PHENOTYPIC CHARACTERISATION OF ACTIVATED PLASMACYTOID DENDRITIC CELLS AMONG ANTI-RETROVIRAL THERAPY-NAÏVE UGANDANS WITH CHRONIC HIV-1 CLADE A OR D INFECTION

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NOVEMBER 2011
DECLARATION

I, Kyabaggu Denis Senkandwa, declare that this is my own original work which has not been presented to any University or institution before for any degree or award.

Signature…………………………………………..        Date……………………………………..

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To my entire family especially Dad, Mum, Winnie, Darren and Denise.
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<td>Acquired Immune Deficiency Syndrome</td>
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<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
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<td>ART</td>
<td>Antiretroviral therapy</td>
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<td>B-Cell</td>
<td>Bone-marrow-derived lymphocyte</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<td>EDTA</td>
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<td>ELISA</td>
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<td>FBS</td>
<td>Fetal Bovine serum</td>
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<td>HAART</td>
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<td>HIV</td>
<td>Human Immunodeficiency virus</td>
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<td>IRB</td>
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<td>mDC</td>
<td>Myeloid Dendritic Cell</td>
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<td>MDDC</td>
<td>Monocyte –derived dendritic cell</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MHRP</td>
<td>Military HIV Research Program</td>
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<td>MUWRP</td>
<td>Makerere University Walter Reed Project</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid Dendritic Cell</td>
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<td>©</td>
<td>Internationally registered trade mark</td>
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<tr>
<td>RCCS</td>
<td>Rakai Community Cohort Study</td>
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<td>RHSP</td>
<td>Rakai Health Sciences Program</td>
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<tr>
<td>T-Cell</td>
<td>Thymocyte Cell (a type of lymphocyte)</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>US</td>
<td>Unites States of America</td>
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<td>WIV</td>
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ABSTRACT

Plasmacytoid dendritic cells (pDC) are the most potent producers of interferon alpha (IFN-α) and tumor necrosis factor alpha (TNF-α) in response to enveloped viruses providing a critical link between the innate and adaptive immune responses. The loss of peripheral blood pDC function and numbers has been linked to HIV-1 disease progression. Studies have demonstrated that both acute and chronic HIV-1 infections resulted in the alteration of pDC frequency, phenotype, functional impairment of IFN-α-release and T-cell activity in both adult and pediatric individuals.

50 samples of chronically HIV-1 infected Ugandans and 10 HIV-1 negative samples were analyzed for the phenotype and function of pDCs by flow cytometry. Five HIV-1 negative PBMC samples from Thai donors were also analysed. pDCs were stained for surface, co-stimulatory and activation markers, followed by intracellular cytokine staining to measure the expression of IFN-α and TNF-α after stimulation with different antigens over night.

The frequency of pDCs in HIV-1 infected Ugandans was reduced compared to uninfected individuals. There was a significant inverse correlation between pDC percentages and the absolute CD4+ T cell count. A significant down regulation of CD80 in HIV-1 subtype D infected subjects when compared to HIV-1 subtype A infected subjects was also observed.

A strong increase in IFN-α production was seen in HIV-1 positive cells after stimulation with TLR7/8 agonist but not observed after stimulation with either whole inactivated virus HIV-1 subtype A or D. After stimulation with influenza A virus, the expression of IFN-α, but not TNF-α, increased in both HIV-1 positive and in HIV-1 negative subjects.

Overall, the phenotype of pDC’s remains relatively unchanged in chronic HIV-1 A or D infection, although the surface expression of CD80 appears down regulated during chronic HIV-1 subtype D infection relative to HIV-1 subtype A. The ability of pDCs to produce IFN-α and TNF-α after certain in vitro stimulation seems to be inhibited in chronic HIV-1 A or D infections.
CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

According to WHO and UNAIDS, by December 2009, there were at least 33.3 million people living with the HIV virus worldwide, of which 2.6 million were new infections. In 2009 alone, at least 1.8 million deaths were associated with AIDS (UNAIDS, 2010). HIV causes more deaths than any other infectious disease worldwide (Klimas et al., 2008).

Sub-Saharan Africa accounts for over two thirds (22.5 million or 68%) of the people living with HIV/AIDS worldwide and 72% (1.3 million people) of the AIDS-related deaths (UNAIDS, 2010). It was estimated that by 2010, 18 million children were orphaned by the AIDS epidemic in Sub-Saharan Africa, the region that also has most of the world’s AIDS orphans (Andrews et al., 2006). The vast majority of AIDS deaths are attributed to inadequate access to HIV prevention and treatment (Klimas et al., 2008). Such figures continue to paint a grim picture of the ongoing global burden of HIV/AIDS, highlighting the need for continued efforts to find effective curative and/or preventative strategies against this pandemic.

There are two distinct genotypes of HIV: HIV-1 and HIV-2. HIV-1 is the virus responsible for the vast majority of AIDS worldwide; and is also the most diverse, grouped into M (major), O (outlier), and N (novel or non-M and non-O) subtypes (Butler et al., 2007; Requejo 2006). Most HIV-1 infections worldwide are due to group M viruses (Requejo, 2006). It has been demonstrated that HIV-1 subtype D infected Ugandans show a faster progress to disease than those infected with HIV-1 subtype A, and/ or with the other HIV-1 subtypes (Kaleebu et al., 2002). It is also striking that some studies have showed that HIV-1 subtype A is more transmissible than HIV-1 subtype D (Conroy et al., 2010).

The predominant HIV-1 sub-types in Uganda are clade A and clade D, together with recombinants of both subtypes (Guwatudde et al., 2009; Lihana et al., 2006; Dowling et al., 2002; Harris et al., 2002). The distribution of these two clades is such that in some parts, such as in Kayunga district in the central region of Uganda, clade A is more common than D (Guwatudde et al., 2009) whilst clade D is the more predominant than A in others, for instance in...
the south western region, where Rakai district is located (Harris et al., 2002). A study in 2006 found that Ugandans infected with subtype D or recombinant strains of subtype D developed AIDS sooner than those infected with subtype A, and died sooner, if they did not receive antiretroviral treatment (Laeyendecker et al., 2006). The authors further suggested that subtype D was more virulent than A because it bound more effectively to immune cells. A similar study in Kenya among HIV-1 subtypes A and D infected women showed twice as high the risk of progression to AIDS and death among subtype D than A infected individuals (Baeten et al., 2007). A study of sex workers in Senegal found that women infected with subtype C, D or G were more likely to develop AIDS within five years of infection than those infected with subtype A (Kanki et al., 1999). All the above studies seem to point towards subtype variations in viral fitness, virulence, or both, that enables temporal differences in the pathology and outcome of infection.

The most common route of HIV infection is across mucosal barriers as a result of sexual exposure. HIV primarily infects vital cells in the human immune system such as T cells (specifically CD4+ T cells), macrophages, and dendritic cells using combinations of CD4 and chemokine receptors, the most common being CCR5 and CXCR4 (Williams et al., 2009; Zack et al., 1990). Within a week following initial infection, the HIV virus is detectable in the mucosal lymph nodes where it interacts with the tissue Dendritic Cells (DC), the Langerhans cells, which carry virus on their surface to lymph nodes (Williams et al., 2009). Virus production is increased in draining lymph nodes where DCs, and perhaps monocytes, interact with viral antigen specific CD4 T cells resulting in amplification of infection through CD4 T cells. This interaction can also result in maturation and activation of monocytes (Ancuta et al., Jan 2006 and May 2006).

Dendritic cells are professional antigen presenting cells (APC) specialized to capture and process antigens in vivo (Buonaguro et al., 2009; Guermonprez et al., 2002) and converting proteins to peptides that are presented on major histocompatibility complex (MHC) molecules and recognized by T cells. DCs also migrate to T-cell rich areas of lymphoid organs, where the two cell types interact to bring about clonal selection (Buonaguro et al., 2009; Steinman et al., 2007;
Randolph et al., 2005; Itano et al., 2003). In particular, two main DC types are present in human peripheral blood, the major subset known as myeloid DCs (mDCs) representing around 80% of blood DCs (Dzionek et al., 2000), and plasmacytoid DCs (pDCs) (Buonaguro et al., 2009).

pDCs are a type of APC and the plasma cell-like morphology and localization of these cells led to their designation as “plasmacytoid T cells,” or “plasmacytoid monocytes.” Subsequently, these cells were found to correspond to a subset of circulating blood DCs, an immature CD11c-population, now referred to as pre-pDCs (an immediate precursor of pDCs), and to be distinct from “conventional” myeloid CD11c+ DCs (mDCs) (McKenna et al., 2005). Pre-pDCs typically mature into pDCs, which produce large amounts of alpha/beta interferons (IFN-α/β), in response to activation by viral and bacterial stimuli, or by ligation of CD40 (McKenna et al., 2005). These cells are thus the “natural type I IFN-producing cell” in blood, a rare CD4+, MHC class II+ (HLA-DR+) cell with potential to secrete significant amounts of IFN-α (McKenna et al., 2005).

There is a reduction of circulating pDC in both acute (Chehimi et al., 2010) and chronic HIV-1 infection in humans, which is coupled with a decrease in their IFN-α production, despite a reported increased accumulation of IFN-α mRNA in these remaining circulating pDCs in some populations (Chehimi et al., 2010). This loss of pDCs likely contributes to disease development in HIV-1 infection though altered innate and adaptive immune responses and their aberrant production of IFN-α may play a role in driving chronic immune activation (Benlahrech et al., 2011). Such evidence seems to point to the functional impairment of the pDCs that remain in circulation during chronic HIV-1 infection (Chehimi et al., 2010).

