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IMPACT OF MALARIA ON B-CELL HOMEOSTASIS AND EPSTEIN-BARR VIRUS REACTIVATION

Endemic Burkitt's Lymphoma Pathogenesis

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Stockholm 2009

To my Parents

ABSTRACT

Over recent years, the concept that many diseases can be aetiologically linked to infection by more than one pathogen has gained increased attention and awareness. *Plasmodium falciparum* (*P.f*) and Epstein-Barr virus (EBV) are recognized co-factors in the genesis of endemic Burkitt's lymphoma (eBL), a high grade B cell malignancy, accounting for up to 74% of childhood cancers in equatorial Africa. In this thesis, we have investigated the interactions existing between *P.f.* and EBV that could lead to the emergence of eBL. A special emphasis was given to the effect of malarial antigens on B cell homeostasis and EBV reactivation.

During intra-erythrocytic proliferation of *P.f*, parasite-derived proteins are successively expressed, exported and presented at the surface of the human red blood cell membrane. The cysteine-rich interdomain region 1 alpha (CIDR1α) of the *P.f* erythrocyte membrane protein 1 (PfEMP1) harbors a multi-adhesive phenotype able to bind to CD36, CD31 and immunoglobulins (Ig). This led us to investigate the effect of *P.f* infected erythrocyte (IE) and CIDR1α, on purified human B cell preparations. Both IE and CIDR1α bound to non-immune B cells and induced polyclonal activation accompanied by the production of cytokines and antibodies. This effect was partially mediated by B cell surface immunoglobulins (a constitutive part of the B cell receptor). Nevertheless, the different gene expression profiles obtained comparing the "activation signature" of CIDR1α and anti-Ig stimuli suggested that other molecules/signaling pathways were implicated in this activation process. CIDR1α preferentially activated the memory B cell compartment and was capable of rescuing germinal center B cells from spontaneous apoptosis, promoting cell cycle entry.

Polyclonal B cell activation is a prominent feature of malaria. We have identified the CIDR1α domain of PfEMP1 as a T cell-independent antigen that induces polyclonal B cell activation, shedding light on a possible molecular mechanism leading to hyper-gammaglobulinemia during malaria infection.

After primary infection, EBV establishes a life long persistence in the host, residing in a latent state in memory B cells. *P.f* infection is associated with elevated EBV loads. Different but not exclusive causative effects have been proposed: i) the impairment of the EBV-specific T cell response resulting from malaria-induced immunosuppression and ii) the expansion of the pool of EBV-carrying B cells resulting from malaria-induced polyclonal B cell activation. We and others have recently demonstrated that children living in malaria endemic areas have elevated EBV levels in the plasma indicating that the high EBV loads observed during malaria infection could also result from active viral replication. This led us to assess the impact of CIDR1α on EBV reactivation using the EBV positive BL cell line Akata as a model. Upon stimulation with CIDR1α, quantitative determination by real time PCR revealed an increased EBV load in the Akata cell cultures. The increased viral load resulted from lytic cycle reactivation as confirmed by experiments performed using an Akata line-based system in which the induction of lytic cycle was reflected by an enhanced expression of green fluorescent protein (GFP). CIDR1α stimulation led to an augmentation of GFP positive cells. Moreover, the virus production in CIDR1α-exposed cultures was directly proportional to the number of GFP-positive Akata cells (lytic EBV) and to increased expression of the EBV lytic promoter BZLF1. CIDR1α also induced the production of EBV in peripheral blood mononuclear cells derived from healthy donors and from children with eBL. Our results demonstrate that *P.f* antigens, such as CIDR1α, can directly drive an EBV latently infected B cell into lytic cycle.

We subsequently monitored the activity of all known herpes viruses (HHVs) infecting humans in saliva and plasma samples from children having acute malaria (day-0) and 14 days after they received anti-malaria treatment (day-14). Children with acute *P.f* malaria infection had elevated levels of circulating EBV, these levels being cleared after recovery. Acute malaria infection was not associated to an increased plasma load of HSV-1, CMV, HHV.6 or HHV-7, as compared to the control groups (malaria day-14 and malaria negative). However, we observed a profound reduction of HSV-1 levels in the saliva after anti-malarial treatment whereas the salivary loads of other HHVs, including EBV, were unchanged. Due to the low detection rates of HSV-2, VZV and HHV-8 in our study, we couldn't draw any significant conclusions on their activity during *P.f* infection. Taken together our results suggest the existence of an intimate link between malaria and EBV. The elevated EBV loads observed during malaria infection seems to result not only from an impairment of the EBV-specific T cell response and polyclonal B cell activation but also from viral reactivation directly driven by malarial antigens.

In conclusion, this thesis provides unique insights on the molecular mechanisms underlying polyclonal B cell activation and EBV reactivation during *Plasmodium falciparum* malaria infection and on how two pathogens can co-operate in lymphoma pathogenesis.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their roman numerals.

I.   Donati D, Zhang LP, **Chêne A**, Chen Q, Flick K, Nystrom M, Wahlgren M and Bejarano MT. Identification of a polyclonal B-cell activator in Plasmodium falciparum.
     Infect Immun (2004) 72: 5412-5418

II. Donati D, Mok B, **Chêne A**, Xu H, Thangarajh M, Glas R, Chen Q, Wahlgren M and Bejarano MT. Increased B cell survival and preferential activation of the memory compartment by a malaria polyclonal B cell activator.
     J Immunol (2006) 177: 3035-3044

III. **Chêne A**[*], Donati D[*], Guerreiro-Cacais AO, Levitsky V, Chen Q, Falk KI, Orem J, Kironde F, Wahlgren M and Bejarano MT. A molecular link between malaria and Epstein-Barr virus reactivation.
     PLoS Pathog (2007) 3: e80

IV. **Chêne A**, Nylen S, Bejarano MT, Wahlgren M and Falk KI. Effect of acute malaria on herpes viruses reactivation and shedding.
     Manuscript

[*] These authors contributed equally to this work

RELATED PUBLICATIONS

❖ Flick K, Ahuja S, **Chêne A**, Bejarano MT and Chen Q. Optimized expression of Plasmodium falciparum erythrocyte membrane protein 1 domains in Escherichia coli.
Malar J (2004) 3: 50

❖ Rasti N, Namusoke F, **Chêne A**, Chen Q, Staalsoe T, Klinkert MQ, Mirembe F, Kironde F and Wahlgren M. Nonimmune immunoglobulin binding and multiple adhesion characterize Plasmodium falciparum-infected erythrocytes of placental origin.
Proc Natl Acad Sci U S A (2006) 103: 13795-13800

❖ Moll K, **Chêne A**[*], Ribacke U[*], Kaneko O, Nilsson S, Winter G, Haeggstrom M, Pan W, Berzins K, Wahlgren M and Chen Q. A novel DBL-domain of the P. falciparum 332 molecule possibly involved in erythrocyte adhesion.
PLoS ONE (2007) 2: e477

[*] These authors contributed equally to this work

CONTENTS

LIST OF ABBREVIATIONS

ATS	Acidic terminal segment
BCR	B cell receptor
BL	Burkitt's lymphoma
BM	Bone marrow
CD	Cluster of differentiation
CIDR1α	Cysteine-rich interdomain regions 1 alpha
CLP	Common lymphoid progenitor
CMV	Cytomegalovirus
CRS	Class switching recombination
DBL	Duffy binding like domains
DC	Dendritic cell
EBER	Epstein-Barr encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EPO	Erythropoietin
FACS	Fluorescence-activated cell sorter
FDC	Follicular dendritic cell
GALT	Gut associated lymphoid tissue
GC	Germinal center
GFP	Green fluorescent protein
HHV	Human herpes virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
ICAM	Intercellular cell adhesion molecule
IE	Infected erythrocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LCL	Lymphoblastoid cell lines
LMP	Latent membrane protein
MZ	Marginal zone
MLP	Myeloid lymphoid progenitor
NTS	N-terminal segment
PECAM	Platelet/endothelial cell adhesion molecule
PEM	Progenitor erythroid myeloid
PfEMP1	*Plasmodium falciparum* membrane protein 1
SHM	Somatic hyper mutation
TM	Trans-membrane
TNF	Tumor necrosis factor
Ups	Upstream flanking sequence
VSA	Variable surface antigen
VZV	Varicella zoster virus

1. INTRODUCTION

Over recent years, the concept that many diseases can be aetiologically linked to infection by more than one pathogen (polymicrobial diseases) has gained increased attention and awareness. *Plasmodium falciparum* and Epstein-Barr virus (EBV) are recognized co-factors in the genesis of endemic Burkitt's lymphoma (eBL).

1.1 Historical aspects of the discovery of Burkitt's lymphoma and EBV

Denis Parsons Burkitt (1911-1993), a British surgeon, served with the Royal Army Medical Corps in Kenya and Ethiopia during World War II. After the war, he established himself in Uganda to work as a governmental surgeon at the Mulago hospital in Kampala where he remained for almost 20 years. In 1957 Burkitt examined a young boy in the paediatric ward. The boy had tumors in the head and neck, and a few weeks later he saw a girl with the same spread of cancer. The tumors proved to be very fast growing and the children died within weeks. After studying hospital records he discovered that jaw tumors were common in Uganda. Burkitt kept numerous notes and concluded that this childhood cancer had not been previously recognized. He published his observations in the British Journal of Surgery in 1958 (Burkitt, 1958). Later, Burkitt and two associates, Ted Williams and Cliff Nelson, conducted a 16.000 km research travel visiting some 60 hospitals in East and Southern Africa to assess the geographical distribution and the incidence of the disease. They found it to be correlated with the same temperature and rainfall zones as malaria. It suggested that the occurrence of the disease may be linked with the distribution of certain insect vectors. Today, Denis Burkitt's lymphoma survey is regarded as one of the pioneering studies of geographical pathology (Burkitt, 1962).

