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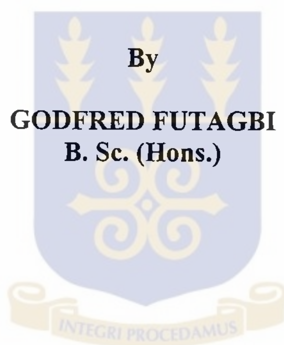
PHENOTYPIC CHARACTERIZATION AND *IN VITRO* RESPONSE OF LYMPHOCYTES OF GHANAIAN CHILDREN WITH BURKITT'S LYMPHOMA TO *PLASMODIUM FALCIPARUM* MALARIA ANTIGENS

A Thesis submitted to the Board of Graduate studies, University of Ghana, Legon, Ghana.

In partial fulfillment of the requirements for the award of the Master of Philosophy degree in Zoology (Applied Parasitology)



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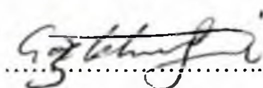
DECLARATION

The experimental work described in this thesis was done by me, at the Immunology Unit, Noguchi Memorial Institute for Medical Research, University of Ghana under the supervision of Prof. B. D. Akanmori (Immunology Unit, NMIMR), Dr. D. A. Edoh (Department of Zoology, University of Ghana).

References cited in this work have been fully acknowledged.



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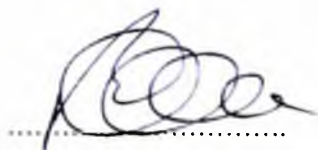

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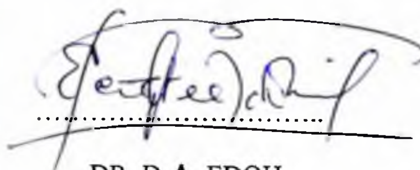

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DEDICATION

THIS WORK IS DEDICATED TO MY LORD JESUS CHRIST IN WHOM I LIVE AND MOVE AND HAVE MY BEING AND MY FAMILY

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ACKNOWLEDGEMENT

I am profoundly grateful to my supervisors, Prof. B. D. Akanmori and Dr. D. E. Edoh, for their encouragement, invaluable contributions and inspiration that made this work possible. My debt to Mr. Alfred Dodoo of Electron Microscopy Unit of the NMIMR for allowing me to use his samples and also helping with the laboratory work. I gratefully appreciate the help of John A. Tetteh in the flow cytometric analysis.

I am thankful to the Director of the NMIMR for permission to work in the institute. My thanks go to Dr. Daniel Dodoo and Mr. Michael F. Ofori both of Immunology Unit of the NMIMR, for their contribution to this work. I appreciate the help of Mr. Eric Kyei-Baafour for assisting with the ELISA work. I am also indebted to Prof. Julius. A. Mingle of the Department of Microbiology, University of Ghana Medical School, who is in charge of the Burkitt's Tumour Project for permission to recruit the BL patients into this study. My sincere thanks to Mr. Isaac Ankrah also of the Department of Microbiology, University of Ghana Medical School, Mrs. Emelia Boadu (social worker with the Burkitt's Tumour Project), doctors and nurses of Department of Child Health, Korle-Bu Teaching Hospital who assisted in the sampling.

My thanks also go to the staff of Immunology Unit, of the NMIMR, namely William Vanderpuije, John Arko-Mensah, Mark Addae, Alex Danso-Coffie, Caroline Danquah, Francis Owusu and Judith Antwi. My debt to the guardians and parents for the permission to use the blood samples from the children for the study, without which this work have been impossible.

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I salute my colleagues and friends, Samuel Kwapong, Nancy Duah, Gideon Helegbe, Gordon Akanzuwine Awandare, Osbourne Quaye, Regina Appiah-Oppong, Sena Matrevi, Nicholas Israel Nii-Trebi, Stephen Kyeremateng, Victor Owusu, Raphael Ndondo Abanja, Uriel S. McAkakpo and Selormey Adukpo.

My gratitude to the Staff of Department of Zoology, University of Ghana for their contribution.

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LIST OF ABBREVIATIONS

ADCC	Antigen-dependent Cellular Cytotoxicity
AICD	Activation-induced Cell Death
AIDS-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
CPM	Complete Parasite Medium
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
FasL	Fas ligand
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
FL	Fluorescence channel
FSC-H	Forward Scatter Height
G-6PD	Glucose-6-phosphate dehydrogenase deficiency
HHV	Human herpesvirus
HI	Heat-inactivated

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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	Lymphokine-activacted Killer
LCL	Lymphoblastoid cell lines
LMP	Latent membrane protein
LPAR	Live parasite
LRBC	Live Red Blood Cells
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

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PPD	Purified Protein Derivative
RPE	R- Phycoerythrin
SSC-H	Side Scatter Height
TNF	Tumour Necrosis Factor
CTLs	Cytotoxic T Lymphocytes
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T helper
VCA	Viral Capsid antigen
WBC	White Blood Cells

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Abstract

It has been shown in epidemiological studies that malaria may play a role in the pathogenesis of endemic Burkitt's Lymphoma (eBL). The contribution of malaria to the pathogenesis of eBL is believed to be due to the imbalances in the immune regulation during malaria infection. Studies have shown a loss of CTL function due to a shift of the immune responses from Th1 towards Th2 T-cell function during malaria infection. This study sought to investigate the phenotypes of peripheral blood lymphocytes from eBL patients and their responses *in vitro* to malaria antigens. Lymphocyte subset distributions and activation in the peripheral blood were studied in 22 BL patients and 15 healthy Ghanaian children by flow cytometry. Plasma and supernatant levels of TNF-α and IL-10 were measured by ELISA and compared between the two groups. The results show that lymphocytes from BL patients have significantly low frequencies of $CD3^+$ ($p=0.003$) and $CD8^+CD3^+$ ($p=0.013$) and both the frequency and the absolute counts of $\gamma\delta^+$ T cells ($p=0.005$ and 0.007 respectively) compared to the controls. The frequency of $V\delta1^+$ $\gamma\delta^+$ T cells was significantly higher in the patients compared to the controls ($p=0.047$). The data also indicates that lymphocytes from BL patients were significantly more activated than those from the controls with regard to the expression of the activation markers, CD95 and HLA-DR by $CD3^+$ and $\gamma\delta^+$cells. Plasma level of TNF-α was lower ($p=0.002$) whereas that of IL-10 was higher in BL patients than in controls ($p=0.042$). Peripheral blood mononuclear cells (PBMC) from BL patients produced significantly less TNF-α compared to the controls when stimulated with *Plasmodium falciparum* schizonts ($p=0.007$) and phytohaemagglutinin (PHA) ($p=0.050$). Similarly, PBMC from BL patients secreted significantly less IL-10 in response to PHA than cells from controls

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(p=0.016) but with regard to the cells stimulated with *P. falciparum* schizonts there was no significant difference in secretion of IL-10 between the two groups. Taken together, the data show that there are imbalances in the immune system of BL patients similar to those found in *P. falciparum* malaria infection suggesting that recurrent *P. falciparum* infection can be an additive risk factor for the development and persistence of eBL.

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CHAPTER ONE

1.1 INTRODUCTION

Malaria is a major childhood vector-transmitted parasitic disease that cause loss of lives, high medical bills and labour lost. It is estimated that each year there are over three hundred (300) million clinical cases of the disease world-wide and in absolute numbers it kills three thousand (3,000) children under five years of age per day. The causative agents in humans are four species of a protozoan parasite known as *Plasmodium*; *P. falciparum, P.malariae, P. vivax, and P. ovale* . The dominant and most lethal form of malaria is caused by *P. falciparum*. (Smyth, 1976; Roll Back Malaria, 2001).

The mainstay of combating malaria involves reducing human-infected-vector contact, chemoprophylaxis and chemotherapy. When DDT and chloroquine proved very potent in combating malaria, the former by killing the vector and the later by eliminating the parasite, in 1955, the World Health Organization began a malaria eradication program in many parts of the world (WHO, 1955; Farid, 1980). The campaign succeeded in wiping out malaria from Europe, North America and Russia but failed in the tropics and subtropics, mainly due to difficulty in reducing vector abundance sufficiently enough to

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decrease the transmission potential below the critical level for sustained transmission, and eventual development of insecticide resistance in Anopheles mosquitoes.

The improper use of antimalarial drugs has also resulted in the emergence of drug resistant strains of *Plasmodium falciparum* in the tropics and subtropics. As a result in 1976, the program was officially declared a failure (Farid, 1980). Thus malaria remains a public health problem in many developing countries.

In Ghana, malaria is hyperendemic with mainly two species of the parasite, *P. falciparum* and *P.ovale*, involved. It accounts for at least 25% of all clinical health care attendance, with young children under 5 years of age accounting for about 40% of all cases. All over the country, it is the predominant cause for seeking medical care by all groups. The mortality rate has been estimated as 6.3 per 1000 in infants and about 10.7 per 1000 in children aged 1-4 years (MOH, 1991).

Attempts by the Ministry of Health to combat malaria has focused on prompt medical care with chemotherapy, manpower development, research, surveillance, strengthening of health care institutions for correct diagnosis and adequate treatment of patients and referral of severe disease to Teaching or Regional hospitals (MOH, 1991).

The high mortality and morbidity caused by falciparum malaria, particularly in children, has motivated many researchers around the globe to find an effective antimalarial vaccine to combat the disease.

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The pathogenesis of malaria involves invasion, alteration and destruction of erythrocytes by malaria parasites, local and systemic circulatory changes and immune mechanisms. These manifest clinically as severe anaemia, cerebral malaria, glomerulonephritis and pulmonary oedema among many others. Although researchers have made many strides to elucidate the phenomena of malaria development, there is still lack of adequate information on the development of the disease and its complications. It is believed that severe malaria may result from immune-mediated damage but the exact mechanisms are not fully grasped (Grau *et al.*, 1989; Abdalla and Weatherall, 1982).

Burkitt's lymphoma (BL), on the other hand, is a malignant monoclonal B-cell tumour that has worldwide distribution but with much higher incidence in areas of holoendemic or hyperendemic malaria, especially, coastal and lakeside regions (Allen, 1999; Epstein and Achong, 1979; Kafuko and Burkitt, 1970). BL is found to be strongly and consistently associated with Epstein-Barr virus (EBV); also known as human herpesvirus 4 (HHV4), which is believed to be the main cause of the disease. The incidence rate of the disease ranges from zero to 3.6% per year worldwide (Cook-Mozaffari et al, 1998). In Africa, endemic BL (eBL) occurs predominantly in children below the age of 16 years. A peak incidence is seen between five and ten years of age (Nkrumah, 1984). It is reported that eBL accounts for 30-70% of childhood cancers in equatorial Africa. eBL also accounts for about 40% of all childhood malignancies in East Africa (Allen, 1999). Male predominance in the incidence has also been reported, where boys are affected 2.5 times as often as girls (Ernberg, 1999). In Ghana, 485 cases of eBL were seen at Korle-

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Bu teaching hospital over a period of 15 years (1969-82). They were cases referred to the Burkitt's Tumour Project at the hospital (Nkrumah, 1984).

Whereas the pathogenesis of severe malaria remains a mystery, Morrow's summary of epidemiological studies strongly suggest involvement of malaria in the pathogenesis of Burkitt's lymphoma (Morrow, 1985). However, the mechanisms by which malaria contributes to the development of BL tumour are not well understood. Some, therefore, suggested that the established relationship between malaria and BL is only because both happened to occur in the same geographical locations (Allen, 1999).

T cells have been implicated in immunity to both malaria and BL. Reports have shown increases in the frequencies of γδT cells in individuals following clinical challenges of *Plasmodium falciparum* malaria (Carding *et al*, 1990; De Paoli *et al*, 1990; Hviid *et al*, 2001). It has also been shown that T cells keep surveillance on the expansion of B cells (Biggar *et al*, 1981) and if this is the case then eBL should not be mentioned among people, especially children, from malaria endemic areas where the proportion of γδT cells is found to be relatively high (Hviid *et al.*, 2000). On the contrary, reports have shown loss of control of EBV^+ cells by T cells during malaria (Casorati *et al.*, 1989). However, the mechanism by which the effector functions of T cells are inhibited during malaria is yet to be understood. Moreover, most of the reviewed studies have been carried out on individuals with little or no challenges of malaria.

