Sex Differences in the Kinetics of Immune Reconstitution under Antiretroviral Therapy

By

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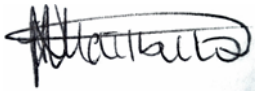
Signed (Student):  **Date: 28/09/2017**

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Dedication

I dedicate this thesis to my present and future family.

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired immune defeciency syndrome
APC	Antigen Presenting Cell
ART	Antiretroviral Therapy
BSA	Bovine Serum Albumin
CA	Capsid (p24)
CCR5	Chemokine Receptor 5
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
CTL	Cytotoxic T Lymphocyte
CXCR4	CXC Chemokine Receptor 4
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme linked Immunosorbent Assay
Env	Envelope
FCS	Fetal Calf Serum
Gag	Group-specific antigen
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal Tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAART	Highly Active Antiretroviral Treatment
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
ICS	Intracellular Cytokine Staining
IFN-γ	Interferon gamma
IFN-α	Interferon alpha
IL	Interleukin
ISG	Interferon stimulated genes

IRIS	Immune reconstitution inflammatory syndrome
kDa	Kilodalton
KIR	Killer Immunoglobulin-like receptor
LRR	Leucine-rich repeat
MA	Matrix (p17)
MAPK	Mitogen-activated protein kinase
mDCs	Myeloid dendritic cells
MHC	Major Histocompatibility Complex
MFI	Mean Fluoroscence intensity
MIP-1b	Macrophage Inflammatory Protein-1b
mRNA	Messenger Ribonucleic Acid
MTCT	Mother-to-child transmission
NC	Nucleocapsid (p7)
Nef	Negative regulatory factor
NK cell	Natural Killer cell
ORF	Open Reading Frame
PAMPs	Pathogen–associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCA	Principle component analysis
pDCs	plasmacytoid dendritic cells
P24	P24 Capsid Protein
Pol	Polymerase
PRRs	Pattern recognition receptors
PrEP	Pre Exposure Prophylaxis
Rev	Regulator of virion expression protein
RNA	Ribonucleic Acid
SHIV-1	Simian/Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
Tat	Transactivator of transcription
TCR	T-cell Receptor

TLR 4/7/8 and 9	Toll like receptor 4/7/8 and 9
TNF- α	Tumor necrosis factor-alpha
TP	Time point
tRNA	Transfer Ribonucleic Acid
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

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Abstract

The human immunodeficiency virus type 1 (HIV-1) remains a global health threat and is increasingly becoming a female epidemic due to gender inequalities. The introduction of antiretroviral therapy (ART) has greatly increased the life span while reducing AIDS-related deaths in HIV-1-infected people. However, some patients experience adverse effects during ART due to an acute inflammatory response termed immune reconstitution inflammatory syndrome (IRIS), which is a paradoxical clinical worsening upon initiation of ART that is thought to be due to a hyperactive or uncontrolled immune restoration. Studies have shown that females infected with HIV-1 elicit a stronger immune response and faster disease progression compared to men with the same viral load. Given the above mentioned higher baseline levels of immune activation in HIV-1 infected females, we hypothesized that immune restoration during ART will have significant sex-based differential outcomes.

The aim of this project was to understand the impact of immune reconstitution in HIV-1-infected men and women during ART by investigating antigen presenting cells (monocytes, myeloid dendritic cells (mDCs) and plasmacytoid dendritic (pDCs) cells) phenotype and function. In total, we investigated cryopreserved samples from eleven HIV-1-infected males and thirteen HIV-1-infected females from which peripheral blood mononuclear cells (PBMCs) and plasma samples were longitudinally collected at defined time points, including before the initiation of ART (TP01), after 1-4 months (TP02) and after 5-8 months of treatment initiation. Changes in toll-like receptor (TLR) responsiveness were analyzed following stimulation with the following toll-like receptor (TLR) ligands: TLR4 ligand Lipopolysaccharide, TLR7/8 ligand CL097, and the TLR9 ligand ODN2216/ CpG. Multiparameter flow cytometry was used to analyze the cytokine production upon TLR stimulation as well as the level of immune activation by analyzing phenotypic characteristics of antigen-presenting cells including monocytes subsets. In addition, multiplex analysis was used to determine levels of plasma pro-inflammatory cytokines (IFN-ɣ, IFN-α, IL-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, TNF-α, IL-12p70, GM-CSF, MIP-1β and IP-10), in males and females at all time-points.

Our results show that the expression of immune activation markers (measured by HLA-DR^+ and $CD38^+$ on T cells) did not significantly differ between males and females, although females showed elevated levels of both activated $CD8^+$ and $CD4^+$ T cells at baseline, the activation levels decreased upon ART initiation. Furthermore, our results demonstrated an increase in the proportion of classical monocytes ($CD14^{++}CD16^-$) in females compared to males at baseline ($p=0.03$). We did not observe any significant differences in the percentage of intermediate ($CD14^{++}CD16^+$) and non-classical monocytes ($CD14^+CD16^{++}$) between males and females at any time point analyzed. Similarly, no sex differences

were evident after TLR ligand stimulation on monocytes in terms of cytokines measured (IFN-α, TNF-α, MIP-1β and IL-12). Interestingly, upon TLR9 stimulation, a significantly higher percentage of pDCs from females produced IFN-α ($p=0.001$, TP03), MIP-1β ($p=0.001$, at TP02) and TNF-α ($p<0.01$, $p<0.001$, TP02 and TP03 respectively) during ART compared to males. In addition, females had increased IFN-α ($p=0.01$) and TNF-α ($p=0.004$) production on pDCs during ART compared to baseline following TLR9 stimulation.

Taken together, our data suggest sex-specific differences in the level of immune reconstitution during ART, as females show signs of elevated immune response and inflammation compared to males Therefore, these findings may provide a basis for future studies in larger cohorts aimed at adapting ART therapy based on sex differences in disease progression rates in men and women.

CHAPTER ONE: LITERATURE REVIEW

1.1 Global overview of the HIV epidemic vs. South Africa

The human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS) which was initially oberseved in 1981 in homosexual men who presented with depletion of T-lymphocytes and opportunistic infection (Friedman-Kien et al., 1981). HIV has since been described as one of the world's leading health challenges and is considered the leading global infectious killer with 39 million estimated deaths reported since the 1980s. It has been reported that 1.1 million people have died as a result of AIDS-related diseases in 2015 (UNAIDS, 2016). According to the World Health Organization (WHO), approximately 36.9 million people were living with HIV/AIDS worldwide despite having the number of new infections declining from 4.8 million in 2004 to 2.1 million in 2016 (UNAIDS, 2016). Moreover, Sub-Saharan Africa is the epicenter of the epidemic, with 25.8 million people living with HIV-1 in 2014, followed by Asia with 5 million HIV infections. Middle East and North Africa had the least cases of HIV-1 infections at approximately 240,000 (Figure1.1).

Even though new infections have declined, South Africa continues to have the highest prevalence of HIV-1 compared to other countries in the world, where 7 million people are reported to be infected by the virus. Furthermore, 380,000 new infections were reported in 2016 and in the same period 200,000 people have been reported to have died of AIDS related illnesses (Zuma et al., 2016). The prevalence of HIV disease differs by geographical region and is reported to be higher in rural informal areas than in urban areas. The KwaZulu-Natal (KZN) province has the highest HIV-1 infection prevalence (16.9%), closely followed by Mpumalanga (14.1%), Free State (14%) and North West (13.3%) provinces, respectively. The Western Cape had the lowest HIV-1 prevalence (5%) followed by the Northern Cape (7.4%) and Limpopo (9.2%) (Simbayi et al., 2014). Although KZN has the highest HIV-1 prevalence, this province has achieved notable reductions in mother to child transmission (MTCT), decreasing from 9.6% in 2008 to less than 2.0% at the end of 2014 (AVERT, 2017).

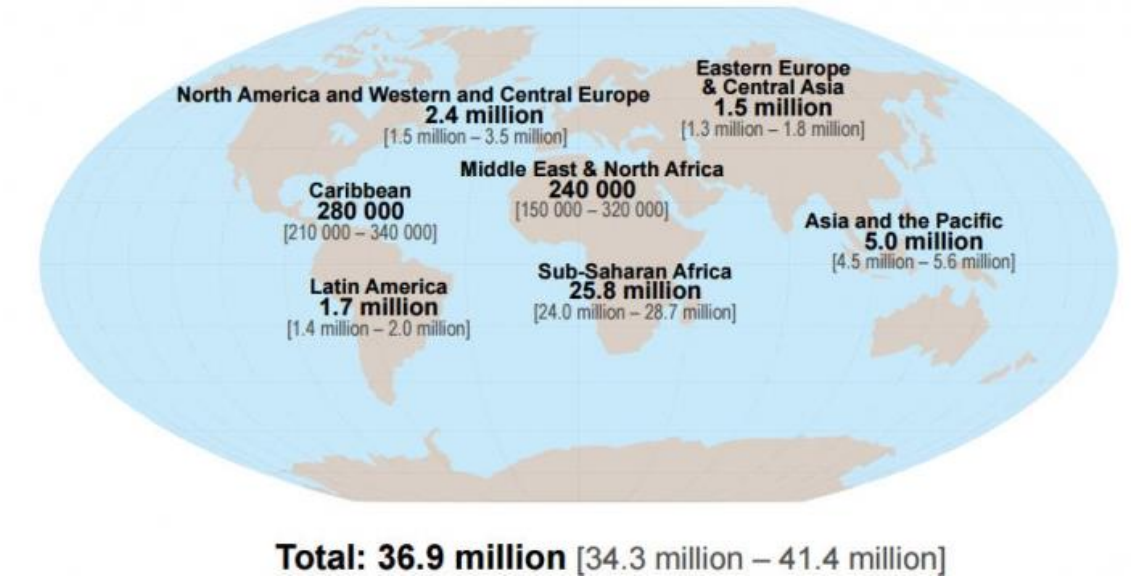

Figure 1.1: Global view of children and adults living with HIV in 2014. Sub-Saharan Africa experiences the great burden of HIV (UNAIDS, 2016).

1.1.1 Sex differences in HIV-1 infection in South Africa

The epidemic continues to have a profound effect on women accounting for 60% of people infected with HIV-1, particularly young women, since they represent a vulnerable population due to factors such as sexual violence, limited economic opportunities, sex with older men and inability to negotiate condom usage. As a result, of these factors, HIV-related risks have become acute for young girls and women in South Africa (HIV/AIDS, 2015). The prevalence of HIV-1 was particularly highest among females aged 30–34 and among males aged 35–49 (Figure 1.2). Moreover, the prevalence of HIV remains significantly higher in females than males in all age groups but remains highest at age 20-44. This shows a 10% difference between the sexes which highlights that the burden of the epidemic is mainly seen among females (Simbayi et al., 2014).

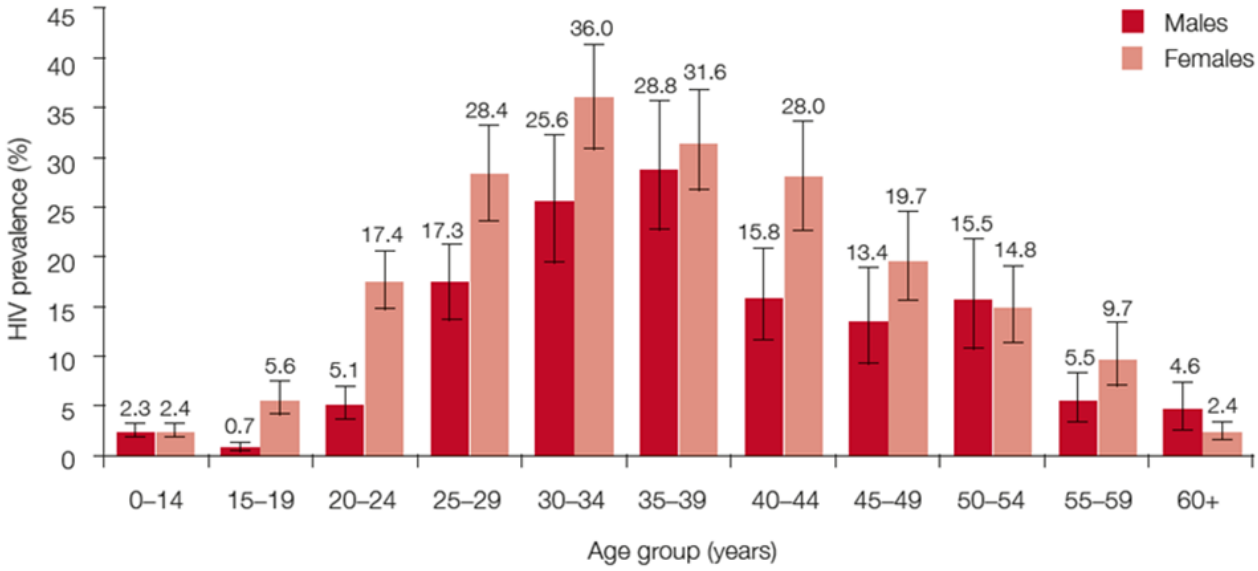

Figure 1.2: Overall HIV prevalence in gender of all age groups in South Africa. In all age groups females have high prevalence of HIV (Simbayi et al., 2014).

1.3 Molecular Biology of HIV

1.3.1 Structure of the HIV

HIV is a *Lentivirus* of the *Retroviridae* family and it is surrounded by a lipid bilayer membrane derived from the human host cell. Each viral particle consists of glycoprotein complexes (gp41 trans-membrane protein and gp120 on the surface) that are anchored into the lipid membrane (Figure 1.3). The matrix protein p17 reside on the inner surface of the viral membrane, following this is the capsid protein (p24) on the core of viral particle, the capsid encloses two copies of (+) single stranded HIV-1 RNA which is the part of protein nucleic complex that consists of p7, reverse transcriptase (RT) and other crucial viral enzymes and proteins (Goto et al., 1998).

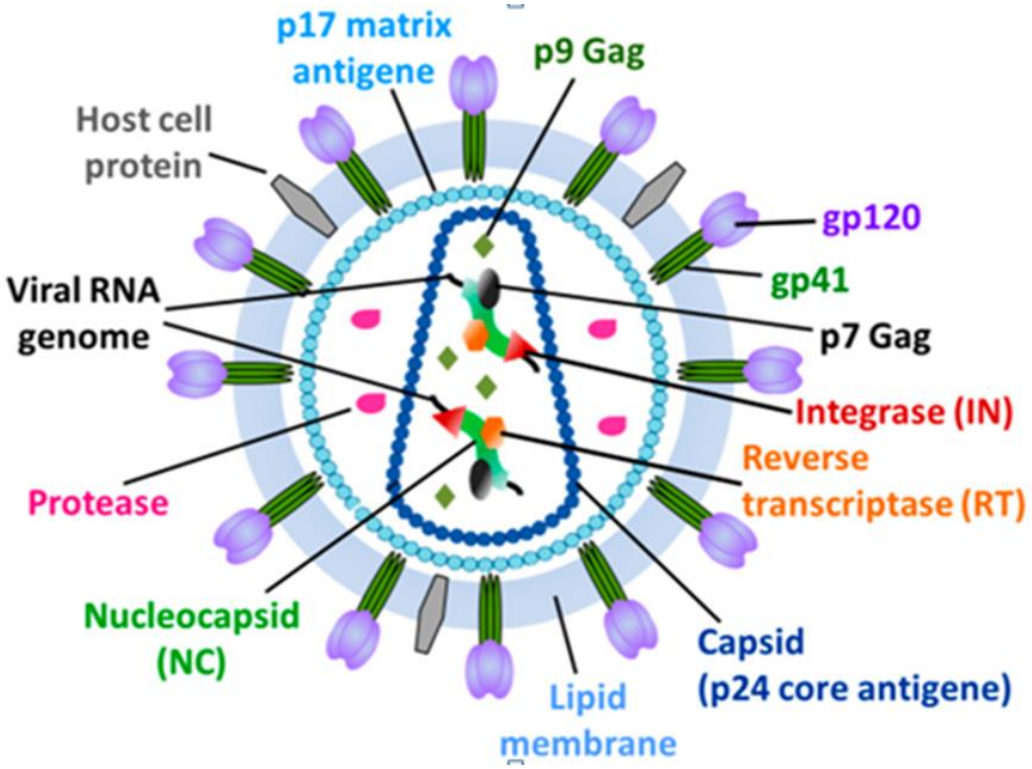

Figure 1.3: Structure of HIV virion particle. The capsid is surrounded by lipid membrane. HIV viral particles have diameter of 100nm and each viral particle contains 72 glycoprotein spikes which are embedded into the lipid membrane (Musumeci et al., 2015).

The HIV genome is 9.2kb long with a long terminal repeat (LTR) at each end of its genome and consists of nine open reading frames (ORF) that produce viral proteins which are divided into 3 classes: 1) the structural proteins, including Gag, Pol and Env which are essential for structural formation and replication, 2) the regulatory proteins Tat and Rev which accelerate production of the HIV virus and 3) the accessory proteins Vpu, Vpr, Vif, and Nef ensure virus survival and transmission (see Figure 1.4) (Frankel and Young, 1998).

Gag proteins are first synthesised as a polyprotein precursor p55, upon budding of the viral particle from the infected cell, p55 is cleaved by protease into four smaller matrix p17 proteins, which provide stability to the virion (Freed, 1998). The p24 capsid protein plays a crucial role in assembling of an infectious virus particle. Nucleocapsid is located at the virion core where it is tightly bound to RNA (Levin et al., 2010). These proteins can be generated from Gag polyprotein or Gag-Pol precursor polyprotein which is generated through ribosomal frame shift (Könnyű et al., 2013).

The *pol* gene encodes for the polyprotein which is cleaved by protease to produce viral enzymes, namely, protease, integrase, reverse transcriptase and RNase H. Reverse transcriptase facilitates the translation of HIV ssRNA into dsDNA, which can then be integrated into human genome (provirus) by integrase. Protease cleaves both Gag and Gag-Pol polyprotein to create mature protein component of an infectious HIV virion. The *env* gene encodes a single protein gp160, which is normally cleaved into gp120 surface subunit and trans membrane gp41 glycoprotein by cellular enzymes (Schwartz and Nair, 1999). In addition, there are 2 regulatory genes and 4 accessory genes which are flanked at the 5' to 3' end region that codes for the proteins that modulate HIV's ability to infect cells, replicate or cause disease (virulence) (Freed, 1998).

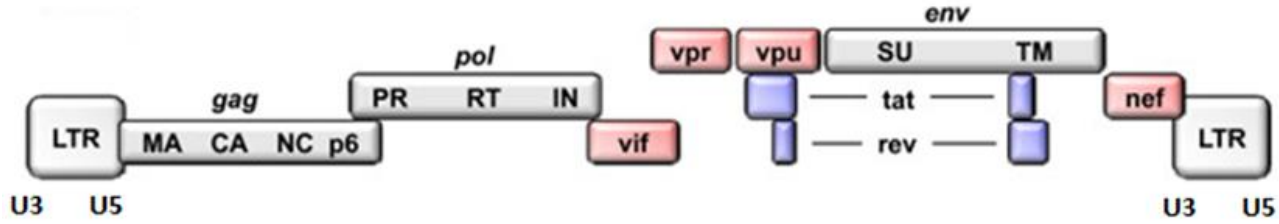

Figure 1.4: The HIV-1 genome. Schematic representation of the HIV-1 gene products encoded by the HIV-1 genomic sequence (Ayinde et al., 2010).

1.3.2 HIV-1 replication

HIV-1 infection takes place when the glycoprotein gp120 binds to the CD4 receptor and co-receptor chemokine receptor 5 (CCR5) or chemokine receptor type 4 (CXCR4) on the surface of the targeted cell. This interaction triggers conformational change in gp120 causing fusion of the viral and cellular membrane, permitting uncoating and release of the viral nucleic component into the cytoplasm of the host cell. Following fusion, the viral ssRNA is reverse transcribed into double stranded DNA by viral reverse transcriptase (RT) in the cytosol (Musumeci et al., 2015). Once synthesised, the viral DNA is transported to the nucleus as part of pre-integration complex (PIC). The viral DNA is inserted into the host DNA (provirus) inside the chromosomes by integrase. The provirus is transcribed using host's RNA polymerase into messenger RNA (mRNA), these transcripts are then translated to viral protein outside the nucleus. The viral RNA and synthesised protein move towards the cell membrane and bud off as immature virus particles. The viral polyproteins are cleaved by protease to form infectious viral particles (Turner and Summers, 1999).

