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MALARIA, B LYMPHOCYTES AND EPSTEIN-BARR VIRUS
EMERGING CONCEPTS ON BURKITT'S LYMPHOMA PATHOGENESIS

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ABSTRACT

Plasmodium falciparum and Epstein-Barr virus (EBV) infections are recognized co-factors in the genesis of endemic Burkitt’s Lymphoma (eBL), the most common paediatric cancer in equatorial Africa. This thesis work examines and discusses interactions between P. falciparum and EBV that could promote the emergence of eBL. Special focus is given to the effect of malarial antigens on the B cell compartment and of P. falciparum infection on EBV persistence.

Plasmodium falciparum infected erythrocytes (IE) express P. falciparum membrane protein 1 (PfEMP1) on their membrane, conferring them with multi-adhesive properties. The demonstration that one of the domains of PfEMP1, the cysteine-rich interdomain region l[] (CIDR1[]), binds to non-immune immunoglobulins (Igs), led us to investigate the interaction between IE and human B cells. IE directly adhere to and activate purified B cells from malaria-naïve individuals, an interaction involving CIDR1[]. We have identified and characterized CIDR1[] as a T cell-independent polyclonal B cell activator that can: (i) induce proliferation and release of IgM, IL-6 and TNF-[]; (ii) rescue tonsillar B cells from apoptosis; (iii) favour the activation and expansion of the B cell memory compartment, where EBV persists after primary infection; and (iv) bind human IgM and IgG at low affinity but sufficient to lead to B cell receptor signalling.

The B cell activation is partially mediated by binding of CIDR1[] to surface Igs. However, comparison of the gene expression profiles induced by CIDR1[] and anti-Ig activation, using a cDNA microarray, demonstrated a low degree of homology in the signatures imposed by both stimuli suggesting that CIDR1[]-activation arises from multiple receptor signalling.

Acute malaria infection impairs EBV-specific immune responses and increases the number of EBV-carrying B cells in circulation. Whether this reflects proliferation of previously infected B cells, and/or enhanced virus production and bystander infection of B cells, is not understood. Thus we analysed the effect of malaria on the EBV load in children living in malaria endemic areas. To this end, we studied the occurrence and quantified cell-free EBV-DNA in plasma from Ghanaian children with and without acute malaria infection. Viral DNA was detected in 40% of samples (47% in the malaria-infected and 34% in the non-malaria group, respectively) but was absent in plasma from Ghanaian adults and healthy Italian EBV sero-positive children. The impact of malaria on the control of EBV persistence seems to be evident only before immunity to malaria is fully acquired, in fact adults living in the same area did not have detectable EBV-DNA in the plasma. These findings indicate that viral reactivation is common among children living in malaria-endemic areas, and may contribute to the increased risk for eBL. They also suggest different mechanisms of EBV persistence in these children as compared to adults living in the same region or to children never exposed to malaria. To evaluate the direct relation between EBV viral load and the course of malaria infection, we quantified EBV DNA in plasma and in saliva from Ugandan children with acute malaria (M⁺) before (day 0) and 14 days after receiving anti-malaria treatment. Controls included children without malaria (M⁻) and children with eBL. EBV DNA was detected in 31% of the plasma samples and in 79% of the saliva samples collected from M⁺ on day 0, anti-malaria treatment cleared the viral load in plasma without affecting saliva levels. The plasma load varied significantly between the groups; the lowest levels were detected in the M⁻ group, increased in the M⁺, and reached the highest values in eBL patients. The same trend was evident in the frequency and levels of anti-BZLF1 antibodies, indicative of viral reactivation. In the M⁺ group, the positive plasma samples clustered in a children age group of 7-9 years, the peak-age incidence of eBL. The clearance of circulating EBV after malaria treatment indicates a direct relation between active malaria infection and viral reactivation.
In conclusion, this thesis provides unique insight into the mechanisms by which two pathogens, *Plasmodium falciparum* and EBV, exploit the immune system by subverting homeostatic control of B cell proliferation, apoptosis and differentiation thus favouring EBV reactivation.

**KEYWORDS:** malaria, *Plasmodium falciparum*, B lymphocytes, polyclonal activation, hypergammaglobulinemia, Epstein-Barr virus, endemic Burkitt’s lymphoma.

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**LIST OF PUBLICATIONS**

This thesis is based on the following papers, which will be referred in the text by their roman numerals:

I. **Donati D.**, Zhang L-P., Chen Q., Chêne A., Flick K., Nyström M., Wahlgren M., and Bejarano M. T.
   Identification of a Polyclonal B cell activator in *Plasmodium falciparum*.
   *Infection and Immunity* 2004; 72:5412-5418

    Increased B cell survival and preferential activation of the memory compartment by a malaria polyclonal B cell activator. *Manuscript*.

    Circulating Epstein–Barr Virus in Children Living in Malaria-Endemic Areas
    *Scandinavian Journal of Immunology* 2005; 61: 461-465

    Clearance of circulating Epstein-Barr virus DNA in acute malaria patients following anti-malaria treatment. *Manuscript*
INTRODUCTION

Over the past few years, the concept that many diseases can be aetiologically linked to infection by more than one pathogen has gained increased attention and awareness. Endemic Burkitt’s lymphoma (eBL) belongs to this group of diseases, often called polymicrobial diseases. Endemic Burkitt’s lymphoma pathogenesis is associated with the infection of two ubiquitous pathogens: Epstein-Barr virus (EBV) and Plasmodium falciparum. Enedmic BL is a childhood disease. Among children living in malaria endemic areas, the combined effect of these two pathogens, both profoundly affecting the B cell compartment, is likely to result in an increased risk of eBL.

MALARIA INFECTION

Plasmodium falciparum and its life cycle
Malaria is caused by a protozoan parasite included in the genus of Plasmodium. Of the four species of Plasmodium that infect man (P. falciparum, P. vivax, P. malariae and P. ovale), P. falciparum is the most “malignant” causing the most severe disease and essentially all mortality associated with malaria. The Plasmodium life cycle includes several developmental stages characterized by asexual reproduction in the vertebrate host and sexual reproduction in the anopheline mosquito vector. The process begins with the inoculation of the parasites in the form of sporozoites into the blood of the host by the mosquito. The sporozoite, which is the infective form, circulates in the blood stream and, within 20 to 30 minutes, attaches and invades the parenchymal cells of the liver. Within the liver cells the sporozoites divide and multiply to exo-erythrocytic and pre-erythrocytic schizonts, each containing thousands of separated, nucleated parasites. Within one to two weeks, the mature schizonts burst and release into the blood a large number of merozoites some of which invade erythrocytes to generate ring stage parasites (trophozoites), and thus initiate the erythrocytic phase of the life cycle. After a period of growth, the trophozoite undergoes an asexual mitotic division process, which leads to the rupture of the erythrocyte and to the release of merozoites, which then quickly reinvade fresh erythrocytes and the cycle is repeated. The asexual erythrocytic stage of the life cycle lasts ~ 48 hours. After several generations, merozoites give rise to sexually differentiated forms (male and female gametocytes). These intra-erythrocytic sexual stages, in the peripheral circulation, are taken up by the mosquito
during a meal. The gametocytes then differentiate into male and female gametes in the insect mid-gut, where fertilization takes place. The resulting zygote transforms into a motile ookinete, which then penetrates the gut epithelium and forms an oocyst. The oocyst matures into elongated sporozoites, which migrate to the salivary glands of the mosquito, ready for transmission to a new host (Figure 1).

Figure 1. Plasmodium falciparum life cycle.

Malaria predominantly affects populations living in tropical and subtropical Africa. Many aspects contribute to malaria epidemiology, among them socio-economic status, changes in global climate and anti-malarial drug resistance. In an epidemic area, malaria occurs episodically in time-periods and is usually found in large cities only during raining seasons. Endemic areas, on the base of parasite prevalence, are characterized by regular incidents that can be classified as hypoendemic, mesoendemic, hyperendemic and holoendemic regions. A parasite prevalence of less than 10% is indicative of hypoendemic areas, a prevalence between 11% and 50% define mesoendemicity, whereas a prevalence of 50% or more can be found in areas of hyper- and holoendemic malaria (1).
PATHOGENESIS

The pathogenesis and particular virulence (severity) of *P. falciparum* malaria is attributed to the ability of the parasite to infect erythrocytes at all development stages, causing very high parasitemia, and by its capacity to adhere to vascular endothelium through a process called sequestration. In fact, a characteristic of *P. falciparum* malaria infection is that, trophozoites and schizonts, are abundant in capillaries and venules of vital organs, while relatively few mature are seen in the peripheral blood. Sequestration, which results from the adherence of parasitized cells to vascular endothelium (2), may serve the parasite twofold, as resistance in the hypoxic post-capillary vessels allows optimal growth, and it also prevents the parasitized red blood cells from circulating to and being destroyed in, the spleen. Adhesion to endothelial cells (cytoadherence) and to uninfected erythrocytes (rosetting), as well as the aggregation of uninfected cells, directly (agglutination) or mediated by platelets (clumping), are adhesive traits of *P. falciparum*-infected erythrocytes that have been studied in vitro and are thought to reflect the events preceding sequestration and the enhanced accumulation of parasites in vascular beds. The parasite ligands for cytoadherence and rosetting described to date are all found in different domains of the variant adhesin *Plasmodium falciparum* membrane protein 1 (PfEMP1).

