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MOLECULAR UNDERSTANDING OF PATHOGENESIS AND IMMUNE RESPONSES IN ENDEMIC BURKITT LYMPHOMA

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DOCTOR OF PHILOSOPHY IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

BY

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Declaration

This dissertation is the result of research work undertaken by Cecilia Smith in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Department of Immunology of the Noguchi Memorial Institute for Medical Research, as well as and Department of Immunology and Microbiology, University of Copenhagen, Denmark under the supervision of Dr. Michael F Ofori (Laboratory work in Ghana), Prof. Lars Hviid (Laboratory work in Denmark), Prof. Lorna Renner (Clinical work in Ghana), and Prof. Richard Gyasi (Pathology laboratory work in Ghana).

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Dedication

This work is dedicated to my family, husband (James Togobo), children (Jeremy Mawulorm Togobo and Cyril Enam Togobo), my parents, siblings and all children in eBL endemic areas.

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List of abbreviations

Abd ..... Abdomen

AHC ..... Afrancho Health Centre

AID ..... Activation-induced deaminase

ANOVA ..... Analysis of variance

BCR ..... B-cell receptor

BL ..... Burkitt lymphoma

C (C_H, C_L) ..... Constant region (of heavy chain, light chain)

CD ..... Cluster of differentiation

cDNA ..... Complementary DNA

CALLA ..... Common acute lymphoblastic leukaemia antigen

CDR ..... Complementarity-determining

CFIM ..... Center for Integrative Microscopy

CLL ..... Chronic lymphocytic leukaemia

CM ..... Central memory (cell subset)

CMP ..... Centre for Medical Parasitology

CPEC ..... Circular polymerase extension cloning

CSR ..... Class-switching recombination

Da (kDa) ..... Dalton (kiloDalton)

DMSO ..... Dimethyl sulfoxide

DN ..... Double-negative

DNA ..... Deoxyribonucleic acid

DP ..... Double-positive

DPX ..... Distreen Plasticizer Xylene

DTT ..... Dithiothreitol

EBER .....Epstein-Barr-encoded RNA

eBL..... endemic Burkitt lymphoma

EBNA ..... Epstein-Barr nuclear antigen

EBV .....Epstein-Barr virus

EM .....Effector-memory (cell subset)

EMRA ..... Terminally differentiated EM (cell subset)

EPCR .....Endothelial protein C receptor

F/M ..... Female/male (ratio)

FACS ..... Fluorescence-activated cell sorting

FFPE..... Formalin-fixed paraffin-embedded

FISH .....Fluorescence *in situ* hybridisation

FNA .....Fine-needle aspirate

H&E .....Haematoxylin-eosin

HCl .....Hydrochloric acid

HEK ..... Human embryonic kidney

HHV .....Human herpes virus

HIV ..... Human immunodeficiency virus

HLA .....Human leukocyte antigen

IE .....Infected erythrocyte

Ig (A, D, E, G, M).....Immunoglobulin (A, D, E, G, M)

ig (*igh*, *igl*) .....Immunoglobulin-encoding genes (heavy, light chain)

KATH .....Komfo Anokye Teaching Hospital

KBTH ..... Korle-Bu Teaching Hospital

LMP .....Latent membrane protein

MHC ..... Major histocompatibility complex

MIC (A, B, C) ..... MHC class I polypeptide-related sequence (A, B, C)

MYC (*myc*) .....Master regulator of cell cycle entry and proliferative metabolism

ND .....Not determined

NHL .....Non-Hodgkin lymphoma

NK .....Natural killer

NMIMR .....Noguchi Memorial Institute for Medical Research

PBMC ..... Peripheral blood mononuclear cells

PCR .....Polymerase chain reaction

P. falciparum ..... *Plasmodium falciparum*

PfEMP1 .....*P. falciparum* erythrocyte membrane protein 1

PRR ..... Pattern recognition receptor

RNA .....Ribonucleic acid

RT .....Reverse transcription

SHM .....Somatic hypermutation

SP .....Single-positive

TCR ..... T-cell receptor

T_{FH} .....Follicular helper T (cell)

TLR .....Toll-like receptor

V (V_H, V_L) .....Variable region (of heavy chain, light chain)

VDJ / V(D)J .....Variable-Diversity-Joining

WACCBIP .....West African Centre for Cell Biology of Infectious Pathogens

Abstract

Endemic Burkitt lymphoma (eBL) is a malignant B-cell lymphoma in children living in Sub-Saharan Africa, including Ghana. Where it occurs, eBL is often the most common cause of cancer death in children under 15 years, although it is potentially curable if diagnosed early and treated appropriately. The disease is associated with exposure to Epstein-Barr virus (EBV) and *Plasmodium falciparum* malaria parasites, but the molecular details of how the combination of these infections can precipitate eBL are not well understood. The mechanisms of the host immune response in the pathogenesis and control of eBL are similarly unclear. It has been suggested that *P. falciparum*-specific B cells are particularly prone to malignant transformation by activated-induced cysteine deaminase-dependent *c-myc* translocation in EBV-infected B cells, and that many eBL cells therefore encode a *P. falciparum*-specific antibody. It has furthermore been proposed that auto-regulatory Vδ1$^+$ T cells play an important role in the normal control of activated B cells, including malignant eBL cells. The physiologic role of this rather enigmatic cell subset is currently essentially unknown.

This PhD research project was undertaken to investigate the above hypotheses. It has resulted in the production of antibodies from the BCR of the tumour cells that is an important new knowledge regarding the antigen specificity of eBL tumour cells that support the hypothesis that particular antigens (likely *P. falciparum*-derived) are important drivers of eBL pathogenesis. There was a highly biased V(D)J rearrangements observed, as most tumour-derived B cells expressed sequences homologous to VH3 (3-7*01, 3-9*03, 3-23*04, and especially 3-30*04) and VL2 (2-14*01), when compared to peripheral B cells from the same patients. It has also provided empirical support for the hypothesis that Vδ1$^+$ T cells are critically involved in the immune response to this devastating tumour. Interestingly, high frequency of Vδ1$^+$ T cells were found in the cancer patients (both eBL and non-eBL). However, there was marked differentiation and maturation of Vδ1$^+$ T cells in the eBL patients compared to the other

study groups (non-eBL, malaria and healthy). Most cells (80%) from the tumour were B- cell confirming previous studies that eBL is a B cell lymphoma. Nonetheless, atypical memory B cells were of higher frequency in eBL as compared to malaria and health controls. In comparing the Giemsa stained FNA (fine needle aspiration) and haematoxylin and eosin stained FFPE (formalin fixed Paraffin embedded) samples, there was a good agreement with the original diagnoses of eBL made in Ghana compared to that in Denmark for the FNA samples (Accuracy, Sensitivity and Specificity were all 100%) . However, there were some disparities with the diagnosis with the FFPE tissues due to poor tissue preservation (Accuracy- 84%, Sensitivity - 83% and Specificity- 84%).

The research undertaken has so far resulted in one manuscript (Reliable Cell and Tissue morphology based diagnosis of endermic Burkitts lymphoma in a resource constrained setting in Ghana) submitted for publication. Another manuscript is currently being prepared for submission with at least one additional original research paper and one additional review paper expected as a result of this work.

In conclusion the data supports that $V\delta1^+$ T cells are important modulators of the B-cell proliferation in eBL, there is a state of chronic B-cell activation, and that eBL tumour cells are have been activated by an antigen that caused them to be differentiated in germinal centres. It is also concluded that the original diagnoses, which involved laboratory assessment of tumour cell morphology, were reliable, when evaluated by independent retrospective analysis of specimens similar to those available at time of the original diagnosis.

1. Introduction

1.1. Background

Patients with eBL have high titres of EBV-specific IgG (Henle & Henle, 1966; Henle *et al.*, 1969; Henle, Henle, & Diehl, 1968), and high EBV-specific antibody titres are a risk factor for development of eBL (de-Thé *et al.*, 1978). This has been known for a long time. The striking co-endemicity of eBL and *P. falciparum* malaria was also recognized many years ago (Burkitt, 1969). Taken together, these findings suggest that eBL is a polymicrobial disease that is the unfortunate result of early infection with a B-cell-specific virus with oncogenic potential, compounded by infection with a parasite causing massive antigen-specific and possibly polyclonal B-cell activation (Chene *et al.*, 2009; Greenwood & Vick, 1975; Thorley-Lawson, Deitsch, Duca, & Torgbor, 2016). In this scenario, intense exposure to *P. falciparum* parasites increases the number of antigen-stimulated B cells that enter germinal centres to undergo AID-dependent somatic hyper-mutation (SHM) and class-switching recombination (CSR), eventually emerging as memory B cells and antibody-secreting plasma cells. In addition, malaria parasites also appear to induce over-expression of AID in germinal centres, which may destabilize genomic integrity and increase the risk of mutation and cancer (Robbiani *et al.*, 2015; Thorley-Lawson *et al.*, 2016; Torgbor *et al.*, 2014). Concomitant massive exposure to the oncogenic virus EBV further increases the risk that germinal centre B cells accidentally experience an AID-dependent translocation of *c-myc* into antibody-encoding loci during the rearrangement and class-switching, and loose normal control of cell division. The EBV in addition produce nuclear proteins: EBNA1 that prevent the mutated cells from being recognised by the immune system to induce the death of the cells via apoptosis. In consonant with latent protein; Latent Membrane protein 1 (LMP1) secreted by the virus enhance the proliferation of the mutated cells leading to eBL (Frappier, 2012; Kieser & Sterz, 2015). Finally, it has been proposed that *P. falciparum* malaria compromises protective cell-mediated immune responses

against EBV-infected B cells and enhances virus replication (Whittle *et al.*, 1984). It thus appears that infection with EBV and *P. falciparum* creates a permissive environment that predisposes to the development of eBL (Moormann, Snider, & Chelimo, 2011).

In this thesis it is hypothesized that *P. falciparum*-specific B cells are particularly prone to such transformation, and that many eBL cells therefore encode a *P. falciparum*-specific antibody. It is normally assumed that the malignantly transformed B cells somehow escape deletion by apoptosis, although the mechanism is not well understood. We hypothesize that auto-regulatory T cells play an important alternative or additional role in the regulation of activated B cells, and that $V\delta1^+$ γδ T cells are of major importance in that respect, and therefore constitute important anti-tumour effector cells in eBL.

1.2. Hypotheses

This study has two basic hypotheses (Figure 1.1):

(i) $V\delta1^+$ T cells are important regulators of the B-cell proliferation in eBL.

(ii) *P. falciparum* antigens are important drivers of the malignant B-cell transformation in eBL.

1.3. Objectives

The overall objective of this thesis work was to improve the understanding of molecular aspects of pathogenesis and immune responses in eBL.

1.3.1. Specific objectives and rationale

The overall thesis objective was pursued under four specific objectives:

1. To assess the accuracy of morphology-based diagnosis of eBL in Ghana

The ability to conduct the proposed research requires that study participants are diagnosed and categorized correctly. In Ghana – as in most eBL-endemic settings, diagnosis is based mainly on the clinical presentation, supported by basic microscopic assessment of tumour cell morphology. To assess the accuracy of the clinical diagnoses made at the time of admission of

the patients included in the study, the diagnoses were retrospectively and independently re-assessed by microscopy of cell morphology, immunohistochemistry, and fluorescence-*in situ* hybridization (FISH). A series of archival tumour biopsies was assessed in a similar manner.

2. To characterize the phenotype of circulating and tumour-infiltrating Vδ1$^+$ T cells in Ghanaian eBL patients

The function of the Vδ1$^+$ subset of T cells, which generally constitutes a very minor proportion of the T cells in the peripheral circulation, is largely unknown. It is similarly unclear, what antigens are recognized by these cells, but recognition appears to resemble that of conventional TCR-αβ$^+$ more than that of the complementary TCR-γδ$^+$ cell subset, Vγ9 (Davey, Willcox, Baker, Hunter, & Willcox, 2018; Willcox, Davey, & Willcox, 2018). The Vδ1$^+$ cell subset is expanded in individuals exposed to *P. falciparum* malaria and in patients with several diseases causing pronounced B-cell activation (Hviid *et al.*, 2000; Hviid *et al.*, 2001; Hviid, Smith-Togobo, & Willcox, 2019), and very preliminary, indirect evidence suggests that it is expanded in eBL also (Futagbi, Welbeck, Tetteh, Hviid, & Akanmori, 2007). To assess the putative role of Vδ1$^+$ T cells in the immune response to eBL, the phenotype of circulating and tumour-infiltrating Vδ1$^+$ T cells was determined in patients with eBL and non-eBL tumours, in children with *P. falciparum* malaria, and in healthy children.

3. To characterize the phenotype of circulating and tumour B cells in Ghanaian eBL patients

Only limited information – most of it fairly old – exists regarding the phenotypes of eBL tumour cells, and characterization according to the current understanding of B-cell maturation is not available. To close this gap, the phenotype of circulating and tumour B cells was determined in patients with eBL and non-eBL tumours, in children with *P. falciparum* malaria, and in healthy children.

4. To characterize the B-cell receptor diversity and antigen specificities of eBL tumour cells from Ghanaian eBL patients

The geographic distribution of eBL is remarkably restricted to areas of stable and intense transmission of *P. falciparum* (Burkitt, 1962a). This suggests that this malaria parasite species drives eBL pathogenesis, and that eBL tumour B cells often encode antibodies that are *P. falciparum*-specific. As a first step towards a direct testing of this hypothesis, the diversity of BCR-encoding genes were determined, and human recombinant monoclonal antibodies were generated.

The work conducted towards achieving these objectives paves the way for future studies on the biological samples collected for the present work, including characterization of T-cell receptor diversity and antigen specificities of circulating and tumour-infiltrating Vδ1$^+$ T cells, as well as single-cell RNA sequencing studies of the collected tumour aspirate material.

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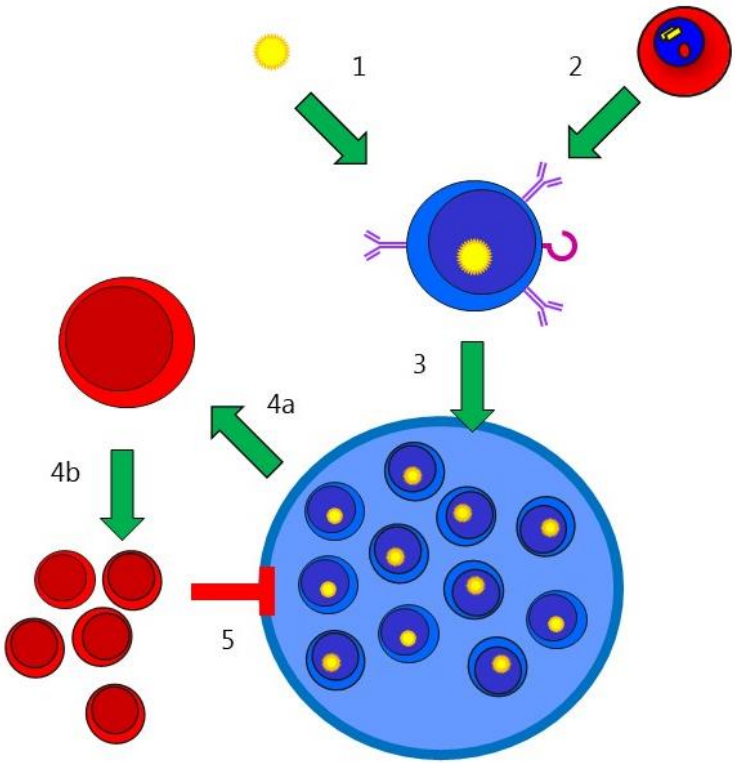


Figure 1.1. Schematic presentation of study rationale and hypothesis
A B-cell clone, which have been infected by EBV (1), is activated and induced to expand by exposure to *P. falciparum* antigens (2). Translocation of *c-myc* into one of the immunoglobulin-encoding genes causes malignant transformation of the activated B-cell clone, leading to

formation of an eBL tumour (3). The expanded population of tumour B cells activates (4a) and induces expansion (4b) of $V\delta1^+$ cells that specifically recognize antigen(s) on the tumour cells. The expanded clone(s) of $V\delta1^+$ cells inhibit the proliferation of the tumour (5). Study hypothesis (*i*) is mainly related to steps 4a-5, whereas hypothesis (ii) is mainly related to step 2

2. Literature review

Endemic Burkitt lymphoma (eBL) is an extremely aggressive solid tumour of the immune system, caused by the malignant transformation and subsequent massive proliferation of a post-germinal B-cell clone. It mainly affects children in tropical Africa and is uniformly fatal if left untreated.

The affected B-cell clone in eBL is usually EBV-positive, and this ubiquitous virus infection evidently plays a key role in the pathogenesis of the disease. The geographical distribution of eBL is furthermore remarkably restricted to areas with intense transmission of the malaria parasite, *P. falciparum*. It is therefore almost exclusively found in tropical Africa, although Papua New Guinea is/has been another focus.

In addition to being a disease of the immune system (a B-cell lymphoma) that involves exposure and immune responses to EBV and *P. falciparum*, the immune system is also involved in the host defence against eBL. The main effector cell types appear to be cytotoxic T cells and NK cells, but the target antigens recognized by these cells remain uncertain.

An exhaustive literature review of the eBL disease, and of the infections and immune system components it involves, is beyond the scope of this section. Instead, it aims to provide sufficient background to enable appreciation of the rationale underpinning the hypotheses investigated in this thesis. EBV and *P. falciparum* are key elements in the pathogenesis of eBL, and those pathogens will therefore be described first.

2.1. The eBL-associated pathogens

2.1.1. Epstein-Barr virus (EBV)

EBV is a double-stranded DNA herpes virus (HHV 4) that infects B cells and epithelial cells. It is the cause of B-cell lymphoproliferative disease called infectious mononucleosis (glandular fever), and has been associated with a number of infections and neoplasms. The virus was originally discovered in *in vitro* cultures of lymphoblasts from a Burkitt lymphoma tumour

biopsy (Epstein, Achong, & Barr, 1964), and its role in the aetiology of infectious mononucleosis was only realized several years later (Gerber, Hamre, Moy, & Rosenblum, 1968; Henle *et al.*, 1968).

EBV is a very common and widespread virus, and a substantial proportion of humans are infected shortly after the protective immunity that is passively acquired by transfer of maternal antibody in utero has waned. The virus has the capacity to immortalize B cells, probably because virus latent membrane protein 1 (LMP1) acts as an antigen-independent mimic of the B-cell activation and proliferation antigen CD40 (Gires *et al.*, 1997; Kilger, Kieser, Baumann, & Hammerschmidt, 1998).

Despite the ability of the virus to immortalize B cells and induce lymphoproliferative disease, the majority of infections are symptomless – especially if acquired early in life. Exposure to the virus leads to acquisition of protective immunity, which results in suppression of EBV-induced B-cell proliferation, but does not eradicate the virus, and once an individual has become infected, the virus persists latently in memory B cells for life (Babcock, Decker, Volk, & Thorley-Lawson, 1998). The mechanism controlling proliferation of EBV^+ B cells is not fully understood.

2.1.2. P. falciparum parasites

Malaria is a mosquito-borne disease that is caused by single-celled eukaryotic parasites of the genus *Plasmodium*. Infection is initiated, when an infected mosquito takes a blood meal and injects sporozoite-stage parasites into the skin. In natural infections, the number of injected sporozoites is usually low (<<100). The sporozoites are transported by blood circulation to the liver, where they invade and infect hepatocytes. Once inside the hepatocyte, the sporozoite transforms to a trophozoite that starts dividing, resulting after a period of seven to ten days in a hepatic schizont that contains thousands of merozoite-stage daughter parasites. Eventually, the hepatic schizont bursts, releasing the merozoites into the blood stream. This marks the

beginning of the blood-stage part of the infection. The merozoites invade erythrocytes and immediately transform into trophozoites, which are initially called “ring-stages” due to their prominent vacuole that give them signet ring-like appearance. These mature to trophozoites, which start to divide mitotically, resulting in up to 32 merozoites that break out of the infected erythrocyte (IE) after approximately 48 hours. Each released merozoite rapidly re-infect another erythrocyte to repeat the 48 hours asexual multiplication cycle.

While the initial liver stage of the infection is clinically silent, disease symptoms appear as increasing numbers of erythrocytes are infected and destroyed. Parasite multiplication in the blood continues until it is curbed by acquired immunity or chemotherapy. At some point, a small minority of the parasites break away from the cycle and differentiate into male and female gametocytes. These divide no further, and instead passively await being taken up by a female anopheline mosquito. Once inside the mosquito, the gametocytes differentiate into gametes and complete the sexual phase of the parasite life cycle, which ends by sporozoites migrating to the mosquito salivary glands, ready to be injected into a new host during the next blood meal of the mosquito.

Of the five species of malaria parasites that infect humans, *P. falciparum* causes the most serious form of the disease, and this species is responsible for most malaria-related serious morbidity and essentially all malaria-related deaths. The particular virulence of *P. falciparum* is related to the ability of this parasite to express adhesive proteins (mainly members of the so-called PfEMP1 family) on the surface of IEs (Hviid & Jensen, 2015). The other species of malaria parasites infecting humans do not possess genes encoding PfEMP1-like molecules.

PfEMP1 allows the IEs to adhere to host receptors on vascular endothelium, whereby they evade destruction in the spleen (Hommel, David, & Oligino, 1983; Leech, Barnwell, Miller, & Howard, 1984). The *P. falciparum* genome encodes approximately 60 *var* genes, each of which encodes a PfEMP1 variant that is functionally different (in terms of receptor specificity and

affinity) and/or antigenically different from the others (Baruch *et al.*, 1995; Gardner *et al.*, 2002; Smith *et al.*, 1995; Su *et al.*, 1995). This intra-clonal ("within clone") diversity, compounded by inter-clonal ("among clones") diversity, results in theoretically almost unlimited variability. This, and the ability of the parasite to express only one PfEMP1 variant at a time, and to switch among them from one 48-h cycle to the next, are probably important reasons for the sluggish, fragile, and incomplete development of acquired immunity to *P. falciparum* infection (see section 2.4.2).

2.2. Endemic Burkitt lymphoma (eBL)

Burkitt lymphoma (BL) is an aggressive, monoclonal non-Hodgkin-type B-cell lymphoma, which was originally described as a common facial tumour of African children (Burkitt, 1958). Three variants of BL exist according to the current WHO classification: endemic BL (eBL) that corresponds to the originally described BL, sporadic BL, and HIV-related BL. Only eBL is geographically restricted, and is found exclusively in areas of sub-Saharan Africa and Papua New Guinea, where it mostly affects children (particularly boys) below 15 years-of-age (Orem, Mbidde, Lambert, de Sanjose, & Weiderpass, 2007).

2.2.1. Burkitt lymphoma in Africa

African Burkitt lymphoma (eBL) was first reported in Uganda by the missionary doctor Alex Cook. Dennis Burkitt, an Irish surgeon, later provided the first detailed description of this type of tumour (Burkitt, 1958). The disease is remarkably restricted to geographical areas that are characterized by perennially hot and humid climates (always >15°C and with an annual rainfall >500 mm) (Burkitt, 1962b). The clinical appearance of the tumour in African children is different from that of BL in Europe, and it was initially thought that it was a jaw sarcoma because the tumour was most often found in the jaw (Magrath, 2012). However, it was soon realised that eBL has similar morphological features to the BL seen in Europe, although the clinical manifestations were different (Burkitt, 1958). Further investigations in Uganda

revealed that eBL is uniquely associated with EBV and *P. falciparum* infection (Dalldorf, Linsell, Barnhart, & Martyn, 1964; Epstein *et al.*, 1964; Kafuko & Burkitt, 1970).

Years after the discovery of eBL, another variant of BL was identified that is associated with immunosuppression, predominantly in HIV patients, and therefore called HIV-associated BL (Ferry, 2006). This tumour occurs in HIV patients of every age, especially in the early stages of the infection. Only 30-40% of the tumours are associated with EBV infection (reviewed in ref. Moormann & Bailey, 2016).

The eBL disease is the most common of all Non-Hodgkin lymphoma (NHL) in Africa. The incidence and mortality of NHL is highest in Eastern Africa. The incidence rate is 7.5 per 100,000 with a mortality rate of about 5.7 per 100,000 (Orem *et al.*, 2007).

2.2.2. Burkitt lymphoma in Ghana

Ghana is known to be endemic for eBL, and the disease is a leading cause of paediatric cancer morbidity and mortality (Nkrumah & Perkins, 1976; Segbefia, Renner, Dei-Adomakoh, & Welbeck, 2013). The intensity of the eBL led to the establishment of an NIH-supported eBL Centre at Korle-Bu Teaching Hospital, where all eBL patients from across the country were referred for diagnosis and treatment (Nkrumah & Perkins, 1973, 1976; Wosornu, Nkrumah, & Perkins, 1971). Although the tumour has been recorded in Ghana for many decades, there are no published data on its nationwide prevalence. A recent retrospective study of 495 paediatric cancer admissions to Korle-Bu Teaching Hospital 2008-2011 showed that lymphomas were the commonest cancer type (31% of all), and that the lymphomas were predominantly eBL (72% of the lymphomas, 22% of all cancers) (Segbefia *et al.*, 2013). The study did not provide an annual breakdown, but the annual average number of eBL cases 2008-2011 was about 27. More recent data have not yet been published, but summary data from the KBTH admissions database show that 18, 22, and 16 eBL patients were admitted in 2014, 2015, 2016, respectively, corresponding to an annual average of about 19 (personal communication, Professor Lorna

Renner, KBTH). A recent similar study of 309 paediatric cancer admissions to Komfo Anokye Teaching Hospital between 2012 and 2014 also showed a predominance of lymphomas (54% of all cancer cases) with eBL as the commonest type (76% of the lymphomas, 41% of all cancers). The study did not provide an annual breakdown, but the annual average 2012-2014 was about 32 cases of eBL (Paintsil *et al.*, 2015).

The incidence of eBL in Ghana appears to have declined in recent years. Clinicians attribute this to the effectiveness of malaria control measures implemented to achieve the WHO Millennium Development Goal 4, which was to reduce the rate of child mortality rates by two-thirds 1990-2015. This reduction has not led to a concomitant reduction in eBL mortality. Although effective treatment is available, the parents/guardian of the affected children often default fully or partially, because they cannot pay its cost, which is not covered by national health insurance. They often take the tumour remission seen after the initial administration of cyclophosphamide as (false) evidence of cure, and decide to default on remaining treatment. That aside, some patients are brought in too late, when the tumour has already spread to the central nervous system, bone marrow, and other organs. At that time, chemotherapy is no longer effective and the patient is put on palliative care.

2.2.3. Pathogenesis of endemic Burkitt lymphoma (eBL)

Heavy exposure to EBV and *P. falciparum* are required for development of eBL, but it is still uncertain how these pathogens cause the development of the tumour (Moormann & Bailey, 2016). The tumour originates from germinal centre or post-germinal centre B cells, as evidenced by somatically mutated immunoglobulin variable regions (Amato *et al.*, 2016; Klein, Klein, Ehlin-Henriksson, Rajewsky, & Kuppers, 1995).

EBV infects resting B cells through the viral capsid protein gp350 and gp42. These viral proteins respectively interact with complement receptor 2 (CD21) and HLA class II proteins on the surface of B cells (Nemerow, Houghten, Moore, & Cooper, 1989; Urquiza, Lopez, Patino,

Rosas, & Patarroyo, 2005). The binding of gp42 viral protein and other viral proteins such as gH, gB and gL to B cells aids in the membrane fusion leading to invasion of the virus into the cell. The survival of the virus in the B cells are due to the production of viral nuclear proteins (EBNA 1-6), latent membrane proteins (LMP 1-2) and small noncoding viral RNAs (EBERs) which are expressed at different stages of the infection (Li, Turk, & Hutt-Fletcher, 1995). The expression of EBNA2 in the initial stage of the infection leads to transcription of cellular genes that contributes to activation and immortalization of EBV-infected B cells (Wang *et al.*, 1987). The malignant transformation of an EBV-infected B-cell clone is the result of a translocation of the *c-myc* oncogene from its usual location on chromosome 8 (where its transcription is tightly regulated) to either the immunoglobulin heavy chain locus (t(8;14); 80% of cases) or one of the two light chain loci (κ (t(2;8) or λ (t(8;22); 10% of cases each) (Boxer & Dang, 2001). In eBL, the breakpoints are usually upstream (5') of the *c-myc* gene (which is therefore intact) and in the VDJ region of the affected immunoglobulin gene (Boxer & Dang, 2001; Shiramizu *et al.*, 1991). The translocation initiates eBL tumour genesis, as it deregulates *c-myc* expression and causes unrestricted proliferation of the mutated cell (Brady, Macarthur, & Farrell, 2007; Leder *et al.*, 1983) The translocation also causes the clone to differentiate, proliferate, and avoid apoptosis (Brady *et al.*, 2007).

The role of *P. falciparum* in eBL tumour genesis is much less clear than that of EBV, although the geographical restriction of eBL to areas where *P. falciparum* infection is common implies that it is critical. *P. falciparum* malaria has been reported to lead to suppression of T-cell control of EBV replication and proliferation of EBV-infected B cells (Moormann *et al.*, 2007; Njie *et al.*, 2009; Whittle *et al.*, 1984). *P. falciparum* infection may also cause a deregulation of AID, which regulates VDJ recombination, class switching, and somatic hyper-mutation, potentially increasing the risk of *c-myc* translocation (Moormann & Bailey, 2016; Thorley-Lawson *et al.*, 2016; Torgbor *et al.*, 2014). More generally, *P. falciparum* is a potent polyclonal

B-cell activator (Banic, Viana-Martins, De Souza, Peixoto, & Daniel-Ribeiro, 1991; Greenwood, 1974), and it has been proposed that this may be mediated by particular domains in PfEMP1 that promote B-cell activation and lytic EBV replication (Chene *et al.*, 2007; Donati *et al.*, 2006; Donati *et al.*, 2004).

2.2.4. Diagnosis of endemic Burkitt lymphoma

A rapid and reliable diagnosis is obviously critical to the correct management of the patient. However, a reliable diagnosis is also of key importance to any research aiming to clarify the pathogenesis of the disease, and the molecular mechanisms and antigenic targets involved in it and in the immune responses to the tumour. Ideally, diagnosis involves a combination of clinical and laboratory assessments, but in the resource-constrained setting where the disease occurs this is often not possible, due to the high cost and/or technical complexity of assays. Thus, the attending physician will usually make the diagnosis on the basis of clinical assessment combined with available ultrasound, cytology, and/or histology findings (triad).

2.2.4.1. Clinical presentation

eBL presents as a very fast-growing extra-nodal swelling, mostly in the jaw, neck, or abdominal region (Burkitt, 1958; Ogwang, Bhatia, Biggar, & Mbulaiteye, 2008). In the abdomen, the disease may affect the gastrointestinal tract, as well as the kidneys and ovaries. The tumour can spread to the central nervous system, leading to nerve damage, paralysis and weakness. Some patients in addition will show signs of lymphadenopathy (Ogwang *et al.*, 2008).

2.2.4.2. Diagnosis by cytology and histology

This type of diagnosis involves microscopic examination of cell smears (cytology) or tissue sections (histology). Cytology is the fastest laboratory investigation available, it is minimally invasive, and can be repeated if required. It is based on examination of smears of cells from tumour fine-needle aspirates (FNAs) or pleural or ascites fluid, or of cell imprints of biopsy

material (Orem, Mbidde, & Weiderpass, 2008; Troxell, Bangs, Cherry, Natkunam, & Kong, 2005). Histology involves examination of tissue sections made from core or excision biopsies of the tumour.

Histology has been the gold standard for the diagnosis of cancer/tumours, but is increasingly being replaced by cytology, immunohistochemistry, and molecular techniques, where available (Cogliatti *et al.*, 2006; Ogwang, Zhao, Ayers, & Mbulaiteye, 2011). A part of the tumour is obtained by core or excision biopsy, stabilized, and cut on a microtome to produce thin tissue sections mounted on slides. The section is then stained with haematoxylin and eosin (H&E) and analysed by microscopy (Lukande, Wabinga, & Tumwine, 2008).

In both types of evaluation, the diagnosis involves demonstration of characteristic medium-sized lymphocytes (B cells) with scanty cytoplasm, large round nuclei with uniform chromatin, and several to multiple nucleoli. Mitotic figures are often discernible, due to the high proliferation rate of the eBL cells (Ferry, 2006). "Starry sky" appearance (scattered unstained macrophages on a background of densely stained tumour cells) (Figure 4.1B) is one of the distinguishing features of eBL preparations (Chuang *et al.*, 2007).

2.2.4.3. Immunological diagnosis

The tumour B cells in eBL express many antigens of normal B cells, such as CD19, CD20, CD22, and CD79a. They also express various other broad, tumour, and activation markers such as BCL6, CD10 (CALLA), CD38, CD43, CD77, Ki 67 and p53 (Ferry, 2006; Gregory *et al.*, 1987). In contrast, eBL cells are reported not to express BCL2, CD5, CD23, CD30, CD39, CD138, Ki-24, or TdT (Ferry, 2006; Gregory *et al.*, 1987). The expression of more recent markers of B-cell maturation, differentiation, activation, and functionality (e.g., CD21, CD27, CD62L, and CXCR5) (Ampomah, Stevenson, Ofori, Barfod, & Hviid, 2014; Moir *et al.*, 2008; Weiss *et al.*, 2009) is not well established. The majority of eBL cells express IgM (but not IgD) on their surface, although some express IgG (Magrath, 1990). Regardless of the isotype

expressed, the antibody-coding genes are often rearranged and carry the extensively mutated V regions typical of post-germinal centre memory B cells (Chapman, Mockridge, Rowe, Rickinson, & Stevenson, 1995; Klein *et al.*, 1995). A recent report propose that expression of either IgM or IgG represents two distinct subsets that reflect the timing of the translocation event relative to normal B-cell receptor rearrangement and class switching (Eason *et al.*, 2016).

