ASSESSING THE PHENOTYPIC AND MIGRATORY BEHAVIORS OF HIV-INFECTED LATENT CD4$^+$ T CELLS

by

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ABSTRACT

Antiretroviral therapy (ART) has helped Human Immunodeficiency Virus (HIV) become a manageable chronic infection by repressing viremia to undetectable levels in People living with HIV (PLWH). However, this treatment is not curative as treatment interruption causes viremia to rebound to pre-treatment levels due to ***viral latency***, a state of reversible non-productive infection of individual cells established early during acute infection. These latently infected cells serve as ***HIV reservoirs***, residing in various anatomical sites and presenting a significant obstacle to achieving a complete HIV cure. Notably, central and effector memory $CD4^+$ T cells are regarded as the major component of the long-lived HIV reservoirs. Memory $CD4^+$ T cells play an essential role in the adaptive immune response. Guided by chemokine cues, memory $CD4^+$ T cells continually recirculate between lymphoid and non-lymphoid tissues, enabling them to carry out immunosurveillance of the body for pathogens. These long-lived cells are sustained by tonic signalling through the T cell receptor (TCR) and cytokines within the secondary lymphoid organs (SLOs), where they can undergo clonal expansion. Therefore, the SLOs are important tissue reservoirs that allow for persistent viral replication in PLWH under suppressive ART. However, an unaddressed question is whether latently infected memory $CD4^+$ T cells retain their ability to recirculate between lymphoid and non-lymphoid organs, allowing them to seed various tissues and access the same survival signals as uninfected memory $CD4^+$ T cells to persist. Therefore, to address this question, I developed a fluorescent protein-based HIV reporter system that allows for the assessment of the phenotypic and migratory capacity of latently infected $CD4^+$ T cells.

The full-length, R5-tropic dual-fluorescent HIV reporter, ***HIV TosZy***, encodes two fluorescent markers: Nef-ZsGreen fusion protein under the control of the HIV-LTR and dTomato protein driven by constitutively expressed EF1α-HTLV1 promoter. Infection of primary $CD4^+$ T-cells with HIV TosZy allows the visualization of latently infected cells. Evaluations of receptors and molecules essential for T cell homing and migration demonstrate that viral latency does not alter their expression. Live-cell *in vitro* imaging studies in a 3D collagen matrix showed that latently $CD4^+$ T-cells displayed robust migration similar to uninfected $CD4^+$ T-cells and retained normal migratory behaviours. These *in vitro* observations were also recapitulated in the popliteal draining lymph node of humanized (hu-PBMC) mice using intravital imaging. The results of my research study indicate that latently infected memory $CD4^+$ T cells maintain the expression of homing and migratory markers and retain their ability to migrate within the lymph node,

potentially enabling them to continuously access survival signals, thus facilitating their persistence in ART-treated PLWH. This may also allow these cells to recirculate constantly between lymphoid and non-lymphoid organs, allowing them to continually seed various tissues. These findings provide valuable insights into how the homing and recirculation of latently infected cells may be targeted to prevent reservoir establishment and persistence in PLWH.

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I recognize that this dissertation is not just a product of my work alone. It reflects the collective efforts, support, and belief of all who have been a part of this journey. For that, I am eternally grateful!

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This dissertation is dedicated to God Almighty, the One who is above all things and the source of all wisdom and strength, for giving me the privilege of completing this amazing research project. His guidance and grace have been constant and have led me through the hurdles I encountered on this journey.

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I present this work in memory, love, and dedication to God, my parents, and my cherished friend.

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LIST OF ABBREVIATIONS

℃	Celcius
ACK	Ammonium–chloride–potassium
ADCC	Antibody-Dependent Cell Cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
AIM	Activation-induced marker
AML	Acute myeloid leukaemia
APCs	Antigen-presenting cells
APOBEC3	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic subunit 3G
ART	Antiretroviral therapy
AZT	Azidothymidine
BALT	Bronchial-associated lymphoid tissues
BBB	Blood-brain barrier
BLT	Bone marrow-liver-thymus
BRD4	Bromodomain-containing protein 4
CA	Capsid
cART	Combination antiretroviral therapy
CCL19	Chemokine (C-C motif) ligand 19
CCL21	Chemokine (C-C motif) ligand 21
CCL3	C-C Motif Chemokine Ligand 3
CCL4	C-C Motif Chemokine Ligand 4
CCL5	C-C Motif Chemokine Ligand 5
CCR5	CC-chemokine receptor 5
CCR7	CC chemokine receptor-7
CD	Cluster of differentiation
cDNA	Complementary viral DNA
CM	Central memory

CMs	Cynomolgus macaques
CMV	Cytomegalovirus
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeat
Cas9	CRISPR-associated protein 9
CSF	Cerebrospinal fluid
CTLs	Cytotoxic T lymphocytes
CXCL10	C-X-C motif chemokine ligand 10
CXCL11	C-X-C motif chemokine ligand 11
CXCR3	C-X-C chemokine receptor type 3
CXCR4	C-X-C-chemokine receptor 4
CXCR5	C-X-C-chemokine receptor 4
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
dCA	Didehydro-cortistatin A
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
EF1α	Elongation Factor 1α
HTLV	Human T-cell Leukemia Virus type 1
EM	Effector memory
ER	Endoplasmic reticulum
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FDCs	Follicular DCs
FIREs	frequently interacting regions
FMDV	foot-and-mouth disease virus
FMO	Fluorescence-minus one
FRCn	Fibroblastic reticular cell networks
FRCs	Fibroblastic reticular cells

GALT	Gut-associated lymphoid tissue
gDNA	genomic DNA
GPCRs	G protein-coupled receptors
GvHD	Graft versus host disease
HATs	Histone acetyltransferases
HDAC	Histone deacetylase
HDACi	HDAC inhibitor
HEVs	High endothelial venules
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
HTLV	Human T-cell Leukemia Virus
Hu-PBMC	Humanized PBMC
IFN	Interferon
IL	Interleukin
IL2R	Interleukin-7 receptor
iLN	Inguinal lymph node
IN	Integrase
IP	Intraperitoneally
IPDA	Intact proviral DNA assay
kB	Kilobyte
LADs	Lamin-associated domains
LFA-1	lymphocyte function-associated antigen 1
LN	Lymph node
LPS	Lipopolysaccharide
LRA	Latency reversal agents
LTR	long terminal repeat
MA	Matrix

MAdCAM-1	Mucosal vascular addressin cell adhesion molecule-1
MALT	mucosa-associated lymphoid tissue
mDCs	Myeloid DCs
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major histocompatibility complex
mL	Millilitre
mM	Millimolar
mm	Millimeter
MOI	Multiplicity of infection
MP-IVM	Multi-photon intravital microscopy
mRNA	Messenger Ribonucleic Acid
MSM	Men who have sex with men
nAB	Neutralizing antibody
NCRs	Natural cytotoxicity receptors
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cell
ng	Nanogram
nm	Nanometer
NHP	Non-human primate model
NK	Natural Killer
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NOD-SCID	Non-Obese Diabetic-Severe Combined Immunodeficiency
NRTIs	Nucleoside reverse transcriptase inhibitors
NSG	NOD/SCID common gamma chain null
ORFs	Open reading frames
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells

PCR	Polymerase Chain Reaction
PD-L1	Programmed death-ligand 1
pDCs	Plasmacytoid DCs
PFA	Paraformaldehyde
PIC	Pre-integration complex
PKC	Protein kinase C
pLN	Popliteal LN
PLWH	People living with HIV
PNAd	Peripheral node addressin
PopLN	Popliteal lymph node
PR	Protease
PrEP	Pre-exposure prophylaxis
PTCs	Post-treatment controllers
PTMs	Pig-tailed macaques
QVOA	Quantitative viral outgrowth assay
rh	Recombinant human
RMs	Rhesus Macaques
RNA	Ribonucleic Acid
RNAPII	RNA polymerase II
RPII	RNA polymerase II
RRE	Rev-response element
RT	Reverse transcriptase
RTC	Reverse-transcription complex
S_1P	Sphingosine-1-phosphate
S_1PR1	Sphingosine-1-phosphate receptor
SAMHD1	SAM domain and HD domain-containing protein 1
SCID	Severe combined immunodeficiency
SCS	subcapsular sinus

SCT	Stem cell transplantation
SHIV	Simian/human immunodeficiency virus
SIV	Simian immunodeficiency virus
SLOs	Secondary lymphoid organs
SMACs	Supramolecular activation clusters
TADs	Topologically associated domains
TCP	Thrombocytopenia
TCR	T cell receptor
TDF	Tenofovir disoproxil fumarate
TDR	Transmitted drug resistance
TLR	Toll-like receptor
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
T_{TM}	Transitional memory T cell
TM	Transitional memory
TT	Tetanus toxoid
VS	Virological synapses
µg	Microgram
µL	Microliter
µm	Micrometer
ZFNs	Zinc finger nucleases

CHAPTER 1

INTRODUCTION

1.1 HIV/AIDS: Epidemiology

Over four decades ago, Acquired Immune Deficiency Syndrome (AIDS) emerged as a new health concern when a growing population of young homosexual men succumbed to opportunistic infections that their immune system would have typically been able to combat and display rare malignancies [1,2]. Human Immunodeficiency Virus (HIV), a retrovirus, was subsequently identified as an etiologic cause of one of the most devastating pandemics, AIDS, in 1983, which causes high mortality in infected individuals if left untreated [1,2]. Human Immunodeficiency Virus is classified into two strains: HIV-1 and HIV-2. HIV-1, which this dissertation primarily focuses on, is more prevalent and pathogenic than HIV-2 as a result of the marked genetic diversity due to the high mutation caused by the error-prone function of the viral reverse transcriptase[3]. Topographically, HIV-1 infection occurs globally, whereas HIV-2 is mainly found in West Africa and European communities with socioeconomic links to West Africa[2,4]. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), approximately 38.4 million people live with HIV, with 1.5 million newly infected individuals in 2021 and 63% of this infection is accounted for by women and girls in sub-Saharan Africa[5]. HIV can be contracted through different routes: sexual, perinatal, intravenous, and percutaneous[4]. However, 80% of adults contract HIV after being exposed at mucosal surfaces, making HIV predominantly a sexually transmitted disease, with heterosexual transmission accounting for a majority of these incidences[4,6]. Some key populations at risk of acquiring HIV/AIDs include sex workers, injection-drug users, men who have sex with men (MSM), and transgender people[4,7]. Due to advances in prevention, testing and treatment for the virus, global AIDS-related death has significantly reduced by 68% since the peak in 2004, when over 2 million people died[5]. However, HIV/AIDS continues to be a significant global health tragedy despite the intense efforts. In December 2020, UNAIDS set a new 95-95-95 strategy. This ambitious target aims to have 95% of People living with HIV (PLWH) aware of their status, provide antiretroviral therapy (ART) for 95% of those diagnosed and 95% of those treated become virally suppressed by 2025[5]. However, recent data from 2022 suggests that these targets may not be within reach. According to UNAIDS, approximately 86% of all people living with HIV were aware of their status. Among these individuals, 89% had access to treatment, and of those receiving

treatment, 93% had achieved viral suppression[5]. These figures indicate the need to intensify research towards HIV cure and prevention studies.

1.2 HIV Genome Structure

HIV is an enveloped virus whose RNA genome is approximately 9.8Kb long and is enclosed in a lipoprotein membrane[8,9]. The HIV genome is contained within the viral core, consisting of two identical single-stranded (ss) RNA molecules, which encode multiple viral proteins[10]. The viral core comprises a portion of the Gag polyprotein, called the Capsid (CA) and contains essential viral protein necessary for the viral lifecycle.

The HIV genome features a 5' and 3' long terminal repeat (LTR) region, and the 5' LTR serves as a promoter responsible for driving the transcription of viral genes (**Figure 1. 1**). Additionally, within the HIV genome, there are nine open reading frames (ORFs) that encode 15 different proteins. These proteins, whose functions are enlisted in **Table 1**, are classified into structural, enzymatic, and accessory/regulatory proteins[9-11]. Structural proteins comprise the viral core and outer membrane and include four Gag proteins [Matrix (MA, p17), Capsid (CA, p24), Nucleocapsid (NC, p7), and p6)] and two Env proteins [Surface (SU, gp120) and Transmembrane (TM, gp41)]. Enzymatic proteins, which are involved in vital viral processes and replication, consist of three Pol proteins [Protease (PR, p12), Reverse transcriptase(RT, p51), and Integrase(IN, p32)]. Accessory and regulatory proteins include Rev, Tat, Vpu, Vpr, Vif, and Nef, which help modulate viral interactions with the host immune system and other cellular processes.

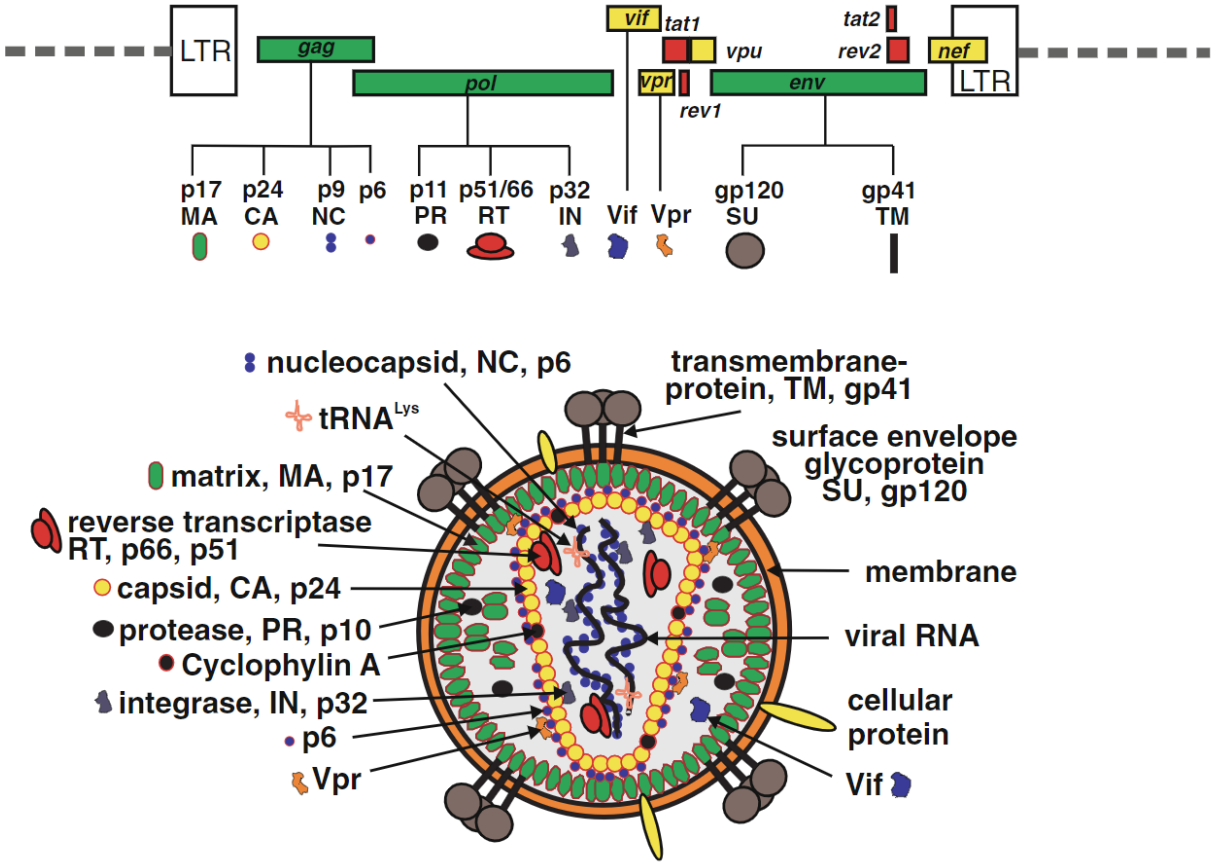


Figure 1. 1: The organization and structure of the HIV genome. The HIV genome is flanked on the 5' and 3' ends by the long terminal repeats (LTRs), which regulate integration and viral gene expression. Enclosed within the flanked ends are the open reading frames that encode 15 viral proteins with diverse functions. *Figure from journal article*[12].

Table 1. Overview of the functions of different HIV genes encoded in the HIV genome. ***Modified from[13]***

Classification	Gene	Protein	Size*	Function
Structural	*gag*	Matrix	p17	Forms the inner membrane layer of the virus
		Capsid	p24	The most abundant protein that protects the viral core
		Nucleocapsid	p9	Aids the assembly of the nucleoprotein/RNA complex
		p6	p6	Involved in viral particle release
	env	Surface	gp120	Allows viral attachment to the target cell via the entry receptor and co-receptor
		Transmembrane	gp41	Facilitate the fusion of the virus envelope and the host cell membrane during viral entry
Enzymatic	*pol*	Protease	p12	Proteolytic cleavage of Gag and Gap-Pol precursor protein, resulting in the release of viral proteins
		Reverse transcriptase	p51	Mediates the transcription of HIV RNA into proviral DNA
		Integrase	p32	Allows for the insertion of proviral DNA into the host genome
Regulatory	*rev*	RNA splicing regulator	p19	Regulates the export of non-spliced and partially spliced viral mRNA from the host cell nucleus
	tat	Transactivator protein	p14	It serves as an activator of the transcription of viral genes and enhances viral transcription.
Accessory	*vpu*	Virus protein Unique	p16	Mediates immune evasion and facilitates the release and dissemination of virus
	vpr	Virus protein r	p15	Plays a role in HIV replication and facilitates viral infectivity
	vif	Viral infectivity protein	p23	Enhances viral infectivity by preventing hypermutation during reverse transcription through APOBEC3G inhibition

	nef	Negative regulating factor	p27	Influences HIV replication, mediates immune evasion, downregulates CD4 on target cells and increases virion infectivity
***Numbers correspond to the size of the proteins (p) or glycoproteins (gp) in kDa**				

1.3 HIV Life cycle, pathogenesis, and host immune responses

1.3.1 HIV lifecycle

CD4 is the primary receptor for HIV and is expressed on the surface of $CD4^+$ T lymphocytes, monocytes, macrophages and dendritic cells[3,14]. To gain entry into the host cell, HIV also requires a co-receptor, generally the chemokine receptors, CCR5 and CXCR4[3,14]. Different tropisms for either or both of these chemokine receptors exist, ranging from CCR5-tropic (R5 viruses), CCR5 and CXCR4 utilizing (R5X4 viruses), and the less frequent CXCR4-tropic (X4 viruses) HIV-1 viruses[3,14,15].

The main target of HIV is activated $CD4^+$ lymphocytes, and the initial recognition of the CD4 receptor and CCR5 and/or CXCR4 co-receptor by the Env glycoprotein allows for a cascade of conformational rearrangement that ultimately results in viral fusion and the release of the viral core into the cytoplasm in host cells[4]. The complex of the viral core that is comprised of the RNA genome, Matrix (MA), Integrase (IN), Reverse transcriptase (RT), and Vpr is referred to as the reverse-transcription complex (RTC), which is transported through the cellular cytoskeleton from the cytoplasm to the host cell nucleus[16,17]. HIV reverse transcriptase, which lacks proofreading capability and results in a high mutation rate (1 error per 10,000 nucleotides), facilitates the reverse transcription of the viral ssRNAs into a linear complementary viral DNA (cDNA) during the transport of the RTC, and this process is mainly completed by the time the RTC reaches the host nucleus[17,18]. Earlier reports have shown that uncoating the capsid-encased RTC in the cytoplasm is necessary for entry into the nucleus, as the intact viral core (~61 nm) exceeds the reported size limit of the nuclear pore (~39nm)[19-23]. New evidence challenges these conclusions and has shown that intact (or near intact) viral cores can enter the nucleus and uncoat less than 2 hours before chromosomal integration, as in situ measurement of the nuclear pores of HIV infected and uninfected T cells and primary $CD4^+$ T cells lines measured at ~64nm[24,25]. Additionally, a time course nuclear pore blockade study in various HIV-1 target cells showed that reverse transcription

and uncoating of the capsid is completed in the nucleus[26]. Uncoated RTC is referred to as the pre-integration complex (PIC), which can integrate preferably in transcriptionally active sites within the host chromatin with the aid of viral integrase, now referred to as a provirus[27,28]. It is important to note that when there is a failed integration of the PIC into the host genome, a circular DNA is formed with 1 or 2 copies of the HIV LTR called 1-LTR and 2-LTR, respectively[29].The host cellular nucleases eventually degrades these circularised DNAs[30]. However, once the provirus is formed, it utilizes the host transcriptional machinery to transcribe viral genes, and this is strictly dependent on the availability of the host cellular transcription factors, such as NF-κB, NFAT, and AP-1[12]. Moreover, the viral regulatory protein Tat plays a crucial role in the efficient transcription of viral genes into viral transcripts by binding to the trans-acting response (TAR) element located on the HIV 5' LTR, thereby enhancing the transcriptional process[12]. These viral transcripts are then exported from the host cell nucleus to the cytoplasm, where they are translated by the host cell's translation machinery, leading to viral protein production[9]. Following export, the Gag protein localizes to the plasma membrane, aided by a myristoyl group at its amino terminus; this localization is succeeded by the budding of immature virions containing intact Gag polyproteins[9]. The final stage of the viral life cycle involves virion maturation, during which the viral protease cleaves the Gag-Pol polyprotein into smaller, functional components[9]. This maturation results in a fully infectious virion capable of infecting other host cells.

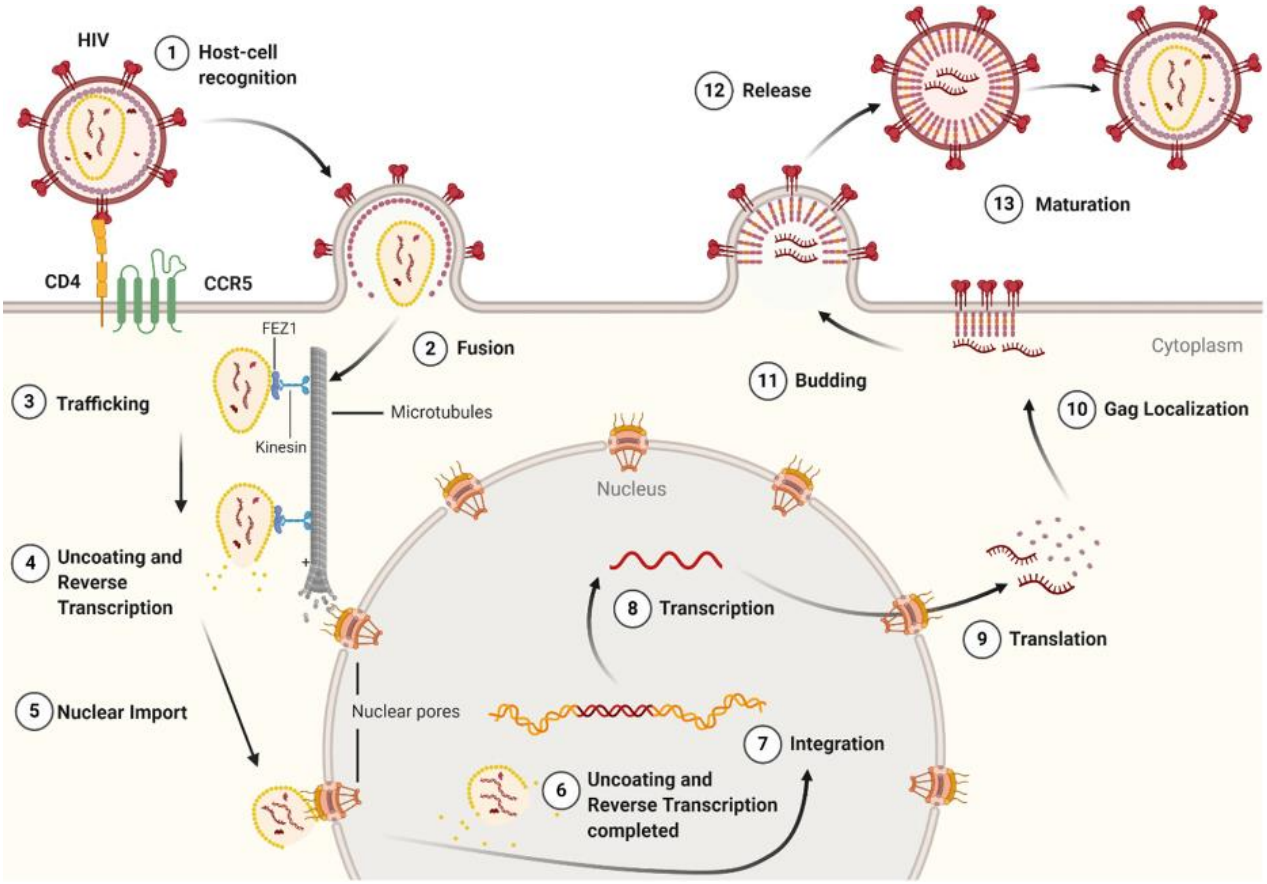


Figure 1. 2: An overview of the lifecycle of HIV-1 in a host cell. HIV gains entry into target cells by interacting with CD4 and either CC-chemokine receptor 5 (CCR5) or CXC-chemokine receptor 4 (CXCR4) with its envelope (Env) glycoprotein. This interaction allows for the fusion and uncoating of the viral RNA into the cell's cytoplasm. The viral RNA is reverse-transcribed into viral DNA either in the cytoplasm or nucleus, and the reverse-transcribed DNA is integrated into the host genome. Mediated by the host transcriptional mechanisms, the viral DNA is transcribed into viral mRNA, which is exported to the cytoplasm, where translation occurs to make viral proteins. These viral proteins are eventually packaged into mature virions—*Figure from journal article*[9].

1.3.2 HIV Pathogenesis

The clinical progression of HIV infection typically follows three stages: acute infection, chronic infection and AIDS. The early or acute phase of HIV infection develops within the first 2 to 4 weeks after infection, where infected individuals display mononucleosis-like clinical symptoms[31]. During acute infection, there is a decline in $CD4^+$ T cells due to *viremia*, which is the exponential rise in viral titre in the plasma of infected individuals due to the rapid increase in viral replication[32]. The immune system mounts an early response before peak viremia (>1 million virus copies/ml of plasma), and this includes the innate immune response, antibody response and the first T cell response of activated $CD8^+$ cytotoxic T lymphocytes (CTLs), which helps control viral replication[6,33]. However, following the acute infection, the inability of the immune system to completely eradicate the virus leads to a "chronic phase" of HIV infection, also known as the asymptomatic or clinical latency phase[31]. The chronic phase of infection is marked by a lower level of viral replication in the secondary lymphoid organs (SLOs). This ongoing viral replication causes a gradual decline in $CD4^+$ T cell count, causing further immune dysregulation in infected individuals and impairing the immune system's ability to fight infections and malignancies[31]. If HIV infection remains untreated, the infection progresses and eventually leads to a severe decline in $CD4^+$ T cell count, resulting in immunodeficiency. When the $CD4^+$ T cell count drops below 200 cells/mm^3, typically within 6 to 10 years after HIV infection, the individual becomes susceptible to opportunistic infections and certain cancers, leading to the clinical diagnosis of AIDS[31].

1.3.3 Host immune response to HIV Infection

The host immune response to HIV involves a complex interplay between innate and adaptive immunity that functionally limits but cannot completely eliminate viral replication in infected individuals.

1.3.3.1 **Innate immune system**

The innate immune system is the first line of defence during HIV infection, contributing to early detection and control of HIV replication. The two most important innate immune cells involved in controlling HIV pathogenesis are Dendritic cells (DCs) and Natural Killer (NK) cells.

Dendritic cells

The strategic localization of dendritic cells (DCs) at mucosal surfaces places them as one of the first immune cell populations to come into contact with HIV during sexual transmission[34]. These sentinel cells are specialized antigen-presenting cells (APCs) that use dendrite-like extensions to efficiently sample the extracellular environment and subsequently process and present antigens by using the major histocompatibility (MHC) molecules on the cell surface[35]. As such, DCs are a crucial bridge between innate and adaptive immune responses. The two primary subsets of DCs are plasmacytoid DCs (pDCs) and myeloid DCs (mDCs), which both express HIV receptors, CD4 and co-receptors, CCR5 and CXCR4[34,36].

Plasmacytoid DCs (pDCs) primarily contribute to innate immunity and facilitate a robust antiviral response by secreting high amounts of type I interferons (IFNα) upon encountering HIV[37]. pDCs internalize HIV through endocytosis, where viral genomic RNA is recognized by TLR-7, triggering a signalling cascade that leads to cellular maturation[34,36]. This maturation process initiates a series of events, including the production of various cytokines (IFNα, IFNβ, IL-6, and TNFα) and upregulation of chemokine receptor, CCR7 and co-stimulatory molecules, CD40, CD80 and CD86 expression[34]. Upregulation of CCR7 enables pDCs to migrate to secondary lymphoid organs (SLOs), where they present processed antigens to adaptive immune cells, such as $CD4^+$ T cells. Upon maturation, pDCs also produce a variety of soluble factors, including chemokines like CCL3, CCL4, CCL5, CXCL10, and CXCL11, as well as the pro-inflammatory cytokine IL-18[37]. The production of IL-18 contributes to the activation and proliferation of natural killer (NK) cells, enhancing their cytotoxic capabilities to eliminate target cells[37].

Myeloid DCs (mDCs) are the more specialized subset of DCs vital in bridging the innate and adaptive immune response due to their exceptional ability to present antigens to T cells[34]. mDCs have a high expression of TLR-3, TLR-9 and TLR-10, enabling them to recognize the presence of intracellular viral dsRNA and DNA, which in turn leads to the production of type 1 IFNs and IL-12[34]. The production of IL-12 by these cells facilitates the activation of Th1 and NK cell responses to counteract HIV infection. Moreover, mDCs possess a superior capacity for cross-presentation, efficiently priming $CD8^+$ T cells through MHC class I molecules to execute cytolytic functions on infected cells[38]. However, the viral protein Nef causes the downregulation of MHCI, which could impair the ability of DCs to cross-presenting antigens to $CD8^+$ T cells[39].

Natural killer (NK) cells

Natural killer (NK) cells play a pivotal role during the acute stage of HIV infection and possess both cytotoxic and immunomodulatory functions through cytokine/chemokine production that help orchestrate and bridge innate and adaptive immune responses. They are regulated by a balance of activating and inhibitory signals mediated by three germ-line encoded receptors. These receptors help determine the activation state of the NK cells, and they comprise[40,41]:

i. *Killer cell immunoglobin-like receptors (KIRS):* Includes activating and inhibiting receptors that recognize MHC class I through optimal immune surveillance.
ii. *Natural cytotoxicity receptors (NCRs):* Recognize viral-derived products and virally infected cells.
iii. NKG2/CD94

These and other receptors, such as the FcγRIIIA (CD16) receptor, allow NK cells to recognize virally infected cells and mount an effective immune response by antibody-dependent cell cytotoxicity (ADCC) through the secretion of cytotoxic granules containing granzymes and perforin, which causes cell lysis[40-42]. The apoptotic bodies formed during cell lysis can be picked up by DCs, causing their maturation and allowing them to present viral antigens to T cells[40]. Also, DC maturation can regulate NK cell effector function by producing IL-12 and IL-18, which promote IFN-γ production in NK cells[40]. Olivia *et al.* showed that when NK cells from HIV-infected individuals were stimulated with IL-2 and IL-15, there was a measurable increase in the production of β-chemokines, namely CCL3, CCL4, and CCL5[43]. These chemokines serve as natural ligands for the CC-chemokine receptor 5 (CCR5), indicating that NK cells may indirectly inhibit HIV entry and replication by increasing β-chemokine expression that competes with HIV gp120[43].

