PLASMODIUM FALCIPARUM MALARIA TRANSMISSION EFFECTS ON NATURAL KILLER CELL FUNCTION IN THE ETIOLOGY OF ENDEMIC BURKITT LYMPHOMA IN CHILDREN FROM WESTERN KENYA

BY

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DECLARATION

I declare that the work presented herein is my original work and has not been presented for award of any degree elsewhere. No part of this thesis may be reproduced without prior written permission of the author and or Maseno University.

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DEDICATION

I dedicate this thesis to my dad Kitungulu, mum Nechesa, my wife Rhodah and my daughters Vanessa, Angel and Janelle. They have unrelentingly offered encouragement and support in my academic pursuit and endeavors. God bless you all.

ABSTRACT

The exact etiological mechanism underlying endemic Burkitt lymphoma (eBL) remains unknown. Past studies show T and B cells are immuno-deregulated in children from malaria holoendemic regions. However, no study has examined the effect of *P. falciparum* transmissions on Natural Killer (NK) cell, which shape T and B immune responses. NK cells are critical in immunosurveillance, elimination of cancerous cells and controlling EBV viremia. This is by production of anti-viral cytokines like Interferon gamma (IFN-γ), cytotoxic degranulation molecules (CD107a and Granzyme B, GrB), and expression of pro-apoptotic markers e.g. programmed death-1, (PD-1). This study investigated how malaria impacts on NK cell function in eBL etiology. Thus 42 children aged 3½ years from areas with diverse malaria transmission and eBL incidence, (Kisumu n=16, malaria holoendemic region of high eBL incidence), Nandi, n=16, malaria hypoendemic region, low EBL incidence) and eBL n=10, (cancer children at Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) were enrolled. To investigate differences in Epstein Barr Virus (EBV) antigen specific NK cell frequencies, peripheral blood mononuclear cells were stimulated with Epstein Barr Nuclear Antigen 1 (EBNA1). The frequencies of EBNA1 and MSP1 specific cells were evaluated by flow cytometry. Merozoite surface protein 1 (MSP1) was a control antigen for malaria exposure. To correlate viremia with NK cell activity, EBV burden was determined by qPCR. Results show that Kisumu children had high parasitemia ($p=0.0180$) and viral load ($p=0.0006$) compared to Nandi. Moreover, Kisumu children presented with low EBNA1 specific IFN-γ NK response ($p=0.0262$) but high MSP-1-specific IFN-γ NK cell response ($p=0.0174$), EBNA1 PD-1 ($p=0.0130$) and CD107a ($p=0.0293$) specific responses. However, there was no significant difference in the frequencies of GrB specific cells among the three study groups in response to either EBNA1 ($p=0.9150$) or MSP-1 stimulation ($p=0.8911$). It was observed that high viral loads led to low NK EBNA1 specific GrB response in Nandi children ($p=0.0490$, $r^2=0.3390$) while there was no association between EBV viral load and EBNA1 specific IFN-γ and CD107a NK cell across the study groups ($p > 0.5$). High viral burden was weakly associated with high EBNA1 specific PD-1 expression ($p=0.05$, $r^2=0.02$) in Kisumu children. This shows that *P. falciparum* transmission affects EBV viral burden and NK cell function. Thus a relationship exists between holoendemic malaria and NK cell function in the presence of high EBV burden. Therefore, malaria perturbation of EBV specific NK, T and B cells could have synergistic effect in eBL etiology.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
DNA	Deoxyribonucleic Acid
eBL	Endemic Burkitt lymphoma
EBV	Epstein Barr Virus
EBNA1	EBV Nuclear Antigen 1
GC	Germinal Center
Hb	Haemoglobulin
INF-α	Interferon alpha
INF-β	Interferon beta
INF-γ	Interferon gamma
IL	Interleukins
JOOTRH	Jaramogi Oginga Odinga Teaching and Referral Hospital
KEMRI	Kenya Medical Research Institute
NK	Natural Killer
MCMV	Murine Cytomegalovirus
MSP1	Malaria surface protein
PTLD	Post-transplant lymphoproliferative disorder
P. *falciparum*	*Plasmodium falciparum*
RTQ-PCR	Real Time Quantitative Polymerase Chain Reaction
PBMC	Peripheral blood mononuclear cells
RPMI 1640	Roswell Park Memorial Institute 1640 media
TNF-α	Tumour necrosis factor alpha
WHO	World Health Organization
SIV	Simian Immunodeficiency Virus
UK	United Kingdom

UMMS	University of Massachusetts Medical School
USA	United States of America

DEFINATION OF TERMS

Endemic: (of a disease) the frequency or how regularly found among people or in a certain area

Endemicity: The constant presence of diseases or infectious agents within a given geographic area or population group. It may also refer to the usual prevalence of a given disease with such area or group.

Genesis: The origin or mode of formation of something

Holoendemic: This is a stable transmission of a disease affecting all or characterized by the infection of essentially all the inhabitants of a particular area all year long. A holoendemic disease is one for which a high prevalent level of infection begins early in life and affects most of the child population, leading to a state of equilibrium such that the adult population shows evidence of the disease much less commonly than do children (malaria in many communities is a holoendemic disease).

Hyperendemic: A hyperendemic disease is one that is constantly present at a high incidence and/or prevalence rate and affects all groups equally.

Hyperendemic: Intense, but with periods of no transmission during dry season.

Hypoendemic: Very intermittent transmission

Lymphoma: cancer of lymphatic system

Mesoendemic: Regular seasonal transmission (of malaria)

Pathogen: A bacterium, protozoan, virus or other microorganisms that cause disease

Transmission: The act of passing or transferring a pathogen from one host to the other. In this study transmission refers to the frequency at which a human host is exposed to *P. falciparum* malaria parasite by infective anopheles mosquitoes

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Endemic Burkitt lymphoma (eBL) is the most common pediatric cancer in equatorial Africa accounting for over 74% of pediatric cancers (Brady *et al.,* 2007), especially in regions where malaria transmission is holoendemic (Mwanda *et al.,* 2004). The characteristic high cell proliferative potential doubling of tumour size within 24 hours makes it a great public health concern. This rapidly dividing cancer is fatal in untreated children especially those from sub-Saharan Africa where medical infrastructure is underdeveloped or lacking all together (Jayasooriya *et al.,* 2012). Although a long-standing epidemiological link between Epstein Barr Virus (EBV) and chronic *Plasmodium falciparum* infection during childhood has been established as the infectious agents required for eBL etiology, the mechanism of the malaria parasite and EBV in the development of eBL remains undefined (Andrew, 2005). Although past immunological studies in western Kenya have shown that both T and B cells are modulated during EBV-malaria co-infection (Asito *et al.,* 2008; Chattopadhyay *et al.,* 2013; Moormann *et al.,* 2007) the role of innate immunity remains uninvestigated. In their study, Asito *et al.,* (2008) showed that the frequencies of B cells are perturbed during malaria infection, leading to an increase in naïve B cells, which are easily infected by EBV. Moreover, Chattopadhyay and others (2013) showed that there was a decrease in the frequencies of EBV antigen specific T cells in children aged 5-9 years living in malaria holoendemic regions, an age that coincides with the peak incidence. Earlier on, a cross sectional study by Moormann in the same region had shown that the frequencies of EBNA1 specific cells decreased in children aged between 5-9 years, an age when eBL is observed (Moormann *et al.,* 2007). Taken together, these studies show that malaria interferes with the cells of adaptive immunity setting a situation that leads to eBL. However, the responses by T

and B cells depend on the signals received from innate arm of immunity and especially NK cells (Hislop *et al*., 2007). Despite these early studies, no information exists on the possible immunomodulatory role of malaria transmission on NK cells function and EBV load control. This study therefore sought to understand how *P. falciparum* transmission pressure affects natural killer (NK) cells function and possibly explains how this increases the risk of eBL in children from malaria holoendemic regions.

The role of NK cells in controlling viral infections is well documented (Williams *et al.,* 2005) in which NK cells not only limit primary EBV infection by production of cytolytic granules (such as CD107a and granzymes such as Granzyme A and B) (Pappworth *et al.,* 2007) but also augment antigen specific T cell responses by release of cytokines such as IFN-γ and IL-2 (Williams *et al.,* 2005). NK cells are further known to exhibit abilities of killing tumor cells without activation (Wu and Lanier, 2003). However, the immunomodulatory role of *P. falciparum* on NK cell function has not been studied in the context of EBV infection and eBL lymphomagenesis. Children aged below 5 years of age from malaria holoendemic regions have been shown to have deficient IFN-γ response to EBV antigens and especially EBNA1 as well as high EBV viral loads *(*Moormann *et al.,* 2007; Piriou *et al.,* 2012*)* compared to children from malaria hypoendemic region. EBNA1 is the only EBV antigen expressed by EBV associated tumors as well as infected cells (Leight and Sugden 2000, 1) and hence the quality of immune responses to this antigen may shed important clues in etiology of eBL. Studies in Kenya have shown that over 99% of the population are EBV seropositive by six months of age (Piriou *et al.,* 2012). Whether this deficiency is limited to cells of adaptive immunity or transcends the cells of innate immunity such as NK cell remains to be investigated. Thus this study investigated the effect of differences in malaria transmission on

the frequencies of EBNA1 specific NK cells and the relationship between the frequencies and viral burden.

1.2 Problem Statement

Although past studies have shown that malaria affects cells of adaptive immunity with respect to EBNA-1, the main EBV antigen expressed by all EBV tumors and infected cells, no study has investigated the effect of malaria transmission on NK cells. The role of NK cell in control of primary EBV infection and augmenting adaptive immunity is well studied (Williams *et al.*, 2005), but there is paucity of data on its role in eBL lymphomagenesis. There is no study that has examined the effect of *P. falciparum* transmission in modulating NK cell function with respect to production of cytokines such as IFN-γ or cytotoxic granules such as CD107a and Granzyme B and viral burden. Further there is no study that has examined the relationship between NK cell function and viral burden.

1.3 Objective of The Study

1.3.1 Main objective

To investigate the effects of *Plasmodium falciparum* malaria transmission and Natural Killer cell function in the etiology of endemic Burkitt lymphoma in children from western Kenya.

1.3.2. Specific Objectives

1. To investigate and determine the frequencies of EBNA1-specific NK cell function (IFN-γ, Granzyme B, PD-1 and CD107a) due to *P. falciparum* transmission patterns in holoendemic and hypoendemic regions of western Kenya.

2. To investigate and determine the correlation between NK cell activity and EBV viral loads.

1.4 Research Questions

i. What are the frequencies of EBNA1-specific NK (IFN-γ, Granzyme B, PD-1 and CD107a) cells due to *P. falciparum* transmission patterns in holoendemic and hypoendemic regions of western Kenya?

ii. What is the correlation between NK cell activity and EBV viral loads?

1.5 Justification of the Study

Endemic Burkitt lymphoma, the most common pediatric cancer in sub-Saharan Africa, occurs at a higher incidence in malaria holoendemic regions such as western Kenya compared to areas with low malaria transmission. Child mortality rate is therefore high in these regions due to eBL development and malaria infection. Thus there is need to understand its etiology in order to generate effective prophylactic and therapeutic strategies.

Although past epidemiological studies have shown that EBV and malaria are the two main co-factors in etiology of eBL, the exact association on how each contributes to eBL development is not clearly understood. The role of malaria and EBV in modulating the expression of some cytokines suggests that cytokine pathways are important in the development of eBL. A better understanding of the molecular events of EBV and malaria linkage in eBL pathogenesis may aid in expanding our understanding of eBL lymphopathogenesis. NK cells are not only crucial during primary EBV infection, but also in

shaping downstream molecular events of adaptive immunity. Understanding the association between malaria transmission and NK cell function as it relates to control of EBV viremia is urgently needed. Findings from this study will also shed more light on the relevance of Natural Killer cell function in understanding the relationship between malaria transmission and eBL.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of eBL in Kenya

Endemic Burkitt lymphoma (eBL) is the most common childhood cancer in equatorial Africa (Chene *et al*., 2009; Mwanda *et al.,* 2004). The observation that eBL occurs at high prevalence in the tropical regions where malaria is holoendemic led to the theorem that malaria and eBL were linked (Mwanda *et al.*, 2004). This tumor presumably has a multifactorial etiology with two ubiquitous microbes EBV and *P. falciparum* etiologically linked in the oncogenic process (de Thé, 1997). The tumor has a very high proliferative index (Magrath, 1990; Parkin *et al.*, 1989) and thus of great public health significance.

Worldwide over 90% of all people become infected with EBV at some point during their lifetime (Münz and Moormann, 2008), but what differs is the temporal kinetics of infections. In equatorial Africa, most children convert to EBV seropositive by six months of age *(*Piriou *et al.,* 2012*;* Piriou *et al.,* 2009) while in developed countries most people become infected during adolescence (Pagano *et al.*, 2004). Though most infected individuals remain healthy, EBV is capable of a variety of pathologic conditions, being linked to several human diseases and malignancies (Bonkamm, 1999). The incidence rate of EBV malignancies in Kenya is much higher around L. Victoria and coastal regions where malaria transmission is hyperendemic than other parts of the country (Rainey, Mwanda, *et al.,* 2007; Rainey, Omenah, *et al*., 2007). Despite the fact that the national incidence rates of this cancer is 1 out of every 100, 000 live births, this tumor has a high incidence rates along the Lake Victoria

which is a malaria holoendemic region reporting incidence rates of over 10 in 100, 000 live births (Mwanda *et al.,* 2004; Rainey, Mwanda, *et al.,* 2007*;* Rainey, Omenah, *et al.,* 2007*).*

Despite the long-standing epidemiological link between EBV and persistent *P. falciparum* in the etiology of eBL (Burkitt, 1983), the exact molecular mechanism of this tumor remains undefined. Further the relative effect of the malaria parasite on the host immune cells is only recently being unraveled *(*Asito *et al.,* 2008; Chattopadhyay *et al.,* 2013; Moormann *et al.,* 2007; Snider *et al.,* 2012*).* However, despite the above seminal studies, none has addressed how malaria imparts immunological function of NK cells and how this may be a predisposing factor in eBL etiology.

2.2 The Link Between *P. falciparum* malaria, EBV and B Cells in The Etiology of Endemic Burkitt Lymphoma

Studies have shown that EBV is a potent B cell transforming virus that has remarkable ability to immortalize B cells (Henle et al., 1967). It has been shown that EBV infects B cells while in the germinal center. It is believed that eBL has its origin from germinal center (GC) B cells (Klein et al., 2008) and is characterized by a typical translocation of the c-myc oncogene into one of the immunoglobulin loci (Hecht *et al.,* 2000; Klein, 1983). The subsequent deregulation of c-myc expression would normally lead to rapid apoptosis of the cell, but the presence of EBV rescues these cells and allows them to be immortal (Thorley-Lawson *et al*., 2008*).* Endemic BL represents an intriguing situation in which interactions between *P. falciparum* parasite and EBV combine to cause a lymphoma, a cancer of B lymphocyte origin. While the mechanisms linking EBV to lymphoma development are becoming better

understood in the context of B and T cells, the link between *P. falciparum* malaria transmission and NK cell leading to eBL has remains completely unexplained.

The first piece of evidence to explain the molecular role of *P. falciparum* in eBL lymphomagenesis was the demonstration of the induction of DNA-mutating and double-strand-breaking enzyme known as activation-induced cytidine deaminase (AID) (Torgbor *et al.*, 2014). This enzyme is normally responsible for the somatic hypermutation and class-switch recombination of immunoglobulin genes that occur in B cells when they enter the germinal center (Muramatsu *et al.,* 2000) and occasionally mutates off targets such as oncogenes (Liu *et al*., 2008*).* Studies have shown that malaria infection causes deregulation of AID and hence becoming a risk factor for lymphoma development, including the c-myc translocation, which is a characteristic of eBL (Ramiro *et al.,* 2004). Studies by Torgbor and colleagues utilizing tonsils from individuals either chronically infected or uninfected with *P. falciparum*, showed that individuals chronically infected with *P. falciparum* malaria had higher numbers of germinal center B cells with elevated AID expression levels and extremely high level of EBV infected germinal center B cells (Torgbor *et al.,* 2014). It was observed that extracts from *P. falciparum*-infected red blood cells directly causes a strong activation of AID in tonsil B cells in vitro and this was due, at least in part, to the action of hemozoin, the metabolic product of hemoglobin digested by *P. falciparum* parasites (Torgbor *et al.,* 2014). Together, these results allowed the (Torgbor *et al.,* 2014) to conclude that *P. falciparum* infection increases two major risk factors for lymphoma development. The increased combinatorial risk of these two events explains the increased prevalence of eBL in *P. falciparum*-endemic areas (figure 2.1), but whether the continuous *P. falciparum* malaria transmission in the holoendemic region suppresses and affects the functions or rather activities of NK cells still uninvestigated.

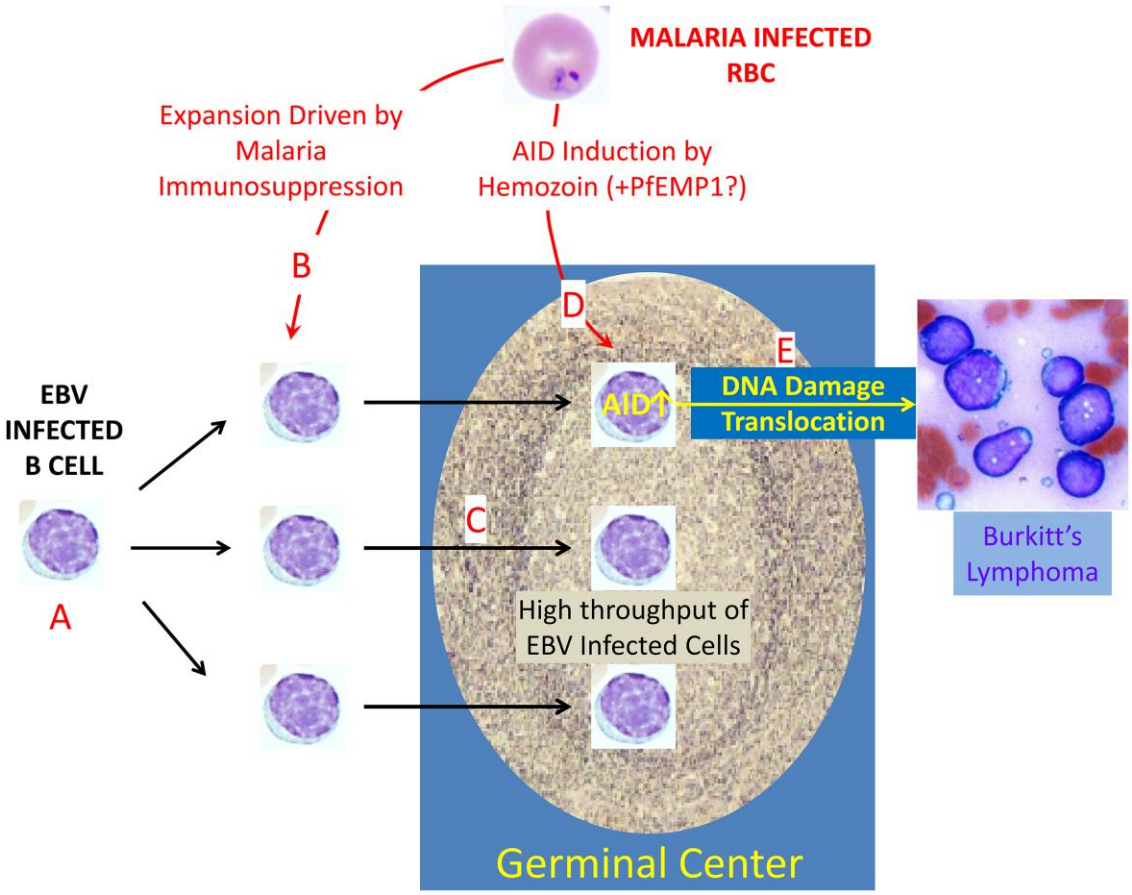


Figure 2.1: Schematic representation of the interaction between *P falciparum*, B cells and EBV in the etiology of eBL (adapted from Thorley-Lawson *et al.*, 2016).