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This study, therefore, set out to investigate the effect of malaria on the effector functions of peripheral blood lymphocytes in children suffering from eBL, who are also hardest hit by malaria. The aim was to phenotypically characterize peripheral blood lymphocytes and look at their cytokine profile when stimulated *in vitro* with *Plasmodium falciparum* schizonts and to test the specific hypothesis that *the response to Plasmodium falciparum, and phenotypic and functional characteristics of lymphocytes from children with eBL differ from those of age- and sex -matched healthy children in the same population as a result of malaria induced immune responses.*

Specific objectives were;

* To phenotypically and functionally characterize lymphocytes from BL patients and age- and sex -matched healthy controls.
* To examine response of lymphocytes from the same categories to *P. falciparum* schizonts and mitogens.
* To provide information on the possible role of malaria in the development of Burkitt's Lymphoma.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria as a disease

Malaria is not a novel disease. It has been with humanity since antiquity and was given names with respect to what were believed to be its cause. For example, it was referred to as paludial derived from a Latin word *palus*, which means marshy ground. Italian writers believed malaria was caused by offensive vapours from marshy areas. Thus the name "malaria" was 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. Ross then demonstrated the role of mosquitoes as vectors (Farid, 1980). The disease causes severe anaemia, cerebral malaria and many 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).

2.2 The parasite

2.2.1 Taxonomy.

The malaria parasite belongs to the family Plasmodiidae of the phylum Apicomplexa. The *italics* in figure 1 traces the classification of the parasite. The family Plasmodiidae has only one genus although there are remarkable differences between the various species. This is because the degree of similarity is so great that they could not be divided

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into separate genera without difficulty. Garnham, (1966, 1980) realizing the problem, thought it is more appropriate to retain the genus Plasmodium and introduce subgeneric names. He therefore divided them into ten subgenera based on erythrocytic stages, exo-erythrocytic stages, sporogonic stages and vertebrate host specificity. Thus we have; Plasmodium (primates), Laverania (primates), Vinckeia (non-primate mammals), Haemamoeba (birds) Giovannolaia (birds), Novyella (birds), Huffia (birds), Sauramoeba (lizards), Carinamoeba (lizards), and Ophidiella (snakes). In this light, the correct names of the four species that infect man are *Plasmodium (Plasmodium) vivax, P. (P.) ovale, P. (P.) malariae and P. (Laverania) falciparum.* However, the subgeneric names are not used in practice. It has been established that it is *P. falciparum* malaria that is dominant in areas where BL is endemic (Morrow, 1985)

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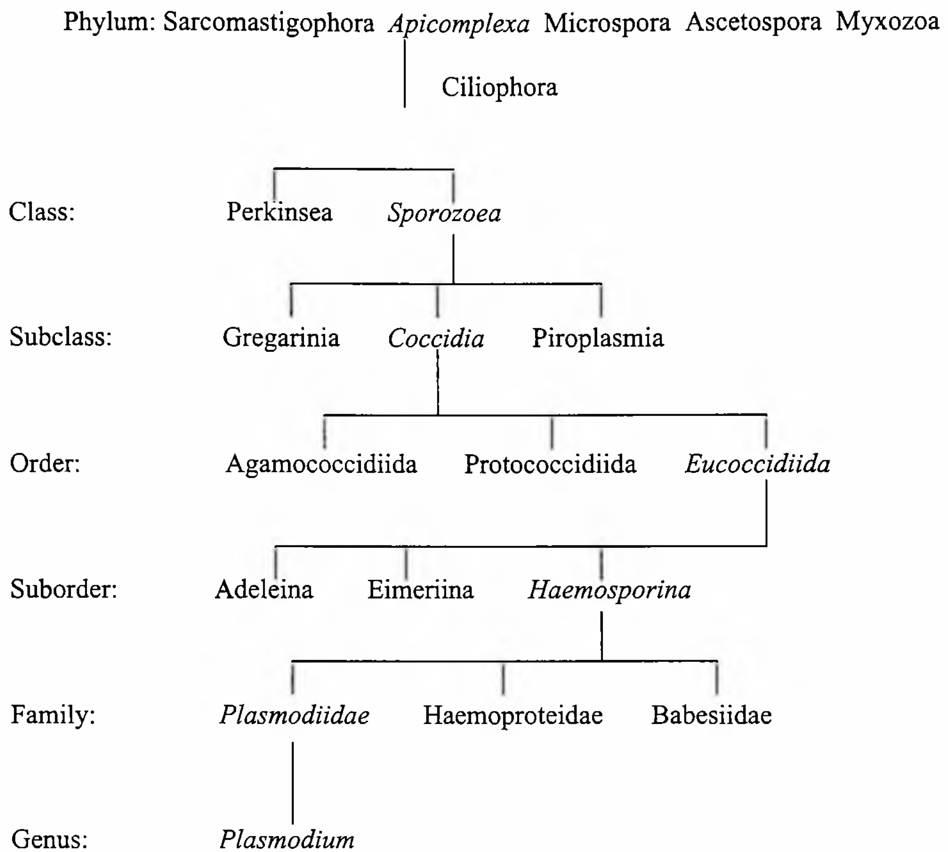
Figure 1. A chart showing the classification of *Plasmodium species*



Species: *Plasmodium vivax, P. ovale, P. falciparum, P. malariae,* P. *knowlesi*, P. *berghei*, P. *yoelii*, P. *chabaudi*, P. *lophurae*, etc. (Levine, *et al.*, 1980; Levine, 1988)

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2.3 Life Cycle of *Plasmaodium.*

Infection commences when an infected female anopheline mosquito inoculates plasmodial sporozoites into the human body when taking a blood meal. The life cycle comprises of three phases of development: Exo-erythrocytic stage, Erythrocytic schizogony and Sexual stage

2.3.1 Exo-erythrocytic stage

The numerous sporozoites that are injected into the body eventually enter the blood circulation. They remain in the blood stream for about 45 minutes and then disappear. Their disappearance is due to the fact that many of the sporozoites are destroyed by the immune system while the rest invade the hepatic parenchymal cells. Once inside the hepatocytes, the parasite multiplies rapidly by schizogony -a phase of asexual reproduction referred to as pre-erythrocytic schizogony. This takes five to fifteen days in *P. falciparum*, after which hepatic schizonts rupture to liberate merozoites into the blood stream (Garnham, 1966).

In *P ovale* and *P. vivax* infections, some of the sporozoites on invading the hepatocytes do not develop, instead remain dormant in the cells for some time. At this stage they are termed as 'hypnozoites'. They undergo schizogony later to cause relapse of disease.

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2.3.2 Erythrocytic schizogony

The erythrocytic cycle begins with the invasion of the erythrocytes by the merozoites released from pre-erythrocytic schizogony. This involves attachment of the merozoites to the erythrocytes, a mechanism believed to be mediated by a specific erythrocyte surface receptor (Hadley *et al*, 1986; Bruce-Chwatt, 1988). The merozoites are finally internalized by endocytosis (Aikawa and Seed, 1980). Predilection of merozoites for erythrocytes of a certain age is found in some species: merozoites of *P. vivax* invade reticulocytes or young erythrocytes, those of *P malariae* attack older ones and *P. falciparum* invades all ages of erythrocytes indiscriminately.

In the erythrocytes, the parasite develops into a ring 'form' called 'trophozoite'. The trophozoite later divides to form the schizont, which matures to form 'meront' which ruptures to release 6-36 merozoites. This takes two days for *P. falciparum, P. ovale* and *P vivax* and three days for *P malariae* (White, 1996) Reinvasion of erythrocytes then follows. However, after a series of asexual cycles some of the merozoites proceed to the sexual stage.

2.3.3 Sexual Stage

This stage is believed to be triggered by rising asexual parasitemia, nutrient depletion, effect of drug suppression and /or rising immunity to asexual stage (Sinden, 1983). The sexual stage begins in the human host but once the gametocytes are formed they are inactive and the sexual process cannot continue in the human host.

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When the gametocytes are taken up by a female anopheline mosquito, they become activated. (Smith and Sanford, 1988) In gametogenesis, the female gametocyte undergoes only few structural changes to form a female microgamete. The male gametocyte on the other hand, goes through elaborate alterations to give rise to eight microgametes in a process referred to as 'exflagellation'. In about 24 hours the zygote is formed, when the gametes fused and it is transformed into a motile ookinete. The ookinete penetrates the wall of the mosquito's gut and encysts as an oocyst. When mature, the oocyst will burst releasing myriads of sporozoites, which then migrate to the salivary gland ready to be inoculated into the next human host.

Each stage in the parasite life cycle presents a distinct surface antigen that the host's immune system has to react to. Sporozoites have a well-defined surface antigen called the circumsporozoite protein (CSP), which is found to trigger the production of T cell-dependent antibodies (Zavala, *et al*, 1983. Merozoites and gametocytes also have surface antigens; merozoites surface proteins (MSPs) and gamtocyte antigen 11.1, repectively, that are immunogenic (Holder, 1988; Koenen *et al.*, 1984; Targett, 1990). However, it is the asexual blood stages of the parasite that are responsible for clinical manifestations of malaria. It has been found that infected erythrocytes express variant antigen called *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) and that though each parasite genome contains about forty (40) PfEMP1 genes, only one PfEMP1 gene is expressed at a given time. PfEMP1 has been demonstrated to be a key element of malaria immunity and found to elicit protective immunity in children (Dodoo *et al.*, 2001; Bull *et al.*, 2000; Marsh and Howard, 1986).

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2.4 The Vector

The vectors of human malaria are female anopheline mosquitoes. Factors that explain their capability to transmit malaria include their habit of feeding on and attraction to human blood, which is necessary for maturation of eggs and completion of the gonotrophic cycle, and ability of the parasite to survive and complete its life cycle in the vector. Male anophelines do not feed on blood and therefore cannot transmit malaria.

Among about 400 species of anopheline mosquitoes only about 105 species were naturally or experimentally found to habour sporozoites. Out of 67 species that are naturally infected with *Plasmodium* species only 27 species are established to have significant degree of transmission of malaria. *Anopheles gambiae* (complex) is a group of anopheline mosquitoes that are most efficient in human malaria transmission and are associated with stable malaria (Wernsdorfer, 1980). The life cycle of the *P. falciparum* is illustrated in Figure 2.

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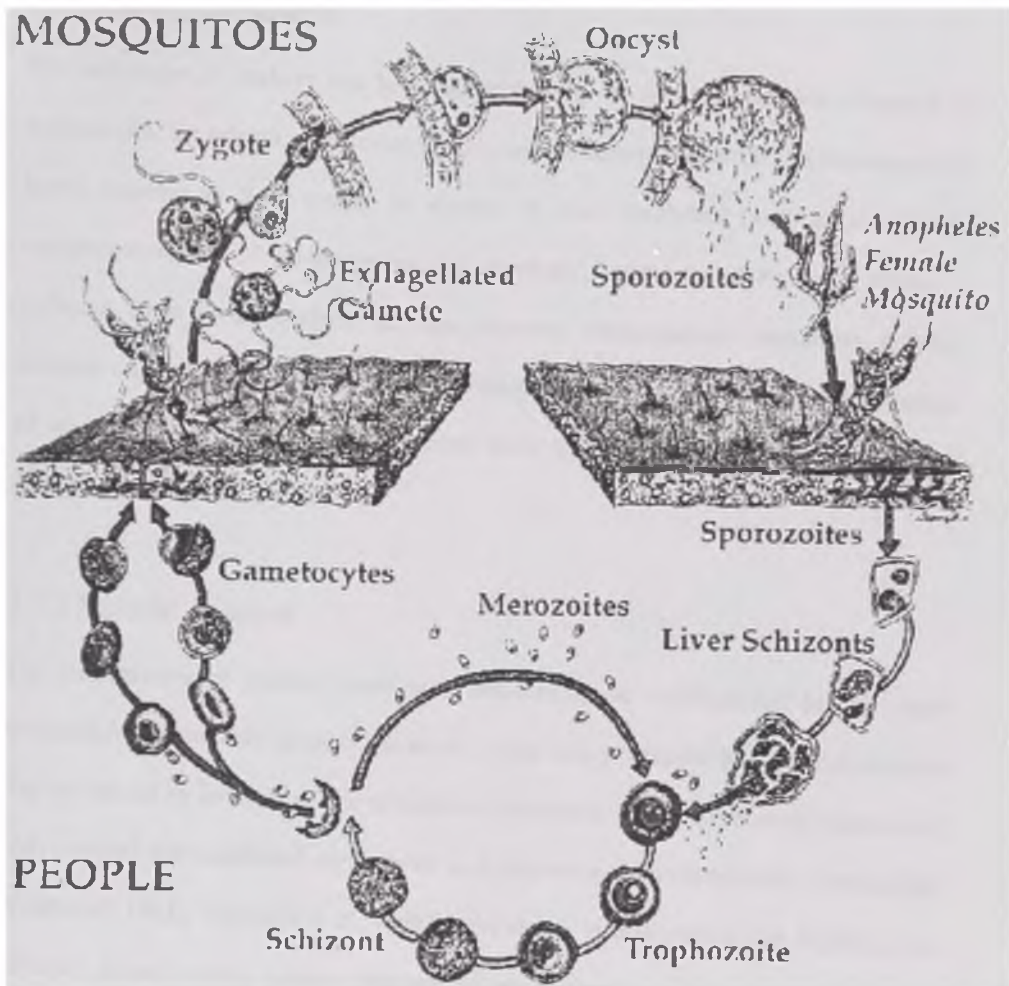
Figure 2. Life Cycle of *Plasmodium falciparum* (Courtesy of malariatest.com)



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2.5 Pathology of Malaria

The pathology of malaria has been established to be due to enhanced clearance of erythrocytes, the release of erythrocyte and parasite materials into the circulation and the host's response to these events. In absence of other confirmed cause 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 fatally are severe anaemia and cerebral malaria (WHO, 2000).