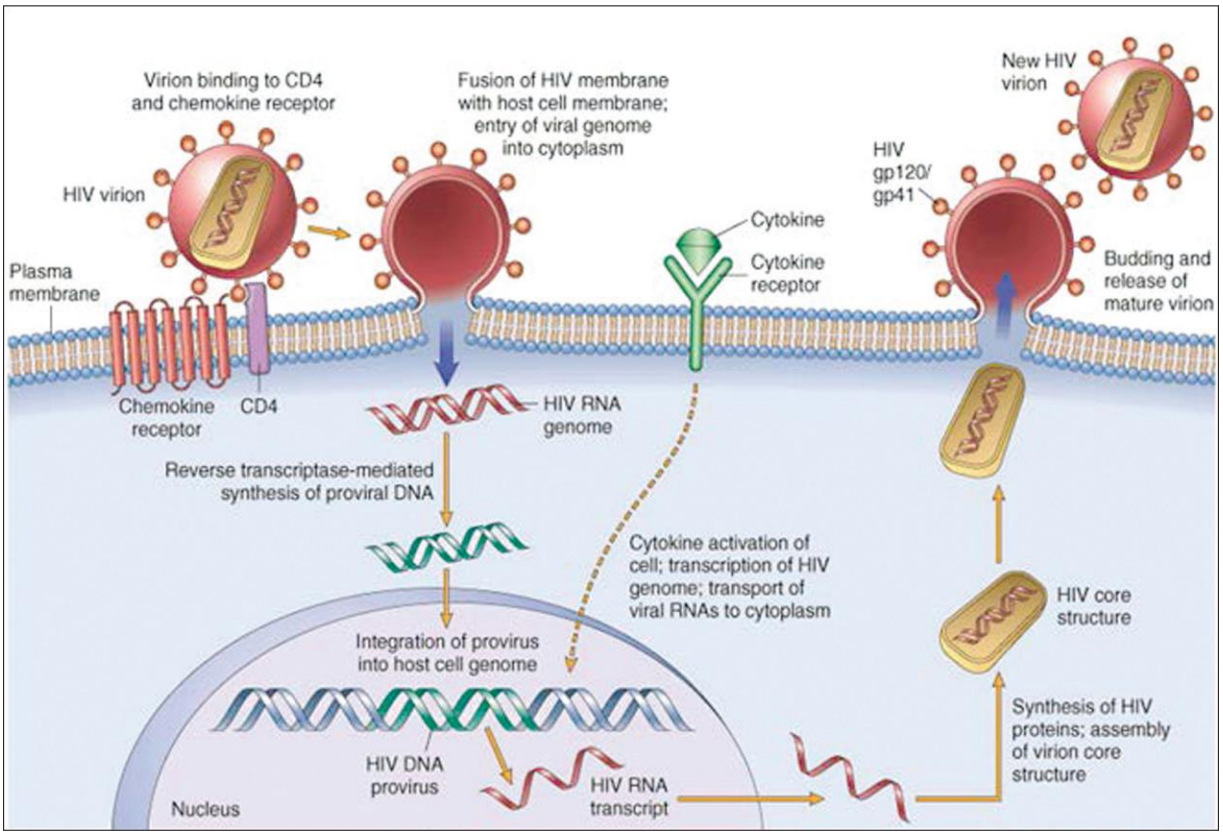

Figure 1.5: Schematic representation of the replication cycle (accessed from online material (MEDICINE, 2011).

1.4 HIV Pathogenesis

1.4.1 Clinical course of HIV-1 infection

HIV is mostly transmitted through sexual intercourse, where the infection is established when a virion binds to CD4 T cells and co-receptors (CCR5 and CXCR4) at the mucosal surface. This is also mediated by dendritic cells at the surface of mucosa, where they bind the virus and migrate to lymphoid organ, mainly gut-associated lymphoid tissue (GALT) to present the virus to the susceptible T cells, thus causing systemic spread and triggering adaptive immune response (Fauci, 2007, Yuki et al., 2007). The course of untreated HIV infection can differ significantly amongst patients. Different people progress at different rates through stages of HIV disease progression due to various factors such as genetic makeup among others. The course of HIV infection has been well documented and is characterized by an acute phase which usually last for a few weeks, followed by a chronic phase which last for 3-10 years and finally an AIDS phase which is caused by immune collapse (Figure 1.6).

Acute HIV-1 infection is characterized by an exponential increase in the viral RNA in the first three to six weeks, when infected individuals are highly infectious. At this stage of infection, there is no antibody formation but the viral RNA can be detected in serum and plasma. Dramatic changes take place in the immune system such as a rapid decline of $CD4^+$ T cells and cytotoxic killing of infected cells by $CD8^+$ T cells (Fauci et al., 1996). Following the acute phase, virus replication reaches a steady state called the viral set point, which takes place during the asymptomatic phase infection. As this stage comes to an end, the viral load increases and the CD4 count continues to steadily decrease thus resulting in full blown AIDS (Fanales-Belasio et al., 2010).

The ability to control HIV replication and the rate of disease progression to AIDS is variable amongst individuals. A small percentage of individuals fall under the category of long-term non-progressors (LTNPs), these individuals infected with HIV maintain their CD4 count within the normal range for more than 7 years in the absence of ART. However, many individuals fall under the category of typical or rapid progressors. Following primary infection, these individuals progress to AIDS within 2 years to 10 years (Pantaleo and Fauci, 1996) .

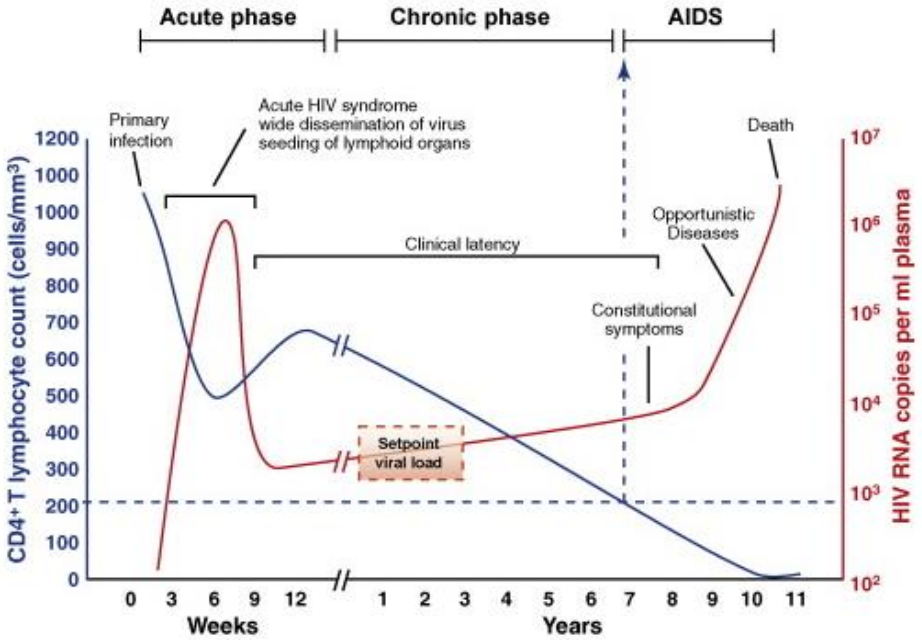

Figure 1.6: A course of HIV infection, depicting acquisition of HIV-1 infection up to the development of AIDS, the dynamics of the viral load in red and CD4 T cell in green is shown (An and Winkler, 2010).

1.5 The role of immune responses in the control of HIV

The innate and adaptive responses both have significant roles in controlling HIV infection. Cells of the innate immune system such as natural killer (NK) cells, dendritic cells and macrophages are the first line of defence against HIV infection. NK cells plays a key role in controlling HIV-1 infection during early stages of infection and shape the adaptive immune response (Chakrabarti and Simon, 2010). They recognize and kill HIV-infected cells by direct recognition of viral proteins or virus induced stress ligands by activating NK cell receptors. HIV-1 infection results in up-regulated expression of stress ligands in infected cells or reduced expression of HLA class I molecules, thus causing infected cells to be more susceptible to NK cell-mediated lysis. Killing of HIV-1 infected cells by killer immunoglobulin receptors (KIR)+ NK cells is mediated by secretion of perforin and granzyme (Altfeld and Gale Jr, 2015). In addition, NK cells are involved in the clearance of infected cells by induction of antibody-dependent cellular cytotoxicity (ADCC), through the potent elimination of antibody-opsonized material via the activation of an Fc-receptor (FcR) on innate immune cells (Berger and Alter, 2011).

Innate immune activation is accompanied by the induction and production of pro-inflammatory cytokines which subsequently leads to activated adaptive immune response, this includes B and T host cell mediated responses. T cells are the second subset of lymphocytes that mediate the adaptive immune response. $CD8^+$ T cells, also referred to as cytotoxic T lymphocytes (CTL) plays an important role in antiviral immunity, they also have greater effect in controlling infection during acute HIV-1 infection (Perreau et al., 2013). HIV-specific T cell responses play an important role in decreasing viral load during acute infection as well as in being able to influence viral load set point. During primary infection, HIV-specific $CD8^+$ T cells coincide with a decrease in viremia (Pantaleo and Fauci, 1996). One of the crucial role of HIV-specific $CD8^+$ T cells in controlling infection was demonstrated in macaques infected with SIV, where depletion of $CD8^+$ T cells resulted in high Simian/Human Immunodeficiency (SHIV) viremia and disease progression (Saez-Cirion et al., 2014). Furthermore, studies have shown that protective HLA class I alleles mediate the initial $CD8^+$ T cell response during acute HIV-1 infection (Chakrabarti and Simon, 2010).

$CD4^+$ T cells, known as T helper' cells ensure crucial support to dendritic cells and B cells in order to induce HIV-specific $CD8^+$ T cells and antibodies. Infection with HIV causes functional impairment of the immune system, subsequently resulting in immune deterioration (Chakrabarti and Simon, 2010). Regulatory $CD4^+$ T cells (Tregs) accumulate in the gut during HIV-1 infection and might contribute to reduced pathogenesis by controlling chronic immune inflammation, and they could also exacerbate infection by suppressing the activation of effector T cells (Saez-Cirion et al., 2014).

HIV infection elicits an antibody or humoral response that targets HIV envelope protein anti-gp41 IgG and lack neutralizing activity during the early stages of infection (Saez-Cirion et al., 2014). Neutralizing antibodies with broad neutralizing activity that recognize conserved regions of the virus envelope protein are only generated after 2-3 years after infection in minority (20%) of individuals. However, the presence of these broadly neutralizing antibodies does not show efficacy in the control of viremia *in vivo*, but has been proven to strongly reduce viremia when administered to SIV-infected macaques and it was dependent on titer and quality of the antibody (Perreau et al., 2013). Studies have found IgA neutralizing antibodies in the genital tract of highly exposed seronegative females in different cohorts, indicating protection by these antibodies from AIDS acquisition in those subjects (Hirbod and Broliden, 2007). The potential role of non-neutralizing antibodies in the control of HIV-1 infection has been demonstrated. Non-neutralizing antibody achieve this by binding to infected cells and recruit activated effector cells (NK cells and macrophage), which induce cytolysis or apoptosis of infected cells. This is accomplished by the formation of immune complex between the IgG Fab portion of the antibody with the viral protein on the cell surface and binding of the Fc portion to the Fc receptors (FcγRs) on effector cells which results in Antibody-dependent cellular cytotoxicity (ADCC) (Overbaugh and Morris, 2012).

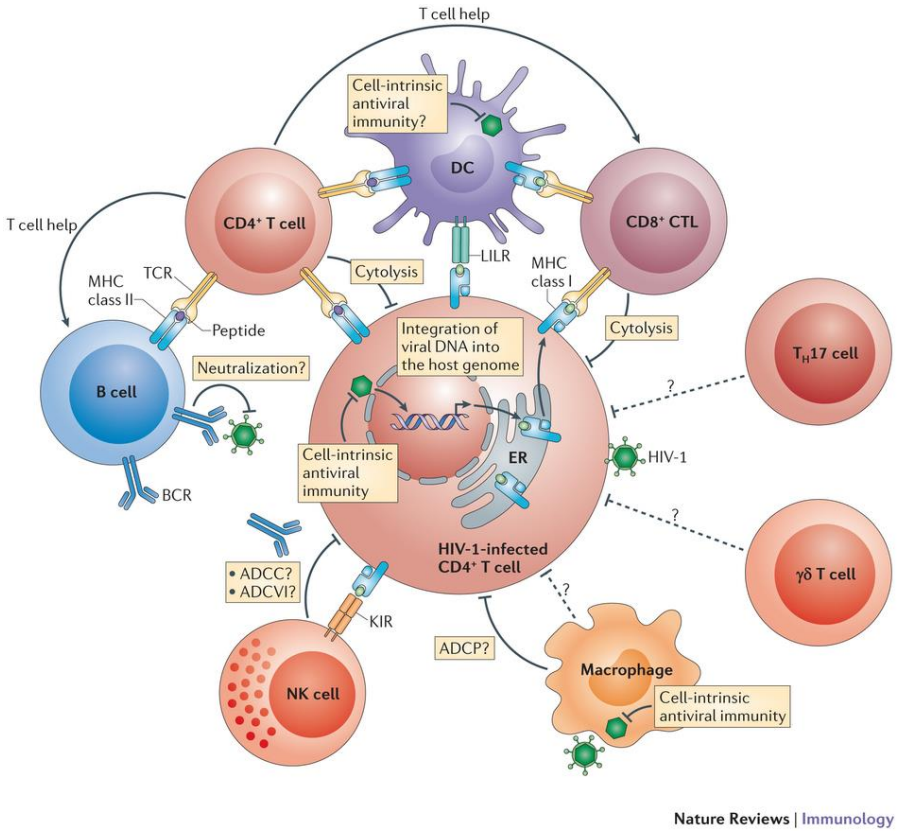

Figure 1.7: Innate and adaptive immune responses contributing to HIV-1 control (Walker and Xu, 2013).

1.6 Role of antigen presenting cells in HIV Pathogenesis

Antigen-presenting cells (APCs) are important immune sentinels that recognize invading pathogens like HIV-1. They play a crucial role as a bridge between innate and adaptive immune systems as they constitute both adaptive and innate immune cells that mediate the cellular immune response by processing and presenting antigens to T cells and induce pro-inflammatory response against antigens (Nagy et al., 2013). APCs of the innate immune system serve as the first line of defense against infection, they recognize and respond to infection using pattern recognition receptors, these receptors bind to ssRNA of HIV-1 and mediate immune response activation (Chang and Altfeld, 2010). Furthermore, classical APCs which include dendritic cells, macrophages and monocytes act as a reservoir of latent HIV infection that can possibly be resistant to antiretroviral therapy, however, this is still an area of debate (Chougnet et al., 2002).

1.6.1 Plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs)

Dendritic cells (DC) are cell population of strong antigen presenting cells derived from the bone marrow and are essential in initiating and regulating both innate and adaptive responses (Fauci et al., 1996). Two populations of DCs have been identified in blood, myeloid dendritic cells (mDCs) which are characterized as $CD11c^+$ and plasmacytoid dendritic cells (pDCs) defined by $CD123^+$. Myeloid dendritic cells (mDCs) are the most common DCs that are circulating in the blood while pDCs are present at low levels (0.1-1%). DCs respond to infection by recognizing pathogen-associated molecule patterns (PAMPs), HIV-1 ssRNA encodes for multiple PAMPs, which can be recognized by Toll-like receptors (TLRs) on the surface of mDCs and pDCs. As a result, DCs present HIV antigen to $CD4^+$ T cells and induce immune responses (cytokine production). During chronic HIV infection, low counts of circulating DCs exist, and are only recovered by the initiation of antiretroviral therapy (Coleman and Wu, 2009). Plasmacytoid dendritic cells (pDCs) are the main natural IFN-α producers *in vivo* (Chakrabarti and Simon, 2010). Induction of IFN-α is one of the earliest antiviral defense mechanism which decreases HIV-1 infection of several cells and impairs HIV-1 transmission from dendritic cells (DC) to $CD4^+$ T-cells (Tavel et al., 2010).

1.6.2 Monocytes

Monocytes make up 3-8% of blood leukocytes in humans (Sassé et al., 2012), and they exhibit a significant immunological role during HIV-1 infection. This include, responding to inflammatory signals by moving to the site of infection where they will divide or differentiate into macrophages. Moreover, Monocytes play a vital role in presenting HIV antigen to T cells and they have also been suggested to serve as the reservoir of HIV-1 during highly active antiretroviral therapy (HAART), however, this topic

is still controversial (Spivak et al., 2011, Kandathil et al., 2016). Monocytes exist as three major subtypes characterized by CD14 and CD16 expression; classical monocytes ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical or inflammatory monocytes ($CD14^{+}CD16^{++}$). It has been reported that CD16 expressing monocytes exhibit great potential for HLA-restricted antigen presentation and pro-inflammatory cytokine production (Zawada et al., 2011). The proportion of $CD14^{+}CD16^{++}$ and $CD14^{++}CD16^{+}$ are found in abundance in HIV-1 infection (Funderburg et al., 2012). As a result, there has been a great interest in $CD14^{++}CD16^{+}$ due to their co-expression of CCR5 which permits HIV infection. These cell subsets are capable of transferring HIV-1 infection across genital mucosal barrier as well as the central nervous system (Castley et al., 2014).

1.6.3 Sensing of viral pathogens by APCs

The APCs of the innate immune system play a significant role in sensing of viral pathogens and initiating antiviral immune responses. APCs sense viral pathogens through recognition of pathogen–associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and RIG-1-like receptor (Nagy et al., 2013). Furthermore, several studies have shown that two additional intracellular PRRs (interferon inducible protein I6 (IFI16) and cyclic GMP-AMP synthase (cGAS) sensed the HIV-1 infection by recognizing the viral reverse transcriptase product during the early stage of viral replication (Jakobsen et al., 2013, Li et al., 2013, Wu et al., 2013). Following pathogen recognition, rapid immune responses are initiated via signaling pathways, involving NFkβ, MyD88-IRF7 and mitogen-activated protein kinase (MAPK), through phosphorylation of transcription factors which subsequently induce the production of pro-inflammatory cytokines such as IL-6, IL-12, and TNF-α and type I interferon is induced (Takeda and Akira, 2005). This inflammatory response to pathogens results in recruitment of both innate and adaptive immune cells to the site of infection (Nagy et al., 2013).

One of the first events that takes place during interaction of APCs with an invading microorganisms is the engagement of cellular TLRs. TLRs are a family of pathogen sensors of innate immune system that trigger local inflammation, recruitment of effector cells, and secretion of cytokines that modulate both the innate and adaptive immune responses (Brichacek et al., 2010). These are membrane-bound receptors localized at the cellular or endosomal membranes, recognizing PAMPs via the leucine-rich repeat LRR domain and transducing signals to the intracellular environment through the Toll-interleukin-1 receptor TIR domain (Mogensen, 2009).

TLR family members are expressed in wide variety of cells and have developed to recognize diverse range of pathogens. TLR pathways have been found to be involved in the persistent immune activation observed in chronically HIV-1– infected individuals (Chang and Altfeld, 2010). Furthermore, it has been

well established that HIV-1 encodes for multiple TLR7/8 ligands that mediate activation of the innate immune response (Mogensen, 2009). Additionally, TLR 3 and 9 have been described to sense several nucleic acid intermediates generated during the viral life cycle (Mogensen et al., 2010). APCs have played a significant role in inducing innate immune responses via TLR against HIV-1, however this study will focus on TLR7/8 and 9 inducing plasmacytoid dendritic cells, since they have been implicated on contributing to major sex differences in HIV-1 infection.

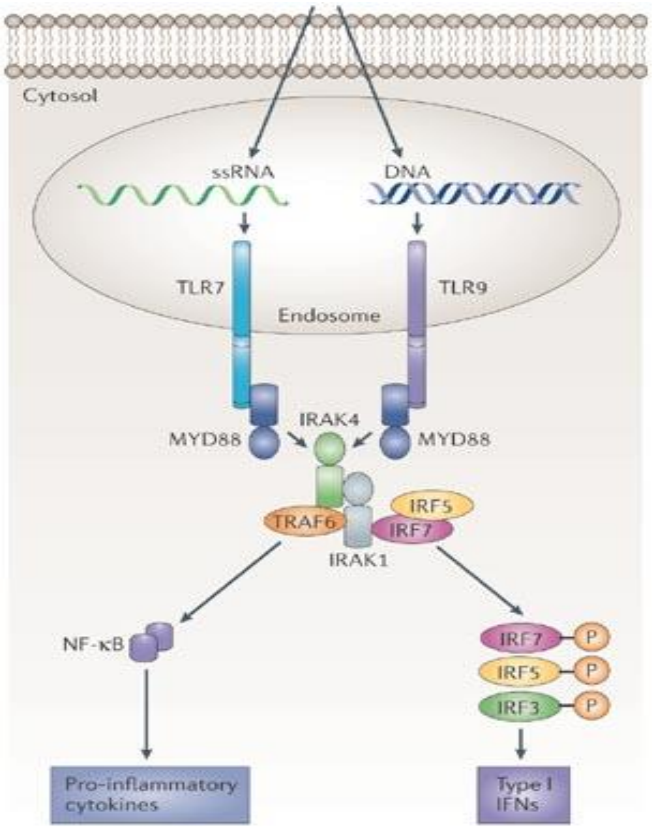

Figure 1.7: Toll-like receptors (TLR) embedded on endosome that senses viral ssRNA, dsRNA and dsDNA. Signaling pathway showing activation of adaptor molecule MYD88 that recruits IRAK and TRAF 6 molecules which results in phosphorylation and activation of NFKB molecules causing induction of Type1 IFN-α and pro-inflammatory cytokines (Gregersen and Behrens, 2006)

1.7 Sex differences in HIV pathogenesis and IFNs anti-viral response

Sex differences in HIV-1 disease progression have been reported previously (Moore et al., 2001). The sex differential outcomes of disease progression have been contradictory, initially HIV infected females were reported to live long following HIV seroconversion, they also had least chances of progressing to AIDS.

Moreover, one study found that women who were infected heterosexually had slow HIV progression rates (Jarrin et al., 2008). However, Mosha et al. (2013) showed similar rates of disease progression between females and males and found out that sex differential disease progression outcomes have changed over the years. Recently, intensive research has been done on determining differences in markers of disease advancement and the distinctive risk between opposite sex progressing to AIDS.

