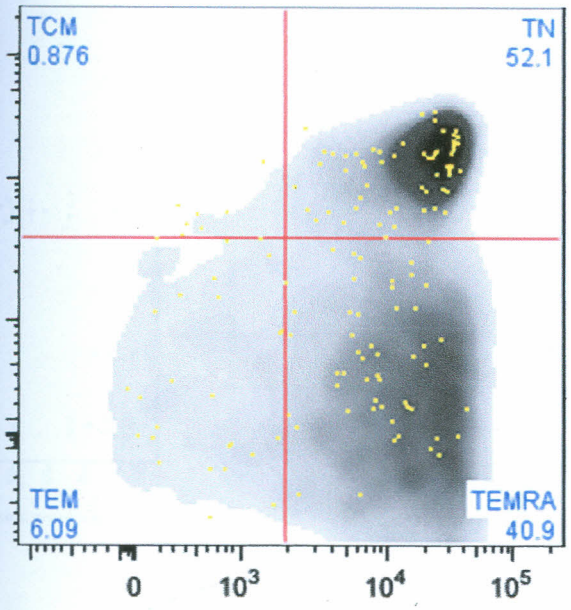
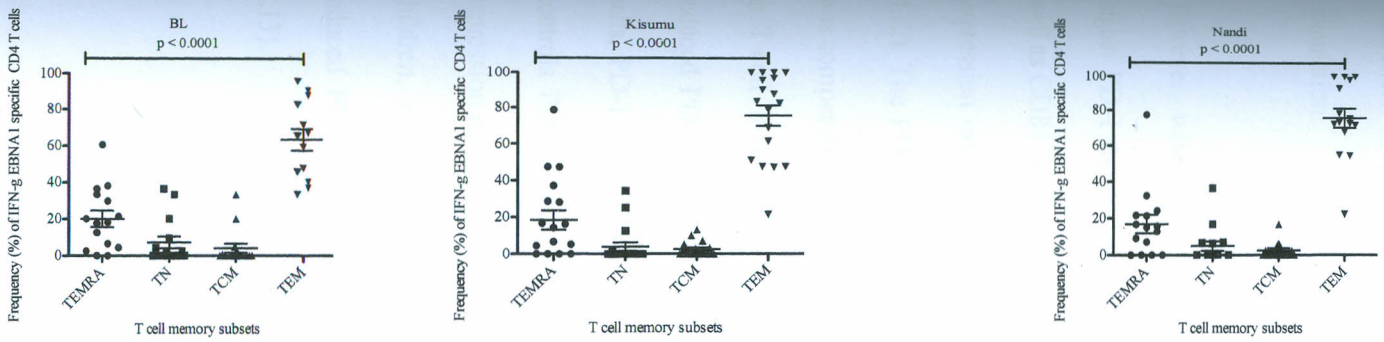


Fig 4.11(A) A Representative Dot Plot Diagram Showing Presence of EBNA-1-specific IFN-γ-naïve-like CD8+ T-cell phenotypes
EBNA-1 IFN-γ-producing positive gated dot plot graph of events was superimposed on a density plot of T-cell memory phenotypes to identify the phenotype of antigen specific cells.

i) CD 4+ T cells



ii) CD8+ T cells

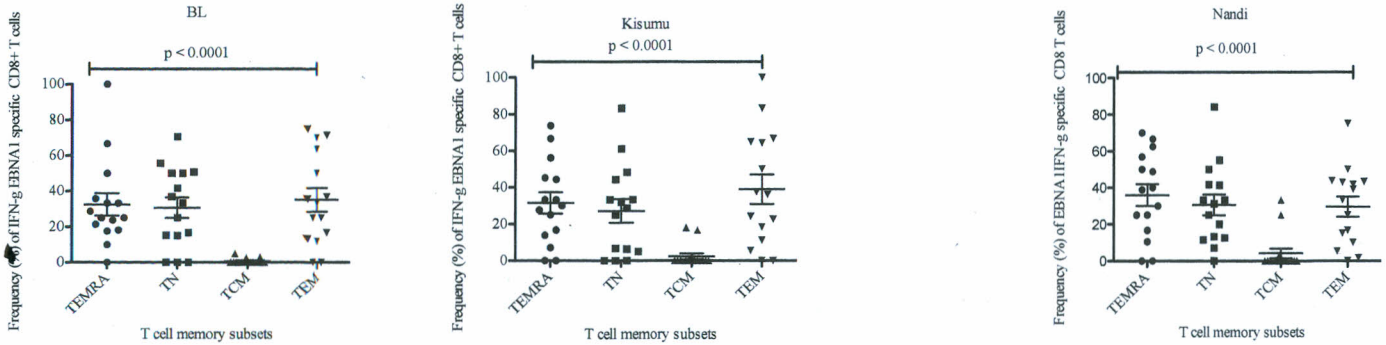


Fig. 4.11(B): Frequency of IFN-γ EBNA-1-specific CD4+ and CD8+ T cells across study populations

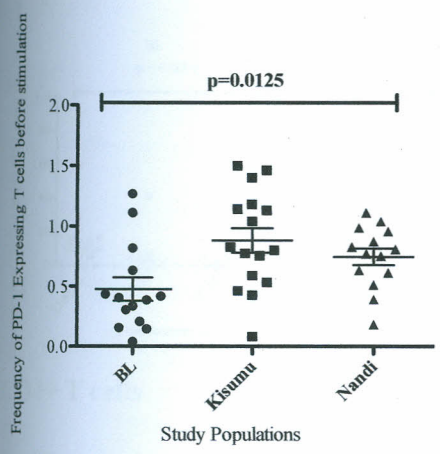
The memory phenotype of EBNA-1-specific, IFN-γ-producing T cells were characterised based on two T-cell lineage markers CCR7 and CD4+5RA. T_N= naïve T-cell, T_{CM}- central memory T-cell; $T_{EMR}A$= T effector memory re-expressing CD4+5RA molecule T-cell; T_{EM}= Effector memory T-cell. In all study groups, there were significant differences in the proportion of effector cells

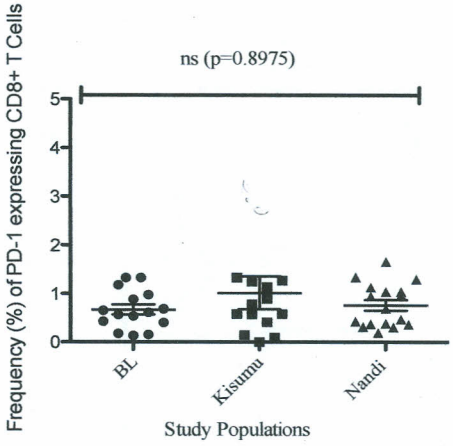
4.6 EBNA-1 and PD-1 Expression

4.6.1 EBNA-1 Specific PD-1 Expression across Study Populations

To investigate the immunoinhibitory effect of *P. falciparum* on T cell function, the frequencies of PD-1 upon EBNA-1 stimulation were analysed. Programmed Death 1 (PD-1) can be considered as both a marker of exhaustion as well as of activation. Baseline PD-1 expression on CD4+ T cells was higher in malaria holoendemic exposed children (Kisumu) compared to both Nandi and BL children (p=0.0125). It was observed that there were no differences in PD-1 expression on CD8+ T cells across study populations before *ex vivo* stimulation.

