

**Pre-HIV Circulating T Cell Profiles as Predictors of HIV Acquisition and Early Disease
Progression**

by

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Abstract

Heterogeneity exists in HIV susceptibility, with some individuals more susceptible and others resistant despite multiple exposures. Factors including genetics and immunological variables, contribute to this varying risk. Previous research highlighted a link between higher pre-HIV $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells and increased HIV acquisition and faster disease progression among South African women. However, contrasting results emerged in different populations, raising questions about $\alpha 4\beta 7$'s role as a predictor of HIV. Additionally, genetic variants were identified in *CD101* which correlated with heightened risk of HIV. We sought to validate these findings considering the impact of demographic variables and investigating the predictive role of additional immune subsets. We also aimed to assess immune phenotype correlating with *CD101* variants and their role in HIV risk.

Pre-HIV PBMCs from different cohorts, HVTN 503 and PP/COS, were analyzed, comprising of heterosexual participants from South and East Africa, respectively. Genotype data for *CD101* variants were available only for PP/COS and the participants categorized based on their variant's status. Flow cytometry was used to quantify immune cells in a blinded fashion and analyzed statistically to establish correlations with HIV.

The association between $\alpha 4\beta 7^{\text{hi}}$ expression and HIV acquisition exhibited variability between cohorts, with opposite trends observed. Sub-group analysis revealed differences in association by sex and country. $\alpha 4\beta 7^{\text{hi}}$ frequency inversely correlated with peak VL in PP/COS ($r = -0.38, p=0.024$), whereas in HVTN 503, it inversely correlated with mean CD4/CD8 ratio ($r = -0.32, p=0.042$) and predicted rapid CD4 decline (aHR=2.72, 95% CI=1.13–6.58, $p=0.026$). Likewise, Th17 cells were associated with faster CD4 decline (aHR=4.18, 95% CI=1.36–12.84, $p=0.013$) in HVTN 503. *CD101* variants were associated with altered levels

of CD101-expressing T cells, which potentially mediated their relationship with increased HIV acquisition.

Our data suggest that $\alpha 4\beta 7^{\text{hi}}$ and Th17 CD4⁺ T cells may predict HIV progression more consistently. The predictive value of these HIV-susceptible cells may be contingent on the characteristics of the studied population, and this should be carefully considered when defining their roles in HIV infection. Lastly, we provided an immunologic link between CD101 genotype and phenotype to better understand the relationship between *CD101* variants and HIV risk.

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Dedication

To my incredible husband and amazing daughter, Oluwafemi and Oluwaferanmi Oludimu, your unwavering support, love, and encouragement have been my greatest source of strength through this journey. Thank you for holding things down at home, for stepping in when I had to prioritize my research, and for motivating me to keep going, even when the path was challenging. This achievement would not have been possible without your sacrifices and understanding.

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List of Abbreviations

Ad5 – Adenovirus Type 5

AGM – African Green Monkey

AHR – Aryl Hydrocarbon Receptor

AIDS – Acquired Immune Deficiency Syndrome

ALIVE – AIDS Linked to the Intravenous Experience cohort

APC – Antigen Presenting Cells

APOBEC3G – Apolipoprotein B mRNA Editing Enzyme Catalytic subunit 3G

ART – Antiretroviral Therapy

BATF – B cell Activating Transcription Factor

Bcl-6 – B-Cell Lymphoma 6

bNAbs – Broadly Neutralizing Antibodies

BV – Bacterial Vaginosis

CCL – C-C Motif Chemokine Ligand

CCR – C-C Motif Chemokine Receptor

CD – Cluster of Differentiation

CNS – Central Nervous System

COS – Couples Observational Study

CRF – Circulating Recombinant Form

CXCR – C-X-C Motif Chemokine Receptor

DC – Dendritic Cell

DC-SIGN – Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

DMPA – Depot Medroxyprogesterone Acetate

DNA – Deoxyribonucleic Acid

dNTP - Deoxynucleoside Triphosphate

E1 – Early gene 1

ECM – Extracellular Matrix

Env – Envelope Glycoprotein

ER α – Estrogen Receptor Alpha

FMO – Fluorescence Minus One

FN - Fibronectin

FoxP3 – Fork-head Box P3

FSW – Female Sex Worker

TDF/FTC – Tenofovir Disoproxil Fumarate / Emtricitabine

FTM – Female to Male Transmission

Gag – Group-specific Antigen

GALT – Gut-associated Lymphoid Tissues

GI – Gastrointestinal Tract

GM-CSF – Granulocyte-Macrophage Colony-Stimulating Factor

gp – Glycoprotein

GRID – Gay-Related Immune Deficiency

GVHD – Graft versus Host Disease

GWAS – Genome-wide Association Study

HAART – Highly Active Antiretroviral Therapy

HEV – High Endothelial Venules

HIV – Human Immunodeficiency Virus

HLA – Human Leukocyte Antigen

HLA-DR – Human Leukocyte Antigen – DR

HSCT – Hematopoietic Stem Cell Transplantation

HSV-2 – Herpes Simplex Virus – 2

HTLV-III - Human T-Lymphotropic Virus Type-III

HVTN – HIV Vaccine Trial Network

IBD – Inflammatory Bowel Disease

Idd10 – Insulin-Dependent Diabetes 10

IDS – Isoleucine-Aspartic Acid-Serine

IFN – Interferon

Ig – Immunoglobulin

IL – Interleukin

IL-17RA/RC – Interleukin-17 Receptor A / Receptor C

IL-1RN – Interleukin-1 Receptor Antagonist

IL-1 β – Interleukin-1 Beta

IL-23R – Interleukin-23 Receptor

IL-2R α – Interleukin-2 Receptor Alpha

ILC – Innate Lymphoid Cells

IRB – Institutional Review Board

IRF4 – Interferon Regulatory Factor 4

ISG – Interferon Stimulating Gene

LAV – Lymphadenopathy-associated virus

LDI/L – Lysine-Aspartic acid-Isoleucine-Leucine

LDT – Leucine-Aspartic Acid-Threonine

LDV – Leucine-Aspartic Acid-Valine

LPAM-1 – Lymphocyte Peyer's Patch Adhesion Molecule-1

LPS – Lipopolysaccharide

mAb – Monoclonal Antibody

MAdCAM-1 – Mucosal Addressin Cell Adhesion Molecule-1

MALT – Mucosal-associated Lymphoid Tissue

MHC – Major Histocompatibility Class

MRK-rAd5 – Merck Recombinant Adenovirus Type 5

MSM – Men who have sex with men

MTF – Male to Female Transmission

Nef – Negative Regulatory Factor

NHP – Non-Human Primates

NK – Natural Killer Cells

NSC – Non-Seroconverters

P/SDI/V – Proline-Serine-Aspartic acid-Isoleucine-Valine

PBMC – Peripheral Blood Mononuclear Cell

PCR – Polymerase Chain Reaction

pDC – Plasmacytoid Dendritic Cells

PEP – Post-exposure Prophylaxis

PLDH – People later diagnosed with HIV

PLWH – People living with HIV

PTM – Pigtail Macaques

Pol – Polymerase

PP – Partners PrEP

PrEP – Pre-exposure Prophylaxis

PWID – People who inject Drugs

p21 - Cyclin-dependent Kinase Inhibitor 1

RA – Retinoic Acid

Rev – Regulator of Expression of Virion Proteins

RM – Rhesus Monkey

RNA – Ribonucleic Acid

RORC2 – Retinoic Acid Receptor-Related Orphan Receptor C2

ROR α – Retinoic Acid Receptor-Related Orphan Receptor Alpha

ROR γ t – Retinoic Acid Receptor-Related Orphan Receptor Gamma t

SAMHD1 – Sterile Alpha Motif and Histidine-Aspartic domain-containing Deoxynucleoside

Triphosphate Tri phosphohydrolase 1

sCD14 – Soluble Cluster of Differentiation 14

sCD40L – Soluble Cluster of Differentiation 40 Ligand

SIV – Simian Immunodeficiency Virus

SM – Sooty mangabey

SNP – Single Nucleotide Polymorphism

SNV – Single Nucleotide Variants

STAT3 – Signal Transducer and Activator of Transcription 3

STI – Sexually Transmitted Infection

T1D – Type 1 Diabetes

TasP – Treatment as Prevention

Tat – Trans activator Protein

T-bet – T-box Transcription Factor

Tc – Cytotoxic T cell

TCM – Central Memory T cell

TCR – T cell Receptor

TDF – Tenofovir Disoproxil Fumarate

TEM – Effector Memory T cell

Tfh – Follicular T Helper cell

TGF- β – Transforming Growth Factor Beta

Th – T Helper

TN – Naïve T cell

TNF- α – Tumor Necrosis Factor Alpha

Treg – Regulatory T cell

TRIM5 α – Tripartite Motif Protein 5 Alpha

TRM – Tissue Resident Memory

TSCM – Stem Cell Memory

TTD – Terminally Differentiated cell

TTM – Transitional Memory cell

UBE2V1 – Ubiquitin-Conjugating Enzyme E2 V1

URF – Unique Recombinant Form

UTR – Untranslated Region

VCAM-1 – Vascular Cell Adhesion Molecule 1

Vif – Viral infectivity factor

VL – Viral Load

VLA – Very Late Antigen

Vpr – Virus protein R

Vpu – Viral protein U

Vpx – Viral Protein X

VRCO1 – Viral Receptor-Co-Receptor 1

Contributions of Authors

1. Chapter 4: Pre-HIV $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells and HIV risk among heterosexual individuals in Africa

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2. Chapter 5. Pre-HIV Infection CD4⁺ Th17 cells as predictors of HIV disease progression

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- Data is yet to be published; manuscript from this chapter is under review with co-authors in preparation for publication.

3. Chapter 6. Genetic variation within *CD101* regulates surface expression of CD101 on circulating T cell subsets, which in turn mediates associations with HIV acquisition

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- Data is yet to be published; manuscript from this chapter is under review with co-authors in preparation for publication.

Chapter 1. Introduction

1.1. HIV

1.1.1. Overview of HIV Discovery

The discovery of Human Immunodeficiency Virus (HIV) dates back to 1981, emerging from the observation of an unusually high rates of rare opportunistic disease such as *Pneumocystis carinii* pneumonia and Kaposi's sarcoma in young gay men. Hence, HIV was initially called Gay-Related Immune Deficiency (GRID), because it was thought to only affect gay men (CATIE, 2024; Lange & Ananworanich, 2014). In 1982, the disease was renamed Acquired Immune Deficiency Syndrome (AIDS), and the first case of AIDS was reported in Africa (CATIE, 2024). By 1982 and 1983, AIDS was established as a sexually transmitted disease, and was recognized to affect a broader population, including women through heterosexual sex, people who inject drugs, and blood transfusion recipients (CATIE, 2024). In 1983, Doctors at the Pasteur Institute in France isolated a novel retrovirus from a patient with lymphadenopathy, which they named Lymphadenopathy-associated virus (LAV) (Barré-Sinoussi et al., 1983; Lange & Ananworanich, 2014). In 1985, Doctors in the United States (US) National Cancer Institute identified a similar virus, which they called Human T-Lymphotropic Virus Type-III (HTLV-III) (Lange & Ananworanich, 2014; Popovic et al., 1984). In 1986, LAV and HTLV-III were confirmed as the same virus and renamed HIV (Lange & Ananworanich, 2014). Subsequent studies conclusively established HIV as the etiological agent of AIDS by demonstrating its specific targeting and depletion of CD4⁺ T cells, leading to immune suppression (Gallo, 1987).

1.1.2. Overview of HIV/AIDS in Sub-Saharan Africa

HIV, the causative agent of AIDS (Pau & George, 2014; Rossi et al., 2021; Zayas & Mamede, 2022), remains a major public health issue and a leading cause of morbidity and mortality globally (Dwyer-Lindgren et al., 2019; Haeuser et al., 2022). Over four decades since

its first isolation in 1983 (Dwyer-Lindgren et al., 2019; Ghosn et al., 2018; Pau & George, 2014), nearly 86 million people have become infected, with a recorded deaths of 40.4 million people dying from AIDS-related complications (WHO, 2024). In 2023, 39.9 million people were reported to be living with HIV worldwide, with the adult population aged 15 years or older making up 38.6 million of the total infections (WHO, 2024).

The prevalence of the disease varies widely across regions, with sub-Saharan Africa bearing nearly 70% of the total HIV burden globally (Dwyer-Lindgren et al., 2019; Kharsany & Karim, 2016; Rosenberg et al., 2023). Within sub-Saharan regions, substantial variations in HIV prevalence and epidemiology exist across localities, age groups and sexes (Dwyer-Lindgren et al., 2019; Haeuser et al., 2022). Of the 26 million people estimated to be living with HIV in sub-Saharan Africa in 2023, 20.5 million are concentrated within East and South Africa compared to other regions (WHO, 2024). African countries with reported highest incidence or burden of HIV include South Africa, Uganda, Mozambique, Kenya, Nigeria, Tanzania, Zimbabwe, Zambia, Malawi and Ethiopia (Kharsany & Karim, 2016).

Heterosexual transmission is identified as a major transmission route of HIV in sub-Saharan Africa (Kharsany & Karim, 2016; Okano et al., 2020). In addition, young adults, especially women within ages of 15 to 24 years are at the most risk of HIV infection in East and South African regions (Ghosn et al., 2018; Kharsany & Karim, 2016; Rosenberg et al., 2023). This contrasts with other geographical regions where transmission routes and other key populations prevail including intravenous drug users, men who have sex with men (MSM) and transgender people (Ghosn et al., 2018). With the disproportionate and heterogenous HIV prevalence within sub-Saharan Africa, focusing research on this region has a potential benefit of reducing HIV burden globally.

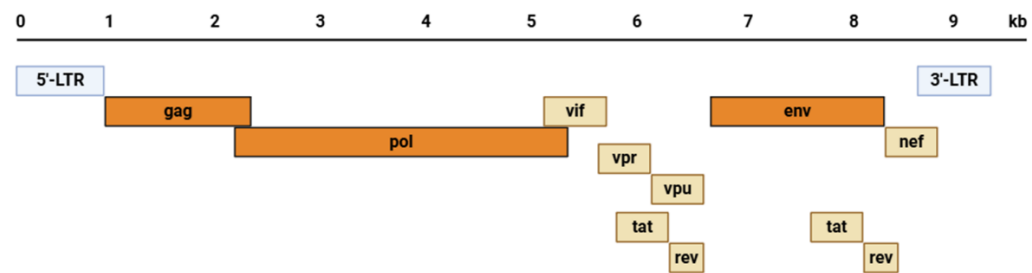
1.1.3. HIV Genome Structure

The genome structure of HIV (**Figure 1**), like other *Retroviridae* family, is composed of two identical single-stranded RNA molecules (Fanales-Belasio et al., 2010; Zayas & Mamede, 2022). The HIV genome surrounded by lipoprotein membrane, contains genes encoding various viral proteins essential for its replication and infection (Turner & Summers, 1999). The viral genome begins and terminates with non-coding long terminal repeats (LTRs) crucial for integrating the virus into the host cell's genome and regulating transcription. Three structural genes, *gag*, *pol*, and *env*, and accessory or regulatory genes, *nef*, *vif*, *vpr*, *vpu*, *tat*, *rev*, *vpx*, play crucial roles in the virus' replication (Andrew & Strebel, 2014; Kogan & Rappaport, 2011; Rossi et al., 2021).

Gag encodes the core structural proteins of the virus including matrix protein p17, capsid protein p24, nucleocapsid protein p7, and p6 protein, all essential for viral assembly and maturation (Rossi et al., 2021). **Env** encodes the viral envelope glycoproteins, gp120 and gp41 responsible for viral entry into host cells (Prabakaran et al., 2007). Surface glycoprotein gp120, is responsible for receptor binding on target cells and facilitates viral entry (Prabakaran et al., 2007). This subunit is made up of five conserved domains, C1 – C5, and five variable loops, V1 – V5 (Prabakaran et al., 2007; Wilen et al., 2012), where the variable loops particularly the V3 loop, play crucial roles in coreceptor binding and immune evasion (Wilen et al., 2012). The transmembrane glycoprotein gp41, mediates the fusion of viral and host cell membranes, allowing entry of the viral core into the host cell (Prabakaran et al., 2007). **Pol** encodes viral enzymes including Reverse transcriptase that convert viral RNA into DNA (W. S. Hu & Hughes, 2012), Integrase that integrates viral DNA into the host cell genome (Maertens et al., 2022), and Protease responsible for processing viral proteins for maturation (Weber et al., 2021).

Tat encodes protein that play multiple functions in HIV replication including promoting efficient viral gene expression (Ali et al., 2021). ***Rev*** encodes protein that regulates the nuclear export of unspliced and partially spliced viral RNA transcripts to the cytoplasm, to produce fully functional viral particles (Shum et al., 2013; Turner & Summers, 1999). ***Nef*** encodes protein involved in modulating host cell responses to enhance viral replication and evade host immune system (Foster & Garcia, 2007; Staudt et al., 2020). ***Vif*** and ***Vpu*** encode proteins responsible for counteracting host cell restriction factors targeted to inhibit viral replication (Andrew & Strebel, 2014). ***Vpr*** encodes protein involved in nuclear import of viral DNA (Kogan & Rappaport, 2011). ***Vpx***, present in some HIV strains , facilitates efficient viral replication (Andrew & Strebel, 2014).

HIV-1 genome



HIV-1 virion

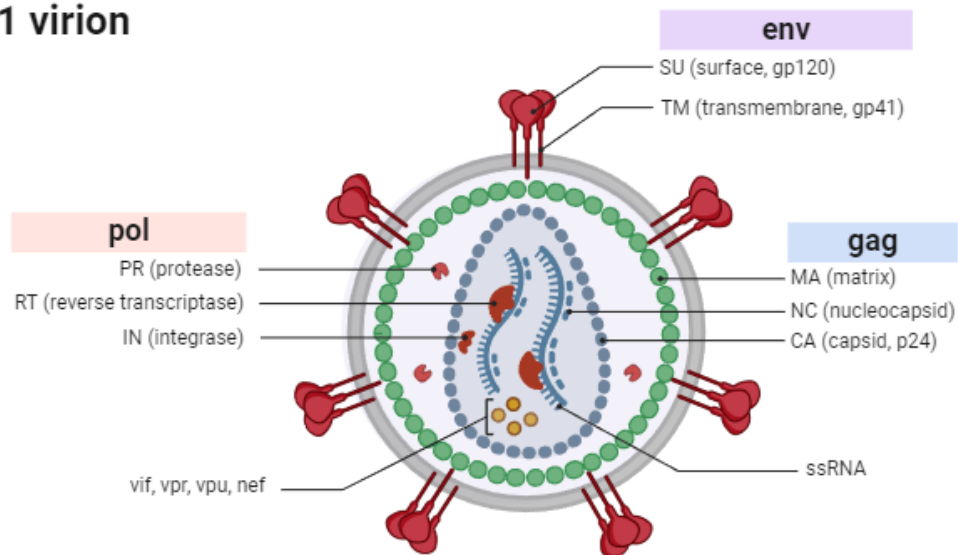


Figure 1. The HIV Genome and Virion Structure

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1.1.3. HIV Replication Cycle

HIV replication is a multistep process, allowing the virus to gain entry into host target cells, replicate its genetic material, and subsequently produce new virus particles to continue the chain of replication (Freed, 2015; Wilen et al., 2012). The steps involved in HIV replication include (1) Binding and Entry, (2) Reverse Transcription, (3) Integration, (4) Transcription and Translation, (5) Assembly and Budding, (6) Maturation, and (7) Release (**Figure 2**) (Fanales-Belasio et al., 2010; Freed, 2015; Rossi et al., 2021; Wilen et al., 2012).

HIV primarily targets CD4⁺ T cells, binding it surface via *env* glycoproteins gp120 and gp41 (Wilen et al., 2012). The binding of HIV glycoproteins is facilitated by CD4 receptors and co-receptors, CCR5 or CXCR4, expressed on the target cells (Prabakaran et al., 2007; Rossi et al., 2021; Wilen et al., 2012). After viral attachment, the envelope fuses with host cell membrane for the viral core to enter host cell (Rossi et al., 2021). The virus then releases its single-stranded RNA genome into a double-stranded DNA sequence via a process called reverse transcription (W. S. Hu & Hughes, 2012; Rossi et al., 2021). This step is very crucial and a unique replication step for retroviruses like HIV (W. S. Hu & Hughes, 2012). The proviral DNA which is the newly synthesized DNA then enters the host cell nucleus, where it is integrated into the host cell's chromosomal DNA via the help of Integrase enzyme (Fanales-Belasio et al., 2010; Rossi et al., 2021; Turner & Summers, 1999). After integration, the provirus becomes a part of the host cell's genetic material. The integrated DNA may either remain dormant causing HIV to persist in latent reservoirs, or it may become active (Fanales-Belasio et al., 2010). When active, the host cell's machinery transcribes the proviral DNA into messenger RNA to produce viral proteins needed for replication (Fanales-Belasio et al., 2010; Freed, 2015). Newly synthesized viral proteins and RNA molecules move to the host cell's surface to assemble into new virus particles (Fanales-Belasio et al., 2010; Rossi et al., 2021). The virus buds from the host cell's membrane, taking a portion of the cell membrane with it,

so it escapes the host immune system without immediately killing it (Fanales-Belasio et al., 2010). Newly formed virus particles are non-infectious and undergo maturation where Protease enzyme cleaves long protein chains into their functional forms to become infectious (Rossi et al., 2021). After maturation, the virus particles are released from the host cell and can infect other target cells continuing the replication cycle (Rossi et al., 2021).

HIV Replication Cycle

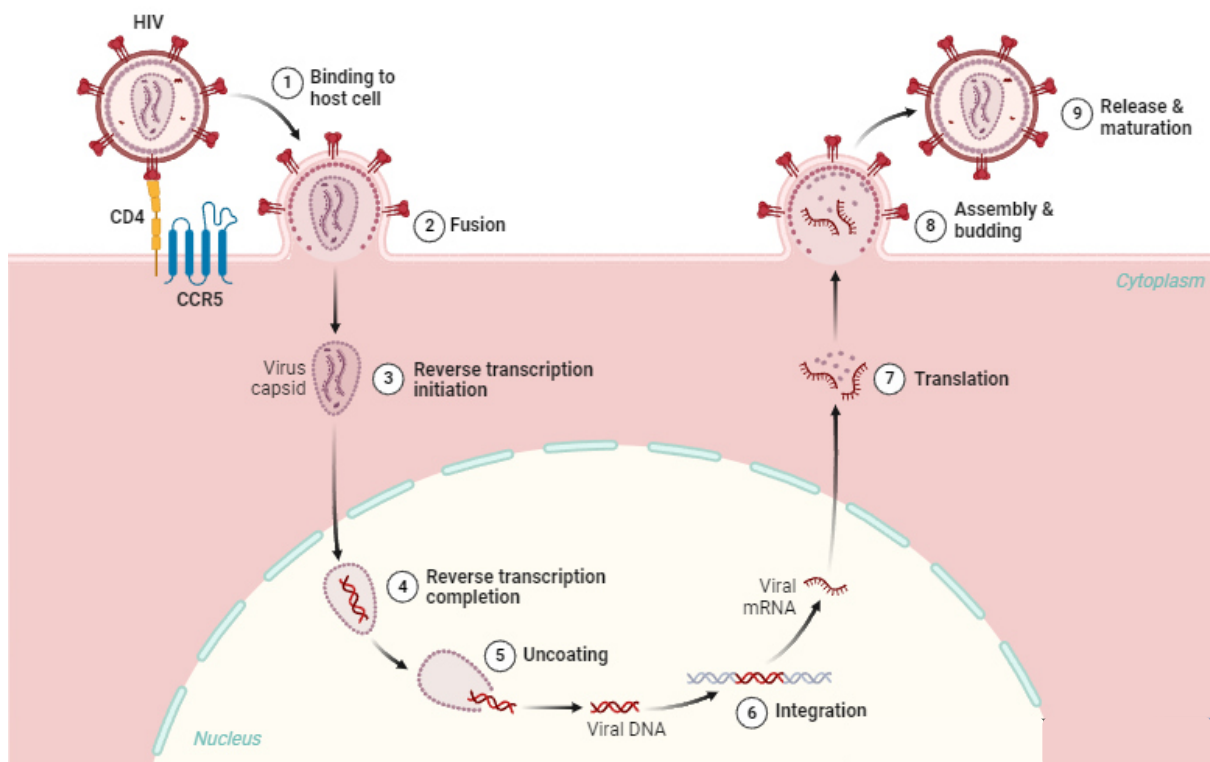


Figure 2. The HIV Replication Cycle

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1.1.4. HIV Subtypes

HIV is known for its high genetic variability, a characteristic which enables it to evade host immune responses and resist treatments and vaccines (Fanales-Belasio et al., 2010; Hemelaar et al., 2019). This variability is due to factors like the virus's error-prone replication, rapid replication rate and recombination when different HIV strains infect the same person (Fanales-Belasio et al., 2010; Spira et al., 2003).

There are two types of HIV: HIV type -1 (HIV-1) and HIV type-2 (HIV-2). HIV-1 is the most prevalent, accounting for approximately 95% of HIV infections globally, while HIV-2 is mostly found in Western and Central Africa, with some cases in Europe and India (Fanales-Belasio et al., 2010). HIV-1 and HIV-2 share similar genome structures but have differences in certain genes, affecting their virulence and progression to AIDS. Specifically, instead of *Vpr* found in HIV-1, HIV-2 has *Vpx*, both proteins involved in the integration of viral DNA into the host genome. Both HIV-1 and HIV-2 can lead to AIDS, however, HIV-2 tends to be less virulent and progresses more slowly to AIDS compared to HIV-1 (Fanales-Belasio et al., 2010).

HIV-1 is further categorized into four groups: M (Major), O (Outlier), N (New), and P (Pending) (Ng'uni et al., 2020). Group M is widespread worldwide and divided into nine subtypes or clades (A, B, C, D, F, G, H, J, and K), each with distinct geographical distributions (Akahome, 2021; Gartner et al., 2020; Hemelaar et al., 2019; McKinnon et al., 2012). Group O and N are mostly found in Gabon and Cameroon (Fanales-Belasio et al., 2010). Clades A and F are further grouped into A1, A2, A3, and A4 and F1 and F2 (Fanales-Belasio et al., 2010). There are also recombinant HIV strains which are formed by the genetic recombination of two or more different HIV strains within a single host (Fanales-Belasio et al., 2010). These recombinant strains can either be unique recombinant forms (URFs) when the newly formed strain is confined to the individual in which it was formed and not spread widely through the

population, or the circulating recombinant forms (CRFs) characterized by their significant and onward transmission among many individuals, making them prevalent in specific regions or globally (Akahome, 2021; Fanales-Belasio et al., 2010; Hemelaar et al., 2019).

HIV clades and recombinant forms (CRFs and URFs) exhibit significant genetic diversity and geographic distribution. Sequence variability within clades can range between 20% – 35% in the *Env* protein (Gartner et al., 2020; McKinnon et al., 2012) whereas Inter-clade sequence variability can vary as high as 42% (Ng'uni et al., 2020). Sub-Saharan Africa shows the highest HIV-1 heterogeneity, having all known clades, groups and recombinant forms present (Akahome, 2021; Fanales-Belasio et al., 2010; McKinnon et al., 2012). Clade C is the most prevalent HIV clade worldwide accounting for almost 50% of all HIV-1 infections globally, but it is particularly dominant in South and East Africa, the regions with the highest global HIV infection rates (Akahome, 2021; Fanales-Belasio et al., 2010; Gartner et al., 2020; Hemelaar et al., 2019; Tyor et al., 2013). Clade C is also prevalent in India and other parts of Asia (Fanales-Belasio et al., 2010; Gartner et al., 2020; Tyor et al., 2013). Clade B, responsible for about 12% of global infection, is common in the Americas, Europe, and Australia (Hemelaar et al., 2019). Clade D, along with clade A, which accounts for nearly 3% and 10% of HIV global infections, respectively, are widely distributed in East Africa including Uganda and Kenya (Fanales-Belasio et al., 2010; Hemelaar et al., 2019). Clade A is also found in Eastern Europe (Akahome, 2021). Recombinant forms are widespread in sub-Saharan Africa and Southeast Asia and are increasing in circulation in diverse regions like Eastern Europe and South Americas (Fanales-Belasio et al., 2010). CRF02_AG and CRF01_AE are among the most common CRFs of HIV-1 worldwide, accounting for 8% and 5% of global infections, respectively (Akahome, 2021; Hemelaar et al., 2019).

The different HIV clades may impact various aspects of the virus, including transmission routes (Fanales-Belasio et al., 2010; Souto et al., 2021), disease progression rates (Baeten et al., 2007; Kaleebu et al., 2002; Kiwanuka et al., 2008; McKinnon et al., 2012; Souto et al., 2021; Vasan et al., 2006), and responses to treatment (Gupta et al., 2012; Spira et al., 2003). For example, HIV clade C is known for its rapid spread and high transmission rates, contributing to the extensive HIV epidemics in the regions where it is predominant (Gartner et al., 2020). The widespread nature of subtype C makes it a significant focus of HIV research, treatment, and prevention efforts. HIV clade B may be transmitted more effectively through homosexual intercourse and intravenous drug use, while clade C and E may be transmitted more frequently through heterosexual route (R. Hunt, 2016; Spira et al., 2003). Recombinant form CRF03_AB also seem more common among injecting drug users in Eastern Europe (Fanales-Belasio et al., 2010). This variation in transmission routes may be attributed to differences in viral replication in specific cells. For example, Clade C and E were shown to replicate better in Langerhans' cells in the mucosa of the cervix, vagina, and penis, whereas clade B replicates better in rectal mucosa (R. Hunt, 2016). While all types of HIV seem to react similarly to antiviral drugs, some strains from specific regions or subtypes might become resistant to certain drugs more easily (Gupta et al., 2012; Spira et al., 2003).

There is substantial evidence that suggest that HIV clade may influence the rate of disease progression. Infection with HIV clades other than clade A were eight times more likely to progress to AIDS than those infected with HIV clade A (Kanki et al., 1999). Among Kenya women, infection with clade C was associated with higher plasma viral RNA levels and significantly lower CD4 cell counts compared to women infected with clades A or D (Neilson et al., 1999). Clade D have been confirmed by numerous studies to exhibit greater virulence compared to clade A, resulting in a more rapid CD4 decline and faster progression of the disease in infected individuals (Baeten et al., 2007; Kaleebu et al., 2002; Kiwanuka et al., 2008;

McKinnon et al., 2012; Vasan et al., 2006). Clade differences in coreceptor usage had been reported as well. Kaleebu *et al.* (2007) in a small sample size Ugandan cohort showed that clade D infections showed a higher likelihood of X4 virus presence compared to clade A (Kaleebu et al., 2007). In their study they showed infection with R5 viruses were linked to higher median CD4 counts than X4 or X4/R5 viruses (Kaleebu et al., 2007). While HIV during early infection predominantly uses the CCR5 coreceptor, as disease progressed, the virus expands its coreceptor usage to include CCR5, CCR3, CCR2b, and CXCR4 (Connor et al., 1997). Viruses using CXCR4 coreceptor have been linked to faster CD4 decline, higher plasma HIV RNA levels and overall increased risk of progressing to AIDS (Daar et al., 2007). Hence, coreceptor usage differences between clades may explain the faster disease progression seen in clade D infections compared to clade A (Kaleebu et al., 2007; Kuritzkes, 2008).

These variations also pose challenges for developing an effective vaccine against HIV, as vaccine efficacy may vary across different HIV clades (Hemelaar et al., 2019). For instance, a candidate vaccine tested against clade B and CRF01_AE in Thailand exhibited promising results. However, when tested against clade C in South Africa, no vaccine efficacy was observed (Gray et al., 2021). This underscores the importance of considering HIV clade diversity in HIV-related studies and vaccine development.

1.2. HIV Immune Response: The Role of Immune Cells in HIV Infection

The immune system, composed of a complex network of immune cells divided into innate and adaptive immune cells, is designed to recognize invading pathogens and mediate responses to tackle the invaders (Bergthaler & Menche, 2017; Howell & Shepherd, 2021; Medina, 2016; Tomar & De, 2014). In general, the innate immune system composed of immune cells including dendritic cells (DC), natural killer (NK) cells, monocytes, macrophages, neutrophils, etc., act as the non-specific first-line of defense against invaders (Board et al.,

2022; Carrington & Alter, 2012; Howell & Shepherd, 2021; Medina, 2016; Tomar & De, 2014), while the adaptive immune system, further categorized as humoral (B lymphocytes) or cell-mediated (T lymphocytes) immunity, act as a specific and more coordinated response to invading pathogens (Howell & Shepherd, 2021; Tomar & De, 2014). The T cell adaptive immune arm is further categorized as T helper cells (Th or CD4⁺ T cells) or cytotoxic (Tc or CD8⁺ T cells), each playing distinct roles in immunity against infection (Howell & Shepherd, 2021; Tomar & De, 2014).

Within the context of HIV, both immune arms play important roles in orchestrating immune response against the virus, although the role of innate immune system is less-explored in HIV compared to the adaptive immune system (Board et al., 2022; Espíndola et al., 2016). Innate immune cells including DCs, and macrophages are key players in the pathogenesis of HIV, while others such as monocytes, neutrophils, NK cells, unconventional T cells and innate lymphoid cells (ILCs) have been implicated in the disease (Barber-Axthelm et al., 2020; Board et al., 2022; Carrington & Alter, 2012; Espíndola et al., 2016; Sidell & Kane, 2022). Within the adaptive immune compartments, CD4⁺ T lymphocytes are the key players in HIV immune responses (Chen et al., 2020).

HIV primarily infect cells expressing CD4 receptors on their surface including CD4⁺ T cells, monocytes, macrophages, DCs, eosinophils, and microglial cells in the central nervous system (CNS) (Fanales-Belasio et al., 2010; Prabakaran et al., 2007). In addition to been infected, these cells are capable of harboring the intact provirus of the virus, making them crucial long-term reservoirs of HIV (Board et al., 2022; Fanales-Belasio et al., 2010). The CD4 receptor helps the immune system's recognize foreign invaders by binding to MHC class II (Fanales-Belasio et al., 2010). When HIV gp120 attached to CD4, it causes a conformational change that allows the virus to bind to chemokine receptors on cell surface to further facilitate viral entry (Espíndola et al., 2016; Fanales-Belasio et al., 2010). HIV most commonly uses two

chemokines as coreceptors, namely CCR5 or CXCR4. CXCR4 is found on various cells, including T cells, while CCR5 is present on monocytes or macrophages, dendritic cells, and activated T cells, rendering these cells conducive for HIV infection (Espíndola et al., 2016; Fanales-Belasio et al., 2010). Differences in the types of chemokine receptors found on target cells are crucial factors in determining how HIV infects cells (Fanales-Belasio et al., 2010; Zayas & Mamede, 2022). Most HIV strains preferentially attach to CCR5 receptor for entry and are called M-tropic or R5 viruses while some strains called T-tropic or X4 viruses preferentially bind to CXCR4 receptor for entry (Fanales-Belasio et al., 2010; Wilen et al., 2012; Zayas & Mamede, 2022). Some HIV strains are dual tropic or X4R5 viruses as they are able to bind to both CCR5 and CXCR4 receptors (Fanales-Belasio et al., 2010; Wilen et al., 2012). Additionally, other non-essential binding receptors on host cells, such as integrin $\alpha 4\beta 7$ or the pattern recognition receptor DC-SIGN, have been identified (Arthos et al., 2008; Carrington & Alter, 2012; Cicala et al., 2009; Wilen et al., 2012).

1.3. Dynamics of HIV infection: Acute, Chronic, and AIDS

Although varying between individuals, a typical course of HIV infection occurs in multiple stages beginning with acute infection, followed by a chronic infection phase and subsequent progression to AIDS if left untreated (Ford et al., 2009; NIH, 2021; Sabin & Lundgren, 2013).

1.3.1. Acute Infection

This phase otherwise known as “primary infection”, or “seroconversion” is the earliest stage, and it typically develops within the first two to four weeks of infection (Hicks et al., 2007; Sax et al., 2014; Zayas & Mamede, 2022). The acute infection phase encompasses the time from virus entry to the detection of antibody response, seroconversion and establishment of set-point plasma viral load, typically lasting three to six months post-infection (Cao et al.,

2015; Sax et al., 2014). This stage is characterized by low CD4⁺ T cell counts, as low as < 200 cells/mm³, with clinical manifestations presenting as flu-like symptoms such as fever, chills, headache, sweating, sore throat, weight loss, rash and swollen lymph nodes (Cao et al., 2015; Hicks et al., 2007; Koch Bentzon, 2020; Sax et al., 2014; Zayas & Mamede, 2022).

During the acute phase, the virus multiplies at a rapid rate, reaching a transient peak, called peak viral load, and spread throughout the body while simultaneously attacking and destroying CD4-expressing cells in the immune system (Sax et al., 2014). The attack of HIV on the immune system leads to rapid depletion of CD4⁺ T cells in the peripheral blood and a more drastic depletion of these cells within the mucosal compartments, particularly in the gut-associated lymphoid tissues (GALT) (Cao et al., 2015; Shacklett et al., 2003). The GALT is widely recognized as the primary anatomical site for HIV replication, playing a pivotal role particularly in the early stages of infection (Ford et al., 2009; Shacklett et al., 2003; Shimada et al., 2014). The high concentration of CD4⁺ T cells in the GALT contributes to the rapid and extensive depletion of these cells in the GALT during acute infection and this depletion is believed to play a major role in the progression of the disease as well as the impairment of the immune system (Ford et al., 2009; Hecht et al., 2006; Shimada et al., 2014). Aside the GALT, HIV can be found in other lymphoid tissues such as the lymph nodes, spleen and the peripheral blood (Orenstein, 2008; Shimada et al., 2014).

The acute infection phase is a critical phase in HIV infection. First, because the risk of HIV transmission during this phase is very high due to the high amount of the virus multiplying in the blood and mucosal sites (Hecht et al., 2010; Pilcher et al., 2007; Pinkerton, 2008). Secondly, the acute infection phase can influence the course of HIV progression, where a robust control of HIV replication coupled with lower setpoint viral loads, has been associated with slower disease progression (Carrington & Alter, 2012). Hence, this stage offers a “window of opportunity” for initiating treatment and this strategy has been demonstrated to have beneficial

effects including improving disease outcomes and preserving immune functions when treatment is begun during the acute infection phase (Cao et al., 2015; Hecht et al., 2006; Hicks et al., 2007; Sabin & Lundgren, 2013).

1.3.2. Chronic Infection

This is the second phase of HIV infection and is otherwise known as “asymptomatic” or “clinical latency” stage (Coffin et al., 1997; Sax et al., 2014). This stage is characterized by CD4⁺ T cell counts > 500 cells/mm³ and it relatively span over an extended period of time with mild to no clinical manifestations (Coffin et al., 1997). During this phase, the virus continues to multiply at a very low rate with the amount of CD4⁺ T cells decreasing at a steadier rate (Sax et al., 2014). The steady setpoint viral load at the end of the acute phase of infection is often used as a correlate of progression to AIDS, with individuals having higher viral loads experiencing faster progression versus individuals having lower viral loads experiencing longer progression time (Coffin et al., 1997; Hecht et al., 2006). Other important HIV progression markers include level of plasma viral load (i.e. the concentration of HIV RNA) (Hecht et al., 2010), the percentage or absolute number of circulating CD4⁺ T cells, and the ratio of CD4 to CD8 count in the blood (Mellors et al., 1997; Sabin & Lundgren, 2013). Chronic HIV can last up to ten years before progressing to AIDS if treatment is not initiated (Sax et al., 2014). However, with treatment, HIV patients can remain in this stage without advancing to AIDS (Koch Bentzon, 2020). The risk of transmission during this stage is relatively low and with treatment, patients can maintain undetectable viral load which equates to less transmissibility potential, hereto, reducing the risk of transmission through sexual contact (Eisinger et al., 2019; Rodger et al., 2019).

1.3.3. AIDS

This is the final and the most severe phase of HIV infection, defined as $CD4^+$ T cell count < 200 cells/mm³, $< 14\%$ $CD4^+$ T cell percentage of total lymphocytes, or the presence of AIDS-related opportunistic infections (Sax et al., 2014). The extensive depletion of infection-fighting cells caused by the virus severely damages the immune system such that it is weakened to tackle invading pathogens (Hicks et al., 2007; Sax et al., 2014). This immunocompromised state renders people living with HIV (PLWH) susceptible to opportunistic infections like herpes, candidiasis, bacterial pneumonia, tuberculosis and cancer (Sax et al., 2014). The clinical manifestations of AIDS include fatigue, night sweats, prolonged diarrhea, joint pain and weight loss (Koch Bentzon, 2020). HIV transmission risk during this phase is extremely high (Pilcher et al., 2007) and without treatment, the chances of patient's survival drastically reduce (Walensky et al., 2006). Various factors influence the rate of HIV progression to AIDS, including plasma HIV RNA levels, CD4 cell count, degree of immune activation, HIV coreceptor usage and subtype, age, socioeconomic status, and host genetics (Kuritzkes, 2008).

With antiretroviral therapy (ART), the course of HIV infection can change profoundly ensuring an effective management of the disease. Effective ART can suppress HIV replication, promote immune recovery, and reduce disease progression, therefore, extending the lifespan of PLWH (Cao et al., 2015; Ka'e et al., 2023). However, ART is ineffective against persisting viruses in latent reservoirs, which can cause a rebound if treatment is interrupted (Ka'e et al., 2023). Hence, there is no definitive cure for HIV yet and PLWH have to be placed on lifelong ART (Koch Bentzon, 2020). In addition to establishing latency in reservoirs, the virus is able to mutate rapidly to escape host immune mechanisms, hence, hindering efforts to develop cure or vaccine against the infection (Falcinelli et al., 2023; Ghosn et al., 2018; Williams et al., 2023).

1.4. Factors Influencing HIV Acquisition and Disease Progression

HIV gains entry into the body via mucosal and parenteral routes, transmitted through blood, genital secretions, and breast milk (Campo et al., 2006; Hicks et al., 2007; Page-Shafer et al., 2006; Tebit et al., 2012; Zayas & Mamede, 2022). The transmission modes of HIV include sexual contacts (vaginal, anal or oral) (Campo et al., 2006; Sax et al., 2014), from mother living with HIV to child during or after childbirth or via breastfeeding (Kourtis & Bulterys, 2010; Page-Shafer et al., 2006) or via intravenous drug use (Sax et al., 2014). Heterosexual HIV transmission is a predominant mode of HIV transmission, especially in sub-Saharan Africa (Kharsany & Karim, 2016; Okano et al., 2020). Compared to other routes, heterosexual transmission is a relatively inefficient route, with the per-act risk ranging from 4 per 10,000 exposures for insertive penile-vaginal intercourse to 138 per 10,000 exposures for receptive anal intercourse (Griesbeck et al., 2016; Patel et al., 2014). The likelihood of HIV transmission is contingent upon various factors involving both partners and is influenced by behavioral, biological, genetic, and immunological risk factors. Sexual practices including unprotected or condomless receptive anal or vaginal intercourse and oral-genital contacts pose a high risk of sexual HIV transmission (Campo et al., 2006; Hughes et al., 2012; Sax et al., 2014; Tebit et al., 2012). In addition, factors including presence of STIs, number of sexual partners or encounters, male circumcision status and age are modifiable risk factors for HIV transmission (Hughes et al., 2012; Lingappa et al., 2011; Sax et al., 2014). A high plasma HIV viral load in the seroconverted partner is associated with increased risk of HIV acquisition (Baeten et al., 2011; Hughes et al., 2012; Quinn et al., 2000). A longitudinal study of African HIV serodiscordant heterosexual couples found that the per-act risk of transmission increases almost three-fold for each $\log_{10}$ increase in plasma HIV RNA (Hughes et al., 2012), underscoring the significant impact of viral load on transmission rates. The “quantity and quality” of HIV-susceptible target cells in the genital or rectal mucosa are also crucial

determinants of HIV susceptibility (McKinnon & Kaul, 2012). Factors that increase the number of mucosal CD4⁺ T cells, such as genital infections, significantly enhance HIV transmission likelihood (Griesbeck et al., 2016; Hughes et al., 2012; McKinnon & Kaul, 2012; Yi et al., 2013). Increased immune activation and expression of HIV-binding molecules such as CCR5 and $\alpha 4\beta 7$ play key roles in facilitating HIV transmission and replication (McKinnon & Kaul, 2012). The Th17 and Th22 cell subsets within mucosal compartments are particularly susceptible to HIV infection (McKinnon & Kaul, 2012). Hence, having more of these susceptible targets at site and time of HIV exposure, may modify HIV acquisition rates and this is discussed extensively at the later part of this chapter.

1.4.1. Sex Differences in HIV

Significant differences have been described between men and women in relation to HIV epidemiology and pathogenesis. Biological women are disproportionately affected by HIV compared to men, particularly in HIV-endemic regions like sub-Saharan Africa, where heterosexual transmission is predominant (Griesbeck et al., 2016; Yi et al., 2013). Initially, this disproportionate burden was largely attributed to socio-economic and behavioral factors, however, extensive research has shown that biological factors also play a crucial role in driving these sex differences. Sex differences extend to various aspects of HIV pathogenesis, from acquisition or transmission to latency establishment, disease progression, response to ART, and even cure and preventive strategies, explained in detail as follows.

1.4.1.1. Epidemiology and Transmission: Women tend to acquire HIV at a much younger age than men, with young women aged 15 – 24 years being significantly more likely to be infected (Yi et al., 2013). There is a general consensus that male-to-female transmission (MTF) is more efficient than female-to-male transmission (FTM) (Griesbeck et al., 2016; Scully, 2018), possibly attributable to the lower infectious potential of women living with HIV noted in earlier

studies of HIV serodiscordant couples (Nicolosi et al., 1994). In later studies, however, HIV transmission rates were comparable between MTF and FTM partners after accounting for viral loads (Hughes et al., 2012; Quinn et al., 2000). Baeten *et al.* (2011) evaluated serodiscordant couples in heterosexual relationships, examining endocervical samples from women and semen samples from men. They showed a strong correlation between plasma and genital HIV RNA concentrations, with each $\log_{10}$ increase in genital HIV RNA associated with 2.20-fold increase in transmission risk for endocervical swabs and a 1.79-fold increase for semen (Baeten et al., 2011). A key finding was the distinction between MTF and FTM transmission dynamics. For women, both genital and plasma HIV RNA levels independently predicted transmission risk, while for men, only genital HIV RNA levels predicted transmission risk, with plasma HIV RNA not showing a significant association in men (Baeten et al., 2011). A similar study by Hughes *et al.* (2012) initially found a higher risk for MTF transmission than that of FTM, but after they adjusted for critical factors such as plasma HIV RNA levels, HSV-2 serostatus, and the age of the uninfected partner, the relative risk for MTF versus FTM transmission were comparable, indicating that the higher observed risk of MTF transmission was primarily due to higher viral loads in men and other confounding risk factors (Hughes et al., 2012).

1.4.1.2. Pathogenesis and Disease Progression: Studies show that during acute HIV infection, women have significantly lower viral loads than men, an effect that diminishes as the disease progresses (Anastos et al., 2000; Sterling et al., 2001). Likewise, women compared to men are more likely to achieve a smaller HIV reservoir size after long-term ART attributable to their lower viral loads during early infection (Cuzin et al., 2015). Despite lower initial viral loads, women are at a greater risk of progressing to AIDS compared to men, possibly because women tend to experience higher immune activation which contribute to disease progression (Cohn et al., 2020; Santinelli et al., 2020). In contrast, men tend to have higher HIV-related mortality rates than women, attributable to differences in treatment access, adherence issues, and higher

prevalence of comorbidities among men (Griesbeck et al., 2016). In a cohort study in Tanzania, Mosha *et al.* (2013) revealed that women tend to have a higher baseline CD4 cell count compared to men. However, as the disease progresses, women experienced a similar CD4 cell count to men, indicating a worse CD4 cell reconstitution despite better virological response (Mosha et al., 2013).

1.4.1.3. Prevention and Treatment: Sex-based differences in effectiveness of preventive strategies such as PrEP have been described. Women tend to have lower tenofovir concentrations in vaginal tissues compared to rectal tissues in men (Louissaint et al., 2013; Patterson et al., 2011), indicating that adherence is more critical for women to achieve the same level of protection as men (Griesbeck et al., 2016). Likewise, women might be more prone to adverse events from ART due to higher drug concentrations leading to increased toxicity (Griesbeck et al., 2016; Umeh & Currier, 2006).

1.4.1.4. Socioeconomic and Behavioural Differences: Sexual behaviour and patterns are critical determinants of the disproportionate high HIV burden in young women. Young women may often engage in sexual relationships with older partners (Yi et al., 2013). This age differences may raise the younger partner's risk of HIV acquisition. For instance, the less-experienced partner may struggle to effectively negotiate condom use, further elevating their risk (Yi et al., 2013). Other behavioural factors also contribute to this risk. For instance, douching is associated with altered vaginal flora and an increased risk of HIV acquisition (Yi et al., 2013). Rates of intravaginal practices may be higher in young women, further complicating their HIV risk. Socioeconomic parameters, including but not limited to gender inequities, cultural practices, poverty, educational level, access to healthcare, sexual violence, and injustice, contribute to disparity between sexes in relations to HIV (Griesbeck et al., 2016; Rodriguez-Garcia et al., 2021).

1.4.1.5. Biological Mechanisms of Sex-Related Differences in HIV

Several biological mechanisms contribute to the sex-related differences observed in the context of HIV. Biological factors contributing to these differences between men and women include anatomical differences, immune cell expression, sex hormones, genital tract microbiome, and genetic differences (Moran et al., 2022; Rechten & Altfeld, 2019; Scully, 2018).

Women have a larger mucosal surface area exposed to HIV during heterosexual intercourse compared to men (Yi et al., 2013). The vagina, penis, and foreskin are all rich in HIV target cells (McKinnon & Kaul, 2012), the cervicovaginal mucosa, however, may present a larger target for HIV infection than the penile and foreskin surface area. Women showed increased mucosal expression of the HIV co-receptor CCR5, particularly in young women (Shacklett & Greenblatt, 2011; Yi et al., 2013). Additionally, systemic innate immune activation is generally higher in women than in men, which correlates with an increased risk of HIV acquisition and disease progression (Cohn et al., 2020). Santinelli *et al.* (2020) demonstrated that immune activation levels are significantly elevated in women compared to men, both in the gut and PBMCs, in a population of individuals on long-term suppressive ART (Santinelli et al., 2020). Women also showed more pronounced alterations in the gut mucosal T cell repertoire, particularly in the Th1, Th17, and Th1/Th17 cell subsets with central or effector memory phenotypes, compared to their male counterparts (Santinelli et al., 2020). These may explain sex differences observed in HIV infection. Lower levels of immune activation in the blood are associated with reduced HIV incidence, suggesting that the heightened immune response in women is a key mechanism driving their increased vulnerability to HIV (Griesbeck et al., 2016; Yi et al., 2013). Women with untreated HIV infection exhibit higher levels of CD8⁺ T cell activation and a greater risk of progressing to AIDS compared to men, even with similar viral loads (Cohn et al., 2020; Santinelli et al., 2020). Furthermore, semen

can induce inflammatory responses in the female genital tract (FGT) and can remain there for up to three days post-coitus, thereby prolonging HIV exposure (Shacklett & Greenblatt, 2011; Yi et al., 2013).

Sexually transmitted infections (STIs) also play a role in the disproportionate HIV risk between men and women (Hughes et al., 2012; Yi et al., 2013). Bacterial STIs and viral infections such as HSV-2 are more prevalent in women, particular young women, and are associated with a higher risk of HIV acquisition (Griesbeck et al., 2016; Moran et al., 2022; Yi et al., 2013). These infections facilitate the recruitment of HIV target cells to mucosal sites, thereby increasing the risk of HIV infection upon exposure (Griesbeck et al., 2016; Hughes et al., 2012; McKinnon & Kaul, 2012). The genital tract microbiome plays a role in the sex difference observed in HIV. The high presence of non-lactobacillus bacterial species in vaginal tissues is often associated with increased genital inflammation and can lead to conditions such as bacterial vaginosis, the most common vaginal dysregulation in women of reproductive age (Rodriguez-Garcia et al., 2021). These conditions contribute to an increased risk of HIV infection.