Strong evidence show that females infected with HIV-1 elicit stronger immune responses and faster disease progression in comparison with men who have the same viral loads (Farzadegan et al., 1998). The mechanism underlying these sex differences is not completely understood. However, it has been reported in previous *in vitro* studies that sex differences in HIV-1 infection induced immune activation are associated with high production of IFN-α by plasmacytoid dendritic cells (pDCs) in response to TLR7/8 from females (Meier et al., 2009, Chang et al., 2013). Although other cells, including macrophages, also secrete IFN-α under certain conditions, the IFN-α levels produced by these cell types is several folds lower than that produced by pDCs. TLR7, 8 and 9 play a central role in sensing viral pathogens such as HIV-1 and induce secretion of IFN-α by pDCs, thus contributing to higher levels of immune activation in women compared to men (Farzadegan et al., 1998, Seillet et al., 2012, Torcia et al., 2012).

HIV-1 infected women express significantly increased levels of some markers of immune activation in comparison to men (Gianella et al., 2016). This induction of markers of immune activation has been associated with sex hormones (Meier et al., 2009). Sex hormones have great impact on immune cells functionality, as determined by transcriptome analysis done on PBMCs isolated from both men and women. Sex steroids carry out their function through binding to specific receptors which has been expressed by myeloid and plasmacytoid dendritic cells, receptors like estrogen (ER), androgen receptor (AR) and progesterone receptor (PR). Therefore, this indicates that these hormone receptors have significant immunoregulatory functions on APCs (Fischer et al., 2015).

High production of IFN-α is beneficial for women during acute infection since it results in low viral loads and high CD4 counts (Figure 1.8) (Addo and Altfeld, 2014). Although beneficial during acute infection, the immunomodulatory effect of IFN-α during chronic infection results in persistent chronic inflammation in women compared to men (Addo and Altfeld, 2014). Type I IFNs display numerous biological effects. They have a strong antiviral action but can also cause detrimental action in the host by inducing chronic persistent immune activation in HIV-1 infection (Hosmalin and Lebon, 2006). They activate proliferation of natural killer (NK) cells and T cells and nitric oxide synthesis by macrophages, and enhance lysis of infected cells by cytotoxic T cells or T helper type 1 cells (Hosmalin and Lebon, 2006). IFN-α in particular, plays a central role in activation and maturation of DCs to generate effective APCs with

increased ability to stimulate $CD4^+$ and $CD8^+$ T cells; high levels of IFN-α are strong predictors of HIV-1 clinical progression (Meier et al., 2009). However, immunological effects of IFN-α during immune reconstitution on ART has not been investigated between sexes.

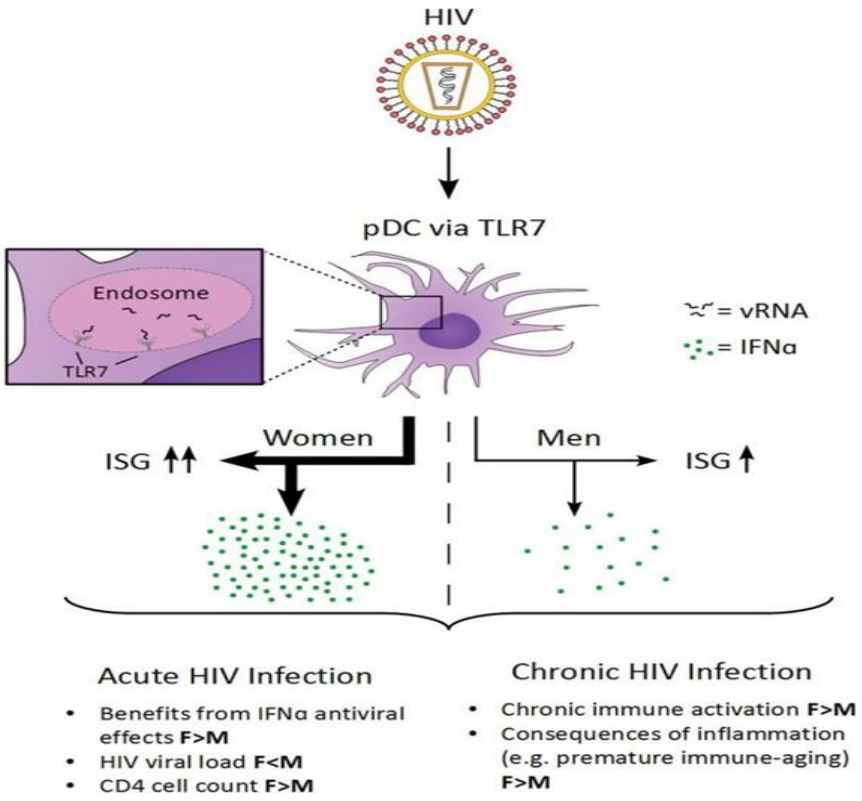

Figure 1.8: IFN-α stimulated gene (ISG) induction following stimulation of plasmacytoid dendritic cells (pDCs) by HIV through Toll-like receptor 7 (TLR-7) between women and men thus resulting in upregulated levels of IFN-α (Addo and Altfeld, 2014).

1.8 Sex differences in response to antiretroviral therapy

South Africa implemented its first national treatment programme in 2004, and currently has the largest ART programme in the world with an estimated 3.5 million people on ART in March 2016 (report, 2015/16). South Africa became the first country in sub-Saharan Africa to completely approve the use of pre-exposure prophylaxis (PrEP) in HIV-1 negative people who are at risk of contracting the virus in December 2015 (AVERT, 2017). Many people will be on ART, since SA has implemented universal treat and test initiative, whereby people living with HIV or testing positive will be initiated on ART immediately after diagnosis, regardless of their CD4 counts as of September 2016 (AVERT, 2017). Despite this intervention of reducing AIDS related mortality and morbidity, the ART programme is

limited by several factors such as co-infection with other pathogens, treatment adherence, drug toxicity, adverse effects and drug resistance.

According to Moore et al. (2003), HIV-1 drug therapy study has shown that equivalent access to care, precise administration and adherence to ART has no significant sex based differences in treatment response. A study showed comparable proportion of females and males attained undetectable viral loads after 6 months of ART (Meditz et al., 2011). In contrast, previous studies proposed that side effects of ART and clinical deterioration could differ by gender. In these studies, the outcomes were mostly seen in first and second generation antiretroviral therapy, where women experienced skin rashes, mitochondrial toxicity, gastrointestinal intolerance and lipodystrophy (Ofotokun and Pomeroy, 2003). These toxicities and side effects may lead to non-compliance from women on ART. Furthermore, clinical studies reported sex-based effects such as nausea in women using Efavirenz compared to men using the same drug (Hodder et al., 2012). However, sex-based adverse effects and immunological response after initiation of ART remains unclear.

1.8.1 Immune reconstitution after anti-retroviral therapy

The introduction of antiretroviral therapy (ART) in Africa has made a significant contribution in minimizing AIDS associated deaths due of immune recovery as a result of decreased HIV-1 replication (El-Sadr et al., 2006). However, in some patients, ART can cause adverse events such as enormous activation of immune response that results in immune reconstitution inflammatory syndrome (IRIS), which is an immune restoration disease that is normally triggered by initiation of ART (Murdoch et al., 2008, El-Sadr et al., 2006). The majority of IRIS cases develop within 3 months of ART initiation during early rapid immune recovery and it can also occur when a failing ART regimen is switched with an effective treatment or when ART is resumed after interruption (Meditz et al., 2011). The most common forms of IRIS are related to underlying opportunistic infections with mycobacteria, cryptococal infections or viruses. The utmost forms of IRIS manifestation can be either an inflammatory response that is caused by unmasking of previously untreated infection or the paradoxical clinical deterioration whereby immune cells target dead or dying organism in a patient that is on appropriate antimicrobial therapy (Dhasmana et al., 2008).

No gold standard characteristics of IRIS exists; currently IRIS is defined as an exaggerated and atypical inflammatory reaction associated with a decrease in HIV RNA concentration from baseline or an increase in $CD4^+$ T cell counts from baseline in recipients of effective ART (French et al., 2004). Although most cases of IRIS occur in patients with low $CD4^+$ T cell counts and high viral load levels prior to antiretroviral therapy, IRIS can occur at any $CD4^+$ T cell count (Müller et al., 2010). Most studies have

reported an increased risk of IRIS in patients initiating ART who have advanced disease (Müller et al., 2010, Zaidi et al., 2012). The proportion of patients that initiate ART and develop IRIS is not well known, but it is estimated to vary between 7 to 50%. Two South African prospective studies showed that 10% of patients starting ART developed all-cause IRIS and TB-IRIS developed in 23% of patients who had started TB therapy prior to ART (Murdoch et al., 2008, Haddow et al., 2009).

1.9 Study rationale

Several studies have enlightened us about the disease progression and the existing sex based differential outcomes between males and females. Antiretroviral therapy (ART) has played a significant role in controlling disease progression and transmission by reducing viral load as the only available lifesaving intervention for people living with HIV-1 infection. However ART has adverse effects on some patients, such as aberrant immune response leading to immune reconstitution inflammatory syndrome (IRIS) (Wilson and Sereti, 2013). Additionally, women are more susceptible to antiretroviral drug-induced adverse effects (Fischer et al., 2015).

There are different underlying biological mechanisms leading to pronounced sex differences in response to HIV-1 infection and immune reconstitution under ART; these include genetic, immunological and hormonal determinants of immunity. Antigen presenting cells especially pDCs of the innate immune system have been implicated in contributing to biased immune shift seen between males and females. Data have shown that women infected with HIV-1 have higher TLR7 mediated IFN-α responses of pDCs that results in higher persistent chronic inflammation in females (Meier et al., 2009). However, very little is known about ART in reversing this immune defect and whether these differ between males and females.

Sex differences have been investigated in vaccine development and studies show that women elicit robust immune responses compared to men (Klein et al., 2010). With the unsuccessful development of an effective HIV vaccine so far, dissecting the biological factors resulting in sex differences in HIV-1 infection is essential to minimize adverse effects on people who are put on ART. This study enabled us to study the consequences of ART on immune reconstitution between sexes through the assessment of antigen presenting cells and pro-inflammatory cytokines in males and females following initiation of ART.

1.10 Aims of the study

Study Aim 1:

Assessment of sex-based differences by analysis of pro-inflammatory function of antigen-presenting cells (APC) in peripheral blood mononuclear cells (PBMCs) in women compared to men.

Objectives

- To assess immune activation during immune restoration under ART by assessing the phenotype and quantification of cytokines (IFN-α, IL-12, MIP-1β and TNF-α) produced by APCs (monocytes, mDCs and pDCs) using multicolor flow cytometry in women and men.
- To determine inflammatory response by evaluating the frequency of activated T cells and monocyte subsets ($CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$) in women and men before and after ART initiation using phenotypic stain.

Hypothesis 1:

We hypothesized that females will have higher levels of immune activation during immune reconstitution under ART that is due to rapid reconstitution of TLR7 based IFN-α production by pDCs in response to HIV compared to men.

Study Aim 2:

Assessment of pro-inflammatory cytokines in plasma using Luminex in women and men before and after initiation of ART.

Objective

- To determine systemic cytokine profiles, the following cytokines: IFN-γ, IL-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IFN-α, TNF-α, MIP-1β, GM-CSF, IL-12p40, IL-12p70 and IP-10 were measured in plasma from males and females using multiplex analysis (Luminex).

Hypothesis 2

We hypothesized that sex differential levels of immune activation would result in increased systemic cytokine production in females due to rapid immune reconstitution.

CHAPTER 2: METHODOLOGY

2.1 Study Ethics consideration

This study was approved by the Biomedical Research Ethics committee (BREC) of the University of KwaZulu-Natal (Ref number: BE148/15), informed consent was obtained from all participants. Ethical approval was obtained for the studies (Ref number: NK-E118/06; SK- E028/99 and AI- E036/06) from Edendale, Sinikithemba and Acute infection study.

2.2 Study Participants

Samples from 24 black South African treatment experienced participants enrolled in HPP studies; Edendale, Sinikithemba and Acute infection, which were recruited from Pietermaritzburg and Durban areas in KwaZulu-Natal, were used for this study. Of these 24 patients, 11 were males and 13 were females, which were sampled before ART initiation and following ART. Clinical characteristics of the study participants are described below. In brief, peripheral blood mononuclear cells (PBMCs) and plasma samples were obtained and analyzed at one time-point before initiation of ART (at baseline) and at two time-points within 8 months of ART start date. All the patients enrolled had a $CD4^+$ T cell count of between 50 and 400 cells/mm^3 at initial sampling. A significant difference was observed in $CD4^+$ T cell counts ($p<0.02$) and viral load ($p<0.04$) at baseline between males and females (Appendix II).

2.3 Viral load and CD4 count measurement

Absolute CD4 counts were measured by TruCount technology using flow cytometry. Plasma viral loads were measured using Roche Amplicor version 1.5 test and NucliSens HIV-1 QT assays, as previously described (Koofhethile et al., 2016). The sensitivity of the both tests was 50 RNA copies/ml.

2.4 Isolation and preparation of cryo-preserved PBMCs

The Ficoll density gradient method was employed for the isolation of PBMCs from whole blood samples as previously described (Thobakgale et al., 2007). This process entails overlaying of blood onto Ficoll Histopaque (Sigma), which can then be centrifuged to obtain gradients of specified densities. After centrifugation, cells with greater density such as granulocytes and erythrocytes sediment at the bottom of the tube while PBMCs form a layer in the middle. Cells of interest are then harvested, counted and cryopreserved in liquid nitrogen at (-210 ˚C -346 ˚F) for long term storage until further use.

2.5 Thawing of cryo-preserved cells

Cryopreserved PBMCs were used in our assays. Cells were thawed before use, this process entails holding the cell vial into a water bath at 37 ˚C until small amount of frozen pellet remained. The outside of the cryo-tube was swabbed with 70% ethanol before it was quickly transferred to a 15ml tube, followed by addition of 10ml of pre-warmed R0 medium (RPMI 1640) drop wise to the cells and centrifuged for 7 minutes, 1500 rpm at 25 ˚C. After centrifugation, the supernatant was discarded to ensure that all the freezing solution containing dimethyl sulfoxide (DMSO) was removed to minimize cell toxicity. The cell pellet was re-suspended in warm R10 medium (500 ml RPMI 1640 (Sigma) supplemented with 50 ml heat inactivated & filter sterilized fetal calf serum, 5 ml L-glutamine, 5 ml Penstrep fungizone (100x) & 5 ml HEPES) and centrifuged again for 7min, 1500 rpm at 25 ˚C. The cell pellet was then re-suspended in 10 ml of R10 medium followed by cell counting. Cells were counted manually by performing a 1 in 10 dilution of cells in trypan blue; 10 µl of the mix was then pipetted on the haemocytometer counting slide (HYCOR Biomedical, USA) and the cells were counted using Olympus CH20 microscope (Olympus Optical, Japan). The cells were then reconstituted to a desired concentration of 1.5 million/ml in R10 before use in subsequent in vitro stimulation assays or phenotypic characterization using multiparameter flow cytometry assays.

2.6 Flow cytometry assays

BD LSR Fortessa was used in this study for phenotypic and intracellular cytokine staining assays. Flow cytometry is made of 3 basic components, which include: fluidic system, optics (lasers) and electronics system. It consists of 5 lasers, however only 4 were used for our experiments; (blue laser 488nm, yellow green laser 560nm, red laser 640 and violet laser 405) and can be used to detect up to 18 colours simultaneously. Flow cytometry is defined as the simultaneous analysis of multiple physical characteristics of a single cell, as the cell flows in the stream of fluid through a laser. This technique involves the use of labeled cell components with the antibody fluorochromes, which are excited by the laser to emit light and fluorescence collected by lenses and filters to appropriate detectors while collecting data which is stored in the computer. Generated light scatter distinguishes between cells by size and internal complexity, whereas light emitted from fluorescently labeled antibodies identifies a wide array of cell surface and cytoplasmic antigens.

2.6.1 Phenotypic assessment of antigen presenting cells and quantification of T cell activation

For phenotypic assays, 250 to 500 hundred thousand PBMCs were stained with viability dye (1µl/ml) (Invivogen) and surface makers using anti-CD3 BV650, anti-CD123 BV421, anti-HLADR PE-CF594, anti-CD19 BV510, anti-CD56 BV510, anti-CD11c PE Cy5, anti-CD14 APC-Cy7, anti-CD38 PE, anti-

CD4 APC, anti-CD69 PerCP/Cy5.5 (all from Biolegend), anti-CD86 PE Cy7, anti-CD8 FITC and anti-CD16 BV786 (from BD Biosciences) at optimal staining concentrations, (Table 1). The cells were incubated in the dark for 20min at room temperature (RT), and then washed with 2%PBS-FCS to block non-specific binding. Cells were fixed with medium A (Invitrogen) for 15 minutes at room temperature and then washed and resuspended in 120µl of PBS before flow cytometry acquisition. Initial gating was on lymphocytes SSC-A versus FSC-A, followed by removal of doublets by forward scatter height (FSC-H) versus forward scatter area (FSCA). Live/dead, B cells and NK cells were discriminated by the use of viability dye, CD19 and CD56 respectively; subsequently the CD3 negative ($CD3^-$) and T cells population were gated on, followed by identification of $CD8^+$ and $CD4^+$ T cells, before activated $CD8^+$ and $CD4^+$ T cells were identified. From the $CD3^-$ population, monocytes subsets were gated on based on expression of $CD14^+$ and $CD16^+$ and specifically classified as (classical monocytes $CD14^{++}CD16^-$, intermediate monocytes $CD14^{++}CD16^+$ and non-classical monocytes $CD14^+CD16^{++}$). Gates were standardized using fluorescence minus one (FMO) controls. The stained cells were acquired on a LSR FORTESSA (BD Biosciences). Spectral overlap was corrected by suitable compensation and rainbow beads were employed to retain the consistency of the fluorescence intensity between experiments. A minimum of 100 000 to 500 000 events were acquired for phenotypic assays. The gating strategy is shown in Figure 3.2.

2.6.2 In vitro Stimulation of PBMCs with TLR ligands

Antigen-presenting cells (APCs): monocytes, plasmacytoid dendritic cell (pDCs) and myeloid dendritic cells (mDCs) from PBMCs were analyzed for their ability to secrete cytokines in response to TLR stimulation (immune activation) using multiparametric flow cytometry (FORTESSA, BD Biosciences). For ICS assays, between $1.5x10^6$ - $2x10^6$ cells/ml PBMCs resuspended in R10 medium were used. The cells were rested for 2 hours before cell stimulation with the following conditions: TLR7/8-CL097, TLR4-LPS and TLR9-CpG from Invitrogen for 18h hrs. The cells were treated with a protein transport inhibitor Brefeldin A (BFA, Sigma) to assess intracellular cytokine production (Figure 2.1). CpG stimulant was prepared prior to stimulation, the stimulant was freshly prepared each time by adding 5.5ul of DOTAP transfection reagent (Roche) to 64µl of RPMI followed by incubation for 10 minutes at room temperature (RT). After incubation, 10µl of CpG and a further 27.5 µl of RPMI were mixed and incubated for 15 minutes at RT. Following CpG preparations, a total of 1.5 x 10^6 to 2 $x10^6$ of PBMCs per ml were stimulated with: 1µl/ml CL097 (Invivogen), 1µl/ml LPS (Invivogen) and 10^7 µl/ml CpG/DOTAP/RPMI mix in duplicate. Unstimulated cells with media alone were used as a control. BFA 5µl/ml was added immediately to the tubes to inhibit release of cellular cytokine before incubating for 18 hrs at 37^0C.

2.6.3 Analysis of cytokine production by multiparameter flow cytometry

After 18hrs of incubation, BFA tubes were washed with PBS and centrifuged at 1500 rpm for 7 minutes at 25˚C. The supernatant was discarded followed by addition of viability dye (1µl/ml) (Invitrogen) and surface antibody master mix of: anti-CD3 BV650, anti-CD123 BV421, anti-CD19 BV510, anti-CD56 BV510, anti-CD11c PE Cy5, anti-CD14 APC-Cy7, anti-CD38 PE, anti- CD4 APC, anti-HLA-DR PE-CF594 (all from Biolegend) (Table 1) and incubated for 20 minutes at RT in the dark. While the incubation was taking place, the supernatant was collected from tubes without BFA for LUMINEX and the pellet was dissolved in 1ml Trizol (Life technologies) for PCR in each condition per time point; then all tubes were frozen down at -80˚C for downstream assays beyond the scope of this masters work.

Once incubation was done in BFA tubes, cells were washed with 2%FBS/PBS and then fixed with 60µl Fix & Perm medium A (Invitrogen) for 15 minutes at RT, followed by washing with 2%FBS/PBS. Next, the cells were permeabilized using 60µl Fix & Perm medium B (Invitrogen) and stained intracellularly with anti-IFN-α PE, anti-TNF-α BV655, anti-IL-12 APC Cy7 and anti-MIP-1β FITC for 20 minutes at RT in the dark. The cells were washed and re-suspended in 140ul of PBS and analyzed on the FORTESSA (as mentioned above). Spectral overlap was corrected by suitable compensation and rainbow beads were employed to retain the consistency of the fluorescence intensity between experiments. A minimum range of 500 000 to 1 000 000 events were acquired for ICS assays. Cell populations of interest assayed for intracellular cytokines were defined as follows: monocytes (lineage-HLA-DR^{+}CD11c-CD123-CD14^{+}), mDCs (lineage- HLADR^{+}CD11c^{+}CD123^{-}CD14^{-}) pDCs (lineage HLADR^{+}CD11c^{-} CD123^{+}CD14^{-}). After successful acquisition the data files were saved to the BD FACS database then exported for further analysis with FlowJo version 10 (TreeStar, Inc.). The gating strategy is shown in (Figure 3.5, 3.7 and 3.9) and positive responses of (TNF-α, IFN-α, MIP-1β and IL-12) were reported after background subtraction.