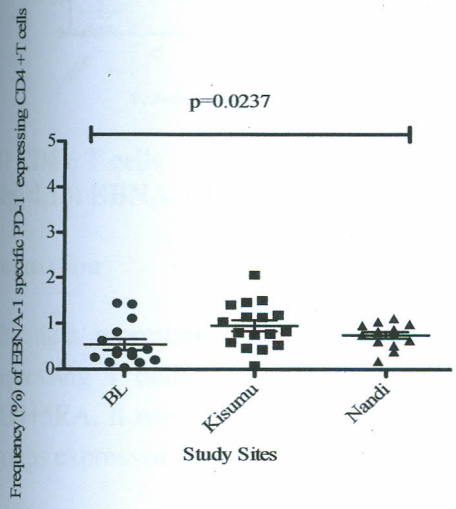
However, when cells were stimulated with EBNA-1, Kisumu children had significantly higher PD-1 expression compared to Nandi and BL group on CD4+ T cells (p=0.0237) as well as CD8+ T cells (p=0.0423). There was no significant increase in the frequency of PD-1 expression on both CD4+ and CD8+ T cells in BL groups after *ex vivo* stimulation (Fig. 4.12). The PD-1 expression in respect to EBNA2 and EBNA3 on CD4+ and CD8+ T cells was comparable across the three study groups, further supporting an EBNA-1-specific defect in T-cell immunity associated with malaria co-infections. Characterization of EBNA-1 induced PD-1 expression on memory T-cell subsets showed that for BL patients CD4+ T-cells, PD-1 was almost exclusively expressed by T_{EM} (p<0.0001), whereas for the healthy controls PD-1 appeared to be on all other T cell memory phenotypes. In contrast, PD-1 expression pattern on CD8+ T cells did not appear to differ between eBL patients and healthy children, regardless of malaria exposure. However, BL children had greatest EBNA-1-induced PD-1 expression on T_N cells compared to other memory subsets (p<0.001) (Fig. 4.13).

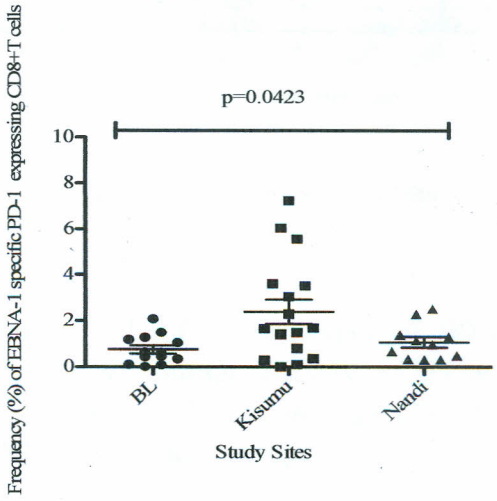




CD4+ T cells **CD8+ T cells**

A) Baseline PD-1 Expression Levels before *ex vivo* EBNA-1 stimulation



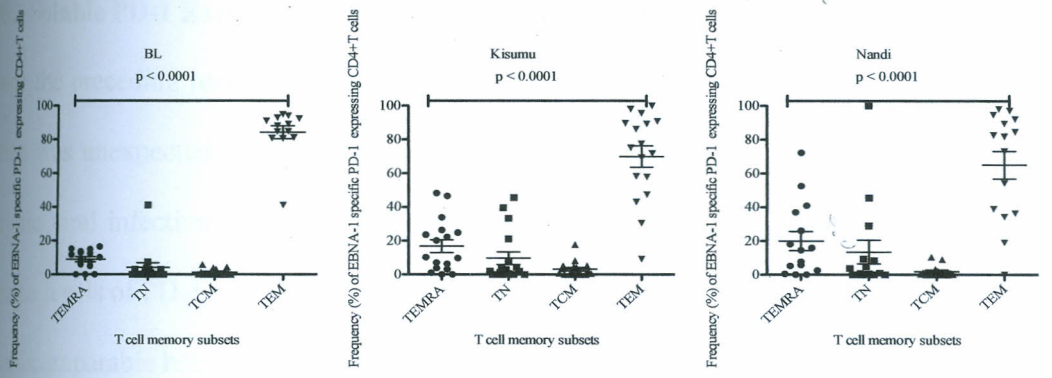


CD4+ T cells **CD8+ T cells**

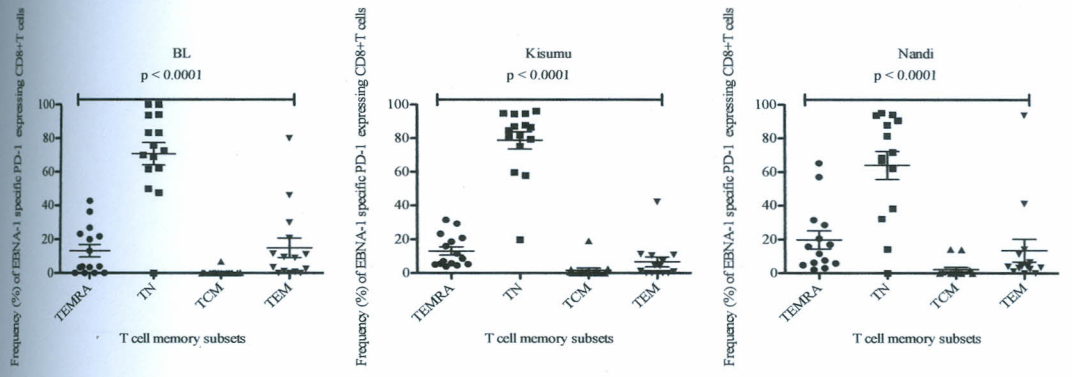
B) EBNA-1 induced PD-1 Expression

Fig. 4.12: PD-1 Expression Levels for CD4+ and CD8+ T cells across Study Populations

Frequencies of PD-1 expressing T cells before (A) and after (B) stimulation by EBNA-1 antigen. BL children had the least PD-1 expression on T cells for both resting and stimulated conditions. Kisumu had significantly higher EBNA-1 induced PD-1 expression after ex vivo stimulation on CD4+ T cells compared to BL and Nandi groups (p=0.0237) as well as CD8+ T cells (p=0.0423).



A) CD4+ T cells



B) CD8+ T cells

Fig. 4.13: EBNA-1 Induced PD-1 Expression on T-cell Memory Subsets across Study Population

The data represent frequencies of PD-1-expressing T cells. The phenotypes of PD-1-expressing T cells were characterized by use of two T-cell lineage markers: CCR7 and CD4+5RA. It was observed that BL CD4+T exclusively expressed PD-1 on T_{EM} while other groups expressed PD-1 on all other cell types (p<0.0001) for all study groups.

4.6.2 Soluble PD-1 Expression across Study Populations

Since the preceding results on PD-1 expression on T cells before *ex vivo* stimulation in BL group was unexpected based on previous studies that have shown high PD-1 expression in chronic viral infection (Day *et al.*, 2006; Greenough *et al.*, 2010; Trautmann *et al.*, 2006), plasma levels of PD-1 was investigated. It was observed that the concentration of soluble PD-1 was comparable between the three (p=0.1377) (Fig. 4.14).

