



**NATURAL KILLER CELL PHENOTYPIC,
FUNCTIONAL, AND NUTRIENT TRANSPORTER
PROFILES DURING SPONTANEOUS CONTROL
OF HIV-1 INFECTION IN BLACK SOUTH
AFRICANS**

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A Dissertation submitted to the Faculty of Health Sciences, University of
the Witwatersrand, Johannesburg, in fulfilment of the requirement of the
degree of Master of Science in Medicine

Sandringham, 2023

DECLARATION

I, Nikayla Batohi, declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

A handwritten signature in black ink, appearing to read 'Nikayla Batohi', is positioned below the declaration text.

24th day of February in 2023

Dedicated to my grandmother

Moveen Doris Fothergill

18-11-1928 – 03-05-2022

PRESENTATIONS

Natural killer cells phenotypic and functional profiles during spontaneous control of HIV-1 infection in South Africans

NICD Scientific Forum

20 April 2022

National Institute for Communicable Diseases

Oral presentation

Natural killer cells phenotypic and functional profiles during spontaneous control of HIV-1 infection in South Africans

Wits Faculty of Health Sciences Biennial Research Day 2022

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Oral presentation

Natural killer cell phenotypic, functional, and metabolic profiles are not different in HIV-1 elite controllers compared to uninfected individuals

South African Immunology Society

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The role of natural killer cell responses to natural control of HIV-1 infection in South Africans

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ABSTRACT

Despite the devastation caused by the human immunodeficiency virus (HIV) for over four decades, a subset of individuals termed HIV-1 elite controllers (ECs) can control the virus in the absence of antiretroviral therapy (ART) and may provide a model for a functional cure. This study aimed to understand the role of natural killer (NK) cells as important innate effector cells in the control of HIV-1 infection in African populations. We measured the phenotypic, functional, and nutrient transporter profiles of NK cells on cryopreserved peripheral mononuclear cells from HIV-1 ECs (n=15), viraemic progressors (VPs) (n=19), antiretroviral therapy-treated individuals (ART-treated) (n=20), and HIV-1 uninfected donors (HCs) (n=21) from Johannesburg, South Africa using multicolour flow cytometry. Functional and metabolic profiles were assessed after stimulation with a major histocompatibility complex-devoid cell line. The frequency of NK cells and their subsets in ECs were similar to HCs and ART-treated and altered compared to VPs. Total NK cells in ECs had similar expression of NKG2A (inhibition), NKG2C (activation), PD-1 (exhaustion), and CD57 (maturation) markers compared to ART-treated and increased expression of CD38 and CD69 (activation markers) compared to ART-treated individuals. CD107a (cytotoxicity) was reduced in total NK cells in all people living with HIV-1 compared to HCs, whereas IFN- γ (cytokine production) was comparable across the ECs, HCs, and ART-treated groups and significantly lower in VPs compared to the other study groups. Metabolic profiles (glucose transporter 1 (Glut1), CD98, and CD71) were similar in ECs compared to ART-treated and significantly increased in VPs compared to other study groups. Together these findings show that NK cells from ECs have an inhibitory, mature profile with low levels of immune exhaustion and reduced metabolic phenotype suggesting functional competence. These new insights could be employed in novel immunotherapeutic strategies for the treatment of HIV-1 in an African population.

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ABBREVIATIONS

ADCC	Antibody-dependent cell cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Allophycocyanin
ART	Antiretroviral therapy
ART-treated	ART-treated individual
BD	Becton, Dickinson and Company
BP	Bandpass
BV	Brilliant violet
CCR5	Chemokine receptor 5
CD	Cluster of differentiation
CO ₂	Carbon dioxide
CS&T	Cytometer setup and tracking
Cy	Cyanine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dPBS	Dulbecco's phosphate buffered saline
ds	Double stranded
EC	Elite controller
<i>env</i>	Envelope gene
Env	Envelope protein
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one

FSC	Forward scatter
<i>gag</i>	Group-specific antigen gene
Gag	Group-specific antigen protein
GFP	Green fluorescent protein
Glut1	Glucose transporter 1
HC	Healthy control
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN	Interferon
IFN- γ	Interferon-gamma
IL	Interleukin
IQR	Interquartile range
KIR	Killer immunoglobulin-like receptor
LDL	Lower than the detectable level
LP	Long-pass
MFI	Median fluorescent intensity
MHC	Major histocompatibility complex
Nef	Negative factor
NICD	National Institute for Communicable Diseases
NK	Natural killer
NKG	Natural killer group
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein 1
PE	Phycoerythrin

PerCP	Peridinin chlorophyll protein complex
PLWH	People living with HIV-1
PMT	Photomultiplier tube
<i>pol</i>	DNA polymerase gene
Pol	DNA polymerase
RBD	Receptor binding protein
Rev	Regulator of expression of virion proteins
RNA	Ribonucleic acid
SIV	Simian immunodeficiency viruses
ss	Single stranded
SSC	Side scatter
Tat	Trans-activator of transcription
UNAIDS	Joint united nations programme on HIV/AIDS
USA	United States of America
Vif	Virion infectivity factor
VP	Viraemic progressor
Vpr	Viral protein R
Vpu	Viral protein U

CHAPTER 1: INTRODUCTION

Human immunodeficiency virus (HIV) induces a transient symptomatic seroconversion illness, in some people, associated with high viral replication and strong immunologic responses to the invading pathogen (Maartens et al., 2014). This is followed by an asymptomatic stage, and, if untreated, a symptomatic stage with progression to acquired immunodeficiency syndrome (AIDS) (Maartens et al., 2014). It continues to threaten global health and represents a significant burden for healthcare systems (Faria et al., 2014). Despite intensive research, no vaccine is available for the prevention of HIV. Vaccine efforts are hampered by the different HIV subtypes as well as the lack of understanding of the correlates of protective immunity. Furthermore, although ART is successful, lifelong therapy is required as it does not eradicate the virus due to the capacity of HIV to remain latent within infected cell reservoirs (Falcinelli et al., 2019).

1.1 CLASSIFICATION AND DISTRIBUTION OF HIV

HIV was first discovered by Barré-Sinoussi et al and is grouped within the Lentivirus genus in the Retroviridae family (Barre-Sinoussi, 1983). HIV can be separated into two major types based on their genetic variation and viral antigens: HIV-1 and HIV-2 (Bbosa et al., 2019). HIV originated from the Simian immunodeficiency virus (SIV) found in primates through many cross-species transmissions (Sharp and Hahn, 2011). The viral transmission began in the Democratic Republic of Congo and spread to sub-Saharan Africa, West Africa, and the rest of the world (Faria et al., 2014). The global spread of the virus resulted in a geographically defined distribution of genetically different viruses. While HIV-1 subtype B is endemic in Europe and the Americas; almost all subtypes are found in Africa (Bbosa et al., 2019). In South Africa, the HIV-1 subtype C is predominant. HIV-2 is endemic in West Africa and is present in small proportions in a few countries outside West Africa (Nyamweya et al., 2013). Worldwide, HIV-1 is mainly responsible for the subsequent progression of HIV into AIDS (Giovanetti et al., 2020). Most lentiviral infections typically show long periods of latent, persistent viral replication leading to chronic disease progression (Fanales-Belasio et al., 2010).

1.2 PREVALENCE OF HIV-1 INFECTION

1.2.1 The global prevalence of HIV-1 infection

The HIV/AIDS epidemic is an ongoing worldwide public health concern and continues to evolve both in magnitude and diversity. Since the virus was identified nearly 40 years ago, the joint united nations programme on HIV/AIDS (UNAIDS) estimates 84 million people have been infected with HIV, of which approximately 40 million have died from AIDS-related illnesses (UNAIDS, 2022). Almost 38 million people are living with HIV in 2021, of which 1.5 million were new infections (UNAIDS, 2022). Nearly 700 000 individuals succumbed to AIDS-related illnesses in 2021 (UNAIDS, 2022).

1.2.2 Prevalence of HIV-1 in South Africa

Despite the advances in new medicines and technology, the number of global infections continue to rise every year, with the majority of new infections occurring in South Africa (McKinnon and Karim, 2016). Although South Africa has made immense progress against the HIV epidemic, the virus is still responsible for more than 300 000 new infections and has claimed more than 80 000 lives in 2020 (UNAIDS, 2022). Today, approximately 8 million people are living with HIV-1 in South Africa (UNAIDS, 2022). The majority of these people live in KwaZulu-Natal, Mpumalanga, and Free State provinces (**Figure 1.1**).

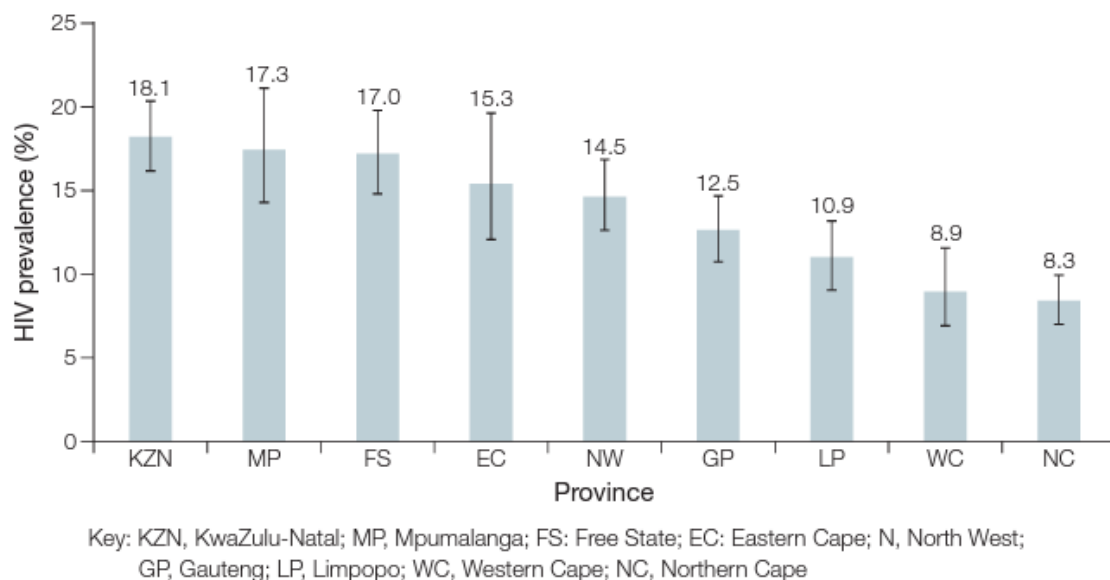


Figure 1.1: The HIV prevalence by the province in South Africa in 2017. KwaZulu-Natal, Mpumalanga, and Free State are the most endemic regions of South Africa, whereas the Northern Cape is the least affected province in South Africa. Image reprinted from Simbayi et al (Simbayi et al., 2019).

1.3 HIV-1 VIROLOGY

1.3.1 The structure of HIV-1

The HIV-1 virion is approximately 100 nm in diameter and is surrounded by a lipoprotein-rich membrane derived from the host membrane (Chen, 2019). Trimeric envelope (Env) glycoproteins, consisting of surface gp120 and transmembrane spanning gp41 heterodimers, are embedded in the virus membrane (**Figure 1.2**) (Chen, 2019). The matrix protein (p17) lines the inside of the viral lipoprotein membrane, connecting the capsid to the membrane (**Figure 1.2**). The capsid (p24) contains two copies of single-stranded (ss) viral ribonucleic acids (RNA), which encodes for envelope (*env*), group-specific antigen (*gag*), and deoxyribonucleic acid (DNA) polymerase (*pol*) genes. (**Figure 1.2**) (Gelderblom et al., 1989, Fanales-Belasio et al., 2010). The *gag* gene encodes for the core structural proteins (p24, p7, and p6) and the matrix (p17) while the *env* gene is responsible for viral envelope glycoproteins (gp120 and gp41) (Korniy et al., 2019) which recognise cell surface receptors. The *pol* gene encodes for enzymes responsible for viral replication: reverse transcriptase, integrase, and protease (**Figure 1.2**).

HIV has many accessory and regulatory proteins that are involved in viral replication, these include trans-activator of transcription (Tat), regulator of expression of the virion (Rev), viral protein R (Vpr), viral protein U (Vpu), virion infectivity factor (Vif), and negative factor (Nef). Tat protein promotes the expression of other HIV genes and is expressed early after infection (Spector et al., 2019) whereas Rev protein ensures genomic viral RNA is transported from the nucleus to the cytoplasm (Jackson et al., 2020). Vpr is involved in cell cycle arrest (Zhang and Bieniasz, 2020) and allows the viral DNA access into the nucleus of non-dividing cells (Zhang et al., 2021). Vpu is responsible for virion release from the cell, and the Vif protein augments the virulence of progeny viral particles (Khan and Geiger, 2021, Salamango et al., 2019). Lastly, the Nef protein is involved in cellular signal transduction and ensures virion budding in the later stages of the virus replication cycle (Kwon et al., 2020).

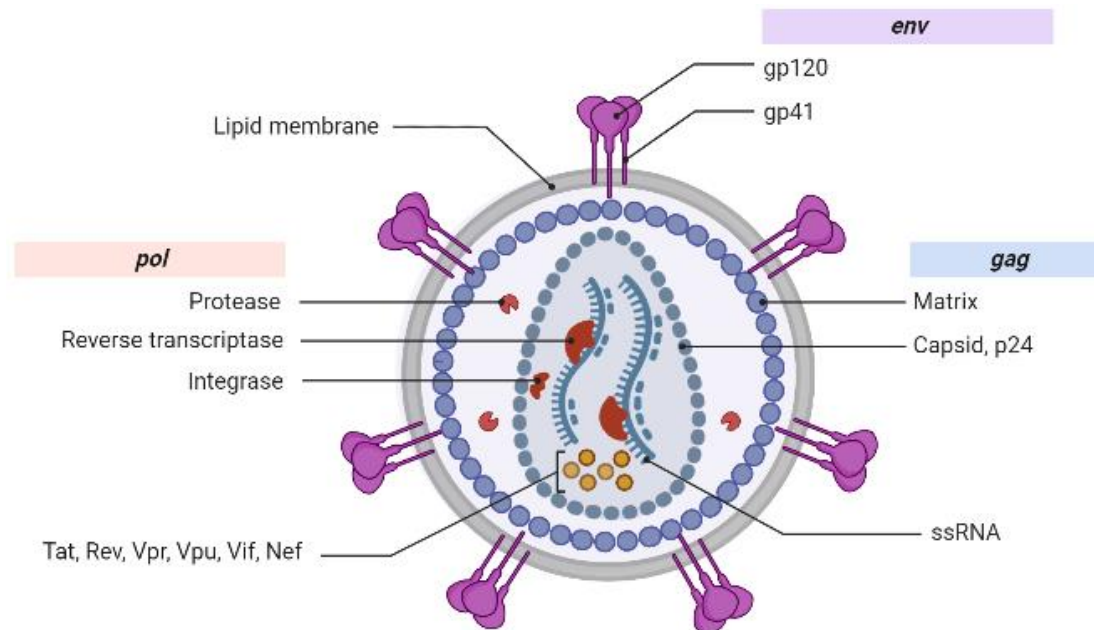


Figure 1.2: The structure of an HIV particle. HIV is enveloped with a lipid membrane embedded with surface glycoproteins, gp120, and gp41. Beneath the envelope is the matrix protein, p17. The viral capsid, p24, contains two identical copies of viral ssRNA. HIV genome encodes for various enzymes (protease, reverse transcriptase, and integrase) and proteins (Tat, Rev, Vpr, Vpu, Vif, and Nef) (Created with BioRender.com).

1.3.2 HIV-1 life cycle

The entry of HIV-1 into host cells is mediated by the Env protein (gp120 and gp41) and host cell receptor, the cluster of differentiation (CD) 4 (**Figure 1.2**) (Ward and Wilson, 2017). This allows for HIV-1 to bind to other host's co-receptors including the chemokine receptor 5 (CCR5) and C-X-C receptor 4 (Rao, 2009) fusing the viral and host membranes (**Figure 1.3**) (Wilen et al., 2012). HIV-1-infected cells can also infect nearby cells via membrane protrusions or the formation of syncytia (Urbano et al., 2018). This method is more efficient than the fusion of cell-free virions as it bypasses the initial attachment of Env proteins (Ward and Wilson, 2017).

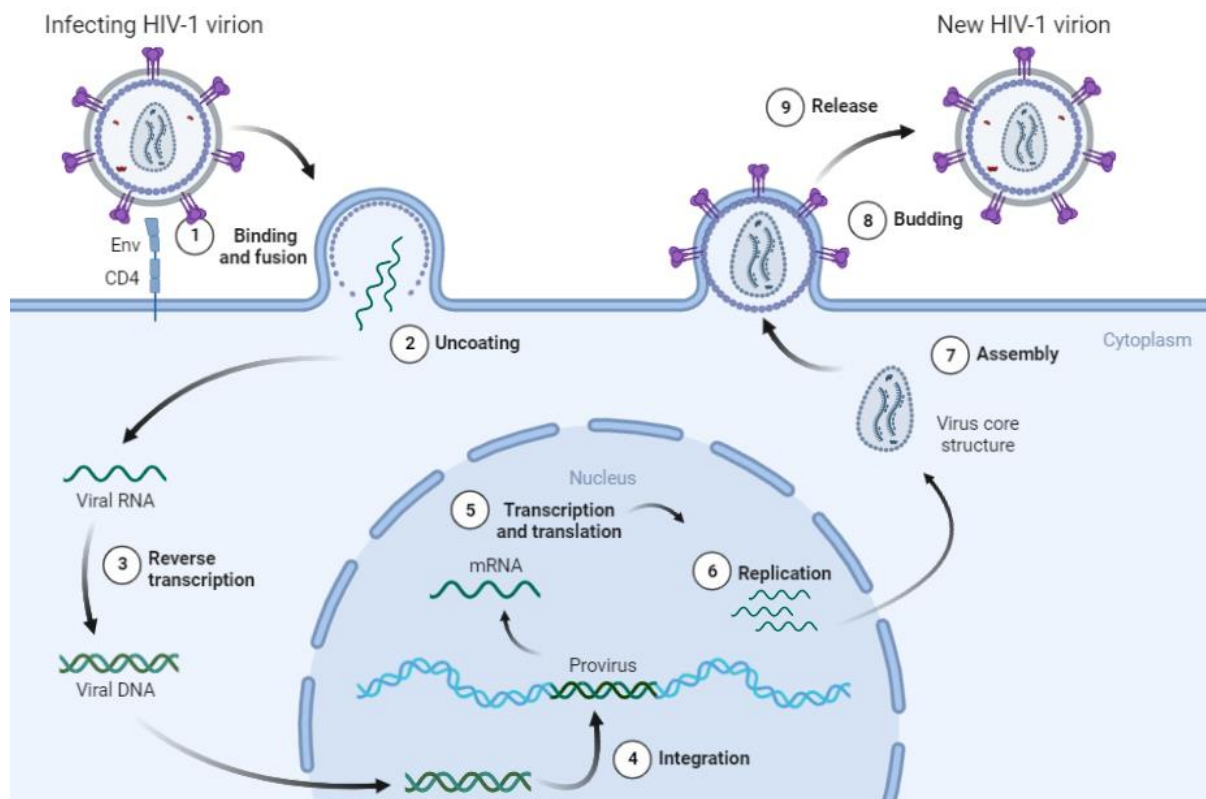


Figure 1.3: The HIV-1 life cycle. HIV-1 Env proteins bind to the CD4 receptor and enter the cell, followed by the uncoating of the virus. Viral DNA is transcribed and integrated as a provirus into the host's DNA. New viral proteins assemble into a new virion that buds from the host cell membrane (Created with BioRender.com).

Viral entry results in the uncoating and the release of the viral capsid proteins into the host's cells (**Figure 1.3**). Viral ssRNA is synthesised into viral double-stranded (ds) DNA by viral enzyme reverse transcriptase (**Figure 1.3**) in three steps: synthesis of the antisense complementary DNA strand, digestion of the RNA template, and the synthesis of the sense complementary DNA strand (Urbano et al., 2018). Reverse transcriptase converts viral RNA into viral DNA. Reverse transcriptase is the key enzyme responsible for the extensive sequence variability of HIV-1. The enzyme is extremely error-prone and lacks proofreading abilities resulting in a dsDNA daughter transcript that is highly variable from its originator. Variability can also occur due to recombination of two different RNA templates during strand transfer (Smyth et al., 2012). This promotes viral escape from the host's immune system and antiretroviral therapy (ART) (Hu and Hughes, 2012).

Cellular factors stabilise and translocate the viral DNA towards the nuclear membrane, where it actively passes through the nuclear pore complexes into the host cell, where

it is then integrated into the host's genome (Ghavami et al., 2016). Integrase binds to the viral DNA and cellular repair enzymes complete the integration of the viral DNA into the host's DNA completing the formation of the provirus (**Figure 1.3**) (Grandgenett, 2005).

The formation of the provirus enables the transcription and translation of viral proteins and subsequent viral genome replication (**Figure 1.3**). The viral proteins converge together in a region on the plasma membrane to assemble into an immature virion (**Figure 1.3**). Immature virions undergo proteolysis to form mature virions that can infect other cells. Proteolysis of group-specific antigen (Gag) proteins induces a conformational change resulting in the compression of the viral genome and reconstruction of the viral capsid into a conical shape. During the budding process, the mature virions acquire membrane proteins from the host. These may include human leukocyte antigen (HLA) class I and II proteins and adhesion proteins which will assist with the adhesion to other target cells from the host (Chen, 2019). The virions bud from the host and are released to infect nearby cells (**Figure 1.3**) (Fanales-Belasio et al., 2010).

1.4 THE PATHOGENESIS OF HIV-1 INFECTION

1.4.1 The natural course of HIV-1 infection

1.4.1.1 Primary infection

HIV-1 transmission requires direct exposure to body fluids from people living with HIV-1 (PLWH). This includes blood, vaginal secretions, semen, and breast milk (WHO, 2022). HIV-1 mother-to-child transmission can also occur during pregnancy and delivery (WHO, 2022). Transmission is dependent on the HIV-1 viral load and the host's susceptibility (Martin and Sattentau, 2009). HIV-1 RNA can be detected 10-12 days from exposure in peripheral blood by reverse transcriptase polymerase chain reaction amplification. HIV-1 viraemia increases exponentially and peaks a few weeks after infection at over 100 million RNA copies/ μ L. The CD4⁺ T cell count decreases substantially following primary infection (**Figure 1.4**).

The high levels of viraemia are normally short-lived as the host generates innate type I interferon (IFN) and natural killer (NK) cells and adaptive immune responses (CD8⁺ T cells and binding antibodies) that contribute to viral suppression. In the following

weeks, the viremia decreases until it reaches a viral setpoint (**Figure 1.4**) (Fanales-Belasio et al., 2010). Many factors related to innate, cell-mediated, and humoral immunity may influence viral replication and the host's establishment of the viral setpoint (Robb and Ananworanich, 2016). Reports have shown that CD8⁺ T cell cytotoxic activity is important for the initial viral control (Borrow et al., 1994, Collins et al., 2020).

Once the innate and adaptive immune responses are generated, levels of HIV-1 viraemia decline, and the CD4⁺ T cell count rebound partially. The combination of both innate and adaptive immunity leads to the asymptomatic period (**Figure 1.4**).

1.4.1.2 The asymptomatic phase

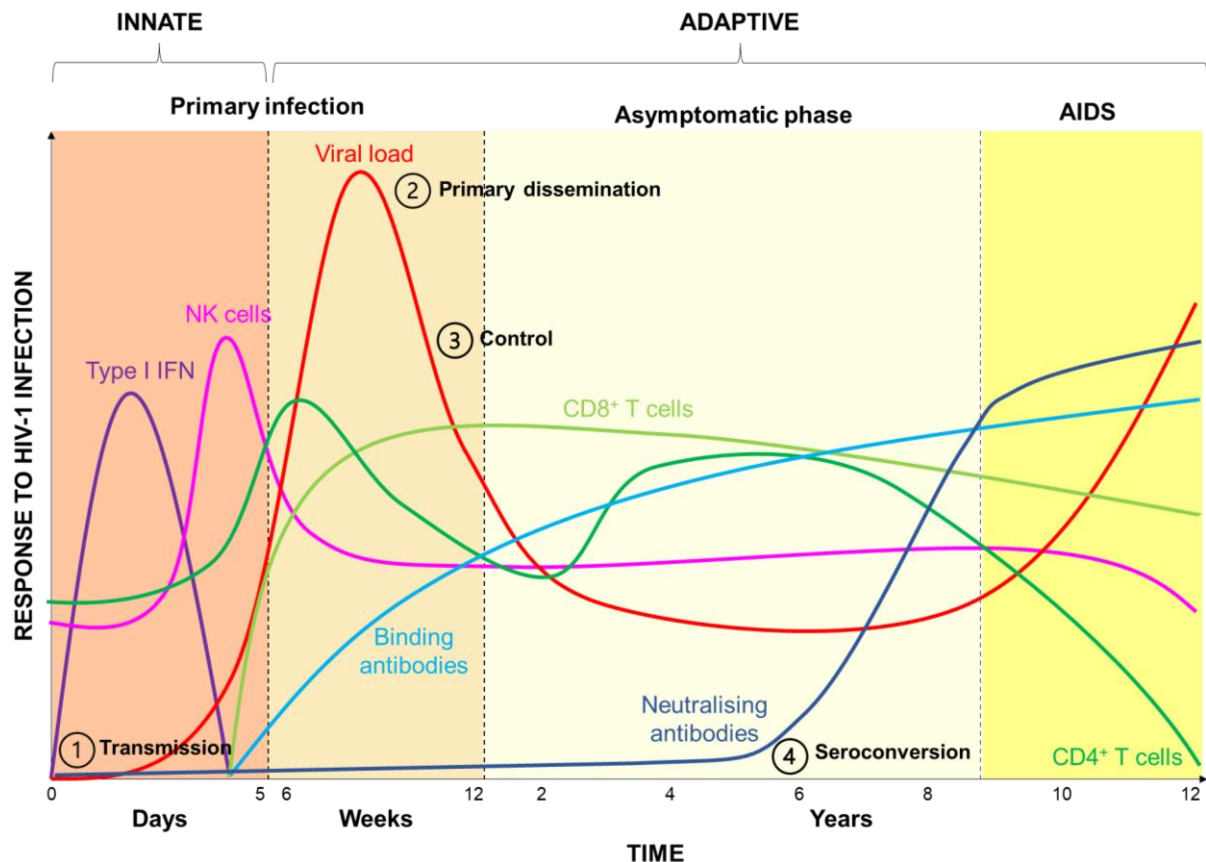
The asymptomatic period is associated with the decline of viral RNA and the expansion of HIV-1 neutralising antibodies (seroconversion) (Fanales-Belasio et al., 2010). However, during this phase, HIV-1 continually replicates within the host and becomes more adept at evading antiviral immunity leading to a state of chronic systemic infection.

Consistent viral replication in lymphoid tissues gives rise to reservoir compartments. Reservoir pools facilitate a highly mutated HIV-1 genome while expressing low levels of viral antigens and are some of the reasons for antiviral host evasion (Urbano et al., 2018). HIV-1 infection causes architectural changes in the lymphoid tissues through chronic immune activation leading to the virus spreading to proximal CD4⁺ T cells (Xu et al., 2013). During the asymptomatic phase, a constant decline in CD4⁺ T cells impairs the immune system.

1.4.1.3 AIDS

In the absence of viral containment, the subsequent decline of CD4⁺ T cells (< 200 cells/ μ L) results in the onset of AIDS (**Figure 1.4**). This period is characterised by opportunistic infections (bacterial, viral, fungal, or parasitic) and irregular tumour growth; both of which are indicative of an extremely impaired immune system. UNAIDS approximated that without the use of ART, the average time from HIV-1 primary infection to AIDS-related death is 11 years (UNAIDS, 2022). HIV-1 disease progression is variable and depends on the infecting virus as well as the host. The rate of disease progression can be predicted soon after infection by the viral set point six months after HIV-1 exposure and by the upregulation of CD38 and HLA-DR and CD8⁺

T cells. Moreover, T cell activation is a stronger marker for disease progression than levels of viraemia and is predictive even before seroconversion (Leng et al., 2001).



IFN, interferon; NK, natural killer; AIDS, acquired immunodeficiency syndrome

Figure 1.4: Schematic of the effect of HIV-1 on the innate and adaptive immune systems during natural infection. Image modified from Sego et al and Judge et al (Sego et al., 2020, Judge et al., 2020).

1.4.2 Antiretroviral therapy

Antiretroviral therapy has significantly improved the life expectancy of PLWH. It inhibits HIV-1 replication thereby reducing the plasma HIV-1 viral load to undetectable levels allowing circulating CD4⁺ T cell counts in peripheral blood to increase (Cheret et al., 2015). Although ART cannot cure HIV-1 infection, it has reduced mortality and morbidity associated with HIV-1 infection and AIDS. The benefits of early initiation of ART include reduced viral reservoir size, partially restored gut damage associated with HIV-1 infection, and decreased systemic immune activation (Ananworanich et al., 2016).

The classes of current antiretroviral drugs include nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, integrase strand transfer inhibitors, CCR5 antagonists, post-attachment inhibitors, and fusion inhibitors (Gandhi et al., 2023). Initial combination therapy regimes usually include two nucleoside reverse transcriptase inhibitors and a third active antiretroviral drug (Gandhi et al., 2023).

Since ART is a lifetime of therapy, it does present major challenges including persistent viral replication in reservoirs that continues to drive pathogenic disease, nonadherence can reduce the effectiveness of therapy, poor drug tolerability, and adverse drug interactions; all of which can lead to drug resistance followed by virologic failure (Martinez-Picado and Deeks, 2016).

1.4.3 Spontaneous control of HIV-1 infection

The majority of people with untreated HIV-1 infection have uncontrolled viraemia and increasing immune impairment that eventually progresses to AIDS. However, an extremely rare group of PLWH can spontaneously suppress viral replication without the use of ART. These individuals are termed HIV-1 controllers and can suppress viral replication to below 2000 RNA copies/mL while maintaining a CD4⁺ T cell count > 500/μL (Woldemeskel et al., 2020). HIV-1 controllers can further be subdivided into elite controllers (ECs) (viral load < 50 RNA copies/mL) and viraemic controllers (viral load ≥ 50, but < 2000 RNA copies/mL) (Gebara et al., 2019). Spontaneous control begins early in HIV-1 controllers. Peak viraemia during the acute phase of infection in HIV-1 controllers is typically lower than in progressors (Saez-Cirion et al., 2014). A schematic illustrating the viral load setpoints and CD4⁺ T cell counts of different categories of HIV-1 infection is shown in **Figure 1.5**. HIV-1 controllers can give us important insight into what is considered to be a functional cure. They can provide genetic, virological, and immunological clues to the mechanisms of viral control.

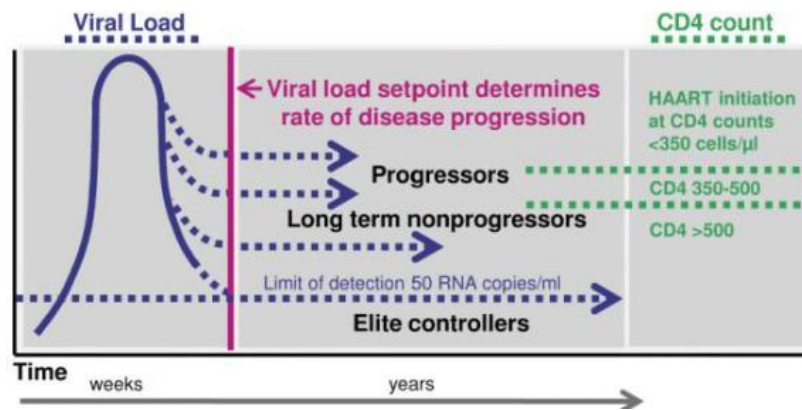


Figure 1.5: Schematic illustration of the different categories of PLWH. Their viral set point (RNA copies/mL) and CD4⁺ T cells/μL determine their rate of disease progression. Image reprinted from Tiemessen and Martinson (Tiemessen and Martinson, 2012).

1.4.3.1 Factors responsible for spontaneous control

1.4.3.1.1 Viral factors

Infection with less virulent strains of HIV-1 may contribute to successful host control and slower disease progression. HIV-1 controllers were thought to be infected with a replication-incompetent virus; however, isolation of the virus from ECs without polymorphisms, showed the opposite (Tiemessen and Martinson, 2012). This is indicative that viral suppression is host-mediated, rather than a result of infection with an unfit virus.

1.4.3.1.2 Intrinsic antiviral factors

Intrinsic host factors block viral replication directly in a process different from pattern recognition receptors which prevent viral infection by releasing interferons and other antiviral molecules (Yan and Chen, 2012). An example is the upregulation of p21 cyclin-dependent kinase inhibitor which decreases the susceptibility of CD4⁺ T cells to HIV-1 in a process associated with viral control (Chen et al., 2011).

Genetic factors are commonly associated with spontaneous control. HLA class I receptors and the interaction between NK killer immunoglobulin-like receptors (KIR) alleles (Goulder and Deeks, 2018). Specifically, the interactions of HLA-B27 and HLA-B57 with KIR3DL1 and KIR2DS1 have previously been associated with disease control (Martin et al., 2002, Wong et al., 2010, Martin et al., 2018). HLA and KIR are the two most polymorphic regions of the human genome, thus viral control is associated with more diverse HLA and KIR alleles. Interestingly, spontaneous control of SIV has been shown in at least two macaque species, independent of major

histocompatibility complex (MHC) genotypes (Saez-Cirion et al., 2014). This suggests that viral control is partly immune-mediated.

Host adaptive and innate immune factors are also linked to spontaneous control of HIV-1. Preserved T cell function with elevated cytotoxicity and proliferation together with polyfunctional Gag-specific cytotoxic T cells are also associated with viral control (Laher et al., 2017, Boppana and Goepfert, 2018). In addition, C-X-C receptor 5 expressing CD4⁺ T cells from ECs can promote greater B cell maturation inducing immunoglobulin class switching better than in viraemic progressors (Buranapraditkun et al., 2017). In addition, enhanced antigen-presenting capabilities of dendritic cells coupled with reduced proinflammatory cytokine production have also been associated with spontaneous control. Another component of innate immunity includes NK cells, which can also be activated by antibody-dependent cellular cytotoxicity (ADCC) activity. This involves the direct binding of specific antibodies that bind to the Fc receptor (CD16). Enhanced ADCC activity has been associated with spontaneous control of HIV-1 (Forthal and Finzi, 2018).

1.5 THE INNATE IMMUNE RESPONSE TO HIV-1 INFECTION

HIV-1 is primarily transmitted through the mucosal surfaces of the genital or gastrointestinal tract which are lined with macrophages, dendritic cells, and lymphocytes (Tebit et al., 2012).

1.5.1 Monocytes and macrophages

Monocytes and macrophages are professional antigen-presenting cells that connect the innate and adaptive immune systems (Campbell et al., 2014). When monocytes differentiate into macrophages, they become more susceptible to HIV-1 infection. They further enhance infection as they are disseminated into almost all tissues (Kruize and Kootstra, 2019). Moreover, since macrophages have a relatively long lifespan and reside in anatomical sites that cannot be accessed by ART, they are important viral reservoirs (Kruize and Kootstra, 2019).

