LACK OF ASSOCIATION BETWEEN INTERLEUKIN-6 (-174G/C), INTERLEUKIN-10 (-1082A/G, -819C/T, AND -592C/A) POLYMORPHISMS AND SUSCEPTIBILITY TO ENDEMIC BURKITT LYMPHOMA IN CHILDREN AGED BETWEEN 5-8 YEARS FROM WESTERN KENYA

By

Oduor Cliff Isaya

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Department of Biomedical Science and Technology

Maseno University

ABSTRACT

Endemic Burkitt lymphoma (eBL) is the most common paediatric cancer in sub-Saharan Africa and occurs at a high incidence in western Kenya. The tumour has the highest proliferative rate of any other human cancer. Although EBV and malaria co-infections appear to be primary factors in the aetiology of eBL, host genetic contributions that renders some children permissive to this pathologic pathway remains to be elucidated. Cytokine signals have the potential to be deletious in the context of lymphoma development by acting as growth facts and promoting tumour cell survival. Over expression of IL-6 and IL-10 is suspected to play a role in the pathogenesis of eBL. It has been shown that genetic variants within the IL-6 and IL-10 gene promoters correlate with elevated levels of these cytokines, but whether these SNPs confer risk to eBL development is unknown. Children genetically predisposed to produce elevated levels of these cytokines may be more susceptible to eBL development. This case-control study investigated the association between the IL-6 (-174G/C) and IL-10 (-1082A/G, -819C/T and -592C/A) promoter polymorphisms and susceptibility to eBL and whether these polymorphisms influence EBV loads in children (n=205) from western Kenya. DNA samples from 117 eBL cases and 88 healthy age-matched controls were used in the analysis. A real-time polymerase chain reaction (PCR) using TaqMan allelic discrimination assay was used for genotyping. The EBV loads were measured using quantitative real-time PCR. The distribution of IL-6 and IL-10 genotypes was compared using $\chi 2$ test. Across group comparisons were evaluated using Kruskal-Wallis test, while the association between the polymorphisms/haplotypes and susceptibility to eBL was determined using logistic regression analyses. Results showed comparable frequencies for IL-6 and IL-10 genotypes between the cases and controls. Logistic regression analysis demonstrated that the individual IL-6 -174G/C (P=0.080), IL-10 -1082A/G (P=0.900), -819C/T (P=0.541) and -592C/A (P=0.541) genotypes and the -1082A/-819C/-592C (ACC) [P=0.671], ATA [P=0.356] and GCC [P=0.708] haplotypes were not associated with susceptibility to eBL or increased EBV load. Results from this study do not eliminate a role for IL-6 and IL-10 in eBL pathogenesis however these common polymorphisms do not predispose to eBL development. The baseline information from this study necessitates future studies to explore other genetic variants regulating immune mediators that could contribute to eBL carcinogenesis.

CHAPTER ONE

1.0. INTRODUCTION

1.1. Background information

Endemic Burkitt lymphoma (eBL) is an aggressive form of B cell lymphoma that accounts for at least 74% of all childhood cancers in equatorial Africa (Brady *et al.*, 2007). It has the highest proliferative index of any human cancer and has the ability to progress rapidly with tumours doubling in size every 24-26 hours (Chêne, 2009). The cancer has a multifactorial aetiology, including environmental factors (i.e. Epstein Barr virus (EBV) and malaria infection) and immune system disturbances (Epstein, 1984; Hecht and Aster, 2000; Chêne *et al.*, 2007). However, the role of human genetic variants in eBL aetiology has not been explored.

Among immune modulating agents are cytokines that are produced by activated cells of the immune system. Accordingly, the potential roles of the cytokines in development of various malignancies have been determined (Cooper and Caligiuri, 2003). Cytokines produced by B cell malignancies are thought to contribute to Burkitt lymphoma (BL) pathogenesis by acting as growth factors as well as by initiating and sustaining the reactive inflammatory infiltrate within the tumour. Alternatively, cytokines produced by inflammatory cells may also support the proliferation and survival of BL cells (Cooper and Caligiuri, 2003; Ogden *et al.*, 2005).

The systematic overexpression of pro-inflammatory cytokines such as IL-6 and anti-inflammatory cytokines such as IL-10 have been suggested to be responsible for the initiation and maintenance of B cell malignancies (Cooper and Caligiuri, 2003). Both IL-6 and IL-10 have been shown to be highly expressed in BL tumour microenvironment, suggesting they may be giving signals that are beneficial in the tumour genesis and development (Yokoi *et al.*, 1990; Ogden *et al.*, 2005).

Cytokines such as IL-6 have been demonstrated to be a growth factor for EBV immortalized B cells resulting in increased immunoglobulin production and B cell immortalization (Yokoi *et al.*, 1990). It has also been shown to be highly expressed in EBV immortalised cells promoting tumour development (Cordano *et al.*, 2005). Like IL-6, high levels of IL-10 in BL patients also appear to be important in the development of the disease (Ogden *et al.*, 2005). IL-10 has been well characterized as a pro-tumourigenic cytokine with immunosuppressive effects by way of inhibition of interferon-γ, pro-inflammatory T-helper-1 lymphocytes and cytotoxic T-cells (Liu *et al.*, 2010) hence contributing to EBV and tumour escape from immune surveillance and enhancing tumour growth.

The observation that inter-individual variations in IL-6 and IL-10 production are genetically determined by single base substitutions at the gene promoter region (Ferrari *et al.*, 2003; Wang *et al.*, 2011) implies they may influence susceptibility to eBL tumorigenesis and/or host response to EBV infection. The human IL-6 gene is located on the short arm of chromosome 7 (7p21). There are four common single nucleotide polymorphisms (SNPs) in the promoter region of IL-6 gene at position -597(G/A), -572(G/C), -373(A/G) and -174(G/C) (Gourley *et al.*, 2002). The -174G/C polymorphism has been shown to influence IL-6 production *in vivo*: with associated decreased and increased expression for the G and C allele, respectively (Gourley *et al.*, 2002). The gene encoding IL-10 is located on the long arm of chromosome 1 (1q31-1q32). The promoter region contains three common SNPs at positions -1082(A/G), -819(C/T) and -592(C/A). Only -1082 appears to affect cytokine levels with A and G alleles associated with lower and higher IL-10 levels respectively (Baran *et al.*, 2008). Specific haplotypes of these three SNPs are also associated with IL-10 production levels, for example, the IL-10 -1082G, -819C, -592C (GCC) haplotype of this promoter region has been associated with high while the ATA haplotype with low IL-10 levels in children (Ouma *et al.*, 2008).

Since increased IL-6 and IL-10 expression has been associated with lymphoproliferative diseases, this study hypothesized that polymorphisms in the IL-6 and IL-10 promoter which increase their production may demonstrate predisposition to eBL along with higher EBV viremia. Given that there have been no investigations on the association of the IL-6 and IL-10 promoter polymorphisms, which influence expression levels of these cytokines, with susceptibility to eBL, this study was conducted on children residing in *P. falciparum* malaria holoendemic region in western Kenya.

1.2. Statement of the problem

Endemic Burkitt lymphoma (eBL) is the most common paediatric cancer in sub-Saharan Africa and occurs at a high incidence in western Kenya, a region that is already burdened by high prevalence of *P. falciparum* malaria (Rainey *et al.*, 2007). This tumour has the highest proliferative rate of any other human cancer with a doubling time of 24 - 26 hours (Chêne, 2009) and has 100% mortality rate if not treated. Although this cancer is responsive to chemotherapy the drugs are expensive, have cytotoxic effects, are not readily available and there have been cases of relapses (Levine, 2002; Molyneux *et al.*, 2012). The mortality rates of eBL could be compounded by limited data on how genetic variations in children would determine susceptibility to eBL development in a malaria holoendemic environment. Such information on the molecular events in eBL development would lead to development of better therapy with no cytotoxic effects and reduced resistance to treatments that has resulted in a high incidence of relapses.

