DEVELOPMENT OF A CELL CULTURE, SCREENING, AND SEQUENCING METHOD FOR HIGH-THROUGHPUT ISOLATION OF HIV-POSITIVE CD4$^+$ T-CELLS FROM HIV-INFECTED PATIENTS

by

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DEDICATION

I dedicate this thesis in loving memory of my father. Although he is not here with me today, my memories of his support, love and warmth have guided me into who I am today.

ACKNOWLEDGEMENTS

First and foremost, I am forever thankful for my husband for always being my number one fan. Without your unwavering love, support, patience, and encouragement, I would not have gotten to where I am today.

I am very fortunate and grateful for my PI, Dr. Tomozumi Imamichi for giving me this opportunity and my supervisor and thesis advisor, Dr. Sylvain Laverdure for never giving up on me. You both have been truly instrumental in my growth as a scientist. I am truly grateful for the support, guidance, reassurance and inspiration of my past and present colleagues, and professors and classmates at Hood College.

Last but certainly not least, I am truly grateful for my children, mom and sisters for their unwavering love and support.

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that targets immune cells critical to innate and adaptive immune responses in infected patients. Despite viremia suppression by antiretroviral drug treatments, chronic immune activation persists in HIV-1-infected patients, increasing their risk of HIV-associated chronic comorbidities. Defective HIV-1 proviruses harboring genetic mutations may contribute to persistent immune activation in suppressed HIV-1-infected patients through expression of canonical or novel viral proteins. In this study, we presented the steps taken to develop optimal culture conditions for $CD4^+$ T cells from suppressed HIV-infected patients, and an unbiased nested polymerase chain reaction (PCR) approach for screening of HIV-1 positive cells. Of the 507 potential HIV-1 positive clones detected, we chose three for further bioinformatic analysis that resulted in observation of two novel open reading frames within HIV-1 integrase and reverse transcriptase genes.

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LIST OF ABBREVIATIONS

AI	attachment inhibitor
AIDS	acquired immunodeficiency syndrome
APOBEC	apolipoprotein B mRNA-editing enzyme catalytic polypeptide
ART	antiretroviral therapy
BST-2	bone marrow stromal cell antigen 2
CA	capsid
CCR5	c-c motif chemokine receptor 5
CCRA	chemokine coreceptor antagonist
CDC	center for disease control and prevention
CXCR4	c-x-c motif chemokine receptor type 4
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
Env	envelope
FBS	fetal bovine serum
FI	fusion inhibitor
Gag	group-specific antigen
gp	glycoprotein
HEK293T	human embryonic kidney 293T cell line

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	human immunodeficiency virus type 1
HLA-DR	major histocompatibility complex, class II, DR
HVS	herpesvirus saimiri
IN	integrase
IL	interleukin
INR	immunological non-responder
INSTI	integrase strand transfer inhibitor
LTR	long terminal repeat
LN2	liquid nitrogen
Luc	luciferase
MA	matrix
MI	maturation inhibitor
MHC-I	major histocompatibility complex class I
NC	nucleocapsid
Nef	negative factor
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside/nucleotide reverse transcriptase inhibitor
ORF	open reading frame

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PI	protease inhibitor
PLWH	people living with HIV-1
Pol	polymerase
Post-AI	post-attachment inhibitor
PR	protease
Rev	regulator of expression of viral proteins
RNA	ribonucleic acid
RNaseH	ribonuclease H
RPMI	Roswell Park memorial institute medium
RT	reverse transcriptase
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SIV	simian immunodeficiency virus
Tat	trans-activator of transcription
TCR	T-cell receptor
UNAIDS	joint united nations programme on HIV and AIDS
usHIV-RNA	unspliced HIV-RNA

Vif	virion infectivity factor
Vpr	viral protein R
Vpu	viral protein U
VSV-g	vesicular stomatitis virus glycoprotein

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that targets immune cells of both myeloid and lymphoid lineages in infected patients, severely impacting their ability to fight infection through the loss and deregulation of immune system functions (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983). HIV-1 is the causative agent of acquired immunodeficiency syndrome (AIDS). Since the start of the HIV epidemic in the 1980s, an estimated 84 million people have been infected with HIV and 40 million have died from AIDS-related illnesses worldwide (UNAIDS, 2022). An estimated 39 million people globally are living with HIV, 1.3 million new infections were reported, and 630,000 people died from AIDS-related illnesses in 2022 (UNAIDS, 2022). In the United States alone, 1.1 million people are living with HIV, with 30,635 new HIV cases in 2020 (CDC, 2022).

Host cells that express CD4 and chemokine receptors, CCR5 or CXCR4, primarily $CD4^+$ T cells (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984) and monocyte-derived cells (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996) are susceptible to HIV-1 infection. HIV-1 is an enveloped virus with surface and transmembrane glycoproteins gp120 and gp41, that bind to receptors on the host cell surface triggering membrane fusion, and subsequent release of the viral capsid and its contents (Figure 1, steps 1-3) (Maddon *et al.*, 1986; McDougal *et al.*, 1986; Stein *et al.*, 1987). Two single-stranded positive-sense viral genomic RNA molecules, Nucleocapsid (NC) and viral enzymes: Reverse transcriptase (RT), Integrase (IN), and Protease (PR), are encapsulated in the core of the virion, as depicted in Figure 1 (Frankel & Young, 1998; Freed, 1998). The viral genomic RNA is reverse transcribed into double-stranded DNA by HIV-1 RT (Figure 1, step 4). Human immunodeficiency virus type 1 (HIV-1) RT lacks 3'-exonuclease proofreading activity contributing to approximately 10^{-5}–10^{-3}

errors/bp/cycle and 10^{-4}–10^{-2} mutants/clones during positive and negative-strand synthesis (Yeo *et al.*, 2020). The HIV-1 DNA is then integrated into the host cell genome by Integrase (Figure 1, step 5), thereby creating the provirus. The provirus is transcribed through host cell mechanisms, allowing for multiple copies of viral RNA to be transcribed under control of the 5'-long terminal repeat (LTR) promoter region that may be translated into viral proteins or packaged as a viral genome (Figure 1, steps 6-7). Synthesized HIV-1 proteins and HIV-RNA assemble at the cell membrane into immature particles (Figure 1, steps 8-9). Subsequently, the precursor Gag molecules are cleaved by Protease during or after budding, forming the mature form of the virion (Figure 1, step 10) (Gottlinger *et al.*, 1989).

The HIV-1 proviral genome contains nine genes: three structural and enzymatic genes (*gag, pol, env*), two regulatory genes (*tat, rev*), and four accessory genes (*vif, vpu, vpr, nef*) and is flanked by identical 5'- and 3'-LTR regions (Figure 2) (Frankel & Young, 1998). The 55-kDa group-specific antigen (Gag) precursor is cleaved into matrix protein p17 (MA), capsid protein p24 (CA), NC protein p7, P6 protein, and spacer peptides p1 and p2 (Freed, 1998). A regulated -1 ribosomal frameshift near the 3'-end of *gag* generates precursor protein, GagPol (Wills *et al.*, 1994). Viral enzymes RT, IN, PR, and RNaseH are encoded by HIV-1 *pol* (Katz & Skalka, 1994) . Structural gene *env* encodes gp160, which is cleaved into surface and transmembrane glycoproteins gp120 and gp41 (Allan *et al.*, 1985; Robey *et al.*, 1985; Veronese *et al.*, 1985). Regulatory gene, trans-activator of transcription (Tat) binds to the stem-loop secondary structure within the 5'-LTR region to regulate transcription of the integrated proviral genome (Dayton *et al.*, 1986; Rosen *et al.*, 1985). Another regulatory gene, regulator of expression of virion proteins (Rev) harbors a nuclear import and export signal, allowing Rev to export unspliced and partially spliced mRNAs to the cytoplasm for translation (Meyer & Malim, 1994). Although

given the name as accessory genes, Vif, Vpr, Vpu, and Nef collectively assist in important roles in viral infectivity and persistence by regulating expression of viral and cellular proteins. Accessory protein, Vif is critical for *in vivo* HIV-1 replication by inducing degradation of APOBEC cytidine deaminase proteins (apolipoprotein BmRNA-editing catalytic polypeptide) (Sheehy *et al.*, 2002). Viral protein Vpr induces G2/M cell cycle arrest to enhance HIV-1 gene expression through depletion of host chromosome periphery protein CCDC137 (Zhang & Bieniasz, 2020). Host transmembrane protein BST-2 that tethers virions to the plasma membrane of infected cells, inhibiting virus release is downregulated by HIV-1 Vpu (Neil *et al.*, 2008; Van Damme *et al.*, 2008). Negative factor (Nef) downregulates surface expression of CD4 (Aiken *et al.*, 1996) and major histocompatibility complex class I (MHC-1) (Schwartz *et al.*, 1996) to evade innate and adaptive immune responses, among other crucial roles.

$CD4^+$ T cell counts, and viral load (HIV RNA copies/mL of plasma) are used to monitor and predict disease progression, evaluate treatment efficacy, and assess risk of opportunistic infections in HIV-1-infected patients (Mellors *et al.*, 1997). Viral suppression is achieved in many people living with HIV-1 (PLWH) through the use of antiretroviral therapy (ART), a combination of HIV-1 inhibitor-containing regimens that prevent productive infection and production of new infectious virions (Fischl *et al.*, 1987; Gulick *et al.*, 1997; Mitsuya *et al.*, 1985). Viral suppression is defined as 200 or fewer copies of HIV RNA per milliliter of blood (HIV.gov, 2023). Antiretroviral agents are divided into drug classes and classified based on the viral life cycle step they inhibit. The classes consist of viral entry inhibitors including fusion, attachment (AI) and post-attachment inhibitors (post-AI), and CCR5 antagonists (CCRA), nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase strand transfer inhibitors (INSTIs), protease

inhibitors (PIs), and capsid inhibitors (Link *et al.*, 2020) (Figure 1) (HIVinfo.NIH.gov, 2021). A combination of antiretroviral agents directed against at least two distinct targets are necessary for HIV-1 suppression due to emergence of drug resistant HIV-1 strains from high cell turnover (Coffin, 1995; Wei *et al.*, 1995). Although ART is not a cure for HIV-1, consistent treatment decreases the risk of mortality and morbidity and the frequency of opportunistic infections in HIV-1-infected patients. Additionally, viral suppression in HIV-1-infected patients prevents sexual transmission of HIV-1 and reduces the risk of HIV-1 transmission through pregnancy and breastfeeding (HIV.gov, 2023). Global health emergencies, such as the recent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic and monkeypox virus outbreak, and racial disparities and inequitable access to HIV prevention and treatment, creates new challenges in advances of treatment and prevention of HIV (Gandhi *et al.*, 2023).

Despite prolonged suppression of plasma viral loads in many patients on ART, diverse HIV-1 reservoirs continue to persist in suppressed HIV-1-infected patients creating a significant obstacle in HIV-1 eradication (Finzi et al., 1997; Wong et al., 1997). Integrated full-length intact proviruses make up less than 10% of HIV-1 proviruses in suppressed patients and are considered latent due to lack of viral gene expression. Latent HIV-1 reservoirs are established early in infection and although the frequency of latently HIV-1-infected CD4 T cells is low (approximately 1 to 10 cells per 10^6 cells in the peripheral circulation), they may contribute to lifelong persistence of HIV-1 in patients (Chun *et al.*, 1997; Finzi *et al.*, 1999). Some full-length intact proviruses are replication-competent and can contribute to rebound viraemia if treatment is interrupted (Davey *et al.*, 1999; Harrigan *et al.*, 1999). Latent HIV-1 reservoirs harboring intact proviruses were found to express surface markers that may reduce exposure to host immune responses, promoting cell survival (Sun *et al.*, 2023). Although intact proviruses may be able to

evade host immune responses, they have been shown to decline more rapidly than cells containing nonintact or defective HIV-1 proviruses (Peluso *et al.*, 2020). Defective HIV-1 proviruses contain insertions, deletions, and point mutations that are introduced into HIV-1 sequences through lack of viral RT proofreading activity and recombination events (Coffin, 1986; Preston *et al.*, 1988; Roberts *et al.*, 1988). Over 90% of all HIV-1 proviruses in the peripheral blood of suppressed HIV-1-infected patients on ART are considered defective (Bruner *et al.*, 2016). Clonal expansion of HIV-1-infected cells harboring defective proviruses in prolonged HIV-1-suppressed patients have been shown to persist more than 10 years *in vivo*, suggesting that persistence of antibodies to multiple HIV-1 proteins could develop due to release of virus or expression of viral proteins from defective HIV-1 proviruses (Imamichi *et al.*, 2014; Singh *et al.*, 2023).

Although viremia can be suppressed to undetectable levels, chronic immune activation persists in ART-suppressed HIV-1-infected patients. Some suppressed patients do not regain normalization of $CD4^+$ T cell count levels despite persistent virological suppression, and are referred to as immunological non-responders (INRs) (Kelley *et al.*, 2009; Robbins *et al.*, 2009; Valdez *et al.*, 2002). The normal $CD4^+$ cell count in adults range from 500 and 1500 cells/mm^3. HIV-1-infected patients with baseline $CD4^+$ cell counts <200cells/mm^3 at the start of ART were less likely to achieve normal $CD4^+$ cell counts in comparison to patients with baseline $CD4^+$ cell counts >350cells/mm^3, despite prolonged virological suppression. Initiation of treatment in the early stages of HIV-1 infection may result in full restoration of $CD4^+$ cell counts more often than when treatment begins at a later stage of infection, regardless of suppression of HIV-1 replication (I.S.S Group *et al.*, 2015).