(EBV; Epstein Barr Virus**, AID;** Activation-Induced cytidine Deaminase**, RBC;** Red Blood Cells, **pfEMP1;** *Plasmodium falciparum* erythrocyte membrane protein-1) and (**A, B, C, D,& E** refers to sections). Essentially all adults are persistently infected with EBV (A). As a consequence, newly infected B cells are continually being produced that transit the germinal center on their way to becoming latently infected memory B cells (the site of viral persistence) *(*Robbiani *et al.,* 2015). Malaria is immunosuppressive (B) (Moormann *et al.,* 2005; Moormann *et al.,* 2007*)*, and has shown that this results in a highly elevated throughput of EBV-infected cells in the Germinal center (C) (Torgbor *et al.,* 2014), also showed that *P. falciparum* induces deregulated expression of the DNA-mutating and -cutting enzyme AID in germinal center cells (D). Subsequently showed in a mouse model that this deregulated expression led to DNA damage, translocations, and, ultimately, lymphoma (E) *(*Robbiani *et al.*, 2015*).* Thus, infection with *P. falciparum* malaria has been shown to have two effects on the germinal center, where eBL originates. Together, these increase the risk that a germinal center cell will undergo a c-myc translocation and that this cell will also be EBV-infected and, therefore, able to tolerate the translocation, synergistically increasing the likelihood of eBL cancer development.

2.3 The Immunomodulatory Effects of Malaria on Immunity as a Risk Factor in the eBL Etiology

The immunopathophysiological effects of malaria infection are possibly due to the polymorphic nature of the parasite antigens, the parasites' complex life cycle, its varied antigens and the host immune response *(*Voller, 1974*;* Yazdani *et al.*, 2006). Malaria parasite has been shown to possess numerous evasive and host immune subversion mechanisms. It has been shown that the parasite can modulate the host defenses both specifically and globally, giving itself an advantage to survive and be transmitted from one human to another (Jayasooriya *et al.*, 2012). It is this combined immunomodulatory effect of malaria on the immunity of young children infected with EBV that could lead to eBL lymphomagenesis. The immuno-bystander effect *P. falciparum* infection on unrelated infections (Nikiforow *et al.*, 2003; Xu *et al.*, 2002) such as EBV has gained prominence with discovery of polymicrobial diseases (Chene *et al.*, 2009; Rochford *et al*., 2005)

P. falciparum malaria has numerous mechanisms that can affect B cells during the course of infection. One such molecule is *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) molecule, which is a polyclonal B cell activator resulting in enhanced B cell proliferation and increased cytokine and antibody secretion or hypergammaglobulimia (Donati *et al*., 2004). *P. falciparum* is also capable of inducing B cell activation through innate immune receptors such as toll like receptors (Hisaeda *et al.*, 2008; Leoratti *et al*., 2008). The byproduct of hemoglobin metabolism breakdown, hemozoin, bound to DNA of *Plasmodium* parasites is capable of stimulating B cells through TLR9 (Ayash-Rashkovsky, Bentwich, and Borkow, 2005). Furthermore *Plasmodium* glycosylphosphatidylinositol (GPI) anchors are capable of stimulating TLR2 (Geurts *et al.*, 2011). The effects of these B cell

activators could be even greater in a population that is chronically exposed to malaria. Studies in Kenya have shown that residences in malaria holoendemic region have been associated with deregulated T cell function with respect to EBV antigen (Moormann *et al*., 2009; Chelimo *et al*., 2011). Although several studies have examined the effects of malaria on B and T cell subsets in malaria exposed individuals (Dent *et al.,* 2009; Spring *et al.,* 2010), there has been no study that has examined NK cell function in children living in a holoendemic region as well as those presenting with eBL.

EBV is a ubiquitous virus that is found in >90% of people worldwide, and is present in nearly all cases of endemic African BL (Burkitt, 1983; Magrath, 1990). A common feature of eBL is the translocation of the oncogene c-myc to the control of the immunoglobulin promoter leading to constitutive expression of c-myc (Ruf *et al*., 1999). Translocations of c-myc in the presence of EBV are sufficient to produce transformed cells (Ruf *et al.,* 1999), but the etiology of the c-myc translocations and whether malaria plays a role in inducing these translocations is unknown.

Further the relative effect of the malaria parasite on the host immune cells is only recently being unraveled (Asito *et al.,* 2008; Chattopadhyay *et al*., 2013; Moormann *et al.,* 2007; Snider *et al.,* 2012*).* Children residing in malaria holoendemic regions have been found to harbor high EBV viral loads (Moormann *et al.,* 2005; Moormann *et al* 2011). Malaria in these studies was shown to lead to perturbations in B cell memory subsets (Asito *et al.,* 2008). High EBV viral loads could increase the likelihood of a B cell with a c-myc translocation getting rescued from cell death (Njie *et al.,* 2009). The ability of the immune

system to control EBV infection is important in controlling the infection and decreasing the likelihood of EBV associated malignancy (Münz and Moormann, 2008; Moormann *et al.*, 2009; Njie *et al.*, 2009; Jayasooriya et al. 2012; Snider *et al.*, 2012). During acute clinical malaria, there is concomitant loss of T cell control over EBV-infected B cells, and a higher viral load (Münz and Moormann, 2008)

However, despite the above seminal studies, none has addressed how malaria imparts immunological function of NK cells and how this may be a predisposing factor in eBL etiology. NK cells are effector lymphocytes of the innate arm of immunity and control several types of malignancies, transformed cells as well as microbial infections by limiting their spread and subsequent histopathology (Ferlazzo and Münz, 2004). NK cells perform these functions by production of cytotoxic granules such as CD107a, GrB as well as effector cytokines such as IFN-γ (Vivier *et al.*, 2008). The exact mechanism by which this parasite exacerbates host immunopathology and how this is related to eBL remains to be described. The mechanism through which this immunomodulation influences the body's ability and, especially the NK cell function was the basis of this study. This study therefore sought to investigate the role of *P. falciparum* on the frequency of EBNA-1-specific NK cells (CD107a, Granzymes B, PD-1 and IFN-γ) and how this relates to viral control.

2.3 The Role of NK cells in *P. falciparum* and EBV Burden Control

Natural killer cells as part of the innate immunity play an important role in microbial control especially during the primary stages of the infection (Vivier *et al.*, 2008). NK cells derived effector mechanisms have been reported in controlling viral infections such as HIV,

influenza, cytomegalovirus among many other infections (Jost and Altfeld, 2013). The function and activation of NK cells to viral infection is controlled by an interplay between inhibitory and activating factors such as PD-1 or expressed cytokines or the cytotoxic potential as seen by the degranulation potential (Brandstadter and Yang, 2011). Despite this critical role of NK cells in controlling viremia burden, it is not known whether there is differential induction of cytokine secretion due to exposure to *P. falciparum* infection, which may affect the effectiveness of NK cell in controlling viremia. Therefore, this study sought to investigate the relationship between *P. falciparum* exposure and the frequencies of EBV antigen specific NK cells and how this is related with EBV viral burden management.

Apart from infection induced proinflammatory cytokines production, NK cells also do produce cytotoxic granules that assist in killing virally infected cells as well cells presenting with tumor signals (Biron *et al.,* 1989) keeping viral replication under control. One of the well-characterized mechanisms is the release of cytoplasmic granules-complex that combines specialized storage and secretory functions with generic degradative functions of lysosomes (Moormann *et al*., 2005). These granules contain a number of proteins such as perforins and granzymes that lyse target cells. Some of the well-known cytotoxic granules include CD107a and CD107b, granzymes A, B and C, which are secreted at target cells helping to control the infection as well as tumors (Gong *et al*., 2012). However, the role of these granules in eBL etiology and specifically granzyme B remains poorly defined. Whether the same mechanism is operational in children living in malaria holoendemic regions or is nonexistent remains to be elucidated. Further it is unknown if the NK cell activity is correlated with EBV viral load control. It has been shown in laboratory studies that NK cells can kill cancer cells through release of cytotoxic protease granzyme B. Therefore, this study sought to examine whether

differences exist in NK cell cytotoxic granules activity due to malaria exposure and whether these difference can explain the propensity of children residing in malaria holoendemic malaria regions towards eBL tumorigenesis.

2.4 The Relationship Between EBV Viral Burden and NK Cell Activities

The effector function of NK cells is a delicate balance between inhibitory and activating signals (Brandstadter and Yang 2011). This function is affected by secreting a number of pro-inflammatory cytokines such as IFN-γ and TNF-α cytokines (Williams *et al*., 2005). Recently another marker, lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) has been described as a marker of NK-cell degranulation following stimulation (Alter, Malenfant, and Altfeld, 2004). Although the frequencies of these molecules can be assayed to investigate the activities of both T and NK cells, there is no study to the best of our knowledge that has investigated the relationship between the production of these molecules and control of viral burden. Studies in humans have shown that NK cells play a critical role against members of the herpes virus, poxvirus and papillomavirus families (Biron *et al.,* 1989). It has been shown that patients with identified NK cell deficiencies have been predisposed to particularly severe, recurrent viral infections. Studies in murine models have provided additional evidence that the NK cells give a critical help to control several viral infections, most notably murine cytomegalovirus (MCMV), poxviruses and influenza ((Biron *et al.,* 1989; Henle *et al*., 1967). Whether the same deficiency exists in people living in malaria holoendemic region which could be a risk factor in the etiology of eBL remains to be studied.

In primary human EBV infection, NK cells control and eliminate infected B cells by release of anti-viral cytokines such as INF-α, INF-β, INF-γ, IL-2, and IL-12 (Williams *et al*., 2005). Tonsillary NK cells have also been shown to be high producers of INF-γ and can delay latent EBV protein expression *in vitro* in the presence of dendritic cell induced IL-12 (Strowig *et al*., 2008). Whether the same effect can be seen in peripheral blood derived NK cells remains to be elucidated. IFN- γ control malaria infection by limiting parasitemia by inducing and activating macrophages that kill intraerythrocytioc parasites by release of reactive oxygen species (Ockenhouse, Schulman, and Shear, 1984). Using the same mechanisms, it has been shown that NK cells can limit the development of clinical malaria by impairing the development of the parasites (Clark *et al.*, 1987). There is need to investigate the relationship between NK cell activities and viral burden control.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The current study was carried out in western Kenya at two sites that differ in malaria endemicity and intensity. Mosoriot (highlands) ($0^0$19'19N, $35^0$10'24E or 0.322078,35.172429) found in, Nandi County is in a region of sporadic (low and unstable) malaria endemicity and located at an altitude of over 3000m above sea level. It is characterized with high rainfall and humidity, but moderately low temperatures. This region has low incidence rates of eBL (Rainey *et al.,* 2007a). Chulaimbo (lowlands) (-$0^0$2'15'N, $34^0$84'17E or -0.037972, 34.638299), located near the shores of Lake Victoria in Kisumu County, is a region experiencing holoendemic (chronic and intense) transmission patterns and has high prevalence rates 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 through-out the year. (Appendices 1-3 for study sites and malaria endemicity). JOOTRH (005'19'5, 34046'19E or -0.088697, 34.772016) is the regional referral centre for all pediatric cancers in Western Kenya.

3.2 Sampling Methods

Purposive sampling technique was used to obtain samples from eBL cancer patients admitted at JOORTH. Two healthy control study populations aged 3½ years old were randomly selected for this study as per the inclusion criteria to ensure that the findings were representative of the general population. This design was chosen so as to compare the frequencies EBNA-1 specific NK cells in both healthy populations living in geographically

different areas and eBL without biasness. The eBL study group was the clinical group presenting with the diseases while the Kisumu healthy and Nandi groups were the control group for eBL.

3.3 Study Populations

The first two study groups were age-matched but from regions with divergent *P. falciparum* exposure. They were aged three and half years. On the other hand the eBL study group (which is a subset of Kisumu children but presenting with cancer) was recruited from Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH), which is the regional referral centre for all cancers in Western Kenya. The samples for the present study were drawn from a prospective pediatric cohort of children that had been enrolled and maintained by one of the research collaborators with the Kenya Medical Research Institute since birth. The measurement parameters investigated in this study included frequency of EBV specific NK cells, *P. falciparum* parasitemia and EBV viral loads.

3.4 Inclusion Criteria

3.4.1 Healthy Children

Before a child was enrolled in the study, the following characteristics were fulfilled:

i. Should be aged 3½ years.
ii. Auxiliary temperatures below ≤37.5^{o}C.
iii. Permanent residents of Kisumu or Nandi County at the time of the study,
iv. Written informed consent was obtained from parents or guardians before blood was drawn.

v. For children presenting with Burkitt lymphoma, blood sample was obtained before commencement of chemotherapy and these children had to be admitted at the JOOTRH.

3.4.2 Cancer Patients

i) They must be from Luo ethnicity

ii) Must be permanent residence of Kisumu County

iii) Must be eBL cases confirmed by paediatric oncologist

3.5 Exclusion Criteria

The following criteria was used for exclusion of children from study:

i. Presence of diseases that compromise immune responses such as HIV infection or severe malnutrition
ii. Evidence for etiology of fever, such as lower respiratory infection, bacterial or viral infection
iii. Anemic (haemoglobin (Hb) less than 5g/dL)

3.6 Ethical Considerations

Approval for this study was obtained from the KEMRI Ethical Review Committee. Written informed consent, in local languages of communications in Western Kenya (Dholuo, local language); Kiswahili (national language); Kalenjin (local language used in Nandi) as well as English (official language in Kenya) was obtained from parents or guardians of all study participants. The purpose of the study was explained to guardians or parents of study participants. Risks and benefits of enrolling them in to the study was also provided. Participants were also assured that the information they were giving was going to be treated with confidentiality. Further they were informed that the findings garnered from the study were important for prevention programs against eBL. Participants were also informed of their

right to withdraw from the study at any time and for any reason without fear of any negative penalty or loss of benefits. To minimize any adverse effect resulting from procedures during sample collections, a trained phlebotomist and a clinical officer attached to the project were responsible for all sample collection. All the samples collected were stripped of personal identity and coded using unique study identification numbers to protect study participants' privacy and only the principal investigator, Co-investigators and a few core members of staff had access to personal details of the study participants, which was kept under key and lock. (Appendix 4, 5 and 6 for study Ethical Approval, consent forms and sample collection forms)

3.7 Demographic Characteristics and Parasitological Examinations

During the enrolment of all study participants' characteristics such as gender and age were taken before a sample was drawn. The project clinical officer examined all the prospective study participants to determine if they met the inclusion criteria. clinical *P. falciparum* malaria diagnosis was determined by microscopic examination of Giemsa-stained, thick and thin blood smears. Study participants who tested positive for *P. falciparum* were treated by the medical staff attached to the project following the Kenya Ministry of Health guidelines in treatment of uncomplicated malaria.

3.8 Sample Size and Power Calculation

To achieve a statistical power of over 80% (β=0.80) and a critical limit of 95% (α = 0.05) for significance, a minimum of 24 study participants was sufficient for this study. However, to take care of some samples that may not have enough cells or those that may not stain properly and further to increase the study power, a total of 42 study participants were recruited as described below. This sample size was calculated using EBV viral loads as one of the measurement variables based on an earlier study done in the same area by Moormann and colleagues (Moormann *et al.*, 2005) that examined on differences on viral loads due to

malaria exposure. The sample size was derived from the following formulae for medical case-control studies for continuous antigen exposure (*Jay et al, 2006*).

$$n = (\frac{r+1}{r}) \frac{\sigma^2 (Z_\beta + Z_{\alpha/2})^2}{(d\text{ifference})^2}$$

Where:

Z_β = 80 (for study power of at least 80% which is acceptable for most biological studies)

Z_α =1.96 or 0.05 which stand for significance level

r =2 (one cases versus 2 controls since the incidence of eBL is rare. Nationally, the incidence of eBL is one for every 100,000 live births (Magrath *et al.,* 1990)

σ=6.0 (3 standard deviations below and above the mean to capture at least 75% of the population distribution)

Differences = 4.69, between mean of EBV viral loads in Kisumu and eBL (According to *Moormann et al., 2005*).

Hence minimum sample size was to be:

$$n = (\frac{2+1}{2}) \frac{(6)2(.8+0.98)^2}{(4.69)^2}$$

n = 1.5(5.18557) = 8 therefore, (8 case and 16 controls {8 from each study site})

n = 24

Although a minimum of 8 children were to be enrolled from each study site, but to take care of study participants who may have poor cell yields or non-respondent sat least 10% or more of the calculated sample size was added bringing the final sample size to 42.

3.9 Sample Collection and Processing

A volume of 2-5 ml of venous blood samples was collected by venipuncture by a qualified phlebotomist following sterile techniques to minimize contamination and harm to the study participant. The blood was collected in heparinized green top vacuitainer tubes (BD vacuitainer, UK) for PBMC isolation and purple top microtainer tubes (BD microtainer UK) for EBV viral load determination. Trained and qualified phlebotomist carried out venipuncture to minimize harm to the study participants. Furthermore, sterile butterfly blood collection needles were used and all sharps were stored in the appropriate biohazard sharp containers before they were disposed following the standard procedures of disposing biohazard material. The samples were transported to UMASS/KEMRI laboratories located at Kisian and processed within 3 hours of post-drawal. The peripheral blood mononuclear cells (PBMCs) were separated from the whole blood by Ficoll-hypaque (GE Healthcare, Sweden) density centrifugation method, where the buffy coat of PBMCs was used for subsequent flow cytometric analysis while plasma was used for serological investigations and erythrocytes discarded.

3.9.1 PBMC Isolation and Proliferation Assay

To achieve Objective 1, PBMCs were separated from sodium heparin anticoagulated whole blood by standard Ficoll-Hypaque density gradient centrifugation. In this procedure, the anti-

coagulated 2.5 ml of blood was layered carefully onto 5 ml of Ficoll-paque (GE Healthcare, Sweden) in a 15ml tube and then centrifuged at 1500 rpm for 30 minutes. Plasma was transferred into sterile tubes and stored at -80^{0}C while the PBMCs was collected using a sterile 10ml pipette and transferred into a 15 ml tube. The cells were washed by adding sterile 1× PBS, pH 7.0, to bring a total volume in the tube to 12 ml followed by centrifugation for 15 min. at 1200 rpm at room temperature. The supernatant was aspirated off, the pellet broken by gentle flicking of the tube and then washed again as described above and centrifuged for 10 min at 1200 rpm. The supernatant was aspirated, the pellets broken by gently flicking the tubes and the cells re-suspended in 1mL of sterile 1×PBS, pH 7.0. To count cells, 10µL of 0.4% Turk's solution was used to dilute the cells in a 1:1 ratio and placed in a haemocytometer and examined under a microscope. The yield was calculated using the formula (cell count in 1ml = (number of cells counted in 4 squares) ×2×10^4). Cells were subsequently washed at least twice in complete RPMI 1640 culture medium containing 1% Penicillin/Streptomycin, 1% L-glutamine, and 10% fetal bovine serum.

3.9.2 PBMC Stimulation and Culture Conditions

The PBMCs were counted and plated at a concentration of 5 × 10^6 cells per well at 37°C in a 5% CO2 humidified incubator. The cells were cultured in complete RPMI1640 media (10% human serum AB, 2mM L-glutamine, 20mM HEPES, and 100µg/ml per/strep). The cultures were then stimulated using the following conditions with the indicated final concentrations in parenthesis: overlapping EBNA-1 peptides (10µg/ml) and MSP-1 (10µg/ml) for 24 hours. Positive control wells were stimulated with 2µg /ml of SEB while the negative control wells had 1x PBS. Malaria recombinant protein (MSP-1) was used to test whether the modulation was specific to EBNA1 or was global. After 18 hours of incubation, the cells were plugged

(blocked) by addition of 6µg/mL Golgi StopTM (BD Biosciences, San Jose, California, USA) to trap the intracellular cytokines. The cells were then harvested and stained with a panel of fluorochrome-conjugated antibodies for flow cytometer analysis.

