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IMMUNOPHENOTYPIC CHARACTERIZATION OF LYMPHOCYTE SUBSETS EXPRESSING PROGRAMMED DEATH-1 (PD-1) IN INDIVIDUALS EXPOSED TO DIFFERENTIAL MALARIA TRANSMISSION PATTERNS AND ENDEMIC BURKITT'S LYMPHOMA

BY

Ogolla Sidney Onyango

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree in Master of Science in Medical Immunology.

Department of Biomedical Science and Technology

Maseno University

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ABSTRACT

Repeated challenge of the immune system by chronic exposure to high antigen levels due to persistent infection may lead to development of impaired T-cells that are not effective in mediating immune functions. Malaria and EBV are two agents that have been implicated in the aetiology of Burkitts lymphoma. However, the precise mechanism is unknown but exhaustion/impairement of immune cells has been implicated. Programmed death-1 (PD-1) is an immune inhibitory molecule that negatively regulates activated immune cells upon interacting with its ligands, programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2) resulting in down-regulation of immune responses. Previous studies in murine and primate viral and parasitic diseases have reported the up-regulation of PD-1 and soluble PD-1 (sPD-1) but no studies have reported the expression of PD-1 in individuals from areas with divergent malaria transmission dynamics or in children presenting with endemic Burkitt's lymphoma (eBL). A cross-sectional study was carried out in three distinct populations; Kokwet (unstable *P. falciparum* transmission), Kanyawegi (malaria holoendemic) regions of Western Kenya and children with eBL from New Nyanza Provincial General Hospital. PBMC's were stained for lymphocyte and PD-1 expression markers and data acquired using a four color flow cytometer. In addition, soluble PD-1 in plasma was quantified by Enzyme Linked Immunosorbent Assay (ELISA) in individuals from Kanyawegi, Kokwet and BL. This study reports an increased expression of PD-1 in Kanyawegi compared to Kokwet [($CD4^+$; $p<0.0001$), ($CD8^+$; $p=0.0078$), ($CD19^+$; $p<0.0001$) and ($CD56^+$; $p<0.0001$)] and a significant elevated surface expression of PD-1 in children with BL [($CD4^+$; $p<0.0001$), ($CD8^+$; $p=0.0418$), ($CD19^+$; $p<0.0001$) and ($CD56^+$; $p<0.0001$)] when compared to age matched children from Kanyawegi and Kokwet. Concentration of soluble PD-1 was significantly increased in BL compared to Kanyawegi and Kokwet ($p=0.0074$). These data indicate that continuous exposure to malaria upregulates PD-1 expression and this upregulation provides an insight on how malaria modulates immune functions and in turn may contribute to the pathogenesis of eBL.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Malaria, a disease caused by parasites of the genus *Plasmodium* is one of the deadliest infectious diseases and has proved to be a significant threat to human health especially in children under the age of five. It is estimated that 247 million clinical cases of malaria and nearly 1 million deaths due to malaria are reported each year most, of them in children in equatorial Africa (WHO, 2008). Most of malaria-related deaths are caused by *Plasmodium falciparum* infection, which accounts for 91% of all malaria cases worldwide, with children under the age of five being the most vulnerable (WHO, 2008).

Studies have shown that immunity to malaria develops gradually following repeated exposure and it has been suggested that immunity to malaria in children living in endemic areas is acquired only after a few infections (Gupta *et al*., 1999). The contribution of B-cells, T-cells and NK-cells together with their antibodies and cytokines is essential to the development of natural immunity (Winkler *et al.,* 1999). T-cells are important in the induction and maintenance of immunity to malaria and the cytokines they produce are important mediators of cellular effector functions (Perlmann and Troye-Blomberg, 2002). B-cells plays a crucial role in the development of immunity to malaria and the antibodies they produce are also an important component of naturally acquired immunity that develops following repeated malaria exposure (Perlmann and Troye-Blomberg, 2002, Gupta *et al*., 1999).

In Kenya, the pattern of malaria transmission varies across the country with the Lake Victoria region experiencing high, periennial transmission (holoendemic malaria) while the highlands of western Kenya experience low transmission intensities (hypoendemic malaria) (Snow *et al.*, 1997). Malaria infection causes a complex pattern of immune modulation and this may have an impact on the immune response to other infections like EBV.

Epstein Barr Virus (EBV) is a gamma-herpes virus that belongs to the family of Gammaherpesviridae (Babcock *et al.*, 1998). Epstein Barr virus, one of the aetiological agents of Burkitt's lymphoma, is a B-cell lymphotropic virus and has the ability to immortalize B-cells. EBV was first discovered in cultures of BL tumors in tropical Africa and over 90% of Burkitt's lymphoma tumors contain EBV (Epstein *et al.*, 1964).

Endemic Burkitt's lymphoma (eBL) is a cancer that affects children aged between 2 and 15 years of age (Mwanda *et al.*, 2004). It accounts for up to 74% of all childhood malignancies in Africa and is the most prevalent pediatric cancer in Kenya (Makata *et al.*, 1996; Mwanda *et al.*, 2004). The geographic distribution of eBL incidence coincides within malaria holoendemic regions where *P. falciparum* malaria infections are often chronic or repeated early in life (Rainey *et al.*, 2007). Malaria co-infection with EBV have been implicated in the pathogenesis of eBL (Rochford *et al.*, 2005) although the precise mechanism by which these two agents lead to the pathogenesis of eBL is poorly understood, however, down regulation of immune responses is one mechanism that has been implicated. A number of molecules like CTLA-4, BTLA-4, and PD-1 have been shown to down regulate immune responses during infection (Greenwald *et al.*, 2005).

Programmed death-1 (PD-1) is a cell surface molecule that is mainly expressed by activated B-cells, $CD4^+$ T-cells, $CD8^+$ T-cells and myeloid cells (Riley, 2009). PD-1 binds to its ligands, programmed death ligand-1 (PD-L1) and programmed death ligand-2 (PD-L2) and the engagement of PD-1 to its ligands transduces a signal that inhibits T-cell proliferation, cytokine production and cytolytic activity (Freeman *et al.*, 2000; Latchman *et al.*, 2001). PD-L1 and PD-L2 are upregulated upon activation or interferon-gamma (IFN-γ) treatment of monocytes and dendritic cells (Freeman *et al.*, 2000). PD-1 and its ligands are negative regulators of T-cells as *in vitro* and treatment of T-cells with anti-CD3 results in impaired T-cell proliferation and IFN-γ production (Freeman *et al.*, 2000).

Studies have reported upregulation of PD-1 in both murine and primate viral infections (Petrovas *et al.*, 2006; Trautmann *et al.*, 2006 and Barber *et al.*, 2006). In murine models, lymphocytic choriomenengitis virus (LCMV) specific $CD8^+$ T-cells express high levels of PD-1 and *in vivo* blockade of this pathway reverses the "exhausted" $CD8^+$ T-cells and reduces the viral loads (Barber *et al.*, 2006). In human, it has been shown that PD-1 is upregulated on HIV specific $CD8^+$ T-cells and that blocking this pathway leads to an increased T-cell proliferation and effector functions (Petrovas *et al.*, 2006; Trautmann *et al.*, 2006).

1.2 Problem Statement

The reason why children from malaria endemic regions have a higher incidence of eBL compared to children from regions where malaria is hypoendemic is not fully understood but previous studies have demonstrated that children residing in malaria holoendemic areas have impaired EBV-specific IFN-γ production measured by ELISPOT (Moormann *et al.*, 2007) and an abnormal

distribution of B-cell subset (Asito *et al*., 2008). These observations implicate chronic malaria exposure as a mechanism leading to lymphocyte exhaustion in the pathogenesis of eBL.

Repeated challenge of the immune system following persistent infection leads to T-cells that become progressively dysfunctional and not effective in mediating immune functions (Wherry and Ahmed, 2004). Persistent viral infections have been reported to be associated with functionally impaired T-cells, showing reduced proliferative potential and effector functions (Wherry *et al*., 2004). Following this observation, it has been suggested that this might be the reason for the inability of the host to eliminate persistent pathogens (Barber *et al*., 2006). Such T-cells have been termed as "exhausted" and have a reduced immunologic function. PD-1, a cell surface immune inhibitory molecule, is reportedly upregulated on the surfaces of activated cells in individuals having viral infections such as HIV, HCV, HBV in human, and in murine disease models, but it's expression in individuals with diverse *P. falciparum* transmission and exposure histories or those presenting with endemic Burkitt's lymphoma has not been investigated.

1.3 Justification

P. falciparum malaria causes a complex pattern of immuno-pertubation with modulation of T-cells having consequences in the immune responses to other infection like EBV. The mechanism by which malaria leads to eBL is still unknown but expansion of B-cell and suppression of specific T-cell immunity have been proposed as two possible mechanisms. By comparing IFN-γ and IL-10 responses in healthy children from two regions of western Kenya with differing malaria transmission patterns (Moormann *et al*., 2007), it was reported that there was an age related loss of T-cell responses to EBV in children from malaria holoendemic region compared to children from epidemic prone area. This observation provides evidence that holoendemic malaria modulates EBV-specific T-cell immunity and also explains its role as a co-factor in the

pathogenesis of Burkitt's lymphoma. However, what is not known is why there is an impaired immune response but immune inhibitory molecule PD-1 has been implicated as a possible cause of the impairment.

Therefore determining and understanding the role of this immune inhibitory molecule in individuals from two epidemiologically distinct areas that differ in transmission intensity of malaria and in children presenting with endemic Burkitt's lymphoma may further give us insight into mechanism that disrupt immune surveillance and permit pathogenesis of eBL.

1.4 Objectives of the study

1.4.1 General objective

To investigate the activation induced exhaustion of lymphocyte populations in individuals from areas with differential malaria transmission patterns (holoendemic compared to hypoendemic) and in children presenting with endemic Burkitt's lymphoma.

1.4.2 Specific objectives

1. To determine the frequency of PD-1 expression in lymphocyte subsets in individuals from areas with differential malaria transmission patterns and in children with endemic Burkitt's lymphoma.
2. To determine and compare the plasma levels of soluble PD-1 in age-matched children from two areas differing in malaria transmission pattern and in children with endemic Burkitt's lymphoma.

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1.5 Null Hypotheses

1. There are no differences in PD-1 expression on lymphocyte subsets in individuals from divergent malaria transmission regions and endemic Burkitt's lymphoma.
2. There are no differences in the concentration of plasma PD-1 in individuals from divergent malaria transmission regions and endemic Burkitt's lymphoma.

1.6 Research Questions

1. What are the differences in PD-1 expression in lymphocyte subsets in individuals experiencing divergent malaria transmission patterns and in children with endemic Burkitt's lymphoma?
2. What are the concentration levels of soluble PD-1 in children with endemic Burkitt's lymphoma and in age matched controls from areas with divergent malaria transmission patterns?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria, Malaria Transmission, Morbidity and Mortality

Malaria is a vector borne disease that is caused by protozoan parasites of the genus *Plasmodium* and is transmitted from person to person by the bites of an infected female *Anopheles* mosquito. There are five species of *Plasmodium* that cause malaria to humans; *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* (White, 2008). About 247 million clinical cases of malaria are reported yearly (WHO, 2008) and it is estimated that 86% of all clinical cases of malaria occur in tropical and subtropical regions of Africa with children under the age of five being the most affected, reporting an annual mortality of 881,000 with Africa reporting 90% of all the deaths (WHO, 2008). In sub-Saharan Africa, most severe clinical cases of malaria are attributed to *P. falciparum*.

In addition to acute infections with malaria and deaths in Africa, malaria also contributes significantly to anemia in children and pregnant women which may intern lead to adverse birth outcomes such as abortion, stillbirth, premature delivery and low birth weight, hence increasing overall child mortality (WHO, 2005). Malaria endemicity in Africa is defined on the basis of parasite prevalence and spleen rates into hypoendemic, mesoendemic, holoendemic and hyperendemic regions. In holoendemic areas, there is stable transmission of malaria that is characterized by recurrent exposure to the infective bites of mosquitoes throughout the year, in mesoendemic areas, there is seasonal transmission under normal rainfall conditions but declines with rainfall, in hypoendemic areas, there is periodic transmission following unusual or

unexpected rainfall and in hyperendemic areas, the transmission is seasonal but very intense (Snow *et al*., 1997).

2.2 Immunity to malaria

Malaria transmission intensity influences the course of development of immunity to the parasite. Children under the age of five years living in malaria holoendemic areas initially suffer severe or clinical malaria but following repeated infection, immunity to the parasite develops and the disease becomes less severe and often asymptomatic. This semi-immunity or partial protection limits high density parasitemia later in life (Perlmann and Troye-Blomberg, 2002). In areas of low malaria endemicity, both children and adults suffer from malaria and symptomatic parasitemia since there is less repeated exposure to the parasite and thus less immunity (Snow *et al*., 1997). Immunity to malaria develops slowly and the immunity reduces after an individual moves away from the malaria endemic region (Langhorne *et al*., 2008). This suggests that the generation and maintenance of effector and memory cells require continued exposure to the malarial antigens, "immunologic boosting". However an alternate hypothesis suggests that the malaria parasite induces host mechanism that prevents the development of immunologic memory.

The contribution of T-cell subsets and their cytokines to the development of protective immunity is essential both in regulating antibody formation and in inducing antibody-independent protection (Winkler *et al*., 1999). Cell mediated immunity involves inhibition of parasite growth and development in the hepatocytes by $CD8^+$ cytotoxic T-cells, macrophage activation by NK cells and production of IFN-γ for enhanced clearance of parasitized erythrocytes (Tsuji and Zavala, 2003). It has been suggested that IFN-γ production by T-cells and nitric oxide produced by macrophages has anti-parasitic effect and that the nitric oxide has been shown to kill *P.*

falciparum and *P. chabaundi in vitro* at high concentration (Balmer *et al.*, 2000). However, other studies in murine malaria suggest that nitric oxide is not required for parasite killing (Favre *et al.*, 1999).