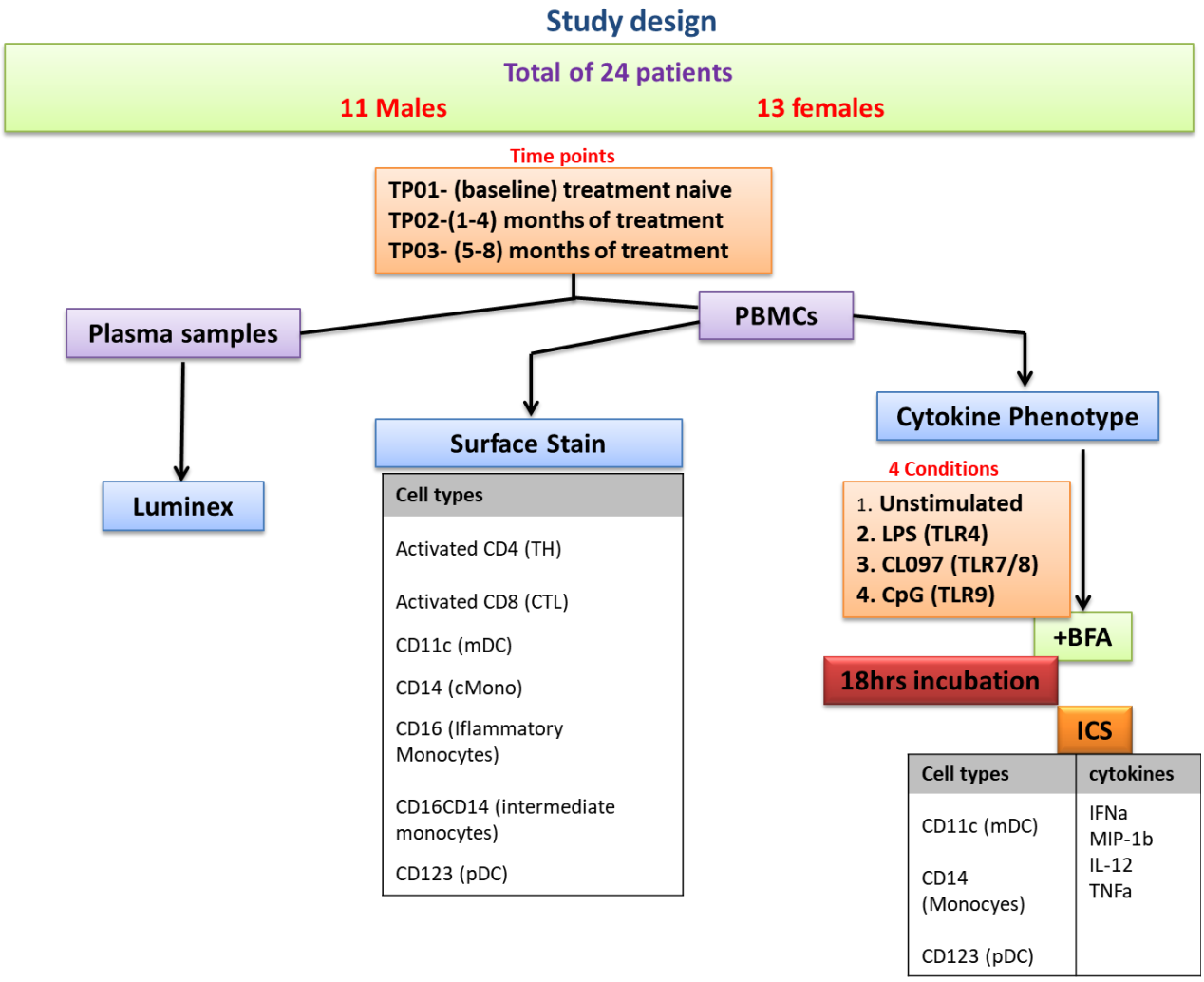

Figure 2.1: Schematic overview of study design for phenotypic and ICS and staining illustrating samples size and panels. PBMC samples (n=24) from males (n=11) and females (n=13) were used in flow cytometry assays.

Table 2.1: Flow cytometry panels of the Phenotypic and ICS stains showing Fluorochromes used for analysis.

Fluorochrome/ antibody	Volume (1x)µl	PANEL 1: Ex vivo		volume (1x)µl	Panel 2: ICS
Per-CP-Cy5.5	2.5	CD69	Surface markers		
PE-Cy7	1	CD86			
PE-Cy5	1	CD11c		1	CD11c
PE-CF594	1	HLA-DR		1	HLA-DR
APC-Cy7	1	CD14		1	CD14
BV421	1	CD123		1	CD123
BV786	1	CD16			
BV650	2.5	CD3	Excluded	2.5	CD3
BV510	1	Zombie aqua		1	Zombie aqua
	2.5	CD19/56		2.5	CD19/56
FITC	1	CD8	Intracellular Cytokines	2.5	MIP-1b
PE	2	CD38		1	IFN-α
BV605				1	TNF-α
APC	1	CD4		1	IL-12

2.7 Quantification of soluble cytokines in plasma using Luminex multiplex Assays

Cytokines were measured in the plasma of samples with matched timepoints as the PBMCs. Luminex (Bio-Plex Pro assays) was used to simultaneously measure the levels of both anti and pro-inflammatory cytokines in 13 females and 8 males (only 8 male plasma samples were analysed due sample unavailability) before initiation of treatment, during 1-4 months and 5-8 months of treatment. The Bio-Plex assay involves the use of microspheres which are 6.5 microns in size and fluorescently dyed to create 100 distinct colours of bead. Two fluorescent dyes are used and the precise concentration of these fluorescent dyes creates 100 distinctly coloured bead sets. These beads are coated with a specific capture antibody, therefore this methodology has the potential to analyse 100 different analytes simultaneously.

The technique of Luminex was invented from the traditional ELISA assay with the objective of quantifying low levels of different cytokines in one sample at the same time. This method involves the use of beads coated by the captured antibody. Coupled beads react with the analyte containing the biomarker of interest. After a series of washes to remove unbound protein, a labelled streptavidin detection biotinylated antibody which is specific for the captured antibody-cytokine complex is added to create sandwich complex. When the microspheres are passed through a laser which excites the fluorescent

dyes within the microsphere, the red laser classifies the bead according to what is being analysed (cytokine) and the green laser classifies the assay result according to the concentration of the analyte. The results are therefore quantified based on the fluorogenic emission. An overview of the technique is shown in (Appendix I).

Plasma concentration of IFN-ɣ, IL-1α/β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, TNF-α, IL-12p70, IL-17, G-CSF, GM-CSF, MCP-1, MIP-1β, IFN-α, IL-12 p(40) and IP-10 was determined in duplicate by (Bio-Plex Pro Human Cytokine 17-Plex Panel: 1x96-well, Bio-Plex Pro Human IFN-Alpha 2 Assay: 1 x 96 well, Bio-Plex Pro Human IL-12 (p40) Assay: 1 x 96 well and Bio-Plex Pro Human IP-10 Assay: 1 x 96 well) kits (Bio-Rad, Laboratories GmbH). Luminex was done according to manufactures manual (BIO-RAD Laboratories, Inc). Plasma samples were prepared by making 1:4 dilutions, adding 1 volume of sample to 3 volume of sample diluent, prior to assay setup. Standards and quality controls are included in each kit. The standards were reconstituted and serially diluted to 1:4 in an assay buffer. These standards were used as a reference for the quantification of the analytes.

To briefly explain the method; wash buffer was added to the microtiter filter plate and placed on shaker at 850±50 rpm for 10 minutes at room temperature. The detection beads (microspheres) were added to the wells and then washed to remove all unbound beads. The standards, assay buffer and samples were added and incubated for 30 min on shaker at 850±50 at RT. After incubation, the plate was washed again and the detection antibody was added. After 30 min of incubation at RT, the plate was washed again and the Streptavidin-PE conjugate was added, which acted as the reporter dye. This was incubated for 10 min on shaker at 850±50 rpm at RT. After incubation, plate was washed twice and the beads were resuspended in assay buffer, then the samples were quantified using Bio-Plex 200 system (Bio-Rad). Cytokines measured beneath the detection limit of the assay were given a value of the midpoint between zero and the detection limit value of the assay and they were included in the analysis.

2.8 Data and statistical analysis

2.8.1 Intracellular cytokine staining and phenotypic stain

The percentage of pDCs, mDCs and monocytes producing cytokines was determined by subsequent analysis using FlowJo software, version 10 (Treestar, Inc). FlowJo results were then exported to Graph Pad Prism, version 5 and analyzed by Two-way ANOVA for multiple comparisons across the time points for both groups. One Way ANOVA (Kruskal-Wallis test) and t- test (Mann-Whitney test) was used to asses cytokine production in different cell population between the groups. Results are expressed as means±SEM percentage of the cell population producing the following cytokines: TNF-α, IFN-α, MIP-1β and IL-12. A p value of <0.05 was considered significant.

For phenotypic analysis, One way Anova (Kruskal-Wallis test) was used to calculate the frequency of monocytes subset and T cell activation across the time points in each group and t- test (Mann-Whitney test) was used to compare males and females at each time point. The correlation of activated T cell ($CD8^+$ and $CD4^+$) with viral load (copies/ml) in females and males at baseline was performed using Spearman's non-parametric correlation. A smooth line was generated by linear regression. The Benferroni correction method was used to correct for multiple comparisons in all analyses.

2.8.2 Cytokine profiling using Luminex

Different methods of statistical analysis were used to determine if there were any sex differences between males and females producing pro- and anti-inflammatory cytokines before and after treatment initiation. The Bio-plex Manager software was used to acquire the data from the multiplex and singleplex assay plate. The data was analysed by Graph Pad Prism, version 5. Non-parametric t test (Mann-Whitney test) was used to determine the differences between males and females for each plasma cytokines in each time point. Kruskal-Wallis test was used to determine if there were any changes in each plasma cytokine level at baseline, during 1-4 months of treatment and 5-8 months of treatment in each group (males or females). The Spearman's correlation test was used to determine if there was an association between plasma cytokine concentration (TNF-α, IFN-α and MIP-1β) and monocytes, mDCs and pDCs producing (TNF-α, IFN-α and MIP-1β) after stimulated with CL097, CpG and LPS. All the tests were two tailed and tests for significance were assessed at a 0.05 level of significance. The Benferroni correction method was used to correct for multiple comparisons in all analyses. Twenty cytokines were tested but only 15 were detected and these were; IFN-ɣ, IFN-α, IL-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, TNF-α, IL-12p70, GM-CSF, MIP-1β and IP-10.

2.8.2.1 Principal Component analysis of Luminex results

Luminex data was analysed using multivariate techniques, specifically principal component analysis (PCA). Data was exported to an excel spread sheet and saved as CSV file. STATA was used to identify principal components which accounted for the majority of the variation in our dataset with the factor selection based on an eigenvalue > 1.0 (see Appendix VII for the scree plot). PCA is a data reduction procedure that permits the major sources of variation in a multi-dimensional dataset to be analyzed without bias being introduced. PCA analysis was primarily performed to evaluate variation in the cytokine data set and to determine presence of clustering. Secondly, heat map was generated using components, to demonstrate contribution of each cytokines responsible for any of the clustering present (Appendix VIIII). Heat map was generated using the Miner Suite of bioinformatics software (CIMminer clustered image map Institute, from Genomics and Bioinformatics group, NIH). The Mann-Whitney U test was used to compare differences in cytokine production in each PC score between females and males infected with HIV-1. All the tests were two tailed and assessed at a 0.05 level of significance**.**

CHAPTER 3: RESULTS

3.3 The clinical characteristics results of participants

A total of 11 males and 13 females subjects were recruited from the Sinikithemba, Acute infection and Endendale studies from Pietermaritzburg and Durban areas in KwaZulu-Natal were used in the assays. The subjects were analyzed at baseline (before treatment), during 1-4 months of treatment and 5-8 months of treatment. Assays were done in matched time points on plasma and PBMCs samples. The clinical characteristics of study participants at baseline were as follows: median age of 31 years (IQR, 27-38 years) for females and a median of 39 years (IQR, 32-51 years) for males. The median $CD4^+$ T cell count for females was 179 cells/mm^3 (IQR, 107-271 cells/mm^3) and a median of 297 cells/mm^3 (IQR, 203-353 cells/mm^3) for males. The median viral load for females was 79 167 RNA copies/ml (IQR, 15 750-131 152 copies/ml) and 188 500 RNA copies/ml (IQR, 83 000-750 000 copes/ml) for males. The viral load significantly decreased in both males ($p=0.001$) and females ($p<0.0001$) upon initiation of antiretroviral treatment (1-4 months of treatment). Furthermore, the levels of viremia markedly decreased at 5-8 months of treatment compared to baseline between males ($p<0.0001$) and females ($p<0.01$). However, most women achieved full viral suppression during the study period, whereas a much smaller percentage of the men achieved this during the study period. Reconstitution (restoration) of $CD4^+$ T cells was minimal in males and significantly higher in females ($p=0.02$: Figure 3.1). Furthermore, several females had clinically defined AIDS (CD4<200) at the start of the study, while only one of the male participants had a CD4<200 and a significant difference was observed in viral load ($p<0.04$) at baseline, with males showing high levels of viremia compared to females (Appendix II).

3.2 Characterization of activated CD8 and CD4 T cells and monocytes subsets

Immune activation was first measured by assessing $CD8^+$ and $CD4^+$ T cells. $CD8^+$ and $CD4^+$ T cell activation was evaluated by co-expression of human leukocytes antigen (HLA)-DR^+ and $CD38^+$ phenotypic (activation) markers. Therefore, T cell activation was characterized by measuring changes in the frequency of $CD8^+$HLA-DR^+CD38^+ and $CD4^+$HLA-DR^+CD38^+ at baseline and following ART initiation in males (n=11) and females (n=10), (see gating strategy: Figure 3.2). There were no significant differences observed in both $CD8^+$ and $CD4^+$ activated T cells as determined by the frequency of HLA-DR^+ and $CD38^+$ co-expression between males and females at all time-points measured (Figure 3.3 A + B). We next determined if there was an association between viral loads and activated T cells as previously shown by other studies (Meier et al., 2009, Scully et al., 2016). We observed a positive correlation of HIV viremia with both $CD8^+$ ($r=0.72$, $p=0.02$) and $CD4^+$ ($r=0.66$, $p=0.04$) activated T cells in females at baseline (Figure 3.3 C & D). In contrast, no correlation with viral load was observed in $CD8^+$ ($r=0.0173$,

p=0.52) and $CD4^+$ (r=0.18, p=0.61) T cells of males co-expressing $HLA\text{-}DR^+$ and $CD38^+$ (Figure 3.3 C and D).

We next evaluated the innate immune response by assessing the inflammatory status of monocytes in males and females since previous reports have indicated that different subsets of monocytes, in particular the intermediate (defined as $CD14^{++}CD16^+$) and the non-classical (defined as $CD14^+CD16^{++}$) monocytes are contributors to inflammation in HIV-1 infection (Campbell et al., 2014, McCausland et al., 2015). We found significant differences in the proportion of classical monocytes (defined as $CD14^{++}CD16^-$) between males and females at baseline (p=0.03) where females showed a higher frequency of this subset compared to males (Figure 3.4 A). However, no significant differences were seen between males and females during the treatment phase (Figure 3.4 A). Similarly, no significant differences were observed in the frequency of intermediate and non-classical monocytes between males and females at baseline (before treatment), during 1-4 (TP02) and 5-8 months (TP03) of treatment (Figure 3.4 B & C). Taken together, these data reveal a possible inflammatory status and immune activation in females that is not observed in males.

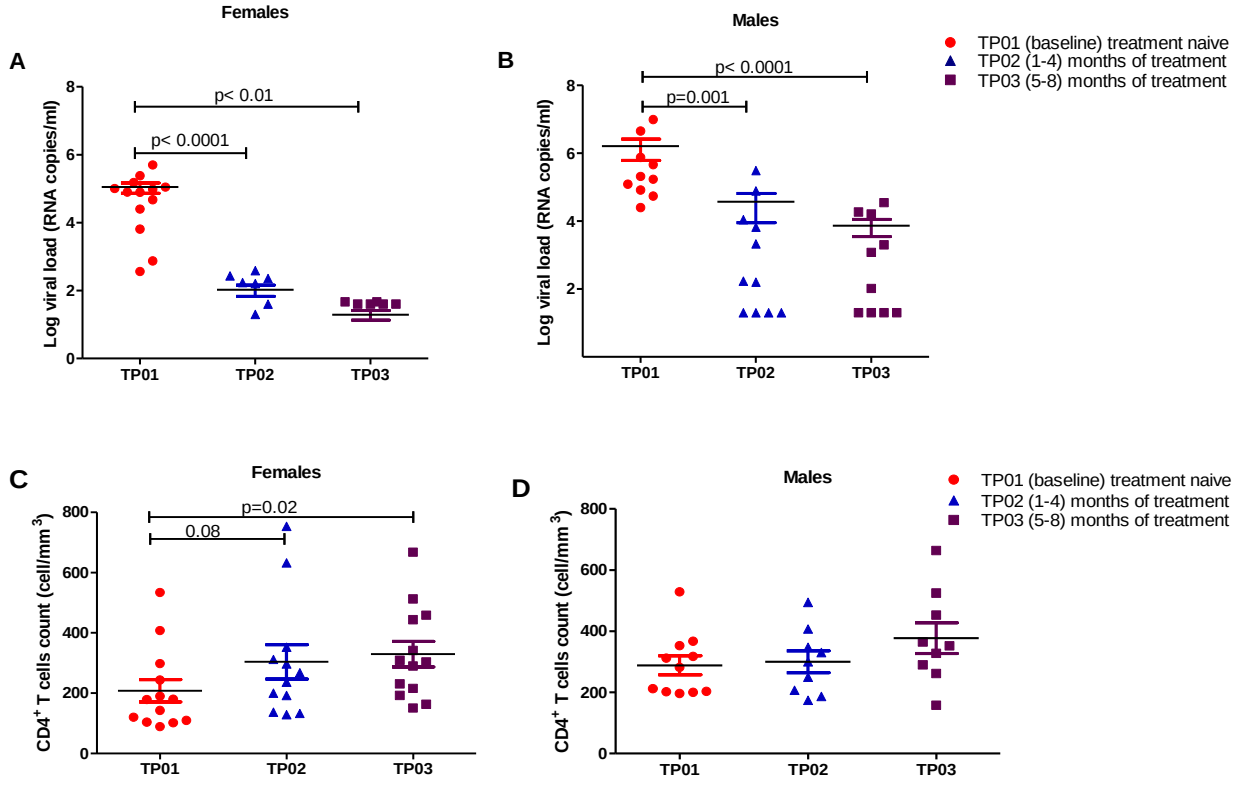

Figure 3.1: Levels of HIV viremia (A-B) and $CD4^+$ T cell counts (C-D) at baseline, 1-4 and 5-8 months of antiretroviral treatment in females (n=13) and males (n=11). Each color and shape represents time point. Data presented as Mean with SEM.

