AN *IN VITRO* MODEL OF ENDEMIC BURKITT'S LYMPHOMA (eBL) PATHOGENESIS; COOPERATION OF *PLASMODIUM FALCIPARUM* AND EPSTEIN BARR VIRUS IN DNA DAMAGE MEDIATED VIA ACTIVATION INDUCED CYTIDINE DEAMINASE.

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Declaration

I hereby declare that this submission is my own work towards the MPhil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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Dedication

This work is dedicated to Madam Agnes N. P. Djanie and Miriam Ayivor.

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Abstract

Plasmodium falciparum (*P. falciparum*) and Epstein-Barr virus (EBV) infections are contributors in the pathogenesis of endemic Burkitt's lymphoma (eBL), although the precise mechanism of their synergy remains elusive. Reports suggest that the role of *P. falciparum* is indirect, creating a permissive environment for the outgrowth of EBV. EBV on the other hand invades and immortalizes lymphocytes *in vitro* and upregulates activation-induced cytidine deaminase (AID), a DNA repair enzyme responsible for diversifying the antibody repertoire and a potent mutagen capable of inducing the genetic damage characteristic of eBL. It is yet to be shown how exposure to *P. falciparum* affects the expression of AID in lymphocytes. The aim of this work was to investigate the possible direct role of *P. falciparum* in eBL lymphomagenesis by exploring parasite-lymphocyte interactions and AID expression after exposure to *P. falciparum* and/or EBV. Malaria positive slides were examined for parasite-lymphocyte interactions and primary tonsillar mononuclear cells (MNCs) were co-cultured with RBCs infected with up to 5% parasitemia of the 3D7 strain of *P. falciparum.* Geimsa stained thin smears were made from these co-cultures and examined for parasite-MNC interactions over a five day period. No direct parasite-MNC interaction was observed from all slides examined. The levels of AID mRNA in MNCs was measured by qPCR after *in vitro* exposure to *P. falciparum* and/or EBV, and in the presence or absence of 2µg/ml cyclosporine. *P. falciparum* induced up to a 6-fold increase in AID over unstimulated controls, EBV induced a 13-fold maximum increase, and both pathogens together induced up to a 22-fold increase in AID. With cyclosporine, AID mRNA levels in the *P. falciparum* stimulated cultures remained unchanged. EBV alone induced a 22-fold increase in AID and both pathogens together induced a 42-fold increase in AID. DNA damage was estimated by Comet Assay and quantified with an algorithm from the Comet Assay Project Lab (CASP). DNA comets revealed that *P. falciparum* induced moderate DNA damage in MNCs with up to 5.6% and 10% DNA in tails of comets with and without cyclosporine respectively. Cultures stimulated with EBV recorded DNA damage of up to 16% and 13% DNA in tails of comets with and without cyclosporine respectively; and both pathogens induced DNA damage with up to 11% and 16% DNA in tails of comets with and without cyclosporine respectively. The levels of DNA damage in these cells correlated with AID levels and demonstrate that *P. falciparum* plays a direct role in eBL pathogenesis, by inducing AID expression to levels similar to that expressed in BL cells and cooperating with EBV to induce abnormally high levels of AID and DNA damage.

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CHAPTER ONE

INTRODUCTION

1.1 Background and Significance

Burkitt's lymphoma (BL) is a subgroup of non-Hodgkin's Lymphomas (NHL) with distinct epidemiological, clinical, pathological, immunological and molecular cytogenetic characteristics (Magrath, 1991). It was first described in 1958 by an Irish surgeon working in Uganda who noted a unique, rapidly growing jaw malignancy in children. It is especially common in low altitude, high rainfall areas, with a mean temperature over 16^{o}C (Burkitt, 1962). Its distribution is concentrated 10^{o} North and South of the Equator; a distribution that overlaps with that of holoendemic malaria (Burkitt, 1988), implicating malaria in the aetiology. There are three subtypes of BL; the endemic/African BL (eBL) is found predominantly in equatorial Africa, the sporadic BL (sBL) is in the United States and Europe and the HIV-associated subtype which is found worldwide. eBL affects mainly children, and boys seem to be more susceptible than girls (Parkin *et al.*, 2003). Data from Globocan (2002) suggests that BL is the second most common pediatric cancers accounting for 20% of all pediatric cancers in the lymphoma belt (Ferlay *et al.*, 2004).

The tumours are highly aggressive and may double in size every 24 hours (Kearns *et al.*, 1986 and Zeigler, 1977). It is potentially curable and is extremely responsive to small doses of chemotherapy and with modern combination chemotherapy, 85-100% of those with early stage disease and 75-85% of those with advanced disease will survive for at least three years without the need for treatment (Sandlund *et al.*, 1996). Relapse is however common.

eBL is of greatest importance in sub-Saharan Africa, where it is the most common childhood cancer, accounting for up to 36% of childhood cancers and 70% of childhood lymphomas (Parkin, 1998). Despite the good prognosis for the disease in general, survival is very poor in developing countries due to factors that include the high cost of treatment, late presentation and limited access to health care. eBL, therefore, remains an important health issue in many African countries.

Although it is claimed that sustained and intense exposure to *Plasmodium falciparum* malaria and Epstein–Barr virus (EBV) infection are two cofactors of the eBL, the mechanisms of interaction between these two pathogens is still unclear, (Rockford *et al.,* 2005). In developing countries, primary infection with EBV is asymptomatic and occurs in young infants soon after decrease in the concentrations of maternal antibodies (Henle & Henle, 1970, Biggar, *et al.* 1978 a & b) and EBV infection rates in children are uniform regardless of the intensity of malaria transmission (Geser *et., al,* 1980). The difference then for the increased risk of eBL in equatorial Africa may not be EBV infection early in life but the presence of holoendemic malaria; as children tend to have high parasite densities in peripheral blood and are at the greatest risk of morbidity and mortality associated with *P. falciparum* infections. Moreover, the highest parasite loads are found among children 6–11 months of age, which is also the age at which primary EBV infection is most likely to occur (Rochford *et al.,* 2005).

Unlike many infectious diseases, *P. falciparum* malaria only elicits protective immunity in children after several years of cumulative exposure. So, exposure to holoendemic malaria is characterized by recurrent (perhaps chronic) *P. falciparum*

infections, and it is likely that during this period the child's immune system is under constant stress. Studies suggest that *P. falciparum* malaria could increase EBV infection of B cells (Chene, *et al.*, 2007) and also reduce the immune response against EBV (Greenwood, 1974). The combined effect of these pathogens is likely to result in an increased risk of endemic BL for children living in malaria holoendemic regions.

The defining characteristic of BL is an oncogenic translocation that juxtaposes c-myc and the immunoglobulin heavy chain locus (IgH). This chromosomal translocation results in deregulated c-myc expression. The nature of the deregulation depends on the exact break point location and the orientation of the insertion. DNA breaks in IgH leading to c-myc/IgH translocations are facilitated by activation induced cytidine deaminase (AID) during antibody class switch recombination or somatic hypermutation (Ramiro, *et al.*, 2004 and Robbiani, *et al.*, 2008).

In order to directly implicate *P. falciparum* in the genesis of eBL, it is crucial to establish the role the parasite plays in events that lead to the genetic lesion. Although it is known that EBV induces the expression of AID as part of its lytic cycle, it is yet to be shown if *P. falciparum* can induce the expression of the enzyme and whether there is a synergy when the two pathogens (*P. falciparum* and EBV) are together.

1.2 Problem Statement

P. falciparum malaria is recognized as a risk factor for eBL, as both anti-malarial treatment and migration of human hosts to areas of low malarial infection reduce the risk for eBL. However, there is limited information on the direct role *P. falciparum* plays, whether alone or in cooperation with EBV, in the events that lead to the development of the cancer. This work was based on the premise that *P. falciparum*

plays a more direct role in the pathogenesis of eBL other than just creating a permissive context for the events leading to the cancer to take place.

1.3 Justification

P. falciparum infection induces hypergammaglobulinaemia, indicating that *P. falciparum* is a B-cell mitogen (Greenwood, 1974) and extracts derived from cultured *P. falciparum* can induce B-cell proliferation and antibody production *ex vivo* (Greenwood, *et al.*, 1979, Gabrielsen, *et al.*, 1982 and Kataaha, *et al.*, 1984). This suggest that proteins from *P. falciparum* have the capacity to stimulate B-cell proliferation and could facilitate the expansion and transition of high numbers of naïve B-cells, EBV positive or negative, into germinal centers, where somatic hypermutation and class switch recombination takes place; processes that require the expression and action of AID.

Secondly, data suggest that *P. falciparum* could have the potential to directly interact with B-lymphocytes on the surface via toll-like receptors. *P. falciparum* expresses a Toll-like receptor 9 (TLR9) ligand during erythrocytic schizogony (Pichyangkul, *et al.* 2004), and B-cell also respond to pattern-recognition stimuli such as bacterial CpG motifs through TLRs (Bernasconi, *et al.*, 2003). In addition, the parasites have the ability to bind to *ras* containing lipid rafts of the red blood cell, a known signaling domain in the B-cell (Murphy, *et al.*, 2007). The expression of AID is a downstream event to activation of ras and TLR9, consequently, *P. falciparum* schizonts and infected erythrocytes have the potential to directly interact with B-cells on the surface and thereby induce AID expression which leads to proliferation and differentiation into antibody-secreting cells (Rochford *et al.*, 2005).

Thirdly, some close relatives of *P. falciparum*, like *Theileria parva* and *Theileria annulata*, members of the apicomplexa family, are known to have the ability to invade leukocytes; T-cells and B-cells for *Theileria parva*, and B-cells and macrophages for *Theileria annulata* (Dobbelaere and Kuenzi, 2004), and this infection activates these cells to produce cytokines and proliferate in an uncontrolled manner, usually via the deregulation of the proto-oncogen *c-MYC* (Dessauge *et al*., 2005). It is therefore reasonable to postulate a possible direct internalization of *P. falciparum* into B lymphocytes during its asexual life cycle in its human host.

Finally, *P. falciparum*-infected red blood cells (iRBCs) bind to EBV-positive B-cells (Akata cell line) through the CIDR1α domain of PfEMP1. This increases the number of cells switching to the viral lytic cycle (Chene, *et al*., 2007). This interaction accounts for the higher viral loads of EBV found in children with malaria, which in turn is thought to be a risk factor for eBL development. Moreover, *P. falciparum* is known to have general immunosuppressive effects (Clark, *et al*., 2003) and EBV replication is induced when the immune system is compromised (Greenwood, 1972).

1.4 Aim of Study

This work sought to investigate the roles that *P. falciparum* plays in eBL lymphomagenesis based on the hypothesis that some factor from malaria is critical in the initiation of the eBL genetic lesion.

1.4.1 Specific Aims

The specific aims of this work were to;

I. **To probe for direct of interactions between *P. falciparum* and lymphocytes *in vitro* and *ex vivo:*** In order to postulate direct effects for the parasite, it is important to first establish that some factor from the parasite directly interacts (by internalization into the B-cell or binding on the surface of B-cell) with the B cell or otherwise stimulates it in a way to increase the risk of creating the genetic lesion. Parasite-lymphocyte interactions were therefore probed. Clinical malaria smears at all degrees of parasitemia were examined for possible internalization events in non-erythrocytes. Tonsillar mononuclear cells were stimulated *in vitro* with a high density of *P. falciparum* and smears were made to search for parasite-MNCs interactions.

II. **To measure the levels of AID mRNA in mononuclear cells after exposure to *P. falciparum* in the presence or absence of EBV.** As a follow-up to aim I, it was determined by qPCR if AID was overexpressed in the *P. falciparum*-stimulated cells. By infecting cultures of MNCs with *P. falciparum* and/or EBV, it was possible to determine if there are synergies when both pathogens are present or whether one or the other is primarily responsible for aberrant AID expression.

III. **To assess DNA breakage/damage in mononuclear cells after exposure *P. falciparum* in the presence or absence EBV.** AID is a genome mutator that can induce inappropriate mutations in non-Ig genes when aberrantly expressed. The level of DNA damage in MNCs after exposure to *P. falciparum* and/or EBV was estimated by the Comet Assay and correlated with the level of AID mRNA.

CHAPTER TWO

REVIEW OF LITERATURE

2.0 General background

Lymphomas are cancers that begin in the lymphatic system and manifest as solid tumors of lymphoid cells. These malignant cells often originate in lymph nodes, presenting as an enlargement of the node. Lymphomas are closely related to lymphoid leukemias, which also originate in lymphocytes but typically involve only peripehral blood and the bone marrow and do not usually form static tumors (Parham, 2005). Lymphomas account for about 10% of all malignant diseases in children below age 15 and 60% of these are non-Hodgkin's lymphomas (NHL) (Young *et al.,* 1986). In the developed world, lymphomas are the most common form of hematological malignancy, or "blood cancer". Taken together, lymphomas represent 5.3% of all cancers in the United States, and 55.6% of all blood cancers (Horner *et al*., 2006).

A study conducted in 2000 revealed that malignant neoplasms accounts for 2.6% of all 34,598 admissions, and 5.6% of all 2,501 deaths at the Korle-Bu Teaching Hospital (KBTH) in the year 1996 (Biritwum *et al*., 2000). In a related study, a review of the cancer register in the Department of Child Health, KBTH, over a 40-month period revealed that malignancies accounted for 1.67% of all admissions, with lymphomas (mainly Burkitt's lymphoma) being the commonest tumour (67%), followed by retinoblastoma (8.6%), leukaemia (8.2%) and Wilm's tumour (7.8%) (Welbeck and Hesse,1998). This trend is similar to the picture seen in West Africa and other developing African countries (Welbeck and Hesse, 1998), with the exception of Namibia (Wessels and Hesseling, 1997).