The contribution of B-cells and their antibodies is essential to the development of natural immunity (Winkler *et al.,* 1999). B-cells play a crucial role in the development of immunity to malaria and the antibodies they produce are also an important component of naturally acquired immunity that develops following frequent malaria exposure (Perlmann and Troye-Blomberg., 2002, Gupta *et al.*, 1999). Immunity to malaria is associated with protective antibodies of certain classes and subclasses and changes in the antibody subclass composition may affect the pattern of immune response to malaria (Ferreira *et al.,* 1998). Cytophilic antibodies of the IgG1 and IgG3 subclasses are considered the most important for protection against *P. falciparum* malaria (Aucan *et al.,* 2001). They act in collaboration with effector cells to mediate opsonization and Ab-dependenT-cellular inhibition.

2.3 EBV and Burkitt's Lymphoma

EBV is a gamma-herpes virus that belongs to the family of Gammaherpesviridae (Babcock *et al.*, 1998). EBV is estimated to infect 90% of adult population worldwide and in Africa, it has been suggested that by the age of three, about 80% of the children are EBV seropositive (Biggar *et al.*, 1978). Primary infection occurs horizontally during childhood through the saliva and this coincides with the period at which maternal immunity diminish (Biggar *et al.*, 1978). After primary infection, EBV establishes a lifelong latent infection and rarely causes disease unless the host-virus immune balance is upset (Donati *et al*, 2006).

EBV has been associated with a number of malignancies and cancer. The most common association is with Burkitt's lymphoma. Burkitt's lymphoma is a distinct form of non-Hodgkin's lymphoma and it is the most common pediatric cancer in equatorial Africa, accounting for 74% of all childhood malignancies (Burkitt, 1983). Burkitt's lymphoma can be classified as endemic, sporadic or HIV-associated (Freedman and Friedberg, 2006). Sporadic Burkitt's lymphoma accounts for 20-30% of non-Hodgkin's lymphoma in children in developed countries, affecting the abdominal region and can be detected at any age. Endemic Burkitt's lymphoma is almost exclusively found in Africa (Freedman and Friedberg, 2006), affecting mainly the facial skeleton in children aged between 2 to 9 years and is the most common pediatric cancer in equatorial Africa (Orem *et al.*, 2007). In Kenya, there is evidence that there is uneven geographical distribution in the incidences of eBL (Rainey *et al.*, 2007). In young adults, BL manifest as acute infectious mononucleosis (AIM) that is characterized by rapid expansion of virus-specific $CD8^+$ T-cells in peripheral blood (Callan *et al.*, 1998). In children in developing countries such as Kenya, primary EBV infections occur within the first few years of life and are often asymptotic infections (de-The, 1977; Moormann *et al*, 2005).

EBV and holoendemic malaria are two agents that have been implicated in the etiology of eBL. Malaria causes a complex pattern of immune modulation accompanied by polyclonal B lymphocyte activation leading to increased numbers of circulating EBV-infected B-cells (Whittle *et al.*, 1984). The highest density parasitemia is observed in children of age between 6-11 months old and it is at this age that primary EBV infection is likely to occur (Rochford *et al.*, 2005). It has been reported that acute malaria causes impairment of EBV-specific T-cell immunity (Gunapala *et al.*, 1990) and that this impaired EBV-specific T-cell responses is indicated by the loss of IFN-γ-

mediated killing of virus infected cells (Moss *et al.*, 1983). The deficiency of EBV-specific T-cell immunity and expansion of latently infected B-cell pool are two possible mechanisms that have been proposed to explain how holoendemic malaria impacts on EBV latency and how this increases the risk to eBL (Rochford *et al.*, 2005).

2.4 Programmed Death-1 (PD-1)

Programmed death-1 (PD-1) is a cell surface protein, a member of the CD28/cytotoxic T-cell antigen-4 (CTLA-4) family of T-cell receptors that negatively regulates antigen receptor signaling. This inhibitory effect has been shown to be effective both in $CD4^+$ and $CD8^+$ T-cells (Carter *et al.*, 2002). PD-1 (or CD279) was initially cloned as a molecule that was over-expressed in cells undergoing cell death (Ishida *et al.*, 1992) and hence named programmed death-1. Although PD-1 was initially known to be a death receptor due to its preferential over-expression by dying cells, further studies have shown that its expression is associated with negative lymphocyte activation (Agata *et al.*, 1996; Vibhakar *et al.*, 1997). PD-1 binds to PD-L1 (CD274) and PD-L2 (CD273) that are both members of the B7 homologues. The engagement of PD-1 to its ligands results in both inhibition of T-cell activation and cytokine production (Freeman *et al.*, 2000; Latchman *et al.*, 2001). PD-L1 is expressed on resting B-cells, T-cells and dendritic cells (Latchman *et al.*, 2001) and this expression is up-regulated upon activation by both type I and type II IFN's. It is also expressed on a wide range of non-hematopoietic cells and at immuno-privileged sites such as the placenta and the eye (Sharpe *et al.*, 2007). The expression of PD-L1 has been reported on many solid tumors and high levels of PD-1 expression have been associated with poor prognosis of the disease (Latchman *et al.*, 2001). PD-L2 is inducibly expressed on dendritic cells, macrophages and masT-cells (Greenwald *et al,* 2006).

2.4.1 Structure of PD-1

Structural and biochemical analyses have shown that PD-1 is a monomer both in solution and on cell surfaces (Zhang and Strome, 2004). PD-1 is a 288 amino acid type I transmembrane protein that is encoded by *Pdcdl* gene on chromosome 1 in humans. It is composed of one immunoglobulin super family domain, a 20 amino acid stalk that separates the IgV domain from the plasma membrane, a transmembrane domain (Keir *et al*., 2008). It also has two tyrosine molecules that are located in its cytoplasmic tail. There is an intra-cellular domain of approximately 95 residues containing an immunoreceptor tyrosine based switch motif (ITSM) and immunoreceptor tyrosine based inhibitory motif (ITIM) located at the C terminal end (Parry *et al*., 2005).

2.4.2 T-cell exhaustion and PD-1 expression in viral and parasitic infections

During an acute viral infection $CD8^+$ T-cells undergoes an expansion phase resulting in the generation of effector $CD8^+$ T-cells that participate in viral clearance (Wherry and Ahmed, 2004). This is followed by a death phase where 90-95% of the effector CD8 T-cells die (Kaech *et al*., 2002) and the remaining 5-10% of the effector $CD8^+$ T-cells differentiate further to generate a pool of long lived memory $CD8^+$ T-cells and these are maintained for long period of time in the absence of antigen stimulation (Lau *et al*., 1994; Murali-Krishna *et al*., 1999). These maintained number of memory CD8 T-cells are highly functional and provide an important component of protective immunity (Wherry and Ahmed, 2004). On the other hand, in chronic infections, functional effector $CD8^+$ T-cells are generated during early stages of the infection but they lose their function during the course of the infection (Wherry *et al*., 2004). This loss of function is referred to as "exhaustion" (Zajac *et al*., 1998) and is a defining characteristic of many chronic infections and factors such as the availability of CD4 T-cell help, level of antigen exposure and

the duration of exposure determines the level of exhaustion (Freeman *et al.*, 2006). Exhaustion comprises of a range of dysfunctions from mild to extreme and occurs in a hierarchical manner with functions such as IL-2 production and proliferative potential being lost first, while other functions such as IFN-γ production occur later. In a previous study, Wherry *et al*, (2003) identified three levels of exhaustion in virus-specific $CD8^+$ T-cells; mild (partial exhaustion I) where there is little IL-2 production and TNF-α production started to be impaired and lytic capacity started reducing, to moderate (partial exhaustion II) consisting of modestly defective IFN-γ production, little IL-2 or TNF-α production and cytotoxicity, to severe or extreme exhaustion (full exhaustion) where the $CD8^+$ T-cells lack all the effector functions, *i.e.* IFN-γ, IL-2 or TNF-α production and cytotoxic activity (Wherry *et al.*, 2003). Impaired proliferative potential is a key feature of exhaustion and it has been shown that it occurs when other functions of the T-cells such as cytokine production and cytotoxicity are intact (Wherry *et al.*, 2003). It has been shown that the proliferative potential of T-cells decreased alongside the loss of these functions while apoptosis increased and as the antigen load increased or CD4 T-cell help decreased, the virus-specific T-cells became more exhausted (Freeman *et al.*, 2000).

Lymphocytic choriomenengitis virus (LCMV) is a natural pathogen in mice and has been used to elucidate the function of PD-1 and it's ligands in immunity and infection. There are two strains of LCMV that can cause either an acute or chronic infection in mice; the Armstrong strain causes an acute infection that is cleared within 8-10 days after infection while clone 13 causes chronic infection that overwhelms the immune system (Wherry *et al.*, 2004). Surprisingly, these two strains differ in only two amino acids in their entire genome (Matloubian *et al.*, 1993). During an acute infection, it was found out that PD-1 was briefly expressed on early effector $CD8^+$ T-cells

but was rapidly down-regulated (Barber *et al.*, 2006) . On the other hand, during chronic infection, there was continued expression of PD-1 on LCMV specific $CD8^+$ T-cells and that the high levels of PD-1 expression were sustained during the infection (Barber *et al.*, 2006). PD-L1 was also up-regulated on infected cells suggesting that this ligand together with PD-1 may be involved in regulation of T-cell function during chronic LCMV infection.

The blockade of the interaction between PD-1 and its ligand has been shown to rejuvenate the exhausted T-cells and restore their function (Okazaki and Honjo, 2006). (Barber *et al.*, 2006) treated chronic infected mice with blocking antibody specific for PD-L1 and monitored T-cell responses and viral control. They discovered that in contrast to untreated mice, a higher proportion of virus-specific $CD8^+$ T-cells from chronically infected mice had expanded and had an increased ability to produce IFN-γ and TNF-α. PD-L1 blockade also resulted in a striking reduction in viral loads in mice treated with PD-L1-specific antibody while the untreated mice still had high levels of the virus that was maintained even after the anti-PD-L1 was stopped (Barber *et al.*, 2006).

In mice infected with *Schistosoma mansoni,* there was increased expression of PD-1 on splenic $CD4^+$ and $CD8^+$ T-cells compared to naïve T-cells and the macrophages expressed high levels of PD-L1 (Smith *et al.*, 2004). Similarly, during *Taenia crassiceps* infection in mice, a high proportion of $CD4^+$ T-cells express PD-1 and both PD-L1 and PD-L2 were upregulated on macrophages (Terrazas *et al.*, 2005). Studies in mice infected with *P. yoelii* demonstrated low expression levels of PD-1 on naïve CD4 and CD8 T-cells and an increased expression of PD-1 on activated CD4 and CD8 cells after infection (Chandele *et al*, 2010). Taken together, these two

studies suggest that parasitic infections may also exploit the PD-1/PD-L pathway to down-regulate specific anti-parasitic immunity and establish a chronic infection.

2.5 PD-1 Pathway in Cancer

The PD-1 pathway has previously been suggested to be involved in the evasion of antitumor immunity by the host immune system (Keir *et al.*, 2008). Indeed recent studies have shown the expression of PD-1 in a number of solid tumors including cancer of the larynx, lung, stomach, colon, breast, cervix, ovary, renal cell, bladder and liver, as well as in melanoma (Blank and Mackensen, 2007). PD-L1 is expressed on many tumors and its expression strongly correlates with poor outcome in these cancer patients (Blank and Mackensen, 2007; Greenwald *et al.*, 2005). It has been shown that it binds to PD-1 on T-cells, resulting in down-regulation of antitumor immunity (Iwai *et al.*, 2002) and blockade of this pathway results in a more robust cytolytic activity and increased cytokine production.

The expression of PD-L1 on tumor cells has been suggested as a possible tumor evasion strategy. Studies by Iwai *et al*, (2002) showed that PD-L1 is expressed on tumor cells. Further, studies have described a correlation between PD-L1 expression and cancer progression on renal cell carcinoma (Thompson *et al.*, 2004) and a correlation between PD-L1 expression and poor prognosis in oesophegal cancer patients (Ohigashi *et al.*, 2005). Together, these studies concluded that the PD-1/PD-L1 interaction negatively regulates immune functions and indicate a possible mechanism for tumor evasion.

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted at Kanyawegi and Kokwet villages and at new Nyanza Provincial General Hospital, Kisumu (Appendix 1). Kanyawegi is an area with holoendemic malaria in the lowlands of western Kenya. Kokwet is an area with hypoendemic malaria situated 100 kilometers northeast of Kisumu in the highlands of Rift Valley Province. Nyanza Provincial General Hospital is within Kisumu town and is the main referral hospital in Western Kenya for children with Burkitt's lymphoma (Moormann *et al.*, 2007).

3.2 Study Population

3.2.1 Inclusion criteria

Inclusion criteria into the study included individuals aged 6 months to 18 years who were residents of the two study areas, hemoglobin of $\geq$5g/dL, body temperature of $\leq$37.5°C and may be *P. falciparum* malaria parasitemic but asymptomatic. Children diagnosed with eBL but not yet on chemotherapy were also included.

3.2.2 Exclusion criteria

Individuals who had hemoglobin (Hb) of less than 5g/dL, parasitemia with fever or evidence of another etiology of fever and individuals who were generally unwell (not including a diagnosis of eBL) due to other unconfirmed health conditions.