3.9.3 Immunofluorescence staining and flow cytometric analysis

Flow cytometric analysis was performed on BD Biosciences FACSCanto^{TM} instrument (BD Biosciences, San Jose, CA, USA) to determine the proportions of CD56 cells. To this end, PBMC (0.5×10^6 cells) were stained with flourochrome-conjugated monoclonal antibodies against CD3 (APC Cy7), CD19 (APC Cy7), CD14 (APC Cy7) and CD56 (PE Cy5). CD3, CD14 and CD19 markers were used in the dump channel. The Live/Dead® vivid violet molecular probe was used as a live marker. To investigate EBNA1-specific immune modulation by *P. falciparum*, the cells were stained with monoclonal antibodies against surface marker expression of CD107a (Brilliant Violet) and PD-1 (PE Cy7) and intracellular IFN-g and Granzyme B expression after *ex vivo* stimulation. At least 10,000 events were acquired using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA). Lymphocytes were gated by forward and sideward scatter and data acquired using the FACSDIVA software program BD Biosciences, San Jose, CA, USA). Data was analyzed using FlowJo software (FlowJo v9.5 Inc. USA) (see Appendix 7).

3.9.4 EBV Viral Loads Determination

To achieve objective 2, it was necessary that viral load burden be determined for study participants. EBV viral loads were determined using real time quantitative polymerase chain reaction (RTQ-PCR) from whole blood. Primers and probes were designed using Primer

Express software (version 2.0;PE Applied Biosystems), to detect a 70-bp region of the EBV BALF5 gene (Kimura *et al.,* 1999). EBV primers, 5'd CGGAAGCCCTCTGGACTTC 3' Forward Primer (FO); 5'd CCCTGTTTATCCGATGGAATG 3' Reverse Primer (RE). A standard curve was generated by extracting DNA from Namalwa BL cell lines (ATCCCR1-1432) that contains 2 integrated copies of EBV genome per cell. β-actin was run as positive PCR control using commercially available probes and primers (PE Applied Biosystems) to standardize the method. EBV viral load was normalized to the number of β-actin copies and then calculated on the basis of copies of EBV genome per microgram of DNA as previously described (Moormann *et al.,* 2005). (Appendix 11 for full protocol of EBV viral load determination).

3.10 Data Analysis

Non-parametric tests were used to analysis the data since the normality of the data was not known and neither did the data follow a specific distribution. Mann Whitney U test was used to analyze for differences between medians in parasitemia densities as well as frequencies of EBV specific NK cells and EBV viral loads. Kruskal-Wallis was used to investigate for differences between groups. To answer objective two, the relationship between median EBV viral loads and the median frequencies of EBNA1 specific NK cells was analyzed by linear regression test. All tests were two tailed with the critical limit being $p \leq 0.05$ for significance.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics of Study Participants

A total of 42 study participants were recruited for this study: Kisumu (n=16), Nandi (n=16) and eBL (n=10) that were above the calculated minimum sample size of 24 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 culture and stimulation. The highest median parasitemia density was observed among the Kisumu children with 3800 parasites per microliter of blood, consistent with children residing in a malaria holoendemic region (Snow *et al.*, 1998), followed by Nandi and eBL with 77.89 and 47.45 parasites per microliter, respectively (p=0.018). Although for the two study sites, purposeful efforts were made to randomly recruit equal number of study participants from each gender, for eBL more males than females presented with the cancer as previously reported in Africa (Aka *et al*., 2012; Stefan, 2015) (Table 4.1).

Table 4.1 Summary of Study Participants' Demographics and Characteristics

Study Site	***P. falciparum* Exposure**	**Sample size (n)**	**n (%)**		**Gender**	**Median parasitemia density**
			Male		Female	
JOORTH	Holoendemic	10	7 (70%)		3 (30%)	44ul
Kisumu	Holoendemic	16	8 (50%)		8 (50%)	3800ul
Nandi	Hypoendemic	16	8 (50%)		8 (50%)	78 ul
Total		42	23 (55%)		19 (45%)	
p value						**0.018**

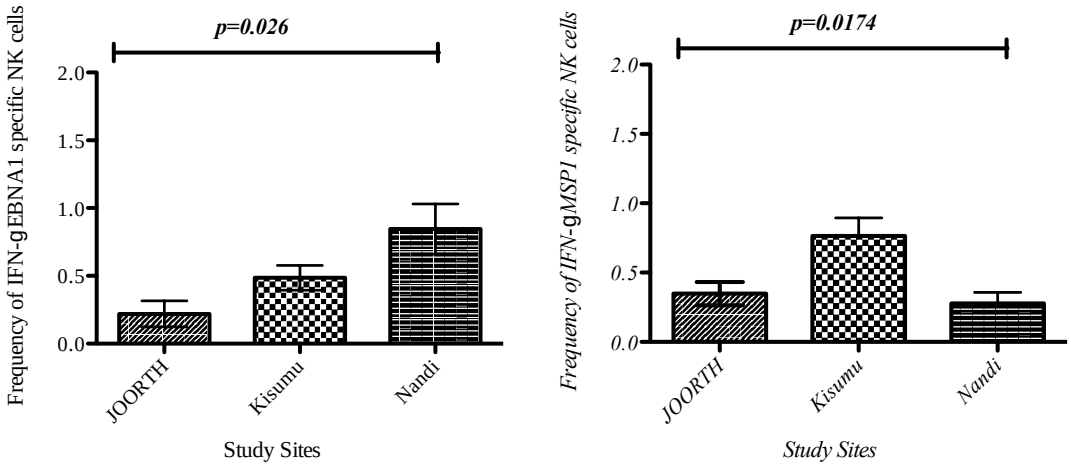
Data presented as summary of demographic characteristics of study participants and parasitemia (median parasitemia) density per study site. Differences in parasitemia densities were analyzed by Kruskall-Walis test. Significant values ($p<0.05$) are in bold. Gender represented as raw scores with percentages in parenthesis

4.2 The Effect of *P. falciparum* Transmission of EBNA1-specific NK Cell Function Across study Populations

To investigate the modulatory effect of *P. falciparum* on EBV specific NK cell function, the frequencies of EBNA1 specific NK cell responses were measured by flow cytometry. These responses were compared to those from a malaria recombinant protein (MSP-1). This study observed significant differences in the frequencies of both EBV and malaria specific NK cell according to malaria exposure.

4.2.1Frequencies of IFN-γ Producing NK Cells Across Study Groups

It was observed that children from Nandi had a higher median frequency of EBNA1 specific IFN-γ producing NK cells (0.6990 percent) response compared to other study groups (p=0.0261). The least response was observed in children presenting with eBL who recorded 0.0800 percent followed by those from Kisumu (0.3110 percent). However, for MSP-1, the highest median frequency of IFN-γ producing NK cells were observed in children from Kisumu (0.6590) followed by the eBL (0.3480) and lastly the Nandi (0.2300) children. This difference was statistically significant (p=0.0174) (Figure 4.1).



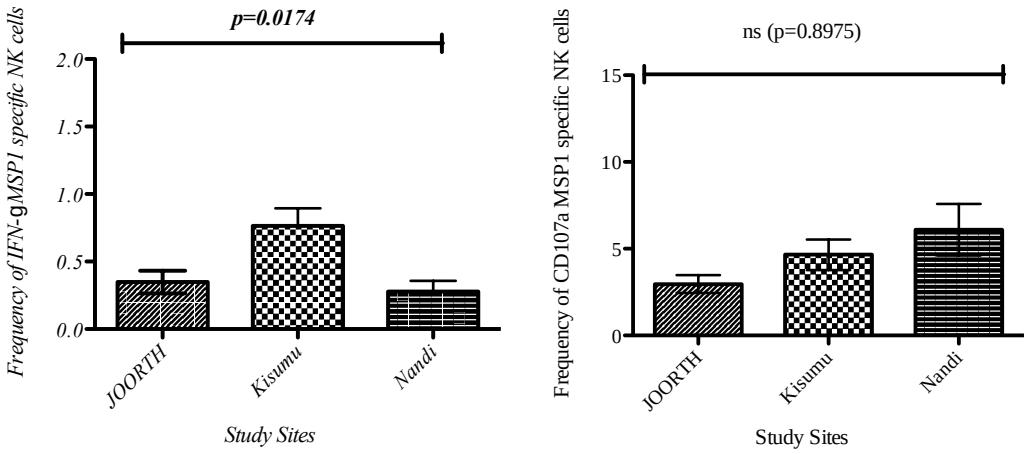
a) EBNA-1 specific NK cell response **b) MSP-1 specific NK cell response**

Figure 4.1 Median Frequencies of IFN-γ Producing NK cells across study groups

Data presented show median frequencies of producing IFN-γ NK cells against EBNA1 (panel a) and MSP-1 (panel b). The differences in median were analyzed by Kruskall-Wallis test. It was observed that children presenting with eBL had the least median NK cell specific response to EBNA1 followed by Kisumu and lastly Nandi (p=0.0260). On the other hand, children living in malaria holoendemic region had robust NK cell MSP-1 specific IFN-γ response compared to other study groups (p=0.0174).

4.2.2 Frequencies of CD107a Producing NK Cells Across Study Groups

To determine the effect of *P. falciparum* transmission on degranulation capacity of NK cell, the intracellular level of lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) was assayed by flow cytometry. Analyzing the median frequencies between different groups, it was observed that children from Nandi region recorded the highest median frequencies of CD107a EBNA1-specific NK cell response (4.68 %) compared to other groups (p=0.0291). The lowest response was recorded in children living in Kisumu (2.41%) followed by eBL (2.56%) children. However, for MSP-1, although the Nandi study group recorded the highest median frequency (0.678%) of MSP-1-specific CD107a producing NK cells, this difference was not significant across study groups (p=0.8975) (Figure 4.2).



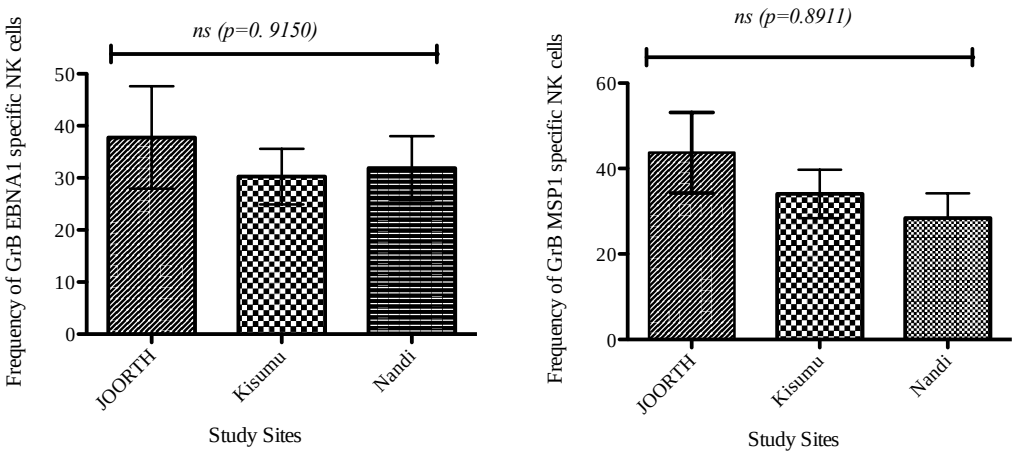
a) CD107a EBNA1-specific NK cells **b)CD107a MSP-1-specific NK cells**

Figure 4.2 Frequencies of CD107a Producing NK cells across study groups

The presented data are median frequencies of producing CD107a specific NK cells producing against EBNA1 (panel a) and MSP-1 (panel b). The differences in median were analyzed by Kruskall-Wallis test. It was observed that children presenting with eBL had the least CD107a EBNA1-specific NK cell response followed by Kisumu and lastly Nandi (p=0.0291). On the other hand, there were no significant differences in median MSP-1 specific CD107a NK cell response across the study groups (p= 0.8975)

4.2.3 Frequencies of GrB Producing NK Cells Across Study Groups

To investigate the ability of NK cells to induce pro-apoptotic activity in target cells as well as the cytotoxic activity, the frequencies of Granzyme B (GrB) specific to EBNA1 were measured by flow cytometry. It was observed that there were no significance differences in the median frequencies of GrB EBNA-1 specific NK cells responses in all the three study groups (p=0.9150). The eBL study group recorded the highest median frequency of 29%, followed by Nandi (25%) and lastly the Kisumu children with a frequency of 23%. On the other hand, although children presenting with eBL showed a higher GrB specific MSP-1 median frequency response (36%), followed by Kisumu (27%) and lastly Nandi (24%), these differences were comparable (p= 0.8911) (Figure 4.3).



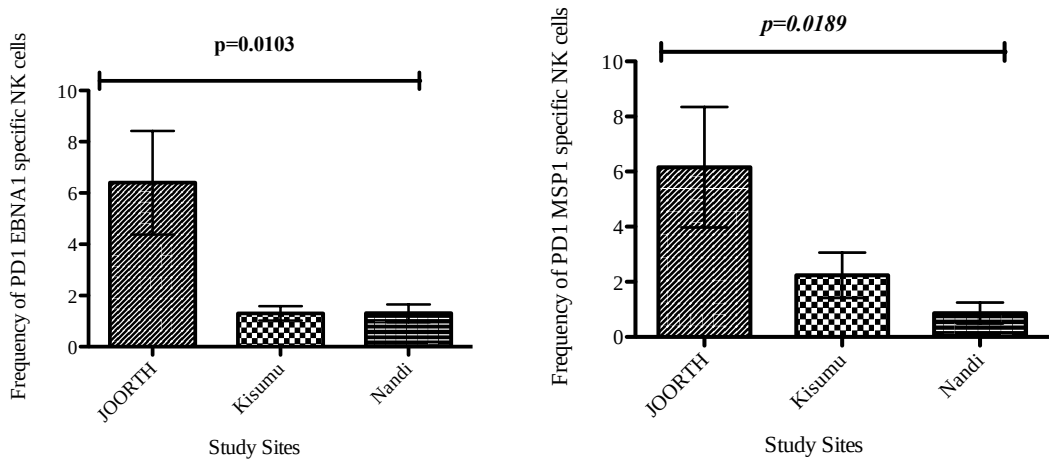
a) GrB-EBNA1 specific NK cells **b) GrB-MSP1 specific NK cells**

Figure 4.3 Frequencies of GrB Producing NK cells across study groups
The data show median frequencies of GrB producing NK cells response against EBNA1 (panel a) and MSP-1 (panel b) after culture for 24 hours. The differences in median were analyzed by Kruskall-Wallis test. It was observed that, there were no significant differences across the three study groups in response to either EBNA1 (p=0.9150) or MSP-1 (p=0.8911).

4.2.4 Frequencies of PD-1 Producing NK Cells Across Study Groups

To investigate the effect of malaria transmission on induction of immune dysregulation or exhaustion of NK cells, the levels of programmed cell death protein 1 (PD-1) was evaluated after *ex vivo* stimulation with EBNA1 and MSP-1 antigen in the three study populations. Children presenting with eBL who are also residents of a malaria holoendemic region, had significantly higher median of MSP-1specific PD-1 NK cell frequency (3.7%) compared to other study groups (p=0.0189), followed by Kisumu children (0.7%) who are also residents from a malaria holoendemic region. The least response was observed in children from Nandi (0.4%), which is a malaria hypoendemic region. On the other hand (panel b), congruently, children presenting with eBL had significantly higher median frequency of PD-1 EBNA-1specific NK cell responses (4.9%) (p=0.0103), followed by Kisumu children (1.89%). Again

the least response was observed in children from Nandi (1.10%) (Figure 4.4).



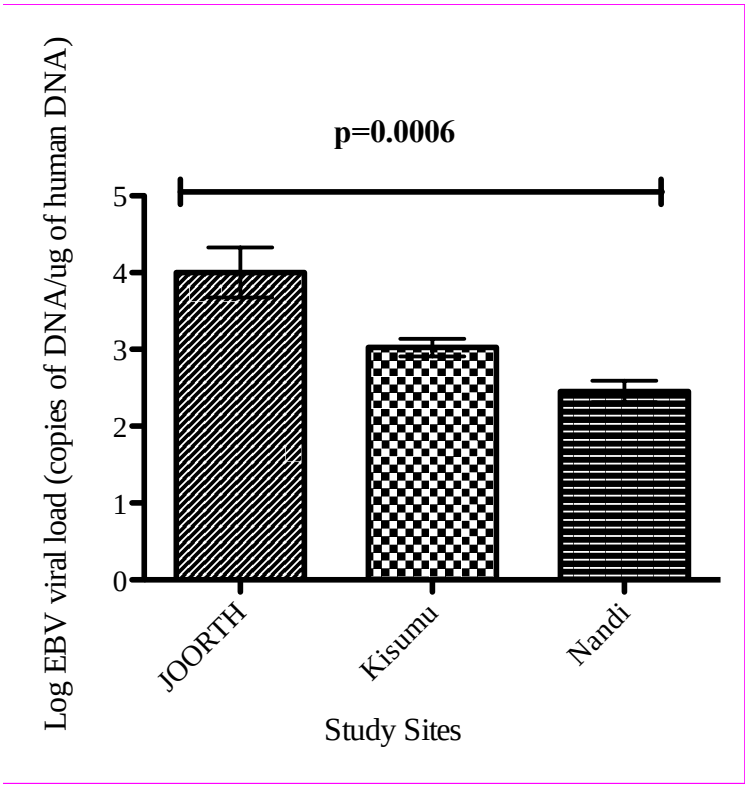
a) PD1-EBNA1 specific NK cells **b) PD1-MSP-1 specific NK cells**

Figure: 4.4 Frequencies of PD-1 Producing NK cells across study groups
Frequencies of PD-1 specific NK cells response against EBNA1 (panel a) and MSP-1 (panel a) were evaluated across the three study groups by flow cytometry. Differences in median frequencies between study groups were analyzed by Kruskall-Wallis test. It was observed that Nandi Children recorded the least median PD-1 MSP-1 specific NK cell response compared to other groups (p=0.0189 (**Panel b**). The highest MSP-1 specific PD-1 response was recorded in the eBL children, followed by Kisumu children, all who are residents of a malaria holoendemic region. Similarly, children presenting with eBL had the highest PD1 EBNA-1 specific NK cell response followed by Kisumu and lastly Nandi. This difference was statistically significant, **p=0.0103** (panel a).

4.3The Effect of *P falciparum* Malaria Transmission on EBV Viral Loads

To investigate the effect of persistent malaria transmission on EBV viral burden as well as NK cell function, cellular EBV viral copies were evaluated using RTQ-PCR in the three study groups. It was observed that children presenting with eBL had markedly elevated viral loads (median, 13054 EBV copies/μg human DNA) followed by those from Kisumu (median, 380 copies /μg human DNA) while those from malaria hypoendemic region recorded the lowest viral burden (median, 109 EBV copies/ μg human DNA), p=0.0006 (Figure 4.5). Evaluating intergroup differences showed that eBL study group was higher than Kisumu

(p=0.0025) as well as Nandi (p=0.0001). Further the Kisumu children were statistically different in viral load burden compared to Nandi (p=0.012).