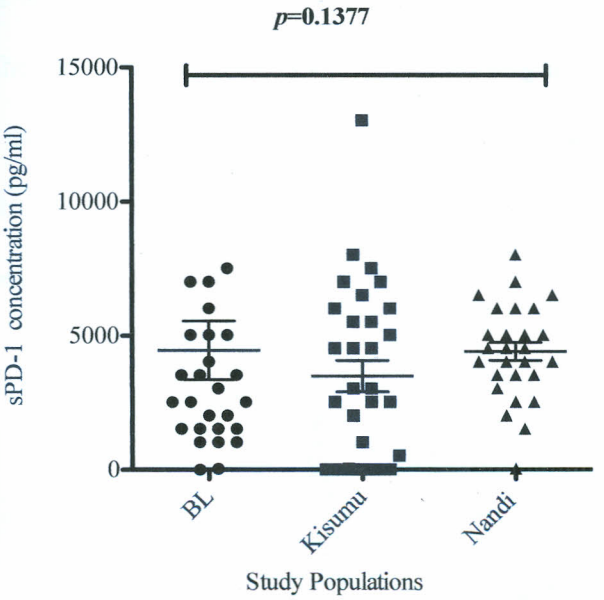


Fig. 4.14: sPD-1 Expression Across Study Populations

Plotted data represent values after background compensation. It was observed that soluble PD-1 levels were comparable across the study groups (p=0.1377)

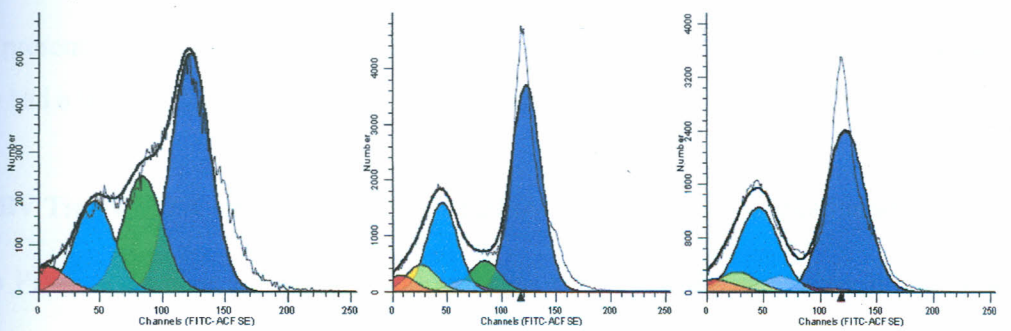
4.6.3 PD-1 Blockade and EBNA-1- IFN-γ-Specific, T-cell Response

Clinical trials have shown that blocking PD-1-PDL1 pathway reverses the immune dysfunction associated with many disease states (Bhadra *et al.*, 2011; Butler *et al.*, 2012; Dulos *et al.*, 2012; Hofmeyer *et al.*, 2011; Parry *et al.*, 2005; Peng *et al.*, 2012; Weber, 2010). To investigate whether it was possible to reverse the above-observed EBNA-1-specific IFN-γ-dysfunctional response in children residing in malaria-holoendemic regions, PD-1 blockade

experiments using anti-CD274 antibody (anti-PD-1 L1) were set in parallel with the other cell cultures. Although there was a marginal increment in the quantity of EBNA-1-specific IFN-γ T cells in all the study groups, this increase was not significant (p=0.4000).

4.7 EBNA-1-specific, T-cell Proliferation across the study Populations

To further asses functional quality of EBNA-1-specific T-cell response across populations, the rate of proliferation was evaluated after stimulation *ex vivo*. The difference in the rate of proliferation between the three study groups was compared using a Proliferative Index (PI). Proliferative Index is the total number of divisions divided by the number of cells that underwent division. This parameter takes into account, at least, cells that underwent one division and, thus, favourable for comparing sample to sample (Fig. 4.15).



BL Kisumu Nandi

Fig. 4. 15: Representative Schematic Diagrams of Mean Proliferation Rates of the Study Populations
The number of peaks on the x-axis shows the number of cycles of cell division while the number of cells on the y-axis shows the actual number of dividing cells. Although BL had the highest number of cell cycle, it had the least number of EBNA-1-specific proliferating cells.

It was observed that although the BL study population exhibited a slightly higher EBNA-1-specific PI compared to other study populations when cells were stimulated with EBNA-1 peptide, this difference was not statistically significant (p=0.1988). The net PI was made above the background levels. Further, it was observed that the number of EBNA-1-specific,

IFN-γ-proliferating cells was the lowest compared to other groups ($p < 0.0001$). The same inference was made when other antigens were compared across the study groups (Fig. 4.16).

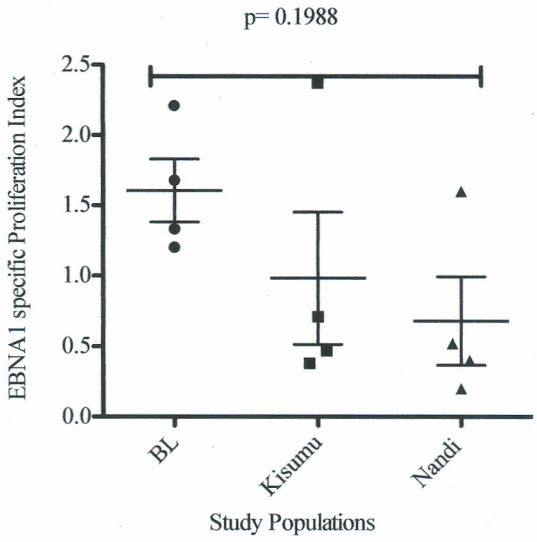


Fig. 4.16 Comparative Proliferative Index across Study Populations

Data presented show mean Proliferative Indices of different study groups. Although BL group had a high PI compared to other groups this difference was not significant.

4.8 EBV Titers, PD-1 and the Quality of EBNA-1-specific, IFN-γ Response

When PD-1 expression in Kisumu children was correlated against IFN-γ-production it was observed that the two events were mutually exclusive and inversely correlated (ρ=-0.06013, p=0.0229). It was also observed that Kisumu children who presented with both high EBV titers and PD-1 expression had the lowest frequencies of EBNA-1 specific IFN-γ levels in comparison to the Nandi children. BL children who had high viral titers but low levels of PD-1 expression had very low frequencies of EBNA-1-specific IFN-γ levels. This observation implicates PD-1 mediated mechanism in EBNA-1 immune dysfunction and EBV viral control as reported by seminal studies in western Kenya (Moormann *et al.*, 2009, 2005; Piriou *et al.*, 2012). However, the exact temporal kinetics needs to be carried out with more replicative as well as longitudinal studies. Similarly it was observed that the Kisumu and BL children who

had significantly higher viral loads had lower frequencies of EBNA-1-specific, IFN-γ T-cell responses both for CD4+ and CD8+ (Fig. 4.17).