Right after conducting his survey on the incidence and distribution of the newly described tumor (BL) affecting young children in East and South Africa (Burkitt, 1958; Burkitt, 1962), Denis Burkitt's went back to Great Britain to share his observations with the medical and scientific community. In 1961, he gave a lecture at the Middlesex Hospital in London entitled "The commonest children's cancer in tropical Africa. A

hitherto unrecognized syndrome". This lecture was of a particular interest for a member of the audience; a virologist named Michael Anthony Epstein. At this time, Epstein's research interests focused mainly on the Rous sarcoma virus (Epstein, 1956). Burkitt's epidemiological findings relating the tumor distribution to climatic factors, such as temperature and rainfalls, led Epstein to hypothesize that an arthropod vector could be involved in the spreading of an oncogenic virus. He was determined to investigate the presence of viruses in such tumors. Burkitt's lymphoma (BL) cell lines were soon after established, giving Epstein an opportunity to validate his hypothesis. In 1964, he confirmed by electron microscopy the presence of virus particles in BL cell lines. As a collaborative work accomplished with two of his colleagues, Bert Achong and Yvonne Barr, he published his discovery in The Lancet the same year (Epstein et al., 1964). From his first observations, Epstein noticed that the viral particles shared similar morphological features with known herpes viruses. The identification of a new herpes virus, namely the Epstein-Barr virus (EBV), was reported in the Journal of Experimental Medicine in 1965 (Epstein et al., 1965).

1.2 Malaria

The malaria parasite is a parasitic protozoa belonging to the genus *Plasmodium* within the phylum apicomplexa. Over 100 *Plasmodia* species have been described but only 5 naturally infect humans (*Plasmodium vivax, Plasmodium malariae, Plasmodium ovale, Plasmodium knowlesi* and *Plasmodium falciparum*). Malaria causes 350-500 million clinical cases and about 1-3 million deaths each year (WHO, 2008), *Plasmodium falciparum* being the main responsible of severe clinical outcomes and fatal cases. Due to the very recent evidence that *Plasmodium knowlesi* can infect humans (Cox-Singh et al., 2008; Cox-Singh and Singh, 2008; Ng et al., 2008), its associated clinical manifestations are still not clearly defined.
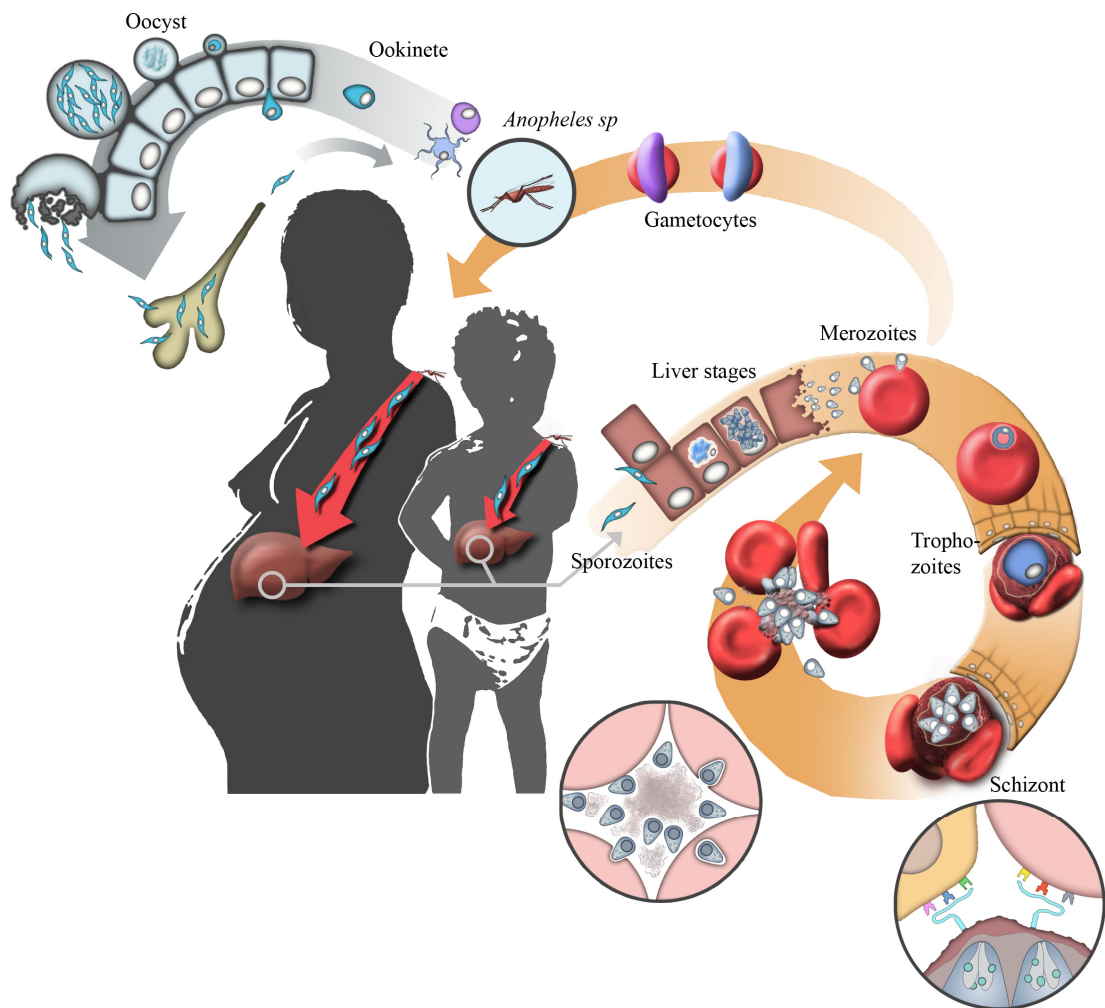
1.2.1 Life cycle

Figure 1. *Plasmodium falciparum* **life cycle**
(Courtesy of Johan Normark)

The malaria parasite has a complex life cycle (Figure 1) as it involves a number of developmental stages both in the human host and in the mosquito vector (Garnham, 1963). The parasite gets transmitted to human by female Anopheles mosquitoes. When the mosquito takes a blood meal, the infective stages of the parasite, called sporozoites, get expelled into the circulation and rapidly migrate to the liver (20-30 minutes) where they infect hepatocytes. Experiments performed in mice suggest that sporozoites need to traverse several cells to activate signaling pathways necessary for further development (Mota et al., 2002; Mota et al., 2001). Inside hepathocytes, the sporozoites start to

differentiate into merozoites, a process taking 5-15 days for *Plasmodium falciparum*. As an infected hepathocyte bursts, 10 to 30 thousands of merozoites are released into the blood stream and swiftly invade erythrocytes starting the 48 hour erythrocytic stage (asexual stage). After invading a red blood cell, the parasite matures through ring and trophozoite stages into a segmented schizont. Upon schizont rupture, 8-32 merozoites are released in the circulation initiating a new cycle. Occasionally, some merozoites undergo an alternate differential pathway and differentiate into gametocytes (male or female) that may be ingested by new feeding mosquitoes, initiating the sexual reproduction stage of the parasite life cycle. Female macrogametocytes and male microgametocytes become gametes and fuse in the midgut of the insect. The fertilization results in zygotic ookinetes that penetrate the gut wall and form oocysts in which develop numerous sporozoites by asexual replication. Upon rupture of the oocyst, sporozoites migrate to the salivary glands and become infectious.

1.2.2 Clinical manifestations

Although most individuals living in *Plasmodium falciparum* endemic areas have multiple episodes of infection, not all of these result in clinical symptoms. Only a minority will develop into severe disease, the nature of the symptoms depending very much upon the pattern and the intensity of transmission that determine the degree of protective immunity. The distribution of malaria cases due to *Plasmodium falciparum* is depicted in Figure 2.

1.2.2.1 Uncomplicated malaria

The most common clinical manifestation of uncomplicated malaria (also known as mild malaria) is a non-specific febrile illness accompanied by fever that rarely follows the classic cycling fevers with chills observed in severe cases. Other general symptoms can occur (cough, abdominal pain, vomiting headache). An accurate diagnosis of uncomplicated malaria can therefore not be based on these unspecific clinical features that could result from other types of infections, and requires additional investigations

such as blood smear examination by microscopy to assess the presence of *Plasmodium falciparum* infected erythrocytes in the circulation.

1.2.2.2 Severe malaria

Cerebral malaria

Cerebral malaria is characterized by the presence of circulating *Plasmodium falciparum* infected erythrocytes and by an unrousable coma (not attributed to other type of infections) that persists for at least 30 minutes after a general convulsion. Post mortem studies revealed an accumulation of mature infected erythrocytes in the brain capillaries associated with endothelial damage and blood barrier breakdowns (Grau et al., 2003; Newton et al., 1998). Even if most children (80-85%) recover from cerebral malaria with 3 days after appropriate treatment (Gordeuk et al., 1992; Newton and Krishna, 1998) about 10% of them will have residual neurological sequelae and permanent disabilities (Holding et al., 1999; Newton et al., 2000; Senanayake and de Silva, 1994).

Severe anemia

Severe anemia is characterized by the presence of parsitemia and a heamoglobin level lower than 5g/dl or a hematocrit level lower than 15%. Severe anemia has been proposed to result from the suppression of response to erythropoietin (EPO) (Phillips and Pasvol, 1992) accompanied by a decrease of erythropoiesis. In addition to the marrow suppression (Abdalla et al., 1980), the red blood cell count is diminished by merozoite invasion and by uptake and destruction of non-infected erythrocytes by macrophages (Evans et al., 2006; Layez et al., 2005). The severe anemia associated mortality is relatively low.

Respiratory distress

Respiratory distress is a very severe clinical manifestation of malaria. It is usually characterized by an increased respiratory rate resulting most likely from metabolic acidosis (English et al., 1996). The decreased hematocrit levels featuring malaria infection reduces the oxygen delivery capability. In addition, sequestration of infected

erythrocytes in the lung capillaries could cause endothelial damage and reduce perfusion. Respiratory distress is associated with a relatively high mortality rate.

1.2.2.3 *Pregnancy associated malaria*

Pregnancy associated malaria, also known as placental malaria, could lead to severe complications for both the mother and the child. During pregnancy, a new organ appears in the maternal body, namely the placenta, displaying new receptors for infected erythrocytes (Fried and Duffy, 1998). A massive sequestration of infected erythrocytes accompanied with infiltration of monocytes in a placenta usually results in low birth weight, maternal anemia and substantial rates of morbidity and mortality.