(EBV, Epstein Barr Virus; DNA, Deoxyribonucleic Acid; eBL, endemic Burkitt lymphoma)

Figure 4.5:EBV Viral Burden Across Study Populations

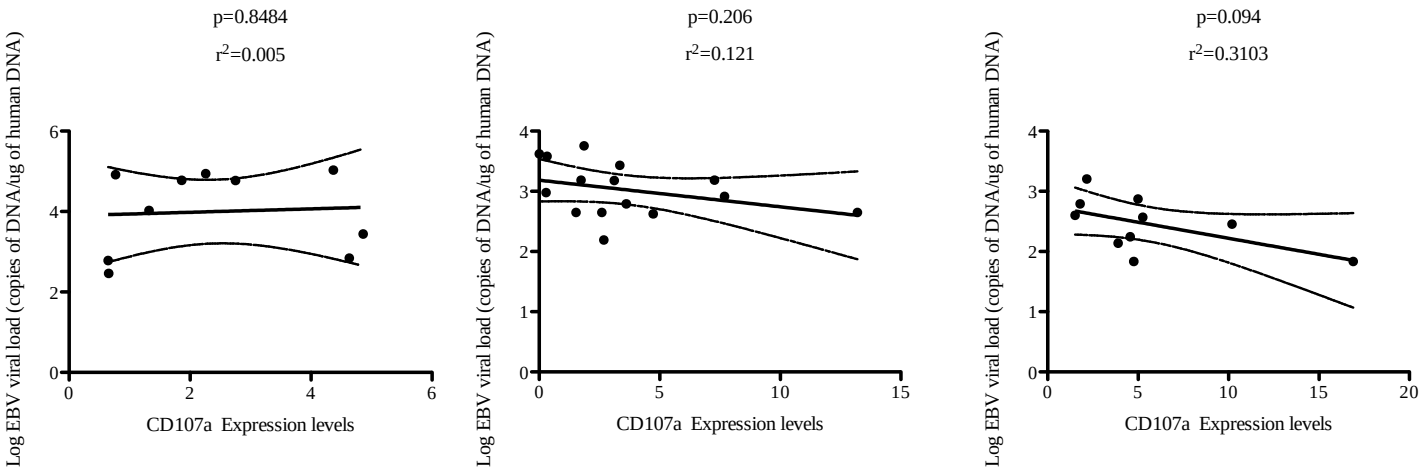
Cellular EBV viral burden in the three study populations was evaluated by RTQ-PCR. Values obtained are plotted to log 10 since the eBL viral burden was highly elevated compared to other groups for presentation purposes, otherwise raw scores were used for Kruskall-Walis test. Children presenting with eBL highest median viral loads (median 1354) EBV copies/µg human DNA), followed by Kisumu children (380 copies /µg human DNA) and the least burden was recorded in the Nandi children (109 EBV copies/ µg human DNA). The differences in median viral burden were highly significant (p=0.0006).

4.3: The Relationship Between EBV Viral Loads and NK Cell Function

To investigate the relationship between malaria transmission pressures and NK cell function, the viral loads generated above (see section 4.5) were analyzed against the eBNA1 specific NK cell frequencies for IFN-g, CD107a, GrB and PD-1 expression by linear regression analysis.

4.3.1 The Relationship Between EBV Viral Loads and EBNA1 specific CD107a NK Response

The association between EBV viral loads and the degree of NK cell CD107a degranulation in response to ex vivo stimulation with EBNA1 was done by linear regression analysis. It was observed that in all the three study populations, there was no statistical relationship between high viral loads and EBNA1 specific NK cell degranulation ($p>0.0050$). However, it was observed that in both Nandi and Kisumu study group, a trend was evident where higher viral loads had a positive regression with low CD107a levels as opposed to eBL group. This linear regression trend reduced with exposure with exposure to malaria and was least observed in the eBL study group (Figure. 4.6).



a) eBL-JOORTH b) Kisumu c) Nandi

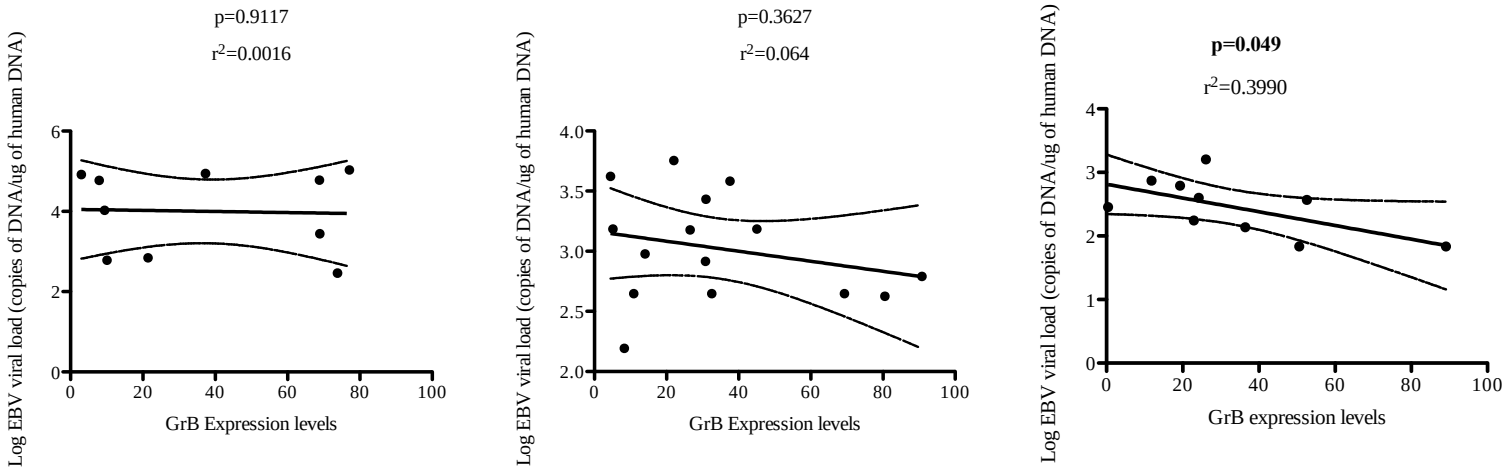
(EBNA-1, Epstein Barr Nucleotide antigen 1; CD107a, Cell of Differentiation 107a; NK, Natural Killer Cell; EBV, Epstein Barr Virus; JOOTRH, Jaramogi Oginga Odinga Teaching and Referral Hospital; eBL, endemic Burkitt Lymphoma)

Figure 4.6 Regression analysis of the effect of EBV viral loads on EBNA1 specific NK cell CD107a degranulation

The effect of EBV viral loads on EBNA1 specific NK cell CD107a degranulation was investigated by linear regression analysis of EBV viral loads on frequency of EBNA1 specific CD107a NK cells after *ex vivo* stimulation and flow cytometry. Although a trend was observed in both Kisumu and Nandi study groups, where low viral loads were associated with a higher degree of degranulation, this was not statistically significant. However, the least regression coefficient was observed in eBL study population who had high viral load.

4.3.2 The Relationship Between EBV Viral Load and EBNA1 specific GrB NK Response Across Study Population

To investigate the association between the levels of cytotoxic granules formed in NK cells after EBNA1 stimulation and EBV viral loads, a linear regression was performed. It was observed that in the Nandi study group, a significantly relationship existed between viral burden and GrB levels ($p=0.0490$). However there was no significant relationship between viral loads and GrB levels in children from malaria holoendemic region. The coefficient of determination was lowest in children presenting with eBL followed by Kisumu study participants (Figure 4.7).



a) eBL-JOORTH b) Kisumu c) Nandi

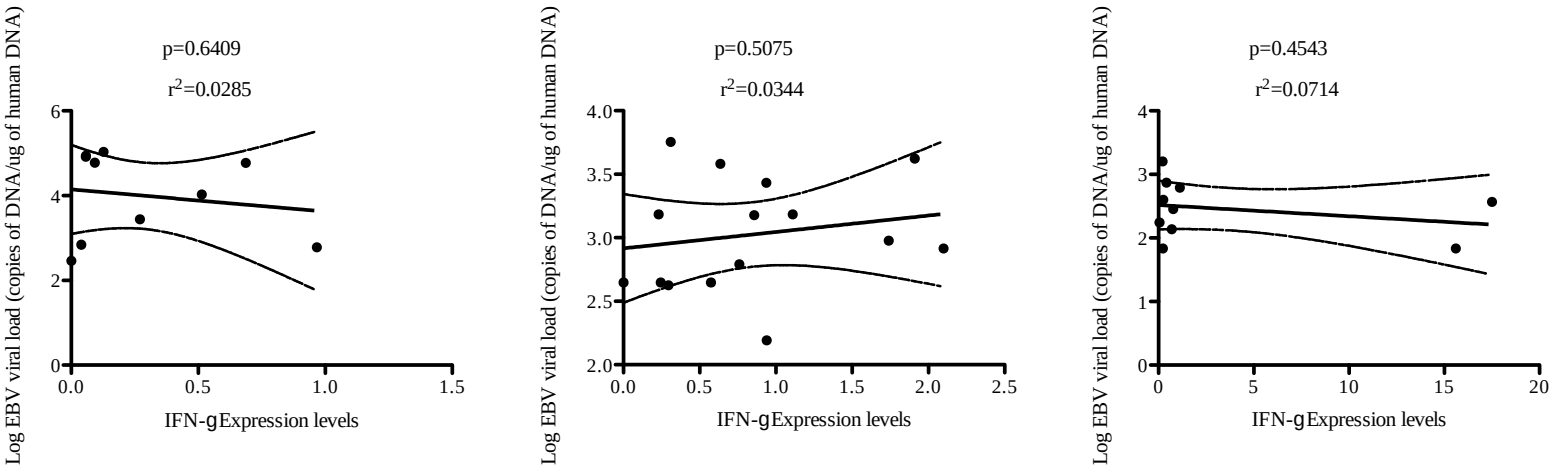
(EBNA-1, Epstein Barr Nucleotide antigen 1; GrB, Granzyme B; NK, Natural Killer Cell; EBV, Epstein Barr Virus; JOOTRH, Jaramogi Oginga Odinga Teaching and Referral Hospital; eBL, endemic Burkitt Lymphoma;)

Figure 4.7: Regression analysis of the effect of EBV viral loads on EBNA1 specific NK cell GrB response levels

To investigate the association between EBV viral loads and EBNA1 specific NK cell GrB response levels, the viral burden was analyzed by linear regression analysis against the frequency of EBNA1 specific GrB response levels after *ex vivo* stimulation and flow cytometry. It was observed that a significantly relationship existed between low viral loads and levels of GrB in Nandi study population **(p=0.049**). However in the children from malaria holoendemic region, although this relationship was not significant.

4.3.3 The Relationship Between EBV Viral Loads and EBNA1 specific IFN-γ NK Response Across Study Population

To investigate the relationship between viral load burden and EBV viral control, regression analysis between EBV burden and the frequencies of EBNA1 specific IFN- γ NK producing cells in the three study populations. In all the three study populations, it was observed that that there was no association between the frequencies of EBNA1 specific INF-γ producing NK cells and EBV viral loads ($p>0.05$). However, it was observed that there was higher coefficient of regression in Nandi study groups compared to Kisumu and eBL group (Figure. 4.8).



a) eBL-JOORTH b) Kisumu c) Nandi

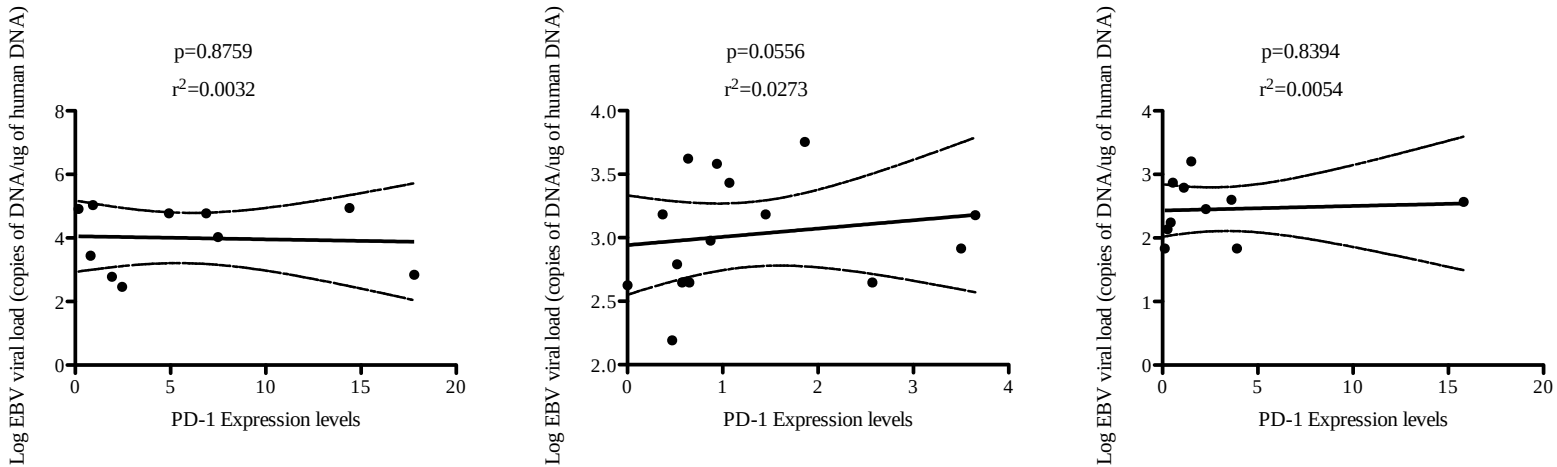
(EBNA-1, Epstein Barr Nucleotide antigen 1; INF –γ, Interferon gamma; NK, Natural Killer Cell; EBV, Epstein Barr Virus; JOOTRH, Jaramogi Oginga Odinga Teaching and Referral Hospital; eBL, endemic Burkitt Lymphoma;)

Figure 4.8 Regression analysis of the effect of EBV viral loads on EBNA1 specific NK cell IFN-γ response levels

The association between IFN-γ and EBV viral loads in response to EBNA1 in NK cells was analyzed by linear regression analysis. It was observed that these two events were mutually independent despite the children from malaria holoendemic regions having high viral loads concomitant with low EBNA1 specific IFN-γ response.

4.3.4 The Relationship Between EBV Viral Loads and EBNA1 specific PD-1 NK Response Across Study Population

To investigate the relationship between EBV viral loads and the level of exhaustion and/or activation on NK cells linear regression analysis was performed to show the relationship between PD-1 expression levels and EBV viral loads. It was observed that these two events were not dependent on each other ($p>0.05$) in all the three study groups under investigation. However it was observed that a borderline relationship existed between PD-1 expression and EBV viral loads in Kisumu children where an increase in EBV viral loads was associated with high PD-1 expression (Figure. 4.9).



a) eBL-JOOTRH b) Kisumu c) Nandi

(EBNA-1, Epstein Barr Nucleotide antigen 1; PD-1, Programmed Cell Death; NK, Natural Killer Cell; EBV, Epstein Barr Virus; JOOTRH, Jaramogi Oginga Odinga Teaching and Referral Hospital; eBL, endemic Burkitt Lymphoma)

Figure 4.9 Regression analysis of the association of EBV viral loads and EBNA1 specific NK cell PD-1 response levels

The association between EBNA1 specific PD-1 expression and EBV viral loads in response to EBNA1 in NK cells was analyzed by linear regression analysis. It was observed that these two events were mutually independent in both eBL and Nandi study groups. However in the Kisumu children, it was observed that high viral loads was concomitantly associated with high EBNA1 specific PD-1 response ($p=0.05$, $r^2=002$).

CHAPTER FIVE

DISCUSSION

This study sought to investigate the association of *P. falciparum* malaria transmission dynamics on EBV specific NK cell function as a risk factor in the etiology of endemic Burkitt lymphoma. The study investigated the relationship between EBV viral loads and EBNA1 specific NK cell function. EBNA1 was chosen as the antigen of focus because it is the only EBV antigen expressed by all EBV-associated tumors as well as EBV-infected cells during latency (Leight and Sugden, 2000, 1) and hence an important biomarker towards the etiology of eBL as a loss of EBNA1-IFN-γ specific immunity has been associated with increase in EBV associated tumors in Post-transplant lymphoproliferative disorder (PTLD) (Gulley and Tang, 2010). As previously shown (Moormann *et al*., 2005), this study found out that children living in malaria holoendemic regions had high EBV viral burden compared to those from malaria hypoendemic regions. Further this study shows that children living in regions with chronic and persistent malaria transmission have EBV specific aberrant NK cell function as compared to those from malaria hypoendemic regions. Lastly the current study demonstrates that high EBV viral loads are associated with high PD-1 expression on NK cells modulating their function.

5.1 Demographic Characteristics of Study Participants

Healthy children from Kisumu presented with high parasite densities of *P. falciparum* as opposed to those living in malaria hypoendemic region, which is consistent with residence in a malaria holoendemic region *(*Snow *et al.*, 1998*;* Ndenga *et al.*, 2006*;* John *et al.*, 2005*).* People living in malaria holoendemic regions have high parasitemia throughout the year due

to favorable weather patterns that ensure availability of the vector for malaria transmission. Kisumu region lying at the equator experiences and near Lake Victoria experiences humid weather ensuing high productivity of the vector. Thus both health control children as well eBL patients are likely to present with high parasitemia. Although children presenting with eBL were from, malaria holoendemic regions, they presented with low parasitemia densities. This low parasitemia could be due to the fact that most of them are usually seen by other clinicians and usually treated for other diseases prior to being admitted at the referral centre (Mulama *et al.*, 2013). Nandi study group had low parasitemia since they live in the highland, which are much colder and unfavorable for Anopheles mosquito's survival. This study shows that the incidence of eBL is higher in males than in females. The higher incidence of eBL in males remains uninvestigated, but could be possibly due to genetic and other male related traits. Published reports from elsewhere have shown discrepancies in factors such as gender, genetic makeup, environment and nutritional status ("Genetic Susceptibility to Cancer. ICRP Publication 79. Approved by the Commission in May 1997. International Commission on Radiological Protection" 1998; Dorak and Karpuzoglu, 2012). A report by Dorak and Karpuzoglu shows that males are more likely to present with cancers of hematological origin than females (Dorak and Karpuzoglu, 2012) which could be due to hormonal changes and other X-linked characteristics that remains to be elucidated.

5.2 The Effect of *P. falciparum* Transmissions Pressure on NK Cell Function

The current study has shown that there are differences in EBV specific NK cell function based on past malaria exposure, It was observed that malaria modulates the quality of response to EBNA1 where children from malaria hypoendemic region had higher IFN-γ frequencies to EBNA1 while maintaining low response to MSP-1. This modulation is

selectively specific to EBV antigens and not malaria antigens explaining the specificity to EBNA-1 and not MSP-1 antigen. This selective immunosuppression can explain the high incidence of eBL in malaria holoendemic regions. The children from malaria holoendemic region exhibited low EBNA1 specific NK response while maintaining high MSP-1 response consistent with past studies (Dent *et al.*, 2009). The children from malaria holoendemic region have higher MSP-1specific IFN-γ responses as a function of memory due to high malaria transmission (Chelimo *et al*., 2011). Constant exposure to *P. falciparum* leads to chronic stimulation of immune cells hence the higher MSP-1 specific responses as compared to EBNA-1 antigens. The low EBNA-1 specific responses evident in Kisumu study participants could be as a result of by-stander deletion of EBV specific cells due to malaria exposure (Xu *et al.* 2002). Xu and others have reported such phenomenon where cytolytic CD4+ T cells to EBV antigens have been known to inhibit proliferation of B cells that have been transformed by EBV (Xu et al. 2002). The low IFN-γ responses to EBNA-1 in malaria holoendemic children could be due to progressive loss of EBV specific immunity as previously reported (Angelosanto *et al*., 2012) due to persistent high EBV viral loads (Moormann *et al*., 2005). On the other hand, children from malaria hypoendemic region do not have sufficient memory to malaria and hence the low MSP-1 specific immune response. However, they do have high EBNA1 specific IFN-γ NK cell response as a function of memory (Piriou *et al*., 2009) and further they are free from high malaria transmission which modulates immunity to EBV.