*Plasmodium falciparum* membrane protein 1

PfEMP1, the main parasite-encoded protein present on the surface of infected red blood cells (iRBC), was first described as a membrane protein with cytoadherence properties (3, 4). The PfEMP1 protein contains functionally conserved domains that are, nevertheless, antigenically highly variable. Rapid, mutually exclusive expression of different variants of PfEMP1 not only allows the appearance of heterogeneous adhesive phenotypes within a clone, but also allows evasion of clinically protective humoral immune responses (5-7). PfEMP1 is a large protein (200-350 kDa) composed of a number of distinct domains (3, 8-10). The extracellular part is segment comprised of conserved N-terminal head structure, the N-terminal segment (NTS), a semiconserved duffy-like binding ligand (DBL1) domain and a less conserved cystein-rich interdomain region 1 (CIDR1) of >300 amino acids. This domain is followed by a variable number of polymorphic DBL domains (Δ, Δ, Δ, or Δ) interspersed with highly diverse sequences, and the domains C2 and CIDR2, which are not present in every variant (Figure 2).
Figure 2. General structure of PfEMP1.

PfEMP1 is mainly expressed during the trophozoite stage, the stage most likely involved in the process of sequestration. PfEMP1 is an important virulence factor involved in various phenomena related to the adhesion of iRBC to human cells and receptors. The individual domains have been shown to possess different affinities for numerous receptors. CIDR1α demonstrate the capability to bind to CD36, CD31/PECAM and non-immune immunoglobulins (Igs) (11). DBL2γ binds ICAM-1 and IgG/IgM (12), while DBL2β binds CD31/PECAM (11). The multi adhesive phenotype of iRBC contributes to the severity of the malaria episodes and to the interaction of the parasite with immune cells.

Several studies have established components of the human serum as crucial factors for cytoadherence and rosettes formation (13-15). Among these proteins are the Igs, in particular IgM and IgG (15-18). These observations, led to hypothesize an interaction between iRBCs and B lymphocytes. Hypothesis explored in this thesis work.

IMMUNITY AND Plasmodium falciparum

The immune system has for many decades been seen as a machine, designed to fight foreign invaders. Lymphocytes were considered the harmed soldiers organized in lymphoid organs. Lymphocytes were known to be capable of dissociating themselves from the lymphoid organs organization to travel alone in lymph and blood as single cells detached from their environment. Current opinion is that the immune system is a complex machinery, finely regulated, that works in symbiosis with the outside infectious environment. There are growing observations
documenting that infectious agents have earned to interact with cells of the immune system and divert their function for their own interest. *Plasmodium falciparum* is not an exception with its capability to elicit and divert immune responses. When a patient, contracts malaria his/her immune system is first activated, then suppressed or subverted by the parasite.

**IMMUNITY TO *Plasmodium falciparum***

Immunity to *P. falciparum* malaria is developed as result of long term exposure to the parasite and is dependent on immunological memory. The key directors in immune recognition and regulation of the immunological responses, are the T cells. These T cells mediate immunity by regulating macrophage and B cell activity, but they may also act directly as cytotoxic cells on infected hepatocytes and through production of parasite-toxic cytokines. The potential immune effector mechanisms against *P. falciparum* are many, though the relative importance of each of them in the immunity to malaria is unknown. Protection seems to be mediated through different mechanisms, according to the degree of exposure to malaria and the pattern of disease transmission. Since immunity to malaria is not an absolute phenomenon, many effector mechanisms are probably working together in (partially) protected individuals. Immunity to *P. falciparum* is acquired after years of exposure to the parasite, and several disease episodes.

**HUMORAL IMMUNITY**

While only a relatively small fraction of antibodies is specific to parasite antigens, malaria is characterized by high levels of Igs (19, 20). The possible action of a polyclonal B cell activator has long been suggested (21), particularly after recognizing that malaria culture supernatants had a stimulatory effect on total peripheral lymphocytes (22). Recently we identified a polyclonal B cell activator as the CIDR1* gene structure of PlfEMP1 (Paper I). A number of studies have demonstrated that antibodies from immune individuals recognize a large diversity of conserved and variable antigenic determinants in *P. falciparum* parasites, from different geographical regions (23-26). Anti-malaria immunity can be acquired by the transfer of IgG from immune adults, conferring protection, illustrating the importance of generating malarial-specific antibody response (27). Titors of membrane antigen-specific antibodies have been found to correlate with protection against clinical malaria (28), although later studies suggest that this protection was strain specific (29, 30). The mechanisms by which anti-malaria Igs confer protection are not clear. Antibody-mediated immunity can: (i) block parasite invasion of the RBC by agglutination of the merozoites, or by blocking the interaction ligands-receptors on the RBC surface (31, 32); (ii)
promote antibody-dependent cytotoxicity of iRBC mediating their opsonization and their targeting to macrophages and other phagocytic cells (33, 34); (iii) block the activity of parasite toxins (35), and (iv) inhibit the development of intra-erythrocytic parasites (36).

**CELL-MEDIATED IMMUNITY**

The arms of innate immune response, such as monocytes/macrophages, polymorphonuclear leukocytes and dendritic cells (DC), participate in the clearance of parasitized cells which primarily occurs in the spleen and liver, and which is not dependent on previously acquired anti-malarial immune response. The cell-mediated immune effector mechanisms associated with protection from malaria include: (i) macrophage activation by interferon-γ (IFN-γ) derived from CD4+ T cells, natural killer cells (NK) or T helper 1 (Th1) cells for enhanced phagocytosis or killing of iRBCs (37); (ii) and inhibition of parasite growth and development inside hepatocytes mediated by CD8+ cytotoxic and IFN-γ producing T cells (38). Nitric oxide (NO), released by macrophages in response to parasitic components and T cell-derived IFN-γ production, can exert anti-parasitic effects. However, *in vivo* data are somewhat contradictory (39, 40). Severe malaria has long been associated with high levels of inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6). On the other hand, IFN-γ has been clearly linked to the onset of pathology in mice as well in humans. The detrimental effects of IFN-γ are believed to be due to its ability to activate macrophages that, in turn, produce endogenous pyrogens (TNF-α, IL-1 and IL-6) leading to an inflammatory cascade (41, 42). The anti-inflammatory cytokines, such as tumour growth factor-β (TGF-β) and interleukin-10 (IL-10), are required to down-regulate the pathological effects of high concentrations of pro-inflammatory cytokines. High levels of TGF-β and IL-10 too early in the infection compromise cell-mediated effector mechanisms, while low levels later in infection lead to a failure to control the inflammatory cytokine cascade with subsequent development of severe pathology. A dynamic equilibrium seems to be required with pro-inflammatory effector mechanisms targeting and controlling the parasite, and anti-inflammatory cytokines suppressing immunopathology.

CD4+ T cells are considered to play an essential role in the immunity to *P. falciparum* blood stages, production of IFN-γ and for providing adequate help to the humoral component of the immunity (43, 44). CD8+ T cells are also key effectors against parasite liver stages (45, 46), but do not seem to participate in the clearance of blood stage parasites.
DEVELOPMENT OF IMMUNITY IN MALARIA ENDEMIC AREAS

In endemic areas, infection with *P. falciparum* results in a range of outcomes from asymptomatic infection, through mild disease, severe disease and death. Immunity is exposure-related and therefore age-related, and develops rapidly to severe non-cerebral disease, more slowly to mild disease and probably never to asymptomatic infection (47, 48). The prevalence of infection within a population is still rising in those age groups in which the incidence of severe disease is already declining (49). High levels of TNF-α, IFN-γ IL-6 and IL-1 are more frequent in children suffering from severe malarial disease than in those suffering from mild disease or asymptomatically infected children (41, 49-52). These observations have led to the hypothesis that infections early in life result in inflammatory immune responses effective against the parasite, but are also associated with immune-pathology. With increasing exposure, immunodeviation and immunoregulation may reduce immunopathology during infection, with the exception of cerebral malaria.

