CXCR4 and CCR5 Receptor Expression by CD4+ T Cells and CD14+ Monocytes in Paediatric Plasmodium falciparum-HIV-1 Co-Infection within Western Kenya

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CXCR4 and CCR5 receptor expression

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DECLARATION

This thesis is my original work and has not been submitted for a degree to any other university or for any other award.

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I dedicate this work to my Family members; my mum Christabel Pande, brothers Amos Ochieng and Josephat Omune, sister Judith Akoth among others and my great friends Rose Ochieng and Florence Adhiambo.
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<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine receptor type 5</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DBS</td>
<td>Dried blood spot</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMC</td>
<td>Division of Malaria Control</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EIR</td>
<td>Entomological inoculation rate</td>
</tr>
<tr>
<td>HC</td>
<td>Healthy control</td>
</tr>
<tr>
<td>HG3PDH</td>
<td>Housekeeping gene 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type 1</td>
</tr>
<tr>
<td>Hz</td>
<td>Haemozoin (malaria pigment)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MOH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PCM</td>
<td>Pigment containing monocytes</td>
</tr>
<tr>
<td>PCN</td>
<td>Pigment containing neutrophils</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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</table>
RANTES  Regulated upon activation, normal T-cell expressed and secreted
RBCs    Red blood cells
RBM     Roll Back Malaria
RT      Room temperature
SDF-α   Stromal derived factor alpha
SDH     Siaya District Hospital
SMA     Severe malarial anaemia
TNF-α   Tumour necrosis factor-alpha
UNM     University of New Mexico
WHO     World Health Organization
ABSTRACT

HIV-1 and malaria pose the greatest health problem in holoendemic areas of malaria infection affecting about four million people world-wide, especially in sub-Saharan Africa. The molecular interactions involving the relative expression of CXCR4 and CCR5 receptors by CD14+ monocytes and CD4+ T cells are important immune networks for HIV and malaria interaction. However, very little have been performed to assess the molecular mechanism underlying these interactions in children from Western Kenya where both malaria and HIV-1 infection are common. To determine the levels of expression of CXCR4 and CCR5 by CD3+CD4+ T cells and CD14+ monocytes, and their association with parasitological and haematological measures during paediatric P. falciparum-HIV-1 exposure and co-infection within Western Kenya, the parameters were examined through flow cytometric analyses on cells collected from children (age, <5 years; n=72) from western Kenya categorized into the following five groups: P. falciparum negative and HIV-1 negative {mal[-]-HIV-[], n=13} as the healthy control; P. falciparum positive and HIV-1 negative {mal[+]-HIV-[], n=30}; P. falciparum positive and HIV-1 exposed {mal[+]-HIV-1[exp], n=17}; P. falciparum negative and HIV-1 positive {mal[+]-HIV-1[+], n=5}; and P. falciparum positive and HIV-1 positive {mal[+]-HIV-1[+], n=7}. Age differed significantly across the groups (P=0.016) with the HIV-1-positive groups having older children relative to the other groups. Proportions of CD3+CXCR4+ and CD3+CD4+CCR5+ cell subsets did not differ significantly across the groups (P=0.082 and P=0.099, respectively). In addition, proportions of CD14+CCR5+ cells were comparable (P=0.065). Comparing mal[+]-HIV-1[+] to mal[+]-HIV-1[-], and mal[+]-HIV-1[+] to mal[-]-HIV-1[-] revealed that only CD14 cells increased significantly (P=0.001 for both). Comparing mal[-]-HIV-1[+] to mal[+]-HIV-1[exp] showed a significant decrease for proportions of CCR5+CD14+ (P=0.023), while comparison of mal[-]-HIV-1[+] to mal[-]-HIV-1[-] revealed that CCR5+CD14+ significantly decreased (P=0.017). Spearman rank correlation test in the combined population of malaria-infected children revealed that CD3+CCR5+ population was inversely correlated significantly with age (r=-0.620, P=0.018); and positively the CD3+ (r=0.363, P=0.008) and inversely CD3+CXCXR4+ (r=-0.711, P=0.021) populations were correlated significantly with intra-monocytic pigment (PCM), while CD14 cells were positively correlated significantly with CD3+CXCXR4+ (r=0.636, P=0.048), CD4+CCR5+ (r=0.875, P<0.0001) and inversely to CD4+ cells (r=-0.271, P=0.036). Further correlation analyses revealed that CD14+CXCXR4+ was highly inversely correlated with the proportion of PCM (%) (r=-0.403, P=0.003) and PCN (%) (r=-0.432, P=0.002). Thus, an increase of CD14+ due to malaria-HIV-1 co-infection may be influencing an increase of CCR5 and CXCXR4 receptors which are being down regulated by both PCM and PCN. In conclusion, the results presented here suggest a dysregulation of CXCXR4 and CCR5 receptors expression on CD4+ and CD14+ cells in children co-infected with malaria and HIV-1, and that altered expression may be driven, at least in part, through acquisition of PfHz by both neutrophils and monocytes. In order to curb the ingestions of these malaria pigments, malaria diagnosis and treatment should be done promptly. The study shows that early treatment of malaria will reduce chances of exacerbation of HIV-1 disease progression.
CHAPTER 1: INTRODUCTION

1.1 Background information

Human immunodeficiency virus (HIV)-1 and *P. falciparum* malaria are major global health burdens with significant morbidities and mortalities resulting from immunological interactions that are only partially characterized. Both diseases overlap geographically, with HIV-1 exacerbating progression of malaria leading to enhanced malaria disease severity, and increased viral loads and transmissibility of HIV-1 (Herbein and Varin, 2010). Additionally, the presence of anti-inflammatory cytokines due to malaria parasites differentially activates macrophages playing an important role in HIV-1 replication and pathogenesis (Herbein and Varin, 2010). Understanding the interaction between malaria and HIV-1 in sub-Saharan Africa is important based on the high prevalence of malaria that affects approximately 225 million people, predominantly children, and HIV that affects nearly 2.25 million children (Schwarzer *et al.*, 1998; WHO, 2010).

It is well established that HIV-1 infection of CD4+ cells requires co-receptor use, initiated by either CCR5 expressed by monocytes and memory T cells (Fackler and Peterlin, 2000; Marechal *et al.*, 2001; Daecke *et al.*, 2005) and CXCR4 expressed primarily by germ cells and T cells (Feng *et al.*, 1996). Together, these surface receptors play an essential role in the multiplication and pathogenesis of HIV-1 (Jiao *et al.*, 2012). CC and CXC chemokines are the natural ligands for the CCR5 and CXCR4 receptors, respectively (Deng *et al.*, 1996; Dragic *et al.*, 1996; Blanchet *et al.*, 2012).
The immunological basis of HIV-1 and malaria co-infections is poorly understood. However, induction of chronic inflammation that activates type-2 cytokine regulatory pathways promotes increased HIV-1 pathogenesis (Harms and Feldmeier, 2002; Herbein and Varin, 2010). A pro-inflammatory cytokine, TNF-α, also appears to play a fundamental role in the entry of HIV-1 virus particles (Cotter et al., 2001; Maheshwari et al., 2006). Pre-treatment of macrophages with TNF-α is associated with inhibition of virus entry into macrophages, likely through its ability to induce secretion of β-chemokines, such as regulated upon activation, normal T-cell expressed, and secreted (RANTES), and macrophage inflammatory protein (MIP)-1α and MIP-1β (Herbein and Varin, 2010). Tumour necrosis factor-alpha (TNF-α) also stimulates RANTES transcripts in glial cells (Marfaing-Koka et al., 1995; Li et al., 2001). RANTES is reduced at the later stages of malaria disease due to increased IL-10 production by human monocytes and microglial cells (Marfaing-Koka et al., 1996; Hu et al., 1999). In addition, it has been shown that acute malaria suppresses circulating RANTES levels in paediatric populations in western Kenya (Were et al., 2006). However, increased TNF-α inhibits stromal cell-derived factor type 1 alpha (SDF-1α) expression (Salvucci et al., 2004) but up-regulates CXCR4 mRNA from intrahepatic cholangiocarcinoma cells (Ohira et al., 2006), suggesting increased CXCR4 surface expression during malaria/HIV-1 -exposure or co-infection.

Malaria infection has been associated with significant increases in TNF-α transcripts (Xiao et al., 1998; Ochiel et al., 2005). During malaria in pregnant women, HIV viral load and CCR5 expression on intervillous maternal and foetal villous macrophages increases (Tkachuk et al., 2001; Mwapasa et al., 2004) indicating an increased risk for
foetal HIV-1 infection but not malarial infection. However, recent studies indicate that placental malaria is not associated with increased risk of mother to child transmission of HIV at birth (Msamanga et al., 2009). Furthermore, studies by Jenkins et al. (2006) reported that paediatrics acute *P. falciparum* malaria may increase expression of CCR5 by monocytes.

HIV-1 envelope glycoprotein gp120 induces TNF-α production and has been associated with down-regulation of both surface and total CD4 expression, while increasing CCR5 expression in primary human macrophages, thereby resulting in enhanced HIV-1 replication within human mononuclear cells (Xiao et al., 1998; Herbein and Varin, 2010). Previous studies by Perrault et al. (2009) in Siaya District found that the HIV-1 co-infected women had a 33-fold increase in malaria parasite burden over HIV-negative women demonstrating the adverse effect of HIV-1 in increasing the malaria burden in areas where the two diseases co-exist.

Previous studies of paediatric malaria in a hyper-endemic region of Central Africa (Gabon) showed increased levels of MIP-1α and MIP-1β, while RANTES levels decreased significantly when malaria pigment (haemozoin, Hz) was added to peripheral blood cultures (Ochiel et al., 2005). Induction of RANTES has been shown to down-regulate CCR5 expression on activated monocytes, thereby inhibiting R5 HIV-1 entry (Herbein and Varin, 2010). Since acquisition of naturally-acquired PfHz by monocytes (pigment-containing monocytes, PCM) reduces expression of RANTES (Were et al., 2009), this may enhance HIV-1 viral entry by decreasing the natural ligand of CCR5.
Previous investigations aimed at deciphering the cellular immunopathogenic mechanisms governing increased SMA development indicated that phagocytosis of the malaria pigment (Hz) by CD14+ monocytes contributes to cytokine and effector molecule dysregulations (Jaramillo et al., 2004; Nti et al., 2005; Ochiel et al., 2005; Awandare et al., 2007; Were et al., 2009). However, the molecular mechanism(s) involving expression of CXCR4 and CCR5 receptors by which malaria-HIV-1 co-infection enhances the pathogenesis of both diseases is largely unknown.

1.2 Statement of the problem

HIV-1 and malaria are amongst the top global health threats with, at least, 4 million deaths annually, primarily in developing countries of sub-Saharan Africa. Based on the co-endemicity of the two infections within Siaya County, HIV-1 infection can increase the severity of malaria, while malaria infection can promote activation of CD4+ cells, thereby providing a conducive environment for replication and transmission, as well as over-production of pro-inflammatory cytokines, such as TNF-α that are known to enhance HIV-1 viral replication and consequently, transmission of the virus. Since malaria infection exacerbates HIV-1 (e.g. increased viral load), there is likely an increased risk of vertical and sexual transmission of the virus.