Sex hormones represent a significant biological mechanism contributing to sex-related differences in HIV pathogenesis. The primary sex hormones – estrogen and progesterone in women and testosterone in men – exert both direct and indirect effects on epithelial and immune cells, as these cells express receptors for sex hormones, thereby are able to modify risk of HIV acquisition (Hagen & Altfeld, 2016; Rodriguez-Garcia et al., 2021; Wira et al., 2010). Immune activation and IFN α responses are identified as key factors in sex differences in HIV pathogenesis (Griesbeck et al., 2016). Persistent immune activation drives HIV disease progression by maintaining a pool of activated CD4⁺ T cells (Griesbeck et al., 2016). Notably, plasmacytoid DCs (pDCs) from women produce more IFN α in response to HIV, resulting in higher immune activation levels compared to men (Griesbeck et al., 2016; Hagen & Altfeld,

2016; Scully, 2018b; Ziegler & Altfeld, 2016). This difference in IFN α production is partly regulated by estrogen receptor alpha (ER α) and linked to progesterone levels (Griesbeck et al., 2016; Santinelli et al., 2020). Moreover, an increase in interferon-stimulating genes (ISGs) has been observed in women living with HIV compared to men at different phases of HIV infection (Hagen & Altfeld, 2016; Ziegler & Altfeld, 2016). Progesterone may increase the expression of markers like CCR5 and CXCR4 on CD4⁺ T cells, thereby increasing the susceptibility of these cells to HIV (Hagen & Altfeld, 2016; Ziegler & Altfeld, 2016). Hormonal contraceptives, particularly progesterone-based ones like depot medroxyprogesterone acetate (DMPA), may further complicate matters by potentially increasing the risk of HIV acquisition (Griesbeck et al., 2016; Scully, 2018a). Mechanisms proposed for the DMPA-HIV effect include decreased epithelial barrier integrity, increased presence of HIV target cells at sites of genital HIV exposure, and alterations in microbiome and genetic profiles (Rodriguez-Garcia et al., 2021). This concern is particularly relevant in sub-Saharan Africa, where DMPA is extensively utilized (Griesbeck et al., 2016).

Genetic factors, including variations in genes related to immune function, can affect susceptibility to HIV and disease progression. Among these, certain variants of the CCR5 gene have been identified as significant contributors to HIV acquisition, with potential differences observed between men and women. One of the most studied variants is the CCR5- Δ 32 variant, which leads to a truncated or non-functional CCR5 protein (Ronsard et al., 2019). Individuals carrying this variant exhibit high resistance to HIV infection, as the virus primarily uses the CCR5 co-receptor for cell entry (Dean et al., 1996; Kulkarni et al., 2019; Sullivan et al., 2001). With a defective co-receptor, HIV is unable to efficiently enter and infect target cells (McLaren & Fellay, 2021; Ronsard et al., 2019; van Eekeren et al., 2022). While research confirms that the presence of CCR5- Δ 32 variant confers strong protection against HIV infection in both men and women, there are noteworthy sex-specific distinctions in how this protection manifest (Hu

et al., 2012; Philpott et al., 2003). Men harboring the CCR5-Δ32 variant experience a significantly reduced risk of HIV acquisition compared to those without it (Dean et al., 1996; Huang et al., 1996). In contrast, although women with the CCR5-Δ32 variant also exhibit a diminished risk of HIV acquisition, the effect may not be as pronounced as observed in men (Philpott et al., 2003). Genetic variations in CCR5 ligands, such as CCL3, as well as variations in other chemokine genes like CCR2, also impact HIV susceptibility and disease progression (Hu et al., 2012; Ioannidis et al., 2003; Kulkarni et al., 2019). For instance, single nucleotide polymorphisms (SNPs) in the CCL3 gene (such as rs34171309A) are associated with an increased risk in HIV acquisition, especially in women (Hu et al., 2012). Sex chromosomes represent additional genetic mechanisms contributing to sex differences in HIV. These chromosomes carry immunoregulatory genes that play a role in shaping the immunological disparities observed between sexes (Cohn et al., 2020; Hagen & Altfeld, 2016; Moran et al., 2022; Santinelli et al., 2020).

Furthermore, socioeconomic and biological factors can intersect to contribute to disparities in HIV infection between sexes, as seen in the case of sexual violence and trauma (Griesbeck et al., 2016). Sexual violence has been linked to HIV acquisition risk. Women who have experienced intimate partner violence were more likely to acquire HIV compared to those who had not experienced such violence (Jewkes et al., 2010; Kouyoumdjian et al., 2013). Research presented by Pollock *et al.* (2024) at the Canadian Association for HIV Research (CAHR) in April 2024, highlighted a robust correlation between recent incidents of forced sexual activity within the previous week and increased genital inflammation, independent of other biological risk factors, such as bacterial vaginosis (Pollock et al., 2024). Forced sex often leads to genital injury (Slaughter et al., 1997) resulting in systemic and local inflammation, which can increase susceptibility to HIV infection (Griesbeck et al., 2016).

1.4.2. Role of Host Genetics in HIV Pathogenesis

1.4.2.1. Overview

Genetics plays a crucial role in HIV pathogenesis by influencing susceptibility to infection, disease progression, and treatment response. Variations in genes that encode viral receptors, immune response elements, and host restriction factors contribute to the complex interplay between the virus and the host. For example, polymorphisms in the CCR5 gene, which encodes an HIV co-receptor, can impact an individual's risk of infection. One of the most well-known genetic variations affecting HIV is the 32-base pairs deletion in the CCR5 gene (CCR5-Δ32 mutation) (McLaren & Fellay, 2021). Individuals who are homozygous for this mutation – having two copies – are highly resistant to HIV infection (Dean et al., 1996; Ni et al., 2018; Philpott et al., 2003; Sullivan et al., 2001) because the mutation renders CCR5 non-functional, preventing the virus from using the co-receptor to enter cells (Kulkarni et al., 2019; McLaren & Fellay, 2021; Ronsard et al., 2019). Those who are heterozygous – having only one copy of the mutation – typically experience lower setpoint viral loads and slower disease progression compared to individuals without this mutation (Dean et al., 1996; Huang et al., 1996; Ni et al., 2018; Sullivan et al., 2001). This discovery based on the observation that individuals with the CCR5- Δ32 mutation are resistant to HIV led to the development of Maraviroc, a CCR5 antagonist antiviral drug used as an ART. (108). Additionally, a few cases of sustained HIV remission in the absence of ART following hematopoietic stem cell transplantation (HSCT) from donors homozygous for the CCR5Δ32 mutation have been reported, most notably the “Berlin patient” (Hütter et al., 2009) and “London patients” (Gupta et al., 2019), suggesting the possibility of a functional cure. The CCR5- Δ32 mutation is found predominantly in populations of European descent, with homozygosity occurring at frequency of about 1% and heterozygosity at approximately 10% (McLaren & Fellay, 2021). In contrast, African and Asian populations typically lack this mutation, contributing to differences in

susceptibility to HIV across populations (Dean et al., 1996; Ioannidis et al., 2003; Philpott et al., 2003; Sullivan et al., 2001).

In addition to CCR5, certain HLA alleles, such as HLA-B*57, are linked to better control of HIV infection, lower viral loads, and slower disease progression due to their role in presenting viral antigens to immune cells (Fellay et al., 2009; McLaren & Fellay, 2021). Whereas HLA alleles, such as HLA-B*35, are associated with rapid progression to AIDS (Fellay et al., 2009). Other genetic variations such as those genes encoding host restriction factors that inhibit HIV replication, like SAMHD1, p21, APOBEC3G and TRIM5 α , as well as genes involved in innate and adaptive immune responses can influence the body's response to HIV in terms of susceptibility and disease progression (Javanbakht et al., 2006; McLaren & Fellay, 2021). For instance, SAMHD1, a dNTP hydrolase, restricts HIV replication by depleting intracellular nucleotide pools necessary for reverse transcription (Laguette et al., 2011), while p21, a cyclin-dependent kinase inhibitor, has been shown to enhance SAMHD1 activity and directly interfere with HIV integration (Chen et al., 2011). Both factors have been implicated in natural resistance to HIV observed in elite controllers and HIV-exposed seronegative individuals (Chen et al., 2011).

1.4.2.2. Focus on *CD101* genetic variants and HIV Acquisition

Recent research continues to identify new genetic factors and mechanisms relevant to HIV to enhance our understanding and inform the development of more effective prevention and treatment strategies. An example is a study by Mackelprang et al. (2017) which identified rare genetic variants in the CD101 and UBE2V1 genes associated with an increased risk of HIV acquisition (Mackelprang et al., 2017). Individuals with a higher number of polymorphic functional sites in these genes showed an increased risk of HIV seroconversion. These genes are involved in immune system regulation, and their variants may lead to increased

inflammation, making individuals more susceptible to HIV (Richert-Spuhler et al., 2021). The researchers used whole genome sequencing data from a cohort of heterosexual sub-Saharan Africans with quantifiable HIV exposure to investigate the association of rare or intermediate-frequency variants with HIV acquisition. They identified fourteen functional variants in the CD101 gene, categorized into four sub-groups based on their structural location: five missense variants in the extracellular immunoglobulin-like (Ig-like) domain, five missense variants in the CD101 cytoplasmic-like (cyto-like) domain, two 3'-UTR variants, and two splice site variants (Mackelprang et al., 2017).

Variants in the Ig-like domains of *CD101* were significantly associated with a higher risk of HIV acquisition, with a hazard ratio (HR) of 4.3. Three of the Ig-like variants (rs17235773, rs3754112, rs12093834) were individually significant and at least one of them were found in 18% of the Kenyan population studied. Additionally, *CD101* Ig-like variants correlated with altered levels of plasma cytokines, evidenced by reduced plasma levels of IL1RN and sCD40L compared to individuals without these variants, suggesting these variants may impact systemic immune response. Variants in the cytoplasmic domain of *CD101* were significantly associated with increased HIV risk (HR of 2.8), but this significance was lost after adjustment for multiple comparison. A UBE2V1 5'-UTR variant (rs6095771) significantly increased HIV risk in women (HR of 6.4), but not in men. None of the identified variants were significantly associated with plasma HIV RNA setpoint among those who became infected, suggesting the variants do not directly impact viral replication. Albeit Mackelprang *et al.* (2017) provided evidence that individuals with these genetic variants, despite their rarity in the general population, were at a higher risk of HIV acquisition compared to those lacking them (Mackelprang et al., 2017). These observations are particularly relevant for the present study, especially concerning the *CD101* genetic variation.

Wanga *et al.* (2020) revealed a differential impact of BV on HIV acquisition based on the presence of *CD101* Ig-like variants. Their study found that BV was associated with a 2.5-fold higher risk of HIV infection in the absence of Ig-like variants, whereas in the presence of these variants, BV did not increase HIV risk, suggesting that *CD101* variants might modulate HIV susceptibility through pathways independent of the microbiota, including BV (Wanga *et al.*, 2020). Following up on the findings by Mackelprang *et al.* (2017), Richert-Spuhler *et al.* (2021) explored the mechanisms by which *CD101* gene variants increase HIV risk. They showed that *CD101* variants are associated with a heightened proinflammatory immune environment and impaired Treg function, which likely contributed to the increased HIV risk observed in those with *CD101* variants. Individuals with these variants exhibited differential T cell phenotypes and functions, characterized by increased proportions of CD8⁺ and Tregs expressing CD101 versus decreased proportions of antigen presenting cells expressing CD101 including DCs, monocytes, and B cells, compared to those without the variants. They observed an increased expression of proinflammatory T cell phenotypes as well as Treg cells with diminished ability to suppress cytokine production by effector T cells (Richert-Spuhler *et al.*, 2021a). Transcriptomic data revealed a differential expression of genes related to antiviral responses and inflammation based on *CD101* variant status. This included downregulation of ISGs and HIV resistance genes, potentially rendering CD4⁺ T cells more susceptible to HIV infection and CD8⁺ T cells less cytotoxic (Richert-Spuhler *et al.*, 2021a). They also observed that different sub-groups of *CD101* variants have varying impacts on immune cell subsets and functions. Specifically, Ig-like variants tend to have a stronger effect on immune cell activation and proinflammatory profiles compared to cyto-like variants (Richert-Spuhler *et al.*, 2021a).

1.4.2.3. CD101 – An Immunoregulatory Marker

The *CD101* gene encodes CD101, a type 1 transmembrane cell-surface protein known for its immunoregulatory functions. Structurally, CD101 is a disulfide-linked glycoprotein

composed of seven Ig-like domains and seven glycosylation sites on its extracellular portion, along with a short cytoplasmic tail that has several serine/threonine phosphorylation sites (Bagot et al., 1997; Boulloc et al., 2000; Wrage et al., 2021). CD101 is expressed on the surface of a variety of immune cells, including regulatory T cells (Tregs), effector and memory CD4⁺ and CD8⁺ T cells, DCs, granulocytes, monocytes, and macrophages in humans and in mice (Fernandez et al., 2007; Rainbow et al., 2011; Strongin et al., 2022). Typically, CD101 is weakly expressed on resting or naïve T cells, but upon activation, most T cells significantly increase their expression of CD101 (Jovanovic et al., 2011; Strongin et al., 2022). Additionally, the expression of CD101 is more pronounced for T cells in intestinal mucosal tissues (Mattner et al., 2019; Schey et al., 2016a; Wrage et al., 2021) and are considered a marker of tissue residency (Girard et al., 2024; Holz et al., 2020). While the ligand for CD101 has not yet been identified (Rainbow et al., 2011; Wrage et al., 2021), many studies have shown its role in T cell activation, DC function, immune suppression, and regulation of immune responses in conditions such as inflammatory and autoimmune diseases.

CD101 acts as a negative co-stimulatory and anti-inflammatory molecule, modulating T cell activation either directly or indirectly through CD101-expressing DCs (Mattner et al., 2019; Schey et al., 2016a; Wrage et al., 2021). CD101 expression on DCs has been shown to significantly reduce T cell proliferation and activation in a TCR/CD3-dependent manner, supported by decreased IL-2 receptor alpha (IL-2R α) expression and reduced IL-2 cytokine secretion (Bagot et al., 1997). Boulloc *et al.* (2000) demonstrated that activating CD101 on DCs induces IL-10 production, which suppresses T cell proliferation, highlighting the immunoregulatory function of CD101 via the IL-10 pathway (Boulloc et al., 2000). Further confirmation of the immunoregulatory role of CD101 was observed in a graft-versus-host disease (GVHD) model, where splenic Tregs expressing high levels of CD101 (CD101^{high}) were more suppressive of T cell proliferation compared to CD101^{low} Tregs (Fernandez et al.,

2007). Hence, CD101 expression on Tregs directly correlates with its potency at controlling immune responses. Mice treated with CD101^{high} Tregs had reduced expansion of donor T cells, better survival rates, lower proinflammatory cytokine levels, and less organ damage in the GVHD model (Fernandez et al., 2007). In contrast, Jovanovic *et al.* (2011) did not observe a marked difference in the suppressive capacity of CD101-expressing Tregs versus CD101-lacking Tregs in the peripheral blood. Rather, they showed that CD101 expression in the peripheral blood was inversely correlated with the cytotoxic activity of CD8⁺ T cells (Jovanovic et al., 2011).

CD101 regulatory function extends to chronic diseases such as inflammatory bowel disease (IBD) (Schey et al., 2016a), type 1 diabetes (T1D) (Penha-Gonçalves et al., 2003; Rainbow et al., 2011), celiac disease (Åkesson et al., 2015), and certain cancers (Liu et al., 2022; Zhang et al., 2019; Zhou et al., 2020). In IBD patients, CD101 expression on myeloid and T cells is protective against enterocolitis and is associated with better disease outcomes due to reduced production of pro-inflammatory cytokines like IL-17 and inhibition of T cell expansion (Schey et al., 2016). Patients with IBD and T1D often exhibit lower levels of CD101, which may be due to genetic variations in the CD101 gene observed in some T1D patients (Mattner et al., 2019). The CD101 gene within the insulin-dependent diabetes (*Idd10*) region is considered a candidate gene for T1D susceptibility, with the risk of T1D linked to CD101 expression on various immune cells (Mattner et al., 2019; Penha-Gonçalves et al., 2003; Rainbow et al., 2011). In a study of liver autoimmunity, reduced CD101 expression was associated with more severe disease, prolonged bacterial persistence, and excessive T cell responses characterized by increased IFN- γ and IL-17 production (Mohammed et al., 2011). In cancers such as pancreatic, ovarian, and brain cancers, CD101 expression on immune cells predicted unfavourable clinical outcomes, including disease severity, poor treatment response, and worse overall survival (Liu et al., 2022; Zhang et al., 2019; Zhou et al., 2021).

In the context of HIV, CD101-expressing CD4⁺ T cells were more susceptible to HIV/SIV infection, particularly in the gut mucosa, marked by high expression of CCR5 and integrin $\alpha 4\beta 7$ (Strongin et al., 2022). During acute SIV infection, CD101⁺ CD4⁺ T cells were preferentially depleted, especially within the Treg compartment and mucosal tissues, and this depletion were associated with higher viral loads and increased level of proinflammatory cytokines. Although partially restored during long-term ART, reconstituted CD101⁺ CD4⁺ T cells exhibited a phenotype marked by elevated inhibitory receptors, production of immunosuppressive cytokines, and high levels of Ki67, indicating potential for homeostatic proliferation. Moreover, the study found no significant difference in the levels of HIV proviral DNA between CD101⁺ and CD101⁻ memory CD4⁺ T cells, indicating that CD101-expressing cells are equally capable of contributing to the latent HIV reservoir during suppressive ART that could potentially reignite infection (Strongin et al., 2022). These findings suggest a crucial role for CD101-expressing CD4⁺ T cells at different stages of HIV infection and highlights the potential of targeting CD101 to mitigate HIV pathogenesis and persistence.

1.5. HIV Intervention and Impact on Immune Biomarkers

1.7.1. HIV-1 Vaccine

There are no effective vaccines against HIV yet since its first discovery 40 years ago, nor has a cure been developed. While ART is effective in suppressing viral loads to undetectable levels, it does not eradicate HIV latent reservoirs, hence there is a risk of viral rebound shortly after ART interruption (Ng'uni et al., 2020). Therefore, an effective vaccine is needed for the complete eradication of the HIV pandemic. Major challenges face the development of a vaccine for HIV despite numerous efforts. Key obstacles to developing an effective vaccine against the virus include the rapid mutation rate of the virus, leading to high genetic variability among strains, the integration of the virus into the host's DNA, creating a

persistent infection that is hard to target, and the ability of the virus to evade and weaken the host's immune response, making it difficult to develop a vaccine that elicits a strong and effective defense (J. H. Lee & Crotty, 2021; Ng'uni et al., 2020; Nkolola & Barouch, 2024).

Most vaccines against other infectious diseases offer protection by inducing efficient antibody responses (J. H. Lee & Crotty, 2021). However, in the case of HIV, the envelope protein is covered by sugar molecules (glycans) that shield it from being recognized by the immune system (J. H. Lee & Crotty, 2021; Ng'uni et al., 2020). This makes it extremely challenging to induce broadly neutralizing antibodies that can neutralize the wide variety of HIV strains (J. H. Lee & Crotty, 2021; Nkolola & Barouch, 2024). Moreover, immunological correlates of protection are not fully elucidated, and many vaccine candidates have focused on eliciting T cell responses, particularly CD8⁺ T cell responses, aimed at halting early viral replication and spread (J. H. Lee & Crotty, 2021; Ng'uni et al., 2020; Nkolola & Barouch, 2024; Shimada et al., 2014). To date, none of these strategies have met with significant success. The only vaccine candidate that has shown some degree of efficacy in preventing HIV acquisition is the prime-boost vaccine regimen involving a combination of ALVAC-HIV (a canarypox vector vaccine) and AIDSVAX B/E (a recombinant gp120 vaccine) tested in the RV144 Thailand trial (Rerks-Ngarm et al., 2009). The trial showed modest efficacy in reducing the risk of HIV infection among Thai participants, particularly against clade B and CRF01_AE, with ~31% reduction in HIV infection among vaccinees compared to the placebo group (Rerks-Ngarm et al., 2009).

At least five distinct HIV-1 vaccine strategies have been explored over several years in various clinical trials with none yielding significant efficacy. An HIV vaccine trial of more relevance to the present study is the HVTN 503, otherwise known as the Phambili trial. It was a Phase IIb study conducted in South Africa to evaluate the safety and efficacy of the Merck Adenovirus serotype 5 (Ad5)-vectored HIV-1 *gag/pol/nef* vaccine (Gray et al., 2011). The

MRK-Ad5 vaccine consists of a 1:1:1 mixture of three Ad5 vectors expressing *gag*, *pol*, and *nef* genes from HIV-1 strains of clade B sequences, inserted in the adenoviral E1 region (Gray et al., 2011; Hopkins et al., 2014). The vaccine was designed to induce cell-mediated immunity to protect against HIV infection or control early plasma HIV viral loads (Buchbinder et al., 2008). The trial was initiated in 2007 and enrolled around 801 participants across five sites, predominantly involving a heterosexual population in regions with a high prevalence of HIV clade C (Gray et al., 2011). The trial was stopped prematurely in September 2007 following interim results from the HVTN 502/STEP study, a parallel trial involving primarily MSM and at-risk women in the Americas and Australia. The study showed that the vaccine was not effective in preventing HIV infection, despite eliciting strong HIV-specific CD8⁺ T cell responses (Buchbinder et al., 2008; Gray et al., 2011, 2014; Hopkins et al., 2014). Moreover, data suggested a potential increase in HIV acquisition risk among certain subgroups, particularly Ad5-seropositive and/or uncircumcised men (Buchbinder et al., 2008; Duerr et al., 2012; Masek-Hammerman et al., 2010). Subsequent analyses of the HVTN 503 trial data confirmed these findings, prompting the permanent cessation of vaccinations and unblinding of the study (Gray et al., 2011, 2014; Moodie et al., 2015).

Post-hoc analyses of the HVTN 503 and STEP trials have tried to elucidate the possible mechanisms underlying the potential increased in HIV acquisition observed among vaccine recipients, and these have generated some conflicting reports. The use of rAd5 as a vaccine vector is popular because of its high immunogenic nature, ability to express large amounts of antigens, and ease of manufacturing (Gray et al., 2014). However, in the case of the MRK rAd5 vaccine, pre-existing immunity to Ad5 was among the mechanisms proposed for the increased risk of HIV acquisition among the vaccinees (Cheng et al., 2012; Qureshi et al., 2012; Überla, 2008). It was shown that the rAd5 vaccine may have activated and expanded Ad5-specific CD4⁺ T cell responses, which then migrated to mucosal sites and increased the pool of HIV

target cells available at infection sites in Ad5-seropositive individuals (Benlahrech et al., 2009; Fauci et al., 2014; Qureshi et al., 2012; Überla, 2008). However, other studies disprove this theory as they showed similar levels of total and vector-specific CD4⁺ T cell responses in the blood or mucosal samples of Ad5-seropositive versus Ad5-seronegative individuals after receiving the Merck rAd5 vaccination (Hutnick et al., 2009; Masek-Hammerman et al., 2010; O'Brien et al., 2009). Other proposed mechanisms include increased mucosal CD4⁺ T cell responses, increased inflammatory responses, and increased immune activation, all of which potentially increase the presence of target cells that could have facilitated HIV acquisition (Benlahrech et al., 2009; Fauci et al., 2014; Überla, 2008). Notably, Benlahrech *et al.* (2009) found an expansion of Ad5-specific memory CD4⁺ T cells, especially in Ad5-seropositive individuals, and these cells showed a mucosal-homing phenotype with increased expression of integrin $\alpha 4\beta 7$ and CCR9 (Benlahrech et al., 2009). These cells also expressed more CCR5 levels and were more susceptible to R5-tropic HIV infection (Benlahrech et al., 2009). Their study therefore suggests that in people with existing immunity to Ad5, the Ad5-vectored vaccine increased the number of HIV-susceptible activated CD4⁺ T cells in mucosal tissues, raising the risk of HIV acquisition (Benlahrech et al., 2009).

In addition, the more recent HVTN 702 trial, large-scale Phase II/III HIV vaccine trial conducted in South Africa and based on the RV144 regimen, was halted in early 2020 after an interim analysis found that the vaccine did not prevent HIV acquisition, underscoring the continued challenge of eliciting protective immunity against diverse HIV subtypes (Gray et al., 2021). A detailed summary of major HIV-1 vaccine clinical trials already completed is provided in (**Appendix Table A1**).

1.7.2. Non-Vaccine HIV Prevention Strategies: Pre-exposure Prophylaxis (PrEP)

The advent of lifelong ART has significantly improved the quality of life of PLWH by suppressing viremia and enabling longer, healthier lives (Ng’uni et al., 2020). Although no cure or vaccine for HIV has been developed yet, various prevention strategies are implemented worldwide to curb the incidence of HIV infection. These strategies include condom use, male circumcision, treatment as prevention strategy (TasP) – which involves administering ART to PLWH not only for their health benefit but also to reduce their risk of transmitting the virus to others (undetectable = untransmissible infection, U=U), – post-exposure prophylaxis (PEP) – an emergency administration of ART to potentially HIV-exposed individuals, – and pre-exposure prophylaxis (PrEP) (Nkolola & Barouch, 2024; Pialoux et al., 2016).

First approved in 2012 (Mushtaq & Kazi, 2022), PrEP is a preventive strategy for HIV that involves administering ART medication to HIV-uninfected high-risk individuals before potential exposure to the virus to reduce the risk of infection (Majid et al., 2012; Pialoux et al., 2016). The concept of PrEP is that ART drugs provide a protection at the earliest stage of infection, making it harder for HIV to spread throughout the body. There is typically a small “window of opportunity” within the first three days of HIV exposure where the virus first infects a small number of cells, which then replicate and spread the virus to more cells (Majid et al., 2012). By taking PrEP, antiretrovirals (ARVs) are already present in the body and can act quickly to block HIV from infecting these initial cells. This early intervention prevents the virus from establishing itself and multiplying, thereby stopping the infection before it can become systemic and harder to control (Majid et al., 2012).

The main PrEP drug regimens tested and used in humans include tenofovir disoproxil fumarate (TDF), emtricitabine (FTC), maraviroc, cabotegravir, rilpivirine, and dapivirine (Majid et al., 2012; Mushtaq & Kazi, 2022; Pialoux et al., 2016). A more recent drug regimen

with very promising results, Lenacapavir, showed a 100% reduction in HIV incidence among young women in South Africa and Uganda (Bekker et al., 2024). A table summarizing ARV drug classes, their targeted steps in the HIV replication cycle, and the timeline of approval is presented in (**Appendix Table A2**). However, the most commonly used PrEP regimen are TDF, either alone or in combination with FTC. Both TDF and FTC are reverse transcriptase inhibitors with long half-lives, allowing them to persist in tissues for prolonged periods of up to 40 hours or more (Majid et al., 2012; Pialoux et al., 2016). They can be administered either orally as a single-dose daily pill or via the mucosa as a topical gel (Majid et al., 2012). They are among the most potent, safe, and well-tolerated HIV medications used worldwide (Comstock et al., 2021; Majid et al., 2012).

The efficacy of PrEP in preventing HIV infection, mainly using TDF with or without FTC has been tested and confirmed in many large clinical trials involving high-risk HIV-negative individuals (Baeten et al., 2012; Grant et al., 2010; Karim et al., 2010; McCormack et al., 2016; Molina et al., 2015; Thigpen et al., 2012), with very few studies reporting a lack of efficacy (Delany-Moretlwe et al., 2018; Marrazzo et al., 2015; Van Damme et al., 2012).

One notable study, the Partners PrEP trial, was a randomized clinical trial of oral once-daily TDF, either alone or in combination with FTC (TDF/FTC, also called Truvada) (Baeten et al., 2012; Murnane et al., 2013). This study enrolled 4,758 HIV serodiscordant heterosexual couples (one partner is living with HIV and the other is uninfected) (Kahle et al., 2013; Mujugira et al., 2011) from multiple sites in East Africa (Kenya and Uganda). Participants were randomly assigned to receive either TDF, TDF/FTC, or a matching placebo and were followed up to 36 months. The study recorded a total of 82 HIV infections among the seronegative partners: 17 in the TDF arm, 13 in the TDF/FTC arm, and 52 in the placebo arm, indicating a significant reduction of 67% and 75% in HIV incidence in the TDF and TDF/FTC arms, respectively. There was no significant difference in the protective effects of TDF and

TDF/FTC, and no differences in efficacy were reported between men and women (Baeten et al., 2012). High adherence to these drug regimen was associated with increased reduction in HIV incidence, up to 86% with TDF and 90% with TDF/FTC (Baeten et al., 2012).

Another clinical trial, CAPRISA 004, assessed the effectiveness of PrEP as a 1% vaginal gel formulated with TDF (Karim et al., 2010). This randomized trial enrolled 889 uninfected heterosexual women from rural and urban areas in KwaZulu-Natal, South Africa and randomized to receive the tenofovir gel or placebo gel and were followed up to 30 months. A total of 38 HIV infections were reported in the tenofovir gel arm, while 60 were reported in the placebo gel arm. Therefore, HIV incidence was reduced up to 39% in the intervention group compared to the placebo arm. With high adherence ($> 80\%$), HIV incidence was reduced by up to 54% in the tenofovir gel arm, while with low or intermediate adherence ($< 80\%$), HIV incidence was reduced by 28% and 38%, respectively (Karim et al., 2010).

A PrEP study conducted in West Africa, which included 936 high-risk heterosexual women from Ghana, Cameroon, and Nigeria, tested oral TDF and showed no significant difference in HIV incidence between the TDF versus the placebo arms (Majid et al., 2012). A detailed summary of major clinical trials testing the efficacy of PrEP, either as an oral or topical prescription, is provided in (**Appendix Table A3**). A major challenge to PrEP efficacy is poor adherence. Therefore, one plausible explanation for non-efficacy in some studies is the lower adherence to PrEP observed (Pialoux et al., 2016). Adherence levels, typically determined by plasma concentration of the drugs or by self-reports from study participants, are strongly associated with PrEP efficacy (Baeten et al., 2012; Grant et al., 2010; Karim et al., 2010). Hence, as with ART for maintaining long-term control of viremia, strict adherence to PrEP is required for its sustained efficacy in preventing HIV.

Although data on the impact of PrEP on immunity are limited, some studies have hinted at its potential influence on the immune system. Richert-Spuhler *et al.* (2019) examined the immunological effects of oral tenofovir-based PrEP in HIV-uninfected but HSV-2 positive heterosexual African women (Richert-Spuhler *et al.*, 2019). They found that PrEP may alter the immune profile differently in the genital tract compared with the systemic compartment in HSV-2 positive women. Specifically, PrEP was associated with reduced CCR5-expressing CD4⁺ T cells, Tregs, and macrophages in the genital tracts, versus increased T cell activation and trafficking profiles observed in the peripheral blood during PrEP usage compared to its discontinuation (Richert-Spuhler *et al.*, 2019). In contrast, Pattacini *et al.* (2015), using data from the Partners PrEP study, found no evidence of alteration in the immune response to HIV as a result of PrEP, as they showed comparable frequency and magnitude of HIV-specific immune responses, including CD4⁺, CD8⁺, and NK cells, between those receiving PrEP and those receiving placebo (Pattacini, 2015).

1.8. CD4⁺ T cells and HIV

1.8.1. CD4⁺ T cell subsets and differentiation

The cell-mediated immune arm of the adaptive immune system represents T cell immunity, generally divided into two effector functions: cytotoxic and helper (Howell & Shepherd, 2021; Tomar & De, 2014). Cytotoxic T (Tc) cells express the CD8 molecule and are primarily involved in the killing of infected cells (Howell & Shepherd, 2021; Tomar & De, 2014). On the other hand, helper T (Th) cells express the CD4 molecule and are crucial for orchestrating immune responses during infection, guiding other immune cells to infection sites and curbing overactive immune reactions (Chatzileontiadou *et al.*, 2020; Gagliani & Huber, 2017; Luckheeram *et al.*, 2012; Ruterbusch *et al.*, 2020). In the context of HIV infection, these cell types play distinct roles. CD4⁺ T cells are central in susceptibility to HIV as they serve as

primary targets for the virus (Fanales-Belasio et al., 2010; McKinnon & Kaul, 2012). Conversely, CD8⁺ T cells play a protective role by controlling viral replication and eliminating infected cells and are elevated in HIV-infected long-term non-progressors highlighting their protective role in HIV (Collins et al., 2020; Pattacini, 2015; Rerks-Ngarm et al., 2009).

CD4⁺ T cells constitute about 60% of peripheral blood lymphocytes (Fanales-Belasio et al., 2010; Prabakaran et al., 2007). These cells exhibit significant heterogeneity influenced by various factors including cytokine signalling and transcriptional factors (Gagliani & Huber, 2017; Luckheeram et al., 2012). They differ in their phenotypes, cytokine profiles, metabolic behaviors, tissue affinities, immune reconstitution, and persistence (Gagliani & Huber, 2017). These cells differentiate into specialized subsets from antigen-inexperienced naïve T cells activated by antigens presented on MHC class II of APCs such as DCs (Kitagishi et al., 2012; Luckheeram et al., 2012; Ruterbusch et al., 2020). This activation begins in the thymus, where T cells develop specificity for foreign antigens. Upon circulation, they recognize antigens presented on MHC II of APCs via TCR, initiating activation (Chatzileontiadou et al., 2020; Prabakaran et al., 2007; Tomar & De, 2014). Co-stimulatory molecules like CD28 enhance this activation and proliferation (Prabakaran et al., 2007). Cytokines then guide the differentiation into subsets with distinct functions (Luckheeram et al., 2012). These subsets include Th1 (induced by IL-12, T-bet), Th2 (induced by IL-4, GATA3), Th17 (induced by IL-6, IL-23, RORγt), Tregs (induced by TGF-β, Foxp3), Th22 (induced by IL-6, AHR), Tfh (induced by IL-6, IL-21, Bcl-6), and Th9 (induced by IL-4, TGF-β, IRF4) (Gagliani & Huber, 2017; Kitagishi et al., 2012; Ruterbusch et al., 2020; Saravia et al., 2019; Tolomeo & Cascio, 2024). Each subset secretes unique cytokines mediating specific effector functions (Chatzileontiadou et al., 2020; Hopkins et al., 2014; Kitagishi et al., 2012; Ruterbusch et al., 2020). CD4⁺ T cells also exhibit plasticity, allowing them to switch characteristics between lineages, adding to their

complexity (Gagliani & Huber, 2017; Saravia et al., 2019). A detailed summary of CD4⁺ T cell differentiation is provided in **(Figure 3, Appendix Table A4)**.

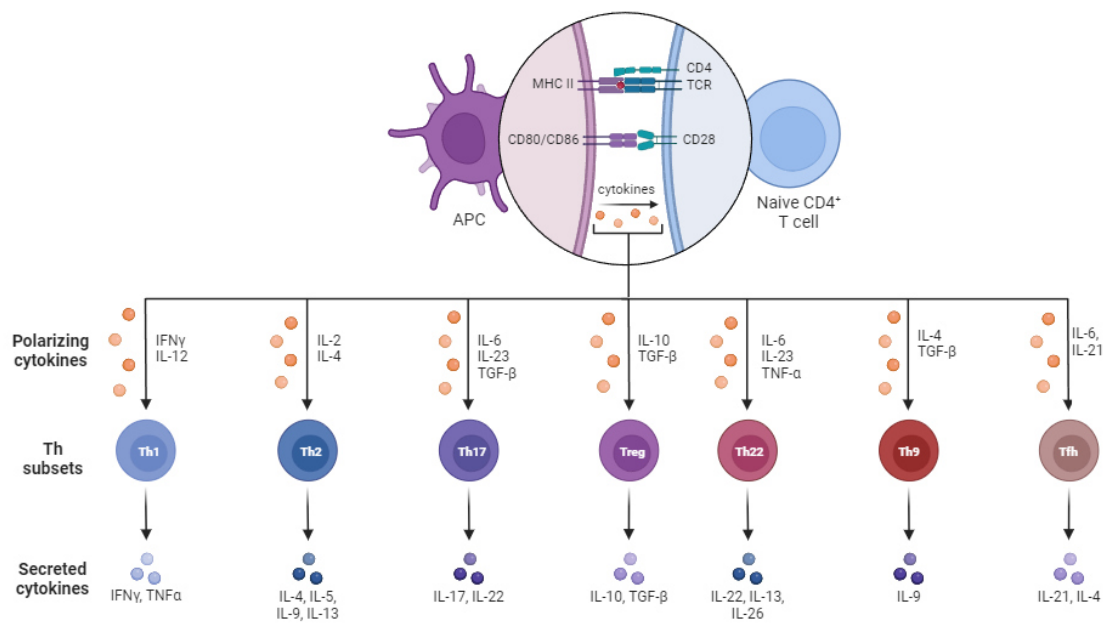


Figure 3. A Schematic Summary of CD4⁺ T Cell Activation and Differentiation.

Created in <https://BioRender.com>

CD4⁺ T cells can be further characterized in terms of their memory phenotype: central memory (TCM), effector memory (TEM), transitional memory (TTM), and stem-cell memory (TSCM) (Künzli & Masopust, 2023; Reeves et al., 2023; Ruterbusch et al., 2020). Memory T cells, derived from antigen-specific effector T cells that survive antigen clearance, respond more rapidly and efficiently to previously encountered antigens (Gagliani & Huber, 2017; Künzli & Masopust, 2023). TCM are confined to secondary lymphoid tissues, while TEM migrate between peripheral tissues and circulate between the blood and spleen (Gagliani & Huber, 2017; Tolomeo & Cascio, 2024). Heterogeneity of CD4⁺ T cells also include migratory potential, as different subsets express various homing markers that facilitate their movement to different tissues and compartments (Masopust & Schenkel, 2013). The migratory markers can be broadly classified into chemokine receptors (such as CCR7, CCR4, CCR6, CCR9, CXCR3) and integrins (such as $\alpha 4\beta 7$, $\alpha 4\beta 1$).

1.8.2. Differential Susceptibility of CD4⁺ T cell subsets to HIV

Despite the heterogeneity within the CD4⁺ T cell compartment, not all subsets are equally susceptible to HIV (McKinnon & Kaul, 2012; Rodriguez-Garcia et al., 2021; Tolomeo & Cascio, 2024; Zayas & Mamede, 2022). Highly susceptible subsets include those expressing CCR5, integrin $\alpha 4\beta 7$, and Th17 cells, all of which facilitate HIV replication (McKinnon et al., 2011; McKinnon et al., 2015; Rodriguez-Garcia et al., 2021; Tolomeo & Cascio, 2024). Immune activation, indicated by the upregulation of CD69, CD38, HLA-DR, and Ki67, also promotes HIV replication (Griesbeck et al., 2016; Rodriguez-Garcia et al., 2021). As reviewed by McKinnon and Kaul (2012), both the quantity and quality (phenotype) of HIV-susceptible target cells at sites of sexual HIV exposure, such as the genital or rectal mucosa, are crucial determinants of HIV acquisition (McKinnon & Kaul, 2012). Additional insights into the impact of specific CD4⁺ T cell phenotypes on HIV susceptibility and progression are outlined below.

1.9. Integrin $\alpha 4\beta 7$ in HIV Pathogenesis

1.9.1. Structure and Function of Integrin $\alpha 4\beta 7$

Integrins are cell adhesion receptors responsible for mediating the interactions between cells or between cells and extracellular matrix (ECM) (Mezu-Ndubuisi & Maheshwari, 2021; Zeller et al., 2001; Ziani et al., 2021). Comprising heterodimeric transmembrane glycoproteins, integrins are formed through stable non-covalent pairings between an alpha (α) and a beta (β) subunit (Mezu-Ndubuisi & Maheshwari, 2021; Perciani et al., 2018; Zeller et al., 2001; Ziani et al., 2021). There are eighteen and eight known α ($\alpha 1 - \alpha 11$, αD , αE , αL , αM , αV , αX , and αIIb) and β ($\beta 1 - \beta 8$) subunits, respectively, which pair with each other to form diverse integrin receptors, each playing distinct roles in cell adhesion, cell trafficking, and signaling processes (Cimbro et al., 2012; Mezu-Ndubuisi & Maheshwari, 2021). Twenty-four different integrin heterodimer pairings have been identified in humans (Mezu-Ndubuisi & Maheshwari, 2021). The $\alpha 4$ subunit (also known as CD49d) can couple with either the $\beta 1$ or $\beta 7$ subunits, resulting in the formation of integrins $\alpha 4\beta 1$ (very late antigen-4; VLA-4) and $\alpha 4\beta 7$ (lymphocyte Peyer's patch adhesion molecule-1; LPAM-1), respectively (Cimbro et al., 2012; Liu & Lusso, 2020; Perciani et al., 2018; Zeller et al., 2001; Ziani et al., 2021). The $\beta 7$ subunit, on the other hand, can pair with either $\alpha 4$ or αE to form $\alpha 4\beta 7$ or $\alpha E\beta 7$, respectively (Cimbro et al., 2012; Perciani et al., 2018). All these three integrin pairings, $\alpha 4\beta 7$, $\alpha 4\beta 1$, and $\alpha E\beta 7$, play significant roles in T cell localization (Perciani et al., 2018).

Integrin $\alpha 4\beta 7$ is a gut-homing integrin of particular importance in HIV pathogenesis. This integrin is expressed on the surface of various immune cells, including lymphocytes, NK cells, monocytes, basophils, and eosinophils (Zeller et al., 2001). $CD4^+$ T cells, however, predominantly expressed this integrin (Calenda et al., 2019; Cicala et al., 2009; Zeller et al., 2001; Ziani et al., 2021) and it facilitates their trafficking to the GALT. The natural known

ligand of integrin $\alpha 4\beta 7$ is mucosal addressin cell adhesion molecule-1 (MAdCAM-1), expressed in high endothelial venules (HEV) of mucosal associated lymphoid tissues (MALT) (Byrareddy et al., 2016; Richardson et al., 2015; Sivro et al., 2018; Zeller et al., 2001). Other identified ligands for $\alpha 4\beta 7$ include Fibronectin (FN) and vascular cell adhesion molecule-1 (VCAM-1) (Richardson et al., 2015; Zeller et al., 2001). In addition, the $\alpha 4$ subunit itself on adjacent cells can function as ligands for $\alpha 4\beta 7$ binding (Altevogt et al., 1995; Zeller et al., 2001). The principal contact sites for $\alpha 4\beta 7$ binding to its ligands is contained on the $\alpha 4$ subunit of the integrin (Altevogt et al., 1995; Arthos et al., 2008; Richardson et al., 2015). The binding motifs for $\alpha 4\beta 7$ -MAdCAM-1 interaction comprise of a tripeptide amino acid residues with a central aspartic acid residue, leucine-aspartic acid-threonine (LDT), isoleucine-aspartic acid-serine (IDS), and leucine-aspartic acid-valine (LDV) sequences (Richardson et al., 2015; Zeller et al., 2001). LDV-containing peptides of $\alpha 4$ can also function as additional ligands for $\alpha 4\beta 7$ binding (Altevogt et al., 1995; Zeller et al., 2001).

1.9.2. Regulators of $\alpha 4\beta 7$ Expression on T Cells

While most T cells in circulation express $\alpha 4\beta 7$, a majority of these heterodimers are expressed on cell surface in an active form (Cimbro et al., 2012). The activation and expression of $\alpha 4\beta 7$ is enhanced in the presence of retinoic acid (RA) (Iwata et al., 2004; Vimompatranon et al., 2023; Wilen et al., 2012). RA is a metabolite of vitamin A, produced primarily by gut-associated DCs (Hall et al., 2011; Iwata et al., 2004; Sidell & Kane, 2022; Vimompatranon et al., 2023). RA plays a key role in regulating the expression of $\alpha 4\beta 7$ on T cells, enhancing the expression of the integrin and facilitating their preferential homing to the gut (Hall et al., 2011; Iwata et al., 2004). This gut-tropism effect imprinted by RA was confirmed in vitamin A-deficient mice, where the depletion of RA resulted in reduced numbers of $\alpha 4\beta 7^+$ memory and activated T cells in secondary lymphoid organs and depletion of T cells from the intestinal lamina propria (Iwata et al., 2004). Additionally, Vimompatranon *et al.* (2023) reported that

unlike memory CD4⁺ T cells, MAdCAM-1-dependent proliferation of naïve CD4⁺ T cells require the presence of RA (Vimonpatranon et al., 2023). The combination of MAdCAM-1 and RA induces a unique differentiation pathway in naïve CD4⁺ T cells, resulting in the emergence of CD4⁺ T cells characterized by $\alpha 4\beta 7^{\text{hi}}$ phenotype (Vimonpatranon et al., 2023). Cimbrow and colleagues identified IL-7 as an additional regulator of $\alpha 4\beta 7$ expression on T cells (Cimbrow et al., 2012). In Cimbrow's study, IL-7 – known for its role in T cell homeostasis and immune reconstitution – induces the expression and functional activation of $\alpha 4\beta 7$ in human T cells primarily in naïve T cells. This induction of $\alpha 4\beta 7$ is associated with enhanced binding activity to its ligand MAdCAM-1 and promotes the preferential homing of treated naïve T cells to the intestinal compartment (Cimbrow et al., 2012). Furthermore, IL-7 triggers a unique activation program in naïve T cells, leading to memory-like phenotypic features and proliferation independent of classic T cell activation markers. The study suggests that IL-7-driven induction of $\alpha 4\beta 7$ is a critical step in T cell reconstitution in lymphopenic hosts and an alternative pathway for T-cell intestinal homing independent of RA-mediated induction (Cimbrow et al., 2012).

1.9.3. Expression Dynamics of $\alpha 4\beta 7$

The expression of integrin $\alpha 4\beta 7$ on CD4⁺ T cells appear to remain relatively stable overtime in certain individuals, especially among those of African descent (Ling et al., 2019; Q. Liu & Lusso, 2020; Martin, Sivro, et al., 2020; Sivro et al., 2018). Kader *et al.* (2009) explored the dynamics of $\alpha 4\beta 7$ cells in the peripheral blood of healthy rhesus macaques and showed that nearly half of blood CD4⁺ T cells were $\beta 7^+$ while the remaining half were $\beta 7^-$ (Kader et al., 2009). Among the $\beta 7^+$ CD4⁺ T cells, about 20% expressed $\beta 7$ at high levels ($\beta 7^{\text{hi}}$) while about 30% expressed it at intermediate levels ($\beta 7^{\text{int}}$), with 95% of the $\beta 7^{\text{hi}}$ and 65% of the $\beta 7^{\text{int}}$ CD4⁺ T cells co-expressing $\alpha 4$ subunit of the integrin $\alpha 4\beta 7$ (Kader et al., 2009). Similarly, other studies have shown that a majority of $\beta 7^{\text{hi}}$ CD4⁺ T cells in the peripheral blood

are positive for $\alpha 4$ subunit (Cicala et al., 2009; Ling et al., 2019; Mijiti et al., 2022; Perciani et al., 2018; Sivo et al., 2018). Likewise, it's been shown that a majority of $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{lo}}$ CD4^+ T cells are central or effector memory cells, respectively, whereas $\alpha 4\beta 7^{\text{int}}$ CD4^+ T cells were of naïve phenotype (Cimbri et al., 2012; Kader et al., 2009). Moreover, within the three integrin $\alpha 4\beta 7$ subsets, $\alpha 4\beta 7^{\text{hi}}$ cells exhibit a greater efficiency in trafficking to mucosal tissues (Kader et al., 2009).

Perciani *et al.* (2018) examined the expression of $\alpha 4\beta 7$, $\alpha 4\beta 1$, and $\alpha \text{E}\beta 7$ integrins on CD4^+ and CD4^- T cells in systemic, cervical, and rectal tissues of healthy Kenyan women and reported that blood and cervical tissues exhibited higher frequencies of $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 1^+$ CD4^+ T cells, while the rectum showed similar levels of $\alpha 4\beta 7^{\text{hi}}$ and $\alpha \text{E}\beta 7^{\text{hi}}$ CD4^+ T cells (Perciani et al., 2018). Investigations from Sivo *et al.* (2018) and Perciani *et al.* (2018) also confirmed a positive correlation between $\alpha 4\beta 7$ frequencies in the peripheral blood and mucosa tissues, validating the use of peripheral blood $\alpha 4\beta 7$ as surrogate where mucosal sampling is not available (Perciani et al., 2018; Sivo et al., 2018). Wittner *et al.* (2019) observed $\alpha 4\beta 7$ dynamics in blood and tissue compartments among healthy individuals versus disease state in the context of HIV and ulcerative colitis and observed minimal variations in $\alpha 4\beta 7$ frequency on total CD4^+ T cells across disease and treatment statuses in both peripheral blood and examined tissues (Wittner et al., 2019). Specifically, $\alpha 4\beta 7^+$ CD4^+ T cells showed heightened activation levels and were more frequently CCR5^+ in healthy individuals and UC patients compared to $\alpha 4\beta 7^-$ counterparts. In contrast, HIV-infected individuals exhibited significantly lower frequencies of $\alpha 4\beta 7^+$ CD4^+ T effector memory T cells compared to healthy controls, a reduction that persisted despite ART (Wittner et al., 2019). Although $\alpha 4\beta 7^+$ CD4^+ T cells are partially reconstituted following ART initiation, they typically are not restored to the normal range (Wang et al., 2018; Wittner et al., 2019).

Byrareddy *et al.* (2015) observed variations in the expression pattern of $\alpha 4\beta 7$ among non-human primate (NHP) species. Specifically, HIV susceptible species like Asian Pigtailed macaques (PTM) and rhesus macaques (RM), demonstrated elevated levels of $\alpha 4\beta 7$ expression on CD4⁺ T cells compared to sooty mangabeys (SM), known for their resistance to disease progression. These differences in $\alpha 4\beta 7$ expression could potentially play a role in the varying susceptibility and disease progression observed among NHP models of SIV infection (Byrareddy *et al.*, 2015).

Racial differences in $\alpha 4\beta 7$ expression on CD4⁺ T cells have been reported. Specially, Kelley *et al.* (2016) found that among black MSM, memory CD4⁺ T cells in the blood showed heightened levels of $\alpha 4\beta 7$ compared to their white counterparts. This increased expression of $\alpha 4\beta 7$ on blood CD4⁺ T cells correlated with a greater proportion of proliferating $\alpha 4\beta 7^{+}$ CD4⁺ T cells in rectal tissue, suggesting an enhanced migration of potential HIV target cells to the rectal mucosa among black MSM, potentially elevating their susceptibility to HIV infection (Kelley *et al.*, 2016). Similarly, Martin *et al.* (2020) demonstrated racial disparities, but not differences based on sex, in $\alpha 4\beta 7$ expression in the blood using a cohort of people who inject drugs (PWID) comprising both men and women. Their findings indicate a higher proportion of $\alpha 4\beta 7^{+}$ CD4⁺ T cells among black individuals, regardless of gender, compared to white individual (Martin, Sivro, *et al.*, 2020).

1.9.4. The Role of Integrin $\alpha 4\beta 7$ in HIV Infection

Several studies have emphasized the critical roles of integrin $\alpha 4\beta 7$ in HIV pathogenesis. In particular, given its role in homing CD4⁺ T cells to the GALT – a key site for HIV replication and dissemination (Calenda *et al.*, 2019; Richardson *et al.*, 2015) – integrin $\alpha 4\beta 7$ may increase the pool of HIV target cells available for infection at these critical sites, highlighting its importance in HIV (Liu & Lusso, 2020; Ziani *et al.*, 2021). Secondly, CD4⁺ T cells expressing

$\alpha 4\beta 7$ serve as highly susceptible targets for HIV infection, as they frequently co-express other HIV-susceptible markers such as CCR5 and Th17 phenotypes (Cicala et al., 2009; Kader et al., 2009; McKinnon et al., 2011; Mijiti et al., 2022; Tokarev et al., 2020). These $\alpha 4\beta 7^+ CD4^+$ T cells are preferentially infected by HIV, evident early in HIV infection as they are among the first cells to be depleted in individuals living with HIV (Tokarev et al., 2020; Wang et al., 2018). However, studies have shown that $\alpha 4\beta 7$ expression alone may not be sufficient for HIV permissiveness; rather, the coexpression of CCR6 and $\alpha 4\beta 7$ identifies a subset of memory $CD4^+$ T cells with heightened susceptibility to HIV infection (Monteiro et al., 2011; Planas et al., 2017).