2.1 Geographical distribution of Burkitt's lymphoma

BL is commonly distinguished into two forms: the "endemic" or African form (eBL), found in equatorial Africa and Papua New Guinea, and the "sporadic" form (sBL), found in North America, Northern and Eastern Europe and the Far East. These distinctions are generally based on geographic location, association with EBV, clinical presentation, incidence, age of onset and sex ratio. An "intermediate" form may also be distinguished, and this occurs in areas such as Southern Europe, the Middle East and parts of South America. Some authors have distinguished a BL subtype associated with AIDS (Wright, 1999).

Besides geographic distribution and clinical manifestation, eBL and sBL differ in EBV infection status: eBL is almost always (>95%) associated with EBV, whereas sBL has a more irregular association, ranging from 10% to 30% positivity in different areas (Bellan *et al.*, 2005). The incidence of BL has recently increased because it is a common neoplasm in HIV-infected patients (Carbone, *et al.*, 1995), and these lymphomas are currently listed as AIDS-related BL according to the World Health Organization (WHO) classification of 2001. The association with EBV is variable among AIDS-related BL with most AIDS-related BL in Western countries being EBV-negative, whereas in Africa they are strongly associated with EBV (Subar, *et al.*, 1988 and Lazzi, *et al.*, 1998)

The area of highest risk for eBL appears to be between 10° north and 10° south of the equator and in Papua New Guinea, where eBL is very common in relation to other types of childhood cancers. Although figures as high as 80% have been quoted (de The, 1985), data extracted from the International Incidence of Childhood Cancer, Vol II,

(Parkin *et al* 1998), suggests that between 19 and 36% of childhood cancers in high-risk areas like Malawi, Nigeria, Uganda and Papua New Guinea are accounted for by BL. This compares with 0-2% in low-risk areas (registries from the Far East, South Africa and Eastern and Northern Europe), and 3-7% in intermediate-risk areas (registries from North Africa and Southern Europe).

2.2 Age and gender specific incidence of Burkitt's lymphoma

BL is primarily a pediatric disease, particularly in high-risk areas. Otieno et al., (2001) reported that in Kenya between 1992 and 1996, 96.5% of BL cases (total n=796) were aged under 16 years. Most cases in equatorial Africa are in the 5-9 year age group whereas in Europe, childhood cases are approximately evenly distributed across a group (Stiller and Parkin, 1990). There are indications of a slightly younger age distribution in the Middle East. For Kuwaitis in Kuwait during 1983-89 and 1992-93, 77% of cases were aged less than five years; the figure was somewhat lower for non-Kuwaitis at 53% (Memon et al., 1998). Similarly, 63% of BL in non-Jews in Israel (1980-89) were aged under five years, although cases in Jews were more evenly distributed; 37% under five years, 38% between five and nine years and 25% between 10 and 14 years (Barchana *et al*., 1998). Shapira and Peylan-Ramu have also reported a weighted median ages of 6.1 for Africa, 19.2 for North America, 6.2 for the Middle East and 7.5 for Europe whiles in Hong Kong and Japan, the disease primarily affects young adults (Shapira and Peylan-Ramu, 1998). In low risk areas, BL has been shown to occur more often in boys than girls. In Europe, the male to female ratio ranges from 1.3:1 (Czech Republic 1980-89) to 8.8:1 (former German Democratic Republic 1981-89) while Shapira and Peylan-Ramu give a ratio of 3:1 for Europe as a whole (Shapira and Peylan-Ramu,

1998) and data from the Surveillance Epidemiology and End Results (SEER) program, North America (1975 - 2009), indicates that white males are at greatest risk (Howlader *et al.*, 2011).

In high-risk areas, the ratio of boys to girls is generally lower at between 1.5:1 and 2.5:1 (Stiller and Parkin, 1990). In intermediate risk areas the ratio is very variable, ranging from 1:1 (Kuwaiti nationals) to 7:1 (Italy, Piedmont). In apparent contradiction to the general pattern of a lower male to female ratio where there is a younger age distribution (i.e. areas of high incidence), some studies from areas of low or intermediate risk report a higher ratio of males to females in the younger age groups. Data from the American Burkitt's Lymphoma Registry show a male to female ratio of 3.3:1 in the under 13s, whereas males and females were almost equally affected in older children and adults (Levine, 1982). Similarly, a small study in the Middle East (n=34) found a male to female ratio of 2.2:1 in those less than ten years, compared with 0.75:1 in older patients (Anaissie, 1985).

A review of all out-patient clinical records of patients histologically and/or clinically diagnosed with BL from January, 2000 to December, 2007 at the Komfo Anokye Teaching Hospital, Ghana by Owusu *et al.*, (2010), revealed an age range of 1-16 years with a mean of 6.9 and males were dominant in incidence. They also observe the age range of 4–8 years as the high risk range for both sexes. Of the 551 cases reviewed, 48.3% were facial and 32.7% were abdominal presentation while 15.8% were combined abdominal and facial. The remaining 3.3% accounted for either facial or abdominal with central nervous system (CNS) involvement (Owusu *et al.*, 2010).

2.3 Clinical presentation

BL can present in a wide range of anatomical sites although the head, neck and abdomen are most commonly involved. The head and neck are the primary sites of presentation in 50-70% of cases in high-risk areas (Burkitt and Wright, 1970) , but in only 8-30% of cases in areas of intermediate and low risk, where abdominal tumors predominate (Anaissie 1985 and Madanat, 1986). American Burkitt's Lymphoma Registry data suggest that the disease tends to present in organs undergoing rapid growth. This includes the jaw in young children, and the breast and ovary in females in the early reproductive years (Levine, 1982 and Levin 1998). The different patterns of presentation in areas of low and high-risk may therefore be, at least in part, a function of the age distribution.

2.4 Time patterns for BL incidence

It is difficult to assess temporal patterns in BL incidence, particularly in high-risk regions, due to the lack of high-quality cancer registration and population data. Apparent trends may be due to slight fluctuations in the number of cases or to factors that alter case ascertainment. Many of the longest data series which have been reported derive from searches of hospital records or case-finding activities in the community, and the methods of ascertainment have changed over time. An apparent decline in incidence in Ghana during the late 1970s and early 1980s may be a consequence of difficulties in ascertainment caused by political unrest (Smith, 1985) and a decrease in the proportion of childhood lymphomas accounted for by BL in Papua New Guinea, from 75% in 1967-71 (Wilkey, 1973) to 55% in 1979-83, may be due to less complete cancer registration for the earlier period (Jamrozik *et al.*, 1988).

2.5 Lymphomagenesis of Burkitt's lymphoma

BL tumors are highly aggressive and may double in size every 24 hours (Kearns *et al*., 1986 and Zeigler,1977) however, BL is potentially curable; 85-100% of those with early stage disease and 75-85% of those with advanced disease will survive for at least three years without the need for treatment (Sandlund, *et al.*, 1996). BL cells are likely derived from lymph node germinal center regions where rapidly proliferating B-cell blasts (centroblasts) differentiate into centrocytes following hypermutation of the immunoglobulin gene variable (IgV) region and transform into malignant BL and/or DLBCL (Diffuse large B cell lymphoma). Chromosomal rearrangement of the proto-oncogene c-MYC is the genetic hallmark of BL with over 80% of BL cases having a translocation of MYC at band q24 from chromosome 8 to the Ig heavy chain (IgH) regions on chromosome 14, t(8;14).

Less frequent rearrangements translocate *c-myc* to a position close to the antibody genes in chromosome 2 or 22 (Orem, 2007), but in every case, *c-myc* finds itself in a region of vigorous gene transcription. These translocations contribute to lymphoma genesis through alterations in the cell cycle regulation, cellular differentiation, apoptosis, cellular adhesion, and metabolism. In eBL cases the *c-myc* translocation involves the heavy chain joining regions while in sBL or in HIV-associated-BL the translocation involves the Ig switch region. MYC translocations are not exclusive of BL and can also be detected in other lymphoma subtypes such as T-cell lymphomas or multiple myeloma. The WHO classification requires for a diagnosis of BL a high growth factor, with Ki67 staining over 99% and evidence of *c-myc* rearrangement when

the analysis is possible (Orem 2007). Table 1, below, summarizes the distinct characteristics of the three subtypes of BL.

Table 1: General overview of clinical variants of Burkitt's lymphoma

Characteristics	eBL	sBL	HIV-associated BL
Epidemiology	- Equatorial - Median age 7 yrs - Associated with malaria/climate - Children in Africa	- Median age 30yrs - Children (30%) - Older adults (1%) - Low Socio Economical Status	- HIV risk groups - Med. age 10-19 yrs
Clinical Presentation	Facial skeleton (50%) CNS (33%) Other organs also affected	Abdominal, ileo-coecal (80%) Bone marrow (20%) Other organs also affected	Organ and nodal presentation
Geographic regions	Malaria belt	Worldwide	In endemic HIV areas in Africa
Pathology/Morphology: *Germinal centre B-cell* *Monomorphic medium sized B cells with basophilic cytoplasm and multiple mitotic figures.*			
Chromosomal translocations: t(8;14)(q24;q32) in >80% of cases; t(8;22)(q24;q11) in 10-15% cases; t(2;8)(p12;q24) in 2-5% cases			
Ig region involved	Ig heavy chain joining region (early B-cell)	Ig switch region (late stage B-cell)	Ig switch region (late stage B-cell)
EBV association	95%	30%	30-50%

Parkin *et. al.,* (2003), Ferlay *et al.,* (2004), Rochford *et al.,* (2005)

2.6 Predisposing factors for BL pathogenesis

Although most epidemiological data indicate that EBV and holoendemic malaria caused by *Plasmodium falciparum* infections are two microbial stimuli necessary to initiate malignant progression in endemic BL, data is limited on the mechanisms that underlie the interaction of these ubiquitous pathogens (Rochford *et al.,* 2005). The following section reviews the potential roles these pathogens play to start the lymphoma.

2.6.1 Epstein–Barr virus (EBV) in the pathogenesis of Burkitt's lymphoma

EBV is a human □-herpes virus that is estimated to infect a vast majority of the human population worldwide (National Cancer Institute, 1982). In developing countries, primary infection with EBV is asymptomatic and occurs in young children soon after the concentrations of maternal antibodies decrease (Henle & Henle, 1970, Biggar, *et. al.,* 1978, and de The 1997) but, if the age of infection is delayed (at adolescence), it gives rise to infectious mononucleosis, a lymphoproliferative disorder characterized by abnormally high levels of lymphocytes in blood and lymph nodes. This implies that the viral infection leads to the proliferation of lymphocytes in the lymph nodes and an increase in EBV positive memory B lymphocytes in the blood.

The association between EBV and BL was firmly established when zur Hausen and colleagues demonstrated that the viral genome is present in BL cells from fresh tumor biopsies (zur Hausen *et al.*, 1970a). Most importantly, they were able to show that BL tumors and cells in culture still carry the viral genome (zur Hausen *et al.,* 1970b). EBV has thus been identified as the first human tumor virus in 1970: a virus whose genome is present in the tumor cells of a given entity *in vivo* and that has strong transforming potential *in vitro*.

Despite asymptomatically infecting most of the human population, EBV is linked to the etiology of at least three important B cell cancers: endemic Burkitt's lymphoma (eBL), post-transplant lymphoproliferative disease (PTLD) and 30–50% of Hodgkin's lymphoma. Each of these tumors has a distinct cellular phenotype (or range of phenotypes) and generally expresses a characteristic pattern of EBV latency proteins (Young and Rickinson, 2004). The precise role played by EBV in the development of

these B-lymphomas is still not completely understood; particularly in the case of eBL (O'Nions and Allday, 2004). *In vitro*, EBV can very efficiently induce the continuous proliferation of primary human B-cells. The resulting 'immortalized' lymphoblastoid cell lines carry the viral genome as extra chromosomal episomes and express only nine EBV 'latency' proteins (Bornkamm and Hammerschmidt, 2001).

It is generally believed that the long-term maintenance of latent EBV infection is in memory B-cells (Laichalk *et al.,* 2003 and Rose, *et al.*, 2001) and relevant to the discussion of the etiology of endemic BL is a study that showed that cycling memory B cells express the EBV latent gene, EBNA-1 (Hochberg, *et al.,* 2004). This reflects the pattern of latent gene expression in endemic BL (Rowe, *et al.* 1987), and the shift from latency to lytic replication and production of infectious virus is thought to occur when memory B cells undergo the final step of differentiation into plasma cells (Crawford & Ando, 1986 and Rochford & Mosier 1995). Throughout the life of the healthy host, CD8+ cytotoxic T lymphocytes (CTL) that are specific for both EBV lytic and latent proteins can be isolated, and loss of EBV-specific CTL responses is associated with an increased risk of development of EBV associated lymphoproliferative disorders, indicating an important role for CTL immunosurveillance in the control of EBV infection (Khanna & Burrows 2000). As a result in cases where there is immunosuppression and the impairment of EBV specific CTL activity, like in the case of a malaria episode, the virus is reactivated and infects new cells. This reactivation is however transient and brought under control when the immune system is boosted and the number of EBV positive B-cells is kept at the barest minimum.