HIV-1 and malaria pose a significant public health problem in holoendemic areas of malaria such as Siaya District. The number of children infected by malaria and HIV-1 each year in Africa has been on the rise mainly due to poor health systems, increasing drug and insecticide resistance and global warming (Ayisi et al., 2003; Rowe et al., 2006; Alemu et al., 2013). However, the molecular basis of co-infection, particularly the
expression of CCR5 and CXCR4 on CD14+ monocytes and CD4+ T cells which appears to represent an important interaction between malaria and HIV-1 in children under the age of 5 years of age, is largely unexplored.

1.3 Justification of the study

In regions of Africa with holoendemic malaria transmission, malaria causes significant economic losses and decreased gross domestic product by as much as 1.3% (2001-2010 UN decade Roll Back Malaria). In Kenya, malaria is further associated with mortalities of approximately 34,000 among children under 5 years of age (DMC, 2009). Similarly, HIV-1 causes high economic constraints and enhanced mortality, particularly in Siaya District which is one of the most affected regions in Kenya with an HIV-1 prevalence of 38.4% in a population of 493,326 people (Otieno et al., 2006; WHO, 2010). The prevalence of HIV-1 amongst mothers attending antenatal care is about 25% (Dabis and Ekpini, 2002; Obonyo et al., 2007).

Characterizing and analyzing the relative expression of CCR5 and CXCR4 on CD14+ monocytes and CD4+ T-cells will provide an increased understanding of the interaction between malaria and HIV-1. Defining this molecular interaction may facilitate the design of therapeutic agents and/or intervention strategies that could reduce the morbidity and mortality associated with malaria and HIV-1 co-infections.
1.4 Research questions

i. What are the effects of *P. falciparum* malaria and/or HIV-1 on the surface expression of CCR5 and CXCR4 by CD3+CD4+ cells and CD14+ monocytes in children co-infected with malaria and HIV-1?

ii. What is the relationship between age, erythrocytic outcomes and the acquisition of intra-phagocytic haemozoin and the expression of CXCR4 and CCR5 on CD3+CD4+ T cells and CD14+ monocytes in children co-infected with HIV-1 and *P. falciparum* malaria?

1.5 Hypotheses

i. The surface expression of CXCR4 and CCR5 receptors by CD3+CD4+ and CD14+ cells is not different in children under 5 years of age with *P. falciparum* malaria and HIV-1-co-infection.

ii. There is no association between expression of CXCR4 and CCR5 on CD14+ monocytes and neutrophils and CD4+ cells and age, erythrocytic outcomes and the acquisition of intra-phagocytic haemozoin in children with *P. falciparum* malaria and HIV-1-co-infection.

1.6 Objectives

1.6.1 General objective
To determine the levels of expression of CXCR4 and CCR5 by CD3+CD4+ T cells and CD14+ monocytes, and their association with parasitological and haematological
measures during paediatric *P. falciparum*-HIV-1 exposure and co-infection within Western Kenya.

1.6.2 Specific objectives

i. To determine the CXCR4 and CCR5 expression by CD3+CD4+ and CD14+ cells during paediatric *P. falciparum* malaria and HIV-1 co-infection.

ii. To examine the relationship between CXCR4 and CCR5 expression with age, parasitological and erythrocytic outcomes in children with *P. falciparum* malaria and HIV-1 co-infection.

1.7 Limitations of the Study

i. Staging for HIV-1 in children were never done, being important in determining the expression of receptors CXCR4 or CCR5, however all study participants had an intact immune system with CD4 counts of above 500 cells/µL.

ii. The sample size for some of the categories was less than the calculated sample size for each group due to the fact that it was difficult getting the samples. This may be because of HIV-1 disease stigmatization and a limited time frame.

1.8 Significance of the Study

The research study has shown that prompt diagnosis and treatment of malaria would help reduce chances of paediatrics HIV-1 infection and exacerbation in areas where the two diseases are common.
CHAPTER 2: LITERATURE REVIEW

2.1 Public health importance of malaria

High incidence of malaria-related mortality has led to increased use of key malaria control measures, particularly insecticide treated nets (ITN) (Noor et al., 2009) and development of policies to sustain the provision of efficacious Artemisinin-based combination therapies (ACT) for malaria case-management (RBM, 2008). This has resulted in reduction of malaria mortality rates, a 25% reduction globally and a 33% reduction in the WHO African region (WHO, 2011b). However, malaria still remains the world’s leading parasitic disease with approximately 216 million cases of acute malarial infections and up to 655,000 deaths every year, predominantly among African children (Hay et al., 2010; WHO, 2011a) accounting for 2.23% of the deaths worldwide.

In Africa, malaria cases are approximately 78 million (Gething et al., 2010) and account for 20% of all childhood deaths, and more than 85% of the world’s malaria deaths which translates into about 556,750 deaths per year in children under 5 years of age in sub-Saharan Africa (WHO, 2009; WHO, 2011a). In Kenya, the malaria burden is high with 25 million people are at risk of contracting malaria (DMC, 2009). Each year, there are 8 million outpatient malaria treatments recorded at the health facilities throughout the country and ~34,000 malaria-related deaths among children under 5 years of age (DMC, 2009). Moreover, malaria is a frequent cause of prenatal abortions, premature deliveries, growth retardation, low birth weight and maternal and infant anaemia in the country (Steketee et al., 2001; Obonyo et al., 2007), suggesting that malaria contributes to poor clinical outcomes in both children and pregnant women in malaria endemic regions.
2.2 Immunopathogenesis of malaria

The life cycle of the *Plasmodium falciparum* malaria parasite involves an endogenous asexual stage in humans and an exogenous sexual stage in the mosquito. The human phase has 2 stages: exoerythrocytic (pre-erythrocytic) and erythrocytic (blood stage) with the clinical manifestations of malaria caused by the blood-stage of disease. Warmer temperatures of about 27°C favor a short extrinsic phase of the *Plasmodium* life cycle, as well as survival of the *Anopheline* mosquito (Pampana, 1969; Li et al., 2009; Minakawa et al., 2012). The *Anopheline* mosquito is the most inherent mosquito species in Western Kenya region and their larval development is higher in warmer areas (Pampana, 1969; Luz et al., 2011). Moreover, higher temperatures accelerate the rate of blood meal uptake, laying of eggs by the mosquitoes and transmission of malaria (Martens, 1998; Mohammed and Chadee, 2011; Thomas et al., 2012). Seasonality is an important determinant of mosquito density, and hence, the entomological inoculation rate (EIR) peaks in May during the wet season each year in Western Kenya (Amek et al., 2012).

Following infection with *P. falciparum*, pro-inflammatory cytokines such as TNF-α and IFN-γ are produced during the early stages of the host immune response to infection for eliciting anti-parasitic activities (Scragg et al., 1999). However, excessive induction of these host-immune responses can contribute to increased immunopathogenesis and enhanced severity of disease (Kremsner et al., 1995). Thus, during a *P. falciparum* malaria infection, enhanced production of cytokines, such as IFN-γ, promote both enhanced protection against the parasite and immunopathogenesis (Luty et al., 1999; Kwiatkowski, 2005). This premise is underscored by the fact that IFN-γ, at least in part,
is responsible for a reduction in parasitaemia through generation of effector molecules such as nitric-oxide (NO) from monocytic cells (Gradoni and Ascenzi, 2004). However, excessive levels of endogenous NO can drive the molecular pathways leading to more profound malarial anaemia (Clark and Cowden, 2003).

It has also been shown that levels of the protective cytokine, IL-12, are reduced in children with severe malaria through an IL-10 dependent mechanism (Casals-Pascual et al., 2006; Keller et al., 2006). In addition, IL-12 levels can also be affected by high levels of TNF-α (Ma et al., 2000), suggesting that high levels of TNF-α may promote anti-inflammatory responses. This premise supports previous studies in which high levels of IL-1β, IL-6 and TNF-α enhance the development of severe malaria.

Additional studies have shown that cytokines, such as TGF-β1, are suppressed in children with severe malaria further supports immunopathogenesis as a central feature of disease since TGF-β1 is important for mediating the proliferation and effector functions of immune cells (Perkins et al., 2000). A study has further shown that a higher ratio of TGF-β1/IL-12 is associated with enhanced development of SMA in Gabonese children, relative to those with mild malaria (Perkins et al., 2000), suggesting that suppression of the protective actions of IL-12 promotes enhanced disease severity. Taken together these reports suggest that dysregulation of cytokines and effector molecules may impact on malaria disease outcomes. This suggests that the change in the host-immune response to malaria also affects HIV-1 pathogenesis and the enhanced pathogenesis witnessed during co-infection may be through modulation and activation of HIV-1 receptor expression.
2.3 Public health importance of HIV-1

HIV-1 is still a major public demise with approximately 40 million people suffering from HIV/AIDS with over 70% of the cases in Sub-Saharan Africa in late 1990s. More recently, 2.5 million children have been reported to be infected, 91% being new infections; translating to ~370,000 new paediatric infections in 2009 (UNAIDS, 2010; WHO, 2011a). The high prevalence of malaria, tuberculosis and helminths represent an additional threat to HIV-1-infected individuals (Chandramohan and Greenwood, 1998; WHO, 2011b). Given the fact that 90% of the paediatric infections are found in Africa, and especially resource poor settings in sub-Saharan Africa (WHO, 2009), exploration of malaria endemic area co-infections with HIV-1 is of great necessity.

Epidemiological studies indicate that of the 40 million people infected with HIV-1, women of childbearing age in sub-Saharan Africa are being the most affected (HIV/AIDS, 2006). Antiretroviral prophylaxis during gestation has lowered the number of children born with HIV-1 virus (Connor et al., 1994), however, this premise has resulted in the increase in the number of HIV-1-exposed uninfected children (Agangi et al., 2005). The World Health Organization currently recommends initiation of highly active antiretroviral therapy (HAART) for HIV-infected breastfeeding women with less than 350 cells/μL CD4+ cell counts, or stage 3 or 4 disease (WHO, 2009). Increased in utero exposure to HIV without transmission induces virus-specific adaptive immune responses where HIV-specific cytotoxic T-cell directed against several viral antigens are detected in 20-100% of HIV-exposed but uninfected infants at birth and during the first few months of life (Rowland-Jones et al., 1993; De Maria et al., 1994; Kuhn et al.,
Further studies have also shown that there is an increased proliferation of regulatory T-cells in HIV-1-exposed but uninfected infants and the in vitro depletion of these cells increased HIV-specific effector T-cell responses (Legrand et al., 2006) suggesting that immune responses due to exposure to either viral antigens or immune cells specific to viral antigens during pregnancy may affect the child’s immunity to HIV-1 and other related infections.

It has been reported that the risk of perinatal acquisition is between 25-40% without intervention and that of all the paediatric HIV disease cases in the United States, 80% occur through perinatal transmission which is much higher in developing countries of Africa (WHO, 2011a). Even though recent interventions with anti-retroviral therapy (ART) has significantly reduced perinatal transmission (Brady et al., 2010), HIV-1 remains a significant public health risk that also negatively impacts on malaria disease outcomes (Cohen et al., 2005).

2.4 Aetiology and Diagnosis of Malaria and HIV-1

Human malaria is caused by the unicellular obligate intracellular protozoan parasites of the genus plasmodium (WHO, 2008). Malarial disease follows an infective mosquito bites and it is not acquired in utero. Children ≤ 4 years old have the highest parasite prevalence rate at 38.8%-62.62.8% (Munyekenye et al., 2005). In western Kenya, the overall annual entomological inoculation rate (EIR) has increased since 2002 with upto 9.6 infectious bites per annum in 2004. Malaria diagnosis involves microscopic
observation on thin and thick blood smears stained with Giemsa reagents (Slutsker et al., 1994; Lyke et al., 2003).