Furthermore, integrin $\alpha 4\beta 7$ may act as a non-essential binding receptor for HIV, potentially providing a selective advantage for viral entry during the initial stages of HIV infection (Arthos et al., 2008; Richardson et al., 2015). Arthos *et al.* (2008) demonstrated that HIV *Env* gp120 can bind to $\alpha 4\beta 7$ in a manner that mimics its binding to MAdCAM-1. The study revealed that HIV gp120 can bind to integrin $\alpha 4\beta 7$ on T cells as well as on NK cells. This interaction was observed to occur specifically on activated form of $\alpha 4\beta 7$ (Arthos et al., 2008). Furthermore, the study uncovered that gp120 binds to $\alpha 4\beta 7$ at sites near the epitopes recognized by its natural ligands (Arthos et al., 2008). The V2 domain of HIV gp120 contains a tri-peptide motif centered around aspartic acid at position 180, accompanied by two other amino acid residues at positions 179 and 181, resembling $\alpha 4\beta 7$ -MAdCAM-1 binding motifs (He et al., 2011; Richardson et al., 2015; Tassaneetrithep et al., 2014). While the aspartic acid residue remains highly conserved across HIV isolates, the other two amino acid residues at the first and third positions exhibit significant variability across clades (He et al., 2011; Richardson et al., 2015). The interaction between gp120 and $\alpha 4\beta 7$, though not necessary for viral entry or replication, was found to promote replication by mediating the activation of lymphocyte function-associated antigen-1 (LFA-1) on T cells, which facilitated the formation of virological

synapses and enhanced cell-to-cell transmission of HIV (Arthos et al., 2008). Moreover, they examined the binding of gp120 from different HIV subtypes to $\alpha 4\beta 7$, revealing that while gp120 from various HIV-1 subtypes (A, B, C, D) exhibited affinity for $\alpha 4\beta 7$, significant variations in reactivity were observed (Arthos et al., 2008).

Similarly, Richardson *et al.* (2015) found that both viral and host factors can influence the interaction between HIV and $\alpha 4\beta 7$. They used a viral replication assay to demonstrate that $\alpha 4\beta 7$ antibodies partially inhibited HIV replication, with the extent of inhibition varying widely among different strains of the virus and over time within individuals (Richardson et al., 2015). Furthermore, they observed that Transmitter/Founder viruses with a specific viral motif, particularly the P/SDI/V motif, exhibited higher reactivity with $\alpha 4\beta 7$ and a dependence on it for replication compared to strains with LDI/L motifs. Notably, they found that the P/SDI/V motif was more common in HIV clade C viruses from South Africa compared to clade C from other regions or other HIV clades, suggesting a geographical dimension to the interaction between $\alpha 4\beta 7$ and HIV (Richardson et al., 2015). Additionally, factors like bacterial vaginosis and elevated genital tract cytokines enhance $\alpha 4\beta 7$ dependence, especially for viruses with the P/SDI/V motif (Richardson et al., 2015). Sivo *et al.* (2018) demonstrated that in South Africa, infection with HIV strains characterized by a V2 loop containing the P/SDI/V motif was associated with a higher frequency of pre-HIV $\alpha 4\beta 7^{\text{hi}}$ cells (Sivo et al., 2018). This increased frequency of $\alpha 4\beta 7^{\text{hi}}$ on blood CD4^+ T cells was predictive of greater risk of HIV acquisition and faster disease progression (Sivo et al., 2018). Similarly, frequency of memory CD4^+ T cells expressing heightened levels of $\alpha 4\beta 7^{\text{hi}}$ pre-SIV and post-SIV challenge was associated with greater susceptibility to infection and disease progression in rhesus macaques (Martinelli et al., 2013). Mijiti *et al.* (2022) showed in the context of chronic HIV infection, using post-HIV samples, that higher frequencies of $\alpha 4\beta 7^+$ CD4^+ T cells in the peripheral blood negatively correlated with disease progression (Mijiti et al., 2022). Moreover, the elevated levels of $\alpha 4\beta 7$

expression have been linked to an augmented risk of HIV acquisition in individuals with prevalent HSV-2 infection (Goode et al., 2014; Martinelli et al., 2011). Conversely, some studies demonstrated no correlation between heightened levels of $\alpha 4\beta 7$ cells with neither HIV acquisition nor disease progression, probably due to racial differences and differences in HIV strains in the cohort (Martin, Patel, et al., 2020; Martin, Sivro, et al., 2020).

Research on $\alpha 4\beta 7$ as a therapeutic intervention in HIV has shown promise in mitigating HIV transmission and disease progression. However, discrepancies in replicating these findings present challenges in utilizing $\alpha 4\beta 7$ -targeted therapies as part of HIV cure strategies. Ansari *et al.* (2011) demonstrated the efficacy of inhibiting CD4⁺ T cell migration to the gastrointestinal mucosa using recombinant anti- $\alpha 4\beta 7$ monoclonal antibody (mAb), administered before and during acute SIV infection. Their study revealed a significant decrease in both plasma and mucosal viral loads, along with a reduction in proviral DNA levels within GI tissue in an SIVmac239 rhesus macaque infection model (Ansari et al., 2011). Byrareddy *et al.* (2016) also demonstrated the effective mitigation of mucosal transmission of SIV in macaques through targeted intervention against $\alpha 4\beta 7$ (Byrareddy et al., 2016). In their study, animals were treated with an anti- $\alpha 4\beta 7$ monoclonal antibody in combination with ART before and after repeated low-dose challenge of rhesus macaques with SIV intravaginally. Notably, animals treated with anti- $\alpha 4\beta 7$ mAb showed significant protection from SIV transmission and sustained virologic control, along with immune reconstitution after ART discontinuation in infected animals (Byrareddy et al., 2016). However, controversies arose when it was discovered that the virus used in the Byrareddy *et al.* (2016) study was an attenuated strain with a stop codon in the SIV *Nef* gene (SIVmac239-nef-stop) rather than a wild-type pathogenic strain, potentially influencing the significance of the findings (Sills, 2019). Calenda *et al.* (2019) demonstrated that a combination of bNAbs (VRCO1) and anti- $\alpha 4\beta 7$ therapy significantly delayed vaginal

SHIV acquisition, maintained CD4⁺ T cell counts, and reduced SIV-DNA loads post-infection compared to untreated macaques (Calenda et al., 2019).

On the other hand, several studies were largely unable to replicate the therapeutic efficacy of the anti- $\alpha 4\beta 7$ antibody treatment in animal models and human studies after ART interruption. Two concurrent studies using the SIVmac239-nef-stop virus (Di Mascio et al., 2019; Iwamoto et al., 2019), along with another study using a wild-type pathogenic SIVmac251 strain (Abbink et al., 2019), all demonstrated no therapeutic benefit of anti- $\alpha 4\beta 7$ antibody treatment in preventing SIV transmission or reducing viral reservoir or rebound after ART discontinuation. Ziani *et al.* (2020) reported that neither $\alpha 4\beta 7$ nor $\alpha 4\beta 7/\alpha 4\beta 1$ blockade resulted in suppressed viral replication or reduced viral reservoir size in blood or lymphoid tissues of rhesus macaques after SIV infection (Ziani et al., 2021). Ling *et al.* (2019) demonstrated that Vedolizumab – a humanized analog of anti- $\alpha 4\beta 7$ mAb used in the treatment of IBD including Ulcerative colitis and Crohn’s disease (Byraredddy et al., 2016; Johnson et al., 2022; McGuinty et al., 2020; Ziani et al., 2021) – failed to prevent HIV-1_{SF162} infection or control viremia or prevent viral rebound after treatment discontinuation in humanized mice models (Ling et al., 2019).

The failure to replicate the therapeutic efficacy of the antibody therapy against integrin $\alpha 4\beta 7$ dashed hopes for its use in HIV cure strategies. Nevertheless, $\alpha 4\beta 7$ remains a viable target contributing to viral pathogenesis and warrants further research.

1.10. Th17 Cells in HIV Pathogenesis

1.10.1. Function and Expression Dynamics of Th17 Cells

Th17 cells was first described in 2005 as an additional T helper cell subset distinct from Th1 and Th2 subsets (Harrington et al., 2005; Park et al., 2005). This subset of CD4⁺ T cells form an integral component of the immune system involved in defense against extracellular

pathogens, particularly at mucosal barrier surfaces (Dunay et al., 2016; Renault et al., 2022). Mostly enriched on mucosal surfaces and also present in peripheral blood, Th17 cells are particularly important in mucosal immunity, where they contribute to the maintenance of barrier integrity and homeostasis (Fernandes et al., 2017; Renault et al., 2022; Rodriguez-Garcia et al., 2014; Zayas & Mamede, 2022). They are key players in host defense mechanisms against bacteria and fungi mediating immune responses such as recruitment of neutrophils and macrophages to effector sites to combat these pathogens (Elhed & Unutmaz, 2010; Renault et al., 2022; Zayas & Mamede, 2022). These cells are also potent mediators of tissue inflammation, secreting proinflammatory cytokines including IL-17A, IL-17F, IL-22, IL-21, CCL20, IFN- γ , TNF- α , and GM-CSF (Dong, 2021; Klatt & Brenchley, 2010; Luckheeram et al., 2012; Muranski & Restifo, 2013; Wacleche et al., 2016; Wiche-Salinas T. R. et al., 2021).

1.10.2. Regulators of Th17 Expression

The differentiation of Th17 cells is regulated by a network of cytokines, including IL-6, IL-21, TGF- β , IL-23, and IL-1 β (Ancuta et al., 2010; Luckheeram et al., 2012; Renault et al., 2022; Wacleche et al., 2017; Zayas & Mamede, 2022). Transcriptional factors and signalling molecules including RORC2 (the human ortholog of ROR γ t in mice), ROR α , STAT3, IRF4, AHR, and BATF, have been implicated in the differentiation of Th17 cells (Ancuta et al., 2010; Dong, 2011; Luckheeram et al., 2012; Paiva et al., 2021; Wacleche et al., 2016, 2017; Wiche-Salinas T. R. et al., 2021). However, RORC2 is the master transcription factor for Th17 differentiation (Ancuta et al., 2010; Renault et al., 2022; Wiche-Salinas T. R. et al., 2021)

1.10.3. Intracellular Cytokine Identification of Th17 Cells

The IL-17 cytokine family comprises six members including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. IL-17A is the major cytokine produced by Th17 cells; hence,

Th17 cells are predominantly characterized by IL-17A production, otherwise referred to as IL-17 (Muranski & Restifo, 2013; Renault et al., 2022). “IL-17A signals through IL-17RA and IL-17RC receptors, expressed on epithelial and mesenchymal cells” (Luckheeram et al., 2012; Wacleche et al., 2017). IL-17A plays key functions in host immune mechanisms including regulating mucosal epithelial cell permeability, inducing antimicrobial peptides, recruiting innate immune cells and facilitating antibody responses (Muranski & Restifo, 2013; Wacleche et al., 2017). Th17 cells also produces IL-17F, an early marker of Th17 differentiation. IL-17F shares many functional and genetic similarities with IL-17A, however, IL-17F is not as potent as IL-17A in signaling through IL-17RA/RC to trigger responses in cells (Luckheeram et al., 2012; Wacleche et al., 2017).

1.10.4. Extracellular Marker Identification of Th17 Cells

In addition to cytokine identification of Th17 cells, surface markers including CCR6, CCR4, CXCR3, IL-23R, CD161, CCL20, CD90 have been used in Th17 identification (Dunay et al., 2016; Guillot-Delost et al., 2012; Renault et al., 2022). Surface markers such as CCR6, CCR4, and CXCR3 are used to identify distinct subsets of Th17 cells (Ancuta et al., 2010; Wacleche et al., 2017). The co-expression of these markers defines subsets with unique functional characteristics, including CCR6⁺ CCR4⁺ (Th17) phenotype producing IL-17, CCR6⁺ CXCR3⁺ (Th1/Th17) phenotype producing IL-17 and IFN- γ , as well as newly identified subsets, CCR6⁺ double-negative (CXCR3⁻CCR4⁻) and CCR6⁺ double-positive (CXCR3⁺CCR4⁺) cells (Ancuta et al., 2010; Paiva et al., 2021; Wacleche et al., 2016, 2017). These subsets exhibit differential susceptibility to HIV infection and contribute to the heterogeneity of Th17 cell responses (Wacleche et al., 2016). Additionally, Th17 cells exhibit extreme plasticity, allowing them to “acquire new effector features and convert toward other lineage subsets” (Renault et al., 2022; Zayas & Mamede, 2022).

1.10.5. The Role of Th17 Cells in HIV Infection

Th17 cells play a critical role in HIV pathogenesis, particularly in the gastrointestinal tract, where they are preferentially depleted during infection (Renault et al., 2022). Th17 cells represent a subset of CD4⁺ T cells that are extremely permissive to HIV infection, possibly due to their high expression of HIV co-receptors like CCR5 or CXCR4 and co-expression of mucosal migration receptors including integrin $\alpha 4\beta 7$ and CCR6 (Loiseau et al., 2016; Renault et al., 2022; Zayas & Mamede, 2022). HIV exploits Th17 permissiveness for its replication and persistence (Renault et al., 2022), leading to their depletion and contributing to chronic immune activation and disease progression. Th17 cell depletion in HIV infection is attributed to various mechanisms, including direct HIV infection (Wacleche et al., 2017; Zayas & Mamede, 2022), altered expression of Th17 polarizing cytokines (Wacleche et al., 2017; Zayas & Mamede, 2022), altered trafficking of Th17 cells (Loiseau et al., 2016; Wacleche et al., 2017), expansion of regulatory T cells (Tregs) (Loiseau et al., 2016; Wacleche et al., 2017), and overexpression of negative regulators of Th17 differentiation (Renault et al., 2022; Wacleche et al., 2017; Wiche-Salinas T. R. et al., 2021).

Preferential Th17 cell depletion early in HIV infection is well documented, in the intestinal and genital mucosal as well as in the peripheral blood (Klatt & Brenchley, 2010; McKinnon et al., 2011; McKinnon et al., 2015; Renault et al., 2022; Rodriguez-Garcia et al., 2014). Gosselin *et al.* (2010) revealed Th17-like CCR6⁺ CD4⁺ T cell subsets, particularly CCR4⁺CCR6⁺ and CXCR3⁺CCR6⁺ cells are highly permissive to HIV replication, exhibit strong gut and lymph node homing potential, and are enriched for integrated HIV DNA, suggesting a key role in sustaining viral reservoirs and promoting tissue dissemination (Gosselin et al., 2010). Likewise, El Hed *et al.* (2010), reported a marked decrease in circulating Th17 cells in ART-treated HIV⁺ patients compared to uninfected controls (El Hed et al., 2010). Cecchinato *et al.* (2008) reported a decrease in both systemic and mucosal Th17 cell frequency

early in SIV infection (Cecchinato et al., 2008). McKinnon *et al.* (2011) observed a preferential depletion of cervical but not blood Th17 cells in HIV infected female sex workers (FSWs) compared to HIV uninfected FSWs (McKinnon et al., 2011). Guillot-Delost *et al.* (2012) showed that circulating Th17 cells, characterized by CD90 expression, were depleted in HIV infected patients relative to uninfected individuals (Guillot-Delost et al., 2012). Brenchley *et al.* (2008) showed that blood Th17 cells were infected by HIV but not preferentially infected (Brenchley et al., 2008). Ndhlovu *et al.* (2008) showed a marked decrease in circulating Th17 cells in children living with HIV, particularly in children with high plasma viremia (Ndhlovu et al., 2008). Favre *et al.* (2009) showed a marked and preferential depletion of Th17 cells in SIV-infected pig-tailed (PTs) macaques coupled with an Imbalanced Th17/Treg dynamics in blood, lymphoid organs and mucosal tissues in PTs but not in African green monkey (AGMs) (Favre et al., 2009). Macal *et al.* (2008) showed Th17 cells were restored to normal levels in GI tracts and peripheral blood in PLWH on long-term HAART (Macal et al., 2008). Th17 cells were identified among the earliest cells infected by HIV/SIV infection in macaques' genital tissues (Maric et al., 2021; Stieh et al., 2016). Depletion of Th17 cells during HIV infection might also affect the body's natural ability to fight HIV. Brenchley et al. (2008) found that T cells specific to HIV, which produce IL-17, weren't present in the blood of HIV-infected individuals when stimulated with HIV antigens outside the body (Brenchley & Douek, 2008). However, Yue *et al.* (2008) showed that during early infection, HIV-specific Th17 cells were present in the blood but disappeared during chronic or non-progressive stages of infection (Yue et al., 2008).

Furthermore, a number of studies have demonstrated an association between Th17 cell frequency and HIV disease outcome. Ndhlovu *et al.* (2008) observed a significant negative correlation between Th17 frequency and HIV plasma viral load (Ndhlovu et al., 2008). El Hed *et al.* (2010) showed that Th17 reduction were correlated with increased immune activation in

ART-treated HIV patients with undetectable viral load (El Hed et al., 2010). Raffatellu *et al.* (2008), showed a marked depletion in Th17 CD4⁺ T cells in the gut mucosa of SIV-infected Rhesus macaques, which in turn led to a blunted immune response against *Salmonella typhimurium* and an increased systemic bacteria translocation, suggesting a loss or impaired mucosal barrier function (Raffatellu et al., 2008). Cecchinato *et al.* (2008) showed that mucosal Th17 frequency negatively correlated with plasma viral load (Cecchinato et al., 2008).

Th17 share similar differentiation pathways with regulatory T cells, making the balance between these two subsets important for immune homeostasis (Ancuta et al., 2010; Paiva et al., 2021; Romagnani et al., 2009). Brenchley *et al.* (2008) showed that an imbalance in Th17/Treg dynamics was predictive of increased immune activation and disease progression (Brenchley et al., 2008). Similarly, independent studies by Favre *et al.* (2009) and Hartigan-O'Connor *et al.* (2012) confirming an imbalance in Th17 and Treg cells ratios in blood and mucosal tissue in macaques were correlated with markers of SIV progression (Favre et al., 2009; Hartigan-O'Connor et al., 2012). In terms of restorative effect of ART on Th17 cells, it's been shown that Th17 CD4⁺ T cells frequency and function are not completely normalized or restored even with ART and the extent of restoration may depend on timing of ART initiation – early ART initiation resulting in better Th17 restoration – and anatomical compartments – circulating Th17 cells are more effectively restored compared to the mucosal Th17 cells (Fernandes et al., 2017; Loiseau et al., 2016; Renault et al., 2022; Wacleche et al., 2017). Hence, based on evidence, the depletion of Th17 cells can have profound consequences on HIV infection. Primarily, the reduction in Th17 cell numbers impairs the integrity of the mucosal barrier, allowing for microbial translocation (Fernandes et al., 2017; Renault et al., 2022; Wacleche et al., 2016; Wiche-Salinas T. R. et al., 2021). This process enables the entry of microbial products into systemic circulation, triggering immune activation and tissue inflammation (Fernandes et al., 2017; Renault et al., 2022; Wiche-Salinas T. R. et al., 2021). These events exacerbate HIV

infection by promoting viral replication and accelerating disease progression. In HIV/SIV natural hosts, AGMs and sooty mangabeys (SMs), in which Th17 cells are preserved, an absence of microbial translocation and systemic inflammation is observed, explain why this species do not progress to AIDS. (Elhed & Unutmaz, 2010; Loiseau et al., 2016; Wacleche et al., 2017; Zayas & Mamede, 2022). Similarly, studies in humans have shown that the frequency of Th17 cells is preserved in HIV-infected long-term non-progressors (Wacleche et al., 2017). Long-lived Th17 cells also contribute to viral reservoir in PLWH, thereby ensuring HIV persistence (Gosselin et al., 2017; Renault et al., 2022; Wiche-Salinas T. R. et al., 2021).

Chapter 2. Study Rationale

2.1. Preliminary Investigation and Rationale

HIV outcomes vary significantly among individuals, influenced by genetic and immunological factors. Some people are more susceptible to HIV, while others remain resistant despite multiple exposures. Similarly, among those infected, some resist disease progression while others progress rapidly. Understanding these variations involves identifying the complex interplay of viral and host factors, which is essential for developing effective treatments and prevention strategies.

The present study builds on previous research investigating the predictive role of pre-HIV $\alpha 4\beta 7$ -expressing CD4⁺ T cells in HIV pathogenesis. Prior work by Sivo *et al.* (2018), linked higher pre-HIV frequencies of $\alpha 4\beta 7$ -expressing CD4⁺ T cells with increased HIV acquisition and faster disease progression in South African women. The women having higher pre-HIV $\alpha 4\beta 7$ frequencies exhibited higher viral loads and more rapid declines in CD4 counts after HIV acquisition (Sivo *et al.*, 2018). Follow-up studies of the CAPRISA 004 cohort revealed that elevated levels of non-Th1 CD4⁺ T cell subsets including Treg and cytokine-expressing CD4⁺ T cells (IL-17, IL-22, and IL-4), were associated with higher viral loads (McKinnon Lab, unpublished). These findings underscore the importance of $\alpha 4\beta 7$ -expressing CD4⁺ T cells and other immune phenotypes in HIV pathogenesis.

However, a subsequent study produced contrasting findings. Research involving MSM and transgender individuals in the USA (HVTN 505) and PWID (ALIVE cohorts) found no significant association between elevated pre-infection $\alpha 4\beta 7$ CD4⁺ T cell levels and increased HIV susceptibility (Martin, Patel, *et al.*, 2020; Martin, Sivo, *et al.*, 2020). Interestingly, Martin *et al.* (2020) revealed a protective effect of $\alpha 4\beta 7$ against HIV acquisition among PWID (Martin, Patel, *et al.*, 2020).

These disparities indicate that the predictive role of pre-HIV $\alpha 4\beta 7$ in HIV may not be universally applicable and warrants further investigation. Additionally, another study identified an increased risk of HIV acquisition in individuals with functional variants in the *CD101* gene among serodiscordant couples from sub-Saharan Africa (110).

2.2. Gaps-in-Knowledge

Despite similar study designs employed by Sivo *et al.* (2018) (Sivo *et al.*, 2018) and Martin *et al.* (2020) (Martin, Patel, *et al.*, 2020), differences among cohorts studied may have influenced the conflicting conclusions regarding the role of pre-HIV $\alpha 4\beta 7$ in HIV susceptibility. While Sivo *et al.* (2018) focused on heterosexual South African women, Martin *et al.* (2020) included MSM, transgender individuals, and PWID in the USA. Variances in sex, geographical regions, HIV clades, and HIV prevention interventions are critical variables that could impact HIV outcomes.

Specific gaps in knowledge include:

- 1. Sex-specific Differences:** Variations in routes and mechanisms of HIV infection between men and women necessitate exploration into whether pre-HIV $\alpha 4\beta 7$ predicts HIV outcomes in heterosexual men as in heterosexual women.
- 2. Geographical and Clade Variations:** Differences in HIV clades across regions may influence the interactions of HIV with $\alpha 4\beta 7$. Further investigation is needed to understand the association between pre-HIV $\alpha 4\beta 7$ and HIV outcomes across various clades, particularly clades A and D.
- 3. Impact of HIV Prevention Interventions:** Understanding the effect of HIV prevention measures, such as candidate HIV vaccines and PrEP, on the relationship between pre-HIV $\alpha 4\beta 7$ and HIV susceptibility is crucial for optimizing prevention strategies.

4. **Other HIV-Susceptible Target Cells:** Identifying additional HIV-susceptible target cells or immune subsets that can predict HIV outcomes is essential for enhancing our understanding of the diverse mechanisms underlying HIV infection and disease progression.
5. **Mechanisms Behind CD101 Variants:** The mechanisms through which CD101 genetic variants are associated with increased HIV risk need to be further elucidated.

2.3. Overarching Aim

Therefore, the current study aims to address these gaps by validating the role of pre-HIV $\alpha 4\beta 7$ -expressing CD4⁺ T cells and exploring other potential immunological markers in predicting HIV outcomes across diverse cohorts. Additionally, we seek to investigate the mechanisms underlying the association of genetic factors, such as *CD101* variants, with HIV susceptibility. By elucidating these inconsistencies and expanding our exploration to include additional immune markers, the current study aims to provide a comprehensive understanding of immunological correlates influencing HIV susceptibility and disease progression, which ultimately contributes to knowledge that may be useful in the development of more effective prevention and treatment strategies for HIV/AIDS.

2.4. Hypotheses

Based on the rationale and identified gaps in knowledge, the following hypotheses were formulated:

1. **HIV-Susceptible CD4⁺ T Cell Phenotypes:** We hypothesize that a higher pre-infection frequency of HIV-susceptible CD4⁺ T cell phenotypes correlate with increased HIV acquisition and faster disease progression. This relationship is expected to be influenced by factors such as sex, virus clades, and HIV prevention interventions.

- 2. Role of CD101-expressing T Cells:** We hypothesize that CD101-expressing T cells mediate the association between CD101 genetic variants and HIV acquisition. Specifically, individuals with more functional variants in the CD101 gene are at heightened risk of HIV acquisition, potentially due to the modulatory effects of CD101-expressing T cells on immune responses.

2.5. Specific Objectives

Objective 1: To assess the consistency of pre-HIV $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells in predicting HIV acquisition and diseases progression across cohorts that differ in sex, HIV clades and HIV prevention interventions. This is addressed in **Chapter 4**.

- To quantify frequency of $\alpha 4\beta 7^{\text{hi}}$ on pre-HIV infection CD4⁺ T cells in peripheral blood of participants enrolled in HVTN 503 and Partners PrEP cohorts.
- To examine whether the association between pre-HIV $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells and HIV outcomes is consistent between heterosexual men and women.
- To investigate whether the association between pre-HIV $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells and HIV outcomes is consistent across different geographical regions with varying HIV clades (e.g., clades A, C, and D).
- To determine whether different HIV prevention methods modulate the predictive role of pre-HIV $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells.

Objective 2: To evaluate the role of other HIV-susceptible CD4⁺ T cells subsets, specifically Th17 cells, in predicting HIV acquisition and diseases progression. This is addressed in **Chapter 5**.

- To quantify frequency of pre-HIV infection Th17 CD4⁺ T cells in peripheral blood of participants enrolled in HVTN 503 and Partners PrEP cohorts.

- To investigate the association between pre-HIV Th17 CD4⁺ T cell frequency and HIV outcomes.

Objective 3: To examine the influence of circulating CD101-expressing T cell subsets in mediating the association between CD101 variants and HIV acquisition. This is addressed in **Chapter 6.**

- To quantify frequency of pre-HIV CD101-expressing T cells in peripheral blood of participants in Partners PrEP cohort.
- To investigate whether circulating T cell subsets, particularly those expressing CD101, mediate the relationship between CD101 variants and the risk of HIV acquisition.

Chapter 3. Materials and Methods

3.1. Study Approval

All participants in the parent cohorts provided informed written consent to have specimens stored for future immunological research, and the sub-studies reported here were approved by the Institutional Review Board (IRB) at the University of Manitoba and at local IRBs where the study was conducted where required.

East African Study Sites (PP/COS)

- University of Washington Institutional Review Board
- Kenya Medical Research Institute Ethics Committee
- Kenyatta National Hospital Ethics Committee
- Kilimanjaro Christian Medical College
- Moi University Ethics Committee
- Republic of Botswana Ministry of Health
- South Africa Medicines Control Council
- Uganda National Council for Science & Technology
- Uganda National AIDS Research Committee
- Uganda Virus Research Institute
- University of Witwatersrand Ethics Committee
- University of Cape Town Institutional Review Board

South African Study Sites (HVTN 503)

- Fred Hutchinson Cancer Research Center, Seattle, Washington, USA
- University of the Witwatersrand
- University of Cape Town

- University of Limpopo
- University of KwaZulu Natal

3.2. Sex as a Biological Variable

This study included male and female at risk of HIV infection, and we present sex-stratified analyses within this work. In the analysis presented in this thesis, the terms “male/female” and “men/women” are used interchangeably to refer to biological sex.

3.3. Study Cohorts

3.3.1. Partners PrEP (PP): The Partners Pre-exposure Prophylaxis study (ClinicalTrials.gov number, NCT00557245) was a randomized, double-blind, placebo-controlled, phase III clinical trial that assessed the safety and efficacy of oral PrEP in the form of Tenofovir (TDF) alone or in combination with emtricitabine (FTC/TDF) for HIV prevention. The study enrolled heterosexual men and women in HIV serodiscordant relationship from nine sites in Kenya (Kisumu, Nairobi, Thika, Eldoret), and Uganda (Kampala, Jinja, Kabwohe, Mbale, Tororo) between 2008 to 2010. The serodiscordant couples were partners in which one partner was living with HIV and the other was not. All participants were between the ages of 18 and 65 years.

3.3.2. Partners in Prevention HSV/HIV Transmission Study (HSV): The HSV study (ClinicalTrials.gov number, NCT00194519) was a randomized, double-blind, placebo-controlled, proof-of-concept phase III trial that assessed the efficacy of HSV-2 suppression with twice daily oral acyclovir on preventing HIV transmission between discordant partners, whom the partner living with HIV is also co-infected with HSV-2. The study enrolled heterosexual serodiscordant couples between 2005 – 2010 from Kenya (Kisumu, Nairobi, Thika, Eldoret), and Uganda (Kampala). All participants were 18 years of age and older.

3.3.3. Couples Observational Study (COS): The COS study was a longitudinal observational study designed to define immune correlates associated with HIV infection in HIV-serodiscordant couples. COS was part of a broader effort that included other studies, such as the Partners PrEP study and the Partners in Prevention HSV/HIV Transmission study, to evaluate the risk of HIV transmission and acquisition. In other word, the study was conducted as an extension from registered trials and enrolled HIV serodiscordant couples from South Africa (Soweto) and Uganda (Kampala), all above the age of 18 years and older.

Since the PP, COS and HSV studies were similar in enrollment criteria, regions and timeline, we integrated participants data from these cohorts and analyzed them together where applicable. We, therefore, renamed these cohorts PP/COS to reflect this data integration.

3.3.4. HIV Vaccine Trial Network (HVTN) 503: The HVTN 503 (ClinicalTrials.gov number, NCT00413725) study was a randomized, double-blind, placebo-controlled, phase II clinical trial that assessed the efficacy of an Adenovirus-vectored vaccine, MRK rAd5 trivalent HIV-1 *gag/pol/nef* vaccine to prevent HIV. The study enrolled heterosexual men and women aged between 18 and 35 years of age from multiple sites in South Africa (Cape Town, Soweto, MEDUNSA, eThekwin, and Klerksdorp). Due to concerns arising from the STEP trial (HVTN 502), which used the same vaccine regimen and showed no efficacy and a potential increased risk of HIV acquisition in certain subgroups, enrollment in the HVTN 503 trial was terminated early (with accrual between January and September 2007), and participants were subsequently followed up for 3.5 years.

3.4. Study Design and Population

We carried out a nested, retrospective, matched case-control study to evaluate HIV-susceptible CD4⁺ T cells, particularly those expressing integrin $\alpha 4\beta 7$, CD101, and Th17 cells, and their association with HIV outcomes, defined as HIV acquisition and disease progression,

using pre-infection samples from the African cohorts mentioned above. From each cohort, HIV-free samples from participants later diagnosed with HIV (PLDH) and non-seroconverting participants (NSC) during the study periods were analyzed. For HIV acquisition analyses, PLDH were sampled at the last available pre-HIV infection visit (RT-PCR negative) and matched to corresponding NSC within the same cohort based on pre-specified criteria. Whereas, for HIV progression analyses, only pre-infection samples from PLDH were analyzed.

Briefly, for PP/COS, pre-HIV infection samples from PLDH ($n = 36$) were sampled within 1 year before HIV diagnosis and had a sampling median of 234 days [interquartile range (IQR) 147·0 – 321·5] pre-infection. These participants were matched with NSC controls ($n = 144$), based on enrollment time, specimen time in freezer, cohort, and baseline risk score. The baseline risk score is a validated risk assessment tool designed to identify HIV-serodiscordant couples at the highest risk of HIV transmission. This score was calculated in the original clinical trial, with a maximum possible score of 12. Couples with a risk score of ≥ 5 are considered as being at higher risk for HIV transmission. The parameters included in the risk score calculation are age of the HIV uninfected partner, married and/or cohabiting status, number of children, frequency of unprotected sex, whether the male HIV-uninfected partner is uncircumcised and plasma HIV RNA levels in the HIV-infected partner.

For HVTN 503, we processed pre-HIV samples from PLDH ($n = 60$), median sampling time of 177 (IQR 141·0 – 218·5) days prior to infection matched with NSC controls ($n = 180$) based on the enrollment time (3-month bins), specimen collection visit, and controls still being at risk at the time of corresponding cases' HIV infection diagnosis date.

3.5. HIV Outcome Readouts

The primary readout for HIV acquisition was a positive HIV diagnosis within the periods the clinical trials were ongoing, and if no positive diagnosis were made, participants

were considered not to have acquired HIV. The primary readouts for disease progression were (1) peak viral load (VL) defined as the highest VL in the first 180 days of infection before ART initiation, (2) set point VL defined as the average VL after the initial 180 days of infection before ART initiation, (3) mean pre-ART CD4/CD8 ratio before and after the initial 180 days following infection, (4) pre-ART CD4/CD8 ratio at the last available sampling before and after the initial 180 days following infection, and (5) decline in CD4 counts below 500 copies/mm³ measured after infection but prior to the initiation of ART.

Low quality samples defined as samples with viability less than 50% were excluded from all analyses. Likewise, samples collected within periods more than 1 year before HIV infection were excluded from the final analyses reported in this study.

3.6. General Reagents

The list of general reagents, including media, buffers, staining kits and control beads, used in this study are itemized on **Table 1**, while reagents used for stimulating cells for Th17 immunophenotyping are presented on **Table 2**.

Table 1. List of general reagents used for sample processing and data acquisition

Reagents	Catalog Number	Company
BD Cytotfix/Cytoperm	554722	BD Biosciences
BD Perm Wash	554723	BD Biosciences
Brilliant Stain Buffer	563794	BD Biosciences
BD Cytometer Setup and Tracking (CS&T) beads		BD Biosciences
Rainbow Calibration Particles, 8 peaks (3.0 – 3.4 µm)	422903	BioLegend
Anti-rat and anti-hamster Ig, k/Negative control Compensation particles set	552845	BD Biosciences
Anti-Mouse Ig, k/Negative control Compensation particles set	552843	BD Biosciences
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation	L34957	Thermofisher Scientific
LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit, for 405 nm excitation	L34959	Thermofisher Scientific
Arc Amine Reactive Compensation Bead Kit	A10346	Thermofisher Scientific
Roswell Park Memorial Institute (RPMI)- 1640	11875093	Thermofisher Scientific
Gibco™ Phosphate Buffered Saline (PBS)	10010023	Thermofisher Scientific
Fetal Bovine Serum (FBS)	MT35077CV	Thermofisher Scientific
Dimethyl Sulfoxide (DMSO)	BP231100	Thermofisher Scientific
Penicillin-Streptomycin (PS)	450-201-EL	Wisent
Trypan Blue solution	609-130-EL	Wisent
Ficoll/Lymphocyte Separation Medium	305-010-CL	Wisent

Table 2. List of stimulation reagents used for Th17 immunophenotyping

Reagents	Catalog Number	Company
Phorbol 12-myristate 13-acetate (PMA)	P8139	Sigma-Aldrich
Ionomycin	I0634	Sigma-Aldrich
BD GolgiPlug	555029	BD Biosciences
BD GolgiStop	554724	BD Biosciences

3.7. *Ex vivo* Immunophenotyping Panels

Six different flow cytometry panels were designed and optimized to characterize various immune cell subsets *ex vivo* in peripheral blood mononuclear cells (PBMC) samples. A summary of these immunophenotyped subsets are shown in (**Figure 4**). However, only panels with their corresponding antibodies for which results were reported in this thesis are shown in (**Tables 3 – 5**).

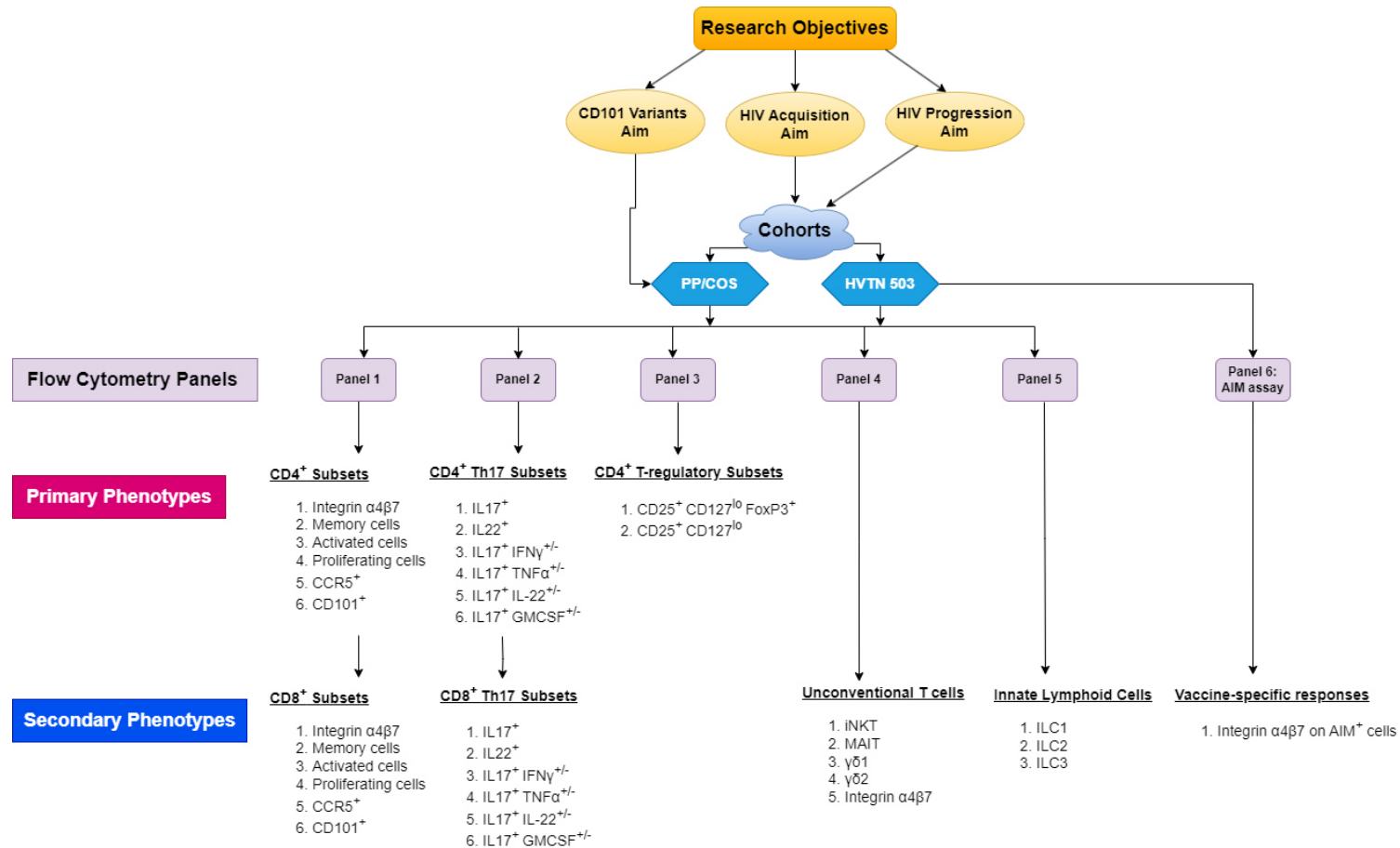


Figure 4. Schematic diagram showing all immune cell subsets characterized. Primary phenotypes refer to cell subsets of main interest to the research questions posed in this study, while secondary phenotypes were additional cell subsets characterized. Memory cells were characterized by CCR7, CD45RA and CD27 expression, activated cells by CD38 and HLA-DR expression, while proliferating cells by Ki67 expression. AIM represents Activation Induced Markers assay used for the cytokine-independent measurements of HVTN 503 vaccine-specific T cell responses based on expression of CD25, CD40L, CD69 and OX40. Image is created on *Drawio.com*.

Table 3. Primary surface staining panel used for immunophenotyping and assessing integrin $\alpha 4\beta 7$ and CD101 expression on CD4⁺ T cells in PBMC samples

Antibody	Fluorochrome	Volume or dilution (μl)	Clone	Catalog Number	Company
CD38	BB515	1	HIT2	564498	BD Biosciences
CD101	PERCP-Cy5.5	0.25	BB27	331016	BioLegend
CD27	PECy7	0.25	M-T271	560609	BD Biosciences
CCR7	PE-CF594	2	150503	562381	BD Biosciences
ITGβ7	PE	1	FIB504	555945	BD Biosciences
CCR5	BV421	2	2D7/CCR5	562576	BD Biosciences
Live/Dead Aqua	V500	1:500	–	L34957	Thermofisher Scientific
HLA-DR	BV650	1	G46-6	564231	BD Biosciences
CD3	APC-H7	1	SK7	560176	BD Biosciences
CD69	APC-R700	1	FN50	565154	BD Biosciences
Ki67	AlexaFluor647	1	B56	558615	BD Biosciences
CD8	BUV395	0.25	RPA-T8	563795	BD Biosciences
CD4	BUV496	1	SK3	564651	BD Biosciences
CD45RA	BUV737	1	HI100	564442	BD Biosciences

Table 4. Secondary surface staining panel used for immunophenotyping and assessing integrin $\alpha 4\beta 7$ expression on CD4⁺ T cells in PBMC samples

Antibody	Fluorochrome	Volume or dilution (μl)	Clone	Catalog Number	Company
γδ1	FITC	1	TS8.2	TCR2730	Thermofisher Scientific
CD49d	PeCy5	1	9F10	559880	BD Biosciences
iNKT	PE-CF594	3	6B	342920	BioLegend
INTβ7	BV421	0.5	FIB504	564283	BD Biosciences
CCR4	V500	0.5	L291H4	359416	BioLegend
Live/Dead Yellow	BV605	1:500	Not Applicable	L34959	Thermofisher Scientific
CD4	BV785	1	RPA-T4	300554	BioLegend
CD8	APC/Fire 750	1	RPA-T8	301066	BioLegend
CD3	APC-R700	1	UCHT1	565120	BD Biosciences
MR1 TET	APC	3	MR1 5-OP-RU	34536	NIH Tetramer Core
γδ2	BUV395	0.5	B6	743754	BD Biosciences
CCR6	BUV496	1	11A9	612948	BD Biosciences
CD45RA	BUV737	1	HI100	564442	BD Biosciences

Table 5. Primary intracellular cytokine staining panel used for immunophenotyping and assessing Th17 CD4⁺ T cells expression in PBMC samples

Antibody	Fluorochrome	Volume or dilution (µl)	Clone	Catalog Number	Company
TNF-α	FITC	4	MAb11	11-7349-41	Thermofisher Scientific
IFN-γ	PECy7	1	B27	557643	BD Biosciences
GM-CSF	PE-CF594	1	BVD2-21C11	562857	BD Biosciences
IL-22	PE	2	22URTI	12-7229-42	Thermofisher Scientific
IL-17A	BV421	4	N49-653	562933	BD Biosciences
Live/Dead Aqua	V500	1:500	Not Applicable	L34957	Thermofisher Scientific
CD8	BV650	0.5	SK1	344730	BioLegend
IL-10	BV711	4	JES3-9D7	564050	BD Biosciences
IL-4	BV786	4	MP4-25D2	564113	BD Biosciences
CD3	APC-H7	1	SK7	560176	BD Biosciences
CD45RO	BUV395	2	UCHL1	564291	BD Biosciences
CD4	BUV496	1	SK3	564651	BD Biosciences
INTβ7	BUV737	0.5	FIB504	565604	BD Biosciences

3.8. Sample Processing and Data Acquisition

3.8.1. PBMC Thawing Procedures

PBMCs were isolated from blood samples drawn from participants in the study cohorts and cryopreserved. The cryopreserved PBMCs were thawed, washed and rested in warm media (RPMI-1640 supplemented with 10% FBS and 1% PS) for 3 hours at 37°C and 5% CO₂ incubating conditions. The cells were resuspended in media, and an aliquot was stained with trypan blue to count cells and estimate cell viability using a hemacytometer cell counter. Low viable samples with less than 50% of live cells were excluded in subsequent analyses. Cells were stained and prepared for flow cytometry analysis, with all experiments performed by experimenters who were blinded to the clinical data. Sample processing and acquisition were carried out longitudinally in batches (12 batches for HVTN 503 and 16 batches for PP/COS) and matched samples were acquired within the same batch. At the start of our experiment, samples were initially acquired on BD LSRII™ (BD Biosciences) (n = 19 for PP/COS) before switching to a BD LSR-Fortessa™ (BD Biosciences) for later data acquisition. Data from stained cells were acquired within 24 hours of staining. A sensitivity analysis excluding samples acquired on the BD LSRII™ was conducted to investigate the impact of machine variation on our results. We included PBMCs from healthy donors to track variations in the measurements of the immune phenotypes and account for potential batch effects. BD CS&T beads (BD Biosciences) were run at every experiment for performance tracking of the flow cytometer, and periodically, Rainbow beads (8 peaks 3.0 – 3.4µm, BioLegend) were run to ensure voltage settings on the instrument were within the optimal range.

3.8.2. PBMC Isolation Procedures

PBMCs from whole blood samples from healthy donors were isolated using density gradient centrifugation method and cryopreserved as follows: whole blood was spun at 1500

revolutions per minute (rpm) and the plasma aliquoted into cryovial and stored at -80°C . The remaining whole blood were diluted with PBS + 2% FBS and carefully layered over Ficoll and centrifuged at 1400 rpm for 26 minutes with brake off, allowing the blood components to separate based on their densities. After centrifugation, distinct layers are formed, with PBMCs (including lymphocytes) located in a thin, whitish layer between the plasma and the Ficoll layer. The PBMC layer was carefully collected into fresh tubes, washed with PBS + 2% FBS and counted manually by trypan blue-hemocytometer method. The isolated cells were frozen in freezing medium (FBS + 10% DMSO) at 8×10^6 cells per ml for further use. The isolated PBMCs from healthy volunteers were used as quality control (QC) samples, as well as for the optimization of panels and protocols.

3.9. Flow Cytometry Procedures

Thawed PBMCs were analyzed by flow cytometry to quantify the expression of immune cells of interest including integrin $\alpha 4\beta 7$, CD101-expressing CD4^+ T cells and Th17 CD4^+ cells. The staining protocol for each panel of antibodies used depend on the nature of the markers on the panels. Specifically, $\alpha 4\beta 7$ and CD101 are extracellular markers and as such were stained following a surface staining protocol. Whereas cytokines used for Th17 characterization are intracellular and as such were stained using an intracellular cytokine staining (ICS) protocol.

3.9.1. Surface Staining Procedures for *ex vivo* Characterization of Integrin $\alpha 4\beta 7$ and CD101

At least $5 \times 10^5 - 1 \times 10^6$ cells were resuspended in fluorescence-activated cell sorting (FACS) buffer consisting of 1X PBS (pH 7.2) and 1% FBS. Samples were first stained with LIVE/DEAD™ Fixable Aqua or Yellow Stain Kit, where applicable, to differentiate between live and dead cells. The Live/Dead dyes were diluted as 1 μl in 500 μl PBS and a 100 μl volume

of the diluted dye is used to stain cells for 20 minutes. This was followed by staining with a premixture of fluorochrome-conjugated monoclonal antibodies to identify integrin $\alpha 4\beta 7$ or CD101 subsets on CD4⁺ T cells. The antibodies were premixed in BSB and PBS and incubated at 4⁰C in the dark. Additionally, the primary panel of antibodies used for the characterization of these cells (**Tables 3**) comprised of immune markers to profile different attributes of T cells, including the homing, memory, and activated T cell markers. Intracellular staining for Ki67 was performed using fixation/permeabilization method with BD Cytofix/Cytoperm. In a separate experiment, samples with high cell counts (n = 183 for HVTN 503, n = 161 for PP/COS) were stained with a secondary flow cytometry panel consisting of markers to characterize integrin $\alpha 4\beta 7$ (**Table 4**). The final step in the surface staining protocol involved treating cells with 25% BD cytofix solution to fix the cells before acquisition. Data from stained samples were acquired on a flow cytometer within 24 hours of staining and the phenotypic analysis using hierarchical gating strategy was performed on FlowJo™ version 10.6.1 (BD Life Sciences). Hierarchical gating involved progressively applying a series of gates to isolate specific subpopulations of interest. This strategy is hierarchical because each gating step depends on the results of the previous gate, gradually narrowing down from broad to specific populations. The percentage of cells positive for a specific marker were exported from FlowJo and used for statistical analyses.

3.9.2. ICS Procedures for *ex vivo* Characterization of Th17 cells

Following sample processing as described above in **section 3.8.1.**, cells were stimulated with PMA (100 ng/ml) and ionomycin (1 μ g/ml) for 4 hours at 37⁰C. Protein secretion was inhibited by the addition of pre-titrated amount of BD GolgiStop and GolgiPlug during stimulation. Following stimulation, cells were first stained with pre-titrated amount of LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit for 20 minutes to discriminate between live and dead cells. This was then followed by surface staining with a premixed cocktail of

extracellular fluorochrome-conjugated monoclonal antibodies, including CD3 APC-H7, CD4 BUV496, CD8 BV650, CD45RO BUV395 and ITG β 7 BUV737, for 20 minutes at 4⁰C. Stained cells were washed, permeabilized using BD Cytofix/Cytoperm at 4⁰C for 20 minutes and stained with a premixed cocktail of intracellular cytokine antibodies (**Table 5**), including IL-17A BV421, IL-22 PE, GM-CSF PECF594, IFN- γ PECY7, TNF- α FITC, IL-4 BV786 and IL-10 BV711, at 4⁰C for 30 minutes. Cells were then washed and acquired on a BD LSR FortessaTM flow cytometer. Immunophenotyping analysis was performed on FlowJoTM version 10.6.1 (BD Life Sciences).

All experimental procedures were performed in a blinded fashion, with experimenters blinded to clinical data all through the period of data acquisition.

3.9.3. Flow Cytometry Panels Optimization

To ensure the reliability and reproducibility of the flow cytometry data, each panel was optimized through a series of essential procedures. The optimization process involved several critical steps including antibody titration, voltage titration with both unstained and single-stained cells, the use of rainbow beads, and the application of Fluorescence Minus One (FMO) controls, all designed to optimize the performance of the antibodies and the flow cytometer settings. Below are details of each procedure:

3.9.3.1. Antibody Titration

Serial dilutions of each antibody were prepared to determine the optimal concentration for staining. Cells were stained with each dilution and analyzed to identify the concentration that provided the best signal-to-noise ratio. This step was crucial to ensure that sufficient antibody binding to the target antigen occurred without excess antibody contributing to non-specific binding or background noise. The antibody concentrations were selected based on the highest mean fluorescence intensity (MFI) with minimal background staining calculated as

staining index. A higher staining index indicated better separation of the positive population from the negative population, reducing background noise and improving the reliability of positive cell identification.

$$\text{Staining Index} = \frac{\text{MFI of positive population} - \text{MFI of negative population}}{2 \times \text{rSD of Negative Population}}$$

3.9.3.2. Voltage Titration with Unstained Cells

Unstained cells were run on the flow cytometer, and the detector voltages were adjusted to set the baseline fluorescence. The goal was to adjust the voltages for each channel until the robust standard deviation (rSD) of the fluorescence signal was three times the rSD of the electronic noise. Voltages were increased or decreased as need to achieve this baseline across all detectors. This step ensures that the instrument's detectors are properly calibrated, allowing for the distinction between background noise and true cellular events.

3.9.3.3. Voltage Titration with Single-Stained Cells

Voltage titration with single-stained cells was performed to optimize the detector sensitivity of each fluorochrome, ensuring accurate and precise detection by the specific fluorescence signal without interference from other channels. Cells were stained individually with each fluorochrome-conjugated antibody and analyzed on the flow cytometer. Detector voltages were adjusted to ensure that each fluorochrome's fluorescence was detected at its optimal intensity, with minimal overlap into adjacent detectors. The spillover spread matrix was also evaluated during this process.

3.9.3.4. Validation of Instrument's Settings with Rainbow Beads

Rainbow beads are beads containing a mixture of fluorophores that emit fluorescence across a wide range of wavelengths when excited by the lasers in the flow cytometer. Each

bead has a consistent and known fluorescence intensity, making them ideal for calibrating and standardizing the performance of flow cytometers. These beads were used occasionally in between experimental batches to verify that the instrument's fluorescence signals were consistent and reproducible overtime between different experiments.

3.9.3.5. Fluorescence Minus One (FMO) Controls

FMO controls were prepared by staining cells with all antibodies in the panel except for one fluorochrome at a time. Each FMO control was used to establish the gating boundary for the fluorochrome that was omitted, helping to differentiate between true positive and negative populations. FMO controls were essential to account for spillover fluorescence and set accurate gates, reducing the risks of false positives and ensuring that the gating strategy accurately identified the population of interest.

3.9.4. Compensation Controls Preparation

Compensation control was performed to correct for spectral overlap between fluorochromes. Compensation tubes were prepared for each fluorochrome used in a panel. To do this, 1 drop of positive compensation beads and 1 drop of negative compensation beads were combined in a single tube, followed by the addition of 400µl of PBS to reach an approximate volume of 420µl. This mixture was then split into four tubes, each receiving approximately 110µl of the solution. Next, 1µl of the appropriate antibody was added to each labeled tube, corresponding to a specific fluorochrome. The tubes were incubated for 10 minutes at room temperature in the dark after incubation, the volume in each tube was adjusted to 300µl by adding ~ 190µl of PBS.