HIV-1-infected patients with sustained viral suppression show heightened $CD4^+$ and $CD8^+$ T cell activation in comparison to non-HIV-infected patients (Hunt *et al.*, 2008; Hunt *et al.*, 2003; Kaufmann *et al.*, 2002). Lower $CD4^+$ T cell gains were observed in ART-suppressed patients with persistent T cell activation (Hunt *et al.*, 2003). In addition, compared to non-HIV-infected counterparts, persistently higher serum immune activation markers and coagulation-associated biomarkers were observed in suppressed HIV-1 infected patients (French *et al.*, 2009; Neuhaus *et al.*, 2010; Sandler *et al.*, 2011). Induced activation of inflammatory and coagulation biomarkers in virologically suppressed HIV-1-infected patients may suggest ongoing immune activation and association with increased risk of disease and death from non-AIDS related complications (Phillips *et al.*, 2008). Chronic immune activation in suppressed HIV-1-infected patients increases the risk of non-AIDS related cancers (Chaturvedi *et al.*, 2007; Kirk *et al.*, 2007; Patel *et al.*, 2008), liver disease, cardiovascular disease (Currier *et al.*, 2003; Triant *et al.*, 2007), and other HIV-1 associated chronic comorbidities (Phillips *et al.*, 2008). Higher levels of cell-associated HIV RNA and $CD4^+$ T cells expressing activation marker HLA-DR were observed in defective proviruses isolated from INRs compared to responders, suggesting defective proviruses may play a role in the immunological nonresponse of INRs (Scrimieri *et al.*, 2024).

Although the cause for persistent HIV-associated immune activation in prolonged HIV-1-suppressed patients is still not fully understood, our theory is that defective HIV-1 proviruses may play a role in persistent immune activation and chronic inflammation. Proviral DNA-unspliced HIV-RNA (usHIV-RNA) pairs were identified from clonally expanded defective proviruses, supporting the theory that some replication-incompetent defective proviruses remain transcriptionally active (Imamichi *et al.*, 2016). Furthermore, it was observed that some

replication-incompetent defective proviruses also remain translationally active through detection of HIV-1 viral proteins, Gag and Nef (Imamichi *et al.*, 2020). Defective proviruses that remain transcriptionally and translationally active could induce activation of the host's innate and adaptive immune responses (Singh *et al.*, 2023).

Expression profile and function analysis of viral proteins and novel chimeric proteins translated by defective proviruses may provide further insight into the persistence of immune activation observed in suppressed patients. However, only one direct demonstration of HIV-1 protein expression from a defective provirus has been observed in clonally expanded $CD4^+$ T cells collected from an HIV-1-suppressed patient thus far (Imamichi *et al.*, 2020). Therefore, we propose developing an *in vitro* model for generation of $CD4^+$ T cell clones to gain a broader scope of canonical and potentially novel viral gene expression and function from defective proviruses obtained from suppressed HIV-1-infected patients. Here, we present our approach in developing optimal cell culture growth conditions for $CD4^+$ T cell expansion, a reproducible unbiased screening approach for detection of HIV-1 positive cells, and an analysis pipeline linking HIV-1 open reading frames (ORFs) obtained from LTR-to-LTR sequencing to predicted protein expression of defective proviruses.

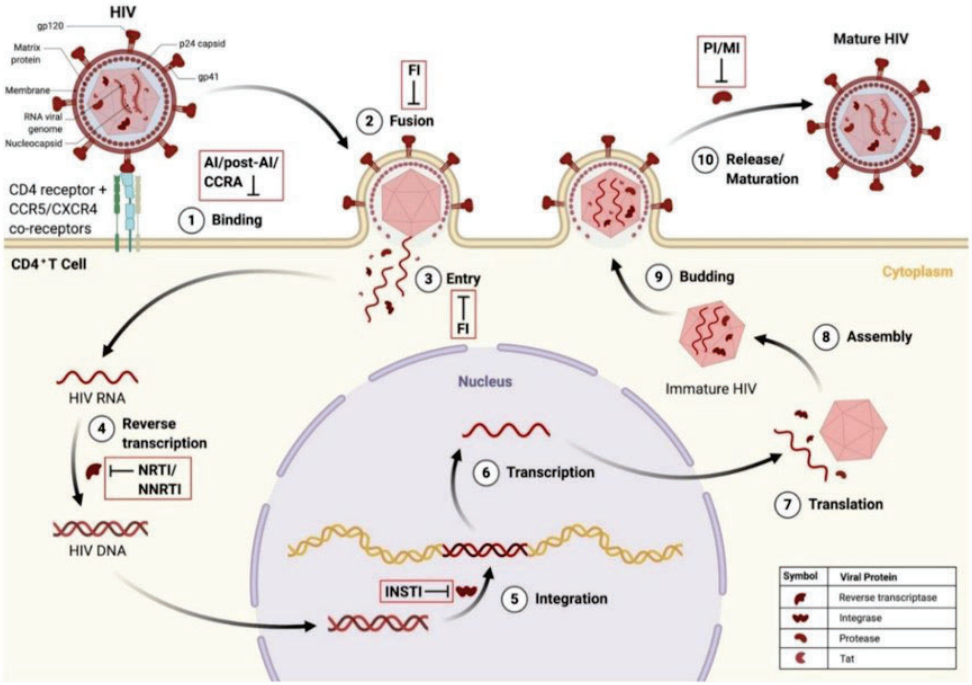

Figure 1. HIV-1 viral life cycle and antiretroviral therapy intervention.
Illustration reproduced from Rudd and Toborek (2022).

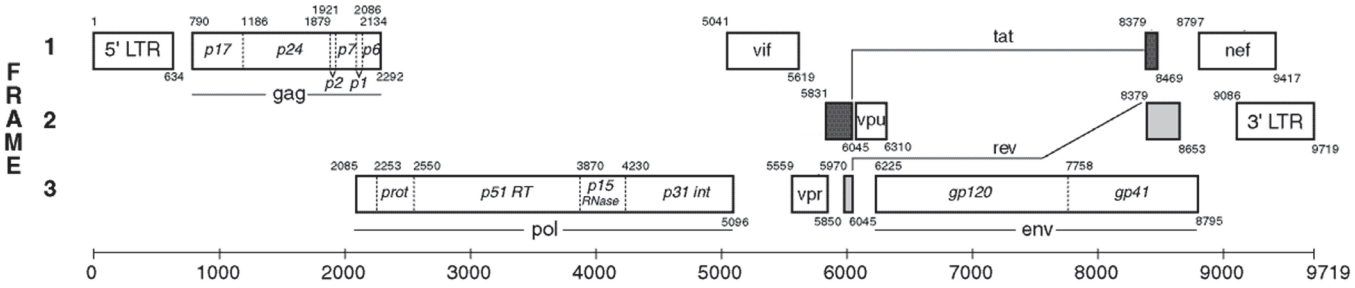

Figure 2. HIV-1 genome.
The HIV-1 genome contains nine genes and is flanked by two long terminal repeats at the 5'- and 3'-ends and is approximately 9.8kb in length. Regulatory proteins, Tat and Rev are encoded by two exons. The start and end coordinates of each gene and encoding regions are indicated. Illustration modified from Los Alamos National Laboratory (2023).

MATERIALS AND METHODS

$CD4^+$ T Cell Isolation, Activation, and Expansion

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor's apheresis using lymphocyte separation medium (MP Biomedicals) and $CD4^+$ T cells were isolated from the PBMCs using EasySep human $CD4^+$ T cell isolation kit (Stemcell Technologies). One day before isolation, a T-25 flask was coated with 10ug/mL purified NA/LE Mouse Anti-Human CD3 antibody and 10ug/mL purified NA/LE Mouse Anti-Human CD28 antibody (BD Biosciences) in 1X PBS (Quality Biological) overnight at 37°C; the coated T-25 flask was washed three times with 1X PBS before plating. Isolated $CD4^+$ T cells were transferred to the coated flask at $2x10^6$ cells per mL of RP-AIMV10 culture medium, 1:1 ratio of RPMI 1640 medium (Thermo Fisher Scientific) and AIM-V medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (R&D Systems, Bio-techne), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 (Quality Biological) and 5 µg/ml of Gentamycin (Thermo Fisher Scientific). $CD4^+$ T cells were bulk activated for three days at 37°C. Activated $CD4^+$ T cells were then plated in 96-well round-bottom plates at a 1:2 bead to cell ratio of anti-biotin MACSiBead particles coated with 10ug/mL biotinylated anti-CD3 and anti-CD28 antibodies (Miltenyi Biotec) in RP-AIMV10 medium supplemented with 50U/mL human recombinant interleukin-2 (IL-2) (R&D Systems). Once confluent, cells were transferred to 96-well flat-bottom plates and then subsequently to larger vessels in wells pre-coated with 10ug/mL purified NA/LE Mouse Anti-Human CD3 antibody and 10ug/mL purified NA/LE Mouse Anti-Human CD28 antibody (BD Biosciences) in 1X PBS (Quality Biological) one day prior.

Recombinant HIV-1 Viruses

HEK293T cells were co-transfected with pNL4-3.Luc.R-E- (hereafter referred to as pNL4-3.Luc.E-) and pCMV-VSV-G using TransIT-293 transfection reagent (Mirus) (Connor *et al*., 1995; He *et al*., 1995). Supernatant of cultured HEK293T cells was collected at 48 hours post transfection and filtered with a 0.22-µm filter unit prior to ultracentrifugation at 28,000 RPM for 2 hours at 4ºC on a 20% sucrose layer. A pseudotyped VSV-G envelope was used to supplement the envelope-deficient NL4-3.Luc.E- virions, containing a luciferase reporter gene in the position of Nef. Viral particles were resuspended in DMEM and quantified by Simoa p24 HIV assay kit (Quanterix).

HEK293T cells were transfected with pNL(AD8) (hereafter referred to as pNL4-3.AD8) using TransIT-293 transfection reagent (Mirus) and co-cultivated with PHA-stimulated CD4 T cells 24 hours post transfection. Cells were harvested after 24 hours, centrifuged at 1500 RPM for 5 minutes at room temperature, and resuspended in RP10 medium with 10U/mL IL-2 in a T-25 flask and incubated for 7 to 10 days at 37ºC. The culture supernatant was layered onto a 20% sucrose cushion and ultracentrifuged at 28,000 RPM for 2 hours. Viral particles were resuspended in DMEM and quantified by viral stock titration (Zhang et al., 1997).

HIV-1 Infection

Bulk activated $CD4^+$ T cells were transferred to 1.5mL screw cap tubes at $2x10^6$ cells per mL in RP-AIMV10 medium. Cells were infected with 100ng/mL replication-incompetent VSVg-pseudotyped HIV-1 (NL4-3.Luc.E-). The infectivity of NL4-3.Luc.E- virus stock was measured by Simoa p24 HIV assay kits (Quanterix). Tubes were placed on a constant rotator and placed at 37°C for 2 hours. Infected cells were washed three times with warm RP-AIMV10 medium and cultured at 37°C. Cells infected with replication-competent HIV-1 (NL4-3.AD8) at

a multiplicity of infection of 1 infectious particle per 1000 cells (Zhang *et al.*, 1997) were cultured in RP-AIMV10 medium supplemented with 10nM dolutegravir, 10nM darunavir, and 10nM efavirenz (Selleckchem). Cells were infected and washed as previously described. For evaluation of luciferase activity in cells infected with NL4-3.Luc.E-, an aliquot of cells were plated in quadruplicate wells at $25x10^4$ cells/well and were lysed in 1X Passive Lysis Buffer (Promega). Luciferase activity was measured using the Luciferase Assay System (Promega) and protein concentrations by BCA protein assay kit (Thermo Fisher Scientific).

HIV-1 Copy Number

DNA isolation was performed with QIAamp DNA purification and isolation kit (Qiagen) to evaluate infection rates of cells infected with NL4-3.Luc.E- and NL4-3.AD8. DNA concentration and purity were measured by a Denovix spectrophotometer/fluorometer. Samples were run for comparative CT using quantitative real-time PCR instrument QuantStudio3 (Applied Biosystems) with Taqman RNase P Detection reagents (Applied Biosystems), and customized Late RT (FWD: 5'-TGTGTGCCCGTCTGTTGTGT-3', REV: 5'-GAGTCCTGCGTCGAGAGAGC-3', PROBE: 5'-(FAM)-CAGTGGCGCCCGAACAGGGA-(TAMRA)-3') and Late RT NL4-3 (FWD: 5'-TGTGTGCCCGTCTGTTGTGT-3', REV: 5'-GAGTCCTGCGTCGAGAGATC-3', PROBE: 5'-(FAM)-CAGTGGCGCCCGAACAGGGA-(TAMRA)-3') (Butler *et al.*, 2001) probes with Taqman Universal PCR Master Mix (Thermo Fisher Scientific) at an initial incubation of 50ºC for 2 minutes and 95ºC for 10 minutes, followed by 40 cycles of amplification at 95ºC for 15 seconds and 60ºC for 1 minute. Known concentrations of human genomic DNA (Thermo Fisher Scientific) and HIV-1 plasmids were used as standards for determining the absolute HIV-1 copy numbers per cell.