The main route by which children acquire HIV infection is through vertical transmission of HIV-1 from mother-to-child that occurs either in utero, during the peripartum period (at birth) or from breastfeeding (intrapartum) (WHO, 2011a). HIV-1 diagnosis in paediatrics involves serological test and confirmation through PCR. The gp41 is mostly chosen since it introduces its fusion peptide into the CD4 cytoplasmic membrane (Melikyan et al., 2000) and HIV-1 gp41 primers are selected for highly conserved HIV-1 group M, N, and O sequences for use in western Kenya (Cappadoro et al., 1998; Ruwende and Hill, 1998). HIV-1 exposed children most likely acquire HIV-1 antibodies from their mothers during pregnancy and/or through breastfeeding (Mock et al., 1999; Mwapasa et al., 2004; Gay et al., 2010).

2.5 HIV-1 interaction with the immune system

HIV-1 infection involves entry into a number of immune cell types which include CD4+ and CD8+ lymphocytes, CD14+ monocytes and macrophages, thymocytes and dendritic cells (DCs). HIV-1 binding and entry occurs through the CD4 receptor in conjunction with the chemokine receptors, CCR5, CXCR4 and CCR2 (Dragic et al., 1996; Sonza et al., 1996). Co-receptor use by HIV initiates an interaction that leads to the progressive damage of peripheral and recently activated lymphoid CD4+ T cell populations eventually resulting in AIDS, with CD4+ lymphocyte and CD14+ macrophage populations being the most commonly targeted cell types (Dragic et al., 1996; Sonza et
al., 1996). To a lower extent, HIV-1 also infects CD8+ lymphocytes and DCs with DC populations being primarily involved in the transmission of HIV-1 to CD3+ lymphocytes. Although macrophages play an important role during co-infection with malaria, their level of infection with HIV-1 is less relative to CD4+ T lymphocytes (Tsai et al., 1996; Benaroch et al., 2010). T-tropic (X4) HIV-1 viruses have been shown to use the coreceptor, CXCR4, during infection (Feng et al., 1996). Recently, the orphan receptor CXCR7, primarily expressed in the vasculature of tumour cells, has been identified as a second receptor for HIV-2 and Simian Immunodeficiency Virus (SIV) strains (Shimizu et al., 2000; Miao et al., 2007), suggesting that in case of depletion of the CXCR4 receptors, HIV may resort to use of this second receptor for entry, however, the biological role of CXCR7, at present, remains controversial.

Studies have shown that multipotent progenitor cells and mast cells are also infected by the HIV virus (Li et al., 2001; Carter et al., 2010), suggesting that disruption of these immune cell populations may contribute to further immunosuppression. Since HIV viruses are intracellular pathogens, they are able, at least in part, to avoid some immune surveillance mechanisms and thereby utilize the host’s cellular genome for reverse transcription of the viral genome (Telesnitsky and Goff, 1997). It is estimated that 0.1% of the $10^{10}$ virions produced daily within individuals are infectious virus particles that undergo high mutational rates during reverse transcription that allow the virus to quickly evolve and escape both immune pressure and antiretroviral treatment (Layne et al., 1992; Bourinbaiar, 1994; Jetzt et al., 2000). Such high mutational rates create challenges for the immune response to eliminate the disease and develop immunity to new infections.
2.6 HIV-1 and malaria interaction

Although there is increasing evidence of interactions between transmittable pathogens, such as HIV-1 and plasmodium, lack of knowledge about the specific mechanism(s) through which these interactions occur forms the basis of the research efforts in this thesis. The geographical overlap between HIV-1 infection and malaria have been widely explored since 1999 in which several studies have indicated that HIV-1 influences greater incidence of severe Plasmodium falciparum induced-malaria cases, mainly through the immunosuppression observed in HIV-1 patients (Grimwade et al., 2003; Cohen et al., 2005; Van Geertruyden et al., 2006). In contrast, some epidemiological studies have failed to demonstrate that malarial infections have an impact on HIV-1, particularly in terms of HIV-1 viral replication (Kublin et al., 2005; Whitworth and Hewitt, 2005; Cuadros et al., 2011).

Studies conducted in murine models using mice depleted of CD4 T cells showed that these animals lacked the ability to make sufficient levels of immunoglobulin that provide immunity against malarial sporozoites (Weiss et al., 1993), suggesting that reduced CD4 cell counts in humans due to HIV-1 infection may also impair the ability to produce immunoglobulins and thereby compromise immunity to malaria. Consistent with this premise, studies performed in Mumbai, a low malaria transmission region, showed that individuals with severe malaria had a higher prevalence of HIV-1 (Khasnis and Karnad, 2003). Taken together, these observations suggest that co-infection with HIV-1 and malaria leads to a worsening of both diseases. The negative interaction witnessed during co-infection is supported by a mathematical model showing that the two diseases
accelerate the spread of either disease (Abu-Raddad et al., 2006). Understanding the molecular interaction through CXCR4 and CCR5 receptors expression between HIV-1 and malaria will help in the rational design of specific therapeutic agents and intervention strategies to manage people (especially children) who are exposed to both HIV-1 and malaria. Such interventions may reduce the high burden of anaemia, low birth weight, morbidity and death associated with HIV-1 and malaria co-infection.

2.7 CCR5 and CXCR4 expression during malaria and HIV-1 infections

Chemokines are the largest group of the cytokine family categorized into different subclasses based on the position and conservancy of the four cysteine residues: CXC- (α); CC- (β) and CX3C- (γ) chemokines. The three are cognate chemokines that form the natural ligands for the CCR5 and CXCR4 receptors that are important in HIV-1 transmission and pathogenesis. Stromal derived factor (SDF)-1α [CXC12- (α)], an alpha (α)-chemokine binds primarily to the CXCR4 and CXCR7 receptors (Bleul et al., 1996; Burns et al., 2006; Cruz-Orengo et al., 2011), while a β-chemokine, RANTES, binds to CCR5 receptors (Alkhatib et al., 1997).

Stromal derived factor (SDF)-1α regulates B- and T-cell maturation, as well as recruitment of macrophages, and various other immune cells important in inflammation (Zou et al., 1998; Rossi and Zlotnik, 2000; Welford et al., 2011), and is inhibited by TNF-α (Salvucci et al., 2004). Conversely, SDF-1α can also enhance the production of IL-1β, IL-10, IL-6, and TNF-α; cytokines that regulate clinical outcomes of malaria (Sherry et al., 1995; Lillard et al., 2003; Lyke et al., 2004). Additional studies have
shown that plasma levels of SDF-1α are elevated in children with cerebral malaria (Shao
et al., 2008; Gyan et al., 2009), illustrating a potential interaction between SDF-1α and
TNF-α, particularly during advanced stages of malaria when TNF-α is known to be
elevated.

Previous studies also show that a functional single nucleotide polymorphism (SNP) in the
3' untranslated region of the SDF-1α gene (like 801G/A) inhibits the progression of HIV-
1 infection to AIDS (Hedrick and Verrelli, 2006; Reiche et al., 2006), suggesting that
levels of SDF-1α regulate clinical outcomes during an HIV infection. Furthermore, a
homozygous mutation at position 881 of the 3' untranslated region of the SDF-1 gene
(SDF-1 3' A) in the mother, but not the child, conditions the rate of MTCT of HIV (John
et al., 2000). Studies have also shown that increased SDF-1 transcripts (SDF1-3'A) cause
a deleterious impact on the protective effect of the heterozygous form of the Δ32 CCR5
polymorphism in HIV-1 infected children, predisposing them to infection (Sei et al.,
2001).

The CD4 molecule used by the viral envelope generates the gp41 transmembrane region
and plays an essential role in HIV viral entry, in conjunction with two principal co-
receptors: CC-chemokine receptor 5 (CCR5 or R5) and CXC-chemokine receptor 4
(CXCR4 or X4) (Deng et al., 1996; Dragic et al., 1996). HIV-1 viral particles are
capable of switching from utilization of the CCR5 receptor to the CXCR4 receptor,
particularly during the advanced stages of diseases. For example, the CXCR4 receptor
occurs as either dual-tropic (R5/X4) or mono-tropic (X4) for viral variants as HIV-1
advances resulting in an expansion of infected cellular repertoires (Connor et al., 1997; Philpott, 2003). However, individuals who do not exhibit the switch for variants have been shown to attain the symptomatic stage of HIV-1/AIDS (van Rij et al., 2000; Regoes and Bonhoeffer, 2005), indicating that it is not only the switching to X4-using variants that causes the disease to advance to AIDS.

The binding of virus to CD4 results in a conformational change exposing the co-receptor binding site, and thereby, bringing the viral and cellular membranes together upon protrusion of the fusion peptide, and consequently, viral entry (Moore and Doms, 2003). Several studies show that changes in variable (V)-3 region of gp120 molecules is associated with alterations in co-receptor usage (Fouchier et al., 1992; Fenouillet et al., 1994; Ogert et al., 2001). The glycosylation of HIV-1 gp120 defines the correct folding and processing of the molecule which determines viral infectivity (Pollakis et al., 2001). Furthermore, studies show that the V2 loop of gp120 contains α4β7 binding site which is the gut homing receptor, co-localized with CCR5 and CD4 on CD4+ T cells, causing damage to the immune system (von Andrian and Mackay, 2000; Ritola et al., 2004; Arthos et al., 2008; Cicala et al., 2011; Nawaz et al., 2011).

The chemokine receptors, CCR5 and CXCR4, functioning as HIV-1 co-receptors are expressed in combination with CD4+ (primary CD4+ T cells) on macrophages and dendritic cells with macrophages playing a central role in HIV-1 entry via the CCR5 receptor located on the surface of macrophages. Other studies have shown that TNF-α, IFN-β, and IFN-γ inhibit R5 and R5/X4 HIV-1 variant entry into macrophages by down-
regulation of both cell surface CD4 and CCR5 molecules (Capobianchi et al., 1998; Lane et al., 1999; Stantchev and Broder, 2000). However, it is important to note that previous studies in malarial endemic regions indicate that RANTES production is suppressed during times in which TNF-α increase during the later stages of malarial disease (Ochiel et al., 2005; Were et al., 2009) suggesting that natural ligand for CCR5 is reduced.

Studies in a malaria endemic area carried out by Kfutwah et al. (2009) indicate that the median TNF-alpha/IL-10 mRNA ratio was significantly higher among HIV-1 positive compared to HIV-1 negative placentae, suggesting that co-infection with malaria may accelerate HIV-1 entry and pathogenesis through interactions with receptors influenced by TNF-α. Moreover, studies in Kenyan women using mother-infant pairs showed that elevated levels of MIP-1 beta (MIP-1β) and SDF-1α were associated with reduced mother-to-infant HIV-1 transmission risk, whereas elevated levels of RANTES in breast milk were associated with increased risk of transmission, independent of HIV-1 RNA levels in breast milk (Farquhar et al., 2005). However, the relative expression of CXCR4 and CCR5 receptors in paediatrics co-infected with malaria and HIV-1 from endemic regions are largely unexplored.