1.5.2 Dendritic cells

Mature dendritic cells are rare, potent, antigen-presenting cells that link the adaptive and innate immune responses aiding in viral control (Altfeld et al., 2011). Although

dendritic cells express CD4 and other HIV-1 coreceptors, HIV-1 primarily enters dendritic cells through a C-type ligand called dendritic cell-specific intercellular adhesion molecule (Wu and KewalRamani, 2006).

HIV-1 replication occurs due to the cell-cell infection enabling viral dissemination (Altfeld et al., 2011). Dendritic cells migrate towards the lymph and gut, both of which are highly concentrated with T cells, which would get infected, causing an even further decline in T cell counts. AIDS is characterised by a decline in circulating dendritic cells. Chronic HIV-1 infection results in continuous stimulation of dendritic cells and the induction of proinflammatory cytokines, increasing the number of immune cells and therefore new viral replication sites (Malleret et al., 2008).

1.5.3 Natural killer cells

Natural killer cells promote targeted cell killing by producing cytolytic proteins, cytokines, and chemokines that lyse virus-infected and tumour cells. NK cells do not have specific receptors to recognise HIV-1-infected cells, rather they rely on a careful balance of inhibitory and activating signals sent to the NK cell by virus-infected cells (Altfeld et al., 2011). The production of cytokines and chemokines allows for other immune modulation via the crosstalk with dendritic cells and T cells.

1.5.4 Trained immunity

Innate immune cells are primed by an initial antigen and later produce off-targeted protection against unrelated pathogens in a process known as trained immunity (Sviridov et al., 2022). When innate immune cells are activated by the first stimulus, they undergo metabolic changes causing epigenetics changes, which maintains cells in a “trained” state (Arts et al., 2016). Trained immunity allows for tolerance of subsequent antigens, or in the case of HIV-1, enhances the response to later presenting stimuli (Zubair et al., 2021).

1.6 NATURAL KILLER CELLS IN HIV-1 INFECTION

1.6.1 The biology of natural killer cells

Natural killer cells are large, granular innate immune cells that comprise 5-15% of total peripheral blood lymphocytes. They stem from the same progenitor as T cells and form a vital role in the early response to infections and tumour surveillance (Abel et al.,

2018). NK cells are regulated by many inhibitory and activating germ-line encoded receptors found on the surface of the cell membrane. These include natural cytotoxicity receptors, KIRs, C-type lectin receptors, signal lymphocyte activation family receptors, and Fcγ receptors (Martinet and Smyth, 2015). The response to potent interleukins (IL), such as IL-2 and IL-15, drives proliferation and activation initiating their effector functions (Abel et al., 2018). NK cell effector functions (cytotoxicity and cytokine secretion) are dependent on the overall balance of the inhibitory and activation signals (Sun and Lanier, 2011).

Characteristically, NK cells lack the common T cell marker, CD3 but have CD56 and CD16, which are used to define these cells. NK cells can further be divided into subsets based on the relative expression of CD56 and CD16 (**Figure 1.6**). The primary NK cell subset in peripheral blood possessing the highest cytotoxic activity is the CD56^{dim}CD16⁺ NK cell subset. CD56^{bright}CD16⁻ NK cells are the less mature precursor to CD56^{dim}CD16⁺ NK cells (Poli et al., 2009). They are often referred to as immunoregulatory because of their increased cytokine production upon stimulation. The expansion of CD56^{neg}CD16⁺ NK cells is associated with chronic viral infections. They are considered to be a dysfunctional subset of NK cells due to their poor cytotoxic capabilities and lack of cytokine production (Cao et al., 2021).

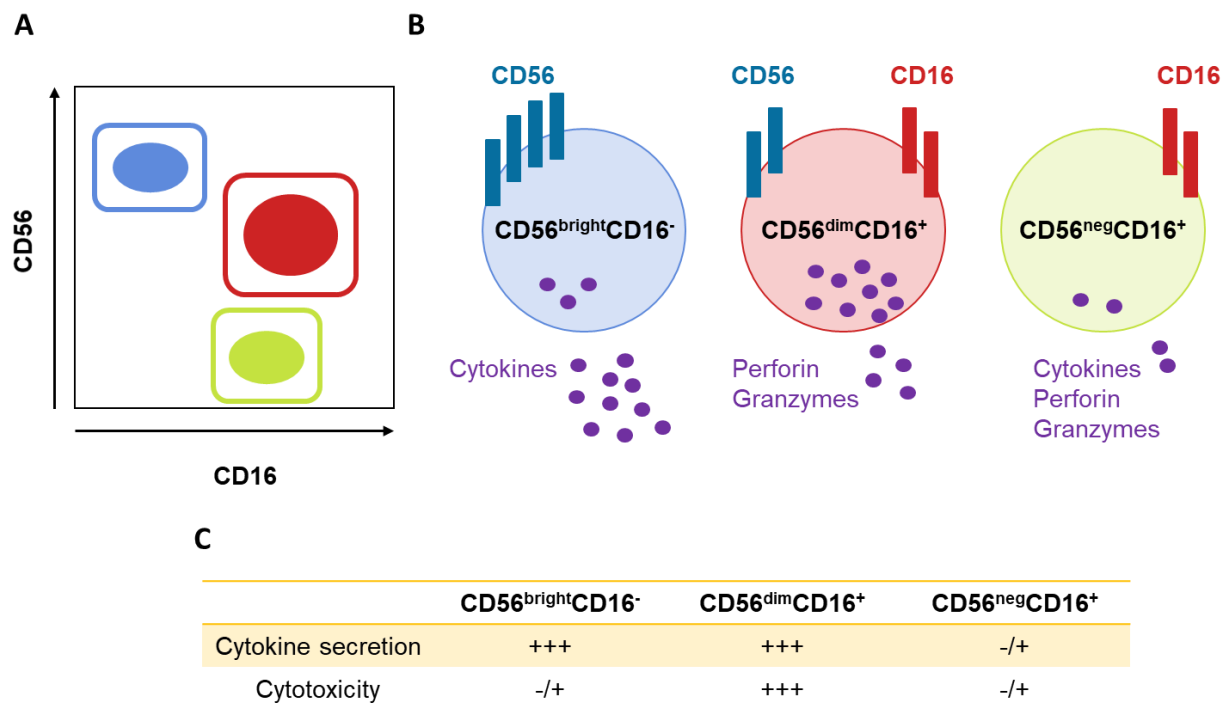


Figure 1.6: (A) Schematic illustration of NK cell subsets based on the expression of CD56 and CD16. (B) Classification of NK cell subsets based on the relative expression of CD56 and CD16. (C) Summary of functional characteristics of NK cell subsets. Image adapted from Bjorkstrom et al (Bjorkstrom et al., 2010).

1.6.1.1 Natural killer cell distribution, phenotype, and function during HIV-1 infection

Acute viral infection causes an expansion of NK cells, accompanied by a redistribution of NK cell subsets (Alter et al., 2005). The expansion is caused by the increase of the CD56^{dim}CD16⁺ NK cell subset. Chronic viral infection appears to reduce the number of circulating total NK cells but induces an expansion of the dysfunctional CD56^{neg}CD16⁺ NK cell subset.

Chronic HIV-1 infection alters the phenotypic profiles of NK cells (Phaahla et al., 2019). Continual viral stimulation causes up and down regulation of cellular receptors driving activation and the expansion of exhausted NK cells (Mikulak et al., 2017). HIV-1 infection causes decreased NK cell education and differentiation ultimately leading to reduced NK cell cytotoxicity and cytokine production (Hong et al., 2010, Parsons et al., 2014). Furthermore, the expansion of the dysfunctional CD56^{neg}CD16⁺ negatively impacts the crosstalk between NK cells and dendritic cells. Dysfunctional dendritic cells fail to release IL-15, which further inhibits NK cell priming (Tomescu et al., 2015).

Limited data show the alternations in NK cell phenotype and function are caused directly by a viral infection or by a constant chronic inflammatory state. Further

investigation of NK cell frequency, phenotype, and function may aid in understanding the intricacies of HIV-1 infection and pathogenesis.

1.6.2 Natural killer cell licensing/education

Natural killer cell development involves a process known as education which produces highly functional, self-tolerant innate immune cells (Cronk et al., 2021). An educated NK cell can interact with both self-HLA class I and specific NK inhibitory molecules, which initiates the signal to the licensing of functionally competent NK cells that can recognise and kill cells that have downregulated HLA class I molecules (Boudreau and Hsu, 2018). NK cells that do not undergo the education process, have diminished functional activity in comparison to fully licensed NK cells (Altfeld et al., 2011). NK cell licensing seems to be a dynamic process, rather than the binary “all or nothing” approach. NK cells that interact with more inhibitory receptors and a range of HLA class I alleles are more functionally competent toward a given stimulus than NK cells with fewer HLA class I contacts (Abel et al., 2018).

During primary HIV-1 infection, NK cell education is encouraged due to the upregulation of HLA-C and KIR2DL on NK cells (Korner et al., 2014). These NK cell subsets show superior polyfunctional characteristics due to the licensing effect (Korner et al., 2014). During chronic HIV-1 infection, Vpu causes the downregulation of HLA-C on immune cells resulting in reduced binding of KIRs to HIV-1 infected cells. (Apps et al., 2016, Holzemer et al., 2017). The remaining HLA-C expression is sufficient to license NK cells, producing potent effector cells able to suppress viral replication (Holzemer et al., 2017).

1.6.3 Natural killer cell memory

A subset of NK cells show adaptive characteristics and can clonally expand with the recognition of an antigen, unlike their conventional, short-lived counterparts. They typically express reduced amounts of CD7, CD161, and FcRγ but increased amounts of CD57 (Nikzad et al., 2019). During Human cytomegalovirus (HCMV) infection, NK cells upregulate the activation marker, natural killer group 2C (NKG2C), in response to HLA-E presenting HCMV viral peptides. This subset of memory NK cells is found in HCMV seropositive individuals (Bernard et al., 2022). Furthermore, NK memory responses were noted in individuals who had prior exposure to HIV-1 Env vaccinations

(Liu et al., 2016). These NK memory cells specifically targeted cells presenting the HIV-1 Env antigen (Nikzad et al., 2019). These results show that NK cells can be characterised by their memory-like responses, redefining their role in immunity.

1.6.4 Natural killer cell metabolism

Cellular metabolism influences the effector functions of immune cells. Cells process glucose to acquire energy or adenosine triphosphate (ATP) in two ways: oxidative phosphorylation or glycolysis. Oxidative phosphorylation is an oxygen-dependent process in which one molecule of glucose is converted into 30 molecules of ATP. Glycolysis is an oxygen-independent process whereby glucose is broken down producing 2 molecules of ATP and other fundamental building blocks required for the cell (Maciver et al., 2008).

In a resting state, NK cells rely on oxidative phosphorylation, however, when activated and stimulated with HIV-1, they undergo a metabolic switch to glycolysis, even if there is sufficient oxygen supply. Glycolysis is favoured, even though it is far less efficient in producing ATP, as it produces the precursor molecules important for the manufacture of lipids, amino acids, and nucleotides (DeBerardinis et al., 2007). These molecules are essential for NK cell proliferation and effector functions.

While degranulation of NK cells is independent of metabolic changes, interferon-gamma (IFN- γ) production, in response to cytokine stimuli, is influenced by oxidative phosphorylation and glycolysis (Keating et al., 2016). Oxidative phosphorylation is sufficient when NK cells are activated with IL-15 (early activation), however, since IL-15 is crucial for the proliferation and survival of NK cells, the concentration of the cytokine drives cellular metabolism (Kobayashi and Mattarollo, 2019). Lower concentrations of IL-15 support oxidative phosphorylation, whereas long-term high concentrations push the cell toward glycolysis. IL-2 increases the rate of both oxidative phosphorylation and glycolysis, whereas IL-18 does not influence NK cell metabolic regulation (Donnelly et al., 2014).

In addition to being phenotypically and functionally distinct, NK cell subsets are also metabolically distinct. CD56^{bright}CD16⁻ reacts to metabolic change by upregulating various metabolic markers to a greater extent than CD56^{dim}CD16⁺ cells (Keating et al., 2016).

Investigation of nutrient transporters, Glut1, CD98, and CD71, showed increased Glut1 expression and decreased CD98 expression in CD56^{bright}CD16⁻ from peripheral blood compared to those from tissue-derived NK cells (Salzberger et al., 2018). Similarly, Glut1 expression was higher and CD98 and CD71 were lower in CD56^{dim}CD16⁺ from peripheral blood compared to CD56^{bright}CD16⁻ tissue-resident NK cells (Salzberger et al., 2018). The CD56^{dim}CD16⁺ subset can further be divided into a licensed, functional subset and an unlicensed, hyporesponsive subset. Unlicensed NK cells favour oxidative phosphorylation when activated. In contrast, licensed NK cells tend to increase the rate of both oxidative phosphorylation and glycolysis (Schafer et al., 2019).

Altered NK cell metabolism has been linked to chronic diseases, such as cancer and obesity (Leone and Powell, 2020, McKinney and Smith, 2018). In a mouse model with the Friend retrovirus, an infection caused increased NK cell metabolic reprogramming and nutrient uptake followed by an increased rate of oxidative phosphorylation and glycolysis (Littwitz-Salomon et al., 2021). HIV-1 pathogenesis is likely causing similar NK cell metabolic dysfunction; however, few studies have shown a link between the two. HIV-1 is known to selectively infect metabolically active cells. The virus takes advantage of the increased metabolic by-products of glycolysis and uses them to make its DNA and complete its life cycle (Saez-Cirion and Sereti, 2021). Other reports have shown that HIV-1, itself, dysregulates immune metabolism by upregulating glycolysis to complete its life cycle (Saez-Cirion and Sereti, 2021). There is a limited amount of data on NK cell metabolism and the role it may play in the control of HIV-1 infection. Since immunometabolism has become a valuable area of research for drug targeting, there is a need for further investigation into the importance of NK cells and the role they play in HIV-1 pathogenesis.

1.7 THE ROLE OF NATURAL KILLER CELLS IN HIV-1 CONTROL

The significance of NK cells during the natural control of HIV-1 infection was first attributed to their ability to suppress viral replication, *in vitro*, just as well as their adaptive immunity equivalents, CD8⁺ T cells (Kottlil et al., 2003). Thereafter, many reports confirmed that host genetics may be an important determinant of HIV-1 control,

such as the interaction of certain HLA class I alleles with specific KIR receptors of NK cells (Kuniholm et al., 2011).

Several studies have investigated the role of KIR receptors and their mechanisms of action during natural HIV-1 control. In PLWH, the KIR3DS1 receptor, in concert with HLA-Bw4^{I80}, resulted in delayed progression to AIDS (Martin et al., 2002). Compared to KIR3DS1⁻ NK cells, KIR3DS1⁺ NK cells had a more functionally competent response to HIV-1 infected HLA-Bw4⁺ CD4⁺ T cells (Alter et al., 2007). Importantly, Long et al showed that KIR3DS1⁺ NK cells displayed more cytotoxic activity even in the absence of HLA-Bw4^{I80}, compared to KIR3DS1⁻ NK cells (Long et al., 2008). This study demonstrated evidence that KIR interactions were important for the functional response of NK cells and could play a role in the spontaneous control of HIV-1 infection (Long et al., 2008).

Reports show that HIV-1 can adapt to host KIR genotypes at a population level and the selection of viral sequence polymorphisms associated with KIR/HLA genotypes associated with escape further highlights the role of KIR in NK cell-mediated immune pressure (Alter et al., 2011, Holzemer et al., 2015). A follow-up study in a South African cohort showed that HIV-1 subtype C can evade NK cell immunity through a mutation in the p24 Gag protein (Ziegler et al., 2021). This disabled the binding of KIR2DL2 to HLA-C-03:04 and diminished NK cell effector functions in individuals with this genotype. Not only does this show that HIV-1 evades NK cell immunity by acquiring mutations within epitopes presented by HLA class I molecules, but also emphasizes the importance of NK cells as effector cells in HIV-1 control and harnessing NK cells as an additional arm of very rapidly responding cells for innovative vaccine design.

Natural killer cell licensing and education may also be contributing factors in the elite control of HIV-1 infection. This ensures self-tolerance and leads to a more educated, functionally competent NK cell, which is activated when exposed to missing/altered self. A study showed PLWH who carried the inhibitory KIR3DL1 allele as well as HLA-B57 had increased polyfunctional NK cells and progressed towards AIDS slowly, compared to PLWH carrying other inhibitory alleles. (Kanya et al., 2011). The continual signalling through inhibitory KIR receptors promotes NK cell survival (Felices et al., 2014), thus the increase in educated NK cells expressing many inhibitory

receptors during chronic viral infection may be associated with delayed disease progression.

Another factor attributed to the spontaneous control of HIV-1 infection is the conservation of the NK cell subset distribution. The maintenance of the CD56^{bright}CD16⁻ NK cell subset was described in long-term non-progressor PLWH (O'Connor et al., 2007). Furthermore, CD56^{bright}CD16⁻ cells are considered to be more metabolically active, shown by large quantities of IFN- γ production during an immune response, adding to the idea that they may play an essential role in controlling HIV-1 infection (Keating et al., 2016). In addition, phenotypic and functional profiles, that are otherwise known to be altered during HIV-1 infection (Zulu et al., 2017), appear to be conserved during natural control (Ravet et al., 2007).

There is insufficient data on HIV-1 ECs in African populations. A study by Moosa et al recently described two ECs in Kwa-Zulu Natal, South Africa. They showed that the ECs from this cohort were infected with a replication-competent virus, and both ECs expressed many HLA class I haplotypes that have been previously associated with delayed disease progression and increased levels of p21, a host restriction factor linked to viral control (Moosa et al., 2018). Adaptive immunological findings showed increased CD8⁺ T cell responses, and interestingly, evidence of HIV-1 specific CD4⁺ T cell responses, pre-infection, was also reported in this study (Moosa et al., 2018). Although the clinical, virological, genetic, and adaptive immunological characteristics were described, the innate immunological findings were not characterised. Thus, to date, limited innate immunological characteristics of ECs in a high-incidence setting have been described.

1.8 PROBLEM STATEMENT

Many genetic and functional studies have highlighted the role that NK cells play in delaying HIV-1 disease progression. Few reports, however, have focussed on the phenotypic and functional characteristics of NK cells in a high-incidence setting, such as South Africa. Crucial regulatory mechanisms of NK cells during elite control of HIV-1 infection in an African population remain unclear. Further research in understanding NK cell phenotypic, functional, and metabolic profiles during spontaneous control of

HIV-1 infection may provide important insights for the development of novel therapeutic strategies, especially in a setting where HIV-1 infection is most prevalent.

1.9 AIM

This study aimed to assess NK cell frequency, phenotype, function, and nutrient transporter profiles in HIV-1 controllers and compare them to treated and untreated PLWH and uninfected controls.

1.10 OBJECTIVES

1. To immunophenotype and compare NK cells from PLWH (ART-treated individuals, elite controllers, and viraemic progressors) and uninfected individuals.
2. To determine NK cell function by measuring the expression of CD107a and IFN- γ in PLWH and uninfected individuals.
3. To determine nutrient transporters profiles in NK cells and their subsets in PLWH and uninfected individuals.

1.11 HYPOTHESIS

We hypothesised that the NK cell frequency, phenotype, function, and nutrient transporter profiles will be similar in HIV-1 uninfected, ART-treated individuals and elite controllers and altered in viraemic progressors.

Table 2.1: Optical configuration of the BD LSRFortessa™ X-20

Laser	λ (nm)	Parameter	Mirror	Filter
Blue	488	PerCP Cy5.5	655 LP	670/30 BP
		FITC	505 LP	530/30 BP
Red	640	APC Cy7	750 LP	780/60 BP
		Alexa Fluor 700	710 LP	710/50 BP
		APC	-	670/14 BP
Violet	405	BV786	750 LP	780/60 BP
		BV711	690 LP	710/50 BP
		BV650	630 LP	660/20 BP
		BV605	595 LP	610/20 BP
		BV510	505 LP	525/50 BP
		BV421	-	450/50 BP
Yellow-green	561	PE Cy7	735 LP	780/60 BP
		PE Cy5.5	685 LP	710/50 BP
		PE Cy5	635 LP	670/30 BP
		PE CF594	600 LP	610/20 BP
		PE	570 LP	586/15 BP

λ , wavelength; PerCP, peridinin chlorophyll protein complex; Cy, cyanine; FITC, fluorescein isothiocyanate; APC, allophycocyanin; BV, brilliant violet; PE, phycoerythrin; LP, long-pass; BP, bandpass

2.1.2 Marker selection

The stain index can be affected by several factors, including the brightness of the reagent, the antigen density, the PMT voltage, the cytometer performance and setup, cell autofluorescence, non-specific binding of the antibody, and spillover. **Table 2.2** shows the relative brightness of fluorochromes ranked by their stain index.

Table 2.2: Relative brightness of fluorochromes ranked by their stain index

Laser / λ (nm)	Very bright	Bright	Moderate	Dim
Violet / 405	BV421 BV650 BV711	BV605 BV786	BV510	
Blue / 488			FITC GFP	PerCP
Yellow-green / 561	PE PE CF594 PE Cy5 PE Cy7			
Red / 640		APC		Alexa fluor 700 APC Cy7

λ , wavelength; BV, brilliant violet, PE, phycoerythrin; APC, allophycocyanin; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; PerCP, peridinin chlorophyll protein complex; Cy, cyanine

Antigens can be classified into three main groups:

1. Primary: are well characterised and easily classified as positive or negative populations. These antigens typically define broad subsets or lineages and are classically bound to dim fluorochromes.
2. Secondary: are well characterised and are typically expressed at a higher density, often over a continuum. These antigens are typically bound to moderately bright fluorochromes.
3. Tertiary: are considered critical. Their expression levels may be low or unknown, or variable on activation. These antigens should be bound to bright and very bright fluorochromes.

Markers of interest considered for the phenotypic panel were markers of activation (NKG2C, CD69, and CD38), inhibition (natural killer group 2A) (NKG2A), exhaustion (programmed cell death protein 1) (PD-1), and maturation/differentiation (CD57). While degranulation (CD107a), cytokine production (IFN- γ), and nutrient transporters (Glut1, CD98, and CD71) were considered in the functional panel. A viability dye was included because frozen peripheral blood mononuclear cells (PBMCs) were used.

As best as we could, fluorochromes were assigned to markers of interest based on their classifications, relative brightness, and the best separation of positive and negative populations. However, because fluorochrome availability was a limiting factor in the panel design, in some cases well characterised primary antigens were assigned to bright fluorochromes and critical markers were assigned to dim/moderate fluorochromes. The proposed phenotypic and functional panels are shown in **Table 2.3**. Antigens of interest were classified and assigned a fluorochrome.

Table 2.3: Proposed phenotypic and functional panel design

<i>Laser / λ (nm)</i>	<i>Fluorochrome</i>	<i>Phenotypic panel</i>			<i>Functional panel</i>		
		<i>Antigen</i>	<i>Classification</i>	<i>Relative brightness of fluorochrome</i>	<i>Antigen</i>	<i>Classification</i>	<i>Relative brightness of fluorochrome</i>
Blue / 488	PerCP Cy5.5	CD14/19	Primary	Dim	CD14/19	Primary	Dim
	FITC/GFP	CD69	Tertiary	Moderate	Glut1	Tertiary	Moderate
Red / 640	APC-Cy7	CD16	Primary	Dim	CD16	Primary	Dim
	Alexa Fluor 700	CD56	Secondary	Dim	CD56	Secondary	Dim
	APC	NKG2A	Tertiary	Bright			
Violet / 405	BV786						
	BV711				CD71	Tertiary	Very bright
	BV650						
	BV605	CD57	Tertiary	Bright			
	BV510	Viability	Primary	Moderate	Viability	Primary	Moderate
	BV421	PD-1	Tertiary	Very bright	CD98	Tertiary	Very bright
Yellow-green / 561	PE Cy7	CD38	Tertiary	Very bright	IFN- γ	Tertiary	Very bright
	PE Cy5.5						
	PE Cy5				CD107a	Tertiary	Very bright
	PE CF594	CD3	Primary	Very bright	CD3	Primary	Very bright
	PE	NKG2C	Tertiary	Very bright			

λ , wavelength; PerCP, peridinin chlorophyll protein complex; Cy, cyanine; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; APC, allophycocyanin; BV, brilliant violet, PE, phycoerythrin; CD, cluster of differentiation; NKG, natural killer group; PD-1, programmed cell death protein 1; Glut1, glucose transporter 1; IFN- γ , interferon gamma

2.2 PHOTOMULTIPLIER TUBE VOLTAGES

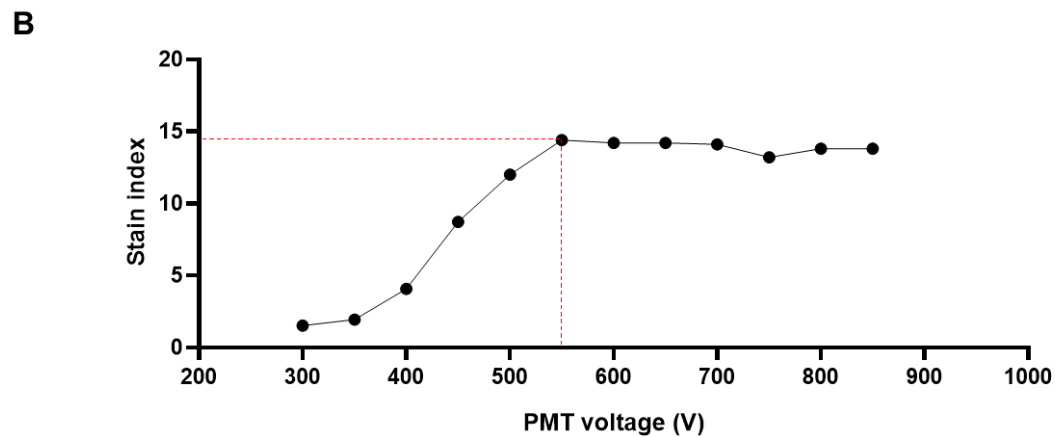
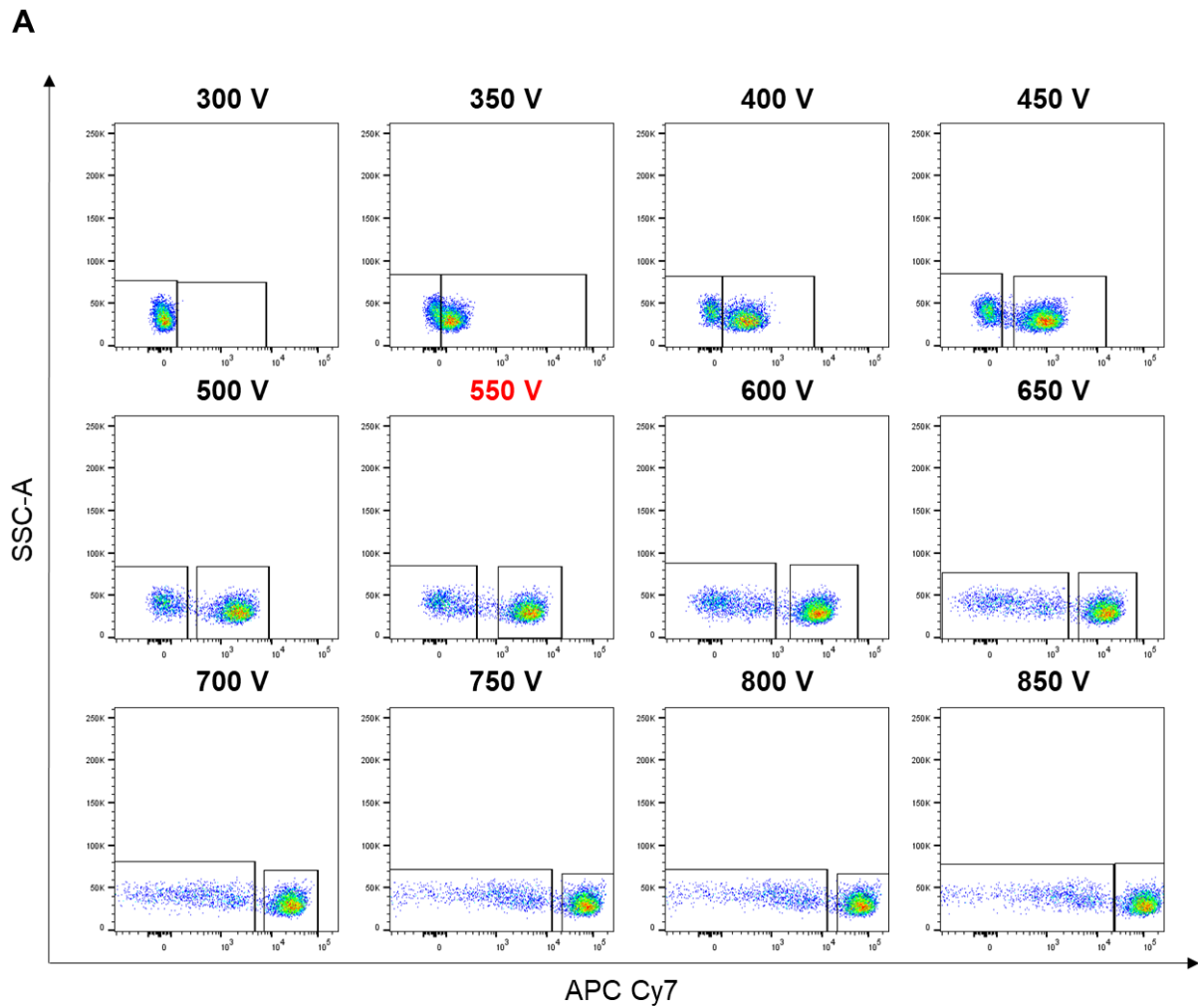
2.2.1 Voltration

A voltration (voltage titration) was performed to determine the optimal photomultiplier tube (PMT) voltages at which the best separation of positive and negative populations could be obtained while maintaining the smallest spread of the negative population. As beads have lower autofluorescence than cells, voltages set using beads may be lower than optimal for cells. Thus, PBMCs were used to perform the voltrations. Voltrations were performed using 1×10^6 cells stained with appropriately titrated antibodies for each channel to be used in the phenotypic and functional panels.

Cells were first acquired at the cytometer setup and tracking (CS&T) PMT voltage. Subsequently, the PMT voltage was reduced until no separation of positive and negative populations was seen (approximately 300 V). The voltage was then increased in 50 V increments until the positive population was off-scale (**Figure 2.2 A**). Approximately 10 000 events were recorded for each change in voltage. The optimal voltage of a given channel is determined by the maximum separation of the negative and positive populations with the least spread in the negative population. This can be calculated using the stain index (**Equation 2.1**). An example of a stain index vs PMT voltage curve is shown in **Figure 2.2 B**. As the voltage increases, the stain index increases until it reaches a maximum of 14.2 at 550 V and then decreases. Where the PMT voltage correlates to the highest stain index and then levels off, is indicative of the optimal voltage for that fluorescent channel.

$$\text{Stain index} = \frac{(MFI_{\text{positive population}} - MFI_{\text{negative population}})}{2 \times SD_{\text{negative population}}}$$

Equation 2.1: The stain index



APC Cy 7; allophycocyanin cyanine 7

Figure 2.2: (A) Optimisation of voltages (voltration) for the APC Cy7 channel. Lymphocytes were gated, followed by singlets. The same tube of PBMCs was run at 15 different voltages. These voltages (V) are indicated above each pseudocolour plot. The voltage in red was the calculated optimal voltage for the APC Cy7 channel. **(B)** Stain index vs PMT voltage curve. A red dotted line shows the optimal voltage of 550 V.

2.3 TITRATION OF ANTIBODIES

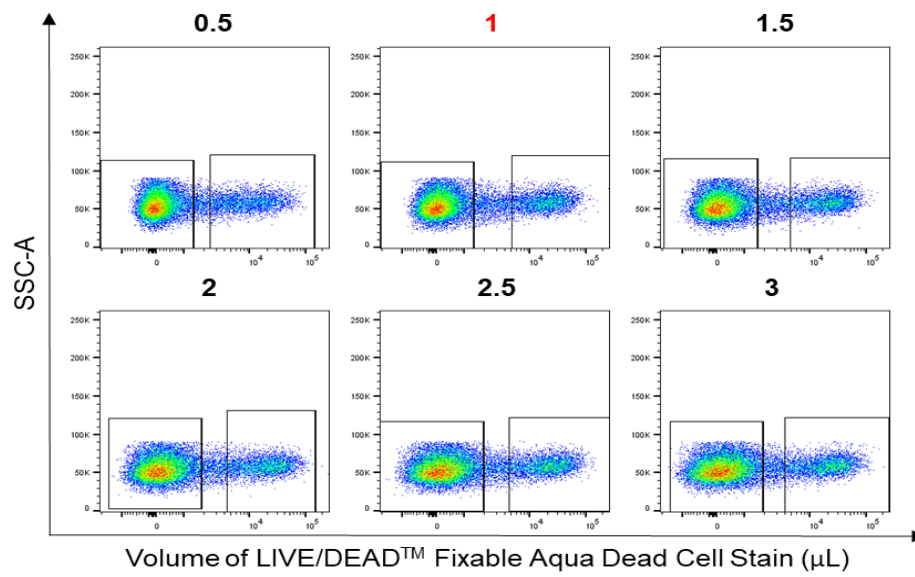
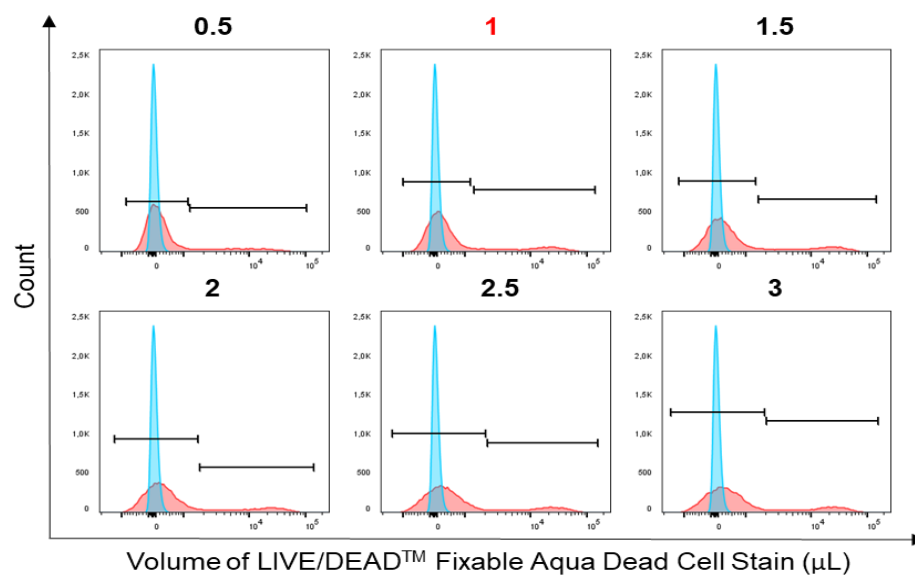
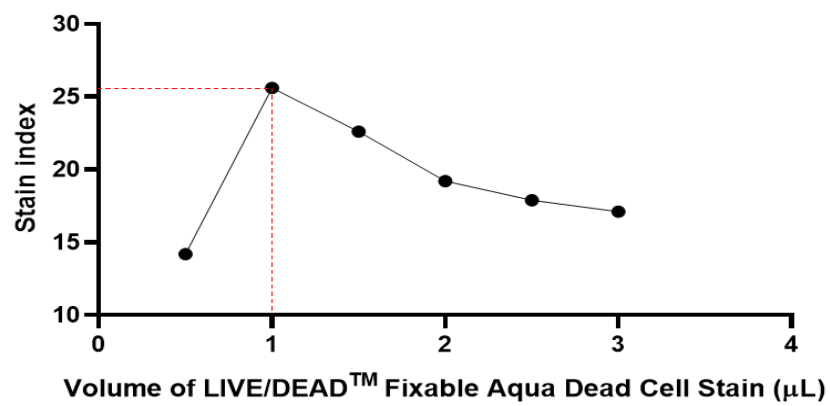
Optimal antibody titration is important to reduce the volume of antibodies used, fluorochrome spillover, as well as background noise. Concentrations of antibodies that are too high may lead to increased nonspecific binding resulting in increased spread of the negative population and decreased resolution. Optimal antibody titres will give the best separation of the positive and negative populations (Ferrer-Font et al., 2021). The optimal concentration of an antibody is determined primarily by the stain index which is defined as the concentration of the antibody that produces the brightest positive population with the least background noise (**Equation 2.1**) (Busuttil-Crellin et al., 2020). A titration curve can be calculated by plotting the stain index against the antibody concentrations. Titrations were performed for all antibodies in both phenotypic and functional panels.