A) CD4+ T cells **B) CD8+ T cells**

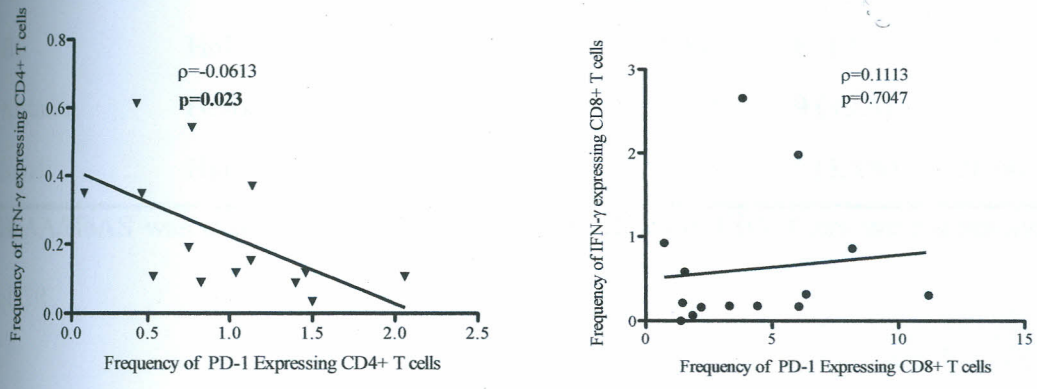


Fig.4.17: Correlation between IFN-γ response and PD-1 expression in Kisumu children.
The matched frequencies of IFN-γ and PD-1 T cells for very samples were plotted together on the same graph. It was observed that there was a significant inverse correlation between PD-1 expression and IFN-γ expression for CD4+ T cells (p=0.023, ρ=0.0613).

4.9 HbAS beta Haemoglobin Genotype, EBV Loads and eBL

The HbAS genotype was chosen as it is one of the most common heamoglobinopathies in Kisumu region that is extensively studied (Foy *et al.,* 1954; Hall, 1959; Aluoch and Aluoch, 1993; Moormann *et al.,* 2003) and its role in malaria protection is well established (Kreuels *et al*, 2010; Scott *et al*, 2011). It was observed that frequency of HbAS trait was highest among the Kisumu children (30%) compared to Nandi (3.5%) children or BL study population (19%) (χ^2 23.42, df=2, p<0.0001) (Table 4.4). Using Kisumu as healthy controls and estimating the relationship between HbAS genotype and eBL, it was observed that HbAS was not associated with eBL [OR=0.5473 (0.2835-1.057 95% CI), p=0.0996)]. This observation implies that HbAS is not a risk factor in the etiology of eBL.

Table 4.4: Frequency of HbAS genotypes and EBV Loads Across Study Populations

Study Population	*P. falciparum* exposure	Sample size (n)	Frequency of beta Haemoglobin Genotypes		Median EBV viral Loads
			HbAA	HbAS	
BL	Holoendemic	31	25 (81%)	6 (19%)	5381.00
Kisumu	Holoendemic	30	21 (70%)	9 (30%)	504.00
Nandi	Hypoendemic	28	27(96.5%)	1 (3.5%)	26.40

HbAA/HbAS was determined by RFLP based PCR while EBV titers were determined by qPCR.

There were no significant differences in viral loads based on HbAS genotype within each study group. However, Kisumu HbAS children had lower viral loads compared to those with HbAA genotype [76.37 EBV copies/nano gram human DNA to 708.5 for those with HbAA genotype (p=0.3005) and 25.86 and 78020 EBV copies/nano gram human DNA for HbAS to HbAA respectively for BL (p=0.3153)]. The statistical analysis could not be done for the Nandi study group, as the frequency of HbAS was very low. Mean parasitemia levels were comparable irrespective of HbAS genotype (p=0.1817) (Fig 4. 18).

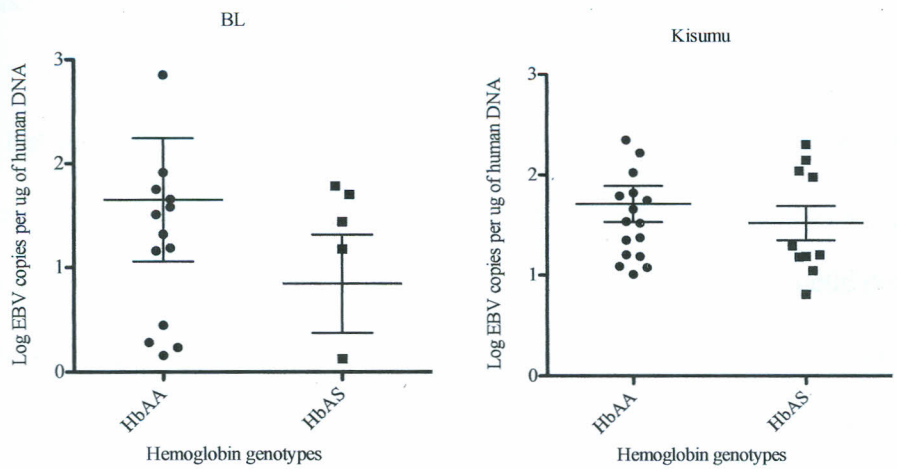


Fig. 4.18: Comparison between EBV loads and beta haemoglobin genotypes

CHAPTER FIVE

5.0 DISCUSSION

This study set out to investigate the effect of holoendemic *P. falciparum* malaria on the quality and function of T cells with respect to EBNA-1, leveraging populations living in areas with divergent malaria transmission and exposure patterns, as compared to those children presenting with eBL. The study further investigated the role of a well-established host genetic factor in malaria protection, HbAS genotype, to EBV titre control as well as influence on the quality of immune response to EBNA-1 antigen. The current study demonstrates that an EBNA-1-specific, IFN-γ producing T-cell deletion is associated with holoendemic malaria exposure and is not due to skewing the immune response towards the dominance of an alternate cytokine profile. Thus, children presenting with eBL retained their EBNA-1-specific producing IL-10 and IL-17 T cells while IFN-γ-producing T cells were selectively depleted. This selective depletion was specific to EBNA-1 and not to other EBV latent antigens (EBNA2 and EBNA3A). Furthermore, it was evident that this depletion of EBNA-1 specific IFN-γ T cells was not due to differences in the quality of T-cell effector-memory phenotypes between study populations but due to chronic *P. falciparum* exposure, which led to increased T-cell activation and exhaustion marking these cells for apoptosis. Further, it has been observed that HbAS genotype is not associated with EBV load control, T-cell function or eBL diagnosis. From this study, it was evident that the frequency of sickle cell trait does not confer protection against eBL. However other human genetic determinants could influence response to EBV or malaria infections and contribute in a multifactorial process to eBL tumorigenesis. The failure of this study to show an association between HbAS and eBL does not entirely delineate the role of this genetic factor in the etiology of eBL.

Recent rapid technological advancement in the field of molecular biology has redefined and facilitated the numerous avenues for robustly exploring more aspects of the immune system architecture never thought of possible before. This becomes even more apparent when analysing cellular phenotypes as well as molecular signatures of immune response. This study exploited these recent high throughput technologies of multiparameter flow cytometry and multiplex ELISA among other techniques.

5.1 Cohort Characteristics According to Study Population

The three main study populations investigated in the present study had distinctive characteristics consistent with different *P. falciparum* transmission pressures. Kisumu children had higher parasitemia levels as expected from residence in a malaria holoendemic region (Bloland *et al.*, 1999, 1999; Snow *et al.*, 1998). The lower parasitemia levels observed in eBL patients is most likely due to the fact that these children were usually referred to the JOORTH from other sub-district hospitals or general ward in the hospital where they may have been treated for blood stage malaria infections. The haematological indices were comparable across the study populations and were within the normal clinical ranges for children living in the same regions (Kibaya *et al.*, 2008; Zeh *et al.*, 2011). This similarity in haematological indices could be due to the fact that the children enrolled in the current study were healthy controls and were age matched. The slightly higher haematological indices observed in the Nandi study populations is consistent with residing at higher altitude as compared to Kisumu and BL groups who lived at lower altitudes. Haematological values are affected by factors such as environment, sex, age, clinical states, nutrition status and ethnicity among other factors (El-Hazmi and Warsy, 2001). A limitation of this study was the failure to measure nutritional status in the healthy appearing children that may be a contributing factor in the discrepancies of the above haematological indices.