Amplification Of HIV-1 Proviruses

To screen wells for HIV-1 positive cells, cells were transferred to 96-well V-bottom plates and lysed with 120uL DirectPCR lysis reagent and proteinase K (Viagen Biotech) and placed on a shaker at 56°C for 5-6 hours. Cell lysates were then transferred to 96-well PCR plates and placed on a thermocycler at 85°C for 45 minutes. The near full-length PCR was performed with VeriFi high fidelity DNA polymerase (PCR Biosystems) with the initial denaturation at 95°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 10 seconds, annealing at 64°C for 15 seconds, and extension at 72°C for 5 minutes, with the final extension at 72°C for 10 minutes with primers (FWD 756 5'-CGGCGACTGGTGAGTACGCCAA-3', REV: 8966 5'-CCTCCTCCTCTTGTGCTTCTAGCC-3'). Second round near full-length amplification was run with PCR primers (FWD: 796 5'-GCGAGAGCGTCAGTATTAAGC-3', REV: 8538 5'-CAATCAAGAGTAAGTCTCT-3') as previously described with the exception of annealing temperature of 60°C. The short-length PCR primers were performed as previously mentioned with an extension at 72°C for 1 minute with outer PCR primers (FWD: 756 5'-CGGCGACTGGTGAGTACGCCAA-3', 5'- REV: 2302 CTAATAGAGCTTCCTTTAGTTGCC-3'), followed by nested amplification with inner PCR primers (FWD: 796 5'-GCGAGAGCGTCAGTATTAAGC-3', REV: 1960 5'-CTTTGCCACAATTGAAACACTT-3'). PCR reactions were performed in volumes of 25uL in 0.2mL 96-well PCR plates. The final concentrations of both primers were 400nM. The PCR products were purified with QIAamp purification kits (Qiagen).

HIV-1 Sequencing

PCR products of near full-length amplicons or the longest amplicons obtained from the series of 24 reverse primers were sequenced with PCR primers spanning the HIV-1 genome

(Table 1, Figure 3) using the 3500xL Genetic Analyzer (Applied Biosystems) with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Sequences were assembled to HXB-2 HIV-1 reference sequence in Sequencher and sent to our in-house bioinformatics group for analysis of predicted viral protein expression from HIV-1 ORFs.

Table 1. PCR primers used for HIV-1 sequencing.

Coordinates based on HXB2 reference sequence. The symbol "+" indicates sense orientation, and antisense as "-".

Primer Location	Sequence
+796	GCGAGAGCGTCAGTATTAAGC
+836	GGGAAAAAATTCGGTTAAGGCC
+1127	AAAAGGCACAGCAAGCAGCAGCT
-1631	TTTGGTCCTTGTCTTATGTCCAGAATGC
+1546	AATCCACCTATCCCAGTAGGAGAAAT
-1960	CTTTGCCACAATTGAAACACTT
+2078	AGGCTAATTTTTTAGGGA
+2165	CAGAAGAGAGCTTCAGGTTTGGG
-2138	TGTTGGCTCTGGTCTGCTCT
-2302	CTAATAGAGCTTCCTTTAGTTGCC
-2603	GGCCATTGTTTAACTTTTGGG
+2603	CCCAAAAGTTAAACAATGGCC
-2691	TATGGATTTTCAGGCCCAATTTTTGA
-2872	TGCATCACCCACATCCAGTA
-3246	CCATTTATCAGGATGGAGTTC
-3299	TGTCATTGACAGTCCAGCTG
+3194	CACACCAGACAAAAAACATCAG
+3379	RGCAATTATGTAAACTCCTTAGGGGA
-3501	TAAGTCTTTTGATGGGTCATAATA
+3869	CTATGTAGATGGGGCAGCTA
-4176	TCTACTTGTTCATTTCCTCC
+4024	AAGTAAACATAGTAACAGACTCAC
+4162	CACACAAAGGAATTGGAGGAAATG
+4745	TAAGACAGCAGTACAAATGGCAG
-4956	TACTGCCCCTTCACCTTTCCA
-5195	TAGTGGGATGTGTACTTCTGAAC
+5550	AGAGGATAGATGGAACAAGCCCCAG
-5562	TGGTCTTCTGGGGCTTGTTC
-5833	AGGGCTCTAGTCTAGGATCTACTGGCTCCA
-5857	GGCTGACTTCCTGGATGCTTCCAGGGC
+5861	TGGAAGCATCCAGGAAGTCAGCCT
-6207	CTCTCATTGCCACTGTCTTCTGCTC
+6210	CAGAAGACAGTGGCAATGA
-6214	CACTCTCATTGCCACTGTCT
-6342	TTCCACACAGGTACCCCATA
+6445	GTGTACCCACAGACCCCAACCCACAAG
-6952	TTCCATGTGTACATTGTACTGTG
+6952	GCACAGTACAATGTACACATGGAA
-7515	GGCATACATTGCTTTTCC
+7515	GGAAAAGCAATGTATGCC
-7795	ATAGTGCTTCCTGCTGCTC
+7626	TTCAGACCTGGAGGAGGAGATATG
+7795	GAGCAGCAGGAAGCACTAT

Primer Location	Sequence
+7850	ACAATTATTGTCTGGTATAGTGCAACAGCA
-8538	CAATCAAGAGTAAGTCTCT
+8277	TTCATAATGATAGTAGGAG
-9146	CTGCCAATCAGGGAAGTAGCCTTGTGT
-9604	GCACTCAAGGCAAGCTTTATTGAGGCTTA

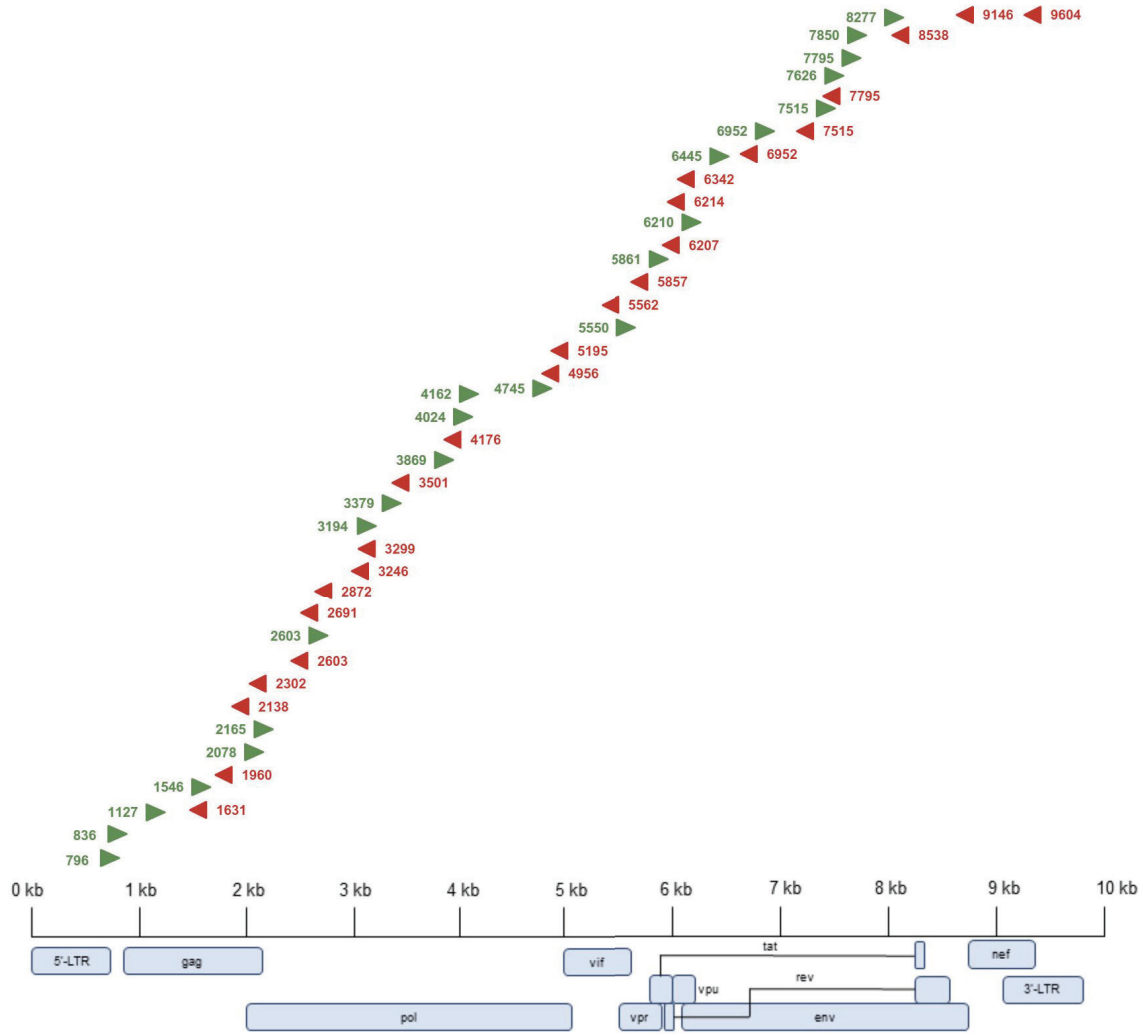

Figure 3. Coordinates of PCR primers used for HIV-1 sequencing.
A representative illustration of PCR primer coordinates based on HXB2 reference sequence. Primer locations and arrows that are green indicate sense orientation, and antisense as red. Created with Biorender.

RESULTS

RP-AIMV10 Culture Medium is Optimal for $CD4^+$ T Cell Growth

We expected to work with an infection rate of less than 1% HIV-infected $CD4^+$ T cells isolated from peripheral blood obtained from ART-suppressed HIV-1-infected patients, similar to previously published metrics (Eriksson *et al.*, 2013; Imamichi *et al.*, 2020; Wu *et al.*, 2023). To account for this infection rate and reduce risk of more than one infected cell per well, we required a starting cell number of 1000 cells or less. To account for this limitation, we first established composition of culture media optimal for $CD4^+$ T cell culture. $CD4^+$ T cells isolated from PBMCs collected from healthy donors were cultured in RP10 medium or a combination of RP10 medium and serum-free medium AIM-V (RP-AIMV10 medium). The addition of AIM-V was recommended in a methods publication by Fleckenstein and Ensser (2004) for long-term expansion of $CD4^+$ T cells. Cells were seeded in triplicate ranging from 100 to 50,000 cells per well in 96-well round-bottom plates with anti-CD3/CD28-coated beads, as depicted in Figure 4A. Cell proliferation was monitored by total live cell counts collected at four separate time points: day 6, 9, 16 and 27 of culture. Cells cultured in RP-AIMV10 medium had significantly more cell growth in comparison to cells plated in RP10 medium (Figures 4B, 4C). The greatest cell counts were obtained at day 16 of culture in wells initially seeded at 5,000, 10,000, and 50,000 cells in RP-AIMV10 medium. Although wells were initially seeded at different cell concentrations, there were no significant differences in average cell counts collected at this time point. In contrast, cell counts were significantly reduced by five- to forty-fold in cells cultured in RP10 medium. A similar trend was observed in both donors. In either cell culture media, cell proliferation could not be monitored from wells initially seeded at 100 or 500 cells due to insufficient cell counts.

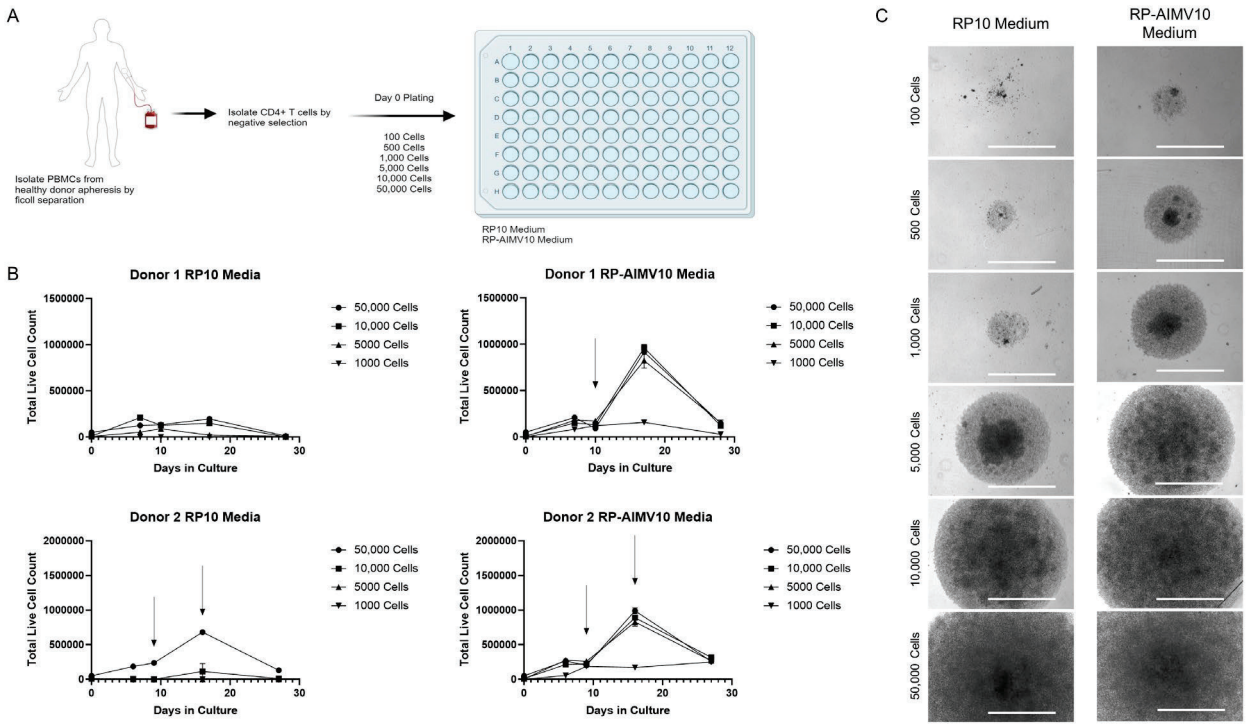

Figure 4. RP-AIMV10 medium is optimal for CD4$^+$ T cell proliferation.
(A) Isolation and culture conditions of CD4$^+$ T cells isolated from healthy donor apheresis. Cells were seeded in triplicate wells at several starting cell numbers. Created with Biorender. (B) Cell growth curves of cells cultured in RP10 medium versus RP-AIMV10 medium in two representative donors. Arrows indicate transfer of cells to subsequently larger vessels. (C) Representative images of cells cultured in RP10 or RP-AIMV10 medium 48-hours post plating. Scale bars: 80µm.