Compensation tubes were run prior to each panel on the flow cytometer to ensure accurate compensation of fluorescence signals. Running these compensation tubes is critical for adjusting the flow cytometer's settings to account for the overlap in emission spectra of

different fluorochromes, thereby allowing for accurate measurement of each fluorochrome's signal in multicolor flow cytometry.

3.9.5. Live/Dead Dye Reconstitution and Compensation Preparation

The Live/Dead reactive dye and DMSO were brought to room temperature before use. To reconstitute the dye, 50µl of DMSO was added to the vial containing the Live/Dead dye pellet. The mixture was thoroughly mixed to ensure the complete dissolution of the dye. The reconstituted dye solution was used immediately, with any remaining solution aliquoted into small volumes, protected from light, and stored at $\leq -20^{\circ}\text{C}$ until next use.

To prepare Live/Dead dye for compensation control, one drop of positive amine-reactive beads was added to FACS tubes. 1µl of the reconstituted Live/Dead stain was added to the beads and incubated at room temperature in the dark for 30 minutes. The beads were washed with PBS containing 2% FBS, centrifuged and resuspended in PBS. Just before data running the Live/Dead compensation control on the flow cytometer, 1 drop of the negative amine-reactive beads were added to the tube.

This compensation step was essential to accurately correct for the spectral overlap of the Live/Dead dye, ensuring reliable discrimination between live and dead cells in the flow cytometry analysis.

3.10. General Statistical methods

Descriptive statistics including medians, interquartile ranges (IQR), and percentages were used to summarize continuous and categorical variables, respectively. Mann-Whitney test was used to compare immune cell levels between groups while Kruskal-Wallis' test was used if more than two groups were compared.

For $\alpha 4\beta 7$ -HIV acquisition analysis, conditional logistic regression (CLR) was used to predict HIV acquisition, with the primary explanatory variables being the three $\alpha 4\beta 7^+$ subsets. Each integrin subset was treated as a continuous variable and modeled separately in unadjusted and multivariable analyses, which adjusted for potential confounders. We next performed stratified CLR to compare the association between $\alpha 4\beta 7$ and HIV acquisition within sub-groups based on sex, country, and treatment arms within each cohort.

For HIV progression analyses, Spearman rank correlation test was used to evaluate the correlation between immune cell frequencies and HIV peak and set point viral loads (VL) as well as CD4/CD8 ratio post-HIV infection, described in **section 3.5**. Unadjusted and multivariable Cox regression was conducted to predict time to CD4⁺ T cell decline. The endpoint was defined as CD4⁺ T cell counts falling below 500/mm³ before initiation of ART. CD4⁺ T cell counts measured during the first 180 days of follow-up were censored to exclude the short-lived CD4 drops during the acute infection. In these cox regression analyses, the main explanatory variables are the specific cells levels, that is, $\alpha 4\beta 7$ or Th17 cells levels, dichotomized as above or below their median frequency of expression.

CLR was performed on SAS 9.4 while other statistical analyses and data visualizations were computed in RStudio 4.2.2. All statistics are two-tailed and p values ≤ 0.05 were considered statistically significant. P values were adjusted for multiple testing using the Benjamini-Hochberg method with *p.adjust* method in R.

3.11. Sample Selection and Statistical Methods for CD101 Analysis

3.11.1 CD101 Sample Selection

CD101 analyses were limited to PP/COS cohorts as these were the cohorts we had *CD101* genotype data. Participants with quantified level of HIV exposure, denoted by baseline risk score, were sampled based on previously defined *CD101* genotype categories. Briefly, we

selected participants having either immunoglobulin-like or cytoplasmic-like variants in their *CD101* gene or having a combination of both variants. Participants in the Ig-like variants group were either homozygous or heterozygous for only one of the three candidate *CD101* Ig-like single nucleotide variants (SNVs) (rs12093834, rs17235773, or rs3754112), while participants in the cyto-like variants group were homozygous or heterozygous for only one of two *CD101* cyto-like SNVs (rs150494742 or rs34248572). Individuals whose *CD101* sequence had no functional SNVs were considered as the control group. All samples were obtained from the participants before the evidence of HIV infection.

3.11.2. CD101 Statistical Methods

Descriptive statistics and comparisons between groups were done as described in the general statistical methods **section 3.10**. Comparisons of the frequencies of CD101 on T cell subsets were made separately between (a) individuals having “any” functional *CD101* Ig-like variants (“any” Ig-like group refers to the combined groups of participants having Ig-like variants only and those having both Ig-like plus cyto-like variants) versus the control group, (b) participants having *CD101* Ig-like variants only versus the control group, and (c) participants having *CD101* cyto-like variants only versus the controls. To evaluate potential mediating effect of CD101-expressing T cells on the relationship between *CD101* variants and HIV risk (**Figure 5**), we adopted two methods, namely, the multiple regression models (MRM) and the structural equation modelling (SEM) methods. We performed logistic regression (LR) analysis to confirm the association between *CD101* variants and HIV acquisition in our dataset. The predictor variable in all models included the variant categories (i.e., any Ig-like or cyto-like variants), each modeled separately using both unadjusted and multivariable models while adjusting for sex at birth as a possible confounder. Statistical analyses including LR and MRM were performed on SAS 9.4 software while other analyses including SEM and figures were

computed in RStudio 4.2.2. All statistics were two-tailed and p values ≤ 0.05 were considered statistically significant.

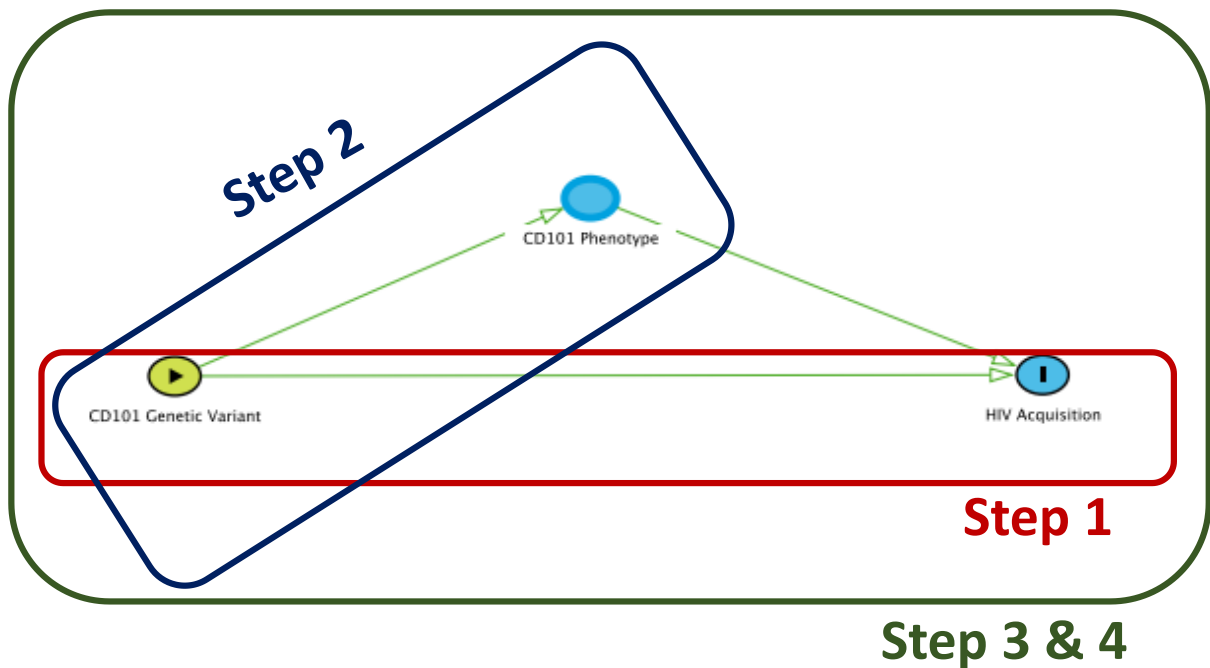


Figure 5. Mediation analysis framework. The steps are the conditions to satisfy to statistically establish a mediating effect. **Step 1.** Regression of the outcome variable (HIV acquisition) against the predictor variable (*CD101* variants). **Step 2.** Regression of the mediating variable ($CD101^+$ phenotype) against the predictor variable (*CD101* variants). **Step 3 and 4.** Regression of the outcome variable against the predictor variable while adjusting for the mediating variable.

Chapter 4. Pre-HIV $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells and HIV risk among heterosexual individuals in Africa

4.1. Introduction

The integrin $\alpha 4\beta 7$ is expressed on the surface of immune cells including CD4⁺ T cells where it mediates immune cell homing to gut-associated lymphoid tissues (GALT) (Guzzo et al., 2017; Q. Liu & Lusso, 2020). Several studies have suggested important roles for $\alpha 4\beta 7$ in HIV pathogenesis with contradictory findings reported. During acute infection, $\alpha 4\beta 7$ -expressing cells are highly susceptible to HIV/SIV and preferentially depleted; this being attributed to the fact that these cells highly co-express other HIV-susceptible markers such as the main HIV co-receptor, CCR5 and Th17 (Cicala et al., 2009; Kader et al., 2009; Wang et al., 2018). Some studies have proposed that $\alpha 4\beta 7$ may act as a non-essential receptor that aids viral attachment via interaction with gp120 of some HIV-1 strains (Arthos et al., 2008; Guzzo et al., 2017; Richardson et al., 2015). As a potential cure strategy, one study combined blocking $\alpha 4\beta 7$ using a monoclonal antibody with antiretroviral therapy (ART) and reported significant virological control in rhesus macaques infected with SIVmac239-*nef-stop* virus (Byraredddy et al., 2016). Two subsequent studies that tested this $\alpha 4\beta 7$ -blockade strategy in a similar infection model did not replicate this effect (Di Mascio et al., 2019; Iwamoto et al., 2019). While this lack of validation suggests targeting $\alpha 4\beta 7$ may not be a viable cure approach, CD4⁺ T cells expressing this molecule are nonetheless important targets of lentiviral infection.

Human cohort studies examining the role of integrin $\alpha 4\beta 7$ as a predictor of HIV acquisition or progression have also generated mixed results. In a previous study involving heterosexual South African women, we showed that women with higher pre-HIV $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells had a higher risk of HIV infection and progressed faster compared to women with lower $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells (Sivro et al., 2018), an observation not replicated in a follow up US-based

study amongst MSM and PWID (Martin, Patel, et al., 2020). Although both studies adopted similar study designs and methods, they differed in the characteristics of the populations examined, which we hypothesized may have contributed to the discrepancies between the conclusions of these studies. Therefore, gaps still exist regarding the consistency of associations between $\alpha 4\beta 7$ and HIV outcomes across multiple populations, clades, and geographies.

4.2. Results

4.2.1. Participants Characteristics

Study participants included both male and female humans at risk of HIV infection with a median age of 32 years (IQR 27 – 40) for PP/COS and 22 years (IQR 20 – 26) for HVTN 503. Of the 180 samples processed for PP/COS, 6 (3.3%) samples did not meet the 50% threshold for cell viability and were excluded from analyses. For HVTN 503, we excluded 19 (7.9%) samples due to low viability or because they were > 1 year prior to HIV infection. In PP/COS, 75% of participants had a baseline risk score ≥ 5 , reflecting seronegative partners with greater risk of HIV exposure. Due to the abrupt end of the HVTN 503 trial, 52 (24%) and 157 (71%) of participants received only one or two doses of the vaccine regimen, respectively, compared to 12 (~5%) who completed three vaccine doses as per the original protocol (**Table 6**).

Table 6. Baseline characteristics of participants

Characteristics	HVTN 503		PP/COS	
	PLDH (<i>n</i> = 47)	NSC (<i>n</i> = 174)	PLDH (<i>n</i> = 35)	NSC (<i>n</i> = 139)
Age (years) ^a	23·0 (21·0 – 27·0)	22·0 (20·0 – 26·0)	30·3 (24·6 – 39·7)	32·2 (28·1 – 40·1)
CD4 count (cells/mm ³) ^a	416 (290·0 – 496·8)	NA	491 (422·2 – 632·5)	NA
Sampling to infection (days) ^a	177 (141·0 – 218·5)	NA	234 (147·0 – 321·5)	NA
Female Sex, <i>n</i> (%)	26 (55·3)	72 (41·4)	19 (54·3)	61 (43·9)
Male Sex, <i>n</i> (%)	21 (44·7)	102 (58·6)	16 (45·7)	78 (56·1)
Treatment Arm, <i>n</i> (%)				
Placebo	24 (51·1)	86 (49·4)	15 (53·6)	60 (53·6)
Vaccine	23 (48·9)	88 (50·6)	NA	NA
PrEP	NA	NA	13 (46·4)	52 (46·4)
Cohort, <i>n</i> (%)				
COS	NA	NA	7 (20·0)	27 (19·4)
Partners PrEP	NA	NA	28 (80·0)	112 (80·6)
HSV2 Positive, <i>n</i> (%)	22 (46·8)	50 (28·7)	18 (72·0)	66 (63·5)
Treatment Status, <i>n</i> (%)				
Completed	3 (6·4)	9 (5·2)	NA	NA
Off treatment early	44 (93·6)	165 (94·8)	NA	NA
Ad5 Titre, <i>n</i> (%)				
≤ 200	19 (40·4)	69 (39·7)	NA	NA
> 200	28 (59·6)	105 (60·3)	NA	NA
Baseline Risk score ^b , <i>n</i> (%)				

< 5	NA	NA	9 (25.7)	34 (24.5)
≥ 5	NA	NA	26 (74.3)	105 (75.5)
Sites: South Africa, <i>n</i> (%)				
Cape town	10 (21.3)	40 (23.0)	NA	NA
eThekweni	11 (23.4)	14 (8.0)	NA	NA
Klerksdorp	8 (17.0)	32 (18.4)	NA	NA
Medunsa	5 (10.6)	22 (12.6)	NA	NA
Soweto	13 (27.7)	66 (37.9)	NA	NA
PHRU	NA	NA	2 (5.7)	4 (2.9)
Sites: Uganda, <i>n</i> (%)				
Jinja	NA	NA	7 (20.0)	29 (20.9)
Kampala	NA	NA	14 (40.0)	55 (39.6)
Sites: Kenya, <i>n</i> (%)				
Kisumu	NA	NA	4 (11.4)	32 (23.0)
Thika	NA	NA	2 (5.7)	8 (5.8)
Nairobi	NA	NA	6 (17.1)	11 (7.9)

All data are presented as sample sizes (*n*) and corresponding column percentages (%) unless otherwise stated. ^aData are presented as median (Interquartile Range).

^bPart of the matching criteria. Abbreviations: PLDH = People later diagnosed with HIV, NSC = HIV non-seroconverters, PrEP = Pre-exposure Prophylaxis, COS = Couples Observational Study, HSV2 = Herpes Simplex Virus 2, AD5 = Adenovirus Type 5, PHRU = Perinatal HIV Research Unit (Soweto, South Africa), NA = Not Applicable.

4.2.2. Effect of pre-HIV Frequencies of Integrin $\beta 7^{\text{hi}}$ CD4⁺ T Cell Subsets on HIV

Acquisition

We characterized three subsets of integrin $\alpha 4\beta 7$ on CD4⁺ T cells based on their expression of integrin $\beta 7$ and CD45RA: $\beta 7^{\text{hi}}$ CD45RA⁻, $\beta 7^{\text{int}}$ CD45RA⁺ and $\beta 7^{\text{lo}}$ CD45RA⁻ (**Figure 6A**). Given that previous reports showed that majority of $\beta 7^{\text{hi}}$ CD4⁺ T cells in the blood co-express $\alpha 4$ subunit of integrin $\alpha 4\beta 7$ (38,207,216,217) and that these cells were mostly memory cells (207,216,217), we considered $\beta 7^{\text{hi}}$ CD45RA⁻ CD4⁺ T cells to be $\alpha 4\beta 7^{\text{hi}}$. As a quality control measure, relatively consistent measurements of $\beta 7^{\text{hi}}$ expression on CD4⁺ T cells from the same healthy volunteer PBMCs were obtained across multiple experimental batches (**Appendix Figure A1**). We compared proportions of $\beta 7^{\text{hi}}$ CD4⁺ T cell subsets by treatment arms, sex, and country (**Figure 6, B – G**). However, the frequency of $\beta 7^{\text{hi}}$ CD4⁺ T cells was only significantly different with treatment arms, specifically, higher in the vaccine arm in HVTN 503 ($p = 0.015$; **Figure 6B**) and in participants who received oral PrEP in PP/COS ($p = 0.026$; **Figure 6E**) compared to the placebo groups. Pre-HIV $\beta 7^{\text{hi}}$ CD4⁺ T cells expression was not significantly associated with HIV acquisition in either cohorts. In HVTN 503, pre-HIV $\beta 7^{\text{hi}}$ odds ratio (OR) was greater than 1 [OR 1.06, 95% confidence interval (CI) 0.96 – 1.18, adjusted OR (aOR) 1.06, 95% CI 0.93 – 1.19], while PP/COS trended towards a protective effect [OR 0.92, 95% CI 0.83 – 1.01, aOR 0.93, 95% CI 0.84 – 1.03; **Table 7**]. In a sex-stratified CLR analyses, the odds of HIV acquisition among female participants [aOR 1.15, 95% CI 0.93 – 1.43] was higher compared to male participants [aOR 1.01, 95% CI 0.78 – 1.29] in HVTN 503 (**Figure 7, A and B**). Interestingly, in PP/COS pre-infection $\beta 7^{\text{hi}}$ CD4⁺ T cell frequency was significantly associated with reduced HIV acquisition among participants enrolled in Uganda (**Figure 7A**), but the association was diminished in an adjusted analysis [aOR 0.86, 95% CI 0.69 – 1.06, $p = 0.16$; **Figure 7B**]. Integrin $\beta 7^{\text{hi}}$ expression was not statistically different between PLDH and NSC (**Figure 7C – D**).

These results above were confirmed with a second flow cytometry panel containing integrin $\beta 7$ and CD45RA, which revealed consistent effect estimates and correlation trends, indicating the reproducibility of these results (**Appendix: Figure A2**). Likewise, sensitivity analyses revealed consistent results (**Appendix: Table A5 – A6**).

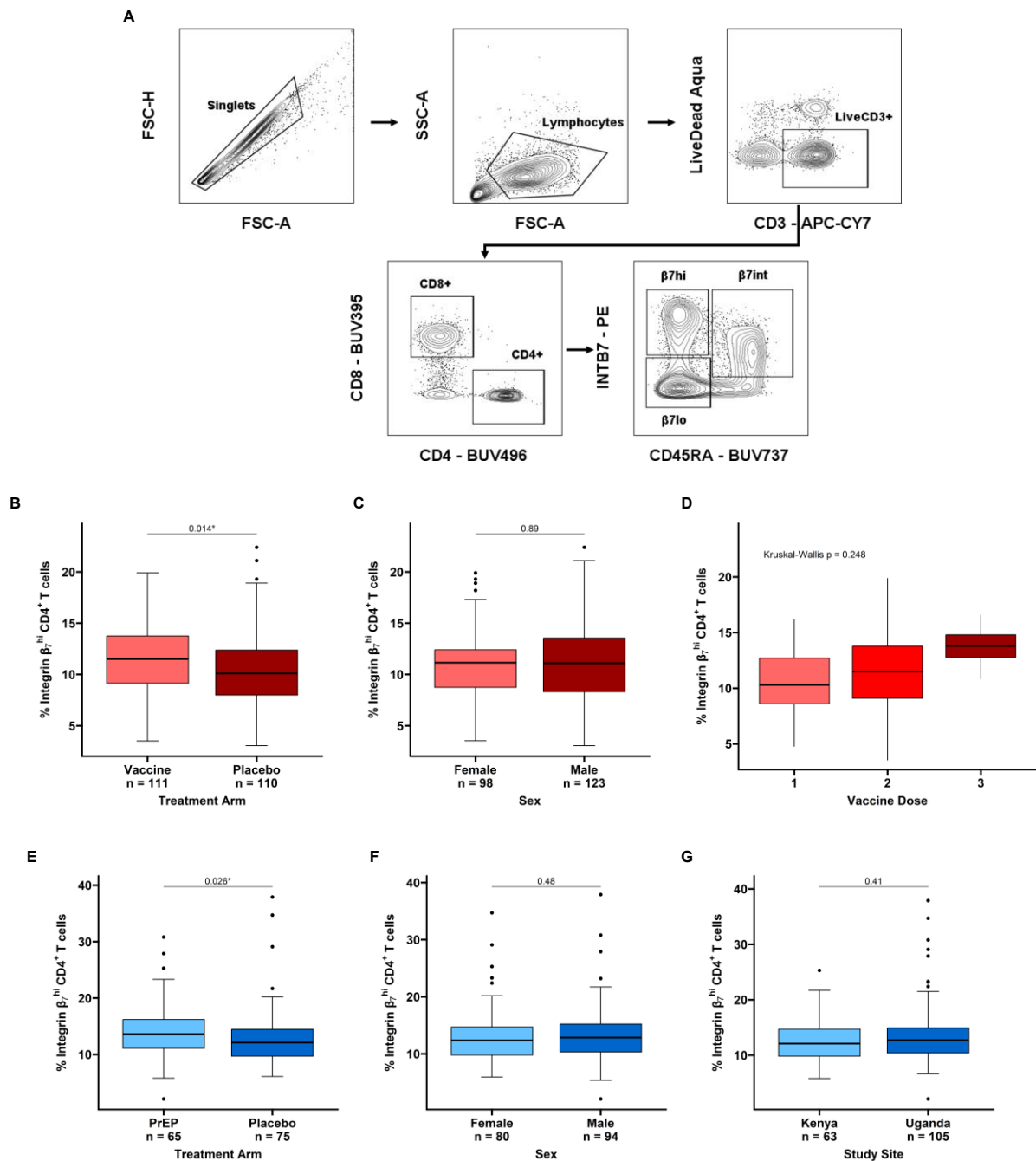


Figure 6. Expression pattern of pre-HIV frequency of integrin $\beta 7^{\text{hi}}$ CD4^+ T cells. (A) Representative gating strategy for flow cytometry analysis of integrin $\beta 7$ subsets from frozen PBMCs. **(B – D)** Comparison of pre-HIV frequency of $\beta 7^{\text{hi}}$ CD4^+ T cells between treatment arms, sex and vaccine doses in HVTN 503 (Dose 1: $n = 25$, Dose 2: $n = 82$, Dose 3: $n = 4$). **(E – G)** Comparison of pre-HIV frequency of $\beta 7^{\text{hi}}$ CD4^+ T cells between treatment arms, sex, and study sites in PP/COS. Plots colored “red” represent analyses from HVTN 503, while those in “blue” represent analyses from PP/COS. Comparison between two groups was analyzed using the Mann-Whitney test, while the Kruskal-Wallis’ test was used for comparison between more than two groups. ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

Table 7. Association of pre-HIV integrin β 7 CD4⁺ T cells with HIV acquisition

Integrin β7	Cohorts	Univariable		Multivariable	
Subsets		Models		Models	
		OR (95% CI)	P value	aOR (95% CI)	P value
Hi	HVTN 503	1.06 (0.96 – 1.18)	0.254	1.06 (0.93 – 1.19)	0.388
	PP/COS	0.92 (0.83 – 1.01)	0.082	0.93 (0.84 – 1.03)	0.182
Int	HVTN 503	0.99 (0.96 – 1.01)	0.379	1.01 (0.98 – 1.05)	0.377
	PP/COS	1.05 (1.01 – 1.08)	0.011*	1.05 (1.01 – 1.10)	0.027*
Lo	HVTN 503	1.01 (0.98 – 1.04)	0.508	0.98 (0.95 – 1.01)	0.187
	PP/COS	0.96 (0.93 – 0.99)	0.026*	0.96 (0.91 – 1.00)	0.061

The multivariable model for HVTN 503 was adjusted for age, sex, study sites, Adenovirus type-5 titer and HSV2 status at baseline while PP/COS was adjusted for age, sex, study sites and HSV-2 status at baseline. Abbreviations: OR = Odds Ratio, aOR = Adjusted Odds Ratio. Odds Ratios were estimated using conditional logistic regression models. ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

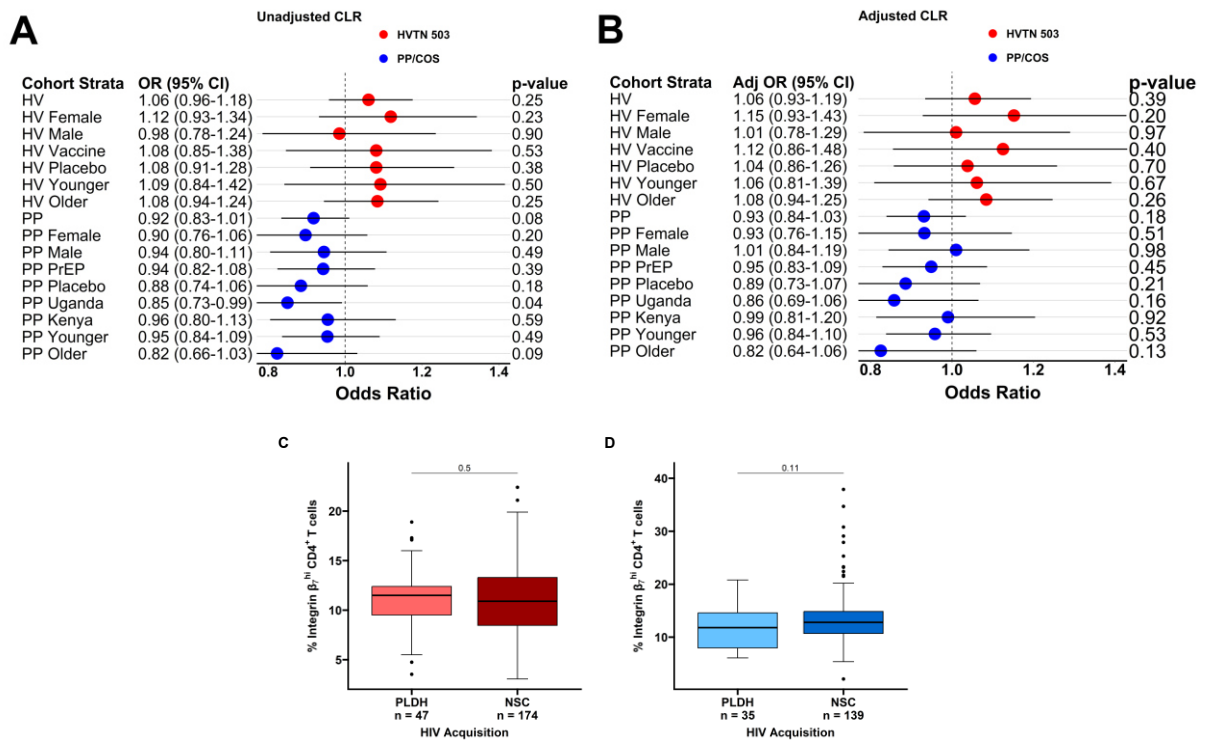


Figure 7. Correlation between pre-HIV frequency of integrin $\beta 7^{\text{hi}}$ CD4⁺ T cells and HIV acquisition. (A and B) Stratified univariable and multivariable analysis estimating the association between pre-HIV integrin $\beta 7^{\text{hi}}$ CD4⁺ T cells and HIV acquisition, reported as odds ratio. **(C and D)** Comparison of pre-HIV integrin $\beta 7^{\text{hi}}$ CD4⁺ T cell frequency between HIV acquisition status in HVTN 503 and PP/COS. Plots colored “red” represent analyses from HVTN 503, while those in “blue” represent analyses from PP/COS. Comparison between two groups was analyzed using Mann-Whitney test. Odds ratios were computed using conditional logistic regression, stratified by sex, treatment arm, and HIV prevention intervention where applicable. The multivariable analysis for HVTN 503 was adjusted for age, Adenovirus type-5 titer, and HSV2 status at baseline, while that for PP/COS was adjusted for age and HSV2 status at baseline. Abbreviations: OR = Odds Ratio, Adj = Adjusted, HV = HVTN 503, PP = PP/COS.

4.2.3. Effect of pre-HIV Frequencies of Integrin $\beta 7^{\text{hi}}$ CD4⁺ T cell subsets on HIV Progression

To investigate the role of pre-HIV frequencies of $\beta 7^{\text{hi}}$ CD4⁺ T cells and the measures of HIV disease progression, we tested associations with viral loads (peak and set point VL; **Figure 8, A – D**), and time to CD4⁺ T cell decline to < 500 cells/mm³ post-infection prior to ART initiation. In addition, we correlated pre-HIV $\beta 7^{\text{hi}}$ CD4⁺ T cell frequency with pre-ART CD4/CD8 ratio within intervals of < 180 days or > 180 days since infection in HVTN 503 (**Figure 8, E – H**). In PP/COS pre-HIV $\beta 7^{\text{hi}}$ cells was inversely correlated with peak VL ($p = 0.024$; **Figure 8B**) while in HVTN 503, it correlated inversely with mean CD4/CD8 ratio after the first 180 days post-infection ($p = 0.042$; **Figure 8F**), and a similar correlation was seen with CD4/CD8 ratio at last visit (**Figure 8H**).

In univariable Cox regression, pre-HIV $\beta 7^{\text{hi}}$ CD4⁺ T cells predicted faster CD4⁺ T cell decline < 500 cells/mm³ in HVTN 503 but not in PP/COS (**Figure 9, A and B**). Specifically, participants in HVTN 503 with $\beta 7^{\text{hi}}$ cells above the median experienced CD4⁺ T cell count < 500 cells/mm³ at more than twice the rate at which those with $\beta 7^{\text{hi}}$ cells below the median value progressed [HVTN hazard ratio (HR) 2.30, 95% CI, 1.10 – 5.00, $p = 0.03$; **Figure 9A**].

Pre-HIV $\beta 7^{\text{hi}}$ CD4⁺ T cells remained a strong predictor of faster CD4⁺ T cell decline < 500 copies in HVTN 503 after adjusting for variables including viral load, age, sex, study site, Ad5 titer, study arm and HSV-2 status at baseline [HVTN adjusted HR (aHR) 2.72, 95% CI 1.13 – 6.58, $p = 0.03$; **Table 8**].

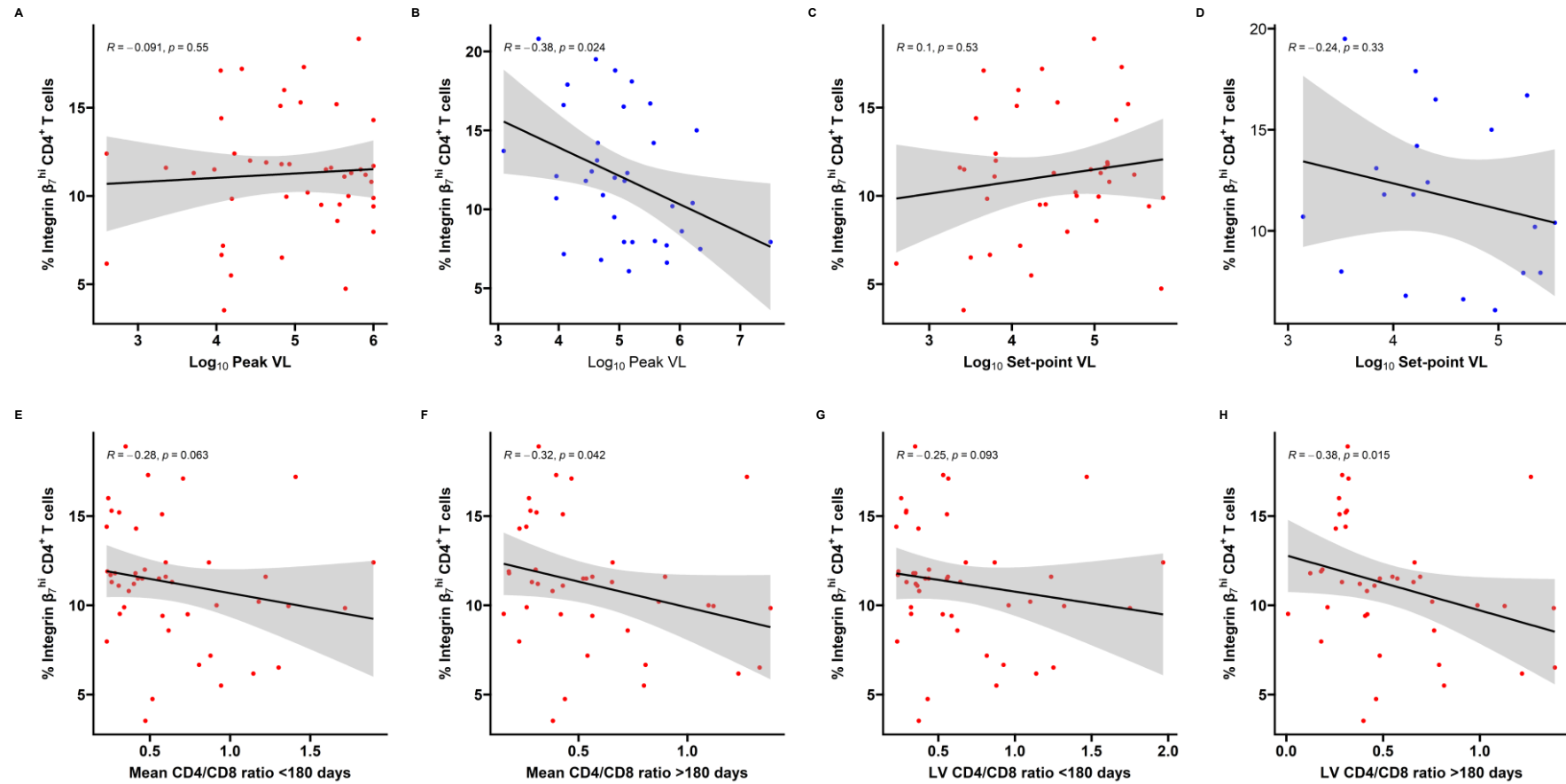


Figure 8. Correlation between pre-HIV frequency of integrin β_7^{hi} CD4⁺ T cells with viral loads and CD4/CD8 ratio. (A and B) Correlation between pre-HIV integrin β_7^{hi} CD4⁺ T cells and peak viral load for HVTN 503 ($n = 45$) and Partners PrEP ($n = 35$). **(C and D)** Correlation between pre-HIV integrin β_7^{hi} CD4⁺ T cells and setpoint viral load for HVTN 503 ($n = 40$) and Partners PrEP ($n = 19$). **(E and F)** Correlation between pre-HIV integrin β_7^{hi} CD4⁺ T cells and mean CD4/CD8 ratio for HVTN 503 at < 180 days ($n = 45$) and > 180 days ($n = 40$). **(G and H)** Correlation between pre-HIV integrin β_7^{hi} CD4⁺ T cells and CD4/CD8 ratio at the last available sampling visit for HVTN 503 at < 180 days ($n = 45$) and > 180 days ($n = 40$). Scatter plots colored in “red” represent analyses from HVTN 503, while those in “blue” represent analyses from PP/COS. Correlations were analyzed using Spearman’s rank correlation test. Abbreviations: VL = Viral Load, LV = Sampling at last available visit.

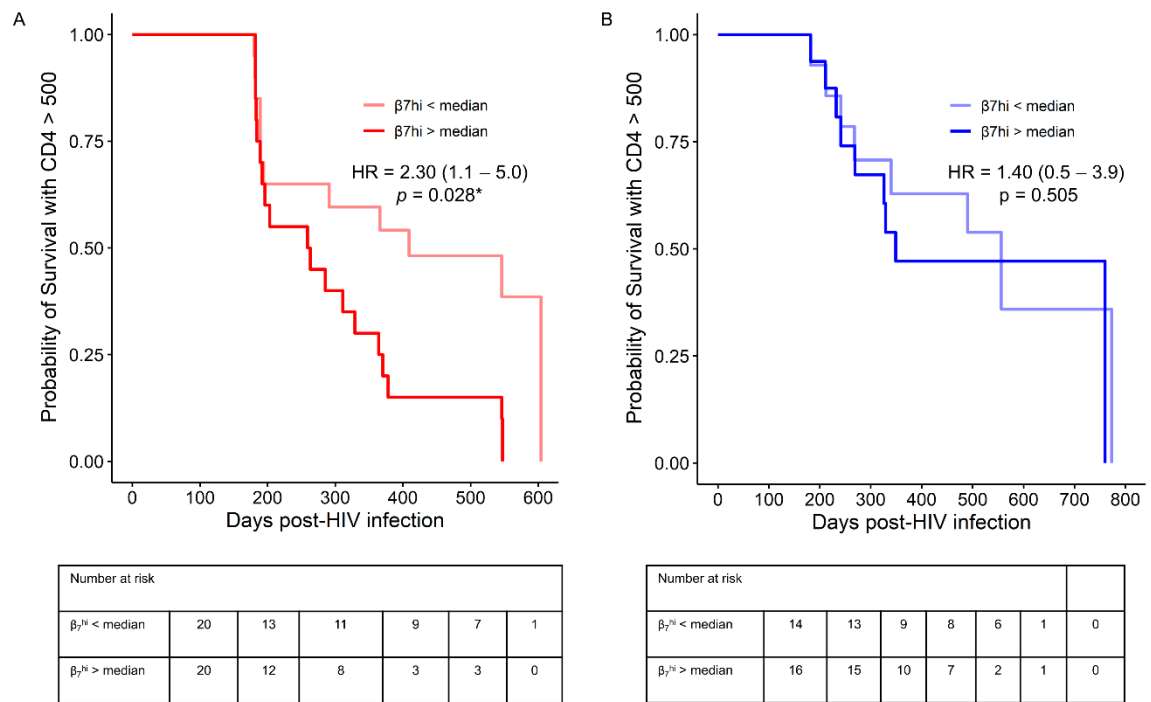


Figure 9. Association of pre-HIV integrin β_{7hi} CD4⁺ T cells with CD4⁺ T cell decline below 500 cells/mm³. (A) HVTN 503 ($n = 40$). (B) PP/COS ($n = 30$). Curves colored in “red” represent analyses from HVTN 503, while those in “blue” represent analyses from PP/COS. Unadjusted hazard ratios analyzed by Cox regression are reported as HR within the plots. Abbreviation: HR = Hazard Ratio. ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

Table 8. Association of pre-HIV $\beta 7^{\text{hi}}$ CD4⁺ T cells with CD4⁺ T cell decline below 500 cells/mm³ (HVTN 503)

Variables	Units	aHR (95% CI)	P value
$\beta 7^{\text{hi}}$ CD4 ⁺ T cells	$\beta 7^{\text{hi}} < \text{median}$	Ref	NA
	$\beta 7^{\text{hi}} \geq \text{median}$	2.72 (1.13 – 6.58)	0.026*
Sex	Female	Ref	NA
	Male	1.37 (0.54 – 3.45)	0.506
Site	Cape town	Ref	NA
	eThekwin	1.45 (0.44 – 4.77)	0.540
	Klerksdorp	1.28 (0.32 – 5.19)	0.730
	Medunsa	1.91 (0.32 – 11.30)	0.475
	Soweto	1.18 (0.32 – 4.32)	0.798
AD5 titer	>200	Ref	NA
	≤ 200	0.98 (0.37 – 2.63)	0.974
HSV2 at baseline	Negative	Ref	NA
	Positive	1.52 (0.39 – 5.87)	0.546
Treatment arm	Placebo	Ref	NA
	Vaccine	1.03 (0.41 – 2.63)	0.947
Peak VL	NA	1.87 (1.00 – 3.51)	0.050
Age (years)	NA	0.94 (0.81 – 1.09)	0.430

Abbreviations: aHR = Adjusted Hazard Ratio, VL = Viral Load, AD5 = Adenovirus Type 5, HSV2 = Herpes Simplex Virus 2, Ref = Reference category, NA = Not Applicable. Hazard Ratios were estimated in multivariable cox regression model. ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

4.2.4. Associations between pre-HIV frequencies of naïve and central memory CD4⁺ T cells mirror the associations of integrin $\beta 7^{\text{int}}$ and $\beta 7^{\text{hi}}$ CD4⁺ T cell subsets on HIV acquisition.

We characterized CD4⁺ T cell memory phenotypes defined based on the expression of CCR7, CD45RA and CD27 (**Figure 10A, Appendix Figure A6**). Similar to the findings with $\beta 7^{\text{hi}}$ CD4⁺ T cells, central memory (TCM) cells did not predict HIV acquisition in HVTN 503 but were associated with lower odds of HIV acquisition in PP/COS (PP OR 0.94, 95% CI 0.90 – 0.98, $p = 0.005$). As observed with $\beta 7^{\text{int}}$ CD4⁺ T cells, naïve (TN) cells were associated with higher rates of HIV acquisition in PP/COS (PP OR 1.04, 95% CI 1.01 – 1.07, $p = 0.006$; **Figure 10B**). In HIV progression analyses, pre-HIV TCM cells were associated with lower CD4/CD8 ratio (**Figure 10, C – D, Figure 11, A – B**). In contrast, TN cells, like $\beta 7^{\text{int}}$ CD4⁺ T cells, positively correlated with CD4/CD8 ratio (**Figure 10, E – F, Figure 11, C – D**). Additionally, pre-HIV frequency of TCM inversely correlated with peak VL ($p = 0.05$; **Figure 10G**) in PP/COS, in line with what was observed for $\beta 7^{\text{hi}}$ CD4⁺ T cells. Considering both TCM and $\beta 7^{\text{hi}}$ CD4⁺ T cells are CD45RA⁻ while TN and $\beta 7^{\text{int}}$ CD4⁺ T cells are CD45RA⁺, we estimated correlations between these subsets, observing positive correlations between TCM and $\beta 7^{\text{hi}}$ CD4⁺ T cells ($p = <0.001$), effector memory (TEM) and $\beta 7^{\text{lo}}$ CD4⁺ T cells ($p = <0.001$) and between TN and $\beta 7^{\text{int}}$ CD4⁺ T cells ($p = <0.001$; **Figure 11, E – F**). In summary, the associations between pre-HIV frequencies of naïve and central memory CD4⁺ T cell subsets mirror the patterns observed with integrin $\beta 7^{\text{int}}$ and $\beta 7^{\text{hi}}$ subsets, both in HIV acquisition and disease progression, emphasizing their interconnected roles.

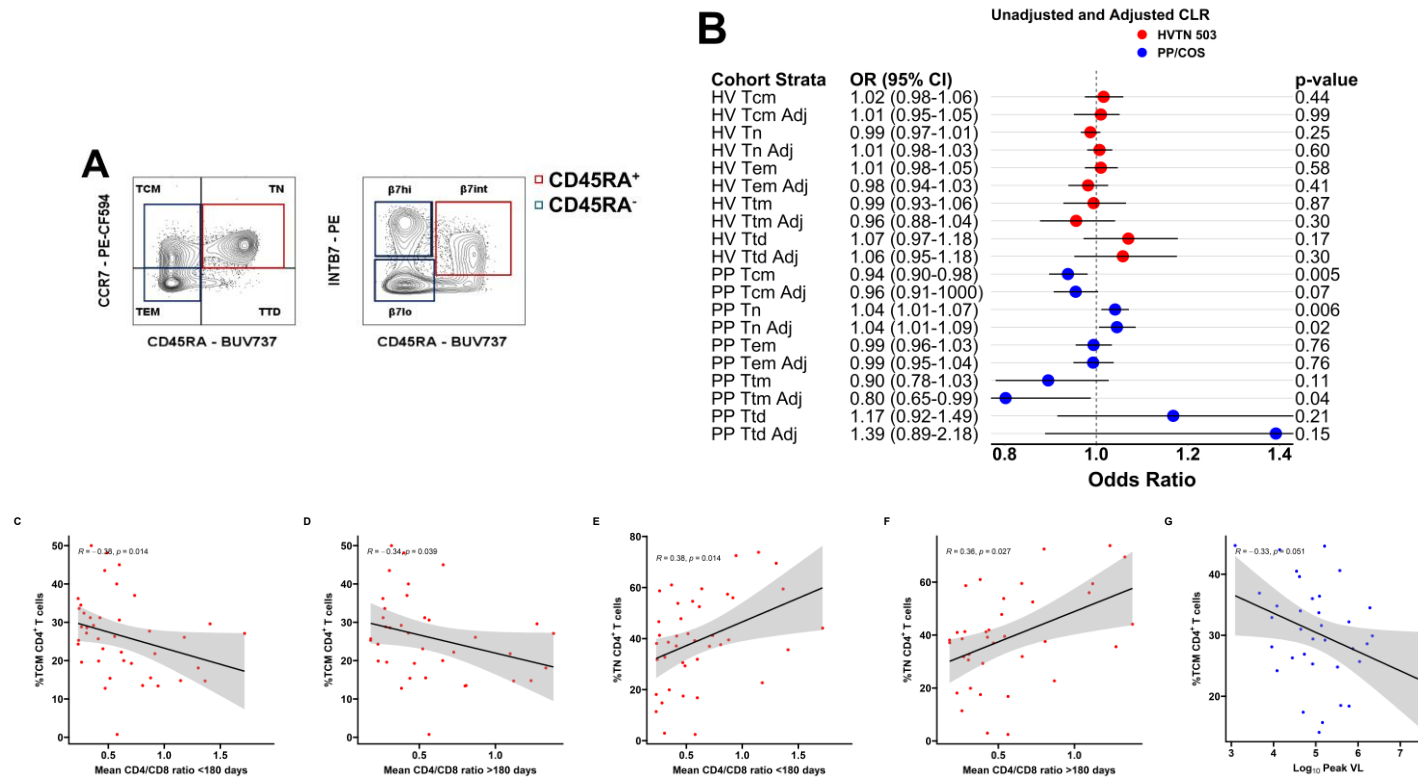


Figure 10. Correlation between pre-HIV frequency of memory CD4⁺ T cell subsets and HIV outcomes. (A) Representative plots illustrating the gating strategy similarities between TCM and $\beta 7^{\text{hi}}$ CD4⁺ T cells, TN and $\beta 7^{\text{int}}$ CD4⁺ T cells. **(B)** Univariable and multivariable analyses estimating the association between pre-HIV memory CD4⁺ T cells and HIV acquisition, reported as odds ratios. **(C and D)** Correlation between TCM and mean CD4/CD8 ratio for HVTN 503 at < 180 days (n = 45) and > 180 days (n = 40). **(E and F)** Correlation between TN and mean CD4/CD8 ratio for HVTN 503 at < 180 days (n = 45) and > 180 days (n = 40). **(G)** Correlation between the frequency of TCM and peak viral load in Partners PrEP (n = 35). Plots colored in “red” denote analyses from HVTN 503, while those in “blue” denote analyses from PP/COS. Odds ratios were computed using conditional logistic regression. The multivariable analysis for HVTN 503 was adjusted for age, Adenovirus type-5 titer, and HSV2 status at baseline, while that of the PP/COS cohort was adjusted for age and HSV2 status at baseline. Correlations were analyzed using Spearman’s rank correlation test. Abbreviations: [TCM = central memory, TN = naïve, TEM = effector memory, TTM = transitional memory, TTD = terminally differentiated] CD4⁺ T cells

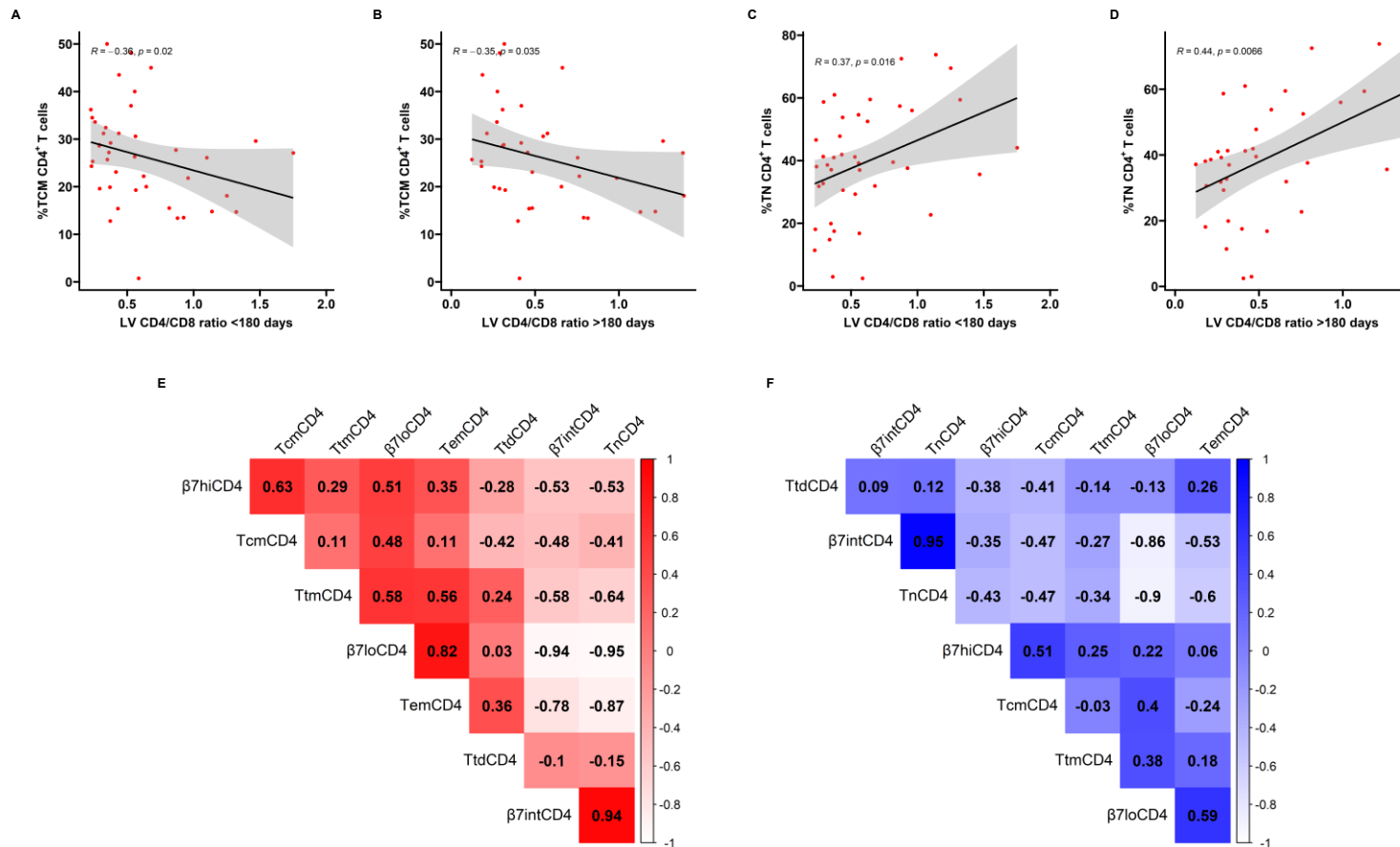


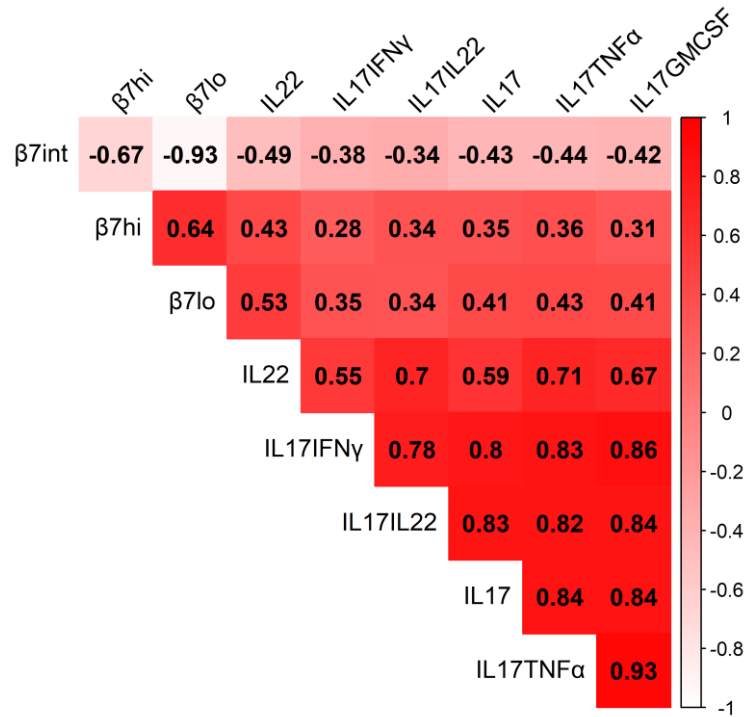
Figure 11. Illustrating the overlapping similarities between the effects of memory and integrin $\beta 7$ CD4⁺ T cells. (A and B) Correlation between pre-HIV TCM cells and CD4/CD8 ratio at the last available sampling at < 180 days ($n = 45$) and > 180 days ($n = 40$). (C and D) Correlation between pre-HIV TN cells and CD4/CD8 ratio at the last available sampling at < 180 days ($n = 45$) and > 180 days ($n = 40$). (E and F) Correlation between integrin $\beta 7$ and memory CD4⁺ T cell subsets in HVTN 503 and PP/COS. Plots colored in “red” represent analyses from HVTN 503, while those in “blue” represent analyses from PP/COS. Correlations were analyzed using Spearman’s rank correlation test. Abbreviations: TCM = central memory CD4⁺ T cells, TN = naive CD4⁺ T cells, TEM = effector memory CD4⁺ T cells, TTM = transitional memory CD4⁺ T cells, TTD = terminally differentiated CD4⁺ T cells.

