PATHOGENESIS OF ENDEMIC BURKITT'S LYMPHOMA: THE ROLE OF T-CELL RESPONSES

BY

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THIS THESIS IS SUBMITTED TO THE DEPARTMENT OF ANIMAL BIOLOGY AND CONSERVATION SCIENCE, UNIVERSITY OF GHANA,

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DECLARATION

I declare that the findings of this thesis are the result of my own research work carried out in the Department of Animal Biology and Conservation Science (DABCS) and Department of Immunology of Noguchi Memorial Institute for Medical Research (NMIMR), all of University of Ghana, Legon under the supervision of Professor Dominic A. Edoh and Professor Ben Gyan. References cited in this work have been duly acknowledged.

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DEDICATION

This work is dedicated to the Almighty God for His grace and goodness, and also to the memory of the late Mr Gerald Laryea, an affable technician who provided assistance in diverse ways, however had been call to glory before this work could be concluded. May he rest in the bosom of the Lord till we meet again.

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LIST OF ABBREVIATIONS

AICD	Activation-induced Cell Death
HIV-BL	Acquired immunodeficiency syndrome-related BL
APCs	Antigen Presenting Cells
BL	Burkitt's Lymphoma
CD	Cluster of Differentiation
CM	Cerebral malaria
CPD	Citrate-phosphate dextrose
DBCL	Diffuse large B-cell lymphoma
EBER	Epstein-Bar Early RNA
eBL	Endemic Burkitt's Lymphoma
EBNA	Epstein- Bar Virus Nuclear Antigen
ELISA	Enzyme-Linked Immunosorbent Assay
EBV	Epstein- Bar Virus
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
FSC	forward and side scatter
G-6-PD	Glucose-6-phosphate dehydrogenase
HI	Heat-inactivated
hIL	Human Interleukin
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IARC	International Agency for Research on Cancer
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

LAK	L ymphokine-activacted Killer
LCL	Lymphoblastoid cell lines
LMP	Latent membrane protein
MA	Membrane antigen
MHC	Major Histocompactibility Complex
NHS	Normal Human Serum
NK	Natural Killer
NMIMR	Noguchi Memorial Institute for Medical Research
OD	Optical Density
OPD	Ortho-Phenylenediamine
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered Saline
PE	Phycoerythrin
PHA	Phytohaemaglutinin
PPD	Purified Protein Derivative
TNF	Tumour Necrosis Factor
CTLs	Cytotoxic T Lymphocytes
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T helper
VCA	Viral Capsid antigen
WBCs	White Blood Cells

Abstract

The mechanisms underlying the deficiency in cellular immunity to eBL and its development have not been conclusively investigated. This study sought to examine T cell phenotypes and responses in eBL. Participants recruited for this study included; 19 healthy controls, 21 eBL patients and 26 malaria patients, PBMCs were isolated from peripheral blood samples collected from participants and combinations of T-cell subset, activation marker (CD25, CD69, CD95, HLA-DR), IFN-γ-, IL-4- or FoxP3-specific monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or PE-Cy5 were used to in surface or intracellular staining of the PBMCs. Intracellular staining was done using FoxP3 staining buffer set. For intracellular staining for cytokines, PBMCs were stimulated with EBNA1 peptide pool and PHA according to the protocol for intracellular cytokine analysis. Cytokine levels in plasma were measured by ELISA. Both absolute numbers and frequency of lymphocytes were significantly lower in malaria patients compared to eBL patients ($p<0.0001$ and $p<0.0001$, respectively) as well as healthy controls ($p<0.0001$ and $p<0.0001$, respectively). The percentage T cells in population of lymphocytes were lower in both BL and malaria patients compared to healthy controls (BL vs Controls, $p=0.003$; Malaria patients vs Control, $p=0.002$). It was also found that the CD4/CD8 ratio was significantly higher in BL patients compared to controls and malaria patients (BL vs Controls, $p=0.007$; BL vs Malaria patients, $p=0.033$). Whereas the median frequency of CD4CD95+ cells did not differ between malaria patients and controls ($p= 0.462$) nor between eBL and malaria patients ($p=0.054$), it was higher in eBL patients compared to controls ($p=0.014$). Generally, CD4+ and CD4FoxP3+ cells expressed more IFN-γ in controls than in eBL patients**.** CD4+ and CD4+FoxP3+ T cells from healthy controls had higher IFN-γ REI to EBNA1 compared to those from eBL patients. Malaria patients were found to have higher frequency of γδ+ cells compared to eBL patients ($p=0.001$) and healthy controls ($p=0.014$). The frequency of the CD3+ γδ+ cells was lower

in eBL patients compared to healthy controls (p=0.004), making it the lowest among the categories. Similar to CD4+ T cells, Vδ1+ γδT cells in healthy controls expressed more IFN-γ to all stimulants than those from eBL patients. But unlike CD4+ cells, IFN-γ REI to EBNA1 by Vδ1+ γδT cells was similar between patients and controls. Additionally, higher frequency of Vδ1+ γδT cells expressed IFN-γ compared to CD4+ cells. Examining the frequency of T reg cells, it was observed that frequency of CD4+CD25hi+FoxP3 was higher in both malaria (p=0.000) and eBL (p=0.022) patients compared to healthy controls. The frequencies of CD4+CD25hi+Foxp3- cells were also higher in both malaria (p=0.012) and eBL (p=0.035) patients compared to controls. Th1 and Th2 profile of CD4+ and Vδ1+ cells were also examined. Whereas REI for IFN-γ and IL-4 in CD4+ cells were similar in health controls, the frequency of IFN-γ expression was higher than IL-4 expression to all stimulants. Likewise REI for IFN-γ and IL-4 in CD4+ cells were similar in eBL patients, just as in health controls but the expression of IL-4 by CD4+ cells to EBNA1 was higher than IFN-γ. The frequency of Vδ1+ cells expressing IFN-γ to EBNA1 and REI for IFN-γ, were higher than those for IL-4 in controls. Similarly, REI for IFN-γ to EBNA1 was higher than REI for IL-4 in eBL patients. However, the frequency of cells expressing IL-4 to both EBNA1 was higher than those expressing IFN-γ in the patients. Levels of peripheral blood TNF-α was significantly lower in eBL patients compared to healthy controls (P=0.002). Conversely, plasma level of IL-10 was significantly higher in eBL patients than in healthy controls (p=0.036). Put together, the data suggest that reduced Th1 response in eBL might be generally due to the prevailing Th2 micro-environment, elevated levels of T reg cells and specifically upregulation of CD95 expression by CD4+ cells. It also indicates that malaria may contribute to the development of eBL by skewing of immune responses in favour of Th2, generation of T reg cells and T cell exhaustion.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Lymphomas are one of the most common forms of cancer that affect children in Ghana (Gyasi and Tettey, 2007). Common paediatric lymphomas are of the non-Hodgkin type with Burkitt's (BL) and diffuse large B-cell lymphoma (DBCL) as common examples. Both BL and DBCL are mature aggressive B-cell lymphomas which if left untreated, progress rapidly and kill the patient within months. BL has worldwide distribution but with much higher incidence in areas where malaria is either holoendemic or hyperendemic (Kafuko and Burkitt, 1970). Epstein-Barr virus (EBV) is consistently found to be strongly associated with human malignancies including endemic Burkitt's Lymphoma (eBL) and it is believed that EBV and malaria are co-factors in the development of eBL (Epstein *et al.*, 1964; Oyama *et al.*, 2007; Shimoyama *et al.*, 2008).

There are two main types of BL, the African type, which is endemic (eBL), and the general type, which is non-endemic or sporadic (sBL). Endemic BL is restricted to areas where malaria is endemic whereas the sporadic type occurs in areas where malaria is non-endemic. HIV-related BL (HIV-BL) has also been identified (Wright, 1999). Cure rates for eBL are about 90 percent in children but there are relapses after treatment. Endemic BL which occurs predominantly in the equatorial belt and/or malaria holoendemic region of Africa as well as Papua New Guinea (Morrow, 1985) and is the most common childhood cancer in equatorial Africa (Goldstein and Bernstein, 1990). Out of every 100,000 African children, at least twenty will develop eBL (Rochford *et al*., 2005).

Aside the speculations that malaria could play a role in early events such as providing an additive risk for development of B-cell clones with chromosome translocations associated with the tumour, there is also the need to understand the immunological mechanisms by which infection with *Plasmodium falciparum* may contribute to lymphomas, especially eBL. For there is no conclusive explanation for the fact that about ninety percent (90%) of the world population is latently and permanently infected with EBV (Magrath, 1990) and yet only a few individuals suffer from BL. This may be due to the fact that in healthy immunocompetent EBV-carrying host; there is an efficient immune surveillance of EBV-carrying B-cells in place. During *P. falciparum* malaria the immune surveillance may be disturbed as a result of imbalances in the immune regulation. Cytokines are very important in immune regulation and function. In malaria infection, the outcome is known to be associated with Th1/Th2 cytokine balance. Th1–Th2 cytokines are of particular interest as targets for studies since they are the fundamental messengers of adaptive immunity and, as such, are likely to be involved in pathogenic mechanisms.

Although humoral immune response against some viral antigens has been identified however, response to EBNAs is poor (Bhende *et al.*, 1997). The role of cellular immunity is therefore imperative and the need to analyze the cellular immune responses to the disease.

It has been found that although EBV specific cytotoxic T lymphocytes (CTLs) are capable of recognizing many viral antigens (Brooks *et al.*, 1993), including EBNA1 when it is added externally or exogenously processed (Blake *et al.*, 1997). EBNA1 appears to be the only viral antigen expressed in BL cells. In some studies, it has been demonstrated that CD4+ T

lymphocytes from healthy adults respond to EBNA1 and that among the virus-encoded antigens that stimulate CD4+ T cells, EBNA1 is preferentially recognized. In both in vitro system and a mouse model EBNA1-specific CD4+ T cells have been shown to have the capacity to prevent the development of BL (Munz *et al.*, 2000; Nikiforow *et al.*, 2001; Paludan *et al.*, 2002; Nikiforow *et al.*, 2003). However, there is *in vivo* loss of the EBNA1–specific T cell responses in patients with eBL, EBV-associated nasopharyngeal carcinoma and AIDS-related non-Hodgkin lymphoma (Piriou *et al.*, 2005; Fogg *et al.*, 2009; Moormann *et al.*, 2009). It has also been found that there is diminished EBV-specific Th1 responses in children living in malaria-holoendemic areas (Moormann *et al.*, 2007) and deficiency of EBNA1-specific IFN-γ T cell responses in children with eBL (Moormann *et al.*, 2009). The mechanism is not clear and it could be due to T-cell exhaustion, suppression by regulatory T cells (Treg cells), skewing of responses towards Th2 at the expense of Th1 responses and/or apoptotic deletion of specific responder cells as a result of activation.

Findings also indicate that $V\delta1^+$ gamma delta (γδ) T cells may be capable of controlling abnormal proliferation of EBV+ B cells and have a crucial role to play in protection against pathogenesis of BL (Hacker *et al.*, 1992). Data from a recent study strongly suggest anti-tumour properties to $V\delta1^+$ T cells (Schilbach *et al.*, 2008). However, if $\gamma\delta^+$ or $V\delta1^+$ T cells control the expansion of B cells, then eBL should not be mentioned among people, especially children, from malaria endemic areas where the proportion of $\gamma\delta^+$ or $V\delta1^+$ T cells is found to be relatively high.

1.2 Problem statement

There is no conclusive explanation for the fact that about ninety percent (90%) of the world population is latently and permanently infected with EBV (Magrath, 1990) and yet only a few individuals suffer from BL. This may be due to the fact that in healthy immunocompetent EBV-carrying host; there is an efficient immune surveillance of EBV-carrying B-cells in place. During *P. falciparum* malaria the immune surveillance may be disturbed as a result of imbalances in the immune regulation. Cytokine are very important in immune regulation and function. In malaria infection, the outcome is known to be associated with Th1/Th2 cytokine balance. This makes Th1–Th2 cytokines important targets for studies.

It has also been found that there is diminished EBV-specific Th1 responses in children living in malaria-holoendemic areas (Moormann *et al.*, 2007) and deficiency of EBNA1-specific IFN-γ T cell responses in children with eBL (Moormann *et al.*, 2009). These results strongly suggest immunodysregulation as a factor in eBL development. Reduced responses to EBNA1could be due to up-regulation of activities of T reg cells, which are known to inhibit activities of cytotoxic T cells. These cells are also known to cause Th1/Th2 cytokine imbalance mainly by production of IL-10. The role of malaria in eBL might be its effect on the activities of Treg cells, which inhibit Th1 responses against tumours. This also needs to be investigated.

1.3 Justification

Endemic BL occurs predominantly in the equatorial belt and/or malaria holoendemic region of Africa as well as Papua New Guinea (Morrow, 1985) and it is the most common childhood cancer in equatorial Africa (Goldstein and Bernstein, 1990). Out of every 100,000 African children, at least twenty will develop eBL (Rochford *et al.,* 2005). The cancer is fatal if not treated early and the current cost of treatment is not within the reach of the poor. It may take 2 months to treat stages I and II BL and treatment is less expensive but most patients report with the later stages. Currently, it costs GH₵ 700 to treat a stage III BL and a duration of about 9 months to treat. Stage IV takes about a year and a higher price to treat (Dr. Segbefia, pers. comm., 2013). Cure rates are about 90 percent in children but there are relapses after treatment. The possible link between the pathogenesis of malaria and that of Burkitt's lymphoma has also not been completely explored.The same is true of most lymphomas. The goal of our work is therefore to clarify the state of cellular responses during development of eBL. Results from this study could provide effective means of preventing Burkitt's lymphoma, as well as developing better therapeutic interventions. The output from this work will also be invaluable in structuring public heath policy and advising the populace about less obvious risks from malaria.

1.4 Hypotheses

Our hypotheses are:

I. the effects of infection with P. falciparum on cellular immunity such as suppression of Th1 responses lead to inability to control abnormal expansion of EBV-infected cells or BL cells and hence the development of Burkitt's tumour.

II. the loss of EBNA1-specific Th1 responses is due to activities of immunosuppressive T cells and cytokines.

1.5 Overall Objective:

The main objective of this project is to characterize T cells in Ghanaian children with eBL and to gain an understanding of the mechanistic events that trigger eBL.

1.6 Specific Aims:

i. To phenotypically characterize T lymphocytes of patients and controls by directing monoclonal antibodies against surface and intracelluar markers.

ii. To compare the frequency of T reg cells in eBL patients, malaria patients and healthy controls

iii. To determine the effect of infection with *P falciparum* on the frequency of mononuclear cells and T reg cells of malaria patients and healthy controls

iv. To determine the response of T cells to EBNA1 by stimulating them with EBNA1 peptides

v. To determine the mechanism that account for the loss of specific T cell responses in eBL.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Burkitt's Lymphoma as a disease

BL was identified, for the first time, by Dr Burkitt in 1957 while working in Uganda (Burkitt, 1958). It is a malignant lymphoma that affects, primarily, the upper and lower jaws, abdomen, bone marrow, ovaries, central nervous system, salivary glands and thyroid (Ziegler *et al.*, 1970; Aderele *et al.*, 1975; Durodola, 1976; Ordonez *et al.*, 1984; Magrath, 1991). The most common presenting features in BL patients from equatorial Africa are those involving the jaw and the abdomen with the jaw being the most frequently involved site (Burkitt and Wright, 1963). Some presenting features are shown in the Figure 1 below.

There are two main types of BL, the African type, which is endemic (eBL) and the American type, which is non-endemic or sporadic (sBL). eBL tumour is found to be the fastest growing tumour known in history. Acquired immunodeficiency syndrome-related BL (AIDS-BL) has also been identified (Wright, 1999). Just like other non-Hodgkin lymphomas, BL has four stages. It is Stage I (early disease) when the tumour is found only in a single lymph node, in one organ or area outside the lymph node. Stage II (locally advanced disease) is when the tumour is found in two or more lymph node regions on one side of the diaphragm. At Stage III (advanced disease), the cancer involves lymph at both sides of the diaphragm, above and below it. At Stage IV (widespread disease), in addition to the lymph nodes, the tumour is found in several parts of one or more organs or tissues or it is in the liver, blood or bone marrow (Ziegler *et al.*,1969).

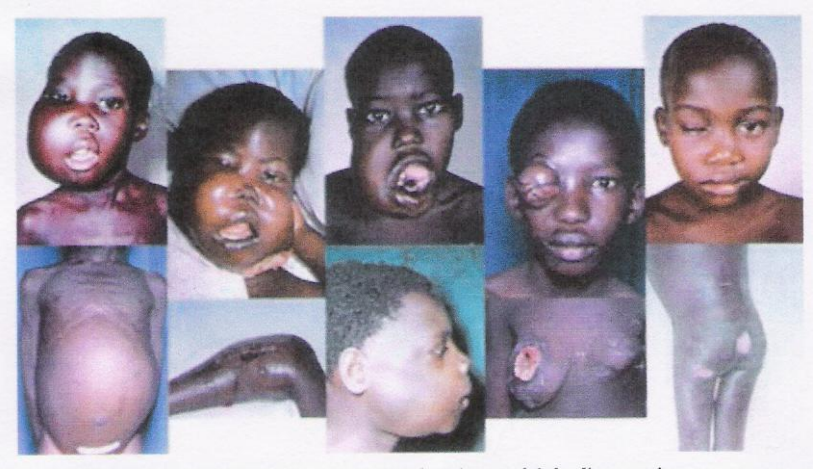


Figure 1: Disease sites of children with eBL (Source: The newletter of the international network for cancer treatment and research. Volume 8, Number 2, Special Issue: Burkitt lymphoma. Ian Magrath 2008)

2.2 Epstein Barr Virus and *Plasmodium falciparum* infections

EBV, also known as human herpes virus 4 (HHV4), was for the first time isolated by Epstein and Barr in cultured BL cells (Epstein *et al.*, 1964). It is virtually ubiquitous in the human population (≈90% prevalence) and the vast majority of individuals who harbour it show no apparent disease. However, EBV is consistently found to be strongly associated with human malignancies such as BL, lymphoproliferative disease, infectious mononucleosis and undifferentiated form of nasopharyngeal carcinoma (Epstein *et al.*, 1964). EBV is transmitted by saliva and from mother to child (Meyohas *et al*., 1996) and is acquired early in life. Just like HIV, EBV has as its strategy the ability to live and persist in the lymphocytes of the immune system itself. The virus is found to transform and 'immortalize' B-cells so that an infected individual carries B-cells containing EBV genome for life. EBV is the most potent growth-transforming agent known (Zerbini and Ernberg, 1983).

EBV has been found to develop a multiple strategy to perpetuate its existence in infected B-lymphocytes of immunocompetent hosts. This involves establishment of cell phenotype specific programs of viral gene expression and the transduction of cellular genes that enables it evade immune surveillance. Four of such programs have been demonstrated in EBV^+ cells, which are latently infected (Ernberg, 1999**)**. Figure 2 below shows the latency programmes and persistence of the virus.

A type III program, also known as latency III has been demonstrated in lymphoblastoid cell lines (LCLs) obtained by *in vitro* immortalization of normal B-cells and in immunoblastic lymphomas (Young *et al.*, 1989). The cells at Latency III express all EBV proteins associated with latency: EBV nuclear antigens (EBNAs), EBNA1-6, and virus encoded latent membrane proteins (LMPs), LMP1, LMP2A and -2B and Epstein-Bar early ribonucleic acids (EBERs). In type II program, at least one, and possibly three of the LMPs (LMP1, LMP2A and -2B) are expressed in addition to EBNA1 and EBERs. This has been demonstrated only in *in vitro* system in transfected B cells (Rowe *et al.*, 1992). However, it has been detected *in vivo* in other cell types (Pallesen, *et al*, 1993). The viral products that have been detected in type I latency are EBNA1, EBERs and LMP2A. The type I program is established in BL biopsies and some BL-derived cell lines (Rowe *et al.*, 1987). Thus viral products that are expressed in all the three programs are EBNA1 and EBERs. It has now been shown that some of the EBV infected B lymphocytes in blood express only EBNA1 (Chen *et al.*, 1995). This may facilitate immune evasion, as there will be no alternative antigens if EBNA1 is not immunogenic. However, in a recent study, five of clones CD4+ T-cell that recognize endogenously processed and presented antigens on EBV-transformed lymphoblastoid cell lines (LCL) has been identified (Demachi-Okamura *et al.*, 2008). This raises the question whether immune evasion plays role in development of eBL.

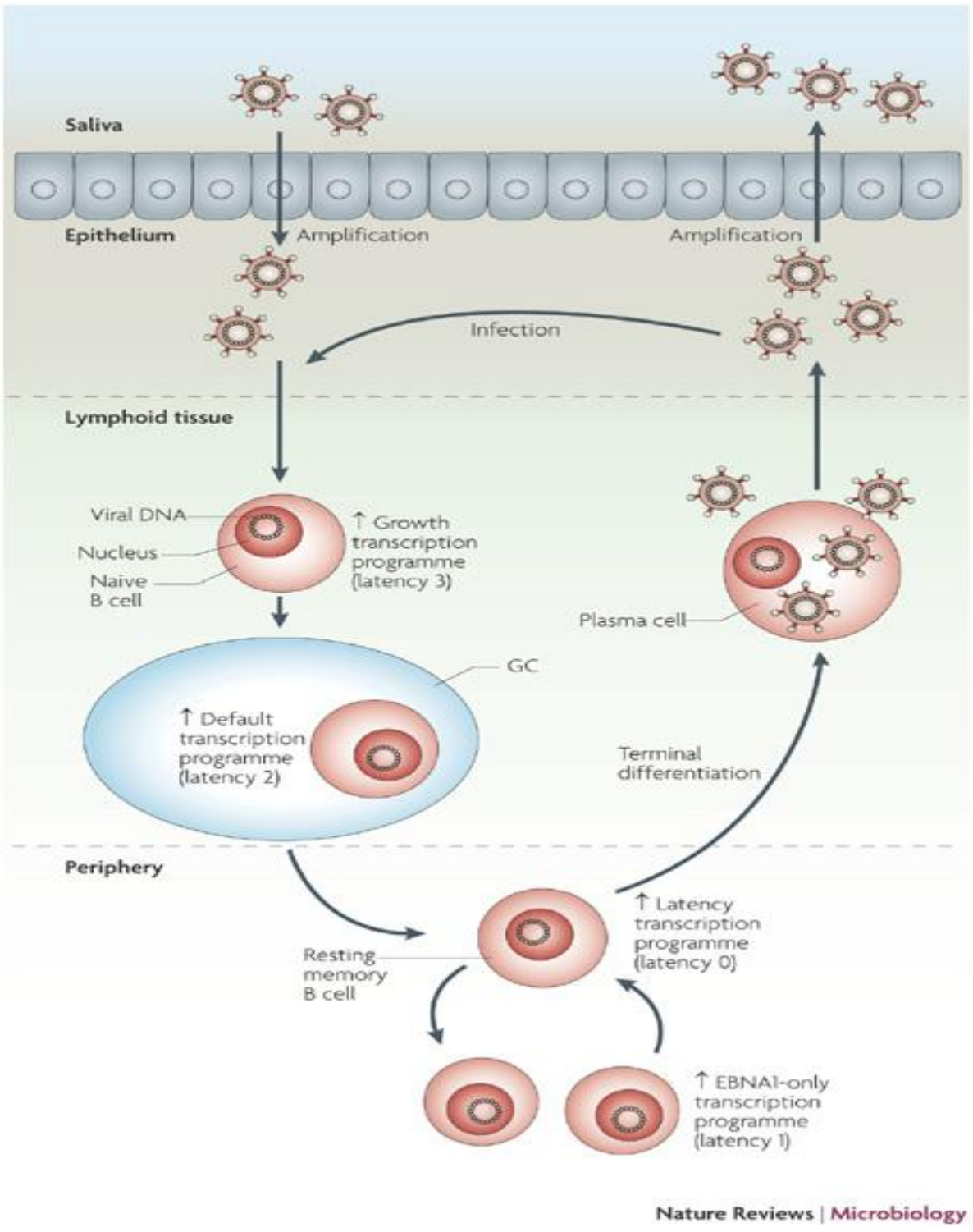


Figure 2: EBV persistence in the immune system and latency programmes (Adapted from Thorley-Lawson and Allday, 2008, *Nature Reviews Microbiology*)

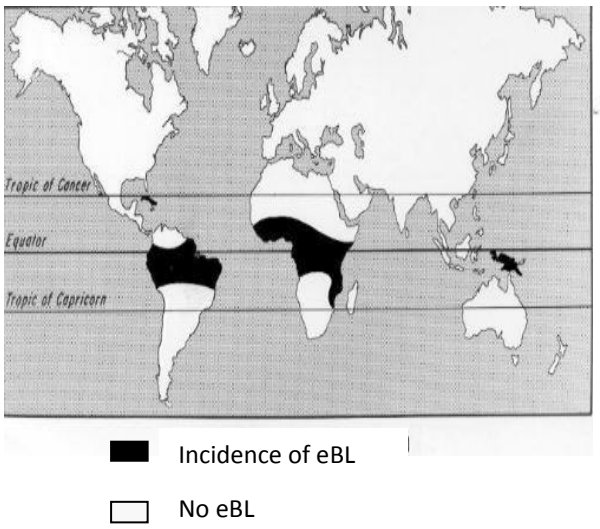
2.3 **Geographical overlap of malaria and eBL**

Along with EBV, Morrow's review of epidemiological studies has shown beyond doubt that *P. falciparum* malaria is a factor in the pathogenesis of eBL (Morrow, 1985). One important finding that implicated malaria in the development of eBL was the correspondence in the geographic distribution of eBL and that of holoendemic or hyperendemic malaria (Wright, 1971; Magrath *et al.*, 1992). Figure 3 below shows the geographic association between malaria and eBL. There is also a close association between the peak age of eBL and the age of acquiring maximum levels of anti-malarial antibodies such that individuals who relocated from low malaria to high malaria regions have older age of onset of eBL (Geser *et al.*, 1989, Donati *et al*., 2006). Intervention programme carried out in Tanzania led to reduction in the the number of eBL cases, strongly indicating the role of malaria in the pathogenesis of eBL (Geser *et al.*, 1989). Additionally, it has been reported that children (<7 years) were more likely to report at the hospital with eBL in the months immediately following the rainy season (June to September in Uganda). This was suggested to be due to the possible increased risk for BL associated with the peak malaria season (Day and Geser, 1974). This seems to suggest that the situation of eBL patients is worsened during and/or just after malaria season.

Malaria could contribute to development of eBL at early, later or all stages. There are speculations that malaria could play a role in early events such as providing an additive risk for development of B-cell clones with chromosome translocations associated with the tumour. Malaria is also known to cause B-cell activation and as the number of B cells rises the number of transformed B cells will also increase. Additionally, acute *P. falciparum* infection is associated with immunosuppression (Geser *et al.*, 1989; Whittle *et al.*, 1984, 1990). Factors that may account for the immunosuppression observed in *P. falciparum*

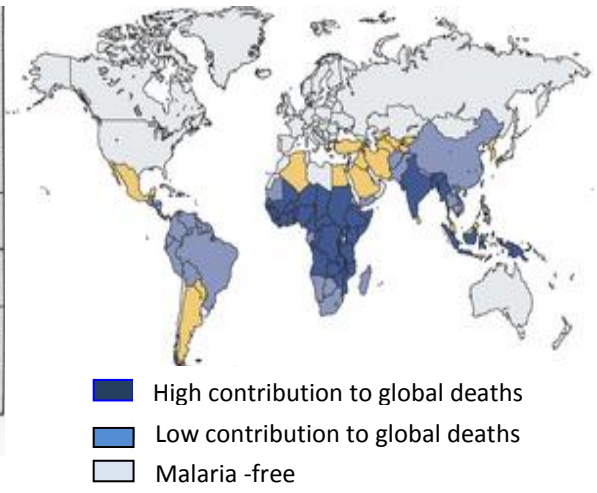
malaria include, a shift in helper T cell response towards Th2 in chronic malaria (von der and Langhorne, 1993) leading to suppression of T-cell function, particularly CTL function and activity of Treg cells which is promoted during *P. falciparum* infection (Yang *et al.*, 2006).

Geographical distribution of eBL



Source: *Tropical* Medicine Central Resource http://tmcr.usuhs.edu/tmcr/chapter41/geographical.htm

Geographical distribution of malaria



Source: Nature Medicine19, 150–155 (2013) (http://www.rbm.who.int/).

Figure 3: Similar geographic distribution of eBL malaria in the tropics

2.4 Pathology of Burkitt's Lymphoma

The tumorigenesis of BL is not clear but it is believed that constitutive activation of *c-myc* by translocations between chromosome 8 and chromosomes 14, 2 and 22 in BL tumour cells, (that is, transfer of the *c-myc* oncogene to chromosomes bearing the immunoglobulin genes), may be involved (Adams, *et al.*, 1983; Croce, *et al.*, 1979; Dalla-Favera, *et al*, 1982; Manolov and Manolova, 1972; Taub, *et al*, 1982). These chromosomal translocations are found to result in increased B-cell proliferation (Baumforth *et al.*, 1999) especially in

lymphoid tissues, which are located in the upper and lower jaws, abdomen, bone marrow, central nervous system, salivary glands, thyroid, breast, and infrequently, cardiac muscles (Ziegler *et al.*, 1970; Aderele *et al.*, 1975; Durodola, 1976; Ordonez *et al.*, 1984; Magrath, 1991)

2.5 Malaria as a Disease

Malaria has been with humanity since antiquity and was given names with respect to what were believed to be its cause. The name "malaria" is coined from two Latin words *mal* and *aria*, which means "bad air" (Bruce-Chwatt, 1988). The Greeks also recognized the association between periodic fevers and exposure to swamps, in the 4th century BC. In the 19th century, Laveran first identified plasmodia as the causative agents of malaria. Five species of malaria parasites belonging to the family Plasmodidae, of the Phylum Apicomplexa, are known to cause human malaria. These are *Plasmodium falciparum*, *P. vivax. P. ovale, P. malariae and P. knowlesi. P. falciparum* is responsible for most lethal form of malaria and dominant in areas where eBL is endemic (Morrow, Jr., 1985). Ross then demonstrated the role of mosquitoes as vectors of malaria (Farid, 1980). Human malaria is transmitted by female anopheline mosquitoes. Malaria causes severe anaemia, cerebral malaria and other malignancies in humans throughout the world with children and expectant mothers being the most affected (Abdalla *et al.*, 1980; Berendt *et al.*, 1994; WHO, 1997).

2.6 Pathology of Malaria

The pathology of malaria is to be due to enhanced clearance of erythrocytes, the release of erythrocyte and parasite materials into circulation and the host's response to these events. In

absence of other confirmed causes of the clinical manifestations, any of the symptoms and laboratory features that show that a patient is suffering from severe malaria includes impaired consciousness, respiratory distress, multiple convulsions and severe anaemia among others. The major clinical manifestations of severe malarial pathology that are more likely to end fatal are severe anaemia and cerebral malaria (WHO, 2000).

