

Identifying novel compounds to eliminate latent HIV infection

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Abstract

There are currently an estimated 36.9 million people living with human immunodeficiency virus (HIV) (PLWH) worldwide. In the past few decades, the advent of antiretroviral therapy (ART) has significantly reduced the number of deaths associated with this virus. However, ART is not curative. The persistence of HIV latently infected CD4⁺ T-cells presents the major barrier towards a cure for HIV. Latently infected T-cells are formed when the virus integrates into the host genome of infected cells without ensuing productive infection. Due to these latently infected cells, viral gene expression and production infection rebounds from the integrated viral DNA if ART is ceased. Thus, ART must currently be taken life-long, posing a tremendous economic burden.

The “shock and kill” approach is an extensively studied cure strategy that involves the use of pharmacological agents termed latency reversing agents (LRAs) to reactivate or “shock” the latent virus to express viral RNA and proteins. Following the reactivation of latently infected cells, the production of HIV proteins and viral particles was proposed to result in the elimination of these cells through immune-mediated clearance or cytopathic events. Results from clinical trials that involve a single LRA to reactivate latently infected cells in PLWH have not yielded any significant impact on the HIV DNA reservoir. This can be attributed to a number of different reasons that include the potency of the LRAs to reactivate latency, the failure to elicit an effective immune response and the inhibition of T-cell clearance by cytopathic viral proteins. There is clearly a need for more potent LRAs as well as novel strategies that will result in the clearance of these latently infected cells once reactivated. In this thesis, we investigate several novel pro-apoptotic compounds in isolation as well as in combination with LRAs to clear latently infected cells. We have also developed two new methods in which to study the effects of LRAs and pro-apoptotic drugs on latently infected cells.

Dual-fluorescent reporter viruses have proven to be useful tools in studying latent HIV infection *in vitro*. Here we have modified a dual-fluorescent reporter HIV aiming to improve its functional characteristics in a pre-activation model of HIV latency. The new virus termed, DuoAdvance, contains two fluorescent viral reporters: a latent GFP reporter driven by

elongation factor 1 α (EF1 α) and a productive E2 Crimson reporter driven by the HIV long terminal repeat (LTR) (Chapter 2). Using DuoAdvance, we demonstrate that DuoAdvance can successfully infect Jurkat T-cell lines. In a pre-activation model of HIV latency in primary resting CD4⁺ T-cells, DuoAdvance infection resulted in little to no latent GFP expression. Subsequent analysis of the GFP negative population of cells revealed DuoAdvance infection can result in the production of latently infected cells carrying latent provirus but the expression of the GFP latency reporter was perturbed. Due to the partial expression of this GFP latent reporter in primary resting T-cells, DuoAdvance is limited to use in dividing T-cell lines and potentially a post-activation model of HIV latency using activated CD4⁺ T-cells, where better expression of the GFP latency and E2 Crimson reporters were seen.

Latency reversing agents can reactivate latent HIV but the effects on decreasing HIV DNA in PLWH has been less encouraging. In this thesis, we examine the effects of different pro-apoptotic drugs combined with different LRAs on decreasing HIV DNA in cultures of CD4⁺ T-cells from PLWH on ART *ex vivo*. Here we tested a number of LRAs together with several phosphoinositide-3 kinase (PI3K) inhibitors: IPI-443, IPI-3063 and wortmannin, as well as a B-cell lymphoma-2 (Bcl-2) inhibitor venetoclax as our pro-apoptotic drugs. The LRA romidepsin combined with all pro-apoptotic drugs resulted in synergistic decreases in the levels of integrated HIV DNA in the PLWH CD4⁺ T-cells *ex vivo* (Chapter 3). Additionally, several other LRA and pro-apoptotic combinations also decreased integrated HIV DNA in CD4⁺ T-cells *ex vivo*. All drugs were able to induce HIV viral transcription. Interestingly, we show that the pro-apoptotic drugs alone also led to an increase in HIV transcription and a decrease in HIV DNA.

These data demonstrated the select combinations of pro-apoptotic drugs and LRAs together or pro-apoptotic drugs alone can result in a decrease in HIV integrated DNA in CD4⁺ T-cells from PLWH on ART *ex vivo*. However, we were unable to distinguish if there was selective death of the reactivated latently infected cells with minimal impacts on uninfected T-cells also in the cell cultures. In order to explore this, we developed a new approach to detect selective cell death (Chapter 4). This method involves the use of PrimeFlow, a HIV RNA *in situ* hybridisation method combined with branched-DNA technology, together with a cell death stain and analysis of stained cells using flow cytometry. Using this approach, we were able to

demonstrate selective cell death in ACH2 T-cell lines treated with a combination of the PMA LRA, and venetoclax or IPI-443 PI3K inhibitor pro-apoptotic drug in a latently infected T-cell line. However, due to the elaborate staining procedure and large cell loss from the multi-step staining procedure, further investigation is required to move this staining approach into testing these drugs upon inducing the selective death of latently infected CD4+ T-cells from PLWH *ex vivo*.

In summary, we have developed two new methods to investigate the effects of LRAs and/or pro-apoptotic drugs on HIV latency. Although further work is required to optimise these methods for use of the novel DuoAdvance fluorescent reporter virus with primary resting CD4+ T-cells for drug testing, or for use of the novel PrimeFlow assay to study the selective impact of these drugs upon latently infected CD4+ T-cell samples from PLWH *ex vivo*. Most importantly, our work demonstrates novel combinations of pro-apoptotic drug and LRA combinations that can decrease HIV integrated DNA in cultures of CD4+ T-cells from PLWH on ART *ex vivo*. This has important therapeutic implications for using these drug combinations to deplete latently infected cells in PLWH on ART and additional studies that investigate these combinations in a clinical setting is warranted. In conclusion, our work demonstrates that latency reversal combined with a drug-based strategy to promote apoptosis can eliminate HIV latently infected CD4+ T-cells from PLWH on ART *ex vivo* and thus this approach holds important potential to lead to HIV remission off ART in PLWH.

Declaration

I declare that this thesis:

- I. Comprises of my original work towards the Doctor of Philosophy except where indicated in the Preface
- II. Due acknowledgment has been made in the text to all other material used
- III. This thesis is less than 100,000 words in length, exclusive of tables, figures, references and the appendices

Youry Kim

Preface

Work for this thesis was carried out under the supervision of Prof Sharon R Lewin, with co-supervision from Dr. Jenny L Anderson in the Lewin Laboratory at The Peter Doherty Institute for Infection and Immunity, Melbourne, Australia. Youry Kim is a student within the Department of Microbiology and Immunology, Faculty of Medicine, Dentistry and Health Sciences at The University of Melbourne. This Ph.D. thesis contains contributions from other individuals and their contributions are detailed below.

Chapter 1 includes material that has been published in the following review, which can be found in the Appendix:

- **Youry Kim**, Jenny L. Anderson and Sharon R. Lewin. 2018. **Getting the “Kill” into “Shock and Kill” Strategies to Eliminate Latent HIV.** *Cell Host & Microbe*. **23**(1):14-26

Chapter 2 has been accepted to The Journal of the International AIDS Society on the 8th November 2019 (Published paper can be found in the Appendix):

- **Youry Kim**, Paul Ulysses Cameron, Sharon Ruth Lewin and Jenny Louise Anderson. 2019. **Limitations of dual-fluorescent HIV reporter viruses in a model of pre-activation latency.** *The Journal of the International AIDS Society*.

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Chapter 2 has been included as the submitted document but formatted in line with the rest of the thesis. References in this chapter although separate in the document submitted to the journal, here they have been included in the bibliography for the whole thesis and as such will be differently numbered to the published journal.

Chapter 3 includes work conducted together with Ajantha Rhodes, with additional help from Ashanti Dantanarayana. Jennifer Zerbato also contributed intellectually in the analysis portion of this chapter.

Chapter 4 includes work contributed by Dr. Jenny Anderson. Dr Jenny Anderson contributed directly to results obtained for Figure 4.1.

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Abbreviations

βME	β-mercaptoethanol
ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
APOBEC3G	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
ART	Antiretroviral therapy
ATI	Analytical Treatment Interruption
BACH2	BTB domain and CNC homolog 2
Bcl-2	B-cell lymphoma 2
BD	Becton Dickenson
bDNA	Branched DNA
BET	Bromodomains and extra-terminal
BIR	Baculoviral IAP repeat
BiTEs	Bispecific T-cell engagers
bNAbs	Broadly neutralising antibodies
BRY	Bryostatin
bsAbs	Bispecific antibodies
BTK	Bruton's tyrosine kinase
CA	Capsid protein
CA	Cell associated
CA US RNA	Cell associated unspliced RNA
CAR	Chimeric antigen receptor
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CF	Cell-free
ciAP	Cellular inhibitors of apoptosis
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
CNS	Central Nervous System
CPE	Cytopathic events
cPPT	Polypurine tracts
csGFP	Codon switched GFP
CTLA4	Cytotoxic T-lymphocyte-associated antigen 4
CTLs	Cytotoxic T-lymphocyte
CyTOF	Cytometry time of flight
DART	Dual-affinity Re-targeting
DC	Dendritic cells
ddPCR	Digital droplet PCR
DIABLO	Direct inhibitor of apoptosis protein-binding protein with low pI
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNTMs	DNA methyltransferase
DSF	Disulfiram
E2CRM	E2 Crimson
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid

EF-1 α -HTLV-1	Elongation factor 1 α -human T-cell leukemia virus type 1
EF1 α	Elongation factor 1- α
ELISA	Enzyme-linked immunosorbent assay
EMT	Effector-to-memory transitioning T-cells
Env	Envelope
FADD	Fas-associated protein with death domain
FBS	Fetal Bovine serum
FLIPS	Full-Length Individual Proviral Sequencing
FOXO1	Forkhead Box O1
GALT	Gut Associated Lymphoid Tissue
GBP5	Guanylate binding protein 5
GFP	Green Fluorescent Protein
GSK-3 β	Glycogen synthase kinase-3 β
HDAC	Histone deacetylase
HDACi	Histone Deacetylase Inhibitors
HIV	Human Immunodeficiency Virus
HMTases	Histone methyltransferases
HMTi	Histone methyltransferase inhibitors
Hsp90	Heat shock protein 90
HTLV-1	Human T-cell leukemia virus type 1
IAP	Inhibitors of Apoptosis
IC	Immune checkpoint
IFI16	Interferon-gamma-inducible protein 16
IFN	Interferon
IL-1 β	Interleukin-1 β
IN	Integrase
IngB	Ingenol B
IONO	Ionomycin
IPDA	Intact proviral DNA assay
IRES	Internal Ribosome entry site
ISH	In situ hybridisation
LAG-3	Lymphocyte activation gene-3
LAV	Lymphadenopathy-associated virus
LRA/s	Latency Reversing Agents
LTR	Long-terminal repeat
MA	Matrix protein
mAbs	Monoclonal antibodies
MATR3	Matrix-associated RNA binding matrix 3
mDCs	Myeloid dendritic cells
MDDCs	Monocyte-derived dendritic cells
MHC	Major histocompatibility complex
MKL2	Myocardin-like protein 2
mKO2	Kusabira Orange 2
MS	Multiply spliced mRNA
MVOA	Murine viral outgrowth assay
NC	Nucleocapsid Protein
NEAT1	Nuclear Enriched Abundant Transcript 1

NF- κ B	Nuclear Factor κ -light chain-enhancer
NFAT	Nuclear factor of activated T-cells
NGS	Next genome sequencing
NHL	Non-Hodgkin's lymphoma
NHP	Non-human primate
NIK	NF- κ B-inducing kinase
NSG	NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1Wjl} /SzJ
nuc-0	Nucleosome-0
nuc-1	Nucleosome-1
NVP	Nevirapine
P-TEFb	Positive Transcription Elongation Factor b
pAkt	Phosphorylated Akt
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Primer binding site
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein-1
pDC	Plasmacytoid dendritic cells
PET	positron emission tomography (PET)
PF	PrimeFlow
PHA	Phytohaemagglutinin
PI3K	Phosphoinositide 3-kinase
PKC	Protein Kinase C
PLWH	People living with HIV
PMA	Phorbol 12-myristate 13-acetate
PMA	Phorbol 12-myristate 13-acetate
PNB	Panobinostat (LBH589)
PR	Viral protease
PRRs	Pattern recognition receptors
PTEN	Phosphate and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
qPCR	Quantitative PCR
RA	Retinoic acid
RGH	Red Green HIV
RIPK	Receptor interacting protein kinase
RNAPII	RNA polymerase II
ROS	Reactive oxygen species
RPL13A	Ribosomal protein L13A
RRE	Rev responsive element
RT	Reverse Transcriptase
RT-qPCR	Reverse Transcription qPCR
RTGV	Raltegravir
SAMHD1	Sterile motif and histidine aspartic domain and HD domain-containing protein 1
SEC	Super elongation complex
SHIV	Chimeric Simian Human Immunodeficiency Virus
SiMoA	Single Molecule Assay

SIV	Simian Immunodeficiency virus
Smac	Second mitochondria-derived activator of caspases
Sp1	Specificity protein 1
SS	Singly spliced mRNA
SU	gp120 external glycoprotein
TAR	Tat-responsive region
TCM	Central memory T-cells
TCR	T-cell receptor
TEM	Effector memory T-cells
TFH	Follicular helper T-cells
TIGIT	T-cell immunoreceptor Immunoglobulin and ITM domain
TILDA	Tat/Rev Induced Limiting Dilution Assay
TLR	Toll-like receptors
TM	gp41 transmembrane protein
TN	Naïve T-cells
TNF	Tumour necrosis factor
TRAF3	TNF receptor-associated factor 3
TRM	Tissue-resident memory T-cells
TSCM	Stem-cell memory T-cells
TTE	Terminally differentiated T-cells
TTM	Transitional memory T-cells
US	Unspliced mRNA
VNX	Venetoclax
VOR	Vorinostat
VPA	Valproic Acid
VSV-G	Vesicular Stomatitis Virus Envelope Glycoprotein
WN	Wortmannin
XIAP	X-linked inhibitors of apoptosis

Chapter 1

Literature Review

Disclaimer:

Sections of this literature review (including figures), written in partial fulfilment of Doctor of Philosophy at The University of Melbourne has been published previously (Appendix).

Chapter 1

Literature Review

1.1 Introduction

Human immunodeficiency virus (HIV) infection remains one of the world's leading health problems on an unprecedented scale. Following the recognition of acquired immunodeficiency syndrome (AIDS) in 1981 [1] and the discovery of HIV-1 as the etiologic agent of this syndrome in 1983 [2], an estimated 36.9 million people of the world's population are now living with HIV infection and approximately 1.8 million individuals are newly infected every year [3]. Death due to AIDS accounts for the loss of 940,000 lives per annum [4]. Even with the introduction of combination antiretroviral therapy (ART) during 1996 and 1997 [5] that has improved patient life expectancy close to that of uninfected individuals, HIV infection continues to be a global pandemic.

Despite the benefits of ART, this treatment must be maintained for life and is a great economic burden, with the cost of ART by 2030 estimated to be 30 billion dollars [6]. Moreover, ART can be difficult to access for the millions infected globally. Importantly, ART is not curative. The emergence of drug resistant HIV strains [7] and overall stigma towards people living with HIV (PLWH) make finding a cure for HIV a priority.

1.1.1 HIV Pathogenesis

HIV is classified in the *Retroviridae* family that are characterised by their ability to reverse transcribe viral RNA into a DNA intermediate using a viral reverse transcriptase enzyme during the viral lifecycle. The DNA is then integrated into the host genome. HIV is further located in the subfamily *Orthoretrovirinae* [8] and genus *Lentivirus* that is characterised by slow progression to disease and death. There are two broad types of HIV based on sequence homology and disease manifestations and these include HIV-1 and HIV-2 [8]. HIV-1 is the more pathogenic, commonly acquired, viral type responsible for the world pandemic, whereas HIV-

2 is largely confined to West Africa [9]. In this thesis I have used the term HIV which refers to HIV-1, unless otherwise stated.

The main cellular targets of HIV are the CD4+ T-cells of the immune system with other cell types such as macrophages also being infected by HIV [8]. CD4+ T-cells are a vital part of adaptive immunity and their significant decrease in numbers during untreated infection ultimately leads to a deteriorating immune system and an increase in susceptibility to opportunistic infections [1].

When an individual becomes infected with HIV, the individual may begin to display “flu-like” symptoms [10]. This is termed the “acute phase” and is defined by the decline in the number of CD4+ T-cells, while the number of viral RNA copies in the blood begin to peak. The immune system counteracts this increase in viral particles by mounting a robust immune response, resulting in the rapid rise of HIV-specific cytotoxic CD8+ T-cells [11]. The increase in HIV-specific CD8+ T-cells is temporally associated with a reduction in plasma HIV RNA and an increase in the number of CD4+ T-cells. It is during this time that antibodies to viral antigens are also produced and detected [12]. Months after the primary infection, PLWH enter a period that can span many years. During this time there is constant turn-over of CD4+ T-cells with destruction of cells through multiple mechanisms and production of cells but there is progressive decline of roughly 50 cells per year. The final stage of HIV infection is the onset of opportunistic infections of acquired immune deficiency syndrome (AIDS)-associated malignancies or decline of CD4+ T-cells to 200 cells per μ l blood [10]. This leads to a clinical or epidemiological definition of AIDS (Figure 1.1).

The mechanisms responsible for the loss of CD4+ T-cells during untreated infection is multifactorial [13]. HIV replication leads to the death of productively infected CD4+ T-cells within one to two days [5, 14] in the absence of ART (Figure 1.1). In the absence of ART, the number of productively infected cells is low, suggesting that the loss of CD4+ T-cells is attributed to the death of bystander resting CD4+ T-cells [15]. Another mechanism by which HIV can deplete CD4+ T-cells is through the formation of syncytia [16] or through bystander CD4+ T-cell apoptosis [15]. It was also demonstrated that 95% of the resting CD4+ bystander

T-cells die following HIV infection through pyroptosis [17]. Pyroptosis occurs in these non-activated CD4+ T-cells as they have undergone abortive infection. This is where incomplete HIV DNA reverse transcripts remain in the cytoplasm, which are detected by receptors such as interferon-gamma-inducible protein 16 (IFI16) lead to the activation of caspase-1 and secretion of the highly inflammatory cytokine, interleukin-1 β (IL-1 β) [18].

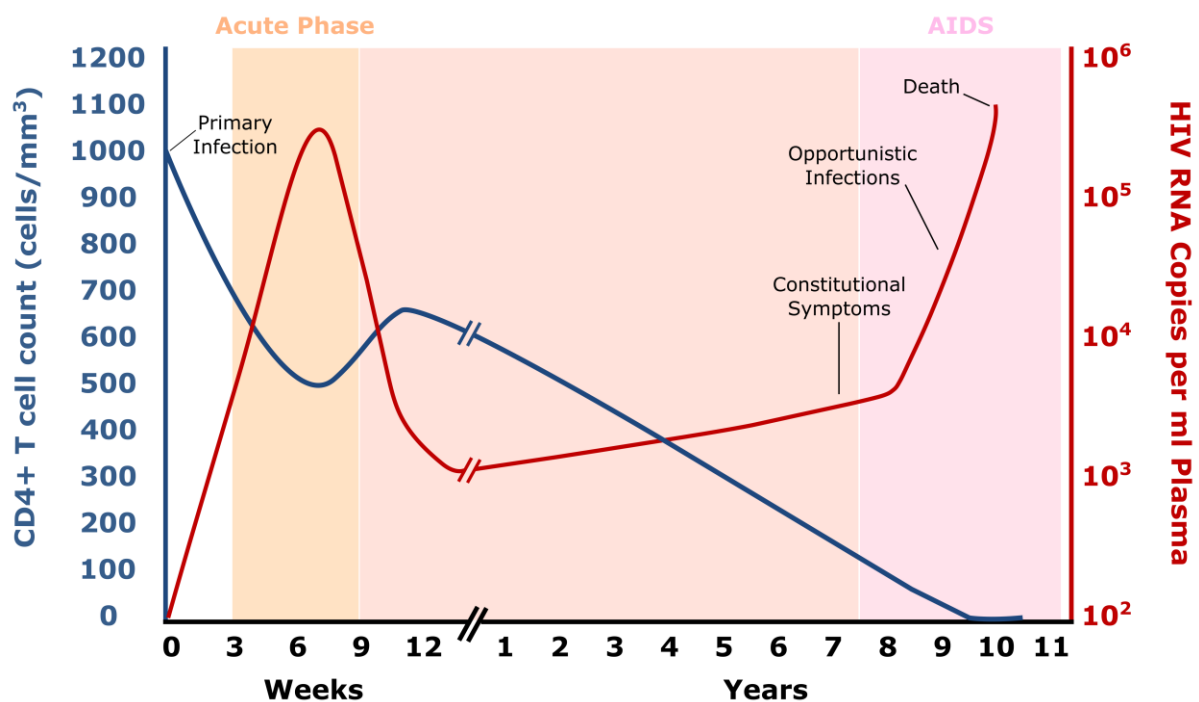


Figure 1.1. Natural history of people living with HIV in the absence of ART

Following infection with HIV (primary infection), there is a rapid exponential increase in plasma HIV RNA and decline in circulating CD4+ T-cells. This is followed by a robust immune response leading to a reduction in plasma HIV RNA and temporary increase in CD4+ T-cells. In the absence of ART, there is a progressive decline in CD4+ T-cells and increase in plasma HIV RNA over time. The definition of AIDS is based on the emergence of opportunistic infections, AIDS-related malignancies or a reduction in CD4+ T-cells to less than 200 cells/mm³. Adapted and reconstructed from [19].

1.1.3 Human Immunodeficiency Virus Genome

HIV is comprised of two single stranded, positive-sense RNA genomes about 9.7 kilobases in length [8]. Each strand of HIV RNA contains the nine viral genes that encode: structural proteins (*gag*, *pol* and *env*), regulatory proteins (*rev* and *tat*) and accessory proteins (*vpu*, *vif*,

vpr and *nef*) [8] (Figure 1.2). Fusion of HIV virions to target cells releases the viral core into the cell cytoplasm and the viral RNA genome inside the core is reverse transcribed into a complementary, double-stranded, HIV DNA (cDNA) intermediate that fully reconstitutes the long terminal repeat (LTR) sequences at either end of the viral sequence (Figure 1.2). This viral DNA then integrates into the host gene, from which viral gene expression occurs. The LTR sequences contain U3, R and U5 regulatory segments for viral transcription [8]. In addition to the HIV LTRs, the viral RNA encodes for *cis* acting sequences such as Rev response element (RRE) that is involved in cytoplasmic export, a primer binding site (PBS) and polypurine tracts (cPPT) for reverse transcription and viral RNA packaging and dimerization signals (ψ) for successful encapsidation of viral RNA into the newly forming viral particles [8].

The *env* gene is transcribed and translated into a precursor envelope (Env) protein gp160 that is cleaved to form the mature gp41 transmembrane protein (TM) and gp120 external glycoprotein Env components. The surface protein (SU) gp120 and the TM gp41 assemble into a trimeric structure on the surface of viral particles, enabling binding and co-receptor binding to the host cell. These proteins are also responsible for the fusion of the membrane of the viral particle with the host cell member and reviewed in [8].

The *gag* gene is translated to produce a Gag precursor protein (Pr55) that is cleaved by the viral protease to generate: p17 matrix protein (MA), p24 capsid protein (CA), p7 nucleocapsid protein (NC) and p6 structural proteins. The matrix protein binds and associates envelope proteins within the inner membrane of the viral particle; capsid forms the viral core that encapsulated the single stranded viral RNA; nucleocapsid is found within the viral core bound to the viral RNA and is involved in reverse transcription after entry into the target cell; p6 binds Vpr protein allowing for the packaging of this protein into the core as well as promoting budding of newly formed viral particles at the end of the replication cycle. In approximately 1 in 20 translation events, ribosomal frameshifting near the end of the *gag* gene enables translation of the *pol* gene to produce a Gag-Pol polyprotein (termed Pr160). This polyprotein is cleaved into the mature Gag proteins above plus the three viral enzymatic proteins encoded by *pol*: protease (PR) responsible for cleavage of the Gag-Pol polyprotein, reverse

transcriptase (RT) responsible for converting the viral single stranded RNA into DNA and integrase (IN) which facilitates HIV DNA integration into the host genome [8].

The regulatory protein Tat, encoded by the *tat* gene is a critical transcription protein that binds to the Tat-responsive region (TAR) in newly transcribed RNA, increasing the activity of RNA polymerase II to promote the elongation of viral RNA transcripts, thereby amplifying the amount of viral RNA and by extension, the amount of viral protein generated. The regulatory Rev protein encoded by the *rev* gene functions to shuttle unspliced or singly-spliced viral mRNA from the nucleus into the cytoplasm before it undergoes further splicing [8].

In addition to the structural and regulatory genes, HIV also encodes the accessory genes: *vpu*, *vif*, *vpr* and *nef*. Vpu protein promotes CD4 downregulation on the host's cell membrane and enhances the release of newly formed viral particles; Vif protein counteracts the host genome defence protein Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) which can hypermutate the HIV genome effectively making the virus non-functional; Vpr protein aids in the transport of HIV into the nucleus and can arrest the cell in the G2 cell cycle phase; Nef protein has many functions but briefly can also downregulate CD4 and major histocompatibility complex (MHC) Class I on the cell surface and is critical for disease progression [8].

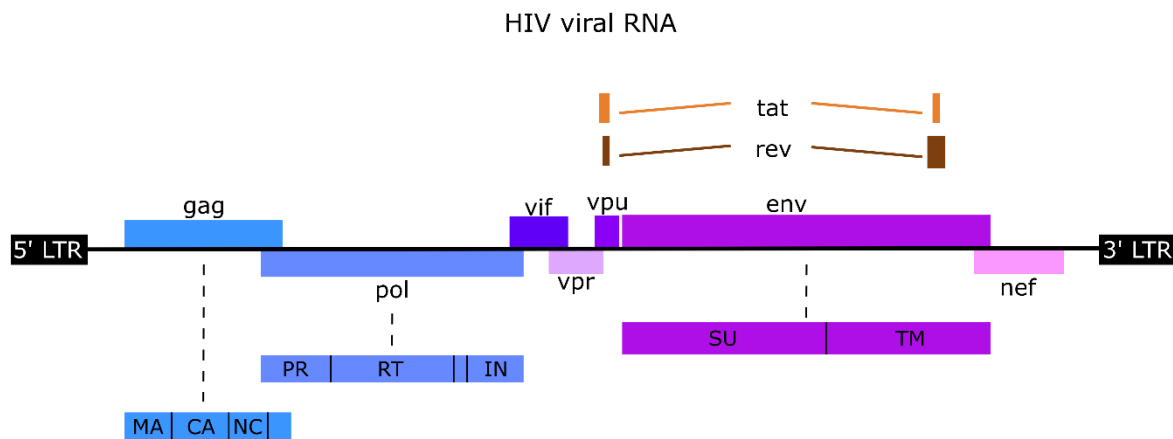


Figure 1.2. HIV Genetic Organisation

Each HIV positive sense RNA genome has a copy of the nine viral genes encoding structural proteins (*gag*, *pol* and *env*), regulatory proteins (*rev* and *tat*) and accessory proteins (*vpu*, *vif*, *vpr* and *nef*). The LTR sequences contain U3, R and U5 regulatory segments for viral transcription. The *env* gene is transcribed and translated into a precursor protein, Env gp160, that is cleaved to form gp41 transmembrane protein (TM) and gp120 surface glycoprotein (SU). The *gag* gene is also translated to produce a precursor protein, Gag Pr55, that is cleaved by viral protease (PR) into: p17 matrix protein (MA), p24 capsid protein (CA), a p2 spacer peptide (not shown), p7 nucleocapsid protein (NC), a p1 spacer (not shown) and a p6 protein that enables budding of the virus. The *pol* gene is initially translated as an extension of *gag* gene by ribosomal frameshifting to produce a Gag-Pol polyprotein (termed Pr160). The Pol portion of this polyprotein is cleaved into the enzymatic viral components: protease, reverse transcriptase (RT) and integrase (IN).

1.1.4 Structure of mature HIV Virions

Mature HIV virions contain two copies of the HIV positive-sense HIV RNA genome. The viral RNA is coated by viral p7 NC and surrounded by a conical-shaped capsid core made up of viral p24 capsid protein (Figure 1.3). The capsid core also contains the viral enzymes IN, PR and RT[8]. The conical-shaped capsid core resides within a larger spherical-shaped particle that is formed from the lipid bilayer of the host cell's plasma membrane. Thus, it also contains proteins that are normally found on the surface of the host cell such as MHC molecules. Additionally, embedded on the surface are viral envelope proteins gp120 and gp41 [8].

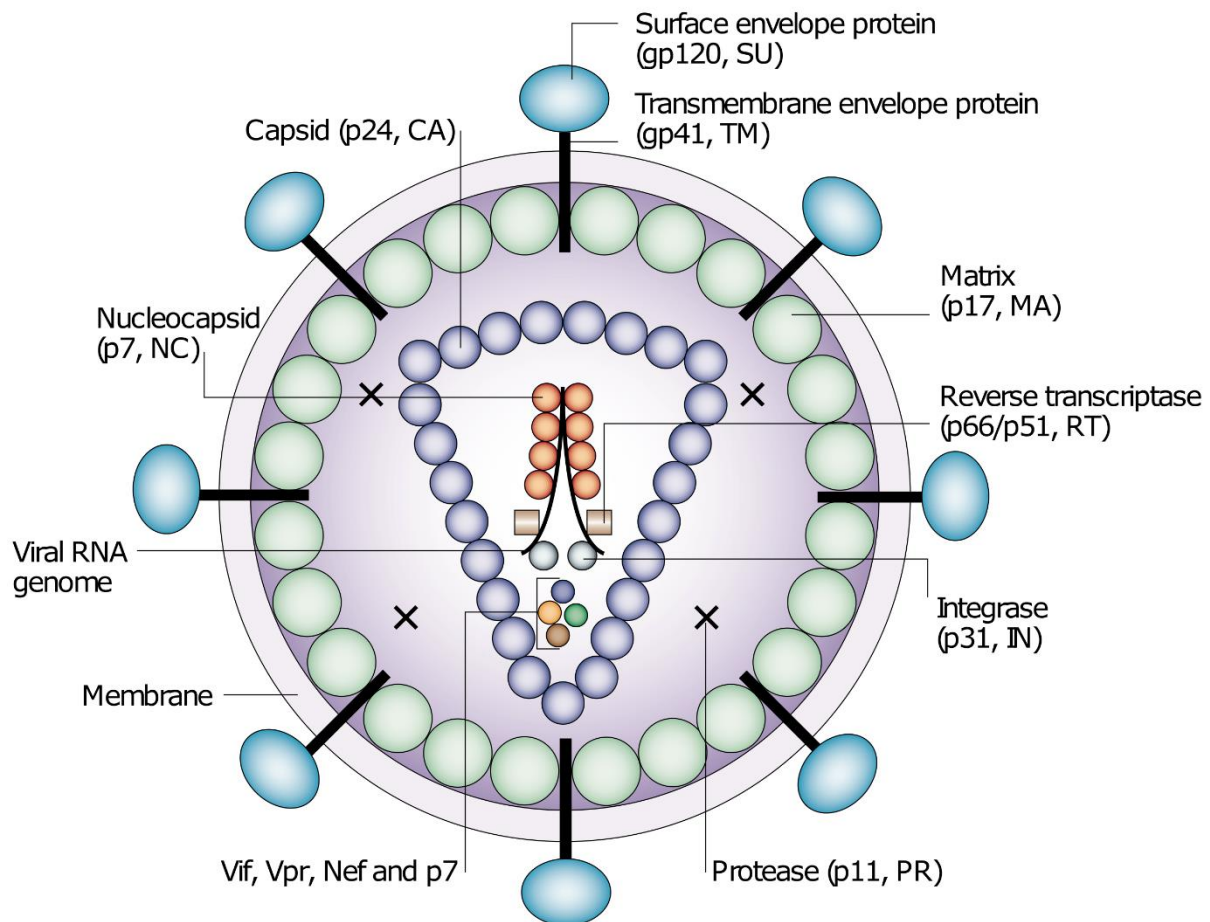


Figure 1.3. Structure of a mature HIV viral particle

Each mature viral particle contains two copies of a single stranded, positive-sense RNA genome. The viral RNA genome is coated in nucleocapsid (NC; p7) and the whole genome including viral enzymes such as integrase (IN; p31), reverse transcriptase (RT; P66/p51) and protease (PR; p11) is encompassed within the capsid (CA; p24). The viral envelope comprises of two viral envelope proteins; a surface envelope protein (SU) gp120 and transmembrane envelope protein (TM) gp41 embedded in the cell membrane. Entry into a host cell involves the interaction between gp120 and the CD4 receptor on host cell membranes, facilitated by chemokine receptors CCR5 and CXCR4. Adapted from [20].

1.1.5 HIV Replication Cycle

HIV enters CD4+ T-cells through interactions of the virus Env protein, gp120, with the CD4 cell receptor and either CCR5 or CXCR4 co-receptor [21]. Fusion of the viral particle envelope and host cell ensues, and the virus conical shaped capsid is released into the cytoplasm. Once in the cytoplasm, the virion core partially uncoats capsid protein and the polymerase and ribonuclease activity of viral reverse transcriptase initiates the conversion of the single-stranded RNA genome into double stranded, cDNA [8].

The double stranded cDNA is in a complex with host-cell proteins, termed the pre-integration complex (PIC), that is actively transported into the nucleus through the nuclear pore and integrates into the host genome, where it is now termed a provirus [8]. Transcription initiates from the HIV LTR promoter at a basal level until sufficient amounts of viral Tat are produced. Binding of Tat to the TAR RNA element in early HIV transcripts greatly enhances transcription elongation, leading to efficient transcription of HIV mRNA in a manner also requiring RNA polymerase II and positive transcription elongation factor b (P-TEFb) [8].

Early in replication, multiply spliced (MS) mRNAs are produced that encode viral Tat, Rev and Nef protein. When there is an adequate amount of Rev, Rev binds to the Rev responsive element found on unspliced (US) or singly spliced (SS) mRNAs and facilitates their transport from the nucleus to the cytoplasm, generating US and SS mRNA. These mRNAs lead to the translation of viral Gag, Gag-Pol (US mRNA) and Env (SS mRNA) structural polyproteins. The structural viral proteins assemble at the plasma membrane with 2 copies of US mRNA and bud off the cell generating new immature virions. Cleavage of Gag-Pol polyproteins by the viral protease within the immature virions lead to structural rearrangements and production of mature virions that can infect new host cells [22, 23] (Figure 1.4).

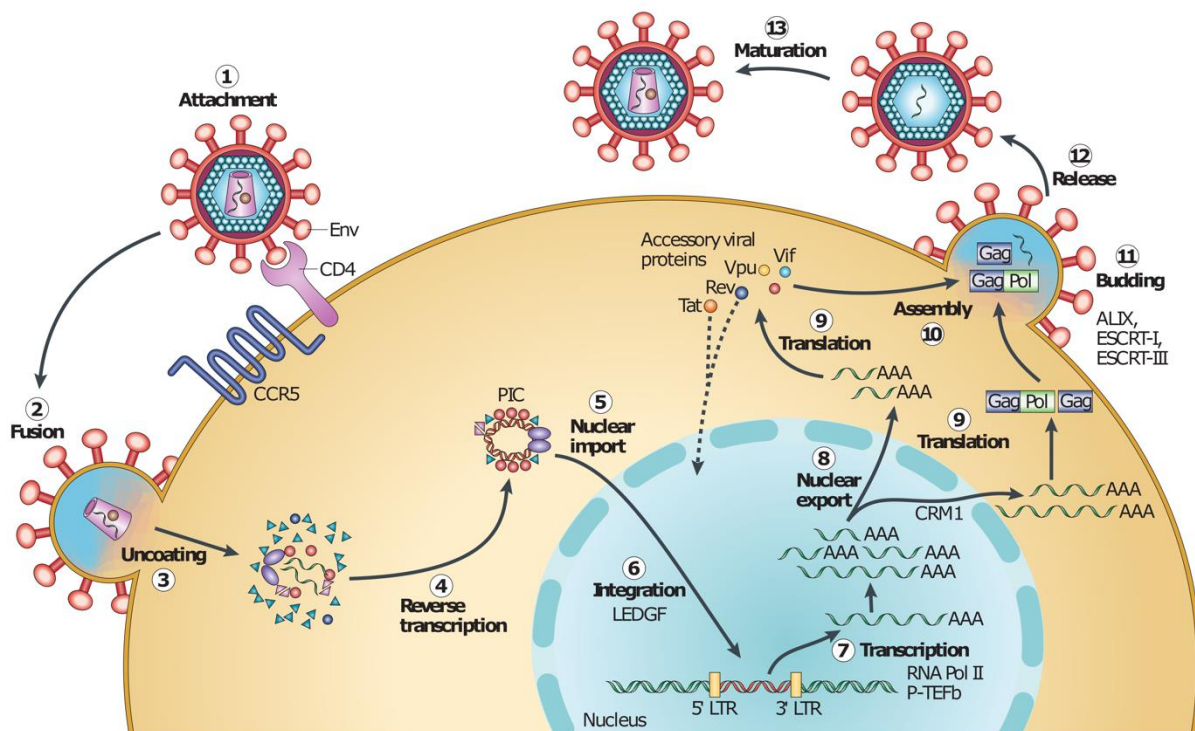


Figure 1.4. The HIV Replication Cycle

HIV binds to its target host cell through interactions between the viral gp120 receptor and CD4 cell surface molecule (Step 1), followed by interaction with CCR5 or CXCR4 chemokine receptor. This enables fusion of the virion and plasma membrane of the target cell (Step 2), releasing the virion core into the host cell. The capsid protein shell of the virion core partially uncoats (Step 3) and through polymerase and ribonuclease activity of reverse transcriptase (Step 4), conversion of the viral RNA into complementary double stranded DNA (cDNA) takes place. The viral cDNA remains in a complex of proteins termed the pre-integration complex (PIC) that then traffics into the nucleus (Step 5). Once the viral cDNA is in the nucleus, linear viral cDNA integrates into the host genome (Step 6). Transcription mediated by host RNA polymerase II, P-TEFb and viral Tat (Step 7) results in viral mRNA. The viral mRNA is exported to the cytoplasm (Step 8) and then used as a template for protein production (Step 9). When levels of Rev protein are low, the full-length viral mRNA is multiply spliced (MS) and exported to the cytoplasm for translation of viral regulatory and accessory proteins: Tat, Rev, Nef, Vpu and Vif. When sufficient levels of Rev accumulate, Rev protein binds an element in singly spliced (SS) or full-length unspliced (US) viral mRNA and exports these mRNA to the cytoplasm for translation of viral Gag, Gag-Pol (US mRNA) or Env (SS mRNA) structural proteins. The viral structural proteins assemble at the plasma membrane, incorporating 2 copies of the US mRNA

form (Step 10), and these new virions bud from the cell (Step 11) and are then released, generating immature virions (Step 12). Proteolytic cleavage of Gag and Gag-Pol polyproteins in the immature virions lead to protein rearrangements in the virions to generate mature infectious particles (Step 13) that can infect new host cells. Figure adapted from [24].

1.2 HIV Latency

Antiretroviral therapy can effectively suppress virus replication leading to undetectable levels of plasma HIV RNA (<20 copies/ml) and prevent infection of new cells [25]. However, virus persists on ART in multiple forms including long lived and proliferating latently infected cells that contain transcriptionally silent but intact virus that can be reactivated to produce infectious virions [26, 27] that can persist for many years [16]. Upon interruption of ART [26, 28, 29], these cells can be stimulated to produce infectious virions [28-30]. Persistence virus presents as latently infected cells either that express no viral RNA or low levels of US RNA [31, 32] but there is limited expression of viral proteins and thus these cells escape immune recognition. ART alone is not able to cure HIV and latency remains the biggest barrier to curing HIV and thus other strategies to surmount latency are required.

1.3 Sources of persistent HIV in PLWH

HIV persists in PLWH on ART due to the contribution of multiple sources including cellular and anatomical reservoirs, low level residual viral replication and clonal expansion.

1.3.1 Cellular Reservoirs

For an infected cell to be considered a latent reservoir, several criteria need to be met. The latently infected cell must be: a) long lived (persist for many months), b) restrict viral replication so that viral antigens are not presented and c) the cell must harbour replication competent virus that is able to resume replication following reactivation [33]. Latent HIV can persist in different subsets of memory CD4⁺ T-cells in individuals on ART [34]. CD4⁺ T-cells can be classified into subsets based on phenotypic markers, function or expression of specific transcriptional factors [35].

Using a classification of phenotypic markers, cellular differentiation is thought to occur from naïve T-cells (T_N) upon activation. Naïve T-cells differentiate into effector T-cells that are short-lived or they differentiate into memory T-cells. Central memory T-cells (T_{CM}) are located in the secondary lymphoid tissue while effector memory T-cells (T_{EM}) are found in sites of recent infection [36]. T_N , T_{CM} , T_{EM} , stem-cell memory (T_{SCM}), terminally differentiated (T_{TD}) and tissue-resident memory (T_{RM}) T-cells can be differentiated based on the expression of extracellular markers CD45RA, CCR7, CD28 and CD95 (reviewed in [35]). T-cell effector function is based on the expression of transcriptional factors, chemokine receptors and cytokine secretion [37].

T_{CM} T-cells are a key reservoir in PLWH on ART and these cells are maintained by a long cellular lifespan [34]. Transitional memory (T_{TM}) T-cells also constitute a key reservoir in individuals on ART and have been shown to make a larger contribution to the overall reservoir in PLWH with low CD4⁺ T-cell counts and persistent immune activation [38]. T_{TM} are primarily maintained through homeostatic proliferation [39] which is driven by cytokines such as IL-7 [34]. T_{EM} and T_{TD} also harbour persistent virus and often contain expanded clones of virus with identical sequences or integrations sites [40, 41]. This finding suggests that there is significant proliferation of these cells, potentially in response to antigen. Other cell types that can harbour HIV on ART include T_{scm} which are rare but have a high proliferative capacity [42]. T follicular helper (T_{FH}) T-cells express high levels of the immune checkpoint protein programmed cell death protein-1 (PD-1) and CXCR5 and are preferentially found in B-cell follicles in lymph node tissue which were found to be preferentially enriched for replication-competent HIV [43].

Several chemokine receptors and markers are upregulated in persistently infected T-cells. Cells that express the chemokine receptor CCR6, CCR7 and CXCR3 in the blood were found to harbour HIV DNA in PLWH [44], indicating CCR6 as a marker of persistent HIV infection. Cells that were enriched for CXCR3 also contained inducible replication-competent virus [44]. Additionally, immune checkpoint markers that mark exhausted T-cells such PD-1, lymphocyte activation gene 3 (LAG-3) and T-cell immunoreceptor Immunoglobulin and ITIM domains (TIGIT) were found to be expressed on HIV infected T-cells [34, 45].

In addition to memory CD4+ T-cells, there is evidence that other cell types harbor latent provirus including $\gamma\delta$ T-cells [46], tissue macrophages [47] and hematopoietic progenitor cells [48].

1.3.2 Macrophages and monocytes as a source of persistent HIV virus

Persistence of HIV in monocytes and macrophages in PLWH on suppressive ART remains a controversial area. Several groups have detected HIV DNA in both monocytes and macrophages isolated from PLWH on ART [49, 50], but whether this is replication-competent virus is unclear. Monocytes and macrophages express host-cell restriction mechanisms such as sterile motif and histidine aspartic domain and HD domain-containing protein 1 (SAMHD1) and APOBEC3G that hinder the infection of these cells by blocking HIV reverse transcription [51]. HIV DNA has been found in monocytes during ART in virally suppressed individuals [50, 52]. Despite multiple studies demonstrating monocytes and macrophages carry HIV provirus, the status of replication competence has yet to be elucidated.

1.3.3 Anatomical Reservoirs

In addition to blood, tissue sites such as gut-associated lymphoid tissue (GALT), lymph nodes and the central nervous system (CNS) contain a higher frequency of latently infected cells as well as infected cells that express HIV RNA and protein [53], these represent a major hindrance to viral eradication.

1.3.3.1 Central nervous system

Evidence of persistent HIV infection in the central nervous system (CNS) in PLWH on ART is also controversial. *In vitro*, HIV can infect astrocytes [54, 55] and human microglia cells [56]. In PLWH on ART, HIV can be found in microglial cells and macrophages in brain tissue [57, 58] consistent with a persistent reservoir. HIV can also be detected in the cerebral spinal fluid (CSF) where viral load is undetectable in the blood [59]. Recently, a study demonstrated an association between the presence of HIV DNA in the CSF and an increased risk of HIV associated neurocognitive disorder (HAND) [60]. Moreover, in non-human primate models of

HIV, viral RNA was found in brain tissue in animals both on and off ART [53]. Due to the difficulty in obtaining tissue samples of the brain, whether the brain represents an anatomical site of persistent HIV infection remains an area of active investigation.

1.3.3.2 Secondary lymphoid tissue

Secondary lymphoid tissues such as lymph nodes and the spleen are highly enriched for infected cells in PLWH on ART. In lymph nodes T_{FH} are a subset of memory T-cells that are highly enriched with HIV replication-competent DNA despite ART treatment [61, 62]. The extended life of these cells has been attributed to the anti-apoptotic protein B-cell lymphoma gene 2 (Bcl-2) [63]. These cells are localised to the B-cell follicles which are a sanctuary site given the limited penetration of cytotoxic T-cells [43, 61, 62]. Additionally, T-cells that circulate in the lymphoid tissues can come in contact with HIV infected dendritic cells (DCs) and endothelial cells (ECs) [64, 65], thereby increasing the chances of latent infection.

1.3.3.3 Gut-associated lymphoid tissue (GALT)

Gut-associated lymphoid tissue comprises of lymphoid aggregates in the small intestine and stomach, lamina propria, Peyer's patches and tonsils as well as containing the highest concentration of activated CD4+ T-cells that also express CCR5 [66], making it an ideal candidate as a site of viral persistence. Several studies have demonstrated HIV DNA and RNA can be detected in rectal tissue from PLWH on suppressive ART including work from our laboratory (Manuscript in press) [67] [68] and another study reporting 5-fold higher levels of HIV DNA in the gut than circulating CD4+ T-cells [69]. Moreover, another study estimated that the gut harbours 1.2×10^9 infected CD4+ T-cells [70], suggesting that the GALT is an important source of persistent viral infection.

Anatomical reservoirs may persist due to the low concentrations of drug in tissue "sanctuary sites", where the drug cannot fully penetrate and suppress viral production or replication [71, 72]. Additionally, secondary lymphoid organs can be considered as a sanctuary site due to the limited access of cytotoxic CD8+ T-cells [61].

1.3.4 Low level Viremia and Residual Virus Production and Replication

In all PLWH on ART, there is persistent low-level viremia at levels <20 copies/ml and detected by assays that detect down to one copy per ml. The source of residual viremia in patients on ART is unclear but multiple studies have demonstrated that drug resistance mutations do not emerge and there is limited or no evolution of virus sequence suggesting that the source of this virus is not from ongoing virus replication [73, 74]. Furthermore, sequencing of low-level plasma HIV RNA, demonstrates multiple identical sequences consistent with a persistent plasma clone [47]. Potential sources of low level plasma HIV RNA include 1) reactivation of viral expression from circulating latently infected resting CD4+ T-cells that become activated or 2) the release of virus from other unidentified sites such as the CNS [75] or GALT [69, 70]. Given the common detection of cell associated US HIV RNA in circulating and tissue resident CD4+ T-cells [76] as well as spontaneous production of viral RNA in CD4+ T-cells from sites such as lymph node [77], it is highly likely that this is some residual virus production in PLWH on ART. This is now commonly referred to as the “active reservoir”.

Whether there is true residual virus replication or just virus production (without infection of uninfected cells) has been a long-standing area of controversy. HIV viral evolution occurs through selection of variants that harbour advantageous mutations that allows for escape from immune mediated clearance [78]. If there was ongoing viral replication, we would expect viral sequence diversification over time in samples collected from PLWH on ART. However, most studies have not found evidence of viral evolution when multiple samples have been collected on ART, in either tissue or blood [73]. This was also the case when sequences from HIV positive individuals were compared pre- and post-ART [79]. Intensification of suppressive ART with additional antiretrovirals has been one way to test whether residual virus replication occurs. The addition of the integrase inhibitor raltegravir to a stable ART regimen in PLWH on ART resulted in an increase in 2-LTR circles in two separate clinical trials [80, 81] but was not observed following the addition of the integrase inhibitor dolutegravir [82]. An increase in this viral episomal DNA product following raltegravir treatment suggested that prior to inhibition of integrase, there was on-going cycles of HIV infection in PLWH suppressive ART [83]. However, in a study with intensification with dolutegravir, there was no increase in 2-LTR circles in treated versus placebo groups [82].

Potential explanations for these different results include the study population. In the study performed by Rasmussen *et al.* [82], only 12.5% of participants were taking protease inhibitors compared to 30-68% in the raltegravir studies [80, 81]. Moreover, this study was powered to detect a 3-fold change in the 2-LTR circles, therefore giving rise to the possibility that smaller increases could not be detected. In summary, it appears unlikely that there is residual virus replication on ART, rather there is persistent virus production on ART and there is a spectrum of transcriptional activity that persist on ART. This means that different strategies may be required to target transcriptionally silent and transcriptionally active CD4+ T-cells on ART.

1.3.5 Clonal Expansion

Multiple lines of evidence now demonstrate that latently infected CD4+ T-cells undergo clonal expansion in addition to having a long half-life. Initial evidence of clonal expansion came from the finding of plasma virus from PLWH receiving ART [47, 84]. However, confirmation of true clonal expansion of infected cells, came from the analysis of different infected cells with identical integration sites [85, 86]. In the absence of ART, HIV can randomly integrate throughout the human genome [87], however in PLWH on ART, there is a preference for integration in particular genes [85, 86]. HIV integration sites can be used to determine whether HIV infected cells arise from a single progenitor through clonal expansion. In one study in PLWH on ART, it was shown that approximately ~58% of all the HIV-infected cells derived from one single cell in one study participant [85]. Additionally, this study demonstrated the longevity of these clonally expanded cells during ART as one participant had at least 13 different clones in samples taken 11 years apart.

Moreover, multiple studies have found that in clonally expanded CD4+ T-cells, HIV integrates into specific genes that are associated with cell proliferation and apoptosis [85, 86]. In both studies, HIV integration into the BTB domain and CNC homolog 2 (BACH2) gene occurred at a higher frequency compared to integration of HIV in Jurkat or HeLa cell lines, suggesting that the site of integration may play a role in cell survival.

Clonal expansion of HIV infected cells can occur with either defective [88] or intact and replication-competent virus [39, 89, 90]. Using single-genome sequencing analysis as well as *ex vivo* culture experiments, numerous groups have demonstrated that provirus in clonally expanded cells are replication-competent and can also produce infectious virions [39, 89, 90]. Recently, it has been demonstrated that defective proviruses that lack the ability to produce functional HIV proteins can accumulate over time [91]. These defective proviruses were also shown to be clonally expanded clones [91]. This data demonstrates an important role of clonal expansion in HIV eradication.

1.4 Pathways to the Establishment of Latent Infection

HIV latency can be established and maintained through a number of mechanisms that are discussed below. There are two forms of latency - pre-integration or post-integration latency. Pre-integration results from the inhibition of the viral cycle, typically at the step of reverse transcription prior to the integration of the viral genome [92]. Upon cell activation, the viral genome translocates into the nucleus for integration [93]. In contrast, post-integration latency refers a state in which the viral genome has already integrated into the host genome but fails to productively produce HIV proteins and virions [94].

Post-integration latent infection can be established by two mechanisms: pre-activation or post-activation latency (Figure 1.5). Post-activation latency begins when a naïve T-cell encounters an antigen. The cell becomes activated and highly permissive to HIV infection. The majority of activated infected T-cells fully replicate HIV and die through virus-mediated cytopathic events [95] but a small population convert to a resting memory T-cell [96]. Recently it has been demonstrated that CD4⁺ T-cells that undergo effector-to-memory transition are highly permissive to HIV infection. Infection of these effector-to-memory transitioning (EMT) cells resulted in integration of the HIV provirus, but inhibition of gene transcription allowed for the establishment of latency [97]. These cells harbour integrated HIV DNA but do not express viral proteins due to the resting cell state and lack of critical cell transcription, RNA processing and translation factors, thus establishing latency [98] (Figure 1.5).

Direct infection of quiescent T-cells of HIV *in vitro* is inefficient due to the presence of multiple blocks to the viral lifecycle [99]. However, multiple studies demonstrate that resting CD4+ T-cells can also be directly latently infected with HIV, termed pre-activation latency. Studies by our laboratory (and confirmed by many other groups [100, 101]) find that pre-treating resting CD4+ T-cells with tissue chemokines: CCL19, CCL20, CCL21, CXCL9 or CXCL10 enhance direct latent infection [102, 103] (Figure 1.5). The addition of chemokines to a resting cell was shown to modify the actin cytoskeleton allowing for enhanced efficiency of HIV nuclear localisation and integration [103]. Additionally, infecting resting CD4+ T-cells with a high virus inoculum [104] and/or in the presence of spinoculation to increase virus infection [105] can also enable direct latent infection of resting CD4+ T-cells. Furthermore, co-culturing resting CD4+ T-cells with myeloid dendritic T-cells [106], SLAN+ DCs, CD14+ monocytes [107] or endothelial cells [108] also enable pre-activation latency (Figure 1.5). Each of these approaches enhance the permissiveness of resting CD4+ T-cells to infection and integration, without causing T-cell activation.