1.2 Problem Statement

There is still need for continued efforts to find effective curative and/or preventative strategies against the HIV pandemic through sustained and cost-effective basic research. Despite the recent very modest success in research towards the development of a safe and effective preventive HIV-1 vaccine (Rerks-Ngarm et al., 2009), a lot more remains to be done, especially in completely
understanding the innate immune response to HIV in general, and in regard to clades A or D in particular. HIV-1 clade A and D have hitherto been documented as the most prevalent in Uganda. A lot of research efforts are focused on HIV-1 clades circulating in the Americas, Europe and in East Asia, and yet the greatest disease burden occurs in sub-Saharan Africa, where Uganda lies.

1.3 Justification for this work
PDCs, through their role of combating viral infections, are key in limiting the effects of HIV-1 that lead to immune dysregulation, and subsequent progress to AIDS. They are classic APCs that also happen to be very potent producers of various type-1 interferons in response to HIV-1 stimulation of their toll-like-receptor (TLR)-7. There is speculation that HIV disease progression may result in part from the failure of pDCs to limit viral replication (Meyers et al., 2007). However, not much information is available about the phenotypic profile of activated pDCs among anti-retroviral therapy-naïve Ugandans, despite a lot of research geared towards Th1-related, CD4 and CTL-mediated immunity among Ugandans.

1.4 Study Objectives
The primary aim of this study was to profile and characterise the phenotype(s) and frequency of activated peripheral blood plasmacytoid dendritic cells (pDCs) from the archived PBMCs of individuals who were chronically infected by HIV-1 clades A or D and had never been started on ART. This study also aimed at elucidating the magnitude of IFN-α and TNF-α cytokines expressed by pDCs induced in the innate immune response against antigens among chronically infected, ART-naïve Ugandans.

1.5 Hypothesis
We worked with the hypothesis that there were differences in the frequencies, phenotype and functional profiles of pDCs of ART-naïve Ugandans infected with HIV-1 subtype A or D when compared to sero-negative controls.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HIV/AIDS

It is scientifically documented that the acquired immune deficiency syndrome (AIDS) in humans is caused by the lentivirus known as the human immunodeficiency virus (HIV). About 85% of the HIV isolates from humans are grouped into two types, HIV-1 and HIV-2. The worldwide main agent of AIDS is HIV-1, while the occurrence of HIV-2 is largely restricted to some regions of Western and Central Africa (Requejo, 2006). HIV-1 is grouped into M (major), O (outlier), and N (novel or non-M and non-O) subtypes (Butler et al., 2007; Requejo 2006) and most HIV-1 infections worldwide are due to group M viruses (Requejo, 2006). Groups M, N and O viruses belong to the primate Lentivirus lineage that also includes the Simian Immunodeficiency Virus (SIV) strains that infect chimpanzees (Requejo, 2006).

The earliest case of HIV-1 infection identified in human blood specimen was collected in 1959 in Kinshasa, Democratic Republic of Congo (Requejo, 2006; Zhu et al., 1993 and 1998), however, the virus was only first discovered about 30 years ago (Barre-Sinoussi et al., 1983). In Uganda, the earliest case of HIV-1 infection was reported in 1981, in the rural south-western district of Rakai (Serwadda et al., 1985).

2.2 Course of HIV disease progression

Following primary infection via the mucosal or parenteral route, the patient may suffer from a flu-like illness and a rash in the first few weeks. The combination of these first symptoms, termed acute HIV infection syndrome (Klimas et al., 2008; Levy, 2006), is also associated with high viraemia due to active viral replication (Weiss, 2008). The patient may also have a fever, diarrohoea, and lymphadenopathy (Weiss, 1993 and 2008).

The high viral load in blood subsequently falls to a lower set point, the level of which is predictive of the rate of progression to AIDS in untreated individuals (Weiss, 2008). HIV has the ability to infect CD4+ lymphocytes and a variety of other cells in the body, including monocytes and thymocytes (Ho Tsong Fang et al., 2008; Nilsson et al., 2007). A gradual deterioration of
immune function occurs after the acute phase of HIV infection, culminating in AIDS several months or years later.

2.3 Human Dendritic Cells

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) that sense the presence of pathogens and serve as a link between the innate and adaptive immune systems (Figure 1). As part of the innate immune system, DCs present antigens to T-cells and B-cells, resulting in efficient adaptive immune responses, which include priming MHC Class I and II restricted T-cell responses (Dillon et al., 2008). With regard to innate immunity, DCs function by recognising pathogen-associated molecular patterns (PAMPs) on microbial pathogens via pattern recognition receptors, such as toll-like receptor (TLRs) (Dillon et al., 2008).

![Fig.1: Functional and Antiviral Properties of pDCs. Figure shows a summary of interaction between innate and adaptive immune responses to a pathogenic virus. The DCs are positioned in the critical intersection between these two systems (courtesy of Zhang et al., 2005).](image-url)
DCs exist in blood and tissues in an immature state before encountering invading microbes, microbial antigens, or before being exposed to pro-inflammatory cytokines (like IL-12), upon which they take up antigen and undergo a highly regulated maturation process (Huang et al., 2001; Dillon et al., 2008). The maturation process results in increased expression of MHC and co-stimulatory molecules (CD80/86, CD40) and production of cytokines (IL-12, IFN-α) that allow for efficient T-cell activation (Dillon et al., 2008). The activated DCs then migrate to lymphoid organs, homing to T-cell areas, and thereby stimulating naïve, antigen-specific T-cells to proliferate (Dillon et al., 2008).

Human DCs are of two main subsets: myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs) (Beignon et al., 2005; Fonteneau et al., 2004; MacDonald et al., 2007). These subsets differ in morphology, phenotype and function (Dillon et al., 2008). The two DC subtypes circulating in blood have different patterns of TLR expression (eg. mDCs express TLR4 whereas pDCs express TLR 9), have different migratory properties, and produce distinct cytokine profiles upon stimulation; suggesting that the activation state of the DC, the type of activation signal it encounters, and the cytokine environment all may contribute to the type and extent of T-Cell activation induced by DCs (Dillon et al., 2008).

2.4 HIV-1 infection of DCs

The exposure to virus or viral proteins can result in the pathological triggering of many immune system cells, limiting the effectiveness of immune responses by killing the cells, impairing their function, or inducing pathways that suppress antiviral responses.

Given their role in bridging innate and adaptive immunity and in priming antigen-specific T-cell responses, DCs are thought to play an important part in HIV-1 pathogenesis (Dillon et al., 2008). During both acute and chronic infection, both mDCs and pDCs have been shown to be depleted from the blood, this finding being associated with high viral loads (Barron et al., 2003), and directly correlated with CD4+ T cell counts in both adults (Schimdt et al., 2006) and children (Zhang et al., 2006). The depletion of blood pDCs has thus been associated with HIV-1 disease progression and the development of opportunistic infections (Soumelis et al., 2001).
In addition to the numeric defects observed in peripheral blood, functional defects and an altered phenotype have also been reported for both DC subsets from HIV-1-infected subjects, both in vitro and in vivo (Dillon et al., 2008). Studies also show that in viremic HIV-1-infected individuals there is an increased expression of CD86 and CD40 on blood DCs and that costimulatory molecule expression correlated directly with viral load (Barron et al., 2003). However, despite all these studies, there is a general consensus that the overall consequences of altered blood DC activation on T cell function in the setting of HIV-1 infection is still not completely understood (Dillon et al., 2008).

Patterson (2001) showed that mDC express CD4 and CCR5, hence supporting HIV entry and replication, whereas pDC allow binding of HIV to DC-SIGN without viral replication, except possibly by CXCR4 strains during maturation (Weiss, 2008). Nevertheless, the attachment of HIV to DC-SIGN allows pDC to deliver HIV to susceptible CD4 T cells upon migration to the lymph nodes (Weiss, 2008). The immunological synapse between pDC and CD4+ cells not only activates the T helper lymphocyte (making it more permissive to HIV replication) but also delivers the HIV particles across the synapse (Weiss, 2008).

2.5 Characteristics of pDCs

PDCs are the dominant producers of type 1 IFNs, and are found in CD4-rich regions of (secondary) lymphoid tissues and in low numbers in the blood (Cohen et al., 2008). These cells express CD4 and CD123 (the interleukin-3 [IL-3] receptor alpha chain) but lack expression of myeloid-related markers, such as CD11b, CD11c, CD13, and CD33 (Fonteneau et al., 2004). PDCs participate in innate immune responses to different types of viruses (especially enveloped viruses like the influenza virus, herpes simplex virus, HIV-1) by producing large amounts of IFN-α, -β and, -ω (Soumelis et al., 2002). These cells release IFN-α in response to HIV-1 and HIV-1-infected cells (Cohen et al., 2008)

On exposure to HIV-1, pDCs produce cytokines such as IFN-α and TNF-α, up regulate the DC maturation markers CD83 and CCR7, and, to a lesser extent, up regulate the co-stimulatory molecules CD80 and CD86 (Fonteneau et al., 2004). HIV-1-activated pDCs also gain migratory
capacity toward CCL19, a chemokine produced in secondary lymphoid organs (Fonteneau et al., 2004). Very early following infection with HIV-1, presentation of antigens by DCs leads to the development of HIV-1–specific CD4+ T cells (Cohen et al., 2008). Fonteneau and colleagues propose that pDCs exposed to HIV-1 stimulate naïve CD4+ T cells to preferentially produce Th1 cytokines could impact on their trafficking, virus uptake, and cellular transmission in trans and on the HIV-1–specific immune response. It is also postulated that the homing of pDCs towards CCL19, produced in secondary lymphoid organs, may affect the capacity of the immune system to fight other pathogens in the periphery and may participate in the eventual development of AIDS since it is in the secondary lymphoid organs where HIV-1 replication is the most active (Fonteneau et al., 2004).