2.5.1 Malarial Anaemia

The pathogenesis of malarial anaemia is believed to be multifactorial but the exact mechanisms are not fully grasped. However, it has been postulated that malarial anaemia may be caused by haemolysis due to rupture of schizonts, immune-mediated clearance of both infected and uninfected erythrocytes and suppresion of erythropoiesis (Abdalla and Weatherall, 1982). Kurtzhals *et al.*, (1997) 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 (Akanmori *et al.*, 2000; Grau *et al.*, 1989).

2.5.2 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 (Berendt *et al.*, 1994). However, CM is found to be associated with high plasma levels of TNF (Grau *et al.*, 1989) and Perlmann, *et al.*, (1997) postulated that

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elevated IgE levels, leading to overproduction of TNF, might be a contributor to the pathogenesis of cerebral malaria. Another mechanism that is believed to contribute to the pathogenesis of cerebral malaria is microvascular obstruction, with accompanying local hypoxia and nutrient depletion (i.e. ischaemia). Sequestration of erythrocytes containing mature stages of parasites in the deep vascular beds of vital organs including the brain, cytoadherence and rosette formation, and increased deformability of the infected erythrocytes are suggested to be 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 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, central nervous system, salivary glands and thyroid (Aderele *et al*, 1975; Burkitt, 1958, 1970; Durodola, 1976; Magrath, 1991, 1997; Ziegler, 1970). 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, 1958,1970; Burkitt and Wright, 1963)

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 and the patient's death is as a result of blocking of most of the throat (Allen, 1999). Acquired immunodeficiency syndrome-related BL (AIDS-BL) has also been identified (Wright, 1999).

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2.6.1 The Epstein Barr Virus

EBV, also known as human herpesvirus 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. In an endemic African region, in a total of 191 BL cases, compiled from 10 different studies, 184 were EBV positive (96%) and also in 395 BL cases from non-endemic regions, 212 were positive (53.5%) (IARC, 1998). Other clinical manifestations of EBV infection are a lymphoproliferative disease, infectious mononucleosis and undifferentiated form of nasopharyngeal carcinoma (Epstein *et al*, 1964; Hanto,*et al.,*1985; zur Hausen, *et al*, 1970).

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 evolved 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 modulates immune responses. Four of such programs have been demonstrated in EBV^+ cells, which are latently infected (Ernberg, 1999).

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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 if EBNA1 is not immunogenic.

2.6.2. 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,

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bone marrow, central nervous system, salivary glands, thyroid, breast, and infrequently, cardiac muscles (Aderele *et al.*, 1975; Burkitt, 1958, 1970; Durodola, 1976; Magrath 1991, 1997; Ziegler, 1970).

2.7 Immunity to Malaria

2.7.1 Non-specific (Innate) 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).

2.7.2 Acquired 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).

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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).

2.7.2.1 Humoral Immunity to Malaria

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. Antibodies are secreted by activated B-lymphocytes. Antibodies bind to malaria antigens on the surface of parasitized erythrocytes resulting in destruction and/or enhanced phagocytosis of those cells and the parasites in them (Jakobsen *et al.,* 1997).

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.*,

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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. 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 malaria 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

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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.

2.7.2.2 Cellular Immunity to Malaria

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. Lymphocytes are divided into two broad categories: B-lymphocytes, which are precursors of antibody secreting cells, and T-lymphocytes, 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.

2.8 T-cells and Malaria

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).

2.8.1 αβT-cells and Malaria

Two main types of αβT cells are recognized. These are $CD4^+$ T cells and $CD8^+$ T cells, which recognize antigens, presented on major histocompactibility complex (MHC), MHC

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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). 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), 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). $CD8^+$ 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. Some suppressor $CD8^+$ T cells have also been identified (Koide and Engleman, 1990).

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). The balance between Th1 and Th2 subsets of the αβT-cells would determine the state of the immune regulation. 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 Weid and Langhorne, (1993), which may lead to immuno-incompetence.

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2.8.2 γδT-cells and Malaria

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 (Augustin *et al.*, 1989; Bluestone and Matis, 1989; Born 1990 Janeway, 1988), an involvement during infection with viruses (De Paulo 1990; Carding *et al.*, 1990), parasites (Georlick *et al.*, 1991) and γδT cells that produce Th1-like and Th2-like cytokines have also been demonstrated (Ferrick *et al*, 1995). 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 are 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).

On the contrary, a number of studies have shown significant increase in the levels of γδT cells during *P. falciparum* infection in adults. However, most of these studies were conducted on non-immune donors and in a study, the elevation of γδT cells was found not to be associated with disease severity (Ho *et al*, 1990; 1994; Perera *et al*, 1994). But no significant increase was found in *P vivax* infection (Worku *et al*, 1997) indicating the role of parasite-related factors. Hviid *et al.*, (1996, 2001) observed increase in γδT cells in children from malaria endemic areas.

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; Seixas and Langhorne, 1999). Growth inhibition of 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

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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). Both in acute *P. falciparum* infection and *in vitro* system, the elevated subset of γδT-cells was $V\gamma9^{+}V\delta2^{+}$T cells (Goodier *et al.*, 1995; Langhorne 1996;).

Now, it has been established that $V\delta1^{+}$T cells also expand in response to malaria antigens *in vitro* as well as in acute infection (Ho *et al*, 1994; Schwartz *et al*, 1996). A recent studies has shown that the rise in γδT cells during *P. falciparum* malaria infection, in individuals from malaria endemic areas, is mainly due to increase in $V\delta1^{+}$T cells (Hviid *et al*, 2001) and in malaria endemic areas levels of $V\delta1^{+}$T cells were higher than that of $V\gamma9^{+}V\delta2^{+}$T cells in healthy donors (Hviid *et al*, 2000). This suggests, at least, that $V\gamma9^{+}V\delta2^{+}$T is not the only subset responding to malaria infection. It also implies that the immune status of the host has a bearing on the response of these subsets of γδT cells.

It has also been established that healthy donors from malaria endemic areas have higher levels of $\gamma\delta^{+}$T cells (>10% of T cells) compared to Caucasians (<5% of T cells) mainly due to expansion of $V\delta1^{+}$ cells. Since no significant association has been found between $\gamma\delta^{+}$ cells or $V\delta1^{+}$ cells and malaria antibodies or parasitemia, the role of these cells in antimalaria response is not clear (Hviid *et al*, 2000). Lymphokines secreted by $\gamma\delta^{+}$T cells are known to stimulate macrophages (Goodier *et al*, 1995), thus may assist in primary infection when there are no specific memory cells.

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).

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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, TNFα has been associated with severe and cerebral malaria (Goodier *et al.*, 1995; Grau *et al.*, 1989).

The $\gamma\delta$T cell response is 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 $\gamma\delta$T cells are $CD4^-CD8^-$ cells and as $\gamma\delta$T cells increase, percentage of $CD4^+$ cells declines (Worku *et al.*, 1997), a scenario suggested to be either due to proliferative response or selective recruitment of $\gamma\delta$T cells into the circulation (Ho *et al.*, 1994). Cytotoxic activity of natural killer (NK) cells against erythrocytic stage of *P. falciparum* has also been reported (Phillips, 1994).

It has been well established that acute *P. falciparum* malaria leads to lymphopenia before initiation of chemotherapy (Hviid *et al.*, 1997), a phenomenon that can adversely affect protective immunity to the disease. The mechanisms that result in this turn of events have not been fully established. Some researchers have pointed to disease-induced reallocation of T cells to sites of inflammation (Elhassan *et al.*, 1994). Others have experimental evidence that suggest FasL-mediated programmed cell death as the cause of lymphopenia of malaria but have failed to provide direct relationship between Fas expression and lymphopenia of malaria (Balde *et al.*, 1995; Kern *et al.*, 2000; Matsumoto, et al., 2000 Toure-Balde *et al.*, 1996). Although activation of lymphocytes is consistent with lymphopenia in non-immune, non-exposed or less exposed individuals (Chougnet *et al.*, 1992; Elhassan *et al.*, 1994; Worku *et al.*, 1997), no report has pointed to activation-

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induced cell death (AICD) as the cause of decreased lymphocyte numbers in acute *P. falciparum* malaria.

2.9 Immunity to BL

2.9.1 Non-specific (Innate) Immunity to BL

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

2.9.2 Specific (Acquired) Immunity to BL

2.9.2.1 Humoral Immunity to BL

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 (Jones *et al*, 1985; Niederman and Evans, 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). Whereas antibodies to viral envelope antigens (MA, VCA) are able to neutralize viral activity through ADCC, BL cells lack expression of VCA and other antigens except EBNA1 and

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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).

2.9.2.2 Cellular Immunity to 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 no cytotoxic response has been

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detected against EBNA1. EBNA1 has also been observed in an experimental mouse model to be non-immunogenic (Trivedi *et al.*, 1994). The inability of the CTLs to recognize the EBNA1 must be of concern 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 B-lymphocytes in BL.

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). However, normal levels of EBV-specific CTL precursors were demonstrated in BL patients (Rooney *et al.*, 1997). In a recent study, 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 response of CD4+ cells is believed to be protective because of secretion of IFN-γ and direct cytolysis after encounter with transformed B lymphocyte cell lines (Münz *et al.*, 2000). This implies that CD4+ dysfunction is likely to be one of the main factors in lack of B cell control in BL patients. 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).

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.

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However, no responses to EBNA1, EBNA LP, or LMP1 were observed (Murray *et al*, 1992). Khanna and his colleages (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 Mũnz and his colleagues (2000) shows that it is instead presented on MHC class II molecule. Thus, the subset of T cells that may help in controlling B cells that express only EBNA1 are γδT cells, for they are not MHC-restricted.

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 were enhanced (Hacker *et al.*, 1992). 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 responsible for controlling abnormal proliferation of B cells and have a crucial role to play in protection against pathogenesis of BL. However, an elevated levels of $V\gamma9^{+}$ cells in peripheral blood during EBV infection in humans has been reported instead (De Paoli, 1990). This makes the protective role of $V\delta1^{+}$ cells in BL unclear.

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

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control of EBV^{+} cells by T cells during malaria (Dalldorf, 1962). This may be due to immunosuppression, which is characteristic of malaria infection. However, the mechanism by which the effector functions of T cells are inhibited during malaria is yet to be fully unraveled. There is therefore speculation that effector functions of Vδ1^{+} T cells might be lost during malaria thus, rise in EBV^{+}B cells and hence development of eBL in malaria endemic regions.

2.10 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 BL. 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. 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 BL. 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 Weid and Langhorne, (1993). 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

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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). B-cell activation also occurs in malaria and the number of B cells rises with the general number of lymphocytes (Geser *et al.*, 1989; Whittle *et al*, 1984, 1990). Thus everything is in favour of expansion of B cells.

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).

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 (Mc Gregor, 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.

Therefore as a consequence of all these, 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

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may amplify the pool of EBV^+ B cells prone to accumulate oncogenic changes and undergo transformation, which are major events in BL pathogenesis. The course of the major events in BL pathogenesis in children, it is believed to be: EBV-infection early in life, followed by persistent exposure to malaria also in early life and then the oncogenic process.

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.

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Human Subjects Samples and Study Design

Study subjects comprised children with BL referred to the Burkitt's Tumour Centre at the Korle-Bu Teaching Hospital from all parts of Ghana. The patients were clinically examined by consultant paediatricians of the Department of Child Health, Korle-Bu Teaching Hospital. Inclusion of the patients in the study was based on clinical information as well as cytological examination of tumour aspirates by a pathologist. Informed consent was obtained from all parents or guardians before the children were enrolled in the study. Healthy Ghanaian children with comparable age and sex were included as controls. A total of 10ml of blood was taken from each patient or subject. The study was therefore a case-control one in which BL patients were compared with age and sex-matched healthy non-BL Ghanaian children.

3.2 Blood Collection

Blood samples were collected in sterile 10ml heparinized vacutainer tubes using sterile butterfly needles. The tubes were heparinized to prevent coagulation. The samples were immediately taken from the hospital to the Immunology Unit of the Noguchi Institute for Medical Research where they were processed. All the samples were processed within six hours after collection.