2.6.1 Malarial Anaemia

The pathogenesis of malarial anaemia is believed to be multifactorial but the exact mechanisms are not fully grasped. It has been found that malarial anaemia may be caused by haemolysis due to rupture of schizonts, immune-mediated clearance of both infected and uninfected erythrocytes and suppression of erythropoiesis. Kurtzhals and his colleagues have shown in their studies that *P. falciparum* infection indeed causes reduced response of bone marrow to erythropoietin but it is reversible. Cytokine dysregulation has also been shown to contribute to severe malarial anaemia. For instance, tumour necrosis factor (TNF) is known to stimulate erythrophagocytosis and depress erythropoiesis in bone marrow and high levels of pro-inflammatory cytokines are associated with severe anaemia in African children. (Abdalla and Weatherall, 1982; Grau *et al*., 1989 and Kurtzhals *et al*., 1997).

2.6.2 Cerebral Malaria

Cerebral malaria (CM) is caused by *P. falciparum* infection and is one of the most prominent manifestations of severe malaria in humans but its pathogenesis is not clearly understood. However, CM is associated with high plasma levels of TNF (Grau *et al*., 1989) and Perlmann

et al., (1997) postulated that elevated IgE levels, leading to overproduction of TNF, might be a contributor to the development of CM. Another mechanism that is found to contribute to the pathogenesis of CM is microvascular obstruction, with accompanying local hypoxia and nutrient depletion. Sequestration of erythrocytes containing mature stages of parasites in deep vascular beds of vital organs including the brain, cytoadherence of parasitized erythrocytes to the endothelium of microvasculature and rosette formation, and increased deformation of infected erythrocytes are important in the sequence of events that lead to the microvascular obstruction (Berendt *et al*., 1994; MacPhersen *et al*., 1985; Maguire *et al*., 1991).

2.6.3 Other Complications of *P. falciparum* malaria

Other complications of *P. falciparum* malaria include damage to the kidney and placenta, also due to cytoadherence and obstruction. Increased rate of removal of parasitized and deformed erythrocytes from circulation is also known to cause splenomegaly (Greenwood et. al., 1979). As stated above, studies have shown beyond doubt that *P. falciparum* malaria contributes to the development of eBL and eBL could be regarded as complication of *P. falciparum* malaria.

2.7 Immunity to BL

2.7.1 Innate (Non-specific) Immunity to BL

Studies have convincingly established the involvement of EBV in development of eBL. Elevated antibody titres to EBV-coded antigens have been reported in several studies of eBL cases from endemic African regions (Nkrumah and Perkins, 1976; Magrath, 1990). Non-

specific, early immune responses to EBV^+ immunoblasts involving natural killer cells (NK), lymphokine-activated killer (LAK), antibody dependent cellular cytotoxicity (ADCC) and macrophage-mediated components have also been identified. These are followed by a persistent specific T-cell immunity.

2.7.2 Adaptive (Specific) Immunity

Adaptive immune responses are vital for clearance of pathogens during primary infection and for protection against re-infection. Lymphocytes are known to be the principal architects of adaptive immune responses. Lymphocytes are made up of B cells and T cells. Lymphocytes can specifically respond to vast numbers of pathogens and can selectively expand, and can be maintained in the form of memory cells during and after immune responses. This feature of memory cells contributes to the ability of the host to mount more rapid and more robust immune responses when the same pathogen is encountered the second and subsequent times.

The two arms of adaptive immunity are humoral immunity and cellular immunity.

2.7.3 Humoral Immunity

Humoral Immunity, also known as antibody-mediated immunity, functions primarily to control extra-cellular infectious agents. It is known to play a major role in acquired resistance to infections. Antibodies, specialized proteins, are the immune effectors in humoral immunity. The mechanism involves prevention of attachment of infectious agents to the host cells, triggering of complement-mediated destruction, opsonization for enhanced uptake by phagocytes or neutralization of toxins produced by the parasites. Here the immune system

triggers or activate specific B-lymphocytes to expand and produce specific antibodies. Antibodies bind to foreign antigens on the surface of parasitized cells resulting in destruction and/or enhanced phagocytosis of those cells and the parasites in them (Jakobsen *et al.*, 1997).

2.7.3.1 Humoral Immunity to BL

B-lymphocytes are the main architects in humoral immunity and precursors of antibody secreting cells. Specific antibody responses to EBV have been found to involve immunoglobulins (Igs); IgG, IgM and IgD. These antibodies are produced early during infection and whereas IgM and IgD are transient, IgG antibodies persist throughout life and are found to control recurrence of EBV infection. Production of IgG and IgM antibodies to viral capsid antigen (VCA) has been demonstrated (Bhende *et al.*, 1997). Also, some of the Igs are neutralizing antibodies that recognize EBV membrane antigen (MA) (Errand, 1992). The last antibodies produced are against EBNAs, which may or may not be detected due to poor response by certain individuals (Jones *et al.*, 1985). Antibodies to viral envelope antigens (MA and VCA) are able to neutralize viral activity through ADCC. But BL cells lack expression of VCA and other antigens except EBNA1 and therefore, are not affected by natural humoral responses. However, an elevated antibody titre against EBV (VCA) has been observed in BL patients (Evans and Mueller, 1997).

2.7.4 Cellular Immunity

The immune system basically comprises of a range of cell types, which participate in direct effector functions, in immune regulatory mechanisms, antibody secretion, or antigen presentation. However in specific cellular immunity, T-lymphocytes are paramount. T-

lymphocytes (T cells) comprised of many cell types, some of which are mainly cytotoxic and others that regulate immune responses through production of cytokines. Cytokines are regulatory proteins secreted by white blood cells and various cell types in the body. Cytokines are different from hormones in that a cytokine can be produced by more than one cell types and has a broad spectrum of action but within a short range whereas hormones are secreted by one type of specialized cells and have a specific action, which is at a distant site.

T cells are divided into two groups. The first group that expresses γ/δ receptor (γδTCR) is called γδT cell group. The second group that expresses α/β receptor (αβTCR) is known as αβT cell group. Majority of peripheral blood lymphocytes (>90%) are αβT cells (Haas *et al.*, 1993).

2.7.4.1 γδ T cells

Studies have shown the main role of the minority group of peripheral blood T cells, γδT cells as a first line of defense to infectious pathogens such as viruses and parasites (Bluestone and Matis, 1989; Born *et al.*, 1990; Carding *et al.*, 1990; De Paoli *et al.*, 1990; Ho *et al.*, 1990) and are particularly known to play role in malaria protection (Taniguchi *et al.*, 2007; Weidanz *et al.*, 2010; Costa *et al.*, 2011). γδT cells that produce Th1-like and Th2-like cytokines have also been demonstrated (Ferrick *et al*, 1995; Dunne *et al.*, 2010) and are now considered not only as first line of defense but also regulatory cells that form a link between innate and adaptive responses (Holtmeier and Kabelitz, 2005). They are the first line of defense because they respond rapidly to infection. Though they have innate responses, they have immunological memory which traditional effectors of innate immunity such as monocytes, macrophages, NK cells, NKT cells lack. They display features of both adaptive and innate immune systems and are therefore at the border between and connecting the innate and the

adaptive immune systems. They are also regard as regulatory cells because of their ability to modulate the activities of other cells of the immune system. The γδ T cell response is also not MHC-restricted (Langhorne, 1996) and has been found to be dependent on $CD4^{+}\alpha\beta^{+}$ T cells (Elloso *et al.*, 1994). It has also been established that the majority of γδT cells are $CD4^{-}CD8^{-}$ cells and as γδT cells increase, percentage of $CD4^{+}$ cells of declines (Worku *et al.*, 1997), a scenario suggested being either due to proliferative response or selective recruitment of γδT cells into circulation (Ho *et al.*, 1994).

Human γδT cells are divided into sub-groups depending on the subset of TCR V-segments expressed. The majority sub-group in Caucasians (about 70 to 90%) expresses both TCR variable segments Vγ9 and Vδ2 and is called $V\gamma9^{+}V\delta2^{+}$T cells. The second most frequent sub-group expresses Vδ1TCR V-segment and is known as $V\delta1^{+}$T cell (Casorati *et al.*, 1989) and in malaria endemic areas levels of $V\delta1^{+}$T cells were higher than that of $V\gamma9^{+}V\delta2^{+}$T cells in both adults and children (Goodier *et al.*, 1993; Hviid *et al.*, 1996; Hviid *et al.*, 2000; Hviid *et al.*, 2001).

2.7.4.2 αβT cells

Two main types of αβT cells are also recognized. These are $CD4^{+}$ T cells and $CD8^{+}$ T cells, which recognize antigens, presented on major histocompactibility complex (MHC), MHC II and MHC I respectively of antigen presenting cells (APCs). When activated, $CD4^{+}$T cells secrete cytokines that define their main function of regulating the immune system (Janeway

et al., 1988). Clones of CD4+T cells have also been identified that have cytotoxic function (Langhorne *et al.*, 1990; Amante and Good, 1997; Paludan *et al.*, 2002).

Based on the type and function of cytokines produced, $CD4^+$ cells can be categorized into two subsets, CD4+ T helper 1 (Th1) and CD4+ T helper 2 (Th2) cells. Th1 cells are mediators in cellular immunity but also regulate certain B cell responses. They are known to produce predominantly the cytokines, interleukin-2 (IL-2), Tumour necrosis factor (TNF-β), gamma interferon (IFNγ) and lymphotoxin, triggering expansion and maturation of T cells, and hence cellular immunity. Th2 cells on the other hand produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, promoting maturation of B cells and antibody production (Mosmann *et al.*, 1989). Th1–Th2 cytokines are of particular interest as targets for studies since they are the fundamental messengers of adaptive immunity and are therefore likely to be involved in pathogenic mechanisms. The balance between Th1 and Th2 response would therefore determine the state of the immune regulation. Additionally, Th1 cytokines such as IFN-γ and TNF, have cytotoxic function and are considered necessary for combating internalized pathogens and Th2 cytokines such as IL-4 and IL-5, on the other hand, have been considered essential for protection against large extracellular parasites (Abbas *et al.*, 1996; Allen and Maizels, 1997). IFN-γ enhances phagocytosis and destruction of pathogens by upregulating expression of the necessary receptors, adhesion molecule and chemokines, which get effector cells to site of infection (Boehm *et al.*, 1997). IL-4 enhances production of antibodies, for example immunoglobin G (IgG) and IgE, effectors of humoral immunity. IL-4 receptor is expressed on Th1 cells and this provides mechanism for regulation of Th1 activity by IL-4. IL-4 is known to trigger a switch of immune responses from Th1 to Th2 and is therefore important in Th1/Th2 balance. In a murine model it has been found that chronic malaria leads

to a shift in helper T cell response towards Th2 cells (von der and Langhorne, 1993b), indicating the effect of malaria on Th1/Th2 balance.

Another Th subset has also been discovered which, like Th1 cells, promote inflammatory responses. These Th cells produce IL-17 and are known as Th17 cells. They are known for their role in the pathogenesis of autoimmune diseases and protection against fungal and bacterial infections (Kolls, 2004; Milner and Paul, 2008).

CD4+ T regulatory cells have also been identified. Experimental results, so far, put together suggest that both natural and inducible Treg cells are required for effective protection against disease (Bilate and Lafaille, 2012). However, Treg cells have, also, the capacity to directly inhibit T cells or indirectly by production of anti-inflammatory cytokines, induce apoptosis of T cells through suppressive activity on cytokine secretion (Pandiyan *et al.*, 2007; Miyara and Sakaguchi, 2007). Although these may be important in regulating excessive immune responses and prevent immunopathology, it may lead to immunosuppression and persistence of infection.

$CD8^+$ T cell group comprises of cytotoxic T cells (TCLs) that are able to destroy target cell through direct contact and/or through production of toxic cytokines. Destruction of target cells through direct contact is mainly mediated by Fas and perforin release. Cytotoxic cytokines secreted by $CD8^+$ T cells include, IFN-γ and TNF (Wong and Pamer, 2003), which are secreted by CD4+ T cells as well. Though majority of CD8+ T cells from peripheral blood in humans have Th1-like phenotype, Th1/Th2 polarized responses have been demonstrated in certain diseases and also in response to *Mycobacterium leprae* antigens

(Paliard *et al*., 1988; Salgame *et al.*, 1991). This implies that Th1 or Th2 responses may not be mediated by CD4+ cells only. CD4+ T cells are also known to have cytotoxic effector function (Nikiforow *et al.*, 2001). Some suppressor $CD8^+$ T cells have also been identified (Koide and Engleman, 1990).

Additionally, CD4/CD8 ratio is used to ascertain how strong one's immune system is. High CD4/CD8 ratio implies not only strong immune system but also indicative of blood cancer or major infection (Health Encyclopedia, 2013). CD4/CD8 ratio is also used to monitor infections such as infectious mononucleosis, which is a contagious disease caused by the infection of B cells by EBV and is associated with sore throat, swollen lymph nodes in the neck and armpits among others.

2.7.4.3 T cell activation and Memory T cells

Activation of T cells occurs when the T cell receptor and a costimulatory molecule on T cells such as CD28 are simultaneous engaged by the MHC costimulatory molecules on antigen-presenting cells (APC). The initial signal is elicited by binding of the T cell receptor to its associated peptide presented on MHC on an APC. The second signal occurs when the co-stimulatory receptor (CD28, the only co-stimulatory receptor expressed by naïve T cells) interacts with co-stimulatory molecules such as CD80 and CD86 on APC. The second signal is crucial to complete the activation process and without it the T cells that have received the first signal may not be able to activate or respond to the antigen subsequently (Appleman and Boussiotis, 2003).

Activated cells express various markers of activation. These include CD25, CD69 (early activation markers), CD95 or HLA-DR (late activation markers). Activated T cells are

antigen-experienced cells. After activation and resolution of infection some activated cells are deleted while some are converted to memory cells. Two types of memory T cells can be identified. These are central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells). T_{EM} cells have activated phenotype, rapid turnover and closely resemble effector cells. They express the early activation markers such as CD69 and CD25 of effector cells. T_{CM} cells, on the other hand, have a relatively slow turnover, lack activation markers, and closely resemble naive T cells (Sprent and Surh, 2002). Data indicate that either overstimulation under certain conditions or activation of all available precursors within a few days favour exhaustion (Sprent and Surh, 2002). Insufficient T cell help including inadequate cytokine supply can also enhance exhaustion of memory cells and T reg cells contribute to reduced T cell help by this mechanism (Ropke *et al.*, 1996; Lee *et al.*, 1999). The length of exposure, amount of the antigen and kinetics of antigen-elimination determine the frequency and activation of memory T cells. It has been found that certain unrelated infections can cause reduction in memory cells for an infection or disease (Schmidt and Harty, 2011) but the mechanisms involved are not clear.

Naive T cells do not express activation markers but having adequate numbers of them also, is essential for the immune system if it should continuously respond to unfamiliar pathogens. Memory T cells are the originators of robust secondary immunity. This is because being antigen-specific experienced cells, they quickly expand to form large numbers of effector T cells upon re-exposure to the same specific antigen and can adequately deal with a previous infection at an early stage.

Although CD95 (or Fas) is an important effector molecule for cytotoxic T cells, its expression also means death of the cell. CD95 is also known to be non-apoptotic. It is found

that high concentration of CD95 agonists lead to inhibitory effect on cellular immunity while low concentration drastically upregulates expression of activation markers and increases production of Th1 cytokines such as IFN-γ, TNF-α and IL-2 (Paulsen and Janssen, 2011; Paulsen *et al.*, 2011).

2.7.5 αβT-cells and BL

The T-cell immunity has been found to be predominantly mediated through reactivation of cytotoxic T cell responses (Svedmyr, *et al*, 1984). The EBV-specific CTL memory is found to be mainly HLA class 1 restricted and is directed against viral products expressed at latency III program (Gavioli *et al.*, 1992; Murray *et al*, 1992). Aside the expression of these potential target antigens, the EBV^+ B cells express lymphocyte activation markers such as CD23, CD30, CD39 (Gordon *et al.*, 1984) and secrete lymphokines such as IL-10 (Burdin *et al.*, 1993). When BL cell lines and Lymphoblastoid B cell lines expressing the III latency program established from peripheral blood of normal donors were screened, the majority produced significantly, more of human interleukin-10 (hIL-10) than mature normal human B-lymphocytes. hIL-10 is not only found to suppress lymphokine production by Th1 T cells but also known to act in an autocrine fashion, enhancing the expansion of B cells. This would invariably lead to increase in EBV transformed cell line in the B cell pool. IL-10 also down-regulates the activation of CTLs.

CTLs are found to keep surveillance on the reappearance of transformed B-lymphocytes healthy virus carriers (Lin and Askonas, 1981) but this function appears to be suppressed once the tumour has set in. It has been found that although EBV specific CTLs are capable of recognizing viral nuclear antigens EBNA3, 4, 6, and to some extent, EBNA2, 5, LMP1 and LMP2 (Brooks *et al.*, 1993); Burrows *et al.*, 1990), yet cytotoxic response has been effective

against EBNA1 in individuals from malaria endemic areas (Moormann, *et. al*., 2007). Other researchers have classified the six virus-encoded nuclear antigens (EBNAs) found in LCLs as EBNAs 1, 2, 3A, 3B, 3C and leader protein (EBNA LP) in addition to the two latent membrane proteins (LMPs 1 and 2) (Murray *et al*, 1992). It has been found that EBNA3A, 3B, 3C have epitopes that are immunodominant among the different latent proteins and CD8+ CTL responses were markedly skewed toward these epitopes. However, no responses to EBNA1, EBNA LP, or LMP1 were observed (Murray *et al*, 1992). Khanna and his colleagues (1992), in a study to localize EBV CTLs epitopes established that epitopes for EBNA3A and EBNA3C were recognized more frequently than any other epitopes whilst no CTL epitopes were localized in EBNA1. The invisibility of the EBNA1 to CD8+ cytotoxic T lymphocytes is now known to be due to prevention of processing and presentation of EBNA1 on MHC class I molecule by its Glu/Ala repeat domain. The result obtained by Münz and his colleagues (2000) shows that it is instead presented on MHC class II molecule. For that matter in an experimental mouse model, EBNA1 was not recognized by the CTLs (Trivedi *et al*., 1994). But Blake and his colleagues found that MHC class I presentation can occur if EBNA1 is exogenously processed (Blake *et al.*, 1997). In that regard, EBNA1-specific CD8+ CTLs that do recognize EBV-transformed cells when EBNA1 is added externally or exogenously processed have been identified (Blake *et al.*, 1997).

The inability of the CTLs to recognize the EBNA1 when processed endogenously will have serious implication for protective immunity since EBNA1 appears to be the only viral antigen expressed in BL cells. This may explain why the CTLs lack the capability to check the abnormal expansion of BL cells. Results obtained from other experiments have suggested destruction or dysfunction of a subset of CD4+ T cells, which are responsible for the

induction of CD8+ CTLs (Whittle *et al*., 1990). In recent studies, it has been demonstrated that CD4+ T lymphocytes from healthy adults respond to EBNA1 and that among the virus-encoded antigens that stimulate CD4+ T cells, EBNA1 is preferentially recognized. This is possible because the Gly/Ala repeat domain does not inhibit processing of EBNA1 onto MHC class II for CD4+ T cells. In both in vitro system and a mouse model EBNA1-specific CD4+ T cells known to secrete IFN-γ after encounter with transformed B lymphocyte cell lines, inhibit EBV-induced B-cell proliferation and/or kill BL cells and (Munz *et al.*, 2000; Nikiforow *et al.*, 2001; Paludan *et al.*, 2002; Nikiforow *et al.*, 2003). However, there is in vivo loss of the EBNA1–specific T cell responses in patients with eBL, EBV-associated nasopharyngeal carcinoma and AIDS-related non-Hodgkin lymphoma (Piriou *et al.*, 2005; Fogg *et al.*, 2009; Moormann *et al.*, 2009). It has also been found that there is diminished EBV-specific Th1 responses in children living in malaria-holoendemic areas (Moormann *et al.*, 2007) and deficiency of EBNA1-specific IFN-γ T cell responses in children with eBL (Moormann *et al.*, 2009). These results strongly suggest immunodysregulation and could be due to T-cell exhaustion, suppression by Treg cells-which affects both cellular and humoral immunity, skewing of responses towards Th2 at the expense of Th1 responses and/or apoptotic deletion of specific cells as a result of activation. However, a study has shown that CD4+ T cells can induce Fas-mediated apoptosis in BL B cells; especially B cells with CD40 ligation at their surfaces. But the persistence expansion of the malignant cells suggested that this Fas-mediated apoptosis is not functioning. There is the suggestion that the Fas-mediated death signal might be modulated by some activation markers at the cell surface (Schattner *et al.*, 1996).

2.7.6 Regulatory T cells

Various types of Treg cells have been described on the basis of the mechanisms employed in suppression, where they originate from or how they can be generated. These include natural thymically-derived CD4+CD25+ Treg cells (nTreg cells) which express the transcription factor, forkhead box P3 (Foxp3). Another type, known as IL-10-producing type 1 regulatory T cells (Tr1) are CD4+ cells that are induced by antigen-specific activation in presence of IL-10 and express CD25. TGF-β-producing helper 3 T (Th3) cells which are believed to have been derived from CD25+ CD4+ Treg cells have also been identified. CD4+FoxP3+ Treg cells which do not necessarily express CD25 have also been identified. However, there are two main subsets of T regulatory cells. The first subset comprised the naturally occurring Treg cells ($CD4^+$ $CD25^+$ $FoxP3^+$). The second subset is made up of CD4+ T cells that acquire regulatory function in the periphery due to exposure to cytokines, infectious agents and their products among others. This type of regulatory cells is known as adaptive or inducible Treg cells (iTreg cells). iTreg cell populations include the Tr1 cells, TGF-β-producing (Th3) cells and inducible Foxp3+ Treg cells (Sakaguchi *et al*., 2006; Finney *et al.*, 2010b).

A study has shown that CD4+ T cells can be induced to express the Foxp3 and phenotype of Foxp3+ T regulatory cells (Chen *et al.*, 2003). This shows that Foxp3 is an important functional phenotypic characteristic of T regulatory cells. Another marker for T regulatory cells is CD25 but it is not a definitive marker since it can be expressed by activated T cells. Moreover, only few Foxp3+ T regulatory cells express CD25. It has been suggested that CD25 expression is a measure of activation status rather than a marker of identification (Belkaid and Tarbell, 2009; Finney *et al.*, 2010a).

Treg cells have the capacity to directly inhibit T cells or indirectly by production of anti-inflammatory cytokines, induce apoptosis of T cells through suppressive activity on cytokine secretion (Pandiyan *et al.*, 2007; Miyara and Sakaguchi, 2007). Although these may be important in regulating excessive immune responses and prevent immunopathology, it may lead to immunosuppression and persistence of infection. The mechanisms employ by T reg cells include: cell-to-cell contact and in part, a cytokine signaling by CD4+CD25+ and FoxP3+ T reg cells, secretion of IL-10 and TGF-β by Tr1 and TGF-β by Th3 cells. A study has shown that by a portion of natural and adaptive Foxp3+ Treg cells can acquire IL-10 competency extrathymically and that both Foxp3+ and Foxp3– precursor cells acquire this competence by a mechanism that does not required IL-10 signaling but dependent on TGF-β (Maynard *et al.*, 2007). Tr1 cells lack an identification marker. They also normally express CD25 but unlike CD25+ CD4+ Treg cells that continue to express CD25 in their resting state, Tr1 cells do not express high levels of CD25 in resting phase

2.7.7 Regulatory T cells and Tumour immunity

One of the underlying factors in tumour immunology is immunological tolerance. Immunological tolerance refers to specific non-reactivity of the immune system to a particular antigen, which the system is capable of under other conditions, due to anergy, clonal deletion of specific responder cells and/or immunodysregulation. Treg cells have been implicated in immunological tolerance. Studies have pointed to a role of Treg cells in development of tumours but their role in the pathogenesis of eBL is not yet clear. In an immunotherapy model involving melanoma, transfer of CD4+ CD25+ T reg cells inhibited CD8+ T cell-mediated tumour destruction (Antony *et al.*, 2005). Additionally, it has been shown that intratumoral T reg cells inhibit proliferation and function of autologous

intratumoral CD8+ T cells in both in vivo and in vitro experiments (Yang *et al.*, 2006). Depletion of CD25 was also found to improve the efficacy of anti-tumour vaccines (Golgher *et al.*, 2002). Studies have also shown the involvement of indoleamine 2,3-dioxygenase 1 (IDO) in tumorigenesis as it has been found that IDO expression in tumor cells is associated with accumulation Tregs (Wainwright *et al.*, 2012). It has also been demonstrated that IDO expression in astrocytes is stimulated by IFN-γ, indicating that IFN-γ may contribute indirectly to tumour development (Grant *et al.*, 2000).

CD4+CD25+FoxP3+ T reg cells were also found to be recruited into ovarian carcinoma and patients' survival inversely associated with their numbers (Curiel *et al.*, 2004). However, in a study with patients with mycosis fungoides and cutaneous T-cell lymphoma (CTCL) the presence of FOXP3+ Tregs in CTCL is found to be associated with disease stage and improved patient survival (Gjerdrum *et al.*, 2007). This is not contradictory because inhibition of Th1 responses by the Tregs includes suppression of CTCL growth. Tr1 cells which require IL-10 for their development are also known to induce tolerance through secretion of IL-10 and TGF-B (Roncarolo *et al.*, 2001). EBV-infected B cells also produced IL-10 (Burdin *et al.*, 1993) which, firstly, contributes to the level of IL-10 in the environment and secondly, promotes generation of Trl cells. Moreover, persistent stimulation of naive CD4+T cells by EBV+ B and tumour cells can lead to production of more Tr1 cells. This implies that there is a positive feed-back mechanism for IL-10 and TGF-β production. IL-10 and TGF-β promote Th2 responses and tumour growth while down-regulating Th1 responses and hence tolerance (Daly *et al.*, 2005).

In a study by Voo and his colleagues, when EBNA1–specific $CD4^+$ T-cell lines and clones were analyzed it was shown that they were $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells that are capable of suppressing the proliferative responses of naïve $CD4^+$ and $CD8^+$ T cells but do not secrete IL-10 and TGF-ß. They mediate their suppressive activity in the manner typical of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells- through cell-cell contact and partly by cytokine signaling. These Treg cells were also shown to suppress IL-2 secretion by $CD4^+$ effector T cells specific for EBNA1 as well as a melanoma antigen, (Voo *et al*., 2005). It has also been shown that in recurrent tumours, therapeutic tumour-specific CD4+ T cells could be converted to $CD4^+$ $FoxP3^+$ Treg cells (Jensen *et al.*, 2012). This shows that tumours could increase the frequency of T reg cells that consequently downregulates immune responses, particularly, Th1 responses and promote tumour survival.

2.7.8 $\gamma\delta^+$ T Cells, EBV and Tumours

It has been demonstrated that when EBV-transformed B cell line were used as stimulating cells they caused a striking expansion of only $V\delta1^+$T cells of T cells obtained from healthy donors and patients suffering from a chronic HLA-B27+ mono-arthritis. And in absence of $V\delta2^+$ cells, proliferative response was enhanced (Hacker *et al.*, 1992). A study has also demonstrated ability of $V\delta1^+$T cell clone from human allogeneic haematopoietic stem cell transplant patients to expand and exhibit cytotoxicity *in vitro* against stimulation with autologous EBV–lymphoblastoid cell lines (LCL). These patients exhibit skewed TCR repertoires of $V\delta1^+$ T cells just like in infections with human immunodeficiency virus (HIV) and malaria. EBV-infected cells were also found to stimulate *in vitro* oligoclonal expansions of autologous $V\delta1^+$T cells from healthy EBV-seropositive donors (Fujishima *et al*., 2007). In *in vitro* system, EBV^- BL cells also have the ability to stimulate $V\delta1^+$ cells and that this becomes enhanced in presence of EBV. These findings indicate that $V\delta1^+$ cells may be

capable of controlling abnormal proliferation of EBV+ B cells and have a crucial role to play in protection against pathogenesis of BL. However, elevated levels of $V\gamma9^+$ $V\delta2^+$ cells in peripheral blood during EBV infection in humans have been reported instead (De Paoli *et al.*, 1990). This might be due to the fact that majority of $\gamma\delta^+$ cells in individual without history of malaria are $V\gamma9^+$ and these cell expand upon stimulation. Moreover, just like studies on role of $\gamma\delta^+$ cells in immunity to malaria, most of the studies that show that $\gamma\delta^+$ cells have anti-tumour potential were done with $V\gamma9^+$ $V\delta2^+$ cells (Fisch *et al.*, 1997). From the results, there is an indication that primary exposure and response, aside the cytokine environment, of both $V\gamma9^+$ $V\delta2^+$ and $V\delta1^+$ T cells might determine their subsequent responses, both in vivo and in vitro.

Data from a recent study strongly suggest anti-tumour properties to $V\delta1^+$ T cells. It was found that both $V\delta1^+$ and $V\delta2^+$ T cells secrete both Th1 and Th2 cytokines, and tumour growth promoting factors such as angiogenic growth factors such as angiogenin (ANG), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and Insulin-like growth factor (IGF)-I but when stimulated by neuroblastoma tumor cells, $V\delta2^+$ cells down-regulated the production of Th1 cytokines and strongly up-regulated tumour growth-promoting factors whereas $V\delta1^+$ cells stopped production of TGF-B and tumour growth-promoting factors, maintained IL-2 production and strongly up-regulated other Th1 cytokine production (Schilbach *et al.*, 2008).

However, if $\gamma\delta^+$ or $V\delta1^+$ T cells control the expansion of B cells, then eBL should not be mentioned among people, especially children, from malaria endemic areas where the proportion of $\gamma\delta^+$ or $V\delta1^+$ T cells is found to be relatively high. Reports have shown loss of

control of EBV^+ cells by T cells during malaria (Dalldorf, 1962). However, what account for the lack of effective immunosurveillance by $V\delta1^+$ T cells during malaria is yet to be fully unraveled. Though there is speculation that effector functions of $V\delta1^+$ T cells might be lost due to immunosuppression, which is characteristic of malaria infection; thus, rise in EBV^+B cells due to lack of effective control and hence development of eBL in malaria endemic regions. It could also be that the repertoire of $V\delta1^+$ cells during malaria, are specific for malaria but not EBV+ B cells or BL. Another possibility is that $V\delta1^+$ cells are overwhelmed by rise in the number of EBV+ B cells, activated EBV- B cells and BL cells that they have to deal with during persistent malaria. B cell activation is known in malaria and activated $\gamma\delta^+$ cells, particularly $V\delta1^+$ cells that are believed to respond to self-antigens express by activated B cells in malaria has also be found (Freeman and Parish, 1978; Banic *et al.*, 1991; Hviid *et al.*, 2001).

2.8 Immunity to Malaria

2.8.1 Innate (Non-specific) Immunity to Malaria

Certain host factors are found to confer some resistance to malaria infection. Absence of the Duffy blood group is known to protect against *P. vivax* infection. Genetic factors such as β-thalassaemia, which influences the rate of haemoglobin synthesis; glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, an important erythrocyte metabolic enzyme and sickle cell trait are also found to impair intraerythrocytic developmental stages of the parasite. The reticulo-endothelial system in the liver and spleen assists in this regard by clearing parasitized cells from circulation through phagocytosis (Bruce-Chwatt, 1985; Friedman, 1978). However, it is believed that this clearance involves unparasitized erythrocytes as well, thus leading to severe anaemia (Dondorp *et al.*, 1999).