PDC levels are reduced during HIV-1 infection in individuals who have progressed to AIDS, but are higher in long-term non-progressors (LTNPs) than in healthy controls, (Soumelis et al., 2001). The authors proposed that the drop in pDC number, together with the decrease in their induced IFN production were associated with the presence of opportunistic infections and active Kaposi sarcoma, suggesting that pDCs might play a big role in restricting disease progression (Cohen et al., 2008). It is also plausible that pDCs may facilitate virus transfer while at the same time processing and presenting viral antigens to T cells.

It has been proposed that the continuous exposure to HIV-1 antigens in the chronic state may induce dysregulation of DC function since it has been observed that the remaining DCs in blood of HAART-naïve patients have a defect in their capacity to stimulate allogeneic T-cell proliferation (Fonteneau et al., 2004; Donaghy et al., 2003).

2.6 Immune activation of pDCs in chronic HIV-1 infection

Some of the latest findings show that pDC (and mDC) levels decline very early in acute HIV-1 infection; however, those pDCs that remain in circulation retain their function and are able to stimulate allogeneic T-cell responses, and up-regulate maturation markers as well as produce cytokines/chemokines in response to stimulation with TLR7/8 agonists (Sabado et al., 2010). These studies demonstrated that despite the reduction in circulating DC numbers, those that remain in the blood displayed hyperfunctionality implicating a possible role for pDCs (and
mDCs) in promoting chronic immune activation (Sabado et al., 2010).

HIV-1 infection can lead to immune activation via TLR stimulation (Chang et al., 2009). Immune activation in chronic HIV-1 infection can occur through several mechanisms which include the stimulation of pDCs by HIV-1–encoded TLR7 ligands inducing production of IFNα and other proinflammatory cytokines (Fig 2.) (Chang et al., 2009).

**Fig. 2: Immune activation mechanisms in DCs.** HIV-1 infection can lead to immune activation via TLR stimulation. Immune activation in chronic HIV-1 infection can occur through the stimulation of plasmacytoid dendritic cells by HIV-1–encoded TLR7 ligands inducing production of IFNα and other pro-inflammatory cytokines. Additionally, microbial translocation in the gut has been well described in chronic HIV-1 infection, and elevated serum levels of LPS can cause immune activation via TLR4 stimulation. (Courtesy of Chang et al., 2009).

The stimulation of the pDC toll-like receptor (TLR) pathway by HIV-1 has been implicated in contributing to the persistent immune activation observed in chronically HIV-1–infected individuals. However, the phenotypic profile of activated pDCs among ART-naïve Ugandans was yet to be characterized. Using multiparameter flow cytometry techniques we simultaneously compared expression of a range of maturation and or activation markers and chemokine/cytokine

10
receptors on pDCs in thawed PBMCs from untreated HIV-1-infected individuals, using seronegative subjects as controls.
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

This study was developed from a larger study, the molecular epidemiology study (MER) (Fig.3), which was a nested cross-sectional study from the Rakai community cohort study (RCCS) carried out by the Rakai Health Sciences Programme (RHSP) in collaboration with the United States Military HIV Research Program (MHRP). The RCCS was a rural, population-based open cohort in which annual HIV-1 disease surveillance was conducted from 1994-2002 among persons aged 15-49 years residing in the Rakai district of southwestern Uganda. This was conducted prior to the introduction of ART in 2004 (Conroy et al., 2010).

All subjects provided written consent for their samples to be stored and used for research purposes. The protocol was reviewed by institutional review boards (IRBs) in both the United States of America (US) and Uganda. Samples for the MER study were collected by the RHSP between 1997 to 2002, processed and stored at the Makerere University Walter Reed Project (MUWRP) laboratory in Mulago. The design and implementation of the MER study, as well as the ethical reviews and approvals have previously been described (Kiwanuka et al., 2008).

3.2 Study Area and Population Characteristics

This study analysed archived frozen PBMC samples collected from Rakai district of South Western Uganda. The study population and participant characteristics have previously been described (Kiwanuka et al., 2008) as follows: HIV-1 incident seroconverters, who were recruited during the annual sero-surveys among 56 communities in the district of Rakai, under the RHSP, and participants who tested HIV-1 positive between 1997 and 2002 were recruited into the ethically approved MER study. A total of 488 were enrolled in MER and HIV-1 subtype for 350 participants has since been availed and found to be predominantly subtypes D (59.1%) and A (15.1%), and recombinants of these two (Kiwanuka et al., 2008; Arroyo et al., 2006; Hoelscher et al., 2002;). Among the 349 persons with incident HIV-1 infection 60% were female, and 76% were <35 years old (Kiwanuka et al., 2008). Study subjects made visits after 1, 3, 6, and 12
months from date of serocoversion and followed up annually after the first year. Venous blood was collected for HIV-1 viral load, sub-typing and CD4+, CD8+ T lymphocyte immunophenotyping. PBMC were isolated and kept frozen in the vapor phase of liquid nitrogen.

**Fig. 3: MER study scheme.** Samples were collected from an HIV-1 annual sero-survey cohort. *Incident seroconverters between 1997 and 2002 were recruited into the MER study and followed up for up to 5 years. 488 seroconverters were enrolled in MER and the HIV-1 subtype could be obtained for 350 participants of those (Kiwanuka et al., 2008).*

This work involved the use of 25 MER PBMC samples from individuals who were chronically infected with HIV-1 subtype A and 25 samples from participants who were infected with HIV-1 subtype D. 10 seronegative samples from the same population were used as controls. Five Thai sero negative samples were also included for added quality assurance to ensure correct assay optimization.
3.3 Study Sample Characteristics

The samples used for this particular work had had some prior demographic, immunophenotypic, and serological results compiled during the MER study. Viral load, and HIV-1 subtyping data had also been compiled by previous research. In brief, during the MER protocol, three blood samples were collected in EDTA, ACD and serum separator tubes at each visit in volumes of 3ml, 8.5ml and 10ml respectively. The EDTA blood was used for CD4/CD8 immunophenotyping using monoclonal antibodies against surface markers and analyzed on a flow scan cytometer (Becton Dickinson, San Jose, California, USA). Both absolute numbers and percentages of CD4/CD8 immunophenotypes were obtained and results compiled. Plasma was aliquotted and stored at -80°C in 1.5 ml NUNC cryotubes. PBMCs were isolated from Acid Citrate Dextrose (ACD)-anti coagulated peripheral whole blood within 8 hours of collection, by the Ficoll-hypaque technique (Pharmacia, Sweden) and stored in the vapour phase of liquid nitrogen (-130°C).

3.3.1 HIV-1 Viral Load estimation

The MER study also ensured that all samples that tested positive for HIV-1 by ELISA also had HIV-1 viral load testing performed on the frozen EDTA anticoagulated plasma using the Roche Amplicor HIV-1 Monitor test, version 1.5 (Roche Diagnostics, Indianapolis, Indiana, USA) according to manufacturer’s instructions. Briefly, HIV-1 RNA was extracted from EDTA plasma using viral lysis buffer containing pre-added quantitative standard and incubated at room temperature. The lysis reaction was stopped by addition of absolute isopropanol followed by centrifugation to pellet the RNA. The pellet was washed and concentrated by vortexing in 70% ethanol followed by centrifugation. The resultant RNA pellet was resuspended in HIV-1 diluent provided in the kit before subsequent amplification was then performed on GeneAmp 9600 or 9700 thermocyclers (Applied Biosystems, Foster City, CA, USA).

Detection was done by the enzyme immunoassay micro-well plate method. PCR products were denatured before addition to test detection plates and the plates were incubated at 37ºC in a Fisher Scientific Isotemp incubator. Plates were washed using the ELx50 Auto Strip Washer (BIO-TEK Instruments, Inc, Highland Park, Vermont, USA) and Optical Densities (ODs) were
read on an *ELx 800* Universal Microplate Reader (BIO-TEK Instruments, Inc, Highland Park, Vermont, USA). The derived ODs were used to calculate viral load according to manufacturer’s instructions.

### 3.3.2 Enumeration of CD4+ T-helper cells

Absolute CD4 counts were also compiled on all MER participants at each visit. Lymphocyte immunophenotyping was performed on EDTA anti-coagulated whole blood using the FACS MultiSET System. Samples were run on a dual laser benchtop flow cytometer using the single platform Multi-test 4-color reagent in combination with TruCount tubes for evaluation of lymphocyte subsets and analyzed using MultiSET software (Becton Dickinson, San Jose, CA).