This study investigated the effect of *P. falciparum* pressure on CD107a degranulation capacity. CD107a is an excellent marker of cellular activity (Alter, Malenfant, and Altfeld 2004) and was thus used to assay for NK capacity of intracellular degranulation. The

intracellular level of CD107a EBNA1-specific NK cell was assayed by flow cytometry. It was observed that children from malaria hypoendemic region (Nandi study group) had an increased EBNA1-specific NK cell degranulation activity or capacity compared to holoendemic and eBL children. This reduced degranulation capacity in malaria holoendemic regions shows that children living in this region have impaired immune function that can be a panacea to increased risk of developing eBL. The deregulation appears to target EBV antigens. However this was not the case for MSP-1 specific CD107a producing NK cells since the differences were not significant in malaria holoendemic, hypoendemic and in eBL children which seems to be consistent with previous studies that investigated the functional activities of CD107a in T cell immunity in malaria infections (Bijker *et al*., 2014).

The study has also shown that there was no difference in pro-apoptotic activity in target cells as well as the cytotoxic activity as measured by the frequencies of Granzyme B (GrB) to EBNA1 in the three study populations. Moreover children presenting with eBL showed a higher GrB specific MSP-1 response, the frequencies differences were comparable across the study groups. Granzyme can either exist as cell associated or soluble granzymes. Studies that have examined the circulating soluble granzymes have found higher concentrations in individuals presenting with clinical malaria unlike healthy ones (Hermsen *et al*., 2003). Our study did not measure released soluble granzymes *ex vivo* after EBNA1 or MSP-1 stimulation. This lack of differences may be due to the important role played by circulating granzymes over cell associated ones. Further, granzyme B release is concomitant with perforin during the cytotoxic activity (Salti *et al.* 2011) and thus should have been investigated in the current study. Although this is a weakness in the current study, it does not in way negate the fact that there was a trend of children from malaria holoendemic region

exhibiting high levels of granzymes B in response to EBNA1 and MSP-1 compared to those from the hypoendemic region. This finding is consistent with low levels of degranulation and increased apoptotic activity in NK cells of children residing in malaria holoendemic regions which possibly leads to increased risk to eBL. It has been shown that GrB as a cytotoxic marker is evident in mature and antigen experienced cells (Bijker *et al.* 2014), which are lacking in malaria holoendemic region due to selective deletion.

To compensate for the weakness where the levels of released granzyme B and perforin were not investigated, this study instead measured the levels of NK cell associated programmed cell death protein-1 (PD-1) in response to EBNA1 and MSP-1 stimulation. It is known that PD-1 can either act as a marker of exhaustion or activation (Hofmeyer, Jeon, and Zang 2011, 1). It was observed that children presenting with eBL who were residents of malaria holoendemic region had significantly higher MSP-1 and EBNA-1 PD-1 specific NK cell activity than those from a malaria hypoendemic region (Nandi County). This means that these children have most of their antigen specific cells marked for deletion thus leaving them immunologically naked. Thus elevated PD-1 expression is suggestive of the adverse effects of continuous malaria infection as well as high viral loads, which is a key mechanism on NK cells activation. This increased exhaustion/activation of NK cells marks them towards apoptosis, leading to poor signaling of cytotoxic T lymphocytes (Hofmeyer *et al.*, 2011) leading to possible eBL lymphomagenesis.

5.3.1 The Association Between *P falciparum* Malaria Transmission and EBV viral loads

The present study has shown that residence in malaria holoendemic region is associated with high EBV loads. This finding is consistent with previous studies that have associated the chronic immune stimulation and dysregulation with high EBV viral loads (Bagni and Whitby 2012; Moormann *et al*., 2009). Chronic over stimulation of the immune cells by malaria parasite leads to increased number of B cells which are the reservoirs of EBV leading to increased viral burden. Further, children in malaria holoendemic region have selective deletion of immunosurveillance cells such as NK cells and T cells leading to aberrant proliferation of EBV infected B cells. It is the combined activity of these activities that leads to increased viral burden. African children are usually infected with EBV early in life (Piriou *et al.,* 2012) and those from malaria holoendemic regions show high viral loads which have been indicated as one of the biomarkers towards eBL development. The high EBV viral loads observed in the Kisumu children could possibly be due to the lytic activation of chronic *P falciparum* infection which increases the number of EBV infected B cells (Asito *et al*., 2008) as well as increasing the replicative activity of EBV *(*Donati *et al.,* 2004) and hence high viral loads. EBV infected cells due to cmy-c translocation have uncontrolled replicative potential augmenting the viral burden (Chêne *et al.,* 2007). The Nandi children are exposed to low malaria transmission burden and hence are not likely to have high viral loads. These high viral loads are responsible for high PD-1 expression due to immune overstimulation and activation by both *P. falciparum* as well as EBV. These findings are consistent with past published studies from Kenya *(*Moormann *et al.,* 2005; Piriou *et al.,* 2012*)* as well as from elsewhere (Aka *et al*., 2012; Mbulaiteye, 2013). It could be this immune overstimulation that deregulates NK cells leading to low production of IFN-γ, degranulation as well as GrB expression as seem in the children from malaria holoendemic region. These results support the hypothesis that repeated malaria infections in very young children modulate the

persistence of EBV and increase the risk for the development of eBL due to immune dysregulation (Asito *et al.,* 2008; Moormann *et al*., 2009; Snider *et al.,* 2012). These previous studies have shown that there is both T and B cell immune modulation in children living in malaria holoendemic region who have abrogated EBV control increasing their risk of eBL lymphomagenesis.

5.3.2 The Relationship Between EBV Viral Loads and NK Cell Function

To investigate the relationship between EBV viral loads and NK cell activity in response to EBNA1 and MSP-1 stimulation, linear regression was performed between viral burden and the frequencies of EBNA-1 specific NK cells in each of the three study populations. This study observed that repeated malaria infections in young children were associated with NK cell non-responsiveness with respect to EBNA1 stimulation. Earlier studies had suggested that chronic malaria modulate the persistence of EBV and increase the risk for the development of eBL (Chattopadhyay *et al.,* 2013; Njie *et al.,* 2009; Williams *et al*., 2005).

High viral loads in Simian Immunodeficiency Virus (SIV) infected macaques studies have been associated with NK cell dysfunction (Albrecht *et al*., 2014) which ultimately leads to AIDS-like syndrome seen in humans infected with HIV. In human HIV infections, high frequencies of polyfunctional NK cells have been associated with slower progression towards AIDS (Ahmad *et al.,* 2014). Further clinical studies have associated rapid progression to AIDS with reduced NK cell activity and a decrease in NK cell numbers (Bruunsgaard *et al*., 1997). The effect of high HIV viral loads have not only been observed in NK cells *per se* but in other cells of the immune system (Fu *et al.,* 2015). The same observation have been documented in Kenyan studies where high EBV viral loads have been associated with

dysregulated immune responses(Moormann *et al*., 2007; Moormann *et al*., 2009; Moormann, Snider, and Chelimo, 2011). Compounded with holoendemic exposure, the immune system of children living in malaria holoendemic regions is selectively modulated to EBV antigens (Snider *et al*., 2012). Taken together, the above data shows that holoendemic malaria and associated high EBV viral loads are associated with dysfunctional NK cell activity, which may be a panacea to eBL oncogenesis.

CHAPTER SIX

SUMMARY OF STUDY FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Study Findings

This study shows that children living in regions with chronic and persistent malaria transmission have EBV specific aberrant NK cell function as compared to those from malaria hypoendemic regions. It was observed that children from malaria hypoendemic region that is Nandi had an increased EBNA1-specific NK cell degranulation activity or capacity of compared to holoendemic and eBL children.

It also found out that children living in malaria holoendemic regions had high EBV viral burden compared to those from malaria hypoendemic regions. Thus the current study demonstrates that high EBV viral loads are associated with high PD-1 expression on NK cells modulating their function. It was observed that the incidence of eBL is higher in males than in females. Taken together, the above data shows that holoendemic malaria and associated high EBV viral loads are associated with dysfunctional NK cell activity, which may be a panacea to eBL oncogenesis. What is not clear from the above studies is the exact mechanisms that malaria and EBV viral loads lead to c-myc translocation, another molecular event in eBL development.

6.2 Conclusions

1. Holoendemic *P. falciparum* malaria transmission imparts selectively and negatively on NK cell function.

2. Holoendemic *P. falciparum* exposure is associated with high viral loads and dysfunctional NK cell activity

6.3 Recommendations From The Current Study

1. There is need to control and reduce the exposure of children to malaria infections as persistent malaria imparts negatively on the cells of the immune system.

2. There is need to monitor the EBV viral burden especially in children living in malaria holoendemic regions who may be at risk of developing eBL.

6.4 Suggestions For Further Studies

1. There are is need to progressively follow up immune development in children living in malaria holoendemic region to categorically identify the kinetics in NK cell

2. There is need for an in-depth study on immuneprofiling and characterization of NK cells in children living in malaria holoendemic regions.

3. Children exposed to malaria who are likely to be at risk of developing eBL should be treated with anti-viral agents against EBV, to control viral load surge hence reducing the likelihood of developing this cancer.

4. There is urgent need for development of EBV vaccine and other immunotherapeutic agents to protect children and post-transplant patients who may develop EBV associated lymphomas.

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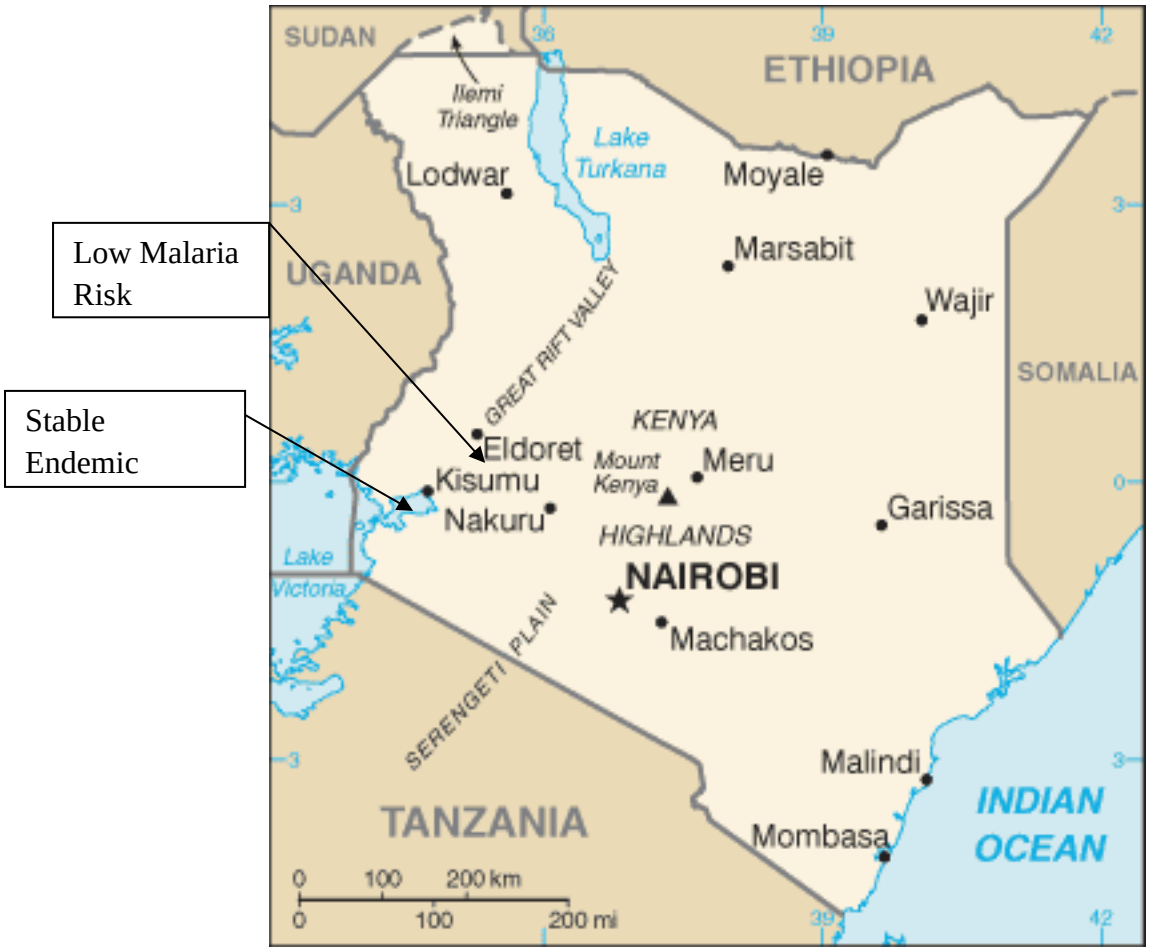
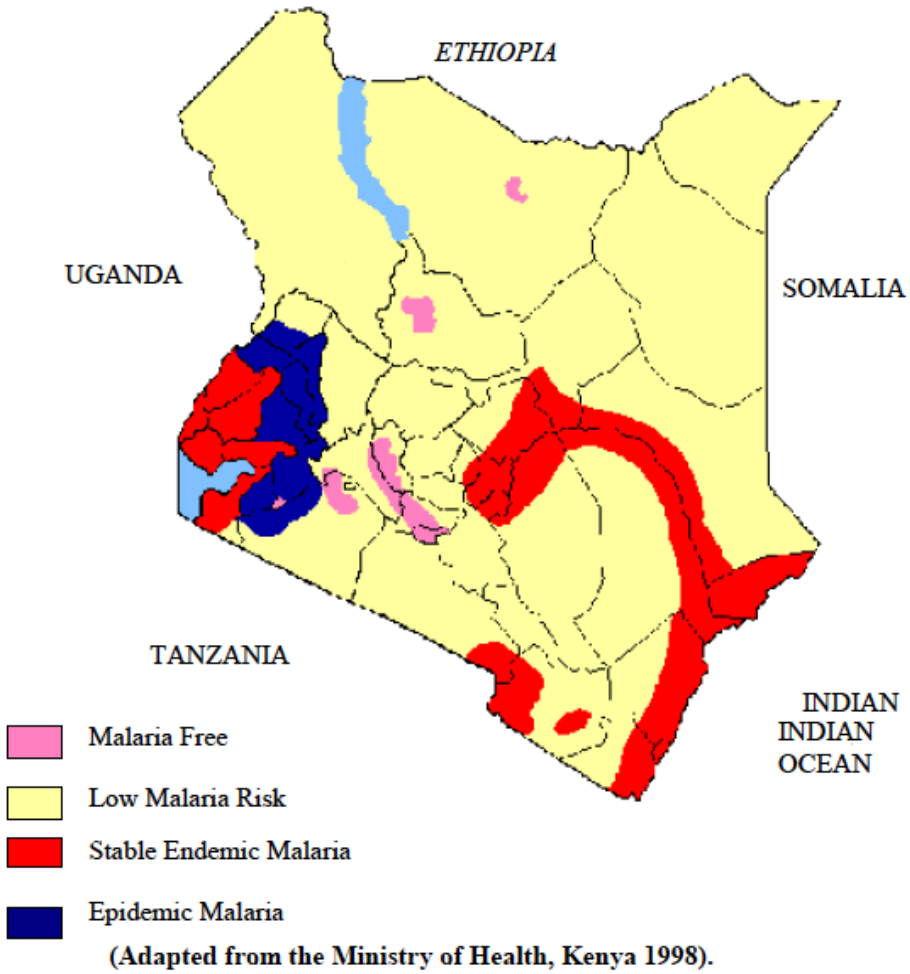
APPENDICES

Appendix 1: A map of Kenya showing the Study Sites



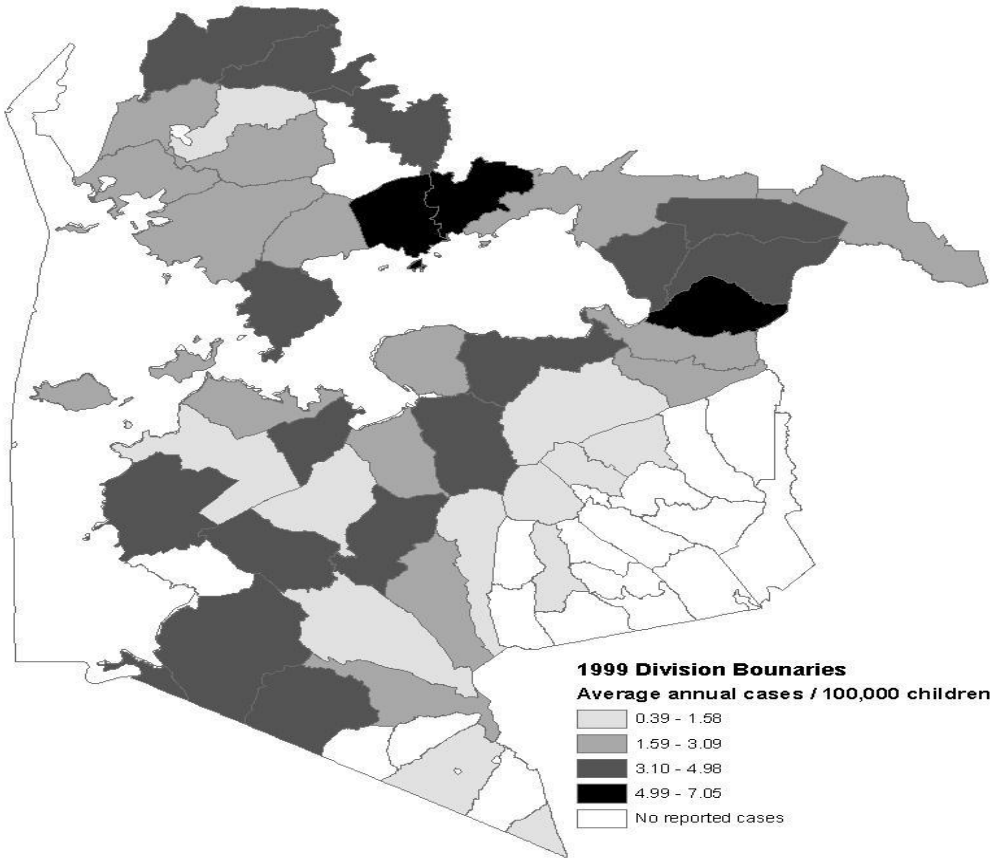
Kenya map showing the two study sites where samples are to be drawn from. Downloaded courtesy of the National Geographic Maps (http://maps.nationalgeographic.com/maps)

Appendix 2: Malaria Endemicity in Kenya



(Adapted from the Ministry of Health, Kenya 1998).

Appendix 3: Map of Annual Distribution of endemic Burkitt Lymphoma in Nyanza Province



Endemic Burkitt lymphoma six-year (1999-2004) average annual incidence rates by Division, Nyanza Province (*Courtesy*, Jeanette Jane Rainey, 2005).

Appendix 4: Approved Ethical Consent from Kemri

KENYA MEDICAL RESEARCH INSTITUTE

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

KEMRI/RES/7/3/1 **October 01, 2013**

TO: **PROF ANN MOORMANN,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. STEPHEN MUNGA,**
ACTING DIRECTOR, CGHR,
KISUMU

FORWARDED
9/10/2013
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

Dear Madam,

RE: **SSC NO. 1381 (*RATIFICATION OF EXPEDITED-REQUEST FOR ANNUAL RENEWAL*): THE EFFECT *OF PLASMODIUM FALCIPARUM* MALARIA ON T CELL IMMUNITY AND ENDEMIC BURKITT LYMPHOMA.**

This is to inform you that at the 219th meeting of the KEMRI Ethics Review Committee held on Tuesday, 24th September 2013, the full Committee ratified the conditional approval granted by the ERC Chair on 16th September 2013.

The Committee concluded that due consideration has been given to the ethical issues that may arise from the conduct of the study and granted approval for implementation effective. Consequently, the study is granted approval for implementation effective this **24th September 2013** for a period of one year. Please note that authorization to conduct this study will automatically expire on **September 23, 2014.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **August 13, 2014**.

You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received.
Please note that any unanticipated problems resulting from the conduct of this study should be brought to the attention of the ERC and you should advise the ERC when the study is completed or discontinued.