2.8.2 Humoral Immunity to Malaria

Epidemiological studies conducted in areas of stable malaria transmission have shown an age-related increase in malaria specific antibodies and consequent decrease in morbidity. It has been established that repeated exposures to infection over the years leads to acquisition of antimalarial antibodies, which can be lost if infection is not regular, due to loss of immunological memory (Deloron and Chougnet, 1992; Egan *et al*, 1996; Sarthou *et al.*, 1997).

In malaria endemic regions, human foetuses and newborn babies are found to be protected from malaria attack by a substance believed to be an immune-mediator transferred from their immune mothers across the placenta (Bruce-Chwatt, 1952; Reinhardt *et al.*, 1978). Studies have also established protection of infants from malaria in early life through passive transfer of antibodies from their immune mothers through breastfeeding (Akanmori *et al.*, 1995; McGregor, 1984). The overall level of antimalarial antibodies is found to have strong association with degree of exposure to infection (Marsh, *et al.*, 1989) and in areas of persistent malaria transmission; it increases with age reaching a plateau during early adult and remains high for the rest of life (McGregor *et al.*, 1970). Thus general Ig and total antimalarial antibodies are found to be high in residents of malaria endemic areas (Bolad and Berzins, 2000). However, it has been established that antibody responses of children and adults differ regardless of degree of exposure (Baird, 1995). Antibody responses induced during malaria infection are, so far, found in immunoglobins (Ig); IgA, IgG, IgM (Collins *et al.*, 1971; Targett, 1970), and more recently, IgE (Perlmann *et al.*, 1999). No antimalarial antibody has yet been demonstrated in IgD.

Studies have shown that IgG is more persistent than other antimalarial immunoglobulins and has a strong correlation with malarial precipitins in plasma of donors at all ages over a year (McGregor, 1970). Moreso, passive and artificial transfers of IgG confer protection against *P. falciparum* infection (McGregor *et al.*, 1963). The persistence and association of IgG and malaria antigens suggest that IgG may play an important role in immunity to malaria parasites. Protective malaria-specific IgG1 and IgG3 responses have now been demonstrated(Farouk *et al.*, 2005). On the other hand, it has been found that IgM levels rose sharply in association with parasitemia but declined drastically when chemotherapy was completed, although malarial antigens were still in circulation (Targett, 1970). This may suggest that IgM response may be more to disease than to parasite.

Malaria parasites have also evolved ways of inducing immunosuppression and diverting immune responses to repeated regions of surface antigens, eliciting production of redundant non-protective B-cell responses (Anders, 1986). It has also been reported that certain immunodominant epitopes divert responses away from more important targets in the antigenic variation (Howard, 1987). In children, antibodies to these critical antigenic targets are not fully developed making them more vulnerable to malaria attack (Baird, 1995).

Recently, elevated levels of both total IgE and antimalarial IgE antibodies have been shown in malaria patients (Perlmann *et al*, 1999) and its levels are found to be significantly higher in patients with cerebral malaria than those with uncomplicated falciparum malaria. This makes researchers believe that IgE may play a role in the pathogenesis of cerebral malaria. Moreover, TNF-α, a cytokine found to correlate with severity of *P. falciparum* malaria attack (Grau *et al.*, 1989), is found to be associated with IgE.

2.8.3 αβT-cells and Malaria

Whereas the cytotoxic activities of $CD8^+$ against blood stage of the parasite seems to be non-existent, they appear to protect against pre-erythrocytic stage with their activities directed against infected hepatocytes (Hockmeyer and Ballou, 1988). Th1 cells are found in some rodent malaria to produce IFNγ and IL-2 and are important in controlling infection at its early stages. Th2 cells on the other hand, secrete IL-4 and IL-10 and by these cytokines, induce B-cells to produce antibodies. These Th2 responses are found to be vital for protection against malaria parasites in late phase of infection (Troye-Blomberg *et al.*, 1994). Brain-sequestered CD4+ and CD8+ T cells and cytokines such as IFN-γ produced during infection are known to be involved in mediation or development of experimental cerebral malaria (Yanez *et al.*, 1996; Hermsen *et al.*, 1997; Belnoue *et al.*, 2002)**.** Early production of IFN-γ is associated with protection from murine CM (Mitchell *et al.*, 2005), a later production of IFN-γ is related to the development of CM (Grau *et al.*, 1989). A study has shown that CD4+ T cells stimulated by malarial antigens produce cytokines that activate γδ T cells to proliferate and provide protective cellular immunity against the malaria parasite (Elloso *et al.*, 1996). Protective immunity against *P. yoelii* in resistant mice due to a CD4+ Th1 response has been found to involve IFN-γ and TNF-α together (Steveson *et al.*, 1995). Depletion of CD4+ T cells has been found to adversely affect the clearance of *P. chabaudi in vivo* showing the important role of these cells in antiparasite immunity (Langhorne *et al.*, 1990). Though antibody immunity to *P. falciparum* antigens is promising in the development of vaccine, studies over decade have shown that CD4+ T cells, independent of antibody, can also control parasitemia. In this regard, adoptive transfer of specific CD4+ T cells have been demonstrated to confer protection in murine malaria models (Langhorne *et al.*, 1990; Amante and Good, 1997).

However, Th1 cytokines have been implicated in malaria and adoptive transfer of CD4+ Th1 cells and found to promote disease in mice (Hirunpetcharat *et al.*, 1999). Individuals who have not been exposed to malaria before have CD4+ Th1 cells with an activated phenotype that respond strongly in vitro to malaria parasites (Currier *et al.*, 1992). It is therefore hypothesized that these cells having Th1 cytokine profile could contribute to pathology in individuals that are exposed to malaria for the first time (Good *et al.*, 2005).

2.8.4 Regulatory T cells and Immunity to Malaria

2.8.4.1 CD4+CD25+ T Regulatory Cells

Since Long and his colleagues (Long *et al.*, 2003) reported the involvement of these T regulatory cells in immune responses to murine *P. berghei* malaria, several studies have demonstrated the role of CD4+CD25+ T regulatory cells in the pathogenesis of malaria. In murine models, they are known to increase in response to malaria and their depletion is associated with contradictory results of decreased, no effect on or increased parasitemia, reduced, no effect on or increased disease severity, no effect on mortality or reduced mortality, enhanced IFN-γ responses and increased proliferation of CD4+ T cells (Finney *et al.*, 2010b).

CD4+CD25+ FoxP3+ T regulatory cells are known to increase in number and are associated with high IL-10 and low IFN-γ levels in murine malaria. Just like CD4+CD25+ T regulatory, CD4+CD25+ FoxP3+ T regulatory cells depletion experiments are also known for conflicting results of decreased, no effect on or increased parasitemia, reduced, no effect on or increased disease severity, no effect on mortality or reduced mortality, enhanced IFN-γ production,

reduced IL-10 secretion and higher pro-inflammatory responses and higher levels of apoptosis (Finney *et al.*, 2010b).

In human studies of malaria, it was observed that CD4+CD25+ T regulatory cells are associated with decreased pro-inflammatory responses and enhanced parasite growth in vivo and their depletion led to increase in IL-2 production and CD8+CD25+ cells. Genetic resistance to *P. falciparum* malaria observed in Fulani is known to be associated with fewer CD4+CD25+T regulatory cells. A study has shown that individuals who have high levels of CD4+CD25+ T regulatory cells prior to infection with *P. falciparum* have increased susceptibility to malaria (Walther *et al.,* 2005; Torcia *et al*., 2008; (Stevenson *et al.,* 2011). These studies therefore confirmed the immunosuppresive role of CD4+CD25+ T regulatory cells. CD4+CD25- FoxP3+ Treg cells have also been identified. Analyses revealed that they phenotypically and to a certain extent functionally resemble conventional Treg cells (Bonelli *et al.,* 2009) whereas Foxp3 is expressed on effector cells that are not Tregs following activation (Godfrey, 2005)

It is becoming clear that the inconsistencies observed in both murine and human malaria are as a result of incomplete characterization of the Treg cells (Finney *et al.,* 2009). It is clearly established that Treg cells are induced and expand in response to *P. falciparum* malaria. Perturbations in Th1/Treg ratio have been demonstrated between acute disease and during convalescence in both severe and uncomplicated malaria but no significant difference were observed between severe and uncomplicated malaria. It is therefore suggested that loss of homeostasis as a results of outpacing of Treg response by Th1 response leads to development of clinical disease (Finney *et al.,* 2009). This suggestion indicates that immunosuppressive

activity of T reg cells may not play a role in the development of severe malaria. It is not clear whether they prevent effector T cells from developing into memory cells through suppression of parasite induced IL-2 secretion or through their control of Th1 responses that promotes persistence of asymptomatic malaria and maintenance of immunological memory. A study has shown that high proportions of circulating FOXP3+ Treg cells are associated with low fractions of CD4+ T cells with memory phenotype in infants (Rabe *et al.*, 2011) and in a recent study, it was observed that individuals living in regions with holoendemic malaria transmission have more EBV-specific CD8+ T-cell populations with fewer central memory cells than individuals living in malaria-free or hypoendemic malaria regions (Chattopadhyay *et al*., 2013).

It has been found that $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells suppress phosphoantigen-mediated Vγ9Vδ2 T-cell line proliferation in vitro via a cell–cell contact-independent mechanism by secreting of a soluble non-proteinaceous factor. This mechanism was found not to have effect on the proliferative activity of αβ T cells (Kunzmann *et al.*, 2009). This seems to suggest that upregulation of $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells during malaria may account for low percentage of Vγ9Vδ2 T cells in individuals from malaria endemic region.

In conclusion, FoxP3 expressing Treg cells such as CD4+FoxP3+ and CD4+CD25+FoxP3+ cells, in humans, are known to play an important role in maintaining immune homeostasis. They are found to influence the Th1/Th2 immunological balance, which can tip either way leading to susceptibility to infection or immunopathology (Finney *et al.*, 2009).

2.8.5 γδT-cells and Malaria

In malaria endemic areas levels of $V\delta1^{+}$T cells were higher than that of $V\gamma9^{+}V\delta2^{+}$T cells in both adults and children (Goodier *et al.*, 1993; Hviid *et al.*, 1996; Hviid *et al.*, 2000; Hviid *et al.*, 2001). It has been shown that gammadelta-T cells, not NK cells, are the predominant producers of innate IFN-γ when stimulated with live schizont-infected red blood cells (iRBC). These malaria-responsive γδT cells cells were also shown to express NK receptors (D'Ombrain *et al.*, 2007) indication that they do not only compliment the activities of natural killer T cells (Choudhury, *et al*., 2000), which are major plays in th innate immune responses, but also share some characteristics with them.

A number of studies have shown significant increase in the levels of γδT cells during *P. falciparum* infection in adults (Hviid *et al.*, 2000). But the elevation of γδT cells was found not to be associated with disease severity (Ho *et al.*, 1990). It has been shown in a mouse model that γδT cells proliferate in response to rises in parasitemia and play an important role in controlling it (Langhorne, 1996). Growth inhibition of *P. falciparum* by γδT-cells *in vitro* has also been confirmed and their response has been found to be associated with products from schizont rupture (Elloso *et al*, 1994). It has been suggested that cytotoxic activities of these cells may take place in the spleen, since they are found to be localized in the spleen (Troye-Blomberg *et al*, 1994; Langhorne, 1996). They were also shown to control liver stage of the parasite in experimental mice (Langhorne, 1996). Some researchers have shown that in both acute *P. falciparum* infection and *in vitro* system, the elevated subset of γδT-cells was $V\gamma9^{+}V\delta2^{+}$T cells *in nonimmune patients* (Goodier *et al.*, 1995). In vitro experiments have demonstrated polyclonal expansion of γδ T cells obtained from unexposed non-immune

individuals in response to stimulation with *P. falciparum* merozoites, schizonts, and circumsporozoites (Goerlich *et al*., 1991; Bender *et al*., 1993).

It seems the parasite do not stimulate γδ T cells only directly but also induce production of IL-2 which then stimulate activation and expansion of γδ T cells. Moreover, it has been shown that live parasite but not parasite extract induced significant IL-2 production in vitro and the researchers suggested that a labile product of schizonts that mimic IL-2 might be involved in early response of γδ T cells to live schizonts instead of PfSE. Response to PfSE was observed after 7 days of stimulation (Waterfall *et al*., 1998).

Costa and his colleagues (Costa *et al.*, 2011) have also shown that both intra-erythrocytic parasites and the extracellular red blood cell-invasive merozoites specifically activate Vγ9Vδ2 γδ T cells but the γδ T cell-mediated anti-parasitic activity only targets the extracellular merozoites. They have also demonstrated that granulysin is essential for the *in vitro* anti-plasmodial activity and its levels increase in association with high levels of granulysin-expressing $V\delta2^{+}$ T cells endowed with parasite-specific degranulation capacity in patients with *P.falciparum* infection. Therefore the stimuli inducing γδT-cell response are not only parasite antigens but also products of other cells such as granulysin produced by CD8+ (cytotoxic) T cells, natural killer (NK) and natural killer T (NKT) cells, IL-2 by mainly T cells and products of parasite metabolism.

Most of these results were obtained from experiments involving samples from donors that were unexposed or non-immuned to malaria. Therefore the findings, such as potential protection against malaria, are based on the activities of $V\gamma9^{+}V\delta2^{+}$T cells, which are majority of γδT cells of donors that are non-immuned to malaria. However, in earlier studies in non-

immuned patients, an increase in the percentage and absolute number of both Vδ1$^-$ γδT and Vδ1$^+$ cells of γδT cells in the peripheral blood during acute infection has been observed (Ho *et al*, 1994). Although there was no in vitro proliferative response by the γδT cells of these patients to various malarial antigens, they increase in number when stimulated with IL-2 in vitro and the results show that there was a proportional expansion in Vδ1$^+$ cells, though the main source of expansion of the γδT cells was Vδ1$^-$ γδT cells (Schwartz *et al.,* 1996). This shows that Vδ1$^+$T cells also expand in response to clinical malaria, at least, in acute infection.

It has also been established that healthy donors from malaria endemic areas have higher levels of γδ$^+$T cells (>10% of T cells) compared to Caucasians (<5% of T cells) and the levels of these cells rise further during *P. falciparum* malaria infection. The expansion of γδ$^+$T has been found to be mainly due to expansion of Vδ1$^+$T cells which are also majority subset of γδ$^+$T cells in donors from malaria endemic areas; both adults and children (Goodier *et al.*, 1993; Hviid *et al.*, 1996; Hviid *et al.*, 2000; Hviid *et al.*, 2001). This buttresses the earlier findings that suggest that Vγ9$^+$Vδ2$^+$T –cell is not the only subset responding to malaria infection. It also implies that the history and frequency of exposure to *P. falciparum* infection of the host has a bearing on the response of Vδ1$^+$T cells subsets of γδT cells. However, the role of γδ$^+$T cells in pathogenesis of malaria and eBL is still not clear.

A study has demonstrated that NK and γδT were critical in survival and protection from *Plasmodium yoelii* infection in mice (Taniguchi *et al.*, 2007). The protective role of γδT cells has been buttressed by another study that has shown that it is γδT cells not NK cells are essential for cellular protective responses against *P. chabaudi* malaria (Weidanz *et al.*, 2010).

Lymphokines secreted by γδ^{+}T cells are known to stimulate macrophages (Goodier *et al.*, 1995), thus may assist in primary infection when there are no specific memory cells. In a study where the productions of IFN-γ and IL-4 by γδ^{+}T cell clones were investigated, various patterns emerged. Some clones produce about the same amount of both cytokines, therefore exhibiting Th0 phenotype; some produce only either IFN-γ or IL-4 showing Th1 or Th2 phenotypes respectively. These patterns were found not to be γδ Vδ1 phenotype –restricted as they were seen in clones of only Vγ9Vδ2 T cells. However, most of the clones produce IFN-γ and in high amounts and low mean levels of IL-4 were produced by less than twenty percent of the clones. Vδ1^{+} cells were found to produce high levels of IFN-γ and low levels of IL-4. In comparing secretion of the two cytokines between γδ and αβ T cells, they found that γδ^{+}T cell clone produce lower levels of IFN-γ and higher levels of IL-4 than αβ T cell clones (Chomarat *et al*., 1994). On the contrary, intracellular secretion of intracellular IFN-γ was found in almost twice as many TCR-γδ cells and Vδ1+ cells as among CD3+ cells in children with malaria from a malaria endemic region. However, TCR-γδ cells and Vδ1+ cells produced substantially less TNF-α than CD3 cells (Hviid *et al.*, 2001). Just like Vδ1^{+} cells, Vγ9Vδ2 T cells also produce TNF-alpha (Dunne *et al.*, 2010). From the above it can be seen that both Vδ1^{+} cells and Vγ9Vδ2 T cells produce IFN-γ, TNF-α and IL-4. Vδ1^{+} cells were found to produce less IFN-γ and TNF-α compared to Vγ9Vδ2 T cells from donors that not immuned to malaria (Christmas and Meager, 1990). But γδ^{+} cells from children that are exposed to malaria have higher IFN-γ+ Vδ1^{+} cells than IFN-γ+ Vγ9Vδ2 T (Hviid *et al.*, 2001).

Study has also indicated that γδT cells from donors that are not immune to malaria can be polarized into either Th1 or Th2 cytokine pattern depending on the cytokines in the

environment in which contact with antigen by γδ+ cells occurs as well as the type of antigen they are responding to and can produce IFN-*γ* and TNF-*α,* IL-4, IL-5, IL-10, IL-13 and IL-17 (Wesch *et al*., 2001; Caccamo *et al*., 2006; Vermijlen *et al*., 2007; Dunne, 2010).This phenomenon has also not been adequately study in donors from malaria endemic areas in which the majority of γδ cells is $V\delta1^+$ cells. Moreover, most of these studies were in vitro and whether the same pattern can be obtained in vivo is yet to be unravelled. $\gamma\delta^+$T cells are also known to produce TGF-β and the main producer being $V\delta1^+$ subset. In *in vitro* system these $\gamma\delta^+$T cells were found to have more regulatory potential than CD4+ CD25+ cells (Kuhl *et al*., 2009).

However, $\gamma\delta^+$T cells are implicated in the pathogenesis of malaria due to their stimulative response to a stage of parasite associated with disease development (Goodier *et al*, 1995). Also, $\gamma\delta^+$T cells are implicated in the pathogenesis of malaria because $\gamma\delta^+$T cells are pronounced during infection in non-immune donors who are susceptible to severe disease (Miossec *et al*, 1990; Perera *et al*, 1994). Moreover, cytokines produced by $V\gamma9^+V\delta2^+$T cells have been associated with pro-inflammatory response and especially, TNF-α has been associated with severe and cerebral malaria (Goodier *et al.*, 1995; Grau *et al.*, 1989). It has been shown that treating cerebral malaria susceptible mice with anti-γδT cell antibody, results in failure of the mice to develop the disease, indicating that γδT cells play a role in the development of cerebral malaria (Yanez *et al.*, 1999). Studies have demonstrated that presence of parasitized erythrocytes and infiltration of lymphocytes in cerebral vessels preferentially, accumulation of γδT cells in the brain is associated with signs of endothelial damage (Haque *et al*., 2001).

Although studies have shown that activation and early production of IFN-γ and TNF-α by $\gamma\delta$T cells, complementing the activity of natural killer T cells, in the early control of parasitemia (Choudhury, *et al*., 2000) and also in pathogenesis of CM (Yanez *et al*., 1999), the mechanisms are yet to be fully understood. The expansion and phenotypic characteristics of $V\delta1^+$ T cells in AIDS patients suggest that these cells may be involved in the pathogenesis of AIDS too (Boullier *et al*., 1997). It could also mean that they expand to augment the shortfall in the activity of CD4+ cells. However, proliferative response was found to diminish in $V\gamma9/V\delta2$ T lymphocytes obtained from HIV^+ donors (Wallace *et al*., 1997).

Although Vδ2 + cells account for most of the expanding cells in non-immune adult patients, those of patients of endemic regions are the minority and do not expand further after re-infection (Goodier *et al*., 1993) and it has been suggested that $V\delta1^+$ malaria-reactive cells may suppress the $V\delta2^+$ response in these patients (Schwartz *et al*., 1996) .

2.9 The Role of Malaria in the pathogenesis of eBL

It has now been established beyond doubt that malaria is a cofactor in the pathogenesis of eBL and there are speculations that suggest that one of the major roles of malaria and EBV infections may be to provide an additive risk for development of B-cell clones with chromosome translocations leading to constitutive *c-myc* activation. This is based on the background that neither malaria alone nor EBV alone provides sufficient B-cell stimulation to result in a noticeable increased risk for eBL. However, the existence of EBV^- and non-malaria related BLs (Adams, *et al.*, 1983; Dalla-Favera, *et al.*, 1982; IARC, 1998) suggest that each factor can be replaced by other mechanisms.

Malaria could contribute to eBL development in various ways. B-cell activation also occurs in malaria and the number of B cells rises with the general number of lymphocytes and at the same time malaria confer protection against B cell apoptosis (Whittle *et al.*, 1984; Donati *et al.*, 2006). Thus everything is in favour of expansion of B cells. Studies have also shown that malaria promotes EBV reactivation and one of such studies has *P. falciparum* antigens such as PfEMP1 can directly induce EBV reactivation and increase viral load during malaria infections (Rasti *et al.*, 2005; Chene *et al.*, 2009). This also increases the risk for eBL development. A study has also shown that hemozoin, the end-product of haemoglobin metabolism by intraerythrocytic malaria parasites, is an important factor in malaria-associated immuno-incompetence. It is found to affect both antigen processing and immunomodulatory functions of macrophages (Scorza *et al.*, 1999). Plasmodial infection is associated with rise in the level of IgE in the blood of the majority of people living in malaria endemic areas and only up to five percent (5%) are anti-malarial antibodies. Fc-IgE is known to interact with IgE receptor (CD23) and increases the expansion of B cells (Perlmann *et al*, 1999). Additionally, children with malaria are found to have very high serum levels of IgG and IgM, most of which are not anti-plasmodial antibodies. The levels plateau after the age of five to six years (McGregor, 1970) coinciding with the peak age of incidence of BL in holoendemic malarious areas (Molineaux and Gramiccia, 1980) but how abnormal levels of IgG and IgM could contribute to development of BL is not clear.

There is no explanation for the fact that about ninety percent (90%) of the world population is latently and permanently infected with EBV (Magrath, 1990) and yet only a few children suffer from BL. This may be due to the fact that in healthy immunocompetent EBV-carrying host; there is an efficient immune surveillance of EBV-carrying B-cells in place. During *P.*

falciparum malaria the immune surveillance may be disturbed as a result of imbalances in the immune regulation. Children already have underdeveloped immunity (Baird, 1995) and therefore their immune mechanisms can easily be derailed making them more vulnerable to BL. The contribution of malaria is believed to be due to the imbalances in the immune regulation during malaria infection but this is yet to be fully proven. Several studies have pointed to immunosuppression (Geser *et al.*, 1989; Whittle *et al.*, 1984, 1990), which is a common feature in acute *P. falciparum* infection, as an important factor that could lead to increased susceptibility to eBL. Several factors may account for the immunosuppression observed in *P. falciparum* malaria. It has been found in a murine model that chronic malaria leads to a shift in helper T cell response towards Th2 cells (von der and Langhorne, 1993a; von der and Langhorne, 1993b) It has also been shown in a study that *in vitro* stimulation of lymphocytes with malaria antigens induces secretion of cytokines with Th2 profile such as IL-10 and TGFβ (Wahlgren *et al.*, 1995). The cytokines secreted by Th1 are very vital in mounting protective immunity especially, against intracellular infectious agents. Skewing of the helper response towards Th2 implies a rise in IL-10 secretion by Th2 cells, and IL-10 is known to suppress the functions of T cells, particularly CTL function. IL-10 is also found to act as an autocrine growth factor for B cell (Mosmann and Coffman, 1989).

Malaria could contribute to the pathogenesis of eBL by way of Treg cells as well. Malaria is known to promote the development and function of Treg cells which are known to promote immunological tolerance. Immunological tolerance is one of the underlying factors in tumourigenesis. It has been shown that intratumoral T reg cells inhibit proliferation and function of CTLs and their depletion improves the efficacy of anti-tumour vaccines (Yang *et al.*, 2006). The reported diminished specific protective responses against eBL development in

children living in malaria-holoendemic areas (Moormann *et al*., 2007) and deficiency in such responses in children with eBL (Moormann *et al.,* 2009) could be promoted by T reg cells. Treg cells such as CD4+FoxP3+ and CD4+CD25+FoxP3+ cells, in humans, can suppress Th1 responses which are vital for immunity against EBV-infected and BL cells. Increase in immunosuppressive activities of these cells during malaria is known (Long *et al.*, 2003) and high levels of these cells in individuals from malaria endemic regions, due to persistent malaria could contribute to development of eBL. A recent study has shown that high proportions of circulating FOXP3+ Treg cells are associated with low fractions of CD4+ T cells with memory phenotype in infants (Rabe *et al.*, 2011). Memory cells are very important in mounting adequate immunity against any subsequent infection after first encounter. Activities of malaria-induced Tregs could account for diminished EBV-specific Th1 responses observed in children living in malaria-holoendemic areas (Moormann *et al*., 2007). This may be through cytokine –driven apoptosis of effector CD4+ T cells (Pandiyan *et al*., 2007) but the possible role and the mechanisms of involvement of T reg cells are yet to be unraveled. Figure 4 below shows the suppressive effects on Th1 responses and development of memory cells. Therefore, hypothetically, as a consequence of all the above, the number of B-lymphocytes latently infected with EBV will increase while the ability of T cells to suppress the outgrowth of EBV-infected lymphoblastoid cells is impaired. This implies that acute *P. falciparum* malaria may amplify the pool of EBV^+ B cells prone to accumulate oncogenic changes and undergo transformation, which are major events in eBL pathogenesis and also suppress the ability of CTLs to kill the tumour cells.

The course of the major events in eBL pathogenesis in children is believed to be EBV-infection early in life, followed by persistent exposure to malaria also in early life and then the oncogenic process.

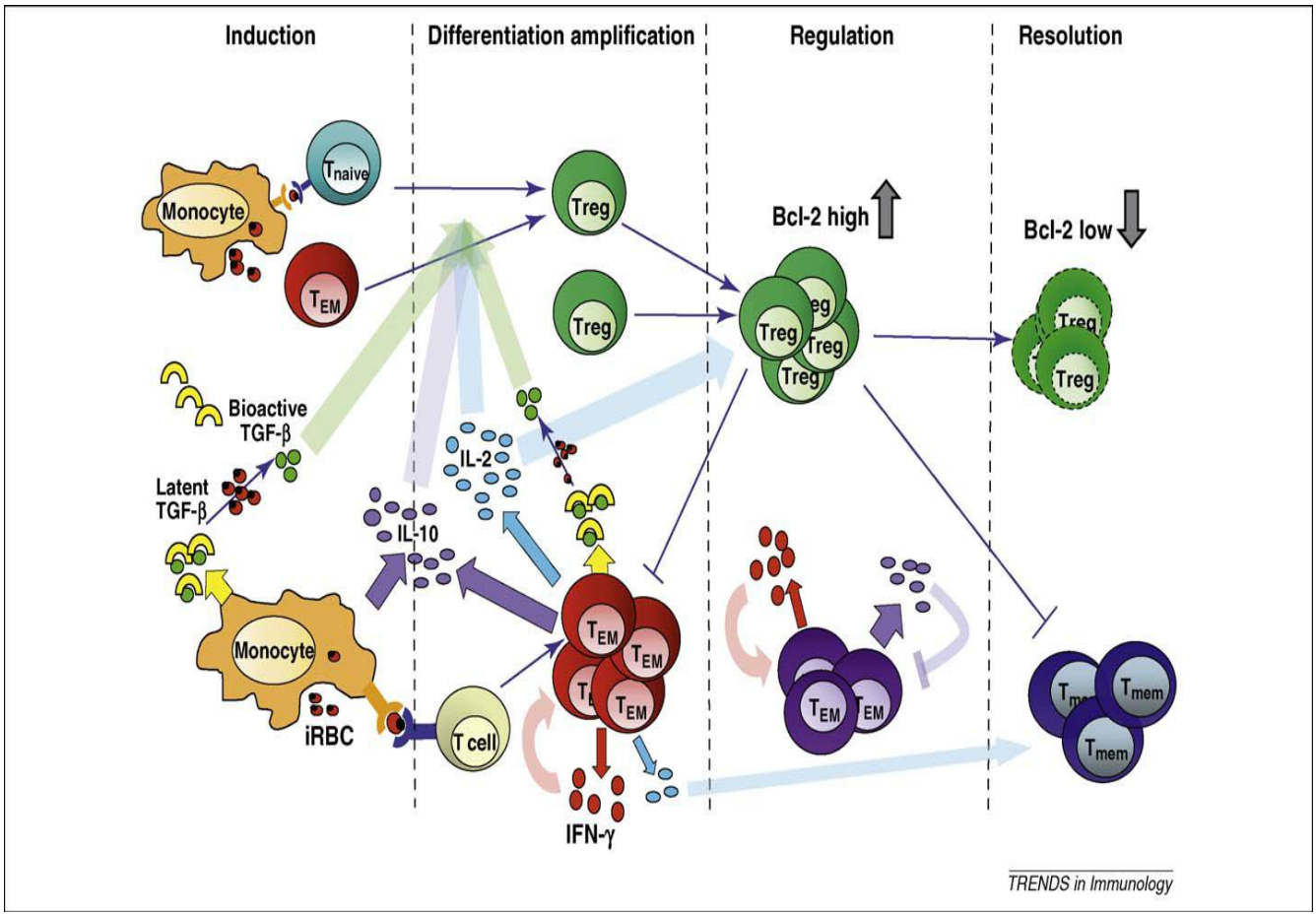


Figure 4: Proposed model for the role of Treg cells in malaria infection. Induction, Differentiation and Amplification (Finney *et al.*, 2009). (iRBC) - malaria-infected red blood cells, TEM- effector memory T cells, Tmem- memory T cells.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Population and Inclusion Criteria

The study was conducted in children with endemic Burkitt's lymphoma. The ages of study participants range between 1-16 years old. Patients were enrolled at the Korle-Bu Teaching Hospital. Age and sex matched healthy controls without parasitaemia and children with malaria were also enrolled for the study. Healthy control subjects and children with malaria were enrolled from the University of Ghana staff village school, and University of Ghana hospital and the Ghana Atomic Energy Commission (GAEC) clinic. The University of Ghana hospital is attended primarily by students and staff of the university as well as people living within the surrounding communities. Permission was sought from the Ghana Education service (GES) health inspectorate to enroll student from the University of Ghana Staff village school. Parents of pupils enrolled as controls gave their signed informed consent before they were enrolled for the study. The Institutional Review Board at Noguchi Memorial Institute, and the University of Ghana Medical School Scientific Research and Ethical Committee both granted ethical approvals for the study.

3.2 Sample Collection and Processing

Blood was collected by qualified personnel (pediatricians for patients and a technician for healthy controls) from children into sterile heparin and EDTA tubes (BD Vacutainer™) using sterile safety-lok™ blood collection set. The samples were then transported to the Immunology Department of Noguchi Memorial Institute for Medical Research (NMIMR) and

processed within two hours. Additional data on the frequency of stages of eBL presentation were obtained from patients' folders.