$CD4^+$ T Cell Proliferation is Enhanced by Activation with Anti-CD3/CD28-Coated Beads and Bulk Activation

RP-AIMV10 medium cultured $CD4^+$ T cells had increased cell growth in comparison to cells cultured in RP10 medium, although significant growth required a minimum starting cell concentration of 5,000 cells per well. In an aim to lower the number of cells required for initial seeding, we sought to establish the best approach for activation of $CD4^+$ T cells optimal for cell proliferation and expansion. Freshly isolated $CD4^+$ T cells were activated with anti-CD3/CD28 antibodies, either coated in PBS or coating buffer, or adsorbed on bead particles. Antibodies were used at 1ug/mL or 10ug/mL. Cells were seeded in quadruplicate at 1,000 cells per well in 96-well flat- or round-bottom plates and counted at day 7 of culture. The highest cell counts were observed in cells seeded in 96-well round-bottom plates with 10ug/mL anti-CD3/CD28-coated beads (Figure 5A). Cell proliferation was 10- to 16-fold lower in cells activated with 10ug/mL anti-CD3/CD28 antibodies plated in PBS or coating buffer in 96-well round-bottom plates. In cells plated in 96-well flat-bottom plates, high cell proliferation was observed in wells coated with 10ug/mL anti-CD3/CD28 antibodies in PBS. The average cell count of cells activated in 96-well round-bottom plates with coated beads were two times higher than for cells plated in wells coated with antibodies in PBS, although the difference was not significant ($p = 0.3073$). Cell proliferation was seven-fold lower in cells activated with 10ug/mL anti-CD3/CD28-coated beads or antibodies plated in coating buffer in 96-well round-bottom plates. In all activation methods, cell proliferation was significantly reduced by one- to fourteen-fold in cells activated with 1ug/mL antibodies.

In addition, we evaluated cell proliferation of $CD4^+$ T cells activated through bulk activation in comparison to cells plated immediately following isolation as described in previous

experiments (Figure 5B). $CD4^+$ T cells were isolated from aliquoted frozen PBMCs collected from a healthy donor and plated in a T-25 flask coated with anti-CD3/CD28 antibodies in PBS. The cells were collected after a three-day activation. At this time point, another aliquot of frozen PBMCs from the same donor were processed for $CD4^+$ T cell isolation. Bulk activated cells and newly isolated $CD4^+$ T cells were seeded at 100 cells per well in 96-well round-bottom plates cultured in RP-AIMV10 medium. The cells were seeded with anti-CD3/CD28-coated beads and stimulated with 50U/mL IL-2. We evaluated the initial growth of cells by observing the cells 48-hours post plating (Figure 5C). The cells activated in bulk were notably more confluent.

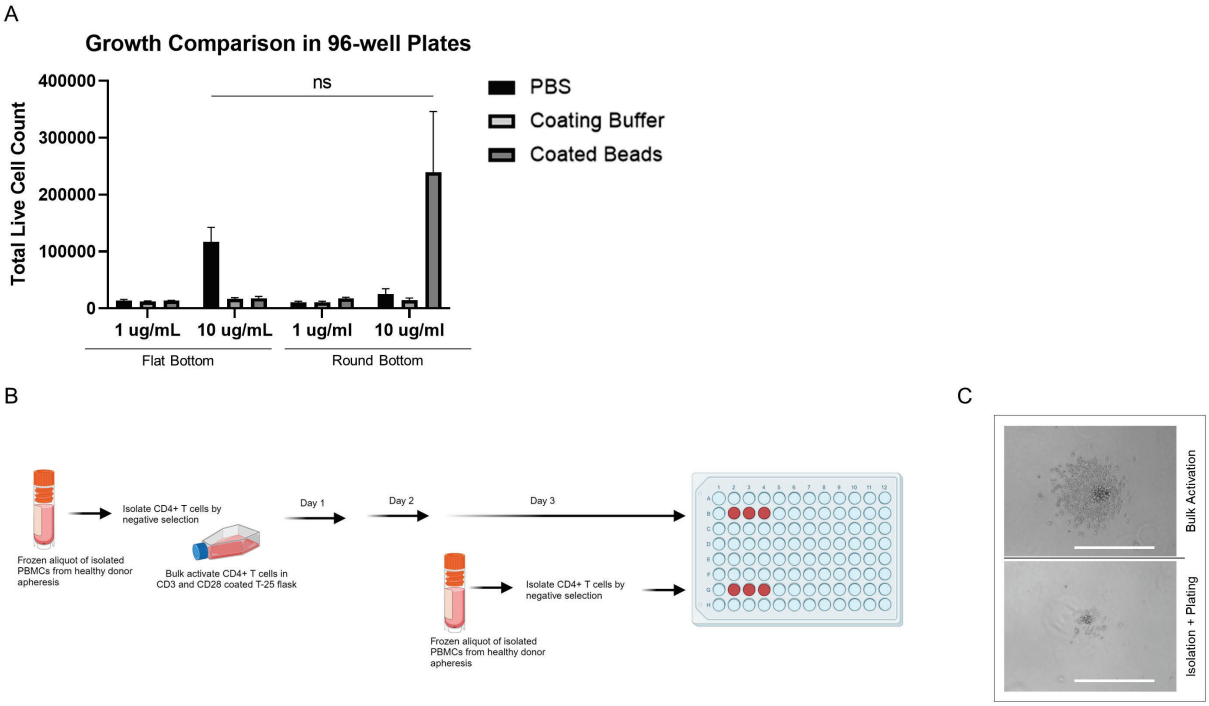

Figure 5. Anti-CD3/CD28-coated beads and bulk activation enhances CD4⁺ T cell proliferation.

(A) Growth comparison of activation methods and culture vessels for CD4⁺ T cell proliferation. Cells were seeded in quadruplicate and cell counts were obtained at day 7. Result of student's t-test are indicated, ns = non-significant. (B) Comparison of CD4⁺ T cells plated in triplicate with anti-CD3/CD28-coated beads either after a three-day bulk activation or directly after isolation. Created with Biorender. (C) Representative images of cells obtained from both methods are being displayed at two days post-plating. Scale bars: 80µm.

An Initial Seeding Concentration Of 1000 Cells Per Well Stimulated With 50u/mL IL-2 Is Optimal for $CD4^+$ T Cell Expansion

In continuation, we assessed effects of cytokines shown to induce $CD4^+$ T cell proliferation *in-vitro* (Geginat *et al.*, 2001). $CD4^+$ T cells were bulk activated and plated in triplicate in 96-well round-bottom plates at 100 cells per well with anti-CD3/CD28-coated beads in RP-AIMV10 medium (Figure 6A). Cytokines IL-2, -7, or -15 were added to cell culture at plating and maintained throughout the duration of culture. Cell counts were obtained at three timepoints: day 14, 28, and 35. The greatest cell proliferation was observed in cells stimulated with 50U/mL IL-2 (Figure 6B, 6C). An increase in growth was observed at each time point resulting in a 3.5-log increase at day 35. Significant cell proliferation was also observed in cells stimulated with 10U/mL IL-2 to day 35 but did not exceed a 1.5-log increase. Cell proliferation was significantly reduced in cells stimulated with IL-7 and IL-15.

Although we were able to successfully culture bulk activated $CD4^+$ T cells in RP-AIMV10 media with 50U/mL IL-2 at 100 cells per well, our last step was to determine the optimal initial seeding concentration to reach sufficient cell numbers required for screening of HIV-positive wells and expansion for further downstream experiments. As noted previously, we anticipate an infection rate lower than 1% in $CD4^+$ T cells obtained from ART-suppressed HIV-infected patients, resulting in our decision to compare wells seeded at 100 or 1000 cells per well. We bulk activated $CD4^+$ T cells and plated in a 96-well round-bottom plate cultured in RP-AIMV10 medium. We added anti-CD3/CD28-coated beads to wells and stimulated cells with 50U/mL IL-2. The cells were cultured until day 24 and cell counts were obtained at day 11 and day 24 (Figure 6D). A two-log increase was observed in both starting cell concentrations at day 11. Wells seeded at 1000 cells per well further increased one-log to day 24, with average cell

counts of 441,400 cells in donor 1 and 1,060,667 cells in donor 2. A 0.5-log increase from day 11 was observed in wells seeded at 100 cells per well at day 24.

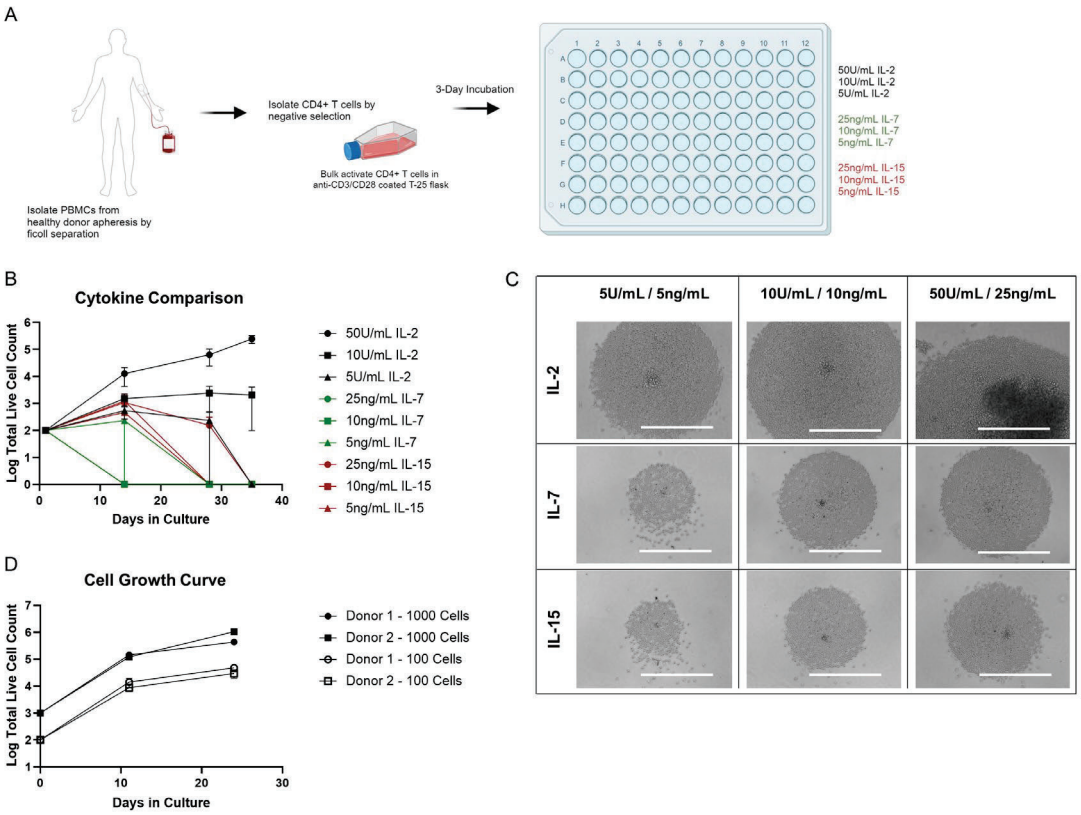

Figure 6. Optimal CD4$^+$ T-cell proliferation in 50U/mL IL-2.
(A) Isolation, activation, and culture conditions of CD4$^+$ T cells isolated from healthy donor apheresis. Created with Biorender. (B) Growth curve comparison of CD4$^+$ T cells stimulated with different concentrations of IL-2, -7, and -15. (C) Representative images of cells cultured with IL-2, -7 or -15 at eight-days post plating. Scale bars: 80µm. (D) Comparison of cell proliferation in CD4$^+$ T cells seeded at 100 or 1000 cells per well.