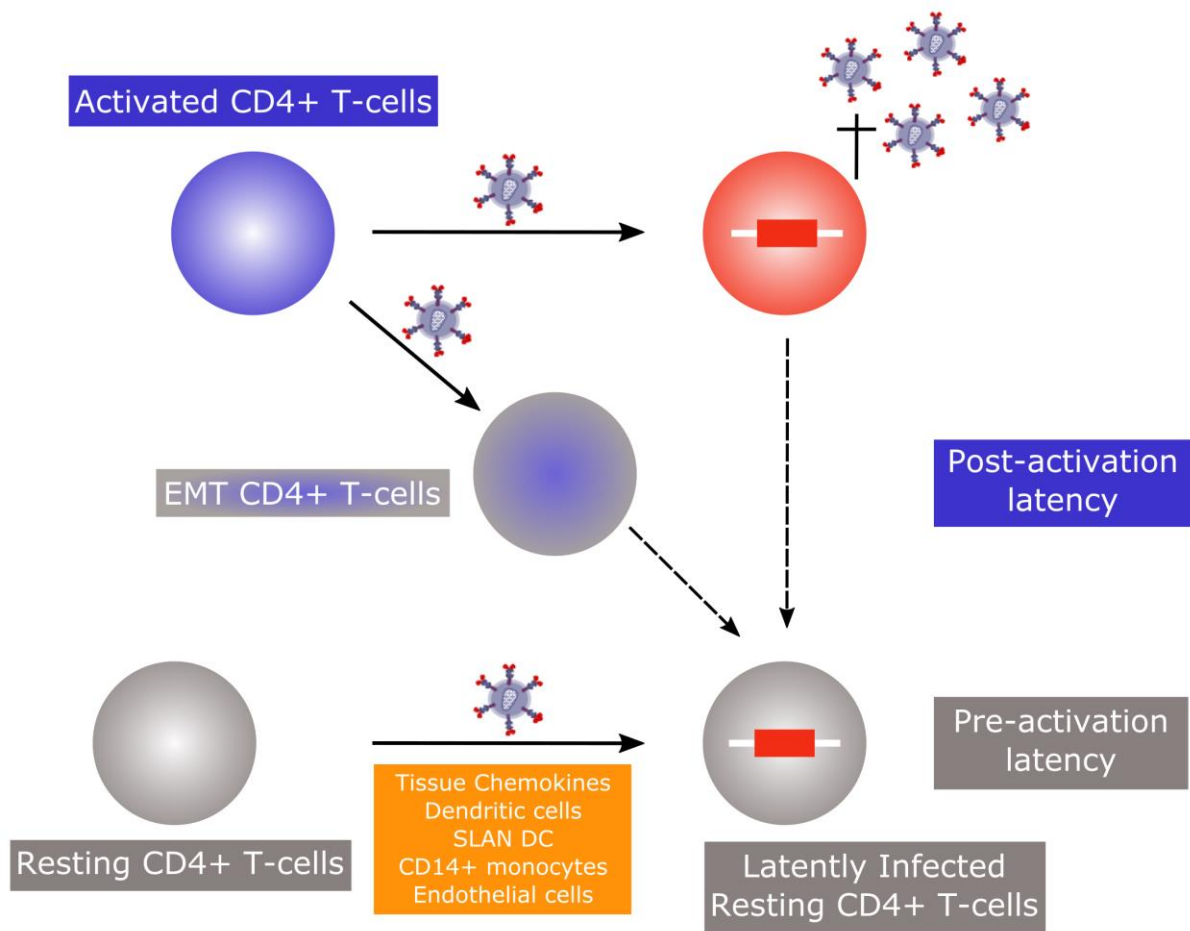


Figure 1.5. Establishment of latent infection of resting T-cells

Establishment of latency can arise by two key mechanisms. First, activated T-cells that become infected with HIV through direct infection can undergo two fates: cell death or revert back to a quiescent state with integrated HIV that fails to express viral proteins termed latently infected resting CD4+ T-cells. This mechanism is termed post-activation latency. Activated CD4+ T-cells are also permissive to infection as they transition to a resting state and leads to establishment of latency. An alternate mechanism termed pre-activation latency, is where resting CD4+ T-cells are directly infected with HIV, enhanced by tissue chemokines, or co-culture with myeloid or SLAN+ dendritic cells, CD14+ monocytes or endothelial cells. Viral DNA integrates into the cell while it is still in its resting state, but viral proteins are not expressed, also generating latently infected resting CD4+ T-cells. Adapted from [109].

1.5 Maintenance and Outcomes of HIV Latency

The molecular mechanisms that contribute to the maintenance of HIV latency are most likely multifactorial and involve a diverse range of cellular processes.

1.5.1 Site and orientation of integration

The site and orientation of integration can affect the transcriptional state of a latently infected cell. In most instances, HIV proviral DNA integrates into actively transcribed regions of the host genome [87]. Transcriptional interference refers to the direct negative impact of one gene on another. One mechanism of transcriptional interference in the context of HIV maintenance is promoter occlusion (Figure 1.6), where the provirus integrates downstream of a host gene that is in the same transcriptional orientation or polarity [110]. This may result in the displacement of constitutively expressed transcription factors that bind to the HIV LTR and are crucial for gene expression [111]. Another mechanism is referred to as convergent transcription. This occurs when the provirus integrates into the opposite orientation or polarity relative to the host gene causing the collision of RNA Polymerase II (RNAPII) from the host and viral promoters and ultimately leading to early arrest of transcription [112]. Thus, the site and orientation of integrated provirus may prevent HIV promoter transcription to favour latency.

1.5.2 Epigenetic modification

Cellular DNA, including integrated HIV DNA, bind histone proteins, which arrange into nucleosomes and package the DNA into higher order structures termed chromatin. Chromatin structure may be affected by modifications to histone tails. These modifications affect the contact between different histones in adjacent nucleosomes (a structural unit of a chromosome, consisting of a DNA coiled around a core of histones [113]) or the interaction of histones with DNA. Well understood histone modifications include histone acetylation [114] and methylation [115]. Histone acetylation plays a critical role in the transition and maintenance of the two chromatin environments; active euchromatin and silent heterochromatin [116, 117].

The compaction of the HIV proviral DNA and viral transcription directly depend on epigenetic modifications such as hypoacetylation and hypermethylation of histones (Figure 1.6). These modifications recruit various regulatory proteins and transcription factors to the HIV LTR, leading to the downregulation of HIV gene expression [118] resulting in silencing of active gene transcription [119].

In addition to histone modification, DNA methylation also regulates gene transcription. DNA methylation is achieved by DNA methyltransferases (DNMTs) and occurs at 5-methyl cytosine predominantly in the setting of CpG dinucleotides [92]. DNA methylation in regions that undergo active transcription is associated with gene silencing. During HIV latency, the HIV LTR promoter was found to be hypermethylated at two CpG islands that surround the HIV transcription start site in *in vitro* models of HIV latency [120]. Additionally, regulation of CpG methylation was found as a reactivator of HIV latency [121]. However, it was reported that CpG methylation is rare in the HIV LTR from aviremic individuals on ART [122]. Thus, there is controversy surrounding the degree of methylation of the HIV LTR *in vivo* and if this plays a role in maintaining HIV latency *in vivo*.

1.5.4 Transcription initiation factors

Transcription factors bind to the HIV LTR and can either promote or repress viral transcription. Important transcription factors that activate the HIV LTR do so through the formation of the pre-initiation complex. This complex binds to the U3 region of the LTR and is comprised of numerous cellular factors including: nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) [123], specificity protein 1 (Sp1) [123] and nuclear factor of activated T-cells (NFAT) [124]. In resting cells, these transcription factors are sequestered in the cytoplasm thereby maintaining HIV latency [96, 125, 126].

1.5.5 Transcription elongation factors

HIV Tat protein is produced early in HIV replication [127]. After the binding of cellular transcription factors and RNAPII to the HIV LTR promoter to initiate transcription, the complex pauses early after initiation and Tat is crucial to the continuation of transcriptional elongation (reviewed in [128]). Tat acts in tandem with P-TEFb and the super elongation complex to

facilitate elongation of the HIV transcript and overcome RNAPII pausing [129]. Activity of Tat and P-TEFb are tightly regulated through post-translational modifications such as acetylation, deacetylation and low levels of the cyclin T1 component of P-TEFb [130] (Figure 1.6). The downregulation of cyclin T1 and thus active P-TEFb, together with the lack of Tat expression from latent HIV has been suggested to contribute to HIV latency due to their role in viral transcription [131-133].

1.5.6 RNA Processing and Translation factors

HIV Rev protein mediates the nuclear export of HIV unspliced and partially spliced mRNA transcripts in infected cells [134]. Co-factors such as matrix-associated RNA binding matrix 3 (MATR3) can interact with Rev and is essential for viral RNA processing [98]. Recent evidence has emerged demonstrating that blocks in post-translational factors such as MATR3 can impact the maintenance of HIV latency [98]. CD8⁺ depleted T-cells from PLWH *ex vivo*, expressed very low levels of translational factors MATR3 and polypyrimidine tract-binding protein-associated splicing factor [98]. It is likely that the low levels of translation factors restricted the reactivation of HIV latency using well-known latency reversing agents.

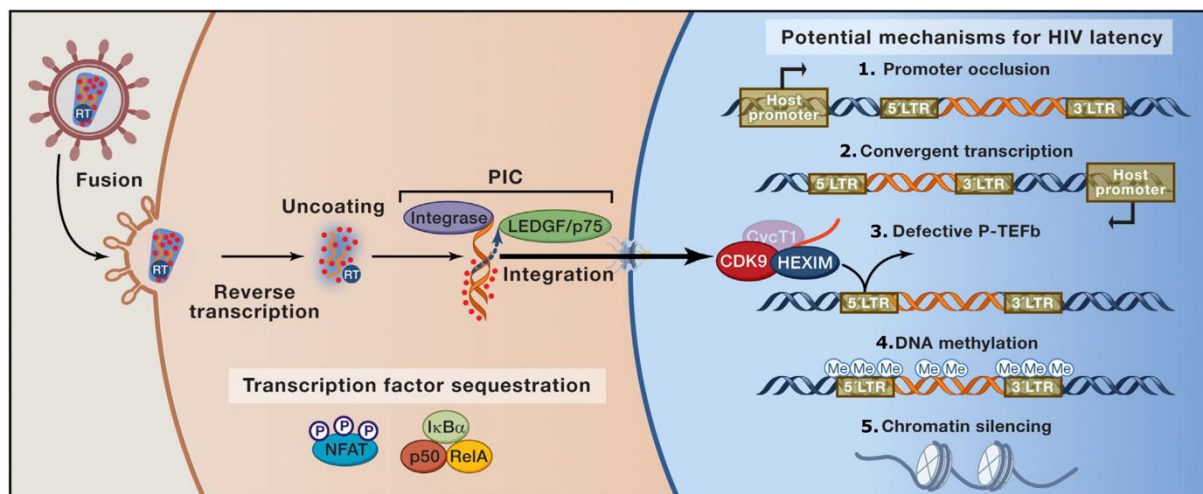


Figure 1.6. Molecular maintenance of HIV latency

Multiple mechanisms contribute to the maintenance of HIV latency. These include the site of integration and 1) promoter occlusion, in which the positioning of the provirus downstream of the host promoter causes the displacement of necessary transcription factors from the HIV promoter. 2) Convergent transcription is another mechanism that is described as the collision

of RNA Polymerase II complexes due to the opposite orientation of the host gene and provirus. Both integration site orientations perturb HIV transcription and foster latency. 3) Alternatively, the sequestration of essential transcription factors like NFAT and NF- κ B in the cytoplasm can prevent transcription and contribute to latency. Low levels of cyclin T1, which forms positive transcription elongation factor-b (P-TEFb), can be low or sequestered in the cytoplasm resulting in pausing of HIV transcription and thus latency. Finally, 4) DNA methylation and 5) chromatin silencing by histone acetylation and histone methylation can also lead to HIV latency maintenance [110]. Adapted from [110].

1.5.7 Cellular factors maintaining latency

HIV latency can also be maintained through a range of other mechanisms that are characteristic of T-cell homeostasis [135, 136]. These include the long half-life of some T-cell subsets, homeostatic proliferation, antigen-driven clonal expansion and T-cell differentiation and reversion. Naïve and central memory CD4⁺ T-cells have long half-lives, which enables them to carry HIV provirus for extended periods of time. In fact, upon re-examination of the stability of the latent reservoir in PLWH on ART, it was found that latently infected cells can survive up to 43 months [137].

Clonal expansion of infected cells with identical integration sites and/or virus sequence has also been demonstrated on ART [85, 86] (section 1.3.5). These clones wax and wane over time [138] and given that the number of latently infected cells doesn't expand over time, must be under some homeostatic control [39]. Potential factors that might control the total pool of latently infected cells over time include recognition of cognate or cross-reactive antigen. Antigen-driven T-cell proliferation was observed in infected T-cells enriched in cancer metastases from a PLWH on ART who had squamous cell carcinoma [39]. Additionally, cytokine-driven homeostatic proliferation may contribute to the persistence of latently infected T-cells. The cytokine IL-7 increases the number of CD4⁺ T-cells by promoting proliferation [139]. In a clinical trial of IL-7, proliferation of HIV latently infected cells without virus reactivation was observed, leading to the expansion of latently infected cells [34, 140, 141].

The site of HIV integration may also determine the probability of proliferation [142]. HIV proviruses selectively integrate into some specific genes (section 1.5.1), such as BACH2 and myocardin-like protein 2 (MKL2) gene which have been associated with cell survival [85, 86]. Additionally, our laboratory has demonstrated integration sites in clonally expanded cells from PLWH on suppressive ART were in pathways associated with apoptotic evasion and cell proliferation [143].

It has now become clear that there are numerous mechanisms that control and maintain latency, and these may differ for different T-cell subsets making elimination of latency complex (Figure 1.7).

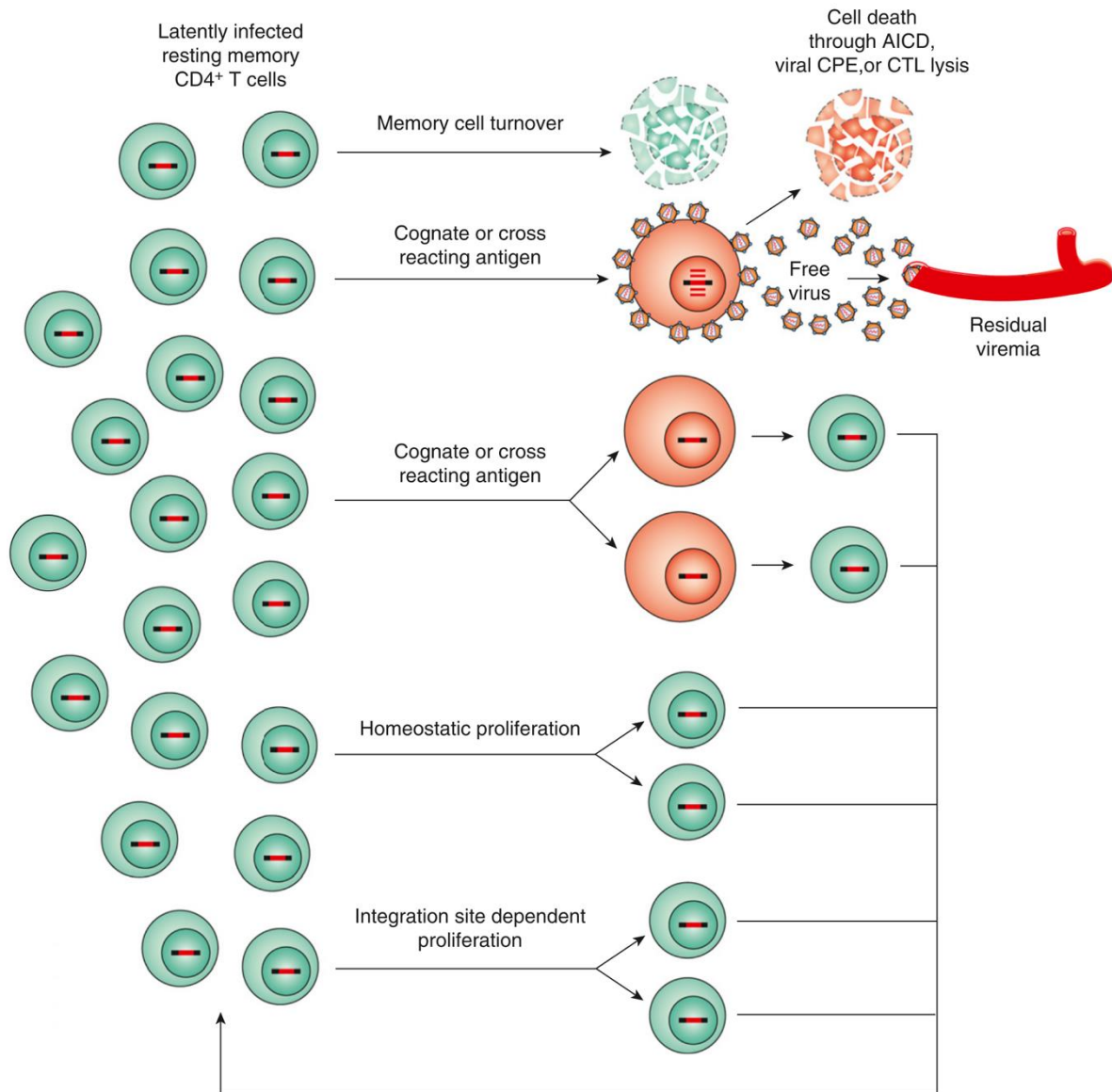


Figure 1.7. Other additional mechanisms of maintenance of the viral HIV reservoir

Latently infected resting CD4⁺ T-cells with integrated provirus can have multiple fates allowing for long term persistence. Normal cell turnover results in the death of some latently infected cells. Cognate or cross-reacting antigens can reactivate a small number of latently infected cells. These reactivated viruses then begin to produce viral particles which may contribute to low level viremia. The reactivated cell can then die through viral cytopathic events (CPE) or cytotoxic T-cell lysis (CTL) or through activation-induced cell death. Additionally, cognate or cross-reacting antigens can drive proliferation. Homeostatic proliferation and integration site dependent proliferation both contribute to the maintenance of the latent HIV reservoir. Image from [144].

1.6 Measuring the latent reservoir

The low frequency of latently infected cells with intact replication-competent virus *in vivo* has made quantification of the reservoir a major challenge. Proviruses are defined as replication-competent if the provirus is capable of producing viral particles upon re-stimulation [145]. Several assays described below measure the frequency of intact, replication-competent provirus while others measure the number of intact proviruses.

1.6.1 Measuring the frequency of infected cells

1.6.1.1 Replication-competent virus

The quantitative viral outgrowth assay (QVOA) is considered to be the gold standard for measuring the frequency of latently infected CD4⁺ T-cells that harbour replication-competent provirus. In this assay, resting CD4⁺ T-cells are isolated from PLWH on ART and plated in a serial dilution format. As the cells are resting and do not produce virus, the cells are activated with a stimulus such as the mitogen PHA and co-cultured with irradiated PBMC to amplify virus replication. Following latency reversal, CD8-depleted lymphoblasts are added to cell culture to propagate released virus for 2-3 weeks. Culture supernatants are then analysed for p24 antigen using ELISA [30, 146]. Some modifications to improve the throughput and sensitivity of the QVOA have included using CD3/CD28 antibody-coated beads [147] or PMA with ionomycin [148] in place of PHA with irradiated feeder PBMCs. Substitution of the CD8-depleted lymphoblasts with a T-cell line that expresses CD4 and CCR5, such as MOLT4/CCR5, can also reduce the labour-intensive virus expansion step. Utilisation of the MOLT-4/CCR5 T-cell line is advantageous as these cells can be left to culture for long periods of time without the need for additional cells, purification or activation [146].

Traditionally, outgrowth of virus was measured using enzyme-linked immunosorbent assay (ELISA) for the HIV p24 antigen 2-3 weeks after addition of CD8-depleted lymphoblasts to cell culture. An alternative approach that can reduce the time to complete the QVOA assay is to use quantitative reverse transcription PCR (RT-qPCR) to detect HIV RNA. This method of detection allows for measurement of viral outgrowth as early as 7 days following cell activation [69]. An additional modification the use of Single Molecule Arrays (SiMoA or digital

ELISA), which has allowed for the detection HIV p24 from PLWH, was 1000-fold more sensitive than traditional ELISA [149].

The QVOA assay has traditionally used a single round of T-cell activation. Studies have demonstrated that the QVOA assay underestimates the number of replication-competent proviruses in the latent reservoir due to the presence of intact, non-induced proviruses [145]. Additional stimulation of wells negative for viral outgrowth resulted in the activation of some intact, but previously non-induced proviruses, increasing the number of proviruses detected using QVOA [90, 145].

Further modifications to enhance sensitivity of replication-competent virus has included the murine viral outgrowth assay (MVOA) [150]. This assay involves the adoptive transfer of CD4⁺ T-cells from PLWH on ART into NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice. After 4-57 days plasma is collected, and qRT-PCR is used to quantify HIV RNA. Cells can be activated with anti-CD3 in some circumstances or CD8⁺ T-cell depleted [150] prior to infusion in the NSG mice, to increase the amount of virus detected. Using the MVOA, virus was successfully amplified from cells taken from PLWH on ART and elite suppressors with undetectable viral loads [150]. Additionally, cells taken from simian immunodeficiency virus (SIV)⁺ macaques with undetectable viral loads were also amplified in the NSG mice. Although MVOA is not quantitative, this method presents as a sensitive assay for the detection of residual virus in the blood that would not be measured using QVOA [150]. Moreover, MVOA could potentially be used to study the location of viral reservoirs in tissues.

1.3.1.2 Intact virus

Despite being the gold standard approach to measuring the latent HIV reservoir, the QVOA assay is laborious and time-consuming. An alternative approach to detect intact proviruses is to use sequencing. The Full-Length Individual Proviral Sequencing (FLIPS) assay [151] is based on Next Genome Sequencing (NGS) to sequence intact and defective full-length proviruses [151]. Previous full-length sequencing assays used primers that target the highly conserved 5' and 3' U5 LTR regions, rather than targeting sub-genomic regions of the full-length genome. FLIPS is a high-throughput NGS assay that follows a process by which proviruses that contain

inversions, large internal deletions, stop codon/s and hypermutations, frameshift mutations and packaging signal or major splice donor site defects are eliminated and determined to be defective or replication-incompetent [151]. Using FLIPS, it was observed that PLWH on long-term ART contain very few intact, replication-competent proviruses (5%) and the proviruses are unequally distributed within CD4⁺ T-cell subsets [151]. It is important to note that detection of intact proviruses does not mean that the virus is replication-competent [151].

Recently, a higher throughput method to detect intact virus has been described [152]. The intact proviral DNA assay (IPDA) can distinguish intact and defective proviruses, by using primers that bind to regions of the virus that commonly contain hypermutation or large deletions. IPDA distinguishes proviruses using a droplet digital PCR (ddPCR) that contains amplicons in the 5' ψ packaging signal and 3' *env* region [152]. Moreover, the *env* region PCR also allows for the identification of hypermutated proviruses. Using this novel detection method, it was found that the frequency of intact proviruses in blood from PLWH on ART or elite suppressors was approximately 100 per 10⁶ resting CD4⁺ T-cells and were vastly outnumbered by defective proviruses (97.6%) [152]. The assay correlated strongly with QVOA but the frequency of intact proviruses was 100-fold greater than what was detected using QVOA [152].

1.6.1.3 PCR-based assays – Total and integrated DNA

A common approach to measure the latent reservoir is to perform a quantitative PCR (qPCR) to detect viral DNA from CD4⁺ T-cells [153]. These assays will detect unintegrated linear proviral DNA 2-LTR circles and integrated DNA.

Integrated DNA can be quantified using Alu-LTR PCR [154]. The Alu-PCR includes a primer that specifically targets Alu elements that are found in genomic DNA and a second primer that targets an HIV gene, commonly HIV *gag* and is followed by a second round PCR with primers to the HIV LTR [154, 155]. PCR-based assays result in overestimation of the replication-competent reservoir [156] because PCR-based assay cannot differentiate between defective and intact, or replication-competent proviruses [145]

1.6.2 Measuring the transcriptionally active reservoir

1.6.2.1 PCR based assay- Unspliced and Multiply spliced RNA

Alternative methods that measure the inducible latent reservoir have been developed and these assays measure the levels of HIV RNA from cells or cell supernatants. HIV RNA taken from cells or cell extracts are termed as cell-associated (CA) RNA, whilst RNA from cell supernatants are termed cell-free (CF) RNA [157]. Alternative splicing of HIV US RNA can result in the production of over 100 RNA transcripts, including SS and MS RNA transcripts [158].

Measurements of CA HIV RNA can provide information on the frequency and transcriptional status of the latent HIV reservoir. An advantage of HIV RNA assays to measure the transcriptionally active reservoir is that they do not rely on a virus propagation step. Thereby reducing the cost and time associated with the QVOA. A single or semi-nested RT-qPCR is performed to quantify the RNA in either CA or CF samples, which enables quantification of RNA in samples with low copy numbers [159]. Measuring CA HIV RNA can also result in overestimations of the latent HIV reservoir. Moreover, transcriptional competency does not always equal replication competency as other post-translational blocks may affect the ability of a provirus to produce viral particles. Finally, these assays when done without limiting dilution are unable to distinguish between one cell producing abundant HIV RNA or many cells producing low levels of HIV RNA.

1.6.2.2 RNAscope/ViewRNA

One strategy to identify the number of cells producing HIV RNA is to use *in situ* hybridization (ISH) and immunohistochemistry. RNAscope also known as viewRNA builds upon the ISH techniques and combines it with branched DNA (bDNA) technology [160] to simultaneously achieve single-molecule visualisation and increase specificity and sensitivity [161, 162]. RNAscope [161] involves staining of target RNA sequences with a set of RNA probes that have a novel double-Z design strategy. Each target probes consists of an 18-25 base sequence that is complimentary to the target RNA, a spacer sequence and a 14-base tail sequence. A pair of target probes that each have a different tail sequence will hybridise to the target region and form a hybridisation site for the pre-amplifier. The use of a pair of target probes that bind

adjacent to each other significantly increases the specificity of this staining strategy. The pre-amplifier contains a 20-base binding region to which the amplifier can bind, which also has a 20-base binding region for the label probe. Labelled probes will amplify the RNA target signal up to 8000-fold [161].

Using RNAscope technology, the quantification of transcription-competent cellular HIV reservoirs were found across a range of tissues in both untreated and ART-treated non-human primate (NHP) animal models [53, 163]. The RNAscope technology can be modified to detect HIV DNA, known as DNAscope [163]. The combined RNAscope and DNAscope methods enabled the distinction of transcriptionally active cells (RNA+ DNA+) from transcriptionally inactive (RNA- DNA+) cells, which are potentially latent [163]. Using RNAscope and DNAscope to examine lymph node and rectal tissue sections from PLWH collected before and after ART and in NHP on ART, it was shown that while, the level of HIV DNA decreased in the lymph nodes following ART, RNA remained detectable [53]. These studies therefore demonstrated the persistence of a transcriptionally active reservoir in tissue sites on ART.

1.6.2.3 Inducible virus- TILDA

The Tat/Rev Induced Limiting Dilution Assay (TILDA) measures the frequency of CD4+ T-cells from blood that carry inducible latent HIV proviruses [164]. CD4+ T-cells are maximally stimulated with PMA/ionomycin and plated in limiting dilutions. The levels of *tat/rev* multiply spliced RNA are then measured using RT-qPCR. TILDA is a sensitive and specific assay that measures the level of inducible virus from virally suppressed individuals. Analysis of CD4+ T-cells from PLWH, the mean frequencies of latently infected T-cells that carried inducible virus was 24 cells/ 10^6 CD4+ T-cells [164]. This is 48 times higher than the frequency measured by QVOA and 6-27 times lower than the frequency measured by PCR-based assays [164]. An advantage of TILDA is that the assay does not require the amplification of the virus unlike the QVOA which can take up to seven days to culture, only a small volume of blood is required, and sample plates can be stored for long periods of time, making it an attractive choice for longitudinal clinical studies [164].

1.6.3 Measuring the translationally competent reservoir

1.6.3.1 Flow Cytometry based

When measuring transcriptionally competent provirus, not all cells that are HIV RNA positive will contain provirus that is able to produce HIV proteins and infectious virus particles. Moreover, it has been hypothesised that HIV infected cells carrying defective provirus have increased susceptibility to reactivation [165]. Thus, several groups have focused on measuring translationally competent cells. This is achieved by the combination of PrimeFlow RNA staining with an intracellular HIV Gag protein antibody stain [166, 167]. Using this combined technique, several groups have identified RNA positive, HIV protein positive cells in CD4+ T-cells from PLWH on suppressive ART [166, 167]. Baxter *et al.* [166] reported an average of 3.56 RNA positive, HIV Gag protein positive (HIV^{RNA+/Gag+}) cells per million cells and Grau-Exposito *et al.* [167] reported 6 RNA and Gag protein positive cells per million CD4+ T-cells. These values are higher than the number of replication-competent cells determined using QVOA but are less than PCR-based methods. Crucially, the detection of false positives was decreased compared to PrimeFlow staining alone.

Another approach is to measure HIV protein alone using antibodies to HIV p24. HIV-Flow is an assay that has been recently described [168] that allows for the detection of HIV capsid protein with high sensitivity and specificity using two HIV p24 antibodies; p24 KC57-PE and p24 28B7-APC. Comparing HIV-Flow to PrimeFlow, a significant correlation between both assays was observed [168], indicating HIV-Flow estimates the frequency of translationally competent cells (4.3 p24-producing cells per 10⁶ CD4+ T-cells) similar to PrimeFlow in cells from PLWH [166, 167].

1.6.3.2 Single cell assays- LURE

Latent cell capture or LURE is a recently described method that isolates and enriches for reactivated latent cells using a combination of antibody staining, magnetic enrichment and flow cytometry [88]. Enrichment of cells was dependent on the activation with PHA [88]. Although LURE can capture a subset of latently infected cells that contain translationally competent HIV provirus, the use of PHA for reactivation limits the detection of the entire latent population of

cells as not all latently infected cells will be reactivated following a single round of reactivation [145].

1.6.3.3 Imaging

One major limitation in the eradication of latent HIV is the inability to characterise the burden of HIV infection in tissues and therefore newer *in vivo* imaging approaches are of great interest. Immuno positron emission tomography (PET) (immunoPET) imaging using an antibody to envelope labelled with ^{64}Cu could detect infected cells in tissue sites in macaques infected with SIV [169]. Detection of SIV env were found to be localised in the gut, genital tract and lymphoid tissues in chronically infected, aviremic, ART-treated and elite controller animals [169] and imaging signals were verified using qRT-PCR on tissue biopsies. Whether these technologies can also be applied to PLWH on ART remains to be determined.

1.7 Models of HIV Latency

To study HIV latency and investigate drug compounds that could be used to purge latently infected cells in patients, models of HIV latency are typically used. This is because while *ex vivo* CD4⁺ T-cells from PLWH on ART would be the best source of latently infected CD4⁺ T-cells, this is challenging due to the low frequency of infected cells [170]. This minor population of latently infected cells (approximately 60-70 per 1 million CD4⁺ T-cells) [145, 157] also do not have distinct cell surface markers or other characteristics from uninfected cells [171]. To perform any laboratory-based experiments on these cells from patient donors; millions of highly purified CD4⁺ T-cells must be obtained by leukapheresis, which is expensive but possible and has been successfully performed in this thesis [171]. However, for many studies such as understanding the establishment of latency or assessing multiple compounds for latency reversal or silencing, latency is typically studied using T-cell lines, primary T-cells and animal models. Animal models of latency are not used in this thesis and are described elsewhere [172, 173].

1.7.1 T-cell line models of HIV latency

Latently infected transformed cell lines have been extensively used to understand mechanisms of latency establishment and reactivation. Discussed below are some of the T-cell line models of HIV latency used in this thesis.

1.7.1.1 J-Lat T-cell line model of HIV latency

J-Lat T-cells allow for quantification of HIV transcription using detection of GFP by flow cytometry. These cell lines were generated through infection of the lymphocytic Jurkat T-cell line with viral particles containing the molecular clone (HIV-R7/E-/GFP pseudotyped with vesicular stomatitis virus envelope glycoprotein (VSV-G)). This clone contains a GFP open reading frame in place of the *nef* gene and has a frameshift mutation in the *env* gene to restrict infection to a single round [174]. Several different clones were isolated from the infection, one of which the 6.3 T-cell clone was used in this thesis. The different J-Lat clones described (Table 1.1) all contain a frameshift *env* mutation, thus making them replication-incompetent and GFP in place of *nef* [174]. Work from our laboratory demonstrated that the single integration site in J-Lat cells was stable over time [175].

1.7.1.2 ACH2 T-cell line model of HIV latency

ACH2 T-cell lines carry replication-competent latent HIV provirus and were generated from infection of the A3.01 cells with the lymphadenopathy-associated virus (LAV) strain of HIV and cloned using limiting dilution. ACH2 cells represent a clone that survived the infection and constitutively expressed low levels of reverse transcriptase and this cell line is also CD4-negative [176-178]. Recently, our laboratory has demonstrated that the ACH2 T-cell line are not clonal [175]. In ACH2 T-cells which has a reported HIV integration site in chromosome 7 [177, 178], additional integration sites in chromosome 9 were detected [175]. Additionally, the number of diverse integration sites increased over time in culture with the ACH2 T-cells, demonstrating that there is some ongoing virus replication. Although latently infected T-cells can undergo clonal expansion [85, 86], HIV integration into the genome is heterogenous [136]. Treatment of the ACH2 cells line with either tumour necrosis factor- α (TNF- α) or the mitogen phorbol 12-myristate 13-acetate (PMA), is able to successfully reactivate HIV [177]. The advantage of the ACH2 T-cell line over J-Lat6.3 T-cell line is the presence of full-length HIV

that also results in the production of Envelope and Nef protein when reactivated. Moreover, less than 5% of the total cells are latently infected with HIV [176], which better recapitulates latent HIV infection *in vivo*. Although ACH2 T-cells do not reflect the cellular environment of HIV infection *in vivo*, ACH2 T-cells are easy to use, readily available and can be used for large drug screening experiments.

1.7.1.3 Other latently infected T-cell lines

Multiple chronically infected T-cell lines have been described (Table 1.1). U1 T-cells were generated from the infection of the parent U937 promonocytic T-cells with HIV_{LAV} virus. Infected cells that were negative or showed low expression of HIV were isolated and cultured [176, 179]. These cells could be reactivated using tumour necrosis factor or PMA [177]. The U1 T-cells carry two integration sites [179] and carry a mutation in the *tat* gene, which maintains latency [180, 181].

J89EGFP latently infected T-cells, often referred to as J89 cells were made from a clonally expanded population of Jurkat T-cells infected with the 89.6 HIV viral strain modified to contain GFP protein [182]. The unique factor of the 89.6 HIV viral strain is the dual-tropic nature of the virus, allowing the use of both CXCR4 and CCR5 co-receptors for viral entry. The incorporation of the GFP gene allows for fast and easy detection of the expression of HIV reactivation using flow cytometry or fluorescent microscopy.

Latently infected J1.1 T-cell line was established after infection of Jurkat T-cells with HIV_{LAV} virus [183]. Recent work has demonstrated that two major HIV integration sites are present in these cells [175]. Moreover, the J1.1 T-cells have defects in the production of interleukin-2 (IL-2) and calcium (Ca²⁺) mobilisation following CD3 stimulation [183].

Table 1.1. HIV chronically infected T-cell lines and their characteristics

Cell Line	Clone	Parent cell line	Replication-Competent virus	Mutation	Comments	Ref
ACH2		A3.01 cells	Yes	Mutation in TAR region, impairing response to Tat	Low CD4 expression	[178]
U1		U937 cells	Yes	Mutation in Tat	Low CD4 expression	[179]
J89EGFP		Jurkat T-cells	Yes	Wildtype, insertion of GFP into <i>env</i>	Dual-tropic virus	[182]
J1.1		Jurkat T-cells	Yes	Wild type	Defects in IL-2 production and Ca ²⁺ after CD3 signalling	[183]
J-Lat	J-Lat10.6	Jurkat T-cells	No	<i>env</i> frameshift mutation	Contains GFP reporter Deficient in <i>nef</i>	[174]
	J-Lat6.3	Jurkat T-cells	No	<i>env</i> frameshift mutation	Contains GFP reporter Deficient in <i>nef</i>	[174]
	J-Lat15.4	Jurkat T-cells	No	<i>env</i> frameshift mutation	Contains GFP reporter Deficient in <i>nef</i>	[174]
	J-Lat8.4	Jurkat T-cells	No	<i>env</i> frameshift mutation	Contains GFP reporter Deficient in <i>nef</i>	[174]
	J-Lat9.2	Jurkat T-cells	No	<i>env</i> frameshift mutation	Contains GFP reporter Deficient in <i>nef</i>	[174]

1.7.1.4 Disadvantages of T-cell line models of HIV latency

While T-cell line models can help illustrate signalling pathways and search for reactivation of latent provirus, they do not recapitulate HIV infection *in vivo*. T-cells lines are immortalized and clonal, which means that the site of viral integration is often the same in all cells [175, 184]. Latently infected T-cell lines also are unable to cycle from active to quiescent phases unlike primary models of HIV latency. Therefore, primary CD4⁺ T-cell models of HIV latency are preferred. Primary CD4⁺ T-cell models of HIV latency have a diverse numbers of integration sites [185, 186], and are not clonal or immortalised, which is similar to what is seen *in vivo*.

1.7.2 In vitro primary T-cell models of HIV latency

Currently several different primary T-cell models are used to investigate HIV latency, each modelling one or more aspects of latency [187]. Since HIV mainly infects resting CD4⁺ T-cells, many groups have tried to mimic the generation of these latently infected cells *in vitro* via both post-activation as well as pre-activation latency pathways (summarised in Table 1.2).

1.7.2.1 Post-Activation Latency Primary T-Cell Models

The first post-activation latency primary T-cell model was developed by Sahu *et al.* [188]. This model involved activation and infection of CD4⁺ T-cells using replication-competent HIV and co-culture of cells with brain tumour-derived feeder cell line H80 cells to establish latency. Similarly, the Tyagi model activates primary CD4⁺ T-cells from healthy donors with α -CD3/ α -CD28 antibodies, infects with a HIV vector and co-cultures with the H80 feeder cell line and IL-2 [125] to induce latency. Marini and colleagues describe a model of HIV latency that utilises antigen loaded monocyte-derived dendritic cells (MDDCs) to activate CD4⁺ T-cells [189]. The Bosque and Planelles [96] model involve activating naïve CD4⁺ T-cells with α -CD3/ α -CD28 antibodies in the presence of IL-2. In the Yang/Siliciano model [190], primary CD4⁺ T-cells were activated with α -CD3/ α -CD28/IL-2 and transduced with a lentiviral vector expressing the Bcl-2 gene enabling cells to survive a long culture period.

1.7.2.2 Pre-Activation Latency Primary T-Cell Models

The first *in vitro* model to demonstrate direct latent infection of resting CD4⁺ T-cells was reported by Swiggard *et al.* [191], where spinoculation of resting CD4⁺ T-cells with high inoculums of HIV allowed for direct latent infection.

The **chemokine model** of HIV latency, developed by our laboratory, involves stimulation of resting CD4⁺ T-cells with tissue chemokines such as CCL19 and CCL21 to enhance direct latent infection [102, 103]. Our group demonstrate that pre-treatment of resting CD4⁺ T-cells *in vitro* with chemokine ligands for chemokine receptors: CCR7 (CCL19, CCL21); CCR6 (CCL20) or CXCR3 (CXCL9, CXCL10) allows for efficient HIV integration but little viral reverse transcription, consistent with latent infection [102, 103, 192]. This occurs through the rapid dephosphorylation of cofilin, leading to changes in the filamentous actin polymerisation that allowed for efficient nuclear localisation and integration of the HIV provirus [103]. The addition of CCL19 facilitated changes to the target cells before HIV infection.

Our laboratory [106, 107] has also demonstrated that co-culturing resting CD4⁺ T-cells with myeloid dendritic cells, or SLAN⁺ dendritic cells or CD14⁺ monocytes can dramatically increase latent HIV infection in memory CD4⁺ T-cells. Direct cell-to-cell contact of infected myeloid dendritic cells (mDCs) and non-proliferative (eFLUOR^{hi}) CD4⁺ T-cells resulted in the production of latently infected T-cells [106]. The production of latently infected T-cell was also greater in memory T-cells than naïve T-cells. Recently, we have also shown that myeloid dendritic cells can induce latency in proliferating CD4⁺ T-cells, which were identified as eFLUOR^{lo} [193]. Proliferating cells expressed markers of activation including CD69, HLA-DR, Ki67 and immune checkpoint markers TIM-3 and PD-1 [193]. A subset of latently infected T-cells was long-lived. This model suggests that latent infection may not be connected to the proliferation and activation status of the CD4⁺ T-cell.

Additionally, several groups report that stimulation of resting CD4⁺ T-cells with endothelial cells can lead to direct productive and latent infection of these cells [65, 108, 194]. Co-culture of CD4⁺ T-cells with EC resulted in the production of latently infected cells that remain unactivated [108].

1.7.3 Comparison of primary T-cell models of HIV Latency

Each primary T-cell model of HIV latency has advantages and shortcomings (summarized in Table 1.2). Despite the benefits of *in vitro* models of HIV latency, it is important to determine that results from *in vitro* models align with latently infected cells from PLWH on ART *ex vivo* and ideally with effects observed *in vivo*. Spina and colleagues [195] performed a cross-model comparison of 10 different *in vitro* models of HIV latency to *ex vivo* latently infected patient cells using a panel of known LRAs that reactivate the latent virus. No single *in vitro* model of HIV latency completely recapitulated the response of *ex vivo* latently infected cells from PLWH on ART to the known stimuli [28]. However, the CCL19 chemokine model and J-Lat 5A8 T-cell line model clustered more closely with cells from PLWH more than other models [195]. This suggests that these two models may represent the biological properties of latently infected cells from PLWH on ART *ex vivo*. The CCL19 chemokine model only had a single false negative result compared to the J-Lat 5A8 model, which had six false negative results [195]. As the likelihood of the CCL19 chemokine model to miss compounds that reactivate latency in *ex vivo* patient cells is lower, this may indicate that the CCL19 model is an appropriate *in vitro* surrogate model to screen for new agents that reactivate latency. Additionally, the tissue chemokine treatment of resting cells in the CCL19 model to establish latency may better mimic aspects of latency establishment in tissue. The presence of tissue chemokines in the CCL19 model of HIV latency mimics the environment in which CD4+ T-cells are infected. Other models of HIV latency such as the Yang/Siliciano model may not reflect HIV infection *in vivo* due to the overexpression of the pro-survival Bcl-2 gene, rendering the cells more resistant to cell death and not recapitulating what is seen *in vivo* [190].

Table 1.2 Comparison of Primary T-cell HIV latency Models*

Model Type	Model	Starting Population	cell	Phenotype of resting CD4+ T-cell	MOI	Virus/Virus Vector for infection	% of latently infected cells	Time to establish the model	Advantages	Disadvantages
Ex vivo patient cells	Margolis	Latently infected resting CD4+ T-cells		All T cell subsets CD25 ⁻ /DR-	N/A	Endogenous HIV	Roughly 1-60 per 1x10 ⁶	N/A	Patient cells closely mimic reactivation of latent virus <i>in vivo</i>	Rare population of cells Hard to obtain large numbers
Primary Post-activation Latency Models	Tyagi/ Karn [125]	Activated CD4+ T-cells		T _{CM}	N/A	Δgag/Δtat	20%	2 months	Generates a large population of cells, which can be sustained for a long time	Model requires feeder cell line Doesn't allow study of transitional memory cells Cells are not in resting state
	Sahu/ Cloyd [188]	Activated CD4+ T-cells		T _{CM}	1-10	Replication-competent	>5%	2 months		Cells are not in resting state Require feeder cells
	Marini/ Romerio [189]	Activated CD4+ T-cell		T _{CM}	0.002	Replication-competent	2%	2 months	Uses wild type HIV	Uses IL-7 to reactivate cells, which could also activate proliferation in uninfected and infected cells
	Bosque/ Planelles [96]	Activated CD4+ T-cell		T _{CM} , T _{EM}	50	Δenv or ΔenvΔnefGFP	NP 45%/ Th1 ^g 11%/ Th2 ^h 40%	1 month	Large number of latently infected cells in multiple T-cell subtypes	Does not allow for the study of naïve cells observed in patients. Due to activation step, cells express lower levels of CCR5, making it difficult to study R5 viruses.
	Yang/ Siliciano [190]	Activated CD4+ T-cell		T _{EM}	<0.01	Δgag/Δvif/Δvpr /ΔvpuΔenv	1-3%	~4 months	Cells can remain long-lived without cytokine stimulation	Generates T _{EM} cells, but not T _{CM} , T _{TM} or naïve subsets. Overexpression of Bcl-2 results in resistance to cell death

Pre-activation Latency Models	Saleh/ Lewin [192]	Resting cells	CD4+	T-	Naïve, T _{CM}	0.5-1	WT HIV ^{NL4.3}	~1%	Several days	Chemokines enhance infection of cells	Model does not generate all subsets of latently infected CD4+ T-cells seen in patients
	Swiggard/ O'Doherty [191]	Resting cells	CD4+	T-	Naïve, T _{EM}	22-150	Replication-competent	4.5%	Several days	Cells are in resting state, similar to patient cells <i>ex vivo</i>	Limited number of cells generated

* This table summarises various properties of primary CD4+ T-cells of HIV latency and *ex vivo* latently infected patient cells and was adapted from [196] [197].

T_{CM}: Central memory T-cell, T_{EM}: Effector memory T-cell, Th1: Type 1 T helper cell, Th2: Type 2 T helper cells, NP: Non-proliferating, WT: wildtype, MOI: multiplicity of infection, N/A: not applicable, WT: wildtype, DR-: HLA-DR negative, Δ: deletion.

1.8 Dual Fluorescent Reporter Virus Models of HIV Infection

1.8.1 Red Green HIV (RGH) model of HIV latent and productive infection

In currently used models of HIV latency (Table 1.2), the absence of a positive marker for latently infected cells restricts the ability to differentiate latent and productive infection [100]. Moreover, the use of a single marker of viral reactivation, such as LTR-driven green fluorescence protein, limits the identification of early latency establishment [198]. In order to understand the establishment of HIV latency, Dahabieh and colleagues constructed a dual fluorescent HIV virus termed Red Green HIV (RGH).

The RGH virus contains two fluorescent markers inserted into the HIV genome that can distinguish between latent and productive HIV infection (Figure 1.8A) [198]. The first fluorescent marker, GFP, is placed in the *gag* gene and is dependent on the HIV LTR promoter (and HIV Tat expression) for transcription and production. Gag is a product of late HIV gene expression; hence expression of GFP is a likely indicator of active HIV production [198]. The second fluorescent reporter, mCherry, replaces the *nef* gene and is driven by a cytomegalovirus (CMV) promoter (Figure 1.8A). As mCherry transcription is driven by a CMV promoter, it was assumed that mCherry would be constitutively expressed after viral cDNA integration. Thus, the combination of the two fluorescent proteins allow the identification of latent cells that only express mCherry, and productive infection where both mCherry and EGFP are expressed. RGH virus also contains a frameshift disruption in the *env* gene, limiting replication to a single-round and requiring co-expression of viral envelope protein in *trans* (pseudotyping) to successfully infect cells [198].

Infection of the Jurkat T-cell line with RGH virus pseudotyped with VSV-G demonstrated that this reporter virus can distinguish between latent and productive infection in Jurkat T-cells and primary CD4⁺ T-cells [198]. One day post-infection, mCherry only and GFP+mCherry+ populations of cells were observed consistent with latent and productively infected cells respectively. However, GFP only cells were also observed, resulting from GFP labelled virions in the initial inoculum still bound to the cells. This GFP only population decreased with increasing days in culture in the Jurkat T-cell line due to turnover of the virus inoculum but was stable when the virus was added to activated and resting CD4⁺ T-cells, indicating these

primary T-cells still contained plenty of GFP labelled virus inoculum adherent to the cells [198]. This high GFP background from the virus inoculum will complicate the measurement of productive infection in primary T-cells as GFP+mCherry+ cells could be a mix of bona fide productively infected cells expressing both GFP and mCherry reporters, plus mCherry latent cells with GFP+ virus inoculum still adhered to the surface. This is an important limitation of using this system in primary T-cells (Table 1.3).

1.8.2 Duo-Fluo I model of HIV latent and productive infection

A different dual fluorescence reporter designed by the Verdin laboratory [199] is termed Duo-Fluo I [100]. Duo-Fluo I derives from the HIV HXB2-based R7/3 clone. It has a deletion in the *env* gene and contains GFP inserted in the *nef* gene (Figure 1.8B). GFP is expressed using the *nef* translation start codon, thus acting as the HIV productive infection reporter [199]. While GFP denotes productive infection, due to the deletion of *env* in this virus, there is only a single round of virus expression and further cells cannot be infected. mCherry driven by EF1 α -promoter was also inserted between GFP and the virus 3' LTR in this construct (Figure 1.8B). Being driven by the EF1 α -promoter, mCherry is thus constitutively expressed upon viral cDNA integration. Similar to RGH virus, Duo-Fluo I discriminates between latently infected cells that express mCherry only, and productively infected cells that express GFP and mCherry. Importantly, this construct does not label the virus particle inoculum with GFP, thus avoiding a high background of GFP only cells from the virus inoculum adhered to primary T-cells after infection [100].

Duo-Fluo I has been shown to infect primary CD4+ T-cells activated with α -CD3/ α -CD28 dynabeads [199], and more recently, resting CD4+ T-cells [100]. It has been used to investigate the kinetics of HIV latency and the role of T-cell activation on latency. Importantly Duo-Fluo I can identify latently infected cells after infection of resting CD4+ T-cells pre-treated with CCL19 chemokine and thus can be used in the chemokine model of HIV pre-activation latency [100].

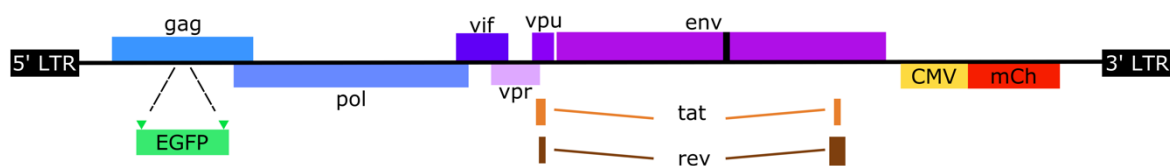
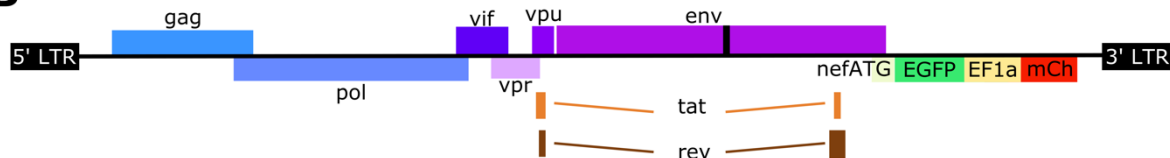
A**B**

Figure 1.8. Schematic representation of Red Green HIV and Duo-Fluo I HIV dual fluorescent reporter viruses

(A) Schematic representation of the Red Green HIV (RGH) construct. Two fluorescent reporter proteins are inserted into the HIV viral genome. EGFP is inserted into the gag gene, flanked by two protease cleavage sites (inverted triangles) and is under the control of the endogenous HIV LTR promoter. The second reporter mCherry, driven by a CMV promoter for constitutive expression after viral DNA integration, replaces the nef gene. (B) Schematic representation of the Duo-Fluo I construct. In this construct, EGFP replaces part of the nef gene and its' expression is under the control of the HIV LTR promoter and the nef ATG translation start codon. A whole transcriptional unit was inserted downstream of the nef ATG-EGFP, encoding mCherry driven by the EF1 α promoter for constitutive mCherry expression after viral DNA integration. Both constructs contain a mutation in the HIV env gene (black bar). Thus, to make infectious virus particles, a viral envelope protein must be co-expressed in trans with either construct. EGFP; enhanced green fluorescent protein, mCh; mCherry, EF1 α ; elongation factor 1 α , CMV; cytomegalovirus. Diagrams adapted from [198, 199].

1.8.3 Comparison of RGH and Duo-Fluo I dual fluorescent reporters of HIV infection

A major advantage of the dual fluorescent reporter virus is the ability to label latently infected cells. When comparing the two constitutively expressed promoters that mark cells with integrated viral cDNA, RGH has a slight disadvantage as this construct contains the CMV promoter to drive mCherry expression, while Duo-Fluo I uses EF1 α promoter (Table 1.3). The

use of the CMV promoter might not be optimal for resting CD4⁺ T-cells as CMV promoter expression appears dependent on the cell cycle phase of transduced cells [200], while EF1 α is less affected by the cell cycle [200].

Another caveat of the RGH construct is the location of the HIV LTR-driven GFP reporter. In RGH, GFP is inserted into the *gag* gene. Although this location in *gag* is a marker of late HIV replication, viral particles also incorporate GFP via the Gag polyprotein. The presence of this GFP only population of cells may underestimate the population of latently infected cells (as mCherry only cells may also be positive for GFP due to virions bound to the cells) and overestimate the GFP productive population of cells for the same reason. In addition, it has been reported that the insertion of GFP into the *gag* gene has cell-type-dependent effects on replication [201], thus causing low infectivity. The addition of wildtype Gag protein *in trans* however, can rescue the function of attenuated Gag, which has been demonstrated by several groups [202, 203]. This issue was avoided in Duo-Fluo I by Verdin and colleagues through placement of the GFP productive reporter in the *nef* gene.

1.8.4 Recently improved HIV_{GKO} and Hlfate-E dual fluorescent reporters of HIV infection

Recently, a new and improved Duo-Fluo I has been reported [204] (Table 1.3). One of the caveats of the original Duo-Fluo I reporter virus is the use of both GFP and mCherry proteins in proximity to each other. Shared nucleotide sequences at the N- and C- termini of GFP and mCherry can result in the recombination of these proteins during reverse transcription when the virus infects target cells [205]. The resultant recombination may allow mCherry to be expressed from the *nef* ATG as a productive infection reporter in place of GFP as the constitutively expressed latent infection reporter in Duo-Fluo I. This would lead to the underestimation of the mCherry latent and GFP productive populations of cells. To address this, Battivelli and colleagues modified Duo-Fluo I, swapping the GFP productive infection reporter to a codon switched GFP (csGFP), and replacing the mCherry latency reporter to an unrelated fluorescent protein, Kusabira Orange (mKO2), to produce HIV_{GKO} [204]. Compared to the previous version of the virus (Duo-Fluo I), HIV_{GKO} resulted in a higher detection of

double positive (csGFP+mKO2+) cells in activated primary CD4+ T-cells infected with each respective construct [204].

Building upon the design of Duo-Fluo I, HIFate-E, another dual-fluorescent reporter virus was recently created [206]. In this construct, a far-red E2 crimson reporter was placed under the control of the HIV LTR to label productively infected cells. Then, EGFP was placed under the control of an elongation factor 1 α -human T-cell leukemia virus type 1 (EF-1 α -HTLV-1) composite promoter for constitutive expression after viral cDNA integration to label latently infected cells. Use of this new reporter virus has only been studied in activated T-cells and thus whether this reporter can successfully infect non-activated cells has yet to be elucidated.

