Understanding the Role of Dendritic Cells During HIV Transmission and Latency Expansion

By

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ABSTRACT

HIV dissemination from the genital mucosal tract to the lymphoid organs is the first critical step towards systemic infection. We and others have previously shown that upon sexual HIV exposure, a local pool of infected cells is found in the genital mucosae, followed by rapid HIV spread into the draining lymphoid tissues before detection in the blood. We extended these observations by addressing the role of DCs in the transfer of HIV particles to susceptible $CD4^+$ T cells through trans-infection. Our laboratory previously showed that $CD4^+$ T cells engaged in prolonged contacts with HIV-bearing DCs that was dependent on gp120:CD4 and LFA-1: ICAM-1 adhesive contacts. Abrogating stable DC: T cell contacts significantly reduced HIV transmission. However, a knowledge gap is a molecular mechanism that facilitates the efficient transfer of HIV through HIV^+ DC: T cell contacts. Here, using a custom human kinomics array platform, I identified distinct signaling pathways that are activated in $CD4^+$ T cells during in vitro co-culture with DCs bearing wildtype HIV, but not in HIVΔEnv. Utilizing functional network analysis programs, such as IPA and InnateDB, I demonstrated that mTOR, ERK, and Ca^{2+} pathways are upregulated during DC: T cell contacts in the presence of wildtype, but not Env-deficient HIV. These data suggest that T cells accumulate signals downstream of the CD4 molecule that impacts their motility and function as they contact HIV-bearing DCs, some of which are prerequisite for efficient viral infection and ultimately systemic spread.

The persistence of HIV-infected $CD4^+$ T cells in individuals under suppressive ART therapy is the main obstacle towards achieving HIV cure. The clonal expansion of infected $CD4^+$ T cells harboring latent provirus is the main driver of HIV maintenance in ART-treated individuals. Previous studies have shown that DCs generate latency in $CD4^+$ T cells, requiring close cell-cell contact. However, the contribution of DCs to the maintenance of HIV latency in $CD4^+$ T cells is unclear. Using our full-length dual-fluorescent HIV latency reporter (HIV-Nef-CRIMZY) and *in vitro* DC: T cell co-culture models, I showed that DCs dramatically increase latently-$CD4^+$ T cell proliferation that is dependent on cell-cell interactions. I further showed that DCs induce the proliferation of the latent reservoir in an antigen-dependent and-independent manner. However, the proliferation of latent T cells significantly reduced after CD80 and CD86 dual blockade, suggesting that CD80/CD86-induced pathways in $CD4^+$ T cells play an essential role in driving HIV persistence in T cells. I further showed that DC-soluble factors have a minimal effect on the

expansion of the latent reservoir. These studies demonstrate that DCs are the primary driver of HIV maintenance.

Collectively, this thesis project demonstrates the role of DCs in both HIV transmission and the expansion of latently-infected $CD4^+$T cells through stabilized DC: T cell interaction. My results show that DCs can provide specific signals that reprogram $CD4^+$ T cells to increase susceptibility to HIV infection and for infected T cells to undergo proliferation that contributes to long-term HIV persistence. Together, these studies show that HIV can take advantage of normal DC: T cell behaviors to favor HIV transmission and persistence in ART-treated individuals. These results suggest new avenues for HIV prevention and cure strategies.

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DEDICATION

This thesis is dedicated to my parents for constantly reminding me that: "**WHERE THERE IS A WILL, THERE IS A WAY**"

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LIST OF ABBREVIATIONS

°C	Celsius
AIDS	acquired Immune Deficiency Syndrome
AIM	activation-induced marker
AMPs	antimicrobial peptides
APCs	antigen-presenting cells
AP-1	activator protein-1
APOEC3G	apolipoprotein B mRNA editing enzyme catalytic subunit
ART	antiretroviral therapy
BCL-2	B-cell lymphoma 2
CCL19	chemokine (C-C motif) ligand 19
CCL21	chemokine (C-C motif) ligand 21
CCR5	C-C chemokine receptor-5
CCR-7	C-C chemokine receptor-7
CXCR4	C-X-C chemokine receptor type 4
CXCL10	C-X-C motif chemokine ligand 10
CD	cluster of differentiation
CD62L	cluster differentiation sixty-two Ligand
CD40L	cluster differentiation forty ligand
CPI	cytomegalovirus, influenza, and parainfluenza
DAG	diacylglycerol
DMEM	dulbecco's modified eagle medium
CMV	cytomegalovirus
DAMP	damage-associated molecular pattern
DC	dendritic cell
dCA	didehydro-cortistatin A
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
E.coli	Escherichia coli

EBV	Epstein-Barr virus
ELIspot	enzyme-linked immune absorbent spot
Env	envelope
ERK	extracellular-signal-regulated kinase
FGT	female genital tract
FMO	mean fluorescent minus one
FRT	female reproductive system
GM-CSF	granulocyte-macrophage colony-stimulating factor
HAART	highly active antiretroviral therapy
HDAC	histone deacetylase
HIV	human immunodeficiency virus
HLA-DR	human leukocyte antigen-DR isotype
HSV-1	herpes simplex virus type 1
ICS	intracellular cytokine staining
ImDC	immature dendritic cell
ICAM-1	intracellular adhesion molecule 1
IFN -γ	interferon gamma
IFN-α	interferon alpha
IL	interleukin
IL-7R	interleukin-7 receptor
INSTI	integrase strand transfer inhibitor
IP3	inositol 1,4,5- triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
ITK	interleukin-2 inducible T cell kinase
KLF6	Krüppel-like factor 6
LAT	linker of activated T cells
Lck	lymphocyte-specific protein tyrosine kinase
LFA-1	lymphocyte function-associated antigen-1
LPS	lipopolysaccharide
LRA	latency-reversal agent
LTR	long terminal repeat

MAPK	mitogen-activated protein kinase
MCP-1α	monocyte chemoattractant protein-1 alpha
MDDC	monocyte-derived dendritic cell
MHC	major histocompatibility complex
MIP-1β	macrophage inflammatory protein -1 beta
mL	milliliter
mm3	cubic millimetre
mTORC	mammalian target of rapamycin complex
NFAT	nuclear factor of activated T cells
NF-kB	nuclear Factor kappa-light-chain-enhancer of activated B cell
NK	natural killer
NNRTI	nonnucleoside reverse transcriptase inhibitor
NRTI	nucleotide reverse transcriptase inhibitor
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PD-L1	programmed death-ligand 1
PFA	paraformaldehyde
PHA	phytohemagglutinin
PI	protease inhibitor
PI3K	phosphoinositide 3-kinase
PLC-γ1	phosphoinositide-specific phospholipase C-gamma 1
PLWH	people living with HIV
PrEP	pre-exposure prophylaxis
P-TEFB	positive transcription elongation factor
RASGPR1	RAS guanyl releasing protein 1
RNA	ribonucleic acid
SAMHD1	SAM domain and HD domain containing protein 1
S.aureus	staphylococcus aureus
SEB	staphylococcus enterotoxin B
SIV	simian immunodeficiency virus

SLO	secondary lymphoid organ
ssRNA	single-stranded ribonucleic acid
STAT	signal transducer and activator of transcription
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EM}	effector memory T cell
Th1	T helper type-1
Th2	T helper typer-2
Th17	T helper type-17
TJ	tight junction
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
T_{RM}	tissue-resident T cells
T_{TM}	transitional memory T cell
U3	unique 3 sequence
U5	unique 5 sequence
VI	virological synapse
ZAP-70	zeta-chain-associated protein kinase-70
ZDV	zidovudine
µg/mL	microgram/milliliter
µL	microlitre

Chapter 1- INTRODUCTION

1. HIV epidemiology

Human Immunodeficiency Virus-1 (HIV) is classified as a retrovirus and has been a major burden on global public health since the 1970s. HIV infects and depletes $CD4^+$ T cells, its primary target cell type, leading to the dismantling of cell-mediated and increased risk of opportunistic infection[1]. Without early treatment, acute HIV infection gradually develops into a chronic infection that finally progresses to Acquired Immune Deficiency Syndrome (AIDS). Patients are diagnosed with AIDS when they exhibit CD4 T cell counts of less than 200 cells/mm^3 in contrast to 500 to 1,600 cells/mm^3 $CD4^+$ T cells in healthy individuals.

AIDS-related deaths have decreased by 47% since 2010 and further reduced to 1.5 million in 2020 [2] due to the expanding availability of different tools to manage the HIV epidemic, including antiretroviral (ART) therapy and pre-exposure prophylaxis (PrEP). ART therapy consists of multiple antiretroviral drugs that block HIV replication [3], while PrEP is the use of medications to reduce HIV acquisition in uninfected people at high risk of getting HIV [4]. Therefore, the global prevalence of HIV has increased from 31 million in 2002 to 35.3 million in 2012 [5], as antiretroviral therapy increases the lifespan of people living with HIV (PLWH) and prevents progression to AIDS. Despite this, approximately 37 million people currently live with HIV, with a disproportionately high number of new infections among adults across Sub-Saharan Africa, estimated at roughly 7.5 million people [6, 7]. Most HIV infections worldwide occur across the genital mucosa through sexual transmission, and women are more susceptible than men [8-10]. Despite significant research efforts focused on controlling and preventing HIV infections worldwide, HIV epidemic continues to be the major contributor of mortality and morbidity globally [11-13].

2. Advances in HIV prevention and treatment

2.1 Successes of ART therapy

The advent of antiretroviral drugs in the last two decades has greatly declined global HIV- incidence and viral transmission. When taken regularly, ART can suppress HIV-1 viremia

to undetectable levels and increase life expectancy, and partially restore immune function in PLWH soon after early therapy initiation [14]. Despite global efforts to provide treatment to all PLWH, only 73% currently have the access to ART therapy which must be taken lifelong [2, 15]. More than 20 licensed antiretroviral drugs belonging to six different classes are used for managing HIV infection[16]. Each drug class specifically inhibit key steps within the HIV life cycle to impair viral replication inside target cells. Until 1995, nucleotide reverse transcriptase inhibitors (NRTIs) were the only available antiretroviral drugs which target the reverse transcriptase enzyme that is crucial for HIV replication in the early steps[17]. Of these, zidovudine (ZDV) was the first NRTI to receive approval for clinical use. Although potent efficacy was achieved at first, viral resistance emerged. Therefore, monotherapy was not sufficient to achieve sustained viral suppression [18]. Later, different antiretroviral classes were introduced, including nonnucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), fusion inhibitors, and CCR5 antagonists. A question was further raised whether dual regimens would be more effective in managing infection than a triple regimen. While some studies have shown low effectiveness and increased toxicities due to drug interaction and emerging resistance [19-21], other studies reported no difference between dual and triple treatment [22-24]. However, it was subsequently reported that triple therapy is more efficient in restoring the $CD4^+$ T cell population and attenuating high immune activation[25, 26]. Hence, a minimum of three different antiretroviral drugs from each class – also called highly active antiretroviral therapy (HAART)- is recommended as the first-line treatment for PLWH to take every day. These medications generally consist of two NRTIs as backbone therapy, combined with another class of drugs, such as PIs, INSTIs, and NNRTIs [16, 27, 28].

2.2 Limitations of ART therapy

Despite successful advances in antiretroviral drugs, many challenges still exist because ART does not eliminate HIV in individuals. ART also fails to fully restore the health and immune function of PLWH. While ART can control pathological chronic immune activation and inflammation, a driver behind $CD4^+$ T cell depletion and development of AIDS[29], PLWH still exhibit elevated levels of proinflammatory cytokines [30], and sustained immune dysfunction even after years of therapy[31, 32]. Moreover, some studies reported a decline in total HIV-DNA within

the first year of initiating ART, followed by a steady phase of total HIV-integrated DNA after four years[33-35]. This steady phase is mainly caused by the ability of HIV to establish the pool of infected cells harboring silent transcriptional HIV-integrated DNA known as a latent reservoir [36]. The latent reservoir is predominately established in resting memory $CD4^+$ T cells at various sites in the body, such as the tissues, lymph nodes, and gut mucosa[37]. However, these silent proviruses can revert to being transcriptionally active, rapidly producing infectious virions upon HAART removal [38-40]. Sadly, the latent reservoir cannot be targeted even by optimal ART, as ART only arrests cells that undergo productive infection, i.e., those that actively produce viral particles [41-46]. Additionally, HIV-infected T cells have been found in various tissues, and the penetration of antiretroviral drugs into tissue compartments remains insufficient for the low levels of viral replication in deep tissues that can seed and replenishes the latent reservoir [47, 48]. Subsequent studies have demonstrated that the latent reservoir decays slowly, with a half-life of 44 months [44, 49], indicating that more than 70 years of therapy is required to eradicate the latent reservoir. Additionally, initiation of ART early after infection does not prevent the formation of the latent reservoir, as several studies have shown that a pool of latently-infected cells is already established within days of infection [50, 51]. Hence, ART therapy is insufficient as a curative strategy, given that the latent reservoir is the main obstacle towards HIV elimination [52].

Another hallmark of HIV is its fast mutation rate resulting in vast genetic diversity, allowing the virus to escape immune system recognition and develop drug-resistant variants [53-55]. The accumulation of high genetic diversity can largely be attributed to the function of HIV reverse transcription that lacks a proof-reading mechanism during viral replication [56, 57]. Due to selective pressures imposed by ART administration, these drug-resistant HIV variants can readily accumulate in high numbers, diversifying the viral population within a month if drugs are not taken routinely [58]. Another constraint of ART is that it exposes PLWH to many side effects within three months, affecting the central nervous system, liver, gastrointestinal and metabolism, which can impede continued ART adherence for some individuals [59-61]. Taken together, ART alone is not sufficient to eliminate HIV-1, and deeper insights into mechanisms of anti-HIV-1 immunity mechanisms are needed to yield new potential strategies for HIV cure.

3. The human immune system and response to HIV infection

The immune system is armed with two primary defenses: innate immunity and adaptive immunity. Innate immunity is characterized as the first-line and non-specific defense after initial exposure to invading pathogens. It generates a rapid but similar response to all non-self (foreign) proteins without the capacity to retain the memory of previous infection (immunological memory). It also plays a crucial role in activating and engaging the other arm of the immune system, known as adaptive immunity. Adaptive immunity is highly specific, and after the initial pathogen encounter, it provides immunological memory resulting in a more rapid immune response to the previously-encountered pathogens.

The pool of immune cells that comprise innate immunity is diverse and includes mast cells, neutrophils, natural killer cells (NK cells), macrophages, and dendritic cells (DCs). Macrophages and DCs serve as antigen-presenting cells (APCs) in innate immunity. The main function of APCs is to take up antigens, break them down into small peptides, and finally present peptides in the context of the major histocompatibility complex (MHC) on their surface to initiate an antigen-specific immune response [62]. Therefore, APCs are central links between innate immunity and adaptive immunity [63-65].

Adaptive immunity is made up of two branches: cell-mediated immunity and humoral immunity. Cell-mediated immunity is governed by T lymphocytes which recognize antigen: MHC complex and initiate an antigen-specific response to kill pathogens. On the other hand, humoral immunity is mediated by the production of specific antibodies by B lymphocytes that bind to viral particles and neutralize their binding to host cell receptors. B cells can also function as APCs to activate T cells *in vitro*[66] and *in vivo* [67]. In this thesis, the primary focus will be on T lymphocyte activation and function, as they are the main targets of HIV infection.

3.1 Immunological barriers against sexual HIV infection

As mentioned earlier, women are more vulnerable to HIV infection compared to their male counterparts [11], where vaginal transmission accounts for the majority of HIV infection worldwide. Several characteristics of the female reproductive tract (FRT) make it prone to HIV infections and is the fist focus point of infection before spreading systemically. The FRT is an

immunologically unique structure that is equipped with both physical and immunological barriers that serve to preserve female genital health against infectious agents, while maintaining fertility [68, 69]. To reach its target cells, HIV must overcome significant innate immune defenses comprised of physical, chemical, and cellular components of the immune system in the FRT.

3.1.1 Physical barriers of the FRT

The physical barriers of the FRT include epithelial cells and mucus. In contrast with the upper FRT, which is lined by a monolayer of columnar epithelial cells, the lower FRT is lined by layers stratified squamous epithelium. Mucosal epithelial cells are covered with a dense mucosal membrane and the gel-like mucus that traps and prevents the pathogens from ascending to deeper tissues [70]. The stability of epithelial cells in lower FRT is primarily dependent on the networks of tight junctions (TJs) [64] protein complexes consisting of adhesion molecules, transmembrane proteins, and adaptors proteins that hold cells together like velcro [71]. Following HIV transmission, TJs are often disrupted and increase permeability within 2-4 hours [72], resulting in the infiltration of HIV virions into the deeper submucosal tissues and eventually their target cells at the interface between the basal epithelium and lamina propria [64, 73-75]. Besides the role of epithelial cells in the physical barrier, they participate in forming a chemical barrier by secreting a wide range of immunomodulatory molecules, such as antimicrobial peptides (AMPs), chemokines, cytokines, transporting IgA, growth factors, enzymes and toll-like receptors (TLRs) which makeup of a crucial facets of the chemical barrier [76-78].

3.1.2 Chemical barriers of the FRT

3.1.2.1 Cytokines and chemokines

Cytokine and chemokines function as central mediators to develop an effective immunological response against infection. Cytokines are small soluble proteins secreted by many immune cells, and are crucial for cell development, differentiation, and cell-cell communication. They can be subdivided into two general functions: those that are anti-inflammatory and those that are pro-inflammatory. While acute inflammation helps protect the body from pathogens, repeated stimulation, or ineffective regulation results in chronic inflammation that causes damage to the

body and different diseases[79]. In an inflammation state, the immune cells, mainly monocytes, macrophages, and T cells, become activated and produce a wide range of both pro- (e.g., IL-1β, IL-6, and TNF-α) and anti-inflammatory (e.g., IL-4, IL-10, and IL-11) cytokines to balance the immune response, and ultimately dysregulate pathogen infections [80, 81]. Furthermore, epithelial cells can also secret various cytokines, such as IL-6 or granulocyte-macrophage colony-stimulating factor (GM-CSF) to help clear pathogens during microbial infections [77].

Chemokines are also conserved small proteins that serve multiple purposes, including coordinating cell trafficking [82, 83] and hematopoiesis [84]. In addition to cytokines, epithelial cells can also produce chemokines, such as IL-8 and monocyte chemoattractant protein-1 (MCP-1) that promote leukocyte trafficking [85]. Although mucosal inflammation and immune activation are crucial for initiating an immune response, HIV can subvert these defenses[86]. During inflammation at the genital mucosal site, the increased pro-inflammatory cytokines and chemokines lead to immune cell recruitment, including activated $CCR5^+$ $CD4^+$ T cells (the main target) to the site of infection, as well as disruption of epithelial barrier, resulting in increased HIV acquisition rates [87, 88]. Using simian immunodeficiency virus (SIV) infection in macaques as a model for HIV infection, the production of pro-inflammatory cytokines, such as MIP-1α, MIP-1β and IL-8 led to the accumulation of $CD4^+$ T that enhance infection across the vaginal mucosa of rhesus macaques *in vivo* which is crucial for establishing productive infection[89]. Following this, the virus spreads across the vaginal mucosa of rhesus macaques [89], but blocking immune activation inhibits SIV transmission in this animal model. These studies define a step-wise mechanism of sexual HIV transmission across the FRT, and that underlying genital inflammation increases HIV acquisition risk.

3.1.2.2 Toll-like receptors (TLRS)

During immune recognition of pathogens, TLRs are an essential class of pattern recognition receptors (PRRs) that initiate innate immune responses by recognizing microbe-specific signatures. The recognition of these pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by TLRs, plays a crucial role in linking innate with adaptive immunity [90-93]. TLRs are widely distributed in cells, including epithelial cells[94] and immune cells, such as DCs and macrophages [91]. The expressions and locations of

TLRs can be situated either on the cell surface or intracellular. 13 TLRs have been reported, ten of which are expressed on human cells [95, 96]. Each TLR can sense specific PAMP ligands on the pathogenic microbe. For example, TLR8 in DCs mediates the recognition of HIV single-stranded RNA (ssRNA) or TLR4 recognizes lipopolysaccharide (LPS) [97], both of which activate internal signaling cascades culminating in host defense reactions within cells [98]. These signaling cascades induced by different TLRs cause the expression of inflammatory mediators, including cytokines and chemokines, leading to increased risk of HIV vaginal acquisition due to the recruitment of the immune cells to the site of infection [89, 99-101].

3.2 Cellular defenses of the FRT

3.2.1 Dendritic cells (DCs)

Beyond physical and chemical barriers, the FGT also provides both innate and adaptive immunity against pathogens in the form of cellular defenses. A specialized class of APCs that serve to link the innate and adaptive arms of our immunity are dendritic cells (DCs) [104]. Known for their tree-like morphology, these professional APCs are ubiquitously distributed in many body tissues, especially at mucosal surfaces[102, 103]. With the help of their finger-like projections, the primary function of DCs is to capture, process, and present protein antigens to naïve T cells, facilitating antigen-specific activation and differentiation into effector cells. DCs produce distinct cytokines that coordinate the immune response against the invading antigens[63, 104-107]. Since the investigation of DCs has been limited by their scarcity in peripheral blood mononuclear cells (PBMCs), our current understanding of human DCs biology has been obtained from differentiating monocyte into monocyte-derived DCs (MDDCs) by adding a combination of cytokines such as GM-CSF and IL-4 [108].

In the absence of pathogens, mucosal DCs remain in their immature states. Immature DCs (imDCs) are characterized by their circular shape and low expression of MHC molecules and B7 costimulatory molecules (e.g., CD80, CD86) and CD40, which impedes their ability to activate antigen-responsive naïve T cells [104, 109, 110]. However, imDCs are highly phagocytic and constantly searching for foreign antigens in their immediate environment, leading to the presentation of peptides onto MHC I or II [111-113]. The initiation of the immune response depends on the activation and maturation of DCs which conversely enhances HIV capture and

transmission to $CD4^+$ T cells[114-116]. HIV targets different immune cells in the FRT to establish systemic infection. It was demonstrated that DCs are the first cells that encounter HIV-1 virions in the mucosa, accounting for 90% of HIV transmission from the FRT to $CD4^+$ T cells [103]. Upon sensing PAMPs by their surface receptors, imDCs undergo maturation. During chronic HIV infection, DC stimulation is also induced by the microbial translocation from the intestinal tract, resulting in elevated levels of circulating LPS that triggers DC maturation[117]. Mature DCs undergo structural changes that impact their phenotypic and functional properties. The maturation process involves the induction of finger-like projections (dendrite extension), increased surface expression of costimulatory molecules[118], secretion of cytokines (IL-12, IL-10 and IL-13), and chemokine receptors (CCR7) [104, 113, 119]. Upregulation of CCR7 is crucial for DC migration from peripheral tissues towards the draining lymph nodes which express high levels of CCL19 and CCL21, two chemokine ligands for the CCR7 receptor, in the T-cell zone [120]. The interaction between CCR7 with its ligands enables DCs to enter the T-cell zone in lymph node where antigen-presentation to T cell populations or other immune cells occurs[107, 121]. We previously showed that capture of HIV particles by DCs does not abrogate their chemotactic migration toward lymph-node homing chemokines CCL19/CCL21 compared to uninfected controls [122]. Additionally, using BLT humanized mice, blocking leukocyte egress from the FRT significantly delayed widespread infection and plasma viremia [123, 124], indicating that migratory cells actively carry virus out of the FRT. Therefore, the spread of HIV from the FRT to draining lymph nodes is the first critical step in establishing systemic viral spread, possibly by DCs [123, 124].

3.2.1.2 DC subsets involved during viral infection

Like other immune cells, DCs are also a heterogeneous population with distinct phenotypic markers and functions regulated by specific gene expression profiles. They consist of two major populations based on their surface expressions, immunological function, and distribution: resident lymphoid tissue DCs and migratory non-lymphoid tissue DCs, both of which can be divided into different subsets[125]. Migratory DCs are broadly subdivided into two major subtypes, namely plasmacytoid DCS (pDCs; HLA-DR^+ $CD123^+$ $CD11c^-$), and myeloid cells DCs (HLA-DR^+ $CD123^-$ $CD11c^+$) [126]. Langerhans cells are tissue-resident DCs that are located in

the skin and their roles include responding to pathogens within the epidermis [104]. Myeloid DCs and pDCs reside in both lymphoid and non-lymphoid tissues, but pDCs are more abundant in non-lymphoid tissues under inflammation conditions [127]. In general, pDCs are specialized for antiviral function by producing potent antiviral cytokine called Interferon alpha (IFNα) during HIV infection [128], and myeloid DCs release high levels of IL-12 for direct Th1 effector immunity polarization [126]. Since DC subsets are populated at low abundance, MDDCs are used in *in vitro* studies for modeling DC and HIV interaction[108], as they are functionally similar to myeloid DCs and have high surface expression of MHC class II and CD11c [107]. Together, DCs are a critical player in the development of anti-HIV T cell immunity but can also facilitate HIV spread through a specialized mechanisms called *trans*-infection, which is described later.

3.2.2 T lymphocytes

T lymphocytes (T cells) are an essential component of adaptive immunity which coordinate antigen-specific responses after activation by interactions with APCs. Cytotoxic T cells ($CD8^+$ T cells) along with helper T cells ($CD4^+$ T cells) make up the main T cell subsets, recognizing and responding to cell presenting antigens in the context of MHC I and II, respectively[129, 130]. T cells are generated in the bone marrow and then mature in the thymus. In the thymic compartment, T cells are educated by positive and negative selections, allowing them to discriminate foreign antigens from self-antigens These mature T cells, which have not yet encountered their cognate antigen (i.e. naïve T cells), constantly recirculate between blood and secondary lymphoid organs. The trafficking of naïve T cells to the T-cell zone in lymph node is mediated by CCR7 expression. Here, the trafficked naïve T cells spend 6-12 hours in the lymph node searching for APCs presenting cognate peptide and will move onto the next chain of lymph nodes if there is no recognition [129, 131]. T cells express an unique T cell receptor (TCR) on its surface that recognizes a limited range of antigens in the context of MHC molecules [129, 132], and roughly one T cell in 10^5-10^6 is specific for its cognate peptide in a sea of as many as 100,000 other peptides displayed by a single APCs [129, 133, 134]. The TCR complex is comprised of mainly TCR α/β heterodimer (or γδ chains in a different T cell subset) and CD3γ/δ/ε/ζ subunits, referred to as TCR-CD3 complex. Following TCR bindings to the peptide-MHC (pMHC) complex, the cytoplasmic domain of the CD3 molecule initiates signaling transduction in T cells

that regulates their proliferation, differentiation, and effector responses [135]. The strength of the TCR signaling cascade is dependent on the binding affinity between the TCR molecule and pMHC complex interactions, as the high affinity antigens induce strong and more prolonged TCR signaling transduction, causing optimal activation of cellular immune response[136-138].

