MALARIA, EPSTEIN-BARR VIRUS AND THE GERMINAL CENTER: CLUES FOR ENDEMIC BURKITT'S LYMPHOMA PATHOGENESIS

By

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Declaration

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

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Dedication

This thesis work is dedicated to The LORD God Almighty, the source of divine wisdom and inspiration, and my strength. This work is also dedicated to my dear mother, Ms. Margaret Tagbor, and my siblings.

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Abstract

Endemic Burkitt's lymphoma (eBL) is the commonest childhood cancer in equatorial Africa. eBL is tied to EBV and malaria infection and it is characterized by the proliferation of EBV-infected B cells. eBL tumor cells appear to have participated in the germinal center (GC) reaction where the characteristic c-Myc/Ig translocation which seeds the tumor is believed to occur. The translocation occurs via the action of the enzyme activation induced cytidine deaminase (AID). This thesis examines the interaction of EBV with the various B cell sub-populations to further appreciate EBV persistence and how it could contribute to lymphomagenesis. *In vivo* experiments were also carried out to test a new hypothesis of eBL pathogenesis. The levels of factors that could increase the probability that the c-Myc/Ig translocation will occur in GC B cells of tonsils from a malaria-endemic region were determined, and compared to levels in control tonsils. Finally, *in vitro* experiments were carried out to ascertain the induction of AID mRNA by *Plasmodium falciparum*. Two models generated from *in vitro* EBV-infected B cells revealed that GC B cells consist of an equal mix of cells that have undergone at most 0 to 3 divisions and cells that have undergone rapid divisions (χ^2= 2.8). Memory B cells from the peripheral blood of chronic EBV carriers; infectious mononucleosis (IM) patients; and those from the tonsils of chronic EBV carriers did not show compelling signs of direct EBV infection. They have undergone more than 20-25 divisions and might still be dividing. The EBV-infected tonsillar naïve B cells were a complex mix of newly infected cells and cells that have undergone rapid divisions. They seem to be a self-sustaining directly EBV-infected B cell sub-population. B cells carrying high EBV episome copy numbers could be tumor-prone. It was interesting to find that whereas the percentage total B cells of tonsils from the non-malaria and the malaria-endemic region was not different (p = 0.184), GC throughput in tonsils from the malaria-endemic region was 1.5 fold higher compared to tonsils from the non-malaria region (p< 0.001). The level of EBV-infected GC B cells from the malaria-endemic region was 42 fold higher compared to the non-malaria region (p= 0.0041). AID mRNA expression was 5 fold higher in GC B cells from the malaria-endemic region compared to the non-malaria region (p= 0.0046). It was also found that c-Myc mRNA and protein are indeed expressed in GC B cells. c-Myc mRNA expression was 4 fold higher in GC B

cells from the malaria-endemic region compared to the non-malaria region (p= 0.0307). *Plasmodium falciparum* extracts sustainably induced AID by 15, and 40 fold, on day 3 (p= 0.0303), and day 5 (p= 0.0001) respectively as compared to controls. Finally this thesis reports that *Plasmodium falciparum* pigment hemozoin which together with *Plasmodium falciparum* DNA act as TLR9 agonist is internalized by B cells. To end with, this thesis throws more light on EBV persistence and provides clues of co-operating factors that could increase the probability of eBL occurring in a malaria-endemic region.

List of abbreviations

AID	Activation induced cytidine deaminase
AP-1	Activator protein-1
APE	Apurinic/ apyrimidic endonuclease
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic peptide like
ATM	Ataxia telangiectasia mutated
BCR	B cell receptor
BIM	Bcl-2-interacting mediator of apoptosis
BL	Burkitt's lymphoma
CBF1	centromere promoter factor 1
CIDR1-α	Cysteine interdomain region-1α
CSA	Chondroitin sulphate A
CSR	Class switch recombination
CtBP	C-terminal-binding protein
DARC	Duffy antigen receptor for chemokines
DC	Dendritic cell
DSB	Double stranded break
eBL	Endemic Burkitt's lymphoma
EBNA	EBV encoded nuclear antige
EBV	Epstein-Barr virus
ERK	Extracellular-signal-regulated-pathway
FOXP3	Forkhead box P3
GC	Germinal center
GPI	Glycosylphosphatidylinositol
GSK3	Glycogen synthase kinase 3
HDACs	Histone deacetylases
HEV	High endothelial venules
HIV-BL	HIV-related Burkitt's lymphoma
HMS	Hyper-reactive malaria splenomegaly
ICAM-1	Intracellular adhesion molecule 1

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Ig	Immunoglobulin
ITAMs	Immunoreceptor tyrosine-based activation motif
JAK3	Janus activated factor 3
JNK	c-Jun N terminal kinase
LMP	Latent membrane protein
LPS	Lipopolysaccharide
MAPK	Mitogen-associated protein kinase
MDM2	Murine double minute 2
MHC	Major Histocompatibility complex
MLV	Maloney leukaemogenic virus
MRN	Mre11/Rad50/Nbs1
MZMs	Marginal zone macrophages
NHEJ	Non-homologous end joining
NO	Nitric oxide
PALS	Periarteriolar lymphoid sheath
PFEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1
RAG 1 & 2	Recombination activation genes 1 & 2
RBC	Red blood cell
RBP-Jk	Recombination signal binding protein J kappa
RESA	Ring-infected erythrocyte surface antigen
RPA	Replication protein A
RSS	Recombination signal sequence
sBL	Sporadic Burkitt's lymphoma
SEER	Surveillance, Epidemiology and End Result
SHM	Somatic hypermutation
SLVL	Splenic lymphoma with villious lymphocytes

SSB	Single stranded break
STATs	Signal transducers and activators of transcription
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR receptor associated death domain
TRAF	TNF receptor-associated factor
UNG	Uracil-DNA glycosylase
VCAM-1	vascular cell-adhesion molecule 1

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CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Burkitt's lymphoma, malaria and Epstein Barr virus

Burkitt's lymphoma (BL) is a rapidly proliferating B cell tumor characterized by a translocation of the c-Myc oncogene to a region downstream of the promoter/enhancer of an Ig locus. BL is classified into three main types; sporadic (sBL), found world-wide, primarily in teenagers and young adults, HIV-related (HIV-BL), found globally in HIV+ individuals, and endemic BL (eBL), which is found in tropical zones where malaria is holoendemic, almost exclusively in children. Many etiological factors have been suggested in the causation of eBL, *viz.*, malaria, Epstein-Barr virus (EBV), arboviruses, tumorigenic phorbol esters in plants (van den Bosch, 2004), HIV infection (Epeldegui *et al.*, 2007b), and *Helicobacter pylori* infection (Shannon *et al.*, 2000; Grewal *et al.*, 2008). Epidemiological evidence points strongly to a correlation between EBV, malaria, and endemic BL (Burkitt, 1962). Endemic BL, which occurs predominantly in the equatorial belt and/or malaria holoendemic region of Africa, accounts for 74% of all paediatric cancers on the continent (Morrow, 1985; Goldstein and Bernstein, 1990). The tumors almost invariably are latently infected with the EB virus. EBV is a lymphotropic γ-herpes virus (DNA genome) which generally establishes a non-symptomatic, persistent infection in 90% -100% of the world's population (Bornkamm, 2009). It is transmitted by saliva exchange, usually in early childhood. When contracted in adolescence, it may lead to acute, infectious mononucleosis (IM).

Malaria remains one of the most difficult diseases in Africa to eradicate and control.

There is also increasing evidence, although still unclear, that *Plasmodium falciparum* and its antigens could either contribute to or cause other diseases apart from malaria, including cancer (Bedu-Addo and Bates; 2002, Bellen *et al.*, 2003). Part of how it accomplishes this is by selective immunosuppression (Asito *et al.*, 2008; Minigo *et al.*, 2009) and B cell activation. The c-Myc translocation and/or c-Myc dysregulation that characterises all BL is mediated by a DNA mutating (editing) enzyme known as activation-induced cytidine deaminase (AID) (Ramiro *et al.*, 2004; Takizawa *et al.*, 2008) that is critical for antibody diversification. It is expressed by germinal centre B-cells undergoing somatic hypermutation and/or class switch recombination. Thus B-cells undergoing the germinal centre (GC) reaction are the most likely precursor cells for BL. Upon appropriate stimulation, AID becomes phosphorylated and translocates into the nucleus where targeting factors bring it to an Ig gene. Less than 10% of the total cellular AID is actually in the nucleus (Honjo *et al.*, 2005; Cattoretti *et al.*, 2006). When it is overexpressed [attainable through severe and persistent infection (Muramatsu *et al.*, 1999), for example], however, it loses its specificity for immunoglobin loci (Petersen-Mahrt *et al.*, 2002). Non-Ig genes, including c-Myc and other proto-oncogenes, are then targeted (Vallur *et al.*, 2007). It should be noted that AID's affinity for non-immunoglobin genes is ten-fold lower than for its intended targets, Ig loci (Robbiani *et al.*, 2008).

Endemic BL can be fatal within six months if untreated and the tumor has a doubling time of 24hrs (Ziegler, 1977; Kearns *et al.*, 1986). Fifty-two percent of eBL patients from Ghana had a relapse of their tumor and the overall predicted survivals at 2yrs and at 4yrs were 44% and 38% respectively (Nkrumah and Perkins 1976).

The precise etiology and pathogenesis of Burkitt's lymphoma remain elusive. It has yet to be shown how the two pathogens (EBV and malaria) specifically interact to contribute to the formation of the genetic defect (c-Myc translocation) (of which t(8:14) is predominant) that is the hallmark of the eBL disease state. To date, little work has been done on the effect of malaria and EBV on AID which mediates processes implicated in initiating eBL. This work attempts to fill that gap.

Ghana has always had a high rate of leukemia and lymphoma, as tropical zones present more pathogens to those living there. Consequently, lymphocytes proliferate more and receive more stimulation. Other cancers are also on the rise throughout Africa due to changes in diet, lifestyle, and toxin exposure (Shibuya *et al.,* 2002). The end result of a rigorous investigation into the molecular pathogenesis of eBL would be the development of new treatments for this cancer and possibly others. Ultimately prevention strategies could also be devised. Basic knowledge about the immune disruption caused by malaria and EBV would be very helpful in understanding oncovirus-induced cancers, as well as how malaria is a co-factor in other pathologies.

Recently a report has shown that the co-operation between two pathogens results in the direct re-activation of the EB oncovirus production in latently-infected B-cells (Chene *et al.,* 2007; Thorley-Lawson and Duca, 2007). Currently accepted models of eBL causation are not backed with specific mechanisms that explain how the critical c-Myc translocation arises, and specifically what role malaria plays. Mainly they propose how various aspects of malarial immunosuppression might permit the malignancy to develop.

1.2 Problem statement

This will be the first attempt (to our knowledge) to delineate precisely how two pathogens interact to cause a genetic lesion that gives rise to eBL. This thesis moves beyond the idea of the creation of a permissive context for tumorigenesis due to malarial immunosuppression. Results from this research could provide effective means of preventing eBL. It will also be invaluable in public health policy structuring and advice to the populace about less obvious risks from malaria as well as shedding new light on the potential of the malaria parasite to cause harm in more subtle ways than just acute infection.

1.2.1 Project rationale

The effect of malaria infection and EBV (co-factors of eBL) on the GC/ or memory B-cells (proposed origin of BL) and activation-induced cytidine deaminase (AID), the enzyme which mediates processes that seed the eBL translocation is not known. The precise etiology and pathogenesis of BL remains a mystery and current models are not backed with precise mechanisms (evidence). The goal of this thesis is therefore to ascertain and show that normal behaviours in the GC create a pre-disposing, context in which eBL occurs, with EBV and malaria being co-conspirators.

1.3 Main objectives

1.3.1 Hypothesis and rationale

Based on an extensive review of the BL and EBV literature, as well as observations at the Komfo Anokye teaching hospital (KATH), Ghana, two new hypotheses have been formulated which are not mutually exclusive.

Conjecture A: AID is a very potent mutagen when overexpressed and it tends to target oncogenes. It is normally turned on in GC B cells. Chronic antigen exposure and the resulting B cell stimulation from malaria may lead to prolonged or excessive AID expression and/or activation. With a highly active potent mutagen in the nucleus, there is a higher probability of accidental c-Myc translocation. Malaria also increases EBV loads and therefore elevated numbers of EBV+ B cells passing through the GC en route to memory also increases the probability of an accidental translocation resulting from AID's actions occurring in a cell that will tolerate the translocation, *i.e.*, the EBV infected. The cells harbouring the translocation may escape immunosurveillance due to malaria's perturbation of T-cell killing.

Conjecture B: Malaria may act more directly to cause the genetic lesion in B cells. The Plasmodium parasite, a rhoptry (specialized secretory organelles that are uniquely present in protozoans of the phylum apicomplexa including *Plasmodium falciparum*), or a single malarial protein may get into the cell and directly assist AID, either by activation via phosphorylation, or association with the AID mutasome to direct the cuts. Malaria may also potentiate EBV somehow, beyond increasing viral loads, potentially via signalling from lipid rafts on the cell surface.

The conjectures can be rationalized in a number of ways. Malaria has been reported to interact with the B cell compartment and cause lytic replication of EBV. Endemic BL tumors display a germinal centre (GC) phenotype, having undergone class switch recombination (CSR) and somatic hypermutation (SHM). These two processes occurring in the GC have been reported to seed the t(8:14) translocation. AID is the enzyme mediating CSR and SHM, so AID is implicated. A report by Robbiani *et al.,* (2009) on a mouse model for BL demonstrated that AID was essential for the equivalent of the human eBL translocation. Secondary enzymes were also implicated, but an absence of AID failed to yield the precise translocation.

Toxoplasma gondii, an obligate intracellular parasite is also a member of the apicomplexan family and is capable of entering lymphoctyes to manipulate the host immune response. It infects fibroblasts and introduces a secretory protein into the host as a mechanism of virulence (Taylor *et al*., 2006). It also has a protein, rop18, with kinase activity that enters the B cell (El Hajj *et al.,* 2007; Sibley *et al.,* 2009). This presents the opportunity for AID phosphorylation and concomitant hyperactivation. It must however be stated that the specificity of rop18 for AID phosphorylation has not been determined. Bioinformatics scans have identified homologous candidate proteins in *Plasmodium falciparum* that likely perform the same function as rop18 (Haase *et al.,* 2008, Wu *et al.,* 2009). The intracellular parasite *Theileria* immortalizes its host B cells via c-Myc activation. When it no longer needs the B cell for replication, it exits and c-Myc expression returns to normal (Dessauges, 2005). The B cell stage is analogous to the liver stage for malaria and it later infects red blood cells just as malaria.

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EBV alone has also been shown to upregulate AID (Epeldegui *et al.*, 2007a). It also has the capability of immortalizing B cells. EBV proteins (EBNA 3A and 3C) act to down regulate the pro-apoptotic Bcl-2-interacting mediator of apoptosis (BIM) at the RNA level to allow the cells to escape apoptosis (Anderton *et al.*, 2008).

1.3.2 Specific aims

Malaria and EBV infection are tied to eBL pathogenesis. The focus of this thesis was on EBV dynamics and the germinal center (GC), the impact of malaria on the GC B cells and the effect of malaria on AID and c-Myc expression. Clearly, understanding the "normal" interaction of these factors with B cells will give leads as to how disease arises. Invariably, there should be detectable early events that lead to tumorigenesis because the "normal" interactions have gone awry; these are key in determining mechanistic causation. The goal is to prove that normal behaviours in the GC create a pre-disposing context in which eBL occurs. This work lays the groundwork to eventually prove the formulated hypotheses which is a very long-term project. Specifically, the relationship between the proliferation and expansion of EBV infected GC B cells and GC throughput, is explored. The levels of AID and c-Myc in GC B cells from Kumasi with those from control patients in Boston, USA were also compared. The following specific aims will enable us to understand the "normal" interactions between malaria and EBV within the GC, suggesting early events that could lead to tumorigenesis. The specific aims were;

- Determination of EBV episome copy numbers in GC B cells, memory B cells and naïve B cells (as a function of proliferation and expansion). This will help us
    - To understand the interaction of EBV with the various B cell sub-populations and may also suggest mechanisms of oncogenesis
    - To validate the GC hypothesis of EBV persistence
- Determination of GC throughput and the level of EBV infected GC B cells of tonsils from a malaria-endemic region (Ghana) and a non-malaria region (US)
- Determination of AID mRNA levels in GC B cells of tonsils from the malaria-endemic region (Ghana) and the non-malaria region (US)
- Determination of c-Myc expression in GC B cells, and subsequently investigate the level of c-Myc mRNA expression in GC B cells of tonsils from the malaria-endemic region (Ghana) and the non-malaria region (US)
- Determination of AID mRNA induction by *Plasmodium falciparum* in *in vitro* cultures

1.4 Project justification

Endemic Burkitt's lymphoma (eBL) accounts for 74% of all pediatric cancers in equatorial Africa (Morrow, 1985; Goldstein and Bernstein, 1990). Out of every 100,000 African children, at least twenty will develop endemic BL (Rochford *et al.*, 2005; Thorley-Lawson and Allday, 2008). There is cause to believe that the incidence of eBL is higher than reported in the literature. At the Korle-Bu BL clinic in Accra, and Komfo Anokye BL clinic in Kumasi, for example, the price of treatment is subsidized by NGOs at both places, but it is still a problem (Duca, K.A., personal communication). While

together they see closer to 200 children or less yearly, it is likely that there are many more dying in the villages that have never used formal health care systems. The cancer is generally fatal within six months if not treated, and despite successful initial treatment, few affected children reach adulthood.

The furthest effort to show the co-operation of *Plasmodium falciparum* and EBV in the causation of the lymphoma has been the direct lytic re-activation of EBV in B cells. From literature so far gathered this thesis seeks to show that malaria and EBV individually and co-operatively lead to the over-expression of activation induced cytidine deaminase (AID) which leads to the DNA breaks that seed the chromosomal translocation observed in BL. Again this thesis work proposes that malarial and/or EBV factors could aid a specific and directed chromosomal translocation.

There is an urgent need to research into the precise etiology or pathogenesis of BL as information gathered in this vein will not only ensure elucidation of the pathogenesis but will set the tone for specific and more effective means of preventing BL or other EBV, malaria related diseases. A new perspective on the role of AID in disease and healthy states will be established.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Characteristics of BL subtypes and epidemiology

Burkitt's lymphoma is a subgroup of non-hodgkins lymphoma which consists of high grade, diffuse, small non-cleaved lymphocytes with round to ovoid and uniformed size nuclei (National Cancer Institute, 1982). BL is classified into three main types and these include; endemic (eBL), sporadic (sBL), and HIV-related (HIV-BL). Table 1 presents an overview of the characteristics of all three subtypes of BL; these include; incidence, regions affected, likely co-factors, percentage of tumors which are EBV+ and usual immunoglobulin break point. BL presents in a broad range of anatomical sites, the head and neck (jaw) being the predominant sites in the high risk areas or endemic form (50-70% of cases) (Burkitt's and Wright, 1970; Nkrumah and Perkins, 1976; Hesseling *et al.*, 1989; Owusu *et al.*, 2010). According to Adatia (1966), 100% of cases of children at age 3 have jaw presentation and there is a steady drop in the rate afterwards. Jaw lesions account for only 15-18% of cases in non-endemic regions (Adatia, 1966; Adatia, 1978). Abdominal presentation accounts for 70-90% of non-endemic BL (Philip, 1985; Magrath and Sariban, 1985). Interestingly the anatomical presentation is linked to the age of the patients, whereby children preferentially show cases in the jaw with adults showing more of abdomen presentation although not exclusively. The genetic argument for the pathogenesis and etiology of BL suffers a set-back in the face of the finding in the Surveillance, Epidemiology and End Results (SEER) program, North America; that white male are at a greater risk (age-adjusted incidence rate of 1.4/100,000) than non-white males, white females and non-white females (0.4/100,000). Also Black Americans

originating from high-incidence areas of sub-Saharan Africa, have a lower risk of BL than their white American counterparts (Reviewed by Cardy *et al.*, 2001). The correlation of geographical origin of tumor with breakpoint location on chromosome 8 and 14 (see Figure 1) as well as epidemiological differences speaks to the fact that the environment is likely to be the primary determinant (Hesseling *et al.*, 1989; Shiramazu *et al.*, 1991; Gutierrez *et al.*, 1992).

Table 1: Characteristics of BL subtypes

	eBL	**sBL**	**HIV-related BL**
Incidence	1-20/100,000	0.01/100,000	Variable
Regions Affected	Equatorial Africa, Papua New Guinea (South America, but less)	United States, Europe	Worldwide
Likely Cofactors	EBV, malaria	Unknown	HIV infection
EBV+ Tumors	> 95%	<20%	~30%
Usual immunoglobulin break point	VDJ region	Switch region	Switch region
Age range	2-16 years	All ages	All ages
t(8:14) c-myc translocation is characteristic of all subtypes and antigen loads are relatively high in all subtypes			

(Rochford *et al.*, 2005; Thorley-Lawson and Allday, 2008)

2.1.1 Ascertaining BL cases

It is imperative that BL is clearly defined both for deciding treatment regimens and to ascertain the precise etiology and pathogenesis which remain enigmatic. Atypical BL

has the immunophenotype and the hallmark of BL, the genetic abnormality, but are unique in morphology. It is yet to be explained whether these "BL-like" lymphomas are distinct in terms of their biology or an interesting variant in terms of morphological presentation. Diffuse large B cell lymphomas which are easily confused with BL cases and vice-versa are said to carry the characteristic c-Myc translocation in about 5-10% of reported cases (Kramer *et al.,* 1998; Dave *et al.,* 2006). Ideally the diagnosis of BL should be based on morphology, the presence of the characteristic c-Myc translocation, a Ki67 score>90%, CD10 and/BCL6 positivity, and CD20 or CD19 positivity (Dave *et al.,* 2006). Arthur *et al.*, (2011) have recently reported independent prognostic biochemical markers for both progression free and overall survival significance. These include; serum lactate dehydrogenase (HR=2.04; 1.25–3.32; log rank=8.3; p =0.004), serum creatinine (HR=3.59; 1.62–7.98; log rank=15.4; p =0.002) and St. Jude stage (HR=1.74; 1.11–2.73; log rank=8.0; p =0.015).

2.1.2 Models of eBL pathogenesis

Two main models for the pathogenesis of eBL have emerged since its identification in 1958. Both models hold that endemic BL is an example of multistep carcinogenesis (Klein, 1979; Goldstein and Bernstein, 1990) with each step governed by a different agent (Goldstein and Bernstein, 1990; de-The, 1993). In the first model, EBV with oncogenic potential (de-The, 1993) infects B cells, causing proliferation (Goldstein and Bernstein, 1990). Alternatively, heavy infection with EBV causes immortalization of B-lymphocytes and some immune tolerance, thus permitting additional proliferation (Klein, 1979). Malarial infection further enhances proliferation of B cells, via polyclonal activation (Chene *et al.,* 2009). In any case, large numbers of B cells are now available

for the next step (Klein, 1979; Goldstein and Bernstein, 1990). Malaria reactivates the lytic cycle of virus (Chene *et al*., 2007), while also reducing cytotoxic T lymphocytes that control the polyclonal proliferation of EBV infected B cells (Klein, 1979; de-The, 1993; Asito *et al.,* 2008; Minigo *et al*., 2009). The last step is the chromosomal translocation and tumorigenesis step. The proto-oncogene c-Myc lands downstream of a promoter/enhancer region of an Ig gene; this leads to constitutive activation. c-Myc is thereby deregulated and this results in a cell capable of unlimited growth, eventually becoming the progenitor cells of the tumor (Klein, 1979; Goldstein and Bernstein, 1990; de-The, 1993). A recent report by Toujani *et al*., (2009) using comparative genomic hybridization and spectral karyotyping has established that eBL tumors have multiple translocations, including some involving the oncogene *rel* and BIRC6, an anti-apoptotic protein believed to predispose to cancer. This coarse model evolved over a number of years, a review by Thorley-Lawson and Allday, (2008) summarizes the model, adding new molecular and paradigm shifting insights and these will be later discussed.

In the second model, chromosomal re-arrangement in B cells occurs first. This is suggested by the fact that the breakpoints in the chromosomes are different (and consistent) in the three BL sub-types (see Figure 1 for a summary of the various break-points in BL). Intense immunological stimulation from holoendemic malaria leads to a large pool of B cells. Sheer numbers lead to an increased risk of c-Myc translocation, which eventually occurs. EBV infection then immortalizes a cell that has the translocation, as EBV can override normal apoptosis that must happen when c-Myc is deregulated (Lenoir and Bornkamm, 1987).

van den Bosch (2004) has proposed another model that involves three infectious agents and a tumor promoter found in African bushes. While there is no mechanistic evidence to date for this unusual hypothesis, it is suggested by epidemiological data that localizes clusters of eBL to areas where the bushes are. Moreover, few African children have only one infection when they present with malaria, so other agents may be involved.

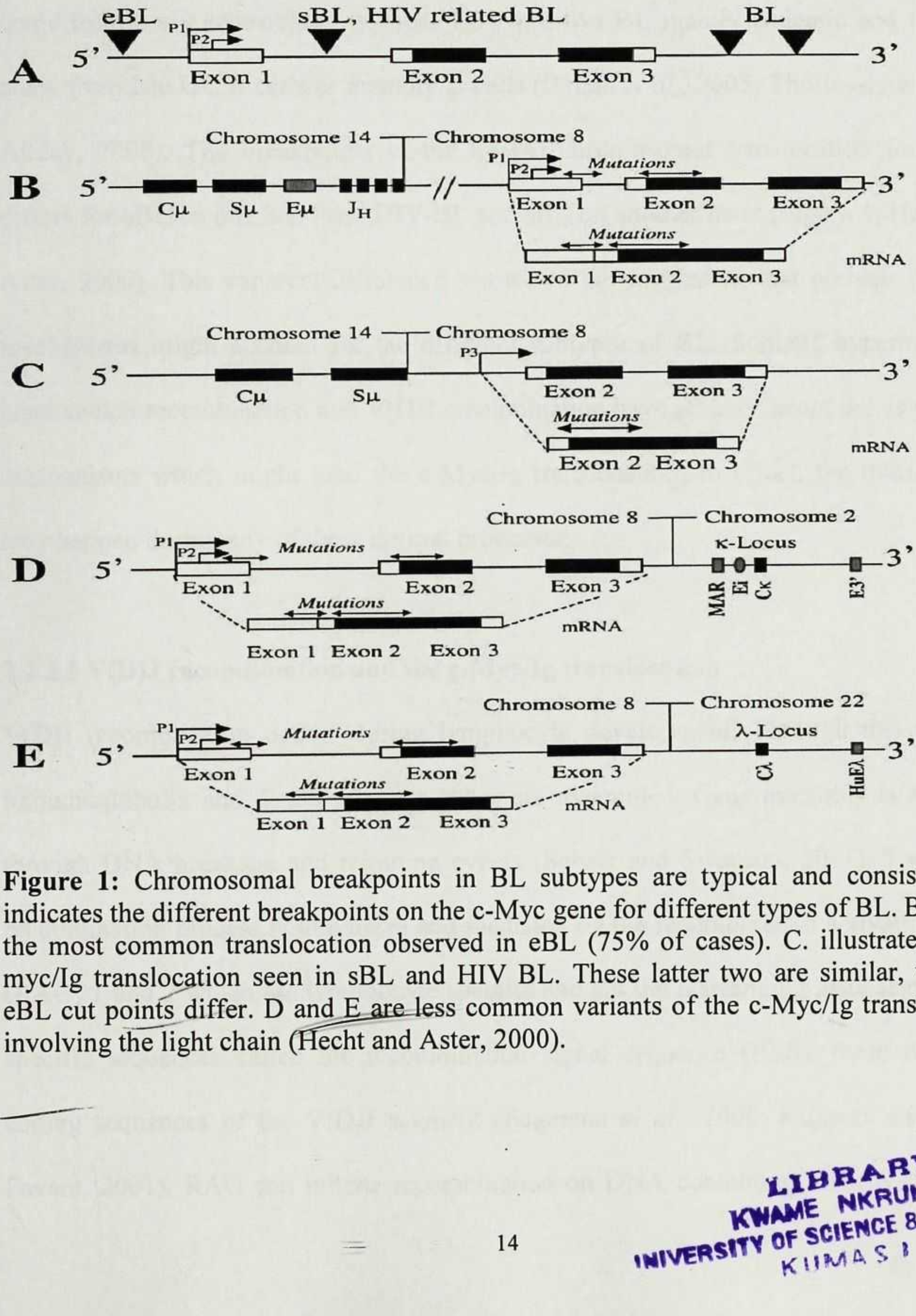

Figure 1: Chromosomal breakpoints in BL subtypes are typical and consistent. A. indicates the different breakpoints on the c-Myc gene for different types of BL. B. shows the most common translocation observed in eBL (75% of cases). C. illustrates the c-myc/Ig translocation seen in sBL and HIV BL. These latter two are similar, whereas eBL cut points differ. D and E are less common variants of the c-Myc/Ig translocation involving the light chain (Hecht and Aster, 2000).

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2.1.3 Possible mechanisms of chromosomal translocation in BL

Evidence so far generated from literature points to the fact that sporadic Burkitt's lymphoma (sBL), HIV-BL and endemic BL (eBL) may represent different entities of disease in terms of pathogenesis although they present the same immunophenotype and proliferative ability. Most sBL (in the form of EBV-negative BL) have been proposed to come from early centroblasts whereas EBV-positive BL mainly endemic and HIV-BL come from late GC B cells or memory B cells (Bellan *et al.,* 2005; Thorley-Lawson and Allday, 2008). The breakpoints in the t(8:14) chromosomal translocation for c-Myc differs for eBL on one hand and HIV-BL and sBL on another hand (Figure 1; Hecht and Aster, 2000). This apparent difference has led to the suggestion that perhaps different mechanisms might account for the different subtypes of BL. Somatic hypermutation, class switch recombination and V(D)J recombination have all been proposed as possible mechanisms which might seed the c-Myc/Ig translocation. In effect, the translocation may happen during any of these normal processes.

2.1.3.1 V(D)J recombination and the c-Myc/Ig translocation

V(D)J recombination occurs during lymphocyte development. Through this process, immunoglobulin and T cell receptor genes are assembled. Gene assembly is achieved through DNA breakage and rejoining events (Schatz and Swanson, 2011). The V(D)J recombination process is initialized and mediated by the recombination activating genes (RAG) 1 and 2 which are lymphocyte specific and cut the rearranging gene segments at specific sequences called the recombination signal sequence (RSS), these flank the coding sequences of the V(D)J segment (Fugmann *et al.,* 2000; Kuppers and Dalla-Favera, 2001). RAG can initiate recombination on DNA containing RSS (McBlane *et*

al., 1995). RSS are short DNA sequences that contain a conserved heptamer and nanomer separated by either 12 or 23 base pairs, these flank the recombining gene segments (Bassing *et al.*, 2002).

According to Haluska *et al.*, (1987), the nucleotide sequence surrounding the eBL breakpoint, i.e the V(D)J region, implicates the V(D)J recombination process. They further state that the presence of similar RSS found on the c-Myc chromosome (chromosome 8) adds impetus to their assertion that V(D)J recombination has a role in the c-Myc/Ig chromosomal translocation. Reports of the existence of an AID-independent pathway for translocation between the Ig switch region and c-Myc although rare (Unniraman *et al.*, 2004) seems to give this point of view some leverage. Dorsett *et al.*, (2007) however argue based on the finding of Yu *et al.*, (1999) that translocations occurring in eBL involving the V-JH or V-JL region, occur in mature B cells expressing AID but not RAG1/2. They argue that since the translocations in the case of eBL indicate hypermutation in the Ig variable region; AID lends itself as the best candidate) and hence CSR and/or SHM should be the mechanisms of interest.

2.1.3.2 Somatic hypermutation, class switch recombination and the c-Myc/Ig translocation

SHM and CSR are two processes by which antibody refinement or fine-tuning is carried out to ensure an efficient or competent immune response. Both occur in GC B cells. These constitute the second wave of genetic alteration after antigenic stimulation (Honjo *et al.*, 2002). During SHM nucleotide substitutions are carried out within the antibody-binding/receptor variable region of the antibody thereby increasing its affinity for

antigen. In CSR, there is substitution of the Ig constant region for one of the downstream constant regions thereby generating an antibody with new and refined effector functions. AID mediates both SHM and CSR. In these processes cytidine nucleotides in the variable and switch region are deaminated leading to the formation of U: G mismatches in the Ig locus, which must be repaired. Repair generates mutations in SHM whereas double strand breaks are rejoined by non-homologous end joining (NHEJ) in CSR. Double strand breaks present viable substrates for translocation. Dorsett *et al.,* (2007) stress that translocations seen in sBL involve the switch region so should occur around the time of CSR whereas the breaks in eBL have been argued to occur around the time of SHM. Both SHM and CSR are initiated by AID which is in fact required for the c-Myc/Ig translocation (Ramiro *et al.,* 2004; Ramiro *et al.,* 2006; Robbiani *et al.,* 2008; Robbiani *et al.*, 2009). Dorset *et al.,* (2007) reported that in an IL-6 transgenic mouse model, aberrant AID expression caused c-Myc/Ig translocations similar to those in EBV positive BL. It was evident that both the Ig and c-Myc genes had undergone SHM indicating that dsDNA breaks induced by AID during SHM are intermediates in the translocation process. It is important to also bear in mind that translocations have equally been identified with physiologic levels of AID but the frequency is a thousand times lower than in over-expressed situations; indeed c-Myc/Ig translocations occur in normal mice and two percent of healthy humans (Muller *et al.,* 1995; Roschke *et al.,* 1997). SHM hotspots have been identified and AID targets G- rich sequences which occur in both Ig and c-Myc genes (Duquette *et al.,* 2005).

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2.2 ACTIVATION-INDUCED CYTIDINE DEAMINASE (AID)

AID is a 24 kDa molecule of the APOBEC family (Muramatsu *et al.,* 1999; Cattoretti *et al.,* 2006). It is located largely in the cytoplasm as shown through cellular fractionation (Pasqualucci *et al.,* 2004). There is less than 10% localization in the nucleus (Honjo *et al.*, 2005; cited by Cattoretti *et al.,* 2006). AID is primarily expressed in the GC where it mediates SHM and CSR. Cattoretti *et al.,* (2006) have reported that AID is expressed in the dark zone, where GC cells proliferate and SHM and CSR occur, as well as in extrafollicular large B cells, and this was realized in tonsils and lymph nodes. Again large and medium sized AID positive cells were identified in the immediate environs of the follicular mantle as well as interfollicular areas and within tonsil epithelium. Basal and apical light zones have few weakly AID positive cells. Dark zone AID positive cells are CD20 and Bcl6 positive whereas AID positive cells outside the GC were CD20+, BCL2-, BCL6- and Ki 67+. Extrafollicular AID+ cells were occasionally c-Myc positive. AID positive cells in the GC were negative for c-Myc whereas extrafollicular CD30+ cells were almost 100% c-Myc positive (Cattoretti *et al.,* 2006). Induction of AID is possible through CD40 engagement and stimulation through IL-4. BCR crosslinking as well as Toll-like receptor signaling further enhances AID induction (Reviewed by Cattoretti *et al.,* 2006; Reviewed by Xu *et al.,* 2007). Indeed AID expression, B cell proliferation and CSR are also induced and/or enhanced by microbial LPS and/or unmethylated CpG DNA. LPS and CpG DNA activate TLR4 and TLR9 signal transduction pathways respectively. It is highly conceivable that *in vivo* there is crosstalk between the signal transduction pathways of the TLRs with pathways activated in B cells by the host immune system, namely CD40 ligand and IL-4, this will induce optimal AID expression to generate a robust immune response (Reviewed by Xu *et al.,*

2007). TGF-β induces AID expression in B cells essentially mediating CSR to IgA, and inhibiting B cell proliferation and CSR to other isotypes when it is present at high concentrations. Higher steady state AID mRNA is seen in B cells undergoing a higher rate of proliferation; this is consistent with the higher level of expression in centroblasts which are more proliferative than centrocytes, meaning AID regulation is cell-cycle dependent. It seems AID induction and enhancement lies in the balance between CD40 and CD30 both TNFR family proteins expressed on the B cell surface (with their ligands CD40 ligand and CD153 respectively expressed on the T cell surface). Interestingly expression of CD30 on human B cells is upregulated by CD40 ligand: CD40 engagement. CD30 negatively modulates AID expression and this is likely through its repression of CD40-Traf2 signalling thereby hampering NF-κB activation. CD40 ligand: CD40 engagement-mediated CD30 induction could be inhibited by BCR crosslinking. The crosstalk between CD40 and BCR activated signal transduction pathways is therefore critical for AID induction in GC B cells (Reviewed by Xu *et al.*, 2007). As earlier stated, AID expression is also mediated by the intracellular TLR9 in B cells stimulated with CpG DNA. TLR9 is localized in the endoplasmic recticulum and is recruited to lysosomes after acidification of endosomes and lysosomes. Chloroquine and bafilomycin, both agents that block endosomal acidification completely terminates CpG DNA induced signaling (Reviewed by Xu *et al.*, 2007). Primary human B cells that are stimulated with CD40 ligand, BCR crosslinking and CpG DNA respond with a higher rate of proliferation, stronger AID induction and higher efficiency of CSR when compared to B cells stimulated with CD40 ligand and BCR crosslinking only. TLR9 thus plays an essential role in enhancing the CD40 and BCR signals that invariably leads to AID induction and CSR (Reviewed by Xu *et al.*, 2007).

2.2.1 Role of AID in CSR and SHM

There are two main schools of thought concerning the role of AID in CSR and SHM namely; RNA editing enzyme or initiator of single stranded (SSB) and double stranded DNA breaks (DSB) as well as repair.

AID has been postulated to behave as an RNA editing enzyme which edits the endonuclease responsible for the DNA lesions observed in both CSR and SHM (Honjo *et al.*, 2002). It is possible that AID edits mRNA of factor(s) responsible for undertaking DNA repair (Papavasiliou and Schatz, 2002). Papavasiliou and Schatz (2002) have shown contrary to other findings that DSBs were present in both GC B cells from wild type and AID-deficient mice. Doi *et al.*, (2003) have reported that protein synthesis appears needed for AID function to buttress the fact that AID is probably an RNA editing enzyme. That is to say AID edits mRNA of an unkown endonuclease which is responsible for generating the DNA DSBs observed in both CSR and SHM and therefore protein synthesis must occur at the time of CSR and SHM. Petersen *et al.*, (2001) have shown that repair factors are detectable in the vicinity of switch regions in wild type but not AID-deficient B-cells which have been stimulated to undergo CSR. They conclude that there is a lack of CSR- related DSBs in the absence of AID. AID could therefore act in repair and also initiate DSBs. Prior to the transport/translocation of AID into the nucleus, it is domiciled in the cytoplasm and held by restriction factors. This state, which is unphosphorylated, represents the inactive stage of AID (Mcbride *et al.*, 2006). Upon stimulation, AID is phosporylated and it moves into the nucleus where targeting factors direct it to the Ig gene. This is followed by the recruitment of the repair-polymerase complex. AID deaminates cytidine to uridine to initiate CSR (or SHM). Uracil produced

as a result of AID initiated deamination can either undergo replication and become fixed as a transition mutation, or will be processed by pathways dependent upon uracil excision or mismatch recognition (Reviewed by Reynaud *et al.,* 2003; Vallur *et al.,* 2007).

2.2.1.2 Targeting of AID

AID may target via transcription, since this provides the ssDNA which AID prefers in the deamination of C to U. It is possible that AID could interact directly with the transcription machinery of transcribed genes (Dickerson *et al.,* 2003; Ramiro *et al.,* 2003; Nambu *et al.,* 2003). In fact *in vitro* results indicate that AID has the ability to deaminate any transcribed substrate (Chaudhuri *et al.,* 2003; Dickerson *et al.,* 2003; Pham *et al.,* 2003; Ramiro *et al.,* 2003; Shen and Storb, 2004) and acts on selected highly transcribed genes when it is overexpressed (Martin and Scharff ,2002; Yoshikawa *et al.,* 2002; Okazaki *et al.,* 2003). No strand bias has been observed *in vivo*, and mutations in C occur in both strands (Milstein *et al.,* 1998). Two possibilities stand out, a SSB could be converted to DSB upon replication, or AID could attack both strands (Reviewed by Vallur *et al.,* 2007). The latter seems more likely since in ung-/- Msh-/- mice which are not able to repair the U/G mismatch created by AID, there are equivalent amounts/levels of mutagenesis of C to U on both strands (Xue *et al.,* 2006). AID must be phosphorylated in order to interact with replication protein A thereby modulating the activity of AID on transcribed DNA (Chaudhuri *et al.,* 2004; Basu *et al.,* 2005; Pasqualucci *et al.,* 2006).

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2.2.1.3 Summary of AID's life cycle

Upon antigen activation, AID which predominantly localizes in the cytoplasm diffuses into the nucleus (active transport is possible) after phosphorylation via protein kinase A (PKA). Targeting of AID to the Ig locus depends upon, replication protein A (RPA), RNA polymerase II, and chromatin status. When PKA localizes to the Ig locus AID is phosphorylated at Ser 38, this modification is essential for AID co-factor RPA to be recruited (Vuong *et al.,* 2009). Three additional AID phosphorylation sites whose roles are unclear include, Thr 27, Thr 140, and Thr 184 (Mcbride *et al.,* 2008). AID is loaded on the transcription initiation complex and follows RNA polymerase II as elongation proceeds. Indeed AID binds to RNA polymerase II (Nambu *et al.,* 2003) and its intimate association with the transcription complex (Besmer *et al.,* 2006) is a glowing proof that transcription is tied to CSR. AID's activity is followed by UNG/MRN or Mut Sα/ EXO mutagenic repair followed by nick creation by Apurinic/ apyrimidic endonuclease (APE). AID is excluded from the nucleus via a (chromosome region maintenance/ exportin 1) CRM1-mediated mechanism that mediates its active degradation. Actually, nuclear AID has been shown to be polyubiquitinated when cells were treated with a proteosome inhibitor (Aoufouchi *et al.,* 2008) suggesting that proteasomal degradation of nuclear AID limits its half-life in the nucleus. Overall, AID expression is regulated in a number of ways including; regulation of AID protein levels and nuclear/cytoplasmic localization and regulation by miRNAs (including miR-155 and miR-181b) (Stavnezer, 2011).

2.2.1.4 AID and the c-Myc translocation

Over-expression of AID *in vitro* in spleen B cells activated to switch leads to the Ig/c-Myc translocation within 24hrs (Ramiro *et al.,* 2006). Ramiro *et al.,* (2004) have indeed shown *in vivo* using a mouse model that AID was an important agent for the c-Myc/IgH translocation. In the event of AID overexpression [attainable through severe and persistent infection of infectious agents e.g. Hepatitis C virus and EBV [(Muramatsu *et al.,* 1999; Machida *et al.,* 2004; Machida *et al.,* 2005; Epeldegui *et al.,* 2007a] and malaria infection, AID loses its specificity (Petersen-Mahrt *et al.,* 2002) and non-Ig genes including c-Myc and other proto-oncogenes (like BCL6, *Rho*H, PIM1 and PAX5) are targeted (Johnston and Carrol, 1992; Shen *et al.,* 1998; Pasqualucci *et al.,* 2001; Reviewed by Vallur *et al.,* 2007).

It is worthy of note that, Ramiro *et al.* (2006) and Dorsett *et al.,* (2007) have indicated that resolution of AID created lesions into translocations occur in a distinct pathway from CSR. This conclusion was arrived at since the process leading to translocations does not require H2AX, p53 binding proteins or the NHEJ proteins Ku70/Ku80 and PKcs otherwise needed in CSR. It has been reported that during AID overexpression alone, translocation is inhibited by the tumor suppressors ATM (which activates p53 in response to DSBs) Nbs, p19 (Arf) and p53 (Ramiro *et al.,* 2006). Disruptions in these factors produce strong enhancement of translocation although all do not have equal effects. Defects in ATM and p53 seem to provide the strongest enhancement of translocation (Ramiro *et al.,* 2006).

2.3 GENOMIC INSTABILITY AND ONCOGENESIS; THE ROLE OF c-MYC

Genetic instability refers to genetic changes that affect the normal organization and function of genes and chromosomes. Genomic instability has been linked with c-Myc expression, since c-Myc has been shown to induce the formation of reactive oxygen species leading to DNA breakage (Vafa *et al.*, 2002). It is conceivable that c-Myc is able to modify the genome structurally since abnormal c-Myc expression leads to an increase in gene amplification (intra and extra-chromosomally) and an increase in gene rearrangements. c-Myc should consequently be regarded as a structural modifier of the genome that ultimately could permit the initiation and progress of tumors (Mai and Mushinski, 2003). Evidence is rife that the GC serves as a platform for certain Hodgkins and non-Hodgkin's lymphoma including Burkitt's lymphoma; which is characterized by deregulated expression of c-Myc due to translocation involving c-Myc and the Ig locus. It has been argued as to whether c-Myc is originally expressed in these GC cells prior to the deregulated expression or it only turns on during the translocation; either direction has implications for pathogenesis and there is a need to resolve this mystery. Klein *et al.*, (2003) has it that the c-Myc proto-oncogene is not expressed in centroblasts according to their microarray hybridization profile and immunohistochemistry results. On the other hand Martinez-Valdez *et al.*, (1996), using RT-PCR and Cutrona *et al.*, (1997), using immunoflorescence, flow cytometric analysis, immunohistochemistry and RT-PCR report that c-Myc is highly expressed in GC B cells. The theory of the high expression of c-Myc in GC B cells has it that, this may bestow on centroblasts the ability to proliferate at the very high rates reported (~6 to 10 hrs per division). Again c-Myc would position the highly proliferating centoblast for death in an environment where Bcl-2 is known to be absent (this prevents the neoplastic transformation of GC B cells). It is also believed

that the high expression of c-Myc in the GC could account for the high apoptotic tendency of centroblast and centrocytes. Differentiation of centroblasts to centrocytes is marked by downregulation of c-Myc (Martinez-Valdez *et al.*, 1996). In the GC models without c-Myc (Klein *et al.,* 2003), the apoptotic behaviour of GC B cells remains enigmatic. c-Myc is a sequence specific DNA-binding transcription factor responsible for directly or indirectly regulating approximately 15% of all genes (Dang *et al.,* 2006). The genes regulated by c-Myc include those responsible for processes such as cell division, cell growth and proliferation and metabolism. Others include apoptosis, angiogenesis and adhesion, immortalization and stem/progenitor cell differentiation (Hecht and Aster, 2000; Zeller *et al.,* 2006). Apart from being a regulator of transcription, c-Myc has also being shown to have a role in the DNA replication process and consequently deregulated expression leads to genomic instability (Review by Kuttler and Mai, 2006).

The translocation of c-Myc to an Ig locus leads to the constitutive expression of c-Myc RNA and accumulation of c-Myc protein. Constitutive activation of c-Myc alone in transgenic mouse models causes B cell lymphomas which are usually derived from early B cells (Adams *et al.,* 1985) and do not resemble BL. It seems that to replicate BL the transgene must of a necessity consist of a complete c-Myc/Ig translocation (Kovalchuk *et al.*, 2000) since all BL is characterized by the c-Myc/Ig translocation. Regulation of proliferation and apoptosis especially are what c-Myc is best known for (Shi *et al.,* 1992; Evan *et al.,* 1992; Dang *et al.,* 2006) and therefore the net effect of c-Myc deregulation is the activation of cell growth and uncontrolled proliferation which increases genomic instability and increased susceptibility to apoptosis (Wade and Wahl, 2006). The c-Myc

gene contains a G rich region which may be an equivalent to the G-rich heavy chain S regions which are the targets of AID for CSR. The formation of a G-loop (DNA structures) within a transcribed region contributes to AID targeting of a specific group of transcribed genes and indeed a G loop forms upon c-Myc transcription and AID binds to these G-loops within c-Myc. In other words c-Myc is a target of AID (Duquette *et al.*, 2005).

2.3.1 Regulation of c-Myc

Regulation of c-Myc occurs at multiple levels. The half-life of c-Myc protein has been reported to be 30 minutes (Welcker *et al.*, 2004; Yada *et al.*, 2004). c-Myc protein can be modified at the post-translational level prominently by phosphorylation and two well studied phosphorylation sites of c-Myc include Thr-58 and Ser-62. Myc box protein 1 and 2 (MB1 and MB2) are two highly conserved sequence elements in the transactivation region of c-Myc and the phosphorylation of Thr-58 and Ser-62 in MB1 is an important determinant of c-Myc stability (Lutterbach and Hann, 1994; Sears *et al.*, 1999; Sears *et al.*, 2000).

The level of c-Myc increases when cells enter G1 and this occurs upon activation by mitogens. c-Myc activates the transcription of various genes that are important for progression into S phase. c-Myc is subsequently phosphorylated on Thr-58 and Ser-62 (Sears *et al.*, 2000) and this will lead to immediate degradation by the ubiquitin-proteasome pathway. In fact the phosphorylation of Ser-62 stabilizes c-Myc and the subsequent phosphorylation of Thr-58 promotes c-Myc ubiquitylation and degradation (Sears *et al.*, 1999; Sears *et al.*, 2000). c-Myc abundance decreases gradually to a low

steady state level, and this level stays the same as long as the cells are continuously proliferating.

The phosphorylation of Thr-58 is mediated by glycogen synthase kinase 3 (GSK3) (Welcker *et al.*, 2004; Yada *et al.*, 2004) and this is dependent on the prior phosphorylation of Ser-62 (Lutterbach and Hann, 1994) which is likely mediated by the Ras-ERK (extracellular signal-regulated kinase) pathway (Sears *et al.*, 2000). The Ras proto-oncogene encodes a small GTP-binding protein and this is critical in the control of cell growth (White *et al.*, 1995). The activation of Ras initiates a complex array of signal transduction which includes the Raf/ MAPK (ERK) pathway. The Raf/ MAPK (ERK) pathway is primarily involved in plasma membrane to nucleus signaling that is crucial for mitogen-induced cell proliferation (Seger and Krebs, 1995; Lavoie *et al.*, 1996). Raf activation also initiates the PI3 kinase/ AKT (also known as protein kinase B, PKB) pathway which is involved in cell survival signaling (Kauffmann-Zeh *et al.*, 1997). The activation of Ras-signaling is essential for cells to leave a quiescent state and pass through G1 phase of the cell cycle (Peeper *et al.*, 1997).

Phosphorylation at Thr-58 and Ser-62 can be controlled by the two Ras effector pathways, Ras/ ERK and Ras/ PI3K/ AKT. The Ras/ Raf/ ERK pathway extends the half-life of the c-Myc protein which enhances the accumulation of c-Myc activity (Sears *et al.*, 2000). Indeed, the ERK inhibitor PD098059 blocks Ras-mediated c-Myc stabilization (Sears *et al.*, 1999). The role of GSK-3 in the phosphorylation of Thr-58 and thus promoting c-Myc degradation is quite intriguing since GSK3 activity is regulated by the PI3K/ AKT arm of the Ras pathway (Cross *et al.*, 1995) and this

provides a second mechanism for Ras to control c-Myc protein stability. Indeed the chemical inhibitor Wortmannin which specifically inactivates PI3K (but has no effect on MAPKs), specifically inhibits the ability of Ras to increase c-Myc accumulation (Sears *et al*., 2000). It seems that as Ras initiates the PI3K/ AKT pathway, GSK-3 activity is held in check thereby preventing the phosphorylation of Thr-58. This is so because it is only when AKT activity declines that GSK-3 has the capacity to phosphorylate Thr-58 in order to induce degradation of c-Myc (Sears *et al*., 2000).

The mechanism of transient c-Myc accumulation, proposed by Sears *et al*., (2000) is as follows. Growth stimulation will lead to a rapid activation of Ras and therefore the accumulation of both Raf/ ERK and PI3K/ AKT kinase activities. c-Myc is consequently induced and the initial c-Myc product will be stabilized through the ERK mediated phosphorylation of Ser-62. The phosphorylation of Ser-62 then primes c-Myc for degradation since the ability of GSK3 to phosphorylate Thr-58 is dependent on the prior phosphorylation of Ser-62, but, GSK3 will be held in check by AKT. As the activity of Ras subsides, there is a concomitant decline of the AKT activity which will allow GSK3 to become active. This leads to the phosphorylation of Thr-58 which triggers the degradation of c-Myc.

2.4 MALARIA INFECTION, PATHOLOGY AND IMMUNITY

The protozoan parasites of the genus *Plasmodium* cause malaria infection. In spite of the fact that there is repeated exposure to the malaria parasite in endemic areas, protective immunity against infection requires many years for development (Snow *et al.*, 2005). Overall protection might be a consequence of an immune response to the various stages

of the parasite's cycle.

An encounter with malaria begins when an infected female anopheles mosquito bites the vertebrate mammalian host thereby inoculating sporozoites (seeding parasites) into the dermis, not directly into the blood stream as perceived (Sidjanski and Vanderberg, 1997; Yamauchi *et al.,* 2007; Amino *et al.,* 2008). The sporozoite stage is transient and is without pathology. If sporozoites are not bound and blocked by antibodies or drained by the lymphatic circulation after an infectious mosquito bite (Amino *et al.,* 2008), they will proceed to infect the liver cells within the first 15 minutes to few hours of infection (Sidjanski and Vanderberg, 1997; Yamauchi *et al.,* 2007). Differentiation and replication of the parasites inside the hepatocytes occurs for about a week culminating in the lysis of the hepatocytes (Nardin and Nussenzweig, 1993). Cytotoxic cells and those equipped with the secretion of IFN-γ enable the destruction of intracellular parasites (Good and Doolan, 1999). The sporozoites are now in a parasitophorous vacuole whose membrane is derived from the host cell membrane; this membrane will undergo rapid remodeling as parasite proteins are inserted into it. The parasite moves close to the host nucleus and is associated with the host endoplasmic reticulum, at this stage there are multiple nuclear divisions which culminate in cytokinesis and the individualization of progeny whose membranes might be derived from deep invaginations of the initial plasma membrane that earlier were converted into parasitophorous vacuole membranes (Bano *et al.,* 2007). These progeny are termed merozoites and the process of formation is termed schizogony. Thousands of uninucleate merozoites are released from each infected hepatocyte which can invade RBC and begin the blood stage cycle; this is the main cause of disease. Erythrocyte surface molecules sialic acid, on glycophorin A, B and C

are receptors for merozoite invasion (Miller *et al.,* 1977; Perkins, 1981; Maier *et al.,* 2003). Complement receptor 1 is a sialic acid-independent erythrocyte receptor for *Plasmodium falciparum* (Spadafora *et al.,* 2010; Tham *et al.,* 2010). It has recently been shown that the Duffy antigen receptor for chemokines (DARC) is not the sole receptor for *Plasmodium vivax* entry into RBC (Menard *et al.,* 2010). This means that in the absence of DARC *Plasmodium vivax* can invade RBC. In the RBC each merozoite will undergo asexual reproduction called the erythrocytic schizogony to form the erythrocytic schizont. Each schizont ruptures after about 48 hrs (for *Plasmodium falciparum* and *Plasmodium vivax*) to release about 16 to 32 merozoites to start a new infection cycle. Otherwise merozoites will differentiate into male and female gametes which can be ingested by a mosquito upon a blood meal. Fusion of male and female gametes occur in the midgut of the mosquito resulting in the generation of infectious sporozoites which colonize the salivary gland of the mosquito ready to be transmitted (Good and Doolan, 1999).