Table 1.3. Dual-fluorescent HIV reporter viruses and their properties

Name	Latent reporter	Productive reporter	Advantage	Disadvantage	Ref
Red Green HIV (RGH)	CMV driven mCherry	LTR-driven GFP		-GFP reporter inserted into the <i>gag</i> gene -CMV promoter can vary in different cell types	[198]
Duo-Fluo I	EF1 α -driven mCherry	LTR-driven GFP	-Detected latent and productive cells in resting CD4+ T-cells	-Possible recombination of proteins -Mutation in <i>vpr</i> -Loss of mCherry signal over time	[100, 199]
HIV _{GKO}	EF1 α -driven Kusabira orange	LTR-driven codon switched GFP	-Codon switched GFP -Kusabira orange for latent reporter – no possible recombination of proteins	-Mutation in <i>vpr</i> -Infection of only activated T-cells	[204]
Hlfate-E	EF1 α -HTLV-1 driven GFP	LTR-driven E2 Crimson	-E2 Crimson for latent reporter – no possible recombination of proteins	-Infection of only activated T-cells	[206]

EF1 α - elongation factors 1 α ; GFP- green fluorescent protein; CM- cytomegalovirus

1.8.5 Further improving dual fluorescent reporter viruses

Although Duo-Fluo I appears more beneficial as a dual fluorescent reporter virus in resting CD4⁺ T-cells compared to RGH, there are still a number of improvements that could benefit the use of this construct to identify latent and productively infected cells. One limitation of the current construct is the use of mCherry as the latent reporter. Substituting the latent mCherry reporter for the brighter EGFP reporter with higher photostability [207] could readily be used to sort latently infected cells on most flow cytometers. This would enable enrichment of latently infected cells for subsequent studies testing potential reactivation compounds, which would be of great benefit. Additionally, in two previous studies using an EGFP marker of latency, high levels of latent cells were detected and allowed for differential cell sorting for subsequent analysis [48, 208], demonstrating the benefits of EGFP labelled latent cells.

If EGFP is used as the latent reporter, a different productive infection reporter is therefore required. Using a brighter red (Tomato) or far-red (E2 Crimson) fluorescent protein [209] as the productive infection reporter should allow for easier detection of productive infection on a flow cytometer compared to the weaker fluorescence of the mCherry option. Duo-Fluo I also contains a frame shift mutation in the HIV *env* gene. Therefore, virus particles must be pseudotyped with HIV envelope proteins to infect resting CD4⁺ T-cells, which can be inefficient and reduce the infectivity of virus preparations. Thus, altering Duo-Fluo I to express a wildtype HIV *env* gene for optimal, wildtype envelope protein expression would avoid pseudotyping and may enhance the infectivity of virus stocks for experiments. Finally, we found that Duo-Fluo I also contains a *vpr* frame shift mutation as it is derived from the HXB2 strain of HIV. Given the importance of HIV Vpr protein for early viral replication, transcription and cell apoptosis [210], changing Duo-Fluo I to express wildtype Vpr would also benefit studies looking for drugs that impact HIV latency establishment, latency reactivation and death of these cells.

Therefore, modifying Duo-Fluo I by swapping to an GFP latency reporter, an E2 Crimson or tomato productive infection reporter [205], and changing to wildtype *env* and *vpr* genes may generate more infectious virus particles that can be used to study HIV latency establishment, reactivation and preferential cell death, as well as screen for novel compounds that impact

these processes. Combined with flow cytometry detection, this could also generate a superior, high-throughput, CCL19 primary T-cell model of HIV latency for such novel compound screening.

1.9 Reversing HIV latency – “Shock and Kill”

Latently infected CD4⁺ T-cells are a major barrier to HIV eradication in patients on ART. Once latency is established, the expression of the proviral promoter is tightly regulated at a number of levels, all of which need to be overcome to restart viral production [211]. One approach to eliminate latently infected cells is to activate HIV transcription, also known as “shock and kill” [212], or “kick and kill”. Compound/s are used to reactivate (shock or kick) latent integrated provirus to induce viral transcription and protein expression in resting CD4⁺ T-cells [213] (Figure 1.9). In theory, the reactivated cell may then die through viral-mediated cytopathic events or by immune-mediated clearance that recognises the newly produced HIV proteins. Although this approach seems counterintuitive as viral reactivation and subsequent release of HIV viral particles would lead to infection of new cells, patients would also remain on ART to prevent new rounds of infection while purging the latently infected cells.

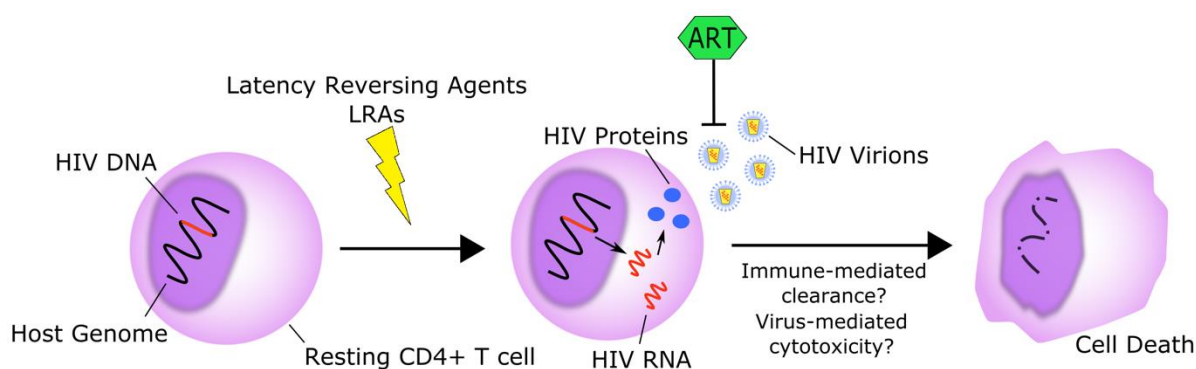


Figure 1.9. The “Shock and Kill” strategy

The “shock and kill” strategy to eliminate HIV latently infected cells involves the addition of compounds to reactivate integrated HIV DNA in the host cell genome to transcribe HIV RNA and express HIV proteins. This is the “shock” part of the strategy. The “kill” comes from either the cytopathic effects of the HIV viral proteins, and/or from the immune-mediated recognition and clearance of the viral protein expressing cells. To prevent further transmission of the viral

particles, antiretroviral therapy is given in conjunction with compounds that reverse latency. Adapted from [214].

1.10 Latency Reversing Agents (LRAs) to “shock” latently infected cells

Latency reversing agents aim to “shock” latently infected cells to reverse blocks in HIV transcription that keep the infected cell in a state of latency. Initial attempts at reactivating latency relied on global T-cell activation and included the use of anti-CD3 antibodies and IL-2, however this approach was associated with significant toxicity [215-217]. Therefore, alternative LRAs were sought. In latently infected cells, a number of molecular mechanisms maintain the constant state of latency including epigenetic modifications and sequestration of transcriptional factors [110] (section above). LRAs targeting different latency molecular mechanisms are discussed below and summarised in Table 1.3.

1.10.1 HDAC inhibitors (HDACi)

Histone deacetylase (HDAC) proteins have a wide range of functions and importantly, target histones that condense cellular DNA into chromatin, leading to gene silencing [218]. A number of different HDAC classes and protein members in these classes exist [219]. Multiple mechanisms recruit HDACs to the HIV promoter resulting in transcriptional silencing, with Class I HDACs primarily implicated in HIV latency (reviewed in [220]). HDAC inhibitors (HDACi) can antagonise all classes of HDACs and can target the repressive effects of HDACs on latent integrated HIV DNA to reactivate viral transcription initiation [221].

The human HDAC proteins are classified into 4 classes based on their sequence homology to yeast HDACs, subcellular localisation and enzymatic function [222]. Class I HDACs comprise of HDAC1, -2, -3 and -8 and localise to the nucleus [223]. HDAC1 and -2 mediate the recruitment of HDACs to promoters required for modulating their deacetylase activity [116]. Class II HDACs are present in cytoplasm and are imported to the nucleus when needed [224]. Class II HDACs are further classified into two subclasses: Class IIa (comprising of HDAC4, -5, -7 and -9) and class IIb (including HDAC6 and -10) [225]. Class III, also known as sirtuins (SIRT1-7) target various histone and non-histone proteins (reviewed in [226]). HDAC11 is the only member of Class IV and shares similar structure to HDAC3 and -8 [227].

1.10.1.1 HIV LTR and histone modification

Epigenetic modifications of the HIV-1 promoter are crucial components in the silencing of viral transcription. Regardless of the site of integration, in the transcriptional silent state, the 5' LTR of the HIV provirus has been found to consistently form within two nucleosomes [228]. The two nucleosomes, nucleosome-0 (nuc-0) and nucleosome-1 (nuc-1) define two large nucleosome-free regions positioned in respect to the *cis*-acting regulatory elements [228, 229]. The first region is composed of the core promoter (nt 200-465) and the second region is located at the site of transcription initiation (nt 610-720) [228]. The positions of the nucleosomes overlap with binding sites for several transcription factors that are key in HIV gene expression. It is thought that the presence of a nucleosome downstream of the transcription initiation site in the HIV promoter, such as nuc-1 could interfere with transcription or elongation [228]. Therefore, HIV gene expression is thought to be governed by the epigenetic modifications of nuc-0 and nuc-1.

Reactivation of latent provirus by HDACi can occur through multiple pathways, including through the hyperacetylation of histones [230] as well as non-histone proteins [231]. HDACs are recruited to the HIV LTR by host transcriptional factors and acetylated nuc-1 prevents proviral transcription [230]. HDACi disrupt the recruitment of HDACs, which in turn cannot silence viral transcription [232]. HDACi have several advantages over other latency reversing agents due to their extensive evaluation in clinical trials as anti-cancer drugs [233] and investigation of their pharmacological and toxicological properties [234]. Multiple selective- and pan- HDACis (HDACis that inhibit numerous classes of HDACs) have been studied for their potency in reactivating HIV.

1.10.1.2 Valproic Acid

Valproic acid (VPA) is a weak, non-selective HDACi that targets HDAC1 and HDAC2 and is approved for the treatment of epilepsy and bipolar disorder [235]. The first clinical trial with VPA showed that in 3 out of 4 participants, the administration of VPA resulted in a decline in the frequency of replication-competent HIV in resting CD4⁺ T-cells [236]. However, subsequent studies failed to validate these initial results. In an ensuing clinical study, in 9 participants, latently infected cells were readily detected, and the number of cells were

comparable to participants that did not receive VPA treatment [237]. Sagot-Lerolle *et al.* also drew the same conclusion that prolonged VPA treatment did not significantly reduce the size of the HIV reservoir in individuals on ART compared to the matched control group [238]. The discrepancy and non-reproducibility between the clinical studies suggests that the other more potent HDACi be investigated.

1.10.1.3 Vorinostat

Suberoylanilide hydroxamic acid (SAHA, also termed Vorinostat, VOR) is a well-studied HDAC inhibitor with respect to HIV latency [239]. Vorinostat is an FDA-approved oral drug used in the treatment of cutaneous T-cell lymphoma [240]. This HDAC inhibitor has micromolar potency, is selective for Class I HDAC 1 and 3 proteins, and has been shown to activate latently infected T-cells *in vitro* [241] as well as latently infected T-cells from PLWH on ART *ex vivo* [242, 243]. In clinical trials in PLWH on ART, vorinostat also induced transient increases in the transcription of cell associated, HIV unspliced RNA (CA US RNA) [242] [243]. This was the first demonstration by multiple groups that HIV latency could indeed be reversed and was a significant advance in our understanding of latency.

1.10.1.4 Panobinostat

Panobinostat (PNB, LBH-589) is a more potent, pan-HDAC inhibitor than VOR that reactivates latent HIV at nanomolar concentrations [244]. In a clinical trial involving panobinostat [244] in HIV infected patients on ART, panobinostat was able to induce both cell-associated and plasma HIV RNA. This is in contrast to vorinostat, where increases in plasma HIV RNA were not observed [243, 245].

1.10.1.5 Romidepsin

Romidepsin (RMD, FK228) is a potent HDAC inhibitor selective for Class I, HDAC 1 and 2 proteins [246] that also reactivates latent HIV at nanomolar concentrations [247]. In light of the finding that romidepsin was capable of activating HIV transcription in latently infected *ex vivo* patient cells as well as inducing the release of HIV virus particles, the effects of romidepsin on the reactivation of latent HIV in PLWH on ART was evaluated *in vivo* [248].

Romidepsin led to increases in both HIV CA US and plasma RNA but did not reduce HIV DNA. A recent randomised placebo controlled clinical trial of romidepsin in PLWH on suppressive ART did not demonstrate any significant change in CA US or plasma HIV RNA, despite increases in histone acetylation [249]. Further work is needed to fully understand the varying results from these clinical trials of romidepsin.

1.10.1.6 Other HDAC inhibitors

Chidamide is a novel benzamide class of HDAC inhibitor that has selective inhibition of HDAC-1, -2, -3 and -10 [250]. In numerous latently infected T-cell lines such as ACH2, U1 and J-Lat T-cells, chidamide resulted in the reactivation of latent HIV in a dose-dependent manner and time-dependent manner with minimal toxicity [251]. Further studies of this compound using *in vitro* T-cell models and *ex vivo* T-cells from PLWH are warranted.

While valproic acid, vorinostat, panobinostat and romidepsin could all induce CA US HIV RNA in some study participants, none of these compounds induced a significant decline in HIV DNA. Therefore, while these clinical trials provide evidence that *in vivo* latency reversal is possible with HDACi, clearly more potent LRA(s) and new complementary kill strategies are required to collectively decrease the latent HIV reservoir.

1.10.2 Histone Methyltransferase Inhibitors (HMTi)

Histone methylation is a stable post-translational modification and is involved in long-term maintenance of the cellular genome [115]. Histone methyltransferases (HMTases) modify a single lysine residue on a single histone protein and this post-translational modification can either lead to activation or repression of transcription [252].

Chaetocin is a fungal mycotoxin and is a competitive inhibitor of S-Adenosyl methionine [253]. Another HMTi is BIX-1294, which is a (1H-1,4-diazepin-1-yl)-quinazolin-4yl amine derivative that has been demonstrated to be a specific inhibitor of euchromatic G9a HMTase [254]. These two compounds were able to induce HIV expression from resting CD4+ T-cells isolated from PLWH on long-term ART [255]. Unfortunately, chaetocin and BIX-1294 cannot be safely

administered to humans due to their toxicity [256]. However, these studies provide evidence to support the concept for using HMTis as a latency reversing agent.

1.10.3 Protein Kinase C (PKC) Activators

Aside from epigenetic modifications, various transcription factors are down-regulated in latently infected cells such as active NF- κ B, NFAT and the cyclin T1 component of P-TEFb [229, 257]. Up-regulation of these active transcription factors is therefore another approach to purge the latent HIV reservoir. Compounds that target the protein kinase C (PKC) signalling pathway and downstream induction of active NF- κ B [211] have emerged as important targets given the importance of this pathway for inducing active NF- κ B factors critical for HIV transcription. Multiple PKC activators have been reported to reactivate latent HIV in primary T-cells and T-cell lines [258] [259].

Prostratin and Bryostatin-1 are two of the most studied protein kinase C activators used to reactivate latent HIV infection. Prostratin reactivates HIV expression in two latently infected cell lines [260] and latently infected resting CD4⁺ T-cells [261, 262]. Prostratin acts through PKC mediated activation of the NF- κ B signalling pathway [263, 264]. Bryostatin-1 also modulates HIV latency through PKC signalling [259]. Further investigation revealed prostratin and bryostatin-1 stimulate the phosphorylation and degradation of the I κ B α inhibitor of NF- κ B, leading to the nuclear translocation of active NF- κ B. Liberated NF- κ B is free to engage cognate NF- κ B binding sites in the HIV LTR promoter or indirectly induce the expression of secondary messengers leading to subsequent reactivation of latent HIV [264] [265].

An emerging group of compounds that have been characterised to activate latent HIV through the activation of the NF- κ B pathway are ingenol esters. Ingenol esters are structurally analogous to phorbol esters such as PMA, a widely studied PKC activator [266]. An ingenol derivative, Ingenol-3-hexanoate (also called Ingenol B, IngB), has been shown to effectively promote reactivation of HIV LTR-induced gene expression in both J-Lat A1 cell lines and CD4⁺ T-cells isolated from PLWH on long-term ART [267]. Recently another ingenol derivative, ingenol-3-angelate (PEP005), demonstrated the ability to reactivate latent HIV in an *in vitro* T-cell line model and cells isolated from PLWH on suppressive ART [268]. Both IngB and

PEP005 had synergistic effects with JQ1 (a bromodomain inhibitor, see below), which further supports the notion that the administration of a combination of latency reversing agents that act through different but complimentary mechanisms to reverse latent HIV will be able to more effectively reactivate and eliminate the latent reservoir.

Benzolactam-related compounds are known to be potent activators of the PKC pathway [269]. Several benzolactam derivatives were recently tested in models of HIV latency [265, 268, 270]. In latently infected T-cell lines, the benzolactam derivative BL-V8-310 demonstrated superior reactivation than the other tested derivatives and resulted in similar levels of HIV reactivation seen with prostratin [271]. When combined with other LRAs, BL-V8-310 and JQ1 potently reactivated latent HIV in J-Lat T-cell lines. This combination was then assessed in CD4⁺ T-cells from PLWH on ART. In CD4⁺ T-cells treated with either drug alone, both JQ1 and BL-V8-310 resulted in increases in the transcription of HIV mRNA in some but not all donors [271]. Interestingly, treatment of latently infected cells with BL-V8-310 enhanced apoptosis through the up-regulation of caspase-3 signalling (section 1.14.2). These results indicate that BL-V8-310 is a potential candidate as an LRA but also a compound that can kill the cells once reactivated.

1.10.4 Positive transcription elongation factor b (P-TEFb) activators

Recruitment of Tat and P-TEFb to the HIV LTR to enhance viral transcriptional elongation is a limiting step during HIV latency. A major obstacle in latency reactivation is the bromodomain containing protein BRD4, which directly competes with Tat for binding to the P-TEFb component of the super elongation complex (SEC), thereby inhibiting Tat-mediated transcriptional elongation [272]. However, the small molecule inhibitor of bromodomain and extra-terminal (BET) protein family, JQ1, competitively binds BRD4 and prevents BRD4 from interacting with P-TEFb, liberating P-TEFb to bind Tat and stimulate HIV transcriptional elongation [272, 273]. JQ1 has been shown to reactivate latent HIV in a primary T-cell model of HIV latency [274] as well as induce virus outgrowth from resting CD4⁺ T-cells from one of three patients on suppressive ART [273]. Recently, another bromodomain protein BRD2 was identified as a Tat-independent suppressor of HIV transcription and this highlights the potential of BET inhibitors as latency reversing agents [274].

1.10.5 PI3K/Akt pathway activator- Disulfiram

The FDA-approved drug disulfiram, used to treat alcoholism was identified as an HIV latency reversing agent using a high throughput drug screen [275]. This drug was reported to induce HIV gene transcription without global T-cell activation or cytokine release in a primary T-cell model of latency [276]. At a molecular level, it was demonstrated that disulfiram significantly reduced the levels of phosphate and tensin homolog (PTEN) protein levels in U1 cell lines and in resting CD4+ T-cells from uninfected donors [276]. Disulfiram (DSF) was only able to induce reactivation of HIV expression in the U1 cell line, but not in ACH2 and J89GFP cells. This was thought to be attributed to the fact that the ACH2 and J89GFP cell lines lack PTEN. PTEN negatively regulates intracellular levels of phosphatidylinositol-3,4,5-triphosphate (PIP₃) in cells (section 1.14.5.3) and functions as a tumour suppressor by negatively regulating the Akt signalling pathway and in turn NF-κB pathway.

In the first clinical trial of disulfiram in PLWH on suppressive ART, disulfiram was administered for 14 days. The drug was well tolerated but did not result in any change in the levels of plasma HIV RNA or have an effect on HIV DNA levels [277]. In a follow up clinical trial of increasing doses of disulfiram, an increase in CA US HIV RNA and plasma HIV RNA was observed, however there were no significant changes in HIV DNA [278].

1.10.6 Acylaminothiazole

Recently, a new class of LRA has been defined and demonstrated the ability to reactivate latent HIV in both an *in vitro* model of HIV latency and CD4+ T-cells from PLWH *ex vivo* [279]. Using a new latency reporting cell line, a high-throughput drug screen identified 2-acylaminothiazole compounds as a hit compound that was able to enhance HIV LTR activity [279]. Several derivative compounds were generated and tested for their ability to reactivate latent HIV in T-cell lines as well as CD4+ T-cells from PLWH. Of the tested compounds, compound 90 displayed comparable reactivation capabilities as vorinostat and JQ1 in a J-Lat T-cell model. Compounds 37 and 96 also resulted in an increase in unspliced HIV RNA in latently infected CD4+ T-cells *ex vivo*. Moreover, compound 37 acted synergistically with JQ1 in two T-cell line models and CD4+ T-cells from PLWH *ex vivo* [279], further demonstrating that a combination of LRAs may be needed to increase HIV reactivation efficiency. Although

the exact mechanism of 2-acylaminothiazoles is unknown, the study provides evidence of a potential new LRA that can reactivate latent HIV in PLWH.

1.10.7 SMAC mimetics

PKC agonists activate the NF- κ B pathway, which is a key transcription factor that can bind to the HIV LTR (section 1.10.3). Compounds such as PMA, bryostatin and ingenol activate the canonical NF- κ B pathway [280, 281]. The canonical NF- κ B pathway is characterised by short-lived transcriptional activation resulting in a broad inflammatory response [282]. In contrast, the noncanonical pathway is characterised by a long-lasting transcriptional response that impacts a select number of cellular processes. In the absence of stimulation, several inhibitors of apoptosis (IAPs) including baculoviral IAP repeat (BIR) 2 (BIRC2, also known as cellular inhibitor of apoptosis; cIAP1) and BIRC3 (cIAP2) and TNF receptor-associated factor 3 (TRAF3), form a complex and constitutively degraded NF- κ B-inducing kinase (NIK) [283]. Receptor stimulation results in the ubiquitination and degradation of TRAF3 by BIRC2 and BIRC3, permitting the accumulation of NIK [282]. Accumulation of NIK leads to the formation of a p52:RelB heterodimer that translocates to the nucleus and induces the expression of target genes (reviewed in [284]).

Second mitochondria-derived activator of caspase (Smac) also known as direct inhibitor of apoptosis protein-binding protein with low pI (DIABLO) binds to IAPs and inhibits their function [285]. Using Smac mimetics, cIAP1 was identified as a regulator of viral transcription in HIV. Smac mimetic SBI-0637142 was able to successfully reverse latent infection in J-Lat10.6 T-cell lines, which was attributed to the degradation of cIAP1 and stabilisation of NIK protein [286]. Two different Smac mimetics combined with panobinostat led to the reactivation of latent HIV in these cells demonstrating comparable results to the T-cell activation using anti-CD3/CD28 [286].

Recently, another Smac mimetic AZD5582 enhanced HIV transcription and virion production in both *in vitro* and *ex vivo* models of HIV latency [287]. These studies demonstrate the potential of Smac mimetics as LRAs and the combination of Smac mimetics with other LRAs requires further evaluation.

1.10.8 Toll-like Receptor (TLR) agonists

The innate immune system can recognise cell invasion by infectious organisms by detecting pathogen-associated molecular patterns (PAMPs) expressed by foreign organisms, which are detected through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) [288]. The recognition of PAMPs by toll-like receptors leads to a cascade of signalling pathways, some which result in the production of type I interferons (IFN) and some pathways that end in the production of inflammatory cytokines [288]. TLRs can be divided into two subgroups depending on their cellular localisation. TLR1, 2, 4, 5, 6 and 10 are localised on the cell surface while TLR3, 7, 8 and 9 are localised in intracellular organelles (reviewed in [288]). Several TLRs can recognise bacterial and viral nucleic acids, such as TLR7 present on antigen presenting cells such as plasmacytoid dendritic cells (pDCs) that recognise HIV single stranded RNA (ssRNA) [289]. Bacterial and viral DNA that are rich in unmethylated CpG motifs are recognised by TLR9. Engagement of TLR9 results in the stimulation and secretion of type I IFN- α , which then goes on to activate natural killer (NK) cells [290]. Initial studies using cell lines revealed that TLR agonists were able to successfully reactivate the HIV LTR [290-292]. Additionally, TLR7 agonist GS-9620 (Vesatolimod) treatment of PBMCs from PLWH on ART *ex vivo* resulted in an increase in HIV RNA, and also activated HIV-specific cytotoxic CD8+ T-cells [293]. In a recent double-blind, placebo-controlled dose-escalation study with vesatolimod in PLWH, the drug was well-tolerated with no reported severe adverse effects. At the higher doses of this TLR7 agonist, immune activation was recorded [294].

Two different TLR7 agonists, GS-9620 and GS-986 (an analogue of GS-9620) were able to induce transient viremia in SIV-infected rhesus macaques on ART [295]. Repeated dosage of TLR7 agonists was associated with decreases in SIV DNA in both tissue and blood, with the most significant decrease observed in T_{TM} and T_{CM} cells. In two of the tested macaques, plasma viremia remained undetected despite the cessation of ART for more than 700 days [295]. This suggests that nearly all replication-competent virus was cleared from these two macaques following TLR7 agonist treatment, resulting in the reductions of viral RNA.

Finally, TLR9 agonists can activate HIV replication in latently infected T-cell lines [291] and the TLR9 agonist MGN1703 resulted in a significant increase in the levels of CA US HIV RNA in CD4+ T-cells from PLWH by a median of 1.4-fold [290]. Similar to the TLR7 agonist, Offersen *et al.* also observed increases in NK cell degranulation activity and IFN- α production. In a clinical trial of MGN1703 in PLWH on ART [296], an increase in plasma HIV RNA was observed in a subset of participants [296].

1.10.9 Immune checkpoint blockers

A hallmark feature of chronic viral infections such as HIV is T-cell exhaustion. During chronic viral infections, to attenuate the effector functions of T-cells and protect against tissue damage, the expression of inhibitory molecules such as PD-1 and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) are upregulated [297]. The collective term of these molecules is immune checkpoint (IC) molecules and activation of these molecule can result in inhibition of cytokine production and activation of apoptosis [298].

Multiple studies have demonstrated a correlation between the frequency of latently infected cells in PLWH on ART, measured as HIV DNA or integrated DNA, and expression of the IC molecule PD-1 [34, 45, 68]. CD4+ T-cells expressing high levels of PD-1 are enriched for HIV on ART and PD1^{hi} cells have over 10-fold more HIV DNA than PD1^{lo} cells [34]. Additionally, cells from PLWH on ART that co-express other IC molecules, TIGIT and LAG-3 were enriched with HIV compared to cells that did not express the IC molecules [45, 62]. Our laboratory has also demonstrated using an *in vitro* model of HIV latency that PD-1 expression favours the establishment of latency [299]. Together these data support the notion that PD-1 could potentially be a target for HIV reversal.

Immune checkpoint blockade (ICB) has been proposed to either reverse HIV latency, by blocking inhibitory signals in cells that harbour latent HIV increase production of viral proteins or through the enhancement of HIV-specific CD8+ T-cells that also express high levels of PD-1 [300]. Indeed, in CD4+ T-cells from virally suppressed individuals, PD-1 blockade with the monoclonal antibody pembrolizumab enhanced HIV reactivation when cells were treated with bryostatin [301], demonstrating latency reversal. Moreover, in an HIV+ individual

receiving treatment for metastatic melanoma, our laboratory has demonstrated that multiple rounds of anti-CTLA4 (ipilimumab) and a single round of an anti-PD-1 blocker (nivolumab) increased in cell-associated HIV US RNA [299, 302]. The data suggests that the increase in HIV transcription could potentially lead to latency reversal.

1.10.10 IL-15 agonists

IL-15 can also activate latent HIV in an *in vitro* and *ex vivo* model of HIV latency [101]. IL-15 and the superagonist ALT-803 could reverse latent HIV with no observed cell toxicity. In CD4+ T-cells from PLWH on ART, ALT-803 induced significantly high levels of supernatant RNA compared to the negative control and this was accompanied by model levels of T-cell activation and no induction of reservoir expansion [101]. Using CD8+ T-cells as bio-detectors of latency reversal, the same group demonstrated that two IL-15 superagonists (IL-15SA and ALT-803) could reactivate latent HIV and prime these cells for HIV-specific CD8+ T-cell recognition [101]. The upregulation of CD137 on CD8+ T-cells co-cultured with IL-15 superagonists in addition to an increase in IFN- γ production indicated that latently infected cells had been reactivated and primed for recognition by HIV Gag-specific CD8+ T-cells. HIV infected T-cells isolated from PLWH and treated with ALT-803 resulted in significant induction of IFN- γ production from CD8+ T-cells, confirming that ALT-803 induced detectable antigen from infected cells that allowed them to be targeted by HIV-specific CD8+ T-cells [101].

Other than the LRAs mentioned above, there are many additional LRAs that target different mechanisms that maintain latency (Figure 1.10), summarized in Table 1.4.

1.11 Identifying LRAs that efficiently induce mature HIV mRNAs and protein expression

Understanding the limitations of LRAs is critical to finding ways to enhance efficacy. It has recently been demonstrated that HDACi can initiate viral transcription but do not lead to complete viral reactivation [157]. Using a novel panel of reverse transcription ddPCR that can measure different HIV transcripts, the major reversible blocks in CD4+ T-cells isolated from PLWH on suppressive ART were found to be blocks to proximal elongation, transcription and polyadenylation (indicative of HIV transcriptional completion) and multiply splicing [303].

Unlike the prevailing theory that the major reversible block in HIV latency is transcriptional initiation, the investigators observed high proportions of total HIV transcripts (indicative of transcriptional initiation).

CD4+ T-cells from ART suppressed individuals reactivated with a number of clinically relevant LRAs revealed that the HDACi panobinostat and romidepsin resulted in an increase in the total and elongated transcripts but had little to no effect on polyadenylated and multiply spliced transcripts, indicating successful HIV transcription but failure of viral reactivation [303]. Moreover, using a highly sensitive HIV p24 ELISA that can detect the production of p24 to the single-cell level, Passaes *et al.* also demonstrate other classes of LRAs also fail to induce complete HIV reactivation [149]. The inability of a single LRA to induce all species of HIV transcripts or produce viral p24 protein offers an explanation as to why clinically tested LRAs failed to decrease the levels of HIV DNA in all participants but resulted in an increase in CA HIV RNA. The varying degree of HIV transcription and the production of HIV viral proteins to induce cytopathic cell death or activate apoptosis by latency reversing agents suggests that optimal reversal of HIV latency will require more potent drug compounds or likely a combination of different classes of LRAs.

It is important to note that some LRAs can have pro-survival effects through the phosphoinositide-3 kinase (PI3K) pathway. For instance, the anti-alcohol drug disulfiram acts as an LRA via depletion of the PTEN regulator of the PI3K/Akt pathway, thus promoting pro-survival PI3K/Akt signalling as well as NF- κ B driven HIV transcription [275, 304]. Additionally, the HDACi vorinostat and the PI3K α isoform agonist 55704 also promote pro-survival PI3K/Akt signalling [305]. The activation of the PI3K/Akt pathway by these particular LRAs may enhance cell survival and explain why clinical trials with vorinostat [242] and disulfiram [278, 306] both failed to reduce the levels of HIV DNA in PLWH on ART.

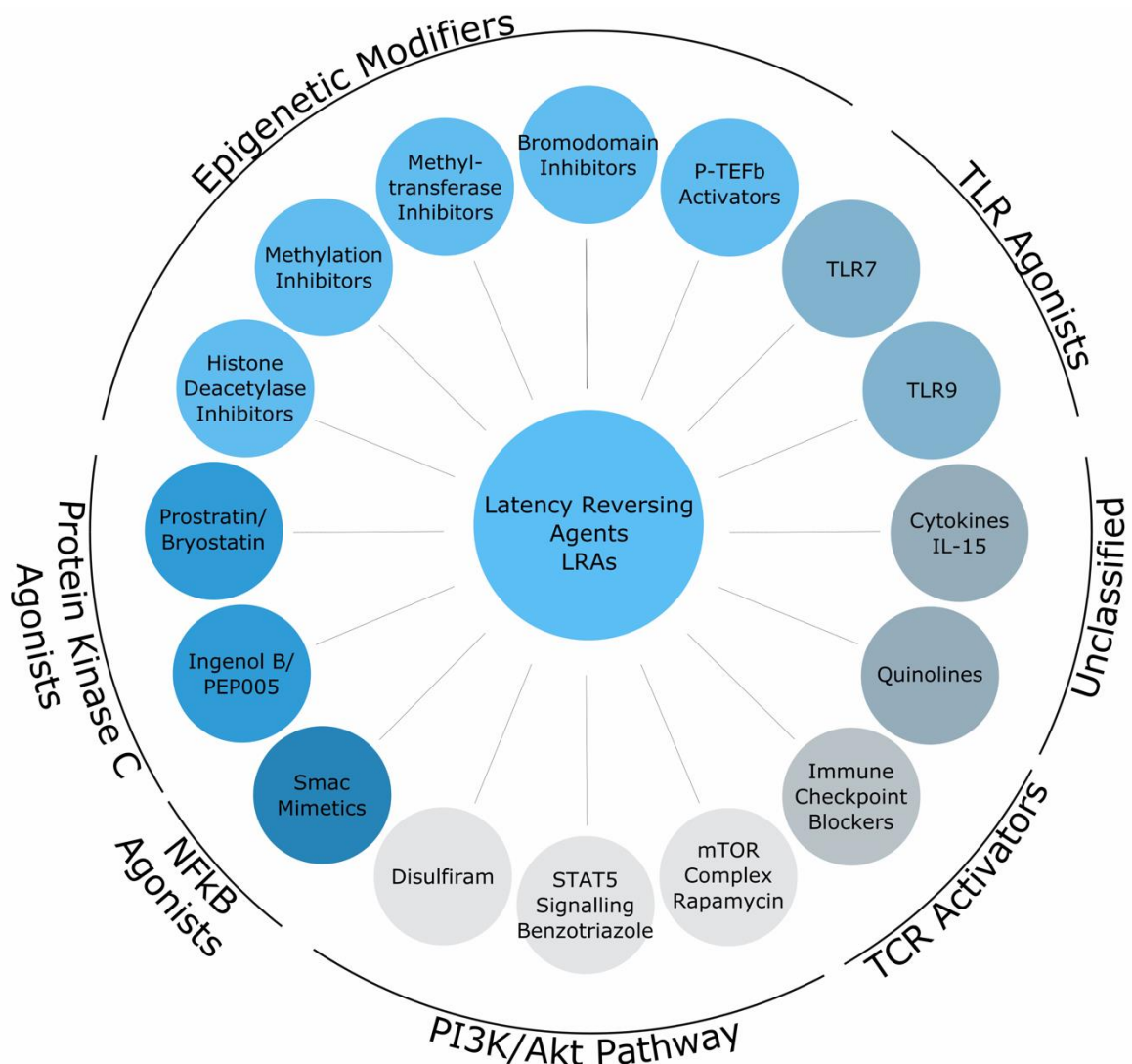


Figure 1.10. Different classes of latency reversing agents

Latency reversing agents (LRAs) can act through a multitude of different pathways that result in the reactivation of latent HIV. This diagram summarises key classes of LRAs in the coloured inner circles, and their main mechanism to reactivate latent HIV written on the outer circle. P-TEFb, positive transcription elongation factor b; TLR, toll-like receptor; mTOR, mechanistic target of rapamycin; STAT5, signal transducer and activator of transcription 5; IL-15, interleukin-15; TCR, T-cell receptor, PI3K, phosphoinositide 3 kinase; Smac, second mitochondria-derived activator of caspases. Adapted from [214].

Table 1.4. Summary of Latency Reversing Agents that reactivate latent HIV

Type of compound	Compound	Mechanism of Action	Comments	Ref
Histone deacetylase (HDAC) inhibitors	Vorinostat	Histone and non-histone protein acetylation Activate Akt via PI3K pathway Increase apoptosis Decrease pro-inflammatory cytokines	Activity against both class I, II and IV HDACs Licensed for treatment of cutaneous T-cell lymphoma Two clinical trials that assessed whether vorinostat can induce virus production from HIV latently infected cells <i>in vivo</i> demonstrated that vorinostat administration resulted in increases of viral RNA but had no effect on viral DNA	[241, 243]
	Panobinostat (LBH589)	Histone and non-histone protein acetylation	Several studies in phase I/II clinical trials found panobinostat to reactivate latent HIV measured by an increase in cell-associated unspliced HIV RNA	[244]
	Romidepsin (FK228)	Histone and non-histone protein acetylation	More potent than vorinostat by one thousand-fold Recent clinical trial with romidepsin demonstrated increases in HIV RNA but total HIV DNA levels did not significantly change	[246, 248, 307]
	Chidamide	Histone protein acetylation	Reactivation of latent HIV in T-cell lines such as ACH2, U1 and J-Lats	[251]
Histone Methyltransferase inhibitors	Chaetocin	Specific inhibitor of the SU(VAR)3-9 class of histone methyltransferases	Chromatin-mediated repression Shown to induce HIV reactivation in 86% of resting CD4+ T-cells	[308, 309]
	BIX-1294	Specific inhibitor of G9a histone methyltransferases	Chromatin-mediated repression Able to reactivate 21.2% of latent virus in cell lines	[309, 310]
DNA Methylation inhibitor	5-aza-2' deoxycytidine (5-aza-CdR)	FDA-approved inhibitor of DNA methylation	Synergized with TNF- α and prostratin to promote viral gene expression	[120, 311]

Protein Kinase C (PKC) Activators	Prostratin	Activation of PKC, resulting in phosphorylation of I κ B α , which releases NF- κ B that translocates to the nucleus, binding HIV LTR & activating transcription	Down-regulates CD4 receptor Potent anti-tumour agent Activates cells without cell cycling Reactivated latent HIV in both pre- and post-integration latency in resting CD4+ T-cells	[261, 262]
	Bryostatin	Activation of PKC, resulting in phosphorylation of I κ B α , which releases NF- κ B that translocates to the nucleus, binding HIV LTR to activating transcription	Has immunomodulatory activity including induction of cytokine release and expansion of tumour specific lymphocyte populations Reactivation of latent HIV from monocytic and lymphocytic T-cell lines	[259, 265]
	Ingenol esters (Ingenol-3-angelate/PEP005, IngB)	Activation of PKC, resulting in phosphorylation of I κ B α , which releases NF- κ B that translocates to the nucleus, binding HIV LTR to activating transcription	Approved for clinical use Activator of NF- κ B pathway but does not increase NF- κ B protein Potent reactivation of latently infected T cells from HIV+ individuals on ART ex vivo	[268] [303, 312]
	Benzolactam-related BL-V8-310	Activation of PKC, resulting in phosphorylation of I κ B α , which releases NF- κ B that translocates to the nucleus, binding HIV LTR to activating transcription	In HIV latently infected T-cell lines, similar reactivation levels to prostratin Combined with JQ1 results in potent reactivation in J-Lat T-cell lines and CD4+ T-cells from PLWH Apoptosis was enhanced through upregulation of caspase-3 signalling	[271]
P-TEFb Activators	HMBA	Phosphorylation of HEXIM1 and release of active P-TEFb to enhance HIV transcription elongation	Potent cell differentiator Similar in structure to SAHA Reactivated HIV viral production in latently infected T-cell lines	[313]
	JQ1		Removes P-TEFb from inhibitory 7SK snRNP	[273, 274, 314]

		Inhibitor of bromodomain and extra-terminal (BET) protein family, which liberates P-TEFb for binding to HIV Tat to enhance HIV transcription elongation	Showned synergistic suppression of T-cell proliferation with prostratin Reactivated latent HIV from J-Lat T-cell lines	
PI3K/Akt pathway modulators	Disulfiram	Role in PI3K/Akt pathway by depleting PTEN inhibitor of PI3K, promoting PI3K pathway and NF-κB production for HIV transcription initiation.	Used to treat chronic alcohol abuse Clinical trials to test HIV reactivation found a transient increase in viral load that was statistically significant with high dose	[276, 278]
Unclassified	Acylaminothiazole Compounds 37/90	Unknown mechanism of action	Reactivation of latency in T-cell lines and CD4+ T-cells from PLWH Comparable levels of reactivation with vorinostat and JQ1	[279]
TLR Agonists	TLR7- GS-9620 TLR9- MGN1703	Speculated to reactivate latent HIV by inducing the production of IFN-α and active NF-κB for HIV transcription initiation. Also increases activity of NK cells, CD4+ and CD8+ T-cells.	MGN1703 (TLR9 agonist) recently completed a Phase 1b/2a clinical trial (NCT02443935) MGN1703 was able to induce US HIV RNA and plasma RNA GS-9620 (TLR7 agonist) in a clinical trial for chronic hepatitis B virus (NCT02166047)	[293, 296]
SMAC Mimetics	SBI-0637142 AZD5582	Binds to IAP proteins to inhibiting the formation of a complex that degrades NIK. This allows NIK to form a heterodimer that translocate to the nucleus to induce expression of target genes	SBI-0637142 reverses latency in J-Lat T-cell line Attributed to the degradation of cIAP Combined with panobinostat, reactivation was comparable to anti-CD3/CD28 AZD5582 reversed latency in both <i>in vitro</i> and <i>ex vivo</i> models	[286, 287]
Immune Checkpoint Blockers	PD-1 blocker/ CTLA4 blocker	Antibodies bind to immune checkpoint molecules that are markers of T-cell exhaustion. Activation of molecules can result in apoptosis and inhibition of cytokine production	PD-1 monoclonal antibodies with bryostatin reversed latency in CD4+ T-cells <i>ex vivo</i> Multiple rounds of anti-CTLA4 and anti-PD-1 increased US HIV RNA	[301, 302]
IL-15 Agonists	IL-15S			[101]

ALT-803	Unknown mechanism of action relating to latency reversal Can activate NK cells and CD8+ T-cells	Increased production of supernatant viral RNA in CD4+ T-cells from PLWH Enhanced CD8+ T-cell killing of latently infected cells

1.12 Strategies to Kill Latently Infected Cells

Clinical trials using latency reversing agents such as vorinostat [243] or romidepsin [248] did not lead to a decrease in the frequency of latently infected cells. Moreover, panobinostat only showed a small decrease in HIV DNA in a subset of patients [244]. These findings collectively demonstrate that reactivation with HDACi in isolation may not be enough to eliminate the latently infected cells in all patients. Several reasons these interventions have not yielded successful viral clearance include: limited potency and delivery of these drugs, and post-translational blocks to viral transcription [315], clonal expansion of latently infected cells [39], and the failure of patient CD8⁺ T-cells to recognise and clear the cells with reactivated viral protein expression due to viral escape mutants [316]. The first generation LRAs did not induce viral eradication in these initial clinical trials, thus points to the need to investigate complimentary methods to kill latently infected cells reactivated by LRAs.

Complimentary methods being pursued to eliminate latently infected cells that are induced to express viral proteins with LRAs include immune-based strategies such as: therapeutic vaccination [317, 318], administration of neutralising antibodies [319, 320] (reviewed in [321]), infusion of bispecific antibodies, rejuvenation of HIV-specific CD8⁺ T-cells with drugs such as immune checkpoint blockers [322, 323] or replacement of HIV-specific cells with interventions such as chimeric antigen receptor (CAR) T-cells that recognise viral proteins on the surface of an infected cell [324, 325]. Additionally, novel pharmacological strategies that enhance apoptosis can be used and is the main focus of this thesis. This will be discussed in detail in section 1.14.5.

1.13 Immune-based strategies to kill latently infected cells

1.13.1 Therapeutic vaccines

The main objective of a therapeutic vaccine is to induce the host's immune response to HIV antigens using a planned exposure [317]. In the ideal scenario, vaccination would lead to targeting and elimination of infected cell as well as lead to long term control of any residual virions so that plasma HIV RNA is <20 copies/ml in the absence of ART. Therapeutic vaccines should aim to induce anti-HIV CD8⁺ CTLs and/or neutralising antibodies [326]. Development in recent years have resulted in new approaches in therapeutic vaccinations based on innovative

vectors such as recombinant virus, DNA-based or dendritic cell-based vaccines [317]. In a recently described study, a synthetic p24 Gag peptide therapeutic HIV vaccine, Vacc-4x and recombinant human granulocyte macrophage colony-stimulating factor (rhuGM-CSF) as an adjuvant was combined with the LRA romidepsin to investigate the effect on the latent HIV reservoir [327]. Three important findings were described. The first was the finding that the combination of Vacc-4x and rhuGM-CSF with romidepsin resulted in a significant reduction in total HIV DNA from participants [327]. A decrease in integrated DNA was also observed but was not significant. The second finding was that histone acetylation and cell-associated HIV RNA was increased after each romidepsin administration. Only 4 out of 17 participants had quantifiable plasma RNA after one of the romidepsin treatments suggesting that an effective elimination of latent cells was achieved resulting in limited CA US HIV RNA production [327]. Lastly, closely monitored antiretroviral therapy interruption (ATI) was performed in the study and it was observed that the combination did not prolong time to viral rebound [327]. Subsequent analysis of this study demonstrated an increase in the number of CD8+ T-cell proliferation in participants with a reduction in total HIV DNA [328]. Moreover, there was an observed reduction in the number of replication-competent provirus measured with QVOA, indicative of elimination of latent provirus [328].

In SIV-infected rhesus macaques, the combination of Ad26/MVA therapeutic vaccine with the TLR7 agonist GS-986 improved virological control of SIV and delayed viral rebound subsequent to discontinuation of ART [329]. Moreover, 3 out of 9 animals demonstrated virological control to undetectable levels during the discontinuation of ART. This study provided proof-of-concept that a combination of a therapeutic vaccine and immune stimulation could impact viral rebound following the cessation of ART [329].

1.13.2 Broadly neutralising antibodies (bNAbs)

Broadly neutralising antibodies (bNAbs) bind HIV Env proteins and act to block the interaction between Env on the surface of virions and cellular receptors on the host cell, thus preventing the entry of virus into the host cell [320]. bNAbs can also bind Env protein expressed on the surface of infected target cells and recruit immune effectors like CD8+ T-cells and NK cells to kill infected target cells [330, 331]. The use of bNAbs is of high interest and an area of active investigation.

Broadly neutralising antibodies target HIV Envelope protein on the surface of the virus. bNAbs aim to block transmission of the virus by neutralising the virus through direct inhibition of the viral Envelope protein and thus blocking entry of the virus into uninfected target cells. Additionally, bNAbs can also facilitate non-neutralising functions through binding of the antibodies to Envelope on infected cells and activate the complement pathway or antibody-dependent cell-mediated cytotoxicity (ADCC) [330, 331]. Fc-receptor (FcR) expressing cells such as NK cells have been demonstrated to bind to HIV-infected cells bound with bNAbs resulting in elimination by ADCC [330]. Additionally, effector cells can be stimulated to secrete cytokines and other soluble factors that can affect viral spread. Combined with ADCC and neutralisation, this has been termed antibody-dependent cell-mediated viral inhibition [332].

There are four main categories that classify bNAbs based on the location of conserved epitopes of the Envelope protein (reviewed in [333]). Several bNAbs are in advance clinical development and include VRC01 and 3BNC117 (both recognise the CD4 binding site [334, 335]); 10-1074 and PGT121 (both recognise the base of the V3 loop [336, 337]); 10E8 (binds to the membrane proximal region [338]) and PGDM1400 and CAP256 (both bind to the hypervariable loops V1/V2 region and the surrounding glycans [339]).

Chimeric SIV that contains the HIV envelope (SHIV) animal models have been used to explore prolonged viral remission following bNAb treatment. Passive immunotherapy treatment of macaques during acute infection can result in changes to the course of SHIV infection [340]. In the absence of ART, SHIV-infected macaques that were administered with a 2-week course of bNAbs 3BNC117 and 10-1074. Of the 13 treated macaques, 6 monkeys were able to control their levels of virus to undetectable levels and maintain viral control for 1 year and another 4 monkeys showed reduced viral loads with normal CD4⁺ T-cell counts. Control of viremia was mediated by CD8⁺ T-cells as the elimination of these cells with an anti-CD8 monoclonal antibody resulted in viral rebound [340]. In contrast to ART-naïve macaques, monkeys that received 15 weeks of ART 3 days after infection demonstrated rebound plasma viremia when ART was discontinued. This study demonstrated passive immunotherapy can facilitate emergence of controller monkeys that can suppress viral replication.

In macaques infected with SHIV, the combination of a potent bNAb PGT121 combined with the TLR7 agonist vesatolimod (GS-9620) was used to target the viral reservoir [341]. Following the cessation of ART in monkeys treated with both PGT121 and vesatolimod, 5 animals did not show viral rebound for 6 months. This was in contrast to monkeys that received PGT121 or vesatolimod alone and rebounded at 112 days and 21 days respectively [341]. Additionally, cellular RNA was undetectable following treatment, suggesting that the combination of bNAbs with immune stimulation by the TLR7 agonist reduced the viral reservoir. Overall, these data demonstrate the potential success of a dual pronged approach to “shock and kill” through the use of the TLR7 agonist as the “shock” LRA and the bNAb PGT121 as the “kill”.

Clinical trials of bNAbs in humans was first reported for 3BNC117, VRC01 and 10-1074 [342-344]. A single infusion of 3BNC117, administered to both infected and uninfected individuals resulted in reduced viral plasma and significantly delayed viral rebound for 28 days [342]. In a subsequent study, the use of 3BNC117 to delay viral rebound during ATI was investigated in 13 PLWH who were on ART [345]. Two groups, one received two infusions of the bNAb while the others received 4 infusions were evaluated. It was demonstrated that two infusions of 3BNC117 resulted in an average time to viral rebound of 6.6 weeks while those that received 4 infusions suppressed viral for a longer 9.9 weeks [345]. Trials with VRC01 demonstrated a delay in viral rebound with a single VRC01 infusion in viremic individuals [346]. In ART-treated PLWH, infusion of VRC01 did not lead to a delay in viral rebound 4 weeks after the bNAb infusion [346]. This was also found by another group [343]. In PLWH undergoing ATI, the infusion of VRC01 resulted in a slight delay in plasma viral rebound but this was not sustained beyond 8 weeks [343]. Further analysis revealed most study participants had pre-existing baseline resistance to VRC01.

10-1074 is a more potent bNAb with a similar half-life to 3BNC117 and VRC01 [344]. Administration of 10-1074 to PLWH resulted in a 1.52 log reduction in viremia [344], demonstrating similar potency to 3BNC117, but there was a higher frequency of escape mutants detected with 10-1074 [344]. In these clinical trials, despite transient delays in viral rebound with single infusions subsequent analysis of escape mutants showed sensitivity to other bNAbs [343, 344, 346]. For example, escape mutants that showed resistance to 10-1074 were sensitive to

3BNC117 and VRC01 [344]. Thus, it is conceivable that a combination of two or more different bNAbs could significantly delay viral rebound and warrants further study in human clinical trials.

1.13.3 Bispecific antibodies

The encouraging results from bNAbs studies have prompted the development of bispecific antibodies (bsAbs) that allow a single therapeutic agent to target two epitopes by essentially linking two antibody epitope recognition domains (single chain antibody fragments) together [347]. bsAbs can target two different Envelope epitopes or antigens with varying neutralising actions or can link an infected cell to an effector cell such as cytotoxic T-lymphocytes to elicit an immune response [347] (Figure 1.11). Bispecific T-cell engagers (BiTEs) comprise of a specific variable fragment and a CD3-specific fragment joined by a peptide linker [348]. The first FDA-approved BiTE, blinatumomab (for use in refractory acute lymphoblastic leukemia) has demonstrated the efficacy and safety of these engineered antibodies to treat cancer [349]. Recently, BiTEs specific for HIV Env have shown potential in eliminating infected cells *in vitro*. Bispecific antibodies that target a HIV Env epitope and the CD3 epitope induce cross-linking of CD3 on T-cells to both activate the cell to induce Env expression and also increase CD8⁺ cytolytic function [350]. The addition of LRAs may then allow for the cross-linking of cytotoxic CD8⁺ T-cells to initiate cytolysis and subsequent death of the reactivated cells (Figure 1.11).

Improvement of BiTEs has led to the production of HIV-specific bispecific T-cell engaging antibodies termed Dual-Affinity Re-Targeting (DART) antibodies with wider recognition to a diverse range of viral strains [351]. One such DART incorporates non-neutralising monoclonal antibodies (mAbs) that recognise Env (A32 and 7B2) epitopes [352] with an anti-CD3 moiety. Another paired the bNAb VRC07 with an anti-CD3 moiety [353], or with PGT121 [354]. Laboratory generated antibodies that include non-neutralising antibodies have the advantage of recognising epitopes that are not normally present on the surface of infected cells and thus may be beneficial against latently infected cells that do not display any distinguishing epitopes. These DARTs were all able to direct T-cell mediated cytolysis CD4⁺ T-cells collected from PLWH on ART [352-354]. Although these bsAbs have yet to be tested *in vivo* in humans, a study performed in ART-treated

macaques demonstrated safety of bsAbs [353]. Taken together, this data prompts further investigation of anti-HIV bsAbs and their use *in vivo* to purge the latent reservoir.