T cell populations reside in lower FRT near the basal epithelial cell layer at stromal interface. Although $CD8^+$ T cells are more abundant than $CD4^+$ T cells in the FRT [139], the majority of $CD4^+$ T cells populate in the lower FRT, specifically ectocervix[73]. However, multiple independent studies showed that $CCR5^+$ $CD4^+$ T cells, the main target for HIV-1, reside in the transformation zone where columnar epithelial cells lining endocervix change into the squamous epithelial cells lining the ectocervix[73, 139-141]. Furthermore, recent studies have shown that T-helper 17 (Th17) are the preferential target for HIV in the cervix[142, 143], as these cells express HIV co-receptor (CCR5) and integrin α4β7, which binds to HIV envelope and facilitates viral transmission[144]. Additionally, these cells are almost depleted in HIV^+ women, indicating that they might be a major target for HIV infection[142].

3.2.2.1 Formation of the immunological synapse during T cell priming

DC: T cell interaction leads to the formation of crosstalk (the immunological synapse; IS) between them through which naïve T cells can receive three signals from DCs to become activated, and then migrate to the appropriate site of infection. Signal 1 is generated when TCR recognizes the pMHC complex on the surface of DCs [145]. Signal 2 is provided via the engagement between B7 costimulatory molecules on DCs (e.g., CD80 and CD86) with their cognate ligands (e.g., CD28) on T cells triggering TCR signaling downstream, which is crucial for clonal expansion. Full T cell activation production of pro-inflammatory cytokines IL-2 and IFN -γ, which further propagate T cell expansion and activation, respectively [146]. Moreover, it was reported that adhesion molecules or glycoproteins expressed on cell surfaces, can maintain DC: T cell interactions and enhance HIV transmission to CD4+ T cells[115]. Specifically, intracellular adhesion molecule 1 ICAM-1; primarily on DCs) and leukocyte function-associated function 1 (LFA-1; primarily on T cells) can stabilize DC: T cell contact and support T cell activation [147, 148]. Signal 3 is mediated by cytokines secreted from mainly activated DCs which trigger the polarization of naïve T cells to responding cells called effector T cells[106]. Once $CD4^+$ T cell

become activated, they differentiate into specific subsets such as T helper (Th) 1, Th2 and Th17 and provide a more specialized immune responses against microbes[130] and that this is highly dependent on the microenvironment or nature of stimuli [149] and provide a more specialized immune responses against microbes[130]. Each Th subset produces their own signature cytokine profile that serve different immunological functions[150].

In the context of HIV infection, activated Th cells are the preferred target for HIV productive infection. However, these cells are short-lived and undergo apoptosis (programmed-cell death) due to cytopathic effects of the virus or $CD8^+$ T cell-mediated killing[151]. Some fraction of $CD4^+$ T cells revert into long-lived and quiescent memory cells that are distinguished from recently activated counterparts by their smaller size and absence of surface activation markers such as CD69, CD25, and HLA-DR[152]. Once they encounter the same antigen, they have a higher and faster magnitude response due to several characteristics, such as the need for a lower activation threshold and less dependency on costimulatory molecules during reactivation [153].

3.2.2.2 TCR intracellular signaling pathway during priming

A defined series of signaling events occur after TCR engagement with cognate peptide in the context of MHC complex that regulate downstream effector responses. The first is the recruitment of Src family kinase Lck and Fyn to the TCR complex in the center of the immune synapse and interactions with co receptors CD4 or CD8. These early events are followed by phosphorylation of activation motifs (ITAMs) located in the cytoplasmic domain of CD3 and ζ-chains of the TCR complex. It has been demonstrated that increasing the abundance of Lck can enhance T cell response to lower pMHC affinity, suggesting that Lck recruitment to the TCR complex is the crucial step for initiating T cell downstream signaling [154]. The ITAMs generate a docking site for translocation of ZAP-70 from the cytoplasm to the plasma membrane through its Src homology2 (SH2) domain. ZAP-70 also requires additional phosphorylation by Lck to amplify its activation and stabilization [155, 156]. ZAP-70 subsequently phosphorylates the adaptor molecules LAT and SLP-76 serving as scaffold proteins that bind to several proteins simultaneously to propagate signaling events [157, 158]. LAT contains four phosphorylation sites for ZAP-70, among which Y132 serves as a docking site for PLC-γ1 [157, 159]. Additionally, ITK is another kinase that is phosphorylated by Lck, which activates PLC-γ1; PLC- γ 1 hydrolyzes

phosphatidylinositol 4,5-bisphosphate (PIP2), a minor but essential constituent of the plasma membrane to generate secondary messengers IP3 and DAG; IP3 binds to its receptor IP_3R in the endoplasmic reticulum (ER) to release calcium into the cytoplasm [160]. Calcium-calmodulin interactions activate calcineurin, triggering IL-2 gene transcription through transcription factor NFAT translocation into the nucleus. DAG phosphorylates PKCθ and RASGPR1, the latter binds to threonine kinase Raf-1 to initiate MEK and MAPK/ERK kinase pathway[161]. In resting cells, the ERK 1/2 cascade is localized in the cytoplasm because of the interaction with specific anchor/scaffold proteins[162]. However, upon stimulation, MEK and ERK 1/2 are usually released from their cytoplasmic anchors, translocating to the nucleus to regulate several transcriptional factors that are crucial for T cell activation, such as NF-kB and NFAT[163, 164]. Co-stimulatory signals also function to enhance the strength of TCR signaling, where CD28 molecules on the cell surface of T cells interact with CD80/CD86 to activate the PI3K pathway. Subsequent phosphorylation of AKT ultimately activates mTORC1 and mTORC2 that are crucial regulators of cell growth, apoptosis, and cell metabolism [165, 166]. Together, specific kinase pathways are stimulated in an antigen-specific manner that ensures activation of specific T cell subsets against pathogens.

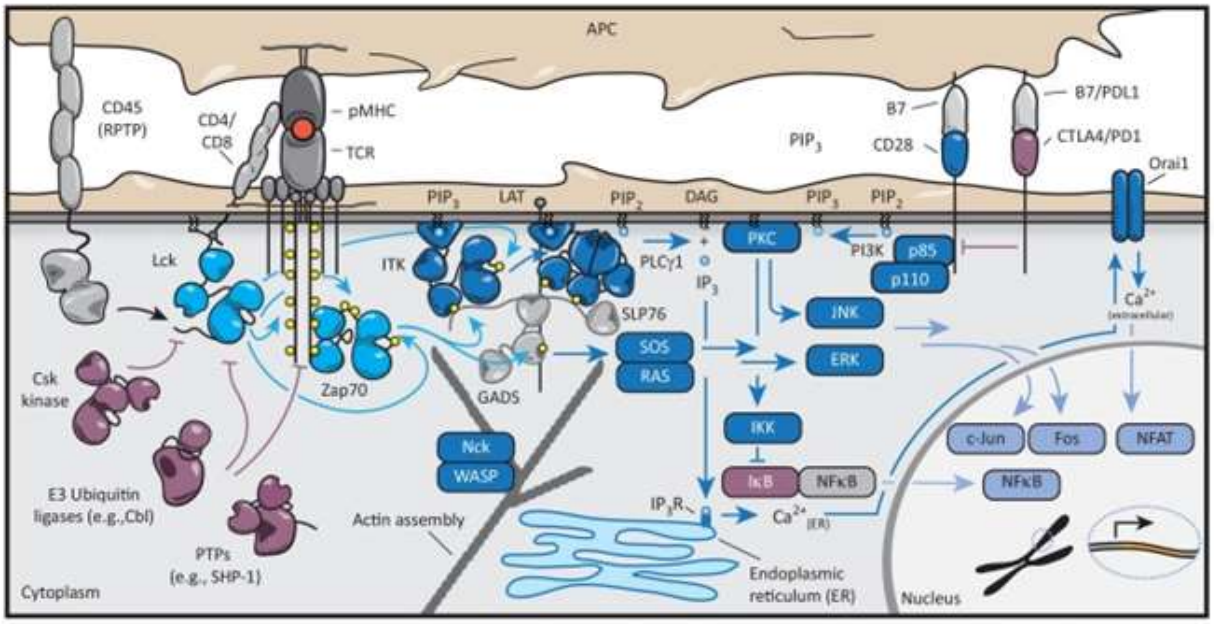


Figure 1. Schematic representation of TCR signaling cascade in T cells. TCR engagement with an antigen in the context of MHC class II triggers signaling events by recruiting Lck to the centre of synapse that phosphorylates ITAMs of the TCR associated CD3 and zeta proteins (TCRζ). ZAP-70 is then recruited and become activated which in turn phosphorylates adaptor proteins, LAT and SLP-76. LAT serves as a docking site which triggers RAS-ERK, PLCγ1 pathways and calcium pathways. All signals lead to activation of different transcription factors and gene expressions in the nucleus. Figure from [167].

3.2.2.3 Formation and maintenance of memory CD4⁺ T cell subsets

Memory $CD4^+$ T cells are divided into subpopulations based on their surface expression of markers, function, and distribution within the body. These subsets include central memory T cells (T_{CM}), effector memory T cells (T_{EM}) and transitional memory T cells (T_{TM}), tissue-resident memory T cells (T_{RM}) [168]. Memory $CD4^+$ T cells are characterized by the surface expression of CD45RO, CCR7 and CD62L, like naïve T cells[168, 169]. These receptors allow T_{CM} migrate toward and into secondary lymphoid organs (SLOs) to access a large population of antigen-presenting DCs [169]. They typically do not display high proliferation but can survive for decades. T_{EM} cells typically express low CCR7 and CD62L, are likely derived from T_{CM} cells, and have

immediate effector capabilities upon antigenic stimulation; T_{EM} cells migrate between lymphoid and non-lymphoid organs [169, 170]. T_{TM} cells possess functional characteristics between T_{CM} and T_{EM} cells[171]. Finally, T_{RM} cells are found almost in all organs, including epithelial, muscle, connective tissues, and some in SLOs[172, 173]. Unlike other memory T cell subsets, T_{RM} cells lack C62L and CCR7 receptors required for the entry into lymphoid tissues; therefore, they are commitment to the tissues of residence [173].

Memory T cell homeostasis is maintained by TCR: self-pMHC interactions, referred to as tonic signaling, and multiple cytokines mainly IL-7 and IL-15 [174-177]. For memory $CD4^+$ T cells, IL-7 is the major factor driving homeostatic proliferation and survival, IL-15 plays a dominate role over IL-7 in maintaining memory $CD8^+$ T cells[178]. IL-7 is produced by cells, such as thymic epithelial cells and lymph node fibroblastic reticular cells [179, 180], and is upregulated once immune challenge subsides. IL-7 binds to its receptor that is composed of two chains: the IL-7 receptor α-chain (IL-7Rα; also known as CD127) and the common cytokine-receptor γ-chain (IL-7γ_c; also known as CD132). IL-7 induces PI3K/AKT pathway through IL-7Ra expression, augmenting the expression of genes such as anti-apoptotic proteins (Bcl-2) to enhance T cell survival [181, 182]. Moreover, IL-15 is produced by a wide variety of cell types, including epithelial cells, fibroblasts, macrophages and DCs[183, 184]. IL-15 induces the expression of signal transducer and activator of transcription (STAT)-3 and STAT-5, both of which then translocate to the nucleus where they activate Bcl-2 to enhance T cell survival[185]. In addition to cytokines, the previous studies have shown that basal TCR signaling or tonic signaling is required for both $CD4^+$ and $CD8^+$ T cells, as TCR-deficient T cells gradually lost over time, with half-lives of 46 and 16 days for $CD4^+$ and $CD8^+$ T cells, respectively[186]. Moreover, tonic signaling can modulate gene expression levels which are important for T cell survival[187, 188]. For instance, it was demonstrated that tonic signaling induces the expression of PI3K, calcineurin, and MEK, leading to the basal phosphorylation of ERK 1/2 that is necessary for survival in resting memory T cells [187]. Overall, memory T cells play a crucial role in adaptive immune response due to their rapid response and are maintained by different mechanisms. However, the exact mechanism that drives memory T cells homeostasis is not yet well understood.

4. HIV structure and viral life cycle

4.1 HIV structure

The HIV genome is composed of two identical ssRNA that is converted into linear double stranded DNA (dsDNA) by reverse transcriptase within the cytoplasm of target cells upon viral entry. The proviral DNA is flanked at both ends by Long Terminal Repeat (LTR), which has binding sites for host and viral transcription factors, serving as the promoter and control center of transcriptional initiation. The LTR is subdivided into three different regions: The Unique 5 sequence (U5), Repeated sequence, and the Unique 3 sequence (U3). HIV-1 transcription is initiated from the 5' region of the LTR, followed by transcription elongation of the encoded structural (Gag, Pol, and Env), regulatory (Tat and Rev), and accessory (Vpr, Vpu, Vif, and Nef) proteins. Transcription is finally terminated at the 3' end, leading to the production of viral mRNA that is subsequently packed into newly budding virus.

4.2 HIV entry and replication

In order to infect $CD4^+$ T cells, the virus must first establish contacts with the host cell membrane prior to entry [189]. This attachment is mediated by the interaction of viral gp120 with the CD4 receptor which is expressed at high levels on the surface of T cells, and to a lower extent on DCs, and macrophages. To complete viral fusion with the host membrane and entry into its target cells, HIV also requires co-receptor binding either with CCR5 or CXCR4[190] used by CCR5-tropic (R5) and CXCR4-tropic (X4) viruses, respectively[189]. CCR5 is highly expressed on memory T cells compared to naïve T cells, thereby, preferentially infecting resting memory T cell subsets. [191]. The CCR5 co-receptor accounts for most viral spread via cell-cell interaction, as shown during early infection [192-194], whereas the X4 HIV infects a broad range of T cells and are predominately detectable at later stages of infection [195]. When both CD4 and the appropriate co-receptors are engaged, the viral envelope and host cell membrane undergo conformational changes that eventually facilitate virus-cell fusion. The viral genomic contents are then released into the cytoplasm, and Vif, an accessory protein, downregulates host transcription factor APOBEC3G by proteasomal degradation[196] to set up the stage for reverse transcription that converts genomic ssRNA into linear dsDNA. After import of viral dsDNA into the nucleus, the integrase (IN) enzyme enables insertion of the viral genome into, preferentially, transcriptionally

active chromatin within the host cell chromosome[197, 198]. After integration, the HIV DNA is referred to as provirus, and its expression is under strict control by the availability of host cell transcriptional machinery, including NF-κB, NFAT, and AP-1. Following translocation to the nucleus, these transcription factors bind to their respective binding sites within the HIV-1 LTR promoter, often resulting in stochastic HIV-1 transcription. Tat, which is a regulatory protein, interacts with host transcription factor like P-TEFB to utilize host RNA polymerase II (RNA pol II). After proper docking on the promoter, the host RNA pol II transcribes unspliced and multispliced viral mRNA that are exported out of the nucleus to be translated into polyprotein precursors, such as the HIV Gag polyprotein (Gag-Pro-Pol) [199, 200]. Upon proteolytic cleavage of this precursor, the mature HIV-Gag traffics to the plasma membrane where it coordinates assembly of viral components, maturation, and ultimately budding of new infectious viral particles through the action of protease [201].In some cases, HIV dsDNA may fail to integrate into the host chromosome leading to the formation of circular DNA- containing one or two HIV LTRs. Since this circular DNA does not undergo viral replication, these defective replicons have a short half-life of roughly 24 hours and are gradually titrated out with each cell division[202].

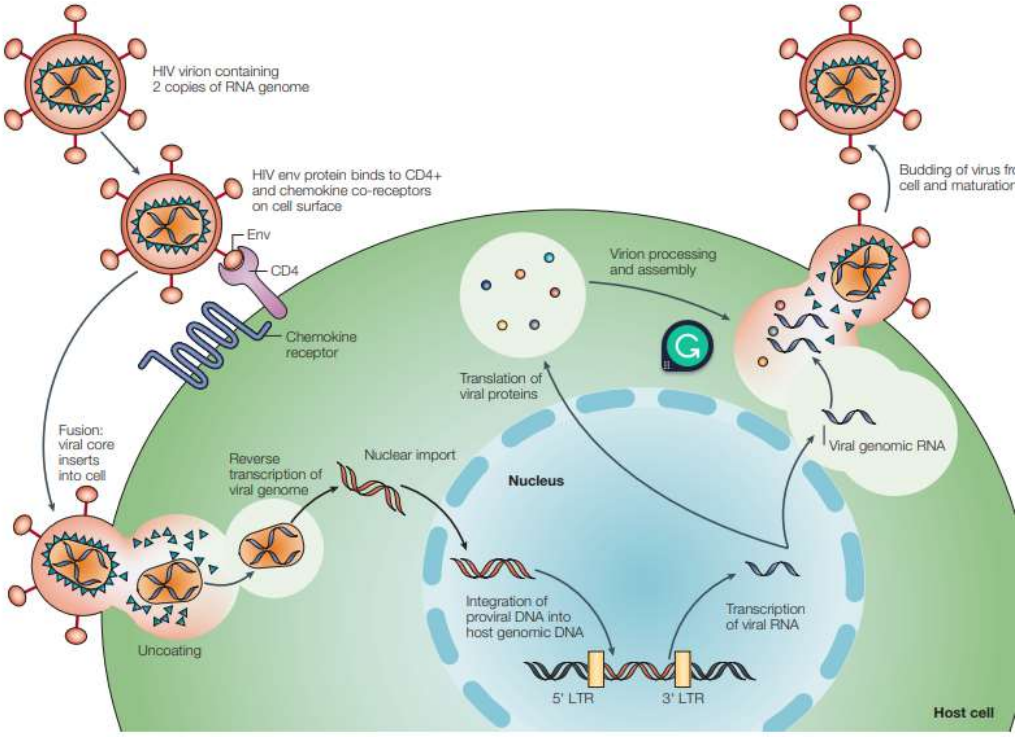


Figure 2. HIV life cycle in CD4$^+$ T cells. HIV enter its target cells via the attachment of HIV envelope (Env) glycoprotein to CD4 and either CCR5 or CXCR4 receptors on the cell surface, thereby fusion and uncoating of viral components occur. The viral RNA is then reverse-transcribed into DNA which is then imported to the nucleus and integrated into the host genome. HIV DNA is transcribed to viral mRNA depending on the cellular transcription machinery. Viral mRNA is then transported to the cytoplasm to eventually make mature viral particles. Figure from [203].

4.2.1 Modulation in TCR signaling events by HIV proteins

After infection, the production of a large amount of HIV proteins can exploit a plethora of host cell factors, pathways, and behaviors, in addition to the downstream TCR signalosome, to ensure efficient viral transcription and budding. HIV Nef, a critical multifunction accessory protein, co-opts various host proteins to tune cellular activation and survival signals in favor of viral infectivity. Nef-driven downregulation of MHC I and CD4 helps infected cells evade clearance by cell-mediated immunity [204]. Nef can also limit activation-induced T-cell death by downregulating co-stimulatory receptor CD28[205, 206]. More importantly, Nef can delay apoptosis by directly stimulating and disabling the function of pro-apoptotic factor, Bad [207].

Given that AKT is involved in the activation of multiple transcription factors such as NF-κB, Nef-mediated modulation of this pathway plays a crucial role in initiating HIV-1 transcription replication[208].

Initial characterization of HIV infection was described as cell-free virus fusing with target cells to produce productive infection, but recent studies have shown that HIV can be transmitted from one cell to another through cell-cell contacts. Initial studies described T cell: cell interactions as a conduit for HIV particle transfer[209], but later studies also describe macrophage: T cell and DC: T cell interactions as vessels for HIV transfer events[114, 210, 211]. Studies have shown that over 200 host cell factors are differentially regulated during viral transmission between target cells[212, 213], indicating that the virus may also alter the behavior of infected cells. Cell-cell HIV transmission occurs at the site of contact, called the virological synapse (VS), which involves interactions between gp120 (from the infected cell side) and CD4 (from the target cell side). Additional adhesion molecules, such as LFA-1: ICAM-1 interactions further stabilize these contacts that, in this way, is analogous to the immunological synapse. Gp120:CD4 engagement recruits phosphorylate Lck to the central supramolecular activation complex (cSMAC), resulting in the trafficking and activation of ZAP-70, SLP76 and PLC-γ1 in target T cells [214]. Indeed, successful viral spread from productively infected $CD4^+$ T cells is enhanced by the presence of phosphorylated ZAP-70 within uninfected cells, while its deficiency impairs viral replication[214]. HIV gp120 and CD4 clustering also induces transient stop signals that arrest target cells, further stabilizing interactions for efficient cell-cell HIV transmission[215-217]. Finally, gp120engagement also modulates downstream signaling in target T cells that maintains their high activation status and increased survival by upregulating the MAPK/ERK pathway, the PI3K/AKT/mTOR pathway and calcium flux [218]. Overall, these data suggest that HIV-1 exploits may host cell factors and signaling networks to perpetuate activation and T-cell survival in order to complete its viral life cycle.

4.2.2 HIV capture and trans-infection to target T cells

Infected T cell can transmit HIV particles to target cells through cell-cell contacts. However, DCs have been shown to perform the same function without becoming infected, as they are 10- to 100-fold less susceptible *in vivo* [219]. This resistance of HIV infection is ascribed to

several DC-inherent characteristics, such as low CD4 and CCR5/CXCR4 expression, or that viral particles are rapidly degraded after engulfment [122, 220]. Other intracellular DC restriction factors are known to block HIV infection, including expression of APOBEC3G, which hypermutates the nascent HIV-1DNA during its synthesis, and SAMHD-1 driven deprivation of dNTP substrates in the cytoplasm required for reverse transcription [221]. Several independent studies reported that Siglec-1 (CD169) is fully responsible for viral capture in a gp120-independent manner, as Siglec-1 blockade abrogates HIV capture by DCs[222-225]. Siglec-1 binds to ganglioside GM3, a member of glycosphingolipids that are present on the HIV membrane. This lectin-like receptor is significantly upregulated in DCs stimulated with LPS[226] or in the presence of interferon alpha (IFNα) produced by pDCs during HIV-challenge [128], and helps explain why mature DCs are more efficient at facilitating viral capture and transmission compared to immature DCs. Upon HIV capture, DCs compartmentalize many viral particles into endocytic compartments that are enriched with tetraspanin proteins, such as CD81, CD82, and CD9 [227, 228]. However, a recent study showed that DCs cluster the viral particles into the compartments that lack the markers of early endosomes, lysosomes, and antigen-processing vesicles[220]. Instead, HIV trafficking occurs in a pocket-like structure at the plasma membrane colocalizing with the CD81-positive subcellular compartment and are not degraded. *In vitro* studies have shown that upon forming DCs and $CD4^+$ T cell crosstalk, HIV-bearing DCs polarize HIV virions toward the site of cell-cell junction, termed *trans*-infection [116, 229]. Given the large numbers of DCs and T cells within the genital mucosa, it is hypothesized that DC: T cell clusters may fuel early viral replication and spread [114]. Cell-cell transmission offers several advantages to the virus. First, cell-cell contact helps the virus to circumvent immune recognition, antiviral factors, neutralizing antibodies, and antiretroviral drugs [230-233]. Second, the formation of the virological synapse between cells allows a large number of viral virions to be transferred at the site of cell-cell interaction [192, 234]. A better understanding of the molecular mechanisms involved in this process may yield a potential therapeutic strategy to limit systemic infection. Together, HIV utilizes multiple ways to subvert the immune response and take advantage of normal immune cell behaviors to increase infectivity and spread in the body.

5. Establishment and maintenance of HIV latency

As previously mentioned, activated $CD4^+$ T cells are the preferred target for HIV infection, supporting all steps of viral replication for successful productive infection. The majority of infected cells will produce a large number of HIV particles and undergo apoptosis cytopathic effect of the virus or killing by cytotoxic $CD8^+$ T cells[235-237]. However, a smaller portion of activated $CD4^+$ T cells revert to a memory phenotype T cell, where they are permissive to HIV infection and favors the establishment of HIV latency [235, 238]. Latently-infected T cells are defined as cells harboring stable and intact HIV provirus that is transcriptionally silent, and can persist over time [38, 44, 239, 240], and reactivate viremia upon cellular stimulation or ART removal [38, 39, 44, 52, 241]. However, it is worth mentioning that this definition does not extend to all HIV provirus, as full genome sequencing has revealed that 95%-98% of proviruses are defective with only a small number of cells harboring intact proviral genomes in PLWH [242, 243]. It is challenging to functionally characterize latently-infected $CD4^+$ T cells *in vivo* given their scarcity (~ 1 in 1 $x10^6$ cells) [244], and there are inherent challenges in differentiating this population from uninfected $CD4^+$ T cells [245]. Studies from Fromentin *et al* showed that PD-1 and LAG-3 are enriched in T cells harboring provirus[246], and there is considerable debated whether CD32 is a marker for T cell latency[247, 248]. Latent T cells remain long-lived with a half-life of 44 months, indicating that natural decay of the reservoir in a person's lifetime is not possible [44, 239]. As such, the eradication of this reservoir presents a critical challenge, and requires intense research endeavors that can specifically target cells these rare populations to achieving HIV cure [45].

5.1 Dynamics of HIV latency during ART therapy

The mechanism underlying latency establishment is not well-understood. While ART therapy can limit the size of the latent reservoir [249, 250], it does not suppress latency generation[251]. The precise timing of latency generation in humans is difficult to determine, as symptoms do not typically appear during the early acute stages of infection. Additionally, roughly 2% of total T cells reside in peripheral blood, while the remaining population resides in various tissue compartments in the body, most notably in lymphoid tissues of the gut and lymph nodes [243, 252]. Thus, analysis of peripheral blood T cells is a small fraction of all T cells in the body,

and it is challenging to collect and survey infected cell compartment within tissues from PLWH. Interestingly, Henrich *et al*. studied two extremely early diagnosed cases of HIV who had a positive viral load on the day of PrEP initiation [51]. Even after two years of ART therapy, both participants experienced viral rebound within 225 days following therapy cessation. In agreement with this observation, initiation of ART in eight individuals at the early stage of HIV infection (Fiebig 1 stage), significantly reduced viremia but did not abrogate viral rebound upon ART initiation [253].