Previous studies have implied that cytokines such as IL-6 and IL-10 are highly expressed in BL tumour micro-environment, indicating that they may have a role in enhancing eBL tumour development and inhibiting anti-tumour immunity (Tanner and Tosato, 1991; Zeidler *et al.*, 1997). Since increased production of these cytokines has been implicated in eBL tumour development, genetic variants that enhance expression of these cytokines would maintain a high EBV viremia once infected thus facilitating eBL development. Despite the fact that children genetically

predisposed to have a high expression of these cytokines (IL-6 and IL-10) would likely be at risk of developing this aggressive malignancy, the association between IL-6 (-174G/C), IL-10 (-1082A/G, -892C/T and -592C/A) promoter polymorphisms and susceptibility to endemic Burkitt lymphoma in resident in western Kenya has not been determined. Since studies have shown that children aged 5-8 years are the most affected in holoendemic regions such as in western Kenya, they form the target group of interest since they are likely to be the first beneficiary of a vaccine developed against eBL. As such, the current study tested for the association between IL-6 (-174G/C), IL-10 (-1082A/G, -819C/T and -592C/A) promoter polymorphisms and susceptibility to endemic Burkitt lymphoma in children (aged 5-8 years) resident in western Kenya.

1.3. Objective of the study

To determine the association between IL-6 (-174G/C), IL-10 (-1082A/G, -819C/T and -592C/A) promoter polymorphisms and susceptibility to endemic Burkitt lymphoma in children aged 5-8 years resident in western Kenya.

1.3.1. Specific objectives

i. To determine the association between IL-6 -174G/C and IL-10 (-1082A/G, -819C/T and -592C/A) promoter variants and susceptibility to eBL in children aged 5-8 years diagnosed with eBL compared to healthy controls from western Kenya.

ii. To determine the association between IL-10 promoter haplotypes and susceptibility to eBL in children aged 5-8 years from western Kenya.

iii. To determine the association between IL-6 (-174G/C) and IL-10 (-1082A/G, -819C/T and -592C/A) promoter variants and EBV load in children aged 5-8 years diagnosed with eBL compared to healthy controls from western Kenya.

1.3.2. Research questions

i. What is the association between IL-6 -174G/C and IL-10 (-1082A/G, -819C/T and -592C/A) promoter variants and susceptibility to eBL in children aged 5-8 years from western Kenya?

ii. What is the association between IL-10 promoter haplotypes and susceptibility to eBL in children aged 5-8 years from western Kenya?

iii. What is the association between IL-6 -174G/C and IL-10 (-1082A/G, -819C/T and -592C/A) promoter variants and EBV load in children aged 5-8 years from western Kenya?

1.4. Justification of the study

Although EBV and malaria co-infections appear to be primary factors in the aetiology of eBL, host genetic contributions to this pathologic pathway remains a gap that needs to be explored. Genetic variants that would contribute to modulate risk of eBL could act as targets to determine populations at risk of developing this malignancy. Cytokine signals have the potential of being both deletious and beneficial in cancer development. IL-6 and IL-10 have been shown to promote the growth, survival and immune evasion of B cell tumour cells and EBV immortalised B cells. These molecules may therefore be targets for drug development that would repress there functions in enhancing tumour survival. Children genetically predisposed to produce elevated levels of these cytokines may be at risk of developing eBL. Some genetic variants, more so in the promoter of a gene, influence expression of the IL-6 and IL-10 genes, leading to either increase or decrease in expression of these cytokines. Varied expression among individuals may contribute to susceptibility to eBL development and also increased EBV due impaired viral clearance initiated by overexpression of these cytokines.

This study investigated the association between IL-6 and IL-10 promoter polymorphisms and EBV loads as a functional measure of viral control and as a potential biomarker for eBL development.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Endemic Burkitt lymphoma

Burkitt lymphoma (BL) is an aggressive B cell malignancy with endemic, sporadic and immunodeficiency associated variants according to geographical distribution (Orem *et al.*, 2007). Endemic BL is the most common childhood cancer in Africa and is associated with Epstein Barr virus (EBV) in over 95% of the cases (God and Haque, 2010). In Africa this paediatric malignancy has the highest incidence rates in regions where *P. falciparum* malaria is highly prevalent, hence suggesting that the two are linked (Rainey *et al.*, 2007). This concept is reinforced by the observation that eBL disappears from the areas where malaria has been controlled. The devastating effect of this disease, especially to children, motivated studies on the epidemiology and aetiology of the disease (reviewed in (Magrath, 1991).

The basic molecular event in Burkitt lymphoma is translocation of the oncogene (*c-myc*) from its normal position on chromosome 8 to either chromosomes 2, 14 or 22, always in close proximity to the regions that regulate the expression of immunoglobin (Ig) genes (Chêne, 2009). In BL this translocation results in juxtapositioning of the DNA coding sequences for *c-myc* with enhancer sequences in the Ig genes. Because Ig enhancer elements are specifically active in mature B cells, their juxtaposition to *c-myc* in BL cells drives inappropriately high levels of *c-myc* mRNA and protein expression (Hecht and Aster, 2000). As a direct consequence of the chromosomal translocation in BL cells, negative regulatory sequences residing with *c-myc* gene are removed or mutated through other mechanisms, further contributing to increased *c-myc* activity seen in BL cells. The *c-myc* plays major role in cellular homeostasis and it activity is normally tightly regulated. Posttranscriptional control of this gene are usually overcome in BL through over

expression of *c-myc* protein and other secondary genetic events that enhance transforming activities, antagonize the activity of negative regulatory factors and/or downregulate *c-myc* activities that counteract cellular transformation (Hecht and Aster, 2000).

Endemic Burkitt lymphoma accounts for over 74% of all childhood cancers in equatorial Africa (Brady *et al.*, 2007) and has an incidence of 5-15 cases/100,000 children (Moormann *et al.*, 2007). It occurs mainly in male children with a sex ratio of about 2:1 and the majority of the cases are children aged between 2-14 years with a median peak incidence of 6 years.

Its tumours can present in a wide range of anatomical sites, although the head, neck and abdomen are commonly involved in 50-70% of the cases. It tends to present in organs undergoing rapid growth including the jaws in children and the breast and ovary in early reproductive years (Magrath, 1991; Magrath, 2010). Although therapy of eBL has a high survival fraction, it is still a great public health concern. Even though the affected children are getting treatment, there is need to do just more than just conventional chemotherapy, which is quite costly, and identify potential cytokine modulators to help resolve the tumour or help immune cells kill the tumours.

2.2. Endemic Burkitt lymphoma and infectious microbes

Up to 30% of all cancers worldwide have been linked to infectious diseases caused by microbes such as viruses, bacteria and parasites (Dave, 2010). Cancer is characterised by uncontrolled growth of cells in the body and microbes have been shown to make this happen, in ways that cause the body's immune system to initiate a cascade of events leading to inflammation, which can be chronic during persistent infection. A state of chronic inflammation may be permissive to the development of the cancer by enhancing tumour development and suppression of tumour surveillance. *P. falciparum* malaria parasite and EBV are the two microbes associated with eBL aetiology (Kafuko and Burkitt, 1970; Brady *et al.*, 2007; God and Haque, 2010).