3.3 Haematological analysis

An automated haematology analyzer (Sysmex KX-21, Japan) was used to determine all the 21 haematological parameters of the patients and the subjects. The absolute counts of lymphocytes were determined from this analysis.

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3.4 Parasitology

Each sample was examined for presence of malaria parasites. 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), for 10 minutes, washed carefully and thoroughly under running tap water. The slides were dried and observed with immersion oil under a light microscope (Olympus BH2, Japan) at 1000x magnification.

3.5 Sample Processing

The blood samples were processed under sterile conditions. Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Nycomed Pharma, As, Oslo) density gradient centrifugation. A volume of 5ml of venous blood was carefully layered on top of 2ml of Lymphoprep and centrifuged at 814xg for 30 minutes. The ring of white blood cells was carefully aspirated and washed (centrifugated at 814xg for 10minutes) three times in RPMI1640 containing 10% heat-inactivated foetal calf serum (FCS) supplemented with gentimycin, and L-glutamine. 25µl of PBMC suspension was stained with leukocyte stain and cells counted using the Neubauer chamber haematocytometer. Table 1 shows how the cell counts were obtained. The PBMC were then aliquoted into four vials (cryotubes) and cryopreserved (frozen at -196C in liquid nitrogen) in RPMI1640 supplemented with gentimycin, L-glutamine dimethyl sulphoxide (10%) and FCS (25%) using a gradient freezing device which yields up to 95% cell viability upon thawing (Hviid *et al.*, 1993). This has been established at Immunology Unit of NMIMR and used over several years. The plasma obtained was stored at –40C. Before use, the cells were thawed quickly in a water bath at 37C and washed (once for surface staining or three times for stimulation) with washing buffer.

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3.6 Counting of cells for viability

A volume of 25µl of cell suspension was stained with Trypan blue (instead of leukocyte stain) to count and also ascertain cell viability after which the cell concentrations were adjusted appropriately.

Table 1. Cell counts from the Neubauer chamber haematocytometer

Each of the four (4) squares (chambers) of the haematocytometer is 1x1mm and the depth is 0.1mm.

⇨ Volume of cell suspension per square $= 1 \times 1 \times 0.1 mm^3$ or 10^{-4}ml.

$$\therefore \text{No of cells per millilitre} = \frac{N \times \text{dilution factor}}{10^{-4}} = N \times \text{dilution factor} \times 10^4$$

where N is the average count per square and the dilution factor depends on the amount of stain, and volume of original cell suspension used.

3.7 Cell surface staining

Briefly, the PBMC were directly stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, R-phycoerythrin (RPE)- and RPE-Cy5-conjugated antibodies for two or three-colour fluorescent analysis. The antibodies were directed against CD3 (UCHT1; DAKO, Glostrup, Denmark), CD4 (MT310; DAKO), CD8 (DK25; DAKO), CD25 (ACT-1; DAKO), CD69 (L78, Becton Dickinson (BD) Biosciences), CD95 (DX2; BD Immunocytometry Systems), HLA-DR (L243; BD Immunocytometry Systems), TCR-γδ (11F2; BD PharMingen (PE) and 11F2; BD Immunocytometry Systems (FITC), Vδ1 (TS8.2; Endogen), Vδ2 (B6; BD PharMingen), Vδ3 (ImmunoTech, Marseilles, France), Vγ9 (B3; BD PharMingen) and B cell (FMC7; DAKO).

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The PBMC were stained in twelve tubes in combinations shown in table 2. Monoclonal antibodies; PE anti-human Vδ2, PE anti-human γ/δ TCR and FITC Vγ9 TCR which were not of working concentration, were diluted according to specification (1 in 4). Before flow cytometric the cells were washed once with washing buffer and re-suspended in PBS supplemented with 2%FCS (FACS buffer). After counting as described earlier, the cell concentrations were adjusted to 1.0×10^6 cells/ml or more.

A volume of 10µl of the antibodies were put at the bottom of the FACS tubes, and then followed by 100µl of cell suspension per tube. The mixture was stirred briefly using vortex and incubated at room temperature for 20 minutes. After incubation, the cells were spun in 3ml FACS buffer at 814xg for 8 minutes. After a second wash the cells were re-suspended in 200µl FACS buffer for acquisition on the same day or fixed in 200µl PBS + 0.5% paraformaldehyde and acquired within three days. 8000 to 20,000 gated lymphocytes were acquired and analysed. The samples were acquired and analysed on a FACScan flow cytometer (BD).

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Table 2: Antibody panel used for surface staining

Tube#			
1.	CD8 FITC	CD4 PE	CD3 Cy5
2.	γδ FITC	CD69 PE	CD3 Cy5
3.	γδ FITC	HLA-DR PE	CD3 Cy5
4.	γδ FITC	CD25 PE	CD3 Cy5
5.	γδ FITC	CD95 PE	CD3 Cy5
6.	B cells	CD25 PE	
7.	γδ FITC	Vδ2 PE	
8.	γδ FITC	Vδ3 PE	
9.	Vγ9 FITC	γδ PE	
10.	CD8 FITC	γδ PE	
11.	γδ FITC	CD4 PE	
12.	Vδ1 FITC	γδ PE	

The functions of some of the T-cell markers are listed in table 3.

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Table 3. Functions of T-cell markers

Marker	*Functions*
CD3	Specific T-cell receptor (TCR) important in signal transduction for T-cell activation.
CD4	Expressed by T helper cells and acts as co-receptor with the TCR for MHC class II recognition.
CD8	Expressed by cytotoxic cells, important in maturation and positive selection of MHC class I restricted T cells.
CD25	Also known as interleukin-2 receptor (IL-2R), is an activation marker associated with T-cell growth.
CD69	An activation inducer molecule (therefore known as early activation marker).
CD95(APO-1/Fas)	An activation marker that transduces an apoptotic signal for clonal deletion of T-cells.
HLA-DR	An activation marker that is part of MHC class II molecule, restricts and regulates the immune responses is a highly specific way.
TCR-γδ	Anti-microbial and cytolytic functions.

3.8 Flow cytometric analysis

Before sample acquisition, colour compensation optimisation was carried out. Data was acquired and analysed using CELLQuest Software (BD, San Jose, CA) after setting appropriate forward and side scatter gates around the lymphocyte population. Negative isotype control were stained with IgG1 and used to draw the cut-off line in the histogram. Lymphocytes were first selected by electronic gating according to forward scatter and side scatter, and then by their expression of surface markers. The proportions of lymphocytes, which were positive for the various markers, were then obtained from histograms (Figure 3).

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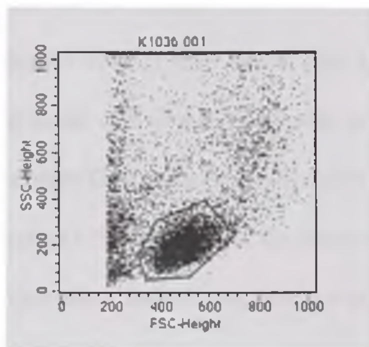
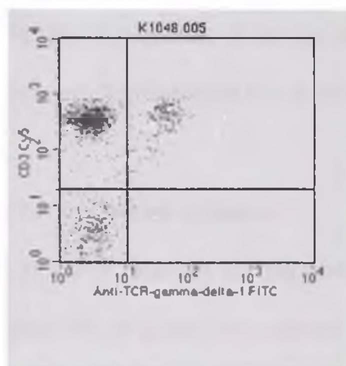
Figure 3. Flow cytometric data showing analysis of lymphocyte surface marker expression. A) Dot plot showing selection of lymphocytes by electronic gating. B) and C) illustrate separation of gated lymphocytes according to expression of surface markers. Positive cells are separated from negative cells by quadrant markers. The distribution and mean fluorescence of cells are shown in the quadrant statistics. UL=upper left, UR=upper right, LL=lower left and LR=lower right.

A.



B.

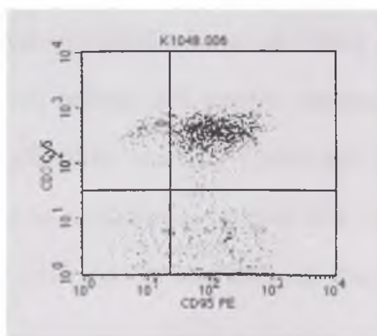


Quadrant Statistics

File: K1048.005
Acquisition Date: 11-Dec-01
Gate: G1
X Parameter: FL1-H Anti-TCR-gamma-delta-1 FITC (Log)
Y Parameter: FL3-H CD3 Cy5 (Log)

Quad	Events	% Gated	% Total
UL	2114	56.45	13.91
UR	620	16.56	4.08
LL	983	26.25	6.47
LR	28	0.75	0.18

C.



Quadrant Statistics

File: K1048.006
Acquisition Date: 11-Dec-01
Gate: G1
X Parameter: FL2-H CD95 PE (Log)
Y Parameter: FL3-H CD3 Cy5 (Log)

Quad	Events	% Gated	% Total
UL	321	8.24	2.08
UR	2462	63.23	15.92
LL	240	6.16	1.55
LR	871	22.37	5.63

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3.9 Stimulation of peripheral blood monuclear cells (PBMC)

3.9.1 Preparation of whole *P. falciparum* (LPAR)

3.9.1.1 Parasite culture

Frozen chloroquine resistant strains of *P. falciparum* malaria parasites (3D7) were taken from the liquid nitrogen tank, thawed quickly in a 37^0C water bath, an equal volume of thawing mix (normal saline-0.9%NaCl) was added and span down at 415xg for 10 minutes. This was repeated twice with complete parasite medium (CPM). The parasites were then added to a 50ml culture flask containing 200µl of washed A^+ red blood in 5ml of CPM, gassed with a gas mixture (2% O_2, + 5.5% CO_2 balanced with N_2) and. incubated in a CO_2 incubator at 37^0C. The culture medium was changed every day and each time, a thin smear was prepared and examined, as described previously, to determine parasitaemia, growth stage and viability of parasites. When the parasitaemia was about 5% subcultures were made, using prepared uninfected red blood cells (RBCs).

3.9.1.2 Separation of *P. falciparum* schizonts

When the majority (75% or more) of the parasites were at the schizonts stage and the parasitaemia was about 2% or higher, the parasites were separated for stimulation on the same day. Briefly, 7ml of fresh isotonic percoll (percoll + 10% 10x PBS) diluted with 28% RPMI1640 was placed in a 15ml centrifuge tube (corning) and carefully layered with 3.5ml of *P. falciparum* culture and span at 1000xg for 25 minutes. Cells at the interface between the percoll solution and parasite medium, which were mainly late stages or schizonts, were withdrawn carefully, pooled and washed three times with stimulation medium. A smear was prepared and stained with Giemsa to determine the percentage of the infected cells harvested that were schizonts. They were then kept at 4°C and ready for use.

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3.9.2 Preparation of Red Blood Cells (LRBC)

Blood from an A^+ donor was buffered with CPD (citrate-phosphate dextrose) and kept overnight at 4°C. The plasma was then removed and an equal amount of red blood cells (RBC) buffer was added and span at 1000xg for 8 minutes. The medium and white blood cells (WBC) were then removed. This was repeated three more times and an equal amount of RBC buffer was added and stored at 4°C for use in parasite culture and stimulation. For stimulation, the LRBC were washed twice with the stimulation medium to avoid contamination of culture with the RBC wash.

3.9.3 Preparation of Mitogens

Working concentrations of phytohaematogglutinin (PHA) and purified protein derivative of *Mycobacterium tuberculosis* (PPD) (10µg/ml) were also prepared using RPMI1640.

3.9.4 Stimulation procedure

Before stimulation, the PBMC were taken from liquid nitrogen tank, thawed quickly and washed as described previously except that here the culture (stimulation) medium (RPMI1640 supplemented with 10%NHS, L-Glutamine, Penicillin/Streptomycin and filtered) was used. The cells were counted, also, as described previously after which the concentrations were adjusted to 1.0 $x10^6$ cells/ml. LPAR and LRBC were also counted and their concentrations adjusted to $7.5x10^7$ cells/ml.

After adding a volume of 60µL per well of parasites (LPAR), red blood cells (LRBC), purified protein derivative (PPD) or phytohaematogglutinin (PHA), 600µL of PBMC suspension from patients and controls were added and incubated in a CO_2 incubator at 37^0C. Culture supernatants were harvested after 24 hours and also on days 3 and 6 for cytokine analysis.

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3.10 Cytokine Assay by ELISA

Levels of the cytokines, tumour necrosis factor-alpha (TNF-α) and Interleukin-10 (IL-10) were determined in culture supernatants of PBMC and plasma of BL patients as well as their healthy counterparts, who served as controls. 96-well microtitre plates (Immulon 4 HBX, Dynex) were coated with 50μl/well of anti-human TNF-α or anti-human IL-10 monoclonal antibody at 2μg/ml (diluted with carbonate buffer: 0.1M $NaHCO_3$, 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 250μl/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).