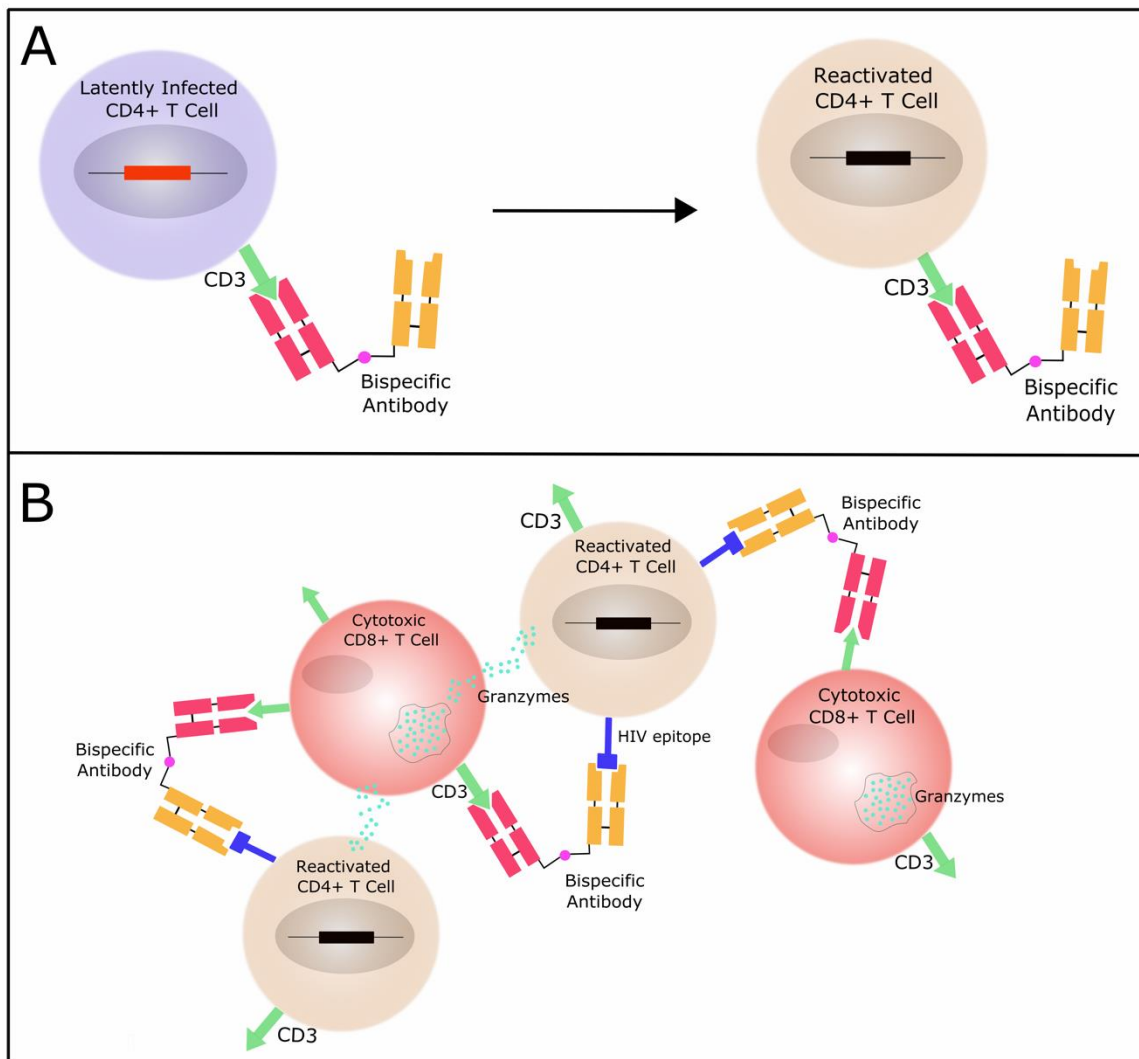


Figure 1.11. Mechanism for using bispecific antibodies to eliminate latently infected resting CD4+ T-cells

A) Bispecific antibodies engineered to recognise CD3 moieties could bind to CD3 receptors present on latently infected CD4+ T-cells, leading to their reactivation. B) Additionally, bispecific antibodies may cross-link reactivated CD4+ T-cells by the Env moiety with immune effectors cells such as cytotoxic CD8+ T-cells by the CD3 moiety as CD8+ T-cells also express CD3 receptors. The bispecific antibody would bind to HIV Env on reactivated cells and then bind to CD8+ T-cells, leading to the death of the reactivated cells through the release of granzymes.

1.13.4 Boosting CD8+ T-cell responses

HIV-specific CD8+ T-cells are generated in response to HIV infection during acute infection and are temporally associated with the decline of plasma HIV RNA [355, 356]. In a subset of PLWH, there are a rare few individuals who can spontaneously control viral load to levels below the limit of detection (viral loads of <50 copies/ml) without ART. These individuals are termed elite controllers [357, 358]. Viremic controllers on the other hand, can maintain levels of viral suppression to an extent and have low but detectable virus below 2000 HIV RNA copies/ml [359]. Viral control in elite controllers has been associated with the MHC and the overrepresentation of protective HLA class I alleles [360, 361]. Another cellular mechanism associated with viral control is the high-frequency of HIV-specific cytotoxic CD8+ T-cells and their superior ability to suppress viral replication [362-364]. In response to HIV-infected autologous CD4+ T-cells, HIV-specific CD8+ T-cells from elite controllers were able to proliferate and expand better than CD8+ T-cells from progressors (those with normal disease progression) [364]. Additionally, the proliferation of the CD8+ T-cells was coupled with an increase in perforin expression and granzyme B, which resulted in the clearance of HIV-infected CD4+ T-cells [363, 364].

Due to the superior ability of CD8+ T-cells to control the HIV reservoir in elite controllers and viremic controllers, it has been proposed that boosting HIV-specific CD8+ T-cell function will be able to effectively clear reactivated latently infected cells. Shan and colleagues have demonstrated that the administration of vorinostat alone did not lead to the death of latently infected cells [365]. However, in the same study, they also show that pre-stimulation of CD8+ T-cells with B57-restricted Gag peptides effectively eliminated HIV infected autologous CD4+ T-cells reactivated with vorinostat [365]. This study illuminates the model of “shock and kill” that combines latency reversal with a kill regime that boosts immune-mediated clearance using CD8+ T-cells. One major caveat of this study is the use of a model of HIV latency that overexpresses Bcl-2. Bcl-2 is an anti-apoptotic protein that correlates with resistance to cell death [366]. This model system likely under-estimates the efficiency of CD8+ T-cell mediated cytotoxicity and thus, further investigation in a model of HIV latency that doesn’t exhibit pre-existing resistance to apoptosis is needed.

The Margolis laboratory are testing the priming and expansion of patient CD8+ T-cells by HIV antigens *ex vivo* (which kill autologous latently infected cells treated with an LRA) with a view to then reinfuse these cells back into the same patient, aiming to clear latently infected cells reactivated by an LRA [352, 367].

Therefore, it is evident that a successful shock and kill strategy will be one that combines latency reversal with an agent that not only reactivates latent HIV, but also does not affect the function and activity of cytotoxic CD8+ T-cells.

1.13.5 Immune checkpoint blockade

Immune checkpoint blockade, specifically inhibition of PD-1 could potentially have a dual role in elimination of latency. The first mechanism is through the reversal of latency, which has been demonstrated in a few studies [299, 302] (section 1.10.9). The second is through the enhancement of HIV-specific CD8+ T-cell effector function. In an SIV-infected animal model, the administration of PD-1 blockade was safe, well-tolerated and led to a rapid expansion of SIV-specific CD8+ T-cells [322]. This expansion of SIV-specific CD8+ T-cells occurred in both acute and chronic SIV-infection and was observed in the blood as well as colorectal mucosal tissue. Importantly, the administration of PD-1 blockade also resulted in the enhancement of antiviral CD8+ T-cell function, generating polyfunctional cells that produced cytokines IFN- γ , TNF- α and IL-2 [322].

The combination of ART and PD-1 blockade has also been investigated in an SIV-infected macaque model [323]. The administration of PD-1 antibodies 10 days prior to ART resulted in improved T-cell response of circulating CD4+ and CD8+ T-cells. There was also an improvement in viral suppression when ART was initiated [323]. Following the initiation of ART, another PD-1 antibody infusion to SIV-infected rhesus macaques was administered. Proliferation in total and antiviral CD4+ T-cells was observed in the blood and in the lymph node, granzyme B+ CD8+ T-cells were found to be localised, suggestive of cytosolic CD8+ T-cell migration into sites that are known to be viral reservoirs.

There have been only a limited number of studies that investigate CD8+ T-cell function following PD-1 blockade in PLWH [368, 369]. Of these, one study has shown a significant increase in HIV-specific CD8+ T-cells [370]. In a clinical trial exploring ICB for the treatment of HIV without malignancies, Gag-specific CD8+ T-cell numbers were increased following a single low dose of an anti-PD-L1 antibody in 2 of the 6 participants [370]. Unfortunately, the trial was ceased due to toxicities associated with the antibodies. Despite the toxicities observed, the above studies all show the potential and contribution of immune checkpoint blockade in enhancing HIV-specific T-cell function.

1.13.6 Chimeric Antigen Receptor (CAR) T-cells

Recent trials with chimeric antigen receptor (CAR) T-cells in the treatment of B-cell malignancies with engineered CAR T-cells [371, 372] has provided valuable knowledge and interest in this approach to clear HIV infected T-cells. CAR T-cells are generated through the combination of an extracellular antigen-binding domain with an intracellular T-cell activation domain [373]. Anti-HIV CAR T-cells combine the CD3 ξ signalling domain of the T-cell receptor (TCR) with the extracellular transmembrane domain of the CD4 receptor (CD4 ξ -CAR) or combine the variable region from monoclonal antibodies (scFv-CAR) [324, 325]. CAR T-cells work in an MHC-independent manner and thus are less susceptible to escape mechanisms used by HIV such as the downregulation of MHC class I molecules [374]. Additionally, as MHC class I recognition is not required, CAR T-cell therapy can be administered to patients with various HLA subtypes.

First generation anti-HIV CD4 ξ -CAR T-cells were shown to be safe and well tolerated in PLWH, however, there was only a modest antiviral effect and control of the virus was not observed [375-377]. A 10-year follow-up study revealed that the modified CAR T-cells have long half-lives of over 16 years [377]. Retrospective analysis found that the CAR T-cells were susceptible to HIV infection [378, 379] and thus new generations of CAR T-cells were developed. Co-stimulatory motifs such as CD28, CD127 and 4-1BB were introduced to improve *in vivo* persistence, potency and specificity in cytotoxicity [380, 381]. To inhibit the infection of the cells by HIV, CAR T-cells have been engineered to include HIV fusion inhibitors, zinc finger nucleases or short hairpin RNA targeting CCR5 (reviewed in [382]). Re-engineering of the original CD4 ξ -CAR T-cell through changes to the CAR expression vector, transmembrane, promoter and co-stimulatory domain, it

was demonstrated in a humanized model of HIV that the re-engineered CAR T-cells expanded in response to antigen and protected CD4⁺ T-cells from infection whilst reducing viral loads, superior to the original CD4 ξ -CAR T-cells [381].

An alternative strategy to engineer CAR T-cells is to use bNAbs as the binding domain, generating CAR T-cells directed to a variety of HIV epitopes [324, 325]. One limitation of bNAb CAR T-cells is the inability to target all HIV epitopes due to HIV escape. The use of a single bNAb moiety limits the breadth and immunogenicity of the modified CAR T-cells. One study that investigated seven different bNAb-based CAR T-cells found that each CAR had antiviral activity in transduced CD8⁺ T-cells specific for HIV infected cells *in vitro* [324]. The CAR T-cells proliferated in the presence of HIV-specific CD4⁺ T-cells and mediated killing of these cells [324]. Another study that generated CD8⁺ T-cells modified with a VRC01 bNAb CAR moiety containing CD28 and 4-1BB co-stimulatory domains (VR-CAR-T cells) demonstrated cytotoxicity of HIV infected T-cells [325]. Additionally, when antiretroviral drugs were removed from HIV infected cell cultures *in vitro*, VR-CAR-T cells significantly suppressed viral production. Importantly, VR-CAR-T cells were able to decrease cell-associated HIV RNA and inhibit viral replication in CD4⁺ T-cells from PLWH that had been reactivated using LRAs [325]. It remains to be seen whether bNAb CAR T-cells will have the same therapeutic effect *in vivo* and requires evaluation.

In summary, immune-based strategies could potentially be combined with LRAs to eliminate latently infected cells. However, these strategies rely on the patient's immune system to effectively clear these cells, which remains compromised and in a state of exhaustion. An alternate approach to elimination of latently infected cells is to activate pro-apoptotic pathways that are triggered by viral proteins. This could be a promising approach to eliminate latently infected cells and was the major focus of this thesis.

1.14 Apoptosis and HIV latency

1.14.1 HIV latency and non-apoptotic cell death mechanisms

Productive HIV infection of CD4⁺ T-cells can lead to cell death through numerous mechanisms. The main proposed form of programmed cell death is apoptosis. However other forms of programmed cell death may contribute. Pyroptosis is a highly inflammatory form of cell death

that features cellular swelling, rupture of the plasma membrane and release of the cell cytoplasmic contents and pro-inflammatory cytokines into the extracellular milieu [17]. During active HIV infection, abortive reverse transcription of the viral RNA genome to DNA can result in incomplete viral DNA reverse transcripts that are sensed by protein recognition receptors such as IFI16. This results in assembly of the inflammasome by caspase-1 activation and ultimately leads to pyroptosis. [18]. Pyroptosis is mediated by caspase-1 and works independently of the other pro-apoptotic caspases. However, it is unclear if pyroptosis plays any role in a HIV latently infected T-cell.

Another mechanism of cell death is necrosis, which is non-apoptotic, passive, accidental and unregulated cell death. Necrosis does not require activation of specific pathways and can occur as a consequence of extensive cell damage. However, necrosis is not always passive and can result from a signal cascade in a regulated manner, much like apoptosis, aptly termed necroptosis [383]. Necroptosis can be triggered by death receptors TNF and TNFR1, which requires the activity of receptor interacting protein kinase-1 (RIPK1) and -3 (RIPK3) [384]. When TNF- α binds to TNFR1, TNF receptor-associated death domain (TRADD) recruits RIPK1. In the absence of caspase-8, RIPK1 assembles with RIPK3 to form a complex termed the necrosome, acting as the signal transducer for necroptosis [385]. Mixed lineage kinase domain like protein is then activated through phosphorylation by the necrosome resulting in the insertion of this protein into the plasma membrane and subsequent expulsion of intracellular contents [386]. Alternatively, other death receptors such as the TLRs and the cytosolic DNA sensor DNA-dependent activator of IFN regulator genes have also been shown to induce necroptosis [387, 388]. There is accumulating evidence that pyroptosis and necroptosis play a role in untreated, active HIV infection leading to the loss of CD4⁺ T-cells [17, 389]. However, there is little evidence that pyroptosis or necroptosis are activated following latency reversal. Although, further work is needed to determine whether these pathways could be exploited to preferentially eliminate latently infected cells.

1.14.2 Cellular apoptosis pathways and regulators

Apoptosis is a cellular process that results in programmed cell death of unwanted or damaged cells [390]. Apoptosis is tightly controlled, and defective regulation of apoptosis is implicated in

many human diseases such as cancer, inflammation and autoimmune disease [390]. There are two main apoptotic pathways, the intrinsic and extrinsic pathways (Figure 1.12).

The extrinsic pathway is mediated by death receptors on the surface of the cell, including members of the TNF receptor family. Binding of ligands to their respective death receptors results in the recruitment and activation of members of the caspase protease family that induce apoptosis [391]. When death receptor Fas binds to Fas ligand, the receptors recruit Fas-associated protein with death domain (FADD). Dimerization of the death effector domain results in association of FADD with procaspase-8 and the formation of a death-induced signalling complex that cleaves procaspase-8 into active caspase-8 [392]. Active caspase-8 promotes activation of the caspase cascade including cleavage and activation of caspase-3 leading to apoptosis [393].

In contrast, the intrinsic pathway is often referred to as the mitochondrial pathway and is initiated by cellular stress, DNA damage, radiation and other stress signals. These stress signals lead to the formation of a pore by cellular Bak or Bax proteins in the mitochondria membrane. This leads to permeabilization of the mitochondrial outer membrane and the release of cytochrome c and Smac and the protease Omi into the cytosol [394]. Once in the cytosol, these mediators trigger apoptosome formation and activate the caspase cascade to induce apoptosis [393] (Figure 1.12).

Apoptosis is regulated by members of the Bcl-2 protein family [395] and these are summarised in Table 1.5. Bcl-2 family members that tightly regulate apoptosis at the mitochondrial level include; the pro-apoptotic proteins Bak and Bax; anti-apoptotic proteins Bcl-X_L, Bcl-2, Bcl-W, A1 and Mcl-1; and BH3-only proteins that act as sensors of apoptotic stimuli including Bad, Bid, Bim, Bik, Bmf, Hrk, Puma and Noxa [396, 397] (Table 1.5). Members of the IAP protein family including X-linked inhibitor of apoptosis protein (XIAP) and cIAP can also regulate apoptosis by inhibiting Smac, preventing caspase activation to suppress apoptosis [397].

Both the intrinsic and extrinsic pathways converge at the stage of caspase-8 activation. When the extrinsic apoptotic pathway is triggered, the formation of active caspase-8 can lead to apoptosis

in a mitochondrial-independent pathway through the activation of the effector caspases (caspase-3 and -7) [398]. Additionally, active caspase-8 can cleave the BH3-only protein Bid producing truncated Bid (tBid) [399]. tBid can induce mitochondrial outer membrane permeabilization leading to the activation of mitochondrial-dependent apoptosis [400]. Therefore, caspase-8 provides a link between the extrinsic “death-receptor” and intrinsic “mitochondrial” apoptotic pathways.

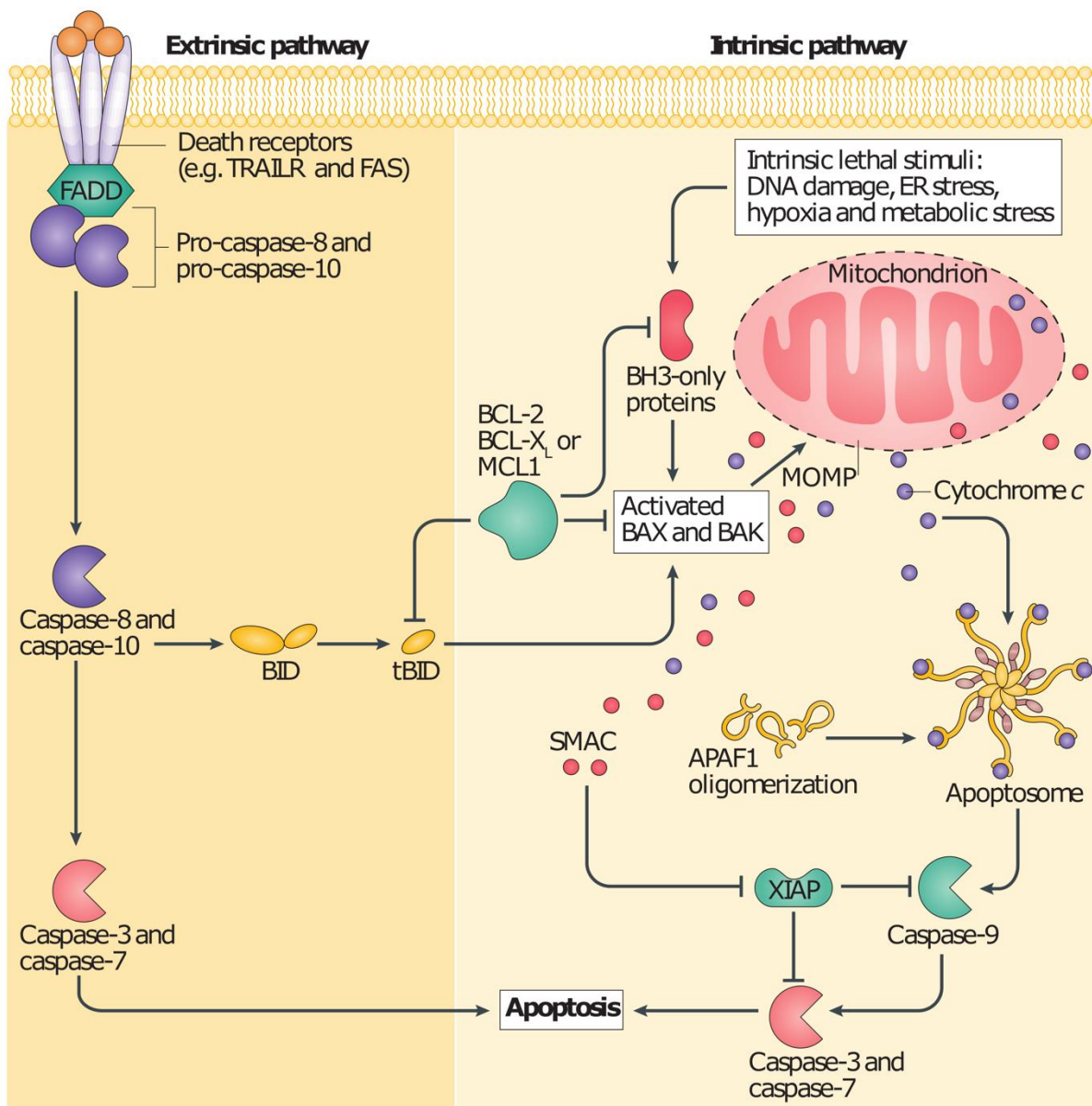


Figure 1.12. Intrinsic and extrinsic pathways converge to activate apoptosis

The extrinsic apoptotic pathway (left) can be activated through the binding of death receptors such as tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptor (TRAILR)

and Fas on the surface of cells. Binding of these molecules leads to the recruitment of the Fas associated death domain (FADD) which cleaves pro-caspase-8 and pro-caspase-10 to their active forms. Active pro-caspase-8/10 cleave and activate the caspase-3/7 which leads to apoptosis. Internal stress stimuli such as DNA damage and metabolic stress initiate the intrinsic apoptotic pathway (right). This pathway, often referred to as the mitochondrial pathway, starts with mitochondrial outer membrane permeabilization (MOMP). MOMP is triggered by Bax and Bak which are in turn activated by Bcl-2 homology domain 3 (BH3)-only proteins. Following mitochondria permeabilization, second mitochondria-derived activator of caspases (SMAC) and cytochrome c are released. Cytochrome c interacts with apoptotic protease activating factor 1 (APAF1) leading to the formation of the apoptosome and activation of caspase-9. Caspase-9 will activate caspase-3/7 leading to apoptosis. Smac can inhibit x-linked inhibitor of apoptosis (XIAP) which negatively regulates this pathway. Caspase-8/10 also cleave the BH3-only protein BH3-interacting death domain (BID) to a truncated form (tBID) that can also activate Bak and Bax. Adapted from [401].

Table 1.5. Bcl-2 family proteins and their main mechanism of action

Pro-/Anti-Apoptotic	Protein	Mechanism of Action	Refs
Anti-apoptotic	Bcl-2	Binds to and sequesters pro-apoptotic proteins Bax, Bim, Puma, Bmf and Bad	[402, 403]
	Bcl-X _L Bcl-W	Binds to and sequesters pro-apoptotic proteins Bax, Bak, Bim, Puma, Bmf, Bik, Hrk and Bad	
	A1 Mcl-1	Binds to and sequesters pro-apoptotic proteins Bak, Bim, Puma, Noxa and Hrk	[404]
Pro-apoptotic	Bax/ Bak	Once activated, Bax and Bak undergo conformational changes, oligomerize and permeabilize the mitochondrial outer membrane, resulting in the release of cytochrome c and Smac/DIABLO	[405-407]
	Activator BH3-only proteins Bid Bim Puma	High affinity for Bax/Bak Binds directly to and activates Bax/Bak Sequestered by all anti-apoptotic proteins	[402, 403, 408]
	Sensitizer BH3-only proteins Bik Bmf Bad Hrk Noxa	Binds to anti-apoptotic molecules, preventing these molecules from sequestering Bax/Bak. Displaces activator BH3-only proteins from anti-apoptotic proteins allowing the activator proteins to bind and directly activate Bax/Bak.	[402, 409, 410]

Bcl-2: B cell lymphoma-2, Bcl-X_L: B cell lymphoma-extra large, Bcl-W: B-cell lymphoma-W, A1: Bcl-2-related gene A1, Mcl-1: myeloid cell leukemia 1, Bax: Bcl-2-associated X protein, Bak: Bcl-2 antagonist killer 1, Bid: Bcl-2 interacting domain death agonist, Bim: Bcl-2 interacting mediator of cell death, Puma: p53-upregulated modulator of apoptosis, Bik: Bcl-2 interacting killer, Bad: Bcl-2 antagonist of cell death, Bmf: Bcl-2 modifying factor, Hrk: Harakiri, BH3: Bcl-2 homology domain 3, Smac: second mitochondria-derived activator of caspases. Adapted from [214].

1.14.3 HIV proteins influence apoptosis

HIV proteins can interfere with apoptosis pathways leading to a range of outcomes including activation, inhibition or delay in cell death [411] (Figure 1.13, 1.14). During the early stages of viral gene expression, the viral proteins Nef, Tat and Vpr can impair apoptosis to promote cell

survival, allowing for viral replication [411] (Figure 1.13). In contrast, in the late stages of viral gene expression, the HIV Env and Vpu proteins can promote apoptosis of infected cells by a variety of mechanisms [411]. Production of the viral protease later in viral gene expression can also lead to the generation of a pro-apoptotic Casp8p41 peptide that promotes apoptosis [412]. HIV Tat, Vpr and Nef also transition to pro-apoptotic roles later in viral gene expression when Tat and Vpr expression increases [411] (Figure 1.14). It is likely that the balance between anti-apoptotic and pro-apoptotic viral and cellular proteins determine the fate of infected cells between survival or apoptosis.

1.14.4 HIV latency and avoidance of apoptosis

While productive HIV infection typically leads to the death of infected cells [413], HIV latently infected CD4⁺ T-cells are long lived [414]. These latently infected T-cells can also proliferate [39, 85, 86] leading to clonally expanded cells that may contain identical and intact provirus [39, 89], as mentioned above. It is possible that latently infected cells avoid death [85] because there is no expression of pro-apoptotic viral proteins like HIV Env, Protease and Vpr [415], or there is an absence of viral RNA detection through the RIG-I PRR [416]. Additionally, latently infected cells may avoid death from the virus integrating into genes like BACH2, which encodes a transcriptional regulator that may promote cell survival [85]. Furthermore, the XIAP protein, which inhibits the protease activity of caspase-3, -7 and -9 [417, 418], is also increased in latently infected cells compared to uninfected cells [417] to favour cell survival. Jones and colleagues have found that some LRAs can have detrimental effects on CD8⁺ T-cell function [101, 419]. The latent HIV reservoir exhibits an inherent resistance to CD8⁺ T-cell mediated killing [420] despite functional CD8⁺ T-cells and thus suggests there are other mechanisms within latently infected T-cells that contribute to the resistance of apoptosis. Finally, inhibition of other pro-apoptotic cellular proteins in latently infected cells may also be involved. For example, knockdown of pro-apoptotic proteins such as Bax and FADD have been shown to prolong cell survival in Jurkat T-cells infected with HIV [421].

These data collectively suggest that the absence of pro-apoptotic viral proteins, together with elevated anti-apoptotic and decreased pro-apoptotic cellular proteins, together promote the survival of HIV latently infected cells.

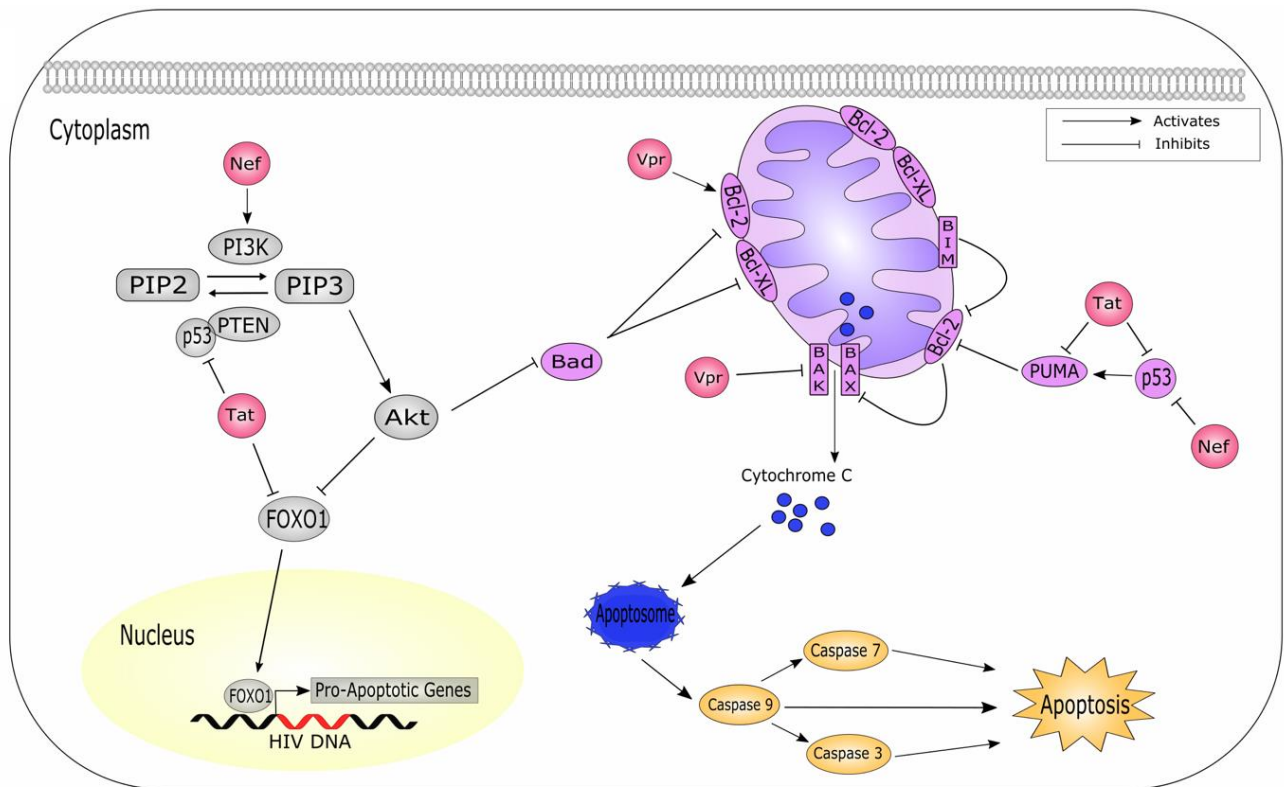


Figure 1.13. HIV proteins expressed early in viral gene expression can lead to cell survival

Anti-apoptotic HIV proteins (shown in red) expressed early in viral gene expression that can promote cell survival include Nef, Tat and Vpr. Nef can bind phosphoinositide 3 kinase (PI3K) proteins to increase the conversion of PIP_2 to PIP_3 . This leads to an increase in Akt protein that subsequently leads to inhibition of pro-apoptotic molecules such as Bad from activating apoptosis thereby promoting cell survival. Additionally, Nef can inhibit p53 from activating the pro-apoptotic molecule PUMA that inhibits Bcl-2. HIV Tat protein can inhibit PTEN, a negative regulator of the PI3K pathway, thereby promoting the PI3K/Akt pathway to promote cell survival. Tat can also directly inhibit FOXO1 transcription factors to prevent the transcription of pro-apoptotic genes, preventing apoptosis. Tat can also inhibit the pro-apoptotic molecule PUMA from inhibiting Bcl-2 to prevent apoptosis. HIV Vpr can lead to the increase of anti-apoptotic molecule Bcl-2 in addition to inhibiting the pro-apoptotic Bak molecule to prevent apoptosis. PIP_2 : Phosphatidylinositol 4,5-bisphosphate or $PtdIns(4,5)P_2$, PIP_3 : Phosphatidylinositol-3,4,5-trisphosphate or $PtdIns(3,4,5)P_3$, PTEN: phosphatase and tensin homolog, Bcl-2: B-cell lymphoma 2 Bcl-XL: B-cell lymphoma-extra large, PUMA: p53 upregulated modulator of apoptosis, FOXO1: forkhead box O1. Adapted from [214].

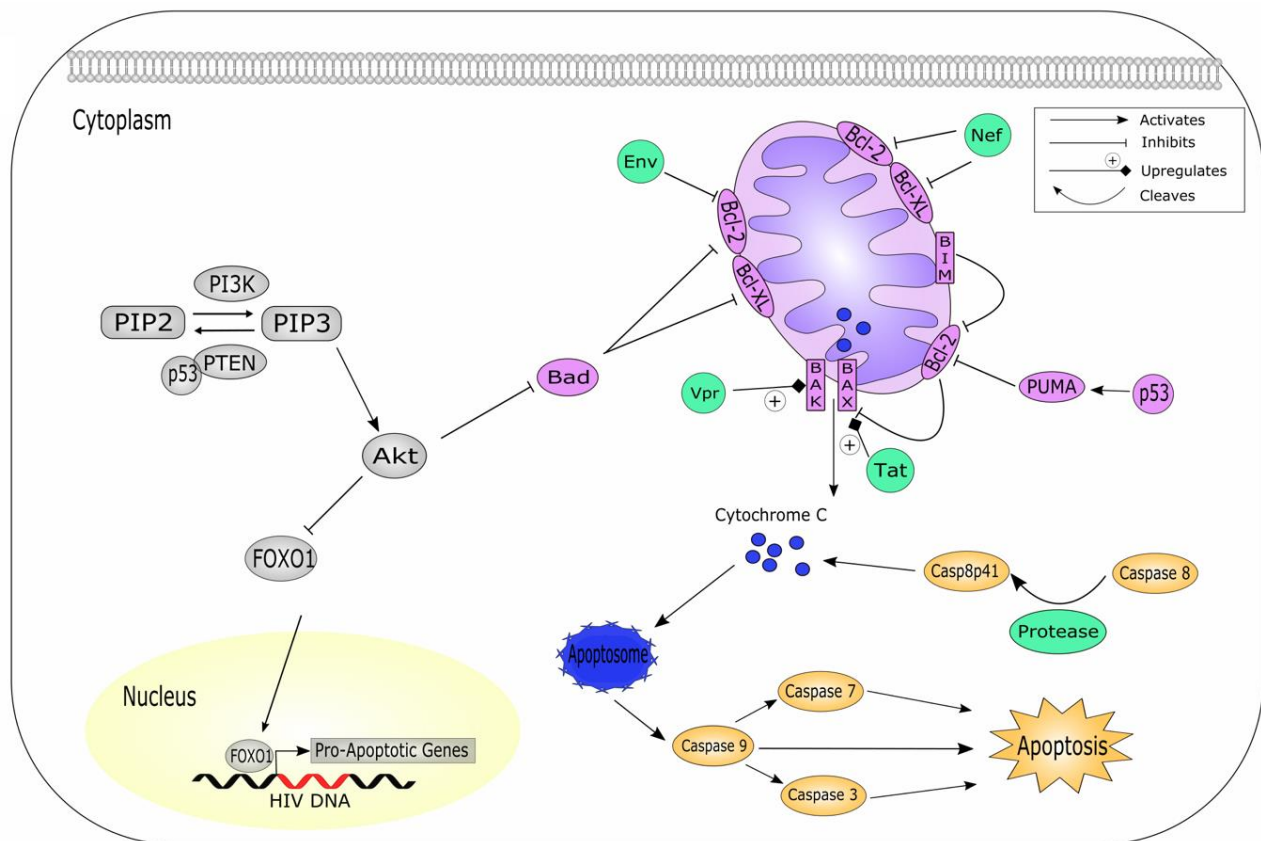


Figure 1.14. HIV proteins expressed late in viral gene expression can lead to cell death

Multiple HIV proteins (shown in green) can interact with the members of the apoptosis pathway later in viral gene expression leading to increased apoptosis. Higher expression of Nef can inhibit both the anti-apoptotic molecules Bcl-2 and Bcl-X_L to favour apoptosis. Higher expression of HIV Vpr can bind and lead to the upregulation of Bak to initiate apoptosis. Higher expression of Tat also leads to the upregulation of Bax, which can result in the release of cytochrome C from the mitochondria, activating the caspase cascade and apoptosis. HIV Env protein can inhibit anti-apoptotic protein Bcl-2 to favour apoptosis. The HIV protease protein also cleaves caspase-8 to the pro-apoptotic Casp8p41 peptide to promote apoptosis. It is likely that the balance of pro-apoptotic versus anti-apoptotic cellular and viral proteins decide the fate of infected cells between apoptosis or cell survival. PIP2: Phosphatidylinositol 4,5-biphosphate or PtdIns(4,5)P₂ , PIP3: Phosphatidylinositol-3,4,5-triphosphate or PtdIns(3,4,5)P₃, PTEN: phosphatase and tensin homolog, Bcl-2: B-cell lymphoma 2 Bcl-X_L: B-cell lymphoma-extra large, PUMA: p53 upregulated modulator of apoptosis, FOXO1: forkhead box O1. Adapted from [214].

1.14.5 Pro-apoptotic compounds to clear HIV latently infected T-cells

The balance of pro-apoptotic and anti-apoptotic cellular and viral factors likely determine if cells live or die. HIV latently infected cells express higher levels of anti-apoptotic XIAP [417] and do not express pro-apoptotic viral proteins favouring cell survival. Therefore, we hypothesised that treating latently infected T-cells with pro-apoptotic drugs to increase cell sensitivity to apoptosis, together with LRAs to drive the expression of pro-apoptotic HIV proteins later in gene expression, could lead to the selective death of latently infected cells.

Multiple pro-apoptotic compounds have been developed, typically for the cancer field (reviewed in [422, 423]), and recent studies suggest that select compounds may hold promise in clearing HIV latently infected cells including: Bcl-2 antagonists, drugs that increase procaspase-8, PI3K/Akt inhibitors, Smac mimetics/XIAP inhibitors and RIG-I inducers (summarised in Figure 1.15 and Table 1.6). The potential activity of these compounds in eliminating HIV latently infected cells is discussed below.

1.14.5.1 Bcl-2 antagonists

The overexpression of Bcl-2 is common in many cancers and thus is a target of cancer therapy [424]. Many of the Bcl-2 antagonists currently being investigated are BH3-mimetics [425]. Compounds such as venetoclax and navitoclax mimic the BH3 binding domain present in pro-apoptotic molecules and bind with higher affinity to anti-apoptotic Bcl-2 family proteins [412, 426], blocking their anti-apoptotic function thereby favouring apoptosis. Venetoclax has recently been licensed for the treatment of chronic lymphocytic leukaemia (CLL) and is in advanced clinical development for non-Hodgkin's lymphoma (NHL) [427].

In vitro, Bcl-2 antagonists have been shown to deplete latently infected T-cells following T-cell receptor activation with antibodies binding to CD3, a co-receptor for the TCR, and CD28, a co-stimulation signal [428]. During active HIV replication, the HIV protease results in cleavage of procaspase-8 to generate the Casp8p41 fragment containing a BH3-like domain that subsequently binds to and activates Bak, triggering apoptosis [429]. However, during full activation with the mitogen PHA, reactivation of HIV latency in resting CD4⁺ T-cells, HIV protease-dependent apoptosis is prevented as the cells contain high levels of anti-apoptotic Bcl-2, which can sequester

the pro-apoptotic Casp8p41 to prevent apoptosis [412]. The authors propose that inhibiting Bcl-2 prevented the Bcl-2-mediated sequestration of pro-apoptotic Casp8p41, liberating Casp8p41 generated by HIV protease to induce apoptosis of activated latently infected cells. However, this observation was in the context of full T-cell activation.

Pre-treatment of CD4⁺ T-cells from PLWH on ART with the Bcl-2 antagonist venetoclax and subsequent activation of the T-cells with anti-CD3 and anti-CD28 T-cell reduced the frequency of latently infected T-cells in cultures from 8 of 11 individuals [412]. However, agents that induce maximal T-cell stimulation are too toxic to administer as therapeutics and the significant changes to the cellular milieu following T-cell activation is quite different to targeted activation of the HIV LTR [215-217]. Whether the combination of Bcl-2 inhibitors like venetoclax with LRAs that do not induce maximal T-cell stimulation will also lead to the death of latently infected cells remains unclear. Furthermore, it is unclear if other HIV proteins expressed following latency reversal synergise with venetoclax to induce apoptosis. Interestingly, venetoclax also leads to the selective killing of HIV infected primary T-cells during productive infection *in vitro* [430].

1.14.5.2 Drugs that increase pro-caspase-8

The generation of pro-apoptotic protein Casp8p41 by HIV protease kills cells through a cleavage event that reveals a BH3 groove domain that binds Bak, leading to the permeabilization of the mitochondrial membrane and eventual apoptosis [431]. Recently, it was discovered that Casp8p41 can bind other BH3 groove domain proteins such as Bcl-2 [412] (see section above). Given the ability of HIV protease to cleave pro-caspase-8 into Casp8p41, if levels of pro-caspase-8 in a latently infected cell were increased, an increase in apoptosis would be observed.

Four FDA-approved drugs flurbiprofen, indomethacin, doxycycline, and bezafibrate were all able to induce pro-caspase-8 substrate expression in uninfected CD4⁺ T-cells and decreased HIV DNA following primary CD4⁺ T-cell infection *in vitro*. These drugs also decreased HIV DNA when added to CD4⁺ T-cells collected from PLWH on ART [432] in the presence of anti-CD3/anti-CD28. However, as mentioned above, compounds that induce maximal T-cell stimulation also confers with toxicity [215-217]. Understanding whether selective cell death of infected cells can occur

with these agents that increase pro-caspase-8 expression and/or synergise with latency reversal remains to be determined.

1.14.5.3 PI3K and Akt inhibitors

PI3K are lipid kinases that produce secondary messengers that control a wide range of intracellular signalling pathways in leukocytes [433]. Activation of the PI3K pathway has the cumulative effect of promoting cell survival [433]. PI3Ks are also important in the apoptosis pathway and the survival of virus-infected cells [434]. Therefore, we hypothesised that PI3K inhibitors could potentially enhance death of HIV infected cells.

Activation of the PI3K pathway leads to generation of serine/threonine kinase Akt (also referred to as Protein Kinase B or PKB) [435] (Figure 1.13). Binding of the Pleckstrin homology (PH) domain of Akt to the phosphoinositide products of PI3K (PIP_3) results in the recruitment of Akt to the plasma membrane and Akt activation [436]. Activated Akt then inhibits pro-apoptotic molecules either through direct inhibitory phosphorylation of the Bcl-2 family member Bad, or indirectly through the phosphorylation of transcription factors such as Forkhead box O1 (FOXO1) that subsequently translocates out of the nucleus thereby no longer promoting transcription of pro-apoptotic genes [437]. This prevents apoptosis and fosters cell survival.

Two HIV proteins expressed early in the virus life cycle have been shown to interact with the PI3K pathway (Figure 1.13, 1.14). The HIV protein Nef activates the PI3K signalling pathway through binding the p85 unit of PI3K [438], which resulted in inhibitory phosphorylation of pro-apoptotic factor Bad blocking premature apoptosis in T-cells [439]. HIV-1 Tat protein also interferes with PTEN [439, 440]. PTEN converts PIP_3 back to PIP_2 , thereby inhibiting activation of Akt. PTEN is regulated by binding to p53. p53 is a pro-apoptotic molecule that enhances PTEN expression, leading to the repression of the PI3K/Akt signalling pathway. However, HIV-1 Tat also binds p53, preventing p53 binding to PTEN and causing downregulation of PTEN. This leads to activation of PI3K/Akt signalling and impaired apoptosis [438, 439]. Therefore, both HIV Nef and Tat can promote the PI3K/Akt signalling pathway to prevent apoptosis.

Given the role of PI3K/Akt signalling in preventing apoptosis, and the ability of HIV Nef and Tat proteins to promote this pathway and prevent apoptosis, inhibition of the PI3K/Akt pathway may lead to apoptosis and death of HIV-infected cells [441]. During HIV infection of macrophages, the HIV Tat protein promotes the stress-induced activation of the PI3K/Akt survival pathway [439]. Activation of this pathway limits cytotoxicity and may be important for long-term survival of infected macrophages. Inhibition of Akt, through the drugs Miltefosine, Perifosine and Edelfosine, prevented HIV infected macrophages from activating the PI3K/Akt signalling pathway upon cell stress and the cells died [441]. This study showed the potential of PI3K/Akt inhibitors to eliminate HIV latently infected macrophages by sensitizing these cells to apoptosis [439]. However, whether these inhibitors will similarly induce death of latently infected T-cells is unclear. Pre-sensitizing latently infected T-cells with PI3K/Akt inhibitors to favour apoptosis followed by reactivation of latent HIV with LRAs to drive the expression of pro-apoptotic viral proteins could lead to the death of these latently infected T-cells, similar to experiments using the venetoclax Bcl-2 antagonist (see section 1.14.5.1).

1.14.5.4 Smac mimetics and XIAP inhibitors

Apoptosis is tightly regulated by proteins such as the IAPs, which are defined by the presence of one to three BIR domains [390]. Eight human IAPs have been identified: NAIP (BIR1), cellular-IAP1 (BIR2), cIAP2 (BIR3), XIAP (BIR4), survivin (BIR5), BRUCE/Apollon (BIR6), livin (ML-IAP1/BIR7) and ILP2 (BIR8) [304]. XIAP, cIAP1 and cIAP2 have major roles in both the intrinsic and extrinsic apoptosis pathways while the other IAPs regulate cell survival through cell cycle control or inflammation [304]. IAPs regulate apoptosis via interactions with Smac. Smac is released from the mitochondria upon activation of the intrinsic apoptosis pathway and binds to and inhibits IAPs [285]. XIAP directly competes with Smac to bind and inhibit caspase-3, -7 and -9 activity, thus preventing the caspase cascade and apoptosis. In contrast, cIAPs do not directly bind caspases but instead interact with Smac, which activates the ubiquitin activity and degradation of cIAP1/2 to promote caspase activation, modulating TNF-mediated apoptosis and NF- κ B pathways [304, 305, 390].

Due to the role of XIAP in apoptotic pathways and the elevated expression of XIAP in HIV latently infected T-cells [417], it was recently investigated whether targeting this protein could result in

the selective killing of latently infected cells. The flavopiridol cyclin-dependent kinase 9 (CDK-9) inhibitor that also downregulates XIAP expression [442] increased apoptosis of the latently infected ACH2 T-cell line compared to untreated cells [417]. Long-lived memory CD4⁺ T-cells were treated with XIAP antagonist GDC-0152, Smac mimetic birinapant, and inhibitor of XIAP embelin [443]. Birinapant, GDC-0152 and embelin resulted in a dose-dependent significant increase in the number of latently infected CD4⁺ T-cells undergoing apoptosis without the intervention of LRAs, demonstrating that HIV latently infected cells can be selectively cleared without reactivation [443]. Interestingly, Birinapant has been shown to clear Hepatitis B virus-infected cells *in vitro* and in a mouse model, through a similar mechanism [444, 445].

1.14.5.5 RIG-I Inducers

The induction of the innate immune response plays a critical role in the host cell defence against invading viruses. Recognition of viral pathogens through the innate immune response PRRs, which include TLRs or retinoic acid-inducible gene I (RIG-I), can also drive the apoptosis of virus infected cells [446]. RIG-I is a DExD/H box RNA helicase that senses viral RNA within the cytoplasm of infected cells [447, 448]. During HIV infection, RIG-I can sense HIV RNA and induce anti-viral signalling and apoptosis [449]. However, the HIV protease can also degrade or sequester RIG-I to abrogate RIG-I mediated apoptosis [450] and circumvent this innate immune response.

Retinoic acid (RA) can induce RIG-I expression as well as activate the p300 acetyl transferase cellular transcription factor, thus leading to HIV transcription [416]. Therefore, treating HIV latently infected cells with drug compounds that mimic retinoic acid might similarly activate both HIV transcription and RIG-I mediated apoptosis to both reactivate latent HIV and tip the balance towards apoptosis of these infected cells despite HIV protease expression impeding RIG-I (Figure 1.15).

Acitretin, an FDA-approved retinoic acid derivative used to treat psoriasis [451], was recently shown to reactivate latent HIV and induce the selective apoptosis of HIV latently infected cells *in vitro* [416]. In the study by Li *et al.*, acitretin induced HIV expression in latently infected T-cell lines and preferential death of HIV infected cells compared to uninfected samples. Moreover,

this effect was enhanced when the LRA HDACi vorinostat was combined with acitretin. Cell death was driven by RIG-I-mediated increases in the pro-apoptotic protein Bax, which can trigger caspase-mediated apoptosis [416]. Unfortunately, these findings were not replicated in a subsequent study that also evaluated latently infected cell lines and patient derived cells [452].

In summary, there are multiple pro-apoptotic compounds used in cancer research that have the potential to also deplete HIV latently infected T-cells, especially when combined with LRAs. It will be critical to distinguish between selective death of HIV-infected cell death, rather than non-specific T-cell depletion of both infected and uninfected cells. We therefore hypothesised that the combination of inhibitors of PI3K inhibitors or Bcl-2 with LRAs that induce viral proteins will selectively promote apoptosis of latently infected CD4⁺ T-cells and could be a potential new strategy to eliminate latency that could be tested clinically.

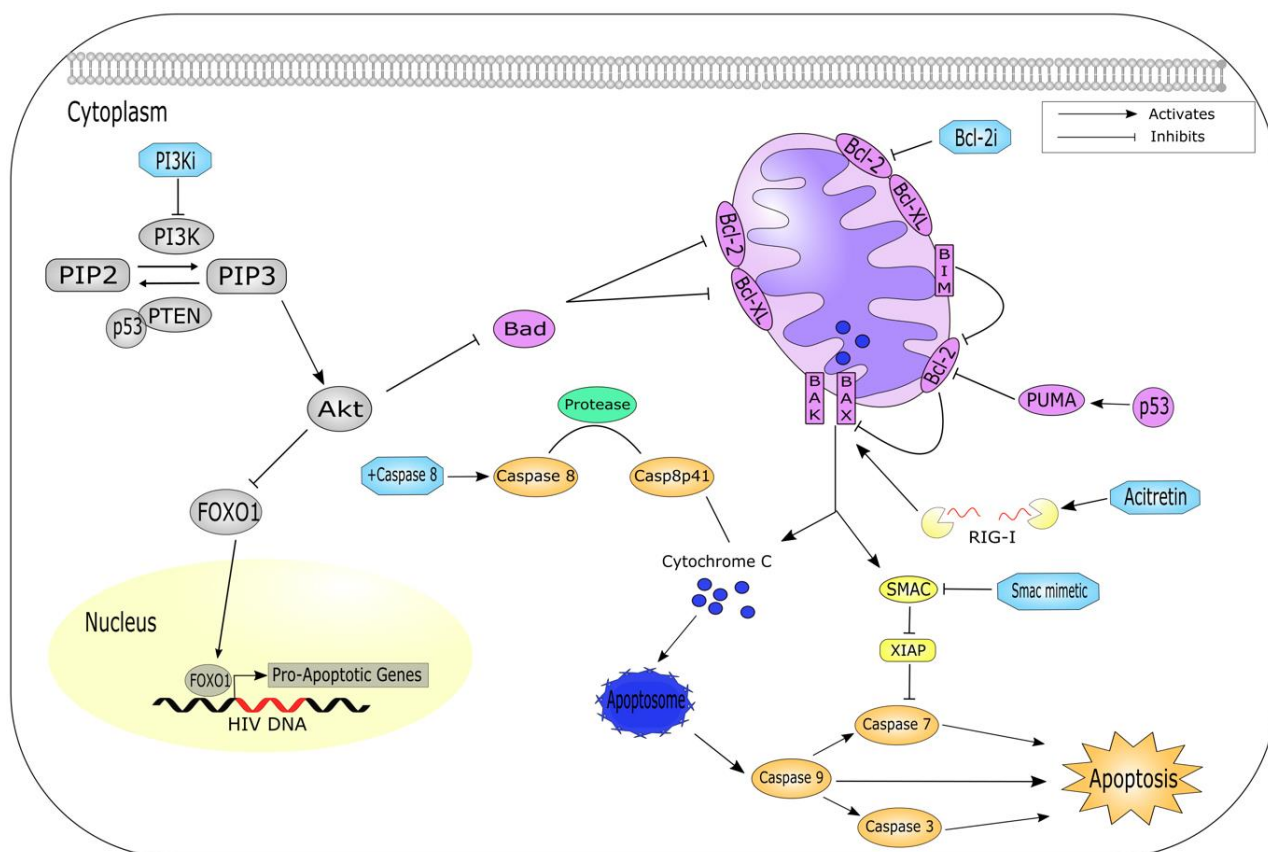


Figure 1.15. Modulation of apoptosis pathways using compounds

Compounds (light blue) that act on different cellular proteins in apoptosis pathways could potentially be used to increase cell sensitivity to apoptosis and tip the balance toward apoptosis of HIV infected cells. PI3K inhibitors block the conversion of PIP₂ to PIP₃, decreasing active Akt within a cell to stop Akt from inhibiting apoptosis. Akt inhibitors directly decrease active Akt to prevent Akt from inhibiting apoptosis to ultimately induce apoptosis. Drugs that increase procaspase-8 increase the procaspase-8 substrate that is cleaved to induce apoptosis, favouring apoptosis. Bcl-2 inhibitors such as venetoclax inhibit anti-apoptotic Bcl-2 to sensitize the cells towards apoptosis. Smac mimetics competitively bind inhibitors of apoptosis proteins like X-linked inhibitor of apoptosis (XIAP) to promote apoptosis. Retinoic acid-inducible gene I (RIG-1) detects viral RNA as well as activating pro-apoptotic Bak/Bax proteins, leading to apoptosis. In addition to viral RNA, RIG-1 inducer compounds like the retinoic acid derivative acitretin can also trigger apoptosis. Drugs that induce the expression of procaspase-8 lead to the increased production of casp8p41, resulting in increased apoptosis when latently infected cells are reactivated. Adapted from [214].

Table 1.6. Summary of compounds that induce apoptosis of HIV latently infected cells

Inducers of Apoptosis	Pathway Description	Compounds to induce apoptosis	Effects on HIV latency in vitro	Clinical Development for non-HIV indications	Ref
Bcl-2 antagonists	Anti-apoptotic cellular Bcl-2 sequesters the pro-apoptotic Casp8p41 fragment generated by HIV protease. Inhibiting Bcl-2 liberates pro-apoptotic Casp8p41 generated by HIV protease to promote apoptosis	Venetoclax	Depletion of latently infected cells using CD4+ T-cells from HIV-infected individuals on ART ex vivo	Licensed- chronic lymphocytic leukemia	[428]
		Navitoclax	Similar results to Venetoclax, but resulted in increased non-specific toxicity	Phase II- chronic lymphocytic leukemia	[428]
Pro-caspase 8 inducers	Pro-caspase 8 proteins can be cleaved into the pro-apoptotic protein Casp8p41	Flurbiprofen Doxycycline Indomethacin Bezafibrate	Decreased the levels of cell associated HIV DNA from CD4+ T-cells taken from HIV+ individuals on ART	N/A	[432]
PI3K/Akt Inhibitors	PI3K leads to activated Akt, which inhibits pro-apoptotic Bad and the transcription of pro-apoptotic genes. Inhibiting PI3K/Akt blocks this process, allowing pro-apoptotic Bad to function and transcription of pro-apoptotic genes, favouring apoptosis	Edelfosine Perifosine Miltefosine	Inhibited Akt activation induced by HIV infection in macrophages, leading to death upon cellular stress	Phase II- non-small-cell lung cancer Phase II- non-small-cell lung cancer Licensed- visceral leishmaniasis	[441]
		Lancemaside A Compound K Arctigenin	Eliminated cytoprotective phenotype of microglial cell lines infected with HIV upon cellular stress	N/A	[453]
Smac Mimetics	Smac mimetics bind and block anti-apoptotic IAP proteins to promote apoptosis	Birinapant GDC-0152 Embelin	Dose-dependent increase in death of HIV latently infected CD4+ T-cells	Phase I/II- Hepatitis B Phase I- Locally Advanced or metastatic malignancies Phase IV- Psoriasis	[443]
RIG-I Inducers	Induce RIG-I that detects viral RNA and promotes apoptosis. Retinoic acid (RA) also activates p300 acetyl transferase cellular transcription factor leading to HIV transcription	Acitretin	Induced HIV expression, reduced HIV DNA and induced preferential death in latently infected CD4+ T-cells from HIV infected individuals on suppressive ART	Licensed- Psoriasis	[416]
			No induction in HIV expression or death in latently infected cells using <i>in vitro</i> models		[452]

Bcl2: B-cell lymphoma 2, PI3K: phosphoinositide-3-kinase, Smac: second mitochondria-derived activator of caspase, RIG-I: retinoic acid-inducible gene I. Adapted from [214].