Most of our current knowledge on the dynamics of HIV latency has been obtained from nonhuman primate models of HIV infection (rhesus macaques challenges with the analogous SIV), but these findings have produced inconclusive results. Whitney *et al* demonstrated that initiation of ART therapy as early as three days after SIV inoculation [254] does not prevent latency establishment, but significantly reduces the detectable viremia[43]. Yet, Okeye *et al* initiated ART therapy after the earliest time identification of infection (4-9 post-infection) followed by 2-year suppressive ART therapy which led to failure in viral rebound after ART removal, possibly due to elimination of the latent reservoir[254]. From this, the authors suggested that the latent reservoir establishes well before early initiation of therapy [255], and since ART treatment profoundly reduces HIV-associated inflammation and T cell activation, it can also reduce the turnover of the cellular reservoir. These studies collectively highlight significant clinical challenges of early ART initiation in HIV-infected individuals.

5.2 Mechanisms of latency formation in memory CD4$^+$ T cells

HIV provirus preferentially resides in memory CD4$^+$ T cells [38, 240, 244] due to their long-lived nature and ability to undergo proliferation upon antigenic stimulation [256]. Chomont *et al* identified T_{CM} and $T_{TM,}$ subsets as the best-characterized HIV reservoir with different decay rates, while naïve T cells or T_{EM} subsets contribute to this pool to a small extent[257]. T_{CM} is characterized by long-term survival and undergo proliferation upon antigenic stimulation. In contrast, T_{TM} is thought to replenish the latent reservoir due to their low level of proliferation and have the highest number of intact or replication-competent provirus resulting in viral rebound upon interruption in therapy [257, 258].

Two models have been proposed for the establishment of latency *in vitro*: the direct infection of memory $CD4^+$ T cells (pre-activation latency) [259-261] or infection of cells transitioning from activated to central memory $CD4^+$ T cells after T-cell activation (post-activation latency) [262, 263]. It was reported that pre-activation latency supports more productive infection and viral reactivation than post-activation latency, suggesting that latency is deeply set in the post-activation model. Several lines of evidence suggest that the nature of infection impacts latency. For example, IL-7 is a cytokine involved in the homeostatic proliferation of cells and modulates SAMHD1 expression, thereby enhancing memory $CD4^+$ T cell susceptibility to HIV infection [264]. Chemokines, CCL19 and CCL21 have been shown to facilitate the integration of HIV-1 DNA through modification of actin cytoskeleton [253], enhancing latent infections in T cells. In post-activation latency, the general consensus is that activated T cells express high levels of CCR5, transcription factors, and dNTP that support productive infection prior their transition to a more quiescent phenotype [256]. However, this may be oversimplification, as recent studies have challenged this view, arguing that latency establishment is a multifactorial phenomenon. It has been documented that TCR strength could influence latency establishment, as engagement of TCR and CD28 with their ligands leads to activation of transcription factors, chromatin remodeling, and increased host RNA pol II expression [265-267]. A recent study by Gagne *et al* showed that low or intermediate signal levels bias the cells toward latent infection resistant to the reversal[268], whereas high affinity signals induce latency that is highly reactivable. The contribution of APCs, particularly DCs, in latency establishment is well-documented, but their ability to do so seem to be highly dependent on the DC subset. For example, the interaction of T cells with myeloid DCs, but not pDCs, can support latency generation in both proliferating and non-proliferating T cells *in vitro* even after 12 days post-coculture[269, 270]. Moreover, Evans *et al* reported that myeloid DCs modulate TCR signaling to downregulate NF-κB pathway and increase the expression of Krüppel-like factor 6 (KLF6) and other signaling pathways that cause T cell quiescence[271]. Finally, other mechanisms contributing to latency establishment could be explained by the epigenetic modifications, chromatin remodeling and impaired RNA splicing or translation[272]. For instance, histone acetylation at HIV LTR plays a crucial role in activating HIV transcription. However, in latent T cells, histone acetylation is absent due to the recruitment of histone deacetylases (HDACs) to the LTR[273]. Overall, a better understanding of the molecular mechanism that governs latency establishment will inform the efficacy and optimal use of

therapies and interventions to extinguish the latent reservoir. The role of cell-cell contacts in the maintenance of T cell latency is still unclear and was addressed in my thesis project.

5.3 Strategies to reduce or eradicate HIV reservoir in vivo

5.3.1 The "Shock and Kill" approach

A promising strategy to deplete the latent reservoir, termed "shock and kill", uses latency-reversal agents (LRAs) to induce reactivation of viral gene expression in the presence of ART so that reactivated cells are cleared by either viral cytopathic effects or by $CD8^+$ T cells [274, 275]. Various LRAs have been tested *in vivo*, *in vitro* and *ex vivo* to "shock" HIV out of latency [276-278] which include histone deacetylases, PI3K/AKT inhibitors, protein kinase C (PKC) agonists[279], and TLR7 agonists [280, 281]. Despite successfully augmenting cellular and HIV transcription, only a limited number of drugs can induce latency reversal or immune clearance of HIV-infected $CD4^+$ T cells, as some latently-infected $CD4^+$ T cells may require even multiple stimulations to achieve full reactivation [282]. Importantly, LRAs tested in clinical trials lead to a spike in viral protein and RNA production in tissues, but no substantial reduction in reservoir size has been demonstrated, suggesting the need for combinatory LRAs or complementation with other strategies [283]. Another limitation of LRAs include potential side effects on healthy cells that become transcriptionally active, owing to the non-specific nature of these drugs. Altogether, these data suggest that further optimization of both the "shock" and the immune response that enhance the "kill" are required to reduce HIV reservoir size in PLWH.

5.3.2 The "Block and Lock" approach

Rather than eliminating cells harboring provirus, the concept behind the "Block and Lock" approach is to permanently maintain proviruses in latent state even after ART removal. This silencing can be achieved by using latency-promoting agents (LPAs) that block viral expression. Specifically, LPAs can suppress viral or cellular factors involved in the transcriptional machinery and the initiation of HIV transcription [284, 285]. Currently, the best-described LPA is didehydro-cortistatin A (dCA), which suppresses Tat-dependent transcription. Tat is the critical viral protein that initiate efficient HIV transcription by recruiting RNA polymerase II to the LTR

promoter[286]. dCA favors epigenetic modeling that represses viral reactivation after therapy cessation Moreover, using humanized mice, it was demonstrated that dCA reduces viremia in both cells and tissues, and hinders viral rebound upon ART interruption [287]. Nevertheless, the efficacy of the "block and lock" strategy is yet to be assessed in PLWH and clinical benefit is not yet known.

5.4 The immunological landscape that supports HIV persistence

The long-lived HIV reservoir, under ART, produces near undetectable viremia, and the size of the reservoir reaches a plateau at roughly 4 years of therapy[33-35]. However, the dynamics of HIV latency is complex and subject of much debate in the field. In general, two different views of latency maintenance under ART have been proposed: 1) continual replenishment of the reservoir by low level of viral replication exclusively within SLOs, such as the lymph nodes and gut-associated lymphoid tissues, and 2) the clonal expansion of T cells harboring provirus that was established prior to initiation of ART. While the former was thought to be the major mechanism due to decreased drug penetration was observed in deep tissues, HIV genome sequencing studies have demonstrated little evidence for viral evolution during ART. This suggested that with optimal ART, very limited viral replication occurs and argues against this model of HIV reservoir maintenance [288, 289].

5.4.1 Clonal expansion of HIV reservoir

Sequencing and integration site analysis of proviruses showed that latently-infected $CD4^+$ T cells undergo clonal expansion during ART[290-292], where cells carrying the same proviral sequence are found through sequencing analysis. This is due to the fact that HIV RT is highly prone to errors during reverse transcription [56, 57], making it highly unlikely that two parallel infection events producing the exact same proviral sequence occurs. Clonal expansion of latent T cells can be detected within a few weeks after HIV infection and continually under ART [292-295]. As such, more than 50% of all T cells harboring provirus are thought to be derived through clonal expansion of a smaller population of cells established at or near the time of ART initiation [292]. While it was thought that only defective proviruses were primarily clonally

expanded, new lines of evidence show that approximately 56% of intact proviruses also undergo clonal expansion[296, 297]. These are three proposed mechanisms that may drive clonal expansion of the HIV reservoir: (i) homeostatic proliferation, (ii) integration-site dependent proliferation, and (iii) antigen-driven proliferation [298]. These mechanisms are described in more detail below.

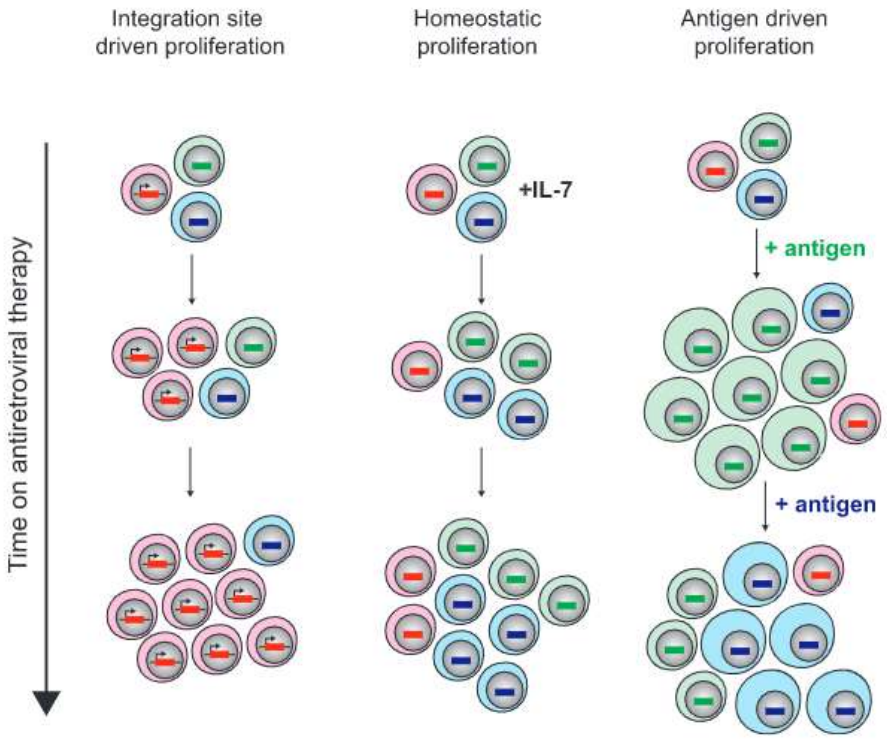


Figure 3. HIV maintenance through clonal expansion in individuals under suppressive ART therapy. Three independent mechanisms have been proposed that drive the proliferation of latent T cells. First, integration into specific genes may provide survival benefits latent T cell clones. Second, homeostatic proliferation such as IL-7 may cause latent T cell expansion without viral reactivation. Finally, re-exposure of antigen-specific T cells harboring HIV provirus to antigens may expand the latent reservoir. Figure from [299].

5.4.1.1 Homeostatic proliferation

Homeostatic proliferation is crucial for the maintenance of $CD8^+$ and $CD4^+$ T cell populations at steady-state [300]. IL-7 is a critical cytokine that helps maintain naïve and memory T cell homeostasis without TCR stimulation [301, 302], while IL-15 is required for the survival of $CD8^+$ T cells over $CD4^+$ T cells. The proliferation of memory $CD4^+$ T cells profoundly decreases due to low expression of IL-7 receptor (CD127) during chronic HIV-1 infection[303]. Conversely, plasma IL-7 levels are high after $CD4^+$ T cell depletion, leading to the hypothesis that IL-7 treatment may be beneficial to restore CD4 T cell counts[304]. To this end, the Chomont lab treated seven ART-suppressed individuals with IL-7 in the presence of antiretroviral drugs[305]. This study showed that IL-7 administration induced viral production in productively-infected T cells but had no effect on latent T cells. However, IL-7 dramatically increased the size of HIV reservoir within 4 days and normalized at day 28. These studies indicated that while IL-7 improved T cell counts in ART-treated individuals, the number of cells harboring latent virus also increased, suggesting that cytokine treatment led to the expansion of the HIV reservoir. Consistent with this, three independent studies *in vitro* have shown that IL-7 drives the proliferation of the HIV-infected $CD4^+$ T cells without inducing viral reactivation diminish the size of the HIV reservoir [305-307]. Taken all together, these data indicate that IL-7 treatment can increase the number of latently-infected cells with little-to-no viral reactivation under ART [306].

5.4.1.2 Integration-site driven proliferation

HIV integrates primarily into actively transcribed regions such as cancer-associated genes[267]. In some cases, the integration of HIV into these regions, including BACH2, MKL2, MKL1, STAT5B is associated with clonal expansion in multiple ART-suppressed individuals [308]. It was also reported that SIV also integrates into BACH2, MKL2 and STAT5B prior to ART, similar to HIV-1 [309]. However, clonal expansion has been observed in the absence of integration into these sites[295], indicating that this mechanism is likely not the sole driver of HV reservoir expansion. The HIV provirus is subject to chromatin and epigenetic regulation by host genes near the integration site[292, 310]. Overall, more investigations are needed to understand the role of integration-induced proliferation in HIV maintenance.

5.4.1.3 Antigen-driven proliferation

HIV co-infections with other viruses, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are common in PLWH. Accumulating evidence show that $CD4^+$ T cells harboring replication competent provirus are enriched in CMV- and EBV-specific T cells in PLWH [32, 51, 311, 312]. In one study, HIV provirus of identical sequences was found in expanded T cells responding to CMV, HIV and Gag proteins in PLWH under long-term ART [313]. Clones of antigen specific $CD4^+$ T cells harboring defective latent HIV provirus were also detected in seven out of eight individuals, while those harboring intact was present at lower frequencies. However, the magnitude of antigenic response varied among the individuals, which is expected [313]. In line with this observation, a recent study by Simonetti *et al* used CMV viral lysate and purified HIV Gag to stimulate antigen-specific T cells from PBMCs of HIV^+ patients on ART [314]. They showed identical proviral sequences within CMV-responding $CD4^+$ T cells and provide evidence for antigen-driven proliferation that was independent of integration site [290]. Other studies have identified proviral clones within influenza- and *Candia albicans*-specific T cells [315, 316]. Finally, HIV+ individuals who received treatment for metastatic squamous cell carcinoma saw an expansion of provirus containing CMV- and EBV-specific T cells [295]. Taken together, these studies suggest that microbial antigens may drive the proliferation of latent reservoir in a way that mimics physiological maintenance of T_{CM} cells. Given those antigenic stimulations occur through APC: T cell interactions, my thesis project focused on cell-cell communication in the proliferation of latent T cell *in vitro*.

6. Thesis, rational, objectives and hypothesis

6.1 Rationale

DCs are specialized APCs that are found throughout the body and are a crucial activator of antigen-specific $CD4^+$ T cell responses [105, 317]. Previous studies in our lab have shown that HIV has evolved multiple mechanisms to subvert cell-mediated immunity in order to enhance systemic viral spread and establish a chronic infection. One emerging mechanism is that HIV takes advantage of normal DC: T cell interactions to efficiently spread between cells. We have evidence that DCs capture HIV particles and display them to migrating $CD4^+$ T cells in a way that enhances

viral spread and susceptibility of T cells. However, how HIV-bearing DCs modulate T cell behaviors is not well understood. At the same time, DC: T cell contacts may also enhance HIV latent infections and facilitate the long-term maintenance of latency in T cells. Given that extensive clonal expansion of the viral reservoir is observed in ART-treated individuals, DC: T cell clusters may be a "hub" that promotes latent T cell proliferation. Whether the presence of microbial antigens leads to the proliferation of T cell subsets harboring HIV provirus, and the role of DCs in this process is unclear. Together, how HIV takes advantage of normal immune cell behaviors to establish a chronic infection is the main gap in knowledge being addressed in this thesis.

6.2 Hypothesis

I hypothesis that DC: T cell interactions stimulate unique signals that enhance HIV dissemination and promote antigen-specific expansion of latently-infected $CD4^+$ T cells over time.

6.3 Objectives

This thesis has four main objectives:

***Objective 1*:** To identify the signaling pathways that are initiated through DC: T cell contacts that function to enhance HIV spread and T cell susceptibility to HIV infection.

Objective 2: To validate activation of specific signaling pathways in T cells during DC: T cell co-cultures in the presence of HIV particles.

Objective 3: To investigate the role of DCs: T cell interactions in the expansion of latently-infected T cells in an antigen-dependent manner *in vitro.*

Objective 4: To evaluate the role of cell-cell contact and soluble cytokines in the proliferation of latent T cells.

6.4 Significance and Impact

Previous studies have highlighted the role of dendritic cells in the establishment of HIV latency in $CD4^+$ T cells, but this study is the first to demonstrate that DCs can also help latent T cell expansion in an antigen-specific and non-specific manner. I utilized a novel full-length latency reporter and a primary *in vitro* DC: T cell co-culture model to show that cell-cell contacts are a requirement for the proliferation of latent T cells without inducing virus reactivation. Specifically, I show that co-stimulatory signaling is crucial for latent T cell expansion. Together, my results provide additional evidence that HIV can hijack normal DC: T cell interactions to access proliferation signals required for their long-term persistence under ART. Our approach helps shed light on the function of DCs during chronic HIV infection and provides a better understanding of the cellular mechanisms behind HIV persistence in PLWH under ART suppressive ART therapy.

Chapter 2. MATERIALS AND METHODS

2.1 Isolation and preparation of human primary cells

2.1.1 Human CD14$^+$ and CD4$^+$ T cell isolation

PBMCs were isolated from healthy donors (NetCAD; Canadian Blood Services) using Ficoll-Paque PLUS density gradient centrifuge. Afterwards, human CD14$^+$ monocytes and CD4$^+$ T cells were isolated using EasySep^{TM} isolation kit (Stemcell Technologies, Cat # 17952 & 17858) following manufacturer's instructions. Primary monocytes were differentiated into human monocyte-derived DCs by seeding into T25 NunclonTM Sphera^{TM} flasks (Thermo Scientific Cat # 12566439) with 50ng/mL each of human granulocyte macrophage colony-stimulating factor (GM-CSF; Biolegend, Cat #578606) and interleukin 4 (IL-4; Biolegend, Cat #574008) in Immunocult^{Tm} -XF T cell Expansion media (Stemcell, cat no. 10981) supplemented with 1% Penicilin/Streptomycin (Pen/Strep). Naïve CD4$^+$ T cells were plated in 24-well plates at 1 x 10^6 per mL and were activated (day 0) by incubation with Dynabeads coated with anti-human CD3ε/CD28 antibody (1:1 bead/cell ratio; Life Technologies; Cat #. 11131D) in complete media. After 48 hours, beads were removed by EasySep^{TM} magnet (Stemcell Technologies, cat no. 18000) and proliferating cells were expanded for additional 4-7 days in complete medium containing 50 IU/mL human recombinant IL-2 (rIL-2; R & D systems, Cat #. 200-02), replacing media and rIL-2 every 2 days. On day 5, immature DCs were converted into mature DCs with 100 ng/mL purified polysaccharide from Salmonella Minnesota *(*Invivo Gen Cat #tlrl-smlps) and incubated under 37°C/5% CO_2 condition for 24 hours. Day 7 T cells and day 5 MDDCs were used for all the experiments.

2.1.2 MAGI-CCR5 and HEK293T Cells

MAGI-CCR5 cells were obtained from the NIH ARRRP Cat #3522) and grown in DMEM supplemented with 10% Fetal Bovine Serum (FBS; VWR Seradigm), 2mM GlutaMax (Gibco), 1mM sodium pyruvate (Corning, cat no. #25-000-CI) and 10mM HEPES (Sigma-Aldriche) under 37°C/5% CO_2 conditions. The parental cell line of MAGI is a HeLa cell clone, which is a female cell line. This cell line has not been authenticated.

HEK293T cells were obtained from ATCC (Cat #. 3522) and grown in DMEM supplemented with 10% fetal bovine serum, 2mM GlutaMax, 1mM sodium pyruvate, and 10mM HEPES under 37°C/5% CO2 conditions.

2.2 Preparation of HIV Stock

All the stocks for R5-tropic HIV-Nef-CRIMZY reporter were produced by transient transfection of HEK 293T cells (ATCC; CRL-3216) using JetOPTIMUS Buffer. An expression plasmid encoding VSVg was used to produce VSVg-pseudotyped HIV. All Viral supernatants were harvested at 48 h post-transfection and were centrifuged at 1300 rpm for 10 mins. Next, the supernatant was mixed with Lenti-X concentrator (Takara Bio Cat # 631232) for 60 mins. The samples were centrifuged at 1300 rpm for 45 mins at 0 °C, and the supernatant was discarded. The pellet was resuspended in 1/100 volume of complete DMEM and virus aliquots were stored at -80 °C. Viral stocks were titrated using MAGI.CCR5 and expressed as blue-forming units (bfu) per milliliter. All cells were infected with a multiplicity of infection (MOI) of 0.1.

2.3 Phospho-flow cytometric assay

Pan $CD4^+$ T cells were isolated from PBMCs of healthy donors (NetCAD, Canadian Blood Services) using a negative selection magnetic column (EasySep^{TM} isolation kit). On day 7, one million $CD4^+$ T cells were aliquoted into round-bottom polystyrene tubes in 1 mL complete RPMI 1640 media containing 10% FBS (VWR Seradigm), 2 mM GlutaMAX (Gibco), 1 mM sodium pyruvate (Corning Cat #25-000-CI) and 10 mM HEPES (Sigma-Aldrich). Cells were then restimulated with PHA (10 µgr/ml) or Dynabeads for 5 mins, 15 mins and 30 mins and 60 mins, and incubated in the 37 °C water bath. Following incubation, the beads were magnetically removed, and the samples were immediately fixed with 2% paraformaldehyde (PFA) for 45 mins at 4°C. Next, samples were washed twice with 2 mL FACS Buffer and centrifuged at 350 xg, 4°C for 5 mins. For permeabilization, cells were resuspended with pre-chilled 100% methanol. Samples were vortexed and incubated on ice for 30 mins. Thereafter, cells were centrifuged and washed twice with FACS buffer. Intracellular staining was performed with following fluorescently-labeled phospho-specific mAbs in the dark at RT for 60 mins: anti-LcK phospho-Tyr394 (Biolegend Cat

933104), anti-ZAP-70 phospho (Tyr319)/Syk phospho-Tyr352 (Biolegend, Cat # 683708), anti-ERK 1/2 phospho Thr202/ Tyr204 (Biolegend, Cat #675504). Finally, samples were washed twice in FACS buffer and resuspended in 200 µl prior to analysis.

2.4 AIM assay

Immature MDDCs were harvested and washed at 350 xg for 5 mins. Cells were pulsed with the indicated antigens in serum-alternative media supplemented with 1% Pen/Strep, followed by incubation at 37 °C for 2 hours. Antigens include viral and bacterial cell lysates: CMV-infected fibroblast cell lysates, EBV-infected B cell lysates (Virusys, 10 µg/ mL*), E.coli* and *S.aureus* lysates (50 µl/ reaction) and CPI (CMV, parainfluenza, influenza) protein pool (Immunospot. Cat # CTL-CPI-001). CMV- and EBV-uninfected lysates (Virusys, 10 µg/ mL) and SEB-stimulated cells (0.5 µg/mL) served as negative and positive controls, respectively. Antigen-pulsed DCs were then cocultured with T cells at ratio of 1:5 (DC/ T cell) and placed in a 37°C/5% CO_2 incubator for the indicated times. The combination of surface activation markers (OX40, CD40L, CD69, and PD-L1) were used for phenotyping antigen-responsive T cells.

2.5 Flow cytometry and antibodies

DCs and $CD4^+$ T cells were stained for surface makers using conjugated antibodies listed in table 1. DCs were first pretreated with 1µL of Fc receptors (FcRs) blockade using human TruStain FcX solution (Biolegend, cat no. 422302), and incubated on ice with 1 µL of the indicated surface antibodies for 30 minutes. Thereafter, cells were washed twice by adding 2 mL FACS buffer and centrifuged at 400 xg for 5 mins. The supernatant was discarded, and cells were resuspended in 500 µL of 2% PFA. Cell acquisition was performed on the LSRFortessa (BD Bioscience, USA). Data was analyzed with FlowJo software (version 10.7.2)

Table 1. The list of cell surface antibodies

Number	Antibody	Catalogue #	Clone	Company
1	anti-human CD3	300318	HIT3a	Biolegend
2	Anti-human CD4	317439	OKT4	Biolegend,
3	Anti-human CD4	317432	OKT4	Biolegend
3	anti-human OX40	350003	ACT35	Biolegend,
4	anti-human PD-L1	329713	29E.2A3	Biolegend,
5	Anti-human CD69	310912	FN50	Biolegend
6	Anti-human CD27	356406	M-T271	Biolegend
7	Anti-human CD45RO	304222	UCHL1	Biolegend
8	Anti-human CD154 (CD40-L)	310826	24-31	Biolegend
9	Anti-human PD-L1	329713	29E.2A3	Biolegend
10	anti-human CD197 (CCR7)	353208	G043H7	Biolegend
11	Anti-human CD197 (CCR7)	353232	G043H7	Biolegend
12	Anti-human CD4	317439	OKT9	Biolegend
13	anti-human CD80	305208	2D10	Biolegend
14	Anti-human CD83	305330	HB15e	Biolegend
15	Anti-human CD86	374210	BU63	Biolegend
16	anti-human CD11c	301614	3.9	Biolegend
17	anti-human CD45RA	12-0458-42	HI100	eBioscience
18	anti-human HLA-DR	1-9952-42	TU36	eBioscience,

2.6 HIV infection of T cells in collagen matrix

To infect T cells on day 7, five million T cells per tube were aliquoted into FACS tubes centrifuged at 1300 rpm for 10 mins and were mixed with HIV- Nef-CRIMZY reporter and then placed in a collagen solution, as we have published that this enhances infection rates [318]. Next, 270 µL of collagen mixture were placed into 48-well plate and incubated at 37°C for 3 hours. Following the incubation, 400 µL of 1x collagenase D (Roche, Cat # #11088866001) was added in each well to digest collagen, and the cells were washed and resuspended at 1 million/ml in serum-alternative media with hrIL-2 (5 µg/mL) in 24-well plate, replacing the media and IL-2 every 2 days. To confirm T cell infection rates, latent and productive infection was measured at day 5 post-infection using flow cytometry.

2.7 T cell proliferation assay

T cells were labelled using Tag-it proliferation dye (Biolegend Cat # 425101) according to manufacturer's instructions prior to the coculture assay. Briefly, T cells were washed with PBS at 1500 rpm (400 xg) for 10 mins and resuspended at 1 milllion cells /mL in a working dye solution (5 µM) at 37°C/ 5% CO2 for 20 mins in the dark. The labelled cells were then washed with PBS and centrifuged at 1500 rpm for 10 mins. The pelleted cells were resuspended in media culture and used for the indicated experiments.