3.3 Study Design and Populations

This study was an age-structured cross-sectional study involving a total of 125 individuals from the three study populations, i.e. from the high (Kanyawegi), low (Kokwet) malaria transmission areas and children with BL. Age stratification of (<1, 1 – 5, 5 – 9, 9 – 14, >18 years) was used based on the development of immunity and the incidences of both malaria and EBV in Kanyawegi and Kokwet and on a previous study that was done in the same populations (Moormann *et al.*, 2007).

3.4 Sample Size Calculation

Sample size was calculated using G-power software (Faul *et al.*, 2007), since no data on PD-1 expression during malaria infection and in endemic Burkitt's lymphoma was available that could be referred to. In order to achieve a statistical power of 80%, a significance of $\alpha=0.05$ with a medium effect size, a minimum of 64 individuals were enrolled (Figure 1).

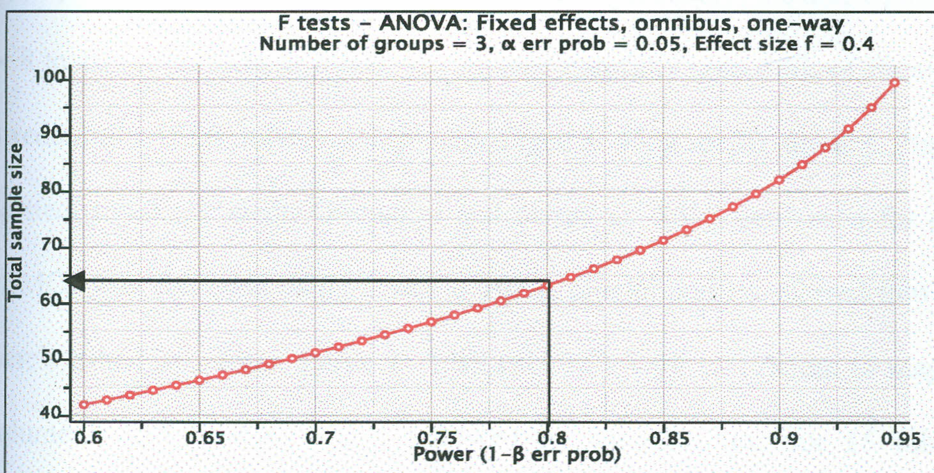

Figure 1: Sample size calculation

2012-0172

3.5 Blood Sample Collection, Microscopy and Complete Blood Count (CBC)

Venous blood (2 – 5ml from children and 8 – 10ml from adults) was drawn by venipuncture into 10ml heparinized Vacutainers™ blood collection tubes and transported within two hours to the UMMS/KEMRI laboratory located at Centre for Global Health Research (CGHR) in Kisumu and processed on the same day. *P. falciparum* diagnosis was determined by microscopic examination of Giemsa-stained thick and thin blood smears. Parasite density was expressed as the number of asexual *P. falciparum* per μL of blood. Individuals with detectable parasites in the smear were treated with Coartem™ for uncomplicated malaria according to the Kenya Ministry of Health guidelines after sample collection. A coulter counter was used to quantify the hematological indices of the study participants.

3.6 Peripheral Blood Mononuclear Cells (PBMC) Isolation

PBMCs were separated from sodium heparin anticoagulated whole blood by standard Ficoll-Hypaque density gradient centrifugation. In this procedure, the anti-coagulated 2 to 5ml (children) or 8 to 10 ml (adults) of blood was layered carefully onto 5mL of Ficoll-paque (GE Healthcare, Sweden) in a 15mL tube and then centrifuged at 450g for 30 minutes. Plasma was transferred into Sarstedt tubes (Sarstedt, Germany) and stored at -80^{0}C until used for sPD-1 ELISA while the PBMCs was collected using a sterile 10mL pipette and transferred into a 15mL tube. The cells were washed by adding sterile 1× PBS (pH 7.0), without calcium or magnesium, to bring a total volume in the tube to 12mL followed by centrifugation for 15 minutes at 350g at room temperature. The supernatant was aspirated off, the pellet broken by gentle flicking of the tube and then washed again and centrifuged for 10 min at 350g. The supernatant was aspirated, the pellets broken by gently flicking the tubes and the cells resuspended in 1mL of sterile 1 × PBS (pH 7.0). A volume of 10µL of 0.4% Turk's solution was used to dilute the cells in a 1:1 ratio to

aid in visualizing the cells under a microscope and using a Haemocytometer to calculate the yield using the formula [cell count in 1ml = (# cells counted in 5 squares)$\times 5 \times 2 \times 10^4$]. The calculations of the cell count were carried out in Microsoft Excel sheets.

3.7 Staining for Flow Cytometry

Half a million freshly isolated PBMCs were aliquoted into numbered 5mL polystyrene tubes (Becton Dickinson, USA). Appropriate monoclonal antibodies specific for different surface molecules were added (Appendix 2). The tubes were then vortexed and incubated at room temperature for 20 minutes in the dark. After incubation, 2mL of cold 0.5% BSA in PBS (flow buffer) was added to each tube and vortexed gently and then spun at 450$\times$g for 5 minutes at 4^0C. The supernatant was aspirated off and cells fixed using 0.5mL of 2% paraformaldehyde added to all the tubes, vortexed gently and incubated in the dark for 20 minutes at room temperature.

3.8 Flow Cytometry Acquisition and Analysis

Data was acquired within 24 hours using CELLQuestPro software on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, CA). The fluorescent intensity measurement was done using logarithmic amplifiers whereas the forward scatter and side scatter measurement was made using linear amplifiers. Flowjo software (Tree star Inc., USA) was applied to collect data for compensation and processing. Proportions of cells in the negative tube were subtracted from the positive tube to geT-cell population expressing PD-1 before statistical analysis was done.

3.9 Soluble PD-1 ELISA

A 96-well microplate was coated with 100μL of 1.0μg/mL anti human PD-1 capture antibody, sealed and incubated overnight at room temperature. After a three step wash with wash buffer

(0.05% Tween 20 in PBS pH 7.2), non specific binding was blocked for one hour using 300μL of blocking buffer (1% BSA in PBS, pH 7.2). These were then washed twice and 100μL of standards (starting at a concentration of 10,000pg/mL and serially diluted seven times) or the samples added to the wells, covered using a plate sealer and incubated for two hours at room temperature. After the incubation period, the plates were washed twice with wash buffer; 100μL of 200ng/mL biotinylated goat anti human PD-1 detection antibody added to each well, covered using a plate sealer and further incubated for two hours at room temperature followed by addition of 100μL of streptavidin HRP to each well. HRP activity was detected using 3,3',5,5'-tetramethylbenzidine (Organon Teknika) in H_2O_2 (1:1) and the reaction stopped by adding 2*M* sulfuric acid (H_2SO_4). The optical density was determined at 450nm (Anthos 2001 reader, Anthos Labtec Instruments, Salzburg, Germany). Soluble PD-1 concentrations were determined by extrapolation from the standard curves.

3.10 Data Analysis

All acquired and processed data were analyzed using Graphpad 5.1 (GraphPad Software, Inc, La Jolla, CA) software. Differences in the median PD-1 expression and concentration of soluble PD-1 between two groups were compared by Mann-Whitney U test, while differences in PD-1 expression across the age groups and among the three study populations (Kanyawegi, Kokwet and BL) were compared using Kruskal-Wallis test. Any detected statistical difference between the groups was further tested using Dunn's post hoc tests to identify which groups differed from the others. A $p \leq 0.05$ was considered statistically significant.

3.11 Ethical Considerations

Approval for the study was obtained from the Kenya Medical Research Institute (KEMRI), National Ethical Review Committee and Ethical Review Board of University of Massachusetts Medical School (UMMS), USA. Written informed consent was obtained from adult participants and parents or guardians of all study participants who were less than 18 years of age. Qualified phlebotomists collected venous blood under sterile conditions in order to minimize the risk of infection and discomfort.

CHAPTER FOUR

4.0. RESULTS

4.1. General Characteristics of the Study Populations

The general characteristics of the study population are summarized in Table 1. Individuals from the holoendemic malaria transmission area are referred to as Kanyawegi while individuals from the hypoendemic malaria transmission area are referred to as Kokwet. They included 51 individuals from Kanyawegi with 63% being males and mean hemoglobin of 10.80 g/dL, 53 from Kokwet with 34% being males and mean hemoglobin of 12.40g/dL and 21 children with endemic Burkitt's with mean hemoglobin of 9.73 g/dL. More males (62%) than females had endemic Burkitt's lymphoma as had been reported elsewhere (Mwanda *et al.*, 2004).

As expected, the highest percentage of *P. falciparum* parasitaemia was among individuals from Kanyawegi as twenty nine percent of these individuals had asymptomatic *P. falciparum* blood stage infection. In contrast, no individual from Kokwet was found positive for malaria by blood smear. Children having endemic Burkitt's lymphoma did not have malaria parasites in their blood.

Table 1: General characteristics of the study population

Parameter	Kanyawegi	Kokwet	BL
Number of participants	51	53	21
Gender			
Male (n[%])	32 (63)	18 (34)	13 (62)
P. falciparum +(n[%])	15 (29)	0 (0)	0 (0)
Temperature, ^{0}C	36.55	36.64	36.27
Hemoglobin (g/dl)	10.80	12.40	9.73

Data for temperature and hemoglobin are presented as means.
Abbreviation; BL- Burkitt's lymphoma (enrolled at the Nyanza provincial general hospital).

4.2 Hematological Indices

Complete blood counts were generated to determine if there were any overt differences in cell composition between study groups and the results are summarized in Table 2. The three study populations differed in a number of hematological indices.

There was a significant difference in lymphocyte counts in the three populations (p=0.0002). Further analysis revealed that there was a difference when Kanyawegi vs BL and Kokwet vs BL were compared. However, there was no significant difference when Kanyawegi and Kokwet were compared. There was a significant difference in monocyte counts (p=0.0013) and red blood counts (p=0.0034), however, on further analysis the difference was only between Kokwet and BL in both cases. Hemoglobin differed significantly in the three groups (p<0.0001) but on further analysis, there was no difference when Kanyawegi and Kokwet were compared. Hematocrit count differed significantly (p=0.0001) in the three groups, but on further analysis the difference was significant when Kanyawegi vs BL and Kokwet vs BL were compared. There was a significant difference in mean corpuscular (p=0.0312) and on further analysis, the difference was only significant when Kanyawegi vs Kokwet were compared. The mean corpuscular haemoglobin concentration and platelet count were significantly different (p=0.0001 and p=0.0001 respectively) and on further analysis, there was a significant difference when Kanyawegi vs Kokwet, Kanyawegi vs BL and Kokwet vs BL were compared.

There was no significant difference in white blood cells count (p=0.4437), mean platelet volume (p=0.1911) and red cell distribution width (p=0.7636) when the three groups were compared.

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Table 2: Hematological indices of the study population.

	Kanyawegi	Kokwet	BL	*p* value	*Post test*		
					Kany vs kok	Kany vs BL	Kok vs BL
White Blood cell count WBC(×10*3/µl)	7.400	7.950	7.700	0.4437	ns	ns	ns
Lymphocyte counts LY(%)	52.9	58.50	41.00	**0.0002**	ns	*	***
Monocyte counts MO(%)	5.900	5.150	8.700	**0.0013**	ns	ns	***
LY#(×10*3/µl)	3.70	4.50	3.07	**0.0371**	ns	ns	*
MO#(×10*3/µl)	0.400	0.400	0.840	**0.0069**	ns	*	**
Red blood cell count RBC(×10*6/µl)	4.740	4.905	4.310	**0.0034**	ns	ns	*
Hemoglobin Hgb(g/dL)	10.80	12.40	10.10	**<0.0001**	**	ns	***
Hematocrit Hct(%)	36.20	39.45	31.20	**<0.0001**	ns	***	***
Mean Corpuscular Volume MCV(fL)	77.20	81.35	71.00	**0.0007**	ns	*	***
Mean Corpuscular Haemoglobin MCH(pg)	22.80	24.55	23.40	**0.0312**	*	ns	ns
Mean Corpuscular Haemoglobin Concentration [MCHC (g/dL)	29.30	30.40	32.30	**<0.0001**	**	***	***
Red cell distribution width RDW(%)	15.80	15.30	18.80	0.7636	ns	ns	ns
Platelet count Plt(×10*3/µl)	447.0	648.5	350.0	**<0.0001**	***	*	***
Mean Platelet Volume MPV(fL)	8.700	8.300	8.200	0.1911	ns	ns	ns

Data are presented as medians. [a] Kruskal-Wallis test was used to determine differences in medians between Kanyawegi, Kokwet and BL. Dunn's post test was used to determine which of the three groups differ from the other. Statistically significant $p \leq 0.05$ are in bold. Abbreviation: BL= Burkitt's Lymphoma. ns - not significant, * (p= 0.01 to 0.05), ** (p=0.001 to 0.01) and *** (p<0.001).

4.3 Expression of PD-1 on lymphocytes between Malaria Holoendemic and Hypoendemic Individuals.

In order to determine if there was a difference in the expression of PD-1 on lymphocytes in individuals from areas with divergent malaria transmission pattern, helper T-cells ($CD4^+$), cytotoxic T-cells ($CD8^+$), B-cells ($CD19^+$) and natural killer cells ($CD56^+$) cells expressing PD-1 from individuals from Kanyawegi (holoendemic malaria exposure) and Kokwet (hypoendemic malaria exposure) were quantified. A representative flow cytometric analysis that was used to quantify the expression of PD-1 on $CD4^+$ and $CD8^+$ T-cells in peripheral blood is illustrated in Figure 2a whereas Figure 2b is the gating strategy used to quantify the expression of PD-1 on B-cells and NK cells.