1.15 Conclusion

Latently infected resting CD4⁺ T-cells remain a major barrier to a cure for PLWH on long-term ART. One approach being pursued to eliminate latently infected cells is to activate HIV transcription and protein expression. Several clinical trials to date have demonstrated that this is possible but there is not clearance of infected cells, in the absence of an additional intervention to kill the infected cells. This has been demonstrated to successfully occur in macaques with the combination of a TLR7 agonist and the bNAb PGT121 and is yet to be tested in human clinical trials. An alternative approach to pharmacologically eliminate latently infected cells is to selectively trigger apoptosis of infected cells. To achieve this, there is a need for better *in vitro* models to successfully screen for novel LRAs, assessment of combinations of agents that drive virus production and enhance cell death and finally new assays to quantify the proportion of infected cells that undergo apoptosis *ex vivo*.

1.16 Hypothesis and Aims

We **hypothesize** that the combination of pro-apoptotic drugs that inhibit the PI3K pathways and/or Bcl-2 with latency reversing agents will selectively deplete latently infected cells. To achieve this, several new tools will be required including

1. a high throughput model of latently infected primary T-cells that can detect latent and productive infection and
2. an assay that can quantify the proportion of infected and apoptotic cells using CD4⁺ T-cells from PLWH.

This will be examined in the 3 following aims:

Aim 1: To establish a high throughput chemokine model of HIV latency using a dual fluorescent virus for detection of latent and reactivated (productive) infection for identifying novel compounds that activate latent virus.

Aim 2: To assess whether inhibiting the PI3K and Bcl-2 pathways in combination with LRAs can kill latently infected cells using latently infected cell lines and blood from PLWH on suppressive ART

Aim 3: To establish a flow cytometric method to identify selective death of latently infected cell using latently infected cell lines and blood from PLWH on suppressive ART

Chapter 2

Limitations of dual fluorescent HIV reporter viruses in a model of pre-activation latency

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Chapter 2

Limitations of dual-fluorescent HIV reporter viruses in a model of pre-activation latency

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HIV, dual-fluorescent reporter, latency, pre-activation model of HIV, latency establishment

2.1 Abstract

Introduction: HIV latency can be established *in vitro* following direct infection of a resting CD4+ T-cell or infection of an activated CD4+ T-cell which then returns to a resting state. We modified a previously published dual-fluorescent reporter virus seeking to track the establishment and reactivation of pre-activation latency in primary CD4+ T-cells.

Methods: A previously published dual-fluorescent reporter virus was modified so that expression of enhanced green fluorescent protein (GFP) was under control of the elongation factor 1 alpha (EF1 α) promoter to detect latent infection, and E2 crimson, a far-red fluorescent protein (E2CRM) was under control of the *nef* promoter to detect productive infection. NL4.3 that expressed GFP in place of *nef* was used as a positive control. We infected the Jurkat T-cell line and primary CD4+ T-cells that were either unstimulated or stimulated with either the chemokine CCL19 or phytohemagglutinin (PHA)/IL-2 and quantified the expression of both fluorescent proteins by flow cytometry. The study was carried out over a period of 2 years from September 2016 to October 2018.

Results and Discussion: Expression of both fluorophores was detected following infection of the Jurkat T-cell line while only low levels of the latent reporter were observed following infection of primary CD4+ T-cells. In unstimulated and CCL19 treated CD4+ T-cells, expression of the GFP latent reporter, increased after further activation of the cells with PHA/phorbol 12-myristate 13-acetate (PMA).

Conclusions: Our findings demonstrate that the EF1 α promoter has poor constitutive expression in resting CD4+ T-cells. Therefore, dual-fluorescent reporter viruses with the EF1 α promoter may underestimate the frequency of latent infection in resting CD4+ T-cells.

Word Count

256

2.2 Introduction

The main barrier to finding a cure for HIV infection is the persistence of latently infected cells in HIV-infected individuals on antiretroviral therapy (ART) [26, 29, 454]. *In vitro*, latent infection can be established through multiple pathways: direct infection of resting CD4⁺ T-cells (pre-activation latency [192]), infection of an activated cell which survives and reverts to a resting state (post-activation latency) [455] or infection of a cell transitioning from an effector memory to central memory phenotype [27, 456]. Given the low frequency of latently infected cells *in vivo* [457] insights into latency are often obtained through use of *in vitro* models of latent infection established in primary T-cells or T-cell lines (reviewed in [197]).

In recent years, dual-fluorescent reporter viruses have emerged as a common tool to study latency *in vitro* [100, 198, 199] by including two fluorescent reporters - a latent reporter that is under the control of an HIV LTR-independent promoter that is constitutively expressed following integration and a productive reporter that is expressed following transcription from the HIV LTR. Duo-Fluo is a previously reported virus that contains mCherry under the control of the elongation factor 1 α (EF1 α) promoter as the latent reporter and enhanced green fluorescent protein (GFP) as the reporter for productive infection, both inserted into the *nef* gene [199]. One potential limitation of the Duo-Fluo virus is the possibility of recombination of the two reporters during reverse transcription due to the matching amino and carboxyl termini [205]. This limitation was recently addressed in a new virus HIV_{GKO}, whereby mCherry was replaced with Kusabira orange (mKO2) and GFP was switched to a codon-switched GFP protein (csGFP) [204].

In order to enhance infectivity in primary cells and to enhance the detection of latent virus established by direct infection (pre-activation latency), we designed a new dual-fluorescent reporter virus. We modified Duo-Fluo and restored *env* and *vpr* to wild type sequences and changed the reporter proteins to GFP (latent) and E2 crimson (productive infection), naming it DuoAdvance. Following direct infection of resting cells stimulated with CCL19 [102, 192], we found low constitutive expression of GFP (the latent reporter), despite successfully establishing latent infection. We conclude that dual-fluorescent reporter viruses may underestimate the frequency of latently infected cells in resting CD4⁺ T-cells.

2.3 Methods

2.3.1 Cell line culture

The Jurkat T-cell line was cultured in RPMI-1640 (RF10) and human embryonic kidney (HEK) 293T cells and TZM-bl cells (cell lines: NIH AIDS Reference Reagent Program) were cultured in Dulbecco's modified eagle medium (DMEM10) at 37°C, 5% CO₂. All media was supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin and 29.2mg/ml L-glutamine.

2.3.2 Preparation of E2 crimson fluorescent protein plasmids, NL4.3-EGFP

E2 crimson fluorescent plasmid, NL4.3-EGFP and Duo-Fluo plasmids were transformed in Stbl-2 cells and plated on Luria broth (100µg/ml ampicillin) or terrific broth (TB) (100µg/mL kanamycin for Duo-Fluo). Single colonies were cultured for 16hrs and plasmids prepared using QIAprep Spin Miniprep Kits (QIAGEN, Hilden, Germany) as per manufacturer instructions.

2.3.3 DuoAdvance plasmid generation

A 6668bp DNA plasmid insert containing the modified DuoAdvance sequence was synthesized by GenScript (GenScript, Piscataway, NJ). The DuoAdvance DNA plasmid insert was ligated into the Duo-Fluo plasmid using T4 DNA ligase. Plasmids were prepared using a Maxi Kit (QIAGEN, Hilden, Germany) and ligation confirmed using combined *NdeI* and *PacI* digestion for 2.5hrs at 37°C followed by 1hr at 50°C with *BssHII*, resolved on a 1.2% gel.

2.3.4 Preparation of virus stocks and determining infectivity

Viral stocks of DuoAdvance, Duo-Fluo and NL4.3-EGFP were prepared from transfection of HEK293T cells with each respective plasmid, concentrated and used in all experiments as previously described [102].

The infectivity of viral stocks was performed using a TZM-bl assay. TZM-bl cells were plated and infected with 10-fold dilutions of viral stocks in triplicate. Negative and positive control wells using media and a reference virus with known infectivity were also included, respectively. 50% tissue culture infectious dose (TCID₅₀) was measured using luciferase readout and calculated using the Reed-Muench method [458].

2.3.5 Infection of Jurkat T-cell line

Jurkat T-cells were infected with concentrated DuoAdvance virus, Duo-Fluo virus, NL4.3-EGFP virus at a TCID₅₀ of 0.5 in an infection volume of 100µl. The integrase inhibitor Raltegravir (1µM) or non-nucleoside reverse transcriptase inhibitor Nevirapine (10µM) (Selleck Chemicals, Houston, TX) were added to cells 30mins before infection and then maintained in cultures throughout the remaining experiment. Cells were infected for 2hrs, washed, cultured for 3 days and then fixed in 1% formaldehyde for flow cytometry. Samples were analysed on a BD LSR Fortessa using FACSDIVA software (BD Biosciences, San Jose, CA). Data was analysed using FlowJo v10 (FlowJo LLC, Ashland, OR).

2.3.6 Isolation and infection of resting CD4+ T-cells

Peripheral blood mononuclear cells (PBMCs) and resting CD4+ T-cells were isolated from whole blood from healthy donors as previously described [192] and cultured in three different conditions; unactivated, 100nM recombinant human CCL19/MIP-3β (In Vitro Technologies, Vic, AUS) treated and PHA/IL-2 activated. Untreated and treated primary CD4+ T-cells were typically infected with 100µl of DuoAdvance or NL4.3-EGFP (both at TCID₅₀ 0.5-1) per 1x10⁶ cells for 2hrs. Cells were also spinoculated (1200xg, 90mins, room temperature) followed by a further 30min incubation. Cells were then washed and resuspended in RF10 plus 1U/mL IL-2 and cultured 6 days.

For reactivation studies, the E2CRM- fraction of cells were sorted out from CCL19 treated CD4+ T-cells infected with DuoAdvance using a BD FACS Aria Fusion (BD, Franklin Lakes, NJ) and then reactivated by the addition of 10µg/ml PHA+10nM PMA, or DMSO diluent negative control, for 3 days in the presence of 1µM raltegravir. After reactivation, cells were fixed in 1% formaldehyde and run on a BD LSR Fortessa using bandpass filters for E2CRM (670/30 Yellow) and GFP (530/30 Blue) plus FACSDIVA software. Results were analysed using FlowJo v10.

2.3.7 Data Analysis

FlowJo results were graphed using GraphPad Prism (version 7, GraphPad Software, La Jolla, CA). Statistical testing was not appropriate as the small sample size (n<6) precluded the use of non-parametric tests. Due to the small sample size, testing normality cannot be performed, thus

parametric t-tests were also not used. Additionally, as the percentage fluorescence data points appeared skewed, this further suggests that a parametric t-test was not appropriate. Therefore, we have only reported the mean, range and fold-difference between means.

2.4 Results and Discussion

2.4.1 DuoAdvance HIV reporter virus can establish latent and productive infection in a T-cell line

We first compared infection of the Jurkat T-cell line using DuoAdvance, the original Duo-Fluo (Fig 1A) and NL4.3-GFP as a comparison for GFP productive infection reporter expression. Cells were infected at 0.5 TCID₅₀ per cell as titration studies found comparable levels of latent infection of CCL19-treated CD4⁺ T-cells using DuoAdvance at 0.5-2 TCID₅₀ after 72 hours (data not shown). Following infection of the Jurkat T-cell line with DuoAdvance, latently infected (GFP+E2CRM-) and productively infected cells (E2CRM+) were detected (Fig 1). All E2CRM+ cells were considered productively infected irrespective of GFP expression. The mean fluorescence intensity of GFP as a latency reporter following DuoAdvance infection [mean(range)=8.43(3.92-12.60)%] was below the intensity observed after infection with NL4.3-EGFP where GFP is a productive reporter [mean(range)=21.70(14.40-28.40)%] (Fig 1B, D). Infection with DuoAdvance and Duo-Fluo (Fig 1A) resulted in a mean frequency of 8.43% (range=3.93-12.60%) and 5.02% (range=4.36-5.62%) latently infected cells respectively (Fig 1B-E). The number of productively infected cells was also comparable between both viruses (Fig 1D-E). Nevirapine or raltegravir successfully inhibited infection of Jurkat T-cells by both viruses (Fig 1B-E). As DuoAdvance and Duo-Fluo were comparable in their infection of the Jurkat T-cell line, DuoAdvance therefore showed promise as a new dual-fluorescent reporter virus.

2.4.2 Minimal constitutive expression of EF1 α following infection of resting cells

We next used DuoAdvance to infect CCL19 treated and PHA/IL-2 activated CD4⁺ T-cells. E2CRM was detected in both models and this declined when nevirapine was added to the cell culture prior to, during and after infection. As expected, the frequency of E2CRM+ cells was higher in PHA/IL-2 activated [mean(range)=2.55(0.83-5.14)%] compared to CCL19 treated cells [mean(range)=0.11(0.01-0.28)%] (Fig 2A, C and D). Low frequencies of GFP (latent infection) were

detected in both CCL19 treated [mean(range)=0.01(0.00-0.01)%] and PHA/IL-2 activated CD4+ T-cells [mean(range)=0.034(0.02-0.05)%] (Fig 2A-B). These GFP frequencies were higher than mock infection and when infection was performed in the presence of nevirapine. Spinoculation of the DuoAdvance virus at the time of infection of primary T-cells was also tested to enhance viral binding and infection [105] however we found little change in the number of infected cells following spinoculation (Fig 2B and C, closed symbols) or when we increased the TCID₅₀ from 0.5 to 1 (data not shown) to enhance infection.

The low frequency of latently infected cells observed following infection of primary resting CD4+ T-cells with DuoAdvance may be explained by inefficient expression of the EF1 α -driven GFP latent reporter in primary resting CD4+ T-cells, in contrast to expression in the Jurkat T-cell line (Fig 1). Promoter silencing may account for the low expression levels in resting CD4+ T-cells compared to the actively dividing Jurkat T-cell line. A key feature of resting CD4+ T-cells is sequestration of transcription factors NF- κ B and nuclear factor of activated T-cell (NFAT) [459]. These transcription factors are important for the initiation of transcription of HIV and may also be important for initiation of transcription of the latent GFP reporter from the EF1 α promoter. The downregulation of antiretroviral defence genes such a SAM domain and HD domain-containing protein 1 (SAMHD1) and guanylate binding protein 5 (GBP5) in Jurkat T-cells could contribute to the success of viral infection in cell lines compared to primary CD4+ T-cells, where these genes are not downregulated [460]. Additionally, the recognition of CG dinucleotides in viral RNA by the cellular zinc-finger antiviral protein (ZAP) could have contributed to depleted cytoplasmic viral RNA and impaired viral expression [461].

2.4.3 Inducible virus is detected in E2CRM negative cells

We next performed a reactivation experiment (Fig 3A) to test whether the E2CRM- cells at 6 days post-infection with DuoAdvance contained inducible virus (consistent with latent infection), even if there was poor constitutive expression of GFP. Using cells from different donors, at six days post-infection with DuoAdvance, low levels of GFP expression were again detected in all conditions [unactivated cells: mean(range)=0.19(0.00-0.52)%; CCL19 cells: mean(range)=0.29(0.01-0.69)%; PHA/IL-2 cells: mean(range)=0.37(0.05-0.78)%] that was higher than mock infection [mean(range)=0.01(0.00-0.01)%] (Fig 3B and C *left panel*). There was also an

increase in E2CRM productive infection in all conditions compared to mock infection [unactivated cells: mean(range)=1.17(0.00-2.44)%; CCL19 cells: mean(range)=1.83(0.01-3.52)%; PHA/IL-2 cells: mean(range)=5.21(0.10-14.30)%] (Fig 3B and C *right panel*). Infection of PHA/IL-2 activated cells resulted in the highest levels of latent and productive infection (Fig 3B-C).

To test for inducible virus, the E2CRM⁻ population was isolated on day 6 from CCL19-treated infected cells and reactivated with PMA/PHA for 3 days. PMA/PHA led to an increase in the number of E2CRM⁺ cells **3.1-fold** versus the DMSO diluent control (Fig 3D). This revealed that the E2CRM⁻ cells sorted 6 days post-infection harboured inducible virus, even though these cells had low to absent GFP expression prior to stimulation. Interestingly, the level of GFP+E2CRM⁻ latent cells also increased **2.4-fold** with PMA/PHA reactivation compared to the DMSO diluent control (Fig 3D), suggesting that the latent reporter may need the cell to be in an activated state to effectively express GFP protein. We don't believe that this increase in GFP was a result of integration of unintegrated DNA following T-cell activation as reactivation experiments were performed in the presence of an integrase inhibitor.

In our experiments, the expression of the EF1 α -driven GFP clearly increased following T-cell activation. It is worth noting that we were unable to determine if the inefficient expression of GFP in resting T-cells was a feature of the EF1 α promoter itself or a consequence of the EF1 α promoter in the background of the DuoAdvance construct. However, we propose that the use of the EF1 α promoter in a dual reporter virus might significantly underestimate the frequency of latently infected cells, particularly when using a pre-activation model of resting CD4⁺ T-cell latency.

In activated primary CD4⁺ T-cells low expression of the latent reporter GFP was observed despite efficient expression of the HIV LTR-driven E2CRM (Fig 2) might be explained by promoter competition. This can occur when a promoter that has a higher affinity for a limiting factor competitively inhibits transcription from an adjacent promoter [462]. For example, once the HIV LTR becomes activated by Tat, there may be preferential recruitment of transcription factors to the HIV LTR promoter driving E2CRM expression at the expense of the EF1 α promoter and GFP expression, thereby suppressing GFP and resulting in a higher proportion of E2CRM⁺ cells. Similar

promoter competition has been reported in other model systems such as human embryonic stem cells transduced with a dual-promoter vector [463] and HeLa cells co-transfected with HIV and HIV Rev binding aptamer under the control of the human cytomegalovirus (CMV) promoter [464].

Collectively, these experiments demonstrate that while DuoAdvance can successfully infect the Jurkat T-cell lines and establish latent and productive infection, following infection of primary CD4⁺ T-cells, only E2 Crimson expression was detected and there was poor constitutive expression of the EF1 α promoter as detected by GFP expression. Expression of E2 Crimson and GFP could be induced following activation of sorted E2CRM- CCL19 CD4⁺ T-cells suggesting integration did occur in these cells, but GFP had poor constitutive expression and was not expressed in all cells that contained inducible virus.

Interestingly, Chavez and colleagues noted using Duo-Fluo, that in activated T-cells returning to a resting state, the expression of both latent and productive infection fluorescent markers were progressively lost over time [100]. The second generation HIV_{GKO} variant of Duo-Fluo has only been used to infect activated T-cells to date and expression of the new EF1 α -driven mKO2 latency reporter has not yet been examined in resting CD4⁺ T-cells [204]. Another dual-fluorescent reporter virus (Hlfate-E) has some similarities to DuoAdvance [206]. In this construct the latency reporter was changed to GFP and the productive reporter to E2 Crimson and a composite EF1 α -HTLV-1 promoter was used in place of the EF1 α promoter to drive the GFP latency reporter. Following infection of activated CD4⁺ T-cells with Hlfate-E, insufficient latently infected cells were generated to assess the response to further activation [206], suggesting a very low yield of latently infected cells despite successful infection of activated CD4⁺ T-cells similar to this study. Further investigation with these two new viruses, HIV_{GKO} or Hlfate-E, in direct infection of resting CD4⁺ T-cells will elucidate whether these viruses are suited to studying HIV latency in pre-activation models.

Developing a sensitive and specific marker of latently infected cells using an in vitro model of a dual reporter virus to infect primary CD4⁺ T-cells is of high interest as this would allow for examination of host gene changes following latency using techniques such as scRNAseq as these studies can't be performed with immortalised cell lines, and activation of the cell to express viral

proteins changes the host transcriptome, as recently reported [88]. If latency is defined by GFP expression but not all latently infected cells express GFP, this would underestimate the frequency of latently infected cells. If these cells were being used to screen for LRAs, this would potentially overestimate the efficiency of a particular LRA. In addition, screening would miss latently infected cells that don't react to an LRA.

Finally, adding an internal ribosome entry site (IRES) between the EF1 α promoter and the latency reporter might lead to the improved expression of the latency reporter in resting CD4+ T-cells by enhancing the reporter gene activity as previously described [465]. This approach could be pursued to drive better expression of the latency reporter in resting CD4+ T-cells to study HIV latency and reactivation in resting CD4+ T-cells [34].

A limitation of this study was the number of donors analysed. We examined 6 or fewer donors for each experiment and therefore could not determine whether the data was normally distributed. In preference to using parametric tests, as is often used for small samples, we have only reported the mean fold changes in different experimental conditions and not reported p values.

2.5 Conclusions

We generated a modified dual-fluorescent reporter virus that was able to successfully infect a Jurkat T-cell line and generate latent and productively infected cell populations. However, DuoAdvance resulted in low levels of expression of the latency reporter in resting CD4+ T-cells, using a pre-activation model of HIV latency. As current dual-fluorescent reporters are usually studied by infecting activated CD4+ T-cells [100, 199, 204], and expression of latent and productive infection reporters can be progressively lost as activated cells return to a resting state [100], dual-fluorescent reporter viruses may significantly underestimate the frequency of latently infected cells *in vitro*.

Competing interests

SRL has received investigator-initiated, industry funded research support from Merck Sciences, Gilead Sciences and ViiV, and provision of reagents from Infinity Pharmaceuticals, Merck Sciences

and BMS for investigator-initiated research. SRL and JA have research collaborations with Merck Sciences unrelated to this work.

Authors' contributions

Y.K., S.R.L., and J.L.A. designed the research study and Y.K. and J.L.A. performed the research. Y.K., P.U.C. and J.L.A analysed the data. Y.K., S.R.L and J.L.A wrote and edited the paper. All co-authors have read and approved the paper.

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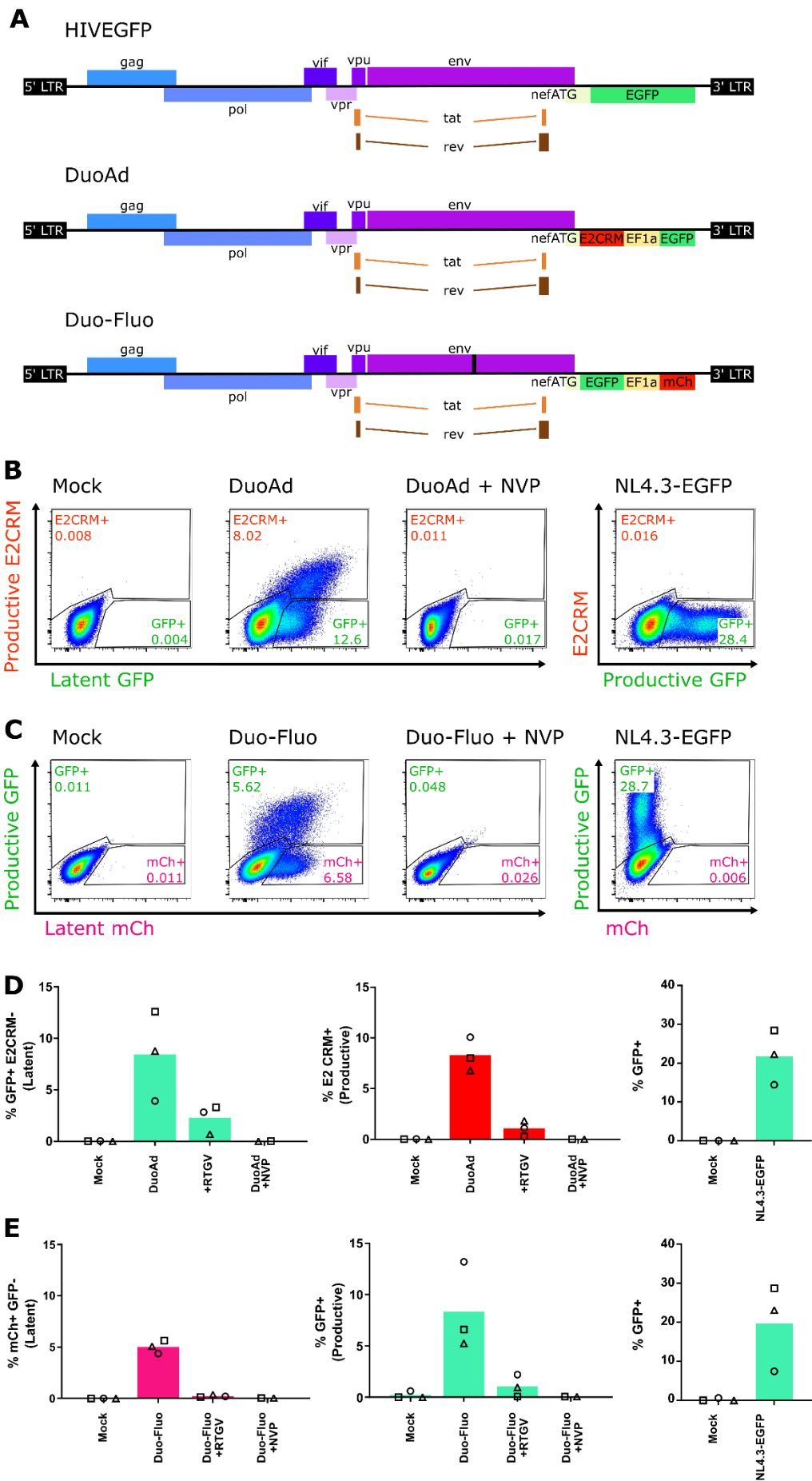


Figure 2.1. DuoAdvance infection of the Jurkat T-cell line generates latent and productively infected cells similar to Duo-Fluo

(A) Schematic of HIVEGFP (top), the DuoAdvance (DuoAd) dual-fluorescent reporter construct virus (middle) and Duo-Fluo dual-fluorescent reporter construct (bottom). DuoAd contains an EF1 α promoter driven enhanced green fluorescent protein (EGFP) marker for latent cells and an E2 Crimson (E2CRM) marker under the control of the HIV LTR that serves as the productive marker. This construct also contains wildtype HIV env and vpr sequence. Jurkat T-cells were infected with either DuoAd (B, D) or Duo-Fluo (C, E) for 3 days in the presence or absence of nevirapine (NVP) or raltegravir (RTGV) and expression of EGFP (GFP), E2CRM or mCherry (mCh) quantified by flow cytometry. NL4.3-EGFP virus was used as a positive control for productive infection and expression of GFP (B-E). Representative flow plots and gates are shown in B and C. In D-F, each symbol represents an independent experiment and columns represent the mean.

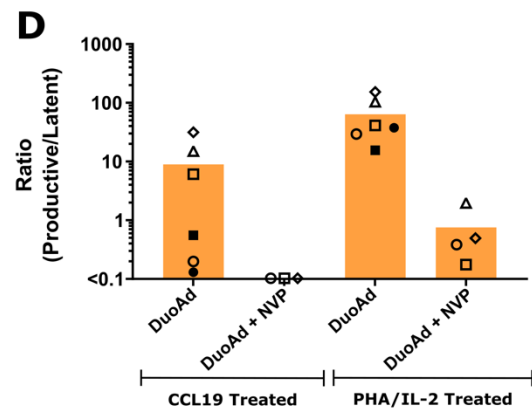
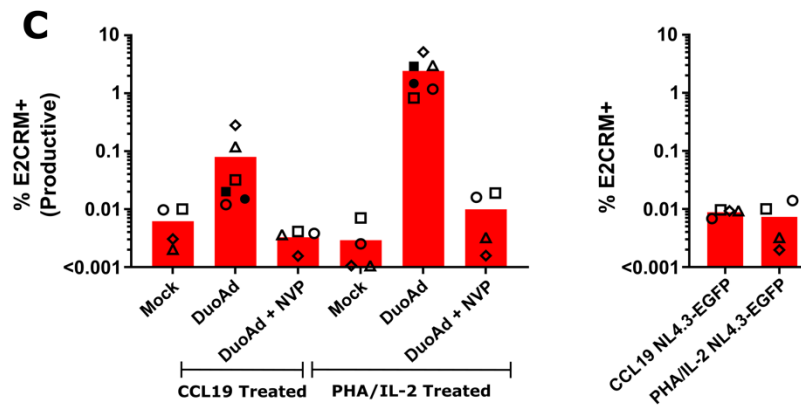
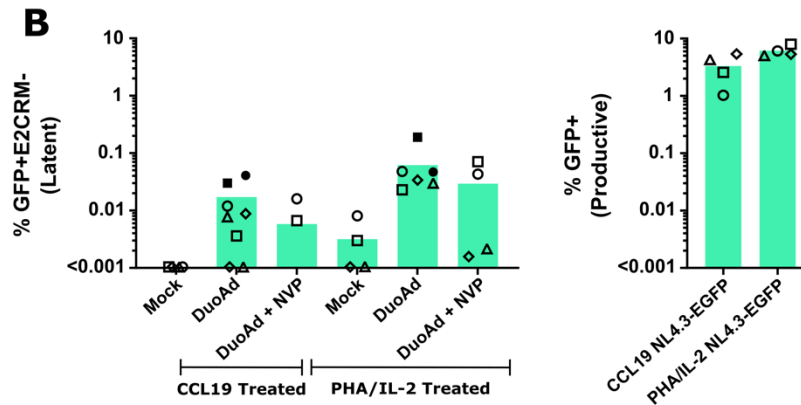
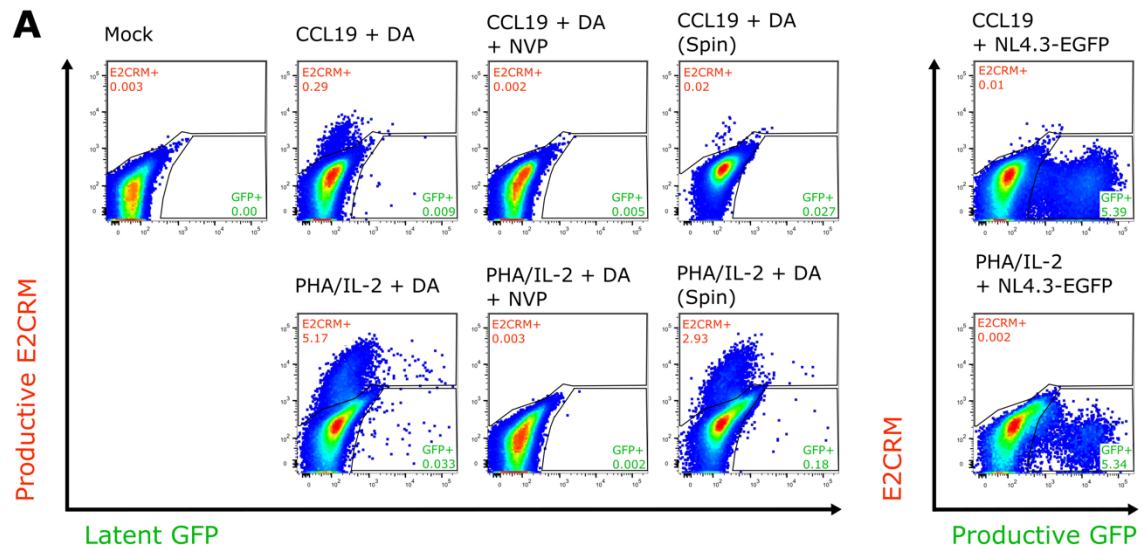


Figure 2.2. DuoAdvance can infect activated and resting primary CD4+ T cells

DuoAdvance (DuoAd, DA) was added to CCL19-treated or PHA/IL-2 activated primary CD4+ T-cells at a TCID₅₀ of 0.5 for 2hrs, cells washed and cultured a further 6 days. Uninfected cells (mock), cells infected with DuoAd in the presence of Nevirapine (NVP) to block infection, and cells infected with 0.5 TCID₅₀ NL4.3-EGFP as a comparative control were included. In some experiments, spinoculation (spin, closed symbols) was included. (A) FACS dot plots from a representative experiment is shown and includes the gating used to determine GFP+E2CRM- (latent) and E2CRM+ (Productive) cells. (B, C) Resulting levels of GFP+E2CRM- latent and E2CRM- productive cells from n≤6 experiments are shown. On a separate graph, the levels of GFP+ (productive infection reporter) and E2CRM+ (background) from the NL4.3-EGFP infection positive control are also shown. (D) The ratio of productive to latent infection was calculated using the data from panel B and C, with fluorescence background in mock sample subtracted from the DuoAd infected samples before calculating productive to latent infection ratio. Each symbol represents a different donor and the height of the column represents the mean of the experiments.

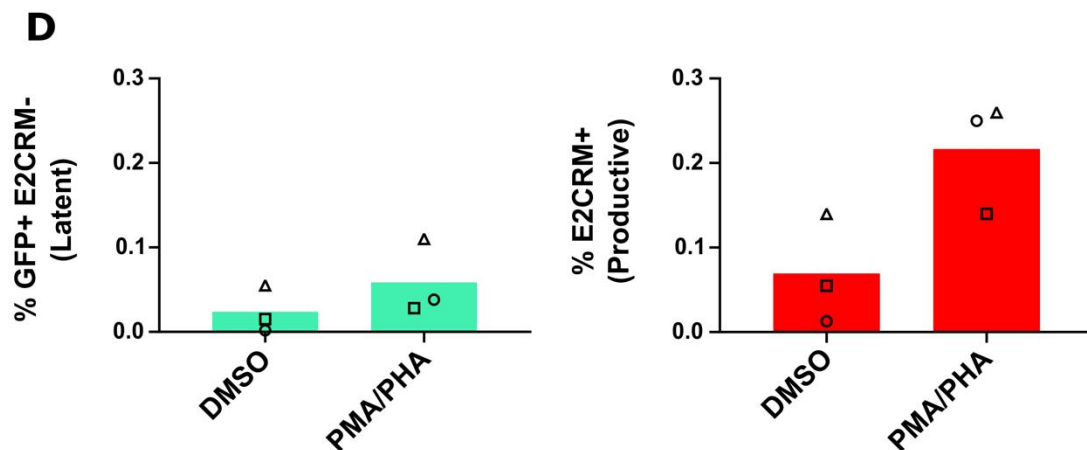
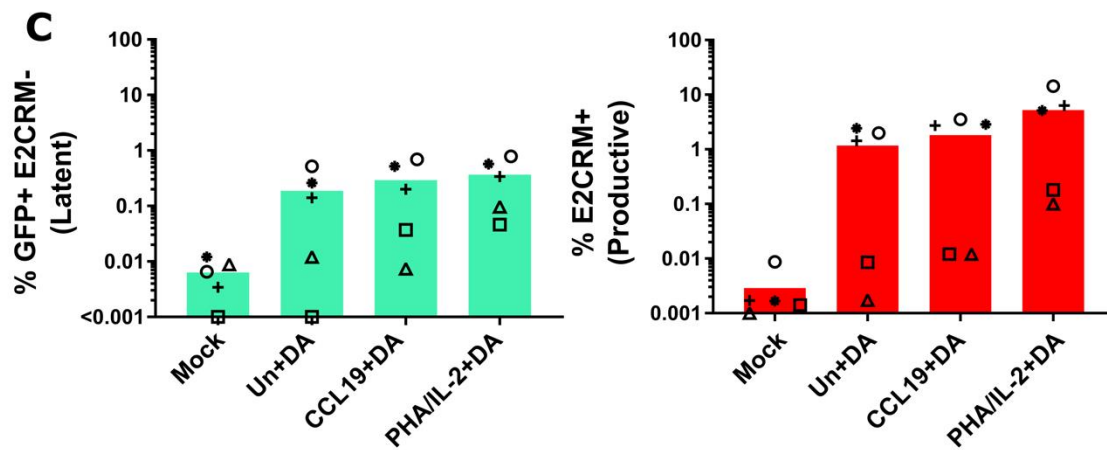
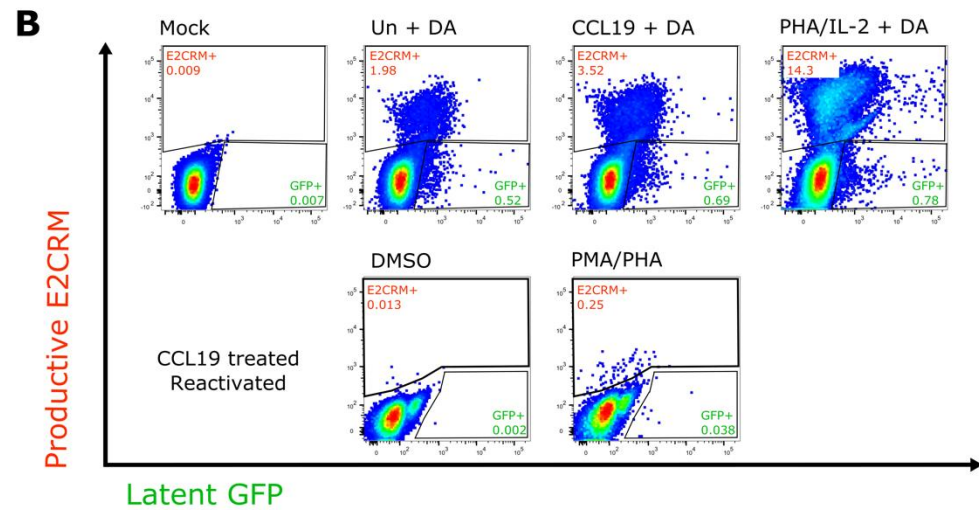
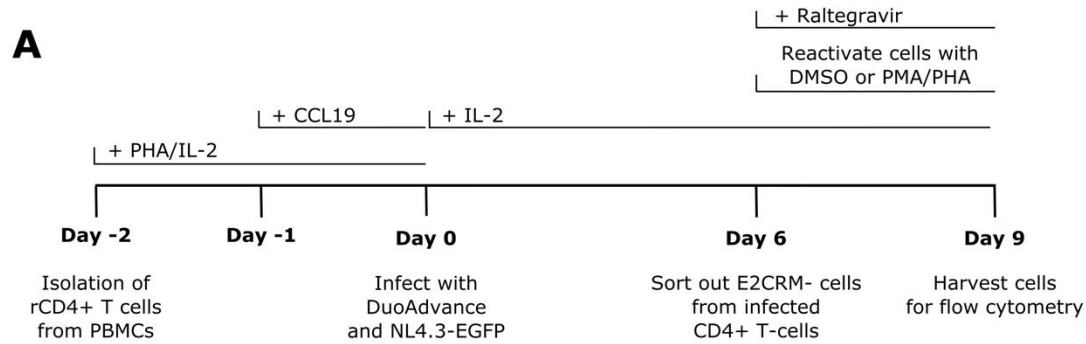
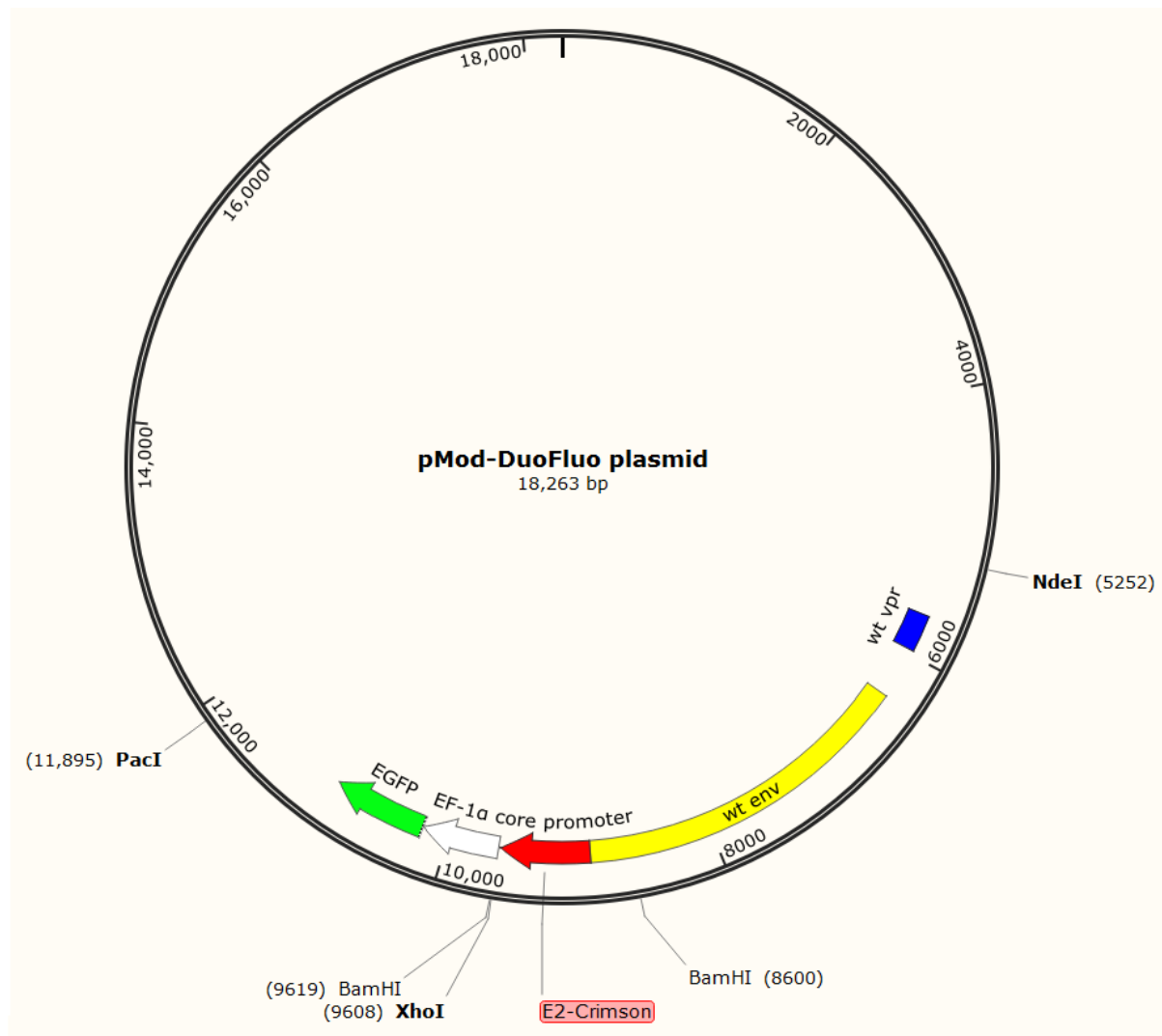


Figure 2.3. E2 Crimson negative population of cells harbour inducible latent virus

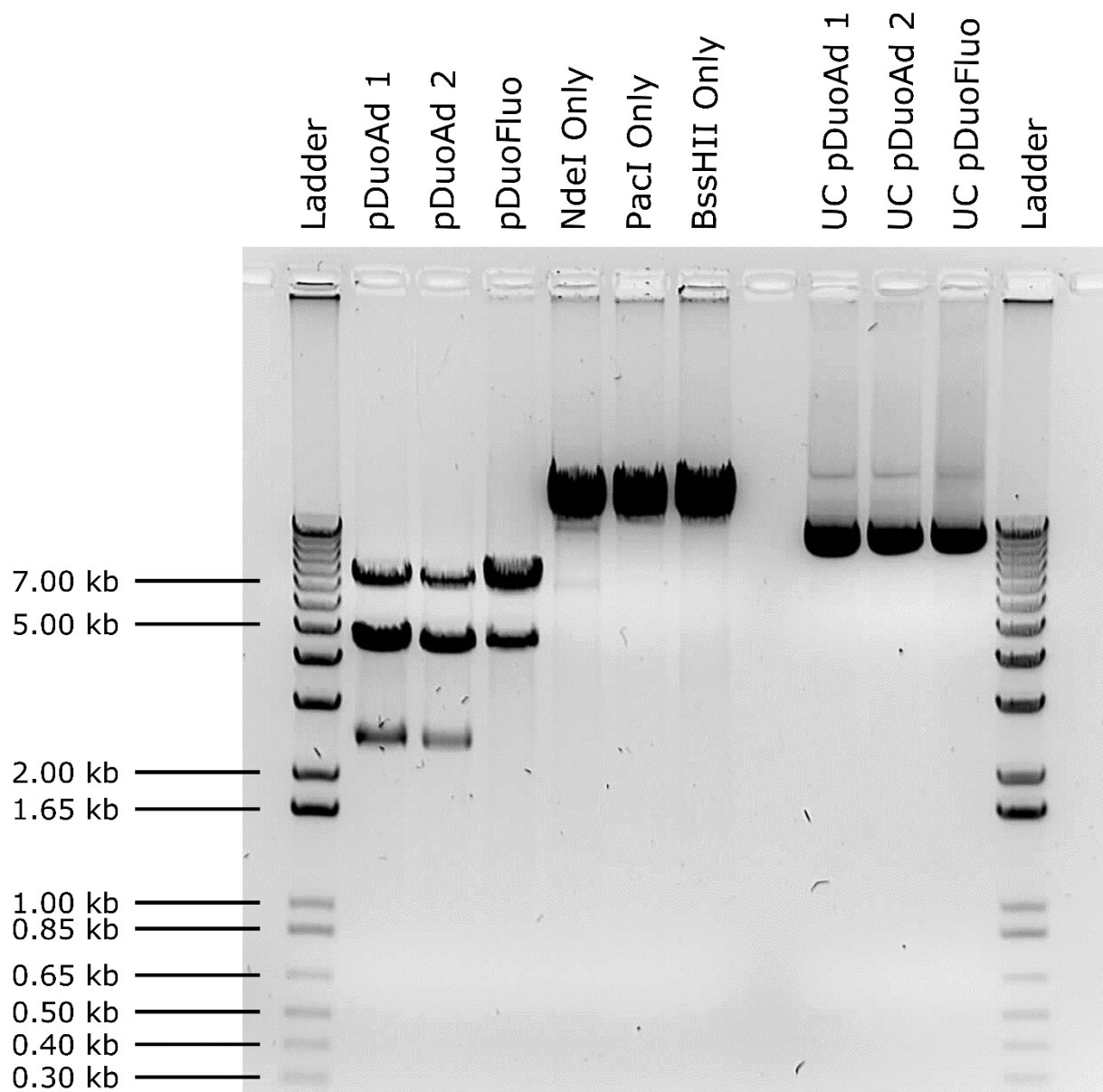
CD4⁺ T-cells were isolated from PBMC and left resting (unstimulated, Un) or stimulated with either CCL19 or PHA/IL-2 and then infected with either DuoAdvance (DA) virus or media only (mock). After 6 days, expression of both fluorescent proteins was quantified by flow cytometry (B, C, D). The E2 Crimson negative cell population (cells that were not present in the E2CRM⁺ gate in B top panel) was sorted from the CCL19 treated samples six days after infection and then stimulated with either DMSO or PMA/PHA in the presence of 1 μ M raltegravir and expression of both fluorescent proteins quantified by flow cytometry (D). Each symbol represents a different donor and the column represents the mean.

2.6 Supplementary Figures



Supp Figure 1.1. Schematic representation of the modified construct including restriction sites used to move the CCL19 resting CD4⁺ T-cell model into a high-throughput screen

The modified Duo-Fluo I reporter virus has four changes from the original construct. The first and second modifications remove the mutation in env and vpr for expression of wildtype protein. Thirdly, the EGFP fluorescent marker has been changed to the latent reporter, driven by EF1α promoter. Finally, the productive infection marker has been changed to E2 Crimson. Upon infection, latently infected cells will only express GFP whereas productively infected cells will express E2 Crimson and EGFP. Uninfected cells will not express any fluorescent proteins. An insert spanning from the NdeI restriction site to the PacI restriction site was synthesized and cloned into the backbone of the original Duo-Fluo plasmid.



Supp Figure 2.2. Restriction enzyme and gel electrophoresis analysis of DuoAdvance plasmid clone

DuoAdvance (DuoAd) plasmid was synthesized and cloned into the analogous region of the original Duo-Fluo plasmid using *NdeI* and *PacI* restriction enzymes. The ligated plasmid was transformed into *Stbl-2* *E.coli* cells and individual clones cultured, plasmids extracted and digested using *NdeI*, *PacI* and *BssHII* restriction enzymes and then run on an agarose gel electrophoresis to identify plasmid clones where the DNA insert had ligated into the Duo-Fluo backbone in the correct orientation to generate DuoAdvance. Two DuoAdvance clones (pDuoAd 1 and pDuoAd 2) with the correct orientation by restriction enzyme analysis are

shown. pDuoAd 1-plasmid DuoAdvance Prep1; pDuoAd 2-plasmid DuoAdvance Prep2; pDuoFluo - plasmid Duo-Fluo original; NdeI Only- plasmid DuoAdvance Prep1 cut with NdeI enzyme; PacI Only- plasmid DuoAdvance Prep1 cut with PacI enzyme; BssHII Only- plasmid DuoAdvance Prep1 cut with BssHII enzyme; UC pDuoAd 1- uncut plasmid DuoAdvance Prep1; UC pDuoAd 2- plasmid DuoAdvance Prep2; UC pDuoFluo - Uncut plasmid Duo-Fluo original; Ladder- Life Technologies 1Kb plus DNA ladder.

Chapter 3

**Ability of pro-apoptotic drugs and LRAs to
reactivate and kill HIV latently infected T-cells**

Chapter 3

Ability of pro-apoptotic drugs and LRAs to reactivate and kill HIV latently infected T-cells

3.1 Abstract

One strategy to eliminate latently infected CD4⁺ T-cells in PLWH on ART is to use LRAs to reactivate virus expression. However, clinical trials with LRAs have not led to a decrease in the frequency of infected cells or time to viral rebound once ART is stopped. Thus, additional strategies to kill reactivated cells are needed. Phosphoinositide-3 kinases (PI3K) are lipid kinases that act as secondary messengers in numerous intracellular signalling pathways. PI3K promote cell survival by preventing apoptosis. Thus, inhibitors of PI3K (PI3Ki) could promote cell death. Additionally, Bcl-2 is an anti-apoptotic cellular protein expressed in T-cells. Therefore, inhibitors of Bcl-2 (Bcl-2i) could also sensitize cells towards death. Numerous HIV proteins, such as viral envelope, protease and high levels of Vpu and Tat can promote apoptotic pathways. The expression of these proteins in a reactivated cell could result in cell death. We hypothesised that treatment with pro-apoptotic drugs to increase the sensitivity of latently infected cells to apoptosis, in combination with LRAs to drive the expression of pro-apoptotic viral proteins, will tip the balance towards apoptosis and ultimately lead to the clearance of these cells. In this chapter, we demonstrate that treatment of CD4⁺ T-cells from PLWH on ART *ex vivo* with the LRA romidepsin combined with one of three different PI3K inhibitors or a Bcl-2 inhibitor led to larger declines in levels of HIV integrated DNA versus either drug alone. Additionally, panobinostat or JQ1 combined with Bcl-2 inhibitors could clear latently infected T-cells taken from PLWH on ART. Taken together, the data suggests that sensitisation of latently infected CD4⁺ T-cells with pro-apoptotic compounds combined with latency reversal to drive expression of viral pro-apoptotic proteins can enhance the clearance of latently infected cells.

3.2 Introduction

The persistence of long-lived latently infected CD4⁺ T-cells during ART is the main barrier to finding a cure for HIV [29, 457, 466]. These cells evade the immune system as well as clearance

by ART, and thus present as a reservoir of HIV within infected individuals. Latency refers to a state of transcriptionally silent replication arrest [27] and the absence of viral proteins allows the latently infected cell to be indistinguishable from uninfected cells, thereby evading the immune system. When ART is stopped, HIV virus rebounds rapidly [466]. In order to purge the latently infected cell reservoir, multiple eradication studies are being explored. The prevailing strategy and perhaps the most defined and studied, is the “shock and kill” approach [213]. Once the latently infected cell has been reactivated through the administration of LRAs, which constitutes the “shock”, the once latent cell is no longer transcriptionally silent and actively transcribes and translates HIV RNA and viral proteins, which may lead to the production of newly formed viral particles. The subsequent “kill” was proposed to arise from cytolysis as a result of reactivation of viral protein expression, or through viral protein expression making the cells visible for immune-mediated clearance [214].

Notable classes of LRAs include epigenetic modifiers such as HDACi, P-TEFb activators and bromodomain inhibitors; PKC agonists; TLR agonists and T-cell receptor activators [214]. The administration of these LRAs to PLWH on ART *in vivo* resulted in transient increases in HIV RNA transcription in all cases except for the PK C agonist bryostatin [270]. However, they did not lead to a decrease in HIV DNA, and by extension the latent HIV reservoir (reviewed in [467]). Thus, there is need to understand why these LRAs alone have not led to declines in infected cells in PLWH on ART. Importantly, it is critical to identify complimentary strategies that enable the death of the cells once they have been reactivated.

One understudied approach to clear the latently infected cells once reactivated is to use pro-apoptotic drugs to activate cell death via the apoptotic pathway. Apoptosis is a cellular process that results in clearance of damaged or unwanted cells [398]. Apoptotic pathways can be initiated by intracellular stimuli such as cellular stress, DNA damage and radiation [393], converging at the stage of caspase activation, leading to the caspase cascade and ultimately cell death by apoptosis. This programmed method of cell death is tightly regulated by numerous families of proteins such as the Bcl-2 family, which contain both pro-apoptotic and anti-apoptotic proteins [397] and the PI3K family of proteins [441]. The intracellular balance

of pro-apoptotic and anti-apoptotic factors likely determines whether cells live or die by apoptosis.

The Bcl-2 protein is an anti-apoptotic protein that binds and sequesters pro-apoptotic proteins such as Bak and Bim [397]. Antagonists to Bcl-2 exert their function by binding with higher affinity to Bcl-2 proteins than the pro-apoptotic proteins [426]. Studies of Bcl-2 antagonists/inhibitors have shown that administration of the compound venetoclax can sensitise cancerous cells towards apoptosis [427]. More recently, venetoclax has been shown to clear HIV latently infected T-cells from PLWH on ART *ex vivo* when combined with T-cell activation as an LRA [412], serving as a precedent that combining pro-apoptotic drugs and LRAs can clear HIV latently infected cells. More clinically suitable LRAs need to be tested for their ability to deplete HIV latently infected T cells when combined with a Bcl-2 inhibitor like venetoclax as T-cell stimulation as an LRA is toxic [215, 216].