4.2.5. Differential Phenotypic Characteristics of Integrin $\beta 7$ Subsets in HVTN 503 versus PP/COS Cohorts

Given the divergent point estimates for HIV acquisition risk observed between cohorts, we subsequently investigated the phenotypic characteristics of each integrin $\beta 7$ subset in both cohorts to elucidate the underlying factors contributing to these differences. First, we examined the correlation between integrin $\beta 7$ subsets with IL-17-expressing $CD4^+$ T cells and observed a modest correlation between $\beta 7^{hi}$ cells and IL-17 $^+$ $CD4^+$ T cells in HVTN 503 ($p = <0.001$; **Figure 12A**) while $\beta 7^{hi}$ cells were not associated with IL-17 $^+$ $CD4^+$ T cells in PP/COS cohort ($p = 0.334$; **Figure 12B**).

Furthermore, we observed similar levels of $\beta 7^{hi}$ expression between the two cohorts, however, the frequency of $\beta 7^{int}$ cells were significantly lower and $\beta 7^{lo}$ cells were higher in PP/COS compared to HVTN 503 (**Figure 13A**). $CCR5^+$ cells were not significantly different on the $\beta 7^{hi}$ subsets between the cohorts. However, the frequency of activated ($CD38^+$ $HLADR^+$), proliferating ($Ki67^+$) and central memory $CD4^+$ T cells were higher in PP/COS $\beta 7^{hi}$ $CD4^+$ T cells compared to HVTN 503, while $\beta 7^{hi}$ cells in HVTN 503 were higher in their frequency of effector and transitional memory phenotypes (**Figure 13, B and C**). The integrin $\beta 7^{int}$ in PP/COS samples had significantly higher expression of naïve cells than that in HVTN 503 (**Figure 13D**).

A



B

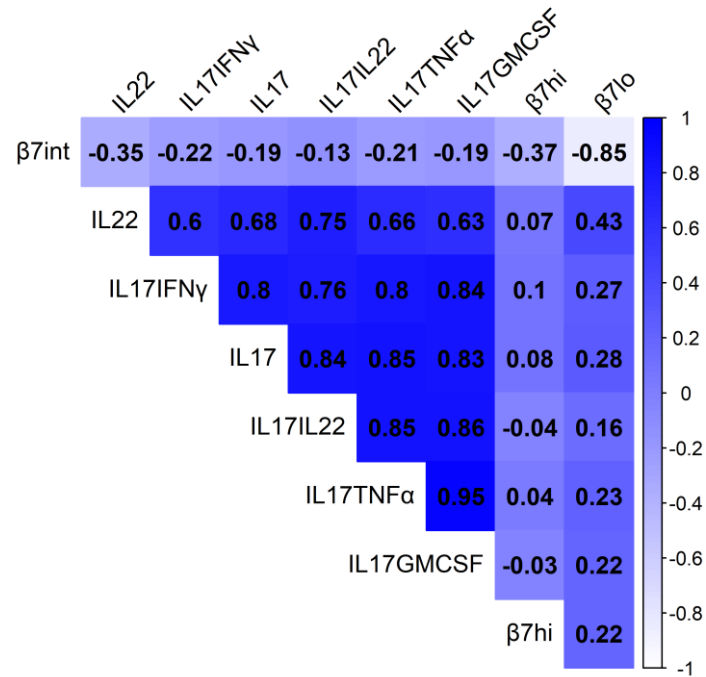


Figure 12. Correlation analysis of integrin $\beta 7$ CD4⁺ T cells with Th17 CD4⁺ T cells subsets. (A) HVTN 503 (B) PP/COS. Plots colored in “red” represent analyses from HVTN 503, while those in “blue” represent analyses from PP/COS. Correlations were assessed using Spearman’s rank correlation test.

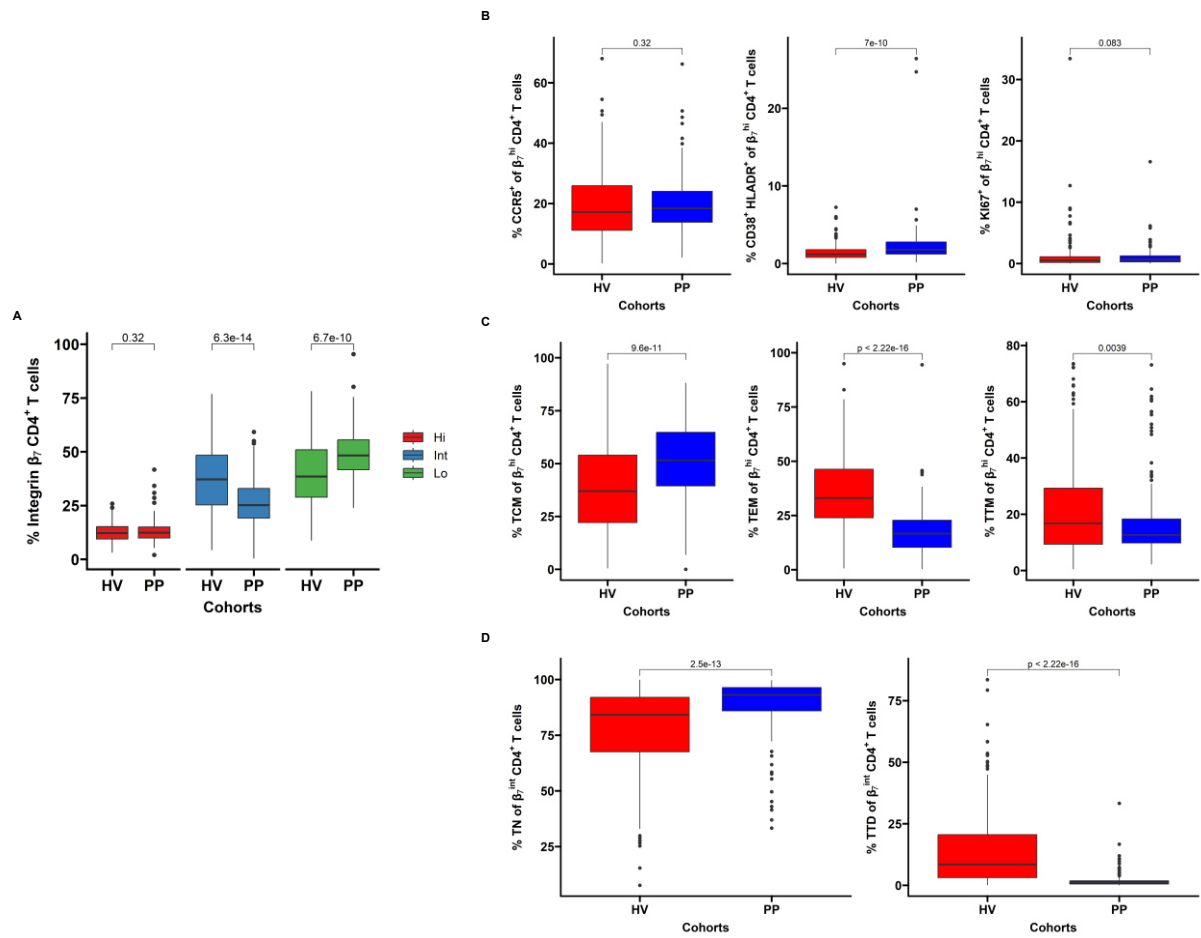


Figure 13. Comparative analysis of integrin β_7 subsets between cohorts. (A) Distribution of integrin β_7 CD4⁺ T cell subsets across cohorts. (B) Phenotypic comparison of integrin β_7^{hi} CD4⁺ T cells expressing CCR5, Activation, and proliferation markers between cohorts. (C) Phenotypic comparison of integrin β_7^{hi} CD4⁺ T cells expressing memory phenotype between cohorts. (D) Phenotypic comparison of integrin β_7^{int} CD4⁺ T cells expressing memory Phenotype between cohorts. Group comparisons were analyzed using the Mann-Whitney test. Abbreviations: HV = HVTN 503, PP = PP/COS.

4.3. Discussion

The role $\alpha 4\beta 7^{\text{hi}}$ in HIV pathogenesis and transmission, including its utility to predict HIV acquisition and disease progression, have generated conflicting findings across various studies. The aim of the present study was to further explore associations of $\alpha 4\beta 7^{\text{hi}}$ expression with HIV outcomes focusing on men and women exposed to HIV through heterosexual transmission. Through analyses of two large cohorts from South and East Africa, comprising high-risk individuals, our study did not observe statistically significant associations between integrin $\alpha 4\beta 7^{\text{hi}}$ expression and HIV susceptibility in either cohort. However, we noted divergent trends between cohorts, with varying degrees of association observed in subgroup analyses. It is noteworthy that these cohorts exhibit important differences in the association of $\alpha 4\beta 7$ with HIV acquisition relative to the associations we have previously reported. We have undertaken an exploration of potential reasons for these disparities, including factors such as sex, HIV clade, and the correlation of $\alpha 4\beta 7$ with Th17 and memory phenotypes. Of note, we did replicate the association between $\alpha 4\beta 7^{\text{hi}}$ expression and disease progression in South Africans, confirming that $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells were associated with faster disease progression within HVTN 503, with a similar effect size as was observed in CAPRISA 004 (Sivro et al., 2018).

Our previous study showed a modest association between systemic $\alpha 4\beta 7^{\text{hi}}$ expression and increased HIV acquisition and faster progression in heterosexual South African females enrolled in CAPRISA 004 (Sivro et al., 2018). The present analyses are in general agreement with the acquisition finding in South Africa women in terms of observing a similar point estimate, e.g. a 15% increase in HIV risk per 1% increase in $\alpha 4\beta 7^{\text{hi}}$ expression (albeit with 95% confidence intervals that overlapped with 1). It should be noted that the sex-stratified sample size was not powered to find HIV acquisition associations in either cohort, hence, these findings require further confirmation. Similar validation studies showed no association between $\alpha 4\beta 7^{\text{hi}}$ and HIV risk in MSM (Martin, Patel, et al., 2020). Males and females exhibit major differences

in their sexual route of HIV transmission (Baeten et al., 2011; Yi et al., 2013) which may impact the interaction of target cells with HIV. Taken together, one interpretation of our data is that the association between high $\alpha 4\beta 7$ expression on CD4⁺ T cells and HIV acquisition may be particularly relevant in the context of South African females.

In addition to sex and route of exposure, the stronger associations observed between integrin $\alpha 4\beta 7^{\text{hi}}$ and HIV outcomes in South Africa may be partly due to differences in HIV clades. HIV clades demonstrate distinct geographic distribution patterns, with regional variation in subtype prevalence (Hemelaar, 2012; Hemelaar et al., 2006). While clade C is the predominant HIV clade in South Africa, clades A and D are mostly predominant in Kenya and Uganda, while subtype B is mostly found in the Americas (Bbosa et al., 2019; Hemelaar et al., 2006). Some data suggest that HIV clades may interact differently with $\alpha 4\beta 7$ (Nawaz et al., 2011; Richardson et al., 2015). For example, $\alpha 4\beta 7$ cells may bind directly to HIV gp120 second variable loop (V2) of some strains of HIV and act as a non-essential receptor for the virus (Arthos et al., 2008; Richardson et al., 2015). However, the strongest binding affinity of $\alpha 4\beta 7$ to HIV has been observed with viral strains containing the P/SDI/V motif, which is most commonly found in HIV clade C strains from South Africa compared to other clade C strains and other HIV clades (Richardson et al., 2015). We have also previously reported that in South Africa, being infected by HIV strains with a V2 loop containing the P/SDI/V motif was associated with a higher frequency of pre-HIV $\alpha 4\beta 7$ (207). The type of infecting virus may also influence the rate of HIV progression after infection (Hemelaar, 2012; McKinnon et al., 2012). The differences in the interaction between $\alpha 4\beta 7$ and different HIV clades may contribute to why studies based in South Africa are associated with increased HIV susceptibility (Sivro et al., 2018). Interestingly, our data support the hypothesis that the association between $\alpha 4\beta 7^{\text{hi}}$ and HIV acquisition may be influenced by differences in HIV clades, with stronger $\alpha 4\beta 7^{\text{hi}}$ effects in women from South Africa where clade C predominates.

Our data from HVTN 503 demonstrated that vaccination was associated with an increase in the frequency of $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells in peripheral blood compared placebo recipients. The vaccine used in HVTN 503 was an adenovirus type-5 (Ad5) vectored trivalent vaccine designed to induce T cell immunity (Gray et al., 2011; Hopkins et al., 2014). Unfortunately, the vaccines failed to offer protection against HIV infection and may have increased HIV susceptibility of vaccinated individuals seropositive for Ad5 (Gray et al., 2011, 2014), possibly due to the expansion of activated Ad5-specific T cells, including those expressing $\alpha 4\beta 7^{\text{hi}}$, thereby increasing the number of HIV target cells (Benlahrech et al., 2009; Bukh et al., 2014; Gray et al., 2014). Similarly, in PP/COS, there was a higher expression of $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cells among participants in the PrEP intervention compared to the placebo arm of that trial. The impact of PrEP on immune cell frequency and distribution have been examined, particularly in mucosal sites where HIV transmission commonly occur (Richert-Spuhler et al., 2019). While some studies have shown that PrEP does not significantly alter the number or distribution of immune cells (Pattacini et al., 2015), others have shown transient changes in the frequency of immune cells after initiating PrEP (Richert-Spuhler et al., 2019; Taiwo et al., 2013). Overall, our data showed that HIV prevention interventions may influence the expression levels of $\alpha 4\beta 7^{\text{hi}}$ on CD4⁺ T cells but in neither case did these appear to impact associations between $\alpha 4\beta 7^{\text{hi}}$ and HIV risk, which in both cohorts were similar between treatment arms.

Our examination of the phenotypic differences of integrin $\beta 7$ subsets between the HVTN 503 and PP/COS cohorts provides valuable insights into the factors contributing to variations in HIV acquisition risk. These insights encompass correlations with IL-17-expressing CD4⁺ T cells, demographic parameters, and distinctive profiles of T cell phenotypes. Central memory CD4⁺ T cells were more strongly correlated with $\alpha 4\beta 7^{\text{hi}}$ cells in HVTN 503 compared to PP/COS. Interestingly, our findings indicate distinct subsets within

memory cells, distinguishing $\alpha 4\beta 7^{\text{hi}}$ from $\alpha 4\beta 7^{\text{lo}}$. Specifically, $\alpha 4\beta 7^{\text{hi}}$ cells predominantly exhibit a central memory phenotype, while $\alpha 4\beta 7^{\text{lo}}$ cells tend to display an effector memory phenotype (Kader et al., 2009; Mijiti et al., 2022). This observation prompts the intriguing possibility that $\alpha 4\beta 7^{\text{hi}}$ cells may exhibit a preference for migration not solely to the gut but potentially to gut lymphoid tissue. Likewise, we observed an association between integrin $\beta 7^{\text{int}}$ and naïve CD4^+ T cells and increased HIV acquisition in PP/COS but not in HVTN 503, despite strong correlations between these two subsets in both cohorts. Given the relationships between the integrin $\beta 7$ and memory CD4^+ T cell subsets, it's perhaps not surprising that memory and naïve cell-HIV associations mirror the subsets with which they are associated. We also showed that $\alpha 4\beta 7^{\text{hi}}$ cells correlated with Th17 subsets in HVTN 503 but not in PP/COS. Th17 cells are also highly susceptible to HIV and have been implicated in both HIV acquisition and disease progression post-HIV infection (Fert et al., 2022; Rodriguez-Garcia et al., 2014; Wacleche et al., 2017). Taken together, the phenotypic differences in $\alpha 4\beta 7^{\text{hi}}$ cells between cohorts may contribute to the associations between $\alpha 4\beta 7$ and HIV outcomes that have been observed.

A strength of our study is that we adopted similar designs and analyses as previous studies (Martin, Patel, et al., 2020; Sivo et al., 2018). In addition, we adopted multiple quality control procedures to ensure consistency of experiments over time and reproduced our main flow cytometry results using a second panel. One limitation of our study is that we did not have access to corresponding mucosal samples to correlate the effects of $\alpha 4\beta 7^{\text{hi}}$ at the mucosal level. However, the frequency of $\alpha 4\beta 7^{\text{hi}}$ CD4^+ T cells in the blood have been shown to correlate with that in the genital tract (Perciani et al., 2018; Sivo et al., 2018). A second limitation was our inability to match cases and controls on exposure risk levels for HVTN 503 as we did in PP/COS (Kahle et al., 2013). Exposure to HIV may be relatively low among some serodiscordant couples due to low viremia in the HIV positive partner (Attia et al., 2009; Cohen et al., 2016; Rodger et al., 2016) and the risk levels tend to be highest at the early stages of the

relationship, waning as the relationship progresses (Lasry et al., 2014). While matching on baseline risk score in PP/COS ensured that HIV exposure level is comparable between cases and controls, this may have dampened associations in PP/COS compared to HVTN 503. We were unable to examine the effect of our immune phenotypes on CD4/CD8 ratio in PP/COS due to lack of data on the absolute CD8⁺ T cells count in this cohort. However, we were able to test HIV progression associations within this cohort by using CD4 counts and VL as HIV progression parameters. We acknowledge that our sample size limitations may have constrained our ability to detect significant effects in our HIV acquisition analyses. Additionally, due to the limited sample size, we were unable to conduct subgroup analyses in our progression analyses as we did in the acquisition analyses.

In conclusion, our study suggests that associations between pre-HIV $\alpha 4\beta 7^{\text{hi}}$ expression on CD4⁺ T cells and HIV outcomes may vary between different populations. Our data suggest that $\alpha 4\beta 7^{\text{hi}}$ may more consistently predict disease progression compared to acquisition, where the interplay of exposure, susceptibility, and various co-factors makes prediction of acquisition more complex. While $\alpha 4\beta 7^{\text{hi}}$ cells are reliable targets for HIV, their abundance alone may not consistently predict HIV acquisition, which in turn depends on external factors and the specific population being studied. Our research emphasizes the need to recognize the population-specific nature of $\alpha 4\beta 7$ associations with HIV.

Chapter 5. Pre-HIV Infection CD4⁺ Th17 cells as predictors of HIV disease progression

5.1. Introduction

HIV continues to pose a global health challenge, necessitating the need to enhance our understanding of pathogenesis to improve therapeutic strategies. The host immune profile near the time of HIV infection could significantly influence the early stages of HIV infection and the associated immune damage, which is a key predictor of HIV pathogenesis (Hartigan-O'Connor et al., 2012; Koning et al., 2005; Ngcobo et al., 2022; Sivro et al., 2018). Prior studies have revealed that the abundance of HIV target cells before infection may accelerate HIV disease progression (Ngcobo et al., 2022; Sivro et al., 2018). Pre-HIV immune profiles characterized by high expression of HIV-susceptible migratory marker integrin $\alpha 4\beta 7$ (Sivro et al., 2018), increased immune activation markers (Koning et al., 2005) and increased plasma cytokine responses (Ngcobo et al., 2022; Roberts et al., 2010) were predictive of either HIV susceptibility and/or accelerated HIV progression post-infection.

Th17 cells, a subset of CD4⁺ T cells are an important target cell for HIV, and they play a crucial role in mucosal immunity, particularly in defending against bacterial and fungal infections (Klatt & Brenchley, 2010). Th17 cells are enriched on mucosa surfaces where they help in maintaining the mucosal barrier integrity and are involved in inflammatory immune responses which are essential for preventing infections (Paiva et al., 2021; Rodriguez-Garcia et al., 2014). These cells produce a variety of pro-inflammatory cytokines, predominantly interleukin-17 (IL-17) which are involved in a number of host immune mechanisms including neutrophil recruitment to infection sites, regulation of mucosal epithelial cell permeability, as well as induction of antimicrobial peptides (Muranski & Restifo, 2013; Wacleche et al., 2017).

The high expression of Th17 cells in the gastrointestinal mucosa, the primary replication site for HIV (Ancuta et al., 2010; Mckinnon et al., 2015), underscores the

significance of these cells in the context of HIV infection. HIV primarily attacks CD4⁺ T cells, otherwise known as T Helper (Th) cells, and HIV progression in the absence of treatment is characterized by the progressive loss of CD4⁺ T cells, particularly in mucosal environments (Ancuta et al., 2010; Mckinnon et al., 2015). Th17 cells represent a subset of CD4⁺ T cells that are highly permissive to HIV infection and are depleted early on during infection (Mckinnon et al., 2015; Renault et al., 2022; Schuetz et al., 2014). Th17 cells are among the first set of cells to be infected during HIV infection (Maric et al., 2021; Stieh et al., 2016) possibly due to their location in mucosal tissues and high expression of CCR5 or CXCR4, two coreceptors used by HIV to enter cells (Loiseau et al., 2016; Renault et al., 2022; Zayas & Mamede, 2022). This early loss of Th17 cells leads to the disruption of mucosal barrier and correlates with increased microbial translocation from the gut, which in turn drives systemic immune activation (Fernandes et al., 2017; Renault et al., 2022; Wacleche et al., 2016; Wiche-Salinas T. R. et al., 2021). This persistent immune activation is a major driver of HIV disease progression (Roberts et al., 2010; Schuetz et al., 2014). Hence, Th17 cell depletion is a hallmark of HIV infection, leading to compromised mucosal immunity and increased susceptibility to secondary infections (Fernandes et al., 2017; Renault et al., 2022; Wacleche et al., 2016; Wiche-Salinas T. R. et al., 2021). Interestingly, the HIV-induced Th17 cell depletion observed in HIV-susceptible hosts such as humans and rhesus macaques, are not observed in the natural hosts such as sooty mangabeys and African green monkeys as well as in HIV-infected long term non-progressors, all of which do not actively progress to AIDS (Schuetz et al., 2014).

Although existing knowledge have shown the contribution of Th17 cells to HIV disease progression and their role as favorable HIV targets, it is not established whether the abundance of these cells in circulation before HIV infection could influence HIV progression outcomes. Here, we demonstrate that higher pre-HIV Th17 cell frequency in the peripheral blood were

predictive of faster disease progression after infection in a cohort of heterosexual individuals from Africa.

5.2. Results

5.2.1. Participants Characteristics

We analyzed 67 PBMC samples from participants enrolled in HVTN 503 (N = 35) and PP/COS (N = 32) who were HIV seronegative at the time of blood draw but later seroconverted. Participants from HVTN 503 were younger, with a median age of 23 compared to Partners PrEP, with a median age of 30 years. The average sampling days to infection was 177 (Interquartile range [IQR] = 136 – 219) for HVTN 503 and 225 days (IQR = 116 – 328) for PP/COS. 100% of our study participants from HVTN 503 did not complete the originally planned number of doses of the treatment regimen as a result of the abrupt ending of the trial due to lack of efficacy. In PP/COS, participants were selected from two trials involving serodiscordant couples that ran concurrently, namely, Partners PrEP and Couples Observational Study (COS), which made up 78% and 22% of the study participants, respectively. Hence, we renamed the analysis from these cohorts as PP/COS for ease of understanding. Additionally, more than 50% of PP/COS participants had a baseline risk score greater than 5, reflecting serodiscordant couples with higher risk of HIV transmission. Details about the baseline characteristics of study participants are shown in (**Table 9**).

Table 9. Baseline characteristics of study participants

Variables	PLDH	
	HVTN 503 (<i>n</i> = 35)	PP/COS (<i>n</i> = 32)
Age (years) ^m	23 (22 – 27)	30 (25 – 40)
Estimated sampling to infection (days) ^m	177 (136 – 219)	225 (116 – 328)
Sex		
Female	17 (48.57)	19 (59.38)
Male	18 (51.43)	13 (40.62)
Treatment Arm		
Placebo	17 (48.57)	14 (43.75)
Vaccine	18 (51.43)	N/A
PrEP	N/A	11 (34.38)
Cohort		
COS	N/A	7 (21.88)
PrEP	N/A	25 (78.12)
HSV2 Status (Positive)	20 (57.14)	17 (53.13)
Circumcision (Male)	6 (17.14)	0 (0.00)
Off treatment early	35 (100.00)	N/A
AD5 Titre at Baseline		
≤ 200	12 34.29)	N/A

> 200	23 (65.71)	N/A
Baseline Risk score		
< 5	N/A	13 (40.62)
≥ 5	N/A	19 (59.38)
Sites: South Africa		
Cape town	9 (25.71)	N/A
eThekwini	7 (20.00)	N/A
Klerksdorp	6 (17.14)	N/A
Medunsa	4 (11.43)	N/A
Soweto	9 (25.71)	N/A
PHRU	N/A	2 (6.25)
Sites: Uganda		
Jinja	N/A	6 (18.75)
Kampala	N/A	13 (40.63)
Sites: Kenya		
Kisumu	N/A	4 (12.50)
Thika	N/A	2 (6.25)
Nairobi	N/A	5 (15.62)

Data are presented as sample sizes (n) and corresponding column percentages (%) unless otherwise stated. ^mData are presented as median (Interquartile Range). Abbreviations: PLDH = People later diagnosed with HIV, PrEP = Pre-exposure Prophylaxis, COS = Couples Observational Study, HSV2 = Herpes Simplex Virus 2, AD5 = Adenovirus Type 5, PHRU = Perinatal HIV Research Unit (Soweto, South Africa), N/A = Not Applicable.

5.2.2. Pre-HIV IL-17⁺ CD4⁺ T Cells Abundance was not Correlated with Viral Loads

We characterized Th17 cells as IL-17A⁺ cells using flow cytometry. Representative gating strategy used to quantify IL-17A⁺ cell populations within CD4⁺ T cells is shown in **(Figure 14A)**. In addition, we explored other cytokines produced by Th17 cells, including IL-22, TNF- α , IFN- γ and GM-CSF, and gated these subsets in relation to IL-17A expression **(Appendix Figure A8A)**. We then estimated the correlation between pre-HIV IL-17⁺ CD4⁺ T cells frequency and viral loads, including peak and set point viral loads. Our results showed that IL-17-expressing CD4⁺ T cells were not significantly associated with either peak or set point viral loads in both cohorts **(Figure 14, B – E)**. Additionally, there was no significant correlation between viral loads and the other cytokines evaluated independently of IL-17 expression **(Appendix Figure A8, B – G)**.

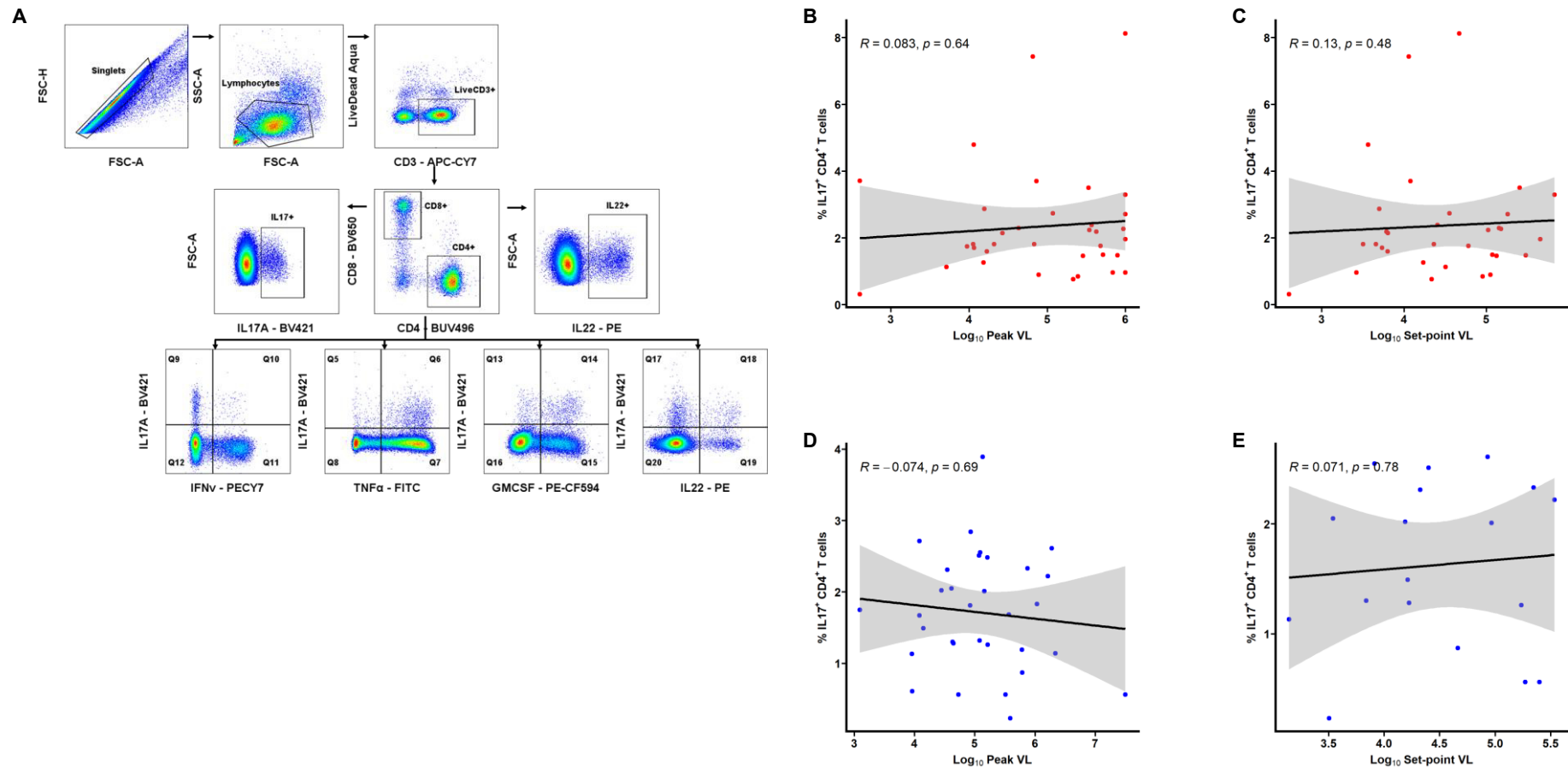


Figure 14. Correlation analysis between viral loads and pre-HIV IL-17⁺ CD4⁺ T cells. (A) Representative flow cytometry plots showing the gating strategy for IL-17⁺ CD4⁺ T cells. (B and C) IL-17⁺ CD4⁺ T cells correlation with peak (n = 35) and set-point (n = 32) viral loads in HVTN 503 shown in “red”. (D and E) IL-17⁺ CD4⁺ T cells correlation with peak (n = 32) and set-point (n = 18) viral loads in PP/COS shown in “blue”. Peak viral load is defined as the highest viral load in the initial 180 days post-infection while set-point viral load is defined as the average viral load post 180 days of infection. Viral loads measured after initiating ART and taken after 1 year of HIV diagnosis were excluded from the analysis. Correlation was analyzed using Spearman’s rank correlation test.

5.2.3. Pre-HIV IL-17⁺ CD4⁺ T cells abundance inversely correlated with CD4/CD8 ratio in HVTN 503

We performed spearman correlation test to determine the association between pre-HIV IL-17⁺ CD4⁺ T cells abundance and CD4/CD8 ratio, another marker of HIV progression. We calculated mean CD4/CD8 ratio both before and after the initial 180 days post-infection (**Figure 15, A and B**). We also determined association with CD4/CD8 ratio at the last available pre-ART sampling visit (**Figure 15, C and D**). Our results indicated a significant inverse correlation between IL-17⁺ CD4⁺ T cells and mean CD4/CD8 ratio at < 180 days ($r = -0.42$, $p = 0.012$; **Figure 15A**), a correlation which became progressive beyond 180 days post-infection ($r = -0.55$, $p = 0.001$; **Figure 15B**).

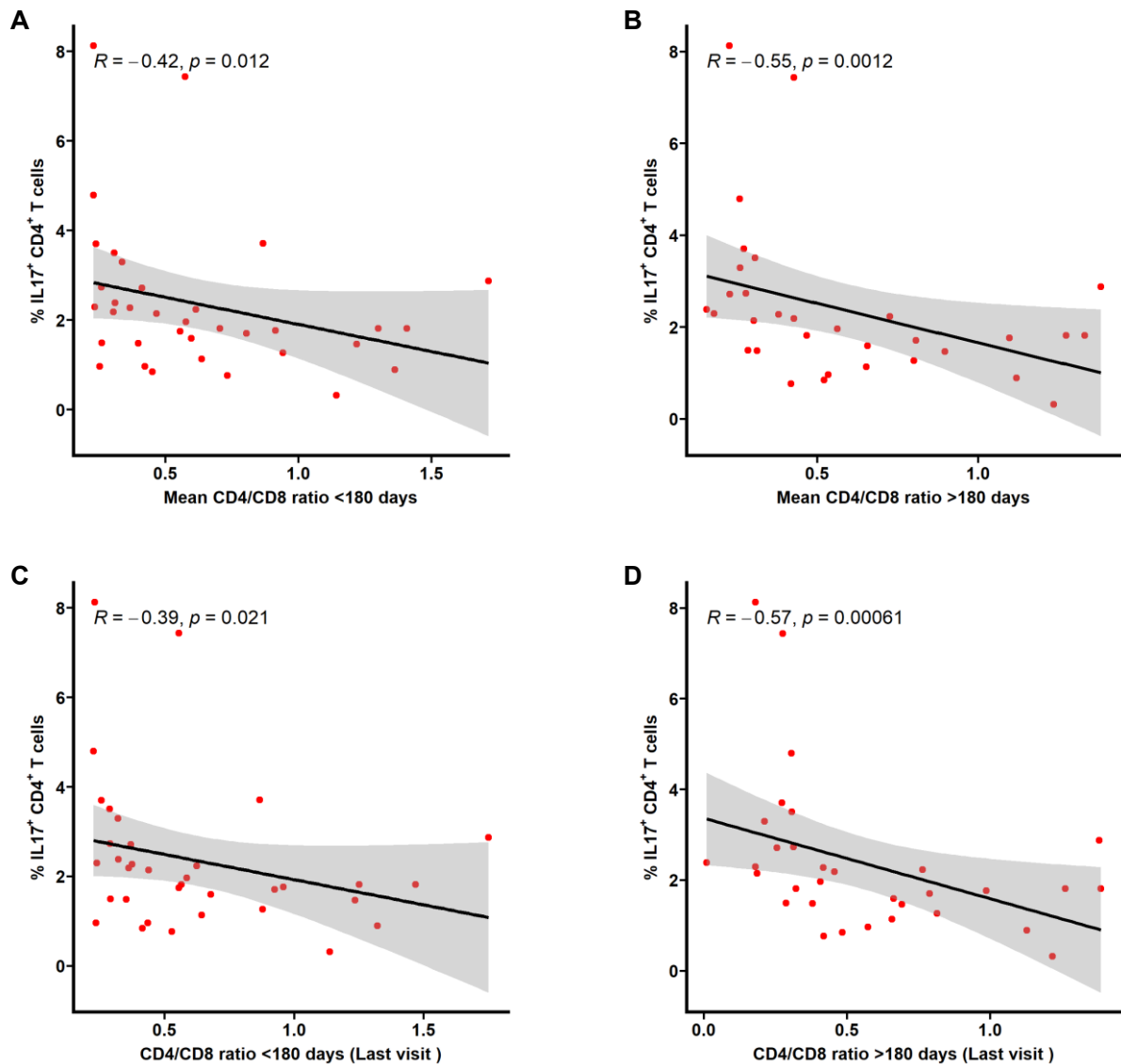


Figure 15. Correlation analysis between CD4/CD8 ratio and pre-HIV IL-17⁺ CD4⁺ T cells. (A and B) Correlations with mean CD4/CD8 ratio. (C and D) Correlations with CD4/CD8 ratio at last sampling visit. CD4/CD8 ratio was calculated from CD4 and CD8 counts taken within the initial 180 days ($n = 35$) and beyond the initial 180 days ($n = 32$) post-infection. CD4 and CD8 counts measured after initiating ART and after 1-year post-infection were excluded from the analysis. These analysis are from HVTN 503. Correlation was analyzed using Spearman's rank correlation test.

Additionally, within the initial 180 days following infection, IL-17⁺ CD4⁺ T cell subsets including IL-17⁺ IFN- γ ⁺, IL-17⁺ IFN- γ ⁻, IL-17⁺ IL-22⁻ and IL-17⁺ GM-CSF⁺ were all significantly correlated with mean CD4/CD8 ratio (**Figure 16, C – E, H**). After the initial 180 days post-infection, all the IL-17⁺ CD4⁺ T cell subsets investigated were significantly correlated with mean CD4/CD8 ratio except IL-17⁺ TNF- α ⁻ while IL-17⁺ GM-CSF⁻ was only marginally significant (**Figure 17, A – H**). These results remained consistent when we determined the association with CD4/CD8 ratio at the last sampling visit. Furthermore, we explored if other cytokines independently correlate with CD4/CD8 ratio and showed that IL-22⁺, IFN- γ ⁺ and GM-CSF⁺ CD4⁺ T cells inversely correlated with CD4/CD8 ratio in a time-progressive manner as observed with IL-17⁺ CD4⁺ T cell subsets (**Appendix Figure A9**). In summary, IL-17⁺ CD4⁺ T cell subsets exhibited a negative correlation with CD4/CD8 ratio, an association that became increasingly pronounced with increasing time. Overall, we showed a poorer HIV progression outcome as a result of increased pre-HIV Th17 cell levels.

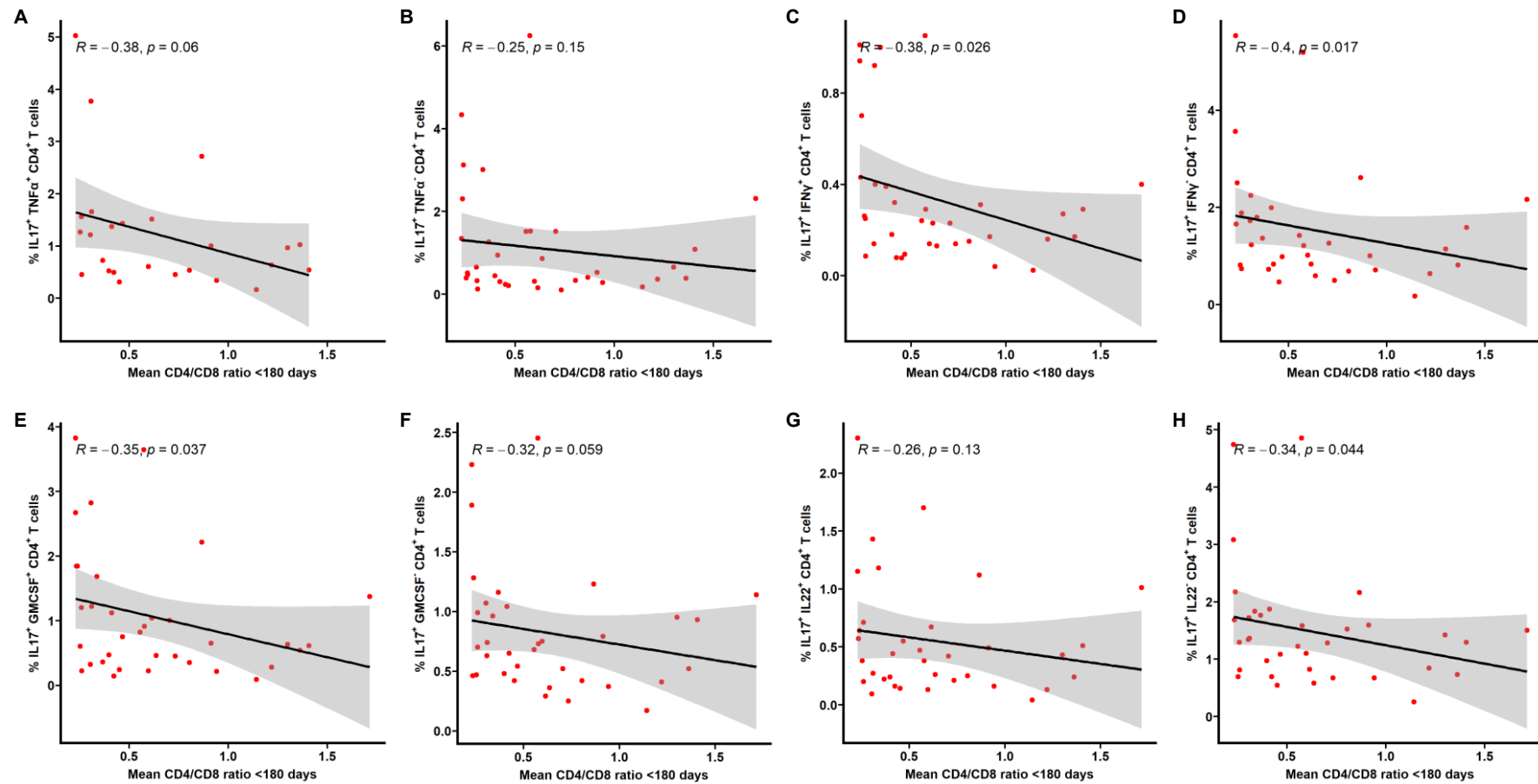


Figure 16. Correlation analysis between mean CD4/CD8 ratio within the first 180 days post-infection and pre-HIV IL-17⁺ CD4⁺ T cell subsets. (A) IL-17⁺ TNF- α ⁺ subset (B) IL-17⁺ TNF- α ⁻ subset (C) IL-17⁺ IFN- γ ⁺ subset (D) IL-17⁺ IFN- γ ⁻ subset (E) IL-17⁺ GM-CSF⁺ subset (F) IL-17⁺ GM-CSF⁻ subset (G) IL-17⁺ IL-22⁺ subset (H) IL-17⁺ IL-22⁻ subset. CD4/CD8 ratio was calculated from CD4 and CD8 counts taken within the initial 180 days and beyond the initial 180 days post-infection. CD4 and CD8 counts measured after initiating ART and after 1-year post-infection were excluded from the analysis. These analysis are from HVTN 503 (n = 35). Correlation was analyzed using Spearman's rank correlation test.

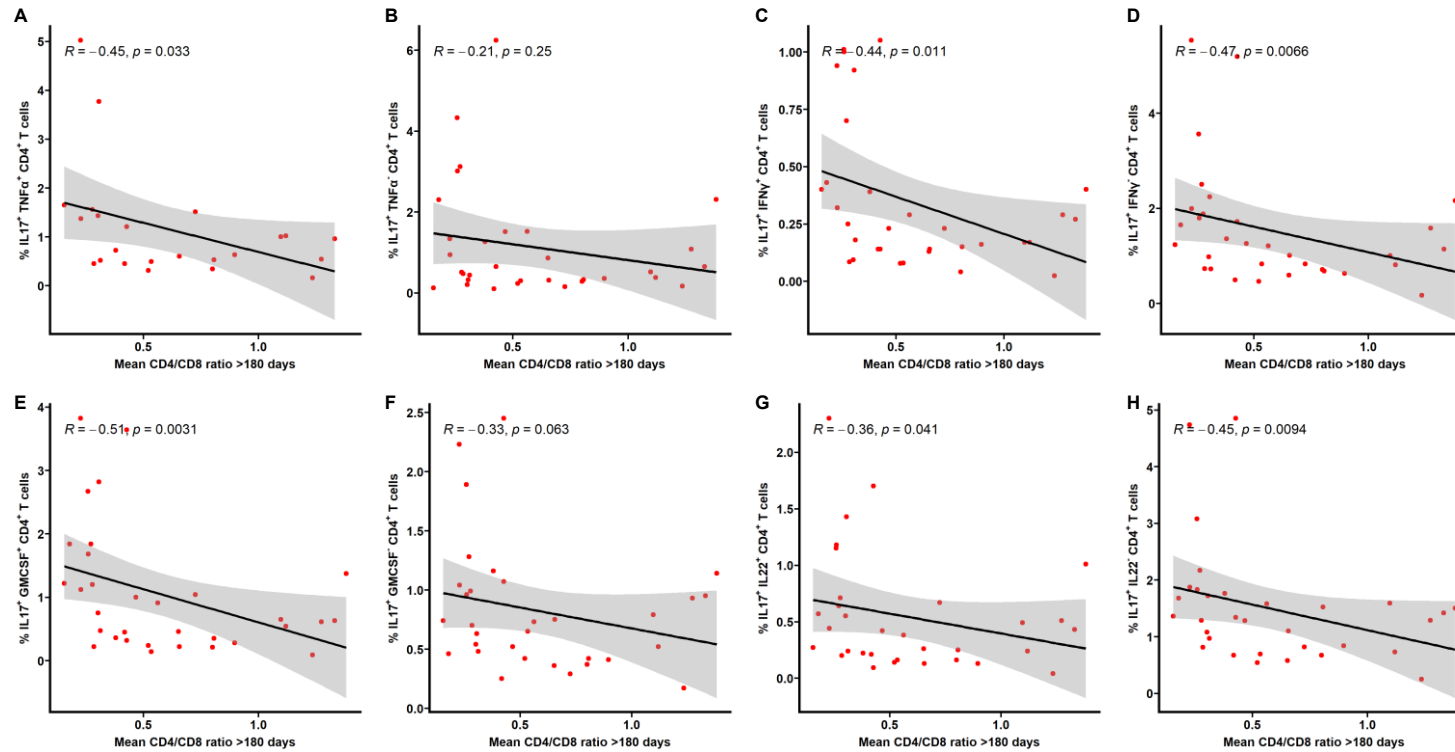


Figure 17. Correlation analysis between mean CD4/CD8 ratio beyond the first 180 days post-infection and pre-HIV IL-17⁺ CD4⁺ T cell subsets. (A) IL-17⁺ TNF- α ⁺ subset (B) IL-17⁺ TNF- α ⁻ subset (C) IL-17⁺ IFN- γ ⁺ subset (D) IL-17⁺ IFN- γ ⁻ subset (E) IL-17⁺ GM-CSF⁺ subset (F) IL-17⁺ GM-CSF⁻ subset (G) IL-17⁺ IL-22⁺ subset (H) IL-17⁺ IL-22⁻ subset. CD4/CD8 ratio was calculated from CD4 and CD8 counts taken within the initial 180 days and beyond the initial 180 days post-infection. CD4 and CD8 counts measured after initiating ART and after 1-year post-infection were excluded from the analysis. These analysis are from HVTN 503 (n = 32). Correlation was analyzed using Spearman's rank correlation test.

5.2.4. Pre-HIV IL-17⁺ CD4⁺ T Cells Abundance are associated with Faster CD4⁺ T Cell Decline Post-Infection

We explored the association of pre-HIV Th17 cells and CD4⁺ T cell decline, another parameter used to predict HIV progression. Our analysis revealed a significant correlation between higher levels of pre-HIV Th17 cells, and faster CD4⁺ T cell decline in HVTN 503. Specifically, individuals with pre-HIV IL-17⁺ CD4⁺ T cell frequency above the median experienced almost a 3-fold faster CD4 decline in an unadjusted model (Hazard ratio [HR] = 2.9, 95% Confidence Interval [CI] = 1.2 – 6.9, $p = 0.015$; **Figure 18A**). Similar results were obtained after adjusting for peak viral load (Adjusted HR [aHR] = 2.5, 95% CI = 1.1 – 6.1, $p = 0.038$; **Appendix Table A7**). In the full adjusted model including covariates such as sex, Ad5 titer, HSV2 status, age and peak VL, pre-HIV IL-17⁺ CD4⁺ T cells remained a significant predictor of faster CD4 decline in HVTN 503 (aHR = 4.2, 95% CI: 1.4 – 12.8, $p = 0.013$; **Table 10**). Conversely, we did not observe an association between pre-HIV IL-17⁺ CD4⁺ T cells and faster CD4 decline in PP/COS (HR = 1.2, 95% CI = 0.4 – 3.4, $p = 0.795$; **Figure 18B**). In the analyses that combined both cohorts, we observed that higher pre-HIV IL-17⁺ CD4⁺ T cells remained a significant predictor of faster CD4 decline in the unadjusted model (HR = 2.2, 95% CI = 1.1 – 4.3, $p = 0.023$) and was marginally significant in the fully adjusted model (aHR = 2.2, 95% CI = 0.98 – 5.0, $p = 0.057$; **Table 10**).

We, next determined if the correlation with CD4 decline is specific to IL-17⁺ cells or cytokines in general. To do this, we estimated the association between other cytokines including TNF- α , IFN- γ , and GM-CSF, independent of IL-17 expression. Our result showed no significant association between these other cytokines independently with faster CD4 decline, suggesting that this association is specific for IL-17 expression by Th17 CD4⁺ T cells (**Table 11**).

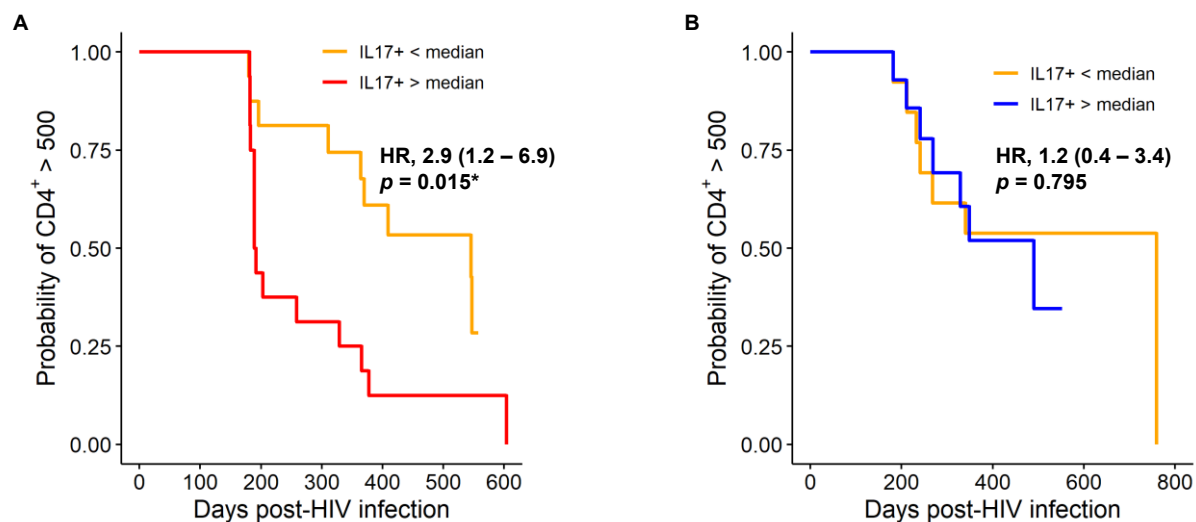


Figure 18. Association of pre-HIV IL-17⁺ CD4⁺ T cells with CD4⁺ T cell decline below 500 cells/mm³. (A) HVTN 503 (n = 32) (B) PP/COS (n = 27). CD4 counts measured after initiating ART and after 1-year post-infection were excluded from the analysis. CD4 counts sampled within the initial 180 days post-infection was also excluded from the analysis. Plots shown in “red” represent analysis from HVTN 503 data only while plots shown in “blue” represent analysis from Partners PrEP data only. Unadjusted hazard ratios (HR) were analyzed by Cox Regression models. ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

Table 10. Effect estimates of the association between pre-HIV IL-17⁺ CD4⁺ T cells and CD4 decline below 500 cells/mm³

Cohort	HR (95% CI)	<i>P</i> value	aHR (95% CI)	<i>P</i> value
HVTN 503	2.9 (1.2 – 6.9)	0.015*	4.18 (1.36 – 12.84)	0.013*
PP/COS	1.2 (0.39 – 3.4)	0.795	1.14 (0.32 – 4.09)	0.844
Combined Cohorts	2.2 (1.1 – 4.3)	0.023*	2.2 (0.98 – 5.01)	0.057

Multivariable model for HVTN 503 was adjusted for as sex, adenovirus type 5 (Ad5) titer, herpes simplex virus-2 (HSV2) status, study arm, age and peak viral load; PP/COS and combined cohorts were adjusted for age, sex, HSV2 status, and peak viral load. Abbreviations: HR = Hazard Ratio, aHR = Adjusted Hazard Ratio. ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

Table 11. Unadjusted cox regression analysis of CD4⁺ T cells cytokines independent of IL-17 expression and CD4 decline below 500 cells/mm³

Cohort	Variables	HR (95% CI)	<i>p</i> value
HVTN 503	TNF- α ⁺ CD4 ⁺ T cells	1.40 (0.48 – 4.05)	0.533
	IFN- γ ⁺ CD4 ⁺ T cells	1.68 (0.70 – 4.04)	0.242
	GM-CSF ⁺ CD4 ⁺ T cells	1.35 (0.57 – 3.21)	0.498
	IL-22 ⁺ CD4 ⁺ T cells	1.38 (0.61 – 3.15)	0.440
PP/COS	TNF- α ⁺ CD4 ⁺ T cells	1.1 (0.36 – 3.50)	0.837
	IFN- γ ⁺ CD4 ⁺ T cells	2.3 (0.73 – 7.29)	0.153
	GM-CSF ⁺ CD4 ⁺ T cells	0.79 (0.27 – 2.37)	0.677
	IL-22 ⁺ CD4 ⁺ T cells	1.1 (0.38 – 3.38)	0.821
Combined Cohorts	TNF- α ⁺ CD4 ⁺ T cells	0.52 (0.24 – 1.15)	0.106
	IFN- γ ⁺ CD4 ⁺ T cells	1.1 (0.57 – 2.18)	0.758
	GM-CSF ⁺ CD4 ⁺ T cells	1.4 (0.74 – 2.83)	0.286
	IL-22 ⁺ CD4 ⁺ T cells	1.4 (0.72 – 2.64)	0.331

Percentage expression of phenotype below the median value was used as the reference category. Abbreviations: HR = Hazard Ratio.