In Vitro HIV Infection Does Not Reduce Cell Proliferation

To confirm the previous culture conditions were suitable for infected cells, we first infected a subset of $CD4^+$ T cells from healthy donors with replication-incompetent VSVg-pseudotyped HIV-1 (NL4-3.Luc.E-) virions, following bulk activation (Figure 7A). Due to the ease of use of VSVg-pseudotyped infection, we were able to rapidly and accurately track LTR activity over time. To evaluate infection efficiency, we performed a copy number assay on NL4-3.Luc.E- infected cells 24-hours post infection. The HIV-1 copy number per cell was measured by quantitative real-time PCR with RNase P and HIV-1 probes, of DNA isolated from infected cells. Infected and uninfected cells were mixed at different infected to non-infected ratios (1:10, 1:100 and 1:1000) and seeded in 24-wells at 1000 cells per well in a 96-well round-bottom plate as described previously. Cell counts were obtained at three time-points: day 7, 15, and 26. No significant reduction was observed in cell counts obtained for 100% HIV-1-infected cells in comparison to uninfected cells ($p = 0.3385$) at day 26 (Figure 7B, 7C), indicating that our optimal $CD4^+$ T cell culture conditions are suitable for replication-incompetent infected cells. An unpaired two-tailed t-test was used in determining whether NL4-3.Luc.E- infection had an effect on cell proliferation.

Furthermore, we assessed the impact of infecting $CD4^+$ T cells with replication-competent HIV-1 (NL4-3.AD8) virions. A subset of cells were infected with HIV-AD8 at a multiplicity of infection of 1 infectious particle per 1000 cells. At 24-hours post infection, infected and a subset of uninfected cells were treated with a combination of HIV-1 antiretroviral drugs (protease inhibitor (Darunavir), integrase inhibitor (Dolutegravir), and non-nucleoside reverse transcriptase inhibitor (Efavirenz)) to prevent further productive infection, maintaining clonality of infected cells. Cells were seeded in 24-wells at 1000 cells per well in 96-well round-

bottom plates and cultured as described previously. At day 26 post plating, no reduction was observed in cell counts obtained for drug-treated uninfected and infected cells ($p = 0.4009$). Furthermore, there was no reduction in drug-treated uninfected cells in comparison to non-treated uninfected cells ($p = 0.1842$), indicating that antiretroviral drug treatments had no impact on cell growth in our culture conditions (Figure 7D).

Although NL4-3.Luc.E- and NL4-3.AD8 infection did not reduce cell proliferation in $CD4^+$ T cells cultured up to day 26, we questioned whether the infection rate of infected cells remained consistent throughout growth. We addressed this question by performing a short time-course copy number assay, as described previously. The number of HIV-1 copies in NL4-3.Luc.E- infected cells remains proportional, suggesting that infected and uninfected cells are growing at a similar rate (Figure 7E). The copy number rapidly declines in NL4-3.AD8 infected cells despite maintaining cell proliferation, indicating infected cells are being outgrown by uninfected cells (Figure 7E). Furthermore, we performed a long time-course and assessed whether infection rates remained consistent in NL4-3.Luc.E- infected cells plated at 100% infected and different infected to non-infected ratios (1:10, 1:100). The infection rate of NL4-3.Luc.E- infected cells remained consistent throughout the time course, representative of two independent donors, and a 10-fold difference was observed in cells infected at 100% and 10%, and similarly in 10% to 1% (Figure 7F).

The infection rate of NL4-3.Luc.E- infected cells remained consistent but rapidly declined in NL4-3.AD8 infected cells. Therefore, all further experiments were performed in NL4-3.Luc.E- infected cells. We observed no reduction in cell proliferation or infection rate in cells infected with NL4-3.Luc.E-, prompting our next question regarding active transcriptional activity of the infected cells throughout time of culture. Since a luciferase reporter gene is

inserted in our viral construct, we used luciferase activity as a measurement of LTR transcriptional activity over time. Infected NL4-3.Luc.E- cells were aliquoted in quadruplicate at $25x10^4$ cells/well and lysed, and luciferase activity was measured using a luciferase assay system. Luciferase activity in infected cells increased 15-fold to day 16, then rapidly declined to day 37 (Figure 7G), representative in two independent donors. Although LTR-mediated HIV-1 transcriptional activity of NL4-3.Luc.E- infected cells declined, transcriptional activity remained persistent throughout culture.

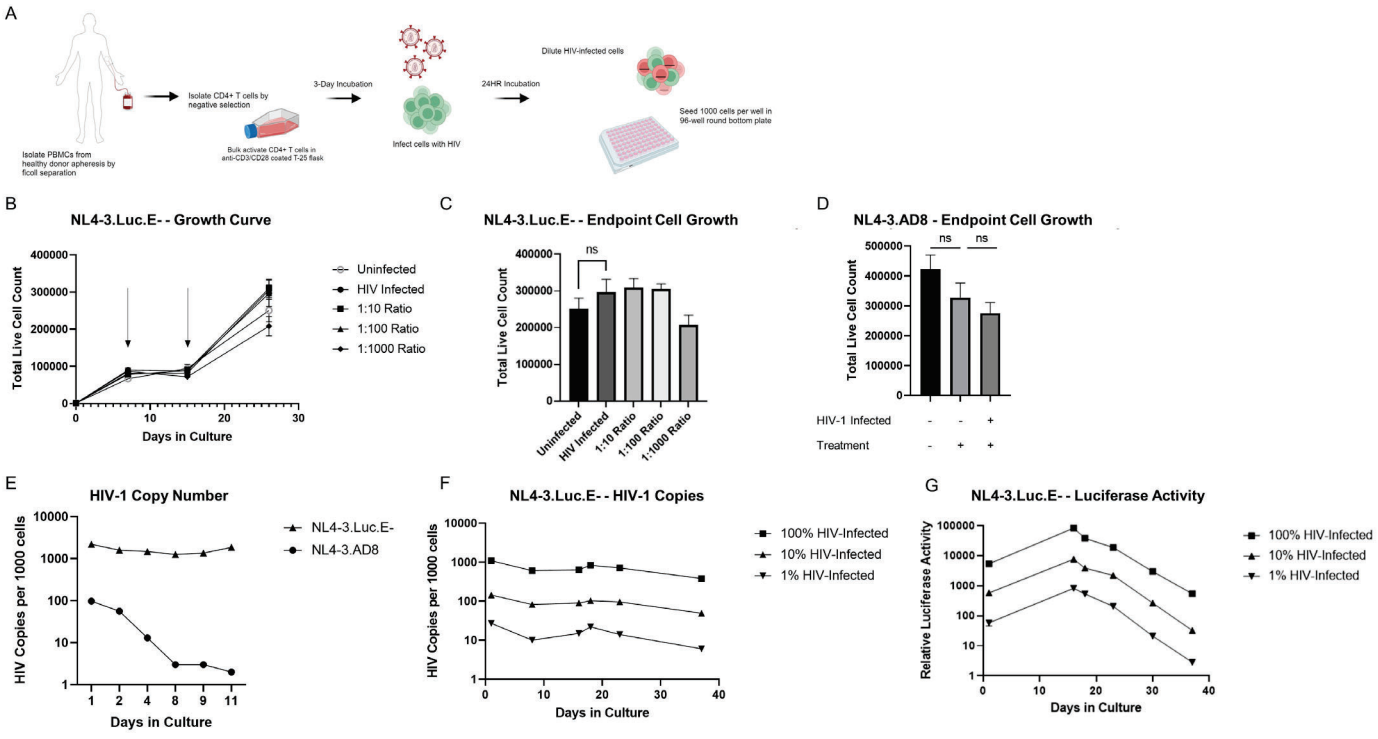

Figure 7. Cell proliferation and infection rates are maintained in NL4-3.Luc.E- infected CD4$^+$ T cells.

(A) *In vitro* HIV-1 infection of CD4$^+$ T cells isolated from healthy donor apheresis. Created with Biorender. (B) Growth curve of CD4$^+$ T cells infected with NL4-3.Luc.E virus and plated at different infected to uninfected ratios (1:10, 1:100, and 1:1000). Arrows indicate transfer of cells to subsequently larger vessels. (C) Bar graph comparison of cell counts of NL4-3.Luc.E- and (D) NL4-3.AD8 infected CD4$^+$ T cells at day 26. Result of student's t-test are indicated, ns = non-significant. (E) Comparison of HIV-1 copy numbers for cells infected with NL4-3.Luc.E- or NL4-3.AD8. (F) Time course of HIV-1 copy numbers and (G) corresponding relative luciferase activities in NL4-3.Luc.E- infected cells mixed at different infected to uninfected ratios (100%, 10%, and 1% infection).

High-Throughput Screening Process for Detection Of HIV-1 Positive Wells

We established that the infection rate in NL4-3.Luc.E- infected cells remains consistent over time in culture, indicating our ability to move forward with a PCR-based screening process to detect wells containing HIV-1 positive cells at any time-point in culture. First, we needed to establish a cost-effective high-throughput lysis method for direct PCR amplification to seed a multitude of wells to accommodate our goal in achieving multiple clones from a single patient. We lysed cells using DirectPCR lysis reagent and a compatible high-fidelity DNA polymerase, to eliminate the need of prior DNA isolation or purification. Next, we tested several combinations of primer sets near the 5'- and 3'-LTR from published (Imamichi *et al.*, 2020) and custom-designed primers, scoring each set based on length, amplification, sensitivity and reduction of non-specific binding (Tables 2-4, Figures 8-9). The highest scoring primer sets were evaluated for detection limits by sensitivity assays of infected to uninfected cell ratios at several concentrations. Detection of NL4-3.Luc.E- infected cells ranging from $1x10^2$ to $1x10^5$ cells were assessed in $1x10^5$ and $5x10^5$ uninfected cells for both near full-length and short amplification (Figure 10C and 10D). We concluded that a nested PCR approach was needed to detect lower concentrations of infected cells. We choose forward primer 756 and reverse primer 8966 for first-round near full-length PCR (Figure 10A). Following a nested PCR approach, we again tested combinations of primer sets internal from the chosen first-round forward and reverse primers (Table 3). We chose forward primer 796 and reverse primer 8538 for second-round near full-length PCR (Figure 10A). Although defective proviruses are truncated, HIV-1 *gag* is primarily retained (Imamichi *et al.*, 2016) prompting our decision to establish a short-length primer set flanking the *gag* region. Again, we chose forward primers 756 and 796 for first- and second-round PCR and tested reverse primers at the gag 3'-end (Table 4, Figure 10B). We chose

reverse primers 2303 and 1960 for short-length first- and second-round PCR (Figure 10B). The amplified cells detected with the short-length primer sets would yield an amplicon of approximately 1200bp within *gag* and would not be suitable for prediction of potential viral protein expression. In preparation of sequencing HIV-1 positive cells obtained using the short-length primer set, we established a series of 24 reverse primers spanning from the 5'-LTR to the 3'-LTR, published by Imamichi *et al.* (2020) (Figure 10E, 10F). The longest amplicon obtained from PCR amplification was used as the template for sequencing. In comparison, all HIV-1 positive wells obtained using the near full-length primer set were used as the sequencing template. HIV-1 positive templates were sequenced using a series of 48 reverse and forward primers spanning the HIV-1 proviral genome within LTR regions (Imamichi *et al.*, 2020). Sanger sequencing was performed using the 3500xL Genetic Analyzer. Obtained sequences from HIV-1 positive clones were aligned to HXB-2 HIV-1 reference sequences using Sequencher. Assembled sequences were sent to our in-house bioinformatics team for ORF analysis, allowing prediction of canonical HIV-1 proteins as well as potential novel chimeric protein expression.

Table 2. PCR primers tested for detection of HIV-1 positive cells

Coordinates based on HXB2 reference sequence. The symbol "+" or "F" indicates forward orientation, and reverse orientation as "-" or "R".