1.3.3.2 Adaptive immune response

The adaptive immune response, which takes place primarily in secondary lymphoid organs (SLOs), such as lymph nodes (LNs), the spleen, tonsils, and mucosa-associated lymphoid tissue (MALT), is a highly specific and coordinated cellular process that plays a prominent role during the acute stage of HIV infection[44,45]. The establishment of immunological memory allows for a

rapid recall response that aids in pathogen clearance and involves two main arms: The cellular and humoral immune response[45].

Cellular immune response

The cellular immune response is mainly orchestrated by $CD8^+$ cytotoxic T lymphocytes (CTLs), as $CD4^+$ helper cells are severely depleted during infection[39].

$CD8^+$ cytotoxic T lymphocytes (CTLs)

$CD8^+$ cytotoxic T lymphocytes (CTLs) confer a strong immune response during HIV infection and are essential for viral control during acute infection[46]. CTLs mount this response by recognizing HIV derived-peptide presented on the MHC class I molecules of virally infected $CD4^+$ T cells by their T cell receptor (TCR), which leads to their activation[46]. The activation of these HIV-specific CTLs causes clonal expansion, generating a large pool of effector cells[46,47]. The effector functions of these cells are classified into lytic (cytotoxicity) and non-lytic (cytokine production) mechanisms[44,48]. The lytic mechanism of effector CTLs involves the high capacity of degranulation of specialized secretory lysozyme, causing the secretion of cytotoxic molecules, granzyme B and perforin, and the upregulation in apoptosis-inducing Fas ligand (CD95L), leading to the apoptosis of virally infected cells, whereas non-lytic mechanism of effector CTLs involves the secretion of cytokines IFN-γ, TNF-α, and IL-2[44,48]. These cytokines are crucial for promoting antiviral and inflammatory responses as well as T-cell survival and proliferation. CTLs primarily function during the acute phase of infection as they rapidly expand to facilitate initial infection control. However, their role diminishes during chronic infection due to cellular exhaustion resulting from continuous antigen exposure. Additionally, immune escape caused by the rapid mutation of the virus further reduces the effectiveness of CTLs in controlling the infection during chronic stages[49].

Humoral immune response (B cells)

The humoral immune response to HIV infection doesn't have as much pronounced effect as the cellular immune response, but it also plays a role in HIV infection control[50]. This immune response is mediated by antibody-secreting cells named B-cells, which produce a variety of antibodies in response to different viral antigens upon HIV infection. Some of the functions of the antibodies are listed below[50-52]:

i. *Neutralization:* Antibodies can neutralize the gp120 and gp41 proteins on the HIV envelope, preventing the virus from binding to and infecting target cells. Thus, this inhibits the spread of infection.

ii. *Antibody-dependent cell cytotoxicity (ADCC):* HIV-specific IgG1 antibodies can tag infected cells by binding to their surface, allowing natural killer (NK) cells to recognize and destroy the infected cells via their FcγRIIIA receptors.

iii. *Opsonization:* Antibodies can coat infected cells, marking them for destruction by phagocytes such as macrophages. The phagocytes then engulf and eliminate the infected cells.

1.4 Current therapies for HIV infection management and prevention

1.4.1 Antiretroviral therapies (ART)

Our detailed understanding of the HIV lifecycle has directly led to the development and testing of antiretroviral drugs and led to HIV infection becoming a manageable chronic disease instead of a progressive, fatal illness. In general, antiretroviral therapy (ART) functions to block the HIV replication cycle at several pivotal steps, thereby reducing viral load to undetectable levels while preventing or reversing immunodeficiency in people living with HIV (PLWH)[53]. Current ART regiments have reduced overall mortality rates by 50%, thus leading to a 5% increase in 5-year survival compared to untreated individuals[54]. Current ART regimens are a combination of at least two different classes of antiretroviral drugs that target different stages of the viral replication cycle, described below[53,55-61] (**Figure 1. 3**):

i. <u>*Entry Inhibitors:*</u>

   a. *CCR5 antagonist:* Maraviroc is the first approved drug in this category that works by binding the HIV co-receptor CCR5, thus changing its conformation and inhibiting the binding of the virus to the surface of the host target cells to prevent viral entry.

   b. *Fusion inhibitor:* Enfurivirtide interferes with viral fusion by binding to a critical region in the viral envelope gp41, thus preventing conformation changes necessary for the fusion of viral and host cell membranes.

ii. *Integrase strand transfer inhibitors (INSTIs):* These inhibitors block the viral integrase enzyme to potently reduce proviral integration and subsequent viral replication and spread. Examples of approved INSTIs include: Raltegravir, Elvitegravir, Dolutegravir, Bictegravir and Cabotegravir.

iii. *Reverse transcriptase inhibitors:*

a. *Nucleoside reverse transcriptase inhibitors (NRTIs):* These drugs are analogues of the natural nucleosides and nucleotides, which commonly lack the 'OH group needed for the DNA growing chain, thus causing DNA synthesis termination. This process halts the transcription of viral RNA into complementary viral DNA. Examples of approved NRTIs include: Zidovudine, Lamivudine, Abacavir, Tenofovir, and Emtricitabine.

b. *Non-Nucleoside reverse transcriptase inhibitors (NNRTIs):* In contrary to the mechanism of actions of NRTIs, these drugs are non-competitive inhibitors for the reverse transcriptase enzyme. NNRTIs bind to a pocket near the enzyme's active site, inducing a conformation change that abrogates its enzymatic activity. Examples of approved NNRTIs include: Nevirapine, Efavirenz, Etravirine, Rilpivirine and Doravirine.

iv. *Protease inhibitors:* Protease inhibitors act late in the viral replication cycle by blocking the protease enzyme that cleaves viral polyproteins into smaller functional proteins during the viral maturation steps. Protease inhibitors function to prevent viral core maturation and infectivity. Examples of approved protease inhibitors include: Saquinair, Lopinavir, Atazanavir, Fosamprenavir, Tipranavir, and Darunavir.

Antiretroviral drugs are also utilized as pre-exposure prophylaxis (PrEP) to protect high-risk individuals from acquiring HIV before being exposed to the virus[61,62]. One of the most extensively studied regimens is the daily oral administration of tenofovir disoproxil fumarate combined with emtricitabine (TDF/FTC)[61-63]. A study demonstrated that this PrEP could reduce the risk of HIV acquisition by up to 86% in men who have sex with men (MSM) and 76% in

serodiscordant heterosexual couples, where one partner is HIV-positive, and the other is HIV-negative[64]. This study highlights the importance and effectiveness of PrEP in curtailing new HIV infection incidence.

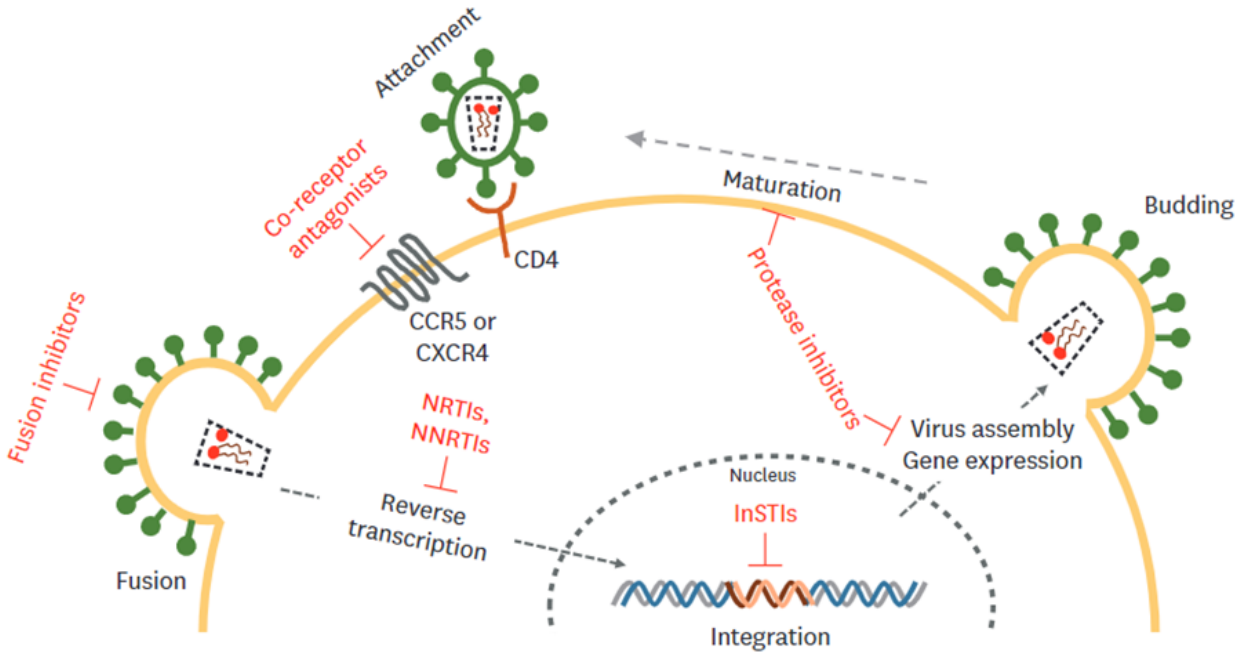


Figure 1. 3: Current antiretroviral drug classes and their targeted stages in the HIV life cycle. The four different classes of antiretroviral drugs interfere with distinct stages in the viral lifecycle, thus inhibiting the virus's ability to replicate and progress —*Figure from journal article*[53].

1.4.2 Challenges/Limitations of current antiretroviral therapy

Antiretroviral therapy (ART) has revolutionized the management of HIV/AIDS and helped substantially increase the quality of life in PLWH. Nevertheless, there are several limitations associated with their use that warrant discussion. First, incomplete adherence to suppressive ART regimens can contribute to the emergence of drug-resistant viral strains[65-67]. The emergence of drug resistance in a population and subsequent transmitted drug resistance (TDR) of drug-resistant strains within newly infected individuals present a considerable clinical challenge by reducing therapeutic options[65,68-70]. Reduced ART adherence may be the result of adverse or toxicity reactions, costs, accessibility to medications or the stigma associated with using antiretroviral drugs[71,72]. Second, current drug regimens are highly effective in blocking new rounds of viral production but do not eliminate infected cells in the body. Upon ART cessation, the vast majority

of individuals will experience a viral rebound, owing to the generation of an ***HIV reservoir*** (*further discussed in section 1.7*) within the body. The HIV reservoir is established early during acute infection and persists throughout ART suppression[73]. Similarly, studies in non-human primates (NHPs) show that ART initiation as early as 1-3 days after simian immunodeficiency virus (SIV) infection did not prevent viral reservoir establishment[74]. Hence, despite significant progress in HIV prevention and treatment, HIV cure remains elusive and is the subject of rigorous research.

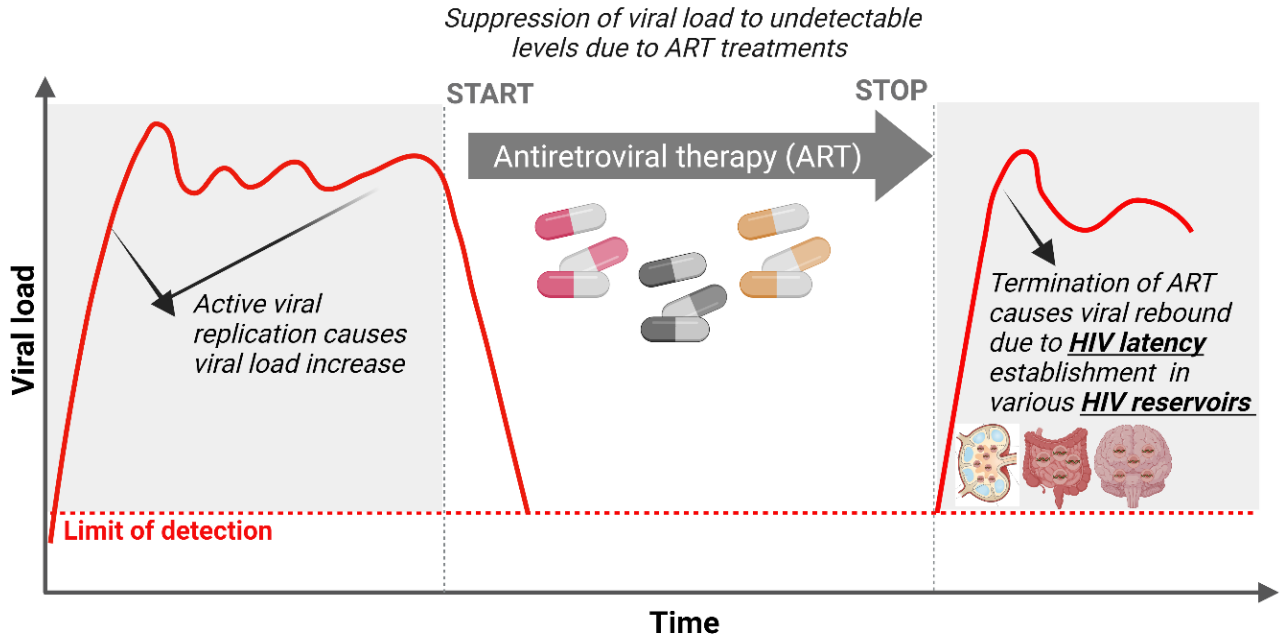


Figure 1. 4: The drawback of HIV-1 management with antiretroviral therapy (ART). The figure illustrates how untreated HIV infection leads to a surge in viral load due to unchecked replication. ART treatment can suppress the viral load to undetectable levels; however, discontinuing ART can cause a viral rebound due to the presence of viral reservoirs within cells and anatomical structures —*Figure created with Biorender.*

1.5 Case reports of long-term HIV control and durable HIV cure

1.5.1 Stem cell transplantation (SCT)

Stem cell transplantation (SCT) has been demonstrated to achieve HIV cure in a few successful individuals. SCT is a medical procedure typically used to treat diseases affecting blood cell production or the immune system, such as sickle cells, leukaemia, multiple sclerosis, etc.[75].

SCT involves replacing a patient's dysfunctional stem cells with healthy ones from a donor, following a conditioning process using chemotherapy or chemoradiotherapy to eliminate the old stem cells[76]. This approach gained attention as a potential form of HIV cure after 3 reported cases of patients who have demonstrated a long-term remission or complete eradication of HIV after receiving SCT and going off antiretroviral therapy (ART)[77-81]. Two of these patients were male and were named the "Berlin, and the "London" patient, both who received an allogeneic hematopoietic stem cell transplant (alloHSCT) from unrelated donors who have a homozygous mutation in the HIV co-receptor CCR5 (CCR5Δ32/Δ32) to treat acute myeloid leukaemia (AML) and Hodgkin's lymphoma, respectively. The Berlin patient was the first individual to be cured of HIV infection and was in remission for approximately 13 years before succumbing to AML, while the London has been in remission for approximately five years and continues to live without ART. More recently, a mixed-race woman who underwent a CCR5Δ32/Δ32 haplo-cord transplant (cord blood cells combined with haploidentical stem cells from an adult) to treat AML with durable remission for 18 months has been reported[79]. In these cases, the immune system of the HIV-infected patient is essentially reset where most of the existing immune cells, including HIV-infected cells, are killed off during conditioning therapy, and the immune system is then replenished with cells derived from stem cells that lack functional HIV co-receptor, CCR5[82]. Consequently, replacing the patient's immune systems with cells devoid of functional CCR5 receptors allowed their bodies to largely resist HIV infection. While these rare cases provide hope for SCT to achieve sterilizing HIV cure, this approach is high risk due to the potential complications such as xenogeneic graft versus host disease (GvHD)[82]. SCT is also very expensive and does not represent a scalable HIV cure strategy for PLWH[83,84]. Moreover, finding compatible donors with the CCR5Δ32/Δ32 mutation is inherently challenging due to its rarity in the general population, especially among individuals of Asian and African descent[83,85-87]. However, these promising instances of durable HIV cures provide important proof-of-principle observations that can be further extended using gene-editing technologies with lower risk profiles and costs that can be accessed by a larger population.

1.5.2 Elite and Post-treatment controllers

In fewer than 1% of individuals infected with HIV, the virus is naturally controlled, with the viral load being undetectable in the absence of antiretroviral therapy (ART), and these individuals are referred to as ***elite controllers (ECs)***[88-91]. Remarkably, ECs can maintain this drug-

free suppression of HIV-1 replication even though an intact replication-competent virus is present. However, most of these replication-competent viruses exist in a "blocked and locked" state because they are integrated at sites with a limited transcriptional activity that hinders replication[88]. Jiang *et al.* employed full genome sequencing and discovered that the integration of intact proviral sequences in ECs occurred at unique regions within the human genome, which allows for a deep latent state compared to those of HIV patients on long-term ARTs[89]. These sequences were reported to be mainly located in centromeric satellite DNA or within the Krüppel-associated box domain-containing zinc finger genes on chromosome 19, which are associated with heterochromatin features. It was also observed that, in elite controllers, the integration sites of the intact proviral sequences showed an increased distance to the transcriptional start sites and were enriched with repressive chromatin marks. A significant proportion of elite controllers were observed to exhibit a potent and targeted immune response against HIV, involving CTLs and NK cells, as well as protective MHC-I alleles, including human leukocyte antigen (HLA)-B27 and HLA-B57 (HLA-B27/B57)[47,48,90,92-96].

Another set of HIV controllers is called the post-treatment controllers (PTCs). Most of these individuals have been reported to have started cART early, and exhibit continued viral suppression for a prolonged period, even after treatment cessation[97]. PTCs were shown to have a smaller reservoir size with their total and intact provirus 7-fold lower than non-controllers, which could delay viral rebound[98,99]. The exact mechanism behind this viral control is not yet completely understood, but control of HIV reservoir expansion is believed to be one contributing factor[88,100]. Recently, it was shown that PTCs have distinct immunological features, which showed that they have more HIV-specific $CD4^+$ responses, lower $CD4^+$ T cell exhaustion and more functional NK cells[101]. The study of ECs and PTCs has provided valuable insights into the mechanisms of viral control and immune response, which is beneficial in the development of novel therapeutic strategies and vaccine research.

Interestingly, it appears that a sex bias may be present in the observed controller status among both adult and pediatric cohorts, as females constitute the majority of these controllers[89,102,103]. However, the exact mechanisms underlying this control in females remain elusive.

1.6 HIV latency

Viruses have evolved various strategies to establish persistent infections in their hosts by evading detection and clearance by the immune system[104,105]. As mentioned earlier, one example that HIV employs is facilitating Nef-mediated MHC-I downregulation to evaluate CTL killing. Another important strategy is the establishment of ***viral latency***, a state of reversible non-productive infection of individual cells that is established early during acute infection[104,106]. In the context of HIV, these cells make up the ***viral reservoir*** that can remain long-lived in various anatomical sites and pose a major hurdle in achieving HIV[107-109].

HIV latency is a complex and coordinated process that is largely regulated at the integration stage of the viral lifecycle and can be established in different cell types after infection[110,111]. However, long-lived resting (memory) $CD4^+$ T cells have been shown to constitute the majority of the important latent reservoir population; this is because HIV preferentially targets activated $CD4^+$ T cells, and while a majority of these cells die as a result of the cytopathic effect of the virus, a small fraction could revert to a resting memory phenotype that can reactivate viral production after exposure to activation cues[112-115]. Chun *et al.* were the first to demonstrate that HIV can establish a non-productive state of infection in resting $CD4^+$ T cells when they observed that a low frequency of these cells contains replication-competent viruses that can produce new virions upon *ex vivo* stimulation[116]. These long-lived, transcriptionally silent cells were also detected in PLWH on cART and were extremely rare (~ 1 in 1×10^6 cells)[117]. A longitudinal study showed that in PLWH on cART, the mean half-life of these cells is about 44 months, and if the latent reservoir consists of only 1×10^6 cells, approximately 73 years of treatment will be needed to completely eradicate the viral reservoir[117,118]. This suggests that in an individual's lifetime, cART alone will not be sufficient to completely eradicate the HIV reservoir. Although cART doesn't completely eradicate the viral reservoir, recent studies in PLWH on suppressive cART for over two decades showed that there was a change in the viral reservoir landscape, with intact proviruses predominantly integrated into heterochromatin regions, specifically in areas of centromeric satellite and microsatellite DNA[119]. Thus, it suggests that long-term cART may act as a selection pressure and put integrated provirus in a ***block-and-lock*** state and induce deep latency when there is limited or no transcriptional activity of the provirus.

1.6.1 Classification and molecular mechanism of HIV latency

Based on the integration status of the viral DNA, HIV latency can be broadly classified into ***pre-integration*** and ***post-integration latency***[114,120,121] . ***Pre-integration latency*** occurs when HIV infects a resting $CD4^+$ T cell. Since these resting cells are in a low metabolic state, they cannot facilitate the energy-dependent import of the pre-integration complex (PIC) into the nucleus, and this state also inhibits the integration of the PIC into the host genome[120,121]. The non-integrated PIC have a finite life span of only 24 hours and are eventually degraded[122]. However, if the infected resting $CD4^+$ T cell becomes activated before the degradation of the PIC, viral integration can proceed, and the subsequent viral lifecycle can proceed[14,121]. ***Post-integration latency*** is a more stable form of HIV latency, which occurs after viral DNA integration[108,121]. Post-integration latency typically occurs when an activated $CD4^+$ T cell reverts to a resting state after productive infection, reaching a cellular state that suppresses viral replication[114,120,121]. This dissertation will mainly focus on the phenotypic and functional characterization of infected cells in post-integration latency.

Following proviral integration, HIV relies on the host transcriptional machinery to initiate viral gene expression[123]. The 5' LTR of the HIV genome, which primarily serves as the core promoter and enhancer for viral gene expression, contains binding sites for various transcription factors, such as NF-κB, NFAT, Sp1, AP-1, and TFIID, which interact with the cellular RNA polymerase II (RPII) to initiate viral transcription[123,124]. However, in resting $CD4^+$ T cells, the transcription factors, NF-κB and NFAT, which bind to the κB sites in the HIV LTR, are sequestered in the cytoplasm and are unable to be translocated to the nucleus to facilitate viral gene expression[110,112]. NF-κB can exist as p50/p50 homodimers or p50/RelA heterodimers and can respectively play inhibitory or activating effects on the HIV-LTR[110,112]. The inhibitory p50 homodimers can promote HIV latency by recruiting histone deacetylase (HDAC) to the HIV LTR, promoting heterochromatin formation and decreasing chromatin accessibility to transcription factors. On the other hand, the p50/RelA heterodimers, which recruit histone acetyltransferases (HATs) like p300/CBP, allow for histone tails acetylation, which leads to a euchromatin state, thus facilitating transcription initiation due to increased accessibility of transcription factors to the chromatin. Notably, the absence or inhibition of the viral transactivator, Tat, which enhances viral transcription, has also been linked to the establishment of HIV latency, as the use of didehydro-

cortistatin A (dCA), a Tat-dependent transcription inhibitor, shows a permanent induction of the viral transcription inactivation[125,126]. As previously mentioned, HIV proviruses mainly integrate into the actively transcribed region of the chromatin; however, transcriptional interference of the viral gene could allow for the establishment and maintenance of post-integration latency[28,112,127]. For instance, suppose the provirus integrates in the same transcriptional direction, the host's RNA polymerase II (RNAPII) starting from an upstream promoter, could potentially displace important transcription factors from the HIV-LTR promoter by reading through it. On the other hand, if the provirus integrates in the reverse direction, the RNA polymerases from the host and viral promoter could clash with each other. This collision could result in the premature termination of transcription from one or both of the promoters.

1.7 HIV reservoirs

1.7.1 CD4$^+$ T cells

$CD4^+$ T cells are highly mobile cells that HIV exploits to promote its spread throughout different tissues and compartments of the host's body. These various tissues and compartments are typically inaccessible to free viral particles moving in the body's extracellular fluids[128]. $CD4^+$ T cells exhibit a distinctive physiological state, allowing them to transition between active and resting stages and undergo the dynamic process of changing from effector to memory cells[129,130]. Resting memory $CD4^+$ T cells make up a significant portion of the long-lived HIV reservoir and are the primary focus of research on viral reservoirs[108,131-134]. The memory compartment of $CD4^+$ T cells can be characterized by different cell surface markers, including CD45RO, CD27, CCR7 or CD62L, and is predominately composed of Effector memory (T_{EM}) and Central memory (T_{CM}) $CD4^+$ T cells[135-137]. T_{CM} express CCR7 (a lymph node homing molecule), has a longer life span (typically years to decades) and exhibits high proliferative capacity upon antigen re-exposure[138]. In contrast, T_{EM} does not express CCR7, has a shorter life span, limited proliferative capacity, and responds rapidly to antigen stimulation[138,139]. Transitional memory $CD4^+$ T cell (T_{TM}) is another memory subset that displays an intermediary phenotype between T_{EM} and T_{CM}[140]. Earlier studies by Chomont *et al.*, characterizing the $CD4^+$ T cells reservoir of virally suppressed PLWH, identified T_{CM} (in patients with high $CD4^+$ count) and T_{TM} (in patients with low $CD4^+$ count) as key reservoirs harbouring HIV proviral DNA[131]. This study showed that infection of these memory T cell subsets and their natural ability to survive long-term may represent important reservoirs in virally suppressed PLWH. Boritz *et al.* showed that in elite controllers, the skewed distribution of

the T cell viral reservoirs from long-lived T_{CM} towards shorter-lived T_{EM} and T_{TM} could be a crucial factor for viral control[141]. Additionally, in the VISCONTI study, which studied 14 PTCs, the T_{TM} subset was shown to make up a majority of their viral reservoir, contributing about 54%, while T_{CM} contributed only 22%[142]. It is important to note that T_{EM} harbours the largest proportion of intact-replication competent provirus, which are highly inducible *in vitro* and *ex vivo*[132,143,144]. Several cellular markers have been reported to help identify cells harbouring integrated provirus. Fromentin *et al.* showed that $CD4^+$ cells harbouring integrated viral DNA had a higher expression of LAG-3, TIGIT, and PD-L1[145]. There have also been conflicting results on if Fc gamma receptor IIa (FcγIIa), CD32a, marks for viral latency in $CD4^+$ T cells[146-149]. Together, these highlight that the mobility, physiological states and longevity of $CD4^+$ T cell memory subsets confer an advantage for HIV to spread and persist within the host's body.

More recent characterization of the T cell reservoir by several groups showed strong evidence for ***clonal expansion*** of HIV-infected $CD4^+$ T cells over time under ART suppression. T cell clonal expansion is a normal immunological response to infection that involves massive proliferative expansion of antigen-specific T cells. In the context of HIV infection, integration site analysis in $CD4^+$ T cells isolated from virally suppressed PLWH revealed that over 40% of provirus was contained within clonally expanded cells, with the majority being defective[150,151]. However, follow-up studies observed the contrary and indicated that 55% of cells harbouring replication-competent HIV-1 proviruses can clonally expand and persist *in vivo*[152-155]. Additionally, a study by Halvas *et al.* on 8 PLWH who had nonsuppressible low-level persistence viremia for >6 months despite adherence to cART indicated that this persistence was a result of large T cell clones[156]. There are three distinct mechanisms by which HIV-infected $CD4^+$ T cells undergo clonal expansion: (1) homeostatic proliferation, (2) antigen-driven proliferation, and (3) integration-site proliferation, and they will be discussed in greater detail below[84,157].

Homeostatic proliferation helps maintain the repertoire of memory $CD4^+$ T cells and is mostly driven by cytokines, such as interleukin (IL)-7 and IL-15[158]. Homeostatic proliferation can drive the proliferation of latently infected $CD4^+$ T cells without viral reactivation, and it has been shown to maintain the T_{TM} $CD4^+$ T cell reservoir in PLWH[131,144,159,160]. ***Antigen-driven proliferation*** was established to help maintain the T_{CM} $CD4^+$ T cell reservoir of PLWH[131]. This form of proliferation is driven by the periodic or persistent recognition and stimulation of a cognate

antigen by an HIV-infected, antigen-specific $CD4^+$ T cell (s)[159,161,162]. Various studies have shown an enrichment of replication-competent harbouring $CD4^+$ T cells specific for antigens such as HIV Gag, cytomegalovirus (CMV), and tetanus toxoid (TT) and influenza in PLWH[163-165]. Thus, in response to the cognate antigen stimulation, these cells can become activated and undergo normal physiological expansion. The activation of these HIV-infected, antigen-specific $CD4^+$ T cell(s) potentially triggers viral reactivation, leading to viral blips, an intermittent increase in viral load[166]. Although most of these activated cells die from the possible viral cytotoxic, a small population reverts to a quiescent state, which remains as the antigen stimulation wanes over time[159,161,162]. Gantner *et al.* provided evidence of antigen-specific proliferation in translation-competent T cells isolated from virally suppressed PLWH using TCRβ sequencing analysis[167]. They showed that the TCR of two expanded clonotypes were CMV and influenza-specific. Proviral integration into specific oncogenic gene sites such as *NFATC3*, *BACH2*, *MKL2*, and *STAT5B* has been associated with clonal expansion in PLWH[150,168-170]. According to Coffin *et al.*, ***Integration-site proliferation*** plays a minor role in viral reservoir persistence as only a small fraction of clonally expanded cells was shown to be driven by this mechanism[171]. Homeostatic and antigen-driven proliferation mainly occurs in the secondary lymphoid organ (SLOs), which could allow for viral persistence in the tissue[172].

1.7.2 Myeloid cells

Myeloid cells, such as monocytes, macrophages and dendritic cells (DCs), are also important cell subsets that can make up the viral reservoirs as they express the primary receptor for HIV entry, CD4 and the co-receptors, CCR5 and CXCR4[173]. Circulating monocytes can become infected during the acute phase of HIV infection[174]. These cells have a relatively short lifespan, typically lasting around 4-7 days, and can differentiate into macrophages or dendritic cells after their extravasation into various tissues. While host-restriction mechanisms, such as SAMHD1 and APOBEC3, limit the efficiency of HIV infection in monocytes, these infected cells can contribute to the spread of the virus to different tissues[173,174]. Upon reaching their destination, monocytes can differentiate into tissue-resident macrophages, like microglia in the brain, which can become long-lived[175]. A recent study by Veenhuis *et al.* demonstrated that HIV produced by monocyte-derived macrophages is capable of infecting bystander cells, which can further perpetuate the formation of HIV reservoirs and the further dissemination of the virus[176].

Dendritic cells (DCs), particularly follicular DCs (FDCs), can directly contribute to the HIV reservoir through a mechanism called *trans-infection*[175,177]. DCs utilize the C-type lectin receptors, DC-SIGN and Siglec-1, to capture and compartmentalize HIV without getting infected, after which they can mediate viral transfer by forming virological/infectious synapses with target $CD4^+$ T cells[35,178,179]. Therefore, by harbouring and transferring infectious virions, DCs act as crucial facilitators in viral reservoir establishment by seeding new cellular targets for HIV infection.