A standard (recombinant) human TNF-α or IL10 was added at serial dilutions (diluent: RPMI + 5% HI AB serum NHS) from 2000pg/ml to 31pg/ml in addition to undiluted plasma or culture supernatants at 50ul/well. The plates were then incubated at room temperature 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 IL10 was diluted (diluent: 5% FCS in PBS) to 1μg/ml and added to the plates at 50μl/ml. The plates were again incubated 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 50ul/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

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H_2O_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_2SO_4 at 50ul/well and read using a microtiter plate reader (Multiskan Ascent V1.24, ThermoLabsystems, Finland) at 492 nm. The OD values of the standards were used to draw the appropriate curves using a statistical software (TBLCurves, Jandel Scientific) and the curves were used to transform the sample OD values to concentrations in pg/ml.

3.11 Ethical Consideration

Ethical approval for this study was granted by the University of Ghana Medical School Scientific Research and Ethical Committee, and the Institutional Review Board of the NMIMR. Participation in the study was strictly voluntary and signed informed consent of parents and guardians was obtained.

3.12 Statistical analysis

Comparison between groups and subsets were done using Student's t-test (t), except when equal variance and normality tests failed, in which case the Mann-Whitney rank-sum test (*T*) was adopted. Confidence intervals for median differences were calculated as described by Conover (1980). Spearman's rank correlation was used to establish association between different parameters. SigmaStat software (Jandel Scientific, San Rafael, CA) was used for all statistical calculations except correlations, SPSS software was used for correlations and Microsoft Excel (Microsoft Corporation) and SigmaPlot software (Jandel Scientific) were used for graphical presentations. P values less than or equal to 0.05 were considered significant.

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CHAPTER FOUR

RESULTS

4.1 Summary

This study involved twenty-two (22) Burkitt's Lymphoma patients and fifteen (15) age- and sex-matched healthy children.

Lymphocytes from eBL patients showed high levels of $CD4^{+}CD3^{+}$ (p=0.004), $CD95^{+}CD3^{+}$ (p=0.008)' $HLA\text{-}DR^{+}CD3^{+}$ (p=0.013), $CD95^{+}\gamma\delta^{+}$ (p<0.001), $HLA\text{-}DR^{+}\gamma\delta^{+}$ (p<0.001), $V\delta1^{+}\ \gamma\delta^{+}$ (p=0.047), and B cells (p<0.001) but lower levels $CD3^{+}$(p=0.003), $\gamma\delta^{+}$(p=0.007), $CD8^{+}CD3^{+}$(p=0.013) and $V\gamma9\ \gamma\delta^{+}$(p=0.001) of lymphocytes compared to the controls. Plasma level of TNF-α was lower in patients compared to controls (p=0.002) and conversely, plasma level of IL-10 was higher in patients than in controls (p=0.042). Stimulation of PBMC with *P. falciparum* schizonts, PHA and PPD showed remarkable reduction in immune response with regard to production of TNF-α and IL-10 in patients compared to controls. *P. falciparum* schizonts seem to induce elevated production of IL-10 in both controls and patients.

The following graphs; figures 4-15 and tables 4 illustrate the results of the study. The whiskers of the bar charts represent the standard errors and in the box plots, the box shows the interquartile range; the line through the box represents the median; the whiskers show 95% confidence interval and the outliers are indicated by individual symbols.

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4. 2 Characteristics of Subjects

Twenty-two (22) BL patients were recruited {13 males and 9 females; Mean age (95%CI): 7.0 (5.5 to 8.0)} and out of this seven died. The sites of involvement of the tumour and their combinations are shown in Table 4. Most of the patients had abdominal (~77%) and jaw(~55%) masses. Fifteen healthy Ghanaian children {9 male and 6 female; Mean age (95%CI): 6.5 (5.0 to 7.5)} were included as controls.

Table 4. Sites and distribution of tumours in BL patients

Sites and their combinations	Number of patients
Abdomen	17
Jaw	12
Eye	3
Neck	1
Jaw & Eye	1
Abdomen & Jaw	7
Abdomen, Jaw &Eye	2

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4. 3 Frequency of T cells is lower in BL patients than in healthy controls

The mean frequency of peripheral blood $CD3^{+}$ cells was significantly lower in BL patients than in healthy controls {Mean difference (95%CI): 15.64(5.75 to 25.56); $p(t)=0.003$ }(figure 4). The mean absolute number of $CD3^{+}$ cells was also lower in BL patients than in the controls but this was not significant {Mean difference (95%CI): 1.16×10^{5} (-14.23×10^{5} to 11.91×10^{5})/ml; $p(t)=0.851$}.

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4. 4 B-cell levels are elevated in BL and show activated phenotype

BL patients showed elevated levels of B cells as compared to age-matched controls in terms of both frequency {Mean difference (95%CI): 4.96 (3.17 to 6.76); p(t)<0.001 } and absolute counts {Mean difference (95%CI): 0.11 $x10^5$ (0.02 $x10^5$ to 2.21 $x10^5$)/ml; p(*t*)=0.047} (figure 4). Moreso, higher counts of B cells in BL expressed the activation marker, CD25 than in controls {Mean (95%CI): 0.29(-0.83 to 1.41) $x10^5$/ml, n=3; 0.033 (0.01 to 0.05) $x10^5$/ml, n=13, respectively}.

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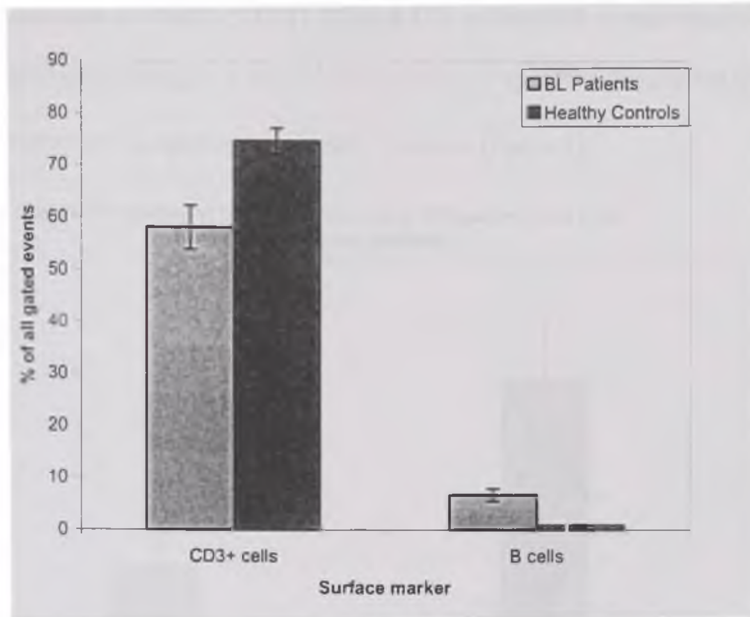
Figure 4. Frequencies of CD3+ and B cells in gated events



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4. 5 Marked low level of T cells expressing TCR-γδ in BL

The lymphocytes bearing TCR-γδ were significantly lower in terms of both frequency {Median difference (95%CI): 5.33 (1.62 to 8.47); $p(T)=0.005$ } and absolute numbers {Median difference (95%CI): 1.82×10^5 (0.29×10^5 to 5.22×10^5)/ml; $p(T)=0.007$ } in BL patients as compared to age-matched healthy children (figure 5).

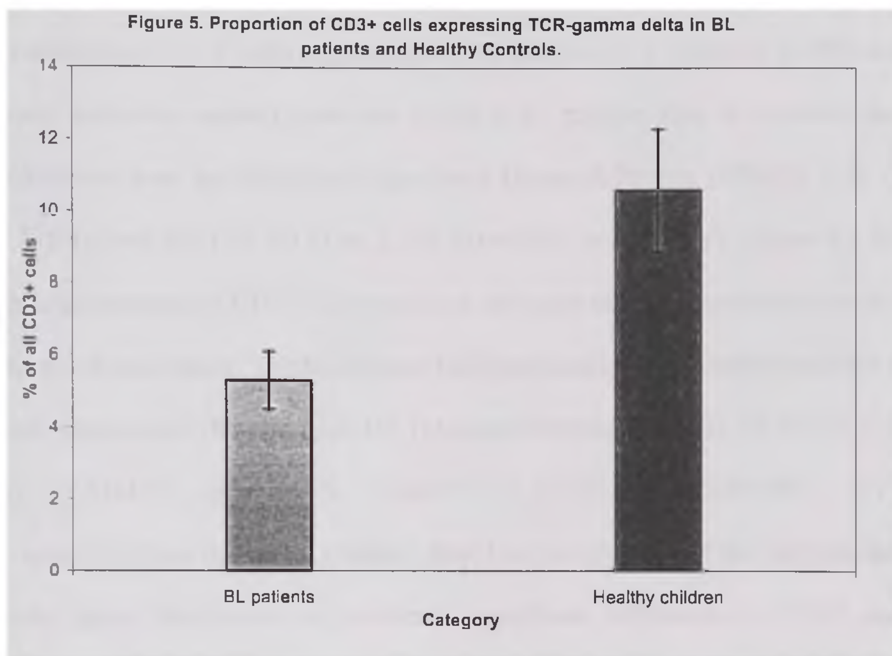

Figure 5. Proportion of CD3+ cells expressing TCR-gamma delta in BL patients and Healthy Controls.

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4. 6 Lymphocytes in BL exhibit an activated phenotypic profile and express high level of the apoptotic marker (CD95)

The proportions of peripheral blood $CD3^{+}$ T cells expressing the activation markers, CD95 (apoptotic marker) and HLA-DR (late activation marker), were significantly higher in BL patients than in healthy children {Mean difference (95%CI): 14.11 (3.97 to 24.26); p (t)=0.008; Mean difference (95%CI): 13.08 (2.98 to 23.19) p (t)=0.013 respectively}. Frequencies of $CD3^{+}$ cells expressing CD25 (interleukin-2 receptor, IL-2R) and CD69 (early activation marker) were also higher in BL patients than in controls, though the differences were not statistically significant {Mean difference (95%CI): 3.28 (-0.72 to 7.27); (p(t)=0.104,1.26 (-0.23 to 2.74); p(t)=0.094 respectively} (figure 6). When the absolute numbers of $CD3^{+}$ cells expressing the same activation markers were compared, the result was similar. The BL patients had significantly higher median number of $CD3^{+}$ cells expressing CD95 and HLA-DR {Median difference (95%CI): 12.95×10^{5} (-1.08×10^{5} to 27.83×10^{5}), p(T)=0.035; 2.28×10^{5} (1.17×10^{5} to 12.30×10^{5}); p(T)=0.026, respectively}than in healthy children. Based on comparisons of the absolute number of cells, again, there were no statistically significant differences in CD25 and CD69 expression by the T-cells between the two groups. {CD25: Median difference (95%CI): 0.49×10^{5} (-8.7610^{5} to 3.83×10^{5}); higher in BL patients, p(T)= 0.805 and CD69: 0.44×10^{5} (-0.65×10^{5} to 1.53×10^{5}); lower in BL patients p(T)=0.401}

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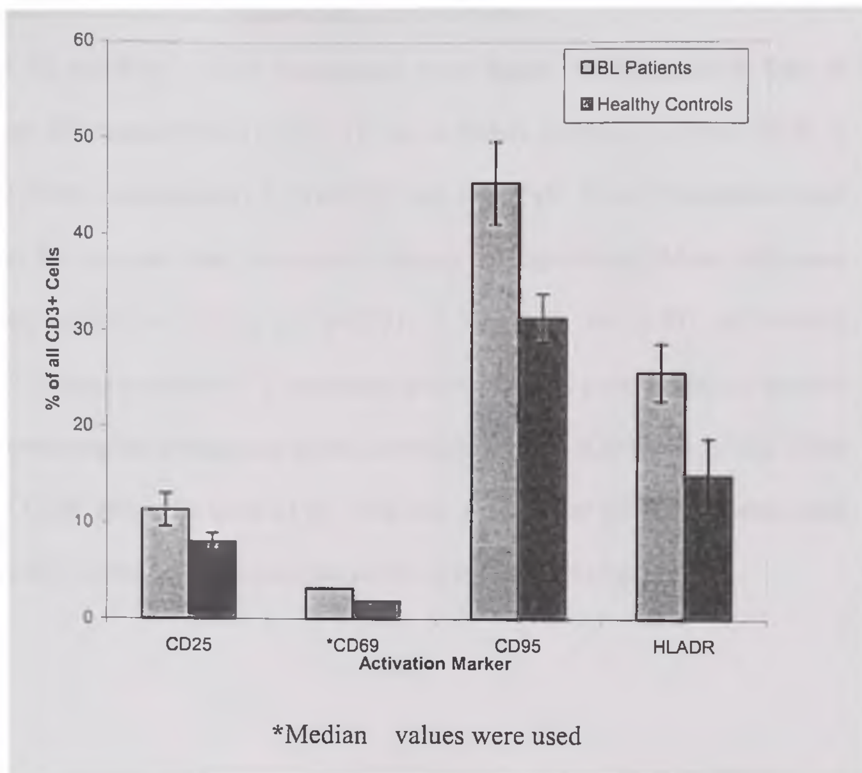
Figure 6. Frequencies of CD3+ T cells bearing various activation markers in BL Patients and Healthy Controls