It is worth noting that 100% of all residents in an endemic area are periodically re-infected by *Plasmodium* parasites (Haffalla *et al.,* 2011). Endemicity classes include hypoendemic- which is characterized by very intermittent and low transmission yet severe clinical malaria in both children and adults with high mortality encompassing all population sectors. Childhood infection prevalence is less than 10%. In mesoendemic malaria areas, there is regular seasonal transmission, cerebral malaria is common and infection is well tolerated in adults; infection prevalence is <20%. Hyperendemic areas are characterized by intense transmission but there are periods of no transmission during the dry season; infection prevalence is >50<70%. Finally holoendemic areas are

characterized by a stable transmission; infection occurs all year long and mortality is highest in 1st and 2nd yrs of life; infection prevalence is between >60% to 70% or more (Adapted from Malaria epidemiology lecture, Shiff, 2006).

2.4.1 Adaptive immunity during malaria

Adaptive immune pathways which play a role in long term protection against malaria include antibody-mediated immunity which targets and blocks invasion of RBC by parasites (Chulay *et al.,* 1981) and antibody mediated cytotoxicity of iRBC (Groux *et al.,* 1990). Antibodies also block the activity of parasite toxins (Schofield *et al.,* 2002). CD4+ T cells play a central role in cell mediated immunity to malaria. They are activated by dendritic cells leading to a number of events including; activation of macrophages, phagocytosis of parasitized red blood cells by these macrophages and lastly a release of nitic oxide and oxygen radicals by macrophages which in turn cause the death of parasites intracellularly. It is worth mentioning that the reports of adaptive immunity to malaria have varied depending on the experimental systems utilized.

2.4.2 Cell-mediated immunity during malaria

Evidence supporting a role for antibody-independent cell-mediated immunity is suggested by the fact that CD4+ T cells and CD8+ T cells recognize the *Plasmodium falciparum* sporozoite antigens, circumsporozoite (CS) protein and the trombospondon related adhesive protein (TRAP) (Hill *et al.,* 1992; Gilbert *et al.,* 1998). The proposal has been that CD8+ T cells are critical effector cells of pre-red blood cell (pre-RBC) stage immunity. IFN-γ secretion as opposed to cytolysis is responsible for arresting liver stage infection (Doolan *et al.*, 1996; Good and Doolan, 1999). Pre-RBC stage immunity

mediated via CD8+ T cells also involves nitric oxide, IL-12 and NK cells (Doolan *et al.*, 1996; Doolan and Hoffman, 1999). CD8+ T cells recognize specific peptide-MHC complexes on the surface of infected hepatocytes, and this leads to the induction of IFN-γ. IFN-γ mediates the production of IL-12 which in turn induces the production of additional IFN-γ by NK cells and other cells (Doolan and Hoffman, 1999). It has however been argued that high levels of IL-10 in the liver and the fact that hepatocytes will normally not express co-stimulatory molecules both hinder T cell activity. IL-10 downregulates MHC class II molecules on antigen presenting cells, it also inhibits nitric oxide production, prevents the priming of T cells and their proliferation. IL-10 also suppresses the production of IFN-γ, TNF-α, GM-CSF and LT by T cells (Akdis and Blaser, 1999). The aforementioned reasons raises questions about the ability of T cells to actually provide liver-stage immunity although aside CD8+ T cells, CD4+ T cells (Weiss *et al.*, 1993) and γδT cells (Rzepczyk *et al.*, 1997) have also been reported to offer IFN-γ mediated liver stage protection. It seems that CD4+ T cells play a crucial role in antibody-independent cell mediated immunity to the RBC stage of malaria infection. In summary CD4+ T cells are activated by mature dendritic cells which have prior endocytosed *Plasmodium* parasites. These activated CD4+ T cells will then release IFN-γ and TNF-α which activate macrophages leading to phagocytosis of parasitized RBC. These macrophages will also release nitric oxide and oxygen radicals which results in the intracellular death of parasites. Evidence for an important role for CD4+ T cells in immunity to malaria includes the following. Cytolytic CD4+ T cell clones produce IFN-γ and IL-2 and passive transfer of these CD4+ T cells from the spleen of mice immunized with irradiated sporozoites offer very high level of protection to naïve mice when challenged with sporozoites (Tsuji *et al.*, 1990). According to Weiss *et al.*,

(1993) mice are not protected by sporozoite immunization if they have their CD4+ T cells depleted. These mice make little antibody towards sporozoites giving a glowing proof to the relevance or T cell dependency of the humoral response. Interestingly hyperimmune serum will not restore immunity in CD4 depleted mice, and IL-2 will not compensate for the absence of CD4+ T cells. Though the number of CD8+ T cells and their activation remained normal in CD4 depleted mice they are not enough for protection against malaria (Weiss *et al.*, 1993). According to Kumar *et al.*, (1989), CD4+ T cells are absolutely necessary for immunity in mice after inoculation with *Plasmodium vinckei*. CD4+ T cell depleted mice that were earlier immune died of malaria. There was a reconstitution of immunity upon transfer of CD4-enriched T cell populations from the spleens of immune animals. Kumar *et al.*, (1989) further argue that CD4+ T cells are most-likely required for cell mediated immune mechanisms rather than for help in antibody production. The reasons which led to their proposition included; mice that make no antibody (μ-suppressed) still developed immunity. Infection and cure kinetics were the same as that of normal mice, that is *in vivo* depletion of CD4 T cells in these B cell deficient mice is what abrogates their immunity. Sera from immune mice transferred to athymic nude mice apparently had no effect on parasitemia (Kumar *et al.*, 1989). Indeed IFN-γ production by CD4+ T cells to specific antigens of the RBC phase protects against re-infection in Africa (Luty *et al.*, 1999). Pombo *et al.*, (2002) report that when ultra low doses of *Plasmodium falciparum* infected RBC was administered to volunteers and left over an extended period without drug treatment and cure, volunteers were protected against malaria; there were no signs of detectable antibodies binding to parasites or infected RBC, but immunity was characterized by proliferative T cell response which involves CD4+ and CD8+ T cells. The cytokine response consisted of

IFN-γ (not IL-4 or IL-10). There were also high concentrations of nitric oxide synthase activity in peripheral blood mononuclear cells (Pombo *et al.*, 2002). Figure 2 summarizes a possible mechanism of antibody-independent cell-mediated immunity.

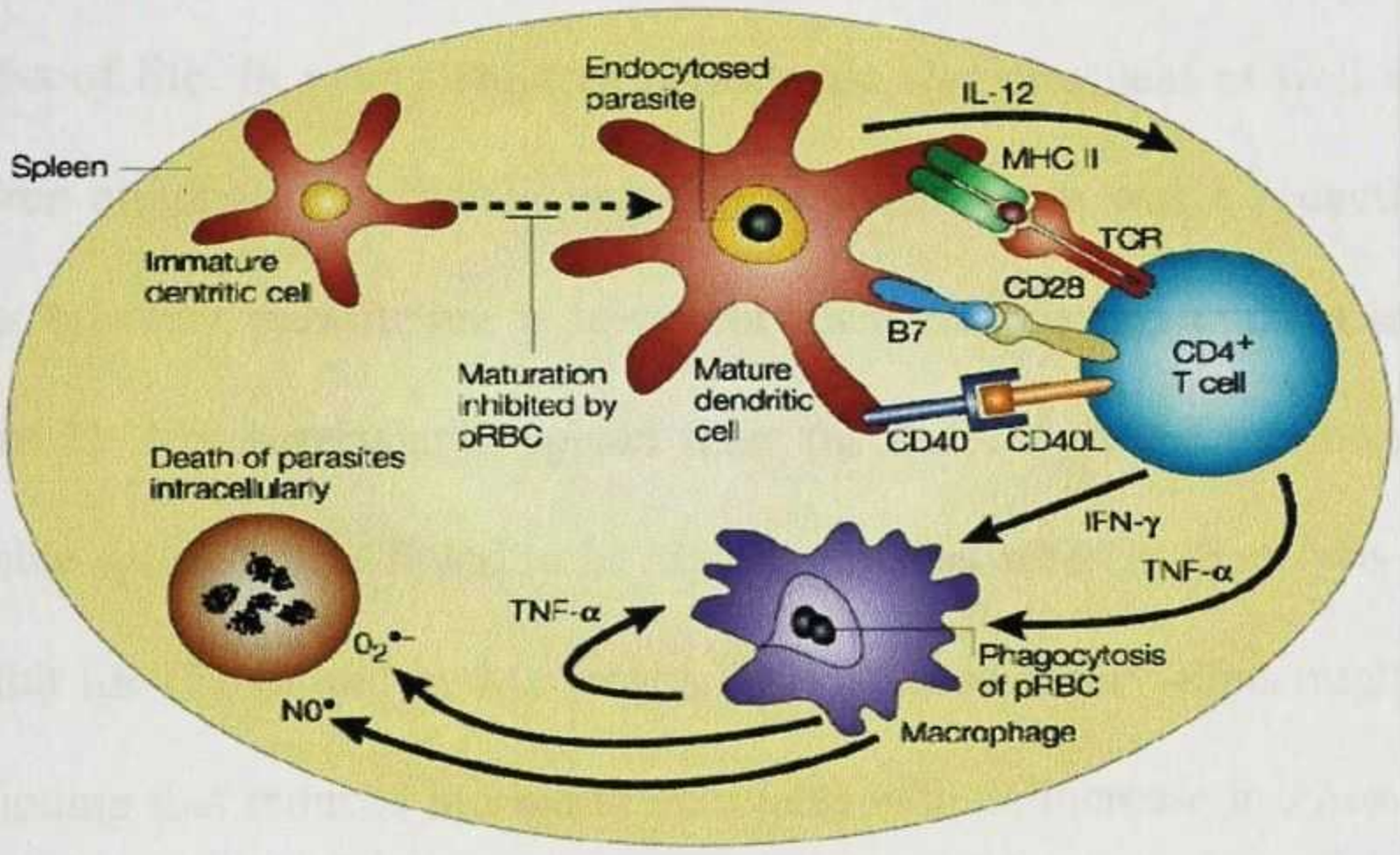

Figure 2: Possible mechanism of antibody-independent cell mediated immunity. CD4+ T cells are activated by mature dendritic cells leading to activation of macrophages, phagocytosis of parasitized RBC, release of cytokines and small inflammatory molecules like nitric oxide and oxygen radicals. T cell immunity is thought to occur largely but not exclusively in the spleen (Good, 2001).

2.4.3 Humoral immune response to malaria

There is yet still an argument for the role of antibodies in malaria immunity and for followers of this proposition the following are their testimonies. High serum concentration of IgG, IgA and IgM are seen simultaneously shortly after establishment of parasitemia. IgG and IgM levels were higher than IgA. Whereas IgA and IgM are more transient and sometimes lost despite parasetemia, IgG antibodies will linger longer (Reviewed by McGregor, 1974). Antibody mediated immunity against the RBC stages of malaria is a critical part of naturally acquired immunity to malaria (Marsh and

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Kinyanjui, 2006; Bull *et al.,* 1998 ;Wipasa *et al.,* 2010; Crompton *et al.,* 2010). Indeed Garraud *et al.,* (2001) have shown that mononuclear cells from blood and tonsils of people from immune populations exposed to *Plasmodium falciparum* antigens demonstrate *in vitro*-driven anti-*Plasmodium falciparum* IgG antibody production. It is rare to find congenital malaria and newborns are resistant to infection during the early months of life. In young children parasitemia was prevalent as well as dense. In older children although parasitemia was still prevalent there was a reduction in density. In adults however parasitemia is low, infrequent and clinical malaria is a rarity (also in Figure 3). Not surprisingly spleen rates (as defined as the number of children with palpable spleen) were found to be high in children whereas there was a marked decline in adult life (Reviewed by McGregor, 1974). These observations might be explained by the finding that reduced morbidity correlates with an increase in *Plamodium falciparum* specific IgG1 and IgG3 (cytophilic antibodies) which increases with age. It is interesting to note that the switch from IgG2 and IgG4 (isotypes which have been proposed to be antagonistic to protection) (Ndungu *et al.,* 2002) and IgM (all of which are non-cytophilic antibodies) occurs late; this further explains the slow development of a protective immune response (Bouharoun-Tayoun and Druihle, 1992; Oeuvray *et al.,* 2000). Total IgG and anti-malaria IgG were high in patients with uncomplicated malaria whereas IgE antibodies were higher in patients with severe malaria and in more than half of the patients (65%) there was a positive correlation between anti-malarial IgE antibodies and parasetemia. Anti-malarial IgG may therefore be essential in reducing the severity of malaria infection while anti-malarial IgE antibodies contribute to or heighten pathogenesis (Perlmann *et al.,* 2000).

In Kenyan children with acute malaria there was an increase in total memory B cells in the peripheral blood, although the overall B cell percentage was significantly decreased. A high percentage of transitional B cells was also observed (Asito *et al.*, 2008). In a longitudinal study carried out in an extremely low malaria transmission area in Northern Tanzania, malarial antigen specific antibodies and malaria specific memory B cells were detected in a significant portion of the population with a history of malaria. Antigen specific antibody and memory responses persist over time even in the absence of re-infection. Interestingly in some cases antigen specific memory B cells could be quantified yet the sera of the patients had no detectable antigen specific antibodies (Wipasa *et al.*, 2010). Finally in a seasonally high transmission area in Mali there was a slow and stepwise acquisition of *Plasmodium falciparum* antibodies and memory B cells in correlation with years of repeated exposure despite intense annual exposure to the *Plasmodium falciparum* parasite (Weiss *et al.*, 2010).

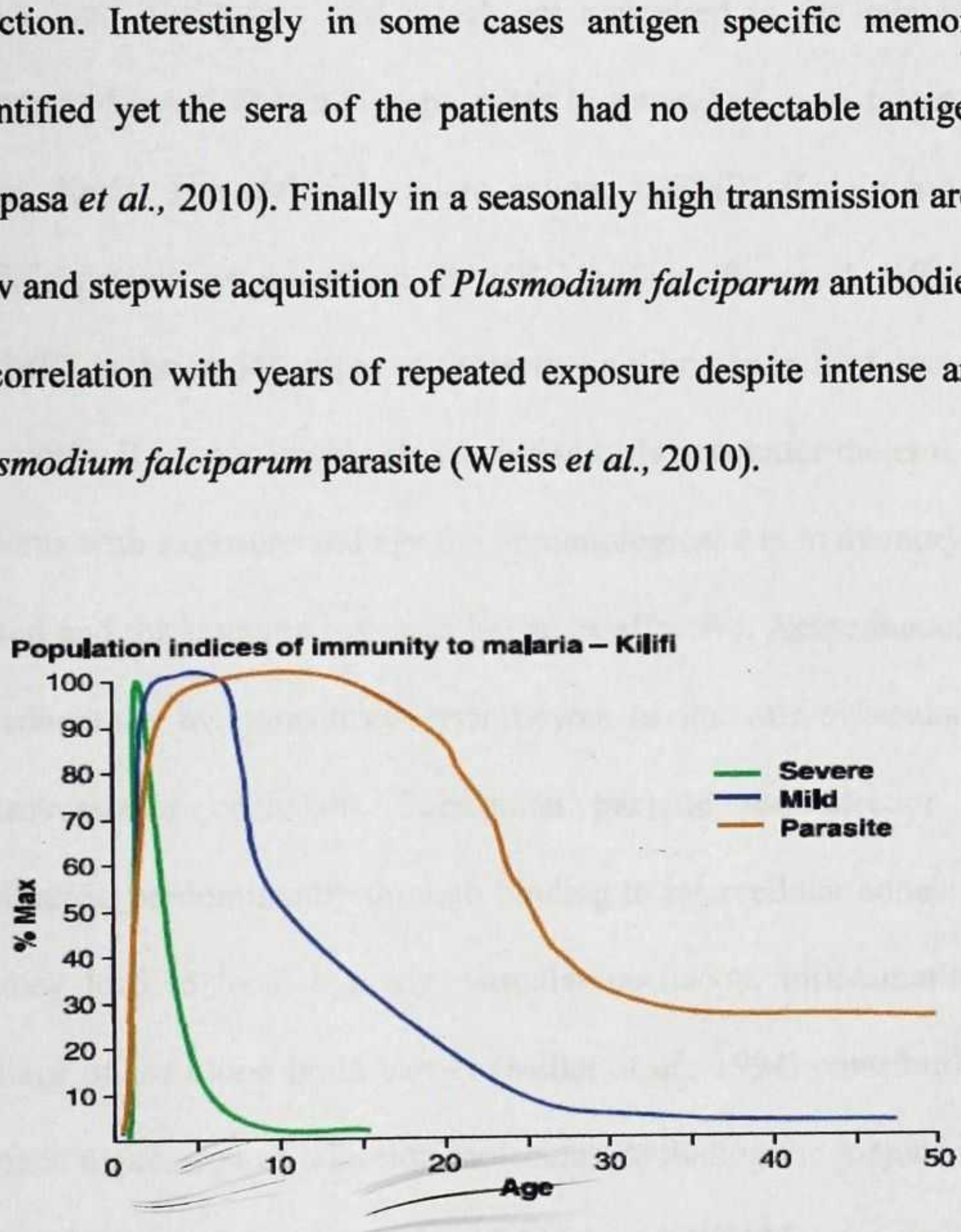

Figure 3: Population indices of immunity to malaria in Kilifi, Kenya. Age pattern of assymptomatic parasite prevalence and the prevalence of both mild and severe clinical malaria (Marsh and Kinyanjui, 2006).

2.4.4 Parasite sequestration and malaria pathology

Plasmodium falciparum infected RBCs are able to avoid spleenic clearance by attaching to the vascular endothelium. PfEMP1 (*Plasmodium falcipaum* erythrocyte membrane protein 1) encoded by *Plasmodium falciparum* enables iRBC to engage a number of receptors including intracellular adhesion molecule 1 (ICAM-1), CD36, thrombospondin (Baruch *et al.,* 1995; Baruch *et al.,* 1996), vascular cell-adhesion molecule 1 (VCAM-1) (Miller *et al.*, 1994), CD31, endothelial cell selectin (E-selectin), chondroitin sulphate A (CSA) and hyaluronic acid which are expressed in the microvascular beds of deep organs and could concentrate parasites in several of these target organs (Schofield and Grau, 2005). The malarial variant antigen PfEMP1 (Udeinya *et al.,* 1983), is a 200-350kda protein expressed on the RBC surface (Su *et al.,* 1995; Smith *et al.,* 1995). PfEMP1 is the major target of protective antibodies in high transmission areas (Bull *et al.,* 1998). It is conceivable that as individuals encounter the entire range of 60 PfEMP1 variants with exposure and age the immunological gap in memory B cells is narrowed or closed and the immune response becomes effective. Acute malaria pathology is a result of adherence by parasitized erythrocytes to the microvascular endothelium causing microsvascular occlusion. Substantial parasite sequestration in the brain micro-capillaries, predominantly through binding to intercellular adhesion molecule 1 (ICAM-1) may lead to local hypoxia, vascular occlusion, inflammation and subsequently a damage of the blood brain barrier (Miller *et al.,* 1994) contributing to cerebral malaria. Surface expression of adhesion molecules, including the major binding receptors on the endothelial microvasculature i.e. ICAM-1 and VCAM-1 (as well as CD36 on platelets) is upregulated when overproduction of type 1 cytokines, especially TNF-α and IFN-γ, occurs in the form of an inflammatory surge.

2.4.5 Immunodeviation due to malaria

Urban *et al.,* (1999) have shown that intact malaria infected erythrocytes adhere to dendritic cells, inhibit their maturation and subsequently reduce their capacity to stimulate T cells. Malaria parasites actively modulate the immune system and this is evident in the fact that T cell responses are suppressed during the course of the infection (Troye-Blomberg *et al.,* 1984; Ho *et al.,* 1986; Ho *et al.*, 1988). Immune stimulation by *Plasmodium* parasite's altered peptide ligands (which are analogues derived from the original antigenic peptide carrying amino acid substitutions at TCR contact residues) lead to defective effector T cells; these will proliferate in response to antigen but will not kill or produce the protective liver stage cytokines TNF-α or IFN-γ (Plebanski *et al.,* 1999). The same altered peptide ligand epitopes are also antagonistic for cytotoxic memory T lymphocytes from malaria exposed donors thereby obliterating their lytic activity (Gilbert *et al.,* 1998; Plebanski *et al.,* 1999). According to Ocana-Magner *et al.,* (2003), infected RBCs (iRBC) affect DC survival and invert the IL12/IL10 ratio. IL-12 is needed for both initiation and maintenance of T cell responses. They further stress that antigen internalization is not affected but there is inhibition of the antigen specific CD8+ T cell activation by DCs since DCs fail to mature and proceed to apoptotic death. Infected RBCs encounter DCs in blood and spleen; there is inhibition of the expression of costimulatory and MHC class I and II molecules and consequently there is an inhibition of the capacity to initiate new immune response. DCs also secrete soluble factors that inhibit activation of CD8+ T cells (Ocana-Magner *et al.,* 2003).

2.4.6 Protective immunity and immune pathology is a balancing act

When volunteers were administered with ultra low doses of *Plasmodium falciparum* and subsequently cured through the administration of drugs, they mounted a considerable protective immune response (Pombo *et al.*, 2002). It was observed that protective immunity was derived from CD4+ T cells, IFN-γ and NO and not antibodies. There was a drop in the level of peripheral NKT cells and the response was not dependent on IL-10 and IL4. Pro-infammatory cytokines are vital in the quest of the host to limit parasitemia, anti-inflammatory cytokines like IL-10 and TGF-β on the other hand are essential once peripheral parasitemia has been curtailed to dampen the protective, yet detrimental, host defense mechanism. The outcome of *Plasmodium* infection depends on the balance between pro-inflammatory and anti-inflammatory responses. T_{regs} (CD4+CD25+FOXP3+) which act to dampen immune responses in malaria are associated with TGF-β secretion during blood stage infection (Walther *et al.*, 2005). Indeed CD4+CD25+ T cells bind TGF-β on their cell surface in order to mediate contact-dependent suppression of other T cells (Nakamura *et al.*, 2001). In fact TGF-β induces and increases the expression of FoxP3, a transcription factor essential for the generation and function of regulatory T cells (Chen *et al.*, 2003; Schramm *et al.*, 2004). CD4+CD25+ T cells also produce high levels of TGF-β and IL-10 (without dividing) when stimulated with anti-CD3 and soluble anti-CD28 and/or IL-2 (Nakamura *et al.*, 2001; Zheng *et al.*, 2004). These CD4+CD25+ cells greatly increase the numbers of CD4+CD25- cells that become CD25+ cytokine dependent suppressor cells (Zheng *et al.*, 2004) which suppress T cell proliferation in an IL-10 dependent fashion (Dieckermann *et al.*, 2002). The result of this is that pro-inflammatory cytokine production is diminished as well as antigen specific responses, and parasite growth is

rapid (Walther *et al.*, 2005).

2.4.7 Important malarial bioproducts and their activities

Three important malarial products with far-reaching consequences on the immune response include; PfEMP1; Glycosylphosphatidylinositol (GPI) and Hemozoin;

As earlier mentioned PfEMP1 binds to a number of ligands which direct the parasites chiefly to the brain and placenta. Engagement of CD36 on DCs will suppress DC and macrophage activation (Reviewed by Schofield and Grau, 2005). The cysteine-rich interdomain region (CIDR) - 1 alpha component of PfEMP1 binds to Ig and acts as a T-independent polyclonal B cell activator preferentially activating the memory B cell compartment (Donati *et al.*, 2006b). It increases the expression of TLR7 and 10 and sensitizes B cells to TLR9 signalling (Simone *et al.*, 2011).

Glycosylphosphatidylinositol (GPI) which acts as both a PAMP (pathogen associated molecular pattern) and a toxin binds to TLR2, TLR4 and c-type lectins on DCs, macrophages, endothelial cells and adipocytes as well as CD1d and V-α 14-V-β 8 TCR on NKT cells. GPI induces the expression of genes encoding proinflamatory proteins such as TNF-α, IL-1, IL-6, IL-12, iNOS, ICAM-1, VCAM-1. GPI will also activate NKT cells and induce the production of TH1 and TH2 cytokines (Reviewed by Schofield and Grau, 2005).

Hemozoin is the crystalline pigment granule produced within the blood stage food granule as a result of haemoglobin digestion by the malaria parasite *Plasmodium falciparum*. Hemozoin consists of 65% protein (a mixture of native and denatured

human globulin in a non-covalent association with metalloporphyrin), 16% ferriprotoporphyrin-IX (hematin), 6% carbohydrate and trace amount of lipids and nucleic acids (Goldie *et al.,* 1990). It is long lived and accumulates in phagocytes. It induces the production of the T Helper 1 cytokines TNF (Sherry *et al.,* 1995) and IL-12 (Coban *et al.,* 2005) or the T Helper 2 cytokine IL-10 and it inhibits the proliferative response of human leukocytes (Deshpande and Shantry, 2004). Hemozoin has been shown to promote monocyte and macrophage dysfunction, it impairs phagocytosis and the expression of MHC II molecules, CD11 and ICAM 1 (Schwarzer *et al.,* 1992). Morakote and Justus (1988) and Scorza *et al.,* (1994) both report that hemozoin is generally immunosuppressive, by affecting both the antigen processing and immunomodulatory functions of macrophages. Hemozoin (albeit synthetic) stimulates naïve murine macrophages resulting in the myeloid differentiation primary response gene 88 (MyD88)-independent activation of NF-κB and ERK as well as the release of the chemokine MCP-1. In macrophages prestimulated with IFN-γ however, hemozoin causes a MyD88 dependent release of TNF-α (Griffith *et al.,* 2009). Hemozoin is reported to bind to TLR9 on DCs and leads to rapid accumulation of NF-κB and nitric oxide overproduction (Jaramillo *et al.,* 2003). There is controversy as to whether hemozoin on its own binds to TLR9 (Coban *et al.,* 2005) or as Parroche *et al.,* (2007) have clearly shown, hemozoin rather presents plasmodial DNA which stimulates the TLR9 of the host, radically enhancing innate responses. This activity was further shown to be DNase sensitive. Hemozoin inhibits the differentiation and maturation of human monocyte derived dendritic cells (Skorokhod *et al.,* 2004).

2.4.8 Crossroads between malaria and Toll-like receptor signaling

TLRs are pattern recognition receptors that are a core constituent of the innate immune system. They initiate innate immune responses against pathogen associated molecular patterns (PAMPs) from bacteria, viruses, parasites and other pathogens. TLR1/TLR2 heterodimers recognize triacylated lipopeptides; TLR2/TLR6 heterodimers recognize diacylated lipopeptides; TLR3 recognizes double stranded RNA; TLR4 recognize lipopolysaccharides (Jide *et al.,* 2009) [it is worthy of note that although mouse B cells express TLR4 and are triggered by LPS, human B cells do not express TLR4 and therefore do not respond to LPS. The LPS response in humans is restricted to dendritic cells (Bourke *et al.,* 2003; Bernasconi *et al.,* 2003)].

2.4.9 Positive TLR signaling during malaria

The intense induction of pro-inflamatory cytokines like TNF-α, IL-1, and INF-γ follows an encounter with either whole parasites and or parasite products released when an infected RBC ruptures. The pro-inflammatory cytokines TNF-α, IL-1, IL-6, IL-12 and IFN-γ have important roles in the control of parasite growth and are linked to TLR stimulation (Reviewd by Jide *et al.,* 2009). Macrophages can be induced by soluble parasite products and release TNF-α (Bate *et al.,* 1988), TNF-α will in turn activate macrophages to produce nitric oxide which is responsible for the death of parasites intracellularly (Figure 3; Reviewed by Good, 2001). TLR-MyD88 pathways are required for the induction of IL-12 during malaria infection (Adachi *et al.,* 2001). Plasmacytoid DC activated by schizonts or soluble schizont extracts will upregulate CD86 expression and produce IFN-α through the TLR9-MyD88 pathway. Interestingly, schizont DNA did very little in terms of the induction of IFN-α when added at extremely high

concentration. Also worth noting is the fact that the activity of the schizont extract was heat labile and precipitated with ammonium sulphate unlike DNA (Pichyangkul *et al.*, 2004). According to Zhu *et al.*, (2005), parasite GPIs will induce macrophages to produce TNF-α, IL-12, IL-6 and nitric oxide (NO). In response to malaria parasite infected RBC, NK cells secrete IFN-α and, additionally macrophages were essential in NK cell function; both cell types utilize the MyD88 pathway en-route to NK activation (Baratin *et al.*, 2005).

2.4.10 *Plasmodium falciparum* escapes immune surveillance through TLR signaling

Through TLR9 signaling, malaria parasites can control the host inflammation thereby promoting parasite immune invasion. The progression of malaria infection brings with it changes in DC response/behavior. Further on in malaria infection, DCs will become non-compliant to the production of IL-12 and TNF-α which is TLR mediated, concurrently increasing the production of IL-10 and yet holding the ability to activate naïve T cells. Early in infection, IL-12 secreting DCs will stimulate an IFN-γ dominated T cell response and, IL-10 secreting DCs in later stages will induce an IL-10 dominated T cell response (Perry *et al.*, 2005). According to Millington *et al.*, (2006) hemozoin when phagocytosed by DCs impairs their function and CD4+ T cells activated by such DCs fail to clonally expand. There is also a defect in the ability of these T cell subsets to migrate to B cell areas and therefore a failure to provide requisite help for B cell expansion and antibody production. In conclusion hemozoin modulates DC function significantly depressing the immune system and allowing for immune evasion (Perry *et al.*, 2005). According to Hisaeda *et al.*, (2008) malaria parasites interact with DCs through TLR9 in order to activate regulatory T cells (T_{regs}) (*in vitro*) enhancing their

suppressive functions. Walther *et al.,* (2005) have also reported that upregulation of T_{regs} correlates with a rapid increase in parasites during human malaria. Indeed when mice were infected with a lethal strain of *Plasmodium yeolii*, those depleted of CD4+CD25+ regulatory T cells (T_{regs}) are protected from death (and cleared parasites completely), whereas untreated mice had severe parasitemia (Hasaeda *et al.,* 2004). This protection is due to an increased T cell responsiveness against parasite derived antigens.

2.4.11 TLRs in malaria pathogenesis/ syndrome

MyD88 pathways mediate IL-12 production during malaria infection which causes liver damage (Adachi *et al.,* 2001). Mice deficient in TLR2 and TLR9 escape cerebral malaria as opposed to those deficient in TLR4, TLR5 or TLR7. TLR9 and TLR2 mediated cerebral malaria is associated with the accumulation of hemozoin, CD8+ Tcells, NK and DC cells in the brain (Reviewed by Jide *et al.,* 2009). According to Lu *et al.,* (2006), ERK1/2 JNK, p38 and c-Jun and activating transcription factor-2 are phosphorylated in *Plasmodium falciparum* GPI stimulated macrophages. They concluded that GPI induces TNF-α secretion through the activation of MAPK pathways, thereby favoring cerebral malaria.

2.4.12 Malaria and the spleen

The spleen is a key site for removal of iRBC and the generation of immunity; it is also a hub of lymphocytes. The spleen is therefore a good model for investigating the interaction between B lymphocytes and *Plasmodium falciparum*.

Blood from the circulation enters the spleen through arterioles into the marginal zone

sinus and the red pulp. The marginal zone separates the red pulp and the white pulp regions and it consists of reticular cells, marginal metallophilic macrophages, and marginal zone macrophages (MZMs), B cells involved in T-independent responses, dendritic cells and T cells. Whereas the marginal zone metallophilic macrophages are in the inner border of the marginal zone (close to the white pulp), the marginal zone macrophages are present at the opposite end of the boundary (close to the red pulp). Marginal zone macrophages will remove bacteria as well as particulate material from the blood as there is a slow flow in the marginal sinus (Reviewed by Engwerda *et al.,* 2005; Figure 4). The marginal zone serves as the traffic conductor as it directs lymphocytes and dendritic cells from the blood into the white pulp (Lyons and Parish, 1995). The red pulp has a reticular cell network; the elaborate red pulp filtration system has highly phagocytic macrophages as key actors (Weiss *et al.,* 1989; Figure 4).

Blood exits the spleen via the venous route after passing through the red pulp. B cell follicles around the T cell zone (periarteriolar lymphoid sheath) comprise the white pulp (van der Eertwegh *et al.,* 1992). B and T cells both sample antigen in immune complexes on the surface of DCs (specifically follicular DCs for B cells), antigen specific B cells then proliferate forming GCs and undergo SHM and CSR. CD4+ T cells recognize peptides associated with MHC class II. Antigen specific CD4+ T cells will proliferate and produce IL-2 which enables them to expand as well as supporting the proliferation of CD8+ T cells. Both CD8+ and CD4+ T cells must migrate to the border of the periarteriolar lymphoid sheath (PALS) and B cell follicles to provide cognate help and cytokines for antigen specific B cells (Jenkins *et al.,* 2001).

Pathogen-specific B and T cell responses are generated in structures in the white pulp of the spleen and therefore the spleen is a useful site for the development of both protective and pathological immune responses following *Plasmodium falciparum* infection. The marginal zone and the red pulp are viable sites where infected RBC can be removed. Neutrophils and macrophages have been shown to be able to remove parasites leaving the RBC intact. The flow of blood in the spleen is slowed down in the marginal zone allowing antigen to be captured with high efficiency by MZMs and DCs (Kraal *et al.*, 1989; Figure 4).

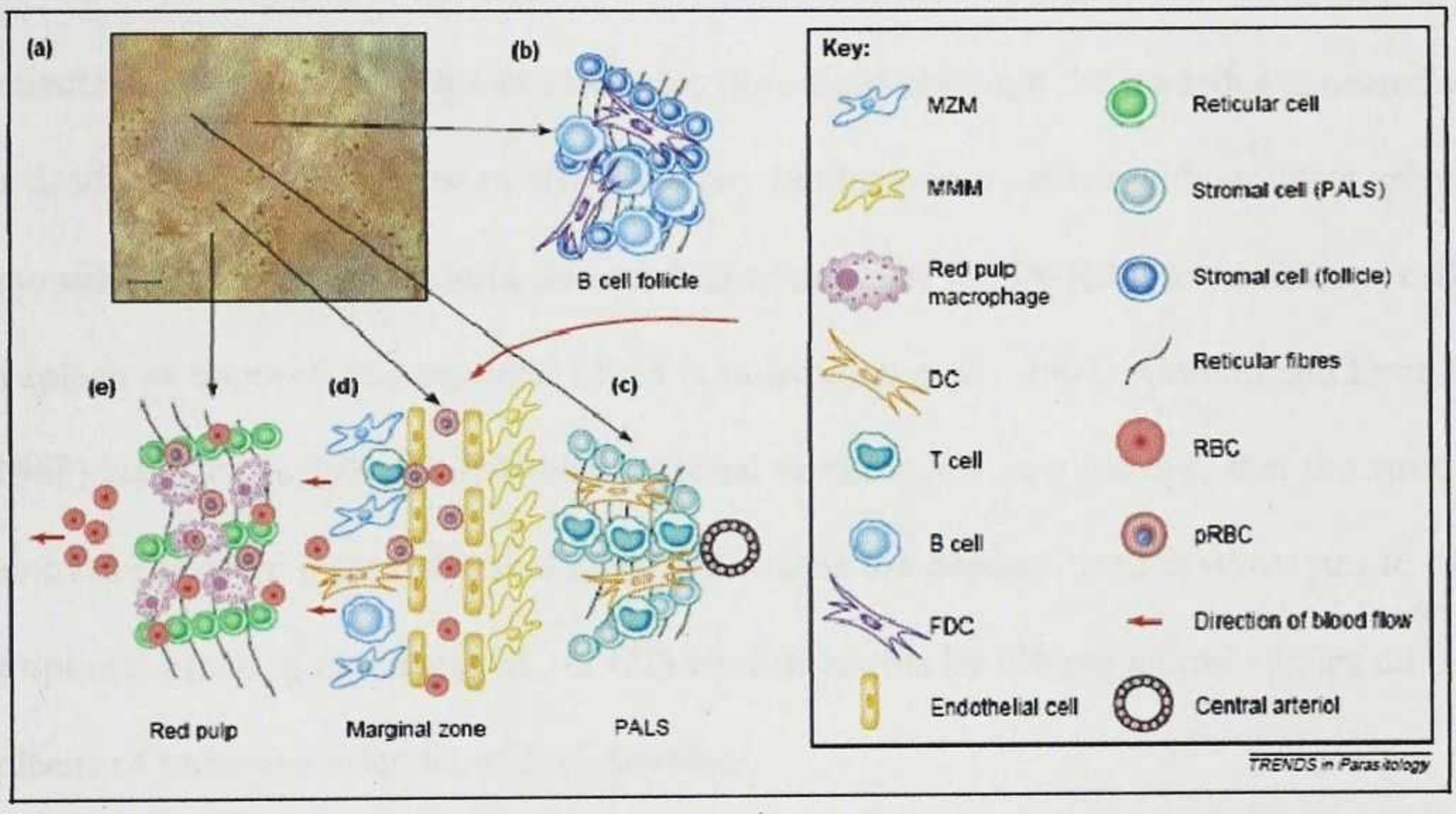

Figure 4: The spleen and malaria

a. A cryosection of a spleen that has been stained for T cells (CD33, blue) and B cells (CD45 B220, red). Mice had been intravenously injected with Indian ink. The Marginal zone Macrophages (MZMs) are black due to uptake of Indian ink.
b. Antigen is presented to B cells on the surface of follicular dendritic cells (DCs) and antibody responses are generated in the B cell follicles
c. After antigen presentation by DCs, T cell responses are primed in the periarteriolar lymphoid sheath (PALS)
d. From the central arteriole, blood flows into the marginal sinus. Blood then travels through the marginal zone (MZ) into the red pulp
e. In the red pulp parasitized red blood cells (pRBCs) are removed by macrophages Scale bar = 100mm (Engwerda *et al.*, 2005).

It remains to be understood how antigens are transported to the white pulp for acquired immune response generation. This notwithstanding, parasite antigen whether free or taken up by DCs or macrophages can be accessed by B cells in the spleen. In humans the spleen is useful for either removing parasitized red blood cells from the circulation or pitting (i.e de-parasitizing the RBC leaving the RBC intact). According to Chotivanich *et al.,* (2002), in splenectomized patients with acute *falciparum* malaria, none of the patients had erythrocyte surface antigen RBC-RESA that is, RBC that had been parasitized but no longer contain the malaria parasite. RESA was however found in all patients with acute malaria and intact spleens. Parasite clearance in splenectomized patients after artesunate treatment was also prolonged although the parasites appeared to be dead and would not grow *ex vivo*. Autopsy findings on a patient with an intact spleen who died from cerebral malaria showed higher ratios of RESA-RBC to parasitized cells in spleen as opposed to peripheral blood (Chotivanich *et al.,* 2002). Conrad and Dennis, (1968) reported, using radiolabbeled infected erythrocytes in monkeys, that the spleen removes parasites from red blood cells and returns the deparasitized erythrocytes to the peripheral blood. Schnitzer *et al.,* (1972) confirmed this by ultrastructural studies on the spleens of monkeys infected with *P. knowlesi*.

Malaria infection can have deleterious effects on the spleen. Hyper-reactive malaria splenomegally (HMS) (sometimes refered to as tropical splenomegaly syndrome), an extreme form of malaria induced pathology is a result of multiple malaria infections and is distinct from the enlargement of the spleen as a direct result of malaria parasitemia. HMS appears to be a result of dysfunctional T cell control in response to recurrent malaria leading to the gross overproduction of both antimalarial and non-malarial IgM

antibodies. The overproduction of IgM leads to the formation of high molecular weight immune complexes, gross splenomegaly, recurrent severe anemia and high succeptibility to infections (Reviewed by Crane, 1986). In malaria endemic regions of Ghana HMS and B-lymphoproliferative disorders were identified as the most common disorders associated with massive splenomegaly. Patients were classified as having HMS if their spleen size, measured every month shrunk sustainably in size of at least 40% after the first line of anti-malarial treatment (3 months) and second line of treatment (3 months), a total of 6 months. Patients with lymphoproliferative disorder had their spleen size reduced up to 25% with anti-malarials (Bedu-Addo and Bates, 2002).

2.4.13 Malaria and cancer

Splenic lymphoma with villious lymphocytes (SLVL) is a type of B cell non-Hodgkin's lymphoma found in West Africa with striking similarities to HMS (a reported prevalence of 2%). As opposed to HMS, SLVL will not respond to anti-malarial treatment and is identical both demographically and clinically to HMS. Just like HMS it is associated with splenomegaly, large B cell proliferation and circulating villous lymphocytes (>30%). Whereas lymphocyte proliferation is polyclonal in HMS, SLVL arises predominantly from single cell clones of cells (Wallacel *et al.*, 1998). Although in the malaria endemic regions of Ghana HMS and B-lymphoproliferative disorders were identified as the most common disorders associated with massive splenomegaly, it is worth noting that 10% of the patients were diagnosed with tropical splenic lymphoma (Bedu-Addo and Bates, 2002).

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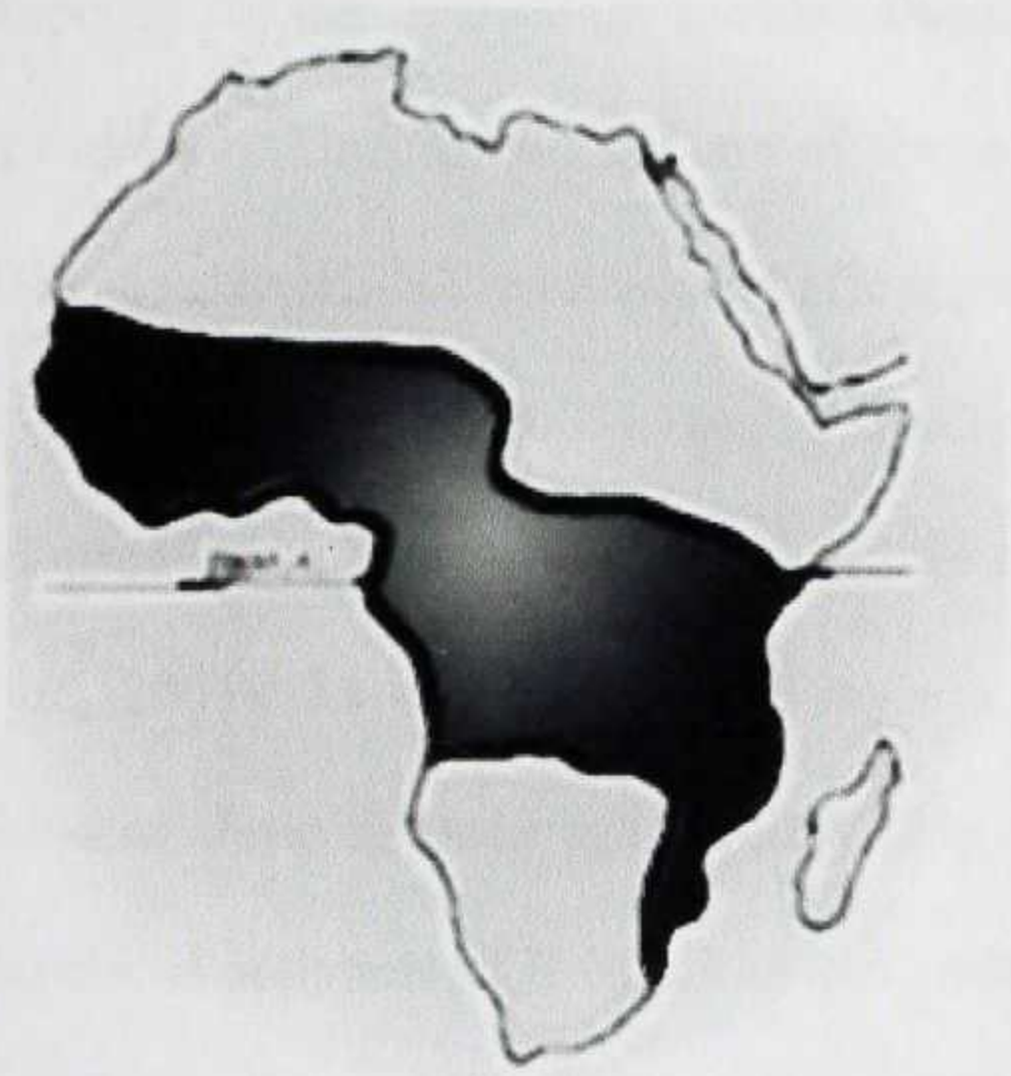
2.4.14 Malaria and BL

There is a strong geographic and climatic correlation between endemic Burkitt's lymphoma (eBL) and malaria infection (Burkitt, 1962; Owusu *et al.*, 2010). BL is mostly found in the lymphoma belt of sub-Saharan Africa (Wright, 1967) which also coincides with the hyperendemic and holoendemic malaria region of Africa (Wright, 1971). An analysis of the relative number of eBL cases in locations across the globe and the percentage of malaria caused by *Plasmodium falciparum* in those areas reveals that in Africa where eBL cases are highest, about 96% of malaria is caused by *Plasmodium falciparum.* In South America where the eBL cases are lowest, about 30% of malaria is caused by *Plasmodium falciparum.* Strikingly, in Papua New Guinea where the relative numbers of eBL cases is considered intermediate between Africa and Asia, the percentage of malaria caused by *Plasmodium falciparum* stands at about 60%. Simply put, where there is high prevalence of eBL, there is a high prevalence of malaria caused by *Plasmodium falciparum*. Furthermore, a chloroquine treatment program in Tanzania carried out for about 6 years seemed to have lowered the number of Burkitt's cases; a drop in Burkitt's prevalence from 4 in 100,000 to 1 in 100,000 (Geser *et al.,* 1989). Also interesting are the following pointers implicating malaria infection in eBL pathogenesis: there is a close correlation between age incidence of eBL and the age of acquiring maximum levels of anti-malarial immunoglobulin; residence in urban areas, where malaria transmission is lower, is associated with reduced risk for eBL; and there is an older age of onset in patients who have migrated from low malaria to high malaria zones (Geser *et al.,* 1989). The sickle cell trait also lowers the incidence of eBL (Pike *et al.,* 1970). Osmond *et al.,* (1990) have also reported that malaria produces a prolonged increase in the level of proliferative activity and cell production by early B lymphocyte

precursor cells in the bone marrow of mice.

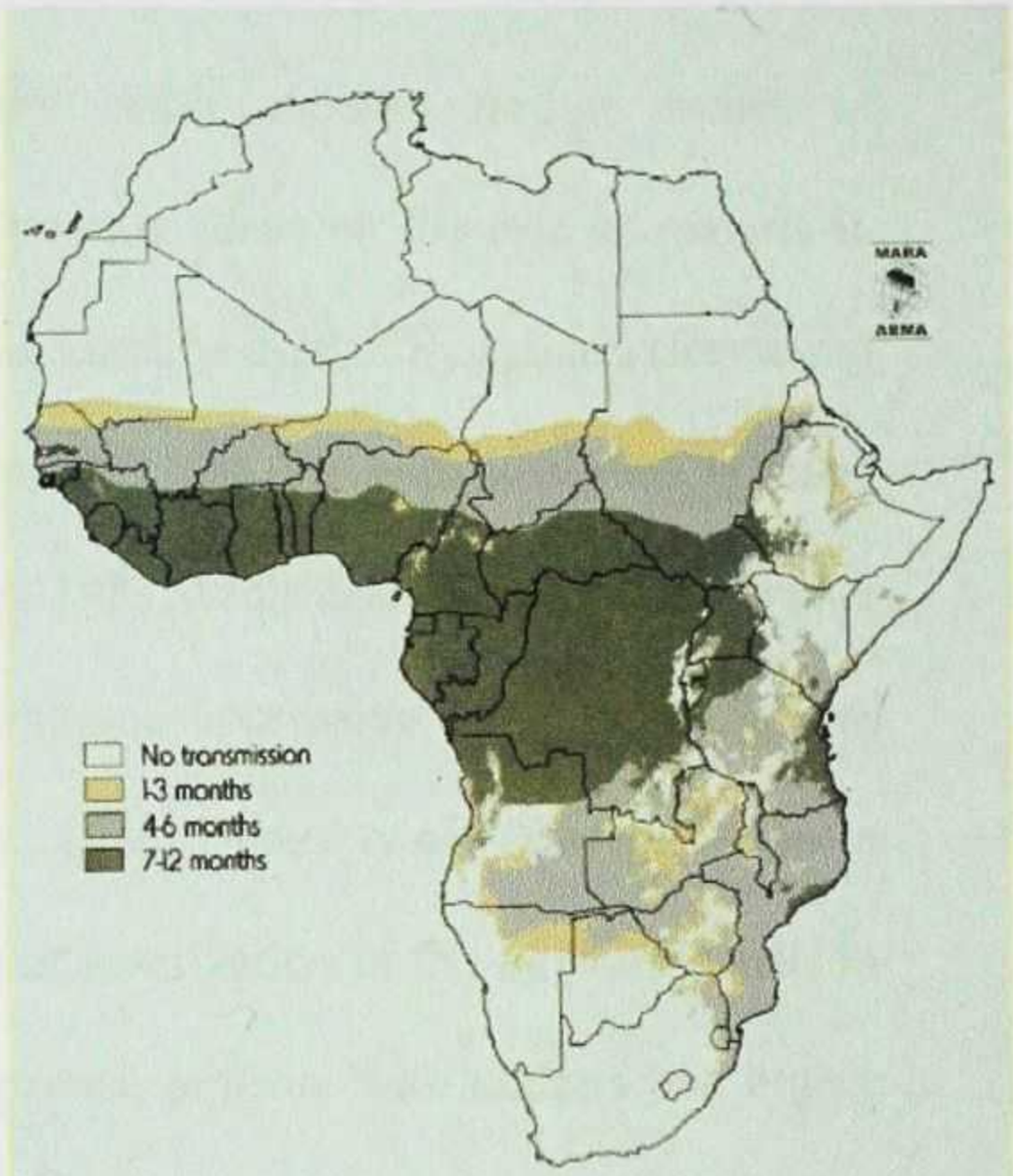
Recently Emmanuel *et al.,* (2011) have shown that there is a strong correlation between the age of eBL patients and the multiplicity of *Plasmodium falciparum* parasites (mixed genotypes). In other words, infection with multiple strains/variants of *Plasmodium* and the consequent immune response it elicits might be a predisposing factor to the development of endemic BL. This might well explain why few eBL cases are recorded among children between 0 to 2 years (Nkrumah and Perkins, 1976; Owusu *et al*., 2010; Arthur *et al*., 2011), although this age group has a high parasite burden, as one must over time acquire a complexity of parasite strains rather than have a heightened burden of parasite per se. Futagbi *et al*., (2007) have also reported that children with eBL showed similar changes in lymphocyte composition just like children with *Plasmodium falciparum* malaria. eBL patients showed a marked reduction in the proportion of CD3+ cells compared to healthy controls and the observed difference was mainly attributed to the perturbation among TCR-γδ+ cells. Thus a link between the pathogenesis of *Plasmodium falciparum* malaria and eBL is suggested. Wedderburn (1970) has described an intriguing work on the effect of concurrent malarial infection with *Plasmodium berghei yeolii* (Pby) on the development of Maloney leukaemogenic virus (MLV) induced lymphoma in BALB/C mice. She showed that with MLV infection, 9% of mice developed lymphoma whereas none of the mice developed lymphoma with Pby. However, when Pby and MLV were introduced into the mice within 5 minutes of each other 83% developed lymphoma, 46% if introduction of MLV was 10 days later.

Lymphoma Belt



Magrath (2008). The newletter of the international network for cancer treatment and research. Volume 8, Number 2, Special Issue: Burkitt lymphoma.

Malaria Belt



Shiff (2006). Malaria epidemiology lecture, John Hopkins Bloomberg School of public health

Figure 5: Co-incidence of lymphoma/ BL belt and the malaria belt of Africa

2.4.15 The role of malaria in BL pathogenesis

Without a doubt, EBV positive Burkitt's lymphoma is quite rare outside the boundaries of Africa and therefore EBV-positive c-Myc-translocation-positive B cells must of a necessity be either rare or efficiently eliminated. It is imperative therefore that there is a dramatic increase in these events in high EBV-positive Burkitt's lymphoma areas. The most likely candidate that will increase the probability of genetic lesion in an EBV positive cell in the malaria endemic regions is malaria infection. In the malaria endemic belt of Africa, children with the highest antibody responses to EBV are the ones most

likely to develop BL (de-The *et al.,* 1978); could this be because the B cell compartment gets so stimulated such that EBV positive memory B cells are forced to re-visit lymphoid tissue including tonsils where they could undergo further rounds of proliferation. Unsurprisingly, one of the most popular ideas on the role of malaria in EBV positive BL is the immense buffeting of the immune system by malaria (Reviewed by Rochford *et al.,* 2005), and secondly how this intense and sustained immune buffeting might increase EBV load (Moss *et al.,* 1983; Whittle *et al.,* 1990; Moormann *et al.,* 2005). As earlier discussed malaria is immunosuppressive for T cell responses including those directed against EBV (Ho *et al.,* 1986; Donati *et al.,* 2004), it is also a polyclonal activator of B cells and can cause lytic reactivation of the virus in B cells in culture (Donati *et al.,* 2004). As might be expected, patients with malaria had higher levels of EBV infection compared to healthy patients (Moormann *et al.,* 2005). The touting of increased EBV viral load as the major malaria associated risk factor for EBV positive BL could be put to a rigorous test in the face of the fact that allograft patients who are highly immunosupressed also carry high loads of EBV (Babcock *et al.,* 1999) yet when these patients occasionally succumb to lymphoma, it is immunoblastic lymphoma as opposed to BL (Thorley-Lawson and Allday, 2008). Immunoblastic lymphoma's from these highly immunosuppressed patients express the growth program (Hopwood and Crawford, 2000), but not the EBNA1 only program expressed in BL (EBV programs will be discussed in detail latter on). It gets even more interesting to note that although AIDS patients are immunosupressed, AIDS-BL is inversely correlated with immunological status, since, as immunesupression increases/ intensifies, EBV loads increase but the risk shifts from BL to lymphoblastic lymphoma (Bigger *et al.,* 2007). By this, immunosupression and elevated EBV loads together should rather be

predisposing factors to lymphoblastic lymphoma. AIDS-BL offers a beautiful clue as to what additional cues set off BL. It has been speculated (Reviewed by Thorley-Lawson and Allday, 2008) that chronic B cell activation occurs during HIV infection, and this is associated with consistent opportunistic infections, strikingly similar to what happens during malaria. In malaria holoendemic areas, children are chronically infected with *Plasmodium falciparum* in such a way that the parasite is present in about 90% of children under the age of 5 years (Bloland *et al.*, 1999).

With the arguments so far adduced the plausible conjecture for how EBV and malaria infection interact or conspire to cause eBL could include; chronic B cell stimulation by malaria provoking a prolonged or atypical AID expression leading to a high probability of the occurrence of c-Myc translocation. Concurrently, immunosupression/ dysfunction caused by malaria permitting a higher number of EBV infected cells through the GC en-route to the memory pool, increasing the probability of a c-Myc translocation within an EBV infected cell. EBV proteins EBNA3A and EBNA3C downregulate the pro-apoptotic BIM (BCL2 interacting mediator of cell death), which allows the tolerance of c-Myc translocations which otherwise would cause apoptosis (Thorley-Lawson and Allday, 2008).