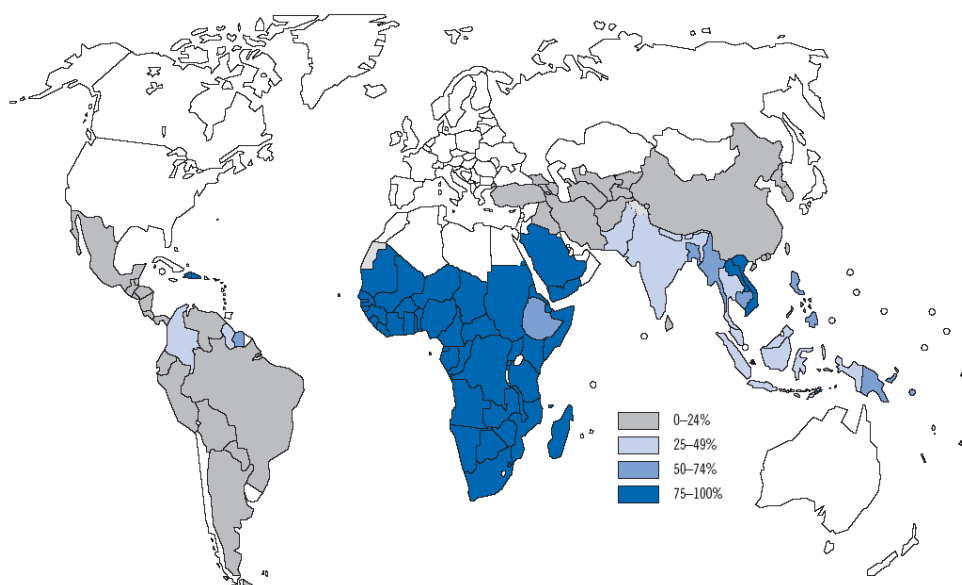**Figure 2. Estimated percentage of malaria cases due to *Plasmodium falciparum*, 2006 (World Malaria Report 2008)**

1.2.3 Pathogenesis

During intra-erythrocytic proliferation of *Plasmodium falciparum*, parasite-derived proteins are successively expressed, exported and presented at the surface of the human red blood cell membrane. By presenting ligands mediating cytoadhesion, the parasite

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also exposes an Achilles' heel, allowing host immune recognition of the altered erythrocyte surface. In order to evade the mounting immune response these polypeptides display extensive antigenic variations concurrently changing the receptor recognition and the tissue tropism of the infected cell. Thus, the properties of the exposed variant surface antigens may determine the outcome of the disease.

Sequestration

Only red blood cells infected with early stages of *Plasmodium falciparum* are detectable in the peripheral circulation. Erythrocytes infected with mature stages have the capacity to adhere to endothelial cells (cytoadhesion) and sequester in the microvasculatures avoiding clearance by the spleen. The hypoxic environment of the post capillary vessels is a favorable niche for optimal growth of the parasites. Cytoadhesion, binding of infected erythrocyte to uninfected red blood cells and/or infected erythrocytes (rosetting and auto-agglutination respectively) and the reduced deformability of infected erythrocytes could lead to complete clogging of the microvasculatures. Rosetting and cytoadhesion of parasitized red blood cells to endothelial receptors is predominantly mediated by the highly variable surface antigen *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). Sequestration most likely represents a virulence factor since it is rarely seen in other *Plasmodium* species infecting human.

1.2.4 *Plasmodium falciparum* erythrocyte membrane protein 1

PfEMP1 is a large protein (200-350 KDa) encoded by a family of approximately 60 *var* genes. Each individual parasite only expresses a single *var* gene at a time, maintaining all other members of the family in a transcriptional silent state following a very precise, but yet to be determined, counting mechanism. The same *var* gene is expressed during many parasite generations. Switching of expression to another variant molecule allows the parasite to avoid immune clearance and to prolong the period of infection.

The configuration of *var* genes is composed of two exons, a conserved intron and a 5' upstream flanking sequence (Ups). Almost all PfEMP1s share some conservation in their structure: an N terminal segment (NTS), variable numbers of Duffy Binding Like domains (DBL), one or two cysteine-rich interdomain regions (CIDR), a trans-membrane

(TM) domain, a C2 domain; and a conserved intra-cellular acidic terminal segment (ATS) (Kraemer and Smith, 2006). Even though PfEMP1s have related protein architectures, they differ extensively in their amino acid composition and binding properties. An example of PfEMP1 organization and binding phenotype (Chen et al., 2000) is displayed in Figure 3, PfEMP1$_{var1}$ from the parasite clone FCR3S1.2 being the variant referred to throughout this thesis.
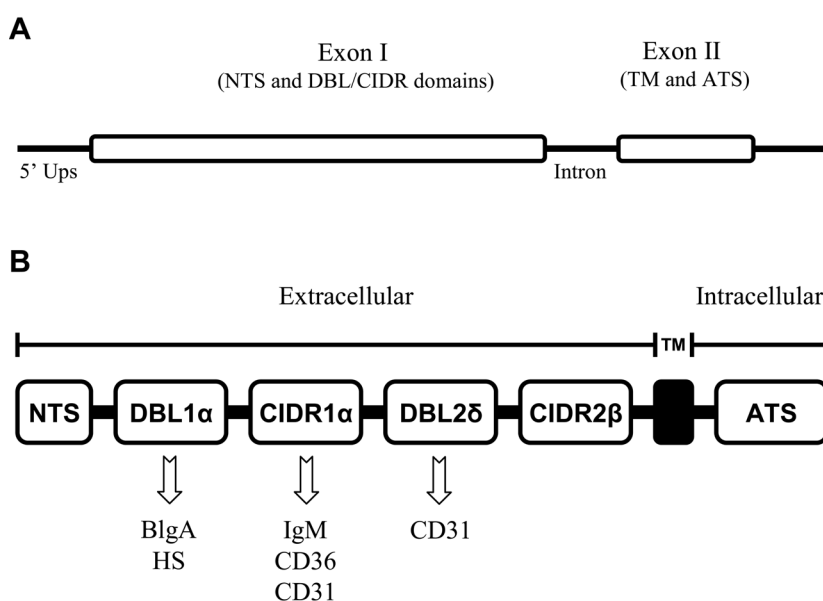

A

Exon I
(NTS and DBL/CIDR domains)

Exon II
(TM and ATS)

5' Ups

Intron

B

Extracellular

Intracellular

TM

| NTS | DBL1α | CIDR1α | DBL2δ | CIDR2β | | ATS |

BlgA
HS

IgM
CD36
CD31

CD31

Figure 3. Gene configuration (A) and protein organization/domain binding phenotypes (B) of PfEMP1$_{var1}$ (parasite clone FCR3S1.2). BlgA: Blood group A antigen, HS: Heparan sulphate

CIDR1α

The recently determined crystal structure of the CIDR1α sub-domain MC179 from the Malayan camp parasite strain (MC) gives new insights on the general structure of PfEMP1 (Klein et al., 2008). The CIDR1α sub-domain MC179 is composed of a bundle of three α-helices that are connected by a loop and three additional helices. Despite the enormous diversity of PfEMP1 sequences, the related structures of CIDR and DBLs (Singh et al., 2008; Singh et al., 2006) can predict that PfEMP1 is a polymer of three-helix bundles.

1.2.5 Immunity

Acquisition of immunity

The intensity and pattern of transmission are very important factors to consider with regards to malaria immunity. In highly endemic malaria areas, clinical symptoms related to *Plasmodium falciparum* infections become increasingly rare with age (with the exception of pregnancy-associated malaria) whereas clinical outcomes can occur throughout a life time in areas of low endemicity. The acquisition of protective immunity takes years of exposure to develop, possibly never leading to sterile immunity as suggested by the fact that parasites can still be found in the circulation of adults living in high transmission settings. Such observations are of great importance for malaria vaccine design suggesting that a strategy aiming at developing a protective vaccine against clinical outcomes may be more feasible than aiming at conferring sterile immunity.

In *Plasmodium falciparum* high endemic areas, children acquire protection from severe manifestations first followed by clinical immunity to uncomplicated cases accompanied by a progressive decreased in number of circulating parasites (McGregor, 1974). Regardless previous exposure to *Plasmodium falciparum*, pregnant women become susceptible again to clinical symptoms (placental malaria). New born babies are usually protected from clinical outcomes for a period of 5 to 9 months by immunoglobulin transfer from the mother during gestation (Campbell et al., 1980; Hviid and Staalsoe, 2004).

Short periods of interruption to exposure can lead to loss of immunity (Riley et al., 1994) implying that constant exposure may be needed for maintaining efficient protection.

Humoral immunity

The passive transfer of IgG isolated from hyper-immune donors to recipient children is conferring protection to *Plasmodium falciparum*, clearly demonstrating the existence of naturally acquired immunity and the central role played by specific antibodies in protection (Cohen et al., 1961). One of the strongest evidence of the importance of humoral immunity may reside in the fact that during subsequent pregnancies, women are gradually protected to the detrimental consequences of placental malaria by antibody

dependant immunity against these particular variants (Fried et al., 1998). Antibodies could protect against *Plasmodium falciparum* infection in different ways: i) by inhibiting merozoite invasion (Wahlin et al., 1984), ii) preventing sequestration by binding to infected erythrocyte antigens involved in adhesion/rosetting (Carlson et al., 1990; Moll et al., 2007), iii) increasing phagocytosis and complement activation by opsonisation of infected red blood cells (Celada et al., 1982; Groux and Gysin, 1990). Malaria is associated with the presence of high levels of antibodies but only a small fraction is specific to parasite antigens. Chronic *Plasmodium falciparum* infection leads to hyper-gammaglobulinemia (Abele et al., 1965) most likely as a result of polyclonal B cell activation, another prominent feature of the infection. It has been suggested that immunoglobulin binding proteins could serve as an immune evasion mechanism to divert the specific antibody response (Leonetti et al., 1999).