One of the most fascinating aspects of virology is how a transforming virus like EBV is able to establish a lifelong infection in its natural host, usually without doing any harm. This is of some significance to the role the virus plays in the pathogenesis of BL. When the virus infects B-cells and epithelial cells, it establishes its latency exclusively in the B-cell compartment and is transmitted in saliva and infects B-cells that are located in the oropharyngeal epithelium (Miller, 1990). From there, the virus is transmitted to the mucosal lymphoid tissue. There are two proposed routes of latent B-cells infection by EBV. The first school of thought suggests that latent infection of naïve B-cells by EBV and subsequent transit through the germinal center are required to seed the memory B-cell compartment (Babcock *et al.,* 2000 and Joseph, *et al.,* 2000), while the second school of thought postulates that EBV can also directly infect naïve germinal center and memory B-cells ex vivo (Ehlin-Henriksson, *et al.,* 2003). The later thought has led some researchers to argue that the germinal center transition is not necessary for generation of latently infected memory B cells but rather that memory B cells are directly infected with EBV in vivo (Kuppers, 2003).

While it is not the goal to prove any of the proposed mechanisms of latent infection of EBV, the current work subscribes more to the idea that the virus establishes persistence in the host by infecting naïve B-cell and shuttling them via the germinal center reaction to generate memory B-cells that are latently infected. This is because EBV encodes among other proteins, latent membranes proteins 1 and 2 (LMP1, LMP2), which mimic CD40 and BCR-like signals and cooperate with BAFF/BLys and APRIL to induce T-cell independent Ig heavy chain class switching (He, *et al.,* 2003, Caldwel, *et al.,* 1998, and Kilger *et al.,* 1998). It is therefore not surprising that EBV is known to

upregulate the expression of AID, an enzyme that is essential for class switch recombination in the germinal centers of lymphoid tissues.

The role of EBV as a cofactor in the genesis of eBL is compelling. The virus may act by increasing the size of the B-cell pool and transforming B lymphocytes, thereby increasing the chances of chromosomal translocations, or it could play a more direct role in tumourigenesis working in concert with the changes induced by the MYC translocation.

2.6.2 *Plasmodium falciparum* malaria in the pathogenesis of BL

Malaria caused by *P. falciparum* has been recognized as a predisposing factor for eBL since the discovery of the cancer in the late 1950s. The lymphoma belt in Africa overlaps the holoendemic malaria regions in Africa and there is a reduced incidence of the cancer in people with the sickle-cell trait. It has also been reported that the incidence of eBL in regions where malaria has been eradicated has decreased and the age of onset of eBL in immigrants from higher, malaria-free altitudes, to lower malarial endemic regions is also delayed. Although these findings are purely epidemiological, they suggest an important and direct role for malaria in the genesis of eBL. There are some data to suggest that *P. falciparum* malaria is not a mere passenger in the lymphomagenesis of eBL; data to suggest that the parasite plays a more direct role than known so far.

Firstly, *P. falciparum* infection induces hypergammaglobulinaemia, indicating that *P. falciparum* is a B-cell mitogen (Greenwood 1974). Extracts derived from cultured *P. falciparum* induce B-cell proliferation and antibody production *ex vivo* (Greenwood *et al.,* 1979, Kataaha *et al.,* 1984 and Donati *et al*., 2004); however, the exact mechanism

for this polyclonal B-cell activation remains unclear. Donati and colleagues have shown that *P. falciparum*-infected erythrocytes induced both proliferation and secretion of immunoglobulin M (IgM) by IgM-positive B cells via the cysteine-rich interdomain region 1 (CIDR1α) of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Donati *et al.*, 2004). Although this work did not directly examine the effects of the PfEMP1 antigen on naive B cells, the ability to stimulate proliferation of IgM-positive B-cells but not IgG-positive B-cells might indicate that PfEMP1 is selective for naive B cells rather than post-germinal center memory B cells. Therefore, the capacity of PfEMP1 to stimulate B-cell proliferation could facilitate the expansion and transition of high numbers of naive B cells, EBV positive or negative, into germinal centers.

P. falciparum has also been shown to express a Toll-like receptor 9 (TLR9) ligand during erythrocytic schizogony (Pichyangkul *et al.*, 2004) and B cells to respond to pattern-recognition stimuli such as bacterial CpG motifs through TLRs (Bernasconi *et al.*, 2003). Human B cells only express a limited number of TLRs, including TLR9 (Bourke *et al*., 2002 and Hornung *et al.*, 2002). Naïve B-cells require BCR engagement before TLR9 expression is induced, whereas memory B-cells constitutively express TLR9 (Bernasconi *et al*., 2003). Memory B-cells rapidly proliferate in response to stimulation with CpG, a TLR9 ligand and following expansion, memory B-cells can differentiate into antibody-secreting plasma cells. Consequently, *P. falciparum* schizonts and infected erythrocytes have the potential to directly interact with B cells and thereby induce proliferation and differentiation into antibody-secreting cells (Rochford *et al*., 2005). In addition, the parasite binds to *ras* containing lipid rafts of the red blood cell, a known signaling domain in the B cell (Murphy *et al*., 2007).

Recently Chene and colleagues showed a molecular link between malaria and EBV reactivation. They showed that *P. falciparum*-infected red blood cells (iRBCs) bind to EBV-positive B Akata cells through the CIDR1α domain of PfEMP1. This increases the number of cells switching to the viral lytic cycle as measured by green fluorescent protein (GFP) expression driven by a lytic promoter (Chene *et al.*, 2007). They propose that this interaction may account for the higher viral loads of EBV found in children with malaria, which in turn is thought to be a risk factor for eBL development.

In addition, *P. falciparum* is known to have general immunosuppressive effects (Clarke *et al.*, 2003) and EBV is activated when the immune system is compromised (Greenwood, 1974). Consequently, the increased viral loads seem more likely to arise from this immunosuppression caused by *P. falciparum* infection. It must be noted, however, that the EBV levels during malarial infection are still lower than in HIV-positive individuals without malaria. Moreover, the observed increase in viral replication seems too small to constitute a risk for eBL. Individuals who are immunosuppressed experience much greater increases in viral load but do not develop eBL. As compelling though they may be, these findings only go a step further beyond epidemiology to implicate *P. falciparum* in the genesis of the cancer. It is yet to be shown what effect the parasite has on the events that lead to the specific translocation seen in eBL.

2.6.3 Models of eBL pathogenesis

eBL can be considered a polymicrobial disease in which the B-cell compartment represents an intersection between malaria and EBV infection (Rochford *et al.*, 2005). In developing countries, EBV infection occurs in young infants although the rates of infection are uniform regardless of the intensity of malaria transmission. The difference

for the increased risk of eBL in equatorial Africa, therefore may not only be EBV infection early in life but the presence of holoendemic malaria as well, because in these areas children are exposed to recurrent, perhaps chronic, *P. falciparum* infections during which time the child's immune system is under constant stress from repeated infections with a high parasite burden that could lead to a reduced immunological response/control to EBV infection. This immunosuppression induced by the malaria parasite and hyper-activation of the B cell compartment in which EBV is latently persisting, potentially leads to increased risk of eBL, although little direct evidence exists to define possible mechanisms involved in eBL formation.

Two possible, but not mutually exclusive, models have been proposed to explain how holoendemic malaria could impact EBV latency and immunity in children and increase the risk of eBL; suppression of EBV specific T-cell immunity and/or expansion of the latently infected B-cell pool (Rochford *et al*., 2005). One model proposes that eBL may potentially be a multistep process that starts with a heavy infection with EBV in children, resulting in the immortalization of B lymphocytes and some immune tolerance, permitting proliferation of infected cells. Holoendemic malaria infection would stimulate expansion of the B-cell pool and suppress T cell responses involved in the control of EBV replication. The final step would be the development of translocations leading to deregulation of MYC and the development of a malignant clone (Klein, 1979).

The other model proposes the BL translocation occurs when B-cells are rearranging their chromosomes, as suggested by the constant involvement of one of the Ig loci. Intense immunological stimulation from holoendemic malaria would give rise to a large

pool of B-cells transiting the germinal center, which would increase the risk of developing translocations. The idea is that once the translocation involving the MYC gene has arisen, the following step would be infection and immortalization of a cell that has already rearranged its Ig genes by EBV (Williams *et al*., 1982).

Another less supported hypothesis considers arboviral infections as a cofactor on the genesis of eBL (Haddow, 1964); since some arboviruses have been shown to have oncogenic properties (Williams *et al*., 1982), and some plant extracts act as tumour promoters (Furstenberger and Hecker. 1985). In this model it is suggested that the rearrangement of Ig genes involved in the eBL takes place during the arboviral infection and that the action of the two potentially oncogenic viruses (i.e. the arbovirus and EBV) are potentiated by plant tumour promoters (van den Bosch, 2004).

The above models only suggest an indirect role for *P. falciparum*, where the parasite creates a permissive environment for the cancer to start. However the fact that *P. falciparum* infection augments the number of $CD10^{+}$ B-cells entering the germinal center implies that the *P. falciparum* infection may have a direct effect on the expression of activation induced cytidine deaminase, AID. In addition, the immune suppression induced by P. falciparum infection may also impede the clearance of cells with the eBL translocation.

2.6.4 Activation-Induced Cytidine Deaminase (AID) and BL

The defining characteristic of BL is an oncogenic translocation that juxtaposes c-myc and the immunoglobulin heavy chain locus (IgH). This chromosomal translocation results in deregulated c-myc expression as it is now in back of a promoter/enhancer.

The nature of the deregulation depends on the exact break point location and the orientation of the insertion. DNA breaks in IgH leading to c-myc/IgH translocations are facilitated by activation induced cytidine deaminase (AID) during antibody class switch recombination or somatic hypermutation (Ramiro *et al*., 2004 and Robbiani, *et al*., 2008).

The activity of AID as a genome mutator in mice leads to the question of whether AID induces inappropriate mutations in non-immunoglobulin genes. Human BL tumours express the AID gene, which should not be on after exit from the germinal centre. Moreover, there is a correlation between high AID levels and the t(8:14) translocation in many lymphomas (Agopian *et al*., 2009). The link between AID expression and unfavorable consequences in various organs was revealed by phenotypic analyses of a transgenic mouse model with AID expression. Constitutive and ubiquitous AID expression in the mice induced the development of lymphomas (Ramiro *et al.,* 2004 and Robbiani, *et al*., 2008). AID-transgenic mice exhibiting ectopic expression of the gene have been shown to develop neoplasia in unusual places, including liver, stomach and epithelium. Inducible AID knockout mice ($AID^{-/-}$) are protected against plasmacytoma (Ramiro *et al*., 2004). These findings indicate that aberrant AID expression might cause tumorigenesis in both lymphoid and non-lymphoid organs, via the accumulation of somatic mutations in oncogenes or tumor suppressors.

AID is a mutation-generating enzyme that is crucial for somatic hypermutation and isotype switching in immunoglobulin genes during affinity maturation in the germinal center. BL tumors in humans, therefore, probably arise from an aberrant expression of AID. The series of events leading to tumorigenesis probably occurs spontaneously, but

rarely, in sBL. The combination of EBV and malaria may then dramatically increase the frequency of AID deregulation, resulting in eBL. This scenario is plausible, since both EBV and malaria act as potent stimulators of B-cell proliferation and EBV is known to turn on AID (He, *et al*., 2003). In order to prove that *P. falciparum* can play a direct role in events that lead to the BL lesion, it would therefore be essential to assay for the level of AID expression in lymphocytes after exposure to *P. falciparum* in the presence and absence of EBV; and as a follow up, assay for the level of DNA damage in these cells.

2.7 The Comet Assay as tool for assessing DNA damage

The Single Cell Gel Electrophoresis assay (also known as Comet Assay) is an uncomplicated technique for the detection of DNA damage at the level of the individual eukaryotic cell. Since its introduction by Ostling and Johanson (1984) and its independent modification by Singh *et al.* (1988), the Comet assay has been widely used for genotoxicity studies and cell biological studies as well as in human biomonitoring studies.

The principle of the Comet assay is the migration of DNA in an agarose matrix under electrophoretic conditions. Under a microscope, a cell with damaged DNA has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands migrating in the direction of the anode. The detection of altered DNA migration is dependent on various parameters such as the concentration of agarose in the gel, the pH, temperature and duration of alkaline unwinding and the pH, temperature, voltage, amperage and duration of electrophoresis (Hartmann *et al*., 2003).

Under total alkaline conditions (alkaline unwinding and electrophoresis buffer pH>13) the method enables detection of the broadest spectrum of DNA damage. It can detect double and single-strand DNA breaks, alkali-labile sites that are expressed as single-strand breaks and single-strand breaks associated with incomplete excision repair (Hartmann *et al*., 2003). On the other hand under neutral conditions (no alkaline unwinding, electrophoresis buffer pH= neutral), the method can detect mainly double strand DNA breaks. A modification of the Neutral Comet Assay would be to unwind the DNA in an alkaline buffer (pH>13) before running electrophoresis at a neutral pH. This procedure would make it possible to detect both single and double stranded DNA breaks.