5.2.5. Interaction and Stratified Analyses

We explored potential interactions between IL-17⁺ CD4⁺ T cells and specific variables including age, sex, and viral load. While we found no significant interactions when each cohort was analyzed separately, we observed a significant p value for the interaction term between high IL-17⁺ CD4⁺ T cells and age ($p = 0.022$) in the analysis that combined data from both cohorts, suggesting the effect of IL-17⁺ levels on the hazard of CD4⁺ T cell decline vary by age. To further understand this interaction, we converted age into a categorical variable with two levels based on the median age (26 years; IQR = 22 – 31 years). In the participants younger than the median age, higher pre-HIV IL-17⁺ CD4⁺ T cell levels was significantly associated with an increased rates of CD4⁺ T cell decline (HR = 3.5; 95% CI = 1.35 – 9.22, $p = 0.010$), whereas in participants older than the median age, the association was not significant (HR = 1.3; 95% CI = 0.48 – 3.49, $p = 0.615$; **Table 12**). In other words, younger participants with higher pre-HIV IL-17⁺ CD4⁺ T cell levels (greater than the median) have a 3.5 times higher rates of CD4⁺ T cell decline compared to older participants with higher pre-HIV IL-17⁺ CD4⁺ T cells levels. Similar results were observed after dichotomizing age at 35 years and showed that participants younger than 35 years are more than twice likely to experience faster CD4 decline (HR = 2.8; 95% CI = 1.37 – 5.83, $p = 0.005$) than the older participants (HR = 0.32; 95% CI = 0.03 – 3.61, $p = 0.359$; **Table 12**).

Table 12. Association of pre-HIV IL-17⁺ CD4⁺ T cells with CD4 decline below 500 cells/mm³ stratified by age (combined cohorts)

Variable	< Median Age		≥ Median Age	
	HR (95% CI)	<i>P</i> value	HR (95% CI)	<i>P</i> value
IL-17 ⁺ CD4 ⁺ T cells	3.5 (1.35 – 9.22)	0.010*	1.3 (0.48 – 3.49)	0.615
	< 35 years		≥ 35 Years	
	HR (95% CI)	<i>P</i> value	HR (95% CI)	<i>P</i> value
IL-17 ⁺ CD4 ⁺ T cells	2.8 (1.37 – 5.83)	0.005**	0.32 (0.03 – 3.61)	0.359

Sample size for < median age (n = 29), ≥ Median age (n = 30), < 35 years (n = 50), ≥ 35 years (n = 9). Abbreviation: HR = Hazard Ratio. (*p* < 0.05*, *p* < 0.01**, *p* < 0.001***).

Sex-stratified analysis revealed the association of IL-17⁺ CD4⁺ T cells with faster CD4 decline was more pronounced in the male participants (HVTN 503: HR = 7, 95% CI = 1.46 – 33.7, $p = 0.015$) than in the female participants (Female strata HVTN 503: HR = 1.8, 95% CI = 0.454 – 7.36, $p = 0.396$; **Table 13**). Likewise, a significant inverse correlation was observed between IL-17⁺ phenotypes and CD4/CD8 ratio in HVTN 503 within the male strata but not in the female strata (**Figure 19**). Taken together, our data showed a sex-specific difference in the association between increased pre-HIV Th17 cell levels and poorer HIV progression outcomes in HVTN 503.

Table 13. Association of pre-HIV IL-17⁺ CD4⁺ T cells with CD4 decline below 500 cells/mm³ stratified by sex (HVTN 503)

Variable	Male Strata		Female Strata	
	HR (95% CI)	<i>P</i> value	HR (95% CI)	<i>P</i> value
IL-17 ⁺ CD4 ⁺ T cells	7.0 (1.46 – 33.7)	0.015*	1.8 (0.45 – 7.36)	0.396

Sample size for male strata (n = 17), female strata (n = 15). Abbreviation: HR = Hazard Ratio. ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

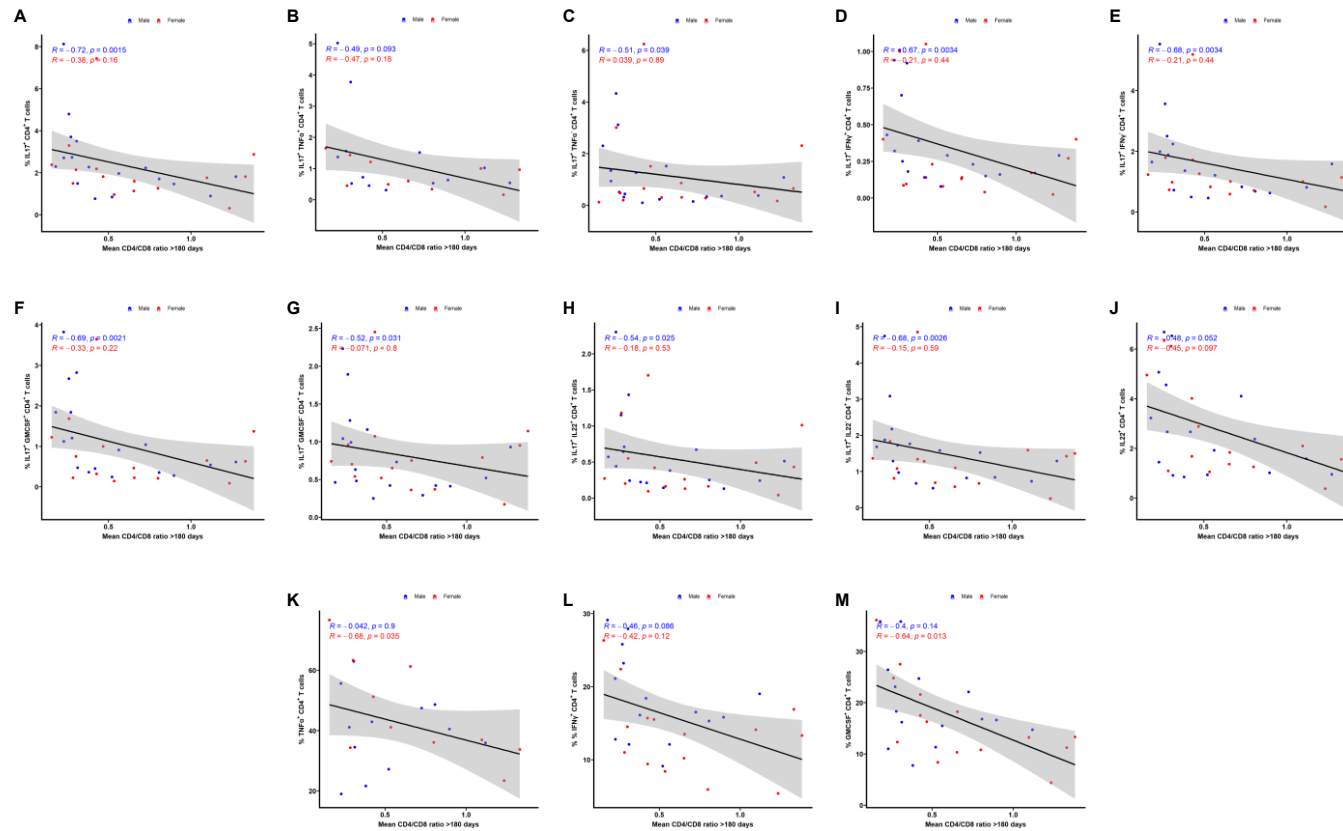


Figure 19. Sex-stratified correlation analysis between mean CD4/CD8 ratio and pre-HIV IL-17⁺ CD4⁺ T cells. (A) IL-17⁺ subset (B) IL-17⁺ TNF- α ⁺ subset (C) IL-17⁺ TNF- α ⁻ subset (D) IL-17⁺ IFN- γ ⁺ subset (E) IL-17⁺ IFN- γ ⁻ subset (F) IL-17⁺ GM-CSF⁺ subset (G) IL-17⁺ GM-CSF⁻ subset (H) IL-17⁺ IL-22⁺ subset (I) IL-17⁺ IL-22⁻ subset (J) IL-22⁺ subset (K) TNF- α ⁺ subset (L) IFN- γ ⁺ subset (M) GMCSF⁺ subset. CD4/CD8 ratio was calculated from CD4 and CD8 counts taken within the initial 180 days and beyond the initial 180 days post-infection. CD4 and CD8 counts measured after initiating ART and after 1-year post-infection were excluded from the analysis. These analysis are from HVTN 503. Correlation was analyzed using Spearman's rank correlation test.

5.3. Discussion

Systemic inflammation and immune activation are major drivers of immune dysfunction associated with HIV, contributing to the rapid progression of disease in the absence of treatment (Koning et al., 2005; Roberts et al., 2010; Schuetz et al., 2014). Many studies have investigated the contribution of HIV-mediated Th17 cell loss to these drivers of HIV progression (Brenchley et al., 2008; Cecchinato et al., 2008; Favre et al., 2009; Hed et al., 2010; Schuetz et al., 2014). Besides, there are substantial evidence suggesting that the immune composition at baseline and during early HIV infection, especially the amount of HIV target cells, may promote subsequent progression of the disease (Ngcobo et al., 2022; Roberts et al., 2010; Sivo et al., 2018). Herein, we investigated the association of Th17 cells in the peripheral blood prior to infection with HIV viremia and CD4⁺ T cell loss to establish their predictive role in early HIV progression.

Our analysis showed compelling evidence of the relationship between a higher pre-infection Th17 cells and faster CD4⁺ T cell loss during HIV infection. Our findings contribute to the existing body of literature emphasizing the significance of Th17 cell frequency in HIV disease progression (Brenchley et al., 2008; Cecchinato et al., 2008; Favre et al., 2009; Hed et al., 2010; Ndhlovu et al., 2008; Raffatellu et al., 2008). HIV infection is marked by the gradual depletion of CD4⁺ T cells and persistent immune activation (McKinnon et al., 2015). Among CD4⁺ T cell subsets, Th17 cells stand out as particularly susceptible to HIV due to their expression of HIV co-receptors, CCR5 or CXCR4 and susceptibility markers including $\alpha 4\beta 7$ (Guillot-Delost et al., 2012; Hed et al., 2010; McKinnon et al., 2011). Thus, a higher pre-infection frequency of Th17 cells may provide an expanded pool of targets for the virus upon infection, potentially accelerating CD4⁺ T cell loss. Notably, Th17 cells play a critical role in maintaining mucosal barrier integrity (Ancuta et al., 2010; Klatt & Brenchley, 2010). Hence, the depletion of Th17 cells can initiate a detrimental cycle in HIV infection. This depletion may

compromise mucosal barrier function, leading to microbial translocation and systemic immune activation, which further exacerbate HIV progression (Cecchinato et al., 2008; Raffatellu et al., 2008). Additionally, Th17 cells are essential for regulating inflammation and coordinating immune responses against bacterial pathogens (Klatt & Brenchley, 2010; Romagnani et al., 2009). The loss of Th17 cells may disrupt this regulatory function, impairing host defenses against bacterial infections and exacerbating local immune activation (Raffatellu et al., 2008). Consequently, these interconnected processes contribute to the progressive loss of CD4⁺ T cells and the eventual development of AIDS.

Viremia contributes to HIV progression by causing significant immune damage and by driving persistent immune activation, which leads to progressive CD4⁺ T cell loss (Roberts et al., 2010). However, our study found no association between pre-HIV Th17 cells and viral loads, contrasting the study by (Hartigan-O'Connor et al., 2012), which showed that higher pre-SIV Th17 cells in the blood and intestinal tissues prior to infection correlated with lower peak and set-point viral load (Hartigan-O'Connor et al., 2012). Nevertheless, this disparity does not diminish the importance of our findings regarding the predictive capacity of pre-infection Th17 cell abundance for disease progression. Our results consistently demonstrate an association with accelerated CD4 decline and altered CD4/CD8 ratio, underscoring the clinical relevance of Th17 cells in HIV pathogenesis. We propose that the association observed between pre-HIV Th17 cell frequency and HIV progression may be independent of HIV viremia and are more relevant in relations to the promotion of immune activation and systemic inflammation role of Th17 cells in HIV, which further contributes to the gradual depletion of CD4⁺ T cells.

While we identified some associations between pre-infection IFN- γ ⁺ (Th1) and IL-17⁺ IFN- γ ⁺ (Th1/Th17) cell profiles and poorer HIV disease outcome, these associations were not as consistent as that observed with IL-17⁺ (Th17) cell profiles. Thereby suggesting that the

predictive role of Th17 cells in HIV progression may be specific or most significant in relations to IL-17 expression characteristic of these cells. Th1 cells, characterized by IFN- γ expression, play a crucial role in antiviral immunity including HIV (Kang et al., 2018; Roff et al., 2013). Our findings further underscore the significance of Th1/Th17 cells, characterized by the concurrent expression of IL-17 and IFN- γ , in HIV pathogenesis.

Furthermore, our results indicate that pre-HIV IL-17⁺ CD4⁺ T cell levels predict CD4⁺ T cell decline differently by age, with a significant effect observed in younger participants. This significant interaction was however only observed when we combined the data from the two cohorts but not when we analyzed each cohort separately. This is likely because our study is underpowered to detect interaction effects in each cohort. Hence, further confirmation of these results requires a larger sample size. We also observed sex-specific differences in the correlation between pre-HIV IL-17⁺ CD4⁺ T cells and CD4/CD8 ratio and CD4 decline in HVTN 503, an effect more pronounced in the males compared to females. HIV disease outcomes vary based on sex differences, with men observed to have lower viral loads than women during acute HIV infection (Anastos et al., 2000; D’Ettorre et al., 2019; Sterling et al., 2001). Likewise, a study observed that men tend to have lower baseline CD4 cell count compared to women (Mosha et al., 2013). The better virological and immunological response observed in women at early infection stages eventually evens out or becomes worse in women as disease progresses (Cohn et al., 2020; D’Ettorre et al., 2019). Albeit our findings provide a possible mechanism for the poorer virological control and greater CD4 cell loss observed in men at early infection stages.

One limitation of our study is that we focused primarily on the frequency of circulating Th17 cells, which may not fully capture the dynamics of Th17 cells at mucosal sites, where HIV transmission primarily occurs. Additionally, the absence of CD8 data for calculating the CD4/CD8 ratio in one of the cohorts limits our ability to assess this progression parameter

comprehensively. The cohorts used in our study may not fully represent the diversity of population living with HIV, particularly those from regions outside of Africa. The sample size in each cohort may also limit the generalizability of our findings to broader populations. Despite these limitations, our study provides robust evidence for the predictive role of pre-infection circulating Th17 cell abundance in HIV disease progression, while also suggesting potential roles for Th1 and Th1/Th17 phenotypes.

In conclusion, our study found a distinct correlation between heightened levels of Th17 CD4⁺ T cells before HIV infection and accelerated disease progression, specifically in HVTN 503. Overall, pre-HIV Th17 cell frequency may serve as valuable predictor of HIV disease progression, particularly in relations to their IL-17 expression. Understanding the dynamics of different CD4⁺ T cell subsets before HIV exposure could inform strategies for early intervention and personalized treatment approaches. Further research is warranted to elucidate the mechanisms underlying the observed associations and to explore potential therapeutic interventions targeting Th17 cell subsets in HIV infection.

Chapter 6. Genetic variation within *CD101* regulates surface expression of CD101 on circulating T cell subsets, which in turn mediates associations with HIV acquisition.

6.1. Introduction

Host inflammation and immune activation are important drivers of HIV risk (Deeks et al., 2013). While inflammation is mainly a protective response of the immune system intended to defend the body against invading pathogens (Xiao, 2017), in the context of HIV, it can aid viral entry and contribute to the initial establishment of infection. Inflammation triggers the activation of immune cells including CD4⁺ T cells, which are primary target cells for HIV (Deeks et al., 2013; Sáez-Cirión & Sereti, 2021). The activation of CD4⁺ T cells increase their expression of HIV co-receptors, CCR5 and CXCR4 (Lucia Contento et al., 2008), which enhances HIV's ability to infect and replicate within these cells. Inflammatory signals can also promote the recruitment of these target cells at the sites of virus entry (Cromarty & Archary, 2020), thereby increasing the chance of their cellular infection. Therefore, the knowledge of host pathways that regulate inflammation is fundamental to understanding host susceptibility to HIV infection.

CD101 is known to impact host inflammatory pathways as it encodes a cell surface type 1 glycoprotein called CD101 (Jovanovic et al., 2011). CD101 contains seven immunoglobulin-like domains with seven extracellular glycosylation sites and a short cytoplasmic tail (Jovanovic et al., 2011). Whole genome sequencing data from HIV serodiscordant couples from sub-Saharan Africa identified functional variants in *CD101* gene, that were associated with an elevated risk of HIV acquisition (Mackelprang et al., 2017). The identified variants were categorized based on their structural location as immunoglobulin-like and cytoplasmic-like variants. Individuals with higher numbers of immunoglobulin-like variants were at greater

risk of HIV acquisition, while those with cytoplasmic-like variants made smaller contributions to predicting the risk of HIV acquisition (Mackelprang et al., 2017).

CD101 is expressed on various immune cell subsets, including T cells, B cells, monocytes and natural killer cells (Jovanovic et al., 2011; Y. Liu et al., 2022; Mattner et al., 2019; Schey et al., 2016a). It is involved in immune regulation and plays a role in modulating immune responses (Fernandez et al., 2007; Jovanovic et al., 2011; Schey et al., 2016a). Its expression on T cells suggests a potential role in T cell function and modulation of T cell activation (Liu et al., 2022; Richert-Spuhler et al., 2021). Increasing evidence suggests CD101 may promote regulatory T cell functions and dampen T cell responses that occurs during inflammation (Mohammed et al., 2011; Richert-Spuhler et al., 2021a; Schey et al., 2016a). CD101 is also involved in tissue residency as it is recognized as a marker of tissue resident memory cells (TRMs) (Holz et al., 2020). Mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), the natural ligand for gut-homing integrin $\alpha 4\beta 7$, is able to induce CD101 expression on blood CD8⁺ T cells in the presence of retinoic acid (RA) and transforming growth factor-beta (TGF- β) (Girard et al., 2024). It has also been implicated in certain diseases such as autoimmune diseases, IBD and cancer (Jovanovic et al., 2011; Liu et al., 2022; Schey et al., 2016b; Zhou et al., 2020). More recently, it was reported that T cells from individuals with *CD101* variants had altered surface expression of CD101 on circulating T cells subsets and exhibited heightened proinflammatory T cell functions and a reduced suppression capability of the regulatory T cells, thereby suggesting a possible contribution of *CD101* genetic variants to homeostatic regulation of inflammation (Richert-Spuhler et al., 2021a).

Given the contribution of CD101 in inflammatory processes and the association of its genetic variants with HIV risk, we aimed to further investigate the impact of CD101 variants on the surface expression of CD101 on circulating T cells, particularly those expressing homing marker, integrin $\beta 7$, and investigate whether CD101 expression on circulating T cells mediates

the association between *CD101* variants and HIV acquisition. We herein showed significant alterations in circulating *CD101*-expressing T cell phenotypes with a specific *CD101* variants and further revealed a possible mediating effect of these phenotypes on the association between *CD101* variants and HIV acquisition risk.

6.2. Results

6.2.1. Participants Characteristics

We analyzed samples from individuals with pre-defined variants within *CDI01* (n = 230), among whom had variants at both the immunoglobulin-like and cytoplasmic-like domains (n = 8), variants within the immunoglobulin-like domain only (n = 86), variants within the cytoplasmic-like domain only (n = 25) and no variants at all (n = 111). Demographic details of the study participants are shown in **Table 14**. Due to the small sample size of individuals with variants in both domains, we combined samples in this group with that of the Ig-like variants-only group, re-named as having “any Ig-like variants” (n = 94).

Table 14. Baseline characteristics of study participants

Variables	Any Ig-like variants* (<i>n</i> = 94)	Cyto-like variants (<i>n</i> = 25)	Controls (<i>n</i> = 111)	Overall (<i>n</i> = 230)
Age (years) median (IQR)	29.1 (25.0, 34.5)	28.5 (25.5, 35.4)	30.4 (26.0, 35.5)	29.5 (25.6, 35.2)
Sex				
Female	52 (55.3)	13 (52.0)	61 (55.0)	126 (54.8)
Male	42 (44.7)	12 (48.0)	50 (45.0)	104 (45.2)
Cohort				
COS	34 (36.2)	7 (28.0)	45 (40.5)	86 (37.4)
HSV	9 (9.6)	3 (12.0)	18 (16.2)	30 (13.0)
PrEP	51 (54.3)	15 (60.0)	48 (43.2)	114 (49.6)
Treatment Arm				
Placebo	43 (45.7)	10 (40.0)	39 (35.1)	92 (40.0)
PrEP	8 (8.5)	5 (20.0)	9 (8.1)	22 (9.6)
HIV Status				
SC	11 (11.7)	4 (16.0)	4 (3.6)	19 (8.3)
Non-SC	83 (88.3)	21 (84.0)	107 (96.4)	211 (91.7)
Sites				
Jinja	7 (7.4)	3 (12.0)	9 (8.1)	19 (8.3)
Kampala	59 (62.8)	13 (52.0)	75 (67.6)	147 (63.9)
Kisumu	16 (17.0)	7 (28.0)	12 (10.8)	35 (15.2)
PHRU	4 (4.3)	N/A	1 (0.9)	5 (2.2)
Thika	3 (3.2)	N/A	6 (5.4)	9 (3.9)

Nairobi	5 (5.3)	2 (8.0)	8 (7.2)	15 (6.5)
HSV2 Status (Positive)	37 (39.4)	12 (48.0)	42 (37.8)	91 (39.6)
Baseline Risk score ^a				
<6	37 (39.4)	10 (40.0)	50 (45.0)	97 (42.2)
≥6	56 (59.6)	15 (60.0)	61 (55.0)	132 (57.4)
Circumcised	5 (5.3)	2 (8.0)	11 (9.9)	18 (7.8)
Pregnancy at Visit	7 (7.4)	2 (8.0)	2 (1.8)	11 (4.8)
STI ^b				
Gonorrhea	-	-	2 (1.8)	2 (0.9)
Chlamydia	-	-	2 (1.8)	2 (0.9)
Trichomonas	3 (3.2)	-	7 (6.3)	10 (4.3)
Contraceptives				
Condom/others	26 (27.7)	5 (20.0)	28 (25.2)	59 (25.7)
Injection	10 (10.6)	8 (32.0)	8 (7.2)	26 (11.3)
Implant	2 (2.1)	N/A	3 (2.7)	5 (2.2)
Oral	2 (2.1)	N/A	N/A	2 (0.9)
IUD	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
BC none	12 (12.8)	2 (8.0)	20 (18.0)	34 (14.8)

Data are presented as sample sizes and corresponding column percentages, unless otherwise stated. *Any Ig-like = Participants with either Ig-like variants only ($n = 86$) or a combination of Ig-like and cyto-like variants ($n = 8$), Cyto-like only = Participants with cyto-like variants only, Controls = Participants with no functional variants. ^a Baseline risk score was calculated according to Kahle *et al.*, 2013 [Ref: 184] and further details are provided in chapter 3.4. ^b Only positive STI results are presented. A dash (-) indicates that no positives were reported for that category. Abbreviations: Ig-like = Immunoglobulin-like, Cyto-like = Cytoplasmic-like, IQR = Interquartile Range, HIV = Human Immunodeficiency Virus, HSV2 = Herpes Simplex Virus, COS = Couples Observational Study, PrEP = Pre-exposure Prophylaxis, SC = Seroconverters, Non-SC = Non-seroconverters, IUD = Intrauterine device, PHRU = Perinatal HIV Research Unit (Soweto, South Africa), BC none = No birth controls, N/A = Not Applicable.

6.2.2. *CD101* Ig-like Variants Alter CD101 Expression on Circulating T Cells

To confirm that variants in either the Ig-like or cyto-like domains of *CD101* differentially alter the frequency of CD101 on circulating T cell subsets, we characterized T cells based on their expression of homing, activated, proliferating and memory markers (**Figure 20**) and compared the frequencies of CD101 on each subset between individuals with *CD101* variants and individuals without *CD101* variants. First, we observed a significantly higher CD101 expression on CD8⁺ T cells (median 16.1%; IQR: 10.0-24.0%) compared to CD4⁺ T cells (median 3.4%; IQR: 2.4-5.5%, **Figure 20B**). When we compared CD101 expression stratified by variant categories, those with Ig-like variants had higher CD101 expression on both CD4⁺ and CD8⁺ T cells while cyto-like variants and control groups had similar levels of CD101 expression on these T cell subsets (**Figure 20C, 20D**).

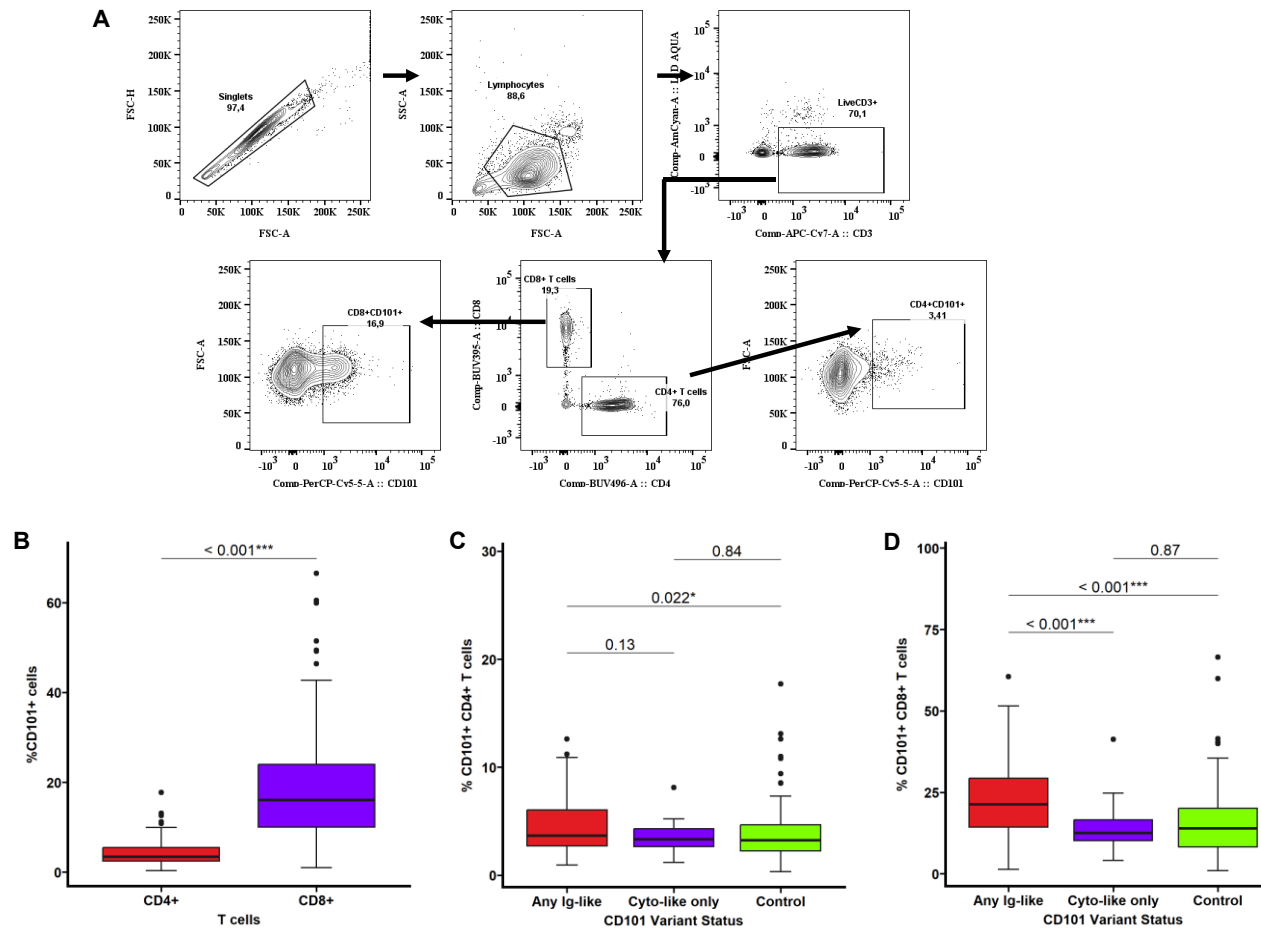


Figure 20. Frequency of CD101⁺ surface expression on overall T cells. (A) Representative flow cytometry plots showing the gating strategy used to quantify CD101⁺ expression on circulating T cells. (B) Comparison of CD101⁺ expression on overall CD4⁺ T cells vs CD8⁺ T cells. (C) Comparison of CD101⁺ expression on CD4⁺ T cells stratified by *CD101* variants. (D) Comparison of CD101⁺ expression on CD8⁺ T cells stratified by *CD101* variants. Comparisons between two groups were analyzed using Mann-Whitney test. Asterisk (*) denotes significance level, where *** < 0.001 , ** < 0.01 , and * < 0.05 .

We next characterized three subsets of homing T cells based on the expression of integrin $\beta 7$ and CD45RA as previously described in **section 4.2.2**, as $\beta 7^{\text{hi}}$, $\beta 7^{\text{int}}$, and $\beta 7^{\text{lo}}$ CD4⁺ and CD8⁺ T cells (**Figure 6A**). Compared to the control group, having any Ig-like variants was associated with significant increases in CD101 frequency on $\beta 7^{\text{hi}}$ and $\beta 7^{\text{lo}}$ T cells but not on $\beta 7^{\text{int}}$ T cells (**Figure 21A, 22A**). Both $\beta 7^{\text{hi}}$ and $\beta 7^{\text{lo}}$ subsets are CD45RA⁻ while $\beta 7^{\text{int}}$ subsets are CD45RA⁺. To determine whether these integrin $\beta 7$ differences merely reflected the alternate memory status of $\beta 7$ subsets, we characterized memory and naïve T cells subsets by their expression of CCR7, CD45RA and CD27 markers, which results in central, effector and transitional memory T cells subsets as CCR7⁺ CD45RA⁻ CD27⁺, CCR7⁻ CD45RA⁻ CD27⁻, CCR7⁻ CD45RA⁻ CD27⁺, respectively, while terminally differentiated and naïve T cells were defined as CCR7⁻ CD45RA⁺ CD27⁻, CCR7⁺ CD45RA⁺ CD27⁺, respectively. Similar to the integrin $\beta 7$ subsets, the CD45RA⁻ central, effector, and transitional memory cells showed higher CD101 frequency in individuals with any Ig-like variants compared to those with cytoplasmic-like variants and controls (**Figure 21C, 22C**). CD101 expression on the CD45RA⁻ memory phenotypes were not statistically significant between the cyto-like variants and control groups. In contrast, CD101 expression on naïve T cells was lower in individuals with *CD101* Ig variants compared to controls. In particular on naïve CD8⁺ T cells, CD101 expression was decreased for those having any Ig-like variants (median 7.1%; IQR: 3.8-17.2%) and cyto-like variants (median 5.0%; IQR: 2.9-8.9%) compared to controls (median 9.3%; IQR: 4.6-17.5%). In summary, the presence of an Ig-like *CD101* variant increase surface expression of CD101 on memory but not naïve T cells. Given the similar *CD101* variants associations observed in integrin $\beta 7^{\text{hi}}$ and $\beta 7^{\text{lo}}$ cells, it is unclear whether this association is driven by the $\beta 7$ or memory status of the T cells.

In addition to characterizing homing and memory T cells, we also examined associations between *CD101* variants and markers of T cell activation, recent proliferation, and

CCR5 expression. Activated T cells were classified as cells expressing both CD38 and HLA-DR, and recent proliferation was defined as T cells expressing Ki67. Those with any Ig-like variants had higher frequencies of CD101⁺CD38⁺HLA-DR⁺CD8⁺T cells (median 9.9%; IQR: 3.6-20.7%) compared to controls (median 5.8%; IQR: 2.9-3.3%, **Figure 22B**). However, we did not observe a significant differential expression of CD101⁺ cells on activated CD4⁺T cells among the variant groups (**Figure 21B**). For T cells expressing Ki67, there was a significant higher CD101 frequencies among individuals with any Ig-like variants than the control group in both CD4⁺ and CD8⁺T cells (**Figure 21B, 22B**). Similarly, having any Ig-like variants was associated with a higher frequency of CD101⁺CCR5⁺T cells (**Figure 21B, 22B**). We did not observe any significant difference in CD101 expression on these T cell subsets between those with cyto-like variants and the controls. Overall, Ig-like *CD101* variants were associated with significant increase in CD101 frequency on multiple T cell phenotypes that are relevant for HIV pathogenesis. Analyses involving the Ig-like only group yielded similar results as with the combined “any” Ig-like variants group (**Table 15**).

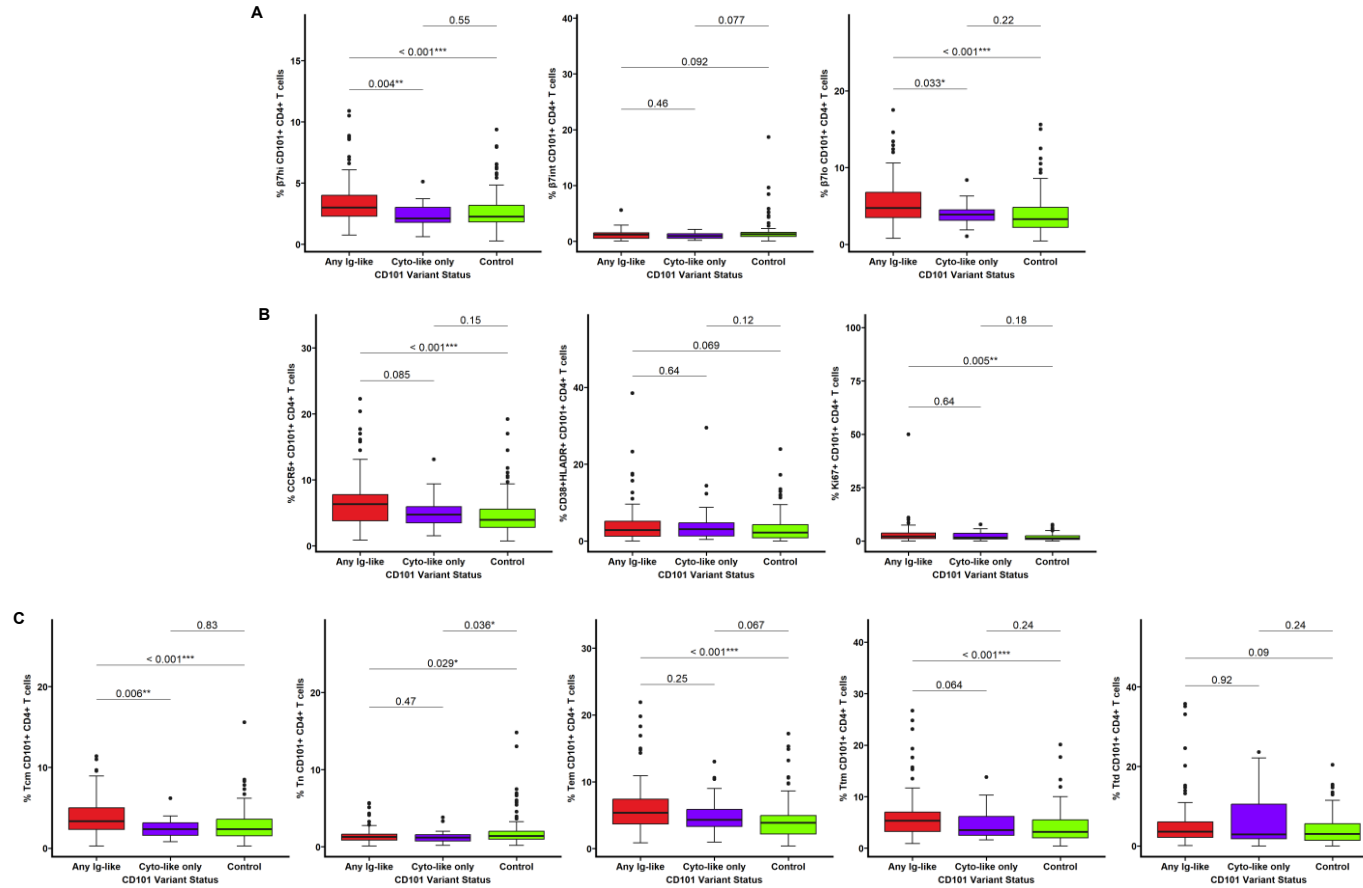


Figure 21. Frequency of CD101⁺ surface expression on CD4⁺ T cell subsets. (A) Frequency of integrin $\beta 7^{\text{hi}}$ CD45RA⁻, $\beta 7^{\text{int}}$ CD45RA⁺, and $\beta 7^{\text{lo}}$ CD45RA⁻ CD4⁺ T cells expressing CD101. **(B)** Frequency of CCR5⁺, activated (CD38⁺ HLADR⁺), and proliferating (Ki67⁺) CD4⁺ T cells expressing CD101. **(C)** Frequency of memory CD4⁺ T cells expressing CD101. Tcm = central memory, Tn = naïve, Tem = effector memory, Ttm = Transitional memory, Ttd = Terminally differentiated CD4⁺ T cells Comparison was made between *CD101* variants and control group. Differences between two groups were analyzed using Mann-Whitney test. Asterisk (*) denotes significance level, where *** <0.001, ** <0.01, and * <0.05.

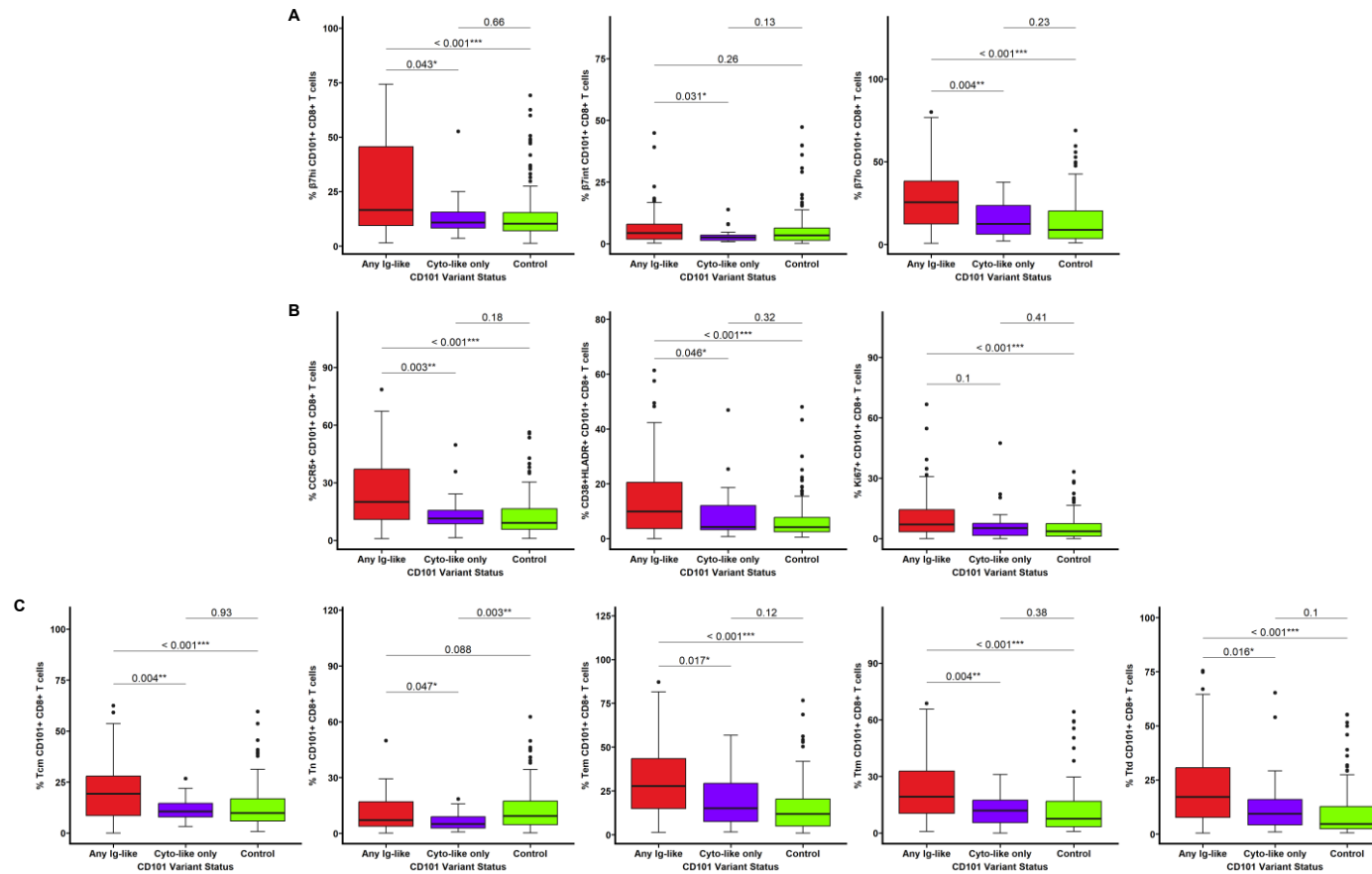


Figure 22. Frequency of CD101⁺ surface expression on CD8⁺ T cell subsets. (A) Frequency of integrin β_7^{hi} CD45RA⁻, β_7^{int} CD45RA⁺, and β_7^{lo} CD45RA⁻ CD8⁺ T cells expressing CD101. (B) Frequency of CCR5⁺, activated (CD38⁺ HLADR⁺), and proliferating (Ki67⁺) CD8⁺ T cells expressing CD101. (C) Frequency of memory CD8⁺ T cells expressing CD101. Tcm = central memory, Tn = naïve, Tem = effector memory, Ttm = Transitional memory, Ttd = Terminally differentiated CD8⁺ T cells Comparison was made between *CD101* variants and control group. Differences between two groups were analyzed using Mann-Whitney test. Asterisk (*) denotes significance level, where *** <0.001, ** <0.01, and * <0.05.

Table 15. Comparison of CD101 expression on T cells between participants with Ig-like variants only vs controls

T Cell types	Ig-like only (<i>n</i> = 86)	Controls (<i>n</i> = 111)	<i>P</i> value
CD4⁺ T cell Subsets			
CD101 ⁺	3.7 (2.7, 6.0)	3.2 (2.2, 4.7)	0.016*
CD101 ⁺ B7 ^{hi}	3.0 (2.3, 4.1)	2.3 (1.8, 3.2)	<0.001***
CD101 ⁺ B7 ^{int}	1.2 (0.7, 1.6)	1.3 (0.9, 1.6)	0.226
CD101 ⁺ B7 ^{lo}	4.8 (3.5, 6.8)	3.3 (2.2, 4.9)	<0.001***
CD101 ⁺ CD38 ⁺ HLADR ⁺	2.8 (1.2, 5.0)	2.2 (0.8, 4.4)	0.071
CD101 ⁺ CCR5 ⁺	6.3 (3.7, 8.1)	3.9 (2.8, 5.6)	<0.001***
CD101 ⁺ Ki67 ⁺	2.1 (1.1, 3.6)	1.3 (0.8, 2.5)	0.005**
CD101 ⁺ TCM	3.4 (2.3, 5.0)	2.4 (1.5, 3.6)	<0.001***
CD101 ⁺ TEM	5.2 (3.7, 7.5)	3.9 (2.1, 5.0)	<0.001***
CD101 ⁺ TN	1.3 (0.9, 1.7)	1.4 (1.0, 2.0)	0.066
CD101 ⁺ TTM	5.2 (3.2, 7.0)	3.2 (2.0, 5.5)	<0.001***
CD101 ⁺ TTD	3.5 (2.0, 5.9)	3.0 (1.4, 5.6)	0.181
CD8⁺ T cell Subsets			
CD101 ⁺	21.4 (14.5, 29.1)	13.9 (8.3, 20.2)	<0.001***
CD101 ⁺ B7 ^{hi}	16.5 (9.6, 43.4)	10.3 (6.9, 15.8)	<0.001***
CD101 ⁺ B7 ^{int}	4.6 (1.8, 8.0)	3.4 (1.4, 6.4)	0.225
CD101 ⁺ B7 ^{lo}	24.7 (12.2, 38.0)	8.9 (3.5, 20.2)	<0.001***
CD101 ⁺ CD38 ⁺ HLADR ⁺	9.7 (3.6, 21.0)	4.1 (2.4, 7.8)	<0.001***
CD101 ⁺ CCR5 ⁺	19.8 (11.2, 36.6)	9.1 (5.7, 16.6)	<0.001***
CD101 ⁺ Ki67 ⁺	7.0 (3.5, 14.0)	3.6 (1.2, 7.6)	<0.001***
CD101 ⁺ TCM	19.4 (10.0, 28.0)	9.7 (5.9, 18.1)	<0.001***
CD101 ⁺ TEM	27.4 (15.0, 42.9)	11.8 (4.8, 20.4)	<0.001***
CD101 ⁺ TN	7.2 (4.1, 17.3)	9.3 (4.6, 17.5)	0.183
CD101 ⁺ TTM	19.2 (10.4, 30.8)	7.6 (3.3, 16.8)	<0.001***
CD101 ⁺ TTD	18.0 (8.0, 29.2)	4.6 (2.4, 12.8)	<0.001***

Data are summarized as median and interquartile range in brackets of percentage CD101 expression. Comparison between Ig-like only vs control group was analyzed using Mann-Whitney test. Asterisk (*) denotes significance level, where *** <0.001, ** <0.01, and * <0.05. Abbreviations: TCM = Central memory cells, TEM = effector memory cells, TN = Naive cells, TTM = Transitional memory cells, TTD = Terminally differentiated cells, Ig-like only = Participants with immunoglobulin-like variants only, Controls = Participants with no *CD101* variants.

6.2.3. CD101⁺ Expression on Circulating T Cells may Mediate the Relationship between *CD101* Variants and HIV Acquisition

Using logistic regression, we confirmed in this sample subset that *CD101* variants were significantly correlated with HIV acquisition as previously reported (110). In unadjusted models, both types of *CD101* variants, i.e., having any Ig-like variants (odds ratio (OR), 3.6; 95% confidence interval (CI): 1.09-11.5; $p = 0.04$) or cyto-like variants (OR 5.1; 95% CI: 1.18-22.00; $p = 0.03$) were significantly associated with elevated risk of HIV acquisition relative to controls. The association remained statistically significant for both variant classes in multivariable models after adjusting for sex (**Table 16**).

Table 16. Logistic regression estimating the association between *CD101* variants and HIV acquisition

Variable	Univariable Models		Multivariable Models	
	OR (95% CI)	P value	OR (95% CI)	P value
Any Ig-like variants*	3.55 (1.09, 11.53)	0.036*	3.59 (1.10, 11.74)	0.035*
Cyto-like variants only	5.10 (1.18, 22.00)	0.029*	5.36 (1.22, 23.47)	0.026*

N = 230; *Any Ig-like variants = combination of participants having Ig-like only (n = 86) and participants having Ig-like plus \Cyto-like variants (n = 8); Cyto-like variants only (n = 25). The multivariable models were adjusted for sex. Asterisk (*) denotes significance level, where *** <0.001, ** <0.01, and * <0.05. Abbreviations: Ig-like = Immunoglobulin-like, cyto-like = Cytoplasmic-like, OR = Odds Ratio, CI = Confidence Intervals

In a three-step method, we showed that CD101-expressing CCR5⁺ CD8⁺ T cells satisfied the conditions needed to establish a mediation using a multiple regression model (**Figure 5**). In essence, Ig-like variants significantly predicted HIV acquisition in step 1 (**Table 16**) and significantly regulated CD101⁺ expression on CCR5⁺ CD8⁺ T cells in step 2 (**Figure 22B**), CD101-expressing CCR5⁺ CD8⁺ T cells was also significantly associated with increased HIV acquisition (**Table 17**). Finally, the effect of Ig-like variants on HIV acquisition was no longer significant while that of CD101⁺ CCR5⁺ CD8⁺ remained significant in step 3 (**Table 18**). Additionally, the effect size of the variants on HIV acquisition was reduced from 3.6 (in step 1, **Table 16**) to 2.4 (in step 3, **Table 18**), while adjusting for CD101⁺ CCR5⁺ CD8⁺ cells. Interestingly, we observed that other CD101⁺ CD8⁺ T cell subsets that were strongly correlated (correlation coefficient, $r \geq 0.5$, **Appendix Figure A10**) with CD101⁺ CCR5⁺ CD8⁺ T cells, including CD101⁺ (TTM, TEM, TTD, $\beta 7_{lo}$ and CD38⁺ HLADR⁺) CD8⁺ T cell subsets also satisfied most of the required conditions for mediation, except their effect was no longer significant in the final step (**Table 18**). Altogether, the results from the multiple regression models indicates a possible partial mediating effect of CD101-expressing CCR5⁺ CD8⁺ T cells and related cells on the relationship between *CD101* Ig-like variants and HIV acquisition.

We observed similar effects between our SEM and MRM results, and a minimal indirect effect of *CD101* Ig-like variants on HIV acquisition that is explained by the mediator (**Figure 23**). In summary, given the strong correlation between the CD101-expressing T cell subsets, we assumed that these subsets partially mediate the relationship between *CD101* variants and HIV risks.

Table 17. The association of CD101-expressing cells with HIV acquisition

CD101 ⁺ Phenotype	Univariable Model		Multivariable Model	
	OR (95% CI)	<i>P</i> value	OR (95% CI)	<i>P</i> value
CD8 ⁺	1.042 (1.004 – 1.081)	0.031*	1.037 (0.997 – 1.078)	0.068
β ₇ ^{lo} CD8 ⁺	1.032 (1.005 – 1.060)	0.019*	1.030 (1.003 – 1.059)	0.030*
TEM CD8 ⁺	1.026 (1.001 – 1.050)	0.039*	1.024 (0.999 – 1.049)	0.063
TTM CD8 ⁺	1.032 (1.004 – 1.062)	0.026*	1.032 (1.003 – 1.063)	0.031*
TTD CD8 ⁺	1.033 (1.007 – 1.060)	0.012*	1.030 (1.003 – 1.056)	0.027*
CD38 ⁺ HLADR ⁺	1.037 (1.001 – 1.074)	0.043*	1.030 (0.995 – 1.067)	0.096
CD8 ⁺				
CCR5 ⁺ CD8 ⁺	1.038 (1.010 – 1.066)	0.007**	1.035 (1.007 – 1.064)	0.013*

Logistic regression was performed to examine the effect of the potential mediating variables (CD101⁺ T cell subsets) on the outcome variable (HIV acquisition). Only phenotypes with significant effects are reported here. Asterisk (*) denotes significance level, where *** <0.001, ** <0.01, and * <0.05. Abbreviations: OR = Odds ratio, CI = Confidence Interval.