Cell mediated immunity

γδT cells, NK cells, and monocytes appear to play an important role in the innate immune response to malaria at an early stage of the infection. γδT cells rapidly expend and subsequently secrete pro-inflammatory cytokines (Pichyangkul et al., 1997). NK cells have the capacity to interact with *Plasmodium falciparum* infected erythrocytes and produce IFNγ (Artavanis-Tsakonas et al., 2003). Moreover, NK cells have been shown to lyse infected erythrocytes *in vitro* (Orago and Facer, 1991) even though the molecular mechanisms involved in the recognition of infected erythrocytes still remain to be determined. γδT cells and NK cells derived IFNγ induces macrophage activation consequently leading to phagocytosis of opsonized infected erythrocytes and released of nitric oxide that has anti-parasitic effects. Nevertheless, *Plasmodium falciparum* is able to modulate the immune response to insure its survival. Acute malaria provoke a profound alteration in the phenotypic profile of NK sub-populations reflected by a reduction of the $CD3^-$, $CD56^+$ NK subset and a concomitant increase of the "non functional" NK subset ($CD3^-$, $CD16^-$, $CD56^-$, $CD57^+$) (Orago and Facer, 1991). Macrophages can phagocyte infected erythrocytes in absence of opsonizing antibodies (McGilvray et al., 2000; Serghides and Kain, 2001; Smith et al., 2003) but this type of phagocytosis, possibly mediated by the binding of PfEMP1 to CD36, does not result in pro-inflammatory cytokines production. The parasitic pigment hemozoin also alters the

function of monocytes/macrophages (Schwarzer et al., 1998; Schwarzer et al., 1992) and inhibit the dendritic cell activity (Urban and Roberts, 2002). Furthermore, intact infected erythrocytes have an inhibitory effect on dendritic cell (Urban et al., 1999). It has also been proposed that PfEMP1 to CD36 could result in dendritic cell modulation (Urban et al., 2001). Antibody to CD36 and CD51 inhibit dendritic cell maturation. The modulated dendritic cells fail to recognize T cells and secrete IL-10 (and not IL-12 as mature dendritic cells usually do) driving a Th2 response. CD4+ T cells are essential for immune protection against the erythrocytic cycle of *Plasmodium falciparum* infection. CD4+ T cells isolated from naturally exposed donors respond to malaria antigens *in vitro* by proliferating and/or secreting IFNγ or IL-4 (Troye-Blomberg et al., 1994). In contrast, CD8+ T cells seem to only play a role during the liver stage of the parasite.

Cytokines

Cytokines play both a protective and a pathological role during *Plasmodium falciparum* infection. The balance between pro-inflammatory cytokines (Th1) and anti-inflammatory (Th2) is key factor in the regulation of an effective immune response to malaria. IFNγ may be beneficial for the host by promoting killing of infected erythrocytes by activated monocytes/macrophages. On the other hand, over-activation of macrophages and consequent release of IL-1, IL-6 and TNFα could lead to an inflammatory cascade detrimental for the host (Day et al., 1999). Furthermore, high levels of TNFα, levels have been reported to positively correlate with disease severity (Kern et al., 1989; Prakash et al., 2006). TNFα also augments the expression levels of adhesion molecules on endothelial cells, such as ICAM-1 (Mackay et al., 1993), increasing the ability of infected red blood cells to sequester. Anti-inflammatory cytokines can re-equilibrate the balance and prevent potential pathological effect resulting from the excessive Th1 response. Nevertheless chronic activation of CD4+ T cells by IL-10 could lead to T cell clones with low proliferative capacity and to a state of anergy (Groux et al., 1997).

1.3 Epstein-Barr Virus

Human herpes viruses (HHVs) can be divided into 3 different sub-families with regards to their genetic and biological characteristics. Viruses belonging to the α sub-family (HSV-1, HSV-2, VZV) have a neurotropism for latency whereas β (CMV, HHV-6, HHV-7) and γ (EBV, HHV-8) viruses establish latency in leukocytes and possibly other cell types which are yet to be determined (Table 1).

Virus	Site of latency
HSV-1	Neuron
HSV-2	Neuron
VZV	Neuron
EBV	Memory B cells
CMV	Monocytes, lymphocytes, ?
HHV-6	T cells, ?
HHV-7	T cells, ?
HHV-8	B cells

Table 1. Latency tropism of herpes viruses

1.3.1 Genome

The EBV genome is composed of a double stranded DNA (≈172,000 bp) encoding about 85 genes (Baer et al., 1984; de Jesus et al., 2003). Present in a linear form in the virus particles, the DNA harbors terminal repeats allowing latency establishment as an episome (circular form) in the nucleus of infected cells.

Even if minor genetic variations can often be observed comparing different genomes, EBV can be classified into two main strains based on the EBNA2 gene sequence (Kieff and Rickinson, 2001); EBV types 1 and 2 (also referred as type A and B). The important biological differences between type 1 and type 2 is the much greater ability of type 1 to convert primary B cells into lymphoblastoid cell lines (LCL) *in vitro* (Cohen et al., 1989; Rickinson et al., 1987). Interestingly, the distribution of EBV strains differs in the world population. Type 1 and type 2 are found equally represented in Africa but type 2 is much less prevalent than type 1 in Europe and in the United States. It is therefore tempting to envisage a link between the different functional EBV types and the occurrence of EBV

related cancers. Despite a few attempts (Edwards et al., 2004; van Baarle et al., 1999) no conclusive studies have been yet able to clearly establish a correlation between EBV types and EBV related diseases even though the possibility of a link remains open.

1.3.2 Infection

EBV is horizontally transmitted by the saliva and enters the epithelium in the nasopharynx. The way in which the virus penetrates the epithelium is not clearly defined. Even though epithelial cells can get infected by EBV (Lemon et al., 1977), it has been reported that the apical side (oral cavity) of the epithelium is not susceptible to the infection (Tugizov et al., 2003). EBV would then passively transit through the gaps of the epithelium. The attachment to underlying B cells is mediated by the interactions of the viral envelop glycoprotein gp350/220 and CD21 (Nemerow et al., 1987). The virus enters B cells by endocytosis (Nemerow and Cooper, 1984). The penetration process involves the fusion of the virion envelop with the B cell membrane, before or during endocytosis. The virus/cell fusion is mediated by glycoproteins; gH, gL, gB and gp42. After release into the cytoplasm EBV rapidly homes to the cell nucleus.

1.3.3 Persistence

Following primary infection, EBV establishes a life long life persistent infection within the human host characterized by frequent mild viral reactivation to allow shedding and transmission. EBV establishes latency exclusively in memory B cells (Babcock et al., 1998). In contrast to other herpes viruses (α and β sub-families) which shut down all viral genes expression after infection of their target, γ viruses (EBV and HHV-8) express a set of latent genes. EBV has 4 latency programs characterized by the differential expression of genes encoding for latent proteins or non-translated RNAs. The mechanisms underlying the infection of memory B cells by EBV are still a matter of discussion.

A model of EBV persistence proposed by Thorley-Lawson links the establishment of EBV latency to early B cell development (Thorley-Lawson and Babcock, 1999; Thorley-

Lawson and Gross, 2004) and emphasizes the intersection of EBV with the germinal center (Roughan and Thorley-Lawson, 2009).

After penetrating the nasopharyngeal epithelium, EBV infects underlying resting naïve B lymphocytes. EBV switches on the growth program (latency III) associated with full expression of latent genes (EBNAs, LMPs, EBERs, BART). Viral latent proteins are able to induce a cellular response (transition from G0 to G1 phase) driving the naïve B cell to an activated blast. The naïve blast expresses chemokine receptors and homes to the follicle. The expression of EBNA3a and EBNA3c represses the expression of EBNA2 and the growth program is turned off. The blast is now free to differentiate and the EBV switches to the default program (latency 2). LMP2 drives SHM and provides a rescue signal for the cell (Caldwell et al., 1998). LMP1 is subsequently expressed and promote CSR (He et al., 2003). LMP1 expression protects the cell from apoptosis and turns off Bcl-6 (Panagopoulos et al., 2004). The cell may exit the germinal center and enter the memory compartment. EBV switches to the latency program (latency 0) with latent gene expression restricted to EBERs. Memory cells circulate in the blood and in the secondary lymphoid organs. During homeostasis cell division, EBV expresses EBNA1 alone (latency 1). Latently infected cells can re-enter the Waldeyer's ring where they can divide upon encounter of bystander T helper cells. Some are re-introduced in the circulation and some differentiate into plasma cells, migrate to the lymphoepithelium and release virions insuring a persistent infection.

Nevertheless this model of EBV persistence has been challenged. *In vitro* EBV infection of naïve B cells leads to the lost of their functional and phenotypical germinal markers and the expression of activation markers normally found on mature B cells (Siemer et al., 2008). These findings support earlier reports showing that EBV infected cells proliferating in the germinal center during acute infection did not participate in the germinal center reaction (Kurth et al., 2003). Studies performed in severely immunodeficient patients with X-linked lymphoproliferative disease (lacking switched class memory cells) revealed that EBV can establish persistence (in non-switched memory cells $IgM^+IgD^+CD27^+$) in absence of functional germinal center activity (Chaganti et al., 2008). Furthermore, EBV is able to infect any B cell type (not only naïve B cells) *in vitro*, transforming them into proliferative blast.

1.3.4 Immunity

Primary infection by EBV can lead to infectious mononucleosis (IM). The early immunological events implicated in the response to acute infection are difficult to assess *in vivo* since the associated clinical symptoms may only appear after several weeks (Hoagland, 1964).

NK cells

NK cells play an important role in the control of primary EBV infection by eliminating infected B cells and augmenting the antigen-specific T cell response via release of cytokines (IFNα, IFNβ, IFNγ, IL-2, IL-12) (Williams et al., 2005). IFN (type I and type II) have been shown to inhibit B cell transformation (Garner et al., 1984; Kikuta et al., 1984), IFNγ exerting a 7-10-fold more potent antiviral effect than IFN type I (Lotz et al., 1985). Tonsillary NK cells are high producers of IFNγ and can delay latent EBV protein expression *in vitro* in the presence of dendritic cell produced IL-12 (Strowig et al., 2008). In addition, the EBV switch from latency to viral replication is accompanied by decreased surface expression of the major histocompatibility complex (MHC) class I and a concomitant sensitization of the infected cell to NK cell killing (Pappworth et al., 2007). Taken together these findings suggest that NK cells can limit primary EBV infection in tonsils until adaptive immunity establishes immune control.