2.8 DC: T cell co-culture studies and analysis of T cell proliferation

As indicated above, T cells infected with HIV- Nef-CRIMZY reporter were labelled with Tagit proliferation dye, then incubated with autologous DCs at a ratio of 1: 5 (DC: T cell) in 24-well plate for 8 days. To prevent subsequent rounds of HIV infection, 10 µM of the integrase inhibitor, raltegravir (NIH ARRRP, Cat # 11680) was added to the culture after 5 days. In some studies, MDDCs were pulsed with 6 µg/mL of protein pool CPI (Immunospot, Cat # CTL-CPI-001). For blockade experiments, MDDCs were pretreated with FcR blockade (Biolegend Cat #. 422302) to inhibit FCR-mediated opsonization and incubated at 4°C for 20 mins. Without washing, cells were then incubated with 10 µg/mL of either isotype or blocking antibodies and incubated at 4°C for 30 mins. Antibody-blocked DCs were then co-cultured with HIV infected T cells in 24

well-plates, as above. For blocking antibodies, anti-CD80 (R&D, Cat # MAB140), anti-CD86 (R&D, Cat # MAB141-100), purified anti-MHC class II (BD Bioscience, Cat #55556), and anti-ICAM-1 (R&D, Cat # AF720-SP) were used. After 2 and 6 days of co-culture, culture supernatant were collected for further analysis, and cell were harvested for flow cytometry analysis.

2.9 Kinome data analysis

The specific responses of each peptide were calculated by subtracting background intensity from foreground intensity. Signal intensities induced by DEnv HIV-iGFP were subtracted from the intensities from the time-matched, wildtype HIV-iGFP biological conditions, and test statistics were calculated. Average intensities were then taken over the three replicate intensities, and these values were subjected to hierarchical clustering analysis using InnateDb (www.innatedb.com) and ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, CA). For our investigations, input data were limited to peptides that showed consistent responses across the biological replicates (black bars), and p-values (red line), calculated with the Fischer's exact test, and reflects the likelihood that the association between a set of active kinases and a biological function is significant [p-value ≤ 0.05].

2. 10 Statistical analysis

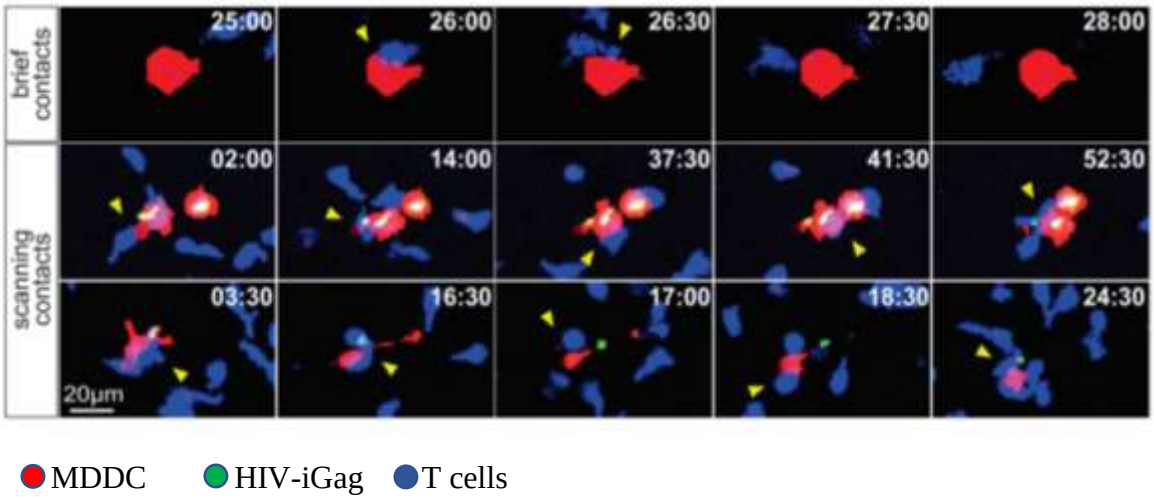
Data analysis was performed using GraphPad Prism (San Diego, CA; software version 9.0d). Respective data represent mean $\pm$ SD, and differences were considered statistically significant when p-value < 0.05. One-way Anova was used for statistical analysis.

Chapter 3. RESULTS

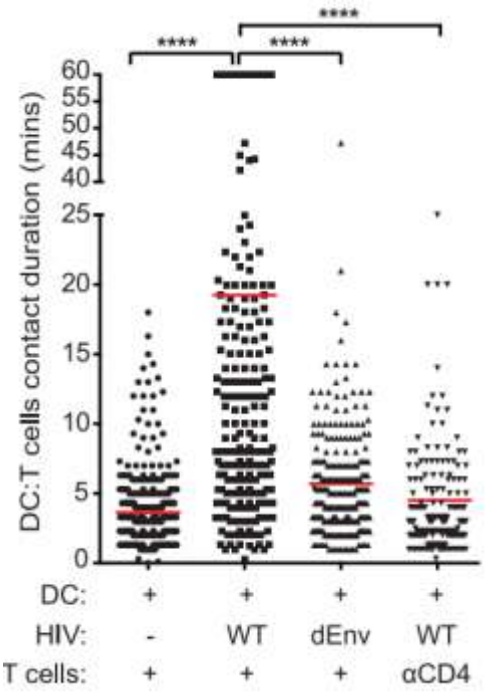
3.1 HIV-bearing DCs engage in prolonged contacts with T cells through CD4:gp120 interactions

Our previous study examined HIV particles captured by DCs and subsequent DC:T cell interaction dynamics in 3D collagen matrix [122]. $CD4^+$ T cells were cocultured with DCs bearing wild type (WT) HIV particles which have envelope gp120 on their surface. Using live-cell microscopy, we observed prolonged interactions between T cells and HIV-containing DCs, but not with control DCs without HIV particles. When contact durations were characterized as brief (< 5 minutes), scanning (5-17 minutes) or stable (> 17 minutes), we observed that more than 70% of all HIV^+DC: T cell conjugates were either prolonged or scanning contacts in the presence of WT virus (Figure 4A). However, the contact time of DC: T cell significantly diminished in the presence of Env-deficient HIV, indicating that Env:CD4 interactions were driving stable DC: T cell interactions. We obtained comparable results when CD4 or LFA-1: ICAM-1 interaction was blocked using neutralizing antibodies in our coculture (Figure 4B & C), consistent with previous reports that LFA-1: ICAM-1 contacts are essential for stabilizing DC: T cell interactions. Together, these data show that HIV-captured DCs can effectively "slow down" T cells to promote the long-lasting cell-cell contacts through an Env-dependent mechanism. This was an unexpected finding and further studies were designed to determine how Env:CD4 interactions modulate T cell behaviors to favor HIV spread between cells.

A


brief contacts
scanning contacts
25:00
26:00
26:30
27:30
28:00
02:00
14:00
37:30
41:30
52:30
03:30
16:30
17:00
18:30
24:30
20μm
MDDC
HIV-iGag
T cells

B


****
****
****
DC:T cells contact duration (mins)
60
55
50
45
40
25
20
15
10
5
0
DC: + + + +
HIV: - WT dEnv WT
T cells: + + + αCD4

C

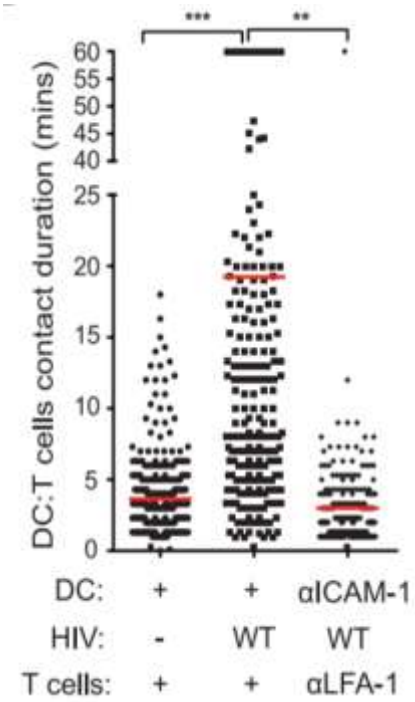

***
**
DC:T cells contact duration (mins)
60
55
50
45
40
25
20
15
10
5
0
DC: + + αICAM-1
HIV: - WT WT
T cells: + + αLFA-1

Figure 4. HIV-bearing DCs prolong contacts with susceptible $CD4^+$ T cells through Env: CD4 and LFA-1: ICAM-1 interactions.

(A) DCs were pulsed with HIV particles for 4 hours (hrs) and washed to remove free virus. HIV-captured DCs were pulsed with Texas Red-conjugated dextran and then co-embedded with T cells at a ratio of 1: 4 or 1:8 (DC/T cell). The representative image shows time-lapse micrographs of DC: T cell contacts that are defined as brief (< 5 mins) or prolonged (> 17 mins) interactions. Time stamp are in min:sec of the movie recordings.

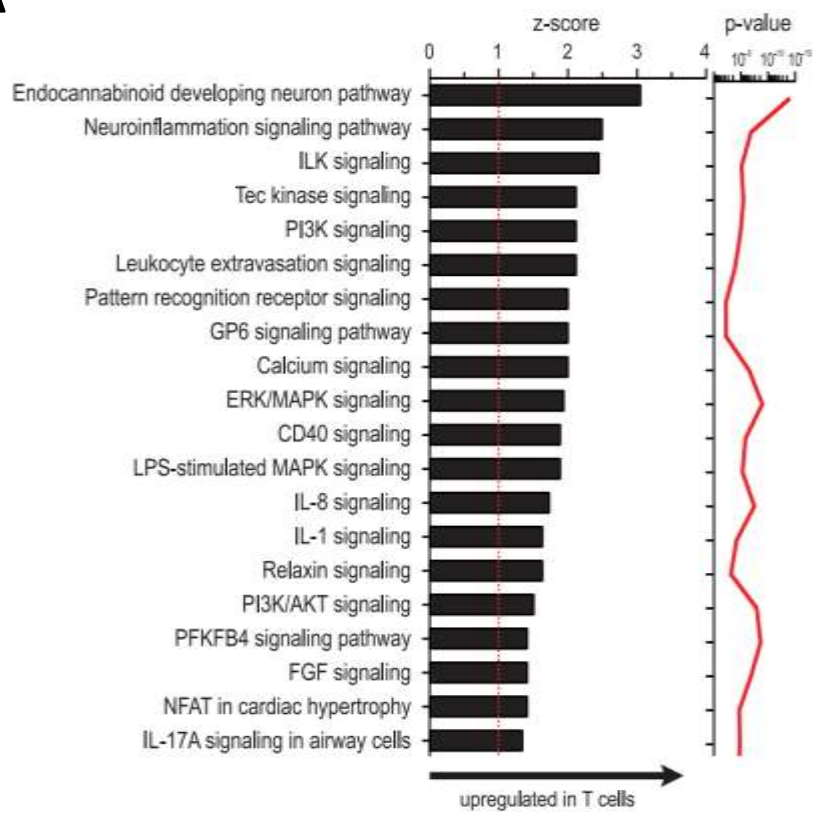
(B) DC: T cell contact duration in the presence of wild-type or dEnV (Env-deficient) HIV. Red lines indicate median values. aCD4, anti-CD4 antibody. ****$p < 0.001$, using Mann-Whitney U test. Data from ten independent experiments are shown (n = 1,172 total DC: T cell contact events).

(C) DC: T cell contact duration (min) in the presence or absence of LFA-1: ICAM-1 interactions. ICAM-1, anti-ICAM-1 antibody; aLFA-1, anti-LFA-1 antibody. Red lines indicate median values. **$p < 0.01$, ***$p < 0.001$, using Mann-Whitney U test. Data from eight independent experiments are shown (n = 957 total DC: T cell contact events). Data from Koh *et al [122]*.

3.1.1 Gp120:CD4 interaction invokes distinct signaling to facilitate viral dissemination

Planar model systems have revealed that gp120:CD4 engagement triggers stop signals in target $CD4^+$ T cells and promotes the phosphorylation of Lck, ZAP-70, and PLC- γ [215, 216]. These and other studies demonstrated that HIV particles can induce signaling in T cells and enhance infectivity. However, whether HIV particles that DCs bind can also stimulate similar signaling pathways in T cells in a way that stabilizes DC: T cell conjugates is ill-defined. Since our findings showed prolonged HIV^+ DC: T cell interactions, we next assessed how T cell signaling pathways were impacted during cell-cell interactions. $CD4^+$ T cells were cocultured with DCs previously pulsed with WT or Env-deficient HIV particles, in a time course experiment (15 and 60 mins). $CD4^+$ T cells were then magnetically removed and lysed. Cell lysates were placed on a custom human kinomics array platform (containing >900 phospho-target motif) to perform a non-biased evaluation of kinase phosphorylation. Ingenuity pathway analysis (IPA) and innateDB analysis predicated similar downstream signaling at both 15 min- and 60-min time points. IPA analysis illustrated upregulation of Tec kinase, calcium signaling, and ERK/MAPK signaling during DC: T cell contact in the presence of wildtype, but not Env-deficient HIV (Figure 5A). InnateDB analysis also revealed the activation of various signaling pathways such as TCR signaling, mTOR signaling and Ca^{2+} pathways (Figure 5B). Many of these signaling pathways directly regulate T cell motility, such as Ca^{2+} or ERK/ MAPK pathways. These data suggest that T cells accumulate signals downstream of the CD4 molecule that impact their motility and function as they contact HIV-bearing DCs, some of which are pre-requisite to facilitate HIV dissemination within lymphoid tissues.

A



B

Upregulated Kinase Pathways	p-value (≤0.05)
Ca^{2+} Pathway	0.0265
MAPK/ERK Pathway	0.000000319
Translocation of ZAP-70 to Immunological Synapse	0.0767
mTORC1-Mediated Signalling	0.00000455
Lck and Fyn Tyrosine Kinases in Initiation of TCR Activation	0.0000922
Calmodulin Induced Events	0.0995

Figure 5. HIV-captured DCs induce distinct TCR signaling events in T cells to facilitate viral dissemination in a gp120: CD4-dependent manner.

(A) IPA analysis identified relevant pathways that were significantly (p-value $\leq$ 0.05) upregulated (z-score > 1) in $CD4^+$ T cells co-cultured with wild type HIV-pulsed DCs for 15 mins (T cells co-cultured with Env-deficient HIV-iGag-pulsed DCs set as background controls). The p-value (red line), calculated with the Fischer's exact test, reflects the likelihood that the association between a set of activated kinases and a biological function is significant (p-value $\leq$ 0.05).

(B) Summary of InnateDB analysis of upregulated kinase pathways in $CD4^+$ T cells after 15-min coculture with HIV-pulsed DCs compared to Env-deficient HIV (p-value $\leq$ 0.05).

3.2 Measuring T cell signaling with the phospho-flow cytometric assay

Since my data demonstrated that HIV-bearing DCs activates unique signaling cascades in T cells through cell-cell contacts, we developed an *in vitro* coculture system to confirm activation of signaling pathways identified by the human kinome array. I decided to use the intracellular phospho-flow cytometry approach to measure Lck, ZAP-70, and ERK1/2 phosphorylation in $CD4^+$ T cells after culture with HIV-bearing DCs. To accomplish this, primary $CD4^+$ T cells were first tested for their responses to different exogenous stimuli such as phytohemagglutinin (PHA; 10 μg/mL) and anti-CD3/anti-CD28 Dynabeads (25μL/mL) at various time points. The stimulation was stopped by fixing both treated and untreated cells with paraformaldehyde (PFA). Then, cells were permeabilized with pre-chilled 100% methanol, described in Materials and Method. I first assessed pLck, pZAP-70 and pERK 1/2, as their roles in HIV pathogenicity have been shown in T cells, and that Lck and ZAP-70 are activated at early time points following T-cell activation[155, 160]. Both PHA (crosslinks the T cell receptor) and Dynabeads stimulations were used as positive controls to maximize T cell response for assay optimization. In my hands, little to no augmentation in phosphorylation of Lck, ZAP-70, and ERK 1/2 were observed after PHA stimulation (Fig 6A). As seen in Figure 6B, no robust response was also detectable in cells stimulated with anti-CD3/CD28 at a concentration that promotes extensive cell activation[319]. Analyzing Mean fluorescent Intensity (MFI) of both populations demonstrated modest change in pLck, pZAP-70, and pERK1/2 (Figure 6C& D).

The early T cell activation markers CD69 and HLA-DR were measured by flow cytometry to confirm T cell activation by PHA. Day 7 cultured T cells stimulated with PHA showed higher expression of CD69 and HLA-DR after 1 hour and 24 hours compared to unstimulated T cells (Figure 7A & B), providing evidence that PHA induces T cell activation in this assay. However, I did not observe T cell proliferation after 24 hours as expected (Figure 7C). Further optimization protocols were tried such as using different fixation/permeabilization buffers, phospho-site specific Abs, and antibody titration. However, I did not observe any significant change in the phosphorylation of these kinases. Thus, these data demonstrated that in my hands, phospho-flow cytometric assay was not a sensitive tool to perform temporal analysis of T cells signaling in our DC: T cell co-culture systems. Therefore, we decided to take on another approach to study signaling transduction in $CD4^+$ T cells after exposure to HIV-captured DCs.

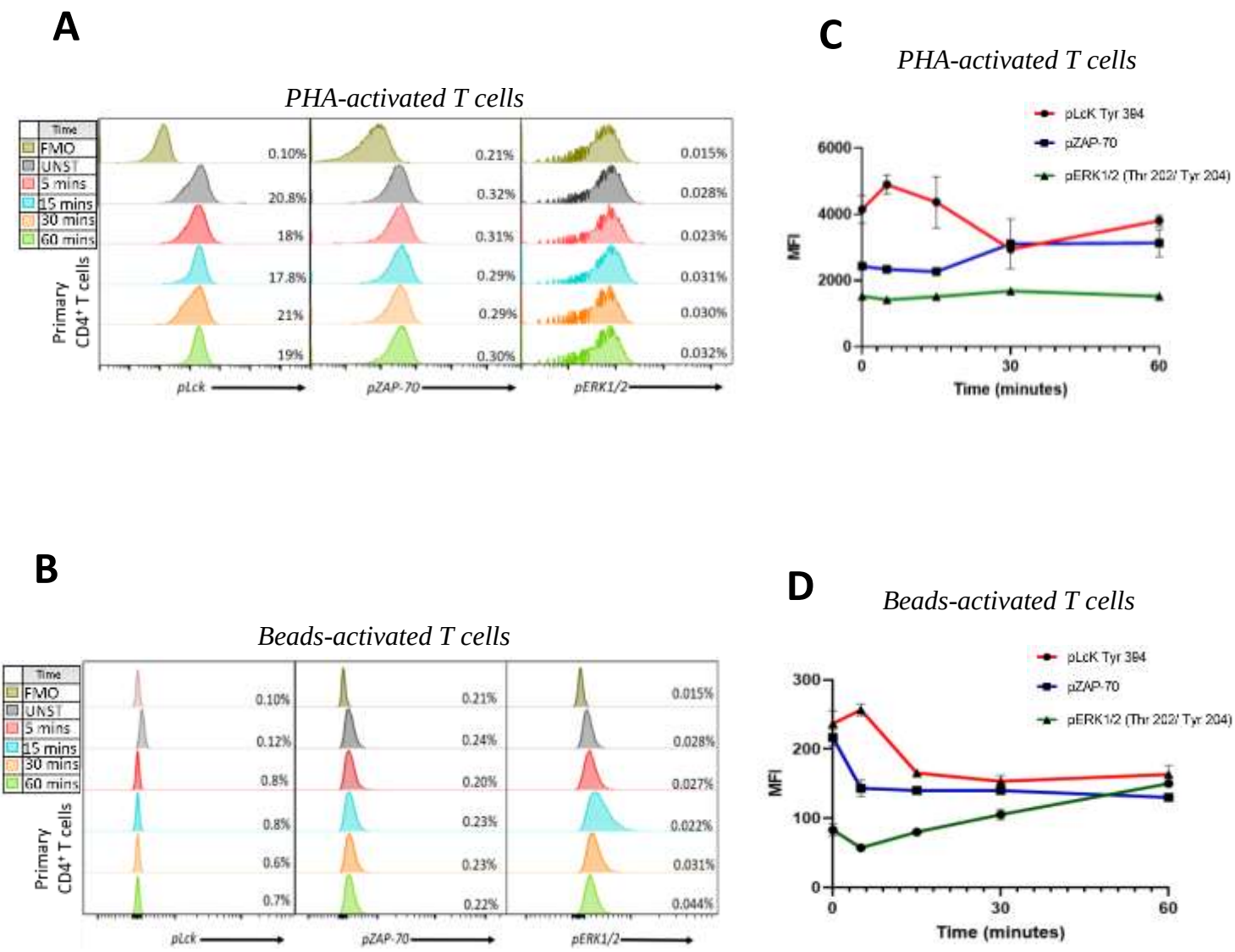
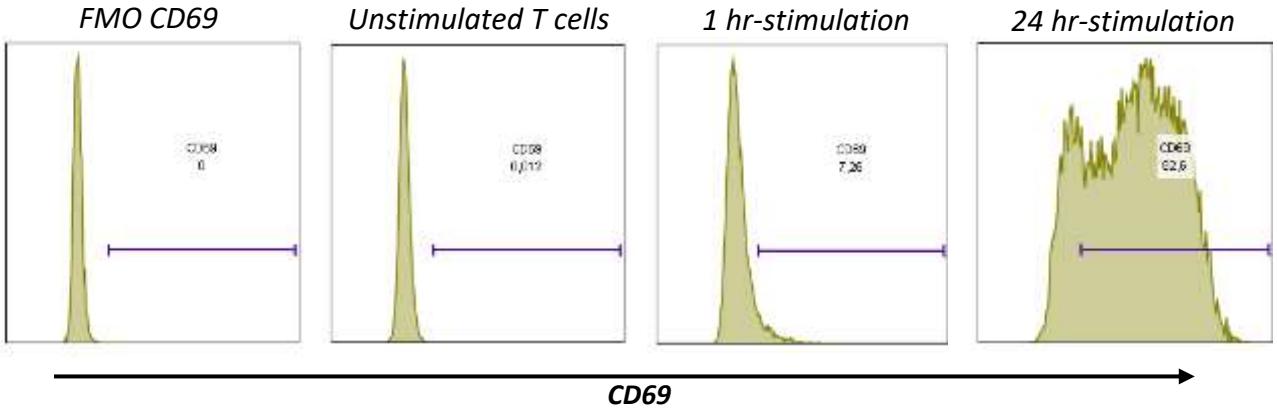


Figure 6. Analysis of primary CD4⁺ T cell kinase pathways using phospho-flow cytometry.

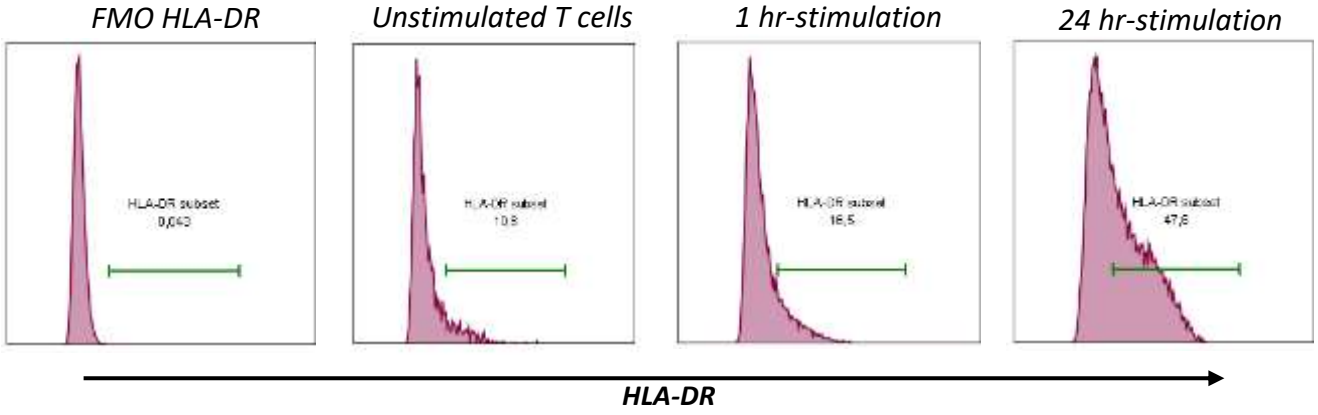
(A-B) CD4⁺ T cells were incubated with either PHA (10 µg/mL) or anti-CD3/CD28 beads (25 µL/mL) for the indicated times and then were fixed with 2% paraformaldehyde for 45 mins. Cells were then permeabilized with methanol and stained for phospho-Lck, phospho-ZAP-70, and phospho ERK 1/2. Kinase phosphorylation was measured by flow cytometry. Data from three independent experiments are shown. Unstimulated T cells served as a negative control and Fluoresce Minus One (FMO) was used to set the proper flow gating.

(C-D) Mean Fluorescent Intensity (MFI) of the phosphorylated kinases in PHA-stimulated T cells and bead-stimulated T cells are displayed. Black bars represent mean ± SEM.

A


FMO CD69
Unstimulated T cells
1 hr-stimulation
24 hr-stimulation
CD69

B


FMO HLA-DR
Unstimulated T cells
1 hr-stimulation
24 hr-stimulation
HLA-DR

C

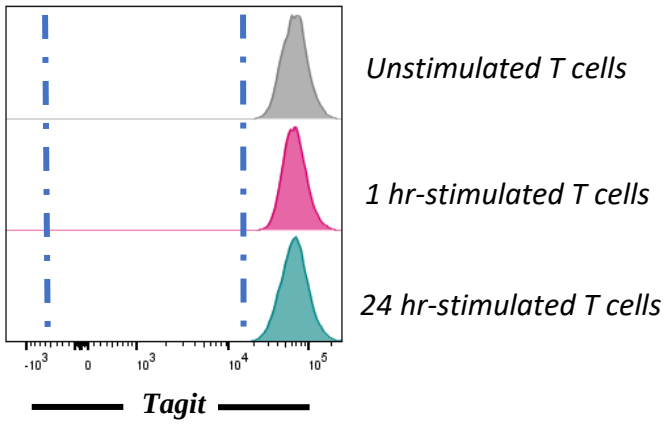

Unstimulated T cells
1 hr-stimulated T cells
24 hr-stimulated T cells
Tagit

Figure 7. Expression of CD69 and HLA-DR increased on primary PHA-stimulated $CD4^+$ T cells.

(A-B) Healthy $CD4^+$ T cells were stimulated with PHA (10 µg/mL) and incubated for 1 hr and 24 hrs. Cells were stained for CD69 and HLA-DR and fixed for flow cytometry analysis. Example histograms show CD69 and HLA-DR expression from primary $CD4^+$ T cells. Unstimulated T cells were used as a negative control. Data are shown from two independent experiments.