2.5 EBV INFECTION

EBV is a gamma herpes virus with the capacity to persist indefinitely in greater than 90% of human adults. Primary infection generally occurs at an early age (Reviewed by Thorley-Lawson and Allday, 2008), and is largely assymptomatic (Joncas *et al.*, 1974). Infectious mononucleosis may develop if infection occurs during adolescence or later

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(Niedermann *et al.,* 1968). In culture, EBV is a very potent B cell transforming virus (Henle *et al.,* 1967) activating and driving the infected B cells to become a continuously proliferating and latently infected line (lymphoblastic cell line) in a matter of days. EBV predominantly infects B cells but sometimes infects other types of cells especially epithelial cells. *In vivo*, EBV infected cells express four transcription programs, defined based on gene expression patterns, which are dependent on the location and differentiation stage of the infected B cell. Lymphoblastic cell lines express nine viral proteins these include six EBV nuclear antigens (EBNA) namely EBNA 1-6, and three latent membrane proteins (LMP) namely LMP1, LMP2A and LMP2B (Thorley-Lawson, 2001a), which are all under the control of EBV encoded nuclear antigen-2 (EBNA2). There are also a large number of non-encoding RNAs including microRNAs. EBNA2 is a viral transcription factor that does not bind directly to DNA but gets tethered to EBNA2 responsive promoter elements through its interaction with cellular RBP-Jk (recombination signal binding protein J kappa) also known as CBF1, PU.1 and AP2 which mediate interaction with other promoter regions (Grossman *et al.,* 1994; Hsieh and Hayward, 1995; Robertson, 2005). EBNA2 therefore acts as a surrogate for the notch intracellular domain (IC) in the notch signaling system (Hofelmayr *et al.,* 2001). A newly infected resting B cell can be driven by all the aforementioned EBV factors to resemble an antigen activated B lymphoblast (Thorley-Lawson and Mann, 1985) and this is called the growth transcription program (Thorley-Lawson, 2001b; Thorley Lawson and Gross, 2004) or latency III. In the default program also referred to as latency II the only protein encoding genes expressed are EBNA1, LMP1 and LMP2A (Frahraeus *et al.,* 1988; Brooks *et al.,* 1992; Thorley Lawson, 2001). In latency 0 there is no protein encoding gene expression whereas in the EBNA1 only program only EBNA1

is expressed and this is typical of dividing memory B cells (Hochberg *et al.,* 2004; Thorley Lawson, 2001). Transcripts of LMP2A are also often detected in these cells (Thorley-Lawson, 2001b). It is worthy of note that the aforementioned programs do not produce infectious virus as this is the preserve of the lytic program. The gene expression profiles have unique functions in the infected B cells; the growth transcription program activates resting B cells allowing them to become proliferating B blasts; the default program provides the requisite signals for infected lymphoblasts to differentiate into memory as well as maintaining the persistently infected memory B cells; the EBNA1 only program allows the replication of the EBV genome when memory B cells divide; the latency program allows a non-pathogenic persistence of the virus in the recirculating memory B cells and makes them "invisible" to the immune system (Thorley Lawson, 2001).

2.5.1 EBV persistence in B cells

In antigen-driven T dependent B cell responses, a resting B cell in the follicle of a lymph node interacts with cognate antigen, becomes activated and proliferates extensively forming a germinal center (GC), B cells in the GC undergo somatic hypermutation (SHM) (a process which, when combined with selection through competition for antigen, fine-tunes immunoglobulin's affinity for antigen by introducing mutations into the antibody combining site). B cells in the GC also undergo class switch recombination (CSR) (a process by which immunoglobulins change their isotype). As mentioned earlier both SHM and CSR are mediated by activation induced cytidine deaminase (AID) an RNA/ DNA editing enzyme. High affinity antibody producing cells are selected whereas all other cells (that are self reactive or have low affinity) undergo apoptotic cell death.

Survival of cells in the GC is dependent on help signals from antigen specific T-helper (T_H) cells as well as the ability to receive signals, through the B cell antigen receptor, from antigen carried on specialized non-hematopoetic stromal cells which are found in the follicles or GC (these are called follicular dendritic cells) (Liu and Arpin, 1997; Maclennan, 1994; Liu *et al.,* 1989).

In parallel with the normal B cell differentiation route, EBV drives B cell through the GC and into the memory compartment where it persists (Babcock and Thorley-Lawson 2000; Thorley Lawson, 2001; Laichalk *et al.,* 2002; Thorley Lawson and Gross, 2004). The source of infectious EBV is saliva, whereby EBV is transmitted from an infected person to other individuals (Hoagland, 1955). Virus is produced continuously by B cells and epithelial cells undergoing lytic replication in the tonsils. The follicular mantle (an anatomical structure immediately beneath the tonsillar epithelium which is formed when proliferating GCs cells push out naïve B cells from the follicles) is where infectious virus is produced- needless to say, this is where a large concentration of B cells reside. In the tonsils of healthy carriers, only naïve B cells express the growth transcription program which is charactristic of directly infected cells (Babcock and Thorley-Lawson 2000; Joseph *et al.,* 2000). In the absence of a good anatomical argument and considering the following facts; that the lymphoepithelium is more like a marginal zone and does not contain naive B cells but rather the naive B cells are in the mantle zone below the lymphoepithelium, the best idea of how naive B cells get infected by EBV is as follows. Naive B cells get infected as they transverse the lymphoepithelium on their way ~~from~~ from the high endothelial venules (HEV), to the mantle zone. This fits with the finding that there are no infected cells in the mantle zone (or naive B cell zone)

(Roughan *et al.*, 2010) since a naive B cell that was infected en route would migrate directly to the GC (Thorley-Lawson, D.A., personal communication).

EBNA gene transcripts initiate from one of two promoters namely, Cp which is located in a unique region upstream of the BamH1 W repeat region or IR1 repeats of the EBV genome (Bodescot *et al.*, 1987; Woisetschlaeger *et al.*, 1989). The Wp promoter is encoded within the major multiple repeats (IR1) of EBV and is present in multiple copies (Sample *et al.*, 1986; Woisetschlaeger *et al.*, 1989). It can be gleaned from cell lines that exclusively utilize Cp and Wp to drive EBNA gene transcription (Woisetschlaeger *et al.*, 1989), that both Cp and Wp promoters are capable of driving the expression of all 6 EBNA genes. The Cp promoter is tightly autoregulated by the EBNA gene products. EBNA1 and EBNA2 upregulate Cp-initaited transcription (Sung *et al.*, 1991; Woisetschlaeger *et al.*, 1991; Jin and Speck, 1992; Rooney *et al.*, 1992; Schlager *et al.*, 1996; Puglielli *et al.*, 1996) and EBNA3 family interfere with EBNA2–mediated transactivation through centromere promoter factor 1 (CBF1) (Marshall and Sample, 1995; Robertson *et al.*, 1995a; Krauer *et al.*, 1996). Wp activity does not appear to be dependent on the Cp EBNA2 enhancer (Yoo *et al.*, 1997; Yoo and Speck, 2000). The existence of lymphoblastic cell lines (LCLs) with deleted Cp demonstrates that Cp is not required for immortalization of B cells *in vitro* (Woisetschlaeger *et al.*, 1990; Yandava and Speck, 1992). It therefore appears that *in vitro*, Wp can fully compensate for the transcriptional function of Cp. It seems that Wp is active during the initial stages of B cell infection, followed by upregulation of transcription from Cp at later times (Woisetschlaeger *et al.*, 1990; Woisetschlaeger *et al.*, 1991; Alfieri *et al.*, 1991). It is possible that Wp has evolved to be responsive to the transcriptional setting in quiescent,

resting B cells whereas Cp is autoregulated by viral latency gene products. DNA methylation of Wp regulatory sequences has been suggested as a mechanism by which the switch from Wp to Cp-driven EBNA gene transcription is regulated (Tierney *et al.*, 2000b). It was observed during the characterization of EBNA1 gene transcription in BL tumor cell lines (which express the EBNA1 only program) that Cp and Wp-initiated transcripts were not detectable (Sample *et al.*, 1991; Schaefer *et al.*, 1991). The Qp promoter has been identified as the promoter from which EBNA1 expression is initiated in these BL tumor cell lines (Schaefer *et al.*, 1995b; Tsai *et al.*, 1995; Nonkwelo *et al.*, 1996). Cp and Wp are hypermythylated in tumor cell lines whereas the region around Qp is not methylated (Schaefer *et al.*, 1991; Jansson *et al.*, 1992; Robertson *et al.*, 1995b; Robertson and Ambinder, 1997a; Schaefer *et al.*, 1997b; Paulson and Speck, 1999; Paulson *et al.*, 2002). It has further being shown that in the long term latency reservoir in seropositive individuals, Cp and Wp are methylated whereas Qp is protected from methylation (Paulson and Speck, 1999). EBNA1 expression in the latency II (default program) and latency I (EBNA1 only) programs is said to be driven by the Qp promoter (Robertson, 2005).

In acute infection, EBV enters the tonsillar crypts (invaginations of the tonsillar epithelium) and crosses the thin layer of epithelium which lies over the bed of lymphocytes beneath to infect naïve B cells. These B cells express the growth transcription program driven by EBNA2 to become B blasts (Thorley-Lawson, 2001b; Thorley-Lawson and Gross, 2004; Thorley-Lawson and Allday 2008). EBNA2 the master transcription factor is the first viral protein to be expressed upon infection employing the Wp promoter (Young and Rickenson, 2004; Thompson and Kurzrock,

2004). Subsequently it transactivates the Cp promoter effecting a switch from Wp to Cp driven transcription (Woisetschlaeger *et al.*, 1991). A switch to Cp turns on the growth program. It is worthy of note that EBNA2 turns on all the other viral latent proteins in the growth transcription program as well as a range of cellular activation associated genes including the proto-oncogene c-Myc (Wang *et al.*, 1987; Wang *et al.*, 1990; Kaiser *et al.*, 1999). Proliferation in part is driven by EBNA2's activation of c-Myc (Reviewed by Thorley-Lawson, 2001b). EBNA2 must interact with RBPJk (or CBF1), a recombination signal binding protein (Ling *et al.*, 1994) in order to bind to and regulate Cp and other genes. Activation of Cp results in the expression of all the EBNA proteins including EBNA3A and EBNA3C. According to Anderton *et al.*, (2008), EBNA3A and EBNA3C interact with and repress BCL2 like protein 11 (BCL2L11) which is also called BIM. Repression of the pro-apoptotic BIM may therefore allow B cells to tolerate activated c-Myc. Since BIM repression is epigenetic, EBNA3A and EBNA3C will autoregulate cell growth and allow B cells to differentiate into germinal center B cells with repressed BIM even after EBNA3A and EBNA3C are turned off (Thorley-Lawson and Allday, 2008). For the transition to GC to occur it is imperative that EBNA2 is turned off since it blocks differentiation (Polack *et al.*, 1996). EBNA3A and EBNA3C autoregulate cell growth by associating with RBP-Jk (CBF1) thereby displacing EBNA2 consequently repressing EBNA2 mediated transactivation and leading to growth arrest (Thorley-Lawson and Allday, 2008). Concurrently, EBNA3A and EBNA3C recruit histone deacetylases (HDACs) and CtBP complexes resulting in the inactivation of Cp and blocking further production of EBNA2. CtBP recruitment leads to deacetylation and methylation of histones that are assembled into chromatin in and around the Cp promoter ensuring that it is stably silenced. Consequently EBNA3A and EBNA3C

together with EBNA2 are able to control RBP-Jk activity and regulate both cellular and viral genes (Young and Murray, 2003; Thorley-Lawson and Allday, 2008). An intriguing cascade could be ignited whereby the silent chromatin state could trigger DNA methylation (Jaenisch and Bird, 2003) spreading downstream to also engulf Wp. It comes as no surprise that the region which covers Cp and Wp is epigenetically silenced and displays CpG methylation in the EBNA1 only program as seen in both EBV positive Burkitt's lymphoma (Day *et al.,* 2007; Tao and Robertson, 2003) and *in vivo* latently infected memory B cell pool (Paulson and Speck, 1999).

The newly EBV infected cell enters a follicle, switches to the expression of the default program (Thorley-Lawson and Gross, 2004) and then undergoes the GC differentiation process (Thorley-Lawson, 2001b; Thorley-Lawson and Gross, 2004; Roughan and Thorley-Lawson, 2009; Roughan *et al.,* 2010). The three viral proteins expressed in the default program are LMP1, LMP2A and EBNA1. It is unclear how LMP1 and LMP2 expression is activated in the abscense of EBNA2. It has been suggested that this might involve cytokines such as IL-4, IL-12 and IL-21 (Kis *et al.,* 2005, Kis *et al.,* 2006; Konforte *et al.,* 2008). LMP1 and LMP2A are vital since they have the necessary signaling capability to drive activated blasts through the GC and onward to differentiation into memory B cells. LMP1 and LMP2 are both multiple membrane spanning molecules (transmembrane proteins). They lack significant extracellular domains but act instead as constitutively active ligand independent receptors (Gires *et al.,* 1997; Caldwell *et al.,* 1998). LMP1 and LMP2 act as constitutively active CD40 and BCR respectively. So, with the aid of LMP1 and LMP2, EBV infected cells transit through the GC and follow the normal B cell differentiation route (Thorley-Lawson,

2001b; Roughan and Thorley-Lawson, 2009; Roughan *et al.,* 2010), turning off all latent gene expression (Babcock *et al.,* 1999) to become memory B cells then exit the tonsils into the peripheral circulation where EBV persists for life. The virus is truly invisible to the immune system in this state since the main cytotoxic T lymphocyte (CTL) targets are not expressed (Hochberg *et al.*, 2004). Memory B cells located in the tonsils express LMP1 and LMP2 (Babcock and Thorley-Lawson, 2000) as opposed to the periphery indicating that perhaps resting recirculating B cells do return to the tonsils (Thorley-Lawson, 2001b). Occasional terminal differentiation of memory B cells in the tonsils is said to be the signal that initiates EBV lytic replication (Anagnostopoulos *et al.,* 1995; Laichalk and Thorley-Lawson, 2005; Bhende *et al.,* 2007; Sun and Thorley-Lawson, 2007). This is the source of virus for either the infection of other B cells or the colonization of host saliva for onward spread to other host.

2.5.2 GC-expressed EBV proteins

2.5.2.1 LMP1

LMP1 protein has a transmembrane domain of 6 loops and its amino (N) terminal cytoplasmic portion fixes it to the membrane of the infected cells whereas its long cytoplasmic tail allows it to interact with cellular sigalling molecules. They interact with multiple members of the tumornecrosisfactorreceptor (TNFR) associated factor (TRAFs) family (Mosialos *et al.,* 1995), as well as TNFR receptor associated death domain (TRADD) (Devergne *et al.,* 1998; Luftig *et al.,* 2003; Schneider *et al.*, 2008). TRAFs act as intermediaries in the signaling by members of the TNFR family of cell surface receptors (Inoue *et al.,* 2000) which includes receptors that provide both survival and death signals (Baker and Reddy, 1996). CD40, which is an important member of this

family, is a key receptor on GC B cells and when engaged by the CD40 ligand (also called CD154), which is found on T_H cells, provides survival signals. Carboxy (C) terminal activating regions (CTARs) of LMP1 are functionally homologous to the cytoplasmic tail of human CD40 (Banchereau *et al.,* 1994; Zimber-Strobl *et al.,* 1996). Although LMP1 and CD40 do not interact with exactly the same set of molecules, the downstream signaling of both of them results in the activation of nuclear factor- κB (NF-κB) (Hammarskjold and Simurda, 1992). LMP1 and CD40 both rescue B cells from apoptosis and drive their proliferations and are indeed interchangeable (Gires *et al.,* 1997). LMP1 and CD40 interact with Janus activated kinase 3 (JAK3) as well as signal trasnducers and activators of transcription (STATs), and through the c-Jun N terminal kinase (JNK) pathway, activate the activator protein-1 (AP-1) complex (Hsing *et al.,* 1997; Hanissian and Geha, 1997; Gires *et al.,* 1999). LMP1 therefore mimics the CD40-CD40L interaction which is usually provided by T cell help (Uchida *et al.,* 1999).

2.5.2.2 LMP2A

LMP2A on the other hand is comprised of a transmembrane domain consisting of 12 membrane-spanning loops with a C terminal cytoplasmic tail (similar to LMP2B). LMP2A has an N terminal cytoplasmic tail which consists of 8 tyrosine residues. Two of these tyrosine residues form an immunoreceptor tyrosine-based activation motif (ITAM) (Longnecker, 2000). These ITAMs are also found in the alpha and beta chains of the B cell receptor (BCR). LMP2A and BCR both associate with Lyn (Miller *et al.,* 1995; Kurosaki, 1999) which is a member of the Src family of tryrosine kinases. Phosphorylation of the tyrosine residues within the ITAM by Lyn will lead to the recruitment of the Syk tyrosine kinase and then downstream BCR signaling events

including entry into lipid rafts (Kurosaki, 1999). The presence of BCR-negative B cells in the periphery of LMP2A transgenic mice suggests that LMP2A can provide survival signals to cells with a defective BCR since such cells should otherwise have been eliminated via apoptosis (Caldwell *et al.,* 1998). LMP2A therefore provides essential signals for B cell survival (Lam *et al.,* 1997; Caldwell *et al.,* 1998), which can replace the survival signal usually provided by an intact BCR (Caldwell *et al.,* 1998). In the case of the BCR, an engagement with specific antigen leads to growth promoting cascade, but in the absence of antigen however, BCR provides a tonic, non proliferative signal essential for the survival of all B cells (Lam *et al.,* 1997). Since LMP2A does not cause B cell growth (Miller *et al.,* 1995) the most probable function of the BCR that it mimicks is the tonic B cell survival signal (Thorley-Lawson, 2001b).

2.5.2.3 EBNA1

EBNA1, also expressed in EBV infected GC B cells, is a DNA binding phosphoprotein. EBNA1 plays a crucial role in the replication and maintenance of the viral genome (Lee *et al.,* 1999). It localizes to the nucleus of the host where it interacts with host chromosomes (Shire *et al.*, 1999). EBNA1 binds to specific sequences within the plasmid origin of replication (OriP) and these include the OriP dyad symmetry (DS) element and the family of repeats (FR) (Rawlins *et al.,* 1985). The DS element and the FR contain 20 and 4 EBNA1 binding sites respectively (Nambo *et al.,* 2007). EBNA1 specifically binds to the FR and tethers the bound EBV episome to the chromosomal sites (Marechal *et al.,* 1999; Sears *et al.,* 2003). The episome is the covalently closed circular form of the genome which persists in the nucleus of latently infected cells (Yates *et al.*, 1985). According to Kapoor *et al.,* (2005), EBNA1 achieves EBV DNA

tethering by associating with specific cellular proteins and then as reported by Sears *et al.,* (2004), it binds to AT-rich cellular DNA. One school of thought is that, through its amino (N) terminal, EBNA1 tethers the replicated viral genome to host cell metaphase chromosomes ensuring that equal numbers of genomes are acquired by each daughter cell (Reedman and Klein, 1973; Ohno *et al.,* 1977; Petti *et al.,* 1990; Mackey *et al.,* 1995; Mackey and Sugden, 1999). According to Nambo *et al.,* (2007) however, only 88% of the duplicated EBV genomes are partitioned faithfully and separate at anaphase, whiles 12% are segregated into a single daughter cell.

2.5.3 EBV and Burkitt's lymphoma

EBV has definite roles in the causation of infectious monucleosis and X-linked lymphoproliferative disorder and there is a strong association between EBV and Burkitt's lymphoma, Hodgkin's lymphoma, B cell lymphoma in immunosuppressed people, nasal natural killer-T cell lymphoma, nasopharyngeal carcinoma and leiomyosarcoma in immunosuppressed people where EBV is found in 100% of the tumor cells (Thorley-Lawson and Gross, 2004). The misconception that Burkitt's lymphoma is caused primarily by immunosupression makes it imperative to clearly delineate it from lymphoproliferative disorders arising from immunosupresssion.

EBV associated lymphoproliferative diseases are a heterogenous set of disorders (Hopwood and Crawford, 2000) that usually carry the EB virus and express the growth program (Thomas *et al.,* 1990). Prominent among these diseases is post-transplantation lymphoproliferative disease. The probable explanation of how this disease arises is that there is an uninhibited growth of EBV infected cells due to the impairment of the

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cytotoxic T cell response. The growth program clearly indicates that these blasts are unable to exit the cell cycle to become resting memory B cells. It has been postulated that post transplantation lymphoproliferative disease arises from the infection of B cell types other than naïve B cells in the Waldeyer's ring. Consequently these EBV infected B cells express the growth program but are unable to exit the cell cycle, and therefore continue to proliferate as a result of defective T cell immunity. It is interesting to note that even in immunosuppressed people only one or two EBV infected cells go on to become tumors. This adds impetus to the fact that there is strict regulation of EBV infection *in vivo* even when the immune response is compromised (Thorley-Lawson and Gross, 2004).

Of a truth, EBV was discovered in Burkitt's lymphoma some 50 years ago but it is not clear how exactly it contributes to the development of this tumor (Thorley-Lawson and Allday, 2008). The discovery of EBV in cultures of the tumor (Epstein *et al.,* 1964) revealed that perhaps EBV is implicated in BL pathogenesis. The only EBV latent protein in Burkitt's tumors is EBNA1 (Gregory *et al.,* 1990). It is a fact that the EBNA1 only program is present in non-tumor memory B cells that expresses the latency program only when they divide (Hochberg *et al.,* 2004). EBNA1 and EBV encoded RNAs expressed in the EBNA1 only program have been suggested to possess anti-apoptotic properties and may contribute to an increased risk of tumorigenesis (Komano *et al.,* 1999; Ruf *et al.,* 1999; Kennedy *et al.,* 2003).

There is strong and ample epidemiological evidence that EBV is involved in the pathogenesis of eBL. Greater than 95% of eBL tumors are EBV positive. The tumor

cells are monoclonal for EBV (so originate from a single cell) and posses multiple copies of the viral genome (Table 1). Primary EBV infection occurs early in equatorial Africa and coincides with the age of susceptibility to severe malaria. EBV loads and anti-EBV antibody titres in eBL patients are very high compared to normal controls (Rasti *et al*., 2005). Increased levels of EBV have been identified in the peripheral blood of children suffering from malaria. There was clearance of plasma viral load upon anti-malaria treatment but the saliva levels remain unchanged. It is also interesting to know that there was practically no difference in the EBV loads in the saliva of children with malaria, children without malaria as well as children with BL (Donati *et al*., 2006a). EBV infected cells expand frequently in the GC of tonsils from areas with a high incidence of EBV associated lymphoma (Araujo *et al.*, 1999). It is interesting to note that intermediate risk areas also have intermediate proportions of BL in which EBV is found, 51-91% for the Middle East, North Africa and South America.

2.5.3.1 EBV in BL pathogenesis

The proliferative activity of c-Myc (which is eventually dyregulated as a result of the c-Myc/Ig translocation) is well counterbalanced by an increased sensitivity to apoptosis (Harrington *et al.*, 1994). In c-Myc overexpression, the tumor suppressor p53 is activated predominantly through CDKN2A (also called p14ARF). CDKN2A in turn interacts with and also antagonizes a negative regulator of p53, MDM2 (Eischen *et al.*, 1999). c-Myc associated apoptosis is induced primarily by p53-mediated transactivation of pro-apoptotic genes. These include PMAIP1 (phorbol-12-myristate-13-acetate-induced protein 1; also called NOXA), BBC3 (BCL2-binding component 3; also called PUMA) as well as BAX (BCL2-associated X protein). About 30% of EBV-positive

Burkitt's lymphoma carry p53 mutations, and those with wild type p53 frequently overexpress MDM2 or loose p14ARF (Lindstrom and Wiman, 2002).

Another c-Myc-activated pathway of apoptosis exists and this involves BCL2L11 (i.e BCL2-like protein 11 also called BIM). In mature B cells, BIM is a major regulator of life and death decisions. It has been proposed that BIM initiates apoptosis by binding and inactivating pro-survival BCL2- family members including BCL2 and myeloid leukemia differentiation protein (Strasser, 2005). The loss of a single allele of BIM accelerates B cell lymphomagenesis (Egle *et al.,* 2004). Human B cell lymphomas have been found to possess mutations and methylation of the BIM locus (Tagawa *et al.,* 2005; Mestre-Escorihuela *et al.,* 2007). Inactivation of any of the several c-Myc effectors of apoptosis (p14ARF, 19ARF, p53 or BIM) can allow cell proliferation and the initiation of tumor development (Dang *et al.,* 2005; Hemann *et al.,* 2005), as it seems these independent apoptosis mechanisms must act cumulatively. Two mutants of c-Myc (P57S and T58A) commonly observed in BL tumors do not induce BIM expression but retain the ability to stimulate proliferation. Consequently overexpression leads to the activation of p53 without promoting apoptosis. Conversely BL tumors with c-Myc that can induce BIM expression generally have genetic lesions in the p14-p53 pathway (Dang *et al.,* 2005; Hemann *et al.,* 2005) and hence apoptosis is avoided.

In latently infected B cells, there is no evidence to suggest that EBV directly impairs the p53 mediated apoptosis pathway (Allday *et al.,* 1995; O'Nions *et al.,* 2006). It is known however that EBNA3A and EBNA3C functionally interact and epigenetically silence the expression of the pro-apoptotic BIM in latently infected cells (Anderton *et al.,* 2008).

This means that, as opposed to other GC B cells, EBV infected cells have the penchant to tolerate a translocated c-Myc without undergoing apoptosis en-route to the memory B cell pool. EBNA2 induces c-Myc expression so if it remains true that c-Myc is generally expressed in GC B cells (Martinez-Valdez *et al.,* 1996; Cutrona *et al.,* 1997) then there could be a scenario where there is an added augmentation of c-Myc expression in EBV infected B cells.

It is believed that chromatin modifications and DNA methylation will lead to the epigenetic suppression of BIM and therefore prevent its induction by a deregulated c-Myc even in the absence of the EBV proteins that ignited the condition (Thorley-Lawson and Allday, 2008). If the features of BL are anything to go by *viz* AID expression and GC phenotype as well as a translocation event predicted to occur in the GC environment, then EBV positive BL must arise from a GC B cell that has left the lymph node with a repressed BIM (by way of EBNA 3A and EBNA3C) and deregulated c-Myc and hence is unable to become a resting B cell. This could offer an explanation for why EBV positive BL are extra nodal (Thorley-Lawson and Allday, 2008). Latent proteins are turned off as per usual with the exception of EBNA1 Q-K (originating from the Qp promoter) because the cell fails to enter a resting state but rather keeps proliferating. This offers an explanation for why EBV positive BL express the EBNA1 only program (Thorley-Lawson and Allday, 2008) and why the SHM rate of EBV positive BL (6.1 per 100 bp) (Bhatia *et al.,* 1993) is strikingly identical to that of EBV positive memory B cells (6.0 per 100bp) (Souza *et al.,* 2005). The SHM rate in EBV negative memory B cells (5.2 per 100bp) is significantly lower than for EBV positive memory and EBV positive BL. It is plausible that since EBV or EBV associated factors

induce AID (Casola *et al.*, 2004; He *et al.*, 2003; Epeldegui *et al.*, 2007a), there is increased likelihood of a c-Myc translocation in these EBV positive B cells (Thorley-Lawson and Allday, 2008). Figure 6 summarizes the current model of Burkitt's lymphoma pathogenesis.

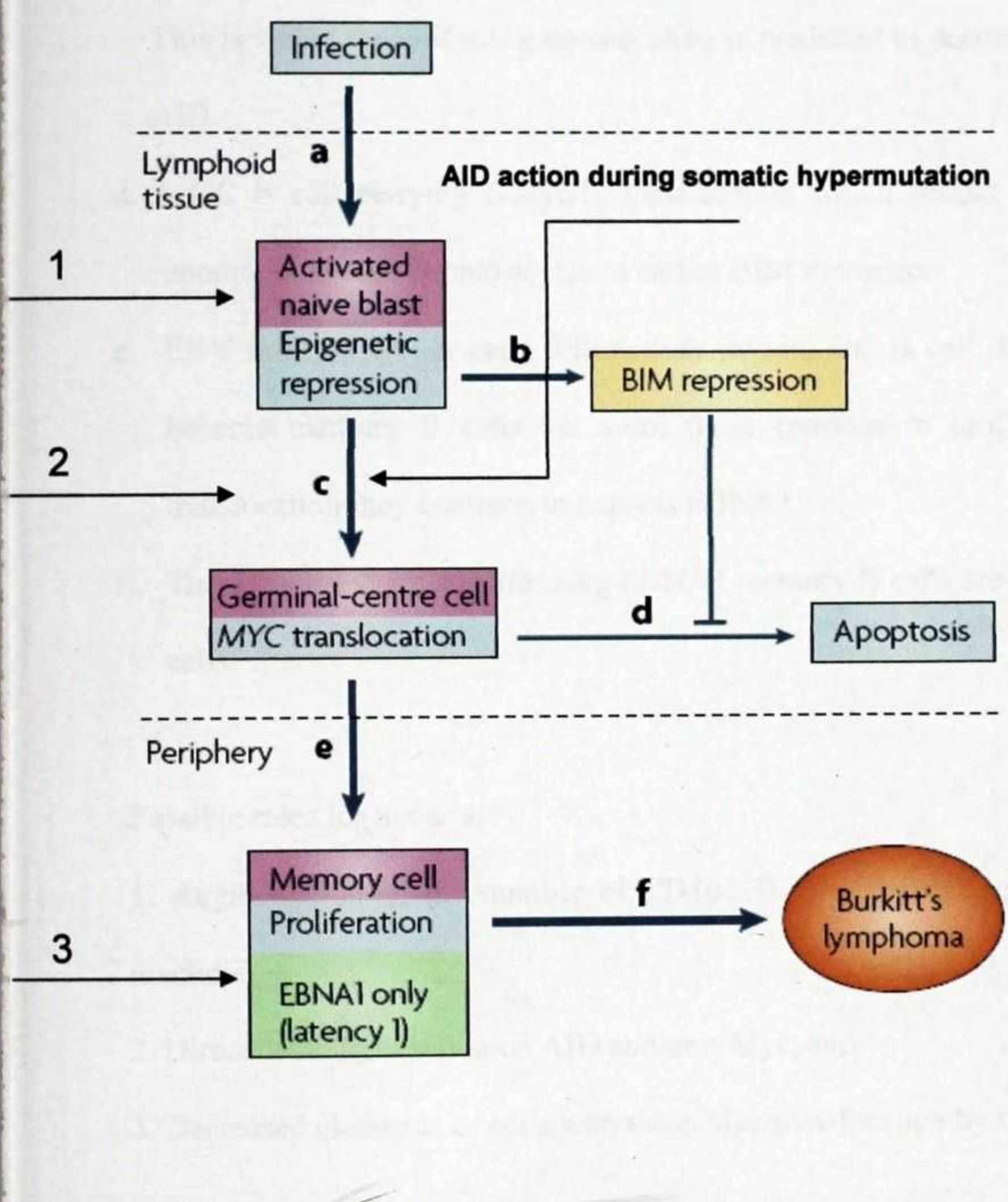

Figure 6: Current model of BL pathogenesis (Adapted from Thorley-Lawson and Allday, 2008). (See explanation below).

a. EBV infects naïve B cells under the lymphoepithelium and drives B cells to become blasts expressing the growth program

b. EBNA3A and EBNA3C repress BIM enabling apoptosis evasion

c. EBNA3A and EBNA3C represses master transcription factor EBNA2A allowing germinal center (GC) differentiation where GC B cells express the default program. This is where the c-Myc/ Ig translocation is predicted to occur under the auspices of AID.

d. A GC B cell carrying c-Myc/Ig translocation which should have otherwise gone apoptotic, evades apoptosis due to earlier BIM repression

e. EBV infected GC B cells follow their normal GC B cell differentiation route to become memory B cells but since these continue to proliferate due to c-Myc translocation they continue to express EBNA1

f. These continuously proliferating EBNA1 memory B cells are the BL tumor seeding cells

Possible roles for malaria:

1. Augmentation of the number of CD10+ B-cells undergoing the germinal centre reaction;

2. Direct or indirect action on AID and/or c-Myc, and

3. Decreased clearance of cells with the c-Myc translocation by CD8+ T-cells

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 CELLS AND CELL LINES

The EBV-positive lymphoblastoid cell line IB4 (gift of Elliott Kieff) and Namalwa Burkitt's lymphoma cell line were used as positive controls for DNA PCR of the W-repeat region of the EBV genomes. IB4, Namalwa and the Burkitt's lymphoma cell line Raji were used in TaqMan reverse transcription-PCR (RT-PCR) for EBER1 (EBV encoded RNA 1). The EBV-negative cell line CB60, a mouse T-cell hybridoma cell line (gift of Miguel Stadecker) was used as a negative control in all W-PCR and EBER1 experiments. The Burkitt's lymphoma cell lines Raji, Rael, Mutu, Daudi, Akata –ve, BL2 and DG75 as well as spontaneous lymphoblastic cell lines Salina, AM (T2), SP-LCL, Angu, and SP-IM 171 were used as positive controls for c-Myc real time RT PCR. All cell lines were cultured at 37°C with 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 2 mM sodium pyruvate, 100 IU of penicillin-streptomycin, and 1 µg/ml ciprofloxacin hydrochloride (RPMI-complete).

3.2 ISOLATION OF TONSILLAR MONONUCLEAR CELLS AND PERIPHERAL BLOOD MONONUCLEAR CELLS

3.2.1 Tonsillar mononuclear cells isolation

Palatine tonsils were obtained from patients receiving tonsillectomy either at Tufts Medical Center, Boston MA, USA, or at the Komfo Anokye Teaching Hospital, Kumasi, Ghana (age range for this project was 14 years or younger). Twenty one (21) Boston tonsils were obtained from the Pathology Department of the Tufts Medical Center. A

total of twelve (12) Kumasi tonsils were used in this thesis work. The Kumasi tonsils were obtained from the EENT Clinic of the Komfo Anokye Teaching Hospital from children undergoing routine tonsillectomy (October 2008 to April 2009). These tonsils were processed at the Kumasi Center for Collaborative Research in Tropical Medicine (KCCR), Kumasi, Ghana, stored in liquid nitrogen and shipped to Tufts University School of Medicine, Boston, USA on dry ice. (Reuben Ayivor-Djanie, Biochemistry and Biotechnology Department, processed the Kumasi tonsils and they were shipped under the supervision of Prof. Karen Duca).

Tonsil tissue was cut into very small pieces in ice-cold PBSA (1x PBS + 0.5% BSA) and then minced. Supernatants were pipetted through a cell strainer into 50 ml conical tubes to avoid debris. Supernatants were centrifuged at 1,600 rpm at room temperature for 10 minutes and then aspirated. Pellets were resuspended and brought to 50 ml with PBSA. About 25 ml of cells was carefully layered onto 20 mls of Ficoll-paque plus (GE Healthcare Biosciences, Philadelphia, USA) and then spun at 2,000 rpm for 30 minutes at room temperature (with no brake). Mononuclear cells were collected from the interface (buffy coat) and cell pellet discarded. The volume of mononuclear cells was adjusted to 50 ml with PBSA (and an aliquot taken for counting); cells were then washed once by spinning at 1,500 rpm for 10 minutes. After counting, cells were frozen in fetal bovine serum (FBS) (Sigma, St. Louis, USA) plus 10% DMSO (dimethyl sulfoxide) at 1 x 10^8 cells/ ml (or lower depending on the amount of cells in total). Cells were aliquoted in cryotubes and kept on ice for about 5 minutes, stored at -80°C overnight and then transferred to liquid nitrogen for long term storage.

3.2.2 Peripheral blood mononuclear cells (PBMC) isolation

Blood samples were collected into BD vacutainer blood collection tubes (BD Biosciences, San Jose, USA) by venipuncture and diluted with 1x PBS at a ratio of 1:1. An equal volume of diluted blood was carefully layered onto 20 mls of Ficoll-paque plus (GE Healthcare Biosciences, Philadelphia, USA) in 50 ml conical tubes and then spun at 2,000 rpm for 30 minutes at room temperature (with no brake). Plasma was aspirated off, mononuclear cells were collected from the interface (buffy coat) and transferred into a new 50 ml conical tube. Volume was made up to 50 ml with PBSA and cells were spun at 1,500 rpm for 15 minutes at 4°C. Supernatant was aspirated and cells were re-suspended again in 50 ml PBSA (and an aliquot taken for counting). Cells were finally spun at 1,200 rpm for 10 minutes at 4°C, supernatants aspirated and frozen in fetal bovine serum (FBS) (Sigma, St. Louis, USA) plus 10% DMSO at 5 x 10^7 cells/ ml, in cryotubes. Cells were aliquoted in cryotubes and kept on ice for about 5 minutes, stored at -80°C overnight and then in transferred to liquid nitrogen for long term storage.

3.2.3 Purification of PBMC B cell and tonsil B cell populations

Tonsil mononuclear cells or PBMCs were thawed in FBS/ RPMI, and spun down at 1,500 rpm for 5 minutes. B cells were either first purified using StemSep according to manufacturer's instruction, or cells were re-suspended in 0.5% BSA in 1x PBS (PBSA) i.e staining buffer. Cells were resuspended at 5x10^6 cells/ 100 µl PBSA either directly into FACs tubes or 15 ml tubes for staining. A list of antibody dilutions has been provided in Appendix A.

3.2.4 Extracellular staining

For extracellular staining only, the appropriate concentration of fluorochrome conjugated antibody was added to cells in appropriate tubes and incubated for 15 minutes at room temperature in the dark, after thorough mixing. Cells were washed once with PBSA, vortexed, and spun down at 1,500 rpm for 5 minutes. Finally cells were re-suspended in 300 µl PBSA and stored at 4°C until FACs.

3.2.5 Extracellular and intracellular staining

Tonsil cells were spun down as per usual. Cells were washed in Dulbecco's PBS$^-$ (without calcium or magnesium), and fixed either with 4% formaldehyde or with BD Cytofix/Cytoperm fixation and permeabilization solution (BD Biosciences, San Jose, USA) and then incubated for 20 minutes at room temperature. Cells were then washed twice either with 1x BD wash/perm buffer (BD Biosciences, San Jose, USA) or with 0.04% saponin based wash buffer and spun down at 1,500 rpm for 5 minutes. Permeabilization buffer (0.5% saponin) was used to re-suspend cells at $5x10^6$ cells/ 100 µl. Two microliters (2 µl) of 2% normal human serum (Thermo Fisher Scientific Rockford, USA) was added in order to block against non-specific antibody binding, and cells were incubated for 30 minutes at room temperature. Primary antibodies for intracellular antigens were added (see Appendix A for dilutions) and incubated at room temperature for 30 minutes. Cells were then washed once and resupended in 0.5% saponin, 2 µl of normal human serum was again added and incubated for 30 minutes at room temperature. Fluorochrome conjugated secondary antibodies were then added at appropriate dilutions and incubated for 15 minutes in the dark. Cells were then washed once in wash buffer, and re-suspended at $5x10^6$ cells/ 100 µl PBSA. Appropriate

amounts of extracellular antibodies were then added and incubated for 15 minutes at room temperature in the dark. Cells were washed once with wash buffer, spun down at 1,500 rpm for 5 minutes and stored in 300 µl PBSA at 4°C until FACs analysis (using FACs calibur), flow cytometry (using MoFLo), or image stream analysis at Tufts University laser cytometry unit. Sorted populations were >90% purity.

3.3 LIMITING DILUTION ANALYSIS

For limiting dilution analysis the following populations were isolated: GC B cells (CD19+CD10+); tonsil memory B cells (CD10-IgD-CD19+); tonsillar naïve B cells (IgD+CD10-CD27-); and peripheral memory B cells (IgD-CD27+ after StemSep B cell purification or CD19+CD27+IgD- without StemSep). Populations were isolated by FACS and usually 10 replicates each of $2x10^5$, $1x10^5$, $5x10^4$, $2.5x10^4$, $1.25 x10^4$, $0.625x 10^4$, $0.3125x 10^4$ (higher or lower dilutions are added when there is the need) were placed in a 96 V bottom plate for subsequent EBV W-repeat DNA PCR. The plate was spun down at 1,500 rpm for 10 minutes and the supernatant aspirated. To each well was then added 20 µl of digestion mix (10X PCR buffer, 100 µl; Igepal (NP-40) 100 µl; Tween-20 100 µl; Proteinase K 50 µl of 20 mg/ml stock and 650 µl of water). The plate was sealed air-tight and incubated at 55°C overnight. This was followed by proteinase K deactivation at 95°C for 10 minutes. Ten microliters (10 µl) of water was then added to all the sample wells.

3.4 W-REPEAT EBV DNA PCR

DNA real-time PCR specific for the W-repeat sequence of the EBV genome was performed on each well. For each reaction, a master mix was prepared, containing 12.5

µl IQ Supermix (Biorad cat 170-8862), 2.5 µl of 900 nM primers and 2.5 µl of 250 nM fluorogenic probe. Five microliters (5 µl) of DNA was added to 20 µl of master mix with a final reaction volume of 25 µl. (See Appendix A for primer and probe sequences). The PCR reactions were performed on a Biorad iCycler. The protocol was as follows: Step 1 (1 cycle): 3' at 95°C; Step 2 (50 cycles): 15" at 95°C, 1' at 60°C.

3.4.1 Calculation of the level of EBV infected cells

The level of EBV infected cells was determined based on the Poisson equation $S = e^{-m}$. S represents the fraction of wells which gave negative W-PCR (EBV PCR) signals and m is the mean number of EBV positive cells/ well at a particular dilution. First, the numbers of wells/ replicates of each dilution which give positive W-PCR signals were determined and the fraction negative at each dilution was calculated. A semi-log plot of fraction negative (y-axis, log scale) versus cell number/ dilution (x-axis, linear) was made with the y-intercept at 1. Where the trend-line intersects fraction negative 0.37 is the cell number that contains, on average 1 EBV infected cell (level of EBV per 10^7 cells is then calculated). Alternatively, the formula of the trend-line can be used and solved for x when y = 0.37. The corresponding x-value for y= 0.37 (fraction negative of 0.37) is the number of cells that contain on average 1 EBV positive cell; *viz* when S= 0.37 m= 1. The error around the mean level of EBV infected cells, of multiple repeated experiments was usually <30%.

To show that limiting dilutions can give a reliable level/ frequency of EBV infected cells, a validation experiment was done. A tonsil with a known level of EBV infected cells, 10 EBV infected cells in 10^7 total cells (as prior determined with limiting dilution

analysis) was selected and the cell number that gave 2 EBV infected cells ($2x10^6$) was taken (40 µl). 100 IB4 cells (110 µl) was sorted and added to tonsil cells (total volume= 150 µl). Limiting dilutions were made from $2x10^6$ to $1x10^4$ (cells were all used up) and W-PCR was then carried out as described above.

3.4.2 Estimation of episomal copy number

EBV episome copy number was estimated in tonsil GC B cells, tonsil memory B cells, tonsil naïve B cells, peripheral blood memory B cells from IM patients and chronic EBV carriers as well as newly infected B cells in culture. Cell populations were isolated by FACS as described above and the level of infected cells estimated by limiting dilution and W-repeat DNA PCR as described above. The appropriate cell populations were subsequently sorted into a 96 well V-bottom plate at numbers predicted to give at most one infected cell per sample/ well prior to analysis. In all, three separate tonsils were analyzed for each B cell sub-population analyzed. For newly infected cells the following protocol was performed. StemSep purified B cells were infected at a density of 10^6 cells per ml by re-suspending into undiluted supernatant from the B95-8 cell line (a lymphoblstoid cell line obtained by infecting marmoset monkey peripheral blood leukocytes with EBV). The supernatant was prepared by collecting cell-free supernatants of B95-8 in culture, these were then spun down and the virus containing supernatant carefully harvested and used immediately or frozen at -80°C. The purified B cells infected with B95-8 EBV virus was then incubated for 2-3 hours at 37°C after which an equal volume of fresh medium was added, and the cells incubated overnight at 37°C. The cells were then spun down and re-suspended at 10^6 cells/ ml in pre-warmed (37°C) PBSA to which was added 2 µl of a 5 mM stock of CFSE (carboxyfluorescien diacetate,

succinimidyl ester) in DMSO per ml of cells (final concentration 10 μM CFSE). The cells were then incubated for 10 minutes at 37°C followed by addition of 2 volumes of culture media and incubation on ice for 5 minutes. The cells were then washed 4 times, once with pre-warmed media and three times with ice cold media. The cells were then harvested at various times post infection and analyzed for the number of cell divisions based on CFSE staining using the FACS. Cells that had undergone 0,1,2,3, and 4 divisions were sorted singly into the wells of a 96 well V-bottomed plate that already contained 10^4 EBV negative CB60 filler cells.

To estimate EBV episome/ genome copy number, W-repeat real time DNA PCR was performed on the samples as described above and the signal compared to that obtained from a standard curve. To generate this curve dilutions of IB4 (4 genomes/ cell) and Namalwa (2 genomes/ cell) were made by FACS sorting that produced five replicates each of 10,000, 100, 10, 5, 3, 2 and 1 cells and the cell number brought to 10^4 when necessary through addition of CB60 cells. W-repeat DNA PCR was then performed as described above. An analysis was accepted if the standard curve yielded a straight line over the entire range of cell numbers tested when the log of the genome copy number was plotted versus the Ct value from the real time PCR.

3.5 PURIFICATION OF RNA, RT-PCR AND REAL TIME RT PCR

RNA was purified by TRIzol extraction (Invitrogen, Life Technologies, Grand Island, USA) and then treated with TURBO DNase (Ambion, Life Technologies, Grand Island, USA) to eliminate DNA prior to RNA amplification (where necessary). cDNA was made from the RNA using a cDNA synthesis kit (Invitrogen iScript cDNA synthesis

kit). For the cDNA synthesis reaction, a master mix was prepared which included 4 µl of 5X iScript reaction mix, 1 µl of iScript reverse transcriptase, and 8 µl of nuclease-free water. Seven microliters (7 µl) of purified RNA was added to 13 µl of master mix. All reactions were performed on an Applied Biosystems PCR machine (Thermal cycler). The protocol was as follows: one cycle that included 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. For real time PCR a master mix was prepared, containing 12.5 µl of IQ Supermix (Bio-Rad), 2.5 µl of 900 nM primers, and 2.5 µl of 250 nM fluorogenic probe, except when Taqman pre-developed assays were being used in which case, 12.5 µl Supermix, 1.25 µl 20x primer-probe mix and 6.25 µl water. Five microliters (5 µl) of cDNA was added to 20 µl of master mix with a final reaction volume of 25 µl. All real time PCRs were performed on a Bio-Rad iCycler. The protocol was as follows: step 1, one cycle of 3 minutes at 95°C; step 2, 55 cycles of 15 seconds at 95°C and 1 minute at 60°C. All our real time PCR assays were optimized to detect down to the single cell level (See Appendix A for a list of primers and probes).

3.6 *PLASMODIUM FALCIPARUM* EXTRACT STIMULATION ASSAY

Plasmodium falciparum 3D7 extracts re-suspended in PBS (obtained by the kind courtesy of Kirk Deitsch) were sonicated on ice 3 times at 5 seconds interval x 5 and the protein concentration determined by the Bicinchoninic assay (BCA assay). Fresh tonsil mononuclear cells (MNC) were isolated as described above and re-suspended at ~$1x10^6$ cells/ ml in pre-warmed PBSA. Two microliters of 5 mM CFSE in DMSO was added per ml of cells (final concentration 10 µM CFSE). The cells were then incubated for 10 minutes at 37°C. Cells were spun down and re-suspended in 2 volumes of ice-cold

culture medium and incubated on ice for 5 minutes. Cells were then re-pelleted and re-suspended in 2 volumes ice cold culture medium (2 times). Washing was done one more time in 2 volumes of pre-warmed medium and cells were finally re-suspended in fresh pre-warmed cell culture medium (described above). A final concentration of 3 μM of CpG-2006 (Hycult Biotech, Plymouth Meeting, USA), 0.25 μg/ml CD40 ligand (eBioscience, San Diego, USA), 5 ng/ml IL-4 (eBioscience, San Diego, USA) and 10 μg/ml of crude parasite extract, were used in the tonsil mononuclear cell stimulation. Cells were harvested on days 3, 5, and 7/10, stained with CD19 and then B cells were sorted with the MoFLO. RNA was extracted, cDNA constructed and then real time RT PCR was carried out as described above.

3.6.1 Hemozoin-CpG internalization assay

Human CpG DNA with a phosphorothionate (PTO) backbone was conjugated onto synthetic hemozoin (obtained from Parisa Kalantari in the Golenboch lab). Synthetic hemozoin (sHz) was sonicated for 1 minute and vortexed for 30 seconds followed by another 1 minute sonication. Hemozoin was quickly dispensed into eppendorf tubes at 100 μg/ tube. Dilutions of CpG-DNA (from 1 μg to 20 μg) were added to 100 μg of synthetic hemozoin (sHz). The mixture was then vortexed briefly after which a quick spin was done. Rotation of the sHz-CpG DNA mix was performed overnight at room temperature (usually 2 hours at room temperature is optimal). The sHz-CpG complex was then spun down at high speed and the supernatant transferred to new eppendorf tubes. Bound and unbound DNA were determined by measuring the DNA concentration in the collected supernatant, using the Nanodrop. BL2 and SP-IM 171 cells were each plated at 1.5 ml and 2 mls (with approximately 100,000 cells per plate). 5 μg of CpG/

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100 µg Hemozoin dilution was added to the cell lines (BL2 and SP-IM 171) and incubated for 2 hours after which confocal microscopy was carried out with the help of Parisa Kalantari of the Golenboch laboratory.

3.7 DETERMINATION OF THE PRESENCE OF *PLASMODIUM FALCIPARUM* IN BLOOD AND TONSILS

3.7.1 Rapid diagnostic test

Ten microliters (10 µl) of peripheral blood, plasma and buffy coat from Ficoll centrifugation of minced tonsil tissue, was placed in the sample well/ lane of *Plasmodium falciparum* rapid diagnostic test (RDT) cassette (ACON Laboratories, Inc., San Diego, USA) and results were read after incubating for 15 minutes at room temperature. Sample(s) move by a lateral flow along the sample pane of the cassette until it reaches a region coated with antibodies specific for the *Plasmodium falciparum* antigen, histidine-rich protein 2 (HRP-2). If the sample contains *Plasmodium falciparum* proteins including the HRP-2, an immunochromogenic reaction between the coated antibody and the parasite-derived antigen would yield a color band. The result is read as positive if the sample band corresponds to a similar band at the control region. The result is read as negative if only the control band appears and is considered invalid if there is no control band. (This was done by the kind courtesy of Stephen Kusi).

3.7.2 Plasmodium microscopy

Thin and thick blood smears were prepared with 10 µl heparinized whole blood samples and stained by standard giemsa staining procedure. Thin smears were prepared by placing the 10 µl drop of blood on a clean slide (towards one end), and using the edge of

another clean slide, a smear of blood was created. The slides were allowed to dry overnight at room temperature. Thick smears were prepared by placing 10 µl of whole blood at the center of a clean slide, and the blood was evenly spread out in a circular fashion. The slides were allowed to dry at room temperature overnight. The next day, prior to giemsa staining, the thin smear was dipped into a jar of absolute methanol for 2 minutes to fix the red blood cells. This prevented the red blood cells from getting lysed during staining. Next, both the thick and the thin smears were stained with 10% giemsa stain for 10 minutes by flooding each of the slides with giemsa stain at the face containing the samples. The slides were then washed by sliding them under gentle running tap water. Finally, the slides were drained and left to dry at room temperature. Immediately after drying, the slides were examined under a light microscope with oil immersion at 1000X magnification. The "non-blood" samples (i.e. buffy coat and plasma from Ficoll centrifugation of minced tonsil tissue) were prepared and examined in a similar manner to the thin blood smears. Parasitemia was estimated for only whole blood (thin smears) by counting the number of parasites per RBCs in10 different fields. (This was done by the kind courtesy of Stephen Kusi, Biochemistry Dept. KNUST).

3.8 WESTERN BLOT

3.8.1 Isolation of nuclear and cytoplasmic extracts

Cells were spun at 1,500 rpm for 5 minutes and the supernatant was aspirated. $5x10^6$ cells were re-suspended in 100 µl TKM 10-10-1 buffer (consisting of 10 mM Tris pH 7.6, 10 mM KCl, 1 mM $MgCl_2$, and deionized water). Ten microliters of 10% Triton-X-100 was then added together with protease inhibitors (Thermo Fisher scientific, Rockford, USA), vortexed vigorously and then incubated on ice for 7 minutes. After

incubation the lysate was spun at 13,000 rpm for 16 minutes after which the supernatant which represents the cytoplasmic protein was collected into a fresh tube. Nuclei were washed with about 200 µl cold TKM 10-10-1 buffer and spun down at 1,500 rpm for 10 minutes (in the cold room). The supernatant was removed and discarded after which ~50 µl of RIPA buffer (Sigma, St. Louis, USA) with protease inhibitors was freshly added. The lysate was incubated on ice for 5 minutes after which sonication was performed to shear the DNA. Finally the lysate was spun down at 13,000 rpm for 30 minutes and the supernatant (nuclear protein extract) was transferred to a new eppendorf.

3.8.2 Protein concentration determination

Protein standards were made with 1 µg/µl BSA in duplicates of the following; 0 µl, 0.5 µl, 1 µl, 2 µl, 4 µl, 8 µl. Two microliters of each sample was assayed in 3 replicates. A 1 to 5 dilution of Bio-Rad protein assay reagent (Bio-Rad cat. number: 500-0006) was made and 1 ml was added to each of the samples and standards in cuvettes. Incubation was carried out for 10 minutes while the spectrophotometer (Hitachi U-2000, Triad Scientific, Manasquan USA) was warmed. The optical density for the standards and samples was then measured and concentrations of the samples determined. Alternatively the bicinchoninic acid (BCA) method of protein concentration determination was used. Triplicates of the following standards were made; 2,000 µg/ml, 1,000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 0 µg/ml. To 25 µl of standard or samples was added 200 µl BCA reagent mix (Thermo Fisher scientific, Rockford, USA) according to manufacturer's instruction. Protein concentrations were read using the SpectraMax Plus-microplate spectrophotometer.

3.8.3 Running gel and protein transfer

To each protein sample was added 12.5% beta mercaptoethanol and 1x SDS sample buffer (Boston Bioproducts, Ashland, USA) to achieve the same concentration. Five microliters of protein standard (Biorad, Hercules, USA) was added to designated well(s). Samples were heated at 95°C for 5 minutes and resolved on 4-20% Tris-Glycine gel 1.5 mm x 15 wells (Invitrogen, Life Technologies, Grand Island, USA) using Xcell SureLock Mini-Cell (Invitrogen, Life Technologies, Grand Island, USA). Novex Tris-glycine SDS Running buffer (Invitrogen, Life Technologies, Grand Island, USA) was used to run the gel at 160 V for 1 hour 30 minutes. Nupage antioxidant (500 µl) containing N, N-Dimethylformamide (Invitrogen Life Technologies, Grand Island, USA) was added to 200 ml running buffer. Immobilon-P polyvinylidene fluoride (PVDF) transfer membrane (Millipore, Billerica, USA) was placed in 100% methanol and then washed with distilled water after which it was allowed to equilibrate in Novex Tris-glycine transfer buffer (Invitrogen Life Technologies, Grand Island, USA) with 20% methanol for at least 2 minutes, together with filter papers and the gel. Nupage antioxidant (300 µl) was added to 300 ml transfer buffer. A semi-dry electrophoretic transfer was performed with an apparatus from CBS Scientific (San Diego, USA), model number EBU-4000. Transfer was performed at 10 V for 45 minutes.