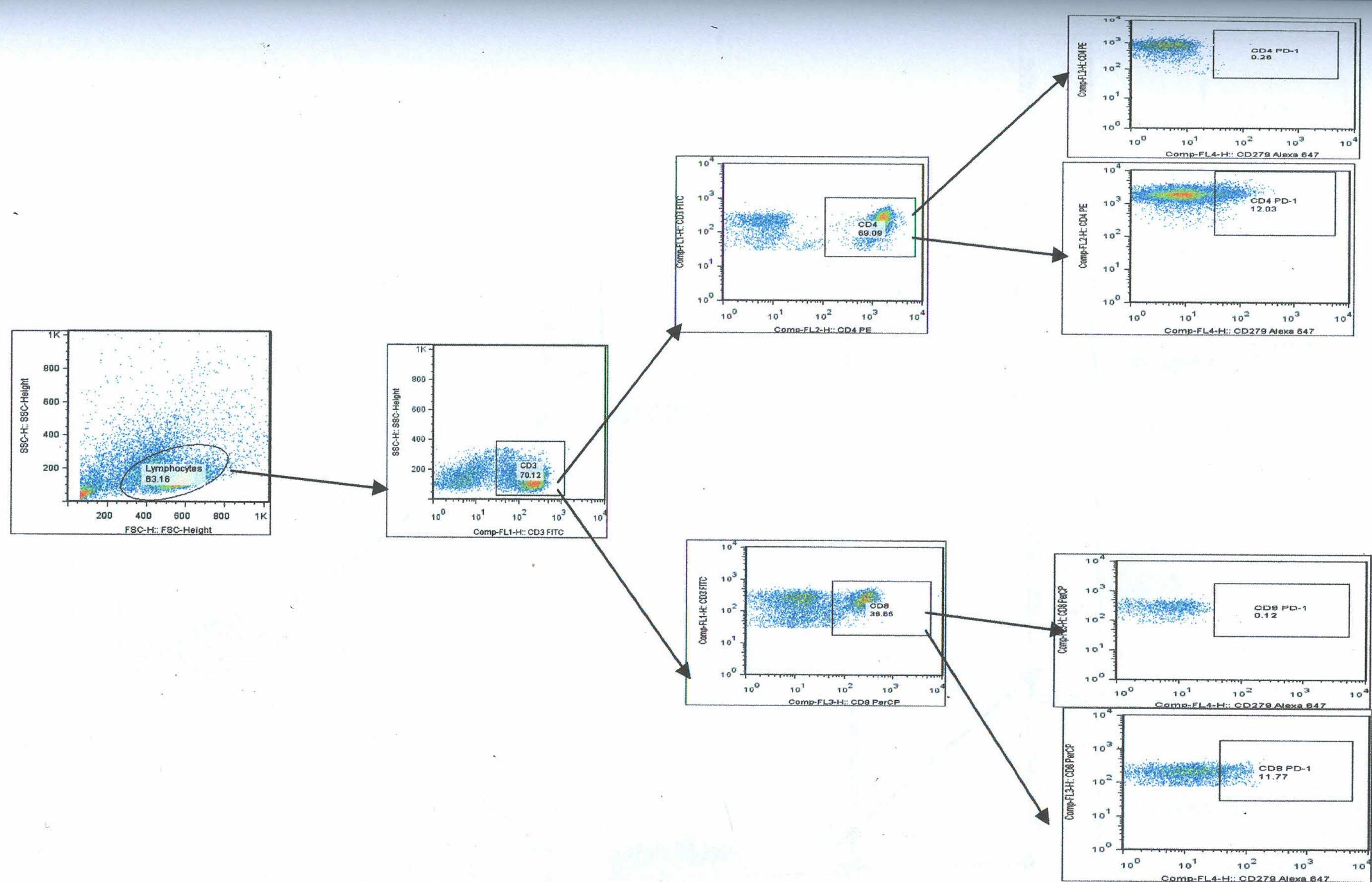


Figure 2A: Representative flow cytometric analysis of $CD4^+$ and $CD8^+$ T-cells expressing PD-1 in peripheral blood.

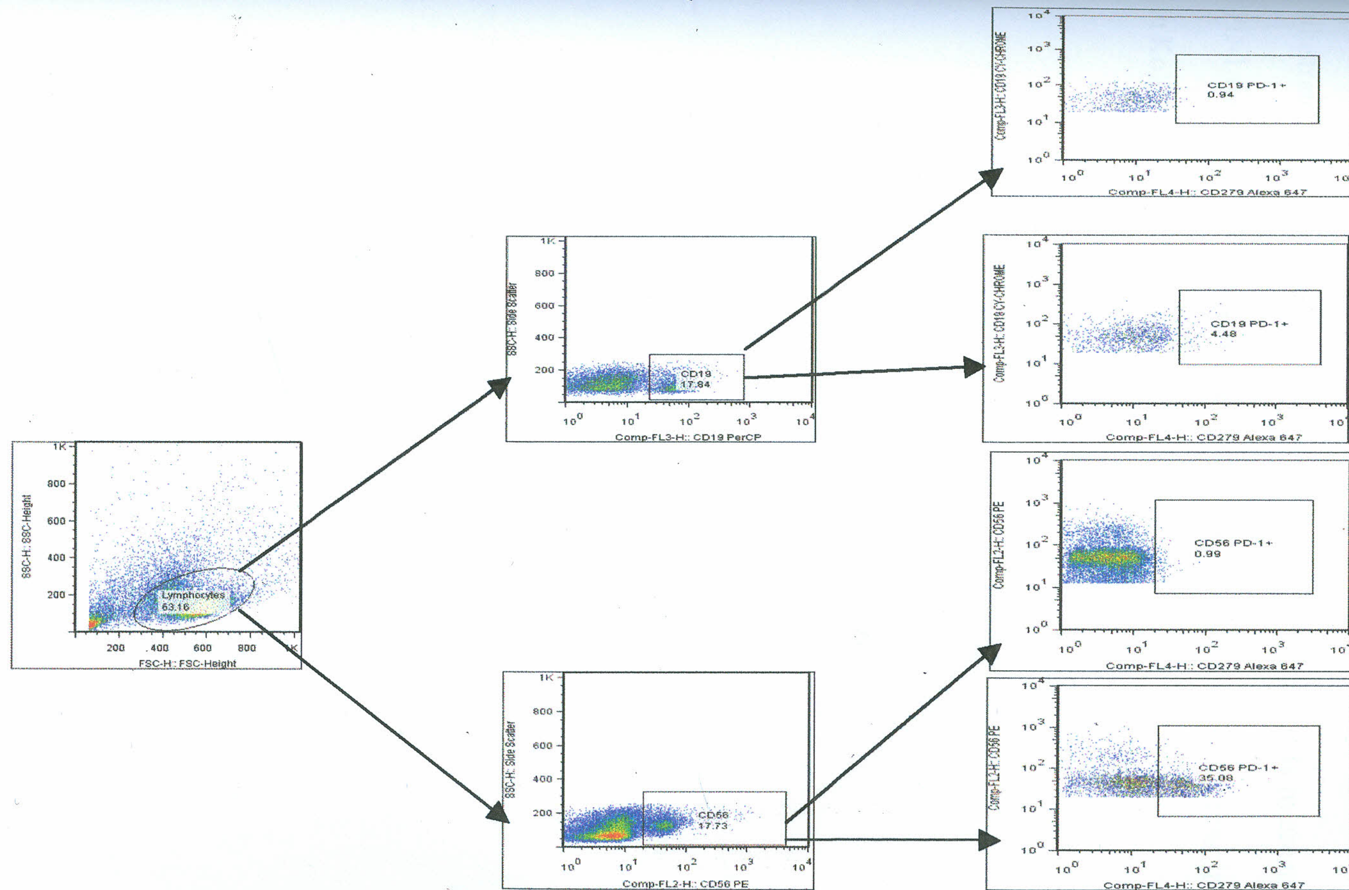


Figure 2B: Representative flow cytometric analysis of B-cells and natural killer cells expressing PD-1 in peripheral blood.

4.3.1 Expression of PD-1 on Total $CD4^+$ T-cells in Kanyawegi compared to Kokwet.

The median percentage of total $CD4^+$ T-cells that expressed PD-1 was significantly higher in individuals from Kanyawegi (6.31%) compared to Kokwet (3.13%) ($p<0.0001$), as shown in Figure 3A. Across the age group comparison shows that there was no difference in the median percentage of CD4 $PD\text{-}1^+$ T-cells in Kanyawegi ($p=0.0556$) and Kokwet ($p=0.1999$) as illustrated in Figure 3B and 3C respectively. However, it is interesting to note that expression of PD-1 on $CD4^+$ T-cells increased with age in Kanyawegi whereas it remained relatively constant in Kokwet.

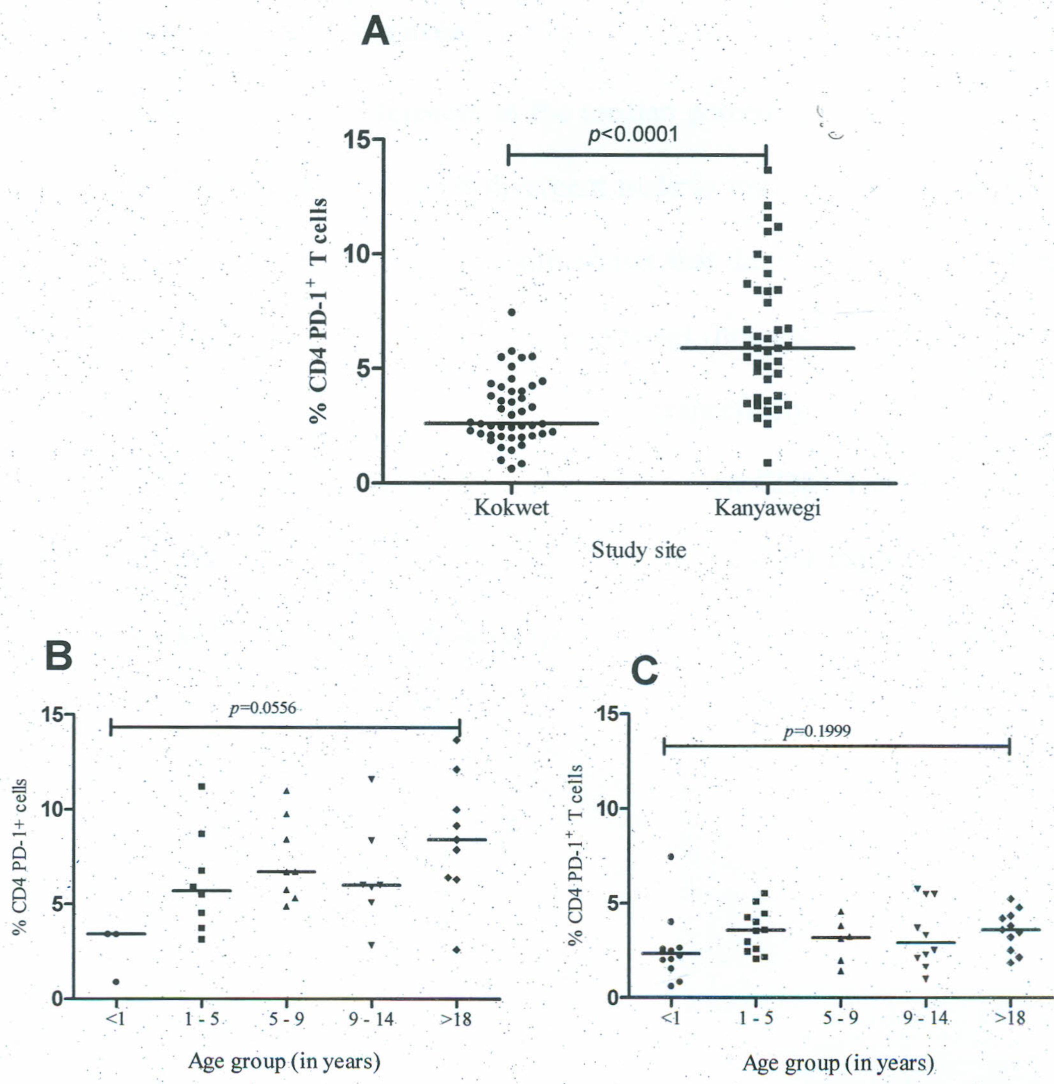


Figure 3: Increased median expression of PD-1 on total CD4^{+} T-cells in Kanyawegi compared to Kokwet (A). Pooled data showing the median expression across age groups in Kanyawegi (B) and Kokwet (C).

4.3.2 Expression of PD-1 on total $CD8^+$ T-cells in individuals from holoendemic compared to hypoendemic malaria transmission area.

To investigate whether there were differences in the median percentage expression of PD-1 on $CD8^+$ T-cells in individuals from areas with divergent malaria transmission dynamics, freshly isolated PBMC's were stained for PD-1. The results shows that the median percentage of PD-1 on total $CD8^+$ T-cells were significantly higher in Kanyawegi (holoendemic malaria) compared to Kokwet (hypoendemic malaria), 10.08% and 7.560%, respectively (p=0.0078), Figure 4A. When comparing the median percentage expression of PD-1 on $CD8^+$ T-cells across the age groups for each study site, there was no significant difference in PD-1 expression on $CD8^+$ T-cells for both Kanyawegi (p=0.1277) and Kokwet (p=0.4297), Figure 4B and 4C respectively.