Primer Location	Sequence
F1	AAATCTCTAGCAGTGGCGCCCGAACAG
F2	TCTCTCGACGCAGGACTCGGCTTG
F3	ACAGGGACCTGAAAGCGAAAG
+711	CGCAGGACTCGGCTTGCTGAAG
+756	CGGCGACTGGTGAGTACGCCAA
+796	GCGAGAGCGTCAGTATTAAGC
+836	GGGAAAAAATTCGGTTAAGGCC
+1127	AAAAGGCACAGCAAGCAGCAGCT
-1631	TTTGGTCCTTGTCTTATGTCCAGAATGC
+1546	AATCCACCTATCCCAGTAGGAGAAAT
-1960	CTTTGCCACAATTGAAACACTT
+2078	AGGCTAATTTTTTAGGGA
+2165	CAGAAGAGAGCTTCAGGTTTGGG
-2138	TGTTGGCTCTGGTCTGCTCT
-2302	CTAATAGAGCTTCCTTTAGTTGCC

Primer Location	Sequence
-2603	GGCCATTGTTTAACTTTTGGG
+2603	CCCAAAAGTTAAACAATGGCC
-2691	TATGGATTTTCAGGCCCAATTTTTGA
-2872	TGCATCACCCACATCCAGTA
-3246	CCATTTATCAGGATGGAGTTC
-3299	TGTCATTGACAGTCCAGCTG
+3194	CACACCAGACAAAAAACATCAG
+3379	RGCAATTATGTAAACTCCTTAGGGGA
-3501	TAAGTCTTTTGATGGGTCATAATA
+3869	CTATGTAGATGGGGCAGCTA
-4176	TCTACTTGTTCATTTCCTCC
+4024	AAGTAAACATAGTAACAGACTCAC
+4162	CACACAAAGGAATTGGAGGAAATG
+4745	TAAGACAGCAGTACAAATGGCAG
-4956	TACTGCCCCTTCACCTTTCCA
-5195	TAGTGGGATGTGTACTTCTGAAC
+5550	AGAGGATAGATGGAACAAGCCCCAG
-5562	TGGTCTTCTGGGGCTTGTTC
-5833	AGGGCTCTAGTCTAGGATCTACTGGCTCCA
-5857	GGCTGACTTCCTGGATGCTTCCAGGGC
+5861	TGGAAGCATCCAGGAAGTCAGCCT
-6207	CTCTCATTGCCACTGTCTTCTGCTC
+6210	CAGAAGACAGTGGCAATGA
-6214	CACTCTCATTGCCACTGTCT
-6342	TTCCACACAGGTACCCCATA
+6445	GTGTACCCACAGACCCCAACCCACAAG
-6952	TTCCATGTGTACATTGTACTGTG
+6952	GCACAGTACAATGTACACATGGAA
-7515	GGCATACATTGCTTTTCC
+7515	GGAAAAGCAATGTATGCC
-7795	ATAGTGCTTCCTGCTGCTC
+7626	TTCAGACCTGGAGGAGGAGATATG
+7795	GAGCAGCAGGAAGCACTAT
+7850	ACAATTATTGTCTGGTATAGTGCAACAGCA
-8538	CAATCAAGAGTAAGTCTCT
+8277	TTCATAATGATAGTAGGAG
-8868	TCCCACCCTATCTGCTGCTGGC
-8882	TCTCGAGATGCTGCTCCCACCC
-8966	CCTCCTCCTCTTGTGCTTCTAGCC
-9039	GGCTAAGATCTACAGCTGCCTTG
-9081	GGAGTGAATTAGCCCTTCCAGTCCC
-9146	CTGCCAATCAGGGAAGTAGCCTTGTGT
-9604/R2	GCACTCAAGGCAAGCTTTATTGAGGCTTA
R3	CTAGTTACCAGAGTCACACAACAGACG
R1	TGAGGGATCTCTAGTTACCAGAGTC

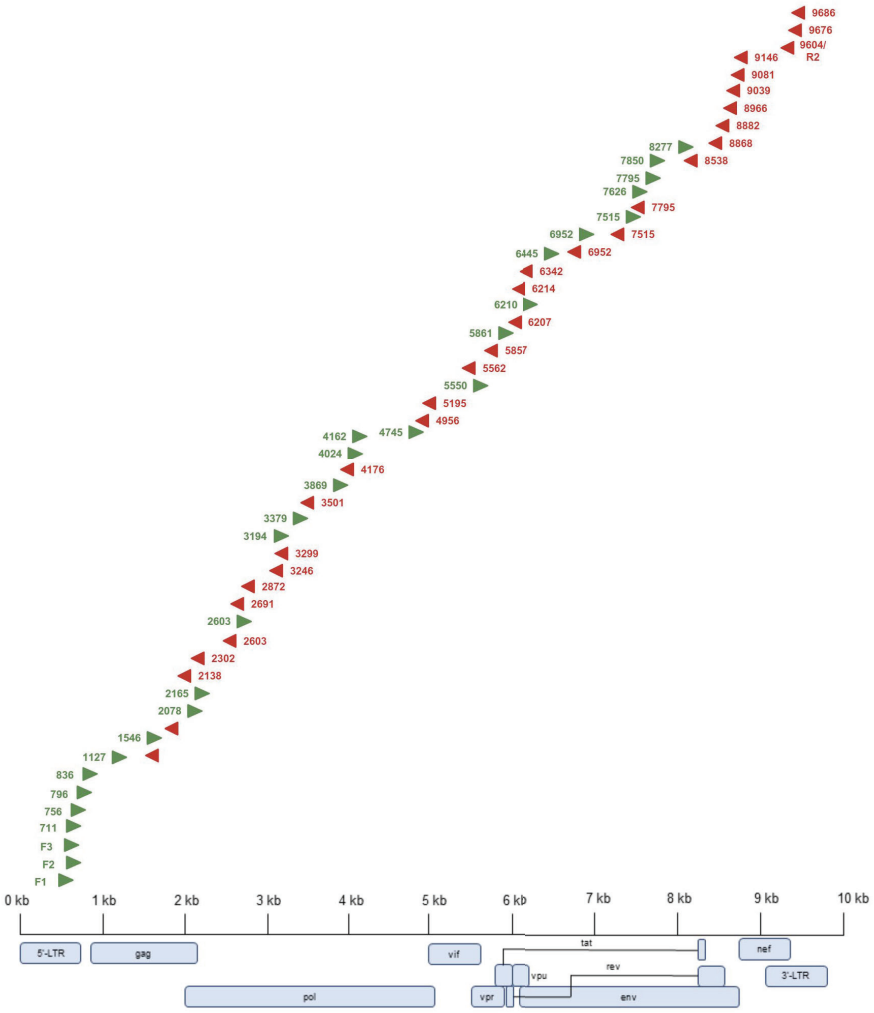

Figure 8. Coordinates of HIV-1 primers tested for detection of HIV-1 positive cells.
A representative illustration of PCR primer coordinates based on HXB2 reference sequence. Primer locations and arrows that are green indicate forward orientation, and reverse orientation as red. Created with Biorender.

Table 3. First round PCR primer sets for near full-length HIV-1 amplification.

Primer sets consisting of HIV-1 published and custom-designed primers were scored based on length of amplicon (bp), and amplification, sensitivity and non-specific binding of NL4-3.Luc.E- and NL4-3.AD8 plasmids as positive controls, uninfected $CD4^+$ T cells and a non-template control. Coordinates of primers are based on HXB2 reference sequence.

Forward	Reverse	Amplicon size (bp)	Amplification	Single Band	Specificity
F1	R1	9064	✓	x	x
F1	R3	9054	✓	x	x
F1	9604	9010	✓	x	x
F1	9146	8549	✓	x	x
F1	8538	7934	✓	x	x
F1	7795	7191	✓	x	x
F1	7515	6910	✓	x	x
F2	R1	9005	✓	x	x
F2	R3	8995	✓	x	x
F2	9604	8951	✓	x	x
F2	9146	8490	✓	x	x
F2	8538	7875	✓	x	x
F2	7795	7132	✓	x	x
F2	7515	6851	✓	x	x
F3	R1	9041	✓	x	x
F3	R3	9031	✓	x	x
F3	9604	8987	✓	x	x
F3	9147	8526	✓	x	x
F3	8538	7911	✓	x	x
F3	7795	7168	✓	x	x
F3	7515	6887	✓	x	x
796	R1	8891	✓	x	x
796	R3	8881	✓	x	x
796	9604	8837	✓	x	x
796	9147	8376	✓	x	x
796	8538	7761	✓	✓	✓
796	7795	7018	✓	✓	x
796	7515	6737	✓	✓	✓
836	R1	8851	✓	x	x
836	R3	8841	✓	x	x
836	9604	8797	✓	x	x
836	9147	8336	✓	x	x
836	8538	7721	✓	x	x
836	7795	6978	✓	x	x
836	7515	6697	✓	x	x
1127	R1	8560	✓	x	x
1127	R3	8550	✓	x	x
1127	9604	8506	✓	x	x
1127	9147	8045	✓	x	x
1127	8538	7430	✓	x	x
1127	7795	6687	✓	x	x
1127	7515	6406	✓	x	x
1546	R1	8141	✓	x	x
1546	R3	8131	✓	x	x
1546	9604	8087	✓	x	x
1546	9147	7626	✓	x	x
1546	8538	7011	✓	✓	✓
1546	7795	6268	✓	✓	✓

Forward	Reverse	Amplicon size (bp)	Amplification	Single Band	Specificity
1546	7515	5987	✓	✓	✓
2078	R1	7609	x	x	x
2078	R3	7599	x	x	x
2078	9604	7555	x	x	x
2078	9147	7094	x	x	x
2078	8538	6479	x	x	x
2078	7795	5736	x	x	x
2078	7515	5455	x	x	x
711	8538	7867	✓	x	x
711	8862	8200	✓	x	x
711	8882	8214	✓	x	x
711	8966	8300	✓	x	x
711	9039	8372	✓	x	x
711	9081	8416	✓	x	x
756	8538	7822	✓	✓	✓
756	8862	8155	✓	x	x
756	8882	8169	✓	x	x
756	8966	8255	✓	✓	✓
756	9039	8327	✓	x	x
756	9081	8371	✓	x	x
796	8538	7761	✓	✓	✓
796	8862	8094	✓	x	x
796	8882	8108	✓	x	x
796	8966	8194	✓	x	x
796	9039	8266	✓	x	x
796	9081	8310	✓	x	x

Second round primer sets for near full-length HIV-1 amplification.

Forward	Reverse	Amplicon size (bp)	Amplification	Single Band	Specificity
756	8966	8255	✓	x	x
756	8538	7822	✓	x	x
796	8966	8194	✓	x	x
796	8538	7761	✓	✓	✓

Table 4. First round PCR primer sets for short (5'-end) HIV-1 amplification.

Forward	Reverse	Amplicon size (bp)	Amplification	Single Band	Specificity
711	6342	5672	✓	x	x
711	6214	5544	✓	x	x
711	6207	5542	✓	x	x
711	5857	5194	✓	x	x
711	5833	5172	✓	x	x
711	5562	4892	✓	x	x
711	5195	4528	✓	x	x
711	4956	4287	✓	x	x
711	4176	3506	✓	x	x
711	3501	2835	✓	x	x
711	3299	2629	✓	x	x

Forward	Reverse	Amplicon size (bp)	Amplification	Single Band	Specificity
711	3246	2577	✓	x	x
711	2872	2202	✓	x	x
711	2691	2027	✓	x	x
711	2603	1934	✓	x	x
711	2302	1636	✓	x	x
756	6342	5627	✓	x	x
756	6214	5499	✓	x	x
756	6207	5497	✓	x	x
756	5857	5149	✓	x	x
756	5833	5127	✓	x	x
756	5562	4847	✓	x	x
756	5195	4483	✓	x	x
756	4956	4242	✓	x	x
756	4176	3461	✓	✓	✓
756	3501	2790	✓	✓	✓
756	3299	2584	✓	x	x
756	3246	2532	✓	✓	✓
756	2872	2157	✓	x	x
756	2691	1982	✓	✓	✓
756	2603	1889	✓	✓	✓
756	2302	1591	✓	✓	✓

Second round primer sets for short (5’-end) HIV-1 amplification.

Forward	Reverse	Amplicon size (bp)	Amplification	Single Band	Specificity
756	2302	1591	✓	x	x
796	2302	1530	✓	x	x
796	2138	1362	✓	x	x
796	1960	1186	✓	✓	✓

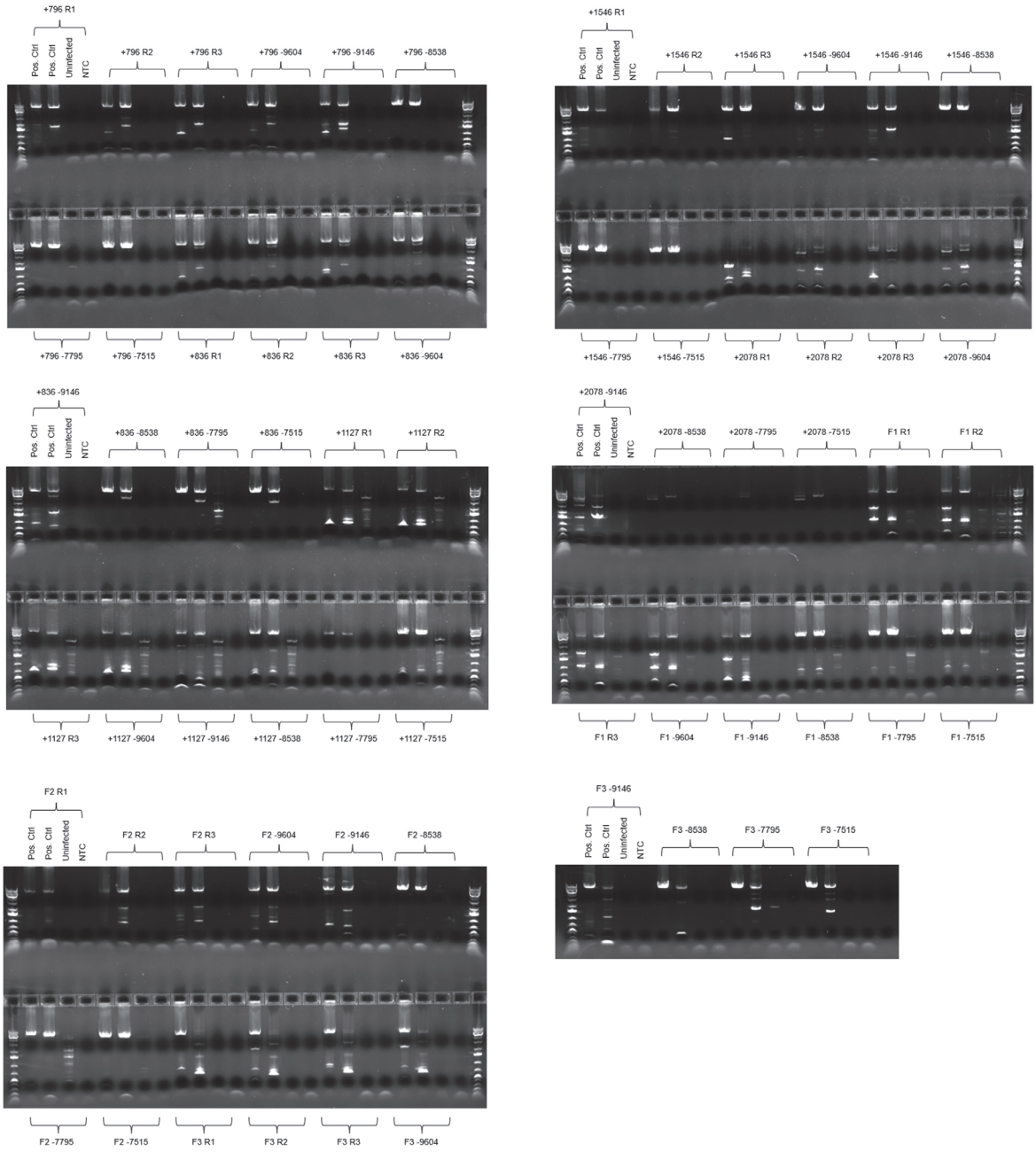

Figure 9. Published and custom-designed primer sets tested for detection of HIV-1 positive cells.