3.8.4 Antibody exposure and membrane development

Membrane was blocked with either membrane blocking solution (Invitrogen, Life Technologies, Grand Island, USA) or 5% milk in 1x Tris buffered saline with Tween 20 (TBST) (Cell signaling, Danvers, USA) at room temperature for 1 hr. The PVDF membrane was then incubated with primary antibody in appropriate blocking buffer at

4°C overnight. The membrane was washed in TBST or Invitrogen wash solution three times, 10 minutes each and incubated with secondary antibody in 5% milk in TBST for 45 minutes at room temperature. The membrane was then washed with Invitrogen wash solution 4 times, 10 minutes each at room temperature and then enhanced chemiluminescence (ECL) was performed. SuperSignal west femto maximum sensitivity substrate (Thermo Fisher scientific, Rockford, USA) was added to the membrane and incubated at room temperature for 3 minutes with gentle rotation to ensure all parts of the membrane are in contact with the chemiluminescence reagent. The membrane was then placed in between a membrane-sized sheet protector and the excess chemiluminescence reagent was blotted with Kim wipe and then held in place in a Fisherbrand autoradiography cassette 20x25 cm (Thermo Fisher scientific, Rockford, USA). Hyblot CL autoradiography film (Denville scientific, Metuchen, USA) was then exposed to the membrane in the dark room for about 1 second to 1 minute and then developed in a Kodak X-MAT 2000 processor.

3.8.5 Stripping the membrane and re-probing

Two stripping agents were used, either 7 M guanidine HCL or Restore plus western blot stripping buffer (Thermo Fisher scientific, Rockford, USA). About 10-20 mls of 7 M guanidine HCL or restore plus western blot stripping buffer was added to the membrane with shaking for about 5 minutes – 10 minutes at room temperature. The stripping buffer was then quickly dumped and 20 ml of wash buffer was added and also dumped immediately. Finally 20 mls of wash buffer was added to the membrane with shaking for 30 minutes. This was repeated for about 2 to 3 times. The membrane was then stored in TBST at 4°C or blocking was performed for 1 hour at room temperature and the

antibody incubation and membrane development protocol followed accordingly.

3.9 ETHICAL CONSIDERATION

This study was approved by the Institutional Review Board of the Tufts Medical Center, Boston, USA and the Committee on Human Research and Ethical Publications of the School of Medical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), and Komfo Anokye Teaching Hospital (KATH), Kumasi Ghana.

3.10 STATISTICAL ANALYSIS

Chi square test was performed to compare models of EBV episome copy number distribution generated from *in vitro* EBV infected cells that had undergone particular number of cell divisions, with the EBV episome copy number distribution of tonsillar germinal center B cells. All other data including comparison of parameters between tonsils from the malaria endemic region and the non-malaria region as well as results of *Plasmodium falciparum in vitro* B cell stimulation assays are expressed as mean ± SD. Data sets were compared by the Student two-tailed, Unpaired *t* test using a software package [Graph Pad prism 4 or online Graph pad and Microsoft Excel (Microsoft office 2007)]. Significance was considered achieved when the *p* value was <0.05. Summit version 4.3 was used to analyze FACs and flow cytometry data. ImageJ was used in western blot analysis. MyiQ was used to analyze real time PCR data.

CHAPTER FOUR

4.0 RESULTS

4.1 Validation of limiting dilution method

The level or frequency of EBV infected cells in any cell population can be determined by the limiting dilution method utilizing the Poisson equation. A cursory validation of limiting dilution analysis was done using a tonsil whose estimated EBV frequency (10 EBV infected cells in 10^7 total cells), had been determined earlier using the limiting dilution method. This tonsil was spiked with IB4 to yield a total EBV frequency of 100 EBV infected cells in 10^7 total cells. Limiting dilution analysis was performed on the tonsil-IB4 cocktail by employing W-PCR. Table 2 indicates the expected and observed fraction negative of W-PCR signals at each dilution/ cell number. The expected values were generated with the Poisson equation (see methods for details).

Table 2: Expected and observed fraction negative of EBV spiking experiment

Cell number	Expected fraction negative	Observed fraction negative
200,000	0.14	0.20
100,000	0.37	0.40
50,000	0.61	0.80
25,000	0.78	1.00
10,000	0.9	0.70

With the aid of a semi-log plot consisting of cell number versus fraction negative, the

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frequency of EBV infected cells was determined. The cell number corresponding to a fraction negative of 0.37 contains, on average, one EBV infected cell (see methods). An experimental frequency of 80 EBV infected cells in 10^7 total cells was obtained. The experimental error of limiting dilution analysis is usually 30% around the mean. This makes the experimental (observed) EBV frequency (i.e 80 EBV infected cells in 10^7 total cells) and expected EBV frequency (i.e 100 EBV infected cells in 10^7 total cells) a very good fit and thus generates renewed confidence in the limiting dilution analysis method for determining the frequency of EBV infection.

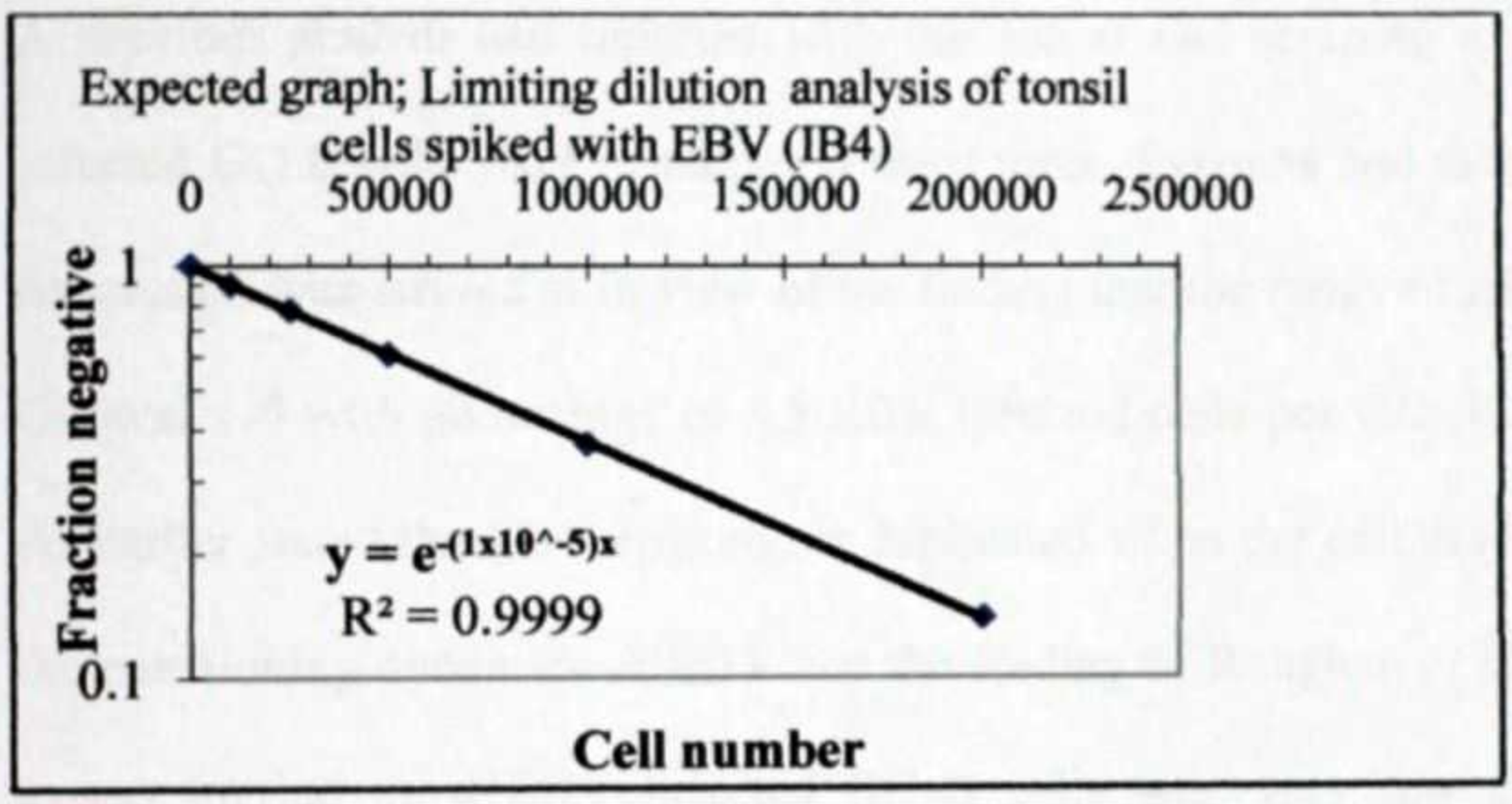


Figure 7: Limiting dilution analysis of a tonsil with a known frequency of EBV infected cells, spiked with IB4 cells. A plot of cell number vs. fraction negative yields the frequency of EBV infected cells for both expected (A) and experimental/observed (B) results.

4.2 EBV infected germinal center (GC) B cells proliferate extensively without increasing in absolute number

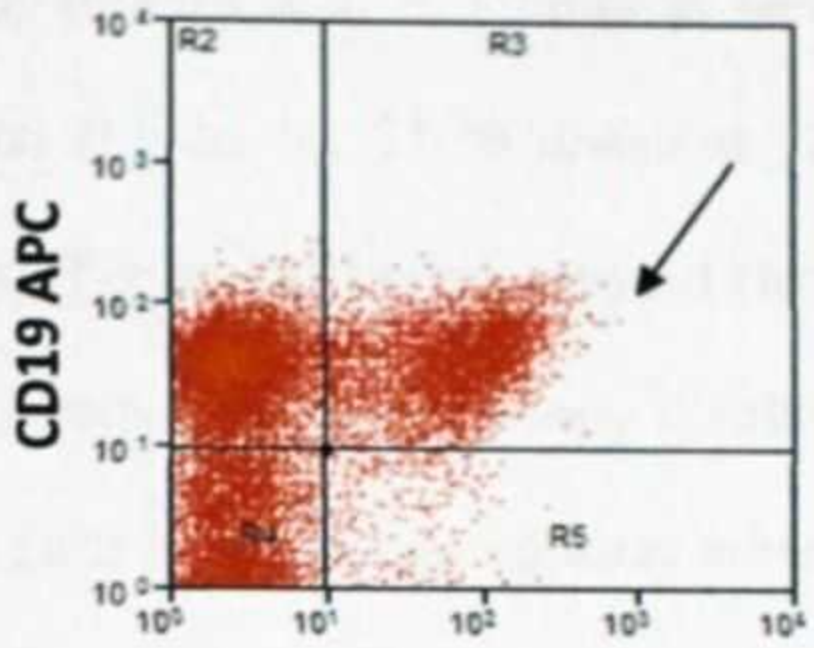
Based on previous data (Hurley and Thorley-Lawson, 1988) and Nambo *et al.,* (2007), the EBV episome (genome) is only replicated when an EBV infected cell divides, i.e only one additional episome is made per cell division. The duplicated EBV episomes are partitioned faithfully 88% of the time whereas 12% of the time both episomes are segregated into a single daughter cell (Nambo *et al.,* (2007).

A previous student had reported with the aid of GC scraping experiments that EBV infected GC B cells likely undergo at most three divisions and then leave the GC. This conclusion was arrived at in view of the finding that the range of EBV infected cells per GC was 1-9 with an average of 3.5 EBV infected cells per GC (Roughan *et al.,* 2010). As earlier stated the EBV episome is duplicated when the cell divides, so judging from the partitioning dynamics of EBV and the finding of Roughan *et al.,* (2010) one should expect to find most EBV infected GC B cells with very few EBV episome copies (mostly between 1-3) and at most 9 episome copies in very few cases if any. The aforementioned finding is very crucial to understanding precisely how EBV establishes persistence through the GC reaction, *viz*, do EBV infected GC B cells actually undergo the GC reaction which is characterized by rapid proliferation? Consequently, this result was verified by employing EBV episome copy number distribution analysis. Again, endemic Burkitt's lymphoma (eBL), is a disease characterized by the proliferation of EBV infected cells that had probably participated in the GC reaction, and secondly the c-Myc/Ig translocation occurs in these EBV infected GC B cells. These aforementioned

reasons make it even more imperative that the EBV dynamics in GC B cells be well understood.

The episome copy number distribution in latently infected tonsillar GC B-cells from three patients was analysed after the level of EBV infected cells had been determined (Figure 8A and 8B). The resulting distribution of EBV episome copy per cell was not uniform with about one third of the cells containing only one episome and the remainder containing a wide range of episome copy numbers (mean 5.5 $\pm$ 5.0 and range of 1-20 episome copies). It appears therefore that latently infected tonsillar GC B cells are a mixture of cells that have undergone very few divisions and cells that have undergone extensive divisions (Figure 8C). EBV episome copy numbers as high as 20 could only be achieved by extensive divisions as per EBV episome duplication dynamics. To assess and clarify this situation freshly isolated peripheral blood B lymphocytes were infected with EBV and their proliferation was tracked with CFSE proliferation dye and flow cytometry.

A



B

Frequency of EBV in Germinal center B cells/ 10^7 GC B cells					
	Number of experiments				
Tonsil Code	1	2	3	4	Mean
GC1	10	10	7	10	9
GC2	100	90	85	85	90
GC3	200	160	170	155	171

C

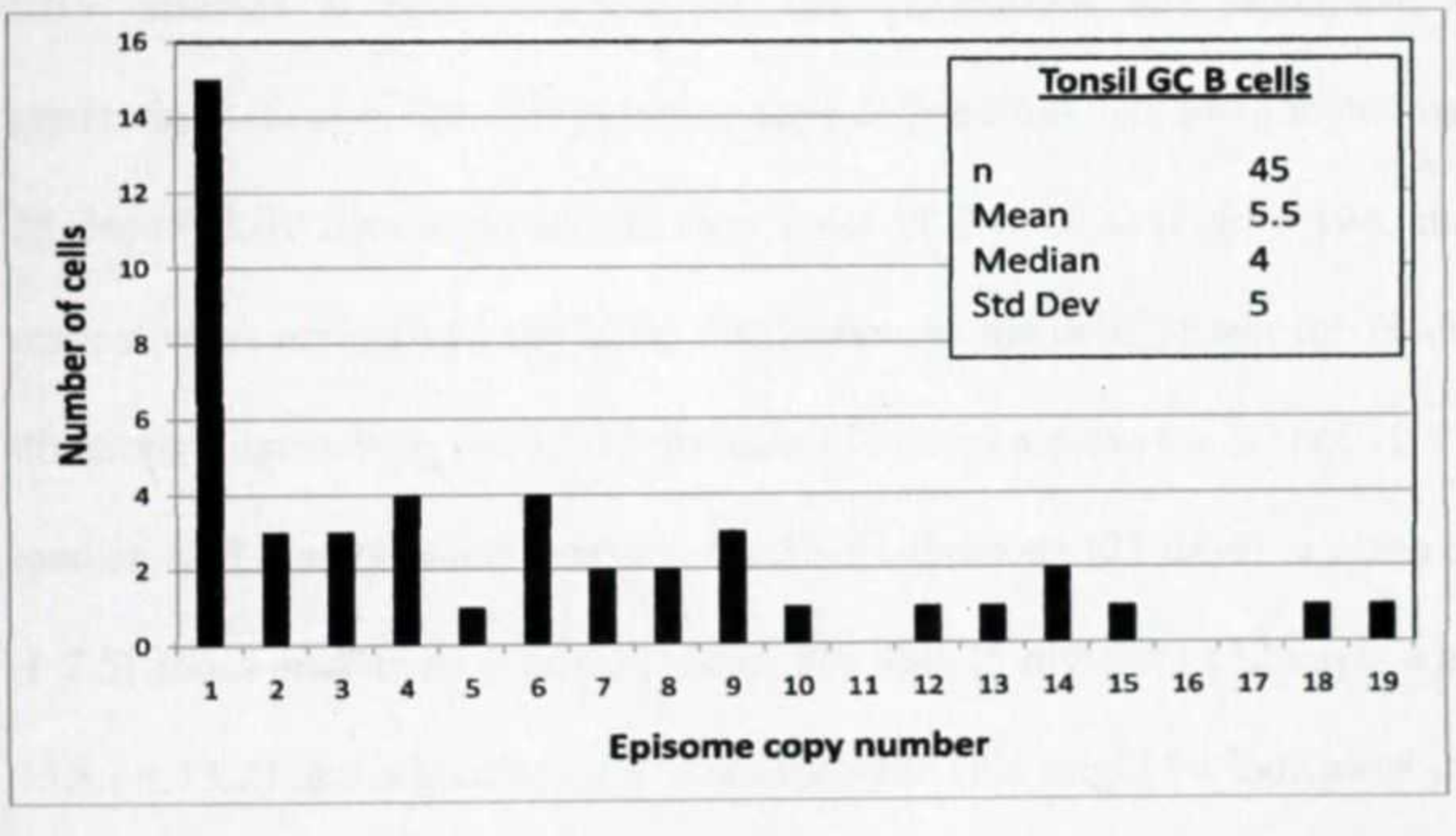


Figure 8: Germinal centre B cells contain a broad distribution of EBV episome copies. Tonsillar germinal center B cells (CD10+CD19+) were sorted by flow cytometry (A). By limiting dilution analysis the frequency of EBV infected germinal centre (GC) B cells was measured (B). GC B cells were subsequently sorted at numbers predicted to give one infected cell per well/sample. Using a standard curve constructed from the IB4 cell line (4 EBV genome copies/cell) and the Namalwa cell line (2 EBV genome copies/cell) the episome copy number/ GC B-cell was determined (C).

Cells that had only divided 0, 1, 2, 3 times as well as cells that had undergone 20-25 divisions (20 days) (Figure 9), 25-30 divisions (25 days) (Figure 10A), and ~30-35 divisions (32 days) (Figure 10B) were analysed (for the purposes of this work) for EBV episome copy numbers. The episome copy distribution after 1 division indicated that about 93% of the cells had only one episome whereas about 7% had two episomes [in another experiment (Appendix B) the percentages of cells with 1 and 2 episomes were 89% and 11% respectively]. This result is in congruence with previous results that only 1 episome forms per infected B cell (Hurley and Thorley-Lawson, 1988). The episome copy distribution in cells that had been grown for 20 days (thus have undergone 20-25 divisions) (Figure 9C) appear to have similar right skew in the distribution profile when compared with the distribution obtained by Nambo *et al.*, (2007). They allowed *in vitro* EBV infected B cells to grow for 100 generations and subcloned for 25 more generations (Figure 12). The episome copy distribution data of cells that were grown for 25 days (25-30 divisions) and 32 days (~30-35 divisions) (Figure 10A and Figure 10B respectively) maintained the same distribution as the cells grown for 20 days i.e. 20-25 divisions (Figure 9C). For 20-25 divisons (20 days) a mean ($\pm$ SD) of 10.1 ($\pm$ 8.7) and a median of 7 was obtained whereas for 25-30 divisions (25 days), a mean $\pm$ SD of 7.9 ($\pm$ 7.5) and a median of 5 was obtained. For ~30-35 divisions (32 days) a mean $\pm$ SD of 13.8 ($\pm$ 13.6) and a median of 8 was obtained. This might be indicative of the fact that the episome copy distribution probably reaches equilibrium after 20-25 divisions.

Results of *in vitro* infections were employed to generate two simple models. In the first case it ~~is assumed~~ that GC B cells followed the simple "three and out" model that a colleague had described earlier (Roughan *et al.*, 2010). This would mean that the tonsil

GC B cells would be a mixture of cells that had undergone 0, 1, 2 or 3 divisions. Given these assumptions a predicted distribution was derived by combining the observed episome distribution curves for cells that had undergone 0, 1, 2 or 3 divisions, giving equal weight to each group. This distribution was then normalised to the total number of cells, 45 in the observed tonsil distribution. The predicted and observed are shown in Figure 11A. From this Figure it is apparent that the simple "three and out" model does not singularly explain the episome distribution found in the tonsil GC B cells (χ^2 <0.001). In the second model (Figure 11B) the distribution from the "three and out" hypothesis was combined with the distribution of cells that had undergone many divisions giving equal weight to both distributions. Again this distribution was normalised to the total number of cells 45, in the observed tonsil distribution. The predicted and the observed distributions are shown in figure 11B. As seen in figure 11B this results approximates what was actually seen for tonsil GC cells (χ^2= 0.28). It can be concluded that the tonsil GC B cells consists of a mixture of cells most of which only undergo approximately three divisions before they die or leave, but a subset of which continue to proliferate without increasing in absolute number, i.e. they must be dying at the same rate they are proliferating.

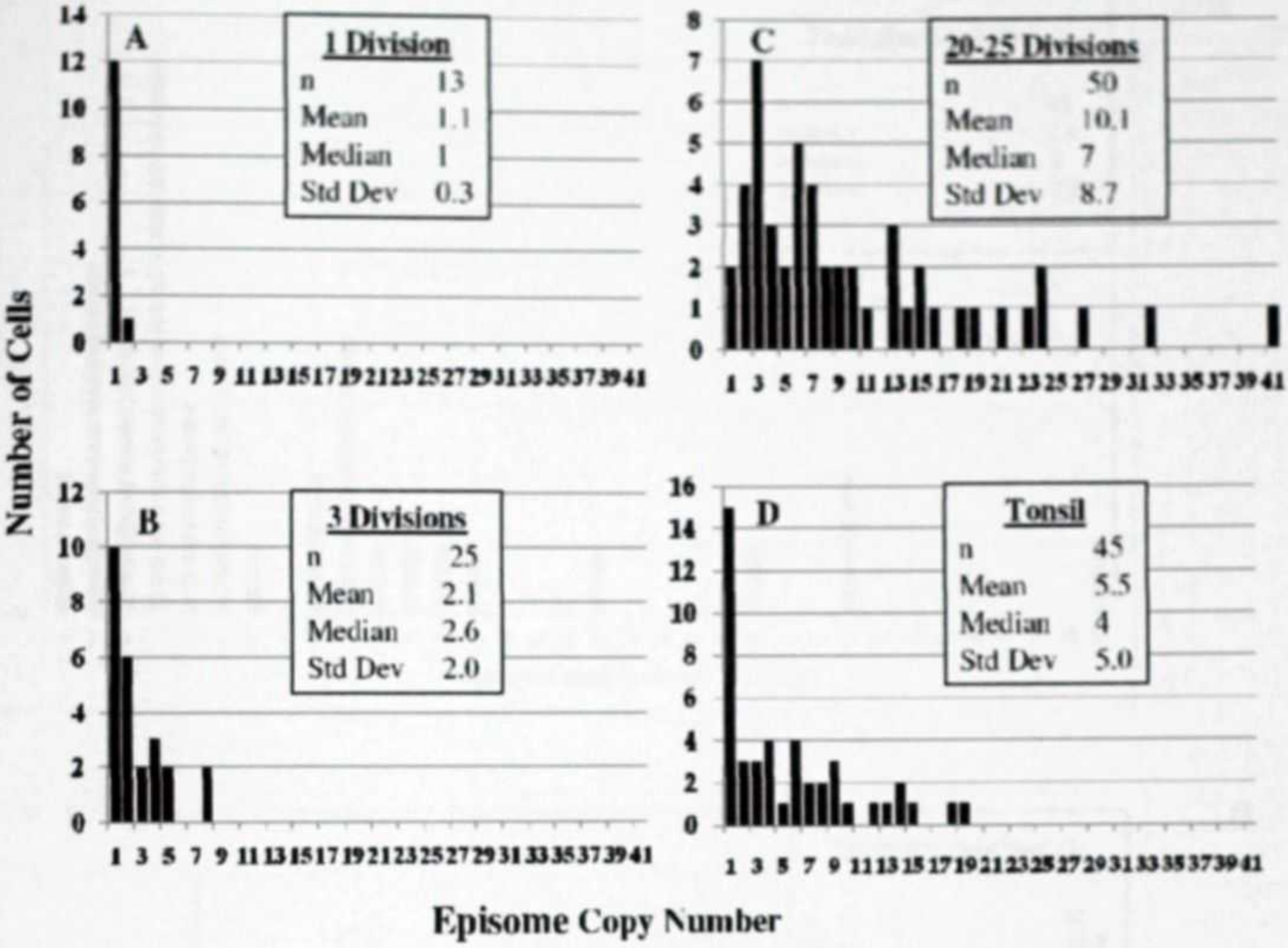
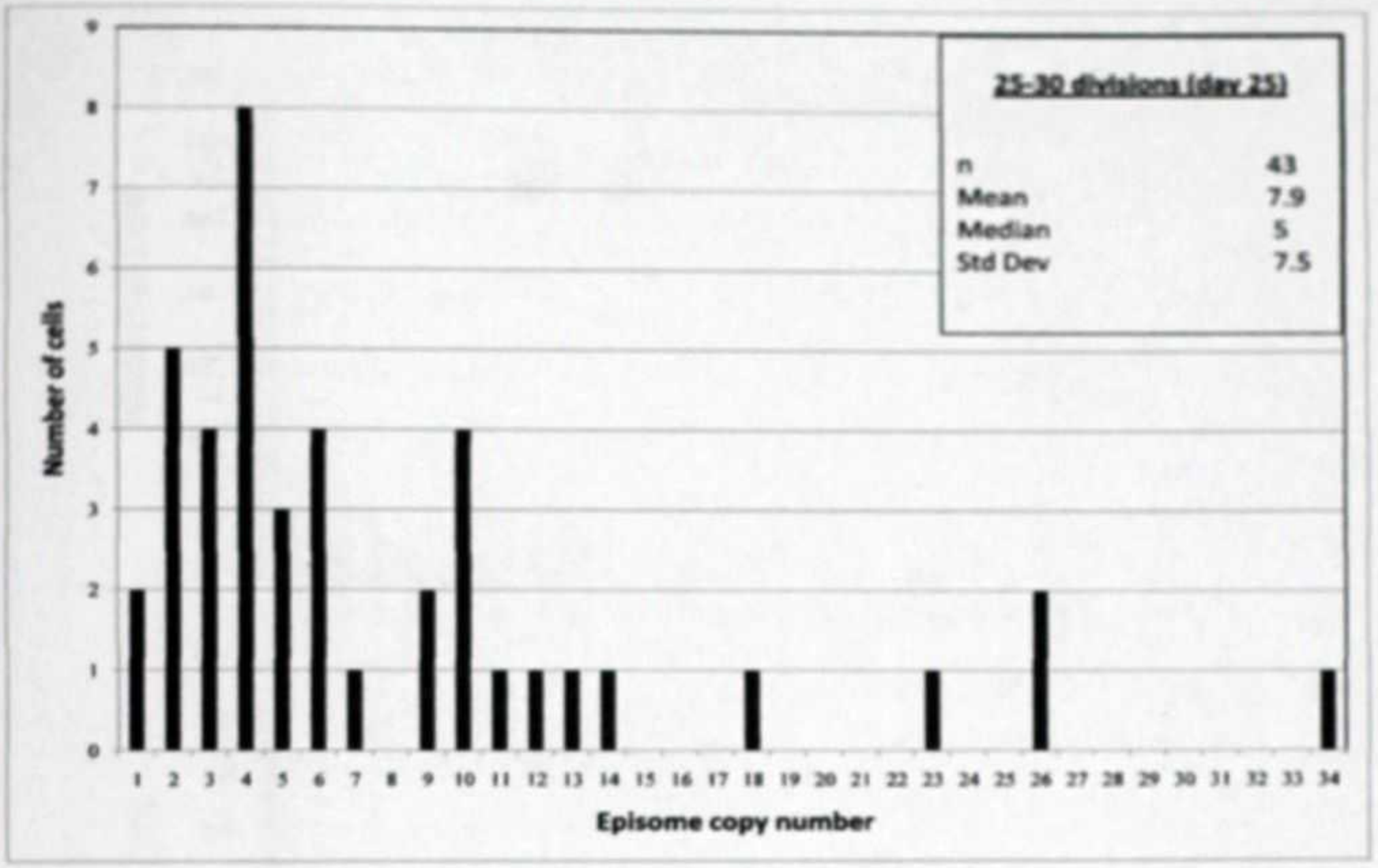


Figure 9: Distribution of EBV episomes in *in vitro* infected B cells. Panel A represents *in vitro* infected peripheral blood B cells that had divided only once based on CFSE staining. Panel B shows *in vitro* infected peripheral blood B cells that had divided three times based on CFSE staining. Panel C depicts *in vitro* infected B cells that had been grown for 20 days (20-25 divisions). D is the combined result for GC B cells from three tonsils. The zero division, 2 division data and CFSE output can be seen in appendix B.

A



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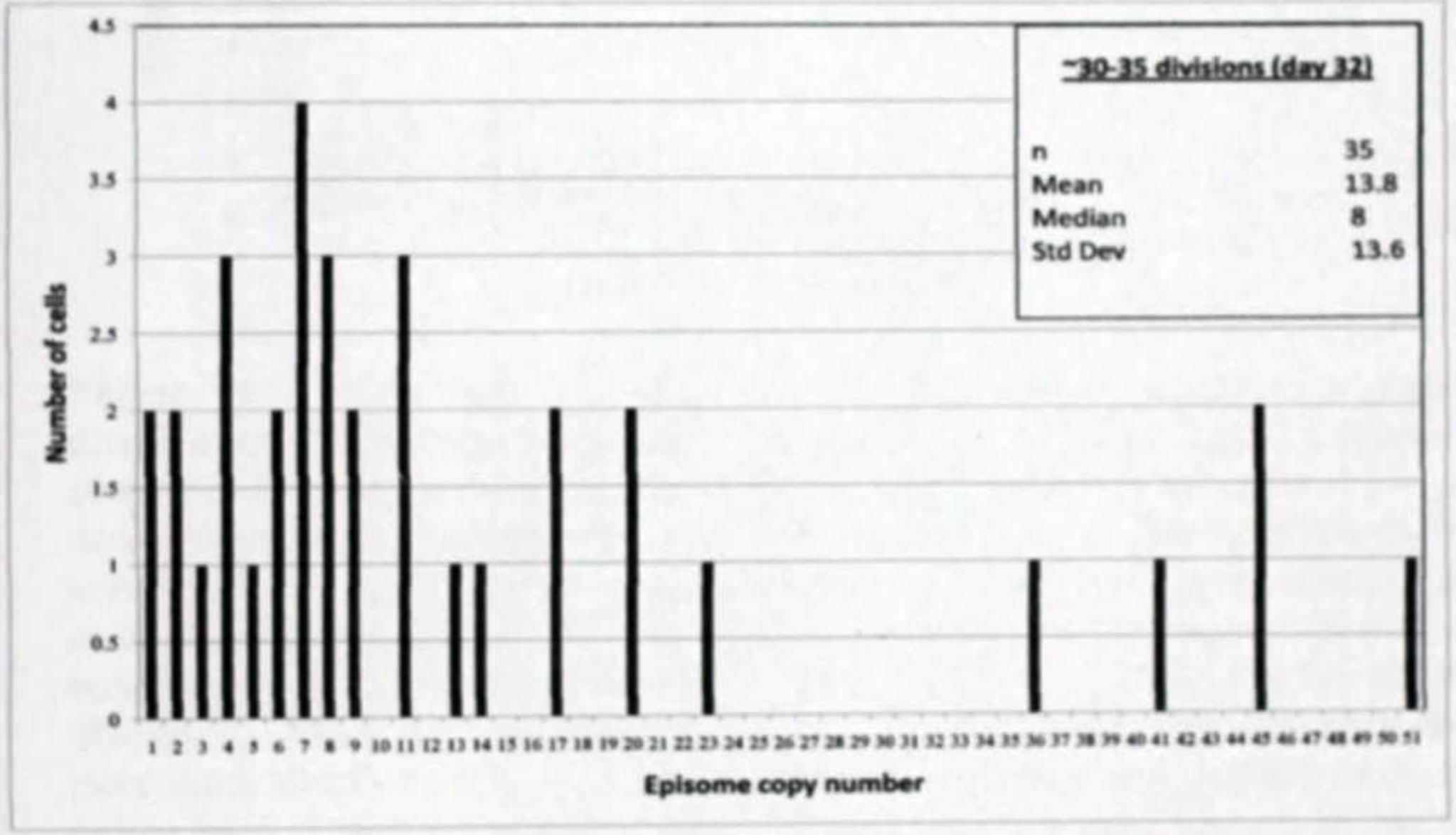

Figure 10: Distribution of EBV episomes in *in vitro* EBV infected B cells that had been grown for 25 days and 32 days. Panel A depicts *in vitro* EBV infected B cells that had been grown for 25 days (20-25 divisions) and panel B shows *in vitro* EBV infected cells that had been grown for 32 days (~30-35 divisions).

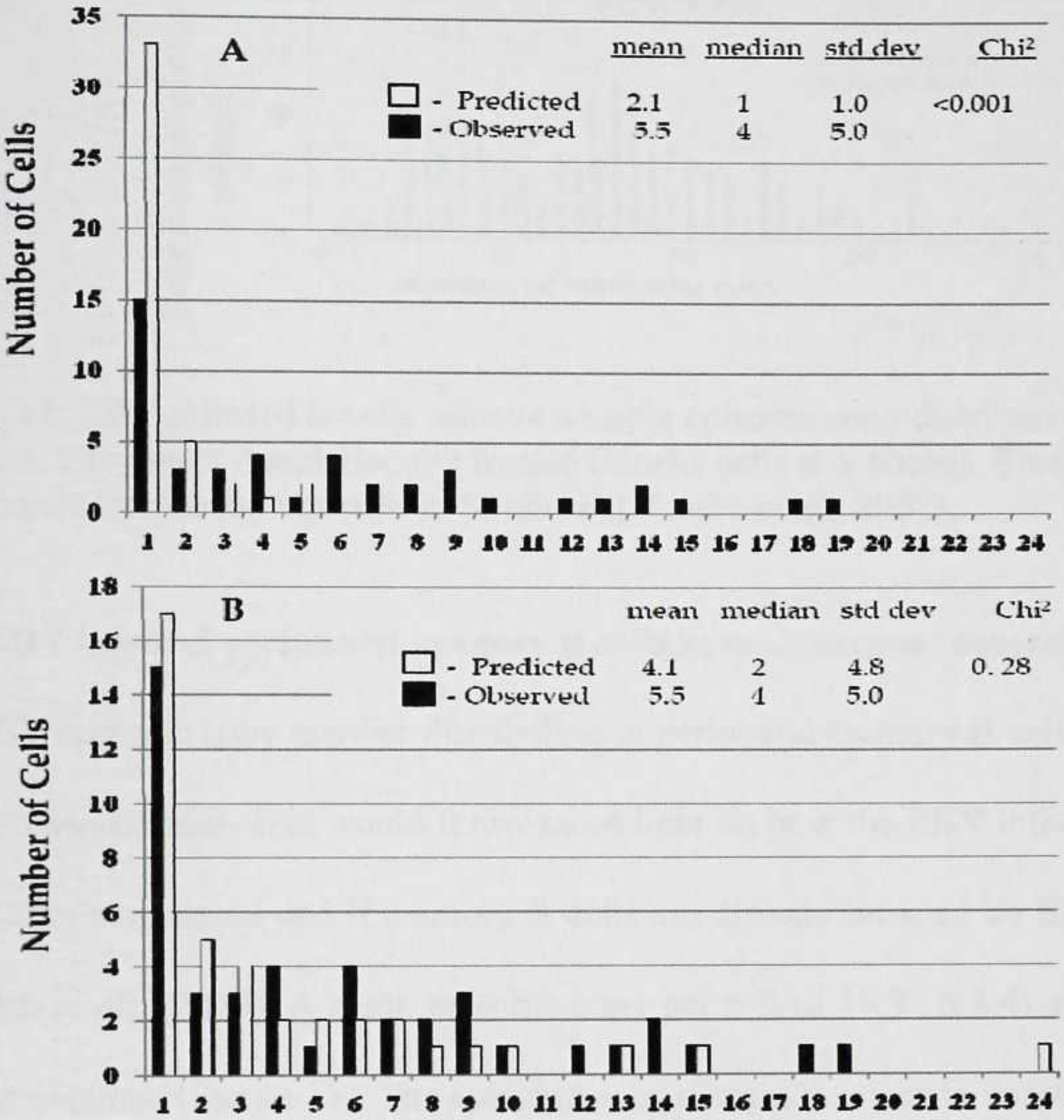

Figure 11: Models were generated from *in vitro* data to predict the observed distribution of viral episome copies in tonsillar GC B cells. Panel A shows the observed distribution (black bars) compared to the expected outcome if the cells divide three times and then either leave or die (white bars). The expected outcome was derived by combining equal portions of the episome distributions obtained from cells that had divided 0, 1, 2 and 3 times and then normalising to 45 (the total number of cells in the observed tonsil distribution). Panel B shows the observed distribution (black bars) as compared to the expected outcome if half the cells divide three times and then either leave or die and half undergo a large number of division (white bars). The expected outcome was derived by combining results from the three division model with data from cells that have undergone 20-25 divisions (in an equal mix), and then normalising to 45 (the total number of cells in the observed tonsil distribution). The bottom panel is a better fit, thus implying that a fraction of the EBV-infected GC B cells are indeed dividing more than three times before exiting into the blood compartment.

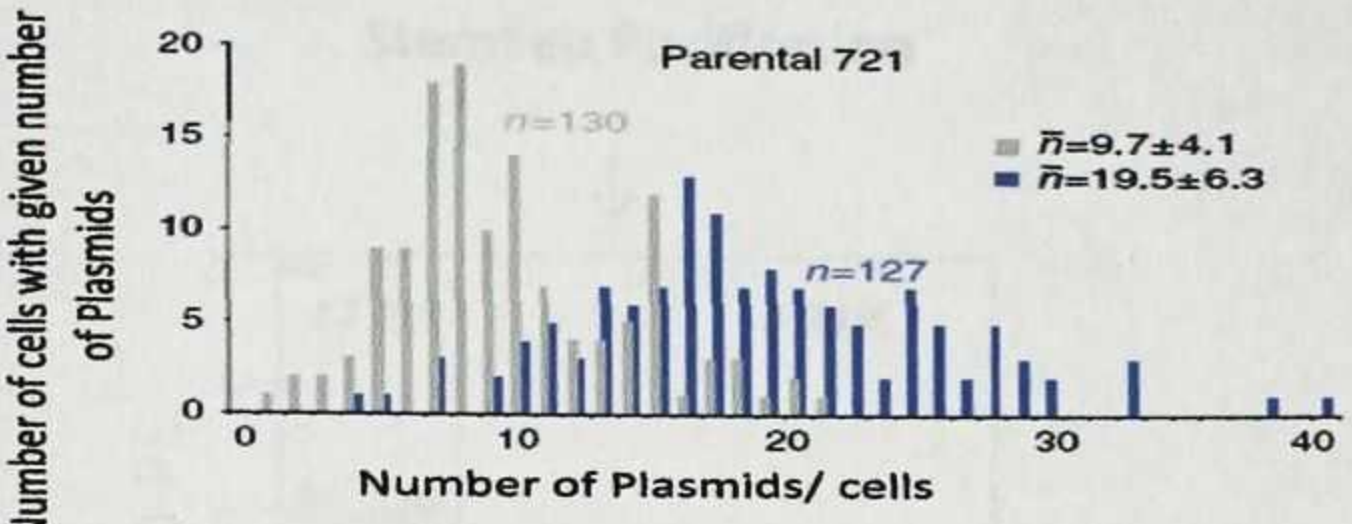

Figure 12: EBV infected B cells achieve a stable episome copy distribution after several divisions. Grey bars; Amphidocolin treated (blocks cells at S phase). Blue bars; Nocodazole treated (blocks cells at M phase) (Nambo *et al.*, 2007).

4.2.1 EBV infected peripheral memory B cells have undergone several divisions

The EBV episome copy number distribution in peripheral memory B cells from 4 adults was also ascertained. This would throw more light on how the EBV infected memory B cell pool is constituted and if memory B cells are directly infected by EBV as reported by Kurth *et al.*, (2003). A mean episome copy per cell of 10.9 (± 8.4) and a median of 9.5 was obtained (Figure 13). The episome copy distribution was in consonance with the distribution of EBV infected cells that had undergone at least 20 divisions (Figure 9C, Figure 10A and Figure 10B). It can be inferred from this result that peripheral blood memory B cells have undergone extensive division or at least not less than 20-35 divisions as per *in vitro* EBV infection data.

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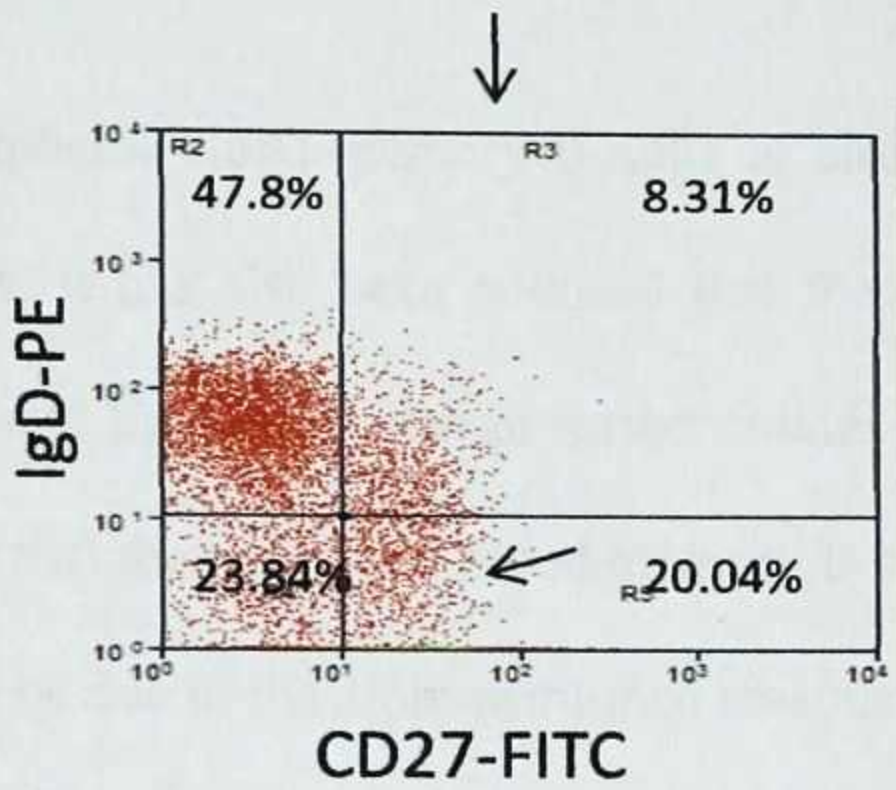


B

Chronic carrier	Frequency of EBV/10^7 Peripheral Memory B cells
CC1	150
CC2	500
CC3	200
CC4	200

C

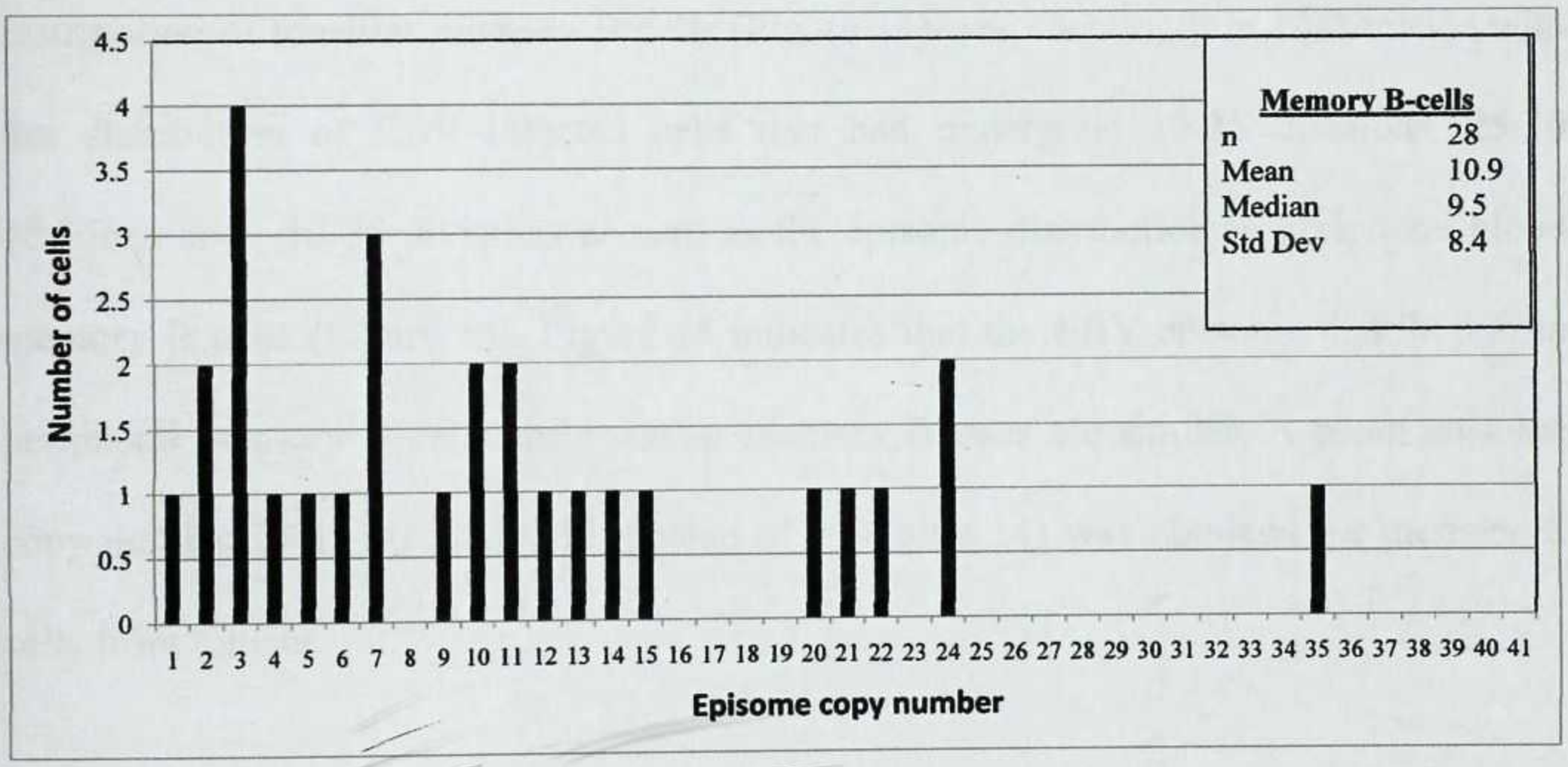
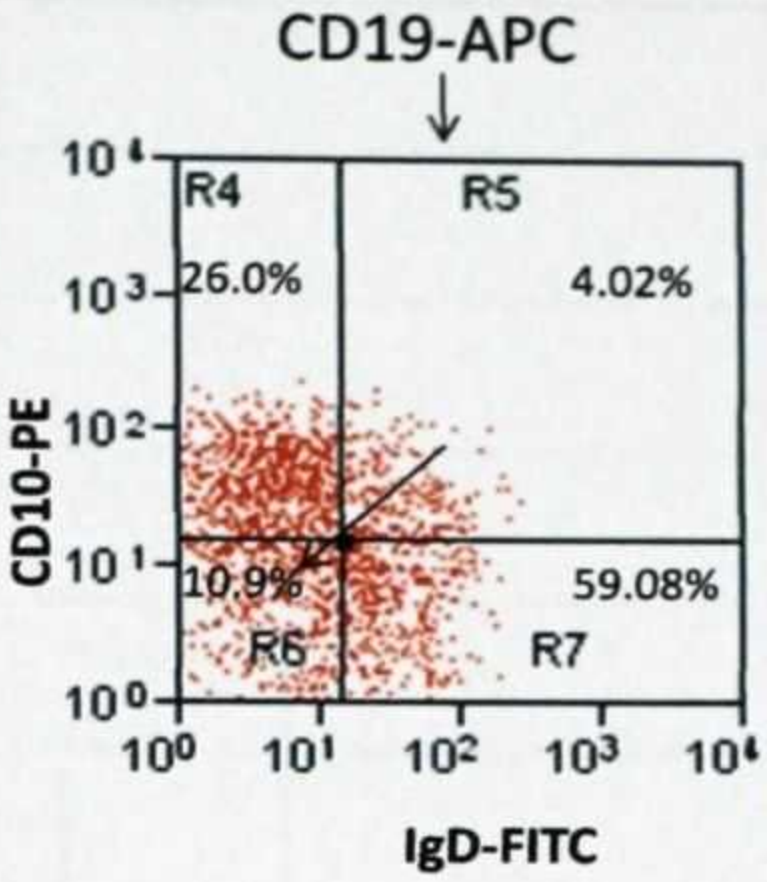


Figure 13: Distribution of EBV episomes in peripheral blood memory B cells
A. Peripheral blood memory B cells (IgD-CD27+) from 4 adult chronic carriers were sorted after StemSep B cell isolation. B. EBV frequency was then determined and C. EBV episome copy number/ cell was then determined as earlier described.

4.2.2 EBV infected tonsillar memory B cells do not show visible signs of direct infection

It is known that peripheral blood memory B cells in circulation do divide in order to maintain homeostasis. It has also been adduced that these peripheral memory B cells could return to lymphoid tissues to undergo further rounds of proliferation. An argument could be propounded that the peripheral blood memory B cell episome distribution result (from 4 adults) could be due to the aforementioned reasons and perhaps this is the reason why there are no signs of direct memory B cell infection. To lay this argument to rest, the episome copy distribution of memory B cells from 3 tonsils (these were from children between the ages of 5-12 years) was also acertained. Thus the premise will be that; if the EBV episome distribution of tonsillar memory B cells parallels that of the peripheral memory B cells then EBV infected memory B cells originate from the GC reaction and not by direct infection. Again it was found that the episome copy distribution of tonsillar memory B cells (Figure 14) was seemingly in consonance with the distribution of EBV infected cells that had undergone 20-25 divisions, 25-30 divisions and ~30-35 divisions as well as the episome distribution in peripheral blood memory B cells (Figure 13). Figure 15 indicates that the EBV episome distribution in peripheral memory B cells and tonsillar memory B cells are similar. A mean episome copy number of 13.3 ($\pm$ 12) and median of 9 (Figure 14) was obtained for memory B cells from tonsils.

A



B

Frequency of EBV in tonsil memory B cells/ 10^7 GC B cells						
	Number of experiments					
Tonsil Code	1	2	3	4	5	Mean
TM1	3,120	2,010	1,005	2,840	2,300	2,255
TM2	2,010	1,005			1,230	1,415
TM3	1,230	1,290			1,500	1,340

C

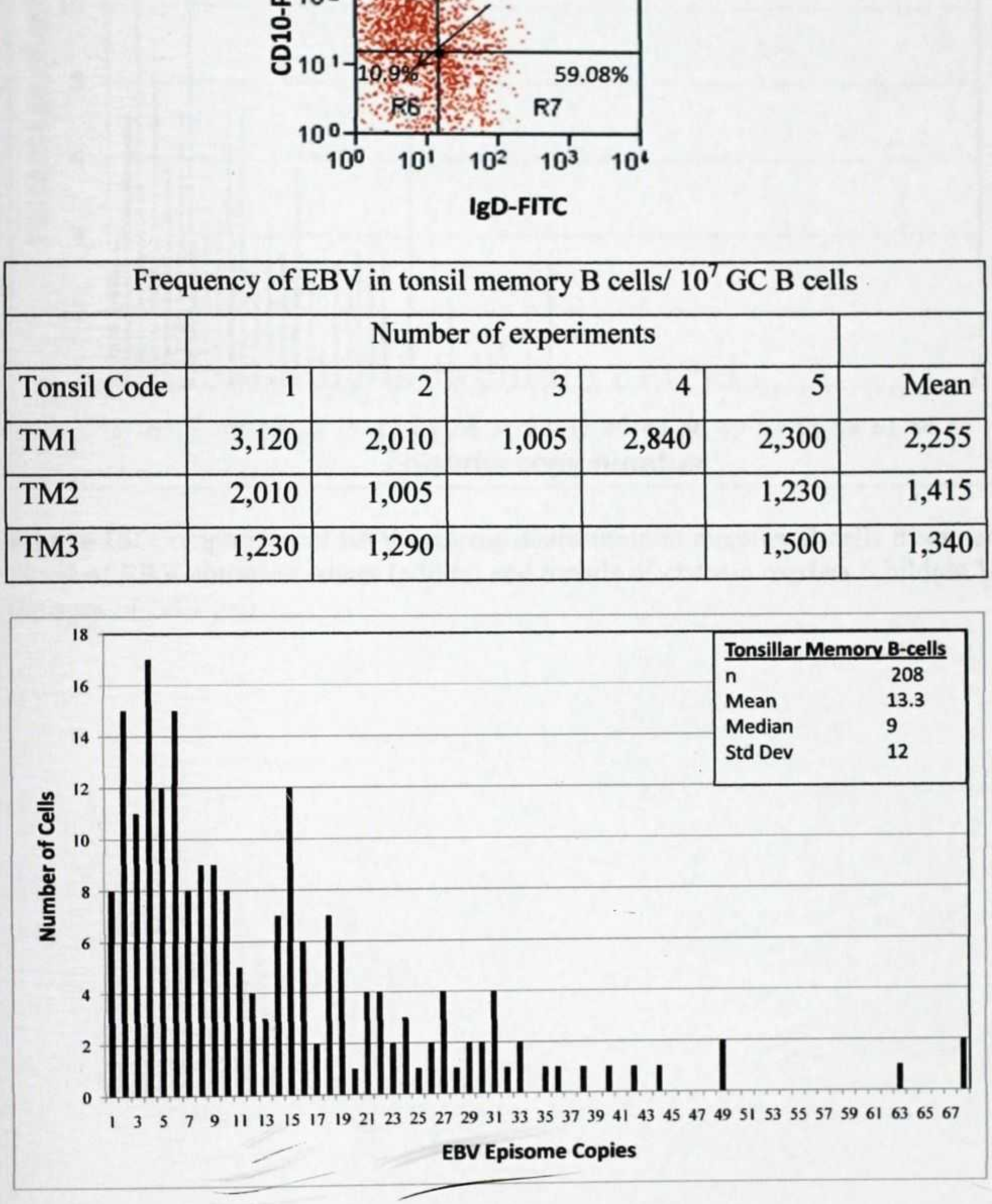


Figure 14: Distribution of EBV episomes in tonsillar memory B cells
Tonsillar memory B cells (CD19+C10-IgD-) from 3 chronic carriers (children between the ages of 2-14) were sorted (A), frequency of EBV was assayed (B) and then EBV episome copy number per cell was determined (C) as usual.