1.7.3 The central nervous system (CNS)

The central nervous system (CNS) is another important reservoir as HIV DNA has been found in brain cells, such as astrocytes, perivascular macrophages, and microglial cells, even in patients with undetectable viral loads and neurocognitive disorders[180]. The CNS has unique mechanisms that suppress viral replication and induce latency, such as the blood-brain barrier (BBB), which limits drug penetration and immune surveillance[180,181]. CNS infection primarily occurs through migrating infected $CD4^+$ T cells and monocytes crossing the BBB[182]. Although circulating monocytes have short lifespans and are not typically HIV reservoirs, they can infect and differentiate into long-lived cells tissue-resident cells like astrocytes, perivascular macrophages, and microglial cells[107,183]. Microglial cells, the major cellular reservoir in the brain, slowly divide and expand, leading to viral persistence and potential reseeding of the blood[184-186]. Viral escape in the CNS, which occurs when the virus is detected in the cerebrospinal fluid (CSF) even when viremia is suppressed, can also contribute to HIV persistence[187-190]. Persistent expression of Tat in the CSF indicates the existence of a CNS reservoir, which can cause viral rebound if ART is interrupted[191]. Studies in animal models, such as BLT mice and pig-tailed macaques, and human samples have provided evidence of persistent HIV or SIV-infected cells in the brain, even under long-term ART suppression, highlighting the importance of the CNS as an important tissue reservoir[185,192-195]. Recent studies have shown the presence of intact HIV proviruses in the CNS in approximately 70% of virally suppressed individuals who were sampled[196,197]. Moreover, latently infected $CD4^+$ T cells but not myeloid cells in the CNS have been shown to be the source of viral rebound upon ART interruption in PLWH[198].

1.7.4 Secondary lymphoid organs (SLOs)

1.7.4.1 *Structure*

Secondary lymphoid organs (SLOs) include the spleen, lymph nodes (LNs), mucosal-associated lymphoid tissues (MALT), and bronchial-associated lymphoid tissues (BALT). The gut-associated lymphoid tissue (GALT) is considered the largest and most well-defined component of MALT[199,200]. The SLOs primarily consist of immune cells, including DCs, monocytes, macrophages, and $CD4^+$ T cells, and serve as the central hub for adaptive immunity largely due to their unique architecture[200]. These organs are often located in areas highly susceptible to pathogen exposure, such as genitourinary, gastrointestinal, and respiratory tracts, to allow for the generation of an effective and timely immune response[201]. This is deemed possible with the aid of the lymphatic system comprised of lymphatic vessels that serve as a conduit to transport lymph fluid throughout the body and facilitate the movement of immune cells and soluble antigens to regional LNs[202]. The initiation and maintenance of the adaptive immune response take place in the LNs[203]. The lymph fluids, which transport antigens and immune cells, go through the LNs through the afferent lymphatic vessels and out through the efferent lymphatic vessels [202,204]. The incoming lymph fluid is directly in contact with the subcapsular sinus (SCS), a macrophage-rich area directly underneath the LN capsule where the initial screening and capture of antigens and pathogens occurs[203,205]. A large portion of the LN is called the cortex, which is divided into the outer (follicular cortex) and the inner cortex (paracortex) area[203,205]. The follicular cortex, also known as the B cell zone, is CXCR5 rich and comprised of B cell follicles containing B cells and follicular dendritic cells (FDCs). The paracortex, also known as the T cell zone, contains circulating/migrating naïve and memory T cells, which come in through specialized blood vessels called the high endothelial venules (HEVs) and DCs[203,206]. A specialized type of stromal cell, the fibroblastic reticular cell networks (FRCn), are found in the paracortex; the FRCn confers essential mechanical support for the LN and provides a scaffold for cell migration and interaction[207]. FRCn expresses chemokine ligands CCL19 and 21, which guide circulating/migrating CCR7-expressing T cells by allowing cells to adhere and crawl along these networks, thus facilitating interactions between T cells and DCs. Maximizing DC: T cell contact frequencies improves the ability of adaptive immunity to become activated[207]. FRCs are also major producers of IL-7 that promote the survival and proliferative capacity of naïve and memory T cells[208]. Together, the stromal and

immune cell networks in secondary lymphoid organs are critical for the long-term survival of T cells, and in the presence of cognate antigen(s), these networks help support their clonal expansion and differentiation.

1.7.4.2 *Secondary lymphoid organs as a viral reservoir*

Various pivotal studies have identified SLOs as major sites for viral replication and reservoir establishment. In one of such study, Brenchley *et al.* showed that in the gastrointestinal tract, there was a severe depletion of $CD4^+$ T cells in all stages of HIV infection in PLWH[209]. Estes *et al.* also highlighted the role of lymphoid tissue as a major HIV reservoir in a study involving ***non-human primates (NHPs)*** and virally suppressed PLWH[210]. The study showed that before ART initiation in Simian Immunodeficiency Virus (SIV)-infected macaques, the lymphoid organs contained >98% of the SIV RNA+ and DNA+ cells. Upon ART treatment, they could still detect viral RNA in the lymphoid organ, albeit at a reduced frequency, suggesting that continued low-level viral production in lymphoid tissues is a critical source of viral rebound after ART interruption[210]. This finding was corroborated by Cadena *et al.,* who observed the persistence of viral RNA in the lymphoid organs of SIV-infected macaques who were on suppressive ART for 1-3years[211]. In a study conducted on virally suppressed PLWH who has been on ART for a decade, it was observed that in the gut (ileum), the levels of viral DNA were 5-6 times higher compared to the peripheral blood[212]. Post-morterm deep tissue and viral transition analysis from four virally suppressed patients indicated that the sources of viral dispersal to different anatomic sites were mainly from the lymph nodes and gut tissues[195]. This observation implicates SLOs as a major hub for viral dissemination in PLWH. Collectively, these studies underscore the need to target SLOs in the development of curative strategies against HIV infection.

1.8 Eradication strategies to date

1.8.1 Shock and Kill approach

The shock-and-kill approach is a strategy that involves the purging and eradication of the HIV reservoir using latency reversal agents (LRAs) to reactivate viral gene expression under ART suppression. The reactivation of viral gene expression, or the “shock”, is then followed by cell death by viral cytopathic effects or immune-mediated clearance[84,213]. Several classes of LRAs have been extensively studied *in vitro, in vivo,* and *ex vivo* to examine their “shock” potential for use in

ART-interruption clinical studies. These include, but are not limited to, Protein kinase C (PKC) agonists, Tat vaccines, SMAC mimetics, epigenetic or chromatin remodelers such as HDAC (Histone deacetylase) inhibitors, MAPK agonists, Toll-like receptor agonists (TLR-7, TLR-9), immunotherapy (IL-7, IL-2), and PI3K/Akt agonists[84,213]. However, one limitation of this approach is its inability to differentiate between replication-competent viruses and defective proviruses[214]. Clinical studies using HDAC inhibitors (HDACi) like vorinostat, panobinostat, romidepsin, and disulfiram have demonstrated increased cell-associated HIV RNA production and/or plasma viremia after *in vivo* administration[215-218]. However, none of these LRAs have yet to yield measurable reductions in the reservoir size in PLWH on ART. One hurdle that has been identified is that the clearance of infected cells, or the "kill", is insufficient or suboptimal[219-221]. This suboptimal killing may partly be due to the significant impairment of HIV-specific CTL function in PLWH; therefore, after LRA reactivation, cells reexpressing viral expression cannot be killed[222,223]. It is also established that three of the HDACi that were tested in clinical trials (vorinostat, romidepsin, and panobinostat) impaired CTL-killing *in vitro* by suppressing IFN-γ production and also inhibited NK cell function[224,225]. Additionally, *in vitro* and *ex vivo* studies have shown that there is differential responsiveness in viral gene reactivation to different LRAs across distinct memory $CD4^+$ T cell subset; thus a need for a combination of different LRAs may be needed to elicit a global reactivation[226-229]. Another hurdle is the limited penetration of ART to sanctuary sites such as the CNS, GALT, lymph nodes, etc.[185,230-232]. Studies involving PLWH and NHPs showed that panobinostat does not penetrate the CNS, and the concentration of romidepsin in the CSF was only 2% of the levels found in the plasma, respectively[233,234]. Additionally, various studies showed that lower ART concentration in the lymphatic tissues of PLWH is associated with persistent viral replication[210,235]. Thus, continued research into potential LRAs in combination with immune-mediated clearance mechanisms and enhancement of the ART delivery system for better tissue penetration is needed to reduce the HIV reservoir size significantly.

1.8.2 Block and lock approach

The "block" and "lock" approach is based on the notion that HIV provirus can be permanently silenced by epigenetic factors[236,237]. This strategy focuses on inducing a deep or irreversible latent state, preventing viral gene expression even after treatment cessation to achieve long-term viral suppression[238,239]. One approach is to modify the epigenetic and transcriptional status of cells harbouring replication-competent provirus. For example, Tat inhibitors such as

didehydro-cortistatin A (dCA), HIV integrase inhibitors such as LEDGINs, RNA-induced epigenetic silencing using short interfering or short hairpin RNA, Jak-STAT inhibitors, mTOR inhibitors, and bromodomain-containing protein 4 (BRD4) inhibitors have all been described to achieve prolonged silencing of the HIV provirus[239,240]. The fact that elite controllers can naturally suppress viral transcription without ART provides important proof-of-concept for the block-and-lock approach to achieve durable HIV control. However, no FDA-approved drug(s) are currently available, and whether this strategy will work in PLWH is still under investigation.

1.8.3 Genome editing

To date, three individuals living with HIV have experienced long-term remission after receiving stem cell transplants from donors with a homozygous mutation in the HIV co-receptor CCR5 (CCR5Δ32/Δ32)[77-81]. Moreover, the CCR5 antagonist, Maraviroc, has been found to significantly improve viral suppression and increase $CD4^+$ cell counts in PLWH[241]. These studies indicate that the blockade or removal of CCR5 represents a powerful approach to preventing or suppressing viremia. There have been many genome editing technologies that have been used to examine the therapeutic potential of blocking/removing CCR5, such as transcription activator-like nucleases (TALENs), zinc finger nucleases (ZFNs), and clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease 9 (Cas9)[242-244]. A 2014 clinical trial in PLWH demonstrated that ZFNs to edit the CCR5 gene in $CD4^+$ cells were proven safe[245]. The majority of patients experienced a decrease in HIV DNA, with one patient showing undetectable HIV RNA levels. Recently, HIV infection was eliminated in virally suppressed humanized mice whose CCR5 was edited with CRISPR-Cas9[246]. Thus, these studies highlight the potential for CCR5-editing technologies as a therapeutic strategy for PLWH as an alternative approach to bone marrow transplantation in order to achieve long-term control of HIV infection.

1.9 Models and molecular tools used to study HIV latency

1.9.1 Primary $CD4^+$ T cell latency model

Several primary $CD4^+$ T cell models have been developed to aid in studies on the mechanisms of HIV latency as well as to screen for potent LRAs[121,247-249]

1.9.1.1 *Generation of latency directly in resting memory T cells*

Resting memory $CD4^+$ T cells make up a significant portion of the $CD4^+$ T cell reservoirs but are not readily susceptible to HIV infection, as described earlier. In order to establish a pool of latently-infected resting memory T cell subsets, Doherty *et al.* demonstrated that spinoculation, a method that increases viral binding to resting $CD4^+$ T cells by ~20-fold, enhances both binding and reverse transcription[250]. Importantly, spinoculation can improve infection of resting $CD4^+$ T cells and increase latently-infected cells that can reactivate the virus upon stimulation[251,252]. Another method explored to improve infection of resting $CD4^+$ T cells was the preincubation of these cells with chemokine ligands constitutively expressed in lymphoid organs. Eckstein *et al.* highlighted the ability of lymphoid tissues to support active HIV-1 replication in resting, naive T cells *ex vivo*[253]. Therefore, Saleh *et al.* explored the effect of preincubating resting $CD4^+$ T cells with chemokine ligands (CCL19 and 21)[254]. They observed that the preincubation with these ligands facilitated an increase in HIV entry and integration in resting $CD4^+$ T cells, and this increase was linked to chemokine-induced changes in the actin cytoskeleton of these cells[254,255]. Despite the fact that the resting memory T cell model generates a very low number of latent $CD4^+$ infected cells, it has been used for LRA screening[256].

1.9.1.2 *Post-activation latency model*

In this model, primary $CD4^+$ T cells isolated from the PBMC of healthy aviremic donors are activated with an activating agent such as anti-CD3/28 for a couple of days, and the activated cells are infected with an HIV vector[121]. These infected cells are then cultured for several days or weeks to allow cells to revert to a resting memory/latent state. An inherent limitation of this model is the gradual cell death that follows the activation and proliferation of $CD4^+$ T cells. Consequently, prolonged cell culture after activation and HIV infection might result in a significantly reduced population of viable cells. Different strategies have been developed to allow for the prolonged survival of these resting latent cells to carry out various studies. The first post-activation latency model was developed by Sahu *et al.,* where they co-cultured HIV-infected $CD4^+$ T cells co-cultured with a feeder cell H80 (a brain tumour-derived cell line) for approximately two months to allow the long-term survival of the infected cells[257]. This method allowed the development of latent central memory cells, which were reactivatable with an LRA[258]. Marini *et al.* cultured activated infected cells with IL-7, a homeostatic cytokine that has a protective effect against cell

death, allowing cell survival for up to two months[259,260]. The Bosque model activated and induced naïve into a T_{CM}-like phenotype before infection and cultured cells in IL-2; this allowed the survival of latently infected cells for up to four weeks[160,261,262]. Yang *et al.* transduced activated cells with Bcl-2, an antiapoptotic protein downstream of the IL-7 signalling prior to HIV infection and established latent cells for up to two months[263]. It is important to note that most of these studies rely on viral protein expression or the use of an HIV vector encoded by a single fluorescent reporter driven by the HIV long terminal repeat (LTR) promoter to distinguish productive infection from latently infected cells[263]. However, the development of a molecular tool called the ***dual fluorescence HIV reporter*** now allows us to distinguish productive infection from latent infection (*further discussed in section 1.9.1.3*).

1.9.1.3 Molecular tool for HIV latency study: Dual fluorescent-protein HIV reporter

In order to distinguish between latently and productively infected T cells in live culture, several studies have described the generation and use of a dual-fluorescent protein HIV reporter[214,247,263-271]. The basic construct is that a fluorescent reporter is under the transcriptional control of the HIV LTR, whereas a second fluorescent marker is under the control of an independent, constitutively active mammalian promoter that is turned on post-integration. This reporter construct can be used in primary or T cell lines and has been used to study LRA potency and proviral integration dynamics. For instance, the reporter HIV_{GKO} was used to further highlight the heterogeneous nature of the latent reservoir and the drawbacks of the shock and kill strategy[214,272]. Using primary $CD4^+$ T cells infected with HIV_{GKO}, Battivelli *et al.* identified that the proviruses within the population of non-reactivated latently infected cells were predominantly integrated into the repressive chromatin environment found within lamin-associated domains (LADs) at the nuclear periphery[214]. This study also identified that the cell-associated HIV RNA quantification used to evaluate the efficacy of various LRAs does not accurately represent the number of cells undergoing viral reactivation. Therefore, the use of dual fluorescent reporters provides a more precise method, enabling the direct quantification of cells undergoing reactivation. Additionally, dual fluorescent reporters allow for real-time monitoring and analysis of this population, which could provide valuable insight into HIV latency dynamics *in vivo* and *in vitro*. However, a significant limitation of published reporter HIV strains is that they lack expression of accessory genes *Nef, Env* and *Vpu*. These accessory genes play crucial roles in HIV pathogenesis

and replication, as summarised in *Table 1*, and their absence in these published reporter HIV strains might limit the ability to fully recapitulate the behaviour of wild-type HIV in experimental settings. Therefore, one of the primary goals of my dissertation project was to develop a new dual fluorescent reporter containing these missing accessory genes that will be used to assess latently infected cells *in vivo* and *in vitro* for my studies.

1.9.2 Humanized mice models to study HIV latency in vivo

The study of HIV infection *in vivo* has been hampered by the fact that HIV does not infect murine cells due to species-specific differences in the receptors and co-receptors required for viral entry and replication. The development of humanized mice has allowed researchers to study viral pathogenesis *in vivo* and provided a platform to test new therapeutic/prevention strategies[273]. In general, humanized mice encompasses the approach of using immunodeficient mice as recipients of human immune cells and tissues to study mechanisms of HIV entry, replication and persistence *in vivo*[274-277]. Different humanized mouse models are used in HIV latency studies, some of which will be discussed below.

i. *SCID-hu model:* The first humanized mouse model was created by the Weissman lab in 1988, using mice that exhibited severe combined immunodeficiency (SCID) to study the human immune system[275,278]. These SCID mice carry mutations in the gene for the catalytic polypeptide of DNA-activated protein kinase (*Prkdcscid*)[275]. This mutation disrupts an essential process for DNA repair and ultimately prevents the successful rearrangement of T cell and B cell receptors. As a result, these mice do not possess mature T and B cells. The SCID mice were implanted with human fetal thymus, a tissue required for T cell education and development. Simultaneously, they were injected intravenously or intrathymically with a source of human progenitor cells (fetal human liver cells). Approximately 6 to 7 weeks following implantation, researchers detected human T cells circulating in the mouse bloodstream. However, a limitation of this model is that the population of human T cells was no longer detectable after the 9th week. The *SCID-hu model* was the pioneer mouse model used to explore HIV-1 infection after infection with HIV-1.sub.JR-CSF (an infectious molecular clone)[279]. In this infection model, viral replication was detected in the thymus and lymph node. Additionally, this model was the first to assess the efficacy of the nucleoside analog

reverse transcriptase inhibitor azidothymidine (AZT) as an antiretroviral treatment for HIV-1, where it was shown to suppress viral replication[280].

ii. *SCID–human thymus/liver (SCID–hu-Thy/Liv) model:* Given the limitation of the SCID-hu model, where the reconstitution of human cells is not sustained beyond nine weeks, Namikawa and colleagues made modifications. In the improved model, they co-implanted syngeneic human fetal thymus and liver tissues underneath the kidney capsule of a SCID mouse[278,281]. This process allows for the production of a new vascularised organ that functions as the human thymus that supports the thymopoiesis of human T cells[282]. In the *SCID–hu-Thy/Liv model*, there was a long-term and stable reconstitution of human cells of multiple lineages for up to about 15 months and minimal graft-versus-host disease (GvHD). However, a significant limitation of this model is that there is no systemic reconstitution with T cells, as most of these cells are confined within the implant[283,284]. Therefore, invasive surgery is required for HIV infection and analysis[285]. Additonally, in this model, there are little or no primary cellular or humoral immune responses[282]. Although this limitation can be partially addressed by using a NOD/SCID common gamma chain null (NSG) strain of immunodeficient mice for xenograft implantation to develop a T cell-only mouse model[286]. In this mouse strain, the NOD mutation affects the development of myeloid cells and mouse NK cell activity, and the IL-2 receptor common gamma chain deletion (IL2Rγnull) impairs the development and function of B-, T- and NK- cells[287,288]. Despite the limitation of the *SCID–hu-Thy/Liv model*, it has been used to test the efficacy of HIV-1 maturation and fusion inhibitors[289,290]. Brooks *et al.* also used the *SCID–hu-Thy/Liv model* to establish that HIV latency can be generated during thymopoiesis[291].

iii. *Human peripheral blood mononuclear cell (hu-PBMC) model:* The *hu-PBMC model*, which I will be utilizing for my experiments, is a method of humanizing mice by introducing whole human PBMC or sorted individual immune cells into immunodeficient mice. These human cells can be delivered via intraperitoneal, intravenous or intrasplenic routes[275]. Upon cell transplantation, this model is well

reconstituted with major cells required for HIV infection studies, such as the human T cells and myeloid cells (to a lesser extent) in various organs[292]. This technique, first developed in 1988, provides a quick and straightforward approach to humanizing mice. The initial model was created by injecting human peripheral blood leukocytes (PBL) into a SCID mouse, either intraperitoneally or intravenously[293]. A human T cell population was detected in the periphery as early as two weeks post-engraftment. The hu-PBMC mouse model has been shown to be susceptible to HIV-1 infection, with approximately half of the infected animals maintaining a persistent infection for up to 16 weeks[294,295]. However, due to the SCID recipient mouse's functional innate immune activity, the engraftment success rate of human cells is relatively low[296-298]. Therefore, Hesselton *et al.* employed NSG immunodeficient mice as recipients for human PBMCs and observed a high level of human cell engraftment four weeks post-injection[299]. These mice were also highly susceptible to HIV-1 infection[299-301]. In addition to the NSG, other immunodeficient mouse strains have been developed to allow for better human cell engraftments, and this includes the NOD mouse strain with the IL2Rγ and Rag1 null mutation[302]. However, a significant challenge with the hu-PBMC model is the rapid onset of xenogeneic graft-versus-host disease (GvHD) as early as three weeks post-engraftment. This occurs because the injected human cells, being mature, quickly activate and start attacking host tissues[282]. This rapid onset of GvHD can limit the duration of experiments. The hu-PBMC model has been used to study HIV latency, the rapid screening of ART, and to test newly developed antiretroviral regimens for HIV studies[303-305]. Additionally, this model has been used for studying approaches to eradicate HIV infection, such as the use of gene-editing technologies[306-309].

iv. *Human-hematopoietic stem cell (hu-HSC) model:* The *hu-HSC model* involves the introduction of purified human $CD34^+$ hematopoietic stem cells (HSC) into an immunodeficient recipient mouse, which are sublethally irradiated to remove existing cells in the bone marrow[302,310-312]. The HSCs used for cell transplant are typically from umbilical blood cords or aborted fetal livers[274]. These cells can be introduced into the recipient's bloodstream through various routes, including intravenous, intracardiac, intrahepatic or facial vein injections. After injection, the human $CD34^+$ HSC cells

migrate and engraft into the mouse's bone marrow, where hematopoiesis occurs[313]. As the human HSCs differentiate, they develop into various types of blood cells, thereby facilitating the formation of comprehensive reconstitution of cells belonging to myeloid and lymphoid lineages in various organs, including bone marrow, thymus, liver, digestive, lymph nodes, spleen, lung, and reproductive tracts[314]. This includes a variety of cell types, such as T cells, B cells, dendritic cells, macrophages, and others. The developing differentiated T and B cells undergo positive and negative selection in the mouse thymus; therefore, GvHD development in this model is significantly delayed to about 14-20 weeks post-cell transplant[313,315]. The hu-HSC model has been used to evaluate the efficacy of broadly neutralizing antibodies (bnAbs) in suppressing viral replication[316-318]. Furthermore, sterilizing cures have been demonstrated in the hu-HSC model in two studies utilizing gene-editing technologies, establishing a proof-of-concept for this approach[246,319].

v. *Bone marrow-liver-thymus (BLT) mice:* This development of the *BLT mice* combines *SCID–hu-Thy/Liv* and *hu-HSC* models. In this model, immunodeficient mice were sublethally irradiated and implanted with human fetal thymus and liver tissue underneath the kidney capsule[320,321]. One to two weeks after the implantation of the human fetal liver and thymus tissues, the mice are sublethally irradiated and injected with autologous human $CD34^+$ hematopoietic stem cells (HSCs) isolated from the fetal liver. Like the hu-HSC model, this humanization process allows for a robust systematic reconstitution of immune cells of multiple lineages in various organs[322]. In comparison to other mice models, the onset of GvHD is delayed in the BLT model to around 4-5 months post-engraftment due to the development of MHC-restricted T cells facilitated by the presence of an autologous human thymic environment[323,324]. In 2013, Lavender *et al.* developed a *BLT mouse strain* by utilizing an immunodeficient mouse with triple gene knockout (CD47, common gamma chain (γc), and Rag 2 null mutations), which is resistant to GvHD[325,326]. The delay or resistance of GvHD in the BLT model allows for long-term HIV latency studies. HIV-infected BLT mice can be treated with cART to model HIV latency establishment similar to that observed in ART-treated PLWH[327-

[329]. The BLT mice model has also been used to evaluate the block and lock strategy[125,126].

Overall, one of the advantages of the mouse animal model is that they are small and cost-effective, allowing a large number of animals to be accommodated in a given study. Also, the short gestational period of mice makes them highly valuable for gene-editing-related experiments for HIV studies. One major disadvantage of this model is the short lifespan of the mice, which will prevent longitudinal studies. Additionally, the process of humanization in certain models relies on the availability of fetal tissue, which may pose a significant challenge.

1.9.3 Non-human primate model

The non-human primary (NHP) model of HIV infection is the gold standard animal model for testing vaccine candidates, new drug therapies or novel cure strategies. These include Asian monkeys such as rhesus (*Macaca mulatta*), cynomolgus (*Macaca fascicularis*), and pig-tailed (*Macaca nemestrina*) macaques are the most used[330]. Macaques are anatomically, physiologically and immunologically similar to humans and can be infected with simian immunodeficiency virus (SIV) or simian/human immunodeficiency virus (SHIV)[331].

Simian Immunodeficiency Virus (SIV) belongs to the lentivirus family closely related to HIV. SIV is prevalent in numerous species of non-human primates native to Africa, including sooty mangabey monkeys, Chimpanzees, African green monkeys, and mandrills, among others[332,333]. Interestingly, despite maintaining a high viral load, these natural hosts of SIV do not get chronically ill due to their infections, making them unsuitable as models for disease pathogenesis. Different SIV strains are classified by the species they are isolated from. For example, SIVagm is isolated from the African green monkeys, SIVcpz is isolated from chimpanzees, and SIVsmm is isolated from the sooty mangabey monkeys. In 1985, another SIV strain, SIVmac, which is not naturally occurring, was unintentionally developed in the lab when SIVsmm was used to infect Rhesus macaques[334]. Two of these SIV strains are closely related to the causative agents of human AIDS: SIVcpz to HIV-1 and SIVmac to HIV-2[335]. The retroviruses, HIV and all SIVs share open reading frames for the *Nef*, *Tat*, *Pol*, *Vif*, *Vpr* and *Rev* genes, but the *Vpu* gene is unique to SICcpz[336]. The gene *Vpx*, thought to have evolved from *Vpr* due to similarities in amino acid sequences, is unique to SIVmac[337,338]. The Vpx protein enhances HIV-1 infection in macrophages and dendritic cells by neutralizing the impact of SAMHD1, a host

protein that significantly reduces HIV-1 reverse transcription[339-341]. There are fundamental differences between HIV-1 and SIV that limit the use of SIV-macaque models for certain studies. For example, the genetic differences of the HIV envelope glycoprotein prevent the study of HIV-1 neutralizing anti-Env responses[342,343]. Additionally, while most ART drugs are effective against SIV, non-nucleoside reverse transcriptase inhibitors (NNRTI), such as nevirapine and efavirenz, are active only against HIV-1 and not against HIV-2 or SIV[60]. Therefore, to address these limitations, a chimeric virus that combines genes from SIV and HIV, SHIV, was developed to study different responses to HIV better[344]. Various SHIVs that have been developed includes: SHIVs expressing HIV-1 *Env*, HIV-1 *Pol* genes, and stHIV-1 (simian-tropic HIV-1), a lab-generated strain of HIV-1 that has been engineered to replicate in NHPs[345,346].

Rhesus Macaques (RMs) are classified into two types based on their geographical origins: India and China[347]. The Indian-origin RMs are the most extensively characterized and commonly utilized for HIV/AIDS research. RMs are commonly challenged with the SIVmac strain and develop infection progression similar to what is seen in humans[348]. Upon infection with either $SIVmac_{251}$ (an uncloned viral isolate) or $SIVmac_{239}$ (an infectious molecular clone), RMs typically exhibit a peak high viral load, often surpassing 10^7 RNA copies/mL of plasma, and maintain these elevated levels during the chronic phase of the infection[330]. $SIVmac_{239}$ causes a more rapid disease progression than $SIVmac_{251}$, as infection of RMs with $SIVmac_{239}$ causes a profound depletion of $CD4^+$ T cells in the GALT, and it takes about 1-2 years for SIV-infected RMs to progress to AIDS[349-351]. AIDS progression induced by SIV infection is characterized by severe CD4 depletion, susceptibility to opportunistic infections, significant weight loss, the occurrence of opportunistic tumours, and widespread granulomatous inflammation in the brain. Pig-tailed macaques (PTMs) are the second most used macaques, next to RMs. Compared to RMs, PTMs rapidly progress to AIDS on an average of 42 weeks after $SIVmac_{239}$ infection, and this progression is often associated with thrombocytopenia (TCP) and severe $CD4^+$ depletion in the GALT[352-355]. PTM lack the host restriction factor, TRIM5α, a protein that in many other species blocks HIV-1 replication; thereby, they can establish low-level viremia when infected with construct derived from HIV-1[356]. Additionally, the absence of TRIM5α makes them suitable models for studying simian-tropic HIV-1 (stHIV-1)[357]. Cynomolgus macaques (CMs), also known as crab-eating macaques, are sourced from the island of Mauritius and are less commonly used for HIV/AIDS research. CMs have a unique genetic characteristic and have only seven identified MHC haplotypes[358]. Depending on

the route of infection, viral load and host, CMs display variable levels of viremia[330]. Bruel *et al.* showed that when CMs with the H6 MHC haplotype are intrarectally infected with a high viral load of $SIVmac_{251,}$ they showed disease progression and viremia levels similar to that seen in humans[359]. However, they spontaneously controlled SIV viremia for up to 5 years due to the MHC haplotype, H6[360].

NHPs have provided fundamental insights into immune responses and the establishment and distribution of viral reservoirs across various tissues/organs[361]. Thanks to physiological similarities with humans, NHP models do not need major surgery to be developed. The physiological similarities enable the study of diverse viral transmission routes- intravaginal, intrarectal, penile, oral, and mother-to-child[362]. Given their longer lifespan and lack of GvHD development compared to smaller animal models, NHPs also facilitate longitudinal studies that would be ethically challenging in humans, such as the evaluation of pharmaceutical interventions or vaccine strategies[122]. Furthermore, NHP models have been pivotal in uncovering viral escape mechanisms from cytotoxic T lymphocytes (CTLs) and the roles of natural killer (NK) cells in controlling the virus[122,363]. Importantly, these models provide access to various tissues and organs that are difficult to obtain from PLWH and allow for the evaluation of various vaccine strategies, thus paving the way for more comprehensive research[364]. A major challenge faced with the NHP model for HIV/AIDs research includes the cost of obtaining and housing the study animals, thereby limiting the number of animals that can be accommodated in a given study[362].

1.10 Overall Dissertation Overview: Rationale, Hypothesis and Objectives

1.10.1 Rationale

It is well established that long-lived cells harbouring replication-competent provirus represent a major barrier to HIV cure. An important component of the HIV reservoir is memory $CD4^+$ T cells (T_{CM} and T_{EM}), which have unique recirculatory migrational behaviours that are instrumental for their long-term survival in the body. Their ability to enter both non-lymphoid and lymphoid organs is critical for immunosurveillance against invading pathogens and can maintain effector functions through repeated cell-cell interactions and access to survival cytokines. Therefore, it is unsurprising that secondary lymphoid organs (SLOs) are important tissue sites for HIV replication and persistent infection, with the blood and lymphatic system acting as conduits

for HIV dissemination. An important and unanswered question is whether latently infected $CD4^+$ T cells retain their recirculation capacity and ability to access the same survival and tonic TCR signals that help maintain normal memory T cell numbers. This has important implications for identifying the nature and location of the HIV reservoir and the fact that this latent reservoir is dynamic and constantly in flux. The **primary objective of my dissertation project** was to generate a visualization-based approach to describe the phenotypic and migratory characteristics of latently-infected primary memory $CD4^+$ T cells and to apply *in vivo* microscopy approaches to study their motility behaviours directly in lymphoid tissues.

1.10.2 Hypothesis

Latently infected $CD4^+$ T cells display similar migratory behaviour as uninfected central memory $CD4^+$ T cells within the T cell zone in the LNs, allowing for access to survival and proliferation signals that facilitate their long-term survival.