### 3.3.3 Determination of HIV-1 subtypes by the MHA assay

Evaluation of HIV subtypes for the MER study samples was done using a validated screening multiplex hybridization assay (MHA) to identify subtype assignment and the possible presence of recombinants. The MHA for HIV-1 subtype determination was first described in 2002 (Hoelscher M *et al.*, 2002). The assay was developed for subtyping HIV-1 using five genomic regions, efficiently discriminating pure from recombinant strains as well as being able to detect dual infections. The principle of the MHA is to amplify different regions of the HIV-1 genome in separate first round reverse transcriptase PCR and a small amount of each amplicon is then distributed into separate second-round PCR, each with a different subtype-specific probe in a Taqman real-time PCR format (Hoelscher *et al.*, 2002).

The MHA$_{acid}$ was performed on all the MER HIV-1 seropositive samples by a previous researcher. Extraction and purification of viral RNA was done using the QIAamp Viral RNA Mini kit (Qiagen Inc., Valencia, California, USA) according to the manufacturer’s instructions. Sample lysates during extraction step were centrifuged in a Micromax ultracentrifuge (International Equipment Company, Needham Heights, MA, USA) at the manufacturer’s recommended speed and time. RNA-MHA was performed on extracted viral RNA. Reverse transcription of viral RNA (RT-PCR) into cDNA and amplification was performed using the
QIAGEN OneStep RT-PCR kit (Qiagen Inc., Valencia, California, USA) according to manufacturer’s instructions.

Amplification of the five short regions along the HIV-1 genome in the gag, pol, vpu, env and gp41, was done each in a separate first round RT-PCR tube. Each first round RT-PCR tube contained 10 µl QIAGEN 5X OneStep RT-PCR Buffer, 1µl Qiagen dNTP mix with 10mM of each dNTP, primers F and R (final concentration of 0.6µM), 1µl of QIAGEN OneStep RT-PCR Enzyme mix, RNase inhibitor (10 U/ reaction), RNase free water 25.75 µl and 10 µl template RNA to make up a 50µl reaction. Amplification conditions were as follows; 1 hold cycle of 60ºC for 30min, 1 cycle of 95ºC for 15min., 45 cycles of 94ºC for 15 sec, 60ºC for 1min. and 72ºC for 1min. and a final extension cycle at 72 ºC for 10min. and final hold at 4 ºC forever.

In the second round real-time PCR, each amplicon from first round PCR was distributed to three second round PCRs, each with a subtype-specific fluorescent probe, and a fourth tube containing SyBR Green, in a Taqman real-time PCR format. The second round PCR reaction contained; 2.5µl of RT-PCR first round product, 6.25µl Taqman 2x Universal real-time PCR Master Mix, No Amperase UNG (Commercially prepared by Applied Biosystems, Foster City, CA, USA), 0.3µl of each forward and reverse inner primers (20mM) and 0.55µl of subtype-specific probe (5pmol/µl).

The second round real-time PCR amplification was performed on a 384-well spectrofluorometric ABI 7900 HT fast Real-Time PCR sequence detection system (Applied Biosystems, Foster City, CA, USA). Amplification conditions were as follows; 1 cycle of 95ºC for 10min., 40 cycles of 95ºC for 15 sec, 55ºC for 1min. and 60ºC for 1min. Fluorescence intensity was monitored during every cycle by the sequence detection system (SDS) software (Applied Biosystems, Foster City, CA, USA).

In the MHAacd, HIV-1 subtype assignment was given when two or more probes hybridized as this criteria was demonstrated to provide an adequate measure of the subtype distribution when compared with full length sequencing genotyping (Hoelscher et al., 2002). Real-Time PCR amplification data was captured and analyzed by the Sequence Detection System (SDS) Software (Applied Biosystems, Foster City, CA, USA). A probe was considered to have hybridized if the
resultant fluorescence was \( \geq 1000 \) fluorescent units on the multi-component graph in the SDS file. Reactivity of different subtype-specific probes in different genes represented a recombinant form of the virus, while reactivity of two or more different probes in the same region of the HIV-1 genome indicated the possibility of dual infection.

### 3.4 Phenotypic Characterisation of pDCs in chronic HIV-1 infection

The cryopreserved PBMCs from 60 participants of the MER study, which were preserved in the vapor phase of liquid nitrogen, were rapidly thawed in a 37\(^\circ\)C water bath with gentle agitation until only a small ice crystal was left.

The PBMCs were then gently decanted into a 50ml conical tube and washed with 10% complete media (CM) (RPMI 1640 supplemented with 10% FBS, 2% L-glutamine amino acid, 1% penicillin/streptomycin [all Biowhittaker] and 2% Hepes buffer [Invitrogen]) by centrifugation at 1200rpm for 10 minutes. The supernatant was decanted, while the cell pellet was re-suspended in 10ml of 10% CM and counted using Guava Viacount\textsuperscript{®} reagent. A cell dilution of 1:20 using the Guava reagent was made for cell counting (380\(\mu\)l reagent+ 20\(\mu\)l PBMCs).

The pDC phenotype panel was then set up as follows: four Falcon\textsuperscript{®} (BD Biosciences Inc., CA) tubes were set up per sample, each containing 5 \( \times \) 10\(^5\) cells. The cells were subsequently centrifuged (300g, 5min), and washed twice with PBS containing 0.1% bovine serum albumin (BSA) (ICS wash buffer) (Pharmacia). For the surface staining, the cells were incubated with the following antibodies for 30 min in the dark at 4 degrees celcius: CD86-V450, CCR7-PE Cy7, Lin-1-FITC, (all BD Biosciences); HLA-DR-APC Cy7, CD11c-APC, CD123-PerCP Cy5.5 and CD80-PE (all BioLegend). Lin-1 consists of a cocktail of antibodies for identifying PBMC lineage markers for the non-DC cells. These are CD3, CD14, CD16, CD19, CD20, and CD56. Live-Dead Aqua viability Dye\textsuperscript{®} was used to identify live cells as these did not take up the dye.

Cells in tube 1 were surface stained using fluorochrome-conjugated antihuman monoclonal antibodies against CD80, CD86, Lin-1, HLA-DR, CD11c, CD123 and CCR7 pDC surface markers (see Appendix I).
Cells in tube 2 were stained with a similar antibody cocktail, but leaving out the antihuman CD80 antibody (fluorochrome minus one, FMO), while cells in tube 3 were stained by a cocktail excluding antihuman CD86 antibody (second FMO). The FMO tubes helped in the setting of the staining gates for the cell population that was positive for the excluded antibody. The 4th tube was unstained. It only contained cells in wash buffer as a negative control.

The cells were washed twice (300g, 5 min) using ICS wash buffer before being preserved in 2% paraformaldehyde in PBS, and acquired within 24 hours on an 8-colour FACSCanto II flowcytometer (BD Biosciences Inc, CA). Compensation tubes were prepared for each of the fluochromes used in the experiments for purposes of optimizing detection on the Facscanto machine. The above described procedure, see Appendix I, was drawn up after several trial runs and optimization of experiments, which included the titration of all the antibodies used.

3.5 Functional characterisation of pDCs in chronic HIV-1 infection

After thawing and counting as described above, and setting up the pDC phenotype panel, the remaining cells were prepared for the pDC function ICS assay. At least 1 million cells in 100 ul of 10 % CM were set up for each well in the pDC function panel in a 96 well polystyrene plate. Briefly, whenever cell yields permitted, the panel for each sample constituted of an Unstimulated control well, containing only cells, a Test well in which the whole pDC surface antibody cocktail was to be added, followed by ICS for IFN-α and TNF-α.

The third and fourth well for each sample were set up as Influenza A virus FMOs for IFN-α and TNF-α production respectively.

Wells 5 and 6 were set up for stimulation with whole inactivated viruses (WIV) HIV-1 clade A, and WIV clade D (all NIH AIDS Research and Reference Reagent Program) respectively. Flu (Influenza A H3N2 Strain A [Aichi/2/68]) was set up in well 7 as an enveloped virus control, while microvesicles were used as a negative control for the WIV in well 8, and toll-like receptor 7/8 agonist (invivogen) experiment positive control stimulant in well 9.

The cells were centrifuged (300g, 5 min), then re-suspended in 10% CM at 100ul/1 x10^6 cells.
The cells were then added to the appropriate wells on the micro well plate, followed by overnight incubation (12 to 18hrs) in 5% CO2 at 37°C. Following the overnight incubation, the cells were washed, and the respective stimulants added. The stimulation was performed for 3 hours in conditions of 5% CO2, 90% relative humidity, and 37°C.

Brefeldin A (BFA), diluted to a final concentration of 3µg/ml in 10% CM was then added to each well, followed by a further 3 hour incubation in the same conditions as described above. This was followed with pDC surface staining for 30 minutes at 4°C in the dark. Subsequent washing steps then followed, after which the cells were fixed, and then permeabilised using Perm Wash buffer. ICS was then performed for IFN-α and TNF-α for 30 minutes at 4°C in the dark. The cells for the pDC function (ICS) panel were then resuspended at 200 µl/well in 2% PFA and stored at 4°C before subsequent acquisition on a FACSCanto II (BD Biosciences Inc. CA) flow cytometer within 24 hours of ICS.