Diagnostic antigens or antigen combinations expressed by eBL cells can be determined by a variety of assays, such as flow cytometry and immunochemistry (which can also be used on histological specimens). The techniques all involve marker-specific antibodies conjugated to either chromophores (for flow cytometry) or enzymes (for immunochemistry) (Ogwang *et al.*, 2011).

2.2.4.4. Molecular diagnosis

Translocation of *c-myc* is a defining feature of eBL. Fluorescent *in situ* hybridization (FISH) is a highly sensitive and specific method that can be used to detect chromosomal breaks and translocations (Frickmann *et al.*, 2017). It can be performed on many different type of biological specimens, including smears and thin histology sections. FISH involves fluorochrome-labelled DNA probes that are complementary to DNA sequences at or on either side of the putative break. The detection of breaks in various type of *c-myc* FISH assays is based on the location, presence, or absence of signal from probes annealing to the target sequence (hybridization). In fusion-probe FISH, translocations are evident when probes that should normally be far apart (e.g., a *c-myc* probe (chromosome 8) and an *igh* (chromosome 14) are found adjacent to each other). In split-probe assays, breakage is evident when probes that should be adjacent (e.g., located near, but on either side of the putative breakage point in *c-myc*) are found far apart (e.g., on chromosome 8 and 14, respectively). Although FISH would potentially help increasing the accuracy of diagnoses such as eBL (Kodet, Mrhalova, Stejskalova, &

Kabickova, 2011), its cost currently prohibits its widespread use in resource-constrained settings.

2.3. The immune system

2.3.1. The organization of the immune system

The immune system is the structures, processes, and mechanisms in the body for protection against infections and cancer. It is essential for the survival of all multicellular organisms. The immune system is found everywhere in the body and is made up of cells that are able to distinguish between "self" and "non-self" antigens. The immune system consists of organs/tissues such as the thymus and the bone marrow, which constitute the primary (or generative) lymphoid organs, and the spleen and lymph nodes (and several other smaller collections of lymphoid tissues, e.g., Peyer's patches), which constitute the secondary lymphoid tissues. These and other tissues are populated by the cells of the immune system, which are either resident or recirculating. Numerous types of immune cells have been delineated, both in the innate and in the acquired immune system. Prominent examples are dendritic cells, natural killer (NK) cells, macrophages, polymorphonuclear granulocytes, and the B and T lymphocytes. Finally, the immune system also includes a range of soluble mediators and effector molecules such as chemokines and cytokines, complement, antibodies and many others

2.3.1.1. The innate immune system

The innate immune system constitutes the first line of defence against infections. It consists of physical barrier (skin, mucosal surfaces, etc.), chemical barriers (clotting factors, gastric acid, defensins, etc.), and cells such as dendritic cells, NK cells, macrophages, granulocytes etc. The characteristics of the innate immune system includes its ability to respond fast to injury (mast cells discharge their contents within seconds of being activated), the non-clonal distribution of antigen receptors on cells (all cells of a particular type express the same set of receptors), and the lack of immunological memory (the response to first exposure to an antigen and the

responses to each subsequent re-exposure to the same antigen are essentially the same, both quantitatively and qualitatively) (Keenihan *et al.*, 2003; Portugal *et al.*, 2014; Yman *et al.*, 2019).

Recognition of microbes is via pattern recognition receptors (PRRs), which recognize molecular signatures on pathogens. Important among these are the Toll-like receptors (TLRs). NK cells are cytotoxic cells that respond according to the balance of activating and inhibitory signals received through several receptors on their surface (Hamerman, Ogasawara, & Lanier, 2005). An important function of NK cells is to detect and kill cells that do not express MHC-I molecules. These are normally present on all nucleated cells in the body, and their absence often indicates viral infection and/or malignant transformation. For this reason, NK cells have been implicated as important in the immune response to eBL (Forconi *et al.*, 2018).

2.3.1.2. The adaptive immune system

The main components of the adaptive immune system are the T cells and the B cells. The function of these cells, and of the adaptive immune system in general, differs from function of the innate immune system and its cells in several important ways. T cells and B cells characteristically express clonally distributed antigen receptors. This means that each of these cells (and the clones formed by their immediate progeny) express receptors that are different from the T-cell receptor (TCR) and B-cell receptor (BCR) expressed by any other T-cell and B-cell clone, respectively. This means that the antigen receptors of these cells are extremely diverse, and can recognize an almost unlimited diversity of antigens, often with extremely high precision (the ability to discriminate between very small differences). How this diversity is generated during the maturation of these lymphocytes is described in more detail below. The clonal distribution of antigen receptors on T cells and B cells also means that the number of T cells and B cells with any given antigen specificity is initially very low. The consequence is that the ability of the adaptive immune system to respond to antigenic challenge is slower

(several days) than that of the innate immune system – at least at the first exposure. However, clonal diversity, combined with selective expansion and functional specialization of antigen-specific cells in response to antigen, affinity maturation and class switching (B cells only), and formation of memory cells that respond faster often better than naïve cells, also results in an immune response that adapts to the challenges faced, both in terms of quality and quantity. The adaptive immune system which therefore responds faster and better to subsequent exposures to an antigen recognized previously.

2.3.2. The T cells

T cells constitute one of the two major classes of lymphocytes in the adaptive immune system (the other is the B cells discussed below). The T cells have two major functions. Helper T cells facilitate the function of other cells of the immune system by receptor- and cytokine-mediated interactions, whereas cytotoxic T cells kill pathogens or cells infected by pathogens. This killing is mediated by cytokines and by the directional release of substances that disrupts the cell membrane of the pathogen or infected cell and promotes apoptosis (programmed cell death).

2.3.2.1. The T-cell receptor

The T-cell receptor (TCR) is a clonally distributed surface molecule that is the defining feature of T cells. The TCR recognizes peptides and determines the antigen specificity of T cells. Peptide recognition occurs at the distal part of the TCR, which is clonally variant (in principle, each clone of T cells has a unique TCR). The most variable part is called the complementarity-determining region (CDR), which can be subdivided into three segments: CDR1, CDR2, and CDR3. The CDR sequence thus largely determines the antigen specificity of T cells. The most variable of the CDR regions is CDR3 (Zeng *et al.*, 2012).

The TCR heterodimer is composed of either an α and a β chain (TCR-αβ), or by a γ and a δ chain (TCR-γδ). The large majority (mostly >95%) of circulating T cells are TCR-$\alpha\beta^{+}$. These

are often called conventional T cells. TCR-$\alpha\beta^+$ are part of the adaptive immune system. They respond exclusively to peptide antigens presented on major histocompatibility complex (MHC) molecules on antigen presenting cells such as dendritic cells, macrophages and B cells. All TCR-αβ cells carry either CD4 or CD8 as part of the TCR complex, where they serve as restriction elements for MHC II and MHC I, respectively. $CD4^+$ TCR-$\alpha\beta^+$ cells thus only recognize peptides presented on MHC-II, while $CD8^+$ TCR-αβ exclusively respond to peptides presented on MHC-I.

A minority of circulating T cells (usually <5%) express TCR-γδ instead of TCR-αβ, but TCR-$\gamma\delta^+$ cells are more abundant in mucosal and epithelial tissues (Brenner *et al.*, 1986). Most evidence suggests that TCR-$\gamma\delta^+$ cells play a role in local immune-surveillance and immune defence against infection and cancer (Kabelitz & Dechanet-Merville, 2015), although they have also been implicated in suppression of anti-tumour immune responses (Rei, Pennington, & Silva-Santos, 2015), and have been proposed to bridge innate and adaptive immunity (Holtmeier & Kabelitz, 2005). The function and antigen specificity of the TCR-γδ is only partially understood, but appears to differ markedly from the TCR-αβ. TCR-$\gamma\delta^+$ cells are not MHC-restricted, and do not seem to require antigen-presenting cells. Nevertheless, some TCR-$\gamma\delta^+$ cells (including some $V\delta1^+$ cells) recognize MHC-like molecules (CD1c, CD1d, EPCR, MICA, MICB,) or non-peptide antigens associated with these molecules (Eleme *et al.*, 2004; Faure, Jitsukawa, Miossec, & Hercend, 1990; Groh *et al.*, 1999; Roy *et al.*, 2016; Spada *et al.*, 2000; Willcox *et al.*, 2012). Essentially all TCR-$\gamma\delta^+$ cells are CD4-negative, while some (mainly $V\delta1^+$ cells, see below) express CD8. The remainder usually the majority are double-negative; express neither CD4 nor CD8)

There are two main subtypes of TCR-$\gamma\delta^+$ cells, which can be distinguished by the expressed δ chain (either Vδ1 or Vδ2) (Triebel & Hercend, 1989). Vδ2 is usually expressed by cells also expressing a particular Vγ chain (Vγ9). The Vγ9Vδ2 subset is normally by far the dominant

subset, and is also by far the most studied type of TCR-$\gamma\delta^+$ cells. These cells recognize non-peptide antigens in an MHC-unrestricted manner (Born, Kemal Aydintug, & O'Brien, 2013; Constant *et al.*, 1994). Most of the remaining TCR-$\gamma\delta^+$ cells express a receptor composed of Vδ1 paired with one of several Vγ chains.

2.3.2.2. T-cell development and generation of clonal diversity

T-cell progenitors are generated in the bone marrow, but they migrate to the thymus, directed by adhesive interaction between P-selectin on the progenitor cells and PSGL1 on thymic epithelium (Rossi *et al.*, 2005). Once in the thymus, the pro-T cells undergo several maturation steps, including positive and negative selection, before the small proportion (usually <5%) that survive these processes are finally released into the circulation as mature, but naïve (foreign antigen-inexperienced) T cells (Gameiro, Nagib, & Verinaud, 2010). The first precursor cells (pro-T cells) do not express TCR. They do not express CD4 or CD8 either, and are therefore called double-negative (DN). The DN cells first begin assembling the β-chain (or, in a minority of cases, δ-chain) of the TCR, which involves a random combination of one of each of the multiple V, D, and J elements that make up the CDR region of the TCR. This process, called clonal VDJ recombination, also involves insertion and removal of nucleotides, and results in an enormous TCR diversity (Ru *et al.*, 2015). If the rearrangement is productive (encodes a functional β- (or δ-) chain), the cell, which is now called a pre-T-cell, is positively selected and can progress to the next maturation step. If not, it is deleted by apoptosis. β-chain$^+$ pre-T cells proceed to assemble the α-chain in a manner similar to that of β-chain assembly (except that no D elements are involved). Cells that are δ-chain$^+$ assemble the analogous γ-chain instead. The successful (VJ) recombination of the TCR, acquires both CD4 and CD8 at this stage the cell, is now called double-positive (DP). (at least in the case of TCR-$\alpha\beta^+$ cells) (Shah & Zuniga-Pflucker, 2014). TCR-$\alpha\beta^+$ DP cells interact through their TCR with self-peptide/MHC complexes expressed by thymus epithelial cells. Those that bind strongly to these complexes

(whether MHC-I or MHC-II) are potentially auto-reactive. Most of them are deleted by apoptosis (negative selection), although some MHC-II complex-binding cells instead differentiate into $CD4^+$ regulatory T cells (Passos, Speck-Hernandez, Assis, & Mendes-da-Cruz, 2018). Cells that show low-affinity interactions are allowed to proceed, whereas cells that do not bind peptide/MHC-complexes at all are deleted ("Death by neglect"). The surviving cells differentiate into single-positive (SP) cells that are either $CD4^+$ or $CD8^+$ by down-regulating expression of the other MHC restriction element. Whether CD4 or CD8 is down-regulated, depends on the type of MHC molecule in the self-peptide/MHC complex recognized by the nascent T-cell. Cells recognizing MHC-I complex down-regulate CD4, whereas those recognizing MHC-II complex down-regulate CD8 (Anderson *et al.*, 2005; Gameiro *et al.*, 2010; Passos *et al.*, 2018). The resulting mature (but naïve) TCR-$\alpha\beta^+$ T cells are released into the peripheral blood and recirculate through secondary lymphoid tissues, ready to differentiate into effector and memory cells when exposed to a foreign antigen.

TCR-$\gamma\delta^+$ T cells undergo a thymic maturation process that is similar to that of TCR-$\alpha\beta^+$ T cells (Vermijlen, Gatti, Kouzeli, Rus, & Eberl, 2018). However, the fact that antigen recognition by TCR-$\gamma\delta^+$ cells is not restricted by MHC shows that some details must be different, although exactly which and how appear not to be known (Ciofani & Zuniga-Pflucker, 2010; Xiong & Raulet, 2007). There are several key differences between the emerging TCR-$\alpha\beta^+$ and TCR-$\gamma\delta^+$ cells. Thus, the majority of TCR-$\gamma\delta^+$ remain DN even at the time of release from the thymus, although some (mainly Vδ1 cells, see below) have become $CD8^+$ (Ciofani & Zuniga-Pflucker, 2010). For some reason, cells that use Vδ2 also preferentially express a Vγ9 chain in their TCRs (Vδ2Vγ9), which furthermore show very limited diversity among clones, very unlike TCR-$\alpha\beta$ cells and more like cells of the innate immune system (Willcox *et al.*, 2018). In contrast, TCR-$\gamma\delta^+$ cells that use Vδ1 rather than Vδ2 in the expressed TCR employ a variety of Vγ chains (including Vγ9). This latter subset, usually referred to as Vδ1 cells

expresses TCRs with very diverse CDR regions, and in that respect they resemble conventional (TCR-$\alpha\beta^+$) T cells more than they do $V\delta2^+V\gamma9^+$ TCR-$\gamma\delta$ T cells (Davey *et al.*, 2018).

2.3.2.3. The T-cell response to infection (differentiation and memory)

Naïve TCR-$\alpha\beta^+$ cells recirculate between the peripheral blood and the secondary lymphoid tissues where they interact with peptide/MHC complexes on antigen-presenting cells. Upon TCR-specific recognition of their cognate antigen, T cells become activated, and start to proliferate (clonal expansion) and differentiate into effector cells. $CD4^+$ cells differentiate into several types of so-called helper T cells that provide a variety of instructive cytokine signals to a wide variety of cells in the immune system, including naïve T cells. $CD8^+$ cells instead differentiate into cytotoxic effector cells that can kill infected and cancerous MHC-I^+ cells directly, or through the release of molecules that induce cell lysis, such as perforin and granzyme. The effector cells have different recirculation patterns than naïve cells, and are more prone to migrate into tissues at sites of infection or inflammation. Upon resolution of the infection that prompted their activation and differentiation, expanded clones contract to pre-infection levels, leaving only a small population of so-called memory cells that are able to respond faster than naïve cells in case of re-infection. These memory cells recirculate much like naïve T cells. Four maturation-stage subsets of T cells (and B cells) are often recognised based on cell surface expression of CD27 and CD45RA (an isoform of the leukocyte common antigen) Figure 2.1). The four subsets are naïve cells ($CD27^+CD45RA^+$), central memory (CM) cells ($CD27^+CD45RA^{neg}$), effector-memory (EM) cells ($CD27^{neg}CD45RA^{neg}$), and terminally differentiated effector-memory cells (EMRA) ($CD27^{neg}CD45RA^+$) (Dunne *et al.*, 2013; Golubovskaya & Wu, 2016).

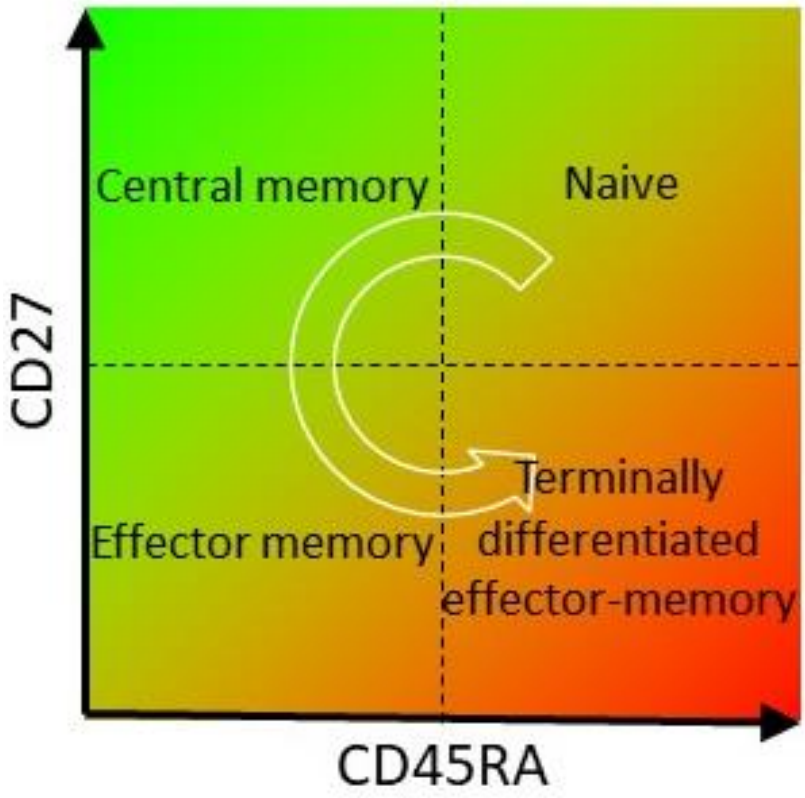


Figure 2.1. T-cell maturation

Schematic representation of the maturation-related changes in expression of CD27 and CD45RA on T cells.

TCR-$\gamma\delta^+$ cells either circulate or reside in various tissues, not least near epithelial surfaces and in lymphoid tissues such as the spleen and the liver. As mentioned above, they recognize antigen directly rather than via MHC, and their TCRs thus function more like the receptor of B cells (see below) than the TCR of conventional (TCR-$\alpha\beta^+$) T cells. (Allison, Winter, Fournié, Bonneville, & Garboczi, 2001; Holtmeier & Kabelitz, 2005). Functionally, they resemble cytotoxic $CD8^+$ TCR-$\alpha\beta^+$ T cells (apart from the lack of MHC-I restriction) and NK cells (although they are not inhibited by MHC-I) (Smyth *et al.*, 1990).

2.3.3. The B cells

B cells constitute the other major class of lymphocytes in the adaptive immune system aside the T cells described above. The role of B cells is to produce antibodies, which are soluble effector molecules that can recognize particular molecular features with extremely high specificity. Antibodies can protect against infection directly by interfering with the infectivity or other vital functions of pathogens (neutralization). They can also enhance the ability of phagocytic and cytotoxic effector cells to destroy extracellular pathogens or cells infected by intracellular pathogens (opsonisation).

2.3.3.1. The B-cell receptor and antibodies

The BCR is a heterodimeric transmembrane protein, expressed on the surface of all normal B cells except plasma cells (terminally differentiated effector B cells). The BCR consists of two identical heavy chains: corresponding to the β-chain of the TCR and two identical light chains which also corresponding to the α-chain of the TCR, and can be either κ or λ (Schroeder & Cavacini, 2010).

The light chains are each composed of an N-terminal variable (V) region and a C-terminal constant (C) region. These regions each contain one ~110 amino acid Ig domain (V_L and C_L, respectively) held together by disulphate bonds. The heavy chains are each composed of a single-domain V region (V_H), followed by a constant region composed of three-four Ig domains (C_H1-C_H4).

The BCR also exists as soluble effector molecules called antibodies. These are produced by terminally differentiated B cells (plasma cells), which downregulate membrane-bound BCR and instead secrete large quantities of the corresponding antibody.

The antigen binding site of both membrane-bound BCR and antibody (soluble BCR) is composed of the V_L and V_H, in particular CDR (as for the TCR).The effector function of antibody is determined by its C_H2-C_H4 domains, and these domains can be used to divide BCRs

and antibodies into five classes (IgA, IgD, IgE, IgG, IgM; IgD only exists in membrane-bound form). IgA and IgG can be further sub-divided in two (IgA1 and IgA2) and four (IgG1-IgG4) sub-classes, respectively.

2.3.3.2. B-cell development, selection, and generation of clonal diversity

B-cell generation and development occur in the bone marrow throughout life. The development of the B cells involves maturation and selection processes (positive and negative) similar to those of T cells. B cells develop from haematopoietic stem cells that have differentiated into multipotent progenitors then to common lymphoid progenitors. In the bone marrow, these cells first develop into pro-B cells, which contain germline BCR-encoding genes and do not express BCR. The pro-B cells develop into pre-B cells through VDJ recombination of the heavy chains. If the recombination is productive, the pre-B-cell expresses heavy chains paired with surrogate light chains (Martensson, Almqvist, Grimsholm, & Bernardi, 2010). Cells with unproductive rearrangements are deleted. This maturation step is followed by V-J rearrangement of the κ light chain genes, which if successful allows the expression of complete and functional BCRs on the surface. If the κ-chain rearrangement is unsuccessful, the process is repeated with the λ genes. If this also fails, the B-cell is deleted. Self-reactive B cells are removed by negative selection, similar to that occurring for T cells, but with the addition of receptor editing, which involves additional modification of the light chains to remove auto-reactivity. The immature B cells migrate to the spleen, where their transition to mature, naïve B cells, which express IgM and IgD on the surface, is completed (Loder *et al.*, 1999; Rolink, Schaniel, Andersson, & Melchers, 2001). They then migrate via the peripheral circulation to secondary lymphoid tissues, where they take up residence.

2.3.3.3. The B-cell response to infection (differentiation and memory)

Naïve B cells are activated in secondary lymphoid tissues when recognizing their cognate antigen via the surface-expressed BCR. Full differentiation, affinity-maturation, and class-

switching of the activated B-cell requires contact- and cytokine-mediated help from particular helper T cells, called follicular helper T cells (T_{FH} cells) (Crotty, 2015). Affinity maturation occurs via a process called somatic hyper-mutation (SHM), which causes daughter cells of the expanding clone to express BCRs that are variants of the parental BCR. Daughter cells that express BCRs with higher affinity for the activating antigen are preferentially expanded. Depending on the specific cytokine-mediated signals received from the T_{FH} cells, activated B cells may also undergo class-switching, which involves replacement of the original IgM-type C_H region-encoding gene with downstream genes encoding the C_H of the other antigen classes. The regulation of SHM and class switching involves activation-induced deaminase (AID). Over the course of repeated antigen exposure and multiple B-cell expansion and contraction cycles, this can result in differentiation into B cells that either express (memory B) or secrete (long-lived plasma cells) antibody with very high antigen-affinity and appropriate effector function (Crotty, 2015; Victora & Nussenzweig, 2012). In the absence of T_{FH} cells responding to the same antigen, B-cell activation results in limited clonal expansion and differentiation into short-lived plasma cells that mainly secrete low-affinity, IgM-class antibody (Hua & Hou, 2013; Vos, Lees, Wu, Snapper, & Mond, 2000). Most antigens involved in T-independent B-cell responses contain ligands for TLR (Alugupalli, Akira, Lien, & Leong, 2007). TLR recognizes molecule that is broadly shared by pathogens but distinguishable from host molecules. TLR modify the innate immune system via single membrane-spanning proteins that recognize ligands with structurally conserved molecular patterns.

B-cell differentiation can be assessed phenotypically, in a similar way to that described above for T cells. Other features can also be assessed phenotypically, such as the proportion of memory B cells that appear to be “atypical” or “exhausted”. The emergence of such cells, which appear to be functionally impaired, have been described in various chronic infections involving

continued B-cell stimulation, such as HIV and *P. falciparum* (Illingworth *et al.*, 2013; Moir *et al.*, 2008; Weiss *et al.*, 2009).

2.4. Immunity to the eBL-related infections and to eBL tumour cells

The pathogenesis of eBL involves infections with EBV and *P. falciparum*, and the immune responses to those infections also impact on the immune response to the tumour cells. As an example, it has been proposed that *P. falciparum*-infection can undermine the cytotoxic function of EBV-specific $CD8^+$ T cells, allowing the virally infected B cells to thrive, thereby increasing the risk of their malignant transformation (Aka *et al.*, 2013; Chattopadhyay *et al.*, 2013).

2.4.1. Immunity to Epstein-Barr virus

The immune response to EBV occurs during the lytic phase of the infection, and involves both innate and adaptive immune responses (Landais, Saulquin, & Houssaint, 2005). Upon infection, EBV proliferates shortly in the B cells of the oropharynx. This leads to recruitment of various types of immune cells such as macrophages, dendritic cells, NK cells and neutrophils. Virus recognition is via receptors (PRRs), in particular TLR3 and TLR9 (Chijioke, Azzi, Nadal, & Munz, 2013), leading to production of antiviral cytokines and phagocytosis of viral particles. Activation of cells of the adaptive immune system follows, via presentation of viral antigens on MHC molecules on antigen-presenting cells and cytokines. Cytotoxic NK cells participate in the destruction of EBV-infected cells, and also secrete cytokines that activate cells of both the innate and the adaptive immune system (Varela-Calvino, Skowera, Arif, & Peakman, 2004). T cells and B cells help in controlling the infection by cytotoxic T cell destruction of infected cells , enhancing innate and adaptive responses ($CD4^+$ T cells), and by secreting virus-specific antibodies (B cells).

The virus can evade immune responses by down-regulating expression of immunogenic antigens and by inducing the production of antagonists to activation of immune effector cells (Shen, Zhang, Sun, Wu, & Qian, 2015).

2.4.2. Immunity to P. falciparum

Naturally acquired immunity to *P. falciparum* malaria takes several years and multiple disease episodes and infections to develop. Substantial clinical protection is eventually acquired, but sterile immunity is probably never achieved following natural exposure to this parasite (Hviid, 2005, 2010). This means that a substantial proportion of individuals in *P. falciparum*-endemic areas carry low-grade, often sub-microscopic, infections for extended periods of time.

Immune responses involve both innate and adaptive responses, mostly targeting the asexual blood stage of the parasite life cycle, which is associated with all the clinical manifestations of *P. falciparum* infection (Artavanis-Tsakonas & Riley, 2002; Bouharoun-Tayoun, Attanath, Sabchareon, Chongsuphajaisiddhi, & Druilhe, 1990; Cohen, McGregor, & Carrington, 1961; Moormann, Nixon, & Forconi, 2019). Cytokine and cytotoxic responses by cells of the innate immune system, such as NK cells and $V\gamma9V\delta2^{+}$ γδ T cells appear to dominate early on (Artavanis-Tsakonas & Riley, 2002; Howard *et al.*, 2018; Moormann *et al.*, 2019). These innate responses are later superseded by adaptive responses, in particular antibodies. Antibodies to antigens on the surface of merozoites and IEs appear to be of particular importance, and although antibody responses are often transient, immunological memory appears more robust (Hviid, Barfod, & Fowkes, 2015; Kinyanjui, Bejon, Osier, Bull, & Marsh, 2009; Recker *et al.*, 2004). Cytophilic IgG1 and IgG3 dominate the antibody response to most parasite antigens (Cavanagh *et al.*, 2001; Chizzolini, Trottein, Bernard, & Kaufmann, 1991; Megnekou, Staalsoe, Taylor, Leke, & Hviid, 2005; Wahlgren, Berzins, Perlmann, & Persson, 1983). Among these, antibodies with specificity for PfEMP1 (and possibly other IE surface antigens)

are assumed to be of particular importance, as they may interfere with the IE sequestration and enable efficient removal of opsonized IEs (Arora *et al.*, 2018; Fried, Nosten, Brockman, Brabin, & Duffy, 1998; Hviid & Jensen, 2015). The chronic nature of *P. falciparum* infection in areas of stable transmission causes persistent activation of the immune system, and high IgG synthesis and hyper-gammaglobinaemia are therefore common (Cohen *et al.*, 1961; Curtain, Kidson, Champness, & Gorman, 1964). As this includes both parasite-specific and non-specific antibody, it has long been speculated that *P. falciparum* infection can cause polyclonal B cell activation due to the presence of parasite mitogen(s) and/or malaria-associated loss of T-cell control of B-cell activation (Greenwood, 1974; Greenwood & Vick, 1975). Some particular PfEMP1 domains appear to constitute examples of the former (Chene *et al.*, 2007; Donati *et al.*, 2006; Donati *et al.*, 2004). Evidence of malaria-induced loss of T-cell control of B-cell proliferation has long been available, but has not been much studied since the early observations (Whittle *et al.*, 1990; Whittle *et al.*, 1984). In addition to the response of $V\gamma9V\delta2^{+}$ γδ T cells mentioned above, γδ T cells using Vδ1 rather than Vδ2 also appears involved in the immune response to *P. falciparum* malaria, possibly as regulators of parasite-activated B cells (Hviid *et al.*, 2019).

Acquisition of immunity to severe and life-threatening forms of *P. falciparum* malaria precedes immunity to uncomplicated, sub-clinical, and asymptomatic infection. This appears to be because those PfEMP1 variants that enable fast parasite multiplication (and severe disease) dominate early in life, and because those variants are gradually replaced by less and less virulent variants as protective immunity to the more virulent types is acquired (Bull *et al.*, 2000; Bull, Lowe, Kortok, & Marsh, 1999; Cham *et al.*, 2009; Cham *et al.*, 2010; Nielsen *et al.*, 2002). The virulent variants furthermore tend to be relatively conserved among different clones, whereas the variants associated with sub-clinical and asymptomatic infections are very diverse (Hviid & Jensen, 2015). These features go a long way towards explaining the relatively

rapid acquisition of immunity to severe malaria, and the *de facto* absence of sterile immunity to *P. falciparum* malaria acquired by natural exposure. Chronic, low-grade parasitaemia may contribute to the maintenance of immunity to clinical disease, but have major consequences for mothers and their newborns (Chen *et al.*, 2016).

2.4.3. Immunity to the eBL tumour cells

eBL is a disease of the immune system, but the immune system is also engaged in restricting the growth of the tumour. This probably mainly involves cytotoxicity against the malignant cells, although the mechanisms, in particular the molecular targets recognized on the tumour cells – are not well known. NK cells appear to be important effectors (Forconi *et al.*, 2018), while cytotoxic attack on the tumour cells by $CD8^+$ T cells is undermined by the low expression of MHC Class I on eBL cells (Andersson, Stam, Klein, Ploegh, & Masucci, 1991). Activation of $CD4^+$ T cells by tumour-associated antigens presented on MHC Class II is also compromised (Amria, Cameron, Stuart, & Haque, 2008), apparently via the EBV protein, BZLF1, which can reduce interferon-γ-induced MHC Class II expression (Morrison, Mauser, Wong, Ting, & Kenney, 2001). A minor population of TCR-$\gamma\delta^+$ cells, which are cytotoxic, but not MHC Class I-restricted like conventional $CD8^+$ T cells, have nevertheless been shown to recognize MHC-like molecules (MHC-Ib, -c, -d), or non-peptide antigens associated with these molecules (Roy *et al.*, 2016). This is of potential direct relevance to this thesis work because some activated and malignant B cells express CD1c and CD1d, and because these antigens appear to be recognised by TCR-$\gamma\delta^+$ cells, including $V\delta1^+$ cells (Delia *et al.*, 1988; Faure *et al.*, 1990; Roy *et al.*, 2016; Small *et al.*, 1987). MICA, another MHC-related molecule, is also expressed by some activated and transformed B cells. MICA is an NKG2D ligand, and MICA may also be involved in NK-cell-specific recognition of eBL tumour cells (Kim & Lee, 2015).

Overall, the ability of the immune system to control eBL tumours appears undercut by the multiple ways they have evolved to evade immunity (God & Haque, 2010). This is compounded

by the ability of the tumour cells to escape apoptosis, underpinning the massive proliferative potential of this cancer (Kelly, Milner, Baldwin, Bell, & Rickinson, 2006).

3. Methodology

3.1. Research ethics of study

The protocols of the study were reviewed and approved by the Institutional Review boards at Komfo Anokye Teaching Hospital (KATH) (Appendix 1 and Appendix 2), Korle-Bu Teaching Hospital (KBTH) (Appendix 3), and Noguchi Memorial Institute of Medical Research (Appendix 4). Only patients whose parent(s) or legal guardian had given written informed consent after the explanation of the aim and contents of the project in the appropriate local language were included in the study. A written assent was obtained from each of the children aged seven years and above before sample collection as recommended by WHO.

3.2. Study sites

All patients included in this study were recruited either at KATH or at KBTH. These two tertiary hospitals are located in Kumasi (Ashanti region) and in Accra (Greater Accra region), respectively. Control samples were collected from a district health centre (Afrancho District Health Centre) and a nearby school in the Ashanti region (Appendix 5), chosen because two of the eBL patients recruited for the study lived in the district.

3.2.1. Child Health Department, KATH

The Komfo Anokye Teaching Hospital (KATH) serves as a referral centre for northern Ghana. The Child Health Department was established to provide the best possible health services to children in the Kumasi metropolis and beyond. The department takes care of all children between the ages of two months and 14 years. About 20,000 out-patients and 11,000 in-patients are seen per year. The department has an Oncology Unit that receives about 150 paediatric cases per year, mainly Burkitt lymphoma patients (personal communication, Dr. Vivian Paintsil).

3.2.2. The Department of Child Health, KBTH

Korle-Bu Teaching Hospital (KBTH) serves as referral centre for southern Ghana. The Department of Child Health provides care for referred medical and surgical cases among children under 13 years-of-age. Cases are referred from all over Ghana, but in particular from the southern half of the country. About 36,000 children per year are seen at the out-patient unit. About 30% of these are admitted to the in-patient wards for further management. The department has an Oncology Unit that receives paediatric cases from the region, including Burkitt lymphoma patients.

3.2.3. Afrancho Health Centre (AHC)

AHC is a district health centre that serves 13 communities and sub-communities in the Afigya Kwabre district of the Ashanti region. AHC receives averagely 100 patients in a day. It runs mainly out-patient clinic with a few detentions. It had only six beds at the time of sample collection. The health centre does not offer admission services.