3.2.1 Isolation of Peripheral blood mononuclear cells (PBMC) from whole blood

Blood was spun at 2000rpm for 7 minutes and some plasma was removed and stored in vials at -80^{o}C. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Histopaque (Sigma-Aldrich) density gradient centrifugation. The venous blood was diluted with equal volume of RPMI1640 supplemented with 1% penicillin/streptomycin and L-glutamine. It was carefully overlaid the frit above Ficoll-Histopaque in a 10ml cell separator tube (LeucoSep™ tube; Sigma-Aldrich) and spun at 2000 rpm for 10 minutes. PBMC layer above the frit at the interface between the Ficoll-Histopaque and the plasma- medium mixture was removed using a sterile transfer pipette and washed three times in RPMI1640 containing 10% heat-inactivated foetal calf serum (FCS) supplemented with penicillin/streptomycin, and L-glutamine. Staining was done with Trypan blue to estimate PBMC concentration and ascertain cell viability. The PBMC were then dispersed in a cold freezing mix (10% DMSO in FCS), aliquoted into cryotubes, placed in Mr Frosty® (Nalgene cryo1°C freezing container, USA) and frozen at -80^{o}C overnight. The cells were removed from the -80^{o}C freezer the following day and cryopreserved in liquid nitrogen. The blood samples and the isolated PBMC, except during staining, were handle under sterile condition in a biosafety hood (Walker Safety Cabinet Limited, U.K, Figure 5).

Figure 5: Processing of sample in a biosafety hood (Walker Safety Cabinet Limited, U.K)

3.2.2 Estimation of Cell Concentration and Examination for Cell Viability

Small volume of cell suspension (25ul) was added to a known volume of Trypan Blue (75ul) in an eppendorf tube and mixed using a vortex. The stained cells were examined for viability and counted using the Improved Neubauer haemocytometer and 40X objective lens of the light microscope. The cell concentration was estimated using the equation below:

$$\text{Cell concentration} = N \times \text{dilution factor} \times 10^4 \text{ cells/ml}$$

Where, N= Average count per chamber. The dilution factor depends on the amount of stain and volume of cell suspension added to the stain.

3.3 Parasitological and Haematological Examinations

An automated haematology analyzer (Sysmex KX-21, Japan) was used to determine all the haematological parameters of the participants. The absolute counts of lymphocytes were determined from this analysis. All the venous blood samples were examined for presence of parasite infected red blood cells to confirm infection with *P falciparum* and also to exclude asymptomatic healthy donors. Thick and thin blood smears were prepared, dried and the thin smears fixed in methanol. The films were then stained with freshly prepared 10% Giemsa (Laboratory Supplies, Poole BH15 ITD, England), washed cautiously and thoroughly under running tap water. The slides were dried and observed with immersion oil under a light microscope (Olympus BH2, Japan) at 100x magnification, for the presence of *P. falciparum* infected red blood cells.

3.4 Cell Phenotyping by Flow Cytometry

3.4.1 Surface Staining

During flow cytometric analysis, the PBMC were quickly thawed in a water bath at 37˚C and washed (centrifuged at 1500rpm) twice in RPMI1640 containing 10% heat-inactivated (FCS) supplemented with penicillin/streptomycin, and L-glutamine. The cells were then stained with Trypan blue to ascertain cell viability and viable cell concentration adjusted to $1x10^6$/ml in a staining buffer, and stained with combinations of T-cell subset, activation marker (CD25, CD69, CD95, HLA-DR), IFN-γ-, IL-4- or FoxP3-specific monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or PE-Cy5. Surface staining was done with antibodies directed against CD3 (HIT3a; BioLegend, San Diego, CA), CD4 (RPA-T4; BioLegend), CD8 (RPA-T8; BioLegend), CD25 (BC96; BioLegend), CD69 (FN50; BioLegend), CD95(DX2; BioLegend), HLA-DR(LN3;

BioLegend), TCR-γδ (B1; BioLegend), Vδ1 (TS8.2; Thermo Scientific), Vγ9 (B3; BioLegend).

Three microliters (3ul) of the antibodies were added to the cells and mixed using a vortex. Stained PBMC were incubated at room temperature in the dark for 15 minutes. After incubation, the cells were washed with FACS Buffer (spun at 1500rpm for 7 minutes) three times with supernatants decanted. Cells were re-suspended in 200ul of FACS buffer for acquisition or re-suspended in 1ml of fixation/permeabilization buffer and taken through intracellular staining.

3.4.2 Intracellular Staining

Where intracellular staining for cytokines was involved, PBMC were first stimulated with antigens or mitogens of interest before any staining was done as described below. Before intracellular staining, the cells were taken through surface staining with antibodies directed against CD4 (RPA-T4; BioLegend), CD8 (RPA-T8; BioLegend), CD25 (BC96; BioLegend), TCR-γδ (B1; BioLegend) and Vδ1 (TS8.2; Thermo Scientific), FoxP3 (PCH101, eBiosciences), IFN-γ (4S.B3; BioLegend) and IL-4(MP4-25D2; BioLegend) as described above. Intracellular staining was done using FoxP3 staining buffer set (cat 00-5523, eBiosciences) and according to manufacturer's instructions.

Surface-labelled PBMC were fixed and permeabilised by adding1ml of freshly prepared fixation/permeabilization buffer and incubated in the dark at room temperature for 20 minutes. After incubation, cells were washed twice with 2ml of 1x Permeabilization Wash Buffer and supernatant carefully aspirated each time. The fixed/permeabilized cells were re-suspended in residual Permeabilization Wash Buffer. The 3ul of antibodies for intracellular

staining; Foxp3 (PCH101, eBiosciences), interferon-gamma (IFN-γ,) and interleukin-4 (IL-4) were then added to the cells, pulse vortexed and incubated in the dark for 20 minutes. After incubation, the PBMC were firstly washed with 2ml of 1x Permeabilization Buffer and secondly with 2ml of Flow Cytometry Staining Buffer. The stained cells were then re-suspended in 200ul of Flow Cytometry Staining Buffer for acquisition.

3.5 In vitro Stimulation

PBMC were cultured for 6 hours in the presence of stimulants such as EBNA1 peptide pool and PHA at 1.5 x 10^6 PBMC/well in 150 uL medium in 96 flat-bottom plates (culture medium: RPMI supplemented with penicillin/streptomycin and 10% human pool AB serum). After 2 hours, 1:1000 brefeldin A was added to allow accumulation of cytokines in the cytosol and incubated for the rest of the 6 hours. 3ml of EBNA-1 peptide pool of stock concentration of 30nmol of each peptide per ml was added to each well making a final concentration approximately 0.6 nmol/ml (or1ug/ml) of each peptide. The pool of EBNA-1peptides consists mainly of 15-mer sequences with 11 amino acids overlap, covering the compete sequence (except the GA region) of the EBNA-1. Therefore the peptides span all the regions (including the entire C-terminal region) of the protein encompassing all possible epitopes for any potential HLA type presentation. PHA was added as a positive control at a final concentration of 5ug/ml. Medium without stimulant was included as a negative control.

After stimulation, the cells were carefully collected by pipetting up and down and wells rinsed with buffer. The cells were then washed in FACS buffer and aliquoted at a minimum of 100,000 cells in100ul for surface and intracellular staining as described above.

3.6 Acquisition

Flow cytometry acquisition was done using a FACScan flow cytometer (Becton Dickinson, Japan). Two or three-color flow cytometry analyses panels were employed. Appropriate isotype controls were also analyzed. Instrument parameters were checked and optimized using CaliBRITE beads (Becton Dickinson) before data acquisition. Data were acquired with Multiset CellQuest software (Becton Dickinson), where Gates were set to record events with forward and side scatter (FSC) characteristics. At least 10,000 events were acquired per tube of antibody combinations. Figure 6 shows flow cytometric acquisition.

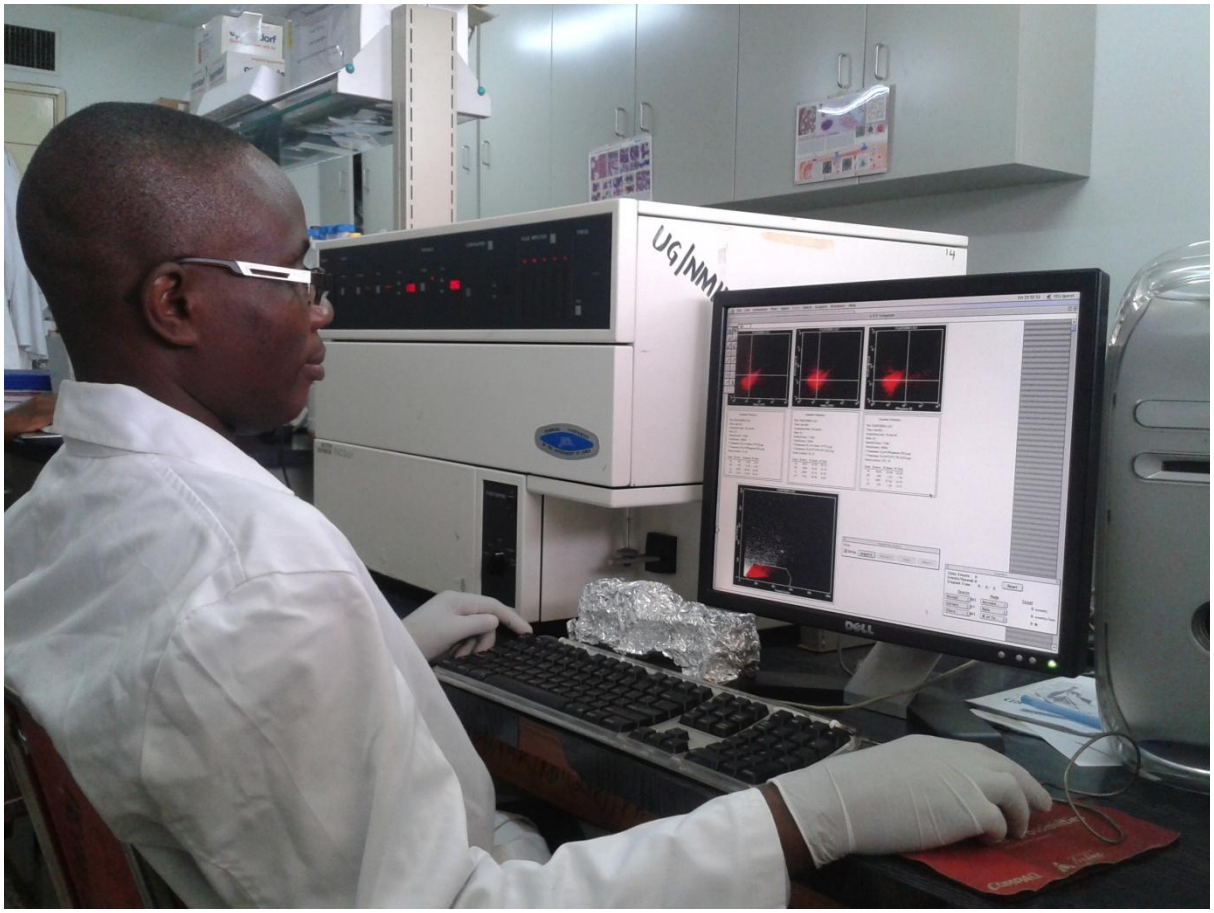


Figure 6: Aquisition of stained PBMC using the FACScan (Becton Dickinson, Japan)

3.7 Measurement of Cytokines by Enzyme-linked Immunosorbent Assay (ELISA)

Levels of the cytokines, tumour necrosis factor-alpha (TNF-α) and Interleukin-10 (IL-10) were determined in plasma of eBL patients as well as their healthy counterparts. 96-well microtitre plates (Immulon 4HBX, Dynex) were coated with 50µl/well of purified anti-human TNF-α or anti-human IL-10 monoclonal antibody at 2µg/ml (diluted with carbonate buffer: 0.1M NaHC03, pH 8.2) and incubated overnight at 4°C. The plates were then washed four times with a washing buffer (0.05% Tween 20 in phosphate-buffered saline (PBS) at 250µI/well. A blocking solution (10% heat inactivated FCS in PBS) was added at 150µl/well and the plates incubated at room temperature for 1 hour. After incubation the plates were washed twice using an automated plate washer (Wellwash AC, ThermoLabsystems, Finland).

Standard (recombinant) human TNF-α or IL-10 was added at serial dilutions (diluent: RPMI + 5% HI AB serum NHS) from 2000pg/ml to 31 pg/ml in addition to undiluted plasma at 50µl/well in duplicates. The plates were then incubated at room perature for 2 hour on a shaker. Following incubation, the plates were washed four times using the plate washer. A biotinylated anti-human TNF-α or IL-10 was diluted (diluent: 5% FCS in PBS) to 1µg/ml and added to the plates at 50µl/ml. The plates were incubated again for 45minutes at room temperature and washed five times as previously described.

An avidin peroxidase conjugate was then added at 2.5µg/ml (diluent: 5% FCS in PBS) and 50µl/well and incubated for 30 minutes. The plates were again washed five times. This was followed by addition of OPD substrate (0.4mg/ml in citrate-phosphate buffer +0.4mg/ml H_20_2 added immediately prior to use) at 100µl/well. The plates were then developed in the dark for 30 minutes, stopped with 2.5N H_2S0_4 at 50µl/well and read using a microtiter plate reader (Multiskan Ascent V1.24, ThermoLab systems, Finland) at 492 nm. The OD values of the

standards were used to draw the appropriate curves using statistical software (TBLCurves, Jandel Scientific) and the curves were used to transform the sample OD values to concentrations in pg/ml.

3.8 Flow Cytometric Analyses

Flow cytometry data was analyzed using FlowJo software (Treestar, Ashland, OR, USA). Lymphocyte population was set using FSC display and gated. Gating strategies and antibody combinations used are shown below (Figures 7 and Appendix 1and2)

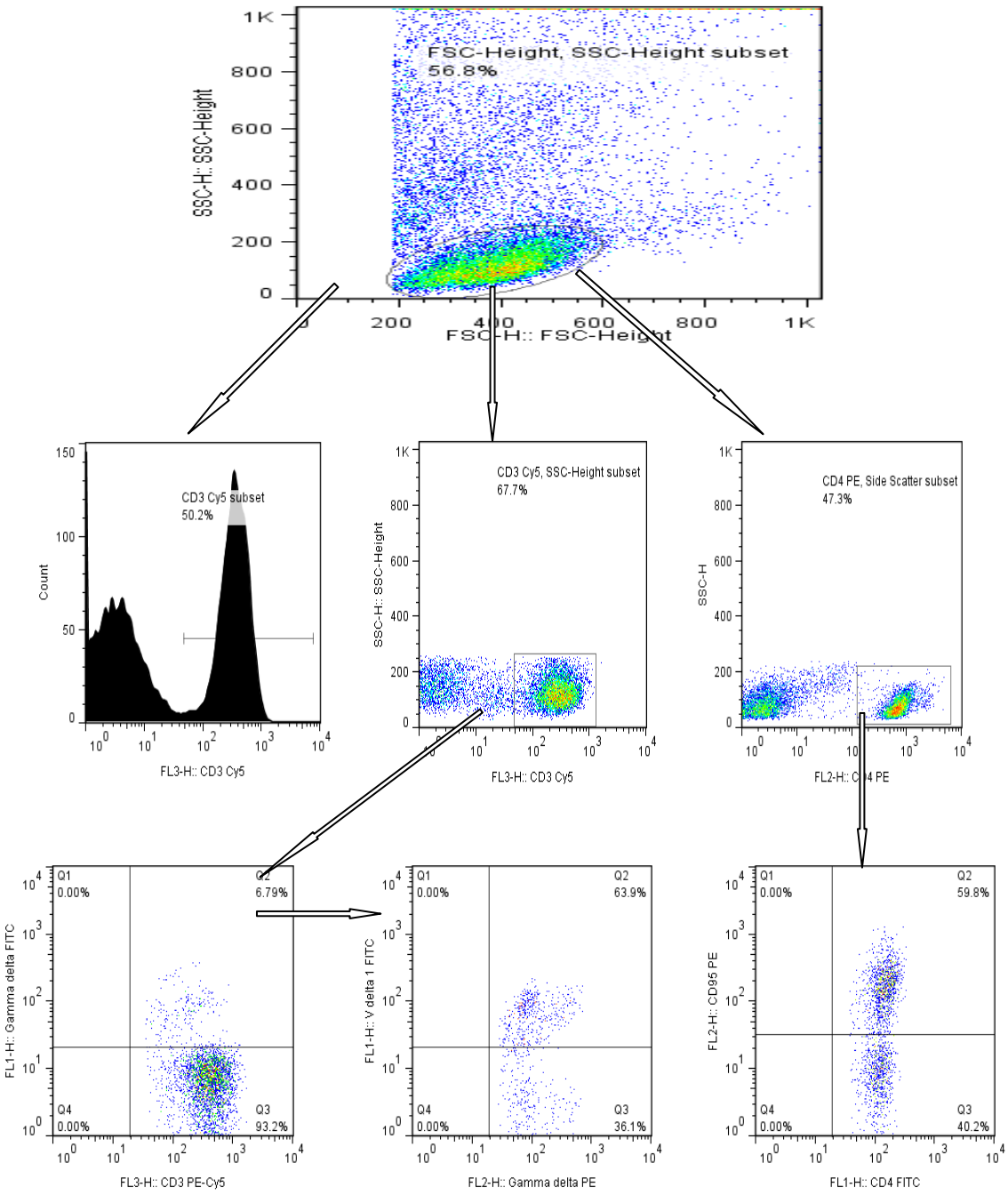


Figure 7: Gating strategies for flow cytometric phenotyping. Lymphocytes were identified by FSC properties and the subsets of T (CD3+) cells and activation status were identified by their respective markers. All numbers represent the percentage of the parent gate.

3.9 **Statistical Analyses**

Data were entered and analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL) and GraphPad Prism (GraphPad Prism, GraphPad Software, San Diego, CA, USA) softwares. Statistical tests were performed using Kruskall-Wallis or Mann–Whitney rank sum nonparametric test. $P<0.05$ was considered significant. Association between parameters were determined using non-parametric Spearman correlation. Relative expression index (REI) was calculated as the specific cell percentage for a marker resulting after culture with a stimulus (EBNA1 or PHA) divided by the cell percentage for the same marker after culture in medium without stimulus

CHAPTER FOUR

4.0 RESULTS

4.1 WBC Subpopulations with special focus on CD4+ and CD8+ T Cells in Patients and Controls

4. 1.1 Demographics and Clinical Data of Study Participants

The characteristics of the study participants are shown in Table 1. Participants recruited for this study included; 19 healthy controls, 21 eBL patients and 26 malaria patients, with mean ages 8.7, 6.9 and 8.6 years respectively. Analysis of their clinical data shows significant difference among the three groups. Except for absolute numbers of lymphocytes that was significantly lower, absolute number of WBCs, frequency of lymphocytes and, absolute number and frequency neutrophils were significantly higher in malaria patients compared to healthy controls ($p<0.0001$, $p=0.0039$, $p<0.0001$, $p=0.0076$ and $p=0.0035$, respectively**).** There were no differences between malaria and eBL patients with regard to absolute numbers of WBCs and both absolute numbers and frequency of neutrophils ($p=0.5518$, $p=0.9408$ and $p=2459$, respectively). However, both absolute numbers and frequency of lymphocytes were significantly lower in malaria patients compared to eBL patients ($p<0.0001$ and $p<0.0001$, respectively) as well as healthy controls ($p<0.0001$ and $p<0.0001$, respectively). The absolute numbers of WBCs and neutrophils were significantly higher in eBL patients compared to controls ($p=0.0017$and $p=0.0115$, respectively), whereas the frequency of MIX was significantly lower in eBL patients compared to controls ($p=0.0003$**)**. There were no significant differences in both absolute number and frequency of lymphocytes between healthy donors and eBL patients ($p=0.0499$ and $p=0.1640$, respectively). Likewise there were no significant differences in the absolute number of MIX and frequency of neutrophils between controls and eBL patients ($p= 0.2859$ and $p=0. 0.2591$, respectively).

Table 1: Demographics and clinical data of the study participants

	Participants			Significance		
	Controls (CON)	**eBL Patients**	**Malaria Patients (MAL)**	**CON vs eBL**	**CON vs MAL**	**eBL vs MAL**
Characteristic	**Mean (95%CI)**			**P-value**		
WBCs# ($x10^3/\mu L$)	**5.95** (5.43 to 6.47)	**8.17** (6.96 to 9.38)	**8.75** (7.36 to 10.15)	**0.0017**	**0.0039**	0.5518
LYMP# ($x10^3/\mu L$)	**2.82** (2.47 to 3.16)	**3.59** (2.81 to 4.36)	**1.79** (1.49 to 2.09)	0.0499	**<0.0001**	**<0.0001**
LYMP (%)	**48.8** (43.8 to 53.7)	**42.9** (36.0 to 49.9)	**22.4** (19.0 to 25.8)	0.1640	**<0.0001**	**<0.0001**
NEUT# ($x10^3/\mu L$)	**2.45** (2.04 to 2.86)	**4.24** (2.01 to 6.47)	**4.14** (2.10 to 6.19)	**0.0115**	**0.0076**	0.9408
NEUT (%)	**40.3** (35.6 to 44.9)	**46.5** (30.5 to 62.5)	**58.9** (40.1 to 77.7)	0.2591	**0.0035**	0.2459
MXD# ($x10^3/\mu L$)	**1.07** (0.18 to 1.95)	**0.21** (-0.10 to .52)	ND	0.2859		
MXD (%)	**11.0** (9.0 to 13.0)	**2.8** (-1.5 to 7.0)	ND	**0.0003**		
HGB (g/dL)	**12.10** (11.5 to 12.7)	**10.78** (7.7 to 13.9)	**11.63** (10.9 to 12.3)	0.2906		
Male#	**13**	**14**	**14**			
Female#	**6**	**7**	**12**			
Mean Age (range, years)	**8.7 (5-14)**	**6.9 (3-11)**	**8.6 (3-14)**			

MXD- Monocytes; Basophilsand Eosinophils together; *ND*- Not done; #-number; LYMP (%), NEUT (%) and MXD (%) are percentage of WBCs (white blood cells) that were lymphocytes, neutrophils and, Monocytes, Basophilsand Eosinophils mixture, respectively.

4. 1. 2 Stages of eBL among patients reporting at the Hospital (n=99)

The frequencies of various stages of eBL are shown in Figure 8 below. Majority (53.5%) of patients reported at the hospital with stage III BL. This was followed by stage IV with 20.2%. Stages I and II accounted for 19.2% and 7.1%, respectively. Stages III and IV, therefore, accounted for nearly 75% of cases reporting at the hospital. This implies that most of the patients report at the hospital with advance stages of the lymphoma, which are more expensive to treat.

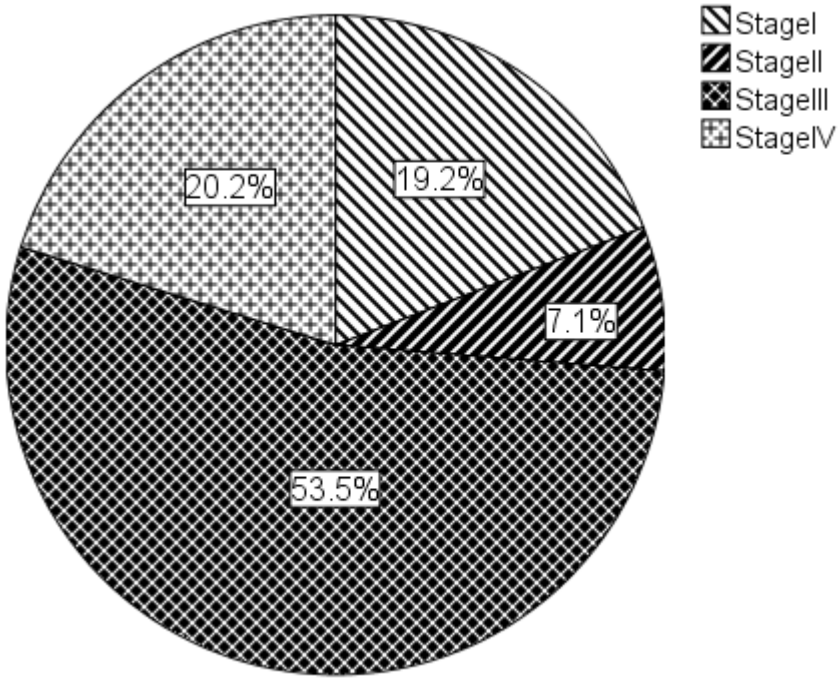


Figure 8 : Frequecy of BL stages at presentation

4.1.3 Frequency of Peripheral Blood T Cells is lower in Patients Compared to Healthy Controls

The proportions of all lymphocytes identified by FSC properties that were T cells were examined. The median percentages of gated lymphocytes that were T cells were lower in both eBL and malaria patients compared to healthy controls (eBL vs Controls, p=0.003; Malaria patients vs Control, p=0.002). No significant different was found between eBL and malaria patients (p=0.451, Figure 9 and Appendix III).

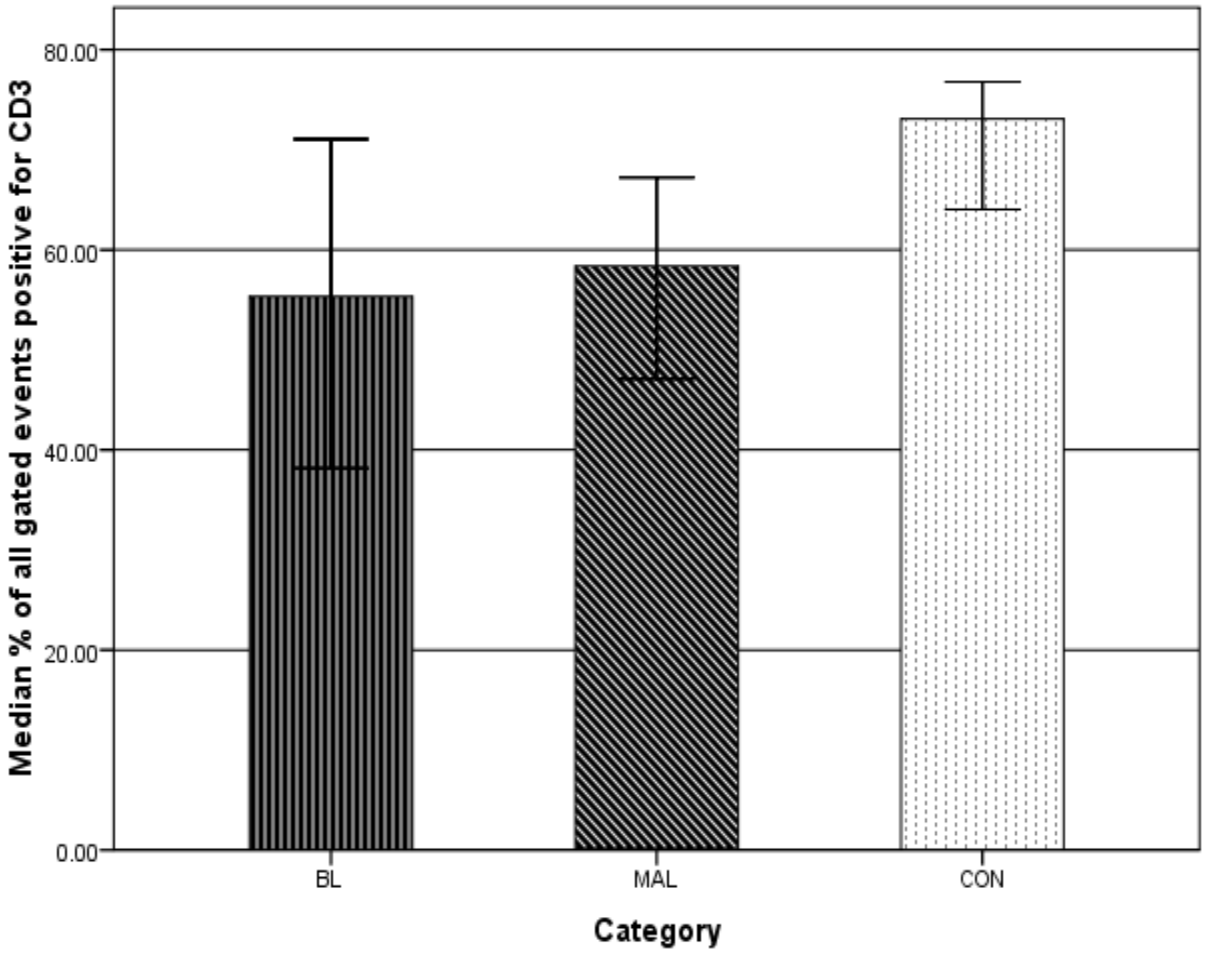


Figure 9: Frequency of gated lymphocytes expressing CD3 in patients and controls. Error Bars: …

4.1.4 Frequencies of CD3+CD4+, CD3+CD8+ and CD3+CD4+CD8+ Cells and CD4/CD8 ratio

The median frequencies of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were not significantly different among the malaria patients, healthy donors and eBL patients ($CD3^+CD4^+$, p=0.225; $CD3^+CD8^+$, p=0.200). On the other hand, the median frequency of $CD3^+CD4^+CD8^+$ cells was significantly lower in both eBL and malaria patients compared to healthy controls but no significant difference was observed between eBL and malaria patients (eBL vs Controls, p=0.001; Malaria patients vs Control, p=0.004; eBL vs Malaria patients, p=0.269). It was also found that the median of CD4/CD8 ratio was significantly higher in BL patients compared to controls and malaria patients (eBL vs Controls, p=0.007; eBL vs Malaria patients, p=0.033). However the CD4/CD8 ratio, though higher in malaria patients compared to controls, it was not significant (p=0.384, Figures 10, 11 and Appendix IV).