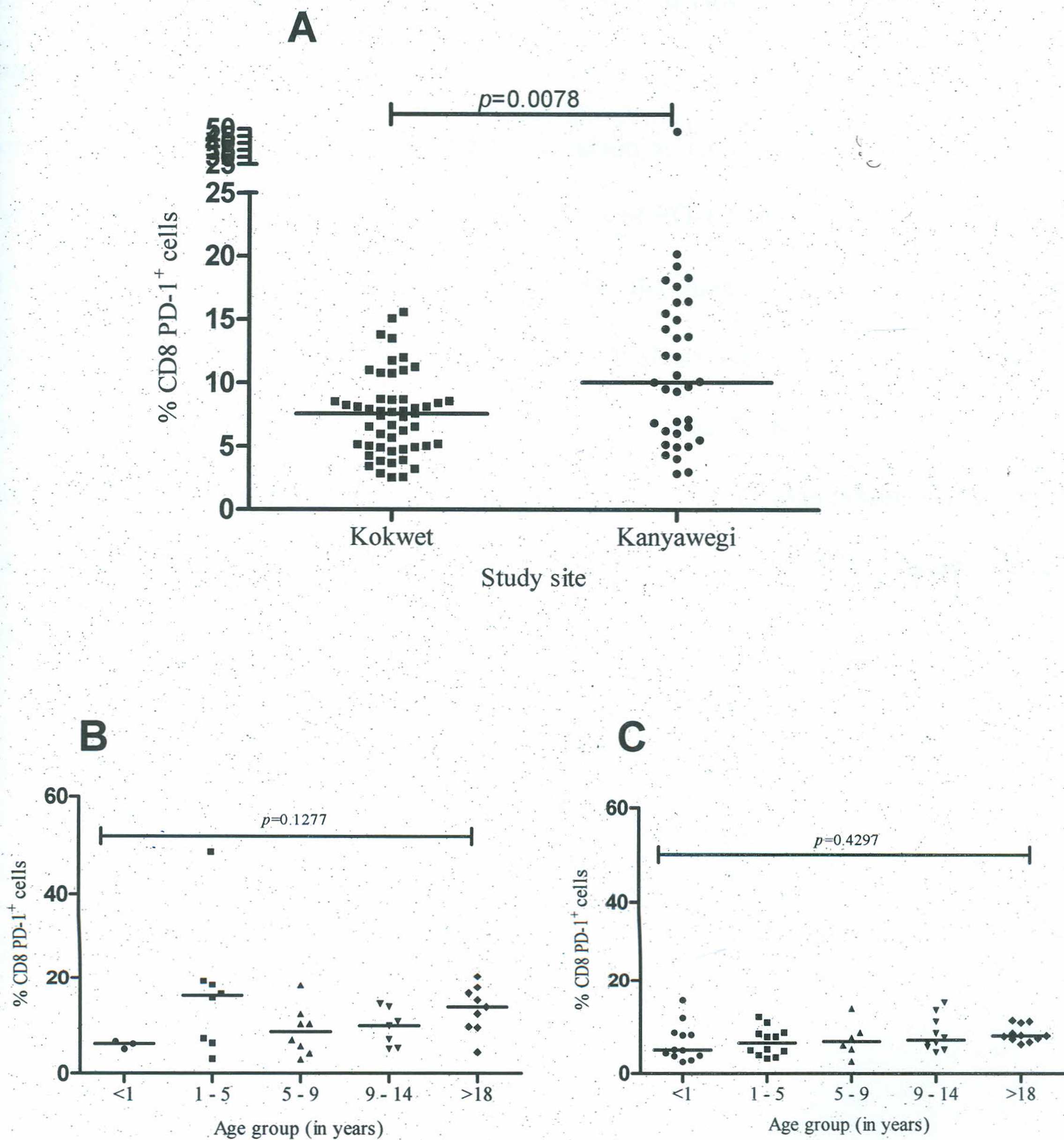
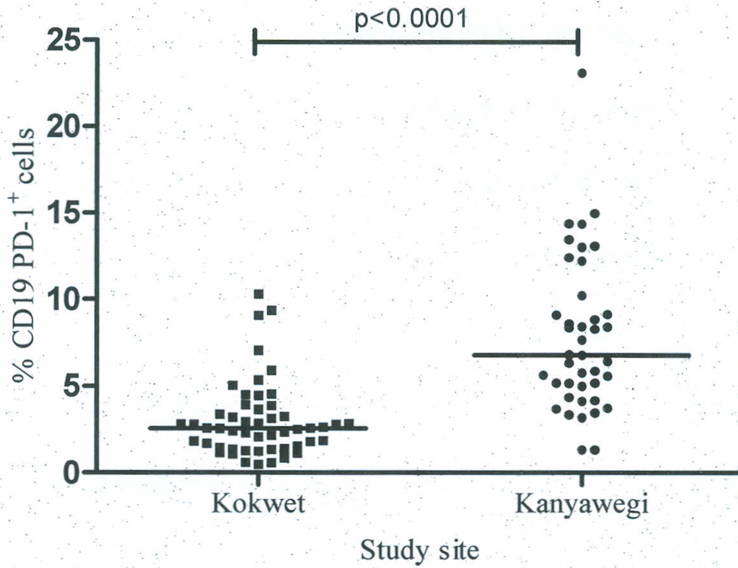
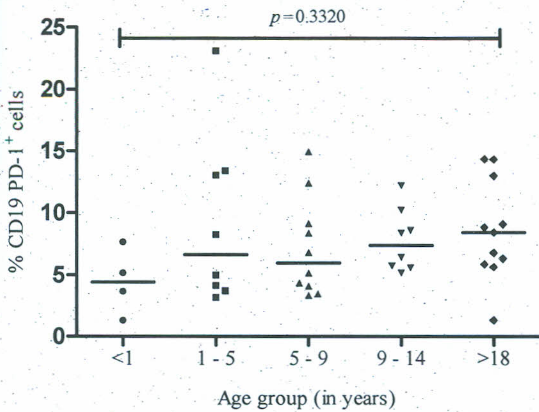


Figure 4: Median expression of PD-1 on CD8^{+} T-cells. The median expression on total CD8^{+} T-cells from Kokwet compared to Kanyawegi (A) and across age group comparison in Kanyawegi (B) and Kokwet (C).

4.3.3 Frequency of PD-1 Expression on B-cells in individuals from holoendemic malaria compared to hypoendemic area.

In order to determine the frequency of PD-1 expression on B-cells in individuals from areas with divergent malaria exposure histories, the frequencies of PD-1 expression on $CD19^+$ cell in these individuals were quantified. As shown in Figure 5A, the median expression of PD-1 expressed by total B-cells was significantly higher in individuals from Kanyawegi (holoendemic malaria) compared to Kokwet (hypoendemic malaria), 6.790% and 2.580%, respectively, $p<0.0001$. However, there were no significant age-related differences in the expression of PD-1 on B-cells in individuals from Kanyawegi ($p=0.3320$) and Kokwet ($p=0.5592$), figure 5B and 5C, respectively.





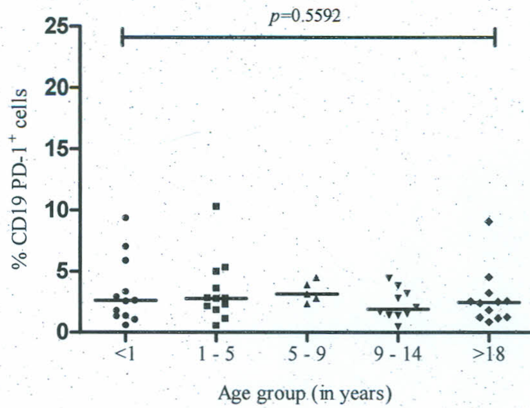


Figure 5: Increased expression of PD-1 on B-cells in Kanyawegi compared to Kokwet (A). B and C show the pooled data for the median expression of PD-1 in Kanyawegi and Kokwet respectively.

4.3.4 Expression of PD-1 on Natural Killer cells in individuals from holoendemic compared to hypoendemic malaria areas.

This study further analyzed the expression of PD-1 on NK cells to investigate if divergent malaria transmission patterns affect the frequency of PD-1 on these cell types. As shown in Figure 6A, the median expression of PD-1 on total $CD56^+$ cells differed significantly between Kanyawegi and Kokwet (6.260% and 3.680%, respectively, p=0.0001). This study also investigated if there was age-related changes in PD-1 expression and as shown in Figure 6B and 6C, median expression of PD-1 on $CD56^+$ cells were not significantly different across the age groups for both Kanyawegi and Kokwet (p=0.7564 and p=0.2648, respectively).

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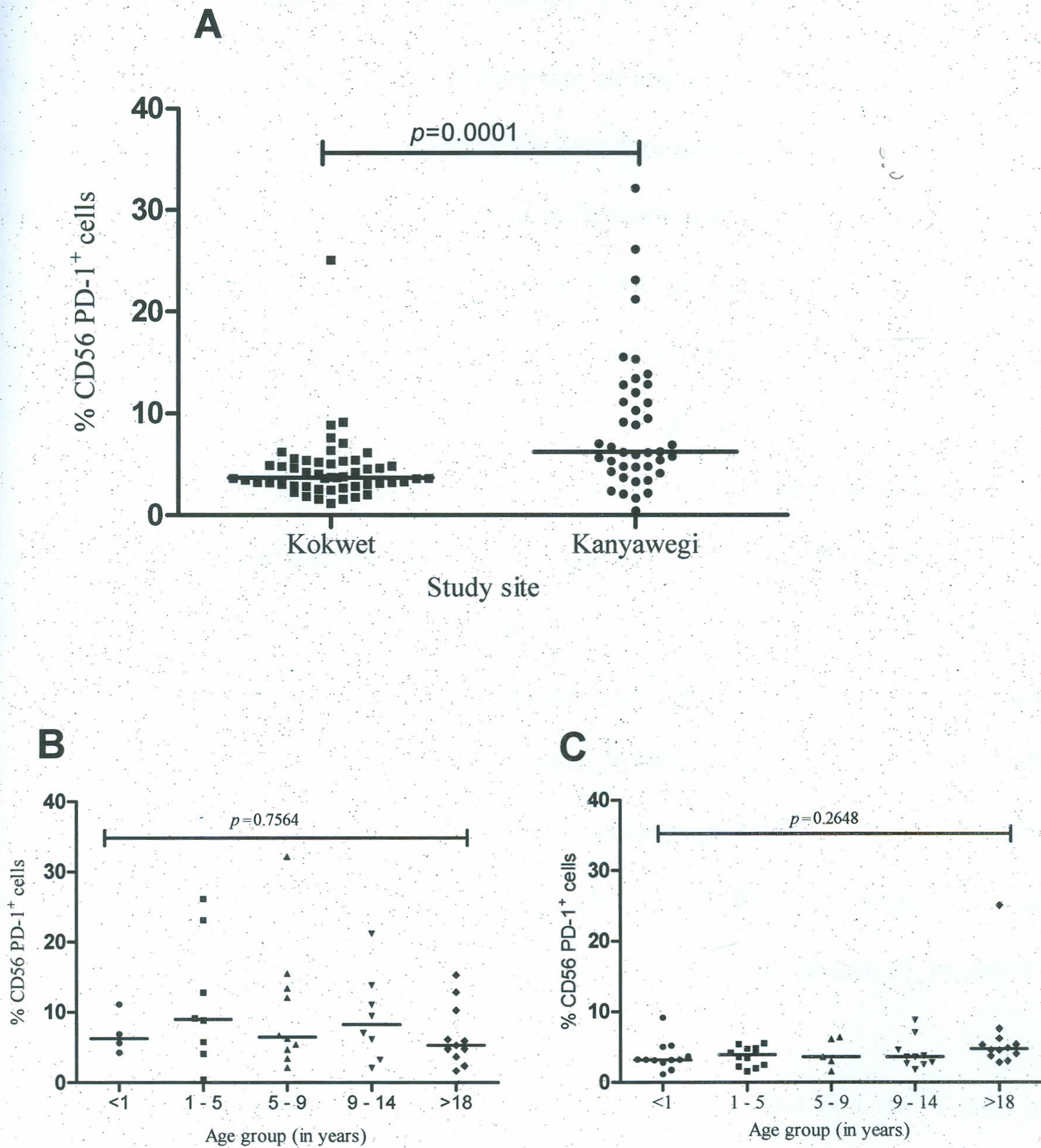

Figure 6: Increased expression of CD56 PD-1^+ cells in Kanyawegi compared to Kokwet. Figure A on total $CD56^+$ cells. Expression across age groups in Kanyawegi (B) and Kokwet (C).

4.3.5 Age related changes in the expression of PD-1 in lymphocytes

This study further wanted to determine if there was an age related difference in PD-1 expression in individuals of different age groups from areas with divergent malaria transmission patterns. As summarized in Table 3, in infants (<1 years old), there was no significant difference in the median percentage of $CD4^+$ T-cells expressing PD-1 in Kanyawegi (3.41%) compared to Kokwet (2.34%), (p=0.6134). On the other hand, the percentage of $CD4^+$ T-cells expressing PD-1 were significantly different between children of age group 1 – 5 years from Kanyawegi and Kokwet (5.70% and 3.57%, respectively, p=0.0122). Similarly, children from Kanyawegi of age group 5 – 9 years showed a significantly higher median percentage of PD-1 expressing $CD4^+$ T-cells compared to children of the same age from Kokwet (6.71% and 3.19%, respectively, p=0.0007). This difference in PD-1 expression was also apparent in the older age groups (9 and 14 years) when comparing Kanyawegi and Kokwet (6.00% vs. 2.93%, respectively; p=0.0068). Similarly, adults from Kanyawegi had a higher percentage $CD4^+$ T-cells expressing PD-1 relative to Kokwet (8.42% and 3.61%, respectively, p=0.0018). This suggests a cumulative overall increase in PD-1 expression on CD4 T-cells associated with prolonged, repeated malaria exposure.