5.2 Common Infectious Agents Sero-Profiles and eBL

The incidence of eBL geographically coincides with *P. falciparum* transmission patterns (Dalldorf *et al.*, 1964; de-The, 1977), however, the causal association has not been conclusively determined. Recent studies have implicated incidences of both chronic and acute malaria in EBV reactivation and host control (Chêne *et al.*, 2007a; Donati *et al.*, 2006; Pagano, 1999). Although it is known that EBV and *P. falciparum* are the main twin infectious agents in the etiology of eBL, the possibility of other mediators has not been ruled out. Previously, eBL has been described as polymicrobial disease (Chene *et al.*, 2009; Rochford *et al.*, 2005). It was on this premises that sero-profiles against other common infectious agents in the resource-limited settings (Petney and Andrews, 1998) of western Kenya were evaluated in the study populations employing multiplex ELISA platform (Luminex). Multiplex ELISA offered a better platform over convectional ELISA due to the fact that up to 100 different analytes could be measured simultaneously from limited sample volumes as are obtained from children. Furthermore, this assay is less laborious, has few reactions steps and is easily reproducible compared to the convectional ELISA. These infectious agents included *P. falciparum* malaria, measles virus, schistosomiasis and EBV.

This study has shown that children from malaria holoendemic region presented with higher antibody titers to *P. falciparum*, EBV as well as schistosomiasis as compared to those from malaria hypoendemic regions. The higher antibody titers recorded in Kenyan children could be a function of multiple variables. First, EBV infection occurs early in life in African populations (de-The, 1977; Piriou *et al.*, 2012), which may be related to the socioeconomic and cultural practices (de-The, 1977; Rainey *et al.*, 2008). The high VCA titers could be a function of higher viral loads/lytic reactivation induced by repeated malaria infections. Recent studies have shown that *P. falciparum* causes EBV lytic reactivation from latency

possibly explaining why Kisumu children who live in a malaria holoendemic region have high anti-VCA titers compared to Nandi. Secondly, apart from age, the differences in *P. falciparum* intensities could be modulating effector T-cell function in populations living in malaria holoendemic regions specific to EBV antigens (Moormann *et al.*, 2009, 2007). Anti-VCA and EAd antigens showed remarkably high response level for Kisumu and BL study populations. Piriou and others (Piriou *et al.*, 2012, 2009), have shown that Kisumu children are more likely to be infected earlier in life than Nandi children although in both populations, most children are seropositive by 12 months of age. Children presenting with eBL or living in malaria holoendemic regions showed evidence of continuously switch from EBV latency to lytic cycle as opposed to the Nandi children as shown after DNAse 1 treatment.

As expected, populations living in malaria holoendemic regions had a more robust humoral response to all the malaria antigens assayed. Children from Nandi as well as the adults had lower titers, which were comparable to US adults consistent with lower exposure to *P. falciparum*. The most interesting observation within the study populations was that Kisumu and BL not only had significantly higher humoral response not only to EBV and malaria antigens they also had higher antibody titers to measles and schistosomiasis antigens compared to the Nandi children. However the high humoral responses to SWAP shown by the malaria naïve North American controls, a crude schistosomiasis extract (Sher *et al.*, 1989), could be a case of high sensitivity but low specificity leading to cross reactivity. There is need to evaluate the implications of the multiple infections found in malaria holoendemic regions where eBL is most common so as to rule out the possibility of several infectious agents acting in synergy to cause eBL tumorigenesis. The low humoral responses to SWAP observed in the Nandi population of both adults and children could be due to the fact that this population is far removed from the Lake Victoria basin which has high prevalence rates of

schistosomiasis and as a result are not likely to exposed to schistosomiasis. Kisumu district is located on the shores of Lake Victoria and schistosomiasis is quite prevalent (Opisa *et al.*, 2011). Schistosomiasis is mostly considered a rural phenomenon (Opisa *et al.*, 2011), similar to eBL (Rainey *et al.*, 2008, 2007a, 2007b).

Although eBL has for long been associated with *P. falciparum* and EBV, the role for other factors such as socio-economic status, exposure to certain plant extracts of the europhorbiacea, exposure to pesticides, and to other infectious agents such as schistosomiasis and arbovirus remain to be elucidated (Chene *et al.*, 2009; Mannucci *et al.*, 2012; Orem *et al.*, 2007). Malaria and schistosomiasis co-infections are common in most tropical regions and studies evaluating the immunological outcomes of these two infections have shown that schistosomiasis indeed influences the quality of acquired immune response towards malaria antigens where the quality of humoral immunity is heightened with polarization of cellular immunity towards Th_2 phenotype (Diallo *et al.*, 2010). More studies need to be carried out to examine how these co-infections modulate pediatric immunity in respect to increasing the risk to eBL.

5.3 EBV Titers Differ By Malaria Exposure History as well as Blood Compartment

Consistent with previous studies in the same geographical regions (Moormann *et al.*, 2005), children from malaria holoendemic regions exhibited elevated EBV titers compared to those from a neighbouring district but with a history of less malaria transmission pressure. BL patients had significantly higher peripheral blood viral load compared to other groups. Most children in developing countries such as Kenya experience their primary EBV infection within the first year of life (Piriou *et al.*, 2012; Rochford *et al.*, 2005). Recurrent *P. falciparum* infection is believed to diminish EBV-specific T-cell responses to lytic and not

latent antigens (Moormann *et al.*, 2007; Snider *et al.*, 2012). This loss of malaria-induced, EBV-specific T-cell immunosurveillance (Njie *et al.*, 2009), coupled with the polyclonal activation of memory B cells (where EBV virus is harbored after infection) increases the number of latently infected B cells and EBV lytic reactivation (Chêne *et al.*, 2007), possibly augments the high viral loads observed in Kisumu children. It is possible that high *Plasmodium* malaria burden coupled with high EBV burden leads to exhaustion or hypo-responsiveness of EBV latent and lytic antigen specific CD8+ T cells, thereby accelerating the probability of EBV associated malignant B-cell clones arising.

Elevated EBV titers in children residing in malaria holoendemic regions coupled with early EBV infection in life (Piriou *et al.*, 2012, 2009) and loss of EBV-specific, cytotoxic T-cell immunosurveillance in solid organ post-transplant patients has been associated with increased risk of EBV associated malignancies (Bingler *et al.*, 2008; Haque *et al.*, 1996; Rowe *et al.*, 2001). The poor immune outcomes observed in children who get infected with EBV early in life in respect to control of EBV and indirectly development of eBL (de-The, 1977; Piriou *et al.*, 2012) can be hypothesized to be a function of ineffective T-cell function towards EBV antigens. Measurement of EBV loads in populations at increased risk of eBL can be a desirable functional readout that can be exploited for clinical management of EBV infection. However, choosing the right compartment will be critical, as we have shown that most of the plasma EBV particles may just be circulating EBV DNA that could be as a result of tumor lysis. However, it is worthy noting that even after DNAse1 treatment, children residents of malaria holoendemic regions still exhibited elevated levels of infective and encapsulated virus pointing to viral reactivation by either CIDR1-α, one of the cluster of extracellular proteins expressed *P. falciparum* infected erythrocytes (*Pf*EMP) (Chene *et al.*, 2009). Furthermore, EBV reactivation may be due to either host physiological factors (Rowe

et al., 2001, 2009) or exposure to environmental agents such as from *Euphorbia triculi* (Mannucci *et al.*, 2012).