Among the many parameters of Comet features, the parameters of the tail are used as indicators of DNA damage. The tail parameters that are the most frequently used are those of the tail moment, the fraction of DNA in the tail, and the tail length (De Boeck *et al.,* 2000). The tail moment and the percentage of DNA in the tail introduced by Olive *et al*. (1990a, 1992) and Muller et al. (1994), have been used by many researchers for genotoxic studies (Anderson *et al*., 2003; Bajpayee *et al*., 2002; Garaj-Vrhovac and Zeljezic, 2002; Kim *et al*., 2002; Schabath *et al.,* 2003). In addition, a metric based on the percentage of migrated DNA, such as the tail moment, has become popular with the increased use of computerized image analysis systems used to collect Comet data (Tice *et al.,* 2000).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Isolation of mononuclear cells (MNCs) from tonsillar tissue

Tonsils, with matched blood, were obtained from a 7 year old boy undergoing routine tonsillectomy at the EENT clinic of the Komfo Anokye Teaching Hospital (KATH), with informed consent. Tonsillar tissue was placed immediately in cold buffer (1X PBS with 0.5% bovine serum albumin (BSA)). Blood was screened for HIV, Hepatitis B and malaria and was negative for all tests. Isolation of MNCs from tonsillar tissue commenced one hour after surgery. Tonsils were teased apart with forceps and scalpels in a petri dish in cold buffer and clumps were dispersed by repeated aspirations through a sterile pipette. Debris were removed by passing the cell suspension through sieve into 50 ml falcon tubes. The process was repeated until the supernatant became clear with only connective tissue left in the petri dish. The cell suspension was centrifuged for 5 minutes at 1600 rpm. The resulting supernatant was decanted off and the pellets were resuspended in buffer and gently layered on ficoll-hypague solution for density centrifugation (Bøyum, 1968). The tubes were then centrifuged at 2000 rpm for 30 minutes without brakes at 4^{o}C. The buffy coats were aspirated with a pipette into a 50 ml falcon tube. The cells were washed, counted and tested for viability by trypan blue exclusion assay. Cells were cryopreserved in 95% fatal bovine serum (FBS) with 5% DMSO at 1x10^{8} cells/ml in liquid nitrogen for future use.

3.2 Culture of 3D7 strain of *P. falciparum* and production of EBV

The 3D7 strain of *Plasmodium falciparum* obtained from the Immunology Department of the Noguchi Memorial Institute of Medical Research (NMIMR) was cultured by standard procedure, (Trager and Jensen 1976). O^+ blood was washed 3 times in wash medium made up of RPMI 1640 (Gibco) supplemented with 0.05mg/ml gentamicin (Gibco), after incubation at 4^oC overnight after which 0.2 ml of packed RBCs was added to 4mL of complete parasite culture medium (RPMI 1640, (Gibco) supplemented with 0.05mg/ml gentamycin (Gibco), 1X (2 mM) L-glutamine (Sigma) and 10% Albumax II (Invitrogen), in T25 tissue culture flasks. Cryopreserved parasites were thawed in 3.5% NaCl solution and washed with RPMI. The pelleted parasites were resuspended in 1ml of complete parasite medium and inoculated in the culture flask. The flask was then flushed with a gas mixture (2% O_2, 5.5% CO_2, 92.5% N_2) for 30 seconds at 1.5 to 2-bar pressure, closed tightly and incubated at 37°C. After 48 hours, the culture was monitored daily for parasite growth and parasitemia. The spent medium was gently removed and thin smears were made from 10 μL of packed RBCs and Giemsa-stained. Fresh, pre-warmed culture medium was added to the flask, flushed with gas as above, and returned to incubation at 37°C. The percent-parasitemia was determined daily and the culture was transferred into a T50 culture flask, to reach a parasitemia of 15%.

Monkey B95-8 cells (EBV producers), donated by Dr. Thawley-Lawson, Tufts University, were cultured in RPMI 1640 supplemented with 10% FBS, 1X (2mM) L-glutamine and 1X Penstrep. The culture was maintained for two weeks and expanded into T-50 flasks. The culture was harvested after five days when the medium had turned yellowish and quick-frozen and thawed three times to lyse the cells and release EBV.

The resulting suspension was centrifuged for 10 minutes at 2000 rpm and filtered through a 0.22 micron filter and stored at -80^{o}C for future use.

3.3 *In vitro* stimulation of tonsillar MNCs with *P. falciparum* and EBV

To investigate parasite-MNC interactions *in vitro*, tonsillar MNCs, from a patient that tested negative for HIV, Hepatitis B and malaria were rested overnight in growth medium (RPMI 1640 supplemented with 10% FBS, 1X (2mM) L-glutamine and 1X Penstrep). The rested cells were counted and 1x10^{6} cells were plated into wells in 6-well plates. The 3D7 strain of *P. falciparum*, cultured above were serially diluted with uninfected RBC to parasitemia of 5%, 2.5%, 1.25% and 0.625% and 3x10^{6} iRBCs from each dilution point was added to five separate wells containing 1x10^{6} MNCs. To find out if the parasite can interact with MNC differently under immunosuppressed conditions, the co-culture was repeated with the addition of cyclosporine A to a final concentration of 2µg/mL. Thin Geinsa-stained smears were made from these cultures at 24 hour intervals for a period of five days. The slides were examined under a light microscope for parasite-lymphocyte interactions.

In a second stimulation experiment, resting MNCs, from the same patient were challenged with *P. falciparum*-infected/uninfected RBC (iRBC/RBC) at a 5% parasitemia at a lymphocyte to iRBC ratio of 1:3. A total of 1x10^{6} resting MNCs were challenged with a total to 3x10^{6} iRBC in 6-well plates with each well having a total volume of 5 ml growth medium. Parallel stimulations of 1x10^{6} lymphocytes with 200 ul of EBV supernatant and both iRBC and EBV supernatant were also carried out. The three paradigms were repeated with cyclosporine A (final concentration of 2ug/ml). A well was harvested, at 24hr intervals for five days. The stimulated MNCs were isolated

by ficoll-Hypaque density centrifugation and counted for viability. The harvested MNCs were split into two, one portion was lysed in 800ul TRizol reagent (Gibco) and the other cryopreserved in liquid nitrogen for Comet Assay analysis. The number of cells harvested from each well and the cell viabilities are presented in figure 2.

3.4 Examination of malaria positive slides from diagnostic centers

Overall, 417 thick Geimsa-stained malaria positive slides were collected from some diagnostic centers (MediLab, KNUST hospital) around Kumasi. The smears were examined under oil immersion objective and 10X eyepiece lenses and scored as + when 1-10 parasites were counted per 100 oil-immersion thick film fields, ++ when 11-100 parasites were counted per 100 oil-immersion thick film fields, +++ when 1–10 parasites were counted per single oil-immersion thick film field and ++++ when more than 10 parasites were counted per single oil-immersion thick film field. The fields were also examined for the relative position of parasite to WBCs, whether a parasite was attached to a lymphocyte on the surface or inside a lymphocyte. A total of 80 thin Geimsa-stained smears from the *in vitro* parasite-MNC co-cultures in section 3.3 were also examined under oil-immersion for parasitemia and parasite-MNC interactions. The percent parasitemia was calculated as the ratio of infected RBCs to total number of RBC counted in all fields examined, expressed in percentages. The cell numbers of MNCs harvested at each time point and their viabilities as well as the parasitemia are represented in figure 1.

In a different experiment to probe for parasite-MNC interactions, iRBCs from an adult diagnosed for malaria were cultured in growth medium (RPMI 1640 supplemented with 10% FBS, 1X (2mM) L-glutamine and 1X Penstrep) for a week

used in a co-culture stimulation of MNCs. Thin smears were also made from this and examined for parasitemia and possible parasite-MNC interactions.

3.5 Isolation and purification of total RNA

A 200 µL aliquot of TRizol reagent was added to thawed cell lysates (from Section 3.3), in 1.5 mL Eppendorf tubes, vortexed and allowed to stand at room temperature for 5 minutes. 200 µl of chloroform was added and shook vigorously for 15 seconds followed by incubation at room temperature for 2 minutes. This was centrifuged at 11,500 rpm for 15 min at 4^{o}C. To precipitate the extracted RNA, the resulting aqueous layer containing RNA was carefully transferred into 1.5 mL Eppendorf tubes with 1 mL isopropanol and vortexed, followed by 10 minutes incubation at room temperature. The RNA was then pelleted from solution by centrifugation at 11,500 rpm for 10minutes at 4^{o}C. The supernatant was decanted and 1mL of 75% EtOH was added without shaking. 1µl of Glyco-blue (Ambion, AppliedBiosystems) was added to aid in visualization of the RNA pellet. This was then centrifuged at 9,200 for 5 minutes and the supernatant was poured off. Trace drops of the supernatant were removed with sterile pipette tip and cotton swap. RNA was allowed to air-dry for about 10 minutes and resuspended in 7 µL of nuclease-free water. The RNA was then purified using TURBO DNA-free™ DNase Treatment kit (Ambion, AppliedBiosystems), according the manufacturer's instructions. It involved addition of 2.5 µl of 10X Turbo DNAse Buffer and 1µl Turbo DNase was added to the RNA and mixed gently followed by incubation at 37^{o}C for 25 minutes. 2.5µl DNase inactivation reagent was added and incubated at room temperature for 2 minutes while mixing to disperse the inactivation reagent. The suspension was then centrifuged at 12,000 rpm for 2 minutes and the supernatant containing the purified

RNA was carefully transferred into fresh tubes. The RNA solution was topped-up to a total volume of 20 µL. 5 µL of the RNA solution was used for determination of RNA concentration and purity on the Eppendorf Biophotometer.

3.6 Quantitative Real-Time PCR

Complementary DNA (cDNA) was synthesized from the purified RNA using the iScript™ cDNA Synthesis Kit from Biorad, following the manufacturer's instructions. The cDNA Master mix per sample was prepared as shown in the table below.

Table 2: cDNA Master Mix preparation

Component	Vol .(µl)	No. of tubes tubes	Total Vol. (µl)
5X iScript Reaction mix	4	1	**4**
iScript reverse transcriptase	1	1	**1**
Nuclease free water	8	1	**8**
			Total vol. = 13

7 µl of RNA solution from samples were added to 13 µl cDNA master mix for a 20 µl reaction per sample and incubated in a thermal cycler for 5 minutes at 25°C, followed by 30 minutes at 42°C, followed by 5 minutes at 85°C and finally at 4°C for 5 minutes. The cDNA were then stored at -20°C for use in real time PCR. Quantitation of Gene Expression using relative standard curve method described by Applied Biosystems was performed on Rotor-Gene 6000 light cycler (Corbett life sciences) using the TaqMan® Gene Expression Assays for both AID and β-actin as endogenous control (catalogue numbers Hs00221068_m1 and 4319413E, respectively; AppliedBiosystems).

AID and β-actin standard curves were made using cDNA synthesized from FirstChoice® Human Burkitts Lymphoma (Raji) Total RNA (Ambion,

AppliedBiosystems; Catalog #: AM7856). cDNA from experimental samples were run in triplicates and the amount of AID and β-actin mRNA in samples were calculated from their relative standard curves. BSA was added to make up a final concentration of 0.05µg/µl per 25µl reaction. The level of expression of AID was normalized to β-actin and expressed relative to AID amount in $1x10^6$ unstimulated control cells.

3.7 β-actin normalization of AID mRNA expression

The expression of β-actin mRNA was used to normalize the expression levels of AID mRNA. Although the general trends in β-actin expression was normal, there are some instances where the amount of mRNA measured for β-actin were significantly lower than expected. Because the amount of RNA isolated from all the samples fell within a very tight range (0.05 to 0.09 µg), the abnormality in the amount of β-actin measured was interpreted as a failure of the PCR runs. Hence the average amount of β-actin in each stimulation paradigm was used to normalize the amount of AID.

3.8 Comet Assay for Estimation of DNA damage

Low Melting Agarose (Trevigen) was melted in of boiling water for 5 minutes and placed in a 37^{o}C water bath for 20 minutes to cool. Cryopreserved cells from the stimulation were thawed, washed in 1X PBS (Ca^{2+} and Mg^{2+}-free) and resuspended in 20µl of 1X PBS (Ca^{2+} and Mg^{2+}-free). The cell suspension was then mixed with 200µL of molten LM Agarose (at 37^{o}C) and 50µl was pipetted onto pre-warmed CometSlides™ (Trevigen).

The slides were then placed flat in refrigerator 30 minutes to enable the gelation of the Low melting Agarose. Slides were then immersed in pre-chilled Lysis Solution

(2.5M NaCl, 100mM EDTA (pH10), 10mM Tris Base, 1% sodium lauryl sarcosinate, 1% Triton x-100) from Trevigen and left in the refrigerator for 45 minutes. Excess buffer was drained from the slides and they were immersed in freshly prepared NaOH solution, pH>13 for 30 minutes at room temperature in the dark. This process helps unwind and expose any break in the DNA. Excess alkaline solution was drained off the slides and the slides were then immersed in 1X TBE buffer for 5 minutes, twice. The slides were then aligned equidistant from electrodes in an electrophoretic tank. 1X TBE buffer was poured into the tank to 0.5 cm above slides, and a voltage of 50V was applied across the electrodes for 10 minutes.

After electrophoresis, excess electrophoresis solution was drained off the slides after which the slides were immersed twice in dH_2O for 10 minutes, then in 70% ethanol (ethanol fixation) for 5 minutes. Slides were then dried at 45^{o}C for 15 minutes to bring all the cells in a single plane to facilitate observation. Samples were then stored at room temperature, with desiccant (silica gel) prior to scoring. 100µL of diluted SYBR® Green I (1µL of 10,000X concentrate SYBR® Green 1 in DMSO, in 10ml 1X TAE) was placed onto each circle of dried agarose and placed in refrigerator for 5 minutes. Gently, excess SYBR solution was tapped from the slides and allowed to dry completely at room temperature in the dark.

The slides were examined at 20X objective and 10X eyepiece, by epifluorescence microscopy, on a Zeiss AxioScope.A1 in the Pathology laboratory of the School of Allied Health Sciences, KBTH. Pictures of cells were taken and examined visually for DNA damage. To compensate for the relativity of visual inspection, the CASP algorithm was used to quantify the level of DNA damage in the cells after the comet

assay. Exactly 50 randomly selected non-overlapping cells were scored per sample using the CASP algorithm for quantitative analysis. The algorithm measures the amount (fluorescent intensity) of DNA in the head and tail of the comets, relative to the background intensity, and calculates the percent DNA in heads and tails of comets. The percentage of DNA in the comet tail, length of the comet tail, and comet tail moment (% of DNA in the tail × length of the tail), which are all measures of DNA migration from the cell, were used as indicators of DNA-damage.