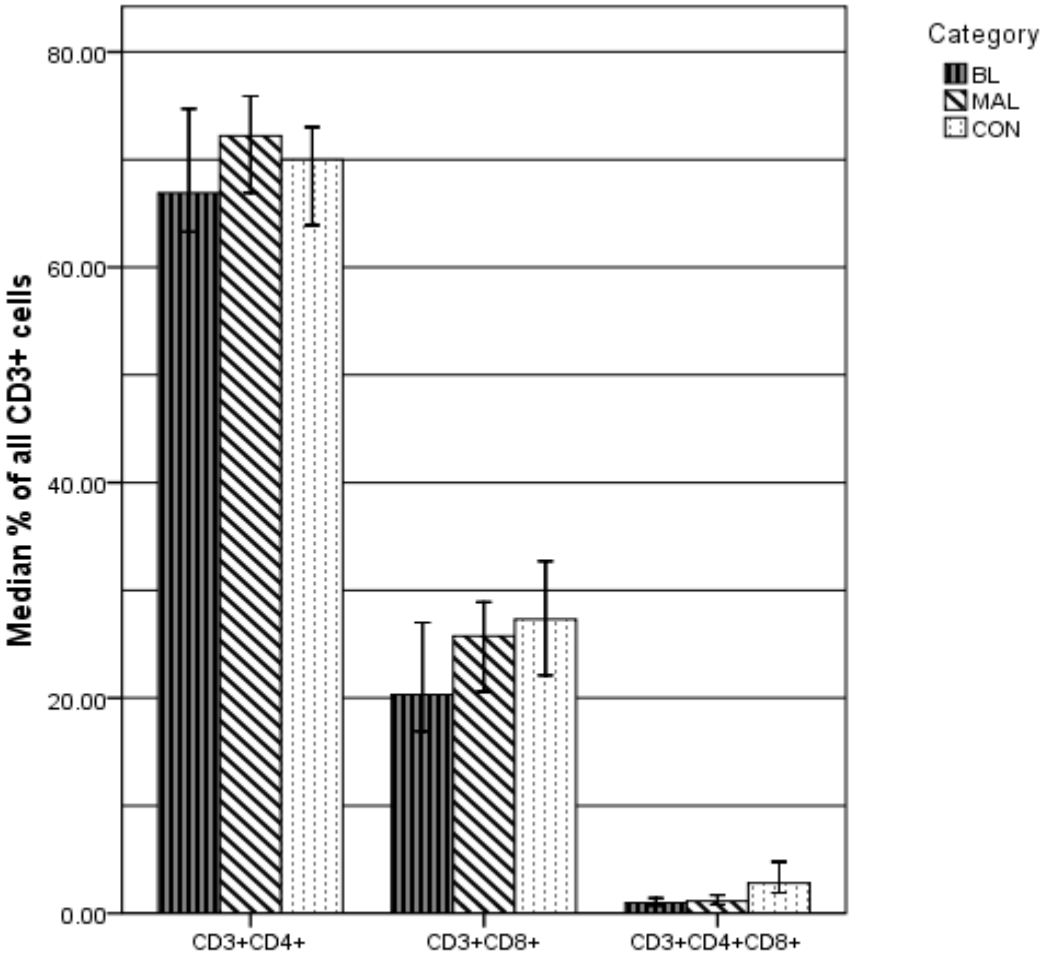


Figure 10: Median frquencies of CD3+CD4+, CD3+CD8+ and CD3+CD4+CD8+ T Cells Compared among eBL and Malaria patients and Healthy Controls. Error Bars: 95% CI

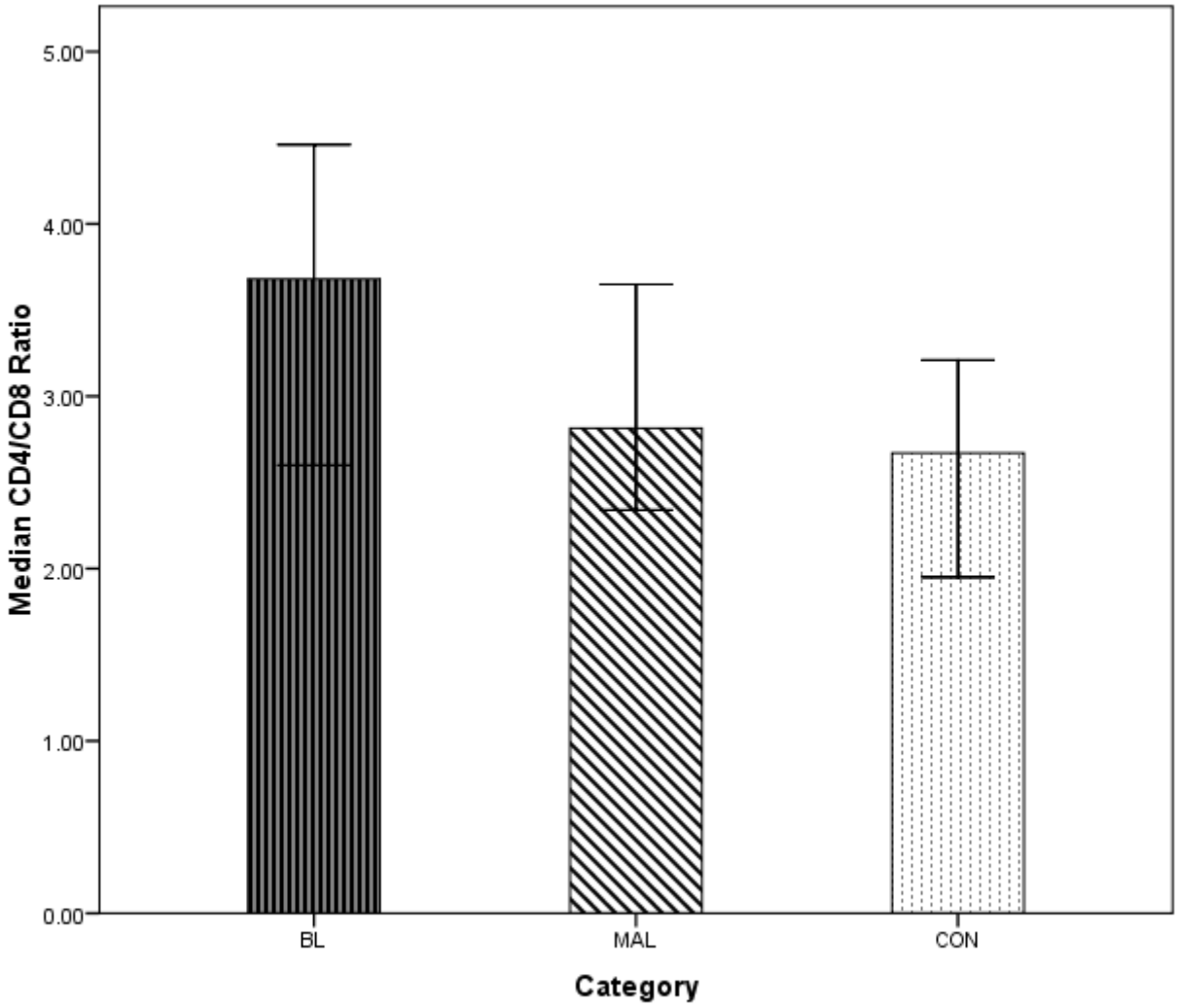


Figure 11: CD4/CD8 Ratio among Patients and Controls. Error Bars: 95% CI

4.1.5 Activation status of CD4+ and CD8+ T Cells

The activation status of T cells was also examined. Median frequency of CD4CD25+ cells were higher in malaria patients compared to controls (p=0.021) but did not differ significantly between eBL and malaria patients (p=0.054) or between eBL patients and healthy controls (p=0.094). Likewise, median frequency of CD4CD69+ cells were higher in malaria patients compared to controls (p=0.026) but did not differ significantly between eBL and malaria patients (p=0.659) or between eBL patients and healthy controls (p=0.111). Whereas the median frequency of CD4CD95+ cells did not differ between malaria patients and controls (p= 0.462) nor between eBL and malaria patients (p=0.054), it was higher in eBL patients compared to controls (p=0.014). Additionally, the frequencies of CD25, CD95 and double expression of CD25 and CD95 by CD8+cells, did not differ between the patient groups and healthy donors (p=0.252 and p=0.123, respectively, Figures 12, 13 and Appendix V).

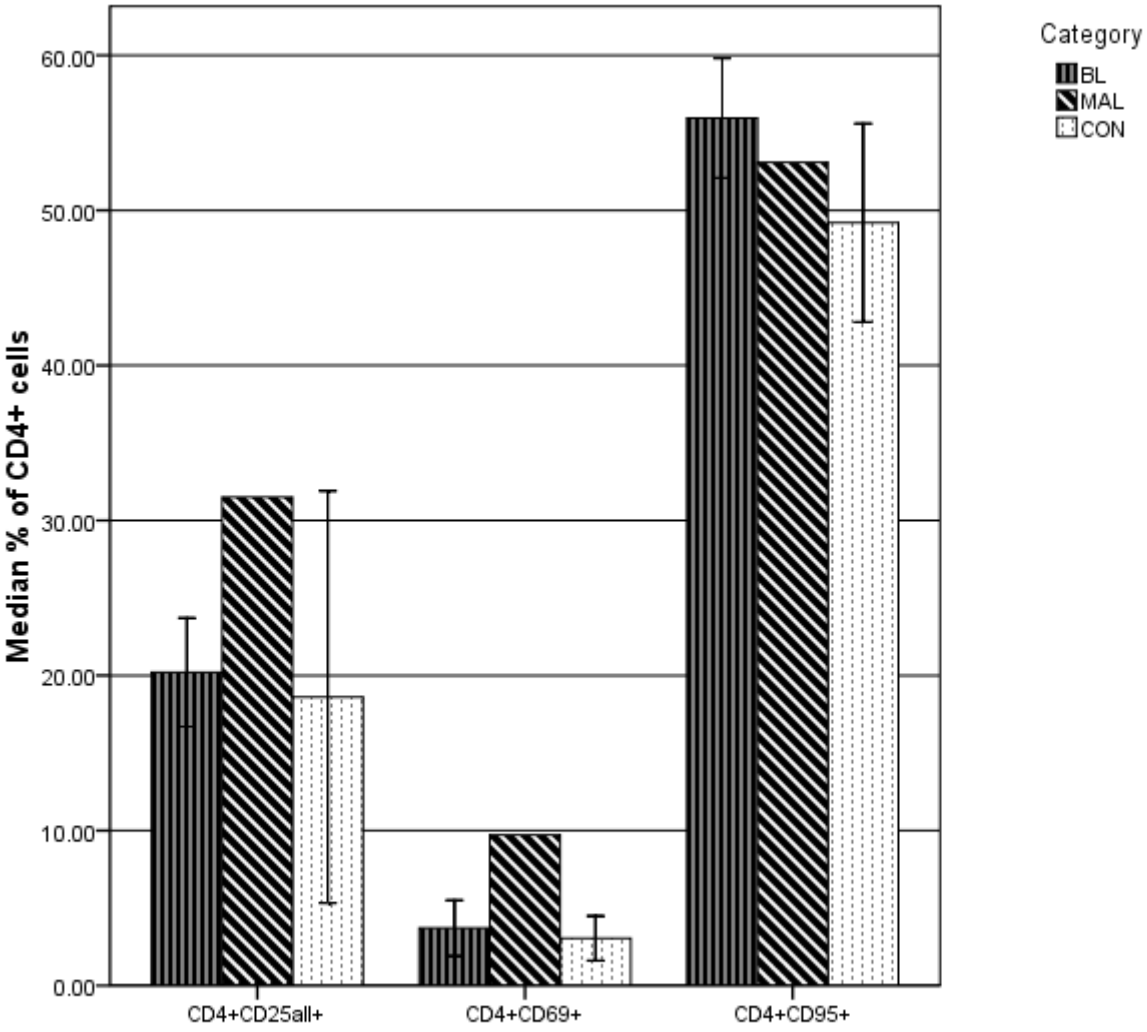


Figure 12: Frequencies of CD4+ T Cells expressing early and late activation markers. CD4+CD25all+ represent both CD4+CD25+ high and low. Error Bars: 95% CI

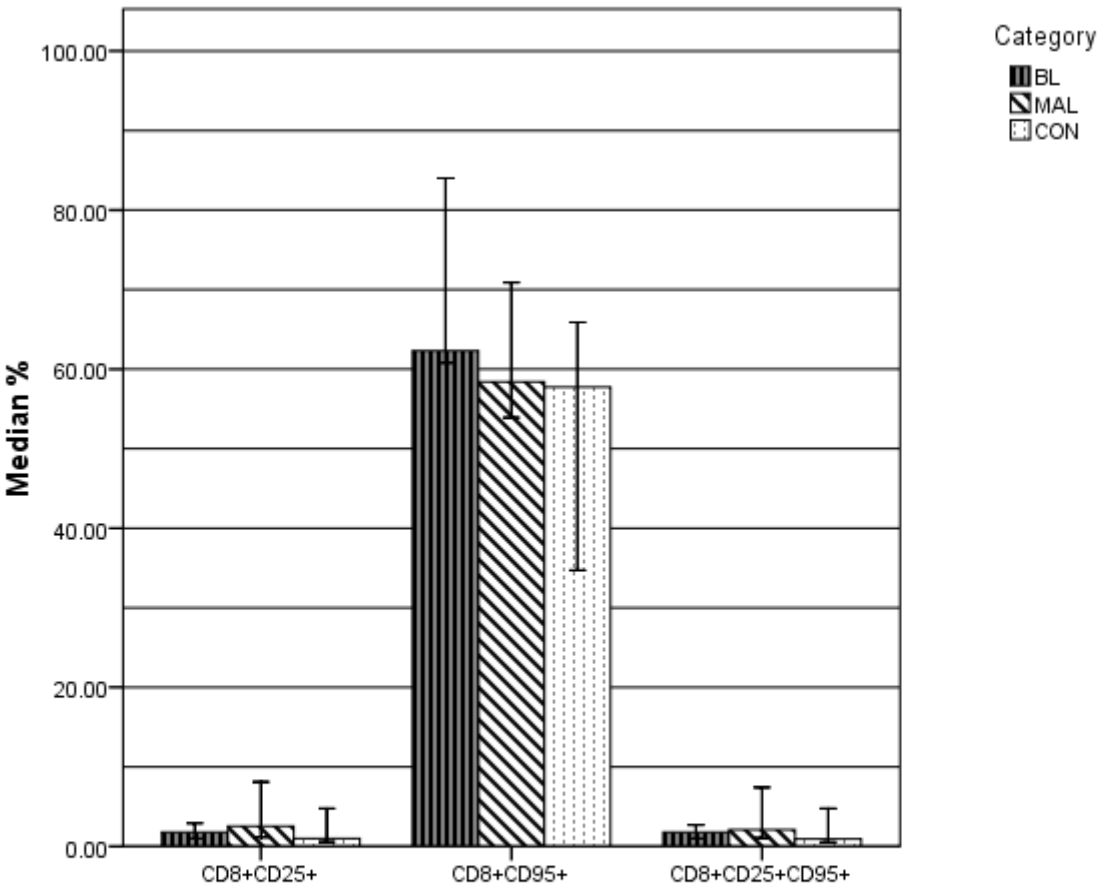
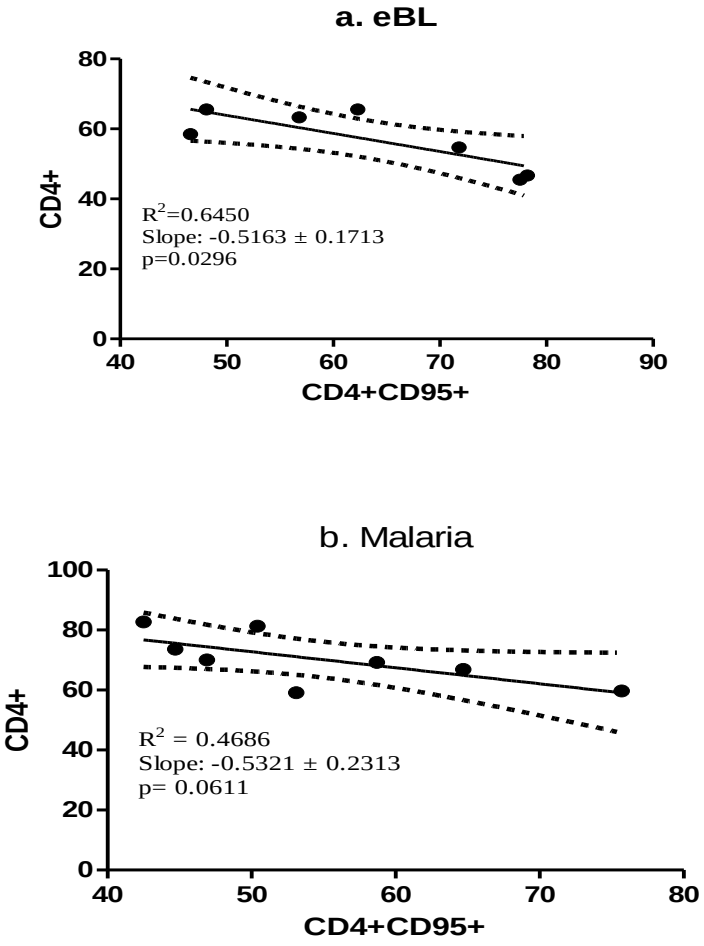


Figure 13: Frequencies of CD8+ T Cells expressing early and late activation markers. Error Bars: 95% CI

4.1.6 An inverse relationship between CD4+ and CD4+CD95+ T cells frequency in eBL patients

The relationship between the frequencies of CD4+ and CD4+CD95+ T cells were examined. The result showed significant inverse relationship between the two cell populations in eBL patients (R^2= 0.6450, p=0.0296). Similar inverse retionship was found in malaria patients but not significant (R^2=0.4686, p=0.611). Conversely, in healthy control, positive association was found between the frequencies of CD4+ and CD4+CD95+ T cells (R2=0.4005, p=0.1271) (Figure 14).



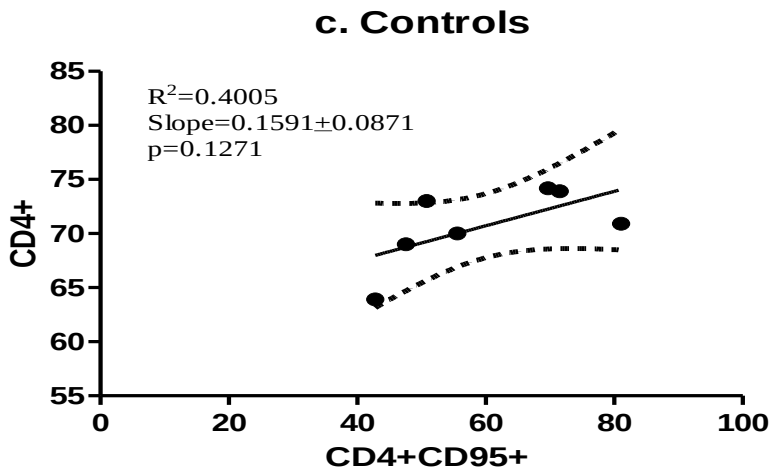


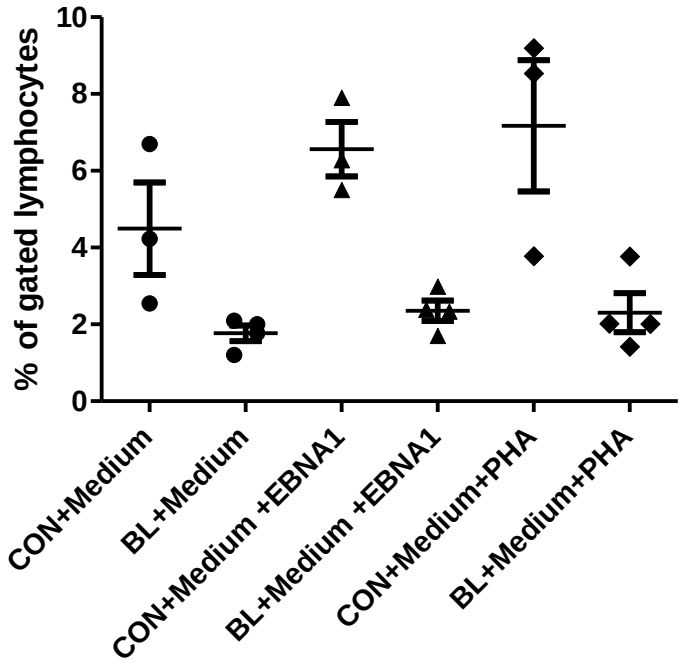
Figure 14: Association between the frequencies of CD4+ and CD4+CD95+ T cells in patients and controls. eBL, malaria and healthy controls are represented as a, b and c, respectively. CD4+ represents percentage of CD3+ cells expressing CD4 and CD4+CD95+ stands for percentage of CD4+ cells expressing CD95. Association between the parameters was determined using non-parametric Spearman correlation. The broken lines show the 95% confidence band of the linear regression line.

4.1.7 EBNA-1-Specific Responses in CD4+ and CD8+ T cells of PBMC of eBL patients and Healthy controls

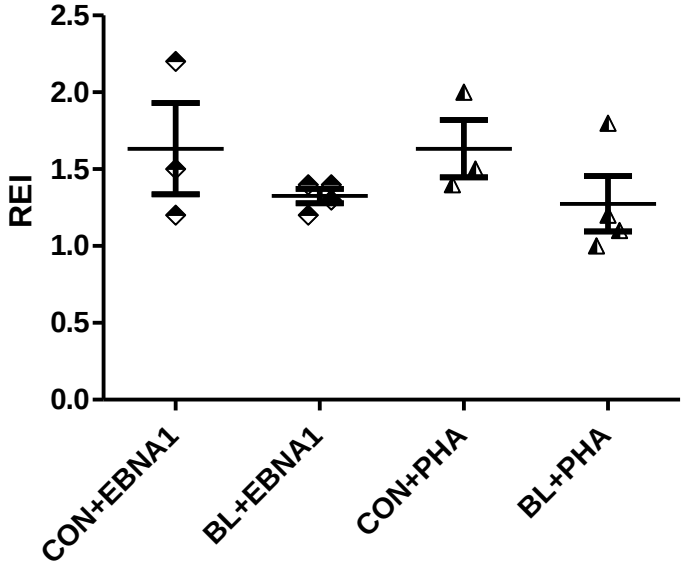
Figure 15 below shows the response of CD4+ CD8+ T cells to EBNA1. PHA and medium without any stimulation were used as positive and negative controls respectively. Generally, CD4+ (Figure 15A and C) and CD4FoxP3+ (Figure 15E) cells expressed more IFN-γ in controls than in eBL patients, to all the stimulants. The low response of CD4+ T cells to PHA, which is a general immune stimulant, is not clear but it could be an indication of a universal reduction in immune response in CD4+ cells in eBL. It could also be seen that CD4+ and CD4+FoxP3+ T cells from healthy controls had higher IFN-γ REI to EBNA1 compared to those from eBL patients (Figure 15B, D and F). This indicates a decrease in EBNA1-specific response by CD4+T cells in eBL. Moreover, whereas in healthy controls the relative expression index (REI) was higher in CD4+FoxP3+ cells than in all CD4+ cells together, the opposite is true for eBL. This also shows that CD4+FoxP3+ cells are a better source of IFN-γ in healthy controls but not in eBL patients.

EBNA1-specific response by CD8+ T cells is presented in Figure 15G-J. Looking at the frequencies of CD8+IFN-γ cells after stimulation, it can be seen that CD8+ cells from patients and controls had similar REI (Figure 15H and J). This indicates that there is no reduction in EBNA1-specific Th1 response by CD8+ T cells in eBL. However, just as in CD4+ cells, the frequency of CD8+ T cells from healthy controls expressing IFN-γ was higher for all stimulants than those from eBL patients (Figure 15G and I). This implies that it is not only EBNA1-specific responses that are reduced in CD4+ and CD8+ cells in eBL but also responses to other stimulants such as PHA.

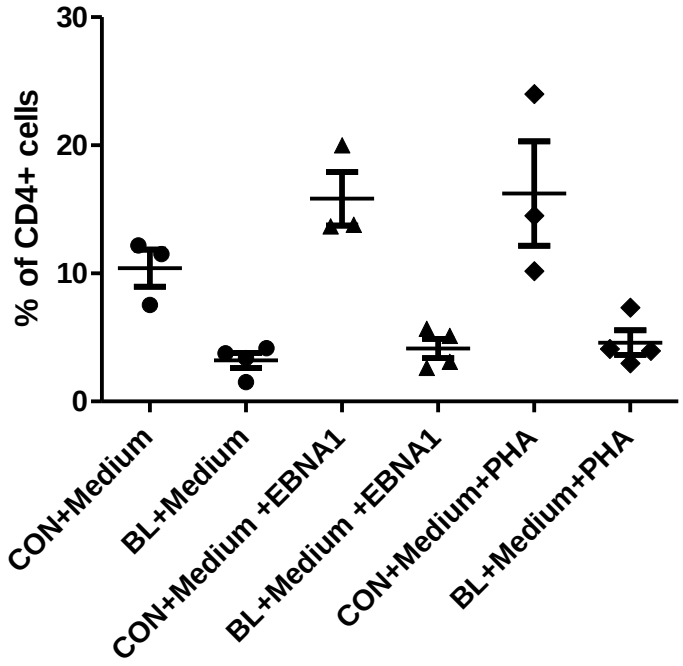
A. CD4+IFN-γ+



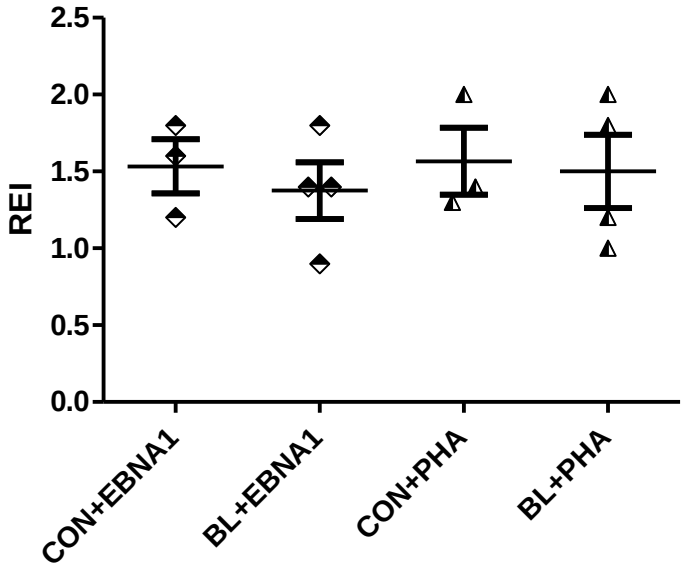
B. IFN-γ REI for CD4+



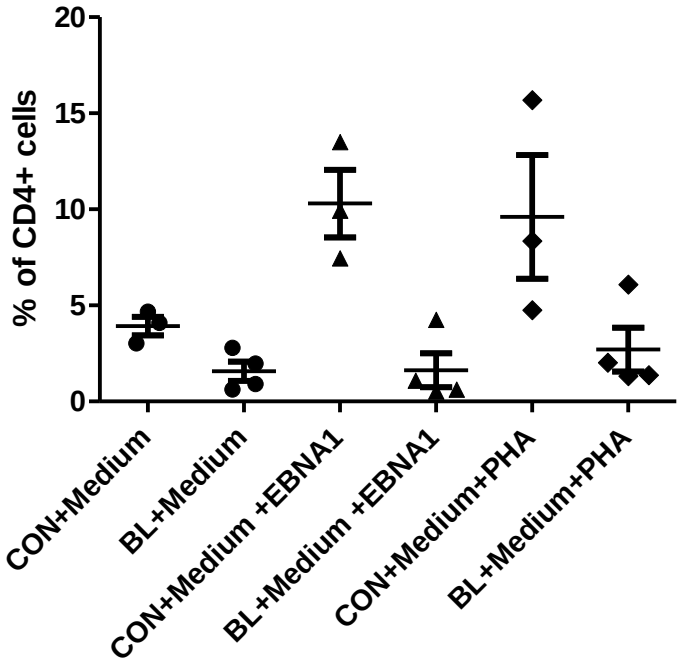
C. CD4+IFN-γ+



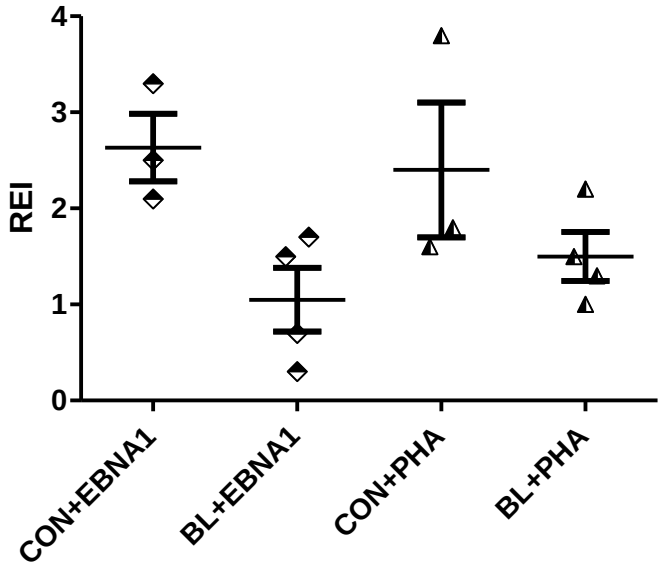
D. IFN-γ REI for CD4+



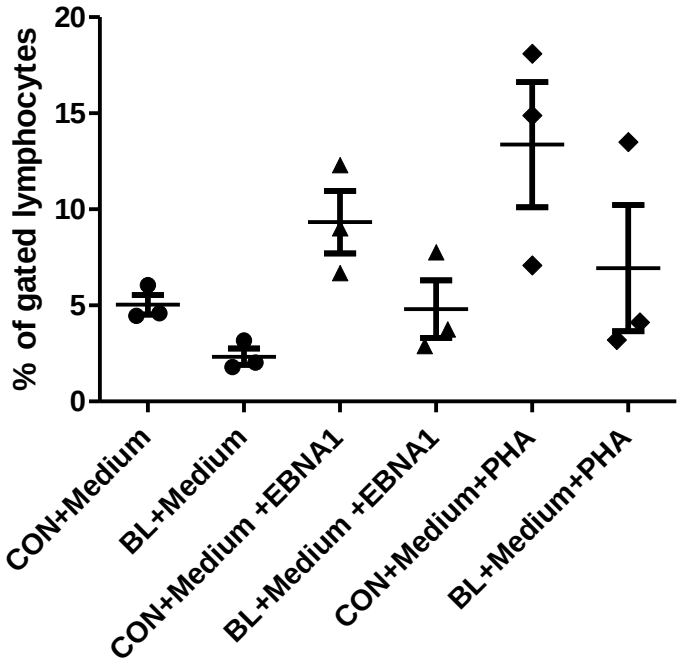
E. CD4+FoxP3+IFN-γ+



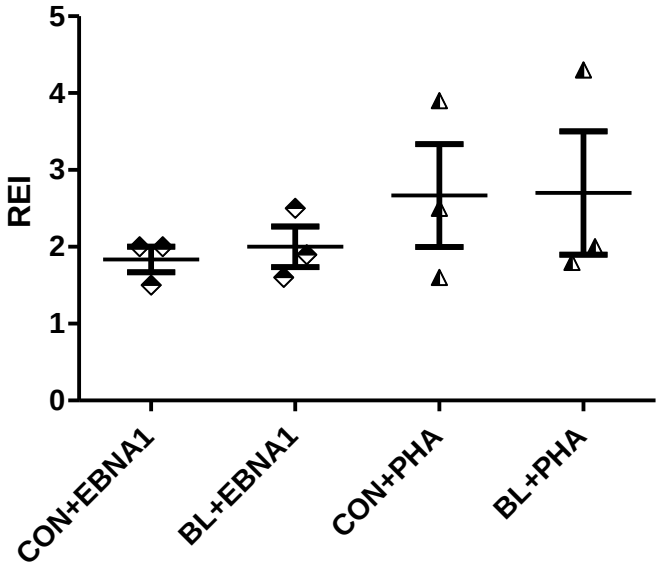
F. IFN-γ REI for CD4+FoxP3+



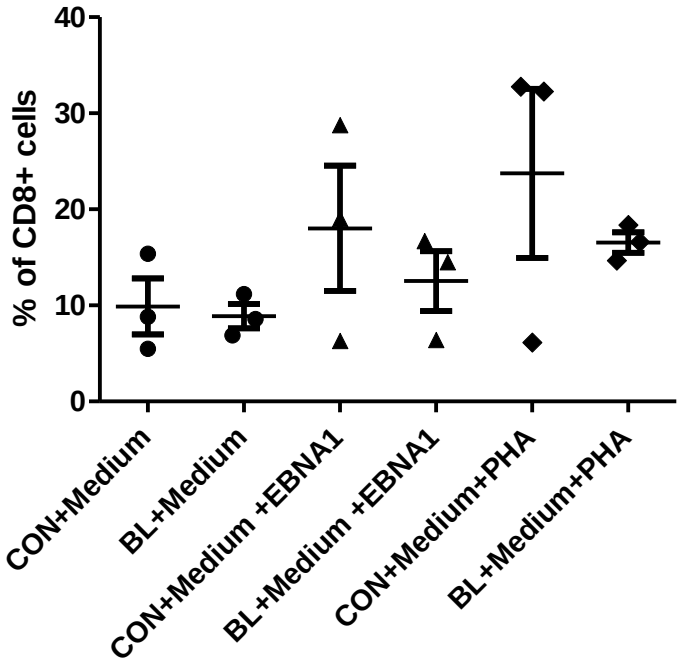
G. CD8+IFN-γ+



H. IFN-γ REI for CD8+



I. CD8+IFN-γ+



J. IFN-γ REI for CD8+

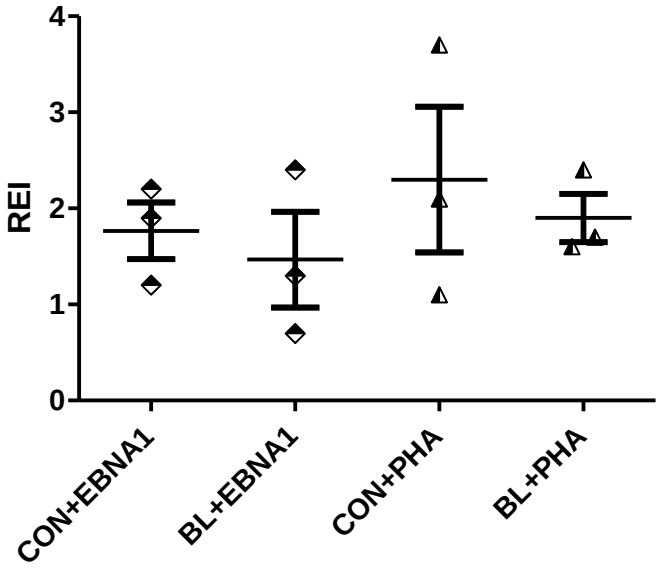


Figure 15: IFN-γ Production by CD4+ and CD8+ T Cells in Response to EBNA1. IFN-γ expression by CD4+ cells (A-F) and CD8+ cells (G-J) compared between BL patients and controls. IFN-γ production by CD4+ cells expressed as a percentage of gated lymphocytes (A) and the corresponding relative expression index (REI) (B). IFN-γ production by CD4+ and CD4+FoxP3+ cells expressed as a percentage of CD4+ and CD4+FoxP3+ cells

respectively (C and E, respectively). Corresponding REIs for C and E were shown in D and F, respectively. IFN-γ production by CD8+ cells expressed as a percentage of gated lymphocytes (G) or percentage of CD8+ cells (I) and the respective REIs (H and J, repectively). REI = percentage of cells of interest expressing IFN-γ after culture with a stimulus (EBNA1 or PHA) divided by percentage of the same cells expressing IFN-γ after culture in medium without stimulus. Bars show mean±SE.