1.10.3 Objectives

This dissertation has two main objectives:

1. To develop and characterize a full-length dual fluorescent-protein HIV reporter, this newly developed reporter will contain all vital HIV accessory genes and allow for the visualization of latently infected $CD4^+$ T cells, enabling me to carry out my studies.
2. To assess the migratory capacity of latently infected $CD4^+$ T cells *in vitro* and *in vivo.*

1.10.4 Significance and Impact

The development of dual fluorescent HIV reporter allows us to distinguish productively infected from latently infected cells, and various studies have utilized this molecular tool to gain a mechanistic understanding of HIV latency. However, these viral constructs do not contain all the essential accessory viral genes and therefore, do not entirely replicate biological HIV infection. My dissertation project focused on developing a newly improved full-length dual fluorescent reporter to visualize and study the migration behaviour of latently infected $CD4^+$ T cells *in vitro* and *in vivo*. Understanding the migratory behaviour and recirculation dynamics of latently infected $CD4^+$ T cells will give us valuable insight into how to develop new therapies to reduce or eliminate viral reservoirs.

CHAPTER 2

MATERIALS AND METHODS

2.1 Primary Cell Culture

2.1.1 Isolation of Human CD4$^+$ T cells

Human CD4$^+$ T cells were routinely isolated from the peripheral blood mononuclear cells (PBMCs) buffy coat fraction of healthy donors obtained from NetCAD (Canadian Blood Services) using Ficoll-Paque PLUS density gradient centrifugation with cell purity >95%[365]. One million naïve CD4$^+$ T cells were activated by adding 25µL of dynabeads coated with anti-human CD3/CD28 antibody (Life Technologies Cat. #11131D) in complete RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS; VWR Seradigm Cat. #1500-500), 2 mM GlutaMAX (Gibco Cat. #3050-061), 1mM sodium pyruvate (Corning Cat. #25-000-CI), 10mM HEPES (Sigma-Aldrich Cat. #H4034) and 1% Penicillin/Streptomycin (Pen/Strep). 2 days post-activation the beads were removed, and cultured in medium containing 50 IU/mL rh-IL-2 (Biotechne Cat #202-IL-500) under 37°C/5% CO_2 conditions for at least 4 days, keeping cell density at 0.2-1×10^6 cells/mL. Days 6 and 7 expanded cells CD4$^+$ T cells were used for experiments. PBMC derived from donors are anonymized, with no associated data provided about the donor's gender, race, or health status. Studies using human blood products were approved by the University of Manitoba Biomedical Research Ethics Board (B2015:030).

2.1.2 MAGI CCR5 and HEK293T culture

MAGI-CCR5 cells were obtained from the NIH ARRRP (Cat. #3522) and cultured in complete DMEM supplemented with 10% Fetal Bovine Serum, 2 mM GlutaMAX, 1mM sodium pyruvate, 10mM HEPES and 1% Penicillin/Streptomycin (Pen/Strep) under 37°C/5% CO_2 conditions. The parental cell line of MAGI, which has not been authenticated, is a HeLa cell clone, a female cell line derived from the uterus.

HEK293T, an immortalized human embryonic kidney cell, was obtained from ATCC (Cat. #CRL-3216) and cultured in complete DMEM under 37°C/5% CO_2 conditions.

2.2 Viral plasmid construction and viral stock preparation

The dTomato DNA insert was amplified from pHAGE-EF1α-dTomato (available in the Murooka's lab) using primers 5'-GCC ACC ATG GTG AGC AAG GGC GAG GAG GTC-3,' and 5'-GCT ACC CGG GTT ACT TGT ACA GCT CGT CCA-3' by PCR and was inserted into the R5-tropic HIV-CRMZY NL4-3 backbone (available in the Murooka's lab) to replace ZsGreen upstream of mammalian Elongation Factor 1 α (EF1α)-HTLV1 chimeric promoter using unique restriction enzyme sites XmaI (NEB Cat. # R0180S) and NcoI (NEB Cat # R3193S). The resulting plasmid was then re-cloned by the insertion of Nef-ZsGreen amplified from HIV-CRMZY using primers 5'-TGC ACG CGT GGA GGG GGC GGT ATG GCC CAG TCC AAG CACG-3' and 5'-AGA TCC GCG GTC AGG GCA AGG CGG AGC CGG-3'. The resulting plasmid, termed 'HIV TosZy,' was isolated using GenElute HP Plasmid Maxiprep Kit (Sigma) and sequenced on both strands using Sanger sequencing before transfection into HEK293T cells. HIV TosZy was pseudotyped with Vesicular stomatitis virus G (VSV G) protein and used for infection studies. HIV stocks were produced by the transfection of HEK 293T cells using JetOptimus reagent (Polyplus Cat. #117-15). Viral supernatants were collected 48 hours after transfection and centrifuged at 1500xg after incubation with Lenti-X™ concentrator (Takara Bio Cat. #631232) for 45mins. Virus pellets were resuspended in 1/100 volume of RPMI, aliquoted and stored at -80℃ until use. Viral stocks were titrated using MAGI.CCR5 and expressed as blue forming units (pfu) per mL.

2.3 HIV infection in 3D collagen matrix

Using an optimized protocol, One million $CD4^+$ T cells were embedded into 270µL of collagen mix at a concentration of 1.7mg/mL [20µL 10X MEM (Thermo Scientific), 10µL sodium bicarbonate (Gibco), 148µL bovine Type I collagen (PureColl, Cat. #5005-100ML), and 92 µL cell/virus suspension in medium supplemented with 50 IU/mL rh IL-2] into 48-well plates for 3 hours under 37°C/5% CO_2 conditions[365]. All infections were done at an MOI of 0.1, apart from the viral titration assay where the MOI used was indicated. The collagen gels were digested with 1mg/mL collagenase D (Roche Cat. #11088866001), and recovered cells were washed and resuspended in 1mL fresh complete media with rh IL-2 (50 IU/mL) in a 24-well plate. After 48 hours, 10nM of anti-retroviral drug (Raltegavair) was added to the media to prevent multiple rounds of infection. In cases where an anti-retroviral drug was used as a control, $CD4^+$ T cells were

co-cultured with 10nM of Raltegavair in the collagen mix and after resuspension of cells. Drugs were all obtained from the NIH HIV Reagent Program. $CD4^+$ T cells were incubated for a total of 5 days before flow cytometry analysis for all experiments, while for time course experiments, cells were analyzed on days 2, 5 and 7, and the culture media was replaced every 2 days and supplemented with rh IL-2. For chemokine or cytokines experiments, $CD4^+$ T cells were preincubated for 24 hours with chemokines CCL19 (10nM; Biolegend Cat. #582102) and CCL21 (10nM; Biolegend Cat. #424902) and rh IL-7 (50ng/µL; Biolegend Cat. #581904), and cells were washed before infection in collagen.

2.4 Live cell imaging in 3D collagen chambers and imaging analysis

Glass slide chambers were constructed as previously described[366]. 2-4 million $CD4^+$ T cells were infected with HIV TosZy for 2 days, washed and resuspended in a bovine Type I collagen to achieve a final concentration of 1.7mg/mL in each chamber. For the control population control, 1 million uninfected $CD4^+$ T-cells were labelled with Celltracker Green (CMFDA; 1.5µM), washed and embedded into collagen. Collagen chambers were allowed to solidify for 45-60 minutes at 37°C/5% CO_2 conditions and placed onto a custom-made heating platform attached to a temperature controller apparatus (Warner Instruments). A thermocouple was used to continuously monitor and maintain the chamber temperature at 37± 0.5 °C. A multiphoton microscope with two Ti:sapphire lasers (Coherent) was tuned to between 780nm and 950-980nm for optimized excitation of ZsGreen and dTomato, respectively. For four-dimensional recordings of cell migration, stacks of 11 optical sections (512×512 pixels) with 13 z-stacks at 4 µm spacing, 30 s per cycle, and 121 total cycles were acquired to provide imaging volumes of 40µm in depth. Emitted light and second harmonic signals were detected through 460/50nm, 525/70nm and 595/50nm dichroic filters with non-descanned detectors. All images were acquired using the 2031.0 N.A. Olympus objective lens (XLUMPLFLN; 2.0mm WD). Time-lapse microscopy images will be processed using available 4D image rendering software, Imaris 8.3 (Bitplane), to generate maximum intensity projections (MIPs) for export as Quicktime movies and to perform automated 3D cell tracking for motility analyses. Motility parameters were calculated as described previously[367]. Mean velocities were defined as the average distance a cell moved within the time period between the two successive frames. Arrest coefficient was defined as the proportion of time

on a track during which a cell moved at a speed less than 4µm/min. This was calculated using a custom script in MATLAB (Mathworks).

2.5 Generation of humanized PBMC (hu-PBMC) Mice

5- to -6 weeks old female NOD-SCID (NOD.CB17-*Prkdc*^{scid}/NCrCrl) mice (Charles River) were treated through daily intraperitoneally (IP) injections with 25µg of anti-asialo GM1 antibody (Wako Chemicals Inc. Cat #986-10001) for 2 days to deplete dysfunctional NK cells. Fresh peripheral blood mononuclear cells (PBMCs) from buffy coat fraction of healthy donors obtained from NetCAD (Canadian Blood Services) using Ficoll-Paque PLUS density gradient centrifugation were injected intraperitoneally (IP) at 50 million cells per mouse. 3-to 5- weeks post PBMC injection, mice were bled by submandibular bleeding and checked for PBMC engraftment, and mice that showed reconstitution with T cells (>400 $CD4^+$ T cells/µl of blood) were used for experiments (**Figure 3. 14A**). Most experiments should reach an endpoint within 8-10 weeks after PBMC engraftment or at the onset of xenogeneic graft-versus-host disease (GVHD) symptoms that can be monitored by weight loss. Mice with a weight loss of >20% indicate GVHD development and are excluded from the study and then euthanized.

2.6 HIV infection of hu-PBMC mice

For tissue localization assay, hu-PBMC mice were injected intraperitoneally with 5×10^6 infectious units of HIV TosZy. Mice were bled by submandibular bleeding, and various organs (Spleen, liver, lungs and lymph nodes) were harvested after mice were euthanized 3 days post-infection, from which cells were isolated and stained for flow cytometry analysis.

For *in vivo* migratory assay in the popliteal lymph node (PopLN), Hu-PBMC mice were injected in the right footpad with 1.5×10^5 of HIV TosZy 24 and 48 hours before analysis by intravital imaging.

2.7 Tissue processing for flow cytometry

Spleen processing: Mice spleens were surgically removed and transferred to a 50mL Falcon tube containing sterile PBS on ice. The spleens were placed on a 40µm cell strainer in a 6-well plate containing 1 mL sterile PBS. Splenocytes were obtained by mechanically disrupting the spleens using a plunger from a 1 mL syringe. The cell suspension was filtered through a 40µm cell strainer and transferred to a FACs tube, and centrifuged at 400xg for 5mins. The red blood cells from the

splenocyte suspension were lysed by adding 5mL Ammonium–chloride–potassium (ACK) lysing buffer, and the cells were collected by centrifugation. Cells were ready for flow cytometry processing.

Lymph node (LN) processing: Mice spleens were surgically removed and transferred to a 50mL Falcon tube containing sterile PBS on ice. LNs were minced with fine forceps in a 6-well plate containing 1 mL sterile PBS and passed through a 40μm mesh cell strainer to obtain single-cell suspensions. Cell suspension was transferred to a FACs tube and centrifuged at 400xg for 5mins. Cells were ready for flow cytometry processing.

Liver and Lungs processing: The liver and lungs were surgically removed and placed in cold PBS, then placed in 1mL PBS containing 500μL Collagenase D [50mg/mL] (Sigma-Aldrich, USA, Cat #11088866001) and 20μL of 2M Calcium Chloride. The tissues were minced with a scissor and placed in a 37 °C water bath for 30 minutes. The tissue and supernatant were meshed with a plunger from a 1mL syringe and filtered with a 40μm mesh cell strainer to obtain single-cell suspensions. Cell suspension was transferred to a FACs tube and centrifuged at 400xg for 5mins. Cells were ready for flow cytometry processing.

2.8 Intravital multiphoton microscopy and image analysis

Hu-PBMC mice were anaesthetized with ketamine/xylazine, and the popLN was microsurgically exposed for MP-IVM as previously described[368]. Imaging depth was typically 90–150μm below the LN capsule. For multiphoton excitation and second harmonic generation, a MaiTai Ti:sapphire laser (Newport/Spectra-Physics) was tuned to between 780 and 950-980nm for optimized excitation of ZsGreen and dTomato respectively. For four-dimensional recordings of cell migration, stacks of 11 optical sections (512×512 pixels) with 13 z-stacks at 4μm spacing, 30s per cycle, and 121 total cycles were acquired to provide imaging volumes of 40μm in depth. Emitted light and second harmonic signals was detected through 460/50nm, 525/70nm and 595/50nm dichroic filters with non-descanned detectors. All images were acquired using the 2031.0 N.A. Olympus objective lens (XLUMPLFLN; 2.0mm WD). Time-lapse microscopy images will be processed using available 4D image rendering software, Imaris 8.3 (Bitplane) to generate maximum intensity projections (MIPs) for export as Quicktime movies and to perform automated 3D cell tracking for motility analyses. Motility parameters were calculated as described previously[367]. Mean velocities were defined as the average distance a cell moved within the time

period between the two successive frames. Arrest coefficient was defined as the proportion of time on a track during which a cell moved at a speed less than 4μm/min. This was calculated using a custom script in MATLAB (Mathworks).

2.9 Transwell chemotactic assay

To investigate the chemotactic migration of the different subpopulations of the HIV-infected $CD4^+$ T cells towards lymph node chemokines, 0.5-1million HIV-infected $CD4^+$ T cells were placed into the top insert at a final volume of 100μL of a Transwell plate (5μm pore size; Corning Cat # 3421). 600μL of media containing human CCL19 (0.5μg; Biolegend Cat #582102) and CCL21 (0.5μg; Biolegend Cat #424902) ass added to the bottom chamber. Transwell plates were incubated for 3 hours at 37°C/5% CO_2, after which migrated $CD4^+$ T cells in the bottom well were collected, analyzed, and counted using counting beads (Biolegend, Cat #424902) by flow cytometry. As a control, $CD4^+$ T cells were pre-treated with CCR7 neutralizing antibody (2μg; R&D Cat #MAB197R-100) for 25mins at 4℃ prior to analysis in transwell assays.

2.10 Flow cytometry and analysis

Phenotypic characterization of $CD4^+$ T cells was performed on the LSRFortessa (BD Bioscience, USA) and analyzed by FlowJo (Tree Star). Cell suspensions from Spleen, liver, lungs and peripheral blood were pretreated with 2μL of Fc-blocker (anti-mouse and anti-human CD16/32, BioLegend, USA, Cat # 101301) for 10mins at 4℃ prior to surface staining. Cells were stained for surface makers using 2μL of conjugated anti-human mAbs listed in **Table 2**. Thereafter, cells were washed by adding 2mL FACS buffer and centrifuged at 400xg for 5 mins. The supernatant was discarded, and HIV-infected cells were fixed with 250μL 1.5% PFA for 20 mins in 4℃ before flow cytometry analysis.

For intracellular staining, cells that have been stained with surface marker were fixed 500uL of 1%PFA for 1 hour in 4℃, cells were centrifuged at 400xg for 5mins, and the supernatant was discarded. While vortexing, Fix/Perm buffer (Biolegend) was added to cells and incubated for 10 minutes, after which cells were washed by adding 2mL of ice-cold perm wash buffer (Biolegend) and centrifuged at 400xg for 5mins. Afterwards, supernatants were discarded, and cells were stained with 1μL of HIV-1 (p24) anti-human mAbs (28B7) APC labelled (MediMabs, Cat# MM-0289-APC). Washed stained cells were resuspended in 250μL of 1.5% PFA, ready for

flow cytometry analysis. Cell viability was assessed by using the SSC-A (granularity) vs. FSC-A (size) profile.

Table 2. Summary of cell surface antibodies and cell dyes.

Antibodies	Clone	Catalog no.	Source
Anti-Human CD4	RPA-T4	300512	Biolegend
Anti-Human CD3	HIT3a	300318	Biolegend
Anti-Human CD3	HIT3a	300312	Biolegend
Anti-Human CD8	SK1	344718	Biolegend
Anti-Human BST-2	RS38E	348410	Biolegend
Anti-Human CD69	FN50	310912	Biolegend
Anti-Human CD45RO	UCHL1	304251	Biolegend
Anti-Human CD45RA	HI100	304127	Biolegend
Anti-Human CCR7	G043H7	353214	Biolegend
Anti-Human CCR7	G043H7	353207	Biolegend
Anti-Human CCR5	2D7/CCR5	562576	BD Bioscience
Anti-Human S1PR1	SW4GYPP	50-3639-42	eBioscience
Anti-Human CD62L	DREG-56	304843	Biolegend
Anti-Human LFA-1	L130	744553	BD Bioscience
β7 integrin	FIB504 (RUO)	551059	BD Bioscience
Anti-Human CD27	0323	302827	Biolegend
CellTracker™ Blue CMAC Dye		C2110	Thermofisher
CellTracker™ Green CMFDA Dye		C2925	Thermofisher

2.11 Quantification and Statistical Analysis

The numbers of the individual donor(s), experiment(s), recording(s), and animal(s) analyzed are indicated in the figure legends as appropriate. The statistical test used was Mann-Whitney U test to compare datasets non-normal distribution, using Prism 9 (GraphPad). Medians and p-values

from statistical analyses are indicated in each graph. P-values greater than 0.05, differences were noted to be non-significant.

CHAPTER 3

RESULTS

3.1 Development and validation of a full-length R5-tropic dual fluorescent protein HIV reporter system, HIV-TosZy

3.1.1 Development of the full-length R5 tropic dual fluorescent protein-based HIV reporter system, HIV-TosZy

A significant limitation with previously published reporters is that they are *Env*, *vpu* and *Nef* deficient, three crucial viral accessory genes important for HIV/AIDS pathogenesis. To overcome these limitations, I developed a full-length dual fluorescent protein HIV reporter, which I name HIV-TosZy, containing all HIV genes (**Figure 3. 1A**). HIV-TosZy is constructed on a CCR5-utilizing NL4-3 backbone, thus allowing for the maintenance of genetic stability due to the variability in the upstream and downstream LTR sequence of the viral genome[369]. The differences in LTR sequences significantly reduce the potential for proviral deletion and recombination between the two LTRs, which has limited the use of the system in the past[214]. HIV-TosZy contains two fluorescent proteins: (i) ZsGreen, a tetrameric green fluorescent protein fused to the Nef protein with a linker protein, whose expression is driven by the HIV LTR promoter, is a marker for HIV-1 gene expression, and (ii) dTomato, a dimeric red fluorescent protein that is transcriptionally controlled by the constitutive EF1α-HTLV chimeric promoter, which is a marker for proviral integration into the host genome (**Figure 3. 1A**). These two fluorescent proteins were chosen due to their brightness and photostability, making them suitable for flow cytometry and imaging analysis. I defined three categories of infected cell populations: latent infection (Red; LTR^{-} $EF1\alpha^{+}$), early productive infection (Green; LTR^{+} $EF1\alpha^{-}$) and late productive infection (Yellow; LTR^{+} $EF1\alpha^{+}$). HIV-exposed but uninfected T cells are negative for both markers (LTR^{-} $EF1\alpha^{-}$). In conclusion, the development of HIV TosZy has overcome the limitations of previously published reporters by including all HIV genes important in HIV pathogenesis.

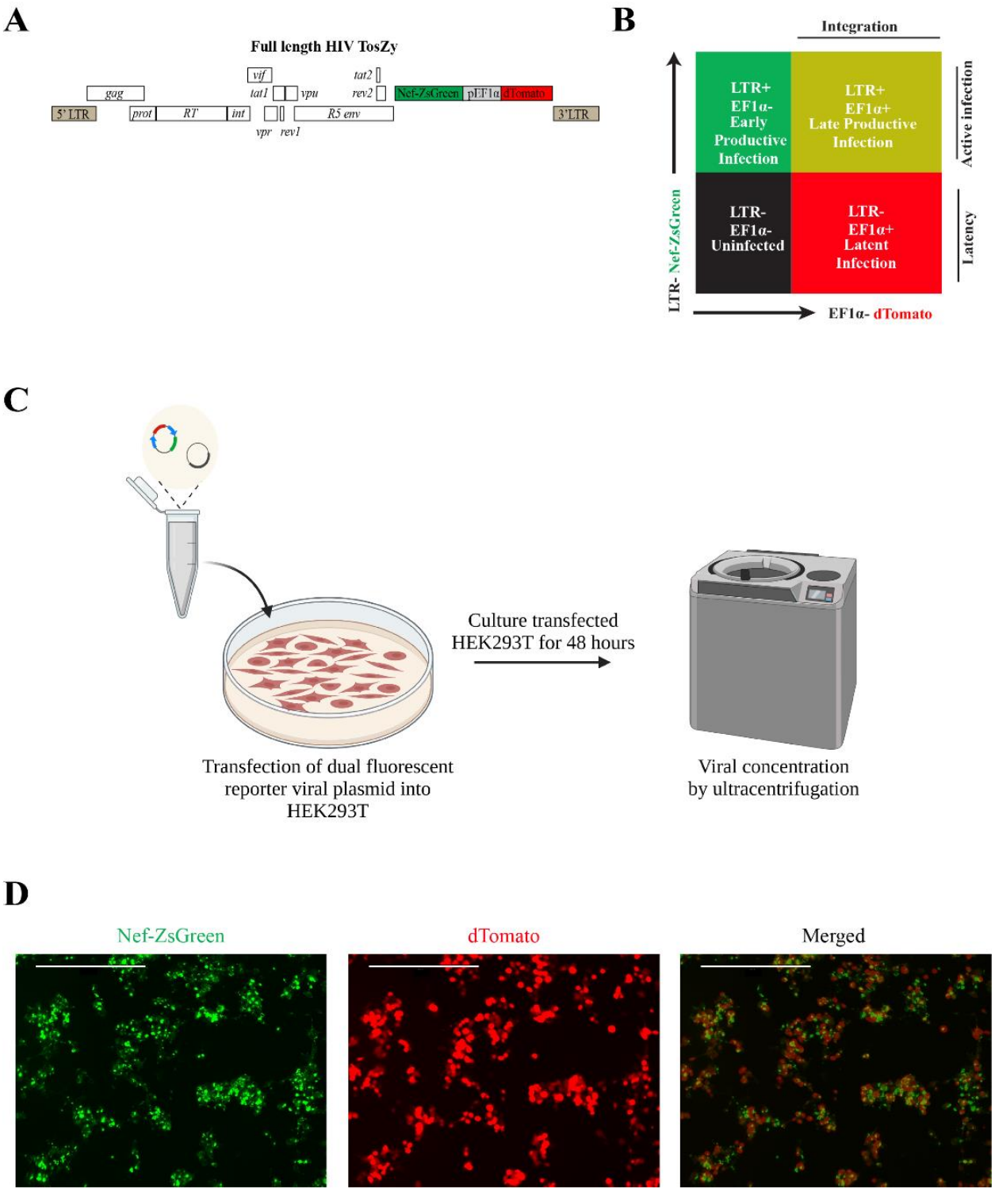


Figure 3. 1: HIV-1 dual fluorescence reporter (HIV-TosZy) to study HIV latency in T cells.

(A) Proviral schematic of the R5-tropic HIV-TosZy reporter. HIV TosZy contains an EF1α promoter-driven dTomato gene and a Nef-ZsGreen fusion protein under the control of the HIV

LTR. This construct is a full-length provirus that contains wildtype HIV Env, Nef and vpu sequences from the NL4-3 backbone.

(B) Schematic depiction of HIV-TosZy infected cell subpopulations detected by flow cytometry analysis —*Figure created with Biorender*.

(C) Experimental schematic of how HIV-TosZy reporter virus is produced for experiments.

(D) Representative fluorescence images of MAGI.CCR5 cells transfected with HIV-TosZy plasmids after 48 hours. LTR-Nef ZsGreen (Green) and EF1α-dTomato (Red), Scale bars: 400μm.

3.1.2 HIV-TosZy can infect primary human CD4$^+$ T cells in vitro

To validate and ascertain that the newly constructed HIV-TosZy is able to infect primary human CD4$^+$ T cells, CD4$^+$ T cells were isolated from PBMC of healthy donors. Cells were activated using anti-CD3/CD28 dynabeads for 2 days and cultured in the presence of rh-IL2 for an additional 4-5 days before infection. Activated primary human CD4$^+$ T cells were infected *in vitro* with HIV-TosZy using an optimized collagen gel method[365] (**Figure 3. 2A**). The different cell populations were identified by flow cytometry analysis using the gating strategy outlined in **Figure 3. 2B**. T cells were infected at a multiplicity of infection (MOI) of 0.1, as we have established that this protocol achieves a high infection rate with minimal cell death[365]. After infection, cells were cultured with rh-IL2 for a 7-day time course study, using uninfected and ART-treated cells as controls. On days 2, 5 and 7 post-infection, flow cytometry was performed on infected cells, where the four different populations were clearly observed: early productive (Green; LTR$^+$ EF1α^-), late productive (yellow; LTR$^+$ EF1α^+), latent (red; LTR$^-$ EF1α^+) infection, HIV-exposed but uninfected T cells (black; LTR$^-$ EF1α^-) *in vitro* (**Figure 3. 3A**). I observed that latently-infected cells increased over time, while there was a decrease in productively infected cell subpopulations (**Figure 3. 3C-D**), presumably due to viral cytopathic effects[370,371]. These studies provide validation that the HIV-TosZy reporter can infect primary human CD4$^+$ T cells and, most importantly, establish and visualize latent infection by flow cytometry. Additionally, the time course study of infected cells revealed that the optimal day for flow cytometry analysis was 5 days post-infection. This is due to the high infection rate, minimal cell death, and distinct separation of the different cell subpopulations at this time point. All subsequent flow cytometry studies were conducted at 5 days post-infection. Notably, increasing MOI did not increase the proportion of latent infection in our primary T cell infection model (**Figure 3. 4A and B**).

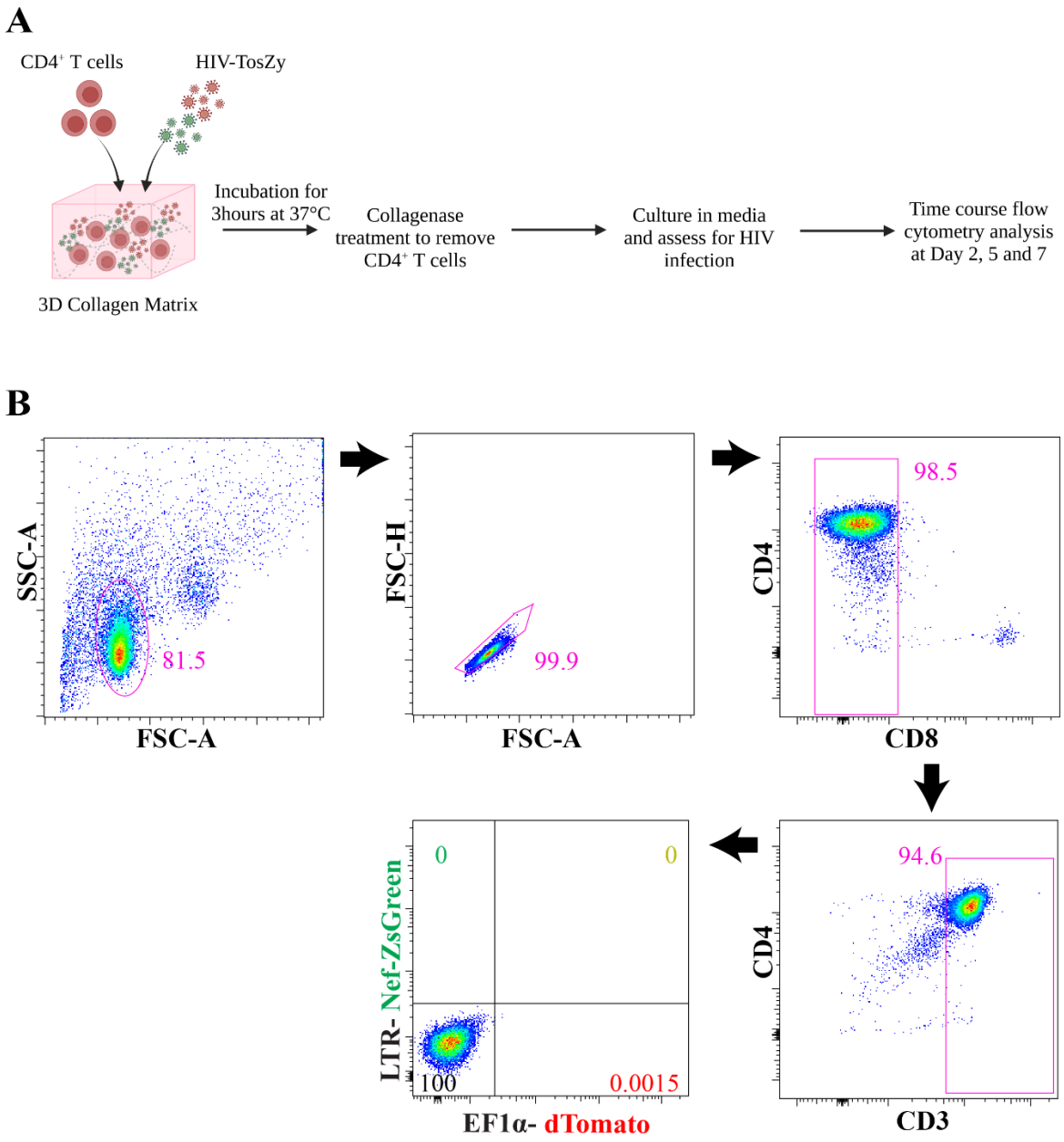
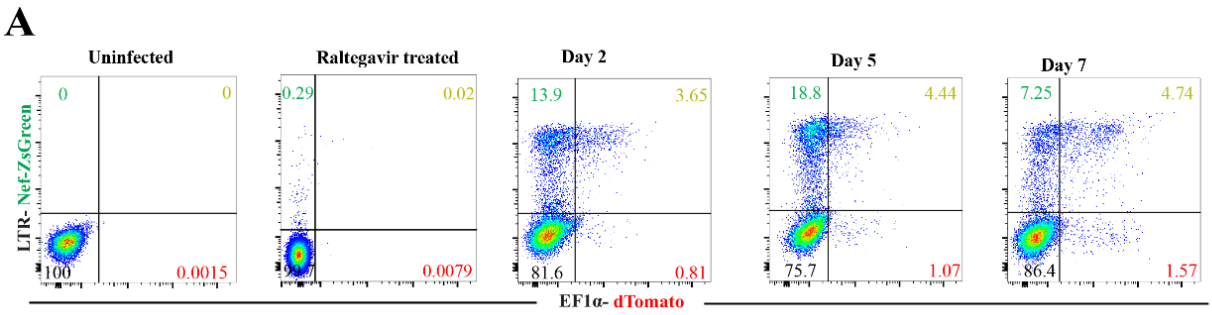


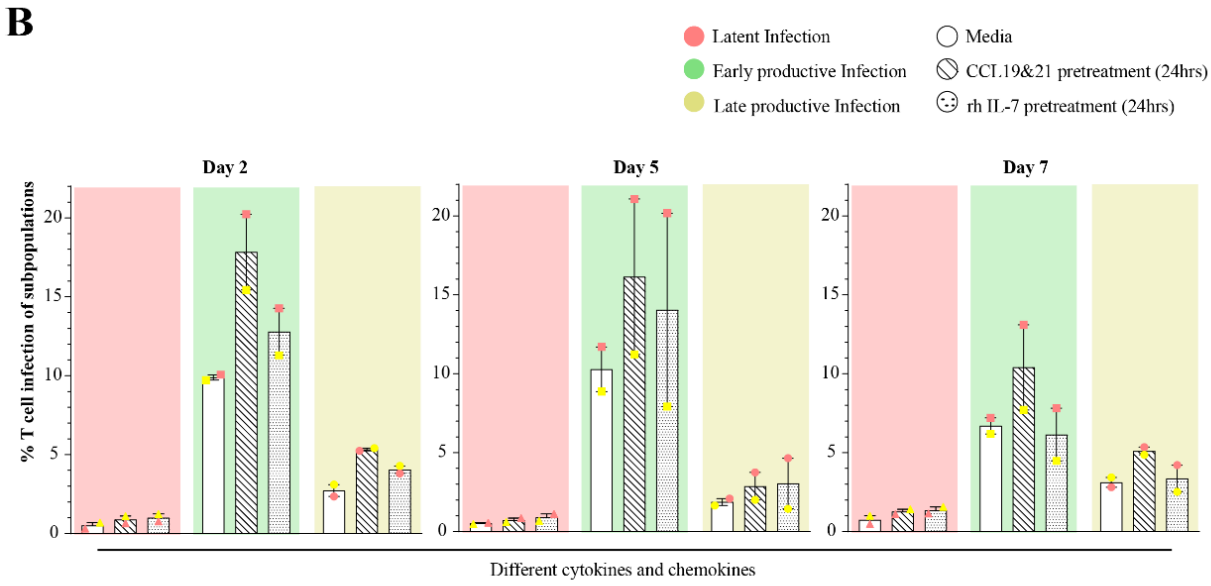
Figure 3. 2: Primary human CD4⁺ T cell infection schematic and gating strategies *in vitro*.