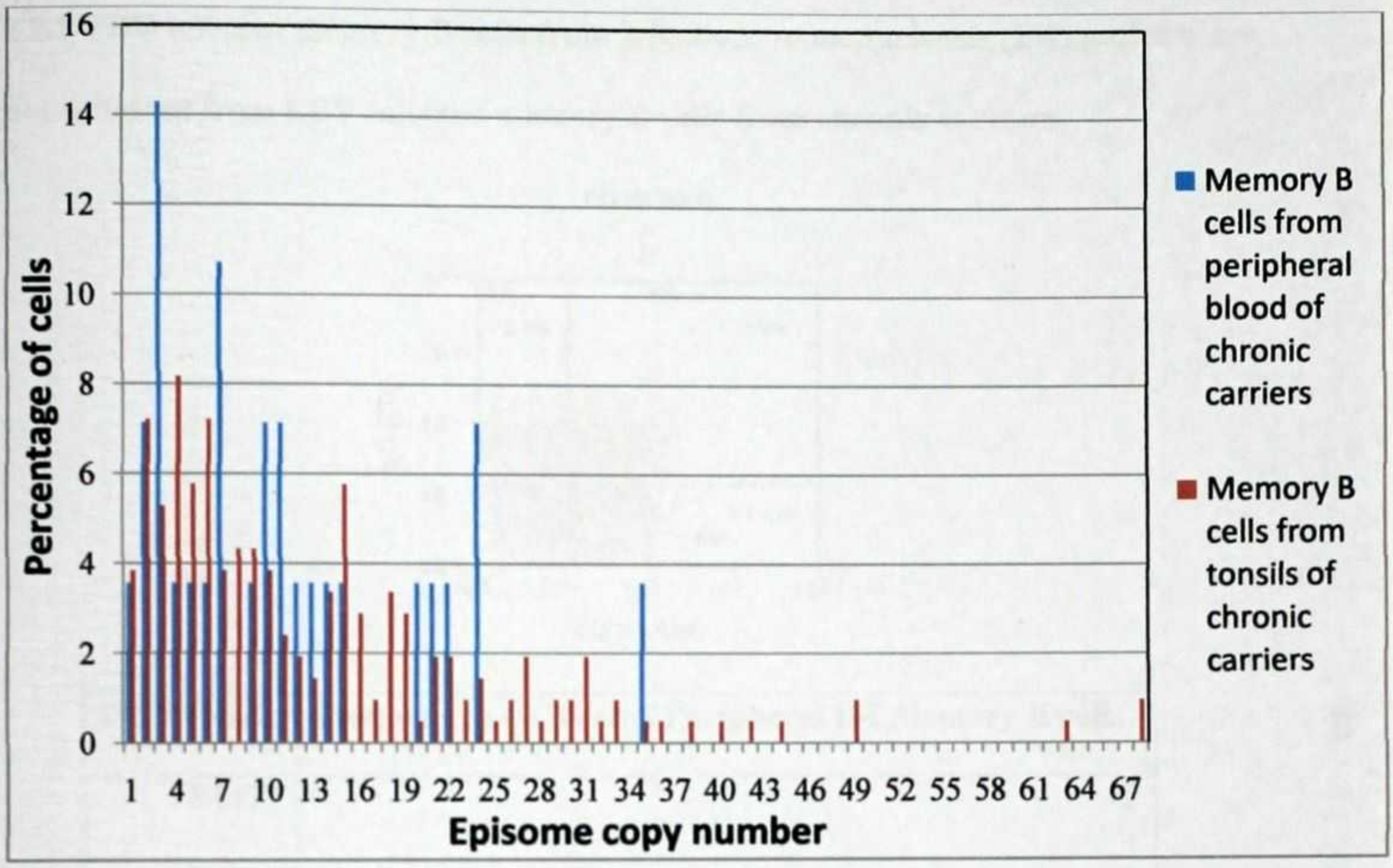
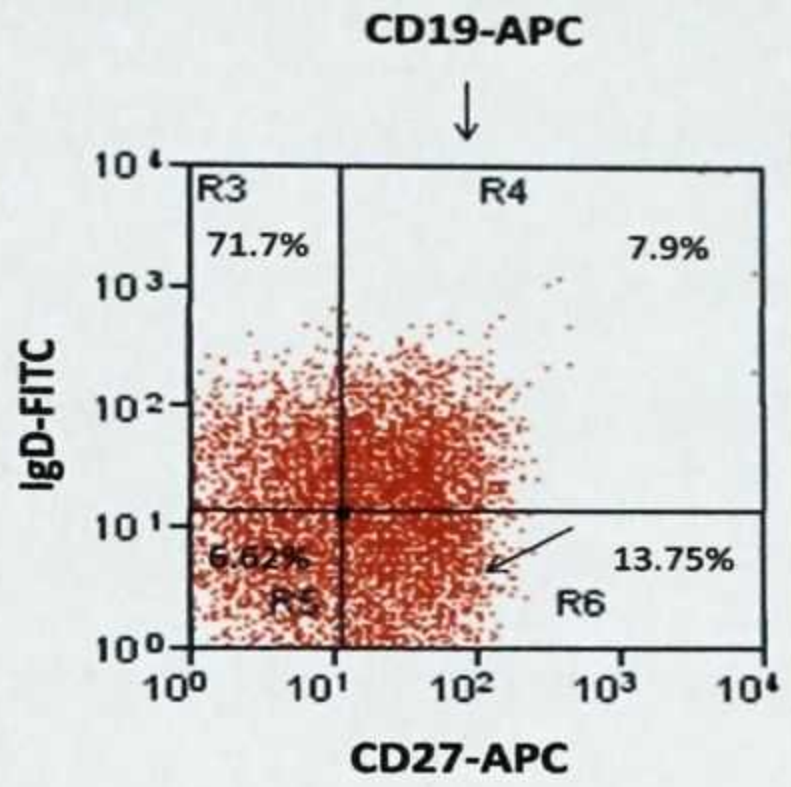


Figure 15: Comparison of EBV episome distribution in memory B cells from peripheral blood of EBV chronic carriers (adults) and tonsils of chronic carriers (children between the ages of 2-14 yrs)

4.2.3 EBV infected memory B cells from infectious mononucleosis (IM) patients are not different from EBV infected memory B cells from chronic carriers

A



B

IM Patients	Frequency of EBV/1/N Peripheral IM Memory B cells
E1437	42
E1433	36
E1434	34

C

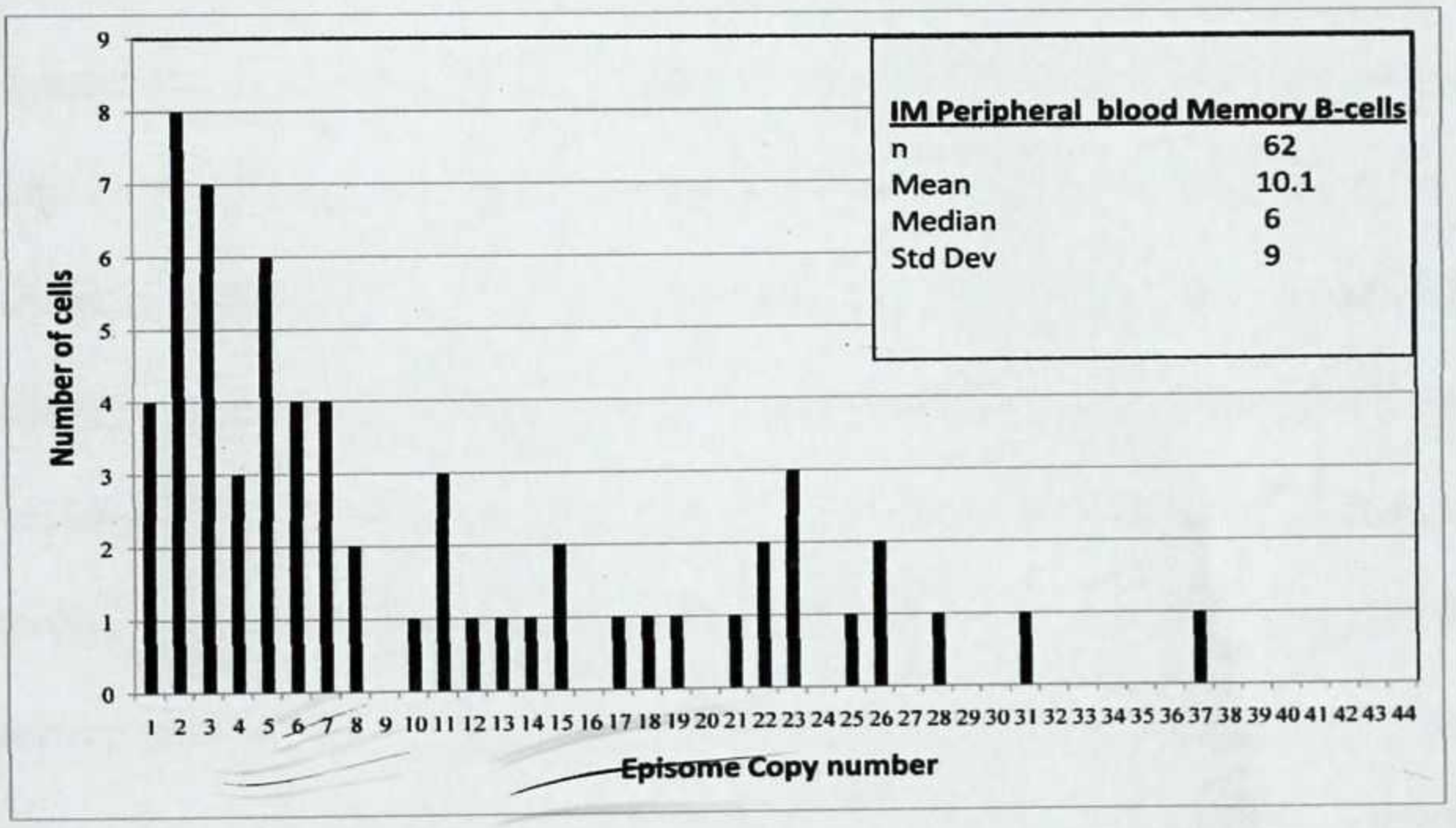


Figure 16: EBV episome copy distribution in peripheral blood memory B cells of IM patients. Peripheral blood memory B cells (CD19+IgD-CD27+) from 3 IM patients (A) with known frequency of EBV infected cells (B) were sorted after which EBV episome copy number/ cell was determined (C).

Patients suffering from infectious mononucleosis (IM) have a very high frequency of EBV infected cells (Figure 16B). IM is a self-limiting lymphoproliferative disorder which arises when primary EBV infection occurs in adults and adolescents. It has been suggested that the pattern of EBV infection in IM patients might be different from the pattern of infection in chronic carriers. It has also been postulated that EBV directly infects memory B cells in this particular condition (Kurth *et al.,* 2003; Chaganti *et al.,* 2009). It was therefore expected that the EBV episome copy distribution profile in EBV infected memory B cells from chronic carriers will be different from the episome copy distribution profile of IM patients. Figure 16C indicates a mean of 10.1 ($\pm$ 9) and a median of 6 obtained for the episome copy distribution in peripheral memory B cells in IM patients. This distribution is not distinct from the episome copy distribution in peripheral memory B cells from chronic carriers (Figure 13). It also follows a similar pattern of episome distribution seen in the tonsillar memory B cells. It is quite interesting to note that there was no difference between the percentages of the B cell sub-populations in either chronic carriers or IM patients (Figure 18), as reported by Chaganti *et al.,* (2009). (It must be said though that the median percentage naive B cell population is slightly higher in chronic carriers (1.45 fold) than in IM patients although the difference is not significant). Table 3 compares the mean and median EBV episome copy number in peripheral memory B cells from chronic carriers and IM patients as well as tonsillar memory B cells from chronic carriers. It is clear that memory B cells irrespective of their source are not different in terms of EBV infection and proliferation dynamics since, aside the EBV epsiome copy distribution profiles, the mean and median EBV episome copy numbers are similar in all the memory B cell categories assayed in this work.

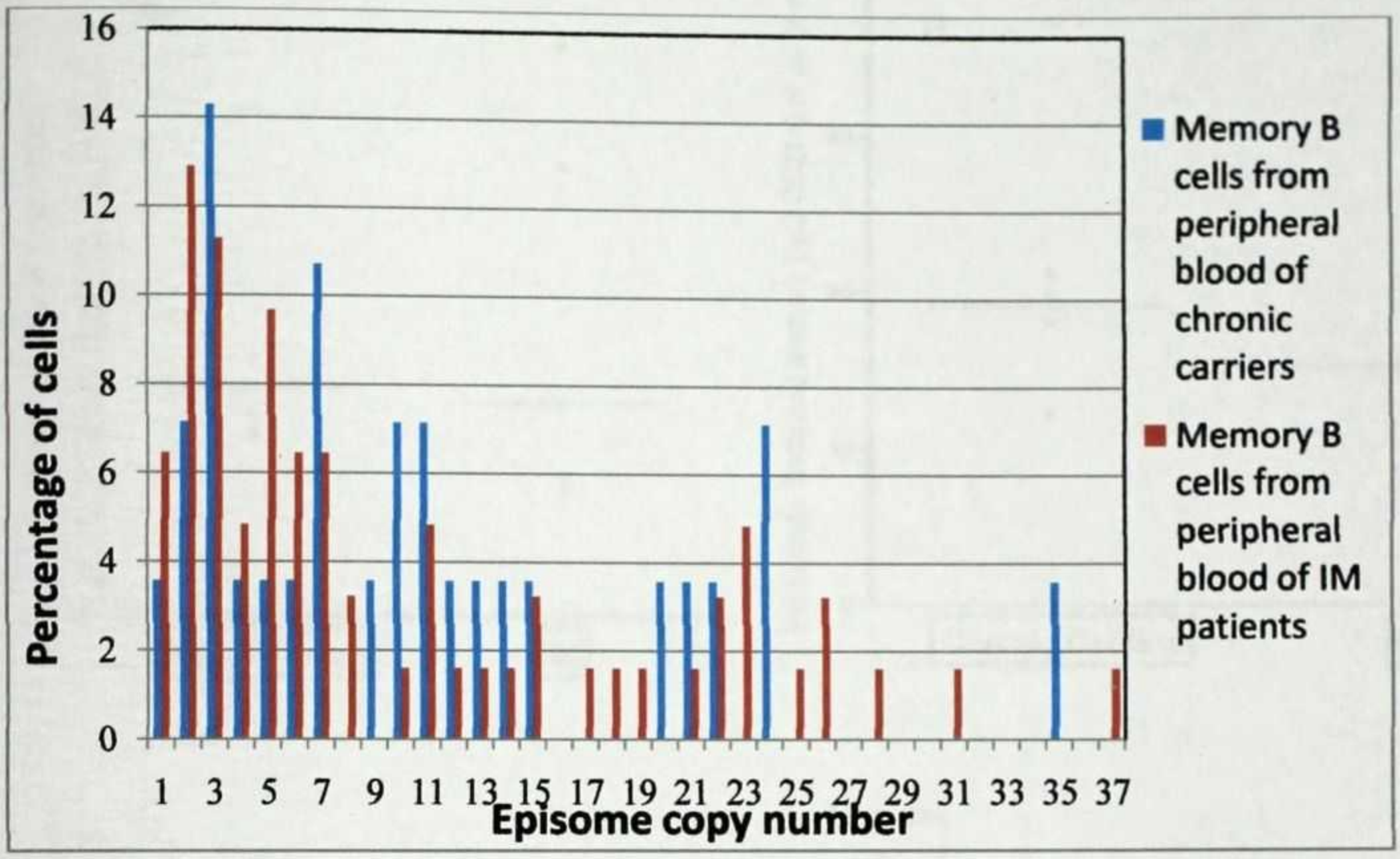
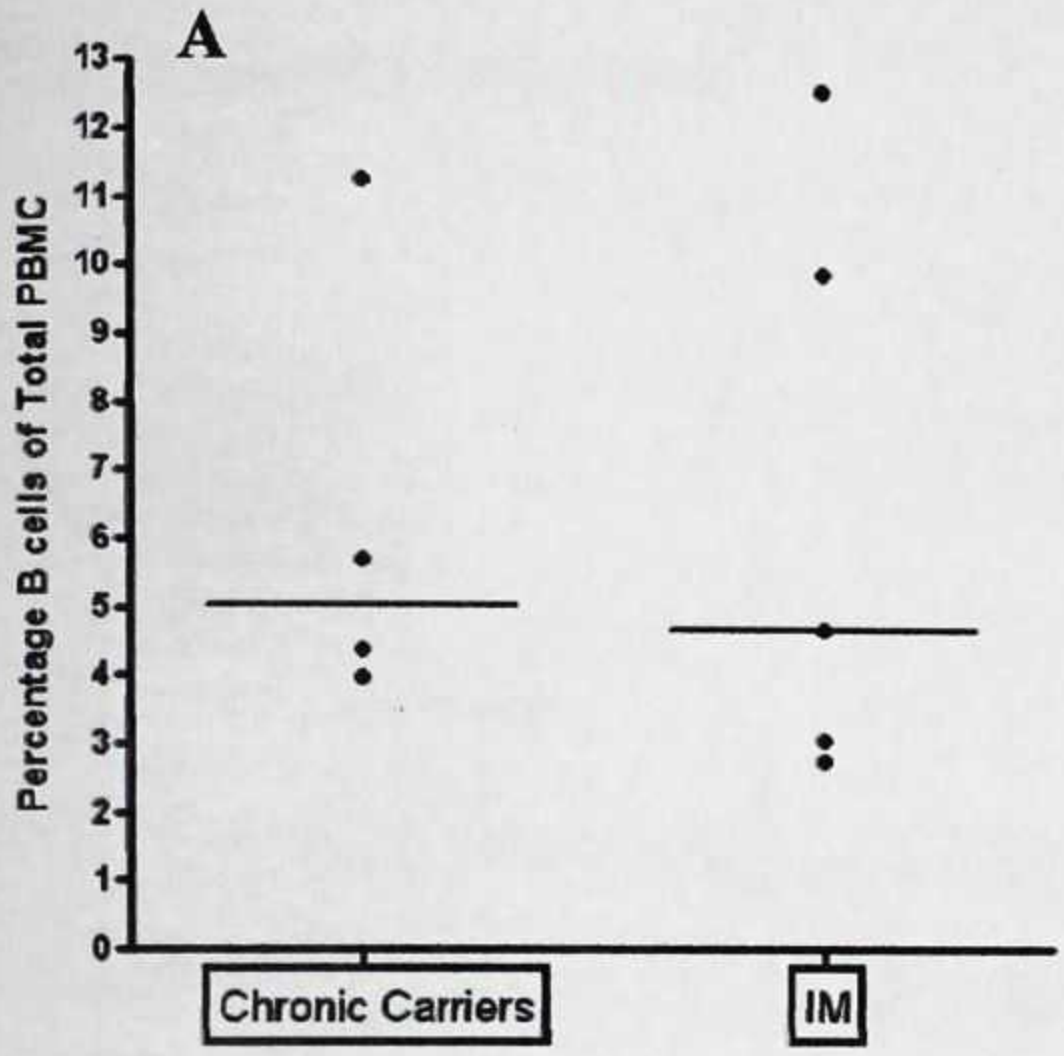
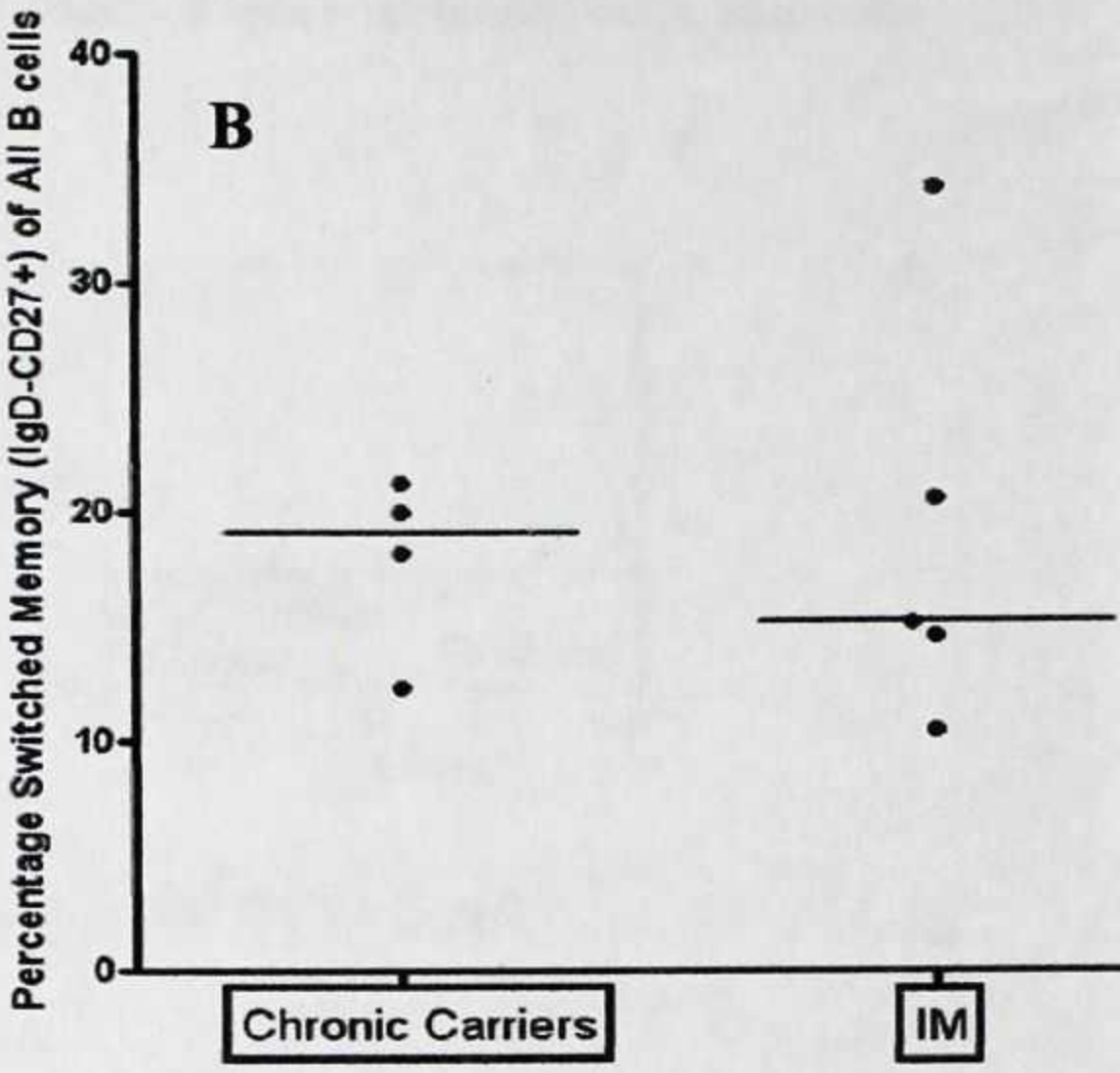


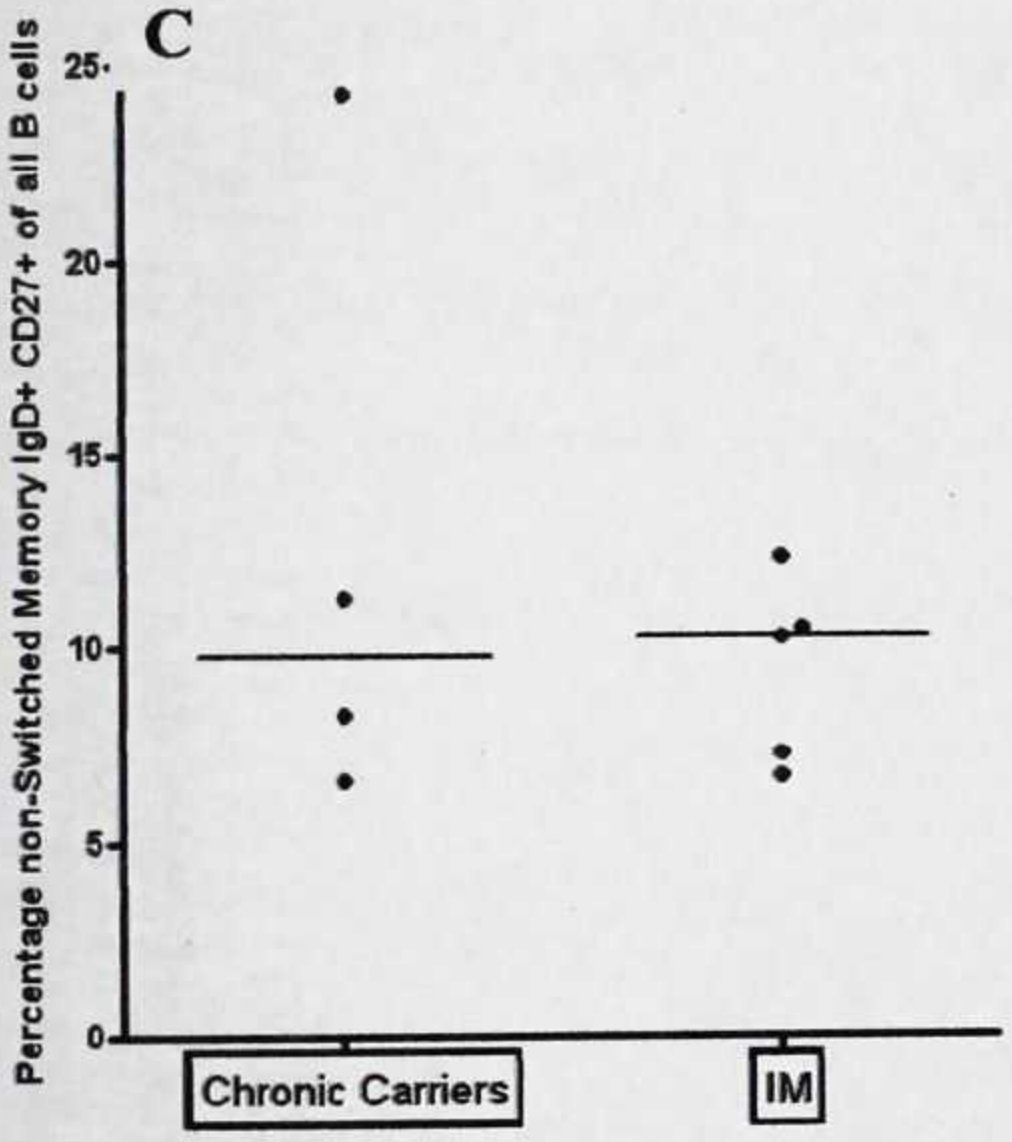
Figure 17: Comparison of EBV episome distribution in memory B cells from peripheral blood of EBV chronic carriers and peripheral blood of IM patients.

Table 3: Comparison of mean and median EBV episome copy number in memory B cells from peripheral blood and tonsils of chronic carriers, and peripheral blood of IM patients

Source of memory B cells	Mean EBV episome copy number	Median EBV episome copy number
Peripheral blood of chronic carriers	10.9 ($\pm$ 8.4)	9.5
Tonsils of chronic carriers	13.3 ($\pm$ 12.0)	9
Peripheral blood of IM patients	10.1 ($\pm$ 9.0)	6







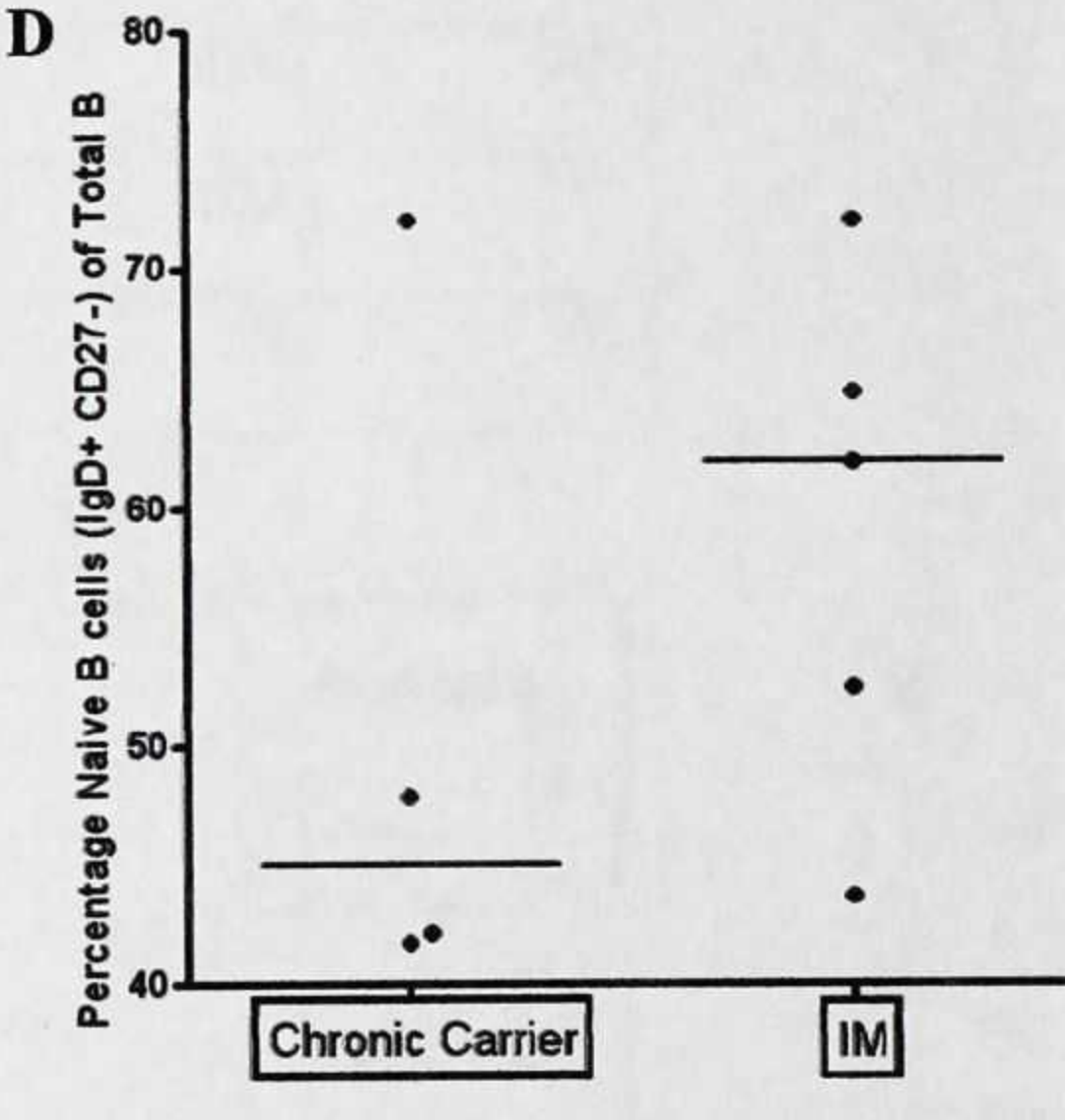
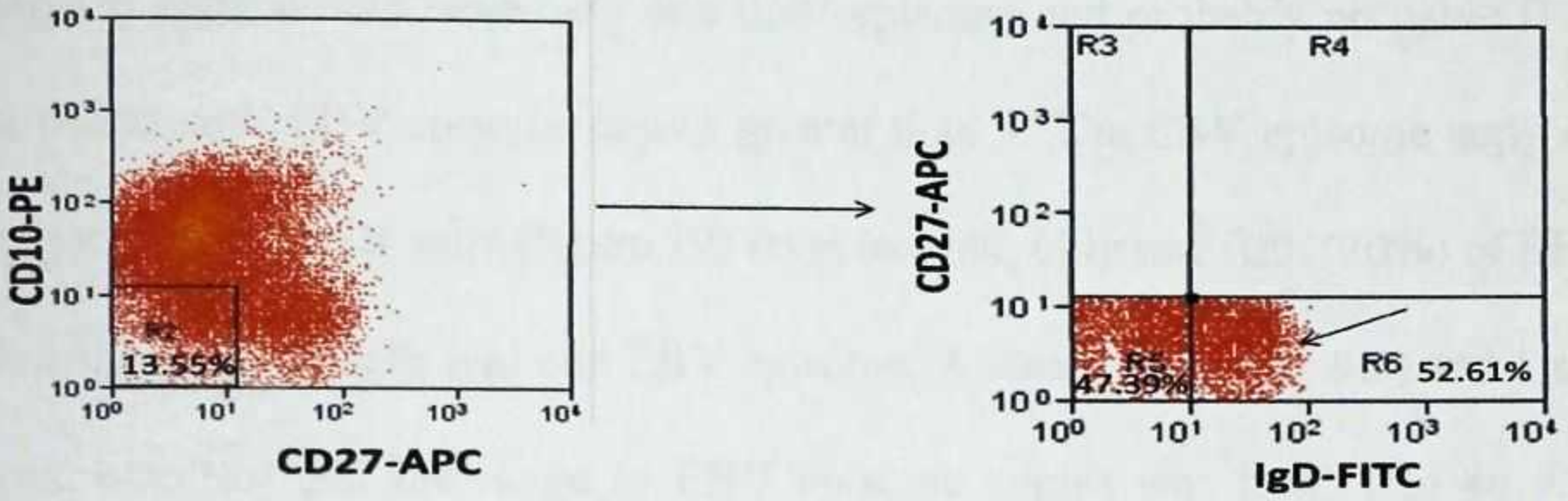


Figure 18: B cell compartments of peripheral blood from chronic EBV carriers and IM patients. B cell populations of peripheral blood from chronic carriers and IM patients were analysed by flow cytometry. A. Percentage B cells of total PBMC (CD19+) B. Percentage switched memory B cells (CD19+IgD-CD27+) of all B cells C. Percentage non-switched memory B cells (CD19+ IgD+CD27+) and D. Percentage naive B cells (CD19+IgD+CD27-). Bars indicate median percentage B cell values.

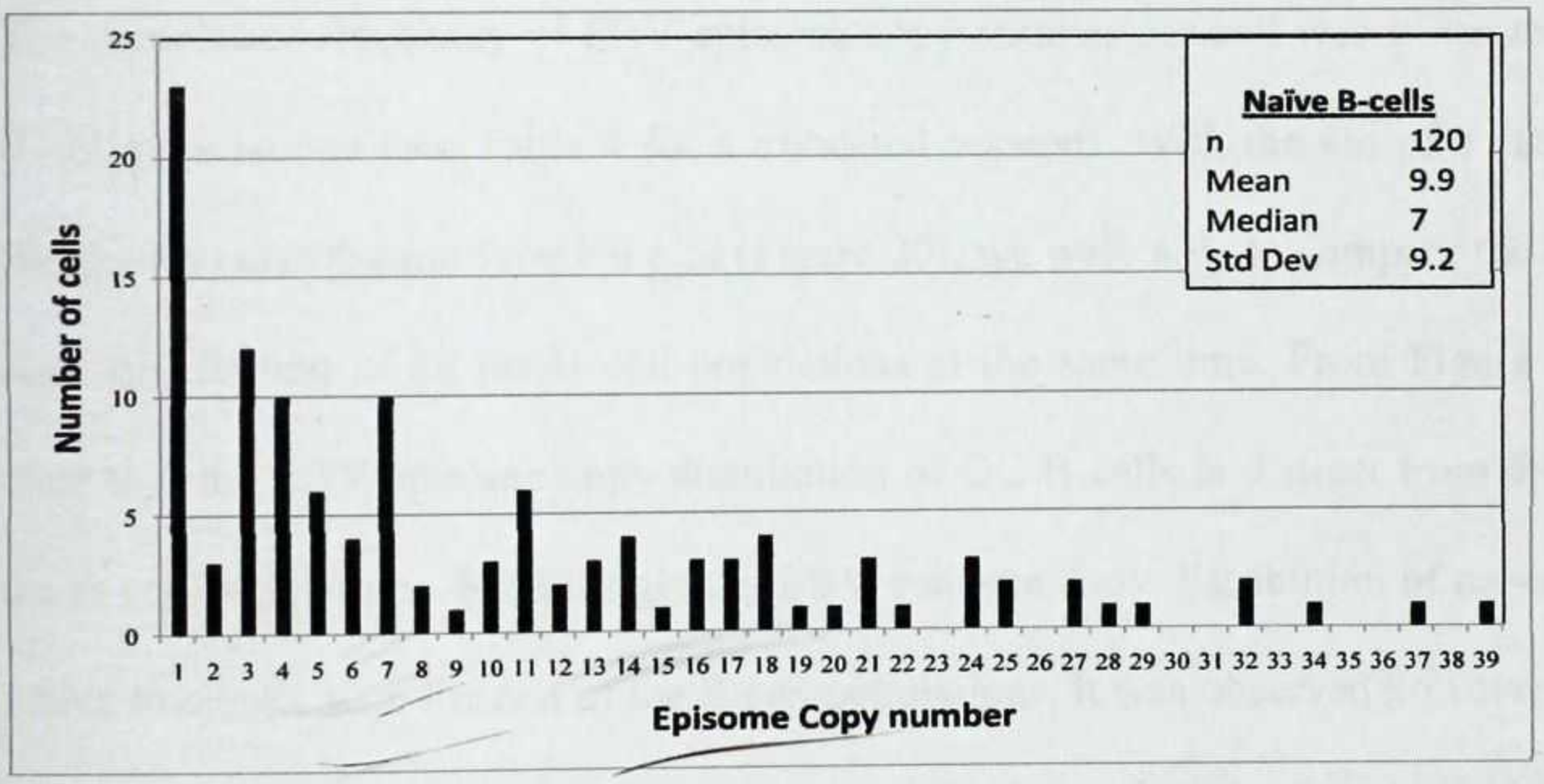
4.2.4 Tonsillar naive B cells might be a complex mix of newly infected cells and cells that are rapidly proliferating



A

Frequency of EBV infection in Naïve B cells/ 10^7 Naïve B cells				
	Number of experiments			
Tonsil code	1	2	3	Mean
N1	200	400	240	280
N2	100	100	120	107
N3	100	70	50	73

B



C

Figure 19: EBV episome copy distribution in naïve B cells from chronic carriers. Naïve B cells (IgD+CD10-CD27-) from 3 tonsils were sorted by flow cytometry (A), frequency of EBV infected cells (B) and episome copy distribution was determined (C).

It is logical to argue that, if naive B cells represent newly EBV infected cells which only divide a few times and then differentiate into GC B cells, then a majority of tonsillar naive B cells should have only one EBV episome and probably no naive B cell should be found with EBV episome copies greater than 9. The EBV episome copy distribution for tonsillar naive B cells (figure 19) revealed that, only one fifth (20%) of EBV infected tonsillar naive B cells had one EBV episome. A mean of 9.9 ($\pm$ 9.2) and a median of 7 were obtained and the range of EBV episome copies was from 1 to 40. One way to rationalize this result is that, the 20% of cells carrying only a single EBV episome, represents newly infected naive B cells. The unexpected high EBV episome copies observed in the right tail of the distribution could be a result of the rapid proliferation of the newly EBV infected naive B blasts.

4.2.5 Cumulative distribution function of EBV episome copy numbers in B cell populations

The cumulative frequency of EBV episome copy number per cell was generated for all B cell populations (see Table 4 for a truncated version). With the aid of a cumulative (frequency) distribution function plot (Figure 20), we were able to compare the episome copy distribution of all the B cell populations at the same time. From Figure 20, it is clear that the EBV episome copy distribution of GC B cells is distinct from the rest of the B cell populations. Surprisingly the EBV episome copy distribution of naive B cells seems to cluster with the rest of the B cell populations. It was observed however that the distinction between the EBV episome copy distributions of all the B cell population was actually seen between EBV episome copy numbers 1-5. Table 4 shows a truncated version of cumulative distributions. In Figure 21 the subtle difference between the

episome copy distribution of naive B cells and the rest of the B cell populations becomes evident. It is therefore safe to say that, the episome copy distribution of GC B cells form one cluster; naive B cells also form another cluster; and finally, peripheral memory B cells, tonsillar memory B cells, IM peripheral memory B cells, *in vitro* EBV infected cells that have undergone 20-25 divisions, and 25-35 divisions, all form one cluster. These clusters must represent different EBV proliferation dynamics or EBV interactions with the various B cell populations.

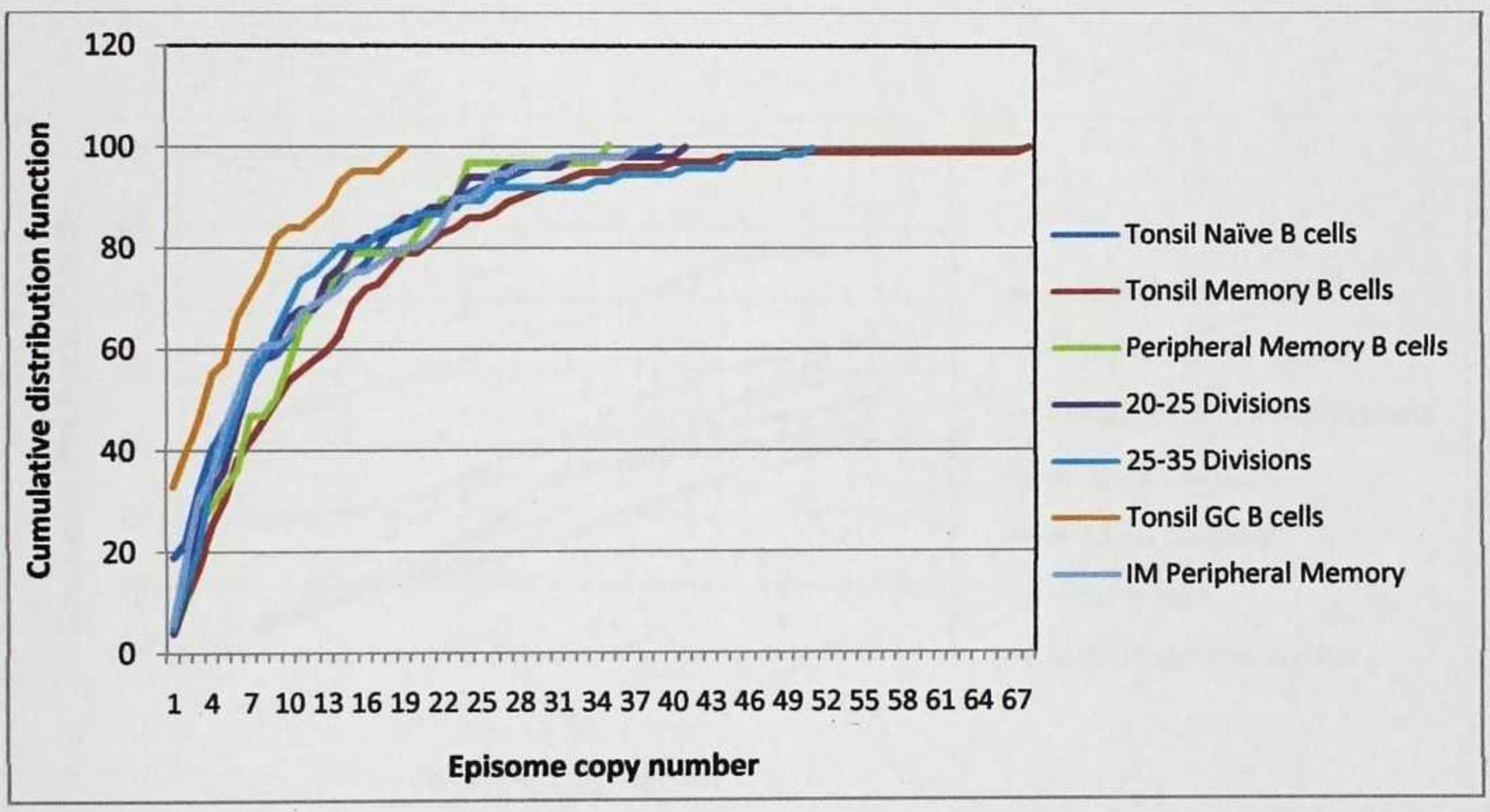


Figure 20: Cumulative distribution function of EBV episome copies in various B cell populations. A cumulative distribution function was generated from the frequency of episome copy number for all B cell populations.

Table 4: Truncated cumulative distribution function of EBV episome copy numbers in B cell populations

Episome copy no.	IM P Memory	T. Memory	P. Memory	20-25 Divisions	25-35 Divisions	GC B cells	Naive B cells
1	6	4	4	4	5	33	19
2	19	11	11	12	15	40	22
3	30	17	25	26	21	46	32
4	35	25	29	32	35	55	40
5	45	30	33	36	40	57	45

T. Memory: tonsillar memory B cells, P. Memory: peripheral blood memory B cells

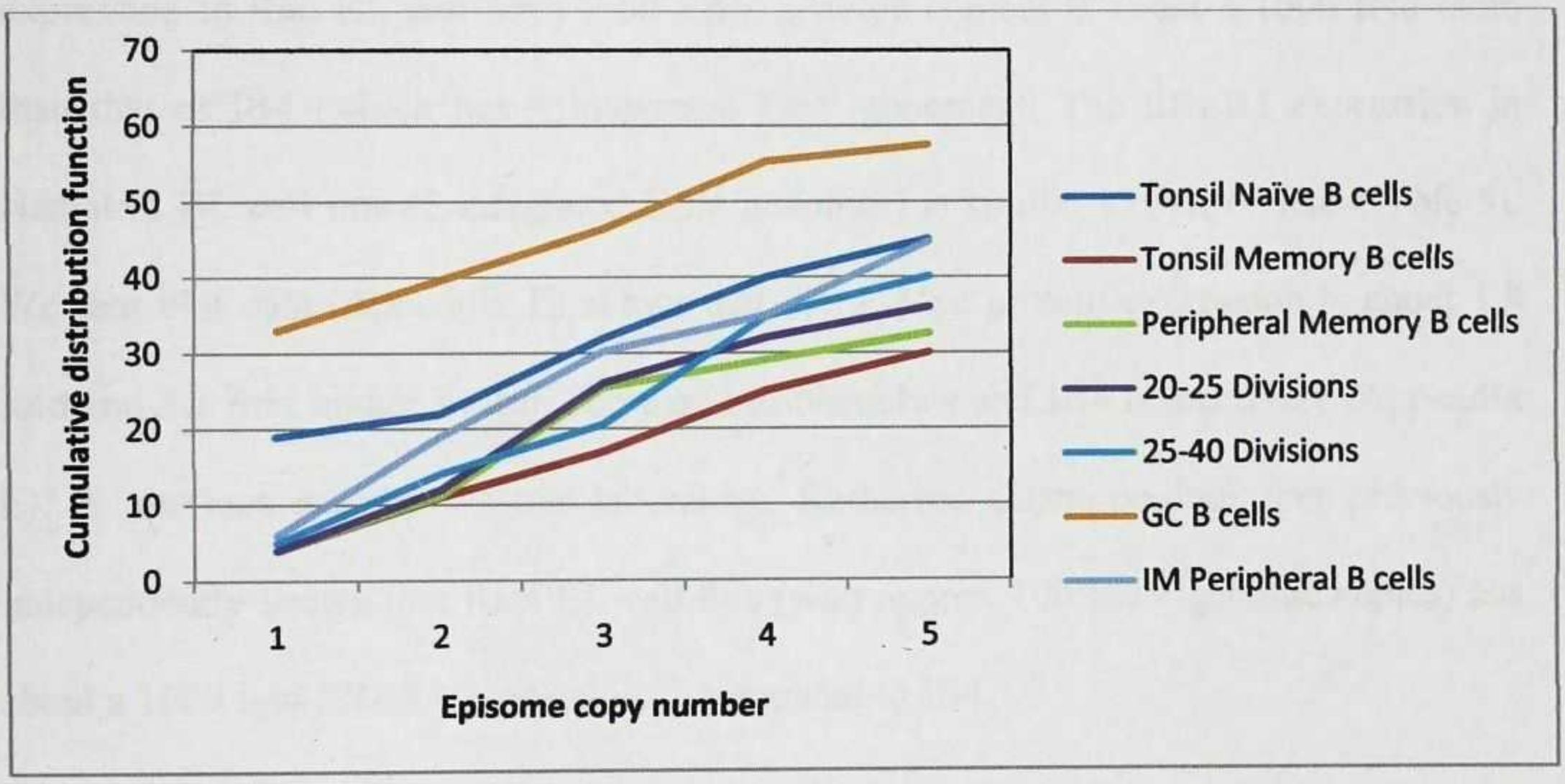


Figure 21: Truncated cumulative distribution function of EBV episome copies in various B cell populations. A cumulative distribution function was generated from the frequency of episome copy number for all B cell populations for up to 5 episome copies since this was the range which fully represented the difference in the various B cell populations.

4.3 Differential expression of the potentially oncogenic EBER1 and c-Myc in cell lines with low (integrated) EBV genomes and a cell line with high EBV episomes

Repellin *et al*., (2010), with the aid of EBER1 transgenic mice, report that EBER1 plays an oncogenic role in EBV associated malignancy, and it is has been reported that there is a c-Myc binding site in the promoter region of EBV encoded RNA (EBER1) (Niller *et al.,* 2003; Niller *et al.,* 2004). This raises the possibility that the expression of the possibly oncogenic EBER1 could be influenced by c-Myc expression (or vice versa) and this may present one way by which EBV could contribute to the pathogenesis of EBV positive lymphoma. A curious question to ask is this, is EBER1 expression linked to EBV episome copy number and c-Myc expression? This thesis reports that EBER1 expression in Raji BL cell lines (~60 EBV genome copies) is about a 1000 fold more than that of IB4 (which has 4 integrated EBV genomes). The EBER1 expression in Namalwa BL cell line (2 integrated EBV genomes) is similar to that of IB4 (Table 5). Western blot data (Appendix E) shows that that c-Myc protein expression is about 1.8 fold and 2.1 fold higher in Raji compared to Namalwa and IB4 respectively (Appendix E). A previous member of our laboratory, Katherine Cosmopoulous had previously independently shown that Rael BL cell line (with approx 100 EBV genome copies) has about a 1000 fold EBER1 expression as compared to IB4.

Table 5: Expression of EBER1 in Raji, IB4, and Namalwa cell lines

Cell line	EBER1 fold expression
Raji	1,000
IB4	1
Namalwa	0.93

4.4 High GC throughput and high level of EBV infected GC B cells from malaria-endemic region as compared to tonsils from non-malaria region

This work proposes that one of malaria's contributions (not paramount) towards the pathogenesis of endemic BL (eBL) is that, immunosupression or immune dysfunction as well as chronic B cell stimulation caused by malaria infection will permit or cause an increased number of B cells to undergo the GC reaction. This will also allow an increased number of EBV infected cells to undergo the GC reaction. Since the pro-apoptotic BIM is hypothesised to already be downreagulated by EBV proteins in EBV infected GC B cells, there is an increased likelihood of a c-Myc/Ig translocation being tolerated in these B cells.

Using flow cytometry (CD19+CD10+ staining) the percentage of total B cells as well as the percentage GC B cells of tonsils from either the malaria-endemic region or the non-malaria region were determined. Tonsils from the malaria-endemic region yielded a mean percentage B cell of 80.42% ± 4.67% and a range of 71%-86% (Table 6). For tonsils from the non-malaria area the mean percentage B cells was 77.1% ± 6.94% with a range between 61%-81% (Table 7). The percentage B cells of tonsils from the non-malaria region is not significantly different from the percentage B cells from the malaria-endemic region ($p = 0.1835$) (Figure 22). The mean percentage GC B cells (of all lymphocytes) of tonsils from the non-malaria region was 24.8% ± 5.84%, whereas the mean percentage GC B cells (of all lymphocytes) from the malaria-endemic region was 38.5%9 ± 8.3% (Table 7 and Table 6 respectively). Thus the mean percentage GC B cells of the malaria-endemic region is 1.5 fold or 50% higher than that of tonsils from the non-malaria region ($p < 0.001$) (Figure 23).