(C) $CD4^+$ T cells were stimulated with PHA (10 µg/mL) for 1 hr and 24 hrs and labelled with Tagit proliferation dye for 20 mins. Cells were then washed and fixed for flow cytometry analysis. Unstimulated T cells were used as a negative control. The data are shown form two independent experiments.

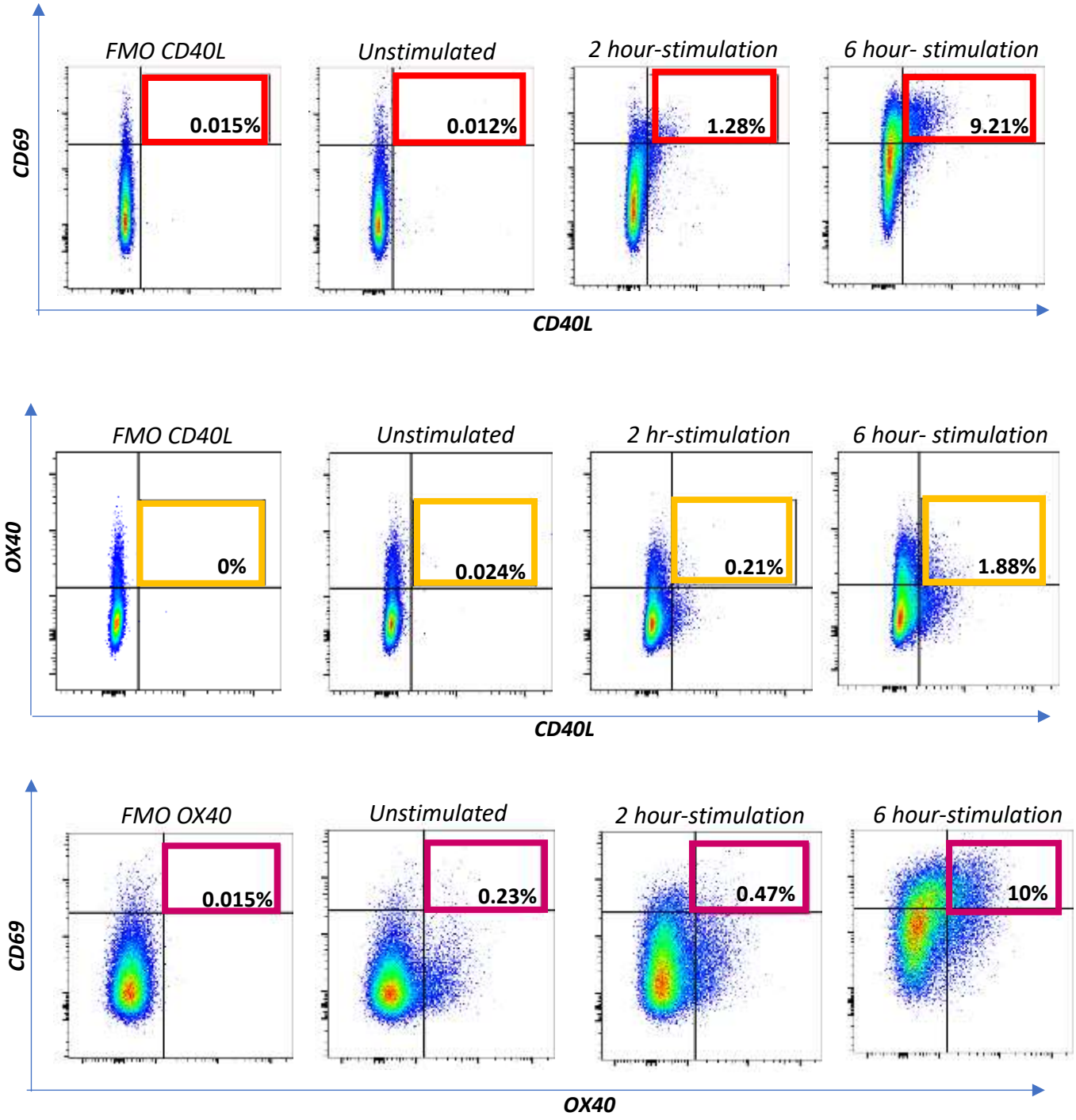
3.3 Detection of antigen-responsive CD4$^+$ T cells using TCR-induced activation markers

Common methods such as enzyme-linked immune absorbent spot (ELISpot) or intracellular Cytokine Staining (ICS) could underestimate the frequency of antigen-responsive T cells[320]. More recently, it was reported that the frequency of antigen-specific T cells could be evaluated by the upregulation of TCR-induced surface activation markers, termed the activation-induced markers (AIM) assay [321, 322]. To determine whether this approach can identify activated T cells with better sensitivity, CD4$^+$ T cells were stimulated with 10 µg/mL of PHA at various time durations. Thereafter, co-expression of T cell surface activation markers was assessed by flow cytometry. I focused on the markers CD69, OX40, CD40L based on previous studies that described these receptors as early indicators of T cell activation [323]. After PHA stimulation, CD69$^+$ CD40L$^+$, CD69$^+$OX40$^+$, and OX40$^+$CD40L$^+$ T cell populations increased gradually after 24 hours (Figure 8A & B). As shown in Figure 8A, the background expression levels of these assays in day seven expanded primary CD4$^+$ T cells were consistently low. However, the frequency of OX40$^+$ CD40L$^+$ T cells was very low after 24 hours of stimulation (Figure 8B). Notably, CD40L gets internalized after interaction with its ligand CD40 on DCs; therefore, this marker was later replaced with PD-L1 which was more stable.

Next, I performed AIM after DC: T cell coculture in the presence of antigens. *In vitro* expanded CD4 T cells were found to have differential expression of CCR7 and CD45RA (Figure 9A), indicating the existence of various T cell subpopulations in culture. Monocyte-derived dendritic cells (MDDCs) were expanded for 5 days (immature DCs), and DCs were matured by treating them with LPS. As expected, both mature and immature DCs uniformly expressed high levels of CD11c (Figure 9B). Immature DCs were defined as lows expression of co-stimulatory molecules (CD80, CD83 and CD86) relative to LPS-stimulated DCs (Figure 9B), confirming both DC phenotypes. To further optimize the AIM assay for detecting antigen-responsive CD4$^+$ T cells, I used CPI protein pool (CMV, parainfluenza and Influenza; 6µg/mL) or CMV infected lysate (10 µg/mL) that require natural antigen processing and presentation by DCs, and induce polyclonal responses by T cells. Both mature and immature DCs were first pulsed with various protein pools for 2 hours at 37°C. Lysates from uninfected cells served as a negative control, and *Staphylococcal* enterotoxin B (SEB) used as a positive control in these studies. Without washing, DCs were then

cocultured with autologous $CD4^+$ T cells for 24 hours. Flow cytometry AIM analysis demonstrated that both DC populations efficiently upregulated CD69/OX40, CD6/PD-L1 and OX40/ PD-L1 double positive populations on $CD4^+$ T cells compared to uninfected CMV lysate. OX40/PD-L1 gave the strongest signal, whereas CD69/ PD-L1 gave the weakest (Figure 10A & B). I observed that imDCs induce more activation of the AIM assay than mature DCs, likely because imDCs are more phagocytic and these viral proteins undergo more efficient processing and presentation in imDCs compared to their mature counterparts. Furthermore, $CD4^+$ T cells response to CPI protein pool is higher compared to CMV cell lysate alone. Altogether, I concluded that AIM assay can successfully detect antigen-specific responses in T cells using imDCs in coculture studies.

A


FMO CD40L
Unstimulated
2 hour-stimulation
6 hour- stimulation
0.015%
0.012%
1.28%
9.21%
CD69
CD40L
FMO CD40L
Unstimulated
2 hr-stimulation
6 hour- stimulation
0%
0.024%
0.21%
1.88%
OX40
CD40L
FMO OX40
Unstimulated
2 hour-stimulation
6 hour-stimulation
0.015%
0.23%
0.47%
10%
CD69
OX40

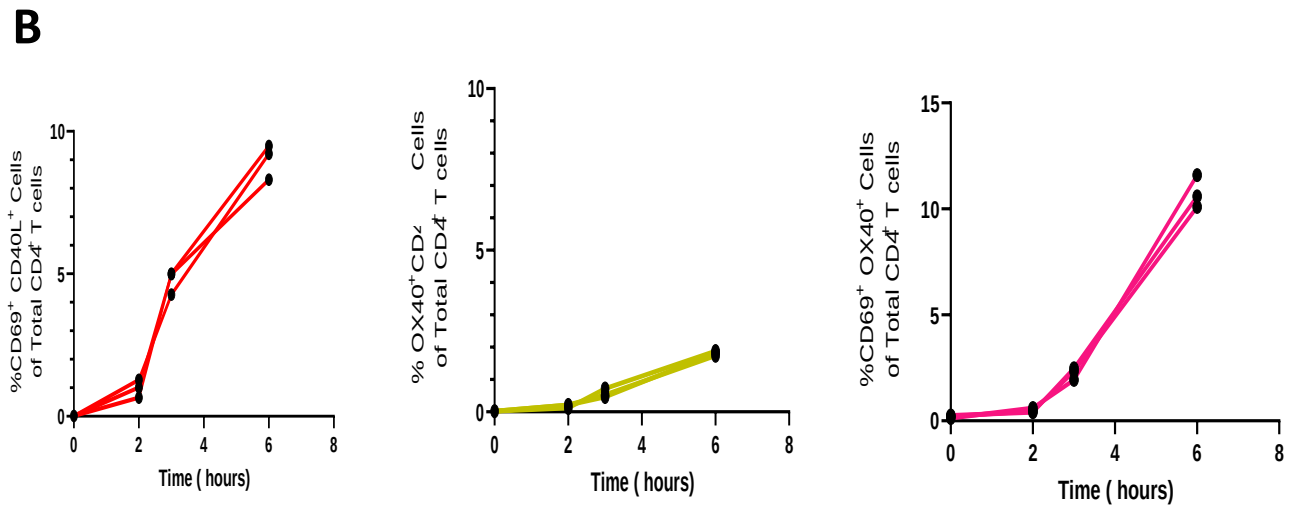
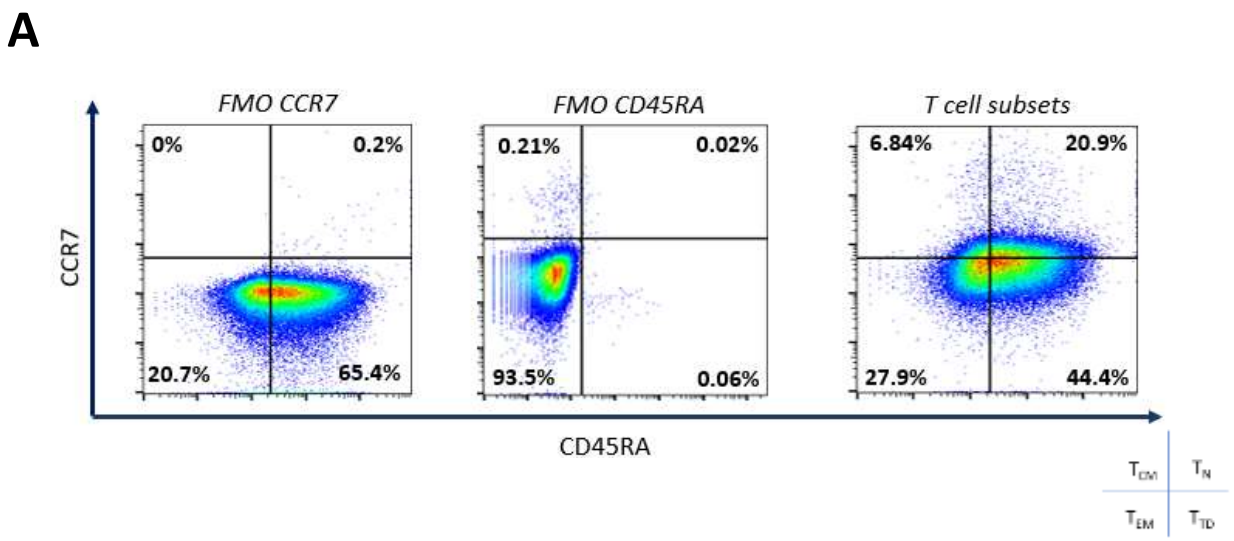


Figure 8. AIM assay optimization in PHA-stimulated primary CD4$^+$ T cells.

(A) Day 7 expanded CD4$^+$ T cells were stimulated with PHA (10 µg/ml) for 2 and 6 hrs. Cells were stained for AIM markers (CD69, OX40 and CD40L) and analyzed by flow cytometry. Example flow cytometry plots of CD69$^+$CD40L$^+$ (red), OX40$^+$CD40L$^+$ (yellow) and CD69$^+$ OX40$^+$ (pink) expression from primary CD4$^+$ T cells. Data from three independent experiments are shown.

(B) Quantification of activated T cells of triplicates from one experiment is shown. Each line indicates an individual replicate.



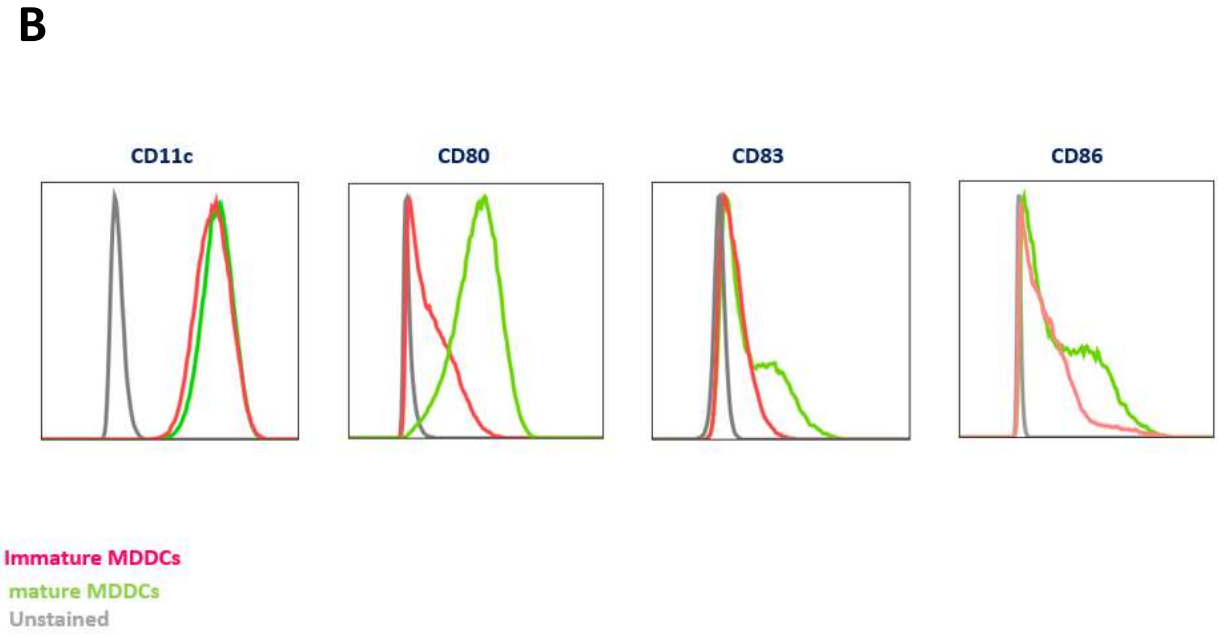


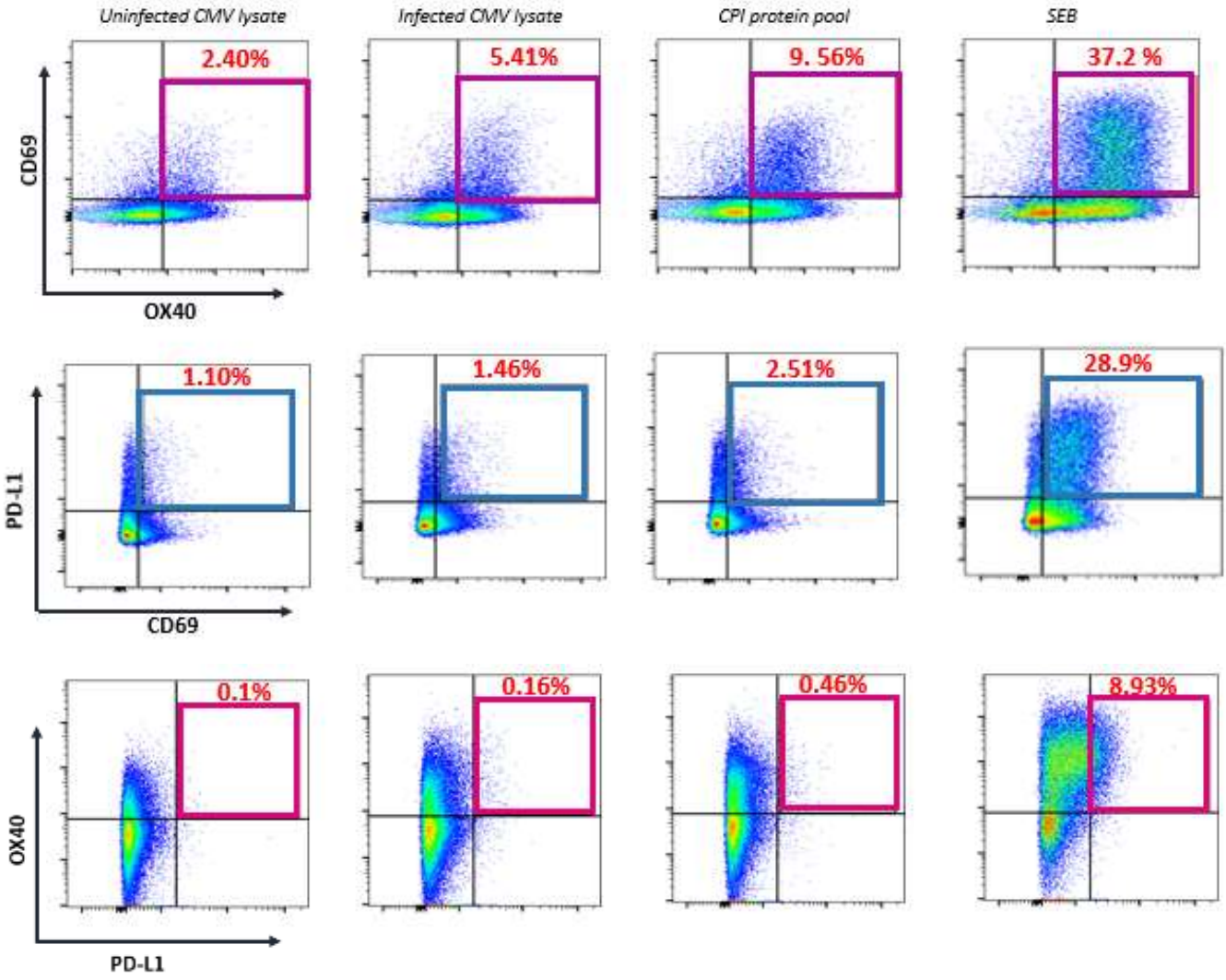
Figure 9. Phenotypic analysis of DCs and CD4$^+$ T cells before in vitro co-culture analysis.

(A) CD4$^+$ T cell subsets were characterized based on the expression of CCR7 and C45RA. Naïve T cells (T_N) are identified as CCR7$^+$ CD45RA$^+$; Central memory (T_{CM}) are CCR7$^-$ CD45RA$^+$; effector memory (T_{EM}) are CCR7$^-$ CD45RA$^-$; transitionally differentiated (T_{TD}) are CCR7$^-$ CD45RA$^+$. FMO was used for the proper flow cytometry gating.

(B) On day 5, monocyte derived dendritic cells (MDDCs) were stimulated with LPS (100 ng/mL) and incubated for 24 hrs. The activation markers, CD80, CD83 and CD86 were compared in LPS-stimulated (green) and immature DCs (red). The flow gating was set on $CD11c^{+}$ DC population.

A

Mature DC : T cell interactions



B

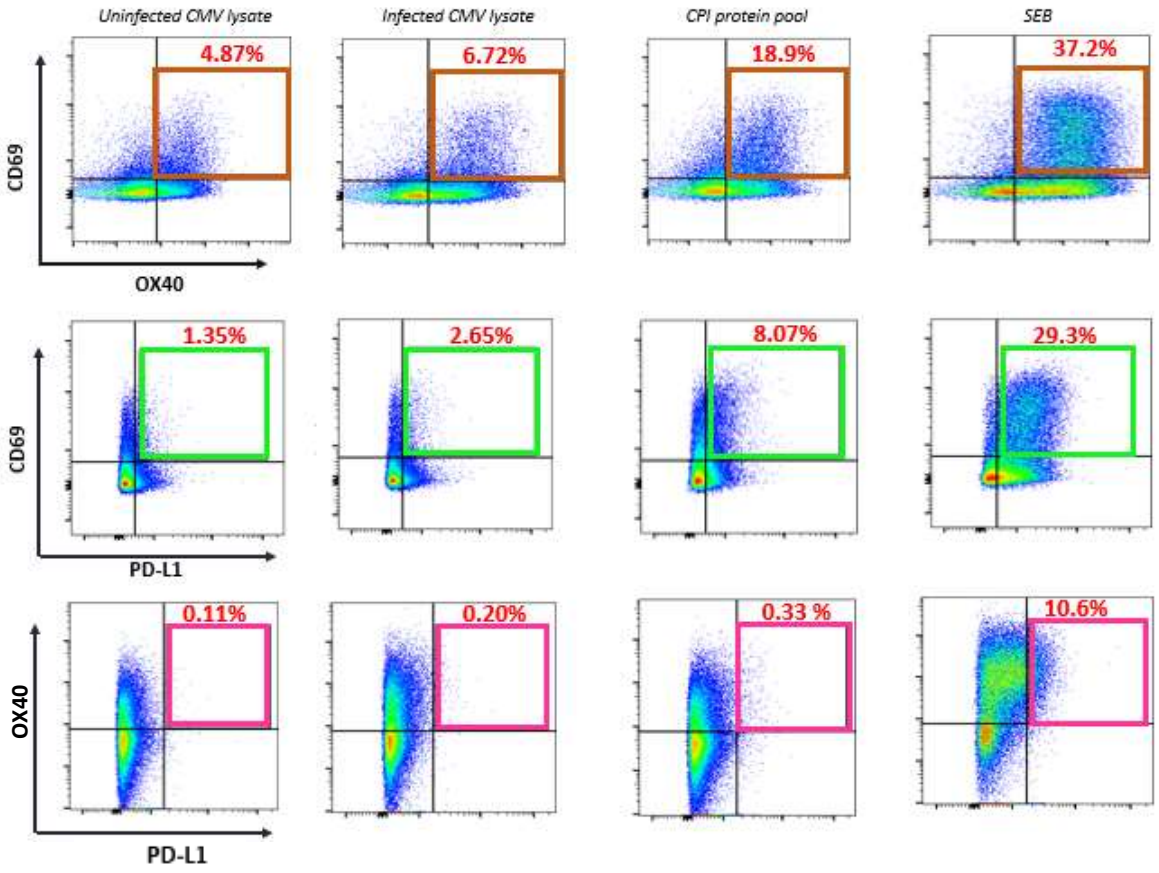


Figure 10. Comparison of AIM markers on T cells after *in vitro* DC: T cell co-culture in the presence of viral antigens

(A-B) Representative flow example plots of $CD69^+$ $OX40^+$, $CD69^+$ $PD\text{-}L1^+$ and $OX40^+$ $PD\text{-}L1^+$ expression from primary $CD4^+$ T cells following 24 hr co-culture with mature (A) or immature (B) DCs. Briefly, both LPS-stimulated and immature DCs were pulsed with infected CMV lysate (10 ug/mL) and co-cultured with autologous day 7 activated T cells. The frequency of antigen-specific $CD4^+$ T cells was measured by flow cytometry. The data shown is representative of three independent experiments.

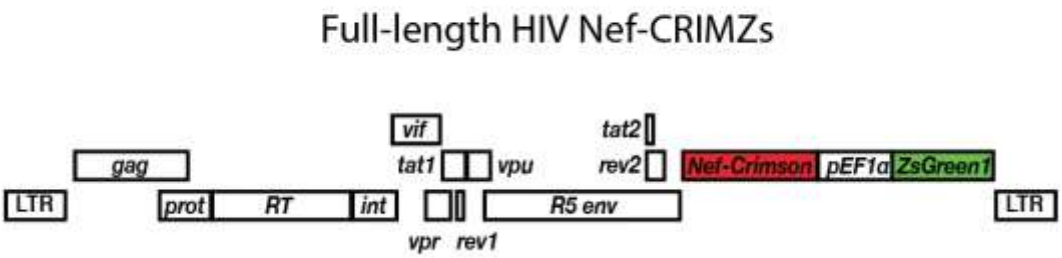
3.4 DCs drive the expansion of the HIV reservoir

While my initial focus was on evaluating T cell signaling during DC: T cell encounters during viral transmission events, I decided to apply the AIM assay and similar techniques to address a different but important question in HIV biology: the maintenance of HIV latency in ART-treated individuals. HIV latency is defined as the stably-integrated provirus in the host's genome which is transcriptionally silent and thus, it can efficiently evade immune clearance or antiretroviral drugs[37, 324]. Previous studies showed that myeloid DCs and monocytes could establish latency in both proliferating and non-proliferating $CD4^+$ T cells [269, 271]. More recent studies have demonstrated that the HIV reservoir is found in T cell clones, indicating that T cells with certainly antigen specificities preferentially carried HIV provirus. Therefore, using the AIM assay, I tested the hypothesis whether DC-mediated expansion of T cell latency would occur in an antigen-specific manner. To test this, dual-fluorescent latency reporter, referred to as HIV- Nef-CRIMZY, containing ZsGreen under the control of elongation factor 1α (EF1α) and Nef-Crimson fusion protein under the control of HIV-LTR was developed in the lab (Figure 11A). I assessed the capacity of our HIV-Nef-CRMZY to infect primary $CD4^+$ T cells and enumerated the number of infected $CD4^+$ T cells at day 5 post-infection using the gating strategy showed in Figure 11B. I observed both productively-infected and latently-infected cells, defined as $Crimson^+$ $ZsGreen^+$ $CD4^-$, and $Crimson^-$ $ZsGreen^+$, respectively (Figure 11C). Interestingly, we observed a dual population of latently-infected $CD4^+$ T cells ($CD4^+$ $p24^-$ and $CD4^-$ $p24^+$ cells) (Figure 11D). Therefore, we further identified the latent population as $ZsGreen^+$ $Crimson^+$ $CD4^+$ $p24^-$ and productive population as $ZsGreen^+$ $Crimson^+$ $CD4^-$ $P24^+$. As expected, the number of productive infections was lower than the latent infections across three different donors (Figure 11E), as the virus causes cytopathic effect on T cells, reducing the number of productively-infected cells over time[230].