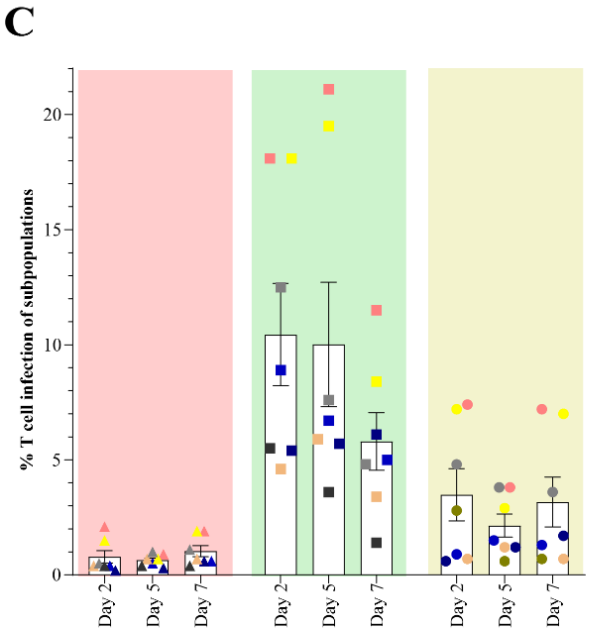
(A) Experimental schematics of T cell infection in bovine collagen gels (1.7 mg/mL) —*Figure created with Biorender*.

(B) Representative nested gating strategy of uninfected CD4⁺ T cells to identify the different cell subpopulations. Lymphocyte population was gated according to SSC-A (granularity) vs. FSC-A (size) properties, after which single cells were gated (FSC-H vs. FSC-A), followed by CD4+ cells gated out, and CD4+ CD3+ were analyzed for Nef-ZsGreen and dTomato expression.

Quadrant gating was used to determine the frequencies of the different subpopulations using FlowJo software.


A
Uninfected
Raltegavir treated
Day 2
Day 5
Day 7
0
0
100
0.0015
0.29
0.02
0.0079
13.9
3.65
81.6
0.81
18.8
4.44
75.7
1.07
7.25
4.74
86.4
1.57
LTR- Nef-ZsGreen
EF1α- dTomato


B
Latent Infection
Early productive Infection
Late productive Infection
Media
CCL19&21 pretreatment (24hrs)
rh IL-7 pretreatment (24hrs)
Day 2
Day 5
Day 7
% T cell infection of subpopulations
Different cytokines and chemokines


C
% T cell infection of subpopulations
Day 2
Day 5
Day 7

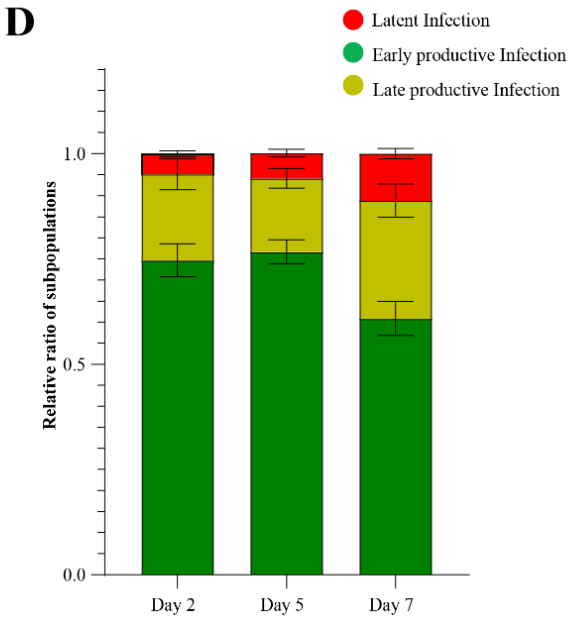

D
Latent Infection
Early productive Infection
Late productive Infection
Relative ratio of subpopulations
Day 2
Day 5
Day 7

Figure 3. 3: Time course infection analysis using HIV-TosZy in primary CD4$^+$ T cells *in vitro*.

(A) Representative flow cytometry plot of HIV-TosZy-infected human CD4$^+$ T cells at different time points post-infection. Distribution of infected T cell subsets is shown: HIV-exposed but uninfected (black), latent (red) and productive (green and yellow) infection in the presence or absence of Raltegravir (HIV integrase inhibitor) is shown.

(B) Percent infection of the different subpopulations at various time points after pre-treatment with rh IL-7 or CCL19/21 24 hours prior to infection in collagen [n=2]. Cells were infected with an MOI (multiplicity of infection) of 0.1.

(C-D) Percent infection (C) and Relative ratio (D) of the different subpopulations at various time points [n=7]. Cells were infected with an MOI (multiplicity of infection) of 0.1.

Each colored dot indicates an individual donor performed in duplicates/triplicates. Error bars represent standard error of the mean.

**There were notable but no significant differences within the groups.*

3.1.3 Pre-treatment with recombinant cytokine IL-7 and chemokines CCL19/21 enhances HIV infection in human CD4$^+$ T cells.

After establishing a primary T cell model system to follow productive and latent HIV infection longitudinally, I next addressed how lymph node cytokines alter infection rates. Previous studies have demonstrated that various cytokines and chemokines can impact CD4$^+$ T cell infection. Specifically, IL-7 can increase the permissively of CD4$^+$ T cells to HIV infection and have an anti-apoptotic effect, promoting infected cell survival, while CCL19/21 was shown to facilitate HIV entry and viral integration in resting CD4$^+$ T cells[254,255,372,373]. To explore this further using my primary T cell model system, I pre-treated activated CD4$^+$ T cells with recombinant human IL-7 (rhIL-7) and rhCCL19/21, washed, and then infected with HIV-TosZy in a time course analysis using flow cytometry. Compared to untreated T cells, CCL19/21 pre-treatment led to a notable increase in productively-infected T cells and a modest increase in latently-infected cells (**Figure 3. 3B**). In contrast, rhIL-7 pre-treatment showed a modest increase in productively-infected cells and a more notable increase in latently-infected T cells (**Figure 3. 3B**). Together, my results were consistent with published literature on the role of cytokine/chemokine in the induction of HIV latency, and all subsequent HIV infection studies were performed with rhIL-7 pre-treated cells to increase the proportion of latently-infected T cells.

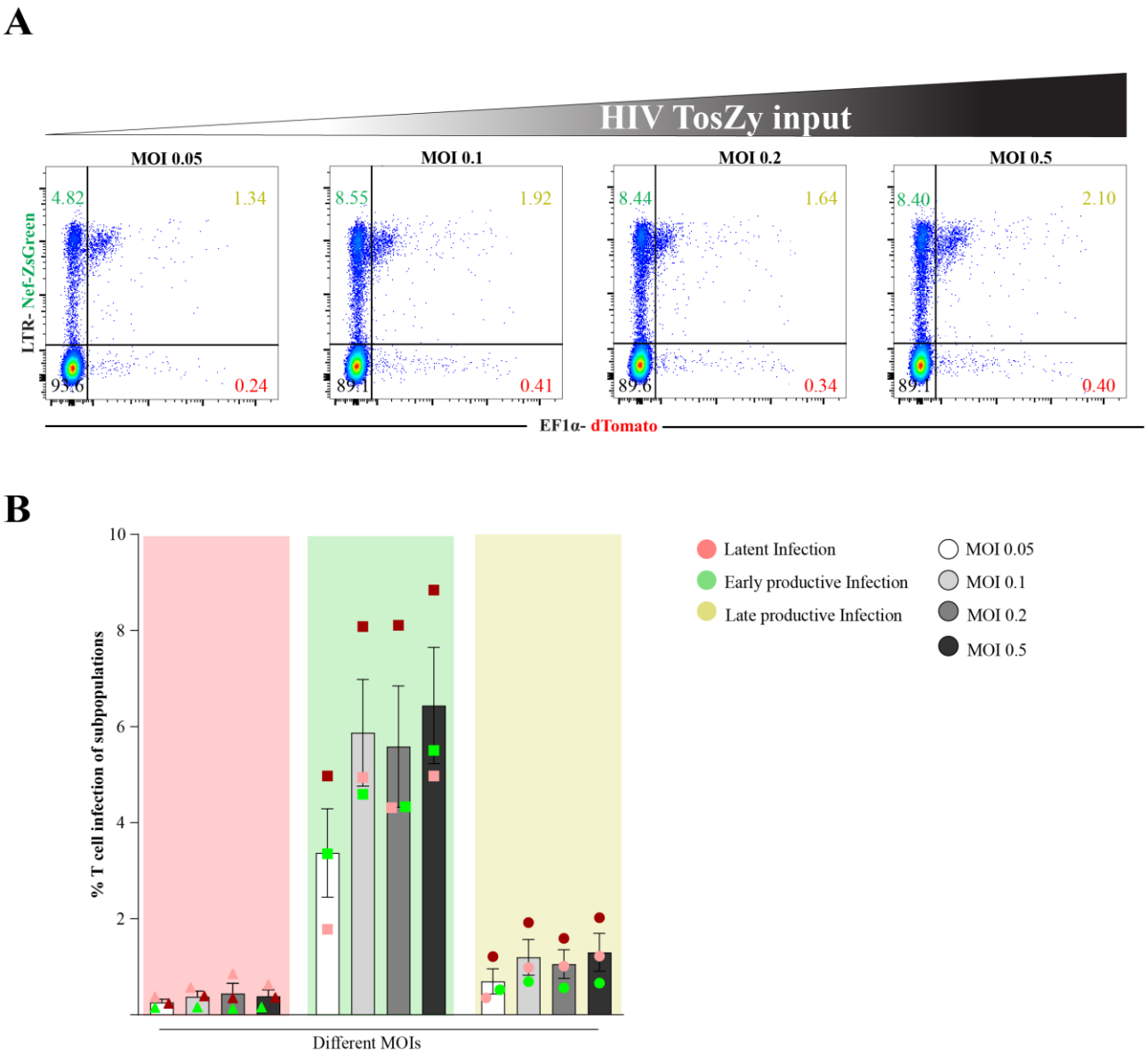


Figure 3. 4: Analysis of primary CD4$^+$ T cell infection with HIV-TosZy at different MOIs.

(A) Representative flow cytometry plots of infected human CD4$^+$ T cells, 5 days post-infection at the indicated MOI of HIV-TosZy.

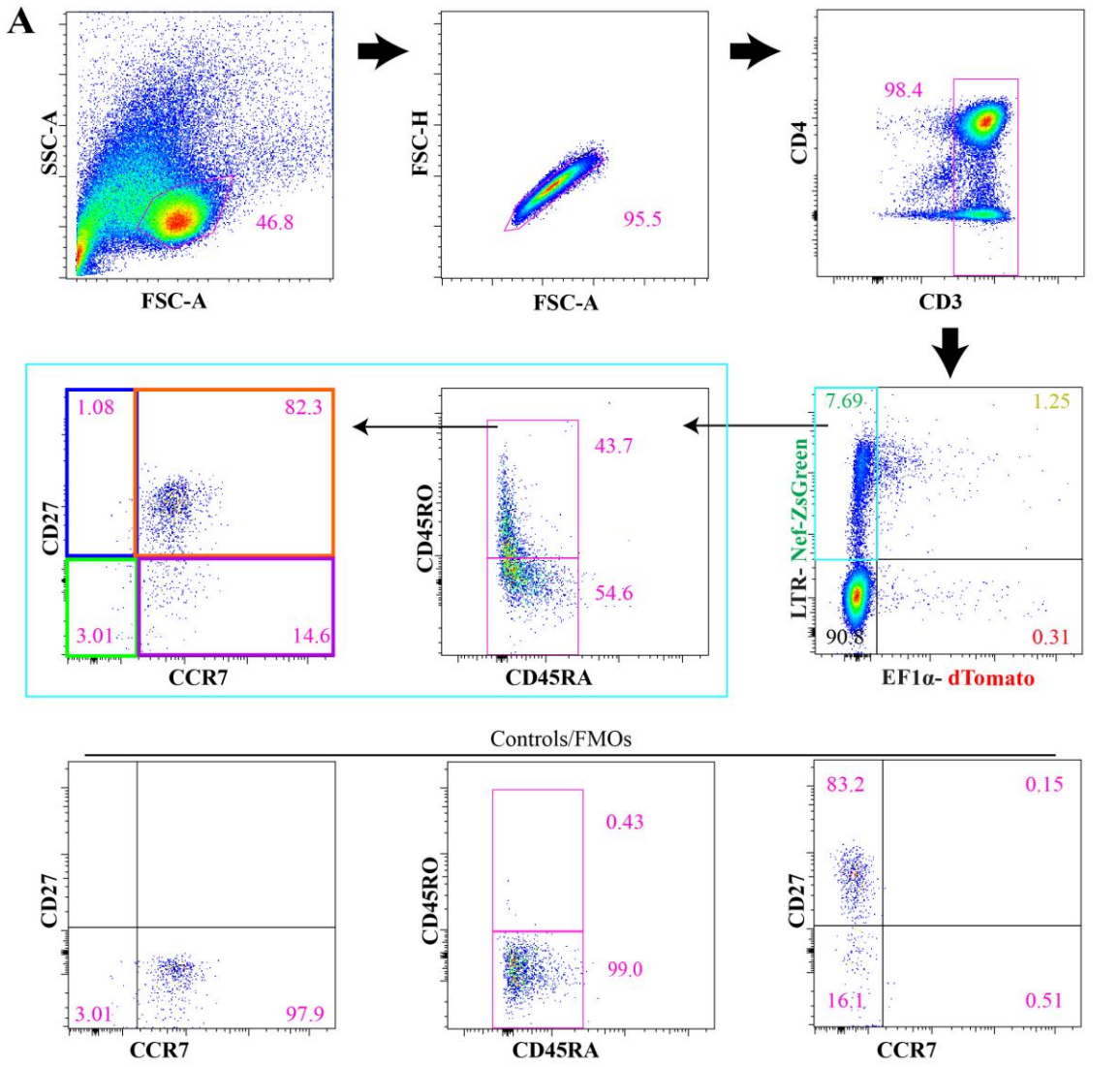
(B) Percent infection of the different subpopulations day 5 post-infection with different MOIs [n=3]. *Each colored dot indicates an individual donor performed in duplicates/triplicates. Error bars represent standard error of the mean.*

**There were notable but no significant differences within the groups*

3.2 Phenotypic characterization of latently-infected T cells marked using HIV-TosZy *in vitro.*

3.2.1 Analyzing the CD4$^+$ T cell subsets within the different cell subpopulations in vitro

To ensure that any differences observed among the various cell subpopulations in this study are not attributed to preferential infection of a particular CD4$^+$ T cell subsets, infected CD4$^+$ T cells were further divided into subsets using defined markers of memory T cells. The different isoforms of the leukocyte common antigen (CD45), RA and RO markers were utilized to differentiate between naïve and memory T-cells, respectively. Memory CD45RO+ cells were further gated based on the expression of CC chemokine receptor-7 (CCR7) and CD27 (**Figure 3. 5A**). These subsets include: Effector (CD45RO+CD27-CCR7-), Effector memory (EM; CD45RO+CD27-CCR7-), Central memory (CM; CD45RO+CD27+CCR7+) and Transitional memory (TM; CD45RO+CD27+CCR7-) (**Figure 3. 5B**). I observed that the distribution of T cell subsets was not significantly different in the latently and productively infected T cells. These data suggest that any observed differences between the cell subpopulations are not attributed to differences in infected CD4$^+$ T cell subsets (**Figure 3. 5B**).


A
SSC-A
FSC-A
46.8
FSC-H
FSC-A
95.5
CD4
CD3
98.4
LTR- Nef-ZsGreen
EF1α- dTomato
7.69
1.25
90.8
0.31
CD45RO
CD45RA
43.7
54.6
CD27
CCR7
1.08
82.3
3.01
14.6
Controls/FMOs
CD45RO
CD45RA
0.43
99.0
CD27
CCR7
3.01
97.9
CD27
CCR7
83.2
0.15
16.1
0.51

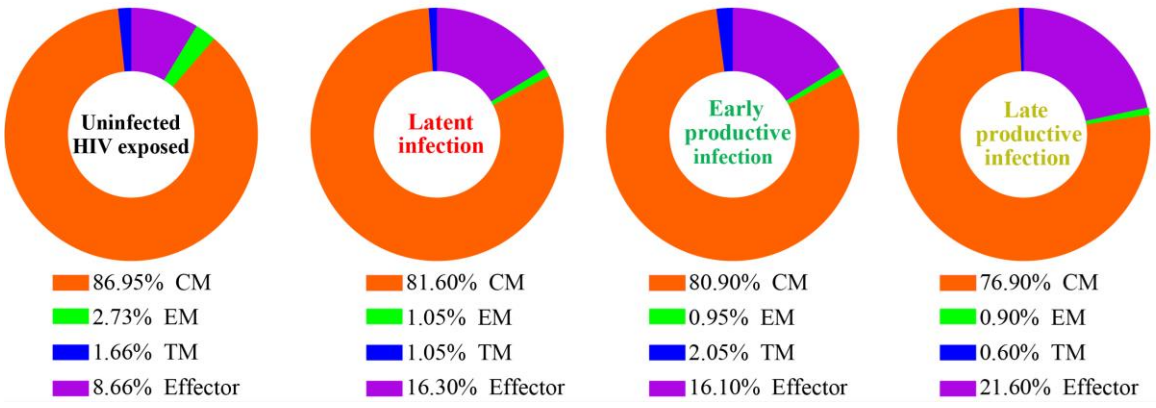

B
Uninfected HIV exposed
86.95% CM
2.73% EM
1.66% TM
8.66% Effector
Latent infection
81.60% CM
1.05% EM
1.05% TM
16.30% Effector
Early productive infection
80.90% CM
0.95% EM
2.05% TM
16.10% Effector
Late productive infection
76.90% CM
0.90% EM
0.60% TM
21.60% Effector

Figure 3. 5: T cell subset characterization of HIV-infected CD4$^+$ T cells *in vitro*.

(A) Representative nested gating strategy of HIV-infected CD4$^+$ T cells used to identify the principal CD4$^+$ T cell subsets. Lymphocyte population was gated according to SSC-A (granularity) vs. FSC-A (size) properties, after which single cells were gated (FSC-H vs. FSC-A), followed by CD4+ cells gated out, and CD4+ CD3+ were analyzed for Nef-ZsGreen and dTomato expression. Each cell subpopulation's quadrants were further gated to identify memory (CD45RO+), and naïve (CD45RA+) cells and memory cells were gated into the various CD4$^+$ T cell subsets using CCR7 and CD27 markers. Fluorescence-minus one (FMO) control was used for the proper flow cytometry gating.

(B) Representative pie chart showing the percent of the different CD4$^+$ T cell subsets 5 days post-infection within the different cell subpopulations. Effector, EM=effector memory, CM=central memory and TM=transitional memory cell populations were defined as follows: EM=CD45RO+CD27-CCR7-;CM=CD45RO+CD27+CCR7+; TM=CD45RO+CD27+CCR7-; Effector=CD45RO+CD27-CCR7+.

3.2.2 Evaluating the functionality of HIV Nef and Vpu protein in HIV-TosZy in vitro

HIV-TosZy was engineered to include crucial viral accessory proteins, Nef and Vpu, which play roles in immune evasion, viral replication and apoptosis. Specifically, Nef potently decreases CD4 cell surface expression in infected T cells as a way to prevent superinfection and dampen further T cell activation[374,375]. When CD4 expression was evaluated among the different infected populations, I observed a significant 7 to 20-fold decrease in CD4 expression in early and late productively infected cell subpopulations, respectively, while I saw a non-significant 1.5-fold decrease in CD4 expression in the latently-infected cell subpopulation (**Figure 3. 6A-C**). Similarly, Vpu downregulates the host restriction factor tetherin (BST-2)[376-378]. A trend of lower BST-2 expression in the productively infected cells was observed, while high BST-2 was maintained in the latently-infected cell population (**Figure 3. 6D-F**). To further evaluate the functionality of Nef and Vpu, I performed intracellular staining using flow cytometry analysis to quantify the amount of HIV protein produced in the different cell subpopulations. Specifically, I used HIV Gag protein, p24, which is the most abundant HIV protein produced during HIV-1 infection and is clinically utilized to diagnose early HIV infection[6]. High p24 expression was observed in productively infected T cells, whereas expression was reduced in latently-infected T cells, as expected (**Figure 3. 7A-B**). Collectively, these results confirm that both *Nef* and *Vpu* genes are functional in the HIV- TosZy reporter construct and allow for the use of CD4 and BST-2 expression to further differentiate between productive and latent infections during longitudinal studies *in vitro*.

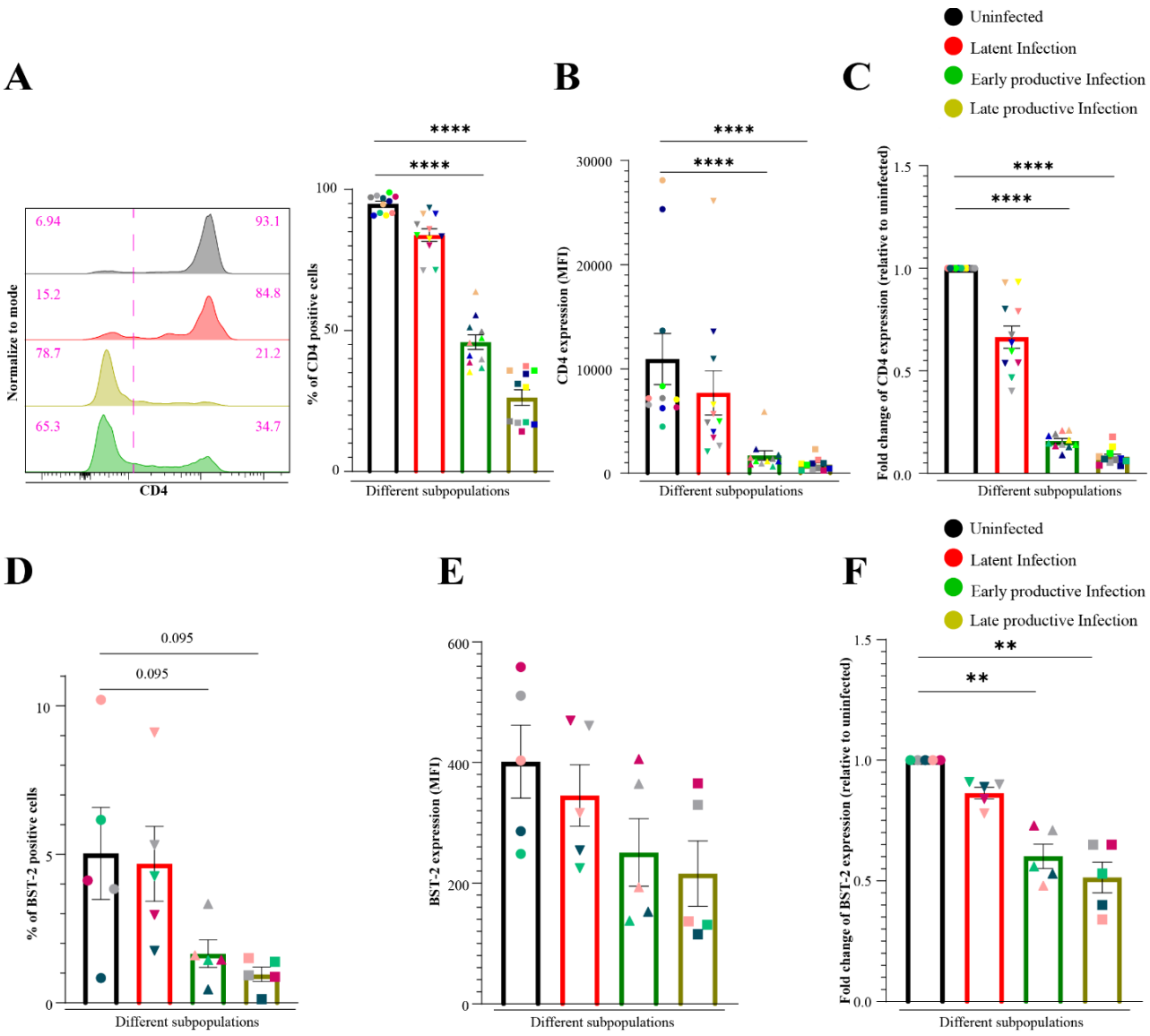


Figure 3. 6 Expression of HIV-1 accessory proteins Nef and Vpu cause downregulation of CD4 and tetherin (BST-2), respectively, in productively, but not latently infected T cells.

(A-C) Percentages (A), Mean fluorescent intensity, MFI (B), and Fold changes (C) of CD4 within the different subpopulations' day 5 post-infection [n=11].

(D-F) Percent (D), Mean fluorescent intensity, MFI (E), and Fold changes (F) of BST-2 within the different subpopulations' day 5 post-infection [n=5].

*Each colored dot indicates an individual donor performed in duplicates/triplicates. Error bars represent standard error of the mean. Statistical analysis was performed using a Mann-Whitney test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$*

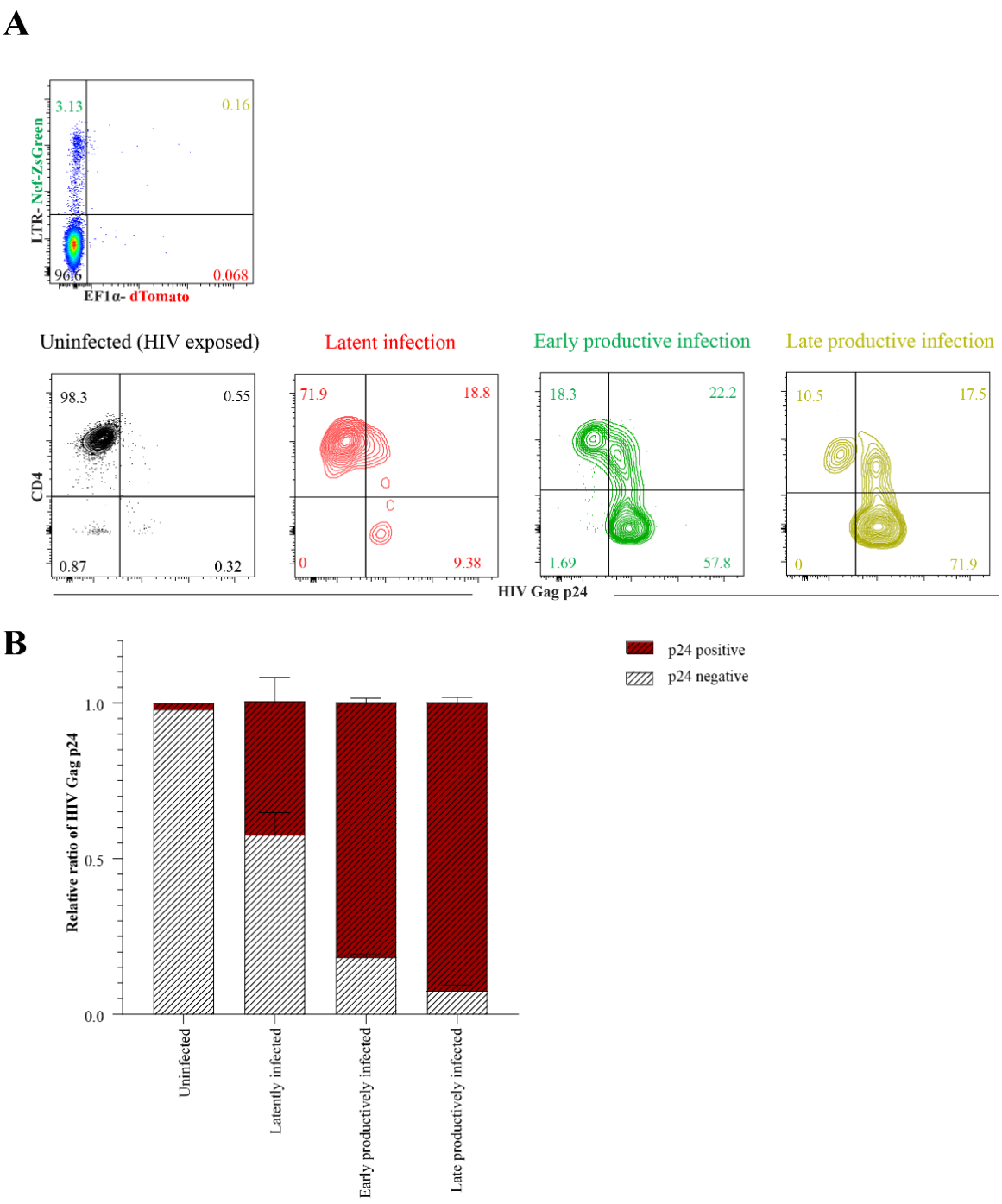


Figure 3. 7: HIV Gag protein expression in HIV-infected CD4$^+$ T cells *in vitro*.

(A) Flow cytometry representative plots of cell surface CD4 and intracellular p24 expression of within the indicated subpopulations' at day 5 post-infection.

(B) The relative ratio of HIV-Gag protein (p24) expression within the indicated subpopulations' at day 5 post-infection.

3.2.3 HIV co-receptor CCR5 is expressed at comparable levels across all infected subpopulations

Since HIV-TosZy is a CCR5-utilizing virus, we next ruled out the possibility that this reporter virus was selectively infecting different T cell subsets, thereby contributing to the differential CD4 and BST-2 expression observed in **Figure 3. 6A-F**. As expected, CCR5 expression in the uninfected HIV-exposed, resting T cell subpopulation was low[379], whereas a 2.3-fold increase in CCR5 expression was observed within the late productively infected subpopulation. However, the expression of CCR5 between the latently and productively infected subpopulations was comparable (**Figure 3. 8A-C**). Thus, I conclude that the observed differences between the latently and productively infected cell subpopulations are not due to differences in their HIV infectivity.