2.3.1 Titration of the viability dye

Amine reactive compounds stain cell-surface amines on live cells. As dead cells have compromised membranes, the viability dyes can also bind to the free amines in the interior of the cell. Thus, dead cells will stain more brightly than live cells and so can be distinguished from live cells.

PBMCs were thawed, washed, rested, and then stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen, Waltham, USA) according to the manufacturer's instructions. The titration increased in increments of 0.5 µL of dye and finished at the recommended volume of 3 µL in 1 mL Dulbecco's phosphate buffered saline (dPBS) (Thermo Fisher Scientific, Waltham, USA) to which 100 µL was added to the cells (**Figure 2.3 A**). Lymphocytes were gated on followed by single cells to remove doublets. Pseudocolour plots were used to calculate the median of the positive and negative cell populations as well as the standard deviation of the negative population (**Figure 2.3 A**). These statistics were used to calculate the stain index.

Figure 2.3 B shows histogram overlays of the stained and unstained samples. This figure is helpful to make a direct comparison of cell populations to see if there is a shift in population due to antibody staining. Usually, the most well-aligned stained and unstained histogram together with the highest stain index (**Figure 2.3 C**) is considered to be the optimal minimum antibody concentration.

A**B****C**

SSC-A, side scatter area

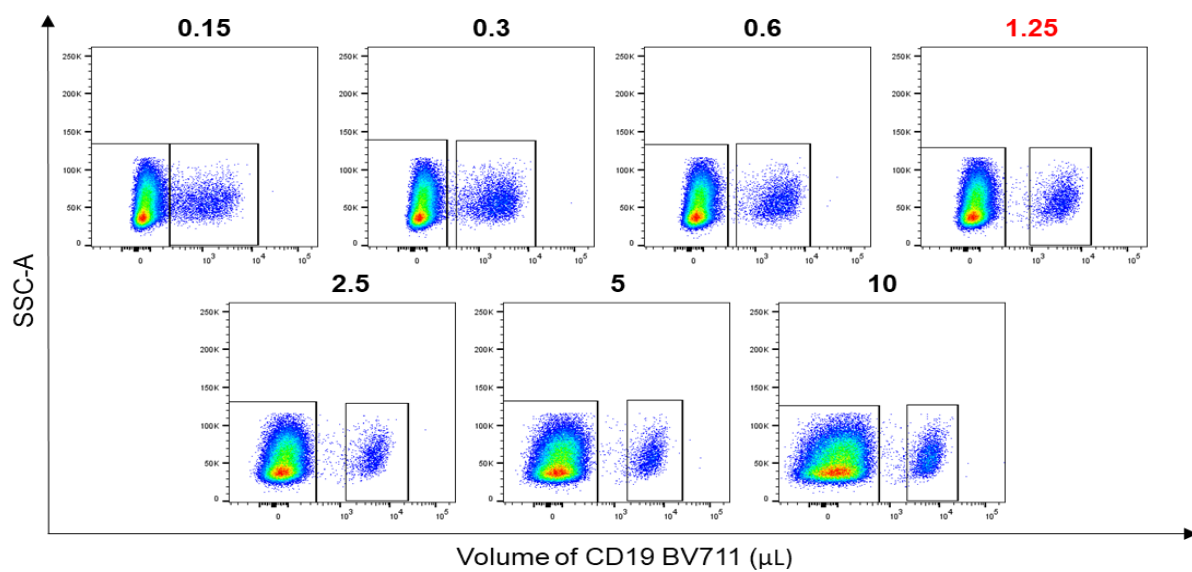
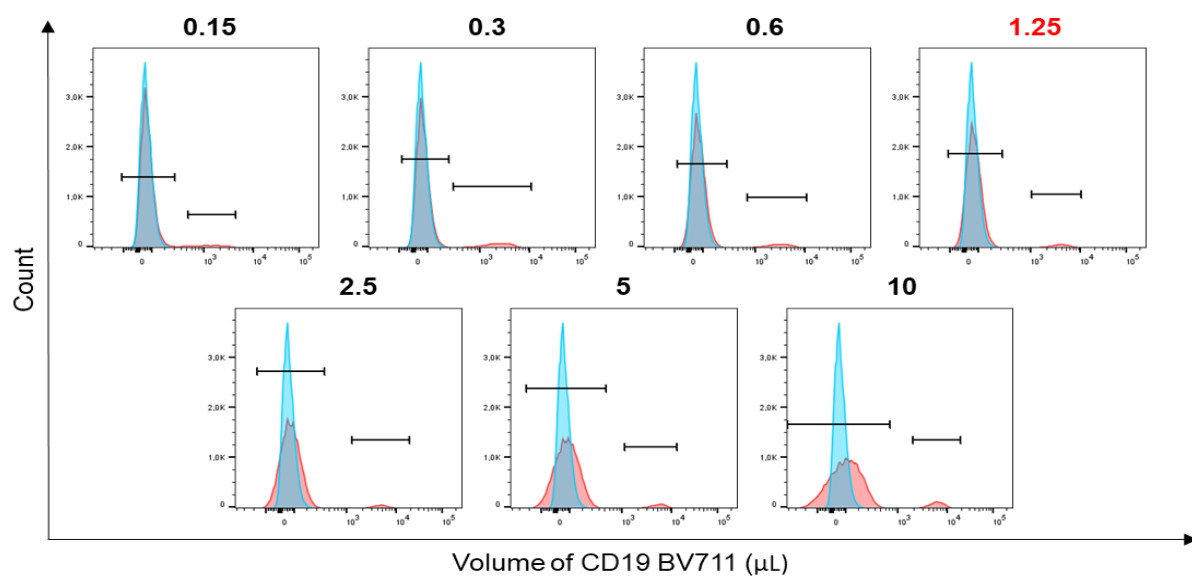
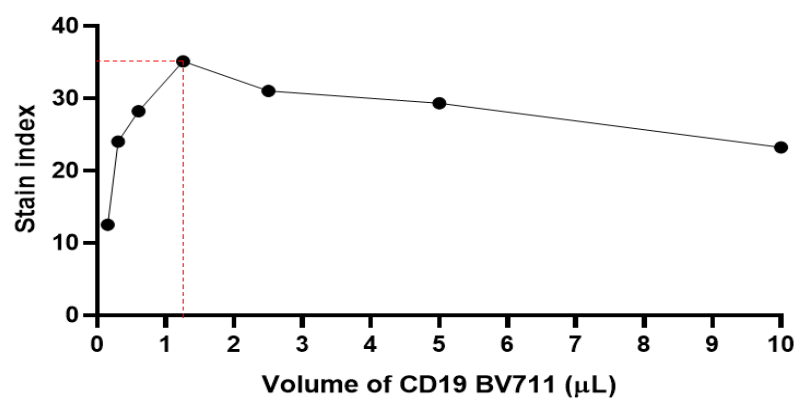
Figure 2.3: (A) Titration of the LIVE/DEAD™ Fixable Aqua Dead Cell Stain. PBMCs were gated on lymphocytes and single cells. The volume of viability dye added is shown above each pseudocolour plot. (B) Histograms showing the LIVE/DEAD™ Fixable Aqua Dead Cell Stain titration with stained (red) and unstained (blue) cells at the indicated volumes of antibody. The volume shown in red was the calculated optimal concentration. (C) The stain index vs volume of antibody curve was used to determine the minimum optimal concentration of stain required. A red dotted line shows the highest stain index which correlated to the optimal volume of 1 μ L of LIVE/DEAD™ Fixable Aqua Dead Cell Stain.

Since the stain index and volume curve suggests that 1 μ L of LIVE/DEAD™ Fixable Aqua Dead Cell Stain is the minimum optimal concentration, referring back to **Figure 2.3 B** confirms that this concentration also presents with minimal shift in populations once stained. For all future experiments, 1 μ L of LIVE/DEAD™ Fixable Aqua Dead Cell Stain was used. The same technique was carried out for all surface and intracellular antibody titrations.

2.3.2 Titration of cell surface antibodies

Cells were stained at room temperature in the dark with different concentrations (0.15, 0.3, 0.6, 1.25, 2.5, 5, 10 μ L) of surface antibody for 20 minutes. Cells were washed with 1 mL dPBS and centrifuged at 2000 rpm for 5 min. The supernatant was discarded, and the pellet was fixed in 100 μ L FIX & PERM™ Medium A from the FIX & PERM™ Cell Permeabilization Kit (Invitrogen, Waltham, USA) (fixation medium) to ensure all morphological characteristics of cells remained intact. Thereafter, cells were suspended in an additional 200 μ L dPBS and stored in foil in a 4 °C fridge until data acquisition. Lymphocytes were gated, followed by gating on singlets. A representative example of a titration of a surface antibody (CD19 brilliant violet (BV) 711) is shown in **Figure 2.4**. Pseudocolour plots, histograms, and the stain index of the antibody at the relative concentrations were used to determine the optimal minimum concentration of the antibody required, as described previously.

Since the stain index was the highest at 1.25 μ L of CD19 BV711 (**Figure 2.4 C**), and the stained and unstained histograms overlay with a minimal spread from the negative population (**Figure 2.4 B**), this concentration was used for all subsequent experiments.

A**B****C**

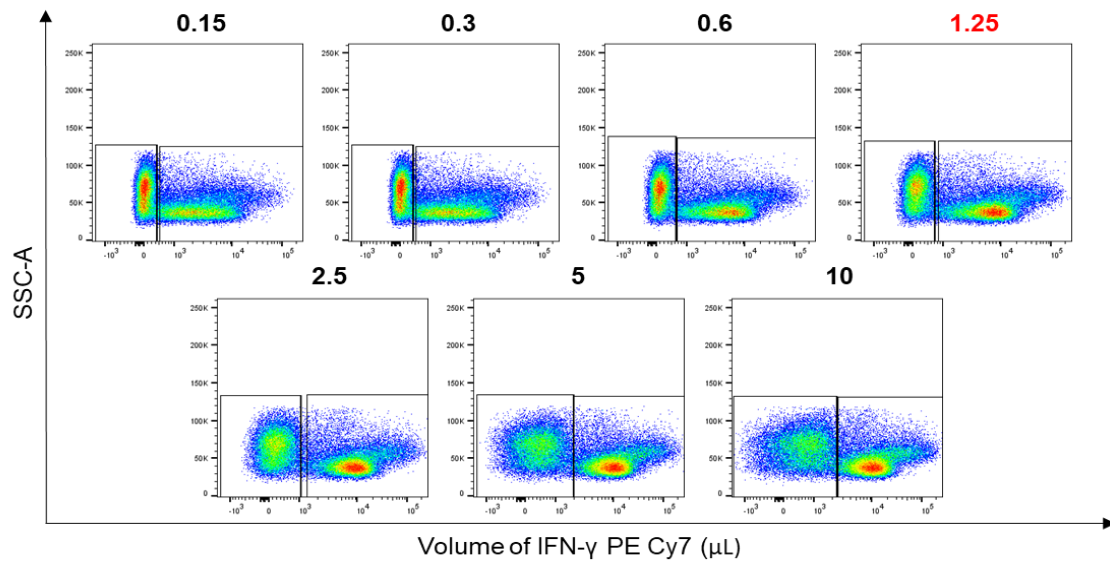
SSC-A, side scatter area; CD, cluster of differentiation; BV, brilliant violet

Figure 2.4: (A) Titration of CD19 BV711. PBMCs were gated on lymphocytes and single cells. The volume of antibody added is shown above each pseudocolour plot. A two-fold dilution series was performed. (B) Histograms showing the CD19 titration with stained (red) and unstained (blue) cells at the indicated volumes of antibody. The volume shown in red was the calculated optimal concentration. (C) The stain index vs volume of antibody curve was used to determine the minimum optimal concentration of antibody required. A red dotted line shows the highest stain index which correlated to the optimal volume of 1.25 μ L of CD19 BV711.

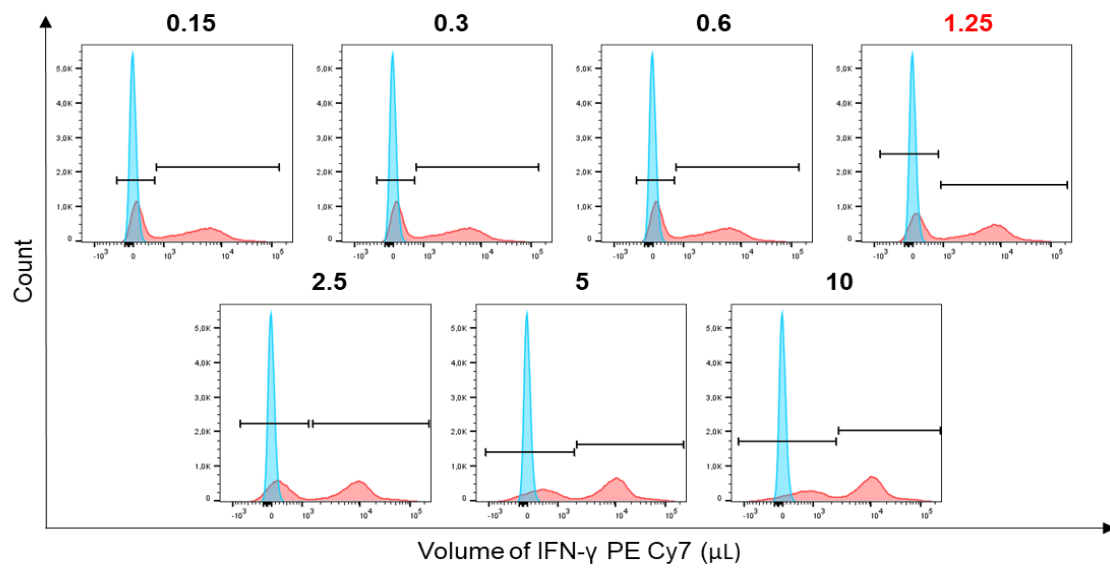
2.3.3 Titration of intracellular antibodies

Markers that determine how well the cell is functioning must be titrated under the same stimulated conditions that will be used for the assay. To match assay conditions, PBMCs were stimulated for 18 hours with a K562 (MHC devoid) cell line at a ratio of 10:1 and IL-2, IL-15, and IL-18 at a concentration of 10 ng/1x10⁶ cells. Following stimulation, cells were washed and fixed in the fixation medium, as mentioned previously. In the presence of FIX & PERM™ (Invitrogen, Waltham, USA) (permeabilising medium) a range of volumes (0.15, 0.3, 0.6, 1.25, 2.5, 5, 10 μ L) of antibody were added to the cells for intracellular staining. The antibody was allowed to stain for 20 min, at room temperature, in the dark. Cells were washed with 1 mL dPBS and centrifuged at 2000 rpm for 5 minutes. After the supernatant was discarded, the pellet was resuspended in 300 μ L of dPBS for acquisition. A representative example of a titration of an intracellular antibody (IFN- γ PE Cy7) is shown in **Figure 2.5.**

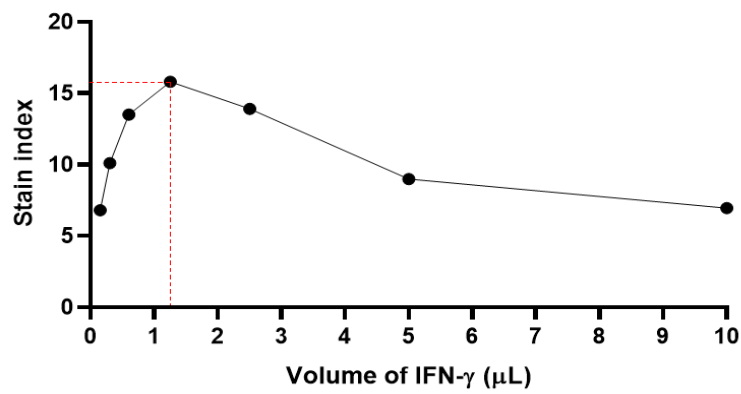
A



B



C



SSC-A, side scatter area; IFN-γ, interferon gamma; PE Cy7, phycoerythrin cyanine 7

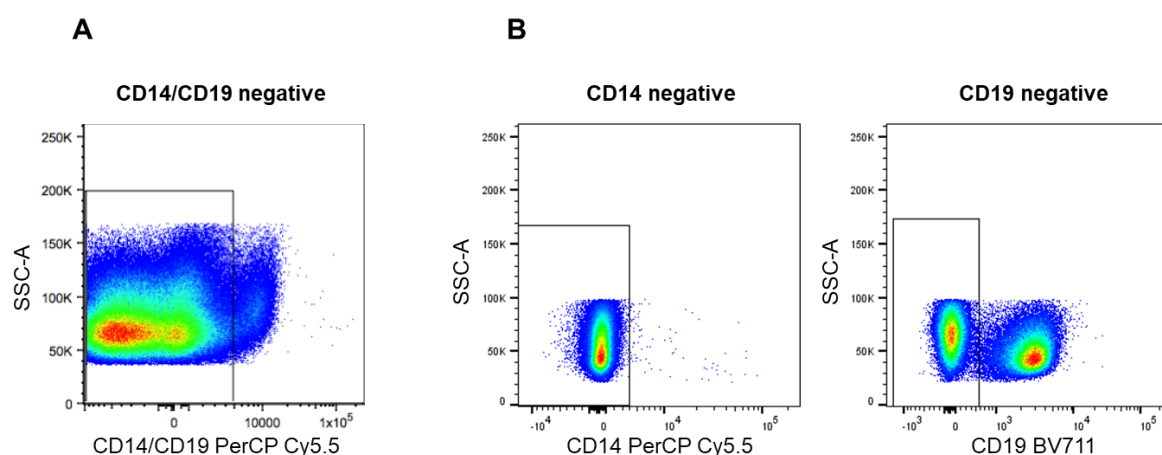
Figure 2.5: (A) Titration of IFN- γ PE Cy7. PBMCs were gated on lymphocytes and single cells. The volume of antibody added is shown above each pseudocolour plot. A two-fold dilution series was performed. (B) Histograms showing the IFN- γ titration with stained (red) and unstained (blue) cells at the indicated volumes of antibody. The volume shown in red was the calculated optimal concentration. (C) The stain index vs volume of antibody curve was used to determine the minimum optimal concentration of antibody required. A red dotted line shows the highest stain index which correlated to the optimal volume of 1.25 μ L of IFN- γ PE Cy7.

Pseudocolour plots (**Figure 2.5 A**), histograms (**Figure 2.5 B**), and the stain index vs volume curve (**Figure 2.5 C**) were used to determine the minimum volume of antibody required for staining of IFN- γ PE Cy7, as described previously. Since the stain index was the highest at 1.25 μ L of IFN- γ PE Cy7, (**Figure 2.5 C**), and the stained and unstained histograms overlay with a minimal spread from the negative population (**Figure 2.5 B**), this concentration was used for all subsequent experiments.

2.4 PANEL OPTIMISATION

2.4.1 Issues in the phenotypic panel

Surface staining of CD14 and CD19 both conjugated to PerCP Cy5.5 (dump channel), showed poor separation of these cells into positive and negative populations (**Figure 2.6 A**). To improve separation, CD14 was left on PerCP Cy5.5, but CD19 was changed from PerCP Cy5.5 to BV711 (**Figure 2.6 B**). As CD14 PerCP Cy5.5 and CD19 BV711 positive and negative cells were easily distinguishable and did not affect the percentage expression of markers during downstream analysis, these changes were implemented.

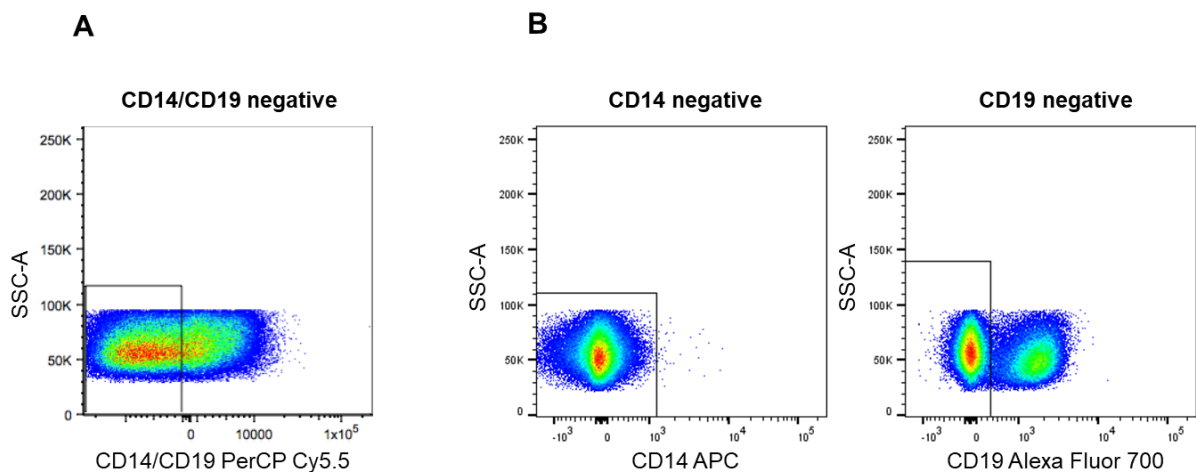


SSC-A, side scatter area; CD, cluster of differentiation; PerCP Cy, peridinin chlorophyll protein complex; Cy, cyanine, BV, brilliant violet

Figure 2.6: Comparison of different fluorochromes for the identification of CD14⁺ and CD19⁺ cells. Pseudocolour plots show PBMCs that have been gated on lymphocytes and single viable cells. (A) CD14/CD19 PerCP Cy5.5 and (B) CD14 PerCP Cy5.5 and CD19 BV711.

2.4.2 Issues in the functional panel

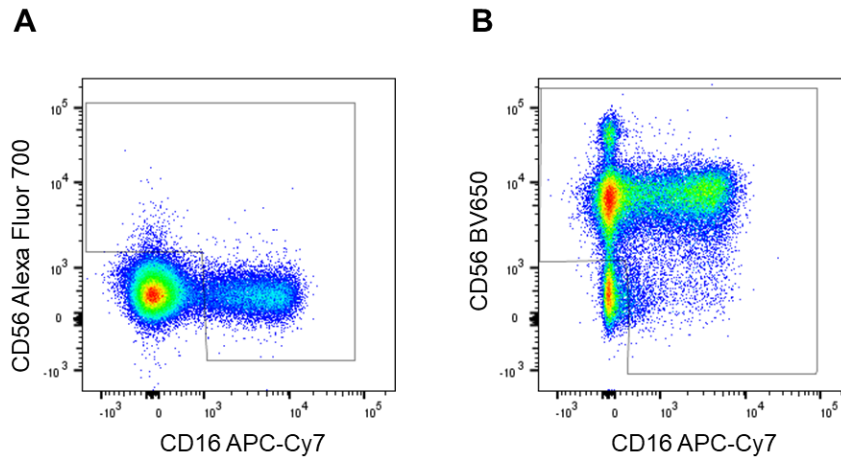
Similar to that observed in the phenotypic panel, poor separation of CD14/19 PerCP Cy5.5 was observed in the functional panel (**Figure 2.7 A**), therefore CD14 was changed to APC, and CD19 was changed to Alexa fluor 700 (**Figure 2.7 B**).



SSC-A, side scatter area; CD, cluster of differentiation; PerCP, peridinin chlorophyll protein complex; Cy, cyanine; APC, allophycocyanin

Figure 2.7: Comparison of different fluorochromes for the identification of CD14⁺ and CD19⁺ cells. Pseudocolour plots show PBMCs that have been gated on lymphocytes, single viable cells. (A) CD14/CD19 PerCP Cy5.5 and (B) CD14 APC and CD19 Alexa Fluor 700.

Additionally, there was a poor separation of NK subsets when CD56 was conjugated to Alexa Fluor 700 (**Figure 2.8 A**). Therefore, CD56 BV650 (a brighter fluorochrome) was substituted for CD56 Alexa Fluor 700. This improved the resolution of the different NK cell subsets (**Figure 2.8 B**). Further analysis of NK cell subsets was imperative to the study and so this newly developed panel was adapted for all experiments.



APC, allophycocyanin; Cy, cyanine; BV, brilliant violet

Figure 2.8: Comparison of different fluorochromes for the identification of CD56⁺ NK cells. Pseudocolour plots showing PBMCs that have been gated on lymphocytes, single viable cells, and CD3⁻, CD14⁻ and CD19⁻ cells. (A) CD56 Alexa Fluor 700 and (B) CD56 BV650.

2.4.3 Panel verification

The panels were tested on at least 5 different HIV-1 uninfected individuals. The experiments were compared to each other to ensure that the expression of each marker of interest was similar to confirm positive staining in their respective channels. Thus, once the two polychromatic flow cytometry panels were developed and optimised, the next step was to apply these to patient samples (part 2).

Part 2: Data collection of clinical samples

2.5 STUDY PARTICIPANTS

Twenty-one HIV-1 uninfected volunteers and 54 PLWH were enrolled in this cross-sectional study. The HIV-1 uninfected volunteers were randomly selected and recruited from the National Institute for Communicable Diseases (NICD), Sandringham, South Africa. The PLWH comprised of 20 ART-treated individuals who were on treatment for at least seven years, 15 HIV-1 elite controllers (viral load <20 RNA copies/mL and CD4⁺ T cell count >450 cell/μL), and 19 HIV-1 progressors who were ART-naïve (viral load >10,000 RNA copies/mL and CD4⁺ T cell count <450 cells/μL). These patients were recruited from Brenthurst clinic, Parktown, South Africa, and were matched for age, gender, and ethnicity. They did not have any other co-morbidities and were not infected with severe acute respiratory syndrome coronavirus 2 or *Mycobacterium tuberculosis*. No female patients were pregnant.

2.6 ETHICAL APPROVAL

All blood donors gave their written informed consent to voluntarily participate in this study and for the use of their blood samples for further scientific studies according to the guidelines set by the Human Ethics Committee of the University of the Witwatersrand. The main study received ethical approval (reference number: M140925; principal investigator: C.T. Tiemessen and so did the sub-study reference number: M210231, principal investigator C. Thobakgale, and C.T. Tiemessen) (Appendix A and B). Where specimens were limited, participants were called back, and up to 60 mL of blood was collected (as approved in the original ethics protocol), and specimens were stored for later use.

2.7 SAMPLE COLLECTION

Blood samples were collected in six ethylenediaminetetraacetic acid tubes from study participants. Samples were transported at room temperature to the Cell Biology Laboratory at the NICD where sample processing took place within 3 to 6 hours.

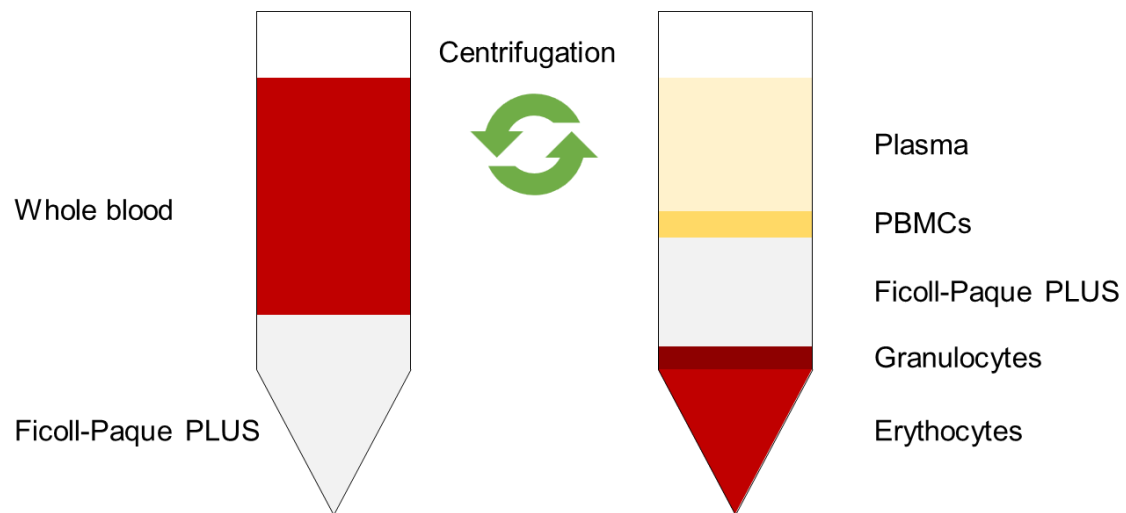
2.8 VIRAL LOAD AND CD4⁺ T CELL COUNTS

Approximately 1 mL of blood was sent to the HIV Sero-Molecular Laboratory at the NICD to quantify HIV plasma viral RNA copies/mL and CD4⁺ T cells/ μ L. Plasma HIV-1 RNA viral loads were measured by using the COBAS® AmpliPrep/COBAS® Taqman® HIV-1 test, v2.0 ultrasensitive tests (Roche Diagnostic Systems, Basel, Switzerland) according to the manufacturer's instructions, and the fluorescence-activated cell sorting (FACS) count system (BD Biosciences, San Jose, USA) was used to determine CD4⁺ T cell counts.

2.9 PREPARATION OF CELLS

2.9.1 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated from approximately 60 mL of fresh blood by density-gradient centrifugation using Ficoll-Paque PLUS (Cytiva, Uppsala, Sweden). In 50 mL falcon tubes (Corning, New York, USA), blood was carefully layered on Ficoll-Paque PLUS, at room temperature, at a ratio of 1:2, respectively. Tubes were centrifuged at 2000 rpm for 30 minutes at room temperature with the brake off. The PBMC layer, at the interface between the plasma and the Ficoll (**Figure 2.9**), was collected and transferred to a 50 mL falcon tube to which 20 mL of dPBS had been dispensed. The tube was topped up with dPBS to a final volume of 45 mL. Cells were washed and centrifuged at 2000 rpm for 10 minutes at 4 °C. The supernatant was carefully poured off and the pellet was resuspended. To lyse contaminated erythrocytes, 5 mL of 4 °C red cell lysis buffer (Roche Diagnostic Systems, Basel, Switzerland) was added and continuously swirled for a duration of five to eight minutes. The greater the erythrocyte contamination, the longer the cells were exposed to the red cell lysis buffer. The falcon tube was topped up with dPBS and the cells were washed and centrifuged at 1800 rpm for 10 minutes at 4 °C. The supernatant was discarded carefully, and the pellet was resuspended in 10 mL of dPBS in preparation for cell counts.



PBMCs, peripheral blood mononuclear cells

Figure 2.9: Schematic showing isolation of PBMCs. Peripheral whole blood was layered onto Ficoll-Paque PLUS (left) and separated into plasma, PBMCs, granulocytes, and erythrocytes following centrifugation (right). Image adapted from Puleo et al (Puleo et al., 2017).

2.9.2 Cell count and viability

The number of viable cells, present in a measured volume, were counted using a light microscope and a haemocytometer in combination with the viability stain, trypan blue.

2.9.2.1 The haemocytometer

The haemocytometer is used to accurately count cells under a microscope. It is a thick glass slide with two standard-size rectangular chambers (**Figure 2.10**). When a coverslip is placed on top, cells in suspension can be loaded via capillary action such that they are one cell layer thick. Haemocytometers are manufactured with grid lines of exact dimensions such that the width and depth of the chambers are known. This allows for an accurate cell count in a given volume of fluid. Thereafter, the concentration of cells in suspension can be calculated.

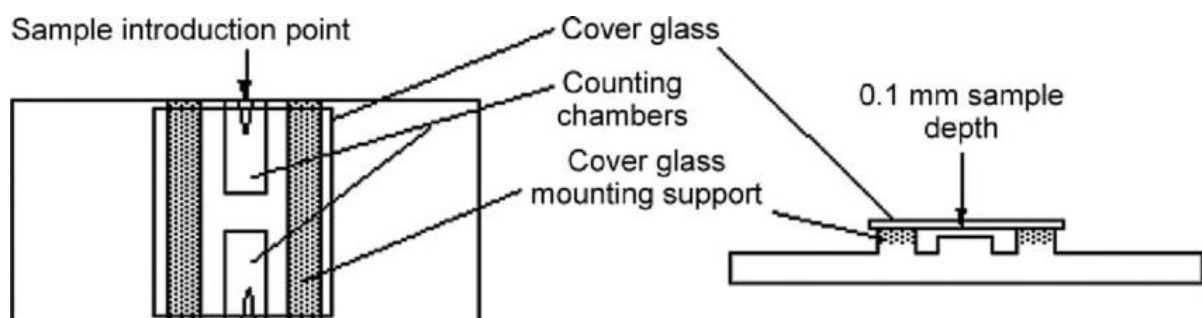


Figure 2.10: A labelled haemocytometer. Image reprinted from Coyle and McLaughlin (Coyle and McLaughlin, 2016).

2.9.2.2 Determining viability using trypan blue

Trypan blue is a stain that is used to discriminate live cells from dead cells. An intact cell membrane excludes dyes such as these, whereas dead cells have permeable membranes and dyes that can penetrate the cell (Strober, 2015). Thus, under a microscope, dead cells will appear blue and viable cells clear (Crowley et al., 2016).

A 20 μ L of cell suspension was added to an equal volume of 0.4% trypan blue (Abcam, Cambridge, United Kingdom) and mixed thoroughly. The haemocytometer coverslip was centred over the haemocytometer chamber to which the cell suspension was dispensed and left to rest for 1 minute to allow cells to settle. The haemocytometer was placed under a light microscope and cells were counted in the centre 1 mm² square with the help of the subdivisions. Cells were counted in a snake-like fashion; from left to right, top to bottom, to avoid counting the same cell multiple times. The number of cells in 1 mL can be calculated by counting the number of cells in a 1 mm² square of a haemocytometer multiplied by the dilution factor and the constant 10⁴ (Equation 2.2).

$$\frac{\text{Cells}}{\text{mL}} = \text{Number of cells counted} \times \text{dilution factor} \times 10^4$$

Equation 2.2: Calculation of the number of cells in 1 mL

The total cell count was calculated by multiplying the number of cells in 1 mL by the total cell volume. The percentage of viable cells was calculated by dividing the number of live cells by the total number of cells in a 1 mm² square of the haemocytometer and multiplied by 100.