The surface expression of CXCR4, CCR5 and CD4, which are vital for the entry of primary R5X4 and X4 HIV-1 viral isolates into alveolar macrophage, are inhibited in vitro by IL-4 and IL-13 (Fenton et al., 1992; Bailer et al., 2000; Wang et al., 2001). However, it is imperative to note that HIV-1 viral entry into T cells is not affected by these molecules (Denis and Ghadirian, 1994), indicating that T-helper2 (TH2) cytokine,
such as IL-4 and IL-13, can only block HIV-1 replication at the transcriptional level in differentiated macrophages, but not in peripheral blood lymphocytes (Montaner et al., 1993; Mikovits et al., 1994; Naif et al., 1997). Macrophage activation by IL-13 and IL-4 typically causes down-regulation of TH1 cytokines, and thereby, limit the expansion of HIV-1 viral reservoirs within macrophages that are created by TH1 cytokine recruitment of CD4 cells. During the later stages of disease, the production of IL-10 targets IL-4 and IL-13 plasma membrane receptors (Zurawski et al., 1993) leading to deactivation of macrophages (Moore et al., 2001; Gregory and Devitt, 2004; Alfano et al., 2008). This partially explains the immune failure observed during the later stages of HIV-1.

One of the viral isolates (R5 variants) use the CCR5 receptor for entry into CD4 cells (Irlbeck et al., 2008) but during later stages of HIV-1 infection it promotes the emergence of the CXCR4 (X4) phenotype (Karlsson et al., 1994; Bjorndal et al., 1997). Additional studies show that the progression to immunodeficiency may also occur in individuals having no detectable CXCR4 viral isolates with the R5 viral phenotype remaining throughout the entire course of disease (de Roda Husman et al., 1999), suggesting that its natural ligand, RANTES, can mediate inhibition. However, some AIDS patients with the R5 viral phenotype display R5 viruses that are resistant to inhibition by RANTES (Koning et al., 2003) and thus compete favourably for the binding sites.
2.8 Effect of haemozoin on the expression of CCR5, CXCR4 and chemokine production

Studies have shown that malarial pigment (haemozoin, Hz) is the key molecule that mediates the malaria-associated immunosupression responsible for the dysregulation in the inflammatory mediators (Jaramillo et al., 2004; Ochiel et al., 2005; Ong’echa et al., 2008; Were et al., 2009). Naturally-acquired Hz and synthetic pigment (β-hematin) both have been shown to have similar effects on pro- and anti-inflammatory cytokines, effector molecules and chemokines such as IL-1β, TNF-α, IL-10, NO, PGE2, and MIP-1α and MIP-1β from peripheral blood mononuclear cells (PBMCs) in children with Plasmodium falciparum malaria. For example, MIP-1α and MIP-1β increase with the severity of the disease, while RANTES decreases as disease severity increases (Ochiel et al., 2005).

Moreover, an increase in NO plasma levels, primarily from PBMCs, during and after acute malaria, have been shown to synergize with TNF-α (Oswald et al., 1994; Kremsner et al., 1996; Weinberg, 1998; Perkins et al., 1999), a cytokine which is responsible for inhibition of SDF-1α (Salvucci et al., 2004). This implies that the levels of the natural ligand for CXCR4 receptors will decrease, thus creating the potential for virus particles to enhance binding opportunities and replicate within cells expressing CXCR4. This premise supports studies which showed that both NO and TNF-α plasma levels were higher in HIV-1 infected children (Morikawa et al., 2000; Gonzalez-Nicolas et al., 2001). Additional study using cultured PBMCs revealed similar results when Hz was added to the cultures (Ochiel et al., 2005). Studies in pregnant women with P. falciparum malaria,
a disease state characterized by suppressed immunity, show that MIP-1α, monocyte chemoattractant protein (MCP)-1 and IL-8 transcripts increase with elevated monocyte concentration (Abrams et al., 2003). Moreover, studies using a murine model of malaria show that inoculation of Hz into the animals induces high levels of chemokine and chemokine (CC) receptor expression which are positively correlated with cellular infiltration (Jaramillo et al., 2004).

Although *Plasmodium falciparum*-derived Hz (*PfHz*) promotes dysregulation of CCR5 and CXCR4 and their cognate ligands in monocytes and CD4+ T cells resulting in increased HIV-1 replication, the mechanism(s) by which dysregulation in the expression of these receptors in children co-infected with malaria and HIV-1 is largely undefined. As such, the current study explored these molecular interactions to determine the relative expression of CCR5 and CXCR4 on CD14+ monocytes and CD4+ T cells in order to more fully characterize the relationship between malaria and HIV-1 co-infections in paediatrics.
CHAPTER 3: MATERIALS AND METHODS

3.1 Study site

The study was conducted at the Siaya District Hospital (SDH), a rural health facility located in a malaria holoendemic region of Western Kenya that provides referral health care for the residents of Siaya County (Appendix I). Siaya District covers an area of 1,520 Km² and has an altitude of 1,140 - 1,430 m above the sea level with an equatorial climate and has a population of about 550,224 people where children below 5 years of age comprise 15% (www.knbs.or.ke/census % 20 Results/2009). The region receives an average annual rainfall of 800 - 2,000 mm. The average annual temperature ranges from 15° - 30°C (Republic of Kenya, 2001). The major public health problems of the area include: malaria, HIV-1, bacteraemia, malnutrition due to poverty, maternal and infant mortality, and respiratory tract infections (Bloss et al., 2004; Brent et al., 2006; Ouma et al., 2010). The most intense malaria transmission occurs during the seasonal rainfalls in April to August and November to January (Beier et al., 1994). At SDH, it is estimated that malaria is responsible for 83% of the admissions, 85% of the severe anaemia cases, 66% of the in-hospital mortality and 53% of the malaria-related deaths (Obonyo et al., 2007; Okiro et al., 2010). HIV-1/AIDS prevalence in Siaya is estimated at 27.7% of the total country population (Perrault et al., 2009). The residents of Western Kenya receive up-to 300 infective bites annually (Beier et al., 1990; Githeko et al., 1993).

3.2 Study design

The study was a hospital-based, cross-sectional study at SDH using convenient sampling. Whole blood were obtained from children aged between 2.6 months to 48.0 months for
performing the following: staining and immunophenotyping, determining parasitemia, complete blood count, PCM and PCN counts, making dry blood spot (DBS) for HIV-1 PCR and for separating plasma for HIV rapid diagnostic test. Five groups of *P. falciparum* positive and HIV-1 negative {mal[+]\-HIV-1[-]}; *P. falciparum* positive and HIV-1 exposed {mal[+]\-HIV-1[exp]}; *P. falciparum* positive and HIV-1 positive {mal[+]\-HIV-1[+]}; and *P. falciparum* negative and HIV-1 positive {mal[-]\-HIV-1[+]} and healthy controls, *P. falciparum* negative and HIV-1 negative {mal[-]\-HIV-1[-]} were classified.

### 3.3 Inclusion and exclusion criteria

#### 3.3.1 Inclusion criteria

All blood samples were obtained before administration of anti-malarial or other treatment measures. Children (age, < 5 years; n=72) from western Kenya were stratified into the following groups: *P. falciparum* negative and HIV-1 negative {mal[-]\-HIV-1[-], n=13} as healthy controls; *P. falciparum* positive and HIV-1 negative {mal[+]\-HIV-1[-], n=30}; *P. falciparum* positive and HIV-1 exposed {mal[+]\-HIV-1[exp], n=17}; *P. falciparum* negative and HIV-1 positive {mal[-]\-HIV-1[+], n=5}; and *P. falciparum* positive and HIV-1 positive {mal[+]\-HIV-1[+], n=7}. The healthy control group were children visiting the hospital for routine vaccination and were included in the study (HC; Hb ≥11.0 g/dL). Children of both sexes were eligible for participation in the study having met the following criteria: Parents/guardian able and willing to sign the informed consent form before enrolling the child, parents/guardian coming from a distance ≤ 25 Km to the SDH, Children participating aged < 5 years. Children presenting with malaria, HIV-1 and
intestinal worms were treated according to the Ministry of Health (MOH), Kenya guidelines which include Coartem® (artemether and lumefantrine) for uncomplicated malaria and intravenous quinine for severe malaria. Pre- and post- test HIV and AIDS counselling was performed and treatment with antiretroviral drugs for HIV-1 positive children was started.

3.3.2 Exclusion criteria

A child was excluded from the study if he or she was above 5 years of age, the parent/guardian declines to sign the consent form, parents/guardian coming from a distance extending above 25 Km to the SDH, the parent/guardian had planned to move/relocate to another town outside the study area, and if the patient had enrolled in another study.

3.4 HIV-1 screening

HIV-1 diagnosis was performed by serological test and positive serological results were confirmed by proviral DNA polymerase chain reaction (PCR) results. Serology was determined by both Determine® (Abbott Laboratories, Abbott Park, IL, USA) and Uni-Gold™ (Trinity Biotech, Carlsbad, CA, USA) assays. Briefly, 2-3 drops (20 μL) of venous blood were used to make each of the four blood spots within the free to air (FTA) card and allowed to air dry for at least four hours. DNA was extracted from the dried blood spots (DBS) using Gentra System DNA extraction protocol for FTA cards where 2 punches of 3 mm diameter disk each were made into a clean and sterile 2 mL eppendorf tube. Purification was performed three times using 150 μL Generation DNA purification
solution (solution 1) incubating at room temperature followed by washing using 150 μL
elusion solution (solution 2) incubating at room temperature. Extraction was performed
using about 100 μL elusion solution incubating at 99°C over a dry block heater to release
the DNA. Each process (step) of incubation took 15 minutes. After heating, cooling was
performed to room temperature, the solutions were pipetted up and down 15 times then
eluted DNA was transferred to a clean labelled eppendorf tube.

HIV-1 exposed [HIV-1(exp) ] was defined by at least one (or two) positive serological
tests with either Determine® and Uni-Gold™ and negative HIV-1 DNA PCR; and HIV-1
positive [HIV-1(+)] was defined as those with at least one (of two) positive serological
result with the Determine® and Uni-Gold™ tests, and positive HIV-1 DNA PCR results
on two consecutive HIV-1 DNA PCR assays three months apart according to the
previously published methods (Otieno et al., 2006). Briefly, a house-keeping gene for
the PCR testing was performed using hG3PDH forward (5' -TGA AGG TCG GAG TCA
ACG GAT TTG GT-3') and reverse (5' -CAT GTG GGC CAT GAG GTC CAC CAC-3')
primers with the initial denaturation at 94°C for 2 min, denaturation at 94°C for 45 sec,
annealing at 62°C for 45 sec, extension at 72°C for 2 min for 30 cycles, a final extension
at 72°C for 7 min and finally stored at 4°C for until required.

Determination of the PCR results was performed by HIV-1 gp40 (5' -TCT TAG GAG
CAG CAG GAA GCA CTA TC-3'), gp41 (5' -AAC GAC AAA GGT GAG TAT CCC
TGC CT-3'), gp46 (5' -ACA ATT ATT GTC TGG TAT AGT GCA ACA-3') and gp47
(5' -TTA AAC CTA TCA AGC CTC CTA CTA TCA-3') primers since HIV-1 gp41
transmembrane protein is a conserved molecule that anchors the gp120 into the membrane of the virion with a cycling condition of initial denaturation at 95°C for 5 min, denaturation at 95°C for 45 sec, annealing at 55°C for 45 sec, extension at 72°C for 1 min for 35 cycles, a final extension at 72°C for 10 min and finally stored at 4°C as it awaits loading on an ethidium bromide-stained 2% agarose gel for electrophoresis and viewed under UV high performance trans-illuminator (UVP, Upland, USA) at 302nm. HIV-1 PCR positive was defined by the presence of the 460bp fragment.