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4. 7 $\gamma\delta^+$ T Cells are more activated than $\alpha\beta^+$ T cells in BL

$CD95^+\gamma\delta^+$and HLA-$DR^+\gamma\delta^+$ T-cell frequencies were higher in BL patients than in controls {Mean difference (95%CI): 32.95 (17.04 to 48.86), p(*t*)<0.001; 30.42 (20.97 to 39.87), p(*t*)<0.001, respectively) } $CD69^+\gamma\delta^+$ and $CD25^+\gamma\delta^+$ T-cell frequencies were also higher in BL patients than in controls, though not significant {Mean difference (95%CI): 4.48 (-2.95 to 11.92), p(*t*)=0.221; 1.56 (-3.63 to 13.97) p(*T*)=0.431, respectively) } This shows that $\gamma\delta^+$ T were more activated in BL patients than in controls (Figure 7). Comparing the frequencies of the activation markers (CD95, HLA-DR, CD69 and CD25) in $CD3^+$ cells with those of $\gamma\delta^+$ cells also showed that $\gamma\delta^+$ T cells were more activated than $\alpha\beta^+$ T cells in BL patients but not so in the controls (figure 8).

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Figure 7. Frequencies of Gamma-Delta T Cells bearing various activation markers in BL Patients and Healthy Controls

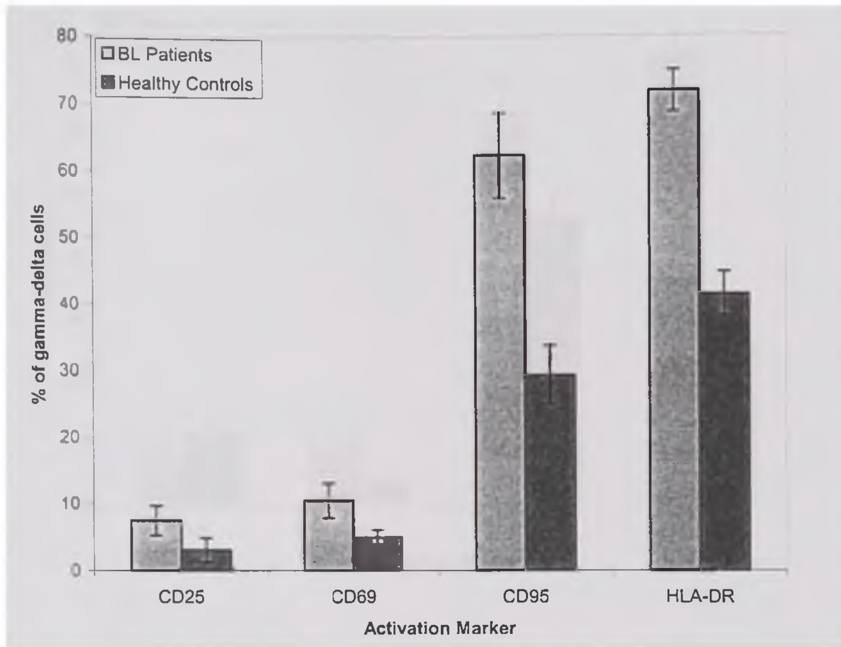

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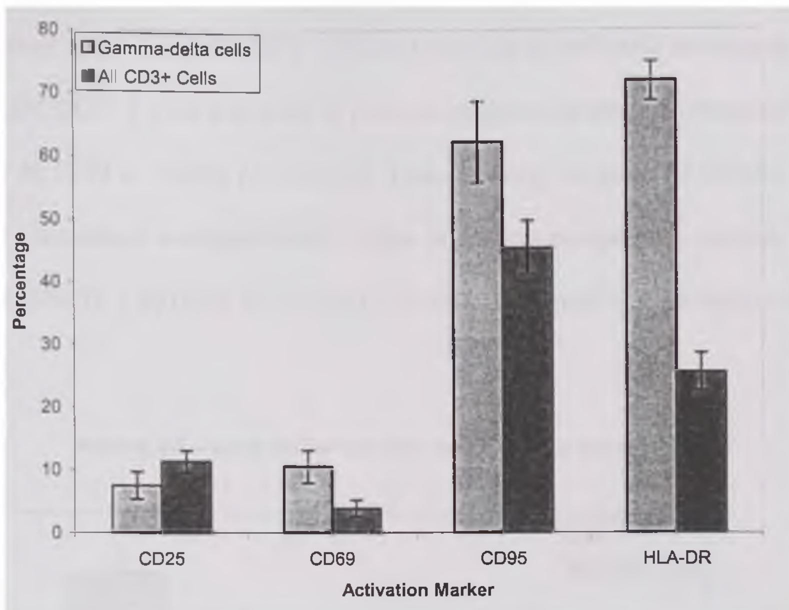
Figure 8. Percentages of activation markers in Gamma-delta cells compared to those in CD3+ cells in BL patients



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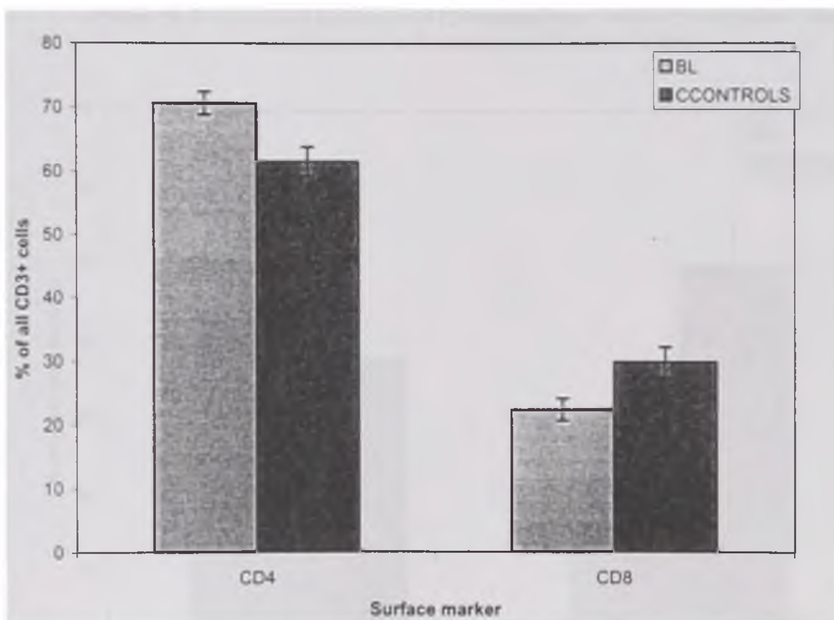
4.8 The ratio of CD4/CD8 in BL patients is higher than in healthy children

The mean percentage of $CD4^{+}CD3^{+}$ was significantly higher in BL patients than in controls {Mean difference (95%CI): 9.17 (3.21 to 15.12); p(t)=0.004} whereas the mean value of $CD8^{+}CD3^{+}$ T cells was lower in patients compared to controls{Mean difference (95%CI): 7.49 (1.73 to 13.25); p(t)=0.013}. Consequently, the mean of CD4/CD8 ratio in terms of percentages was significantly higher in patients compared to controls {Mean difference (95%CI): 1.30 (0.40 to 2.20); p(t)=0.006}. This trend is illustrated in figure 9 below.

Figure 9. Frequencies of CD4+ and CD8+ cells in patients and controls



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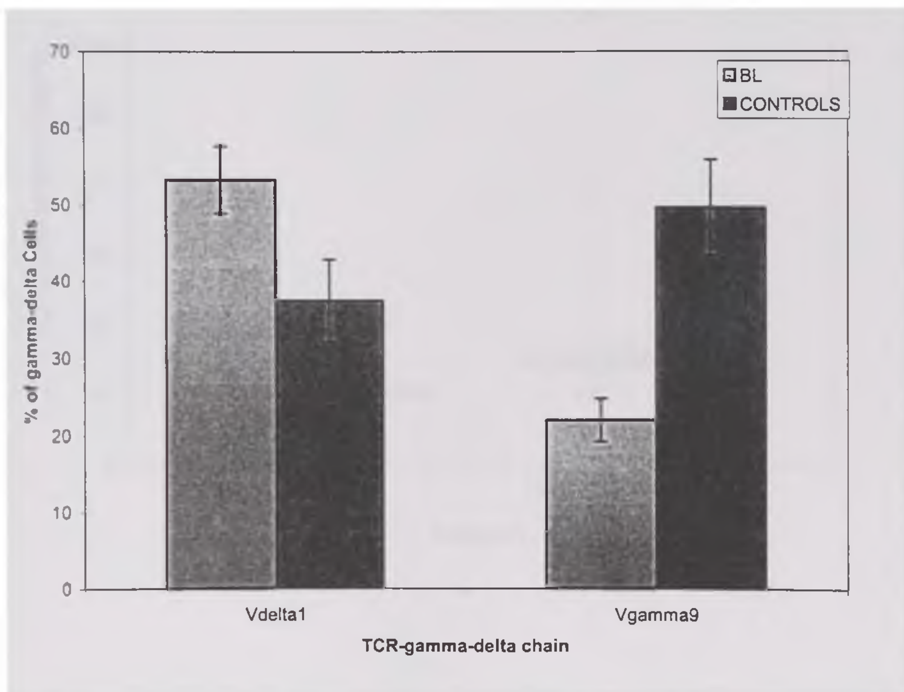
4. 9 Percentages of TCR-$\gamma\delta^+$ cells expressing the variable (V)-segments, Vδ1 and Vγ9, in BL patients and healthy controls

The percentage of V$\delta1^+$ $\gamma\delta^+$ T cells was higher in BL patients compared to controls {Mean difference (95%CI): 15.80 (0.22 to 31.38); $p(t)=0.047$} and conversely, the percentage of V$\gamma9^+$ $\gamma\delta^+$ T cells was lower in BL patients compared to controls { Median difference (95%CI): 36.34 (16.11 to 49.17); $p(T)<0.001$}. Figure 10 illustrates this.

Figure 10. Frequecies of expression of TCR-gamma-delta variable (V)-segments, Vdelta1 and Vgamma9 in BL patients and Healthy Controls



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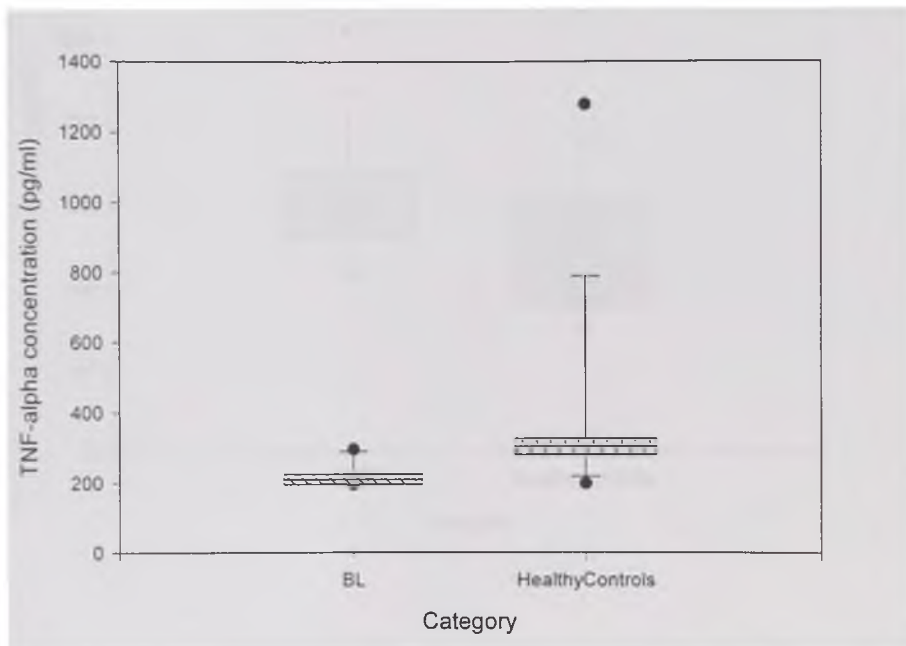
4.10 Plasma levels of cytokines

4.10.1 Tumour necrosis factor-alpha (TNF-α)

The median level of TNF-α in peripheral blood as measured in the plasma by ELISA was significantly lower in BL patients compared to healthy controls {Median difference (95%CI): 101 (24 to 198) pg/ml, p(*T*)=0.002}. The distributions of the plasma levels of TNF-α in study subjects are shown in the box plot (figure 11).