2.9.3 Cryopreservation of cells

The cell suspension, in a 50 mL falcon tube, was topped up to 45 mL with dPBS and centrifuged at 2000 rpm for 10 minutes at 4 °C. The supernatant was gently poured off and cells were resuspended at a concentration of 5 x 10⁶ cells/mL in a 1mL solution of 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA) and 90% foetal bovine serum (FBS) (Hyclone Laboratories Inc, Logan, USA) into a 1.5 mL Nunc® CryoTubes® (Sigma-Aldrich, St. Louis, USA) and immediately transferred to a

Nalgene® Mr. Frosty container and placed in a -80 °C freezer overnight. The Nalgene® Mr. Frosty containers were filled with isopropyl alcohol (Sigma-Aldrich, St. Louis, USA) which provides the essential 1 °C/min cooling rate mandatory for cell cryopreservation. This ensured that no ice crystals formed within the PBMCs, and reduced the chance of cell membrane rupture, and cell death. The next day, the Nunc tubes were transferred to liquid nitrogen at -196 °C for long-term storage.

2.9.4 Thawing of peripheral blood mononuclear cells

Frozen vials of samples were removed from liquid nitrogen and thawed in a 37 °C water bath using aseptic techniques. The samples were reconstituted with 1 mL R-10 media (Roswell Park Memorial Institute 1640, GlutaMax, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% heat-inactivated FBS (Hyclone Laboratories Inc, Utah, USA) and benzonase endonuclease (500 U/mL) (Sigma-Aldrich, St. Louis, USA). Samples were then transferred, in a dropwise manner to avoid bubbles, into a 15 mL falcon tube (Corning, New York, USA) to which 9 mL of R-10 media had already been dispensed. Tubes were gently inverted to wash the cryopreservation media off the cells. Cells were washed twice by centrifuging at 1800 rpm for 8 minutes at room temperature. PBMCs were resuspended in 1 mL of R-10 media and counted using a Neubauer haemocytometer and viability was assessed using trypan blue. The concentration of the cells was adjusted to 2×10^6 cells/mL with warmed R-10 media and left to rest for a minimum of 2 hours at 37 °C in a humidified 5% carbon dioxide (CO₂) incubator before using in experiments.

2.10 FLOW CYTOMETRY

Flow cytometry is a method used to study single cells as they flow past one or several lasers while suspended in a solution (McKinnon, 2018). A flow cytometer can analyse a cell for light scatter and one or more fluorescence parameters. Light scatter is measured in two directions, forward scatter (FSC) and side scatter (SSC) (Maciorowski et al., 2017). FSC correlates to the size of the cell while SSC, measured at a 90° angle, is related to the granularity and complexity of that cell (Buscher, 2019).

Flow cytometers use three systems: fluidics, optics, and electronics. The fluidics system pressurises sheath fluid (a buffered salt solution). The pressurised fluidics

system enables the sample to be delivered to the laser intercept where the sample can be analysed (McKinnon, 2018). The optics consist of lasers (excitation optics), filters, photomultiplier tubes, and photodiodes (collection optics). The optics system produces the light scatter as well as the fluorescent light signals which are used to analyse the sample. Lastly, the electronic system transforms all the light signals received into digital data that can be analysed using the relevant software (McKinnon, 2018).

2.10.1 Controls

2.10.1.1 Instrument controls

2.10.1.1.1 Cytometer setup and tracking beads

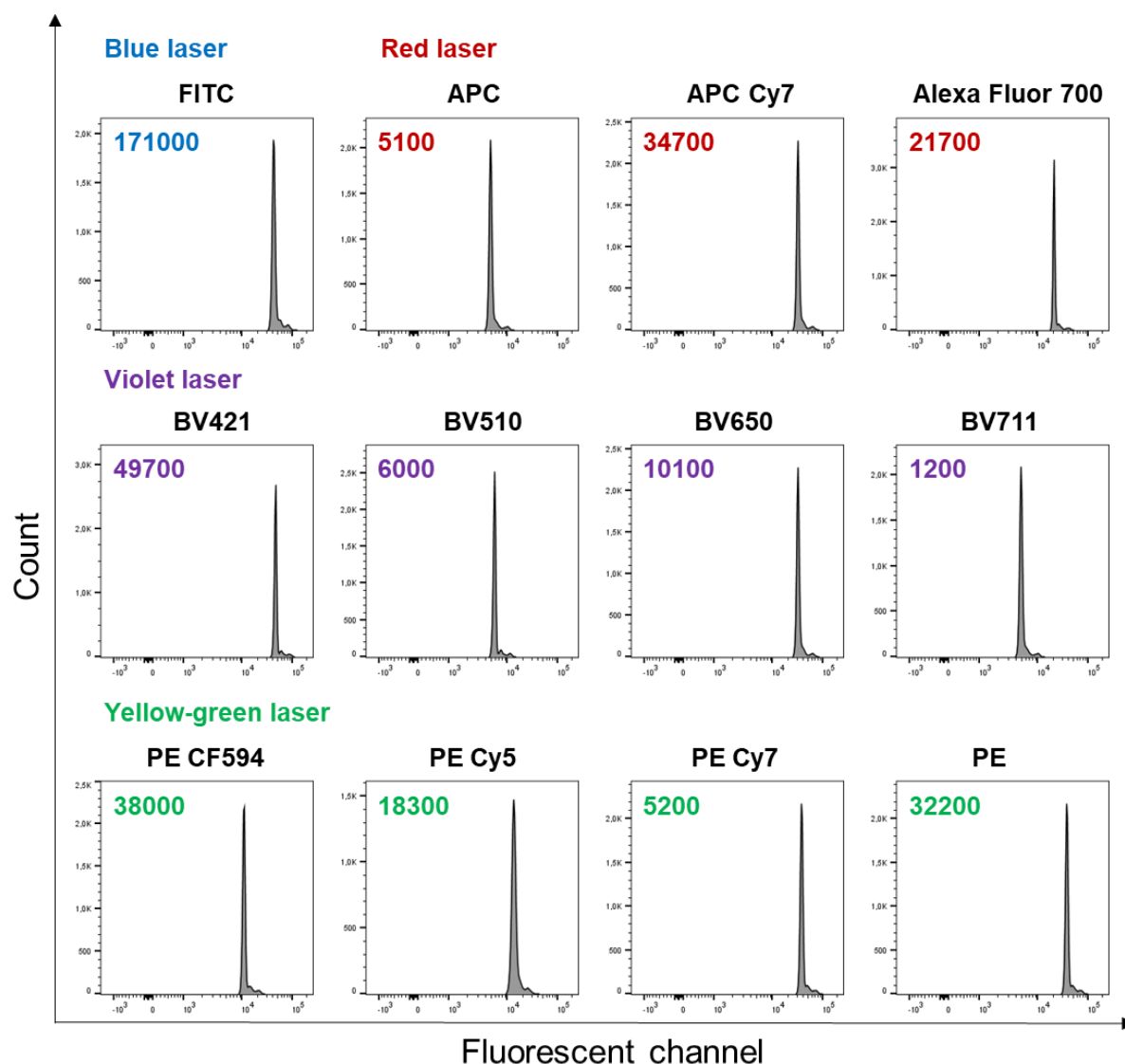
Cytometer setup and tracking (CS&T) beads are made of polystyrene and consist of equal quantities of bright, mid, and dim-stained fluorochromes. Because of this, they fluoresce in all channels and serve as quality control and allow performance tracking over time. CS&T beads were run before every experiment.

2.10.1.1.2 Rainbow beads

The fluorescence intensity of a flow cytometer varies due to changes in laser alignment, laser power, optical efficiency, and temperature as well as after maintenance and alignment. Thus, rainbow beads are important to ensure standardisation and reproducibility. Furthermore, the use of rainbow beads is necessary for assays where the median fluorescence intensity (MFI) is used as a measure for the marker of interest. MFI is a more appropriate measure for markers that are constitutively expressed on all cells and do not separate into negative and positive populations. Thus, the relative shift in MFI is an alternative way to measure expression. This method of standardisation reduces user set-up variation and allows for the comparison of data acquired over different days.

Mid-range rainbow beads are stained with fluorescent dyes and so will produce a single peak in each instrument channel (**Figure 2.11**). These beads were acquired under optimised instrument settings generating a set of baseline target values. For all future experiments, the voltages will be adjusted until the mean fluorescence intensity of the rainbow beads is within 10% of these baseline target values (**Figure 2.11**).

Rainbow beads were prepared by adding 1 drop of Sphero™ Rainbow Fluorescent Particles (BD Biosciences, San Jose, USA) to 350 µL of BD FACSFlow™.



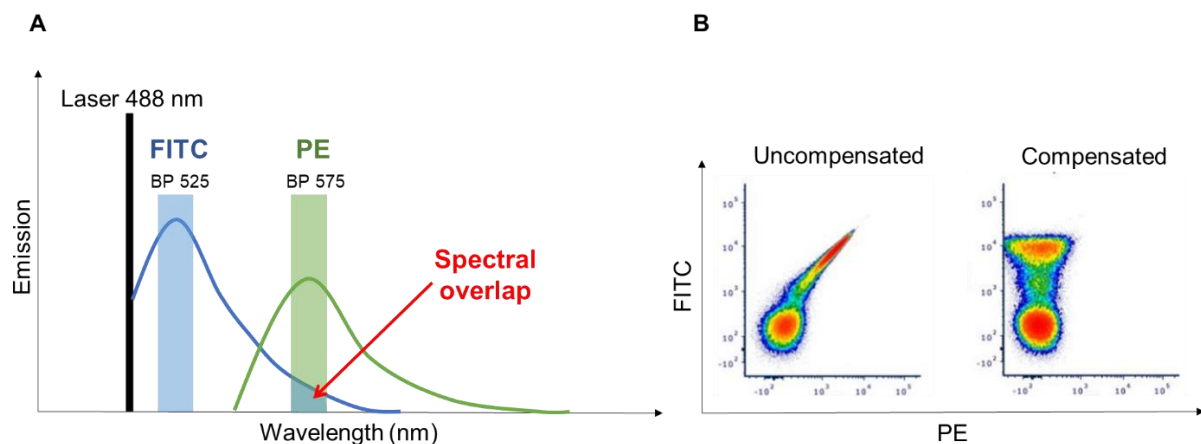
FITC, fluorescein isothiocyanate; APC, allophycocyanin; Cy, cyanine; BV, brilliant violet; PE, phycoerythrin

Figure 2.11: Histograms displaying signal detection from rainbow beads in all channels used in the panel. The baseline target values for their respective channels are shown.

2.10.1.2 Compensation controls

Compensation refers to the process in which spectral overlap, or spillover, is mathematically corrected. In conventional flow cytometry, one detector, known as the primary detector, is assigned to one fluorochrome. However, photons end up in detectors other than the primary detector because the emission spectra from

fluorochromes overlap and this is known as spillover (**Figure 2.12 A**). Compensation means removing the signal of a fluorochrome from all the detectors except for the primary detector. Compensation is specific for a fluorochrome and not a cell type. This means that beads can be used for compensation. Compensation controls must be run for every experiment. The difference between an uncompensated sample and a compensated sample is shown below (**Figure 2.12 B**).



FITC, fluorescein isothiocyanate; PE, phycoerythrin

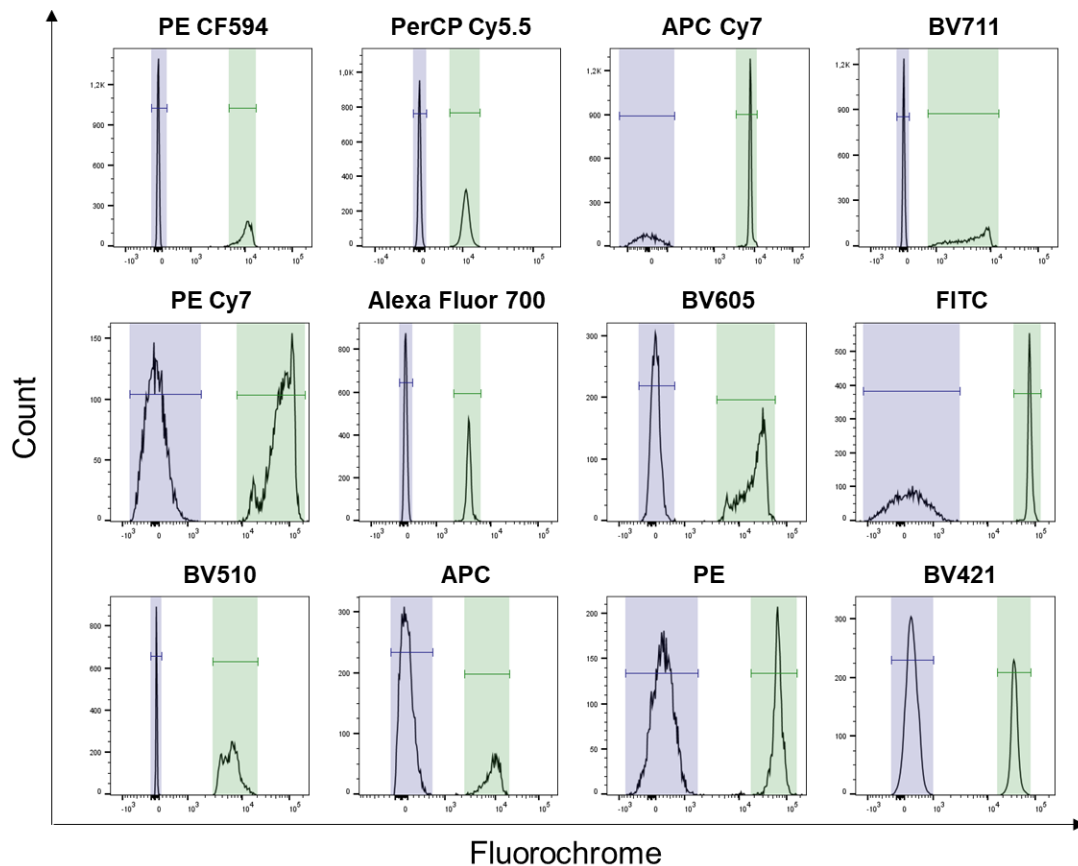
Figure 2.12: (A) Histogram of emission intensity versus emission wavelength. FITC emission spectra overlap with PE. (B) Uncompensated data versus compensated data. Image adapted from NanoCelect Biomedical (NanoCelectBiomedical, 2021).

The viability stain was compensated using the ArC™ Amine Reactive Compensation Bead Kit (Invitrogen, Waltham, USA). To 100 µL of BD FACSFlow™, one drop of ArC reactive beads was added and allowed to reach room temperature. The LIVE/DEAD™ Fixable Aqua Dead Cell Stain was prepared as previously mentioned and 1 µL was used to stain the beads. The beads were incubated in the dark for 30 minutes at room temperature and washed with dPBS. The beads were fixed with fixation medium and resuspended in 200 µL dPBS. One drop of negative beads from the ArC™ Amine Reactive Compensation Bead Kit was added to the tube and mixed thoroughly.

The remaining compensation controls were prepared by adding 100 µL of BD FACSFlow™ together with one drop of BD™ CompBeads Negative Control (BD Biosciences, San Jose, USA) and 1 drop of the BD™ CompBeads Anti-Mouse Igk beads (BD Biosciences, San Jose, USA) to each FACS tube and vortexed. The titrated antibody was added and incubated at room temperature in the dark for 20 minutes. The unbound antibody was washed off with 1 mL dPBS and centrifuged at 2000 rpm

for 5 minutes. The pellet was fixed in 100 μ L of fixation medium and then resuspended in 200 μ L dPBS. All FACS tubes were stored in foil at 4 °C until data acquisition.

Compensation controls (**Figure 2.13**) were included for all fluorochromes in the phenotypic and functional panels.



PE, phycoerythrin; PerCP, peridinin chlorophyll protein complex; APC, allophycocyanin; Cy, cyanine; BV, brilliant violet; FITC, fluorescein isothiocyanate

Figure 2.13: Histograms of unstained (purple) and stained (green) compensation beads for all fluorochromes in the phenotypic panel.

2.10.1.3 Experimental controls

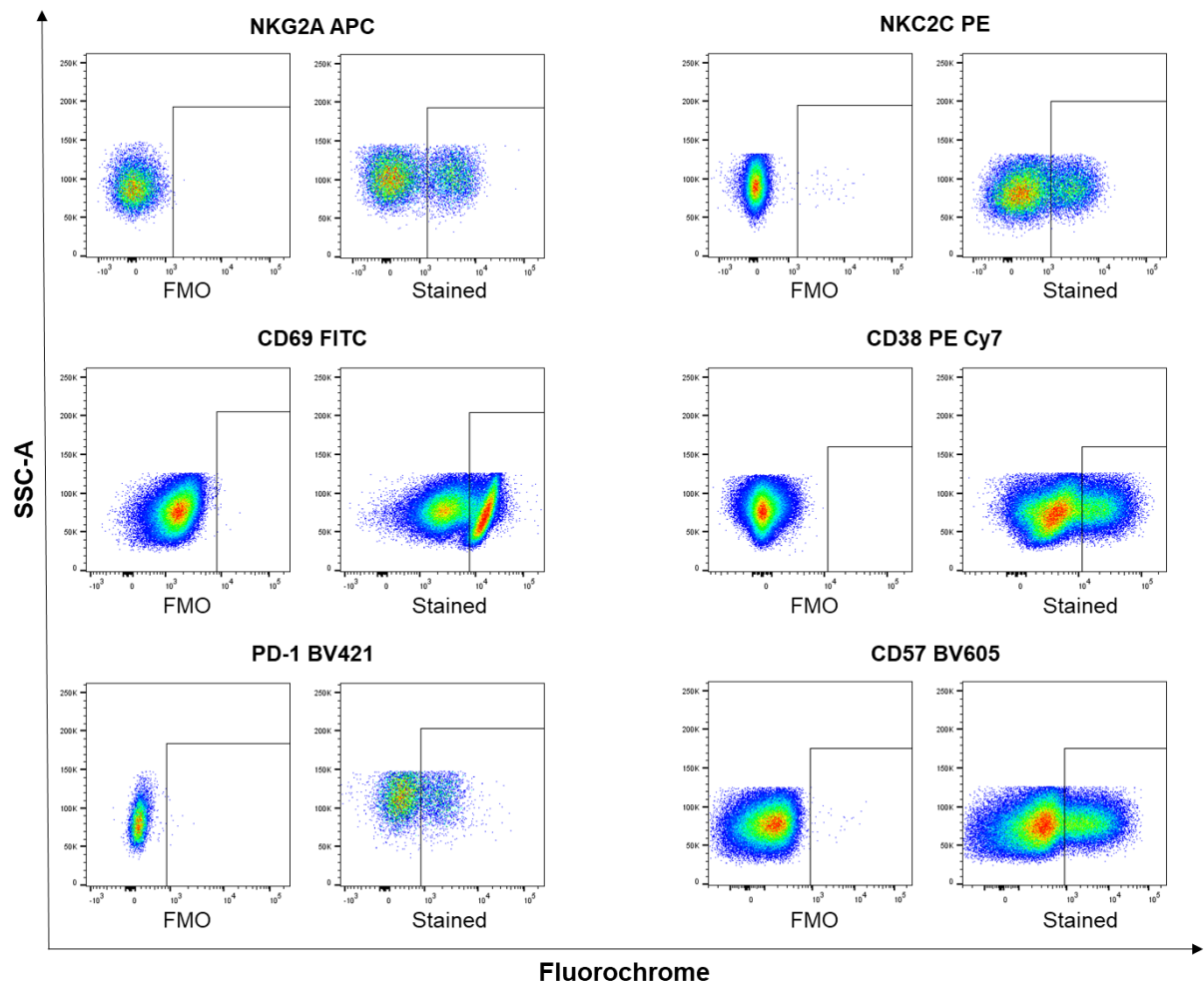
2.10.1.3.1 *Fluorescence minus one controls*

Fluorescence minus one (FMO) controls are cells stained with all the fluorophores in the panel except for one fluorophore. They were used to set the lowest limit of detection from the background fluorescence to allow for accurate gating of the positive population in a multicolour panel (Lee et al., 2019). FMO controls become extremely important when a positive population is not distinctly separate from a negative population due to low or variable expression levels. They are more accurate gating

controls than isotype controls or unstained samples as they take into consideration the spread of the negative population, due to the spillover of fluorochromes, in multicolour panels (Tung et al., 2007). FMO controls are also affected by cellular autofluorescence as well as secondary marker expression and so the same type of cell must be used for the FMO as for the experiment. Beads cannot be substituted for cells (Lee et al., 2019).

When optimising a new panel, each marker should have its own FMO. Once the panel is fully optimised, some of the FMO controls can be omitted. The remaining FMO controls are those for difficult-to-gate populations. These should be run with each batch of fully stained samples for accurate gating.

For each experiment, the FMO controls (CD38, CD57, CD69, NKG2A, NKG2C, and PD-1) were prepared for the phenotypic panel. **Figure 2.14** shows how FMO controls help with gating. The FMO defines the lower limit of the gate. However, the gate is often set higher.



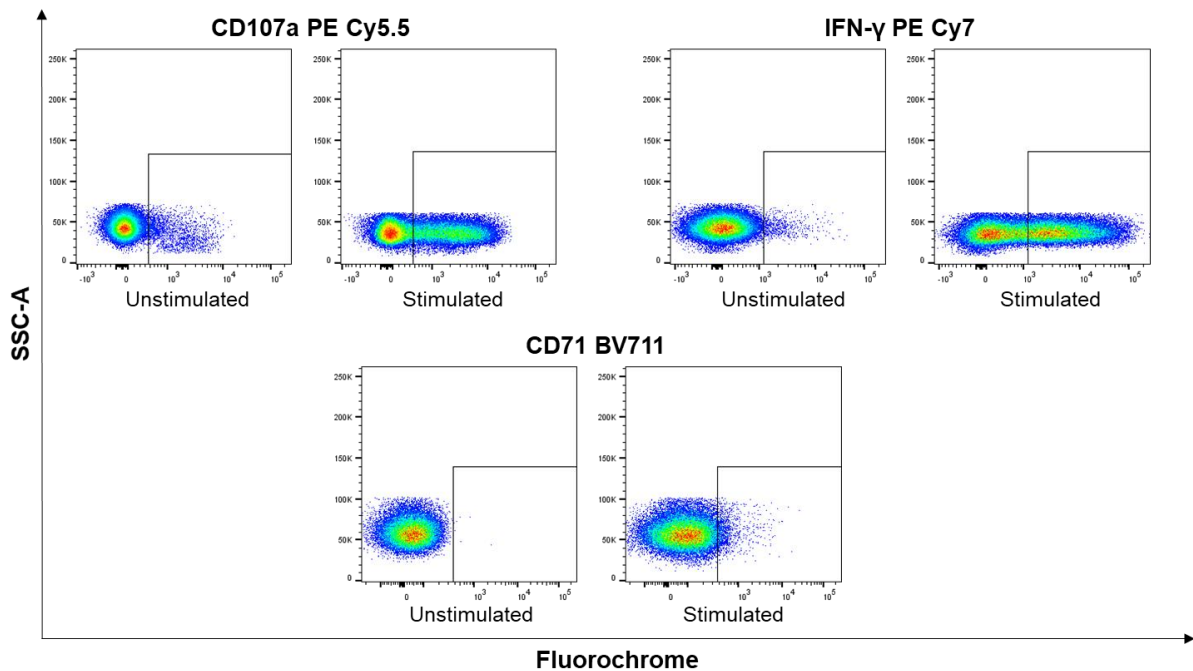
SSC-A, side scatter area; NKG, natural killer group; APC, allophycocyanin; PE, phycoerythrin; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; Cy, cyanine; PD-1, programmed cell death protein 1; BV, brilliant violet

Figure 2.14: FMO controls from the phenotypic panel. CD3⁺, CD14⁺, and CD19⁺ lymphocytes were gated. Thereafter, the total NK cell population was selected based on the expression of CD56 and CD16.

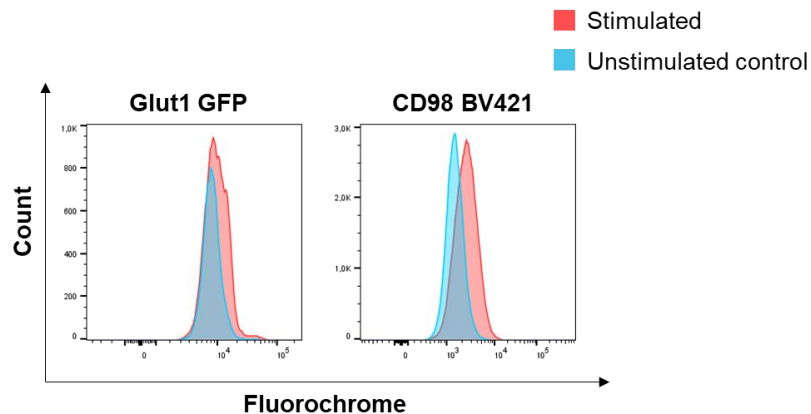
2.10.1.3.2 Unstimulated controls

Unstimulated controls are used as negative controls in functional assays. Unstimulated controls identify background binding of the target antibody since the unstimulated cells should not express the target, thus FMO controls would underestimate the background expression signals and aid in gating (Maecker and Trotter, 2006). Unstimulated controls (CD107a, IFN- γ , Glut1, CD98, and CD71) were prepared for each functional assay. Pseudocolour plots are shown when markers were measured by percentage expression (CD107a, IFN- γ , and CD71) (**Figure 2.15 A**) and histograms were used for markers that were measured using MFI (Glut1 and CD98) (**Figure 2.15 B**).

A



B



SSC-A, side scatter area; CD, cluster of differentiation; PE, phycoerythrin; Cy, cyanine; BV, brilliant violet; Glut1; glucose transporter 1; GFP, green fluorescent protein

Figure 2.15: Unstimulated controls from markers that were measured by (A) percentage expression (B) and MFI from the functional panel. CD3⁺, CD14⁺, and CD19⁺ lymphocytes were gated. Thereafter, the total NK cell population was selected based on the expression of CD56 and CD16.

2.10.2 Assessment of phenotypic properties of natural killer cells

One million cells were dispensed into a FACS tube for each sample and centrifuged at 2000 rpm for 5 minutes at room temperature to remove excess media. Cells were washed with 1 mL dPBS and spun again.

2.10.2.1 Viability staining

LIVE/DEAD™ Fixable Aqua Dead Cell Stain (Invitrogen, Waltham, USA) was reconstituted in 50 µL DMSO as per the manufacturer's instructions. The stain was diluted with dPBS (1:1000) on the day of the experiment. A volume of 100 µL of the diluted viability stain was dispensed into the FACS tube containing the cells and incubated for 20 minutes at room temperature in the dark. Cells were washed with 1 mL dPBS and centrifuged at 2000 rpm for 5 min at room temperature.

2.10.2.2 Surface staining

Cells were stained with a range of antibodies mentioned in **Table 2.4** for 20 minutes, at room temperature, in the dark, in 50 µL of BD Horizon™ Brilliant Stain Buffer (BD Biosciences, San Jose, USA). The buffer is necessary when 2 or more BV-conjugated antibodies are present in a panel. The buffer prevents spill over into other BV channels and improves the separation of populations. Following washing with dPBS and centrifugation at 2000 rpm for 5 minutes, the supernatant was discarded, and the pellet was fixed in the presence of the fixation medium from the FIX & PERM™ Cell Permeabilization Kit (Invitrogen, Waltham, USA) to ensure all morphological characteristics of cells remained intact. Thereafter, cells were resuspended in 200 µL dPBS and stored covered in foil in a 4 °C fridge until sample acquisition.

Table 2.4: The antibody panel used to assess NK cell phenotypic characteristics

<i>Marker</i>	<i>Fluorochrome</i>	<i>Company</i>	<i>Clone</i>	<i>Volume used (μL)</i>	<i>Purpose</i>
<i>Viability</i>	Aqua	Invitrogen, Massachusetts, USA	-	100	Viability
<i>CD3</i>	PE CF594	BD Biosciences San Jose, USA	UCHT1	0.5	T cell exclusion
<i>CD14</i>	PerCP Cy5.5	BD Biosciences San Jose, USA	M5E2	2.5	Monocyte/macrophage exclusion
<i>CD19</i>	BV711	BioLegend San Diego, USA	H1B19	1.25	B cell exclusion
<i>CD56</i>	Alexa Fluor 700	BD Biosciences San Jose, USA	B159	1	NK cell marker
<i>CD16</i>	APC Cy7	BD Biosciences San Jose, USA	3G8	1.5	NK cell marker
<i>CD38</i>	PE Cy7	BioLegend San Diego, USA	HIT2	1.25	Late activation marker
<i>CD57</i>	BV605	BD Biosciences San Jose, USA	QA17A04	0.6	Differentiation marker
<i>CD69</i>	FITC	BD Biosciences San Jose, USA	FN50	5	Early activation marker
<i>NKG2A</i>	APC	Beckman Coulter Brea, USA	Z199	5	Inhibitory marker
<i>NKG2C</i>	PE	R&D Systems Minneapolis, USA	134591	10	Activation marker
<i>PD-1</i>	BV421	BD Biosciences San Jose, USA	EH12.1	2	Exhaustion marker

CD, cluster of differentiation; NKG, natural killer group; PD-1, programmed cell death protein 1; PE, phycoerythrin; PerCP, peridinin chlorophyll protein complex; BV, brilliant violet; Cy, cyanine; APC, allophycocyanin; FITC, fluorescein isothiocyanate

2.10.3 Assessment of functional properties of natural killer cells

Levels of NK cell cytolytic functional activity were determined through the expression of the degranulation marker, CD107a, and cytokine expression of IFN- γ after stimulation. For each sample, 1×10^6 cells/mL were stained with CD107a (BD Biosciences, San Jose, USA) together with BD GolgiStop™ protein transport inhibitor (0.67 μ L/mL media) (BD Biosciences, San Jose, USA) and Brefeldin A (5 μ g/mL) (Sigma-Aldrich, St. Louis, USA).

2.10.3.1 Natural killer cell stimulation

PBMCs were stimulated with the K562 MHC-devoid cell line (effector-to-target ratio of 10:1) and with 10 μ g/mL recombinant human IL-2, IL-15, and IL-18 (R&D Systems, Minneapolis, USA), for 18 hours at 37 °C in a humidified incubator with 5% CO₂. Unstimulated negative control tubes with R10 media were included for each patient sample.

2.10.3.2 Viability stain

Following overnight culture, samples were centrifuged at 2000 rpm for 5 minutes at room temperature and washed with 1 mL dPBS. The supernatant was carefully removed, and cells were stained with LIVE/DEAD™ Fixable Aqua Dead Cell Stain to eliminate dead cells as previously mentioned.

2.10.3.3 Surface staining

PBMCs were stained with CD3, CD14, CD19, CD56, CD16, CD71, CD98, and Glut1 (**Table 2.5**) for 20 minutes, at room temperature in the dark, in 50 μ L of BD Horizon™ Brilliant Stain Buffer; followed by washing with 1 mL dPBS to remove unbound antibody. Cells were centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded, and the pellet was fixed in 100 μ L of fixation medium and incubated in the dark for 20 minutes, at room temperature.

2.10.3.4 Intracellular staining

Following washing with 1 mL dPBS, cells were intracellularly stained with IFN- γ PE Cy7 (BD Biosciences, San Jose, USA) in the presence of 100 μ L of permeabilising medium and incubated at room temperature for 20 minutes followed by washing steps with dPBS. Cells were resuspended in 300 μ L dPBS and stored in foil at 4 °C until data acquisition.

Table 2.5: Antibody panel used to assess NK cell function

Marker	Fluorochrome	Company	Clone	Volume used (μL)	Purpose
<i>Viability</i>	Aqua	Invitrogen, Massachusetts, USA	-	100	Viability
<i>CD3</i>	PE CF594	BD Biosciences San Jose, USA	UCHT1	0.5	T cell exclusion
<i>CD14</i>	APC	BD Biosciences San Jose, USA	M5E2	10	Monocyte/macrophage exclusion
<i>CD19</i>	Alexa Fluor 700	BioLegend San Diego, USA	HIB19	1.25	B cell exclusion
<i>CD56</i>	BV650	BD Biosciences San Jose, USA	NCAM16.2	1	NK cell marker
<i>CD16</i>	APC Cy7	BD Biosciences San Jose, USA	3G8	1.5	NK cell marker
<i>CD107a</i>	PE Cy5	BD Biosciences San Jose, USA	H4A3	1.25	Degranulation marker
<i>IFN-γ</i>	PE Cy7	BD Biosciences San Jose, USA	B27	1.25	Cytokine production
<i>CD71</i>	BV711	BD Biosciences San Jose, USA	M-A712	5	Iron transportation
<i>CD98</i>	BV421	BD Biosciences San Jose, USA	UM7F8	10	Amino acid transportation
<i>Glut1</i>	RBD-GFP	Metafora Biosystems Paris, France	-	10	Glucose transportation

CD, cluster of differentiation; IFN-γ, interferon-gamma; Glut1, glucose transporter 1; PE, phycoerythrin; APC, allophycocyanin; BV, brilliant violet; Cy, cyanine; RBD, receptor binding domain; GFP, green fluorescent protein; NK, natural killer

2.10.4 Sample acquisition

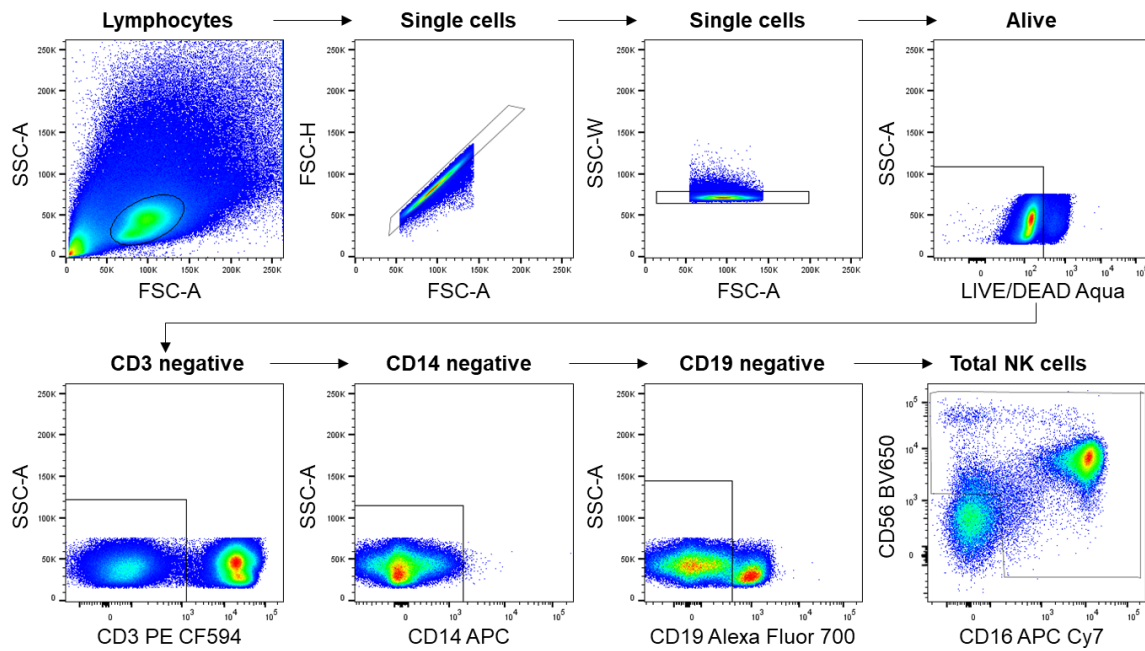
All samples were acquired on the BD LSRFortessa™ X-20 Cell Analyser (BD Biosciences, San Jose, USA). The instrument was switched on at least 30 minutes before use to allow the lasers to warm up. Once CS&T beads were run and rainbow beads were acquired, the compensation controls followed. Once compensation was calculated, each sample was acquired. Approximately 400 000 events were acquired in the lymphocyte gate per sample.