3.9 Statistical Analysis

Student's t-test was carried out to determine the statistical difference between the mean levels of AID mRNA (means of three independent measurements), the percent of DNA in tails of comets, comet lengths and tail moments of the stimulated and unstimulated MNCs. *P*-values lower than 0.05 were considered significant.

CHAPTER FOUR

RESULTS

4.1 No internalization was detected *in vitro* and *ex vivo*

Clinical malaria smears at all degrees of parasitemia were examined for possible internalization events in non-erythrocytes. Out of the 417 slides examined, 24 (5.8%) were scored as ++++, 89 (21.3%) were +++, 176 (42.2%) were ++ and 128 (30.7%) were + (Table 3). No specific direct interaction (i.e. surface binding of parasites or iRBCs to MNCs and internalization of parasites into MNCs) was detected in all the fields examined.

Table 3: Parasitemia and Parasite-lymphocytes interactions

Parasitemia	Number of Slides	Interaction events
+	128 (30.7%)	Nil
++	176 (42.2%)	Nil
+++	89 (21.3%)	Nil
++++	24 (5.8%)	Nil

In the *in vitro* stimulation with wild *P. falciparum* isolates, there seemed to be some events of interactions between some parasites and MNCs. However because the experiment was compromised with bacterial contamination, it was unclear if these interactions were actual parasite-MNC interaction.

4.2 No internalization was detected in *in vitro* stimulated cells

Primary tonsillar mononuclear cells (MNCs) were also stimulated *in vitro* with two fold increasing parasitemia (from 0.625 to 5 %) of the 3D7 strain of *P. falciparum* with and without cyclosporine A for five days. The parasitemia, number of MNCs isolated from the co-culture and their viabilities were determined from these stimulation cultures, Geimsa-stained thin

smears were made and examined for internalized parasites. Figure 1 below summarizes the MNC numbers, viability and parasitemia observed over the five-day period.

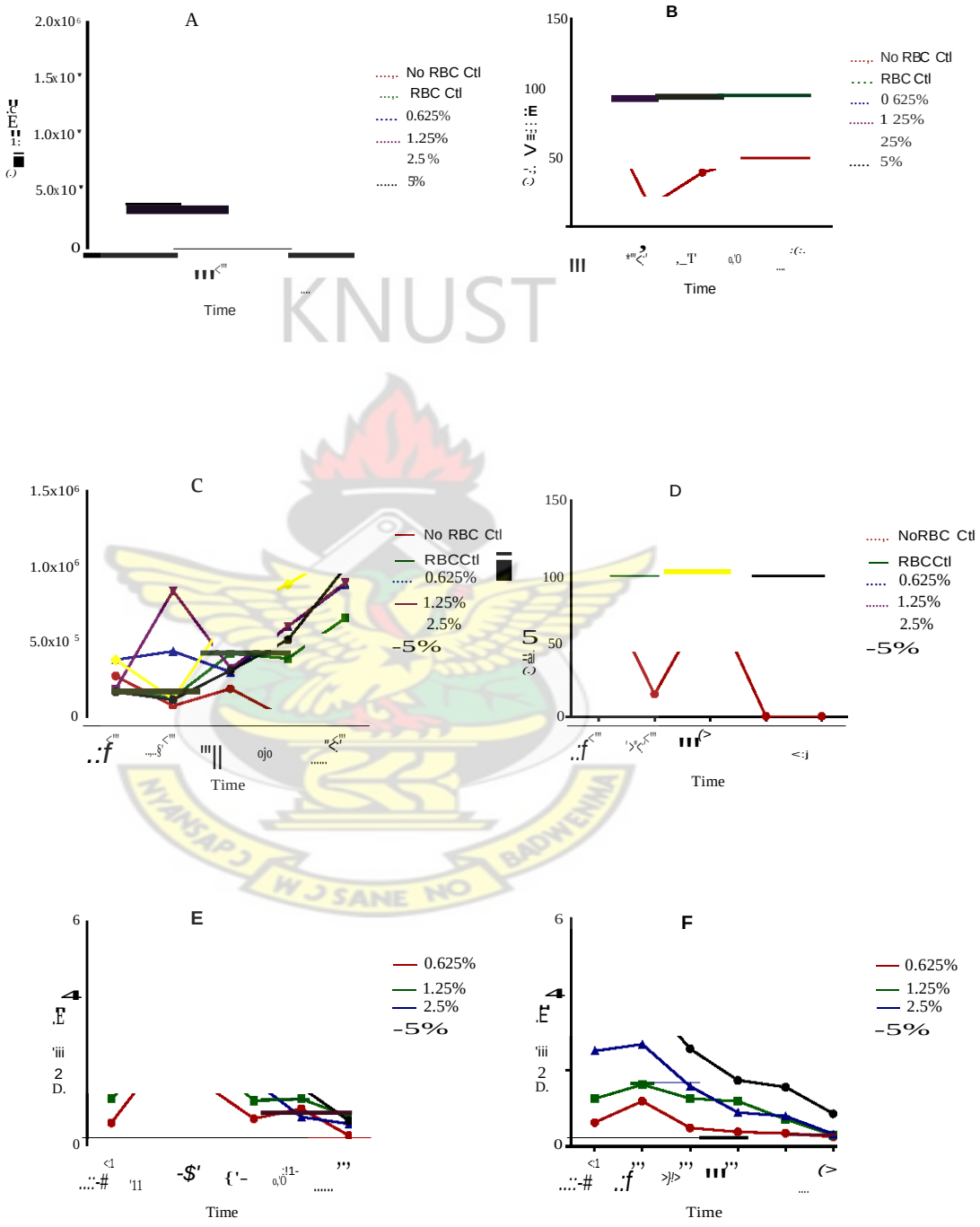

Figure 1: *Cell numbers, viabilities and parasitemia from Pf-M NC co-culture:* Number of MNCs without CyA (A) and viabilities (B). Number of MNCs with CyA (C) and viabilites (D). Percent parasitemia without CyA (E) and with CyA (F).

The parasitemia in all wells decreased from day 1 to day 5. Schizonts, trophozoits and rings were seen between days 1 and 2 and a few ring stages after 72 hours. There were no specific direct interactions observed in all the fields examined.

4.3 *P. falciparum* and EBV induced multiplication and survival of MNCs *in vitro*

In order to determine how the exposure of tonsillar MNCs to *P. falciparum* and EBV affects the expression of AID, $1x10^6$ isolated tonsillar MNCs were challenged with both pathogens, alone or together, over a five-day period. Figure 2A represents the number of cells counted from each stimulation paradigm at 24 hour intervals.

Without cyclosporine, the cell numbers decreased from $1x10^6$ to $3.5x10^5$ after 48 hours for *P. falciparum* stimulated MNCs, $3.8x10^5$ for EBV stimulated cells and $3.2x10^5$ for the dual stimulated MNCs and increased sturdily to $7.9x10^5$, $9.2x10^5$ and $8.1x10^5$ 120 hours post stimulation for *P. falciparum*, EBV and dual stimulated MNCs respectively (Figure 2A). The trend was similar in the presence of the drug with cell numbers increasing after 48 hours from $3.3x10^5$ to $8.2x10^5$ for *P. falciparum* stimulated cells, $3.6x10^5$ to $9.4x10^5$ for EBV stimulated cells and $3.5x10^5$ for the dual stimulated MNCs. On the other hand, the cell numbers from the RBC and RBC+CsA stimulation decreased sturdily over the 120 hour period, $1x10^5$ cells for RBC and $2x10^5$ cells for RBC+CsA. Over the stimulation time period, viability of MNCs stimulated with P. falciparum, EBV and both were high ranging between 85% and 99% with and without cyclosporine. The viabilities of cells from the RBC/RBC+CsA stimulation fell from 94% after 24 hours to 67% 120 hours post stimulation (Figure 2B). The percent parasitemia of all the wells decreased linearly, from the initial 5% at the start of the stimulation assay to a least of 0.6% in the Pf+CsA stimulation (Figure 2C).

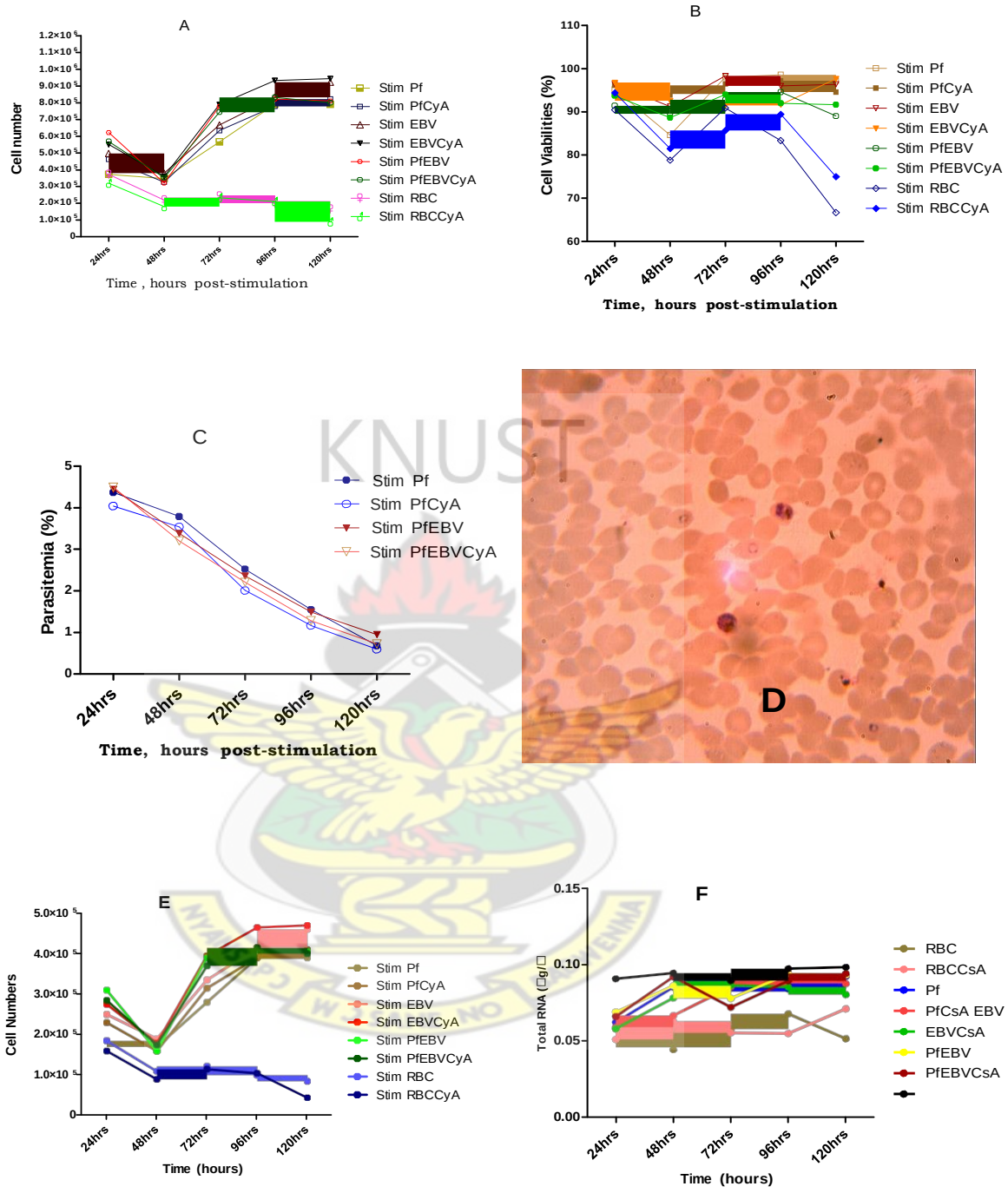

Figure 2: ***Cell counts, viabilities and trend in parasitemia over a five-day stimulation course.*** (A): Number of MNCs counted at each time point after harvest from stimulation culture, (B): Viability of MNCs harvested from stimulation assay, (C): Percent parasitemia of iRBC in each well, over a five-day time course, (D): Geimsa stained thin smear of 5% parasitemia 3D7 *P. falciparum* culture used for stimulation of MNCs. (E) Number of cells used for the PCR reaction and (F) Amount of RNA extracted from MNCs after each time point.

The initial unsynchronized culture used for the stimulation had early trophozoits (rings), trophozoits and schizont stages of the parasite as shown in the Geimsa-stained thin smear in figure 2D.

4.4 AID is down regulated in RBC stimulated relative to untreated MNCs

AID expression in MNCs challenged with uninfected RBC with and without cyclosporine was down regulated over the 120 hour stimulation period. Without cyclosporine, the AID levels in RBC stimulated MNCs decreased linearly from 0.57, 24 hours post stimulation to 0.06 after 120 hour. The level of AID mRNA was significantly lower ($p<0.05$) in RBC stimulated MNCs from 48 to 120 hours. With the drug, the AID levels were slightly higher ranging from 0.73, 24 hours post stimulation, to 0.18 after 120 hours (Figure 3A). Although there were generally low levels of AID mRNA, the means were statistically different ($p<0.05$) 48, 96 and 120 hours post stimulation. Relative to the unstimulated controls, with AID expression level of 0.61 (Figure 3C), the fold change in AID expression in RBC stimulated MNCs with and without cyclosporine showed a downward trend from 24hours to 120 hours (Figure 3B).