4.2 γδT Cells and eBL

4.2.1 Frequencies of γδT Cells in Patients and Healthy Controls

The median percentage of CD3+ T cells that expressed TCR-γδ were different among the patient groups and healthy controls, with malaria patients having the highest frequency compared to eBL patients (p=0.001) and healthy controls (p=0.014). The frequency of the CD3+ γδ+ cells was lower in eBL patients compared to healthy controls (p=0.004), making it the lowest among the categories. The frequencies of γδ+Vδ1+ cells were also analysed. It was higher in both malaria (p=0.000) and eBL (p=0.007) patients compared to healthy controls. Conversely, the frequency of γδ+Vγ9+ cells was higher in healthy controls compared to malaria (p=0.003) and eBL (p=0.001) patients. But frequencies of both γδ+Vδ1+ and γδ+Vγ9+ cells did not differ between malaria and eBL patients (p=0.558, Figures 15 and Appendix VI).

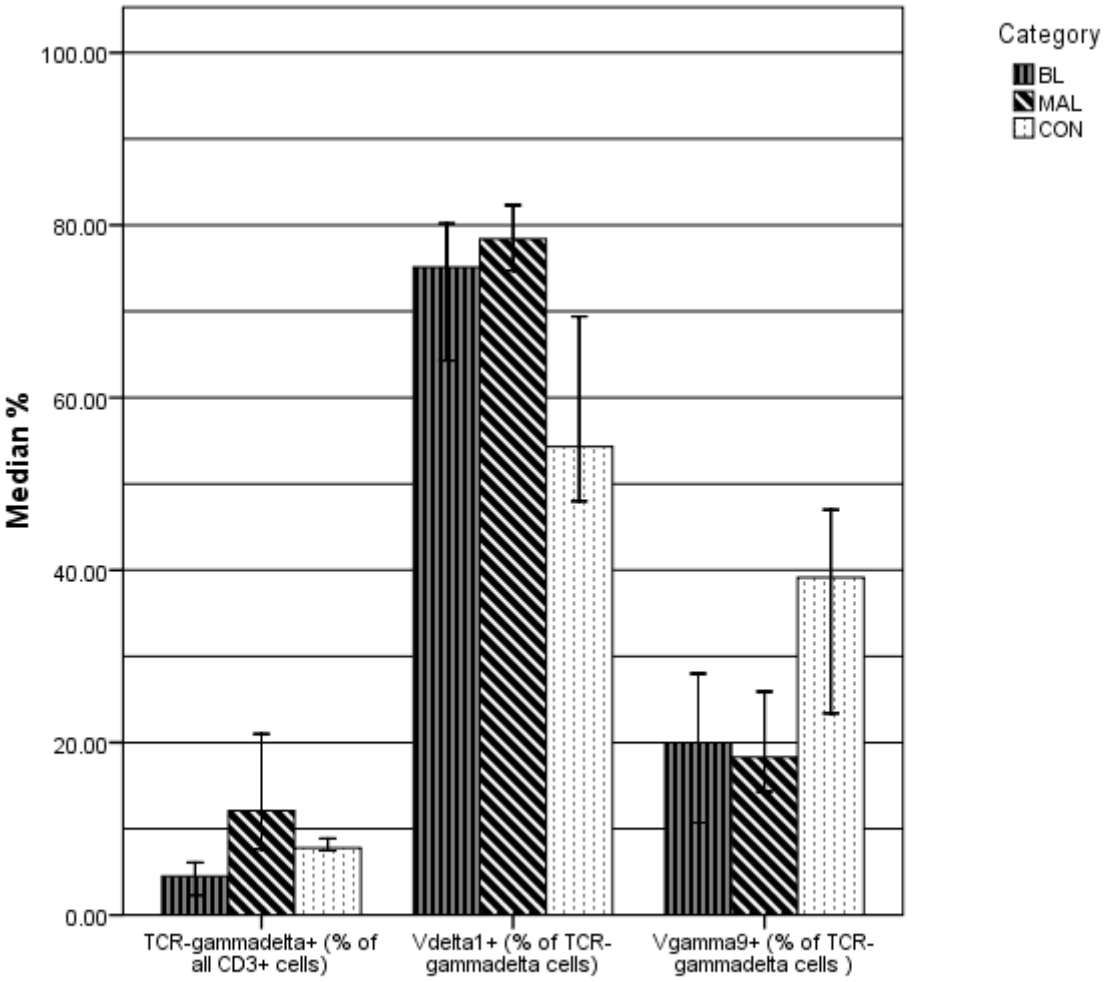


Figure 16: Frequency of gamma delta T cells in patients and controls. Error Bars: 95% CI

4.2.2 Activation Status of γδ+ T cells in eBL patients and Healthy Controls

The frequencies of TCR-γδ+ CD25+ and HLA-DR+ were higher in eBL patients compared to healthy controls, (p=0.007 and 0.003, respectively). There were no significant differences between the two groups with regard to CD69 and CD95 (p=0.601; p=0.072, respectively; Figures 16 and Appendix VII). This shows that TCR-γδ+ T cells are more activated in eBL patients compared to healthy controls.

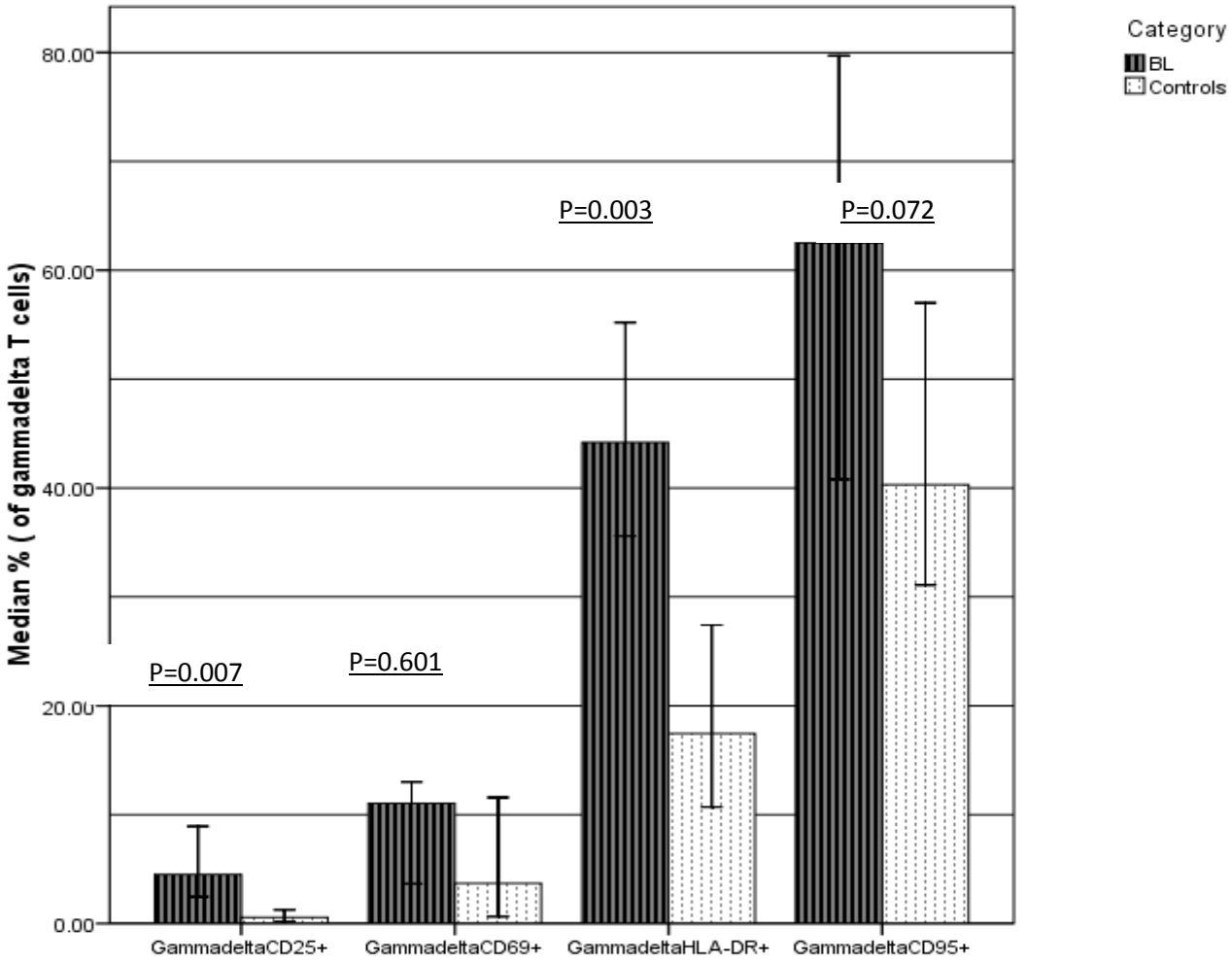
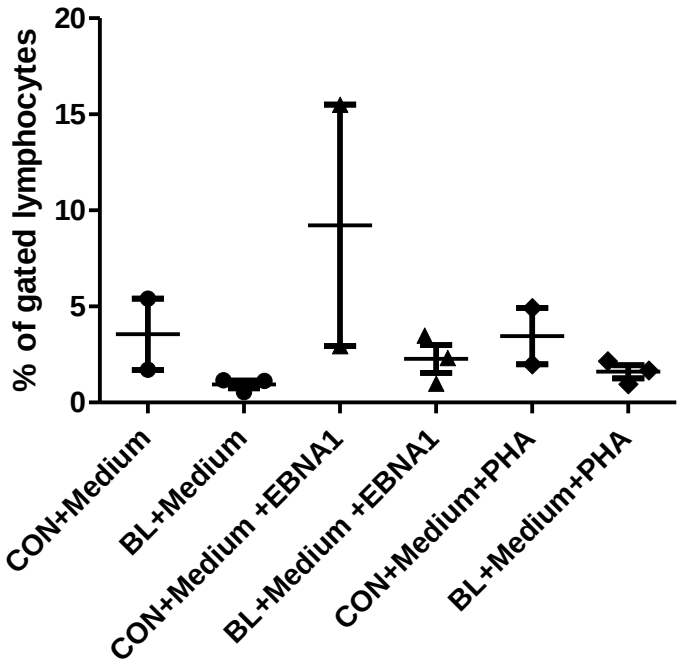


Figure 17: Frequencies of activated cells within TCR-gammadelta+ T lymphocyte subsets in Burkitt's lymphoma patients and healthy control donors. Error Bars: 95% CI

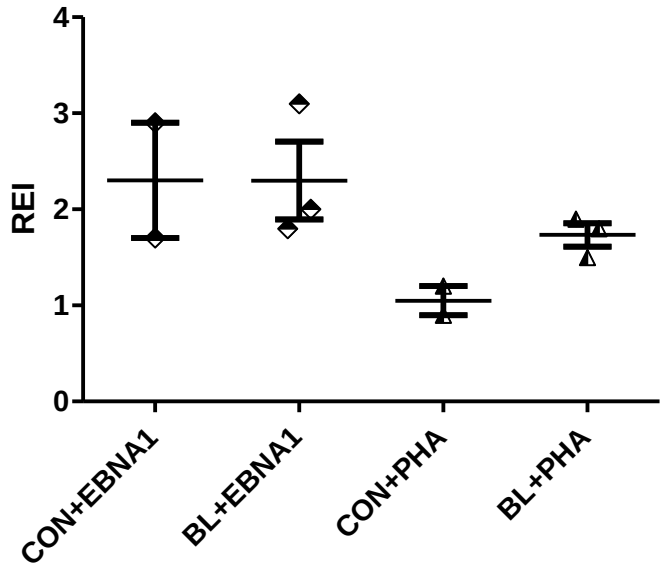
4.2.3 EBNA1-Specific Th1 Response by Vδ1+ γδT Cells

Similar to CD4+ T cells, Vδ1+ γδT cells in healthy controls express more IFN-γ to all stimulants than those from eBL patients (Figure 18A and C), indicating a universal reduction in immune responses in Vδ1+ γδT cells from eBL. But unlike CD4+ cells, IFN-γ REI to EBNA1 by Vδ1+ γδT cells was similar between patients and controls (Figure 18B and D). This suggests that there is no decrease in EBNA1-specific Th1 response by Vδ1+ γδT cells in eBL. Additionally, higher frequency of Vδ1+ γδT cells express IFN-γ compared to CD4+ cells (Figures 15C and 18C).

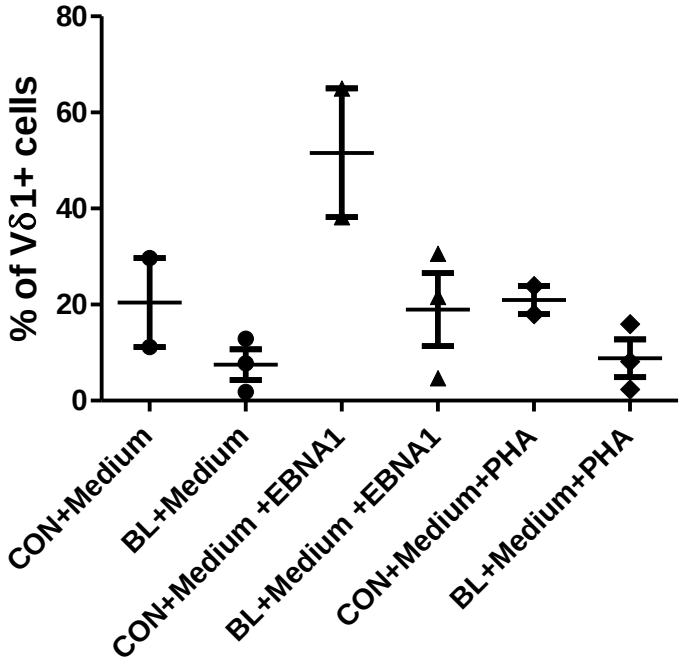
A. Vδ1+IFN-γ+



B. IFN-γ REI for Vδ1+



C. Vδ1+IFN-γ+



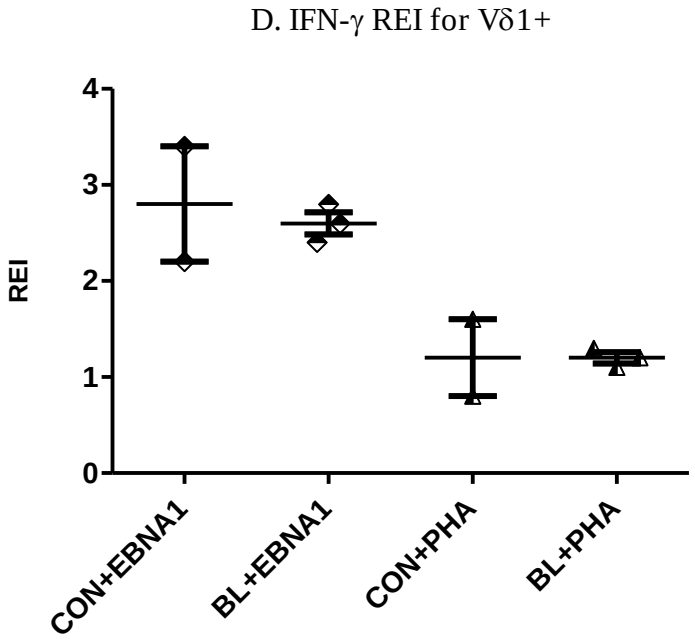


Figure 18: IFN-γ Production by Vδ1+ γδ T Cells in Response to EBNA1. IFN-γ production by Vδ1+ cells expressed as a percentage of gated lymphocytes (A) and the corresponding REI (B). IFN-γ production by Vδ1+ cells expressed as a percentage of Vδ1+cells (C) and the corresponding REI (D). REI = percentage of Vδ1+ cells expressing IFN-γ after culture with a stimulus divided by percentage of Vδ1+ cells expressing IFN-γ after culture in medium without stimulus. Bars show mean±SE.

4.3 Regulatory T Cells and eBL

4.3.1 CD4+ CD25+ Treg cells and other Foxp3 Expressing Cells in Patients and Healthy Controls

The frequencies of Treg cells were also analysed. Frequencies of CD4+CD25hi+ and CD4+CD25hi+FoxP3 were higher in both malaria (p=0.000 and p=0.000, respectively) and eBL (p=0.027 and p=0.022, respectively) patients compared to healthy controls. No significant differences were found between malaria and eBL patients with regard to CD4+CD25hi+ and CD4+CD25hi+FoxP3 cells (p=0.411 and p=0.937 respectivley). The frequencies of CD4+CD25hi+Foxp3- cells were also higher in both malaria (p=0.012) and eBL (p=0.035) patients compared to controls but not different between malaria and eBL patients (p=0.623, Figures 17 and Appendix VIII).

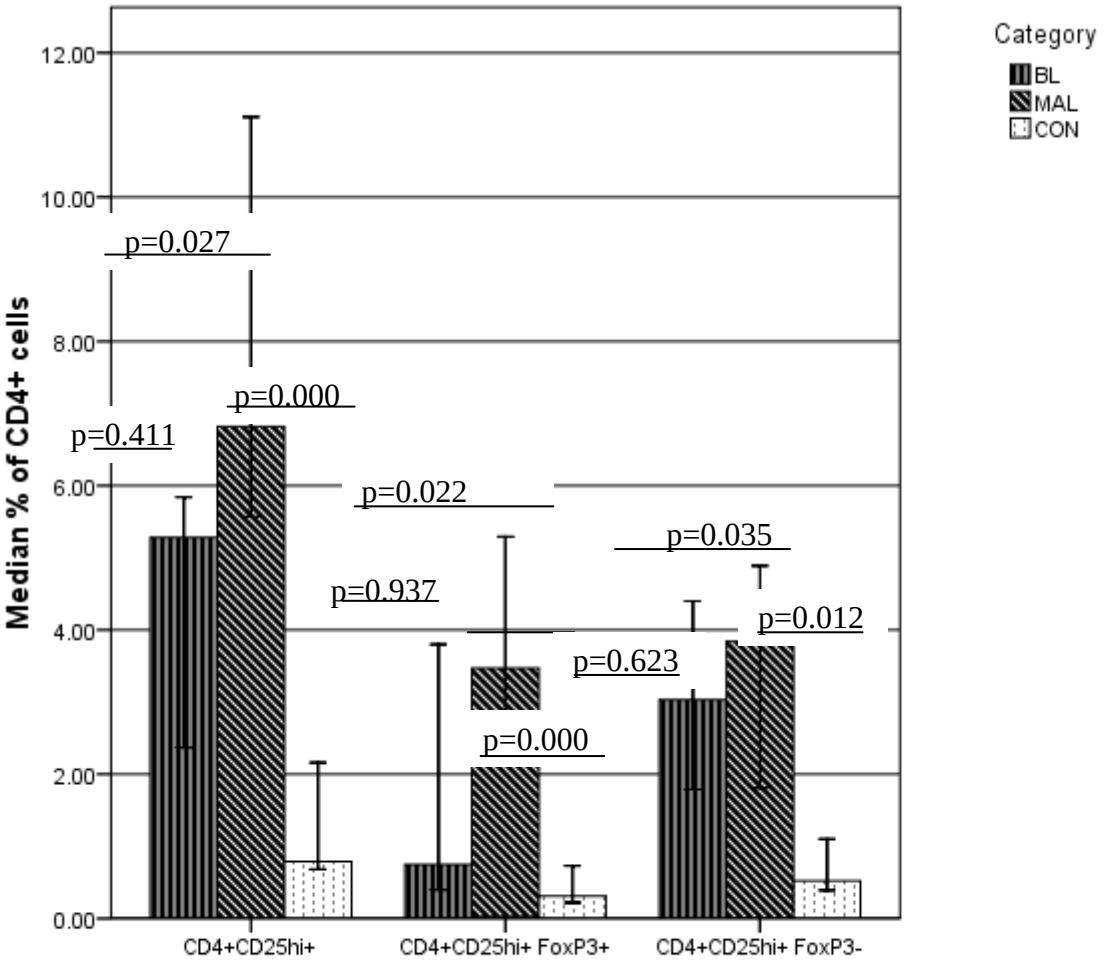


Figure 19: Frequencies of CD4+CD2hi+ and CD4+CD25hi+FoxP3+ regulatory T cells in patients and controls. Error Bars: 95% CI

4.4 Th1/Th2 Responses to EBNA1 in eBL patients and Controls

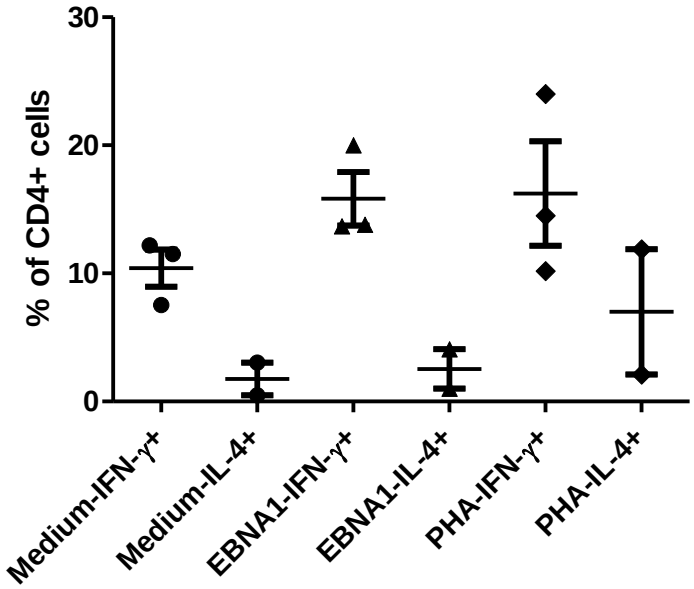
4.4.1 IFN-γ and IL-4 Expression to EBNA1 stimulation by CD4+, CD4+FoxP3+ and Vδ1+ Cells

Whereas REI for IFN-γ and IL-4 in CD4+ cells were similar in healthy controls, the frequency of IFN-γ expression was higher than IL-4 expression to all stimulants. Likewise REI for IFN-γ and IL-4 in CD4+ cells were similar in eBL patients (Figure 20D), just as in health controls but the expression of IL-4 by CD4+ cells to EBNA1 and PHA was higher than IFN-γ (Fifure 20B). This indicates that responses in eBL are not only skewed in favour of Th2 but also the skewing is not specific to EBNA1.

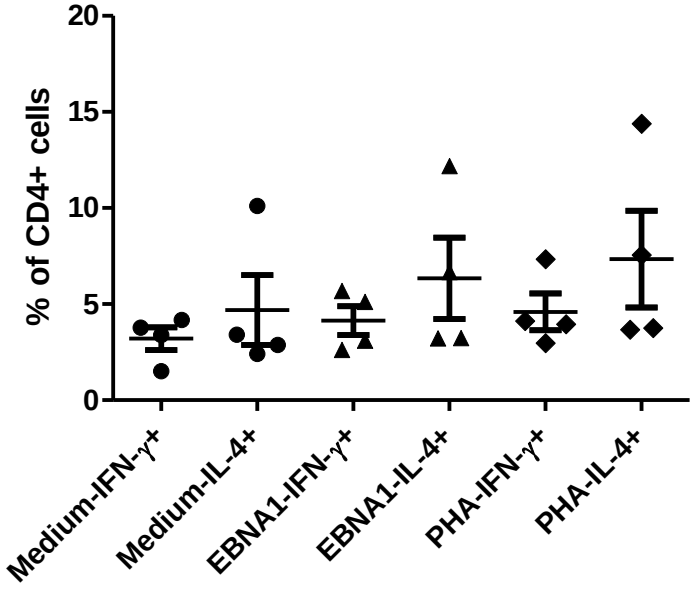
With regard to CD4+FoxP3+ T cells, REI for IFN-γ on stimulation with EBNA1 was higher than REI for IL-4 in controls (Figure 20G). Moreover, the frequency of IFN-γ expression to EBNA1 was higher than that of IL-4 (Figure 20E). This shows that CD4+FoxP3+ cells in healthy controls express more IFN-γ than IL-4 and are therefore pro-Th1. In patients, the REI for IFN-γ and IL-4 in CD4+FoxP3+ T cells were similar (Figure 20H) but just as in healthy controls, higher frequency of CD4+FoxP3+ cells expressed IFN-γ than IL-4, upon EBNA1 stimulation (Figure 20G). This suggests that in both patients and controls, higher frequency of CD4+FoxP3+ cells express IFN-γ than IL-4, however, the Th1 response to EBNA1 was better in healthy controls compared to patients.

The frequency of Vδ1+ cells expressing IFN-γ to EBNA1 and REI for IFN-γ, were higher than those for IL-4 in controls (Figure 20I and K). Similarly, REI for IFN-γ to EBNA1 was higher than REI for IL-4 in eBL patients (Figure 20L). However, the frequencies of cells expressing IL-4 to all stimulants were higher than those expressing IFN-γ in the patients (Figure 20J). This indicates that thoughVδ1+ cells have higher REI for IFN-γ compared to IL-4 in both patients and controls, there is tilting of the responses in favour of Th2 in patients.