It has been documented that EBV may play a role in the origin and development of eBL by dysregulation of *c-myc* through potentiation of *c-myc* activity and clonal expansion, direct mutagenesis and immune inactivation leading to the escape of malignant clones (Dalla-Favera *et al.*, 1982; Hecht and Aster, 2000; Orem *et al.*, 2007). Epstein Barr Virus is known to transform resting B cells into latently infected lymphoblastoid cells (Orem *et al.*, 2007). EBV immortalized cells have been shown to express a myriad of cytokines, which are thought to contribute to B cell immortalization, growth and chemotaxis, as well as inhibit potential immune recognition by effector cells (Baiocchi *et al.*, 2005). IL-6 and IL-10 are two potential cytokines related to EBV infection, since the virus expresses proteins that induce these cytokines (Eliopoulos *et al.*, 1997; Vockerodt *et al.*, 2001). Their elevation in the tumour microenvironment is thought to enhance eBL tumour growth (Vockerodt *et al.*, 2001). It has been documented that Latent Membrane Protein 1 (LMP1) induces IL-10 production in BL cells (Vockerodt *et al.*, 2001). The signalling events leading to the IL-10 expression in BL upon LMP1 expression still remain unclear but the enhanced IL-10 expression by LMP1 may be a mechanism by which the virus enhances tumour development due to the immunosuppressive actions of IL-10 (Vockerodt *et al.*, 2001). The production of an analogue of IL-10 (BCRF1/ viral IL-10), has been proposed to be an evolved mechanism of the virus to promote survival and proliferation of EBV infected cells, through the immunosuppressive properties of IL-10 (Liu *et al.*, 1997; Zeidler *et al.*, 1997; Khatri and Caligiuri, 1998).

The role of *P. falciparum* malaria infection in the pathogenesis of eBL is thought to arise from a combination of immune modulating events and B cell activation (Brady *et al.*, 2007), and is associated with a 100 fold increased risk of eBL (Kafuko and Burkitt, 1970). Previous studies on the effects of malaria on host immunity, which include a mixture of intense proliferation of lymphocytes stimulated by malaria antigens and impairment of immune mechanisms controlling polyclonal proliferation is thought to create a suitable environment for the reactivation of viruses such as EBV (reviewed in (Rochford *et al.*, 2005). During malaria infection, high IL-10 levels are

necessary for suppression of hepatic pathology, cerebral pathology and severe malarial anaemia (Ouma *et al.*, 2008; Niikura *et al.*, 2011) in the host by inhibiting the effects of TNF and IFN-γ (Niikura *et al.*, 2011). During protective effect of IL-10 on the host, it may predispose the host to eBL susceptibility due to IL-10's down regulating effect to the transporter protein 1 (TAP1) (Zeidler *et al.*, 1997), MHC class 1 expression and loading of peptides is decreased, therefore the potential of recognizing and lysing transformed B cells and EBV infected B cells is decreased (Bejarano and Masucci, 1998; Kurte *et al.*, 2004). This down-regulatory effect of IL-10 induced by malaria infection may be one of the links explaining the relationship between malaria and endemic BL development. Despite the availability of this information, the association between IL-10 promoter variants that enhance expression of IL-10 and susceptibility to eBL has not been investigated in children aged 5-8 years resident in *P.falciparum* holoendemic transmission regions of western Kenya. The current study determined the association between the IL-10 polymorphisms and susceptibility to eBL in this study population.

2.3. Cytokines and cancers

It is evident that cytokine signals have the potential to be both deleterious and beneficial in the context of cancer (Balkwill and Mantovani, 2001; Cooper and Caligiuri, 2003). The body's response to inflammation is not unlike that of its response to tumours (Cooper and Caligiuri, 2003). Many similarities have been noted between the processes of wound healing and tumorigenesis and numerous associations have been established between inflammation and tumours. Some of these include links between infection with papillomavirus, *Helicobacter pylori* and hepatitis with cervical, stomach and liver cancers respectively (Balkwill and Mantovani, 2001).

Cytokines and inflammation are intimately linked and in each of these infections, the pro-inflammatory environment created by cytokines and chemokines is thought to play a major role in the promotion of tumour development and progression (Cooper and Caligiuri, 2003). There are multiple mechanisms by which inflammation and cytokines might result in tumour development,

including the promotion of cell survival and proliferation, tumour cell differentiation and cell migration (Balkwill and Mantovani, 2001). A number of cytokines have been directly linked to the development of human cancers, for example IL-2, IL-6, IL-10, IL-15, TNF-α, IL-1β and MCP-1 (Voorzanger *et al.,* 1996). Of these cytokines, IL-6 and IL-10 have been associated with B cell malignancies (Tosato *et al.*, 1993; Khatri and Caligiuri, 1998). Cytokines produced by B cell malignancies are thought to contribute to eBL pathogenesis by acting as autocrine growth factors as well as by infiltrating and sustaining the reactive inflammatory infiltrate within the tumour. Alternatively, cytokines produced by inflammatory cells may also support the proliferation and survival of BL cells (Cooper and Caligiuri, 2003; Ogden *et al.*, 2005).

2.4. Role of IL-6 and IL-10 in the development of endemic Burkitt lymphoma

Networks of cytokines have been demonstrated to exert regulatory effects on the proliferation of normal lymphocytes, but their interaction *in vivo* on the proliferation of malignant lymphoma cells is still poorly understood. Some of the cytokines shown to influence tumorigenesis of lymphatic cells include IL-10, IL-6, IL-2 and TNF-α (Voorzanger *et al.,* 1996).

Previous studies have reported elevated IL-10 production in various B cell malignancies including acute lymphoblastic leukemia, chronic lymphocytic leukemia, Burkitt's lymphoma and non-Hodgkins lymphomas (Khatri and Caligiuri, 1998). Although the significance of IL-10 in the development of B cell malignancies is not clearly understood, there is good experimental evidence suggesting that the cytokine plays a key role in the development of these cancers (Cooper and Caligiuri, 2003). For example, injection of EBV positive human peripheral blood lymphocytes into mice with severe combined immune deficiency (SCID) but with variable IL-10 levels lead to development of spontaneous human EBV associated B cell lymphoproliferative disorder (EBV-LPD) in mice with significantly higher serum levels of human IL-10. The study further showed that serum supplemented with IL-10 was able to prevent programmed cell death and promote cell growth of fresh human tumour from the mice *in vitro* (Baiocchi *et al.*, 1995).

Interleukin-10 has been shown to activate human macrophages to have an enhanced capacity to engulf apoptotic cells *in vitro* and to produce noticeably higher levels of the B cell survival factor (BAFF) (Ogden *et al.*, 2005). These IL-10 activated macrophages have the potential to promote BL pathogenesis through suppression of anti-tumour immunity following enhanced engulfment of tumour cells and through increased production of tumour cell growth factors (Ogden *et al.*, 2005).

Besides IL-10 effects on cell survival and proliferation, other studies have shown that there are several other potential mechanisms in which IL-10 can promote enhanced progression of B cell malignancies (Bejarano and Masucci, 1998; Biron *et al.*, 1998). For control of viral infection, type 1 adaptive T cell immune response such as interferons' (IFN's) secretion is required. However, IL-10 has been known to support type 2 adaptive T-cell response which is characterised by immunosuppression of an effective type 1 antiviral response (Biron *et al.*, 1998). Elevated levels of IL-10 would therefore aid in suppressing type 1 response to EBV infection and may potentially lead to higher levels of EBV loads that increase chances of development of eBL. Outgrowth inhibition assay of $CD4^+$ and $CD8^+$ enriched populations used to analyse the effect of IL-10 on EBV-induced B-cell proliferation showed that IL-10 abrogates the growth inhibitory capacity of the T cells and also affects the growth of EBV-infected B cells. This IL-10 effect was mediated through suppression of T-cell activation induced by IL-2 and IFN-γ production and caused a direct enhancement of EBV infected B cell growth (Bejarano and Masucci, 1998).