3.3. Study participants, collection, and pre-preparation of samples

3.3.1. Patients and healthy controls

Patients, aged 1-15 years, admitted to KBTH or KATH with tumours in the face, neck, or abdomen, and with a presumptive clinical diagnosis of lymphoma were recruited into the study if approved by the attending project physician. Cytological examinations of diagnostic fine-needle aspirates (FNAs; see below) were performed and the children diagnosed and classified as having eBL or non-eBL by the attending Physician. A list with details of all patients included in the study is available (Appendix 6).

Two groups of age-matched children were included as controls. One group consisted of children with *P. falciparum* malaria, while the other consisted of healthy children without *P. falciparum* infection (negative *P. falciparum* blood smears and rapid diagnostic tests). These

children were recruited for participation in the study if approved by an attending project physician.

3.3.2. Collection and processing of peripheral blood samples

A peripheral blood sample (5-10 mL) was collected from patients and control children into Lithium-heparinised tubes by venipuncture using syringes and needles. For control children, an additional sample was collected into EDTA tubes for rapid diagnostic testing for malaria parasites (Smartcare, Somerset USA), and thin and thick blood films were prepared for microscopy of Giemsa-stained slides.

The blood samples were centrifuged (2,000 rpm; 5 min) and aliquots of 1 mL of plasma separated and frozen (-80°C [patients] or -30°C [controls]). The pellet was re-suspended in an equal volume of wash_solution [(RPMI 1640 (Gibco/ScienCell), 10% HI-FBS, 1% 100µM penicillin/streptomycin, 1% 25mM HEPES and 1% L-Glutamine (Sigma-Aldrich, Germany)]. Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Fresenius Kabi Norge AS) density gradient separation according to the manufacturer's instructions, washed ×3 in wash solution, and counted (Trypan blue-stained cells, Improved Neubauer Chamber). Cell concentration was adjusted to 1×10^7 PBMC/mL using wash solution supplemented with 10% DMSO, and 1-mL aliquots gradient-frozen according to a previously described protocol (Hviid, Albeck, Hansen, Theander, & Talbot, 1993). The PBMC were stored in liquid Nitrogen until use.

3.3.3. Collection of fine-needle aspirates from tumours

From each of the participating cancer patients, a fine-needle aspirate (FNA) sample was collected from the tumour for diagnosis by microscopic assessment of tumour cell morphology and for project laboratory investigations. Cells were aspirated with a 23/25G hypodermic needle from the hardest part of the tumour after palpation with or without ultrasound guidance.

Two drops of the aspirate were placed on frosted slides (4-6 slides per patient) and smears were prepared. Two smears were fixed (95% ethanol, 15 min) while wet and used for on-site morphology diagnosis. Remaining smears were air-dried at room temperature. Two of the air-dried smears were fixed in absolute methanol. All slides not used for immediate diagnosis were stored at room temperature for subsequent project-related laboratory analysis.

The remaining cell suspension was centrifuged (1,300 rpm; 10 min), washed 3 times, counted to determine cell concentration, and cryopreserved as described for PBMC above.

3.3.4. Archival tissue samples

Archival paraffin-embedded tissue block samples from patients diagnosed with eBL and other childhood lymphomas from 2010 to 2017 that were available at the time of sample collection were retrieved. All the tissue blocks were re-embedded in paraffin wax. Sections (3-4 µm thickness) were cut and stained with haematoxylin and eosin to confirm the presence of the tumours in the tissue blocks by light microscopy. Sections of the same thickness were mounted on pre-coated slides (Leica BOND^{TM} Plus) and stored at -20°C until use in the project. A list with details of all the donors of the archival samples is available in Appendix 7.

3.4. Laboratory methods

3.4.1. Giemsa-staining of blood films and FNA smears

Air-dried thick and methanol-fixed thin blood smears were covered (2 min) with Stock Giemsa stain (locally prepared), washed with tap water and air-dried. After drying, the smears were cleared by rinsing in xylene for one minute and mounted with DPX (Distreen Plasticizer Xylene) and cover-glass. When set/dried, slides were examined by light microscopy, using the ×10 and ×40 magnification for malarial parasites (blood films) and cell morphology (FNA smears).

3.4.2. Haematoxylin and eosin staining of FFPE sections

Prospective staining of FFPE sections were performed according to the standard haematoxylin and eosin protocol. Briefly, sections were dewaxed in three changes of Tissue-Clear (Sakura Finetek Europe B.V), hydrated in descending grades of ethanol (100% -3x, 96% - 2x, 80% - 2x and 70% - 2x), then finally into water. The dewaxed and hydrated sections were stained in haematoxylin (nuclear stain), blued in Scotch's tap water substitute, and counter-stained with eosin (cytoplasmic stain). The sections were dehydrated again through ascending grades of ethanol (70% - 2x, 80% - 2x, 96% - 2x and 100% - 3x), cleared in 2 changes of Tissue-Clear, and mounted.

3.4.3. Light microscopy of FNA smears and FFPE sections

Morphological assessment of tumour FNA smears and FFPE tumour sections was done blinded to the original diagnosis. For FNAs, the diagnosis of eBL was based on finding of monotonous, medium-sized, blastoid cells with basophilic cytoplasm usually containing lipid vacuoles and round nuclei with finely clumped chromatin containing multiple medium-sized basophilic nucleoli. For the FFPE sections, the eBL diagnosis was based on finding of a solid tumour composed of medium-sized, blastoid cells with diffuse monotonous growth pattern, cohesive with squared-off borders of retracted basophilic cytoplasm (usually with visible lipid vacuoles), and round nuclei with finely clumped chromatin containing multiple medium-sized basophilic nucleoli (Figure 4.1A). Presence of tingible body macrophages, giving rise to a characteristic "starry sky" appearance, and mitotic figures was also considered (Figure 4.1B). Only vital and sufficiently fixed tumour tissues were evaluated, and samples containing solely necrotic tissue and/or autolyzed tumour tissue were discarded.

3.4.4. FISH staining of tissue sections

The frozen tissue sections were brought to room temperature and fixed to the slides (58°C, 45 min). Paraffin was removed in three changes of Tissue-Clear (Sakura Finetek Europe B.V.),

employing 10 dips in the first two changes and 15 min in the third, followed by three changes of 100% ethanol (10 dips in the first two and 1 min in the last change). The sections were subsequently washed in two changes of 96% ethanol followed by two changes of 70% ethanol (10 dips in the first, and 1 min in the second change). The sections were then rinsed in two changes of wash buffer (Tris-HCl buffer, pH 7.6) (3 min each), followed by pre-treatment buffer (MES-buffer, pH 6.55) at boiling temperature (10 min) in a microwave oven (Whirlpool, CRISP) and cooling (15 min) in the pre-treatment buffer. The sections were then rinsed in two changes of wash buffer as above, blotted with tissue paper and digested with pepsin (ZytoVision, GmbH, Germany; 8 min, room temp), rinsed again as above, dehydrated in two changes of 70%, 96%, and 100% ethanol (10 dips in first change of each, and 2 min in the second). To further enhance dehydration, sections were subsequently air-dried (room temp, 15 min). *c-myc/igh* fusion probes (ZytoVision, Germany, z-2105-200) and *c-myc* dual split probes (DAKO, Agilent, USA) were added (1.5-10µL, depending on the size of the section), the sections were then covered with coverslips (12×12 or 22×22 mm^2) and sealed with Fixogum (ZytoVision, E-4005-126). Following incubation (85°C, 5 min followed by 37°C, overnight) of the slides on a hybridiser (ThermoBrite, Statspin, Abbot Molecular), the seals and coverslips were removed, and the sections rinsed (1 min) in Stringent buffer (SSC Buffer 20x concentrate (Sigma), Triton X-100 (Sigma)). The sections were transferred to a Coplin jar two-thirds filled with Stringent buffer and incubated (64°C, 10 min) in a water-bath to remove unspecific binding of the probes. Finally, the sections were rinsed in two changes of wash buffer, dehydrated, and mounted in fluorescence mounting medium (10 µL) containing DAPI stain (Vectashield®, Vector Lab. Inc., Burlingame, CA 94010). The above procedures were done in batches of ten sections plus known positive (BL tissue) and negative (reactive lymph node) controls.

3.4.5. FISH staining of FNA smears

FNA smears were fixed (3.7% formalin, 5 min) and rinsed in two changes of wash buffer (3 min each). The smears were further dehydrated as above and divided into two halves. Probes as above (10 µL each) were added to one half each. The slides were covered, sealed, hybridized and processed further as described above.

3.4.6. Fluorescence microscopy

Each FISH slide (blinded to the original diagnosis of the patient) was evaluated for *c-myc* breaks (Figure 4.4A) and *c-myc/igh* fusion (Figure 4.4B), using an Olympus BX61 fluorescence microscope equipped with an attached Zeiss camera. For the split probe, the sample was scored positive if >10% of the red and green signals were clearly separated. For the fusion probes, the sample was scored positive if >10% of the red and green signals were immediately next to each other or overlapping (yellow signal).

Images were acquired with an LSM710 confocal microscope from Zeiss (Obrekochen, Germany). The images were taken with a 63× 1.4 NA oil immersion objective, and the used excitation laser lines were 405, 488 and 561 nm, respectively, assembled by Zeiss. Three tracks were used for acquisition, with the 405 and 561 channels recorded with PMTs, while the 488 channel was recorded with a GaAsP detector. The matching dichroic mirrors were used for all channels, and the pinhole was set at 1AU for 525 nm. Sampling in X, Y and Z was at Nyquist.

3.4.7. Immunohistochemistry

Expression of C-MYC was evaluated according to the guidelines for routine diagnostic work-up at the Department of Pathology, Herlev Hospital (Pedersen *et al.*, 2019). FFPE tissue sections were pre-treated (pH=9.0) on a PT Link pre-treatment module (Dako), including paraffin removal, rehydration, and epitope retrieval, and subsequently stained in a Dako Autostainer Link 48, using EnVision FLEX+ visualization kits (Dako) and monoclonal C-MYC antibody (Epitomics; clone y69/EP121, 1:100 dilution EnVision Flex Antibody Diluent). All

steps were completed according to the manufacturer's instructions. The stained sections (Figure 4.1C) were evaluated and scored using a double-headed microscope (Olympus BX51), equipped with a colour view camera and analySIS getIT 5.0 software (Soft Imaging Systems Munster, Germany). MYC expression was evaluated on full slide sections in hot spot areas and all staining intensities were included as previously described (Clark Schneider *et al.*, 2016). A section was graded positive when >75% of the nuclear cells in tumour hot spot areas were stained brown.

3.4.8. Multi-parameter flow cytometry

Flow cytometry is a valuable technique to identify and quantify expression of components in and on cells. It relies on specific antibodies or other reagents that are specific for the cellular component of interest, and which can be used to quantitatively detect it. The detecting reagent (whether antibody or not) must either be fluorescent, conjugated to a fluorescent molecule, or labelled with a secondary fluorescent reagent. Analysis of multiple components is often possible by using multiple fluorochromes.

In this study, 14 antibodies, in addition to a live-dead fluorescent stain, were used. PBMC and FNA cells were analysed by 16-colour flow cytometry using an LSR Fortessa 5 instrument (BD), equipped with five lasers. The antigen specificities and other characteristics of the antibody reagents used are listed in Appendix 8. It is important, not least when using multi-parameter analysis as here, to carefully optimise the staining protocols, to ensure concentrations of all reagents that maximize specific labelling (sensitivity), while minimizing unspecific labelling and cross-detection due to fluorochromes with overlapping emission spectra. In this study, the choice of fluorochromes for the selected multicolour panel was made using FluoroFinder software tool, customized to the settings of the instrument used to acquire the data (https://flowcytometry.ku.dk/hardware/fluorofinder/). Furthermore, all antibodies were titrated to maximize sensitivity and minimize spectral overlap. In brief, serial 2-fold dilutions (starting

at the manufacturer's recommended dilution, which is often too low) of each antibody alone, as well as of antibody combinations, were tested until the best concentrations of all antibodies in combination was achieved (Appendix 8).

Frozen cells to be analysed were thawed partly at 37°C and transferred into 10 mL of wash buffer (1% BSA in PBS). The cell suspension was washed (centrifuged ×3 at 2,000 rpm; 6 min each). After the final wash, the cells were suspended in 3 mL of wash buffer and counted in Trypan Blue, using an improved Neubauer Counting chamber. A suspension of 2 million cells/mL was prepared for multi-parameter staining.

Into 96-well plates were pipetted 100 µL of cell suspensions and 100 µL of the pre-diluted antibody mix was added, mixed gently, and incubated (20 min; room temp. in the dark). The cells were then washed ×3 in wash buffer (200 µL, 3 min each) and suspended in PBS (200 µL) for flow cytometry. Cell phenotype data were quantified using FlowLogic software (v. 7, Miltenyi Biotec, Germany), using the gating strategies illustrated in Appendix 9 (peripheral blood) and Appendix 10 (tumours).

3.4.9. Fluorescence-activated cell sorting (FACS)

For single-cell sorting of B cells, frozen PBMC and FNA samples were thawed and gently re-suspended in 1% BSA in PBS and washed (×3; 2,000 rpm; 6 min each). The cells were then stained with Trypan Blue to determine cell counts and viability. The cell concentration was adjusted to one million cells/mL in 1% BSA-PBS containing an antibody cocktail consisting of anti-CD19 (PerCP-Cy5.5, BD 561295, 1:50), anti-CD27 (PECy7, BD 560609, 1:400) together with a viability stain (Fixable viability stain 520, BD 564407). The cells were stained for 20 min at room temperature and washed three times in 1% BSA in PBS (2,000 rpm; 4min each). Single, viable and $CD19^+$ $CD27^+$ lymphocytes were sorted (Figure 3.1) into skirted 96-well plates (Starlab, E1403-5200), containing 10 µL/well of freshly prepared catch buffer [12.5% molecular grade Igepal CA-630 (Sigma, 18896), 2U/ µL RNAsin Ribonuclease inhibitor

(Promega, N2515), 10mM DTT, 0.5X RNase free PBS (Invitrogen, AM9624)], using a FACSJazz FACS instrument (BD Bioscience, Singapore). The sorted cells were immediately covered with aluminium sealer, centrifuged (500 g; 30 sec), snap-frozen on dry ice, and stored at -80˚C.

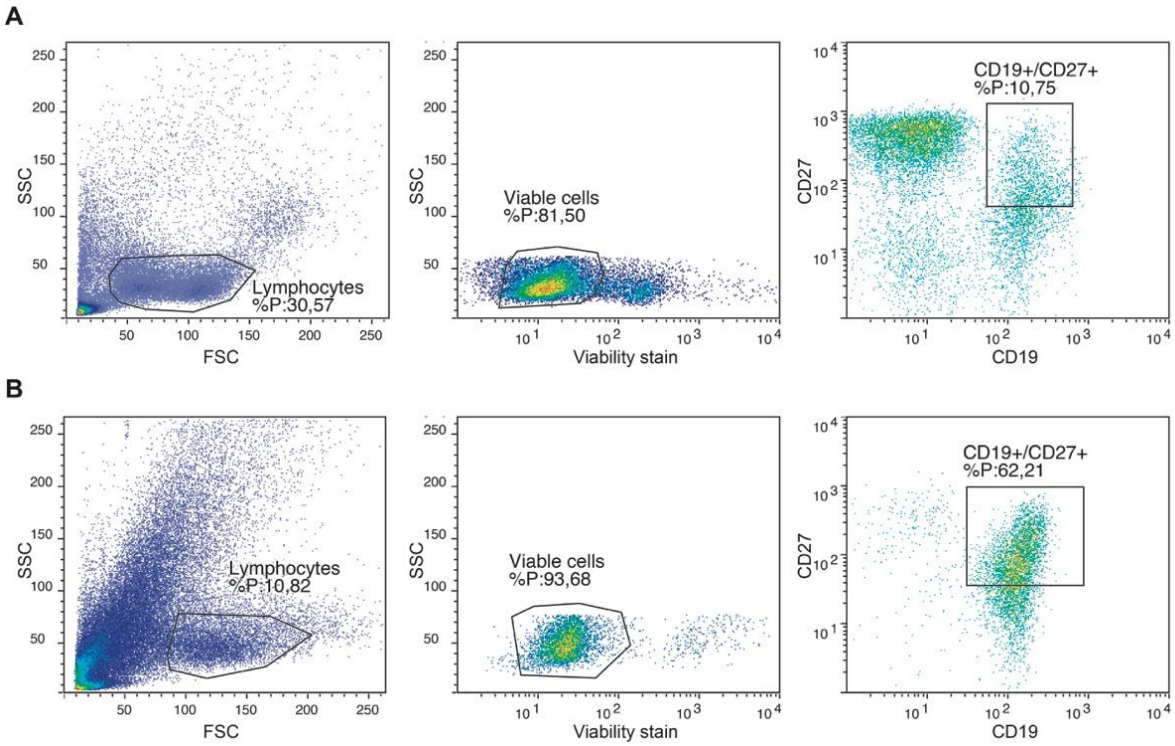


Figure 3.1. Gating strategy for single-cell sorting of memory B cells
Gating strategies for PBMC (**A**) and FNA samples (**B**) are shown.

3.4.10. Single-cell reverse transcription (RT)-PCR and Ig gene amplification

Complementary DNA (cDNA) was synthesised using Superscript™ IV Reverse transcriptase (Invitrogen) following the manufacturer's instructions. Briefly, the frozen sorted cells were thawed on ice and spun down (500 g; 30 sec). Primer master mix (2 µL) (Appendix 12) and deoxyribonucleotide triphosphates (1 µL; 40 nM; Invitrogen, 10297-018) were added to each well and incubated (65˚C; 10 min). After incubation, RT-master mix (5X RT buffer

(4 µL), 100mM DTT (1 µL), RNAsin (1 µL; 40U/µL) and SuperScript IV (1 µL)) was added into each well and RT performed at 55˚C for 10 min followed by enzyme deactivation at 80˚C for 10 min.

Antibody V(D)J genes were amplified from the cDNA by nested PCR (Figure 3.2), using the primer mixes described in Appendix 12 and Appendix 13). First PCR was performed on 1 µL of cDNA and the second PCR on 1 µL of the first PCR product (after a 1:10 dilution in nuclease free water). For the first PCR, master mix (nuclease-free water (7.5 µL), Phusion high fidelity PCR master mix (10 µL; New England Biolabs, M0531), DMSO (0.5µL), and primer mix (1 µL; same as used for RT)) was added to each well. The PCR program used was 98˚C for 30s, 15 cycles of 98˚C for 10 s and 72˚C for 1 min, 15 cycles of 98˚C for 10 s, 68˚C for 30s, and 72˚C for 1 min, followed by a final extension step at 72˚C for 10 min.

For the second PCR, IgH, Igκ and Igλ gene transcripts were amplified independently using CPEC primer mixes specific for each chain (Appendix 13). Separate master mixes were prepared for each of the chains by mixing the corresponding chain-specific primer mix (0.25 µL), DMSO (0.5 µL), Phusion high fidelity PCR master mix (10 µL), and nuclease-free water (8.25 µL). Master mix (19 µL) was dispensed into each well in a new 96-well plate, and the diluted first-PCR product was added (1µL). The PCR program used was 98˚C for 30 s, 40 cycles of 98˚C for 10s and 72˚C for 1min, followed by a final extension step at 72˚C for 10 s.

Amplification success after the second PCR was evaluated by 1.4% agarose gel electrophoresis. Agarose gels were prepared in Tris-Borate-EDTA buffer (TBE, 44.5 mM Tris, 44.5 mM boric acid and 1 mM EDTA) with 0.3µg/mL ethidium bromide. The total volume after the second PCR was run in parallel with a DNA ladder (100 bp of DNA, SM0323, Thermo Scientific) and if positive (band at around 440 bp) both for the heavy and the light chain (κ or λ), the bands were cut and purified from the gel using the NucleoSpin® Gel and Clean-up kit (Macherey-Nagel, 740609) following the manufacturer's instructions.

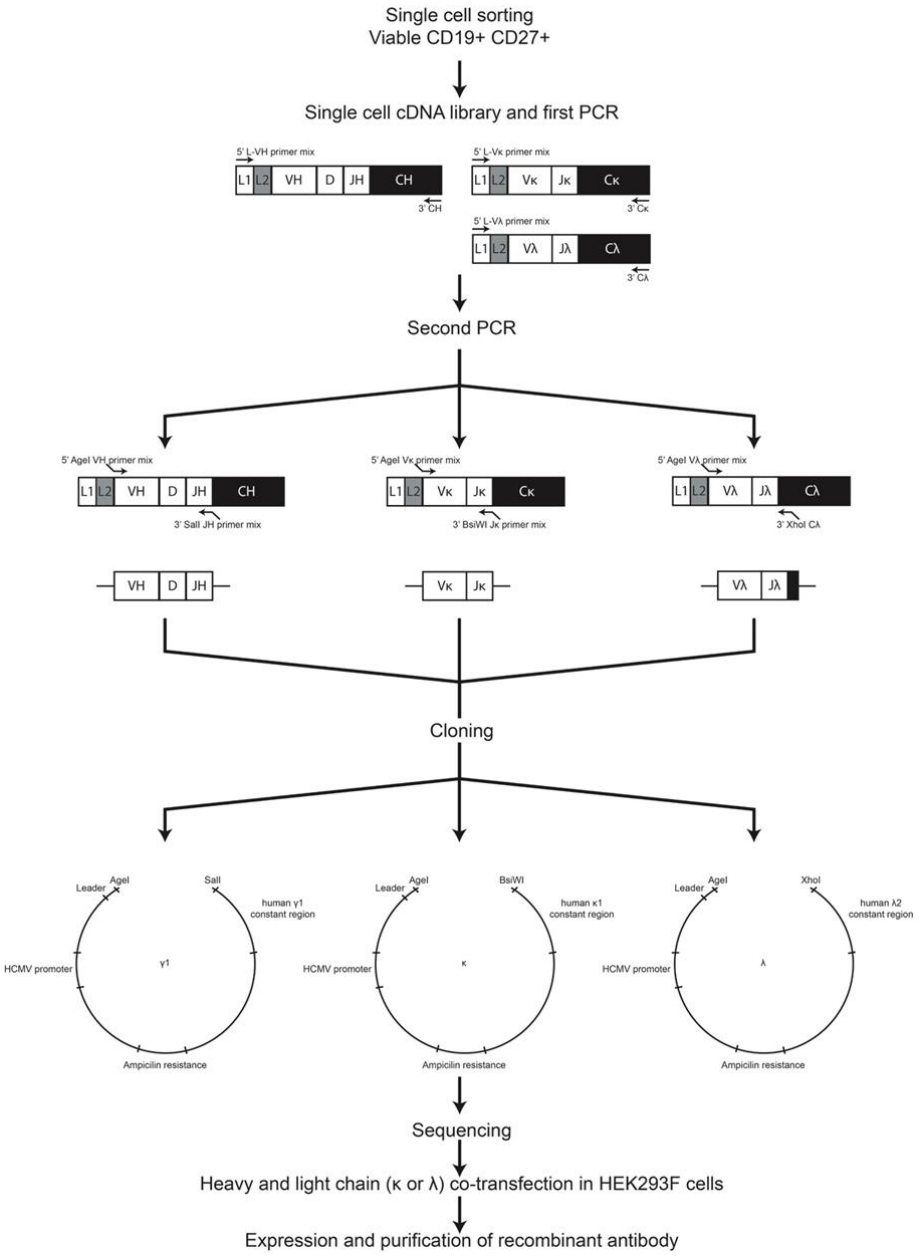


Figure 3.2. Strategy for cloning and expressing monoclonal antibodies
Ig heavy and light chain genes were amplified by nested RT-PCR from single cells. cDNA and first PCR were performed with forward primer mixes specific for the leader region and reverse primers specific for the respective IgH, Igκ or Igλ constant region. The second PCR reactions were performed with forward primer mixes specific for FWR1 and respective nested reverse primers specific for the IgH, Igκ and Igλ J genes or constant regions. The second PCR primers contained restriction sites allowing the direct cloning of the products. All PCR products were sequenced after expression vector cloning to determine the respective V, D, and J gene. For recombinant antibody production, plasmids containing inserts were co-transfected into HEK 293F cells and antibodies were purified from culture supernatants.

3.4.11. Expression vector cloning

The PCR products purified from gels were digested in 96-well plates with the respective Fast Digest restriction enzymes matching the cleavage sites introduced in the CPEC primers (Appendix 13). AgeI combined with SalI, BsiWI or XhoI were used for IgH, Igκ or Igλ, respectively. The digestion reactions were prepared as indicated in Appendix 14. Digestions were performed at 37°C for 1 h, followed by enzyme inactivation at 80°C for 10 min. Digested PCR products (~20 ng) were ligated into the corresponding digested plasmid (50 ng), using the Quick Ligation Kit (New England Biolabs, M2200L) and following the manufacturer's instructions. Ligations were prepared as indicated in Appendix 15 and incubated for 10 min at room temperature. XL10-Gold® ultra-competent cells were transformed at 42°C with 2 µL (~10 ng) of the ligation reaction. Transformed bacteria were plated on pre-warmed (37°C) LB agar plates containing 100 µg/mL of ampicillin and incubated at 37°C overnight. Colonies were screened by PCR using primer pairs flanking the multiple cloning site in each of the vectors (Appendix 16). The master mix for the colony PCR was prepared as in Appendix 17.

Colonies with a positive band of the expected size (600-700 bp for IgH and 550 bp for Igκ/Igλ) were further cultured in LB medium at 37°C overnight and plasmid DNA was isolated using the GeneJET plasmid miniprep kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Fifteen microliters of plasmid DNA at a concentration of 50 ng/µL were sent for sequencing to Eurofins genomics (https://www.eurofinsgenomics.eu/). Similar primers as those used for colony screening were used for sequencing of the cloned variable regions (Figure 3.2 and Appendix 16).

3.4.12. Antibody sequence evaluation

The sequences for the cloned variable regions were evaluated using IgBlast (https://www.ncbi.nlm.nih.gov/igblast/) to determine V(D)J gene family usage by comparison

to the germline. Complementarity determining region (CDR) 3 length and sequence identity to the germline was also determined (Appendix 18).

3.4.13. Recombinant antibody production and purification

Selected human monoclonal antibodies were expressed using the FreeStyle 293 Expression System (Thermo Fisher Scientific) following manufacturer's instructions. Briefly, human embryonic kidney (HEK) 293-F cells were grown in polycarbonate Erlenmeyer flasks with vent cap (Corning, 431144) at 37˚C and 5% CO_2 in FreeStyle 293 expression medium. HEK-293F cells during exponential growth ($1x10^6$ cell/mL in 50 mL) were transfected with equal amounts (25 µg) of IgH and corresponding Igκ/λ expression vector DNA using the FreeStyle TM MAX reagent (Gibco, 16447100) following manufacturer's instructions. Five to six days after transfection, cell culture supernatants were harvested by centrifugation (300 g; 5 min). Supernatants were collected into 50 mL tubes and cellular debris further removed by centrifugation (3,000 g; 15 min; 4˚C). The recovered supernatant was mixed with equal volumes of PBS and filtered with a 0.22µm filter unit (VWR, 5140308). Recombinant antibodies were purified from cell culture supernatant using HiTrap® Protein G High Performance columns (GE Healthcare, 17040401) coupled to an ÄKTAxpress chromatography system. Antibodies were eluted from the Protein G columns using 0.1 M Glycine-HCl buffer (pH 2.7), and pH immediately equilibrated with neutralization buffer (1 M Tris pH 9). The fractions containing eluted antibodies were pooled and buffer was exchanged for PBS using Vivaspin 20 concentration units (Millipore) following the manufacturer's protocol. Recombinant antibody purity and integrity was evaluated by SDS-PAGE under reducing and non-reducing conditions in the presence or absence of DTT respectively. Briefly, 5 µg of antibody were loaded on NuPAGE™ 4-12% Bis-Tris Protein gel (Thermo Scientific) and run in parallel with a molecular weight marker (PageRuler™ Plus Prestained Protein ladder (10-250 kDa, Thermo Scientific, cat. 26619) (150 V; 1 h). After electrophoresis, gels were stained

with Instant Blue protein stain (Expedeon) (15 min) and washed in distilled water until clear bands were visible.

3.4.14. Data analysis

Statistical analysis (including the graphical representation of results) was done in Prism GraphPad v. 8.2.0. Non-parametric statistics were used throughout, due to the non-normal distribution of most of the data obtained. Comparisons of two groups were done using unpaired two-sample Wilcoxon tests (a.k.a. Mann-Whitney tests). Comparisons of more than two groups were done by Kruskal-Wallis tests (a.k.a. non-parametric ANOVA on ranks), supplemented by pairwise analysis (Dunn's post-hoc test). Comparisons were done using the median. P-value < 0.05 was considered as statistically significant (α=0.05). The sensitivity and specificity data (and their confidence intervals) reported in section 4.3 were calculated as described in detail elsewhere (*Statistics with confidence*, 2000), using the MedCalc online calculator (https://www.medcalc.org/calc/diagnostic_test.php/). The gold standard for the sensitivity and specificity was the morphological staining (Giemsa and H&E).

Data are presented as box plots throughout. In those diagrams, the centre line represents the median, the box mark the central 50% of the values, the whiskers mark the central 90% of the values, whereas individual points beyond the central 90% are shown as dots (not present in most diagrams due to the limited size of the data sets). P-values for pair-wise comparisons are shown in diagrams, where relevant.

4. Results

4.1. Basic characteristics of study participants

Fifty paediatric cancer patients with an average age of 7.5 years were recruited into the study from either KATH or KBTH. About half of the patients were admitted to hospital with an eBL diagnosis, the other half with various other tumours. There was no statistically significant difference in the average age of eBL and non-eBL patients (P=0.6). Among the eBL patients, the tumour was located in the jaw in 12 patients, in the abdomen in 17 patients, and in the cervical lymp node in the last. As expected from the literature, there was 60% of males among the eBL patients. The full characteristics of all the above-mentioned prospective study patients are included as Appendix 6. All cancer patients were negative for *P. falciparum* malaria from the clinical data from the hospitals.

A total of 22 age-matched children (average age 7.2 years; P=0.6 relative to patients) were recruited into the study as controls from the same community. Eleven of these donors had *P. falciparum* malaria, while the rest were clinically healthy and without detectable *P. falciparum* parasitaemia (Table 4.1).

4.2. Basic characteristics of archival study samples

A total of 111 archival tissue samples from children with an average age of 9.3 years and admitted to either KATH or KBTH between 2010 and 2017 with diagnoses of paediatric lymphomas were included in the study. About half of these patients (N=55) were admitted to hospital with an eBL diagnosis, the others were with various other tumours. As above, there was no statistically significant difference in the average age of eBL and non-eBL patients (P=0.4). Among the eBL patients, the tumour was located in the jaw in six patients, in the abdomen in 27 patients, and elsewhere in the remaining 23. However, the proportion of males (68%) recruited in the prospective samples (FNA) were similar to that of the archival samples.

The full characteristics of all the donors of the included tissue specimens are included as Appendix 7.

Table 4.1. Characterization of study patients

Characteristics	eBL patients	Non-eBL patients	Malaria patients	Healthy controls	p-value
Participants	n=19	n=9	n=11	n=11	
Age, years	7.2 (2-12)	9.9 (5-12)	7.1 (3-12)	8.4 (2-13)	0.1922
Female/Male (%)	31.6/68.4	53.6/44.4	27.3/72.7	54.5/45.5	0.1731

4.3. The accuracy of morphology-based diagnosis of eBL in Ghana

In this part of the thesis project, the original eBL diagnoses of the study patients, which were based on clinical assessment and cytology at KATH and KBTH, were compared to retrospective diagnoses made at University of Copenhagen and University Hospital Herlev, and based on

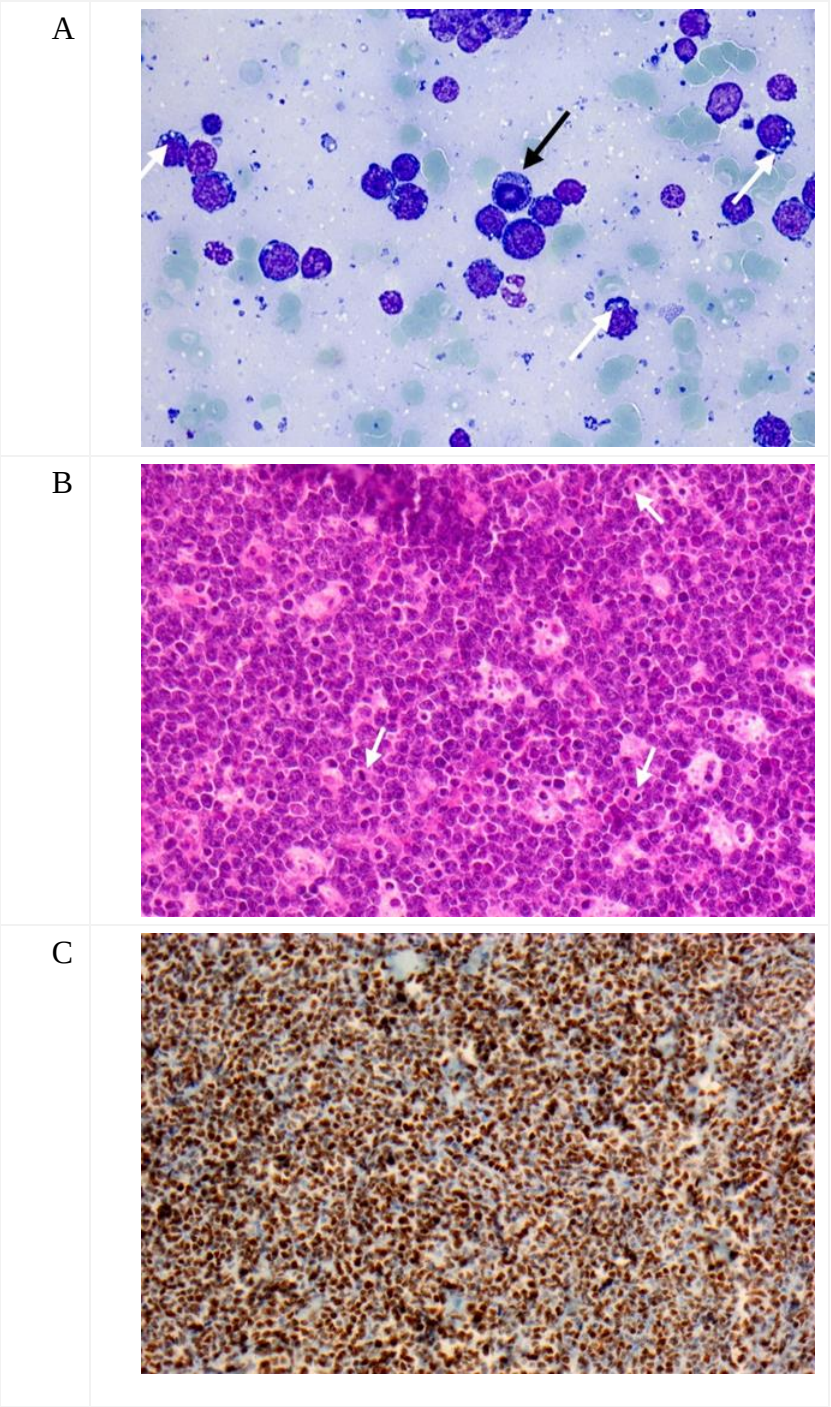


Figure 4.1. Morphology of eBL cells by light microscopy
(**A**) Giemsa-stained FNA smear from an eBL tumour with cells showing characteristic cytoplasmic vacuoles (white arrows) and a cell with a prominent mitotic figure (black arrow). (**B**) Haematoxylin-eosin-stained FFPE tumour tissue section (20× magnification) showing cells with mitotic figures (arrows) and characteristic "starry sky" staining due to weakly stained macrophages among numerous densely stained tumour cells. (**C**) Immunohistochemistry-stained FFPE tumour tissue section showing prominent C-MYC expression (brown).

cytology supplemented by FISH and C-MYC immunohistochemistry. In addition, archival tissue biopsies from KATH and KBTH were evaluated in a similar way.