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Table 6: Percentage of B cell sub-populations (of total tonsillar lymphocytes) in tonsils from malaria-endemic region

Tonsil Code	% GC B-cells	% Non-GC B-cells	% B-cells
1	55.9	30.2	86.1
2	24.05	55.38	79.43
3	36.33	39.64	75.97
4	46.08	25.05	71.13
5	36.19	44.24	80.43
6	36.15	41.58	77.73
7	39.97	40.16	80.13
8	32.86	52.32	85.18
9	38.5	44.84	83.34
10	39.83	44.88	84.71
Mean	38.59	41.83	80.42
SD	8.30	9.08	4.67

Table 7: Percentage of B cell sub-populations (of total tonsillar lymphocytes) in tonsils from non-malaria region

Tonsil Code	% GC B-cells	%Non-GC B-cells	% B cells
1	23.49	53.00	76.49
2	33.28	49.64	82.92
3	23.42	58.04	81.46
4	23.85	51.31	75.16
5	16.18	45.43	61.61
6	29.87	46.82	76.69
7	23.56	51.59	75.15
8	26.67	44.23	70.9
9	28.76	54.74	83.5
10	22.53	55.20	77.73
11	20.74	62.19	82.93
12	40.77	41.69	82.46
13	28.23	42.74	70.97
14	14.75	46.35	61.10
15	21.41	58.38	79.79
16	20.63	66.01	86.64
17	24.50	49.38	73.88
18	25.70	56.36	82.06
19	27.96	55.73	83.69
20	20.24	56.42	76.66
Mean	24.8	52.3	77.1
SD	5.84	6.55	6.94

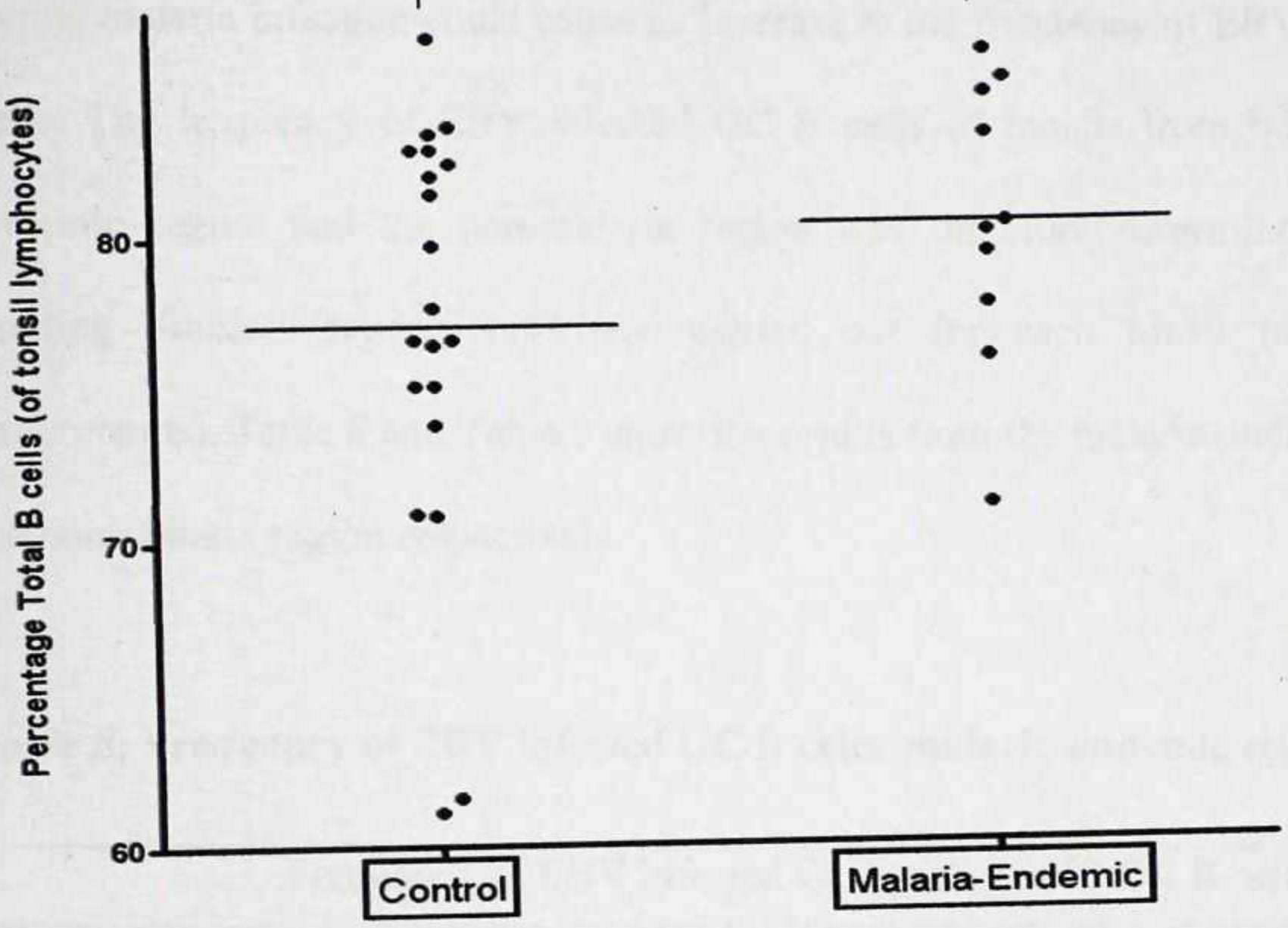

Figure 22: Percentage total B cells of all tonsil lymphocyte; malaria-endemic vs non-malaria region

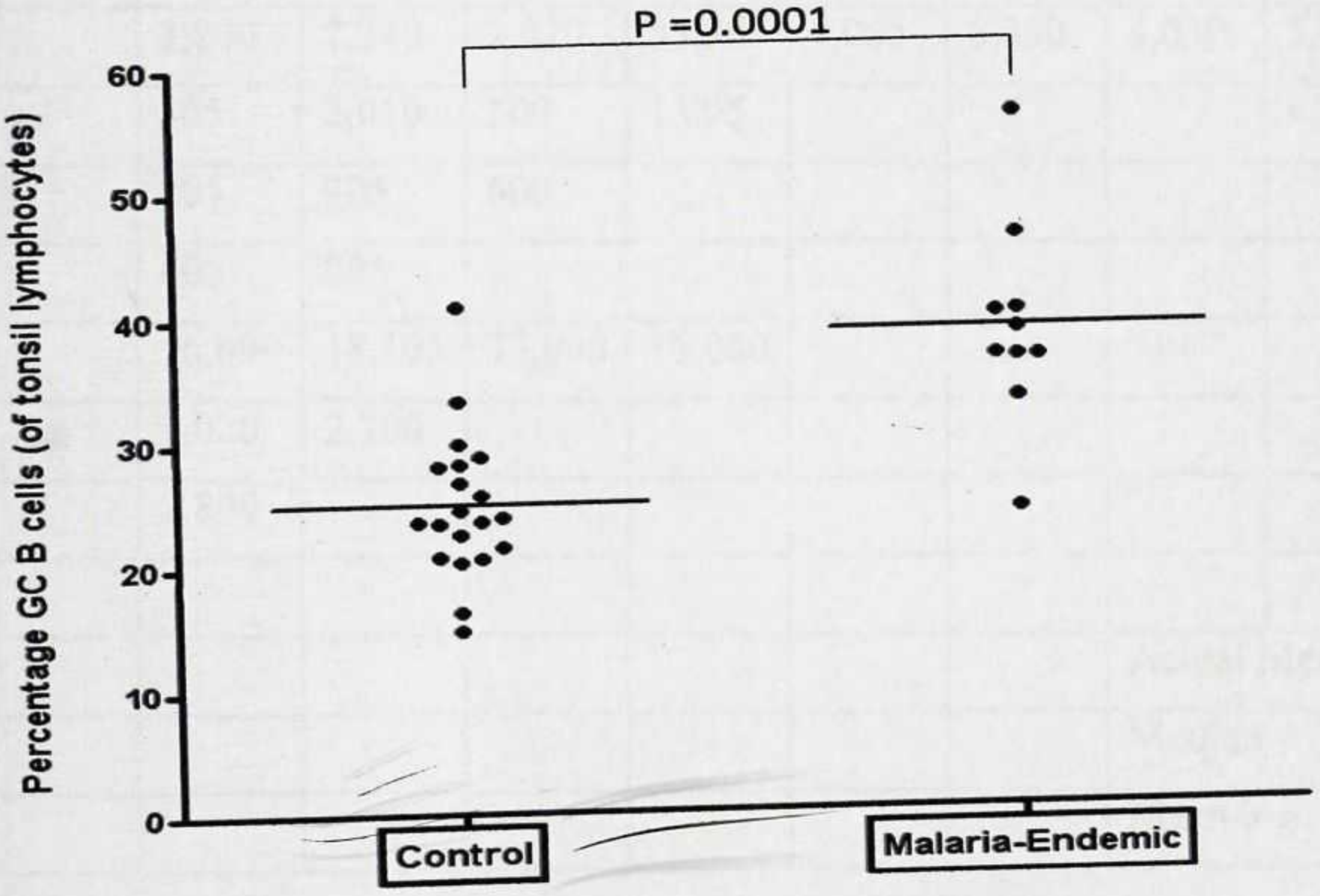

Figure 23: Percentage GC B cells of all tonsil lymphocytes; malaria-endemic vs non-malaria region

As earlier stated, it was proposed that immunosuppression and chronic B cell stimulation during malaria infection could cause an increase in the frequency of EBV infected GC B cells. The frequency of EBV infected GC B cells of tonsils from both the malaria-endemic region and the non-malaria region was therefore determined. Independent limiting dilution experiments were carried out for each tonsil (maximum of 8 experiments). Table 8 and Table 9 show the results from the malaria-endemic region and the non-malaria region respectively.

Table 8: Frequency of EBV infected GC B cells; malaria-endemic region

Frequency of EBV infected GC B cells/ 10^7 GC B cells									
	Number of experiments								
Tonsil code	1	2	3	4	5	6	7	8	Mean
1	3,420	5,030	2,820	2,210	3,220	2,210			2,702
2	3,200	2,240	2,020	5,030	4,025	5,030	4,030	3,020	3,574
3	805	2,010	500	1,005					1,080
4	705	905	600						737
5	605	905							755
6	16,090	18,105	15,090	15,090					16,094
7	2,020	2,700							2,360
8	2,800								2,800
							Actual Mean		3,763
							Median		2,531
							Mean log		2,232

Table 9: Frequency of EBV infected GC B cells; non-malaria region

Frequency of EBV infected GC B cells/ 10^7 GC Bcells					
	Number of experiments				
Tonsil code	**1**	**2**	**3**	**4**	**Mean**
1	100	90	85	85	90
2	200	160	170	155	171
3	40				40
4	80	100	80		87
5	Nil	Nil	Nil		Nil
6	1,005	1,410	1,005		1,140
7	30				30
8	30				30
9	5				5
10	805	500	700		668
11	200	300	200		233
12	10				10
13	80				80
14	100	200	200		167
15	80	60	50		63
16	100	200	100		133
17	5				5
18	10				10
19	10				10
20	10				10
21	Nil	Nil			Nil
				Mean	157
				Median	52
				Mean log	51

The median and the calculated mean log values of the frequency of EBV infection of each sample were chosen for comparisons instead of the "actual" mean because the EBV frequency distribution in both the malaria-endemic and the non-malaria region are not normal distributions (see appendix F for mean log calculation). For the malaria-endemic region, a median frequency of EBV infected cells of 2,531 infected GC B cells per 10^7 GC B cells was obtained. The mean log frequency of EBV infected cells was 2,232 infected GC B cells per 10^7 GC B cells. The median and mean log value of the frequency of EBV infected GC B cells per 10^7 GC B cells for the non-malaria region was 52 and 51 respectively. This is in agreement with a large amount data that our laboratory has previously published pertaining to the frequency of EBV infected GC B cells from chronic carriers (healthy controls). It can therefore be deduced that the frequency of EBV infected GC B cells from the malaria endemic region is about 42 fold more than the frequency of EBV infected GC B cells from the non-malaria region (controls). This fold difference of the level of EBV infected GC B cells between the malaria-endemic and the non-malaria region is significant ($p= 0.004$) (Figure 24) and similar to the fold difference that has been reported between immunosuppressed patients vs controls: 50 (Thorley-Lawson and Gross, 2004). According to Moormann *et al.*, (2005), in whole blood, the log EBV copies/μg of DNA from children in a holoendemic region of Kenya was 2.10 ± 0.38 and in an area of unstable malaria transmission with periodic outbreaks also in Kenya, the log EBV copies/μg of DNA from children was 1.0 ± 0.29. The log EBV copies/μg of DNA from adults in Kenya was 0.34 ± 0.56 and that of US adults was -1.98 ± 0.46. The fold difference of EBV loads between children in the holoendemic region and children in the unstable transmission region is therefore 12.6. On the other hand the fold difference of EBV loads between children in the holoendemic

region and Kenyan adults is 57. Together with the result from this work, it is tenable that exposure to holoendemic malaria results in the elevation of EBV loads in the peripheral blood as well as the GC B cells of children from holoendemic malaria regions.

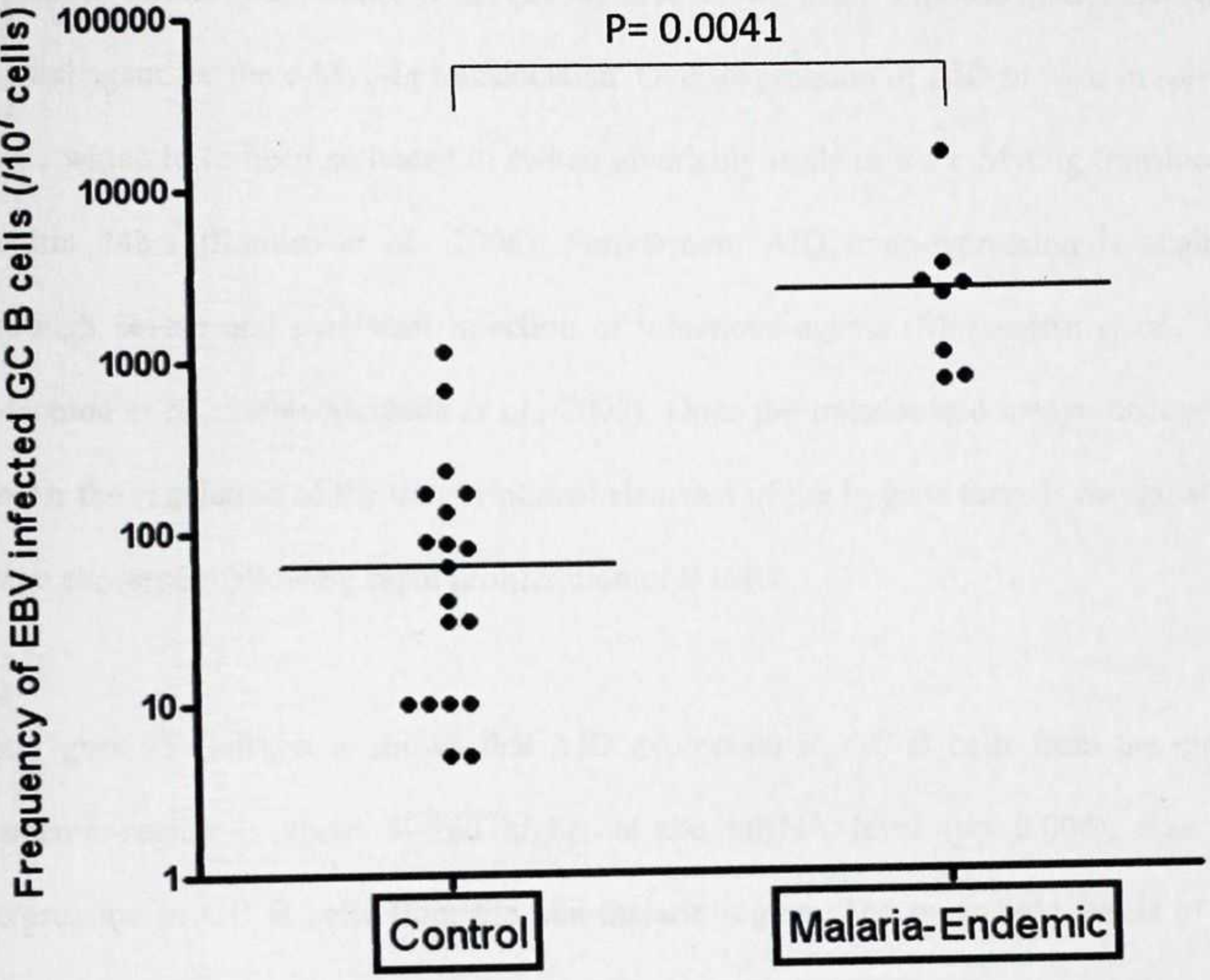

Figure 24: Frequency of EBV infected GC B cells, malaria-endemic vs non-malaria region

4.5 High level of AID expression in GC B cells of tonsils from malaria-endemic region as compared to tonsils from a non-malaria region

Activation induced cytidine deaminase (AID) is a pre-requisite for the c-Myc/Ig translocation (Ramiro *et al.,* 2004; Ramiro *et al.,* 2006; Robbiani *et al.,* 2009), which is a characteristic of BL. Ramiro *et al.,* (2004) have shown using a mouse model that AID is a vital agent for the c-Myc-Ig translocation. Over-expression of AID *in vitro* in spleen B cells which have been activated to switch invariably leads to the c-Myc/Ig translocation within 24hrs (Ramiro *et al.,* 2006). Furthermore AID over-expression is attainable through severe and persistent infection of infectious agents (Muramatsu *et al.,* 1999; Machida *et al.,* 2004; Machida *et al.,* 2005). Once the translocated c-Myc oncogene is under the regulation of the transcriptional elements of the Ig gene there is deregulated c-Myc expression allowing rapid proliferation of B cells.

In Figure 25 (left), it is shown that AID expression in GC B cells from the malaria endemic-region is about 5 fold higher at the mRNA level ($p= 0.004$), than AID expression in GC B cells from the non-malaria region. The mean fold levels of AID expression observed in non-malaria and malaria-endemic GC B cells were 11.01 ± 1.95 and 54.94 ± 34.19 respectively compared to the caliberator, IgD+CD10- tonsillar B cells (which are predominantly naive B cells). According to the data of Smit *et al.,* (2003) (Figure 25 right), AID mRNA expression levels in GC B cells of healthy people is about 13 fold higher than in corresponding tonsillar naive B cells.

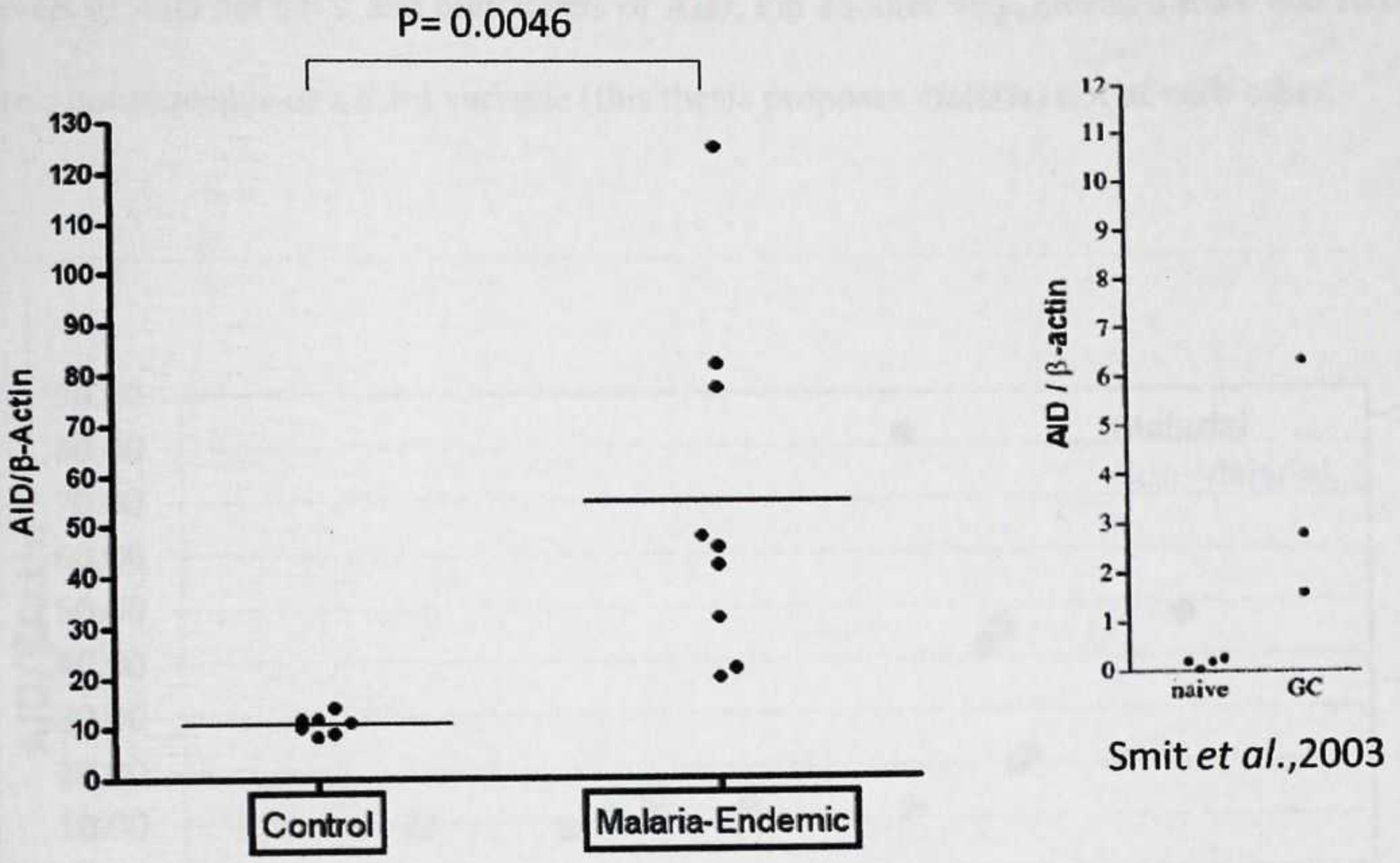

Figure 25: AID mRNA expression in GC B cells from malaria-endemic region and non-malaria region. Real Time (Quantitative) RT-PCR analysis of AID and β-actin was performed on GC B cells from both malaria and non-malaria regions. Each dot represents three independent AID/ β-actin ratio measurements (experiments). IgD+CD10- B cells were used as caliberator.

4.5.1 Level of EBV infection and *in vivo* levels of AID mRNA expression do not correlate

Since EBV infection of B cells *in vitro* as well as EBV associated factors have been shown to induce AID expression (Casola *et al.*, 2004; He *et al.*, 2003; Epeldegui *et al.*, 2007a) the correlation between the level of EBV infection and AID mRNA expression levels was first investigated. In Figure 26, it can be found that rather surprisingly there is no correlation. For non-malaria GC B cells the levels of AID were quite constant over a two log range of EBV infection. Conversely AID levels in malaria GC B cells varied about 10 fold over a 10 fold variation in EBV infection but with no correlation whatsoever between the two. Thus the strong correlation is between malaria and high

levels of AID not EBV and high levels of AID. Put another way, elevated EBV and AID are a consequence of a third variable (this thesis proposes malaria) not of each other.

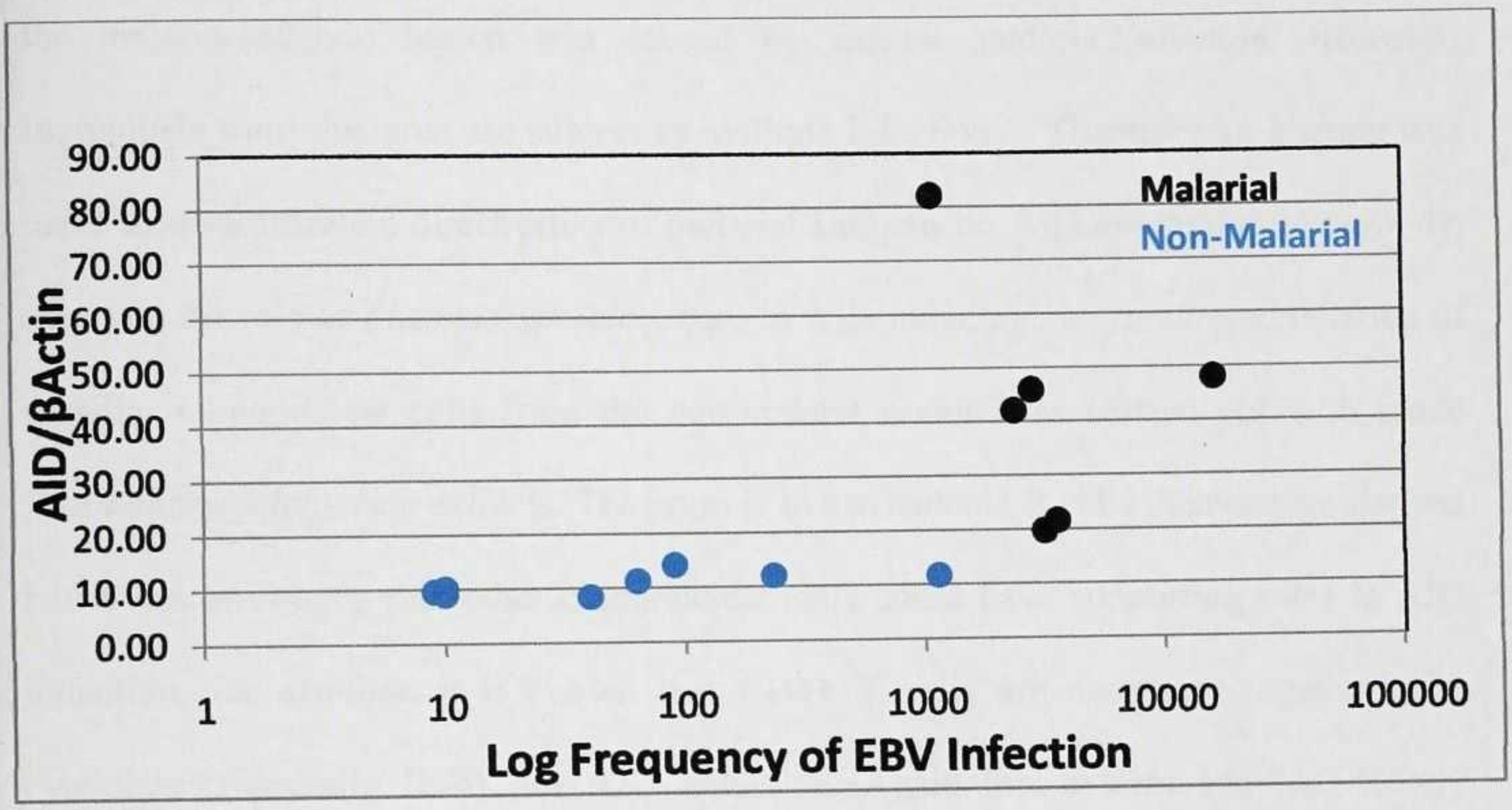

Figure 26: AID mRNA expression and frequency of EBV infection do not correlate

So, per our data, there is no correlation between the level of EBV infected cells and level of AID mRNA expression, it can therefore be hypothesized that the high AID mRNA expression in GC B cells from the malaria-endemic region could be a result of *Plasmodium falciparum*, another co-factor in BL pathogenesis.

It is worth noting that out of 7 patients within the ages of 2-16 years, 6 of them (representing 86%) had detectable *Plasmodium falciparum* parasites either in the blood and/or in the tonsils (this was done by microscopy and *Plasmodium falciparum* rapid diagnostic test (RDT), see methods for details: by the kind courtesy of Stephen Kusi).

4.6 *In vitro* stimulation of tonsillar mononuclear cells with crude *Plasmodium falciparum* extracts

It was also hypothesized that elevated AID levels in the GC B cells of the tonsils from the malaria-endemic region was caused by chronic malaria infection. However, individuals from this area are subject to multiple infections. Therefore an attempt was made to demonstrate a direct effect of malarial antigens on AID expression *in vitro*. To ascertain the role of *Plasmodium falciparum* in AID induction, an *in vitro* stimulation of tonsillar mononuclear cells from the non-malaria region was carried out with crude *Plasmodium falciparum* extracts. The norm is to use isolated B cells in such stimulations but it was envisaged that other mononuclear cells could have supporting roles in AID induction, for example it is known that CD4+ T cells are necessary together with cytokines (especially IL-4), for AID induction. Again this system adopted, closely parallels reality since B cells interact with other cells in the process of activation.

The effect of various stimulants on AID induction in the adopted *in vitro* set up (Figure 27) was first ascertained since this was a different experimental system. In the initial experiments, CpG was used in place of *Plasmodium falciparum* antigens because malarial antigens have been reported to act as TLR9 ligands (just like CpG) (Parroche *et al.*, 2007) and it is known that CpG stimulation of B cells leads to AID induction. Figure 27 shows results of the initial stimulation experiments. B cells were sorted after 3, 5 and 10 days of stimulation and AID and β-actin real time RT PCR were performed. As expected CD40 ligand together with IL-4 successfully induced AID expression in B cells sorted from the stimulated tonsillar mononuclear cells (Figure 27). In the adopted system, CD40 ligand, IL-4 and CpG individually do not induce AID expression. CD40

ligand and CpG together also failed to induce AID; indeed Ruprecht and Lanzavecchia, (2006) have reported that even anti-Ig and CpG alone failed to induce AID in stimulated naive B cells. When CpG was added together with CD40 ligand and IL-4, however, there was a remarkable increase in AID as compared to cells stimulated with CD40 and IL-4 (Figure 27). Indeed, TLR9 ligation (as in the case of CpG) plays an essential role in enhancing the CD40 and BCR signals that invariably lead to AID induction and CSR (Reviewed in Xu *et al.*, 2007). In the initial *in vitro* experimental model peak AID expression was seen on day 5.

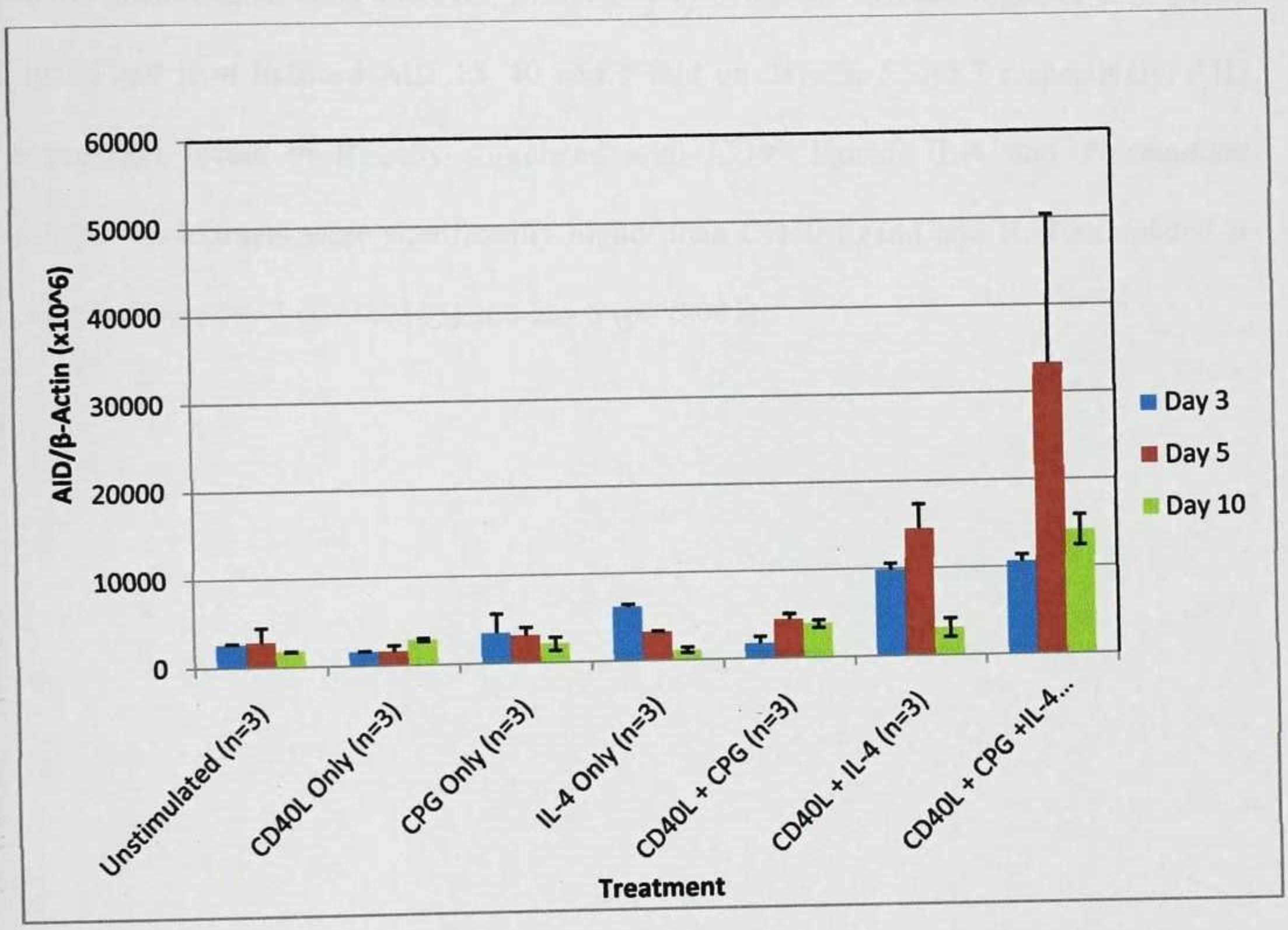

Figure 27: AID mRNA expression in tonsil B cells sorted from whole tonsil mononuclear cell stimulations(A). After 3, 5 and 10 days of stimulating whole tonsillar mononuclear cells with various agents, B cells were sorted and AID and β-actin real time RT PCR was carried out. (See appendix D for flow cytometry data). Error bars represent mean ± SD (n=3).

4.6.1 *Plamodium falciparum* crude extracts induce AID expression appreciably in a CpG-like fashion

Plasmodium falciparum extracts alone induced AID albeit not appreciably (2 fold) in tonsillar B cells (Figure 28) unlike stimulation with CpG only. However when *Plasmodium falciparum* extracts was added together with CD40 ligand and IL-4, AID expression levels increased appreciably on both day 3 (4 fold increase) and day 5 (7 fold increase) compared to cells stimulated with both CD40 ligand and IL-4. When compared to the unstimulated cells however, *Plasmodium falciparum* extracts together with CD40 ligand and IL-4 induced AID 15, 40 and 5 fold on days 3, 5 and 7 respectively. AID expression levels in B cells stimulated with CD40 ligand, IL-4 and *Plasmodium falciparum* extracts were significantly higher than CD40 ligand and IL-4 stimulated B cells, both on day 3 ($p= 0.0303$) and day 5 ($p< 0.001$).

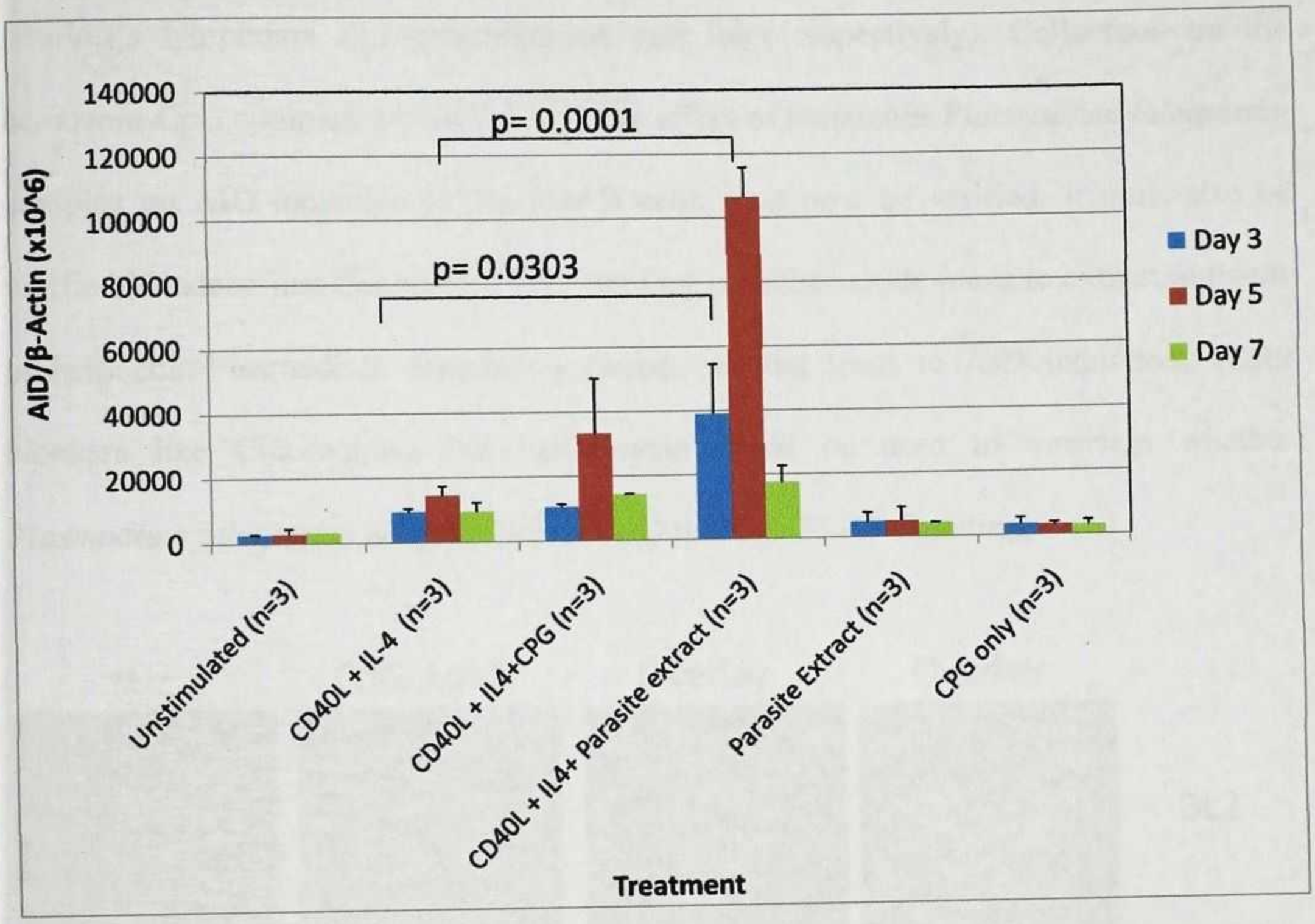

Figure 28: AID mRNA expression in tonsil B cells sorted from whole tonsil mononuclear cell stimulation (B). After 3 days, 5 days and 7 days of stimulating whole tonsillar mononuclear cells with various agents including *Plasmodium falciparum* crude extracts, B cells were sorted and AID and β-actin real time RT-PCR was carried out. (See appendix D for flow cytometry data). Error bars represent mean ± SD (n=3).

It is imperative to ascertain what factor(s) in *Plasmodium falciparum* crude extracts induce or augment AID expression. Since it is known that this parasite factor(s) act in a similar manner as CpG, it was hypothesized that hemozoin bound to *Plasmodium falciparum* DNA could be the factor inducing AID in the tonsillar B cells. Hemozoin bound to *Plasmodium falciparum* DNA has been shown to bind to TLR9 just like CpG. It was imperative to show that B cells can internalize both hemozoin and *Plasmodium falciparum* since TLR9 receptor is intracellular. Figure 29 shows a cursory hemozoin-CpG internalization assay, where CpG replaces the parasite DNA. Hemozoin was first bound to Alexa 488-bound CpG overnight and was introduced onto BL2 and IM-171

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(Burkitt's lymphoma and lymphoblastic cell lines respectively). Cells took up the hemozoin-CpG complex within 2-4 hrs. The effect of hemozoin-*Plasmodium falciparum* complex on AID induction in tonsillar B cells must now be verified. It must also be verified if indeed just like CpG, TLR9 binding by either crude parasite extract antigens or hemozoin-Plasmodium falciparum extract is what leads to AID induction. TLR9 blockers like Chloroquine and bafilomycin could be used to ascertain whether *Plasmodium falciparum* antigens induce AID through TLR9 signaling.

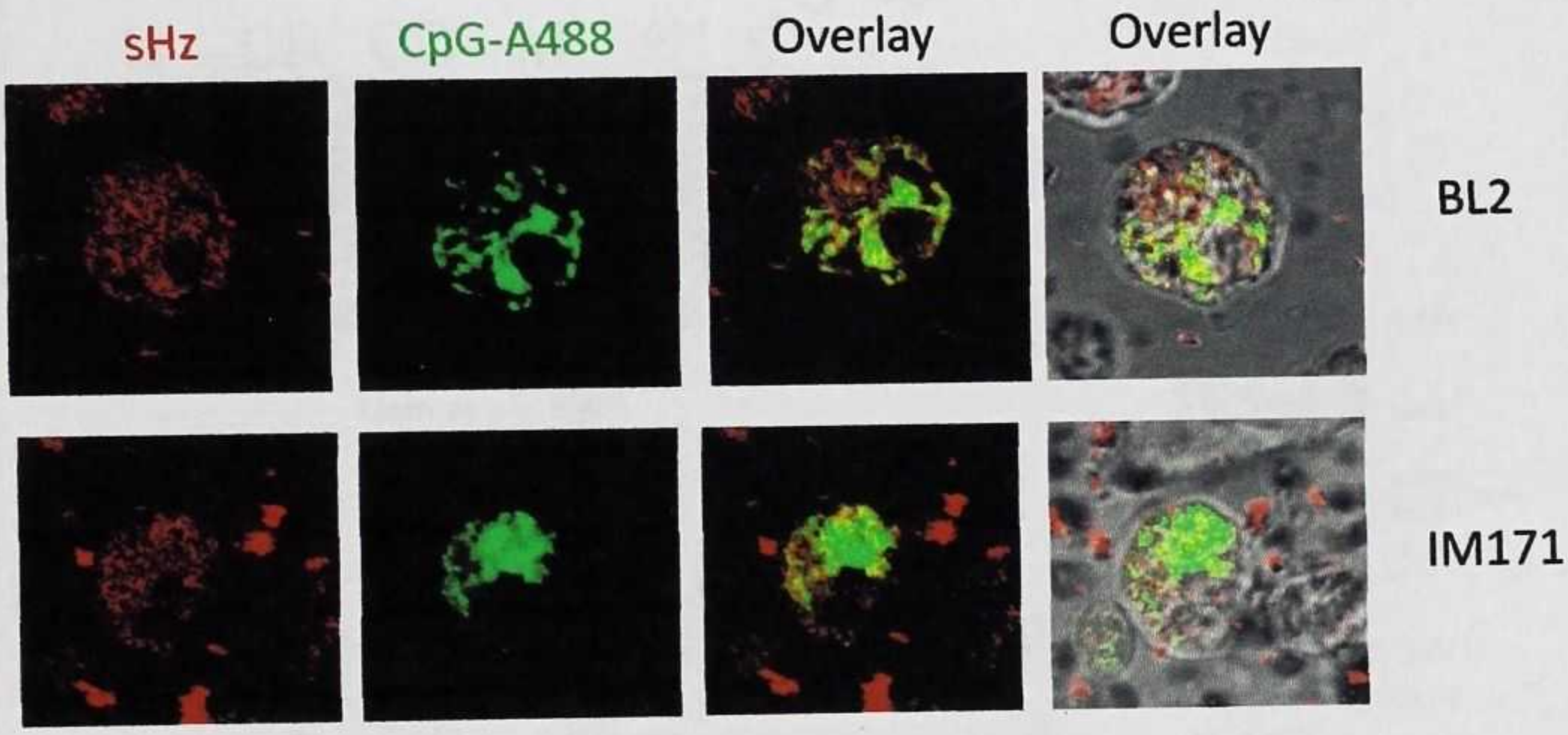
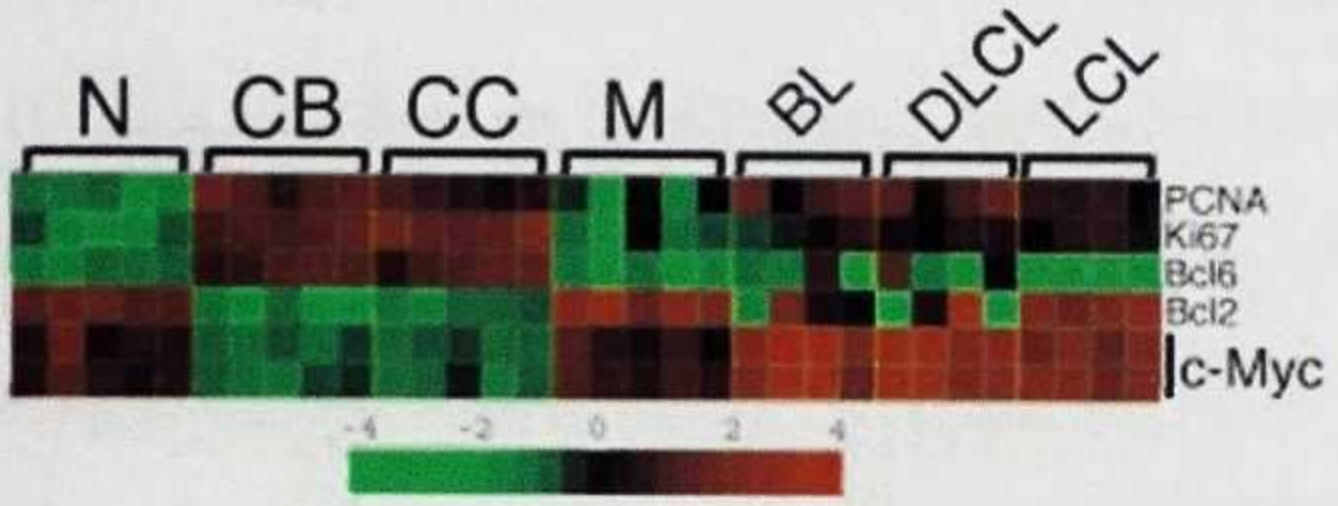


Figure 29: Synthetic hemozoin (sHz) and CpG DNA are internalised by B cells. 5 µg of CpG-Alexa 488 bound to 100 µg of synthetic hemozoin (sHz) was added to BL2, and IM171 cell lines and incubated for 2-4 hours after which confocal microscopy was carried out (Assistance provided by: Pariasa Kalantari, Golenboch Lab)

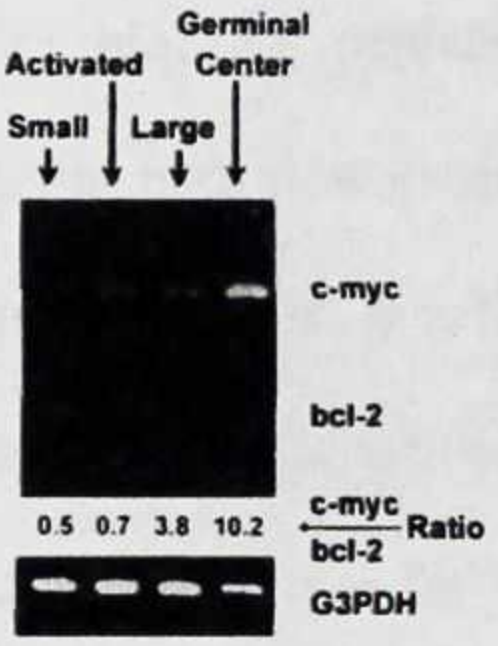
4.7 Oncogenic c-Myc, is expressed in GC B cells

The oncogenic c-Myc protein is a global regulator of transcription in many cell types and it exhibits a very complex regulation. Indeed the expression of c-Myc in GC B cells still remains somewhat controversial, as some reports maintain it is not expressed at all in GC B cells, while others claim it is expressed albeit not very highly (Klein *et al.*,

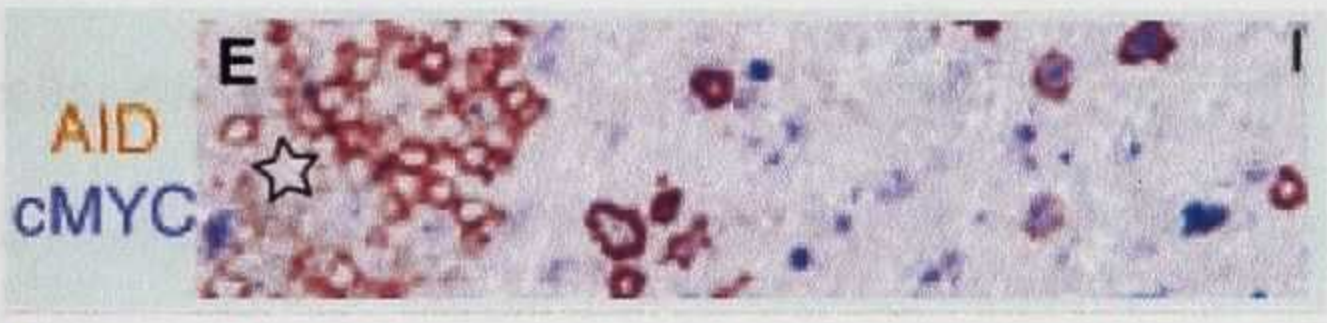
2003; Catoretti *et al.*, 2006) (Figure 30 left, up and down). Martinez-Valdez *et al.*, (1996) and Cutrona *et al.*, (1997), on the other hand report that c-Myc is highly expressed in GC B cells (Figure 30 right). The role of c-Myc in normal GC physiology or in eBL initiation is not fully understood. It has been argued that c-Myc might be turned on (Thorley-Lawson and Allday 2008) and deregulated in EBV infected GC precursor B cells prior to the translocation event.



Klein et al., 2003



Cutrona *et al.*, 1997; Martinez-Valdez *et al.*, 1996

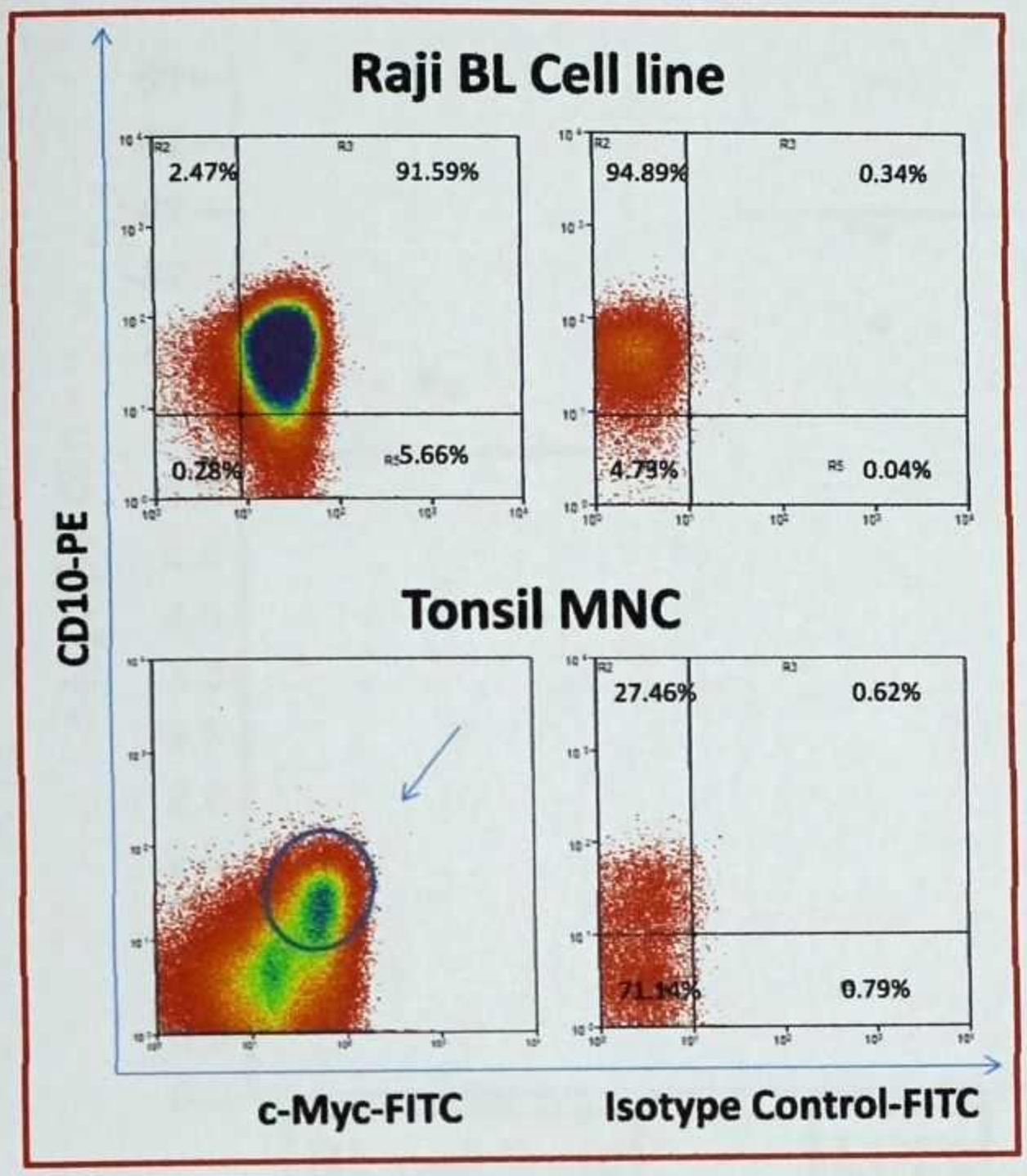


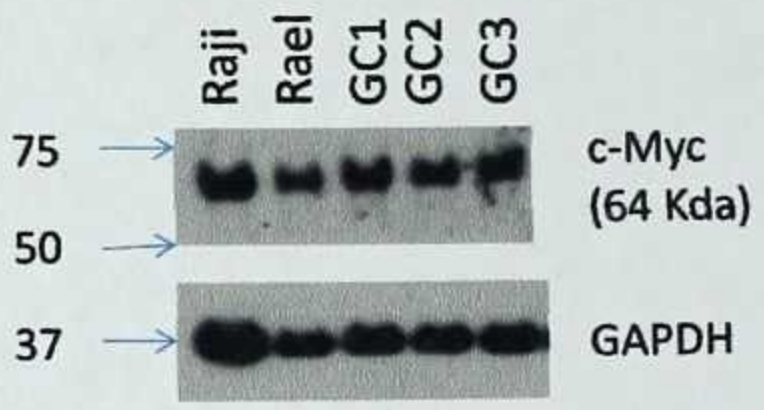
Cattoretti *et al.*, 2006

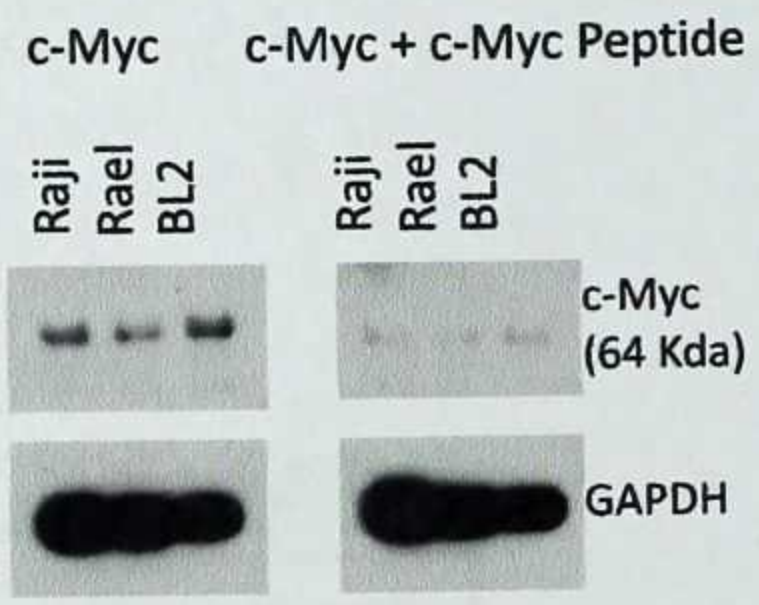
Figure 30: c-Myc expression in germinal center B cells. Klein *et al.*, (2003) and Cattoretti *et al.*, (2006) (Top and bottom left) have both argued that c-Myc is not expressed in germinal center B cells as per their micro-array and immunostaining respectively whereas Cutrona *et al.*, (1997) and Martinez-Valdez *et al.*, (1996) have both argued as per their RT-PCR data that c-Myc is highly expressed in germinal center B cells (left).

Both cases (default expression or ectopic expression of c-Myc) could have very different implications for pathogenesis and therefore must be diligently explored.

Assays for c-Myc expression in GC B cells were performed in order to resolve the apparent contradictions. This thesis work reports that c-Myc is expressed in GC B cells. c-Myc flow cytometry using two c-Myc antibodies (from two different companies) demonstrates that c-Myc is present in GC B cells (Figure 31A). Figure 31B, c-Myc western blot on the BL cell lines Raji and BL2 as well as GC B cells from three tonsils (from non-malaria region) also indicates that c-Myc protein is expressed in GC B cells. A blocking assay with c-Myc peptide specific for the antibody used (Sigma, St. Louis, USA) was also carried out to ascertain the specificity of the western blot result (Figure 31C). It is clear from the blocking assay that the c-Myc western blot antibody is specific for c-Myc as seen by the diminishing of signals when the blocking peptide was introduced. Image stream analysis (Figure 31D), which combines both flow cytometry and confocal microscopy was also carried out for c-Myc expression in GC B cells and again the results are compelling evidence that c-Myc protein is expressed in GC B cells. In Figure 31D, staining for the nuclear Bcl6, a benchmark standard marker for GC B cells, indicates that we do achieve nuclear staining. Real time RT-PCR (Figure 32) of c-Myc expression in BL cell lines and lymphoblastic cell lines was also carried out and this was compared to c-Myc expression in GC B cells from the non-malaria region. Lymphoblastic cell lines (LCL) are the best controls for c-Myc mRNA expression since these do not carry the c-Myc/Ig translocation which is carried by the BL cell lines. c-Myc mRNA expression in the LCLs were all high as expected (as this accounts for the rapid proliferation of LCLs). c-Myc mRNA expression in the BL cells lines was lower on the average than c-Myc mRNA expression in LCLs.