2.10.5 Analysis

Flow cytometry standard files were imported to BD FlowJo™ version 10.7 (BD Biosciences, San Jose, USA) from BD FACSDiva™ version 8.0.2.

2.10.6 Gating strategy

Lymphocytes were gated based on their low FSC and SSC, next doublets were excluded by excluding any cells with a higher FSC area and FSC width by gating FSC area vs FSC height and FSC area vs SSC width. The viability dye was used to exclude dead cells and T cells, monocytes/macrophages, and B cells were excluded by gating CD3⁻, CD14⁻, and CD19⁻ cells, respectively. Finally, the total NK (CD56^{bright}CD16⁻, CD56^{dim}CD16⁺, and CD56^{neg}CD16⁺) cell population was identified by plotting a pseudocolour plot of CD56 vs CD16. This gating strategy (**Figure 2.16**) was implemented for all subsequent data analyses.

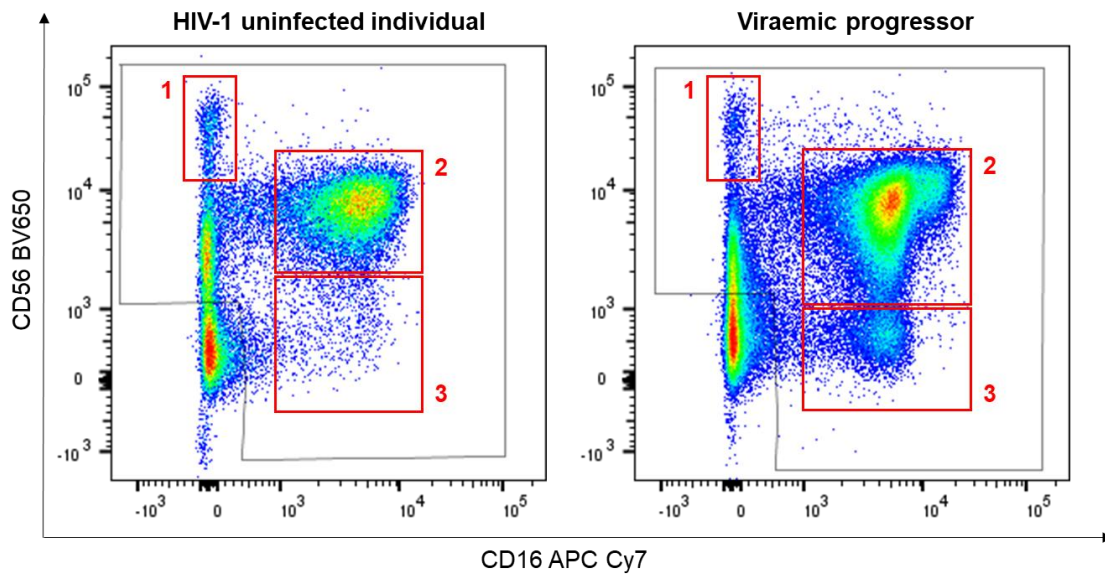


SSC-A, side scatter area; FSC-A, forward scatter area; FSC-H, forward scatter height; SSC-W, side scatter width; CD, cluster of differentiation; PE, phycoerythrin; APC, allophycocyanin; BV, brilliant violet; Cy, cyanine

Figure 2.16: Pseudocolour plots showing the gating strategy for NK cells. Lymphocytes were gated using FSC-A vs SSC-A, following which doublets were excluded using FSC-A vs FSC-H and FSC-A vs SSC-W. Live cells were gated using LIVE/DEAD Aqua, after which T cells (CD3 negative), monocytes (CD14 negative), and B cells (CD19 negative) were excluded. The total NK cell population was identified by the expression of CD56 and CD16.

2.10.6.1 Identification of natural killer cell subsets

Natural killer cell subsets, $CD56^{\text{bright}}CD16^{-}$, $CD56^{\text{dim}}CD16^{+}$, and $CD56^{\text{neg}}CD16^{+}$, were identified based on the relative expression of CD56 and CD16 as shown in **Figure 2.17**. Differences in the distribution of NK subsets in HIV-1 uninfected and HIV-1 viraemic progressors are shown below.



CD, cluster of differentiation; BV, brilliant violet; APC, allophycocyanin; Cy, cyanine

Figure 2.17: Natural killer subsets in an HIV-1 uninfected individual and an HIV-1 viraemic progressor. (1) $CD56^{\text{bright}}CD16^{-}$; (2) $CD56^{\text{dim}}CD16^{+}$; (3) $CD56^{\text{neg}}CD16^{+}$.

2.10.7 Statistical analysis

To confirm there were no differences in sex between the four cohorts, the non-parametric Fisher's exact test was used. The differences between the four cohorts were analysed by using the non-parametric Kruskal Wallis test, followed by the Mann-Whitney U test. Secondary analyses of relationships between variables were determined using non-parametric Spearman's rank correlation coefficient. Statistical analysis was done using GraphPad Prism software version 8.0.1 (GraphPad Prism Software Inc., San Diego, USA). Differences between groups were considered statistically significant at $p < 0.05$.

CHAPTER 3: RESULTS

3.1 CLINICAL DATA OF STUDY PARTICIPANTS

In this study, we examined the phenotypic, functional, and nutrient transporter profiles of NK cells during the spontaneous control of HIV-1 infection. We compared 21 (71% female) HIV-1 uninfected individuals (healthy controls) (HCs) with 20 (65% female) ART-treated individuals (ART-treated), 15 (67% female) HIV-1 elite controllers (ECs), and 19 (53% female) HIV-1 viraemic progressors (VPs). The median CD4⁺ T cell count of the ART-treated, EC, and VP groups was 770 cells/ μ L, 760 cells/ μ L, and 130 cells/ μ L respectively; and the median viral load for the same groups was lower than the detectable limit for the ART-treated and ECs, and 86000 RNA copies/mL in the VP group. The clinical data of all study groups are presented in **Table 3.1**.

Since participants were age and sex-matched and no significant age or sex differences were found between the study groups. CD4⁺ T cell counts were higher and viral load was lower in ECs and the ART-treated group compared to VPs ($p < 0.0001$) (**Figure 3.1 A**). In addition, there was a strong, negative correlation between viral load and CD4⁺ T cell counts in all study participants and VPs (**Figure 3.1 B**).

Table 3.1: Clinical data of study participants

Study groups	n	Age (years) Median (IQR)	Sex (F/M)	CD4 ⁺ T cell count (cells/ μ L) Median (IQR)	Viral load (RNA copies/mL) Median (IQR)
HC	21	41 (37 - 44)	15/6	n/a	n/a
ART	20	45 (40 - 50)	13/7	770 (640 - 800)	LDL
EC	15	46 (40 - 49)	10/5	760 (580 - 1100)	LDL
VP	19	42 (38 - 46)	10/9	130 (84 - 204)	86000 (36000 - 160000)

HC, healthy controls; ART, ART-treated individuals; EC, elite controllers; VP, viraemic progressors; n, number of study participants; IQR, interquartile range; F, female; M, male; n/a, not applicable; RNA, ribonucleic acids; LDL, lower than the detectable limit

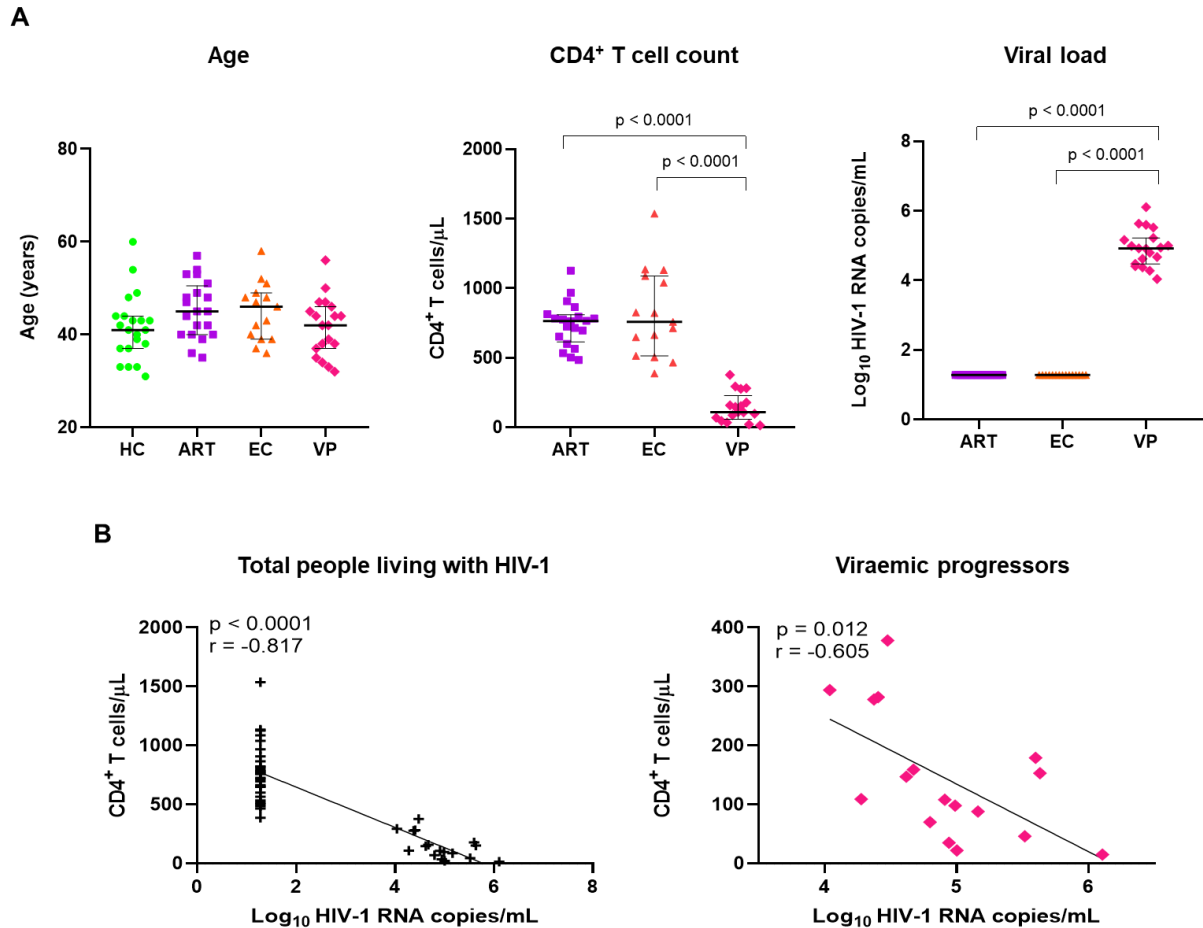


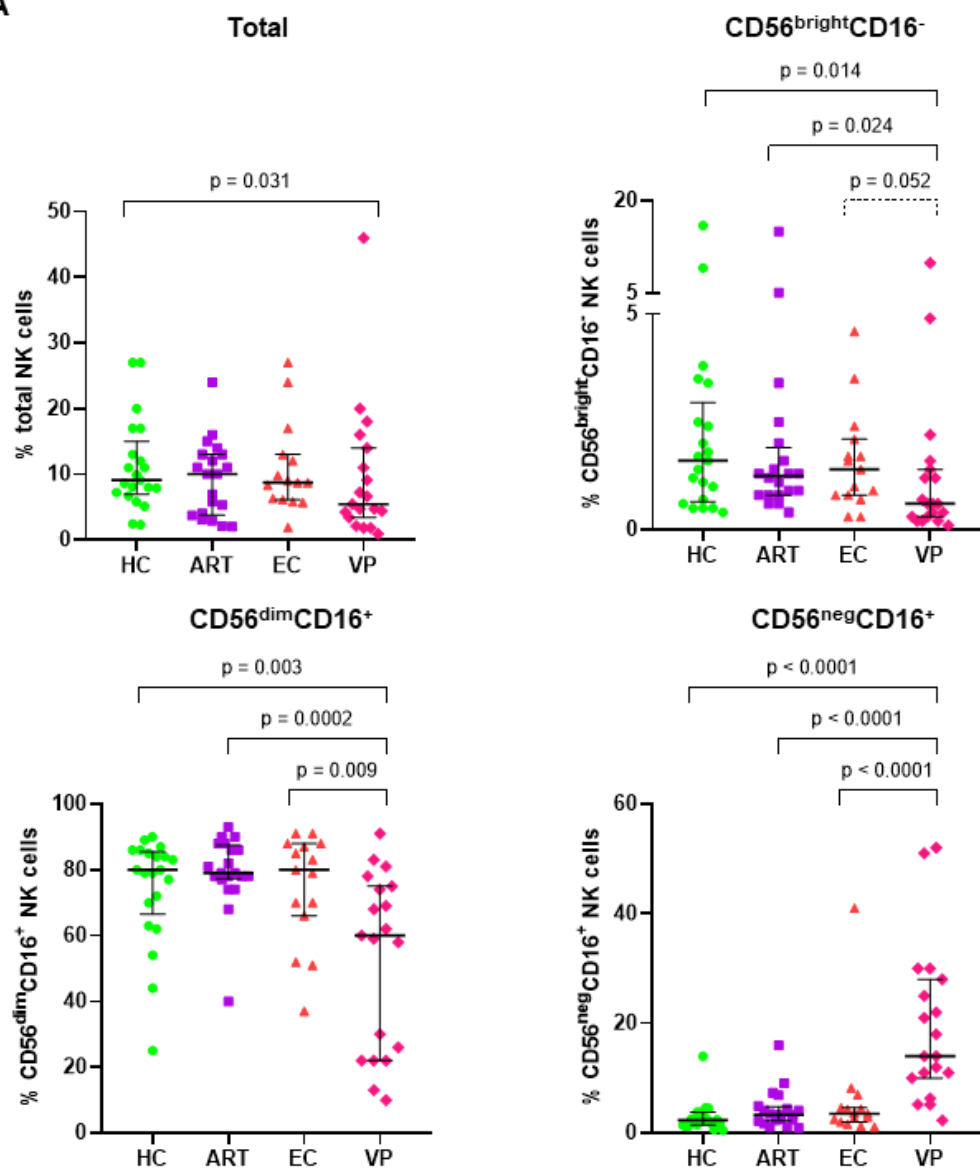
Figure 3.1: (A) Age, CD4⁺ T cell counts, and viral loads of healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines show the medians and error bars represent the 25th and 75th percentiles. **(B)** Correlations between viral load and CD4⁺ T cell counts in total people living with HIV-1 and viraemic progressors. Lines are calculated using linear regression. Each symbol represents a study participant. Significant p values and Spearman rho (r) values are shown.

3.2 NATURAL KILLER CELL FREQUENCY AND DISTRIBUTION

To evaluate the differences in NK cell frequency and distribution of ECs compared to other study groups, we examined frozen PBMCs using multicolour flow cytometry. NK cells were identified as CD3⁻, CD14⁻, and CD19⁻ lymphocytes and were further categorised based on the relative expression of CD56 and CD16 (**Figure 2.8**). As expected, the frequency of the total NK cell population was lower in VPs compared to HIV-1 uninfected individuals ($p = 0.031$), but the total NK cell frequency of ECs, ART-treated group, and HCs did not differ (**Figure 3.2 A**). The frequency of CD56^{bright}CD16⁻ and CD56^{dim}CD16⁺ NK cells was also lower in VPs compared with HCs, ART-treated, and ECs (**Figure 3.2 A**). Consistent with previously reported data, the frequency of CD56^{neg}CD16⁺ NK cells was higher in VPs compared to HCs, ART-treated, and ECs

(**Figure 3.2 A**) (Cao et al., 2021). The shift in the distribution of NK cell subsets is shown in **Figure 3.2 B**. No significant differences were observed in the frequency of any of the NK cell subsets in the EC group compared to HIV-1 uninfected individuals and ART-treated groups. Overall, the frequency of NK cells was similar in ECs compared to HCs and ART-treated groups. In contrast, notable differences were observed in VPs across all NK cell subsets compared to HCs and total PLWH.

A



B

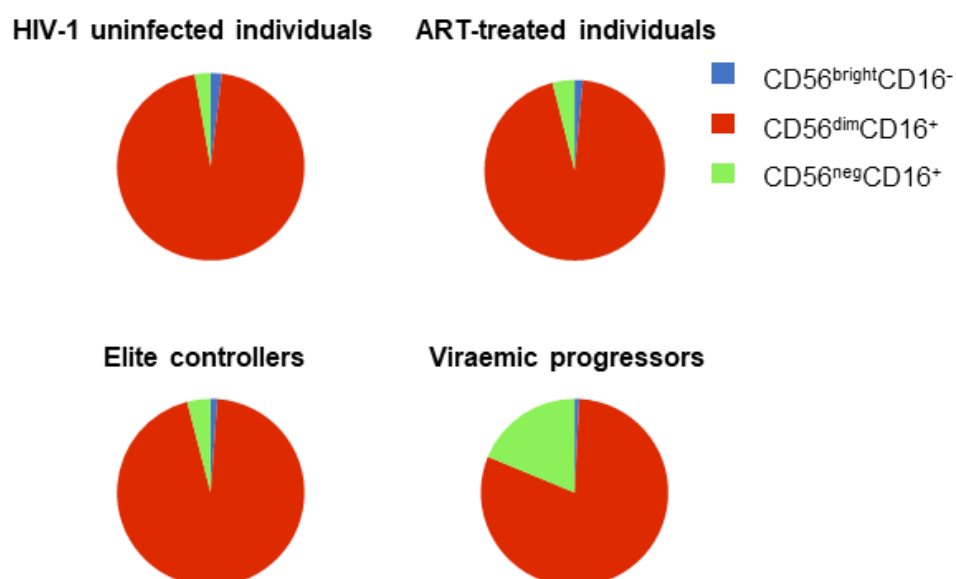


Figure 3.2: (A) Frequencies of total NK cells and their subsets: CD56^{bright}CD16⁻, CD56^{dim}CD16⁺, and CD56^{neg}CD16⁺ in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). Each symbol represents a study participant. The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. Significant p values ($p < 0.05$; solid line) and trends ($p < 0.08$; dotted line) are shown. (B) The distribution of NK cell subsets: CD56^{bright}CD16⁻ (blue), CD56^{dim}CD16⁺ (red), and CD56^{neg}CD16⁺ (green). The areas of the graphs represent the medians of each study group.

3.3 PHENOTYPIC CHARACTERISTICS OF NATURAL KILLER CELLS

We next assessed the impact of HIV-1 infection in ECs, compared to other study groups on phenotypic markers of activation, inhibition, exhaustion, and maturation on total NK cells.

Natural killer cells are known for their careful balance of many inhibitory and activation receptors that ultimately leads to an effector function (Borrego et al., 2006). Once NKG2A/C binds to HLA-E it yields two different responses. The binding of NKG2A – HLA-E inhibits NK cell activation, whereas NKG2C – HLA-E promotes NK cell activation (Cauli et al., 2018). Thus, we assessed NK cell inhibition (NKG2A) and activation (NKG2C) in total NK cells. The expression of NKG2A and NKG2C on total NK cells in the EC group did not differ from the ART-treated groups or HCs (**Figure 3.3 A**). The expression of NKG2A on total NK cells was significantly higher in ECs and HCs compared to VPs, however, the expression of NKG2C on total NK cells was significantly lower in ECs compared to VPs (**Figure 3.3 A**). The expression of NKG2C on total NK cells in HCs and the ART-treated group did not differ compared to VPs. There were no significant correlations between NKG2A or NKG2C expressing NK cells and viral load or CD4⁺ T cell count in total PLWH (**Figure 3.3 B**).

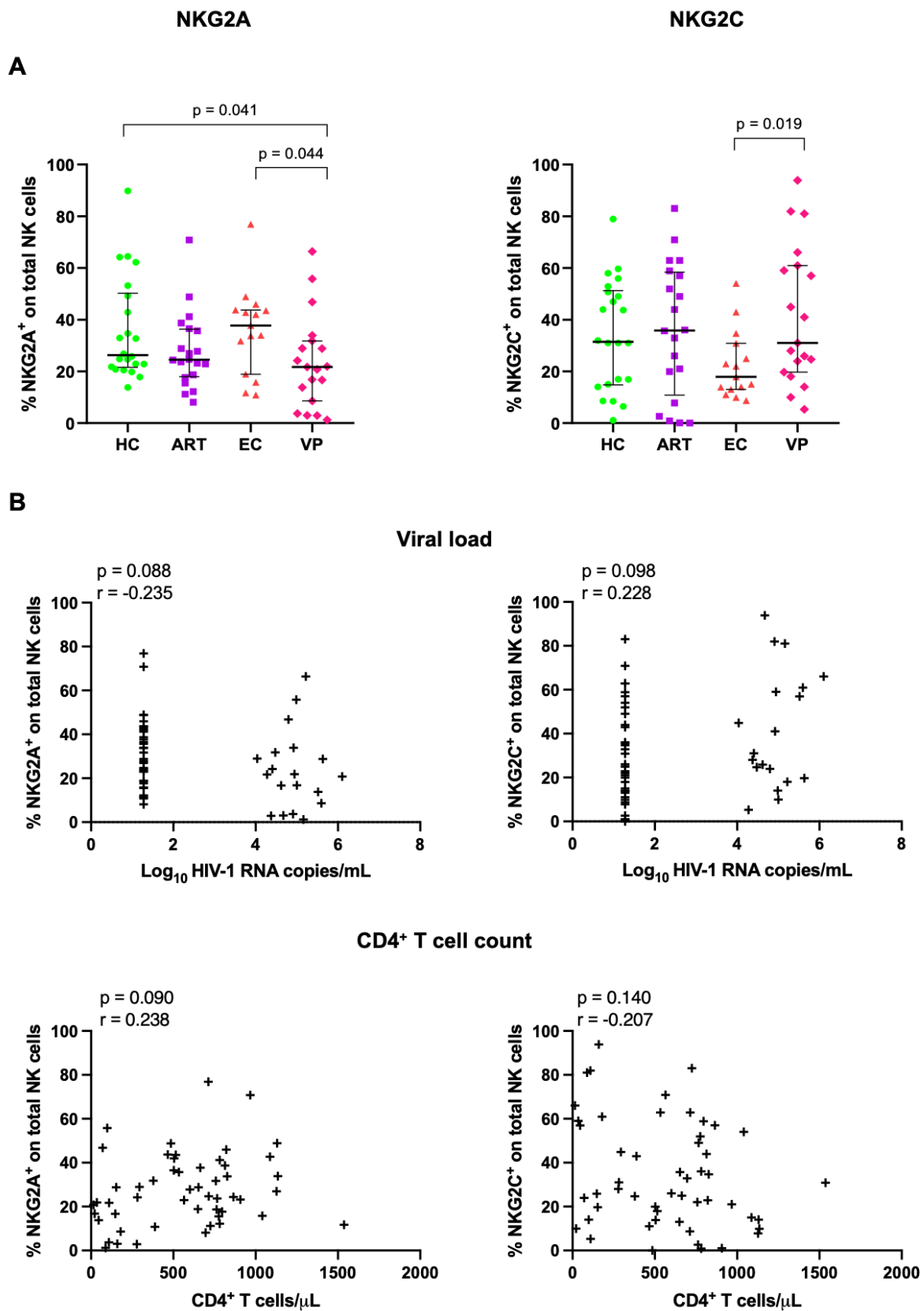


Figure 3.3: (A) The frequency of NKG2A and NKG2C cells on total NK cells in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. **(B)** Correlations between the frequency of NKG2A and NKG2C on total NK cells, and viral load and CD4⁺ T cell counts in total PLWH. Lines are calculated by using linear regression. Each symbol represents a study participant. Significant p values and Spearman rho (r) values are shown.

The ratio of NKG2A/NKG2C cells on total NK cells revealed a more inhibitory NK cell profile in ECs when compared to VPs and was not different in ECs when compared to the ART-treated group and the HCs (**Figure 3.4 A**). We observed no correlation between the ratio of NKG2A/NKG2C expressing NK cells with viral load but observed a positive correlation between the ratio of NKG2A/NKG2C expressing NK cells and CD4⁺ T cell in total PLWH (**Figure 3.4 B**). Taken together, the expression of NKG2A and NKG2C on total NK cells was similar in the EC group compared to the ART-treated group and HCs but was altered in the VP group. This suggests that an inhibitory NK cell profile may be associated with HIV-1 control.

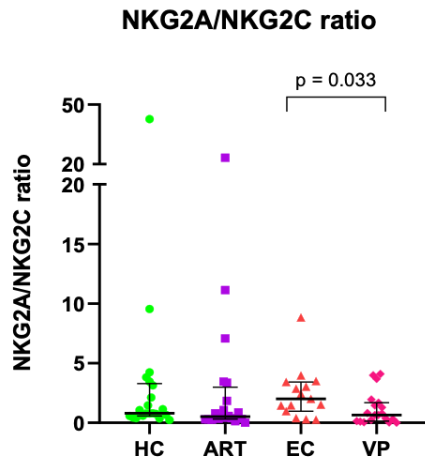
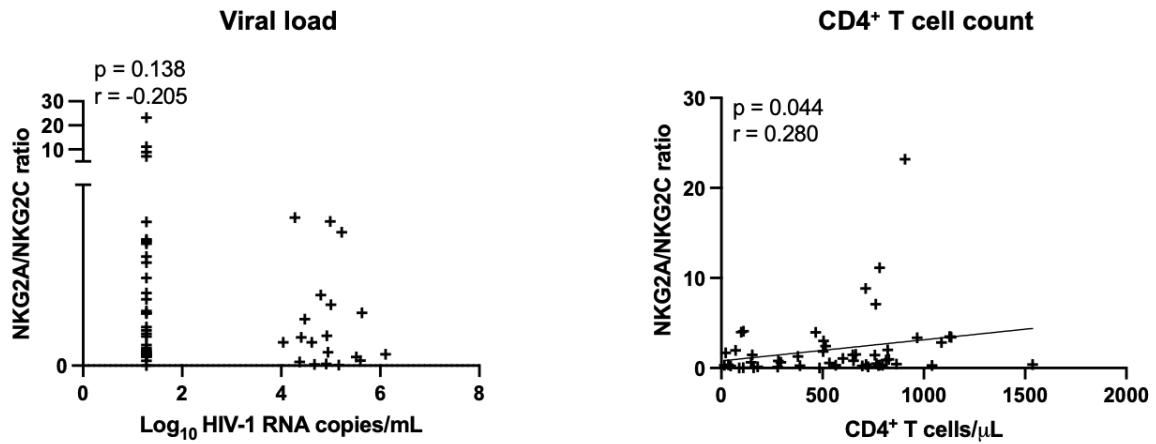
A**B**

Figure 3.4: The ratio of NKG2A/NKG2C on total NK cells in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between the ratio of NKG2A/NKG2C on total NK cells, and viral load and CD4⁺ T cell counts in total PLWH. Lines are calculated by using linear regression. Each symbol represents a study participant. Significant p values and Spearman rho (r) values are shown.

Next, we assessed activation by measuring the expression of CD69 and CD38 on total NK cells. CD69 is expressed shortly after activation and so is considered a marker of early activation (Cibrian and Sanchez-Madrid, 2017), whereas CD38 is expressed during chronic inflammation and activation and is therefore considered a marker of late activation (Hearps et al., 2017). Decreased expression of CD69 on total NK cells was observed in the ART-treated group compared to ECs and the other groups (**Figure 3.5 A**). Similarly, there was decreased expression of CD38 on total NK cells observed in ART-treated compared to ECs and VPs, as well as in HCs compared to VPs (**Figure 3.5 A**). CD38 expression on total NK cells correlated positively with viral

load and negatively with CD4⁺ T cell counts (**Figure 3.5 B**) in total PLWH. However, the expression of CD69 on total NK cells in total PLWH did not correlate with viral load or CD4⁺ T cell counts (**Figure 3.5 B**) Overall, NK cell activation was higher in ECs compared to the ART-treated group and was not different from VPs. The observed increased activation of NK cells may be associated with continuous stimulation by HIV-1.

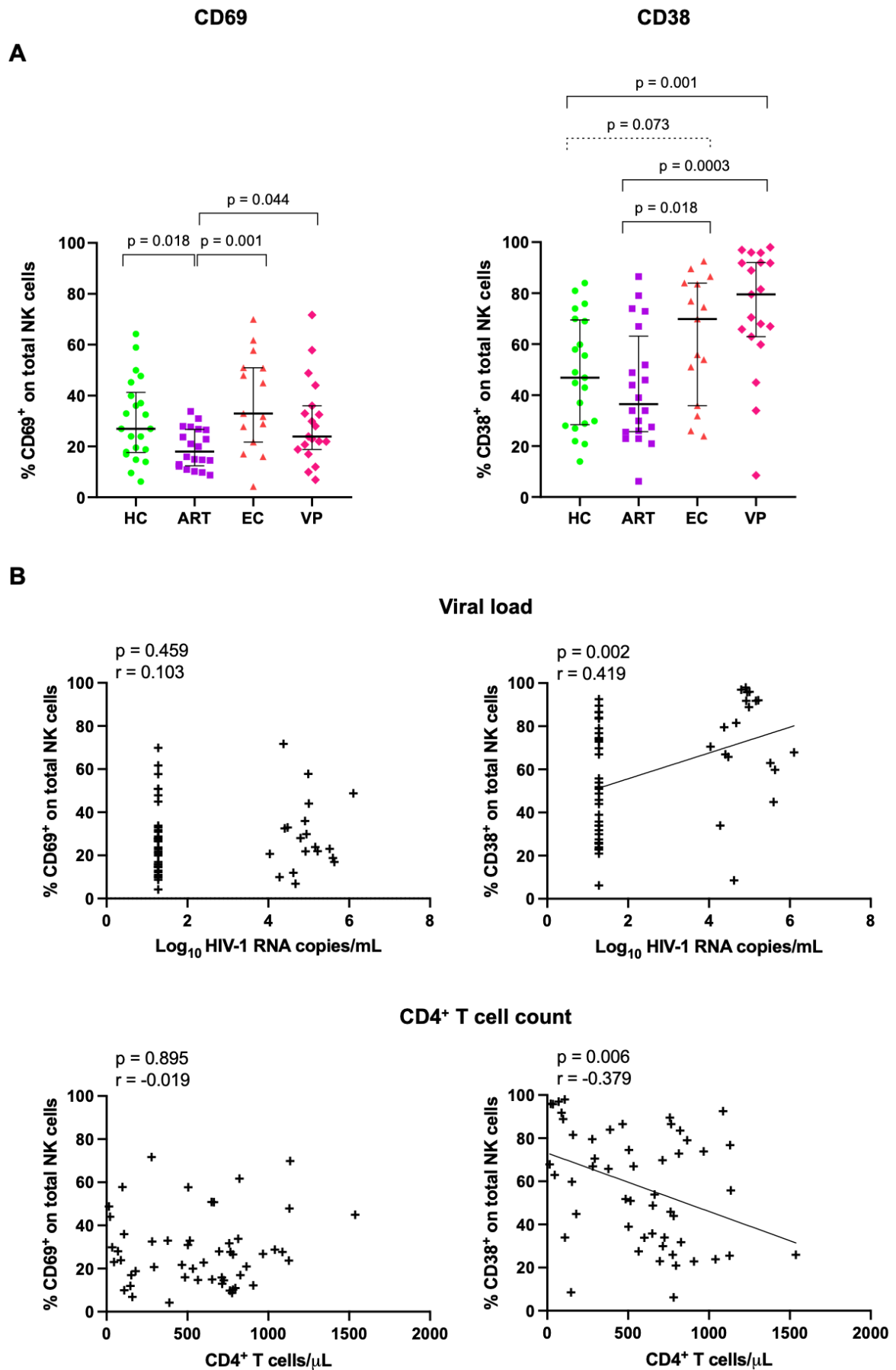


Figure 3.5: (A) The frequency of CD69 and CD38 on total NK cells in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between the frequency of CD69 and CD38 on total NK cells, and viral load and CD4⁺ T cell counts in total PLWH. Lines are calculated by using linear regression. Each symbol represents a study participant. Significant p values ($p < 0.05$; solid line), trends ($p < 0.08$; dotted line), and Spearman rho (r) values are shown.

To assess the exhaustion of NK cells, PD-1 expression was measured on total NK cells. PD-1 is also an immune checkpoint inhibitor and can be upregulated as a consequence of activation (Beldi-Ferchiou and Caillat-Zucman, 2017). Consistent with our hypothesis, the expression of PD-1 on total NK cells did not differ in ECs when compared to the ART-treated group and HCs but was significantly higher in VPs compared to HCs and ECs (**Figure 3.6 A**). There was a negative correlation, and a trend toward a positive correlation between the expression of PD-1 on total NK cells and CD4⁺ T cell counts, and viral load, respectively, in the total PLWH (**Figure 3.6 B**). We noted a similar, stronger, negative correlation between the expression of PD-1 on NK cells and CD4⁺ T cell counts in the EC group (**Figure 3.6 B**). Overall, NK cell exhaustion was not different in ECs compared to HCs and the ART-treated group but was increased in VPs. These findings confirm existing reports that the upregulation of PD-1 expression is associated with HIV-1 disease progression (Lima et al., 2017, Zhao et al., 2020).