There was no significant difference in the median expression of PD-1 on $CD8^+$ T-cells in all the age groups except adults where Kanyawegi had a higher median expression (13.58%) compared to Kokwet (8.01%; p=0.015).

There was no significant difference in the expression of PD-1 on B-cells in infants (less than 1 year of age) from Kanyawegi relative to Kokwet (4.425% and 2.610% respectively, p=0.4306). Kanyawegi children of age group 1 – 5 years had a significantly higher median percentage expression of PD-1 on B-cells compared to the same age group from Kokwet (6.630% and

2.790%, respectively; $p=0.0098$). In children of age group 5 – 9 years, the difference in percentage median expression of PD-1 on $CD19^+$ cells was significantly different between Kanyawegi and Kokwet 5.980% and 3.130%; ($p=0.0193$). In the 9 -14 years age group, the difference in expression between Kanyawegi and Kokwet was also significant 7.415% and 1.890%; $p<0.0001$. The same difference was observed in adults where those from Kanyawegi showed a higher median expression compared to those from Kokwet (8.430% and 2.465%, $p=0.0015$).

The median expression of PD-1 on $CD56^+$ cells in infants differed between Kanyawegi and Kokwet, 6.290% and 3.205%, respectively, $p=0.0249$. The same trend was noted in the 1 – 5 year age group, where Kanyawegi children had a significantly higher median expression of PD-1 relative to Kokwet children (8.990% and 3.905%, respectively, $p=0.0229$). However, there was no significant difference in the median percentage expression of PD-1 on $CD56^+$ cells in age groups 5 – 9 years, 9 – 14 years and in adults > 18 years (6.495 and 3.630; $p=0.1292$, 8.275 and 3.615; $p=0.0676$, 5.300 and 4.750; $p=0.7349$, respectively).

Table 3: Age-dependent frequencies of selected lymphocytes expressing PD-1 in individuals from areas experiencing different malaria transmission patterns.

	<1 years			1 – 5 years			5 – 9 years			9 – 14 years			>18 years		
	Kany	**Kok**	***p value***	**Kany**	**Kok**	***p value***	**Kany**	**Kok**	***p value***	**Kany**	**Kok**	***p value***	**Kany**	**Kok**	***p value***
$CD4^+$	3.410	2.340	0.6134	5.695	3.565	**0.0122**	6.705	3.185	**0.0007**	6.000	2.925	**0.0068**	8.420	3.610	**0.0018**
$CD8^+$	6.050	4.975	0.8286	15.95	6.470	0.0698	8.460	6.760	0.6620	9.720	7.125	0.6691	13.58	8.010	**0.0151**
$CD19^+$	4.425	2.610	0.4306	6.630	2.790	**0.0098**	5.980	3.130	**0.0193**	7.415	1.890	**<0.0001**	8.430	2.465	**0.0015**
$CD56^+$	6.290	3.205	**0.0249**	8.990	3.905	**0.0229**	6.495	3.630	0.1292	8.275	3.615	0.0676	5.300	4.750	0.7349

Freshly isolated PBMCs were analyzed by flow cytometric staining with various combinations of monoclonal antibodies. For each population, the percentages given are the medians of $PD-1^+$ cells within the indicated subset of cells. Statistical differences between the two groups were determined using Mann Whitney test. Statistically significant values at $p \leq 0.05$ are in bold.
Abbreviations; Kany (Kanyawegi)-individuals from holoendemic area, Kok (Kokwet)-individuals from hypoendemic area.

4.4 Evaluation of Expression of PD-1 on Lymphocyte Subsets in Children with endemic Burkitt's lymphoma

In order to determine if there are differences in the expression of PD-1 on select lymphocyte subsets in children presenting with endemic Burkitt's lymphoma, this study compared these children to age-matched children from areas experiencing divergent malaria transmission patterns.

4.4.1 PD-1 Expression on $CD4^+$ T-cells in endemic Burkitt's lymphoma patients

This study reports a significant difference in the median percentage of PD-1 expression on total $CD4^+$ T-cells between children with endemic Burkitt's lymphoma (14.88%), in comparison to age matched children from Kanyawegi (5.89%) and Kokwet (2.42%, $p<0.0001$), as shown in Figure 7 and Table 4. Further analysis showed that the greatest difference in median percentage of PD-1 expression on total $CD4^+$ T-cells was between children from areas experiencing holoendemic malaria and children with Burkitt's lymphoma.

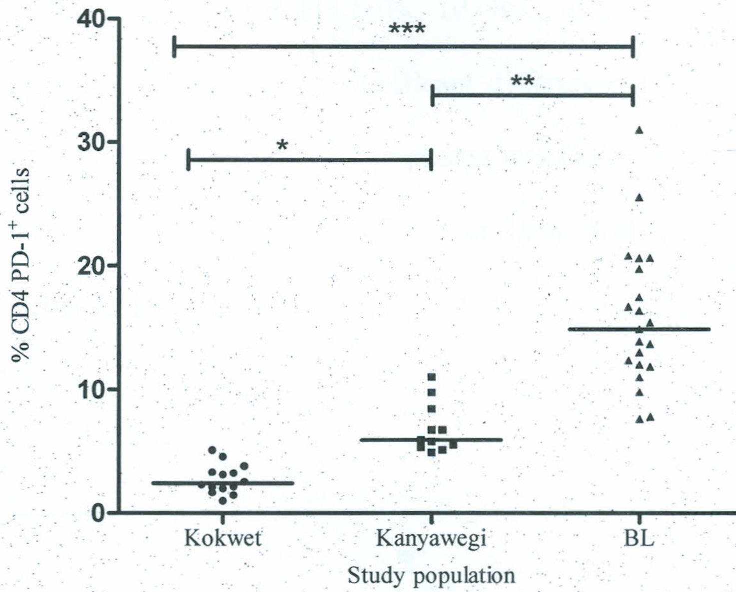

Figure 7: Expression of PD-1 on $CD4^+$ cells in children having Burkitt's lymphoma compared to children from Kanyawegi and Kokwet. Abbreviations BL- Burkitt's lymphoma patients. *** (p<0.001), ** (p=0.001 to 0.01) and * (p=0.01 to 0.05)

4.4.2 Frequency of PD-1 expression on $CD8^+$ T-cells in BL patients compared to controls

As shown in Table 4 and illustrated in Figure 8, there was a difference in the median percentage expression of PD-1 on total $CD8^+$ T-cells when children with BL were compared to age matched children from Kanyawegi and Kokwet (11.56%, 10.08%, 6.550% respectively, p=0.0418). Further analysis revealed that there was no significant difference in PD-1 expression on total $CD8^+$ T-cells between children with Burkitt's lymphoma and children from Kanyawegi. On the other hand, there was a significant difference when children with Burkitt's lymphoma were compared with those from Kokwet (p=0.0121).

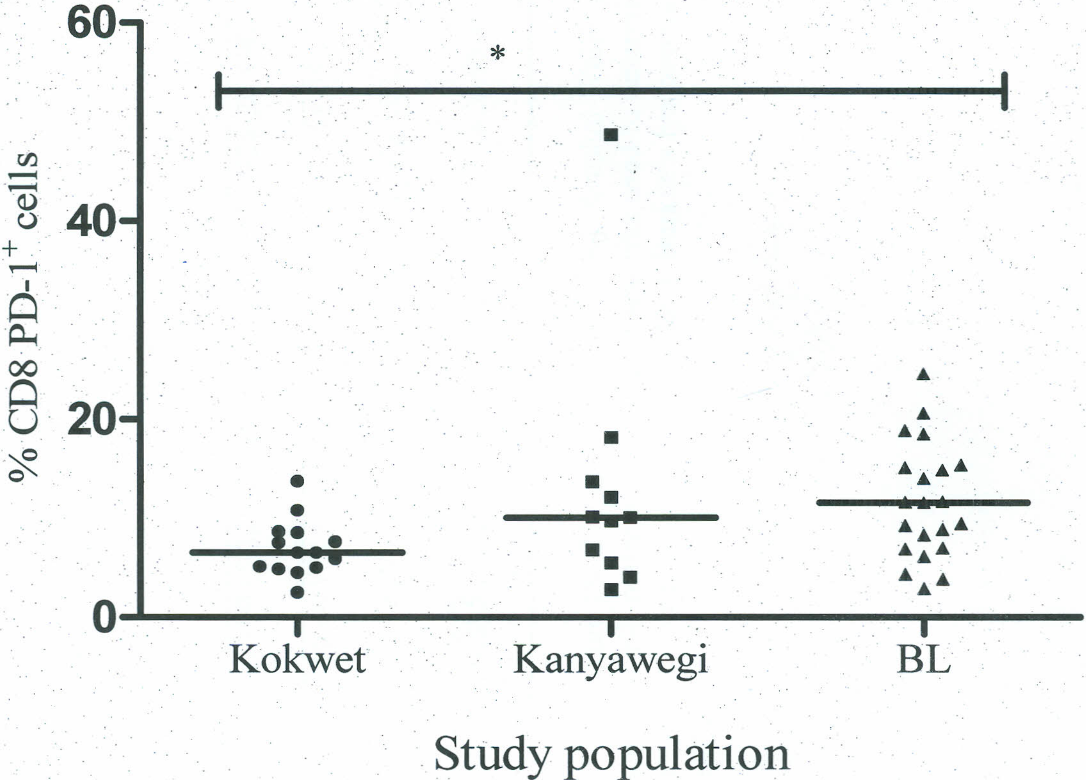

Figure 8: Expression of PD-1 on $CD8^+$ T-cells in children having Burkitt's lymphoma compared to controls. Abbreviations BL- Burkitt's lymphoma patients. * (p=0.01 to 0.05)

Table 4: Expression of PD-1 on different lymphocyte subsets in children with endemic Burkitt's lymphoma relative to children with divergent malaria exposure

	Kokwet (hypoendemic malaria)	Kanyawegi (holoendemic malaria)	BL	*p*-value	*Post test*		
					Kany vs kok	Kany vs BL	Kok vs BL
$CD4^+$ T-cells	2.415 (1.900-3.443)	5.890 (5.320-8.430)	14.880 (11.920-20.190)	**<0.0001**	*	**	***
$CD8^+$ T-cells	6.550 (5.020-8.605)	10.080 (5.530-13.670)	11.560 (6.970-15.270)	**0.0418**	ns	ns	*
$CD19^+$ cells	2.340 (1.505-3.415)	6.615 (4.293-12.270)	6.180 (4.558-8.250)	**<0.0001**	***	ns	***
$CD56^+$ cells	3.620 (2.290-6.270)	6.895 (5.238-13.950)	22.780 (17.900-24.990)	**<0.0001**	ns	**	***

Data are presented as medians (in brackets are the 25th and 75th percentiles). Kruskal-Wallis test was used to determine differences in medians between Kokwet, Kanyawegi and BL patients. Dunn's post test was used to determine which of the three groups differ from the other. Statistically significant $p \leq 0.05$ are in bold. Abbreviation: BL= Burkitt's lymphoma. ns - not significant, * (p= 0.01 to 0.05), ** (p=0.001 to 0.01) and *** (p<0.001).

4.4.3 PD-1 expression on B-cells in children with eBL compared to children from malaria holoendemic area in contrast to those from hypoendemic Area.

This study further reports that there was a significant difference in the expression of PD-1 on CD19$^+$ cells when children with Burkitt's lymphoma were compared to children from Kanyawegi and Kokwet 6.180%, 6.615%, 2.340% respectively, (p<0.0001), Table 4. Further analysis revealed that there is no significant difference in the expression of PD-1 on B-cells between eBL patients and children from Kanyawegi, but there was a significant difference in PD-1 expression between eBL patients and children from Kokwet and between children from Kanyawegi and Kokwet.

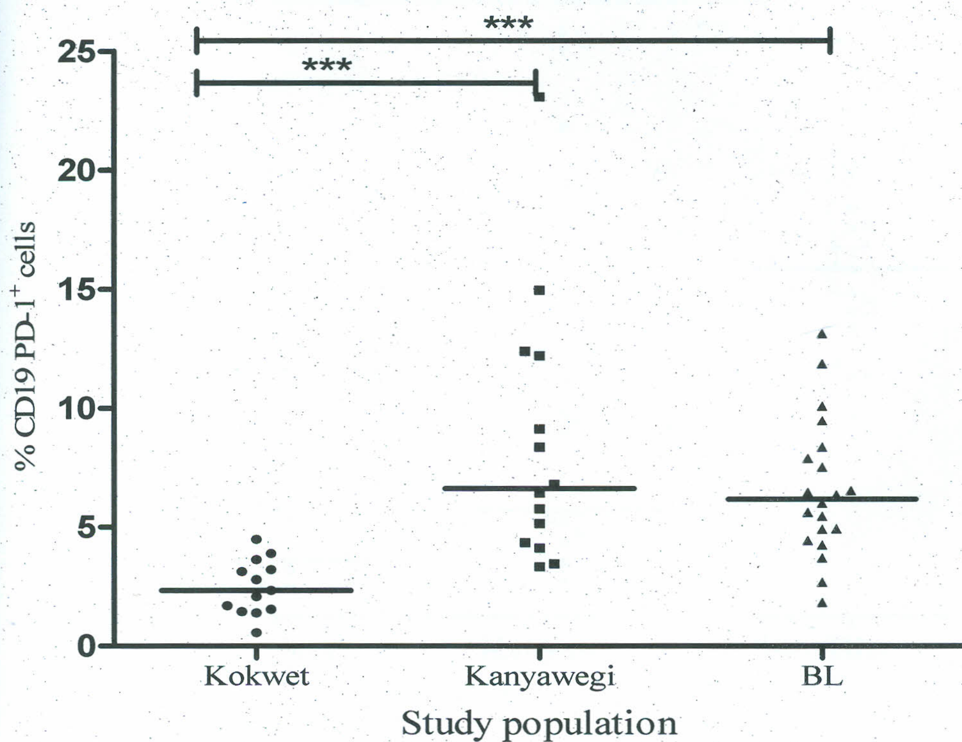

Figure 9: Expression of PD-1 on CD19$^+$ cells in children with BL compared to controls. Abbreviations BL- Burkitt's lymphoma patients, *** (p<0.001).