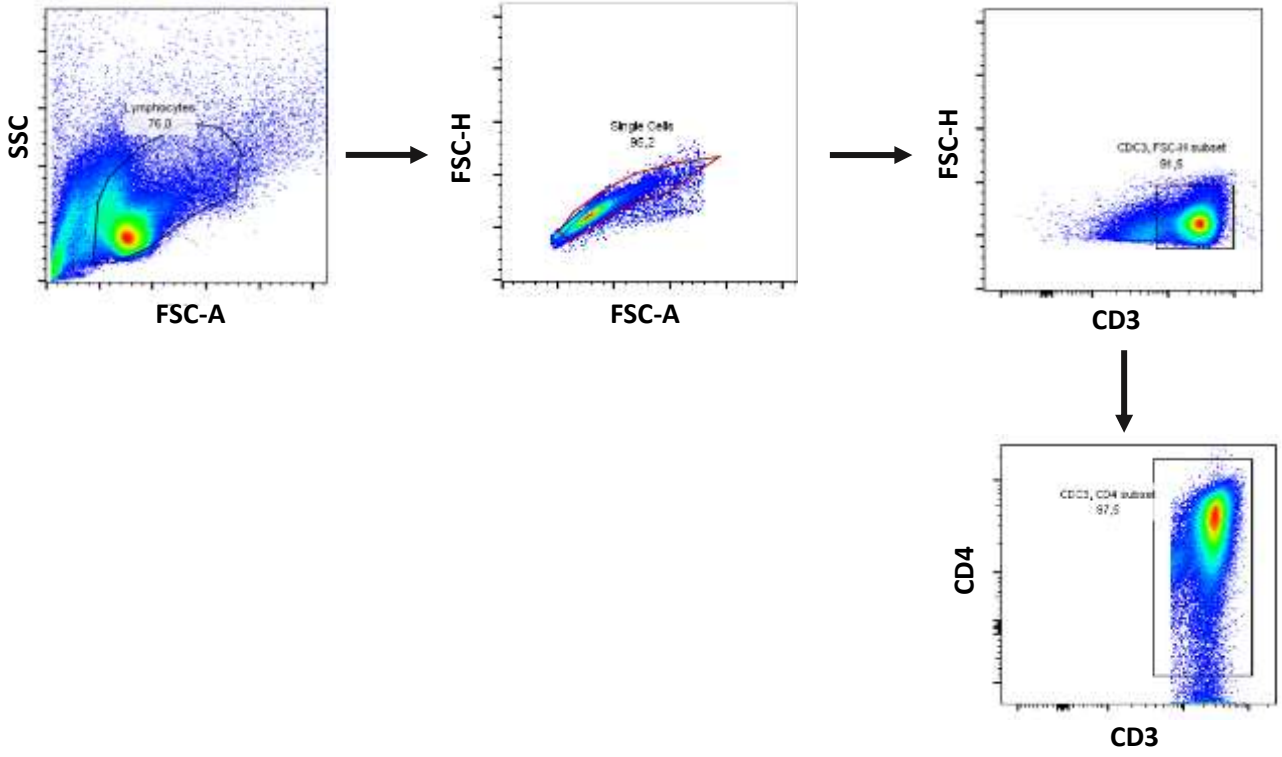
As shown in figure 12A, at day 5 post-infection, T cells were cultured alone or in the presence of immature MDDCs (imDCs) for 8 days (13 days post-infection) with IL-2 to maintain cell viability. Integrase inhibitor raltegravir was included from day 5 post-coculture to block the spread of infection and to allow the quantification of latent population for at least 8 days. I used CD4 surface marker to identify T cells in the coculture and to clearly characterize the true latent population, as described above. Interestingly, I observed that the proportion of latently-infected $CD4^+$ T cells in the presence of imDCs was significantly higher compared to latent T cells alone

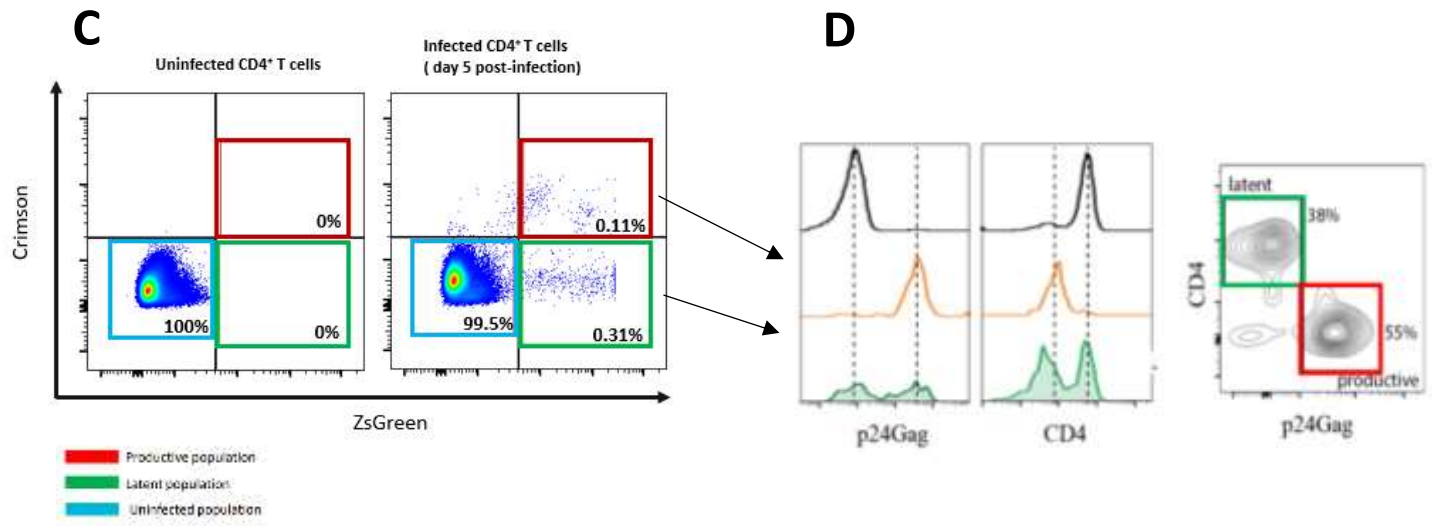
(56.9% versus 85.5%) (Figure 12B & C). At the same time, we observed that $CD4^-$ $ZsGreen^+$ population diminished from 39.7% to 18.9% in the presence of DCs. Together, these data support the idea that DCs can expand a pool of cells harboring HIV in our DC: T cell co-culture system, possibly by providing T cells with survival and proliferative signals.

A


Full-length HIV Nef-CRIMZs
vif
tat2
gag
tat1
vpu
rev2
Nef-Crimson
pEF1α
ZsGreen1
LTR
prot
RT
int
R5 env
LTR
vpr
rev1

B


SSC
FSC-A
FSC-H
FSC-A
FSC-H
CD3
CD4
CD3



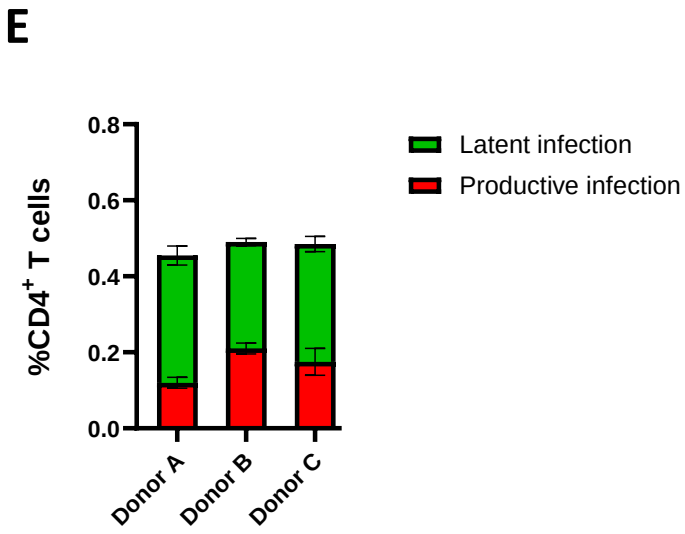


Figure 11. Characterization of primary CD4⁺ T cells infected with HIV-Nef-CRIMZY reporter.

(A) Schematic illustration of the HIV-Nef-CRIMZY reporter vector.

(B) Gating strategy for identification of productive and latent populations. The cells of interest were gated onto a forward scatter (x-axis) versus side scatter (y-axis) plot and then singlet cells

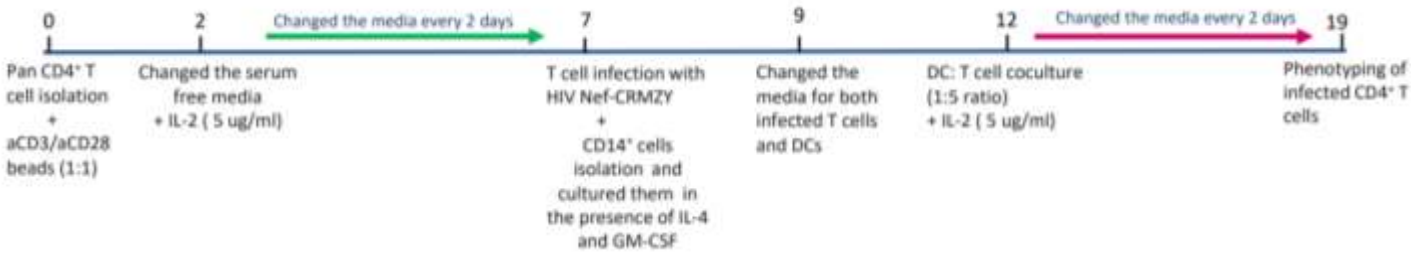
were identified. From CD3 positive cell population, CD4 positive cells were identified to describe $CD3^+$ $CD4^+$ T cells.

(C) Representative dot plots of uninfected T cells and HIV-infected T cells gated on Crimson and ZsGreen. The uninfected T cells are identified as $Crimson^-$ $ZsGreen^-$; productive infection as $Crismon^+$ $ZsGreen^+$ and latent infection as $Crimson^-$ $ZsGreen^+$. The data are shown as the frequency of uninfected, productive and latent populations in each plot. Representative plots of three independent experiments are shown.

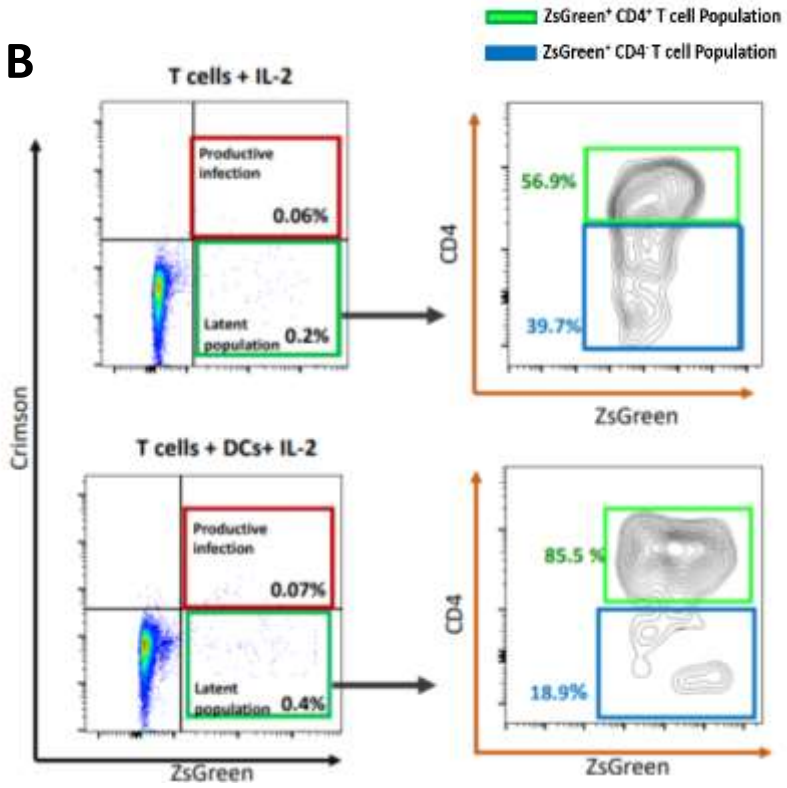
(D) Histograms of CD4 and HIV-iGag (p24) expression in uninfected, productive and latent T cell populations.CD4 expression was used to identify truly latent population ($ZsGreen^+$ Crimson– $CD4^+$ $p24^-$).

(E) Comparison of productive (red bar) and latent (green bar) populations among three different donors. Black bars represent mean ± SEM.

A


0
2
Changed the media every 2 days
7
9
12
Changed the media every 2 days
19
Pan CD4+ T cell isolation + aCD3/aCD28 beads (1:1)
Changed the serum free media + IL-2 (5 ug/ml)
T cell infection with HIV Nef-CRMZY + CD14+ cells isolation and cultured them in the presence of IL-4 and GM-CSF
Changed the media for both infected T cells and DCs
DC: T cell coculture (1:5 ratio) + IL-2 (5 ug/ml)
Phenotyping of infected CD4+ T cells

B


ZsGreen+ CD4+ T cell Population
ZsGreen+ CD4- T cell Population
T cells + IL-2
Productive infection
0.06%
Latent population
0.2%
56.9%
39.7%
CD4
ZsGreen
T cells + DCs+ IL-2
Productive infection
0.07%
Latent population
0.4%
85.5 %
18.9%
CD4
ZsGreen
Crimson
ZsGreen

C

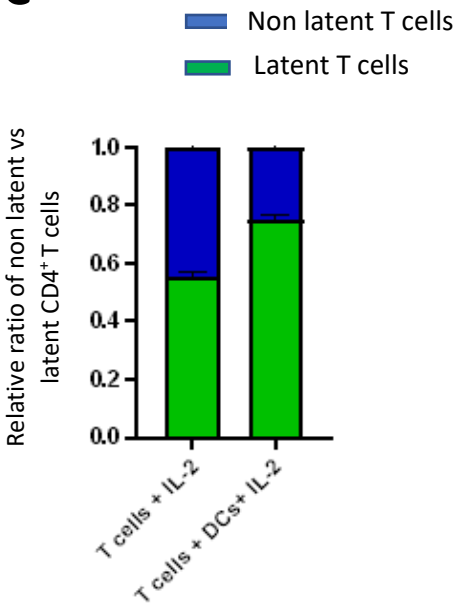

Non latent T cells
Latent T cells
Relative ratio of non latent vs latent CD4+ T cells
1.0
0.8
0.6
0.4
0.2
0.0
T cells + IL-2
T cells + DCs+ IL-2

Figure 12. DCs expand latently-infected $CD4^+$ T cells in a contact-dependent manner.

(A) Experimental design of DC: T cell co-culture in the presence of HIV Nef-CRIMZY.

(B) At day 5 post-infection, infected $CD4^+$ T cells were co-cultured in the presence or absence of DCs at a ratio of 1:5 (DC/T cell). At day 8 post-coculture, the number of latently-infected $CD4^+$ T cells were measured by flow cytometry. CD4 expression was used to identity the proper latent population ($ZsGreen^+$ $Crimson^-CD4^+$). The percentages show the frequency of each cell population.

(C) The relative ratio of the latently-infected $CD4^+$ T cells ($ZsgGreen^+$ $Crimson^-$ $CD4^+$ $p24^{-)}$ co-cultured with DCs compared to non-latent T cells ($ZsGreen^+$ $Crimson^-$ $CD4^-$ $p24^+$) among two different donors.

3.4.1 DCs induce proliferation of latent T cells during DC: T cell co-culture

DC-mediated increase in the proportion of latent T cells populations can be due to dying of productively infected cells or proliferation of latent T cells. To explore this, I performed similar DC: T cell coculture studies, but with Tagit proliferation dye. At day 5 post-infection, primary $CD4^+$ T cells were first labelled with proliferation dye and then cultured alone or in the presence of DCs. 8 days following co-culture, I measured the discrete proliferation peaks of latent T cells by flow cytometry. Of note, I used anti-CD3/ CD28 and SEB as positive controls for these studies. Tagit dilution histogram data was separated from zero (no proliferation) to 3 (high proliferation). High proliferation was defined as T cells that have completed significant cell division and lost Tagit signal, while "low proliferation" was defined as cells that have undergone a few division cycles.

Interestingly, after gating on latent population ($ZsGreen^+$ $Crimson^-$ $CD4^+$), my results revealed that DCs induced both high proliferation (13.8%) and low proliferation (18.5%) of latently-infected $CD4^+$ T cells. In contrast, no significant proliferative response of latent T cells in culture alone was observed (Figure 13A). These studies are consistent with my hypothesis that DCs provide proliferative signals to latently-infected T cells, and help maintain their latent state over time. As expected, SEB caused the significant expansion of latent T cells (Figure 13B), since SEB crosslinks MHC class II on DCs with TCR V beta chains leading to excessive cell T cell activation and proliferation [325-327]. In contrast, stimulation of latent T cells with anti-CD3/CD28 did not cause dramatic proliferation of the latent T cells (Figure13B), indicating that some of the HIV proviruses may have been activated in response to stimulation and undergone apoptosis. Together, these data demonstrate that the presence of DCs leads to some proliferation of latently-infected $CD4^+$ T cells in the absence of any added antigens.

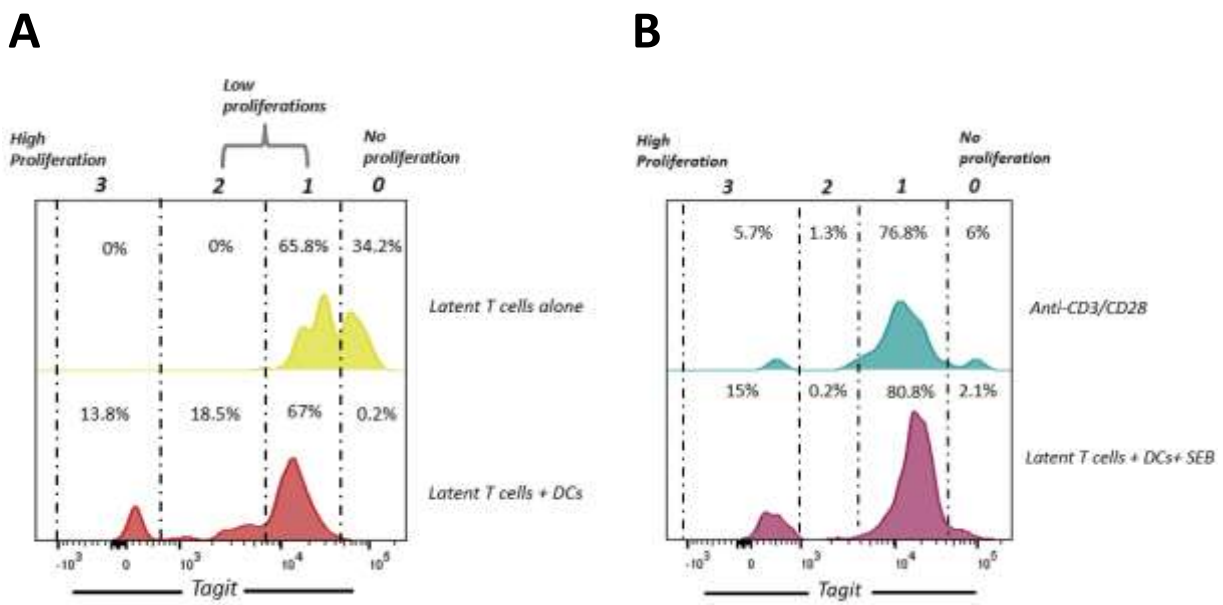


Figure 13. The presence of DCs drive proliferation of latently-infected CD4$^+$ T cells *in vitro.*

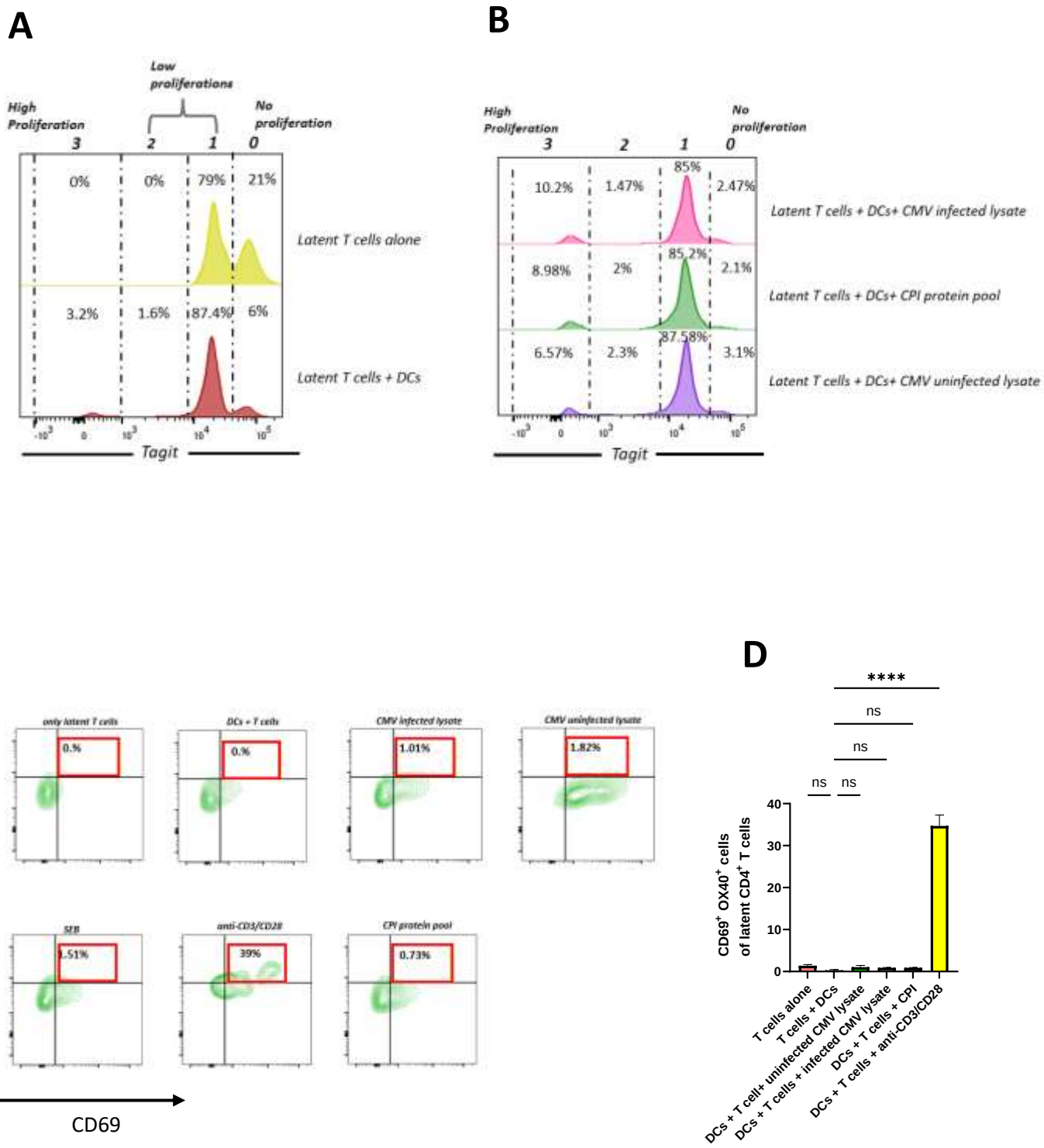
(A-B) CD4$^+$ T cells infected with HIV-Nef-CRIMZY were first labelled with Tagit proliferation dye. T cells were then co-cultured in the presence or absence of DCs at ratio of 1: 5 (DC/T cell) together with the indicated stimulus. The representative histograms indicate latent T cells (ZsGreen$^+$ Crimson$^-$ CD4$^+$) that have undergone low and high proliferation at day 8 post-coculture. The indicated percentages represent the number of the low and high proliferated latently-infected CD4$^+$ T cells. Anti-CD3/CD28 and staphylococcus enterotoxin B (SEB) were used as positive controls. Representative histograms of two independent experiments are shown.

3.4.2 DCs cause the proliferation of antigen-specific T cells harboring HIV reservoir

While DCs in the absence of antigen induced some proliferation of latent T cells, we next examined whether the addition of microbial antigen can further expand this latent population in DC: T cell co-culture. This rational is based on the fact that clonal expansion of latently-infected T cells is observed in ART-suppressed individuals, some of which are specific for microbial antigens such as CMV, EBV, and influenza [313, 314]. For this, we again utilized CMV-infected cell lysate or CPI (CMV, parainfluenza, and influenza) protein pools that require uptake, processing, and presentation by immature DCs. To look at T cell response in DC: T cell co-cultures, I used both AIM and Tagit proliferation dye assays to measure antigen-dependent responses in latent $CD4^+$ T cells. In this donor, no proliferation was detected in the T cells alone condition after 8 days co-culture (Figure 14A & B). In contrast, a modest increase in latent T cell proliferation was detected in the presence of autologous DCs without adding antigens, which is consistent with our previous experiment (Figure 13A). We observed a significant increase in the percentage of T cells undergoing "high proliferation" in the presence of CMV-derived proteins and CPI protein pool after 8 days of co-culture (Figure 14B). Similar results were obtained in three independent experiments from different donors, where both antigen-dependent and -independent mechanisms were stimulating T cell proliferation, consistent with previous reports by Vollbrecht *et al* [328].

The AIM assay was used to evaluate whether T cell responses were detectable in DC: T cell co-cultures. I specifically focused on CD69 and OX40, since these two markers in tandem gave me a robust reading of recent TCR stimulation. However, there was little to no upregulation of $CD69^+OX40^+$ population in $CD4^+$ $ZsGreen^+$ cells, which did not correlate with proliferation results. These data indicate that the AIM assay may not be sensitive enough to detect antigen-specific responses in my hands (Figure 14C & D). Subsequently, we looked at the expansion of the T cell reservoir in response to other microbial antigens such as EBV, *E-coli*- and *S.Aureus.* Interestingly, in this donor, a strong proliferation of latent T cells in the presence of DCs was observed in the absence of microbial antigens, which was a significantly different response compared to previous studies (Figure 14 E & F). This precluded our ability to assess the role of microbial antigens in driving latent T cell proliferation in this donor, although strong proliferative

responses were observed in the presence of *E.coli*. These data indicated that T cell responses to antigen were highly variable between donors, which needs to be considered in the future. Together, I show some data that antigen-pulsed DCs can lead to the expansion of the latent reservoir *in vitro*.