3.6 Statistical Analysis

Flow cytometry analysis was done using FlowJo Software version 9.2 (Tree Star, Ashland, OR). Statistical analysis was performed using Graph Pad Prism Software version 5.01 for Mac OSX (Graph Pad Software, La Jolla, CA). Direct comparisons between two groups were performed using the non-parametric Mann-Whitney U test for continuous data. Correlation between groups was analysed by the Spearman’s rank test.
CHAPTER FOUR

4.0 RESULTS

4.1 Characterization of the Study Population

For this particular study we used 25 study participants chronically infected with HIV-1 subtype A, 25 subjects infected with HIV-1 D, and 10 sero negative samples from the same population as controls to determine the baseline pDC frequency, phenotype and function (Table 1). A further 5 sero negative samples from an East Asian population were included for comparison purposes. 32 (64%) of the HIV-1 infected subjects were female, while 18 (36%) were male. The median age of the combined HIV-1 infected subjects was 25 years, with a range of 15-49 years. On the other hand, the median age, and range of the negative controls was 27 and 19 – 36 respectively. The absolute CD4 counts were significantly reduced in the HIV-1 positive individuals compared to uninfected (p= 0.001). The median absolute count in HIV-1 participants, either infected with subtype A or was 629 cells/ml, ranging from 109 – 1336 cells/ml. In HIV-1 negative subjects the median was almost 2fold higher (1,073 cell/ml) and ranged from 818 – 1282 cells/ml. In HIV-1 positive individuals, the median viral load was 29,952 copies of viral RNA/ml, with a range of 371 – 663,265 copies/ml.

4.2 Numbers of pDCs in PBMCs in chronic HIV-1 infection

Frozen PBMCs from 50 HIV-1 infected and 10 uninfected Ugandans were thawed, washed and analyzed by flow cytometry to determine the phenotype and function of pDCs. PDCs were identified as live, HLA-DR+, Lin-1-, CD123+ and CD11c- cells (Figure 3). The percentage of pDCs out of PBMCs was reduced in individuals with chronic HIV-1 infection compared to HIV-1 negative subjects (Figure 4). Consistent with the literature the median percentage of pDCs in PBMCs of HIV-1 uninfected persons was 0.15%, ranging from 0.06 – 0.43%. In HIV-1 infected individuals those numbers were slightly lower, showing a median of 0.12% (range 0.04 – 0.35%). To compare the basic level of pDCs in PBMCs, the same experiments were performed.
with frozen PBMCs of HIV-1 negative individuals from Thailand, East Asia (Figure 5). The median percentage of pDCs in these samples was 0.24% and the numbers ranked from 0.17 up to 1.02%, and therefore they were slightly higher than the numbers in uninfected Ugandans. Furthermore we analyzed the percentage of pDCs in individuals infected with either HIV-1 subtype A or D. As shown in Figure 6 no statistical significant difference could be seen by comparing the percentage of pDCs in subtype A and subtype D infected samples. Overall these results suggest that infection with either HIV-1 subtype A or D reduces the percentage of plasmacytoid dendritic cells in the peripheral blood, as expected, compared to HIV-1 negative controls.

<table>
<thead>
<tr>
<th>Table 1. Demographic Data of the study Population</th>
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<td>Subjects, n</td>
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<td>Sex, n (%)</td>
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<tr>
<td>Male</td>
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<td>Median age (range), yrs</td>
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<tr>
<td>Immune Phenotype</td>
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<td>CD4 absolute, median cells/ml (range)</td>
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<td>Viral Load, median copies/ml (range)</td>
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<td>HIV-1 subtype, n</td>
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<td>HIV-1 clade A</td>
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<td>HIV-1 clade D</td>
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Table 1.: Demographic data of the study population 60 samples, consisting of HIV-1 positive as well as HIV-1 negative individuals, were analyzed in this study. Samples were taken from a HIV-1 incident seroconverter cohort, established in Rakai, Uganda in 1997.
Fig. 4: Gating strategy to identify human pDCs. Frozen PBMCs were thawed, washed and stained for flow cytometry. pDCs were determined as a subpopulation of PBMCs which consists of HLA-DR+, Lin1-, CD123+ and CD11c- cells. The gating strategy is shown above. Cells in the CD123+, CD11c- gate were then analyzed for their phenotype and function. All analysis was done with FlowJo 9.2.
Fig. 5: Percentage of pDCs in chronic HIV-1 infection. 50 frozen PMBC samples of HIV-1 positive individuals were thawed, washed and stained for flow cytometry. 9 samples from uninfected individuals served as a control. Shown are the percentages of pDCs (referred as HLA-DR+, Lin1-,CD123+, CD11c-cell population) in total numbers of PBMCs. A) Results shown in box and whiskers (min-max) plots (B) as well as scatter plots (B). A 1.2 fold reduction in the numbers of pDCs can be seen in HIV-1 infected individuals (grey box, circles), compared to HIV-1 negative controls (white box, squares). All statistical analysis was performed with the Mann-Whitney U test.

Fig. 6: Numbers of pDCs in HIV-1 positive and negative Ugandans, compared to uninfected Thai donors. In addition to the 60 samples from Ugandan donors, the percentage of pDCs was also determined in 5 PBMC samples of Thai donors. The median percentage of pDCs was slightly higher than in Ugandan negative subjects, but no statistical significant difference could be seen. All statistics were performed with the Mann-Whitney test.
From existing literature a depletion of pDCs in chronic HIV-1 infection is associated with increased viral load and reduced CD4+ T cell counts in those patients. The analysis of 50 samples of HIV-1 subtype A and D infected individuals showed a significant inverse correlation between their viral load (copies of viral RNA/ml) and the CD4+ T cell counts (p= 0.006, r = -0.394; Figure 8.A). Furthermore an inverse correlation between the percentages of pDCs in the PBMCs of HIV-1 infected subjects and the absolute CD4+ T cell count was noted (p= 0.001, r = -0.385; Figure 7.B). Taken together, as disease progresses and CD4+ T cells are depleted in the samples investigated it appears that the number of pDCs is increased, maybe suggesting a conflicting role of those two immune cells in chronic HIV-1 infection.

**4.3 Numbers of pDCs in ART-naive Ugandans chronically infected with HIV-1**

Fig. 7: Percentage of pDCs in PBMCs during chronic infection with two different HIV-1 subtypes. PBMC samples of 50 with HIV-1 infected individuals as well as 10 uninfected were thawed, washed and stained with pDC surface-staining mAbs for Flow Cytometry. 25 samples were infected with subtype A (grey box), the other 25 with subtype D (black box), as determined by MHA assay. No statistical significant difference could be seen by comparing the percentage of pDCs of subtype A and subtype D infected samples, or compared to the HIV-1 negative controls (white box). All statistical analysis was performed with the Mann-Whitney test.
Fig. 8: Relationship between pDCs and HIV-1 replication and progression. CD4 T cell absolute counts (cells/ul) inversely correlate with HIV-1 RNA copy number/ml (Fig. 4A, \( r = -0.394 \), \( p = 0.006 \)). The number of pDCs in HIV-1 positive individuals also correlated with the absolute counts of CD4 T cells (B). There was no association found between number of pDCs and viral load (data not shown). All correlation analysis was done with the Spearman \( r \) rank test.
4.4 The phenotype of pDCs in chronic HIV-1 infection

PBMC samples of HIV-1 infected (n=50) and HIV-1 negative individuals (n=10), as well as 5 PBMC samples of HIV-1 negative Thai donors were analyzed for the expression of the activation markers CD80, CD86 and the C-C chemokine receptor 7 (CCR7). CD80 and CD86 are co-stimulatory markers expressed on mature activated DC to help modulate adaptive immune responses, CCR7 serves as a homing receptor, facilitating the migration of T cells to the center of inflammation. CCR7 has also shown to be taking part in the maturation process of dendritic cells (Fonteneau et al., 2004). As shown in Figure 8, no significant difference in the expression of either of these markers in HIV-1 infection compared to HIV-1 negative controls could be seen. In fact, the phenotype of pDCs seems not to alter during chronic HIV-1 infection in Ugandans.

Fig. 9: Phenotypic characterization of pDCs in chronic HIV infection. Frozen PBMC samples of HIV-1 infected (grey boxes) and uninfected (white boxes) were thawed, washed and stained for the expression of CD80 (A), CD86 (B) and CCR7 (C). No statistical difference between the two groups could be seen. All analysis was done with the Mann-Whitney test. The grey shaded boxes show the expression of the above markers in PBMC samples of HIV-1 negative Thai individuals to compare the basic expression level. (MFI=Mean fluorescence intensity)
To see if the basic expression level of the three surface markers differ from other populations, the expression of CD80, CD86 and CCR7 was determined in pDCs of HIV-1 negative Thai donors. No significant difference could be seen by comparing the two populations (Figure 9). Next the expression of CD80, CD86 and CCR7 in HIV-1 subtype A and D infection was analyzed. Except for CD80, which showed to be significantly down regulated in HIV-1 subtype D infection-the subtype of HIV-1 associated with more rapid progression, no differences could be seen by comparing the two subtypes (Figure 10). The median expression level (in MFI) of CD80 was 559.5 in HIV-1 infected individuals and 672 in HIV-1 negative controls. CD86 median surface expression was 922.5 MFI in HIV-1 positive and 1286 MFI in HIV-1 negative samples. CCR7 median expression was 884 MFI in HIV-1 positive participants and 959 in HIV-1 negative individuals. Altogether, it seems, that in chronic HIV-1 infected Ugandans pDCs are still able to stimulate the T cell compartment.