Figure 11. TNF-alpha levels in plasma of BL patients and heathy controls



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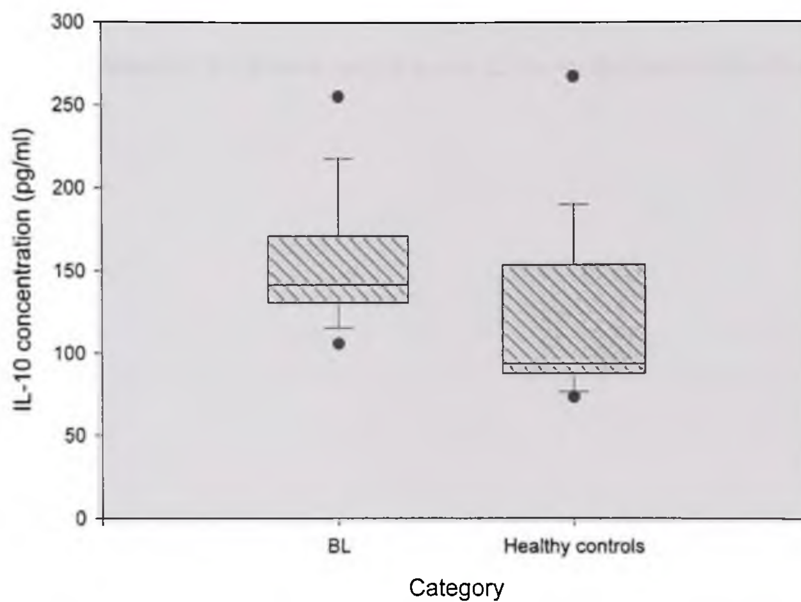
4.10.2 Interleukin-10 (IL-10)

Plasma IL-10 was significantly higher in BL patients compared to healthy controls {Median difference (95%CI): 48(52 to 123) pg/ml, p(*T*)=0.042}. This is illustrated in figure 12.

Figure 12. IL-10 levels in plasma of BL patients and healthy controls



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4.11 Kinetics of TNF-α and IL-10 secretion by *in vitro* stimulated PBMC.

To ascertain the best time point for measurement of TNF-α and IL-10 in culture supernatants PBMC were cultured for Day 1, Day 3 and Day 6 in the presence of malaria parasites and mitogens. In both BL patients and controls, peak TNF-α was produced within twenty-four (24) hours. No detectable levels of TNF-α were found on Day 3. Similarly in, both BL patients and controls, IL-10 secretion generally declined from Day 1 to Day 6. The decline was very profound in PBMC from controls that were stimulated with phytohaematogglutinin (PHA) and purified protein derivative (PPD). Based on this, Day 1 measurements of IL-10 were used. Figures 13 shows the trend of IL-10 secretion.

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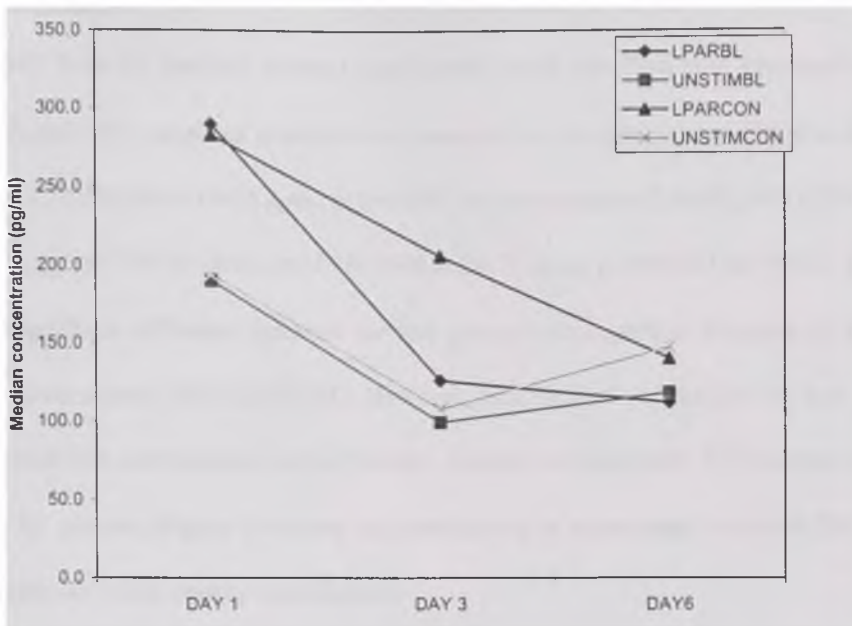
Figure 13. Kinetics of IL-10 production of lymphocytes of BL patients and healthy controls when stimulated with *P. falciparum malaria* parasites



LPARB and UNSTIMBL are PBMC from BL patients stimulated with LPAR, and the unstimulated cells respectively, likewise LPARCON and UNSTIMCON are PBMC from controls stimulated with LPAR and the unstimulated cells respectively.

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4.12 Cytokine levels in supernatants after *in vitro* stimulation.

4.12.1 TNF-α

PBMC from BL patients secreted significantly much less TNF-α in response to LPAR, PHA and PPD compared to controls as measured in the supernatants {Median difference (95%CI): 500(88 to 1443) pg/ml, p(*T*)=0.007 for live parasites (LPAR); 429 (-271 to 1878) pg/ml, p(*T*)=0.050 for PHA and 1739 (598 to 2013) pg/ml, p(*T*)=0.007 for PPD}. There was no significant difference between the two groups with regard to secretion of TNF-α by the unstimulated cells (UNSTIM). However, looking at the spread of the box plot, it is obvious that unstimulated cells of healthy children produce more TNF-α than cells from the BL patients. Figure 14 shows the distributions of supernatant levels of TNF-α in BL patients and their healthy counterparts.

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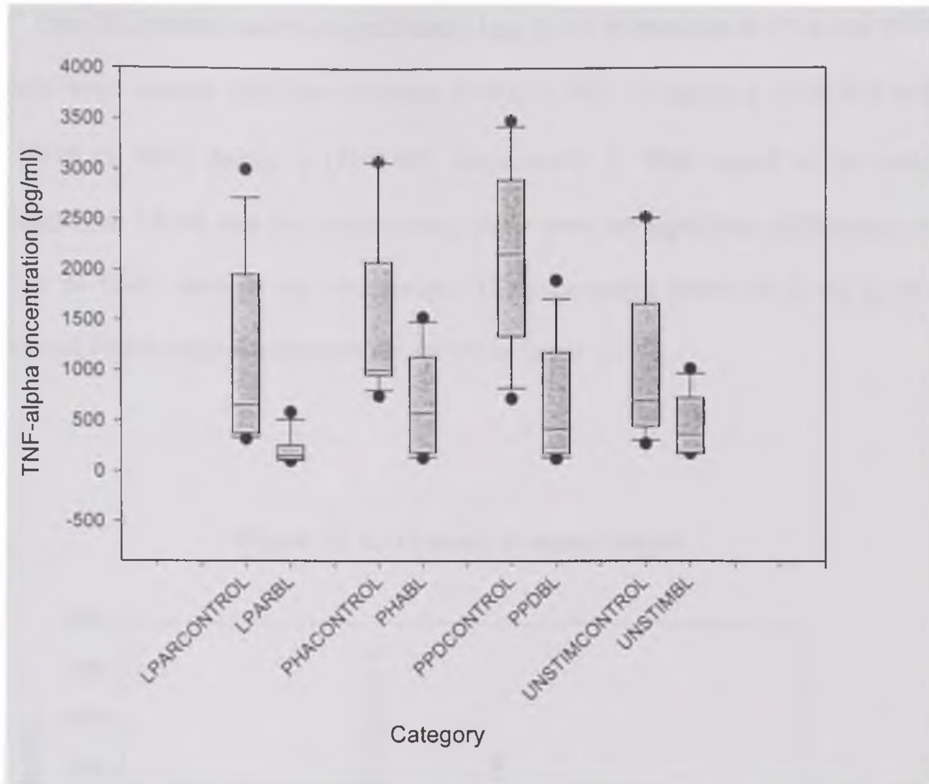
Figure14. TNF-alpha levels in supernatants



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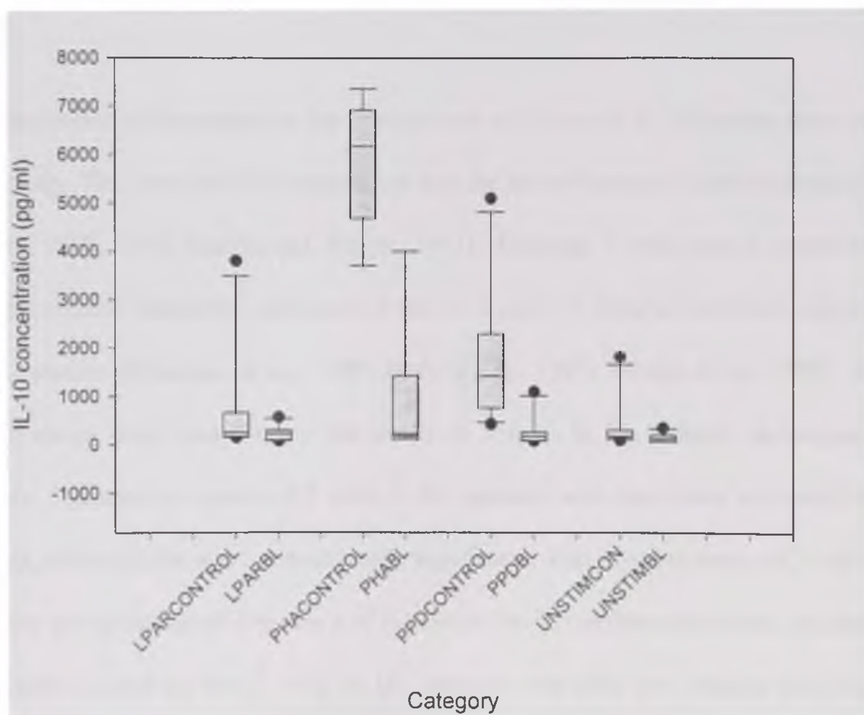
4.12.2 IL-10

PBMC from BL patients secreted significantly less IL-10 in response to PHA and PPD than cells from controls {Median difference (95%CI): 5961 (-) pg/ml, p (*T*)=0.016 and 1250 (-338 to 4883) pg/ml, p (*T*)=0.009, respectively }. With regard to the cells stimulated with LPAR and the unstimulated, there were no significant differences in secretion of IL-10 between the two groups. The supernatant levels of IL-10 in BL patients and their healthy counterparts are shown in figure 15.

Figure 15. IL-10 levels in supernatants



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CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

The link between malaria and endemic (eBL) remains obscure, even though both diseases occur in the same areas of the world. This study therefore sought to find out the role of malaria in the pathogenesis of eBL by comparing, with reference to controls, the characteristics of the lymphocytes from eBL patients with regard to the proportions of lymphocyte sub-groups, expression of lymphocyte surface and activation markers, and pro- and anti-inflammatory responses to *Plasmodium falciparum* malaria parasites, to the already established characteristics of lymphocytes in *P. falciparum* malaria in the same population.