PI3K control a range of intracellular signalling pathways in leukocytes. Akt is the main effector of the PI3K pathway and binding of this protein to the products of PI3K can result in activation of Akt, resulting in direct or indirect inhibition of apoptosis [434]. PI3K/Akt inhibitors combined with cellular stress to induce apoptosis in HIV infected macrophages and microglial cells was demonstrated to activate apoptosis in these infected cells [439, 441]. Whether selective PI3K inhibitors have similar activities on HIV latently infected T-cells stimulated with LRAs has yet to be fully elucidated and thus, will be examined in this thesis.

HIV proteins can affect the apoptotic pathways, having anti-apoptotic and/or pro-apoptotic functions. In particular, late stage HIV proteins expressed during productive replication such as HIV Env, Vpu, protease, high levels of Tat and Vpr can promote apoptosis through a variety of mechanisms. HIV protease is crucial in the production of the pro-apoptotic peptide Casp8p41, unique in the depletion of HIV infected T-cells [468, 469]. The anti-apoptotic Bcl-2 protein can sequester this peptide to inhibit the progression of apoptosis, but venetoclax can release the peptide from Bcl-2 [412], freeing the peptide to continue with the progression of apoptosis. Thus, as HIV proteins expressed later in productive replication can induce apoptosis, we hypothesised that using LRAs to reactivate the expression of the pro-apoptotic

viral proteins from latent infection, combined with a pro-apoptotic drug to increase cell sensitivity towards apoptosis, will drive the death of HIV latently infected T-cells. Such drug combinations may eventually lead to clearance of the HIV reservoir in PLWH on ART.

Based on this hypothesis, here we examined and observed that novel combinations of pro-apoptotic drugs together with LRAs can successfully deplete latently infected cells. Specifically, romidepsin combined with one of three different PI3K inhibitors or the Bcl-2 inhibitor venetoclax could reactivate latent HIV and effectively eliminate these cells compared to either drug alone.

3.3 Materials and Methods

3.3.1 Compounds

The following pharmaceuticals were dissolved in dimethyl sulfoxide (DMSO) to make stock concentrations stored at -80°C: 10mM vorinostat (suberoylhydroxamic acid; SAHA or VOR) (Selleck Chemicals, Houston, TX), 10mM panobinostat (PNB; LBH589) (Selleck Chemicals, Houston, TX), 10mM romidepsin (RMD; FK228) (Selleck Chemicals, Houston, TX), 10mM venetoclax (VNX; ABT-199) (Selleck Chemicals, Houston, TX), 10mM wortmannin (WN; KY12420) (Sigma-Aldrich, St. Louis, MO), 1mM PMA (Sigma-Aldrich, St. Louis, MO), 1mM raltegravir (RGTV) (Selleck Chemicals, Houston, TX) and 10mM disulfiram (DSF) (Selleck Chemicals, Houston, TX). IPI-443 (Infinity Pharmaceuticals, Cambridge, MA) and IPI-3063 (Infinity Pharmaceuticals, Cambridge, MA) were provided by Infinity Pharmaceuticals, constituted in DMSO at a stock concentration of 10mM and stored at -20°C. The following compounds were reconstituted in DMSO and stored at -20°C: 100µM bryostatin (BRY) (Sigma-Aldrich, St. Louis, MO), 1mM JQ1 (Selleck Chemicals, Houston, TX), 1mg/ml phytohaemagglutinin (PHA) (Sigma-Aldrich, St. Louis, MO), 10mM nevirapine (NVP) (Jomar Life Research, Vic, AUS) and 1mM Ionomycin (IONO) (Sigma-Aldrich, St. Louis, MO).

3.3.2 Study Participants

Blood from study participants were provided from the Melbourne Leukapheresis Cohort. Participants were Caucasian males with a mean age of 54 years. Inclusion criteria were that

participants were all on ART for a minimum of three years and had viral load under 20 copies/ml (Supplementary Table 3.1).

3.3.3 Cell culture media and T-cell lines

J-Lat6.3, ACH2, Jurkat T-cells (NIH AIDS Reference Reagent Program) were cultured in RPMI-1640 plus 10% heat-inactivated fetal bovine serum (FBS) (Interpath, Vic, AUS), supplemented with 100U/ml penicillin (Life Technologies, Carlsband, CA), 100µg/ml streptomycin (Life Technologies, Carlsband, CA) and 29.2mg/ml L-glutamine (Life Technologies, Carlsband, CA) (RF10). Human embryonic kidney (HEK) 293T cells and TZM-bl reporter cells (NIH AIDS Reference Reagent Program) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin and 29.2mg/ml L-glutamine (DMEM10). All cells were maintained in a humidified 37°C, 5% CO₂ atmosphere.

3.3.4 Resting CD4+ T-cell isolation from Healthy Donors

3.3.4.1 Isolation of PBMCs from whole blood

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood from healthy donors as previously described [192] using Ficoll-Paque (Amersham Pharmacia Biosciences AB, Sweden) density separation.

In brief, whole blood were diluted in an equal volume of PD and layered over Ficoll-Paque. The samples then underwent density gradient centrifugation at 800xg for 20 minutes at room temperature. The PBMC layer was collected, resuspended in cold PD and then subjected to centrifugation at 500xg for 10 minutes at 4°C. The pellet was resuspended in cold PD and pelleted at 300xg and the above step repeated at 200xg. Finally, the cell pelleted was resuspended in RF10 media and spun down at 50xg for 2 minutes and stored at 4°C until used.

3.3.4.2 Isolation of resting CD4+ T-cells

Resting CD4+ T-cells were isolated from PBMCs using negative bead depletion as previously described [192]. PBMCs were resuspended in a resting CD4 Depletion cocktail consisting of antibodies raised in mice to the following human cell surface markers: anti (α)-CD8 (OKT8 hybridoma; ATCC), α-CD11b (OKM-1 hybridoma: ATCC), α-CD14 (FMC-17 hybridoma: Flinders

Medical Centre, Adelaide, AUS), α -CD16 (3G8 hybridoma: Steinman Lab and Assoc. Prof. Anthony Jaworowski), α -CD19 (FMC-63 hybridoma: Heidi Zola, Flinders Medical Centre, Adelaide, AUS), α -HLA-DR (2.06 hybridoma: Prof. Ken Shortman, WEHI), α -erythrocyte (Gly-A (10FM.N) hybridoma: Prof. Ken Shortman, WEHI) and pure α -CD69 (BD Biosciences). Cells were washed and then resuspended in 120 μ l of goat anti-mouse immunoglobulin (IgG) Miltenyi beads (Miltenyi Biotech, Gladbach Germany) per 1×10^6 cells and incubated for 15 minutes at 4°C. Samples were then sorted in autoMACs running buffer (PD + 1% FBS + 2mM EDTA (Invitrogen, Carlsband, CA)) using an autoMACS Pro Separator (Miltenyi Biotech, Gladbach, Germany) according to manufacturer's instructions. The negative fraction of each donor containing resting CD4⁺ T-cells was resuspended in RF10 for a final concentration of 2×10^6 cells/ml and placed in an incubator at 37°C, 5% CO₂.

3.3.5 Total CD4⁺ T-cell isolation from PBMCs from PLWH on ART

PBMCs were isolated from whole blood taken from PLWH on ART using leukapheresis using Ficoll Histopaque-1077 (Sigma-Aldrich, USA) gradient density centrifugation as above and cells stored in 90% FBS plus 10% DMSO in liquid nitrogen until use. For experiments, PBMCs were thawed in a 42°C water bath and warm FBS added 1:1 in a dropwise manner to frozen cells and then transferred to a larger 50ml Falcon tube. Cryovials were washed with 1ml of warm RF10 and added dropwise to the 50ml Falcon tube. Additional media was added to the tube for a total of 45ml. The tube was inverted carefully, and cells pelleted for 7 minutes at 400xg at room temperature. The supernatant was discarded, and the pellet was resuspended in 45ml of warm RF10 and washed again. The pellet was resuspended in cold PD medium and stored on ice until ready to be used. The number of cells in each 50ml tube was counted using a haemocytometer.

Total CD4⁺ T-cells were sorted from the PBMCs isolated from the blood of PLWH using a Miltenyi Total CD4⁺ T-cell human isolation kit (Miltenyi Biotech, Gladbach, Germany). In brief, thawed PBMCs were pelleted at 300xg for 7 minutes at 4°C and then resuspended in Miltenyi buffer (PD + 1% FBS) at 40 μ l/ 10^7 PBMCs. Biotin-Antibody cocktail was directly added to the cells at 10 μ l/ 10^7 cells, mixed and incubated at 4°C for 5 minutes. Miltenyi buffer at a concentration of 30 μ l/ 10^7 cells was added again. The CD4⁺ T-cell Micro Bead cocktail from

the kit was then added at 20 μ l/10⁷ cells and cells incubated for an additional 10 minutes at 4°C. 10ml of Miltenyi buffer was added to each tube and pelleted at 300xg for 7 minutes at 4°C. The supernatant was discarded, and cells were washed one more time. LS columns (Miltenyi Biotech, Gladbach, Germany) were equilibrated with 3ml of Miltenyi buffer and flow through was discarded. Cell pellets were resuspended in 500 μ l/10⁸ cells and the cell suspension was applied to the column. The flow through containing the unlabelled total CD4⁺ T-cells was collected and pooled into one tube. The unlabelled total CD4⁺ T-cells were pelleted and resuspended in 1ml /10⁶ cells of RF10 containing 1 μ M RTGV and counted for both live and dead cells using trypan blue. Total CD4⁺ T-cells were left overnight in this media to recover at 37°C and 5% CO₂.

3.3.6 Akt Phosphorylation

1x10⁶ resting CD4⁺ T-cell aliquots were treated with different concentrations of PI3K inhibitors: IPI-443 and IPI-3063 for 4 hours or 100nM wortmannin for 1 hour. All samples except the unstimulated control, DMSO control and drugs alone were then activated with 12 μ l PMA (40nM) for exactly 15 minutes at 37°C. Activation was terminated with 300 μ l of warm fixation buffer (BD Biosciences, Franklin, NJ), and incubated for a further 10 minutes. Cells were pelleted at 600xg for 6 minutes at 4°C and supernatant aspirated leaving approximately 50 μ l. 1ml of ice-cold Perm III buffer (BD Biosciences, Franklin, NJ) was added and cells were left to re-hydrate on ice for 30 minutes. Cells were washed in cold PD and then with cold staining buffer (PD + 2% FBS + 1mM final EDTA). 10 μ l Alexa Fluor 647 mouse anti-human phosphorylated Akt antibody (BD Biosciences, Franklin, NJ) was added to each sample and incubated at room temperature for 30 minutes. Cells were washed twice with staining buffer and then fixed with 100 μ l 1% FACs Fix (PD + 1% formaldehyde). Cells were stored at 4°C, protected from light until analysed on a BD LSR Fortessa (BD Biosciences, Franklin, NJ). Data was analysed using FlowJo v10 (FlowJo, BD Biosciences, Franklin, NJ).

3.3.7 J-Lat6.3 T-cell line treatment and stain

J-Lat6.3 T-cells were seeded at a concentration of 0.25x10⁶ cells/500 μ l in a 48 well plate. Three concentrations (100nM, 10nM and 1nM) of both IPI-443 or IPI-3063 were added to the cells and incubated for 48 hours at 37°C and 5% CO₂. Cells were then harvested and stained

with a Live/Dead Fixable Violet Cell Death Stain (Life Technologies, Carlsband, CA) (as per manufacturer's instructions). In a separate set of experiments, after 24 hours of PI3K inhibitor treatment, the LRAs: 1 μ M vorinostat, 30nM panobinostat, 40nM romidepsin, 12.5nM bryostatin or 16nM PMA + 0.5 μ M ionomycin were added and the cells were incubated for an additional 24 hours before being harvested and stained as above. All samples were read on a BD LSR Fortessa using FACs DIVA software and analysed using FlowJo v10 (FlowJo, BD Biosciences, Franklin, NJ).

3.3.8 *Ex vivo* stimulation of total CD4⁺ T-cells

Total CD4⁺ T-cells were seeded at a concentration of 3x10⁶ cells/1000 μ l in RF10 + 1U IL-2 + 1 μ M RTGV. 100nM IPI-443, 100nM IPI-3063, 100nM wortmannin, 5nM venetoclax or 1 μ M venetoclax (collectively termed pro-apoptotic compounds) diluted in a background RF10 + 1U IL-2 + 1 μ M RTGV was added to the cells and incubated for 24 hours at 37°C. After 24 hours, 20nM romidepsin, 30nM panobinostat, 10nM bryostatin or 1 μ M JQ1 was added to each respective well, in the background of pro-apoptotic drug. Four hours after the addition of romidepsin, cells were washed 3 times with RF10 + 1U IL-2 + 1 μ M RTGV, spun at 300xg for 7 minutes at room temperature, resuspended in media that contained pro-apoptotic compounds and incubated a further 20 hours. Twenty-four hours after the addition of panobinostat, bryostatin and JQ1, cells were washed and resuspended as above and incubated a further 48 hours. All samples were then harvested for both RNA and DNA. For the DNA harvest, 1/3 of the total population of cells was pelleted at 350xg for 7 minutes at room temperature. Cells were washed 3 times in 1ml of PD. After the last wash, the supernatant was discarded, and cell pellets stored at -80°C. For RNA, 2/3 of the total population of cells were pelleted at 300xg for 7 minutes at room temperature and washed in PD once. After the supernatant was discarded, 350 μ l of RLT buffer (RNeasy Kit, QIAGEN, Hilden, Germany) containing 1% β -mercaptoethanol (β ME) (Sigma-Aldrich, St. Louis, MO) was added to each sample and vortexed for 10 seconds at top speed. Cells were pulsed to the bottom of the tube at 8000xg for 30 seconds at room temperature. Samples were homogenised through a QIASHredder spin column (QIAGEN, Hilden, Germany) at top speed for 2 minutes at room temperature. The spin column was discarded, the tube was capped and immediately stored at -80°C.

3.3.9 qPCR quantification of HIV Integrated DNA

Stored DNA pellets were thawed at room temperature. 50µl of lysis buffer (10mM Tris HCl pH 8.0, 1mM EDTA, 0.002% Triton-X/1% SDS in H₂O, ddH₂O and 0.8mg/ml Proteinase K) was used to resuspend each cell pellet and the lysate transferred to a sterile 96-well qPCR plate and spun at 1000xg for 2 minutes at room temperature. The plate was then incubated at 56°C for 60 minutes to lyse the cells, followed by a 10 minute incubation at 95°C to inactivate the Proteinase K. The plate was chilled on ice for 5 minutes before being stored at -80°C until ready to use.

Integrated HIV DNA was quantified using a nested qPCR based on a previously described assay [470]. To measure integrated HIV DNA, 5µl of DNA cell lysates were pre-amplified in a 50µl reaction that contained 1x Platinum Taq Buffer (Life Technologies, Carlsband, CA), 50mM Platinum MgCl₂, 2.5mM deoxynucleoside triphosphates (dNTPs) (Promega, Madison, WI), a forward HIV LTR primer ULF1 (refer to Table 3.1 for primer sequences), two human Alu primers (Alu 1 and Alu 2), UltraPure DNase/RNase free distilled water (Invitrogen, Carlsband, CA) and Platinum Taq (Life Technologies, Carlsband, CA). An additional 50µl control reaction was run for each sample that contained all of the above reagents excluding the Alu-LTR primers as a control for amplification from the LTR only primer. The first round of PCR amplification was carried out on a Thermocycler (Eppendorf, Hamburg, Germany) and consisted of: a denaturation step of 8 minutes at 95°C, 12 cycles of amplification (95°C for 1 minute, 55°C for 1 minute and 72°C for 10 minutes), followed by a final step at 72°C for 10 minutes. The amplified DNA was diluted at a ratio of 1:100 in UltraPure water. Then, 5µl of the DNA dilutions were transferred to a new PCR plate for the second-round amplification in a 20µl final reaction volume per well. The LTR forward primer used in the first PCR amplification round contains lambda phage-specific sequence at the 5' terminus of the primer, which adds the lambda sequence to first round PCR products. Therefore, using a lambda phage-specific primer (Lambda T) in the second-round amplification ensures that only products from the first round reaction is amplified [470]. Thus, second round qPCR reactions contained the Lambda T primer together with a primer specific for 5'-LTR (UR2), plus a TaqMan hydrolysis probe that binds to the end of the LTR-R region (UHIV TaqMan) to amplify

and measure the LTR region of the first round PCR products. Second round reactions also contained UltraPure water and Brilliant III reaction mix (Agilent Technologies, Santa Clara, CA). The qPCR was performed on Mx3000P (Agilent Technologies, Santa Clara, CA). The PCR cycle conditions were as follows: a denaturation step at 95°C for 3 minutes and a two-step amplification process that consisted of 40 cycles (95°C for 5 seconds and 60°C for 20 seconds). Samples were analysed in triplicate for Alu-LTR levels and in a single reaction for the LTR only control.

Serial dilutions of ACH2 cells that carry a single copy of an integrated HIV genome were used for the standard curve. Briefly, ACH2 cells were cultured, counted and centrifuged into cell pellets. Cell pellets were lysed as described above and then stored as single aliquot uses at a stock concentration of 8×10^6 cells/ml. Standard dilutions of the ACH2 cells at 2×10^4 , 2×10^3 , 2×10^2 , 2×10^1 , 1×10^1 , 5×10^0 and 1×10^0 diluted in Ultrapure water were then used as standards. Standards were analysed in triplicate.

To normalise integrated HIV DNA copies to cell input, the amount of the CCR5 gene was also quantified using qPCR as a marker of cell input in the same DNA cell lysates. Cell lysates were first diluted 1:100 into UltraPure water and 5µl of this diluted sample was added to a total reaction volume of 25µl containing the primer LK46, primer LK47, UltraPure water and 1x SYBR Green Brilliant II probe (Agilent Technologies, Santa Clara, CA). The qPCR cycle conditions were as follows: a denaturation step for 8 minutes at 95°C, followed by 45 cycles of amplification (94°C for 20 seconds, 58°C for 40 seconds and 72°C for 40 seconds). PBMCs from uninfected individuals were isolated (as described above), 4×10^7 cells/ml counted and centrifuged as cell pellets. The cell pellets were lysed as described above. Ten-fold serial dilutions of the PBMCs from 1×10^4 to 1×10^0 cells per 5µl were used to create a standard curve.

PCR results were analysed on MxPro qPCR software (Agilent Technologies, Santa Clara, CA). Samples that were amplified but were below the lowest limit of dilution were given a value that was halfway between no amplification (zero) and the lowest limit of detection (one), therefore the samples were corrected as 0.5. Samples that were not amplified were given a value of 0. Integrated DNA copy number was divided by CCR5 cell number, and then

multiplied by 10^6 for HIV integrated DNA copies per 10^6 cells. Calculations were performed using Microsoft Excel.

Table 3.1 Primers and primer sequences used for integrated HIV DNA PCRs

Primer	Concentration	Sequence
ULF1	15 μ M	5'-ATGCCACGTAAGCGAAACTCTGGGTCTCTCTDGTAGAC-3'
Alu 1	15 μ M	5'-TCCCAGCTACTGGGGAGGCTGAGG-3'
Alu 2	15 μ M	5'-GCCTCCCAAAGTGCTGGGATTACAG-3'
Lambda T	20 μ M	5'-ATGCCACGTAAGCGAAACT-3'
UR2	20 μ M	5'-CTGAGGGATCTCTAGTTACC-3'
UHIV TaqMan	1:15	6-FAM)CACTCAAGGCAAGCTTTATTGAGGC(3'-BHQ-1
LK46	20 μ M	5'-GCTGTGTTTGCCTCTCTCCAGGA-3'
LK47	20 μ M	5'-CTCACAGCCCTGTGCCTCTTCTTC-3'

3.3.10 RNA Extractions

Cells lysed in RLT buffer with 1% β ME, homogenised using a QIAshredder spin column and stored at -80°C were thawed at room temperature and RNA extracted using a RNeasy Mini Kit (QIAGEN, Hilden, Germany). To this end, 350 μ l of 70% ethanol was added to each lysed and homogenised RNA sample. Samples were then transferred to a RNeasy Spin Column (QIAGEN, Hilden, Germany). The lysate-ethanol mixture was centrifuged at top speed (15,000xg) for 1 minute at room temperature. The flow through was discarded and the RNeasy column was washed with 350 μ l RW1 Buffer. After the flow through was discarded, 80 μ l of DNase I (RDD Buffer + DNase I stock) was added to the column membrane and incubated for 15 minutes at room temperature. The column was then washed with 350 μ l RW1 buffer at 15,000xg for 1 minute at room temperature. The DNase treatment and the RW1 wash was repeated another time. After the last wash, 500 μ l of Buffer RPE was added to the column and centrifuged using the same conditions as above. Columns were once again washed, and the column was placed into a new 2ml collection tube, and centrifuged again for 2 minutes at 15,000xg at room temperature. The RNeasy column was transferred to a new 1.5ml collection tube and 80 μ l of RNase-free water added directly onto the spin column membrane. Columns were incubated for 1 minute, before being centrifuged for 1 minute at

15,000xg, room temperature to elute the RNA. Spin columns were then discarded, and the eluted RNA kept on ice. A small 2µl aliquot of the eluted mixture was added to a separate tube to measure the concentration of RNA using a Nanodrop (Thermo Fisher Scientific, Waltham, MA). All RNA was stored at -80oC until ready for Nanodrop or USRNA RT-qPCR.

3.3.11 RT-qPCR quantification of cell associated HIV unspliced RNA

Cell-associated unspliced (CA US) HIV RNA was measured in RNA purified from drug treated, cell samples from PLWH using a semi-nested, reverse transcription (RT)-qPCR. First, 100ng (or less) of extracted RNA was mixed with 10mM dNTP (Life Technologies, Carlsband, CA), 3µg/µl Random hexamers (Life Technologies, Carlsband, CA) and 0.5µg/µl Oligo dT (Life Technologies, Carlsband, CA) and annealed at 65°C for 5 minutes. The sample was chilled on ice for another 5 minutes. Next the sample was reverse transcribed into cDNA using a 6.5µl mix of 5x First Strand Buffer (Life Technologies, Carlsband, CA), 0.1M DTT (Life Technologies, Carlsband, CA), 40U/µl RNase Out (Life Technologies, Carlsband, CA) and Superscript III Reverse Transcriptase (Life Technologies, Carlsband, CA) (for the positive reactions) or water (for the negative reactions). The mixture was reverse transcribed for at 42°C for 45 minutes and then 80°C for 15 minutes to inactivate the enzymes. Once the cDNA was made, the cDNA was analysed for HIV US RNA in a semi-nested qPCR. The first round PCR reaction contained 11.5µl of cDNA, AmpliTaq Gold DNA polymerase (Life Technologies, Carlsband, CA), the forward MH535 primer (refer to Table 3.2 for primer sequences), reverse SL20 primer in a final reaction volume of 45µl using the PCR conditions: 10 minute denaturing step at 95°C followed by 15 cycles of amplification (94°C for 20 seconds, 55°C for 40 seconds and 72°C for 40seconds). The second round qPCR reaction contained 2µl of first round PCR reaction, 1x SYBR Green Brilliant II (Agilent Technologies, Santa Clara, CA) quantitative PCR (qPCR), forward SL19 primer and the reverse SL20 primer for 40 cycles using the same PCR conditions as above.

Table 3.2. Primer and primer sequences used in cell-associated unspliced HIV RNA RT-qPCR

Primer	Concentration	Sequence
MH535	20μM	5'-AACTAGGGAACCCACTGCTTAAG-3'
SL20	20μM	5'-TCTCCTTCTAGCCTCCGCTAGTC-3'
SL19	20μM	5'-TCTCTAGCAGTGGCGCCCGAACA-3'

Standards for the cell-associated unspliced HIV RNA were made using *in vitro* transcribed SP-Δ33-NL4.3 plasmid. The RNA concentration of the transcribed RNA was determined using a nanodrop and the molecular weight of the RNA transcript was used to calculate the number of molecules/μl for the qPCR.

PCR results were analysed using the MxPro qPCR software. RNA copy numbers generated for samples with specific product melt temperatures (86-87°C) from the MxPro qPCR software were compiled and averaged using Microsoft Excel. Melt temperature of the SYBR green amplified peaks from each sample was analysed first. Samples with specific peaks at 86-87°C on the standard curve were given the value of the copy number generated by the standard curve. Data with a specific peak but copy number values that were below the lowest standard of 1 copy was denoted 0.5. Samples without a specific peak and non-specific peaks was denoted 0 copies. Samples with melt temperature peaks shifted from the wildtype 86-87°C temperatures were also denoted 0. Samples with dual or triple peaks were excluded from analysis as the assay only quantifies specific wildtype product peaks at 86-87°C melt temperature. Samples with <50ng RNA input were removed from analysis due to potential inaccuracy in US RNA measured with less input RNA. Additionally, samples that contained low levels of qPCR products with dual peak melting temperatures were excluded from analysis as these values cannot be quantified. US RNA copy numbers in RNA inputs between 50ng to <100ng were scaled to the equivalent of 100ng RNA input so that all data is shown as US RNA copies per 100ng input RNA. Data was compiled and averaged in Microsoft Excel.

3.3.12 Bliss Independence Model of Synergy

The Bliss Independence Model for synergy between the pro-apoptotic drug and LRAs was calculated as previously described [471].

3.3.13 Data Analysis and Statistics

Graphs were generated on GraphPad Prism v8. Statistical analysis was not performed. Previous work [68] has demonstrated that *ex vivo* data generated from PLWH is not normally distributed. Therefore, the use of parametric analyses is not correct. Here the appropriate statistical method to analyse the data would be to use non-parametric analysis. However, low sample numbers ($n > 6$) precludes non-parametric statistical analysis. Thus, the means, fold change and range were reported.

3.4 Results

To examine the effect of combining pro-apoptotic drugs and LRAs on the decline of HIV latently infected CD4+ T-cells, we tested four different pro-apoptotic drugs (IPI-443, IPI-3063, wortmannin and venetoclax) combined with the HDACis (romidepsin, panobinostat), PKC agonist (bryostatin) and a bromodomain inhibitor (JQ1) (Table 3.3).

Table 3.3 Pro-apoptotic and LRA drug compounds and their mechanism of action

Drug Type	Drug Compound	Mechanism of Action	Reference
Pro-apoptotic	IPI-443	Potent dual inhibitor of PI3K δ/γ isoforms	N/A
	IPI-3063	Potent PI3K δ isoform inhibitor	[472]
	Wortmannin	Non-specific pan-PI3K inhibitor	[473]
	Venetoclax	Inhibitor of anti-apoptotic molecule Bcl-2	[412]
LRA HDACi	Romidepsin Panobinostat	Remodel chromatin structure to reactivate latent HIV, displace histone acetylase	[246, 248] [244]
Protein Kinase C (PKC) agonist	Bryostatin	Stimulate the PCK pathway leading to the recruitment of NF- κ B to reactivate latent HIV	[270]
Bromodomain Inhibitor	JQ1	Dissociates a bromodomain ⁴ from the HIV protomer, allowing stimulation of HIV elongation	[272, 274]

3.4.1 Selective PI3K inhibitors can inhibit the phosphorylation of Akt^{Ser473}

The ability of wortmannin to inhibit pan-PI3K enzyme isoforms and PI3K/Akt signalling in CD4+ T cells is well described (Reviewed in [474]). Additionally, the ability of IPI-443 or IPI-3063 to selectively inhibit PI3K p110 δ/γ isoforms or PI3K p110 δ isoform respectively and subsequent PI3K/Akt signalling in B-cells is reported [472]. However, the impact of these selective PI3K inhibitors on inhibiting PI3K signalling in resting CD4+ T-cells is not clear. Thus, the capability of IPI-443 and IPI-3063 to prevent PI3K/Akt signalling in resting CD4+ T-cells and inhibit phosphorylation of Akt was assessed using flow cytometry for phosphorylated Akt^{Ser473}.

Both IPI-443 and IPI-3063 inhibited phosphorylation of Akt^{Ser473} in a donor-dependent manner in some (Figure 3.1) but not all donors (Figure 3.2). In resting CD4+ T-cells from one donor

sensitive to the PI3Ki drugs, all IPI-443 and IPI-3063 doses decreased Akt phosphorylation compared with cells stimulated with PMA+DMSO diluent control (Figure 3.1, PMA+PI3Ki black peaks versus PMA+DMSO blue peaks in each histogram). Additionally, when measuring the mean fluorescence intensity of the phosphorylated Akt^{Ser473}, the highest 5µM dose of IPI-443 and IPI-3063 most potently inhibited Akt phosphorylation 2.64-fold and 2.50-fold versus cells stimulated with PMA+DMSO diluent control (Figure 3.2, Donor 96). In contrast, other donors were not sensitive to inhibition of phosphorylated Akt^{Ser473} by IPI-433 and IPI-3063 (Figure 3.2: Donor 02 and Donor 44, compare PHA+DMSO versus drug+DMSO). Combining the results of 4 donors, the average mean fluorescence intensity of phosphorylated Akt^{Ser473} in resting CD4+ T-cells incubated with all IPI-443 and IPI-3063 concentrations modestly decreased to approximately 80% of the levels in cells stimulated with PMA+DMSO diluent control (Figure 3.2). Additionally, a pan selective PI3K inhibitor, wortmannin, which acted as a positive control also inhibited Akt phosphorylation but to a greater extent than the selective PI3K inhibitors (Figure 3.2, 6.1% of the phosphorylated Akt^{Ser473} level in the PMA+DMSO diluent control).

These data suggest that selective inhibition of PI3K γ and/or δ isoforms by IPI-443 or IPI-3063 can decrease PI3K signalling in resting CD4+ T-cells in some but not all healthy donors, while inhibition of all PI3K isoforms with wortmannin led to a higher and more consistent degree of PI3K inhibition.

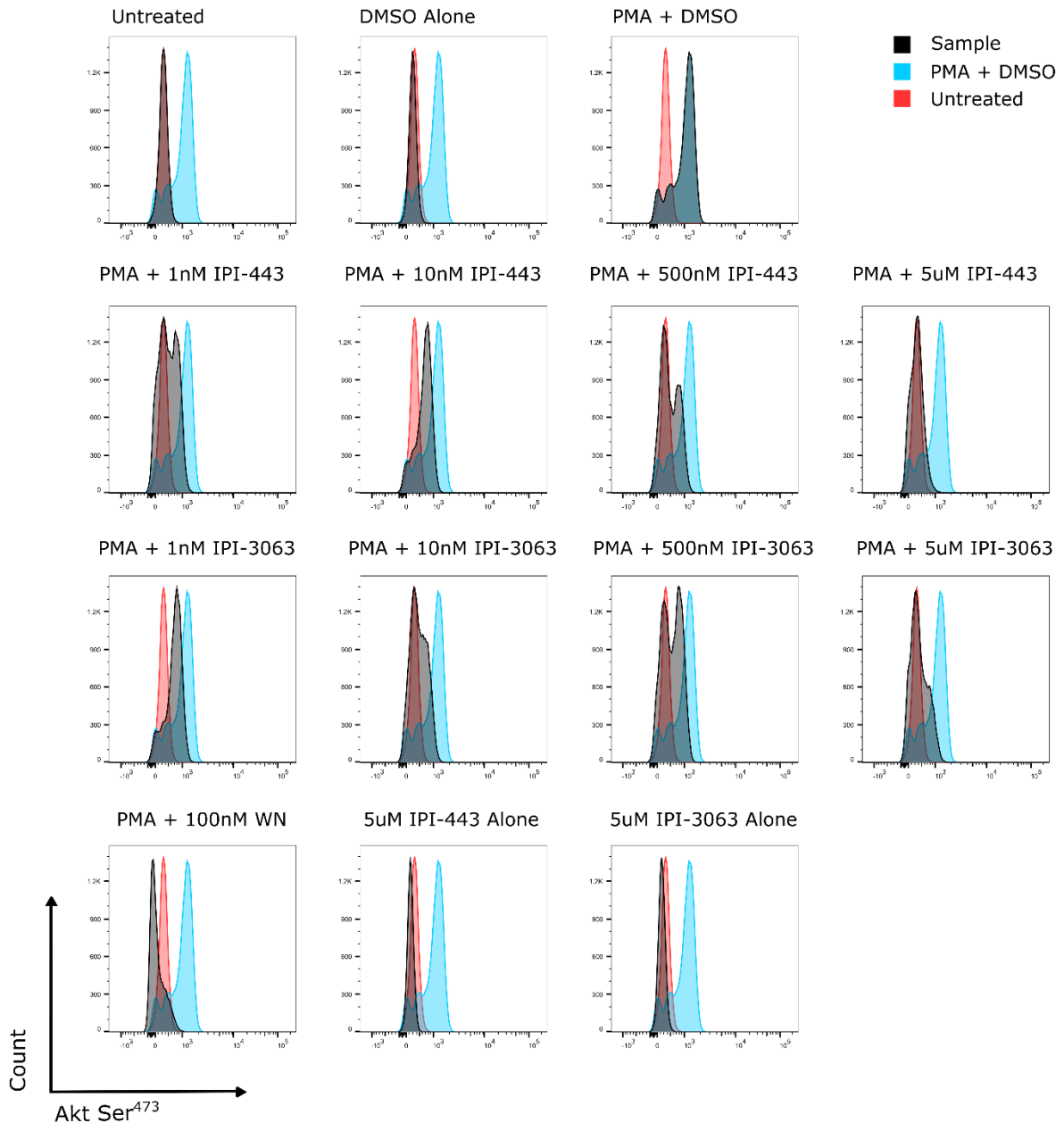


Figure 3.1. PI3K inhibitors IPI-443 and IPI-3063 both inhibit the phosphorylation of Akt^{Ser473}
 Resting CD4⁺ T-cells were isolated from one healthy donor and cultured unstimulated for two days. Cells were incubated for four hours in the presence or absence of PI3K inhibitors: IPI-443 or IPI-3063 at varying concentrations, or 100nM wortmannin as a comparative control. Cells were then stimulated with PMA for 15 minutes to induce PI3K/Akt signalling and Akt^{Ser473} phosphorylation. Cells were then fixed and permeabilized for Akt^{Ser473} antibody staining. Stained samples were analysed using flow cytometry. Untreated and DMSO drug diluent treated samples were included as controls. Histograms of phosphorylated Akt^{Ser473} are shown, including unstimulated cells (baseline phosphorylation, red), cells treated with PMA and

DMSO (PMA induced phosphorylation, blue), and cells treated with the drugs labelled above the histogram (black).

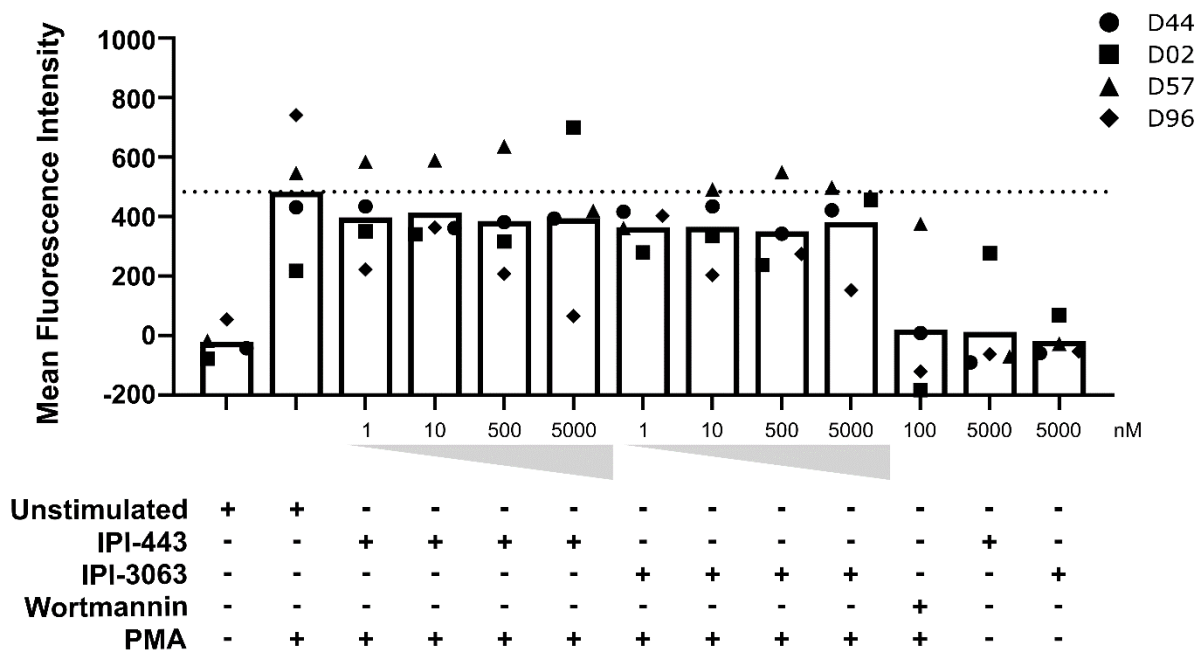


Figure 3.2. IPI-443 and IPI-3063 PI3K inhibitors decrease phosphorylated Akt^{Ser473} in resting CD4+ T-cells in a donor-dependent manner

The mean fluorescence intensity of Akt^{Ser473} phosphorylation in resting CD4+ T-cells from 4 healthy donors treated for 4 hours with different concentrations of PI3K inhibitor IPI-443 or IPI-3063, or 100nM wortmannin, or DMSO drug diluent control, and then treated for 15 minutes with or without PMA stimulation to activate PI3K signalling. Cells were then stained with an antibody for phosphorylated Akt^{Ser473}. The average mean fluorescence intensity across 4 donors is shown for each condition (bars, n=4), with each symbol representing a separate donor.

3.4.2 PI3K inhibitors alone do not have an impact on cell death in J-Lat6.3 T-cell lines

Next, we tested whether the two selective PI3K inhibitors alone: IPI-443 and IPI-3063, had any effect on latent HIV reactivation and if reactivation occurred, whether this pro-apoptotic drug alone might lead to the death of latently infected cells using a T-cell line model of HIV latency.

In the J-Lat6.3 latently infected cell line, the PI3K inhibitors alone did not significantly increase the percentage of GFP+ cells at any of the time points analysed: 24 hours (Figure 3.3A), 48 hours (Figure 3.3B) or 72 hours (Figure 3.3C). In contrast, the PMA positive control led to an increase in the percentage of GFP+ reactivated cells approximately 210.81-, 178.44- and 111.48-fold change over DMSO at 24, 48 and 72 hours, respectively (Figure 3.3). Thus, PI3K inhibitors alone did not lead to reactivation of latent HIV in this J-Lat 6.3 cell line. Additionally, culturing J-Lat6.3 cells with the PI3K inhibitors alone did not result in increase the percentage of dead cells after 24 hours (Figure 3.4A), 48 hours (Figure 3.4B) or 72 hours (Figure 3.4C) of culture compared to the DMSO drug diluent negative control.

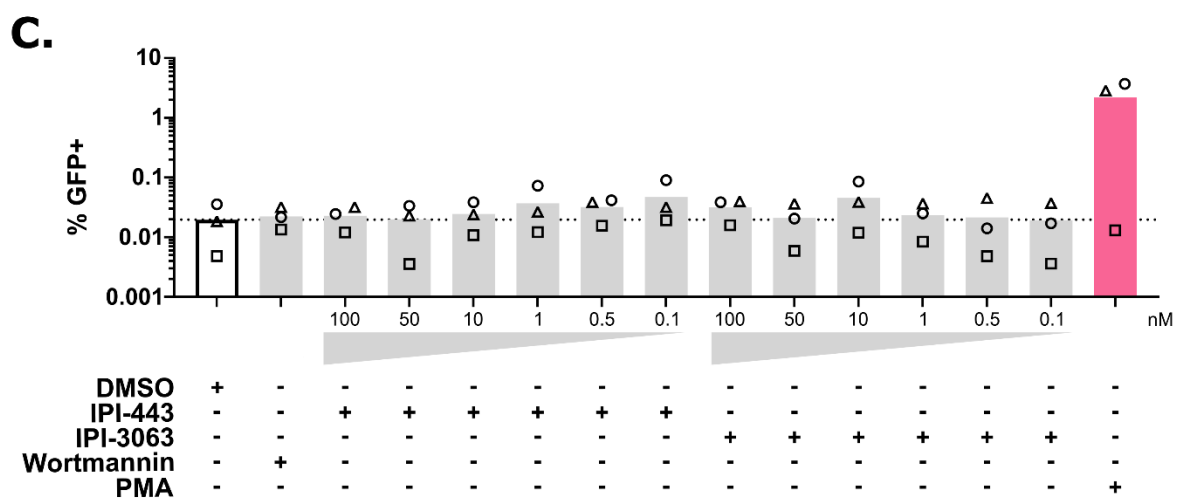
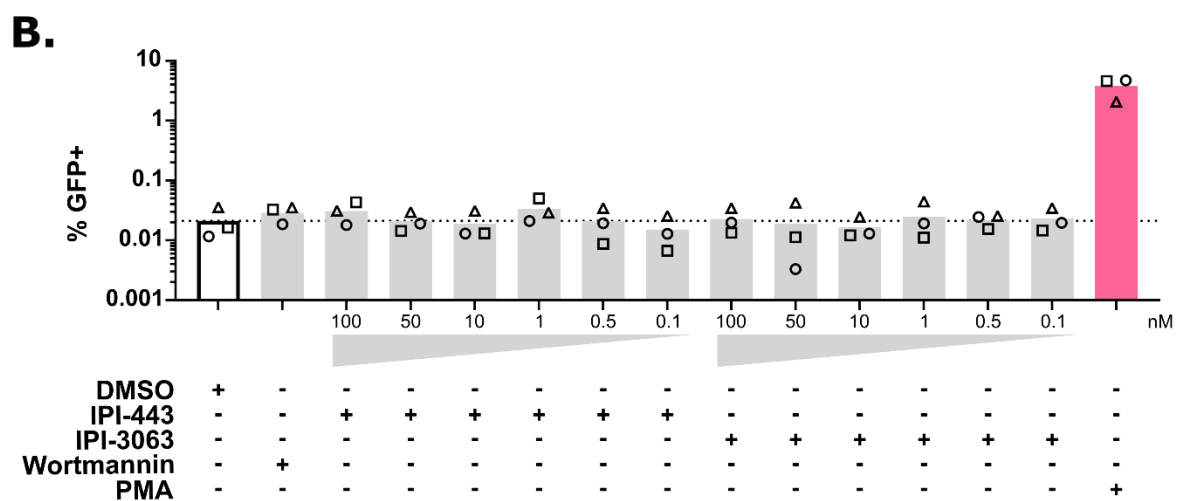
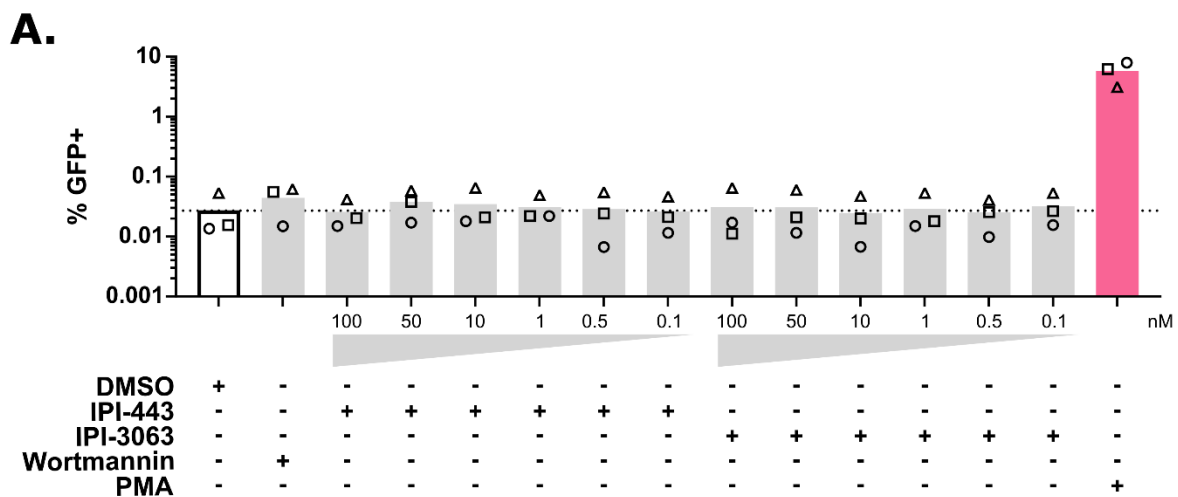


Figure 3.3. PI3K inhibitors IPI-443 and IPI-3063 do not result in virus expression in J-Lat6.3 T-cells

J-Lat6.3 T-cells were cultured with varying doses of the PI3K inhibitors; IPI-443 and IPI-3063 (0.1 to 100nM). Controls included: DMSO diluent negative control, 100nM wortmannin (a pan-PI3Ki) comparative control and 16nM PMA LRA positive control. Cells were cultured for A) 24 hours, B) 48 hours or C) 72 hours, after which cells were harvested. Reactivation of latent virus expression was measured using the viral GFP reporter with the percentage of GFP+ cells shown for 3 independent experiments (n=3). Each symbol represents an independent experiment and is the mean of two replicate wells. The bars represent the mean of the three experiments.

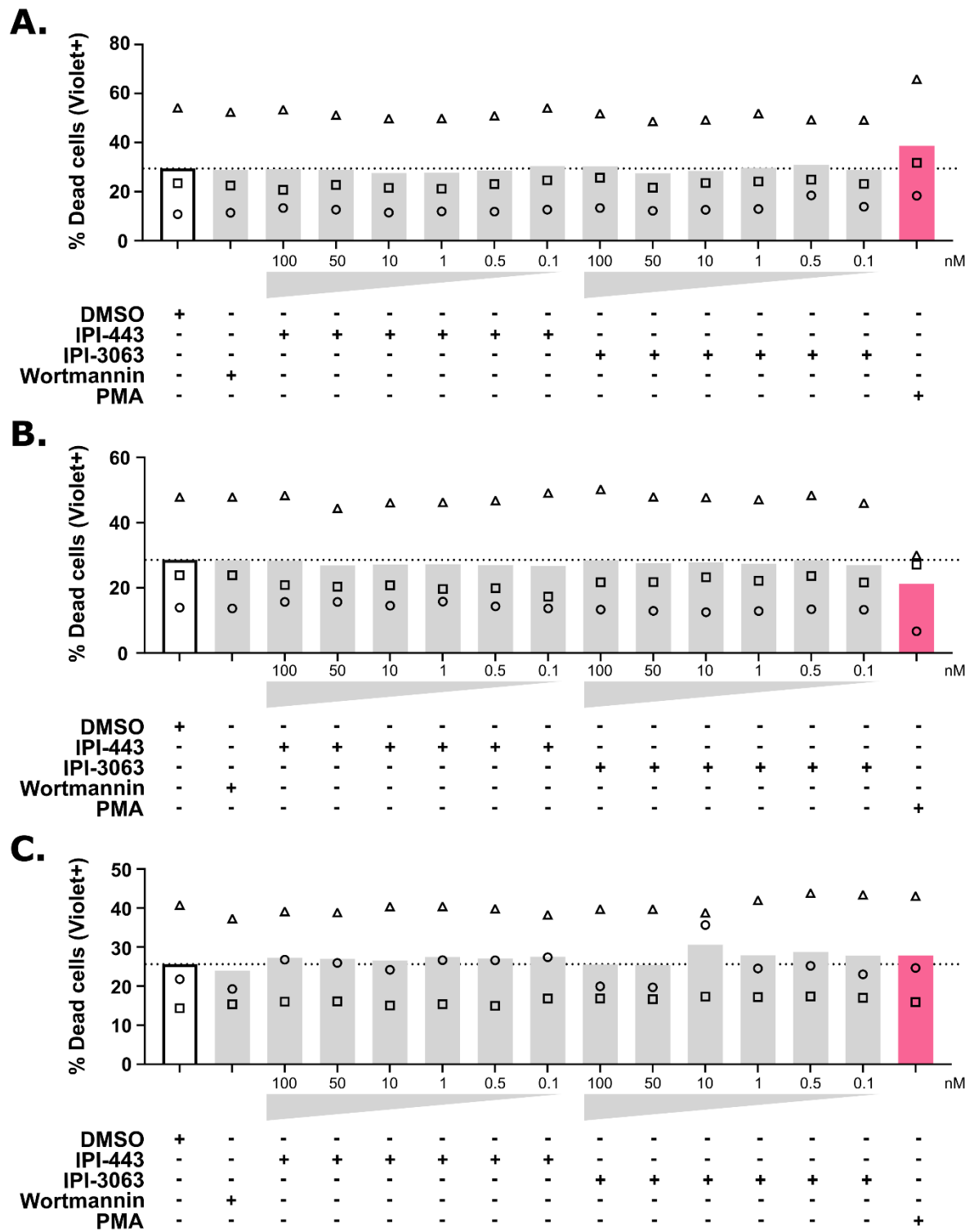


Figure 3.4. PI3K inhibitors do not have toxic effects on J-Lat6.3 T-cells at either 24, 48 or 72 hours

J-Lat6.3 T-cells were cultured with 0.1 to 100nM concentrations of the PI3K inhibitors; IPI-443, IPI-3063 or 100nM wortmannin for A) 24 hours, B) 48 hours or C) 72 hours. DMSO diluent negative control and 16nM PMA LRA positive control was included for comparison. Cells were harvested after each time point and stained with a Live/Dead Violet Fixable Dead Cell Stain and analysed using flow cytometry. Shown is the overall cell death in each sample. Each symbol represents an independent experiment and is the mean of two replicates. The bar represents the mean of all three experiments (n=3).

3.4.3 J-Lat6.3 T-cells treated with PI3K inhibitors together with latency reversing agents

To investigate whether the combination of PI3K inhibitors together with LRAs to drive the expression of pro-apoptotic viral proteins would lead to an increase in virus expression and an increase in cell death, J-Lat6.3 T-cells were treated for 24 hours with either IPI-443 or IPI-3063 PI3K inhibitors to sensitize the cells to apoptosis. The LRAs: vorinostat, panobinostat, romidepsin or bryostatin were then added to cultures to reactivate latent HIV (Figure 3.5). The collected cells were stained with a Live/Dead Fixable Violet Death Cell Stain and analysed using flow cytometry. For flow cytometry analysis, cells were first gated on forward scatter area versus side scatter area to exclude debris, then forward scatter height versus width to exclude cell doublets. Next, cells were gated into either GFP+ or GFP- cell populations. The percentage of live (violet-) or dead (violet+) cells within sample was also determined (Figure 3.6).

The PI3K inhibitors alone did not induce GFP expression compared to the DMSO negative diluent control (Figure 3.7A), as shown previously (Figure 3.3). Combining panobinostat with either PI3K inhibitor markedly decreased the levels of GFP+ cells compared to the LRA alone (100nM IPI-443+PNB mean=0.162%; 100nM IPI-3063+PNB mean=0.181%, PNB alone mean=1.31%) (Figure 3.7A). In contrast, adding vorinostat, panobinostat, romidepsin or bryostatin alone increased the % GFP+ cells 3.42-, 77.82-, 1193.07- or 60.50-fold respectively versus the DMSO control consistent with reactivation of latent HIV. Combining 100nM IPI-443 or IPI-363 together with vorinostat had little impact on the levels of GFP+ cells (100nM IPI-443+VOR mean=0.021%; 100nM IPI-3063+VOR mean=0.027%) compared to VOR alone (mean=0.058%) apart from one outlier value in cells treated with 10nM IPI-443+VOR (mean=0.154%, Figure 3.7A). A similar trend was also observed with bryostatin and romidepsin. The overall mean level of GFP+ cells were higher in J-Lat6.3 T-cells treated with romidepsin alone than cells treated with the PI3K inhibitors alone (mean=20.08%), but again, this was comparable to cells treated with romidepsin+IPI-443 or IPI-3063 at 100nM (mean=16.97% and mean=17.52%, respectively).

We then investigated whether the PI3K inhibitors combined with LRAs led to a decrease in latently infected cells using the Violet Live/Dead Fixable Death Cell Stain. As seen in Figure

3.7B, the PI3K inhibitor alone did not impact cell death but all the LRAs alone increased cell death to various magnitudes versus the DMSO diluent control. However, combining panobinostat, romidepsin or bryostatin with either PI3K inhibitor did not increase overall cell death above LRA alone (mean percentage of dead cells =51.34%, 68.93%, 18.43%, respectively, Figure 3.7B).

When cells were treated with PI3K inhibitor together with vorinostat, there was a modest decline in the percentage of dead cells (100nM IPI-443+VOR mean=18.55%; 100nM IPI-3063+VOR mean=20.02%) compared to the LRA alone (mean=31.55%). Analysis of the levels of cell death in the GFP+ and GFP- cell populations revealed that the majority of dead cells were in the GFP negative (latent) cell population (Figure S3.2). In cells treated with romidepsin alone, we observed small increases in the number of dead cells in the GFP+ population. This increase was also seen when romidepsin was combined with IPI-443 or IPI-3063 with comparable levels of cell death compared to romidepsin alone. This suggests that there was no selective death of the reactivated J-Lat6.3 T-cells when treated with a pro-apoptotic and LRA drug combination (Figure S3.2).

When the time of LRA culture was increased from 24 hours to 48 hours, similar trends were seen for GFP expression (Figure S3.1A). We observed no increase in virus expression in J-Lat6.3 T-cells treated with the PI3K inhibitor and LRA compared to samples treated with the LRA alone. J-Lat6.3 T-cells treated with HDAC inhibitors romidepsin resulted in overall lower levels of GFP expression after 48 hours of treatment compared to its 24 hour counterpart. Vorinostat and panobinostat treatment for 48 hours showed comparable levels of GFP expression compared to their 24 hour treated counterparts except for 2 samples treated with PI3K inhibitor and bryostatin. Due to an outlier in samples treated with 1nM or 100nM IPI-3063 PI3K inhibitor and bryostatin, the levels of virus expression appear higher than their 24 hour counterparts (Figure S3.1A).

However, treatment with the LRAs romidepsin and panobinostat for 48 hours resulted in an unacceptably high level of cell death, reaching a maximum 100%, irrespective of the addition of the PI3K inhibitors (Figure S3.2B). In contrast, the combination of bryostatin with IPI-443

at all concentrations had comparable levels of dead cells to bryostatin alone. The combination of bryostatin and IPI-3063 at two concentrations, 100nM and 1nM resulted in an increase of dead cells (100nM IPI-3063+BRY mean=29.97%; 1nM IPI-3063+BRY mean=32.70%) than the bryostatin alone treated cells (mean=18.78%). This increase in cell death, although at first seems promising, it was only observed in one of the three independent experiments and oddly was not seen with 10nM IPI-3063. We observed a decrease in the number of dead cells in J-Lat6.3 T-cells treated with both PI3K inhibitor and vorinostat, which was the same trend as the 24hr time point (Figure 3.7B). We observed a decrease in the mean of dead cells with IPI-443/IPI-3063 (100nM IPI-443+VOR mean=22.08%; 100nM IPI-3063+VOR mean= 23.23%) than cells treated with vorinostat alone (mean=53.20%).