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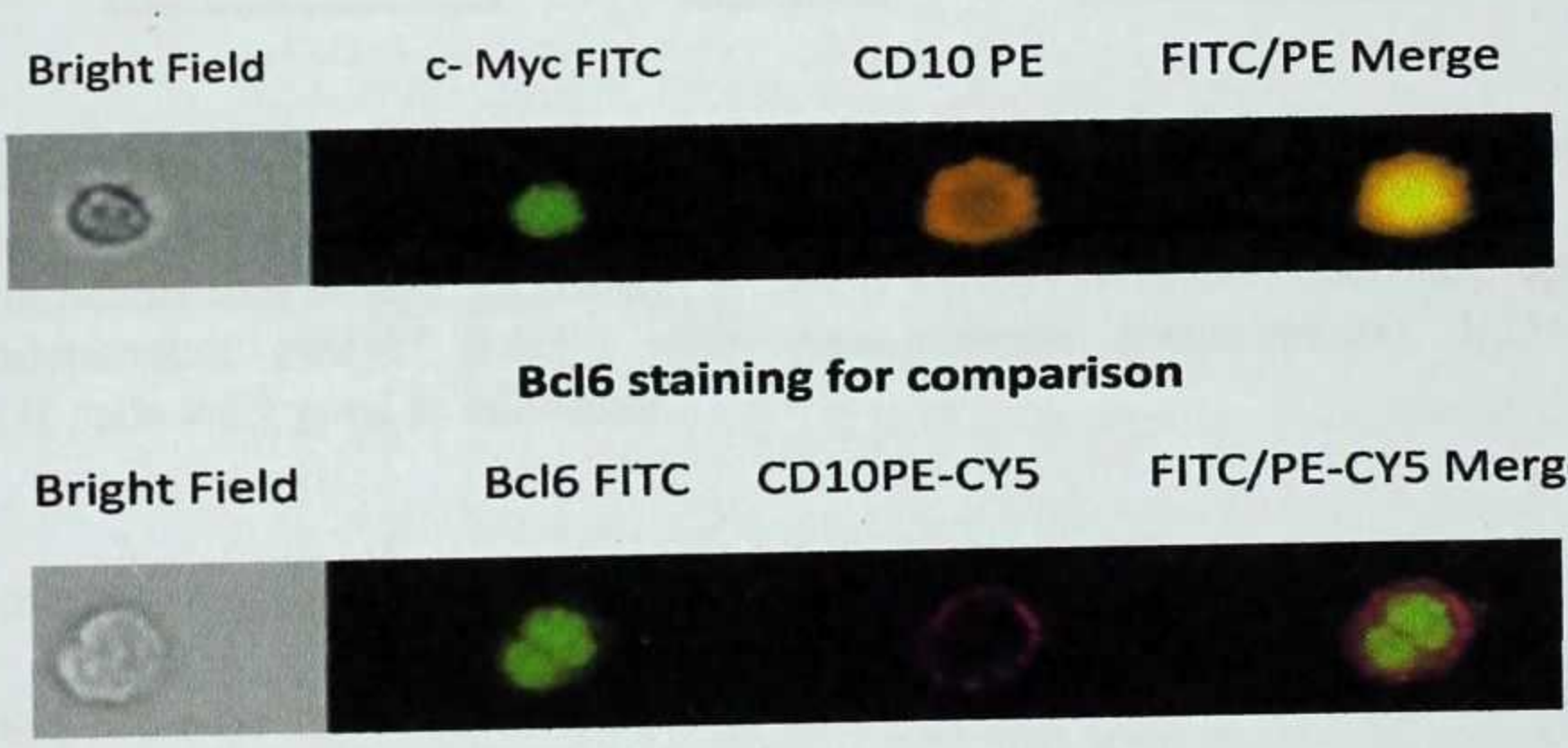

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Figure 31: Germinal center B-cells clearly express c-Myc protein. Panel A shows Raji BL cell line and tonsil mononuclear cells that were stained with the extracellular CD10 antibody and intranuclear c-Myc antibody; two different c-Myc antibodies were used. Panel B depicts western blot for c-Myc using 2 BL cell lines Raji and Rael and GC B cells of three separate US tonsils. Panel C is a c-Myc blocking western blot assay that was carried out for three positive control BL cell lines, Raji, Rael and BL2; the right represents c-Myc western blot without blocking peptide whereas the left is c-Myc + c-Myc peptide. Panels D shows image stream analyses carried out on CD10 and c-Myc stained whole tonsil lymphocytes (top) and CD10 and Bcl6 stained whole tonsil lymphocytes, respectively (bottom). Bcl6 represents an established GC B cell marker.

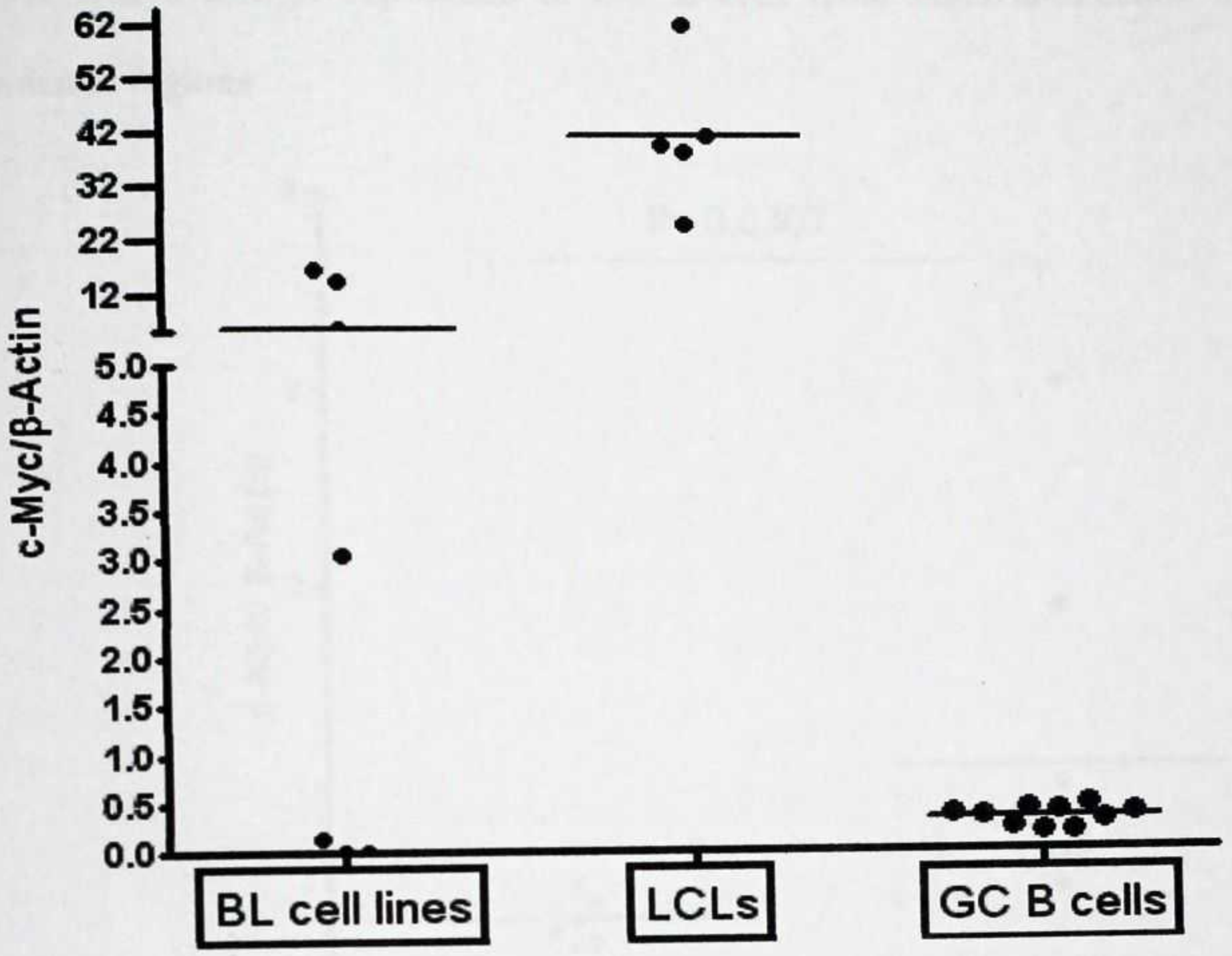

Figure 32: c-Myc mRNA is expressed in GC B cells albeit at very low levels compared to BL cell lines and Lymphoblastic cell lines (LCLs). Real Time RT PCR of c-myc mRNA indicates that c-Myc expression in GC B cells is transient. Each dot represents three independent c-Myc/ β-actin ratio measurements (experiments). IgD+CD10- tonsillar B cells were used as calibrator.

c-Myc micro-array data of Klein *et al.*, (2003) (Figure 30 top left), indicate a low expression of c-Myc mRNA in GC B cells compared to naive B cells. The expression of c-Myc in GC B cells offers one means by which AID expression might be increased in view of c-Myc's role as a global regulator of transcription.

4.7.1 c-Myc mRNA expression in GC B cells from malaria-endemic and non-endemic regions

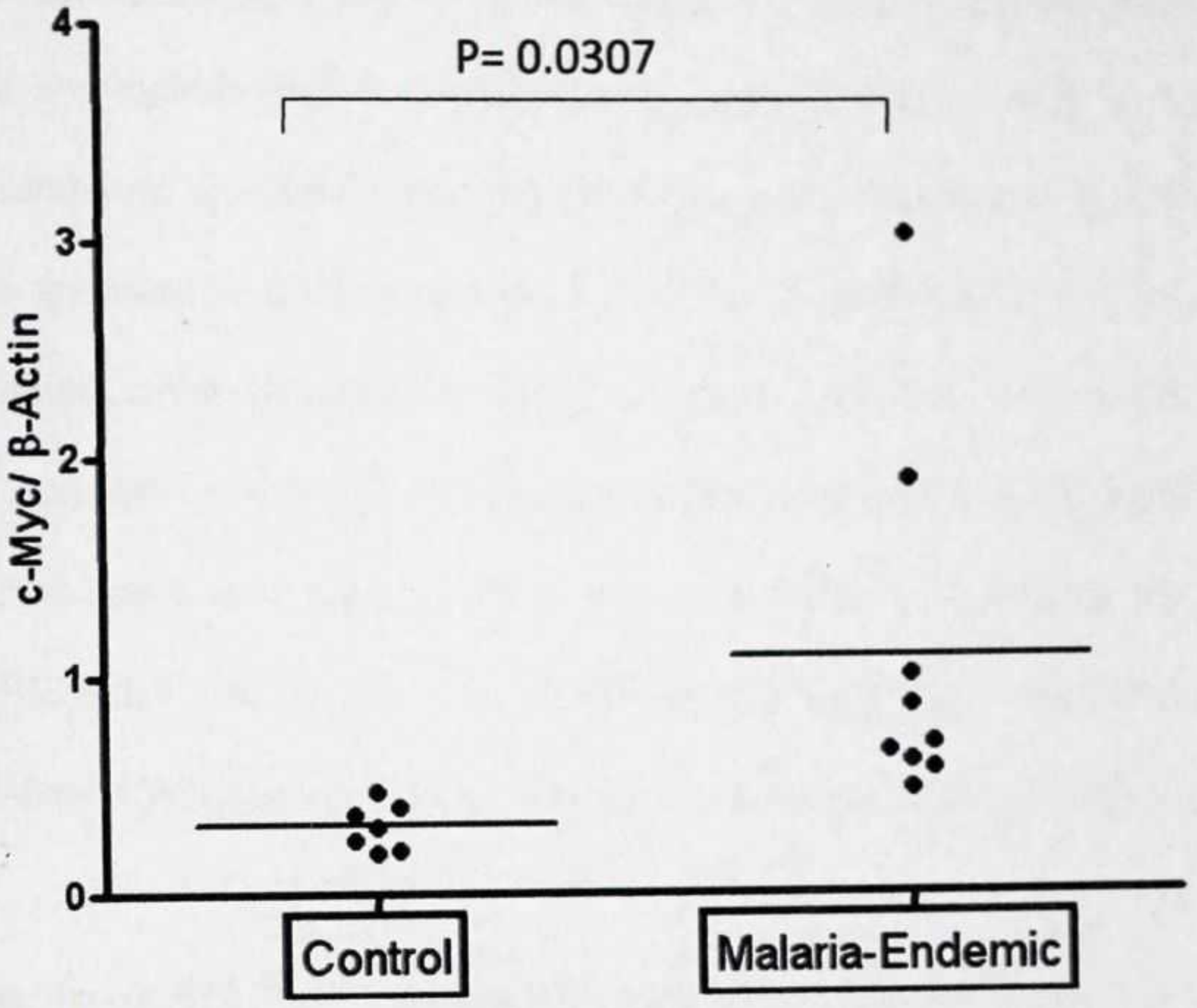

Figure 33: c-Myc mRNA expression in GC B cells of tonsils from malaria endemic region (Kumasi, Ghana) is slightly higher than c-Myc RNA expression in a non-malaria region (Boston, US). As usual each dot represents three independent c-Myc/ β-actin ratio measurements (experiments). IgD+CD10- tonsillar B cells were used as calibrator.

After being convinced that c-Myc is expressed in GC B cells, c-Myc expression in GC B cells from the malaria-endemic and the non-malaria regions was compared. It was found that c-Myc expression in GC B cells from the malaria endemic region was 4 fold higher than the c-Myc mRNA expression in GC B cells from the non-malaria region (Figure 33). The difference in c-Myc expression in the two groups was significant (p= 0.0307) but not nearly so striking the difference with EBV or AID.

A cumulative fold risk of ~2,000 was arrived at based on the fold differences in

parameters between the malaria endemic and non-malaria regions (Table 10). This cumulative risk of 2,000 can be described as the risk or probability of a c-Myc/ Ig translocation event occurring in the GC B cells from the malaria-endemic region as opposed to the non-malaria region. Needless to say this estimate is based on several speculative assumptions specifically that the risk of the c-Myc translocation is directly proportional to the increase in these paramters and that the cumulative risk is simply additive (or multiplicative). Nevertheless given these major caveats it is noteworthy that the calculated cumulative fold risk is strikingly similar to the risk or probability of acquiring eBL as opposed to sBL, which is also ~2,000 fold [i.e. judging from the incidence of eBL which ~20 cases in 100,000 children and incidence of sBL 0.01 cases in 100,000 children (Rochford *et al*., 2005; Thorley-Lawson and Allday, 2008)].

Table 10: Cumulative fold risk of BL: malaria-endemic vs. non-malaria

Assuming fold increases represents risk of a translocation event

Parameter	**Fold difference: Malaria-endemic vs non-malaria**
GC Throughput	2 fold
Frequency of EBV infection	42 fold
AID mRNA expression	5 fold
c-Myc mRNA expression	4 fold
Cumulative fold risk	2,000 fold risk of a translocation event

CHAPTER FIVE

5.0 DISCUSSION

Malaria, Epstein-Barr virus (EBV) and germinal center (GC) B cells lie at the center of the endemic Burkitt's lymphoma (eBL) enigma. So far, this work has tried to understand the interaction of malaria infection and EBV on the one hand with GC B cells (where the c-Myc/Ig translocation that seed Burkitt's lymphoma is proposed to occur). Undoubtedly, understanding the "normal" interaction of the aforementioned factors with GC B cells will give us leads as to how GC B cell derived tumors arise or specifically how eBL arises. These results therefore throw light on how normal interactions between malaria infection, EBV and the GC either create a permissive context in which eBL can occur or how these interactions directly cause eBL.

5.1 EBV episome copy number distribution as a tool for understanding EBV dynamics in EBV infected B cell populations

To begin with, this work set out to understand the interaction of EBV with the various B cell sub-populations. The EBV proliferation dynamics in various EBV infected B cell populations from tonsils and peripheral blood was investigated. The EBV episome copy number distributions of the various B cell sub-populations were generated as a function of proliferation. In other words the EBV episome distribution of any B cell population indicates approximately how many times cells in that particular B cell population have divided. This endeavour has multi-faceted benefits, chief of which is that we are able to further appreciate how EBV establishes persistence ultimately in memory B cells. In another vein although subsidiary, it is believed that endemic Burkitt's lymphoma is a

disease characterized by the proliferation of EBV infected cells which have once upon a time participated in the GC reaction. Furthermore, endemic Burkitt's lymphoma cell lines notably Jijoye, and Raji (Pulvertaft, 1964; cited by; Drexler and Minowada, 1998; Karpova *et al*., 2005) have been reported to contain high EBV genome copy numbers (~50-100 genome copies) (Hurley and Thorley-Lawson, 1988; Delecluse *et al*., 1993). B cells with high EBV episome copies could therefore be candidates for eBL pathogenesis. Understanding how EBV establishes persistence through EBV proliferation dynamics therefore has benefits for understanding BL pathogenesis as well.

As stated earlier, a study of the number of EBV infected cells per GCs per tonsil indicated that these EBV infected GC B cells might have undergone few divisions and then leave the GC (alias the three divisions and out model). It is also known from the same study that there is an average of 3.5 EBV positive cells (range 1-9) in an EBV positive GC (Roughan *et al*., 2010). When the EBV episome distribution in GC B cells was compared to two models generated from the *in vitro* data, it became clear that GC B cells latently infected with EBV proliferate extensively without increasing in absolute number. Practically half of the GC B cell population seem to have undergone about three divisions whereas the other half has undergone at least 20 divisions. In effect, this work together with Roughan *et al*., 2010, makes a strong case for the assertion that EBV infected GC B cells undergo extensive proliferation without increasing in absolute number. This work therefore provides a description of the proliferation and expansion dynamics of EBV infected GC B cells en-route to the memory compartment in which the virus persists. Thus it has been shown that there is equilibrium between cell death and cell growth with respect to EBV infected GC B cells. This is apparently because

there isn't an increase in absolute numbers of GC B cells although there is extensive proliferation. This result therefore agrees with the fact that EBV infected GC B cells are indeed participating in the GC reaction, and that these cells are not just shuttling through the GC without dividing. Further proof that EBV infected GC B cells are participating in the GC reaction is provided by the fact that EBV infected GC B cells are AID positive (Appendix C). AID is the key enzyme for SHM and CSR which occur in GC B cells. The apparent non-increase in the absolute number of EBV infected GC B cells should most likely be due to cytotoxic T-cell killing. This opens another chapter in the control of EBV infected GC B cells since there is currently no evidence of cytotoxic T cell activity in the GC (Quingley *et al.*, 2007). It remains to be grasped where and how the immune checks on GC B cells happen. It is also possible that limitation of survival factors for these EBV infected GC B cells might contribute to the limitation of their expansion. How EBV infected GC B cells evade immune checks on them could lend priceless clues in the pathogenesis of EBV related cancers of GC origin including eBL.

In another vein GC B cells with high EBV episome copy numbers which succeed in escaping immune checks may be highly prone to lymphoma or eBL. This is because endemic Burkitt's lymphoma cell lines have been reported to contain high EBV genome copy numbers (Hurley and Thorley-Lawson, 1988; Delecluse *et al.*, 1993). It is important to keep in mind that during malaria infection T cell activity is perturbed and this could diminish immunesurveillence of EBV infected GC B cells.

Results of the episome copy distribution in peripheral blood memory B cells indicate that these cells have divided many times, this inference or conclusion is arrived at

because the episome copy distribution of memory B cells from peripheral blood, closely maps the distribution of *in vitro* data for cells that have undergone several divisions (20-35 divisions at least). It is not far-fetched to envisage that these EBV infected memory B cells could still be dividing (Hochberg *et al.,* 2004). There is no resemblance of the peripheral memory B cell distribution with cells that have undergone 1 division; mean 1.1 (±0.3) and median of 1, or cells that have undergone 3 divisions; mean 2.1 (± 2) and median 2.6. It is therefore safe to say that there is no evidence from the episome distribution in peripheral blood memory B cells to indicate that memory B cells are indeed directly infected by EBV. If these EBV infected peripheral memory B cells have divided several times (and/ or are dividing), then this fits with the finding in this thesis that a fraction of the GC B cells have undergone several divisions. This can be rationalized based on the fact that GC B cells in general and EBV infected GC B cells eventually differentiate into memory B cells. This therefore buttresses the necessity of the GC reaction for EBV to access the memory B cell compartment. If memory B cells are dividing and, as proposed, they return to the lymphoid tissue to undergo further rounds of division, then there is an increased probability of a genetic hit in EBV infected memory B cells, especially during malaria, if these memory B cells are forced to re-visit the lymphoid tissue to undergo further rounds of division. It is important to note that BL tumors typicaly have additional genetic lesions aside the c-Myc/Ig translocation (Lindstrom and Wiman, 2002; Lones *et al*., 2004; Dang *et al.,* 2005; Hemann *et al.,* 2005). Memory B cells containing high EBV episomes could accumulate genetic lesions as they divide in the periphery and also as they undergo further divisions in the lymphoid tissue. This situation could seed BL because of the increased likelihood of genetic lesion(s) occurring in these cells. This might present another twist to the

mechanism of eBL pathogenesis.

The EBV episome copy number distribution in memory B cells of tonsil origin is quite similar to the distribution in peripheral blood memory B cells. This might be pointing to the fact that EBV infected memory B cells in the periphery originate from EBV infected memory B cells from the tonsils. The assertion that EBV can directly infect memory B cells in tonsils of IM patients (Kurth *et al.,* 2003) must be a unique situation in these patients since this work sees no evidence of direct infection of EBV infected tonsillar memory B cells as per the EBV episome distribution data. Whereas EBV episome distribution in both tonsillar memory B cells and peripheral blood memory B cells from chronic carriers appear strikingly similar (Figure 15), IM peripheral memory B cells appear comparatively slightly (indeed subtly) skewed to the left compared to memory B cells from peripheral blood of chronic carriers (Figure 17). It could be envisaged that "unorthodox" direct EBV infection of memory B cells could occur during IM albeit subtly and not the norm. Perhaps the pattern of EBV infection in IM patients is only an exacerbation of an otherwise usual process observed in chronic carriers (Thorley-Lawson and Allday, 2008). The striking similarity between the EBV episome distribution in peripheral memory B cells from IM patients and the distribution in memory B cells from tonsils (both closely matching *in vitro* infected cells that have undergone 20-35 divisions, as well as EBV infected peripheral memory B cells) gives impetus to the assertion that memory B cells are not continuously directly infected by EBV under normal circumstance. EBV episome distribution in all the memory B cell populations do not show clear indication of continuous direct infection as per the *in vitro* EBV infection data and work done by Nambo *et al.,* (2007). Babcock and Thorley-

Lawson (2000) have shown that both GC B cells and memory B cells from the tonsils do not express EBNA2 as in the case of newly EBV infected cells. GC B cells and tonsillar memory B cells rather express EBNA1 (Q-K), LMP1 and LMP2 as opposed to directly infected B cells which express all the latency proteins including EBNA2. It is difficult to believe that EBV infected memory B cells from IM patients are any different from the EBV infected memory B cells of chronic carriers. This is because this thesis observed that the percentages of the B cell sub-populations in chronic EBV carriers and IM patients are the same and this has been confirmed by Chaganti *et al.,* (2009). Hochberg *et al.,* (2004) report that IM is characterized by EBV infection of up to 50% of all memory B cells, yet EBV persistence is tightly regulated just like in chronic carriers. Thus the main difference between IM and chronic EBV infection should be the age of infection and the immune response thereof. The rapid cytotoxic T cell response that characterises IM and culminates in pathology should represent an exaggerated form of what happens when children are first infected (chronic EBV infection). All in all it must be stressed that as per this work, EBV infected memory B cells from IM patients are not different from chronic carriers, in that there is no compelling sign of continued direct infection of these memory B cells and their proliferation patterns are the same. Enchantingly, Table 3 indicates that the mean and median EBV episome copy number in memory B cells from peripheral blood and tonsils of chronic carriers as well as peripheral blood of IM patients is strikingly similar. This would cluster EBV infected memory B cells irrespective of their source into one group. Judging from the similarity between EBV episome distribution in the *in vitro* infected B cells that have undergone not less than 20 divisions and the *in vivo* memory B cells, one can safely propound a plausible conjecture that EBV infected B cells continue to proliferate once they enter

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memory.

Although it is hard to think that memory B cells or IM peripheral memory B cells for that matter are continuously directly infected by EBV, the slight left skew in episome distribution seen in IM peripheral memory B cells as compared to peripheral memory B cells from chronic carriers (Figure 17) might indicate the rare possibility of a subtle and subsidiary infection of memory B cells. Granted that this occurs, the fate of these unusual EBV infected memory B cells will have to be determined. This means that EBV infected memory B cells from the periphery most probably came from the GC and they also do not show visible signs of continuous direct infection.

The puzzle in the EBV episome distribution data is the naive B cell episome distribution. If naive B cells represent the initial EBV infected cells which then divide say at most three times and differentiate into GC B cells then one would expect to see an episome distribution which parallels the *in vitro* three divisions data. Rather, what was found was that the EBV episome distribution data of naive tonsillar B cells seems to almost parallel the GC B cell episome distribution. It is possible that a subset of the newly infected and rapidly proliferating naive B cells, could receive secondary signals that drive them through the GC reaction. Another possibility, although quite radical could be that the EBV episomes are not always copied only once per cell division. It is also possible that most of the high episome copy numbers in these tonsillar naive B cells could be contributed by linear EBV genome that is yet to be lost. Usually EBV linear genome lingers for about a week before it is lost and gives way to the singular circular episome (Hurley and Thorley-Lawson 1988).

Whether the unexpected high episome copy numbers represent the yet to be lost linear episome (since linear episomes are lost after about a week *in vitro*) or there is multiple copying per division, remains to be seen. If the unexpected high episome copy numbers in naive tonsillar B cells remains true, it is possible that these extensively proliferating EBV infected naive B cells could be escaping cytotoxic T cell predation due to the apparent lower frequency/ level of EBV infected cells in the tonsillar naive B cell compartment (compared to GC B cells and memory B cells). Simply put, a low threshold of EBV antigens due to the comparatively lower level of EBV infected cells in the tonsillar naive B cell compartment might limit cytotoxic T cell predation. It will be interesting to see the EBV episome distribution in IgD+CD10+ B cells from tonsils, since these are said to be cells that eventually seed GC B cells. This might clarify the transition of EBV infected cells from naive (IgD+CD10-CD27-) B cells through the GC en route to the memory compartment.

Taken together, the accrued EBV episome distribution data lends credence to the GC model of EBV infection and persistence which starts with primary EBV infection of a naive B cell, which is then driven through the GC reaction to become an EBV infected memory B cell, where EBV persists. Figure 34 is a sketch of probable events in EBV infection of naive B cells based on the accrued EBV episome distribution data.

As stated earlier, typically BL tumors have other genetic lesions or genetic abnormalities aside the c-Myc/Ig translocation which speaks to a sequential accumulation of mutations or aberrations prior to or alongside the c-Myc translocation (Lindstrom and Wiman, 2002; Lones *et al*., 2004; Dang *et al.,* 2005; Hemann *et al.,* 2005). Indeed, 92% of BL

patients/ biopsies were found to have additional chromosomal abnormalities (Lones *et al.*, 2004). A point in case is that about 30% of EBV-positive Burkitt's lymphoma carry p53 mutations, and those with wild type p53 frequently over-express MDM2 or loose p14ARF (Lindstrom and Wiman, 2002). Another example is that BL tumors with c-Myc that can induce BIM expression generally have genetic lesions in the p14-p53 pathway (Dang *et al.*, 2005; Hemann *et al.*, 2005).

It is possible that the additional genetic lesions observed in BL (or eBL) might have been accrued throughout the B cell differentiation process of an EBV infected B cell. Naïve B cells with high EBV episome copy numbers could therefore be at high risk of eBL as they might have undergone several divisions prior to their participation in the GC reaction. It is possible that these EBV infected naïve B cells might have accumulated some mutations as a result of rapid proliferation due to EBV even before they undergo the GC reaction. EBV infected naïve B cells express EBNA2 which is known to induce c-Myc expression (together with other cellular activation associated genes) (Wang *et al.*, 1987; Wang *et al.*, 1990; Kaiser *et al.*, 1999). The prolonged expression of EBNA2 in these naïve B cells could lead to over-expression or deregulated expression of c-Myc and this could be a potential source for the acquisition of genetic lesion(s) since c-Myc expression is linked to genomic instability (Vafa *et al.*, 2002; Kuttler and Mai, 2006). It is will be interesting to know if high episome copy numbers correlate with higher expression of EBV genes since this will imply that the level of possibly oncogenic EBV proteins or factors will also be high.

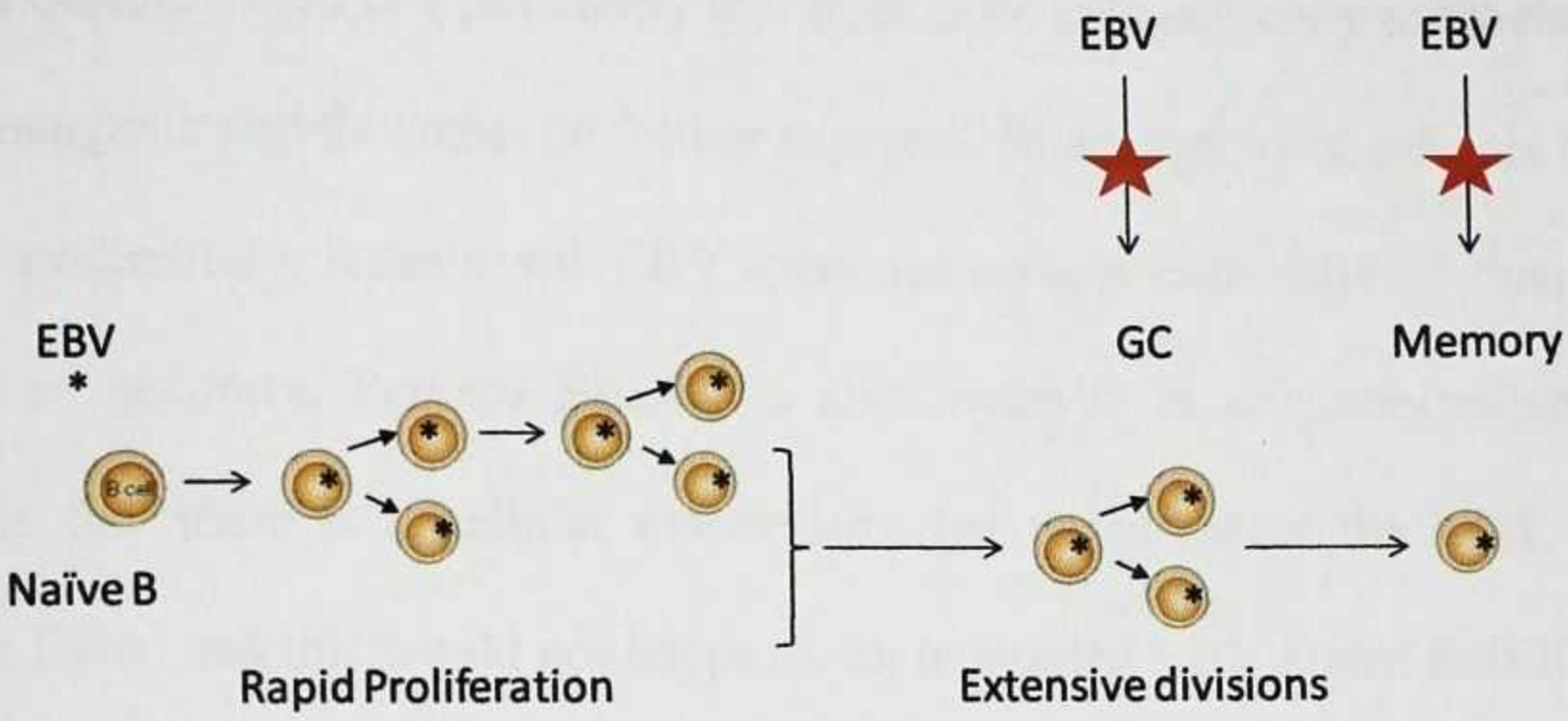

Figure 34: Probable events in EBV infection of naive B cells en route to the memory B cell compartment. EBV infects naive B cells and activates them to become rapidly proliferating blasts. These rapidly dividing EBV infected naive B cells might need a secondary signal which will then allow any of the subsets of both newly infected and proliferated cells to differentiate into GC B cells. It is probable that episome copy number per cell might influence which cells undergo the GC reaction; that is, if the cell has a mechanism for sensing EBV episomes as foreign DNA. As shown it is not the norm for GC B cells and memory B cells to be continuously directly infected by EBV.

5.2 EBER1, c-Myc and EBV episome copy number

A curious but very preliminary work was performed to assess the level of expression of the possibly oncogenic EBER1 in cell lines with varying EBV genome copy numbers. It was realized that the expression of EBER1 in Raji BL cell line [~60 EBV genome copies, according to Hurley and Thorley-Lawson, (1988)] was about a 1,000 fold more than that of IB4 lymphoblastic cell line (4 integrated EBV genome copies) and Namalwa BL cell line (2 integrated EBV genomes copes). Preliminary results in this thesis showed that c-Myc protein expression is about 1.8 fold and 2.1 fold higher in Raji compared to Namalwa and IB4 respectively (Appendix E). Indeed Niller *et al.*, (2003) and Niller *et al.*, (2004) indicate that there is a c-Myc binding site in the promoter region of EBV encoded RNA (EBER1). It could be said albeit cautiously that high EBV genome or episome copies correspond to high expression of the potentially oncogenic EBER1 as

well as c-Myc. There is a possibility that high EBV episome copy numbers could make cells oncogenic and this must be further explored. In another vein, per this results which is very preliminary, B cells with EBV episomes express more EBER1 than B cells with integrated genomes. Perhaps EBER1 is also involved in episome maintenance, it is possible that there is a cellular mechanism that could detect the EBV episomes as foreign DNA, and this would not happen with integrated EBV DNA. EBER1 might play a role in suppressing this cellular response(s) to EBV episomes (Thorley-Lawson D.A., personal communication). More needs to be done to verify this result to ascertain if it has any relevance to disease pathology.

5.3 High GC throughput and high level of EBV infected GC B cells from the malaria-endemic region; implications for eBL

It was also found that GC throughput, i.e. the percentage of GC B cell per tonsil, is significantly higher in tonsils from the malaria-endemic region as opposed to the non-malaria region (fold difference of 1.5, $p < 0.001$). The percentage of total B cells per tonsil from both regions was apparently the same ($p = 0.1835$). Boyaka *et al.,* (2000) report that the mean percentage total B cells of tonsil of healthy people is 76% which corresponds with the finding in this thesis (Table 7). This means that malaria infection (not ruling out other infections) does not generally cause an increase in the B cell pool but rather it might be permitting more B cells and EBV infected GC B cells to undergo the GC reaction and this increases the chance that some EBV infected cells will develop deleterious mutations and/or translocations which will eventually end up in lymphoma. This might be one way in which malaria might be contributing to the higher occurrence of lymphoma and eBL in equatorial Africa as compared to the Western world or non-

malaria regions. As stated earlier this cannot be the only deciding factor in eBL pathogenesis (Thorley-Lawson and Allday, 2008) else nearly every child in the malaria endemic region will develop BL which is not the case.

Results so far gathered are consistent with results elsewhere (Asito *et al.*, 2008) albeit in the periphery that *Plasmodium falciparum* infection results in perturbations of the B cell compartment. Again although it has been shown that *Plasmodium falciparum* binds B cells and causes lytic replication (Chene *et al.*, 2007), this is the first time an attempt has been made to evaluate the GC environment (where the c-Myc/Ig translocation occurs), vis-à-vis malaria. This brings us closer to reality in the attempt to decipher how malaria might contribute to or cause eBL via its effect on the GC. This work has also shown that as expected, the mean frequency/ level of EBV infected GC B cells from the malaria-endemic region is about 42 fold more than the frequency of EBV infected GC B cells from the non-malaria region ($p= 0.0041$). The fold difference of the frequency of EBV infected cells in patients who are immunosuppressed compared to controls has been reported to be 50 (Thorley-Lawson and Gross, 2004). It is luring to draw parallels and say that children from the malaria-endemic region are immunosuppressed, yet, it is unlikely that these children are immunosuppressed in a similar manner as transplant patients because, then they would be at risk to post-transplant lymphoproliferative disorder (PTLD)-like disease i.e. immunoblastic lymphoma. Assuming without admitting that children in the malaria-endemic region are immunosuppressed as per the results presented, immunosuppression alone has not been shown to be enough to cause eBL, but rather it creates a permissive context or congenial environment for eBL to occur. It must be quickly stated though that malaria has been reported to be

immunosuppressive for T cell responses including those directed against EBV (Ho *et al.*, 1986; Donati *et al.*, 2004), and also as expected, children with malaria (or from holoendemic malaria regions) had higher levels of EBV DNA in their peripheral blood compared to controls (Moormann *et al.*, 2005; Rasti *et al.*, 2005). The reason why immunosuppression alone is not enough to cause eBL is this; allograft patients who are highly immunosupressed, who also carry high loads of EBV (Babcock *et al.*, 1999) occasionally succumb to immunoblastic lymphoma as opposed to BL (Thorley-Lawson and Allday, 2008). It must be reiterated that immunoblastic lymphoma's from these highly immunosuppressed patients express the growth program (Hopwood and Crawford, 2000), but not the EBNA1 only program expressed in BL.

The final lesson can be drawn from AIDS patients who are also immunosupressed. Indeed, AIDS-BL is independent of immunological status, but rather, as immune suppression increases or intensifies, EBV loads increase and these patients are then susceptible to lymphoblastic lymphoma (Bigger *et al.*, 2007). It can therefore be safely surmised that immunosupression and elevated EBV loads together should rather be predisposing factors for lymphoblastic lymphoma. As earlier stated it is not surprising that the malaria endemic belt coincides practically with the lymphoma belt. AIDS-BL probably stems from chronic B cell activation during HIV infection which is associated with consistent opportunistic infections (Thorley-Lawson and Allday, 2008). Consequently parallels can be drawn between the events leading to AID-BL and eBL. According to Bloland *et al.*, (1999), children in malaria holoendemic areas are chronically infected with *Plasmodium falciparum* in such a way that the parasite is present in about 90% of children under the age of 5years. Haffalla *et al.*, (2011) also

report that about 100% of all residents in an endemic region are periodically re-infected by *Plasmodium spp*. This work also reports that out of 7 children between the ages of 2-16 years, 86% show the presence of *Plasmosdium falciparum*. The common denominator between AIDS-BL and eBL is chronic B cell activation. Thorley-Lawson and Duca, (2007) as well as Thorley-Lawson and Allday, (2008), have postulated therefore that for eBL to occur, chronic B cell activation due to malaria infection must lead to an increase (or excessive) expression and or activation of the enzyme AID which generates the BL translocation.

To end with, this work has shown that chronic B cell activation contributed by malaria infection engenders high GC throughput and high frequency/ level of EBV infected GC B cells from the malaria-endemic region as compared to GC B cells from the non-malaria region and this will increase the probability of a c-Myc/Ig translocation in an EBV infected GC B cell which could seed eBL.

5.4 High AID mRNA expression in GC B cells from malaria-endemic region; implications for BL

As earlier argued, the main crux of BL pathogenesis lies in the increased/ excessive AID expression or activation which is attained through chronic B cell activation. Consequently the level of AID mRNA expression in GC B cells from the malaria-endemic region and GC B cells from the non-malaria region was determined. It was found that AID mRNA expression is about 5 fold higher in GC B cells from the malaria endemic region as compared to GC B cells from the non-malaria region ($p= 0.046$). It is worth noting that Smit *et al.,* (2003) (Figure 25 right) report that AID mRNA expression

levels in GC B cells is about 13 fold higher than in naive B cells of healthy people and this compares to 11.01 ± 1.95 found in this thesis. One sample with extremely high AID mRNA expression (about 18 fold) could easily be a candidate for the c-Myc/Ig translocation. Indeed over-expression of AID *in vitro* in spleen B cells leads to the c-Myc/ Ig translocation within 24hrs (Ramiro *et al.,* 2006) and AID is necessary for the c-Myc/ Ig translocation (Ramiro *et al.,* 2004). EBV infection of B cells *in vitro* as well as EBV associated factors, have been shown to induce AID expression (He *et al.,* 2003; Casola *et al.,* 2004; Epeldegui *et al.,* 2007a). AID over-expression generally can occur through severe and persistent infections (Muramatsu *et al.,* 1999; Machida *et al.,* 2004; Machida *et al.,* 2005), and this could cause AID to lose its specificity (Petersen-Mahrt *et al.,* 2002) allowing non-Ig genes including c-Myc and other proto-oncogenes to be targeted (Reviewed by Vallur *et al.,* 2007). Rather surprisingly, there was no correlation between the level of EBV infection and AID mRNA expression levels. A second method in which the level of EBV infection in both AID high GC B cells and GC B cells in general was studied confirmed that AID mRNA expression levels and levels of EBV infection do not correlate (Appendix C). It is still possible that the frequency of EBV infected cells in all the samples used in this thesis; averages of 52 in 10^7 GC B cells and 2232 in 10^7 GC B cells for non-malaria and malaria regions respectively are relatively too small to effectively ascertain the effect of EBV on AID expression using bulk GC B cells. As argued earlier in the absence of the ability to directly study AID mRNA expression in *in vivo* EBV positive B cells it could be suggested based on the two methods that, there is probably no link between EBV and AID expression *in vivo*.

In any case the belief has always been that chronic B cell activation by malaria infection

is the crucial factor for AID mRNA over-expression in GC B cells from the malaria-endemic region. Thus *in vitro* stimulation of tonsil mononuclear cells was performed with *Plasmodium falciparum* extracts. AID mRNA induction in B cells from crude parasite extract stimulation was in a CpG like manner, i.e. parasite extracts will appreciably induce AID levels in the presence of CD40 ligand and IL-4 just like CpG. This work has therefore successfully shown that *Plasmodium falciparum* crude extracts can appreciably augment and sustain the expression of AID in the presence of the classical AID inducers, CD40 ligand and IL-4. This suggests a GC location for a possible augumentation of AID expression by *Plasmodium falciparum* antigens since AID induction by CD40 ligand and IL-4 occurs in GCs. Indeed, Parroche *et al.,* (2007) have reported that malaria antigens (*Plasmodium falciparum* DNA bound to hemozoin) act just like CpG since it binds to TLR9. In the presence of parasite extracts AID mRNA expression increased from 4 fold on day 3 ($p= 0.03$), to 7 fold on day 5 ($p< 0.001$), over the background (CD40 ligand and IL-4 only) (and levels drop by day 7). This result means that, malarial factors and factors from the immune system must of a necessity work together to induce and sustain high AID expression which may eventually cause eBL. According to Parroche *et al.,* (2007), *Plasmodium falciparum* DNA alone is not sufficient to stimulate TLR9 but very potent if it is bound to hemozoin. If hemozoin is sporadically associated with *Plasmodium falciparum* DNA *in vivo,* then this adds an additional dimension to the relative rarity of eBL (eBL incidence ~20 in 100,000 children) although malaria infects the majority of children in the malaria-endemic area. Put another way, *Plasmodium falciparum* DNA must associate with hemozoin (which is not always the case) in order to be internalized by B cells in the GC and these B cells must receive CD40 ligation (or T cell help) and IL-4 at the same time. This work has

now shown that hemozoin bound to CpG (mimicking hemozoin-*Plasmodium falciparum* DNA complex) is internalized by B cells. It remains to be determined if hemozoin-*Plasmodium falciparum* DNA complex results in AID mRNA induction and over-expression.

To end with, high AID mRNA expression in GC B cells from the malaria-endemic region could lead to an increased probability of a c-Myc/Ig translocation occurring in GC B cells, which could seed eBL. EBV infected GC B cells are most primed to tolerate a translocated c-Myc because as earlier stated EBV factors (EBNA3A and EBNA3C) repress the pro-apoptotic BIM and therefore allow escape from apoptosis. It remains to be seen however, how the AID protein expression levels compare with the mRNA data.

5.5 c-Myc is expressed in GC B cells albeit there is discord between mRNA and protein expression levels

This work shows that GC B cells express the proto-oncogene c-Myc which is the translocation counterpart of Ig in BL. This result is corroborated by Martinez-Valdez *et al.,* (1996) and Cutrona *et al.,* (1997) who both report that c-Myc is highly expressed in GC B-cells. High c-Myc expression in the GC could account for the observed high rate of proliferation, as well as the high apoptotic tendency of GC B cells. c-Myc is a non-traditional transcription factor with a very complex regulation and we do not yet understand its role in normal GC physiology or in eBL initiation. It has been argued that c-Myc might be deregulated in GC precursor B cells prior to the translocation event and this could have very different implications for pathogenesis. Considering the fact that c-Myc is a transcription factor that is also linked to genomic instability (Kuttler and Mai,

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2006), it is important to answer the question, does c-Myc go awry before or after the translocation, this will be valuable in trying to determine the mechanism of pathogenesis of BL as well as other lymphomas which involve c-Myc. A further question which could now be asked in the face of this result is, does c-Myc turn on AID. These questions will however be non-existent when we consider the opposing result that c-Myc is not expressed in GC B cells (Klein *et al.,* 2003; Cattoretti *et al.,* 2006). In view of the fact that *Plasmodium falciparum* malaria may induce lymphocyte apoptosis, (Matsumoto *et al.,* 2000) a process which is also central to c-Myc, the finding that c-Myc is expressed in GC B cell makes one curious to find out the effect of malaria on c-Myc expression. The intimate association of AID with the transcription complex (Besmer *et al.,* 2006) is a glowing proof that transcription is tied to AID's activity. Indeed, AID is loaded on the transcription initiation complex; it binds and follows RNA polymerase II as elongation proceeds (Nambu *et al.,* 2003). Simply put, for AID to target c-Myc in GC B cells, c-Myc must be expressed in GC B cells since AID deaminates any transcribed substrate (Chaudhuri *et al.,* 2003; Dickerson *et al.,* 2003; Pham *et al.,* 2003; Ramiro *et al.,* 2003; Shen and Storb, 2004) and acts on selected highly transcribed genes when they are over-expressed (Martin and Scharff, 2002; Yoshikawa *et al.,* 2002; Okazaki *et al.,* 2003). The puzzle however is why this thesis observed a high level of c-Myc protein expression compared to a low level of c-Myc mRNA expression in normal GC B cells. It is also intriguing to see that the c-Myc mRNA expression in a few of the BL cell lines parallel the c-Myc mRNA expression in GC B cells, this might be indicative of the normal expression of c-Myc in these BL cells before the c-Myc/ Ig translocation event. The discord between c-Myc mRNA and protein expression that this thesis has observed in GC B cells might be due to differential regulation of c-Myc mRNA and protein levels.

Perhaps as observed in this thesis and also confirmed by Klein *et al.,* (2003) using micro-array, transcription of c-Myc mRNA in GC B cells is kept at a low (even lower than c-Myc expression in naive B cells) to avoid targeting by AID, while protein levels are kept high enough for c-Myc to be able to regulate proliferation and apoptosis in GC B cells. It is not inconceivable that chronic B cell activation will lead to high expression of both c-Myc and AID, and this will result in the targeting of c-Myc by AID which in turn increases the probability of a c-Myc/Ig translocation occurring in these GC B cells.

A quick review of the c-Myc literature throws more light on the results obtained in this thesis. The human c-Myc proto-oncogene encodes three isoforms (namely c-Myc1, c-Myc2, c-MycS, with the c-Myc1 being most abundantly expressed). These three isoforms differ in their N-terminal region (Benassayag *et al*., 2005) and the c-Myc primers used herein can target two of the isoforms -Myc2, c-MycS. It is uncertain if the c-Myc isoform (c-Myc1) that the primers fail to pick up is expressed at the same level or higher than the isoforms ampliflied in this work. It is also uncertain what the differential expression of the c-Myc1 isoform is in GC B cells, BL cell lines and LCLs. Also, in BL cell lines the normal c-Myc allele is usually silent or is expressed at very low levels (Eick *et al*., 1989) and this could indicate that the normal and translocated alleles in BL are differentially regulated. According to Taub *et al*., (1984), one might have expected a high level of c-Myc expression in BL cell lines but this is not the case. Rather, in BL cell lines, the c-Myc transcript is marginally increased in some cases and not at all increased in other cases when compared to LCLs. Taub *et al*., (1984) also confirm that there is a suppression of the normal c-Myc gene in Burkitt's lymphoma cells and that the c-Myc mRNA is transcribed predominantly from the translocated allele. Granted that our

primers are amplifying only the normal c-Myc allele, then the aforementioned reasons might explain why higher c-Myc mRNA expression is seen in the LCLs as opposed to the BL cell lines (Figure 32) since LCL's do not habor the c-Myc translocation. We will also have to tread cautiously as we draw parallels and say that, granted that the primers are amplifying only the normal c-Myc allele then c-Myc mRNA expression in GC B cells might be tightly regulated. The fact that c-Myc mRNA has a half-life of 10-15 minutes (Dani *et al.*, 1984) whereas the c-Myc protein has a half-life of 30 minutes (Welcker et al., 2004; Yada *et al.*, 2004) might lend credence to this argument.

Perhaps the best way to compare c-Myc expression in BL cell lines and "normal" B cells (and in the case of this thesis GC B cells) is by protein expression. The ratios of c-Myc protein expression i.e c-Myc/ GAPDH for Raji BL cell line (1.31) (consistent with a c-Myc/ β-actin ratio of 1.33), Rael BL cell line (1.17), GC1 (1.13), GC2 (1.13), GC3 (1.09) are not different. The question thus remains; does deregulated c-Myc expression amount to its over-expression in BL cells? (Especially since the level of c-Myc protein expression is the same as that of GC B cells) and how do we define c-Myc deregulation in these BL cells. Perhaps the role of the translocation is constitutive c-Myc expression which leads to continuous proliferation and yet the protein is kept at the same physiological level of expression.

After showing that c-Myc is indeed expressed in GC B cells, it was also observed that the c-Myc mRNA expression in GC B cells from malaria-endemic region is 4 fold higher compared to GC B cells from non-malaria region ($p= 0.03$). The similarity of the AID and c-Myc fold increases (5 and 4 fold respectively) in GC B cells from the malaria

endemic region could speak to the fact that both might be turned on by malaria's chronic B cell activation. This will allow AID to target c-Myc due to its high rate of transcription, and eventually increase the probability of a c-Myc/Ig translocation occurring in especially the EBV infected GC B cells which could then seed BL.

5.6 High cumulative fold risk of BL occurring in GC B cells from malaria-endemic compared to the non-malaria region

Assuming fold increases in the parameters measured or compared between GC B cells from malaria-endemic region and the non-malaria region represent the risk of a translocation event then; GC throughput, 2 fold; frequency of EBV infection, 42 fold; AID mRNA expression, 5 fold; and c-Myc expression, 4 fold, represents a cumulative risk of 2,000. That is to say, the risk of a translocation event in GC B cells from the malaria-endemic region is 2,000 fold more as compared to the risk of a translocation event in GC B cells from the non-malaria region. It is intriguing to find that this cumulative fold risk parallels the difference in incidence between eBL and sBL (~20 in 100,000 children and 0.01 in 100,000 children respectively), i.e eBL occurs 2,000 times more frequently than sBL. All in all, the high level of risk factors for a c-Myc/Ig translocation event in the GC B cells of children from the malaria-endemic region puts them at risk for eBL. Figure 35 is a schematic summary of our hypothesis/ findings.

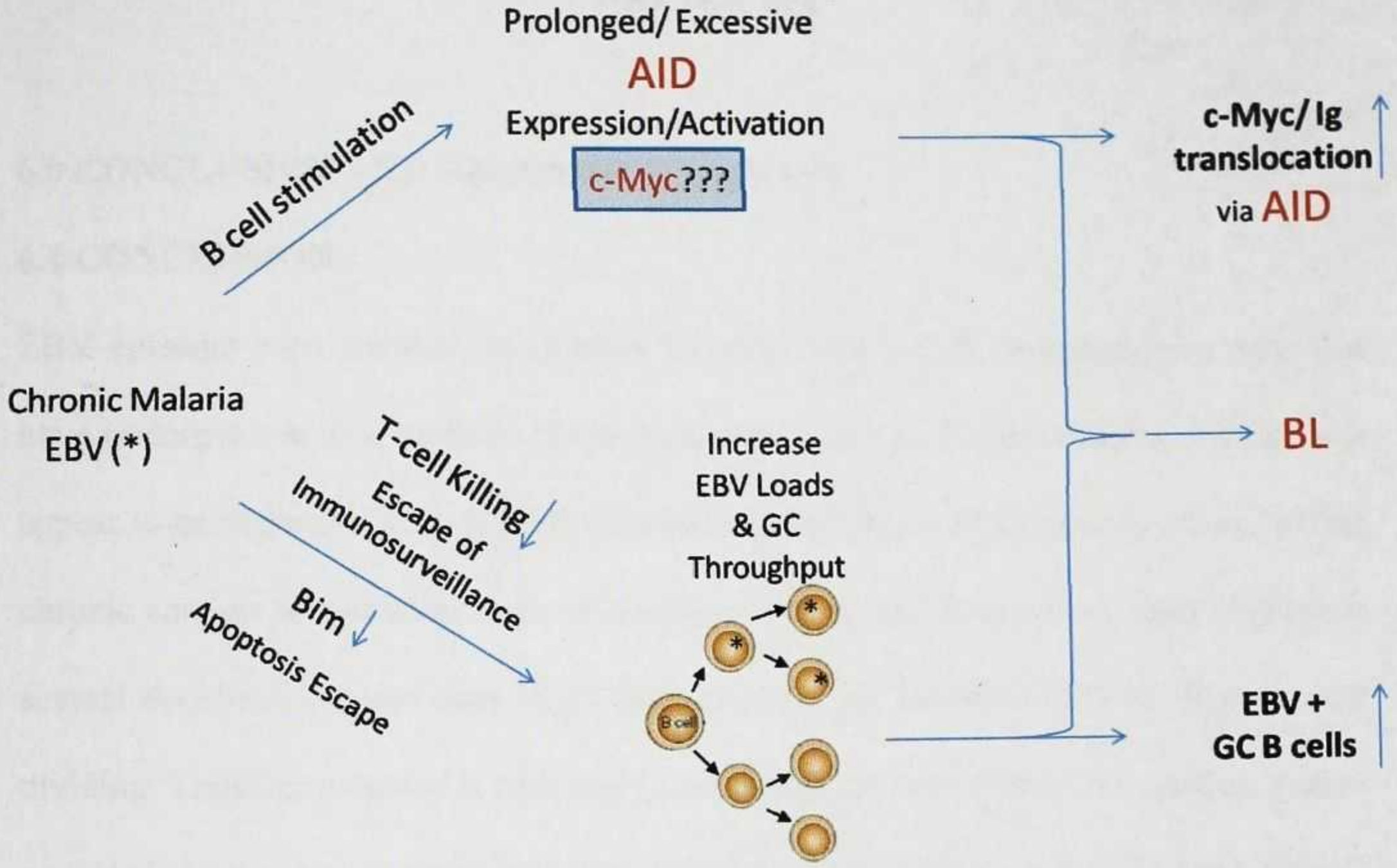

Figure 35: A Summary of hypothesis/ findings. At the heart of our eBL hypothesis is chronic malaria and EBV infection. In one vein (upper half), chronic malaria infection leads to chronic B cell stimulation and this culminates into prolonged or excessive AID expression or activation. c-Myc is also expressed above the usual threshold as a consequence of chronic malaria infection. With a potent mutagen (AID) in the nucleus of GC B cells, there is a high probability of a c-Myc/Ig translocation occurring which can then seed BL. In another vein (lower half), concurrently, chronic malaria infection leads to perturbation of T cell killing and escape of immunesurveillance. EBV factors (EBNA3A and EBNA3C) bind and suppress the pro-apoptotic BIM thereby permitting apoptosis escape. This allows increased numbers of B cells to undergo the GC reaction. As a consequence there is an increase in the numbers of EBV infected cells taking part in the GC reaction and this increases the probability of a c-Myc/Ig translocation occurring in these EBV infected cells which also express high levels of AID (and c-Myc) as described in the upper half of the figure and this will eventually seed BL.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

EBV episome copy number distribution indicates that GC B cells comprise cells that have undergone as few as three divisions and as many as 20-35 divisions. GC B cells appear to be undergoing the normal GC reaction. Peripheral blood memory B cells from chronic carriers do not show signs of continuous direct infection. They have undergone several divisions, no less than 20-35 divisions and the likelihood is that they are still dividing. Tonsillar memory B cells have also undergone several divisions and also show no convincing signs of direct infection. Peripheral blood memory B cells from chronic EBV carriers are not different from tonsil memory B cells from chronic carriers as well as peripheral memory B cells from IM patients, in terms of EBV episome copy distribution. It must be said though that the EBV episome distribution in IM memory B cells is slightly left skewed and therefore subtle and "unorthodox" direct infection cannot be completely ruled out in this particular condition. Memory B cells from the peripheral blood of either chronic carriers or IM patients as well as tonsillar memory B cells belong to the same category in terms of EBV infection dynamics. Naïve B cells seem to be a complex mix of newly infected cells and cells that have undergone several divisions but the contribution of linear EBV genomes to this result must be ascertained first before firm conclusions are made. Thus naïve B cells, germinal center B cells and memory B cells all belong to distinct groups in terms of EBV infection dynamics. As per the *in vitro* data presented in this work it seems the duplicated EBV episomes faithfully partition into each daughter cell about 91% of the time and both episomes are segregated

into one daughter cell 9% of the time (average of two independent experiments). It appears that EBV episome copy number per cell reaches a stable distribution after 20-25 divisions. Why EBV episomes segregates in this manner remains to be understood. The *in vivo* EBV episome distribution data from the various B cell sub-populations buttress the resolve that EBV exploits the normal B cell differentiation pathway to establish persistence, *viz*; EBV primarily infects naive B cells and drives them to become activated blasts. These EBV infected blast participate in the GC reaction as per usual and follow the normal B cell differentiation route to become resting memory B cells where EBV persists.