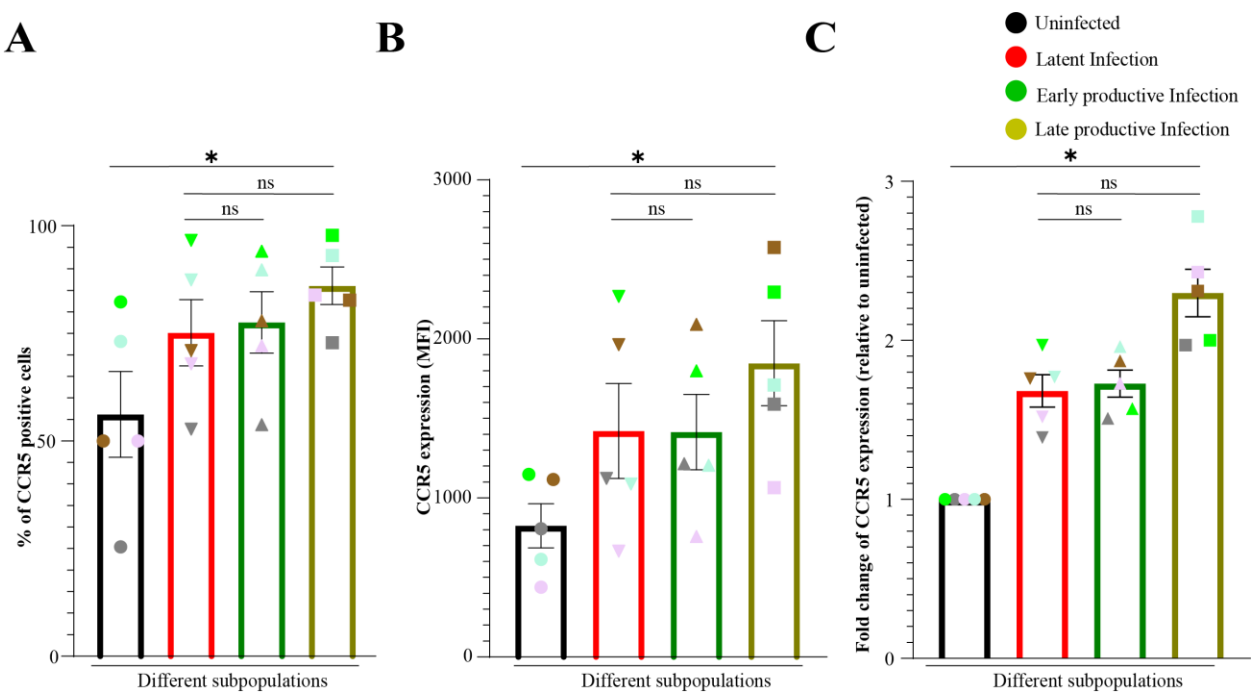


Figure 3. 8: Expression of HIV co-receptor CCR5 in HIV-infected CD4⁺ T cells *in vitro*.

(A-C) Percent (A), Mean fluorescent intensity, MFI (B), and Fold changes (C) of CCR5 expression within the indicated subpopulations at day 5 post-infection [n=5].

*Each coloured dot indicates an individual donor performed in duplicates/triplicates. Error bars represent the standard error of the mean. Statistical analysis was performed using a Mann-Whitney test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ****$p \leq 0.0001$, n.s not significant.*

3.3 Evaluation of receptors involved in T cell trafficking to and within secondary lymphoid organs in latently-infected cells

3.3.1 In vitro expression analysis of receptors on infected CD4$^+$ T cells involved in homing and entry into the lymph node

The lymph node and the gut lymphoid tissue (GALT) are prominent HIV reservoirs whose chemokine-rich environment plays a significant role in HIV latency establishment[380-382]. Here, I aimed to evaluate the expression of chemokine receptors and integrins used for homing, entry and egress from secondary lymphoid organs, including CCR7, LFA-1, CD62L, S_1PR1, and β7 integrins and did a comparative analysis between latently-infected T cells and uninfected HIV-exposed control population (**Figure 3. 9-Figure 3. 11A**). CCR7 was expressed in all T cell subpopulations, with its expression maintained in latently infected cells, whereas a non-significant downmodulation was observed in productively infected cells (**Figure 3. 9B-C**). A higher expression of LFA-1 expression was observed in all infected cells, possibly indicating infection of activated T cells (**Figure 3. 9E-G**). CD62L expression was comparable between latent and control T cells, whereas a significant reduction in expression was observed in productively infected T cells (**Figure 3. 9H-J**). Additionally, S_1PR1 expression, which is involved in the egress of lymphocytes out of the secondary lymphoid organs by sensing S_1P in plasma[383-385], trended towards higher expression in late productively infected cells with no changes observed in latent cells compared to control, although MFI values were similar across all T cell populations (**Figure 3. 10B-D**). Collectively, these data suggest that latently-infected T cells retain expression of lymph node homing (CCR7, CD62L) and exit receptors (S_1PR1) compared to uninfected, resting memory CD4$^+$ T cells, indicating their trafficking and migratory capacity is largely intact.

Lastly, I investigated the expression of gut-homing β7 integrin, which dimerizes with α4 to form a homing receptor essential for the trafficking and retention of CD4$^+$ T cells in the GALT by binding Mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) expressed on the venules of the GALT[386] (**Figure 3. 11A**). Interestingly, compared to the control, I observed an upregulation of β7 integrin expression (MFI and fold increase) in latently-infected cells and not in productively-infected T cells (**Figure 3. 11B-D**). These observations suggest that latently-infected T cells may increase access to the gut lymphoid tissues during the course of the disease, which may have implications on viral dissemination and latency reservoir establishment.

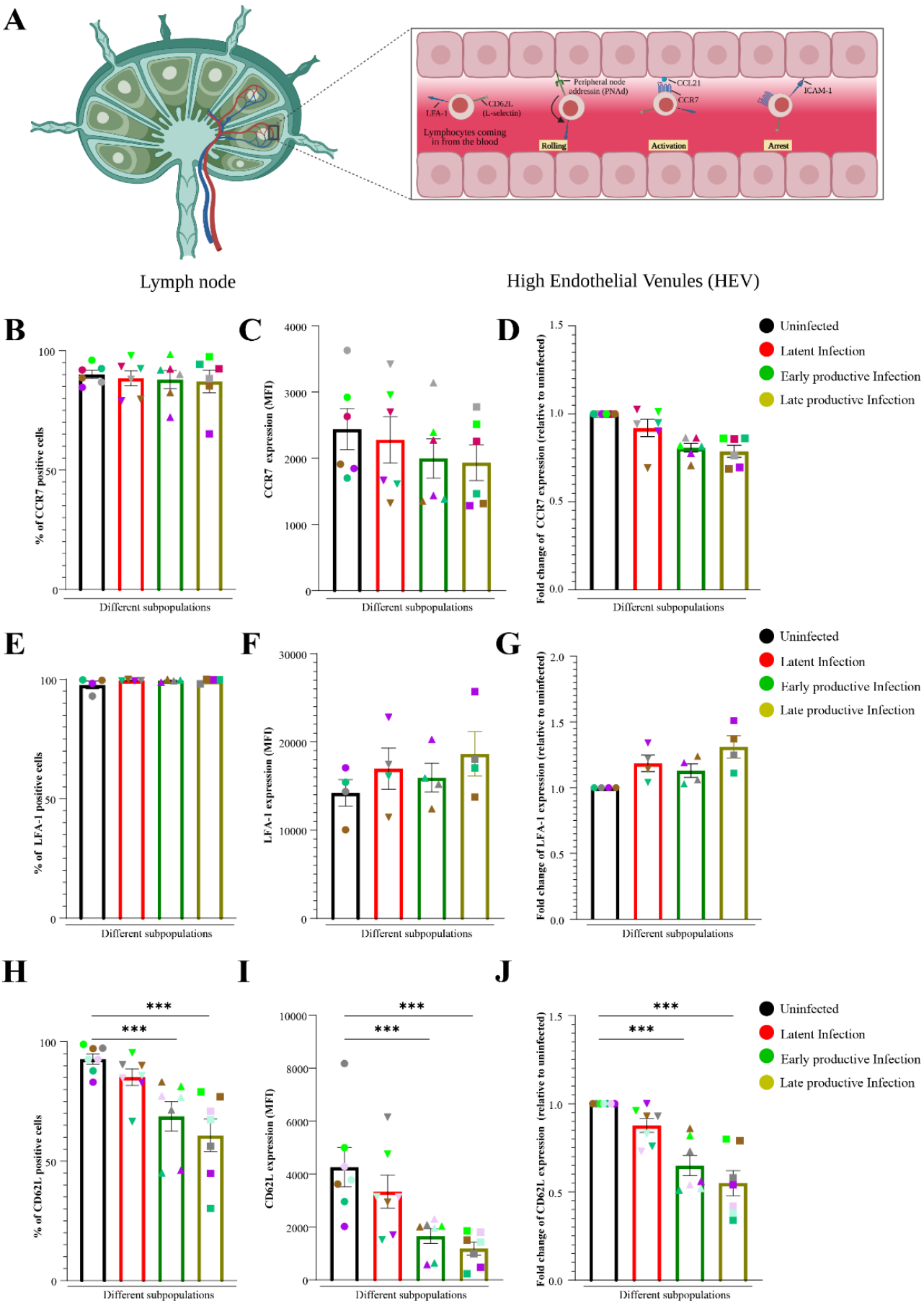

A
Peripheral node addressin (PNAd)
CCL21
CCR7
ICAM-1
LFA-1
CD62L (L-selectin)
Lymphocytes coming in from the blood
Rolling
Activation
Arrest
Lymph node
High Endothelial Venules (HEV)
B
% of CCR7 positive cells
Different subpopulations
C
CCR7 expression (MFI)
Different subpopulations
D
Fold change of CCR7 expression (relative to uninfected)
Different subpopulations
Uninfected
Latent Infection
Early productive Infection
Late productive Infection
E
% of LFA-1 positive cells
Different subpopulations
F
LFA-1 expression (MFI)
Different subpopulations
G
Fold change of LFA-1 expression (relative to uninfected)
Different subpopulations
Uninfected
Latent Infection
Early productive Infection
Late productive Infection
H
***
***
% of CD62L positive cells
Different subpopulations
I
***
***
CD62L expression (MFI)
Different subpopulations
J
***
***
Fold change of CD62L expression (relative to uninfected)
Different subpopulations
Uninfected
Latent Infection
Early productive Infection
Late productive Infection

Figure 3. 9: Expression of lymph node homing markers in HIV-infected CD4$^+$ T cells *in vitro*.

(A) Receptors responsible for T cell homing from the blood to the lymph node parenchyma via the high endothelial venules (HEVs) —*Figure created with Biorender*.

(B-D) Percent (B), Mean fluorescent intensity, MFI (C), and Fold changes (D) of CCR7 expression within the indicated T cell subpopulations at day 5 post-infection [n=6].

(E-F) Percent (E), Mean fluorescent intensity, MFI (F), and Fold changes (G) of LFA-1 expression within the indicated T cell subpopulations at day 5 post-infection [n=4].

(H-J) Percent (H), Mean fluorescent intensity, MFI (I), and Fold changes (J) of CD62L expression within the indicated T cell subpopulations at day 5 post-infection [n=7].

*Each colored dot indicates an individual donor performed in duplicates/triplicates. Error bars represent the standard error of the mean. Statistical analysis was performed using a Mann-Whitney test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$*

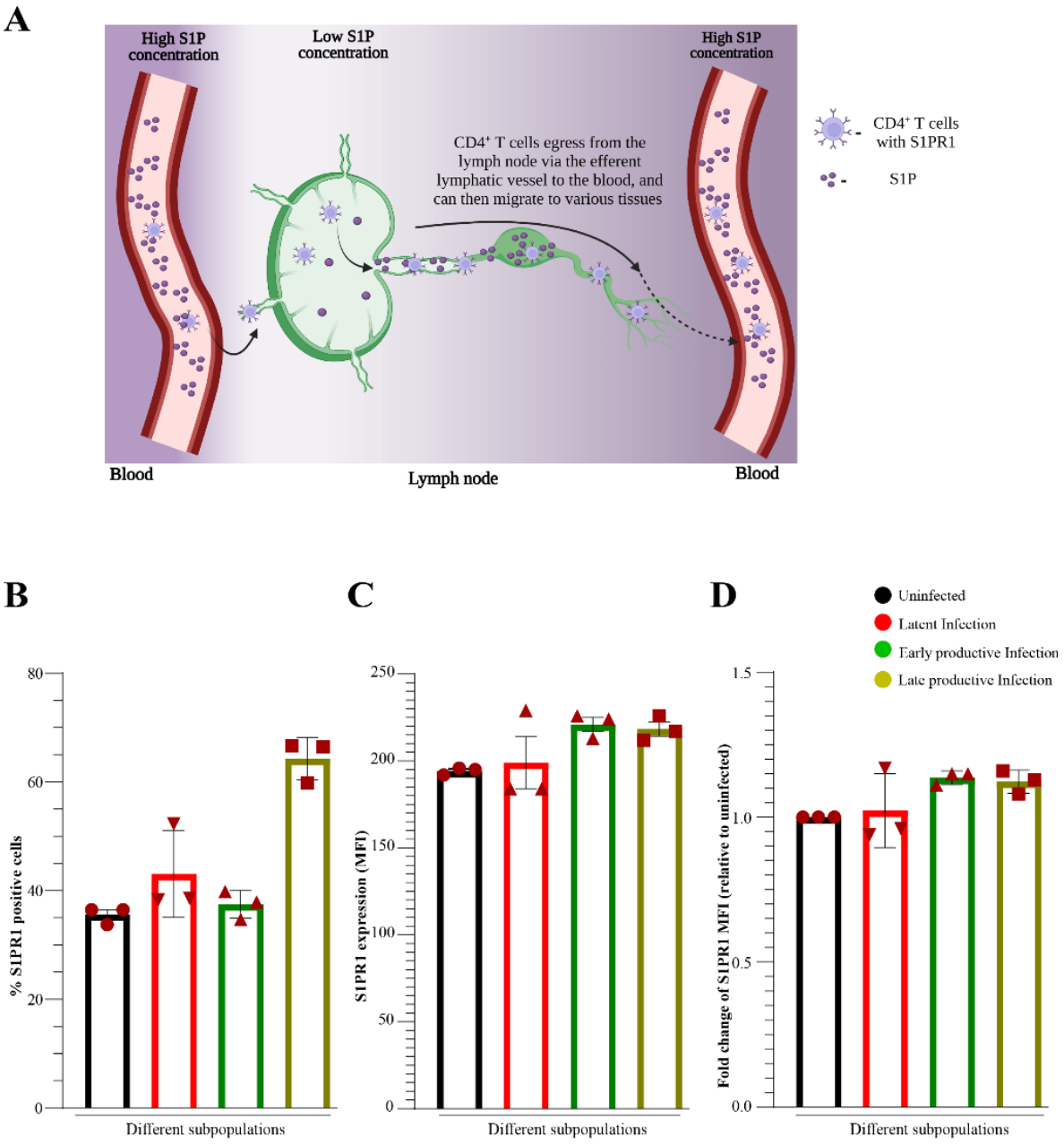


Figure 3. 10: Expression of S1PR1 on HIV-infected CD4⁺ T cells *in vitro*.

(A) T cell egress from the lymph node is regulated by S_1P chemokine gradient and S_1PR1 expression —*Figure created with Biorender*.

(B-D) Percent (B), Mean fluorescent intensity, MFI (C), and Fold changes (D) of S_1PR1 expression within the indicated T cell subpopulations at day 5 post-infection.

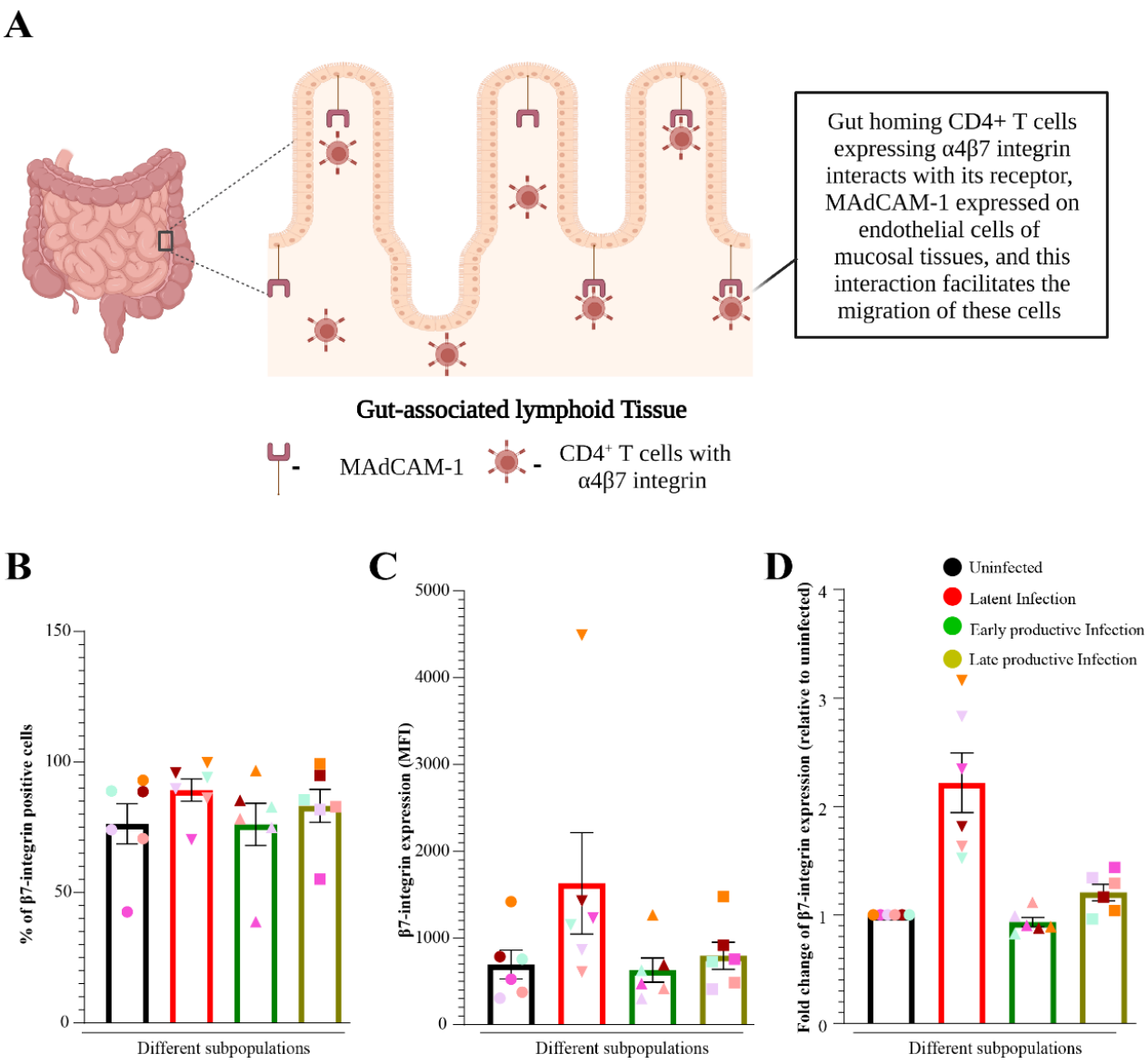


Figure 3. 11: Expression of gut homing marker α4β7 on HIV-infected CD4⁺ T cells *in vitro*.

(A) Schematic representation of CD4⁺ T cells homing in the gut-associated lymphoid tissue.

(B-D) Percent (B), Mean fluorescent intensity, MFI (C), and Fold changes (D) of β7 expression within the indicated T cell subpopulations at day 5 post-infection [n=6].

**There are no significant differences between the subpopulations*

3.3.2 Assessing CCR7 responsiveness to its chemokine ligands CCL19 and CCL21 by latently-infected T cells

CCR7 plays a critical role in T cell homing to the lymph node via high endothelial venules by responding to the high chemokine gradient of CCL19 and CCL21 in the T cell zone, produced by fibroblastic reticular cells (FRCs)[387,388] (**Figure 3. 9A**). To assess the responsiveness and functionality of CCR7 expressed in different infected subpopulations, I performed chemotaxis assays on $CD4^+$ T cells infected with HIV for 2 and 5 days. To determine whether migration was CCR7 dependent, infected cells were treated with anti-CCR7 neutralizing antibody (CCR7nAB; clone 150503R) for 25mins before performing the chemotaxis assay. Next, treated or untreated infected cells were placed in the upper chamber of a transwell plate and allowed to migrate toward either medium alone or media with chemokine ligands, CCL21/CCL19 (0.5µg), in the bottom chambers. Cells that have migrated through the transwell pores (5µm) were then washed and enumerated by flow cytometry. I observed an increased number of migrated cells in the presence of chemokines compared to media alone, as indicated by the cell counts at both time points (**Figure 3. 12A and C**). Fold change analysis between input and migrated cells in this assay revealed similar chemotactic responses in both latently and productively infected cells, although late productive cells did display increased directional migratory responsiveness (**Figure 3. 12B and C**). Surprisingly, CCR7 neutralizing antibody treatment did not abolish CCL19/21-mediated chemotaxis. To confirm that the blockade of the CCR7 receptor was complete, infected T cells were treated with a recombinant monoclonal mouse IgG2A CCR7 neutralizing antibody (clone 150503R) for 25mins and stained with a monoclonal conjugated anti-CCR7 antibody (clone G043H7) for 25mins, and then cells were analyzed by flow cytometry. As shown (**Figure 3. 12E**), there was decreased CCR7 expression in $CD4^+$ T cells treated with the neutralizing antibody compared to the control, even though similar numbers of cells were analyzed. Collectively, these results suggest that CCR7 is responsive to the chemokines CCL19 and 21, and that possibly other chemokine receptors, such as CXCR3, are responsive to either CCL19 or CCL21 and are also involved in T cell migration[389-392].

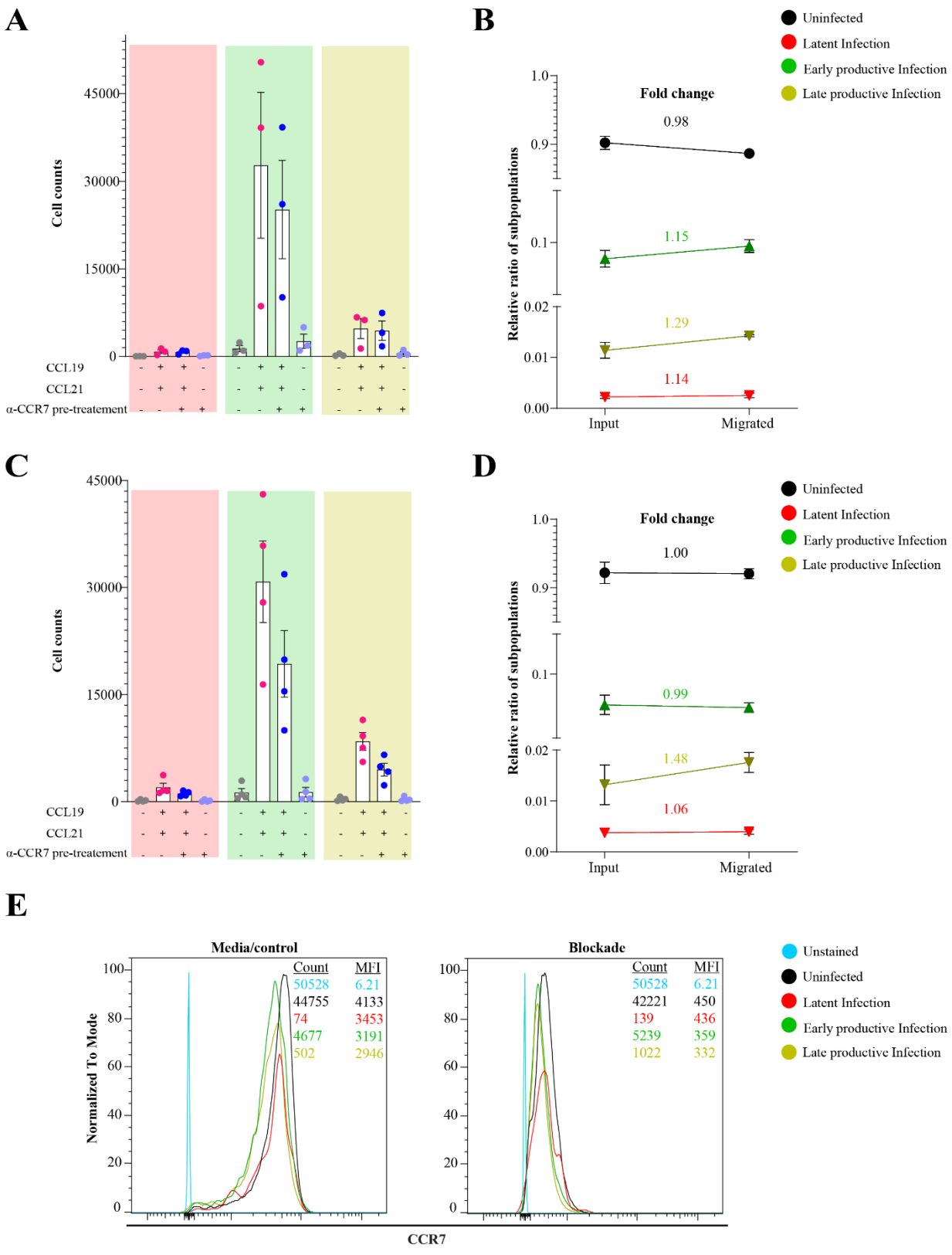

A
Cell counts
45000
30000
15000
0
CCL19
CCL21
α-CCR7 pre-treatement
B
Uninfected
Latent Infection
Early productive Infection
Late productive Infection
Fold change
0.98
1.15
1.29
1.14
Relative ratio of subpopulations
1.0
0.9
0.1
0.02
0.01
0.00
Input
Migrated
C
Cell counts
45000
30000
15000
0
CCL19
CCL21
α-CCR7 pre-treatement
D
Uninfected
Latent Infection
Early productive Infection
Late productive Infection
Fold change
1.00
0.99
1.48
1.06
Relative ratio of subpopulations
1.0
0.9
0.1
0.02
0.01
0.00
Input
Migrated
E
Media/control
Blockade
Count
MFI
50528 6.21
44755 4133
74 3453
4677 3191
502 2946
50528 6.21
42221 450
139 436
5239 359
1022 332
Normalized To Mode
100
80
60
40
20
0
CCR7
Unstained
Uninfected
Latent Infection
Early productive Infection
Late productive Infection

Figure 3. 12: Evaluation of CCR7-mediated chemotactic responses by HIV-infected CD4$^+$ T cells *in vitro*.

A) Absolute cell counts of migrated cells towards the indicated media or chemokine solution 2 days post-infection [n=3]. In some cases, cells were pre-treated with anti-CCR7 antibody for 25 minutes prior to chemotaxis assay.
B) Relative ratio of the indicated T cell populations that have migrated towards CCL19/CCL21 2 days post-infection [n=3]. Fold change was calculated as the ratio of CD4$^+$ T cells responding to chemokine compared to CD4$^+$ T cells added to the transwell system to reveal possible enhancement/reduction in chemotactic responses.
C) Absolute cell counts of migrated cells towards the indicated media or chemokine solution 5 days post-infection [n=3]. In some cases, cells were pre-treated with anti-CCR7 antibody for 25 minutes prior to chemotaxis assay.
D) Relative ratio of the indicated T cell populations that have migrated towards CCL19/CCL21 5 days post-infection [n=3]. Fold change was calculated as the ratio of CD4+ T cells responding to chemokine compared to CD4$^+$ T cells added to the transwell system to reveal possible enhancement/reduction in chemotactic responses.
E) Representative histogram of cell surface CCR7 expression on HIV-infected CD4$^+$ T cells. Infected T cells were treated with CCR7 neutralizing antibody (nAB; Clone # 150503R) for prior to staining with monoclonal conjugated anti-CCR7 antibody (clone G043H7) for 25mins.

Each coloured dot indicates an individual donor performed in duplicates/triplicates. Error bars represent the standard error of the mean.

3.4 Assessing the migratory behavior and tissue localization of latently-infected cells *in vitro* and *in vivo.*

3.4.1 Visual characterization of latently-infected T cell migration in 3D collagen in vitro

Previous studies conducted by Murooka *et al.* showed that HIV infection impacts the migratory ability of productively infected cells by reducing their motility in a Nef-dependent manner[128]. Our phenotypic analysis so far has examined similarities/differences in tissue homing/migration receptors between latently-infected and control T cells, but a direct assessment of their migration capacity in 3-dimensional (3D) environments have not been performed. These have implications on how we view the distribution and recirculation kinetics of latent T cells in the body under ART suppression. We have previously used the 3D collagen matrix imaging platform to characterize T cell migration behaviors and have shown that they have similar kinetics compared to the lymph node of humanized mice[128]. Here, I infected primary $CD4^+$ T cells with HIV-TosZy for two days and then embedded them into collagen gels for live-cell microscopy using the multi-photon microscope (**Figure 3. 13A**). Latent T cell migrational behaviours were compared against uninfected and productively-infected $CD4^+$ T cells. As shown in **Figure 3. 13B**, the fluorescent reporters used were well visualized by multi-photon microscopy: dTomato protein was found throughout the cell because of its soluble nature, whereas Nef-ZsGreen was punctate and localized to the uropod, a protrusion at the rear of the cell involved in cell migration, with its expression found either on the cell surface or within the endoplasmic reticulum as expected. Importantly, we were able to visually distinguish between latently-infected and productively-infected T cells and performed computational analysis on all recorded live-cell videos to describe their migratory behaviours: mean track velocity is the average speed at which T cells are migrating on a per-cell basis, whereas arrest coefficient describes the fraction of time a cell is "arrested" (or migrating at a speed less than 4μm per minute)[367]. We observed robust migratory behaviours within the latently-infected T cell population (12.75μm/min), which is comparable to or even higher than the control population. In contrast, late productively infected T cells displayed lower migration speeds (7.83μm/min), which is consistent with previous studies done in our lab[128]. Additionally, the arrest coefficient of latently infected T cells was not significantly altered compared to control T cells, while productively infected T cells displayed a significantly higher arrested behaviour, indicating impaired migration capacity and the possibility of virological synapses (VS) formation with other non-visualized uninfected $CD4^+$ T cells (**Figure 3. 13D**).

Furthermore, productively-infected T cells displayed abnormally elongated phenotypes (yellow arrowheads in **Figure 3. 13B**), which mirrored similar observations from our earlier study[128], while latently-infected T cells did not exhibit this phenotype but rather displayed a polarized, amoeboid morphology typical of highly migratory cells similar to uninfected cells (**Figure 3. 13B**). Collectively, these data suggest that despite HIV infection, latent T cells retain their migratory ability in a way that is similar to uninfected resting memory $CD4^+$ T cells. These observations have implications on the ability of HIV-infected, but transcriptionally silent $CD4^+$ T cells to access secondary lymphoid tissues and recirculate under ART suppression.