Collectively, these data indicate that treatment of J-Lat6.3 T-cells with PI3K inhibitors followed by reactivation with LRAs at both 24 hours (Figure 3.7) and 48 hours (Figure S3.1), does not increase the death in reactivated (GFP+) J-Lat6.3 T-cells versus LRA alone.

Importantly, the J-Lat6.3 T-cell line is a cancerous cell line deficient in PTEN, which is a negative regulator of the PI3K pathway. Thus, the increased PI3K activity in this cell line may be difficult to overcome with PI3K inhibitors, preventing the impact of PI3K inhibitors and LRAs on purging latently infected T-cells from being assessed using this cancerous cell line. Therefore, the ability of PI3K inhibitors with LRAs to kill latently infected cells was examined in the most biologically relevant source of HIV latently infected cells, CD4+ T-cells from PLWH on ART *ex vivo* that have functional PTEN.

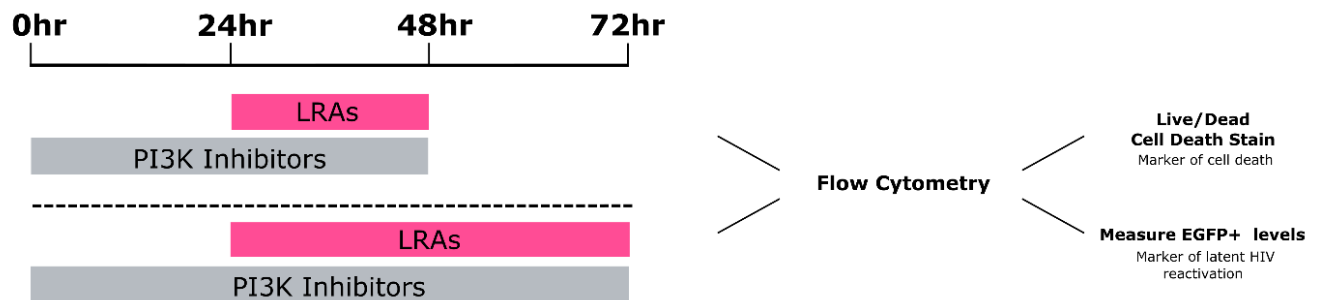


Figure 3.5. Method to assess the ability of PI3K inhibitors combined with LRAs to induce latent HIV expression and deplete latently infected J-Lat6.3 T-cells

The effects of PI3K inhibitors together with LRAs on the reactivation of latent HIV and death of latently infected J-Lat6.3 T-cells was tested. J-Lat6.3 T-cells were first pre-treated with PI3K inhibitors for 24 hours, LRAs added and cells then cultured for an additional 24 hours or 48 hours. At the end of treatment, cells were stained for cell death using a Live/Dead Fixable Violet Death Cell Stain and analysed by flow cytometry for reactivation of the viral GFP reporter and uptake of the Violet dead cell stain.

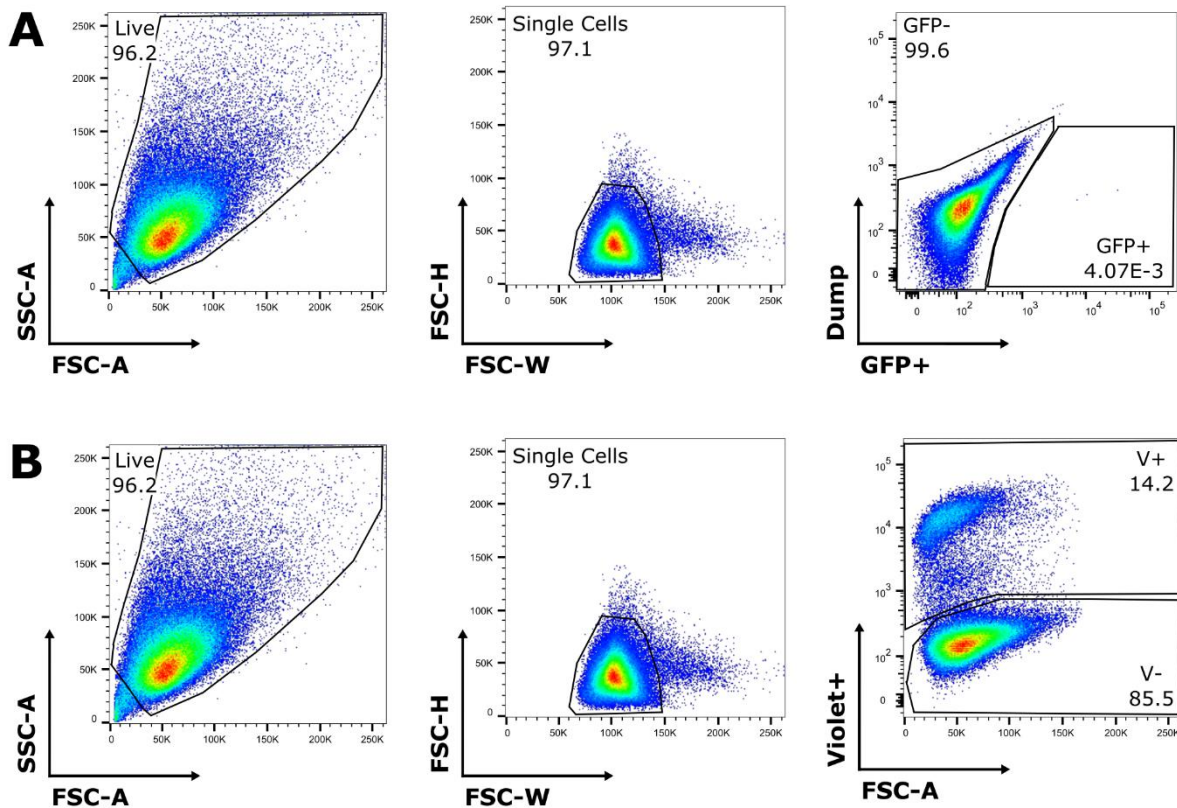


Figure 3.6. Gating strategy used to identify latently infected J-Lat6.3 T-cells that have reactivated expression of the viral GFP reporter and cells that are dead

J-Lat6.3 T-cells were first pre-treated with PI3K inhibitors for 24 hours to sensitise the cells towards apoptosis. LRAs were then added for an additional 24 hours before being stained with a Violet Live/Dead Fixable Dead Cell Stain. Cells were analysed on a flow cytometer, data analysed using Flow Jo software and the following gating strategy used. All cells, excluding the debris were gated on forward scatter area versus side scatter area. Next doublets were excluded using forward scatter height versus width. Cells that were positive for GFP or negative were discriminated (A). Lastly, cells in each of the samples were gated on the Live/Dead Fixable Violet Dead Cell Stain versus forward scatter to determine how many of the latently infected cells had died (B). The flow plots are a representative sample from 1 of 3 independent experiments ($n=3$). FSC; forward scatter, SSC; side scatter, V+; Violet+ (Dead), V-; Violet- (Live).

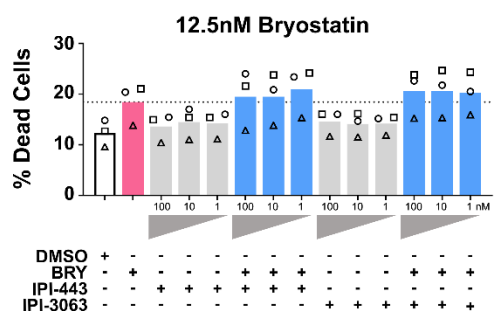
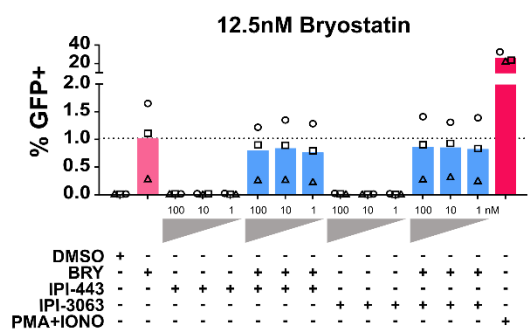
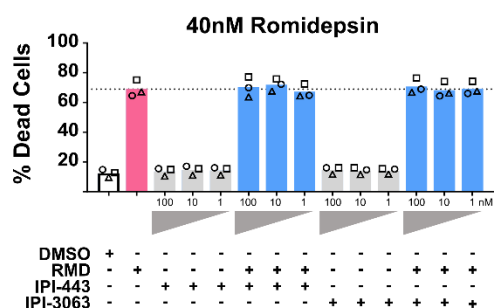
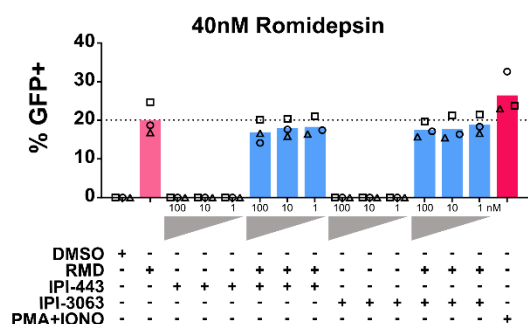
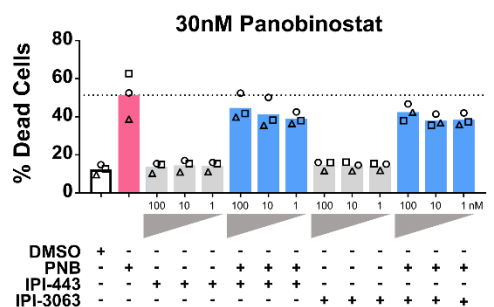
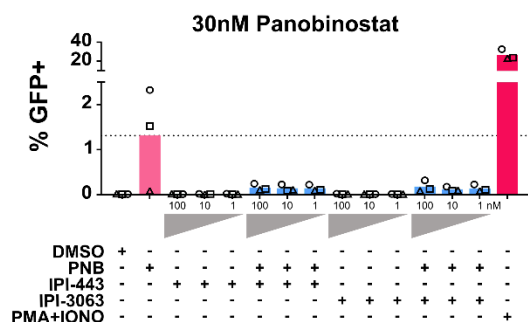
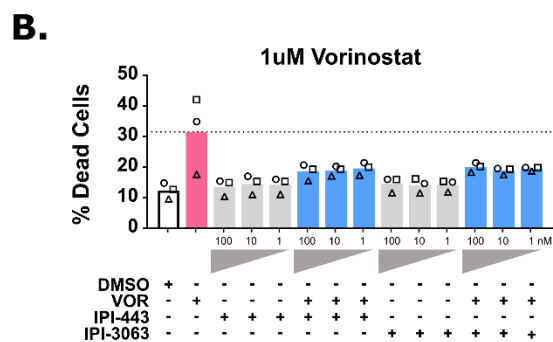
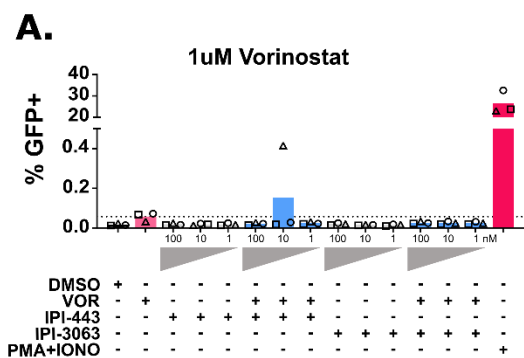


Figure 3.7. J-Lat6.3 T-cells pre-treated with PI3K inhibitors prior to reactivation with LRAs did not enhance cell death versus LRA alone

J-Lat6.3 T-cells were pre-treated with increasing doses of the PI3K inhibitors IPI-443 or IPI-3063 (1, 10 or 100 nM) for 24 hours. Four separate LRAs; 1 μ M vorinostat, 30nM panobinostat, 40nM romidepsin or 12.5nM bryostatin were then added to cell cultures for an additional 24 hours. Cells were harvested 24 hours after the addition of LRAs and stained with a Live/Dead Fixable Violet Dead Cell Stain and analysed using flow cytometry. Reactivation of viral GFP reporter expression (A) was determined with the percentage of GFP+ cells shown. Cells treated with 16nM PMA and 0.5 μ M Ionomycin were included as a positive control for latency reactivation. (B) The percentage of dead cells in the GFP+ populations are shown. Each symbol denotes an experiment performed 3 independent times (n=3).

3.4.4 Pulsing romidepsin and panobinostat for 4 hours with uninfected resting CD4+ T-cells resulted in decreased toxicity when combined with PI3K inhibitors

Before examining the combined effects of PI3K inhibitors and LRAs on CD4+ T-cells from PLWH on ART, we first needed to test the toxicity of these drug combinations on uninfected resting CD4+ T-cells from healthy donors to determine drug doses with minimum toxicity. We tested adding the PI3K and LRA drug doses in four different timing circumstances (Figure 3.8) as the timing of drug administration may also be key in allowing the PI3K inhibitor and LRA to induce the death of latently infected cells.

For the LRA selections, vorinostat was excluded as it is not as potent an LRA as other HDACi like panobinostat and romidepsin [246]. Additionally, disulfiram was added as we were interested in the effect of disulfiram, which reactivates latent HIV through depleting the PTEN inhibitor of the PI3K pathway to promote PI3K/Akt signalling [276], would have on cell death when combined with PI3Ki in resting CD4+ T-cells. Furthermore, another class of LRA, JQ1 was added. JQ1 binds the BRD4 bromodomain protein and liberates P-TEFb that can then interact with HIV Tat, which in turn binds the TAR stem loop of initiated HIV transcripts to stimulate HIV transcription elongation [272]. Therefore, we wanted to examine how different LRAs that reactivate latent HIV in a variety of pathways would impact viral reactivation and cell death.

First, we tested the combination of PI3K inhibitor and LRAs added together in continuous culture for 72 hours (Figure 3.8A). We found that continuous culture of uninfected resting CD4+ T-cells with 30nM panobinostat, 40nM romidepsin and 500nM disulfiram alone resulted in very high levels of cell death irrespective of PI3K inhibitor addition (mean=74.90%, 81.14% and 79.36% cell death respectively). Conversely, continuous culture with 50nM bryostatin and 1µM JQ1 resulted in low levels of cell death (mean= 27.26% and 28.01%) regardless of PI3K inhibitor addition (Figure 3.8A).

To improve the viability of resting CD4+ T-cells, a 24 hour pulse of LRAs was next implemented as previously reported [471] and reduce the toxicity that we observed following 72 hours of continuous culture with the LRAs. Three further timing conditions of PI3K inhibitor and LRA addition were tested as the timing of drug administration may influence the ability of PI3K

inhibitor and LRAs to sensitise the cells to apoptosis and express pro-apoptotic viral proteins respectively to lead to the death of latently infected cells. Overall, all conditions totalled 72 hours incubation. The concentration of bryostatin was also decreased from 50nM to 10nM when testing these three, shorter LRA drug conditions to match previously reported data testing the drug on CD4+ T-cells from PLWH on ART *ex vivo* [471].

Treatment of cells for 24 hours with 10nM bryostatin or 1000nM JQ1 did not severely impact cell viability (Fig 3.8B-D). Overall, using a 24-hour pulse of bryostatin or JQ1 had minor impact on the death of uninfected resting CD4+ T-cells.

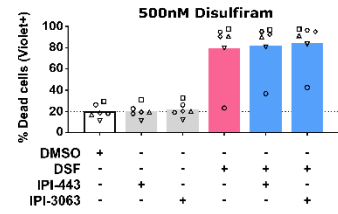
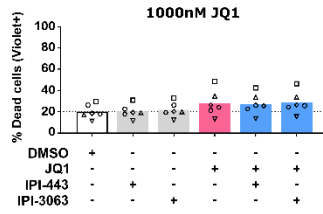
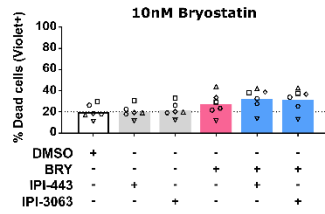
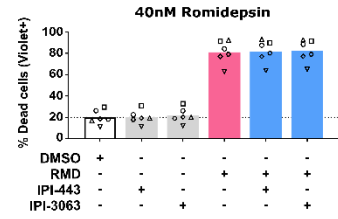
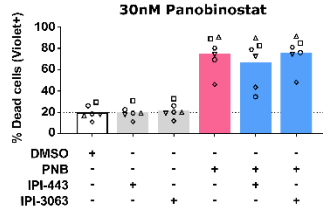
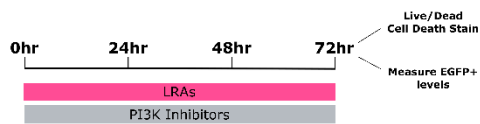
In contrast, a 24-hour pulse of 30nM panobinostat decreased the toxicity of this drug substantially (Figure 3.8B, C and D: PNB alone mean=32.35%, 32.87%, 23.94%, respectively) compared to 72-hour culture (Figure 3.8A: PNB alone mean=74.90%). The addition of the PI3K inhibitor did not have any additive effects on the levels of cell death in samples that were treated with panobinostat plus IPI-443 (Figure 3.8B, C and D: IPI-443+PNB mean=34.18%, 32.73%, 24.50%, respectively) or IPI-3063 (Figure 3.8B, C and D: IPI-3063+PNB mean= 34.73%, 33.65%, 24.75%, respectively).

Similar to panobinostat, a 24-hour pulse of 40nM romidepsin also reduced cell death from continuous 72-hour drug culture (RMD alone mean=81.14%) to between 40-60% dead cells in the 24-hour drug pulse conditions irrespective of PI3K inhibitor addition (Figure 3.8). However, unlike panobinostat, the levels of cell death with 24 hours of 40nM romidepsin still remained high (up to 60%). This could drive unacceptable unselective death of all cells in cultures and may also mask any effect that PI3K inhibitors and romidepsin may have on the selective death of latently infected cells. To further reduce the toxicity of romidepsin, a 4-hour pulse of romidepsin was subsequently examined as previously reported [246]. Reducing both LRA exposure time to 4 hours and the dose of romidepsin from 40nM (Figure 3.9) to 10 or 20nM reduced the drug toxicity (40nM mean=51.60%; 20nM mean=35.36%; 10nM mean=17.28%). Moving forward, a 4 hour pulse of 20nM romidepsin was selected for use on T-cells from PLWH to maximise reactivation activity but minimise non-specific drug toxicity.

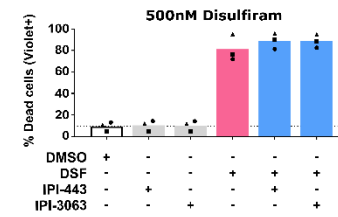
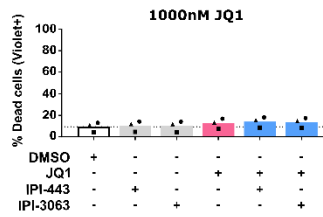
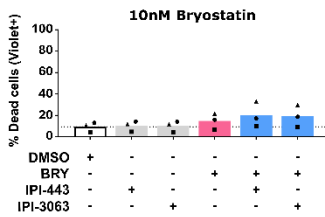
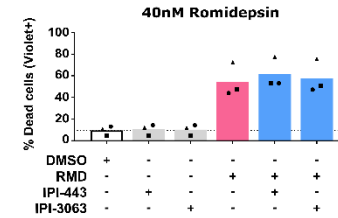
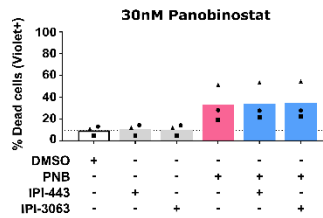
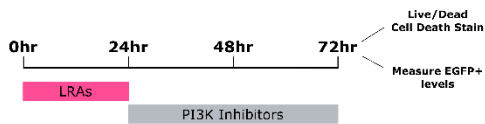
Finally, 500nM disulfiram proved to be very toxic to uninfected resting CD4+ T-cells in continuous 72-hour culture (Figure 3.8A: DSF alone mean=79.36%). Decreasing the exposure time of disulfiram to 24 hours was still unable to reduce the toxicity of this drug on uninfected cells when added irrespective of PI3K inhibitor (Figure 3.8B-C: IPI-443+DSF mean=88.97%, 83.45%; IPI-3063+DSF mean=88.67%, 84.71%). Interestingly, when cells were pre-treated with PI3K inhibitor for 24 hours, then pulsed for 24 hours with disulfiram in the background of PI3K inhibitor and cultured a further 24 hours in PI3K inhibitor alone, this condition decreased cell death in cultures from above 80% in other conditions to a mean of 43.56% for disulfiram alone, and to 48.25% or 43.06% when combined with IPI-443 or IPI-3063, respectively (Figure 3.8D). However, this level of cell death was still high. To decrease the toxic effects of the drug, a concentration lower than 500nM is needed. However, published data using 500nM disulfiram alone in patient derived CD4+ T-cells *ex vivo* show little/no reactivation of latent virus and demonstrated 40% toxicity [471]. Using less than 500nM disulfiram raises concern that we may not reactivate latent virus in patient cells *ex vivo* at this concentration. Therefore, as the risk of lowering toxicity and foregoing effective reactivation of latent virus, moving forward, disulfiram was not included in the panel of LRAs to study in latently infected CD4+ T-cells *ex vivo*.

In conclusion, these data demonstrate that 24-hour pulses of 30nM panobinostat, 10nM bryostatin and 1000nM JQ1, or a 4-hour pulse of 20nM romidepsin are appropriate drug concentrations with low levels of toxicity to test with 100nM PI3K inhibitors in latently infected cells from PLWH on ART *ex vivo*.

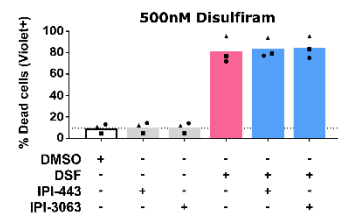
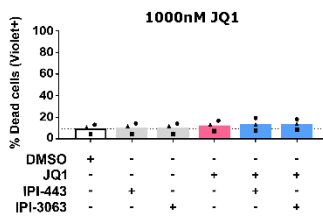
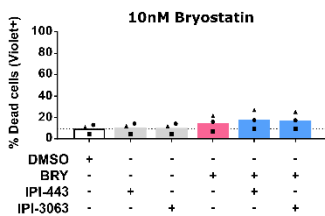
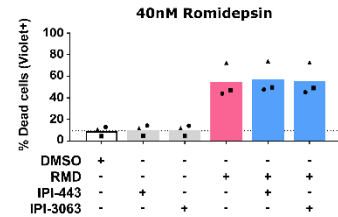
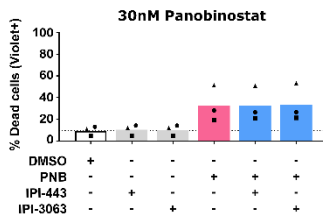
A.



B.



C.



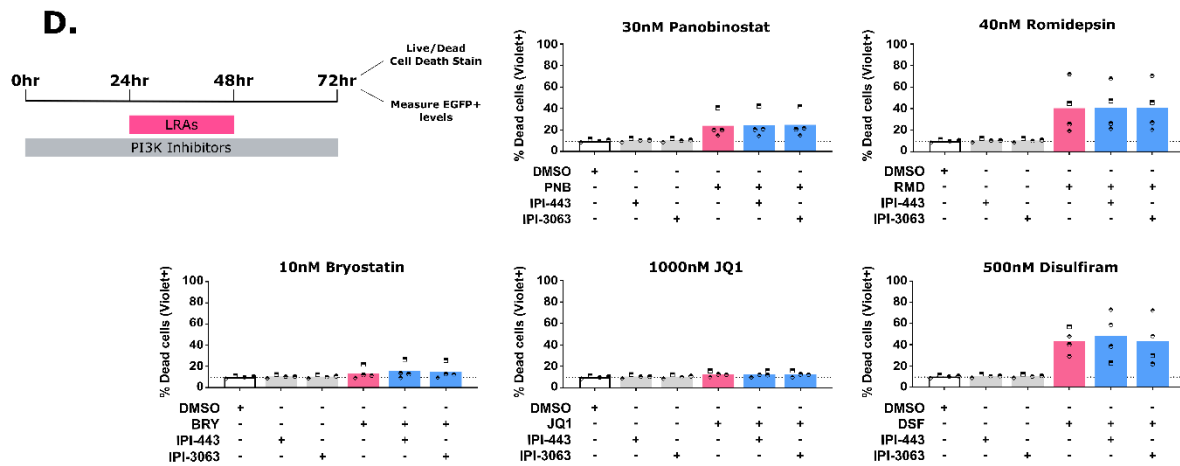


Figure 3.8. 24-hour pulse of LRAs reduced toxicity in uninfected resting CD4+ T-cells irrespective of PI3K inhibitor addition

Resting CD4+ T-cells were isolated from uninfected PBMCs from healthy donors. Cells were treated with PI3K inhibitors and latency reversing agents (LRAs) using 4 different timing conditions. Four different timing conditions were used to test the toxicity of PI3K inhibitors and LRAs in uninfected resting CD4+ T-cells as follows: 1) continuous culture with both PI3K inhibitors and LRA for 72 hours; 2) 24 hour pulse with LRA alone followed by 48 hours with PI3K inhibitors; 3) 24 hour pulse of LRA together with PI3K inhibitors followed by 48 hours of PI3K inhibitors alone; and 4) pre-treatment with PI3K inhibitors alone for 24 hours followed by a 24 hour pulse of LRA plus PI3K inhibitors, followed by PI3K inhibitors for 24 hours. 72 hours after culture, all cells were stained with the cell death stain, Live/Dead Fixable Violet Dead Cell stain and analysed by flow cytometry for the percentage of dead cells. PNB-panobinostat; RMD-romidepsin; BRY- bryostatin; DSF- disulfiram. Each symbol denotes a different donor, $n \leq 6$.

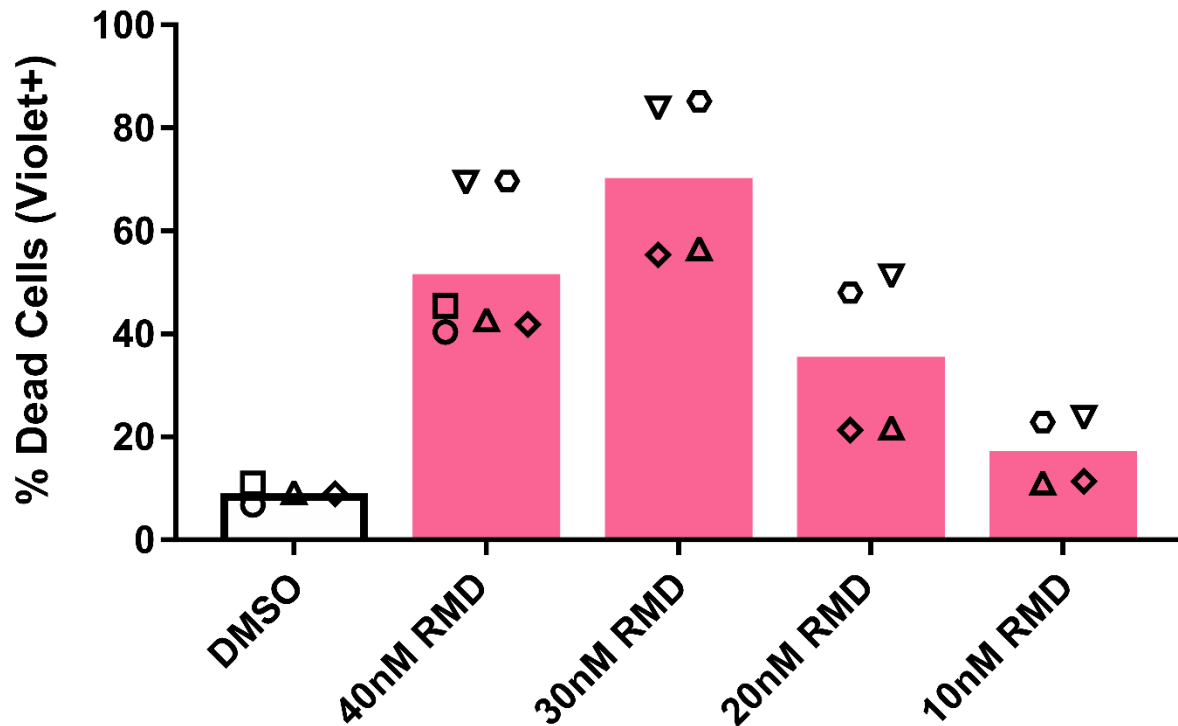


Figure 3.9. Pulsing uninfected resting CD4+ T-cells with 10nM and 20nM romidepsin for 4 hours decreased toxicity of the drug

Uninfected resting CD4+ T-cells were incubated with 40nM, 30nM, 20nM or 10nM romidepsin (RMD) for 4 hours. Cells were then washed and cultured for an additional 48 hours, after which cells were harvested and stained with a Live/Dead Fixable Violet Dead Cell Stain. Cells were analysed for the levels of dead cells (Violet+) using flow cytometry. Each symbol denotes a different donor $n \leq 6$.

3.4.5 Romidepsin combined with pro-apoptotic drugs lead to a decline in integrated HIV DNA

Having determined doses of LRAs and PI3K inhibitors with acceptable low levels of toxicity on primary CD4⁺ T-cells from healthy donors (Figure 3.8), we tested the ability of these drug combinations to reduce infected CD4⁺ T-cells from PLWH on ART *ex vivo*. Past studies find that pre-treating cells with pro-apoptotic drug prior to latency reversal can deplete infected cells *ex vivo* [412, 432], we tested similar timing of drug administration by adding the pro-apoptotic drugs first to pre-sensitize cells towards apoptosis, followed by LRA to drive the expression of pro-apoptotic viral proteins to deplete latently infected cells *ex vivo*.

The impact of four different pro-apoptotic drugs combined with five different LRAs were tested on total CD4⁺ T-cells from PLWH on suppressive ART. All pro-apoptotic drugs alone and romidepsin alone reduced the level of integrated HIV DNA versus the DMSO negative diluent control (Figure 3.11A). When romidepsin was combined with any of the pro-apoptotic drugs, all combinations led to a greater decline in integrated HIV DNA versus either LRA or the matched pro-apoptotic drug alone (Figure 3.11A). For example, RMD alone, IPI-443 alone and RMD+IPI-443 had mean fold changes in HIV integrated DNA of 0.72, 0.78 and 0.58 respectively (Figure 3.11A). Similarly, RMD alone, 5nM VNX and RMD+5nM VNX led to 0.72-, 0.81- and 0.54-fold changes, respectively (Figure 3.11A). The combination of romidepsin and 1 μ M VNX displayed the biggest decrease in fold change of 0.19 (Figure 3.11A), however, it is difficult to draw conclusions as this condition had only 2 donors tested.

In experiments where panobinostat (Figure 3.11B) and JQ1 (Figure 3.11C) were tested with pro-apoptotic drugs, we observed a greater decline in integrated HIV DNA in samples treated with either LRA together with 1 μ M venetoclax (PNB+1 μ M VNX mean fold change=0.47; JQ1+1 μ M VNX mean fold change=0.60), compared to the decline from each drug alone (PNB mean fold change=0.71; JQ1 mean fold change=0.88; 1 μ M VNX mean fold change=0.76, Figure 3.11B, C). No other combination of drugs was able to reduce the integrated DNA levels greater than that of the pro-apoptotic drug alone or LRA alone (Figure 3.11B, C).

In cells treated with bryostatin and PI3K inhibitor IPI-3063, we observed a decline in HIV DNA (BRY+IPI-3063 mean fold change=0.77) below the two drugs alone (IPI-3063 alone mean fold

change=0.83) that was not observed with any of the other LRAs except romidepsin (Figure 3.11D).

Finally, PMA/PHA combined with IPI-443 resulted in a 0.67-fold change in HIV integrated DNA compared the fold change induced by PMA/PHA or IPI-3063 alone, 0.74- and 0.78-fold change respectively (Figure 3.11E). Interestingly, none of the combinations of pro-apoptotic drugs combined with PMA/PHA resulted in declines in HIV DNA.

The two PI3K inhibitors IPI-443 and IPI-3063 alone also resulted in a decline in HIV integrated DNA (mean fold change=0.77; mean fold change=0.83, respectively) than cells treated with the DMSO negative diluent control (Figure 3.11A). This was not observed with the J-Lat6.3 T-cell lines where these drugs had no effect on viral transcription (Figure 3.3) or cell death (Figure 3.4) when administered alone. Venetoclax alone also induced a dose-dependent decline in HIV integrated DNA (5nM and 1uM VNX mean fold change=0.81 and 0.66 respectively, Figure 3.10A).

Synergy between the pro-apoptotic drugs and LRAs to decrease integrated HIV DNA was additionally calculated (Figure 3.12, Table 3.4) using the Bliss Independence Model previously described [471]. If the two combinations of drugs had a Bliss Independence score greater than 0, then the combination was considered synergistic. Consistent with the previous description of changes in integrated HIV DNA data (Figure 3.11), the combination of romidepsin with all pro-apoptotic drugs was synergistic (Figure 3.12A and Table 3.4). CD4⁺ T-cells treated with panobinostat or JQ1 and 1μM venetoclax were also synergistic (Figure 3.12B and C). All other combinations of pro-apoptotic drug and LRA panobinostat or JQ1 did not yield synergistic decreases in HIV integrated DNA. Although the decline of integrated HIV DNA in cells treated with bryostatin and IPI-3063 was modest (Figure 3.11D), the combination of bryostatin and IPI-3063 was also synergistic (Figure 3.12D, Table 3.4).

Taken together, we demonstrated that a synergistic increase in reduction in HIV DNA could be induced with some combinations of LRAs and pro-apoptotic drugs. Romidepsin showed

the most consistent response with a reduction in HIV DNA using a number of different pro-apoptotic drugs.

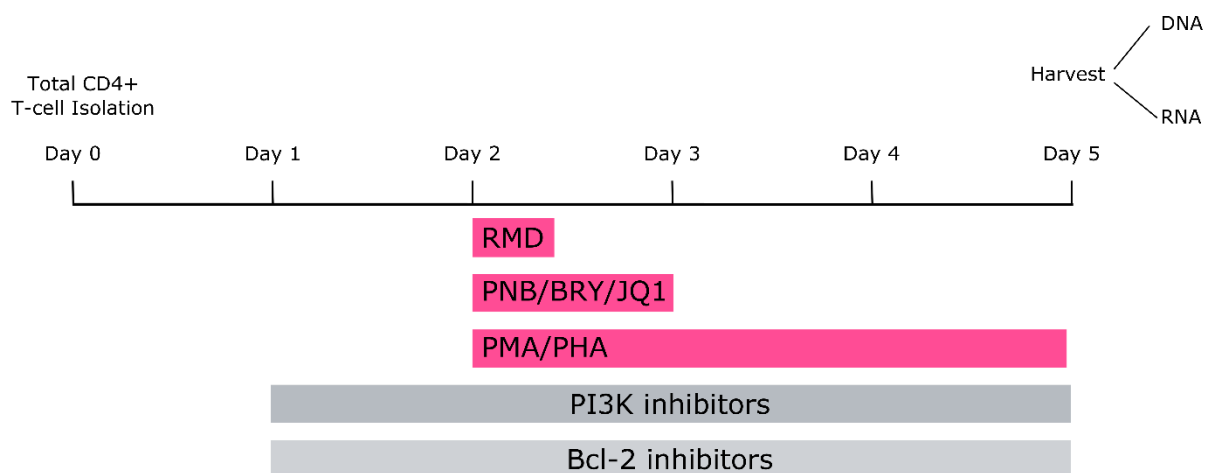
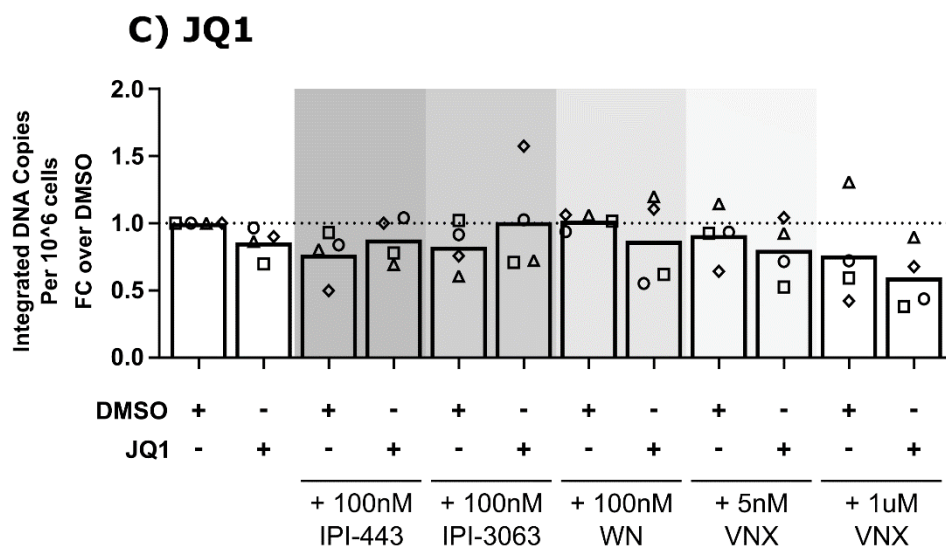
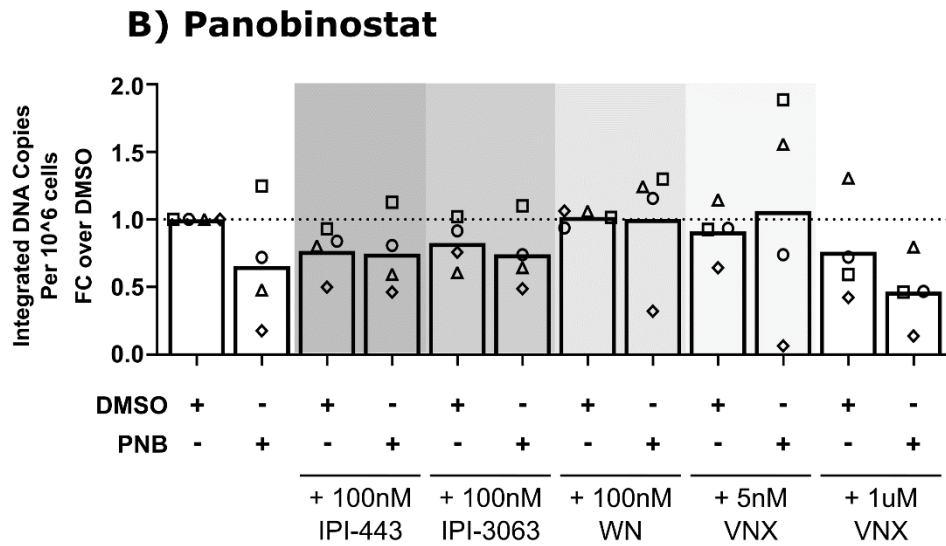
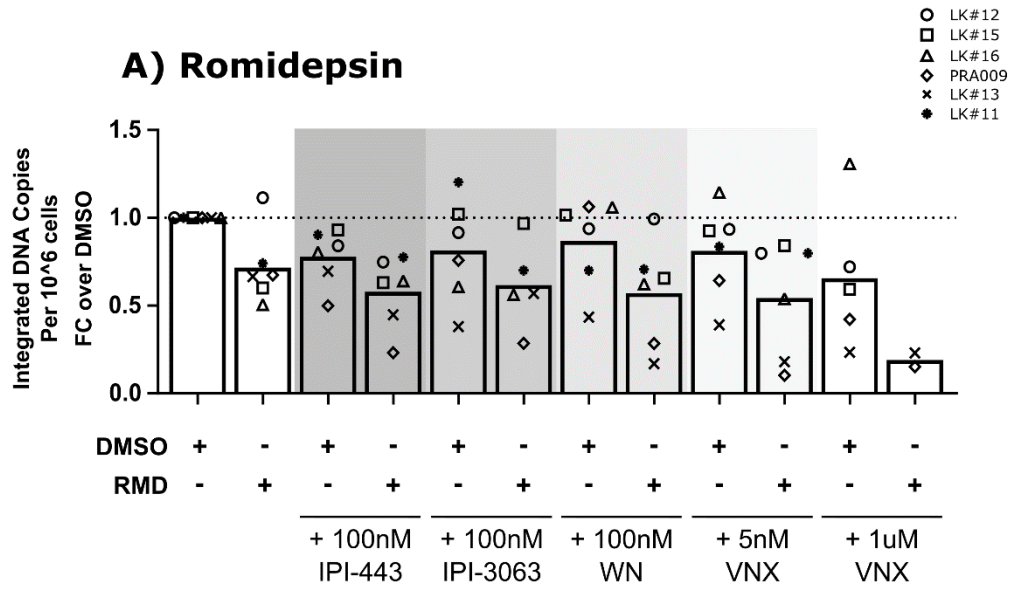


Figure 3.10. Method used to test the impact of pro-apoptotic drug and LRA combinations on total CD4+ T-cells from PLWH on suppressive ART ex vivo

Total CD4+ T-cells were isolated from cryopreserved PBMCs from PLWH on suppressive ART and left to rest for 24 hours. Cells were pre-treated with the pro-apoptotic PI3K inhibitors: IPI-443 and IPI-3063 or the Bcl-2 inhibitor venetoclax for 24 hours prior to latency reversal. Romidepsin was added to the cells for 4 hours and then washed out. Panobinostat, bryostatin and JQ1 were added for 24 hours, and then washed out. Cells were cultured in the background of the matched PI3K inhibitor or Bcl-2 inhibitor for an additional 48 hours. PMA/PHA and DMSO diluent control was added for 72 hours and not washed out. After the culture period, cells were harvested for both DNA and RNA. RMD-romidepsin; PNB-panobinostat; BRY-bryostatin.



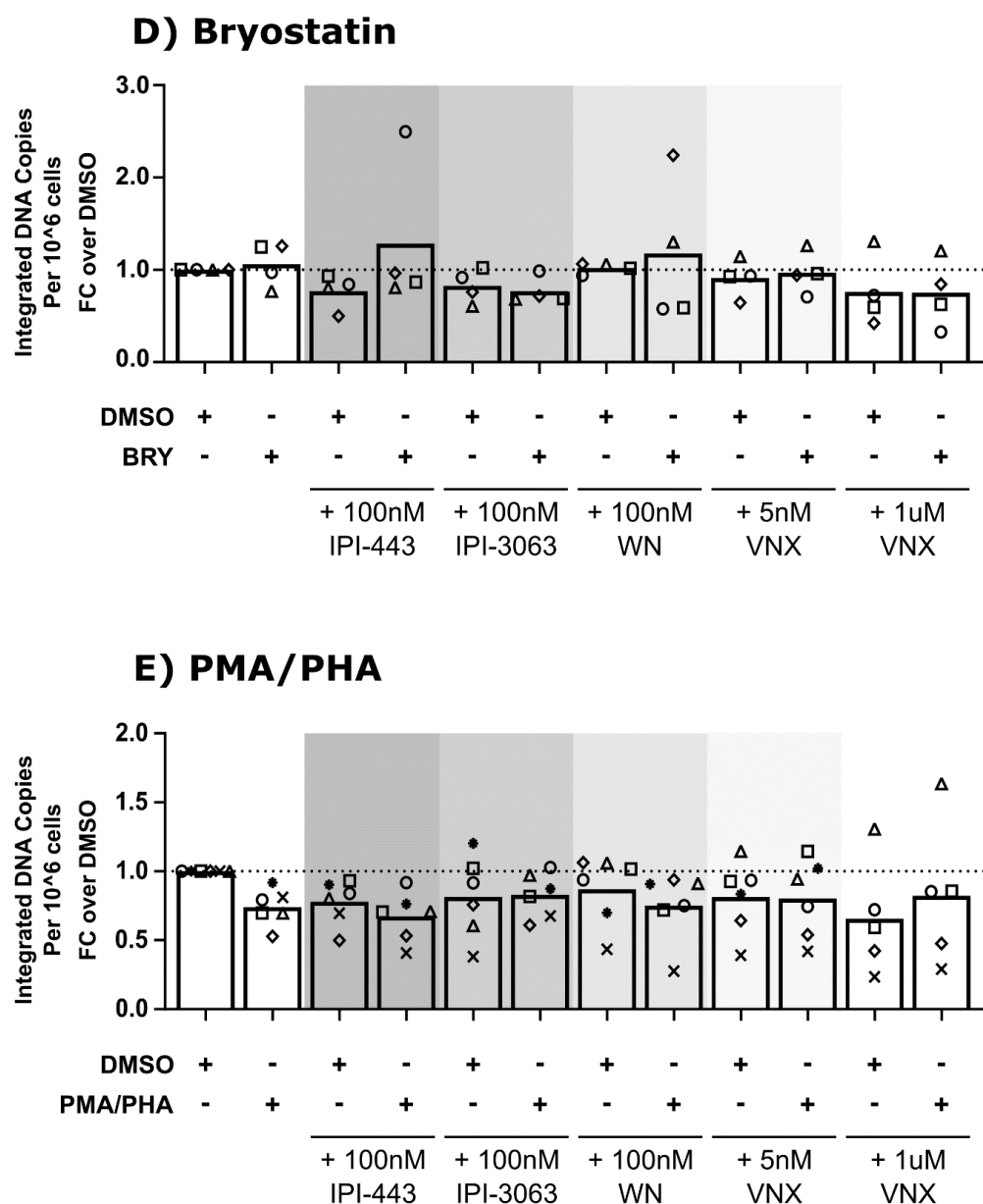


Figure 3.11. Some combinations of pro-apoptotic drugs and LRAs cause greater reductions in the levels of integrated HIV DNA from ex vivo total CD4⁺ T-cells versus either drug alone

Total CD4⁺ T-cells were isolated from PBMCs collected from PLWH on long-term suppressive antiretroviral therapy. Cells were then treated with pro-apoptotic compounds, either 100nM IPI-443, 100nM IPI-3063, 100nM wortmannin, 5nM venetoclax, 1μM venetoclax or DMSO diluent control for 24 hours. Cells were then treated with either: a 4 hour pulse of 20nM romidepsin (A), a 24 hour pulse of 30nM panobinostat (B), 1μM JQ1 (C) or 10nM bryostatin (D), or continuous culture with 10nM PMA + 10μg/ml PHA (E) or DMSO negative diluent control in the background of the pro-apoptotic drug. After the LRA pulse, cells were washed twice in media and cultured for an additional 72 hours in media in the background of the pro-

apoptotic drug. All samples were harvested and analysed for HIV integrated DNA per 10⁶ cells using qPCRs for HIV integrated DNA and CCR5. Fold change (FC) in the levels of integrated HIV DNA versus DMSO is shown, n≤6. RMD-romidepsin; PNB-panobinostat; BRY-bryostatin; WN-wortmannin; VNX-venetoclax. Each symbol represents a different donor. The dotted line represents the DMSO control.

Table 3.4. Bliss Independence scores for the combination of pro-apoptotic drugs and LRAs

		LRAs			
		Romidepsin	Panobinostat	JQ1	Bryostatin
Pro-apoptotic drug	IPI-443	0.19	-0.68	-1.000	-2.86
	IPI-3063	0.64	-0.63	-.126	0.18
	WN	0.50	-1.23	-0.13	-0.39
	5nM VNX	0.32	-0.76	-0.24	-0.45
	1µM VNX	0.90	0.53	0.14	-0.19

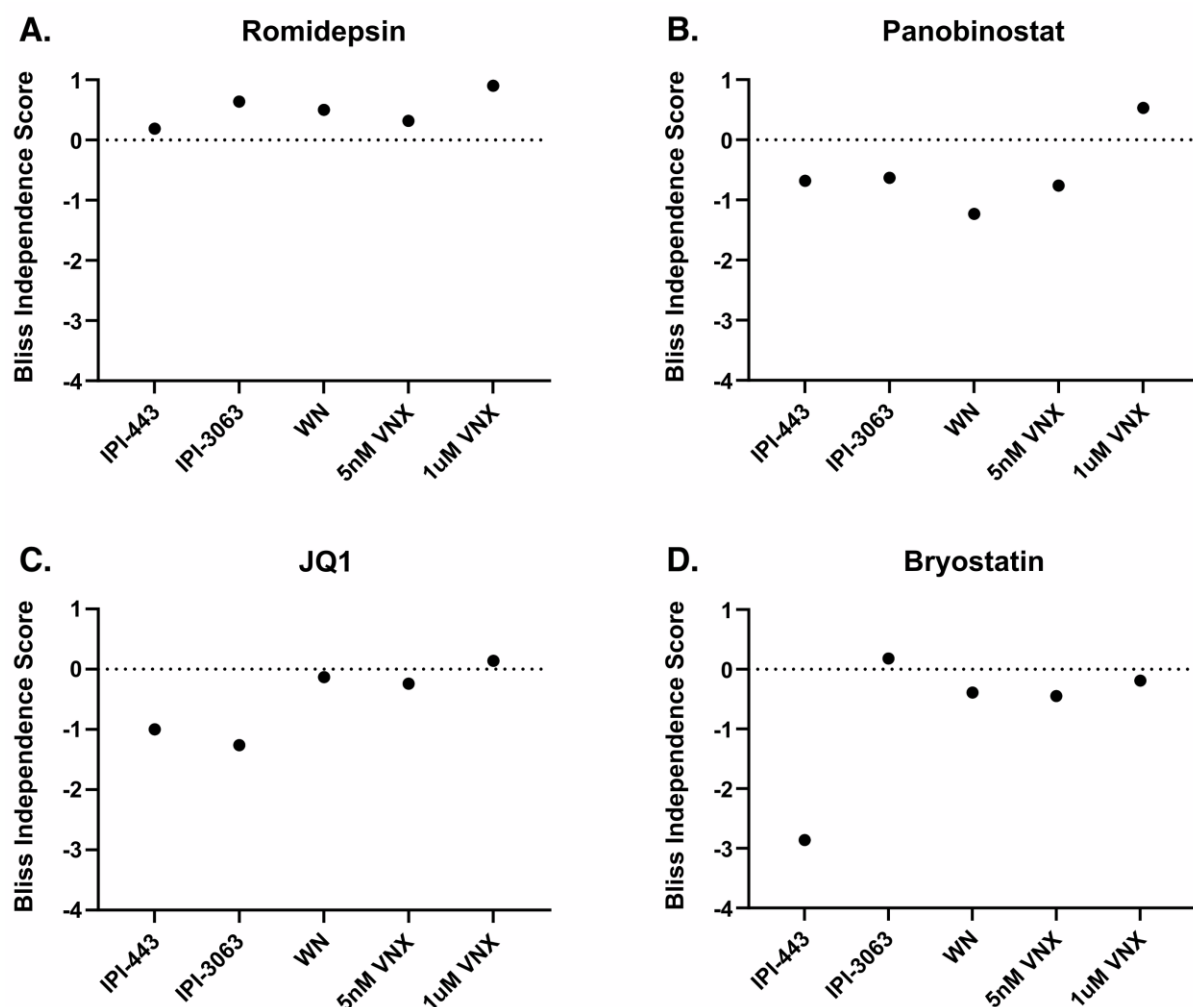


Figure 3.12. Romidepsin together with pro-apoptotic drugs demonstrates synergistic reduction in integrated HIV DNA

Total CD4⁺ T-cells from PLWH were isolated and treated using a combination of pro-apoptotic drug: IPI-443, IPI-3063, wortmannin (WM), 5nM venetoclax (VNX) or 1 μ M venetoclax together with latency reversing agents: romidepsin (A), panobinostat (B), JQ1 (C) and bryostatatin (D). The synergism between the two drugs was calculated used the Bliss Independence model. A Bliss Independence score above 1 indicates synergy, a score of 0 indicates no effect and a score below indicates antagonism.