4.5 *P. falciparum* and EBV induces AID up-regulation in MNCs

Expression of AID mRNA in *P. falciparum* stimulated MNCs was elevated 6 fold higher than in the unstimulated controls, with and without cyclosporin, 24 to 120 hours post stimulation, with AID/β-actin ratios ranging from 3.18, 3.01, 3.22, 3.80 to 3.53 after 24, 48, 72, 96 and 120 hours respectively without cyclosporine (Figure 4A) representing fold changes of 5.22, 4.93, 5.27, 6.22 and 5.78 respectively (Figure 4C) relative to unstimulated controls. There was little variation in the expression of AID

when drug was added with AID/β-actin ratios ranging from 3.95 after 24 hours to 1.81 after 120 hours representing fold changes of 6.47 and 2.96 (Figure 4B&D) relative to unstimulated controls. The expression levels of AID mRNA in P. falciparum stimulated MNCs were significantly higher (p<0.05) than in the unstimulated MNCs with or without cyclosporine throughout the time period.

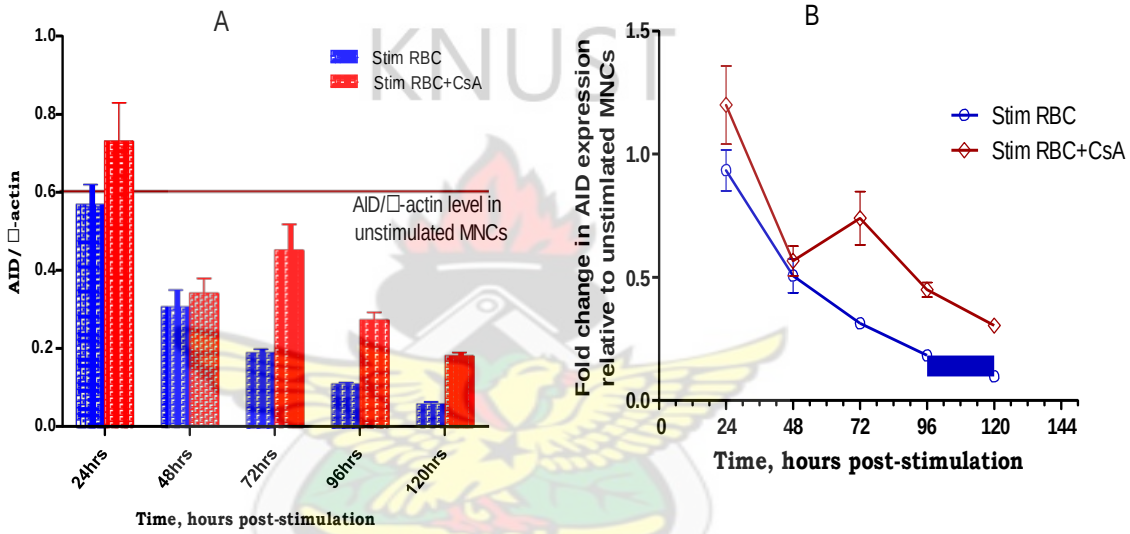

Figure 3: ***AID expression in RBC stimulated MNCs controls*****.** (A)AID/β-actin ratios in MNCs stimulated with uninfected RBC with and without cyclosporine A. (B): Fold change in AID expression in MNCs stimulated with RBC with and without cyclosporine A, relative to unstimulated controls.

Stimulation with EBV induced twice as much AID as compared to the induction caused by *P. falciparum*. The levels of AID mRNA was significantly (p<0.05) higher than in unstimulated MNCs. AID/β-actin ratios were 8.08, 4.99, 6.90, 8.03 and 5.52 (Figure 4A) in the absence of cyclosporine, representing 13.24, 8.17 11.31, 13.16 and 9.04 fold change in AID expression over unstimulated controls from 24 to 120 hours

respectively (Figure 4C). In the presence of cyclosporine, the expression of AID relative to β-actin was 7.32, 4.99, 8.41, 13.2 and 0.42 (Figure 4B) representing fold changes of 11.98, 8.85, 13.76, 21.61 and 0.69 at 24, 48, 72, 96, and 120 hours respectively (Figure 4D).

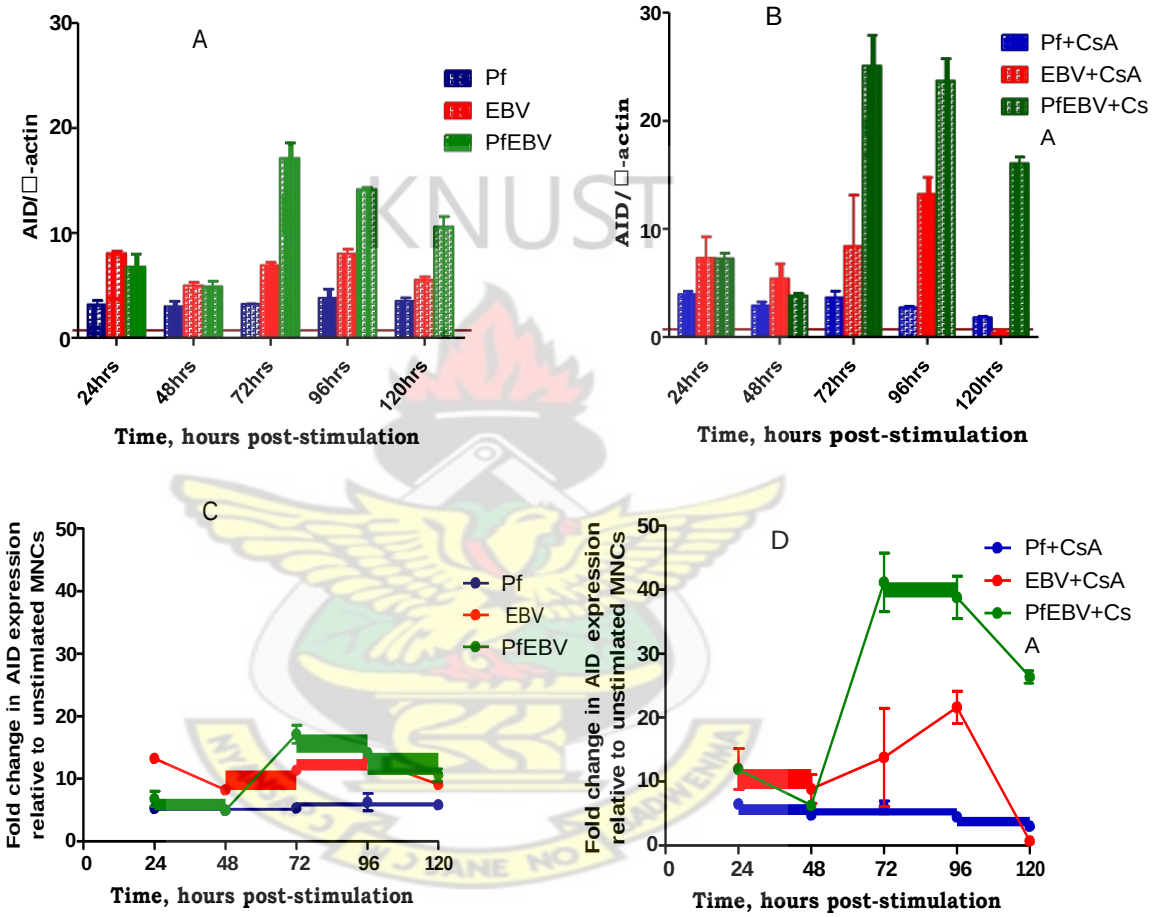

Figure 4*: P. falciparum and EBV induce AID expression:* (A): AID expression in P. falciparum (blue bars), EBV (red bars) and both *P. falciparum* and EBV (green) stimulated MNCs in the absence of cyclosporine. Line represents AID level in unstimulated controls. **(B):** AID expression in *P. falciparum* (blue bars), EBV (red bars) and both *P. falciparum* and EBV (green) stimulated MNCs in the presence of cyclosporine A. Line represents AID level in unstimulated controls. **(C):** Fold change in AID expression relative to unstimulated controls in *P. falciparum* (blue line), EBV (red line) and both *P. falciparum* and EBV (green line) stimulated MNCs in the absence of cyclosporine. (D): Fold change in AID expression relative to unstimulated controls in P. falciparum (blue line), EBV (red line) and both *P. falciparum* and EBV (green line) stimulated MNCs in the presence of cyclosporine.

Together, *P. falciparum* and EBV induced the expression of AID to a higher level than observed when both pathogens were alone and the means at the various time points were significantly higher ($p<0.05$) than that of the unstimulated MNCs. This marked increase was observed after 48 of stimulation. The normalized AID levels in the absence of cyclosporine were observed to be 6.76, 4.91, 17.1, 14.13 and 10.59 (Figure 4A) from 24 hours to 120 hours respectively representing fold changes of 11.07, 8.04, 28.00, 23.16 and 17.31 respectively (Figure 4C).

The highest expression level of AID was observed with cyclosporine, with both *P. falciparum* and EBV, especially after 48 hours. At 24 and 48 hours, the AID expression levels were 7.27 and 3.82 (Figure 4B), 11.9 and 6.26 fold higher over unstimulated controls (Figure 4D). However, at 72, 96 and 120 hours, the expression of the enzyme increased to 25.1, decreasing to 23.7 and 16.07 representing 41.17, 38.8 and 26.37 fold higher over unstimulated controls.

4.6 High AID expression was consistent with DNA damage in stimulated MNCs

The comet assay was used to estimate the level of DNA damage in MNCs after exposure to *P. falciparum* and EBV. By qualitative analysis of comets from the various treatments, there was little or no DNA damage apparent in cells from the untreated controls as well as those from the RBC stimulation. However, there was more cell death, probably via apoptosis after 120 hours of treatment with uninfected RBC with and without cyclosporin, as the DNA in most of the cells was fragmented and more spread-out with unregognisable nuclear DNA. Some level DNA damage were apparent in the *P. falciparum* stimulated cells, 24 hours post-stimulation to 120 hours with most of the comets looking like a level 1 DNA damage. There was much more cells with

damaged DNA in EBV stimulated cells with and without cyclosporin, many of the comets with level 1 and 2 DNA damage. More DNA damage were apparent in the *P. falciparum*+EBV stimulated cells as many more cells (more than oberved in *P. falciparum* and EBV stimulations), formed comets with level 2 DNA damage and few with level 1 DNA damage. Figure 5 is a representation of comet pictures obtained from MNCs after 24 hours of exposure to *P.falciparum*, EBV, both EBV and *P. falciparum*. Also shown are comets from MNCs stimulated with uninfected RBCs with and without cyclosporine and untreated MNCs after the comet assay.

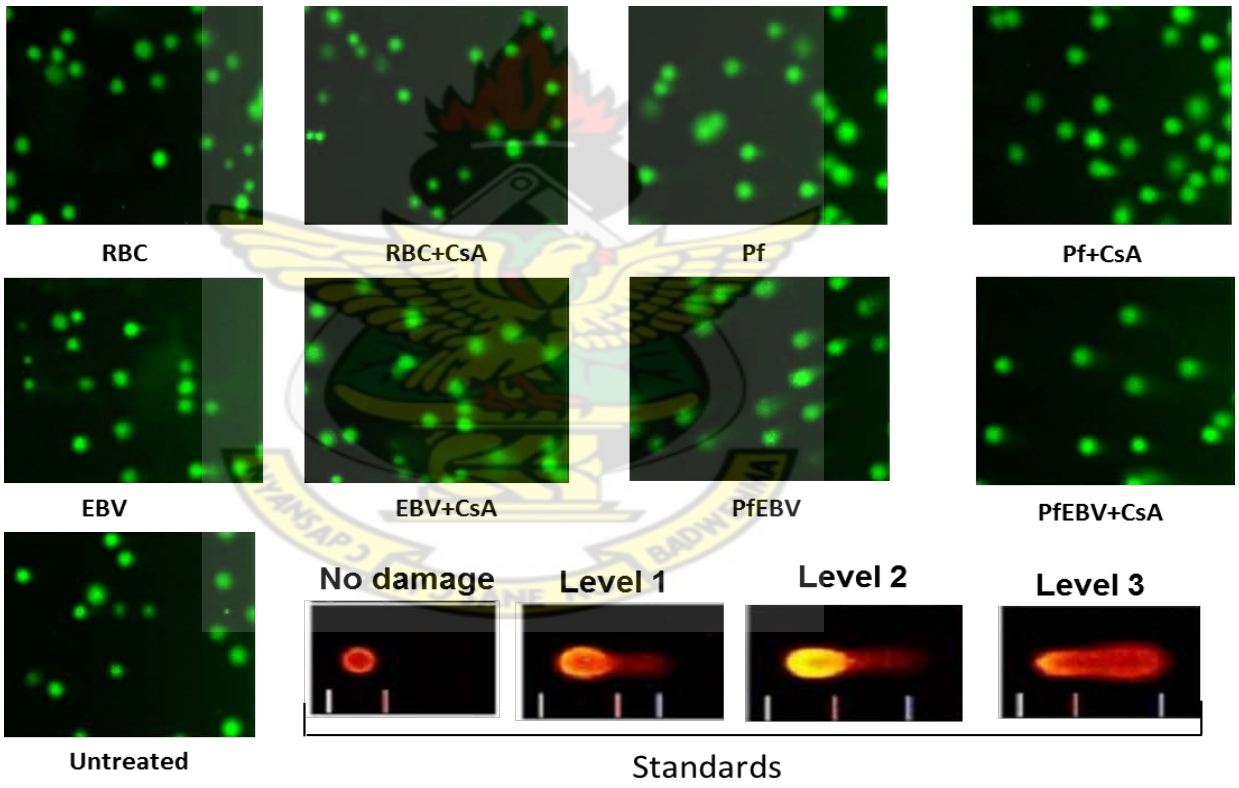

Figure 5: Dual stimulation with *P. falciparum* and EBV induce more DNA damage in MNCs: Top and middle rows: Representative comet assay images from MNCs after 24 hours exposure to purified RBC, *P. falciparum (Pf)*, EBV and both *Pf* and EBV (PfEBV), with and without cyclosporine. **Untreated:** Representative comet images from resting tonsillar MNCs before stimulation with either *Pf*, EBV or PfEBV. **Standards** are reference points for qualitative analysis.