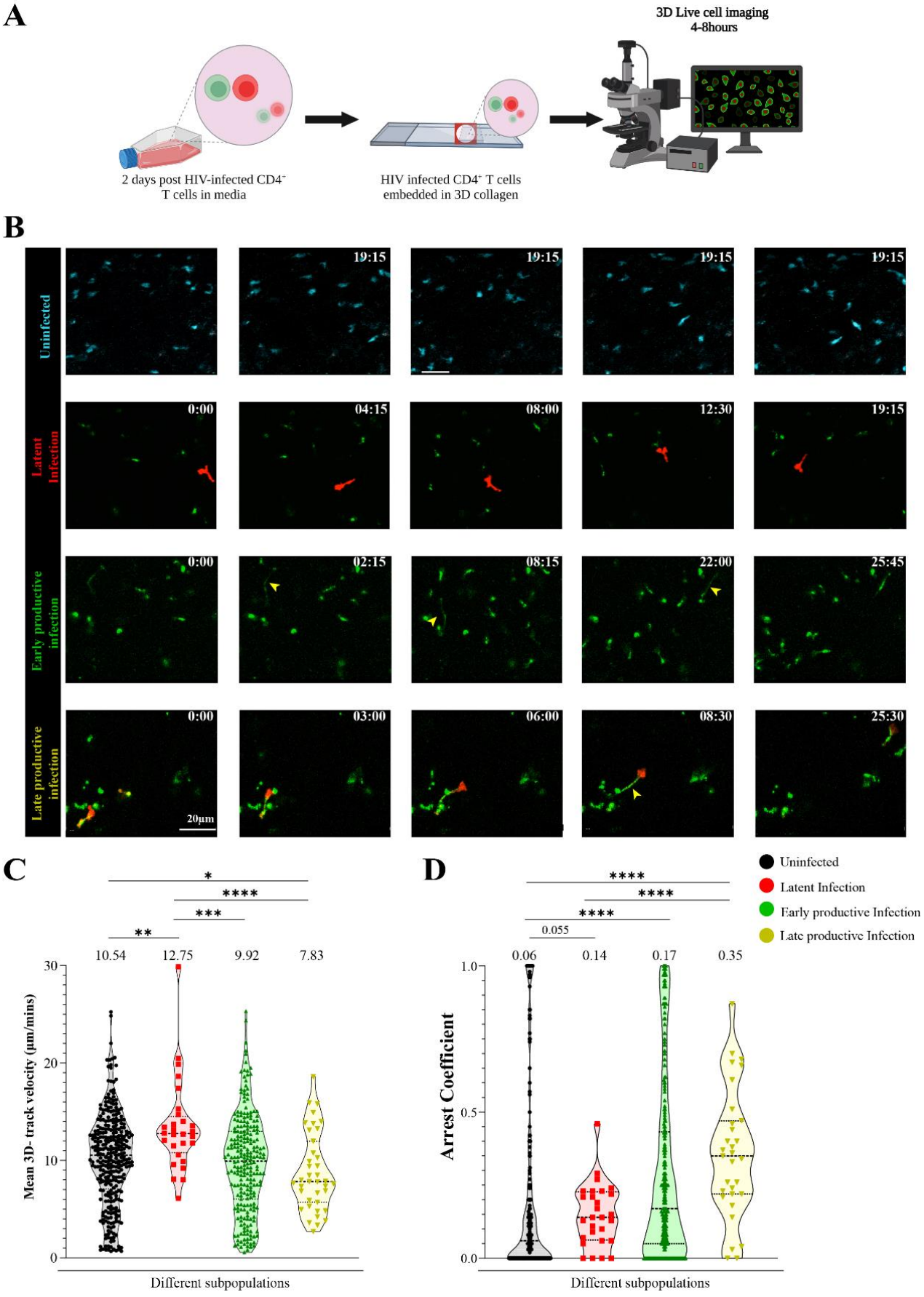

A
3D Live cell imaging
4-8hours
2 days post HIV-infected CD4+ T cells in media
HIV infected CD4+ T cells embedded in 3D collagen
B
Uninfected
Latent Infection
Early productive infection
Late productive infection
19:15
0:00
04:15
08:00
12:30
02:15
08:15
22:00
25:45
03:00
06:00
08:30
25:30
20µm
C
Mean 3D- track velocity (µm/mins)
10.54
12.75
9.92
7.83
Different subpopulations
D
Arrest Coefficient
0.055
0.06
0.14
0.17
0.35
Different subpopulations
Uninfected
Latent Infection
Early productive Infection
Late productive Infection

Figure 3. 13: $CD4^+$ T cell migration of different infected subpopulations in 3D collagen matrix.

A) Schematic design of the experimental setup. Infected $CD4^+$ T cells were embedded into custom chambers to generate 3D collagen imaging platforms for live-cell microscopy —*Figure created with Biorender*.
B) Representative time-lapse multi-photon micrograph of the different subpopulations in 3D collagen. The yellow arrowheads indicate the unique elongated phenotype of a productively infected cell.
C) Mean 3D track velocity of the different subpopulations in 3D collagen.
D) Arrest coefficient of the different subpopulations in 3D collagen.

*The numbers above the graph represent the median. Data was pooled from 39 recordings from a total of 10 independent experiments with 30-minute recordings. Statistical analysis was performed using a Mann-Whitney test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$*

3.4.2 Migratory behavior of latently infected CD4$^+$ T cells in the lymph node in vivo

To confirm observations made using our 3D collagen matrix model *in vitro*, I performed multi-photon intravital microscopy (MP-IVM) of the popliteal lymph node (LN) to examine the migratory capacity of HIV-infected T cells *in vivo* using humanized PBMC (hu-PBMC) mice (*as described in section 2.6*). I decided to use the hu-PBMC model for this study because it is simple to generate, and the T cell zone of the lymph node is largely intact, providing a robust stromal scaffold for T cell attachment and migration (*data not shown*). To generate hu-PBMC mice, NOD-SCID mice were used because they lack mature T and B cells and display dysfunctional NK cells that are ideal recipient hosts for human cells. NOD-SCID mice were injected with anti-asialo GM1 antibody to further remove mouse NK cells 2 days before xenograft implant with healthy human PBMC (**Figure 3. 14A**). Once mice met a reconstitution criterion of >400 CD4$^+$ T cells/µl of blood and the popliteal LN were deemed large enough for imaging, mice were infected with HIV-TosZy via footpad injection and the popliteal LN was surgically prepared for MP-IVM 24-48 hours post-infection as described previously[368] (**Figure 3. 14A**). As depicted in **Figure 3. 14B**, infected T cell populations were visible in the lymph node and allowed for computational analysis of multiple movie recordings acquired, as *described in subsection 3.4.1*. In a separate study, uninfected T cells were stained with Celltracker Green (CMFDA; 1.5µM), injected into the footpad for multi-photon microscopy and used as a reference population for comparative studies with HIV-infected T cells. We observed robust migration within the latent T cell population at comparable mean track speeds as uninfected T cells (9.75µm/min vs 11.17 µm/min), which was consistent with our *in vitro* studies. We only observed one late productively-infected T cell in our lymph node imaging studies thus far, which migrated at 6.72µm/min (**Figure 3. 14C**). This observation likely reflects the fact that productively infected cells may have limited viability in tissues and are rapidly cleared by tissue phagocytic cells. No difference in arrest coefficient between latently and productively infected T cell populations were observed, although few cells were observed in the latter (**Figure 3. 14D**). Together, we confirmed that latently-infected T cells display robust migratory behaviours in the T cell zone of the lymph node parenchyma, making my study the first characterization of latent T cell migration behaviours in living tissues *in vivo*. These studies provide functional proof that HIV-infected but transcriptionally silent T cells retain their ability to enter and migrate within the lymph node, which has implications on how infected T cells continue to seed distant tissues during suppressive ART.

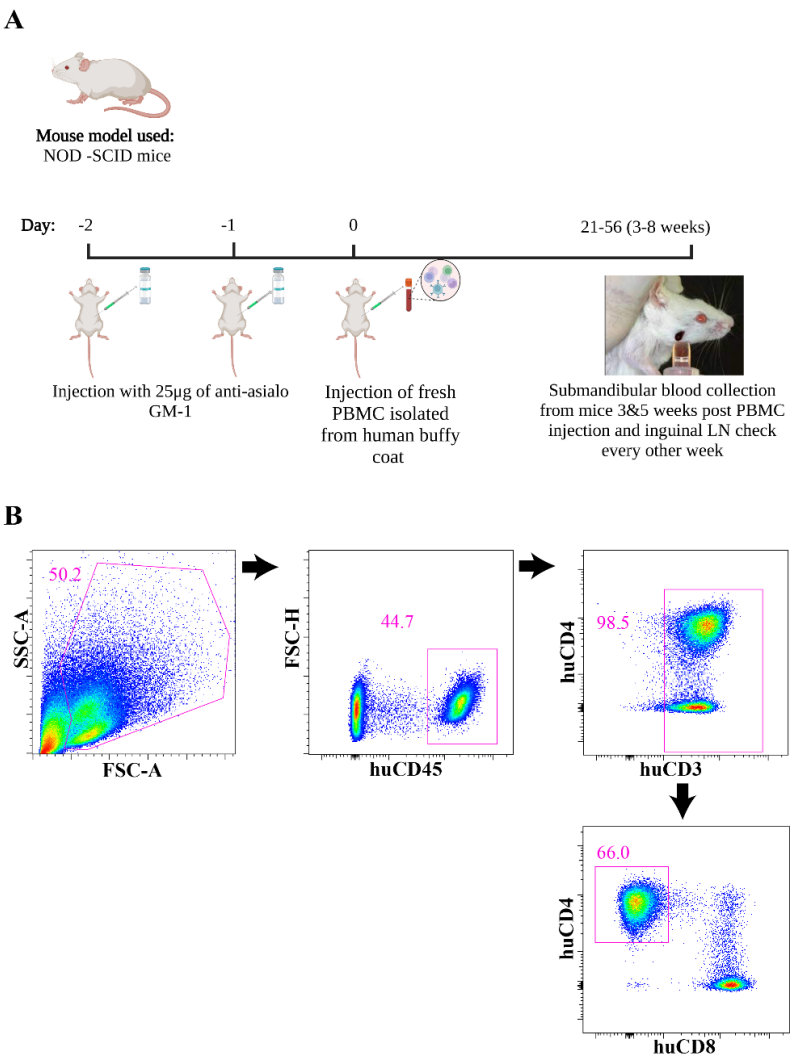
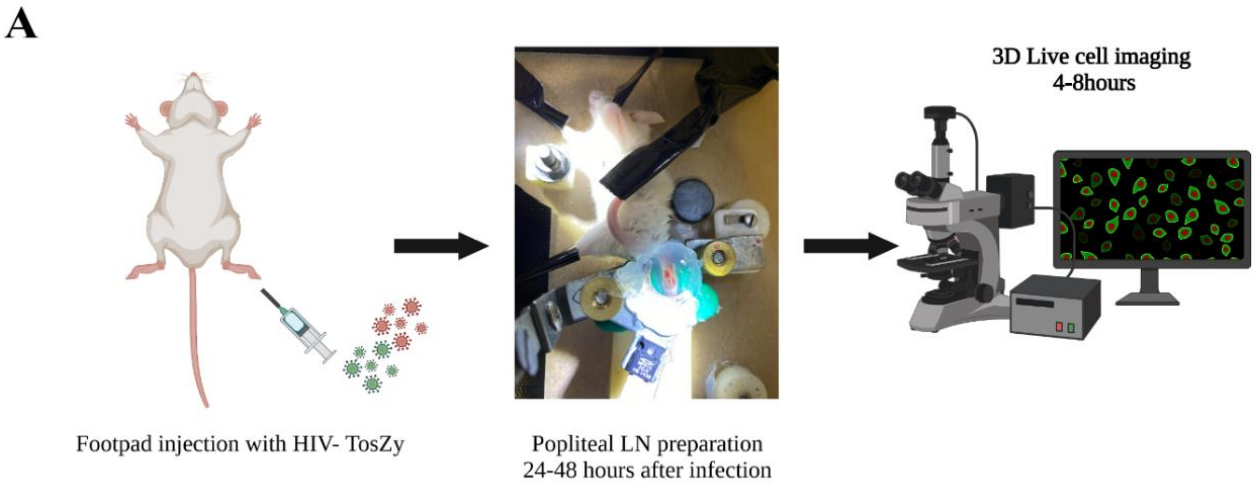
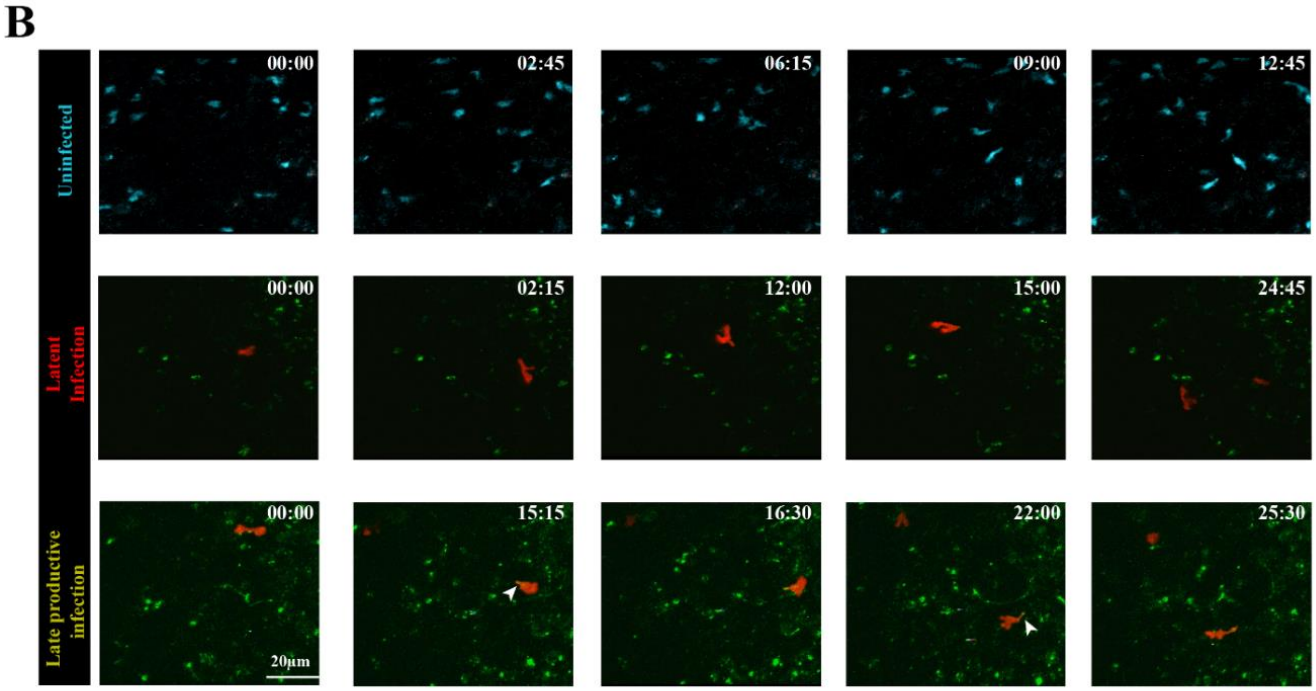


Figure 3. 14: Generation of Humanized PBMC (hu-PBMC) mice.

A) Schematic diagram illustrating the generation of hu-PBMC mice. NOD-SCID mice are implanted with human peripheral blood mononuclear cells (PBMC) after anti-asialo GM1 antibody injection —*Figure created with Biorender*.

B) Representative nested gating strategy of human lymphocytes isolated from peripheral blood at 4 weeks post-engraftment. Live cell population was gated according to SSC-A (granularity) vs. FSC-A (size) properties, after which hematopoietic cells (CD45+) were gated (FSC-H vs. CD4), followed by CD4+ CD3+ cells gated out and CD4+ cell count was used to determine reconstitution levels. Quadrant gating was used to determine the frequencies of the different subpopulations using FlowJo software. Healthy mice reconstitution with >100 $CD4^+$ T cells/µl of blood were used for imaging experiments.


A
3D Live cell imaging
4-8hours
Footpad injection with HIV- TosZy
Popliteal LN preparation
24-48 hours after infection


B
Uninfected
Latent Infection
Late productive infection
00:00
02:45
06:15
09:00
12:45
00:00
02:15
12:00
15:00
24:45
00:00
15:15
16:30
22:00
25:30
20µm

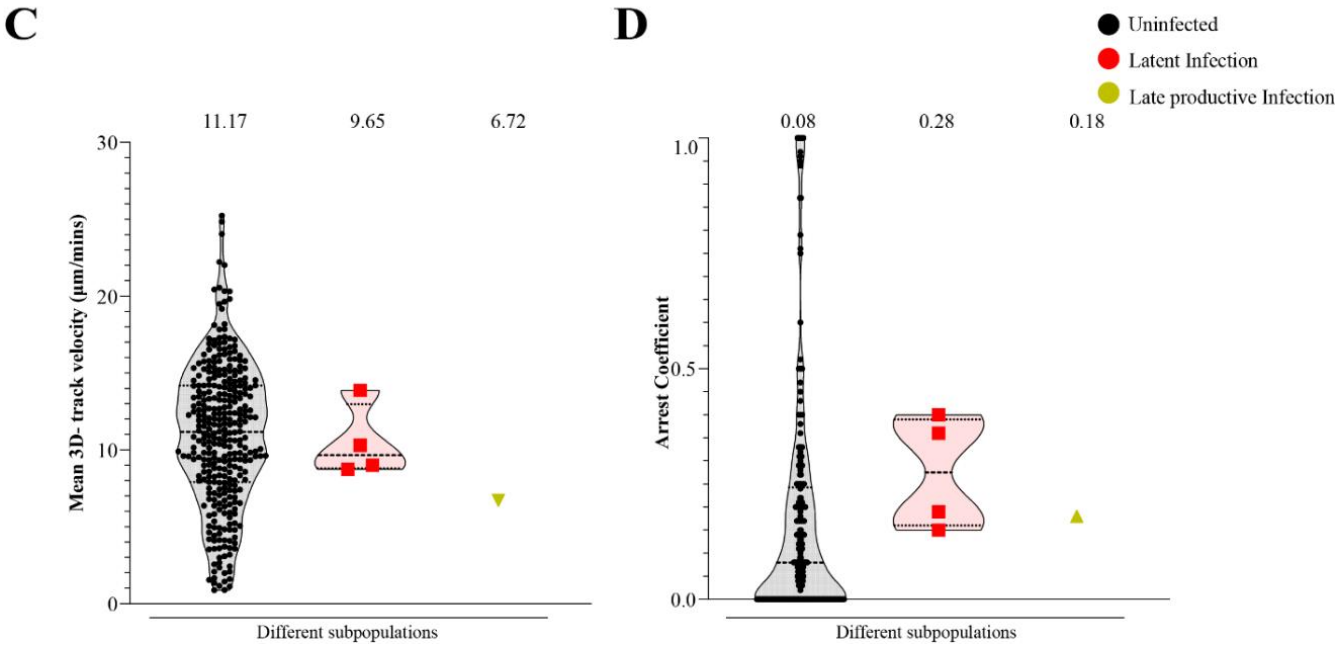

C
D
Uninfected
Latent Infection
Late productive Infection
11.17
9.65
6.72
0.08
0.28
0.18
Mean 3D- track velocity (µm/mins)
Arrest Coefficient
Different subpopulations
Different subpopulations

Figure 3. 15: Visual characterization of latently-infected T cells in the lymph node

A) Experimental design to visualize T cell migration in popliteal LN (pLN) of hu-PBMC mice — *Figure created with Biorender*.

B) Representative time-lapse multiphoton micrograph of the different subpopulations in the popliteal LN at 48 hours post-infection. The white arrowheads indicate Nef-ZsGreen expression in the uropodia of an infected cell.

C) Mean 3D track velocity of the different subpopulations in hu-PBMC popliteal LN at 48 hours post-infection.

D) Arrest coefficient of the different subpopulations in hu-PBMC popliteal LN at 48 hours post-infection.

The numbers above the graph represent the median. Data was pooled from 3recordings/1 experiment.

3.5 Tissue localization of various infected subpopulations *in vivo*

To determine characterize whether the HIV TosZy reporter can infect cells in vivo and identify latently-infected T cells in various tissues other than the LNs, hu-PBMC mice were intraperitoneally infected with $5x10^6$ pfu HIV TosZy to examine the distribution of virally infected cells in various tissues (**Figure 3. 16A**). On day 3 post-infection, mice were sacrificed, and the inguinal lymph node (iLN), spleen, lungs, liver and peripheral blood were collected for flow cytometry analysis. Both latent and productively infected T cells were visible by flow cytometry in various tissues. I observed a relatively low proportion of productively infected cells across the various tissues, with the iLN and the spleen having the highest number of productively infected cells at 0.21% and 0.049%, respectively (**Figure 3. 16B**). The low levels of productively infected cells in the various tissues could be attributed to these cells being cleared by the immune system or these cells undergoing virus-induced cell death, as infected cells actively producing virus have a half-life of 24-48 hours[371]. However, I observed a high proportion of latently infected cells compared to productively infected cells in the tissues analyzed. Notably, the blood had the highest number of latently infected cells at 44%, followed by the lungs, with a latent cell proportion of 12.6%. This could be as a result of the injection route, as the injected virus would need to be absorbed by the bloodstream first before biodistribution to other organs. Overall, these results suggest that HIV-TosZy can cause a systemic infection *in vivo,* and there is a differential expression of the different infected subpopulations across various tissues. This finding is in agreement with Ratnapriya *et al.* study published in Cell Reports, which proposed that extracellular signals may modulate the fate of HIV infection in the different organs of humanized BLT mice, either promoting HIV replication (production) or latency generation[270] . Additionally, consistent with the *in vitro* observation, CD4 expression was relatively maintained in the latently infected cells compared to the productively infected cells whose CD4 expression was extensively downregulated. This suggests that *Nef* and *Vpu* gene function is maintained *in vivo*, and serves as an impetus to study how the distribution of latent T cells in tissues evolves under ART suppression *in vivo* in future studies.

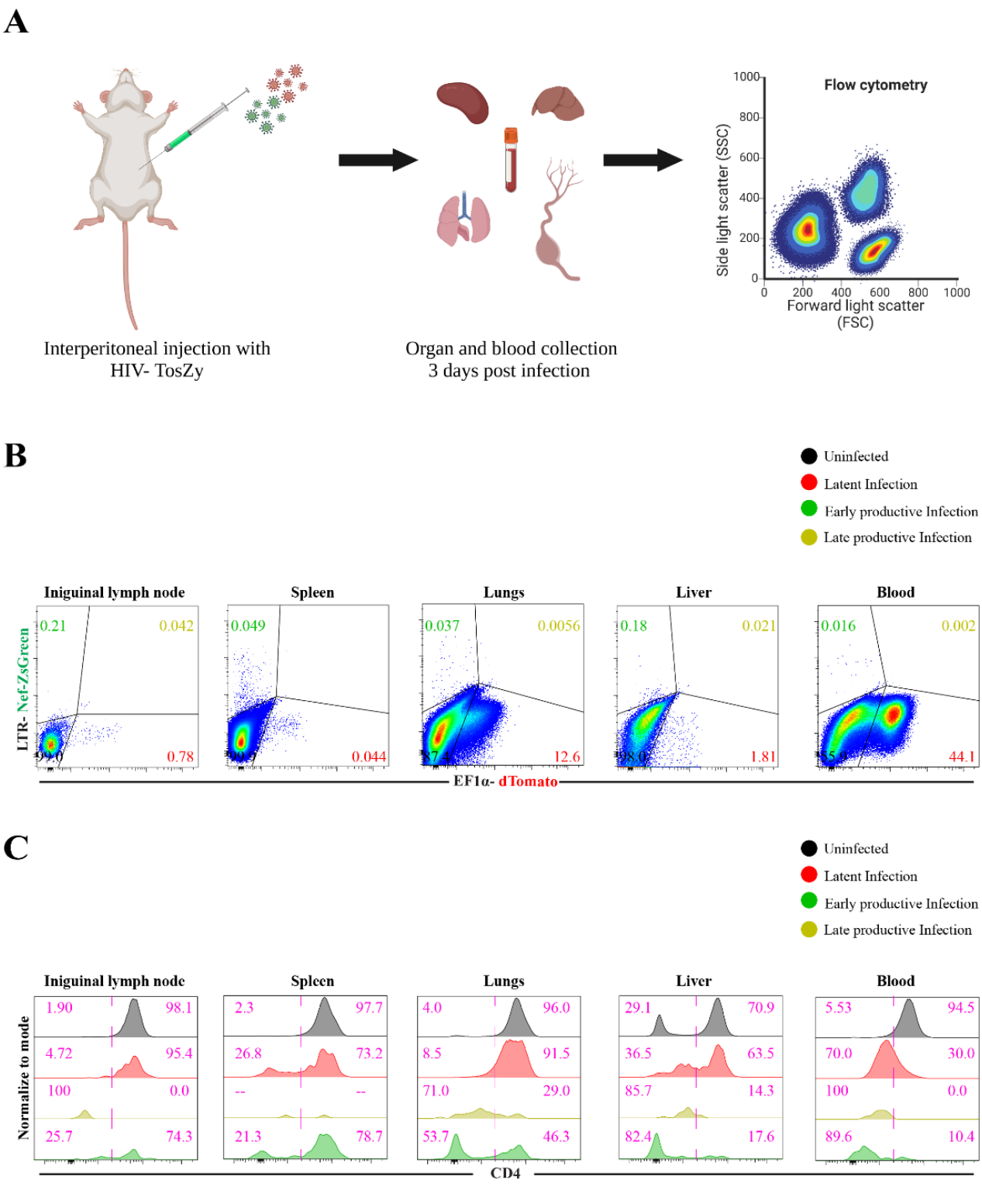


Figure 3. 16: Tissue localization of HIV-infected CD4⁺ T cells in hu-PBMC mice.

A) Experimental design to address the distribution of HIV-infected T cells in hu-PBMC mice. Hu-PBMC mice were injected intraperitoneally (IP) with $5x10^6$ pfu HIV TosZy, sacrificed on day 3 post-infection, and the indicated organs were harvested for flow cytometry analysis —*Figure created with Biorender*.

B) Representative flow cytometry plots of infected human $CD4^+$ T cells isolated from various organs day 3 post-infection.

C) Representative histogram plots showing CD4 expression of infected human $CD4^+$ T cells isolated from various organs day 3 post-infection.

CHAPTER 4

DISCUSSION, CONCLUSION, AND LIMITATION

4.1 The heterogeneous nature of the latent T cell reservoir

The establishment of *viral latency* by HIV-1 presents a significant impediment to achieving a lifelong cure or a durable drug-free remission in infected individuals. It is well documented that the cessation of antiretroviral therapy (ART), which potently inhibits HIV replication and prevents immunodeficiency in people living with HIV (PLWH), causes viral rebound back to pretreatment levels. Therefore, a better understanding of the cellular and molecular mechanism of HIV latency is instrumental to developing new pharmacological therapy and HIV eradication strategies. However, studying latently infected cells that are transcriptionally silent *in vivo* is highly challenging due to their rarity, with only about 1 in 10^5-10^7 $CD4^+$ T cells being latently infected[112,393,394]. Furthermore, there are currently no biomarkers to identify latently infected cells, although studies have shown the upregulation of checkpoint molecules in this population, such as LAG-3, TIGIT, and PD-L1, also the integrin α4β1 was shown to be expressed on 70% of infected cells in both virally suppressed and unsuppressed PLWH[145,395]. Hence, the development of techniques and tools that enable the identification, isolation and enrichment of these latent cells is crucial for advancing research in the field. Various primary cell models have been implored to study HIV latency, but these models have several limitations[251,255,257,259,261,262,396,397]. Mostly, these primary HIV latency models infect cells using an HIV fluorescent reporter (such as green fluorescent protein, GFP) driven by the LTR to establish active infection, followed by cell selection and prolonged cell culture to establish latency, and has led to important discoveries on latency maintenance and reactivation. However, this method hinders the investigation of the early establishment and long-term study of HIV latency since the identification of latently infected cells primarily relies on activating the HIV provirus using anti-CD3/CD28 or PMA/ionomycin stimulations. These activation protocols typically alter phenotypic and transcriptional features of infected cells and can lead to cell death, which prevents longitudinal studies[264]. In the last decade, the development of dual-fluorescent HIV reporters has enabled distinguishing transcriptionally active from transcriptionally silent HIV-infected cells *in vitro* without restimulation protocols[214,247,263-271]. Dual fluorescent reporters allow researchers to investigate various stages of HIV latency and reactivation, including their establishment, maintenance and proviral expression

dynamics. However, a considerable limitation of these previously published reporters is their lack of *Env*, *Vpu* and/or *Nef* genes in order to make the proviral construct smaller. These viral proteins, specifically Nef, have been implicated as a critical factor in the modulation of various T cell functions, including vesicular transport, cytoskeletal remodelling, and signal transduction[398]. Additionally, the lack of the *Env* gene prevents multiple rounds of infection and has been shown to reduce the frequency of defective provirus due to a reduction in the reverse transcription process[399]. Lastly, Vpu orchestrates different processes that allow for viral fitness, such as the degradation of interferon-inducible factor, tetherin (BST-2), thereby allowing for viral dissemination[378,400,401]. Thus, these reporters may not ultimately model latency establishment and maintenance when these viral genes are absent. To this end, I developed a new full-length dual fluorescent reporter construct that allows me to distinguish between productive and latent infection *in vitro* and *in vivo* and longitudinally assess their phenotypic and migratory characteristics. The main objective of my dissertation project was to investigate the similarities and differences between uninfected and latently-infected primary $CD4^+$ T cells in terms of their ability to access and migrate within various tissues as a means to facilitate long-term persistence.

First, I infected and characterized the infection dynamics of HIV-TosZy in primary $CD4^+$ T cells. This led to the identification of four distinct populations: uninfected (LTR^- $EF1\alpha^-$), latent infection (Red; LTR^- $EF1\alpha^+$), early productive infection (Green; LTR^+ $EF1\alpha^-$) and late productive infection (Yellow; LTR^+ $EF1\alpha^+$). In a previous study conducted by our collaborators, no significant difference in the gene expression of early (Green; LTR^+ $EF1\alpha^-$) and late (Yellow; LTR^+ $EF1\alpha^+$) productively infected cells was observed[270]. Due to the constitutive nature of the EF1α promoter, its expression is generally expected to remain active at all times; however, in the population termed "early productive", only the LTR promoter activity is observed (high ZsGreen expression), which may be due to promoter competition or transcriptional interference. Transcriptional interference refers to the disruption or inhibition of the transcription of one gene by another gene located nearby on the same DNA strand[402]. For example, a downstream gene's initiation, elongation, or termination by the transcriptional machinery can be interfered with by a second gene typically found upstream[403]. On the other hand, promoter competition commonly occurs when there is an overlapping promoter region, with transcription preferentially initiated at one of the promoters at a time and is well characterized in prokaryotic organisms[402,403]. However, promoter competition can also occur when two or more promoters compete for the same transcriptional machinery[403].

This phenomenon has been characterized in eukaryotic organisms, chickens, and yeasts[404-406]. In the dual fluorescent reporter system, since EF1α and HIV LTR promoters are likely to compete for the same cellular resources, such as ribosomes and transcription machinery, it is possible that HIV LTR may outcompete the EF1α promoter for these resources, especially in the presence of HIV Tat accessory protein that greatly enhance the transcriptional activity of the HIV LTR. The dampened activity of the EF1α promoter causes a higher expression in the proportion of ZsGreen expressing cells and a reduction in the expression of dTomato in infected cells. The consequence of this is the possible underestimation of the latent population when cells are infected with HIV TosZy. However, even with this limitation of the system, I can continue to clearly distinguish early productively infected cells from those that remain latent, which is the primary goal of this reporter system.