Yours faithfully,

EAB

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

Appendix 5: Consent Form For Venous Blood Collection (in English)

Study number_____-_____-_____-_____-___--
KEMRI/UMMS INVESTIGATIONAL STUDIES
Consent for Human Investigation Studies-English Version
Study Title: Effects of *Plasmodium falciparum* malaria on NK-cell functionand eBL
Principal Investigator: Ann M. Moormann, PhD, MPH
Co-Investigator: David Mulama, PhD
Investigator: Kitungulu Nicholas, BSc
KEMRI SSC 1381
Purpose: This is a malaria immunological study looking at how malaria infections in children affect the child's ability to defend themselves against EBV, leading to a cancer known as endemic Burkitt Lymphoma, which is common in areas with high malaria transmission. Dr. Ann M Moormann from University of Massachusetts Medical School (UMMS-Worcester Campus) in the USA and her colleagues at the Kenya Medical Research Institute (KEMRI) are inviting you to enroll your child to participate in this research study. We are doing this study because we want to understand why children living in areas malaria transmission are at a high risk for a endemic Burkitt lymphoma. This childhood cancer is rear in Kenya however it is more common here than in any other parts of the world. Besides malaria, a virus called Epstein Barr Virus (EBV) is also connected with Burkitt lymphoma. EBV is a common virus that does not normally cause disease. This virus is transmitted by saliva. EBV infects most children by the time they are three years old. Even though this virus does not normally cause disease, it may play a role in the development of Burkitt lymphoma. The goal of this research study is to understand how EBV and malaria infections may increase the risk of getting Burkitt lymphoma. We will do this by looking at your child's defenses against EBV and Malaria. We hope that the results of this study will provide some information as to how to prevent Burkitt lymphoma.

Procedure

If you agree to enter your child in this study, we will draw blood from a vein in your child's arm. We will collect approximately half to one teaspoon (2-5ml) of venous blood. Only trained medical personnel will draw your child's blood. The risks for harm by having amount of blood drawn are very low. There may be slight bleeding at the site, persistent pain, bruising or infection. Bleeding, pain and bruising should disappear after a few days. If infection occurs because of your child's participation in this study, we will cover costs for treatment of the infection.

We will transport your child's blood to UMMS-KEMRI laboratory in Kisumu for further testing. We may also send samples of blood to the University of Massachusetts Medical School for testing. These tests include measuring your child's blood cell defense against EBV and malaria, testing for the presence of EBV, haemoglobin S trait, and genetic factors such as human leukocyte antigen type that play a role in the body's defense against infection and diseases. Your child's blood will also be used to test for antibodies against vaccines routinely given to your child such as polio, measles, tetanus, etc. Any results from these tests will be kept confidential. To protect your child's privacy, a study number will be assigned to the blood samples. This study number will only be linked to your child's name in a database kept by the principal investigator.

Testing for HIV is routinely done as part of your child's medical care in the hospital. As part of this study we would like to know if your child is infected with HIV. The presence of this virus can prevent the body from fighting infection and confuse our study results. If you agree to have your child tested for HIV, an HIV/AIDS counselor will talk to you. The counselor will explain how the disease is passed from one person to another and how you can prevent infection. The counselor will also explain the HIV testing and tell you if your child is infected or not. This is strictly voluntary. The results of HIV testing will be confidential and will not be available to anyone but you. We are requesting permission to record your child's HIV test results as part of the study. You may enroll your child in this study and refuse HIV testing.

Consent to record child's HIV test results for this study:

YES…….NO……..(please circle parent's response).

Benefits

The benefits of participating in this study will include provisions for transportation whenever possible to your residence from hospital. Transport reimbursement will also be available for outpatient follow up visits when your child is recovering.

Storage and use of sample for future studies

Sample of your child will be stored in a freezer and may be used for future testing related to scientific studies not described here including to test related to malaria, schistosomiasis, hookworm and other diseases caused by parasites. Other viral infections not associated with Burkitt lymphoma may also be tested such as cytomegalovirus. Comparing immunity to different persistent viral infections allow us to determine if EBV is truly responsible for some children getting Burkitt lymphoma. However these samples will only be used with the approval from Kenya Medical Research Institute's Ethical Research Board (ERB) and the Principle Investigator's primary Institutional Review Board (UMMS IRB). You will not be contacted for additional consent. You may still participate in this study if you do not consent to us using your samples for future scientific studies about diseases affecting your community. If you check "no" then your sample will be stripped of your study identification number in the database after the completion of this study, and your samples will be destroyed. If you change your mind in future, you may contact ..... or the Director of Center for Global Health Research KEMRI-Kisian or KEMRI National Ethical Review Committee (ERC), P.O. Box 54840, Nairobi 00200 at (020)-272-2541 or the Director of KEMRI P.O. Box 54840.Nairobi at (020)-272-2541.

Consent for use of child's blood samples for future studies:

YES …….NO…….(please circle parent's response)

There is no immediate benefit for enrolling your child in this study. Transport home will be provided by the study if your child participates.

Summary of Your Rights as a Participant in a Research Study

Your participation in this research study is voluntary. Refusing to participate will not alter your child's usual health care or involve any penalty or loss of benefits to which you and your child are otherwise entitled. If you decide to allow your child to join the study, you may withdraw your consent at any time and for any reason without penalty or loss of benefits. If

information generated from this study is published or presented, your child's identity will not be revealed. In the event new information becomes available that may affect the risks or benefits associated with this study or your willingness to participate in it, you will be notified so that you can decide whether or not to continue participating.

Contact Information

Mr…….....or one of his associates/team members ……has described to you what is going to be done, the risks, hazards and benefits involved and can be contacted at 057-202-2989. Further information with respect to illness or injury resulting from a research procedure as well as a research's subject's rights is available from KEMRI/National Ethical Review Committee (ERC), P.O. Box 54840, Nairobi at (020)-272-2541 or contact the committee for Protection of Human Subjects in Research, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655 USA.

Signature

Signing below indicates that you have been informed about the research in which you voluntarily agree to participate; that you have asked any questions about the study that you may have; and that the information given to you has permitted you to make a fully informed and free decision about your participation in the study. By signing this form, you have not waived any of our child's rights and the investigators or sponsors are relieved of any liability they may have. A copy of this form will be provided to you.
If you agree, circle "YES". If you do not agree, Circle "NO"

……

Printed Names of Participant/Child
If participant is a minor, the parent/legal guardian must sign below:
……..
Parent or Legal Guardian signature…….Date…….....
……..
Cell phone contact of parent or nearest neighbor/relative
……
Signature of witness (if needed) printed name of witness (if needed)

Signature*……..Date……
Witnessed by……..Date……
*A person can sign, or verbally state his/her consent in the presence of a witness who will then sign
Sample Collection:
Signature……Date…….
Witnessed by……..Date……
……...Date…….
Signature of Principal Investigator (affirming subject eligibility for the study and that informed consent has been obtained).

Appendix 6: Consent Form For Venous Blood Collection (in Nandi language)

KEMRI/UMMS
FOMITAB CHAMETAB CHII ENG CHIGILET.
Somatnet ko: Borietab cheptigonit eng kimnatetab borto ak kutikab Seriat
Chigilindet neo: Ann Moormann, PhD., MPH
Chigilindet: David Mulama, PhD
Chigilindet: Kitungulu Nicholas, BSc
Fomitab Kayanet nebo ang'wan 4: Chito neo nebo Kenya ne mie

Dr Ann Moormann nebunu univasiti nebo Massachusetts (University of Massachusetts Medical School (UMMS) eng USA ak bik che toretigei chebunu Kenya medical research institute (KEMRI) kotochin eng wenyoni. Eng wenyoni kimachei kekuye amunei si lagok chemengechen eng emet oleo cheptigonit konamei ichek missing seriat ne kekurei Burkitts lymphoma. Kisomin inemu korotik sikeboisie eng tesetabtai eng chigilisiet ak kimachei keger ng'otko terchin bik che echen ak lagok eng chigilosiek che kitiemei eng wenyoni. Seriat nito bo lagok komatao missing eng Kenya, nka kinyorchin emonito missing kosir emotinwek alak eng ng'weny komukul. Kobatei cheptigonit, komi kutik alak chekekurei "Epstain Bar virus" (EBV) chekenyorchin olemi seriat. Kutikab EBV komataibu korot age tugul. Kutichuto kinomtogei kobun ngulek. Kutikab EBV konamei lagok chetindoi kenyisiek somok ak korek. Nka kutikab EBV komataibu korot age tugul kibwatin kele tos ib koroitab seriat (Burkitts lymphoma). Makigen kit ne ibu seriatab lagok. Eng chigiloni ketiemei kenai ole imuch koibunto kutikab EBV ak cheptigonit koroitab seriat. Kichigili kimnatetab bortong'ung koter kutikab EBV ak cheptigonit ak ng'otko imuch koter ak chebo lagok che miachen ak che tindoi seriat. Kimong'u kele walutik alak tugul chebo chigenisioni kotoreti kenai ole tos kigiringta seriat.

Kimachei bik che echen che legit bogol agenge (100) eng wenyoni. Chegiloni kotesei tai eng kenyisiek mut(5) chepwoni/mitei tai. Ngot kesom keboisiek korotikuk kongeny, kesomin isainan fomit age ne lel.

Ng'ot kiyan wenyonito, kinemu korotik eng tigitiot nemi eung'ung. Kinemu korotik che legit kijigesiek somok nebo chaik (16ml). Bik che inemu korotikuk ko bik che kisomonchi boisionito. Matabitu keweluti ye kinemu korotik. Kewelutik che imuch kobit kinemu korotik kou: komong korotik tutigin eng eut, ng'waninto, ak konamin korotwek alak. komong korotik tutigin eng eut ak ng'waninto kobeku eng kasarta ne nwach. Ng'otko bit kewelut age tugul kobun wenyonito kenyoin.

Kitebsenin ng'alek tutikin agobo sabeng'ung ak ng'ot kikonamin cheptigonit. Kinemu korotik ak iwalu tebutik koibe boroindo ne nwach.

Kichigini kutikab cheptigonit eng korotikuk. Walutikab chiginoni inyoru ye ibata betut agenge kong'ete betut ne kinemu korotik. Ng'ot kitindoi kutikab cheptigonet anan kabarunoikab cheptigonit kou, koet mat eng borto konyoin daktari ne kigelewen nebo KEMRI kosubgei ak ng'atutikab konyoisiet chebo baurnatetab emetab Kenya. Bitu daktari eng kebeberta age tugul si kochigilin ng'ot kitindoi miondo age tugul ak konyain kosubgei ak ng'atutikab konyosiet chebo mienwogik eng baurnatetab emetab Kenya. Ng'ot imioni missing kegurei konyoindet neo nebo chigiloni. Ng'ot imioni missing kesomin kometestai eng chiginoni amu kimachei bik che miachen eng chigiloni. Kerichek che kisirun daktari kinyaen inyoru boch eng chiginoni. Ng'enyain koruitojin toretindetab yuto nekigenet ye ibata

betusiek aeng'u. Eng ruitojoni kotebsenin ng'ot kimyeitu ak kochigil oindap mat eng bortang'ung. Ng'otaimyoni ko chigilin daktarindet ne kigelewen. Ng'ot kitindoi cheptigonit neo ketesei tai eng toretet kinyoi inendet. Ng'ot kitindoi miondo age tugul ne kim, keng'ololi kosubgei ak ole imaktoi.

Bikeboisie korotikuk kikwenyi mionwogik alak. Ye ibata dakikaisiek mut kong'ete kakinem korotik, kechigini kimnatetab korotikuk (hemoglobin level). Ng'otko ng'ering korotikuk eng 8 dg/ml (anemic), kekonin kerichek che tesei korotik (iron supplements) kosubgei ak ole kakerto daktari. Koborunoisiek chebo kerichechu kou; kugeni metit, kokutak moet, kang'ung'et, komanta moet, toretigei chito che tuen ak kowasgei moet. Ng'ot kiker kabarunoik che ibwotini ile ng'ete boisietab kerichechu inyoru toretindet ne legit anan ibirchi simet Mr. Odada Peter Sumba eng Kisumu eng numbesiek chemi ng'weny yu.

Kiibe korotik agoi ole kichigilei eng kisumu (UMMS-KEMRI lab). Imuch keyokto korotik alak agoi univasiti nebo Massachusetts (UMMS) eng Amerika. Eng Kisumu kechigini kimnotetab korotik koboryo ak kutikab EBV, cheptigonit, ng'otko mitei kutikab EBV, glucose-6-phosphate dehydrogenase, hemoglobin S trait and human leukocyte antigen. Kichigili kogeny kimnotetab korotik eng mienwokikab lagok kou salomwek (polio), sasarek (measles) ak tetanus. Lokoiywek chebunu chiginoni komo kiborjin chi age tugul. Tugeng'ung komitei amu kiboisie nambaisiek ye kakenemu korotik. Bik che imuch konain eng nambait ko bik che indoijin wenyoni.

Konoret ak kiboisie korotik eng betusiek chemi tai.
Kebeberta nebo korotikuk kekonori eng ole kaitit si tun keboisie eng chigilosiek alak chemakimuch kemwa eng yu. Chigilosiechu kou: cheptigonit, mionwokikab bek, ng'urtonik ak mienwokik alak. Mienwokik alak che ibu kutik che mengechen che legit kou seriat (cytomegalovirus) kechigini. Ng'echigin ole kiringdoi kimnodetab borto kutik che terchin che mengechen, ko imuch koborwech ng'otko ibu kutikab EBV seriat eng lagok. Korotik che kikekonor komo imuch keboisie agoi kesom che indoijin chigilisiet eng Kenya (KEMRI ERC) ak ole bunu chito ne indoijin chigiloni (IRB). Maimuch ketebsanin akobo chameng'ung eng boisioni. Imuch itestai eng wenyoni ang'ut kotaiesie kekonor korotikuk sikeboisie kechigin mienwokik eng emeng'ung eng betusiek chebwoni. Ng'ot iesie, kinemu nabarit ne tinyegei ak kaineng'ung ak keng'em korotik alak tugul che makiboisie. Ng'ot iwal kabwatutikuk eng betusiek che mi tai, ko imuch inyoru Mr. Peter Odada Sumba anan che teleldojin chigilisiet eng emoni (KEMRI ERC), P.O Box 54840, Nairobi 00200 eng (020) 272-2541 anan ne teeldojin KEMRI, P.O. Box 54840, Nairobi eng (020) 272-2541.

Kayanet kekonor korotikuk sikeboisie eng betisuek che mi tai yes no
(kakai ibaru eng serutik mageng'ung)

Keljinet ne inyoru eng chegiloni kou chiginet ak konyosietab boch nebo cheptigonit, ak kikonin kerichek che tesei korotik.

Imantang'ung eng nwagindo eng chiginoni
Keyan iyegu agenge eng chiginoni eng chameng'ung. Ng'ot iesie chiginoni ko maimuch keistoenen ribsetab bortang'ung ne koingeny anan keimin ana kistoenen keljineng'ung nebo betusiek tugul. Ng'ot iyan chiginoni ko imuch istegei eng wei age tugul kosubgei ak mageng'ung ak inyoru imantang'ung. Ng'ot kibarasta kit age tugul ne tinyegei ak chigilani ko maimuch kong'angnta chi age tugul kaineng'ung. Ng'otko bit emo age tugul eng wenyoni

ne imuch kobor asanet anan keljinet kemwoun si ibwatigei inyegei ng'ot imuch itestai anan istegei eng chiginoni.

Nyorunet
Mr. ……anan agenge eng chetoretigei _____ ko kakomwowo akobo kit ni kiyoe, kewelutik, ak keljinet che bunu wenyoni ak imuch anyor eng (057) 20.22989. (0733) 746.854, anan (0720) 766.550. Ng'olio age tugul ne bitu eng chiginoni ak imantanyu ko imuch anyor kong'ete KEMRI (ERC) P.O. Box 54840, Nairobi 00200 eng (020) 272-2541 anan ne teleldojin ng'alekab chigilisiet ak tegelchisiet ab biik eng Amerika anan iyokji baruet kandoik che teleldojin ng'alekab chigilisiet ak tegelchisiet ab biik eng univasiti nebo konyosiet nebo Massachussets, 55 Lake Avenue North, Worcester, MA 01655 U.S.A.

Baornadet
Ye indene sai ngweny yu ko ibaru kole kakemwoun akobo wenyoni ak kaiyan eng chameng'ung; ile kaiteb ng'olio age tugul akobo chegiloni; ak walutik che kinyoru ko imuch kokonin boroindo iyan chigiloni eng mageng'ung. Ye indene baornadet eng fomini ko meiste imantang'ung eng chigilik. Kigoni fomit agenge ye ibata baornadet.

_____
Kaineng'ung

_____ Date _____
Sai neng'ung

_____ Date _____
Nambetab simet neng'ung anan chitab kokwet/tiliet ne legit

_____
Sai nebo ne teleldojin kainenyi

_____ Date _____
Sai nebo chigilindet (kobor kole kakoyanin eng wenyoni ak kaiyan akinyei)

Appendix 7: Consent Form for Venous Blood Collection (in Dholuo language)

KEMRI/ University of Massachusetts Medical School (UMMS)

Kalatas yie mar nonro
Thoro mar Nonro: Kaka kudni makelo malaria mar *Plasmodium Falcipirum* otudore gi ng'ie ng'ie mag remo mageng'o tuoche mar NK cell kod Tuo mar Ningu.
Jatend Nonro: Ann Moormann, Ph.D., MPH.
Jatend: David Mulama, Ph.D
Jatend: Kitungulu Nicholas, BSc
Kalatas Yie mar 2: Nyithindo mani Kod Tuo mar Ningu Esama gi Donjo Enonro

Laktar Ann Moorman moa e Mbalariany mar Massachusetts Medical School (UMMS) mani epiny mar Amerka kod jowadgi mani Kenya Medical Research Institute (KEMRI) kwayi mondo iyie nyathini odonji enonro. Nonroni watimo mondo wang'e gima omiyo nyithindo modak egwenge ma malaria landore ng'aw mar yudo tuo mar Ningu. Tuo mar Ningu en tuo maok ng'eny ahinya e Kenya tokatakamano ong'eny e Kenya mohingo pinje mamoko. Kaopogore gi tuo mar malaria, kudni miluongo ni Epstein Barr Virus (EBV) be otudore gi tuo mar Ningu. Kudni mar EBV en kudni mahinyo bet edel to ok ohiny kelo tuo. Kudni ni landore gi olawo. Kudni mar EBV ni chako donjo kuom ng'eny nyithindo mani e Africa kagin gi higni Adek. Kata obedo ni kudni ni ok hiny kelo tuo kamano to onyalo konyo e dongruok mar tuo mar Ningu. Katakamano pok wang'eyo gima kelo tuo mar Ningu. Thoro mar nonroni en ni mondo wang'e kaka kudni mar EBV kod tuo mar malaria nyalo jiwo landruok tuo mar Ningu. Ma wabiro timo kawang'iyo roteke mag dend nyathini magore gi kute mag EBV kod tuo mar malaria. Wageno ni duoko mawayudi enonroni biro konyowa ng'eyo yore mainyalo geng'o go tuo mar Ningu.

Madirom nyithindo 80 maoyudi gi tuo mar Ningu biro donjo enonroni higa ka higa. Nonroni biro dhi kuom higni Abich. Kadipo ni wakwayo pimo remb nyathini kendo to wabiro kwayi mondo igo seyi e oboke mar yie manyien esechego.

Kaiyie rwako Nyathini enonroni to wabiro kwayi mondo wagol remo eler mani ekor bade Kanyathini niebwo higni Abich to wabiro golo remo madirom Nus kijiko kata kijiko Achiel(2ml- 5ml). To ka nyathini ohingo higa Abich to wabiro golo remo madirom kijiko Achiel nyaka kijiko Achiel gi Nus(5ml – 8ml) eler mani ekor bade. Ng'atno ma otiegi eyore ngima dhano kod thieth kende ema biro golo remo ni. Rach mawuok kuom golo remo madirom kama nok. Richo gi gin kaka; Chwer matin awang' kama ogole, rem ewang kama ogole kod gwarruok ewang kama ogole. Richo gi biro rumo bang' seche manok. Seche moko jomoko nyalo bedo gi wich mayot kod muya marumo bang' golo remo to maokl hiny timore. Kadipo ni hinyruok obedoe kaluwore gi donjo enonroni to wabiro kawo ting' mar thieth mari kaluwore gi hinyruokno.