CD8^{+} T cells

EBV infection elicits a strong cytotoxic T cell response. The primary immune response is associated to a massive expansion of activated specific CD8^{+} T cells to lytic proteins (Callan et al., 1998). CD8^{+} T cell populations committed to single viral epitopes can persist in the circulation of healthy individuals for several years and can account for up to 5% of the total CD8^{+} T cells (Tan et al., 1999). The CD8^{+} T EBV specific response to lytic antigens is preponderant during primary infection compared to the response to EBV latent epitopes. Long term asymptomatic carriers with no history of IM shed low levels of EBV in the saliva and have 1-50 latently infected cells per million of B cells (1000 fold less than individuals with acute IM) (Hislop et al., 2007).

CD4⁺ T cells

CD4$^+$ T cells are crucial for the priming and maintenance of the CD8$^+$ T cells but no massive clonal expansion of the CD4$^+$ T cells has been observed, even during acute infection (Maini et al., 2000). Nevertheless, CD4$^+$ T cells from patients with IM most commonly respond to lytic proteins (BZLF1, BMLF1) and produce IFNγ and TNFα. (Amyes et al., 2003; Precopio et al., 2003; Woodberry et al., 2005).

1.4   B lymphocytes

1.4.1 Haematopoietic development and lymphoid progenitors

Human B lymphopoiesis occurs in multiple tissues (liver, omentum) in early fetal development (Gathings et al., 1977; Solvason and Kearney, 1992) but is strictly restricted to the bone marrow (BM) after mid-gestation. BM hematopoietic stem cells (HSCs) have the capacity to develop into all lymphohematopoietic lineages, but the characterization of early lymphoid progenitor populations is rather difficult to experimentally assess in human.

In 2000, Tucker W. LeBien proposed a developmental model establishing the relationship between HSCs, common lymphoid progenitors (CLPs), and putative early B or T/NK/DC progenitors (LeBien, 2000). In this model, HSCs differentiates into myeloid/erythroid progenitors and into CLPs. Based on work showing that CLPs have the capacity to develop into T, B, NK and (DC) (Galy et al., 1995; Ishii et al., 1999; Kondo et al., 1997; Ryan et al., 1997), LeBien suggested that the CLPs could differentiate into one of the two lymphoid progenitor intermediates: early B cells or T/NK/DC tri-lineage cells and that either the CLPs or the T/NK/DC progenitors would migrate to the thymus to undergo subsequent differentiation (Spits et al., 1998).

The recent identification of new progenitor sub-populations (Adolfsson et al., 2005), namely progenitor with erythroid and myeloid potential (PEM), led to a re-definition of lymphoid progenitors. These findings add an extra level of complexity to the picture suggesting that the haematopoietic development has much more plasticity than previously envisaged.

In 2006, Rolink *et al*. proposed another model where HSCs differentiate into PEMs and into progenitors with myeloid and lymphoid potential (MLPs) (Rolink et al., 2006). Some of the MLPs would migrate to the thymus to undergo subsequent differentiation into T, NK or DC. The remaining MLPs in the BM would differentiate into CLPs mainly giving rise to B cell progenitors (pro-B cells).

1.4.2 Differentiation of B lymphocytes

1.4.2.1 In the bone marrow

B cell progenitors differentiate in the BM by undergoing a complex series of phenotypical and molecular changes.

Pro-B cells

Pro-B cells are phenotypically characterized by the surface expression of CD10, CD34 and the interleukin receptor 7-alpha (IL-7Rα). They also express the terminal deoxynucleotidyl transferase (TdT) and the recombination activating genes RAG-1 and RAG-2 (Gellert, 2002) reflecting ongoing gene rearrangements.

Early pre-B cells

Pro-B cells become early pre-B cells once the D and J gene segments are recombined to in the Ig heavy chain locus. The genes encoding for the light chains are still in germinal configuration. They down-regulate the TdT and CD34 expression.

Late pre-B cells

In late pre-B cells, VDJ-Cμ is fully rearranged and expressed. A functional VDJ rearrangement is essential for the survival of the pre-B cells that would otherwise undergo apoptosis. A subsequent rearrangement of the Ig light chain gene locus leads to surface expression of complete IgM molecules.

Immature B cells

The immature B cells express surface IgM as part of a non functional BCR. RAG-1 and RAG-2 genes are down-regulated and the surface expression of the IL-7Rα is lost.

Transitional B cells

By alternative transcriptional termination and splicing of the Ig heavy chain mRNA, transitional B cells express both surface IgM and IgD. They also acquire a CD24^{high} and CD38^{high} phenotype and are exported from the BM to the periphery heading to the peripheral lymphoid organs; spleen, mucosa-associated lymphoid tissue (MALT) and lymph-nodes (Carsetti et al., 2004).

1.4.2.2 In the secondary lymphoid organs

The spleen and the MALT present a different structural organization than the lymph nodes. They are composed of a central core namely the composite nodule that is surrounded by a marginal zone (MZ), the MZ being absent in the lymph nodes (besides for those located in the mesenterium).

Upon their arrival in the peripheral lymphoid organs, B cells have not yet encounter any antigens and are designated as naïve B cells. They are now mature to complete their differentiation into plasma cells (short or long living) or into memory B cells.

Short living plasma cells

Naïve B cells in the MZ can be activated in a T cell independent manner by bacterial polysaccharides or CpG (Capolunghi et al., 2008) and differentiate in short living plasma cells. By producing natural low affinity antibodies, they provide a rapid first line defense against fast replicating pathogens.

Long living plasma cells and memory B cells

Naïve B cells housing in the composite nodule undergo a much longer and complex differentiation process taking up to a week (compared to hours in the MZ). This will result in the production of high affinity antibodies and the establishment of immunological memory.

Within the composite nodule, a complex network of follicular dendritic cells (FDCs), T cells and B cells compose a structural sub-unit called the primary B follicle. Upon antigen encounter, antigen-specific B cells and primed T cells are recruited to the center of the follicle to initiate a germinal center (GC) reaction. The B cells actively proliferate

and undergo a series of genetic events; somatic hyper mutations (SHM) (Di Noia and Neuberger, 2007) in the Ig variable regions and class switching recombination (CSR) (Stavnezer et al., 2008).

SHM occur randomly. There is therefore a need to select B cell clones with high antigen affinity and eliminate auto-reactive clones that would be a threat for the organism. During the GC reaction, B cells physically interact with FDCs and T helper cells. B cells not binding with sufficient affinity to the antigens presented by the FDCs and/or binding inappropriately to the T helper cells are negatively selected. In absence of surviving signals from both FDCs and T helper cells, B cells die by apoptosis. Positively selected B cells undergo CSR. B cells/accessory cells interactions via CD40 seems to favor a memory phenotype, whereas the absence of CD40 ligand and the presence of IL-2 and IL-10 favor terminal plasma cell differentiation (Calame, 2001).

1.5 Burkitt's lymphoma

Burkitt's lymphoma is an aggressive B cell lymphoma that ranks among the fastest growing human tumors. The malignant cells have an extremely high proliferative index (Ki67>95%) associated with a cell doubling time of 24-26h meaning that the tumor size could almost double in one day and reach up to 10-15 cm of diameter in some advanced cases.

1.5.1 Clinical features

Endemic Burkitt's lymphoma
Endemic BL is diagnosed at an average age of 9 years and frequently involves the head and the neck (60-80% of cases) and less commonly the abdomen and the bone marrow (Tewfik et al., 1996). In the head and neck regions, patients present signs and symptoms of nasal obstruction, facial swelling, unilateral tonsillar enlargement and cervical lympadenopathy. Central nervous system involvement may occur in 30% of cases and cause paraplegia. Tumors in the abdomen involve the mesentery, retroperitoneum, omentum and possibly the gut associated lymphoid tissues (GALT). Interestingly almost

all the children under 3 years of age develop the tumor in the jaw, while at the age of 15 the tumors located in the jaw represent only 10% (Magrath, 1991; Sariban et al., 1984). The presence of small foci of osteolysis and erosion of the thin plate of bones (lamina dura) surrounding unerupted teeth is the earliest radiological feature of jaw involvement (Tosato et al., 1995). Tooth bud/lymphoid structure in the jaw and the environment surrounding the developing teeth could create a favorable context for the establishment of the tumor. EBV is associated to almost 100 % of the endemic BL tumors.

Sporadic Burkitt's lymphoma

In contrast to endemic BL, sporadic BL is associated with slightly older children (average age 12 years) and the tumors usually locates in the abdomen, often involving the terminal ileum, cecum, and/or mesentery (Levine et al., 1982). Jaw as well as other facial bone involvements have been reported but are less frequent. Only approximately one quarter of sporadic BL cases involve the head and neck (Kearns et al., 1986) most commonly in the form of cervical lymphadenopathy which is rarely found in endemic BL. EBV association with sporadic BL varies with the location worldwide. EBV is associated to sporadic BL tumors in 15-20% of the cases in the United States and in 85% of the cases in North Africa and Brazil.

AIDS-associated Burkitt's lymphoma

AIDS-associated BL mainly occurs in adults. Although accounting for less than 1% of adult non Hodgkin's Lymphoma (NHL), BL has been found in as many as 35% of HIV-associated NHL cases (Levine et al., 1982). Like sporadic BL, the AIDS-associated BL tumors often involve the gastrointestinal tract and afflict the bone marrow in 30% of cases. One recent and interesting study performed in Uganda, where BL is endemic, has compared the clinical presentation and outcome of BL in children according to their HIV status. The authors reported no significant difference in the general characteristics of children with BL, irrespective of their HIV status and noticed no difference in their response to chemotherapy treatment (Orem et al., 2009). EBV is associated to 30-40% of the AIDS-associated BL tumors.