Pre- and post-tests HIV counselling were provided for the parents/guardians of all participants. Children positive for one or both HIV-1 serological tests were prophylactically treated with trimethoprim–sulfamethoxazole from the time of enrolment onward until three months after PCR confirmation in which PCR HIV-1 positive were introduced to ART.

3.5 Sample size determination

Sample size was determined by the power and sample size calculation program based on the methods of (Dupont and Plummer, 1990) and studies of clinical and flow-cytometry measures in children (Asito et al., 2008) previously performed with mean CD8 populations at 18.2 and 16.0, respectively, for healthy control (HC) and acute malarial groups, standard error (S.E) of 0.8 and standard deviation (SD) of 3.2 for healthy control. Thus, using a power of 80% and α=0.05, the sample size estimation for test between two independent sample means was calculated as n=20 for each group and the study participants were estimated at 100 individuals using the formula below:
\[ N = \frac{4S^2 (Z_{cv} + Z_{power})^2}{D^2} \]

Where:

- \( N \) = the sample size estimate
- \( Z_{cv} \) = \( Z \) critical value for alpha (0.05\( \alpha \) has a \( Z_{cv} \) of 1.96)
- \( Z_{power} \) = \( Z \) value of 1-beta (80% power has a \( Z \) of 0.842)
- \( S \) = standard deviation
- \( D \) = the expected difference between the two means.

### 3.6 Laboratory procedures

Approximately, 3 mL of venous blood was obtained from children upon enrolment. Thick and thin peripheral blood smears were prepared, then allowed to air dry. The dry thin smears were fixed in methanol, then stained using 10% Giemsa reagent for 5 minutes and examined for malaria parasite identification and quantification using the \( \times 1000 \) (oil immersion) magnification. The number of malaria parasites were counted against 300 leucocytes and the parasite density for each individual was calculated/µL using the total leukocyte counts (Slutsker et al., 1994). Pigment containing monocytes (PCM) and pigment containing neutrophils (PCN) identified in the thin Giemsa-stained blood smears using microscopy technique against 30 monocytes and 100 neutrophils were recorded and the number of PCM and PCN was expressed as a percentage of the total number of cells examined. The total PCM/µL and PCN/µL were calculated using the formulae:

\[ \text{PCM/µL} = \left( \frac{\text{number of pigmented monocytes}}{30} \right) \times \left( \frac{\text{absolute WBC count}}{\% \text{ of}} \right) \]
Expression of surface antigens on PBMC was determined by a four-color FACSCalibur™ machine (Becton, Dickinson and Company, CA, USA) within 24 hours of staining with the antibody panels shown in Appendix III. All the monoclonal antibodies were purchased from BD Biosciences–Pharmingen, (San Diego, CA). Briefly, 8 snap-cap tubes were labelled appropriately according to the panel in Appendix III. Four hundred and fifty (450) μL venous blood was diluted with an equal volume of RPMI-1640 media and mixed thoroughly, aliquots of 100 μL were added in the snap-cap tubes and staining performed where cells were incubated with 5 μL fluorochromed surface antibodies for 30 min at 4°C in the dark, lysed using 2.0 mL lysis buffer (1×; 1:10 dilution of 10× BD lysing buffer) for each tube incubated for 10 minutes at room temperature in the dark, washed twice using 2.0 mL wash buffer (5 g of Bovine serum albumin (BSA) +0.5 g sodium azide (NaN₃) in 500 mL) for each tube, and then fixed in 0.5 mL cold fix buffer (1% paraformaldehyde) (2.5 mL of 40% paraformaldehyde in 100 mL of PBS) solution and analyzed on a FACSCalibur™ (Becton, Dickinson and Company, CA, USA). Acquired data were analyzed with FlowJo software to determine expression of CD4, CCR5 and CXCR4 on CD14+, CD4+ and CD3+ cell subsets. Data
were presented as percent expression of CD4, CCR5 or CXCR4 on CD14+ monocytes or CD3+ lymphocyte populations, and plotted as relative expression (mean ± s.e.m).

3.8 Data analysis

Data were routinely entered into an Excel database. FlowJo was used to determine the levels of expression of cellular markers by the different cell populations. Data were analysed using SPSS (version, 19.0, SPSS Inc.). Pearson’s chi-square tests were used for comparing differences in proportions across groups. Kruskal-Wallis test was used to compare across group differences in medians for continuous variables in: P. falciparum positive and HIV-1 negative {mal[+]-HIV-1[-]}; P. falciparum positive and HIV-1 exposed {mal[+]-HIV-1[exp]}; P. falciparum positive and HIV-1 positive {mal[+]-HIV-1[+]}; and P. falciparum negative and HIV-1 positive {mal[-]-HIV-1[+]}. Significance difference with Kruskal-Wallis test ($P \leq 0.05$) was followed by post hoc Dunn’s Multiple Comparison Test. Pair-wise comparisons were performed using Mann-Whitney U test. Associations were determined using Spearman rank correlation test.

3.9 Ethical consideration

Participation in this study was voluntary and a written informed consent was obtained from the parent or guardian before enrolment of their children into the study. Ethical and scientific approval of the study was obtained from the Centre Scientific Committee, KEMRI/National Ethical Review Committee and the Institutional Review Board of the
University of New Mexico (UNM) and Graduate School of Kenyatta University (Appendix IV).
CHAPTER 4: RESULTS

4.1 Demographic, laboratory and clinical characteristics of the study participants

The relative expression of CXCR4 and CCR5 receptors on CD4+ T cells and CD14+ monocytes was examined in children presenting with malaria and/or HIV-1-exposure or HIV-1 co-infection. The study participants were grouped as follows in table 4.1: mal[+]-HIV-1[+], n=7; mal[+]-HIV-1[-], n=30; mal[+]-HIV-1[exp], n=17; mal[-]-HIV-[+], n=5; and mal[-]-HIV-1[-], n=13 as healthy controls. There were 45 female and 27 male study participants. While gender distribution did not differ significantly across the groups (P=0.410), age was significantly different across the groups (P=0.016) with the mal[-]-HIV-[+] group having older children than the other groups; mal[-]-HIV-1[+] vs mal[+]-HIV-1[exp], (P=0.006) mal[-]-HIV-1[+] vs mal[+]-HIV-1[-], (P=0.003), and mal[-]-HIV-1[+] vs mal[-]-HIV-1[-], (P=0.009). Kruskal-Wallis test across the groups did not conclude significant difference for the axillary temperature (P=0.306), blood glucose levels (P=0.278), median parasitaemia (P=0.077) and geometric mean parasitaemia (P=0.895)(Table 4.1). However, the proportion of children with high density parasitaemia (HDP; ≥10,000 parasites/μL) differed significantly across the groups (P=0.033) and was higher in the mal[+]-HIV-1[+] (71.4%) and mal[+]-HIV-1[-] (70.0%) children relative to the mal[+]-HIV-1[exp] (35.3%) group.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mal[-]-HIV-1[-], n=13</th>
<th>Mal[+] -HIV-1[-], n=30</th>
<th>Mal[+] -HIV-1[exp], n=17</th>
<th>Mal[-]-HIV-1[+], n=5</th>
<th>Mal[+] -HIV-1[+], n=7</th>
<th>P-value</th>
</tr>
</thead>
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<tr>
<td>Female, n (%)</td>
<td>10 (76.9)</td>
<td>20 (67.6)</td>
<td>10 (60.0)</td>
<td>1 (25.0)</td>
<td>4 (60.0)</td>
<td>0.410^a</td>
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<tr>
<td>Male, n (%)</td>
<td>3 (23.1)</td>
<td>10 (32.4)</td>
<td>7 (40.0)</td>
<td>4 (75.0)</td>
<td>3 (40.0)</td>
<td></td>
</tr>
<tr>
<td>Age, mos.</td>
<td>7.6 (9.0)</td>
<td>7.6 (7.8)</td>
<td>6.9 (7.3)^*</td>
<td>35.5 (22.8)</td>
<td>13.0 (36.2)</td>
<td>0.016^b</td>
</tr>
<tr>
<td>Axillary temp., °C</td>
<td>36.6 (1.0)</td>
<td>37.5 (1.9)</td>
<td>37.5 (1.0)</td>
<td>36.9 (0.8)</td>
<td>38.0 (0)</td>
<td>0.306^b</td>
</tr>
<tr>
<td>Glucose (mmol)</td>
<td>5.0 (1.0)</td>
<td>5.3 (1.3)</td>
<td>5.5 (2.0)</td>
<td>-</td>
<td>4.8 (1.1)</td>
<td>0.278^b</td>
</tr>
<tr>
<td>Parasitaemia (μL)</td>
<td>-</td>
<td>16,607 (61,886)</td>
<td>3,578 (41,659)</td>
<td>-</td>
<td>30,438 (66,920)</td>
<td>0.077^b</td>
</tr>
<tr>
<td>Geomean parasitaemia (μL)</td>
<td>-</td>
<td>40,904</td>
<td>24,716</td>
<td>-</td>
<td>41,263</td>
<td>0.895^c</td>
</tr>
<tr>
<td>HDP, n (%)</td>
<td>-</td>
<td>21 (70.0)</td>
<td>6 (35.3)</td>
<td>-</td>
<td>5 (71.4)</td>
<td>0.033^a</td>
</tr>
</tbody>
</table>

Data presented are medians (interquartile range) unless otherwise stated. The study participants were stratified into five categories: mal[+] -HIV-1[+], n=7; mal[-]-HIV-[+], n=5; mal[+] -HIV-1[exp], n=17; mal[-]-HIV-1[-], n=30; and mal[-]-HIV-1[-]; HC, n=13. ^a Pearson’s chi-square test. ^b Kruskal-Wallis (non-parametric ANOVA) test. ^c Parametric ANOVA test. Post-hoc analysis was performed by Dunn’s Multiple Comparison Test. Paired comparisons were examined using Mann-Whitney U test. *P<0.050 (mal[-]-HIV-1[+] vs mal[+] -HIV-1[exp]). Statistical significance are in bold.
4.2 Leukocytic indices of the study participants

To determine levels of the circulating leukocytes during malaria and HIV-1 co-infection or exposure, the leukocyte counts were compared across the five study groups (Table 4.2). The total leukocyte \( (P=0.093) \) and lymphocyte \( (P=0.260) \) counts were not significantly different across the groups. However, the monocyte counts differed significantly across the groups \( (P=0.005) \) such that the mal[+]\text{-HIV-1[-]}, mal[+]\text{-HIV-1[exp]}, and mal[+]\text{-HIV-1[+] groups presented with significantly higher monocyte counts relative to the healthy controls \( (P=0.044; P=0.031; \text{and } P=0.048, \text{respectively}) \). Moreover, the granulocyte counts did not differ significantly across the groups \( (P=0.065) \). Naturally-acquired intra-monocytic pigment (for example pigment containing - monocytes, PCM) and pigment-containing neutrophils (PCN) were only detected in children presenting with mal[+]\text{-HIV-1[exp]}, and mal[+]\text{-HIV-1[+] groups.}