Individuals living in malaria endemic areas have a slow development of protective immunity that may reflect the acquisition of a specific immunity to many non cross-reactive parasite antigen variants. Indeed, CD8+ and CD4+ T cells, that recognize two important antigens of the *P. falciparum* sporozoite (the circumsporozoite protein and the trombospondin related adhesive protein), as well as T cells that recognize most of the known blood stage antigens are largely variant specific (53-56). Specific lack serum antibody responses in malarial infected Kenyan children showed that there is a lack of cross-reactivity to subsequent parasitic infections. Thus, protective antibody responses must be generated against new antigenic variant, thus antigen-specific antibody responses may accumulate with exposure. However, it has been shown that immunity against severe disease may be acquired after a single blood-stage infection, supporting the role for a strain-transcending immunity in early protection.

In areas where malaria is endemic or epidemic, the prevalence and/or level of antibodies to protein expressed on the surface of iRBCs (57) are higher during or soon after infection, than at other times. Because the inhabitants of endemic areas are exposed to parasite infections throughout the year (58, 59), this indicates that antibody levels decrease rapidly after infection. Antibodies are a critical part of immunity to malaria (60) and this frequent loss or diminishment of antibody responses may delay the acquisition of immunity. An alternative explanation for the poor acquisition of malaria immunity in naturally exposed populations is that the parasite actively modulates the immune system of the host, preventing the development of specific immune responses. The loss or delay of malaria specific immunity is accompanied by the loss of specific
immunity towards other infectious agents present in the same area, like EBV. This, phenomenon is probably due to a specific loss of memory B cells (61).

**IMMUNODEVIATION BY *Plasmodium falciparum***

It is clearly in the interest of the parasite, and certainly as a means of selective advantage for strains of *P. falciparum* parasites, to develop ways to inhibit and to evade immune and inflammatory responses potentially harmful to the parasite and the host. Malaria, though, has taken the concept of immune escape to the next level; immunosubversion, being at the same time mediator of immunosuppression and of hyperactivation. In malaria, like in any other infectious disease, there are different ways for the pathogen to escape detection by the host immune system. Still the specific mechanisms of modulation of host cell responses are poorly understood. 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. The extraordinary diversity of antigen phenotypes in the parasite population (antigenic variation) hampers immune recognition that is also obstructed by immunosuppression.

Immunodeviation during *P. falciparum* malaria occurs via acts on different fronts, producing: altered modulation of macrophage and DC functions; impairment of T cell responses; polyclonal B cell activation and hypergammaglobulinemia; production of autoantibodies and increased lymphocyte apoptosis. *Plasmodium falciparum* has an inhibitory effect on T cell responses (62), which results from impairment of IL-2 activity and/or IL-2 production (63), and from the ability of the parasite to inhibit the antigen presentation process.

Macrophage function is reduced during malaria infection (64), and blood stage parasites impair DC maturation and their ability to become efficient antigen-presenting cells (65). Exposure of DCs to parasite-iRBC during maturation fails to stimulate cell proliferation, inhibiting T anti-malaria responses. This phenomenon seems to be mediated by pigments (hemozoin) and adhesion of infected erythrocytes (66).

Malaria infection has been observed to lead to a chronic state of immunodeviation (67, 68) but also to a state of anergy (69, 70). The permanent exposure of the immune system to *P. falciparum* antigens may induce a deletion of reactive T cells in a similar way as described for superantigens (71, 72) and lead to polyclonal B cell activation with hypergammaglobulinemia. The abnormal B cell activation could be mediated by B cell mitogens present on the parasite (21, 73, 74) (Paper I), by its binding to Toll-like receptor-9 (TLR9) (75), also expressed on B cells (76), and could also result from activation mediated by high levels of cytokines such as IL-10 and IL-6 (77).
Polyclonal B cell activation contributes to the characteristic hypergammaglobulinemia, the loss of memory B cells (61) and the impairment of immunological memory seen during malaria. Moreover, *P. falciparum* can induce mononuclear cells apoptosis (78) enhancing the alteration of the protective immune responses and increasing abnormally the levels of IgE (79).

Immune effector mechanisms in the liver and the spleen are avoided by sequestration of the mature parasites to the vascular endothelium. However, the documented destruction of the splenic architecture during malaria infection (80) may play a key role in the increased encounter of malaria antigens with immune cells and, be itself, the result of a repetitive exposure to polyclonal mitogens (81). The interplay between the immune system and the malaria parasite governs the degree of symptomatology, pathology, and the development of immunity to the disease. These interactions are extremely complex and only partially understood.

**EPSTEIN-BARR VIRUS**

Epstein-Barr virus (EBV) is a gamma-herpes virus that infects and persists in over 90% of the human population worldwide. In most cases primary infection occurs subclinically during childhood, when EBV is horizontally transmitted through salivary contact (82). This asymptomatic infection may occur shortly after the disappearance of maternal antibodies during infancy (83). Epidemiological studies carried out in the 1970s showed that primary infection occurs early in non-industrialized countries and in low socioeconomic groups (84, 85). In affluent societies, seroconversion may be delayed until adolescence when infectious mononucleosis (IM), an EBV driven self-limiting proliferative disease, develops in 50-74% of the cases (86, 87) while the rest of the subjects experience “silent” seroconversion (87, 88).

After primary infection, EBV establishes a harmless life-long infection in almost every host and rarely causes disease unless the host-virus balance is upset. The virus persists with latent infection in resting memory B lymphocytes (89) that is accompanied by low-level chronic lytic reactivation in the oral cavity of healthy carriers (90, 91), where the virus is constantly shed at low levels in the saliva. Persistent infection is probably maintained by the continual reactivation of B lymphocytes in the oral lymphoid tissues, which contribute to viral shedding (92). *In vivo*, the virus establishes different forms of latency according to the differentiation stage and origin of the infected cells (82). In B lymphocytes, viral latency is characterized by the lack of production of infectious virus and by the expression of a set of viral latent proteins that depends on the location and differentiation stage of the infected B cells (82). Quantitative analysis has established that in one
million B lymphocytes about 1 to 50 viral genome-carrying cells can be identified (93). EBV-infected cells have been also identified in the oropharyngeal epithelium, suggesting an epithelial-cell tropism for the virus (94, 95). The oropharyngeal epithelium is possibly the first tissue that becomes infected after exposure to the virus, since EBV is horizontally transmitted via saliva (96). Viral replication in epithelial cells could then act as a transit route or an amplification step prior to infection of the main target, which are the B cells residing in the underlying lymphoepithelium. Alternatively, epithelial cells could be used to transit and release virus, produced from infected B cells in the lymphoepithelium, back into the saliva. EBV infection is quite complex and involves at least three compartments: the oral cavity, the peripheral blood lymphocytes, and the cell-free fraction of the plasma, which implies the existence of another distinct, undefined compartment (92, 97).

Infectious virus is produced when memory B cells switch from the latency to the lytic phase, and seems to occur after differentiation from memory to plasma cells (98). Lytic replication begins by expression of the immediate-early transcription factors BZLF1 and BRLF1, followed by translation of late genes that encode for structural components, like viral capsid antigens (VCA). EBV reactivation can be monitored in healthy and immunocompromised patients by the appearance of anti-BZLF1 and anti-VCA antibodies (99, 100). During EBV-related disease (IM, EBV-related lymphoproliferative disease) virus levels increase in the whole blood and appear in the plasma, where viral DNA can be detected. In coalescence, viral DNA is normally not found in the plasma fraction (101). When patients become immunosuppressed, the viral load increases in the peripheral blood and viral DNA can thus be found in the plasma. The expansion of proliferating lymphoblasts due to the suppressed CTL response is believed to account for this increase in viral load, and it is considered to be a major risk factor in post-transplant lymphoproliferative disease (PTLD) and AIDS-associated B cell lymphoma. However, it has been shown that cells detected in the peripheral blood of immunosuppressed patients are not proliferating lymphoblasts but are latently infected, resting, memory B cells, which are the same population of infected cells found in the blood of healthy carriers. The major difference in EBV persistence in immunosuppressed patients seems to be the presence of viral replication in the peripheral blood, not observed among healthy carriers (102). Mechanisms that control viral reactivation and the switch from latency, in vivo, are not yet well characterized. Anti-Ig treatment, which activates B cell antigen receptor (BCR) signalling (103), induces lytic production in vitro and thus may serve as a more physiologically relevant activator.

In vitro, EBV promiscuously infects resting B cells, activating them and almost always transforming them into proliferating lymphoblasts (104, 105). EBV can also infect a variety of
cell types: neutrophils (106, 107), monocytes (108, 109), NK cells (110) and T cells (111), however the biological relevance of this infection is not well understood.