5.4 EBNA-1 Specific, T-cell Deletion is Associated with Holoendemic malaria

Previous studies in western Kenya have shown selective IFN-γ cytokine deficiencies specific to EBV antigens (Moormann *et al.*, 2007) in relation to age, malaria exposure and clinical eBL presentation (Moormann *et al.*, 2009). It is noteworthy to report that Nandi children had significantly higher frequencies of both CD4+ and CD8+ T cells producing IFN-γ compared to Kisumu and BL study populations. This study demonstrated a novel observation of comparable EBNA-1 specific IFN-γ producing CD4+ and CD8+ T cells between the Kisumu and BL study populations when measured by flow cytometric analysis. This observation was a slight deviation from what has previously reported in the same region by Moormann and others that used IFN-γ ELISPOTs to measure EBV-specific responses (Moormann *et al.*, 2009).

In the previous studies it had been observed that Kisumu children had significantly higher frequency of IFN-γ EBNA-1-specific responses compared to BL study group. Since unlike the previous study, this study utilized age-matched controls, it is safe to conclude that the differences observed here are not due to age but rather could be due to other intrinsic factors since the previous study utilized ELISPOT to enumerate EBNA-1-specific, IFN-γ-positive T cells. One of the possible explanations for this discrepancy could be the presence of other contaminating cells. This earlier study used a functional readout that would have diverse sources of origin, which are not limited to monocytes/macrophages or B-lymphocytes, but NK and NKT cells.

Flow cytometry eliminates the possibility of having contaminating cells by exclusive gating and use of a dump channel. In the current study, only CD14 and CD19 cells were excluded. However, other cells that have the capability of IFN-γ responsiveness were taken care of by negative gating, as they do not express CD3 T-cell lineage marker. The decreased number of EBNA-1 IFN-γ-producing T cells can also be due to the influence of malaria (Xu *et al.*, 2002) or the host natural cellular mechanism of immunologic tolerance (Nossal, 1983). One defining observation in the current study was that high viral loads were negatively correlated with EBNA-1-specific IFN-γ T-cell responsiveness and high PD-1 expression. It is this nexus that could possibly explain the immune dysfunction observed in children from the malaria holoendemic region.

The role of antigen-specific T cells (CD4+ and CD8+) in the control of EBV infection is well established (Wherry and Ahmed, 2004). However the role of innate immune responses both at the primary and transient stages EBV infection is a subject that needs to be elevated to the same level of prominence as that of T cells (H. Williams *et al.*, 2005). The NK cells play an important role both at primary stages of infection as well shaping the course of infection until antigen-specific T cells come into play and undergo similar affinity maturation and speciation into mature T and B cells (Ferlazzo and Münz, 2004; H. Williams *et al.*, 2005). The role of NK cells as they relate to malaria in the context of eBL needs further evaluation.

This study did not evaluate the affinity or avidity of the EBNA-1 binding to induce IFN-γ-production from T cells. The effectiveness of an immune response may not just be a function of quantity (that is the frequency of IFN-γ-specific cells) but may also be a function of quality. It is therefore necessary to evaluate the affinity and avidity of the response and to sequentially monitor the functional effectiveness of the respective immune cells over time.

These high throughput studies can be done by first typing the MHC status of study participants and evaluating the avidity binding fluorochrome-conjugated peptide-major histocompatibility complex (pMHC) multimers (tetramers or pentamers) as well as functional and architectural readout as have been described in this study. The same functional studies can be extended to both NK cells as well as B cells.

5.4.1 Frequencies of EBNA-1-Specific IL-10 and IL-17 across Study Populations

Another hypothesis that was tested in the current study was to investigate whether there was malaria induced immunological polarization towards a Th2 (T regs) or Th17 immune response that could prevent the control of EBV, which is best, mediated by IFN-γ response. This study confirmed that there was no immunological swing toward an alternative cytokine production pattern in respect to the frequencies of EBNA-1-specific CD4+ or CD8+ T cells. The frequencies of EBNA-1-specific, IL-10 producing CD4+ T cells were comparable across the three study groups although Nandi had a slightly higher median CD8+ count compared to Kisumu and BL group. The same observation was made when the frequencies of EBNA-1-specific, IL-17-producing CD4+ and CD8+ T cells were compared across the three study populations. Nazaruk and colleagues previously described a Th1-Th2 polarization in EBV-specific CD8+ T cells response similar to the classical CD4+ T-cell pathway (Nazaruk *et al.* 1998) and so it was anticipated that there would be differences between the study populations. Earlier studies in a similar setting using ELISPOT did not show any differences in IL-10 responses to EBV antigens between children living in malaria holoendemic regions and those from malaria hypoendemic regions (Moormann *et al.*, 2007) consistent with the current findings. The fact that no difference was observed in EBNA2 and EBNA3A-specific, IFN-γ and IL-10-producing CD4+ or CD8+ T cells elevates the central role played by EBNA-1 in evading the host immune response to another level that can be explored for

therapeutics. Interestingly, Kisumu children had significantly high EBNA2 and EBNA3A IL-17 producing CD4+ T cells compared to other groups. The increased EBNA2 and EBNA3A IL-17 producing CD4+ T cells among the Kisumu controls could possibly be a function of holoendemic malaria but there is need for more studies to support this hypothesis for *P. falciparum* malaria. Published reports on other forms of malaria (*P. vivax* malaria) show that IL-17 is required for sustenance of proinflammatory responses (Bueno *et al.*, 2012; Ghilardi and Ouyang, 2007). Whether the same principle is operational in *P. falciparum* malaria remains to be described.

5.4.2 Frequencies of EBNA2 and EBNA3A IFN-γ, IL-10 and IL-17-Specific T cells Within the Groups

This study notes selective deletion of EBNA-1-specific, IFN-γ producing CD4+ and CD8+ T cells within populations residing in malaria holoendemic regions and especially the BL group not those from a malaria hypoendemic region. In the healthy Kisumu and Nandi controls, the frequencies of IL-10 and IL-17 producing T cells were comparable. This means that it is this loss of EBNA-1-specific IFN-γ-producing T cells that could be allowing malignant clones to arise putting these children on the path of eBL lymphomagenesis. In Kisumu and BL groups, it was observed that the frequency of EBNA2 and EBNA3A IFN-γ producing CD4+ and CD8+ T cells was much lower compared to IL-10 and IL-17, an observation that was not shared with the Nandi group. The diminished frequency of EBNA2-specific, IFN-γ producing T cells among the Kisumu study populations is intriguing since this antigen together with EBNA-LP are the two nuclear antigens that act as transcription activators and regulate the expression of viral as well as cellular genes involved in initiation and maintenance of cell proliferation (Bornkamm and Hammerschmidt, 2001). EBNA3 group of proteins are required for EBV immortalization and the reduced frequency of IFN-γ specific T cells is telling. More

data needs to be collected on the implications of this loss and the risks of tumorigenesis especially in populations living in malaria holoendemic regions. The dynamics of immune responses to these other EBV latent antigens warrants further investigation.