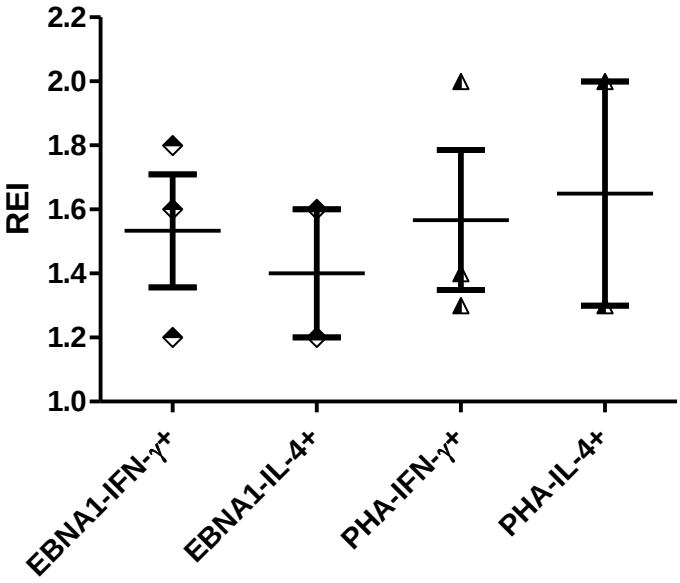
A. CD4+ Cells of controls



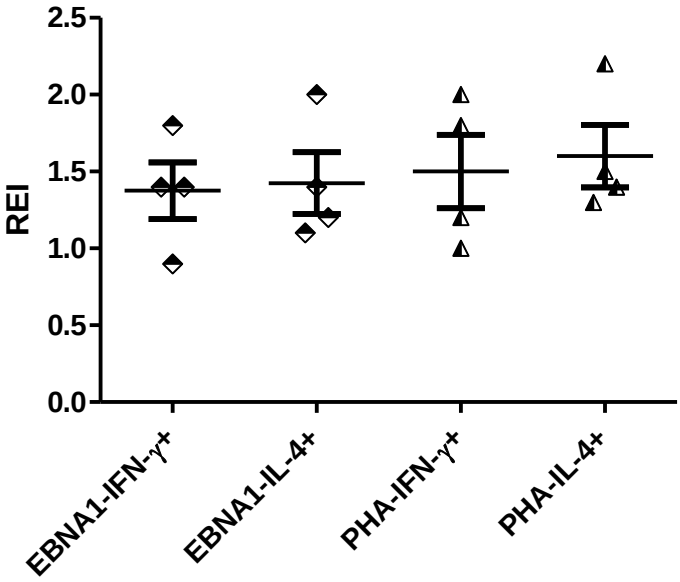
B. CD4+ Cells of BL patients



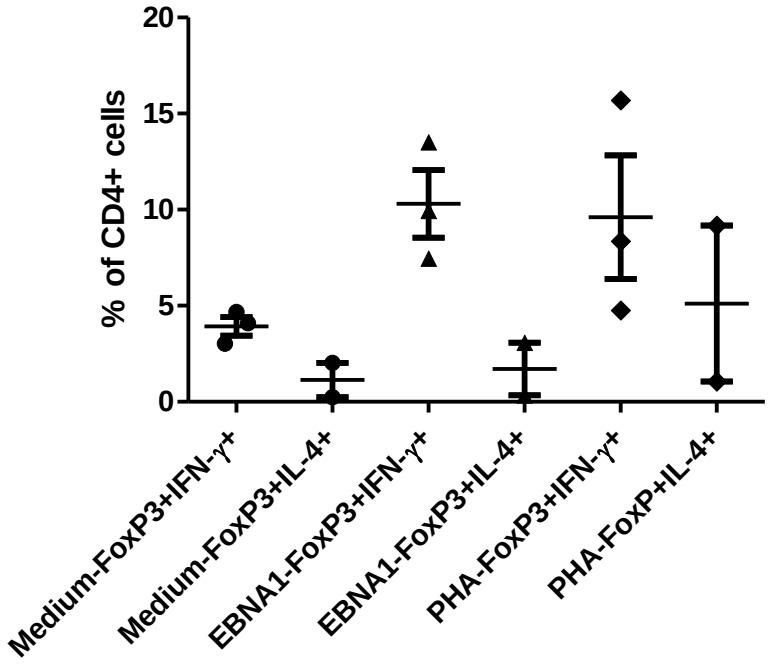
C. Controls



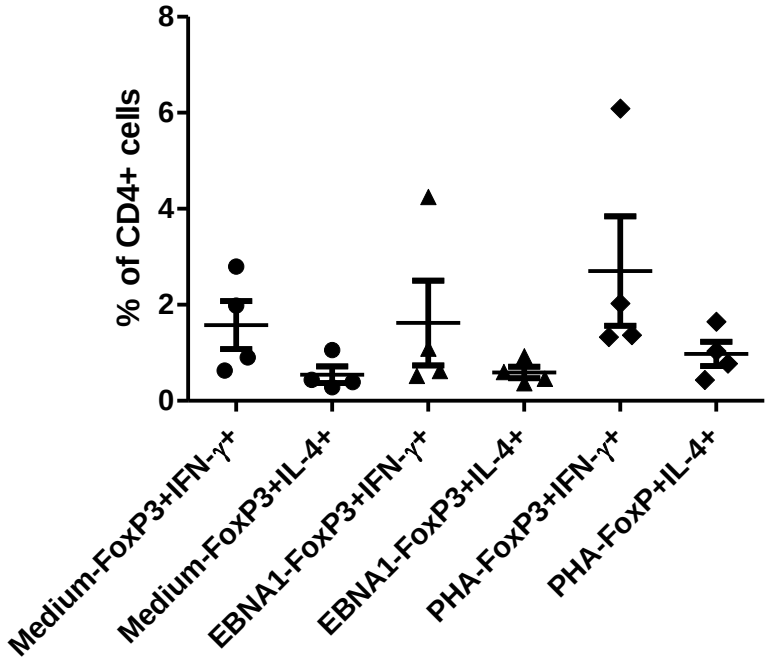
D. BL



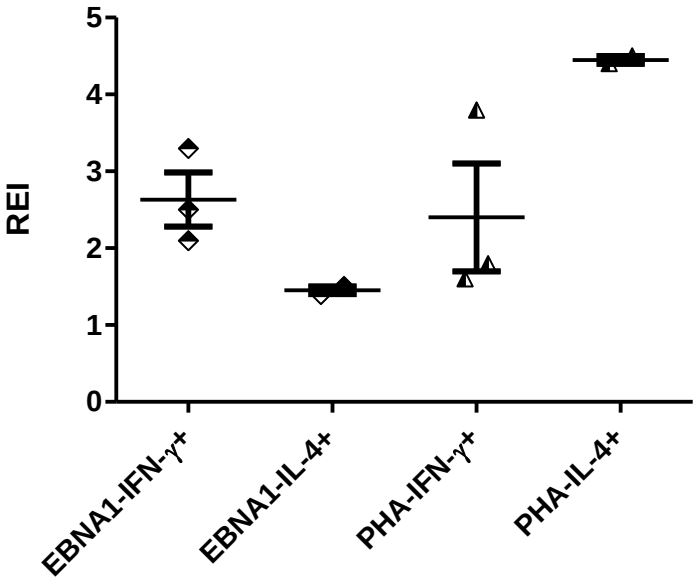
E. CD4+FoxP3+ cells of controls



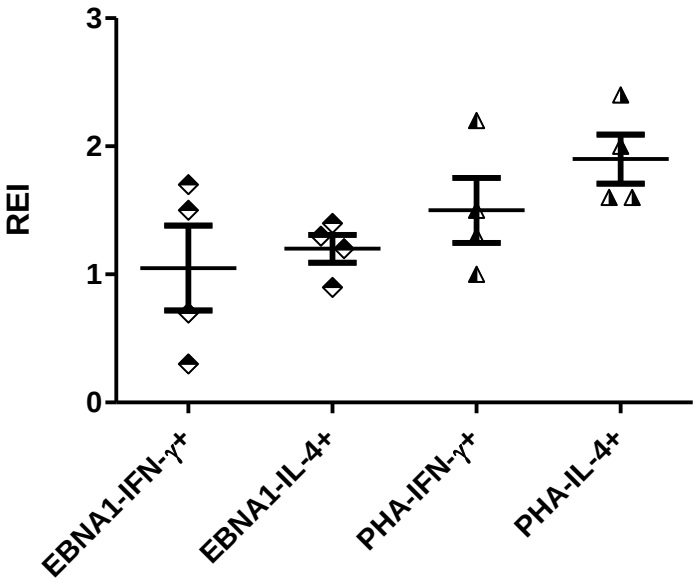
F. CD4+FoxP3+ cells of BL patients



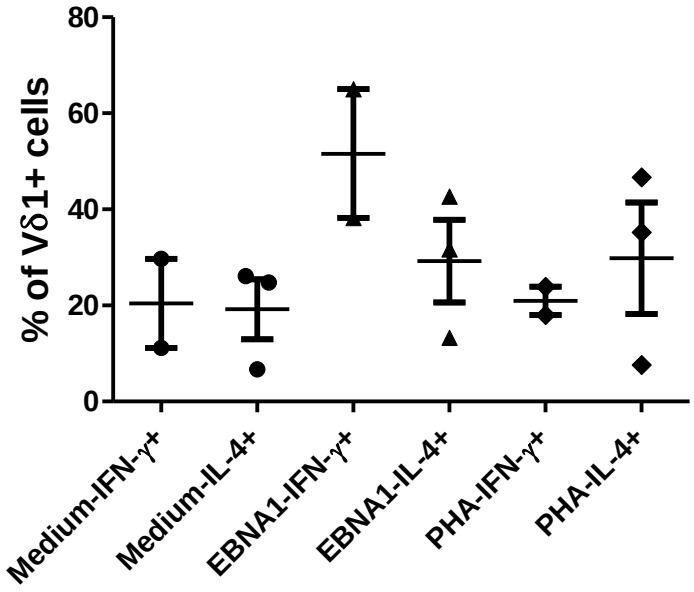
G. CD4+FoxP3+ cells of controls



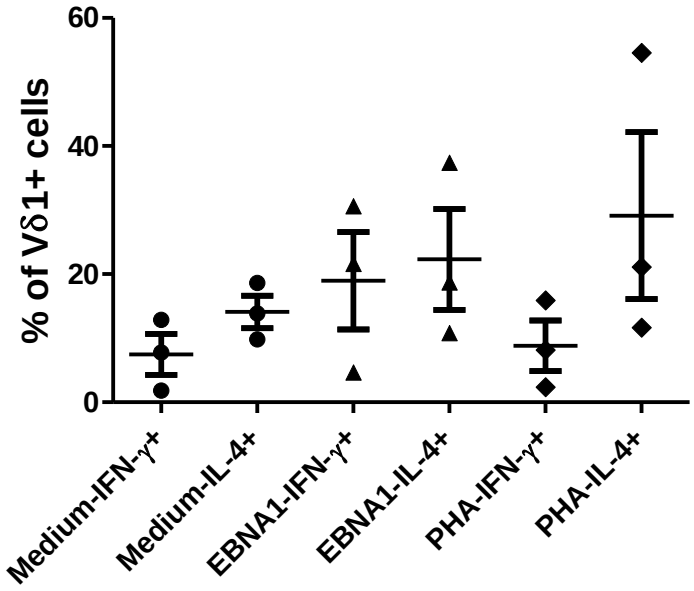
H. CD4+FoxP3+ cells of BL patients



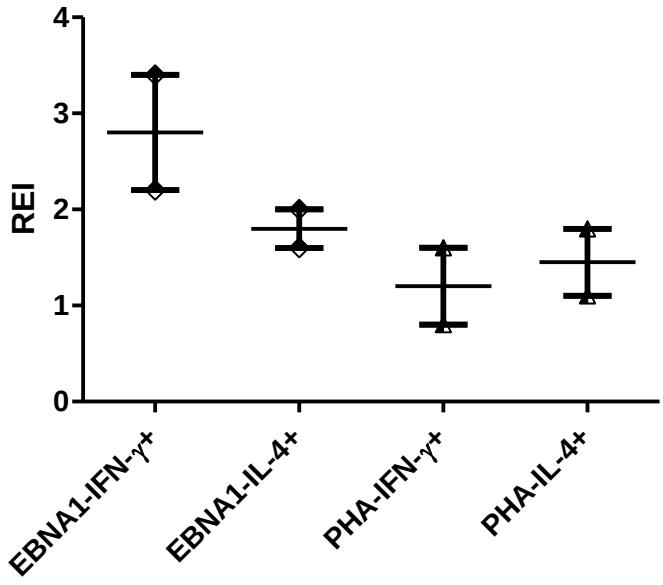
I. Vδ1+ Cells of controls



J. Vδ1+ Cells of BL patients



K. Vδ1+ Cells of controls



L. Vδ1+ Cells of BL patients

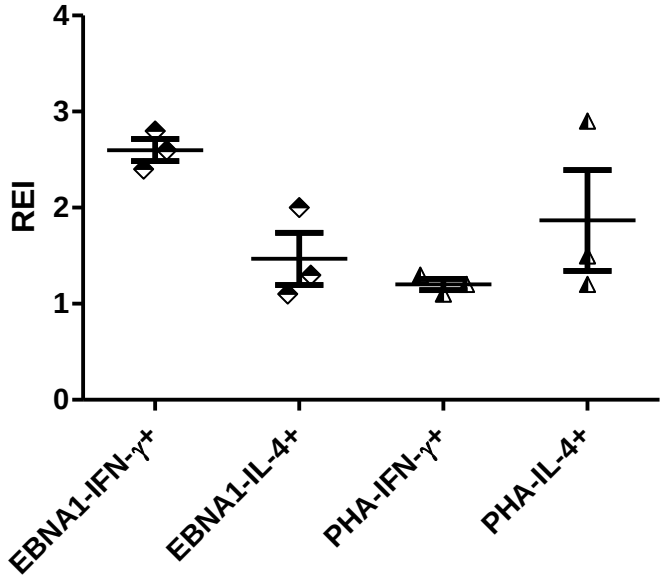


Figure 20: IFN-γ and IL-4 Expression to EBNA1 and PHA by CD4+ and Vδ1+ Cells. Comparisons were made between IFN-γ and IL-4 expression by CD4+, CD4+FoxP3+ and Vδ1+ cells of controls (A, E and I, respectively) and and BL patients (B, F and J, respectively). REIs for IFN-γ and IL-4 among CD4+ (E and F), CD4+FoxP3+ (G and H) and Vδ1+ (K and L) cells of controls, and BL patients, respectively. Bars show mean±SE.

4.4. 2 Plasma levels of Th1 and Th2 Cytokines, TNF-α and IL-10

The median levels of TNF-α in peripheral blood as measured in the plasma by ELISA was significantly lower in eBL patients compared to healthy controls (P=0.002). Conversely, plasma level of IL-10 was significantly higher in eBL patients than in healthy controls (p=0.036). This is presented in figure 18 below.

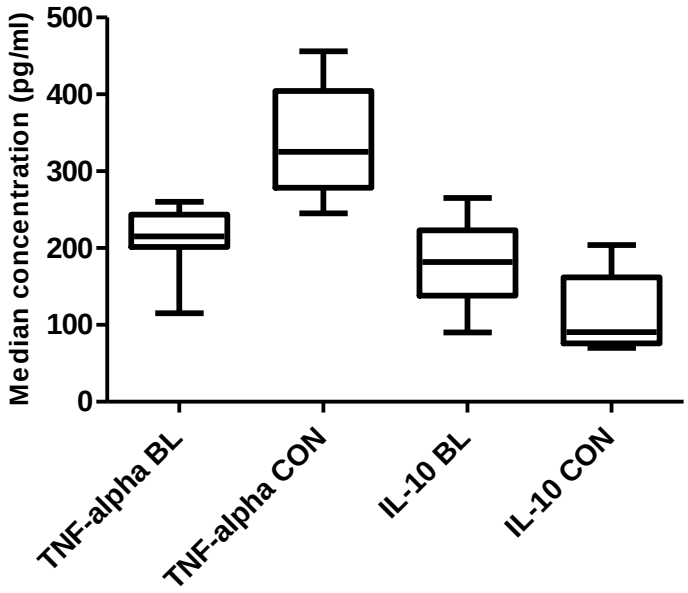


Figure 21: Plasma levels of TNF-alpha and IL-10 of eBL patients and controls

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

WBC Subpopulations with special focus on CD4+ and CD8+ T Cells in Patients and Controls

Children living in malaria endemic regions are the highest risk of having eBL. It has been established that malaria contributes to the development of eBL and it is believed that persistent malaria and dysregulation of the immune system associated with malaria may play an important role in the development of eBL. Recent studies have shown diminished EBV-specific Th1 responses in children living in malaria-holoendemic areas (Moormann *et al*., 2007) and deficiency of EBNA1-specific IFN-γ T cell responses in children with eBL (Moormann *et al.*, 2009). This study aims at throwing light on some of the possible mechanisms that lead to reduced specific responses to EBNA1and how malaria contributes to it.

The high level of WBCs in eBL and malaria patients is obviously due to significant increase in the level of neutrophils in the patient groups. Neutrophils are major players in the defence against infectious agents and higher levels are indications that the body is battling an active infection. Low levels of lymphocyte in malaria patients compared to healthy controls corroborate previous data(Worku *et al.*, 1997). It is expected that the numbers and frequency of lymphocytes increase in eBL patients. This is because eBL is a cancer of lymphocytes and since the frequency of B cells increases in eBL as observed in our earlier study(Futagbi *et al.*, 2007), However both the absolute numbers and frequency of lymphocytes, though higher in eBL compared to healthy controls, they did not differ significantly. These indicate that some components of the lymphocyte population, such as NK and/or T cells, might have declined. A reduction in the frequency of T cells identified.

Reduction in the frequency of T cells in the peripheral circulation of eBL patients could be due to any of the following: increase in B and NK cell frequencies, reallocation of T cells, activation-induced cell death (AICD) or suppression of Th1 responses by other mechanisms including activities of Tregs. Increase in the frequency of B cells in eBL is known but the median difference in frequency of B cells between eBL and healthy controls was 5.4% of all gated lymphocytes in our previous study (Futagbi *et al.*, 2007). However, the difference in CD3+ cell frequency between eBL patients and healthy controls in this study was more than 10%. This means that increase in the frequency of B cells alone could not contribute to the low frequency of T cells in eBL. Activation status of T cells in eBL does not indicate AICD as the cause of lymphopenia. It was observed in our earlier study that CD95 expression by CD3+ T cells was not different between eBL patients and healthy controls (Futagbi *et al.*, 2007). The profile of CD3+ T cells rather suggests reallocation of T cells to the site of endothelium inflammation and possibly to the tumour sites. Lymphopenia is a known phenomenon in *P. falciparum* malaria and is corroborated by this study and data from studies suggest reallocation of T cells to sites of endothelium inflammation as the cause of lymphopenia rather than AICD (Elhassan *et al.*, 1994).

Though the frequencies of CD3+CD4+ and CD3+CD8+ T cells did not differ among the categories, it was observed that the frequency of double positive CD4+CD8+ T cells was significantly lower in eBL and malaria compared to healthy controls but similar between eBL and malaria. CD4+CD8+ formed a very small fraction of T cells but have high cytotoxic activity (Sasahara *et al.*, 1994). Their reduction in the peripheral blood of patients is not clear. Selective recruitment to sites of inflammation or tumour site is a possibility. Higher CD4/CD8 ratio was also observed in eBL compared to malaria patients and healthy controls. The high CD4/CD8 ratio observed in eBL may be due to reduction in the frequency of CD8+

cells, though not significant, with significant a decrease in the frequency of γδ T cells as a contributing factor. In our previous study, we found that in eBL, 36% of γδ T cells express CD8 whereas only 8% of them express CD4. In healthy controls, 31% and 5% of the γδ T cells were γδCD8+ and γδCD4+, respectively. Therefore, significant reduction in the frequency of γδ T cells would lead to decrease in the frequency of CD8+ T cells and consequently high CD4/CD8 ratio. Decrease in the frequency of γδ T cells has been observed in our previous and current data (Futagbi *et al.*, 2007), Figure 18). The normal CD4/CD8 ratio is two (2). The high CD4/CD8 ratio implies that eBL patients are not immunodeficient but there is a sign of major infection or blood cancer. But if a normal or high CD4/CD8 ratio is as a result of reduction in frequency of γδ T cells and consequently decrease in CD8+ cells, then there may be a deficiency of a sort at the periphery.

CD95 is an apoptotic marker that is involved with AICD. Though the frequency of CD4+CD95+ was significantly higher in eBL patients compared to healthy controls, it may not necessarily mean that AICD is occurring in CD4+ T cells because the frequency of CD4+ cells did not differ among the categories. CD95 is also known to be non-apoptotic. It is found that high concentration of CD95 agonists lead to inhibitory effect on cellular immunity while low concentration drastically upregulates expression of activation markers and increases production of Th1 cytokines such as IFN-γ, TNF-α and IL-2 (Paulsen *et al.*, 2011; Paulsen and Janssen, 2011) . The high levels of CD95 expression did not lead to significant increase in expression of early activation markers such as CD25 and CD69, which are even expected to increase during infection. Additionally, the significant inverse relationship between CD4+ and CD4+CD95+ cells in eBL only notwithstanding similar frequencies of CD4+ cells in all categories, indicates that the frequency of CD4+ cells would have been higher in eBL without high levels of CD4+CD95+ cells. Moreover, EBNA1-Th1-specific responses are reduced or

lost in eBL and it is tempting to speculate that the expression of CD95 is having an inhibitory effect on responses, especially Th1 responses, by CD4+ T cells in eBL.

The reduction in CD4+-EBNA1-specific response, and response to PHA, in eBL could also be due activities of Treg cells. The higher IFN-γ REI for CD4+ and CD4+FoxP3+ T cells from healthy donors compared to those from eBL patients corroborate the loss of EBNA1-specific response by CD4+T cells in eBL reported in a recent study (Moormann *et al.*, 2009). Moreover, whereas in healthy controls the REI was higher for CD4+FoxP3+ cells than for all CD4+ cells together, the opposite is true for eBL. This also shows that CD4+FoxP3+ cells are a better source of IFN-γ in healthy controls but not in eBL patients. The low expression of IFN-γ by CD4+ cells in eBL might be as result suppression by T reg cells, in fact it is some of the CD4+FoxP3+ that constitute the FoxP3 expressing T reg cells.

The similarity in REI for CD8+ T cells between eBL patients and healthy controls indicates that there is no reduction in EBNA1-specific Th1 response by CD8+ T cells in eBL. This is also in agreement of what has been observed in a similar study (Moormann *et al.*, 2009). However, in CD8+ T cells, just as in CD4+ cells, the frequency of CD8+ T cells from healthy controls expressing IFN-γ was higher for all stimulants than their counterparts from eBL patients implying reduction in not only EBNA1-specific responses but also responses to other stimulants such as PHA by CD8+ T cells. Since frequency of CD8+CD95+ T cells in eBL was not significantly higher than that of healthy controls, the reduction might not be due to CD95 expression. It could be as a result of reduced EBNA1-specific CD8+ central memory T cells. It has been found in a recent study that individuals living in regions holoendemic malaria have reduced EBV-specific CD8+ central memory T cells compared to individuals living in regions hypoendemic malaria (Chattopadhyay *et al.*, 2013) and this could be due to activities of Treg cells which is persistent feature of *P. falciparum* infection. Another study

has shown that high proportions of circulating FoxP3+ Treg cells are associated with low fractions of CD4+ T cells with memory phenotype in infants (Rabe *et al.*, 2011).

CD69 has been shown to induce TGF-β production in many cells including $CD4^+$ and $CD8^+$ T cells and therefore exhibiting immunoregulatory function(Radstake *et al.*, 2009) and its deficiency has been shown to enhance antitumour immunity (Esplugues *et al.*, 2003) In this study, the frequency of CD4CD69+ cells were significantly higher in malaria patients compared to controls but it did not differ significantly between eBL and malaria patients or between eBL patients and healthy controls. This indicates that regulatory mechanism involving CD69 may be present in malaria and possibly at moderate level in eBL.

γδT Cells and endemic BL

In a previous study, it was reported that frequencies of TCR-$\gamma\delta^+$ cells are selectively increased in *P. falciparum*-exposed healthy Ghanaians in general, and in children in particular. This was mainly due to remarkable expansion of $V\delta1^+$T cells, which also constitute the dominant subset of $\gamma\delta^+$ T cells in both adults and children from malaria endemic areas. (Hviid *et al.*, 2000). Additionally, the $V\delta1^+$ subset further increase during malaria (Hviid *et al.*, 2001). The findings, in this study, regarding the frequencies of TCR-$\gamma\delta^+$ and $V\delta1^+$T cells in healthy controls, malaria and eBL patients, support the earlier data and show a similar lymphocyte composition between with eBL and those with malaria. The increase in the proportion of $V\delta1^+$ cells in both eBL patients and children with *P. falciparum* malaria is of interest because it has been demonstrated that $V\delta1^+$ cells can recognize and kill EBV+ lymphoma cells (Hacker *et al.*, 1992). Data from a recent study also strongly suggest that $V\delta1^+$ T cells have anti-tumour properties (Schilbach *et al.*, 2008). The result also shows that $\gamma\delta^+$ T cells eBL patients are more activated than those in healthy controls, suggesting that eBL patients have

more antigen-experienced, effector or memory cells than healthy controls. The data from this study shows that Vδ1$^+$ T cells from eBL patients have similar EBNA1-specific Th1 response compared to those from healthy controls. If patients have more specific effector cells, they would have expressed more IFN-γ than controls. There is, therefore, no indication that the difference in the frequency of activated cells in eBL is due to differences in EBNA1-specific effector cells between patients and controls. However, the data show that $\gamma\delta^+$ T cells expressed more IFN-γ than CD4+ and CD8+ T cells and are capable of mounting protective Th1 response to the tumour and can do it better than CD4+ and CD8+ T cells. If these cells are redistributed to the tumour sites then it is expected that they check the expansion of the tumour but that would be possible if they are there in adequate numbers and are not suppressed by the micro-environment. It has been shown that intratumoral T reg cells inhibit proliferation and function of CTLs and their depletion improves the efficacy of anti-tumour vaccines (Yang *et al.*, 2006). Analysis of intratumoral T cells in eBL may be required to draw conclusion on the activity of TCR-$\gamma\delta^+$ cells, particularly, Vδ1$^+$ T cells in eBL.

Regulatory T Cells

One of the underlying factors in tumour immunology is immunological tolerance, which is specific non-reactivity of the immune system to a particular antigen due to anergy, clonal deletion of specific responder cells and/or immunoregulation. Treg cells are involved in immunological tolerance and malaria is known to promote the development and function of Treg cells. Studies have also pointed to a role of Treg cells in development of tumours (Yang *et al.*, 2006; Strauss *et al.*, 2007) but their role in the pathogenesis of eBL is not yet clear. In this study, it has been observed that the frequencies of CD4+CD25hi+, CD4+CD25hi+FoxP3 and CD4+CD25hi+Foxp3- cells were significantly increased in both malaria and eBL compared to healthy controls. This result shows involvement of Tregs in immunity to eBL

and confirms the speculation that malaria could contribute to the development of EBV+ tumours through activities of T reg cells. It also indicates that the two mechanisms of suppression by T reg cells are at work in the eBL too. $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells carry out their suppressive activity through cell-cell contact and partly by cytokine signaling. These Treg cells were also shown to suppress IL-2 secretion by $CD4^+$ effector T cells specific for EBNA1 as well as a melanoma antigen (Voo *et al.*, 2005). CD4+CD25+Foxp3- Treg cells, on the other hand, are known to exert their activity through secretion of immunosuppressive cytokines such as TGF-β and IL-10.

It has been suggested that the role of *P. falciparum* malaria in reduced or loss of responses to EBV antigens may be by way of impaired antigen presentation through active IL-10-dependent suppression of DC-mediated priming of T cells, which is known in malaria (Pouniotis *et al.*, 2005) or down-regulation of the IFN-γ T cell response due to T cell exhaustion (Moormann *et al.*, 2009). The high frequencies of CD4+CD25+Foxp3+ and CD4+CD25+Foxp3- Treg cells in malaria and eBL is an indication of parallel between dysregulation of the immune system in the two diseases and suggests that T cell exhaustion is at play in eBL.

It can be hypothesized that at the beginning of lymphomagenesis, malaria may provide a pool of Treg cells that promotes tolerance and once the tumour is formed, it is able to generate its own Treg cells.

Th1/Th2 Responses to EBNA1 in eBL patients and Controls

These results show that except in CD4+FoxP3+ T cells where the frequency of IFN-γ expression to EBNA1 stimulation was higher than IL-4 in both patients and controls, the frequencies of IFN-γ expression to EBNA1 and PHA by CD4+ and Vδ1+T cells were higher than IL-4 in controls only and lower than IL-4 expression in eBL patients. This indicates that responses in controls are pro-Th1 whereas those in eBL are not only skewed in favour of Th2 but also the skewing is not specific to EBNA1. This suggests that there is a general mechanism rather than result of immediate or direct response to EBNA1 in the Th2-weighted responses.

REI for IFN-γ and IL-4 in CD4+ cells were similar in both eBL patients and health controls. With regard to CD4+FoxP3+ T cells, REI for IFN-γ on stimulation with EBNA1 was higher than REI for IL-4 in controls. In patients, the REI for IFN-γ and IL-4 in CD4+FoxP3+ T cells were similar. This shows that CD4+FoxP3+ T cells from healthy controls are pro-Th1 in their response to EBNA1 but not so in eBL patients. This is not surprising because an increased frequency of Treg cells has been observed in the eBL patients. Treg cells inhibit Th1 responses (Finney *et al.*, 2009).

The results also indicate that though Vδ1+ cells have higher REI for IFN-γ compared to IL-4 in both patients and controls, there is weighting of the responses in favour of Th2 in eBL patients as the frequency of Vδ1+ cells expressing IL-4 to both EBNA1 and PHA were higher than those expressing IFN-γ. This suggests that there is a mechanism outside Vδ1+ cells that skewed the Vδ1+ T cell response in eBL patients in favour of Th2. Vδ1+ T cells have been shown to produce both Th1 and Th2 cytokines depending on the environment (Vermijlen, 2007) and their pro-Th2 response might be due to other cells and the cytokine

they secreted. There is therefore contribution by Vδ1+ T cells to tilting of the Th1/Th2 balance in favour of Th2 in eBL.

The low plasma level of TNF-α and conversely high level of IL-10 in patients is an indication of similar dysregulation of the immune response *in vivo*. IL-10 promotes Th2 responses while down-regulating Th1 responses, such as proliferation and activation of CTLs. No significant increases in CD4+CD25+ and CD8+CD25+ cells were observed in eBL but in malaria CD4+ cells significantly expressed higher frequency of the early activation marker, CD25 (Figure 14). This may be an indication that there is suppression of CTLs and the mechanisms of suppression include IL-10 production. IL-10 is also known to trigger anergy in T cell(Akdis and Blaser, 1999). The source of Il-10 in the plasma is not known but possible sources are obvious. Tr1 cells or CD4+CD25hi+ cells, which themselves require IL-10 for their development are known to secrete IL-10 (Roncarolo *et al*., 2001) and EBV-infected B cells also produced IL-10, especially Lymphoblastoid B cell lines expressing the III latency program (Burdin *et al*., 1993). These cells are all present in eBL patients.

TNF-α, as its name connotes, has the ability to kill tumour cells. It has been shown in an islet cancer of the pancreas that TNF as well as IFN-γ can, at least, drive cancer cells into senescence, if they cannot destroy them (Muller-Hermelink *et al.*, 2008; Braumuller *et al.*, 2013). Down-regulation of TNF-α production in eBL patients indicates that the anti-tumour mechanism involving TNF-α is rather reduced in patients where it is most needed. However, role of TNF-α has been found to be paradoxical, involving both protective and destructive or pathological mechanisms, in infectious diseases such as tuberculosis and malaria. In the presence of TNF-α, IL-4 promotes apoptosis of reactive lymphocytes (Grau *et al.*, 1989; Seah and Rook, 2001; Mootoo *et al.*, 2009) and this would consequently suppress protective cellular immunity.

In conclusion, there is tilting of the Th1/Th2 balance in favour of Th1 in addition to reduced production of TNF-α in eBL patients and these might weaken their protective immunity to eBL.

5.2 Conclusions

- There were similar changes in WBCs and lymphocyte composition in eBL and malaria patients.
- Similar T lymphocyte frequency and subsets were observed in eBL and malaria patients.
- The frequency of CD4CD25+ and CD4CD69+ cells were higher in malaria patients compared to controls but similar between eBL and malaria and, conversely, the frequency of CD4CD95+ cells was higher in eBL patients compared to controls but not different between eBL and malaria patients.
- CD4+ and CD4FoxP3+ cells expressed more IFN-γ and had higher IFN-γ REI to EBNA1 in controls than in eBL patients and similarly, the frequency of CD8+ T cells from healthy controls expressed more IFN-γ than those from eBL patients.
- The percentage of CD3+ T cells that expressed TCR-γδ were different significantly among the patient groups and healthy controls, with malaria patients having the highest frequency and eBL patients with the least.
- The frequency of γδ+Vδ1+ cells was higher in both malaria and eBL patients compared to healthy controls.
- TCR-γδ+ cells were more activated in eBL patients than healthy controls.
- Similar to CD4+ T cells, Vδ1+ γδT cells in healthy controls express more IFN-γ to all stimulants than those from eBL patients but higher frequency of Vδ1+ γδT cells express IFN-γ compared to CD4+ cells.
- The frequencies of CD4+CD25hi+Foxp3+ and CD4+CD25hi+Foxp3- T reg cells were higher in both malaria and eBL patients compared to controls
- Whereas the frequency of IFN-γ expression by CD4+ cells was higher than IL-4 expression to all stimulants in healthy controls, the opposite is true for eBL patients.

- Upon EBNA1 stimulation, CD4+FoxP3+ and Vδ1+ cells T cells from controls were pro-Th1 while those from eBL patients were pro-Th2.
- The levels of TNF-α in peripheral blood was significantly lower in eBL patients compared to healthy controls. Conversely, plasma level of IL-10 was significantly higher in eBL patients than in healthy controls.