Table 18. The association of *CD101* variants with HIV acquisition in the presence of CD101-expressing phenotype

Variables	Units	OR (95% CI)	P value
CD8 ⁺ TTDCD101 ^a		1.026 (0.998 – 1.054)	0.0714
	Any Ig-like	2.544 (0.730 – 8.871)	0.1429
CD8 ⁺ CCR5 ⁺ CD101 ^a		1.030 (1.00 – 1.060)	0.0488*
	Any Ig-like	2.376 (0.672 – 8.405)	0.1793
CD8 ⁺ TTMCD101 ^a		1.025 (0.995 – 1.056)	0.1086
	Any Ig-like	2.753 (0.808 – 9.372)	0.1053
CD8 ⁺ TEMCD101 ^a		1.018 (0.991 – 1.045)	0.1908
	Any Ig-like	2.721 (0.778 – 9.518)	0.1172
CD8 ⁺ β ₇ ^{lo} CD101 ^a		1.025 (0.996 – 1.054)	0.0961
	Any Ig-like	2.612 (0.757 – 9.014)	0.1288
CD8 ⁺ CD38 ⁺ HLADR ⁺ CD101 ^a		1.026 (0.988 – 1.065)	0.1878
	Any Ig-like	2.880 (0.840 – 9.878)	0.0925
CD8 ⁺ CD101 ^a		1.034 (0.993 – 1.076)	0.1046
	Any Ig-like	2.861 (0.854 – 9.590)	0.0884

^aCorrelated CD101⁺ subsets with correlation coefficient ≥ 0.5 . Variable boldened met all mediation conditions according to Baron and Kenny, 1986 (319). Asterisk (*) denotes significance level, where *** <0.001, ** <0.01, and * <0.05. Abbreviations: OR = Odds Ratio, CI = Confidence Intervals, Ig-like = Immunoglobulin-like.

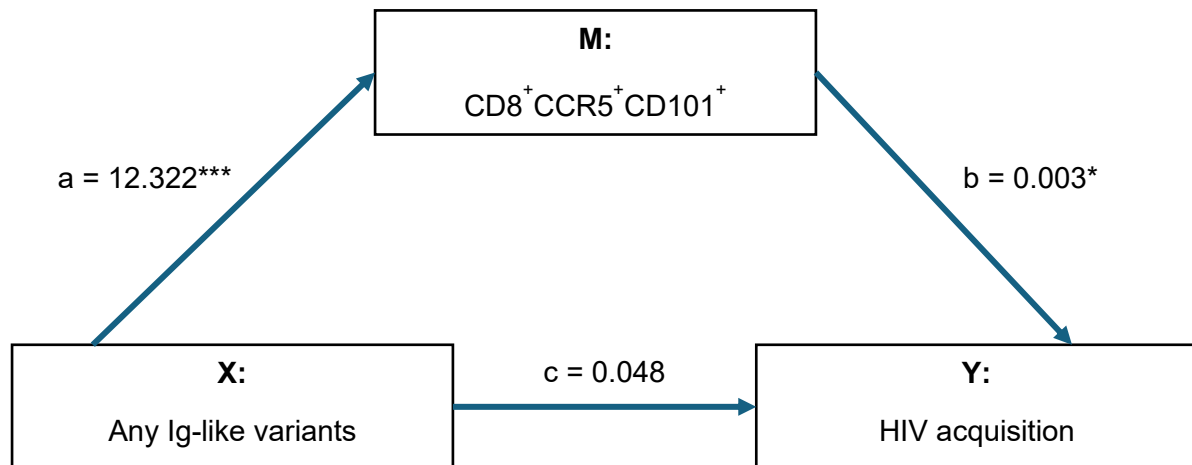


Figure 23. Path diagram of the effect of *CD101* variants on HIV acquisition in the presence of a mediator using structural equation modelling. Where, a = direct effect of X on M = 12.32***, b = direct effect of M on Y = 0.003*, c = direct effect of X on Y = 0.05^{n.s.}, $a*b$ = indirect effect of X on Y = 0.03*, $c+(a*b)$ = total effect of X on Y = 0.08*. Abbreviations: M = Mediating variable, X = Predictor variable, Y = Outcome variable, Ig-like = Immunoglobulin-like. Asterisk (*) denotes significance level, where *** <0.001, ** <0.01, and * <0.05.

6.3. Discussion

Genetic variants in *CD101* gene are associated with increased HIV acquisition risk. The mechanism by which these variants impact HIV risk is not fully clear, but it is known that these variants can modulate immune cell profiles, promoting a proinflammatory immune environments favourable to HIV infection. Our findings suggest that *CD101* variants may modulate CD101 expression on circulating T cells, which could partially contribute to the increased HIV acquisition risk observed in individuals carrying these functional variants. Like others (Mackelprang et al., 2017; Richert-Spuhler et al., 2021), we showed that in heterosexual individuals, *CD101* variants are associated with elevated risk of HIV acquisition and linked with increased CD101 expression on a broad spectrum of circulating T cell types including cells expressing integrin $\beta 7^{\text{hi}}$, CCR5⁺, KI67⁺, activated (CD38⁺ HLA-DR⁺) and memory phenotypes. We further demonstrate that these altered phenotypes contribute to the association between *CD101* variants and HIV acquisition risk.

While CD101 expression distinguishes a more Treg population (Fernandez et al., 2007; Schey et al., 2016; Strongin et al., 2022), CD101 Ig-like variants have been associated with attenuated suppressive ability of Tregs (Richert-Spuhler et al., 2021), suggesting that CD101 variants may promote a state of uncontrolled inflammation and immune activation. The presence of inflammation and a heightened immune activation are notable features of HIV acquisition (Deeks et al., 2013; Hunt, 2012; Mackelprang et al., 2017). Like others have shown, our finding indicate that Ig-like variants seem to be the major driver of altered inflammatory state observed and not the cyto-like variants (Richert-Spuhler et al., 2021). Among those with Ig-like variants were a heightened level of CD101-expressing homing, activated, proliferating and memory T cells. Each of these cell types have been implicated in HIV pathogenesis (Cromarty & Archary, 2020; Lv et al., 2021; Sáez-Cirión & Sereti, 2021; Sivo et al., 2018). We also showed a direct correlation between these phenotypes and increased HIV acquisition in our dataset.

CD101 has been implicated in regulating immune responses and its expression on T cells have been shown to play a role in the modulation of T cell activation (Fernandez et al., 2007; Jovanovic et al., 2011; Y. Liu et al., 2022). T cells play a crucial role in immune response against HIV. The interaction between HIV and T cells involves the binding of the virus to CD4 receptor and co-receptors like CCR5 on T cell surface (Hazenbergh et al., 2000; Okoye & Picker, 2013; Vijayan et al., 2017). Cytotoxic (CD8⁺) T cells on the other hand are responsible for direct killing of HIV-infected cells (Hazenbergh et al., 2000). CD101 expression on CD8⁺ T cells have been previously suggested to be a likely marker of dysfunctional CD8⁺ T cells with less cytotoxic ability to kill virus-infected cells (Hudson et al., 2019; Jovanovic et al., 2011; Richert-Spuhler et al., 2021; Zhang et al., 2019). Our findings of a higher frequency of CD101⁺ CD8⁺ T cells among those with Ig-like variants may indicate inability to mount proper cytotoxic response to HIV thereby enhancing the replication of the virus. CCR5-expressing T cells are highly susceptible to HIV infection and are preferential targets (Gornalusse et al., 2015; van Eekeren et al., 2022), likewise T cells expressing $\alpha 4\beta 7$ (Q. Liu & Lusso, 2020; Sivro et al., 2018; Tokarev et al., 2020). Recently, CD101⁺ CD4⁺ T cells were identified as preferential targets for HIV compared to CD101⁻ CD4⁺ T cells (Strongin et al., 2022) and another study reported a higher percentage of CCR5⁺ CD8⁺ T cells among people living with HIV (van Eekeren et al., 2022). One hypothesis that could emerge from our data is that *CD101* gene variants may alter the functional profile of CD8⁺ T cells by expanding populations with reduced cytotoxic potential and diminished immune surveillance capacity. This may impair early recognition and clearance of HIV-infected cells at mucosal entry sites, thereby facilitating viral establishment and increasing susceptibility to HIV acquisition.

In conclusion, our study contributes to existing knowledge on the role of *CD101* genetic variants in HIV pathogenesis and its function to alter inflammatory environments. Our findings substantiate previous studies indicating a role for *CD101* variants, particularly variants within the Ig-like domain, in altering the expression of CD101 on T cells. Our study builds upon

existing evidence linking *CD101* variants to HIV acquisition risk. Not only did our study confirm this association, but we also identified that the link between *CD101* variants and increased HIV risk may be mediated through altered CD101 expression on immune phenotype such as CCR5-expressing CD8⁺ T cells, which, although not directly infected by HIV, may contribute to a permissive immune environment that facilitates viral acquisition. This novel finding adds depth to the existing understanding of *CD101* genetic variants' involvement in HIV susceptibility.

Chapter 7. Discussion

7.1. General Discussion

HIV remains a significant global health challenge, necessitating an ongoing effort to deepen our understanding of its pathogenesis to improve therapeutic strategies. A critical factor in this pursuit is the immunological events that occur during early HIV infection, which can significantly influence the overall course of the disease. This study was premised on the hypothesis that an individual's immune profile near the time of HIV infection could impact the outcomes of infection, including acquisition and disease progression. We investigated whether specific immune cell subsets, particularly those expressing integrin $\alpha 4\beta 7$ and Th17 cells, could serve as predictors of these outcomes.

Consistent with related studies (Hartigan-O'Connor et al., 2012; Koning et al., 2005; Sivo et al., 2018), our findings reaffirm the theory that the host's immune status prior to HIV infection can significantly influence the course of disease after infection. Our findings demonstrate that higher expression levels of HIV-susceptible immune cells, that is, integrin $\alpha 4\beta 7$ and Th17 cells, may predict HIV outcome, especially HIV disease progression. However, this predictive association is likely influenced by population-specific characteristics, as indicated by variability in the pattern of these associations across different cohorts. In particular, we confirmed that within South African participants, a higher pre-HIV frequency of integrin $\alpha 4\beta 7$ correlates with increased HIV acquisition – a non-significant correlation probably due to a limited sample size – and significantly predicts faster disease progression within this population. However, similar associations were not replicated in the East-African cohort, where an opposite effect was observed in a specific subgroup.

We propose clade differences between regions, particularly the interaction between HIV clade C in South Africa and integrin $\alpha 4\beta 7$, may play a significant role in the variability between these cohorts. Compared to other HIV clades or even clade C from other regions, South African

clade C was shown to most efficiently bind to integrin $\alpha 4\beta 7$ (Arthos et al., 2008; Richardson et al., 2015). It is possible to imply that clade C may utilize integrin $\alpha 4\beta 7$ as an additional attachment factor to facilitate a more productive infection, potentially contributing to the higher prevalence of HIV in this population. Besides, it is not uncommon for the impact of HIV within a specific clade to be completely different from that in another (Tolomeo & Cascio, 2024).

The heterogeneity of HIV poses a considerable challenge in developing a universal cure or vaccine for the disease (Ng'uni et al., 2020; Nkolola & Barouch, 2024). A typical instance is the ALVAC-gp120 vaccine candidate which showed modest efficacy against clade B in Thailand but failed to show efficacy against clade C in South Africa (Gray et al., 2021). Unlike many other infectious pathogens, HIV's pathogenicity is highly variable, influenced by both host differences and viral factors. This variability complicates the replicability of findings across different populations and emphasizes the need for personalized approaches in HIV research. Our observations on the predictive role of pre-HIV integrin $\alpha 4\beta 7$ in HIV within South African population agrees with previous findings by (Sivro et al., 2018). We had previously reported that South African women with higher pre-HIV $\alpha 4\beta 7$ CD4⁺ T cells were more likely to contract HIV and experienced faster disease progression (Sivro et al., 2018). Likewise, our findings in the East-African cohorts agrees with the study by (Martin et al., 2020), which showed that pre-HIV $\alpha 4\beta 7$ CD4⁺ T cells was neither predictive of increased risk of HIV nor faster disease progression. In addition, they reported a protective association between pre-HIV $\alpha 4\beta 7$ CD4⁺ T cells and HIV acquisition among PWID (Martin et al., 2020), similarly to our observations within the East-African participants. The reasons for this protective association will require further elucidation. Albeit, while our current findings validate our previous observations in South African women, it underscores the importance of population-specific factors in HIV research.

A second principal finding of this study is the predictive role of Th17 CD4⁺ T cells in HIV disease progression. In addition to integrin $\alpha 4\beta 7$, we found that pre-HIV Th17 CD4⁺ T

cells levels were strongly correlated with markers of faster disease progression, particularly within the South African cohort. Specifically, a higher pre-HIV Th17 cell profile was significantly associated with lower CD4 counts, independent of viral load. Interestingly, these associations were more pronounced in males, highlighting a potential sex-specific influence on HIV progression.

HIV primarily targets CD4⁺ T cells causing their depletion – a hallmark of the infection (Fanales-Belasio et al., 2010; Tolomeo & Cascio, 2024). However, not all CD4⁺ T cells are equally targeted (McKinnon & Kaul, 2012; Tolomeo & Cascio, 2024). While HIV can infect any CD4-expressing cell (Fanales-Belasio et al., 2010), Th17 cells are among the most dysregulated subsets in HIV infection (Tolomeo & Cascio, 2024; Zayas & Mamede, 2022). It follows that a higher pre-infection frequency of Th17 cells could result in a faster rate of CD4⁺ T cell depletion, as these cells represent a more susceptible target for the virus. The depletion of Th17 cells is particularly detrimental due to their critical functions. Th17 cells are highly expressed in gut tissues, a major site of HIV replication, where they maintain the integrity of the epithelial barrier and protect against opportunistic infections (Renault et al., 2022; Rodriguez-Garcia et al., 2014; Zayas & Mamede, 2022). Therefore, their depletion can lead to barrier disruption, facilitating microbial translocation and subsequent systemic inflammation and immune activation, both of which are key drivers of HIV progression (Cecchinato et al., 2008; Raffatellu et al., 2008; Schuetz et al., 2014). Moreover, Th17 cells share a developmental pathways with regulatory T cells, making the balance between these two populations essential for immune homeostasis (Loiseau et al., 2016; Wan et al., 2020). Although, HIV targets Th17 cells leading to their depletion, HIV does not necessarily cause a decrease in Treg numbers but may increase their immunosuppressive activity (Chevalier & Weiss, 2013; Tolomeo & Cascio, 2024; Yero et al., 2021). This HIV-induced Th17 cell depletion disrupts the Th17 – Treg balance, contributing to a rapid onset of immunodeficiency in people infected with HIV by suppressing immune responsiveness during the early phases of infection (Loiseau et al., 2016).

Further expanding on HIV targets cells, emphasizing that while all CD4-expressing cells are potential targets for HIV, their susceptibility to infection varies (McKinnon & Kaul, 2012). Among these, CD4⁺ T cells expressing integrin $\alpha 4\beta 7$ and Th17 CD4⁺ T cells are particularly HIV-permissive, alongside cells expressing the HIV co-receptors CCR5 or CXCR4 (McKinnon et al., 2011; McKinnon et al., 2015). Our findings highlight that the predictability of HIV acquisition or disease progression depends not only on the “quantity” of target cells but also on their “quality”, a concept coined from McKinnon and Kaul. (2012) (McKinnon & Kaul, 2012). Specifically, among the CD4⁺ T cell subsets we investigated, those known for their high vulnerability to HIV – such as cells expressing integrin $\alpha 4\beta 7$ and Th17 markers – were the ones more predictive of HIV outcomes. This suggests that individuals with higher levels (quantity) of these highly susceptible (quality) immune subsets may be more biologically prone to HIV acquisition and faster disease progression.

Integrin $\alpha 4\beta 7$ -expressing cells and Th17 cells share characteristics that render them particularly susceptible to HIV. Both subsets are characterized by high CCR5 expression, and Th17 cells are also known to co-express integrin $\alpha 4\beta 7$ (Loiseau et al., 2016; Renault et al., 2022; Tokarev et al., 2020). This co-expression suggest that HIV may exploit the trafficking mechanisms of these cells to facilitate its spread. Integrin $\alpha 4\beta 7$ promotes the homing of CD4⁺ T cells to mucosal sites, particularly the gut, where HIV primarily replicates (Byrareddy et al., 2016; Sivro et al., 2018). Th17 cells expressing this homing receptor are likely to migrate to the gut, where they become prime targets for HIV infection and subsequently depleted. Interestingly, in our South African cohort, where both pre-HIV integrin $\alpha 4\beta 7$ and Th17 CD4⁺ T cells significantly predicted faster disease progression, these two immune subsets were strongly correlated with each other. In contrast, in the East African cohort, where these markers were not significantly associated with faster disease progression, integrin $\alpha 4\beta 7$ and Th17 CD4⁺ T cells were not correlated with each other.

Additionally, we observed interesting dynamics within memory CD4⁺ T cell compartments, particularly in relations to integrin $\alpha 4\beta 7$ subsets. In the South African cohort, central memory cells were more strongly correlated with $\alpha 4\beta 7^{\text{hi}}$ cells compared to the East African cohort. Our findings also indicate distinct subsets within memory cells, distinguishing $\alpha 4\beta 7^{\text{hi}}$ from $\alpha 4\beta 7^{\text{lo}}$ cells. Specifically, $\alpha 4\beta 7^{\text{hi}}$ cells predominantly exhibited a central memory phenotype, while $\alpha 4\beta 7^{\text{lo}}$ cells displayed an effector memory phenotype, similar to reports from previous studies (Kader et al., 2009; Mijiti et al., 2022). This observation prompts the intriguing possibility that $\alpha 4\beta 7^{\text{hi}}$ cells may exhibit a preference for migration not solely to the gut but potentially to gut lymphoid tissue (Tokarev et al., 2020; Tolomeo & Cascio, 2024). Memory CD4⁺ T cells have been implicated in HIV pathogenesis (Lee et al., 2019; Tokarev et al., 2020) and both integrin $\alpha 4\beta 7^{\text{hi}}$ and Th17 cells are primarily memory cells (Tokarev et al., 2020). Memory CD4⁺ T cells such as central memory cells express high levels of CCR5 and are therefore, more permissive to HIV infection (Martinelli et al., 2013; Okoye & Picker, 2013; Rullo et al., 2020; Tokarev et al., 2020). These cells not only serve as key targets for HIV but also play crucial roles in the immune responses against the virus (Thibault et al., 2007). For example, effector memory CD4⁺ T cells can provide rapid immune responses upon re-exposure to HIV, contributing to viral control. However, their infection by HIV can compromise overall defense (Okoye & Picker, 2013; Thibault et al., 2007). Given the variability between cohorts in the correlations between integrin $\alpha 4\beta 7$, Th17 cells, and memory subsets, we propose that these phenotypic differences may explain the variation in HIV outcome predictions, where one cohort is characterized by target cells that are highly vulnerable to HIV, while the other is characterized by target cells with lesser susceptibility.

In the East African cohort, we observed an association between integrin $\alpha 4\beta 7^{\text{int}}$, naïve CD4⁺ T cells and increased HIV acquisition. However, this association was absent in the South African cohort, despite strong correlations between these subsets in both cohorts. Given the relationships between integrin $\alpha 4\beta 7$ subsets and memory CD4⁺ T cells, it is perhaps not

surprising that memory and naïve cell – HIV associations mirror those of their associated subsets. Naïve CD4⁺ T cells, unlike memory T cells, are minimally susceptible to direct HIV infection due to their low CCR5 expression (Khoury et al., 2011; Rullo et al., 2020; Tokarev et al., 2020). However, the depletion of CD4⁺ T cells, including naïve cells, due to HIV infection can impact the overall immune function, indirectly influencing susceptibility to HIV acquisition and immune competence (Khoury et al., 2011; McCune, 2001).

In our datasets, we observed an inverse correlation between the frequency of integrin $\beta 7^{\text{int}}$ and naïve T cell subsets with increasing age in the East African cohort. Notably, participants in this cohort were, on average, 10 years older than those in the South African cohort. We propose that age could be a significant confounding factor, potentially linked to the dynamics of naïve versus memory cells as individuals age. It is crucial to note that the $\alpha 4\beta 7^{\text{hi}}$ CD4⁺ T cell variable is a proportion, and thus, its values are influenced by shifts in the proportion of naïve $\alpha 4\beta 7^{\text{int}}$ CD4⁺ T cells. This suggests that as the proportion of naïve CD4⁺ T cell phenotypes decrease with advancing age, it could lead to an increase in both the $\alpha 4\beta 7^{\text{hi}}$ and $\alpha 4\beta 7^{\text{lo}}$ memory compartments, thereby potentially influencing the associations between integrin $\beta 7$ and HIV. A similar dynamic was observed in the Th17 analysis, where the predictive value of Th17 CD4⁺ T cells for the rate of CD4 decline decreased with age. Notably, the age demographic in our initial study (Sivro et al., 2018), where $\alpha 4\beta 7$ significantly predicted HIV outcomes, consisted of younger participants similar in age to our South African cohort. In contrast, the follow-up study showing no effects (Martin et al., 2020) investigated slightly older participants, similar in age to our East African cohort. Altogether, these findings suggest that age is an important factor to consider when using immune cells to predict HIV outcomes. These results further highlight the biological vulnerability of younger individuals typically 15 – 24 years in sub-Saharan Africa, to HIV infection.

Another key message from the present study is that it is more complex to predict HIV acquisition by the amount of HIV target cells present prior to infection than it is to predict the progression of the disease post-infection. While the availability of high-quality HIV target cells, such as CD4⁺ T cells expressing CCR5 or $\alpha 4\beta 7$, and Th17 cells, at the time of HIV exposure is critical to infection, other factors also play crucial roles in ensuring a productive infection. For instance, the viral characteristics, including the strain and viral load of infecting partner, significantly influence whether exposure leads to a successful transmission (Baeten et al., 2011; Hughes et al., 2012; Souto et al., 2021). Additionally, the integrity of mucosal barriers at the site of exposure is a determining factor because any disruption, such as induced by the presence of STIs or local inflammation, can increase susceptibility by facilitating viral entry (Hughes et al., 2012; Moran et al., 2022; Scully, 2018a, 2018b). Moreover, immune activation and the local inflammatory environment can further complicate the prediction of HIV acquisition. Elevated immune activation levels can lead to the recruitment of more target cells to the site of exposure, thus increasing the likelihood of infection (Griesbeck et al., 2016; Santinelli et al., 2020). However, these dynamics vary widely among individuals (Koning et al., 2005; Santinelli et al., 2020), making it challenging to predict outcomes solely based on the presence of target cells. Furthermore, genetic and host factors, such as polymorphisms in the CCR5 gene and hormonal influences (Griesbeck et al., 2016; Hagen & Altfeld, 2016; Ronsard et al., 2019), add another layer of complexity by modulating an individual's inherent susceptibility to infection. In contrast, once HIV is established, predicting disease progression becomes more straightforward. This is primarily because progression is driven by measurable factors like the gradual decline in CD4⁺ T cell counts and persistent immune activation, both of which can be monitored overtime (Ngcobo et al., 2022; Roberts et al., 2010; Sabin & Lundgren, 2013). The contrasting complexity between predicting HIV acquisition and progression highlights the multifaceted nature of HIV transmission and underscores the need for comprehensive approaches in prevention and research.

Lastly, we confirmed the association between rare genetic variants in the *CD101* gene and an increased risk of HIV acquisition, proposing a potential mediation pathway involving HIV target cells. A previous study identified genetic variants in the *CD101* gene that are linked to a higher risk of HIV acquisition (Mackelprang et al., 2017). These variants have been shown to alter immune cells profiles and function, leading to a proinflammatory immune environment and less suppressive Treg populations (Richert-Spuhler et al., 2021). CD101⁺ cells are HIV targets that are preferentially depleted during acute infection, with their depletion associated with increased viral loads and elevated levels of inflammatory cytokines (Strongin et al., 2022). Our findings align with these earlier studies, as we demonstrated that the presence of *CD101* variants, particularly those of the immunoglobulin-like region, are associated with a greater risk of HIV acquisition and altered levels of CD101-expressing T cells in the peripheral blood. Specifically, individuals with these variants exhibited increased levels of CCR5, homing, activated, proliferating, and memory T cells expressing CD101 compared to those without the genetic variants. These cell subsets, modulated by *CD101* variants, play critical roles as HIV target cells, and our data suggest that they may mediate the association between CD101 immunoglobulin-like variants and heightened HIV acquisition risk, particularly through the involvement of CCR5⁺ CD101⁺ cytotoxic T cells.

CD101, as an immunoregulatory marker, plays a role in preventing excessive immune activation and the proliferation of T cells (Bouloc et al., 2000; Schey et al., 2016). CD101 expression on Tregs were associated with high immunosuppressive potential (Strongin et al., 2022), however, *CD101* Ig-like variants were associated with increased levels of less potent Treg populations, thereby fostering a more pro-inflammatory state conducive to HIV replication (Richert-Spuhler et al., 2021). Moreover, CD101 expression on CD8⁺ T cells has been linked to less cytotoxic activity, rendering these cells less effective at killing infected cells (Jovanovic et al., 2011; Richert-Spuhler et al., 2021). Therefore, our data provide compelling evidence that the presence of *CD101* Ig-like variants modulates the immune system in a way

that leads to higher levels of less functional T cells. These cells are less capable of mounting an effective immune response against HIV and promote an immune environment that is highly conducive to the virus replication. This mechanism likely contributes to the increased risk of HIV acquisition observed in individuals with these *CD101* variants.

7.2. Study Strengths

This study provided a detailed analysis of key immune cells in HIV, including Th17 cells, integrin $\alpha 4\beta 7$ -expressing cells, and CD101-expressing T cells, offering significant insights into their roles in predicting HIV acquisition and progression. By incorporating multiple cohorts from South Africa and East Africa, our study was able to explore population-specific variations, revealing the impact of geographical differences on the predictability of HIV outcomes. The inclusion of both male and female participants and conducting sex-stratified as well as other sub-group analyses, allowed for a detailed understanding of the immunological factors influencing HIV outcomes. Although the cohorts differed in certain covariates by design, such as clade differences informed by geographical regions and HIV prevention interventions, they shared important similarities: all participants were heterosexual individuals of African descent, at risk of HIV exposure, and above the age of 18 years, ensuring a degree of comparability between them.

In addition to aligning our experimental design with previous studies that informed this research, we implemented rigorous quality control measures. These included continuous monitoring for batch effects using peripheral blood from healthy volunteers, full optimization of all flow cytometry panels, and the application of appropriate controls such as FMOs and compensation controls. We also used standardization beads to ensure consistent performance of the flow cytometer over time. These measures collectively ensured that our flow cytometry procedures yielded reliable and reproducible results, confirming that the contrasting findings reported in this study reflect true effects rather than methodological issues.

By conducting sensitivity analyses that excluded certain samples based on specific criteria, we demonstrated the consistency of our findings across experiments, even when those samples were included. Additionally, we were able to reproduce our integrin $\alpha 4\beta 7$ findings in a secondary flow cytometry panel, underscoring the robustness of our methodological approach. Importantly, all data were acquired in a blinded manner, without access to participant demographics, which minimized potential personnel bias.

7.3. Study Limitations and Future Directions

Despite its strength, our study is not without its limitations. The sample sizes within each cohort may be too small to detect certain associations and limited us from conducting detailed stratified analyses, especially regarding our HIV progression datasets. We adopted a cross-sectional study design as we only quantified pre-HIV immune cells at a single timepoint, and this limited our ability to observe changes in immune cell dynamic over time. Therefore, our findings are correlational and did not reflect a causal relationship between the target cells and HIV outcomes. Additionally, focusing solely on immune cells expressed in the peripheral blood may not fully capture the complex dynamics of HIV infection in tissue compartments, particularly in mucosal sites where HIV first establishes infection.

Although this study identified correlations between target cell subsets and HIV outcomes, it did not extensively explore the functionality of these cells in the context of HIV infection, which could provide more mechanistic insights. Factors such as age, sex, co-infections, and host genetics may influence immune cell profiles and their association with HIV outcomes. While some of these factors were addressed in our analyses, the full impact of these confounders might not have been entirely accounted for, as we were limited by the information available at the time of primary data collection. Additionally, we used geographical differences as the only marker to infer prevalent HIV clades within a particular region and acknowledge

that this approach may be imperfect for establishing virus strain or clade without laboratory confirmation of viral strains.

We encountered some major technical challenges in optimizing the flow cytometry panel designed for Treg cell analysis, which ultimately hindered our ability to explore the association of these cells with HIV outcomes and their dynamics with Th17 cells. A critical marker used for Treg identification, CD127, was initially placed in the BV711 channel, which we later discovered had sensitivity issues on our flow cytometer. Specifically, it was difficult distinguishing between the positive and negative populations for this marker, a problem that persisted despite the use of FMOs and unstained controls. After moving CD127 to a different channel, BV650, and reoptimizing the panel, the issue improved slightly. However, by then, we were already halfway through processing the study samples, and this led to high variability in the Treg measurements, reducing the quality of the data generated. Since we had only one vial of PBMC per participant, we could not reacquire the initial samples lost due to this problem. Additionally, we had planned to measure vaccine-specific $\alpha 4\beta 7$ immune responses in the HVTN 503 South African cohort using activation-induced marker (AIM) assay. However, due to high background in unstimulated cells, we detected little to no antigen-specific $\alpha 4\beta 7$ responses in most samples, limiting the scope of our findings.

To address these limitations, we propose a larger study that incorporates a longitudinal approach exploring target cell distribution in different anatomical sites, especially mucosal sites. This should involve recruiting a cohort of HIV-negative individuals from multiple regions at high risk of HIV exposure, similar to the populations we investigated in this study, collecting samples from multiple anatomical sites, including peripheral blood and mucosal tissues, such as rectal, vaginal, and GALT, at multiple time points: baseline (pre-exposure), shortly after exposure, and then periodically over a defined period (e.g., monthly for a year) to track changes over time, while collecting relevant demographic data alongside. Participants should be monitored for HIV seroconversion as the primary endpoint, and those who eventually become

infected should be analyzed to track post-infection immune dynamics. In addition to flow cytometry technique strictly used in this study to quantify target cell subsets (integrin $\alpha 4\beta 7$, CD101-expressing cells, and Th17 cells), a future study should adopt other methods such as single-cell RNA sequencing, Imaging techniques, or spatial transcriptomics to explore the functional states of these cells in different compartments, track cell migration from peripheral blood to tissues during early HIV infection and identify changes in immune landscape that may contribute to disease progression. A future research adopting the described design offer greater advantages including providing stronger evidence to establish causality between these target cells and HIV, identifying possible mechanisms for why certain populations are more susceptible to infection due to their specific immune cell profiles and overall will provide a more comprehensive understanding of the early stages of HIV infection.

In relation to Th17 findings, it may be advantageous to include additional markers for the identification of functionally distinct Th17 subsets and investigating how their interplay with regulatory T cells may influence HIV acquisition risk. Th17 cells are not a homogenous population; prior studies have shown that some subsets are pathogenic, characterized by the production of pro-inflammatory cytokines (such as IL-17, IFN- γ , and GM-CSF) and contribute to chronic inflammation and tissue damage. In contrast, non-pathogenic Th17 cells may co-produce IL-17 and regulatory cytokines such as IL-10 and IL-22, supporting mucosal homeostasis and immune regulation. A defining distinction between pathogenic and non-pathogenic Th17 cells is their dependence on IL-23 signalling. While pathogenic Th17 cells rely heavily on IL-23 and express high levels of IL-23 receptor (IL-23R), non-pathogenic Th17 cells do not. Markers associated with pathogenic Th17 cells are often pro-inflammatory such as IL-23, IL-6, IFN- γ , GM-CSF, T-bet, whereas those of non-pathogenic are often immunoregulatory such as IL-10, IL-21, CD39, AHR (Schnell et al., 2023; Stockinger & Omenetti, 2017; Wu et al., 2018).

However, our present study focused on IL-17-expressing cells and their co-expression with several inflammatory markers commonly associated with pathogenic Th17 cells. It is possible that if an individual has a higher proportion of pathogenic rather than non-pathogenic Th17 cells prior to infection, Th17 cells may be more closely linked to disease outcomes due to their capacity to drive inflammation, immune activation, and tissue damage, particularly in the gut. While our study subtly supported this concept, showing that in addition to IL-17⁺ cells, those co-expressing inflammatory cytokines, were also associated with disease progression. Future studies should take advantage of recent advancements in technologies such as spectral flow cytometry, which enables the simultaneous analysis of 40 or more markers, to further characterize Th17 cell heterogeneity.

In addition to considering the heterogeneity of Th17 cells, their dynamics with Treg cell populations are important for HIV pathogenesis, which unfortunately the present study could not explore due to methodological challenges. We therefore propose adding to future studies the simultaneous quantification of Th17 and Treg cells and investigating how their balance affect HIV susceptibility and progression. Our study seems to suggest that the observed association between Th17 cells and HIV progression was independent of viral load, and we suggested that increased microbial translocation and immune activation due to tissue barrier disruption as a result of Th17 cell depletion may have mediated this association. We therefore recommend future studies looking at Th17 cells and their association with HIV to include the measurements of markers of gut barrier integrity, microbial translocation and immune activation such as Zonulin, LPS, sCD14, which could be a consequence of Th17 cell dysfunction. Additional inferences made from our findings is that pre-HIV Th17 cells are predictive of HIV disease progression differently between male and female. The reason for this is unclear and require further investigation. Future studies should explore mechanisms underlying the sex differences in the association of Th17 cells with HIV disease progression, especially the impact of hormonal differences on immune cell expression and dynamics in HIV.

Altogether, these suggestions can be incorporated into the longitudinal study proposed above and can potentially provide answers to why certain populations are more susceptible to adverse HIV outcomes due to their Th17 profile.

Furthermore, the association of *CD101* variants and HIV susceptibility warrants further investigation. We showed that these variants may modulate immune cell expression which in turn may have contributed to their association with HIV. However, our findings here were limited to the East African cohort. We propose a study focused on genotyping these variants and confirming their association with HIV within other populations. This can be done by a genome-wide association study (GWAS) in other regions similarly to the study by (Mackelprang et al., 2017). Additionally, expanding the genetic analysis to include other potential variants associated with immune cell function and HIV susceptibility could uncover more insights into why certain immune markers predict HIV outcomes in some populations but not others. For instance, in relation to integrin $\alpha 4\beta 7$, there might be a genetic predisposition influencing its predictive role in HIV. Africa is known for its high genetic diversity compared to the rest of the world. Therefore, it is not far-fetched to speculate that some genetic polymorphisms may exist in the integrin gene, causing it to promote HIV susceptibility or progression in some individuals but not in others. Studies targeted at investigating genetic polymorphisms in genes regulating integrin $\alpha 4\beta 7$ in the context of HIV in different populations are needed.

7.4. Conclusion and Significance

This study provides valuable insight into various factors influencing HIV susceptibility and disease progression, including immunological, epidemiological, and genetic factors. We identified pre-HIV immune profiles correlating with HIV susceptibility and faster disease progression in a cohort of heterosexual individuals in South Africa. Specifically, our study demonstrated that highly susceptible HIV target cells, including integrin $\alpha 4\beta 7$ -expressing cells

and Th17 cells are strong predictors of HIV pathogenesis in certain populations, revealing the complexities involved in using these cells to predict HIV. We demonstrated the immune alteration effects of *CD101* genetic variants on HIV target cells and the possible mediating role of the modulated cells in HIV susceptibility. Additionally, our study reinforces the notion that HIV is a multifaceted disease, revealing demographic factors such as sex and age as significant players in the immune response to HIV.

The findings in this study are significant as they have broad implications for both HIV research and public health. The identification of predictive immune markers of HIV could be useful in designing personalized care in preventing or managing HIV and provides potential avenues for developing more effective therapeutic strategies. These biomarkers can help in the early identification of individuals at high risk of disease progression, thereby enabling targeted interventions and more effective management strategies. The contrasting findings observed between cohorts highlights the importance of population-specific approaches in HIV research and necessitates the need for a deeper understanding of the interplay between host factors, viral characteristics, and epidemiological factors in relations to HIV susceptibility and progression.

Overall, this study drives the point that HIV research is not a one-size-fits-all disease, as multiple factors are in play in its pathogenesis. Therefore, addressing the complexity of the disease with research will require a multifaceted approach that considers a combination of factors like immune cell dynamics, genetics, viral and demographic variables to HIV prevention and treatment, tailored to the specific needs of diverse populations.

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Appendices

Table A1. Summary of Major Clinical Trials Evaluating the Efficacy of Candidate Vaccines for HIV

HIV Vaccine Trial	Start and Completion Year	Trial design	Location	Clade Targeted	Population	Vaccine intervention	Immune Response Induced	Efficacy	Reference
VAX004	1998 – 2002	Randomized, Phase III, double-blind, placebo-controlled	North America and Europe	HIV-1 clade B	5,403 high-risk individuals, including MSM and heterosexual women	AIDSVAX B/B: A bivalent recombinant gp120 vaccine with alum adjuvant	Non-neutralizing antibodies against HIV-1 gp120	No protective efficacy against HIV acquisition or delay disease progression	(rgp120 HIV Vaccine Study Group, 2005)
VAX003	1999 – 2003	Randomized, Phase III, double-blind, placebo-controlled	Bangkok, Thailand	HIV-1 clade B and E (CRF01_AE)	2,546 high-risk individuals, primarily injection drug users (IDUs)	AIDSVAX B/E: A bivalent recombinant gp120 vaccine with alum adjuvant	Non-neutralizing antibodies against HIV-1 gp120	No protective efficacy against HIV acquisition or delay disease progression	(Pitisuttithum et al., 2006)
HVTN 502 / STEP Trial	2004 – 2007	Randomized, Phase IIb, double-blind, placebo-controlled	Unites States, the Caribbean, South America, and Australia	HIV-1 clade B	3,000 high-risk individuals, including MSM and heterosexual women	MRK Ad5 HIV-1 <i>gag/pol/nef</i> vaccine	T cell responses particularly CD8+ T cell responses	No protective efficacy against HIV acquisition, or delay disease progression.	(Buchbinder et al., 2008)
HVTN 503 / Phambili Trial	2007	Randomized, Phase IIb, double-blind, placebo-controlled	South Africa	HIV-1 clade C	801 high-risk individuals, primarily heterosexual men and women	MRK Ad5 HIV-1 <i>gag/pol/nef</i> vaccine	T cell responses	No protective efficacy against HIV acquisition or delay disease progression.	(Gray et al., 2011, 2014)
RV144 / Thai Trial	2003 – 2009	Randomized, Phase III, double-blind, placebo-controlled	Thailand	HIV-1 clade B and E (CRF01_AE)	16,402 men and women primarily at risk of heterosexual transmission	ALVAC-HIV (vCP1521) prime with AIDSVAX B/E boost using a canarypox vector (ALVAC-HIV) encoding HIV-1 <i>env</i> , <i>gag</i> , and <i>pol</i> genes.	Binding antibodies to HIV-1 <i>env</i> and limited T cell response	Modest protective efficacy with a 31.2% reduction in HIV infection. No efficacy against delay disease progression	(Rerks-Ngarm et al., 2009)
HVTN 505	2009 – 2013	Randomized, Phase IIb, double-blind, placebo-controlled	United States	Clade B	2,504 high-risk MSM and transgender women who has sex with men.	DNA/rAd5 vaccine encoding HIV clade B <i>gag</i> , <i>pol</i> , and <i>nef</i> and <i>env</i> from HIV clades A, B, and C	Binding antibody response and T cell responses	No protective efficacy against HIV acquisition or delay disease progression	(Hammer et al., 2013)
HVTN 702 / Uhambo Trial	2016 – 2020	Randomized, Phase IIb – 3, double-blind, placebo-controlled	South Africa	HIV-1 clade C	5404 men and women	Adapted the RV144 regimen to include ALVAC-HIV and bivalent subtype C gp120 with MF59 adjuvant	Binding antibodies to HIV-1 <i>env</i> and T cell responses	No protective efficacy against HIV acquisition. Was stopped due to lack of efficacy	(Gray et al., 2021)

Table A2: Antiretroviral Drug Classes and Target in the HIV Replication Cycle

Drug Class	Example Drugs	Targeted Step in HIV Lifecycle	Year of First FDA Approval	References
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	Zidovudine (AZT) Lamivudine (3TC) Tenofovir disoproxil fumarate (TDF)	Reverse transcription	AZT: 1987 3TC: 1995 TDF: 2001	(Lange & Ananworanich, 2014)
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Nevirapine (NVP) Efavirenz (EFV) Etravirine (ETV)	Reverse transcription	NVP: 1996 EFV: 1998 ETV: 2008	(Lange & Ananworanich, 2014)
Protease Inhibitors (PIs)	Saquinavir (SQV) Ritonavir (RTV) Lopinavir (LVP)	Viral protein processing	SQV: 1995 RTV: 1996 LVP: 2000	(Lange & Ananworanich, 2014)
Integrase Inhibitors (INSTIs)	Raltegravir (RAL) Elvitegravir (EVT) Dolutegravir (DTG)	Integration of viral DNA into host genome	RAL: 2007 EVT: 2012 DTG: 2013	(Lange & Ananworanich, 2014)
Entry Inhibitors (CCR5 Antagonists)	Maraviroc (MVC)	Binding to CCR5 co-receptor (entry)	MVC: 2007	(Lange & Ananworanich, 2014)
Fusion Inhibitors	Enfuvirtide (T20)	Fusion of viral and host membranes	T20: 2003	(Lange & Ananworanich, 2014)
Capsid Inhibitors	Lenacapavir	Capsid assembly and disassembly	Lenacapavir: 2023	(Bekker et al., 2024)

Table A3. Summary of Major Clinical Trials Evaluating the Efficacy of PrEP (TDF with or without FTC)

PrEP Trial	Enrollment Period	Location	Population	Sample Size	Drug Intervention	Dosage	Trial Design	Efficacy results (95% CI)	Adherence level	Reference
iPREx	2007 – 2009	Multiple countries (USA, Peru, Brazil, Ecuador, South Africa, Thailand)	MSM and Transgender women	2,499	TDF/FTC	Daily oral	Randomized, double-blind, placebo-controlled	44% (15 – 63) reduction in HIV incidence, up to 92% with high adherence	51%; based on drug detection in blood samples	(Grant et al., 2010)
TDF2	2007 – 2009	Botswana	Heterosexual men and women	1,219	TDF/FTC	Daily oral	Randomized, double-blind, placebo-controlled	62% (21 – 83) reduction in HIV incidence	>80%; self-report	(Thigpen et al., 2012)
Partners PrEP	2008 – 2010	Kenya and Uganda	Heterosexual HIV-serodiscordant couples	4,758	TDF and TDF/FTC	Daily oral	Randomized, double-blind, placebo-controlled	67% (44 – 81) reduction for TDF, 75% (55 – 87) for TDF/FTC	Over 80%; based on self-report and drug detection in blood samples	(Baeten et al., 2012; Mujugira et al., 2011)
FEM-PrEP	2009 – 2011	Kenya, South Africa, and Tanzania	Heterosexual women	2,120	TDF/FTC	Daily oral	Randomized, double-blind, placebo-controlled	No significant reduction in HIV incidence	26 – 37%; based on drug detection in blood samples	(Van Damme et al., 2012)
CAPRISA 004	2007 – 2010	South Africa	Heterosexual women	889	1% Tenofovir gel	12 hours before and 12 hours after sex (“BAT24” regimen)	Randomized, double-blind, placebo-controlled	39% (6 – 60) reduction in HIV incidence; up to 54% with high adherence	~60%; based on self-reports and returned applicators	(Karim et al., 2010)
VOICE	2009 – 2011	Uganda, South Africa, and Zimbabwe	Heterosexual women	5,029	1% Tenofovir gel, TDF oral, TDF/FTC oral	Daily oral and topical gel	Randomized, double-blind, placebo-controlled	No significant reduction in HIV incidence	~30%; based on drug detection in plasma samples	(Marrazzo et al., 2015)
FACTS 001	2011 – 2014	South Africa	Heterosexual women	2,059	1% Tenofovir gel	12 hours before and 12 hours after sex (“BAT-24” regimen)	Randomized, double-blind, placebo-controlled	No significant reduction in HIV incidence	~20%; based on self-reports and returned applicators	(Delany-Moretlwe et al., 2018)
PROUD	2012 – 2014	United Kingdom	MSM and transgender women	544	TDF/FTC	Daily oral	Open-label, randomized	86% (64 – 96) reduction in HIV incidence among participants on immediate PrEP compared to deferred PrEP	88%; based on drug detection in blood samples	(McCormack et al., 2016)
ANRS-IPERGAY	2012 – 2014	France and Canada	MSM	400	TDF/FTC	On-demand, taken before and sex	Randomized, double-blind, placebo-controlled	86% (40 – 98) reduction in HIV incidence with on-demand regimen	43%; self-report; 86% AND 82% for TDF and FTC, respectively when measured by plasma drug level in a subset of participants	(Molina et al., 2015)
Purpose 1	2022 – 2024	South Africa, Uganda	Cisgender women and adolescent girls	5338	Subcutaneous Lenacapavir	Once every 26 weeks	Randomized, double-blind, placebo-controlled	100% reduction in HIV incidence	>90% adherence to study regimen	(Bekker et al., 2024)

Table A4. T Helper Cell Subsets and Differentiation

CD4 ⁺ T cell Subset	Triggering Pathogen	Cytokine Promoting Differentiation	Master Transcriptional Regulator(s)	Inhibitory Transcriptional Regulator(s)	Cytokines Secreted	Homing Markers Expressed	Plasticity	Cytokines Responsible for Plasticity	References
Th1	Intracellular bacteria and viruses	IL-12, IFN- γ	T-bet	GATA3	IFN- γ , TNF- α	CXCR3, CCR5	Th1 to Th2, Th17	IL-4, IL-6, TGF-?	(Geginat et al., 2014; Künzli & Masopust, 2023; Luckheeram et al., 2012; Saravia et al., 2019)
Th2	Extracellular parasite e.g., Helminths	IL-4	GATA3	T-bet, Bcl-6	IL-4, IL-5, IL-13	CCR3, CCR4, CCR8	Th2 to Th1, Th9	IFN- γ , IL-1, TGF-?	(Geginat et al., 2014; Künzli & Masopust, 2023; Luckheeram et al., 2012; Saravia et al., 2019)
Th17	Extracellular bacteria and Fungi	IL-6, IL-23, IL-17, TGF-?	ROR γ t	T-bet, GATA-3, FOXP3	IL-17, IL-21, IL-22	CCR6, CCR4	Th17 to Th1, Th2, Treg	IL-12, IFN- γ , IL-4, IL-10	(Geginat et al., 2014; Künzli & Masopust, 2023; Luckheeram et al., 2012; Saravia et al., 2019)
Th22	Extracellular bacteria, skin pathogens	IL-6, TNF- α , IL-23	AHR	Not well-defined	IL-22, IL-13, IL-26	CCR4, CCR6, CCR10	Th22 to Th17	IL-6, TGF-?	(Geginat et al., 2014; Gong et al., 2021; Luckheeram et al., 2012; Saravia et al., 2019)
Treg	Self-antigens, Commensal microbes, Many pathogens	TGF-?, IL-10	FOXP3	ROR γ t	IL-10, TGF-?	CCR4, CCR8	Treg to Th17	IL-6, IL-23, IL-21	(Geginat et al., 2014; Luckheeram et al., 2012; Saravia et al., 2019)
Th9	Allergens, Helminths	IL-4, TGF-?	PU.1, IRF4	Not well-defined	IL-9	CCR3, CCR6	Th9 to Th2, Th17	IL-1, IL-6, IL-21	(Geginat et al., 2014; Humblin et al., 2017; Luckheeram et al., 2012)
Tfh	Many pathogens	IL-6, IL-21	Bcl-6	T-bet	IL-21, IL-4	CXCR5	Tfh to Th1, Th2, Th17	IL-12, IL-4, IL-6	(Geginat et al., 2014; Luckheeram et al., 2012; Saravia et al., 2019)

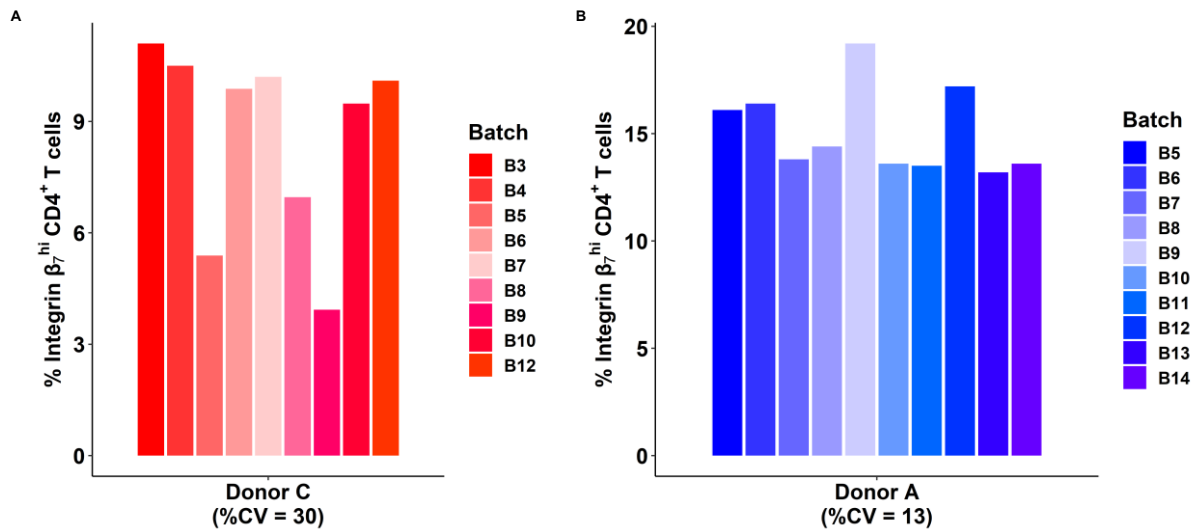


Figure A1. Consistency of integrin β_7^{hi} expression in CD4⁺ T cells across multiple experimental batches. (A and B) Percentage of integrin β_7^{hi} expression on CD4⁺ T cells isolated from frozen PBMCs of healthy volunteer Donor C and Donor A. Bar plots colored in “red” represent experimental batches from HVTN 503, while those in “blue” represent batches from PP/COS. Percent coefficient of variation (%CV) indicates the variability within each batch.