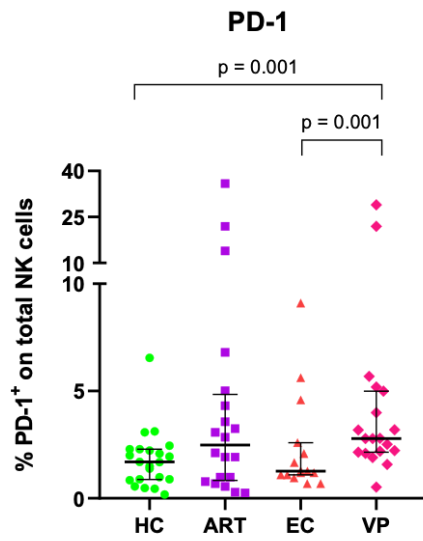
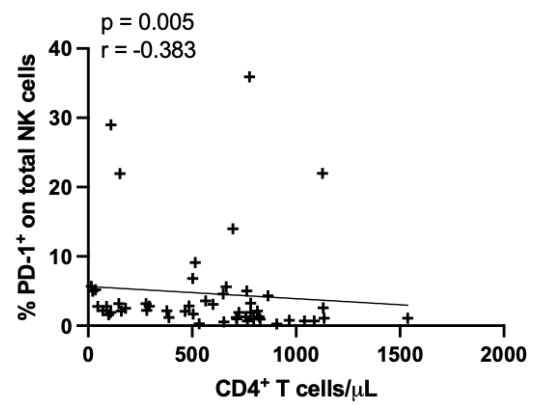
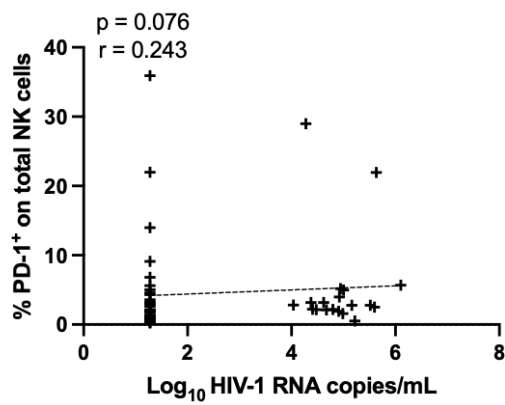
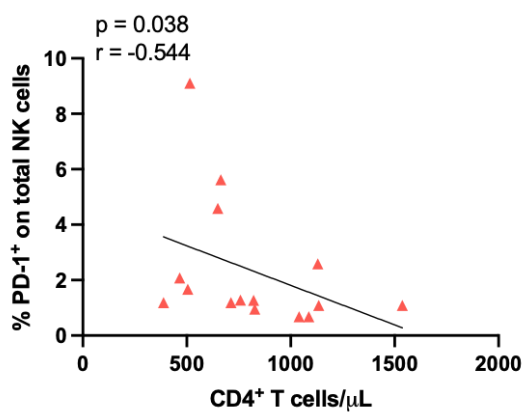
A**B****Total people living with HIV-1****C****Elite controllers**

Figure 3.6: (A) The frequency of PD-1 on total NK cells in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. **(B)** Correlations between the frequency of PD-1 on total NK cells and viral load and CD4⁺ T cell counts in total people living with HIV-1. **(C)** Correlation between the frequency of PD-1 on total NK cells and CD4⁺ T cell counts in elite controllers. Each symbol represents a study participant. Lines are calculated by using linear regression. Each symbol represents a study participant. Significant p values ($p < 0.05$; solid line), trends ($p < 0.08$; dotted line), and Spearman rho (r) values are shown.

Finally, we assessed the maturation status of the total NK cell population by measuring the expression of CD57. Maturation defines a distinct subset of NK cells with superior functional abilities (Nielsen et al., 2013). CD57 expression on total NK cells was similar in ECs compared to HCs and the ART-treated group, whereas VPs had a lower expression of CD57 on total NK cells compared to HCs, the ART-treated group, and ECs (**Figure 3.7 A**). These findings are in line with previously reported data that shows a mature NK cell phenotype is associated with HIV-1 control (Naluyima et al., 2014, Tomescu et al., 2021). CD57 expression on total NK cells correlated negatively with viral load and positively with CD4⁺ T cell counts in total PLWH (**Figure 3.7 B**). Together, CD57 expression was lower in VPs compared to other groups but not different in ECs compared to HCs and ART-treated individuals and may indicate a preserved NK cell function across individuals with non-progressive HIV disease.

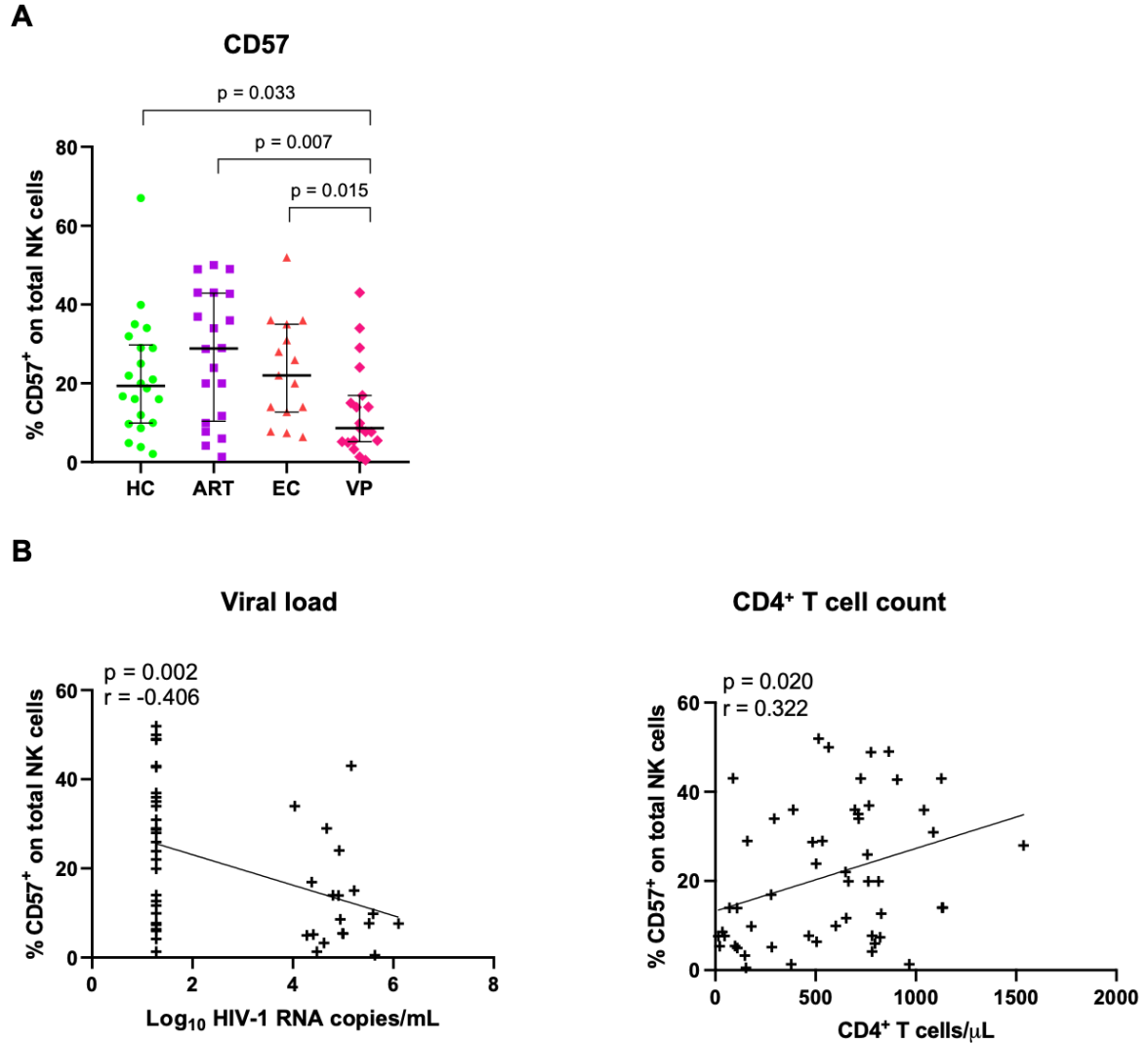


Figure 3.7: (A) The frequency of CD57 on total NK cells in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between CD57 on NK cells and viral load and CD4⁺ T cell counts in total PLWH. Each symbol represents a study participant. Lines are calculated using linear regression. Significant p values and Spearman rho (r) values are shown.

To understand the relationships between activation, inhibition, exhaustion, and maturation, we next assessed the correlations between these markers in all study participants and stratified this by study groups (**Figure 3.8**). Moderate, negative correlations were observed between the frequencies of NKG2A and NKG2C on total NK cells in all study participants ($p < 0.0001$; $r = -0.493$) and total PLWH ($p < 0.0001$; $r = -0.547$). The EC group ($p = 0.020$; $r = -0.600$) and the VP group ($p = 0.008$; $r = -0.591$) were the drivers for this observation. The activation of NK cells has been associated with HIV-1 disease progression (Kuri-Cervantes et al., 2014), thus the positive correlations observed between the expression of CD69⁺ and CD38⁺ on total

NK cells in all study participants ($p = 0.003$; $r = 0.338$) and total PLWH ($p = 0.004$; $r = 0.382$) was not unexpected. Unsurprisingly, the main contributors were the VP group ($p = 0.048$; $r = 0.459$) and the ART-treated group ($p = 0.030$; $r = 0.486$).

Positive correlations were observed between CD38 (activation) and CD57 (maturation) expression on total NK cells in VPs. In contrast, in the EC group, there was a trend towards a negative correlation between the expression of CD69 (activation) and CD57 (maturation) on total NK cells ($p = 0.058$; $r = -0.502$). As expected, positive correlations were observed between CD57 and NKG2C expressing total NK cells in all study participants ($p = 0.015$; $r = 0.278$). This relationship was driven by HCs ($p = 0.030$; $r = 0.463$) and VPs ($p = 0.019$; $r = 0.532$); thus, supporting a more differentiated NK cell profile. We did not observe a significant relationship between the expression of CD57 and NKG2C on total NK cells in our EC group. Taken together, the negative relationship observed between inhibition (NKG2A) and activation (NKG2C) as well as the positive relationship between early (CD69) and late (CD38) activation in total PLWH is likely caused by HIV-1. The positive relationship observed between late activation and maturation may be associated with disease progression. In contrast, the negative relationship observed between early activation and maturation of NK cells may be associated with HIV-1 spontaneous control.

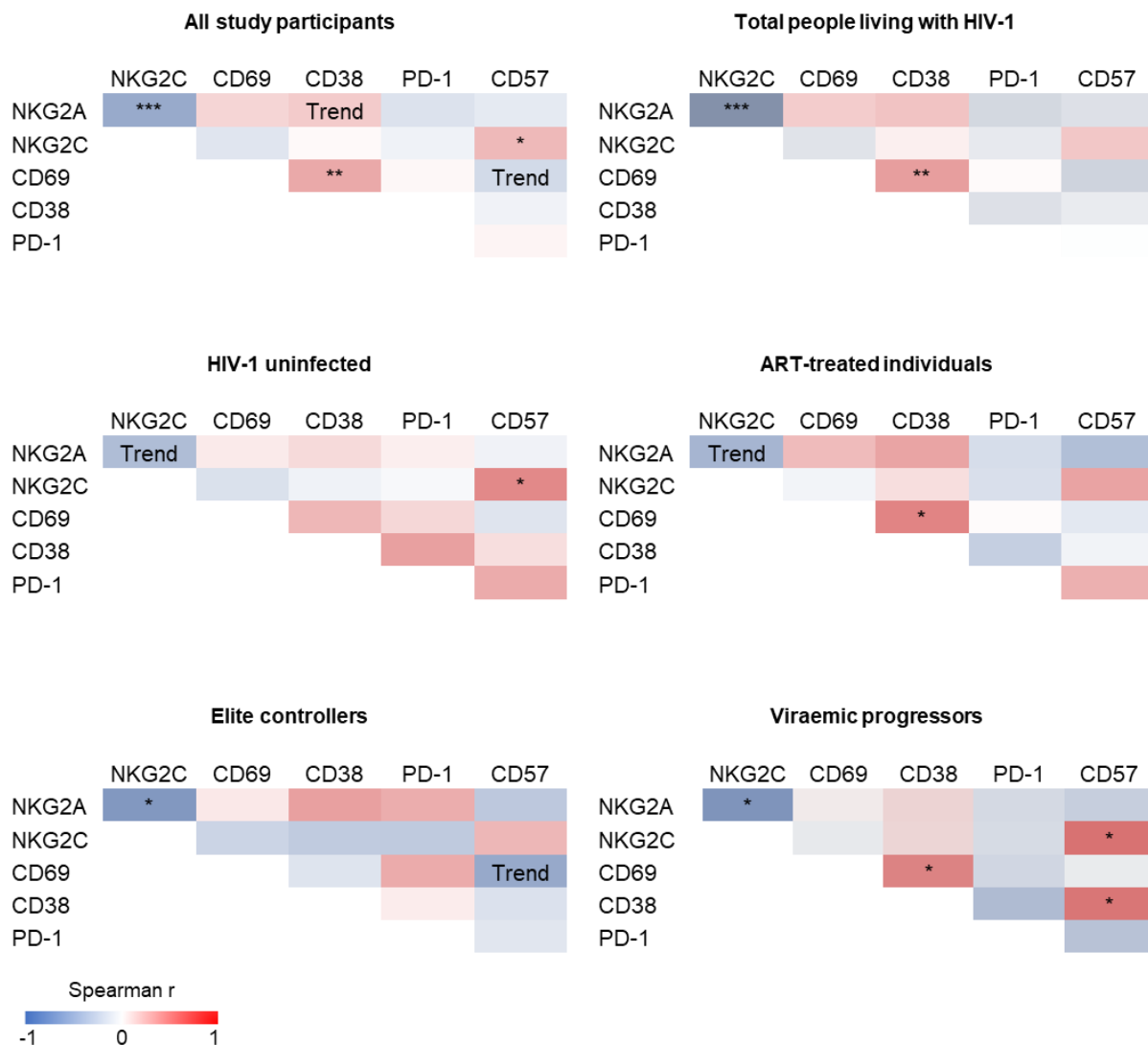


Figure 3.8: Correlations between phenotypic markers in all study participants, total people living with HIV-1, HIV-1 uninfected, ART-treated individuals, elite controllers, and viraemic progressors. *p < 0.001, **p < 0.01, *p < 0.05, trend = p < 0.08.**

Overall, a preserved NK cell population, distribution, and maturation profile similar to ART-treated and healthy individuals together with a more inhibitory NK cell profile was observed in the EC group. Although HIV-1 replication is controlled in our EC group, NK cells remained highly activated. Even with continual stimulation, the exhaustion profile of NK cells from our EC group remained lower than VPs. Furthermore, NK cell differentiation was undisturbed in our EC group but significantly reduced during advanced disease HIV-1 disease progression. In general, ECs phenotypic profile was similar to our HC group.

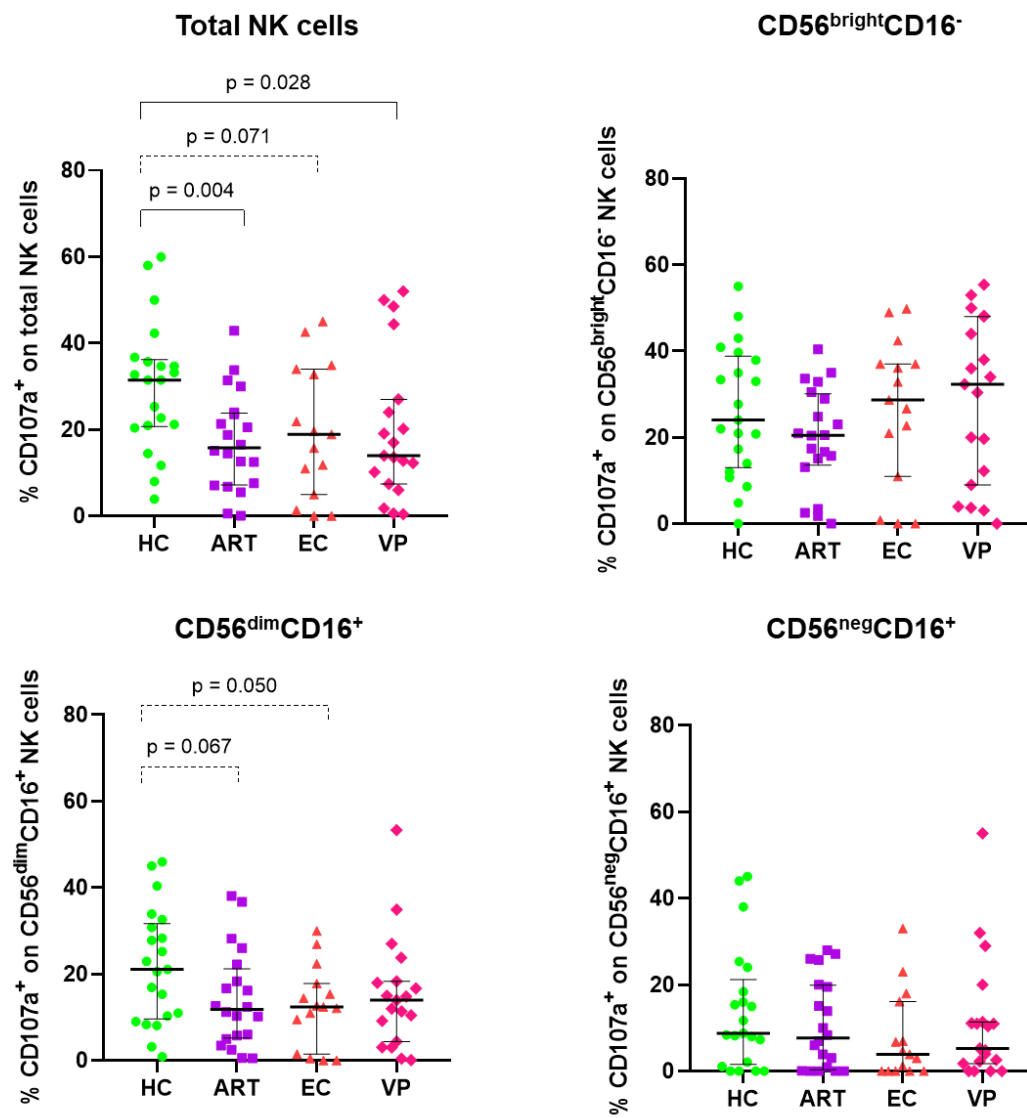
3.4 FUNCTIONAL CHARACTERISTICS OF NATURAL KILLER CELLS AND THEIR SUBSETS

Functional characteristics of the total NK cell population and their subsets (CD56^{bright}CD16⁻, CD56^{dim}CD16⁺, and CD56^{neg}CD16⁺) were determined using the degranulation capacity (CD107a), cytokine production (IFN- γ), and nutrient transporter profiles (Glut1, CD98, and CD71). NK cells were stimulated with an MHC-devoid cell line together with IL-2, IL-15, and IL-18 to mimic an *in vivo* model of HIV-1 stimulation.

We hypothesised that CD107a, a marker of degranulation and cytotoxic activity (Alter et al., 2004, Lorenzo-Herrero et al., 2019) would be lower in the VP group compared to other study groups. It was noted that all PLWH had lower expression of CD107a in total NK cells compared to HCs, however, this was not significant in the ECs (**Figure 3.9 A**). In the CD56^{dim}CD16⁺ NK cell subset, there was a trend towards a lower expression of CD107a in ECs and the ART-treated group compared to HCs (**Figure 3.9 A**). There were no significant differences in CD107a expression between the study groups in the CD56^{bright}CD16⁻ and CD56^{negative}CD16⁺ subsets of NK cells (**Figure 3.9 A**).

Interestingly, the frequency of CD107a total NK cells correlated negatively with CD4⁺ T cell counts in the ART-treated group, but positively in the EC group (**Figure 3.9 B**). Taken together, a significant reduction in total CD107a expression on total NK cells was observed across all PLWH compared to HCs. This was likely driven by the CD56^{dim}CD16⁺ NK cell subset and not by the CD56^{bright}CD16⁻ or CD56^{neg}CD16⁺ subsets.

A



B

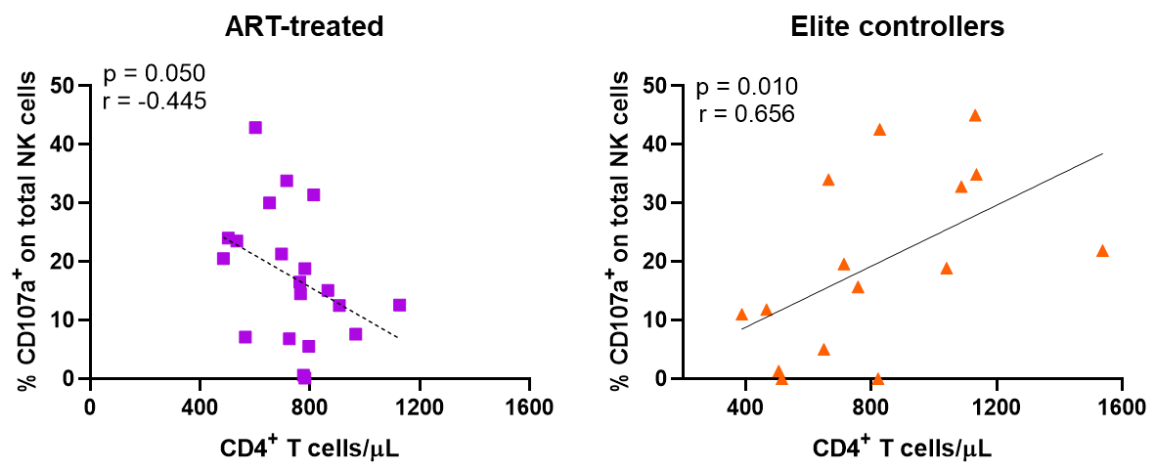


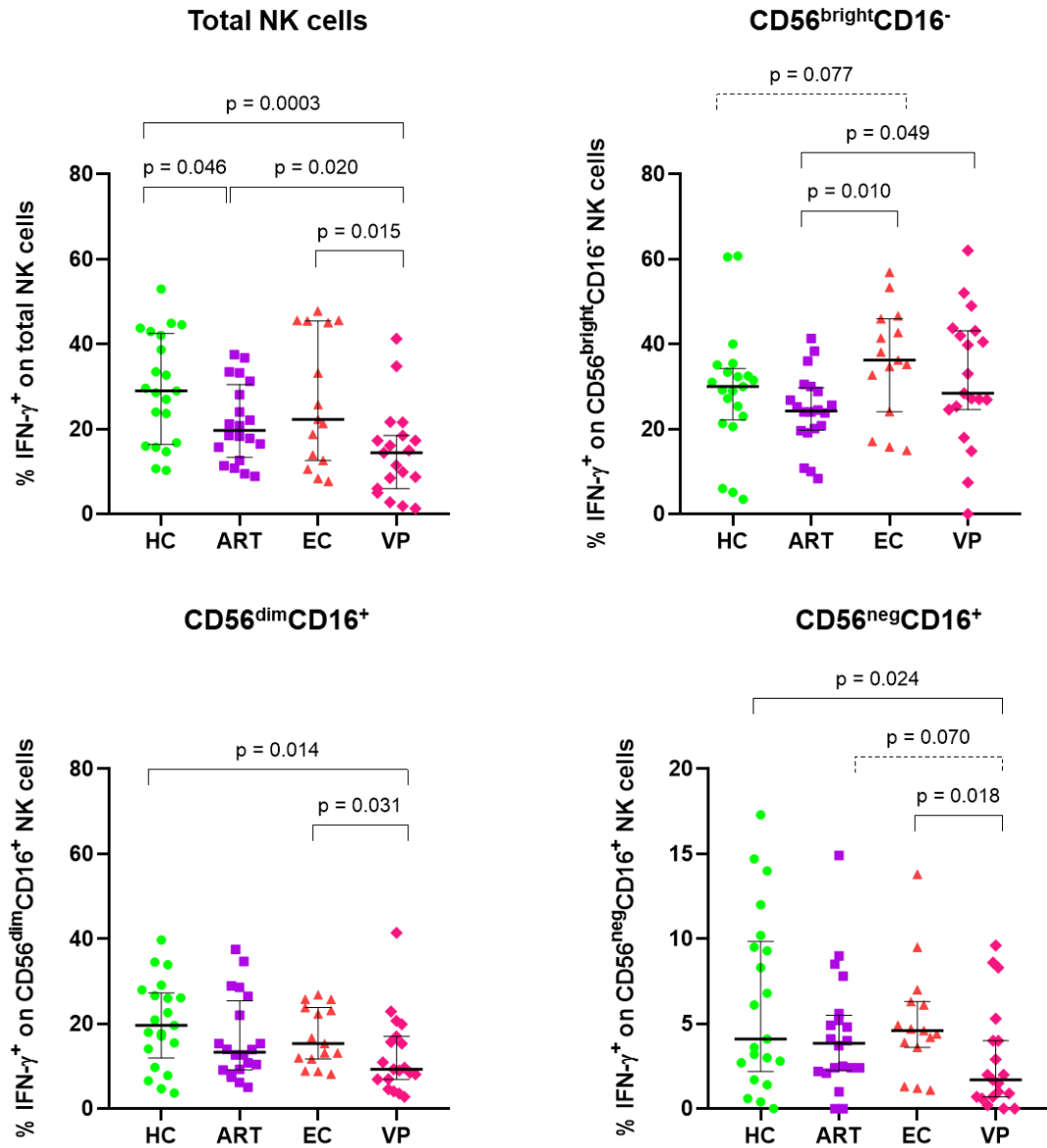
Figure 3.9: (A) The frequency of CD107a on total NK cells, CD56^{bright}CD16⁻, CD56^{dim}CD16⁺, and CD56^{neg}CD16⁺ NK cell subsets in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between the expression of CD107a on total NK cells and CD4⁺ T cell counts in ART-treated individuals and elite controllers. Lines are calculated by using linear regression. Each symbol represents a study participant. Significant p values ($p < 0.05$; solid line), trends, ($p < 0.08$; dotted line), and Spearman rho (r) values are shown.

IFN- γ production, a measure of cell function, did not differ in ECs compared to the ART-treated group and HCs in total NK cells, CD56^{dim}CD16⁺, and CD56^{neg}CD16⁺ cells. In line with a previous study (Jiang et al., 2013), there was lower IFN- γ production in VPs compared to all other study groups as well as in the ART-treated group compared to HCs in total NK cells (**Figure 3.10 A**). Decreased IFN- γ production was noted in CD56^{bright}CD16⁻ in the ART-treated group when compared to ECs and VPs (**Figure 3.10 A**). Lower IFN- γ production in VPs compared to ECs and HCs in both CD56^{dim}CD16⁺ and CD56^{neg}CD16⁺ NK cell subsets were also observed (**Figure 3.10 A**).

IFN- γ production correlated negatively with viral load and positively with CD4⁺ T cell counts in the total PLWH (**Figure 3.10 B**). The positive correlation observed between IFN- γ production and CD4⁺ T cells was mainly driven by the EC group ($p = 0.021$; $r = 0.597$) and the VP group ($p = 0.037$; $r = 0.513$) (**Appendix A**). In contrast, a negative correlation between IFN- γ production and CD4⁺ T cell counts ($p = 0.024$; $r = -0.503$) in the ART-treated group was observed (**Appendix A**).

Overall, IFN- γ production in total NK cells was not different in ECs compared to HCs but analysis of CD56^{bright}CD16⁻ NK cell subset, revealed increased production of IFN- γ in ECs compared to the ART-treated group.

A



B

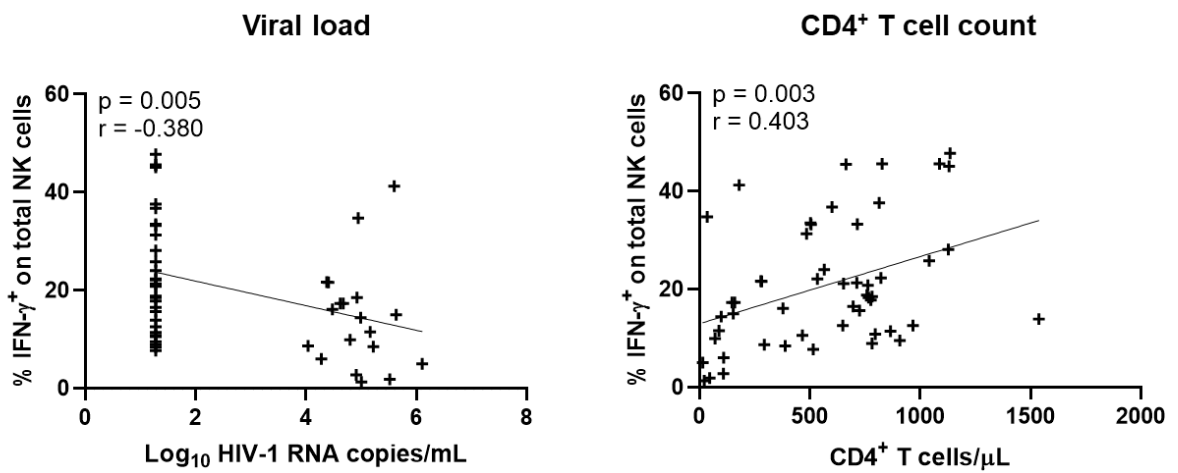


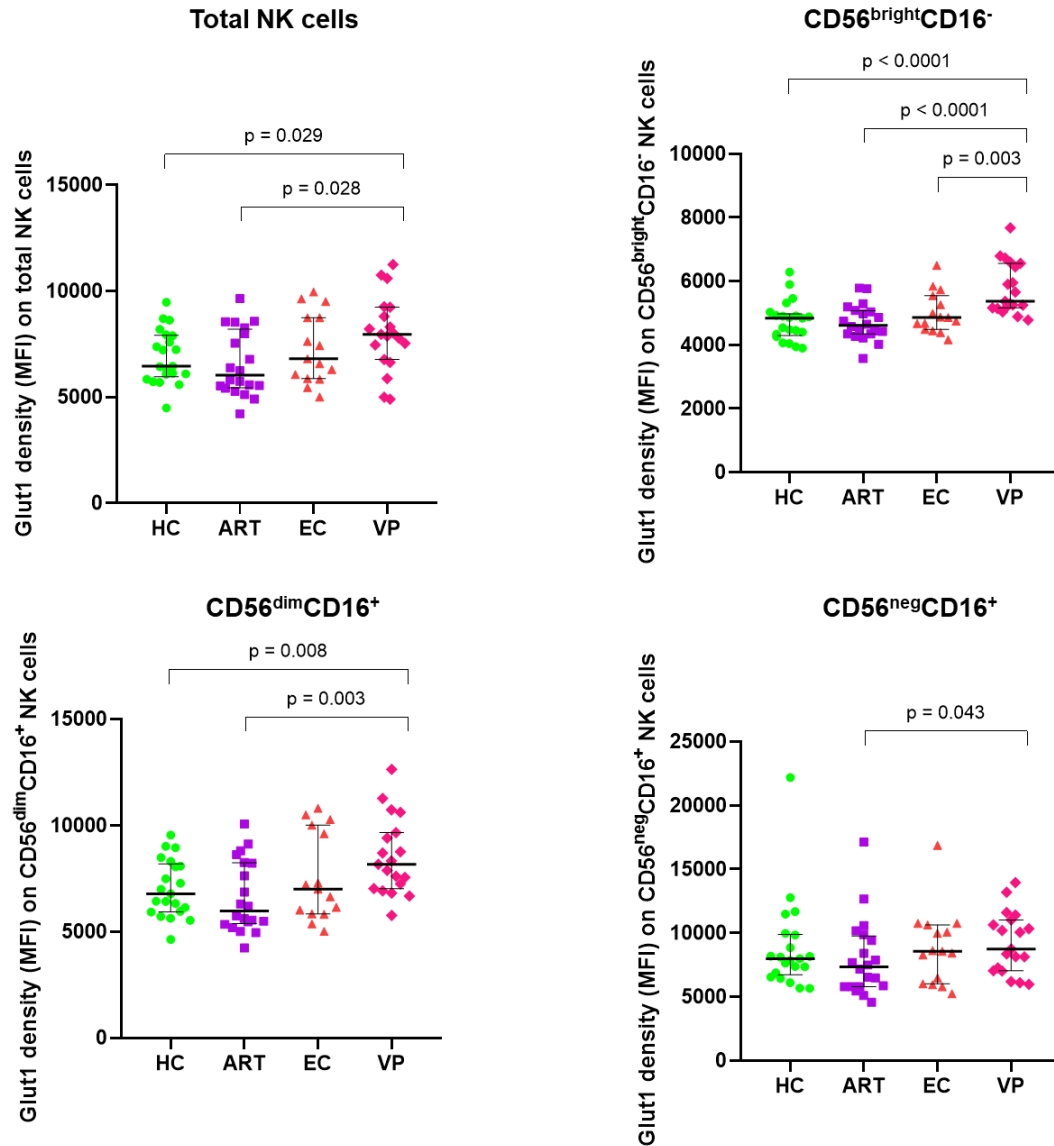
Figure 3.10: (A) The production of IFN- γ by total NK cells, CD56^{bright}CD16⁻, CD56^{dim}CD16⁺, and CD56^{neg}CD16⁺ NK cell subsets in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines show the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between the production of IFN- γ by total NK cells and viral load and CD4⁺ T cell counts in total PLWH. Lines are calculated by using linear regression. Each symbol represents a study participant. Significant p values ($p < 0.05$), trends ($p < 0.08$), and Spearman rho (r) values are shown.

We next investigated immune cellular metabolic function by quantifying the expression of nutrient transporters (Glut1, CD98, and CD71). Although limited reports have demonstrated how cellular metabolism is altered in HIV-1 infection, Salzberger et al reported that NK cell subsets displayed distinct metabolic profiles based on where the NK cell resides (tissue-resident or peripheral blood). They showed that the expression of Glut1, CD98, and CD71 differed relative to their functional requirements and nutrient availability (Salzberger et al., 2018). We hypothesised that HIV-1 infection would influence the nutrient uptake and that the nutrient uptake would differ by NK cell subsets.

Our data shows that when ECs were compared to ART-treated individuals and HCs, the Glut1 density on total NK cells, and their subsets, did not differ (**Figure 3.11 A**). The Glut1 density on total NK cells and CD56^{dim}CD16⁺ was upregulated in VPs compared to HCs and the ART-treated group (**Figure 3.11 A**). The Glut1 density on CD56^{bright}CD16⁻ NK cells was higher in VPs compared to other study groups and was also higher in VPs when compared to the ART-treated group in the CD56^{neg}CD16⁺ NK cell subset (**Figure 3.11 A**).

In addition, there was a trend toward a positive correlation between the Glut1 density on total NK cells and viral load in total PLWH, however, no correlation was found between Glut1 density and CD4⁺ T cell counts (**Figure 3.11 B**). Overall, we observed increased Glut1 density in VPs in total NK cells and across NK cell subsets compared to the other study groups.