5.3 Recommendations

- There is the need to carry out longitudinal investigation into the profile of T cells, importantly of T reg cells and γδ T cells in eBL.
- It is also imperative to analyse and ascertain the phenotype of intratumoural T cells. This would provide solid evidence on recruitment of T cells and immunity in the micro-environment of the tumour.
- It may also be of interest to do futher work on the possibility of involvement of CD95 expression in reduced Th1 response to EBNA1.
- In order to better understand the state of immunity during eBL, expanded cytokine analysis in eBL also need to be looked at.

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APPENDICES

Appendix I: Combinations of fluorochromes and MAbs used during surface staining of PBMC in flow cytometric analyses

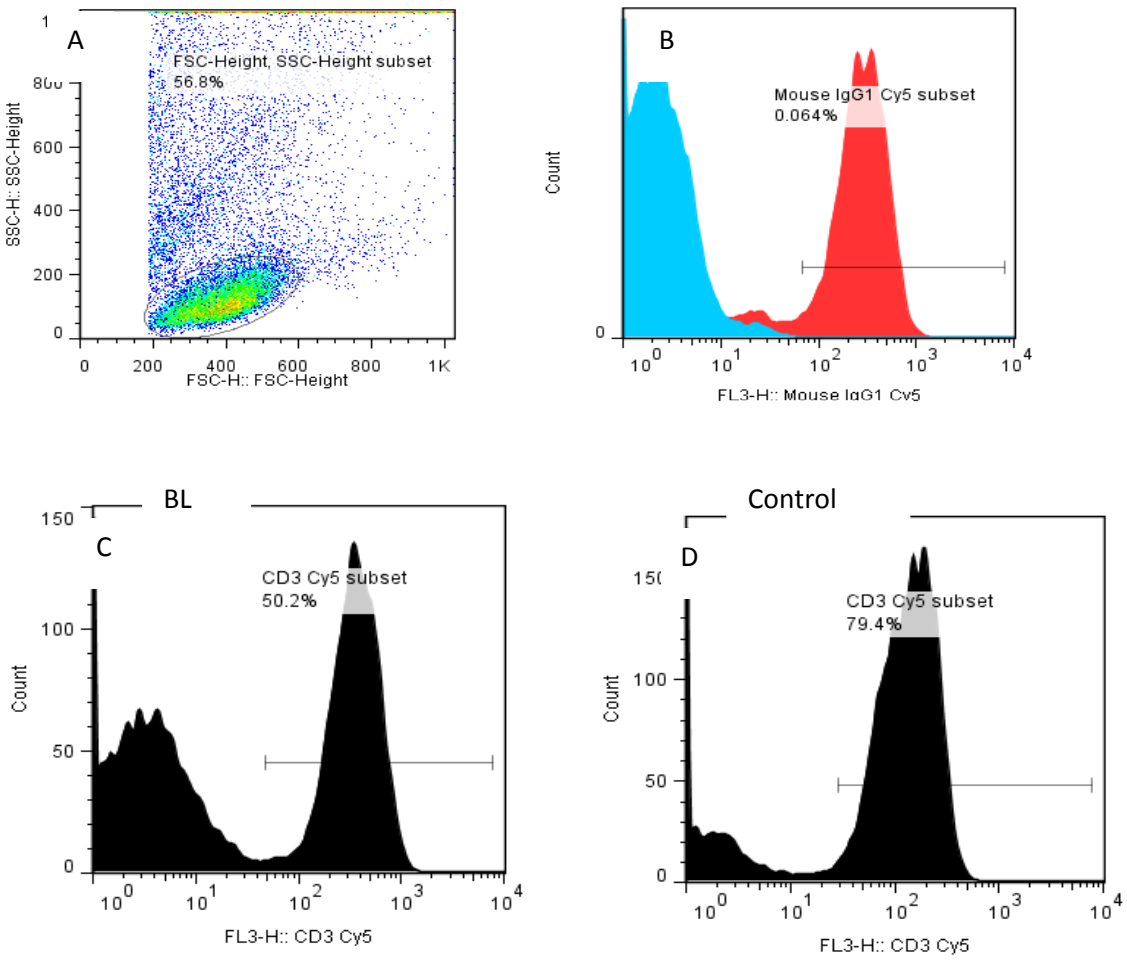
*FITC	*PE	*PE-Cy5
anti-G1	*anti-G1*	*anti-G2a*
anti-γδ	*anti*-CD95	*anti*-CD3
anti-CD8	*anti*-CD4	*anti*-CD3
anti-G1	*anti-G1*	*anti-G1*
anti-γδ	*anti*-CD4	*anti*-CD25
anti-Vδ1	*anti*-CD4	*anti*-CD25
anti-CD8	*anti*-CD95	*anti*-CD25
anti-G1	*anti-G1*	
anti-Vδ1	*anti*-γδ	
anti-CD4	*anti*-CD69	
anti-γδ	*anti*-Vγ9	
anti-CD4	*anti*-CD95	
anti-γδ	*anti*-CD69	
anti-G1	*anti-G2b*	
anti-γδ	*anti*-HLA-DR	

*FITC, fluorescein isothiocyanate; PE, phycoerythrin; PE-Cy5 phycoerythrin-Cy5

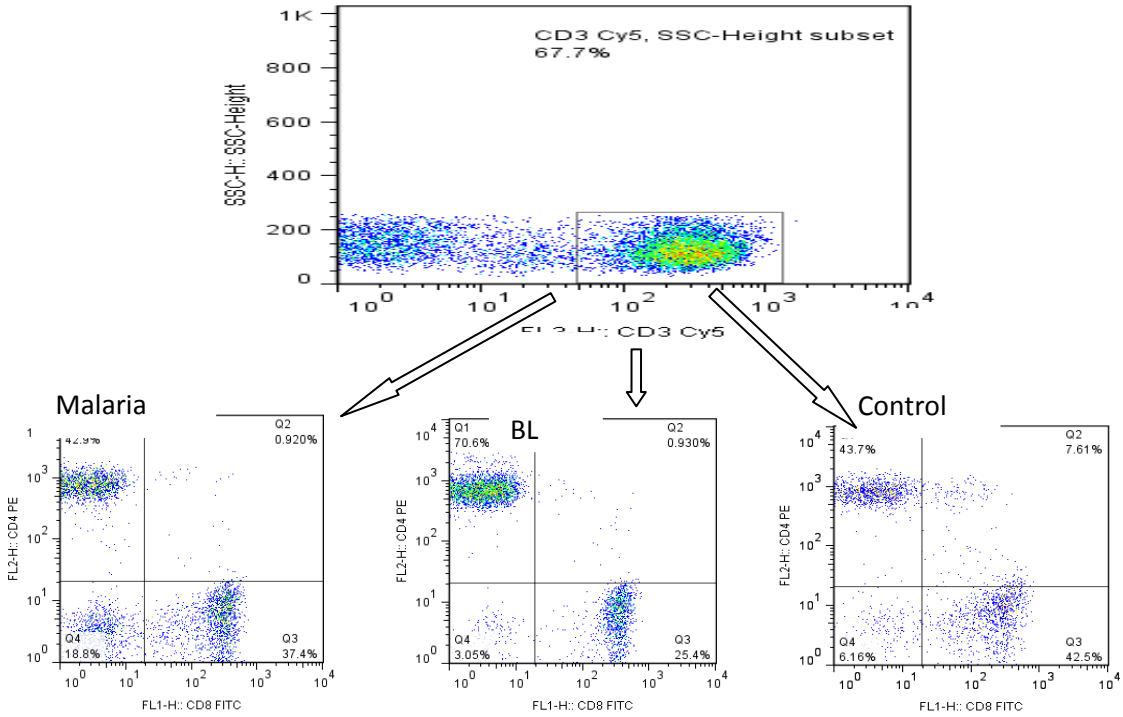
Appendix II: Combinations of fluorochromes and MAbs used in intracellular staining of PBMC in flow cytometric analyses

*FITC	*PE	*PE-Cy5
anti-G1	*anti-G1*	*anti-G2a*
*anti-*CD25	*anti-*CD4	*anti-*FoxP3
*anti-*Vδ1	*anti-*γδ	*anti-*FoxP3
*anti-*CD4	*anti-*IL-4	*anti-*FoxP3
*anti-*CD4	*anti-*IFN-g	*anti-*FoxP3
*anti-*CD8	*anti-*IL-4	*anti-*FoxP3
*anti-*CD8	*anti-*IFN-g	*anti-*FoxP3
*anti-*Vδ1	*anti-*IL-4	*anti-*FoxP3
*anti-*Vδ1	*anti-*IFN-g	*anti-*FoxP3

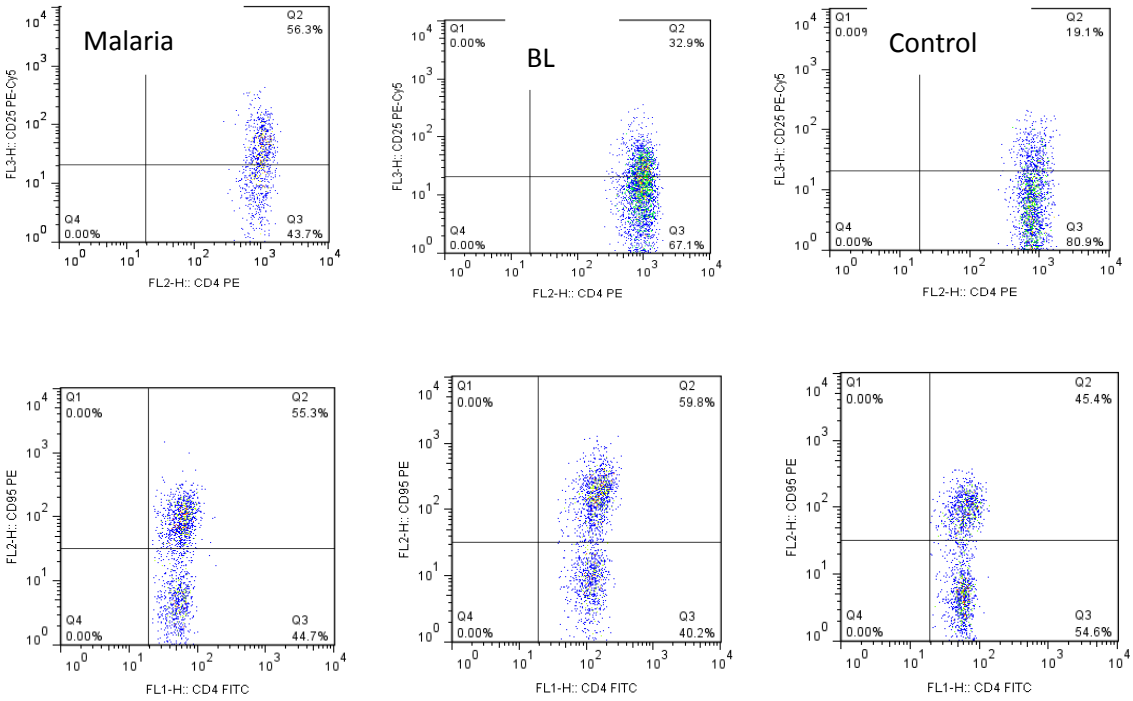
*FITC, fluorescein isothiocyanate; PE, phycoerythrin; PE-Cy5 phycoerythrin-Cy5



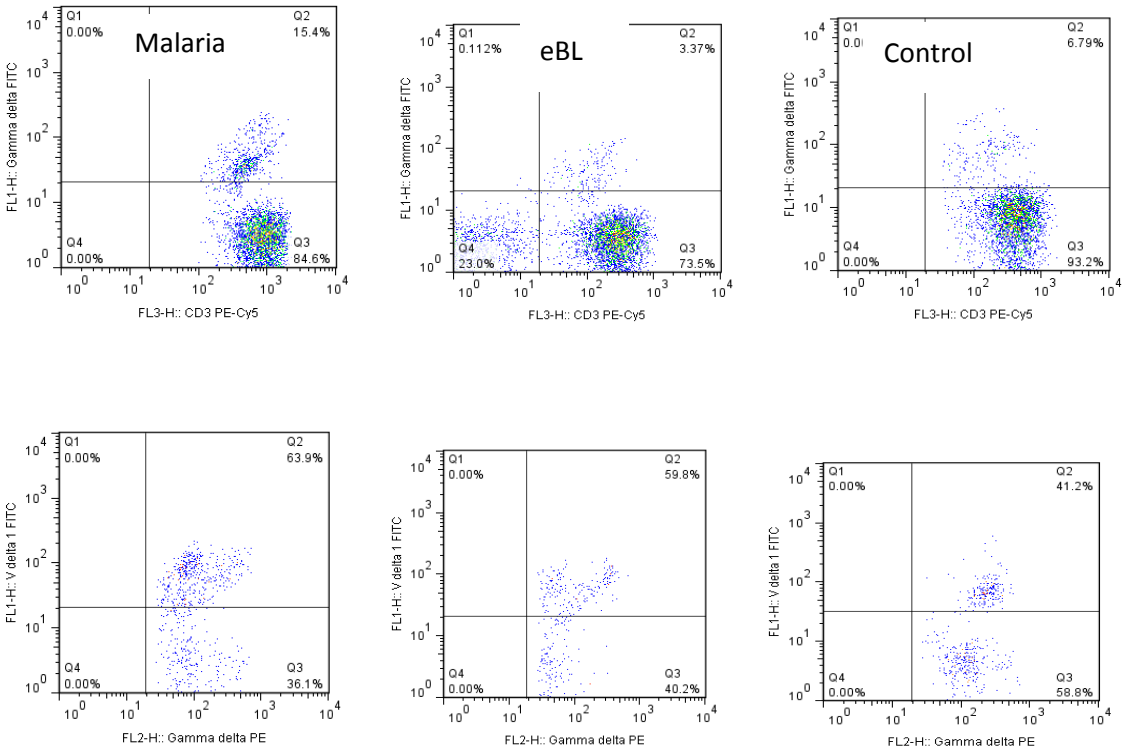
Appendix III: Analysis of T cell counts: Lymphocyte population was set using FSC properties and gated (A), and histogram profiles of isotype controls (B) and T cells on the FSC for eBL patients (C) and and controls (D) are displayed



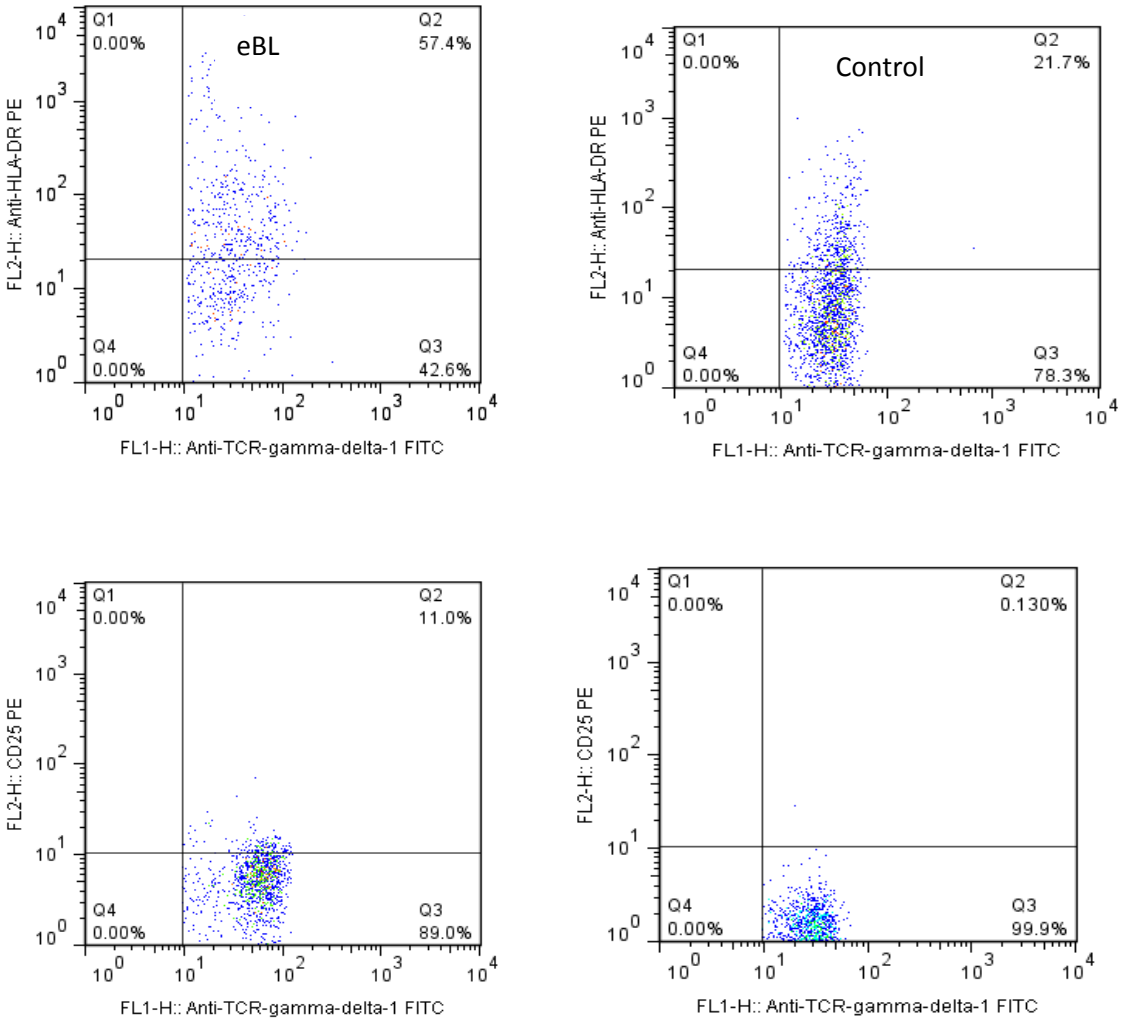
Appendix IV: Analysis for CD4+CD8+ cells. T Lymphocyte population was set using FSC properties and gated (top panel). The lower panels show quadrant plots representative of double expression of CD4 and CD8 by T cells in malaria (left panels), eBL (middle panels) controls (right panels).



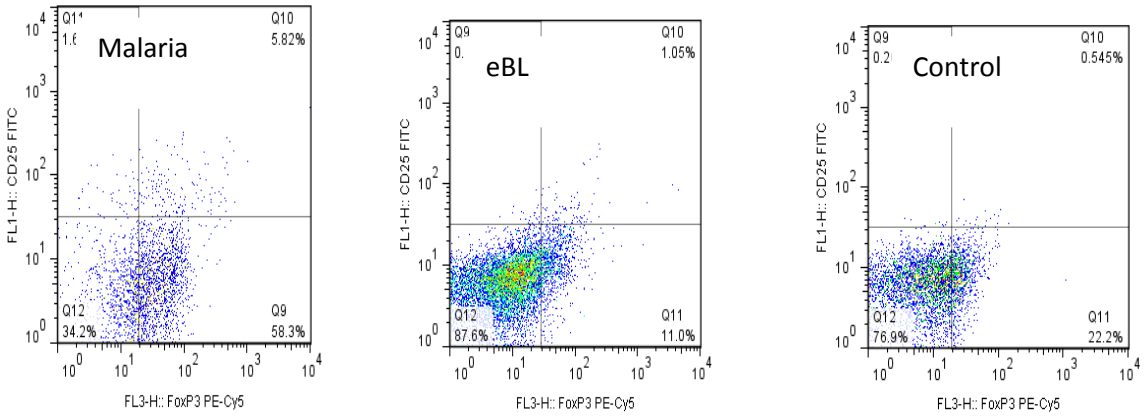
Appendix V: CD25 and CD95 expression by CD4+ cells. Shown are plots representative of malaria (left panels), eBL (middle panels) controls (right panels)



Appendix VI: Analysis of frequencies of γδ+ and Vδ1+ cells. Shown are plots representative of malaria (left panels), eBL (middle panels) controls (right panels)



Appendix VII: Expression of activation markers, CD25 and HLA-DR by γδ+ T cells. Shown are plots representative of malaria (left panels), eBL (middle panels) controls (right panels)



Appendix VIII: CD25 and FoxP3 expression by CD4+ cells. Shown are plots representative of malaria (left panels), eBL (middle panels) controls (right panels)

Appendix IX: IFN-γ Production by CD4+ T Cells in Response to EBNA1

Donor	Stimulant	CD4IFN-γ % of al gated lymp	*REI	CD4IFN-γ % of al CD4+cel	*RE	CD4FoxP3IFN-γ % of all CD4+cells	*REI
FG20	Medium	4.23		11.5		4.67	
	Medium +EBNA1	6.28	1.5	13.7	1.2	9.94	2.1
	Medium+PHA	8.54	2.0	14.5	1.3	8.36	1.8
FG20	Medium	6.7		12.2		4.1	
	Medium +EBNA1	7.9	1.2	20	1.6	13.5	3.3
	Medium+PHA	9.2	1.4	24	2.0	15.7	3.8
FG20	Medium	2.55		7.54		3.02	
	Medium +EBNA1	5.5	2.2	13.8	1.8	7.46	2.5
	Medium+PHA	3.78	1.5	10.2	1.4	4.76	1.6
	Medium +EBNA	**6.56**	**1.6**	**15.83**	**1.5**	**10.3**	**2.6**
Mean	**Medium+PHA**	**7.17**	**1.6**	**16.23**	**1.6**	**9.6**	**2.4**
G03	Medium	1.77		3.77		0.629	
	Medium +EBNA1	2.34	1.3	5.13	1.4	1.09	1.7
	Medium+PHA	2.01	1.1	3.95	1.0	1.37	1.3
G07	Medium	2.1		4.17		2.8	
	Medium +EBNA1	2.99	1.4	5.69	1.4	4.25	1.5
	Medium+PHA	3.77	1.8	7.35	1.8	6.09	2.2
G09	Medium	1.2		1.5		1.98	
	Medium +EBNA1	1.7	1.4	2.63	1.8	0.526	0.3
	Medium+PHA	1.42	1.2	2.98	2.0	2.03	1.0
G10	Medium	2		3.39		0.905	
	Medium +EBNA1	2.39	1.2	3.11	0.9	0.622	0.7
	Medium+PHA	2.02	1.0	4.12	1.2	1.33	1.5
	Medium +EBNA	**2.36**	**1.3**	**4.12**	**1.4**	**2.60**	**1.1**
Mean	**Medium+PHA**	**2.31**	**1.3**	**4.60**	**1.5**	**2.71**	**1.5**

*REI (relative expression index)= percentage of CD4 cells expressing IFN-γ after culture with a stimulus (EBNA1 or PHA) divided by percentage of CD4 cells expressing IFN-γ after culture in medium without stimulus.

Appendix X**: IFN-γ Production by CD8+ T Cells in Response to EBNA1**

Donor	Stimulant	CD8IFN-γ % of all gated lym	*RE	CD8IFN-γ % of all CD8+ cel	*REI
FG202	Medium	6.06		15.38	
	Medium +EBNA1	12.3	2.0	28.8	1.9
	Medium+PHA	14.9	2.5	32.8	2.1
FG203	Medium	4.59		8.82	
	Medium +EBNA1	9.02	2.0	19	2.2
	Medium+PHA	18.1	3.9	32.3	3.7
FG205	Medium	4.46		5.47	
	Medium +EBNA1	6.69	1.5	6.3	1.2
	Medium+PHA	7.1	1.6	6.14	1.1
Mean	**Medium +EBNA1**	**9.30**	**1.8**	**18.0**	**1.8**
	Medium+PHA	**13.4**	**2.7**	**23.75**	**2.3**
G07	Medium	1.79		11.2	
	Medium +EBNA1	2.89	1.6	14.5	1.3
	Medium+PHA	3.21	1.8	18.38	1.6
G09	Medium	3.18		6.87	
	Medium +EBNA1	7.78	2.5	16.7	2.4
	Medium+PHA	13.5	4.3	16.6	2.4
G10	Medium	2.03		8.57	
	Medium +EBNA1	3.76	1.9	6.42	0.7
	Medium+PHA	4.12	2.0	14.7	1.7
Mean	**Medium +EBNA1**	**4.81**	**2.0**	**12.54**	**1.5**
	Medium+PHA	**6.94**	**2.7**	**16.56**	**1.9**

*REI= percentage of CD8 cells expressing IFN-γ after culture with a stimulus divided by percentage of CD8 cells expressing IFN-γ after culture in medium without stimulus.

Appendix **XI: IFN-γ Production by Vδ1+ γδ T Cells in Response to EBNA1**

Donor	Stimulant	Vdelta1IFN-γ % of all gated lym	*REI	Vdelta1IFN-γ % of all Vd1+ cells	*REI
FG203	Medium	5.41		29.70	
	Medium +EBNA1	15.50	2.9	65.00	2.2
	Medium+PHA	4.93	0.9	23.90	0.8
FG205	Medium	1.70		11.20	
	Medium +EBNA1	2.94	1.7	38.20	3.4
	Medium+PHA	1.99	1.2	18.00	1.6
	Medium +EBNA1	**9.22**	**2.3**	**51.6**	**2.8**
Mean	**Medium+PHA**	**3.46**	**1.1**	**20.9**	**1.2**
G03	Medium	0.54		1.82	
	Medium +EBNA1	0.99	1.8	4.70	2.6
	Medium+PHA	0.96	1.8	2.36	1.3
G07	Medium	1.12		12.90	
	Medium +EBNA1	3.49	3.1	30.60	2.4
	Medium+PHA	1.68	1.5	15.94	1.2
G10	Medium	1.16		7.75	
	Medium +EBNA1	2.33	2.0	21.60	2.8
	Medium+PHA	2.16	1.9	8.17	1.1
	Medium +EBNA1	**2.27**	**2.3**	**18.97**	**2.6**
Mean	**Medium+PHA**	**1.60**	**1.7**	**8.82**	**1.2**

*REI = percentage of Vδ1+ cells expressing IFN-γ after culture with a stimulus divided by percentage of Vδ1+ cells expressing IFN-γ after culture in medium without stimulus.

Appendix **XII: IFN-γ and IL-4 Expression to stimulant by CD4+ Cells**

		CD4IFN-γ		CD4IFN-IL-4	
Donor	Stimulant	% of all CD4+ cell	*REI	% of all CD4+ cell	*REI
FG202	Medium	11.5		0.48	
	Medium +EBNA1	13.7	1.2	1.01	2.1
	Medium+PHA	14.5	1.3	2.12	4.4
FG203	Medium	12.2		3.03	
	Medium +EBNA1	20	1.6	4.08	1.3
	Medium+PHA	24	2.0	11.90	3.9
FG205	Medium	7.54			
	Medium +EBNA1	13.8	1.8		
	Medium+PHA	10.2	1.4		
	Medium +EBNA1	**15.83**	**1.5**		
Mean	**Medium+PHA**	**16.23**	**1.6**		
G03	Medium	3.77		3.41	
	Medium +EBNA1	5.13	1.4	6.67	2.0
	Medium+PHA	3.95	1.0	7.55	2.2
G07	Medium	4.17		10.10	
	Medium +EBNA1	5.69	1.4	12.20	1.2
	Medium+PHA	7.35	1.8	14.40	1.4
G09	Medium	1.5		2.40	
	Medium +EBNA1	2.63	1.8	3.24	1.4
	Medium+PHA	2.98	2.0	3.67	1.5
G10	Medium	3.39		2.87	
	Medium +EBNA1	3.11	0.9	3.23	1.1
	Medium+PHA	4.12	1.2	3.75	1.3
	Medium +EBNA1	**4.12**	**1.4**	**6.34**	**1.4**
Mean	**Medium+PHA**	**4.60**	**1.5**	**7.34**	**1.6**

Appendix **XIII: IFN-γ and IL-4 Expression to stimulant by CD4+FoxP3+ Cells**

Donor		CD4FoxP3IFN-γ % of all CD4 cells	*REI	CD4FoxP3IL-4 % of all CD4+ cell	*REI
FG202	Medium	4.67		0.24	
	Medium +EBNA1	9.94	2.1	0.34	1.4
	Medium + PHA	8.36	1.8	1.06	4.4
FG203	Medium	4.1		2.03	
	Medium +EBNA1	13.5	3.3	3.08	1.5
	Medium + PHA	15.7	3.8	9.17	4.5
FG205	Medium	3.02			
	Medium +EBNA1	7.46	2.5		
	Medium + PHA	4.76	1.6		
G03	Medium	0.629		0.44	
	Medium +EBNA1	1.09	1.7	0.60	1.4
	Medium + PHA	1.37	1.3	1.04	2.4
G07	Medium	2.8		1.06	
	Medium +EBNA1	4.25	1.5	0.93	0.9
	Medium + PHA	6.09	2.2	1.65	1.6
G09	Medium	1.98		0.39	
	Medium +EBNA1	0.526	0.3	0.46	1.2
	Medium + PHA	2.03	1.0	0.78	2.0
G10	Medium	0.905		0.28	
	Medium +EBNA1	0.622	0.7	0.37	1.3
	Medium + PHA	1.33	1.5	0.44	1.6
	Medium +EBNA1	**2.60**	**1.1**	**0.67**	**1.2**
Mean	**Medium + PHA**	**2.71**	**1.5**	**0.98**	**1.9**

*REI= percentage of CD4 cells that were positive for FoxP3IFN-γ and FoxP3IL-4 after culture with a stimulus divided by percentage of CD4 cells expressing the FoxP3IFN-γ and FoxP3IL-4, respectively, after culture in medium without stimulus

Appendix **XIV: IFN-γ and IL-4 Expression to stimulant by Vδ1+ Cells**

Donor	Stimulant	Vdelta1IFN-γ % of all Vδ1+ cells	REI	Vdelta1IL-4 % of all Vδ1+ cells	REI
FG202	Medium			24.8	
	Medium +EBNA1			31.7	1.3
	Medium+PHA			35.2	1.4
FG203	Medium	29.70		26.1	
	Medium +EBNA1	65.00	2.2	42.7	1.6
	Medium+PHA	23.90	0.8	46.7	1.8
FG205	Medium	11.20		6.72	
	Medium +EBNA1	38.20	3.4	13.3	2.0
	Medium+PHA	18.00	1.6	7.63	1.1
	Medium +EBNA1	**51.6**	**2.8**	**29.23**	**1.6**
Mean	**Medium+PHA**	**20.9**	**1.2**	**29.84**	**1.4**
G03	Medium	1.82		9.8	
	Medium +EBNA1	4.70	2.6	10.8	1.1
	Medium+PHA	2.36	1.3	11.7	1.2
G07	Medium	12.90		13.9	
	Medium +EBNA1	30.60	2.4	18.7	1.3
	Medium+PHA	15.94	1.2	21.1	1.5
G10	Medium	7.75		18.6	
	Medium +EBNA1	21.60	2.8	37.4	2.0
	Medium+PHA	8.17	1.1	54.6	2.9
	Medium +EBNA1	**18.97**	**2.6**	**22.3**	**1.5**
Mean	**Medium+PHA**	**8.82**	**1.2**	**29.13**	**1.9**

*REI= percentage of Vδ1+cells expressing IFN-γ and IL-4 after culture with a stimulus divided by percentage of Vδ1+ cells expressing IFN-γ and IL-4, respectively, after culture in medium without stimulus.