Figure 3. Hypothetical model of EBV infection and persistence.

a | During primary infection EBV enters through saliva into the crypts of lymphoepithelial structures such as the tonsils. EBV crosses the epithelial barrier, and b | infects the naïve B cells that are in the resting state in the lymphoid tissue of Waldeyer’s ring and activate these cells to become proliferating blasts. This process seems to parallel the activation of a naïve B cell on exposure to an antigen. c | The antigen-activated B cell-blast is rescued through entry into the pool of memory B cells when it receives signals from antigen and antigen-specific helper T cells. Memory B cells occasionally divide, as part of the homeostatic mechanism for maintaining stable numbers of cells. d | EBV latently infected resting memory cells leave the follicles and enter the peripheral circulation, from where they re-enter the tonsil e | In response to unknown signals (perhaps polyclonal activators and/or bystander T cell help), memory cells may differentiate into plasma cells and secrete antibody. This differentiation may be related to the mechanisms that sustain lifetime production of antibody. If such cell contains the virus, it will reactivate viral replication and infectious virus will be produced.
IMMUNITY

EBV has co-evolved with the human immune system. During this long association the virus life cycle has become well adapted to its host so that it is one of our most efficient parasites. Infection by EBV is controlled by both cellular and humoral immune mechanisms. Antibodies limit the spread of infectious virus (112) and cytotoxic T cells (CTL) destroy infected cells that express viral proteins (113). Through the life of the healthy host, CD8+ T lymphocytes that are specific for both EBV lytic and latent proteins can be isolated, and the 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 (114-118).

During primary infection the cellular response can be very vigorous, where up to 40% of all CD8+ cells in the blood are virus-specific, with most of them directed against a single lytic EBV protein epitope, while only 2% of the CD8+ T cells are directed against a single latent protein epitope (114). Almost all T cell responses are directed against EBV lytic antigens, such as BZLF1 and BRLF1 (114, 119). A detailed analysis of the memory T cell repertoire confirmed these findings showing a stronger response against lytic antigens compared to antigens expressed during latency (117).

It is likely that the major targets for the control of EBV infection are memory B cells that have initiated viral replication. By killing these cells and preventing the spread of infectious virus by antibodies, the immune response reduces the level of infection. However, the immune system is unable to eliminate the virus completely, and as a consequence, viral shedding in the saliva and virus-infected cells persist at low levels, approximately 1 in 10^4 to 10^5 memory B cells. It is only during immunosuppression, induced after transplantation, that the number of EBV infected cells rise, increasing the risk of EBV+ PTLD (102).

Much less is known about CD4+ T cell responses to EBV. However, there is an increased awareness of their key role in supporting high affinity antibody production, initiating and maintaining CTL numbers and function, and performing direct effector activity. No CD4+ T cell expansion has been detected, even during the early stages of EBV acute infection (120). However, the presence of EBV-specific CD4+ T cells has been described in healthy seropositive individuals (121), during IM (122) and in BL (123). During primary infection, up to 3% of circulating effector/memory CD4+ T cells are EBV-specific (124). In studies with polyclonal CD4+ T cell lines, the Epstein-Barr virus nuclear antigen 1 (EBNA1) was found to be a main latency-antigenic target (125-127), although the most common responses detected in patients with IM were directed towards the lytic antigens BZLF1, BMLF1 and the latent antigen EBNA3 (124).
EBV-specific CD8\(^+\) and CD4\(^+\) T cell responses are preferentially directed towards the early lytic proteins and, to a lesser extent, towards nuclear antigens, EBNA3, EBNA4 and EBNA6 (128), which are not expressed in most EBV-associated malignancies. EBNA1 appears as an optimal EBV-specific antigen because it is expressed in all proliferating EBV-infected cells to maintain viral episome. Therefore, this antigen is expressed in all EBV-induced tumours. The recent finding that CD4\(^+\) T cells consistently respond to EBNA1 is particularly interesting and suggests that EBNA1 specific CD4\(^+\) T cell responses provide some resistance to the development of BL (127, 129) in a situation in which the same antigen is not recognized by CD8\(^+\) CTL (130).

In contrast, to the well-established role of cell-mediated immunity in controlling persistent EBV infection, little information is available regarding the contribution of antibodies to the overall control of infection. Strong antibody responses to lytic cycle antigens is seen in IM, but their importance has not yet been determined. Clearly, virus-neutralizing antibodies (predominantly anti-gp350 antibodies) (131) have the capacity to prevent generalized spread of the virus as a cell-free viremia and can contribute to antibody-dependent cellular cytotoxicity (ADCC). However, the delayed kinetics of this response in IM patients argues against any primary role of neutralizing antibodies in limiting the spread of the infection (132).

**EBV-SPECIFIC IMMUNITY DURING MALARIA**

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 (133). Peripheral blood lymphocytes from adults and children with acute malaria were unable to control the out-growth of EBV transformed cells in colony regression assays *in vitro* (134, 135). This is thought to reflect an underlying loss of T cell-dependent IFN-\(\gamma\) 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 (133, 135-137). The mechanisms by which recurrent malaria infection impair EBV immunity is not clear. A new hypothesis has emerged proposing that the frequency of infections can have a greater effect on immune function, i.e. the greater the number of antigenically heterologous infections, the greater the decrease in overall memory T cell activity (138). The model was extended when it was shown, in a mouse model, that an acute and persistent infection can induce loss of memory T cell function to previously encountered non-cross-reactive viruses (139). Even though this model focuses on heterologous viral infections, it is
intriguing to speculate on the role of repeated malaria infections on the reduction of T cell responses against EBV in children living in malaria holoendemic areas.

As previously mentioned, IFN-γ-induced CTL responses versus EBV are thought to be crucial for immunosurveillance, and EBV-specific CD8+ T cells recognize most of the viral antigens, although probably not the EBNA1 antigen as a result of a viral immune escape mechanism. EBNA1 is the only EBV antigen expressed both by endemic BL and dividing memory B cells (130, 140) that may have an immunological impact during malaria infection. Moreover BL cells have broad intrinsic defects in class I antigen processing pathways (141), which indicates that the EBV-specific CTL surveillance is likely to be inefficient. *Plasmodium falciparum* malaria severely damages CD4+ T cell responses, which possibly contributes to the loss of CTL control of EBV infections of B cells (134).

Furthermore, high levels of IL-10 during malaria infection have an inhibitory effect on T cell-mediated immune control of B cell transformation by EBV. In addition, IL-10 induces the proliferation of EBV-infected B lymphocytes (142). Activation of T cells, as well as IFN-γ production during early phases of EBV infection, are crucial in determining the outcome of outgrowth inhibition (143). IL-10 can function to: (i) inhibit the production of IFN-γ and (ii) induce antigen specific unresponsiveness (144). It can also be assumed that the reported hindrance of DC maturation by infected erythrocytes would have an impact on the generation and maintenance of EBV-specific responses (65).

**EBV AND MALIGNANCIES**

EBV has been associated with a number of diseases, particularly autoimmune diseases (145, 146) and cancer (147). Although in most individuals EBV persistence and replication proceed unnoticed, EBV is notorious for its association with cancer. The first such association described was with African BL, but EBV infection is now linked to several malignant tumours of lymphoid (BL, Hodgkin disease, PTLD, nasal NK-T cell lymphoma) or epithelial origin (Nasopharyngeal carcinoma, Leiomyosarcoma in immunosuppressed patients) (82). The precise role of EBV in the pathogenesis of these tumours is unknown, but clinical studies confirm that the virus is intrinsically linked to the development of B cell lymphoma in immunosuppressed patients (148). However, the tumour most consistently associated with EBV is undifferentiated nasopharyngeal carcinoma (NPC), an epithelial tumour prevalent in areas of China and south east Asia, (82) and the endemic form of BL.
BURKITT’S LYMPHOMA

Burkitt’s lymphoma is a highly malignant B cell tumour, first described by Dennis Burkitt in 1958 (149). BL can be classified according to clinical subtypes: endemic, sporadic, and acquired immunodeficiency syndrome (AIDS)-related. Endemic BL (eBL) occur almost exclusively in Africa. Sporadic BL comprises 20-30% of non Hodgkin’s lymphoma in children of developed countries and it is rare in adults, while the AIDS related BL accounts for the 30% of all HIV-associated lymphomas. Histologically, it is a poorly differentiated lymphoma in which cells show little variation in size and shape (150); appears as a starry-sky pattern due to the presence of many infiltrated macrophages containing abundant clear cytoplasm (generally macrophages contain cellular debris from necrotic neoplastic cells) and has a very high proliferation index and a tendency to undergo necrosis. EBV is detected in virtually all eBL, while only in 10-20% of the sporadic forms (151).

B cell malignancies seem to be “trapped” at particular stages of normal B cell development. BL tumour cells have the same pattern of Ig gene hypermutation as germinal center (GC) and memory B cells (152), but they have the cellular phenotype of GC cells with high expression of CD10 and CD77, lack of adhesion molecules and activation markers such as CD23 and CD39 (153). The phenotypic characteristics led to the assumption of a GC origin of these tumour cells. The phenotype of the tumour at clinical presentation will influence its clinical behaviour and responsiveness to therapy. In the GC stage, two molecular processes, Ig class switching recombination (CSR) and Ig somatic hypermutation (SHM), are involved in the remodelling of the B cell DNA. Both, CSR and SHM generate DNA breaks (154, 155) and are, therefore, dangerous mechanisms that might predispose to chromosomal translocations. Occasionally, these breaks are resolved aberrantly, leading to chromosomal translocations. In lymphomas, chromosomal translocations typically replace the normal regulatory sequence of a gene with heterologous regulatory elements that drive inappropriate gene expression near the breakpoints. An hallmark of BL are the translocations of the proto-oncogene MYC into one of the Ig loci, resulting in constitutive activity of this transcription factor, the most common of which is the t(8;14) (156). However, many BL tumours share mutations in the gene encoding the tumour suppressor p53 and in the putative tumour suppressor gene retinoblastoma-like (157).
B LYMPHOCYTES

OVERVIEW OF B CELL IMMUNOLOGY AND DIFFERENTIATION

Mature B lymphocytes develop from bone marrow precursors before antigenic stimulation and populate peripheral lymphoid tissues, recirculating in the peripheral blood. Naïve B lymphocytes (IgD⁺IgM⁺) that encounter an antigen, become activated and migrate into the follicle of lymph nodes where the cells form GCs. There, they undergo rounds of proliferation and SHM, followed by selection for cells that have mutated their Ig genes to produce higher-affinity antibodies. All other cells perish by apoptosis. GCs generate memory cells and antibody producing plasma cells (158-160). The B cell activation process consists of a series of responses that lead to proliferation and differentiation, responses characterized by the expression of specific activation markers and the release of cytokines/chemokines and antibodies.

Memory B cells and plasma cells are intermediate and terminally differentiated cells that are responsive for reactive and protective memory, respectively. The decision between memory B cell differentiation and plasma cell differentiation has been described in considerable detail (158, 161, 162). Stimulation trough CD40 ligand (CD40L) and IL-4, sustained expression of B cell determining factor paired box gene 5 (PAX5), and the transcriptional repressor BCL-6, prevents terminal differentiation. By contrast, in the absence of CD40 stimulation, B cell receptor triggering, and IL-2, IL-6, and IL-10 expression leads to the degradation and reduced expression of BCL-6, and the induction of B lymphocytes induced maturation protein 1 (BLIMP1) and other transcription factors that control plasma cell differentiation. Memory cells in the blood are defined by the presence of mutations in the hyper-variable regions of the Ig genes and the expression of CD27 (163). During an immune response only a small fraction of the generated plasma cells can be rescued as long lived plasma cells in the bone marrow. These cells can survive for several months, thereby maintaining the levels of serum antibodies (164, 165). As plasma cells have a finite life span, a mechanism for their replenishment is necessary to maintain constant levels of protective antibodies over a period of many years. It has been recently shown, in vitro, that human memory B cells proliferate and differentiate in response to polyclonal stimuli, such as CpG DNA and bystander T cell help, and can generate plasma cells continuously in vivo in a polyclonal fashion (166). Memory B cells that are not terminally differentiated, can give rise to effector cells in response to polyclonal stimuli and in the absence of antigen stimulation. However, whether persisting antigen is required to maintain serological memory remains debated (167, 168)
Throughout lymphocyte differentiation, B cells put their genomic integrity in danger during the formation and revision of their antigen receptors. A second potentially dangerous event is the response to antigen. When the response functions normally, the clonal expansion of B cells is regulated tightly by homeostatic controls. However, chronic infections can unbalance lymphocyte homeostasis, contributing to lymphoid malignancies.

**Plasmodium falciparum Malaria and the B Cell Compartment**

*Plasmodium falciparum* infection induces hypergammaglobulinemia, polyclonal B cell activation and autoantibody production. Numerous studies have shown that extracts derived from cultured *P. falciparum* iRBCs induce B cell proliferation and antibody production in vitro (22, 74, 169). In animal models of malaria infection, it has been demonstrated that malaria-induced B cell activation is related to parasitemia (170, 171). However, the exact mechanism behind malaria-induced polyclonal B cell activation has remained elusive.

The highly mitogenic capacity of *P. falciparum* is likely to be the result of the concomitant triggering of different immunological mechanisms that lead to hyperactivation of the B cell compartment. Paper I, in the present thesis, identifies and describes for the first time a polyclonal B cell activator expressed on *P. falciparum*-infected erythrocytes, showing how malaria can polyclonally activate B cells in a T cell-independent fashion.

During the erythrocytic phase, *P. falciparum* also express a TLR9 ligand (75) further characterized to be the hemozoin (66). The ability of B cells to respond to pattern-recognition stimuli, such as bacterial derived CpG motifs, through TLRs has been previously described (76). Human B cells express a limited number of TLRs, and their activation produce different responses in the naïve (IgD+CD27) and memory (CD27+IgD) B cell compartments. TLR9 is expressed at very low levels in human naïve B cells, whereas it is rapidly up-regulated after BCR engagement in memory cells, leading to a rapid memory response to CpG, a TLR9 ligand. Following expansion, memory B cells can differentiate in antibody-secreting plasma cells.

The presence of high titers of Igs that are specific for various self-antigens is a prominent feature of malaria infection and is a sign of non-specific polyclonal activation (22, 172). B cells expressing an antigen receptor specific for self-immunoglobulin-\(\square\) (IgG) are responsible for producing autoantibodies. These specific autoreactive cells can be segregated from conventional B cells in peripheral lymphoid tissues: autoreactive B cells tend to localize in the marginal zone of splenic white pulp (173), whereas conventional B cells “home” to the follicular regions. These localization patterns may reflect areas of the preferential autoimmune stimulation. Recent data
provided evidence for a potent synergistic interaction between BCR-TLR signalling events mediated by chromatin containing immune complexes, in particular mediated by TLR9 (174), that are responsible for the autoreactive B cell activation.

The concomitant engagement of the BCR and TLR9 mediated by immunocomplexes and proteins expressed by the parasite, such as CIDR1 and hemozoin, represent a unique pathway that has the potential to enhance the B cell activation phenomenon during malaria infection. In addition, B cell stimulation may be potentiated by the effect of IL-10, IL-6 and TNF-$
\alpha$ (175), whose production is increased after P. falciparum infection, and by the presence of abnormally high levels of IgE (79) that can crosslink the cellular Fc receptor (CD23) expressed on activated B, contributing to the enhancement of autocrine growth. The CD23 activation by IgE contributes to B cell proliferation. The hyperactivation in the B cell compartment must be considered in a wider prospective, framing it in the general disregulatory effect that malaria has on the immune system. Where does this activation take palce? The spleen plays a very important role during malaria infection, with more than 40% of the splenocytes being B cells. It is in the spleen where damaged and iRBC are removed from the circulation, it is a major site for erythropiesis and hematopoiesis and it is where pathogen-specific T and B cell responses are generated. An extreme form of malaria-induced pathology is characterized by hyperreactive malaria splenomegalyn, a distinct form of splenic enlargement resulting from malaria parasitemia and is often associated with B-lymphoproliferative disorders (176, 177). During malaria infection the splenic architecture is profoundly changed (80, 178) and the spleen shows specific pathological features such as splenomegalyn, white pulp hyperplasia, lymphocyte destruction and prominent migration of macrophages into the white pulp regions. Splenic modifications following malaria, potentially make this a major site for B cell stimulation, where B cells have the possibility to directly interact with iRBCs, soluble malarial antigens and specific immune complexes. Furthermore, a recent report showed that repeated exposure to polyclonal activators, such TLR9 ligands, may lead to lymphoid follicle destruction and immunosuppression, possibly contributing to the immunopathology of malaria (81).

EBV FOLLOWING THE STEPS OF B CELL DIFFERENTIATION

Epstein-Barr virus is a human pathogen able to colonize the entire peripheral lymphoid system, persists at low levels, by exploiting the B cell compartment. The current model of EBV infection holds that the replication of the virus activates B cells to become proliferating blasts so that they can then differentiate into resting memory B cells through the process of GC reaction (93).
Memory is a defining property of the immune system and the capability of memory B cells to persist for the life of the host gives the EBV a selective advantage for a life long-persistence. When a resting naïve B cell in the follicle of a lymph node interacts with a cognate antigen, it becomes activated and begins to proliferate as a B cell blast. EBV infection of B cells causes them to differentiate into proliferating B cell blasts due to transcriptional activity of the virus. The antigen-activated B blast then enters the follicle, 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 Th cells. It has been suggested that these signals are mimicked through the expression of the viral default transcription program, which can replace the T cell help and antigen signals. In fact, EBV encodes latent membrane proteins (LMP), LMP1 and LMP2, which can mimic CD40 and BCR-like signals, respectively (179, 180). These make cells latently infected with EBV potentially independent of T cell help and/or antigen. Finally, these cells leave the follicle as memory resting B cells that enter the peripheral circulation. The infected cells enter the memory pool by shutting down the expression of all viral latent proteins that are recognized by the host immune system (89). A small number of memory cells from the pool of latently infected circulating resting memory B cells proceed through to terminal differentiation, and thus initiating viral replication (98). This mechanism is believed to occasionally happen when memory B cells recirculate back to Waldeyer’s ring (89) where infectious virus is released into the saliva (181). EBV reactivation in vivo may occur by two potential mechanisms. One may occur by acute reactivation in resting memory cells in response to stress that allows the virus to escape quickly before the cell dies. This can happen during polyclonal B cell activation in the course of malaria infection. The other may allow replication of EBV in plasma cells located in the epithelium of the tonsil, allowing high-level production of virions that may be shed directly into saliva.

AT THE CROSS-ROADS OF Plasmodium falciparum AND EBV INFECTIONS: ENDEMIC BURKITT’S LYMPHOMA

The endemic form of Burkitt’s lymphoma (eBL) was first clinically described in 1958 by Dennis Burkitt’s, an Irish surgeon working in Uganda (149) that together with his pathologist colleagues, Davies and O’Connor demonstrated that the tumour occurred with high frequency in a broad belt across Africa, the lymphoma belt. This belt stretches from about 10° north to 10° south of the equator. Within the lymphoma belt, eBL accounts for up to 74% of childhood malignant disorders in Africa. Epidemiological studies have linked the lymphoma belt with regions with
holoendemic malaria transmission, suggesting a role of malaria in the pathology of the lymphoma (182). In 1964 Epstein et al. isolated the EBV from BL tumour cell lines and this virus-tumour association was soon demonstrated by several epidemiological studies. Virtually all eBL tumours are EBV positive.

Intensive study on BL led Guy De-The to call eBL the “Rosetta Stone of cancer” (183). Endemic BL was the first human cancer shown to have viral association, the first human cancer for which a chromosomal translocation that resulted in an oncogene activation was identified, and the first cancer to be successfully treated with chemotherapy alone (183). The growing evidences for the direct interaction between EBV and malaria infections enable this tumour to act as a model to understand the mechanisms of polymicrobial interaction that lead to disease. Recently, it has been well established that sustained and intense exposure to P. falciparum and EBV infection are two cofactors of the endemic subtype of BL, however little is known about the potential mechanisms of interaction between these two pathogens.

Endemic BL can be considered a polymicrobial disease where the B cell compartment represents the cross-road between malaria and EBV infection. 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 is not EBV infection early in life but the presence of holoendemic malaria. In these areas children are exposed to recurrent, perhaps chronic, P. falciparum infections. During this period of time, the child’s immune system is under constant stress from repeated infections with a high parasite burden that could lead to a dampening of the immunological response/control of the EBV infection. Malarial-induced immunosuppression and hyperactivation 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 long 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. 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 (184).
The other model proposes the BL translocation (typical of this type of tumour) 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, 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 by EBV that has already rearranged its Ig genes (185). Another less supported hypothesis considers arboviral infections as a cofactor on the genesis of eBL (186); since some arboviruses have been shown to have oncogenic properties (187), and some plant extracts act as tumour promoters (188). 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 the EBV) are potentiated by plant tumour promoters (189).

Despite wide spread acceptance of the first two models, there has been little direct evidence to define a possible mechanisms. 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. Endemic BL is associated with raised concentrations of EBV-specific antibodies, suggesting that EBV is not a mere passenger in the disease. Patients suffering from eBL presented significantly higher titres of IgG antibodies specific to the VCA of EBV up to six years before the onset of the lymphoma (190). However, the VCA antibody titres did not rise after the onset of the lymphoma, suggesting that chronic rather that acute infection with EBV was relevant to tumourigenesis. No other significant EBV antibody differences were shown between pre- and post-BL onset at the time these studies were carried out. EBV early antigen concentrations rise as the disease develops, and decline after treatment (85). These signs of EBV reactivation at a certain stages of BL. genesis couldn’t be explained, if not in the context of a profoundly perturbed immune system, and the association between the lymphoma-belt with holoendemic malaria suggesting evidence for the potential interaction of these two infections. During malaria infection, the virus-host balance is changed in favour of the virus. In vitro studies showed spontaneous out-growth of EBV+ B cells in cultures with supernatants derived from *P. falciparum* infected erythrocytes (191). Whether this spontaneous out-growth is due to an increase in the total viral load and/or to an increase in the proliferative capacity of already infected cells remains to be determined. Limiting dilution analysis of spontaneously established lymphoblastoid cell lines during acute malaria infection revealed an increased number of EBV-carrying B cells in the
circulation. Treatment of the cultures with antiviral drugs established that the increase was partially due to the release of virus and reinfection of bystander B cells upon *in vitro* isolation. Children with acute malaria had a higher frequency of EBV-infected B cells than did children who had recovered from malaria (137). Moreover, during an episode of acute malaria, spontaneous out-growth of EBV-transformed cells occurred at a greater frequency in children suffering from acute malaria (134), which suggests impaired EBV-specific immunity.

Recent reports show that children living in malaria endemic areas have a readily detectable EBV load in the serum compared to healthy children (192)(Paper III). The presence of free EBV in serum is not associated with healthy donors but usually found in immunosuppressed patients and considered a prognostic factor for the development of EBV+ lymphoproliferative diseases (193, 194). Comparison of EBV loads in children living in regions where malaria transmission is holoendemic and regions in which it is sporadic has shown that children from holoendemic areas have significantly higher EBV loads (192). This leads to the hypothesis that high viral burden in children living in malaria holoendemic regions increases the risk for development of eBL.
AIMS OF THE STUDY

The goal of the present work was to characterise the effects of *P. falciparum* infection, and of malarial antigens, on the homeostasis of the B cell compartment and EBV persistence.

Specific aims were to:

- Study the interaction of *P. falciparum* infected erythrocytes and B lymphocytes.
- Characterize the effect of malarial antigens, such as CIDR1, and infected RBCs on the B cell compartment
- Study the effect of malaria infection on EBV persistence in children living in malaria endemic areas
- Characterize the EBV viral load in children with endemic Burkitt’s lymphoma

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

Ethical approval for the studies included in this thesis was obtained from the ethical committee of the Karolinska Institutet and the local ethical committees at the sites where the clinical studies were conducted and samples were collected.
RESULTS AND DISCUSSION

The work of the present thesis focuses on the interaction of malaria with the B cell compartment, and with Epstein-Barr virus, a B lymphotropic herpes virus. The results will be discussed in two sections regarding the two afore-mentioned interactions.

MALARIA AND B LYMPHOCYTES

A B cell polyclonal activator in *Plasmodium falciparum* malaria

PAPER I and PAPER II

Chronic infection with *P. falciparum* malaria leads to a severely deregulated immune system where B lymphocytes are hyperactivated. The identity of the antigens and mechanisms leading to polyclonal activation and hypergammaglobulinemia during malaria infection are not well understood. The demonstration that the cysteine-rich interdomain region 1 (CIDR1) of the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) binds to non-immune IgA, CD36, and to PECAM-1/CD31 led us to investigate the interaction between human B cells and *P. falciparum*-infected erythrocytes (IE). These studies were carried out in peripheral B cells from individuals naïve to malaria exposure. We found that *P. falciparum* IE have the capacity to interact with B lymphocytes, this interaction partially involves the CIDR1 domain of PfEMP1, as shown by binding competition experiments conducted with the recombinant protein, GST- CIDR1 (Figure 4).

![Figure 4. Inhibition of IE-B cell binding by soluble CIDR1.](image)

Ethidium bromide-stained *P. falciparum* IE were co-incubated with peripheral blood B cells stained with acridine orange. The percentage of binding was evaluated by microscopy. Inhibition of the IE-B cells binding was carried out by pre-incubation of B cells with CIDR1 and the control protein GST, at different concentrations. Percentage of inhibition was calculated in relation to the IE-B cell binding in absence of CIDR1.

The interaction between IE and B cells leads to B cell activation and proliferation. Soluble CIDR1 activates B cells in a T cell-independent fashion, inducing increase in cell size, expression of activation markers, CD23 and CD40, of adhesion molecules, CD54 and CD58, and, of
costimulatory molecules CD80, CD86. B cell-induced activation by CIDR1\(\beta\) is also accompanied by B cell proliferation (Figure 5). Furthermore, the coinubcation of B cells with CIDR1\(\beta\) induced cytokine release (IL-6 and TNF\(\alpha\)) and IgM production.

CIDR1\(\beta\) bound to the different regions and classes of human Igs, as well as to Igs purified from different animal species (Figure 6), exhibiting a binding pattern that is similar to that of the Staphylococcus aureus protein A, another immunoglobulin binding protein and a polyclonal B cell activator. The capacity of CIDR1\(\beta\) to stimulate B cells seems to be related to its Ig binding property, since other PfEMP1 domains that lack the Ig binding capacity did not bind to, or induce proliferation of, B cell. Moreover the proliferative effect of CIDR1\(\beta\) was partially inhibited by soluble Igs added to the culture (Paper I).

**Figure 5. CIDR1\(\beta\) induces B cell proliferation.** Primary CD19\(^+\) cells isolated from the peripheral blood, were incubated for 72 hrs with increasing concentration of CIDR1\(\beta\) and the control protein GST. Their proliferative response was measured by thymidine (\(^{3}H\)) incorporation.

**Figure 6. CIDR1\(\beta\) binds Igs.** The mapping of CIDR1\(\beta\) binding to human/non-human Igs and their fragments was evaluated by ELISA. The absorbance values reflect the extent of the binding.

The affinity of the binding plays an important role in the outcome of the activation. The binding affinity (\(K_d\)) of CIDR1\(\beta\) to human Igs (\(K_d \approx 3.7 \times 10^6\) M\(^{-1}\) for IgM and \(K_d \approx 1.2 \times 10^6\) M\(^{-1}\) for IgG) places the protein among the low affinity antigens, though this level of affinity allows BCR triggering (195-197). Given the capacity of CIDR1\(\beta\) to bind human Igs, and its capacity to activate B cells, we analyzed and compared the gene expression profile induced by activation with
CIDR1 and anti-Ig. The two expression profiles were significantly different and suggested that B cell activation, mediated by CIDR1, is probably not only the result of the binding of CIDR1 to sIg, but also due to the interaction of CIDR1 with multiple receptors. It has previously been described that CIDR1 binds to CD31 and CD36 (198), two molecules used by the parasite infected RBC to adhere to the vascular endothelium. CD31 and CD36 are also expressed on human B cells (199-201), therefore, they may play a role in the interaction with CIDR1. Activation mediated by CIDR1 that seems to result from the cooperation of activating/inhibiting signalling delivered by different receptor engagements. The cDNA array analysis of CIDR1 activated-B cells showed that the B cells express a gene profile that reflects an activated status, with the increased expression of genes involved in different activation pathways such as the ERK/MAPK pathway. Further studies need to be conducted to clearly identify which of these pathways plays a key role during CIDR1 mediated activation.

To further characterize the B cell sub-population that is more susceptible to CIDR1-induced activation, we compared the effect of this antigen on memory vs naïve B cells. CIDR1 preferentially activates memory B cells (IgD\(^+\)CD27\(^+\)) increasing the proportion of activated memory B cells within the memory B cell population, and increasing the expression of activation markers, such as CD95, CD70, CD69, HLA-DR, and the costimulatory molecule CD86. On the naïve B cells (IgD\(^-\)CD27\(^-\)) the effect is modest; it induces up-regulation of HLA-DR and down-regulation of HLA-ABC. Furthermore, CIDR1 increases the proportion of activated memory B cells (CD27\(^+\)CD95\(^+\) and CD27\(^-\)CD70\(^-\)) and of the population of memory B cells characterized by the expression of IgD\(^-\)CD27\(^+\), a population known to be responsible for the production of high affinity IgM (202). CIDR1 treatment could also rescue tonsillary B cells from spontaneous apoptosis and increase the proportion of cycling cells (Figure 7) (Paper II).

![Figure 7: CIDR1 rescues tonsillary B cells from spontaneous cell death.](image)
The preferential activation of the memory compartment may relate to its lower threshold for activation, and it is in line with the recent report by Bernasconi et al. (166) where memory B lymphocytes were shown to selectively proliferate and differentiate into plasma cells in vitro, in response to polyclonal stimuli in the absence of BCR triggering. On the other end, naive B cells require specific BCR triggering. These results led to the hypothesis that one of the mechanisms for the maintenance of serological memory involves continuous activation of memory B cells by polyclonal B cell activators (166). Most likely, CIDR1, as a polyclonal B cell activator, contributes in a similar fashion to the hypergammaglobulinemia that characterizes chronic malaria infection (203). The repeated exposure to polyclonal activators can also lead to lymphoid follicle destruction (81), contributing to the damage of the splenic structure seen during malaria (80). During a malaria episode, IE are trapped in the spleen where splenic B cells may be activated by CIDR1 expressed on the IE surface, and presented as soluble antigen, or as immune complexes, by follicular DCs in the presence of cytokines, T cell help, and co-stimulatory signals. Moreover, the low antigenic clearance of CIDR1, due to high malaria endemicity, could result in a long persistence and therefore augment its impact on B cell responses as seen in the impaired maintenance of antigen-specific memory B cells and Ig-specific memory, observed in children from malaria endemic areas (61).

A possible role of malaria, in increasing the risk of eBL through its polyclonal B cell activatory capacity may be to augment the survival of GC B cells that carry translocations. In fact, malaria and EBV have for long been recognized as cofactors in the genesis of endemic BL, although little research has been done to examine the interaction between these two pathogens (Figure 8).

MALARIA AND EPSTEIN-BARR VIRUS

Malarial effect on EBV persistence and reactivation

PAPER III and PAPER IV

Acute malaria infection has been shown to impair the EBV-specific immune responses, which results in increased numbers of EBV-positive B cells in the circulation (137). Whether this increase results from proliferation of previously infected B cells and/or is a consequence of increased virus production and subsequent bystander infection of B cells is not well understood. We therefore analysed the effect of malaria infection on the EBV DNA load in children living in malaria endemic areas.
Figure 8. B lymphocyte activation by infected erythrocytes and CIDR1α

To this end, we studied the occurrence of, and quantity of, cell-free EBV DNA in plasma from Ghanaian children with, and without, acute malaria infection. Viral DNA was detected in 40% of the samples (47% in the malaria-infected and 34% in the non-malaria group, respectively) but was absent in plasma from Ghanaian adults and healthy Italian children previously unexposed to malaria (Figure 9). There was not significant difference in the proportion of samples with detectable EBV DNA between uncomplicated and complicated malaria, although children with complicated
malaria had a median EBV load seven times higher than the group with uncomplicated infection. The impact of malaria on the control of EBV persistence seems to be evident only before immunity to malaria is fully acquired, as adults living in the same area did not have detectable EBV DNA in the plasma. These findings indicate that viral reactivation is common among children living in malaria endemic areas, and may contribute to the increased risk for eBL. They also suggest a different mechanism of EBV persistence in children from malaria endemic areas as compared to adults living in the same regions or to children never exposed to malaria (Paper III).

![Graph showing EBV DNA levels in children and adults](image)

**Figure 9.** Children living in endemic malaria areas have EBV DNA in plasma. Comparison of EBV-DNA in plasma from children living in a malaria holoendemic area (Ghana), with adults (C-adults) leaving in the same region, and children never exposed to malaria (Italian children). Among the children living in malaria holoendemic areas, comparison was conducted between children with acute malaria (M⁺) and children without malaria (M⁻). Bars show the median copy number and the interquartile range of the EBV-positive cases. The dashed horizontal line represents the detection limit of the real-time PCR assay. All samples with <100 EBV-DNA copies/ml were considered negative. The EBV detection rate in each group is outlined at the bottom (EBV negative) and at the top (EBV positive) of the figure.

To evaluate the direct effect of malaria infection on EBV persistence, i.e. the relation between viral load and the course of malaria infection, we studied the prevalence of EBV and quantified viral DNA in plasma and in saliva from children living in a malaria holoendemic region (Uganda) with acute malaria, before and after receiving anti-malaria treatment. This longitudinal study compared children that had experienced acute malarial (M⁺) before and 14 days after receiving anti-malaria treatment, with naïve children living in the same area (M⁻) and children with Burkitt’s lymphoma (BL). In acute malaria patients (M⁺) EBV DNA was detected in 31% of the plasma samples and in 79% of the saliva specimens. Anti-malaria treatment led to the clearance of viral load in the plasma of 85% (36/38) of the cases without affecting the levels in saliva (Figure 10).
Figure 10. Epstein-Barr virus (EBV) loads in children with acute malaria, before and after anti-malaria treatment. The EBV load was quantified by real-time PCR in saliva and plasma samples from children with acute malaria, before (Day 0) and after anti-malaria treatment (Day 14). Data are expressed as log of EBV copies/ml. Boxes depict median values, with 25th- and 75th-percentile values represented by bottom and top edges of lines and medium line showing the median value. Figure 10a and 10b are different representations of the same data, relative to the EBV load in plasma. b) Each dot represents the EBV load from one patient. The lines show the change in EBV load for each child before (Day 0) and after therapy (Day 14), continuous line represent no change in load, dashed line represent decrease in load and dotted line represents absence of load.

There was a significant overall difference in plasma EBV DNA load across the three groups with the lowest levels detected in the M group, increasing in the M+ group, and reaching the highest values in BL patients (Figure 11). In all groups of patients the viral load was higher in saliva than in plasma.

Figure 11. Comparison of Epstein-Barr virus (EBV) loads in children without malaria, with acute malaria, and with Burkitt’s lymphoma. The EBV load was quantified by real-time PCR in saliva and plasma samples. Data are expressed as log of EBV copies/mL. Boxes depict median values, with 25th- and 75th-percentile values represented by bottom and top edges of lines and medium line showing the median value.
In the M+ group all the samples with EBV load in the plasma clustered in an age group of 5-9 years, previously described as the peak age incidence of eBL. In an attempt to define the molecular characteristics of circulating cell-free viral DNA, EBV positive plasma samples were treated with DNase, an enzyme capable of digesting EBV DNA that is in its “naked” form and not-protected by the viral capsid. All the samples treated with DNase showed the presence of “naked” EBV DNA, which probably was not directly derived from viral replication in the periphery, but from malaria-associated reactivation in other compartments or from tumour related events. Even though the plasma EBV DNA did not appear to be derived from EBV reactivation in the peripheral blood, the presence of anti-BZLF1 IgG antibodies was an indication of viral reactivation (204, 205). The frequency and the levels of anti-BZLF1 antibodies in the patient groups were similar with lower levels in the M group, increasing in titers progressively from the M+ to the BL group. These results suggest an ongoing viral reactivation process occurring in another region than the peripheral blood. In fact, elevated titers of early antigens-specific IgGs correlated with increased levels of EBV load and have been associated with an increased risk of EBV+ malignancies (206, 207). This indicates that the disease is preceded by EBV reactivation and that the deregulation of the EBV-host balance precedes the out-growth of a malignant clone (208). Thus, the presence of antibodies directed against BZLF1 in the serum is a good marker for viral reactivation (204, 205).

The clearance of EBV DNA after effective anti-malaria therapy indicates a direct relation between active malaria infection and viral reactivation that may not simply be related to the malarial immunosuppressive effect. The mechanisms by which acute malaria could increase the viral load are not yet defined. Malarial infection could help the expansion of EBV-carrying B cells trough polyclonal B cell activation; it could increase viral replication and/or induce apoptosis of the infected B cell pool leading to the release of EBV DNA. Our results suggest a combination of the last two phenomena. This assumption is supported by the presence of high titers of anti-BZLF1 antibodies, a clear sign of EBV reactivation. However, the presence of “naked” EBV DNA suggests that the EBV load in the plasma, is derived from an apoptotic-related event. EBV DNA is not usually detected in the plasma of healthy subjects (209, 210) and its presence has been not only indicative of viral reactivation (210, 211), but is also prognostic factor for EBV+ lymphoproliferative diseases (193). The mechanisms that may lead to viral reactivation during malaria infection are not well understood. It has recently been demonstrated that terminal B cell differentiation into plasma cells initiate the EBV replicative cycle in vivo (98). In this context, the presence of polyclonal B cell activators on the membrane of P. falciparum IE, such as CIDR1[]}.
and their capacity to induce B cell proliferation and differentiation into antibody secreting cells, suggest a possible role in the viral reactivation.

While anti-malaria treatment led to the clearance of EBV load in the plasma, the levels of virus in saliva were not significantly affected by the treatment and did not vary among the different groups (M⁺, M⁻, BL). After primary infection EBV can be found in three different compartments: the oral cavity, the blood lymphocytes, and the cell-free fraction of the blood plasma. Despite possible exchange among them, infection in the oral cavity appears to be self-renewing and does not require replenishment from peripheral lymphocytes (97). The apparent lack of impact by *P. falciparum* infection on the salivary compartment, where reactivation of latently infected memory B cells is assumed to occur in healthy carriers (98, 212), could imply that acute malaria does not alter the compartment normally associated with EBV persistence. On the other hand, it suggests that malaria elicits reactivation in another distinct, yet undefined, compartment that leads to a pathological condition reflected by the presence of EBV in the plasma. Possible sources of EBV in the plasma are mucosal lymphocytes, lymph nodes and the spleen. During the course of malaria, IE are trapped in the spleen which often increases in size (176, 177) and shows signs of damage (80). It may be in the spleen that the abnormal B cell proliferation leads to the reactivation of latently infected cells with subsequent viral production. The known inhibitory effect of malaria on EBV specific responses (134, 135) may amplify this phenomenon leaving unchecked the cells that start viral production.

In conclusion the results included in this thesis contributed to:

- The identification and characterization of a malaria polyclonal B cell activator, the CIDR1
- A greater understanding of the mechanisms that may contribute to the increased B cell pool and hypergamaglobulinemia characteristic of chronic malaria infection
- Defining the impact of malaria infection on EBV persistence
- Identification of a direct interaction between acute malaria infection and EBV reactivation
- Demonstrating that the epidemiology of EBV infection and persistence varies in different areas of the world.

All together these results shed new light on how malaria infection may contribute to the genesis of eBL.
CONCLUDING REMARKS

It is difficult to determine the aetiology of eBL simply the interaction between malaria and EBV. It is probably the result of many different environmental exposures with convergent pathogenic mechanisms, where malaria and EBV play significant roles. By focusing on the interactions between malaria and the B cell compartment, and then between malaria and EBV in areas where there is a high incidence of BL and a clear epidemiological link between EBV and holoendemic malaria, we can attempt to elucidate the molecular and cellular interactions between these two parasites in the pathogenesis of eBL.

Throughout this thesis, it has been proposed that the B cell compartment lies at the cross-roads between two ubiquitous human pathogens, EBV and P. falciparum, when coinfection occurs. The B cell compartment is under tightly regulated homeostatic controls. When the regulation of B cell differentiation and activation is disrupted, malignancies like BL can occur. The process that normally creates immunoglobulin diversity might be misdirected, resulting in oncogenic chromosomal translocations that block differentiation, prevent apoptosis, and/or promote proliferation. Chronic malaria infection, typical of endemic areas, can wreak havoc on lymphocyte homeostasis producing prolonged and unregulated antigenic stimulation that leads on one hand to hyperactivation, and on the other to immunosuppression, contributing to the development and progression of malignancies. The B cell activation/differentiation process can be easily altered by genetic abnormalities or outside influences, such as EBV.

A simplistic interpretation of the data would explain the endemic lymphoma phenomenon as the result of an EBV reactivation allowed by the immunosuppressive effect of malaria infection. Immunosuppression per se does not seem to be sufficient to explain the increased risk of BL, and it is probably the combination of numerous cause-effect events that lead to the tumourigenic transformation at the origin of the disease. Based on the results of the studies presented in this thesis, it is tempting to propose a model to explain how malaria contributes to the increased risk of eBL. The model considers eBL as a polymicrobial disease resulting from a disregulation of the B cell compartment homeostasis, where P. falciparum and EBV are cofactors. Malaria infection acts as a disruptive factor in B cell differentiation and proliferation, leading to hyperactivation that results in hypergammaglobulinemia and abnormal humoral responses to self-antigens. At the same time, it produces a state of depressed cellular responses that alters the control over EBV-infected B cells and leads to the production and release of new virions that infect bystander B cells, favouring their proliferation. The end result of these events is an increased probability in
the incidence of oncogenic changes as a consequence of increased proliferation. This effect may be further amplified by the protection from cell death mediated by CIDR1, and possibly by other malarial antigens.

It seems reasonable to consider that increased virus production upon malaria infection does not exclusively correlate to the immunosuppression weakening of EBV-specific T cell responses. *P. falciparum* may have a direct effect on inducing viral production as result of the interaction between malaria antigens with polyclonal activity and EBV-infected B cells. This interaction presumably occurs in as yet undetermined compartment, most probably the spleen. It is important to keep in mind that malaria infection occurs in the context of profoundly damaged lymphoid architecture and that repeated antigenic stimulation with polyclonal activators produce lymphoid follicle destruction and suppression of GCs. The immunosuppressive effect imposed by malaria seems to only marginally contribute to the development of eBL. This assumption is based on two facts: (i) BL does not occur in drug-immunosuppressed individuals, and (ii) in HIV-related BL, the lymphoma appears early in the progression of HIV infection in the presence of high CD4+ T cell counts.

Our results also indicate that the pattern of EBV persistence is different among children living in malaria endemic areas. In these regions, seroconversion occurs early in life, and the exposure to continuous and protracted episodes of malaria may result in a higher EBV burden leading to a different pattern of viral persistence. The question remains whether repeated malaria infection affects either the establishment of, or the maintenance of, EBV persistence. Although many of these conclusive considerations are speculative, this thesis, together with the ongoing studies in this field, provides further insights into the possible mechanisms behind the pathogenesis of lymphoid malignancies.

In conclusion, the data presented in this thesis supports our hypothetical model, indicating that the phenomena leading to the oncogenic modification at the origin of Burkitt’s lymphoma may hinge on the ability of the two pathogens, EBV and *P. falciparum*, to efficiently exploit the immune system by subverting the homeostatic control of B cell proliferation, apoptosis and differentiation.
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