The distribution of the tumour in the patients was as typical of BL, affecting many organs of the body. The jaws and the abdomen are still the most frequently involved sites of eBL (Burkitt, 1958, 1970; Burkitt and Wright, 1963). Although T cells play a central role in acquired cellular immunity, decreased levels of T cells in malaria have been reported in several studies (Elhassan *et al.,* 1994; Hviid *et al.,* 1997; Worku *et al.*, 1997). In the present study, there was a lower frequency of T cells in BL patients as compared to controls. The absolute count of T cells in BL patients was also lower compared to the controls, although this was not statistically significant. This low frequency of T cells may be partly due to increased frequency of B cells in the BL patients. However, the fact that the absolute count of the T cells in BL patients was also low implies that the low frequency of T cells in BL patients may not be due to the high frequency of B cells alone;

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other factors such as disease-induced reallocation and/or programmed cell death (apoptosis) of T cells may be involved. Apoptosis may involve a variety of mechanisms, including CD95 (APO-1/Fas)-mediated activation-induced cell death (Alderson *et al*, 1995; Dhein *et al*, 1995), Fas-independent activation-induced peripheral deletion as described in HIV$^+$ individuals (Katsikis *et al.*, 1996), TNF-α-mediated activation as found in glioma cells (Chen *et al.*, 2002) or antibody ligation of the TCR on activated T cells as observed in mice (Kishimoto and Sprent, 1999). The relatively high general activation of T cells and high expression of CD95 (apoptotic marker), both in frequency and absolute numbers in BL patients observed in the present study may suggest CD95 (APO-1/Fas)-mediated activation-induced cell death (AICD) in T cells in acute eBL, though one cannot completely exclude disease-induced reallocation of the cells away from the peripheral circulation. AICD is normal and natural because protective cellular immune response does not only involve activation and expansion of cells but also apoptosis, during activation and effector activity, a phenomenon which is important in regulating cell numbers and ensuring homeostasis (Liu and Janeway, 1990). When cell numbers are reduced at the acute stage of the disease, it may impair protective cellular immunity to the disease. TCR- $\gamma\delta^+$ cells, which in this study were found to have highly activated phenotype and expressed the highest frequency of CD95, also showed the most dramatic reduction in frequency and absolute numbers even during acute stage of the disease, further suggesting the involvement of CD95 in T cell apoptosis in eBL. Although activation-induced cell death (AICD), involving CD95 as the cause of decreased lymphocyte numbers in acute malaria has not been fully established, there is evidence to show that, at least, increases in peripheral CD95-induced apoptosis occur (Balde *et al.*,

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1995; Kern *et al.*, 2000; Matsumoto *et al.*, 2000; Toure-Balde *et al.*, 1996). Thus the role of malaria in the pathogenesis of eBL might be to complement the activation of lymphocytes due to EBV-infection and hence an elevation in the expression of CD95 by T-lymphocytes, consequent deletion of CD95+ T cells and reduction in lymphocyte numbers in individuals with underdeveloped immunity to *P. falciparum* malaria such as children.

The $\gamma\delta^+$ cells are found to respond early and rapidly to certain bacterial and parasitic infections (Born *et al.*, 1999; Halary *et al.*, 1999) and respond to various promiscuous and self-antigens (Hayday, 2000). Studies have also suggested their role as keeping surveillance on expansion of B cells (Biggar *et al.*, 1981). Based on these findings, it is believed that $\gamma\delta^+$ cells play an immuno-regulatory role in immune responses. The decrease in $\gamma\delta^+$ cells in eBL observed in the present study may therefore adversely affect their role of immune surveillance and regulation in eBL because the fast-growing tumour cells might overwhelm them. This may imply that not only is there a lack of an effective control of the abnormal proliferation of the tumour cells but also protection against other infectious agents.

The mean percentage of $CD4^+CD3^+$ was significantly higher in BL patients than in controls whereas the mean value of $CD8^+CD3^+$ T cells was lower in patients compared to controls. As a result the mean ratio of CD4 to CD8 was significantly higher in patients compared to controls. The rise in the CD4/CD8 ratio therefore was not only due to selective increases in $CD4^+$ cells but also an accompanying decrease in $CD8^+$ cells. The

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decrease in $CD8^+$ cells may be due to an apoptotic deletion. A study has demonstrated elevated apoptosis in $CD8^+$ cells upon recognition of self-antigens presented on activated B cells (Bennett, *et al.*, 1998), and the present study has also confirmed activation of B cells in BL patients. B cell activation is also a characteristic of malaria and therefore recurrent malaria may speed-up the removal of the $CD8^+$ cells in children making them more vulnerable to eBL, since $CD8^+$ cells or cytotoxic T lymphocytes (CTLs) are very vital in controlling diseases caused by intracellular infectious agents such as EBV. The low frequency of $CD8^+$ cells may also be due to loss of $CD8^+$ $\gamma\delta^+$ cells from the peripheral circulation as $\gamma\delta^+$ cell numbers declined, because about 30% of the $\gamma\delta^+$ cells in BL patients were $CD8^+$.

This study has also shown that in eBL, majority and significantly higher (compared with controls) proportion of $\gamma\delta^+$ cells are $V\delta1^+$ cells contrary to observed elevation of $V\gamma9^+$ cells during EBV infection in humans in a population non-endemic for malaria (De Paoli, 1990). This may imply that the higher proportion of $V\delta1^+$ cells observed instead of $V\gamma9^+$ cells is due to malaria. Elevated level of $V\delta1^+$ cells is known to be associated with endemicity and severity of *P. falciparum* malaria (Hviid *et al.*, 2000, 2001). None of the study subjects had clinical malaria, so the high proportion of $V\delta1^+$ cells observed may just be a confirmation of the reported high proportion of $V\delta1^+$ cells in healthy children from Ghana. $V\delta1^+$ cell expansion has also been reported in HIV infection (Autran, *et al.*, 1989). However the prevalent rate of HIV infection in Ghana and especially our study age group is negligible (<4%) (Dr. B. Q. Goka, pers. com.), and as such cannot be responsible for the difference. Activated B cells are found to be antigenic target of $V\delta1^+$

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cells (Halary *et al.*, 1999). Dominance of Vδ1$^+$ cells may therefore imply protective immune response to the tumour cells in eBL but in the face of low levels of $\gamma\delta^+$ cells their absolute numbers are much lower and they might be overwhelmed by any fast-growing tumour such as BL.

Our stimulation assay shows that response of PBMC from BL patients was remarkably low compared to the controls with regard to secretion of TNF-α to LPAR and PHA. Cytokine secretion to LPAR represents malaria-specific response while PHA represents non-specific stimulation. This implies that in BL patients, both malaria-specific and non-specific responses were low with respect to TNF-α production. Similarly, PBMC from BL patients produced significantly less IL-10 than controls when stimulated with PHA, again indicating low non-specific response in BL patients. The generally low levels of TNF-α and IL-10 production PBMC of BL patients may be due to many factors. But two important factors that cannot be overlooked are the observed low frequency of T and/or low absolute numbers of $\gamma\delta^+$ T cells in BL patients compared to controls and the fact these cells have a phenotype which indicates that they are poised to undergo AICD (Alderson *et al.*, 1995). PBMC from BL patients were over-activated and expressed high levels of the apoptotic marker (CD95), which may have affected their cytokine production upon stimulation.

Various cell types are known to produce TNF-α but mainly monocytes/macrophages are responsible for TNF-α production. However, monocytes/macrophages require cytokine stimulation from T cells, which also secrete substantial amounts of TNF-α. Therefore the

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low proportion of T cells in PBMC from BL patients may account for the low level of TNF-α measured in the supernatants. A study has shown that lymphokines secreted by $\gamma\delta^+$ T cells activate macrophages, the main producers of TNF-α (Goodier *et al.,* 1995). The reduction in the number of $\gamma\delta^+$ T cells observed in the present study may therefore reduce the function of macrophages and TNF-α production in cells from eBL patients. On the other hand, it would be expected that cells from BL patients should produce more IL-10 than those from healthy children. This is because BL is a B-cell tumour and as the present data have shown, with high levels of activated B cells, it is expected that there will be an increase in production of IL-10, B cells being a major source of the cytokine. The low supernatant level of IL-10 in eBL patients is therefore not clear but it may be accounted for by the same factors that explain the low level of TNF-α such as low levels of $\gamma\delta^+$ T cells numbers. There was slightly higher production of IL-10 in response to stimulation with LPAR in both patients and controls compared to the unstimulated PBMC (Figure 16). Stimulation with *P. falciparum* schizonts-infected erythrocytes, therefore, seems to elicit production of IL-10 in both groups. The elevated level of IL-10 in response to malaria parasites is consistent with what other researchers have found. Studies have shown that stimulation of lymphocytes with malaria antigens induces secretion of cytokines with Th 2 profile such as IL-10 (Wahlgren *et al.*, 1995). This was also found *in vivo* (von der Weid and Langhorne, 1993). A recent study has shown that malarial antigens stimulated PBMC, obtained from malaria patients at acute infection, to produce IL-10. When recombinant human IL-10 was added *in vitro* production of TNF-α was completely abolished in response to malarial antigens (Ho *et al.,* 1995).

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Whereas PBMC from patients produced significantly less amount of TNF-α (even slightly lower than unstimulated PBMC from eBL patients) when stimulated with LPAR, with regards to the production of IL-10 cells from the BL patients produced an amount similar to that of the controls. This implies that the capacity of PBMC from eBL patients to produce IL-10 when challenged by *P. falciparum* malaria parasites is not reduced significantly due to the disease. If the secretion of TNF-α is significantly reduced in BL patients, as the present data suggests, that the shift of the immune response towards the production of anti-inflammatory cytokines (such as IL-10) at the expense of pro-inflammatory cytokines (such as TNF-α) during *P. falciparum* malaria may be more serious in eBL patients than the controls.

Plasma levels of IL-10 were significantly high in BL patients compared to controls. Conversely, plasma levels of TNF-α were significantly low in eBL patients compared to controls. The higher plasma level of IL-10 supports the *in vitro* studies that seem to suggest that elevated level of IL-10 is a characteristic of eBL (Burdin *et al.,* 1993). An elevated production of IL-10 is not an anti-tumour response, rather IL-10 is known to enhance the growth of B cells and hence the tumour cells. Any factor that contributes to elevation of IL-10 level would also contribute to the growth and persistence of the tumour. The high plasma level of IL-10 and the response of PBMC from the BL patients to *P. falciparum* malaria parasites indicate that whereas IL-10 level is already high, during *P. falciparum* malaria the level may be elevated. In this light, another contribution of *P falciparum* malaria to the pathogenesis of eBL aside activation of lymphocytes and

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lymphopenia, may be skewing of the immune responses toward production of anti-inflammatory cytokines through recurrent infection with *P. falciparum* malaria parasites. The low plasma levels of TNF-α also indicate that the reduced number of T cells observed is not due to TNF-α-mediated apoptosis. The cause of low plasma level of TNF-α may be multifactorial but the main factors could be downregulation of its secretion by the high level of IL-10, low T-cell frequency and absolute numbers of $\gamma\delta^+$ T cells in particular.

The hope of controlling the expanding B cells and the fast growing tumour rests mainly with the activities of CTLs (CD8+ cell), $\gamma\delta^+$ T cells (Biggar *et al.,* 1981) and of course TNF-α, a pro-inflammatory cytokine that is very vital for protective cellular immunity as it activates other cells of the cellular immune system essentially CTLs. Unfortunately, as discussed earlier, there is reduction not only in T cell frequency but also in CD8+ and $\gamma\delta^+$ T cells numbers in addition to low plasma levels of TNF-α. Moreover, IL-10 is found to suppress the capacity of CTLs in clearing EBV-infected and cancer cells (Chouaib *et al.,* 1997; von der Weid and Langhorne, 1993). This implies that the T- and γδ-cell lymphopenia, low level of TNF-α and high level of IL-10 observed in BL patients have serious adverse effect on the cellular immune system as a whole with serious implications not only for the ability to mount protective response against BL but also other infectious agents. This turn of events suggest that BL patients at acute stage cannot overcome the disease without medical intervention. This may account for the high death rate among the BL patients recruited for this study.

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5.2 CONCLUSIONS

The findings from the present study show that there is remarkable general activation of lymphocytes and high level of circulating lymphocytes that express the apoptotic marker, CD95 in BL patients. The high expression of CD95 is believed to be caused, at least partly, by *P. falciparum* malaria. The elevated expression of the CD95 would lead to AICD, which may account for the low peripheral levels of T cells and $\gamma\delta^{+}$ T cells in particular. This needs to be further investigated by looking at the expression of the activation markers of the lymphocytes from BL patients when they have malaria. These results also suggest that the shift of the immune response towards production of anti-inflammatory cytokines is a characteristic of eBL and that any factor that shifts the immune responses toward production of anti-inflammatory cytokines such as *P. falciparum* malaria, will contribute to the development and persistence of eBL. Our data also suggest that during *P. falciparum* malaria, the cells from BL patients are likely to produce, at least, as much IL-10 as cells from their healthy counterparts thereby contributing to the already high levels of circulating IL-10 in the BL patients. This would adversely affect the capability of the already beleaguered T cells to mount protective immune responses in the patients. However to fully unravel the trend of the events mentioned, a longitudinal study is also recommended.

These imbalances in the immune system observed in eBL share some similarities with *P. falciparum* malaria in terms of T- and B-cell activation, T-cell lymphopenia, B-cell expansion and an elevated production of IL-10. This confirms our hypothesis that recurrent *P. falciparum* infection is an additive risk factor for the development of eBL.

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APPENDIX

To prepare a litre of phosphate-buffered saline (PBS), the following reagents were used.

NaCl....................................8.0g.

KH_2PO_4...............................0.2g.

$Na_2HPO_4.12H2O$...................2.9g.

KCl.....................................0.2g.