A



B

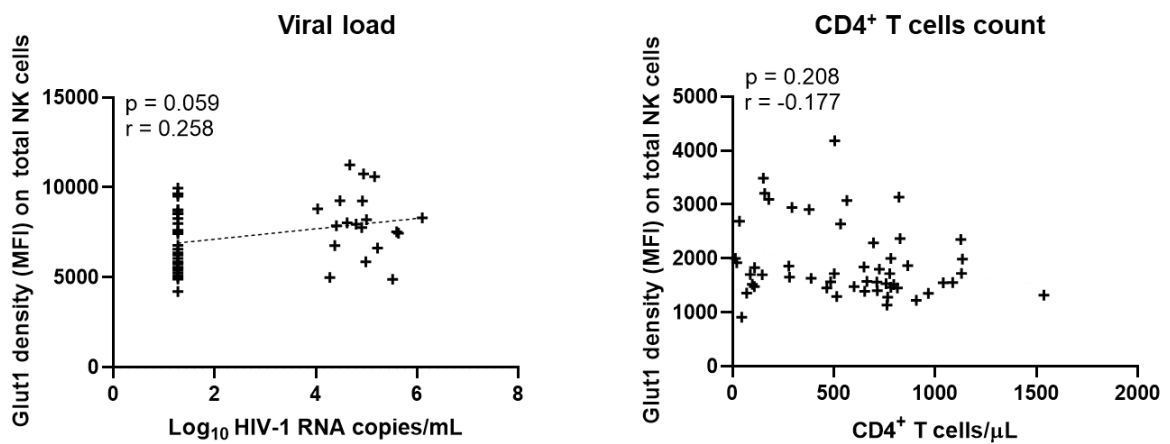
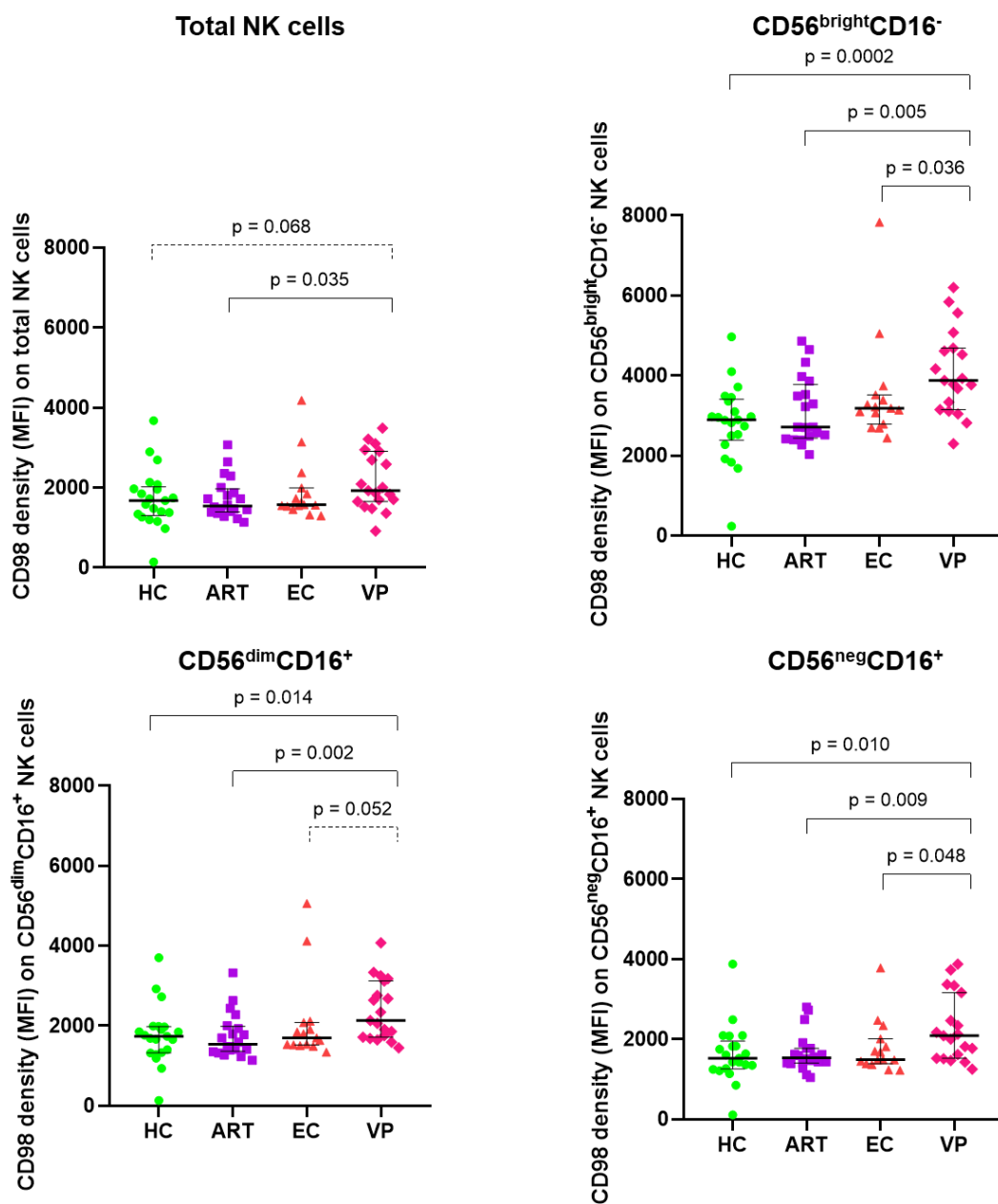


Figure 3.11: (A) Glut1 density, measured by median fluorescent intensity (MFI), on total NK cells, CD56^{bright}CD16⁻, CD56^{dim}CD16⁺, and CD56^{neg}CD16⁺ NK cell subsets in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between the Glut1 density, measured by the median fluorescent intensity (MFI), on total NK cells and viral load, and CD4⁺ T cell count in total PLWH. Lines are calculated by using linear regression. Each symbol represents a study participant. Significant p values ($p < 0.05$; solid line), trends ($p < 0.08$; dotted line), non-significant ($p > 0.05$; no line), and Spearman rho (r) values are shown.

CD98 functions as a coreceptor for amino acid transportation into the cell (Khan et al., 2021). Together with supplying the cell with fuel for immune metabolism, CD98 also regulates cellular proliferation (Kim and Hahn, 2008). Once NK cells are activated (with HIV-1, or other stimuli) CD98 is upregulated to meet energy demands. During inflammation, naïve NK cells upregulate the expression of CD98 to support the proliferation and differentiation into effector cells (Loftus et al., 2018). Our data show similar patterns of CD98 density on total NK cells and across subsets when ECs were compared to HCs and the ART-treated group (**Figure 3.12 A**). As observed with the Glut1 results above, the CD98 density in all NK subsets was higher in VPs compared to other study groups (**Figure 3.12 A**). This pattern, however, was not reflected when analysing the total NK cell population, as CD98 density was only significantly higher in VPs compared to ART-treated individuals, but not other study groups (**Figure 3.12 A**). Although CD4⁺ T cell counts did not correlate with the density of CD98 on total NK cells, a positive correlation was observed with viral load in total PLWH (**Figure 3.12 B**). Overall, the CD98 density on NK cells was not different in ECs compared to HCs and the ART-treated group but was increased in VPs. This data may suggest that the upregulation of CD98 is associated with HIV-1 disease progression.

A



B

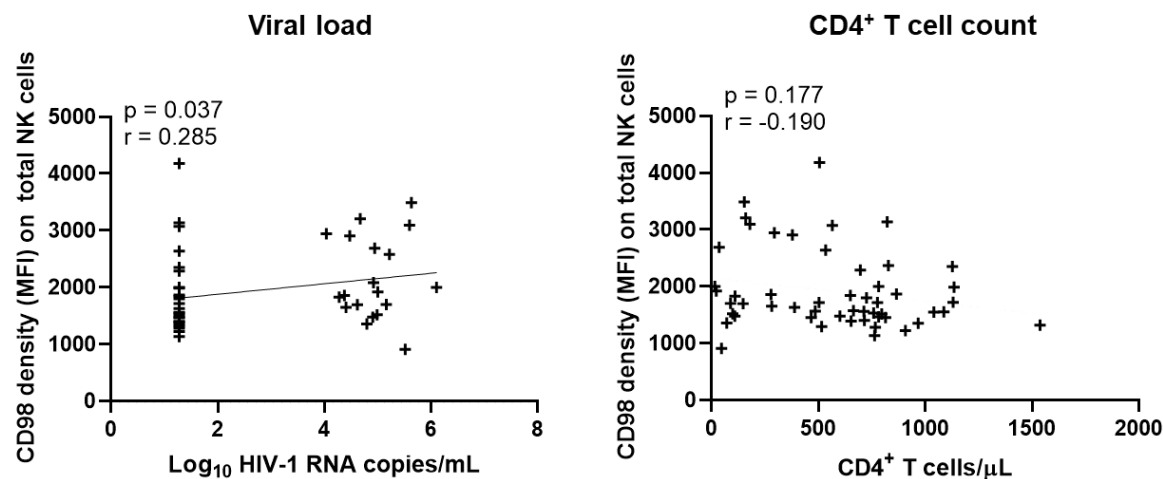
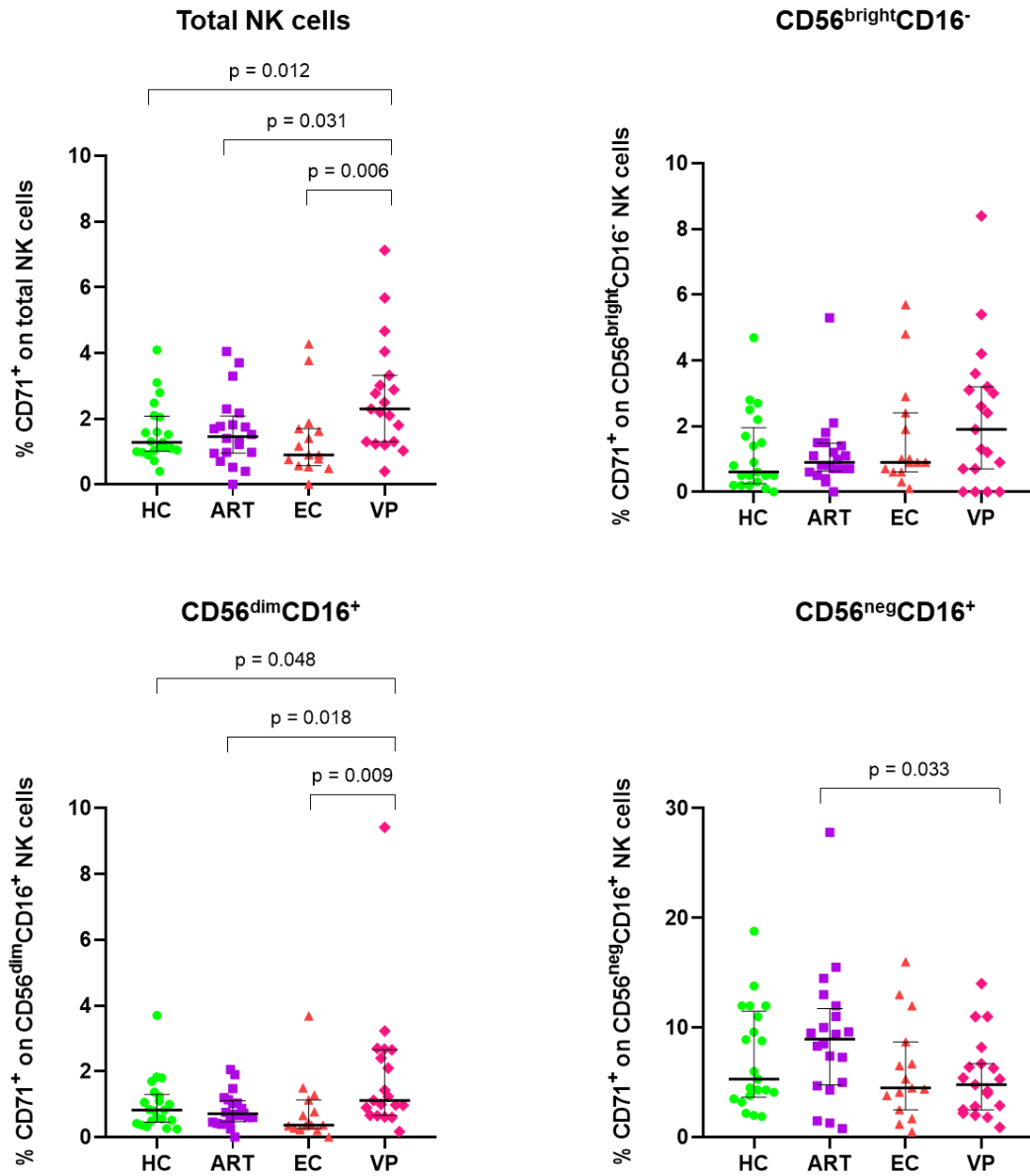


Figure 3.12: (A) CD98 density, measured by median fluorescence intensity (MFI), on total NK cells, CD56^{bright}CD16⁻, CD56^{dim}CD16⁺, and CD56^{neg}CD16⁺ NK cell subsets in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between CD98 density, measured by the median fluorescent intensity (MFI), on total NK cells and viral load, and CD4⁺ T cells in total PLWH. Lines are calculated by using linear regression. Each symbol represents a study participant. Significant p values ($p < 0.05$; solid line), trends ($p < 0.08$; dotted line), non-significant ($p > 0.05$; no line), and Spearman rho (r) values are shown.

Lastly, we assessed iron transportation by measuring the expression levels of CD71 on NK cells and their subsets (Liu et al., 2014). Iron is important for cellular activities including DNA repair and synthesis, epigenetic regulation, mitochondrial function, sensing hypoxia, and effective immune function (Littwitz-Salomon et al., 2021). Since intracellular iron can be toxic at high concentrations, it is strictly regulated (Cassat and Skaar, 2013). Under normal conditions, low levels of CD71 on NK cells is expressed. Once activated CD71 expression increases in response to the need for NK cell proliferation (Littwitz-Salomon et al., 2021). We show that the expression of CD71 on total NK cells and by NK cell subsets did not differ in ECs compared to HCs and the ART-treated group (**Figure 3.13 A**). CD71 expression in total NK cells and CD56^{dim}CD16⁺ NK cells was higher in VPs compared to the other study groups (**Figure 3.13 A**). Even though CD56^{bright}CD16⁻ are considered to be more metabolically active, a similar pattern of high CD71 expression was observed in VPs in this subset, however, the differences were not significant across study groups. Interestingly, CD71 expression in the CD56^{neg}CD16⁺ NK subset was lower in VPs compared to the ART-treated group (**Figure 3.13 A**). Furthermore, the expression of CD71 on total NK cells correlated positively with viral load, and there was a trend towards a negative correlation with CD4⁺ T cell counts in total PLWH (**Figure 3.13 B**). Overall, CD71 expression was similar in ECs compared to HCs and ART-treated individuals but was increased in VPs. These data suggest that CD71 may be associated with progressive HIV-1 disease.

A



B

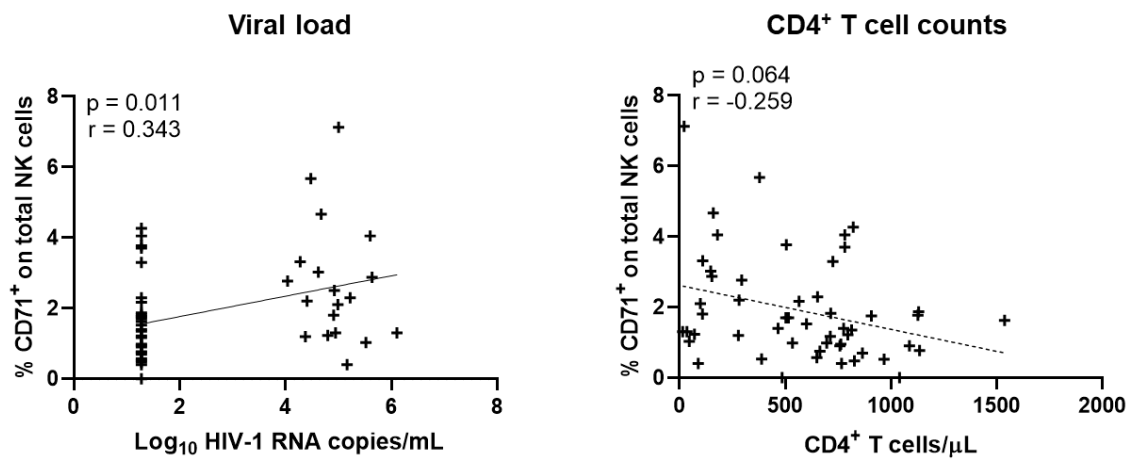


Figure 3.13: (A) The frequency of CD71 on total NK cells, CD56^{bright}CD16⁻, CD56^{dim}CD16⁺, and CD56^{neg}CD16⁺ NK cell subsets in healthy controls (HC), ART-treated individuals (ART), elite controllers (EC), and viraemic progressors (VP). The horizontal lines show the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between the frequency of CD71 on total NK cells and viral load and CD4⁺ T cell counts in total PLWH. Lines are calculated by using linear regression. Each symbol represents a study participant. Significant p values ($p < 0.05$; solid line), trends ($p < 0.08$; dotted line), and Spearman rho (r) values are shown.

To understand the relationship between functional markers (cytotoxicity, cytokine production, and nutrient transporters) we assessed the correlations of the markers in all study participants stratified by study group (**Figure 3.14**). There was a strong, positive correlation between the expression of CD107a and IFN- γ on total NK cells in all study groups except in the VP group (**Figure 3.14**). A positive correlation between the expression of CD107a and CD98 density on total NK cells was noted only in HCs ($p = 0.022$; $r = 0.497$); while a positive correlation between IFN- γ production and CD98 density on total NK cells was observed in both HCs ($p = 0.024$; $r = 0.491$) and ECs ($p = 0.051$; $r = 0.517$).

We observed a moderate, positive correlation between Glut1 and CD98 density on total NK cells in total PLWH ($p < 0.0001$; $r = 0.514$); and a positive correlation between CD98 density and CD71 expression on total NK cells in total PLWH ($p = 0.004$; $r = 0.386$) which was driven by the VP group ($p = 0.012$; $r = 0.566$). Overall, these data indicate nutrient availability requirements vary across the HIV-1 study groups with VPs showing the highest demand for energy transporters. This suggests nutrient transporters modulate the effector functions of NK cells.

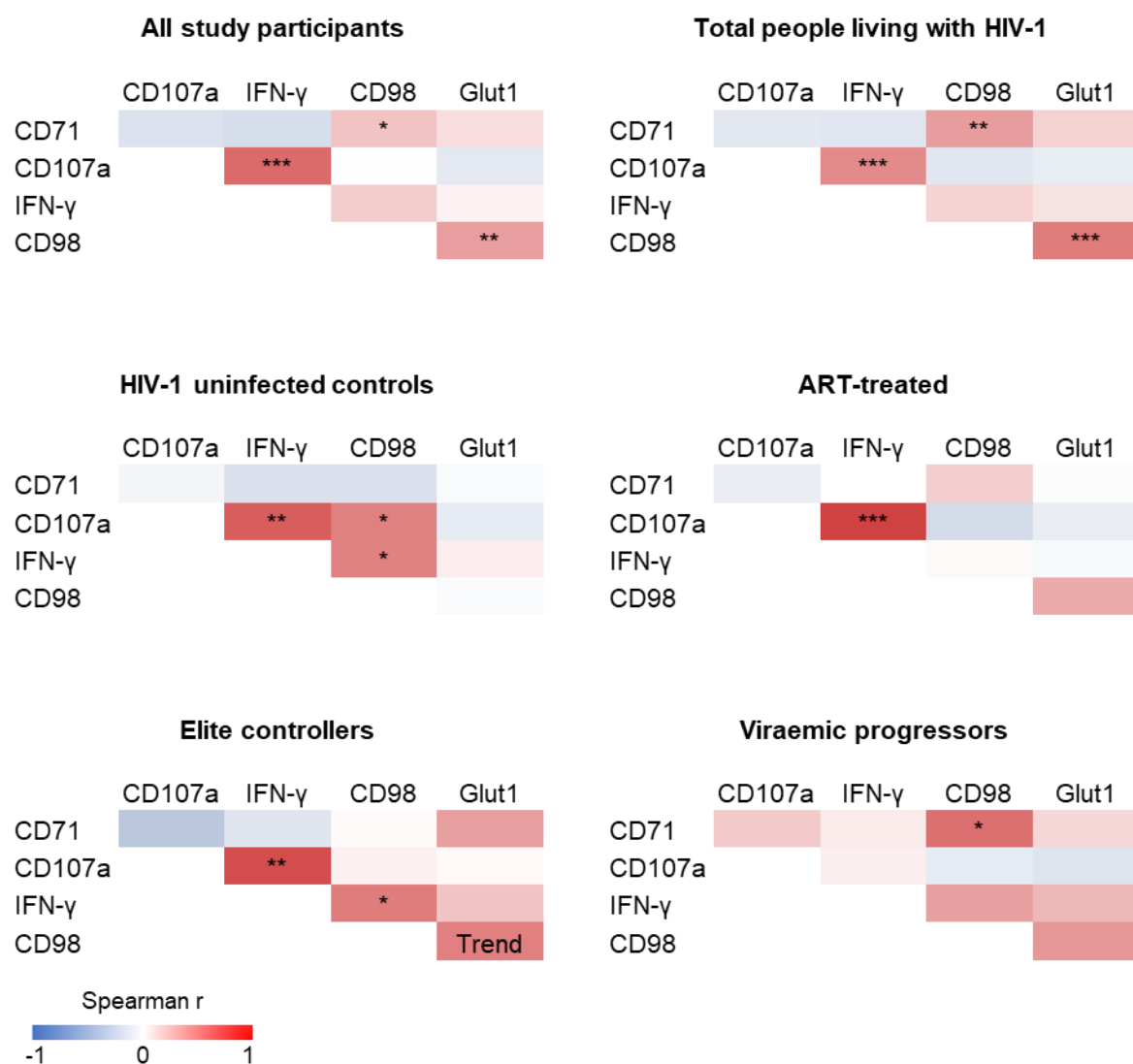


Figure 3.14: Correlations between functional markers on total NK cells in all study participants, total people living with HIV-1, HIV-1 uninfected, ART-treated individuals, elite controllers, and viraemic progressors. *p < 0.001, **p < 0.01, *p < 0.05, trend = p < 0.08.**

In summary, functional analysis shows cytokine (CD56^{bright}CD16⁻) producing capabilities of NK cells do not differ in ECs compared to HCs. A significant reduction in functional activities of total NK cells was shown by the decrease of cytotoxic capacity in all PLWH compared to HCs and a decrease in cytokine production activities in VPs compared to all the other study groups. The metabolic profiles (Glut1, CD98, and CD71) were similar in ECs when compared to HC's and ART-treated individuals, however, were significantly elevated in VPs. Overall, NK cell cytotoxic function, cytokine production, and nutrient transporter profiles do not differ in ECs when compared to HCs.

CHAPTER 4: DISCUSSION AND CONCLUSION

HIV-1 and AIDS-related deaths remain one of the leading causes of mortality in sub-Saharan Africa. South Africa harbours nearly 8 million people living with HIV-1. HIV-1 elite controllers are an extremely rare set of individuals who can suppress viral replication to an undetectable level without the use of ART and so may hold clues as to what constitutes a functional cure. There is a scarcity of data describing HIV-1 elite controllers, especially in African populations from sub-Saharan African countries, where HIV-1 is most prevalent. The factors described in HIV-1 elite control so far include the inheritance of protective HLA class I alleles and superior CD8⁺ T cell responses. This study focused on understanding innate immune immunological mechanisms to address some of the priorities for HIV-1 cure research, mainly how host factors control HIV-1 replication in the absence of therapy.

In particular, this study aimed to determine NK cell frequency, phenotype, function, and metabolic profiles from elite controllers and compare them to HIV-1 viraemic progressors, ART-treated individuals, and HIV-1 uninfected individuals. First, we found the frequency and distribution of NK cells and their subsets were similar in elite controllers compared to ART-treated and HIV-1 uninfected individuals but were overall lower in viraemic progressors compared to other study groups except for the CD56^{neg}CD16⁺ NK cell subset which was higher in viraemic progressors. Second, we found elite controllers had similar NKG2A, NKG2C, PD-1, and CD57 expression on total NK cells when compared to ART-treated and HIV-1 uninfected individuals and showed elevated expression of CD38 and CD69 when compared to ART-treated individuals. Third, degranulation was reduced in NK cells of all PLWH but IFN- γ production was maintained in elite controllers to levels observed in HIV-1 uninfected individuals. Interestingly, IFN- γ production in CD56^{bright}CD16⁻ was higher in elite controllers when compared to ART-treated individuals. Lastly, the NK cell metabolic activity, as measured by Glut1, CD98, and CD71, was comparable in elite controllers, ART-treated, and HIV-1 uninfected individuals but significantly elevated in viraemic progressors compared to other study groups.

Alterations in the frequency and phenotype of NK cells and their subsets during HIV-1 infection have been previously reported (Meier et al., 2005, Zhao et al., 2020), however, there is a paucity of data from spontaneous HIV-1 controllers in African

populations. Determining NK cell redistribution may further our knowledge in understanding some of the factors associated with HIV-1 control since NK cell subsets are linked to key effector functions. We showed that individuals who achieved elite control status were able to maintain their peripheral blood NK cell population, while individuals with progressive disease experienced a significant decrease in NK cells. Furthermore, elite controllers showed no evidence of redistribution of NK cell subsets, which has been reported as a subsequent consequence of chronic viraemic infection (Alter et al., 2005). These data support findings from an American cohort which showed no differences in NK cell subsets between elite controllers, viraemic controllers, ART-treated individuals, and healthy controls (Pohlmeyer et al., 2019). In addition, an earlier report showed no differences in HIV-1 controller NK cell subset frequencies compared to HIV-1 uninfected individuals (Vieillard et al., 2010). However, the study showed an increase in CD56^{bright}CD16⁻ NK cells in HIV-1 progressors compared to HIV-1 controllers, which was not reflected in our data. As expected, individuals with progressive disease had an expanded CD56^{neg}CD16⁺ dysfunctional subset. This is in line with the overall accepted idea that the population of CD56^{neg}CD16⁺ NK cells increases with chronic viral infection, however since these cells are dysfunctional, they provide no overall viral control (Cao et al., 2021). In contrast, the CD56^{neg}CD16⁺ subset was present in low frequencies in elite controllers and ART-treated individuals and was comparable to frequency levels found in HIV-1 uninfected individuals. Vieillard et al whose data showed a similar pattern of CD56^{neg}CD16⁺ expression in HIV-1 controllers compared to HIV-1 uninfected individuals supported our findings (Vieillard et al., 2010). Altogether, we showed that the preservation of CD56^{bright}CD16⁻ and CD16^{dim}CD16⁺ NK cell compartments may be a feature of spontaneous control of HIV-1 infection.

Natural killer cell phenotypic alterations impact cellular function. A better understanding of cell surface markers may provide insight into some of the mechanisms of HIV-1 control. Our study showed the preservation of NKG2A expression in elite controllers, ART-treated, and HIV-1 uninfected individuals. We showed elevated NKG2A expression in elite controllers compared to viraemic progressors and comparable expression in ART-treated individuals. The interaction of NKG2A with HLA-E signals the inhibition of NK cell effector functions and thus the increase in NKG2A expression has previously been associated with HIV-1 viraemic

progression (Zhang et al., 2007). In a study where HLA-A was assessed in South Africans in the context of HIV-1 control, data showed that elevated levels of HLA-A, lead to increased expression of HLA-E and NKG2A-expressing cells. This was associated with impaired HIV-1 control, and thus endorsed the idea of HLA-E blockade as a therapeutic strategy (Ramsuran et al., 2018). In contrast, the interaction of NKG2A with KIRs/HLA-B may promote a fully licensed, functionally competent NK cell (Vieira et al., 2021). While this is possible, we cannot confirm this for our study as we did not measure this interaction. Furthermore, an investigation of NK cell responses after stimulation with HIV-1 infected CD4⁺ T cells showed that a significantly higher frequency of NKG2A was associated with HIV-1 control (Lisovsky et al., 2015). NKG2A⁺ NK cells had increased function supporting a more functionally competent profile (Lisovsky et al., 2015). Interestingly, a recent study showed evidence of an atypical, innate-like T cell subset called virtual memory T cells (CD45RA⁺, KIR⁺, CD8⁺, and/or NKG2A) which may play a role in the clearance of the HIV-1 viral reservoir in ART-treated individuals through dampening KIR-mediated inhibition (Jin et al., 2020). This, in combination with the elevated NKG2A expression in elite controllers compared to viraemic progressors, further promotes the advantageous effects of the presence of NKG2A and supports the idea that it may be beneficial during HIV-1 spontaneous control.

We found that NKG2C expression on NK cells was unaltered in elite controllers compared to ART-treated and HIV-1 uninfected individuals and was decreased in elite controllers compared to viraemic progressors. The interaction of NKG2C with HLA-E promotes the activation of the NK cell effector functions (Gondois-Rey et al., 2017). NKG2C is associated with NK cell memory and hence its presence has been associated with HIV-1 control (Gondois-Rey et al., 2017). Moreover, NKG2C gene deletions were detected in individuals who progressed more rapidly toward a disease state (Thomas et al., 2012). In addition, NK cell memory has previously been associated with CD57, a marker of terminal differentiation. NK cells expressing increased levels of CD57 were described as functionally superior and are associated with HIV-1 control (Lopez-Verges et al., 2010, Tomescu et al., 2021). We found the expression of CD57 on NK cells in elite controllers remained comparable to ART-treated and HIV-1 uninfected individuals but was significantly reduced in viraemic progressors compared to other study groups. In a recent study, HCMV seropositive

PLWH showed an expansion of the NKG2C⁺CD57⁺ NK cell subset with superior effector functions and advanced NK cell memory (Peppas et al., 2018). The NKG2C⁺CD57⁺ subset has also been associated with HIV-1 control and reduced leukaemia relapse (Cichocki et al., 2019). Findings showed that NKG2C⁺CD57⁺ NK cells were expanded in an HIV-1 high-risk group of men who have sex with men and raises the possibility that NKG2C and CD57 are markers for NK cell memory (Florez-Alvarez et al., 2020). Although we did not measure NK cell memory directly or use a comprehensive panel of memory markers to define NK cell memory phenotype, we observed a positive correlation between the expression of NKG2C and CD57 on NK cells in all study participants, but mainly in the HIV-1 uninfected individuals and viraemic progressors, suggesting that these groups may have more NK memory cells present. Since these correlations were not observed in elite controllers or ART-treated individuals, it suggests that the expansion of these cells may be driven largely by HCMV infection and less by HIV-1 infection. Whether NK cell memory subsets observed in this study are due to HCMV infection or to HIV-1 exposure is unknown and requires further investigations, however, given that the prevalence of HCMV in South Africa is above 90% (Cannon et al., 2010) and that almost all PLWH are coinfecting with the virus (Bates and Brantsaeter, 2016), it is likely that NK cell memory responses are HCMV driven and do not play a considerable role in HIV-1 spontaneous control. Prior HCMV exposure could also be inducing trained immunity responses in NK cells. Trained immunity leads to superior cytokine production, inherited by daughter cells, in response to non-related pathogens through epigenetic reprogramming of the innate immune cells (Kleinnijenhuis et al., 2014). We observed a negative correlation between the expression of CD57 on NK cells and viral load and a positive correlation with CD4⁺ T cell count which suggests that NK cell maturation may mark functionally competent NK cells associated with controlled HIV-1 infection as shown in other studies (Lopez-Verges et al., 2010, Nielsen et al., 2013).

Our studies showed that the activation marker, CD38, was increased in viraemic progressors and elite controllers compared to ART-treated individuals, as well as in viraemic progressors compared to HIV-1 uninfected individuals. As expected, the expression of CD38 on NK cells correlated positively with viral load and negatively with CD4⁺ T cell counts (Gonzalez et al., 2009). CD38 has previously been shown to be upregulated on T cells in the presence of HIV-1 infection (Klatt et al., 2013). HIV-1

infection causes chronic immune activation increasing the proliferation and differentiation of naïve and memory CD4⁺ T cells leading to the increased expression of CCR5, making cells more susceptible to the virus (Ta et al., 2022). Thus, chronic immune activation is the force driving the decline in circulating CD4⁺ T cells associated with AIDS and is therefore a stronger marker of disease progression than CD4⁺ T cell counts and/or viral loads (Lu et al., 2021, Froebel et al., 2000). Although elite controllers can suppress viral replication to undetectable levels, the virus still causes chronic T cell immune activation, which is significantly reduced with the administration of ART (Hatano et al., 2013). This pattern of T cell activation is replicated in our chronic NK activation data. We showed no difference in the expression of CD38 on NK cells in individuals with an advanced disease state compared to elite controllers. These data oppose findings from a Mexican cohort that showed elevated CD38 expression in PLWH in a progressive disease state, but not in elite and viraemic controllers (Kuri-Cervantes et al., 2014). It has been well reported that natural control was achieved in individuals who were able to maintain low levels of NK cell activation similar to those of HIV-1 uninfected (Kuri-Cervantes et al., 2014). We noted positive and negative correlations between viral load and CD4⁺ T cells, respectively, with CD38-expressing NK cells. Thus, this brings into question whether CD38 on NK cells is a reliable marker of disease progression as has been established for T cells.

CD69 is another activation marker and is expressed on T cells within 2-4 hours after stimulation in *in vivo* experiments, however from 4-6 hours, CD69 expression slowly diminishes (Cibrian and Sanchez-Madrid, 2017). Intriguingly, our study showed elevated CD69 expression on NK cells in elite controllers compared to ART-treated and HIV-1 uninfected individuals, but not in viraemic progressors. Since immune activation has previously been associated with progressive disease (Joag et al., 2016), we expected the expression of CD69 to correlate with viral load and CD4⁺ T cell counts, however, our data did not correspond with these findings. Previously reported studies from HIV-1 exposed but seronegative cohorts showed that viral control was attributed to an increase in the expression of CD69 on total NK cells (Ravet et al., 2007, Tomescu et al., 2010). In addition, the upregulation of CD69 expression on CD4⁺ T cells was associated with a novel combination of cytokines identified in females who were able to spontaneously suppress HIV-1 viral replication *in vitro* (Jacobs et al., 2017). We noted a decrease in CD69 expression on NK cells in ART-treated

individuals compared to HIV-1 uninfected controls which was supported by data from an English cohort (Goodier et al., 2003). In contrast, in a Ugandan, ART-treated cohort, CD69 expression on NK cells was increased in comparison to HIV-1 uninfected individuals (Nabatanzi et al., 2019). A notable difference between these two cohorts was the difference in the duration of ART. Patients received ART for at least twelve months in the English cohort, while the Ugandan cohort received ART for at least seven years. Taken together, these findings suggest ART may restore/reduce NK cell activation, however, this could be dependent on the duration of treatment. The important presence of CD69 may act as the initial activation required for effector functions to proceed without the constant immune activation and proinflammatory state that the expression of CD38 on NK cells may induce.

Often the consequence of immune activation caused by chronic viral infection is an increase in immune exhaustion which can be measured by the upregulation of the immune checkpoint inhibitor, PD-1. As expected, we found PD-1 expression was lower in elite controllers compared to other studied groups while viraemic progressors showed an elevated PD-1 profile compared to elite controllers and HIV-1 uninfected individuals. Supporting these findings, low levels of PD-1 and other immune checkpoint inhibitors were expressed on NK cells in HIV-1 controllers and HIV-1 exposed-seronegative individuals compared to viraemic progressors (Taborda et al., 2015). In addition, low levels of PD-1⁺CD38⁺ NK cells were reported in an HIV-1-exposed but uninfected cohort (Lima et al., 2017). Zhao et al reported increased PD-1 expression on NK cells during HIV-1 disease progression which was consistent with our data (Zhao et al., 2020). The increase in PD-1 expression on NK cells was associated with limited proliferative capacity and may have contributed to NK cell dysfunction and lack of viraemic control (Zhao et al., 2020). Interestingly, we show similar PD-1 expression on NK cells in elite controllers compared to healthy controls, and ART-treated individuals. Moreover, our data also showed no differences in PD-1 expression on NK cells from ART-treated and viraemic progressors. This in part supports data from Norris et al who showed no difference in PD-1 expression on NK cells in ART-treated PLWH compared to viraemic progressors, however, they did report a significant increase in PD-1 expression in ART-treated compared to a healthy control group (Norris et al., 2012). These findings highlight that NK cell exhaustion may take years to reverse, despite ART, and further depicts the necessity for new

treatment strategies. Immune checkpoint inhibition has been studied in T cells and has been shown to restore function during chronic infection and cancers (Duan and Liu, 2022). Perhaps, more enticing is the use of PD-1 as a blockade in NK cells which has significantly improved function by reversing immune exhaustion in PLWH (Porichis et al., 2018). We showed that individuals who control HIV-1 infection express low levels of PD-1 thereby evading NK cell exhaustion. The use of PD-1 inhibitors, together with ART, could be a novel therapeutic strategy used for the future treatment of HIV-1.