4.4.4 PD-1 expression on CD56$^+$ cells in Burkitt's lymphoma Patients

The expression of PD-1 on natural killer, (CD56$^+$) cells in children presenting with Burkitt's lymphoma was compared with age-matched children from areas that experience divergent malaria transmission patterns. As shown in Figure 10, there was a significant difference in the median expression of PD-1 on CD56$^+$ cells in children with Burkitt's lymphoma (22.78%) compared to children from Kokwet (3.620%) and Kanyawegi (6.895%, $p<0.0001$, Table 4). Further analysis revealed that there was no difference in PD-1 expression on CD56$^+$ cells between Kokwet and Kanyawegi. However, there was a difference in PD-1 expression when Kokwet vs BL and Kanyawegi vs BL were compared.

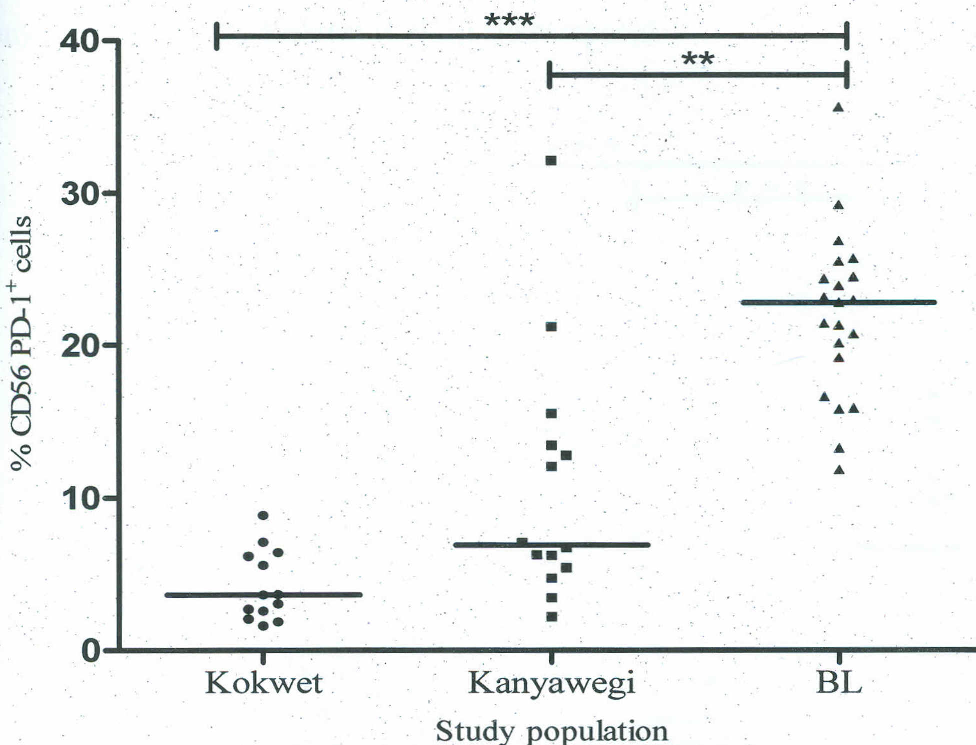

Figure 10: Expression of PD-1 on CD56$^+$ cells in children with BL compared to controls. Abbreviations BL- Burkitt's lymphoma patients, *** ($p<0.001$), ** ($p=0.001$ to 0.01).

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4.5 Levels of sPD-1 in children with endemic Burkitt's lymphoma compared to children experiencing divergent malaria transmission patterns

This study compared the levels of soluble PD-1 in plasma among children from areas with divergent malaria transmission dynamics to those of children with Burkitt's lymphoma. As shown in Figure 11, children with Burkitt's lymphoma had a significantly higher concentration of soluble PD-1 in the plasma [526.3pg/mL, (88.44 – 767.8)] compared to individuals from either Kanyawegi [60.37pg/mL, (0 – 177.4)] or Kokwet [12.63pg/mL, (0 – 204.4)], p=0.0001. Further analysis revealed that there was no difference in the concentration of soluble PD-1 when Kanyawegi and Kokwet were compared. However, there was significant difference when Kanyawegi vs BL and Kokwet vs BL were compared.

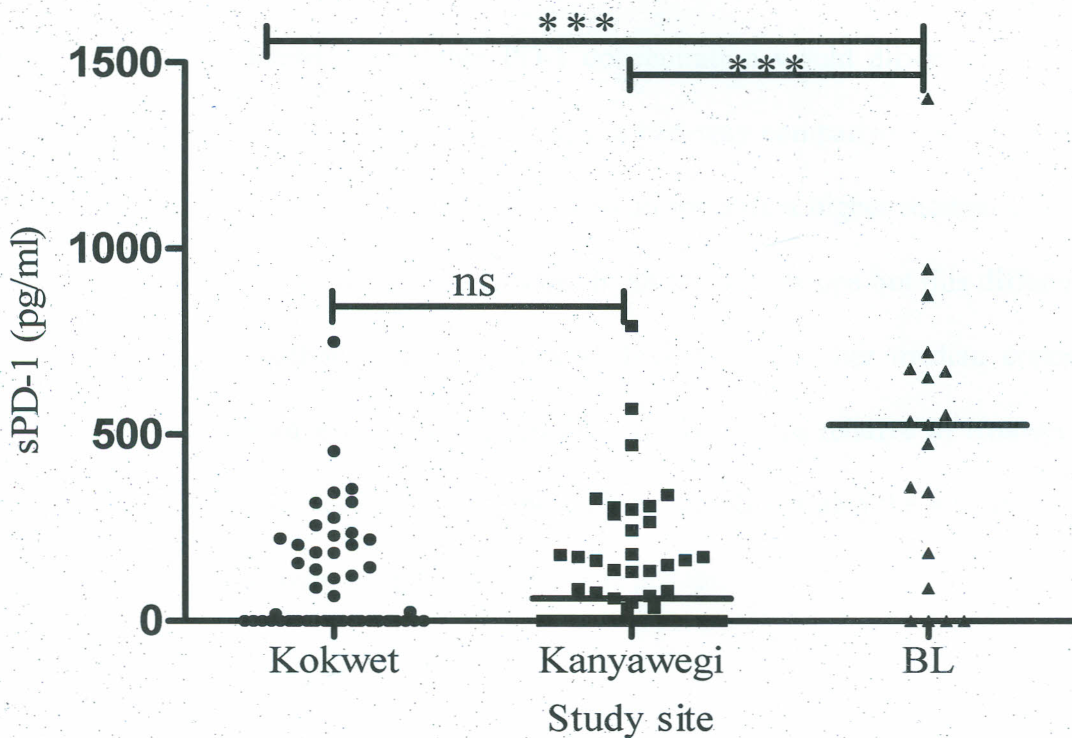

Figure 11: Soluble PD-1 concentration in children with BL compared to age-matched children from Kanyawegi and Kokwet. Abbreviations BL- Burkitt's lymphoma patients, *** (p<0.001)

4.6. Changes in the Concentration of soluble PD-1 in individuals with Divergent Malaria Exposure.

To assess whether continuous exposure to malaria alters the expression of soluble PD-1, results in Figure 12 show that there was no significant difference in median concentration of soluble PD-1 in Kanyawegi compared to Kokwet [60.37pg/ml (0 – 177.4)] and [12.63pg/mL, (0 – 204.4)], respectively (p=0.5168).

Across group comparison shows that the median concentration of soluble PD-1 differ between children of different age groups, both for Kanyawegi and Kokwet (p=0.0028 and p=0.0006 respectively), whereby the young children had higher median concentrations of soluble PD-1 as compared to the adults, Figure 13A and B.

Further, in order to determine whether PD-1 concentration would differ between children of Kanyawegi and Kokwet of different age groups, a two-way comparison was made on each age group. As summarized in Table 5, there was a trend towards a higher median concentration of soluble PD-1 in Kanyawegi relative to Kokwet in all the age groups but this difference was not significant. However, although not significant, there was a higher median concentration of soluble PD-1 among Kanyawegi children in the 1-5 age groups relative to Kokwet children of the same age group. Coincidentally, it is shown here that this is also the age group that has the highest density parasitaemia compared to other age groups.

p=0.5168

1000
800
600
400
200
0

sPD-1 (pg/mL)

Kokwet Kanyawegi

Study site

Figure 12: Concentration of soluble PD-1 in individuals from areas with divergent malaria transmission dynamics

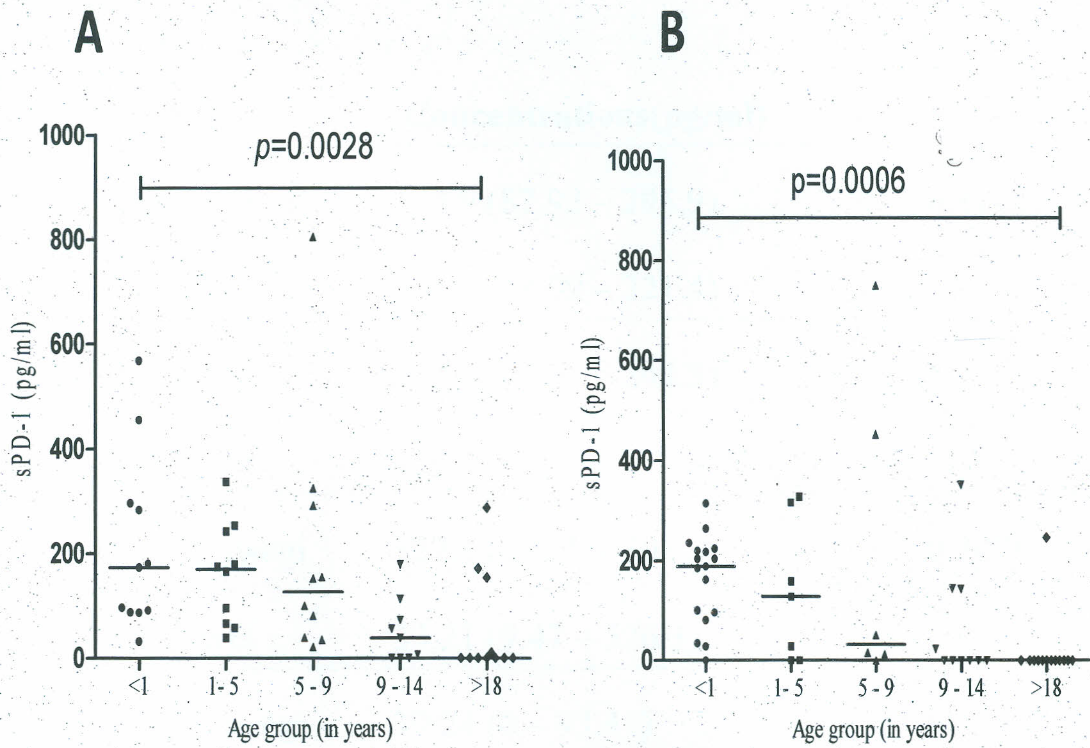

Figure 13: Scatter plots of the levels of soluble PD-1 in plasma in individuals of different age groups from areas with divergent malaria transmission dynamics. (A) Kanyawegi and (B) Kokwet.

Table 5: Concentration of sPD-1 across age groups between Kanyawegi and Kokwet

Age group (years)	**Group**	**Concentrations(pg/ml)**	***p*- value**
<1	Kanyawegi	173.9 (87.92 – 295.9)	0.8141
	Kokwet	188.1 (88.09 – 221.4)	
1 – 5	Kanyawegi	170.2 (64.14 – 244.5)	0.4173
	Kokwet	127.6 (0 – 315.6)	
5 – 9	Kanyawegi	127.1 (39.15 – 300.3)	0.3676
	Kokwet	33.21 (9.47 – 526.3)	
9 – 14	Kanyawegi	39.24 (0 – 93.41)	0.5747
	Kokwet	0 (0 – 143.3)	
18	Kanyawegi	0 (0 – 154.7)	0.1211
	Kokwet	0 (0 – 0)	

Data are presented as medians (in brackets are the 25th and 75th percentiles) unless otherwise noted. Statistical difference between the two groups was determined by Mann Whitney test.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Modulation of the Expression of PD-1 by Exposure to Holoendemic malaria

This study evaluated the expression of immune regulatory molecule PD-1 in the context of differential malaria exposure and in children presenting with endemic Burkitt's lymphoma. The results shows that continuous exposure to high and stable *Plasmodium falciparum* transmission and presentation with eBL are associated with elevated cell surface expression in various cellular components of adaptive and innate immune system and levels of soluble PD-1. Results from this study also demonstrate that infants have lower levels of PD-1 expression on lymphocyte subsets studied as compared to children of 1 - 5 age groups.

The higher levels of PD-1 expression observed in $CD8^+$ T-cells are consistent with previous studies showing two fold increase in PD-1 levels on total $CD8^+$ T-cells in individuals infected with hepatitis C virus (HCV) (Golden-Mason *et al.*, 2008). In contrast, there was significantly higher expression of PD-1 on $CD4^+$ T-cells in children and adults from malaria holoendemic area compared to hypoendemic area. Studies in mice infected with *P. yoelii* demonstrated low expression levels of PD-1 on naïve CD4 cells and an increased expression of PD-1 on activated CD4 cells after infection (Chandele *et al.*, 2010). Taken together, these data suggest that exposure to malaria increases the expression of this inhibitory molecule on the surface of $CD4^+$ T-cells.