Recent studies have identified IL-6 as an important factor in establishment and maintenance of EBV immortalized B cells. The virally infected cells have been shown to express IL-6 surface receptors, secrete IL-6 and use the cytokine as a growth factor (Yokoi *et al.*, 1990).

A possible role of IL-6 in the multistep process of B cell tumorigenesis has been explored by expressing the human IL-6 gene in EBV immortalized B cells and examining the ability of these cells to form tumours (Tanner and Tosato, 1991). The increased tumourigenicity of IL-6 expressing

lymphoblastoid cells *in vivo* may have been dependent on its inhibitory effect on natural killer cells at the tumour site. Interleukin-6 can also down regulate IL-1β and TNF gene expression in human activated monocytes by acting at the transcriptional level. If the tumour-derived IL-6 is capable of similar actions *in vivo*, then it could effectively inhibit a potentially important cytocidal function of the monocyte at the tumour site (Tanner and Tosato, 1991).

2.5. Variations in IL-6 and IL-10 gene in regulating IL-6 and IL-10 levels

In recent years, there have been enormous efforts to identify genetic mutations that play a major role in predisposition and disease progression for particular cancers. In Burkitt lymphoma, the *c-myc* translocation is the hallmark of this cancer (Brady *et al.*, 2007), however, it is likely that a number of low-penetrance genes contribute to this cancer susceptibility in a larger population of patients and may therefore be responsible for a greater proportion of the disease burden (Howell and Rose-Zerilli, 2007a).

Many SNPs and a more limited number of microsatellite polymorphisms have been detected within cytokine gene sequences, particularly within the promoter regions of these genes (Howell and Rose-Zerilli, 2007b). Some of these polymorphisms have been associated with differential rate of gene transcription (Howell and Rose-Zerilli, 2007a), resulting in abnormally high or low cytokine production. Given the important roles that IL-6 and IL-10 appear to have in BL carcinogenesis; it is biologically plausible that the genetic variations of the IL-6 and IL-10 gene may modulate the risk to eBL development.

The gene encoding IL-10 maps on the long arm of chromosome 1 in the q31-q32 region and its 5' flanking region contains numerous polymorphisms. Its promoter is highly polymorphic with two informative microsatellites and three frequent point mutations, -1082 A/G (rs1800896), -819 C/T (rs1800871) and -592 C/A (rs1800872) (Kingo *et al.*, 2003). These mutations have been shown to influence gene transcription and protein production, hence have been highly investigated. It has been reported that the IL-10 SNP at position -1082 and -1082, -819 and -519 haplotypes have

been associated with differential IL-10 expression *in vitro* (Wang *et al.*, 2011), with the -1082G, -819C and -592C haplotype associated with high IL-10 expression (Kingo *et al.*, 2003; Lech-Maranda *et al.*, 2004; Ouma *et al.*, 2008).

It has been shown that both the -1082G and A alleles in the promoter region of the human IL-10 gene physically interact with a nuclear protein which has been identified as poly (ADP-ribose) polymerase 1 (PARP-1) in an allele-specific manner that results in different levels of IL-10 transcription (Wang *et al.*, 2011). It was shown that PARP-1 acts as a transcription repressor. Because the -1082G promoter is bound by a PARP-1 to a lesser extent than the -1082A promoter, the former has a higher transcriptional activity than the latter (Wang *et al.*, 2011).

The other two polymorphisms (-819C>T and -592C>A) most likely do not influence the production of IL-10, but multi-site haplotypes are highly informative allelic markers that can reveal association with disease outcomes not identifiable with single polymorphisms and combinations of different functional polymorphic alleles in the haplotypes indicate how these polymorphisms interact to amplify or moderate individual effects (Ouma *et al.*, 2008). Consistent with this notion, previous studies have demonstrated that the -1082G/ -819C/ -592/C (GCC), ACC, and ATA haplotypes were associated with high, intermediate, and low IL-10 transcriptional activity, respectively (Ouma *et al.*, 2008; Wang *et al.*, 2011).

IL-6 is tightly regulated at the level of expression by several hormones, cytokines and their transcription factors. Among them, IL-1 and TNF-α activate, whereas estradioal and glucocorticoids repress IL-6 gene transcription (Ferrari *et al.*, 2003). Studies have identified several allelic variants in the IL-6 gene promoter region. Among them, a common G→C polymorphism at position -174 involves a DNA binding site for NF-IL-6, a transcription factor that can also interact with estradiol/estrogen receptor complexes to regulate IL-6 gene expression. There has been evidence that this polymorphism produces a functional variant in the -174G allele that results in

lower stimulated IL-6 promoter activity *in vivo* and lower circulating IL-6 concentration compared with the C allele (Gourley *et al.*, 2002; Ferrari *et al.*, 2003).

Given that IL-6 and IL-10 may have an important role to play in eBL tumourigenesis (Yokoi *et al.*, 1990; Tanner and Tosato, 1991), it is biologically plausible that an inherited tendency to produce more IL-6 and IL-10 may confer susceptibility, whereas lower levels may be associated with protection. As such, the current study investigated the frequency of IL-6 (-174G/C), IL-10 (-1082A/G, -819C/T and -592C/A) promoter polymorphisms in children diagnosed with endemic Burkitt lymphoma compared to healthy children residing in *P. falciparum* malaria holoendemic regions.

2.6. Genetic variants and EBV load

Despite the ubiquity of EBV in human population worldwide, the presence of the viral genome within eBL tumours suggest a key role of EBV in the development of this malignant neoplasia. Since not all children develop eBL, it has been postulated that some children might be genetically more susceptible to maintaining a higher EBV viremia and to the development of the EBV associated neoplasias (da Silva *et al.*, 2007). Genetic variations that influence the balance between anti-inflammatory and pro-inflammatory cytokines are likely to contribute to the immunologic dysregulation observed in eBL patients. Not only could the abnormal turnover of cytokines increase the proliferation and survival of eBL cells (Ogden *et al.*, 2005) but it may also contribute to the impairment of the host immune response against them and maintain a higher EBV viremia once infected with EBV.

The observation that some individuals may be genetically predisposed to produce different levels of IL-10 (Ouma *et al.*, 2008), which to some degree may account for maintenance of a high EBV load, may increase their risk to eBL development. A high frequency of the IL-10 (-1082) GG genotype has been found in EBV positive Hodgkin's lymphoma (HL) cases, in comparison with EBV negative cases. Since this genotype has been reported to confer a high IL-10 expression

levels, it suggests that EBV positive HL probably arise more easily in a scenario of higher levels of basal IL-10 synthesis (da Silva *et al.*, 2007). A genetically determined higher expression of IL-10 may be among the factors contributing to the immunologic dysfunction in inhibiting/suppressing EBV infection and the IL-10 promoter SNPs could be putative risk factors for development of eBL due to enhanced viral loads.

The proliferation of EBV transformed B cell is enhanced by IL-6 that acts as one of the autocrine growth factors (Tanner and Tosato, 1991). Elevated expression of this cytokine would enhance the growth of the EBV immortalized B cells and thus would sustain the replication of EBV within these cells resulting in higher EBV loads. IL-6 has also been shown to cause natural killer (NK) cell dysfunction (Tanner and Tosato, 1991) which may illustrate how the virus escapes immune surveillance since the NK cells contribute to innate defence against viral infection (Biron and Brossay, 2001). This study investigated whether individuals genetically predisposed to produce high and low levels of these cytokines (IL-6 and IL-10) would have differential EBV loads that could either increase a child's risk to or protective against eBL development.