HIV-1 infection cripples the host adaptive immunity by depleting $CD4^+$ T cells if left untreated[3]. The HIV-1 accessory protein, Nef, expressed early during infection, has been well established as the primary perpetrator of CD4 depletion in infected individuals, as it induces apoptosis[374]. We confirmed the functionality of the protein in this primary cell model and observed a significant downregulation of CD4 expression within productively infected cells but not in the latently infected cells. As expected, the population actively producing HIV protein, p24 Gag, expressed substantially less cell surface CD4. Vpu has also been implicated in the downregulation of CD4, although its effect is more subtle[407]. Other functions of Vpu were also measured by the assessment of an interferon-induced host restriction protein, tetherin (BST-2)[378], which was downregulated to a greater extent in productive cells but maintained at high levels in the latent cells. Tetherin downregulation aids in viral dissemination by enhancing the release of progeny virions from infected cells; thus, the maintenance of its expression on the latently infected cells may also limit the production and release of viral progeny. These findings from our primary cell latency model align with the known functions of these HIV accessory proteins in other studies, enabling us to mimic HIV infection *in vivo* and to investigate HIV latency in a physiologically relevant cellular context that considers the role of all accessory proteins. Not surprisingly, the lack of viral protein expression in latent T cells contributed to their "normal" phenotype compared to uninfected cells, which has important implications on their ability to persist long-term. However, it is also possible that HIV protein expression can be repeatedly reactivated at low levels during their transit in the body, possibly through engagement with antigen-presenting cells (APCs) or

cytokine exposure. Whether cells that produce low levels of the virus can revert to a latent state remains an unanswered question.

It is important to note that some latently infected cells in our system still express low levels of HIV Gag protein p24, although in a minimal amount compared to the productively infected cells, despite the presence of ART in the culture. Therefore, it is plausible to infer that in the presence of ART, the term latency encompasses a range of transcriptional silencing levels, ranging from integrated viral DNA or proviruses in the deep latent state to those undergoing minimal or transient transcriptional activity, as mentioned above. According to recent seminal work from the Lichterfeld lab, a deep latency state emerges in long-term ART-treated PLWH[408]. *The deep latency state* results from the progressive selection of proviruses within the viral reservoir as a result of the host immune-mediated selection pressure such that selected and accumulated proviruses exhibit limited or no transcriptional activity and exhibit low sensitivity to HIV latency reversal agents (LRA)[89,408-410]. These and other studies collectively showed that the proviral integration site, the epigenetic characteristics of the chromatin, and the epigenetic regulation of the host chromosome could influence the degree of HIV transcriptional silencing[89,408,409,411]. Over time, due to immune pressures, regions resulting in deep latency (for example, nongenic regions such as centromeric satellite DNA) may be preferentially selected. Deep latency has also been shown as a mechanism of viral control in elite controllers[89]. In an *ex vivo* longitudinal study carried out on five ART-treated PLWH where individual proviruses were analyzed, it was observed that the nongenic chromosomal regions harbouring HIV proviruses had increased chromosomal distances to regulatory regions such as the frequently interacting regions (FIREs) and the topologically associated domains (TADs), thereby diminishing transcriptional activity[408]. Interestingly, in two of the participants, it was observed that despite long-term ART, about 34% of the proviruses are transcriptionally active, which is contradictory to the immune-mediated selection hypothesis. In the transcriptionally active population, the proviral integration was in regions of activating chromatin features, and there was a decrease in the chromosomal distance to FIRE, thereby promoting transcriptional activity. A common observation across all patients in both the transcriptionally silent and the transcriptionally active proviruses was the identification of clonal, sequence-identical proviruses derived from the clonal proliferation of these latent T cells. In the transcriptionally silent population, a relatively stable or expansion of defective and intact proviruses in nongenic regions was observed, whereas in the transcriptionally active population,

they see that genes harbouring transcriptionally active clonal proviruses were often involved in the regulation of cell proliferation and oncogenesis. Einkauf *et al.* postulated that these transcriptionally active clonal proviruses, referred to as 'loud minority clones', can survive long-term ART and evade the body's immune system by leveraging high cell turnover rates to continually restore and enlarge their population, thereby overcoming negative selection pressures[408,410,412]. These observations underscore the complex dynamics of HIV latency and the challenges in achieving a functional cure.

4.2 The secondary lymphoid organs (SLOs), an immunological tissue niche that supports viral persistence

Adaptive immunity is generated mainly within secondary lymphoid organs (SLOs), which are strategically located throughout the body to continually survey and respond against non-self antigens and generate immunological memory[413]. This process is driven by the recirculation of various immune cell subsets between lymphoid and non-lymphoid tissues that are guided by the expression of chemokine, integrins and other homing receptors that respond to specific chemokine gradient cues. In the context of HIV-1 infection, the lymph node and the gut-associated lymphoid organ (GALT) have been shown to contain the highest level of viral replication and are preferential sites for viral reservoir establishment in both human and animal studies[195,210-212,382] It is worth emphasizing that the viral reservoir in virally suppressed PLWH consists mainly of $CD4^+$ T cells with a memory phenotype, particularly central and transitional $CD4^+$ T cells, T_{CM} and T_{TM}, while T_{EM} harbours the most intact-replication competent provirus compared to T_{CM} and T_{TM}. In addition, ample evidence in various pioneer studies has shown how lymph node cytokines could play a role in establishing and maintaining the *viral reservoir*[160,372,414]. For example, the interleukin 7 (IL-7) produced by the fibroblastic reticular cells (FRCs) in the lymph node, known to promote the survival and proliferation of memory T cells, has also been linked to the persistence of the viral reservoir in virally suppressed PLWH through, a process known as homeostatic proliferation[131,415]. Additionally, studies have demonstrated that the chemokines CCL19 and CCL21 can augment viral entry and integration in quiescent $CD4^+$ T cells[254,255,373]. This effect was said to be mediated by cytoskeletal rearrangement, cortical actin activation, and the stabilization of the HIV integrase enzyme through the inhibition of proteasomal degradation of the enzyme. Based on this evidence, we postulated that long-lived, latently infected memory $CD4^+$ T cells may co-opt survival and

proliferation signals by maintaining their recirculatory capacity between lymphoid and non-lymphoid organs, which contributes to their long-term persistence. Outstanding questions in the field include: (i) does infection in latently infected $CD4^+$ T cells impact the expression homing, entry and exit markers to SLOs? (ii) what is the migratory capacity of latently infected cells in a three-dimensional (3D) environment? (iii) do latently infected cells retain the ability to enter and migrate within the T cells zone of the lymph node *in vivo?* Some of these questions were addressed in my dissertation, discussed below.

4.3 Impact of HIV latency on SLOs homing and migratory receptors expression in primary $CD4^+$ T cells

Studies have demonstrated that HIV infection can alter the expression of specific integrins and chemokine receptors essential for immune cell trafficking, potentially dysregulating homing and migration[416-418]. For example, cell surface receptors, CC-chemokine receptor 7 (CCR7) and CD62L (L-Selectin) are downregulated in HIV-infected $CD4^+$ T cells[400,417,418]. Here, I addressed whether HIV-infected but transcriptionally silenced provirus also altered the expression of chemokine receptors and integrins involved in T cell homing, entry, and exit from the secondary lymphoid organs.

Naïve and central memory T cells (T_{CM}) perpetually recirculate through the blood, SLOs and lymphatics to maximize the chances of antigen encounter. T cell homing (entry and exit into the LN) is a complex, sequential cascade involving key molecules such as CD62L, CCR7, LFA-1, and S_1PR1[203]. Cells can enter the lymph node via two conduits: the afferent lymphatic vessels, which are in close proximity to the subcapsular sinus (SCS), and the high endothelial venules (HEVs), which are predominately located in the paracortex[203,419]. The HEVs are specialized blood vessels and serve as the primary port of entry for most circulating cells into the LN. As naïve and memory T cells transit from the blood into the LN via the HEVs, they must adhere to the vessel walls to counteract the inherent shear forces within blood vessels[420]. This tethering process is orchestrated by CD62L (L-selection) on circulating T cells and a CD62L reactive ligand, an O-linked glycan called peripheral node addressin (PNAd), on the HEVs[421]. The weak selectin-glycan interactions, combined with the effect of blood flow, enable T cells to roll along the walls of blood vessels[422]. As these cells roll, they encounter CC-chemokine ligand 21 (CCL21), a molecule that is anchored to HEVs due to its binding with heparan glycosaminoglycans, and is recognized by

the G protein-coupled receptors (GPCRs), CCR7, expressed on these rolling cells[423]. Recognition of CCL21 by CCR7 initiates a series of intracellular signals, which in turn trigger the activation and affinity maturation of lymphocyte function-associated antigen 1 (LFA-1)[203,419,420]. The activated LFA-1 interacts with intercellular adhesion molecules (ICAM1/2) on the HEV, leading to the firm adhesion and crawling of T cells along the walls of the blood vessels[424]. This process allows the extravasation of the crawling cells from the vasculature into the LN[425]. T cells' egress, or exit, from the LN back into circulation is primarily mediated by the signalling of a lipid molecule, Sphingosine-1-phosphate (S_1P), and its receptor, S_1PR1, on T cells[426]. The levels of S_1P are relatively higher in the blood/plasma and lymph compared to tissues[426]. S1P in the blood/plasma is mainly produced by red blood cells, erythrocytes, platelets and endothelial cells, while in the lymph, it is majorly produced by the lymphatic endothelial cells[427,428]. It is important to note that the expression of S_1PR1 on circulating T cells is dynamically regulated; the activation of T cells and concentration of S_1P are some of the main regulators. Upon antigen recognition and activation of T cells, they transiently express an early expression marker, CD69, which can interact with S_1PR1 and induce its internalization, allowing cells to temporarily remain in the LN for proliferation and differentiation[429,430]. However, as the expression of CD69 starts to wane, these cells can re-express S_1PR1 again and leave the LN. Additionally, following concentration gradient cues, circulating cells can migrate from a low S_1P concentration environment, such as tissues, to a region of high S_1P concentration, such as the blood[383-385].

CD62L (L-selectin) is expressed by naïve and central memory T cells and is crucial for lymph node entry from the high endothelial venues (HEVs), as demonstrated by studies that show impaired T cell homing in CD62L-deficient mice[431]. The expression of CD62L was similar between the uninfected cells and the latently infected cells, but it was significantly lower in the productively infected cells. This result is expected as HIV proteins, Nef and Vpu, have been shown to impede the transportation of CD62L to the plasma membrane in infected cells[417]. Experiments with CCR7-knockout mice have demonstrated the indispensable role of CCR7 in the trafficking and recirculation of T cells to the lymph node[432,433]. CCR7 expression in the latently infected cells was comparable to the uninfected cells, and it was observed that these cells retain their ability to migrate toward lymph node-homing chemoattractants CCL19 and 21 *in vitro*. LFA-1, whose role have been shown to be significant for T cell homing and residency duration in the lymph node [434-436], was slightly elevated in the latently infected cells compared to the uninfected cells. The

expression of the exit receptor, S_1PR1, in latently infected cells was also expressed in comparable levels to uninfected cells, although productively infected cells showed a higher expression of the receptor.

Strikingly, the expression of a vital GALT homing receptor, β7 integrin, which dimerizes with α4, was shown to be preferentially upregulated in latently infected cells when compared to uninfected cells. Thus, it is likely that this upregulation causes a preferential targeting of homing latently infected cells to the GALT for the continual establishment of a viral reservoir. Collectively, it can be concluded that HIV infection in latently infected cells may not impede the ability of these cells to migrate and recirculate as the expression of homing, entry and exit markers to SLOs are not significantly altered and are expressed in similar levels as that of uninfected $CD4^+$ T cells.

4.4 Migratory behaviour of latently infected $CD4^+$ T cells *in vitro* and *in vivo*

Using *in vivo* imaging approaches, seminal studies by Murooka *et al.* showed that HIV infection alters migratory and phenotypic behaviours of productively infected $CD4^+$ T cells in a way that facilitates viral dissemination *in vivo*[128]. Expression of Env by productively infected $CD4^+$ T cells caused cell-cell fusion events, or syncytia, with other immune cells in the lymph node that remained viable for days[128]. Interestingly, Nef expression was largely responsible for the deceleration of T cell migration through modulation of the host actin polymerization machinery. Inhibition of productively infected T cell exit from locally-infected lymph nodes completely prevented systemic viral dissemination, indicating that recirculation of HIV-infected T cells plays a role in viral spread during acute infection[128,437]. With this knowledge, I addressed whether HIV-infected but transcriptionally silent $CD4^+$ T cells also displayed migratory and recirculatory defects in a way that has implications for their long-term survival under ART suppression.

First, I evaluated latently-infected T cell migratory behaviors in 3D fibrillar collagen gels *in vitro*, which we have shown mimics the interstitial matrix of connective tissues found in the lymph node (1.7mg/mL bovine collagen gels)[366,438-440]. Based on earlier studies demonstrating that latently infected $CD4^+$ T cells lacked measurable or minimal expression of Nef, Vpu and Gag, I hypothesized that latently infected $CD4^+$ T cells would maintain their migratory capacity similar to that observed in uninfected central memory $CD4^+$ T cells. I observed that latently-infected cells were robustly motile and had no impaired migratory capacity as they migrated at an average 3D

velocities of ~13 μm/min, comparable to and even higher than migrating, uninfected memory $CD4^+$ T cells. As expected, migration of productively-infected T cells was reduced in my imaging studies, validating previous observations. Together with data that CCL19/CCL21 also enhance the migration of infected T cells, these data indicate that latently infected $CD4^+$ T cells do not have impaired migratory capacity and have similar migratory behaviours as uninfected central memory $CD4^+$ T cells *in vivo.*

While reductionist systems such as 3D collagen matrices provide complex scaffolding that permits T cell migration, they are devoid of other immune cells and other physiological properties such as innervation, blood and lymph flow. Using a human PBMC (hu-PBMC) mouse model, which recreates the T cell zone of peripheral lymph nodes after successful reconstitution, we addressed whether latently-infected T cells could enter and migrate within the T cell zone of the popliteal lymph node using intravital microscopy. Infection with HIV-TosZy allowed for visualization of both productive and latently-infected T cells and displayed similar migratory behaviors as observed *in vitro*, with an average 3D velocities of ~10 μm/min, comparable to migrating uninfected cells. Taken together, these findings suggest that the latently infected T cells retain their ability to migrate in tissues, which has implications on how we view HIV latency in T cells in individuals on ART. The entry and migration of latently infected T cells within the T cell zone may permit access to physiological survival and proliferative signals in the lymph node, such as IL-7, that contribute to reservoir maintenance. Another possible implication is the interaction of these latent cells with antigen-presenting cells, such as dendritic cells (DCs), posing a risk for possible *antigen-driven homeostatic proliferation/reactivation* and subsequent spread of reactivated HIV to uninfected bystander T cells, perpetuating the viral reservoir, leading to long-term viral reservoir persistence. It could be proposed that the recirculation or entry/exit of these latently infected cells from lymphoid organs to other peripheral tissues could allow for the continual seeding of viral reservoirs in "sanctuary sites", which are tissues that have lower drug penetrations, such as the brain, lymph node, etc. which corroborates the study showing that recirculation of blood could serve as a hub for viral dissemination to various tissues/organs[195]. Further studies using the imaging approaches I have developed will investigate these possibilities *in vivo*.

4.5 Conclusion and impact of the study

In this study, I developed a new full-length dual fluorescent reporter, HIV-TosZy, which contains all essential and accessory HIV genes that permit the longitudinal assessment of HIV latency in primary $CD4^+$ T cells. First, I showed that I could establish a primary $CD4^+$ T cell latency model and visually distinguish latently infected in primary $CD4^+$ T cells infected with HIV TosZy and observed a heterogenous population of these cells. Second, I showed that HIV latency does not affect the expression of lymph node homing and migratory markers in latent primary $CD4^+$ T cells. Third, I observed that HIV latency does not impact the motility of latently infected cells and that they have a migratory behaviour similar to uninfected central memory T cells.

This study highlights that latently infected $CD4^+$ T cells retain their capacity to enter and migrate within the T cells zone in the lymph node, adding to the possibility that their recirculation potential is retained under ART. This has implications on access to survival/proliferative signals that contribute to long-term infected T cell persistence in a way that parallels normal memory $CD4^+$ T cells. My findings have important implications for reducing the size of the T cell reservoir by possibly targeting and blocking the homing to the lymph node and the recirculation of these latent T cells. The disruption of these mechanisms may prevent the reseeding of various tissues and prevent the cells from accessing the necessary survival and proliferative signals that contribute to their persistence, potentially reducing the longevity of the latently infected $CD4^+$ T cells.

4.6 Limitation of Study

1. In this study, we utilized a human peripheral blood mononuclear cell (hu-PBMC) mouse model, which does not fully recapitulate the complexity of HIV infection and latency establishment in humans. As such, conducting *in vivo* studies using human hematopoietic stem cell (hu-HSC) mouse models with a fully functional immune system may be beneficial.
2. It is important to note that *in vivo* lymph node imaging in this model was done at an early time point after infection and also did not take into account the impact of ART therapy in our system; however, it remains to be defined if the migratory dynamics of latently infected cells will change over time and will be impacted by ART therapy. A longitudinal study using our model in the presence of ART therapy may help provide additional clinical relevance.

3. The localized expression of Nef-ZsGreen protein to the uropod of infected cells makes them quite difficult to track using the imaging tracking software; thus, it was impossible to track early productively infected cells *in vivo*. Therefore, it is crucial to develop a reporter system where the *Nef* gene and the ZsGreen fluorescent protein are not linked, allowing for an easier tracking process.

Graphical abstract and proposed model

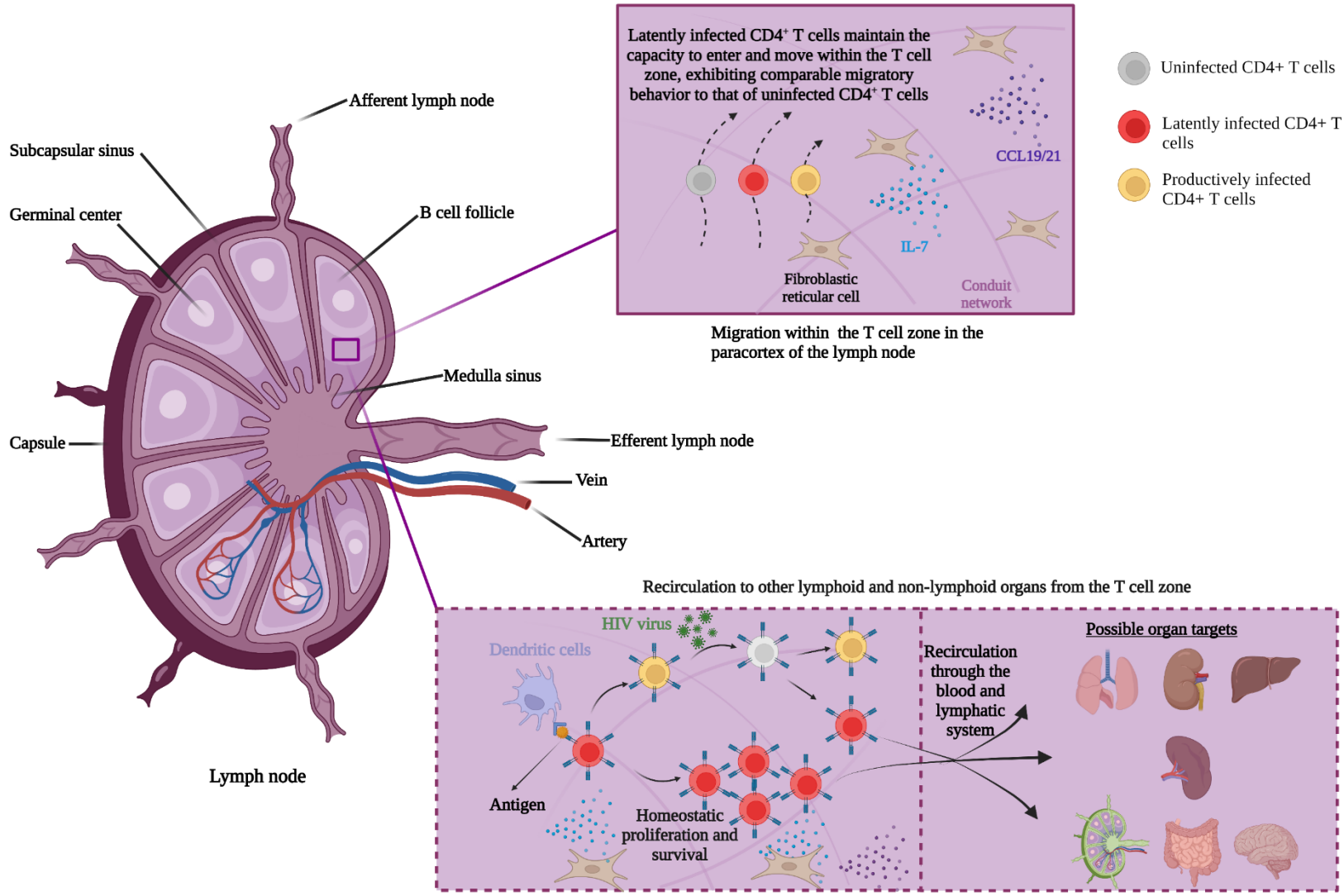


Figure 4. 1: Graphic illustration of the contribution of migrating latent CD4⁺ T cells to HIV viral reservoir persistence and replenishment. Latent CD4⁺ T cells, which are robustly motile, can enter and migrate within the T cell zone of the lymph node. Possible cell-to-cell interaction with dendritic cells allows latent CD4⁺ T cells to either get activated (which can cause viral

rebound, and newly produced virus can infect uninfected bystander $CD4^+$ T cells and cause potential formation of new latent $CD4^+$ T cells) or undergo homeostatic proliferation of these cells. These cells can then migrate to other tissue sites to continually seed the viral reservoir or disseminate the virus. Understanding the recirculation kinetics of latent T cells will give us valuable insight into how to develop new therapies to reduce or eliminate viral reservoirs —*Figure created with Biorender*.

CHAPTER 5

SIGNIFICANCE AND FUTURE DIRECTIONS OF THIS DISSERTATION PROJECT

5.1 Significance

This unprecedented study is the first to visually characterize latently infected $CD4^+$ T cells *in vitro* and *in vivo*, showing that these cells have similar phenotypic and migratory characteristics as memory uninfected $CD4^+$ T cells. In addition, I have established a possible *in vivo* mouse model to carry out various HIV latency imaging studies to help identify "tissue niches" within the LN where latent T cells reside to better understand ways to disrupt latent $CD4^+$ T cell expansion and survival in ART-treated individuals.

5.2 Future directions

5.2.1 Assessing replication-competent proviruses in latently infected cell subpopulations

More than 90% of proviruses in latently infected cells are defective, with less than 1% containing intact, replication-competent proviruses in people living with HIV (PLWH) on long-term ART[399,441,442]. Using an *in vitro* primary T cell model of HIV latency, the Bosque group showed that about half of integrated provirus in latent cells were intact and replication-competent[399]. Thus, validating whether the latent cell population established after infection with HIV-TosZy contains intact, replication-competent proviruses is crucial. The frequency of intact, replication-competent virus in my model system can be further assessed using a limiting dilution, quantitative viral outgrowth assay (QVOA) and PCR-based, intact proviral DNA assay (IPDA)[443,444]. QVOA, the gold standard for estimating the viral reservoir size, involves the serial dilution of latently infected cells into various wells such that a well contains a single latent cell[443,445]. In each well, activating agents such as anti-CD3/CD28 or PMA/ionomycin are added to reactivate viral production and feeder cells, which produce cytokines to support activated cells. After 48 hours, supernatants from stimulated cells are added onto uninfected $CD4^+$ lymphoblasts cultured in separate wells and incubated for 10-14 days to allow for potential viral infection from the supernatant, after which HIV Gag, p24 is quantified. The infection of primary cells with HIV TosZy will allow the latently infected cells to be isolated from the pool of infected cells. A recently awarded CFI to the Murooka lab will enable the purchase of a cell sorter housed in CL2+ containment that will permit the sorting of live, latently-infected T cells for the first time to carry

out these assays. For intact proviral DNA assay (IPDA), the genomic DNA (gDNA) of latent cells are isolated, and then a PCR assay is carried out on the isolated gDNA. Using droplet digital PCR (ddPCR), one of the PCR reactions measures the total HIV DNA by amplifying the region in the HIV *Gag* gene, which is frequently present in both defective and intact proviruses, and the second PCR reaction targets the packaging signal (Ψ) and the Rev-response element (RRE) in the *Env* gene, often mutated in defective proviruses[399,443,446]. Data from ddPCR is analyzed using Poisson statistics. Cells infected with HIV-TosZy to carry out IPDA analysis will prevent the use of Raltegravir in the culture to prevent the formation of 2-LTR circles; instead, different drug cocktails, which include entry inhibitors, reverse transcriptase inhibitors will be used[447]. The results of this assay will help establish whether HIV TosZy can be employed for high-throughput screenings and reactivation assays.

5.2.2 Construction of an HIV-TosZy reporter variant containing a 2A peptide linker

One challenge in multicolour imaging experiments is spectral bleed-through, which occurs when the emission signal from one fluorophore overlaps with another, leading to false signal detection. In the case of the HIV-TosZy, there is potential spectral bleed-through from the ZsGreen channel into the dTomato channel, although this issue was mitigated in our studies by using an optimal dichroic mirror and fluorescent unmixing settings on the multi-photon microscope. My initial thoughts were that by creating a reporter containing a Nef-ZsGreen fusion protein, the subcellular localization within infected T cells would also prevent false identification of infection through spectral bleed-through since dTomato will be widely expressed in the cytoplasm whereas Nef-ZsGreen is localized to the cell surface and ER as green puncta. However, I have realized from imaging experiments that the Nef-ZsGreen fusion proteins caused issues in identifying infected cells versus $CD4^{+}$T cells that have endocytosed Nef-ZsGreen protein, often accumulating in the uropodia of migrating T cells. Instead of a Nef-ZsGreen fusion protein, the self-cleaving 2A sequence, such as those found in the foot-and-mouth disease virus (FMDV) or certain viruses in the Picornaviridae family[448,449] can be inserted between Nef and ZsGreen. The insertion of the 2A peptide will mediate a ribosome skipping event during translation, resulting in the production of two separate proteins: the Nef protein and ZsGreen. This modification will allow ZsGreen to be expressed throughout the cytosol of the infected cell and better identify endogenously infected T cells versus those that have picked up ZsGreen particles *in vivo*.

5.2.3 Antigen specificity of cells upregulating β7 integrin in the latently infected subpopulation

One fascinating finding from my dissertation project is the notable upregulation of the gut-homing marker α4β7 integrin within latently infected cells (**Figure 3. 11B-D**) compared to uninfected and productively infected cells. These observations were found in several repeat studies from different blood donors. However, the underlying cause of this preferential upregulation in this population remains to be determined. One possibility is that latent infection causes an upregulation of this gut-homing integrin. A study conducted by Campbell and Butcher demonstrated that after immunization with LPS and/or OVA, there was a modulation of tissue-specific homing makers in $CD4^+$ T cells[450]. Therefore, since the $CD4^+$ T cells used for infection are polyclonally activated, it would be intriguing to investigate the antigen specificity of these $\alpha4\beta7^{high}$ cells within the latently infected cell population. $\alpha4\beta7^{high}$ latently infected cells will be sorted out, and I will perform an AIM (activation-induced marker) assay using different gut microbial antigens such as bacterial polysaccharides, metabolites, etc.[451,452] in the presence of autologous dendritic cells (DCs), after which I will measure for different activation markers, such as OX40, CD69, CD25, CD40L and PD-L1 to see what antigens these cells are responding to using flow cytometry[453]. During these experiments, I will have positive and negative control; my positive control will include LPS or retinoic acid added in the co-culture, while my negative control will be devoid of any addition in the cell culture[454]. After optimization of antigen responsiveness of latent T cells in these experiments, I will move on to perform tetramer staining with the antigen(s) of interest to confirm antigen specificity by flow cytometry. I can also further phenotype these cells by looking at their clonotypes through single-cell TCR (T cell receptor) sequencing to determine if there was clonal expansion within the $\alpha4\beta7^{high}$ population[167]. If latently infected cells preferentially express α4β7 because they are specific for gut-derived antigens, this could suggest a mechanism by which HIV latency is established or maintained in gut-homing T cells.

5.2.4 Interaction of latently infected cells with antigen-presenting cells (APCs) in the presence and absence of antigen in vitro

Antigen-presenting cells (APCs) such as Dendritic cells (DCs) play a role in the persistence of the viral reservoir by contributing to the clonal expansion of latent T cells by formation of immunological synapses, also known as supramolecular activation clusters (SMACs) with latent T cells. Microbial antigens such as cytomegalovirus (CMV) have been shown to cause an *antigen-*

specific clonal expansion in ART-treated PLWH, which contributes to viral reservoir expansion or maintenance[162]. Additionally, we have previously shown that HIV-captured dendritic cells (DCs) can regulate T-cell migration[179]. Thus, it will be intriguing to see how the presence and absence of different microbial antigens, which have been shown to cause clonal expansion of latent T cells, will modulate the migration of latently infected cells. With the use of the established 3D fibrillar collagen imaging model, latently infected cells can be sorted out and co-cultured with human autologous DCs in the presence and absence of various microbial antigens such as CMV, and we can measure parameters such as contact times between the two cells[179,440,455].

5.2.5 Visualization of lymph node niche(s) occupied by latently infected cells ex vivo

The lymph node (LN) is a complex and well-organized organ composed of diverse niches and cell types that work together to orchestrate immune responses[203]. This structurally complex organ houses many immune cells, such as T cells, B cells, dendritic cells, macrophages, and stromal cells, which play crucial roles in maintaining the lymph node's function and architecture. We have confirmed that latently infected cells can enter and migrate within the lymph node; however, the specific niches these cells occupy within the LN remain to be defined. Thus, investigating the distribution kinetics of latent $CD4^+$ T cells within the lymph node in the presence and absence of ART will be important to determine in future studies. This finding could provide valuable insights into the cellular and molecular mechanisms that underlie viral reservoir dynamics and persistence in the lymph node. Additionally, we can better understand latent $CD4^+$ T cell interactions with other cell types in the different areas of the lymph node. Spatial transcriptomics or confocal imaging can be employed to address this question, as it will enable us to look at the spatial distribution of latently infected cells in relation to other cell types and structures within the LN without disrupting the architecture of the LN[456,457].

5.2.6 Recirculation kinetics of latently infected cells in vivo

I elucidated that the recirculation potential of latently infected cells could potentially lead to the continual seeding of viral reservoirs. Murooka *et al.* previously showed that interference with T cells trafficking in and out of the tissues after infection with a functional S1PR1 antagonist FTY720 (Fingolimod) delayed viremia in BLT mice[128]. Thus, building on this study, we can employ the use of different homing receptor antagonists, such as FTY720 or monoclonal antibodies, such as vedolizumab (a monoclonal antibody against $\alpha4\beta7$ integrin)[458], to study the

recirculation kinetics of latent $CD4^+$ T cells in the presence and absence of ART at different time points, by quantifying the number of latent cells in the different organs. Live sorting and adoptive transfer of latent T cell populations into a hu-PBMC mouse and the use of blocking antibodies can be used to assess the *in vivo* role of these receptors in their homing and recirculation properties.

CHAPTER 6
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