Flow cytometric analyses performed on fresh whole blood samples indicated that the proportions of CD3+ cells did not differ significantly across the groups \( (P=0.087) \), even though children presenting with mal[-]\text{-HIV-1[+] had lower proportions of the CD3+ cells relative to healthy controls \( (P=0.070; \text{Table 4.2}) \). In addition, the absolute CD3+CD4+ T cell counts were comparable across the groups \( (P=0.055) \). No significant differences were observed in the proportions of CD3+CD8+ cells \( (P=0.319) \), absolute CD3+CD8+ counts \( (P=0.624) \), and the CD4:CD8 ratios \( (P=0.480) \) across the study groups. In contrast to the patterns observed for the CD3 cells, the proportions of CD3-CD19+ differed significantly across the groups \( (P=0.031) \) such that mal[+]\text{-HIV-1[exp] presented with lower counts compared to mal[+]\text{-HIV-1[-]} and mal[-]\text{-HIV-1[-]} (healthy
control) groups ($P=0.004$; and $P=0.003$, respectively). Likewise, the proportions of CD14+ cells were significantly different across the groups ($P<0.0001$) with the mal[+]HIV-1[+] group having lower levels compared to the mal[+]HIV-1[exp] group ($P=0.004$), mal[+]HIV-1[-] groups ($P=0.001$) and mal[-]HIV-1[-] groups ($P=0.001$). The mal[-]HIV-1[+] group also had lower proportions of CD14+ cells than the healthy controls ($P=0.054$) (Table 4.2).
Table 4.2 Leukocytic indices of the study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mal[-]-HIV-1[-], n=13</th>
<th>Mal[+]-HIV-1[-], n=30</th>
<th>Mal[+]-HIV-1[exp], n=17</th>
<th>Mal[-]-HIV-1[+, n=5</th>
<th>Mal[+]-HIV-1[+, n=7</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WBC indices</strong></td>
<td></td>
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</tr>
<tr>
<td>Leukocytes (×10³/µL)</td>
<td>10.5 (6.5)</td>
<td>12.0 (7.5)</td>
<td>13.9 (4.5)</td>
<td>7.7 (5.8)</td>
<td>16.9 (2.4)</td>
<td>0.093^a</td>
</tr>
<tr>
<td>Lymphocytes (×10³/µL)</td>
<td>6.4 (4.4)</td>
<td>5.9 (5.6)</td>
<td>6.7 (5.5)</td>
<td>5.5 (3.0)</td>
<td>11.7 (3.0)</td>
<td>0.260^a</td>
</tr>
<tr>
<td>Monocytes (×10³/µL)</td>
<td>0.8 (0.6)</td>
<td>1.1 (0.7)</td>
<td>1.5 (1.6)*</td>
<td>0.4 (0.8)</td>
<td>1.6 (0.4)</td>
<td><strong>0.005^a</strong></td>
</tr>
<tr>
<td>Granulocytes (×10³/µL)</td>
<td>3.0 (1.1)</td>
<td>4.1 (4.1)</td>
<td>4.6 (2.5)</td>
<td>2.9 (3.5)</td>
<td>4.1 (1.2)</td>
<td>0.065^a</td>
</tr>
<tr>
<td><strong>CD3 and CD14 cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+ (%)</td>
<td>61.5 (18.3)</td>
<td>50.4 (14.2)</td>
<td>51.4 (29.5)</td>
<td>27.4 (46.4)</td>
<td>60.3 (40.7)</td>
<td>0.087^b</td>
</tr>
<tr>
<td>CD3+ CD4+ (%)</td>
<td>39.1 (11.9)</td>
<td>30.5 (7.8)</td>
<td>41.0 (36.1)</td>
<td>59.2 (47.8)</td>
<td>38.7 (23.5)</td>
<td>0.059^b</td>
</tr>
<tr>
<td>CD3+ CD4+ (µL)</td>
<td>1,655 (2,291)</td>
<td>1,653 (1,533)</td>
<td>2,732 (3,125)</td>
<td>380 (2,747)</td>
<td>2,795 (3,471)</td>
<td>0.055^a</td>
</tr>
<tr>
<td>CD3+ CD8+ (%)</td>
<td>22.9 (10.7)</td>
<td>18.8 (8.4)</td>
<td>19.3 (14.8)</td>
<td>28.4 (20.0)</td>
<td>35.6 (23.8)</td>
<td>0.319^b</td>
</tr>
<tr>
<td>CD3+ CD8+ (µL)</td>
<td>1067 (1020)</td>
<td>961 (1053)</td>
<td>1185 (2577)</td>
<td>769 (2036)</td>
<td>1865 (3861)</td>
<td>0.624^a</td>
</tr>
<tr>
<td>CD4:CD8</td>
<td>1.9 (1.3)</td>
<td>1.6 (0.7)</td>
<td>2.0 (2.5)</td>
<td>1.7 (2.7)</td>
<td>1.1 (1.7)</td>
<td>0.480^a</td>
</tr>
<tr>
<td>CD3- CD19+ (%)</td>
<td>27.2 (9.2)</td>
<td>32.6 (20.2)</td>
<td>18.8 (11.6)</td>
<td>22.3 (63.7)</td>
<td>22.4 (20.0)</td>
<td><strong>0.031^b</strong></td>
</tr>
<tr>
<td>CD14+ (%)</td>
<td>99.1 (1.5)</td>
<td>97.2 (6.4)</td>
<td>95.7 (28.0)</td>
<td>41.9 (68.6)</td>
<td>12.8 (49.1)</td>
<td>&lt;0.0001^b</td>
</tr>
</tbody>
</table>

Data presented are medians (interquartile range) unless otherwise stated. ^a Kruskal-Wallis test. ^b Pearson’s chi-square test. Post hoc analysis was performed using Dunn’s Multiple Comparison Test. Paired comparisons was conducted using the Mann-Whitney U test. Pearson’s chi-square test significance was not determined for PCN (%) and PCM (%) since n<5. Values in bold denote significant P-values. White blood cells (leucocytes, WBC), pigment containing monocyte (PCM), pigment containing neutrophils (PCN). *P<0.050 (mal[+]-HIV-1[exp] vs mal[-]-HIV-1[+]).
4.3 Erythrocytic and thrombocytic indices of study participants

To determine the influence of malaria, HIV-1 exposure and HIV-1 co-infection on the haematological parameters, erythrocytic and thrombocytic indices were compared across the study groups (Table 4.3). The mean corpuscular haemoglobin (MCH) ($P=0.087$), mean corpuscular haemoglobin concentration (MCHC) ($P=0.081$), mean corpuscular volume (MCV) ($P=0.165$), reticulocyte production index (RPI) ($P=0.244$), absolute reticulocyte number (ARN) ($P=0.217$) and reticulocyte production index (RPI)<2.0 ($P=0.370$) were comparable across the groups. However, severe malarial anaemia (%) (SMA, Hb <6.0 g/dL) ($P=0.008$), Hb levels ($P<0.0001$), hematocrit (Hct) ($P=0.008$), proportions of red cell distribution width (RDW) ($P<0.0001$) and reticulocyte counts (%) ($P=0.021$) differed significantly across the groups with children presenting with mal[+]HIV-1[-],mal[+]HIV-1[exp], and mal[+]HIV-1[+] having the lowest levels of these erythrocytic measures compared to healthy controls ($P<0.01$ for all comparisons).

Across group comparisons in the thrombocytic indices also revealed significant differences in the platelet counts ($P=0.001$), and the degree of thrombocytopenia ($P=0.009$). The platelet counts in the mal[+]HIV-1[-] ($P<0.0001$), mal[+]HIV-1[exp] ($P<0.0001$) and mal[+]HIV-1[+] ($P=0.034$) groups were significantly lower relative to that in the healthy controls. Although the healthy control group had no thrombocytopenia, the proportion of individuals presenting with thrombocytopenia ranged from 40% to 80% in the malaria-positive and/or HIV-1[+] groups.
### Table 4.3 Erythrocytic and thrombocytic indices of study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mal[-]-HIV-1[-], n=13</th>
<th>Mal[+]-HIV-1[-], n=30</th>
<th>Mal[+]-HIV-1[exp], n=17</th>
<th>Mal[-]-HIV-1[+], n=5</th>
<th>Mal[+]-HIV-1[+], n=7</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Erythrocyte Indices</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC (×10^{12}/μL)</td>
<td>5.2 (1.1)</td>
<td>2.9 (1.9)</td>
<td>2.5 (1.5)</td>
<td>4.3 (2.1)</td>
<td>3.7 (1.1)</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Haemoglobin, g/dL</td>
<td>11.3 (1.0)</td>
<td>6.0 (3.3)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>6.1 (3.2)&lt;sup&gt;***&lt;/sup&gt;</td>
<td>11.1 (3.5)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>7.5 (2.9)</td>
<td>&lt;0.0001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>23.0 (5.7)</td>
<td>23.3 (5.4)</td>
<td>23.6 (3.6)</td>
<td>28.1 (7.5)</td>
<td>16.7 (3.5)</td>
<td>0.087&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.7 (1.4)</td>
<td>32.4 (2.8)</td>
<td>32.7 (1.6)</td>
<td>32.2 (1.3)</td>
<td>30.6 (2.1)</td>
<td>0.081&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>69.6 (16.2)</td>
<td>71.8 (14.6)</td>
<td>73.9 (12.1)</td>
<td>87.5 (24.0)</td>
<td>60.2 (13.3)</td>
<td>0.165&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>15.5 (3.8)</td>
<td>22.2 (7.5)</td>
<td>20.9 (5.9)</td>
<td>16.6 (4.2)</td>
<td>21.7 (7.4)</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SMA, n (%)</td>
<td>0 (0.0)</td>
<td>16 (53.3)</td>
<td>8 (47.1)</td>
<td>0 (0.0)</td>
<td>1 (14.3)</td>
<td>0.008&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Reticulocyte indices</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retic count (%)</td>
<td>1.5 (1.2)</td>
<td>3.7 (6.9)</td>
<td>5.0 (7.3)</td>
<td>3.2 (1.6)</td>
<td>15.0 (29.1)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.021&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RPI</td>
<td>5.6 (9.4)</td>
<td>2.3 (1.2)</td>
<td>1.1 (3.1)</td>
<td>1.2 (0.9)</td>
<td>1.2 (1.6)</td>
<td>0.244&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ARN</td>
<td>77.1 (74.3)</td>
<td>65.8 (43.1)</td>
<td>51.3 (189.6)</td>
<td>157.8 (54.0)</td>
<td>505.9 (567.2)</td>
<td>0.217&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>RPI&lt;2.0, n (%)</td>
<td>11 (84.6)</td>
<td>25 (81.8)</td>
<td>12 (68.4)</td>
<td>3 (50.0)</td>
<td>1 (20.0)</td>
<td>0.370&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Platelet indices</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets (×10^9/μL)</td>
<td>313 (95)</td>
<td>166 (110)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>140 (92)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>213 (465)</td>
<td>123 (213)</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thrombocytopenia, n (%)</td>
<td>0 (0.0)</td>
<td>13 (44.1)</td>
<td>9 (52.6)</td>
<td>2 (40.0)</td>
<td>6 (80.0)</td>
<td>0.009&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data presented are medians (interquartile range) unless otherwise stated. <sup>a</sup>Kruskal-Wallis test. <sup>b</sup>Pearson's chi-square test. Post-hoc analysis was performed by Dunn's Multiple Comparison Test. Paired comparisons were conducted using the Mann-Whitney U test. Values in bold indicate significant P-values. Red blood cells (RBC), hematocrit (Hct), red cell distribution width (RDW), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), severe malarial anaemia (SMA, Hb<6.0 g/dL), reticulocyte production index (RPI), absolute reticulocyte number (ARN).