1.5.2 Geographical distribution and incidence

Form of BL	Geographical distribution	Incidence per 100,000/y
Endemic *	Equatorial Africa and Papua New Guinea	5-10
Sporadic	Worldwide	10 to 100 fold less than e-BL
AIDS-associated †	Worldwide	10 to 100 fold more than e-BL

Table 2. Geographical distribution and incidence of BL (Adapted from Kelly and Rickinson, 2007).
* (data for age 3-12 y), † (data for untreated carriers)

1.5.3 Diagnosis

Because of the extremely fast growth of the tumor, rapid, accurate and reliable diagnostics are key factors for early intervention and therapy success. Tissue examination is standard for the diagnosis of non-Hodgkin's lymphomas (Perkins, 2000). The methods commonly employed in obtaining tissue for diagnostic purposes include excision biopsy, cytological investigation from an accessible tumor mass such as touch preparation, fine needle aspiration and cytocentrifugation of body cavity fluids (Ogawa et al., 2005; Stastny et al., 1995). Histopathologically, all forms of BL are characterized by sheets of small to medium sized monomorphic lymphoid cells with prominent basophilic cytoplasm. Nuclei are round and harbor coarse chromatin. Cells are usually interspersed with scattered benign macrophages containing cellular debris from apoptotic neoplastic cells, yielding a classic 'starry sky' appearance when examined under low power magnification (Kelly et al., 1987). Consistent with a rapid growth pattern, mitotic figures are abundant. As a monoclonal B cell lymphoma, BL tumor cells express cell surface immunoglobulins, most commonly IgM and B-cell lineage markers such as CD10, CD20 and CD22. The histological distinction between BL and diffuse large B cell lymphoma could be difficult in practice. This distinction is nevertheless crucial since these two types of lymphoma require different treatments. New advances in molecular diagnostic of BL

including gene expression profiling give promising and accurate results (Dave et al., 2006), but are for the moment very difficult to implement as routine diagnostic tools in BL endemic areas.

1.5.4 Molecular aspects: the c-myc/Ig translocations

Chromosomal translocations involving the human proto-oncogene c-myc on chromosome 8 and the Ig loci located either on chromosome 2, 14 or 22 are characteristic of BL cells and represent a molecular hallmark of the tumor. DNA coding sequences of c-myc are juxtaposed to enhancer elements of the Ig genes leading to a profound deregulation of c-myc expression resulting in an abnormal activity of the proto-oncogene. Even though the role played by c-myc in the mechanisms regulating the cell cycle is not fully characterized, its increased expression is linked to cell differentiation, proliferation and apoptosis (Boxer and Dang, 2001).

In 80% of the tumors, c-myc is translocated to the IgH locus (Ig heavy chain) on chromosome 14, this rearrangement being designated as t(8;14). In 15% of cases, c-myc translocation involves the Ig κ locus (Ig light chain) on chromosome 2 and in 5% of cases, the Ig λ locus (Ig light chain) on chromosome 22. These translocations are referred to as t(2;8) and t(8;22) respectively. The chromosomal breakpoint regions are widely dispersed within the c-myc and the Ig genes.

All forms of BL harbor one of these particular genetic rearrangements; t(8;14), t(2;8) or t(8;22). As seen above, there are distinctive clinical features separating endemic, sporadic and AIDS-associated BL. Interestingly, a correlation can also be established between the c-myc/IgH breakpoint regions and the type of tumors (Hecht and Aster, 2000).

<u>Endemic Burkitt's lymphoma</u>
The breakpoints on chromosome 8 tend to localize in a few bases upstream of c-myc exon 1. On chromosome 14, the breakpoints are often occurring in the IgH joining regions (J_H).

Sporadic and AIDS-associated Burkitt's lymphoma

The breakpoints on chromosome 8 tend to localize between exon 1 and exon 2 of c-myc. On chromosome 14, the breakpoints are often occurring within the switch regions (Sμ).

The presence of c-myc/Ig translocations has been detected in peripheral blood mononuclear cell isolated from HIV positive individuals living in the United States. The t(8;14) translocation was found in about 10% of the HIV positive patients assessed in the study. Most surprisingly, this translocation was also found in 2% of the healthy control donors. Nevertheless, no correlation could be established between the presence of t(8;14) carrying cells and the emergence of a later lymphoma (Muller et al., 1995).

2.   SCOPE OF THE THESIS

The aim of this thesis was to characterize the interactions existing between *Plasmodium falciparum,* the B cell compartment and the Epstein-Barr virus.

Specific aims

Our specific objectives were as follows

- Characterize the molecular mechanism(s) underlying polyclonal B cell activation during *Plasmodium falciparum* malaria by assessing the impact of malarial antigens (CIDR1α) on the dynamic of the B cell compartment.

- Characterize the molecular mechanism(s) underlying the high EBV loads featuring *Plasmodium falciparum* malaria by assessing the impact of malarial antigens (CIDR1α) on EBV reactivation.

- Determine the effect of malaria on the activity of herpes viruses (HHVs) by monitoring the viral loads of all known HHVs infecting humans in plasma and saliva samples from children i) with acute *Plasmodium falciparum* malaria and ii) 14 days after they received anti-malarial treatment.

In the present thesis, the methodological part has been omitted since it is described in detail in the enclosed articles.

3.   ETHICAL CONSIDERATIONS

Ethical approvals for the human components of the studies conducted in this thesis were obtained from the Research Ethical Committee of the Karolinska Institutet, Sweden and from the Research and Ethics Committee in Uganda.

4. RESULTS AND DISCUSSION

4.1 Impact of malaria on B cell homeostasis (Paper I & II)

Polyclonal B cell activation is a prominent feature of malaria infection. Chronic *Plasmodium falciparum* infection leads to hyper-gammaglobulinemia (Abele et al., 1965) and to the production of auto-antibodies (Adu et al., 1982; Guiyedi et al., 2007). The existence of malarial mitogens was suggested decades ago when experiments revealing that antibody production could be induced by stimulation of peripheral blood mononuclear cells with *Plasmodium falciparum* derived products led to the assumption that the polyclonal B cell activation occurring during malaria could result from a direct or indirect process involving T cells and accessory cells (Greenwood and Vick, 1975; Kataaha et al., 1984). Despite these observations, the direct impact of malaria on B cell homeostasis was poorly understood and could not be demonstrated.

To better understand the mechanism(s) responsible of the polyclonal activation that characterizes *Plasmodium falciparum* malaria infection, we analyzed the impact of malaria infected erythrocytes and of purified malarial antigens on B cell preparations.

During intra-erythrocytic proliferation of *Plasmodium falciparum*, parasite-derived proteins are successively expressed, exported and presented at the surface of the human red blood cell membrane. Infected erythrocytes (IE) have the ability to bind to non-immune human immunoglobulins (hIgG and hIgM) (Rasti et al., 2006; Scholander et al., 1996). The CIDR1α domain of PfEMP1-*var1* from the parasite clone FCR3S1.2 harbors a multi-adhesive phenotype able to bind to CD31 (PECAM-1), CD36 and hIgM. This was demonstrated by binding experiments in which CIDR1α was expressed either as a soluble fusion protein or at the surface of transfected COS cells (Chen et al., 2000). CD31 expression on B cells is mainly associated with early developmental stages in the secondary lymphoid organs (Jackson et al., 2000) while CD36 seems to be differentially expressed on B cell subsets during development and in response to antigens (Won et al., 2008). Surface immunoglobulins are expressed on B cells as a constitutive part of the B cell receptor (BCR).

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The CIDR1α binding properties place it as a relevant candidate to assess the impact of malarial antigens on B-cell homeostasis. We first investigated the CIDR1α binding pattern to immunoglobulins in ELISA assays. Our results showed that CIDR1α was able to bind to both non-immune hIgM and hIgG and to IgG from other animal species. The constant of association (Ka) values, reflecting the affinity of CIDR1α to hIgM and hIgG were 3.7×10^6 M^{-1} and 1.2×10^6 M^{-1} respectively. In order to identify the immunoglobulin region(s) involved in the interaction with CIDR1α, we performed similar experiments using different immunoglobulin fragments. CIDR1α bound to the Fc <u>and</u> Fab parts of both hIgM and hIgG. Consequently we have characterized the CIDR1α domain of PfEMP1 as an immunoglobulin binding protein (IBP) presenting a similar binding pattern to that of the bacterial polyclonal B cell activator *Staphylococcus aureus* protein A (Graille et al., 2000).

These results led us to investigate the direct impact of infected erythrocytes/CIDR1α on B lymphocytes. We assessed the physical interactions between infected erythrocytes/CIDR1α on freshly purified B cells from blood donors who had never been exposed to malaria. IE (parasite clone FCR3S1.2) bound to non-immune B cells. To evaluate the role played by PfEMP1 in this interaction, parallel experiments were performed using IE (parasite sister clone FCR3S1.6) expressing much lower levels of PfEMP1. The binding of IE to B cells was abolished. CIDR1α also bound to B cells. This binding could be diminished in a dose dependent manner by competition with soluble hIgM and hIgG. We concluded from these experiments that IE were able to physically interact with non-immune B cells and that the binding was predominantly mediated by the CIDR1α domain of PfEMP1 and involved B cell surface immunoglobulins.

As a next step in our studies, it became of interest to determine if the interaction of IE/CIDR1α with non immune B cells could have a stimulatory effect and trigger a cellular response. IE and CIDR1α were able to induce B cell proliferation in co-incubation assays. Pre-incubation of CIDR1α with soluble hIgM inhibited the cell proliferation in a dose dependant manner. CIDR1α stimulation also led to B cell activation as reflected by an increase in cell size, the up-regulation of HLA-DR, CD23,

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CD40, CD54, CD58, CD80, CD86 and the production of soluble IgM, IL-6 and IL-10. Taken together these results demonstrate that *Plasmodium falciparum* infected erythrocytes and more precisely the CIDR1α domain of PfEMP1 are able to interact with non-immune B cell and induce polyclonal activation.

We subsequently analyzed the impact of the newly discovered malaria B cell polyclonal activator CIDR1α on two different B cell compartments namely on the naïve (IgD$^+$, CD27$^-$) and memory B cell (CD27$^+$) populations. Upon stimulation with CIDR1α we observed an increase in the proportion of memory B cells expressing CD70 and CD95 whereas the proportion of naïve B cells expressing these same markers was unchanged. Interestingly, a recent study reported that children having acute *plasmodium falciparum* infection have an increased proportion of the circulating memory B cells subset with a concomitant decrease of the naïve B cell subset compared to the same children 4 weeks post-recovery (Asito et al., 2008).