Estimating the level of DNA damage by visual inspection of comets is subjective and not very informative. As a result the CASP algorithm, a software from the Comet Assay Project Lab. was used to quantify the percent amount of DNA in the tails of comets, the length of the comets and the tail moment, all of which are indicators of migration of damaged DNA.

The amount of DNA measured in tails of the unstimulated controls ranged from 0.11% to 11.14%, with a mean of 3.50± 2.52% (Figure 6E). The mean amount of DNA measured in tails of the RBC stimulated control MNCs over the 120 hour stimulation period, with and without cyclosporine, was comparable to DNA damage in the unstimulated controls with mean values ranging from 1.67± 0.28% 72 hours post stimulation to 4.0± 0.96%, 24 hours post stimulation, without cyclosporine and 1.16± 0.52% to 4.85±0.52% with cyclosporine (Figure 6A).

In the absence of cyclosporine, the amount of DNA damage in *P. falciparum* stimulated MNCs was slightly higher at the 48 and 72 hour time points (4.85± 0.52% and 4.09± 0.50% respectively). However at 24, 96 and 120 hours post stimulation, the values (2.26±0.44%, 1.28±0.32% and 1.16±0.52% respectively) were slightly lower than measured in unstimulated cells. With cyclosporine in the medium, *P. falciparum* stimulated MNCs had more DNA in tails of comets than observed in unstimulated controls and RBC stimulated MNCs. The mean percent of DNA in tails of comets were 5.91±0.74% 24 hours post stimulation, 2.66± 0.51% 48 hours post stimulation, 9.08±1.9% 72 hours post stimulation, 7.25± 0.82% 96 hours post stimulation and 4.17±0.59% 120 hours post stimulation (Figure 6B).

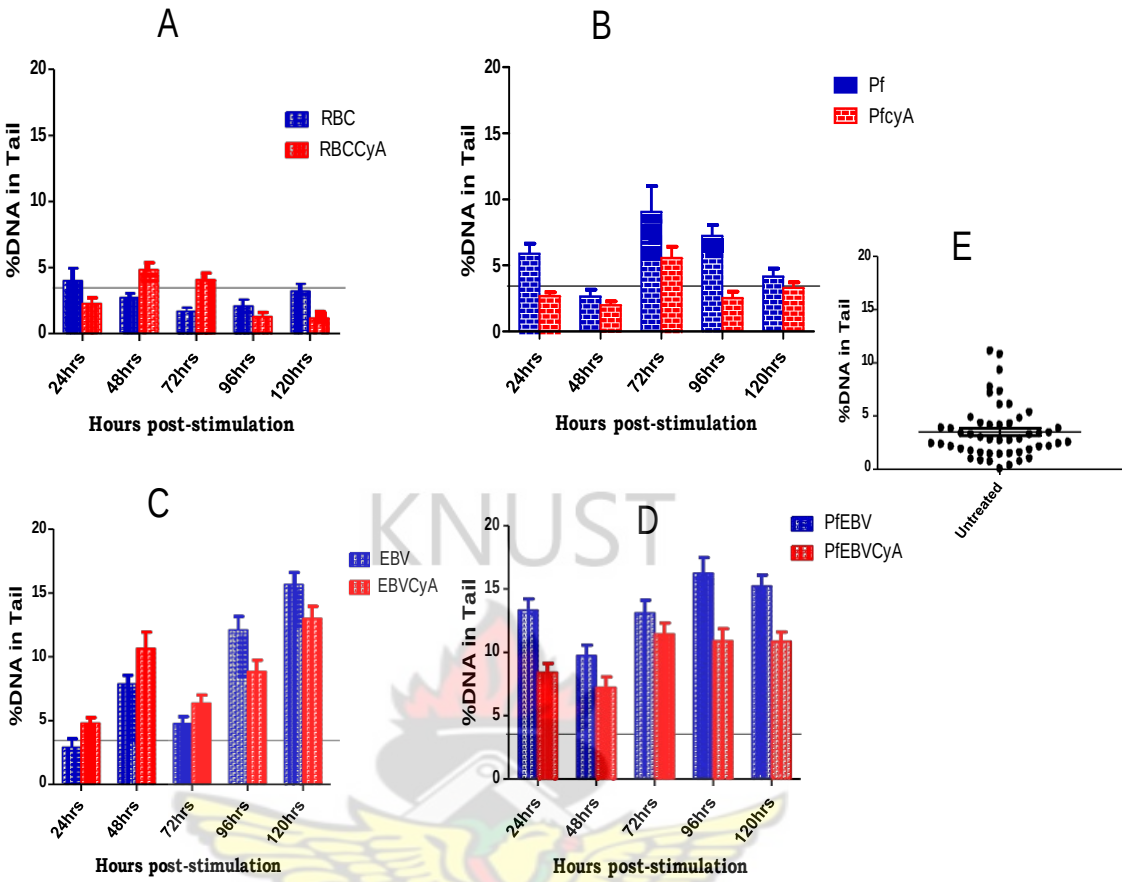

Figure 6: ***Percent DNA in tails of comets from MNCs after stimulation.*** **(A):** Mean percent DNA in tail of comets from RBC/RBCCyA stimulated MNCs. **(B):** Mean percent DNA in tails of comets from Pf/PfCyA stimulated MNCs. **(C):** Mean percent DNA in tails of comets from EBV/EBVCyA stimulated MNCs. **(D):** Mean percent DNA in tails of comets from PfEBV/PfEBVCyA stimulated cells. **(E):** Percent DNA in tails of comets from 50 randomly selected unstimulated MNCs. Line in A, B, C and D represent mean percent DNA in tail of comets from untreated MNCs

More DNA migration was observed in EBV stimulated MNCs, with and without cyclosporine, than in *P. falciparum* stimulated MNCs, RBC stimulated MNCs and unstimulated controls. Without the drug, the mean amount of DNA measured in tails of comets in the EBV stimulated cells ranged from 2.90± 0.65% after 24 hours to 7.88±0.67%, 4.76±0.54%, 12.09± 1.06% and 15.65± 0.95% after 48, 72, 96 and 120

hours respectively (Figure 6C). In the presence of cyclosporine, the trend in DNA migration in EBV stimulated MNCs was similar to that observed in the absence of the drug. The mean amount of percent DNA measured in tails of comets ranged from 4.78±0.45% after 24 hours to 10.66±1.27%, 6.36±0.65%, 8.85±0.87% and 13.00±0.94% after 48, 72, 96 and 120 hours respectively (Figure 6C).

Stimulation with both pathogens, in the presence and absence of cyclosporine, resulted in significantly higher DNA breakage. Without the drug, the mean percentage of DNA measured in comets tails ranged from a lowest of 9.75±0.82%, 48 hours post stimulation to a highest of 16.24±1.25%, 96 hours post stimulation. It is worth noting that after only 24 hours of stimulation with both pathogens, an average of 13.32± 0.90% of DNA was measured in tails of comets from MNCs (Figure 6D). In the presence of the drug, the trends in DNA migration from the nucleus of MNCs treated with both pathogens were similar to that observed when the stimulation was done without the drug; the amount of damage however was a little lower. 8.43±0.70% DNA was measured in tails of comets after 24 hours; the amount dropped slightly to 7.22±0.85% after 48 hours and plateaued at ~11% from 72 to 120 hours (Figure 6D).

Aside the amount of DNA measured in the tail of comets, the CASP algorithm also estimates the length of the tail which can also be used as a measure of DNA damage. The tail length is the distance in pixels from the edge of the head of the comet to the last detectable fluorescent signal in the tail. The unstimulated control cells had a mean tail length of 8.34±4.51. In comparison to the unstimulated controls, the MNCs challenged with only purified RBC without CsA had average tail lengths below 8.34 pixels ranging from 3.9±1.52 to 5.88±3.36 pixels, 72 and 48 hours post stimulation respectively.

Intermediate tail lengths were 5.0±3.34, 3.94±1.70 and 5.4±3.26, 24, 96 and 120 hours post stimulation respectively. With the drug, the tail lengths of MNCs were still below that measured for the untreated cells, with the highest of 7.12±4.02 after 72 hours. *P. falciparum* stimulation on the other hand induced tail lengths higher than in unstimulated controls at 24, 72 and 96 hours post stimulation (8.48±4.14, 9.36±4.28 and 10.04±5.08 respectively) in the absence of cyclosporine. However with the drug in the medium, a mean tail length higher than seen in unstimulated controls was observed only after 24 hours (9.04±4.77).

Longer tails were seen in MNCs stimulated with EBV than unstimulated controls in the presence of cyclosporine from 48 hours to 120 hours with tail lengths ranging from 10.26±5.76 at 72 hours to 14.5±3.11after 120 hours. Without the drug, the tails of EBV stimulated MNCs were higher that unstimulated controls at the 48, 96 and 120 hour time points (20.66±7.01, 11.52±4.15 and 12.94±3.70 respectively).

MNCs stimulated with both pathogens had significantly higher comet tail lengths above the values for the unstimulated controls when compared to that observed in the *P. falciparum* and EBV stimulations, at all time-points. Without cyclosporine, comet tail lengths ranged from a lowest of 14.24±4.99, 48 hours post-stimulation to a highest of 25.16±9.37, 96 hours post-stimulation. With cyclosporine, comet tail lengths ranged from a lowest of 10.86±6.00, 48 hours post-stimulation to a highest of 17.72±5.22, 120 hours post-stimulation. Table 4 summarizes the mean tail length measured of MNCs after stimulation.

Another parameter which is delivered by the CASP algorithm is the Tail moment. The Tail moment is the product of the tail length and the fraction of total DNA in the tail and it incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail). A high tail moment indicates more DNA damage.

In general the tail moment results were consistent with the results from the DNA content in tails and the tail lengths reported earlier in this section. Tail moments significantly higher than background (unstimulated controls) were obtained in the dual stimulations with and without cyclosporine throughout the stimulation period. Table 5 summarizes the tail moments measured from the MNCs after the comet assay procedure and the yellow highlighted values represent higher tail moments than observed in unstimulated controls.

Table 4: ***Length of comet tails form MNCs after stimulation with Pf, EBV and both, with and without CyA***

Time (hours) post-stimulation	Without CyA (Mean ± SD)				With CyA (Mean ± SD)			
	RBC	Pf	EBV	PfEBV	RBC	Pf	EBV	PfEBV
24	5±3.34	8.48±4.14	4.42±2.76	19.02±6.29	3.76±1.52	9.04±4.77	8.22±4.25	13.58±7.61
48	5.88±3.36	5.32±4.05	11.52±4.15	14.24±4.99	5.46±2.49	4.18±1.99	13.46±4.33	10.86±6.00
72	3.9±1.53	9.36±4.28	6.08±3.24	18.06±6.16	7.12±4.02	7.24±4.38	10.26±5.76	16.4±7.39
96	3.94±1.70	10.04±5.08	12.94±3.70	25.16±9.37	4.5±2.86	5.2±2.94	12.34±4.83	16.94±6.04
120	5.4±3.26	7.6±3.93	20.66±7.01	23.62±6.27	3.74±1.31	5.6±2.29	14.5±3.11	17.72±5.22

Untreated (time Zero) = 8.34±4.51, SD = Standard deviation. Yellow highlights= significantly high (*p*<0.05) tail moments than in unstimulated MNCs.

Table 5: ***Tail moment of MNCs after stimulation with Pf, EBV and both, with and without CyA***

Time (hours) Post-Stimulation	Without CyA (Mean ± SD)				With CyA (Mean ± SD)			
	RBC	Pf	EBV	PfEBV	RBC	Pf	EBV	PfEVC
24	0.40±1.13	0.65±0.79	0.22±0.63	2.84±2.00	0.11±0.17	0.25±0.31	0.45±0.45	1.43±1.38
48	0.23±0.29	0.27±0.50	1.05±0.95	1.60±1.49	0.33±0.33	0.11±0.15	1.04±0.95	1.01±1.11
72	0.10±0.15	0.80±0.71	0.38±0.65	2.71±2.44	0.42±0.47	0.62±1.19	0.30±0.30	2.26±2.00
96	0.14±0.43	0.97±1.19	1.76±1.50	4.65±4.20	0.10±0.26	0.19±0.33	1.75±1.51	2.14±1.92
120	0.27±0.47	0.40±0.65	3.53±2.47	3.81±2.29	0.08±0.33	0.23±0.32	3.52±2.47	2.13±1.58

Untreated (time Zero) = 0.37±0.47, SD = Standard deviation, Tail moment = Tail length x fraction of total DNA in tail. Yellow highlights = significantly high (*p*<0.05) tail moments than in unstimulated MNCs.