During chronic viral infections, expression of high levels of PD-1 has been noted on $CD8^+$ T-cells (Barber *et al.*, 2006; Day *et al.*, 2006) and in mice infected with *P. yoelii,* an increased

expression of PD-1 on activated CD8 T-cells after infection was observed compared to naïve CD8 T-cells (Chandele *et al.*, 2010). This study is the first to evaluate the surface expression of PD-1 on $CD8^+$ T-cells in individuals who have repeatedly been infected with malaria parasites compared to those who have not, and found that continuous exposure to malaria antigens may not strongly suppress the effector function of $CD8^+$ cells as in the case of $CD4^+$ T-cells. Further, the observation of increased expression of PD-1 on $CD4^+$ and $CD8^+$ T-cell subsets in chronic viral infection and high malaria transmission suggest that this molecule has an essential role in persistent infections.

A significant dysregulation in B-cell differentiation has been observed in individuals living in areas of holoendemic *P. falciparum* in comparison to those from hypoendemic malaria areas (Asito *et al.*, 2008), but whether this expansion of B-cells is accompanied by changes in expression of suppressive surface molecules has not been evaluated. The present study reports an increase in the expression of PD-1 on B-cells in individuals from malaria endemic area as compared to individuals from unstable malaria transmission area. It is well established that PD-1 is expressed only on activated and not resting B-cells (Agate *et al.*, 1996) further suggesting that *P. falciparum* exposure increases the pool of activated B-cells that could be secreting antibodies evident by the higher levels of total immunoglobulins in children with malaria compared to controls (Asito *et al.*, 2008). However, upregulation of PD-1 molecule on B-cells is reported to inhibit BCR signaling and antigen stimulated B-cells (Okazaki *et al.*, 2001) leading to speculation that continuous exposure to malaria may actually suppress the function of B-cells. This conclusion further support the observation that exposure to *P. falciparum* antigens causes defects in B-cell population (Dorfman *et al.*, 2005).

Natural killer (NK) cells are a group of lymphocytes that can be activated without prior stimulation (Heusel and Ballas, 2003), hence they are important in first line defense against various pathogens and tumor cells as they produce high levels of pro-inflammatory cytokines needed to clear parasites (Horowitz and Riley, 2010). They detect the presence of infections using activating and inhibitory receptors on their surfaces (Papazahariadous *et al.*, 2007). Investigation of expression of the inhibitory molecule PD-1 on NK cells in the present study also shows that persistent malaria exposure upregulates expression of PD-1 on NK surfaces especially in children aged below five years. Since NK cells are a significant source of IFN-γ during early course of malaria infection, the elevated expression of PD-1 on NK cells could impair their function thereby worsening the infection. This idea is supported by a study that associated the upregulation of PD-1 in chronic HCV infection with functional impairment of NK cells (Golden-Mason *et al.*, 2008).

5.2 Age related changes in the expression of PD-1

It is interesting to note that in infants (<1 year), the level of PD-1 expression on $CD4^+$, $CD8^+$, $CD19^+$ and $CD56^+$ cells was low compared to children aged 1 – 5 years within the holoendemic malaria transmission region. Possible explanation for this observation is that children born to mothers exposed to high malaria transmission may be immunologically sensitized to malaria antigens due to prenatal exposure (Malhotra *et al.*, 2009). This exposure may lead to upregulation of suppressive molecules leading to immune anergy or tolerance in this population of infants. The consistently lower PD-1 expression observed in infants independent of malaria exposure compared to children aged 1 – 5 years could be due to the fact that they may still be

immunologically naïve (have not been exposed to *P. falciparum* malaria antigens) or that the exposure has not reached a threshold to start its inhibitory function.

5.3 Expression of PD-1 in children with endemic Burkitt's lymphoma

Tumors have the ability to evade immune surveillance and antitumour immunity is provided by the interaction of negative and positive co-stimulatory signals (Sharpe *et al.*, 2007). Several factors including membrane bound and soluble molecules have been implicated in the tumor escape mechanism. Recently, the role of PD-1 pathway has received attention in the evasion of immunosurveillance by tumors with some studies suggesting the involvement of PD-1 pathway in hematologic malignancies (Kier *et al*, 2008). PD-L1 has been shown to be expressed on a number of solid tumors and increased expression of PD-L1 is associated with poor prognosis (Greenwald *et al.*, 2005; Blank and Mackensen, 2007). It has also been documented that it binds to PD-1 leading to inhibition or attenuations of anti-tumor activity by effector T-cells (Freeman *et al.*, 2000). Further, it has been shown in animal models that PD-1 on tumor cells inhibits T-cell activation and also the lysis of tumor cells (Hirano *et al.*, 2005), and that there is increased expression of PD-1 on $CD4^+$ and $CD8^+$ cells in peripheral blood of melanoma patients (Hino *et al.*, 2010). Collectively, the aforementioned studies support the function of PD-1 as a negative immune regulator in tumor eradication or rejection. The results from this study are congruent with the above observations since there was a higher expression of PD-1 on the lymphocytes.

PD-L1 expression has been associated with poor prognosis in many cancers, including cancer of the larynx, lung, stomach, colon, breast, cervix, ovary, renal cell, bladder and liver, as well as in melanoma (Blank and Mackensen, 2007). Further, it has been suggested that these tumor can evade the immune system by attenuating tumor-specific T-cell responses through the PD-1/PD-

L1 pathway by negatively regulating T-cell proliferation and cytokine production (Dong and Chen, 2003; Iwai *et al.*, 2002). The slight difference in the expression of PD-1 on $CD8^+$ T-cells in BL patients compared to the age-matched controls from both Kanyawegi and Kokwet could negatively impact on responses to EBNA1, the viral antigen that is exclusively expressed by BL cells, are poorly antigenic and has little or no HLA class 1 response (God and Haque, 2010). This therefore, could partially explain the poor $CD8^+$ response in Burkitt's lymphoma patients. Since BL tumor cells display latency program III (Thorley-Lawson and Allday, 2008) and only express EBNA1 and not other EBV antigens, it is thought that cytotoxic $CD8^+$ T-cells do not efficiently recognize BL tumor cells and so are incapable of mounting an effective immune response against them (Staege *et al.*, 2002). Natural killer cells are known to play a crucial role in controlling pathogens that evade the effector CD8 T-cells by down-regulation of essential recognition molecules like MHC 1 (Farag *et al.*, 2002). Further, it has been suggested that NK cells are able to kill many cancer cells while sparing normal cells (Smyth *et al.*, 2003). Therefore, significantly higher PD-1 expression on natural killer cells ($CD56^+$) and not on $CD8^+$ cells observed in BL patients in this study could be one of the means of suppressing the effector activity of NK by tumor cells and further suggesting the crucial role of NK cells in tumor eradication.

Though this study did not measure the viral loads in these samples, it has been reported that an increase in PD-1 expression correlates with the viral loads, decreased CD4 counts and reduced proliferative potential to HIV antigens (Trautmann *et al.*, 2006; Day *et al.*, 2006). It would therefore be interesting to look at the EBV viral loads since viral infections modulate PD-1 expression that may result in persistence of the virus.

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5.4 Levels of soluble PD-1 in Burkitt's lymphoma

This study reports for the first time that there is a significant increase in the median concentration of soluble PD-1 in children diagnosed with eBL compared to children from areas with divergent malaria transmission dynamics. This increase could be attributed to the fact that in endemic Burkitt's lymphoma patients, there is polyclonal activation and expansion of lymphocytes (Weiss *et al.*, 2009) resulting in increased numbers of cells that are secreting PD-1 into the plasma. This observation is congruent with previous studies showing that sPD-1 is elevated in patients with rheumatoid arthritis and that the aberrant function of membrane bound PD-1 is associated with the increased expression of sPD-1 (Wan *et al.*, 2006).

The precise function of sPD-1s in Burkitt's lymphoma pathogenesis is still speculative. sPD-1 may compete for its ligand with surface PD-1 hence blocking the interaction between PD-L1 on APC and membrane bound PD-1 on T-cells thereby interfering with the inhibitory signal sent to T-cells for enhancing immune responses resulting in the dampening of immune responses.

The function of PD-1 pathway is usually altered in viral infections and cancer so findings from this study were not surprising but confirmatory. A number of studies have reported an increase in PD-L1 in solid and hematopoietic tumor which interferes with anti tumor immunity (Thompson *et al.*, 2007; Zhang and Strome, 2004). Consequently, it is interesting to speculate that sPD-1 in plasma of Burkitt's patients bind to its ligand on tumor cells in the absence of strong TCR signaling and positive co-stimulation from activated DC resulting into anergy, instead of recognition and elimination of these neoplastic cells (Keir *et al.,* 2008). Therefore, increased expression of sPD-1 may reflect the dysregulation of T-cell immunity to BL thereby contributing to the pathogenesis of the disease. Future studies should explore PD-1 expression on the B-cell

tumors themselves and determine if *in vitro* anti-PD-1 blocking experiments restore function of EBV-specific T-cells (Barber *et al.*, 2006).

On the other hand, continuous exposure to *P. falciparum* malaria antigens seemingly does not modulate the expression of sPD-1 since there were no significant difference in expression between high and low malaria transmission areas. Although there may have been a prolonged period of higher PD-1 expression with increasing age (up to 5 years which corresponds to the age groups with the higher parasite densities), sample size presented in this study was not powered to verify this trend.

5.5 PD-1 Pathway, a possible EBV immune evasion strategy?

Burkitt's lymphoma is a common childhood cancer and has the highest incidence within malaria holoendemic regions of equatorial Africa (Burkitt, 1958). In addition, it has been demonstrated that by the age of three, nearly 100% of the children in these areas are EBV seropositive (Biggar *et al.*, 1978). Several lines of evidence suggest that an interaction between *P. falciparum* malaria and early-age of primary EBV infection are critical in the emergence of eBL (Rochford *et al.*, 2005; Roughan *et al.*, 2010). Epstein Barr virus, one of the aetiological agents of BL is a B-cell lymphotropic virus that can infect nearly all B-cell subsets (Joseph *et al.*, 2000). However, EBV viral establishment and persistence in malaria endemic areas is still under studied and immune exhaustion has been suggested as a possible mechanism of viral persistence (Moskophidis *et al.*, 1993, Doherty, 1993).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. It has been documented that in self limiting acute infections such as with vaccinia virus, very little or no PD-1 is expressed on T-cells, while in chronic infections such as EBV, HIV and LCMV, high levels of PD-1 is expressed on T-cells (Freemann *et al.*, 2006). Further, data from this study demonstrate that PD-1 was expressed at low levels in the immune cells in individuals from areas with very little malaria exposure, was relatively higher in individuals from malaria holoendemic areas and was significantly elevated in children diagnosed with endemic Burkitt's lymphoma. This may suggest that under conditions of continuous antigen exposure with two pathogens (i.e. repeated, chronic asymptomatic malaria infections in conjunction with high EBV viral loads) the proper regulation of the PD-1 pathway could be pivotal in eBL pathogenesis.
2. The functional antagonism/anergy of the over expressed membrane bound PD-1 on T-cells of BL patients could be mediated by sPD-1 that is secreted in plasma. This conclusion is based on several lines of evidence including the significantly elevated levels of sPD-1 in BL patients compared to children experiencing divergent malaria transmission dynamics. In addition, it has been shown that sPD-1 is able to block the function of membrane bound PD-1 in *vitro* experimental systems (Wan *et al.*, 2006) and therefore it is possible that it attenuates the PD-1 pathway therefore worsening the disease.

6.2 Recommendations

6.2.1 Applications of the observations of current study

1. Since this study has demonstrated an increased expression of PD-1 in individuals from holoendemic area compared to hypoendemic area and the fact that it contributes to dysfunction of immune cells, this suggest a novel strategy for the treatment of parasitic infections.
2. The results from this study have demonstrated elevated expression of PD-1 on lymphocytes in children with Burkitt's lymphoma and other studies have reported overexpression of the PD ligands in various cancers, and interfering with PD-1 or its ligands increases antitumor immunity. Thus blocking PD-1 inhibitory signal by specific monoclonal antibodies has a potential clinical application in the treatment of cancer and other infectious diseases.
3. The observations in this study of the elevated soluble PD-1 in Burkitt's lymphoma patients and the fact that it counteracts the function of membrane bound PD-1 provides a possible explanation for the worsening of the disease. Therefore therapeutic management of BL and other cancers is possible by blockade of the interaction of PD-1 and soluble PD-1.

6.2.2. Recommendation for future studies

1. This study demonstrated an increase in PD-1 expression on lymphocytes in a cross-section of individuals from a holoendemic malaria region, however, further longitudinal studies should investigate PD-1 expression in individuals with repeat episodes of acute clinical (i.e. symptomatic) malaria in order to determine the trajectory of PD-1 expression kinetics and describe temporal variation in PD-1 expression.
2. This study demonstrated the expression of PD-1 on total lymphocyte subsets. Further studies are warranted to look into the specific lymphocyte subsets e.g. naïve, effector or memory cells that express PD-1. In addition, antigen-specific T-cell subsets (at least for EBV-specific CD4 and CD8 T-cells) could be examined for PD-1 expression using HLA-tetramers.
3. Future studies will be necessary to look at the expression of other inhibitory receptors (such as CLTA-4, BTLA, LAG-3, CD160, and FOXp3) and whether these inhibitory receptors are co-expressed on the same cells.

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