Analysis of the expression levels of a broad range of activation markers revealed that memory B cells up-regulated CD70, CD95, CD69 and the co-stimulatory molecule CD86 when co-incubated with CIDR1α. In the same culture conditions, the expression levels of these molecules on naïve B cells remained unchanged. Thus, the CIDR1α domain of PfEMP1 preferentially activates the memory B cell compartment**.**

Following our demonstration that a malarial antigen, such as CIDR1α, could have a direct impact on B cell homeostasis we turned our attention on its potential effect on the dynamic of germinal center (GC) B cells. As a secondary lymphoid tissue, tonsils are rich in GC B cells highly prone to undergo spontaneous apoptosis. Tonsillary B cells were stimulated with CIDR1α for a 24h period. Cell cycle analysis revealed that the proportion of cells undergoing apoptosis (<G0/G1) was diminished upon stimulation with CIDR1α whereas the proportion of cycling cells (S+G2/M) was increased. These results showed that the CIDR1α domain of PfEMP1 is able to rescue B cells from spontaneous apoptosis and promotes cell cycle entry.

The B cell receptor is a complex of membrane immunoglobulins (heavy and light chains) associated with Igα/Igβ heterodimers (CD79a/CD79b) (Reth, 1989). The latter molecules function as signaling sub-units and couple the receptor to intracellular signal transducer elements. The oligomerization of the BCR triggers signal cascades that lead to a cellular response. T cell-independent B cell activation can be initiated by binding of multivalent antigens to the BCR. Soluble CIDR1α is able to activate B cells and induce proliferation in a process partially mediated by surface immunoglobulins. The fact that the binding of CIDR1α to B cells is not fully competed out by soluble immunoglobulins suggests that another (other) molecule(s) could participate in this interaction. This assumption is strengthened by the different gene expression profiles observed when comparing the "activation signature" of CIDR1α and anti-human immunoglobulins stimuli. CD31 has been associated with a naïve B cell phenotype, its expression being lost as the B cells differentiate into memory cells (Jackson et al., 2000). CD31 was shown to act as a co-receptor that serves to negatively regulate the BCR signaling (Henshall et al., 2001). Since CIDR1α is able to bind CD31, it is reasonable to hypothesize that the preferential activation of the memory compartment that we see in our study results from the fact that CIDR1α engaged both the BCR and CD31 on naïve B cells acting anti-synergetically on the cellular response. It could be argued that the modest proliferative response that we observed in our experiment (2.5 fold compared to the control) might not result in polyclonal activation *in vivo*.

In vivo, CIDR1α is expressed at the surface of infected erythrocytes as a constitutive part of PfEMP1. In the case of membrane bound antigens, we couldn't over-emphasize the importance of antigen affinity, avidity and density in relation to the outcome of B cell activation. The threshold of B cell activation by membrane bound antigens in absence of co-stimulation has recently been reported to have a Ka around 1×10^6 M^{-1} (Fleire et al., 2006). According to our results, the affinity of CIDR1α to immunoglobulins (IgM and IgG) classifies it as a low affinity antigen. Nevertheless, in T cell-independent immune responses, large differences in affinity produce only small variation in the intrinsic ability of B cells to mount a protective response to antigens (Shih et al., 2002).

PfEMP1 is most often anchored at the surface of infected erythrocytes in electron dense structures called knobs. It appears as most *Plasmodium falciparum* clinical isolates

display these structures (Kidgell et al., 2006; Ribacke et al., 2007). *In vitro* adapted parasites, such as FCR3S1.2, tend to lose the "knobby" phenotype by deletion of genes located on the left arm of chromosome 2 (Ribacke et al., 2007). Even though FCR3S1.2 has a highly adhesive phenotype, it has been suggested that the biological function of knobs is to augment PfEMP1 adhesion under flow by locally increasing the surface protein density (Cooke et al., 2002; Crabb et al., 1997). *In vivo*, B cell interactions with "knobby" infected erythrocytes could then create a micro-environment where high amounts of PfEMP1 (CIDR1α) are in close proximity of BCR clusters (Depoil et al., 2008). Even though *Plasmodium falciparum* infected erythrocytes and B cells could physically interact in the circulation, it is more likely that the polyclonal activation featuring malaria infection takes place in other compartments. Mature stages of infected erythrocytes sequester in the vasculature to avoid clearance by the spleen. Nevertheless, under the stringent blood flow conditions, a large amount of parasites fail to adhere to the vascular endothelium and end up trapped in the spleen where B lymphocytes can represent up to 50% of the splenocytes. This environment could favor the interactions between B cells and infected erythrocytes.

Importantly, it should be noted that the B cell activation and proliferation observed in our study occurred in absence of accessory cells and cytokines. *In vivo*, the CIDR1α induced polyclonal activation may be enhanced by a multitude of other co-factors. The splenic enlargement featuring malaria infection partially results from a massive expansion of phagocytic cells. Phagocytosis of infected erythrocytes might result in presentation of antigens in soluble forms or as immunocomplexes by follicular dendritic cells or dendritic cells in the presence of cytokines, T helper cells and co-stimulatory signals.

Immunoglobulin binding proteins have been suggested to play an important role during infections by diverting the specific antibody response (Daniel-Ribeiro et al., 1989; Leonetti et al., 1999). We have identified the CIDR1α domain of PfEMP1 as an IBP able to induce polyclonal B cell activation shedding light on a possible molecular mechanism leading to hyper-gammaglobulinemia during malaria infection and to the deflected specific immune response to relevant malarial antigens for protection.

4.2 Impact of malaria on Epstein-Barr virus reactivation (Paper III & IV)

EBV latency is restricted to a specific long-lived B cell compartment, namely the resting memory B cells. In immunocompetent individuals, mild reactivation may occur without concomitant symptoms, when memory cells differentiate into plasma cells upon antigen stimulation (Laichalk and Thorley-Lawson, 2005). This process, allowing viral shedding and transmission, reflects a delicate and fine-tuned balance between viral reactivation and host immune responses. Malaria infection is associated with elevated EBV loads (Donati et al., 2006; Njie et al., 2009; Rasti et al., 2005; Yone et al., 2006) and different but not exclusive causative effects have been proposed. An impaired EBV-specific T cell response and/or the expansion of the pool of EBV-carrying B cells due to polyclonal activation would result in an increased number of latently infected cells. We and others have shown that children living in malaria endemic areas have elevated EBV loads in the plasma (Rasti et al., 2005), and that acute malaria infection leads to increased levels of circulating EBV, levels that are cleared following anti-malaria treatment (Donati et al., 2006). These findings indicate that the augmented EBV load during malaria could also result from active viral replication.

In this study, we aimed at characterizing the molecular mechanism(s) underlying EBV reactivation in *Plasmodium falciparum* malaria.

We have previously demonstrated (Papers I and II) that direct interactions between B lymphocytes and malarial antigens such as CIDR1α could lead to polyclonal B cell activation. CIDR1α was also shown to preferentially activate the memory B cell compartment where EBV may reside in a latent state (Babcock et al., 1998). The aforementioned characteristics of CIDR1α and *Plasmodium falciparum* infected erythrocytes led us to analyze the potential impact of CIDR1α on EBV reactivation. The B cell activation process described in paper I and paper II was partially mediated by surface immunoglobulins. Our assumption was that IBPs (CIDR1α) may trigger viral reactivation in a similar way as anti-human immunoglobulin induces viral production in EBV carrying cell lines (Takada, 1984). The BL cell line Akata has been studied and

described intensively in the literature providing us with a suitable tool/model to test our hypothesis.

We first wanted to confirm that, such as for freshly isolated B lymphocytes, infected erythrocytes and CIDR1α were able to bind to Akata cells. We assessed these interactions by live microscopy and flow cytometry (FACS). Both *Plasmodium falciparum* infected erythrocytes (clone FCR3S1.2) and CIDR1α bound to the EBV carrying cell line.

We subsequently assessed the impact of CIDR1α on EBV production. Upon stimulation with CIDR1α, quantitative EBV determination by real time PCR revealed an increase viral load in the cell cultures. Since we previously showed that CIDR1α was able to protect B cells from apoptosis, this observation could result from a net increase in number of cells carrying EBV. To rule out this possibility we performed cell cycle analysis of CIDR1α stimulated Akata. The proportion of cells in <G0/G1 (necrotic and apoptotic) and S+G2/M (cycling) phases was unchanged compare the controls (unpublished data). Additional experiments co-incubating Akata cells with CIDR1α and the pan caspase inhibitor Zvad (inhibition of apoptotic pathways) led to a similar EBV production than in cell cultures stimulated with CIDR1α alone. We concluded that CIDR1α was able to induce EBV production in the EBV carrying cell line Akata.

To verify that the observed increase in viral load was resulting from lytic cycle reactivation, we used an Akata line-based system in which induction of lytic cycle was reflected by an enhanced expression of green fluorescent protein (GFP). CIDR1α stimulation led to an augmentation of GFP positive cells compared to the controls. A correlation could be established between the proportion of cells undergoing lytic replication and the EBV load monitored in the same cell cultures. Taken together our results show that the CIDR1α domain of PfEMP1 induces EBV reactivation in the model cell line Akata.

We also assessed the impact of CIDR1α on EBV reactivation using freshly isolated leukocytes. CIDR1α stimulation increased the viral load in cultures containing B cells derived from EBV positive healthy individuals and children having Burkitt's lymphoma.

It has been suggested that the augmented EBV load and the increased number of circulating latently cells observed during malaria does not result from viral replication but mainly from an impaired EBV specific-T cell response and/or polyclonal activation (Njie et al., 2009). Nevertheless there is substantial evidence that EBV reactivation often occurs in children living in malaria holoendemic areas. High antibody titers to EBV structural antigens (VSA) in Ugandan children have been associated with a high risk of developing Burkitt's lymphoma (de-The et al., 1978). Moreover, children with acute malaria have also been shown to have higher antibody titers to BZLF1 (that acts as a transcription activator for EBV lytic genes expression) as compared to the same children post recovery.