The results of this part of the thesis work have been submitted for publication. Details (including author contributions) regarding the manuscript can be found in Appendix 11.

4.3.1. Morphology analysis

Blinded Giemsa-stained FNA tumour smears (Figure 4.1A) from 28 of the patients (17 with an original eBL diagnosis) from 2018 (Table 4.2) were available for retrospective morphological review. The results were in good agreement with the original diagnoses made (Figure 4.2)

A reliable retrospective assessment of freshly stained haematoxylin-eosin sections (Figure 4.1B) from archival tissue blocks (Table 4.2) was only possible for 85 (including 42 from patients with an original eBL diagnosis), as the remaining samples had to be discarded because of poor specimen quality (autolysis or no viable tumour tissue due to tumour necrosis) (Appendix 20). The overall sensitivity, specificity, and accuracy estimates were somewhat lower for FFPE sections than for the FNA smears (Figure 4.3A), probably related to the overall lower quality of the archival compared to the fresh samples. The rejection of the archival samples were mainly due to poor preservation but not the duration of storage.

Table 4.2. Characteristics of patients included in the eBL diagnostic accuracy study

			Number	Age (years)	Sex (F/M)	Tumour location (Head/Abd/other)
Study cohort (2018)	eBL	KBTH	7	6.6 (2-12)	4/3	3/4/0
		KATH	22	7.9 (3-13)	6/16	9/13/0
		All	29	7.6 (2-13)	10/19	12/17/0
	Non-eBL	KBTH	8	6.1 (1-13)	3/5	3/5/0
		KATH	13	8.5 (3-15)	7/6	8/5/0
		All	21	7.6 (1-15)	10/11	11/10/0
Archival samples (2009-2017)	eBL	KBTH	35	9.8 (2-25)	11/24	11/18/6
		KATH	20	8.1 (1-15)	7/13	11/9/0
		All	55	9.2 (1-25)	18/37	22/27/6
	Non-eBL	KBTH	37	9.6 (2-15)	8/29	25/6/6
		KATH	19	10.0 (6-15)	2/17	17/2/0
		All	56	9.8 (2-15)	10/46	42/8/6

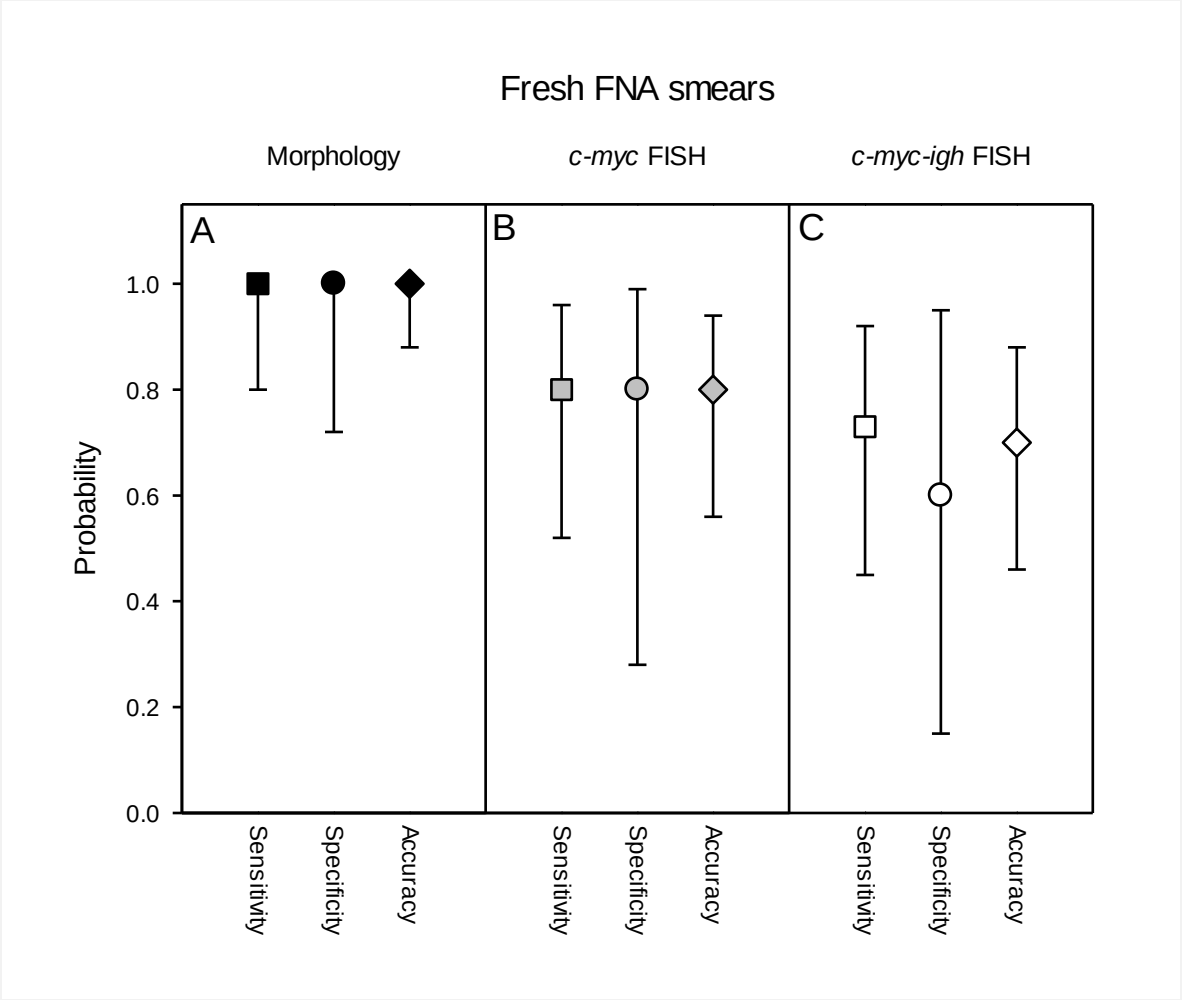


Figure 4.2. FNA review diagnosis - morphology and FISH

Estimated sensitivity (squares) and specificity (circles), and accuracy (diamonds) of original diagnosis, based on retrospective analysis of (**A**; black symbols): cell morphology in Giemsa-stained smears, (B; grey symbols): cytogenetic analysis of *c-myc* breakage and (**C**; white symbols): *c-myc-igh* translocation by FISH. Medians (symbols) and 95% confidence intervals (error bars) are shown (n=28).

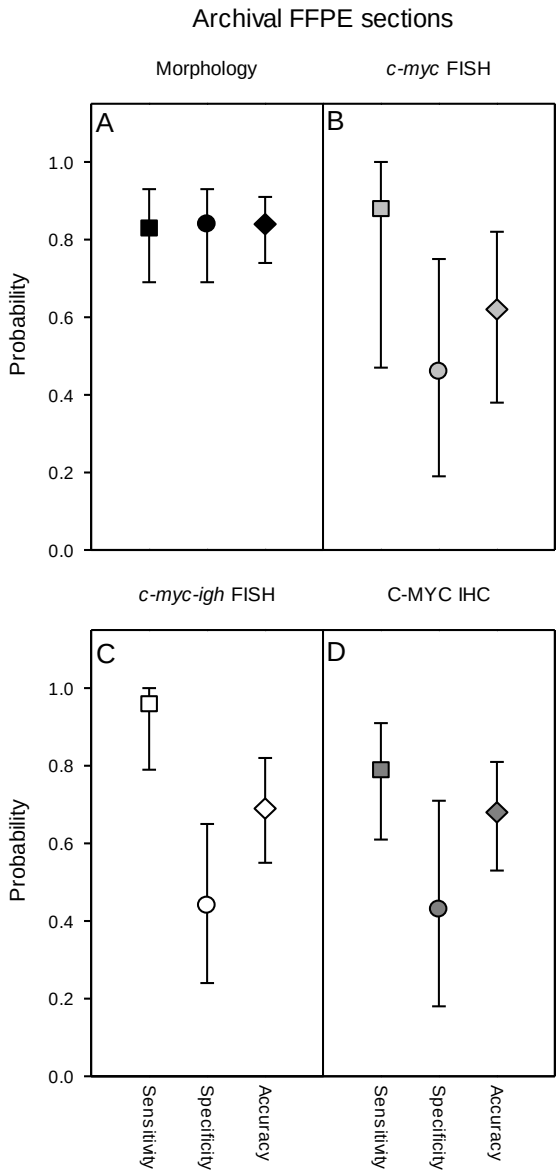
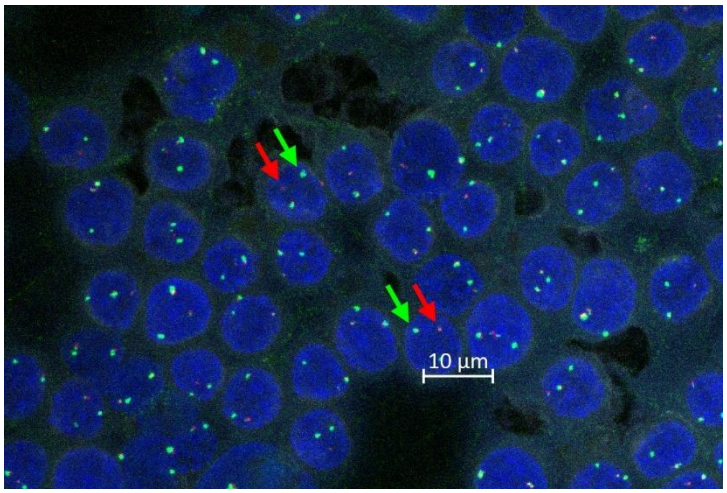


Figure 4.3. FFPE tissue section review diagnosis – morphology and FISH

Estimated sensitivity (squares), specificity (circles), and accuracy (diamonds) of original diagnosis, based on retrospective analysis of (**A**; black symbols): cell morphology in freshly haematoxylin-eosin-stained archival FFPE tissue sections, (**B**; grey symbols): on cytogenetic analysis of *c-myc* breakage, *c-myc-igh* translocation (**C**; white symbols) by FISH, or by C-MYC immunohistochemistry (**D**; dark grey symbols) in sections from the archival samples. Medians (symbols) and 95% confidence intervals (error bars) are shown. (n=85)

A



B

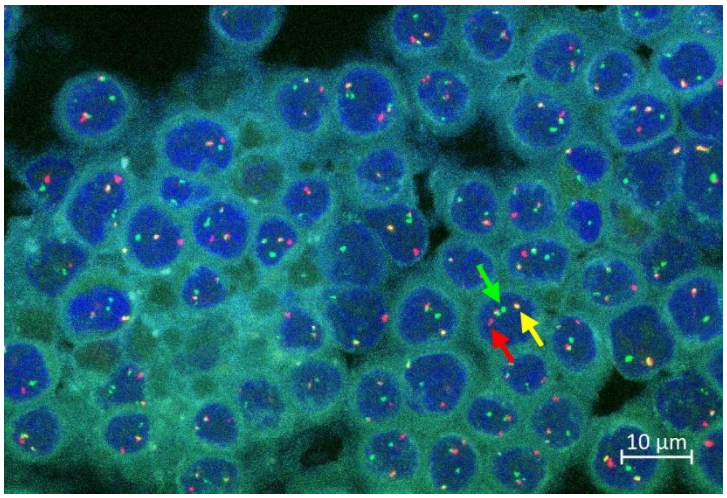


Figure 4.4. FISH micrographs of eBL cells
(**A**) *c-myc* split probe labelling of the upstream (green) and downstream (red) part of *c-myc* gene in an FNA smear from an eBL patient (separation of red and green fluorescence (red and green arrows) indicating *c-myc* breakage . (**B**) *c-myc-igh* fusion probe labelling of *c-myc* (green) and *igh* (red) in an FNA smear from an eBL patient (co-localization (yellow) demonstrates translocation of *c-myc* to *igh*) . (n=28)

4.3.2. Analysis by c-myc FISH and C-MYC immunohistochemistry

To further assess the accuracy of the original cell/tissue morphology-based diagnosis, the FNA smears from 20 of the 2018-patients (including 13 patients originally diagnosed as eBL) were also analysed for FISH evidence of breakage of the *c-myc* oncogene (Figure 4.4A). The

sensitivity, specificity, and accuracy estimates (Figure 4.2B and Appendix 21) were lower than that of the morphology. No evidence of *c-myc* breakage was evident in one of the patients originally diagnosed as eBL, whereas *c-myc* breakage was detected in four patients originally diagnosed as non-eBL. While *c-myc* breakage is not specific for eBL, lack of *c-myc* breakage is rare in eBL, and this analysis thus suggests some over-diagnosis originally. Examination of slides for FISH evidence of *c-myc* translocation into the *igh* locus (Figure 4.4B) yielded similar results (Figure 4.2C and Appendix 22).

Corresponding FISH analysis of archival FFPE sections showed high sensitivity but low specificity

Figure 4.3B-C, Appendix 23, Appendix 24 and Appendix 25). The low specificity was due to the fact that no evidence of *c-myc* breakage was found in half (7/14) of the patients originally diagnosed as eBL. As lack of *c-myc* breakage is rare in eBL, this finding supports the above indication of a degree of over-diagnosis when supportive molecular evidence of eBL is not available. This conclusion is further supported by examination of biopsy sections for immuno-histochemical evidence of C-MYC expression (Figure 4.1C). Breakage and translocation of *c-myc* is often associated with high expression of C-MYC protein (Tapia *et al.*, 2011), and immunohistochemistry is generally less sensitive to sample deterioration than FISH (Nwanze, Siddiqui, Stevens, Saxe, & Cohen, 2017; Wennborg, Altiok, Moore, Ernberg, & Klein, 1991). This analysis

Figure 4.3D) showed low specificity due to absence of C-MYC expression in biopsies from eight of 34 patients originally diagnosed as eBL.

4.4. Phenotypes of circulating and tumour-infiltrating Vδ1⁺ T cells

In this part of the thesis project, the hypothesis that Vδ1$^+$ T cells are important regulators of B-cell proliferation, and therefore likely involved in the immune response to eBL was assessed.

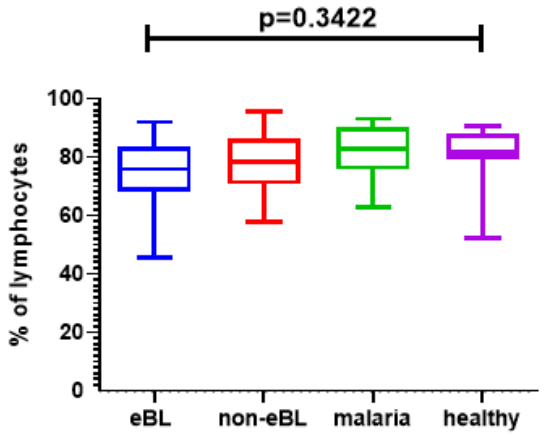
The hypothesis is based on findings of increased frequencies and numbers of circulating Vδ1$^+$ T cells in healthy, *P. falciparum*-exposed individuals and in patients with acute *P. falciparum* malaria, and in general in conditions characterized by chronic B-cell activation. This rationale is described in detail in a review paper written during the thesis work (Hviid *et al.*, 2019). A manuscript describing the results of this part of the thesis work is currently being prepared for submission. Details (including author contributions) can be found in Appendix 11.

4.4.1. Frequencies of T cells in peripheral blood and in tumours

Samples from 19 eBL patients, nine patients with non-eBL tumours, and from 11 *P. falciparum* malaria patients and 11 healthy control children were evaluated. The cancer patients were negative for *P. falciparum* malaria according to the patients records at the time of diagnosis.

The average frequencies of T cells circulating in the peripheral blood of the tumour patients, and and age-matched control children, were not statistically significant (P=0.3422) (Figure 4.5A). However, the frequency of T cells in the eBL tumours were lower compared to that in non-eBL (Figure 4.5B).

A: T cells in peripheral blood



B: T cells in tumours

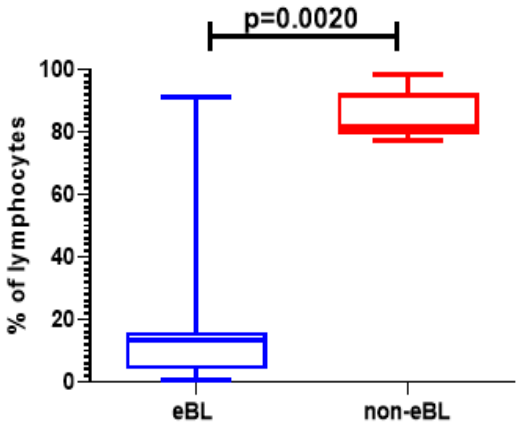


Figure 4.5. T-cell frequencies in peripheral blood and tumours
Frequencies of $CD3^+$ cells (% of all lymphocytes) in eBL patients (blue, n=19), non-eBL patients (red, n=9), and in age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

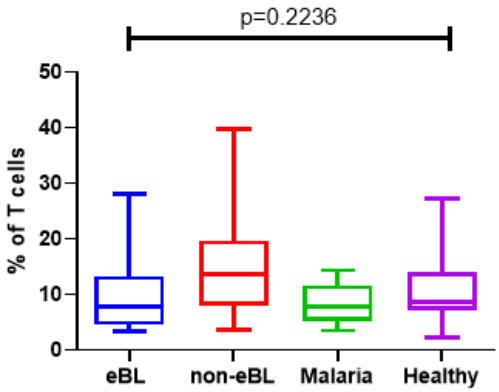
4.4.2. Frequencies of γδ T cells in peripheral blood and in tumours

γδ T cells have been implicated in the immune response in several diseases, including cancers. In this study, a pan-γδ T-cell antibody and an antibody specifically labelling the Vδ1 subset of these cells were employed in combination with various markers of function and maturation. This allowed separate analysis of $V\delta1^+$ and $V\delta1^-$ γδ T cells (Figure 4.6).

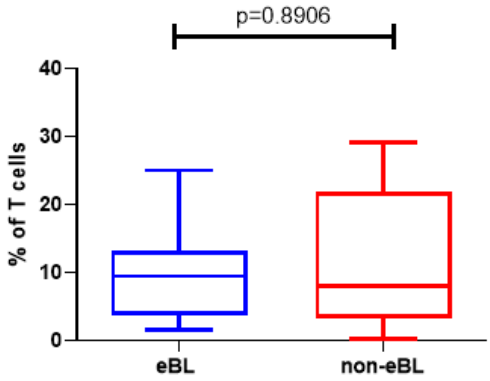
The overall frequencies of TCR-$\gamma\delta^+$ cells (Figure 4.6A), of $V\delta1^+$ cells (Figure 4.6C), and of $V\delta1^-$ cells (Figure 4.6E) in the peripheral blood were not significantly higher in the peripheral blood of the cancer patients compared to the control children.

Within the tumours, the frequencies of TCR-$\gamma\delta^+$ cells (Figure 4.6B), $V\delta1^+$ (Figure 4.6D) and $V\delta1^-$ γδ T cells (Figure 4.6F) were also not significantly different between eBL and non-eBL patients.

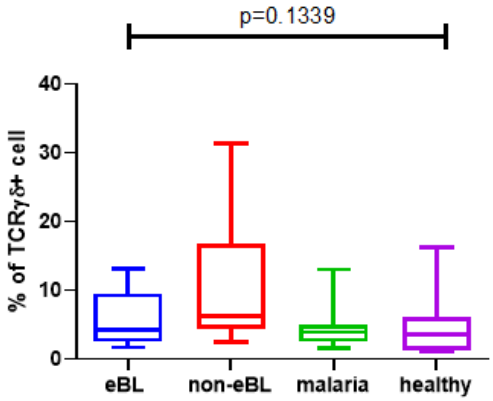
A: TCR-γδ cells in peripheral blood



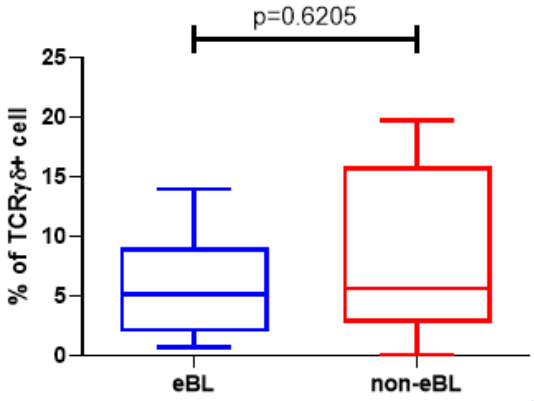
B: TCR-γδ cells in tumours



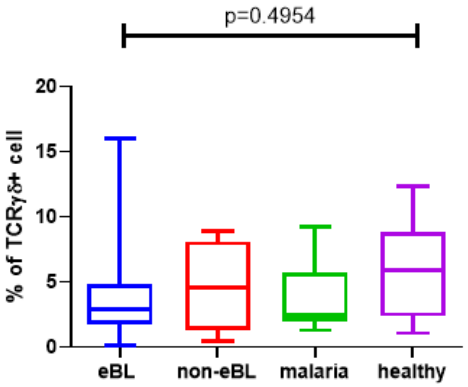
C: Vδ1^{+} cells in peripheral blood



D: Vδ1^{+} cells in tumours



E: Vδ1^{-} cells in peripheral blood



F: Vδ1^{-} cells in tumours

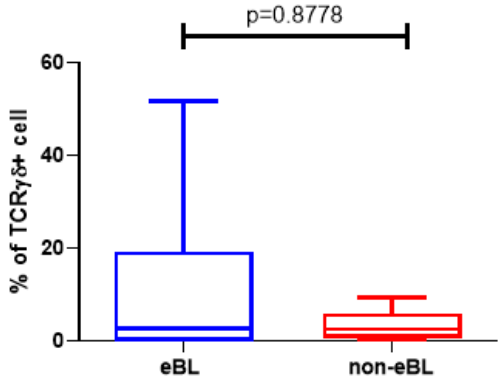


Figure 4.6. γδ T-cell and γδ T-cell subset frequencies in peripheral blood and tumours
Frequencies (% of CD3^{+} cells) in eBL patients (blue, n=19), non-eBL patients (red, n=9), and in age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

4.4.3. Maturation and activation status of circulating and tumour Vδ1⁺ T cells

To pursue this further, the maturation and activation of Vδ1⁺ and Vδ1⁻ γδ T cells was evaluated. T_{EMRA} ($CD27^{neg}CD45RA^{+}$) Vδ1⁺ cells dominated in the peripheral blood and tumours of eBL patients (Figure 4.7A and Figure 4.9A) and non-eBL patients (Figure 4.7B and Figure 4.9B), in contrast to the case of controls with or without malaria, where the dominant Vδ1⁺ maturation subset was T_{CM} ($CD27^{+}CD45RA^{neg}$) (Figure 4.7C) and T_{EM} ($CD27^{neg}CD45RA^{neg}$) (Figure 4.7D), respectively. Dominance of T_{EMRA} cells was much less pronounced in the peripheral blood (Figure 4.7A) than in tumours (Figure 4.9A) of eBL patients, and there was reduced maturation of Vδ1⁻ γδ T cells among non-eBL patients (Figure 4.8B and Figure 4.10B). With respect to the control groups, T_{CM} cells dominated in the peripheral blood of the malaria patients (Figure 4.8C) and healthy children (Figure 4.8D). These findings suggest a selective accumulation of Vδ1⁺ T_{EMRA} TILs, suggesting that they are of functional significance in the immune response to the tumours (Figure 4.11).

To determine the degree of activation of Vδ1⁺ T cells, the cells were labelled with an antibody specific for the α-chain of the interleukin 2 receptor (CD25; a well-established marker of lymphocyte activation) and PD1 (a marker of lymphocyte exhaustion, implicated in the immune response to tumours). CD25 expression on Vδ1⁺ cells was low in all donor groups, and not significantly different among them, neither in the peripheral blood (Figure 4.12A) nor in tumours (Figure 4.12B). There was an indication that Vδ1⁺ T-cell expression of PD1 was higher in the cancer and *P. falciparum* malaria patients compared to healthy, age-matched controls, although the differences were not statistically significant, except for eBL Vδ1⁺ TILs compared to non-eBL TILs (Figure 4.13). Overall, these findings support an anti-cancer effector role for Vδ1⁺ T cells, in particular in the case of eBL.

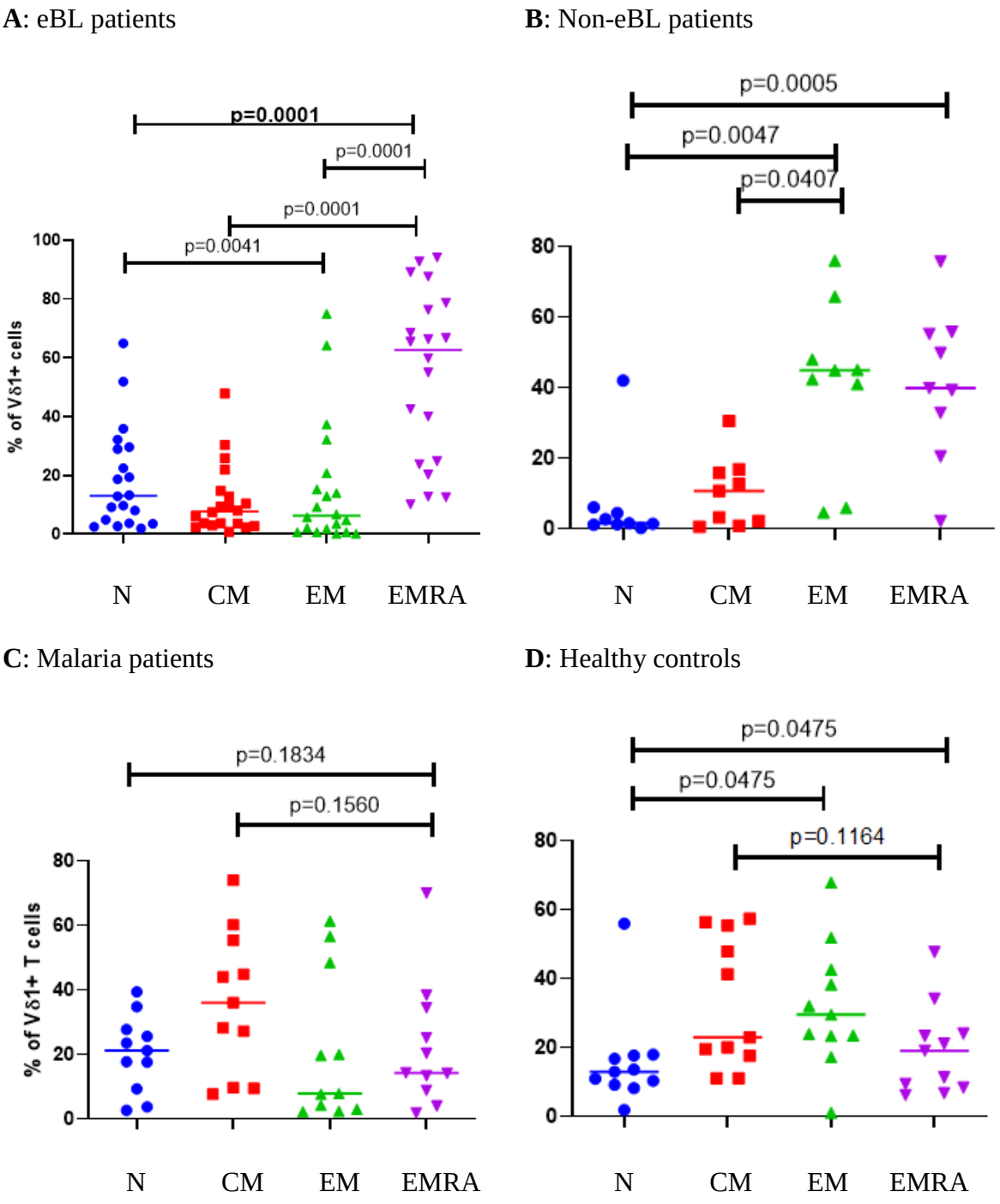
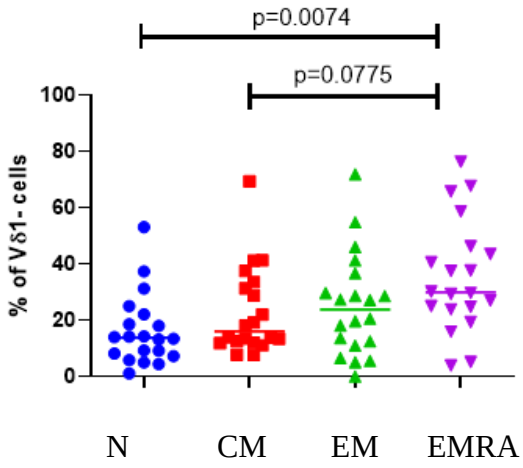
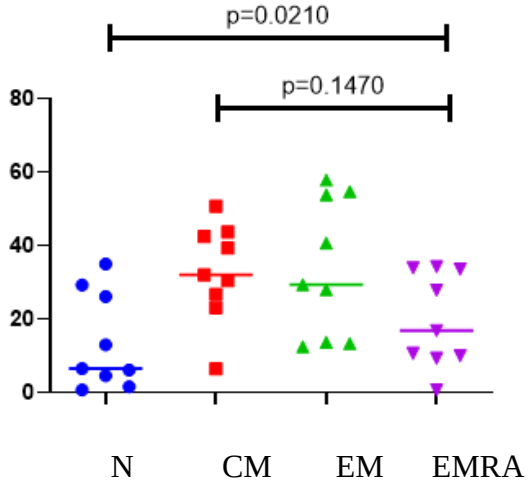


Figure 4.7. Maturation status of peripheral blood Vδ1$^+$ T cells
Distribution (% of Vδ1$^+$ cells) of naïve (blue), central memory (red), effector-memory (green), and terminally differentiated effector-memory cells (purple) in (**A, n=19**) eBL patients, (**B, n=9**) non-eBL patients, and in (**C, n=11**) age-matched children with or (**D, n=11**) without *P. falciparum* malaria.

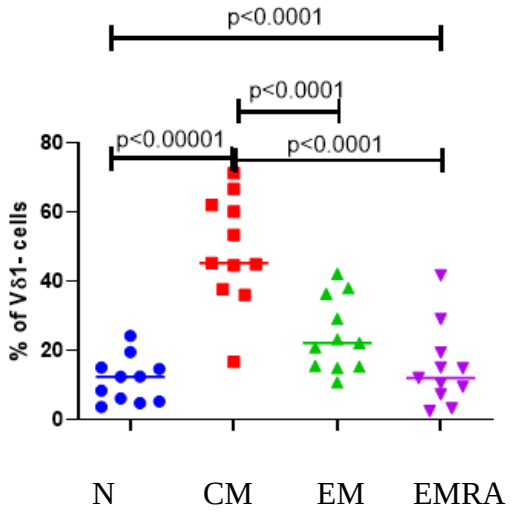
A: eBL patients



B: Non-eBL patients



C: Malaria patients



D: Healthy controls

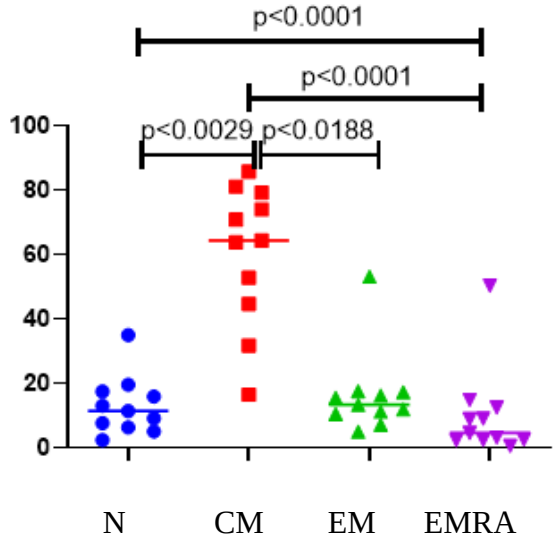


Figure 4.8. Maturation status of peripheral blood Vδ1⁻ T cells
Distribution (% of Vδ1⁻ cells) of naïve (blue), central memory (red), effector-memory (green), and terminally differentiated effector-memory cells (purple) in eBL patients (**A**), non-eBL patients (**B**), and in age-matched children with (**C**) or without (**D**) *P. falciparum* malaria.