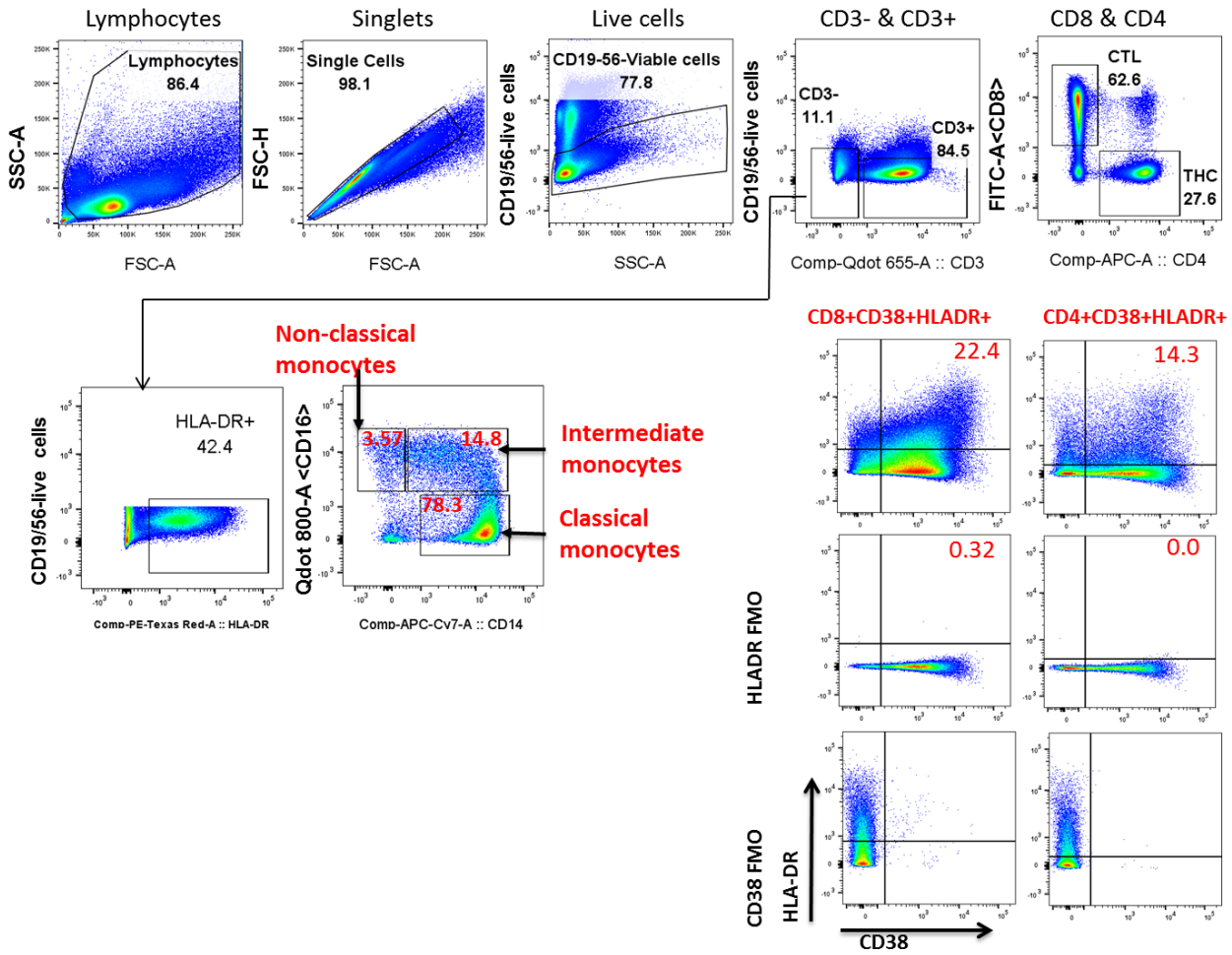

Figure 3.2: Gating strategy showing activated CD8$^+$, CD4$^+$ T cells and monocytes subsets. Identification of total monocytes from peripheral blood mononuclear cells was assessed following exclusion of doublets, dead cells, CD56$^+$ and CD19$^+$ expressing cells. Monocyte subsets were gated from the CD3$^-$HLA-DR$^+$ plot and identified based on the expression of CD14 and CD16 (left side)**.** Three Monocytes subsets were identified as: Classical (CD14^{++}CD16$^-$), intermediate (CD14^{++}CD16$^+$) and non-classical or inflammatory monocyte (CD14$^+$CD16^{++}). Activated CD8$^+$ and CD4$^+$ were from the CD3$^+$ population and defined by CD8$^+$CD38$^+$HLA-DR$^+$ and CD4$^+$CD38$^+$HLA-DR$^+$. The HLA-DR and CD38 fluorescence minus one (FMO's) were used as a control**.** All the T cell plots were gated according to the (FMO's) plot. The flow plots showing data for one representative female patient at time point (TP02) 1-4 months of treatment**.**

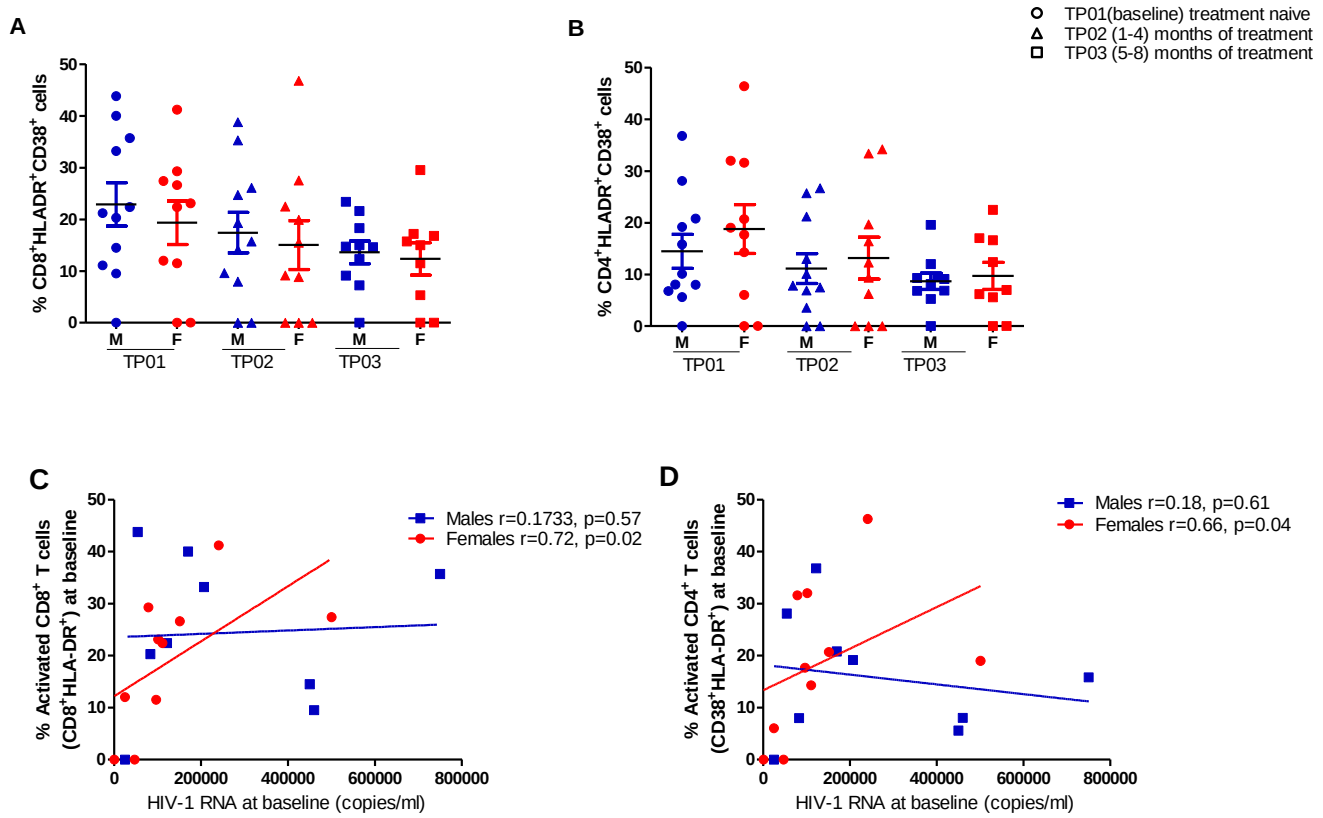

Figure 3.3: CD8$^+$ and CD4$^+$ T cell activation in males and females. The frequencies of CD8$^+$HLADR$^+$CD38$^+$ (A) and CD4$^+$HLADR$^+$CD38$^+$ (B) between males (n=11) and females (n=10) at baseline and during 1-4 and 5-8 months of post ART. t- test (Mann-Whitney test) was used to compare groups at each time point and One way Anova (Kruskal-Wallis test) was used to calculate frequency across the time points in each group. The correlation of activated T cell (CD8$^+$ and CD4$^+$) with viral load (copies/ml) (C and D respectively) in females and males at baseline was performed using Spearman's nonparametric correlation. A smooth line was generated by linear regression.

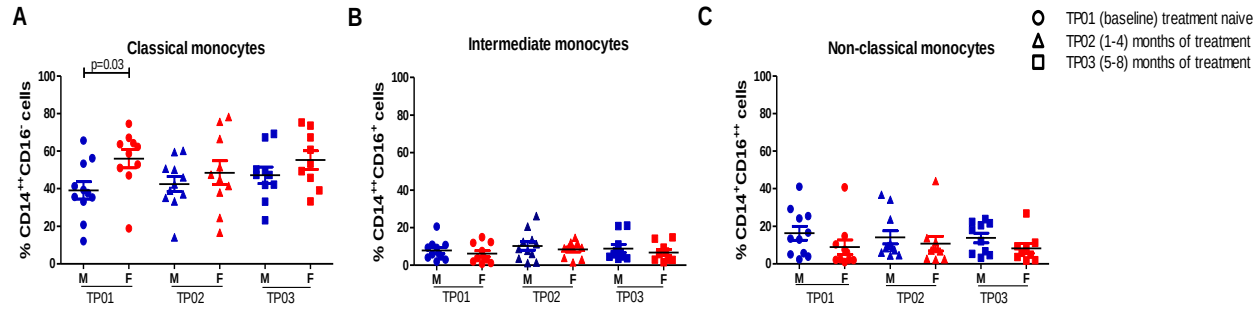

Figure 3.4: Representative plots showing percentage of monocytes subsets: (A) Classical monocytes, (B) intermediate monocytes and (C) non-classical monocytes between males (n=11) and females (n=10) at baseline, during 1-4 and 5-8 months of treatment. Median values and interquartile range are shown. t- test (Mann-Whitney test) was used to compare groups at each time point and one way Anova (Kruskal-Wallis test) was used to calculate frequency across the time points in each group.

3.4 Measurement of TLR responsiveness in females and males by ICS

We next investigated the pro-inflammatory function of the following antigen presenting cells (monocytes, mDCs and pDCs) from cryopreserved PBMCs of study participants at baseline and following ART initiation to determine their polyfunctionality since these cells have been implicated in induction of excess immune activation in HIV-infected patients (Hosmalin and Lebon, 2006). Monocytes were characterized based on their relative expression of ($HLA\text{-}DR^{+}CD14^{+}CD11c^{-}CD123^{-}$; Figure 3.5). No significant differences were seen in the levels of the following cytokines (TNF-α, IFN-α, MIP-1β and IL-12) measured on monocytes following stimulation with CL097 (TLR7/8), CpG (TLR9) and LPS (TLR4) between males and females before ART and after ART initiation (Figure 3.6 A-D). The same observation was made in mDCs (defined as $HLA\text{-}DR^{+}CD14^{-}CD11c^{+}CD123^{-}$; Figure 3.7), indicating no significant differences in the levels of (IFN-α, TNF-α and IL-12) production between males and females (Figure 3.8 A, B and D). However, the levels of MIP-1β-produced by mDCs in response to TLR9 ligand (CpG) were higher at 5-8 months of treatment in females compared to males ($p= 0.01$) and a trend towards higher MIP-1β production was also noted in females at 5-8 months post ART compared to baseline (Figure 3.8 C).

We next assessed pDCs defined as $HLA\text{-}DR^{+}CD14^{-}CD11c^{-}CD123^{+}$ (Figure 3.9). In contrast to the above, we observed sex differences with females demonstrating a higher percentage of pDCs producing TNF-α in response to TLR9 ligand compared to males at 1-4 and 5-8 months of treatment ($p= 0.001$: Figure 3.10) but not at baseline. In addition, females had greatly increased levels of TNF-α producing pDCs following TLR9 stimulation during 5-8 months of treatment compared to baseline ($p= 0.004$: Figure 3.6 A). Although there was a high induction in TNF-α production in response to TLR7/8 ligand (CL097), no significant differences were noted between males and females before treatment and after treatment initiation, similarly for LPS responses (Figure 3.10 A). Regarding IFN-α production by pDCs, females produced higher levels of IFN-α than males in response to TLR9 after 5-8 months of treatment ($p=0.001$) and also there was evidence of reconstitution of IFN-α responses on treatment compared to baseline on pDCs following TLR7/8 (CL097) and TLR9 (CPG) Figure 3.10 B). However, no differences were observed in IFN-α responses between males and females at baseline.

No sex differences were seen in levels of MIP-1β produced by pDCs in response to TLR4 and TLR7/8 ligands before and post ART (Figure 3.10 C). However, females had significantly higher levels of MIP-1β produced by pDCs in response to CpG ligand (TLR9) in comparison to males during 1-4 months of treatment ($p=0.001$) and no significant sex differences were observed at the subsequent time-point (TP03) at 5-8 months of treatment (Figure 3.10 C). In contrast to the above, only minimal IL-12 responses were

induced by the TLR-ligands and none showed any differences between males and females at all time-points measured.

Previous data have shown an inverse relationship between TNF-α production by monocytes and viral load (Scully et al., 2016). In the contrast to previous reports, our data showed no significant association between stimulus-specific cytokine (TNF-α) production from monocytes and plasma viral load at baseline in both males and females (Appendix III). Taken together, our data consistently demonstrate sex differences in cytokine specific responses across mDCs and most evidently on pDCs (TNF-α, IFN-α & MIP-1β pDCs) following stimulation with CpG-TLR9 ligand during ART. Moreover, females produce robust immune responses, demonstrating reconstitution of immune responses in response to TLR9 ligand stimulation during treatment compared to baseline.

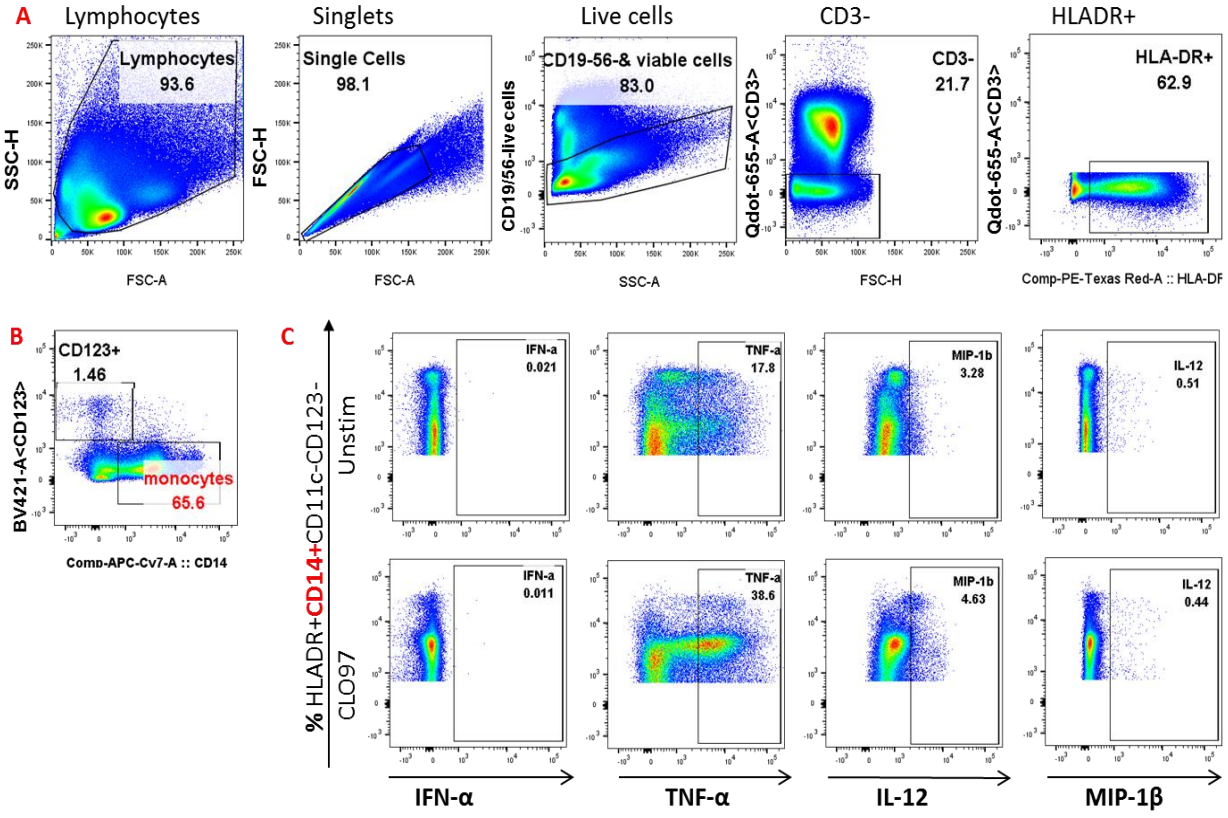

Figure 3.5: A representative scheme showing the flow cytometry gating strategy used for the analysis of monocytes population (CD14) and cytokine production in PBMCs. Panel A: Lymphocytes were gated from the SSC-H/FSA-A gate, followed by exclusion of doublets, dead cells, $CD56^+$, $CD19^+$ and $CD3^+$ expressing cells. $HLADR^+$ cells were gated from the $CD3^-$ cells. Panel B: monocytes population was obtained from the $HLA\text{-}DR^+$ cells. Panel C: representative plot of cytokine production by monocytes, showing unstimulated and one stimulation with CL097 (TLR7/8).

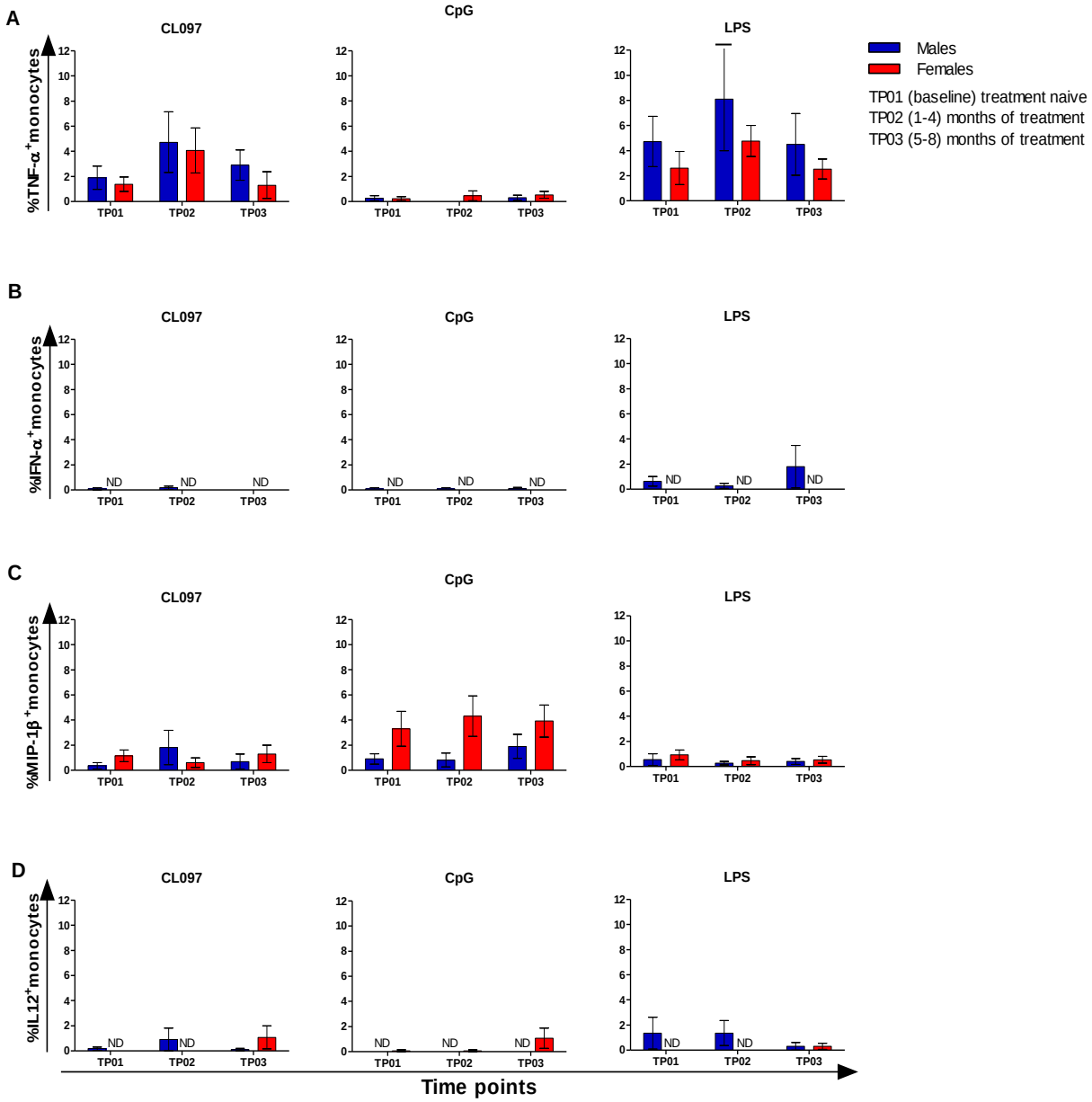

Figure 3.6: Sex differences in cytokine production by monocytes. The above plots shows percentage of (IFN-α, TNF-α, IL-12 and MIP-1β) after background (unstimulated) subtraction produced by monocytes following 18hrs of stimulation with CL097 (TLR7/8), CpG (TLR9) and LPS (TLR4) ligands at baseline, during 1-4 and 5-8 months of treatment between males (n=11) and females (n=13). Two-way ANOVA was used to calculate percentage of cytokines across the time point between males and females and One way Anova (Kruskal wallis test) was used to compare time points in each group, followed by t- test (Mann-Whitney test) comparing groups in each time point. Data presented as mean±SEM. ND represent cytokines which were not detected.

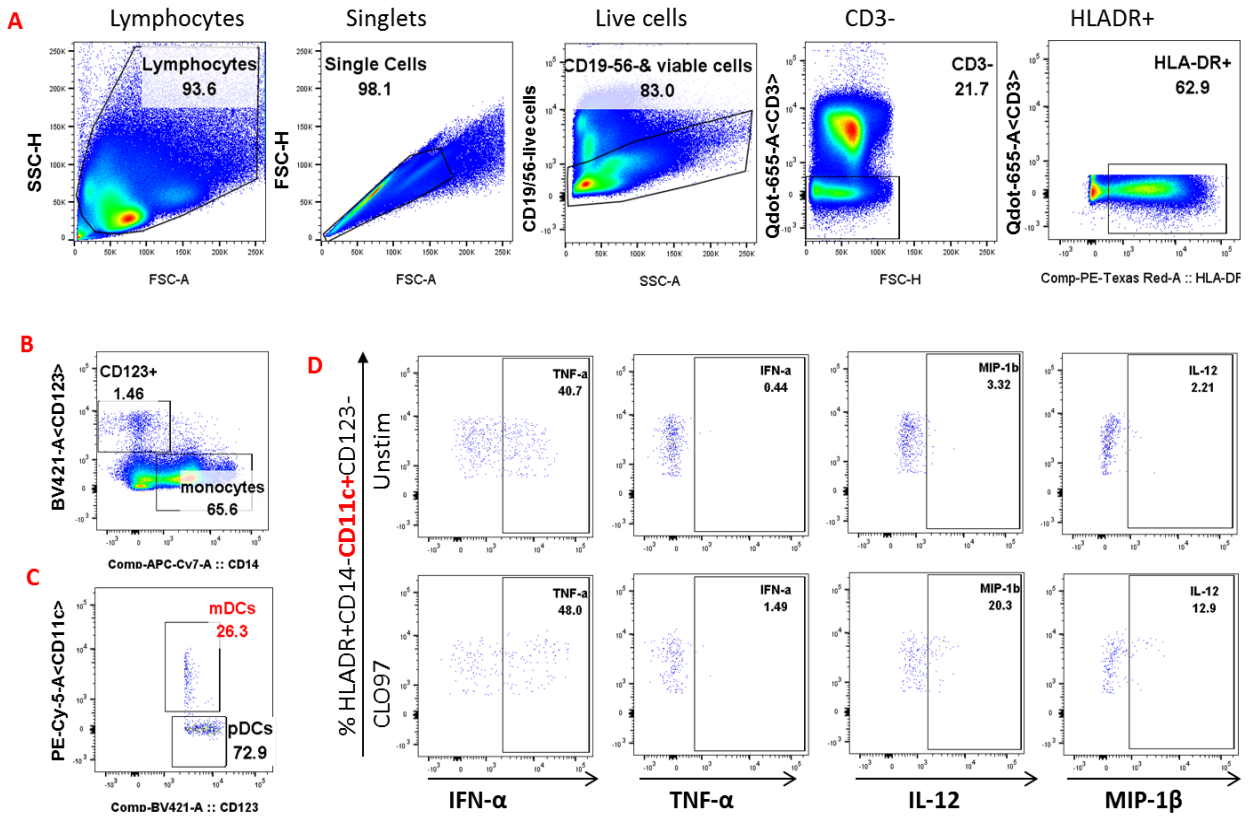

Figure 3.7: A representative scheme showing the flow-cytometry gating strategy used for the analysis of mDCs population (CD11c) and cytokine production in PBMCs. Panel A: Lymphocytes were gated from the SSC-H/FSA-A gate, followed by exclusion of doublets, dead cells, $CD56^+$, $CD19^+$ and $CD3^+$ expressing cells. $HLADR^+$ cells were gated from the $CD3^-$ cells. Panel C: mDCs population was obtained from the $CD123^+$ cells and mDCs were characterized by $CD11c^+CD123^-$. Panel D: representative plot of cytokine production by mDCs, showing unstimulated and one stimulated with CL097 (TLR7/8).

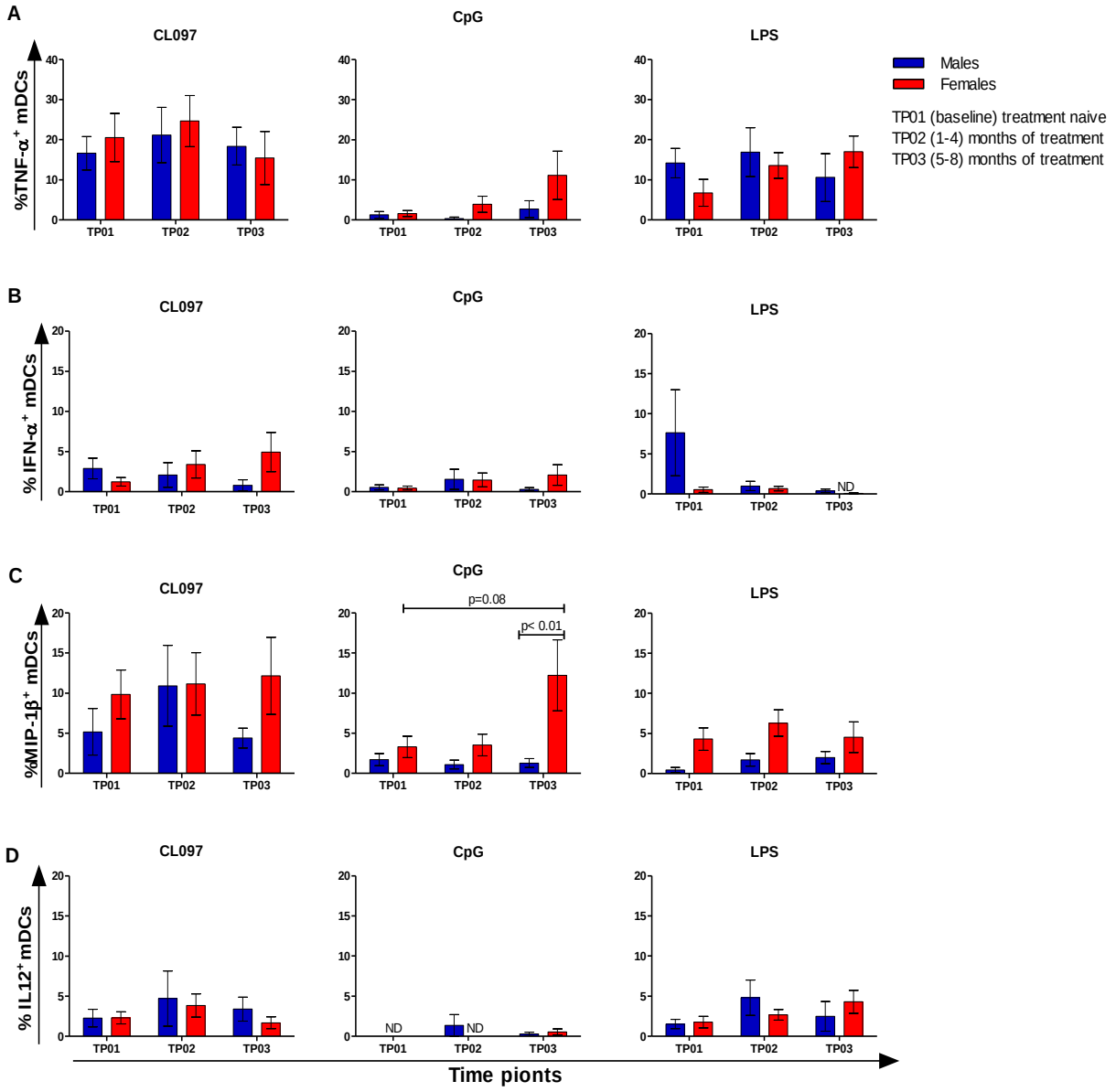

Figure 3.8: Sex differences in cytokine production by mDCs. The above plots shows percentage of cytokines (IFN-α, TNF-α, IL-12 and MIP-1β after background subtraction) produced by mDCs following 18hrs of stimulation with CL097 (TLR7/8), CpG (TLR9) and LPS (TLR4) ligands at baseline, during 1-4 and 5-8 months of treatment between males (n=11) and females (n=13). Two-way ANOVA was used to calculate percentage of cytokines across the time point between males and females and one way Anova (Kruskal wallis test) was used to compare time points in each group, followed by t- test (Mann-Whitney test) comparing groups in each time point. Data presented as mean±SEM. ND represent cytokines which were not detected.

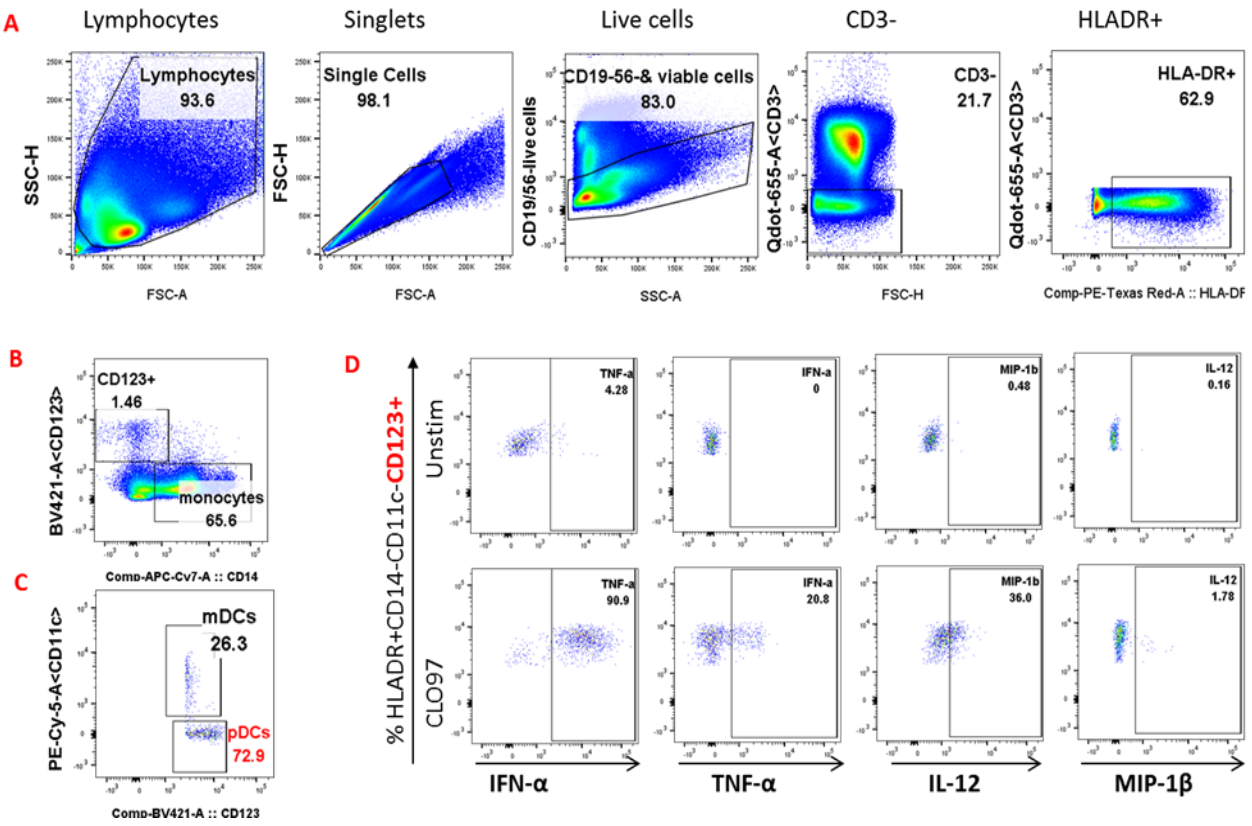

Figure 3.9: A representative scheme showing the flow-cytometry gating strategy used for the analysis of pDCs population (CD123) and cytokine production in PBMCs. Panel A: Lymphocytes were gated from the SSC-H/FSA-A gate, followed by exclusion of doublets, dead cells, $CD56^{+}$,$CD19^{+}$ and $CD3^{+}$ expressing cells. $HLA\text{-}DR^{+}$ cells were gated from the $CD3^{-}$ cells. Panel C: pDCs population was obtained from the $CD123^{+}$ cells and pDCs were characterized by $CD11c^{-}CD123^{+}$. Panel D: representative plot of cytokine production by pDCs, showing example of unstimulated and one stimulated with CL097 (TLR7/8).

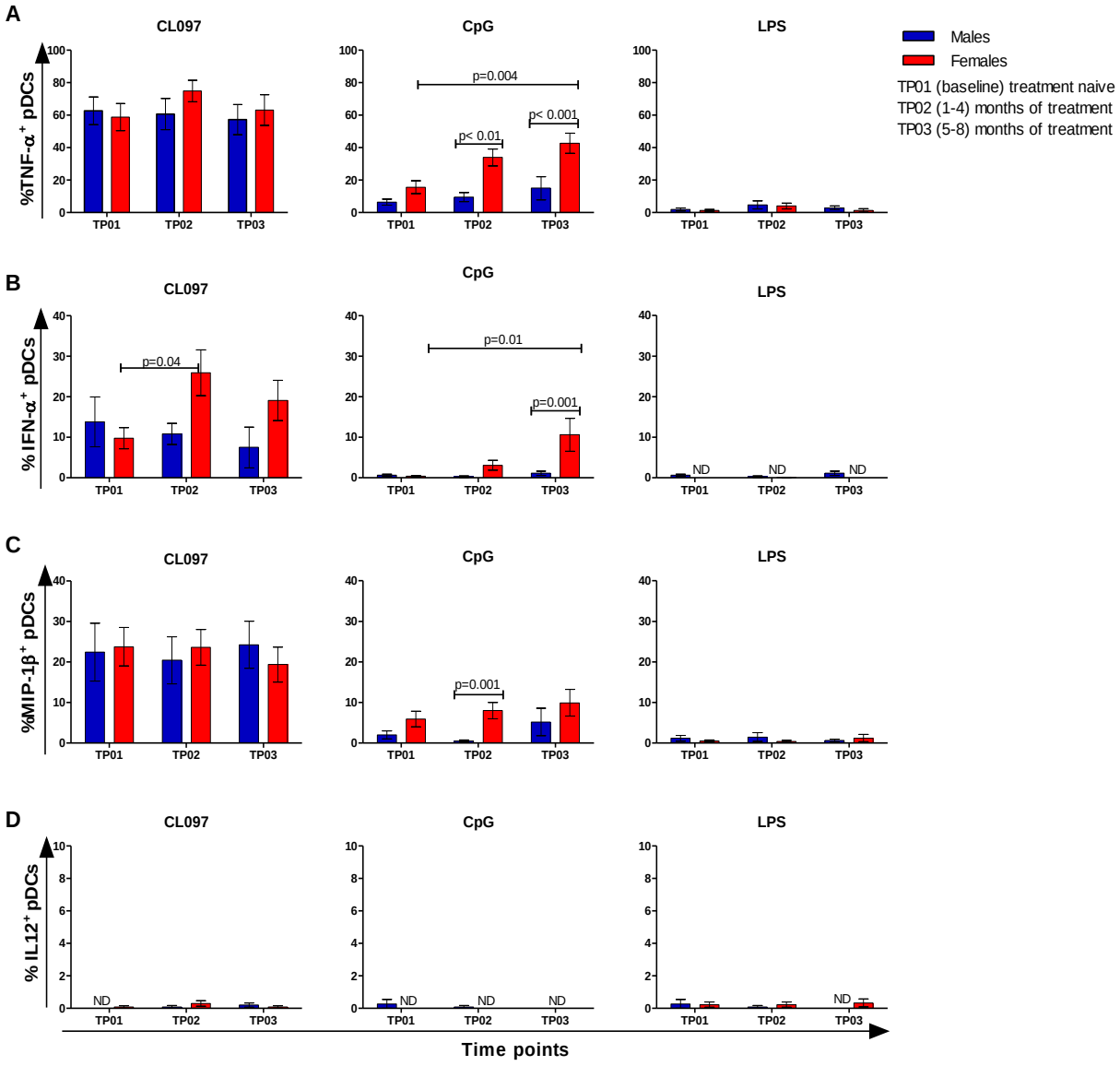

Figure 3.10: Sex differences in cytokine production by pDCs. The above plots shows percentage of cytokines (IFN-α, TNF-α, IL-12 and MIP-1β after background subtraction) produced by pDCs following 18hrs of stimulation with CL097 (TLR7/8), CpG (TLR9) and LPS (TLR4) ligands at baseline, during 1-4 and 5-8 months of treatment between males (n=11) and females (n=13). Two-way ANOVA was used to calculate percentage of cytokines across the time point between males and females and One way Anova (Kruskal-Wallis test) was used to compare time points in each group, followed by t- test (Mann-Whitney test) comparing groups in each time point. Data presented as mean±SEM. ND represent cytokines which were not detected.

3.5 Measurement of plasma cytokine profiles in females and males using Luminex

In order to determine changes in systemic cytokine profiles before and after ART initiation, 15 cytokines profile were quantified: IFN-ɣ, IFN-α, IL-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, TNF-α, IL-12p70, GM-CSF, MIP-1β and IP-10 in males (n=8) and females (n=13) at baseline, during (1-4) and (5-8) months of antiretroviral treatment. No significant differences were observed between males and females in the levels of systemic cytokines at baseline (TP01) and during 1-4 months of ART (TP02) (Figure 3.11 A and B). Similarly, no significant differences were seen in all the cytokines except IL-8 (p=0.03), which was increased in females compared to males at 5-8 months of ART (Figure 3.11 C).

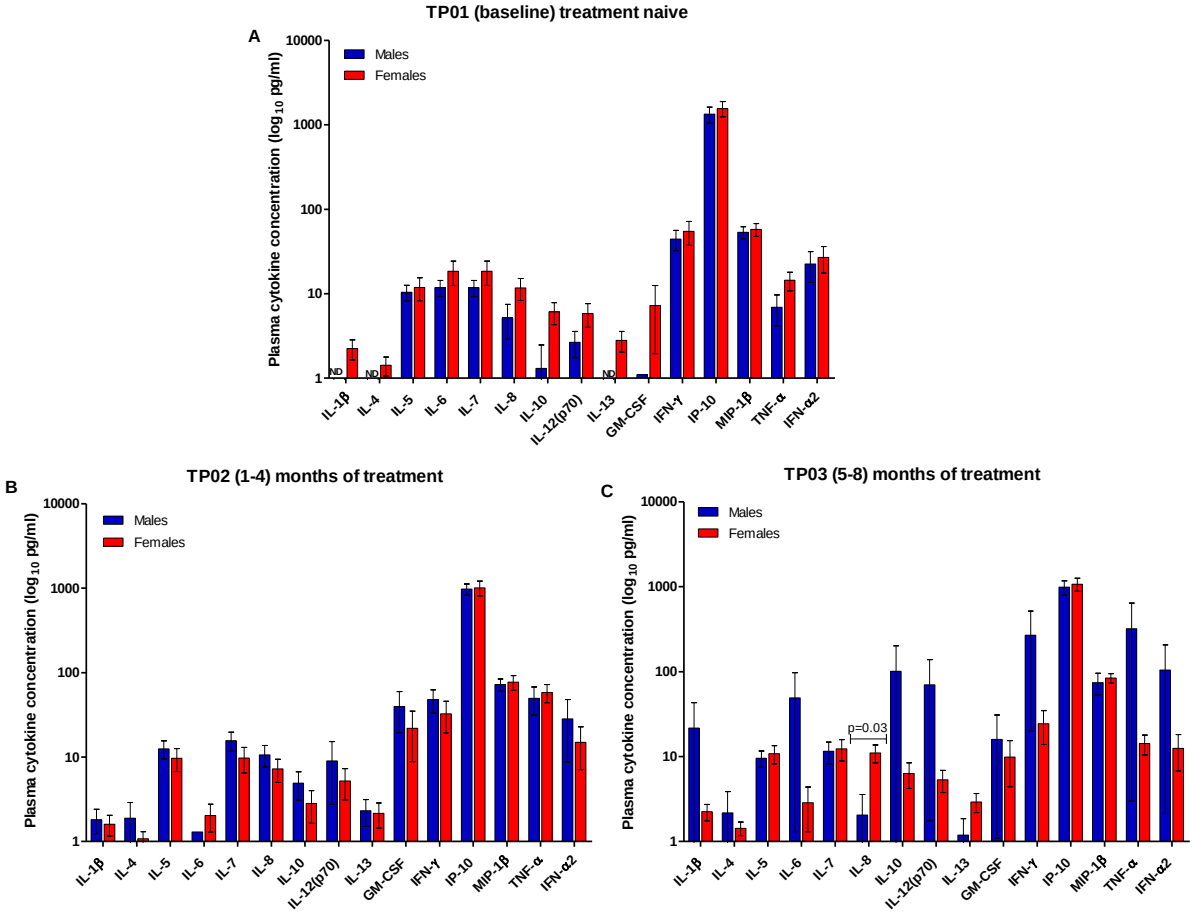

Figure 3.11: Sex differences in systemic cytokine profile of plasma between males (n=8) and females (n=13). t-test (Mann-Whitney test) was used to compare groups in each cytokine. The data represent mean with SEM. ND represent cytokines that were not detected. Only 8 male plasma samples were analyzed due to samples unavailability.

3.5.1 Determining systemic cytokine profile in plasma using Luminex- Principal Component analysis-PCA (exploratory analysis)

Three components were identified from the scree plot with an eigenvalue greater than 1.0 for each time point. The resulting PCA components were used to generate a heat map which determined the presence of clustering and contribution of each cytokine. These three components accounts for the most amount of variance. The heat map (appendix VIIII) shows the relative contribution of each cytokine to the respective component. Thirteen cytokines which include those implicated in a pro-inflammatory role together with anti-inflammatory role contributed positively to most variation seen in component 1 of treatment naive: IFN-ɣ, IFN-α, IL-1β, IL-4, IL-6, IL-10, IL-5, IL-7, IL-8, IL-13, IL-12p40, IFN-α, TNF-α, IL-12p70, GM-CSF, MIP-1β and IP-10. Conversely, GM-SCF, IP-10 and MIP-1β contributed negatively. The same observation was made at component one during ART (appendix VIIII). However, no significant differences were noted between males and females in the cytokine expression profile.

The scores for each observation on Principal Component (PC) 1 and (PC) 2 are shown in appendix VII. The scores were used to identify clustering, which would suggest a common underlying multivariate signal (in this case inflammatory status of plasma) existing for that observed in a particular cluster. An individual with a larger loading score on a PC in a particular direction contribute more heavily to the multivariate signal in that direction and therefore make a greater contribution to observations with high scores on that PC. We suggest that PC1 represents some aspect of the pro-inflammatory state of individuals in contrast to the individuals loading negatively on this axis. PC 1 and 2 demonstrate clustering of females and males according to pro-inflammatory status during treatment naïve and PC1 represents 66.1% of the variance. Additionally, females were separated into two groups with some showing pro-inflammatory status in plasma. However, no significant sex differences were observed between males and females (appendix VIII). PC1 accounted for 55% of variation during (1-4 months of treatment) while PC2 accounted for 14%. Both males and females showed positive and negative contribution to the PCs but no significant sex differences in the pro-inflammatory status in plasma was exhibited. Similarly, no significant differences were seen between males and females at 5-8 months of treatment, although they both showed a decrease in pro-inflammatory status in plasma.

CHAPTER 4: DISCUSSION AND FUTURE WORK

Sex differences have been demonstrated in disease manifestation between males and females in the course of HIV-1 infection. The advent of ART can yield desirable results in lowering viral loads, however excess immune activation can still persist. This ensues as a consequence of different kinetics in the restoration arms of the immune system, remarkably faster restoration of pro-inflammatory innate and adaptive pathways in contrast to regulatory mechanisms. This study aimed to investigate sex based differences kinetics in a cohort of males and females with HIV-clade C infection from Durban, South Africa. We hypothesized that immune restoration following initiation of ART will have significant sex based differential outcomes in immune responses in this African cohort.

Our study results indicated that there was a significant association between activated T cells ($CD4^+HLA\text{-}DR^+CD38^+$ & $CD8^+HLA\text{-}DR^+CD38^+$) with HIV-1 viral load in treatment naive females but not in males. Secondly, females had a higher frequency of classical monocytes (defined by $CD14^{++}CD16^-$) than males at baseline. Evident sex differences in cytokine (TNF-α, IFN-α & MIP-1β) specific responses were higher in pDCs (and less so on mDCs) of females compared to males following stimulation with CpG-TLR9 ligand. Furthermore, females demonstrated robust immune activation in response to TLR9 ligand stimulation during treatment compared to baseline. Finally, our data showed no sex differences in all cytokines measured except for IL-8 that was increased in females during ART.

Although several studies have demonstrated major sex differences in immune activation between males and females in HIV-1 infection, sex based differences in immune restoration during ART remains elusive. To establish the adaptive immune activation characteristics of individuals in this study, T cell activation was evaluated between males and females prior to and during ART using HLA-DR and CD38 markers established to be independent predictive tools for HIV-1 disease progression in the absence of ART (Fahey et al., 1990, Liu et al., 1997, Giorgi et al., 1999). The frequency of $CD4^+HLA\text{-}DR^+CD38^+$ and $CD8^+HLA\text{-}DR^+CD38^+$ at baseline and after treatment were slightly higher in females compared to males, but the differences were not significant.

The study results indicated that there was a significant association between activated T cells and HIV-1 viral load in treatment naïve females but not in males. Our data were in contrast to previous reports that demonstrated an association in both males and females with HIV viremia before ART initiation, although in women it was significantly higher compared to men (Meier et al., 2009). This disparity could be due to the fact that viral load were not matched for both males and females in our study compared to the previous study where controllers and progressors were examined, the participants in our study were selected based on their $CD4^+$ T cell counts. Viral suppression by ART is associated with reduction in T-

cell activation (Sachdeva et al., 2010). Studies have established that the rate of T-cell turnover and the degree of generalized T-cell activation both decrease after initiation of ART, signifying that viral replication directly contributes to heightened levels of T-cell activation (Deeks et al., 2004). Previous reports have shown that immune activation during ART is driven by low-level HIV viremia and microbial translocation in the gut leading to increased LPS (Sachdeva et al., 2010). Studies demonstrated that timing of ART initiation is a crucial factor that influences immune activation with one prospective study showing that cART initiated during acute infection exhibited reduced immune activation to normal levels after 48 week of ART (Rajasuriar et al., 2013). However, we noted a positive correlation of activated CD8+ T cells and viremia even after 3 months of ART treatment in females (appendix III). These observations could indicate the existing sex differences seen in chronic HIV infection, where females show elevated immune response despite initiation of ART as compared to males.

Monocytes can be subdivided into three subsets with distinct cytokine profiles and immune functions in HIV-1 infected patients (Ziegler-Heitbrock et al., 2010). Classical monocytes play a significant role in defending against microbial pathogens; intermediate monocytes are associated with antigen presentation whereas inflammatory and non-classical monocytes exhibit patrolling properties (Chen et al., 2017). In the present study, females exhibited higher levels of classical monocytes compared to males at time point TP01 (treatment naïve). No differences were seen in the other monocyte subsets. Moreover, elevated levels of classical monocytes were correlated with a decrease in $CD4^+$ T cell counts only in females at baseline (appendix IV). Therefore, these data suggest that monocytes and their subsets might contribute to the sex bias seen in innate immune responses.

The composition of intermediate and non-classical monocytes expressing $CD16^+$ differs between individuals but higher in ART naïve in comparison to individuals on ART, indicating that these cells might contribute to disease pathogenesis (McCausland et al., 2015). However, the proportion of these cells in HIV infection and during immune reconstitution between males and females is poorly understood. Our data showed that the proportion of non-classical monocytes and intermediate monocytes did not differ between males and females before treatment and after treatment initiation, although they remained elevated and higher (>5%) than levels normally seen in healthy controls (Han et al., 2009). A study conducted on healthy women and men showed sex differences in non-classical monocytes with females showing significantly higher levels of non-classical monocytes compared to males (Jiang et al., 2014). In contrast, Hearps et al. (2012) showed sex differences in $CD16^+$ expressing monocytes, specifically showing that females have lower proportions of non-classical monocytes compared to males. However, our study differs from previous studies in several aspects, most notably these ex vivo studies generated data based on cross-sectional comparisons of healthy controls with HIV-infected patients prior to and on

ART, they also separate them according to age (Han et al., 2009, Hearps et al., 2012). Therefore, these are likely to cause variations due to different disease stages compared.

Additionally, we evaluated changes in monocyte subsets after the initiation of ART. No significant differences were observed between treatment naive and treated individuals in intermediate and non-classical monocytes in males and females. Similar to our result, Scully and colleagues found no significant differences in the distribution of these subsets between viremic and suppressed patients (Scully et al., 2016). However, several studies have demonstrated an increase in $CD16^+$ expressing monocytes in treatment naïve compared to ART treated individuals (Hearps et al., 2012, Campbell et al., 2014). Hearps and colleagues showed that monocytes phenotype and function can be affected by age, as they showed an increase in the proportion of $CD16^+$ monocytes (considered inflammatory as a result of high TNF-α production) in young viremic HIV-positive individuals compared to elderly (Hearps et al., 2012). The discrepancy seen above in relation to our study might be due to the use of small sample size in the present study and the lack of analyzing a broader age range with sufficient patient numbers.

Interestingly, a previous study comparing inflammatory monocytes between treatment naïve vs individuals on 3 months of treatment in chronic and acute infection showed that early initiation of ART treatment in acute infection can normalize monocytes activation, unlike in patients with chronic infection (Burdo et al., 2011). This irreversible monocytes activation can have adverse effect in HIV-1 infected individuals such as insulin resistance and lipodystrophy (Burdo et al., 2011). Therefore, since South Africa has introduced new ART guidelines which stipulate that all HIV-1 infected people be given ART soon after diagnosis, this early intervention may likely reduce many detrimental effects associated with a lower CD4 T cell count threshold (AVERT, 2017).

Antigen presenting cells of the innate immune system have been implicated in chronic production of IFNs which have been found to be significant in driving the immune activation and disease progression in HIV-1 infection. Furthermore, it has been demonstrated that HIV-1 infected females show a stronger immune activation and faster disease progression compared with males exhibiting the same viral load (Meier et al., 2009). Due to the existing immune activation in females compared to males, we hypothesized that females will have higher levels of immune activation during immune reconstitution under ART due to rapid reconstitution of TLR pathways.

We assessed the consequences of HIV-1 infection and ART initiation on the ability of antigen-presenting cells to respond to TLR stimulation. Our data showed diminished production of all cytokines in monocytes even when it was stimulated with LPS, considered as the major stimulant for monocytes due to their high expression of LPS receptor TLR4 and its related signaling molecule CD14 (Hearps et al.,

2012). Monocytes are also regarded as the primary producers of pro-inflammatory cytokines such as TNF-α and IL-6. The reason behind this dysfunctional monocytes population is not clear but we can speculate that this could be due to that monocytes were exhausted as a result of already existing pro-inflammatory function during exposure to HIV *in vivo*. Nonetheless, our findings confirm the previous reports, which showed no sex differences in the percentage of TNF-α and IFN-α produced by monocytes at baseline during chronic HIV-1 infection (Berghöfer et al., 2006, Meier et al., 2009).

In line with previous reports, our data showed no sex differences in the percentage of IFN-α, TNF-α and IL-12 production by mDCs after stimulation with CL097, CpG and LPS before and after ART initiation (Meier et al., 2009). However, the levels of MIP-1β-produced by mDCs in response to TLR9 ligand (CpG) were higher at 5-8 months of treatment in females compared to males and a trend towards higher MIP-1β production was also noted in females (only) during 5-8 months of ART compared to baseline. This increase in mDCs responses during treatment might be caused by rapid reconstitution of mDCs upon ART, thus causing immune activation.

Surprisingly, no significant sex differences in the frequency of IFN-α-producing pDCs in response to TLR7/8 before and after treatment were detected although females had slightly higher levels of these cytokines compared to males. These results are in contrast to a previous report which showed that pDCs derived from women produced more IFN-α in response to TLR7/8 ligands than pDCs derived from men (Meier et al., 2009). This could be caused by limited number of patients studied here since the previous studies had bigger cohort (>50 patients). Furthermore, these differences might be a result of confounding factors such as age and use of contraceptives. However, *in vitro* stimulation of DCs from HIV-1 infected individuals with TLRs ligands has shown mixed data, with some studies showing reduced levels of IFN-α production during chronic HIV-1 infection than healthy controls (Ferbas et al., 1995, Martinson et al., 2007, Sachdeva et al., 2008).

Sex hormones have potent regulatory effect on innate immune cells (Roved et al., 2016). Studies have shown that sex hormones abundance can modulate the ability of pDCs to produce IFN-α in response to TLR7/8, specifically showing that Progestin modulate pDCs function *in vitro* (Hughes et al., 2008). Seillet et al. (2012), precisely showed a trend towards a lower percentage of IFN-α produced by pDCs stimulated by TLR-7/8 in postmenopausal compared to premenopausal women. Additionally, another study demonstrated that female mice with (estrogen receptor) ERα knockout exhibited impaired capacity to produce IFN-α after stimulation TLR7 ligand (Griesbeck et al., 2015). Therefore, these findings suggest that female sex hormones could regulate the TLR-mediated responses of pDCs.

Although sex differences were not seen in the levels of pDCs producing IFN-α in our study in response to TLR7/8, we noted an increase in induction of TNF-α and IFN-α by females after ART initiation compared to baseline. These results differ from the previous report demonstrating a decrease in IFN-α levels during ART compared to ART naïve (Lehmann et al., 2014). Moreover, primate studies have provided a critical insight into potential mechanisms of immune activation in chronic HIV infection. They have demonstrated that sooty mangabeys which are natural host of SIV lack CD4 T cell activation and depletion in spite of high viremia and their pDCs produce less IFN-α in response to SIV. Conversely, pDCs from rhesus macaques which are similar to human pDCs produce high levels of IFN-α in response to HIV and SIV in the absence of treatment and show high levels of immune activation (Tilton et al., 2006, Mandl et al., 2008). This suggests that type I IFN's play a significant role in mediating HIV induced immune activation during suppressed viremia.

Sex differences have been described in the responses of pDCs to HIV infection, however responses of these cells during ART remain to be elucidated. The study showed significant sex-based difference in the levels of TNF-α during 1-4 and 5-8 months of treatment. Moreover, we showed that pDCs derived from women produce markedly more TNF-α in response to CpG than pDCs derived from men during ART. In contrast neither CL097 nor LPS induced significantly different levels of pDCs producing TNF-α in males and females, although it was slightly higher in females. Furthermore, it was observed that during 5-8 months of ART, pDCs IFN-α response to CpG was higher in females compared to males. The results also showed that during treatment females had elevated levels of TLR responsiveness in comparison to males.

Lastly, pro-inflammatory cytokines in plasma from females and males were assessed. Cytokine levels are indicative of the nature of the immune response for a certain individual (Van der Watt et al., 2014). No significant sex differences were observed between males and females in the levels of systemic cytokines at baseline during 1-4 and 5-8 months of ART except IL-8, which was significantly increased in females compared to males at 5-8 months of ART. A study done by (Pananghat et al., 2016) demonstrated that IL-8 may serve as a potential prognostic marker in conjunction with CD4 counts to monitor disease progression and the efficacy of ART. IL-8 is also known as a mediator associated with systemic inflammation (Calza et al., 2017). The levels of IFN-ɣ, IP10, MIP-1β and TNF-α remained elevated even after 8 months of treatment between both males and females and may indicate a systemic inflammation in these patients that did not demonstrate a declined during ART.

Additionally, Principal component analysis (PCA) was carried out to investigate inflammatory status of plasma between males and females at various time points. The heat map expression profile indicated that both pro-inflammatory and anti-inflammatory cytokines contributed to most of the variation seen, even

though it was not significantly different between males and females. Moreover, females generated two clusters in score plot (appendix VIII), both clusters were indicative of pro-inflammatory status of plasma, while the males only formed one cluster which did not show any inflammation signs. However, no significant sex differences were observed at all-time points (baseline, 1-4 months and 5-8 months of treatment).

The increase in pro-inflammatory response in females as compared to males after initiation of ART seen in this study suggested that TLR-dependent response of pDCs in women might contribute to high levels of immune activation and enhanced risk of immune reconstitution inflammatory syndrome (IRIS). The pathophysiology of IRIS is not completely understood, however, it is believed that IRIS is caused by enhanced dysregulated immune response to disease-specific antigens which results in an overproduction of inflammatory mediators in patients who recently started ART before full reconstitution of their T cells (Stone et al., 2004, Lipman and Breen, 2006, Tilton et al., 2006, Scully et al., 2016).

This study has several limitations related to the heterogeneous nature of our patient's age, control group and ART. No control group (healthy individuals) were included, it was thus difficult to make conclusions with regards to intermediate and non-classical monocytes subsets since it is known that these populations are found in abundance in HIV-1 infection (McCausland et al., 2015). Furthermore, we could not get fresh specimens from the recently recruited volunteers to verify and compare the findings obtained from the cryopreserved PBMCs, since it is known that freezing may have a negative impact on the viability and functionality of the cells. The studied patients were not receiving the same antiretroviral therapy, therefore this difference could also be a confounding factor. We had no information on any hormonal contraceptive used, thus we cannot exclude the possibility that hormones may influence the parameters measured here. It should be noted that the range of $CD4^+$ T cell count used to select the recruits was very broad thus resulting in several females having the clinically defined AIDS (CD4<200) at the start of the study while the males had a CD4>200. Therefore, the variation in disease stages between these two groups could introduce bias. Moreover, the observed sex differences might be driven by sex or may be driven by the variations in disease stage and response to ART between the two groups. Small sample size could also be a result of the trends mostly observed in this study, thus a larger sample size in this cohort of individuals will lead to more conclusive results on the role ART and immune reconstitution.

Nonetheless, the study confirmed previous reports that T cell activation correlates positively with plasma HIV RNA levels but only in females, and showed that CpG and not CL097 induced most of pro-inflammatory cytokine responses in pDCs. The study identified major sex differences in the level of TNF-α pDCs response to TLR9 ligand (CpG) in patients who are on ART. This enhanced immune activation in

women might increase the risk of IRIS. Taken together, these findings provide a basis for future studies with a larger cohort that may provide further insight of adapting the HIV-1 therapy regimen based on differences in disease progression in each sex.

Future work can include:

- Evaluation of the effect of sex hormones on plasmacytoid dendritic cells in HIV-1 infection during immune reconstitution by quantifying plasma progesterone and testestorone levels between pregnant, non-pregnant females and males using Liquid chromatograpy-mass spectrometry (LCMS), in order to determine if sex hormone can further mediate this immune response during ART.
- Assessment of systemic cytokine profiles in cell culture supernatant following TLR stimulation in males, pregnant females and non-pregnant females using Luminex.
- To verify and compare the findings obtained from cryopresrved PBMCs with fresh PBMCs from (Females Rising Through Education, Support and Health (FRESH) study) which are recently recruited volunteers that start treatment following HIV diagnosis. This will be important in order to determine if long term cryopreservation has dentrimental effects on antigen presenting cells.
- Additionally, we would quantify the expression of several interferon-stimulated genes (ISGs) from the pellet which was stimulated with CpG, CL097, LPS and the unstimulated control in HIV-1 infected males, pregnant and non-pregnant females (from FRESH study and cryopreserved samples) using qualitative real-time polymerase chain reaction (qRT-PCR), as previously described by (Singh et al., 2014). This technique is commonly used to detect RNA expression. It is used to qualitatively and quantitatively detect gene expression through creation of complementary DNA (cDNA) transcripts using fluorescent probes and these fluorescent probes allow RT-PCR to measure amplification as it occurs during the experiment. We aim to determine whether males or females would have high ISGs and if these are associated with HIV-1-induced type I IFNs.

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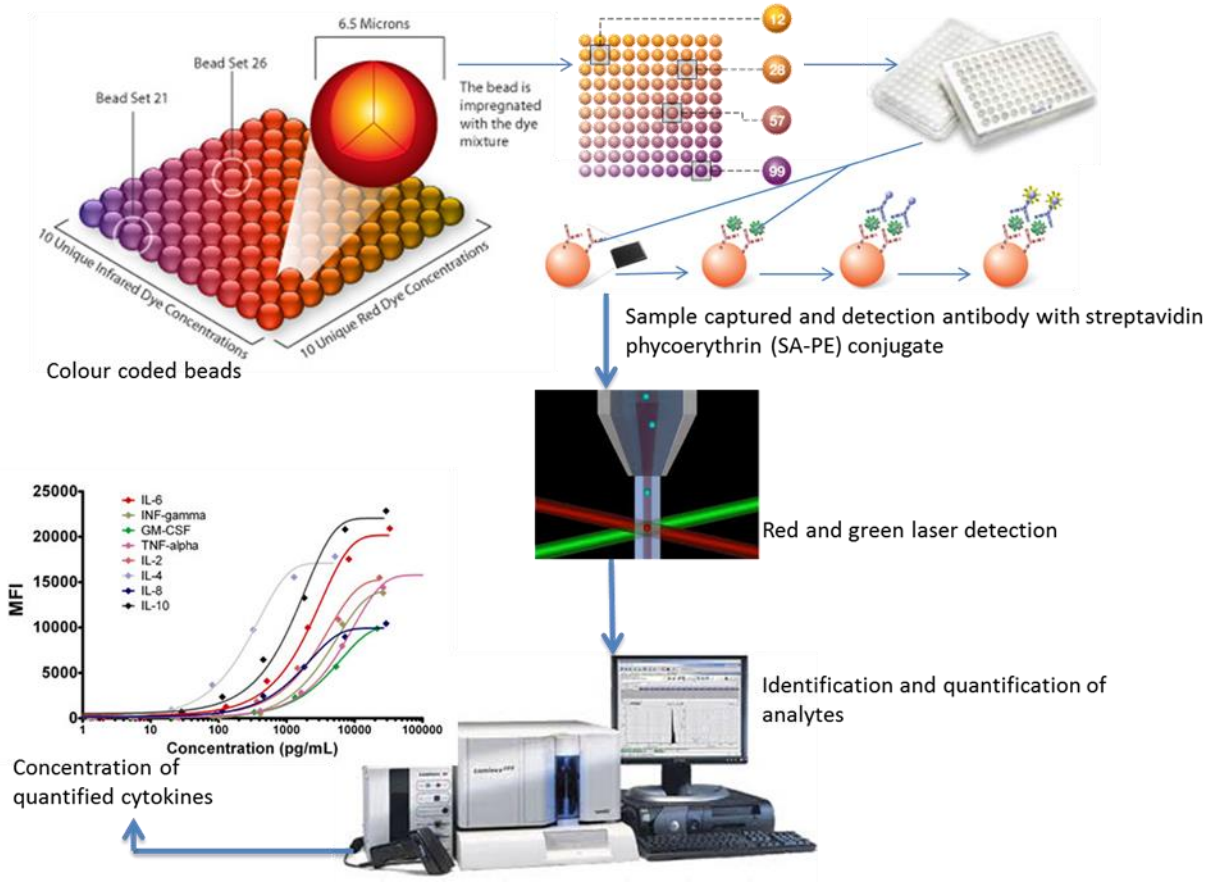
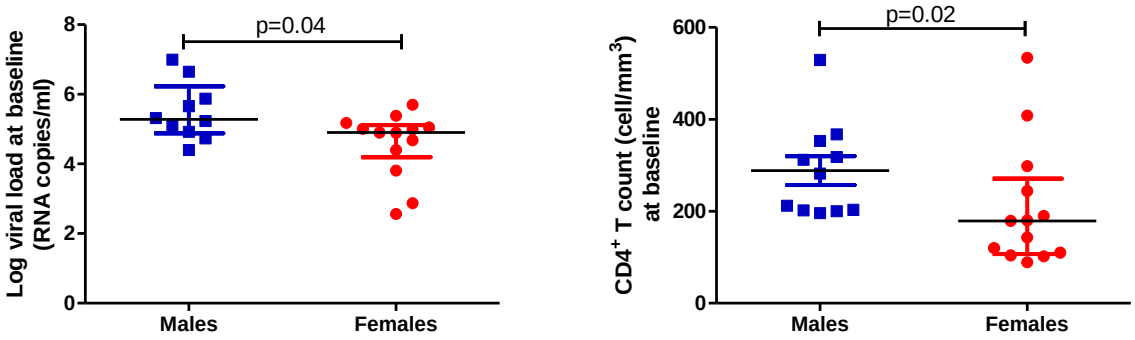
APPENDIX I: Overview of Luminex Methodology.



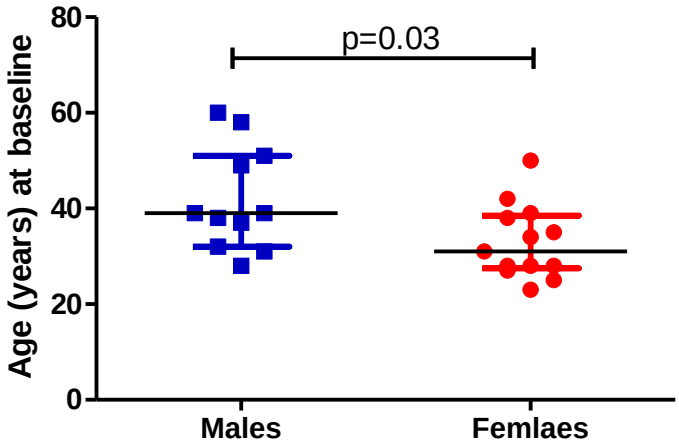
Overview of Luminex Methodology (images taken from www.bioradiations.com). Color-coded capture beads are incubated with the sample (plasma), followed by incubations with detection antibodies and a reporter dye. The Bio-Plex reader classifies the beads by color, and then quantifies the associated reporter signal intensity, which indicates the level of cytokine present.

APPENDIX II: Viral load and CD4 count of males and females at baseline.



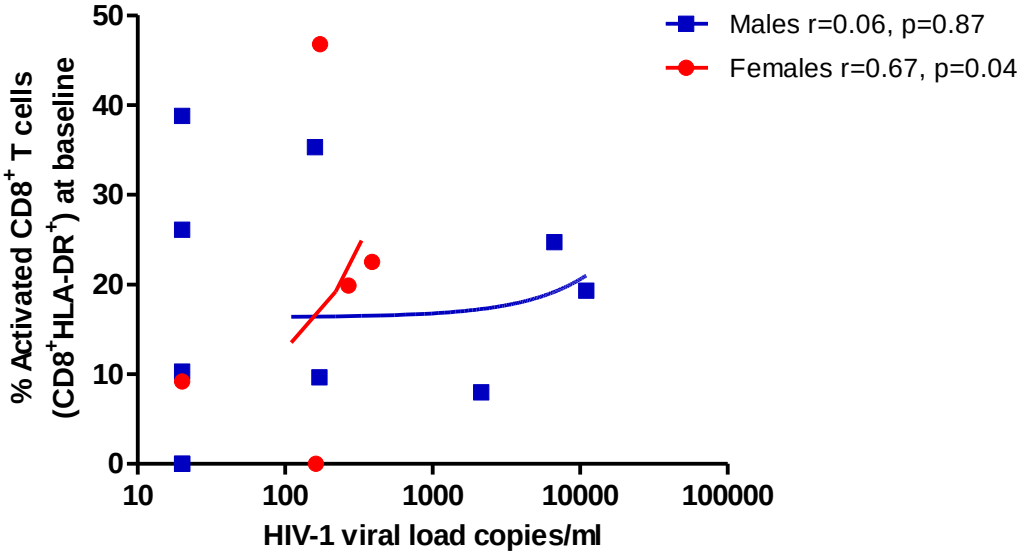
Viral load and CD4 count of males (n=11) and females (n=13) at treatment naïve (baseline). Data is presented as median with interquartile range; t test (Mann-Whitney test) was used to calculate the p value.

APPENDIX III: Age of males and females at baseline.

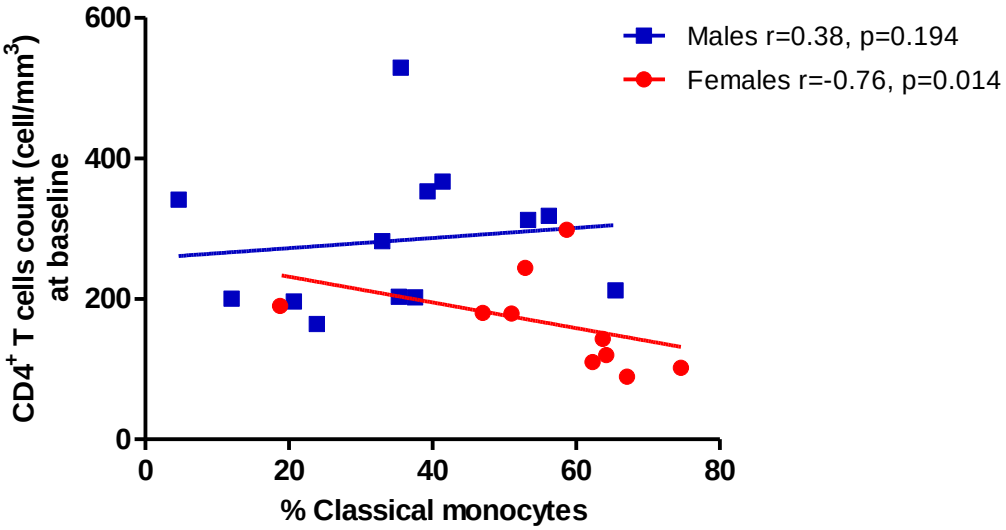


The data showing results of ages between males and females at baseline (before treatment). Data is presented as median with interquartile range, t test (Mann-Whitney test) was used to calculate the p value.

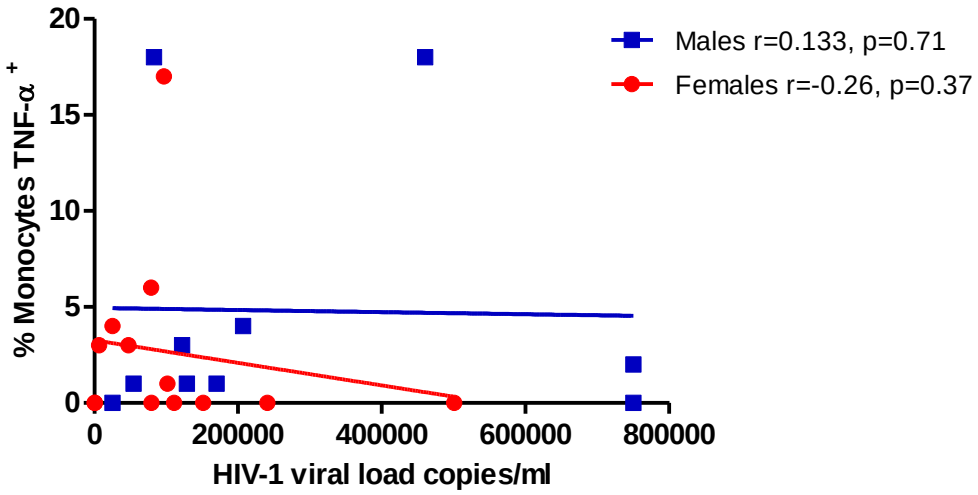
APPENDIX IV: The percentage of activated CD8+ T cells vs viral load in males and females during 8 months of ART.



APPENDIX V: The percentage of classical monocytes correlated with $CD4^+$ T cells count in males and females at baseline.



APPENDIX VI: Monocytes TNF-α production after LPS stimulation correlated with plasma**.**



TNF-α produced by Monocytes after LPS stimulation associated with viral load in males and females. Spearman rank correlation was done and straight line was generated using liner regression.

APPENDIX VII: Results of the principal component analysis (only principal components used in our analysis are shown).

PCA analysis at baseline

Component	Eigenvalue	Difference	Proportion	Cumulative
Comp1	**10.296**	**8.81321**	**0.6864**	**0.6864**
Comp2	**1.48279**	**0.482044**	**0.0989**	**0.7853**
Comp3	**1.00075**	**0.339123**	**0.0667**	**0.852**

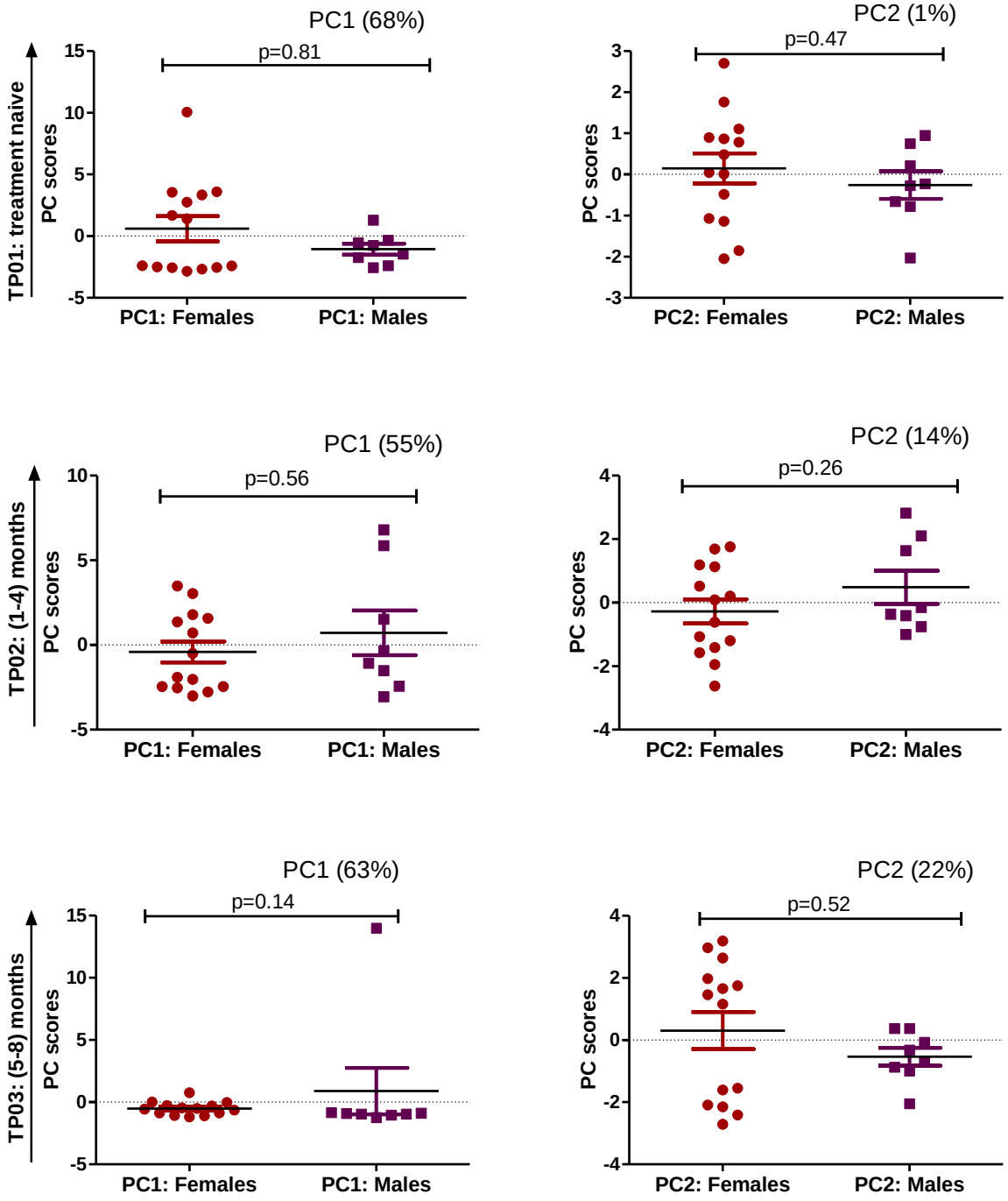
PCA analysis at 1-4 months of treaatment

Component	Eigenvalue	Difference	Proportion	Cumulative
Comp1	**8.28593**	**6.19056**	**0.5524**	**0.5524**
Comp2	**2.09538**	**0.40787**	**0.1397**	**0.6921**
Comp3	**1.68751**	**0.584996**	**0.1125**	**0.8046**

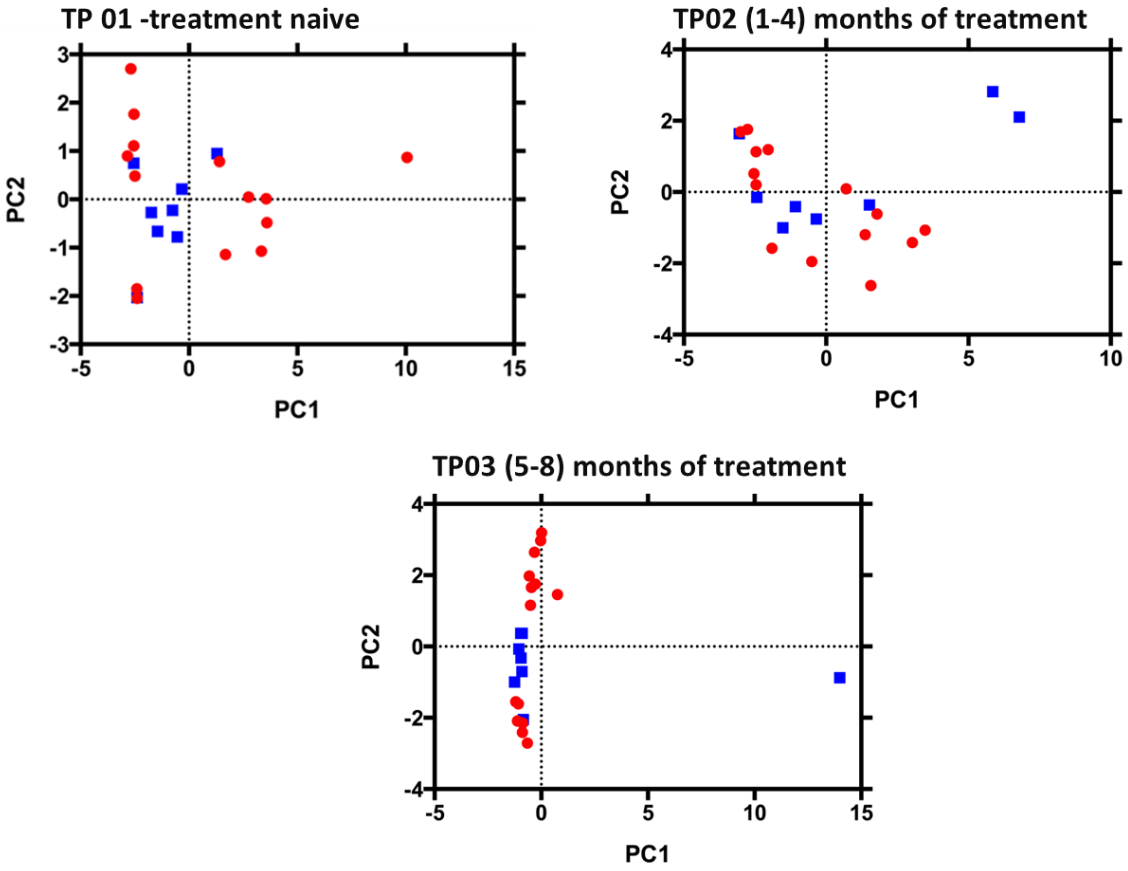
PCA analysis at 5-8 months of treatment

Component	Eigenvalue	Difference	Proportion	Cumulative
comp1	**9.99509**	**6.51203**	**0.6247**	**0.6247**
Comp2	**3.48306**	**2.1168**	**0.2177**	**0.8424**
Comp3	**1.36626**	**0.744926**	**0.0854**	**0.9278**

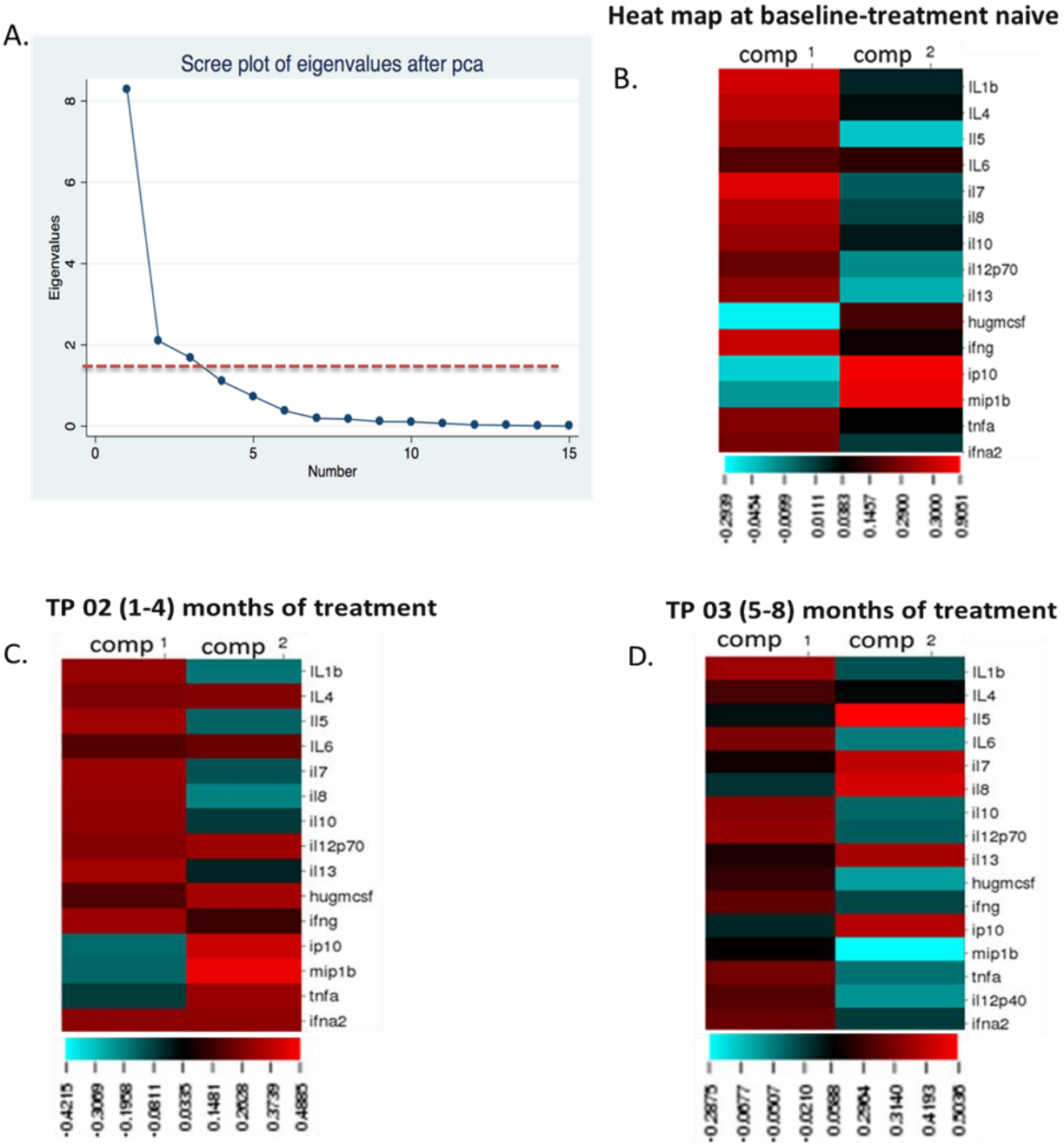
APPENDIX VIII: PCA analysis showing clustering of both males (in blue) and females (in red) at treatment naïve, 1-4 months of treatment and 5-8 months of treatment.



Principal component analysis showing inflammatory clustering status of males and females in PC1 and PC2 at treatment naïve, 1-4 and 5-8 months of treatment. Statistical comparison between males and females was done using Student's t test.


TP 01 -treatment naive
PC2
PC1
TP02 (1-4) months of treatment
PC2
PC1
TP03 (5-8) months of treatment
PC2
PC1

APPENDIX VIIII: The graph showing eigenvalues and component numbers of 15 variables**.**



Only two principle components were selected for further analysis that have an eigenvalue of greater than 1, the successive components were left out since they account for smaller amount of total variation. Heat map comparing 15 plasma cytokine expression profiles of males and females at baseline (B) and 2 TPs (C&D) after treatment. The heat map color scale corresponding to the relative expression of the cytokine relative to the minimum and maximum of all values is shown at the bottom. Blue indicating lowest levels (negative contribution), red and maroon/brown indicating highest levels (positive contribution) and black indicating median levels relative to expression cytokine.