Our studies demonstrate for the first time that malarial antigens such as the CIDR1α domain of PfEMP1 are able to directly drive a latently infected B cell into EBV replication. As for the induced B cell activation (papers I and II) we propose that the molecular mechanisms underlying the EBV switch from latency to lytic cycle result partially from CIDR1α interactions with surface immunoglobulins, and possibly other molecules, mimicking the effect achieved by BCR cross-linking with anti-human immunoglobulins. The outcomes of CIDR1α stimulation on EBV reactivation described in our study were modest but consistent. *In vivo,* the main interactions between B lymphocytes and infected erythrocytes/malarial antigens most likely occur in the secondary lymphoid tissues such as the spleen where memory B cells, potentially latently infected by EBV, could return to undergo additional rounds of maturation (Bende et al., 2007). Furthermore it should be noted that malaria infection is associated with a great disruption of the secondary lymphoid organs. Such an environment would be favorable for malarial antigens to trigger viral reactivation in EBV-carrying lymphocytes consequently leading to production of virions able to infect neighboring target cells. These facts could augment the pool of EBV latently infected cells.

We subsequently monitored the activity of all known herpes viruses infecting humans in samples from children having acute malaria (day-0) and 14 days after they received anti-malaria treatment (day-14).

Detection of HHVs in the plasma of healthy individuals is rare (Hara et al., 2002) but is frequently observed in immunocompromised individuals (Spector et al., 1992; Tanaka et al., 2000). It has been reported that EBV levels are increased in the saliva of HIV positive individuals compared to healthy HIV negative controls (Miller et al., 2006). In our study, children with acute *Plasmodium falciparum* malaria had high circulating EBV levels, these levels being cleared from the plasma after recovery, as previously published (Paper IV; (Donati et al., 2006)). Acute malaria infection was not associated to higher plasma loads of other HHVs as compared to the control groups (malaria day-14 and malaria negative). However, we observed a profound reduction of HSV-1 levels in the saliva after anti-malarial treatment whereas the salivary loads of other HHVs, including EBV, were unchanged.

Taken together these results suggest the existence of an intimate link between malaria and EBV. The augmented EBV load observed during acute malaria infection seems to result not only from an impairment of the EBV-specific T cell response and polyclonal B cell activation but also from viral reactivation directly driven by malarial antigens.

5. CONCLUDING REMARKS

Summary

We have identified the CIDR1α domain of PfEMP1 as an immunoglobulin binding protein able to induce polyclonal B cell activation. Our results shed light on a possible molecular mechanism leading to hyper-gammaglobulinemia during malaria infection.

We have characterized the CIDR1α domain of PfEMP1 as the first microbial-derived polypeptide able to directly drive an EBV latently infected B cell into lytic replication. Our results suggest that the augmented EBV load observed during malaria infection results not only from an impairment of the EBV-specific T cell response and polyclonal B cell activation but also from viral reactivation directly driven by malarial antigens.

Taken together the results of this thesis indicate that EBV and *Plasmodium falciparum* efficiently exploit the immune system by subverting the homeostatic control of B cell proliferation, apoptosis and differentiation, thus favoring EBV reactivation.

Endemic Burkitt's lymphoma pathogenesis

The hallmark of all BL tumors is the translocation of the c-myc oncogene to one of the Ig heavy or light chain loci. C-myc plays a central role in the cell cycle, regulating proliferation, differentiation and apoptosis (Boxer and Dang, 2001). As a result of the translocation, c-myc is constitutively expressed. Over-expression of c-myc in normal cells results in an increased sensitivity to apoptosis. This implies that a key molecular event in BL pathogenesis is the activation of c-myc growth promoting activities and the inhibition of c-myc-induced apoptosis. The finding that healthy individuals carry the translocation without apparent risk of developing lymphomas (Muller et al., 1995) speaks for the fact that the c-myc/Ig chromosomal rearrangement alone does not appear to be sufficient to give rise to a malignancy. This indicates that selection and/or acquisition of multiple genetic lesions might be necessary to allow a c-myc/Ig translocated cell to

survive and to eventually become immortalized. In the context of lymphoma pathogenesis, we could envisage that:

i)   The life span of translocated cells must be prolonged
ii)   The translocated cells must be genetically instable (net effect of myc over-expression) and actively proliferate since genetic lesions are more likely to occur during mitosis

Based on the results presented in this study, it is tempting to propose a model to explain how malaria contributes to an increased risk of eBL (Figure 4).

During malaria infection, the extensive trapping and accumulation in the secondary lymphoid organs, such as the spleen, of malarial antigens (some with mitogenic properties **(Paper I)),** could promote the hyper-activation of the germinal centers. The resulting augmented rate of somatic recombination could lead to an increased occurrence of c-myc/Ig chromosomal rearrangements.

The life span of B cells carrying translocations could be prolonged by malarial antigens via induced protection from apoptosis (**Paper II**) and by an increased frequency of B cell infection by EBV resulting from viral reactivation **(Paper III)**. In addition, the life span of these cells could be augmented in the circulation due to a favorable cytokine environment (IL-10, IL-4, IL-6).

CIDR1α induced proliferation (**Paper I**) could also increase the pool of B cells carrying translocations and favor the occurrence of additional genetic lesions.

Although many of these conclusive considerations are speculative, this thesis provides unique insights on the molecular mechanisms underlying polyclonal B cell activation and EBV reactivation during *Plasmodium falciparum* malaria infection and on how two pathogens can co-operate in lymphoma pathogenesis.

Figure 4.
Hypothetical model of eBL pathogenesis

CIRCULATION

SPLEEN

Proliferation

EBV reactivation

Protection from apoptosis

Proliferation

IL-10
IL-6
IL-4

Activation

Proliferation

Activation

Proliferation
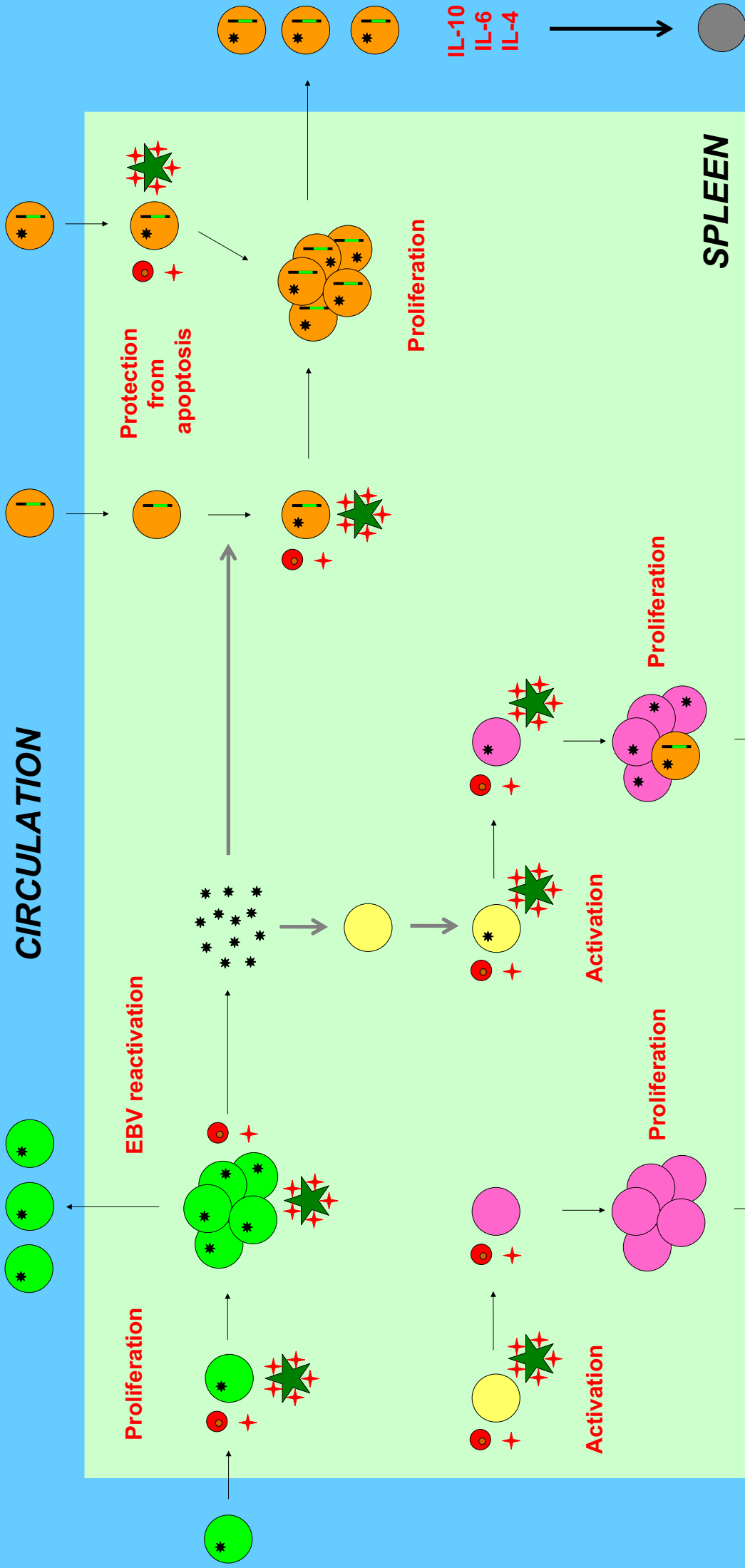
Increased pool of infected cells

Cytokines and antibody production

Naïve B cell

Activated blast

Memory B cell

Translocation carrying B cell

Malignant cell

FDC/DC

Pf. infected erythrocyte

CIDR1α

EBV

6. ACKNOWLEDGEMENTS

- BOP promotion 2006, Woods Hole, USA, and especially to *Patricia Johnson, Chris Hunter, Artur Scherf, Mark, Rachel* and *Nick M.*

- *Sabri and Suleyman* (för god mat!)

AND TO:

Friends who made my stay in Sweden a great and unforgettable time (with some redundancy)
Anders, Anna B.C., Dani, Eva, Gerry, Isabel V., Jessica, Jolle, Johan N., Kirsten, Markus, Mauro, Nicolas, Sophie, Susanne, Ulf M.H., Ulf R., Wilhelm.

Last but not least, I would like to thank *Riina* for her patience and support during these years. Kiitos!

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