5.4.3 Polyfunctionality and Polarization of EBNA-1 Specific CD4+ and CD8+ T cells

Polyfunctionality is a phenomenon where a single cell produces multiple biological molecules such as cytokines, chemokines or antibodies in response to an infectious pathogen (Larsen *et al.*, 2012). Cytokine polyfunctionality has been correlated with protective immunity especially of viral origin (Ning *et al.*, 2011). The current study did not record any polyfunctional cells in response to the EBNA-1, EBNA2 or EBNA3A in respect to the kind of panel that was used. The cytokine secretion pattern of these cells was mutually exclusive. Polyfunctionality is a rare entity and has only been recorded in long term patients with post transplant proliferative disorders with respect to EBV or in long term HIV non-progressors (Ning *et al.*, 2011). The fact that BL children showed lack of polyfunctional cells could be an indicator of clonal selection or deletion of effective T cells that puts them on the path towards lymphomagenesis. Over 98% of EBNA-1-specific CD4+ T cells had a polyfunctionality of 1(mono-specific), either producing IL-10, IL-17 or IFN-γ (albeit in small proportions). The rise of monofunctional T cells could be a function of clonal selection and differentiation marked early in the ontogeny of T cells depending on the cytokine milieu in the bone marrow and other extrinsic signals that a cell receives (Davenport *et al.*, 2002b; Saule *et al.*, 2006; Wherry and Ahmed, 2004).

5.4.4 Characterization of EBNA-1 Specific, T-cell Memory Subsets across Study Populations

The heterogeneity of memory pool architecture is critical in shaping the overall immune response to an invading pathogen first in limiting the infection and further controlling and maintaining the long-term immunity (Lanzavecchia and Sallusto, 2005). This study showed that memory phenotypes across study groups were comparable across the three study populations. The main effector CD4+ T-cell phenotype producing IFN-γ to EBNA-1 was CD4+5RA-CCR7- or T_{EM} while the CD8+ T cells showed great heterogeneity in all the study groups. For the CD8+ T cells, there was great heterogeneity where almost all subsets of T cells T_{EMRA} (CD8+CD4+5RA+CCR7-), T_N (CD8+CD4+5RA+CCR7-) and T_{EM} (CD8+CD4+5RA-CCR7+) were all represented. Thus, the deficit being described in the study manifests in the magnitude of the response and not the quality of the T-cells producing the cytokine responses. The most consistent observation in this study was the presence of naïve-like memory T-cell phenotypes, which have been published elsewhere (Chattopadhyay *et al.*, 2013; Chelimo *et al.*, 2011), and supports that indeed *P. falciparum* could be modulating the host immune system affecting T-cell differentiation and thus imparting their responsiveness to unrelated infectious agents such as EBV (Xu *et al.*, 2002). It could be thus this overstimulation and by-stander effect that leads to deletion of EBNA-1-specific IFN-γ T cells.

5.5 PD-1 Expression, T-cell Proliferation and Possible Role in eBL Etiology

It was noted from the current study that PD-1 expression varied according to *P. falciparum* transmission dynamics with healthy children from Kisumu reporting higher levels than children residing in a malaria hypoendemic region. This finding is consistent with chronic antigenic stimulation that has been observed in many diseases (Day *et al.*, 2006; Su, 2007;

Wang *et al.*, 2013). What was unexpected and intriguing, was the observation that BL children recorded very low levels of PD-1. On analysing the cells expressing this immunoinhibitory molecule, the most important observation was that BL children exclusively expressed PD-1 on their CD4+ effector T cells (T_{EM}) after EBNA-1 stimulation. A further analysis of the CD8+ T cells showed that the BL children highly expressed this molecule on TN after EBNA-1 stimulation.

The high PD-1 expression in Kisumu children could be a signal that marks these cells for deletion due to high antigen burden (high EBV loads). This high expression possibly makes these cells unresponsive to EBNA-1 antigen stimulation, allowing the evasion of immune system by these malignant clones. These dysfunctional clones then do not secrete IFN-γ upon exposure and do not even proliferate and differentiate efficiently making these children susceptible to eBL. Numerous studies in HIV have shown that there is functional impairment accompanying high viremia and elevated PD-1 expression (D'Souza *et al.*, 2007; Day *et al.*, 2006), conditions that are consistent with what is observed in children living in malaria holoendemic regions (Moormann *et al.*, 2009, 2005; Piriou *et al.*, 2012; Snider *et al.*, 2012).

Blockade of this receptor by antibodies (anti-PDL1 and PD-L2) has been marked by reversal of T-cell immune dysfunction and reduced apoptosis (Butler *et al.*, 2012; Peng *et al.*, 2012). In the current study, there was a failure to recover IFN-γ function even after blocking PD-1 ligands. This failure could be due to a shorter incubation period of 24 hours than previously published studies that have used longer periods (Bhadra *et al.*, 2011; Butler *et al.*, 2012; Dulos *et al.*, 2012). The 24-hour incubation period was based on optimization experiments for intracellular cytokine staining flow cytometry. Alternatively these cells had already differentiated into respective subsets and so could not be rescued. Furthermore, only one

antibody against PD-L1 was used leaving PD-L2 intact and hence the whole pathway may not have been completely blocked. The current study was cross-sectional in nature and, thus, cannot provide the exact point in time when children living in malaria holoendemic regions lost their EBNA-1-specific IFN-γ responsiveness.

The current attempt to demonstrate EBNA-1-specific proliferation was not successful, possibly due to the fact that these cells had already differentiated and marked for deletion and thus could not be rescued. It is known that antigen stimulation can lead to T-cell activation, proliferation and differentiation to either become effector or non-responsive cells that die. There have been reports of antigen-specific T-cell deletion due to persistence and overstimulation of the immune system (Allen *et al.*, 2011; Xu *et al.*, 2002). The development of eBL can, thus, be summed up as the consequence of malaria over stimulating the adaptive immunity with resultant dysfunctional T cells marked for deletion leading for the escape of malignant clones that later develop into eBL.

5.6 Sickle-Cell Trait and endemic Burkitt Lymphoma

Having shown that the observed EBNA-1-specific, IFN-γ dysfunction was associated with residency in malaria holoendemic regions, it became apparent to examine some of the host genetic factors that have been known to offer protection against severe forms of malaria, protecting the host against infections but allowing it to be susceptible for eBL. Past studies have shown a high frequency of HbAS trait in regions where malaria transmission pressure is high as compared to regions where malaria transmission is hypoendemic (Beet, 1946; Moormann *et al.*, 2003; Williams, 2006; Kreuels *et al.*, 2010; Scott *et al.*, 2011). The observed high frequency of HbAS trait in Kisumu (30%) and BL (19%) study populations is consistent with previous reports that have shown increased frequency of this

haemoglobinopathy in populations that are exposed to great malaria transmission burdens (Allison, 1954b; Moormann *et al.*, 2003a; Nkrumah and Perkins, 1976), consistent with natural selection for this trait.

Findings from this study showed that ß-haemoglobin polymorphisms (HbAA/HbAS) were not associated with lower viral loads. When stratified by beta-haemoglobin genotype, children with HbAS had statistically comparable cell-associated viral loads to those who had HbAA trait. There was no evidence of fever or anaemia in these individuals at the time of venous sample collection, thus any cases of malaria parasitemia were asymptomatic.