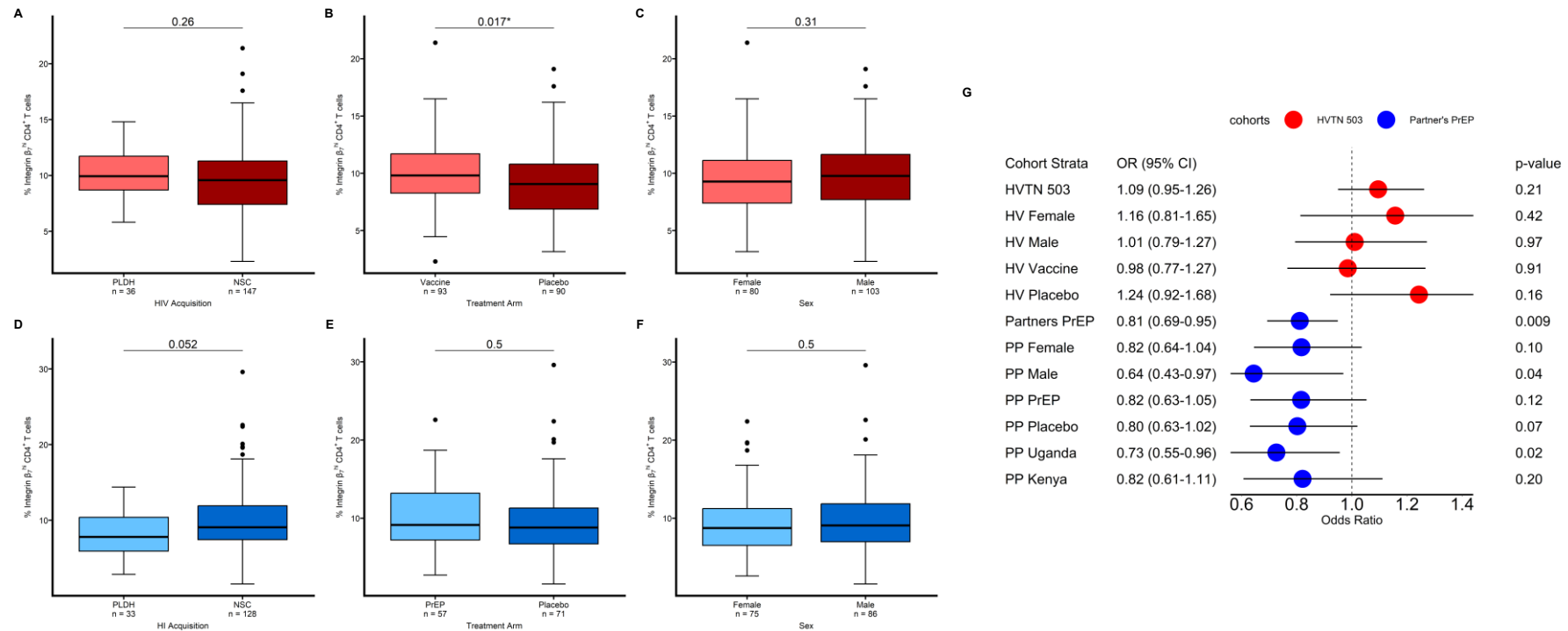


Figure A2. Correlation between pre-HIV frequency of $\beta 7^{\text{hi}}$ CD4⁺ T cells and HIV acquisition analyzed in the secondary flow cytometry panel. (A – C) Comparison of pre-HIV frequency of $\beta 7^{\text{hi}}$ CD4⁺ T cells between HIV acquisition status, treatment arms and sex in HVTN 503. **(D – F)** Comparison of pre-HIV frequency of $\beta 7^{\text{hi}}$ CD4⁺ T cells between HIV acquisition status, treatment arms and sex in PP/COS. **(G)** Stratified univariable analysis estimating the association between pre-HIV frequency of $\beta 7^{\text{hi}}$ CD4⁺ T cells and HIV acquisition, reported as odds ratio. Plots colored in "red" denote analyses from HVTN 503, while those colored "blue" denote analyses from PP/COS. Group Comparisons were analyzed by Mann-Whitney test, and odds ratios were computed using conditional logistic regression, stratified by sex, treatment arm, and HIV prevention intervention, where applicable. Abbreviations: OR = Odds Ratio, Adj = Adjusted, HV = HVTN 503, PP = PP/COS. ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

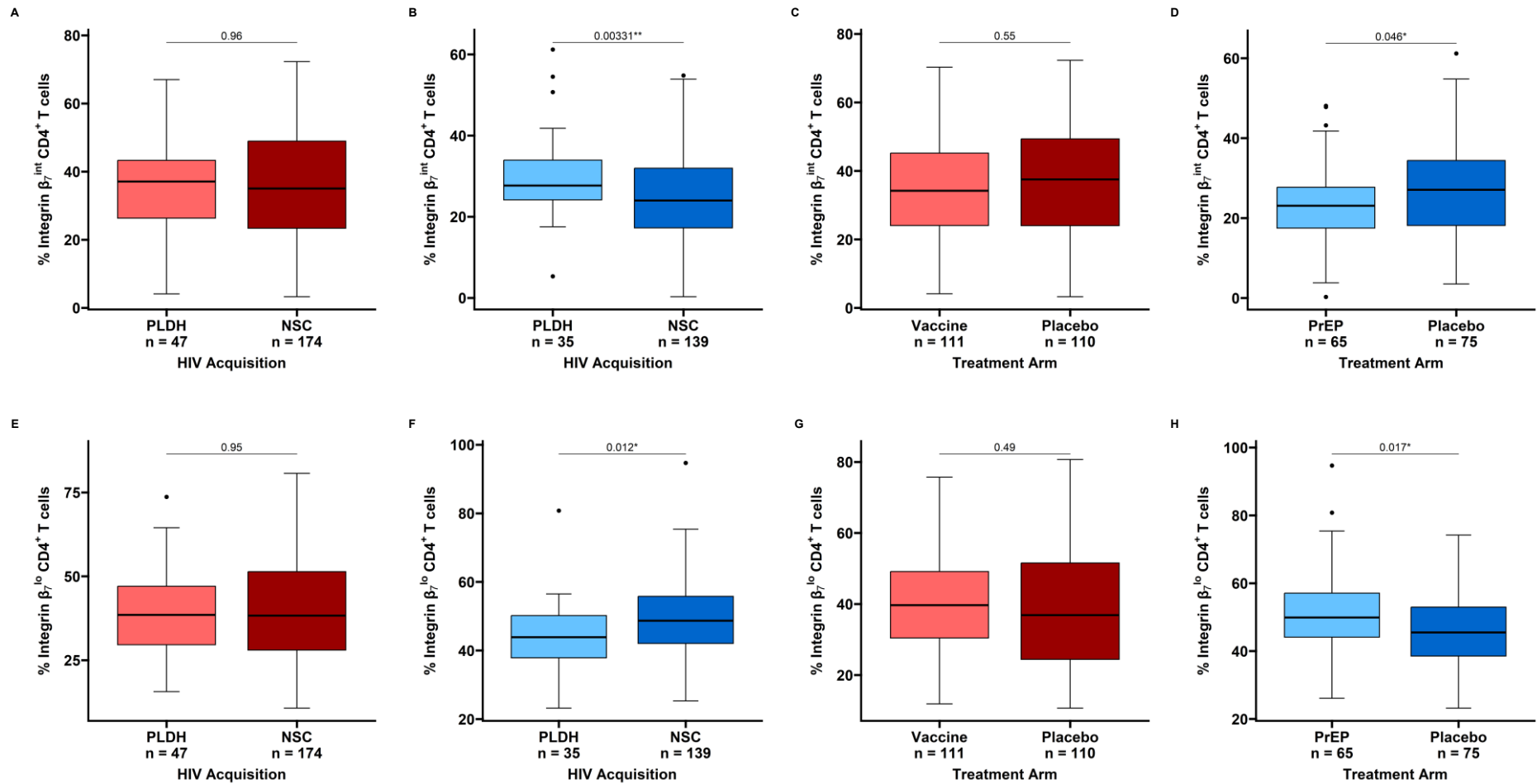


Figure A3. Expression pattern of pre-HIV frequency of integrins β_7^{int} and β_7^{lo} CD4⁺ T cells. Frequency of pre-HIV β_7^{int} CD4⁺ T cells compared by (A and B) HIV acquisition status for HVTN 503, PP/COS, (C and D) treatment arms for HVTN503, PP/COS. Frequency of pre-HIV β_7^{lo} CD4⁺ T cells compared by (E and F) HIV acquisition status for HVTN 503, PP/COS, (G and H) treatment arms for HVTN 503, PP/COS. Boxplots colored “red” denote analyses from HVTN 503 while boxplots colored “blue” denote analyses from PP/COS. Comparison between two groups were analyzed using the Mann-Whitney test. ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

Table A5. Sensitivity analysis excluding experimental batches acquired on a different flow cytometer

Variable	Univariable Models		Multivariable Models	
	OR (95% CI)	<i>P</i> value	aOR (95% CI)	<i>P</i> value
PP/COS, $\beta 7^{\text{hi}}$	0·89 (0·81 – 0·99)	0·038	0·89 (0·79 – 1·01)	0·061

The analysis here excluded samples acquired on BD LSRII flow cytometer. The effect estimates remained consistent as the overall analysis despite excluding these samples. The multivariable model was adjusted for age, sex, study sites. PP/COS ($n = 155$). Abbreviations: OR = Odds Ratio, aOR = Adjusted Odds Ratio. Odds Ratios were estimated using conditional logistic regression models.

Table A6. Sensitivity analysis excluding experimental batches with aberrant $\beta 7^{\text{hi}}$ CD4⁺ T cells measurements in QC samples

Variable	Univariable Models		Multivariable Models	
	OR (95% CI)	<i>P</i> value	aOR (95% CI)	<i>P</i> value
HVTN 503, $\beta 7^{\text{hi}}$	1.04 (0.93 – 1.16)	0.511	1.05 (0.92 – 1.19)	0.486
PP/COS, $\beta 7^{\text{hi}}$	0.91 (0.83 – 1.01)	0.075	0.92 (0.83 – 1.03)	0.138

The analysis excluded batches of samples exhibiting aberrant measurements of $\beta 7^{\text{hi}}$ CD4⁺ T cells. Despite the removal of these samples, the effect estimates remained consistent with those obtained from the overall analysis. The multivariable model for HVTN 503 was adjusted for age, sex, study sites, Adenovirus type 5 titer and HSV2 status at baseline while PP/COS was adjusted for age, sex, study sites and HSV2 status at baseline. HVTN 503 ($n = 188$), PP/COS ($n = 150$). Abbreviations: QC = Quality Control, OR = Odds Ratio, aOR = Adjusted Odds Ratio. Odds Ratios were estimated using conditional logistic regression models.

Table A7. Comparison Between the Effect of Pre-HIV Integrin $\beta 7^{\text{hi}}$ CD4⁺ T Cells and HIV Acquisition Across Different Studies

Cohort	Population	Study Site	HIV Intervention	Univariable Models		Multivariable Models		References
				OR (95% CI)	P value	aOR (95% CI)	P value	
HVTN 503	Heterosexual men and women	South Africa	MRKAd5 HIV-1 gag/pol/nef sub-type B vaccine	1.06 (0.96 – 1.18)	0.254	1.06 (0.93 – 1.19)	0.388	
PP/COS	Heterosexual men and women	Kenya, Uganda	Oral Tenofovir (TDF) or combination Tenofovir–Emtricitabine (TDF–FTC)	0.92 (0.83 – 1.01)	0.082	0.93 (0.84 – 1.03)	0.182	
CAPRISA 004	Heterosexual women	South Africa	1% Tenofovir gel	1.17 (1.05 – 1.32)	0.007	1.16 (1.03 – 1.32)	0.016	(Sivro et al., 2018)
HVTN 505	MSM, Transgender women	USA	DNA prime–recombinant adenovirus type 5 boost (DNA/rAd5 HIV-1 env/gag/pol) vaccine	1.00 (0.89 – 1.11)	0.951	1.10 (0.94 – 1.29)	0.246	(Martin et al., 2020)
ALIVE	PWID	USA	NA	0.85 (0.75 – 0.96)	0.008	0.80 (0.68 – 0.93)	0.004	(Martin et al., 2020)

Abbreviations: OR = Odds Ratio, aOR = Adjusted Odds Ratio, NA = Not Applicable, HVTN = HIV Vaccine Trials Network, PrEP = Pre-Exposure Prophylaxis, CAPRISA = Centre for the AIDS Programme of Research in South Africa, ALIVE = The AIDS Linked to the Intravenous Experience Study, MSM = Men who have sex with men, PWID = People who inject drugs, USA = United States of America, NA = Not Applicable.

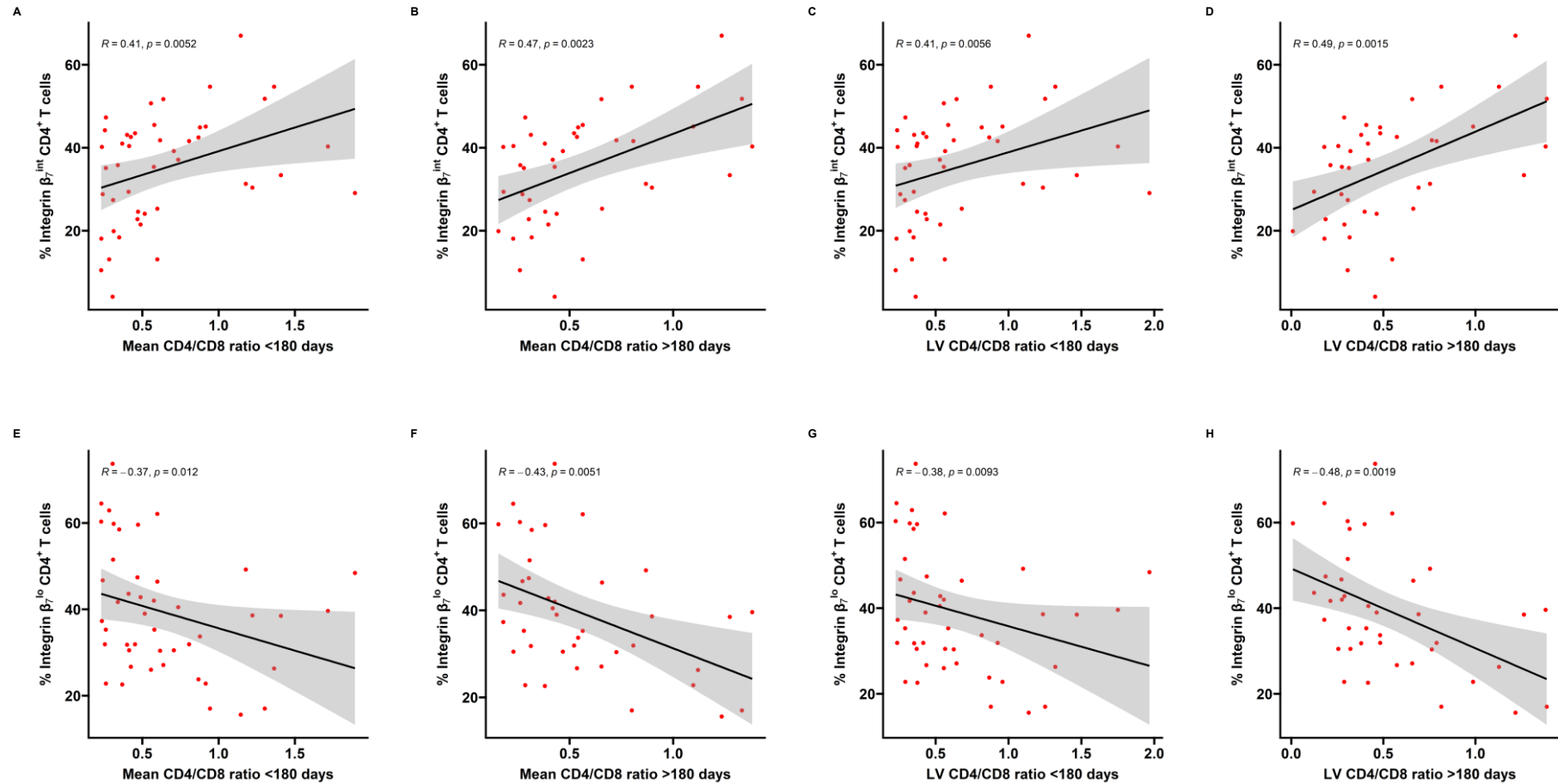
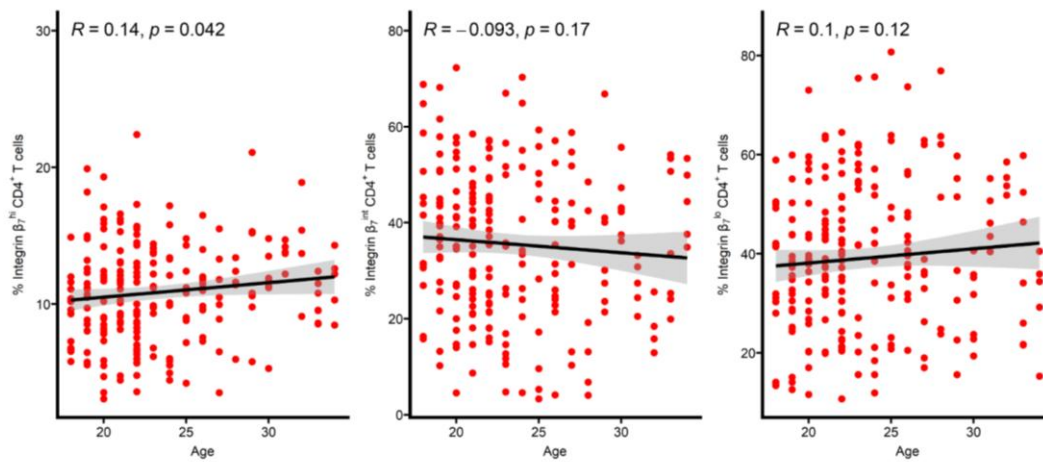


Figure A4. Correlation between pre-HIV frequency of integrins β_7^{int} and β_7^{lo} CD4⁺ T cells with CD4/CD8 ratio. (A and B) Correlation between pre-HIV β_7^{int} CD4⁺ T cells and mean CD4/CD8 ratio at < 180 days ($n = 45$) and > 180 days ($n = 40$). **(C and D)** at last available sampling at < 180 days ($n = 45$) and > 180 days ($n = 40$). **(E and F)** Correlation between pre-HIV β_7^{lo} CD4⁺ T cells and mean CD4/CD8 ratio at < 180 days ($n = 45$) and > 180 days ($n = 40$). **(G and H)** at last available sampling at < 180 days ($n = 45$) and > 180 days ($n = 40$). Scatter plots colored “red” represent analyses from HVTN 503. Correlations were analyzed using Spearman’s rank correlation test. Abbreviations: LV = Sampling at last available visit.

A



B

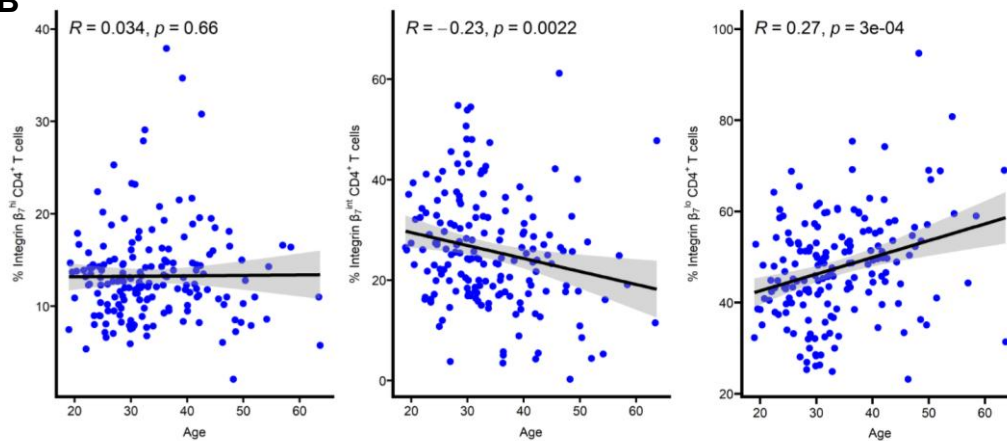


Figure A5. Correlation analysis of integrin β_7 CD4⁺ T cell subsets with age. (A) HVTN 503 (B) PP/COS. Plots colored in “red” represent analyses from HVTN 503, while those in “blue” represent analyses from PP/COS. Correlations were assessed using Spearman’s rank correlation test.

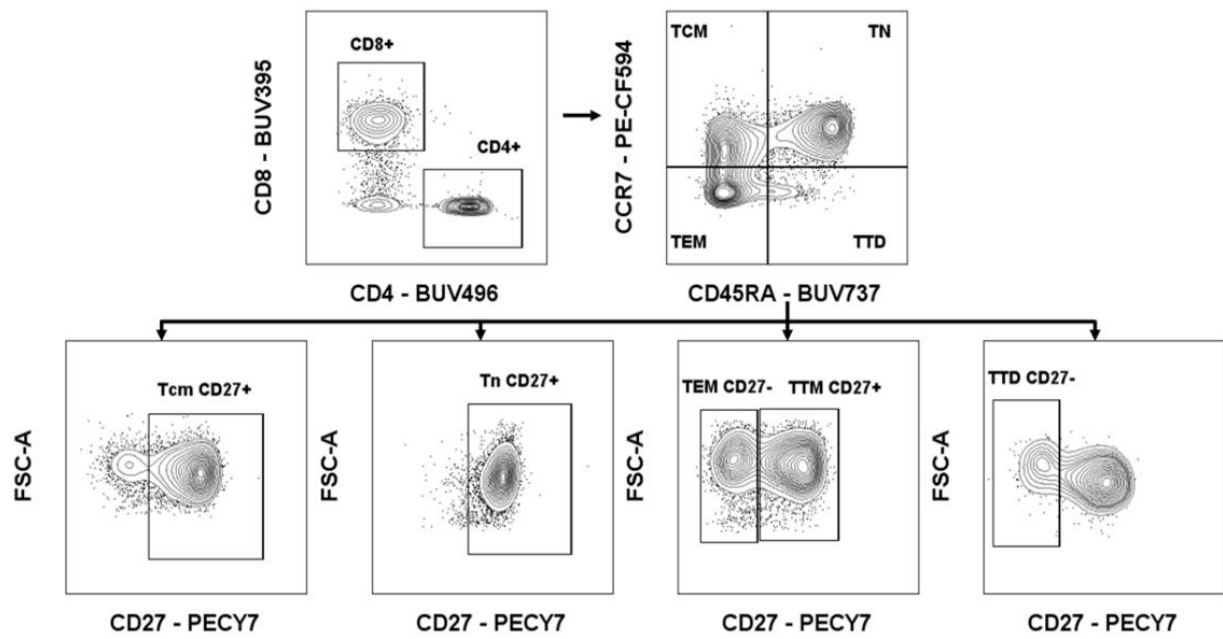


Figure A6. Representative flow cytometry plot showing the gating strategy for memory CD4⁺ T cell subsets.

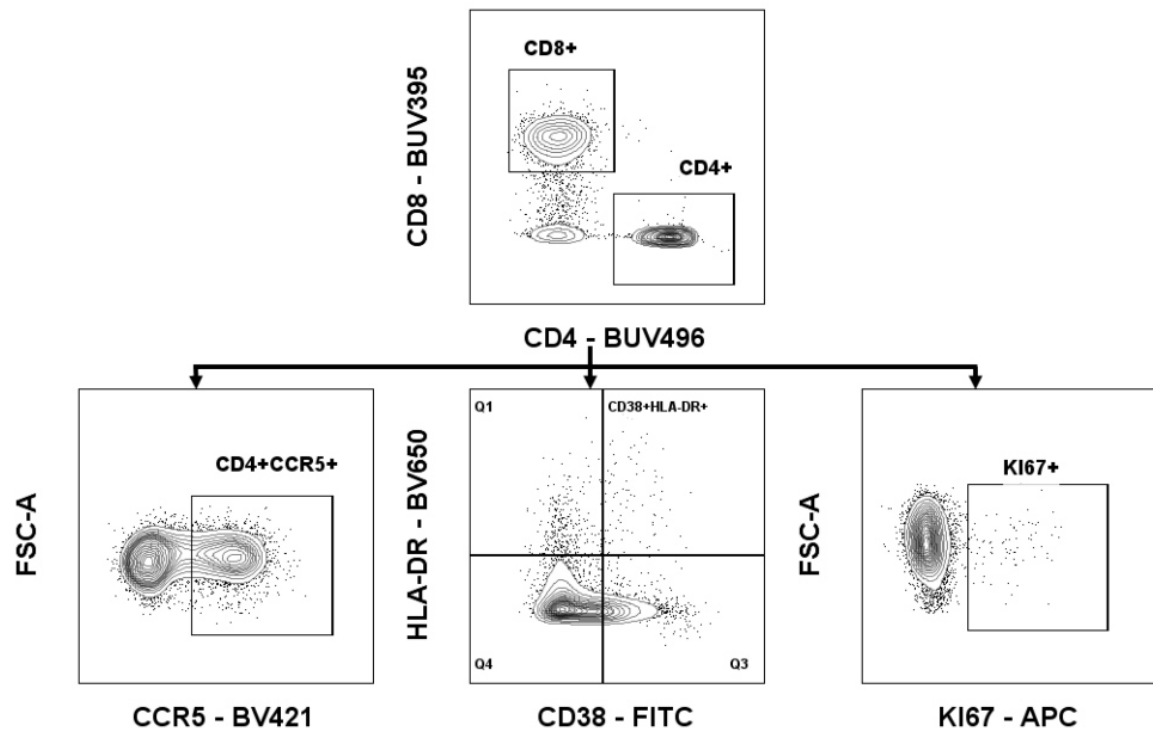


Figure A7. Representative flow cytometry plots showing the gating strategy for integrin $\beta 7$ CD4⁺ T cells expressing CCR5, activated, and proliferating markers.

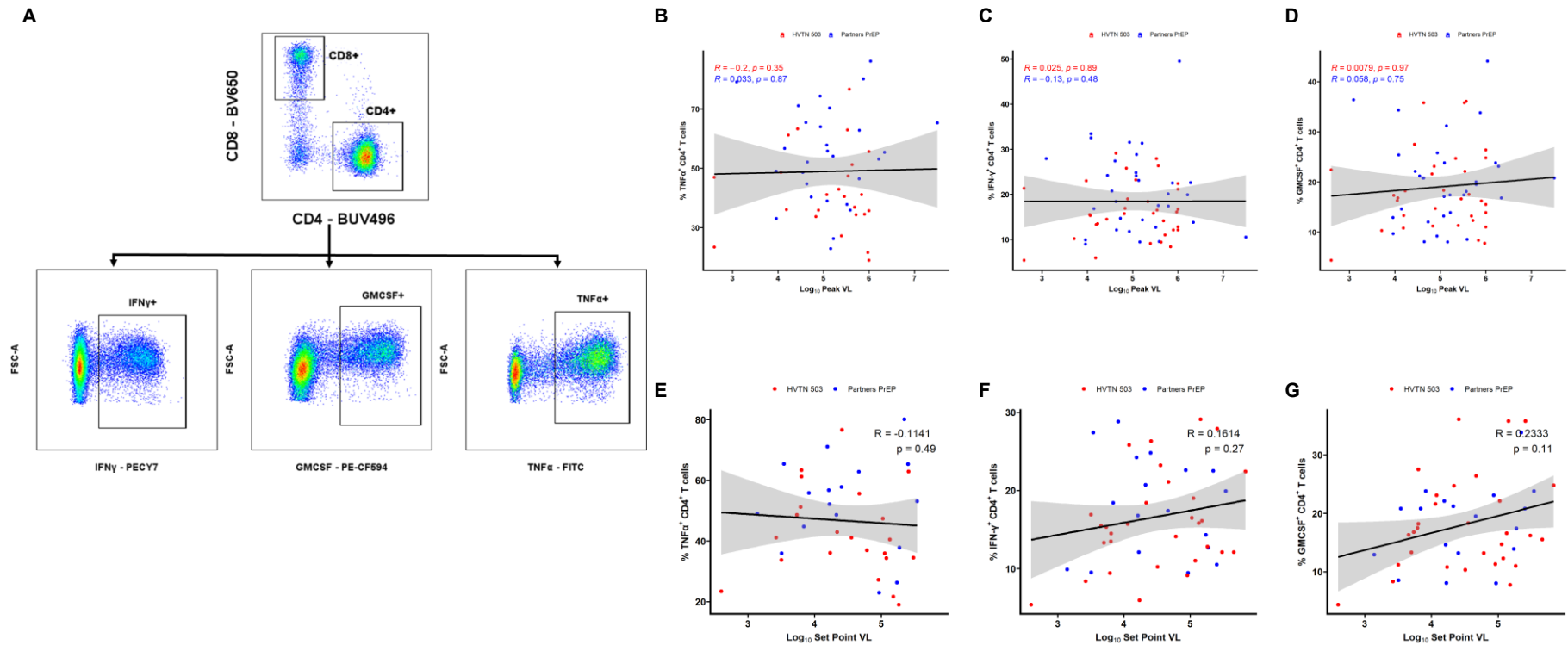


Figure A8. Correlation analysis between viral loads and pre-HIV CD4⁺ T cells cytokines independent of IL-17 expression. (A) Representative flow cytometry plots showing the gating strategy for CD4⁺ T cells cytokines independent of IL-17 expression. (B – D) Correlations with peak viral load. (E – G) Correlations with set-point viral load. Peak viral load is defined as the highest viral load in the initial 180 days post-infection while set-point viral load is defined as the average viral load post 180 days of infection. Viral loads measured after initiating ART and taken after 1 year of HIV diagnosis were excluded from the analysis. HVTN 503 analyses are shown in “red” while PP/COS are shown in “blue”. Correlation was analyzed using Spearman’s rank correlation test.

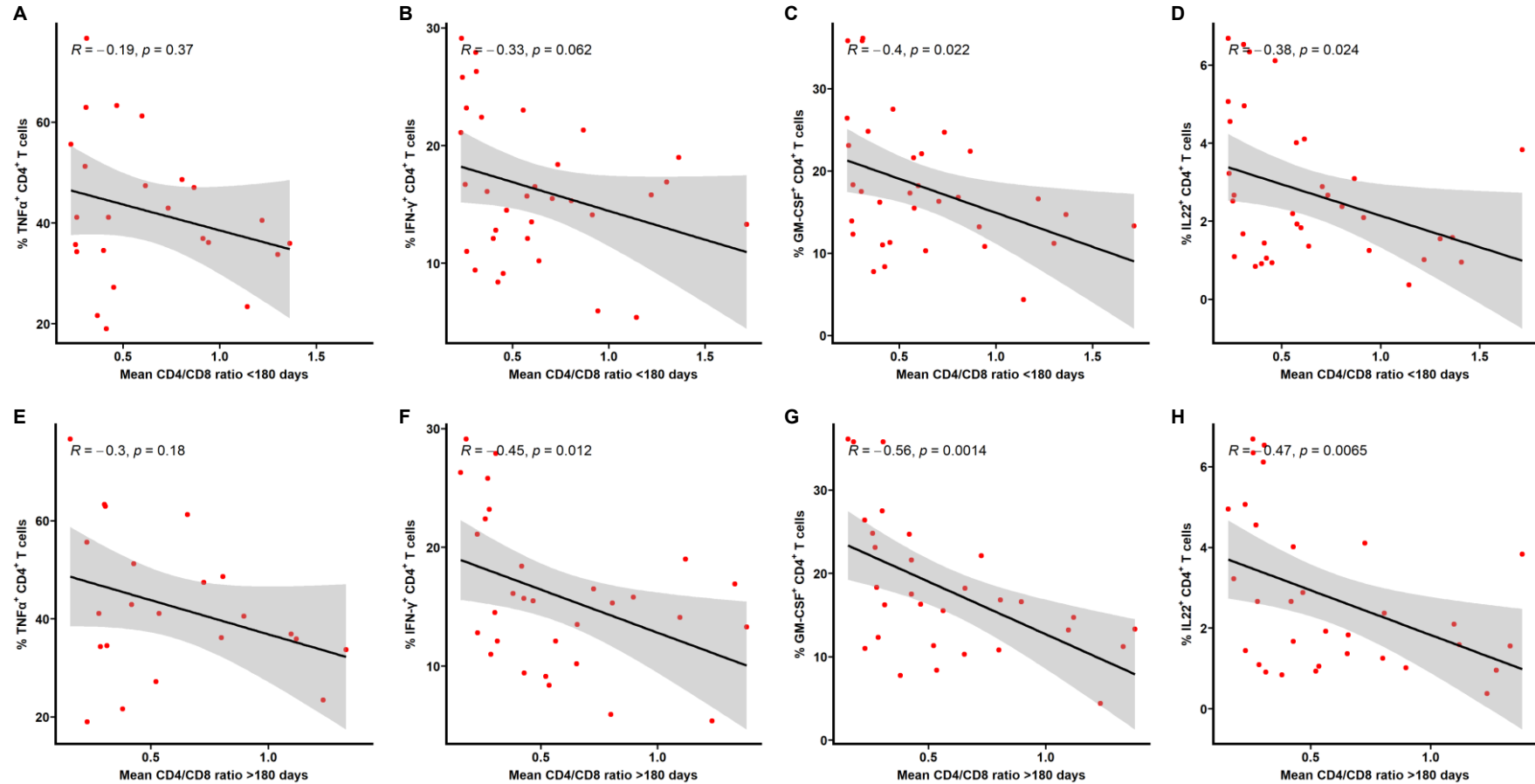


Figure A9. Correlation analysis between mean CD4/CD8 ratio and pre-HIV CD4⁺ T cells cytokines independent of IL-17 expression. (A – D) Within the initial 180 days post-infection. (E – H) After the initial 180 days post-infection subset. CD4/CD8 ratio was calculated from CD4 and CD8 counts taken within the initial 180 days and beyond the initial 180 days post-infection. CD4 and CD8 counts measured after initiating ART and after 1-year post-infection were excluded from the analysis. These analysis are from HVTN 503 (n = 32). Correlation was analyzed using Spearman’s rank correlation test.

Table A8. Cox regression analysis of pre-HIV IL-17⁺ CD4⁺ T cells and CD4 decline below 500 cells/mm³ adjusting for viral load (HVTN 503)

Variables	Units	aHR (95% CI)	<i>P</i> value
IL-17 ⁺ CD4 ⁺ T cells	IL-17 ⁺ < median	Ref	
	IL-17 ⁺ > median	2.54 (1.05 – 6.12)	0.0377*
Peak viral load		1.62 (0.86 – 3.04)	0.1352

Abbreviations: aHR = Adjusted Hazard Ratio, Ref = Reference category. (*p* < 0.05*, *p* < 0.01**, *p* < 0.001***).

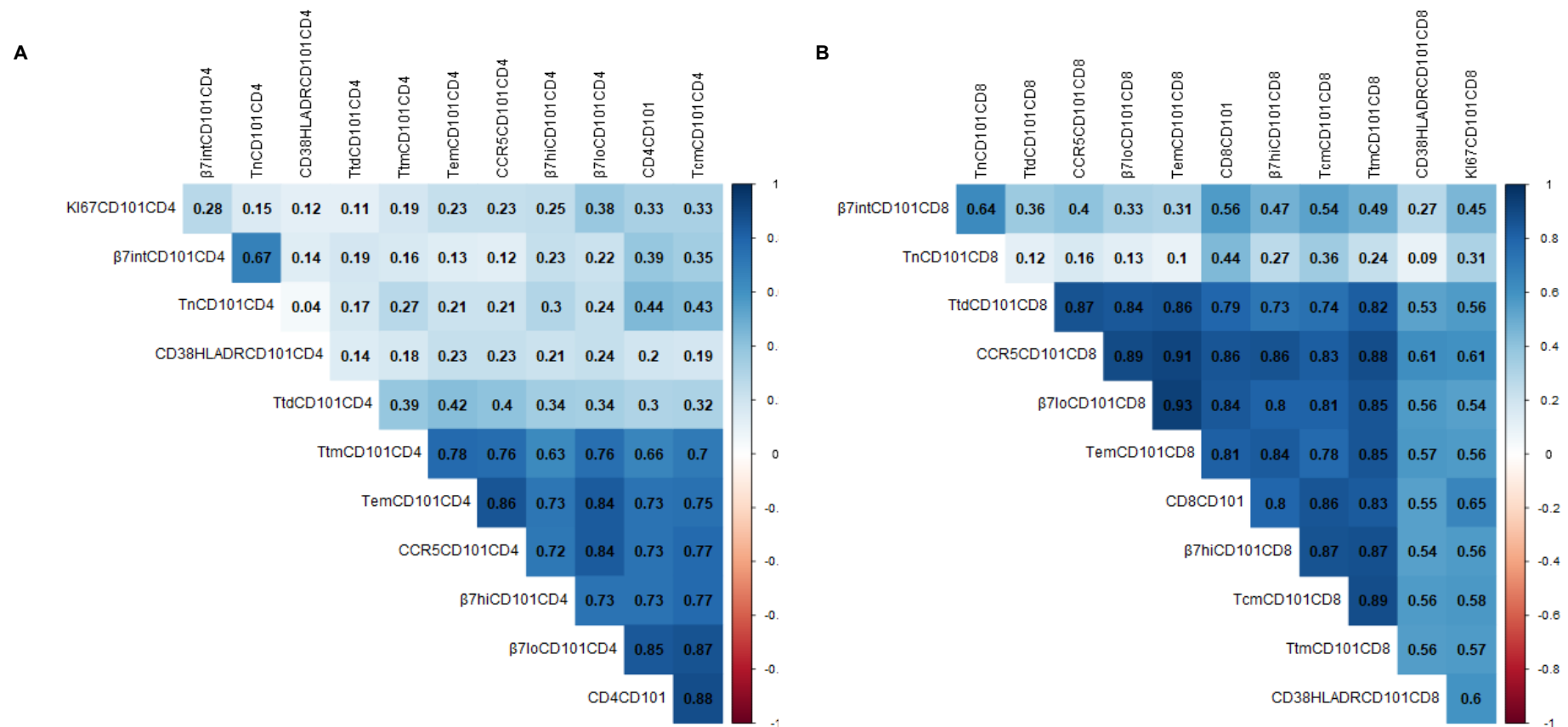


Figure A10. Correlation between C1D101 expressing T cell subsets. (A) Correlation matrix of CD101⁺ CD4⁺ T cells. **(B)** Correlation matrix of CD101⁺ CD8⁺ T cells. Correlation was analyzed using Spearman's rank correlation test. Correlation coefficient $r \geq 0.5$ was considered highly correlated cells and hence, assumed to be in the same mediating pathway

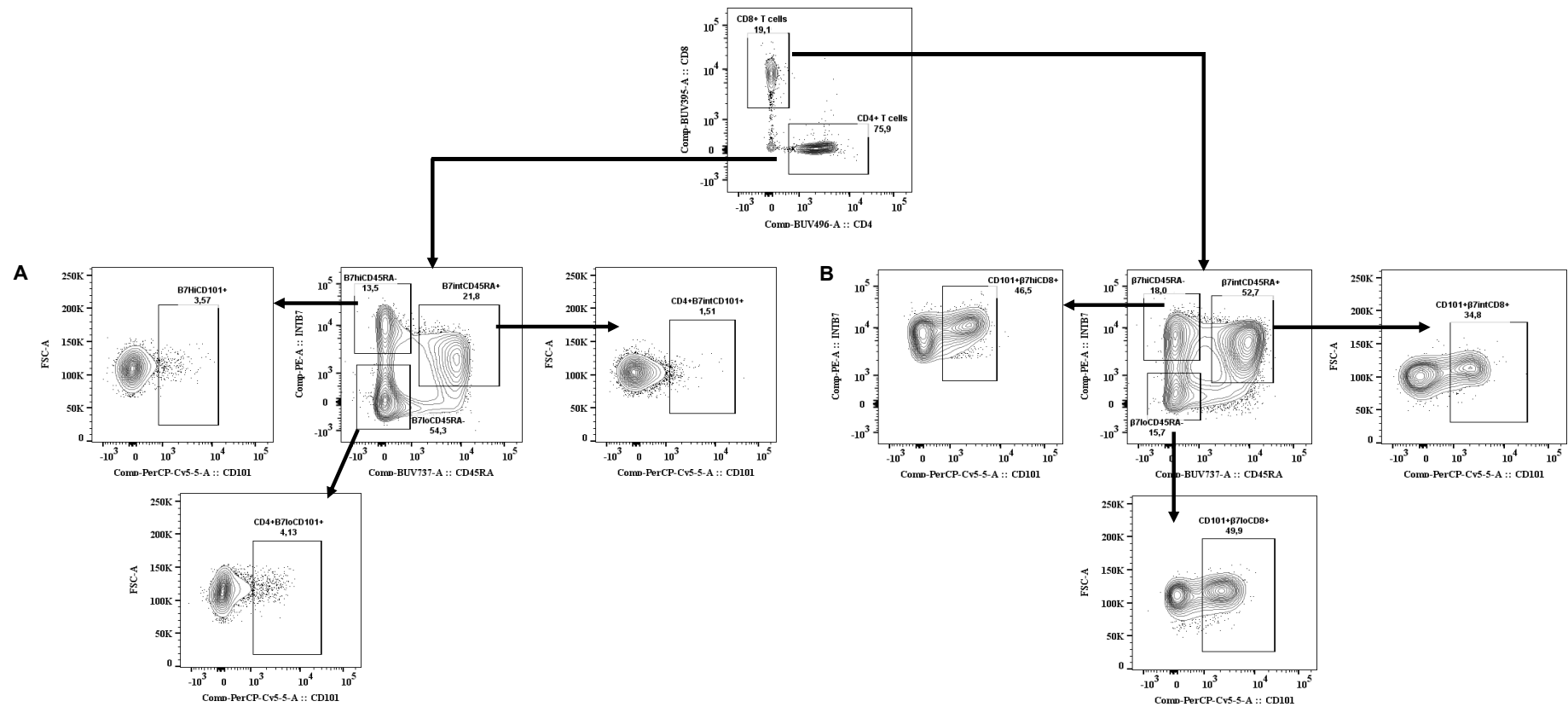


Figure A11. Representative gating strategy to quantify CD101⁺ expression on homing T cells. (A) Integrin $\beta 7$ CD4⁺ T cells (B) Integrin $\beta 7$ CD8⁺ T cells. We used conventional gating to quantify CD101⁺ cells on homing T cells based on the expression of Integrin $\beta 7$ and CD45RA.

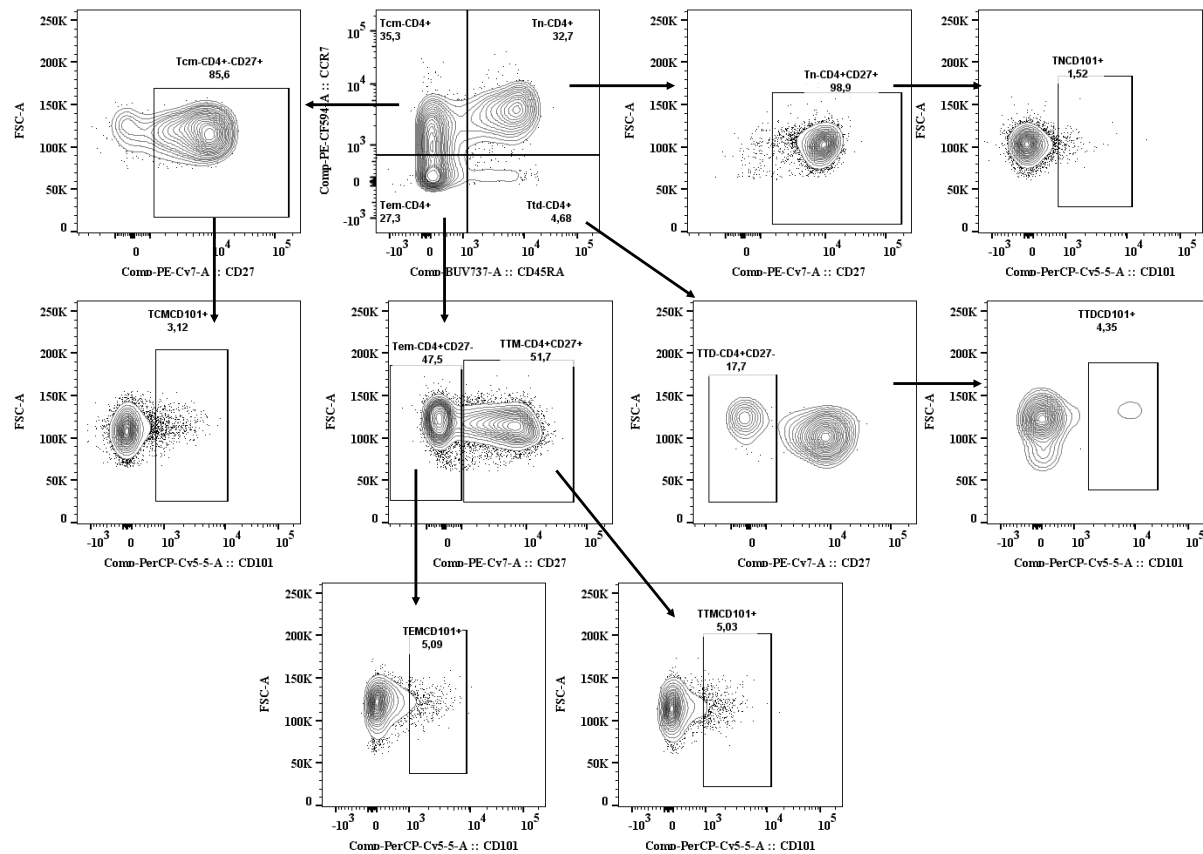


Figure A12. Representative gating strategy to quantify CD101⁺ expression on memory CD4⁺ T cells subsets. We used conventional gating to quantify CD101⁺ cells on memory CD4⁺ T cells based on the expression of CCR7, CD45RA and CD27.

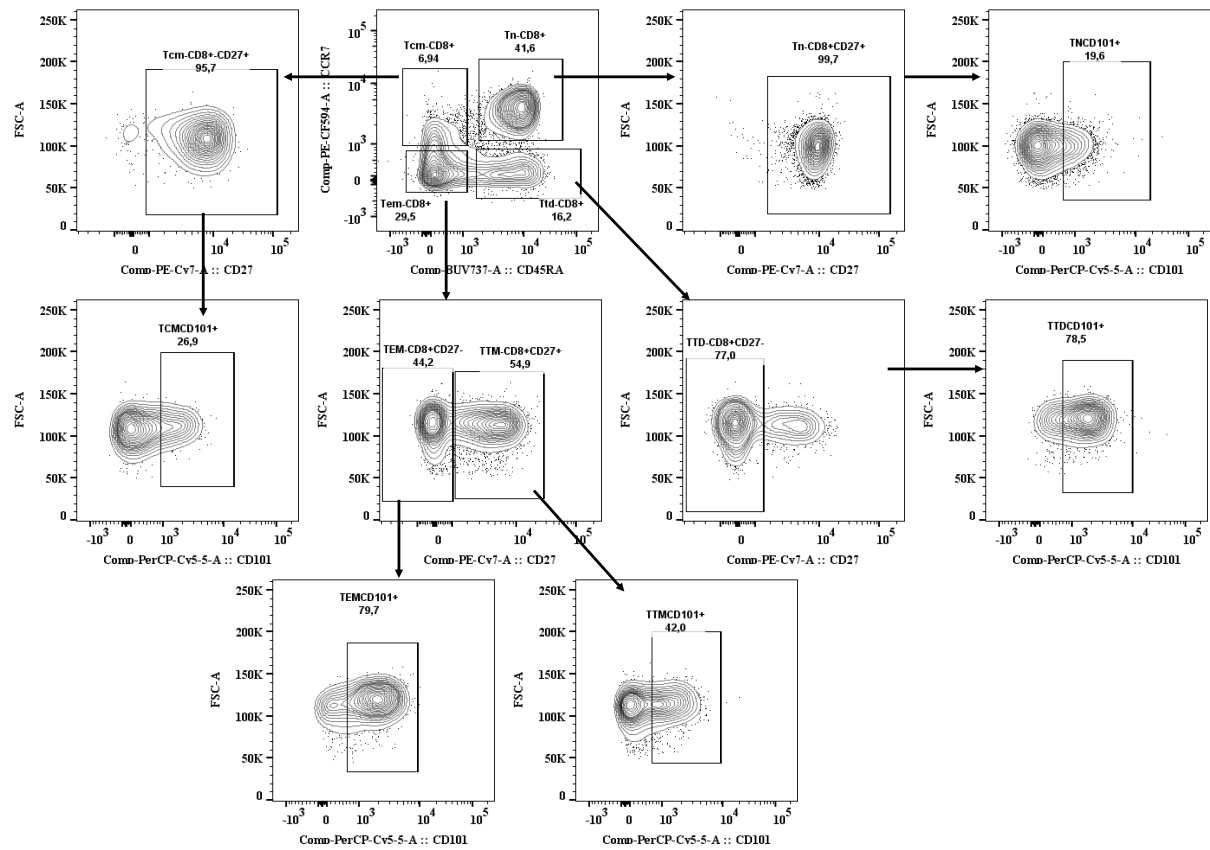


Figure A13. Representative gating strategy to quantify CD101⁺ expression on memory CD8⁺ T cells subsets. We used conventional gating to quantify CD101⁺ cells on memory CD8⁺ T cells based on the expression of CCR7, CD45RA and CD27.

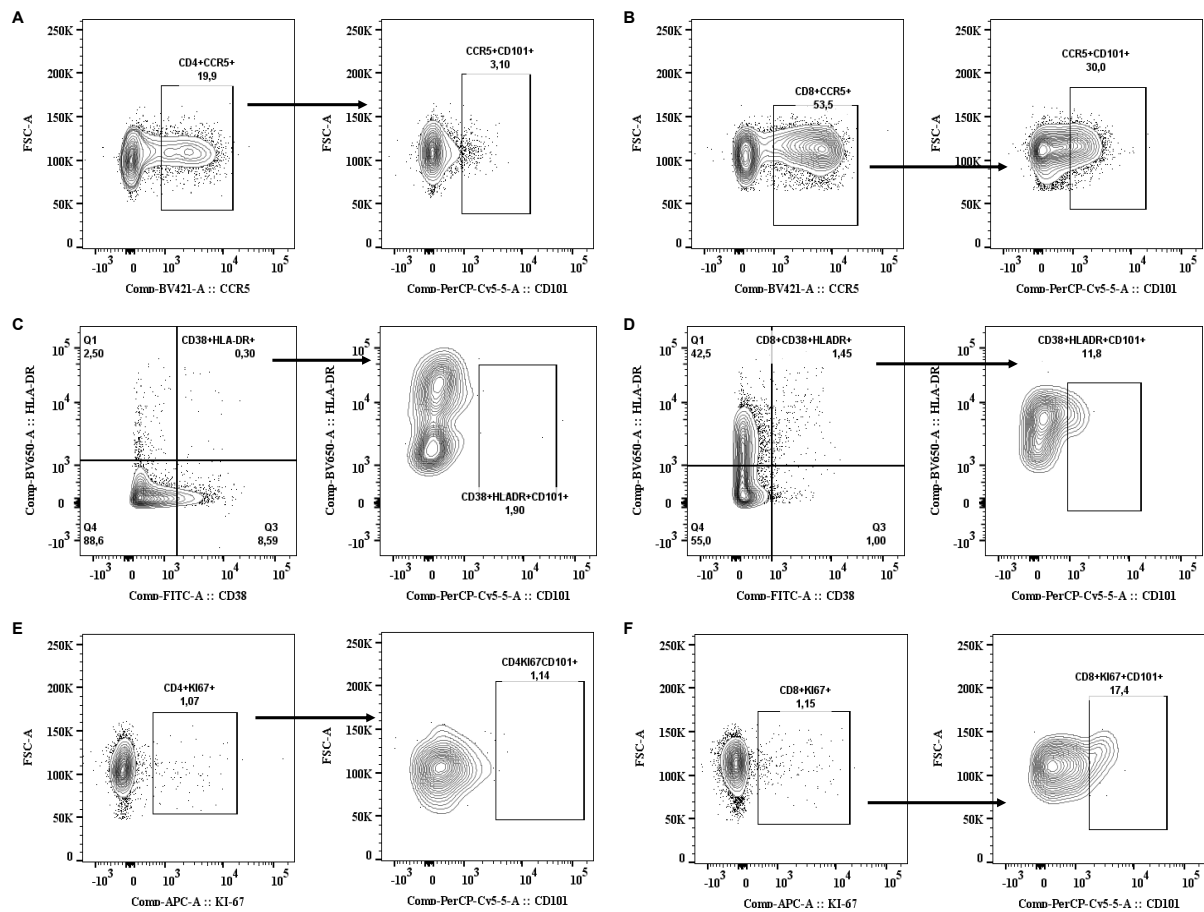


Figure A14. Representative gating strategy to quantify CD101⁺ expression on activated, proliferating and CCR5-expressing T cells. (A) CCR5⁺ CD4⁺ T cells (B) CCR5⁺ CD8⁺ T cells (C) CD38⁺ HLA-DR⁺ CD4⁺ T cells (D) CD38⁺ HLA-DR⁺ CD8⁺ T cells (E) Ki67⁺ CD4⁺ T cells (F) Ki67⁺ CD8⁺ T cells. We used conventional gating to quantify CD101⁺ cells on CCR5⁺, activated and proliferating T cells based on the expression of CCR5, CD38, HLA-DR, and Ki67 markers, respectively.

lavaan 0.6.15 ended normally after 1 iteration

Estimator	ML			
Optimization method	NLMINB			
Number of model parameters	5			
Number of observations	205			
Model Test User Model:				
Test statistic	0			
Degrees of freedom	0			
Parameter Estimates:				
Standard errors	Standard			
Information	Expected			
Information saturated (h1) model	Structured			
Regressions:				
	Estimate	Std.Err	z-value	P(> z)
cd8ccr5cd101 ~				
cd101stts_ (a)	12.322	2.109	5.842	0
hivstatus_num ~				
cd8ccr5101 (b)	0.003	0.001	2.246	0.025
cd101stts_ (c)	0.048	0.038	1.256	0.209
Variances:				
	Estimate	Std.Err	z-value	P(> z)
.cd8ccr5cd101	226.462	22.368	10.124	0
.hivstatus_num	0.065	0.006	10.124	0
Defined Parameters				
	:			
	Estimate	Std.Err	z-value	P(> z)
Indirect (a*b)	0.033	0.016	2.096	0.036
Total c+(a*b)	0.081	0.036	2.246	0.025

Figure A15. R output for simple mediation model with structural equation modelling. CD101⁺ CCR5⁺ CD8⁺ T cell was used as a mediator in a simple mediation model, where *CD101* status (Any Ig-like variant) was the predictor variable and HIV status was the outcome variable.