CHAPTER THREE

3.0. METHODOLOGY

3.1. Study area

Study participants diagnosed with eBL were recruited from Jaramogi Oginga Odinga Teaching and Referral Hospital (Latitude: -0.088697, Longitude: 34.772016) located in Kisumu City which is the largest hospital in the region and is the referral centre for childhood cancer cases. Healthy children serving as controls were recruited from Chulaimbo Health Centre (Latitude: -0.037972, Longitude: 34.638299) which is the regional sub-District Hospital that serves rural residents of Kisumu County. These children reside in malaria holoendemic areas similar to eBL cases and were frequency matched by malaria exposure, age range and year of enrolment.

3.2. Study population

This sub-study was part of an on-going parent study looking at both immunologic and genetic factors in the aetiology of eBL that commenced in 2003. A total of 117 samples from patients with a pathology confirmed diagnosis of eBL were retrospectively availed. A total of 88 malaria exposed yet otherwise healthy controls were also recruited. All the cases and controls were of the Luo ethnic group in Nyanza Province as this provides a homogeneous population for a genetic based study. Although eBL prevalent in children aged between 2-14 years, it has a higher incidence among children aged between 5-8 years, therefore children within this age bracket (5-8) within malaria holoendemic regions are at a higher risk of developing eBL. Cases and controls were matched by age (between 5-8 years) and sex. In total, 117 cases and 88 controls was used for the final analyses.

3.2.1. Inclusion criteria

The eBL cases had to be confirmed by two independent pathologists from needle aspirations stained with May-Grunewald Giemsa. The cases and the controls had to meet the following criteria: Had to be within an age range of 5-8 years, HIV negative, residing in western Kenya were malaria is holendemic and had to be of the Luo ethnic group. Since the Luo ethnic group is the most dominant indigenous community in malaria holoendemic regions in western Kenya, ethnicity matching was done to provide a homogenous population for this genetic study.

3.2.2. Exclusion criteria

Children among the controls that may have had any lymphoma in the past were not considered for this study. The eBL cases that did not have a confirmed diagnosis report from the two pathologists were excluded from this study.

3.3. Sample size determination

The sample size of 205 was based on the allele frequencies of the IL-10 promoter polymorphisms in a western Kenya population which was able to detect significant differences in susceptibility to severe malaria anaemia in 137 cases and 238 controls (Ouma *et al.*, 2008). Since the study was interested in proportion of the alleles and genotypes of IL-6 and IL-10 among the eBL cases and non-eBL controls, sample size determination was based on the following formula:

$$n = \left(\frac{r+1}{r}\right)\frac{(\bar{p})(1-\bar{p})(Z_{\beta}+Z_{\alpha/2})^2}{(p_1-p_2)^2}$$

Where;

n is the sample size.

r is the ratio of cases to controls.(1.3)

p is a measure of variability (similar to the standard deviation)

$(p_1\text{-}p_2)$ is the effect size (difference in proportion)

Z_{β} represents the desired power (typically 0.84 for 80% power)

Z_α represents the desired level of statistical significance (typically 1.96) used to determine sample size (Whitley and Ball, 2002).

The proportion of children possessing the genotypes that enhance cytokine (IL-10) expression in a population that forms part of the control group in western Kenya was found to be 15% in a previous study (Ouma *et al.*, 2008), hence the proportion of cases exposed to eBL and possess the marker was calculated as follows:

$$p_{case\,exp} = \frac{ORp_{controls\,exp}}{p_{controls\,exp}(OR-1)+1}$$

$$p_{case\,exp} = \frac{2.0(0.15)}{(0.15)(2.0-1)+1} = \frac{0.30}{1.15} = 0.26$$

The average proportion of the individuals who possessed the genetic marker was (0.26+0.15)/2=0.205

$$n = 1.752\frac{(0.205)(1-0.205)(0.84+1.96)^2}{(0.26-0.15)^2} = 183.96 \approx 184$$

The calculated sample size was 184. This study had a sample size of 205 in the final analysis. This sample size was divided in to 117 cases and 88 ethnically matched controls.

3.4. Sample processing

DNA was extracted from whole blood samples as per the Qiagen™ DNAeasy kit protocol (QIAGEN Sciences, Germantown, MD, USA). About 4µl of *RNase A* was pipetted into the bottom of a sterile 1.5ml micro-centrifuge tube and 200µl of anti-coagulated blood added. Incubation at room temperature for 2 minutes followed. 20µl of *Proteinase K* was pipetted into the mixture as well as 200µl of *Buffer AL* then vortexed for 10 seconds. The reaction mixture was incubated at 56^0C for 10 minutes and 200µl of absolute ethanol added to each sample then vortexing done to create homogeneity. 630µl of the mixture was pipetted into the center of a DNeasy spin column sitting in a 2ml collection tube and spun at 8,000 rpm for 1 minute in an Eppendorf Centrifuge 5427

R (Eppendorf™ AG Barkhausenweg Hamburg Germany) at room temperature. The flow through was disinfected in 10% bleach and the DNeasy spin column placed in new collection tube. 500µl of *Buffer AW1* was added and incubation done for 5 minutes at room temperature then centrifuged at 8,000 rpm for 1 minute. The flow-through was disinfected in 10% bleach and the DNeasy spin column placed in fresh collection tubes. 500µl of *Buffer AW2* was added and incubation done for 5 minutes. The columns were centrifuged at 13,200 rpm for 3 minutes. Flow-through tubes were discarded and the spin columns transferred to separately labelled 1.5ml micro-centrifuge tubes. 100µl of *Buffer AE* was added to each spin column then incubated for 5 minutes before centrifugation at 8,000 rpm for 1 minute for DNA elution. Another 100µl *Buffer AE* was added for a second elution step. The spin columns were discarded in 10% bleach. The extracted DNA was then quantified using the Nanodrop 2000™ spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) to determine the quantity of DNA extracted for the PCR reactions. The concentrations of the DNA extracted from the samples ranged between 25 - 50 ng/µl, which was adequate for the genotyping assays and determination of the EBV viral loads in each sample. The DNA samples were then stored at -20^{0}C until use.

3.5. IL-10 and IL-6 genotyping

The IL-10 variants (-1082A/G, -819C/T and -592C/A) and IL-6 variant (-174G/C) were genotyped using high through-put TaqMan SNP genotyping assay technology on the StepOne Plus Real–Time PCR system (Applied Biosystems, Foster City, CA, USA). The assay IDs for the SNPs were IL-10 -1082A/G (rs1800896, assay ID, C_1747360_10), -819C/T (rs1800871, assay ID: C_1747362_10) and -592C/A (rs1800872, assay ID: C_1747363_10) (Applied Biosystems, Foster City, CA, USA). The sequences of the custom IL-6 -174G/C SNP assay forward and reverse primers and the two probes to distinguish the alleles are shown in Appendix 1(a). All PCR reactions were carried out in a final reaction volume of 10µl containing 5µl of TaqMan gene

expression master mix, 0.5µl of the SNP assay mix, 3.5µl of molecular grade water and 1.0µl of the sample DNA. The following cycling parameters was used to genotype all the four SNPs investigated in this study; pre-PCR hold stage was done at 60°C for 30 seconds, hold stage at 95°C for 10 minutes, cycling stage at 95°C for 15 seconds and annealing at 62°C for 1 minute. The holding stage to the annealing stage was repeated 40 times. The allelic discrimination plots for the IL-10 (-1082A/G, -819C/T and -592C/A) and IL-6 (-174G/C) promoter polymorphisms are shown in Appendix 2(a), 2(b), 2(c) and 2(d).