To minimize the biasness of *P. falciparum* malaria on viral loads (Chêne *et al.*, 2007a; 2009), all children testing positive by blood smear were excluded from the analysis but this did not change the significance. The low parasitemia and resultant low EBV viral loads could be a product of the protective effect of HbAS, thus minimizing *P. falciparum* replication, resultant B-cell proliferation (Donati *et al.*, 2006, 2004) and EBV viral reactivation (Chêne *et al.*, 2007a; 2009). The fact that HbAS is found in both healthy controls as well as eBL group suggests that HbAS may not be protective against eBL, despite lessening the malaria burden and possibly viral titers in children. A recent longitudinal study in Uganda showed that HbAS protected children against progression of blood stage infection to symptomatic malaria, and this protection increased with age (Gong *et al.*, 2012).

This study although pioneering in showing that HbAS controls one of the surrogate markers (severity of *P. falciparum* infection) towards eBL lymphomagenesis (Bingler *et al.*, 2008), does not explain the exact mechanism how this occurs. It is possible that the combined unfavourable condition offered by sickle cells and the resultant low parasitemia could be

mitigating against B-cell activation and EBV lytic cycle reactivation. The Ugandan study showed that even when a malaria infection occurs in children with HbAS genotype, there is controlled multiplication of the parasite thus leading to low parasite densities (Gong *et al.*, 2012). The current study being cross sectional in nature did not examine at which point in time of infection and growth of the child the effect of HbAS is greatest.

There is need for future longitudinal studies with better controls (either sibling controls or parent sibling controls) and a large sample to be undertaken to examine the human immunogenetics of red blood polymorphisms and eBL. With a lower likelihood of symptomatic infection from *P. falciparum*, HbAS are more likely to harbour parasites in their blood stream for longer periods of time thereby possibly creating a reservoir for EBV reactivation. However both innate and adaptive host mechanisms like production of heme-oxygenase (Ferreira *et al.*, 2011), increased phagocytosis by splenic macrophages of HbAS erythrocytes (Ayi *et al.*, 2004) or the altered expression of PfEMP-1 that dampens cytoadhereance in vasculature (Cholera *et al.*, 2008) among other immune factors could be protective against higher risks of eBL development.

CHAPTER SIX

6.0 SUMMARY OF STUDY FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Findings

This study has shown that there is EBNA-1-specific, T-cell deletion that is associated with EBV-malaria co-infections. Further, it has been observed that HbAS genotype is not associated with EBV load control, T-cell function or eBL diagnosis. The preferential depletion of IFN-γ EBNA-1-specific T-cell function and phenotype observed in pediatric populations living in malaria-holoendemic regions, provides an avenue that can be exploited not only for diagnostic purposes but also for therapeutic purposes if the exact point in time when this defect occurs can be determined. An in-depth understanding of the mechanisms driven by *P. falciparum* and EBV co-infections that result in the depletion of EBNA-1-specific, T-cell immune responses is a pivotal point of intervention. Thus, there is need for more in-depth studies to explore this causal mechanism by studying immune regulatory pathways modulated by *P. falciparum* and which may increase the predisposition to eBL. Understanding how these polymicrobial infections interact and induce dysregulation of T-cell immunity is not only important in the management of this cancer but potentially for other cancers with a viral origin.

6.2 Conclusions

1. The serological profiles to common infectious agents differed according to antigen as well as the study site, which was a function of *P. falciparum* exposure dynamics. Whereas serological profiles to EBV antigens were comparable among the study groups, children from malaria holoendemic regions had more robust responses to malaria,

measles and schistosomiasis antigens compared to those from malaria hypoendemic region.

2. Residency in malaria holoendemic regions was associated with high cellular EBV titers. It was observed that most of the circulating EBV plasma virions were un-encapsulated.

3. It was observed that EBNA-1-specific, cytokine deficiency was specific with IFN-γ and not other cytokines (IL-10 or IL-17). Further, it was observed that these cells were monofunctional and not polyfunctional. Taken together, these observations suggest that the deficiency in EBNA1-specific, IFN-γ T cells (Th1), found in the eBL patients, is not the result of skewing toward Th17 or Treg (IL-10)-producing cells.

4. The IFN-γ T-cell deficiency, observed in malaria-exposed children and eBL patients, is selective for EBNA1 and does not occur for other latent EBV antigens (EBNA2, EBNA3A). The frequency of EBNA1-specific IL-10 and IL-17 responses did not differ by malaria exposure or eBL diagnosis.

5. The T-cell memory phenotypes were comparable across the study groups. This suggests that there is selective loss in the magnitude of EBNA-1-specific, IFN-γ function in populations residing in malaria holoendemic regions.

6. PD-1 and IFN-γ expression in response to stimulation with EBNA1 was mutually exclusive: Kisumu children did not express IFN-γ but demonstrated increased expression

of PD-1 after *ex vivo* EBNA1 stimulation. Children diagnosed with eBL did not express IFN-γ or PD-1 after *ex vivo* EBNA1 stimulation, suggesting a deletion of these cells prior to tumorigenesis.

7. The eBL risk is not significantly predicated on HbAA/AS genotype to the same degree as the protective effect it engenders for malaria in terms of severe disease and hyper-parasitemia.

6.3 Recommendations

6.3.1 Recommendations from the Current Study Findings

1. Additional investigations on the relationship between common infectious agents such as measles and schistosomiasis (which are coincidentally common in regions where eBL incidences is high) would provide more information on their role in eBL pathogenesis.

2. There is need for paradigm shift from use of either plasma or whole blood EBV titers as a biomarker of prognostic to use of cellular EBV viral loads since the latter provides a more realistic biomarker. This will prove useful not only as a utility prognostic marker in monitoring eBL patients once chemotherapy has commenced but can also be used to identify children at increased of eBL tumorigenesis.

3. There is need to study the role of other cytokines that maybe involved in pathogenesis of eBL. This should involve mapping out other cytokines that have been associated

with tumours such as IL-6, IL-4 as well as investigating gene polymorphisms in these immune effector molecules.

4. Elucidation of additional mechanisms by which *P. falciparum* co-infections interferes with EBNA-1 immunity is critical since immunity to other EBV latent proteins (EBNA2 and EBNA3A) is intact.

5. Additional studies on the differences in signalling pathways due to residency in malaria holoendemic regions, to decipher the mechanisms by which these populations loose EBNA-1 specific IFN-γ responsiveness (despite having similar T-cell immune memory architecture to populations living in a nearby region with less intense *P. falciparum* transmission pressure) would provide more information on pathways associated with eBL.

6. Longitudinal, functional studies to address PD-1-mediated mechanisms that lead to deletion of EBNA1-specific T-cell subsets in children repeatedly and/or chronically infected with malaria would provide the opportunity to prevent this cancer.

7. More studies need to be carried out to investigate the role of other host genetic factors that may be associated with eBL.

6.3.2 Recommendations for Applications of Study Findings

1. A sustained long-term effort in malaria control, programs such as, bed net usage and indoor vector control are a necessity to reduce EBV viremia as well as EBNA-1-specific,

T-cell deletion associated with residency in malaria holoendemic regions, which is a risk factor in eBL etiology.

2. The observation that children presenting with eBL have a functional deletion in EBNA-1-specific, T-cell pool, suggests that these children can benefit from adoptive autologous EBNA-1-specific, T-cell immunotherapy as one of the clinical management regimes.

3. A paradigm shift from use of EBV serological load to EBV cellular load as a prognostic biomarker would provide a more realistic marker of true encapsulated viral burden.

4. PD-1-mediated blockade interventions should be explored alongside other chemotherapeutic measures in clinical management of children presenting with eBL to augment functional EBV-specific, T-cell immune reconstitution.

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