Ber mar bedo enonroni en kaka chiwo yor wuoth maoa e kar thieth nyaka dala bang' ka nyathini ose gol e kar thieth nikech ngima mar nyathini ibing'i kod Laktar mar nyithindo maochung' ne nonroni. Pesa maitiyogo ka idwogo nyathini e kar thieth be ibi duokni ka ochiki mondo idwogi e kar thieth.

Wabiro tero remb nyathini ekar pimo remo mani Kisian UMMS - KEMRI mondo watim nonro malach. Remo gi bende wanyalo tero Amerka ekar timo nonro e Mbalariany mar Massachusetts Medical School (UMMS) mondo wapim pim mamoko. Pim gi oting'o ng'iyo ng'ie ng'ie mag remo mageng'o del kuom kute mag EBV kod malaria, ng'iyo kute mag EBV

eremo, ng'ie ng'ie mag remo kotudore gi roteke mag del makonyo del kuom kedo gi touché kod gik makelo tuo. Remb nyathinino bende ibiro ng'iye roteke mag del mageng'o chanjo maimiyo nyithindo go. Duoko moro amora maowuok kuom pim gi ibi kana panda. Mondo ati gi ratiro mar maling'ling mar nyathini, namba mar nonro ibi ndikie eremb nyathini. Nambani ibi tudo gi nying nyathini ekama opandi maong'e mana gi Jatend nonro maduong' kende.

Pimo kute mag Ayaki gin achielo kuom gigo maitimo e kar thieth mondo ong'i go ngima nyathini. Kaka achiel kuom nonroni, wabiro gombo mondo wang'e kadipo ni nyathini ni kod kute mag tuo mar Ayaki. Bedo kutegi erembe nyalo geng'o del mondo kik go tuoché kendo nyalo bago duoko mar nonroni. Kiyie mondo wapim kute mag Ayaki eremb nyathini to ng'at maochung'ne hocho biro wuoyo kodi. Jahochoni biro nyisi kaka kute mag Ayaki landore kendo kaka inyalo geng'e. Jahochono bende biro nyisi kaka pim no obedo kendo nyisi kadipo ni nyathini nikod kutego kata ohoyo. Pimni en kuom hero mari kendo ok ochun ng'ato moro amora. Duoko mar pimni biro bedo maling'ling kendo ibi ng'e mana kodi kende. Katakamano wabiro kwayi mondo wandik duoko mar nyathini kaka achiel kuom nonroni. Inyalo bedo enonroni kata okiyie mondo wapim kute mag Ayaki eremb nyathini.

Yie mar ndiko duoko mar kute mag Ayaki mar nyathini enonroni
Eee Ooyo (Luor duoko mar Janyuol)

Keno kod Tiyo gi Remo Enonoro Mabiro.

Remo gi ibi kan kendo inyal tiyo go epim mabiro maluwore gi nonro mamoko. Magi chal kaka pim mag malaria, kute makelo tuo mar Laremo, Njofni kod tuoché mamoko maikelo gi kute. Kute mamoko ma be ok otudore gi tuo mar Ningu be inyalo pim kaka *Cytomegalovirus*. Ng'iyo roteke mag del gi to gi tuoché mamoko konyo wa ng'eyo ka adier kudni mar EBV nyalo miyo nyithindo yudo tuo mar Ningu. Katakamano remo gi ibi tiyogo mana ka jogo maochung' ne ngima dhano mantie Ekar Timo Nonro ma Kenya (KEMRI – Ethical Review Committee) kod Jotelo mang'iyo nonroni(Institutional Review Board – UH IRB) ochiwo thuolo. Ok bi kwayi mondo igo seyi ekalatas mar yie machielo. Inyalo bedo enonro kata ka ok iyie mondo wakan remb nyathini etimo nonro moko mabiro mag tuoché machando jogweng'u. Kadipo ni idagi to ok wabi kane etimo nonro mabiro bang' pimo pim maondik gi. Kailoko pachi to inyalo tudori kod Mr. Peter Odada Sumba kata Kambi mar Timo Nonro epiny Kenya(KEMRI) Jogo Maochung' ne Ratiro mar Ngima Dhano Epiny Kenya(National Ethical Review Committee) esanduku mar barua 54840, Nairobi kata namba mar simu (020) 272.2541 kata inyalo tudori kod Jatelo maduong' mar Kemri (Director) esanduku mar barua 54840, Nairobi kata (020) 272.2541.

Yie kuom tiyo gi remb nyathini endalo mabiro Eee Ooyo
(Luor duoko mar Janyuol)

Onge ber mapiyo manyathini biro yudo kuom bedo enonroni. Katakamano wabiro konyi eyor wuoth kaoha ekar thieth nyaka e dala.

Ratiro Mari Kaka Janonro Enonro ni Eyo Machuok

Bedo ni enonroni en kuom hero mari maonge achune mari amora. Dagi mari bedo enonroni ok bi loko ngima mari kata ok bi moni yudo ratiro mari. Inyalo wuok enonroni saa asaya maok ilalo gimoro amora. Nyingi kata weche maoluwore kodi ok bi wuok e andiko moro amora mawandiko maowuok enonroni. Kadipo ni weche moko manyien oyudore manyalo bedo gi rach kata ber mar nonroni kata gomboni bedoe to wabiro nyisi mondo iyier kainyalo medo bedo kodwa enonroni kata ohoyo.

Yore Tudruok

Mr. ……kata achiel kuom jowadgi ma gitiyogo _____

Osewachoni kaka nonroni obedo kod gigo maibi tim, richo, yore manyalo kelo hinyruok kod ber mantie kendo inyalo tudori kode enamba mar simu (057) 2022989, (0733) 746.854 kata (0720) 766.550. weche mamoko matudore gi tuo kata hinyruok maowuok enonroni kaluworegi ratiro mar jononro yudore (KEMRI - Migawo Maochung' Ne Ngima Dhano Epiny Kenya(ERC)), esanduku mar barua 54840 Nairobi namba mar simu (020) 272.2541 kata Jatelo maduong' mar kar timo nonro ma KEMRI esanduku mar barua 54840 enamba mar simu (020) 272.2541. Inyalo tudori bende gi jatelo maochung.ne tudo weche mag nonro maoluwore gi ngima dhano epiny Amerka e sanduku mar barua (508) 856- 4261 kata ndikne Bura maochung'ne rito ratiro mar janonro embalariany mar Massachusetts Medical Centre 55 Lake Avenue North, Worcester, MA 01655 U.S.A.

Seyi

Goyo seyi e obokeni nyiso ni osesomni weche duto maoluwore gi nonroni kendo iseyie donjo enonroni, ma isepenjo penjo moro amora kaluwore gi nonroni. Kendo weche duto maosenyisi oyieni mondo iyier ni iyie donjo enonroni. Goyo seyi e obokeni ok bi kedho ratiro mari kendo jotim nonro kata jochiw kony ok oketho gimoro amora. Obokeni achiel ibiro miyi to achiel wabiro dong'go.

_____

(Nying Janonro/ Nyathi) Janyuol kata Jarit emaoyiene go Seyi

_____Tarik _____

(Seyi mar Janyuol kata Jarit Nyathi)

_____Tarik _____

(Namba mar Simu Kata mar Janyuol kata Jarit kata Watne)

_____

(Seyi mar Janeno) (Nying Janeno)

_____ Tarik _____ (Seyi mar Jatend Nonro-Manyiso ni oseyie kendo oyiene ni mondo obed Achiel kuom Jononro).

Appendix 8: Consent Form for Venous Blood Collection (in Swahili language)

KEMRI/UMMS
IDHINI YA KUSHIRIKI KWENYE UCHUNGUZI
Uchunguzi Uliopo: Athari za Malaria aina ya *Plasmodium Falciparum* kwa kinga dhidi ya chembechembe za NK Cell na ugonjwa wa saratani aina ya *eBL*
Mchunguzi Mkuu: Ann Moormann, Ph.D., MPH
Mchunguzi Msaidizi: David Mulam, Ph.D
Mchunguzi: **Kitungulu Nicholas, BSc**
Fomu ya Mapatano 2: Mtoto Mwenye Ugonjwa wa *eBL* anaposajiliwa

Daktari David Mulama kutoka chuo kikuu cha Technologia cha Masinde Muliro University (MMUST) na wenzake kutoka chuo cha utafiti cha madawa (KEMRI) wanakualika kuandikisha mtoto wako kushiriki kwenye uchunguzi. Uchunguzi huu unafanywa kwa madhumuni ya kuelewa sababu zinazosababisha watoto wanaoishi mahala panapo ugonjwa wa malaria kuwa na uwezo mkubwa wa kupata ugonjwa wa saratani aina ya Burkitt lymphoma. Huu ugonjwa wa saratani kwa watoto hauko sana nchini Kenya lakini
unaaminika kuwa hapa nchini kuliko nchi zingine hapa ulimwenguni. Kando na malaria, vijidudu vinavyoitwa Epstein Barr Virus (EBV) pia vina uhusiano na ugonjwa wa saratani wa Burkitt Lymphoma. Vijidudu vya EBV ni vile ambavyo havisababishi magonjwa na vinasambazwa kupitia kwenye mate. EBV linapata watoto wanapofika umri wa miaka mitatu.Ingawa hivi vijidudu havisababishi magonjwa, linauwezekano muhimu kuchangia kuwepo kwa ugonjwa wa saratani wa Burkkitts Lymphoma. Madhumuni ya uchunguzi huu ni kuelewajinsi EBV na malaria yanaweza kuchangia kusambaza ugonjwa wa Burkitt Lymphoma. Tutayafanya haya kwa kuangalia ikiwa mtoto wako anayo yale kinga ya kutosha
dhidi ya virusi vya EBV na malaria. Tunatumaini ya kwatnba matokeo ya uchunguzi huu yatatusaidia kujua jinsi ya kuzuia ugonjwa wa Burkkits Lymphoma.

Takriban watoto 80 kila mwaka waliopatwa na ugonjwa wa saratani aina ya Burkitt lymphoma watashiriki katika uchunguzi huu. Uchunguzi huu unaendelea kwa muda wa miaka mitano. Tukitaka sampuli nyingine ya damu kutoka kwa motto wako, basi utahitajika kujaza fomu nyingine ya mapatano kwa wakati huo.

Ikiwa utakubali kuandikisha mtoto wako kwenye uchunguzi huu, tutatoa damu kutoka kwenye mshipa wa mkono. kabla ya kutoa damu mara ya pili. Ikiwa mtoto wako ni ana umri wa chini ya miaka mitano tutachukua kipimo cha takriban kijiko nusu cha chai hadi kimoja au (2-5ml) cha damu. Ikiwa motto wako anazidi miaka mitano, basi tutatoa damu kipimo cha takriban kijiko kimoja cha chai hadi kijiko kimoja na nusu au (5-8ml) cha damu. Wale ambao wana ujuzi wa kutoa damu pekee ndio watakaotoa damu ya mtoto wako. Madhara yanayotokana na kutolewa damu kiasi hicho ni madogo mno. Kuna uwezekano wa kuvuja panapotolewa damu, uchungu, kugwaruzwa na hata kuambukizwa. Kuvuja, kugwaruzwa na uchungu yanaisha baada ya siku chache. Ikiwa mtoto wako ataambukizwa kutokana na uchunguzi huu, basi tutagharamia malipo ya matibabu.

Kwa vile matibabu yatatolewa na madaktari hospitalini basi faida ya kushiriki katika uchunguzi huu itakuwa uchukuzi wa bure kutoka hospitalini hadi mahali unapoishi mara tu mtoto wako anapotolewa hspitalini. Nauli pia itarudishiwa wale wanaoleta watoto wao hspitalini wakati wanapopona.

Tutasafirisha damu iliyobaki hadi maabara ya UMMS - KEMRI iliyoko kisumu kwa uchunguzi zaidi. Pia tunaweza kusafirisha damu nyingine kule Marekani katika Chuo Kikuu cha Massachusetts (UMMS). Uchunguzi haya yanajumulisha kupima kinga ulio mwilini dhidi ya virusi vya EBV na malaria. Vile vile damu hii itapimwa kuwepo kwa virusi vya EBV na ugonjwa wa malaria, upungufu

wa sukari aina ya glucose- 6- phosphate,chembechembe nyekundu za damu (hemoglobin STrait) na pia kuagalia aina ya chembechembe nyeupei liyo kwenye damu ambayo husaidia mwili kukabiliana na magonjwa (Human Leuokocute Antigen). Damu ya mtoto wako itatumiwa kupima vijidudu vya EBV na kinga mwilini dhidi ya vijidudu hivyo pamoja na chanjo ambayo mtoto wako hupewa mara kwa mara kama vile (*polio*, churua, pepopunda na kadhalika Matokeo hayo yatakuwa siri, na hivyo nambari ya uchunguzi itapewa kwa kila sampuli ya uchunguzi. Nambari hizo zitaambatanishwan a jina la mtoto wako pale tu kwa mitambo ya kompyuta iliyowekwa na wale wachunguzi wakuu. Mara kwa mara kupimwa kwa virusi vya HIV hufanywa kama mojawapo ya njia za kuhakikisha hali bora ya afya ya mtoto wako hospitalini. Hivyo basi katika uchunguzi huu, tungependa kupima ikiwa mtoto wako ana virusi vya HIV. Kuwepo kwa virusi hivi kunaweza kusababisha mwili usiweze kukabiliana na magonjwa hapo basi kuharibu matokeo ya uchunguzi wetu. Ikiwa umekubali mtoto wako aangaliwe virusi vya (HIV) basi mwalimu wa HIV / AIDS atakueleza mengi kuhusu matokeo ya uchunguzi wa damu ya mtoto wako na pia kukuelezea jinsi ugonjwa huu unasambazwa. Atakueleza pia ikiwa mtto wako ana hivyo virusi au la. Uchunguzi hu ni kwa hiari. Majibu baada ya kupimwa (HIV) yatakuwa siri na haitafichuliwa kwa yeyote ila tu wewe. Katika uchunguzi huu, tunaomba kutumia majibu baada ya kupimwa HIV mtoto wako. Unaweza ukamwandikisha mtoto wako kwenye uchunguzi huu na asifanyiwe uchunguzi wa HIV.

Idhini ya kutumia majibu ya Mtoto wako kwa HIV katika uchunguzi huu

NDIO LA
(Tafadhali zungusha jibu la mzazi)

Kuweka na kutumia sampuli kwa siku za usoni
Sampuli za damu ya mtoto wako yatahifadhiwa na yanaweza kutumiwa baadaye kwa utafiti wa kisayansi ambayo hayajatajwa hapa, ikijumulisha uchunguzi zinazohusiana na malaria, *schistosomiasis, hookworm* na magonjwa mengine yanayosababishwa na vijidudu. Maradhi mengine ya virusi ambayo hayana uhusiano na ugonjwa wa saratani ya Burkitt lymphoma pia yanaweza kuchunguzwa kama vile *cytomegalovirus*. Kulinganisha kinga kwa maradhi mengine ya virusi inaweza kutuwezesha kubainisha ikiwa vijidudu vya EBV ndio yanayosababisha mtoto wako kuambukizwa ugonjwa wa Burkitt lymphoma. Hata hivyo, sampuli hizi zitatumiwa tu kama kuna idhini kutoka kwa Kamati ya marudio inayoshoghulikia maadili ya uchunguzi huko KEMRI (ERC), na ile kamati ndogo ya marudio ya Mchunguzi Mkuu. Hautawasilishwa kwa makubaliano zaidi. Bado unaweza kushiriki katika uchunguzi huu hata ukikataa sampuli zako zisitumiwe kwa uchunguzi za baadaye kuhusu maradhi yanayokumba eneo lako. Ukiweka "LA" basi nambari ile ya siri itatolewa na sampuli hizo zitatupiliwa mbali. Ukibadili uamuzi wako hapo baadaye, basi unaweza kuwasiliana na Peter Odada Sumba au Kamati ya marudio inayoshoghulikia maadili ya uchunguzi huko KEMRI (ERC), katika sanduku la barua 54840, Nairobi 00200 au nambari ya simu (020) 272-2541 au Mkurugenzi mkuu wa KEMRI, sanduku la barua 54840, Nairobi au nambari ya simut (020)272-2541.

Idhini ya kutumia sampuli za mtoto wangu kwa uchunguzi za hapo baadaye

NDIO LA
(Tafadhali zungusha jibu la mzazi)

Hakuna faida ya moja kwa moja inayopatikana kwa kushiriki katika uchunguzi huu. Hata hivyo tutatoa usafirishaji hadi nyumbani kwako ikiwa mtoto wako atashiriki

Ufupisho wa haki zako kama mshiriki katika uchunguzi

Kushiriki kwako katika uchunguzu huu ni kwa hiari. Kukataa kushiriki katika uchunguzi haitabadili hali ya afya ya mtoto wako ya kawaida wala hakutakuwa na adhabu ama upotovu wowote wa manufaa ambayo mtoto wako angeyapata. Ukikubali mtoto wako ashiriki katika uchunguzi huu, basi unaweza kutoa idhini yako wakati wowote na kwa sababu yoyote. Jina lako pia haitatumiwa katika maelezo na makala yatakayochapishwa katika uchunguzi huu. Ikiwa kuna maelezo mapya yanayoahusiana na manufaa ama majeraha au kushiriki kwako kwa uchunguzi huu, basi utaelezwa ili kuamua kuacha ama kuendelea kushiriki katika uchunguzi.

.

Mawasiliano

Bwana _____ ashanieleza ni nini kitakachofanywa, athari ubaya na manufaa zilizopo na anaweza kupatikana katika nambari za simu (057) 2022989, (0733) 746854, or (0720) 766550. Maelezo zaidi kwa ajili ya ugonjwa au majeraha kutokana na uchunguzi huu, haki zako,yapo katika kamati inayosimamia haki za binadamu (ERC), Sanduku La Posta 54840 Nairobi au nambari ya simu (020)272-2541 au Mkurugenzi wa taasisi ya utabibu (KEMRl) Sanduku La Posta 54840 Nairobi au nambari ya simu (020)272-2541, au Mratibu wa kamati ya kuzingatia haki za washiriki huko Marekani katika nambari ya simu (518) 856-4261 au andika kwa, kamati ya kuzingatia haki za washiriki katika uchunguzi University of Massachusetts Medical Center, sanduku la posta 55 Lake Avenue North, Worcester, MA 01655 U.S.A.

Sahihi

Kuweka sahihi hapa chini ni dhihirisho kwamba umeshaelezewayo te kuhusu uchunguzi huu na unakubali kushiriki kwa hiari; tena umeshauliza maswali yoyote uliyokuwa nayo na maelezo ambayo umepewa yamekuwezesha kuamua kwa huru na ukamilifu kushiriki katika uchunguzi huu. Kwa kuweka sahihi fomu hii ya mapatano, hautapoteza haki zako kisheria wala hutawapunguzia gharama yoyote waliyonayo wachunguzi. Fomu moja kama hii utapewa.