Fig. 10: Phenotypic characterization of pDCs in chronic infection with HIV-1 subtype A and D. Frozen PBMCs were thawed, washed and analyzed via Flow Cytometry for the expression of CD80, CD86 and CCR7. Shown here are the expression levels of the different markers in samples infected with HIV-1 subtype A (grey bars), D (black bars) and in uninfected individuals (white bars). CD80 shows a significantly higher expression in subtype A infected individuals compared to those infected with subtype D virus (*p=0.041). All statistical analysis was performed with the Mann-Whitney test. (MFI=Mean fluorescence intensity)
4.5 Functionality of pDCs in chronic HIV-1 infection

PBMC samples of HIV-1 positive and HIV-1 negative individuals were analyzed via intracellular cytokine staining for their functionality. Thawed cells were treated with different stimuli and stained for expression/production of the two cytokines IFN-α and TNF-α. Unstimulated cells served as negative controls. Figure 11 shows the expression of IFN-α in the different samples. Figure 11A shows the basic IFN-α level in unstimulated cells. Whereas only 0.1% of the pDCs in HIV-1 positive individuals produce IFN-α, it is about 0.2% in HIV-1 negative. By looking at the different subtypes, there is no IFN-α production in the subtype D infected subjects, but about 0.2% after infection with subtype A. Since pDCs produce IFN-α after stimulation with HIV-1 encoded TLR7 ligands (Chang et al., 2009), a TLR7/8 agonist was used as a positive control in this study. As shown in Figure 11B, the IFN-α production increased after stimulation with the TLR-agonist, in HIV-1 positive (Mean = 15.7%) as well as in HIV-1 negative samples (Mean = 8.9%). The IFN-α expression in the two HIV-1 subtypes showed no significant difference with a mean of 14.5% (subtype A) and 16.7% in subtype D (p = 0.8). PBMCs of either HIV-1 subtype A or D infected subjects were also stimulated with whole inactivated HIV-1 A and D, as well as with microvesicles, which served as negative controls. As shown in Figure 11C and D, a slight but not significant increase in IFN-α production occurs in infected as well as uninfected samples, compared to unstimulated cells (Figure 11A), but this increase can also been seen in cells stimulated with microvesicles, suggesting that this effect is non-specific.

Figure 12 shows the same experiments but this time the production of TNF-α was investigated. Frozen PBMCs were prepared and stimulated with TLR7/8 agonist, WIV HIV-1 A and D and microvesicles for 6 hours. Unstimulated cells served as a control. Cells were then stained with antibodies against surface markers and TNF-α by ICS. In unstimulated pDCs the basic level of TNF-α expression was on average 62% in HIV-1 positive samples, and 32% in HIV-1 negative counterparts. A statistically significant difference in TNF-α expression (with no external stimulant added) can be seen when comparing individuals infected with either subtype A and D (Figure 12A, p = 0.02, n = 6 and 5, respectively). After stimulation with the TLR7/8 agonist the expression slightly increases up to 72% in HIV-1 positive and 69% in HIV-1 negative subjects.
Looking at the samples according to the two HIV-1 subtypes A and D, no difference can be seen (Figure 12B). The difference in unstimulated cells in subtype A and D infection might be due to the fact that a less number of samples was analyzed and the variance in values is therefore higher. 17 and 20 samples, respectively were analyzed after TLR7/8 stimulation, whereas only 6 unstimulated samples for subtype A and 5 unstimulated samples for subtype D were analyzed. After stimulation with either WIV HIV-1 A or D, the expression level of TNF-α in HIV-1 positive subjects shows the same level as in unstimulated cells and is reduced compared to samples after TLR7/8 stimulation (Figure 12A-D). After stimulation with WIV HIV-1 D the TNF-α expression in HIV-1 positive subjects is significantly reduced compared to HIV-1 positive cells stimulated with TLR7/8 agonist (p=0.003). Stimulation with microvesicles served as a negative control for stimulation with WIV and showed almost similar results as the unstimulated cells. Overall, stimulation with WIV HIV-1 did not lead to an increase in TNF-α production but rather a decrease in this cytokine production in WIV HIV-1 D stimulated pDCs.

For nonspecific virus stimulation, cells were also treated with Influenza Virus A (subtype H3N2), which is known to increase IFN-α and TNF-α production in human pDCs. Due to low cell yields of PBMC’s after thawing of samples used in this study, only a certain amount of cells could be stimulated with all agents, and the number of samples treated with Influenza virus A is therefore n=9 (Figure 13B). Compared to unstimulated cells (Figure 13A) IFN-α expression was increased by 14fold (p=0.06) in HIV-1 infected pDCs when stimulated with Influenza A virus. For samples from HIV-1 negative participants the expression of IFN-α was difficult to determine, since only n=2 and n=3 samples could be analysed for both groups (Influenza A stimulated and unstimulated respectively). Both HIV-1 negative samples did not respond to Influenza virus A stimulation. Looking at samples which were infected with HIV-1 subtype A and D, there was a strong increase in the production of IFN-α after stimulation of cells with Influenza virus A (p= 0.08). There was no significant difference in TNF-α production comparing infected and HIV-1 negative cells which were unstimulated or treated with Influenza Virus (Figure 13C and D).

At last the basic expression level of IFN-α and TNF-α in HIV-1 negative samples from Ugandan as well as Thai donors was compared. As shown in Figure 14, there was no significant difference
in the expression of these cytokines *in vitro* after stimulation with different viral and biologic agents or in unstimulated cells. In summary, the ability of pDC’s to produce IFN-α and TNF-α after different stimuli *in vitro* seems to be impaired in chronic HIV-1 infection.

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**Fig. 11: IFN-α production of pDCs in chronic HIV infection.** Frozen PBMC samples were thawed, washed and stained with monoclonal antibodies for the expression of IFN-α. Before intracellular cytokine staining the cells were incubated overnight at 37°C, 5% CO2 and stimulated with either TLR7/8-agonist (B), HIV-1 subtype A (C) and subtype D whole inactivated Virus (D) or microvesicles (E). Unstimulated cells (A) served as a control. Shown are the expression levels of IFN- α in cells of HIV-1 subtype A and D and uninfected individuals after 6 hour stimulation.
Fig. 12: Functionality of pDCs in chronic HIV infection. Frozen PBMC samples were thawed, washed and stained with monoclonal antibodies for the expression of TNF-α. Before intracellular cytokine staining the cells were incubated overnight at 37°C, 5% CO2 and stimulated with either TLR7/8-agonist (B), HIV-1 subtype A (C) and subtype D whole inactivated Virus (D) or microvesicles (E). Unstimulated cells (A) served as a control. Shown are the levels of TNF-α production in cells of HIV-1 subtype A or D infected and in uninfected individuals after 6 hour stimulation. Unstimulated pDCs from patients infected with HIV-1 subtype A significantly produced more TNF-α than those infected with HIV-1 subtype D infection (A, *p= 0.02). A significant difference is also seen when comparing the TNF-α expression level in HIV-1 positive cells stimulated with WIV HIV-1 against stimulation with TLR7/8 agonist (p= 0.03; data not shown). All statistics were performed with the
Fig. 13: Cytokine expression in pDCs after stimulation with Influenza A virus. Frozen PBMCs were thawed, washed and stimulated with Influenza Virus A for 6 hours. ICS for IFN-α and TNF-α was performed on 9 HIV-1 positive and 2 negative samples. Shown are the expression levels of IFN-α and TNF-α in unstimulated (A and C) and Influenza A virus stimulated (B and D) cells of HIV-1 positive (grey bars) and HIV-1 negative (white bars) individuals. In addition the expression of IFN-α and TNF-α in infection with HIV-1 subtypes A (light grey bars) and D (black bars) is shown. A strong increase of IFN-α can be seen in HIV-1 positive cells after stimulation with Influenza A virus, compared to unstimulated cells (p= 0.06). No significant difference was seen in the expression level of TNF-α. All statistical analysis was performed with the Mann-Whitney test.
Fig. 14: Basic cytokine expression in pDCs of Ugandan and Thai donors. To compare the expression levels of IFN-α and TNF-α in Ugandan and Thai individuals, frozen PBMC samples were thawed, washed and stimulated with different virological and biological agents for 6 hrs. Then ICS for IFN-α and TNF-α was performed. No significant difference in expression of both cytokines after stimulation with different agents could be seen comparing Ugandan and Thai donors. All statistic analysis was performed by the Mann-Whitney test.
CHAPTER FIVE

5.0 DISCUSSION

It is now well established that in both acute and chronic HIV-1 infection, there is a progressive decline in pDC numbers (McKenna et al., 2005). The results from the here presented study further support this fact. There was a trend showing a decline in the percentage of pDCs from PBMCs in the chronic HIV-1 positive subjects, regardless of HIV-1 subtype infection, in comparison to the sero negative controls. However, this decline was not significant in our case, probably due to the low numbers of HIV-1 sero negative samples used. The Thai sero negative donors had a slightly higher, but not significantly different, percentage of pDCs than the Ugandan sero negatives. This could possibly be due to a combination of genetic differences between the two populations and exposure to different pathogens, other than HIV-1, in the different spatial environments where these two populations are located. Previous studies showed that the decline in pDCs of HIV-1 infected subjects inversely correlates with their viral load (McKenna et al., 2005). Studies also confirmed an inverse correlation between viral load and the number of CD4+ T-lymphocytes (Soumelis et al., 2001). Our data showed a significant inverse correlation between the copies of viral RNA/ml and CD4+ T-lymphocyte cell counts (p= 0.006, r = - 0.394; Figure 8.A). This was in agreement with previous investigations.