A
Low proliferations
High Proliferation
No proliferation
3
2
1
0
0%
0%
79%
21%
Latent T cells alone
3.2%
1.6%
87.4%
6%
Latent T cells + DCs
Tagit
B
High Proliferation
No proliferation
3
2
1
0
10.2%
1.47%
85%
2.47%
Latent T cells + DCs+ CMV infected lysate
8.98%
2%
85.2%
2.1%
Latent T cells + DCs+ CPI protein pool
6.57%
2.3%
87.58%
3.1%
Latent T cells + DCs+ CMV uninfected lysate
Tagit
C
only latent T cells
DCs + T cells
CMV infected lysate
CMV uninfected lysate
0.%
0.%
1.01%
1.82%
SEB
anti-CD3/CD28
CPI protein pool
1.51%
39%
0.73%
OX40
CD69
D
****
ns
ns
ns
ns
CD69+ OX40+ cells of latent CD4+ T cells
40
30
20
10
0
T cells alone
T cells + DCs
DCs + T cell+ uninfected CMV lysate
DCs + T cells + infected CMV lysate
DCs + T cells + CPI
DCs + T cells + anti-CD3/CD28

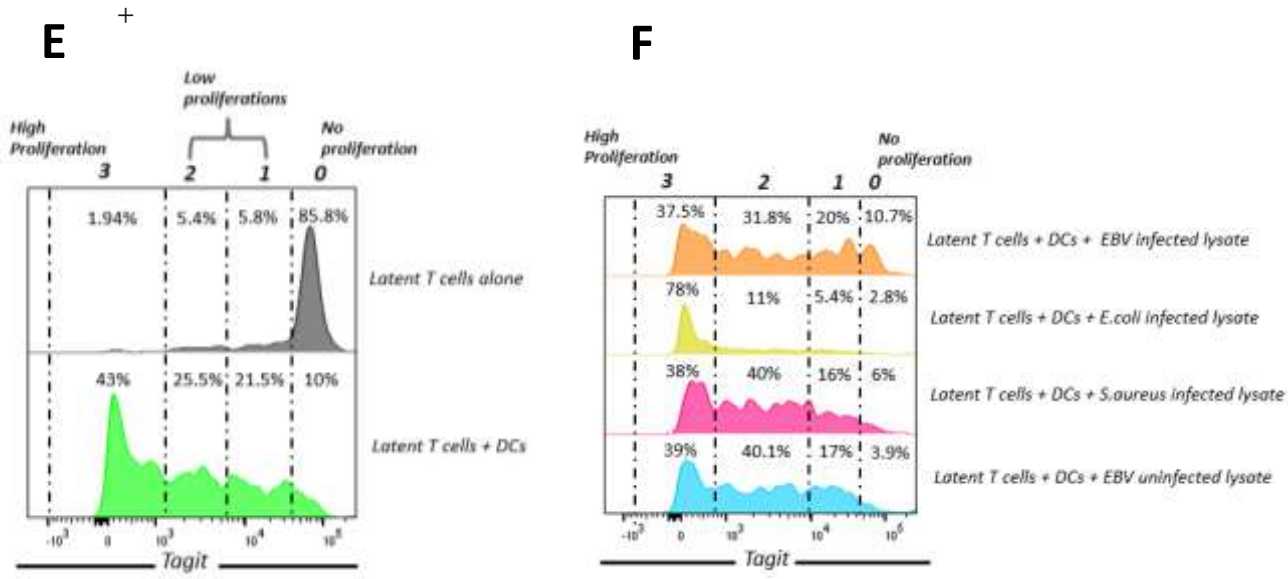


Figure 14. DCs induce proliferation of latently-infected CD4$^+$ T cells in both antigen-dependent and -independent manner.

(A) At day 5 post-infection, DCs were first pulsed with CPI protein pool (6 ug/mL) or CMV cell lysate (10 µg/mL) for 2 hrs. Pulsed-DCs were co-cultured with HIV-Nef-CRIMZY-infected CD4$^+$ T cells that were previously labelled with Tagit proliferation dye. The proliferation of the latent population was quantified at 8 days post-coculture by flow cytometry. Uninfected CMV lysate was used as a negative control. The percentage show the high and low proliferation of latent T cell population.

(B) Representative Flow cytometry plots of AIM assay (OX40$^+$/CD69$^+$) within latently-infected CD4$^+$ T cells

(C) Percentages of AIM$^+$ responses in the triplicate samples. One-way Anova test was performed. n.s., not significant. $^{****}p < 0.001$. Mean ± SEM are shown.

(D) Analysis of latent T cell proliferation in the presence of bacterial *(E.coli* and *S.aureus*) and EBV lysates at day 8 post-coculture with immature DCs.

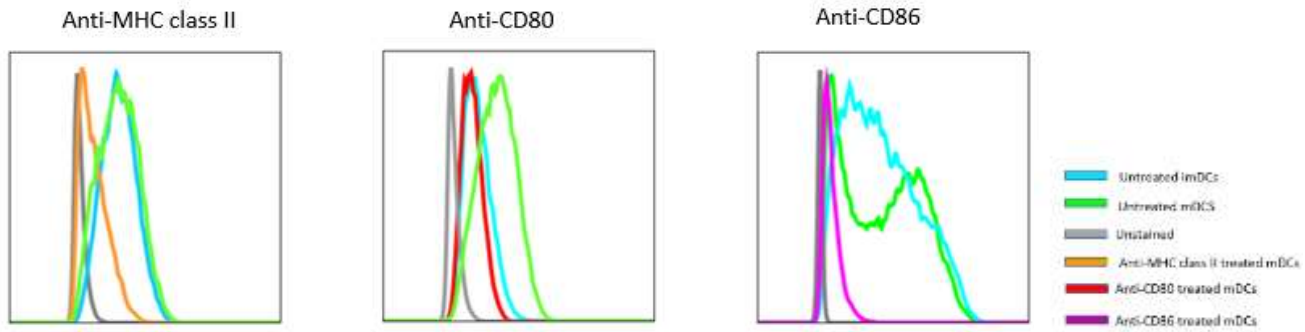
3.4.3 CD80/CD86 blockade abrogates the proliferation of latently-infected CD4+ T cells

With the findings that DC: T cell cocultures led to a progressive increase in the frequency of latent T cells in an antigen-dependent and independent manner (Figure 14A & B), I hypothesized that cell-cell contact was required for this process. Alternatively, soluble cytokines produced by DCs were responsible for T cell proliferation in our assay. I first assessed the requirement of DC: T cell contacts in inducing latent T cell proliferation in our assay. I specifically focused on both TCR: MHC II complex and CD28: CD80/CD86 interactions. I tested a panel of neutralizing antibodies that target MHC-II, CD80 (B7-1), and CD86 (B7-2) molecules. In order to confirm that the blocking antibodies specifically bound to their target molecules, mature DCs with high CD80, CD86, and MHC-II expression, was stained with the indicated neutralizing or isotype control antibodies at 10 μg/mL for 30 mins. Without washing, DCs were then stained with fluorescent-conjugated antibody of the same clone for flow cytometry analysis. As shown in Figure 15A, all blocking antibodies tested reduced expression of their corresponding molecules in both immature and mature DCs. After confirming blocking efficiencies of neutralizing antibodies, I performed DC: T cell co-culture studies in the presence or absence of MHC II, CD80 and CD86 blockade. In these studies, DCs were pulsed with CPI protein pool, then co-cultured with $CD4^+$ T cells previously infected with HIV-Nef-CRIMZY. Of note, I focused on CPI protein pool as our model antigen because a strong T cell proliferative response was observed in my hands. Furthermore, the fact that protein pools from three viruses meant that a broad T cell responses was being measured in my assays.

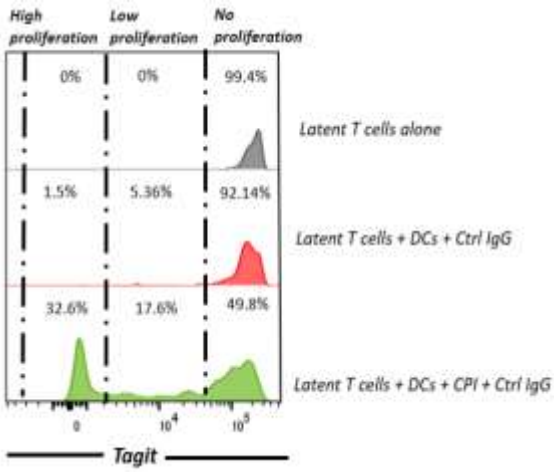
In this donor, DC: T cell co-cultures pulsed with CPI induced strong proliferation of latent T cells (32.6% “high proliferation”, and 17.6% “low proliferation”) while minimal proliferation was observed in the absence of antigen (Figure 15B). Unexpectedly, dual blockade of CD80 and CD86 completely blocked proliferation of latently-infected $CD4^+$ T cells. Furthermore, blocking all MHC II, CD80/CD86 and ICAM-1 limited the proliferation of latently-infected T cells in the presence of CPI protein pool (Figure 15D). However, MHC-II or ICAM-1 single blockade did not suppress T cell proliferation in the presence of viral antigens (Figure15C). Interestingly, I also observed that CD80/CD86 blockade suppressed the proliferation of uninfected

$CD4^+$ T cells ($ZsGreen^-$ $Crimson^-$) (Figure 15E), indicating that CPI also led to the expansion of uninfected T cells in my co-culture experiments. In summary, these data suggest that costimulatory signals through CD28: CD80/CD86 interactions is a crucial driver of DC-mediated proliferation of latent T cell populations and confirms that cell-cell contact is required for this process. The fact that MHC-II did not suppress T cell proliferation in this donor is interesting and requires futures studies in more donors. Overall, this is the first demonstration of DCs inducing proliferation of latent T cells in a contact-mediated manner.

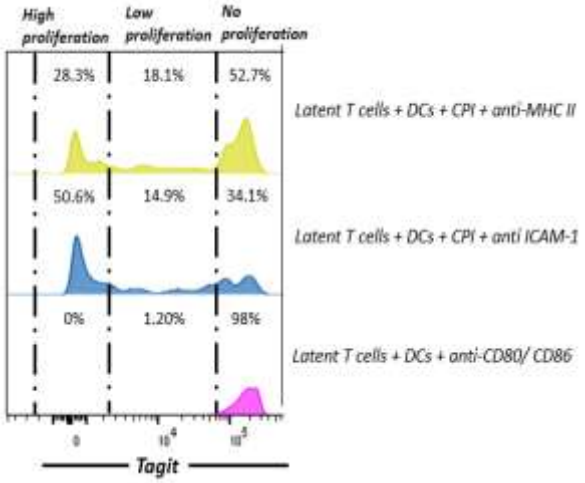
A


Anti-MHC class II
Anti-CD80
Anti-CD86
Untreated imDCs
Untreated mDCs
Unstained
Anti-MHC class II treated mDCs
Anti-CD80 treated mDCs
Anti-CD86 treated mDCs

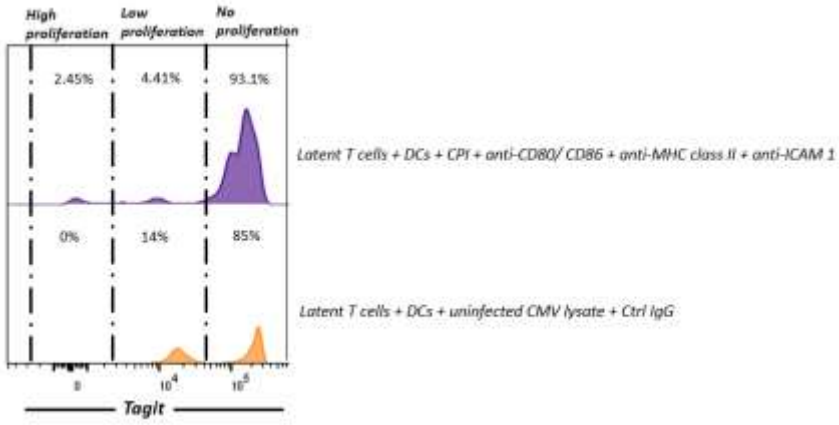
B


High proliferation
Low proliferation
No proliferation
0%
0%
99.4%
Latent T cells alone
1.5%
5.36%
92.14%
Latent T cells + DCs + Ctrl IgG
32.6%
17.6%
49.8%
Latent T cells + DCs + CPI + Ctrl IgG
Tagit

C


High proliferation
Low proliferation
No proliferation
28.3%
18.1%
52.7%
Latent T cells + DCs + CPI + anti-MHC II
50.6%
14.9%
34.1%
Latent T cells + DCs + CPI + anti ICAM-1
0%
1.20%
98%
Latent T cells + DCs + anti-CD80/ CD86
Tagit

D


High proliferation
Low proliferation
No proliferation
2.45%
4.41%
93.1%
Latent T cells + DCs + CPI + anti-CD80/ CD86 + anti-MHC class II + anti-ICAM 1
0%
14%
85%
Latent T cells + DCs + uninfected CMV lysate + Ctrl IgG
Tagit

E

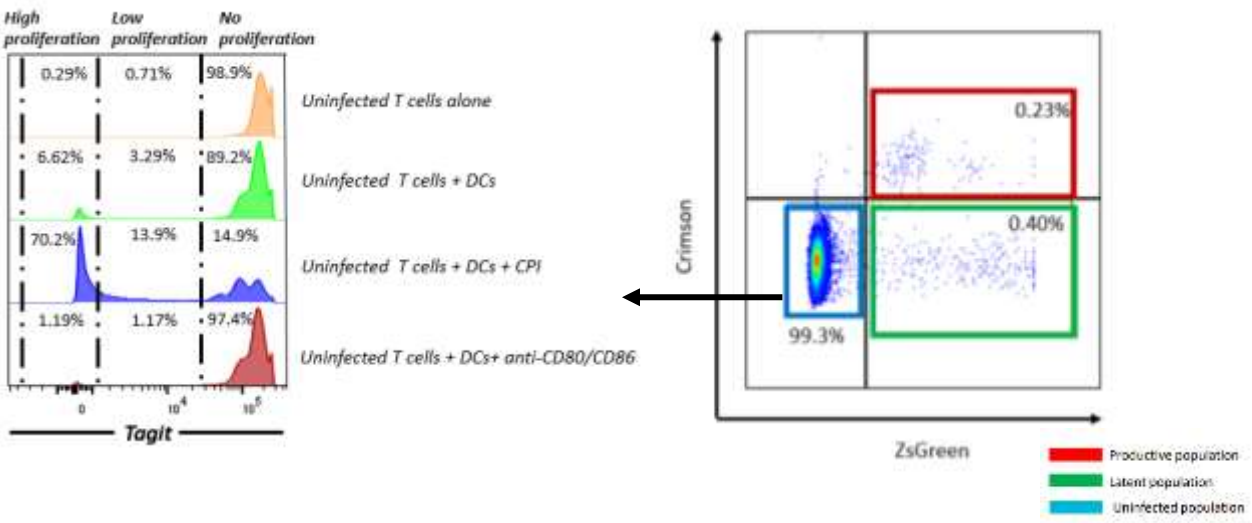


Figure 15. DCs induce proliferation of latent T cell population in a contact-mediated manner.

(A) To confirm the efficacy of the neutralizing Abs, LPS-stimulated DCs and imDCs were treated with 10 µg/mL of blocking Abs, MHC class II, CD80 and CD86, prior to staining with fluorescent conjugated antibodies of the same clone. The histograms show the comparison of the receptor blockades in LPS-stimulated DCs with their immature counterparts**.**

(B-D) DCs were treated with the indicated neutralizing Abs and then co-cultured with HIV-Nef-CRIMZY-infected T cells labelled with Tagit proliferation dye. labelled with Tagit proliferation. Control IgG (10 µg/mL) was used as an isotype.

(E) Representative flow histogram displays proliferative responses in uninfected $CD4^+$ T cell population cell population ($ZsGreen^-$ $Crimson^-$; blue box) within the same DC: T cell co-culture.

3.4.4 Minimal latent T cell proliferation by DC-secreted soluble factors

Next, I examined the role of DC-derived soluble cytokines in directly inducing T cell proliferation. To address this, I collected the culture supernatant from previous DC: T cell co-culture experiments on day 2 and 7, and stored at -80 °C. For the study, T cells from another donor were expanded and infected with HIV-Nef-CRIMZY as described before. AT day 5 post-infection, Tagit labelled T cells alone (without DCs) were incubated with day 2 culture supernatants. After 2 days, cell culture media was replaced with the day 7 collected culture supernatant, and followed for up to 8 days for cell proliferation measurement by flow cytometry. My results showed that culture supernatants induced low levels of proliferation in latently-infected T cells in this donor (35.3%), but no increase in the presence of DCs (35.1%) (Figure 16A). Culture supernatants from the indicated DC: T cell co-cultures in the presence or absence of antibody blockade (studies shown in Figure 15) were also tested. The results showed a slight reduction in latent T cell proliferation when MHC-II, CD80/CD86 were all blocked (19.9%; Figure 16B). Furthermore, the supernatant from ICAM-1 single blockade induced low levels of proliferation in latent T cell population (Figure 16C). Similar results were observed when looking at uninfected T cells in the same study (Figure 16D). Taken all together, DC-derived soluble factors seem to play induce low levels of latent T cell proliferation, whereas DC-contact derived signals may induce high levels of T cell proliferation.

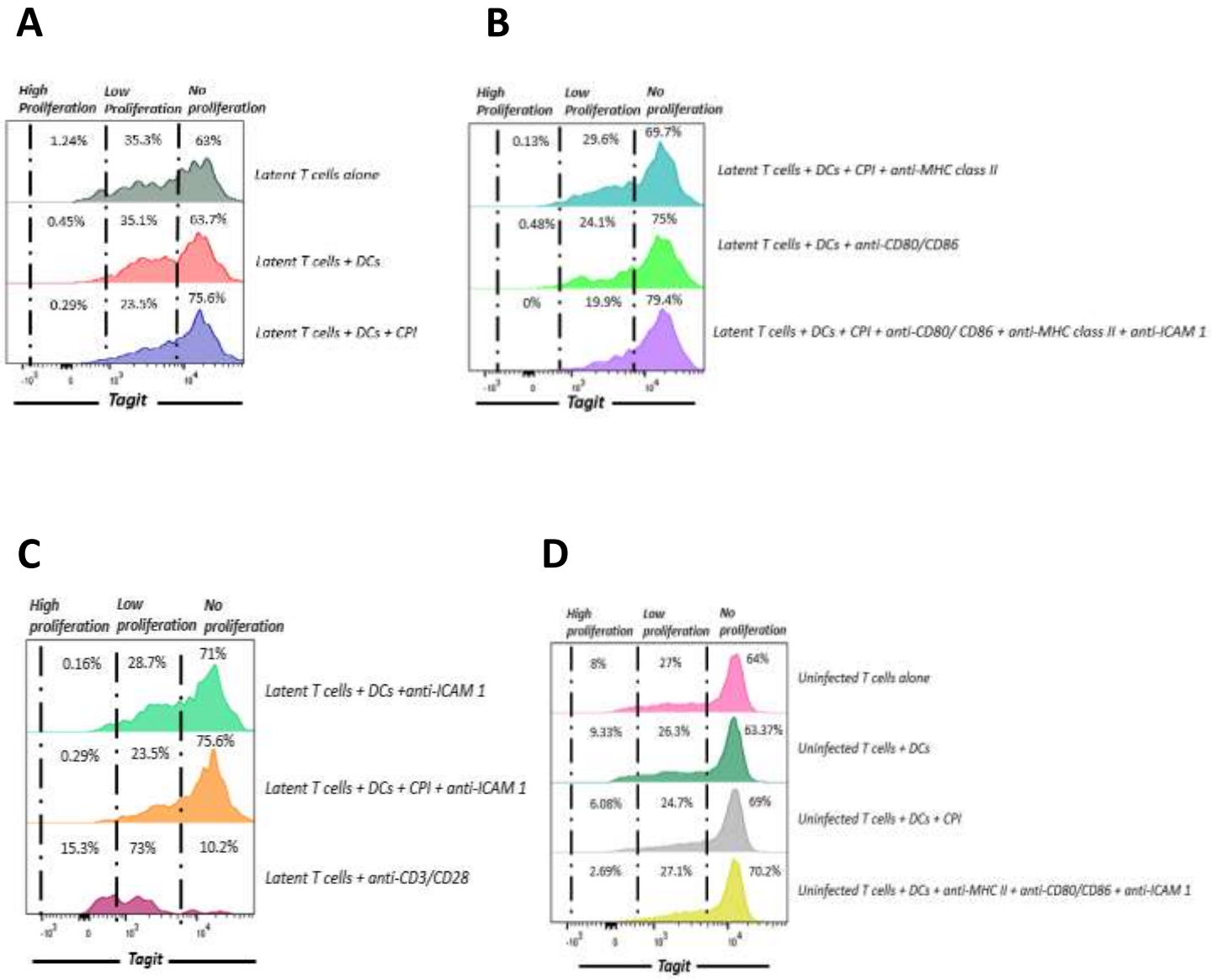


Figure 16. DC-derived cytokines induce low levels of proliferation of the latent T cell.

(A) HIV-Nef-CRIMZY-infected $CD4^+$ T cells alone (without DCs) were exposed to culture supernatants collected from DC: T cell co-cultures (day 2) of a previous experiment using a different donor. After 2 days, media was changed and replaced with day 7 collected DC: T cell co-culture supernatants and incubated for additional 8 days without changing the media. Histograms

show proliferation levels within latent T cell populations in the presence of DC: T cell culture supernatants alone.

(B-C) Similar experiments as in (A), but culture supernatants from the indicated DC: T cell co-culture condition was applied on latent T cells alone for proliferation analysis at day 8.

(C) Similar experiments as in (B),but looking at proliferative responses in uninfected T cells ($ZsGreen^{-}$ $Crimson^{-}$) in the presence of DC-derived soluble factors.

Chapter 4. DISCUSSION

Despite being more than thirty years since the beginning of the HIV-1 epidemic, there is still no effective vaccine or cure strategy for HIV-1 infection. Higher infection rates in women than men are observed worldwide, with sexual HIV transmission through the female genital tract (FGT) being the primary route of infection in young women [8, 9]. The FGT provides both a physical and immunological barrier to protect the host from the outside world. When this physical barrier is disrupted, HIV particles gain access to a large number of $CD4^+$ T cells in the submucosa, which is the most important first step during the early stages of vaginal HIV transmission. Several lines of evidence have shown that dendritic cells, the pivotal decision-making APCs, are the first innate immune cells that encounter HIV particles through the genital mucosae. While DCs are crucial players in the induction of anti-HIV cell-mediated immunity, they are also exploited by virus to enhance viral spread to secondary lymphoid organs prior to viral detection in the blood[329]. It is well-established that cell-to-cell contacts increase the efficacy of HIV spread between cells compared to the transmission of cell-free viruses[329-331]. Using a 3D collagen matrix model to visualize dynamic cell-cell interactions in a lymphoid tissue-like environment , we previously showed that $CD4^+$ T cells engaged in prolonged contacts with HIV-bearing DCs that is dependent on gp120: CD4 and LFA-1: ICAM-1 adhesive contacts[122]. However, Env-deficient HIV or CD4 antibody blockade abrogates the stable DC: T cell contact, suggesting that this virus is actively promoting cell-cell contacts. These data suggest that surface-bound HIV particles arrest T cell migratory behavior through gh120: CD4 interactions, possibly as a prerequisite to enhance viral spread. Understanding the molecular mechanism that governs HIV transmission during cell-cell interactions is vital to develop efficient intervention to limit systemic infection. These data also highlight ways HIV controls the intracellular signaling networks of immune cells to promote various processes and spread in the body, similar to pathogens like *Leishmani*a and herpes simplex virus type 1 (HSV-1)[332, 333]

Following systemic HIV infection, a state of latency, defined as a reversible and non-productive state of infection, in a pool of long-lived memory $CD4^+$ T cells is established within days, and poses a significant barrier toward HIV elimination[324]. The clonal expansion of latently infected $CD4^+$ T cells is thought to be the primary driver of HIV-1 persistence in individuals under suppressive ART therapy [295, 298]. Different mechanisms have been proposed that contribute to

HIV-1 persistence, among which antigen-driven proliferation is hypothesized as an important driver of HIV maintenance and expansion, causing "wax and waning" of latent T cell populations without the reactivation of the HIV reservoir[314, 334]. Kumar *et al.* reported that myeloid DCs and monocytes can directly induce HIV latency infections in memory $CD4^+$ T cells[269, 270]. These data suggest that DCs are involved in facilitating both HIV dissemination and latency generation during the early phase of infection, highlighting an additional ability of HIV to take advantage of normal immune cell functions to establish a chronic infection. However, insights into the role of DCs in the maintenance of HIV-infected T cells and whether cell-cell interactions are required for this process are still lacking. In my thesis, I focused on how DC: T cell communication, and initiation of specific signaling pathways play a role in both HIV transmission and expansion of T cell latency. Specifically, I utilized the human kinome array to demonstrate that gp120: CD4 interactions induced various kinase signaling pathways, some of which were associated with "stop signal" in $CD4^+$ T cells that likely contributed to the formation of stable DC: T cell contacts. I confirmed that HIV Env was involved in this process, as upregulation of these signaling pathways was not observed in T cells engaged with DCs pulsed with Env -deficient HIV in our coculture. Subsequently, I tried to validate phosphorylation of critical kinases identified in the kinome array, namely Lck, ZAP-70 and ERK 1/2, using intracellular phospho-flow cytometry.

Next, I examined whether DCs can expand T-cell latency through DC: T cell contacts. To study this, I took advantage of our new full-length dual-fluorescent HIV latency reporter (HIV-Nef-CRIMZY) to demonstrate, for the first time, that DCs can facilitate the expansion of latently-infected $CD4^+$ T cells *in vitro*. Using T cell proliferation dye as a readout, I showed that DCs induced proliferation of latent $CD4^+$ T cells in an antigen-dependent and -independent manner, and that co-stimulatory signaling was an absolute requirement. I also confirmed that DCs-derived cytokines played a minimal role in latent T cell proliferation and did not on their own explain the robust proliferation observed in DC: T cell cocultures. Collectively, my data demonstrated that DCs play a pathogenic role during HIV infection, both by delivering HIV particles to susceptible T cells in the genital mucosal tissues and lymph nodes, as well as initiating a cascade of intracellular signaling events that drive the clonal expansion of the HIV reservoir.