CHAPTER FIVE

DISCUSSION

5.1 Parasite-lymphocyte surface interactions and/or internalization

Since its discovery, the asexual life cycle of *P. falciparum* in its human host has been known to be restricted to hepatocytes in the liver and circulating erythrocytes in the blood. Although some members of the plasmodium family have the ability to invade and multiply in B lymphocytes, it is yet to be shown if *P. falciparum* has that ability. Although macrophages, monocytes and dendritic cells participate in phagocytosis of iRBCs in the spleen and liver (Groux and Gysin 1990), the documented interactions between the parasite and the B-cell compartment of the immune system are mostly indirect interactions which lead to the polyclonal activation of B-cells.

The closest interaction between B-cells and *P. falciparum* has been reported to be the binding of iRBCs by non-immune B-cells; an interaction partially mediated by cysteine rich interdomain region 1α (CIDR1α) of PfEMP1 on the iRBC membrane and Igs on the B-cell surface (Donati *et. al.*, 2004). This suggests that in the peripheral blood, iRBCs could interact with B-cells through their surface Igs (Scholander, *et al.*, 1998). In addition, Chene and colleagues have also shown that the iRBCs expressing the malaria parasite protein CIDR1α bind to the EBV-positive B-cell line Akata (Chene,*et al.*, 2007). There are several reasons one can postulate as to why these interactions were not observed in this study.

In the first instance, the MNCs used in this study were isolated from the tonsils, a majority of which are undergoing maturation for antibody production and expressing different Igs on the surface than peripheral B-cells. As a result, there was no interaction

between the iRBCs and B-cells as reported by Chene and Colleagues. Alternatively weaker or transient binding could have affected the measurement. These were however not observed at the specific times at which the cells were harvested and fixed.

In addition, the parasite is constantly avoiding detection by the host's immune system. It is in the interest of *P. falciparum*, to develop ways to inhibit and to evade immune and inflammatory responses potentially harmful to the parasite. There are different ways the pathogen employs to escape detection by the host immune system. Firstly *P. falciparum* has the ability to inhibit the antigen presentation process by inhibiting macrophage function (Nielsen, *et al.*, 1986), and DC maturation (Urban,*et al.*, 1999). Exposure of DCs to parasite-iRBC during maturation does not stimulate cell proliferation, inhibiting T-antimalarial responses. Although the specific mechanisms of modulation of host cell responses are poorly understood, it is known that the parasite hides inside host cells during part of its life cycle (hepatocytes and RBCs) avoiding direct interaction with immune cells and exhibiting molecular mimicry of certain host molecules. It is still unclear how *P. falciparum* affects exogenous antigen presentation of MHC molecules; however it has been shown that incubation of DCs with *P. yoelii*-infected erythrocytes inhibits the presentation of exogenous antigen on MHC-I but not MHC-II molecules. If this is true in the case for *P. falciparum*, it could be a mechanism of shutting down the activation of $CD8^+$ T-cells.

In addition to how rare an event of internalization of *P. falciparum* in a lymphocyte would be, the method used in this study might not have been sensitive enough to detect any such interactions. The *in vitro* stimulation conditions used in this study were not optimal for the growth and multiplication of the parasite. Although there were late

trophozoites and schizonts in the culture used for the stimulation, very few new RBCs were infected by the parasite after 48 hours as the parasitemia dropped and more parasite death were seen over the stimulation period (Figure 1). This certainly would have an impact on the ability of the parasite to invade the MNCs if they had such ability. Secondly, Geimsa stain is a nucleic acid stain that stains all nuclei purple. This would make it difficult to differentiate a parasite nucleus from that of a lymphocyte if the two are lying in close proximity, and even worse, when the parasite is actually in the lymphocyte.

The survival of the MNCs depends on stimulation from antigen, which leads to activation and proliferation. Unlike *P. falciparum* and EBV, whole RBCs do not simulate the proliferation of lymphocytes; therefore there is more cell death hence the resultant cell numbers and cell viabilities observed in the study. Under stimulation conditions, *P. falciparum* does not grow and multiply normally, as they are deprived of the required amount of gases needed for optimal growth. As a result, although there were some live parasites after the 120 hour-stimulation period, the parasitemia decreases over the time as the parasites lack the ability to invade new cells and multiply.

5.2 AID expression in *P. falciparum* and EBV stimulated MNCs

Antigen stimulation of activated B lymphocytes triggers somatic hypermutation, which diversifies the variable region of the immunoglobulin genes, and AID expression is essential for this process. The activation of B lymphocytes that leads to proliferation, affinity maturation and memory B-cell production in the germinal centers of peripheral lymphoid tissues is largely dependent stimulation by CD4 T-cells. This is because helper T-cells and the interaction between CD40 and its ligand (CD40L), induced by the

secretion of IL4 by helper T-cells are required for affinity maturation to proceed. Therefore the germinal center reaction that yields high affinity antibody secreting B-cells, via somatic hypermutation and class switch recombination, and the expression of AID in germinal center B cells, is a downstream event in the production of high affinity antibodies in response to a T-dependent antigen and the survival of these cells is dependent on a signal from an antigen. Although the MNCs were isolated from tonsils, they were rested overnight in plain medium and because resting lymphocytes need to be challenged with antigen in other to proliferate and differentiate into antibody secreting cells or memory cells, it is not surprising that AID mRNA was down-regulated in unstimulated MNCs with a mean β-actin normalized AID level of 0.6. Over time if these cells do not encounter any antigen, they begin to die off. As a result AID was down-regulated in RBC stimulated cells with or without the cyclosporine.

The ability of *P. falciparum* to suppress the immune system, inhibit presentation of its antigens and avoid detection does not prevent the activation of resting B-cells and their passage through the germinal center. The fact that exposure to the parasite does not inhibit the presentation of exogenous antigen on MHC-II molecules suggests that $CD4^+$ T-cells could still be activated to help in antibody maturation and differentiation and this is evident in the high titers of immunoglobulin that characterize *P. falciparum* infection. This could account for the 6-fold up-regulation of AID in *P. falciparum*-stimulated MNCs over unstimulated controls. It is also worth noting that the level of AID expression seen in the *P. falciparum* stimulated MNCs were higher than has been observed in normal naïve and memory B-cells from peripheral blood and tonsils in previous studies (Smith, *et al.,* 2003) and when compared to other studies, (Muramatsu

et al., 1999, McCarthy, *et al.*, 2003 and Cerutti, *et al.*, 2002), the level of AID measured in MNCs stimulated with *P. falciparum* in this study was significant and comparable to AID expression levels in germinal center sorted B-cells and Burkitt's lymphoma cells (Smith, *et al.*, 2003).

EBV infection activates B-cells to become proliferating B-cell blasts so that they can then differentiate into resting memory B-cells through the process of GC reaction (Thorley-Lawson, 2001) as part of the transcriptional activity of the virus. This process is crucial for the survival of the virus in its human host, as the virus persist in the latent stage in resting memory B-cells. The antigen-activated B blast enters the follicle of lymphoid tissues, where it expands to form a GC. Within the GC, the survival of the cell depends on its ability to receive antigenic signals presented by follicular DCs and antigen-specific T helper cells. In the case of EBV these antigenic signals are mimicked through the expression of latent viral proteins, which can replace the T-cell help and antigen signals. EBV encodes latent membrane proteins (LMP), LMP1 and LMP2, which can mimic CD40 and BCR-like signals, respectively and cooperate with BAFF/BLys and APRIL to induce T-cell independent Ig heavy chain class switching (He, *et al.*, 2003, Caldwel, *et al.*, 1998, and Kilger *et al.*, 1998).

It is therefore known that EBV up-regulates the expression of AID and this was confirmed in this study. Although the mode of mechanisms are different and unrelated, it is also apparent that EBV is a more potent inducer of AID expression than *P. falciparum* as there was twice as much AID expression in EBV stimulated MNCs as there were in those stimulated with *P. falciparum* with or without cyclosporine. These AID levels are higher than has measured in BL and Diffuse Large B-cell Lymphoma

(DLBCL) cells by Smith and colleagues (Smith *et al.,* 2003). Although there were differences between AID levels at 24 hour intervals with and without cyclosporine, taken together, there were no significant difference between AID expression with and without the drug (p = 0.9210), suggesting the little involvement of T-cells in the induction of AID expression when B cells are infected with EBV.

The imbalances in the immune responses produced by malaria infection directly impact the EBV-specific immune responses. Adults living in malaria holoendemic regions show impaired EBV-specific T-cell responses (Moss, *et al.,* 1983) and peripheral blood lymphocytes from adults and children with acute malaria are unable to control the out-growth of EBV transformed cells in colony regression assays in vitro (Whittle, *et al.,* 1990). This is thought to reflect an underlying loss of T cell-dependent IFNγ responses against EBV. As a consequence, the number of B lymphocytes latently infected with EBV increases, while the ability of T-cells to suppress the out-growth of EBV-infected lymphoblastoid cells is impaired (Moss, *et al.,* 1983, Lam *et al.,* 1991). Therefore in an *in vitro* stimulation using both EBV and *P. falciparum,* the conditions are just right for EBV to replicate and outgrow, increasing the number of dividing B-cells as a result of the reactivation of virus.

Together, antigenic stimulation for the *P. falciparum* and the uncontrolled multiplication of EBV increase the number of cells going through the germinal center reaction with a constitutive expression of AID. The amount AID message measured in cells stimulated with both pathogens were significantly higher than has been measured in all populations of B lymphocytes (naïve, GC and memory B-cells) as well as cells

from various B-cell non-Hodgkin's Lymphomas by Smith and colleagues (Smith *et al.,* 2003).

Cyclosporine A has been shown to suppress humoral and cell-mediated immune responses (Borel *et al.,* 1976a, b) and it has been suggested to specifically inhibit the production of mRNA for interleukin-2 (IL-2) in activated T cells. Imperatively it is expected that with in the presence of cyclosporine the generation of high affinity antibodies and in turn the suppression of AID expression. However, both pathogens induced the expression of the enzyme, suggesting that the expression of AID induced by exposure to both *P. falciparum* and EBV is independent of the T-cell help. The differences in the amount of AID message in MNCs from both *P. falciparum* and EBV stimulation would therefore be as a result of the mechanism of induction and the effect of the drug on the pathogen.

In the case of *P. falciparum* it has been shown that there is a direct toxic effect of cyclosporine A on the parasite (Thommen-Scott, 1981; Nickell, Scheibel & Cole 1982). This could explain in part why the amount of AID measured in the parasite-stimulated MNCs in the presence of cyclosporine was the same as measured when the drug was not in the medium. EBV on the other hand takes advantage of the suppressed humoral and cell-mediated immune responses to replicate and retain its persistence in its human host.

AID is indispensable for the diversification of immunoglobulin (Ig) genes by somatic hypermutation (SHM) through the accumulation of point mutations and class switch recombination (CSR) through double stranded DNA breaks (Delker *et al*., 2009).

The neutral comet assay only allows for detection of double stranded DNA breaks. However with alkaline unwinding/denaturation of DNA, it is possible to detect both single stranded and double stranded DNA breaks although there may be some level of renaturation if the electrophoresis time is prolonged at a neutral pH. In this study, higher tail moments, compared with unstimulated controls, were observed in MNCs stimulated with *P. falciparum* (without cyclosporine), EBV (with and without cyclosporine) and both *P. falciparum* and EBV (with and without cyclocporine). This high level of DNA damage was consistent with higher AID expression levels in MNCs after exposure to the pathogens; this goes to confirm the mutagenic potency of AID. At this stage, it can only be said that cells that expressed high amounts of the enzyme also had more DNA damage, until it is proven that the phosphorylated form of AID is actually in the nucleus of cells.

CHAPTER SIX CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

Although there are compelling data to support a possible internalization of *P. falciparum* into lymphocytes, or direct interactions between *P. falciparum* and lymphocytes, the methods used in this study did not detect any such interactions. The parasite however up-regulates the expression of AID to levels similar to levels in BL and germinal center cells. *P. falciparum* also cooperates with EBV to induce an abnormally high level of AID mRNA, higher than observed in cells from B non-Hodgkin's lymphoma tumors in other studies. Both visual and quantitative analysis of comets revealed that MNCs stimulated with *P. falciparum*, had modest levels of DNA damage with up to 10% DNA in tails of comets. However, together with EBV there was significant DNA damage with up to 16% DNA in tails of comet. The finding in this study corroborates the argument that the two pathogens, *P. falciparum* and EBV seem to cooperate in the pathogenesis of eBL. By the findings here, it can be inferred that one of the results of this cooperation is the up-regulation of AID expression resulting in DNA breaks.

6.2 Recommendations

There is the need to explore further into the types of interactions between the *P. falciparum* parasite and lymphocytes. One possible way to do this would be to differentially stain the parasite's nucleus and that of the lymphocytes. There are strains of *P. falciparum* in the Malaria Research and Reference Reagents Center (MR4) repository that express green fluorescent protein (MRA-568, MRA-569, MRA-570, MRA-576, and MRA-577). After exposure to the parasite, MNCs could be stained with

a different fluorescent dye (DAPI for example) and viewed under fluorescent microscope for MNC-parasite interactions. At this stage it would still be a challenge knowing if the parasite is just on the surface or inside the cell. Confocal microscopy with sectioning can then be used to decipher, at least in part, exactly where the parasite is.

Although measuring mature mRNA levels in cells gives a fair idea about the level of protein being expressed in a cell, it is important to assay for the amounts AID protein in the cells after exposure to the parasite by western blot. Secondly AID is activated by phosphorylation, it would be prudent to assay for the phosphorylated form of AID (pAID) in cytoplasmic and nuclear fractions of MNCs after exposure to *P. falciparum* to ascertain if the enzyme is active. When high amounts of pAID protein is measured in the nucleus of MNCs, then a conclusion of high AID expression resulting in DNA breaks can be made with more confidence.

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