Natural killer cellular function is determined by cytotoxic capabilities and cytokine production (Bjorkstrom et al., 2022). Cytotoxic NK cells release proteins such as perforin and granzymes for targeted cell killing. These proteins are encapsulated within a membrane with high concentrations of lysosomal-associated membrane protein 1/CD107a. By quantifying the expression of CD107a, the degranulation/cytolytic activity of NK cells can be measured indirectly (Alter et al., 2004). Our study found that CD107a/degranulation was reduced in ART-treated individuals and viraemic progressors compared to HIV-1 uninfected individuals in total NK cells. These findings were similar to a recent single-cell transcriptomic analysis of unstimulated NK cells from PLWH which showed a reduction in cytotoxic and cytokine-producing genes compared to healthy individuals (Costanzo et al., 2018). Lower levels of CD107a were noted in perinatally exposed children living with HIV-1 compared to HIV-1 exposed but uninfected, regardless of stimulation (Ballan et al., 2007). We found a positive correlation between CD107a and CD4⁺ T cells in the elite controller group. This supported data from HIV-1 exposed, seronegative cohorts which found an association between elevated levels of CD107a with higher CD4⁺ T cell counts (Ravet et al., 2007, Lima et al., 2017). Overall, these data suggest HIV-1 may reduce NK cellular cytotoxicity while enhanced NK cytotoxic capabilities observed in elite controllers compared to other HIV groups may contribute to HIV-1 spontaneous control.

Another measure of NK cell function is the production of IFN- γ which plays an important role in enhancing T cell recognition by increasing HLA expression, T cell differentiation, macrophage functioning, and the induction of the crosstalk between NK cells and dendritic cells (Paolini et al., 2015). We found that IFN- γ production in total NK cells and CD56^{neg}CD16⁺ was similar in elite controllers compared to ART-treated and HIV-1 uninfected individuals but was reduced in viraemic progressors. IFN- γ

production in the CD56^{bright}CD16⁻ was elevated in elite controllers compared to ART-treated individuals but was reduced in viraemic progressors compared to elite controllers and HIV-1 uninfected individuals in the CD56^{dim}CD16⁺ subset. IFN- γ production correlated negatively with viral load and positively with CD4⁺ T cell counts in all PLWH. A previous report demonstrated increased IFN- γ production, among other cytokines, during HIV-1 viraemic control compared to viraemic progression (Zhang et al., 2021). This is consistent with HIV-1 causing NK cell dysfunction in progressive disease which elite controllers may be able to circumvent. Together, HIV-1 spontaneous control may be achieved by enhanced cytokine production which enables both the innate and adaptive immune systems to work together to promote HIV-1 viral control. Constant exposure to HIV-1 causes reduced IFN- γ production leaving PLWH more susceptible to progressive disease.

There have been many inconsistent reports on the re-establishment of NK cell function on ART. We observed negative correlations between NK cell function (CD107a/degranulation and IFN- γ production) in ART-treated individuals. This suggests that ART may not restore NK cell function and could be one of the lasting effects of HIV-1 infection. Data from a study showed that PLWH on treatment for at most three years showed no correlation between IFN- γ production and CD4⁺ T cell count, however, once they crossed the threshold of treatment of at least 4 years, their IFN- γ production negatively correlated with CD4⁺ T cell counts (Watanabe et al., 2019). Chehimi et al reported incomplete recovery of NK cell function after 52 weeks of ART (Chehimi et al., 2007), while other reports showed a full recovery of NK cell function after 2 years of treatment (Mavilio et al., 2003, Luo et al., 2017). In support of our data, a Ugandan cohort reported suboptimal NK cell function despite 4 years of ART (Bayigga et al., 2014). Given that most of the evidence of NK cell recovery for ART is in cohorts with short treatment periods, emphasis on early treatment following diagnosis is recommended by current treatment guidelines could improve the overall recovery of NK cell function. Collectively, our data suggest that ART does not fully restore NK cell function. Since there are over 10 million PLWH on ART in sub-Saharan Africa who have been on treatment for more than 10 years, it is exceptionally important to know the effects on the innate immune system.

Cellular metabolism is responsible for cell signalling and gene transcription and overall cellular function. Viruses have been reported to disrupt these pathways to further their

replication cycles (Girdhar et al., 2021). Our findings showed augmentation of cellular metabolism during HIV-1 progressive disease compared to other study groups, measured by the increase in the expression of Glut1, CD98, and CD71 on NK cells. Surprisingly, we found no changes in Glut1, CD98, and CD71 in elite controllers compared to ART-treated and healthy individuals. The upregulation of nutrient transporters during progressive disease suggests that HIV-1 activated the NK cells which induced a metabolic switch from oxidative phosphorylation to glycolysis (Cong, 2020). Even though glycolysis is less efficient in the production of ATP, necessary precursors were made during glycolysis that were essential for NK cell effector functions (Butterfield et al., 2020). Our data indicated that during progressive disease, HIV-1 upregulated glycolysis, measured by the upregulation of Glut1, CD98, and CD71, and used the glycolytic precursors to accelerate its replication cycle (Butterfield et al., 2020). This is supported by data that showed the upregulation of CD98 and CD71 on NK cells after retroviral exposure in a mouse model (Loisel-Meyer et al., 2012, Palmer et al., 2014a, Kang and Tang, 2020). In addition, numerous findings showed that the upregulation of Glut1 on CD4⁺ T cells and monocytes is associated with an increased HIV-1 reservoir pool and poor disease outcome (Loisel-Meyer et al., 2012, Butterfield et al., 2017, Kang and Tang, 2020, Tarancon-Diez et al., 2019). Our data on Glut1 expression on NK cells aligned with reports which showed the upregulation of Glut1 on CD4⁺ T cells and monocytes during untreated HIV-1 infection (Palmer et al., 2014b, Palmer et al., 2014a). These studies went on to show that ART partially normalised the expression of Glut1 on CD4⁺ T cells compared to healthy controls but made no difference in the expression of Glut1 on monocytes before and after treatment (Palmer et al., 2014b, Palmer et al., 2014a). In contrast, findings showed Glut1 expression on total NK cells was lower in PLWH compared to healthy individuals and CD98, CD71, and CD36 (fatty acid metabolism) was similar in PLWH compared to a healthy control group (Sun et al., 2022). Together, our data suggested that elite control was achieved by maintaining low viral stimulation resulting in a partially activated state (comparable to ART-treated and HIV-1 uninfected controls), which supports oxidative phosphorylation to meet energy demands for the maintenance of NK cell effector functions. Viral stimulation caused metabolic dysregulation and subsequently reduced viral control. Therefore, we showed the upregulation of certain NK cell pathways may be indicative of progressive disease.

In addition, we observed a significant increase in CD98 expression on all NK cell subsets (CD56^{bright}CD16⁻, CD56^{dim}CD16⁺, CD56^{neg}CD16⁺) in viraemic progressors compared to other study groups, we also found a positive correlation between CD98 expression and IFN- γ production in total NK cells in elite controllers and healthy individuals. We also observed a positive correlation between CD98 and cytotoxicity in healthy individuals. This is similar to data that showed elevated levels of CD98 in NK cell subsets (CD56^{bright}CD16⁻ and CD56^{dim}CD16⁺) which was essential for IFN- γ production (Jensen et al., 2017). It was reported that NK cells with an exhausted profile showed metabolic defects, mitochondrial dysfunction, and defective oxidative phosphorylation, causing a reduction in IFN- γ production, similar to that of CD8⁺ T cells during chronic HIV-1 infection (Cubero et al., 2019). In support, we showed an exhausted NK cell profile with reduced IFN- γ production during viraemic progression, however, we were unable to correlate these findings to mitochondrial fitness. Despite long-term infection, elite controllers evade immune exhaustion while producing significantly higher amounts of IFN- γ compared to viraemic progressors. Since elite controllers show no difference in nutrient transporter expression compared to healthy controls, oxidative phosphorylation may have a role in the spontaneous control of HIV-1, however future studies on NK cell oxygen consumption rates can confirm these findings. We also showed raised levels of CD71 on CD56^{dim}CD16⁺ NK cells during progressive disease compared to other study groups, and significantly higher levels of CD71 on CD56^{neg}CD16⁺ in ART-treated individuals compared to viraemic progressors. In an African cohort, iron accumulation in macrophages was shown to promote HIV-1 replication and was associated with poor patient outcomes (McDermid et al., 2007). In mouse models, NK cells upregulated transferrin receptors in response to retroviral stimulus, and iron deficiency caused activation and metabolic defects which later reduced NK cell degranulation and IFN- γ production (Littwitz-Salomon et al., 2021). Together, these findings demonstrated the link between immune metabolism and function.

This study has several limitations. Our sample size was limited as HIV-1 elite controllers are an extremely rare phenotype of individuals. Our cross-sectional analyses did not allow for NK cell profiling over time. Future studies to increase the sample size, to improve statistical power, could prove hard to achieve as ART is currently recommended to all PLWH. In addition, the majority of NK cells are found in

tissues; and our analysis of peripheral circulating cells may not accurately reflect what is happening in the tissues. Determining the HCMV status of study participants and characterising NK cell education and memory responses during HIV-1 spontaneous control would improve gaps in knowledge in clearly defining memory responses that could be induced in vaccine strategies. Profiling trained immune responses in elite controllers could be a promising area of research as studies are ongoing to determine elite control-like immune responses to inform HIV cure. Although we analysed the expression profiles of glucose, amino acid, and iron metabolism on NK cells, it would be of interest for prospective studies, to determine the role of fatty acid immunometabolism in HIV-1 control. Additional confirmatory assays on NK cells could verify the role of oxidative phosphorylation during HIV-1 spontaneous control and mitochondrial dysfunction during HIV-1 progressive disease. These data would further our understanding of HIV-1 metabolic dysregulation.

Natural killer cell immunity is paradoxical in that early immune activation appears to be valuable during HIV-1 spontaneous control, but chronic immune activation results in altered phenotype, function, and metabolic activity and ultimately poor patient outcomes. While existing HIV-1 treatment halts HIV-1 replication and the progression toward AIDS, the viral reservoir persists, causing NK cell functional dysregulation. Remarkably, our findings show that NK cell frequency, distribution, phenotype, function, and metabolic profiles were preserved during the spontaneous control of HIV-1 infection. Although these findings suggest that the integrity of the NK cell remains intact during HIV-1 elite control, further functional and mechanistic studies are required to answer how spontaneous control is achieved during HIV-1 infection. We showed elite control was supported by an inhibited, mature NK cell profile that maintained low levels of immune exhaustion accompanied by preserved low metabolic activities and superior functional competencies. These new insights could be exploited for novel immunotherapeutic designs for African populations of PLWH.

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APPENDIX A

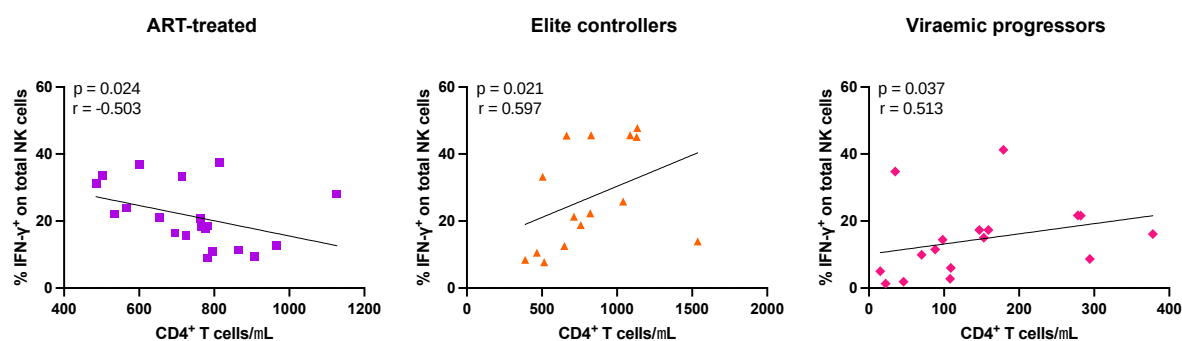


Figure A.1: Correlations between the production of IFN- γ on total NK cells and CD4⁺ T cell counts in ART-treated individuals, elite controllers and viraemic progressors. Lines are calculated by using linear regression. Each symbol represents a study participant. Significant p values and Spearman rho (r) values are shown.

APPENDIX B

ABSTRACT: WITS FACULTY OF HEALTH SCIENCES BIENNIAL RESEARCH DAY

Natural killer cell phenotypic, functional, and nutrient transporter profiles during spontaneous control of HIV-1 infection in South Africans

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Natural killer (NK) cells are innate effector cells that play an important role in the control of HIV-1 infection. HIV-1 elite controllers (EC) have the ability to control the virus in the absence of antiretroviral therapy (ART) and may provide a model for functional cure. Understanding factors associated with spontaneous HIV-1 control in African populations may aid in the development of future drug strategies. We measured the phenotypic, functional, and nutrient transporter profiles of NK cells on cryopreserved peripheral mononuclear cells (PBMCs) from HIV-1 EC (n=16), viraemic progressors (VP) (n=19), ART-treated individuals (n=20), and HIV-1 uninfected donors (n=21) from Johannesburg, South Africa. NK cell phenotypic profiles were characterised by assessing the expression of NK cell receptors (NKG2A and NKG2C), and exhaustion marker (PD-1). Functional (CD107a and IFN- γ) and nutrient transporter (CD98 and Glut1) profiles were assessed following stimulation with MHC devoid cell line. Both phenotypic and functional profiles were characterised using multicolour flow cytometry. The frequency of total NK cells was lower and the frequency of CD56⁻CD16⁺ NK cell subset was higher in VP compared to other patient groups (p<0.05). Compared to VP, HIV-1 EC displayed increased expression of NKG2A⁺ (p=0.048), decreased expression of NKG2C⁺ (p=0.019), and decreased expression of PD-1⁺ (p=0.009) on total NK cells as well as decreased expression of CD98 (p=0.034) and Glut1 (p=0.015) in the bright NK cell subset. Compared to ART-treated individuals, HIV-1 EC displayed higher production of CD107a⁺ (p=0.043) and IFN- γ ⁺ (p=0.007) in the bright NK cell subset. An increased inhibitory versus activation profile in HIV-1 EC

compared to VP may promote a more educated NK cell phenotype with superior functional competencies. Further, a decreased metabolic activity profile in EC may contribute to less progressive disease. Overall, a preserved NK cell phenotype and function may play a role in the spontaneous control of HIV-1.

Natural killer cells from HIV-1 elite controllers have similar maturation and functional profiles compared to HIV-uninfected controls

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Natural killer (NK) cells are innate effector cells that play a crucial role in the control of HIV-1 infection. HIV-1 elite controllers (EC) maintain low viral loads in the absence of antiretroviral therapy (ART) and may hold clues for a functional cure. We investigated innate immune factors associated with spontaneous HIV-1 control in the African population. Multicolour flow cytometry was used to analyse phenotypic markers on NK cells from cryopreserved peripheral mononuclear cells of EC (n=15), viraemic progressors (VP) (n=19), ART-treated individuals (ART-treated) (n=20), and HIV-1 uninfected donors (HC) (n=21) from Johannesburg, South Africa. Functional and metabolic profiles were assessed in the same individuals following stimulation with MHC devoid cell line. EC did not differ in the expression of CD57, IFN- γ , CD71, and CD98 when compared to HC and ART-treated individuals. NK cells of EC displayed increased expression of CD57⁺ (p=0.02) and higher production of IFN- γ ⁺ (p=0.03) when compared to the VP group. In contrast, increased expression of CD71 (p=0.009) and CD98 (p=0.05) was observed in VP compared to EC as well as ART-treated and HC groups. CD57 expression (p=0.002; r=-0.41) and IFN- γ production (p=0.02; r=-0.31) correlated negatively with viral load, whereas CD71 (p=0.01; r=0.37) and CD98 expression (p=0.004; r=0.39) positively correlated with viral load in the total HIV-1-infected group. NK cells of EC were similar to HC and ART-treated in maturation, functional and metabolic profiles. Overall, more mature, and functionally competent NK cells with reduced metabolic profiles may contribute to less progressive disease.

NATURAL KILLER CELL PHENOTYPIC, FUNCTIONAL, AND METABOLIC PROFILES ARE NOT DIFFERENT IN HIV-1 ELITE CONTROLLERS COMPARED TO UNINFECTED INDIVIDUALS

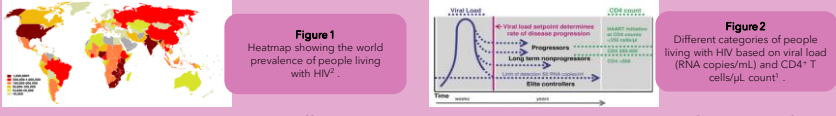


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HIV-1 remains one of the leading causes of death in sub-Saharan Africa (Figure 1). Understanding elite control – a rare phenotype characterised by the ability to spontaneously suppress HIV-1 replication to an undetectable limit in the absence of anti-retroviral treatment (ART), while maintaining a CD4⁺ T cell count > 500/ μ L – serves as the best model for a functional cure (Figure 2)¹.



Natural killer (NK) cells are innate effector cells that play an important role in the first-line defence against infection and have been shown to be crucial in the control of HIV-1³. As the first-line response to foreign pathogens, their effector functions can be harnessed for therapeutic strategies against HIV-1 infection. NK cells can be divided into subsets based on their relative expression of CD56 and CD16⁴. These subsets relate to important effector functions of NK cells (Table 1).

Table 1: Natural killer cell subset classification and characteristics			
	CD56 ^{dim} CD16 ⁺	CD56 ^{dim} CD16 ⁺	CD56 ^{dim} CD16 ⁺
Percentage in peripheral blood	5-10%	80-90%	< 5% in healthy individuals, expanded during chronic viral infection
Cytokine secretion	+++	++	-/+
Cytotoxicity	+	+++	-/+
Metabolic activity	+++	++	-/+

NK cell effector functions are influenced by cellular metabolism. Viral infections cause metabolic reprogramming to support high energy demands in an effort to control infection. Metabolic changes are usually accompanied by an increase in nutrient transporters⁵.

Few reports have characterised NK cells during spontaneous control of HIV-1 in a high-incidence setting, such as South Africa.

To assess natural killer cell **phenotype**, **function**, and **nutrient transporter** profiles associated with HIV-1 elite control.

1 Study participants

Age- and sex-matched

n = 21	n = 20	n = 15	n = 19
HIV-1 uninfected	ART-treated	Elite controllers	Viraemic progressors
CD4 > 500	CD4 > 500	CD4 > 500	CD4 < 350
VL N/A	VL < LDL	VL < LDL	VL > 10 000

CD4, CD4⁺ T cells/ μ L; VL, HIV-1 viral RNA copies/mL; N/A, not applicable; LDL, lower than the detectable limit

2 Isolation of peripheral blood mononuclear cells from whole blood

Whole blood

Centrifugation

Ficoll

Plasma

Peripheral blood mononuclear cells

Ficoll

Granulocytes

Erythrocytes

3 Flow cytometry

4 Assessment of markers

Nutrient transporters

- Iron modulation - CD71
- Amino acid transport - CD98

Phenotypic marker

- Maturation - CD57

Functional marker

- Cytokine production - IFN- γ

Natural killer cell

Additional markers were assessed (data not shown)

Introduction

Conclusion

Aim Methods

Results

Discussion

A more mature and functionally competent natural killer cell with reduced metabolic activity may contribute to an elite control phenotype in HIV-1 infection.

- The **maturation status, functional capacity, and metabolic activity** of NK cells from elite controllers were comparable to ART-treated and healthy individuals.
- A **mature and highly differentiated phenotype promotes functional competence of NK cells** and may be associated with HIV-1 control⁶.
- IFN- γ production allows for immune modulation⁷, enhancing the association of NK cell effector functions with spontaneous control.
- A **significant decrease in NK cell function** in the ART-treated group compared to the healthy individuals shows that even with ART intervention NK cell function was not fully restored.
- NK cells reprogram their cellular metabolism by **increasing their nutrient uptake** after viral infection.
- Increase in nutrient transporters **promotes the expansion of potent, differentiated NK cells to counteract viral infection**⁸.
- Metabolic reprogramming **increases the production of precursor molecules** that HIV-1 uses to its advantage to speed up its own **replication cycle**⁹.
- Thus, the **increase in NK cellular metabolic activity** may be associated with **progressive disease**.

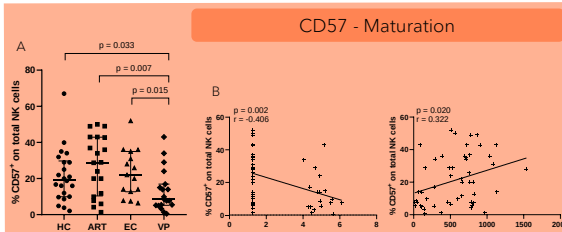


Figure 3: (A) The frequency of CD57⁺ on total NK cells in all study groups. The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between CD57⁺ on total NK cells and viral load and CD4⁺ T cell counts in total HIV-1-infected individuals. Each symbol represents a study participant. Lines are calculated using linear regression. Significant p values and spearman rho (r) values are shown.

CD57 expression on total NK cells did not differ in elite controllers compared to healthy and ART-treated individuals but was lower in viraemic progressors (Figure 3 A). CD57 expression on total NK cells correlated negatively with viral load and positively with CD4⁺ T cells (Figure 3 B).

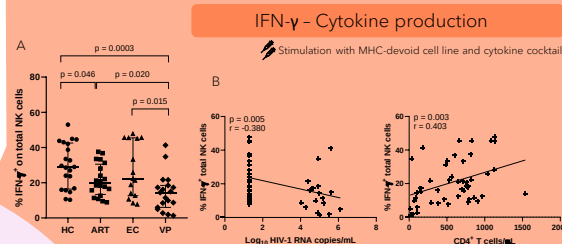


Figure 4: (A) IFN- γ production on total NK cells in all study groups. The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between IFN- γ production on total NK cells and viral load and CD4⁺ T cell counts in total HIV-1-infected individuals. Each symbol represents a study participant. Lines are calculated using linear regression. Significant p values and spearman rho (r) values are shown.

IFN- γ production on total NK cells did not differ in elite controllers compared to healthy and ART-treated individuals but was lower in viraemic progressors compared to the other study groups, and in ART-treated individuals when compared to healthy individuals (Figure 4 A). IFN- γ production on total NK cells correlated negatively with viral load and positively with CD4⁺ T cells (Figure 4 B).

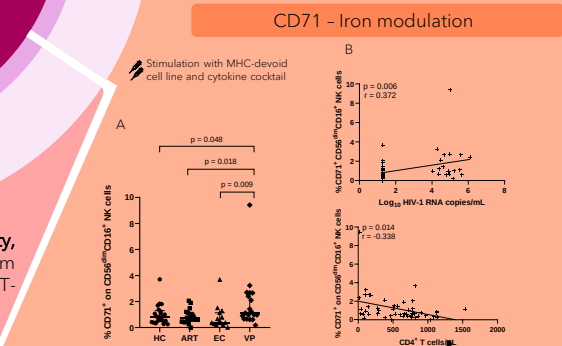


Figure 5: (A) The frequency of CD71⁺ on CD56^{dim}CD16⁺ NK cells in all study groups. The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between CD71⁺ expression on CD56^{dim}CD16⁺ NK cells and viral load and CD4⁺ T cell counts in total HIV-1-infected individuals. Each symbol represents a study participant. Lines are calculated using linear regression. Significant p values and spearman rho (r) values are shown.

CD71 expression on CD56^{dim}CD16⁺ NK cells did not differ in elite controllers compared to healthy and ART-treated individuals but was higher in viraemic progressors (Figure 5 A). CD71 expression on CD56^{dim}CD16⁺ NK cells correlated positively with viral load and negatively with CD4⁺ T cell counts (Figure 5 B).

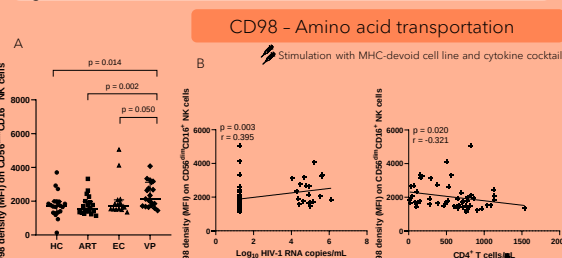


Figure 6: (A) The density of CD98 on CD56^{dim}CD16⁺ NK cells in all study groups. The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. (B) Correlations between CD98 density on CD56^{dim}CD16⁺ NK cells and viral load and CD4⁺ T cell counts in total HIV-1-infected individuals. Each symbol represents a study participant. Lines are calculated using linear regression. Significant p values and spearman rho (r) values are shown.

CD98 density on CD56^{dim}CD16⁺ NK cells did not differ in elite controllers compared to healthy and ART-treated individuals but was higher in viraemic progressors (Figure 6 A). The expression of CD98 on CD56^{dim}CD16⁺ NK cells correlated positively with viral load and negatively with CD4⁺ T cell counts (Figure 6 B).

Acknowledgments

Study participants

Cell biology lab - Centre of HIV & STI



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The role of natural killer cell responses to natural control of HIV-1 infection in South Africans

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A cure for HIV is still deemed a global research priority. HIV elite controllers (ECs) are a subset of people living with HIV (PLWH) who spontaneously control HIV in the absence of antiretroviral therapy (ART). These unique individuals represent a natural model for a functional cure. Since ECs provide continued insights into the mechanisms of host control, we evaluated natural killer (NK) cell phenotypic, functional, and metabolic response to HIV-1 spontaneous control. Multicolour flow cytometry was used to determine NK activation (NKG2C, CD69, and CD38), inhibition (NKG2A), maturation (CD57), exhaustion (PD-1), functional (CD107 and IFN- γ), and metabolic (Glut1, CD98, and CD71) profiles on cryopreserved peripheral blood mononuclear cells in PLWH: ECs (n=15), ART-treated individuals (ART-treated) (n=20), and viraemic progressors (VPs) (n=19), and HIV-1 uninfected healthy controls (HC) (n=21) from Johannesburg, South Africa. Functional and metabolic profiles were assessed after stimulation with the MHC-devoid cell line. The frequency of NK cells and their subsets in ECs were similar to HCs and ART-treated and altered compared to VPs. The expression of NKG2A, NKG2C, PD-1, and CD57 on NK cells were similar in ECs compared to ART-treated, and HCs and CD38 and CD69 were upregulated in ECs compared to the ART-treated group. CD107a expression was reduced in NK cells in all PLWH compared to HCs, whereas IFN- γ production was comparable across the ECs, ART-treated, and HC groups and lower in VPs. Metabolic profiles were similar in ECs compared to ART-treated and HCs and increased in VPs compared to other study groups. Together these findings show that NK cells from ECs have an inhibitory, mature profile with low levels of immune exhaustion and reduced metabolic phenotype

suggesting functional competence. These new insights could be employed in novel immunotherapeutic strategies for the treatment of HIV-1 in African populations.

The role of natural killer cell responses to the natural control of HIV-1 infection in South Africans

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? INTRODUCTION

HIV-1 and AIDS-related deaths remain one of the **leading causes of mortality** in sub-Saharan Africa¹.

HIV-1 **elite controllers** are a **rare** group of individuals able to **supress viral replication** in the **absence of antiretroviral therapy** and represent a model for a **functional cure** (Figure1)².

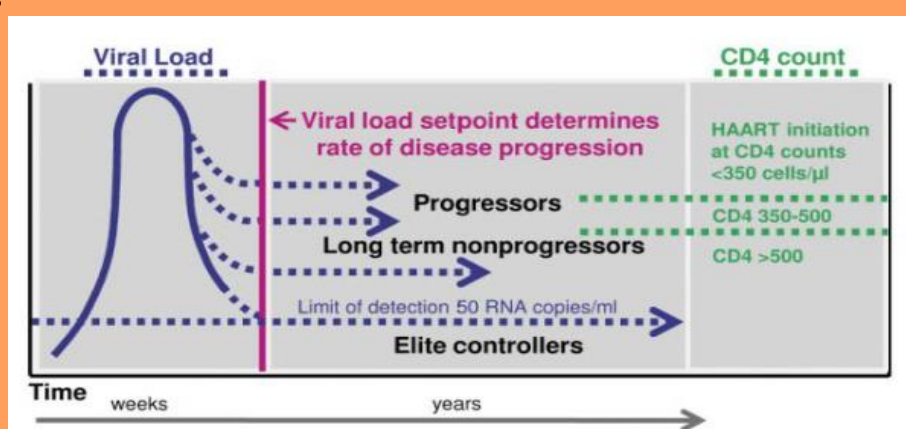


Figure 1: Different groups of people living with HIV-1 based on their viral load set point and CD4 count².

Natural killer (NK) cells are innate, effector cells that are crucial in the **control of HIV-1** infection³. Characterising their **phenotypic**, **functional**, and **metabolic** profiles may provide insight into the mechanisms of **host control**.

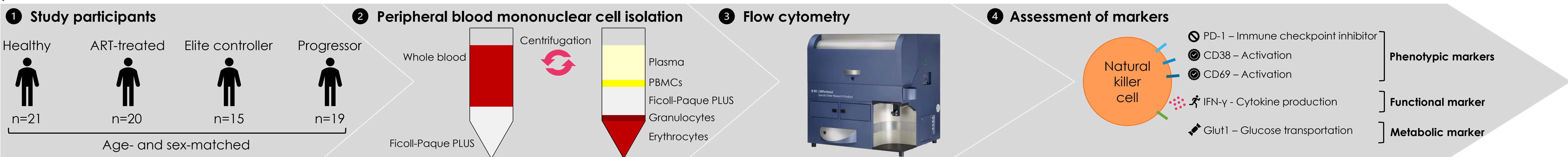
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ACKNOWLEDGEMENTS

Study participants
Cell Biology lab

METHOD



RESULTS

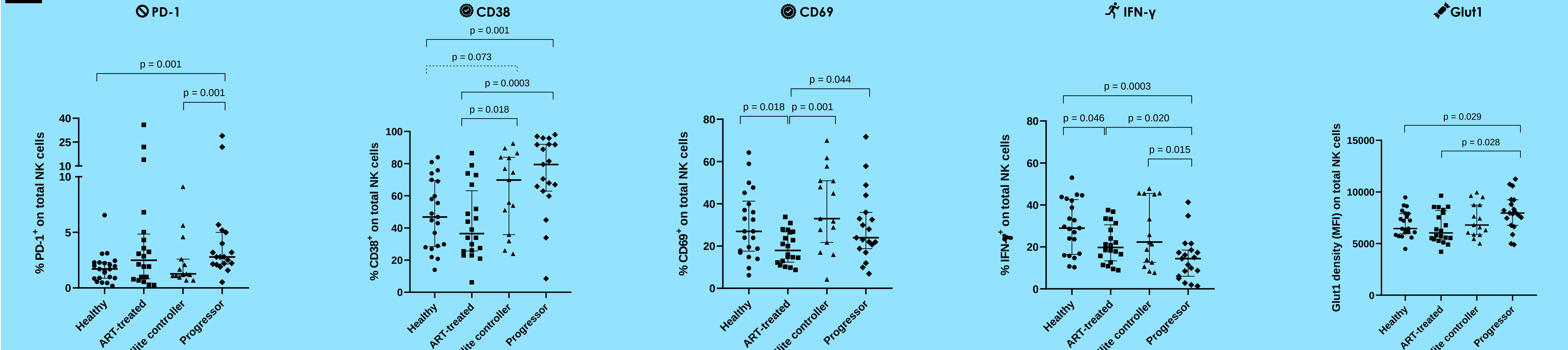


Figure 2: The frequency of PD-1⁺, CD38⁺, CD69⁺, and Glut1⁺ on total NK cells in all study groups. The horizontal lines represent the medians, and the error bars represent the 25th and 75th percentiles. Significant p-values are shown.

- PD-1 NK cell expression was **similar** in elite controllers compared to healthy and ART-treated individuals and **higher** in progressors
- CD38 and CD69 NK cell expression were **upregulated** in elite controllers compared to ART-treated individuals
- IFN-γ production was **comparable** across the elite controllers, healthy, and ART-treated individuals, but **lower** in progressors
- Glut1 was **similar** in elite controllers compared to healthy and ART-treated individuals and **increased** in progressors compared to other study groups

DISCUSSION AND CONCLUSION

Elevated levels of PD-1 are indicative of chronic immune exhaustion⁴. We show that **elite controllers are able to evade NK cell exhaustion**.

Increased expression of CD38 is a marker for chronic immune activation⁵. We show **immune activation in elite controllers in spite of their undetectable viral loads**.

The upregulation of CD69 on NK cells in elite controllers **suggests early immune activation is important for natural control**.

Elevated IFN-γ production in NK cells in elite controllers suggests **enhanced modulation** of the innate and adaptative immune systems and may play a role in **host control**⁶.

IFN-γ is reduced in ART-treated individuals compared to elite controllers This shows **ART does not restore NK cell function**.

HIV-1 causes upregulation of the Glut1 transporter inducing a metabolic shift towards glycolysis⁷. An **increase in Glut1 may be associated with progressive disease**.

A preserved NK cell phenotype with superior functional qualities and reduced metabolic activity may contribute to HIV-1 natural control in the African population.

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R14/49 Dr Christina Thobakgale and Prof Caroline Tiemessen

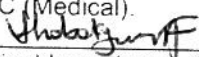
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CLEARANCE CERTIFICATE NO. M210231 MED21-01-069

NAME: Dr Christina Thobakgale and Prof Caroline Tiemessen
(Principal Investigator)
DEPARTMENT: School of Pathology
Centre for HIV and STI, Cell Biology Section
National Institute for Communicable Diseases
PROJECT TITLE: Innate immunological mechanisms of control and factors
driving inflammation in HIV controllers from a high
incidence setting in South Africa (ICIHSA)
DATE CONSIDERED: 26/02/2021
DECISION: Approved unconditionally
CONDITIONS: Sub-study under M190995 (previously M140925)
Prof CT Tiemessen
SUPERVISOR:
APPROVED BY: 
Dr C Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL: 07/05/2021

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

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Principal Investigator Signature

14/05/2021
Date

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R14/49 Professor CT Tiemessen, et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M190995

NAME: Professor CT Tiemessen, et al
(Principal Investigator)
DEPARTMENT: National Institute for Communicable Diseases
Centre for HIV and Sexually-Transmitted Infections
Sandringham

PROJECT TITLE: HIV-1 positive South African Elite and Long-term Controllers:
viral and host targets for functional cure strategies

DATE CONSIDERED: Ad hoc

DECISION: Approved unconditionally

CONDITIONS: Renewal of M140926

SUPERVISOR: Not applicable

APPROVED BY: 
Dr CB Penny, Chairperson, HREC (Medical)

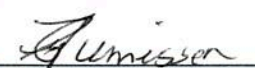
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I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. I **agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **September** and will therefore reports and re-certification will be due early in the month of **September** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).


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