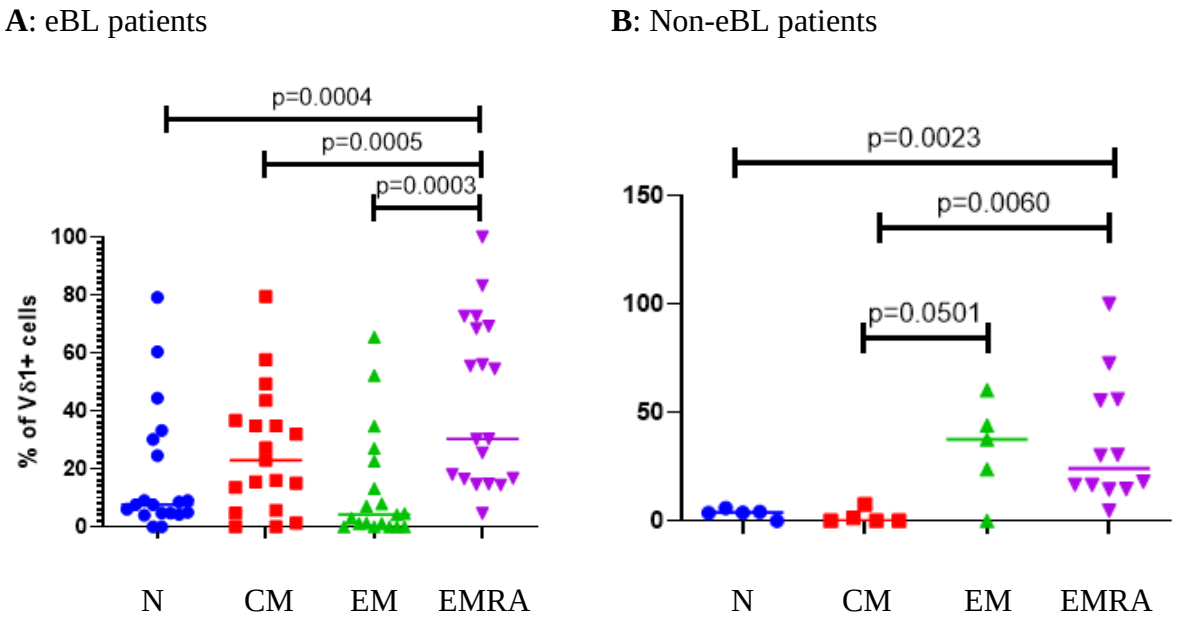


Figure 4.9. Maturation status of tumour Vδ1⁺ T cells
Distribution (% of all Vδ1⁺ cells) of naïve (blue), central memory (red), effector-memory (green), and terminally differentiated effector-memory cells (purple) in (**A, n=19**) eBL and (**B, n=9**) non-eBL patients.

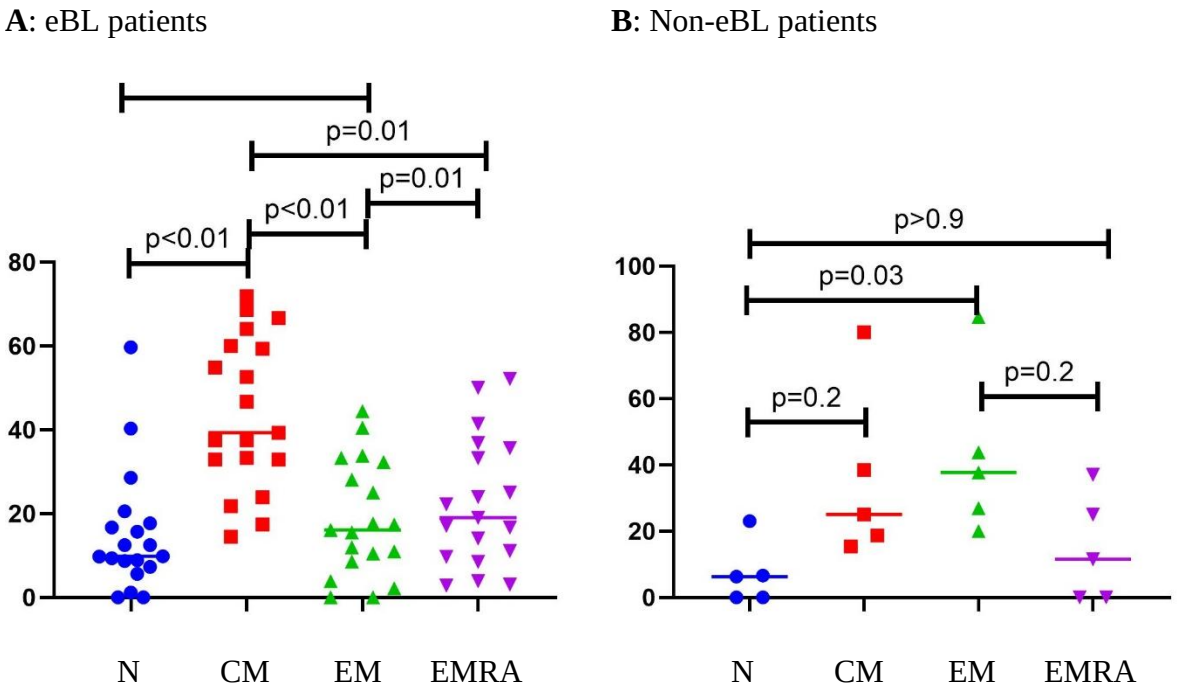


Figure 4.10. Maturation status of tumour Vδ1⁻ T cells
Distribution (% of all Vδ1⁻ cells) of naïve (blue), central memory (red), effector-memory (green), and terminally differentiated effector-memory cells (purple) in (**A, n=19**) eBL and (**B, n=9**) non-eBL patients.

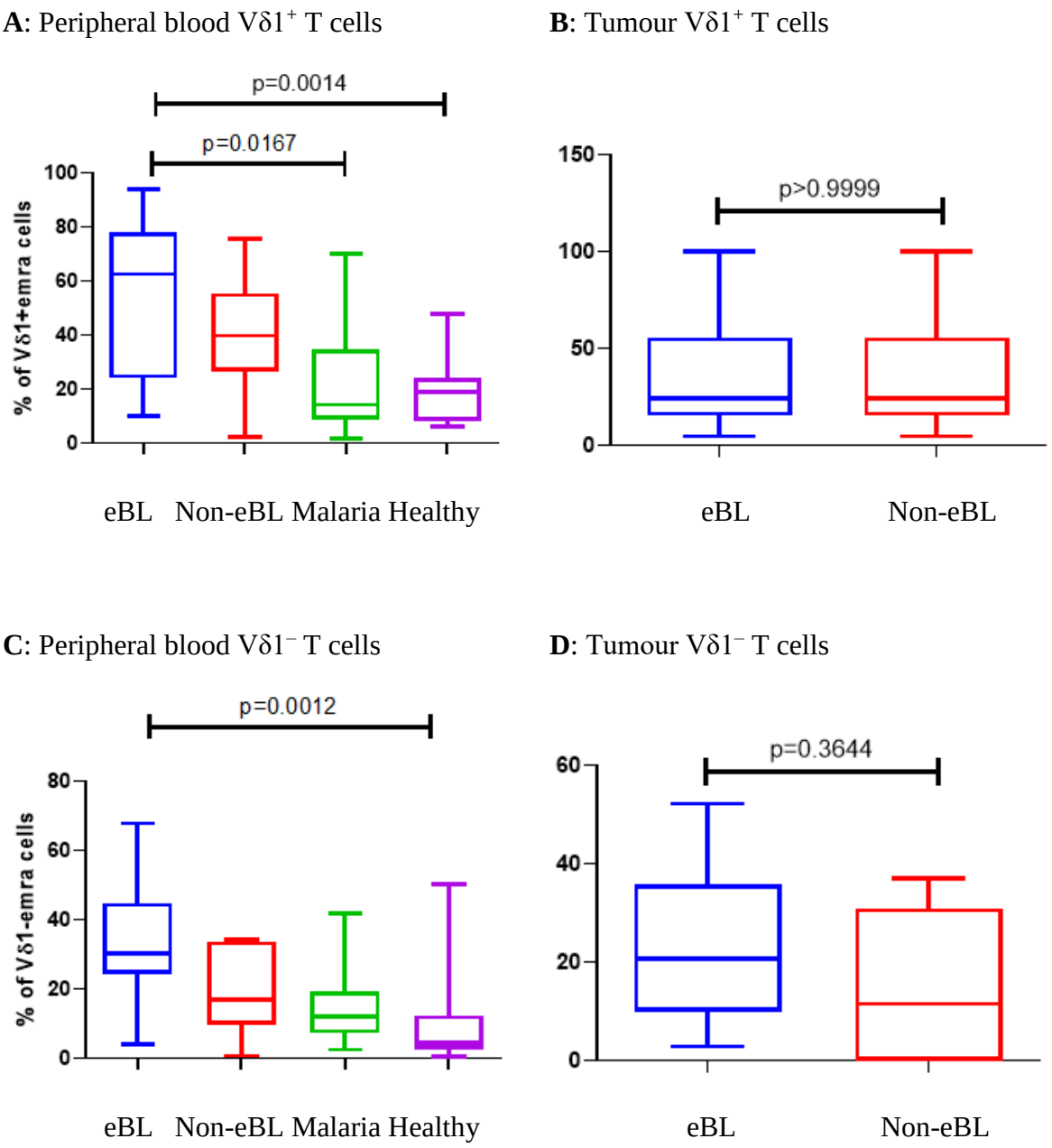
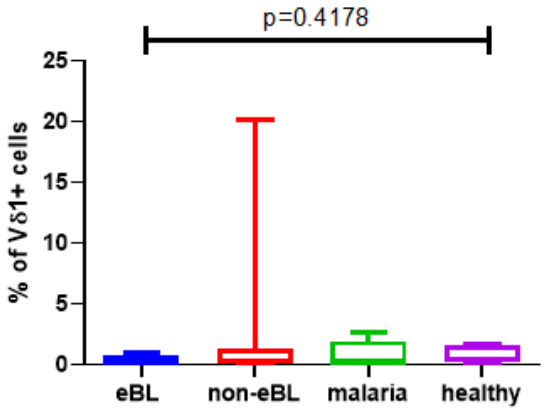


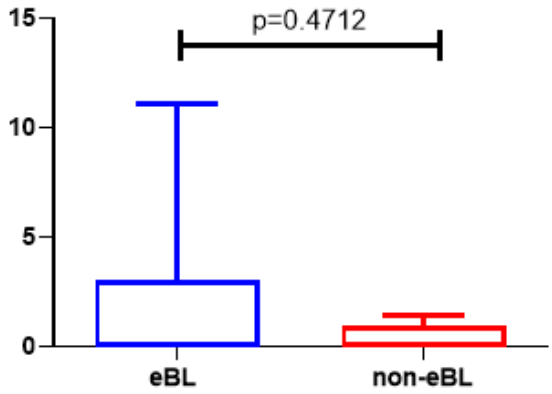
Figure 4.11. Terminally differentiated effector-memory (T_{EMRA}) Vδ1$^+$ and Vδ1$^-$ T cells
Percentage distribution of all (A and B) Vδ1$^+$ or (C and D) Vδ1$^-$cells) in the (A and C) peripheral blood and (B and D) tumours of eBL (blue, n=19) and non-eBL (red, n=9) patients, and in peripheral blood of age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

A: Peripheral blood



eBL Non-eBL Malaria Healthy

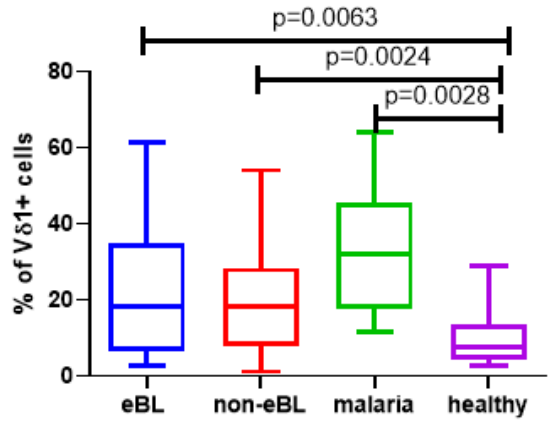
B: Tumours



eBL Non-eBL

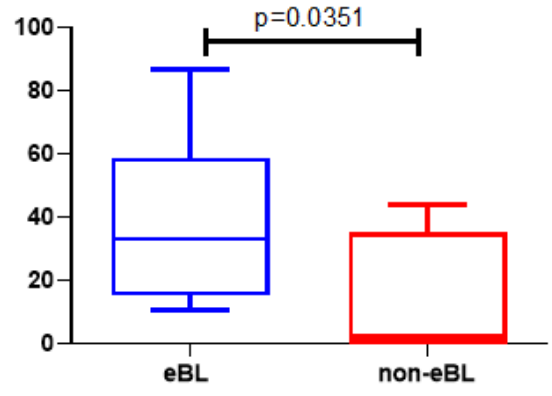
Figure 4.12. Frequencies of CD25$^+$ Vδ1$^+$ T cells
Frequencies of CD25$^+$ cells (% of all Vδ1$^+$ cells) in the (**A**) peripheral blood and (**B**) tumours of eBL patients (blue, n=19), non-eBL patients (red, n=9), and in peripheral blood of age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

A: Peripheral blood



eBL Non-eBL Malaria Healthy

B: Tumours



eBL Non-eBL

Figure 4.13. PD1$^+$ Vδ1$^+$ T cells
Frequencies of PD1$^+$ cells (% of all Vδ1$^+$ cells) in the (**A**) peripheral blood and (**B**) tumours of eBL patients (blue, n=19), non-eBL patients (red, n=9), and in peripheral blood of age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

4.5. Phenotypes of circulating and tumour B cells

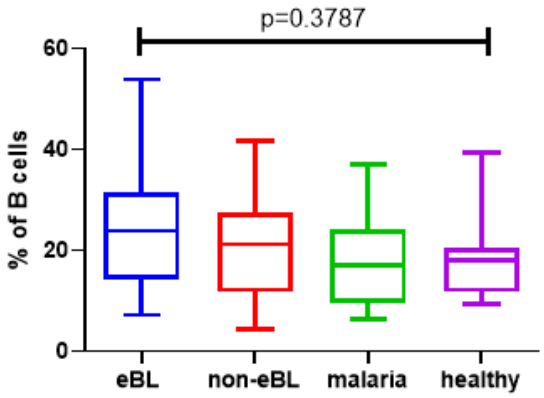
In this part of the thesis project, the phenotypes of circulating and tumour B cells were assessed, in particular with respect to their maturation status, as little information of this kind is available in the literature. The plan is that the data presented here will be incorporated in a manuscript that will focus on the antigen specificity of eBL tumour cells. Details (including author contributions) can be found in Appendix 11 (Manuscript 3).

4.5.1. Frequencies of B cells in peripheral blood and in tumours

B cells are the antibody-producing cells in the body. Antibody secretion is stimulated by the presence of an infectious pathogen. B cells are sources of many lymphomas, including eBL. Though eBL has previously been shown to be a B-cell lymphoma, there is still controversy about their exact phenotype of the tumour cells. B cells were identified by the surface marker CD20, which identify B cells at all maturation stages, except very early (pro-B cells) and terminally differentiated effector B cells (plasma cells).

There were no differences in the frequencies of peripherally circulating B cells among the study groups (Figure 4.14A). As expected, B cell frequencies were very high in eBL tumours, but low in non-eBL tumours (Figure 4.14B).

A: Peripheral blood



B: Tumours

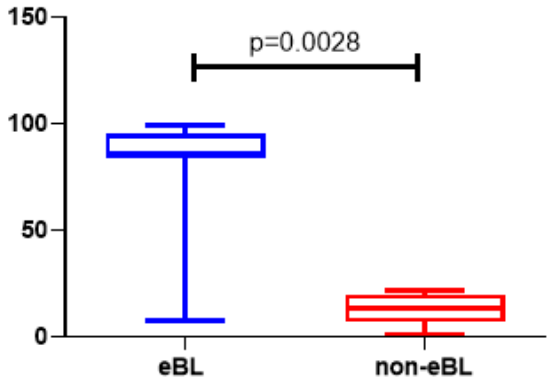


Figure 4.14. B-cell frequencies
Frequencies of $CD20^{+}$ cells (% of all lymphocytes) in the (**A**) peripheral blood and (**B**) tumours of eBL patients (blue, n=19) and non-eBL patients (red, n=9), and in age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

4.5.2. Maturation status of circulating and tumour B cells

Most studies of the phenotype of the tumour cells in eBL predate the current understanding of phenotypic markers of lymphocyte maturation. Here, the surface markers CD10, CD20, CD21, and CD27 were used to investigate the proportions of various B cell subsets in the peripheral circulation and in the tumours. As expected, the majority of CD20+ B cells in the peripheral circulation did not express CD10 (neprilysin, CALLA), which is a marker of immature B cells that is not normally expressed by mature, circulating B cells (Figure 4.15A). In fact, the proportion of circulating CD10+ B cells was even lower in both eBL and non-eBL patients compared to sympatric control children with or without P. falciparum malaria. In stark contrast, the tumour B cells in eBL were almost all CD10+, in accordance with earlier reports (163; 164). Both immature (CD10+) and mature (CD10–) B cells were further classified according to their expression of CD21 (complement receptor 2, EBV receptor) and CD27, which is a member of

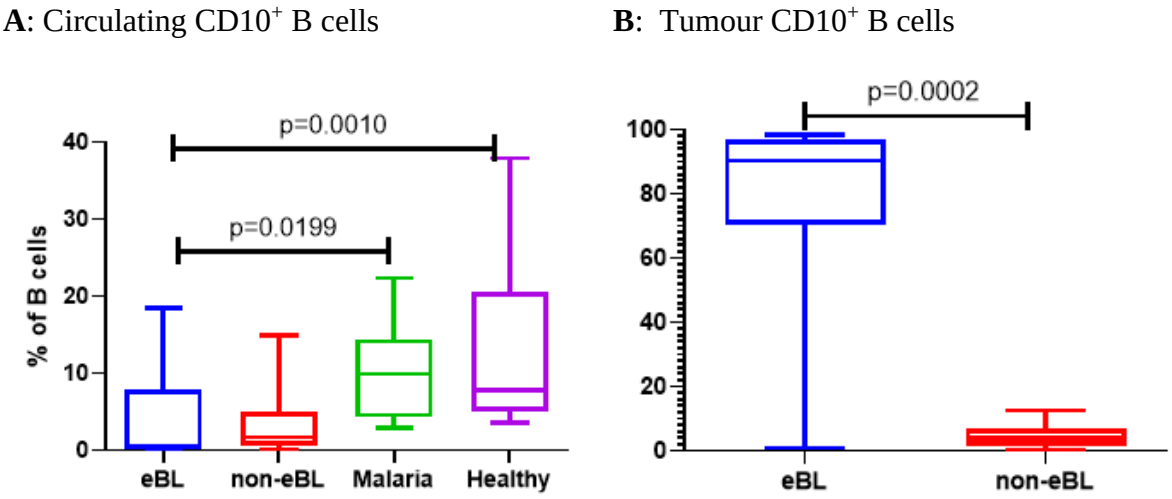


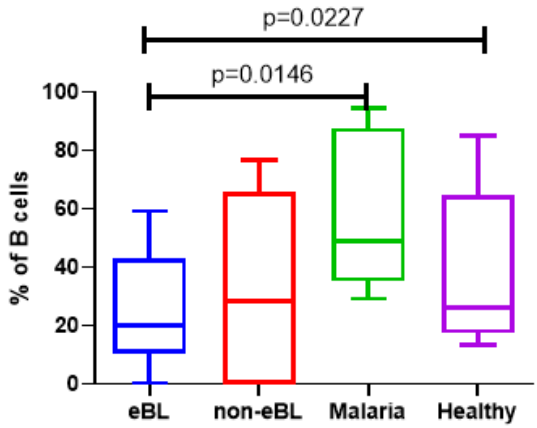
Figure 4.15. B-cell expression of CD10 (neprilysin, CALLA)
Distribution (% of $CD20^+$ cells) of $CD10^+$ B cells in the (**A**) peripheral circulation and in (**B**) tumours of eBL patients (blue, n=19), non-eBL patients (red, n=9), and in age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

the tumour necrosis factor receptor family that regulates B cell activation and immunoglobulin synthesis.

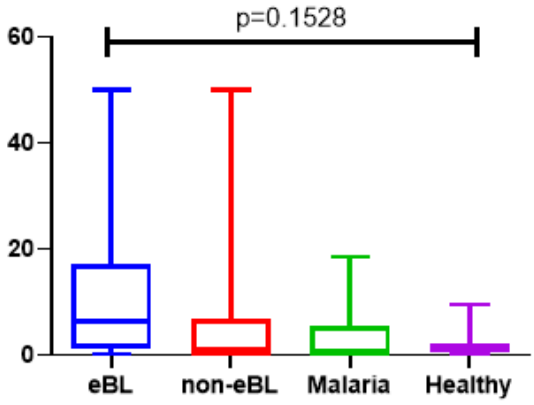
CD10+ cells were sub-divided into CD21–CD27– (immature B cells), CD21–CD27+ (germinal centre cells), CD21+CD27+ (post-germinal centre cells), and CD21+CD27– (transitional B cells), as proposed by Clavarino et al. (165). No marked inter-group differences were noted among these small B cell subsets in the circulation (Figure 4.16), apart from a tendency towards lowered frequencies of transitional-type B cells in the patient groups compared to healthy controls (Figure 4.16D).

In contrast, marked differences were noted among CD10+ cells in the tumours of eBL patients (where these cells dominate completely) versus non-eBL tumours (where they are scarce) (Figure 4.17). In the eBL tumours, most CD10+ cells had a germinal centre (Figure 4.17B) or post-germinal centre phenotype (Figure 4.17C), whereas such cells were rare in non-eBL tumours.

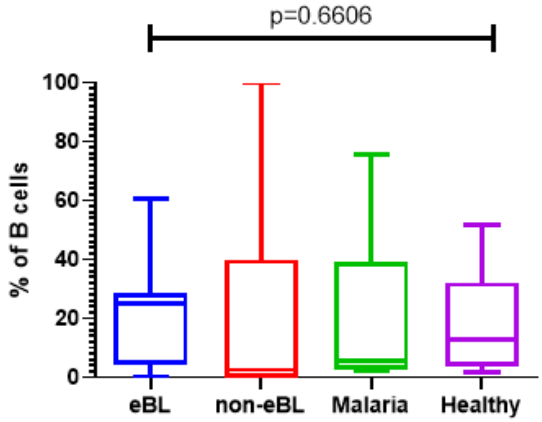
A: $CD21^{-}CD27^{-}$ cells



B: $CD21^{-}CD27^{+}$ cells



C: $CD21^{+}CD27^{+}$ cells



D: $CD21^{+}CD27^{-}$ cells

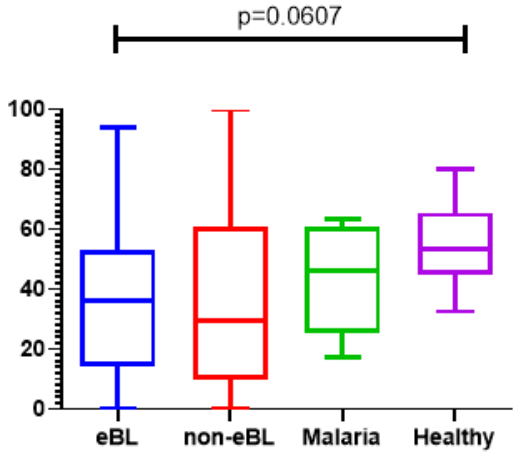


Figure 4.16. Expression of D21 and CD27 by immature, circulating B cells
Distribution (% of $CD10^{+}CD20^{+}$ cells) of cells with a (**A**) classical immature, (**B**) germinal centre, (**C**) post-germinal centre and (**D**) transitional cell phenotype in the peripheral circulation of eBL patients (blue, n=19), non-eBL patients (red, n=9), and in age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

In non-eBL tumors, most $CD10^+$ B cells were immature (Figure 4.17A) and transitional-type cells (Figure 4.17D), thus resembling $CD10^+$ B cells in the peripheral circulation (Figure 4.16).

Mature ($CD10^-$) B cells were similarly classified into $CD21^+CD27^-$naïve cells, $CD21^-CD27^+$ activated B cells, $CD21^+CD27^+$ classical memory B cells, and $CD21^-CD27^-$ atypical memory B cells, as proposed by Illingworth *et al*. (Illingworth *et al.*, 2013). The frequency of atypical memory B cells was markedly higher in the peripheral blood of cancer patients (both

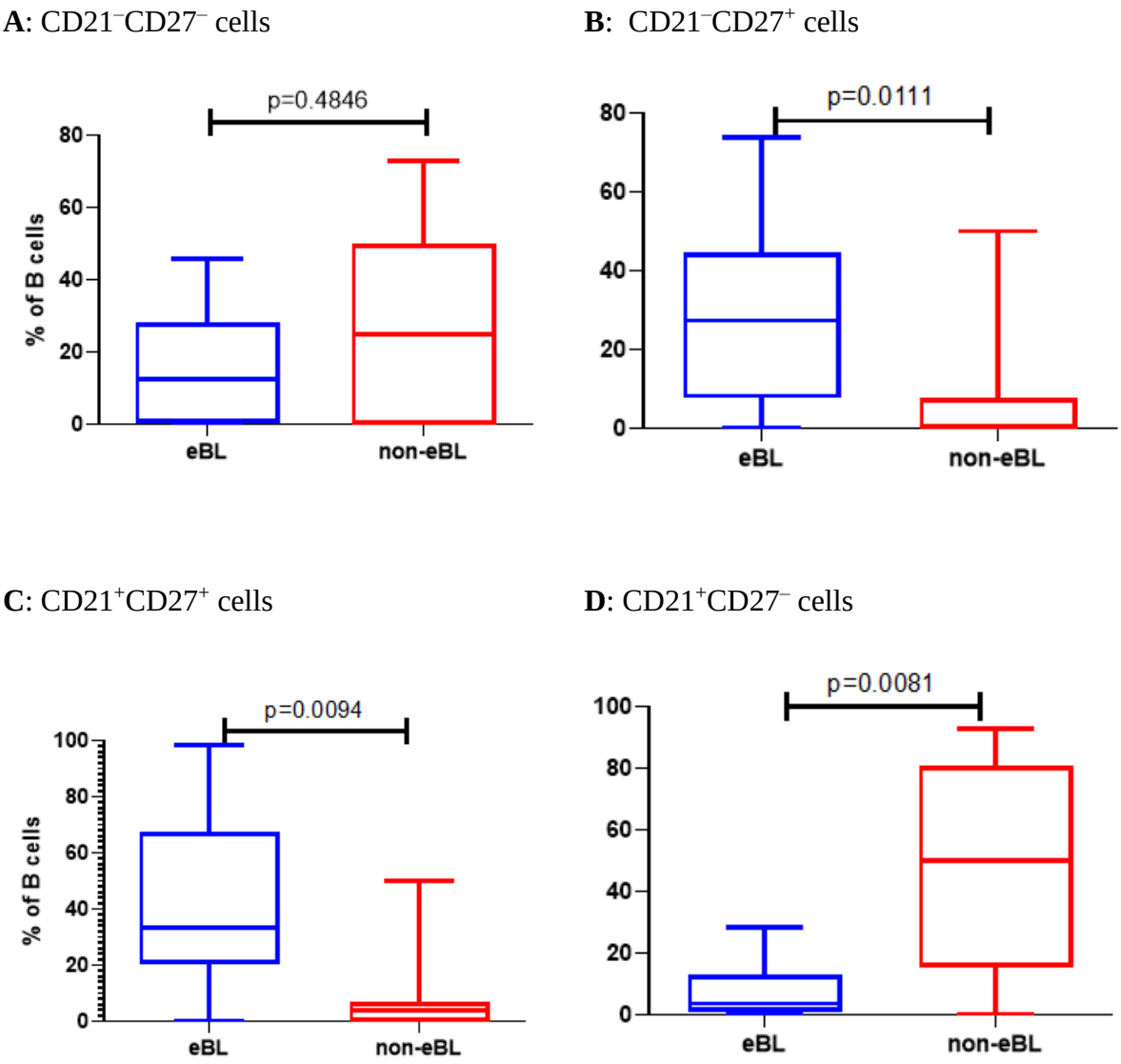
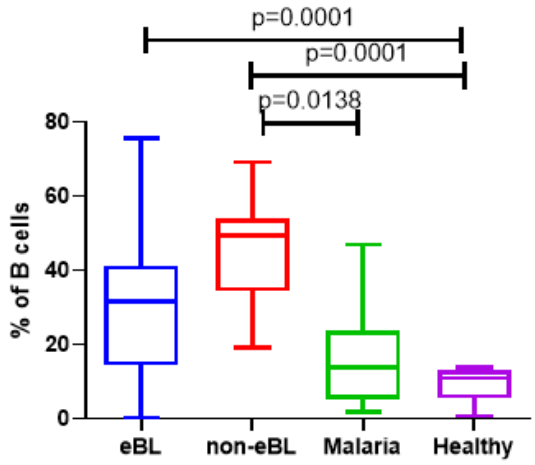


Figure 4.17. Expression of CD21 and CD27 by immature, tumour B cells
Distribution (% of $CD10^+CD20^+$ cells) of cells with a (**A**) classical immature, (**B**) germinal centre, (**C**) post-germinal centre and (**D**) transitional cell phenotype in the tumours of eBL patients (blue, n=19) and non-eBL patients (red, n=9).

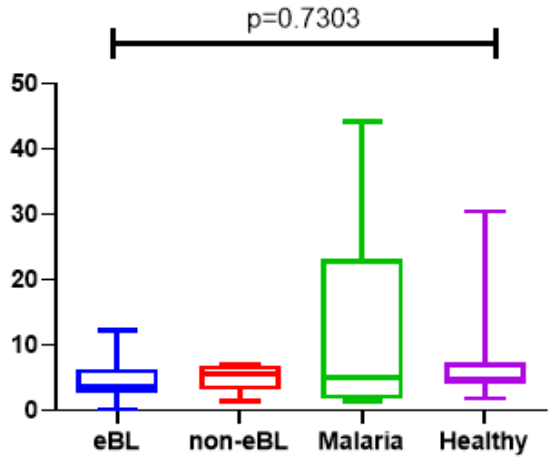
eBL and non-eBL) compared to healthy controls (Figure 4.18A). A similar picture was seen for the malaria patients. Correspondingly, the frequencies of classical memory B cells were lower among the cancer patients compared to healthy controls (Figure 4.18C). This was also the case for the malaria patients, which has not been observed previously. No marked inter-group differences were noted in the activated (Figure 4.18B) and naïve (Figure 4.18D) subsets.

In the tumours (Figure 4.19), the only marked difference observed was a lower frequency of naïve mature B cells in the eBL group, compared to the non-eBL group (Figure 4.19D).