Platelets <150×10^9/μL, **P<0.010 (mal[+]-HIV-1[exp] vs mal[-]-HIV-1[-], HC, **P<0.010 (mal[+]-HIV-1[-] vs HC. While haemoglobin, g/dL, ***P<0.001 (mal[+]-HIV-1[-] vs HC, ***P<0.001 (mal[+]-HIV-1[exp] vs HC and *P<0.05 (mal[+]-HIV-1[exp] vs mal[-]-HIV-1[+]). Retic count *P<0.001 (mal[+]-HIV-1[-] vs HC.
4.4 Gating and analyses of CD4+ and CD14+ subsets

To enumerate the proportions of CD3+CD4+ and CD14+ cells expressing the CXCR4 and CCR5 receptors, flow cytometric analysis was performed on whole blood samples from children presenting with *P. falciparum* malaria-HIV-1 co-infection and -exposure. Figures 4.1 and 4.2 show representative gating dot graphs for CD3+CD4+ and CD14+ cell analyses.
Figure 4.1 Gating of CD4+CXCR4+ and CD4+CCR5+ and cell analyses. The CD4 lymphocytes subsets FACS plots are a representative of percentage (%) of the two receptors in the HIV-1 and malaria co-infection group. (A) Forward and side scatter plots of T lymphocyte gating from leukocytic cell populations. (B) Plots of CD3+CD4+ expressing lymphocytes gated as R1. (C) Plots of CD3+CD4+ and CXCR4 expression from the R1 gate. (D) Plots of CD3+CD4+ and CCR5 expression from the R1 gate.
Figure 4.2 Gating of CD14+CXCR4+ and CD14+CCR5+ and cell analyses. The CD14 monocytes subsets FACS plots are a representative of proportions (%) of the two receptors in *P. falciparum* malaria and HIV-1 co-infection. (A) Forward and side scatter plots of the leukocytic total cell population gating for monocytes at gate R2. (B) Plots of monocyte cells from the R2 gate highlighting CD14+ gated as R3. (C) Plots of CD14+ monocytes and CCR5 expression from R3 gate. (D) Plots of CD14+ monocytes and CXCR4 expression from R3 gate.
4.4.1 CXCR4 and CCR5 expression by CD3+ cell subsets

The expression of the CCR5 and CXCR4 receptors by the CD3+ cell population was examined in different study groups (Figure 4.3A-D). The proportions of the CD3+CXCR4+ population differed significantly across the groups ($P=0.049$), and were significantly lower in the malaria and HIV-1-co-infected individuals relative to the mal[+]-HIV-1[exp] group ($P=0.009$; Figure 4.3A). Children in mal[+]-HIV-1[+] group presented with lower proportions of the CD3+CD4+CXCR4+ relative to mal[+]-HIV-1[exp] individuals ($P=0.047$; Figure 4.3C). Whereas proportions of CD4+CCR5+ did not differ significantly across the groups ($P=0.099$; Figure 4.3B), participants in mal[+]-HIV-1[+] group presented with lower proportions of the CD3+CD4+CCR5+ relative to the mal[+]-HIV-1[exp] individuals ($P=0.009$; Figure 4.3D).
Figure 4.3 CXCR4 and CCR5 expression by CD3+ cell populations. Data are represented as box-plots where the boxes represent interquartile range, the line through boxes represents the median and whiskers show the 10th and 90th percentiles. Across-group analysis was performed using the Kruskal Wallis test. Pair-wise comparisons were performed by Mann-Whitney U test. A) CD3+CXCR4+; B) CD3+CCR5+; C) CD3+CD4+CXCR4+; and D) CD3+CD4+CCR5+ cell populations. Due to low blood samples the CD3+CXCR4+ population was not enumerated in the mal[+]HIV-1[+/-] group. CD3+CXCR4+ in mal[+]HIV-1[+/-] (P=0.148, P=- and P=0.655), mal[+]HIV-1[exp] (P=0.770, P=- and P=0.294) relative to HC, mal[+]HIV-1[-] and mal[-]HIV-1[+], respectively. CD3+CCR5+ in mal[+]HIV-1[+/-] (P=0.373, P=0.325 and P=0.177), mal[+]HIV-1[exp] (P=0.143, P=0.221 and P=0.456) relative to HC, mal[+]HIV-1[+] and mal[-]HIV-1[+], respectively. CD3+CD4+CXCR4+ in mal[+]HIV-1[+/-] (P=0.104, P=0.108 and P=0.219), mal[+]HIV-1[exp] (P=0.380, P=0.278 and P=0.571) relative to HC, mal[+]HIV-1[-] and mal[-]HIV-1[+], respectively. CD3+CD4+CCR5+ in mal[+]HIV-1[+/-] (P=0.766, P=0.137 and P=0.445), mal[+]HIV-1[exp] (P=0.143, P=0.143 and P=0.456) relative to HC, mal[+]HIV-1[-] and mal[-]HIV-1[+], respectively.
4.4.2 CXCR4 and CCR5 expression by CD14+ cell population

The proportions of CD14+CXCR4+ monocytes were comparable across the groups ($P=0.563$; Figure 4.4A). Moreover across group difference for the proportions of CD14+CCR5+ monocytes only neared significance ($P=0.065$; Figure 4.4B). Comparing mal[+]/-HIV-1[+] group to mal[-]/-HIV-1[+] group showed a non-significant increase of CCR5+CD14+ ($P=0.088$; Figure 4.4B). However, mal[-]/-HIV-1[+] individuals showed a significant decrease of CCR5+CD14+ relative to the mal[+]/-HIV-1[exp] group ($P=0.023$; Figure 4.4B). In addition, the proportion of CCR5+CD14+ monocytes were higher in mal[-]/-HIV-1[+] group relative to the mal[+]/-HIV-1[-] group ($P=0.013$), whereas mal[-]/-HIV-1[+] individuals had significantly increased CCR5+CD14+ relative to mal[-]/-HIV-1[-] group ($P=0.017$; Figure 4.4B).
Figure 4.4 CXCR4 and CCR5 Expression by the CD14+ cell population. Data are represented as box-plots where the boxes represent the interquartile range, the line through boxes represents the median while the whiskers show the 10th and 90th percentiles. Across-group analysis was performed using the Kruskal Wallis test. Pair-wise comparisons were performed by Mann-Whitney U test. A) CD14+CXCR4+; and B) CD14+CCR5+ cell populations. CD14+CXCR4+ in mal[+] HIV-1[+] (P=0.861, P=0.336 and P=0.394), mal[+] HIV-1[exp] (P=0.693, P=0.619 and P=0.508) relative to HC, mal[+] HIV-1[-] and mal[-] HIV-1[+], respectively. CD14+CCR5+ in mal[+] HIV-1[+] (P=0.334, P=0.511 and P=0.088), mal[+] HIV-1[exp] (P=0.174, P=0.492 and P=0.023) relative to HC, mal[+] HIV-1[-] and mal[-] HIV-1[+], respectively. HC, healthy controls, n=13; mal[+] HIV-1[-], malaria-positive-HIV-1-negative, n=30; mal[+] HIV-1[exp], malaria-positive-HIV-1-exposed, n=17; mal[-] HIV-1[+], malaria-negative-HIV-1-positive, n=5; and mal[+] HIV-1[-], malaria-positive-HIV-1-positive, n=7.
4.5 Relationship between CXCR4+ and CCR5+ expression and age, parasitaemia, intra-phagocytic pigment, Hb and RPI

Relationship between the CXCR4+ and CCR5+ receptor expression on the CD3+ and CD14+ cells with age, parasitological (parasitaemia and intra-phagocytic pigment) and erythrocytic outcomes (Hb and RPI) was examined in the *P. falciparum*-infected groups only (n=54; Table 4.4). While the CD3+CCR5+ population was inversely correlated with age ($r=-0.620$, $P=0.018$), there was no significant relationship between expression of these receptors/cell populations and circulating parasite levels. However, the CD3+ ($r=0.363$, $P=0.008$) and CD3+CXCR4+ ($r=-0.711$, $P=0.021$) populations were correlated with intra-monocytic pigment, while the other CD3+ cell populations (CD3+CD4+, CD3+CD4+CXCR4+, and CD3+CD4+CCR5+) were not correlated with age, parasitological or erythrocytic outcomes. In contrast, the CD14+ cell population was inversely correlated with intra-monocytic pigment ($r=-0.509$, $P<0.0001$), intra-neutrophilic pigment ($r=-0.469$, $P<0.0001$) and the RPI ($r=-0.394$, $P=0.006$). The CD14+CXCR4+ cell population was inversely correlated with intra-monocytic pigment ($r=-0.403$, $P=0.003$) and intra-neutrophilic pigment ($r=-0.432$, $P=0.002$). However, the CD14+CCR5+ cell population was not correlated with the parasitological or erythrocytic outcomes.
Table 4.4 Relationship between CXCR4 and CCR5 receptor expression and demographic, parasitological and erythrocytic outcomes

<table>
<thead>
<tr>
<th>Receptor expression</th>
<th>Age</th>
<th>Parasitaemia/μL</th>
<th>PCM (%)</th>
<th>PCN (%)</th>
<th>Hb</th>
<th>RPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td></td>
<td></td>
<td>ρ 0.045</td>
<td>P 0.744</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+CXCR4+</td>
<td>-0.539</td>
<td>P 0.108</td>
<td>-0.067</td>
<td>0.855</td>
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<td>1.000</td>
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<td>0.074</td>
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<td>-0.047</td>
<td>0.745</td>
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Spearman’s rank correlations of the CD3+ and CD14+ cell populations expressing CXCR4 and CCR5 and parasitological and erythrocytic outcomes in children presenting with *P. falciparum* infection, n=54. Significant associations are embolden. ρ (rho), Spearman’s rank correlation coefficient. Pigment containing monocytes (PCM), pigment containing neutrophils (PCN).
CHAPTER 5: DISCUSSION

Malaria and HIV-1 co-infection are co-endemic and cause significant paediatric morbidity and mortality in sub-Saharan Africa (WHO, 2011b). Although previous studies from the current study area showed that both HIV-1 infection and HIV-1 exposure are associated with increased SMA cases (Otieno et al., 2006), the cellular correlates of malaria and HIV-1 co-infection and/or -exposure were only partially defined. The current study sought to expand previous findings and characterise CXCR4 and CCR5 expression by CD3+CD4+ and CD14+ cells, and their association with the demographic, parasitological and haematological measures during paediatric *P. falciparum*- HIV-1 co-infection and -exposure.

Assessing the association between age, malaria and HIV-1 co-infection and -exposure illustrated that older children (aged 13 months-48 months) are more affected by HIV-1 and malaria co-morbidities, and as such have greater alterations in the immune response. The patterns of paediatric malaria infections in western Kenya illustrate that the prevalence of clinical malaria decrease with age among children aged 1 to 4 years (Bloland et al., 1999), while children aged 7 months-36 months are the most affected by the primary manifestation of the disease (SMA) (Obonyo et al., 2007).