Representative sample of images of agarose gels obtained for testing of PCR primers for detection of HIV-1 positive cells. NL4-3.Luc.E- and NL4.3.AD8 plasmids were used as positive controls. Uninfected $CD4^+$ T cells obtained from a healthy donor and a non-template control were used as negative controls.

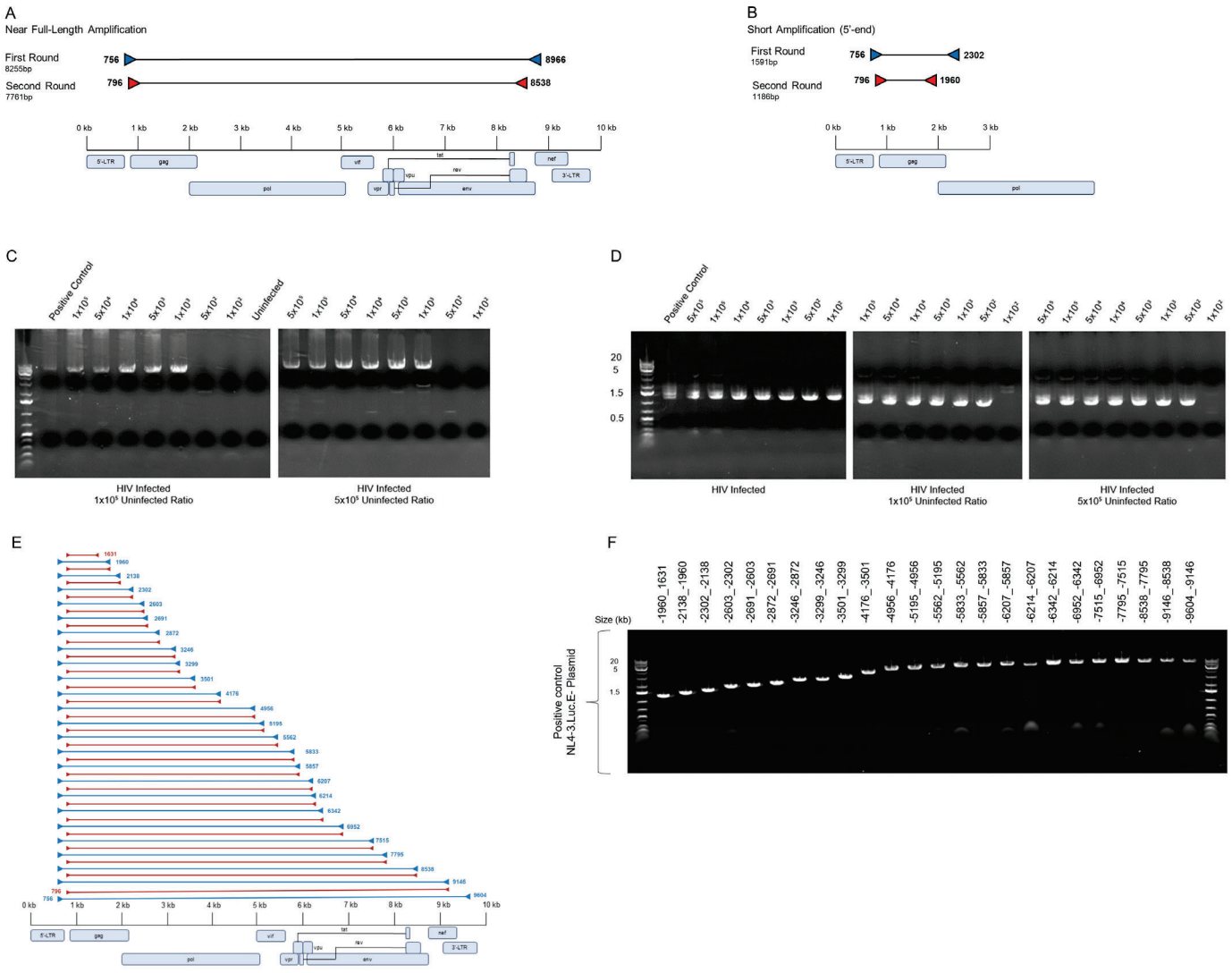

Figure 10. Detection limits and PCR amplification strategies.

(A) Representation of primers selected for first- and second-round near full-length amplification. Coordinates of primers are based on HXB2 reference sequence. (B) Agarose gel images present detection limits of nested PCR amplification of near full-length primer sets. Ratios of NL4-3.Luc.E- infected cells ranging from $1x10^2$ to $1x10^5$ cells spiked in $1x10^5$ and $5x10^5$ uninfected $CD4^+$ T cells. (C) Representation of primers selected for first- and second-round short amplification. (D) Detection limits of short-length primer sets were evaluated as indicated above. (E) A series of 24 reverse primers spanning from the 5'-LTR to the 3'-LTR for first-round and second-round amplification are indicated. First-round reverse primers range from 1960 to 9604, and 1631 to 9146 for second-round amplification. Created with Biorender. (F) An agarose gel image of nested PCR amplification of positive control pNL4-3.Luc.E- utilizing the series of 24 reverse primers.

Isolation of HIV-1 Clones from a Suppressed-HIV-Infected Patient

$CD4^+$ T cells were isolated from PBMCs obtained from an ART-suppressed HIV-1-infected patient, cultured following previous culture conditions and seeded in twenty 96-well round-bottom plates (Figure 11). A media color change indicating high cellular growth was noted in all wells at day 6, and cells were transferred to 96-well flat-bottom plates at day 7. At day 11, half of cell suspension was removed and transferred to 96-well V-bottom plates for washing and addition of DirectPCR lysis reagent. The remaining cell suspension was cultured to day 14, and then transferred to 48-well plates. The lysed wells were screened using both full-length and short-length primer sets for detection of HIV-1 positive cells. The near full-length primer sets detected 24 potential HIV-1 clones, and the short-length amplification yielded 498 positive hits from PCR results observed by gel electrophoresis as depicted in Figure 12A. No HIV-1 sequences could be obtained for any of the 24 near full-length clones, indicating that all bands detected by near full-length amplification may be due to non-specific binding. Twenty wells positive for short-length amplification were randomly selected and run using the pre-established 24 reverse primers noted previously to obtain the longest amplicon detected for sequencing. Full-length amplification (First round: FWD 756, REV 9604, and second round FWD 796, REV 9146) was obtained in three out of the twenty wells (P1C7, P1D9, and P2F9) and were assembled to reference sequence HXB-2 for bioinformatic analysis (Figure 12B). Remaining cell suspension of HIV-1-positive wells were transferred to subsequently larger vessels based on confluency up to day 34, then transferred to 96-well V-bottom plates and stored at -80C for downstream analysis.

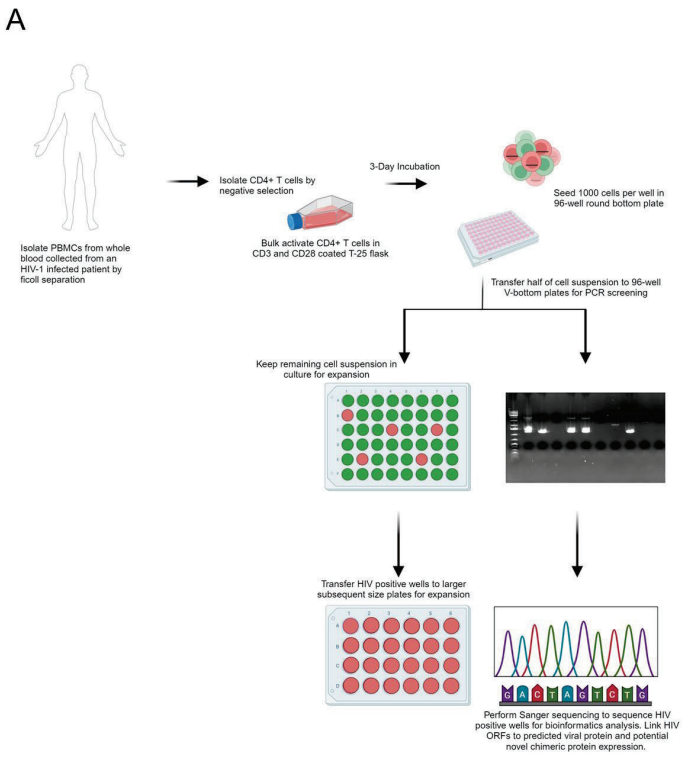

Figure 11. Representation of the cell culture and screening workflow of CD4$^+$ T cells obtained from an HIV-1-infected patient on antiretroviral therapy.
Created with Biorender.

Sequence P1C7 contained a 1000bp deletion spanning from HXB2 coordinates 8600 to 9604, resulting in deletion of HIV-1 accessory gene, *nef* (Figure 12D). P1D9 contained a 4500bp deletion spanning from coordinates 5100 to 9604, resulting in deletion of all HIV-1 accessory genes, regulatory genes and *env*. Similarly, P2F9 had a deletion spanning from coordinates 2700 to 9604, resulting in a 6900bp deletion. Sequences from those three clones were then assessed for presence of two cis-acting elements in the overlapping region of HIV-1 *gag* and *pol* genes essential for regulated -1 ribosomal frameshift. Invariant slippery site heptamer UUUUUUA and downstream structural RNA motif generated stem-loop, separated by an 8-nucleotide spacer were observed in the three clones analyzed. Clones P1D9 and P2F9 had mutations within the 8-nucleotide spacer and within the RNA secondary stem-loop structure (Figure 12C). HIV-1 sequences were then subjected to open reading frame analysis. Clone P1C7 encoded for 75 putative canonical ORFs, no chimeric protein sequences were observed by bioinformatic analysis. Clone P1D9 encoded 43 putative ORFs, among which we found a novel shorter GagPol mutant containing a novel 14aa sequence within IN (Figure 12E). The third clone, P2F9 encoded for an even shorter GagPol mutant containing a novel 25aa sequence within RT (Figure 12F).

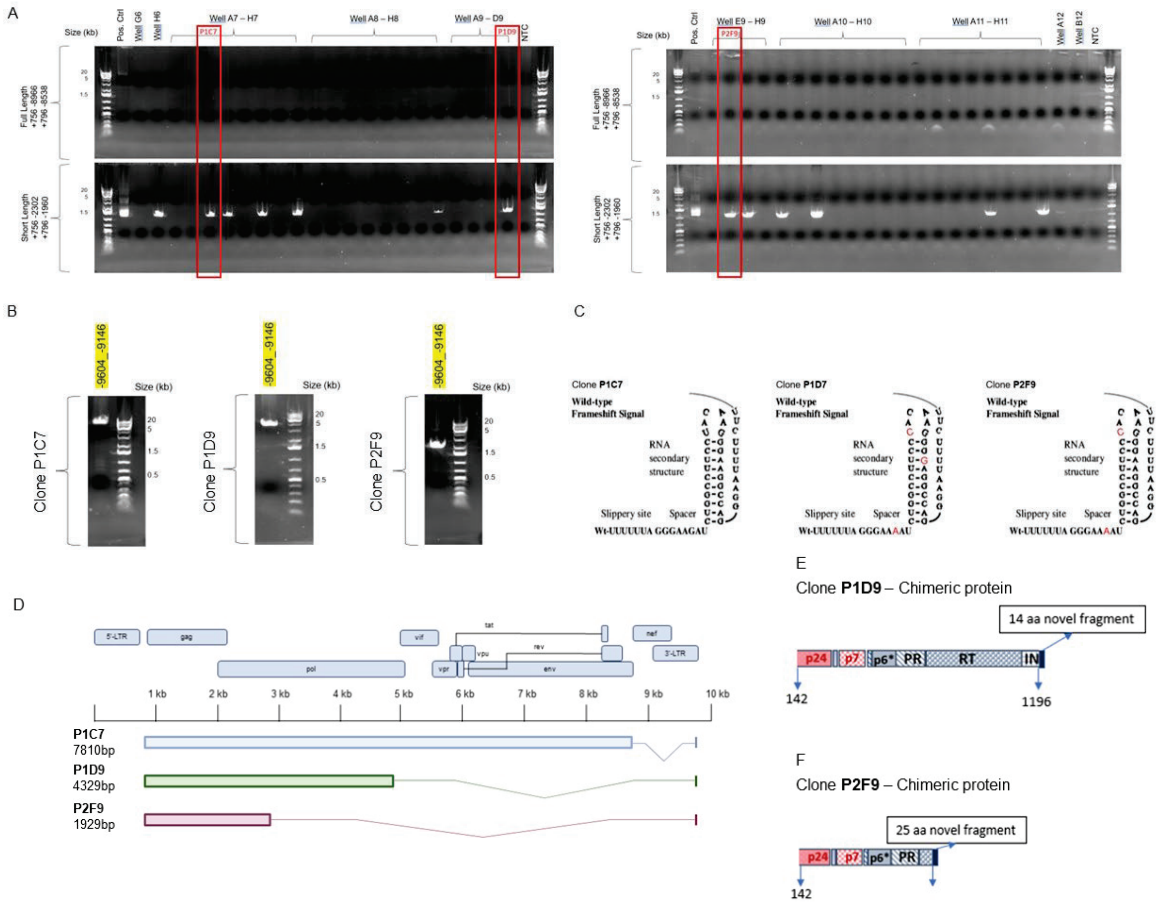

Figure 12. HIV-1 screening and ORF identification.