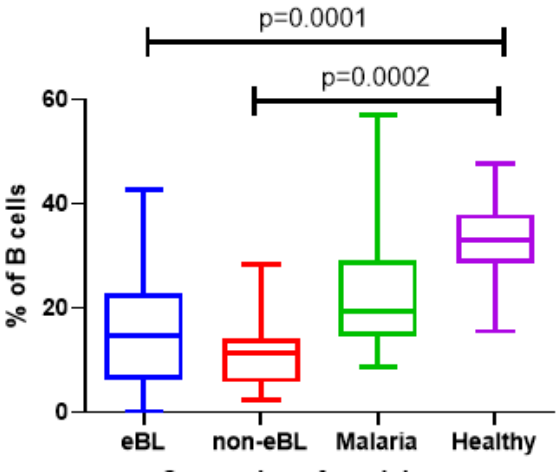
A: $CD21^{-}CD27^{-}$ cells



B: $CD21^{-}CD27^{+}$ cells



C: $CD21^{+}CD27^{+}$ cells



D: $CD21^{+}CD27^{-}$ cells

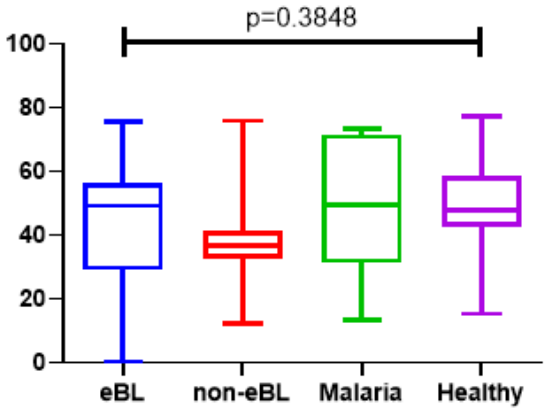


Figure 4.18. Expression of CD21 and CD27 by mature, circulating B cells
Distribution (% of $CD10^{-}CD20^{+}$ cells) of cells with an (**A**) atypical memory, (**B**)activated, (**C**) classical memory, and (**D**) naïve phenotype in the peripheral circulation of eBL patients (blue, n=19), non-eBL patients (red, n=9), and in age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

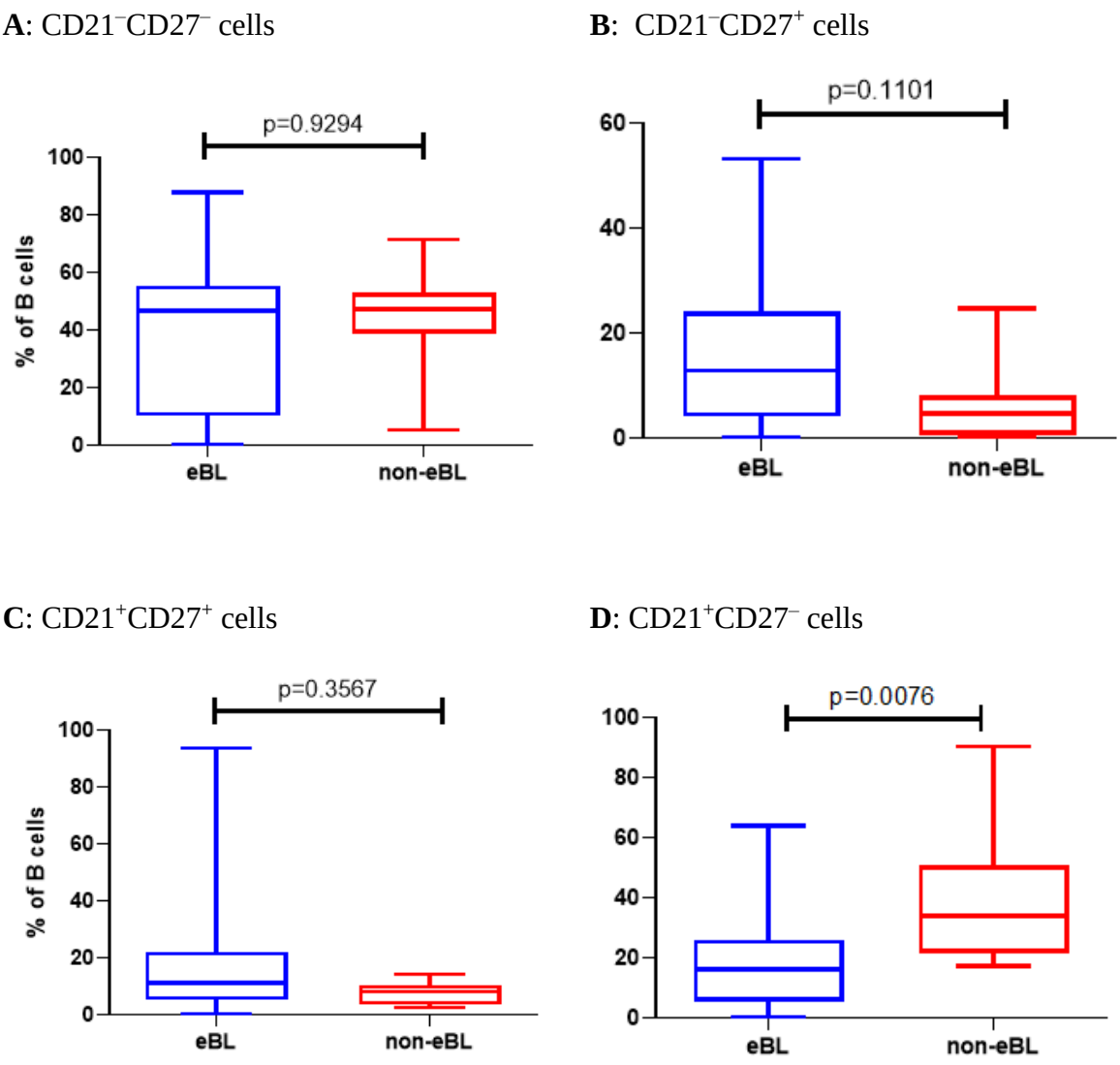
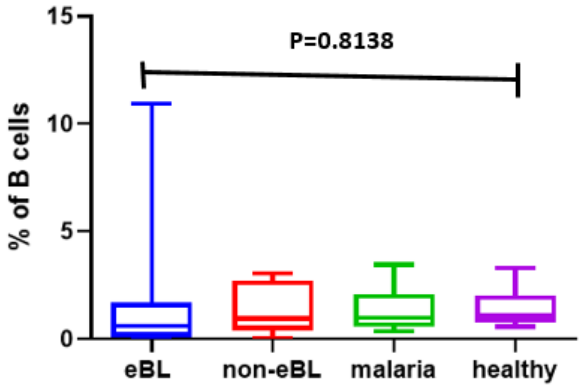


Figure 4.19. Expression of CD21 and CD27 by mature, tumour B cells
Distribution (% of $CD10^{-}CD20^{+}$ cells) of cells with an atypical memory (**A**), activated (**B**), (**C**) classical memory, and (**D**) naïve phenotype in the tumours of eBL patients (blue, n=19) and non-eBL patients (red, n=9).

4.5.3. Activation status of circulating and tumour B cells

To investigate the activation of the B cells, their expression of CD25 and PD1 was evaluated. There was no significant differences in B-cell expression of CD25 among the study groups, neither in the peripheral blood (Figure 4.20A) nor in the tumours (Figure 4.20B) (P=0.4 and

A: Peripheral blood



B: Tumours

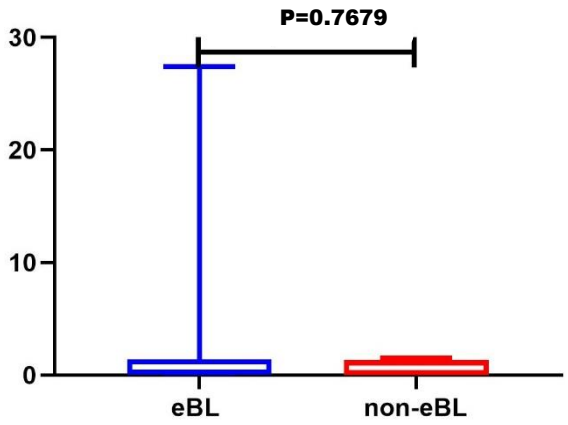


Figure 4.20. Frequencies of CD25$^+$ B cells
Frequencies of CD25$^+$ cells (% of all CD20$^+$ cells) in the (**A**) peripheral blood and (**B**) tumours of eBL patients (blue, n=19), non-eBL patients (red, n=9), and in peripheral blood of age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

P=0.8, respectively). In contrast, B-cell expression of PD1 differed significantly (P<0.01) among circulating B cells in the different study groups, with higher expression in the cancer and malaria patients, compared to healthy controls (Figure 4.21A). The expression of PD1 by B cells in the tumours was generally low and not different between the eBL and non-eBL cancer patients (Figure 4.21B).

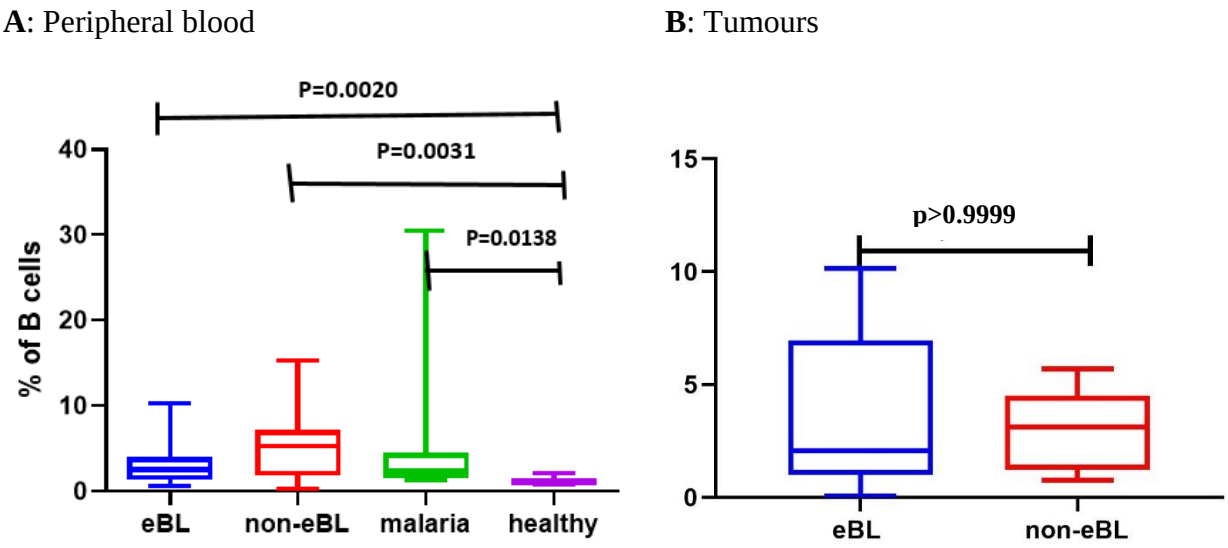


Figure 4.21. Frequencies of PD1$^+$ B cells
Frequencies of PD1$^+$ cells (% of all CD20$^+$ cells) in the (**A**) peripheral blood and (**B**) tumours of eBL patients (blue n=19), non-eBL patients (red, n=9), and in peripheral blood of age-matched children with (green, n=11) or without (purple, n=11) *P. falciparum* malaria.

4.6. B-cell receptor diversity and antigen specificities of eBL tumour cells

In this part of the thesis project, the hypothesis that B-cell activation by *P. falciparum* antigens is an important element in the malignant transformation in eBL was assessed. More specifically, the hypothesis that the BCR repertoire diversity of eBL tumour cells is more restricted and *P. falciparum*-specific than corresponding circulating B cells from the autologous patient is investigated. The hypothesis is based on the well-established co-endemicity of eBL and *P. falciparum* (see section 2.1). A manuscript describing the results obtained as part of this thesis work (which are preliminary and should be augmented by additional similar data), combined with data from section 4.6 and studies of the TCR repertoires of Vδ1$^+$ cells from eBL patients (see section 5.6), is planned. Tentative details regarding the planned manuscript can be found in Appendix 11 (Manuscript 3).

4.6.1. BCR sequence diversity of tumour and circulating B cells

An established method to produce recombinant monoclonal antibodies from single human B cells was used to characterize the BCRs of tumour and circulating B cells from four

representative eBL patients (AS/03, KB/01, KB/04 and KB/13) (Figure 3.2). Full-length Ig gene transcripts were amplified from cDNA after single-cell sorting of memory B cells as described (Smith *et al.*, 2009; Tiller *et al.*, 2008). The presence and amplification of matched immunoglobulin chain genes were verified by agarose gel electrophoresis after the second PCR (Figure 4.22). The overall efficiency for amplification of both heavy and light chain pairs from single cells was fairly low (~20%), compared to previous studies (30-60% efficiency) (Smith *et al.*, 2009; Tiller *et al.*, 2008). This may be due to the necessity of using cryopreserved cells here, as low efficiency has previously been noted when using frozen samples (Wardemann & Kofer, 2013). Although only limited data are presently available, the proportions of *igκ* and *igλ* in patient peripheral B cells (Figure 4.22, lower panel) appear to be similar to that expected in healthy donors (*igκ*: ~60%) and *igλ*: ~40%) (Wardemann *et al.*, 2003). This is in marked contrast to the tumour B cells, which appear to be strongly biased towards *igλ* (Figure 4.22, upper panel). In a few cases (~3%), both *igκ* and *igλ* chain genes were amplified, but in most of those, one band was highly dominant. Functional and productive re-arranged sequences could only be

obtained from the dominant band (e.g. KB/01 5B and 7H, Appendix 18).

4.6.2. Cloning and sequencing of BCR

One eBL patient, where data from both peripheral blood and tumour B cells is currently available, showed dominant use of IGHV3-30*04 combined with IGLV2-14*01 in B cells from the tumour. This is in contrast to the use of a diverse set of heavy and light chain variable segments among peripheral B cells from the same patient (Figure 4.23). Of further notice, strikingly similar tumour B-cell BCRs were detected in the different patients studied so far (AS/03, KB/01 and KB/13), both in terms of CDR3 length (IgH (12 amino acids) and Igλ (10 amino acids)) and amino acid composition (IgH (ARVAVMGPTLHY) and Igλ (SSYSSSSPYV)) (Appendix 18). Expression of recombinant antibodies

Six unique matched heavy and light chain CDR sequence pairs were selected for expression of recombinant monoclonal antibodies. One of these pairs was isolated from sample AS/03

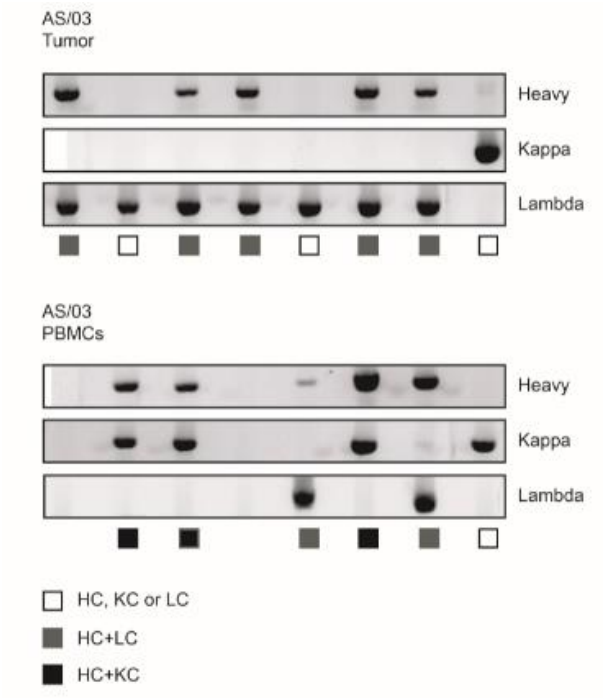


Figure 4.22. Agarose gel electrophoresis of Ig genes
An agarose gel of PCR products from a representative donor (AS/03) is shown. Matched pairs of *igh* and *igκ* (black), or of *igh* and *igλ* (grey) indicate possible *bona fide* hits. Single-positive columns are indicated in white.

(9H), but it was also found in the KB/01 and KB/13 samples. It is therefore representative of those three tumours. Three additional sequences from KB/01, and two from KB/04 were also chosen for expression (Appendix 18; bolded rows). The six antibodies were expressed in HEK 293F cells after liposome-based co-transfection with plasmids encoding the corresponding heavy and light chains. All the transfections were successful and resulted in the production of purified monoclonal antibodies at concentrations from 7-100 mg/L of culture supernatant. The quality of the purified antibodies samples was checked by SDS-PAGE in the presence and absence of the reducing agent DTT (Figure 4.24). All antibodies showed the expected pattern, with a dominant band around 150 kDa (intact IgG) under non-reducing conditions, and two bands at 50 kDa (heavy chains) and 25/35 kDa (light chains) under reducing conditions. The ~35 kDa size of the light chain band (IGLV 2-14*01) in AS/03 9H and in KB/01 7H was higher than expected, but may be explained by post-translational modifications or by low-complexity regions in IGLV 2-14*01. The antigen specificities of these antibodies are currently unknown, but are under ongoing investigation (see section 5.6).

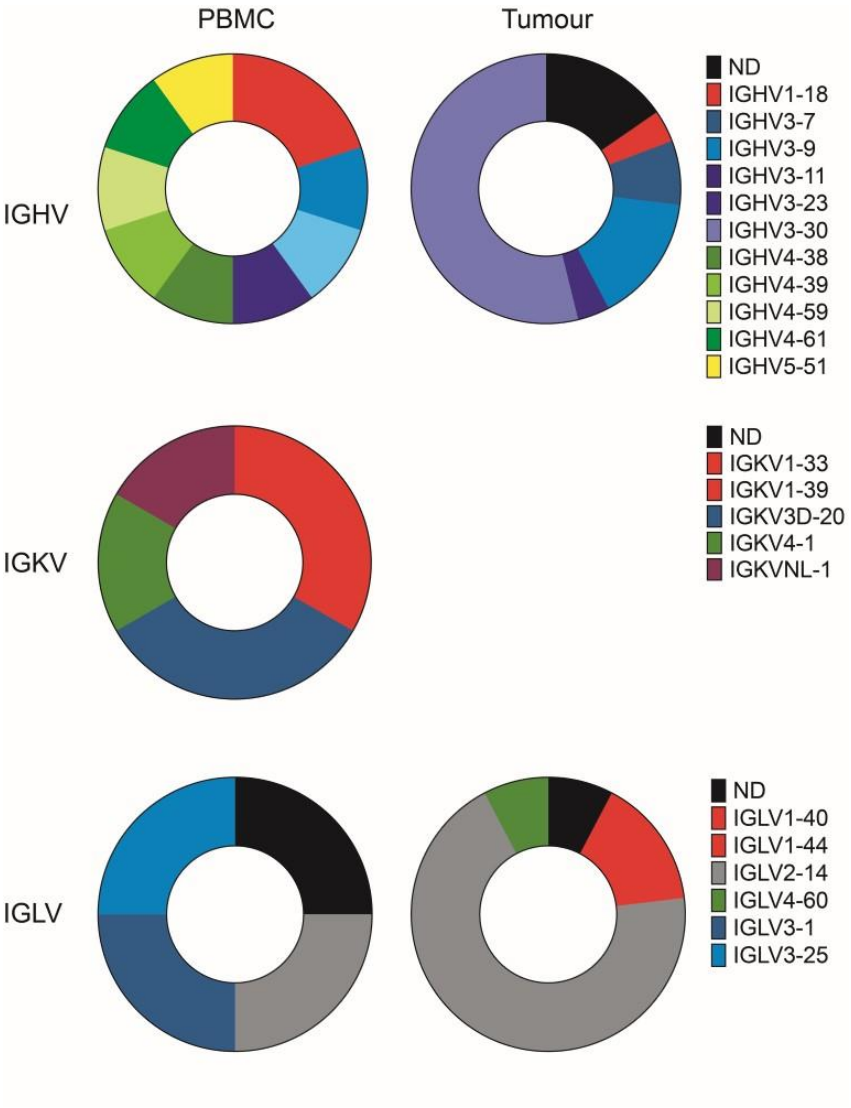


Figure 4.23. BCR receptor usage
IGH, IGK and IGL V-gene family distribution of BCRs encoded by eBL patient-derived B cells, single-cell sorted either from PBMC (left) or the tumour (right). Families are colour coded with family 1 in red, family 2 in grey, family 3 in blue, family 4 in green and family 5 in yellow. ND (black): not determined. The size of the coloured area indicates the frequency (%).

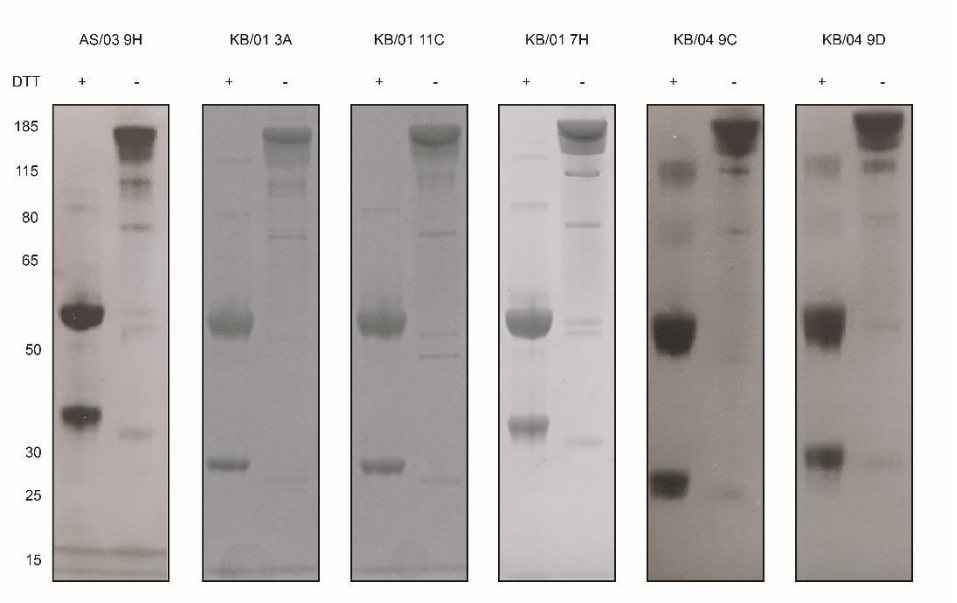


Figure 4.24. Recombinant eBL-derived IgG
SDS gel picture of purified recombinant monoclonal human antibodies under non-reducing (-DTT) and reducing conditions (+DTT). 5 µg of protein were subjected to a 4–12% BisTris polyacrylamide gel electrophoresis. Protein bands were visualized by Coomassie blue staining.

5. Discussion

5.1. Accuracy of morphology-based diagnosis of endemic Burkitt lymphoma

Endemic Burkitt lymphoma is a highly aggressive extra-nodal tumour of children in areas characterized by early and massive exposure to EBV and stable transmission of the malaria parasite *P. falciparum* (Burkitt, 1983; Molyneux *et al.*, 2012). The disease is therefore largely restricted to equatorial Africa, where it is often the most common paediatric malignancy. The prognosis is poor, particularly when diagnosis is delayed and only incomplete chemotherapy is administered, which is often the case in the low-income settings where eBL is most common (Joko-Fru *et al.*, 2018).

In endemic areas, an eBL diagnosis is usually made on the clinical picture and microscopic examination of cell/tissue morphology of tumour aspirates or biopsies (Molyneux *et al.*, 2012). The pathogenesis of eBL characteristically involves a translocation of the *c-myc* gene from chromosome 8 to the immunoglobulin gene locus on chromosome 14 (heavy chain) (about 80% of cases) or less often to either the κ or the λ light chain locus on chromosome 2 and chromosome 22, respectively (Diebold, Jaffe, Raphael, & Warnke, 2001). The translocation drives lymphomagenesis by deregulation of proliferation of the affected B-cell clone, due to overexpression of C-MYC protein. Detection of *c-myc* translocation by FISH and detection of C-MYC expression by immunohistochemistry therefore constitute important additional diagnostic assays (Dave *et al.*, 2006; Molyneux *et al.*, 2012), although these are rarely employed in eBL-endemic settings. In this study, we assessed the sensitivity and specificity of morphologic eBL diagnosis in an endemic setting, and whether diagnostic accuracy could be improved by adding FISH and/or immunohistochemistry.

Retrospective and blinded morphological diagnosis by microscopy of FNA smears showed good agreement with the original diagnoses made after similar examination of separate smears at the pathology departments at the Ghanaian hospitals admitting the patients in Accra and

Kumasi (KBTH and KATH, respectively). Similar assessment of newly made sections of FFPE tissue blocks stored for up to eight years yielded a similar picture, although sensitivity and specificity estimates were lower, likely due to problems with inadequate preparation and/or storage of the archival samples.

Confirmation of the original diagnosis by FISH detection of *c-myc* breakage and *c-myc/igh* fusion (*c-myc* translocation) was generally sensitive, indicating that eBL cases showed generally positive for *c-myc* breakage and translocation which is common with them and a hallmark of eBL pathogenesis. Only translocation of *c-myc* to the heavy chain locus was detected. Specificity was fairly low, as we found no evidence of *c-myc* translocation in samples from a substantial proportion of patients originally diagnosed with eBL. However, some of the non-eBL cases showed positivity for *c-myc* translocation which indicated that *c-myc* translocation is found in other cancers besides eBL. These findings were supported by analysis of c-MYC expression by immunohistochemistry. A few patients originally diagnosed with non-eBL showed retrospective evidence of *c-myc* translocation, but this also occurs, albeit less frequently, in other aggressive B-cell lymphomas such as lymphoblastic lymphoma and diffuse large B-cell lymphoma (Ott, Rosenwald, & Campo, 2013).

The findings of this study suggest that cancer diagnosis can be improved by the addition of complementary tests such as FISH and IHC using specific genes and proteins, respectively peculiar to the particular cancer to be diagnosed.

5.2. Frequencies of tumour-infiltrating and circulating Vδ1$^+$ γδ T cells

T cells are important in the adaptive immune response. The activation of these cells leads to the activation of other immune cells in response to infection and cancer. However, lowered T cell frequencies observed in this study among the cancer patients were expected, as cancer is known to cause an inflammation-related redistribution of T cells away from the peripheral circulation to sites of insult and inflammation (162). Because eBL is a B cell tumour and the

included non-eBL patients were suffering from other childhood cancers, it was also expected that the average frequency of $CD3^+$ T cells in the tumours (tumour-infiltrating T lymphocytes; TILs) was lower ($P<0.01$) in eBL tumour FNAs than in FNAs of non-eBL tumours (Figure 4.5B).

T cells also act as immune effectors, including "non-conventional" T cells such as TCR-γδ cells. Indeed, recent studies advocate use TCR-$\gamma\delta^+$ T cells in adoptive transfer therapy of some cancers (Siegers & Lamb, 2014). In the present study, the role of TCR-γδ T cells, and in particular the $V\delta1^+$ subtype of these cells, in eBL immunity was investigated. Specifically, the frequencies and phenotypes of peripheral and tumour-infiltrating TCR-$\gamma\delta^+$ and $V\delta1^+$ T cells were determined in eBL patients, and compared with non-eBL cancer patients, malaria patients, and healthy controls.

The overall frequencies of TCR-γδ T cells and the $V\delta1^+$ and $V\delta1^-$ subsets in eBL patients were not significantly different from the other study groups, although these were all higher than those generally reported from healthy individuals living outside Africa (Hviid *et al.*, 2000; Hviid *et al.*, 2019). Increased frequencies of $V\delta1^+$ in the peripheral blood of cancer patients, including eBL patients have been reported previously (Futagbi *et al.*, 2007; Reboursiere *et al.*, 2018). The findings obtained here are consistent with those reports, and their conclusions that $V\delta1^+$ cells contribute significantly to the immune response in cancer. More significantly, the data obtained here extend the earlier reports by examining $V\delta1^+$ TILs directly, which documented a specific enrichment of $V\delta1^+$ cells in the tumours. Exactly what the role of these cells in the immune response to cancer remains unclear. Some studies point to a cytotoxic anti-tumour function (Siegers *et al.*, 2011), whereas other studies indicate that these cells can also be immune-suppressive and inhibit anti-tumour immunity (Ma *et al.*, 2012; Peng *et al.*, 2007). The present study does not shed light on this controversy, but does support a direct effector function of these cells in the immune response to eBL, as a remarkable dominance of terminally

differentiated effector Vδ1$^+$ cells was found in the tumours and peripheral blood of the eBL patients (Figure 4.7A and Figure 4.9A) as well as non-eBL patients (Figure 4.7B and Figure 4.9B). Therefore it is speculated that these T_{EMRA} cells in the tumours may play a specific/significant role in the immune response to the tumours depending on the type of tumour. However, that role is was not determined in this current study. It is of note that this was not seen for the complementary Vδ1$^-$ subset (Figure 4.8 and Figure 4.10) where most of the cells were T_{CM} in the other study groups beside eBL. With this observation, we speculate that eBL influence the maturation of Vδ1$^+$ T cells in both peripheral blood and tumour while eBL effect on maturation of Vδ1$^-$ T cells is minimal only in the peripheral blood.

Finally, examination of the activation status of Vδ1$^+$ T cells (assessed by the level of expression of CD25 and PD1) revealed no remarkable differences when comparing circulating cells in the different study groups (Figure 4.12A and Figure 4.13A). However, both markers indicated substantial activation of Vδ1$^+$ TILs in eBL, but not in non-eBL (Figure 4.12B and Figure 4.13B).

5.3. Frequencies of tumour and circulating B cells

eBL tumour cells originate from germinal centre-stage B cells, as evidenced by their expression of somatically mutated BCR V-region genes (Cohen *et al.*, 1987; Klein *et al.*, 1995). Their uniform expression of the common acute lymphoblastic leukaemia antigen (CALLA), which is also a germinal centre marker is compatible with this derivation (Gregory *et al.*, 1987; Ling *et al.*, 1989), was confirmed here (Figure 4.15B). Information on maturational subtypes of these cells is limited, and this study therefore aimed to describe the phenotypes of circulating and tumour B cells in eBL. To that end, the surface markers CD21 and CD27 were used, as they have previously been used to delineate subsets of CD10$^-$ and CD10$^+$ B cells (Clavarino *et al.*, 2016; Illingworth *et al.*, 2013) .

As expected, only a minority of peripheral B cells expressed CD10, with the lowest frequencies found in the cancer patients (Figure 4.15A). This suggest that most of these cells are located in the germinal centres of lymph nodes no wonder their frequency were high especially in the eBL tumours which affect lymph nodes and lymphoid cells. Moreover, the dominance of cells with a germinal or post-germinal centre phenotype among immature B cells in the eBL tumours, in contrast to non-eBL tumours (Figure 4.17) indicates that eBL tumour cells are antigen-experienced.

No major differences among the study groups were identified with respect to expression of CD21 and CD27 by peripheral $CD10^+$ cells, apart from a tendency towards lowered frequencies of transitional-type B cells in the patient groups compared to healthy controls (Figure 4.16D). It is tempting to speculate whether the high frequency of immature B cells found among the studied malaria patients (Figure 4.16A) can be attributed to the polyclonal activation and proliferation of B cells in *P. falciparum* malaria (Whittle *et al.*, 1990).

Among mature ($CD10^-$) B cells, mature B cell in the peripheral blood of the study groups, a markedly increased frequency of atypical memory B cells was evident among the eBL and non-eBL cancer patients (Figure 4.18A). This suggest that most of the B cells though have been exposed to an antigen they have become redundant due to lack of continuous exposure to that same antigen they had experienced. However, the BCR of these cell encode antibody to the antigen they have been exposed to indicating that they can produce neutralizing antibodies (Muellenbeck *et al.*, 2013). This finding is in agreement with earlier evidence of B-cell exhaustion in HIV infection (Moir *et al.*, 2008) and that EBV infect memory B cells (Hochberg *et al.*, 2004). A similar picture was seen for the malaria patients, also in accordance with previous reports (Ampomah *et al.*, 2014; Illingworth *et al.*, 2013). Together with the evidence of increased expression of CD25 (Figure 4.20A) and PD1 (Figure 4.21A)

among peripheral B cells in eBL patients, this is consistent with a chronic state of immune activation in these patients.

It is concluded that eBL is a disease of germinal centre B cells. These cells have been exposed to antigen, and it is hypothesized that *P. falciparum* is a prominent source of the antigen(s) involved.

5.4. BCR repertoires and antibody targets

The BCR is the antigen binding receptor expressed on the surface of B cells. During the development of B cells in the bone marrow, the BCR of each B-cell clone undergoes VDJ recombination to generate clones that express unique receptors. When a B-cell clone encounters its cognate antigen in secondary lymphoid tissues, the clone expands and the BCRs of the daughter cells undergo somatic hyper-mutation, leading to their expression of BCRs with higher affinity than the original B-cell first activated (affinity maturation) (Hoehn, Fowler, Lunter, & Pybus, 2016). The specific role of *P. falciparum* in eBL tumorigenesis is not known, although several hypotheses have been proposed (see section 2.2.3). Of specific relevance to the present study is the possibility that EBV-infected B-cell clones that express BCRs able to recognize *P. falciparum*-specific antigens are particularly prone to the chain of events that ultimately leads to eBL (Moormann & Bailey, 2016). This hypothesis, which is currently not well underpinned by experimental evidence, was investigated here.

Recent studies have looked into BCR repertoires and usage of immunoglobulin variable regions in eBL tumour cells, to examine if particular Ig rearrangements are associated with eBL, and more importantly if the level of BCR sequence diversity suggests that particular antigens (in particular *P. falciparum* antigens) is directly involved in eBL tumour genesis. Most data available so far comes from high-throughput sequencing of bulk DNA and RNA from eBL tumour cells (Lombardo *et al.*, 2017). This type of data is extremely informative, but does not allow the confident matching of the heavy and light chains expressed by clonal B cells, which

is required for further characterization of the BCR specificity. To overcome this difficulty, the present study studied the BCRs of single cell-sorted B cells.

Highly biased V(D)J rearrangements were observed, as most tumour-derived B cells expressed sequences homologous to VH3 (3-7*01, 3-9*03, 3-23*04, and especially 3-30*04) and VL2 (2-14*01), when compared to peripheral B cells from the same patients. Such biased VH usage has been reported in several recent studies of eBL-derived tumour samples (Amato *et al.*, 2016; Grande *et al.*, 2019; Lombardo *et al.*, 2017). Preferential use of a λ-chain of the VL 2-14 family has been also observed in chronic lymphocytic leukaemia (CLL), which is another non-Hodgkin B-cell lymphoma (Tobin *et al.*, 2003).

The data obtained so far, support the clonal nature of eBL tumours, since the majority of the sequences retrieved from single cells in individual donors were closely related. This can confidently be concluded for AS/03 and KB/01, where many individual BCR sequences were evaluated. The presence of spurious and unrelated BCRs suggest circulating B cells that were accidently included in the FNA sample. This hypothesis is supported by the identification of germline BCRs (e.g., 3G, 5C, 7H, and 9F in sample KB/01, typical of naïve B cells. Most interestingly, the analysis of BCR sequences from just a small subset of the eBL tumour samples collected for this thesis work revealed a very restricted BCR repertoire, identifying almost identical BCRs in some individuals (AS/03, KB/01 and KB/13). It is tempting to speculate that this highly restricted and biased repertoire reflects that a similar antigen drove the BCR selection and clonal expansion of the tumour B-cell clone in all three patients. What this antigen might be is not known, but the monoclonal antibodies that have been generated as part of this thesis work should help addressing this question in future investigations. A similar stereotypic and quasi-identical BCR repertoire has previously been reported in CLL tumours (Ghiotto *et al.*, 2004; Stamatopoulos *et al.*, 2007; Widhopf *et al.*, 2004).