3.6. EBV viral loads determination

The EBV viral load was determined according a previous protocol (Hayden *et al.*, 2008) and later modified by Moormann and others (Moormann *et al.*, 2005). For EBV real time quantitative polymerase chain reaction (RTQ-PCR), two PCR primers and Taqman® probes (that detect a 70bp region of the EBV BALF5 gene) combinations were used. The first probe and primer specifically targets and amplifies a 97bp conserved sequence in the EBV EBNA-1 gene while the second probe and primer combinations targets and amplifies a spiked internal control (IC- human beta actin gene) which acts as a house keeping gene designed to prevent false negative results due to inefficient extraction or inhibition of PCR amplification. These previously designed primers amplifying a conserved sequence of viral EBV DNA polymerase (BALF5) gene and a fluorogenic probe for this area (70bp) that have been described targets the amplified sequences (Kimura *et al.*, 1999). The probes were synthesized by PE Applied Biosystems (Foster City, CA, USA) and there sequences are shown in Appendix 1(b). The quantitative (q)-PCR cycle was as follows: 2 minutes at 50 °C, 10 minutes at 95 °C, 42 cycles of 15 sec at 95 °C and 1 minute at 60 °C using a thermal cycler model Bio-Rad CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The IQ Supermix (BioRad laboratories, Hercules, CA) was used for all reactions. To generate a standard curve, two commercial lots of fluorimetrically quantified EBV B95-8 DNA (Advanced Biotechnologies Inc.,

Columbia, MA) were used to create a six point tenfold serial dilution curve ranging from 2.65 copies/µl to 2.62×10^6 copies/µl. All the samples were assayed in duplicate, of which the mean value was taken as the DNA copy number. The EBV viral copy was calculated by extrapolation to a standard curve consisting of serially-diluted EBV DNA standards. The lowest detection limit for this assay was 1 copy of EBV genome per µg of human beta-actin DNA multiplexed in the assay (Piriou *et al.*, 2012). The BL41 lymphoma cell line DNA was used as no template control to check against non-specific genomic amplification as it EBV negative (Farrell *et al.*, 1991).

3.7. Data management and statistical analysis

Data collected was entered into Excel spreadsheet for cleaning and all the data analysis was conducted on SPSS software package version 20.0 (IBM SPSS, Inc, Chicago, IL, USA). The variables collected and recorded were case number, study groups (case/control), age (in years), sex, EBV load, and genotypes for IL-10 (-1082A/G, -819C/T, -592C/A) and IL-6 (-174G/C) polymorphisms.

To determine the distribution of IL-6 and IL-10 promoter variants in the population, Hardy-Weinberg Equilibrium and chi-square analysis were performed on both cases and control samples for the purposes of revealing genetic deviations on all the SNPs. Haplotypes of the IL-10 genetic variants were constructed using the HPlus software (Version 2.5). The results from IL-6 and IL-10 genotypes and haplotypes were analysed using a goodness-of-fit-test to establish whether the relationships visualized during descriptive analysis were significant or were due to chance alone (Lewis and Knight, 2012). Pearson's χ^2 test was used to assess departure from the null hypothesis that cases and controls have the same distribution of the genotype counts.

The association between IL-6 and IL-10 genetic variants/haplotypes and the risk of eBL was determined using multivariate logistic regression while controlling for potential confounders such as age, gender and malaria status (Rainey *et al.*, 2007; Ogwang *et al.*, 2008).

Pairwise comparisons of EBV loads among the cases and controls were done using Mann-Whitney U test. EBV loads for each IL-10 and IL-6 genotypes were described using measures of central tendency and measures of dispersion. Across group comparisons of EBV loads was determined using Kruskal-Wallis test. All statistical significance levels were defined as a probability value less than 0.05 ($p<0.05$).

3.8. Ethical considerations

Approval to carry out this study was provided by the School of Graduate Studies (SGS) of Maseno University. Ethical approval was obtained from the Kenya Medical Research Institute (KEMRI) Ethical Review Committee and University of Massachusetts Medical School Institutional Review Board (Appendix 3). Written informed consent was obtained from the children's parents or guardians (Appendix 4). Confidentiality was maintained throughout the study.

CHAPTER FOUR

4.0. RESULTS

4.1. Clinical, demographic and laboratory characteristics of the study participants

A total of one hundred and seventeen (117) endemic Burkitt lymphoma patients (cases) and eighty-eight (88) controls were included in this study. The demographic, clinical and laboratory characteristics of the study population are presented in Table 1. The results showed that the cases and controls differed significantly with regards to age, *P. falciparum* infection and EBV loads ($p=0.001$, $p<0.001$ and $p<0.001$, respectively), demonstrating that these could be risk factors for eBL development in children population. However, the proportions of males vs. females were comparable between the cases and controls ($p=0.140$). Although age differed between the groups with the controls being older than the cases, both groups were within the age range of which children are at a higher risk of developing eBL in western Kenya.

Table 1: Clinical, demographic and laboratory characteristics of the study participants

	eBL cases	Controls	*p*-value
No. of participants (n = 205)	117	88	
Gender, n, (%)			
Male	77, (65.8%)	49, (55.7%)	0.140[a]
Female	40, (34.2%)	39, (44.3%)	
Age, years; median	5	7	**0.001**[b]
Malaria status, n, (%)			
P.f. positive	16, (13.7)	70, (79.5)	
P.f. negative	101, (86.3)	18, (20.5)	**<0.001**[a]
EBV copies /ng DNA			
Median Rank	133.15	56.7	**<0.001**[b]

[a] Statistical significance determined by the χ^2 analysis. [b] Statistical significance determined by the Mann-Whitney U test. Values in bold were statistically significant at $p<0.05$. EBV=Epstein Barr Virus; *P.f.=Plasmodium falciparum.*

4.2. Association between IL-6 (-174G/C) and IL-10 (-1082A/G, -819C/T and -592C/A) genotypes and endemic BL

Prior to determining the association between the individual genotypes and susceptibility to eBL, the distribution of the genotypes in the cases and controls were carried out (Table 2). Results revealed comparable distributions of the genotype frequencies in controls and eBL cases (Table 2).

Additional analysis using multivariate logistic regression controlling for age and sex (Rainey *et al.*, 2007; Ogwang *et al.*, 2008) was performed to establish the association between the individual genotypes and susceptibility to eBL (Table 2). As shown in Table 2, there was no significant association between the individual IL-6 -174G/C and IL-10 (-1082A/G, -819C/T and -592C/A) promoter variants when comparing eBL cases to controls. The findings presented here suggest that these common variants do not alter susceptibility to eBL.

Table 2: Association between IL-6 -174G/C and IL-10 (-1082A/G, -819C/T and -592C/A) genotypes and endemic Burkitt lymphoma

SNPs	eBL cases n, (%)	Controls n, (%)	OR (95% CI)	*p*-value
IL-10 -1082A/G				0.900[a]
AA	53, (45.3)	39, (44.3)	1.000 (reference)	
AG	53, (45.3)	39, (44.3)	1.229 (0.544-2.778)	0.620[b]
GG	11, (9.4)	10, (11.4)	0.910 (0.24-3.459)	0.890[b]
IL-10 -819C/T				0.541[a]
CC	32, (27.4)	28, (31.8)	1.000 (reference)	
TC	61, (52.1)	39, (44.3)	1.679(0.68-4.146)	0.261[b]
TT	24, (20.5)	21, (23.9)	1.151 (0.391-3.383)	0.798[b]
IL-10 -592C/A				0.541[a]
CC	32, (27.4)	28, (31.8)	1.000 (reference)	
AC	61, (52.1)	39, (44.3)	1.679 (0.68-4.146)	0.261[b]
AA	24, (20.5)	21, (23.9)	1.151 (0.391-3.383)	0.798[b]
IL-6 -174G/C				0.080[a]
GG	113, (96.6)	88, (100.0)	1.000 (reference)	
CG	4, (3.4)	0, (0.0)	-	-
CC	0, (0.0)	0, (0.0)	-	-

Common variants for the IL-6 and IL-10 genes were determined for a population of African children in western Kenya. Chi-square test and multivariate logistic regression was used to test the null hypothesis. [a] Statistical significance determined by the χ^2 analysis. [b] Statistical significance determined by the multivariate logistic regression analysis. Odds ratios (OR) and 95% confidence intervals (CI) were determined using multivariate logistic regression controlling for age and gender. The reference groups in the multivariate logistic regression analysis were the homozygous wild-type genotypes. A striking bias favouring the IL-6 -174G allele over the -174C allele was seen in both the cases and controls which made it difficult to conduct a multivariate logistic regression analysis for the SNP with eBL risk.