(A) Near full-length and short-length PCR amplification of HIV-1 clones obtained from a suppressed HIV-1-infected patient. PCR results are detected on 0.8% agarose gels. (B) Three HIV-1 positive wells detected by short-length amplification were run using a series of 24 reverse primers to obtain the longest amplicon size for sequencing. (C) Bioinformatic analysis of the overlapping region of HIV-1 *gag* and *pol* genes essential for -1 ribosomal frameshift in the three HIV-1 clones. (D) Sequencing results for the previously mentioned HIV-1 positive clones were assembled to the HXB2 reference sequence. (E) Novel ORFs were observed in clone P1D9 and (F) clone P2F9 following bioinformatic analysis.

DISCUSSION

Here we presented the steps taken to develop culture conditions for optimal $CD4^+$ T cell growth and expansion as our first step in evaluating HIV-1 protein expression in persistent $CD4^+$ T cell HIV-1 clonal populations. We first assessed the effect of media composition on $CD4^+$ T cell growth by comparing RP10 media and addition of serum free media, AIM-V (RP-AIMV10 media). In comparison to cells cultured in RP10 media, cell proliferation was significantly higher by five- to forty-fold in $CD4^+$ T cells cultured in RP-AIMV10 medium. We next established the best approach for activation of $CD4^+$ T cells to initiate clonal proliferation and expansion. Initially, we used irradiated PBMCs as feeder cells for $CD4^+$ T cell activation and re-stimulation but quickly found a pitfall in the use of irradiated PBMCs through interference with our ability to visualize cell growth and potential induction of cytopathic effects to the cell culture. We substituted irradiated PBMCs with anti-CD3/CD28 antibodies, either coated in PBS or adsorbed on bead particles to mimic antigen-presenting cells (APCs) for interaction of $CD4^+$ T cell receptors (TCR) and co-stimulatory interactions with CD28. We observed that anti-CD3/CD28-coated beads were best utilized for round bottom plates and anti-CD3/CD28 antibodies coated in PBS were more appropriate for coating of flat culture surfaces, including T-25 flasks, 96-well flat-bottom plates and all subsequently larger vessels. We found that activating $CD4^+$ T cells in bulk, as recommended in a methods publication by Fleckenstein and Ensser (2004) generated a more robust proliferation of $CD4^+$ T cells in comparison to activating small concentration of cells in 96-well round-bottom plates. Anti-CD3/CD28 antibodies in PBS or coated beads were used following activation for restimulation of cells. We concluded that bulk activated $CD4^+$ T cells cultured in RP-AIMV10 medium stimulated with 50U/mL IL-2 and seeded at 1,000 cells per well in 96-well round-bottom plates gave us the number of cells

required for screening of HIV-1 positive wells and cell expansion for further downstream applications.

We confirmed our optimal cell culture conditions were suitable for growth of healthy donor cells infected *in vitro* with replication-incompetent NL4-3.Luc.E- and replication-competent NL4-3.AD8 viruses. Furthermore, we observed no reduction in cell proliferation in cells cultured with HIV-1 antiretroviral drugs Darunavir, Dolutegravir, and Efavirenz. By performing a short time-course copy number assay, we observed that cells infected with NL4-3.AD8 rapidly declined indicating that infected cells were being outgrown by uninfected cells despite no reduction of cell proliferation, advancing all further experiments to be completed with NL4-3.Luc.E- infected cells. We concluded that the infection rate of NL4-3.Luc.E- infected cells remained consistent and LTR-mediated HIV-1 transcriptional activity could be detected throughout culture.

HIV-1 reservoirs harboring latent intact and defective proviruses are the main barrier to HIV-1 eradication in HIV-1-infected patients. Despite prolonged viremia suppression, chronic immune activation persists in HIV-1-infected patients leading to increased risks of HIV-associated chronic comorbidities. Full-length intact proviruses are speculated to contribute to low-level viremia that persist in suppressed HIV-1-infected patients, even though less than 10% of proviruses that persist on ART would be capable of producing infectious virions and are transcriptionally silent. Of the total approximate 300 resting $CD4^+$ T cells per 10^6 cells in the peripheral blood (Eriksson *et al.*, 2013), approximately 1 to 10 cells per 10^6 cells are replication-competent in ART-suppressed HIV-1-infected patients (Chun *et al.*, 1997; Finzi *et al.*, 1999). The remaining $CD4^+$ T cells harbor HIV-1 proviruses that contain insertions, deletions, sequence inversions, hypermutations, point mutations, and defective splice donor and acceptor sites

introduced by proofreading errors and recombination during viral reverse transcription, and activity of host cell restriction factors. Although once thought to be irrelevant, some defective proviruses in HIV-1-suppressed patients remain transcriptionally and translationally active, supporting our theory that defective proviruses may play a key role in persistent immune activation and chronic inflammation.

High frequency of genetic mutations in defective proviruses are often found in HIV-1 *env* and *pol* encoding regions, and less frequently in *gag* (Fisher *et al.*, 2022). Additionally, mutated start codons and premature stop codons in *nef* were frequently observed in hypermutated proviruses (Bruner *et al.*, 2016), indicating that these cells may lack Nef-mediated MHC-I downregulation and immune evasion, inducing cytotoxic $CD8^+$ T cell recognition and elimination (Huang *et al.*, 2018). Consistent with these observations of high frequency of genetic mutations within HIV-1 *pol*, *env* and *nef*, we observed two novel open reading frames within IN and RT and lacked *env* and HIV-1 accessory genes in two of the wells sequenced and sent for bioinformatic analysis. Of the 1,920 wells we plated, we detected 507 potential HIV-1 positive wells from $CD4^+$ T cells isolated from a suppressed HIV-1-infected patient using our optimally designed protocol for $CD4^+$ T cell culture and PCR screening. Of the 24 potential wells detected by full-length PCR, no sequencing results could be obtained, emphasizing the value of a second screening primer set flanking a less frequently mutated *gag* encoding region. Near full-length PCR is favorable for rapid detection of wells containing defective proviral sequences due to variances in genome length but is not conducive in detection of proviral sequences harboring several variable genetic mutations.

The genetic characterization of the proviral landscape that contribute to HIV-1 reservoirs that reside in $CD4^+$ T cells have been well defined through cell-associated HIV-1 RNA or DNA

sequencing, single-cell approaches, *ex vivo* viral outgrowth assays, and quantitative droplet digital PCR. A publication by Nemeth *et al.* (2017) conducted a study to compare the proteome associated with HIV-1 infection of human primary $CD4^+$ T cells infected with HIV-1 *in vitro* and $CD4^+$ T cells collected from ART-treated and untreated HIV-1-infected patients by mass spectrometry. Cells were collected at several time points up to 48 hours post infection, and approximately $1x10^6$ cells were used for the analysis. The proteome of defective HIV-1-infected clones obtained from ART-suppressed HIV-1-infected patients has not yet been assessed due to the lack of a culture system that supports long-term survival of primary $CD4^+$ T cells.

In further, it has been speculated that production of decoy HIV-1 proteins by defective proviruses may distract cytotoxic $CD8^+$ T cell elimination of latent intact HIV-1 reservoirs by inducing T cell exhaustion and depletion (Huang *et al.*, 2018; Imamichi *et al.*, 2020; Kuniholm *et al.*, 2022; Pollack *et al.*, 2017). HIV-1 defective proviral genomes can overcome defects in LTR-mediated transcription and major splice donor sequences by generating intragenic promoters (Kuniholm *et al.*, 2021) and alternative and cryptic slice sites (Purcell & Martin, 1993; Schwartz *et al.*, 1990; Sertznig *et al.*, 2018). Furthermore, point mutations, internal deletions and inversions can also contribute to HIV-1 alternative reading frames in sense and antisense orientations, generating novel open reading frames and enabling translation of chimeric and non-canonical HIV-1 proteins (Cardinaud *et al.*, 2004; Champiat *et al.*, 2012; Imamichi *et al.*, 2016; Imamichi *et al.*, 2020). Therefore, despite their inability to form infectious virions, defective proviruses may play a role pathogenically, contributing to HIV-1 immune evasion, persistence, and pathogenesis.

Further protein characterization of HIV-1 proviruses circulating in peripheral blood of ART-suppressed HIV-1-infected patients is needed to determine the role of defective proviruses

in HIV-1 pathogenesis. As described previously, defective proviruses have the coding potential to express canonical and non-canonical viral proteins through alternative open reading frames and mutations within their HIV-1 genome, but we question whether these proteins are expressed and if so, how are they expressed and what function they may have *in vivo*. We further question whether only translated viral proteins of defective proviruses could contribute to chronic immune activation and inflammation that persists in HIV-1-infected patients or could the formation of HIV-1 defective particles or exosomes (Chen *et al.*, 2021) or infection through cell-to-cell contact (Iwami *et al.*, 2015) play a critical role in HIV-1 pathogenesis. Furthermore, it has been demonstrated that unprocessed *gag*-encoded polyprotein precursors are able to assemble into virus-like particles in the absence of other viral proteins in Simian immunodeficiency virus (SIV) infection (Delchambre *et al.*, 1989) inducing a host immune response.

The aim of our study was to generate $CD4^+$ T cell clones from suppressed HIV-1-infected patients through developing optimal $CD4^+$ T cell culture conditions for $CD4^+$ T cell growth and expansion, screening for HIV-1 positive wells by unbiased PCR amplification, and sequencing and analysis of HIV-1 ORFs for predicted protein expression. Mass spectrometry is commonly used for proteome analysis as used in the study by Nemeth *et al.* (2017) and could confirm detection of HIV-1 viral proteins in our clones through peptide hits matching our predicted canonical or novel open reading frames from *in silico* analysis. In acknowledgement of the limitations in our study, we questioned whether we had sufficient infected cell numbers in our expanded culture for mass spectrometry analysis and further confirmation by western blot. As a result, these uncertainties prompted the adaptation of our cell culture protocol to incorporate immortalization of $CD4^+$ T cells. Immortalization of $CD4^+$ T cells obtained from suppressed HIV-1-infected patients would be an extremely useful *in vitro* model for the study of defective

proviruses in HIV-1 pathogenesis, by overcoming the limitations of primary $CD4^+$ T cell survival and generation of pure clonal cell populations. Primary $CD4^+$ T cells will proliferate after stimulation with mitogen or anti-CD3/CD28, and IL-2 but will gradually reduce viability and further cell proliferation after 4-5 weeks of cultivation, implicating a need for immortalization for long-term survival of $CD4^+$ T cells. In addition, a pure clonal population could not be obtained from primary $CD4^+$ T cells due to the limitations of cell survival, emphasizing the demand for immortalization.

Due to the necessity for immortalized cells going forward, we are developing a protocol to immortalize primary $CD4^+$ T cells with Herpesvirus saimiri (HVS) strain subgroup C488, a member of the Gammaherpesvirinae subfamily that causes peripheral T cell leukemia and lymphoma in non-human primates (Biesinger *et al*., 1992; Fleckenstein & Ensser, 2004). HVS-immortalized $CD4^+$ T cells maintain normal functional phenotype (Mittrucker *et al.*, 1993) and antigen specificity (Weber *et al*., 1993) and can be maintained in culture for extended periods of time without antigen or mitogen stimulation (Meinl *et al*., 1995). Enriched $CD4^+$ T cells from HIV-1-infected patients and HIV-1 *in vitro* infected CD4+ T cells from healthy donors have been successfully immortalized with HVS infection and had no effect on HIV-1 detection (Nick *et al.*, 1993; Saha *et al*., 1997; Saha *et al.*, 1996). After further optimization, we were successful in obtaining a pure clonal population of $CD4^+$ T cells infected *in vitro* with replication-incompetent NL4-3.Luc.E- virus from the culture of a single cell. Furthermore, we could maintain the immortalized cells in culture over five months and were able to regrow immortalized cells that had been previously frozen and stored in LN2. As we continue to optimize this protocol, we are hopeful in generating an *in vitro* model for further analysis in determining the role of defective

proviruses in HIV-1 pathogenesis and chronic immune activation in suppressed HIV-1-infected patients.

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