A negative correlation between the percentages of pDCs in the PBMCs of HIV-1 infected subjects and the absolute CD4+ T cell count (p= 0.001, r = - 0.385; Figure 8.B) was unexpectedly observed. This contradicts earlier studies (Mojumdar et al., 2010), because it has been demonstrated that human pDCs express CD4, CXCR4 and CCR5 that are targeted as receptors and co-receptors for entrance of HIV-1 into the cells (Zhang et al., 2005). Therefore, pDCs are highly susceptible to HIV-1, just like CD4+ T-cells, and a decline in the latter would be expected to correspond to a decline in the former. However, there is a possibility that pDCs, apart from binding, processing and presenting HIV-1 epitopes to the adaptive immune system, are not as productively infected in the chronic state as CD4+ T-cells. Other possible reasons for this negative correlation between pDCs and CD4 T-cells in our study, in contrast to other investigators, could be due to the different HIV-1 subtypes involved, showing varying effects on
the immune system, and the different populations studied. In addition, comparable previous studies (Mojumdar et al., 2010) also studied subjects at different stages of HIV-1 infection, not necessarily in the chronic state, and this, too, may be a factor in the differences in the pDC/CD4+ T-cell correlation observed.

Although literature points to a significant up regulation of CD86 expression on pDCs in early HIV-1 infection (Smed-Sorensen et al., 2005), we found no significant difference in the expression of this co-stimulatory/activation marker in regard to HIV-1 sero status in the chronic state. It is possible that the pDCs in these HIV-1 chronically infected subjects were losing the ability to up regulate CD86 expression.

There was significant down regulation of the CD80 pDC co-stimulatory molecule in subjects infected with HIV-1 subtype D when compared with those infected with subtype A. HIV-1 subtype D has variously been documented as the more aggressive, possibly more virulent and pathogenic of the two subtypes (Baeten et al., 2007; Laeyendecker et al., 2006; Kaleebu et al., 2002; Kanki et al., 1999). This difference in CD80 expression, which seems to be related to subtype variation between A and D in this study, could be due to differences in viral fitness, virulence, or both. Alternatively, perhaps the poor co-stimulatory capacity of pDC in HIV-1 subtype D infection is responsible for faster disease progress.

With regard to the function of pDCs, the significant difference in TNF-α production between HIV-1 subtype A and D infection in unstimulated cells is also quite intriguing, when coupled with the results that no stimulant was able to induce further significant TNF-α production. It is possible that sub-type D leads to a greater dysfunction in pDCs than subtype A, hence the less TNF-α production in subtype D infected pDCs than in the subtype-A infected pDCs. The fact that pDCs produce a diverse array of cytokines and chemokines following activation with pathogens (McKenna et al., 2005), and in this case given that these were mostly symptomatic chronically infected patients, may explain the high background levels of TNF-α in these subjects. The failure of any stimulant to elicit further TNF-α expression might be possibly a result of cell exhaustion due to the chronic HIV-1 immune activation state of these subjects. A significant difference was also seen when comparing the TNF-α expression level in HIV-1 positive cells
stimulated with WIV HIV-1 against stimulation with TLR7/8 agonist (p = 0.03; data not shown). The TLR7/8 agonist stimulated pDCs were able to secrete much higher TNF-α levels, as was expected, and it is for this reason that this reagent was used as the positive control for cytokine production in this study.

For IFN-α, a trend showing an increase of this cytokine was seen in HIV-1 positive cells after stimulation with Influenza A virus, compared to unstimulated cells (p = 0.06). No significant difference was seen in the expression level of TNF-α following stimulation with Influenza virus. This is consistent with studies in which pDCs have been activated using influenza virus (Zhang et al., 2005; McKenna et al., 2005). Influenza viruses (and other enveloped viruses) stimulate the TLR7/8 receptor pathway which results in the secretion of type I interferons (IFNα/β) whose functions are to, among other functions, activate and recruit autologous NK cells and enhance their cytotoxicity (Zhang et al., 2005; McKenna et al., 2005; Mojumdar et al., 2010).

In summary, our findings characterized the effect of chronic HIV-1 subtype A and subtype D disease infection on blood pDC phenotype and function in a Ugandan population for the first time.

**Limitations**

One of the limitations of this study was the fact that a very limited number of subjects (25) was studied for each of the two HIV-1 subtypes. It would also have been good to increase the seronegative samples to about 20 to see if this would have changed the outcome of many of the analyses, such as the cytokine expressions, that showed some trends that were not significant in this study.

It would also have added a poignant dimension to this study if at least 10 Thai samples were used to compare against the Ugandan sero negative samples.

**Conclusion**

The depletion of blood PDCs in chronic HIV-1 infection among Ugandans, in the absence of ART, may be associated with phenotypic changes, which seem to be related to the infecting HIV-1 subtype, but of which further studies are needed to explore this finding. Further studies of this particular cohort would be needed, with a greater number of Thailand donors for instance,
for a more comprehensive comparison of outcomes. It will also be interesting to look at individuals with recombinant HIV-1 infections of subtype A or D in the future.
REFERENCES


HIV Subtype on Rapid Disease Progression in Rakai, Uganda. 13th Conference on Retroviruses and Opportunistic Infections (abstract no. 44LB).


*Zhang Z and Wang Fu-S.* (2005). Plasmacytoid dendritic cells act as the most competent cell


APPENDICES

APPENDIX I: Protocol for Flow Cytometry

PBMC Thaw:

- Do not thaw more than 2 vials at a time
- Label 50 ml conical tube (if thawing 1 or 2 vials) with the sample ID. Add 40ml of 10% warm CM per wash for each 50ml conical tube
- Remove PBMCs from the LN2 freezer
- Immediately Place in a 37°C water bath and agitate gently until only a small ice crystal is visible
- Dry off the outside of the cryovial and wipe with alcohol before opening
- Quickly decant PBMCs into the 50 ml tube
- Centrifuge at 1200 rpm for 10 min. Decant supernatant.
  
  Optional: Wash cells with 10 ml PBS (RT, 1200rpm, 10 min)
- Gently Re-suspend in 10 ml of 10% CM in a conical tube
- Use Dilution tubes to set up the staining
- Add 380 ul of Guava ViaCount to each tube
- Add 20 ul of PBMCs
- Incubate 10 min, at Room temperature in the dark
- Count cells with the Guava Machine.

Set up PBMCS:

- For the pDC Phenotype Panel, set up a minimum of 0.5 million cells/Panel (the following calculation is for one sample)

  4 Panels in 4 Wells (Unstained, Test, FMO1 and FMO2), each with 0.5 million cells = 2 million cells in total.
  
  Calculation: Total number of viable cells (Guava Count) = 5 ml (5000 ul)

  \[ 2 \times 10^6 \text{ million cells} = x \quad \rightarrow \quad (2 \times 10^6) \times 5000 \div \text{Total number} = x \]

- For the pDC Functional Panel (ICS) set up a minimum of 1 Million Cells/Panel (you could use less for Unstimulated and FMOs)
- The left over cells from each sample need to be pooled and used in unstained and FMO wells. There will be 5 wells of pooled cells 0.5 million cells in each well. (5 wells x 0.5 million cells per well = 2.5 million cells total)

- 2 unstained wells (Phenotype, Functional)
- 2 FMO wells for Phenotype (CD80, CD86)
- 2 FMO wells for ICS (IFNα and TNFα)
- Any left over cells should be used to store the Dry pellet for further Molecular Work!

**Flow Assays:**

**Phenotype Panel** (numbers and amounts are for one sample)

- Take out the calculated amount (x) of cells from the conical tube and transfer to a new falcon tube (place remaining cells in the incubator until using it for the functional panel)
- Centrifuge cells down for 5 min at 300g
- Resuspend cells in 400ul of PBS + 0.1% BSA
- Aliquot 100 ul of cells into a 96 well plate (U-bottom)
- Wash plate 1x time with 200ul/well (or 2 ml in Falcon tube) of 0.1% BSA +PBS to remove all media (300g, 5 min)
- Flick plate in order to remove supernatant
- Do Surface Staining (Test Tube, FMOs and Unstained)

**Panel:**

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<thead>
<tr>
<th>Fluorochrome</th>
<th>Test Tube</th>
<th>FMO1</th>
<th>FMO2</th>
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<tr>
<td>PerCPCy 5.5</td>
<td>CD123</td>
<td>CD123</td>
<td>CD123</td>
</tr>
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<td>APC Cy7</td>
<td>HLA-DR</td>
<td>HLA-DR</td>
<td>HLA-DR</td>
</tr>
<tr>
<td>V450</td>
<td>CD86</td>
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<td>CCR7</td>
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<td>CD11c (AF647)</td>
<td>CD11c (AF647)</td>
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<tr>
<td>AmCyan</td>
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<td>Aqua</td>
<td>Aqua</td>
</tr>
</tbody>
</table>

- Stain Cells with 50 ul of Ab Cocktail in PBS+0.1% BSA (see Antibody Distribution Sheet)
For unstained Control add only 50ul of PBS+0.1% BSA
- Incubate 30 min at RT in the dark
- Wash the cells 2x times with 200ul/well (or 2 ml in Falcon tube) of 0.1% BSA +PBS (300g, 5 min)
- Resuspend cells in 200ul 2% Formaldehyde in PBS
- Store at 4 oC and run within 24 hours