4.3. Association between IL-10 (-1082A/G, -819C/T and -592C/A) haplotypes and endemic Burkitt lymphoma

Prior to determining the associations between the IL-10 haplotypes and EBV load, a multivariate logistic regression analysis controlling for covariates was performed to determine whether the IL-10 promoter haplotypes are associated with eBL while controlling for the confounding effects such as age and gender (Rainey *et al.*, 2007; Ogwang *et al.*, 2008). Multi-site haplotypes are highly informative allelic markers for identifying associations with disease

outcomes, not identifiable with single polymorphisms (Ouma *et al.*, 2008). The IL-10 promoter haplotypes were constructed using HPlus software (version 2.5). Of the 9 possible haplotypes generated from the IL-10 promoter SNPs, only 3 haplotypes (ACC, ATA and GCC) were selected for analysis because their frequencies in the study population were greater than 1%. These analyses revealed that there was no significant association between any of the three IL-10 promoter haplotypes and susceptibility to eBL (-1082A/-819C/-592C (ACC), OR; 0.841, 95% CI, 0.378-1.871, p=0.671; ATA, OR; 1.489, 95% CI, 0.640-3.464, p=0.356; GCC, OR; 1.160, 95% CI, 0.534-2.521, p=0.708).

Table 3: Association between IL-10 (-1082/-819/-592) haplotypes and endemic Burkitt lymphoma

Haplotype (-1082/-819/-592)	OR	95% CI	*p*-value
ACC	0.841	0.378-1.871	0.671
ATA	1.489	0.640-3.464	0.356
GCC	1.160	0.534-2.521	0.708

Children were classified based on the presence or absence of eBL. Odds Ratios (OR) and 95% confidence interval (CI) were determined using multivariate logistic regression controlling for age and sex (Rainey *et al.*, 2007; Ogwang *et al.*, 2008). The reference group in the logistic regression analysis were those individuals without the haplotypes.

4.4. Association between IL-6 (-174G/C) and IL-10 (-1082A/G, -819C/T and -592C/A) genotypes and EBV load

To further investigate the possible association of these genetic variations within inflammatory pathways and eBL development, the relationship between the IL-6 and IL-10 promoter SNPs and EBV load was determined. Table 4 presents results on the association between IL-6 (-174G/C) and IL-10 (-1082A/G, -819C/T and -592C/A) genotypes and median EBV loads. No significant difference was observed in the IL-6 (-174G/C) and IL-10 (-1082A/G, -819C/T and -592C/A) promoter polymorphisms and EBV load.

Table 4: Association between IL-6 (-174G/C) and IL-10 (-1082A/G, -819C/T and -592C/A) genotypes and median EBV loads

SNPs	Median (IQR) (EBV copies/ng DNA)	*p*-value
IL-10 -1082A/G		
AA	0.379 (18.8)	
AG	1.107 (9.66)	0.981[a]
GG	0.368 (9.24)	
IL-10 -819C/T		
CC	0.390 (16.41)	
TC	0.676 (14.67)	0.602[a]
TT	0.217 (12.15)	
IL-10 -592C/A		
CC	0.390 (16.41)	
AC	0.676 (14.67)	0.602[a]
AA	0.217 (12.15)	
IL-6 -174G/C		
GG	0.441 (12.6)	
CG	0.63	0.796[a]
CC	-	

EBV load data is represented as medians (interquartile range; IQR). [a] Kruskal-Wallis test, with values being statistically significant at $p \leq 0.05$.

4.5. Association between IL-10 (-1082A/G, -819C/T and -592C/A) haplotypes and EBV loads

In order to determine the association between IL-10 haplotypes and EBV loads, Mann-Whitney U test on EBV loads was performed between those that had the haplotype and those that did not. These analyses showed that the three IL-10 promoter haplotypes are not associated with the EBV load (ACC; p=0.830, ATA; p=0.917 and GCC; p=0.849) (Table 5).

Table 5: Association between IL-10 (-1082/-819/-592) haplotypes and EBV load (EBV copies/ng DNA)

Haplotype (-1082/-819/-592)		EBV load (EBV copies/ng DNA) Median (IQR)	*p*-value
ACC	1	0.474 (23.9)	0.830*
	0	0.423 (9.82)	
ATA	1	0.4871 (12.34)	0.917*
	0	0.39 (16.41)	
GCC	1	0.496 (9.66)	0.849*
	0	0.379 (18.83)	

EBV viral loads are medians (interquartile range; IQR) and the comparisons between carriers and non-carriers of the IL-10 promoter haplotypes were computed using Mann-Whitney U test. 1=carrier of the haplotype, 0=non-carrier. *Statistical significance was determined by Mann-Whitney U test.

CHAPTER FIVE

5.0. DISCUSSION

Human genome sequencing is being used to identify genetic mutations that predispose individuals to many diseases and in particular cancer (Howell and Rose-Zerilli, 2007b). It is highly likely that a number of low-penetrance genes contribute to cancer susceptibility and may be responsible for a greater proportion of disease burden than previously recognized. It was therefore of considerable interest to determine if common polymorphisms may be responsible for a predisposition to eBL. This study selected four SNPs from two cytokine genes (IL-6 and IL-10) known to be involved in inflammatory responses and which have been associated with the development of EBV immortalised B cells and BL (Tanner and Tosato, 1991; Ogden *et al.*, 2005). Although the empirical data imply that enhanced expression of these cytokines would facilitate eBL tumour development and progression (Tanner and Tosato, 1991; Khatri and Caligiuri, 1998; Gilbert and Hemann, 2012), the findings of this study revealed no association of these cytokine gene polymorphisms and susceptibility to eBL.

Interleukin-10 is an anti-inflammatory cytokine that suppresses T-helper 1 (Th-1) response but also regulates growth and differentiation of B cells, natural killer (NK) cells, cytotoxic and helper T cells (Yokoi *et al.*, 1990). Elevated levels of IL-10 have also been shown in various B cell malignancies and appear to mediate survival of tumour cells (Khatri and Caligiuri, 1998). Experimental evidence has suggested that this cytokine may play a key role in the development of eBL (Cooper and Caligiuri, 2003). IL-10 enhances the development of EBV associated B cell lymphoproliferative disorders, by preventing programmed cell death of the immortalized B cells and promoting growth of the tumour (Baiocchi *et al.*, 1995). It has also been shown to stimulate the production of B cell survival factor (BAFF) in BL tumour microenvironment, which mediates survival of the BL tumour cells (Ogden *et al.*, 2005).