Although the data are preliminary at this point, this not only indicates that eBL tumours are largely monoclonal (which was expected), but also that they tend to encode closely related IgH and Igλ sequences arising from the use of the same variable segments; this was in contrast to the polyclonal nature of BCRs expressed by B cells in circulation, where each single B cell analysed used different variable segments with no overlap in sequence (Figure 4.23).

It is concluded that eBL are highly clonal, and the diversity in the rearrangement of the gene segments appears to be strongly influenced by exposure to a particular antigen. We speculate that this might be a *P. falciparum* antigen, but this is not yet known.

5.5. Limitations of the current study

The studies presented here have several limitations. First of all, the number of samples available for analysis was low, mostly because fewer patients were admitted during the period where samples could be collected. For some of the analyses, the number of samples that could be analysed was further reduced by the financial constraints imposed and the high costs of reagents required. This affected both the phenotypic analysis, and the scope of the BCR sequence diversity investigation. While the preliminary BCR sequence data obtained here clearly support the notion of specific B-cell antigen(s) driving tumour genesis in eBL, it was not possible to identify the antigen specificity of the BCRs encoded by the eBL tumours analysed.

With respect to the analysis of archival samples (section 4.3), the poor preservation status of a sizeable proportion of the FFPE biopsies was a challenge.

Finally, some of the assays that were originally planned to form part of the laboratory work, not least the single-cell RNA sequencing of tumour lymphocytes, could not be completed within the time frame of the project for reasons beyond my control.

5.6. Future studies

The data presented in this thesis provide several promising and novel research leads. It will be of great interest to continue the phenotypic characterization of Vδ1$^+$ cells from additional patients, in an effort to improve the statistical power of the findings obtained so far and whether there will be any differences that will influence the probability to accept or reject the null hypothesis. It would also be of interest to add a longitudinal aspect to the study of Vδ1$^+$ cells, to allow comparison of cells obtained during the acute disease with cells obtained after drug-induced remission/cure. Such a study is not currently possible in Ghana, because very few eBL patients receive the treatment that is essential for their survival. We are currently looking for funding to support provision of the eBL chemotherapy that is of vital importance to the patients, and a prerequisite for longitudinal studies of them.

It is the plan to continue the studies of the apparent restriction in the BCR repertoire of the eBL tumour cells, and to pursue identification of the putative conserved antigen(s) driving the malignant B-cell transformation in eBL. In a complementary approach, we wish to investigate whether a similar restriction exists in the TCR repertoire of Vδ1$^+$ TILs. We have already submitted several application for funds to make such studies possible.

6. Conclusions

With respect to the analysis of the diagnostic accuracy of eBL diagnosis in Ghana, it is concluded that the original diagnoses, which involved laboratory assessment of tumour cell morphology, were reliable, when evaluated by independent retrospective analysis of specimens similar to those available at the time of the original diagnosis. However, diagnostic specificity can probably be improved by introduction of immuno-histochemical analysis for evidence of C-MYC expression. Furthermore, the data presented in this thesis support the use of FNA samples for pathology laboratory investigations, in particular when inadequate preservation of biopsy material obtained by surgery makes the justification for this more injurious and complicated procedure questionable.

With respect to the role of $V\delta1^+$ T cells in the immune response to eBL, it is concluded that the evidence obtained supports the first basic project hypothesis, namely that $V\delta1^+$ T cells are important regulators of the B-cell proliferation in eBL. The conclusion is based on the finding of enrichment of $V\delta1^+$ cells in the tumours that have an activated and terminally differentiated effector-memory phenotype in eBL tumours. Evidence of accumulation of $V\delta1^+$ in non-eBL tumours was also observed, raising the possibility that these cells may play both protective and pathogenic roles, depending on the type of tumour.

Findings regarding B-cell phenotypes were generally in agreement with earlier observations. They support the view that eBL tumour cells are cells that have been activated by antigen and differentiated in germinal centres. Of potentially high significance, the preliminary data on the BCR repertoires of circulating and tumour B cells from eBL patients are clearly consistent with the study hypothesis that particular antigen(s) drives the malignant transformation in eBL, although direct evidence regarding the specific antigen(s) involved was not obtained.

7. Recommendations

- The diagnostic specificity of eBL can probably be improved by introduction of immuno-histochemical analysis for evidence of C-MYC expression. Addition of complementary diagnostic test in the diagnosis of cancer in general will improve accuracy of diagnosis. This should be economically feasible.
- FNA samples, as opposed to tissue biopsies, should be collected for diagnostic laboratory investigations of Ghanaian patients suspected of eBL. The evidence obtained here shows that FNA analysis is as good as biopsy analysis, and the collection of FNA material is simpler and less injurious than collection of biopsy material, which requires surgery.
- If biopsy material is collected and archived, increased attention should be paid to adequate preservation and storage.
- The phenotypic characterization of $V\delta1^+$ T cells should be continued on additional patients to increase the robustness of the findings obtained in this project. In addition, it would be very informative, if a longitudinal aspect could be added to such investigations.
- The work on BCR sequence analysis and eBL tumour B-cell-derived monoclonal antibodies should be continued and expanded, to substantiate the preliminary conclusion that particular antigen(s) – possibly from *P. falciparum* parasites – are important drivers of eBL tumour genesis.
- Comprehensive analysis of BCR and TCR repertoires by single-cell RNA sequencing is strongly recommended.

8. References

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Appendices

Appendix 1. Study approval, KATH (2017)

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICAL SCIENCES / KOMFO ANOKYE TEACHING HOSPITAL
COMMITTEE ON HUMAN RESEARCH, PUBLICATION AND ETHICS

CHRPE/AP/175/17 20th March, 20

Ms. Cecilia Smith
Post Office Box LT 190
ACCRA.

Dear Madam,

LETTER OF APPROVAL

Protocol Title: *"Understanding the Molecular Mechanisms of Plasmodium Falciparum and Epstein-Barr Virus Involvement in the Pathogenesis of Endemic Burkitt's Lymphoma and of the Host Immune Responses to this Tumour."*

Proposed Site: *Child Health Directorate, Komfo Anokye Teaching Hospital.*

Sponsor: *Building Stronger University, Office of Research, Innovation and Development, University of Ghana.*

Your submission to the Committee on Human Research, Publications and Ethics on the above named protocol refer

The Committee reviewed the following documents.

- A notification letter of 26th July, 2016 from the Department of Nursing seeking permission from Korle-Bu Teaching Hospital (study site) and it was approved.
- A Completed CHRPE Application Form.
- Participant Information Leaflet and Consent Form.
- Research Protocol.

The Committee has considered the ethical merit of your submission and approved the protocol. The approval is fo fixed period of one year, beginning 20th March, 2017 to 19th March, 2018 renewable thereafter. The Committee m however, suspend or withdraw ethical approval at any time if your study is found to contravene the approved protoco

Data gathered for the study should be used for the approved purposes only. Permission should be sought from Committee if any amendment to the protocol or use, other than submitted, is made of your research data.

The Committee should be notified of the actual start date of the project and would expect a report on your study, annu or at the close of the project, whichever one comes first. It should also be informed of any publication arising from study.

Yours faithfully,

Rev. Prof. John Appiah-Poku.
Honorary Secretary
FOR: CHAIRMAN

Appendix 2. Study approval, KATH (2018)

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICAL SCIENCES / KOMFO ANOKYE TEACHING HOSPITAL
COMMITTEE ON HUMAN RESEARCH, PUBLICATION AND ETHICS

Our Ref: CHRPE/AP/242/18 4th May, 201

Ms. Cecilia Smith
Post Office Box LT 190
ACCRA.

Dear Madam,

LETTER OF APPROVAL

Protocol Renewal: *"Understanding the Molecular Mechanisms of Plasmodium Falciparum and Epstein-Barr Virus Involvement in the Pathogenesis of Endemic Burkitt's Lymphoma and of the Host Immune Responses to this Tumour."*

Proposed Site: *Child Health Directorate, Komfo Anokye Teaching Hospital.*

Sponsor: *Building Stronger University, Office of Research, Innovation and Development, University of Ghana.*

Your submission to the Committee on Human Research, Publication and Ethics on renewal to protocol No. CHRPE/AP/175/17 dated 20th March, 2017 refers.

The Committee reviewed the following documents.

- A notification letter of 26th July, 2016 from the Department of Nursing seeking permission from Korle-Bu Teaching Hospital (study site) and it was approved.
- A Completed CHRPE Application Form.
- Participant Information Leaflet and Consent Form.
- Research Protocol.

The Committee has considered the ethical merit of your proposed renewal and approved it. The approval is for a fixe period of one year, beginning 4th May, 2018 to 3rd May, 2019 renewable thereafter. The Committee may however, suspend or withdraw ethical approval at any time if your study is found to contravene the approved protocol.

Data gathered for the study should be used for the approved purposes only. Permission should be sought from t Committee if any amendment to the protocol or use, other than submitted, is made of your research data.

The Committee expects a report on your study annually or at the close of the project, whichever one comes first. should also be informed of any publication arising from the study.

Yours faithfully,

Osomfo Prof. Sir J. W. Acheampong MD, FWACP
Chairman

Appendix 3. Study approval, KBTH

In case of reply the number
And the date of this
Letter should be quoted

My Ref. No. KBTH/MD/63/17

Your Ref. No......

KORLE BU TEACHING HOSPITAL
P. O. BOX KB 77,
KORLE BU, ACCRA.

Tel: +233 302 667759/673034-6
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Email: Info@kbth.gov.gh
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20th July, 2017

CECILIA SMITH
DEPT. OF BIOCHEMISTRY, CELL AND
MOLECULAR BIOLOGY
UNIVERSITY OF GHANA, LEGON

"UNDERSTANDING THE MOLECULAR MECHANISM OF PLASMODIUM FALCIPARUM AND EPSTEIN-BARR VIRUS INVOLVEMENT IN THE PATHOGENESIS OF ENDEMIC BURKITT'S LYMPHOMA, AND OF THE HOST IMMUNE RESPONSES TO THIS TUMOUR"

KBTH – IRB /00080/2016

Investigator: Cecilia Smith

On 20th July, 2017, the Korle-Bu Teaching Hospital Institutional Review Board (KBTH IRB) reviewed and granted approval to the study entitled "Understanding the Molecular Mechanism of Plasmodium Falciparum and Epstein-Barr Virus Involvement in the Pathogenesis of Endemic Burkitt's Lymphoma, and of the Host Immune Responses to This Tumour"

Please note that the Board requires you to submit a final review report on completion of this study to the KBTH-IRB.

Kindly, note that, any modification/amendment to the approved study protocol without approval from KBTH-IRB renders this certificate invalid.

Please report all serious adverse events related to this study to KBTH-IRB within seven days verbally and fourteen days in writing.

This IRB approval is valid till 30th June, 2018. You are to submit annual report for continuing review.

Sincere regards,

OKYERE BOATENG (MR)
CHAIR (KBTH-IRB)

Cc: The Chief Executive Officer
Korle Bu Teaching Hospital

The Director of Medical Affairs
Korle Bu Teaching Hospital

Appendix 4. Ethical approval, NMIMR

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979A Constituent of the College of Health Sciences
University of Ghana
INSTITUTIONAL REVIEW BOARD

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Post Office Box LG 581
Legon, Accra
Ghana

My Ref. No: DF.22
Your Ref. No:

8th March, 2017

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 083/16-17 **IORG 0000908**

On 8th March, 2017, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **The role of malaria and tumor immunity in the pathogenesisof endemic Burkitt'slymphoma**

PRINCIPAL INVESTIGATOR : **Cecilia Smith, PhD Cand.**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 7th March, 2018. You are to submit annual reports for continuing review.

Signature of Chair: .....
Mrs. Chris Dadzie
(NMIMR – IRB, Chair)

Appendix 5. Introduction letter, Afigya Kwabre District Education Office

GHANA EDUCATION SERVICE

AFIGYA KWABRE DISTRICT EDUCATION OFFICE

(KODIE –ASH.)

P. O. BOX SE
SUAME – KU
TEL : 03220-95007/9

Our Ref GES/ASH/AKD/VOL/48/II/01

Your Ref.....

REPUBLIC OF GHANA

DATE: 13th July, 2018

THE HEADTEACHER
KODIE METH. PRIM. 'A'
KODIE-ASHANTI

LETTER OF INTRODUCTION
CECILIA SMITH

We wish to introduce to you the above named student from the University of Ghana, Legon. She is writing her project on the subject "understanding the role and molecular mechanism of plasmodium falciparum and Epstein-Barr virus in the pathogenesis of endemic Burkitt's lymphoma".

She has chosen Afigya Kwabre District as her place of study.

We would be grateful if you could extend the necessary courtesies to her.

Thank you.

STELLA OFORI-ATTA (MRS)
DISTRICT DIRECTOR

CC:
CECILIA SMITH
UNIVERSITY OF GHANA
LEGON

Appendix 6. Detailed characteristics of study patients

Patient	Age (years)	Sex	Hospital	Diagnosis	Tumour site
AS/01	8	M	KATH	eBL	Abdomen
AS/02	13	F	KATH	LBCL, non-cleaved	Jaw
AS/03	10	M	KATH	eBL	Abdomen
AS/04	10	M	KATH	eBL	Abdomen
AS/05	11	M	KATH	eBL	Jaw
AS/06	6	F	KATH	Spindle cell carcinoma	Abdomen
AS/07	6	M	KATH	eBL	Jaw
AS/08	5	F	KATH	Benign	Sub-mandible
AS/09	7	F	KATH	Benign	Abdomen
AS/10	3	M	KATH	eBL	Abdomen
AS/11	6	F	KATH	Neurofibroma	Cervix
AS/12	11	M	KATH	eBL	Abdomen
AS/13	7	F	KATH	Hepatoblastoma	Abdomen
AS/14	7	M	KATH	eBL	Abdomen
AS/15	10	F	KATH	eBL	Abdomen
AS/16	7	M	KATH	eBL	Abdomen
AS/17	9	M	KATH	eBL	Abdomen
AS/18	5	F	KATH	eBL	Abdomen
AS/19	9	F	KATH	eBL	Abdomen
AS/20	9	M	KATH	eBL	Jaw
AS/21	13	F	KATH	eBL	Jaw
AS/22	9	F	KATH	eBL	Abdomen

Patient	Age (years)	Sex	Hospital	Diagnosis	Tumour site
AS/23	12	M	KATH	Lymphoma (?)	Eye
AS/24	6.5	M	KATH	eBL	Cervix
AS/25	6	F	KATH	eBL	Abdomen
AS/26	5	M	KATH	eBL	Jaw
AS/27	8	M	KATH	eBL	Jaw
AS/28	7	M	KATH	eBL	Jaw
AS/29	11	M	KATH	eBL	Jaw
AS/30			KATH	Small non-cleaved cell lymphoma	Neck
AS/31	15	M	KATH	Non-Hodgkin's lymphoma	Sub-mandible
AS/32	12	M	KATH	Reactive lymph nodes	Abdomen
AS/33	5	M	KATH	eBL	Jaw
AS/34	4	M	KATH	Reactive lymph nodes	Jaw
AS/35	2.5	M	KATH	Small non-cleaved cell lymphoma	Abdomen
KB/01	8	F	KBTH	eBL	Abdomen
KB/02	5	M	KBTH	Rhabdomyosarcoma	Face
KB/03	12	M	KBTH	eBL	Abdomen
KB/04	2	M	KBTH	eBL	Abdomen
KB/05	2	M	KBTH	Hepatoblastoma	Abdomen
KB/06	2	M	KBTH	Malignancy (?)	Abdomen
KB/07	7	F	KBTH	Neuroblastoma	Abdomen

Patient	Age (years)	Sex	Hospital	Diagnosis	Tumour site
KB/08	7	F	KBTH	Malignant	Abdomen
KB/09	8	M	KBTH	eBL	Abdomen
KB/10	1	M	KBTH	Malignancy (?)	Cervical
KB/11	7	F	KBTH	eBL	Jaw
KB/12	12	M	KBTH	Sarcoma	Jaw
KB/13	5	F	KBTH	eBL	Jaw
KB/14	13	F	KBTH	Lymphoma	Abdomen
Kbu/01	4	F	KBTH	eBL	Jaw

Appendix 7. Characteristics of donors of archival tissue samples

Patient	Age (years)	Sex	Hospital	Diagnosis	Tumour site
IHC/AS/40	9	F	KATH	eBL	Retroperitoneal
IHC/AS/41	7	M	KATH	eBL	Mesenteric
IHC/AS/42	8	F	KATH	eBL	Intra-abdominal
IHC/AS/43	3	M	KATH	eBL	Buccal
IHC/AS/44	3	M	KATH	eBL	Neck
IHC/AS/45	7	F	KATH	eBL	Abdominal
IHC/AS/46	9	F	KATH	Burkitt lymphoma	Omental
IHC/AS/47	11	M	KATH	eBL	Cervical
IHC/AS/48	12	M	KATH	eBL	Mandibular
IHC/AS/49	15	M	KATH	eBL	Cervical
IHC/AS/50	5	M	KATH	eBL	Buccal
IHC/AS/51	7	M	KATH	eBL	Abdominal
IHC/AS/52	8	M	KATH	eBL	Buccal
IHC/AS/53	9	M	KATH	eBL	Neck
IHC/AS/54	10	M	KATH	eBL	Abdominal
IHC/AS/55	8	F	KATH	eBL	Intra-abdominal
IHC/AS/56	1	F	KATH	eBL	submandibular
IHC/AS/57	12	M	KATH	eBL	neck
IHC/AS/58	8	F	KATH	eBL	Post auricular
IHC/AS/59	10	M	KATH	eBL	Abdominal
NBL/AS/40	7	M	KATH	Diffuse small cell lymphoma	Neck
NBL/AS/41	12	M	KATH	Non-Hodgkin's lymphoma	Abdominal
NBL/AS/43	10	M	KATH	Non-Hodgkin's lymphoma	Neck
NBL/AS/44	11	M	KATH	Non-Hodgkin's lymphoma	Submandibular
NBL/AS/45	7	F	KATH	Diffuse low grade non-Hodgkin's lymphoma	Submental
NBL/AS/46	10	M	KATH	High grade non-Hodgkin's lymphoma	Intra-abdominal
NBL/AS/47	7	M	KATH	Diffuse low grade non-Hodgkin's lymphoma	neck
NBL/AS/48	10	F	KATH	Non-Hodgkin's lymphoma	left jaw
NBL/AS/49	10	M	KATH	Non-Hodgkin's lymphoma	Submental

Patient	Age (years)	Sex	Hospital	Diagnosis	Tumour site
NBL/AS/50	10	M	KATH	Non-Hodgkin's lymphoma	Intra-abdominal
NBL/AS/51	12	M	KATH	Non-Hodgkin's lymphoma	neck
NBL/AS/52	9	M	KATH	High grade Non-Hodgkin's lymphoma	Left mandibular
NBL/AS/53	12	M	KATH	High grade non-Hodgkin's lymphoma	Cervical
NBL/AS/55	13	M	KATH	Hodgkin's lymphoma.	Left neck
NBL/AS/56	9	M	KATH	Tuberculous lymphadenitis (?)	Submental
NBL/AS/57	15	M	KATH	Hodgkin's lymphoma	Neck
NBL/AS/58	10	M	KATH	Hodgkin's lymphoma	Right cervical
NBL/AS/59	6	M	KATH	Hodgkin's lymphoma	Submandibular
NBL/AS/60	10	M	KATH	Hodgkin's lymphoma	Lymph node
IHC/KB/01	7	M	KBTH	eBL	Jejunum
IHC/KB/02	7	M	KBTH	eBL	Abdomen
IHC/KB/03	8	M	KBTH	eBL	Kidney
IHC/KB/04	11	F	KBTH	eBL	Small bowel
IHC/KB/05	8	M	KBTH	eBL	Abdomen
IHC/KB/06	9	M	KBTH	eBL	Neck
IHC/KB/07	13	M	KBTH	eBL	Buccal mucosa
IHC/KB/08	5	M	KBTH	eBL	Sinonasal
IHC/KB/09	15	M	KBTH	eBL	Abdomen
IHC/KB/10	13	M	KBTH	Diffuse large B-cell lymphoma	Axillary LN
IHC/KB/11	14	M	KBTH	eBL	Breast LN
IHC/KB/12	15	FM	KBTH	eBL	Ileocaecum
IHC/KB/13	4	F	KBTH	eBL	Hemicolon
IHC/KB/14	7	F	KBTH	eBL	Upper arm
IHC/KB/15	10	F	KBTH	eBL	Lesser sac & Omental LN
IHC/KB/16	7	M	KBTH	eBL	Mesenteric LN
IHC/KB/17	5	F	KBTH	eBL	Cervical LN
IHC/KB/18	5	M	KBTH	eBL	Orbit
IHC/KB/19	25	F	KBTH	eBL	Submandibular
IHC/KB/20	9	M	KBTH	eBL	Temporal region
IHC/KB/21	13	M	KBTH	eBL	Abdomen

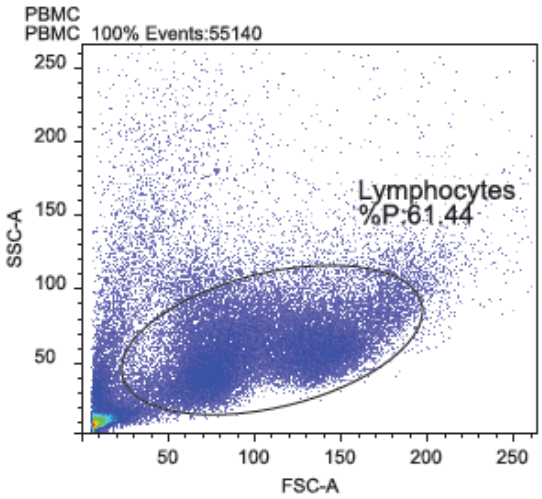
Patient	Age (years)	Sex	Hospital	Diagnosis	Tumour site
IHC/KB/22	13	M	KBTH	eBL	Groin LN
IHC/KB/23	15	M	KBTH	eBL	Ovary
IHC/KB/24	6	M	KBTH	eBL	Submandibular
IHC/KB/25	11	F	KBTH	eBL	Ovary
IHC/KB/26	8	F	KBTH	eBL	Abdomen
IHC/KB/27	13	F	KBTH	eBL	Mediastinum
IHC/KB/28	8	M	KBTH	eBL	Eye
IHC/KB/29	10	M	KBTH	eBL	Retroperitoneum
IHC/KB/30	10	F	KBTH	eBL	Abdomen
IHC/KB/31	2	M	KBTH	eBL	Abdomen
IHC/KB/32	8	M	KBTH	eBL	Axillary LN
IHC/KB/33	5	M	KBTH	eBL	Right distal femur
IHC/KB/34	9	M	KBTH	eBL	Jaw
IHC/KB/35	12	F	KBTH	eBL	Maxillar
IHC/KB/36	15	M	KBTH	eBL	Axillary LN
NBL/KB/01	10	M	KBTH	Non-Hodgkin's Lymphoma	Axillary LN
NBL/KB/02	15	F	KBTH	Non-Hodgkin's Lymphoma	Cervical LN
NBL/KB/03	4	F	KBTH	Non-Hodgkin's Lymphoma	Hemicolon
NBL/KB/04	10	M	KBTH	Non-Hodgkin's Lymphoma	Axillary LN
NBL/KB/05	3	F	KBTH	Non-Hodgkin's Lymphoma	Eye
NBL/KB/06	5	M	KBTH	Non-Hodgkin's Lymphoma	Axillary LN
NBL/KB/07	10	M	KBTH	Non-Hodgkin's Lymphoma	LN
NBL/KB/08	3	M	KBTH	Non-Hodgkin's Lymphoma	Abdomen
NBL/KB/09	10	M	KBTH	T cell lymphoma	Epidura
NBL/KB/10	15	M	KBTH	Diffuse Large Cell Lymphoma	Cervical LN
NBL/KB/11	11	M	KBTH	Hodgkin's lymphoma	Axillary LN
NBL/KB/12	5	M	KBTH	Hodgkin's lymphoma	Cervical LN
NBL/KB/13	12	M	KBTH	Hodgkin's lymphoma	Cervical LN
NBL/KB/14	8	M	KBTH	Hodgkin's lymphoma	LN
NBL/KB/15	6	M	KBTH	Hodgkin's lymphoma	Cervical LN
NBL/KB/16	13	F	KBTH	Mixed lymphoma	Cervical LN
NBL/KB/17	15	M	KBTH	Mixed Hodgkin's lymphoma	Cervical LN

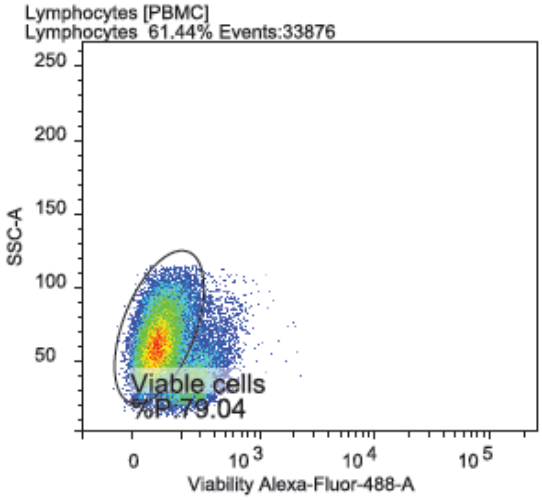
Patient	Age (years)	Sex	Hospital	Diagnosis	Tumour site
NBL/KB/18	14	M	KBTH	Hodgkin's lymphoma	Cervical LN
NBL/KB/19	2	F	KBTH	Lymphoblastic lymphoma	Cervical LN
NBL/KB/20	13	F	KBTH	Hodgkin's lymphoma	Cervical LN
NBL/KB/21	14	M	KBTH	Hodgkin's lymphoma	Cervical LN
NBL/KB/22	10	M	KBTH	Lymphoma	Cervical LN
NBL/KB/23	10	M	KBTH	Anaplastic LCL	Retroperitonium
NBL/KB/24	9	F	KBTH	Lymphoma	Mediastinum
NBL/KB/25	12	M	KBTH	Hodgkin's lymphoma	Cervical LN
NBL/KB/26	5	M	KBTH	Lymphoblastic lymphoma	Cervical LN
NBL/KB/27	7	M	KBTH	Lymphoma	Groin LN
NBL/KB/28	6	M	KBTH	Hodgkin's lymphoma	Cervical LN
NBL/KB/29	13	M	KBTH	Hodgkin's lymphoma	Cervical LN
NBL/KB/30	10	M	KBTH	Diffuse large B-cell lymphoma	Abdomen
NBL/KB/31	11	M	KBTH	Diffuse large B-cell lymphoma	Abdomen
NBL/KB/32	12	M	KBTH	Lymphoma	Cervical LN
NBL/KB/33	7	M	KBTH	Diffuse large B-cell lymphoma	Cervical LN
NBL/KB/34	15	M	KBTH	Hodgkin's lymphoma	Cervical LN
NBL/KB/35	5	M	KBTH	Diffuse large B-cell lymphoma	Submandibular LN
NBL/KB/36	5	M	KBTH	Diffuse large B-cell lymphoma	Axillary LN
NBL/KB/37	12	F	KBTH	Hodgkin's lymphoma	Cervical LN

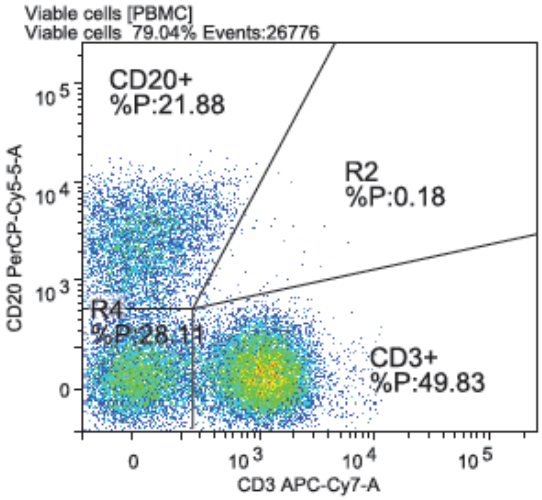
Appendix 8. Antibody reagents used for T cell and B cell phenotyping

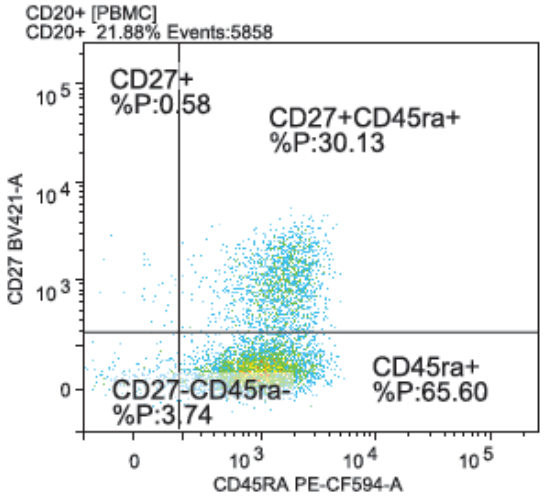
Marker	Fluorochrome	Laser (nm)	Filter (nm/nm)	Company	Catalogue number	Dilution titre
Viability stain 520	FITC	488	530/30	BD	564407	1:16,000
TCR-Vδ1	APC	640	670/14	Miltenyi	130-00-519	1:3,000
CD8	Alexa Flour 700	640	730/45	BD	561453	1:2,500
CD3	APC-Cy7	640	780/60	BD	557832	1:15,000
CD27	Brilliant Violet 421	405	431/28	BD	562513	1:1,000
CD25	Brilliant Violet 510	405	525/50	BD	563352	1:300
CD21	Brilliant Violet 605	405	610/20	BD	742761	1:30,000
IgM	Brilliant Violet 650	405	660/20	BD	740595	1:5,000
CD10	PE	561	586/15	BD	555375	1:6,000
CD45RA	PE-CF594	561	610/20	BD	562298	1:120,000
CD20	PerCP-Cy5.5	561	670/30	eBioscience	35-209-42	1:1,600
IgG	PE-Cy7	561	780/60	BD	561298	1:40,000
CD4	BUV 496	355	515/30	BD	564651	1:600
TCR-γδ	BUV395	355	379/28	BD	564155	1:60
PD-1	BUV737	355	740/35	BD	565299	1:4,000

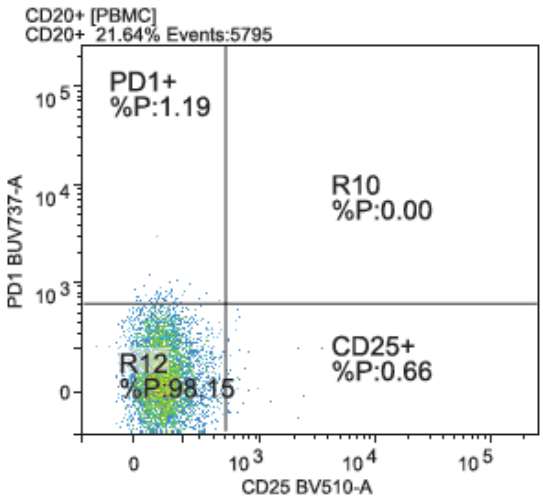
Appendix 9. Gating - peripheral blood lymphocytes