Functional Panel (following amounts and instructions are for one sample)
- After thawing and counting as above prepare the cells for the functional Assay
- Each Panel (1 well in a 96 well plate) requires at least 1 million cells in 100 ul of 10 % Complete media

A1: Unstimulated and Unstained Control
A2: Test Tube (whole Surface Cocktail plus IFNalpha and TNFalpha for ICS)
A3: FMO 1 (whole Surface Cocktail plus IFNalpha for ICS)
A4: FMO2 (whole Surface Cocktail plus TNFalpha for ICS)

B2: Test Tube (whole Surface Cocktail plus IFNalpha and TNFalpha for ICS) \[\rightarrow\] HIV-1 A
B4: Test Tube (whole Surface Cocktail plus IFNalpha and TNFalpha for ICS) \[\rightarrow\] HIV-1 D
B6: Test Tube (whole Surface Cocktail plus IFNalpha and TNFalpha for ICS) \[\rightarrow\] Microvesicles
B8: Test Tube (whole Surface Cocktail plus IFNalpha and TNFalpha for ICS) \[\rightarrow\] TLR Agonist

Calculate Amount of cells as above for the Phenotype Panel. Spin down cells (300g, 5 min).
- Resuspend in 10% CM at 100ul/1 x10^6 cells.
- Unstained/Unstimulated Control and FMOs can have less than 1 million cells, but at least 0.5 million
- Add cells to appropriate wells
- Incubate overnight (12 to 18hrs) at 5% CO2 and 37oC
- Spin cells down (300g, 5 min) and decant supernatant

With Influenza Virus as Stimulants
- Add 100 ul CM to each well
- Add 50ul of stimulant to each well as follows:
  o stimulate cells with HIV-1 WIV subtype A and D for 3 hrs at 37C and 5% CO2
  o Stimulate cells with microvesicle control for 3hrs at 37C and 5% CO2
  o Stimulate cells with Influenza control (already heat inactivated) for 3hrs at 37C and 5% CO2
  o 1x unstimulated Control: add Medium only
  o Stimulate cells with TLR agonist control (in case you have enough cells) for 3hrs at 37C and 5% CO2

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<tr>
<th>Stimulating Agent</th>
<th>Stock</th>
<th>required Conc</th>
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<tbody>
<tr>
<td>1) HIV-1 A07412 Subtype D</td>
<td>247 ug/ml</td>
<td>500 ng/ml of p24</td>
</tr>
<tr>
<td>2) HIV-1 KNH1144 Subtype A</td>
<td>303 ug/ml</td>
<td>500 ng/ml of p24</td>
</tr>
<tr>
<td>3) Microvesicles (Jurkat-TAT-CCR5)</td>
<td>1.94 mg/ml</td>
<td>500 ng/ml of p24</td>
</tr>
<tr>
<td>4) Influenza A</td>
<td>3*10e7 pfu/ml</td>
<td>MOI=1-2</td>
</tr>
<tr>
<td>5) TLR Agonist Control</td>
<td>1 mg/ml</td>
<td>5 ug/ml</td>
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</tbody>
</table>

AFTER 3 HRS:
- Add 50 ul of BFA (Stock 3mg/ml, 3ug/ml final Conc. in 10% CM) and incubate 3hrs at 37C and 5% CO2
- After the 3hrs of BFA
- SPIN down cells (300g, 5 mins). Decant.
- Wash the cells 2x times with 200ul/well 0.1% BSA+PBS (300g, 5 min)
- Stain Cells with 50 ul of Ab Cocktail in PBS+0.1% BSA (see Antibody Distribution Sheet)

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Test Tube</th>
<th>FMO1 (FLU)</th>
<th>FMO2 (FLU)</th>
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<tr>
<td>AmCyan</td>
<td>Aqua</td>
<td>Aqua</td>
<td>Aqua</td>
</tr>
</tbody>
</table>

- For unstained Control add only 50ul of PBS+0.1% BSA
- Incubate for 30 min at 4 degrees in the Dark
- Wash cells 2x using 200ul/well 0.1%BSA+PBS centrifuging at 600rpm 5minutes
- Fix cells with 100ul/well of 2% PFA for 15 min at 4°C
- Spin down cells (600g for 5 min) Decant.
- Wash 2x with 200ul/well 0.1%BSA/PBS at 600g for 5 min
- Add 200ul/well Perm Wash Buffer (10x) at X1 concentration in DH20
- Incubate for 15 min at 4°C.
- SPIN down (600g for 5 min). Decant.
- Wash with Perm Wash buffer 2x at 600g for 5 min
- Stain Cells with 50 ul of Intracellular Ab Cocktail in Perm Wash Buffer (see Antibody Distribution Sheet)

**Panel:**

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<td>IFNalpha</td>
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- For unstained Control add only 50ul of PBS+0.1% BSA
- Incubate for 30 min at 4 degrees in the Dark
- Wash 2x with 200ul/well PERM/WASH at 600g for 5 min
- Resuspend in 200 ul/well 2% PFA
- Store at 4°C and run within 24 hours

*Cell Pellet:*

- Wash cells 2x times for 10 min at 1200 rpm with PBS
- Decant supernatant
- Freeze Pellet down at -80°C
# APPENDIX II: Antibody Distribution Sheet

## Functional Panel

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<td>1/20</td>
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<td>CD11c AF647</td>
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### FMO 1

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<th>Dil Factor</th>
<th>amount (ul)</th>
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<td>CD80 PE</td>
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<td>CD123 Percp-Cy5.5</td>
<td>1/20</td>
<td>20</td>
<td>6.00</td>
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<tr>
<td>CCR7 Pe Cy7</td>
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<td>Aqua AmCyan</td>
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APPENDIX III: Research Approvals

MUWRP
MAKERERE UNIVERSITY WALTER REED PROJECT
Plot 42 Nakasero Road, P.O. Box 16524, Kampala - Uganda
Phone: 256 - 414 - 534 588 Fax: 256 - 414 - 534 586
e-mail: muwrp@muwrp.org Website: www.muwrp.org

To: Dr. Britta Flach, Post-Doctoral Research Fellow MUWRP Laboratory
   Proussy Naluyima, Research Scientist, MUWRP Laboratory
   Denis Kyabaggu, Research Associate, MUWRP Laboratory

From: Prof. Dr. Fred Wabwire-Mangen
       Makerere University- Walter Reed Project
       P. O. Box 16524
       Kampala, Uganda

Date: 29th March 2010

Dear All,

Re: Protocol RV 228 Samples for Further Anonymous Utilization.

As local Associate investigator of protocol RV228, an “Evaluation of Virus and Host Factors of importance to HIV-1 Infection and Disease Progression in Rakai Project, Uganda. An Integrated Community HIV Epidemiological Research (CHER) and Molecular Epidemiological Research (MER) Project”, I grant permission to utilize protocol RV 228 anonymized samples from up to 550 individuals, derived from the above referenced cohort study with known dates of HIV-1 sero-conversion; and anonymized samples from up to 100 HIV-1 sero-negative individuals for the following proposed studies:

1. “Phenotypic and functional characterization of the relationship between T cells and NK cells in Anti-retroviral Therapy naïve chronic HIV-1 infected Ugandans” by Proussy Naluyima and

2. “Phenotypic Characterisation of Activated Plasmacytoid Dendritic Cells Among Anti-Retroviral Therapy naïve Ugandans with Chronic HIV-1 Clade A or D Infection” by Denis Kyabaggu.

Sincerely,

[Signature]
Fred Wabwire-Mangen, PhD
Associate investigator
Protocol RV 228
Our Ref: GC/127/10/01/04
Your Ref: RV 228, version 1.2 dated 7th Aug

11th January 2010

Dr. Francine E. McCutchan, Col. Nelson Michael, Fred Wabwire-Mangen et-al,

RE: UVRI SEC progress report Titled: “Protocol RV 228: Evaluation of virus and host factors of importance to HIV-1 infection and disease progression in Rakai Project, Uganda: An integrated community HIV epidemiological research (CHER) and molecular epidemiological research (MER) project”.

Thank you for submitting the above progress report dated 15th December 2009 and received by UVRI Science and Ethics Committee on 8th January 2010.

This is to inform you after review of your progress report; approval has been given for you to continue with your study for another one year up to 21st January 2011. At that time, SEC would expect you to submit a progress report and request for renewal.

Yours sincerely,

Mr. Tom Lutalo
Chair, UVRI SEC

C.C Ag. Director, UVRI
Secretary, UVRI SEC
Your Ref:.......................... IIS 413
Our Ref:..........................

Date:.............................. 25/08/09

Prof. Fred Wabwire-Mangen
Associate Investigator
Protocol RV 228
MUWRP
P.O. Box 16524
Kampala

Dear Prof. Wabwire-Mangen,

The Uganda National Council for Science and Technology (UNCST) has granted your request for approval to continue with the study entitled, “Protocol RV 228, Evaluation of Virus and Host Factors of Importance to HIV-1 Infection and Disease Progression in Rakai Project, Uganda: An integrated Community”. The approval will expire on August 21, 2010. If, however, it is necessary to continue with the research beyond this expiry date, a request for continuation should be made to the Executive Secretary, UNCST.

Yours sincerely,

Jane Nabbato
for Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY