Impact of Duffy Antigen Receptor for Chemokine (DARC)-null polymorphism on T cell function and pro-inflammatory cytokine responses during HIV-1 infection

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Submitted in partial fulfilment of the requirements for the degree of
Master of Medical Sciences

In the School of Laboratory Medicine and Medical Sciences at the
Discipline of HIV Pathogenesis Programme,
Nelson R. Mandela School of Medicine, College of Health Sciences,
University of KwaZulu-Natal, Durban

2018

PREFACE

The experimental work described in this thesis was conducted at the HIV Pathogenesis Programme Immunology laboratory, Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa, from February 2017 to November 2018, under the supervision of Dr Christina Thobakgale-Tshabalala.

This work has not been submitted in any form for any degree or diploma to any tertiary institution.

Z. Shangase [redacted] Date 04 December 2018

C.F. Thobakgale-Tshabalala [redacted] Date 04 December 2018

PLAGIARISM: DECLARATION

I, **Zesuliwe Shangase,** declare that

(i) The research reported in this dissertation, except otherwise indicated, is my original work.

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RESEARCH OUTPUT

- Awarded a full scholarship based on abstract merit to present poster at the **8th Infectious Diseases in Africa (IDA) Symposium**, 12-17th November 2018. Cape Town, South Africa.

Oral and Poster Presentations

Zesuliwe Shangase, Kewreshini Naidoo, Thumbi Ndungu' and Christina Thobakgale-Tshabalala. Impact of Duffy antigen receptor for chemokine (DARC)-null polymorphism on T cell function and pro-inflammatory cytokine activity during HIV-1 infection. Poster presentation, November 2018. IDA Symposium, Cape Town, South Africa.

Shangase ZB, Naidoo K, Ndung'u T, Thobakgale-Tshabalala CF. Impact of Duffy antigen receptor for chemokine (DARC)-null polymorphism on T cell function and pro-inflammatory cytokine activity during HIV-1 infection. School of laboratory Medicine and Medical Sciences, Oral presentation, 29 August 2018. Durban, South Africa.

DEDICATION

This thesis is dedicated to my loving husband Ndumiso Jule for his unconditional love, support, and constant encouragement and our son Avuyile Jule for his patience.

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who made it possible for me to complete this study.

Firstly, I would like to thank God for his immense blessings and for providing me the strength and courage to complete this study;

Dr. Christina Thobakgale-Tshabalala, my supervisor and mentor, for her time, patience, guidance and support throughout the course of the research.

Prof Thumbi Ndung'u, my co-supervisor for his academic guidance and support.

Kewreshini Naidoo for assistance with laboratory training and results analysis.

Akeem Ngomu and Nontlantla Mdletshe for their kind assistance with laboratory work.

Staff and students from HPP for their patience and support.

I am grateful to National Research Foundation (NRF) and South African Medical Research Council (SAMRC) for providing me with funding during the course of my studies.

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ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
APC	Antigen Presenting Cell
ARG	Arginase
ART	Antiretroviral therapy
CCL5	Chemokine (C-C motif) ligand 5
CCR5	Chemokine Receptor 5
CD107a	Cluster of Differentiation 107a
CD4+ T cells	Human T cells expressing CD4+ antigen
CD8+ T cells	Human T cells expressing CD8+ antigen
CD38	Cluster of Differentiation 38
CD57	Cluster of Differentiation 57
CDK6	Cyclin-dependent Kinase 6
CFR	Circulating Recombinant Forms
CSF3	Colony Stimulating Factor 3
CFSE	Carboxyfluorescein succnimidyl ester
CTL	Cytotoxic T lymphocyte
CXCL2	Chemokine (C-X-C motif) ligand 2
DARC	Duffy Antigen Receptor for Chemokine
DC	Dendritic Cell
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked Immunosorbent Assay
Env	Envelope
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FMO	Fluorescence Minus One
Gag	Group-specific antigen
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony Stimulating
H_2O_2	Hydrogen Peroxide
HAART	Highly active antiretroviral treatment

HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen DR isotype
ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma
IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17	Interleukin 17
MCP-1	Monocyte Chemoattractant Protein 1
MHC	Major histocompatibility complex
MIP-1β	Macrophage Inflammatory Protein 1 beta
mRNA	Messenger ribonucleic acid
Nef	Negative regulatory factor
NETs	Neutrophil Extracellular Traps
NC	Nucleocapsid (p7)
NK	Natural killer
P24	Capsid protein
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed Death 1
PD-L1	Programmed Death Ligand 1
PHA	Polyhydroxyalkanoate
PMN	Polymorphonuclear
Pol	Polymerase
RBC	Red Blood Cell
Rev	Regulator of virion expression protein
RNA	Ribonucleic acid

ROS	Reactive Oxygen Species
RT	Reverse Transcriptase
SA-PE	Streptavidin-phycoerythrin
SEB	Staphylococal enterotoxin B
SIV	Simian immunodeficiency virus
Tat	Transactivator of transcription
TB	Tuberculosis
TCR	T cell receptor
TNF-a	Tumor necrosis factor alpha
UNAIDS	United Nation on AIDS
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
WHO	World health organization

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ABSTRACT

The Duffy-null trait presents as non-expression of the Duffy antigen receptor for chemokines (DARC) on red blood cells and is highly prevalent in African populations. The Duffy-null genotype is the most significant genetic determinant of ethnic neutropenia and has been previously associated with HIV-1 transmission and disease progression. A recent role of the involvement of neutrophils in cross-talk with other immune cells such as natural killer (NK) and cytotoxic T cells has been reported, however these cells are impaired following HIV-1 infection. Some studies have suggested that neutrophils are important in priming of T cells and in mediating pro-inflammatory responses, however, little is known about the impact of DARC-null trait-associated neutropenia on T cell function. We here investigated the association of Duffy-null trait with T cell phenotype and function and proinflammatory cytokine activity in HIV-1 infection.

Antiretroviral therapy (ART) naïve chronically HIV-1 infected individuals (n=20) were recruited from Prince Mshiyeni hospital and HIV uninfected donors (n=20) were from a cohort of young women in Umlazi township in KwaZulu Natal, Durban. Multicolor flow cytometry was used to measure T cell activation (CD38 and HLA-DR expression) and exhaustion (PD-1 expression) in HIV-1 positive and HIV uninfected individuals. T cell function (IFN-γ, TNF-α and CD107a expression) following stimulation with HIV peptides (gag and envelope) was measured by intracellular cytokine staining (ICS) and T cell proliferative capacity by carboxyfluorescein succinimidyl ester (CFSE) was measured following stimulation with gag peptide pool. Lastly, we measured pro-inflammatory cytokine and chemokine plasma levels (G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, MCP-1, MIP-1β, and TNF-α) using Luminex.

Our results showed highly activated CD8+ T cells as measured by HLA-DR and CD38 expression in HIV-1 positive individuals compared to HIV uninfected donors ($p<0.0001$). Our findings also showed high CD8+ T cell responses in HIV-1 infected patients compared to HIV uninfected donors ($p=0.02$ for CD107a and $p=0.03$ for IFN-γ) as well as high proliferative capacity of CD8+ T cells in HIV-1 positive patients compared to uninfected donors ($p=0.05$). Our data showed no significant differences between CD4+ T cell activation and exhaustion in HIV-1 positive individuals and HIV uninfected donors. No differences were also observed in

in cytokine production and replicative capacity in CD4+ T cells between HIV-1 positive individuals and HIV uninfected donors.

Multiplex data showed higher G-CSF levels in HIV-1 infected patients compared to HIV uninfected individuals ($p=0.04$). Unexpectedly, the levels of proinflammatory cytokine IL-8 and chemokine MIP-1β were higher in the HIV-1 negative group compared to HIV-1 positive individuals ($p<0.0001$ and $p=0.0008$ respectively). All these findings in T cells and proinflammatory cytokine expression were not significantly different upon stratification by DARC status.

Collectively, our data suggest that HIV-1 infection results in CD8+ T cell activation and exhaustion with increased cytolytic activity. These data also suggest that despite neutropenia, Duffy-null individuals may have developed evolutionary mechanisms to compensate for possession of this genetic trait with regard to how the immune system responds to infection.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 HIV/AIDS epidemic

1.1.1 HIV/AIDS global epidemic

Human immunodeficiency virus 1 (HIV-1) was discovered three and half decades ago yet still remains a life-threatening disease globally. HIV-1 infections were estimated to be 36.9 million throughout the world with 1.8 million new HIV-1 infections, including 940 000 thousands AIDS related deaths by the end of 2017 (UNAIDS, 2018). According to the 2018 global report, at least 2.2 million new infections were observed globally in 2010, however this number has now reduced to 1.8 million by the end of 2017 (Table 1.1). As seen in Figure 1.1, the number of people living with HIV-1 with access to antiretroviral therapy (ART) continues to rise; 7.5 million HIV-1 infected people were on ART in 2010 compared to 19.5 million by the end of 2016 (Avert, 2018, WHO, 2018). Even though the number of people having access to ARVs has increased, the goal of completely eradicating HIV-1 has not been reached.

Table 1.1 Global and regional reports of HIV-1 epidemic (UNAIDS, 2018)

Regional HIV and AIDS statistics and features | 2017

	Adults and children living with HIV	Adults and children newly infected with HIV	Adult and child deaths due to AIDS
Eastern and southern Africa	19.6 million [17.5 million–22.0 million]	800 000 [650 000–1.0 million]	380 000 [300 000–510 000]
Western and central Africa	6.1 million [4.4 million–8.1 million]	370 000 [220 000–570 000]	280 000 [180 00–410 000]
Middle East and North Africa	220 000 [150 000–300 000]	18 000 [10 000–31 000]	9800 [6400–15 000]
Asia and the Pacific	5.2 million [4.1 million–6.7 million]	280 000 [210 000–390 000]	170 000 [110 000–280 000]
Latin America	1.8 million [1.5 million–2.3 million]	100 000 [77 000–130 000]	37 000 [26 000–51 000]
Caribbean	310 000 [260 000–420 000]	15 000 [11 000–26 000]	10 000 [7100–17 000]
Eastern Europe and central Asia	1.4 million [1.3 million–1.6 million]	130 000 [120 000–150 000]	34 000 [25 000–41 000]
Western and central Europe and North America	2.2 million [1.9 million–2.4 million]	70 000 [57 000–84 000]	13 000 [9900–18 000]
TOTAL	36.9 million [31.1 million–43.9 million]	1.8 million [1.4 million–2.4 million]	940 000 [670 000–1.3 million]

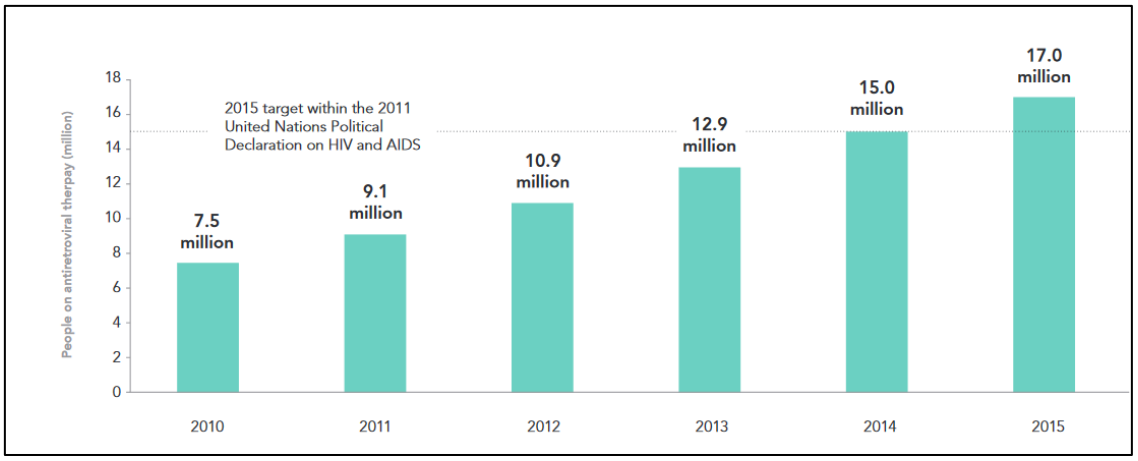

Figure 1.1: The Global number of people living with HIV on Antiretroviral Therapy for 2010 to 2015 (UNAIDS, 2018).

1.1.2 HIV/AIDS in Sub-Saharan Africa

Developing countries are heavily affected by the HIV-1 epidemic, accounting for 66% of HIV-1 infections. A heavier burden of these infections is in Africa, with sub-Saharan Africa (SSA) being the most heavily impacted region with 19.6 million in 2017 (Figure 1.2). In SSA, there were 800 000 reported new HIV-1 infections with 380 000 deaths due to AIDS related causes by the end of 2017, with only 12.9 million of the population on antiretroviral therapy (UNAIDS, 2018). South Africa remains the epicentre of the HIV-1 epidemic, about 7.1 million people were HIV-1 infected by 2016; 270 000 people newly infected and 110 000 people died due to AIDS. At least 56% of the HIV-1-infected people in South Africa were on ART by the end of 2016 (Cornell et al., 2017). South Africa, KwaZulu-Natal (KZN) is the most affected province with a prevalence of 12.2% (Council, 2017a) compared to Northern (Council, 2017b) and Western Cape (Council, 2017c) with prevalence of 6.8 and 5.6% respectively. UMgungundlovu District had a prevalence of 40.7% compared to seven out of nine Districts in KZN with a prevalence of 38.0% including Durban in 2012 (Kharsany et al., 2015).

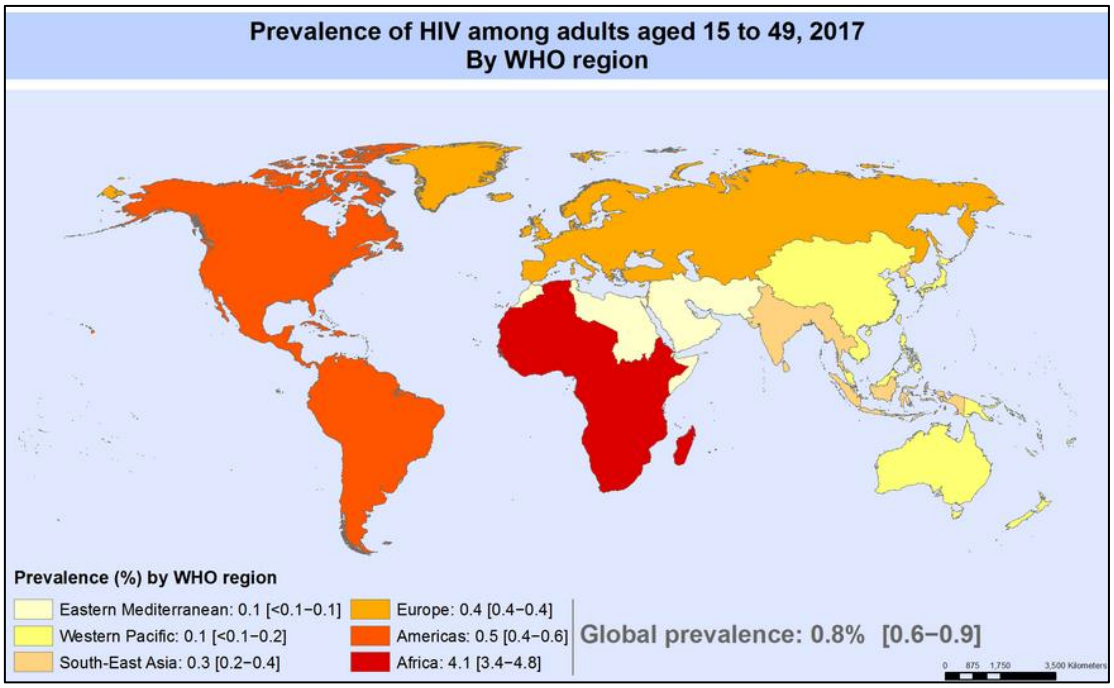

Figure 1.2: The map showing HIV-1 prevalence by the end of 2017 (UNAIDS, 2018).

1.2 HIV Virology

1.2.1 The Classification and structure

HIV, the virus that causes AIDS, was first noticed in 1981 in homosexual men who died from unusual opportunistic infections and uncommon malignancies. HIV is a *Lentivirus* from a *Lentiviridae* family and is divided into HIV-1 and HIV-2 (Barré-Sinoussi et al., 1983). HIV-1 and HIV-2 were transmitted from primates to humans as simian immunodeficiency virus (SIV) (Sharp and Hahn, 2011). HIV-1 is most common in sub-Saharan Africa and worldwide, while HIV-2 is known to be common in Western Africa. HIV-1 is divided into groups M, N, O and P. Group M is the HIV-1 strain that is further classified into nine clades (A, B, C, D, F, G, H, J, and K) and is the cause of global HIV-1 outbreak.

In addition, when distinct subtypes combine their deoxyribonucleic acid (DNA) they form multipartite virus called circulating recombinant form (CRF) (Hemelaar, 2012). High HIV-1 replication rates, viral reverse transcriptase mutations and high recombination rates lead to genetic variability of HIV-1.

The diversity of the HIV-1 sequence makes it a challenge for the immune system to control HIV-1 replication as well as develop an effective HIV vaccine.

Structurally, HIV-1 has a diameter of 119 to 207 nanometre (nm) surrounded by lipoprotein membrane and it has enveloped ribonucleic acid (RNA) virus (Goodsell, 2015, Briggs and Kräusslich, 2011). HIV-1 consist of two strands of RNA packaged within a core of viral proteins for integrity and it is also surrounded by a lipid bilayer (Figure 1.3), in addition to RNA strands, the capsid itself is also composed of viral proteins p24, p6 and p7 (Levy, 1993). The structure also contains enzymes (reverse transcriptase (RT), protease and integrase) to develop new virions. Surrounding the capsid is the matrix, which is composed of viral protein p17. Matrix is surrounded by phospholipid bilayer derived from the host cell. It is also composed of two viral glycoproteins: gp41, the fusogenic protein that pierces the envelope and combines the inside of the virus to the outside, and gp120, the protein on the outside that binds CD4+ T cells (Fanales-Belasio et al., 2010, Gelderblom et al., 1987, Montagnier, 1985).

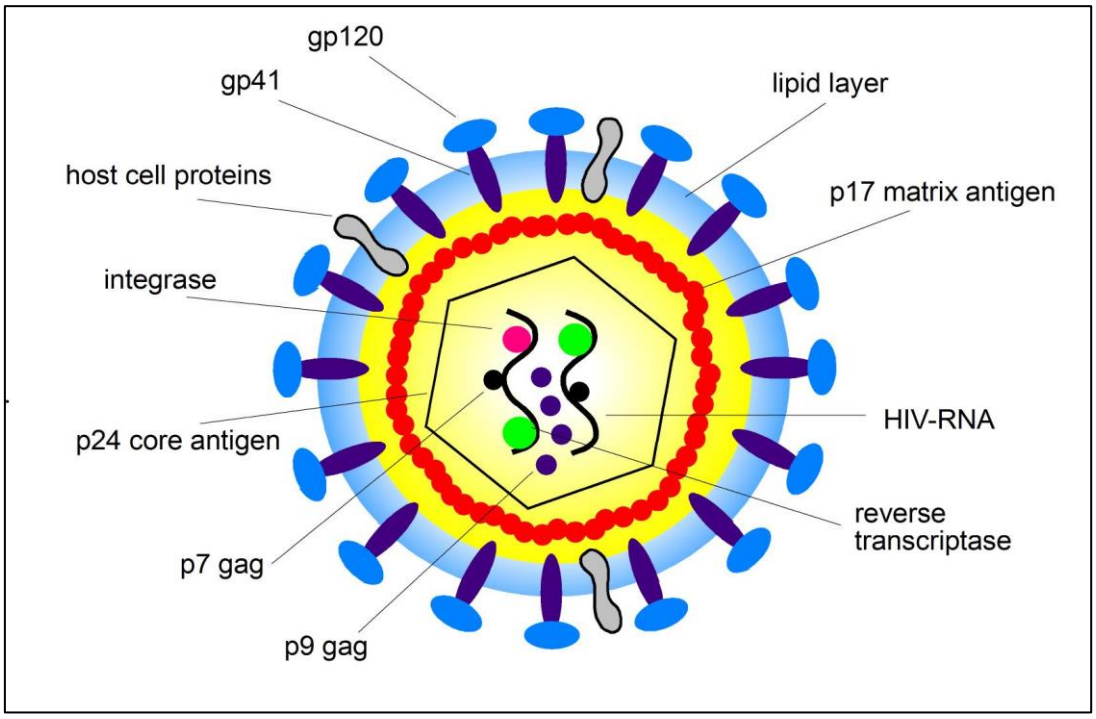

Figure 1.3: The structure of HIV. The viral capsid is enclosed by lipid membrane with the integration of viral glycoprotein and surface proteins. Within virion is the viral capsid made up of a pair of single stranded RNA molecules. The capsids contain viral genome and enzymes (Rockstroh, 2011).

There are nine genes of the HIV-1 virus (Figure 1.4), these can be divided into different types. The structural proteins include Gag, Env and Pol; the regulatory proteins are Tat and Rev, and Vpu, Vif, Vpr and Nef that are the accessory genes (Levy, 1993). Tat and Rev are expressed early in the virus cycle and are responsible for enhancement of viral replication. Vpu, Vif, Vpr and Nef differentiate HIV-1 from other retroviruses, regulate antiretroviral responses, and increase viral replication (Sivro, 2014).

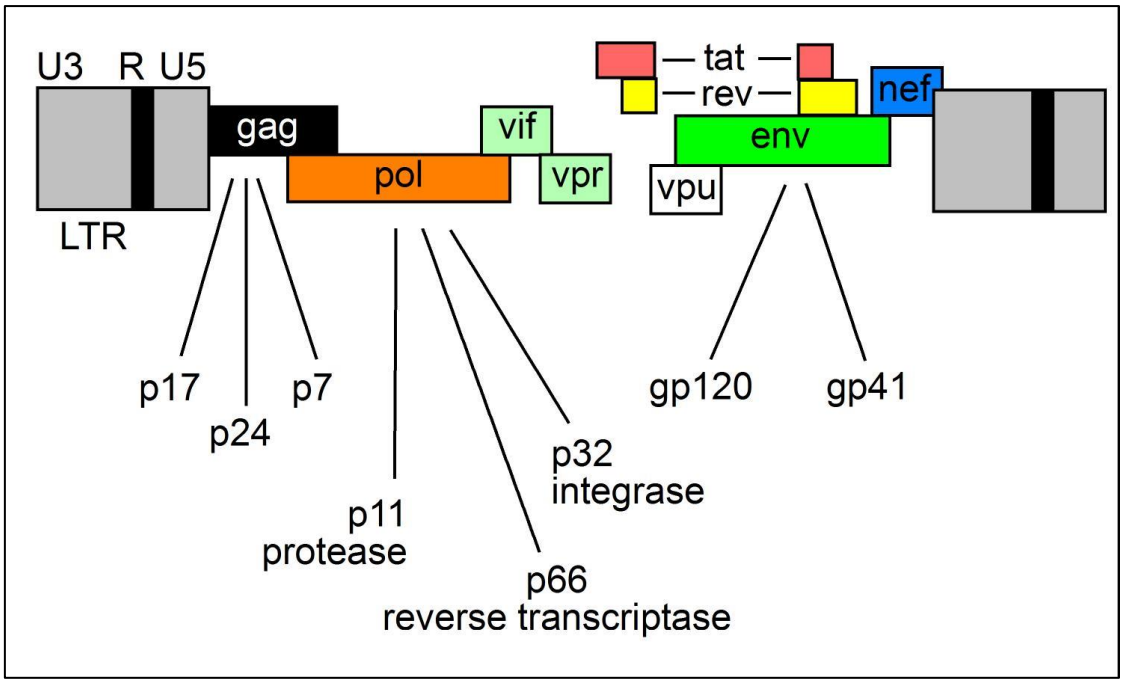

Figure 1.4: Genomic organization of HIV-1. Gag, pol and envelope are shown in rectangles (black, orange and green respectively). Tat, rev, nef, vif, vpr, vpu are represented in squares (red, yellow, blue, light green, and white respectively) LTR (long terminal region) is shown in grey (Rockstroh, 2011).

1.2.2 HIV-1 Life cycle

HIV-1 is a retrovirus, with an outer envelope and two copies of RNA and reverse transcriptase that will convert RNA to DNA. There are 6 steps of HIV-1 replication; binding and entry, HIV replication and integration, assembly, budding and release of new mature virions.

1.2.2.1 Virus attachment and entry

During this process, the virus places itself on the cell surface and uses CD4 T cell receptors to prepare for fusion. Viral fusion is caused by binding of the virus to target cell membrane and this is mediated by gp41 (Weiss, 1993). This is followed by binding of gp120 and CD4 receptors located on the surfaces of T helper cells, macrophages, monocytes and dendritic cells (Fauci, 2007b). This is then followed by a conformational change of the envelope exposing specific domain of gp120 which results in the binding of the C-C chemokine co-receptor 5 (CCR5) (Clapham and McKnight, 2002).

The function of these chemokine co-receptors is to facilitate homing of different types of immune cells to the site of infection (Alkhatib and Berger, 2007). The stock of the gp120 and 41 pieces through into the host cell undergoes conformational change unfolding itself, drawing two membranes together and the binding of the co-receptors triggers pore formation allowing the formation of a helical bundle structure which completes the fusion of the virus and cellular membranes, (Figure 1.5) (Klasse, 2012, Gomez and Hope, 2005).

1.2.2.2 Reverse transcription and Uncoating

Fusion is followed by uncoating where the viral genetic material (two viral RNA strands and three essential replication enzymes) is injected essentially into the target cell and the envelope protein is left at the cell surface. The virus matrix and capsid proteins are digested when the virus enters the cell (Bukrinsky et al., 1993). This then releases viral enzymes and viral RNA into the cell cytoplasm. Reverse transcription takes place when the reverse transcriptase (RT) takes the viral RNA and by using host nucleotide, converts that viral RNA into a single strand of DNA. This single stranded DNA is again transcribed into double stranded DNA (Figure 1.5).

1.2.2.3 Integration

Following reverse transcription, integrase enzyme transports the double stranded DNA and carries it through a nuclear pore into the nucleus of the cell. Integrase then mediates integration of viral DNA to host genome. At this point, the viral DNA is referred to as ‘provirus’. The provirus may stay inactive for longer periods or undergo transcription and translation for new viral proteins (Bukrinsky et al., 1993).

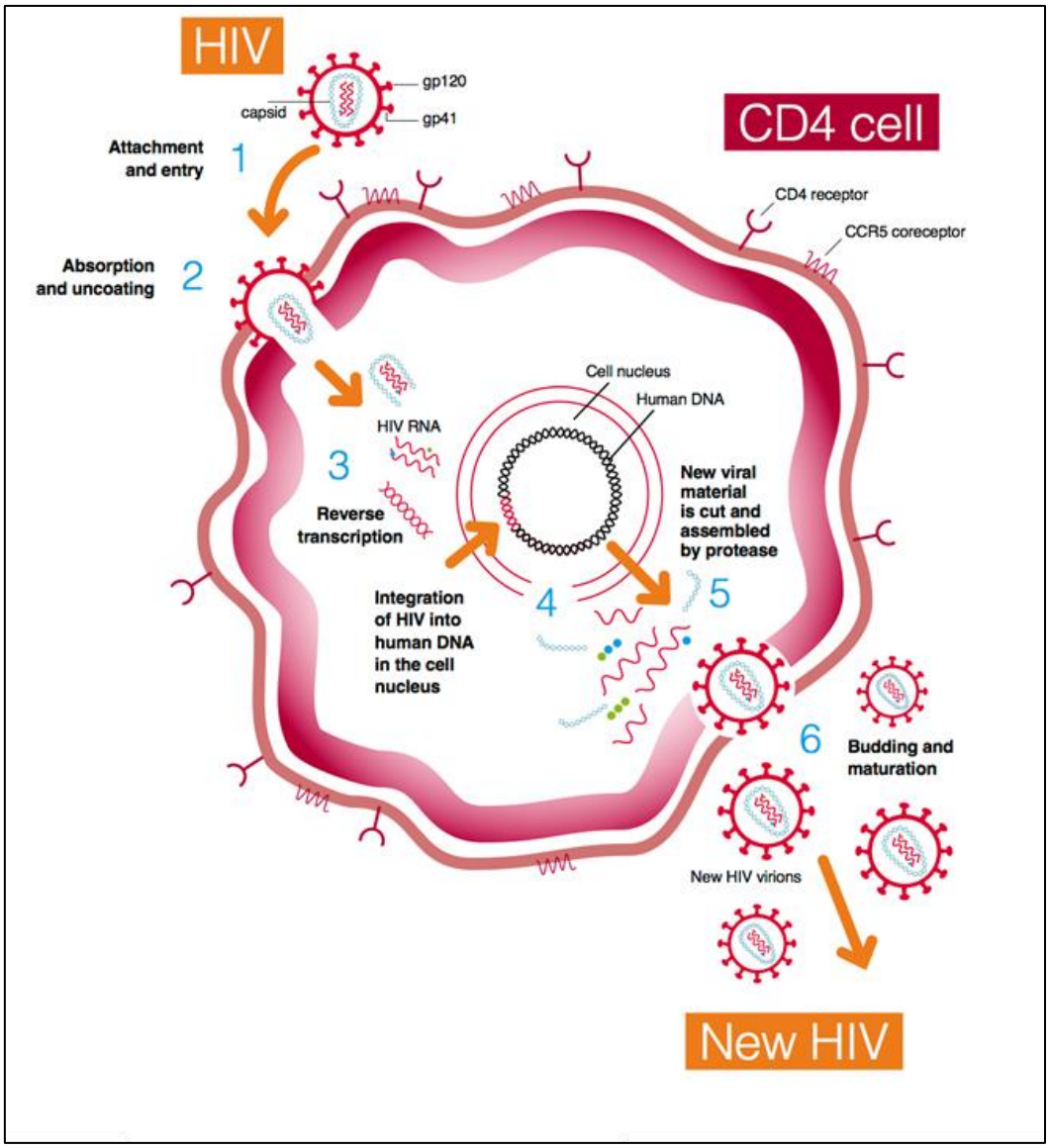

Figure 1.5: Replicative cycle of HIV-1. The HIV virus attaches and enters the cell. 2) Uncoating takes place. 3) Reverse transcription takes place where viral RNA is converted into viral DNA. 4) The integration of Viral DNA into the host genome. 5) The infected cell from the host produces copies of the viral RNA. 6) The host cell assembles new viruses and the newly assembled viruses leave the cell to infect other cells (iBase, 2017).

1.2.3 Assembly and budding

Host genome at this stage contain HIV-1 genetic material. RNA polymerase then comes along and make messenger RNA (mRNA). These mRNAs encode for different viral proteins. They associate with ribosomes at the surface of rough endoplasmic reticulum (ER). Messenger RNA then makes envelope protein directly produced into the ER, shuttled through ER, and taken to the cell surface where it becomes embedded to cell membrane and colonise with other envelope proteins produced (Freed, 2001).

At the same time, there are other mRNAs being produced that allow translation of other viral proteins. Tat and Rev proteins are also synthesized. Tat binds to the TAR site at the beginning of the HIV-1 RNA within the nucleus and stimulates the transcription and the production of longer RNA transcripts. The function of Rev is to facilitate the transcription of longer RNA transcripts and the expression of structural and enzymatic genes. In addition, Rev also inhibits the synthesis of regulatory proteins, promoting the formation of mature viral particles (Fanales-Belasio et al., 2010). These mature viral particles are then released by budding from the host cell surface to continue the cycle of HIV-1 replication.

1.3 The natural response to HIV-1 infection

Immediately after HIV-1 infection, the virus replicates in the mucosal cells, submucosal cells as well as in draining lymphoreticular tissues (Emau et al., 2006, Weiss et al., 2010), and at this time, the virus is unable to be detected in plasma (Keele et al., 2008).

Despite of the route of viral transmission and the first cells infected, within a few days, viral replication occurs on the gastrointestinal tract in gut-associated lymphoid tissue (Brenchley et al., 2004, Mehandru et al., 2004, Veazey et al., 1998). In this tissue, the majority of infected cells are resting CD4+ T cell which lack activation markers and express low levels of CCR5, a chemokine receptor found on T cell surfaces (Li et al., 2005) and other cells including macrophages and Langerhans cells (Derdeyn and Silvestri, 2005). During initial infection, epithelial cells of the mucosa recruit dendritic cells and dendritic cells secrete cytokines to attract activated CD4+ T cells to site of infection (Haase, 2010).

After infection, early immune responses rapidly drives viral replication since the presence of the virus recruits other T cells to the site of infection which in turn lead to more T cells being infected (Li et al., 2009).

During the acute phase of infection, the HIV-1 plasma viral load goes up exponentially and CD4+ T cells are depleted in the first weeks of infection, this phase is also associated with a responsive immune system trying to fight the infection (Figure 1.6) (Fanales-Belasio et al., 2010).

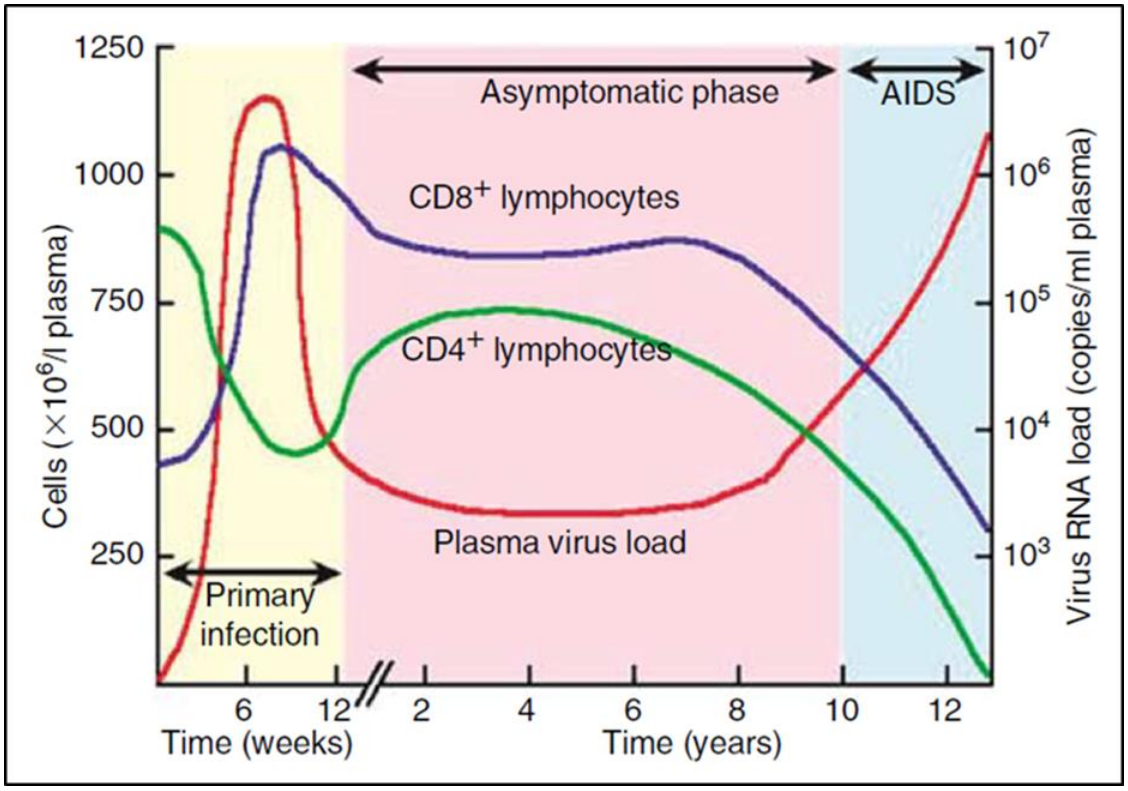

Figure 1.6 HIV clinical course. Diagram of typical course of HIV-1 infection showing changes in CD4+ and CD8+ T-cell counts in peripheral blood and plasma viral load, and three phases of HIV infection (Munier and Kelleher, 2007).

Few weeks after infection, the virus replicates at low levels, developing latent infection (Figure 1.6) (Fauci, 2007a). Latent stage lasts for years, however, some people may progress faster because of the failure of immune response to fight the infection due to persistent viral replication. The virus remains untraceable for years in other patients which indicates that they are able to control the infection (Fanales-Belasio et al., 2010). Following this, HIV-1 spreads to lymphoid tissues leading to further immune destruction and development of opportunistic infections like tuberculosis (TB) and pneumonia (Ford et al., 2009, McMichael et al., 2010b) (Figure 1.6).

The last stage of HIV-1 infection is AIDS where the immune system is totally damaged. At this stage the numbers of CD4+ T cells are very low, below 200 cells per/mm^3, also, during this stage there is increased chances of opportunistic infections (Akram and Inman, 2012, Brooks et al., 2009) and eventually leads to death (Figure 1.6). HIV-1 related mortality and morbidity have decreased since the initiation of ART. After ART initiation, the viral load decreases, however, CD4 T cell counts are not consistent.

Suppression of viremia decreases immune activation and that lowers HIV-1 replication preventing risk of transmission (Yamashita et al., 2001). Despite the effectiveness of ART and improved life expectancy in HIV infected individuals, there are still challenges faced in the new ART era; for example, the occurrence of non-AIDS co-morbidities (UNAIDS, 2018).

1.4 Brief overview of the host immune responses to HIV-1 infection

1.4.1 Innate responses

Skin and the mucosal membrane are physical barriers that act as the first line of defence against invading microbes. Mucosal membrane has mucous that traps invading pathogens. There are also epithelial cells that provide the first line of defence by blocking pathogens from entering the host. However, some pathogens such as HIV-1 can cross the physical barriers and upon doing so they encounter host immune responses where it can be eliminated by natural killer cells and CD8+ T cells (Medzhitov and Janeway, 2000).

The innate immune response is very rapid after pathogen invasion. It also includes cells like neutrophils, macrophages, monocytes, dendritic cells (DCs), natural killer cells (NK cells) and other cell types. Neutrophils, monocytes, and macrophages have phagocytic effects. They engulf infected cells, invading pathogens or dying cells. On the other side, DCs are antigen-presenting cells (APC) that take up the antigen and present it to specific cells depending on the captured antigen. Dendritic cells regulate the adaptive immune response and they bring both arms of the immune system together.

1.4.1.1 Natural killer cells

Natural killer (NK) cells are the main effector cells of the innate immune response. They synthesise and release perforin which is a protein that kills targeted cells by opening pores on their surfaces (Chang and Altfeld, 2010). Natural killer cells bridge the innate and adaptive immune systems in that they directly respond to viruses, develop memory-like responses after initial pathogen encounter or vaccination, and they shape the adaptive immune response of the infection which helps them to clear the infection faster when it comes back (O'sullivan et al., 2015).

The increased sub-population of NK cells in HIV -1 infection has been shown, however these cells are not functional enough to clear the virus (Alter et al., 2005). This impaired functionality is thought to be caused by viral products, suggesting that HIV-1 might have evolved to escape mechanisms against NK cell mediated control (Jost and Altfeld, 2012).

1.4.1.2 Dendritic cells (DCs)

Dendritic cells are in the skin, mucosa as well as in lymphoid tissues. These cells are known to be professionals in terms of presenting antigens to cells such as T cells and NK cells capable of killing pathogens (Vivier et al., 2008, Dustin and Long, 2010). Their important function is to take up the antigen, process it and present it to T or NK cells to create memory as well as prevent these T or NK cells from destroying self-antigens. They do this by expressing antigenic peptides that associates with major histocompatibility complex class II in T cells which recognize these antigens and build immune synapses. Immune synapses are stable intercellular junctions between the cells. To add, DCs also secrete cytokines for regulation of the immune responses (Monks et al., 1998). In HIV-1 infection, the major targeted cells are CD4+ T cells, however DCs as antigen presenting cells, represent a crucial subset in HIV-1 infection by presenting HIV-1 to target cells (Miller, 1998).

1.4.2 Humoral immune response

Humoral immunity is an antibody-mediated immune response. Antibodies are proteins produced by plasma cells and protect the host from infection firstly by neutralization, opsonization and lastly to kill by phagocytosis and activating the complement cascade (Casadevall, 2018). In HIV-1 infection, the humoral immune response antibodies block the virus from entering and infecting target cells (McMichael et al., 2010a). Antibody responses develop four to eight weeks after infection and are principally targeted against virions that are free-floating, whilst some antibodies may eliminate HIV-1 infected cells. Most antibodies cannot prevent the transmission of HIV-1 from one cell to another. Most people infected with HIV-1 develop antibodies to the virus however, a very small number do not, possibly because of individual immune dysfunctions (Alter and Moody, 2010, Ellenberger et al., 1999).

The antibody response is often directed towards the Env variable region of the infecting viral strain. Mutation of the envelope proteins (gp120 and gp41) make it possible for the virus to escape from antibodies (Aasa-Chapman et al., 2004). The rate at which HIV-1 mutate is so high that B cells fail to keep up with mutations and fail to produce enough antibodies and eventually get exhausted. HIV-1 can evade humoral response by selecting escape mutants which are not easily neutralized (Wei et al., 2003). Neutralizing antibodies only arise several months following infection (Aasa-Chapman et al., 2004, Burton, 1997). Even though memory B cells can produce wide range of antibodies to previously encountered virus, there is an impaired response to new antigens due to mutations of the virus.

1.5 CD4+ T cell responses to HIV-1 infection

CD4+ T cells bring together different processes of the adaptive immunity and provide help to B cells and CD8+ T cells (Porichis and Kaufmann, 2011b), however CD4+ T cells are highly targeted by HIV. The HIV-1 specific CD4+ T cell responses are high during acute phase of infection, but these responses decrease after few months of HIV-1 infection (Streeck and Nixon, 2010) and the numbers of CD4+ T cells go down because of increased viral replication within these cells exposes them to death.

As much as the responses of CD4+ T cells are high in many people infected with HIV-1, their magnitude is lower than CD8+ T cell responses (Betts et al., 2001). Stimulation of CD4+ T cells starts when APCs present the processed antigen to CD4+ T cells through HLA class II molecules. Naïve CD4+ T cell requires a co-stimulatory signal to be activated such as the binding of CD28 protein to CD4+ T cells. Activated CD4+ T cells differentiate into T helper 1 (Th1) cells, T helper 2 (Th2), Th17, T follicular helper cells (TFH) and regulatory T cells (Tregs).

T helper 1 cells produce IL-2 for CD8+ T cell proliferation and differentiation (Oxenius et al., 2000), Th2 produce IL-4 and IL-5 to further stimulate B cell proliferation (Zhu et al., 2010) and Th17 cells have been shown to be reduced in the gut-associated lymphoid tissue (GALT) and blood during HIV-1 infection and can predict disease progression (Brenchley et al., 2008, Falivene et al., 2015).

Reduction of CD4+ T helper cells during HIV-1 infection has been reported to affect all immune responses to HIV-1 including CD8+ T cell responses (Porichis and Kaufmann, 2011a).

1.6 CD8+ T cell responses to HIV-1 infection and their role in Acute and Chronic HIV-1 infection

For CD8+ T cells to respond to virus, they recognize HLA class I molecule encoded by the MHC locus on the surface of the target cell. HLA molecule and CD8 molecule interaction is caused by the binding of the epitope-HLA complex to the T cell receptor (TCR). This interaction activates the killing of the infected cells either by lytic or non-lytic mechanisms (Figure 1.7).

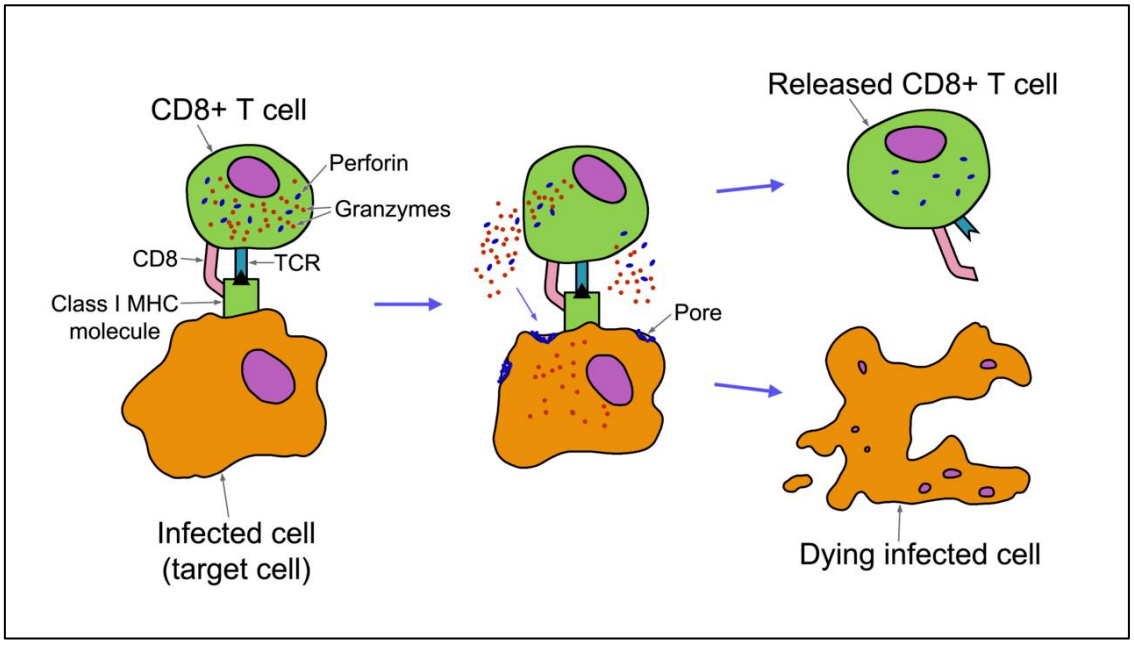

Figure 1.7: Schematic representation of CD8+ T cell recognition. Processed viral peptides from HIV-1 infected cells are presented by MHC class I proteins to CD8+ T cell for recognition and killing (wikiwand).

The principal mechanism in which the CTL kill virus infected cell is through the secretion of granzymes and perforin (Shankar et al., 1999). Perforin open pores on the surface of the target cell allowing granzyme to enter the cell and destroy viral proteins, and the target cell eventually dies through apoptosis (Figure 1.7) (Hudig et al., 1993).

The second mechanism in which CTLs kill virus infected cells is calcium independent Fas mediated pathway. Here the TCR interacts with target cell Fas ligand (FasL) on the surfaces of target cells (Wong et al., 1997) which then triggers the apoptosis cascade then death of the target cell (Broere et al., 2011).

The non-lytic pathway involves soluble molecules secreted by CD8+ T cells to neutralize infected cells. This pathway does not directly kill HIV-1 infected cell but plays a huge role in controlling viral replication (Chang et al., 2003). The CD8+ T cells secrete suppressive factors like β-chemokines which inhibits viral replication by binding to the cognate receptors which blocks viral access to co-receptors important for viral binding and entry into target cells (Copeland, 2002).

Other molecules produced by CD8+ T lymphocytes such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) play a huge role in regulating the host immune responses against HIV-1 infection (Cao et al., 2003, Roff et al., 2013).

Immune responses by CD8+ T cells have been detected in early stages of HIV-1 infection (Fiebig et al., 2003). During acute infection, HIV-1 replication increases exponentially in the peripheral blood then moves to lymphoid tissues such as lymph nodes (Kahn and Walker, 1998). As described in the above sections, this persistent replication of the virus activates HIV-1 virus-specific CTL responses. Several studies have shown the effectiveness of CTLs in HIV-1 infection (Koup et al., 1994a, Borrow et al., 1994, Deng et al., 2015, Lv et al., 2014). In addition, *in vivo* studies done on non-human primates have shown that removal of CD8+ T cells after they were infected with SIV led to increased viral load (Schmitz et al., 2005). This suggest that HIV-1 specific CD8+ T cells play an important role in controlling HIV-1. Other studies had shown that, the appearance of CTLs correlated with low plasma viral load (Koup et al., 1994b). Previous studies also reported that the increased numbers of CTLs specific for HIV-1 in acute infection had an effect in controlling viral load (Ndhlovu et al., 2015b). Even though these studies suggest that CTLs can control HIV-1 virus, these responses are narrowly directed towards certain epitopes and have low magnitude (Altfeld et al., 2001, Dalod et al., 1999, Radebe et al., 2011).

In some cases, the HIV-1 viral activity is strong that they escape CTL responses (Borrow et al., 1997), leading to loss of CTL function and disease progression (Lichterfeld et al., 2004b) despite an increase in the breadth of these responses (Alter et al., 2002, Cao et al., 2003).

In chronic HIV-1 infection, HIV-1 specific CD8+ T cells do not proliferate because of the continuous exposure to the virus that lead to their exhaustion. This is further supported by a study done by Betts and Harari 2008, where they looked at the CD8+ T cell proliferation aspect in HIV-1 viremic controllers with protective HLA class I alleles and found that CD8+ T cells were able to proliferate compared to chronic HIV-1 infected individuals (Betts and Harari, 2008, Koofhethile et al., 2016)

In addition, because of ongoing HIV-1 replication, CD8+ T cells become exhausted and their function is lost. Studies have reported that programmed death-1/programmed death ligand-1 (PD-1/PD-L1) pathway is responsible for the exhaustion of anti-viral CD8+ T cells during chronic infections. CD8+ T cell exhaustion in HIV-1 infection is associated with increased expression of CD57 and PD-1 on the surfaces of T cells (Day et al., 2006). Binding of PD-1 to its ligand PD-L1 expressed on myeloid cells including neutrophils, downregulate proliferation and production of effective cytokines such as IFN-γ, TNF-α and IL-2 by T cells (Keir et al., 2008, Bowers et al., 2014). Impaired functions of virus-specific T cells and B cells leads to immune dysfunction (Palmer et al., 2014).

1.7 Role of neutrophils in immune response

Neutrophils also known as polymorphonuclear leukocytes (PMNs), are a group of leukocytes that control infection and inflammation. They are the first cells to be recruited to the site of infection by inflammatory factors released by macrophages and mast cells, in response to pathogen invasion or danger from infected and necrotic cells. Neutrophils act as the first line of defence against pathogens and as mediators of other immune cells. These cells are short-lived (8–12 hours in the circulation and 1–2 days in tissues) and important cells for both innate and adaptive immune systems (Borregaard, 2010). Neutrophils originate from precursor myeloid cells in the bone marrow, here they become myeloblasts before maturing into neutrophils.

During infection, which may include HIV-1 infection, neutrophils are activated and release neutrophil extracellular traps (NETs), undergo degranulation, and secrete reactive oxygen species (ROS) such as H_2O_2 to fulfil their functions (Amulic et al., 2012, Mócsai, 2013). Although evidence suggests that neutrophils induce human T cell responses through activation of dendritic cells (Kalyan and Kabelitz, 2014), other studies suggest that neutrophils also supress T cell activation by releasing arginase-1, which impairs the T cell responses by blocking the availability of L-arginine. Arginine is involved in wound healing, proliferation of T cells and production of TCR ζ chain that is important in the activation of T cells (Pillay et al., 2013, Rodriguez et al., 2007, Rodriguez et al., 2002). ROS production by neutrophils modifies host molecules, resulting in a weak response to stimuli and eventually apoptosis or phagocytosis by macrophages (Perskvist et al., 2002, Amulic et al., 2012). During HIV-1 infection, chemo attractants released by neutrophils are lost and are then fully restored during HAART (Younas et al., 2016).

1.8 Duffy-null genotype and neutropenia

Given the importance of neutrophils as the first line of defence against pathogens and mediators of other immune cells, when the number of these cells in the blood circulation is reduced, it can lead to severe immunodeficiency. Neutropenia is a condition that results in a decrease in circulating absolute neutrophils counts in the blood to below 1500 cells/µl in children older than one year and adults of any ethnic group (Haddy et al., 1999).

It is characterized clinically as mild (neutrophil counts between 1000 cells/µl and 1500 cells/µl), moderate (500–1000 cells/ml) or severe (below 500 cells/µl) (Newburger, 2016, Thobakgale and Ndung'u, 2014, Haddy et al., 1999, Paz et al., 2011). Individuals with absolute neutrophil count less than 200 cells/µl undergo severe or even fatal infections (Newburger, 2016). Neutropenia can either be congenital or benign. Congenital neutropenia is normally found in Caucasians and these people often experience bacterial infections due to lack of adequate number of neutrophils (Hsieh et al., 2010).

Benign neutropenia is the type of neutropenia where serious infections do not occur even with neutrophil counts below 200 cells/µl (Klein, 2009, Hsieh et al., 2010). Benign ethnic neutropenia has been found to be common in 25-50% of persons of African descent, and is not associated with clinical outcome compared to other ethnic groups (Haddy et al., 1999, Grann et al., 2008, Shoenfeld et al., 1985). The prevalence of benign ethnic neutropenia reported in different ethnic groups is summarized in Table 1.2.

Table 1.2: Prevalence of benign neutropenia by ethnic populations (Thobakgale and Ndung'u, 2014).

Ethnic group	Country of origin	Benign neutropenia prevalence (%)
American Blacks	United States	4.5
American Blacks	United States	10.5
American Whites	United States	0.8
American Whites	United States	0
African Blacks	Uganda	>30
Arabs	Saudi Arabia	10.7
European Americans	United Kingdom	0.8
European Whites	United Kingdom	0
Yemenite Jews	Israel	11.8
Black Ethiopian Jews	Israel	15.4
Dominicans	Dominican Republic	0
Haitians	Haiti	8.2
Jamaicans	Jamaica	2.7

The causes of benign ethnic neutropenia are poorly understood because there is no deficiency in granulocyte forming cells; there is normal myeloid maturation however there is reduced release of mature neutrophils from the vascular endothelium and bone marrow to the periphery (Paz et al., 2011, Reiner et al., 2011).

Even though causes of benign neutropenia are not known, an identified genetic determinant for benign neutropenia has been implicated. Studies have linked benign ethnic neutropenia to Duffy-null genotype (Reich et al., 2009). Using admixture mapping, a region in chromosome 1 was found to be the one that account for differences in white blood cell counts between African Americans and European Americans (Nalls et al., 2008).

It was subsequently confirmed that single nucleotide polymorphism, (rs2814778, -46T→C) of Duffy antigen receptor for chemokines (DARC), that is identified on chromosome Iq22 of the receptor in red blood cells (RBCs) is predictive of neutrophil count in African Americans (Reich et al., 2009). Also there are reports that DARC null trait is highly prevalent in Africa (Figure 1.8) (Howes et al., 2011).

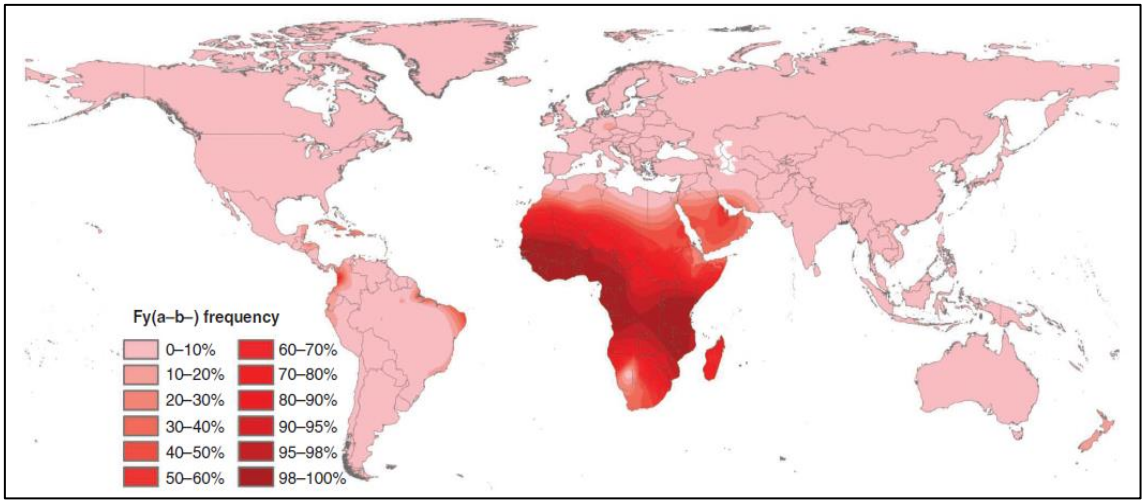

Figure 1.8: Global prevalence of Duffy-null genotype (Howes et al., 2011).

DARC encodes for Duffy antigen on red blood cells, vascular endothelial and neuronal cells. It acts as a sink for pro-inflammatory chemokines, regulating their levels in circulation, affecting neutrophil localization, chemotaxis and migration (Lee et al., 2003, Paz et al., 2011). The DARC null polymorphism selects for DARC null phenotype in which DARC is not expressed on red blood cells. This trait is associated with neutropenia and explains the variations of neutrophil counts between African-Americans and European-Americans (Reich et al., 2009). This is supported by a previous study that confirmed that approximately 59% of African-American males had DARC-null genotype compared to 65% of men and women from South Africa (Julg et al., 2009b). Apart from DARC gene polymorphisms in African Americans, other genetic variants associated with low white blood cells have been discovered. These include genetic variants in CXCL2 on chromosome 4, near CDK6 on chromosome7, and CSF3 on chromosome 17 (Reiner et al., 2011). People with HIV-1 infection suffer from neutropenia (Levine et al., 2006).

Before the association between DARC-null trait and neutropenia was shown, a study by He and colleagues reported that DARC-null might play a role in HIV acquisition and disease progression. It was suggested that DARC-null phenotype was associated with a 40% increased chance of HIV-1 acquisition and may account for up to 11% of HIV-1 burden in Africa. These authors also showed that following HIV-1 infection, DARC-null trait was associated with slower disease progression (He et al., 2008).

The findings by He et al were contradicted by another study done by Walley and others (2009) who demonstrated that, the DARC-null trait had no effect on HIV-1 acquisition or disease progression (Walley et al., 2009). The following year Julg et al, did a study where the effect of the DARC-null genotype on the outcomes of disease progression were examined. The study assessed DARC null, heterozygous and wild-type genotypes and found that these had no effect on CD4 decline or viral load among DARC genotypes, suggesting that if DARC-null trait is associated with disease progression, it was independent of these factors (Julg et al., 2009a). In addition, DARC null has evolved on the African continent as a resistant factor to malaria by preventing invasion on red blood cells by *Plasmodium vivax* (Horuk et al., 1993). However, the impact of this polymorphism on infectious diseases like HIV-1 remains largely unknown.

There are mechanisms proposed to explain how DARC presence influences acquisition and disease progression. Before onset of infection, HIV-suppressive chemokines (CCL5, a chemotactic cytokine for T cells that suppresses HIV-1 replication) associated with DARC-expressing erythrocytes may act as a protective shield on the DARC receptor, preventing HIV-1 to attach to the red blood cell and be transferred to HIV-1 target cells. In this case DARC-null trait increases HIV-1 acquisition risk. However, following HIV-1 acquisition, HIV-1 can bind to DARC receptor on the surface of RBCs thereby allowing transfer of the virus to target cells. This indicates that DARC-expressing RBCs might act as carriers of infectious HIV-1 particles to susceptible cells such as CD4+ T lymphocyte leading to increased disease progression (He et al., 2008).

Furthermore, DARC-positive individuals may be predisposed to a more pro-inflammatory state that promotes HIV-1 replication and spread (Kulkarni et al., 2009).

It is hypothesized that people with neutropenia may experience slow disease progression because of reduced inflammation due to less HIV-1 particles that can bind to DARC-null in red blood cells (Thobakgale and Ndung'u, 2014).

1.9 Interaction of T cells and neutrophils and role in HIV-1 infection

Neutrophils have emerged as important regulators and have been suggested to play a role in cross talk with immune cells such as natural killer cells, B cells, T cells, macrophages, dendritic cells and platelets (Boudaly, 2009, Costantini and Cassatella, 2011, Scapini et al., 2008, Silva, 2010). Neutrophils have been reported to prime CD8+ T cells in the bone marrow and lymphoid organs such as lymph nodes through antigen presenting cells (Maletto et al., 2006). Neutrophils recruit local macrophages to the site of infection and these neutrophils then release IL-1β and TNF-α to recruit more T cells into the infected areas (Hampton and Chtanova, 2016).

Duffy and others (2012) reported that chemokines released by neutrophils also attract T cells to the site of infection. Upon T cells arrival, neutrophils release IFN-γ, TNF-α, cathepsin G, and neutrophil elastase to differentiate T cells. Neutrophils also secrete IL-12 to activate T cells (Duffy et al., 2012b).

Previous studies speculated that neutrophils might inhibit T cells responses in viral infections. The mechanisms of this suppression involve reactive oxygen species (ROS), arginase-I (ARG), and PD-L1 (de Kleijn et al., 2013, Highfill et al., 2010, Schmielau and Finn, 2001).

The first mechanisms in which neutrophils affect survival of T cells is the inhibition mechanism (Figure 1.9). Here, neutrophils undergo degranulation and release serine proteases to inhibit T cell proliferation by stimulating IL-2 and IL-6 and promoting the shedding of IL-2 and IL-6 receptors on the surface of T cells. Neutrophils also inhibit T cell proliferation by the release of reactive oxygen species (ROS) and Arginase-1 as described in the above sections that arginase-1 and ROS downregulate T cell receptor chain (TCRζ) on T cells, which in turn lead to T cell arrest at G0-G1 phase (Figure 1.9). Neutrophils also express PD-L1 to induce T cell apoptosis by binding to interferon-dependent PD-1 that is expressed on T cells (Leliefeld et al., 2015).

The second mechanism involves the activation of T cells by neutrophils. Here, neutrophils activate T cells by presenting antigen to them directly in that, when neutrophils undergo apoptosis after ingesting pathogen, dendritic cells take up antigen from neutrophils and present it to T cells.

Indirect presentation involves presentation of antigen to T cells using MHC class I. An alternate pathway involves the ingestion of antigen and release of bacterial products by neutrophils to activate γδ-T cells (Leliefeld et al., 2015).

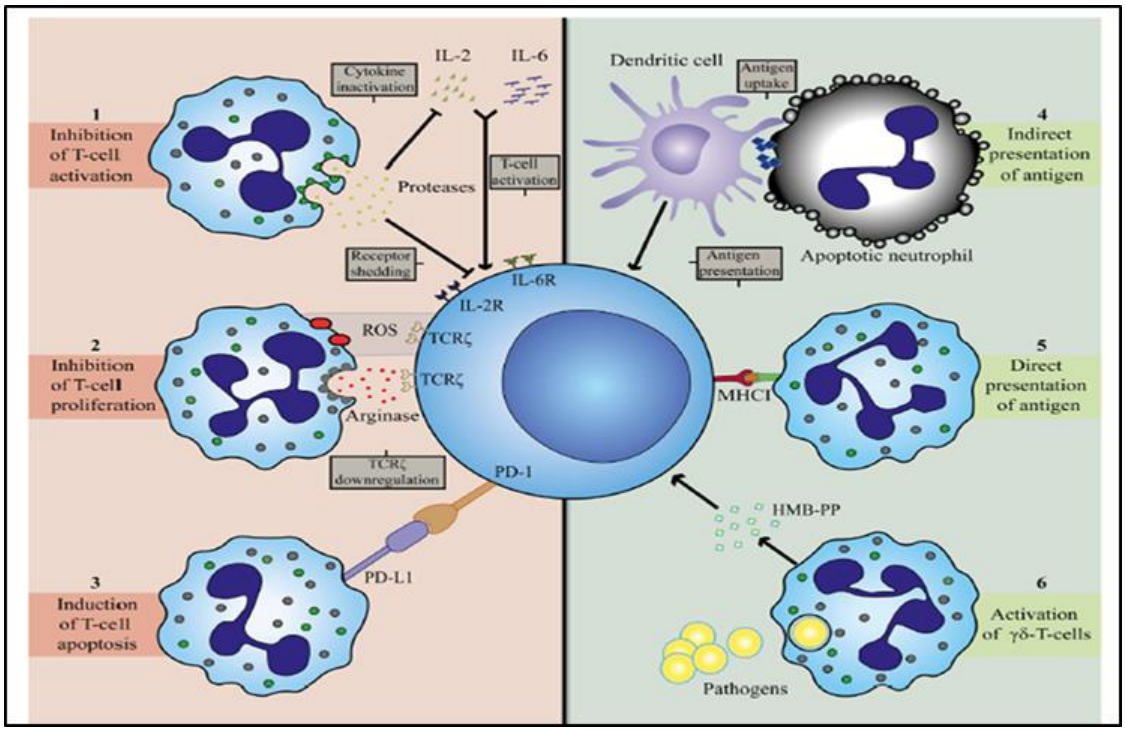

Figure 1.9: Mechanisms involved in T cell inhibition (left panel) and activation (right panel) by neutrophils (Leliefeld et al., 2015).

In HIV-1 infection, the priming of HIV-1 virus-specific T-cells by neutrophils has been reported (Duffy et al., 2012b). A previous study has shown that neutrophils in the blood of HIV-1-infected individuals express high levels of PD-L1 induced by HIV-1 virions. This study also showed that neutrophil PD-L1 levels correlated with the expression of PD-1 and CD57 on $CD4^+$ and $CD8^+$ T cells (Bowers et al., 2014).

This previous study also highlighted that neutrophils purified from the blood of HIV-1-infected patients suppress T cell function via several mechanisms including PD-L1/PD-1 interaction and production of reactive oxygen species (ROS) (Bowers et al., 2014).

The gathered data suggest that chronic HIV-1 infection results in an induction of immunosuppressive activity of neutrophils characterized by high expression of PD-L1 and an inhibitory effect on T cell function. However, the impact of DARC-null on T cell function in Africans with HIV-1 infection is unknown. Studies are needed to understand the role of the DARC-null genotype and low neutrophil counts on various diseases to identify possible biomarkers of disease protection or severity. These could also motivate identification of race-specific reference ranges and therapies for people of African descent to prevent poor outcomes of different illnesses.

We here expanded on the proposed model by Thobakgale and Ndung'u, in 2014 (Figure 1.10), on how neutrophil counts in Africans may affect susceptibility or ability to control diseases (Thobakgale and Ndung'u, 2014) by investigating the role of DARC null on T cell function in Africans from Durban, KwaZulu-Natal.

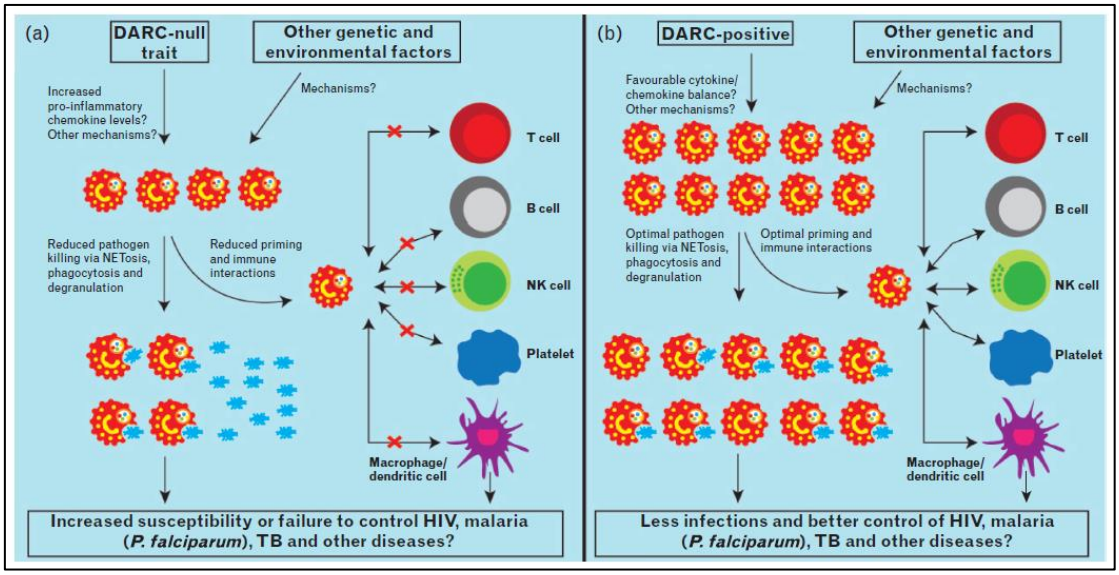

Figure 1.10: A simplified model of how neutrophil counts in people of African descent may affect susceptibility or ability to control diseases. (a) In the context of neutropenia, caused by genetic or environmental factors, neutrophils fail to clear infection and there may be inability to prime T cells, which might increase risk of infection and pathogen spread. (b) When neutrophils count and functions are optimal as dictated by genetic and other undetermined factors, there is increased pathogen clearance, and neutrophil priming and cross-talk with T cells result in HIV-1 infection (Thobakgale and Ndung'u, 2014).

1.10 Aims and Objectives of the study

Here we characterized the mechanisms and the impact that DARC null trait might have on patients' CD8+ T cell profiles and proinflammatory cytokine and chemokine responses. The aims and objectives of the study were as follows:

1. **Aim 1**: To investigate the role of DARC-null linked neutropenia on T cell phenotype during HIV-1 infection.

Objective 1: To assess the T cell activation, senescence and exhaustion (using HLA-DR and CD38, CD57 and PD-1 respectively) using multi-parameter flow cytometry in HIV infected individuals and HIV uninfected donors with and without the DARC-null polymorphism using flow cytometry.

2. **Aim 2:** To investigate the role of DARC-null linked neutropenia on T cell proliferation and function during HIV-1 infection.

Objective 2: To assess the T cell proliferation and cytokine (CD107a, IFN-γ and TNF-α) production following antigen stimulation (gag and envelope) in HIV-1 infected individuals and HIV uninfected donors with and without the DARC-null polymorphism using ICS.

3. **Aim 3:** To investigate the role of DARC-null linked neutropenia on systemic inflammation during HIV-1 infection

Objective 3: To assess whether differences in the pro-inflammatory cytokines (G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, MCP-1, MIP-1β, and TNF-α) levels exist in DARC-positive and DARC-negative HIV uninfected and infected individuals using Luminex assay.

Hypothesis

We hypothesised that the Duffy-null genotype will negatively affect T cell function, phenotype and proinflammatory cytokine levels in people of African descent with HIV-1 infection.

1.11 Candidate's contribution

The candidate did the experiments, analysis and interpretation of results and write-up. Blood collection was done at clinic sites and sample processing was done at the HPP laboratories.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study Participants

In this study we recruited young HIV-1 positive females (n=19) and males (n=1) with median age of 23 years (range 20-24) from the HIV Pathogenesis Programme (HPP) Acute study cohort at Prince Mshiyeni Memorial Hospital, located at Umlazi township, Durban, KwaZulu-Natal, South Africa. The precise time of infection for these participants was unknown but were in the chronic stage of infection for this cross sectional analysis and were antiretroviral therapy (ART) naïve.

In addition, young HIV uninfected individuals (n=20), all women with a median age group of 21 (range 19-22) years were recruited from the Females Rising through Education, Support, and Health (FRESH) cohort located in Umlazi township, Durban, KwaZulu-Natal, South Africa. These women otherwise healthy, however at very high risk of HIV-1 infection, were recruited and closely monitored for HIV infection of as part of HIV prevention curriculum, empowerment, life skills offered through the FRESH study.

This pilot study was exploratory and a sub-study within the HPP studies that received ethical clearance; reference number BF131/11 for the FRESH cohort (for HIV uninfected individuals) and E036/06 for the HPP acute cohort that recruited HIV-1 infected participants. Sample calculation for this exploratory pilot sub-study was not needed. Written informed consent was obtained from each individual for participation in the study. This sub-study was approved by Biomedical Research Ethics Committee (BREC) of the University of KwaZulu –Natal under ethics approval number BE346/17.

2.2 CD4+ T cell count, viral load and polymorphonuclear cells (PMNs) measurements

Absolute CD4 counts were measured using the Tru-Count technology using flow cytometry as previously described (Zulu et al., 2017). Plasma viral loads for the HPP acute cohort were measured using Nuclisens HIV-1 QT assays, the Biomerieux Easy-MAG and Biomerieux Easy-Q platform for nucleic acid extraction with a detection limit of 400 HIV-1 RNA copies/ml plasma according to manufacturer's instructions (Dong et

al., 2018). Sysmex XN system (Sysmex, Canada) was used to measure neutrophil count using full blood count test.

2.3 DARC -46T→C SNP genotyping

A single nucleotide polymorphism DARC T-46C (rs2814778) was genotyped using TaqMan allelic discrimination assays in all subjects as previously described (Julg et al., 2009a, Malkki and Petersdorf, 2012) and the genotypes were verified by direct sequence analysis of samples. Briefly, DNA was isolated from buffy coats (EDTA tubes) using QIAamp DNA Blood Midi Kit from Qiagen. The DNA was then Nano-dropped and standardised at concentration of 50ng/µl with nuclease free water (Ambion). This was then followed by preparation of master mix (Taqman universal PCR master mix, SPN assay mix and nuclease free water). Target sequences were then amplified, and analysis was done on Light Cycler 480 II (Roche). Participants with two copies of C allele (CC) were labelled Duffy null, and those with one allele or none (TC/TT) were labelled Duffy positive.

2.4 Isolation of peripheral blood mononuclear cells (PBMC) and counting

Peripheral blood mononuclear cells (PBMCs) are populations of immune cells such as lymphocytes (T cells, B cells and natural killer cells), monocytes and dendritic cells and were isolated from whole blood by Ficoll density gradient centrifugation. In brief, PBMCs were isolated, frozen and stored as follows:

Whole blood samples were collected from donors using sodium heparin (BD Vacutainer, USA) and were processed within 4 hours of collection. Before processing, reagents were put at room temperature and were wiped with 70% ethanol before being placed inside the class II Biological Safety Cabinet (BSC). Blood samples were poured into 50ml conical tubes for PBMC processing. A 50ml tube of whole blood was then centrifuged at 500xg for 10 minutes at room temperature. After the 10 minutes spin, the supernatant which was plasma, was carefully removed and stored at -80 ºC ultra-freezers. The remaining whole blood was diluted with equal amount of Dulbeco's Phosphate Buffered Saline (DPBS) (Life Technologies, UK) that contained 1% of penicillin-streptomycin solution and was gently mixed by inverting. Diluted blood was overlaid into a 50ml conical tube separation medium (15ml of Histopaque-1077, Sigma Aldrich) at room temperature.

The tube was centrifuged for 30 minutes at 500xg at room temperature. After this, whole blood was divided into different cell layers as seen in Figure 2.1.

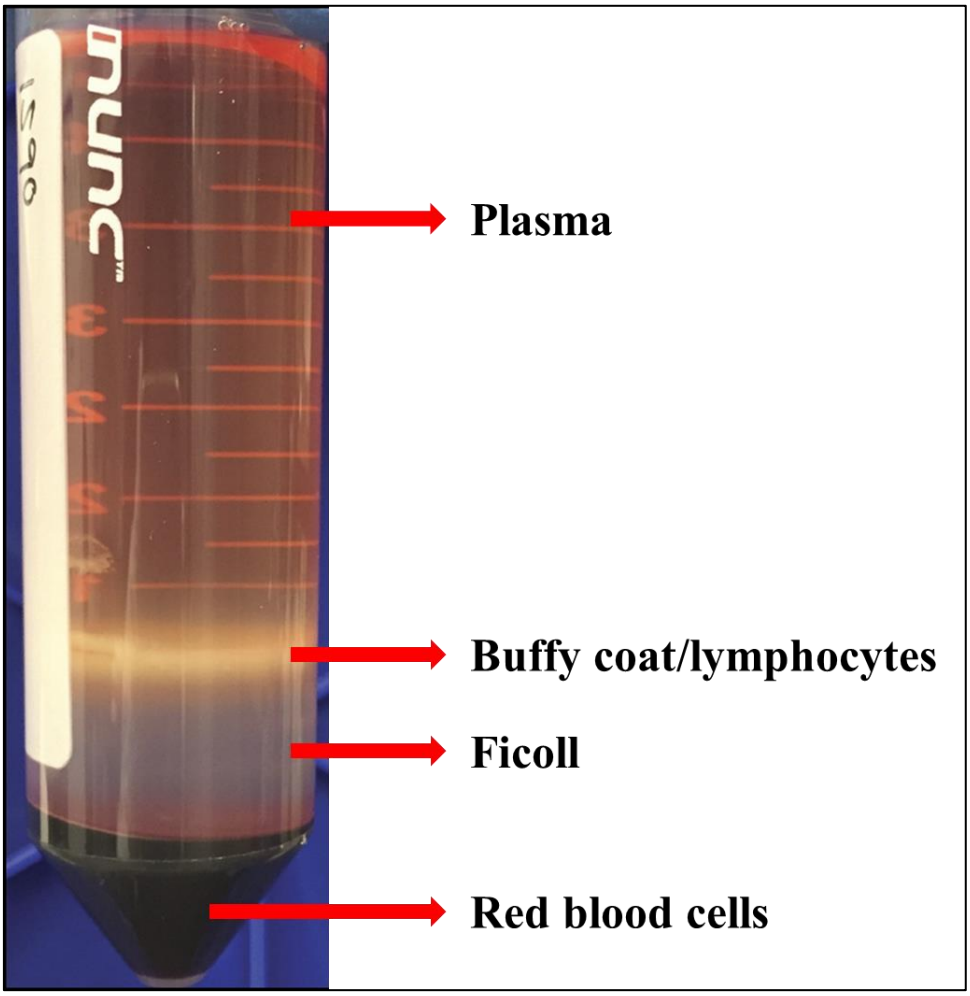

Figure 2.1: Image showing PBMC Ficoll density gradient separation technique after whole blood was centrifuged for 30 minutes (Higdon et al., 2016).

A sterile pipette was used to remove the lymphocytes (buffy coat layer) from the interface and transferred to another sterile 50ml conical tube, followed by addition of DPBS to wash the lymphocytes (cells were centrifuged at 500xg for 10 minutes at room temperature and this step was done twice). After the second wash was complete, the supernatant was decanted, and the lymphocyte pellet was suspended in 20 mL of R10 (RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 100 U/ml penicillin, 1.7Mm sodium glutamate and 1% of Hepes buffer).

From this suspension, isolated PBMCs were counted using trypan blue (Life Technologies, USA) solution (1:10 dilution, mixed 20 µl PBMCs and 180 µl trypan blue). The mixture was vortexed and incubated for 5 minutes before counting. About 10 µl of the mixture was loaded to a haemocytometer slide and counting was done manually using a light microscope before resuspension of the cells at final concentration of ten million per millilitre of R10 medium.

2.4.1 Cryopreservation of PBMCs

After cell counting was done as described above, cells were centrifuged for 10 minutes at 500xg in a chilled centrifuge at 4 ºC. Thereafter the supernatant was discarded and tubes with cells were placed on ice and the pellet was resuspended in 1 ml of fetal calf serum (FCS). Equal amounts of FCS and freezing medium [10% FCS in Dimethyl Sulfoxide (DMSO)] were added. Fetal calf serum was added first and freezing media was added after in a drop wise manner while twirling the tube. Aliquots of 1ml (containing 10 million cells) were then transferred to each cryovial. The cryovials were put into strata coolers and were stored at -80 ºC ultra-freezer overnight. The following day, cryovials were then transferred into liquid nitrogen freezer (-189 ºC) for long term cryopreservation.

2.4.2 Thawing of cryopreserved PBMCs

Before the thawing of PBMCs, R10 was first pre-warmed at 37 °C in an incubator for 1 hour. Cryopreserved PBMC vials were thawed by moving the vials around in a water bath (LabFix, Durban, SA) at 37 °C and removed before the whole ice melts. A small cloth sprayed with ethanol was used to wipe off excess water from the vials. Cells were then poured into 15ml conical tubes. This step was followed by a washing step where 1ml of warm R10 media was used to wash left cells in the vials and this was poured in the 15ml conical tube. About 9 ml of warm R10 was then added gently to the tube for each vial. Tubes were then inverted gently twice and centrifuged at 500xg for 8 minutes at room temperature. Thereafter, the supernatant was decanted, and the pellet was resuspended in 1ml of warm R10 medium. Additional 9ml of R10 was used to again wash the cells and the tube was then further centrifuged at 500xg for 8 minutes at room temperature.

Cells were resuspended in 5ml of R10 and rested for 2 hours at 37º C in 5% CO_2 prior to use. After 2 hours incubation, cells were counted (as described above under PBMC isolation) and used in subsequent assays.

2.5 Flow cytometry assay

Flow cytometry has been used for many years in biological discoveries such as blood. It detects, identifies and enumerate individual cells by analysing their size, shape as well as their properties (Tuchin et al., 2011). Flow cytometer contains several components such as: a) a sample, b) fluidics to move sample to the flow cytometer machine, c) lasers and optics which gather light, d) detectors to sense the light and e) a computer system to analyse data in a digital form. The use of multicolour panels allows researchers to measure lymphocytes precisely and it is less cost effective. In HIV research, flow cytometry allows researchers to identify immune cells affected by the virus (Douek et al., 2002). For example, many intracellular and extracellular markers are available in HIV research specifically to test HIV specific T cells among different groups of patients, including those with acute infection and chronic infection.

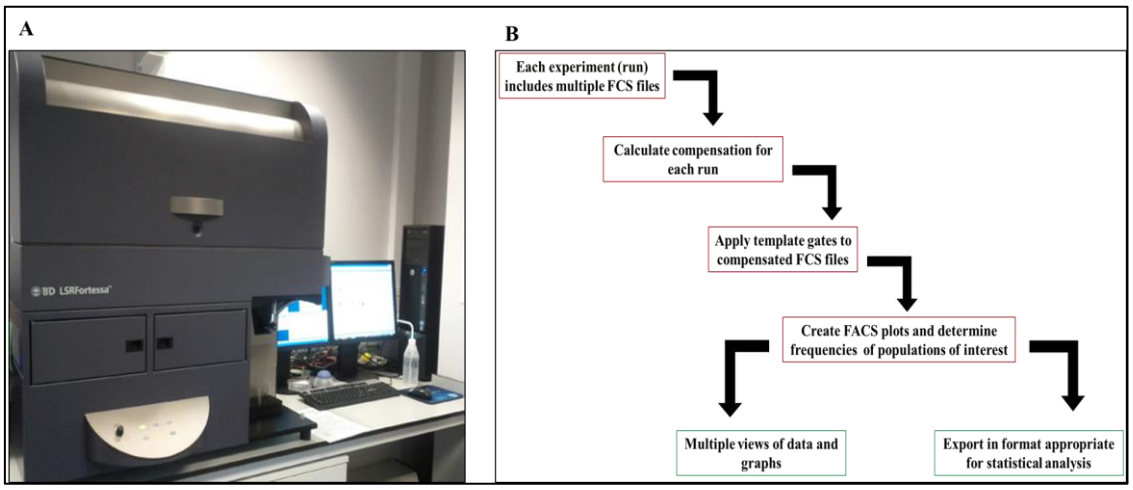

Figure 2.2: Image showing LSRFortessa flow cytometer (BD Bioscience, USA) (A) and (B) flow diagram showing the work flow from each run to data that can be used for statistical analysis.

In this study we used BD LSRFortessa flow cytometer (BD Bioscience, USA; Figure 2.2A) which can measure 16 fluorescent parameters. We used multicolour flow cytometry employing the above principles to assess T cell phenotype, function by intracellular cytokine staining (ICS) and proliferation by carboxyfluorescein diacetate, succinimidyl ester (CFSE) as described in the upcoming sections. As can be seen in the workflow in Fig 2.5B, each run provides many FCS files and compensation is calculated for each run. Template gates are applied to compensated FCS files then graphs and frequencies of the populations of interests can be determined.

2.5.1 Phenotypic characterization of CD8+ T cells

We investigated T cell activation, exhaustion and senescence by examining thawed PBMCs of HIV-1 infected individuals and HIV uninfected individuals for the following markers: CD38, HLA-DR, PD-1 and CD57. Briefly, previously cryo-preserved PBMCs were thawed in warm R10 media and counted as described in section 2.4 and 2.4.2. After counting, about 0.5 x10^6 cells were distributed in a 96-well round-bottomed plate. Cells were then stained with the following markers: anti-CD3- Pe Cy5.5 (BD Biosciences, USA), anti-CD8-Alexa-700 (BD Biosciences, USA), anti-CD14/19-Qdot 655 (Biolegend, USA), anti-CD38-PE (Biolegend, USA), anti-HLADR-PerCP (Biolegend, USA), anti-PD1-BV421 (Biolegend, USA) and anti-CD57-APC (Biolegend, USA) (Table 2.1) as well as aqua viability dye (Life Technologies, UK) for 20 minutes in the dark, at room temperature. For each experiment, the fluorescence minus one (FMO), which is the control that contains all fluorochromes of interest except the one being measured for each marker was included to allow subsequent gating. This was followed by washing of cells with addition of 200µl of 2% of FCS in PBS after incubation. The plate was then centrifuged at 2000rpm (Labotec, Durban, South Africa) for 6 minutes at room temperature and the supernatant was decanted thereafter. Perm A (40µl) was then added to fix the cells for 20 minutes in the dark at room temperature. The cells were washed again, supernatant decanted and cells were resuspended in 200µl of 2% of FCS in fetal bovine serum (FBS) before acquisition on a multicolour BD LSRFortessa flow cytometer (BD Bioscience, USA). Between 200 000 to 300 000 events were acquired and the data was analysed using Flowjo software version 9.8.5 (FlowJo LLC, Ashland, Oregone).

2.5.2 Assessment of T cell function using Intracellular Cytokine Staining (ICS)

Intracellular cytokine staining (ICS) is a flow cytometry technique used in research industry to asssess the function of specific cells through detection of cytokines produced by specific cells such as CD4+ T cells, CD8+ T cells, dendritic cells, neutrophils, natural killer cells, macrophages, B cells, etc. It is thought to be a useful tool in the development of vaccines due to its flexibility and advanced functionality (Smith et al., 2015).

In this study, ICS was also conducted on previously cryo-preserved PBMCs, samples were thawed in warmed R10 media as described above and rested for 2 hours followed by counting. One million cells were distributed in a 96-well round-bottomed plate.

Thereafter, cells were stimulated with Gag (2µg/ml) and Env peptide pools (gp41 and gp120) (2µg/ml). Unstimulated tube (media only) was included in each assay and served as the negative control and while cells stimulated with staphylococcus enterotoxin B (SEB) (1µg/ml stock) and Phorbol-Myristate-Acetate (PMA)/Ionomycin (1µl PMA and 0.5µl ionomycin, both 1mg/ml working solution) served as positive controls. Inhibitor of protein transport, Brefaldin A [(1µ1 of 10µg/ml stock) (Sigma-Aldrich, Missouri, USA)] and Golgi stop [(1µl from dilution 1:10 with R10) (BD Biosciences, USA)] were then added in all the wells. This was then followed by the addition of anti-CD107a PE-Cy5 (BD Biosciences, USA) in each well. Cells were then incubated at 37º C, overnight (16 hours). Following overnight stimulation, the plate containing cells was centrifuged (Labotec, Durban, South Africa) at 2000rpm for 8 minutes and the supernatant was discarded. Cells were then washed with wash buffer 100µl of 2% FCS in DPBS (Life Technologies, UK) buffer and the plate was again centrifuged at 2000rpm for 8 minutes.

Following this, cells were then stained with 100µl of fixable aqua dead dye (Life Technologies, UK) for viability (dilution 1:1000 in DPBS) for 20 min in the dark, (plate was covered with foil), at room temperature. Thereafter, the plate was washed with buffer and centrifuged at 2000rpm for 8 minutes, supernatant was discarded and stained with anti-CD3-PeCy5.5 (BD Biosciences, USA), anti-CD8-Alexa-700 (BD Biosciences, USA), anti-CD14/19-Qdot 655 (Biolegend, USA), CD4-APCy7 (Biolegend California, USA) and anti-CD107a-PE-Cy5 (BD Biosciences, USA) surface antibodies (Table 2.1) for 20 minutes in the dark, at room temperature.

Cells were then washed with 200µl of wash buffer (2% FCS/PBS), spun down at 2000rpm for 8 minutes and fixed in 40 µl Perm A medium (Life Technologies, Frederick, USA) for 20 minutes. Cells were then washed with 200µl of wash buffer and the plate was centrifuged at 2000rpm for 8 minutes. Following this, the supernatant was discarded and 40 µl of Perm B (Life Technologies, Frederick, USA) together with cytokines of interest, IFN-γ-PE-Cy7 (Biolegend California, USA) and TNF-α-PerCPy5.5 (Biolegend California, USA) (Table 2.1) were added and the plate was incubated for 20 minutes in the dark at room temperature. After incubation, the cells were washed with wash buffer and resuspended in 200µl of wash buffer for acquisition on the multicolour BD LSRFortessa flow cytometer (BD Bioscience, US). At least 500 000 events were acquired, and the data was analysed using Flowjo software version 9.8.5 (FlowJo LLC, Ashland, Oregone).

Table 2.1 Table showing flourochrome conjugated antibodies, supplier and volume of antibodies used per well.

Conjugated antibody, Clone	Supplier	Quantity per well
Phenotypic assay		
CD3-PeCy5.5 (UCHT1)	*BD Biosciences, USA*	1.5µL
CD8-Alexa700 (HIT8a)	*BD Biosciences, USA*	1.5µL
CD14/19-Qdot655 (63D3/HIB19)	*Biolegend, California, USA*	1.5µL
CD38-PE (90)	*Biolegend, California, USA*	2µL
HLA-DR-PercP (L243)	*Biolegend, California, USA*	2µL
PD-1-BV421(RMP1-14)	*Biolegend, California, USA*	2µL
CD57-APC (HCD57)	*Biolegend, California, USA*	2µL
ICS assay		
CD3- PE-CF594 (UCHT1)	*BD Biosciences, USA*	1.5µL
CD14/CD19-BV650 (63D3/HIB19)	*Biolegend California, USA*	1.5µL
CD8-Alexa-700 (HIT8a)	*BD Biosciences, USA*	1.5µL
CD4-APCy7 (GK1.5)	*Biolegend California, USA*	1.5µL
CD107a-PE-Cy5 (H4A3)	*BD Biosciences, USA*	3.5µL
IFN-γ-PE-Cy7(AN-18)	*Biolegend California, USA*	2.5µL
TNF-α-PerCPy5.5 (6B8)	*Biolegend California, USA*	2.5µL

2.5.3 Assessment of T cell proliferation using carboxyfluorescein diacetate, succinimidyl ester (CFSE)

Carboxyfluorescein diacetate, succinimidyl ester is a fluorescent dye used to trace lymphocyte migration, cell number and proliferation of cells. It labels the parent cell and passes the stain from parent cell to daughter cells. Carboxyfluorescein diacetate, succinimidyl ester diffuses into cells and its acetyl groups are cleaved to produce a highly fluorescent derivative. This derivative is kept within the cell and can be detected using flow cytometry. As the parent cell divides, the stain intensity within the daughter cells is halved (see Figure 2.3).

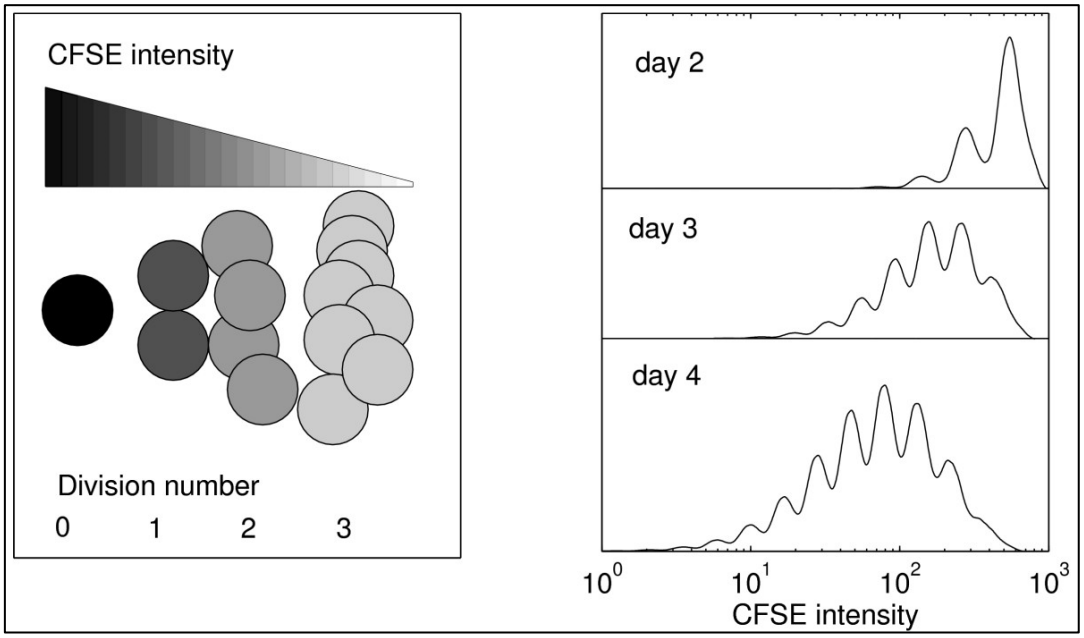

Figure 2.3: Illustration of cell division and CFSE intensity as each cell divides (Luzyanina et al., 2007)

In this study, thawed cryopreserved PBMCs were rested for 2 hours at 37 °C in 5% CO_2 incubator and then 2 x10^6 cells were stained with 0.5µg/ml CFSE (1µl in 4 ml of DPBS) (ThermoFisher Scientific, USA).

From the 2 x10^6 CSFE labelled PBMCs, 0.5 x10^6 cells were plated in each well in a 96 well plate. The plate was then stimulated with Gag peptide (2µg/ml) and PHA (1µ/ml) which served as positive control. Unstimulated well (media only) served as the negative control.

All the surrounding wells were left and filled with DPBS (Life Technologies, UK). This was then followed by incubation (ThermoFisher Scientific, USA) at 37 °C in 5% CO_2 for 7 days. Fresh media (R10) was added to the cells on day 4 (100µl was removed by putting a plate in 45 degrees position and new 100µl of R10 was added). On day 7, the plate was centrifuged at 2000rpm for 8 minutes and cells were then marked with viability marker (100µl of fixable blue dead cell stain, from ThermoFisher Scientific, USA) for 20 minutes, at room temperature. After incubation, cells were washed, and the plate was centrifuged at 2000rpm for 8 minutes and supernatant was decanted. This was then followed by surface staining with anti-CD3 PE-CF594 (BD Biosciences, USA), anti-CD4 APC-Cy7 (Biolegend California, USA) and anti-CD8 Alexa-700 (BD Biosciences, USA) and containing anti-CD14/19 (Bio-legend California, USA) for monocytes and B cell exclusion respectively. For the interest of this study we were only interested in CD4+ and CD8+ T cells. Cells were then incubated in the dark at room temperature for 20 minutes and washed with 2% FCS in PBS buffer (Life Technologies, UK). The supernatant was discarded and 200µl of buffer was added to each well, mixed up and transferred to cluster tubes. Perm A (40ul, Life Technologies, Frederick, USA) was added to each tube before acquisition on LSRFortessa flow cytometer (BD Bioscience, USA). At least 200 000 to 300 000 events were acquired, and data was analysed using FlowJo software version 9.8.5 (FlowJo LLC, Ashland, Oregon).

2.6 Luminex Assays Principle

Luminex is a micro-assay that enables simultaneous detection and quantitation of many secreted cytokines, chemokines, and growth factors from different cell types. These proteins interact with receptors of target cells to mediate physiological responses, which include immunology, growth, inflammation or hemopoietisis. Dysregulation in production of these proteins is associated with disease. Luminex assay is like sandwich ELISA (Figure 2.4), in that the capture antibody against the protein of interest are coupled with beads. These beads then react with the sample containing the protein of interest.

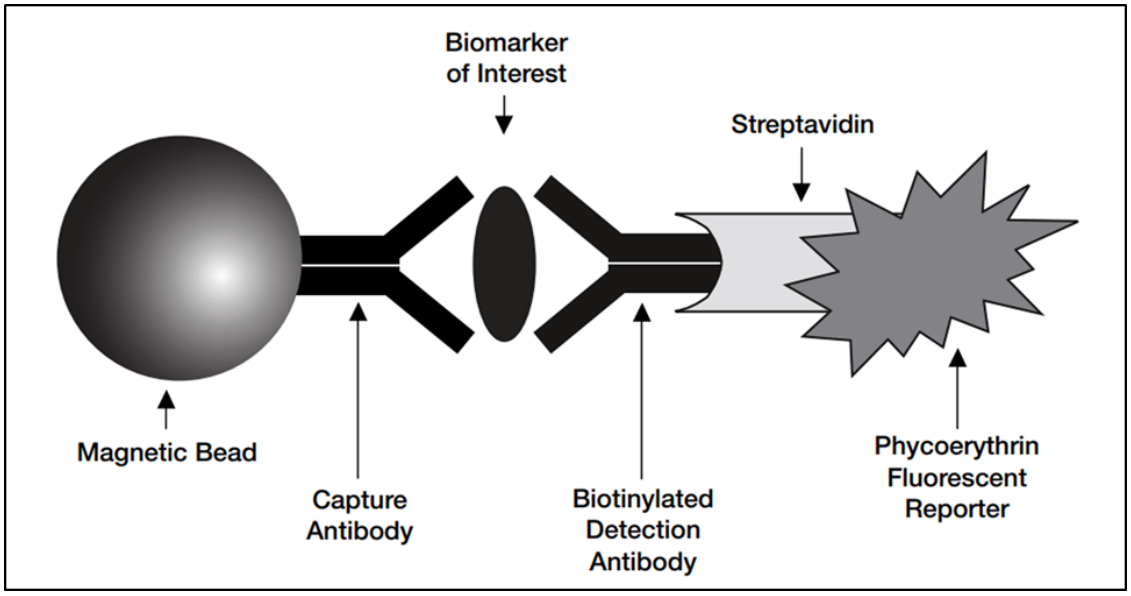

Figure 2.4: Diagram of Bio-plex sandwich immunoassay (Bio-Rad, USA) showing how magnetic beads, capture antibody, detection antibody, and streptavidin combine to make sandwich of the biomarker of interest (Bio-Rad, USA).

Coupled beads react with the sample containing the biomarker of interest (step 1 in figure 2.5). After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate. Phycoerythrin (PE) serves as a fluorescent reporter (step 2, Figure 2.5).

Beads are read on Luminex based reader (Bio-Rad®200 Bio-Plex® system was used in this assay). One laser classifies the bead and determines the analyte that is being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound.

The components of each well are drawn up into the flow-based Bio-Plex suspension array system, which identifies and quantitates each specific reaction based on bead color and fluorescence. The magnitude of the reaction is measured using fluorescently labeled reporter molecules associated with each target protein. Unknown cytokine/chemokine concentrations are automatically calculated by Bio-Plex Manager™ software using a standard curve derived from a recombinant cytokine standard. Luminex assay uses colored beads as the solid phase instead of a coated well, and up to 100 differently colored beads can be mixed and used for quantitating up to 100 different analytes simultaneously (Bio-Rad, USA).

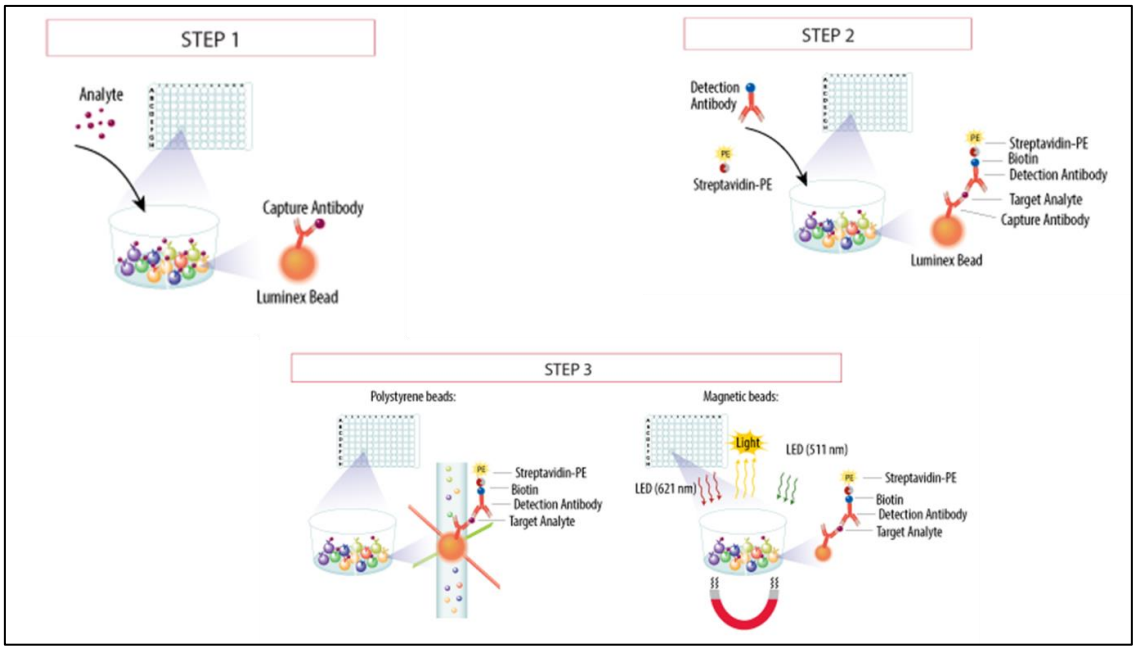

Figure 2.5: Diagram showing steps in Luminex assay. Step 1, magnetic beads with biomarker of interest are added, step 2, detection antibody is added and step 3 is the addition of streptavidin (Bio-Rad. USA).

2.6.1 Luminex for detection of neutrophil and T cell specific cytokines

In this study, a Bio-Rad human 17 multiplex cytokine/chemokine kit was used as per manufacturers' protocol to analyse plasma cytokine/chemokines namely: granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, Monocyte Chemoattractant Protein-1 (MCP-1) (MCAF), MIP-1β, and TNF-α. These cytokines/chemokines include those that are T cell specific and those that are neutrophil specific and were measured to assess if there were differences in the expression of these proteins in HIV positive and negative groups by DARC status. Initially, plate layout was designed. Column 1 and 2 were assigned to standards, with highest concentration in raw A and lowest concentration in raw H. Wells A3 and A4 were assigned to blanks. Columns 3 and 4 (wells B3, B4, C3, C4) were assigned to controls. The rest of the plate was available for samples in duplicates (see Appendix A1).

Bio-Plex R system was warmed 30 minutes prior to use and then calibrated and wash system was primed. Assay buffer, wash buffers and sample diluent were brought to room temperature before use.

Other items were kept on ice until they were required (standards) and the frozen plasma samples were thawed 1 hour before use. Single vial of standards was gently taped, reconstituted in 500µl of diluent, vortexed for 5 seconds and incubated on ice for 30 minutes.

After reconstitution was done; a fourfold standard dilution series and blank was prepared, each tube vortexed for 5 seconds and tips were changed between each dilution. Briefly, 1.5 ml polypropylene were labelled sample 1 (S1) through sample 8 (S8) and blank. Reconstituted standards were vortexed for 5 seconds, volume of 128µl was added to S1 containing 27µl of standard diluent. S1 tube was vortexed for 5 seconds and then new pippete tip was used to transfer 50µl from S1 to S2 tube. This was done up to tube S8 (Appendix A2). Beads were diluted from 10x to 1x in Bio-Plex assay buffer, vortexed for 30 seconds and protected from light. Previously diluted beads were vortexed for 10-20 seconds and 50µl was added to each well of assay plate. The plate was then washed two times with 100µl Bio-Plex wash buffer using the MAG_3X wash protocol. Following washing, 50µl of the samples and standards were vortexed and added to each well and plasma samples were run undiluted. The plate was covered with aluminium foil to protect from light and incubated on a shaker 30 minutes at 850 ± 50 rpm at room temperature. The plate was then washed 3 times and 25µl of detection antibodies were added to each well. Plate was covered with aluminium foil and put on a shaker for 30 minutes. Following this, SA-PE was vortexed for 5 seconds and diluted to 1x and was protected from light 10 minutes before use. The plate was then washed again 3 times to remove any unbound protein.

Streptavidin-Phycoerythrin was then vortexed and added (0.5µl of 100x stock adjusted to a final volume of 50µl in assay buffer) to each well with incubation at room temperature for 10 minutes on a shaker and was then washed 3x with wash buffer. Beads were resuspended in 125µl assay buffer and the plate was covered and incubated on a shaker for 30 seconds at 850± 50 rpm. Finally, the plate was visually checked if all wells were filled with buffer, plate cover was removed before placing plate on the plate carrier and the plate was read on Bio-Plex™ 200 system (Bio-Rad, USA).

2.7 Statistical analysis

The FCS files from flow cytometry were analysed using FlowJo software version 9.8.5 (FlowJo LLC, Ashland, Oregon). Frequencies of cells expressing measured cytokines/markers in T cells in HIV-1 infected and uninfected and stratified by DARC status were measured using two-tailed Mann-Whitney tests and analysed using GraphPad Prism software version 5 (GraphPad Software Inc., USA). Luminex plate was read on Bio-Plex™ 200 system and generated data was saved as excel file, then two-tailed Mann-Whitney test was used, and data was analysed using GraphPad Prism software version 5. All groups of interest were compared and p-values <0.05 were considered statistical significant.

CHAPTER 3: RESULTS

3.1 Clinical characteristics of study participants

This study aimed to examine the impact of the DARC polymorphism on CD8+ T cells in HIV-1 infected individuals and HIV uninfected individuals. The demographics and clinical characteristics for the study participants are presented in Table 3.1 and Fig 3.1. The median age of HIV negative and HIV-1 positive groups was 21 years (IQR 19-22) and 23 years (IQR 20-24) respectively. All HIV negative participants (n=20) were females, whereas 95% of the HIV positive group were females (Table 3.1).

Table 3.1 Clinical characteristics of study participants

	Age (years, IQR)	***Gender (F/M)***	***CD4 Count (cells/mm3, IQR)***	***Viral Load (RNA copies/mL, IQR)***	***Neutrophil counts (cells/mm^3, IQR)***
HIV Negative (CC)	20.5 (19-21.25)	F(n=12) M(n=0)	883 (757-975)	N/A	2560 (2180-3140)
HIV Negative (TT/TC)	21.5 (20.75-23)	F(n=8) M(n=0)	918.5 (721-1065)	N/A	4990 (4600-5720)
HIV Positive (CC)	22.25 (20.25-25.23)	F(n=9) M(n=1)	608 (499.5-740.5)	9300 (4550-14750)	1750 (1660-2090)
HIV Positive (TT/TC)	23 (20-24)	F(n=9) M(n=0)	709 (605-839)	7200 (850-21000)	4340 (3340-5080) *

N/A-not available; CD4-cluster of differentiation 4; IQR-interquartile range; F-Females; M-Males; CC-DARC null; TT/TC-DARC positive; *data for one participant was not available.

Overall, 22 of 39 individuals (56%) in this study had the DARC-null genotype. Within the HIV negative group, 60% (n=12) of the participants had the DARC null (CC) genotype and 40% (n=8) were DARC positive (TT/TC) compared to 53% (n=10) with DARC null and 47% with DARC positive (n=9) genotype amongst the HIV-1 infected group.

The occurrence of the DARC-null allele in similar target group of South Africans from Durban, KwaZulu-Natal was previously reported at 64.8% (247 out of 381 African individuals were DARC genotyped (Julg et al., 2009b). The frequency of DARC-null in other populations including African-Americans and Ugandans are summarized in Table 1.2. In addition, a study examining absolute neutrophil counts from individuals from Nigeria demonstrated that all 69 individuals assayed for DARC were homozygous for the Duffy-null allele (Wonodi et al., 2017). Together, the studies demonstrate high frequencies of the DARC-null allele in African populations.

No differences were noted in absolute neutrophil counts between HIV uninfected and HIV-1 infected groups. However, within the HIV uninfected group, DARC null participants had lower neutrophil counts compared to DARC positive participants (p=0.0004, Figure 3.1, Table 3.1).

The same was observed in the HIV-1 infected group, where DARC null participants had lower absolute neutrophil counts compared to DARC positive participants (p=0.0006, Figure 3.1, Table 3.1). HIV uninfected individuals had higher CD4 counts compared to HIV-1 infected participants (p=0.008, Figure 3.1, Table 3.1), however, this was not significant by DARC status in both HIV negative and positive individuals. HIV-1 positive participants had a median viral load of 9100 RNA copies/mL (IQR, 2650-19500). DARC null participants had lower viral loads compared to DARC positive participants (Table 3.1).

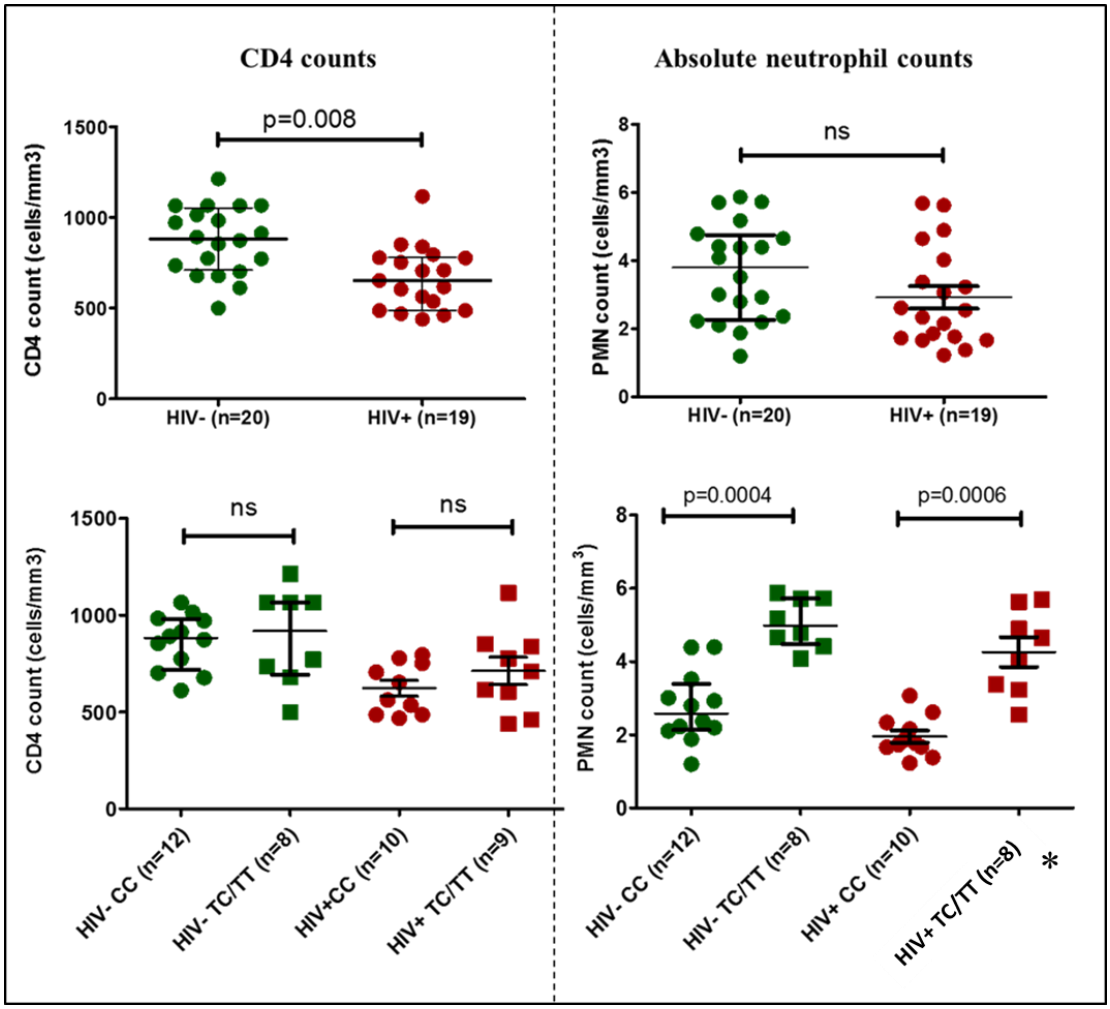

Figure 3.1: Cluster of differentiation-4 (CD4) and polymorphonuclear cells (PMN) counts in HIV uninfected individuals and HIV infected participants and stratified by DARC status within the groups. *data for one participant was not available.

3.2 Levels of CD8+ T cell activation and exhaustion differ by HIV status and not by DARC status

Activation of CD8+ T cells in HIV-1 infected individuals has been reported as a good indication of disease progression (Hazenberg et al., 2003, Lichterfeld et al., 2004a). From these earlier studies, it was clear that the relatively high expression of CD8+ T cell activation as measured by co-expression of CD38 and HLA-DR and exhaustion markers PD-1 (Day et al., 2006) and CD57 (Shankar et al., 2018) detected in HIV-1 infected individuals were elevated in HIV-1 infection. We here assessed whether the expression of these phenotypic markers would vary by DARC status and explain differences in immunological outcomes in HIV-1 infected and uninfected individuals.

To address this question, we used flow cytometry as demonstrated with a gating strategy used in Figure 3.2 (and Appendix B1 for CD4+ T cells) to investigate T cell activation (CD38, HLA-DR), exhaustion (PD-1) and senescence (CD57) *ex vivo* on PBMCs of HIV-1 infected individuals and HIV uninfected individuals with and without the DARC-null polymorphism.

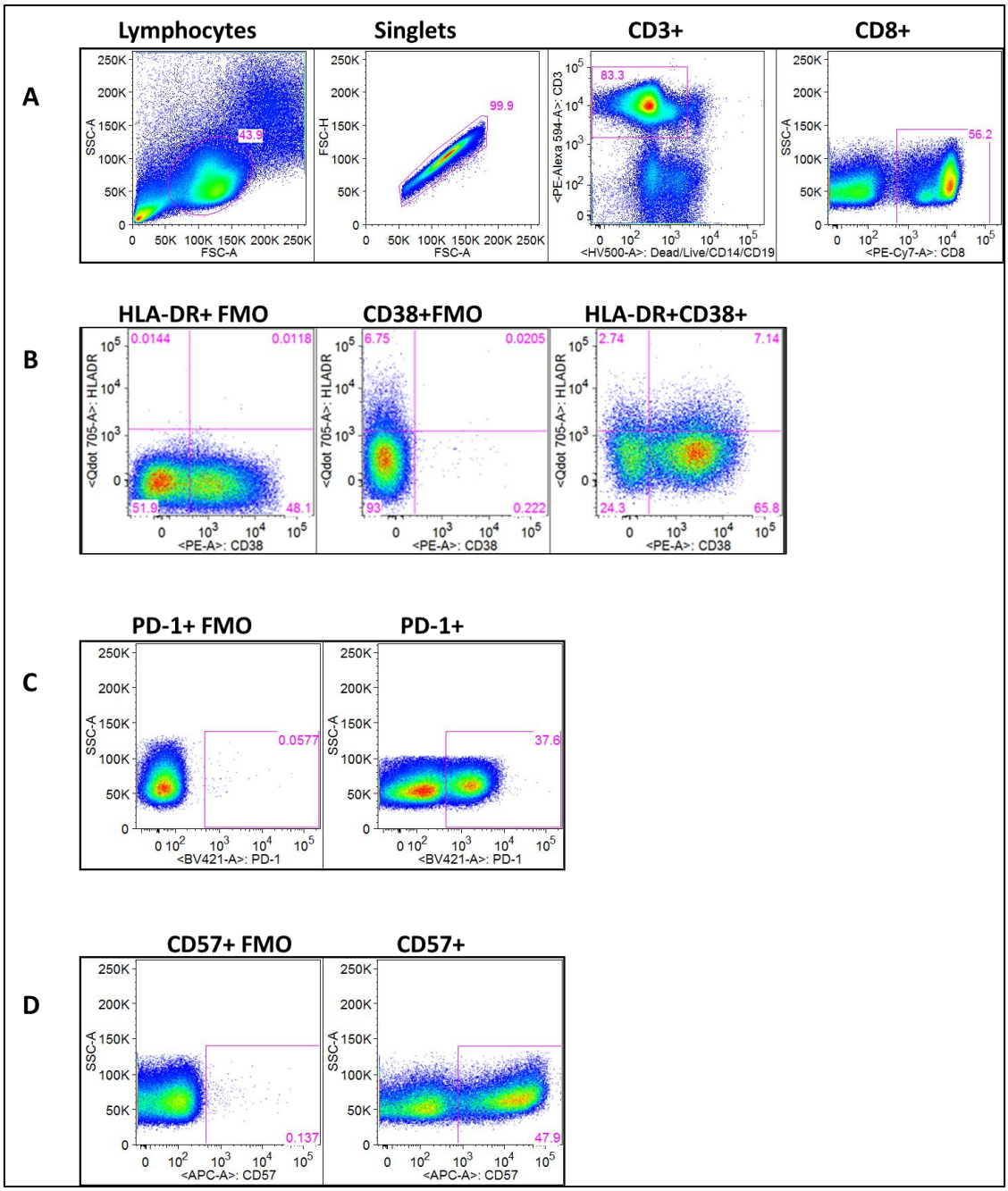

Figure 3.2: Gating strategy for the identification of CD8+ T cells and measurement of activation and exhaustion markers from bulk PBMCs by multicolour flow cytometry from an HIV infected participant. Lymphocytes were first gated on total PBMCs using forward and side scatter followed by singlets then CD14-/CD19- CD3+ cells and finally CD8+ T cells (A). From CD8+ T cells, CD38+ HLA-DR+ (B), PD-1+ (C) and CD57+ (D) cells were gated on using their respective fluorescence minus one (FMO) tubes.

As expected, the levels of CD8+ T cell activation (as assessed by HLA-DR+ and CD38+ T cells) were significantly higher among HIV-1 infected subjects compared to HIV-1 negative subjects (Figure 3.3A, p<0.0001), however there was no significant difference when assessed by DARC status in the HIV-1 positive group (Fig 3.3 B). Unlike CD8+ T cells, there were no significant differences observed in activation levels on CD4+ T cells by HIV and DARC status (Appendix B2).

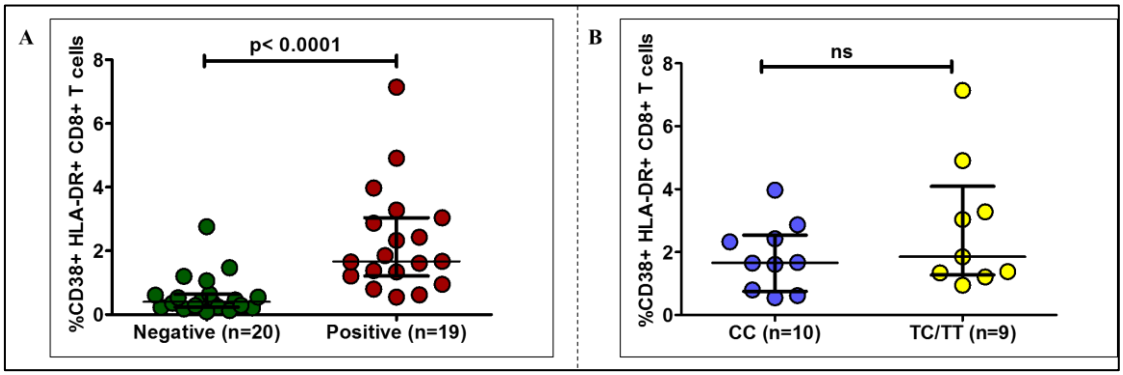

Figure 3.3: Frequencies of CD38+ HLA-DR+ CD8+ T cells in HIV-1 negative individuals and HIV-1 positive individuals. Differences as assessed in HIV-1 negative (green) and HIV-1 positive (maroon) individuals (A), and in HIV-1 positive individuals stratified by DARC status (B).

We next assessed T cell exhaustion marker PD-1 on CD8+ T cells. Our findings demonstrated a trend of higher PD-1 levels in the HIV-1 positive group compared to HIV uninfected individuals (Figure 3.4A, p=0.05). However, a weak trend of higher PD-1 expression on CD8+ T cells was observed in DARC positive individuals in the HIV-1 positive group when stratified by DARC status (Figure 3.4B, p=0.09). There were no differences found in CD4+ T cells by HIV-1 status and by DARC status (Appendix B3).

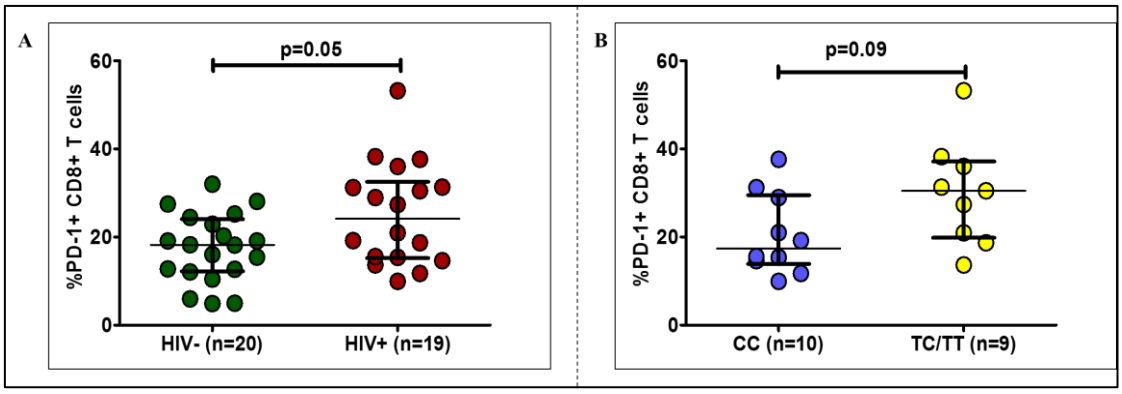

Figure 3.4: Frequencies of PD-1+ CD8+ T cells in HIV-1 negative and HIV-1 positive individuals. Differences in PD-1 expression as assessed in HIV-1 negative (green) and HIV-1 positive (maroon) individuals (A), and in HIV-1 positive individuals by DARC status (B).

No differences were observed in the expression of CD57 by CD8+ T cells in both HIV-1 negative and positive groups (Figure 3.5A) and by DARC status in the HIV-1 positive group (Figure 3.5B). There were also no differences found in CD4+ T cells (Appendix B4). Similarly, no differences were observed in activation, exhaustion and senescence markers on both CD8+ and CD4+ T cells in HIV negative individuals when stratified by DARC (data not shown).

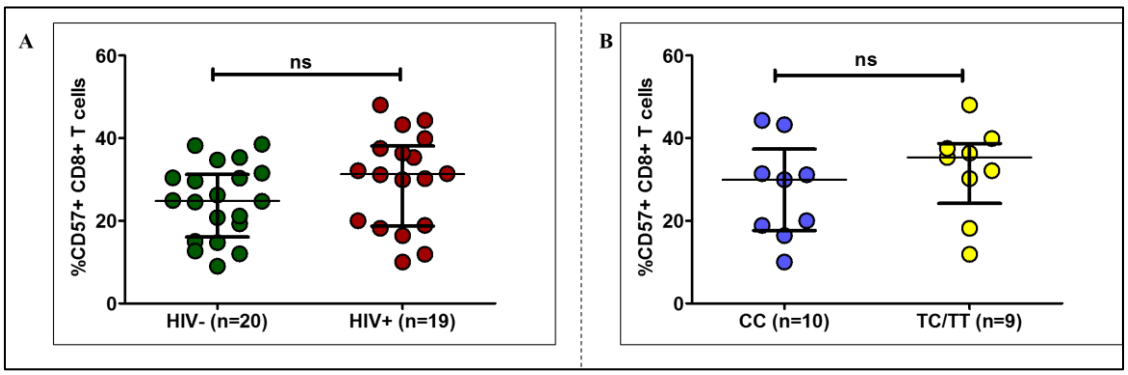

Figure 3.5: Frequencies of CD57+ CD8+ T cells in HIV-1 negative individuals and HIV-1 positive individuals. Differences as assessed in HIV-1 negative (green) and HIV-1 positive (maroon) individuals (A), and in HIV-1 positive individuals by DARC status (B).

There was also no correlation between these phenotypic markers on CD8+ T cells and absolute neutrophil counts in both HIV-1 positive and negative groups (Figure 3.6). Similarly, no differences were observed with regard to the expression of these markers on CD4+ T cells and absolute neutrophil counts (Appendix B5).

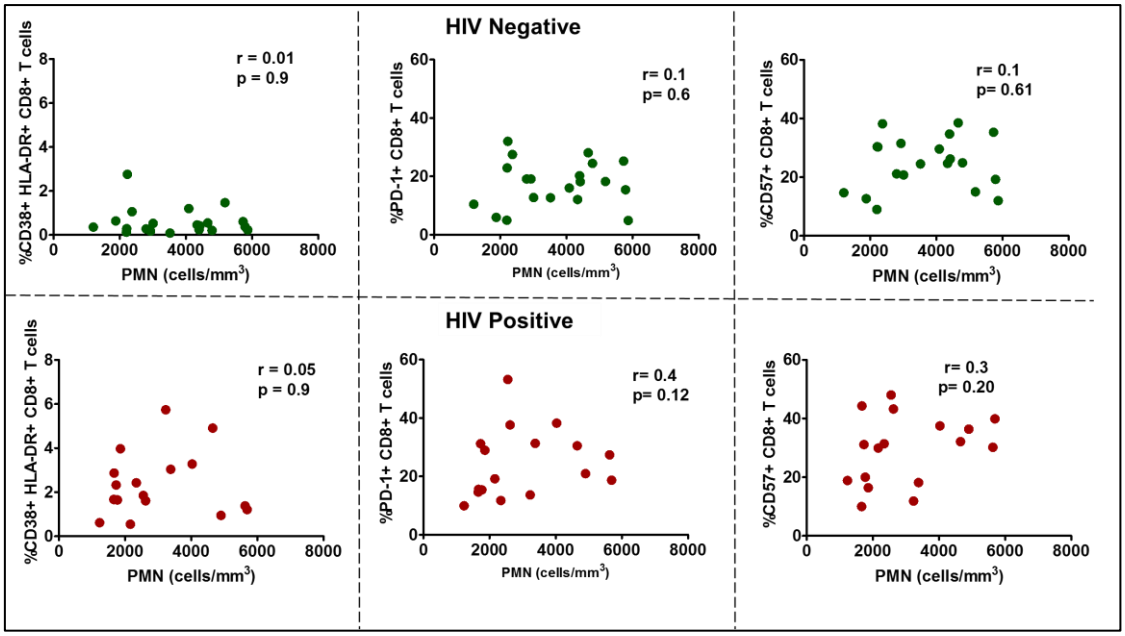

Figure 3.6: The correlations of PMNs and frequencies of CD38+, HLA-DR+, PD-1+ and CD57+ on CD8+ T cells in HIV negative and HIV positive individuals.

Taken together our results confirms previous findings of increased CD8+ T cell activation and exhaustion in HIV-1 positive individuals compared to HIV uninfected individuals. However, our findings did not show any significant differences when assessed by DARC status suggesting that the presence or absence of the DARC polymorphism has no impact (except for PD-1 where a weak trend was noted) on these measured parameters.

3.3 Gag and Envelope specific CD8+ T cell activity in HIV infected and HIV uninfected individuals with or without DARC polymophism

We next investigated if there were differences in the expression of effector cytokines by CD8+ T cells in the study groups by HIV and DARC status following stimulation with HIV antigens. We hypothesised that differences in absolute neutrophil counts in DARC positive and DARC negative individuals would also translate to differences in CD8+ T cell responsiveness given the importance of neutrophils in priming CD8+ T cells.

To assess the functional ability of CD8+ T cells in the study groups, we perfomed ICS on PMBCs stimulated with HIV-1 peptide pools (gag, gp120 and gp41) including the positive control (SEB) and measured cytokines (IFN-γ and TNF-α) of interest and degranulation marker (CD107a) by flow cytometry (as shown in Figure 3.7, and Appendix C1 for CD4+ T cells).

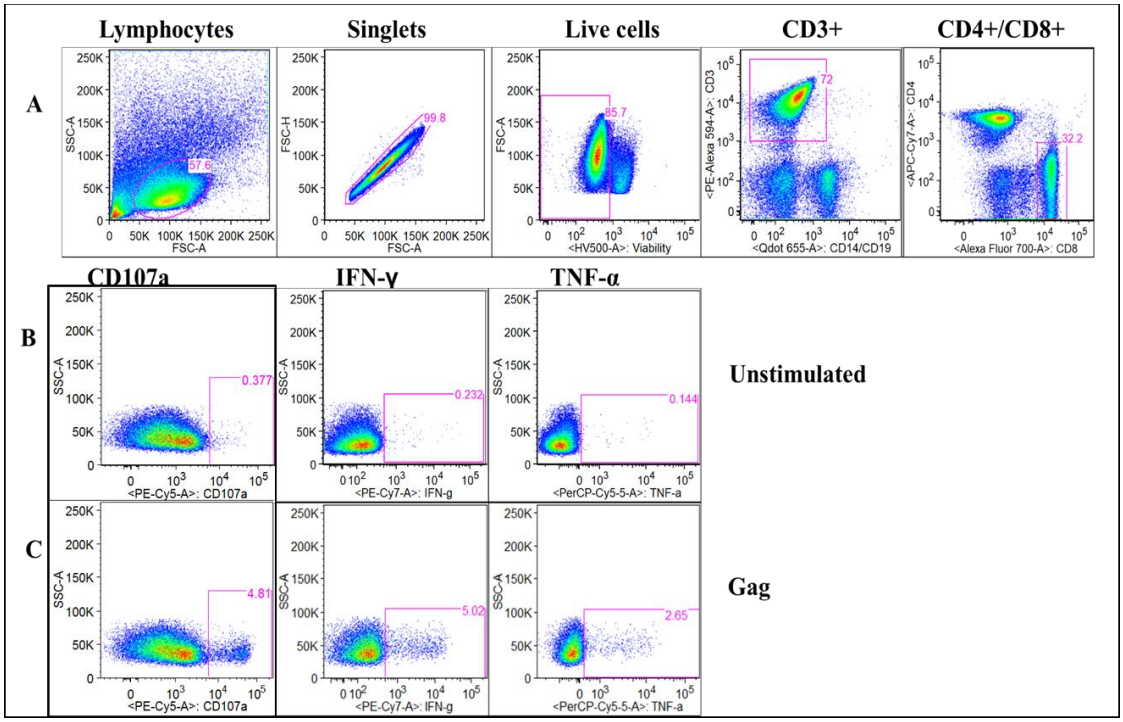

Figure 3.7: Representative gating strategy for the identification of CD8+ T cells from cells from PBMCs by multicolour flow cytometry. Lymphocytes were first gated on total PBMCs using forward and side scatter followed by singlets, live cells then CD14-/CD19- CD3+ cells and finally CD8+ or CD4+ T cells (A). From CD8+ T cells we gated on unstimulated sample (B) and sample stimulated with gag (C) and measured CD107a, IFN-γ and TNF-α.

We noted an increase in CD107a (p=0.03) and IFN-γ (p=0.02) expression on CD8+ T cells upon stimulation with Gag peptide pool in the HIV-1 positive group than in uninfected individuals (Fig 3.8) and no significant difference when stimulated with SEB (data not shown).

However, these were not significant when compared by DARC status in HIV-1 infected individuals (Fig 3.9). The frequencies of CD8+ T cells expressing CD107a and IFN-γ were higher (p=0.03 and p=0.04 respectively) in the HIV-1 positive group compared to HIV-1 negative individuals (Fig 3.8) upon stimulation with HIV-1 gp41 peptide pool, however these did not differ by DARC status (Fig 3.9). There was no significant difference observed in CD107a+, IFN-γ+ and TNF-α+ CD8+ T cells between HIV-1 negative and positive groups when stimulated with HIV-1 envelope gp120 (Fig 3.8) and there was also no difference by DARC status. We also observed no differences in CD8+ T cells expressing TNF-α with all stimulations by HIV-1 or DARC status.

Assessment of these functions on CD3+ CD8- cells (CD4+ T cells) when stimulated with the same HIV-1 peptides pools as above revealed weaker CD4+ T cell responses than those observed for CD8+ T cells, did not differ following stimulation with either gag or envelope peptide pools in HIV-1 negative and positive individuals and also did not differ by DARC status (Appendix C2 and C3). Similarly, no differences were observed in cytokine production on both CD8+ and CD4+ T cells in HIV negative individuals when stratified by DARC (data not shown).

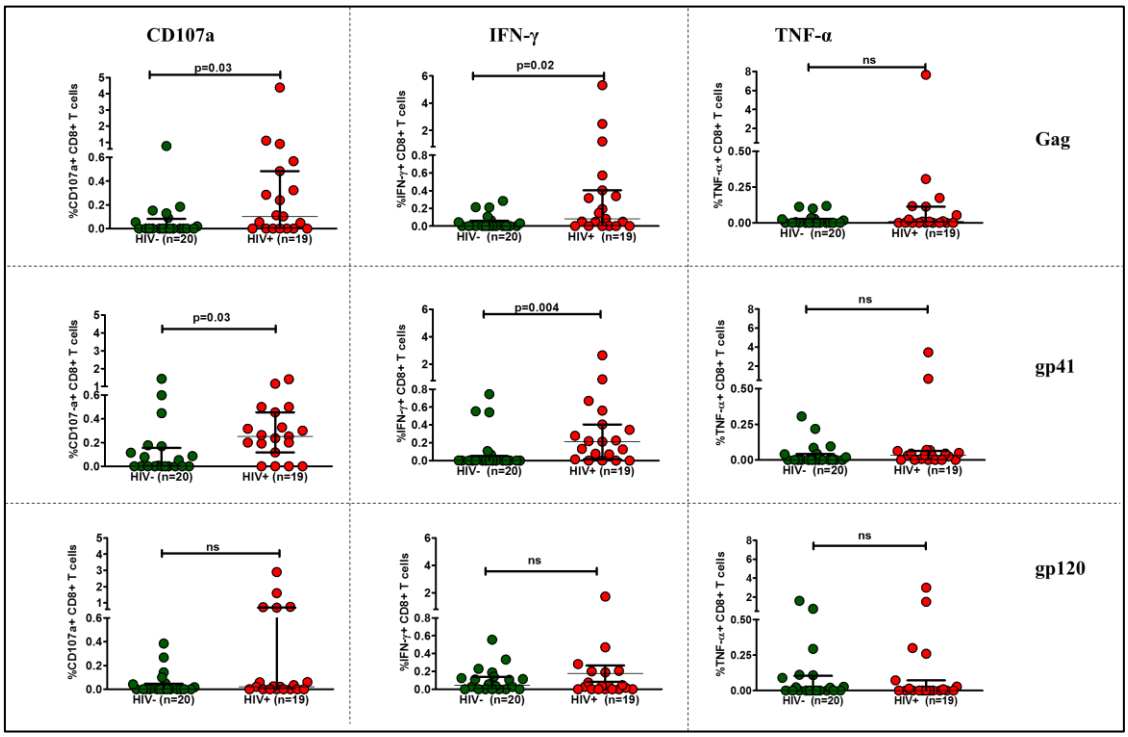

Figure 3.8: Detection of intracellular cytokines following PBMC stimulation. Thawed PBMCs were stimulated with specific HIV antigen (Gag, gp41 or gp120 peptide pools) for 16 hours. Y axes represent the percentage of live, single CD3+ CD8+ T cells that are positive for the indicated cytokine in either HIV negative (in green) or HIV infected individuals (in red).

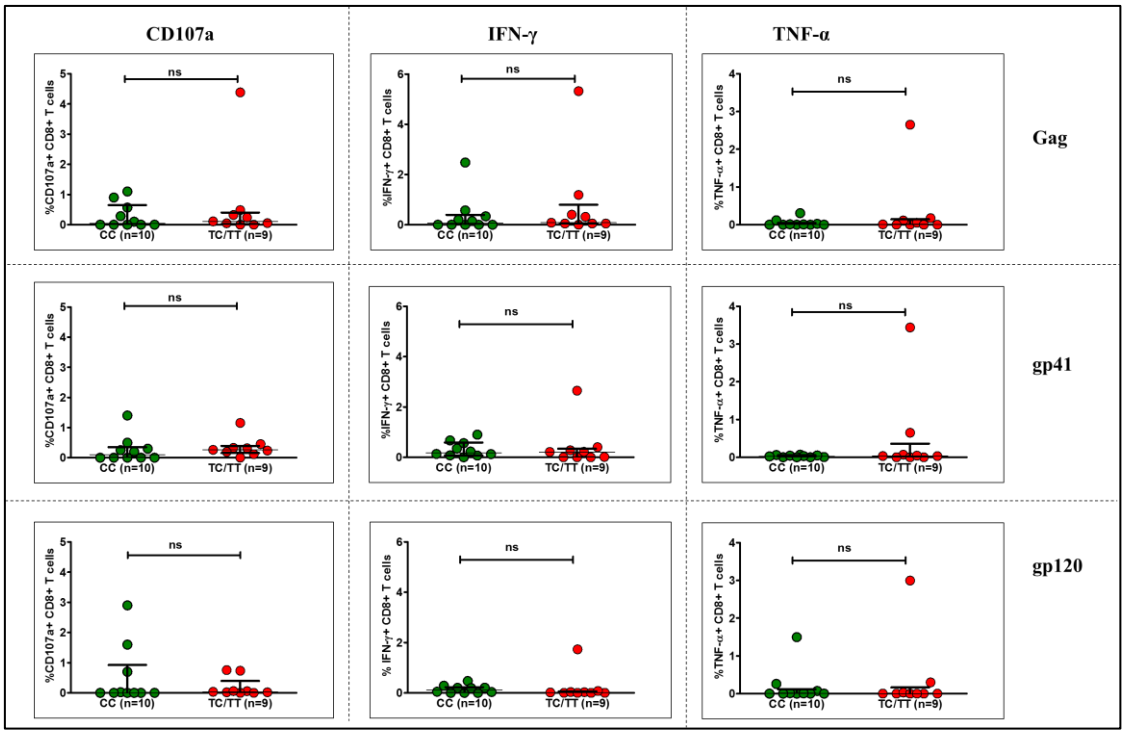

Figure 3.9: Detection of intracellular cytokines following PBMC stimulation. PBMCs were stimulated with specific HIV-1 antigen (Gag, gp41 or gp120 peptide pools) for 16 hours. Y axes represent the percentage of live, single CD3+ CD8+ T cells that are positive for the indicated cytokine and X axes represent DARC null (in green) or positive (in red) within HIV-1 positive group.

3.4 Increased T cells proliferation with HIV-1 infection and not by DARC status

We next investigated the proliferative capacity of CD8+ T cells using CFSE proliferation assay upon stimulation with Gag pool peptides (gating strategy shown in Figure 3.10, Appendix D1 for CD4+ T cells).

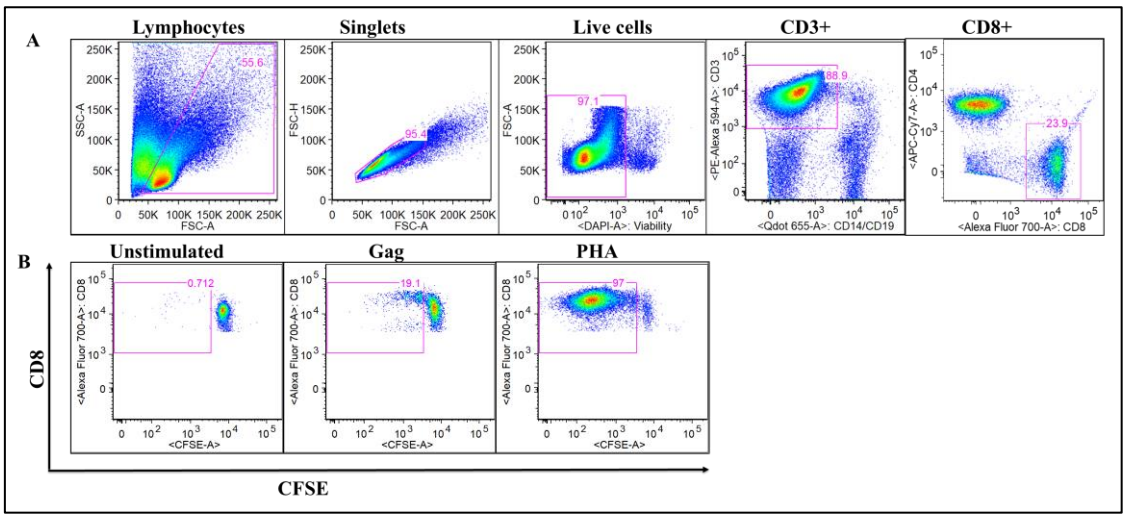

Figure 3.10: Gating strategy for proliferative capacity of CD8+ T cells upon stimulation with Gag peptide pools using multicolour flow cytometry. Lymphocytes were first gated on total PBMCs using forward and side scatter followed by singlets, live cells then CD14-/CD19- CD3+ cells and finally CD8+ T cells (A). From CD8+ T cells we gated on unstimulated sample, sample stimulated with gag or stimulated with PHA as positive control (B).

We hypothesised that the DARC null trait will have an impact on the replicative capacity of T cells. We observed a trend of higher proliferation on CD8+ T cells in HIV-1 infected (p=0.05, Figure 3.11A) than in uninfected participants. There were no differences in the proliferative capacity of CD8+ T cells by DARC status in both HIV negative and positive individuals (Figure 3.11B). Similarly, no significant differences in CD4+ T cells were observed by HIV or DARC status (Appendix D2). Similarly, no differences were observed in replicative capacity on both CD8+ and CD4+ T cells in HIV negative individuals when stratified by DARC (data not shown).

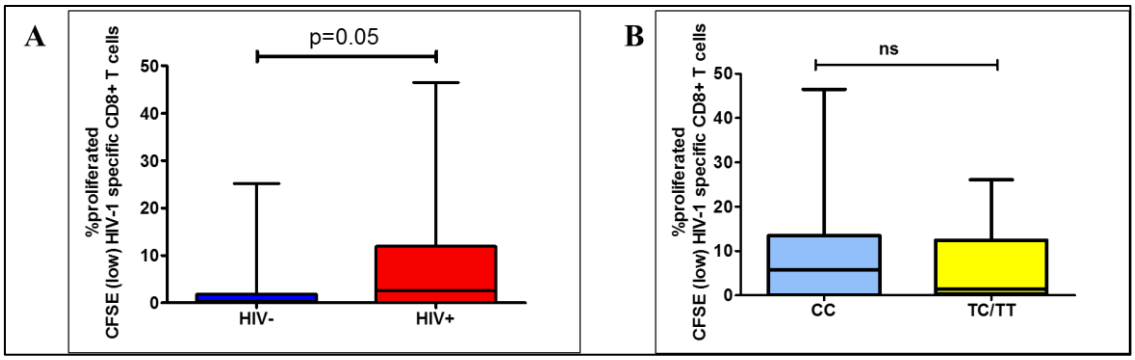

Figure 3.11: Measurement of CD8+ T cell proliferation using CFSE. Thawed PBMCs were stimulated with gag and incubated for 7 days to measure CD8+ T cell proliferation between HIV-1 negative and positive (A) and by DARC status in the HIV-1 positive group (B).

Taken together these results suggest that CD8+ T cells undergo proliferation upon HIV-1 infection. However, as shown with other measured parameters, the DARC null polymophism does not have an impact on the proliferative capacity of CD8+ T cells in HIV-1 infection.

3.5 Decreased IL-8 and MIP1-β and increased G-CSF plasma levels in HIV infection.

We next investigated the role DARC null trait on systemic inflammation assessing differences in the pro-inflamatory cytokine and chemokine levels between DARC null and DARC positive HIV uninfected and HIV-infected individuals. We quantified 17 cytokines [G-CSF, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, MCP-1 (MCAF), MIP-1β, and TNF-α] in plasma. The low limit of detections of the observed concentration of each cytokine are shown in a Table in Appendix A3. Standard curves for each analyte (Appendix A4) indicate that the absorbance of the sample must fall within the range of the standard curve, however if the analytes were not within the range of the standard curve, low limit of detection was used. For those cytokines that were detectable (IL-1, IL-2, IL-8, G-CSF, IL-10, IL,17, IFN-γ, MCP-1, TNF-α and MIP-1β), we observed up to 10-fold higher levels in HIV negative individuals compared to HIV-1 infected individuals whilst others (GM-CSF, IL-4, IL-5, IL-6, IL-7, IL-12 and IL-13) were below the limit of detection (Figure 3.12A).

Among the ones within the range IL-8 and MIP-1β were higher in the HIV negative group ($p<0.0001$ and $p=0.0008$ respectively) compared to HIV positive individuals and G-CSF was higher in the HIV positive group compared to HIV uninfected individuals ($p=0.04$), however, these were not significant when compared by DARC status in both HIV-1 infected individuals (Fig 3.12B) and HIV uninfected individuals (data not shown). The rest that were within the range (IL-1, IL-2, IL-10, IL,17, IFN-γ, MCP-1 and TNF-α) were not different by both HIV-1 and DARC status (data not shown). For those that were not detactable, where the low limit of detection was used, were also not different by HIV-1 or DARC status in both HIV-1 positive or HIV uninfected individuals (data not shown).

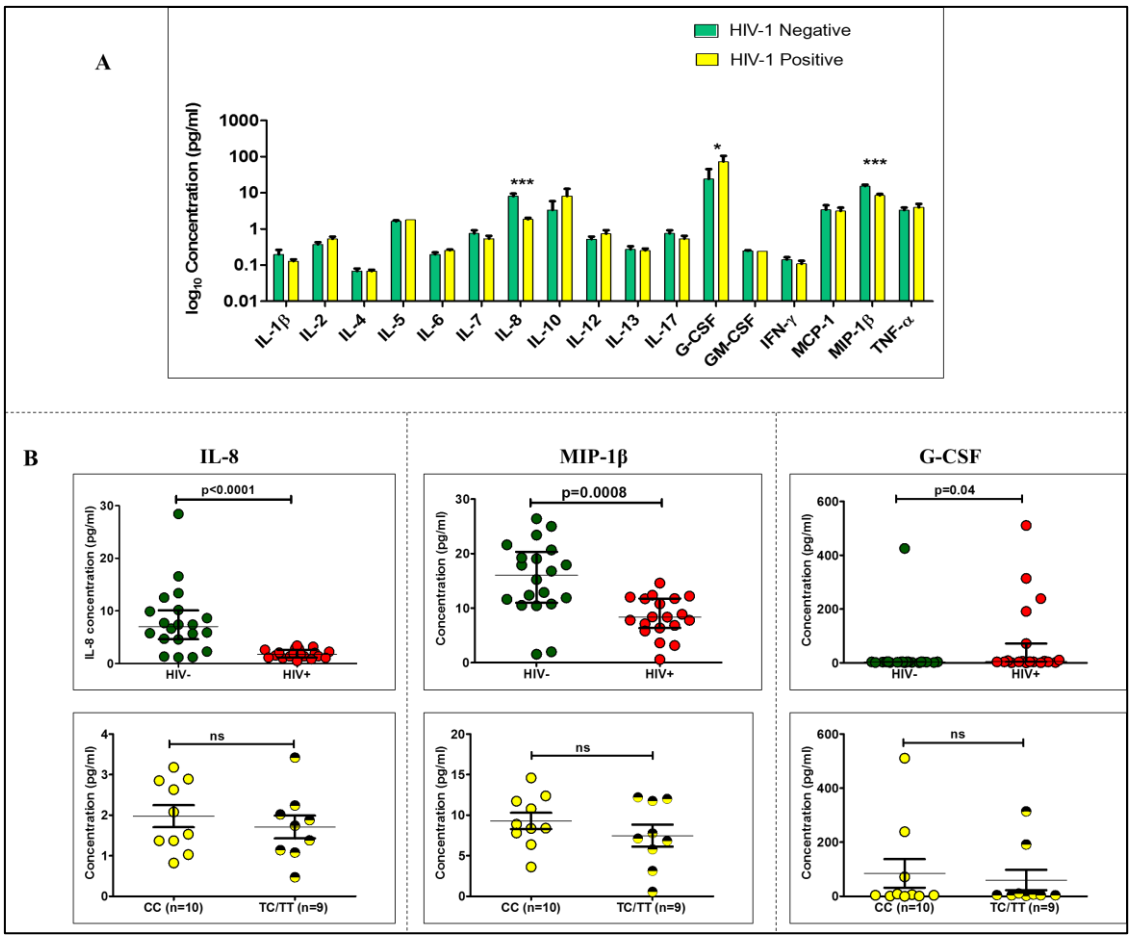

Figure 3.12: Summary of measured cytokine and chemokine concentration levels (A-panel). Cytokine and chemokine levels between HIV negative patients (green) and HIV positive patients (yellow) (top panel B) and cytokine and chemokine levels by DARC status within the HIV positive group (shown in yellow for DARC null and yellow with black for DARC positive bottom panel B).

These data suggest that DARC null trait does not affect cytokine and chemokine levels in both HIV infected patients and HIV uninfected individuals. It was unexpected to note higher plasma cytokine and chemokine levels in HIV uninfected individuals compared to HIV-1 infected individuals.

CHAPTER 4: DISCUSSION

HIV-1 has been studied for decades since its discovery and the availability of ART has resulted in a significant drop in the number of HIV deaths in South Africa and around the world; however, there is still a need for an effective AIDS vaccine to completely eradicate HIV/AIDS. There is also a gap on understanding mechanisms of how the immune system get rid of the HIV-1 virus and the host factors important in mediating differences in disease outcome for infected individuals in the absence of treatment.

In this study we assessed whether the DARC null polymorphism, a genetic trait associated with low neutrophil counts and common in people of African descent, played a role on T cell activation and function in HIV-1 infection. We hypothesized that the Duffy-null genotype will negatively impact T cell function, phenotype and pro-inflammatory cytokine levels in people of African descent with HIV-1 infection.

Our findings demonstrated significantly higher levels of CD8+ T cell activation, and exhaustion in HIV-1 infected individuals compared to HIV uninfected participants, however CD4+ T cell activation was not different between HIV positive and HIV uninfected individuals. Furthermore, investigation of the HIV-1 specific CD8+ T cell response upon stimulation with HIV antigens demonstrated increased levels of CD107a and IFN-γ expression CD8+ T cell responses towards gag and envelope peptide pools in HIV-1 infected individuals compared to HIV uninfected donors and no differences were observed in CD4+ T cells. There were no significant differences observed by DARC status in either T cell activation, exhaustion or function. Finally, our investigation on whether DARC null polymorphism had an impact on systemic inflammation demonstrated no differences in cytokine levels when assessed by DARC status, however we observed higher levels of IL-8 and MIP-1β in HIV negative compared to HIV-1 infected individuals. We also observed significantly higher levels of G-CSF in the HIV-1 infected group compared to HIV uninfected individuals.

As supported by previous findings (Deeks et al., 2004, Douek et al., 2009, Hunt et al., 2008, Ndhlovu et al., 2015a, Hazenberg et al., 2003), our results show increased CD8+ T cell activation in HIV-1 positive patients compared to HIV uninfected individuals. We also noted that activation levels in our cohort was lower compared to other previous

studies (Eggena et al., 2005, Migueles et al., 2002, Betts et al., 2001, Gea-Banacloche et al., 2000). This might be due to low viral loads of the individuals studied here, with a median viral load of 9100 RNA copies/mL compared to general untreated population (Rueda et al., 2012). The small sample size and change in the global HIV treatment guidelines made it difficult to identify more participants for further stratification by DARC status. Our analysis revealed no correlation between CD38 and HLA-DR expression on CD8+ T cells and PMNs, suggesting that PMNs might not play a role in CD8+ T cell activation

In our investigation, we observed a trend of higher PD-1 expression in HIV-1 positive patients compared to HIV uninfected individuals. Several studies have reported that binding of PD-L1 on the surface of monocytes and dendritic cells to PD-1 on T cells negatively regulates T cell function (Carter et al., 2002, Day et al., 2006, Keir et al., 2008). PD-1 has also been shown to be highly expressed in T cells during HIV-1 infection, indicating the exhaustion of these cells due to persistent infection (Wherry, 2011, Day et al., 2006). Although we noted a weak trend, our results were in line with previous studies of higher PD-1 expression on CD8+ T cells in HIV-1 infected group compared to HIV uninfected individuals.

Previous findings by Bowers et al showed that PD-L1 produced by neutrophils in HIV-1 infected patients positively correlated with expression of CD8+ T cell exhaustion marker PD-1 (Bowers et al., 2014). This previous study also highlighted that neutrophils purified from the blood of HIV-1-infected patients suppress T cell function via several mechanisms including PD-L1/PD-1 interaction and production of reactive oxygen species (ROS). This suggested that neutrophils suppress T cell function thereby affecting T cell activation which eventually leads to T cell dysfunction. On the other hand, Bowers and colleagues did not measure CD8+ T cell activation markers (CD38, HLA-DR) in correlation with PMNs. Our findings here show that differences in T cell activation exist in HIV-1 infection and may be driven by viral load and not by DARC status in HIV-1 infected individuals.

We also observed that CD57 expression levels in HIV-1 infected donors were not significantly different to those in HIV uninfected individuals. Furthermore, we did not find any correlation between CD57 and PMNs in both HIV-1 positive patients and HIV uninfected individuals, suggesting that neutrophils were not associated with the expression of this marker. Previous studies have demonstrated that HIV-1 infection is associated with accelerated senescence of CD8+ T cells where CD27 (T cell co-stimulatory protein) expression is lost and CD57 expression is gained in these cells (Brenchley et al., 2003). Some studies have also reported that HIV-specific CD8+T cells might be exhausted because of continuous stimulation by the virus, resulting in poor proliferation of CD57+ T cells and cell death (Dock and Effros, 2011, Wood et al., 2009, Deeks, 2011).

We here hypothesized that Duffy-null genotype will negatively impact CD8+ T cell phenotype in people of African descent with HIV-1 infection. Contrary to our hypothesis, our findings suggest that DARC null does not have an impact on the phenotype of these cells when examining CD38 and HLA-DR together, PD-1 or CD57 expression in both HIV-1 positive and HIV uninfected individuals.

We further assessed whether there would be differences in HIV-1 specific CD8+T cell responses and as expected and previously demonstrated, we observed increased levels of IFN-γ CD8+ T cell responses towards gag and envelope peptide pools in HIV-1 infected individuals compared to HIV uninfected individuals (Ngumbela et al., 2008, Kiepiela et al., 2007). Similarly, we also observed increased levels of CD107a expression in the HIV-1 positive group compared to the HIV-1 negative group as previously reported by others (Kiniry et al., 2017, Aktas et al., 2009). In contrast, our finding of no differences in TNF-α expression by HIV status was not expected as previous studies found TNF-α expression to be elevated in HIV-1 infection (Almeida et al., 2007).

We observed a trend of higher proliferation capacity on CD8+ T cells in HIV-1 infected individuals than uninfected donors as supported by previous studies that reported that T cells during HIV-1 infection become activated and proliferate to clear the virus (Koofhethile et al., 2016, Wherry and Kurachi, 2015).

We also assessed Gag-specific CD4+ T cell responses and proliferative capacity in both HIV-1 positive patients and HIV uninfected individuals and these were not significantly different. A previous study in the same population setting have reported increased IFN-γ CD4+ T cell responses towards gag peptide pools in HIV infected patients (Ramduth et al., 2009). Another study looked at the relationship between tetramer+ CD4+ T cells and cytokine-positive cells following stimulation with Gag peptides and found that only cells secreting IFN-γ correlated with tetramer+ cells (Laher et al., 2017). Our data was not in line with these findings, we attribute this difference to the fact that our HIV positive patients had lower viral loads compared to those reported in previous reports.

It is known that DARC null people have low neutrophil counts and findings from a previous report demonstrated the importance of neutrophils in priming T cells (Wonodi et al., 2017). We thus hypothesized that DARC null genotype will negatively impact T cell function in people of African descent with HIV-1 infection. Contrary to our hypothesis, our findings demonstrated that the DARC null trait does not affect T cell proliferation or function in the absence or presence of HIV-1 infection in people with low absolute neutrophil counts, suggesting that this genetic trait may not have adverse effects in the African population.

We noted that G-CSF chemokine levels were increased in the HIV-1 infected group compared to HIV uninfected individuals. These observations are not in line with previous studies in HIV-1 infection as the levels of G-CSF are reduced because these individuals suffer from neutropenia (Kuritzkes et al., 1998, Bahner et al., 1997, Hermans et al., 1996). This could suggest that levels of G-CSF expression are not associated with mild ethnic neutropenia in our cohort since neutropenia in HIV infection could be caused by many factors such as the use of myelotoxic agents for treatment or even complication with secondary infections (Shi et al., 2014). Other studies observed reduced levels of stimulated G-CSF in cultured marrow cells from HIV positive individuals (Carter et al., 2010, Hensley-McBain and Klatt, 2018). HIV negative patients with neutropenia have been observed to have elevated levels of G-CSF (Cebon et al., 1994).

The findings of higher levels of IL-8 and MIP-1β in HIV-1 negative individuals compared to HIV-1 infected patients were rather unexpected. Cytokines such as IL-2, IL-10, IL-12 and TNF-α were not different in both groups and none of the measured cytokines and chemokines were affected by DARC genotype in both HIV-1 positive group and HIV uninfected individuals.

Previous studies have shown that, during HIV-1 infection, there is elevated expression of type I IFN-regulated genes (Lehmann et al., 2008, Trinchieri, 2010). This could be caused by circulating IFNs, including IFN-alpha, produced by pDCs or activation of proinflammatory pathways, circulating macrophages and T cells (Yan and Lieberman, 2011, Hou et al., 2009). In this study IFN-γ was found to be higher in the HIV-1 infected patients compared to HIV uninfected individuals when PBMCs of these individuals were stimulated with HIV peptide pools, however when measuring cytokine levels in plasma of both HIV-1 and HIV uninfected individuals IFN-γ levels were the same. There could be other factors affecting cytokine levels in plasma than in PBMCs since PBMCs were stimulated with HIV peptides.

Elevated levels of IL-8 and MIP-1β in HIV-1 infection have been reported (Matsumoto et al., 1993, Stacey et al., 2009, Cotter et al., 2001). However, in this study IL-8 and MIP-1β levels were higher in HIV uninfected individuals compared to HIV positive patients. The increased levels of these proteins might be due to other infections that were not tested for in this study from our HIV uninfected individuals, such as influenza previously reported to induce IL-8 production (Ito et al., 2015). Another study looked at MIP-1β levels in HIV uninfected individuals at two time points, day 0 and day 7 and found elevated levels of this protein (Biancotto et al., 2013). In addition, MIP-1β can also be induced by other diseases such as asthma (Alam et al., 1996).

A previous study could not detect IL-2 in PBMCs, however, when using mononuclear cells from lymph nodes, the levels of IL-2 were found to be higher in the HIV-1 positive patients (Graziosi et al., 1996). This group and other studies also found that IL-6 cytokine levels were elevated when patients were chronically infected than acutely infected and associated this with disease progression (Graziosi et al., 1996, Birx et al., 1990, Lafeuillade et al., 1991, Czesnikiewicz-Guzik et al., 2008).

Our findings reveal elevated levels of IL-2 in the HIV-1 positive group compared to HIV uninfected individuals, although these were not significant

Elevated levels of TNF-α (Barcellini et al., 1996, Cotter et al., 2001, Czesnikiewicz-Guzik et al., 2008) and IL-1β (Molina et al., 1989) have been reported in HIV-1 infection; another study found that the levels of TNF-α were stable and progressively increased over time (Graziosi et al., 1996). However, in our studied participants TNF-α was higher in HIV positive participants however did not reach statistical significance. Also, in this study as mentioned above we looked at expression of this cytokine when PBMCs were stimulated with HIV peptide pools and found that expression levels were not different from those of HIV uninfected individuals. We attribute this difference (and other cytokines previously detected in HIV infection) to low viral load in our studied participants.

The absolute neutrophil counts on both HIV-1 infected and HIV uninfected individuals did not have an impact on expression levels of all measured cytokines in this study which might suggest that DARC null may not have adverse effects in the African population. A previous study suggested that DARC-positive individuals may be predisposed to a more pro-inflammatory state that promotes HIV-1 replication and spread as a possible mechanism for the differences in disease outcome in individuals with the different DARC genotypes (Kulkarni et al., 2009). We thus hypothesized that DARC-null individuals of African descent, with HIV-1 infection will have low pro-inflammatory cytokine activity. However, our findings here did not support the proposed mechanism as we showed that the DARC null trait had no influence in the levels of pro-inflammatory cytokines and chemokines in both HIV-1 positive individuals and HIV uninfected individuals. It was also surprising and unexpected that we observed lower levels of IL-8 and MIP-1β in HIV-1 positive individuals and increased levels of these cytokines in HIV uninfected individuals.

Thobakgale and Ndung'u (2014) proposed a simplified model of how neutrophil counts in Africans may affect susceptibility or ability to control diseases. They speculated that, when neutrophil counts were optimal (in DARC positive individuals), neutrophils will effectively prime immune cells including CD8+ T cells as previously suggested (Duffy et al., 2012a), to clear the virus.

However in the case of low neutrophils (e.g. possession of the DARC null genotype), there will be insufficient priming of immune cells resulting in failure to clear HIV-1 (Thobakgale and Ndung'u, 2014). Our study followed up on this model and show that priming of T cells by neutrophils and virus clearance is not solely affected by the number of neutrophils a person has; alternatively our results may also suggest that the absence of the Duffy antigen has no adverse effects on HIV disease in people of African ancestry. This may indeed be the case as a previous study in African individuals from our setting demonstrated that neutrophil counts had no effect on CD4 decline or viral load amongst individuals with the DARC null genotype. The same study suggested that if DARC-null trait is associated with disease progression, this was independent of these factors (Julg et al., 2009b). These findings were in agreement with a separate study carried out in a cohort by the Tri-Service AIDS Clinical Consortium (TACC) HIV Natural History Study (NHS) that demonstrated that the DARC-null trait had no effect on HIV-1 acquisition or disease progression (Walley et al., 2009). Our findings are interesting in light of reports that the DARC null genotype has evolved on the African continent as a resistant factor to malaria parasite (*Plasmodium vivax*) by preventing invasion of red blood cells (Horuk et al., 1993). However, alternate mechanisms have been proposed to explain how DARC presence may influence acquisition and disease progression; HIV-suppressive chemokines associated with DARC-expressing red blood cells may act as a protective shield preventing HIV attachment to the red blood cell and transfer to HIV target cells such as CD4+ T cells. In that case DARC-null might increase acquisition risk. Following HIV-1 acquisition, HIV-1 might bind to DARC on the surface of red blood cells and transfer the virus to target cells, indicating that DARC expression could lead to increased disease progression (He et al., 2008). Our findings do not support or disprove this mechanism as we did not find a clear association between T cell phenotype, function and proinflammatory response with possession of the Duffy genotype.

Our study was limited by the number of patients and thus we could not fully ascertain the impact of T cells on PMN counts and DARC status as this was a pilot study. This study also did not look at STIs. It is possible that STIs if present may affect the levels of CD8 T cells, neutrophils and cytokine production levels.

Another limitation of our study is that we here had HIV infected individuals with low viral loads compared to the general population of untreated individuals and thus the clinical dynamics may not represent a general therapy naïve population. This was a pilot study and we were also limited by the fact we did our assays blinded for the DARC genotype, this was measured after the assays were done, thus we did not have access to many antiretroviral therapy naïve patients with the DARC null genotype. Although it would be meaningful to recruit more patients for follow up studies; this might be difficult to undertake in future since the new universal treatment guidelines requires that every HIV-1 infected individual be initiated on antiretroviral therapy irrespective of CD4 counts (WHO, 2016) .

As far as we know, this is the first study to interrogate the T cell phenotype, function and pro-inflammatory cytokine activity in HIV-1 infected African individuals in the context of the DARC genotype, in a high HIV incidence setting with a high prevalence of the Duffy null genotype. We conclude that HIV-1 infection is associated with immune activation, exhausted and reduced CD8+ T cell cytolytic activity and proliferation and thereby not mediate effector functions; however, in this study, none of these functions were influenced by possession of the DARC null polymorphism. It is also known that ethnic neutropenic is rather mild and individuals with this disorder do not suffer from increased incidents of infection. Thus our data suggest that Duffy-null genotype associated with low neutrophil counts may not have an impact on how the immune system (CD8+ T cell function) responds to infection.

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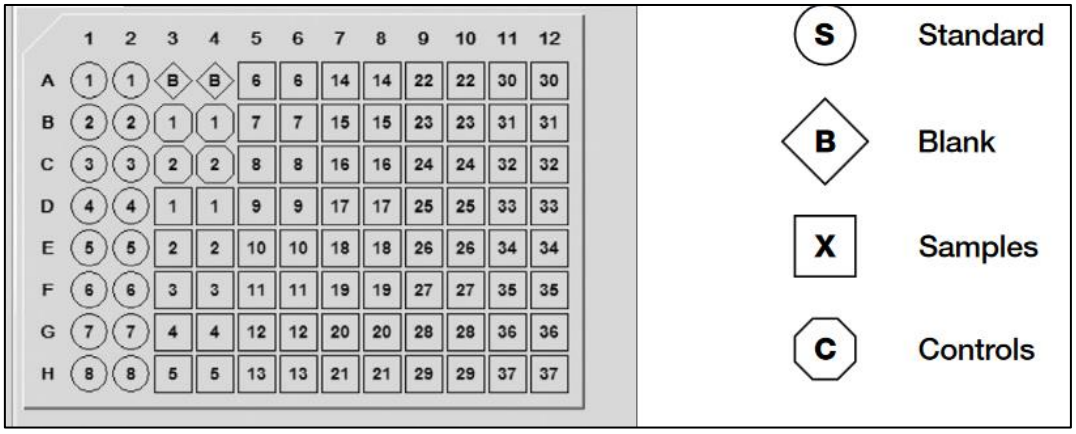
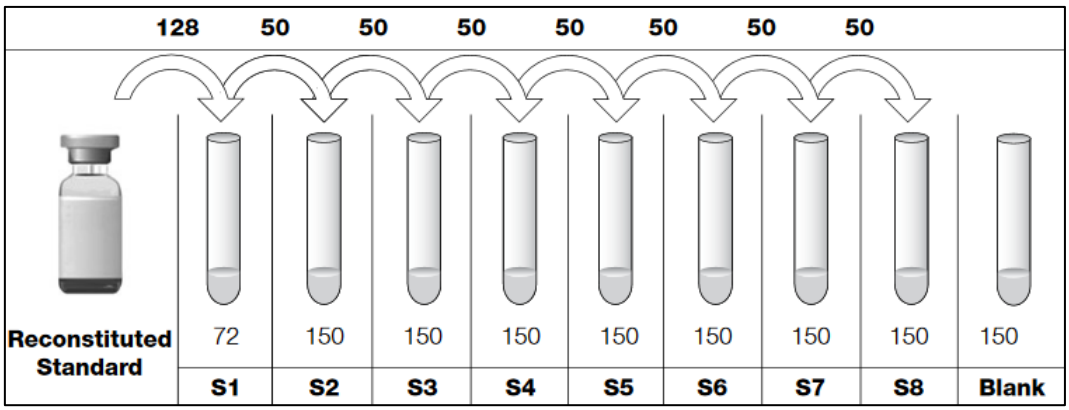
APPENDICES

Appendix A
A1



Appendix A1: Plan plate layout showing standards, blanks controls and samples

A2



Appendix A2: Preparation of a fourfold dilution series of cytokine standards

A3

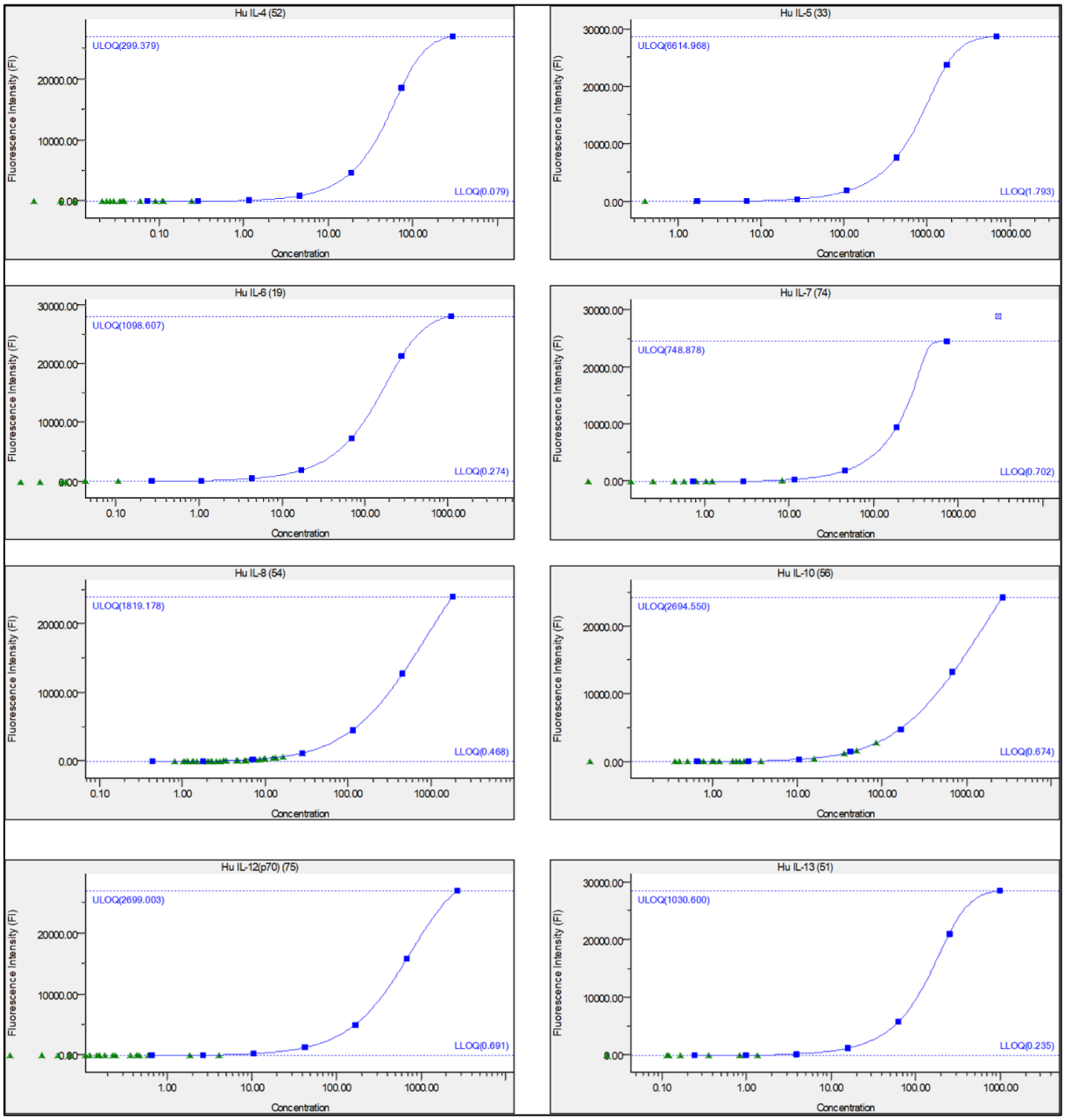
Table A3: Luminex lower limits of detection for each cytokine from Bio-Rad

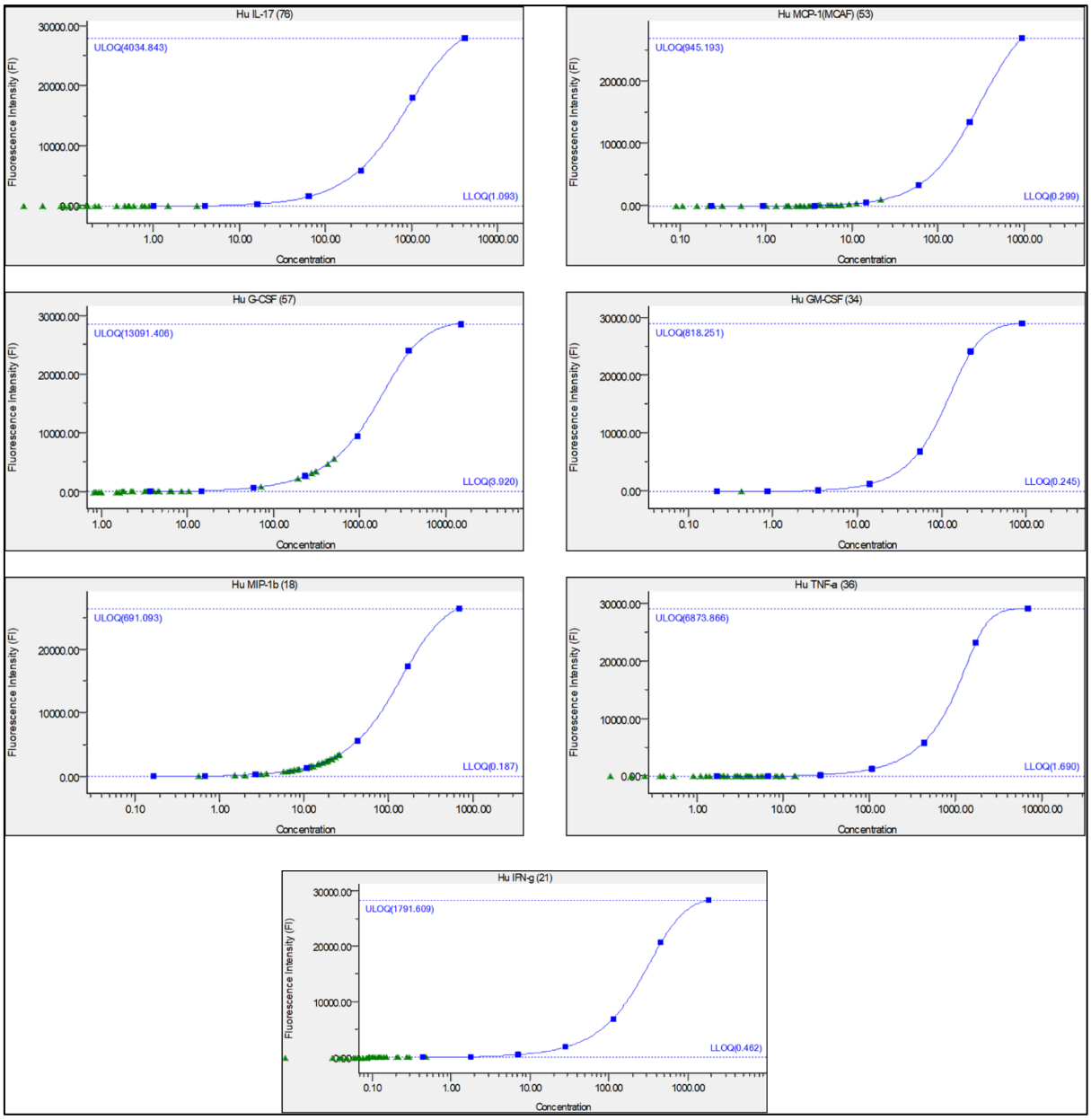
Table 1. Representative performance characteristics.

Analyte	Assay Working Range, pg/ml		Assay Sensitivity, pg/ml	Mean Intra-Assay %CV*	Mean Inter-Assay %CV*	Singleplex Bead Region
	LLOQ*	ULOQ*	LOD*			
Basic FGF	3.26	3,341	2.54	3.1	2.4	44
CTACK	2.10	15,656	0.82	2.7	5.2	72
Eotaxin	0.14	2,281	0.05	4.4	1.2	43
G-CSF	6.35	104,106	3.63	3.1	4.0	57
GM-CSF	0.48	7,846	0.19	4.3	2.2	34
GRO-α	21.05	31,255	13.45	2.6	7.9	61
HGF	8.76	143,513	7.09	2.6	2.9	62
ICAM-1	3.84	62,935	1.51	4.4	2.3	12
IFN-α2	0.95	15,569	0.46	3.3	4.4	20
IFN-γ	1.57	25,665	1.05	3.1	3.6	21
IL-1α	3.73	61,154	6.65	3.5	4.9	63
IL-1β	0.29	4,672	0.24	3.6	3.2	39
IL-1ra	6.21	34,949	3.16	4.7	5.1	25
IL-2	1.29	21,178	0.75	1.7	2.5	38
IL-2Rα	1.48	24,270	1.65	3.4	4.8	13
IL-3	0.13	2,139	0.13	5.0	3.9	64
IL-4	0.19	3,064	0.09	3.2	1.9	52
IL-5	3.63	59,499	0.86	2.3	2.3	33
IL-6	0.38	6,244	0.34	2.2	3.0	19
IL-7	1.92	31,475	1.22	2.7	3.9	74
IL-8	0.85	13,992	0.36	3.2	2.8	54
IL-9	3.62	31,527	1.08	2.6	7.1	77
IL-10	1.06	17,427	0.69	2.3	3.4	56
IL-12 (p40)	14.68	240,562	6.39	4.5	2.4	28
IL-12 (p70)	1.43	23,425	0.78	3.3	2.9	75
IL-13	0.31	5,157	0.22	3.1	2.7	51
IL-15	12.42	203,426	12.82	2.8	4.1	73
IL-16	1.20	19,639	0.25	2.5	3.0	27
IL-17A	2.44	39,972	1.16	2.4	1.4	76
IL-18	0.66	10,892	0.31	2.9	2.2	42
IP-10	3.41	34,953	1.43	2.8	6.0	48
LIF	3.86	53,806	2.05	2.5	4.7	29
MCP-1 (MCAF)	0.53	8,755	0.44	3.2	3.4	53
MCP-3	0.48	4,899	0.24	4.4	4.2	26
M-CSF	0.75	12,290	0.27	2.4	3.6	67
MIF	2.70	44,168	2.45	3.4	4.7	35
MIG	3.16	32,366	1.39	4.4	4.2	14
MIP-1α	0.12	1,218	0.06	4.5	4.2	55
MIP-1β	1.41	1,439	1.41	3.4	2.5	18
β-NGF	0.47	7,655	0.23	2.9	3.9	46
PDGF-BB	7.12	37,133	2.96	3.3	9.7	47
RANTES	16.72	26,467	3.98	3.0	6.7	37
SCF	1.82	29,899	0.99	4.1	2.6	65
SCGF-β	82.11	1,345,200	141.77	2.3	3.8	78
SDF-1α	7.54	9,381	2.44	2.2	5.4	22
TNF-α	3.33	54,566	1.13	3.5	3.0	36
TNF-β	0.80	13,186	0.38	3.0	4.7	30
TRAIL	1.78	29,188	0.89	3.2	4.5	66
VCAM-1	2.91	47,662	2.05	4.1	3.1	15
VEGF-A	16.01	149,830	10.16	2.8	8.5	45

* The LLOQ, ULOQ, LOD, and inter-assay precision %CV are mean data determined from three independent multiplex assays in a serum-based matrix. Intra-assay %CV is derived from one representative assay. LLOQ and ULOQ are defined as the boundary standard curve points within which the performance specifications of individual standard points were met for a 10% intra-assay CV and recovery range of 70–130%. Data were generated using the magnetic workflow with the Bio-Plex Pro Wash Station.