The relationships observed in the present study between leukocytic measures and malaria, and HIV-1 co-infection and -exposure demonstrate that profound leukocytic alterations occur during malaria and HIV-1 co-infection and -exposure. These findings are consistent with previous studies illustrating that HIV-1 co-infection and -
exposure are associated with increased monocytes and lower proportions of CD19+ cells (De Milito et al., 2001; Jaworowski et al., 2007; Fischer-Smith et al., 2008). Unique to the present study, the proportions of CD14+ cells increased in malaria/HIV-1-exposed children, but were reduced in the malaria and HIV-1 co-infection group. While the causal effect of these perturbations remain unknown, placed in the context of previous studies showing increased haematopoietic suppression in both HIV-1 exposure and infection (Jenkins et al., 1998), it appears that this suppression is manifested primarily in infected children when they are co-infected with malaria.

Several studies have identified intraphagocytic pigment as a key molecule that promotes immunosuppression and altered haematopoietic responses in children with falciparum malaria (Casals-Pascual et al., 2006; Awandare et al., 2007; Were et al., 2009). Results presented in the present study extend these findings and demonstrate that both HIV-1 infection and exposure exacerbate levels of PCM and PCN in malaria-infected individuals.

The present study also showed that the erythrocytic measures (like RBC count, Hb and Hct) were lower in the HIV-1-exposed and -infected groups, paralleling previous studies showing an increased probability of SMA in these two groups (Otieno et al., 2006). Moreover, results presented in the present study showing that the reticulocytes (%) were higher in the malaria and HIV-1 exposed and co-infected children demonstrates that erythropoietic responses are altered to an even greater extent than during malaria mono-infection. These results are consistent with previous findings
suggesting that malaria and HIV-1 infections may impact on erythrocytic outcomes (Koka et al., 1998). This phenomenon may be due to compensatory effects of the erythropoietic system attempting to produce more erythrocytes to meet the low levels of RBCs during malaria and HIV-1 infections.

In consistent with previous studies, it is shown that malaria, and malaria and HIV-1 exposure and co-infection are associated with a higher degree of thrombocytopenia (Bierling et al., 1995; Were et al., 2006; Maina et al., 2010) indicating that reduced platelet counts and thrombocytopenia are haematological complications of both paediatric malaria and HIV-1 infections. The present study extends these findings by illustrating that both HIV-1-exposure and infection in children with malaria exacerbates the degree of platelet loss. It is hypothesized that the causal mechanism(s) for such reductions emanate from lineage-specific suppression of thrombocytes just as that characterized for erythroid lineages in malaria and HIV-1 infections (Koka and Reddy, 2004; Redd et al., 2007; Keller et al., 2009).

Although lymphopaenia has been shown to be a haematological manifestation of HIV-1 infections (De Milito et al., 2001), cytometric analyses presenting here showing that CD3+CXCR4+ and CD3+CD4+CCR5+ cells are lowest in the malaria and HIV-1 infected group demonstrates that co-infection promotes an even more profound impact on these cellular populations. Additionally, results presented in the present study showing that CD3+ cells were positively correlated with PCM, while the CD3+CXCR4+ population was inversely correlated with PCM suggest that natural-acquisition of Hz by monocytes, at least in part, has the ability to cross-
regulate surface receptors on this particular circulating lymphocyte subset (CD3+).
Perturbations in the circulating lymphocyte subsets may also result from increased migration and/or homing to the lymphoid tissues since the ligands for these receptors, alpha-chemokines \{SDF-1α [CXCL12], MIP-2α [CXCL2] and MIP-2β [CXCL3]\}; and beta-chemokines \{RANTES, MIP-1α and MIP-1β\} are frequently decreased in both malaria and HIV-1 infections (Jennes et al., 2002; Masuda et al., 2012). In addition, this could be a consequence of Hz-mediated dysregulation in these chemokines and their receptors (Jaramillo et al., 2004; Diou et al., 2010). However, it remains to be shown whether these particular cell populations are functionally involved in the immunopathogenic and/or immunity mechanisms underlying the interaction of malaria and HIV-1 morbidities.

Immunophenotyping studies of the CXCR4 and CCR5 receptor expression on CD14+ cells showing receptor reduction in malaria- HIV-1 -exposed and co-infected children indicate increased pathogen-mediated suppression of these receptors. These findings are consistent with several previous studies showing that HIV-1 infections and acute malaria are associated with decreased surface expression of several chemokine receptors (CXCR4, CCR5, CCR2) (Rodriguez-Frade et al., 2004; Ruibal-Ares et al., 2004; Jenkins et al., 2006). These observations are further supported by the inverse correlations noted between CD14+ and CD14+CXCR4+; and intra-monocytic and intra-neutrophilic pigments. The impact that these intra-phagocytic molecules have on immune activation has been previously described (Awandare et al., 2007; Zhou et al., 2007; Gama et al., 2012; Kramski et al., 2012). In particular, phagocytosis of Hz by monocytes and neutrophils was shown to cause increased suppression of CXCR4 and
CCR5 and associated chemokine ligands in these cells (Jaramillo et al., 2004; Ochiel et al., 2005; Diou et al., 2010). The observations noted in the present study showing inverse correlations between intra-phagocytic Hz, CD14+ cells and RPI are consistent with previous studies implicating intra-phagocytic Hz in the suppression of erythropoietic responses (Skorokhod et al., 2010; Thawani et al., 2013).

In summary, this study shows that natural acquisition of Hz by phagocytic cells is an important molecular event associated with altered receptor expression by the CD4+ and CD14+ cell populations in *P. falciparum* malaria-HIV-1 exposure and co-infections. The implications of these findings include formulation of appropriate treatment strategies for malaria and HIV-1-exposure and co-infections during the early stages of infections to reduce the production of haemozoin. This should be performed through timely malaria-specific prophylaxis and bed-net use with the aim of preventing infection since presence of PCM and PCN, and HIV-associated immunosuppression can lead to treatment failures and severe disease.
CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i. The present study established that *Plasmodium falciparum* malaria-HIV-1 co-infection and/or -exposure is characterized by alterations in the haematological profiles. These include monocytosis and granulocytosis; and the presence of higher loads of intra-phagocytic pigments as well as profound reductions in the haemoglobin and RPI levels. *Plasmodium falciparum* malaria-HIV-1 infections decrease CXCR4 expression by CD3+ lymphocytes in children and conversely leads, at least in part, to increase of CCR5 receptors expression by the CD14+ monocytes and this may be partly playing a role in increased infection and multiplication of HIV-1 in these children.

ii. Age seems to be an important demographic feature governing receptor expression whereby CD3+CCR5+ expression decreases with age. The altered CXCR4 and CCR5 receptor expression in *P. falciparum* malaria-HIV-1 co-infection and/or -exposure is associated with increased uptake of phagocytic Hz by monocytes and neutrophils and this appears to contribute to suppression of erythropoiesis.

6.2 Recommendations

i. Malaria diagnosis should be performed promptly in these malaria/HIV-1 prone areas so as to treat those who are malaria positive before it enters the later phase of the disease in which malaria pigment are produced thus curb the ingestion of these pigment and hence arrest the modulation of the CXCR4 and CCR5 receptors used by HIV-1.
ii. Specific therapeutic agents and vaccines should be designed that target the reduction of the CXCR4 and CCR5 receptors in children who are at risk or exposed to HIV-1 and malaria.

iii. In order to exhaustively describe the role of malaria disease in dysregulating the expression of CXCR4 and CCR5 receptors on CD14+ monocytes and CD4+ lymphocytes, further *in vitro* studies should be conducted using pure cell lines that will enable elucidation of the role played by the parasite, PCM and PCN on expression of CXCR4 and CCR5 receptors.

iv. Further longitudinal studies aimed at examination of expression of CXCR4 and CCR5 receptors in HIV-1-exposed and -infected children with *Plasmodium falciparum* malaria should be conducted for the purposes of determining whether malarial disease and pigments are the key players in the multiplication of HIV-1 within CD4+ cells that leads to HIV-1/AIDS.

v. Genetic studies should also be carried out to establish whether malaria disease and/or the pigments are associated with the mutations that are responsible for dysregulations of the CXCR4 and CCR5 receptors at the gene level.
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APPENDICES

Appendix I: Map of the study area

Map of the former Siaya District (modified from Ong’echa et al., 2006)

KEY

UNM: University of New Mexico

SDH: Siaya District Hospital
Appendix II: HIV-1 PCR SCREENING

In order to categorize the study participants into the following five clinical groups: mal(+)HIV-1(+), n=7; mal(+)HIV-1(-), n=30; mal(+)/HIV-1(exp), n=17; mal(-)/HIV-(+), n=5; and mal(-)/HIV-1(-), n=13 as the healthy control, HIV-1 PCR screening was performed. In the present study, mal (+)/HIV-1(-) group was the modal group (n=30) whereas mal(-)/HIV-(+) category had the least sample size (n=5) group.

a) Plate 1: HG3PDH PCR

In order to confirm the presence of DNA after extraction from FTA-cards housekeeping gene PCR was performed on the extracts.

A representative gel photo showing the presence of house-keeping gene having a size of approximately 1000 base pairs. The six individuals had positive DNA extraction. Low molecular weight (LMW) ladder was used.
b) Plate 2: HIV-1 PCR SCREENING RESULTS

In order to confirm the HIV-1 status for children under the age of 5 years old after a positive serological results, HIV-1 PCR screening were performed in which HIV-1 positive children (n=7) and HIV-1 exposed (n=17) were revealed.

A representative gel photo showing the HIV-1 PCR- screening result. The assay was run in pairs. From the above eleven individuals, three individuals (SMA 082212, SMA 082512 and SMA 083512) were confirmed to be HIV-1 positive, one sample was re-run (SMA 083112). Low molecular weight (LMW) ladder was used.
Appendix III: Flow cytometry working panel

HIV-1/\textit{P. falciparum} malaria co-infection and CCR5/CXCR4 Study

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<td>UNSTAINED</td>
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Appendix IV: Research Approval form

KENYA MEDICAL RESEARCH INSTITUTE

This is to inform that during the 190th meeting of the KEMRI/ERC meeting held on the 14th of June 2011, the Committee conducted the annual review and are of Future plans are to continue enrolment.

This approval is valid from today June 14, 2011 through to June 14, 2012. Please note that authorization to conduct this study will automatically expire on June 14, 2012.

If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the ERC secretariat by April 14, 2012.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SSC and ERC for review prior to initiation.

Yours sincerely,

Caroline Kithinji,
FOR: Secretary
KEMRI/ETHICS REVIEW COMMITTEE

In Search of Better Health
June 14, 2011

TO: JOHN MICHEAL ONG’ECHA,
PRINCIPAL INVESTIGATOR

THRO': DR. JOHN VULULE,
THE DIRECTOR, CGHR,
KISUMU

RE: SSC NO. 696 (REQUEST FOR ANNUAL RENEWAL): GENETIC BASIS OF SEVERE MALARIA ANEMIA.

This is to inform that during the 190th meeting of the KEMRI/ERC meeting held on the 14th of June 2011, the Committee conducted the annual review and are of Future plans are to continue enrolment.

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