EBV interacts differently with the various B cell sub-populations and the EBV infected naïve B cell sub-population seems to be self-sustaining and might need additional cue(s) to proceed through the GC reaction. This could be the place where deleterious mutations begin even before the c-Myc/Ig translocation. The accumulation of the potentially oncogenic EBER1 (which seemingly correlates with c-Myc expression), as a result of increased EBV episomes in rapidly dividing cells, could make high episome containing B cells candidates for genetic lesions. In another vein, since EBV infected memory B cells are dividing, high episome containing memory B cells which are forced to return to the lymphoid tissue to undergo further divisions (possibly contributed to by malaria) could also accumulate deleterious mutations. This could be capped by the c-Myc/Ig translocation in the lymphoid tissue. Finally, high episome containing GC B cells could also be suspect for the c-Myc/Ig translocation because of the aforementioned reasons.

Children between the ages of 2-14yrs from the malaria-endemic region have ~2 fold

higher GC throughput; 42 fold higher frequency/ level of EBV infected GC B cells; 5 fold higher AID mRNA expression in GC B cells; and finally 4 fold higher c-Myc mRNA expression in GC B cells than their counterparts from the non-malaria region. There is a cumulative fold risk of 2000 for a c-Myc/Ig translocation and therefore eBL in children from the malaria-endemic region compared to children from the non-malaria region. There is no correlation between AID mRNA expression and the level of EBV infected cells *in vivo*. Eighty-six percent (86%) of children between the ages of 2-16yrs from the malaria-endemic region have detectable *Plasmodium falciparum* parasitemia. Finally, *Plasmodium falciparum* crude extracts induce AID mRNA expression in a CpG-like fashion and hemozoin-CpG complex (mimicking hemozoin-paraiste DNA complex) is internalized by B cells.

Thus, this thesis work unites the two conjectures, *viz*; malaria infection possibly increases the level of GC B cells and also the level of EBV infected GC B cells. Furthermore, AID is increased in the malaria-endemic background. The second conjecture which points to a more direct involvement of internalized malaria factors is upheld by the finding in this thesis that malaria factor hemozoin is internalized by B cells. Further probing of these two conjectures could capture the entirety of eBL pathogenesis. The requirement of co-incident factors for eBL pathogenesis as indicated in this thesis makes eBL a multi-step process and this could explain why every child in the malaria-endemic regions would not develop eBL. Put another way, the probability of all the cumulative factors enumerated in this thesis occurring in a child in the malaria-endemic regions should be indeed low, yet possible.

6.2 RECOMMENDATIONS AND FUTURE DIRECTIONS

It is imperative that further investigation is carried out to understand how EBV interacts with naïve B cells (primary cells of EBV infection), in terms of the proliferation of these infected naive B cells. This is because, naive B cells are the initial cells infected with EBV and these proceed through the GC reaction, where the c-Myc/Ig translocation is suggested to occur before differentiating into memory B cells. Further experiments must be carried out to rule out the possibility that the naïve B cell episome distribution data might be affected by residual linear EBV genomes. It will be interesting to study the EBV episome copy distribution in IgD+CD10+ B cells, the so-called GC founder cells. Results of EBV episome copy distribution in the various B cell sub-populations (*in vivo*) as well cells grown *in vitro* could better be understood by employing mathematical modeling.

It would be worthwhile profiling the EBV episome copy distribution in the various B cell sub-populations of tonsils from the malaria-endemic regions to further appreciate how EBV's interaction with B cells might be affected by malaria infection. This could leave figureprints leading to EBV related tumorigenesis and eBL.

It is important to verify if AID protein expression levels in GC B cells from the malaria-endemic and the non-malaria region correspond to the mRNA data presented in this work. It must also be ascertained what factor(s) in crude *Plasmodium falciparum* extracts induce AID in a CpG-like fashion. Our prime candidate is hemozoin bound to *Plasmodium falciparum* DNA (and probably PfEMP1) although there is reason to believe this is not the whole story. It must be shown that the *Plasmodium falciparum*

factor(s) indeed induce AID through TLR9 and this can be achieved with the aid of TLR9 blockers like chloroquine and bafilomycin. It will be insightful to know which particular B cell sub-populations are induced by the *Plasmodium falciparum* factor(s).

It is also crucial to ascertain whether there is DNA damage or the characteristic c-Myc/Ig chromosomal translocation in the B cells with high levels of, AID expression, c-Myc expression, or EBV episome copy number.

Finally there is also the need to understand the discord in c-Myc mRNA and protein expression since this will be helpful in understanding the role of c-Myc in normal GC B cell physiology as well as how it gets deregulated en route to disease pathogenesis.

APPENDIX A

ANTIBODIES AND DILUTIONS

Antibodies for Extracellular Staining

Ab	Clone	Species	Source	Cat no.	Dilution /100 µl cells	Cell number up to
CD19APC	HIB19	Mouse	BD Pharmingen	555415	2 µl neat i.e. (1/50)	$5x10^6$
CD10PE	HI10a	Mouse	BD Pharmingen	555375	2 µl neat i.e. (1/50)	$5x10^6$
CD10APC	HI10a	Mouse	BD Pharmingen	340923	2 µl neat i.e. (1/50)	$5x10^6$
CD27APC	MT271	Mouse	BD Pharmingen	558664	2.5 µl neat i.e. (1/50)	$5x10^6$
CD27PE	MT271	Mouse	BD Pharmingen	555441	2.5 µl neat i.e. (1/50)	$5x10^6$
IgD-FITC		Goat	Southern Biotech	2032-02	20 µl of 1/100 i.e (1/500)	$5x10^6$
IgD-PE		Goat	Southern Biotech	2032-09	2.5 µl of 1/100 1/5,000	$2x10^6$

Primary antibodies for Intracellular staining

Ab	Clone	Species	Source	Cat no.	Dilution /100 µl cells
c-Myc	9E11(IgG2ak)	Mouse	Invitrogen	AHO0052	10 µl of 1/ 100
c-Myc-FITC	9E11(IgG2ak)	Mouse	Santa Cruz	Sc-47694	10 µl of 1/ 100
Bcl6	P1F6(IgG1)	Mouse	Abcam Inc	Ab9479	15 µl of (1/100)
Bcl6	PG-B6P(IgG1k)	Mouse	Novus Biological	NB120-17249	15 µl of (1/100)
Isotype ctl	MOPC21(IgG1k)	Mouse	Novus Biologicals		10 µl of (1/25)
Isotype ctl	(IgG1)	Mouse	Caltag /Invitrogen	MG100	15 µl of (1/40)
Isotype ctl	(IgG2a)	Mouse	Caltag /Invitrogen	MG2a00	10 µl of (1/50)
Isotype ctl-FITC	(IgG1)	Mouse	Santa Cruz	Sc-2855	10 µl of (1/25)
Isotype ctl-FITC	(IgG2a)	Mouse	Santa Cruz	Sc-2856	10 µl of (1/25)

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Primary antibodies for western blot

Antibody	Clone	Species	Source	Cat no.	Dilution /100 µl cells
AID	L7E7	Mouse	Cell signaling	4975	1/500 in 4% BSA in TBST
α-Tubulin	B-7 (IgG2a)	Mouse	Santa Cruz	Sc-5286	1/10,000 in 5% milk in TBST
GAPDH-HRP	14C10 (IgG)	Rabbit	Cell signaling	3683	1/5,000 in 5% milk in TBST
Actin-HRP	C-11(IgG)	Goat	Santa Cruz	sc-1615	1/35,000 in 5% milk in TBST
c-Myc-HRP	9E10	Mouse	Santa Cruz	sc-40	1/1,000 in milk in TBST
c-Myc	N-262	Rabbit	Santa Cruz	sc-764	1/1,000 in milk in TBST

c-Myc peptide (cat no. 11667246001 5 mg/ml) specific for mouse monoclonal anti-c-Myc 40ul/20ml Antibody solution

Secondary Antibodies of Intracellular staining and western blot

Antibody	Species	Cat/ Pdt no.	Source	Dilution/ 100 µl cells
Intracellular staining				
Goat Polyclonal anti-mouse IgG-FITC	Goat	NB720-F	Novus Biologicals	15 µl of 1/100
Goat anti-mouse kappa-FITC	Goat	1050-01	Southern Biotech	15 µl of 1/100
Western Blot				
Anti-mouse IgG-HRP	Horse	7076	Cell signaling	1/10,000 in milk in TBST
Anti-Rabbit IgG-HRP	Goat	7074	Cell signaling	1/10,000 in milk in TBST

Primers and Probe

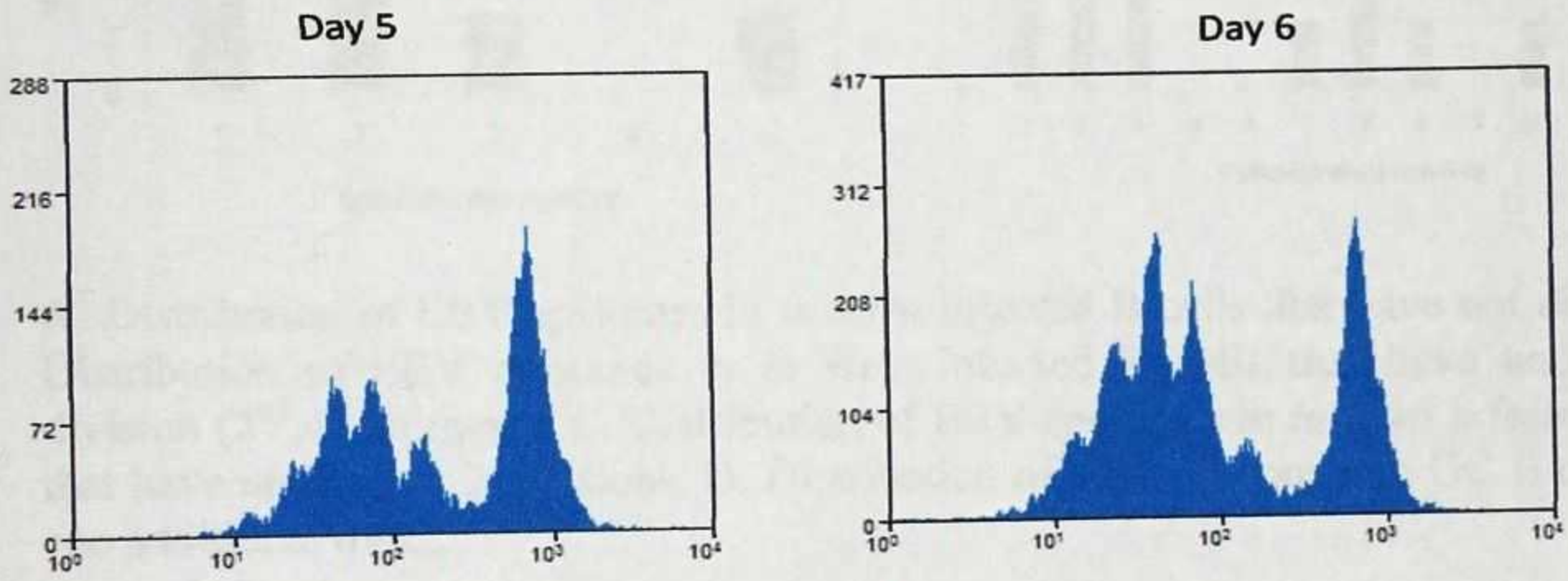
Gene	Forward (For) and Reverse (Rev)	Probe
W	For: AGTGGGCTTGTTTGTGACTTCA	TTACGTAAGCCAGACAGCAGCCAATTGTC
	Rev: GGACTCCTGGCGCTCTGAT	
EBER	For: ACCGAAGACGGCAGAAAGC	ACAGACACCGTCCTCACCACCCG
	Rev: CCTACGCTG CCCTAGAGGTTT	
20x Taqman Pre-developed assay Primer-FAM Probe mix (Applied Biosystems)		
	Assay number	
AID	Hs 00221068_m1 AICDA	
c-Myc	Hs 00905027_m1 MYC	
β-actin	Part no. 4333762T	
18s	Hs 99999901_s1 18s	

All probes are 6-FAM-TAMRA unless otherwise noted

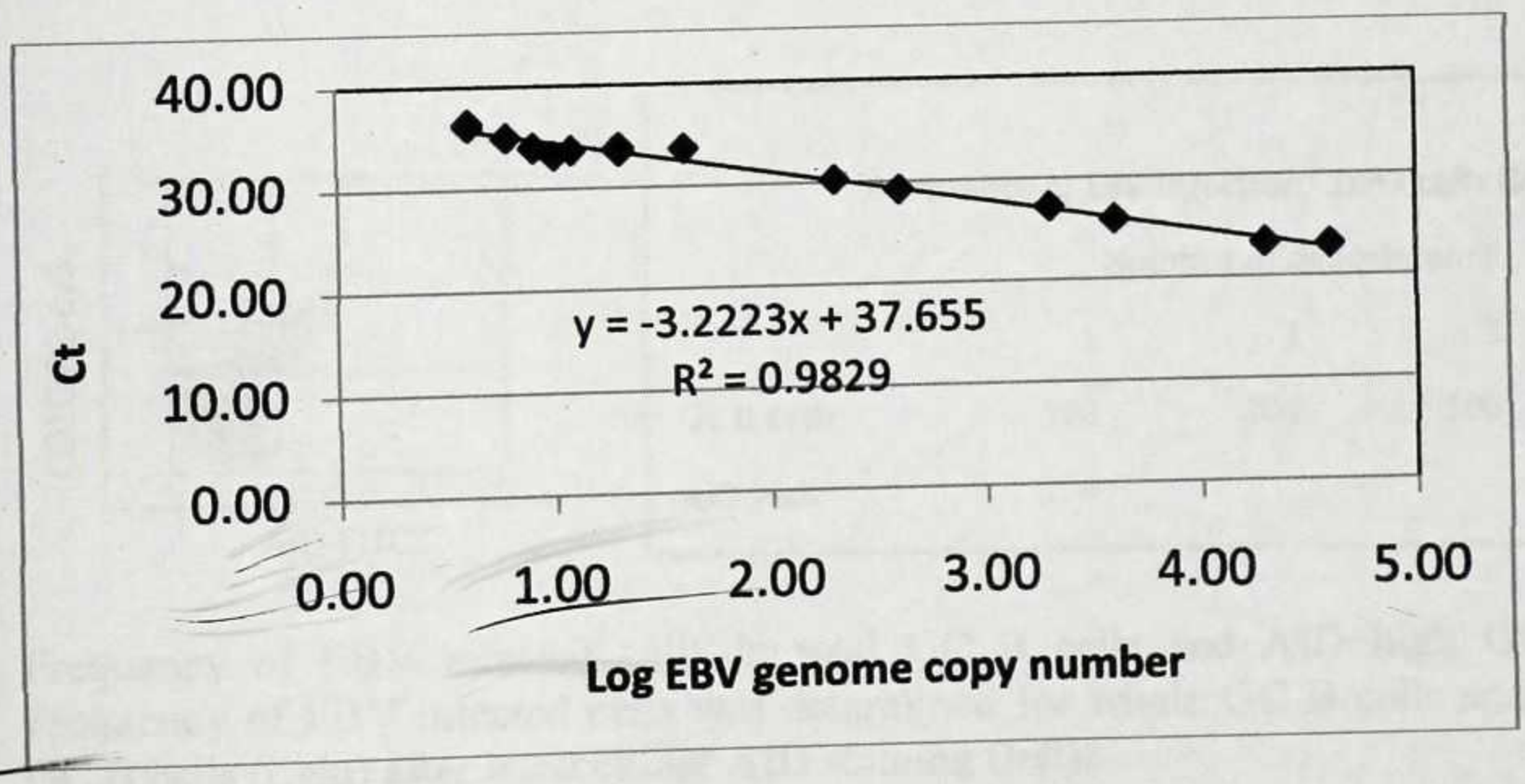
APPENDIX B

EBV EPISOME COPY DISTRIBUTION; AUXILLIARY DATA

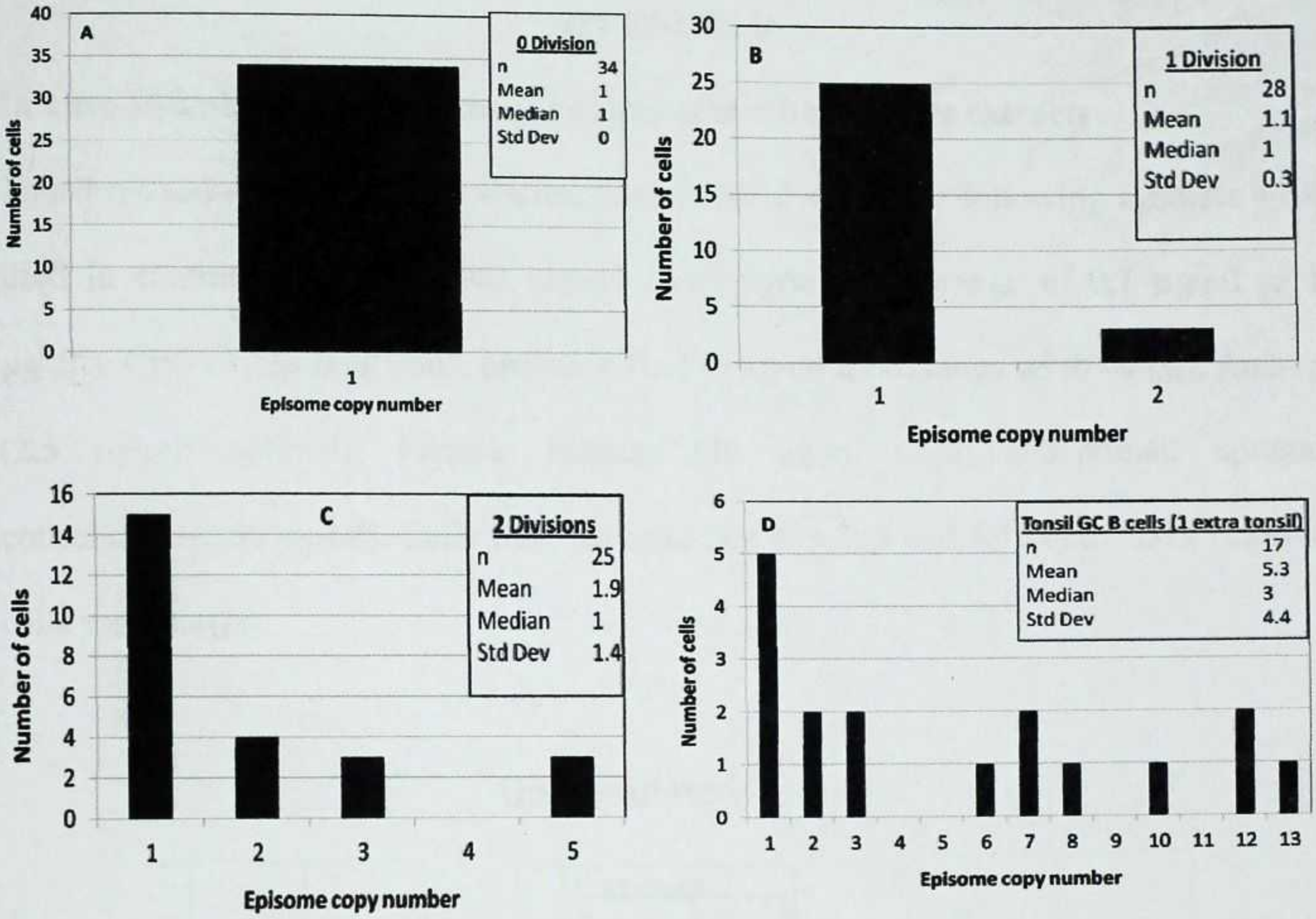
Human B lymphocytes transformed with EBV 200x. The usual clumping of EBV infected lymphoblastic cell lines is observed in our *in vitro* EBV infected peripheral blood B cells



CFSE flow cytometry output of EBV infected B cells after day 5 and Day 6

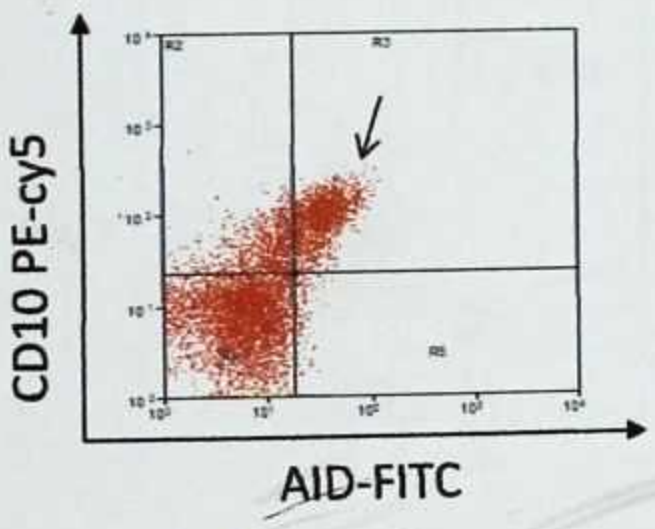


An example of IB4 and Namalwa standard curve



A. Distribution of EBV episomes in *in vitro* infected B cells that have not divided. B. Distribution of EBV episomes in *in vitro* infected B cells that have undergone 1 division (2nd experiment). C. Distribution of EBV episomes in *in vitro* infected B cells that have undergone 2 divisions. D. Distribution of EBV episomes in GC B cells from one additional tonsil.

APPENDIX C



Frequency of EBV infection/ 10^7 cells GC B cells

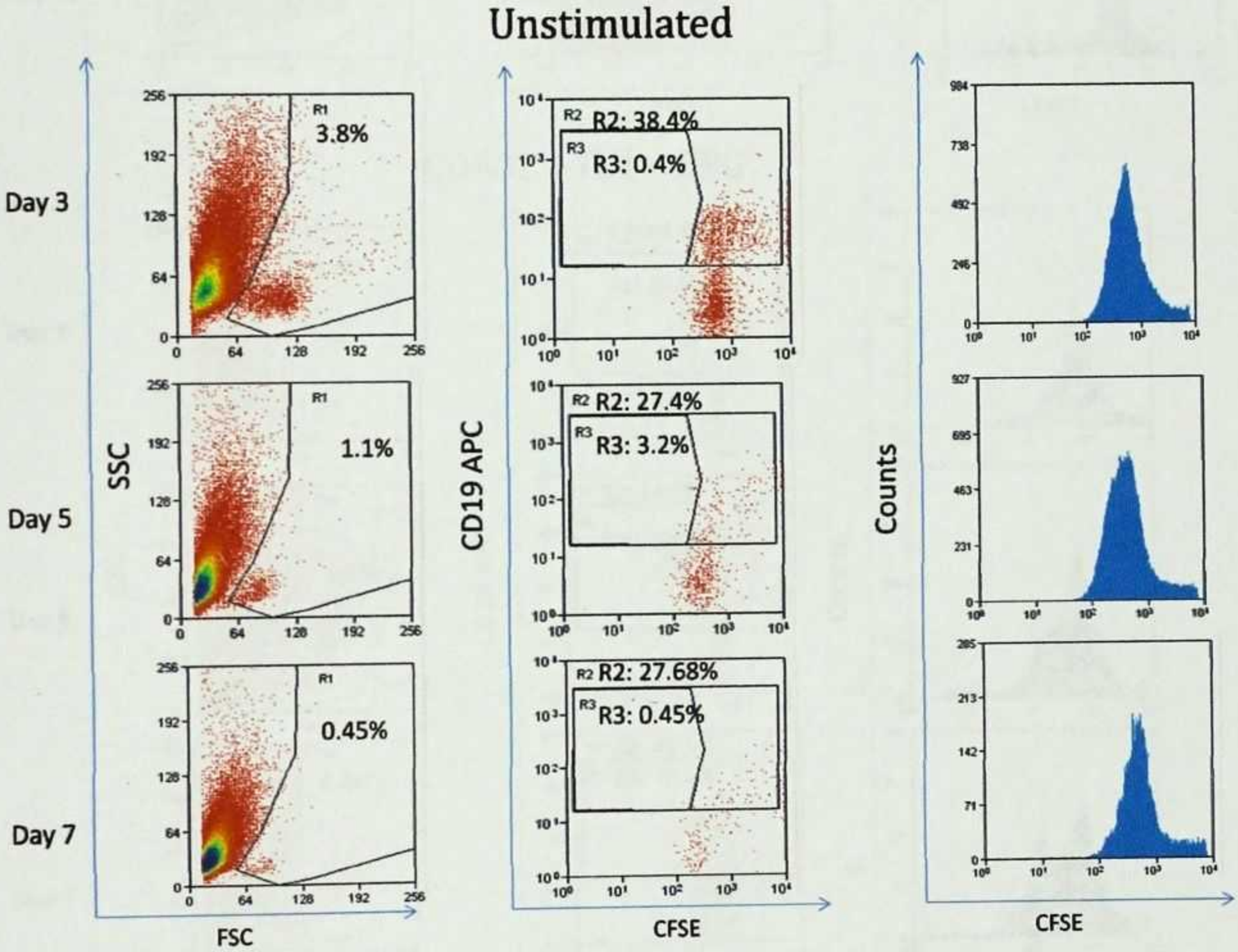
	Number of experiments			
	1	2	3	Mean
GC B cells	100	200	100	133
AID high	200			200

Frequency of EBV infected cells in total GC B cells and AID high GC B cells. Frequency of EBV infected cells was determined for whole GC B cells and AID high GC B cells (right) after intracellular AID staining (left).

APPENDIX D

In vitro stimulation of tonsil mononuclear cells with parasite extracts

Tonsil mononuclear cells were stained with CFSE dye and the following agonists were used in stimulating cells: CD40 Ligand (0.25 µg/ml used; range of 0.1 µg/ml to 1 µg/ml); CPG (3 µM final conc; optimal); IL-4 (5 ng/ml used; range up to 40 ng); Anti-Ig (2.5 µg/ml; optimal); Parasite extracts (10 µg/ml used; suboptimal, optimal concentration; 50 µg/ml). Cells were harvested on day 3, 5 and 7/10 and CD19 positive cells were sorted.



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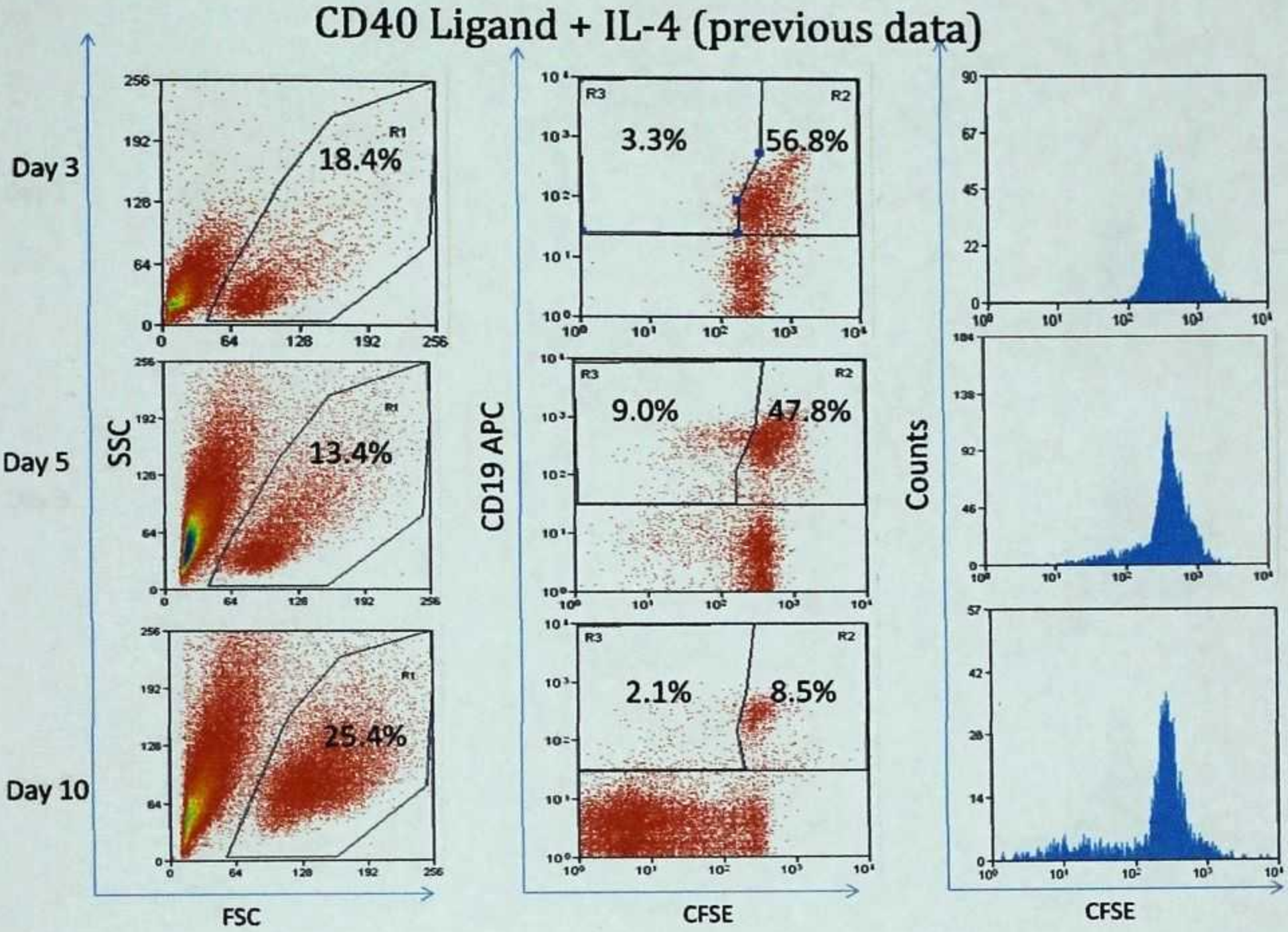
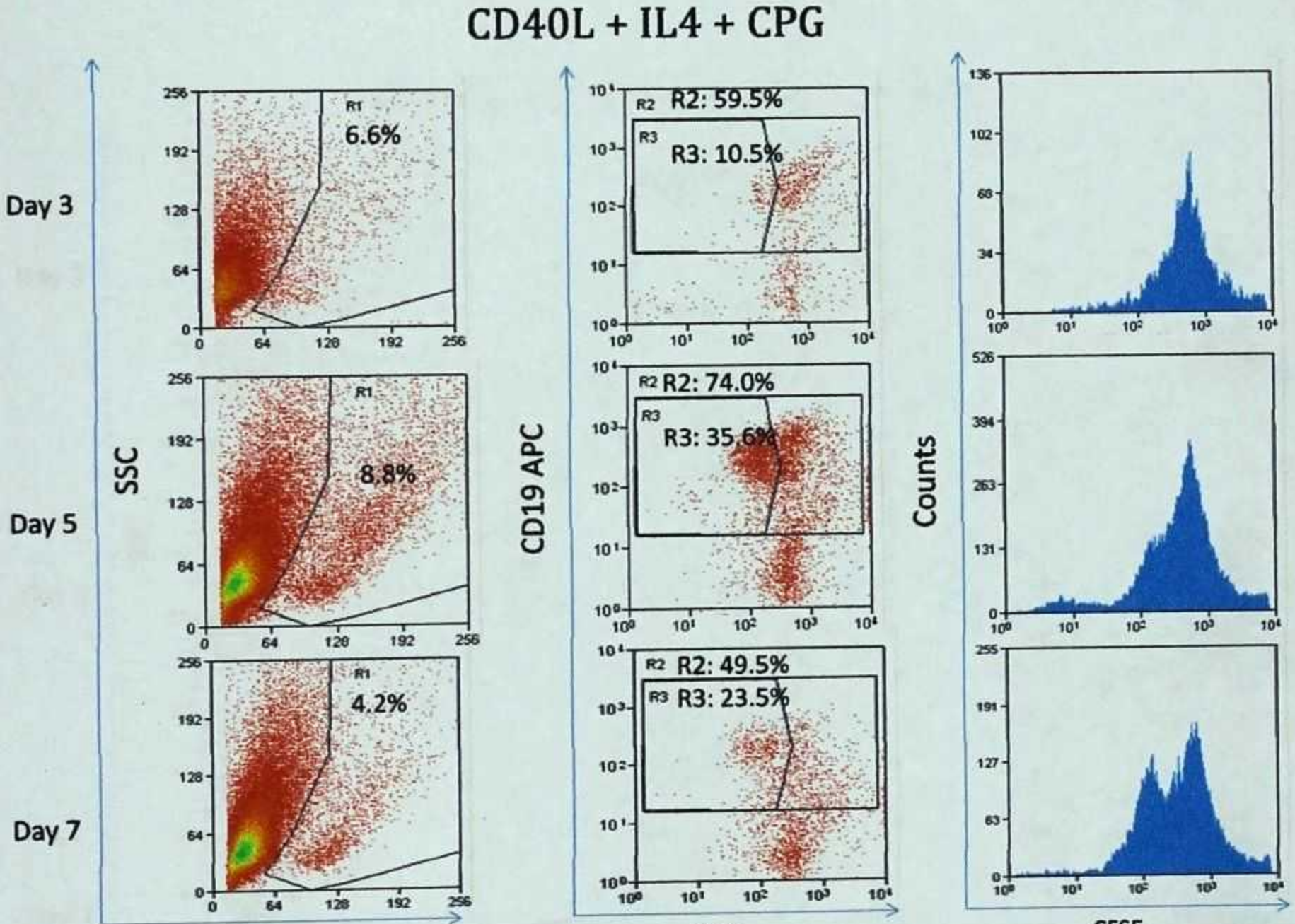
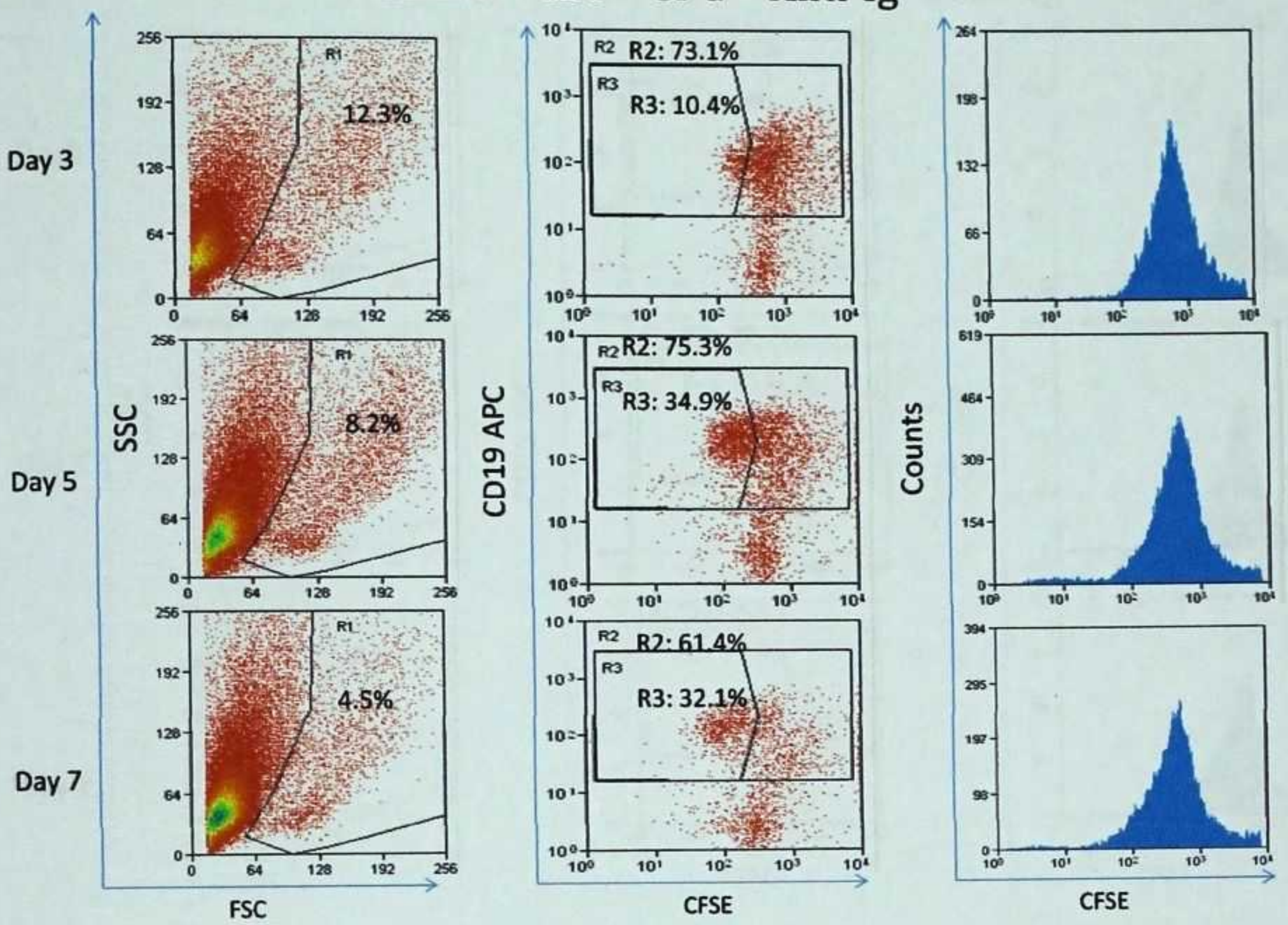
CD40 Ligand + IL-4 (previous data)



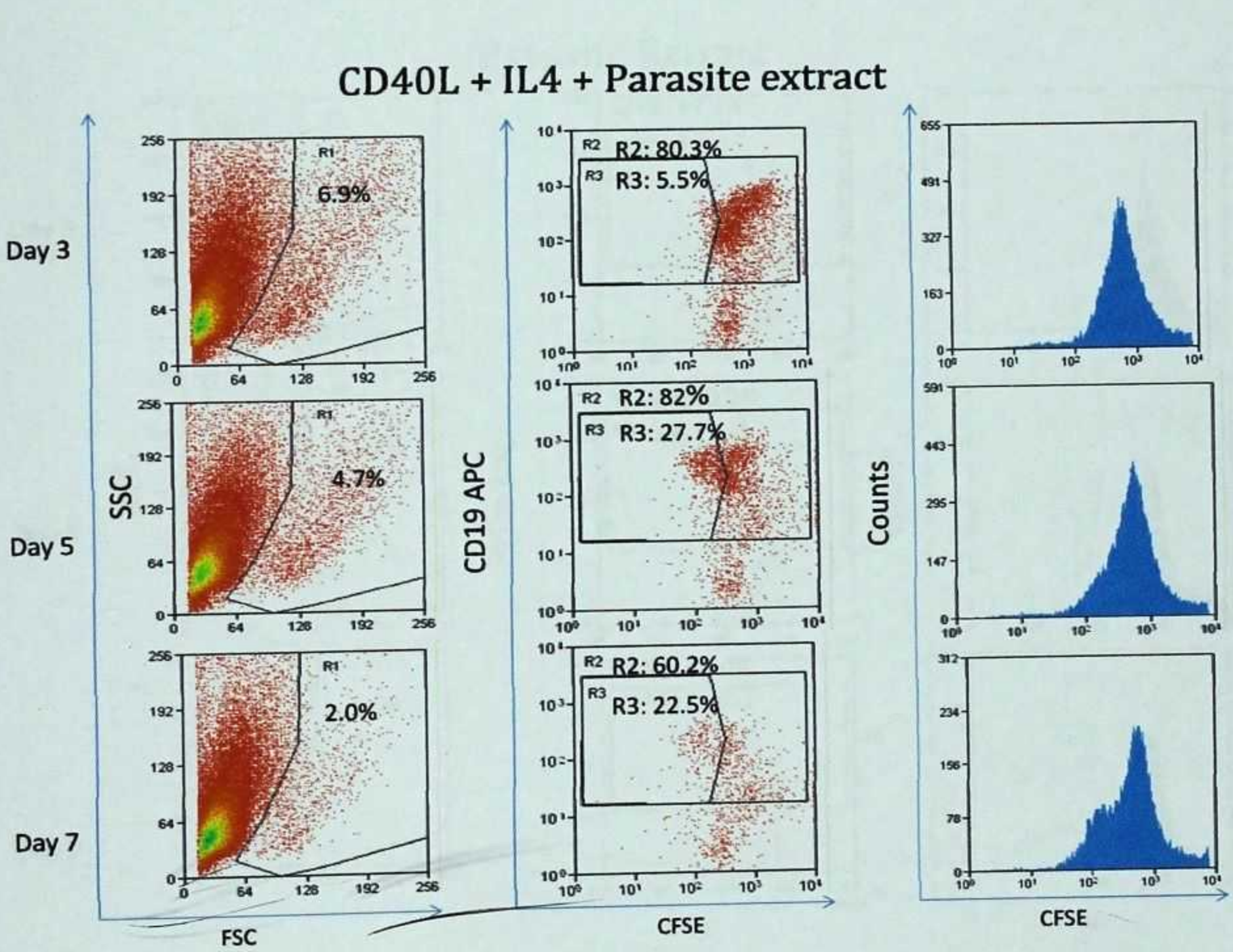
CD40L + IL4 + CPG



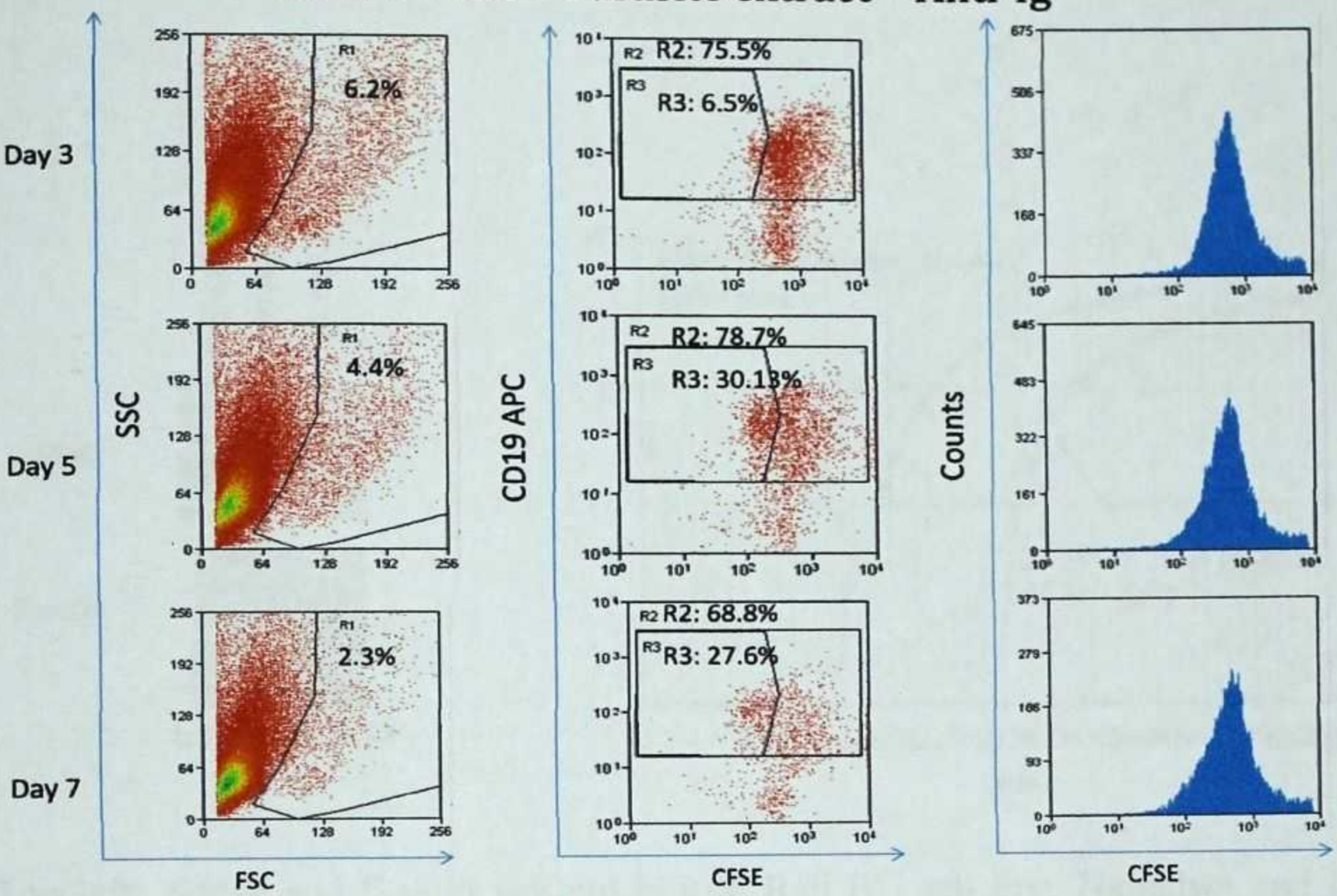
CD40L + IL4 + CPG + Anti-Ig



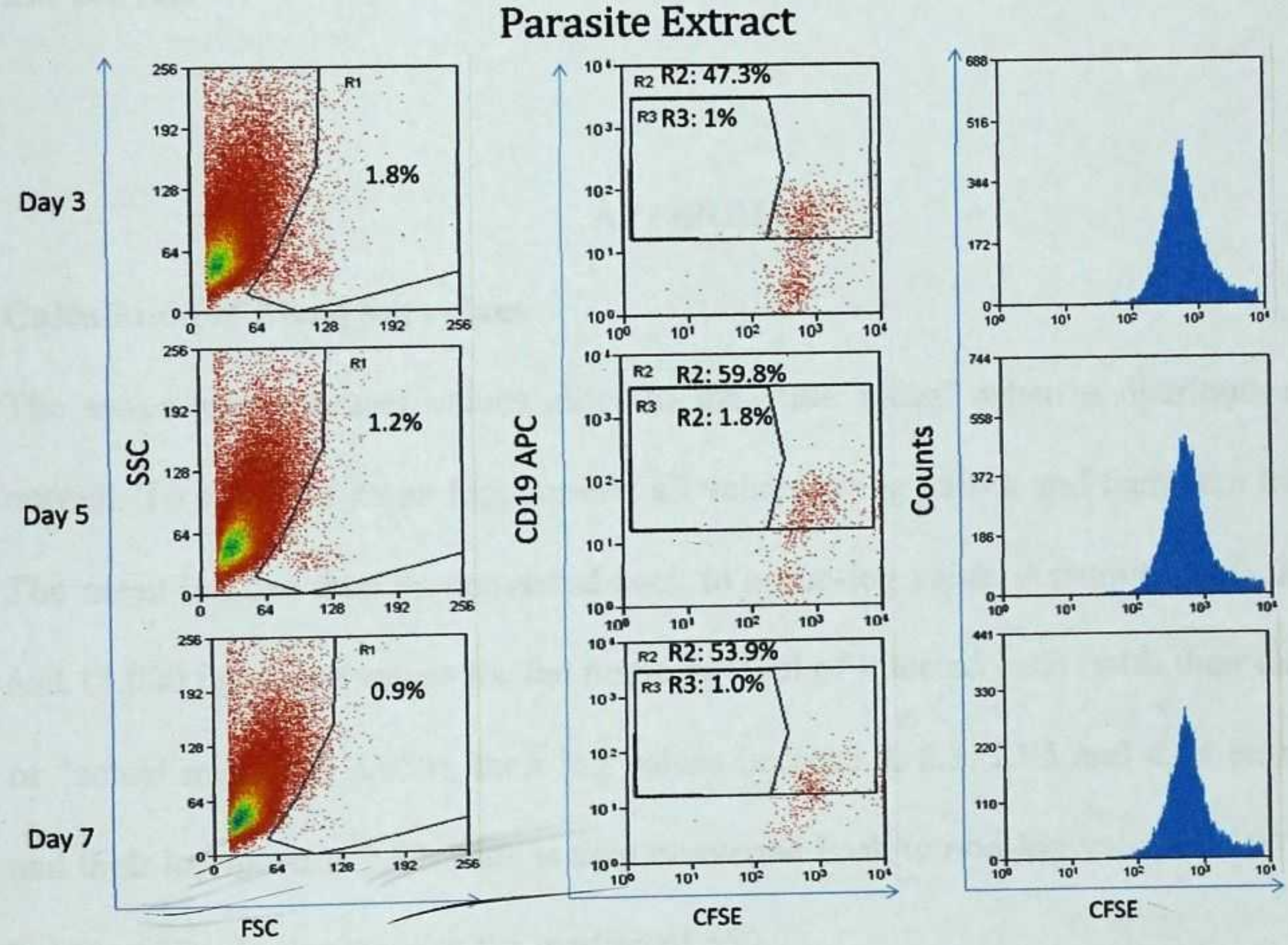
CD40L + IL4 + Parasite extract



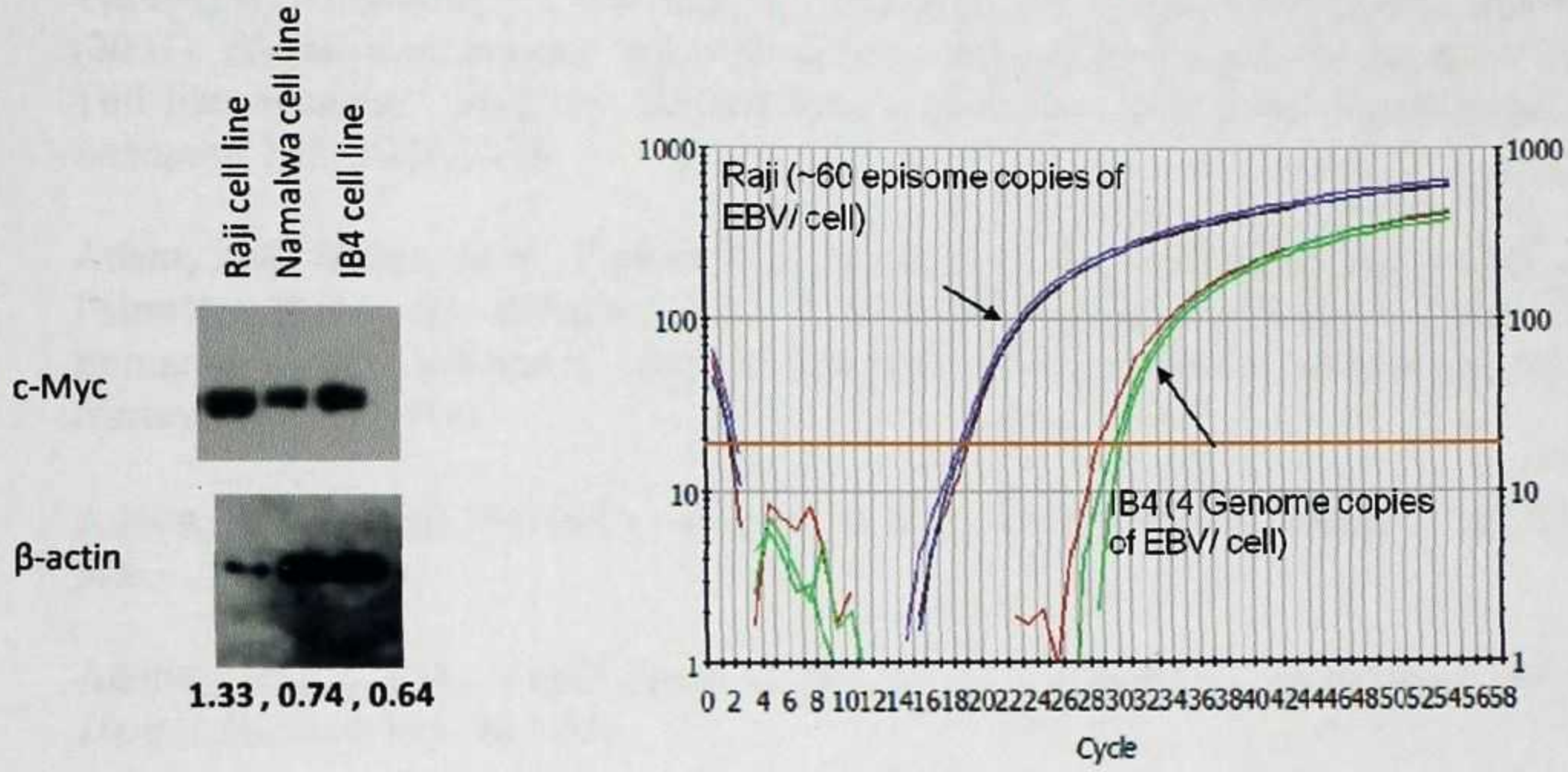
CD40L + IL4 + Parasite extract + Anti-Ig



Parasite Extract



APPENDIX E



Top left: c-Myc and β-actin western blot in Raji BL cell line, Namalwa and IB4 cell lines. Top right: Real Time PCR output of EBER1 expression in Raji BL cell line and IB4 cell line

APPENDIX F

Calculation of Mean log values

The mean log generates values close to the "true mean" when a distribution is log normal. To calculate mean log, convert all values to log values and then take the mean. The mean log can then be converted back to a non-log value. Assuming 100, 200, 900 and 11,000 represent values for the number/ level of infected cells (with their calculated or "actual mean" as 3,050), their log values become 2, 2.3, 2.95 and 4.04 respectively and their log mean is 2.82. This is then converted back to non-log value i.e $10^{2.82}$ which is 660, which is also close to the median of 550.

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