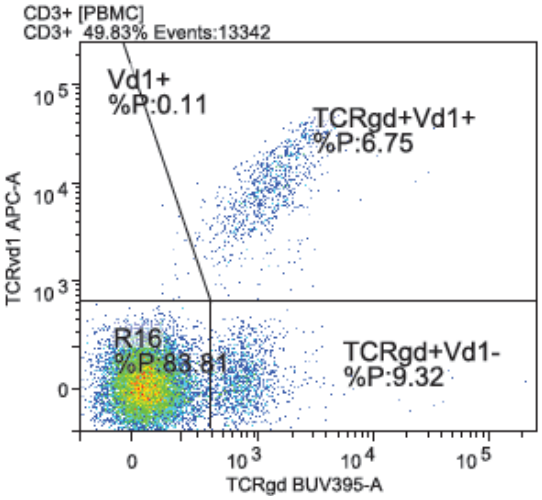


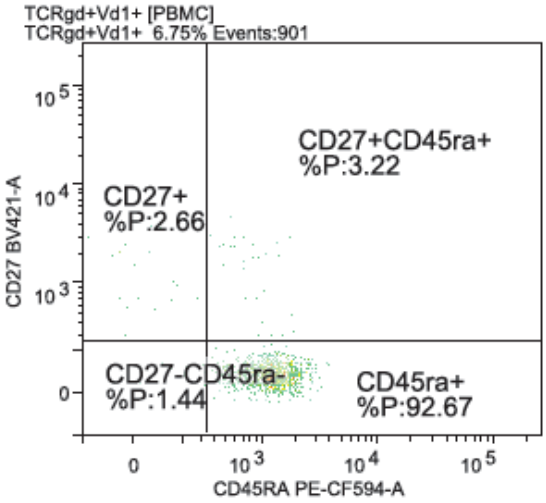


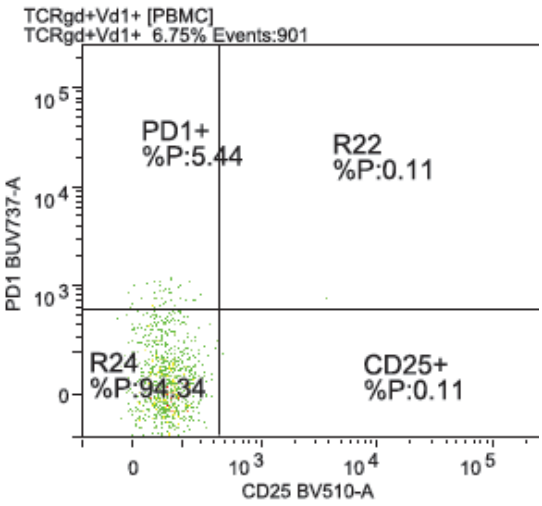


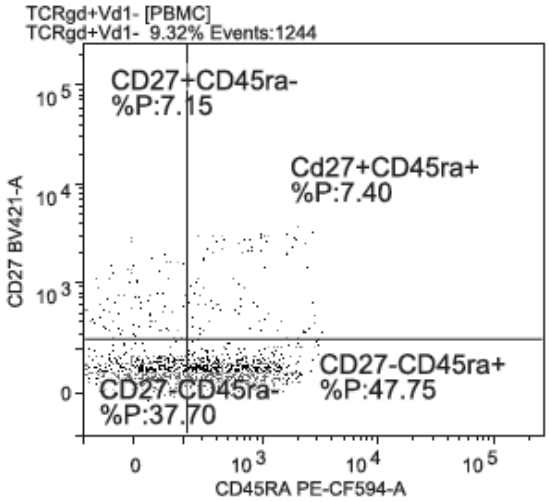


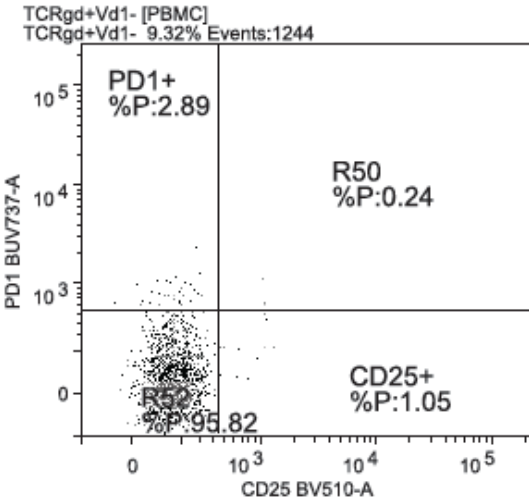




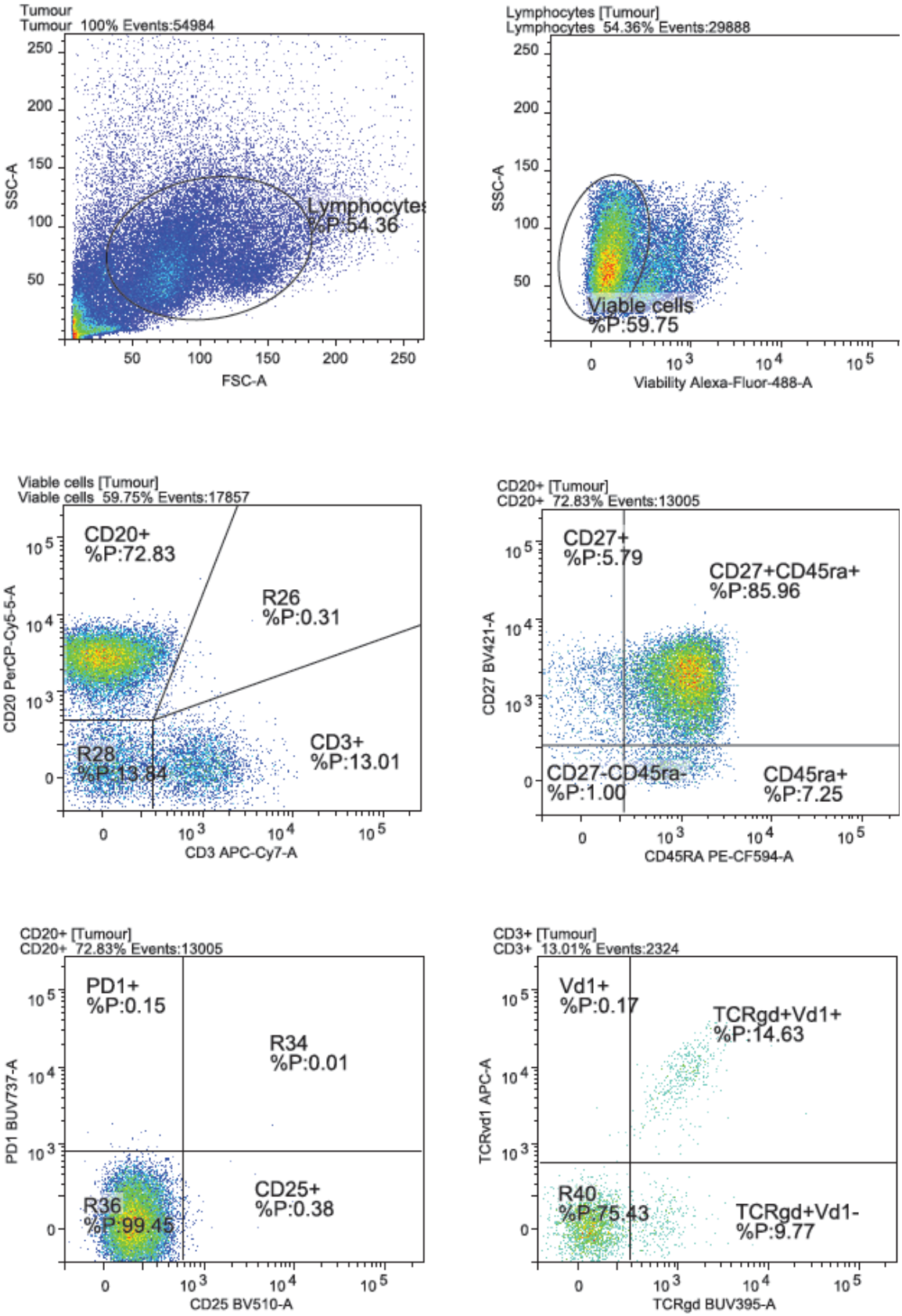


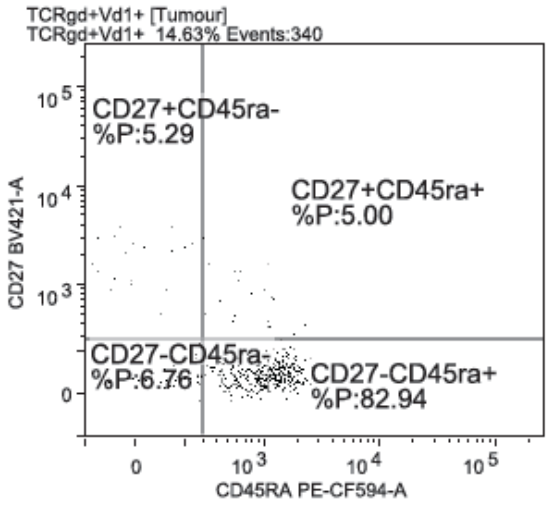


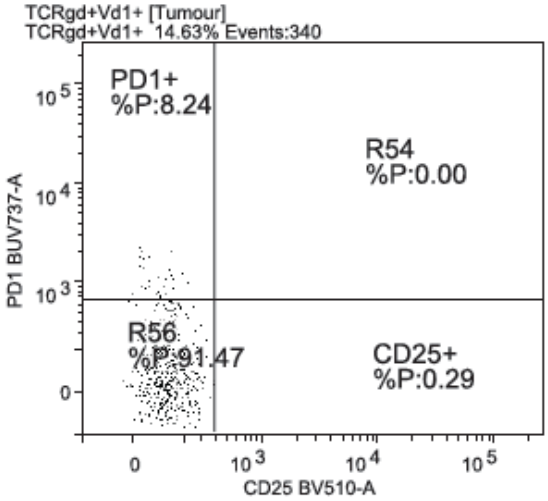


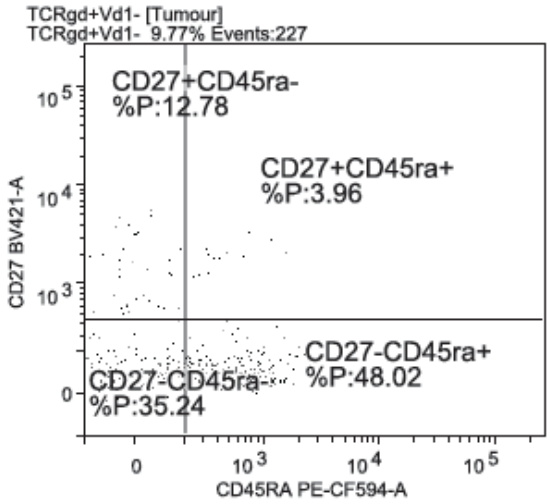


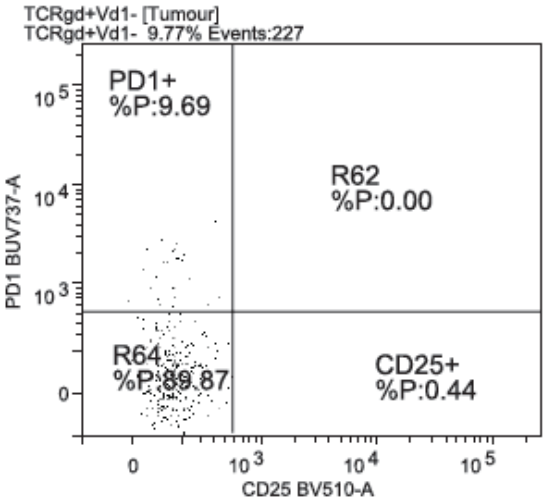
Appendix 10. Gating - Tumour lymphocytes











Appendix 11. Author contributions to manuscripts and published papers

Manuscript 1

Smith-Togobo C, Pedersen MØ, Jensen SG, Duduyemi B, Gyasi RK, Ofori MF, Paintsil V, Renner L, Nørgaard PH and Hviid L. Reliable cell and tissue morphology-based diagnosis of endemic Burkitt lymphoma in resource-constrained settings in Ghana. Submitted for publication.

- Study concept and design: CS, MFO, PHN, LH
- Clinical sample collection: CS, VP, LR
- Archival sample retrieval: CS, BD, RKG
- Laboratory investigations: CS, MOP, SGJ,
- Data analysis: CS, LH

Manuscript 2

Smith-Togobo C, Quintana MP, Ofori MF, and Hviid L. High frequencies of tumour-infiltrating and circulating Vδ1^{+} γδ T cells in endemic Burkitt lymphoma patients. Manuscript in preparation.

- Study concept and design: CS, MFO, LH
- Clinical sample collection: CS
- Laboratory investigations: CS, MPQ
- Data analysis: CS, LH

Manuscript 3

Tentative by-line: Quintana MP, Smith-Togobo C… and Hviid L. Planned manuscript.

- Study concept and design: LH, MPQ, CS
- Clinical sample collection: CS
- Laboratory investigations: CS, MPQ
- Data analysis: MPQ, CS, LH

Review paper

Hviid L, Smith-Togobo C, and Willcox BE. Human Vδ1^{+} T cells in the immune response to *Plasmodium falciparum* infection. Front Immunol *10*, 259, 2019

- Concept and design: LH, CS, BEW

Appendix 12. Primers for cDNA synthesis and first PCR

Forward primers	
AB3 5’ L VH1	ACAGGTGCCCACTCCCAGGTGCAG
AB4 5’ VH3	AAGGTGTCCAGTGTGARGTGCAG
AB5 5’ VH4/6	CCCAGATGGGTCCTGTCCCAGGTGCAG
AB6 5’ L VH5	CAAGGAGTCTGTTCCGAGGTGCAG
AB7 5’ L Vκ1/2	ATGAGGSTCCCYGCTCAGCTGCTGG
AB8 5’ L Vκ3	CTCTTCCTCCTGCTACTCTGGCTCCCAG
AB10 5’ L Vκ4	ATTTCTCTGTTGCTCTGGATCTCTG
AB12 5’ L Vλ1	GGTCCTGGGCCCAGTCTGTGCTG
AB13 5’ L Vλ2	GGTCCTGGGCCCAGTCTGCCCTG
AB14 5’ L Vλ3	GCTCTGTGACCTCCTATGAGCTG
AB15 5’ L Vλ4/5	GGTCTCTCTCSCAGCYTGTGCTG
AB16 5’ L Vλ6	GTTCTTGGGCCAATTTTATGCTG
AB17 5’ L Vλ7	GGTCCAATTCYCAGGCTGTGGTG
AB18 5’ L Vλ8	GAGTGGATTCTCAGACTGTGGTG
Reverse primers	
AB93 3’ Cγ CH1	GGAAGGTGTGCACGCCGCTGGTC
AB94 3’ Cκ 543	GTTTCTCGTAGTCTGCTTTGCTCA
AB21 3’ Cλ	CACCAGTGTGGCCTTGTTGGCTTG

Appendix 13. Primers for second PCR (CPEC primers)

Heavy chain	
AB38 CPEC VH1/5/7	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG
AB39 CPEC VH3	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGAGGTGCAGCTGGTGGAG
AB40 CPEC VH4	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGGAG
AB41 CPEC VH3-23	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG
AB42 CPEC VH4-34	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTGCAGCTACAGCAGTG
AB43 CPEC VH1-18	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTTCAGCTGGTGCAG
AB44 CPEC VH1-24	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTCCAGCTGGTACAG
AB45 CPEC HV3-9/30/33	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGAAGTGCAGCTGGTGGAG
AB46 CPEC VH6-1	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGGTACAGCTGCAGCAG
AB47 CPEC VH4-39	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCCAGCTGCAGCTGCAGGAG
AB48 CPEC VH3-33	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTCAGGTGCAGCTGGTGGAG
AB49 JH1/2/4/5	GATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACCAG
AB50 CPEC JH3	GATGGGCCCTTGGTCGACGCTGAAGAGACGGTGACCATTG
AB51 CPEC JH6	GATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACCGTG
κ chain	
AB53 CPEC Vκ1	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTGACATCCAGATGACCCAGTC
AB54 CPEC Vκ1-9/1-13	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGACATCCAGTTGACCCAGTCT
AB55 CPEC VκID-43/1-8	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTGTGCCATCCGGATGACCCAGTC

AB56 CPEC Vκ2	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATGGGGATATTGTGATGACCCAGAC
AB57 CPEC Vκ2-28/2-30	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATGGGGATATTGTGATGACTCAGTC
AB58 CPEC Vκ3-11/3D-11	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGAAATTGTGTTGACACAGTC
AB59 CPEC Vκ3-15/3D-15	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGAAATAGTGATGACGCAGTC
AB60 CPEC Vκ3-20/3D-20	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAGAAATTGTGTTGACGCAGTCT
AB61 CPECVκ4-1	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCGGACATCGTGATGACCCAGTC
AB62 CPEC Jκ1/2/4	ATGGTGCAGCCACCGTACGTTTGATATCCACTTTGGTC
AB63 CPEC Jκ3	ATGGTGCAGCCACCGTACGTTTGATATCCACTTTGGTC
AB64 CPEC Jκ5	ATGGTGCAGCCACCGTACGTTTAATCTCCAGTCGTGTC
AB65 CPEC Jκ150/3	ATGGTGCAGCCACCGTACGTCTGATTTCCACCTTGGTC
λ chain	
AB 67 CPEC VλI	CTTTTTCTAGTAGCAACTGCAACCGGTTCCTGGGCCCAGTCTGTGCTGACKCAG
AB68 CPEC Vλ2	CTTTTTCTAGTAGCAACTGCAACCGGTTCCTGGGCCCAGTCTGCCCTGACTCAG
AB69 CPEC Vλ3	CTTTTTCTAGTAGCAACTGCAACCGGTTCTGTGACCTCCTATGAGCTGACWCAG
AB70 CPEC Vλ4/5	CTTTTTCTAGTAGCAACTGCAACCGGTTCTCTCTCSCAGCYTGTGCTGACTCA
AB71 CPEC Vλ6	CTTTTTCTAGTAGCAACTGCAACCGGTTCTTGGGCCAATTTTATGCTGACTCAG
AB72 CPEC Vλ7/8	CTTTTTCTAGTAGCAACTGCAACCGGTTCCAATTCYCAGRCTGTGGTGACYCAG
Reverse	
AB73 CPEC Cλ reverse	GGCTTGAAGCTCCTCACTCGAGGGYGGGAACAGAGTG

Appendix 14. Preparation of reaction enzyme mixtures for digestion

	Plasmid for heavy chain-1µg (µL)	Plasmid for κ chain-1µg (µL)	Plasmid for λ chain-1µg (µL)	Master mix for heavy chain (µL)	Master mix for κ chain (µL)	Master mix for λ chain (µL)
Template	0.8	1.1	0.9			
Fast digest buffer	4.0	4	4.0	3.4	3.4	3.4
Fast digest AgeI	1.0	1.0	1.0	0.5	0.L	0.5
Fast digest SaII	1.0	-	-	0.5	-	-
Fast digest BsiWI		1.0	-		0.5	-
Fast digest XhoI		-	1.0		-	0.5
rSAP	2.0	2.0	2.0			
Nuclease free water	31.2	30.9	31.1			
Total volume	40.0	40.0	40.0	4.4	4.4	4.4

Appendix 15. Preparation of reaction mixtures for ligation

	Master mix for heavy chain (µL)	Master mix for kappa chain (µL)	Master mix for lambda chain (µL)
Nuclease free water	15	15.0	15.0
Fast digest buffer	1.5	1.5	1.5
Digested plasmid	2.0	2.0	2.0
Quick ligase	0.5	0.5	0.5
Total volume (µL)	19.0	19.0	19.0

Appendix 16. Primers for colony PCR

AB95	GCTTCGTTAGAACGCGGCTAC
AB96	GGAAGTAGTCCTTGACCAGGC
AB97	GTTATTCAGCAGGCACACAACAG
AB98	TATCTGCCTTCCAGGCCAC

Appendix 17. Preparation of master mix for colony PCR

	Master mix for heavy chain (µL)	Master mix for κ chain (µL)	Master mix for λchain (µL)
DreamTaq Green PCR Master Mix 2X	10	10	10
Forward primer	1 (AB95)	1 (AB95)	1 (AB95)
Reverse primer	1 (AB96)	1 (AB97)	1 (AB98)
Nuclease free water	8	8	8
Fragment length	~600-700bp	~550bp	~550bp

Appendix 18. B-cell CDR3 sequence analysis

[Legend for Table on following page]: Characteristics of the analysed BCR sequences derived from eBL samples. * Heavy or light chains were predicted to be non-productive. ** Data corresponds to control wells were 10 cells were sorted. *** IgH paired with both Igκ and Igλ was detected by PCR but attempts to sequence the two light chain bands were unsuccessful and sequence data were only retrieved for a re-arranged and productive Igλ chain. In bold are sequences selected for expression as recombinant monoclonal antibodies in HEK 293F cells.

		HEAVY CHAIN						LIGHT CHAIN					
Origin	Name	VH-gene	DH-gene	JH-gene	Identity to germline (%)	CDR3 sequence	Length (aa)	VL-gene	JL-gene	germline (%)	CDR3 sequence	Lenght (aa)	Light chain
PBMC	AS/03 2H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	κ
PBMC	AS/03 3B	3-9*03	6-13*01	5*02	99.7	AKDGIAAADDVATGWFDP	18	3D-20*01	4*01	98.9	QQYGSSLT	8	κ
PBMC	AS/03 3C	1-18*01	2-2*01, 2-2*02, 2-2*03	5*02	99.7	ARDRSAAEAGWFDP	14	1-39*01, 1D-39*01	1*01	100	QQSYSTPRT	9	κ
PBMC	AS/03 3E	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	λ
PBMC	AS/03 3F	3-11*04	5-18*01, 5-5*01, 6-6*01	6*04	87.5	ARDEGGYPLVSDV	13	3D-20*01	4*01	91.3	QQYAGSLT	8	κ
PBMC	AS/03 3G	4-38-2*01	3-16*02	4*02	94.5	ARGRGGVVAQTTLDS	15	2-14*01	3*02	92.6		10	λ
PBMC	AS/03 4A*	5-51*03	6-6*01	2*01	86.1	ARRAAAPGFWYFHL	14	4-1*01	3*01	98.3	QQYYSIPLT	9	κ
PBMC	AS/03 4C	4-61*01	3-3*01	5*02	97.3	ARVTPTDPTYYDFWSGYPTPPNW	26	1-NL*01	5*01	99.6	QQYYSTLIT	9	κ
PBMC	AS/03 4D	1-18*01	2-2*01, 2-2*02, 2-2*03	4*02	98.3	AKEGLNTQAWDY	12	3-25*02	2*01, 3*01	98.3	QSADSSDTYV	11	λ
PBMC	AS/03 4F	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	κ
PBMC	AS/03 4H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	κ
PBMC	AS/03 5B	3-30*02, 3-30-	3-3*01	5*02	100.0	AKGGYDFWSGIWGDNNWFDP	20	1-33*01, 1D-33*01	4*01	100	QQYDNLPPN	9	κ
PBMC	AS/03 5E	4-59*01	2-8*01	4*02	98.6	ARYHCPNGVCDSFDY	15	ND	ND	ND	ND	ND	λ
PBMC	AS/03 6B	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	λ
PBMC	AS/03 6C	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	λ
PBMC	AS/03 6D*	4-39*02	5-18*01, 5-5*01	3*02	96.0	ERTGYIYGYWV*Y		3-1*01	2*01, 3*01, 3*02	97.5	QAWDSYTAV	9	λ
PBMC	AS/03 6E	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	κ
PBMC	AS/03 6F**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	κ
Tumo	**AS/03 9H**	**3-30*04, 3-**	**2-21*01**	**4*02**	**91.1**	**ARVAVMGPTLHY**	**12**	**2-14*01**	**1*01**	**95.9**	**SSYSSSSP**	**10**	**λ**
Tumour	AS/03 10A	3-30*04, 3-30*03	2-21*01	4*02	91.2	ARVAVMGPTLHY	12	2-14*01	1*01	95.9	SSYSSSSPYV	10	λ
Tumour	AS/03 10B	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	λ
Tumour	AS/03 10C	3-30*04, 3-30*03	2-21*01	4*02	91.2	ARVAVMGPTLHY	12	2-14*01	1*01	95.9	SSYSSSSPYV	10	λ
Tumour	AS/03 10D*	3-30*04, 3-30*03	2-21*01	4*02	91.1	ARVAVMGPTLHY	12	2-14*01	1*01	95.2	SSYSSSSPYV	10	λ
Tumour	AS/03 10F	3-30*04, 3-30*03	2-21*01	4*02	90.5	ARVAVMGPTLHY	12	2-14*01	1*01	95.5	SSYSSSSPYV	10	λ
Tumour	AS/03 10G	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	λ
Tumour	AS/03 10H	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	κ
Tumour	AS/03 11A	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	λ
Tumour	AS/03 11D*	3-23*04	2-8*02, 3-9*01, 6-13*01	6*01	92.5	AKLVDQGPIGLPPGTLLQEHL	21	4-60*03	3*02	97	ETWDTNTRV	9	λ
Tumour	AS/03 11G	3-30*04, 3-30*03	2-21*01	4*02	90.8	ARVAVMGPTLHY	12	2-14*01	1*01	95.5	SSYSSSSPYV	10	λ
Tumour	AS/03	3-30*04, 3-30*03	2-21*01	4*02	91.1	ARVAVMGPTLHY	12	2-14*01	1*01	95.2	SSYSSSSPYV	10	λ
Tumour	KB/01 1C	ND	ND	ND	ND	ND	ND	2-14*01	1*01	95.9	SSYSSSSPYV	10	λ
Tumour	KB/01 1E	ND	ND	ND	ND	ND	ND	2-14*01	1*01	95.9	SSYSSSSPYV	10	λ
Tumo	**KB/01 3A**	**1-18*01**	**2-2*02, 2-8*01, 2-**	**4*02**	**98.6**	**AKEGLNTQAWDY**	**12**	**1-44*01**	**3*02**	**92.8**		**11**	**λ**
Tumour	KB/01 3C	3-30*04, 3-30*03	2-21*01	4*02	90.8	ARVAVMGPTLHY	12	2-14*01	1*01	95.9	SSYSSSSPYV	10	λ
Tumour	KB/01 3E	3-30*04, 3-30*03	2-21*01	4*02	91.1	ARVAVMGPTLHY	12	2-14*01	1*01	95.2	SSYSSSSPYV	10	λ
Tumour	KB/01 3F	3-30*04, 3-30*03	2-21*01	4*02	91.1	ARVAVMGPTLHY	12	2-14*01	1*01	95.9	SSYSSSSPYV	10	λ
Tumour	KB/01 3G	3-9*03	6-13*01	5*02	100.0	AKDGIAAADDVATGWFDP	18	2-14*01	1*01	95.9	SSYSSSSPYV	10	λ
Tumour	KB/01	ND	ND	ND	ND	ND	ND	2-14*01	1*01	95.9	SSYSSSSPYV	10	λ
Tumour	KB/01 5C	3-9*03	6-13*01	5*02	100.0	AKDGIAAADDVATGWFDP	18	2-14*01	1*01	94.8	SSYSSSSPYV	10	λ
Tumour	KB/01 5E	3-30*04, 3-30*03	2-21*01	4*02	91.2	ARVAVMGPTLHY	12	2-14*01	1*01	95.2	SSYSSSSPYV	10	λ
Tumo	**KB/01**	**3-9*03**	**6-13*01**	**5*02**	**100.0**	**AKDGIAAADDVATGWFDP**	**18**	**2-14*01**	**1*01**	**95.9**	**SSYSSSSP**	**10**	**λ**
Tumour	KB/01 9F	3-9*03	6-13*01	5*02	100.0	AKDGIAAADDVATGWFDP	18	ND	ND	ND	ND	ND	λ
Tumour	KB/01 9G	3-30*04, 3-30*03	2-21*01	4*02	91.1	ARVAVMGPTLHY	12	ND	ND	ND	ND	ND	λ
Tumo	**KB/01**	**3-30*04, 3-**	**2-21*01**	**4*02**	**91.1**	**ARVAVMGPTLHY**	**12**	**4-60*03**	**3*02**	**97**	**ETWDTNTR**	**9**	**λ**
Tumour	KB/04 5B	ND	ND	ND	ND	ND	ND	1-40*01	3*02	96.2	QSYDSSLTRV	10	λ
Tumo	**KB/04**	**3-7*01**	**1-14*01**	**4*02**	**95.3**	**ARESEDNHRRRYLDS**	**15**	**1-40*01**	**3*02**	**97.6**	**QSYDSSLT**	**10**	**λ**
Tumo	**KB/04 9D**	**3-7*01**	**1-14*01**	**4*02**	**95.9**	**ARESEDNHRRRYLDS**	**15**	**1-40*01**	**3*02**	**96.6**	**QSSDSSLT**	**10**	**λ**
Tumour	KB/13 5F	3-30*04, 3-30*03	2-21*01	4*02	90.8	ARVAVMGPTLHY	12	2-14*01	1*01	95.9	SSYSSSSPYV	10	λ

Appendix 19. Supplementary Table 1.

Comparison of original diagnosis and retrospective assessment by microscopy of Giemsa-stained FNA smears

			Retrospective assessment				Validity*		
	Hospital		eBL	Non-eBL	Discarded	Total	Sensitivity	Specificity	Accuracy
Original diagnosis	KBTH	eBL	4	0	0	4	1.0 [.39 to 1.0]	1.0 [.29 to 1.0]	1.0 [.59 to 1.0]
		Non-eBL	0	3	0	3			
		All	4	3	0	7			
	KATH	eBL	13	0	0	13	1.0 [.75 to 1.0]	1.0 [.63 to 1.0]	1.0 [.84 to 1.0]
		Non-eBL	0	8	0	8			
		All	13	8	0	21			
	All	eBL	17	0	0	17	1.0 [.80 to 1.0]	1.0 [.72 to 1.0]	1.0 [.88 to 1.0]
		Non-eBL	0	11	0	11			
		All	17	11	0	28			

*95% confidence intervals for estimates are shown in square brackets.

Appendix 20. Supplementary Table 2

Comparison of original diagnosis and retrospective assessment by microscopy of haematoxylin-eosin-stained tissue sections from FFPE tissue blocks

			Retrospective assessment				Validity*		
	Hospital		eBL	Non-eBL	Discarded	Total	Sensitivity	Specificity	Accuracy
Original diagnosis	KBTH	eBL	21	3	11	35	0.84 [.64 to .95]	0.90 [.74 to .98]	.88 [.76 to .95]
		Non-eBL	4	28	5	37			
		All	25	31	16	72			
	KATH	eBL	14	4	2	20	0.82 [.57 to .96]	0.67 [.35 to .90]	0.76 [.56 to .90]
		Non-eBL	3	8	8	19			
		All	17	12	10	39			
	All	eBL	35	7	13	55	0.83 [.69 to .93]	0.84 [.69 to .93]	0.84 [.74 to .91]
		Non-eBL	7	36	13	56			
		All	42	43	26	111			

*95% confidence intervals for estimates are shown in square brackets.

Appendix 21. Supplementary Table 3.

Comparison of original diagnosis and retrospective assessment by microscopy of FISH-c-myc-stained FNA smears

			Retrospective assessment				Validity*		
	Hospital		eBL	Non-eBL	Discarded	Total	Sensitivity	Specificity	Accuracy
Original diagnosis	KBTH	eBL	2	0	0	2	0.67 [.09 to .99]	n.a.	0.67 [.09 to .99]
		Non-eBL	1	0	0	1			
		All	3	0	0	3			
	KATH	eBL	10	1	0	11	0.83 [.52 to .98]	0.80 [.28 to .99]	0.82 [.57 to .96]
		Non-eBL	2	4	0	6			
		All	12	5	0	17			
	All	eBL	12	1	0	13	0.80 [.52 to .96]	0.80 [.28 to .99]	0.80 [.56 to .94]
		Non-eBL	3	4	0	7			
		All	15	5	0	20			

*95% confidence intervals for estimates are shown in square brackets.

Appendix 22. Supplementary Table 4.

Comparison of original diagnosis and retrospective assessment by microscopy of FISH-c-myc-igh-stained FNA smears

			Retrospective assessment				Validity*		
	Hospital		eBL	Non-eBL	Discarded	Total	Sensitivity	Specificity	Accuracy
Original diagnosis	KBTH	eBL	2	0	0	2	0.67 [.09 to .99]	n.a.	0.67 [.09 to .99]
		Non-eBL	1	0	0	1			
		All	3	0	0	3			
	KATH	eBL	9	2	0	11	0.75 [.43 to .95]	0.60 [.15 to .95]	0.71 [.44 to .90]
		Non-eBL	3	3	0	6			
		All	12	5	0	17			
	All	eBL	11	2	0	13	0.73 [.45 to .92]	0.60 [.15 to .95]	0.70 [.46 to .88]
		Non-eBL	4	3	7	0			
		All	15	5	0	20			

*95% confidence intervals for estimates are shown in square brackets.

Appendix 23. Supplementary Table 5.

Comparison of original diagnosis and retrospective assessment by microscopy of FISH-c-myc-stained FFPE sections

			Retrospective assessment				Validity*		
	Hospital		eBL	Non-eBL	Discarded	Total	Sensitivity	Specificity	Accuracy
Original diagnosis	KBTH	eBL	5	3	4	12	0.83 [.36 to 1.0]	0.67 [.30 to .93]	0.73 [.45 to .92]
		Non-eBL	1	6	1	8			
		All	6	9	5	20			
	KATH	eBL	2	4	4	10	1.0 [.16 to 1.0]	0.0 [.00 to .60]	0.33 [.04 to .78]
		Non-eBL	0	0	0	0			
		All	2	4	4	10			
	All	eBL	7	7	8	22	0.88 [.47 to 1.0]	0.46 [.19 to .75]	0.62 [.38 to .82]
		Non-eBL	1	6	1	8			
		All	8	13	9	30			

*95% confidence intervals for estimates are shown in square brackets.

Appendix 24. Supplementary Table 6.

Comparison of original diagnosis and retrospective assessment by microscopy of FISH-c-myc-igh-stained FFPE sections

			Retrospective assessment				Validity*		
	Hospital		eBL	Non-eBL	Discarded	Total	Sensitivity	Specificity	Accuracy
Original diagnosis	KBTH	eBL	19	10	5	34	0.95 [.75 to 1.0]	0.52 [.30 to .74]	0.73 [.57 to .86]
		Non-eBL	1	11	6	18			
		All	20	21	11	52			
	KATH	eBL	4	4	9	17	1.0 [.40 to 1.0]	0.0 [0.0 to .60]	0.50 [.16 to .84]
		Non-eBL	0	0	2	2			
		All	4	4	11	19			
	All	eBL	23	14	14	51	0.96 [.79 to 1.0]	0.44 [.24 to .65]	0.69 [.55 to .82]
		Non-eBL	1	11	8	20			
		All	24	25	22	71			

*95% confidence intervals for estimates are shown in square brackets.

Appendix 25. Supplementary Table 7.

Comparison of original diagnosis and retrospective assessment by microscopy of immunohistochemistry detection of C-MYC expression on FFPE sections

			Retrospective assessment				Validity*		
	Hospital		eBL	Non-eBL	Discarded	Total	Sensitivity	Specificity	Accuracy
Original diagnosis	KBTH	eBL	15	6	0	21	0.79 [.54 to .94]	0.45 [.17 to .77]	0.67 [.47 to .83]
		Non-eBL	4	5	0	9			
		All	19	11	0	30			
	KATH	eBL	11	2	1	14	0.79 [.49 to .95]	0.33 [.01 to .91]	0.71 [.44 to .90]
		Non-eBL	3	1	0	4			
		All	14	3	1	18			
	All	eBL	26	8	1	35	0.79 [.61 to .91]	0.43 [.18 to .71]	0.68 [.53 to .81]
		Non-eBL	7	6	0	13			
		All	33	14	1	48			

*95% confidence intervals for estimates are shown in square brackets.