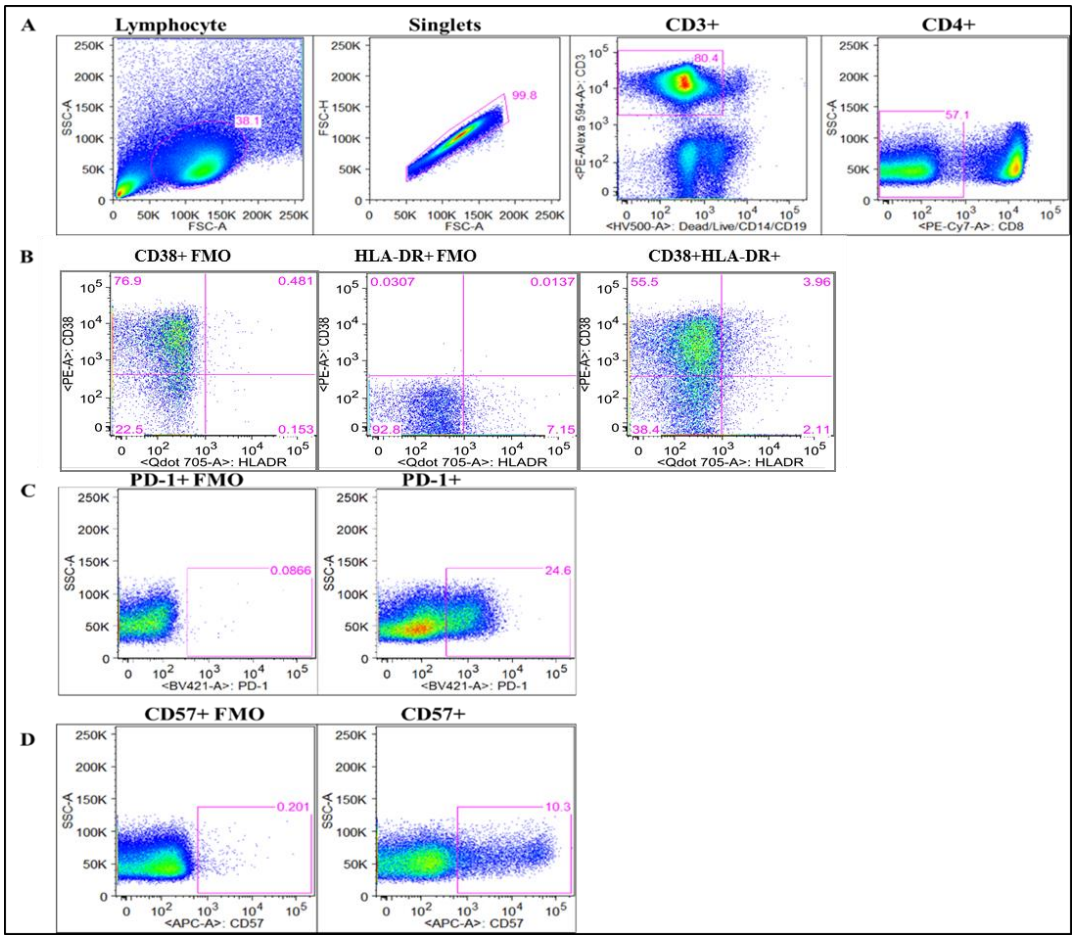
A4. Standard curves of 17 cytokines used in this study




Hu IL-17 (76)
ULOQ(4034.843)
LLOQ(1.093)
Hu MCP-1(MCAF) (53)
ULOQ(945.193)
LLOQ(0.299)
Hu G-CSF (57)
ULOQ(13091.406)
LLOQ(3.920)
Hu GM-CSF (34)
ULOQ(818.251)
LLOQ(0.245)
Hu MIP-1b (18)
ULOQ(691.093)
LLOQ(0.187)
Hu TNF-a (36)
ULOQ(6873.866)
LLOQ(1.690)
Hu IFN-g (21)
ULOQ(1791.609)
LLOQ(0.462)
Fluorescence Intensity (FI)
Concentration

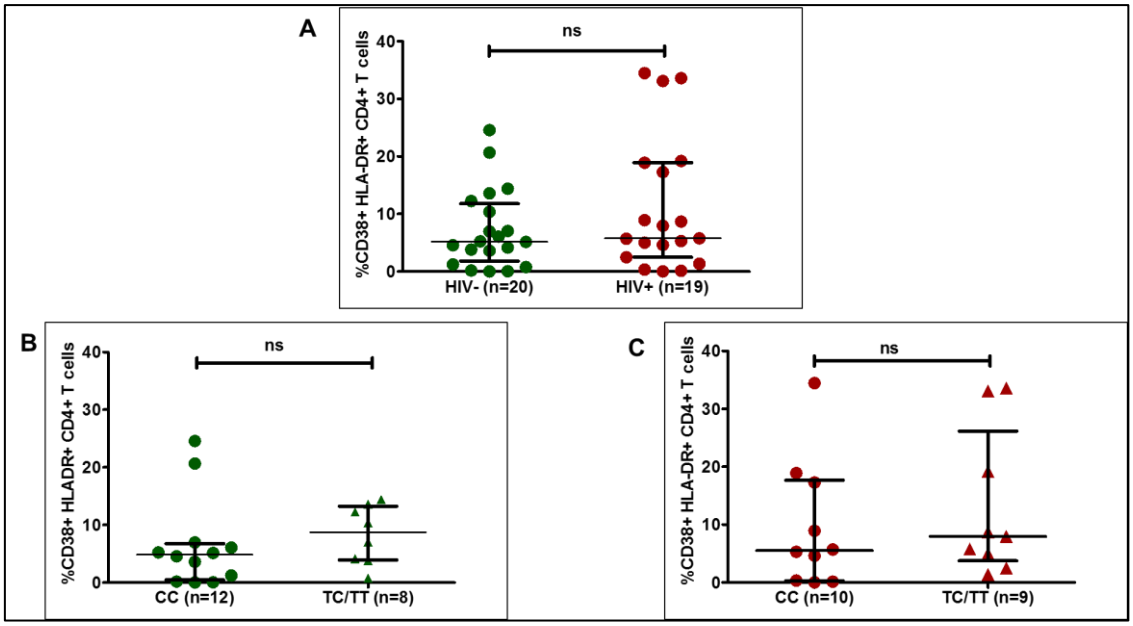
Appendix B

B1



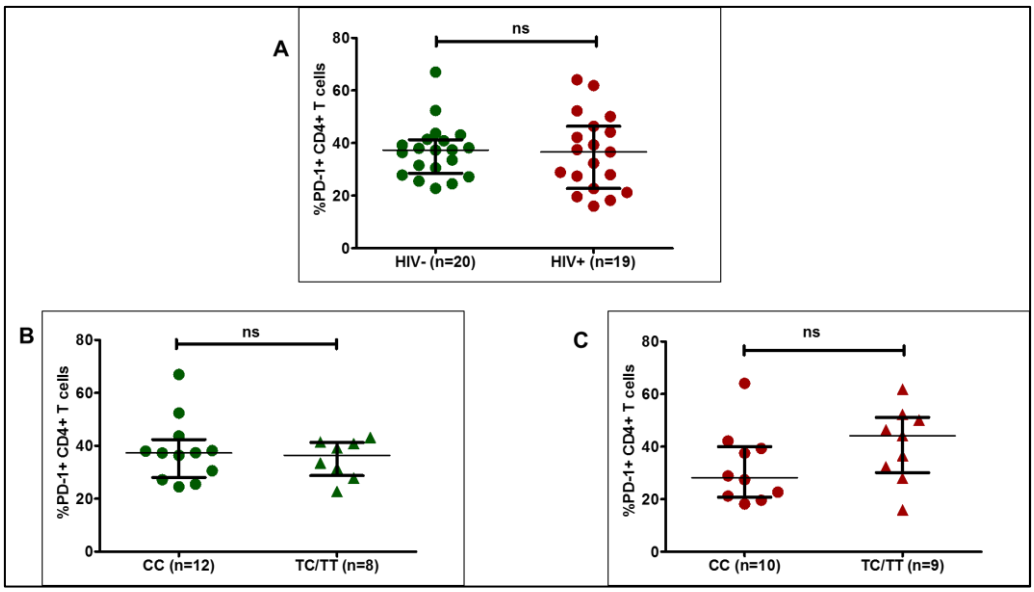
Appendix B1: Gating strategy for the identification of CD4+ T cells and measurement of activation and exhaustion markers from bulk PBMCs by multicolour flow cytometry from a HIV positive patient. Lymphocytes were first gated on total PBMCs using forward and side scatter followed by singlets then CD14-/CD19- CD3+ cells and finally CD4+ T cells (depicted by gating on CD8- T cells). (A). From CD4+ T cells, CD38+ HLA-DR+ (B), PD-1+ (C) and CD57+ (D) cells were gated on using their respective flourescence minus one (FMO) tubes.

B2



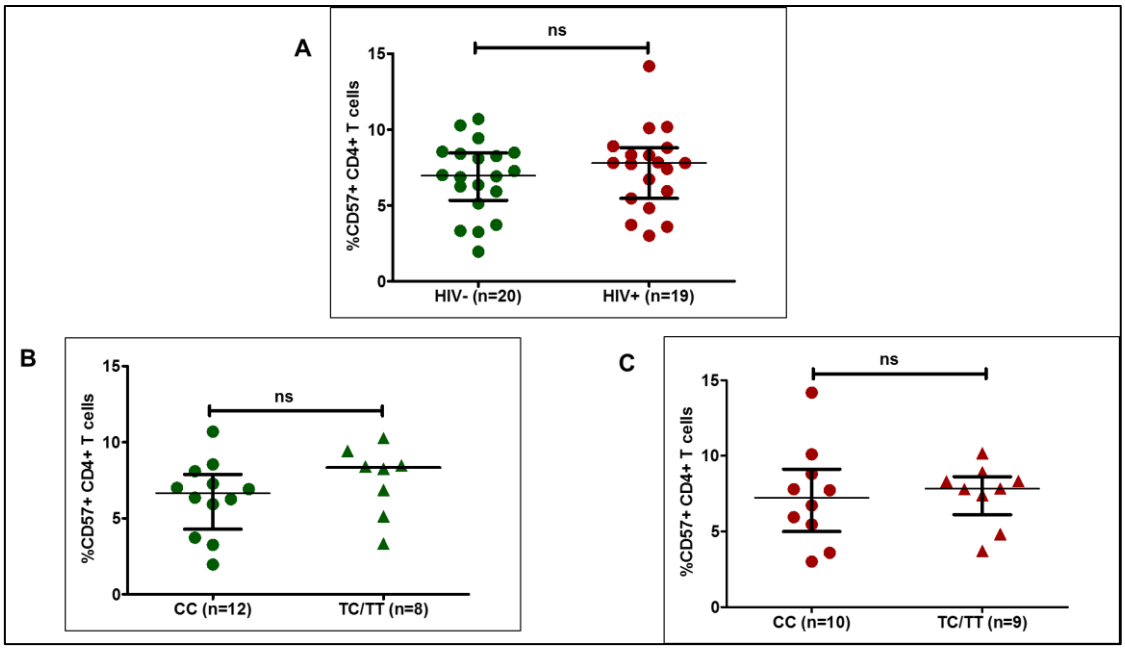
Appendix B2: Frequencies of CD38+ HLA-DR+ CD4+ T cells in HIV negative individuals and HIV positive individuals. Differences as assessed in HIV negative (green) and HIV positive (maroon) individuals (A), and in HIV negative individuals by DARC status (B) and HIV positive individuals by DARC status (C).

B3



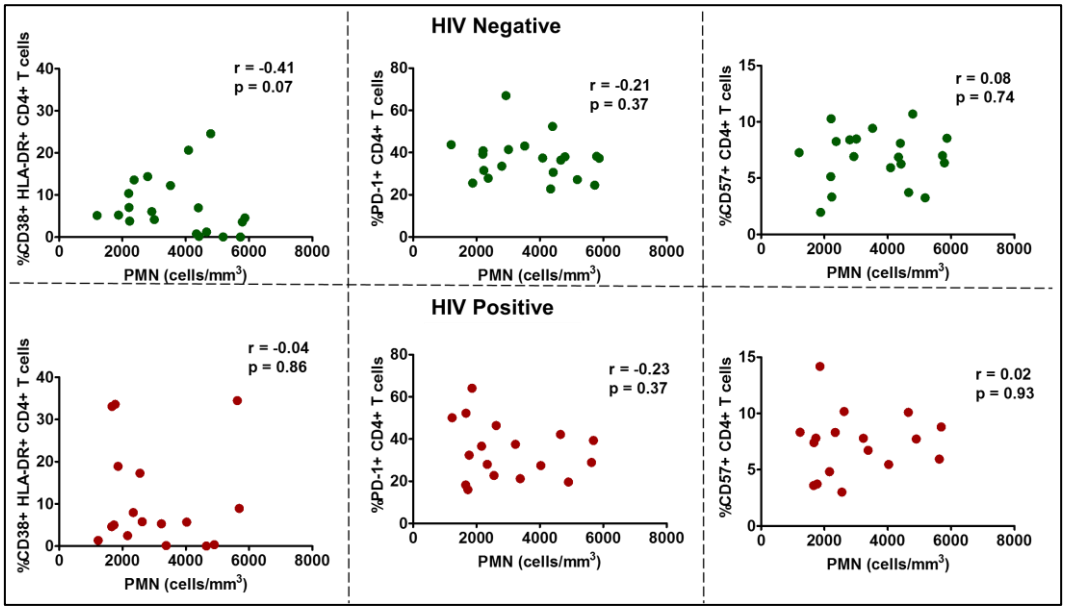
Appendix B3: Frequencies of PD-1+ CD4+ T cells in HIV negative and HIV positive individuals. Differences in PD-1 expression as assessed in HIV negative (green) and HIV positive (maroon) individuals (A), and in HIV negative individuals by DARC status (B) and HIV positive individuals by DARC status (C).

B4



Appendix B4: Frequencies of CD57+ CD4+ T cells in HIV negative individuals and HIV positive individuals. Differences as assessed in HIV negative (green) and HIV positive (maroon) individuals (A), and in HIV negative individuals by DARC status (B) and HIV positive individuals by DARC status (C).

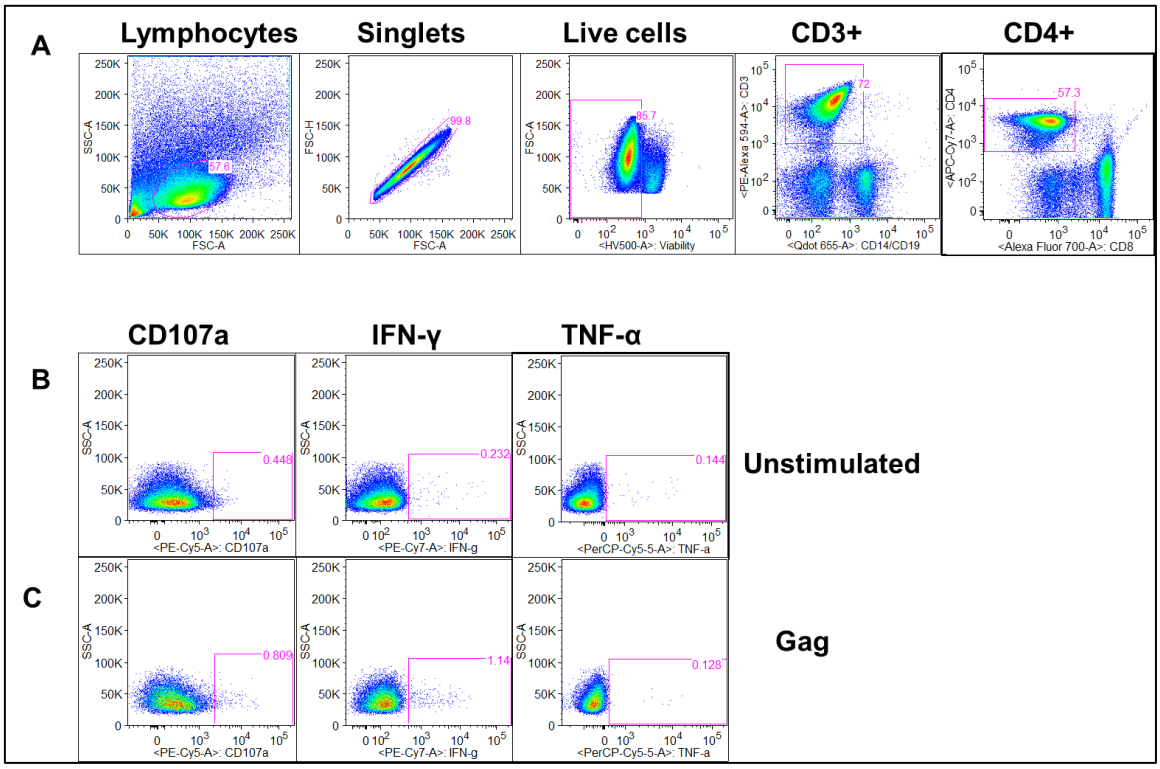
B5



Appendix B5: The correlations of PMNs and frequencies of CD38+, HLA-DR+, PD-1+ and CD57+ on CD4+ T cells in HIV negative and HIV positive individuals.

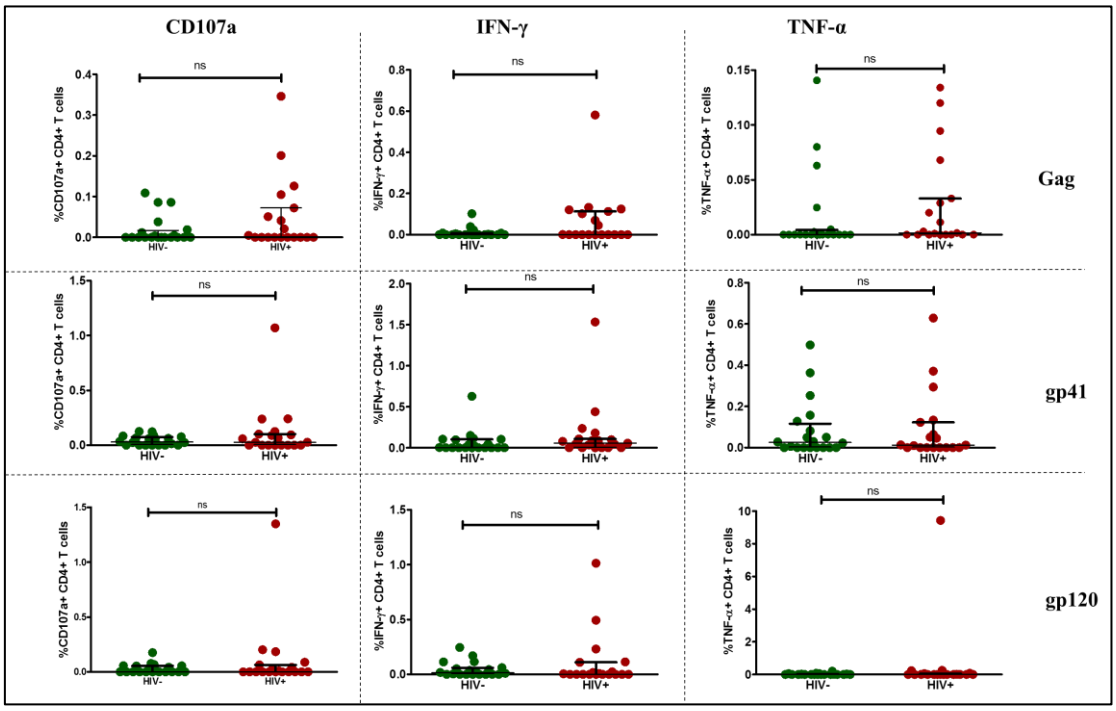
Appendix C

C1



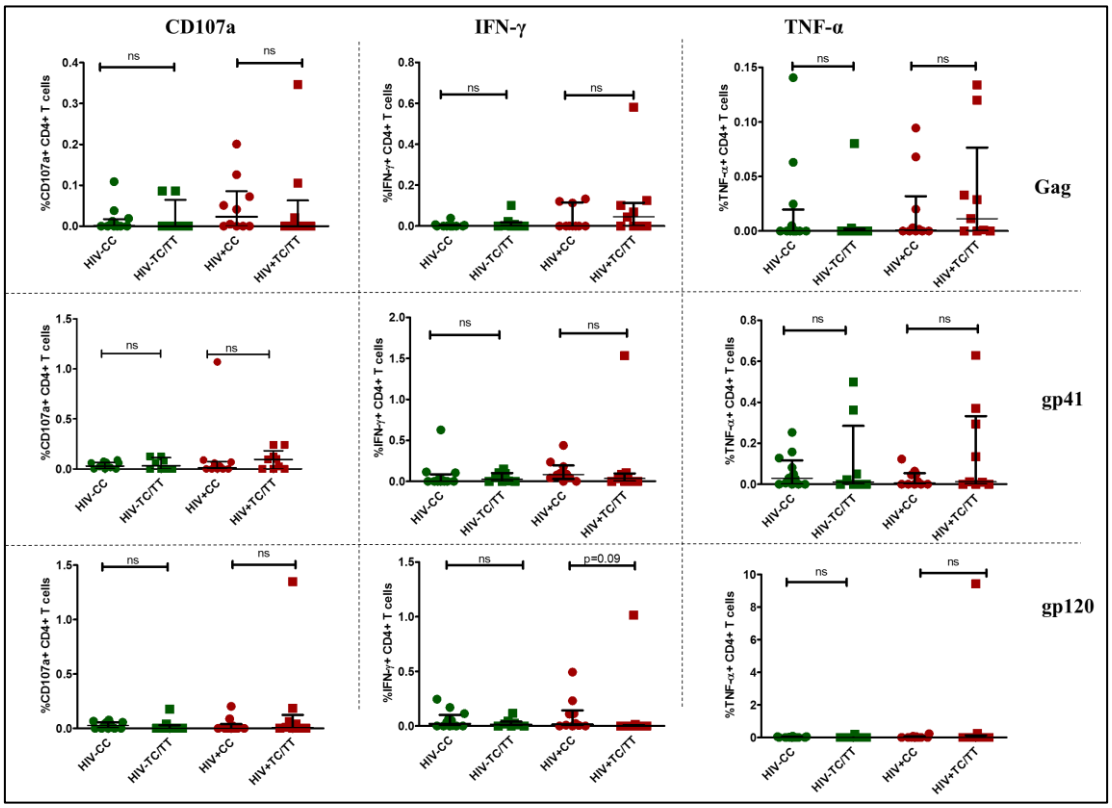
Appendix C1: Gating strategy for the identification of CD4+ T cells from PBMCs by multicolour flow cytometry. Lymphocytes were first gated on total PBMCs using forward and side scatter followed by singlets, live cells then CD14-/CD19- CD3+ cells and finally CD4+ T cells (A). From CD4+ T cells we gated on unstimulated sample (B) and sample stimulated with gag (C) and measured CD107a, IFN-γ and TNF-α.

C2



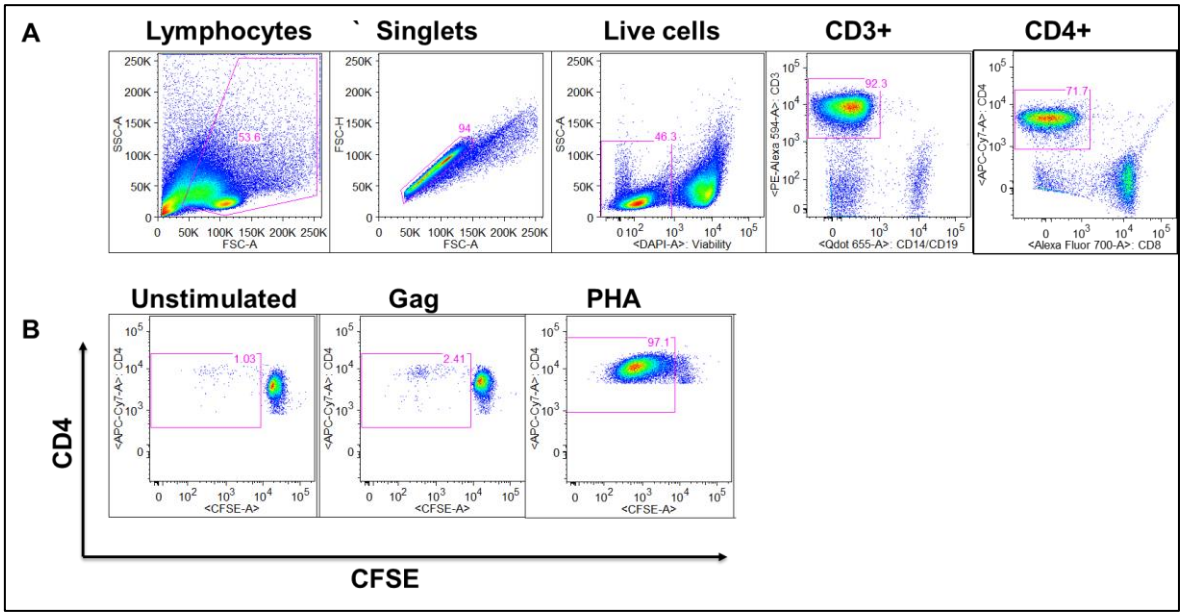
Appendix C2: Detection of intracellular cytokines following PBMC stimulation. Thawed PBMCs were stimulated with specific HIV antigen (Gag, gp41 or gp120 peptide pools) for 16 hours. Y axes represent the percentage of live, single CD3+ CD4+ T cells that are positive for the indicated cytokine.

C3



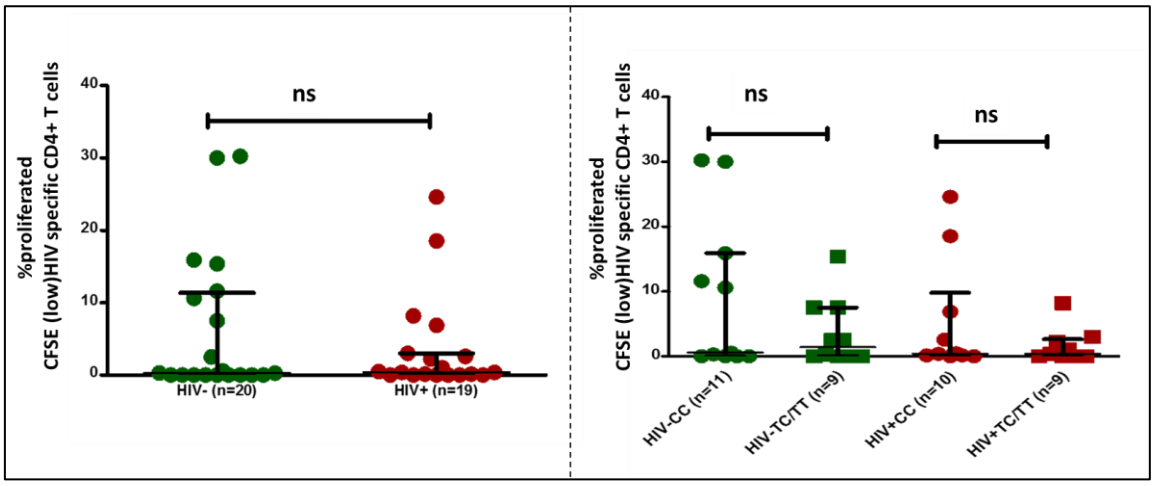
Appendix C3: Detection of intracellular cytokines following PBMC stimulation. Thawed PBMCs were stimulated with specific HIV antigen (Gag, gp41 or gp120 peptide pools) for 16 hours. Y axes represent the percentage of live, single CD3+ CD4+ T cells that are positive for the indicated cytokine and X axes represent DARC null or positive within HIV negative or positive groups.

Appendix D
D1



Appendix D1: Gating strategy for proliferative capacity of CD4+ T cells upon stimulation with Gag peptide pools using multicolour flow cytometry. Lymphocytes were first gated on total PBMCs using forward and side scatter followed by singlets, live cells then CD14-/CD19- CD3+ cells and finally CD4+ T cells (A). From CD4+ T cells we gated on unstimulated sample, sample stimulated with gag and sample stimulated with PHA as positive control (B).

D2



Appendix D2: Measurement of CD4+ Tcell proliferation using CFSE. Thawed PBMCs were stimulated with gag and incubated for 7 days to measure CD4+ T cell proliferation between HIV negative and positive (A) and by DARC status (B).