Based on these findings, IL-10 may play a role in eBL development and progression through its ability to inhibit the production of pro-inflammatory cytokines such as interferon gamma, which suppress type 1 immune response to EBV infection (Yokoi *et al.*, 1990). Due to this action of the cytokine, it is hypothesized that EBV loads would increase, creating an environment that would subsequently increase risk of eBL. Since the expression levels of many cytokines are thought to be influenced by polymorphisms in their promoter region (Hoogendoorn, 2003; Howell and Rose-Zerilli, 2007b), these variations may underlie disease susceptibility. IL-10 promoter polymorphisms have been the subject of past studies on B cell lymphoma's and a plethora of case-control studies have demonstrated positive associations (da Silva *et al.*, 2007; Howell and Rose-Zerilli, 2007b; Minnicelli *et al.*, 2012). Similarly, other investigations have failed to implicate IL-10 promoter polymorphisms in susceptibility, while in other studies results are conflicting (Howell and Rose-Zerilli, 2007b). Though it would be expected that variability of IL-10 expression levels could contribute to lymphoma genesis, this study did not support the role of IL-10 promoter variants -1082A/G, -819C/T and -592C/A in the aetiology of eBL. The differences observed in the current versus previous studies on other B cell malignancies remains unclear, however several possibilities exist. These differences may be due to the small sample size used or the existence of other distinct IL-10 genetic polymorphisms amongst specific populations and geographic regions. Furthermore, cancer is a multifactorial disease. Individual exposure to various environmental factors in combination to genetic susceptibility may have contributed to these varied observations with other B cell malignancies.

A recent case-control study reported a positive association between the IL-10 promoter polymorphisms (-1082, -819 and -592) and BL risk (Minnicelli *et al.*, 2012), results which are inconsistent with the current findings. In the Minnicelli *et al.* only 54.1% of the BL patients included in the study were EBV positive while in the current study all eBL cases were EBV positive. In addition, the fact that they had a smaller sample size of the cases, and the controls from

different genetic background as the cases could have contributed to the discrepancies between the two studies. Furthermore, the previous study did not select age matched controls, as they included adults with a median age of 36 years (Minnicelli *et al.*, 2012).

The IL-6, just like IL-10, may also play a key role in the development of eBL. IL-6 is a B cell growth factor and elevated levels could enhance BL tumour progression, as it has been shown to facilitate the growth of EBV immortalized B cells, which lead to tumour formation (Tanner and Tosato, 1991). Previous studies have also shown that IL-6 is highly expressed by lymphoblastoid cells which are thought to aid in the silencing of NK cells at the tumour site (Tanner and Tosato, 1991). Increased expression of IL-6 in eBL tumour site may promote evasion from the tumour immune surveillance mechanisms.

Since higher serum levels of IL-6 have been correlated to increased tumour burden in previous studies (Tanner and Tosato, 1991), it would be expected that an increased frequency of children bearing IL-6 high expression genotypes would be among the eBL cases. However, a striking bias favouring the IL-6 -174G allele over the -174C allele was seen in both groups which suggests there is a selective advantage for the G allele within this population.

Preceding studies have reported that elevated IL-6 and IL-10 levels facilitate survival of EBV infected B cells, through interference with the innate immune defence against EBV infection (Helminen *et al.*, 1999; Biron and Brossay, 2001). In addition, EBV encodes an analogue of IL-10 (BCRF1 protein) which is thought to modulate the immune system to allow persistent viral infection (Liu *et al.*, 1997; Zeidler *et al.*, 1997). It was therefore speculated that children genetically predisposed to produce high levels of these two cytokines would be unable to clear the virus effectively. However this study did not demonstrate an association between common IL-6 and IL-10 polymorphisms (haplotypes for IL-10) and EBV load.

In an attempt to further determine the association of IL-10 promoter polymorphisms with eBL, IL-10 haplotypes were constructed since, multisite haplotypes are highly informative allelic markers that can reveal association with a disease outcome not identifiable by a single polymorphism (Ouma *et al.*, 2008). Combinations of different functional polymorphic alleles in a haplotype indicate how these polymorphisms may interact to amplify or moderate their individual effects (Ouma *et al.*, 2008). It has been demonstrated that the -1082G/ -819C/ -592C (GCC), ACC and ATA haplotypes are associated with high, intermediate and low IL-10 transcriptional activity respectively (Ouma *et al.*, 2008; Wilson *et al.*, 2008). Since earlier findings show that IL-10 facilitates the development of BL tumours and also aids in EBV survival in infected cells (Liu *et al.*, 1997; Zeidler *et al.*, 1997; Helminen *et al.*, 1999; Biron and Brossay, 2001) it would also be expected that an increased frequency of children bearing the GCC haplotype would be among the eBL cases. However, this study did not observe an association between any of the haplotypes and eBL risk or EBV load.

Even with no observed association existing between the four SNPs, haplotypes and eBL risk, there still exists a possibility that other cytokine SNPs within these genes and many other genes involved in inflammation may be associated with eBL risk and increased EBV load. Further studies on eBL genetics will need to be explored to additionally identify candidate genes that may predispose to eBL in children.

Despite the fact that polymorphisms, particularly within the promoter region, have been associated with differential levels of gene transcription and expression (Hoogendoorn, 2003), cell type and environmental stimulus may also be important in determining how much of the cytokine is being produced. An association may have been missed in this study because of the complexity in gene-gene or environment–gene interactions that may additionally influence the expression of these cytokines. The influence of the different functions related to the polymorphism of the IL-6 -174

site was presumably absent among the population in this study due to the selection observed against the IL-6 -174C allele.

The strengths of this study included an age, ethnicity and malaria exposure frequency matched case-control, data on potential effect modifiers and also a large number of eBL cases. This study was limited to testing the association between common genotypic and haplotypic polymorphisms found within the IL-10 and IL-6 promoter in eBL cases compared to the controls. This study was unable to test the effect of these variants on the circulating levels of IL-10 and IL-6 due to the retrospective nature of this study. Further investigations on IL-10 and IL-6 expression levels that may or may not be associated with human polymorphisms may shed light on the role of these cytokines in eBL risk.

CHAPTER SIX

6.0. CONCLUSIONS

i. Common IL-6 -174G/C and IL-10 (-1082A/G, -819C/T and -592C/A) promoter variants are not associated with eBL.

ii. The three IL-10 promoter haplotypes -1082G/ -819C/ -592C (GCC), ACC and ATA are not associated with eBL.

iii. There was no association between IL-6 -174G/C and IL-10 (-1082A/G, -819C/T and -592C/A) promoter variants and IL-10 promoter haplotypes -1082G/ -819C/ -592C (GCC), ACC and ATA and EBV loads.

6.1. RECOMMENDATIONS FROM THIS STUDY

i. IL-6 and IL-10 promoter single nucleotide polymorphisms or haplotypes of IL-10 promoter may not be leads to follow as targets to determine children in malaria holoendemic regions at risk of eBL development or eBL therapeutic development.

ii. IL-6 and IL-10 promoter single nucleotide polymorphisms or haplotypes of the IL-10 promoter may not be target for determining EBV load in children aged 5-8 years resident in malaria holoendemic.

6.2. RECOMMENDATIONS FOR FUTURE STUDIES

i. Although the study selected controls who were frequency matched to eBL patients by geography as a surrogate for cumulative malaria exposure and ethnicity, future human genetic studies would seek to include sibling and parent-child matches for more definitive genetic comparisons of eBL risk.

ii. Further investigations into the local and systemic IL-10 and IL-6 expression levels and their correlation with host genotypes would elucidate the role of these cytokines in controlling EBV persistence and reducing eBL risk.

iii. Despite there being no observed association between the individual four SNPs, IL-10 haplotypes (based on -1082, -819 and -592) and eBL risk or EBV load, the putative role of other human genetic variants need to be evaluated further to identify candidate genes that may predispose to eBL. These would entail whole genome sequencing to test for genetic association with immune regulatory genes as well as those as yet undetermined.

iv. The IL-6 and IL-10 promoter region may exhibit a high degree of linkage disequilibrium with other polymorphisms in nearby genes involved in inflammatory responses. The analysis of single genetic polymorphisms might be meaningless for determining genetic risk for eBL, but future studies should consider evaluating combinations of different genotypes and genes for possible gene-gene and gene-environment interactions in eBL development.

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