_____

Jina la Mshiriki/Mtoto

Ikiwa Mshiriki ni mtoto basi mzazi/mlinzi lazima aweke sahihi hapa chini:

_____ Tarehe_____

Sahihi ya Mzazi/Mlinzi

_____ Tarehe_____

Nambari ya simu ya mzazi au jamaa/jirani wa karibu

_____

Sahihi ya Shahidi (ikihitajika) Jina la shahidi (ikihitajika)

_____ Tarehe_____

Sahihi ya Mchuguzi Mkuu (Thibitisho kuhusu kushiriki katika uchunguzi na kuwa makubaliano yamefikiwa na mshiriki)

Appendix 9: Healthy Children Enrolment Form

KEMRI-UMMS STUDY

Healthy Child Enrollment

DEMOGRAPHICS

Participant's ID: NAI-HC-1 _____

Today's date: ___/___/_____ (dd/mmm/yyyy)

Date of birth: ___/___/_____ (dd/mmm/yyyy)

Name: _____ Male☐ Female☐

Mother's name _____

Father's name _____

Other caregiver's name _____

Relationship to participant _____

Tribe: Luo☐ Luhya☐ Kalenjin☐ Kikuyu☐ Other☐ specify _____

Highest level of education: Pre unit ☐ None ☐ Lower primary school ☐ Upper primary school ☐

Mother's highest level of education: _____

Father's highest level of education: _____

LOCATION OF RESIDENCE

Residence location _____

Residence sub location _____

Village Name _____

Nearest land mark to residence _____

Participant caregiver's phone# _____

Relationship to participant _____

Secondary contact person _____

Secondary phone# _____

Completed by (initials)_____Cross Checked by (initials)_____Page 1 of 3

KEMRI-UMMS STUDY

Relationship to secondary contact_____

Participant's ID: NAI-HC-1 _____

CLINICAL HISTORY

Any chronic medical conditions?_____
(such as asthma, sickle cell disease…)

Medications routinely taken?_____
(such as albuterol, insulin, multivitamins, iron….)

Is there a bed net in the household? Yes ☐ No ☐

Does the participant use a bed net? Yes ☐ No ☐

Is the bed net treated? Yes ☐ No ☐

Has the household received indoor residual spraying in the last 6 months?
Yes ☐ No ☐

Last time diagnosed with malaria? _____Never or #____weeks ____months___ years ago?
prior to this episode)

Last time anti-malaria medications were taken?____Never or #____wks ____mo ___yr ago?
(prior to this episode)

Symptoms	YES	NO
Is the participant sick today?	☐	☐
Does the participant have a fever today?	☐	☐
Fever in the past two days?	☐	☐
Cough in the past two days?	☐	☐
Headache in the past two days?	☐	☐
Chills in the past two days?	☐	☐
Diarrhea in the past two days?	☐	☐
Stomach ache in the past two days?	☐	☐
Other symptoms in the past two days?	☐	☐

If yes, please describe _____

KEMRI-UMMS STUDY

Participant's ID: NAI-HC-1 _____

CLINICAL ASSESSMENT

Weight (kg) _____ Height (cm) _____

Axillary Temperature (degrees C)_____

Heart Rate (beats per minute) _____

Respiratory Rate (breaths per minute)_____

Blood pressure Systolic_____Diastolic_____

Blood sample should be drawn
Laboratory assessment will be completed on another form linked only by STUID

For Data Entry Use Only	
Entered by: _____	Date: ___/___/_____(dd/mmm/yyyy)
Reviewed by: _____	Date: ___/___/_____(dd/mmm/yyyy)

Appendix 10: Flow Cytometry Protocol

Intracellular Cytokine Staining and Cell Surface Marker Staining by Flow Cytometry
Materials

- **PharmigenCytofix/Cytoperm Plus™ (with GolgiStop™**) BD, San Diego, CA, USA
    - *Cat no. 554715*
    - *Contains fixation/permeabilization and diluent/wash solutions and the protein transport inhibitor (with monensin), GolgiStop™.*
    - *Monensinis toxic – avoid contact with skin, eyes and mucous membranes.*
    - *Cytofix/Cytoperm™ contains formaldehyde, a suspected carcinogen - avoid contact with skin, eyes, mucous membranes, and avoid breathing fumes.*
    - *Perm/Wash™ solution contains saponin and sodium azide – harmful by inhalation, in contact with skin, and if swallowed. Wear gloves and suitable clothing, keep away from eyes. Use in a well-ventilated area.*
    - *Perm/Wash ™ solution is a 10x concentrate. Dilute 1:10 in distilled water prior to use.*
- **GolgiPlug**
- **BD Stabilizing Fixative**
- **Staining/Wash Buffer: PBS with 0.5% BSA**
    - *To prepare: Add 0.5% BSA by weight (for 500 ml, add 2.5 g BSA to one 500 ml bottle of PBS), shake to mix*
    - *Store at 4°C. Use within 2 weeks of preparation.*
- **DNase Thaw media**
    - *To prepare:* Add 0.020 mgs DNase to 10 mL RPMI (good for 1 week at 4°C)
    - Working concentration: Add 0.5 ml to 49.5 mLs RPMI; sterile filter before use.

CD28/49d Costimulatory antibodies
Use 1 ug/ml per 10^6 cells (for 10^5 cells use 0.5ug/500ul of culture media)

Procedure

1. *Thaw cells:*
    a. Add 100 ul working concentration DNase to 9.90 mL thaw media for each sample to be thawed.
    b. Follow thaw protocol
    c. Rest cells overnight before stimulation.

2. *Preparation*:
    a. Warm culture media (CRPMI 1640-Human serum AB) to 37 °C
    b. Thaw peptides and keep at 4 °C

3. *Stimulate*:
    a. Spin down cells at 1300 rpm (300g) for 10 minutes. Resuspend in 1 ml RPMI and count. Seed cells at 0.5 x10^6 cells per FACS tube/96-micotiter plate per well (200ul) (500μL final volume in RPMI 1640-Human serum AB).

b. Add peptide, mitogen, or PBS at the indicated concentration
c. Incubate at 37 °C for indicated time (24hrs)
d. Use SEB/PHA/PMA-I as positive control at final concentration of 2ug/ml and peptides at 5ug/ml final concentration.

4. *Plug/Block*: (the last 6 hrs of stimulation)
    a. Thaw GolgiStop at room temperature (contains DMSO, solid at 4 °C).
    b. Dilute GolgiStop in RPMI: 0.35 ul per 500 ul RPMI per tube
    c. Add 2.5 ulGolgiPlug per 500ul of media per tube.
    d. Aliquot 300 ul of culture media for multiplex ELISA.
    e. Replace with equal amount of media having a cocktail of anti-CD107a (2.5ul per 500ul of media), CD28/CD49d (1ug/ml) and antigen of interest. Use anti-CD28/49d at 1ug/ml (2.5ul per 500ul of media)
    f. Incubate at 37 °C for 6 hours. DO NOT EXCEED 12 HOURS

5. *Surfacestain*:
    a. Wash cells twice with 1ml 0.5% BSA in PBS, centrifuging the tubes at 300g for 5 min. Resuspend cells in ~200 ul PBS remaining in tube after decanting
    b. Add 100 ul 20 mM EDTA (0.02%). Incubate 15 minutes at RT, vortexing halfway through incubation. Wash cells twice.
    c. Make 1:40 dilution Live-Dead NIR; add 5 ul to each tube. Incubate at RT 20 min. Wash cells twice. (Although the protocol says twice at each was step, a third wash improves the resolution especially after staining commences!!
    d. Prepare surface stain cocktail (prepare extra for pipetting error)

Surface Stain Antibody	**Vol. per tube**
CD3 Alexa Flour 700	0.5 ul
CD4 PE Texas Red	1.5 ul
CD8 BD V500	**4.0 ul**
CD45RA PerCp Cy 5.5	2.5 ul
CCR7 PE Cy7	5.0 ul
CD107a Brilliant Violet 421	2.5 ul
CD 56 PE Cy5	2.5 ul
CD14 APC Cy7	2.5 ul
CD19 APC Cy 7	2.5 ul
Volume per sample	**23.5 ul**

Note CD107a antibody is not included in this cocktail as it was added earlier on during plugging!!! For CCR7 stain separately at 37 °C.

    e. Add antibody mix to cells, vortexing cells well before and after addition
    f. Incubate for 20-30 mins in at RT the dark (Mario suggests this step should be done at 37°C).
    g. Wash cells twice with 1ml 0.5 % BSA in 1X PBS, centrifuging the tubes at 300g for 5 min. Remove last drop of PBS with pipet after second decantation of the supernatant.

6. *Permeabilize*:
    a. Vortex/Resuspend cells. Add 500 µL per tube of Cytofix/Cytoperm™ solution
    b. Incubate on ice slurry for 20-30 min, protected from light. Vortex halfway through incubation.
    c. Wash cells twice in 1mL 1x Perm/Wash™ solution and centrifuge at **350g** for 5 min
        i. Note: increased speed required after permeabliziation).
        ii. Note: Perm/Wash ™ solution is a 10x concentrate. Dilute 1:10 in distilled water prior to use.

7. Intracellular Staining:
    a. Resuspend cells in Perm/Wash buffer remaining after decant
    b. Prepare intracellular stain cocktail *(can be done during perm incubation)*

Intracellular Stain Antibody	**Vol. per tube**
IFNy FITC(BD), (2,5 from BL)	0.5 ul
IL-2 APC	2.5 ul
MIP1b PE	2.5ul
TNF-a Brilliant Violet 605	2.5 ul
Volume per sample	8 ul

    c. Add antibody cocktail to tubes
    d. Incubate 30 mins on ice slurry protected from light.
    e. Wash cells twice with 1mL 1x Perm/Wash™ solution and centrifuge at 350 g for 5 min.
    f. Resuspend the cells in 200ul Perm/Wash solution for FACS analysis. Add 100 ul BD Stabilizing Fixative.

8. Single color control beads:
NOTE: Prepare during the intervals while staining cells
    a. Label number of tubes needed for staining controls
    b. Add 200 ul staining buffer (0.5% BSA in PBS). Vortex compensation beads and add one drop of control beads to each tube
    c. Add volume of individual antibody and vortex
    d. Incubate at RT for 20-30 min, dark
    e. Wash twice in staining buffer- 1 mL, 300g, 5 min
    f. Resuspend in remaining buffer (200 ul)
    g. Add one drop negative control beads
    h. Ensure that you use cellular single control tubes for Amcyan or BD V500 and ***NOT*** compensation beads

Appendix 11: Real Time Bi-Plex Q PCR for Cell-Associated EBV Viral Load

By David Mulama

March 15, 2014

Modified from: "Rochford Protocol Used in Moormann Lab, Version 10 June 2010"

I. Primer and Probe Sequences

From: Kimura, H, et al. Quantitative Analysis of Epstein - Barr virus Load By Using A Real-Time PCR Assay. *Journal of Clinical Microbiology*. January 1999. pg 132-136. Vol. 37. No. 1.

EBV primers

5'd CGGAAGCCCTCTGGACTTC 3' Forward Primer (FO)

5'd CCCTGTTTATCCGATGGAATG 3' Reverse Primer (RE)

EBV Probe TMP-EBV-Balf-5 with 5' FAM reporter and TAMRA BHQ-1 3' quencher

5'd FAM -TGTACACGCACGAGAAATGCGCCT-BHQ-1 3' Probe w/ FAM reporter and TAMRA quencher

Human beta-actin primers

5'd TCACCCACACTGTGCCCATCTACGA 3' Forward Primer (FO)

5'd CAGCGGAACCGCTCATTGCCAATGG 3' Reverse Primer (RE)

Human beta-actin Probe with 5'CAL Fluor Orange 560 reporter and TAMRA BHQ-1 3' quencher (read in HEX channel):

5'd CalFluorOrange -ATGCCCTCCCCCATGCCATCCTGCGT- BHQ-1 3'

- Stock tubes: Reconstitute lyophilized primers and probes to 100 nM.
- Working aliquots: Make 10 nM aliquots for personal use. Do not share primer/probe aliquots with others.
- Probes are light-sensitive. Keep stocks and working aliquots in amber tubes. Protect reactions from light (work in hood with light off).

II. Standard Curves

A. EBV Standard Curve: Namalwa DNA.
   a. Stock DNA: Namalwa cell line has two integrated copies of EBV per cell (one copy per genome). Namalwa cells (ATCC, Manassas, VA, and Cat. No. CRL-1432) were grown in culture and genomic DNA was extracted with Qiagen DNeasy Blood & Tissue Kit (QIAGEN Sciences, Maryland 20874, USA. Cat. No. 69504) including RNase treatment (QIAGEN RNase A, Cat. No. 19101). EBV copies per microliter of isolated DNA were quantified with the Roche LightCycler EBV Quantification Kit (Roche Applied Science, Germany. Cat. No. 3330028).
   b. Standard Curve: 6 5-fold dilutions, range ~1E+05 copies/reaction to 1E+01 copies/reaction. Dilutions used for r5r6r9 viral loads: 1. 24242, 2. 4848, 3. 970, 4. 194, 5. 139, 6. 8 copies per reaction. Exact copy numbers may vary in future assays according to starting concentration of Namalwa genomic DNA.
   c. Background DNA: To control for nonspecific amplification of genomic DNA, EBV Standard Curve Reactions were "spiked" with a constant amount of genomic DNA. 1 ul of 50 ng/ul BL41 DNA (EBV-free cell line) was added to each EBV Standard Curve Reaction.
   d. Reactions: 1 ul standard curve DNA + 1 ul background BL41 DNA were added to each EBV Standard Curve reaction. Standards were run in duplicate.

B. Beta actin Standard Curve: Namalwa DNA
   a. Stock DNA: Namalwa DNA contains two copies of beta actin per cell (one copy per genome). In addition, Namalwa DNA contains two copies of EBV per cell, which can serve as a control for cross-amplification of EBV. Namalwa cells were grown in culture and genomic DNA was extracted as above. Results of Roche LightCycler EBV Quantification (1 copy of EBV = 1 genome = 3.3 pg DNA) were compared with quantification of DNA mass using Quant-iT PicoGreen dsDNA Kit (Invitrogen Molecular Probes, Carlsbad CA, Cat. No. P7589) and the conversion factor 3.3 pg DNA = 1 haploid genome to determine beta actin copy number per ul DNA elution.
   b. Standard Curve: 6 3-fold dilutions, range ~ 5E+05 to 2E+02 copies/reaction. Dilutions used for r5r6r9 viral loads: 1. 48485, 2. 16162, 3. 5387, 4. 1796, 5. 599, 6. 200 copies per reaction. Exact copy numbers may vary in future assays according to starting concentration of Namalwa genomic DNA.
   c. Reactions: 2 ul standard curve DNA was added to each beta actin Standard Curve reaction. Standards were run in duplicate.

III. Reagents

A. Master mix: BioRad iQ Supermix, 2x (Bio-Rad Laboratories, Hercules, CA. Cat. No. 170-8864).
B. Water: use PCR-grade water in small aliquots.

IV. Procedure

1. Set up and print templates:
    a. Reaction calculations template: Use Excel template to enter total number of reactions (standard curves + samples). Template calculates master mix with extra volume (10%) for pipetting error.
    b. Plate setup template: Enter standard curves and samples in 96-well template for reference while pipetting.
2. Thaw reagents on ice.
3. Wrap optical plate in foil and add to ice bucket. Be careful not to touch bottoms of optical plate wells with anything – many materials, such as dust, may fluoresce and compromise results. Clean foil is ok.
4. Clean Clinical Sample Template Hood with 70% EtOH.
5. Spray an 8x12 tube rack with 70% EtOH and place in Clinical Sample Template Hood. Pull samples to thaw and arrange in 8x12 rack like plate setup (if samples are in individual tubes). Thaw in Clinical Sample Template Hood.
6. Thaw DNA for standard curves in Clinical Sample Template Hood.
7. Turn on PCR machine and computer. Open Bio-Rad CFX Manager software.
8. In "Clinical Sample Template Hood": make standard curve dilutions and place in position in 96-well rack with clinical DNA samples.
    a. (Details of standard curve dilutions once established)
9. In "No Template" PCR hood:
    a. Clean hood with 70% EtOH
    b. Make master mix according to Excel template
    c. Aliquot into 96 well optical plate.
    d. Check by eye that all wells have equal volume and that there are no air bubbles in the bottom of wells. Air bubbles can prevent accurate detection of fluorescence.
    e. When finished, clean hood with 1) 10% bleach and 2) 70% EtOH.
10. Add template to plate in "Clinical Sample Template Hood:"
    a. Vortex and quick spin all samples and standards.
    b. Pipet up and down 2-3 times when adding sample to well to mix samples with master mix.
    c. Be sure not to introduce a bubble into the bottom of the reaction well. Draw pipet tip up from bottom of well before fully depressing plunger.
    d. Add 1 ul EBV standard or 2 ul beta actin standard to appropriate wells. Standards are run in duplicate.
    e. Add 1 ul "background" DNA spike to EBV standards.
    f. Add 2 ul sample to each sample well.
    g. Add 2 ul H2O to each NTC (no template control) well.
11. Apply optical microseal to plate. Touch only "ears" of microseal with gloves. Use something clean and firm (like a tip box lid) to ensure that microseal is adherent to top of all wells. Seal along each edge of plate. Remove ears from microseal.
12. Click "open lid" on computer to make sure computer and pcr machine are communicating. Load plate in machine. Click "close lid."
13. Set up software:
    a. Go to File > New > Experiment. In "Experiment Setup" window Protocol tab, click "Select Existing" to load saved PCR protocol. Click "Next."

b. Ensure that "QuickPlate_96Wells_All Channels" is listed as selected plate. Click "Next."
c. Click "Start Run."

V. Reaction Setup

EBV Viral Load qPCR Template Updated 23Feb2011

Date:

Plate number:

Number of reactions:				
	conc	Final conc	**per rxn**	**total**
Water			2.5	0
IQSuper Mix	2x	1x	6	0
Actin F Primer	10 uM	50 nM	0.06	0
Actin R Primer	10 uM	50 nM	0.06	0
Actin Probe	10 uM	50 nM	0.06	0
EBV F Primer	10 uM	500 nM	0.6	0
EBV R Primer	10 uM	500 nM	0.6	0
EBV Probe	10 uM	100 nM	0.12	0
Aliquot by:			10	
Total template DNA:			2	

Total rxn volume:			12	

EBV curve: 1 ul Namalwa + 1 ul BL41 1:4 dilution

Serial dilutions: 1:5 (5 ul total volume), 1st standard is stock

Beta actin curve: 2 ul Namalwa

Serial dilutions: 1:3 (9 ul total volume), 1st standard is stock

VI. Reaction Conditions

Reactions were run on a Bio-Rad CFX96 Real-Time System with C1000 Thermal Cycler base.

Reaction volume: 12 ul

Cycling conditions:

1) 3:00 at 95° C
2) 0:10 at 95° C
3) 0:30 at 62.5° C * Plate read
4) Go to 2, 39 more times
5) End

VII. Viewing and Analyzing Data in Bio-Rad CFX Manager

A. Enter standard curve values
   a. Click 'View/Edit Plate'
   b. Select all wells (click upper left corner of grid) and deselect fluorophore channels not used in assay
   c. Select wells containing EBV standard curve
      i. Choose 'Sample Type: Standard' from dropdown menu
      ii. Deselect 'HEX'
      iii. Click 'Replicate Series. Choose Replicate Size = 2, Starting Replicate # = 1, and choose horizontal or vertical to match layout of standard curve on plate. Click 'Apply'
      iv. Select first two wells of standard curve (replicates) and enter starting value under 'Concentration.' Hit Enter or click checkbox under 'Load' (otherwise your concentration will not be entered in the well).

        v. Repeat for remaining wells in standard curve
    d. Repeat for beta actin standard curve (select HEX detection channel)

B. Select negative control wells and choose ‘Sample Type: NTC.’
C. Select well containing sample. Click text area of dropdown under ‘Sample Name’ and type sample id. Hit Enter or click ‘Load’ checkbox.
D. Click ‘OK’ and approve ‘Apply Changes?’
E. Check standard curve Efficiency, R^2, and Slope. Remove outliers by right-clicking well an selecting ‘Exclude Well from Analysis.’
F. Mouse over NTC wells to ensure no amplification
G. Look for samples (‘x’ in standard curve window) that lie outside the range of the standard curve. Check amplification plots for false positives.
H. To export data:
    a. As PDF: Go to Tools > Reports
    b. As Excel file: Go to Tools > Export All Data Sheets to Excel