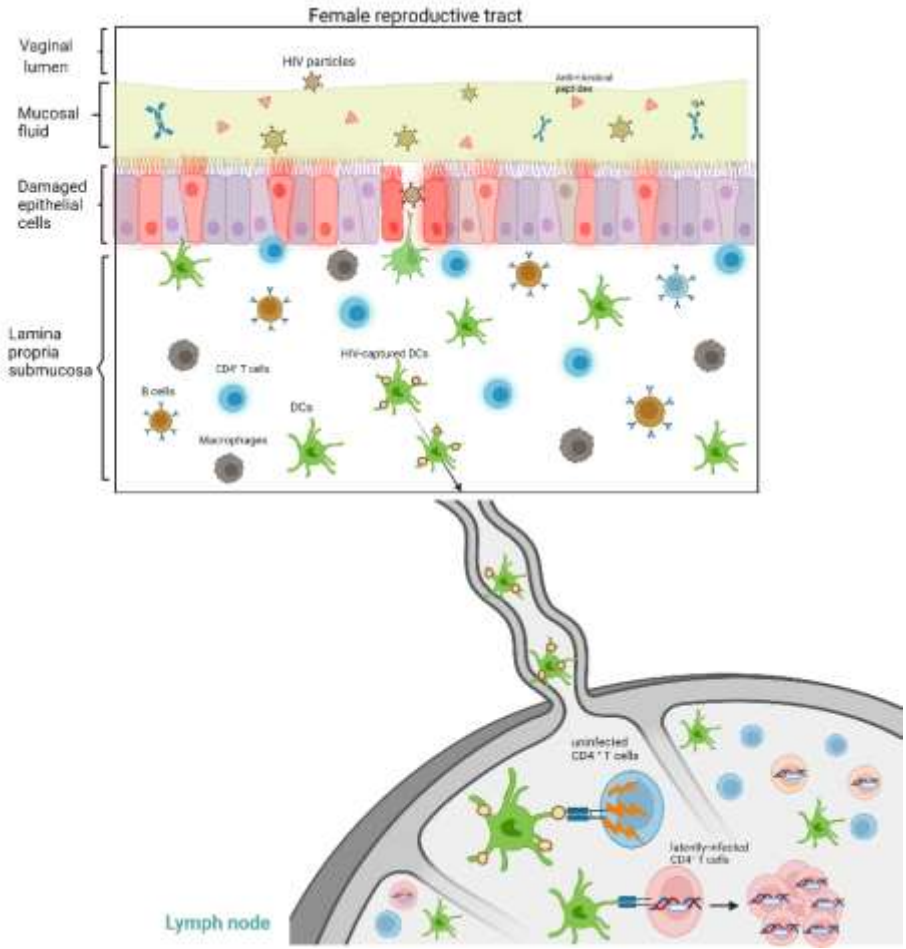


Figure 17. Graphic illustration of the contribution of DCs to HIV transmission and maintenance of latent reservoir. Dendritic cells are the first immune cells in the FRT which encounter HIV particles. DCs capture and retain HIV particles and migrate to the lymph node to transfer viral particles to susceptible $CD4^+$ T cells. Kinome analysis reveal that HIV-captured DCs induce distinct intracellular signaling pathways in T cells to enhance viral dissemination, leading to wide-spread infection. DCs also drive proliferation of $CD4^+$ T cells harboring silent HIV proviral DNA in a contact-mediated manner. Future studies will assess the molecular mechanisms which modulate HIV maintenance through DC: T cell interactions.

4.1 Unbiased analysis of kinase signaling pathways in T cells contacting HIV-bearing DCs

Early studies showed that gp120: CD4 interactions alter CD4 T cell migratory behaviors by activating distinct signaling pathways [212, 215-217]. To address whether DC-bound HIV can induce similar signaling pathways in migrating T cells, DC: T cell co-cultures in the presence of wildtype HIV particles were performed. My human kinome array analysis with IPA and InnateDB analysis align with these previous studies, showing that TCR proximal molecules and downstream kinases such as ERK, PI3K, and Tec signaling pathways are upregulated in the presence of wild type but not in Env-deficient HIV [215]. Tec family kinases (z-score 2.1, p-value $3.4x10^{-6}$) are activated downstream of the TCR and are crucial for T cell maturation, activation, and differentiation [335-337]. IT, RLK, and Tec are the members of Tec family kinase that are expressed only in T cells at different levels in T cell subsets [338]. They contribute to T cell differentiation and lineage commitment [339]. ITK is the predominate member of the Tec family, which is recruited to the plasma membrane following activation of PI3K and accumulation of PIP3 [340, 341]. Next, ITk interacts with SLP-76/LAT via its SH2 and SH3 domains[341, 342], leading to its phosphorylation through the activation loop (Y511) by Lck [343]. ITK is involved in various functions such as governing Ca^{2+} mobilization, MAPK activation, actin mobilization, and cytoskeleton reorganization[335, 344]. Recent studies have also implicated Tec family kinases in the HIV-1 replication. Vasiliver-Shamis *et al* demonstrated that ITK is activated during gp120:CD4 conjugation [215] and that the loss of ITK activity abrogates F-actin rearrangement, resulting in a decrease in the level of intracellular P24 expression during acute infection. This agrees with other studies done by Readinger *et al* that showed that ITK dysfunction impairs viral transcription, assembly, and ultimately viral dissemination[345]. These data suggest that this pathway likely regulates both T cell motility as well as HIV susceptibility. Similarly, the PI3K/AKT/mTOR pathway (*z*-score 2.1, p-value $1.3x10^{-5}$) is a master regulator of various T cell functions, including proliferation, survival, and migration[346, 347]. Recent data suggested that mTOR limits metabolic restrictions in $CD4^+$ T cells, resulting in increased dNTP levels that are required for efficient HIV-1 reverse [348]. The canonical MAPK pathway (z-score 1.9, p-value $1.1x10^{-9}$), also known as the Ras-RAF-MEK-ERK pathway, also modulates T cell survival, proliferation, cell adherence, and motility[349-351]. ERK activation has been previously shown

to facilitate HIV infectivity and was required for the efficient organization of reverse transcription enzyme[352]. It was also reported that this pathway regulates cell adherence, as inhibiting ERK signaling is known to restrain ECM-induced cell motility[350, 353]. Additionally, this pathway has been suggested to directly trigger NF-κB or other transcription required for transactivation of the HIV promoter in infected cells [354, 355]. Lastly, stimulation of Ca^{2+} pathway is involved in inducing the transcription factors that are important in T cell proliferation, activation, and differentiation [356, 357], and Ca^{2+} signaling is also a primary driver of "stop" signals in T cells, and is possibly involved in the stabilization of DC: T cell contacts in the presence of wildtype HIV particles. Taken all together, my analysis of downstream signaling events in T cells provides insights into the signaling cascades that regulate T cell motility, HIV production, and cell survival that has implications on HIV spread.

4.2 Intracellular phospho-flow cytometry to validate TCR signaling network

To validate results from the human kinome analysis, I used phospho-flow cytometry to measure kinase activation in T cells. This approach was ideal because it can quantitatively analyze signaling in single cells and has higher throughput than western blot analysis. I first performed a series of optimization studies to detect changes in the phosphorylation of Lck, ZAP-70, and ERK 1/2, as they are downstream of TCR signaling transduction and are essential in the early steps of HIV replication[167, 212]. These kinase pathways were also upregulated in T cells co-cultured with DCs and HIV based on my human kinome array (Figure 5). In contrast to our study, most insights on TCR signaling pathways have been revealed using human T cell lines such as Jurkat T cells. Although Jurkats have had a long history in biomedical research as a model of TCR downstream cascade, several deficiencies in gene expression have come to light, resulting in misinterpretation of T-cell biology compared to donor-derived primary $CD4^+$ T cells [358]. The most notable defect in Jurkat cells is the absence of PTEN (Phosphotase and Tensin homologue) and SHIP. PTEN dysfunction results in constitutive activation of PI3K/AKT pathway which could provide results compared to primary cells, while the biological implications of SHIP deficiency is unclear [359]. However, it is well-understood that SHIP negatively regulates PI3K pathway by dephosphorylating PIP3[360]. Besides, Gioia *et al* identified additional mutated genes in Jurkat T cells, such as CTLA-4 and Syk, which are an integral part of T cell signaling and function [361].

For these reasons, my work was done exclusively using healthy donor-derived primary $CD4^+$ T cells, which is more challenging and has higher donor-dependent variability. However, despite numerous attempts to optimize intracellular phospho-flow, I saw little phosphorylation of Lck, ZAP-70 and ERK 1/2 in primary $CD4^+$ T cells stimulated with either PHA or anti-CD3/CD28 beads, similar to unstimulated cells. I confirmed that both stimulations were working, as CD69 and HLA-DR increased after one hour of stimulation. Previous studies have shown that phosphorylation of kinases after TCR triggering with anti-CD3/CD28 occurs as early as 60 seconds within T cells [362]. TCR signaling cascade is also transient, as *in vitro* cell stimulation with anti-CD3ε antibody induces early and transient activation of ZAP-70 and PKC-θ in both Tregs and non-Tregs[363]. These data suggest that downstream signaling in primary $CD4^+$ T cells is highly transient, reversible, and thus, it may be challenging to capture phosphorylation in T cells during DC: T cell co-culture studies. Another likely consideration is that that cross-linking of CD3 and CD28 molecules, caused optimal ERK phosphorylation; however, robust signaling was observed in primary naïve T cells relative to memory T cells[364], proposing that naïve and memory $CD4^+$ T cells respond differently to TCR stimulation. My study primarily used memory $CD4^+$ T cells in assays, as they are the main target of HIV infection. Another possible explanation for the unsuccessful phospho-flow assay in my hands is that PFA enters and reacts with the amine group on proteins, resulting in protein-protein cross-linking and blocking any enzymatic activity within the cells. Some phospho-site specific antibodies might be sensitive to PFA, reducing the chance of antibodies to target their proteins. Altogether, modeling TCR signaling cascades posed multiple technical challenges, and further optimization was necessary to obtain consistent and robust intracellular signaling transduction measurements using intracellular phospho-flow cytometry.

4.3 The contribution of DCs in the expansion of latently-infected $CD4^+$ T cells

It is well understood that an extremely stable pool of HIV reservoir cells exists in resting memory T cells, particularly central memory (T_{CM}) and transitional memory (T_{TM}) $CD4^+$ T cells [257, 365]. Long-term persistence of the HI reservoir seems to be contained within T cell clones in ART-suppressed individuals, where cells carry identical proviral sequences integrated into the same chromosomal position of the host genome [295, 366]. This makes it highly likely that

infected T cells continue to proliferate under ART suppression, continuously replenishing the latent HIV reservoir. It has been proposed that expansion of the HIV reservoir could be driven by homeostatic cytokines, antigenic stimulation, or integration-site regulation[257, 292, 310]. Studies have provided evidence for all three mechanisms, but the two latter mechanisms have been the primary focus in the HIV cure field [366]. Biological processes that lead to the expansion of latent memory T cells are likely to do so in the absence of viral production [298].

My interest in studying how DC: T cell contacts help maintain HIV latency was derived from my earlier studies using the same approach to study HIV transmission. The frequent DC: T cell contacts in the lymph node indicated that T cells are always in contact with DCs *in vivo*. Additionally, the role of dendritic cells on the generation of HIV latency in T cells was previously investigated by the Lewin lab based on the fact that DC: T cell interactions frequently occur in secondary lymphoid organs. Myeloid DCs increased the number of latent infections in both nonproliferating and proliferating $CD4^+$ T cells, and that this was largely dependent on close cell-cell contacts [269, 271]. Follow-up studies subsequently supported the notion that other DC subpopulations, such as $CD1c^+$ DCs or $SLAN^+$ DCs can also promote HIV latent infections through DC: T cell interactions [270]. These studies argue that APCs induced signaling in T cells that increased HIV infection rates and established a latent state. However, an open question is whether similar DC: T cell contacts can facilitate the maintenance or expansion of latently-infected T cells. This is similar to physiological maintenance of memory T cells through cell-cell contacts that provide survival and proliferation signals for long-term. This potential mechanism has profound implications on understanding how the HIV reservoir is maintained in PLWH under suppressive ART therapy, and to develop new ways to reduce the size of the HIV reservoir in. To this end, we developed a full-length dual-fluorescent HIV latency reporter system (HIV-Nef-CRIMZY) in our lab, allowing us to simultaneously identify latent ($ZsGreen^+$ $Crimson^-$) and productive ($ZsGreen^+$ $Crimson^+$) T cell populations. Because these cells do not have to be restimulated, I was able to follow these cells over time in culture. Interestingly, within the latent T cell gate, I observed that there were two populations: one that continued to express p24 and downregulate CD4 expression, and those that did not express HIV Gag p24 and had high CD4 expression. The reason why T cells express p24 but not Nef-Crimson is not known, but it would be interesting to examine whether HIV-DNA is integrated into the host's genome or remains unintegrated DNA in the cytoplasm. We used CD4 expression as a marker for p24 expression and

identified truly latent T cells (ZsGreen^{+} Crimson^{-} CD4^{+}). After gating on true latent population, I showed that co-culture with autologous DCs can increase latent T cell proportion compared to latent T cells alone, which did not proliferate. This increase in infected cell numbers was not due to new rounds of infection, as integrate inhibitor, raltegravir, was added to the culture. My findings align with previous studies showing that DCs helped maintain latent CD4^{+} T cells for up to 12 days co-culture[271]. Additional studies also underpin the role of DCs in increasing latency during infection by other viruses. For example, using an *in vivo* model, lymphoid DCs (CD11c^{+} CD8α^{+}) are associated with increased numbers of latently-infected T cells in HSV-1 infected mice[367]. DC depletion in mice with melanoma-associated vitiligo (MAV) resulted in a dramatic drop in the number of memory CD8^{+} T cells, but clustering DCs with T cells recovered their survival[368]. These data suggest that DCs are required for the long-term maintenance of the memory T cell pool and that HIV can take advantage of this to establish infection.

4.4 Extensive proliferation of the latent reservoir through DC: T cell interaction

The increased proportion of latent T cells in DC: T cell cocultures could result from cell death after infection, or an increase in latent T cell proliferation. Using a proliferation assay, I showed that autologous DCs induced proliferation of latent T cells even without adding any exogenous antigen, suggesting that weak interactions through TCR: MHC II, defined as tonic signaling, may be at play [186, 369]. It is important to mention that all studies were performed using healthy-donor derived PBMCs that were expanded in serum-alternative media (XF media, StemCell Technologies) to remove any confounding factors from fetal bovine serum. This finding was surprising, but also found it was highly dependent on the donor. The strength and duration of the contact can produce tonic signaling that induces the expression of genes that upregulate transcription factors favoring T cell maintenance[370]. Previous studies showed that TCR-deficient T cells are gradually lost over time with half-lives of 16 days for CD4^{+} T cells [186]. It was also demonstrated that TCR-mediated tonic survival signals require MHC-II engagement because murine CD4^{+} T cell pools diminished dramatically in Rag 2 $^{-/-}$ MHC class II $^{-/-}$ mice relative to Rag 2$^{-/-}$ MHC class II $^{+/+}$ mice [186]. Although it is believed that naïve T cells require continuous MHC-II interaction for their maintenance compared to memory T cells, studies by Alessandra Riva *et al* showed that murine memory CD4^{+} T cells from MHC class II deficient hosts

have impaired functionality and survival capacity *in vivo* [371]. This is due to reduction in basal phosphorylation of signaling molecules, such as LAT and ERK 1/2, which are crucial for T cell activation and survival, respectively [369]. These studies suggest that tonic signaling may also allow memory T cells harboring provirus to ensure long-term survival in the body.

DC engagement can also reactivate HIV expression from latent T cells through cell-cell contacts, supporting the notion that TCR signaling strength determines the fate of HIV-1 provirus [255]. Interestingly, recent work by Gagne *et al* developed chimeric antigen receptors (CARs) which have different ligand affinities [268]. They showed that weak signals at the time of infection induced more irreversibly latent T cells, while strong signals induced more reactivable latent T cells. However, both strength signals led to similar integration levels, highlighting the possibility that a polyclonal T cell repertoire with different affinities to either self or foreign antigen is the key determinate of latent T cell fate. However, the relationship between TCR signaling, virus reactivation, and cell proliferation is not well understood. The possibility that DCs can reprogram T cells by activating specific gene profiles that favor cell proliferation while silencing HIV transcription [372] is intriguing and subject to future studies.

4.4.1 What is the role of antigen in the expansion of the HIV reservoir?

Recent studies show that the expansion of the latent reservoir may depend on antigenic stimulation, which drives the proliferation and differentiation of memory $CD4^+$ T cells [258, 314, 373]. Evidence is accumulating that coinfections, such as CMV or EBV, are widespread among PLWH and that this could cause the antigen-driven proliferation of latent T cells[311, 312]. Henrich *et al* detected an enrichment of HIV proviral DNA in EBV- and CMV-responsive $CD4^+$ T cells following chemotherapy in an HIV^+ individual [374]. Furthermore, a recent study found both intact and defective proviruses in HIV- and CMV-specific T cells and that they were responsive to chronic viral antigens *in vitro* [313]. HIV proviruses have also been detected in other antigen-specific $CD4^+$ T cells, such as those recognizing influenza [315], *Candida albicans* [316], and tetanus[315, 375]. These data suggest that repeated exposure to antigens may be associated with expansion of antigen-specific T cells harboring the latent reservoir. Based on these findings, I hypothesized that DC-mediated antigen processing and presentation might contribute towards the proliferation of latent T cells in an antigen-dependent manner. Using the same DC: T cell co-

culture approach I have described before, I used the AIM assay to measure upregulation of activation markers, such as OX40, CD69, CD40L, and PD-L1, during DC: T cell co-cultures. The advantage of the AIM assay over other commonly used methods, such as ELISPOT and Intracellular Cytokine Staining (ICS), is that AIM markers can be evaluated on live cells without the need for permeabilization, intracellular staining and use of Golgi transporting inhibitors, which would facilitate live cell sorting. Moreover, cytokine analysis would require pre-determination of the target cytokine, resulting in a biased analysis of T cell subpopulations and underestimating the frequency of total antigen-specific populations. I first optimized the AIM assay in my DC: T cell coculture model and observed that both mature and immature DCs upregulate AIM markers (CD69/OX40, CD69/PD-L1, and PD-L1/OX40) on $CD4^+$ T cells. When I looked for activation markers within responsive $CD4^+$ T cells in the presence of DCs and antigens, there was not a striking increase in double-positive CD69/OX40 latent T cells. This is likely due to transient and early upregulation of these markers after antigen stimulation that was not captured well in my assay. I concluded that the AIM assay may not be sensitive enough for my studies and decided not to use this assay in future experiments.

When proliferation dye was used as a readout in my DC: T cell coculture, I observed proliferation of latent T cells in the presence of DCs. I found that the magnitude of latently-infected $CD4^+$ T cell responses varied considerably among different donors, illustrating the complexity of DC: T cell interactions and signaling. I observed that DC: T cell co-cultures induced proliferation of latent T cells in the absence of exogenous antigen, but in some donors, the addition of viral and bacterial antigens further led to latent T cell expansion. I also observed that uninfected T cells also proliferated in response to bacterial (*E.coli* and *S.aureus*) or viral (CMV) protein lysates, confirming that antigen-dependent effects were not just observed by infected T cells. These data seem to support the hypothesis that the HIV reservoir undergoes continual proliferation without necessarily reactivating viral re-expression, since the number of latent T cells increased in DC: T cell co-cultures. I also observed higher proliferation of latent T cells in response to CPI (CMV, parainfluenza and influenza A) protein pools than CMV cell lysates alone, which could be explained by the fact that Canada has one of the lowest CMV seroprevalence worldwide [376]. Together, we observed both antigen-dependent and independent proliferation of latent T cells that was dependent on DCs although individual responses were variable among different donors.

4.5 Cell-cell contact is required for the proliferation of latently-infected T cells

I next assessed whether close cell-cell contacts were required for the persistence of the latently infected $CD4^+$ T cells. The requirements for TCR signaling, co-stimulation, and cytokine to support T cell proliferation and differentiation have been studied extensively *in vivo* and *in vitro* [377-379]. Naïve T cell activation is initiated through the formation of the immunological synapse between DC and T cells that involves TCR: MHC-II engagement (signal 1) along with interactions of costimulatory molecules (CD28: CD80/CD86) (signal 2), amplifying TCR signaling events [380]. To address whether these and other molecules were involved in latent T cell proliferation, I used antibodies to block MHC-II, CD80, CD86, and ICAM-1 binding, as they are crucial for stabilizing DC: T cell contacts and initiating T cell signaling. My data demonstrated that CD80/CD86 dual blockade, which both interact with their ligand CD28 on T cells, strongly suppressed latent T cell expansion. However, ICAM-1 and MHC II blockades had little-to-no effect on latent T cell proliferation. All neutralizing antibodies were confirmed by flow cytometry. These data indicate that DC: T cell contacts, mainly co-stimulation signaling, is an important factor that allows expansion of infected T cells *in vitro*.

Given that TCR engagement with the peptide: MHC complex drives activation [145], this result was surprising. Future studies with more donors will need to be done to confirm these findings. One possible explanation is that soluble factors secreted by DCs may support T cell proliferation in a CD28: CD80/CD86-dependent manner. For example, IL-12, secretion by DCs, can induce T cell proliferation and prevent apoptosis of activated peripheral T cells[381-383] through induction of the PI3K/Akt pathway [384]. Additionally, it is possible that the recycling of MHC-II molecules on the surface of DCs may be a confounding factor. Newly-synthesized MHC-II complexes that are transported to the DC surface are continually internalized and recycled [385]. Therefore, continuous antibody blockade of this molecule may be required in our 8-day coculture assay. B7 family-CD28 interaction (signal 2) works with signal 1 to strengthen signaling downstream of the TCR[386]. After that, co-stimulation recruits membrane rafts containing various kinases and adapters to the DC: T cell immunological synapse, resulting in amplifying the downstream TCR signaling. The PI3K/Akt pathway is a key regulator of T cell survival and proliferation during priming [346, 347, 387]. Moreover, PI3K/Akt signaling pathway can also

favor the progression and survival of cancer cells. Co-culturing multiple myeloma cells with DCs promotes tumor survival, whereas anti- CD28 blockade reduces tumor survival that is dependent on PI3K/Akt pathway activation[388]. Lastly, CD28 stimulation augments calcium mobilization which is an essential pathway during T cell [389, 390]. These data reinforce the idea that $CD4^+$ T cells, including the cells harboring HIV provirus, require CD28-mdiated engagement to initiate survival and proliferative signals required for their long-term persistence in the body. Whether activation of the PI3K/Akt pathway is involved in latent T cells proliferation is the subject of future studies.

Finally, I investigated the role of cytokines in the proliferation of latent T cells. There is accumulating evidence that IFN-α and IL-15 are the first cytokines increase within 5 days of viremia detection, followed by TNF-α, CXCL10, and IFN-γ, and then by IL-12[391]. Furthermore, increased IL-6, IFN-γ, and IL-12 levels correlate with a higher level of viral replication[392]. IL-7 has been shown to be predominately responsible for the homeostatic proliferation of T_{CM} cells harboring provirus without viral reactivation or cell differentiation [306]. Like IL-7, IL-15 can also contribute to the persistence of latently-infected $CD4^+$ T cells, but to a lesser extent [257, 305]. IL-7 favor homeostatic expansion of T cells through the expression of anti-apoptotic protein Bcl-2 and its family members[393]. Here, I showed that placing culture supernatants from DC: T cell cocultures onto latent T cells alone (from another donor) led to only low proliferative responses, likely due to the presence of secreted soluble factors from cells. It has been demonstrated that both IL-6 and IL-1β, secreted by DCs, induce memory $CD4^+$ T cell but not $CD8^+$ T cell cycling *in vitro* without upregulating survival factor Bcl-2 [394], but whether they can directly increase T cell proliferation is unclear. It has been suggested that TNFα may inhibit HIV expression, as it suppresses viral replication in several cell types, such as infected PBMCs or alveolar macrophages[395, 396]. However, TNFα can also induce the expression of NF-κB followed by promoter activation [397-399], indicating that TNFα has different impacts on HIV-infected T cells during chronic infection. Altogether, these data propose that the cytokine-mediated expansion plays a partial role in latent T cell proliferation that is dependent on initial DC: T cell contacts. Further investigation is required to understand the relationship between cytokine-induced signaling and T cell proliferation during HIV reservoir expansion.

Chapter 5. SIGNIFICANCE AND FUTURE DIRECTIONS

Previous studies have shown that myeloid cells, such as monocytes and DCs, can increase latent T cell infections through DC: T cell co-cultures. However, my work in this thesis is to show that DCs are crucial mediators of latently-infected $CD4^+$ T cell proliferation in both an antigen-dependent and -independent manner. I showed that viral or bacterial protein antigens can induce high proliferation of a subset of latent T cells, and that cell-cell contact is a requirement for this process. Using a new full-length, dual-fluorescent HIV latency reporter, I validated that actual latent T cells can continue to undergo proliferation in the presence of DCs. My results are one step towards understanding the underlying mechanism of T cell clonal expansion observed in ART-treated individuals and show another example of HIV taking advantage of normal immune cell functions.

While my study concentrated on the function of monocyte-derived dendritic cells (MDDCs), mainly because large numbers can be extracted from donor PBMCs, it would be important to study the contribution of other APCs, including myeloid DCs, monocytes, and B cells in the expansion of latent T cells over time. Future studies should also test primary DCs isolated from blood or tissues. These futures studies have implications for whether specific APC subsets have a dominant role in HIV reservoir expansion during suppressive ART. Moreover, no data describes the molecular signals by which APC-mediated latent T cell expansion is regulated. Utilizing human kinome array, as done in Figure 5, would reveal kinase pathways that regulate T cell proliferation, virus reactivation, or potentially both in latent T cells. These studies may identify unique signals that promote latent T cell proliferation, similar to a recently described role of BIRC5 in promoting latent T cell survival[400]. Finally, it would be also interesting to investigate the change in latent T cell metabolism in the presence of the exogenous antigens, and how cellular metabolism can impact on HIV reactivation or expansion during chronic infection.

A limitation of this study is that I was not able to determine whether the various stimulus was inducing HIV reactivation. To do this, latent T cell populations would need to be purified/sorted prior to DC co-culture. Currently, I am not able to do this, but only to follow different T cell subsets within a population. It would be of interest for the future studies to test different antigen concentrations to measure HIV protein production and proliferation simultaneously. Furthermore, it would be interesting to assess whether HIV-specific $CD8^+$ T cells

are able to kill reactivated T cells to clear infected cells. Future studies should focus on how to eliminate T cells containing provirus using a more immunological and specific strategy, to reduce the size of the HIV reservoir in PLWH.

Chapter 6. REFERENCES

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