3.5.6 PI3K inhibitors and Bcl-2 inhibitor venetoclax alone or combined with pro-apoptotic drugs results in increased HIV unspliced RNA

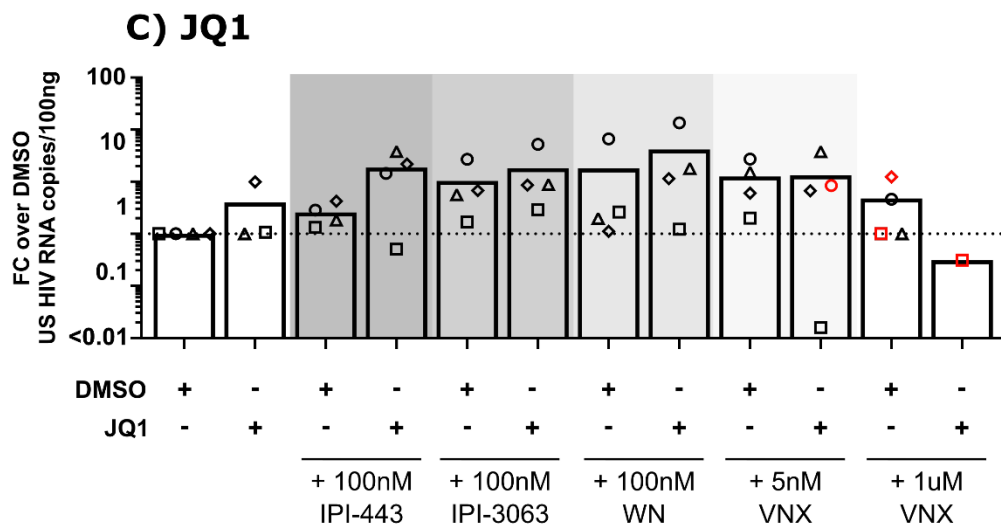
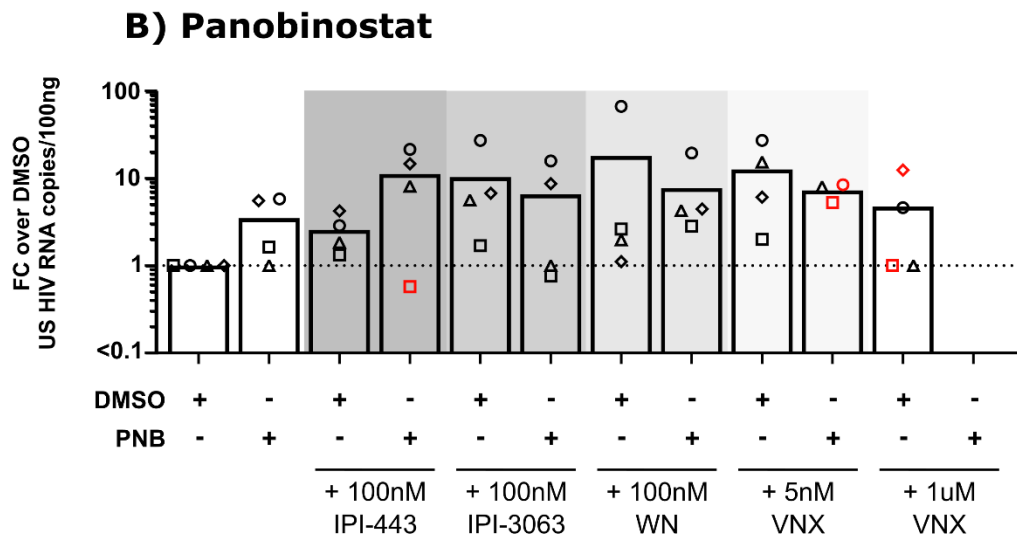
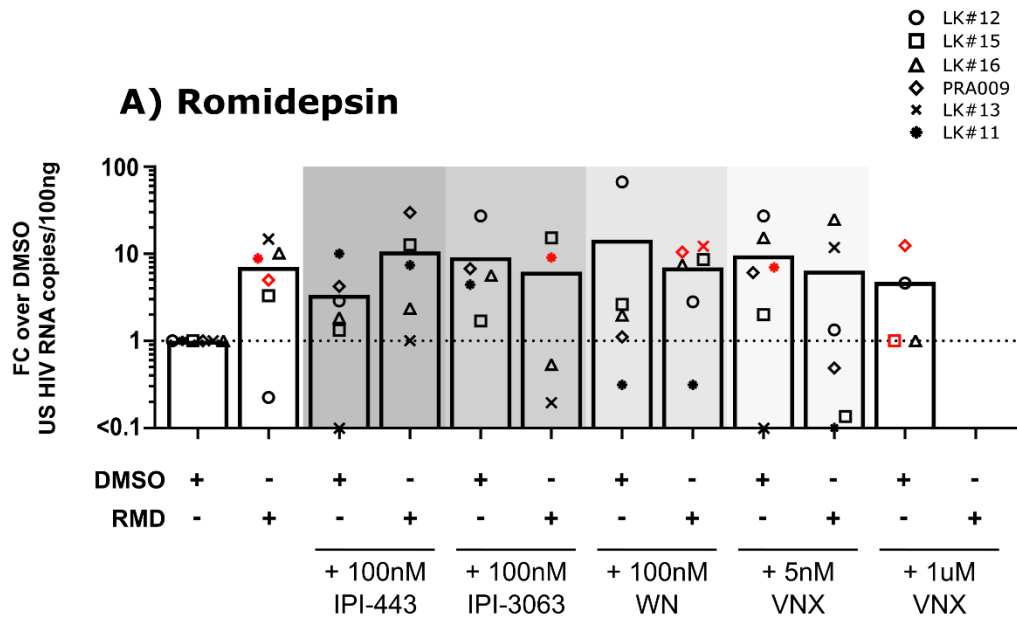
Next, we examined cell-associated HIV unspliced RNA to confirm that the LRAs alone could reactivate HIV RNA expression from latency in the *ex vivo* patient cells and to also assess if the combination of pro-apoptotic drugs and LRAs influenced the reactivation levels of HIV RNA (Figure 3.13). All of the LRAs alone overall increased the mean fold change in copies of US HIV RNA per 100ng RNA input (RMD mean fold change=7.06; PNB mean fold change=3.50; BRY mean fold change=17.73; JQ1 mean fold change=4.01; PMA/PHA mean fold change=8.97 versus DMSO). Although, not all donors individually responded to each LRA treatment (Figure 3.13). Unexpectedly, treatment with the different pro-apoptotic drugs alone also led to increased HIV RNA levels overall and in some individual donors compared to DMSO (Figure 3.13).

When the pro-apoptotic drugs and LRAs were combined, most drug combinations overall showed increased levels of HIV RNA relative to the DMSO control (Figure 3.13). We observed that several combinations of pro-apoptotic drug and LRA treatment resulted in lower levels of RNA reactivation compared to the pro-apoptotic drug alone. For instance, romidepsin combined with wortmannin had a mean fold change in HIV RNA of 6.97 compared to wortmannin alone (mean fold change=14.50), which was also observed with panobinostat plus wortmannin (mean fold change=7.74) versus wortmannin alone (mean fold change=18.04, Figure 3.13A, B). Romidepsin together with IPI-3063 also had reduced levels of HIV RNA (mean fold change=6.23) compared to IPI-3063 alone (mean fold change=9.12). This was also observed with panobinostat (PNB+IPI-3063 mean fold change=6.55) and bryostatin (BRY+IPI-3063 mean fold change=8.70) compared to IPI-3063 alone (mean fold change=10.29).

Venetoclax (5nM) combined with either panobinostat (PNB+5nM VNX mean fold change=7.25) or bryostatin (BRY+5nM VNX mean fold change=2.08) had lower levels of HIV RNA than the samples treated with 5nM venetoclax (mean fold change=12.66). The highest dose of venetoclax combined with bryostatin (BRY+1 μ M VNX mean fold change=1.89), JQ1 (JQ1+1 μ M VNX mean fold change=0.31) was also lower than cells treated with 1 μ M venetoclax alone (mean fold change=4.75). However, there was only one donor value for samples treated with

JQ1 and 1 μ M venetoclax, thus confounding the result. HIV US RNA was not able to be measured in any samples treated with romidepsin or panobinostat and 1 μ M venetoclax due to either low RNA input for the RT-qPCR, low copies that could not be quantified by the standard curve, or samples having low levels of qPCR products with dual peak melting temperatures that cannot be quantified by this qPCR.

In summary, the combination of LRA together with PI3K inhibitor IPI-443 overall lead to an increase in US HIV RNA compared to IPI-443 alone (Figure 3.13). Additionally, other LRA and pro-apoptotic drug combinations increased HIV RNA versus the pro-apoptotic drug alone, which was commonly observed with the PMA/PHA LRA with all pro-apoptotic drugs except when combining with the high 1 μ M dose of venetoclax.



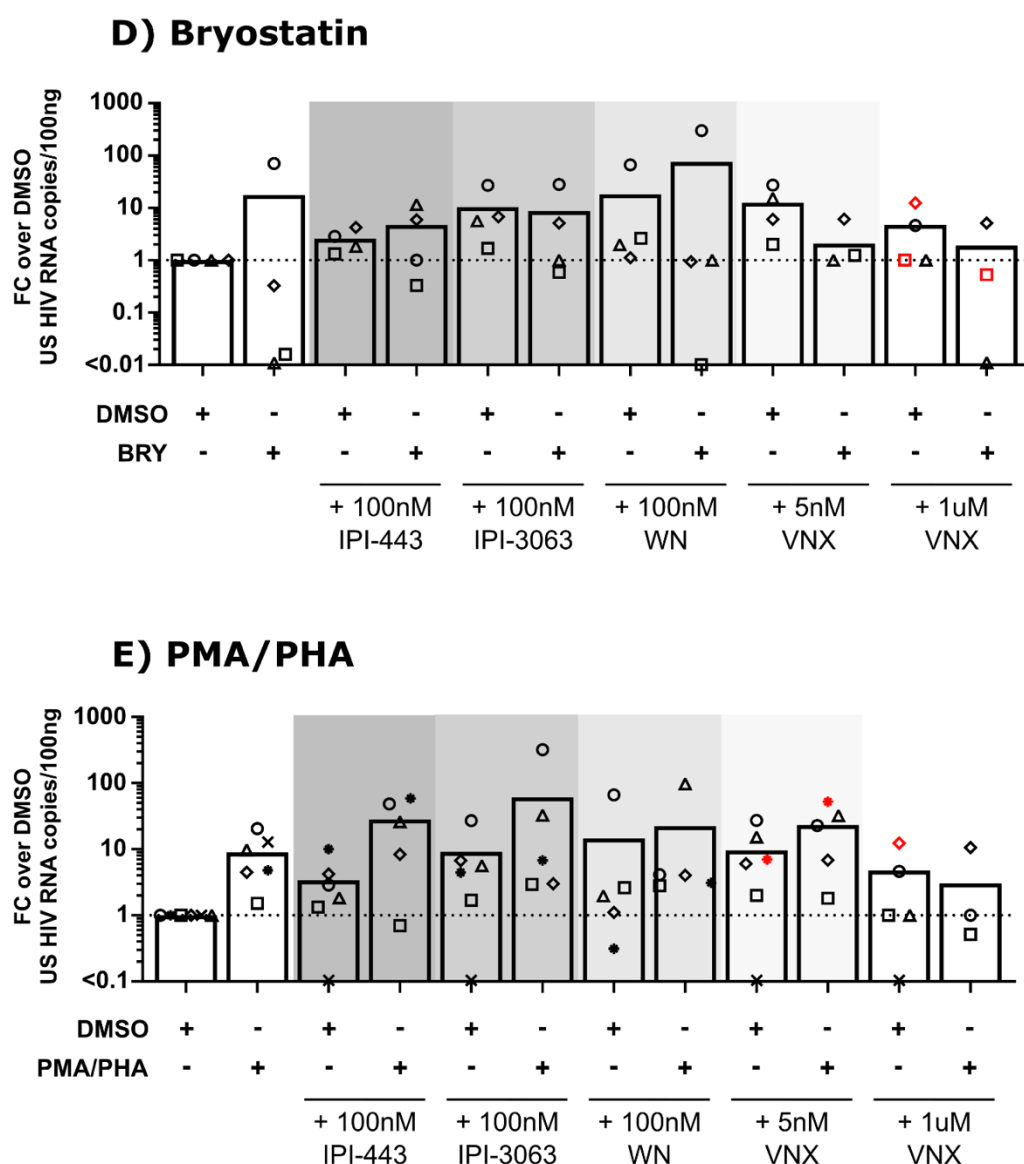


Figure 3.13. LRAs alone or together with pro-apoptotic drugs can reactivate latent HIV and increase the levels of unspliced HIV RNA in total CD4+ T-cells from PLWH on ART ex vivo

Total CD4+ T-cells were isolated from PBMCs derived from PLWH on long-term suppressive ART. Cells were treated with pro-apoptotic drugs: 100nM IPI-443, 100nM IPI-3063, 100nM wortmannin, 5nM venetoclax, 1μM venetoclax or DMSO diluent control for 24 hours. LRAs were then added, including; 20nM romidepsin (A) for 4 hours, or 30nM panobinostat (B) 1μM JQ1 (C) and 10nM bryostatin (D) for 24 hours. After incubation with these LRAs, cells were washed twice and cultured for an additional 48 hours in the background of the pro-apoptotic drugs. PMA/PHA or the DMSO diluent control was added to cells for the full 72 hours without being washed out. All cells were harvested for RNA and unspliced HIV RNA copies per 100ng

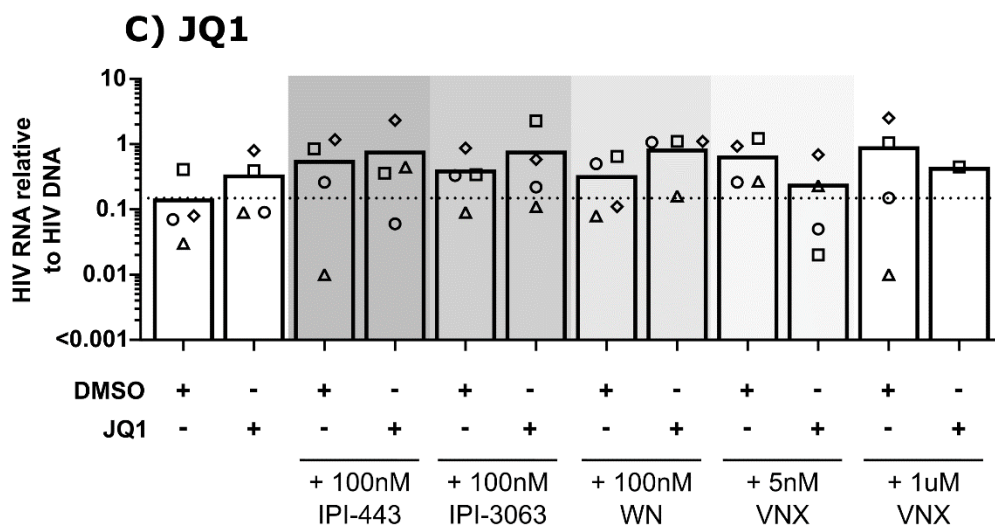
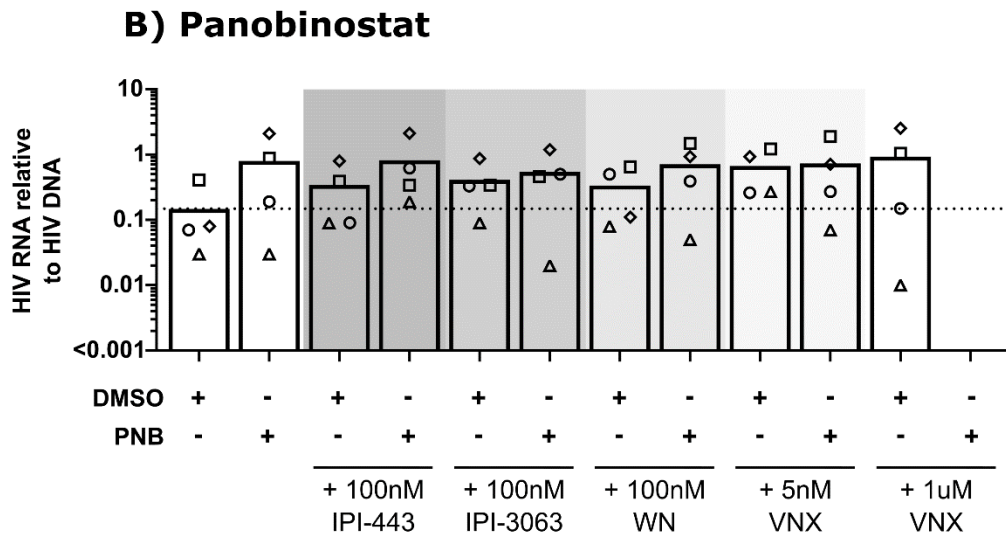
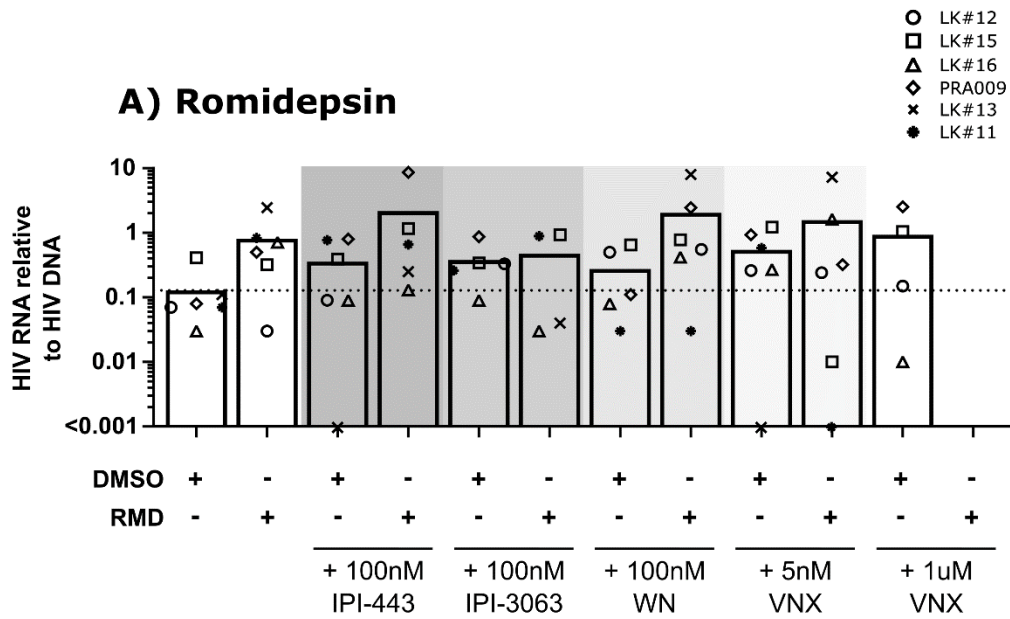
RNA input was determined using RT-qPCR. RMD-romidepsin; PNB-panobinostat; BRY-bryostatin; WN-wortmannin; VNX-venetoclax. Each symbol represents a different donor, $n \leq 6$. Symbols shown in red had $>50\text{ng}$ but $<100\text{ng}$ input RNA in the RT-qPCR, and their values have been adjusted for the equivalent of 100ng RNA input. Samples with less than 50ng RNA input, or with qPCR peaks of incorrect melt temperature were excluded from analysis.

Examining the samples that saw a reduction in the levels of integrated HIV DNA, we observed complimentary increases in cell-associated unspliced HIV RNA with the combination of RMD and pro-apoptotic drugs, bryostatin and IPI-363 and PMA/PHA and IPI-443. There was no data available for cells treated with panobinostat and 1 μ M venetoclax and only one value was obtainable for JQ1+1 μ M venetoclax. Values were excluded during the analysis of the raw data (exclusion criteria can be found in the methods section).

In all samples treated with a pro-apoptotic drug or an LRA alone or in combination, there was an increase in HIV RNA relative to integrated HIV DNA compared to the DMSO negative diluent control (Figure 3.14) The increase in the ratio of HIV RNA to DNA indicates that all drug treatment, whether alone or in combination led to an increase in HIV transcription per cell, assuming there is one copy of integrated DNA per cell.

An important caveat of the unspliced HIV RNA analysis is that it is based on SYBR green detection of amplified products. A number of values were omitted from analysis as the samples did not generate the melt temperature peak expected for the HIV wildtype product in the qPCR (Figure 3.15, Supp Table 3.2), thus potentially impacting the interpretation of results.

Taken together, the data indicates novel combinations of pro-apoptotic drugs and LRAs can successfully reactivate latent HIV transcription (measured by the amount of cell-associated unspliced HIV RNA) whilst at the same time reducing the levels of integrated HIV DNA in total CD4⁺ T-cells from PLWH *ex vivo*. Romidepsin in particular shows promise as an LRA to administer with the pro-apoptotic drugs; IPI-443, IPI-3063, wortmannin or venetoclax as all of these combinations led to an increase in HIV RNA transcription but importantly led to a decline in HIV DNA.



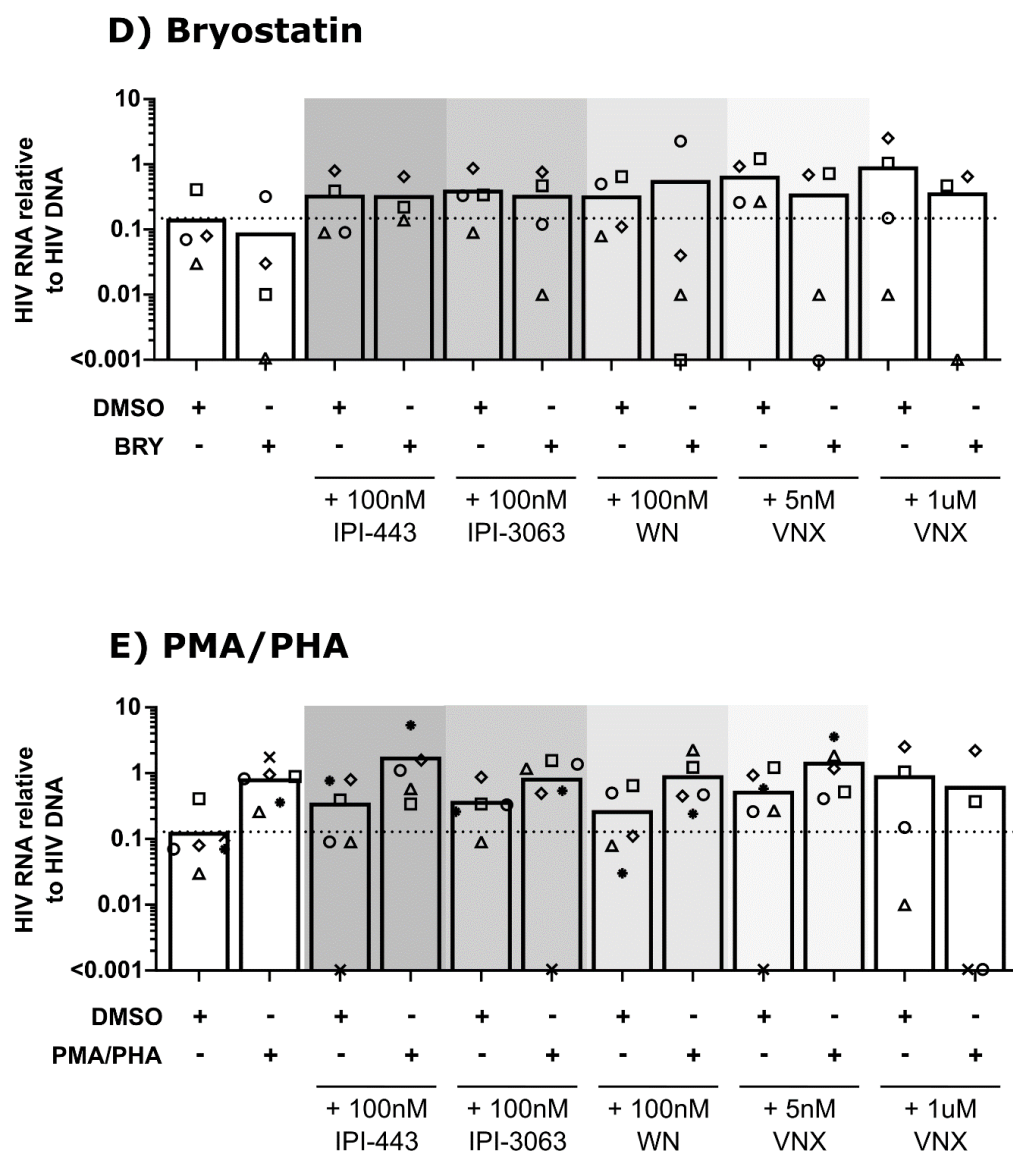
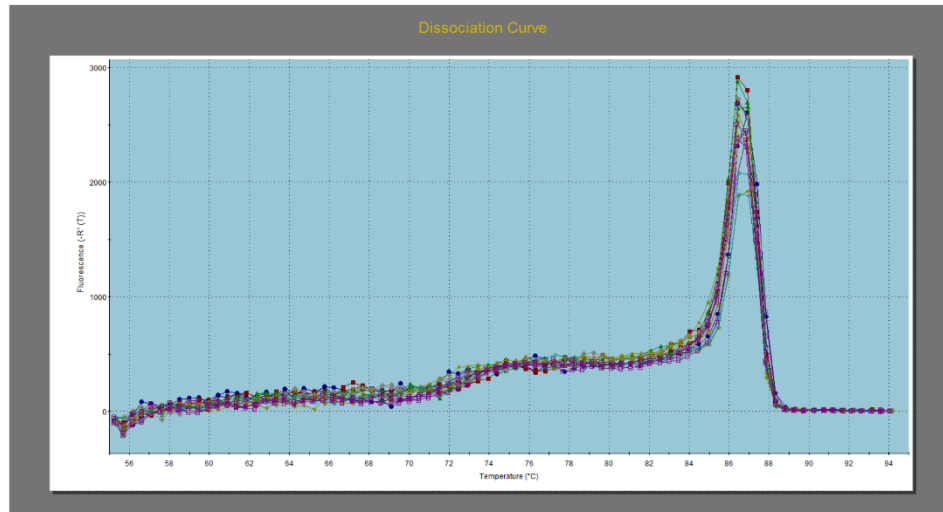


Figure 3.14. All pro-apoptotic and LRA drug compounds administered alone or in combination increased the mean ratio of RNA to DNA compared to the DMSO control

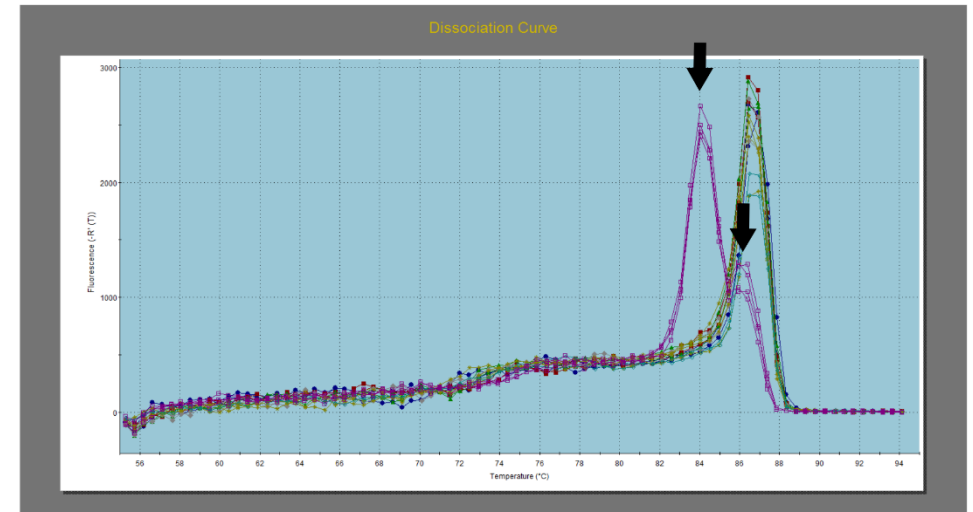
Total CD4⁺ T-cells were isolated from PBMCs of PLWH using negative bead selection. Cells were rested for 24 hours and then treated with pro-apoptotic drugs: IPI-443, IPI-3063, wortmannin (WN) and 2 doses of venetoclax (VNX) (5nM and 1μM) for 24 hours. Cells were then reactivated with LRAs for; 4 hours with 20nM romidepsin (A) or 24 hours with 30nM panobinostat (B), 1μM JQ1 (C) and 10nM bryostatin (D). Cells were put back into pro-apoptotic drug media and cultured for an additional 48 hours. Samples were treated with PMA/PHA (E) received continuous 72 hour drug treatment. Following treatment, cells were harvested, DNA and RNA were extracted and analysed using qPCR and RT-qPCR respectively. The amount of HIV RNA relative to the levels of integrated HIV DNA are shown above. RMD-romidepsin; PNB-

panobinostat; BRY-bryostatin; WN-wortmannin; VNX-venetoclax. Each symbol represents a different donor, $n \leq 6$ and the bar graph represents the mean of the values.

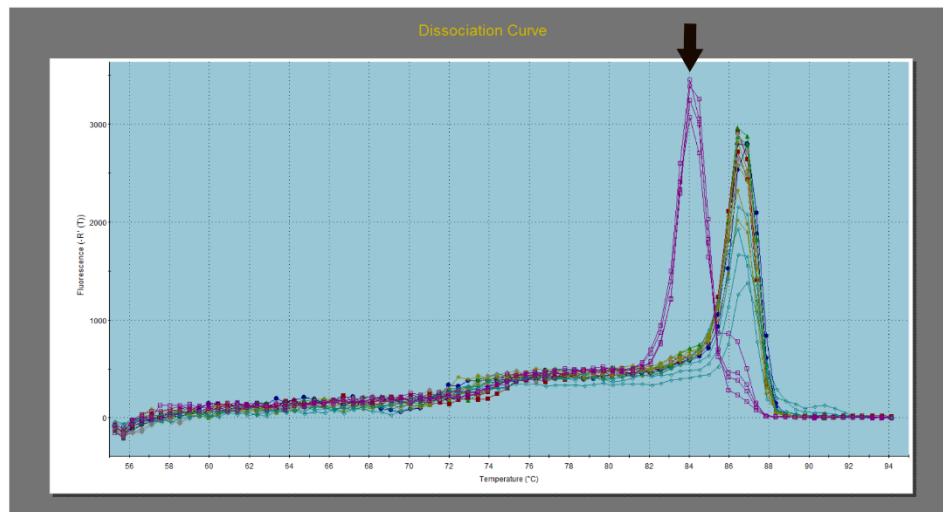
A. Wildtype Peak



B. Dual Peak



C. Shifted Peak



D. Non-specific Binding

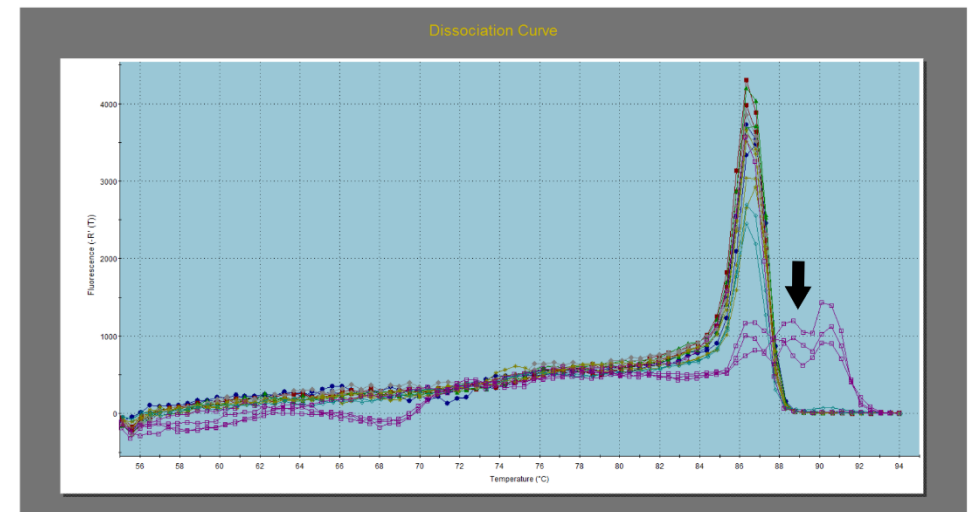


Figure 3.15. Representative melting temperature disassociation curves from cell-associated unspliced HIV RNA qPCR in samples that display wildtype and/or unusual melting curves

Unspliced HIV RNA RT-qPCR results from a representative donor (LK#12) that demonstrates: (A) a “wildtype” disassociation melt curve including RNA standards and a sample treated with panobinostat (in purple), (B) a dual peak (in purple, black arrow) that displays two distinct peaks, (C) a complete shift (in purple, black arrows) in the melt curve from the “wildtype” peak and (D) non-specific binding (in purple, black arrow) .

3.6 Discussion

Previous clinical trials involving LRAs with compounds that reactivate latent HIV transcription, have not led to declines in the frequency of infected cells, measured as HIV DNA [242, 244, 248, 475, 476]. Emerging evidence has revealed that pre-sensitisation of latently infected CD4+ T-cells with pro-apoptotic compounds prior to latency reversal can prime the cell towards apoptosis [412, 432]. Here, we demonstrate that novel combinations of pro-apoptotic drugs and LRAs that can reactivate latent HIV and importantly, reduce the frequency of integrated HIV DNA in CD4+ T-cells from PLWH on ART *ex vivo*, warranting their future clinical investigation. In contrast, these drug combinations did not lead to a reduction of infected cells using the latently infected J-Lat6.3 T-cell line. Thus, these data also demonstrate the importance of evaluating new agents that deplete latently infected cells using primary CD4+ T-cells from PLWH on ART *ex vivo*, rather than using latently infected cancerous cell lines.

When combinations of pro-apoptotic drugs and LRAs were tested in total CD4+ T-cells from PLWH on ART *ex vivo*, treatment with romidepsin and any of the pro-apoptotic drugs successfully resulted in larger declines in the number of integrated HIV DNA copies versus either drug alone (Figure 3.11A) and the effect of the pro-apoptotic drug and LRA combinations were synergistic (Figure 3.12 and Table 3.4). This effect was observed with the romidepsin LRA, however there was a trend towards similar results in some conditions using panobinostat. There may be several explanations for these findings. First, the potency of romidepsin as an activator of HIV transcription may be relevant here. Romidepsin is a more potent LRA than panobinostat when compared using an *ex vivo* assay [246] and as observed in clinical trials in PLWH [244, 248]. Romidepsin also induced 1.75-fold greater increase in CA US RNA compared to JQ1 increase (Figure 3.13). Thus, the increased potency of romidepsin may better drive the production of pro-apoptotic viral proteins such as viral protease, Tat and Env that could contribute to inducing apoptosis [411]. Recently, Yukl *et al.* demonstrated that different LRAs have differing capabilities to overcome numerous blocks in HIV latency and produce mature viral RNA species that would express pro-apoptotic viral proteins [303]. Romidepsin was able to generate mature HIV RNAs in all 5 donors tested similar to panobinostat (4 of 5 donors) but there was little activity by JQ1 alone by comparison [303].

However, this does not explain why bryostatin or PMA/PHA T-cell stimulation, which induced a similar mean fold increase in CA US HIV RNA to romidepsin (Figure 3.13), did not lead to greater declines in infected T-cells. Stimulation with PMA/PHA leads to T-cell activation and is comparable to anti-CD3/CD28 T-cell activation and should generate viral proteins consistent with quantitative and/or viral outgrowth assay studies performed elsewhere [29, 146, 414, 457]. This suggests that romidepsin may have additional, unique properties to help deplete HIV latently infected T-cells when combined with pro-apoptotic drugs. Such unique properties might be the select inhibition of HDAC1 and 2 [246] or the sustained inhibition of cell-associated HDAC activity maintained by romidepsin compared to panobinostat or vorinostat which correlated to persistent activation of HIV induction [246]. Finally, romidepsin is well known to induce cell death on its own. The doses of romidepsin used in these experiments were titrated to ensure little to no toxicity (Figure 3.8), however romidepsin can activate apoptotic pathways through the upregulation of proteins such as caspase-3 and BAK protein [477], which leads to cell death. Despite the effect romidepsin can have on cells, we are confident the reduction in integrated HIV DNA was selective as the frequency of integrated HIV declined (Figure 3.11). If romidepsin caused all cells to die, the frequency of integrated DNA would remain the same.

Apart from romidepsin and any pro-apoptotic drugs tested, we also observed synergistic decreases in integrated HIV DNA using panobinostat or JQ1 LRA combined with 1 μ M venetoclax (Figure 3.11). Cummins *et al.* previously demonstrated that priming of CD4+ T-cells *ex vivo* using 1 μ M venetoclax followed by α -CD28/ α -CD3 T-cell stimulation as an LRA depleted total HIV DNA in total CD4+ T-cells from PLWH on ART *ex vivo* [412]. However, in our hands, venetoclax alone at 1 μ M has >70% toxicity using resting CD4+ T-cells from healthy donors *ex vivo* (Manuscript in preparation), and therefore it is unclear if the decline in infected cells was a result of non-specific cell death which was potentially further exacerbated by adding the LRAs. This is suggested by the HIV US RNA analysis of these drug combination samples, where US RNA data was lacking for many donors (Figure 3.13) due to low RNA input for the RT-qPCR or HIV RNA values being below the limit of detection (1 copy) potentially due to HIV RNA degrading in dead cells. Taken together, these drug combinations could reduce

the frequency of infected cells, although non-specific cell death from venetoclax should be carefully investigated.

A recent paper also found that treatment of CD4⁺ T-cells from PLWH with 1 μ M venetoclax resulted in non-specific T-cell toxicity [478]. However, this is in direct opposition to Cummins *et al.* who found that up to 1 μ M venetoclax for 5 days treatment of CD4⁺ T-cells from uninfected donors did not display reduced viability [412]. In the Cummins study, CD4⁺ T-cells isolated from HIV negative donors were cultured for 5 days in the presence of 1 μ M venetoclax. In contrast, Ren *et al.* [478] co-cultured central memory CD4⁺ T-cells with effector CD8⁺ T-cells, both from HIV negative individuals in the presence of venetoclax and both studies measured viability using the Live/Dead Fixable Cell Death stain. The recommended dose of venetoclax in individuals with relapsed or refractory CLL is a daily dose of 400mg [479]. This calculates to approximately 2.42 μ M for an *in vitro/ex vivo* experiment. There is clearly a disconnect between what is observed in humans treated with venetoclax and what is observed in cultured cells *ex vivo*. This may be related to issues with tissue penetration of the drug, plasma concentration and half-life of the drug in humans all of which would differ in a laboratory setting where drug is added directly to cells in culture. Of note, the use of venetoclax in patients with CLL is reported to have the side effects of nausea, anaemia, thrombocytopenia and fatigue [480].

In our studies, combining 1 μ M venetoclax with either PMA/PHA T-cell stimulation or bryostatin LRA did not induce larger declines in integrated HIV DNA versus either drug alone (Figure 3.11C). Our findings with bryostatin are similar to a recent report from another group [478]. Bryostatin alone and in combination with venetoclax 1 μ M increased HIV US RNA, (Figure 3.13D), but it is possible that there were downstream blocks in generating mature HIV RNA and/or viral proteins. However, this is unlikely to explain the PMA/PHA result as PMA/PHA T-cell stimulation has previously been shown to generate HIV MS RNA viral protein expression and virion release [102, 164, 481]. An alternative explanation is that bryostatin and PMA/PHA can both induce proliferation of T-cells [482, 483] which could also include proliferation of infected cells. Indeed, the combination of PMA/PHA with 1 μ M venetoclax resulted in a small increase in HIV integrated DNA levels (Figure 3.11). The impact of these

specific drug combinations on T-cell proliferation and survival could be tested in future work staining for Ki67 proliferation or a marker of proliferation such as carboxyfluorescein succinimidyl ester (CFSE) together with a Live/Dead stain in a drug time course assay.

We surprisingly observed that 5nM and 1 μ M venetoclax alone led to an increase in CA-US HIV RNA as well as a decline in infected T-cells *ex vivo* in a dose-dependent manner (Figure 3.13-14). This latency reversing property of venetoclax was an unexpected, novel finding. Previous work in latently infected T-cell lines demonstrated cellular Bcl-2 negatively regulated HIV replication [484]. Thus, venetoclax perhaps led to a release of the Bcl-2 block on HIV replication which occurs following translation of proteins, and subsequent increase in HIV RNA (Figure 3.13).

We also observed that the three PI3K inhibitors (IPI-443, IPI-3063 and wortmannin), were also able to induce HIV transcription and reduce the levels of integrated HIV DNA in *ex vivo* patient CD4+ T-cells (Figure 3.13-14). To our knowledge, this is the first time anyone has reported an effect of these three PI3K inhibitors having latency reversing activity and depleting infected CD4+ T-cells from PLWH on ART *ex vivo*. Inhibition of PI3K activity and therefore inhibition of phosphorylated Akt (pAkt) could sensitise the cells towards apoptosis as previously demonstrated [485]. However, we expected that the ability of PI3K inhibitors to inhibit pAkt and subsequent activation of NF- κ B [486] would reduce the recruitment of NF- κ B to the HIV LTR, thereby decreasing HIV transcription from integrated provirus. While the mechanism of this effect on HIV transcription is unclear, one explanation could be the induction of stress in infected CD4+ T-cells. Induction of the heat shock protein 90 (Hsp90) molecular chaperone by cellular stresses like proteasome inhibitors or heat shock can induce HIV transcription in both an *ex vivo* model of CD4+ T-cells from PLWH on ART and in an *in vivo* humanised mouse model of HIV infection on ART [487-489]. Additionally, the PI3K inhibitors could have increased HIV transcription through an effect on cell cycling. Glycogen synthase kinase-3 β (GSK-3 β) phosphorylates cyclin D1 resulting in cell cycle progression [490] and in the presence of pAkt, GSK-3 β is inhibited and in turn so is cyclin D1, resulting in cell cycle arrest. Spontaneous reactivation of HIV latency has been reported in cells that express high levels of Nuclear Enriched Abundant Transcript 1 (NEAT1) [491] which can activate cyclin D1 in cancer cells. Thus, an increase in cyclin D1 in latently infected cells following inhibition of PI3K may have

led to reactivation of HIV transcription (Figure 3.13) [490]. While the exact mechanism by which PI3K inhibitors can reactivate latent HIV and increase death has not been delineated, these findings warrants further investigation. Venetoclax and the PI3K inhibitors duvelisib [492] and idelalisib [493] are already licensed to treat various immune cell cancers and could be readily advanced to clinical testing in PLWH on ART.

We also observed a decrease in US HIV RNA in some experimental conditions compared to the drug treatment alone. The addition of romidepsin or panobinostat to the PI3K inhibitor IPI-3063 led to a decrease of unspliced HIV RNA compared to the IPI-3063 alone, as well as a decrease in the levels of integrated HIV DNA (Figure 3.11, 13). However, when examining the ratio of HIV RNA relative to integrated HIV DNA, all conditions which included the pro-apoptotic drug alone and in combination with LRAs resulted in an increase of HIV transcription per DNA (Figure 3.14). In cancer cell lines, the combination of HDACis such as panobinostat and vorinostat with PI3K inhibitors inhibited the phosphorylation and activation of Akt [494, 495]. This inhibition of pAkt can lead to an increase in apoptosis, but the inactivated Akt protein can also lead to a decrease in the recruitment of transcription factors to the LTR such as NF- κ B [496]. Another explanation could be that with enhanced cell death of infected cells, there is degradation of RNA transcripts within the dead cells [412].

Conversely, we observed an increase in HIV US RNA in samples treated with the PI3K inhibitor IPI-443 combined with an LRA versus IPI-443 alone (Figure 3.13). This might be explained by the different isoforms that the two PI3K inhibitors target. IPI-443 is a potent dual inhibitor of PI3K δ and PI3K γ isoforms and IPI-3063 inhibits the PI3K δ isoform only. While both isoforms are mainly expressed in leukocytes [497], the PI3K γ isoform plays an important role in innate immune cell regulation and inflammatory responses [497, 498]. The impact of PI3K γ on the innate immune system may contribute to the reactivation profile of IPI-443 (Figure 3.13) and warrants further elucidation.

Samples treated with PMA/PHA and all pro-apoptotic drugs except 1 μ M venetoclax also showed an increase in CA US HIV RNA compared to the pro-apoptotic drug or PMA/PHA alone (Figure 3.13). This might be explained by the competing effects of PMA/PHA inducing HIV RNA

transcription and cellular proliferation, overriding the effects of the pro-apoptotic drug. In the absence of cell death, the combined effects of both drugs may have been to just increase CA US HIV RNA. We observed that following treatment with PMA/PHA, we were unable to quantify CA US HIV RNA in a significant number of samples due to the occurrence of dual or shifted melting temperature peaks (Supp Table 3.2). We do not currently understand the reason for this, but one possible cause could be mutated HIV RNA products that lower the qPCR product melting temperature. Why these aberrant HIV RNA products would be more common following activation with PMA/PHA remains unclear. In the future, RNAseq could potentially be used to measure the abundance and production of wildtype versus defective HIV RNAs in these samples.

Finally, an important caveat of the experimental method used here was that HIV integrated DNA was measured as a surrogate for infected T-cells. However, in PLWH on ART, most proviruses are defective and only a small pool of intact, replication-competent provirus exists [145, 499]. Thus, it is unclear whether pro-apoptotic drug and LRA combinations have different effects on infected cells with replication-competent intact provirus or defective virus. In the future, using a quantitative viral outgrowth assay to measure replication-competent provirus [164] and IPDA to measure intact and defective provirus [152] could be used to help elucidate which treatment combinations resulted in the decline of replication-competent or intact virus.

When pro-apoptotic drugs and LRAs were initially tested in J-Lat6.3 T-cells, the PI3K inhibitors IPI-443 and IPI-3063 alone did not reactivate viral transcription or enhance cell death (Figure 3.3-4). In contrast, the LRAs alone increased reactivation of latent HIV as expected and induced some level of cell death to varying magnitudes (Figure 3.7). However, when we combined the PI3K inhibitors with LRAs romidepsin, vorinostat and bryostatin, comparable levels of viral GFP expression and cell death were observed compared to the cells treated with each inhibitor alone (Figure 3.7). Strikingly, the combination of panobinostat+PI3K inhibitor profoundly reduced the levels of GFP+ cells (Figure 3.7).

Alternatively, the combination of panobinostat and PI3K inhibitors may impair latency reversal by panobinostat, perhaps via PI3K inhibitor-mediated inhibition of pAkt, which can activate NF- κ B [500, 501]. If the combination of panobinostat and PI3K inhibitor decreased Akt phosphorylation and NF- κ B activation, a decrease in viral reactivation could be observed, as seen here (Figure 3.7A). However, a decrease in pAkt should also increase apoptosis and cell death [502], which was not seen here (Figure 3.7B), suggesting this explanation is unlikely. As the PI3K inhibitor was administered before panobinostat, a decline in pAkt generation and NF- κ B expression by the PI3K inhibitor may limit the subsequent reactivation of latent HIV by panobinostat, which unravels chromatin for HIV transcription initiation. Reversing the order of drug administration to first unravel chromatin for HIV transcription initiation, permit gene expression with the available active NF- κ B, followed by PI3K inhibitor addition to drive apoptosis might bypass such an issue. It is curious as to why loss of reactivation was not observed when cells were treated with PI3K inhibitors and romidepsin, a more potent HDACi than panobinostat [246].

The failure of the combination of HDACi LRAs and PI3K inhibitor to increase the death of J-Lat6.3 cells in our study (Figure 3.7B) is not in agreement with other studies that have examined cancer cell lines in the absence of HIV. One such study observed an increase in vorinostat-induced cytotoxicity when vorinostat was combined with the PI3K inhibitor LY294002 [495]. This observation may be a consequence of different properties in immortalised cell lines used in our study (J-Lat6.3 T-cells) and the Erlich study (head and neck squamous carcinoma cell lines). In another study, the combination of panobinostat and a PI3K inhibitor BMK-120 also positively synergised to increase FOXO1 transcription factor that drives pro-apoptotic protein expression [494], inhibit pAkt generation and inhibit cell growth of medulloblastoma *in vivo* using a murine model [494]. In our J-Lat6.3 T-cells, an increase in cell death was not observed. To delineate the exact mechanism resulting in a decrease in virus expression but not cell death with panobinostat and PI3K inhibitors, further investigation is needed. The levels of pAkt, FOXO1 and active NF- κ B transcription factors, plus pro-apoptotic and anti-apoptotic proteins should be examined in these cell lines following drug administration.

Although we first optimised the conditions for LRAs and pro-apoptotic drugs using the J-Lat6.3 T-cell line, our findings in the J-Lat T-cell line was quite different from findings using CD4+ T-cells from PLWH on ART. There may be several reasons for these differences.

First, immortalised cell lines have perturbations related to apoptosis and proliferation. Latently infected T-cells are known to upregulate anti-apoptotic proteins such as Bcl-2 [412], making them more resistant to apoptosis. J-Lat T-cell lines also have dysregulated expression of PTEN [174]. Within the J-Lat6.3 T-cell lines, the loss of PTEN protein expression results in high levels of pAkt and in general, constitutive activation of PI3K/Akt signalling pathway [503]. The restoration of PTEN in Jurkat T-cell lines resulted in up to 50% loss of cells, as well as down-regulation of pAkt, decreased activity of Akt and importantly increased sensitivity towards apoptosis [504]. The loss of PTEN in the J-Lat6.3 T-cell line shifts the cells towards a phenotype that is inherently more resistant to apoptosis. Consequently, the addition of PI3K inhibitors may be insufficient to overcome the abundance of pAkt in this T-cell line, explaining why apoptosis was not observed (Figure 3.7B).

The site of HIV proviral integration can influence proliferation and survival of a cell [85, 86], rendering it less susceptible to apoptosis or increase the likelihood of latent reactivation through LRAs [142]. Additionally, the chromatin environment that surrounds HIV integration sites can determine the level of HIV RNA transcription [230]. The efficacy of LRAs that modify the chromatin environment such as histone deacetylase inhibitors may be linked to the site of HIV integration. Most T-cell lines including J-Lat T-cell lines are clonal and contain a single integration site [175, 185]. This is in contrast to CD4+ T-cells from PLWH, in which integration can occur in a multitude of locations, predominantly into actively transcribed chromosomal regions [204, 505]. The degree of transcriptional repression on the integration sites in CD4+ T-cells from PLWH may be due to the different chromatin environments [506], resulting in inducible latent as well as non-induced latent proviruses [145]. The number of diverse integration sites present in CD4+ T-cells from PLWH compared to J-Lat T-cell lines that have a single integration site could contribute to the differing responses to LRAs and pro-apoptotic drugs that was observed here.

Another explanation for the differences observed between T-cell lines and CD4+ T-cell from PLWH is the use of GFP as the measurement of viral reactivation in J-Lat6.3 T-cells. As the cell dies, the GFP protein might be degraded [412, 507] leading to the decrease in GFP+ cells, underestimating the frequency of cells reactivated as observed with panobinostat treatment in J-Lat6.3 T-cells (Figure 3.7A). If the two drug combinations synergised to increase reactivation of cells as well as apoptosis, we would expect to see a concurrent increase in the number of dead cells. However, as this drug combination did not enhance cell death (Figure 3.7B), this explanation is unlikely. The degradation of GFP protein makes this method of measurement unreliable as it is difficult to pinpoint the exact moment GFP protein is or isn't degraded.

Our experiments using a T-cell line did not recapitulate the effects of the drugs in primary T-cells from PLWH on ART *ex vivo*. Other investigators have reported that some LRAs can lead to significant changes in HIV expression in latently infected T-cell lines, but absolutely no response *ex vivo* [242-244, 248, 278, 508]. Additionally, cell lines are immortalised, clonal, and have a single integration site and aberrant cell signalling to allow for proliferation, replication and survival. Depletions in the T-cell line may be balanced by replenishment post-replication. The differences in response of the J-Lat cells line and cells derived from PLWH may be particularly important when evaluated pro-apoptotic compounds and needs to be considered in future work.

3.7 Conclusion

In summary, we have shown that the combination of romidepsin and all pro-apoptotic drugs resulted in a synergistic decrease of integrated HIV DNA compared to each of the drugs alone using total CD4+ T-cells from PLWH on ART *ex vivo*. Other combinations including panobinostat or JQ1 and 1 μ M venetoclax; bryostatin and IPI-3063; and PMA/PHA and IPI-443 also resulted in synergistic decreases in integrated HIV DNA compared with each drug alone. All the drugs tested were able to induce HIV viral transcription, measured as an increase in CA US HIV RNA. Interestingly, we demonstrate that the PI3K inhibitors: IPI-443, IPI-3063 or wortmannin alone, as well as venetoclax alone, can all increase transcription of HIV, as well as led to the decline of integrated HIV DNA in CD4+ T-cells from PLWH on ART *ex vivo*. The

exact mechanism of how these pro-apoptotic drugs reactivate latent HIV has not been elucidated. Here we show the promising potential of not only combinations of pro-apoptotic drugs and LRAs, but the use of pro-apoptotic drugs alone to reduce the frequency of integrated HIV DNA. Further work is needed to understand the relative reduction of intact and defective virus and potentially the effect of these combinations using an *in vivo* animal model.

3.8 Supplementary Figures and Tables

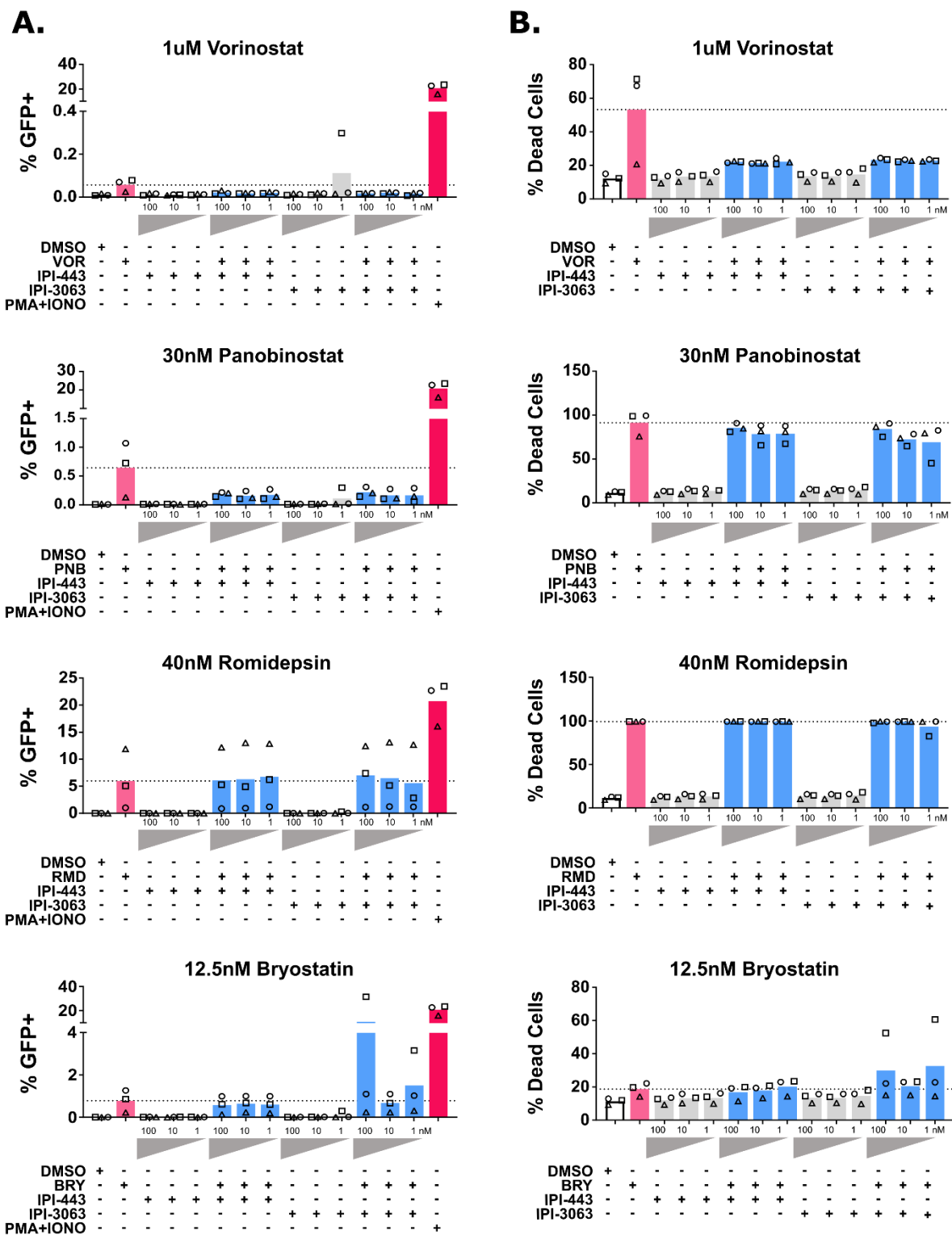


Figure S3.1. Impact of PI3K inhibitors and LRAs on viral GFP reactivation and cell death in J-Lat6.3 T-cells decreased viral GFP reporter expression

J-Lat6.3 T-cells were pre-treated with increasing doses of the PI3K inhibitors IPI-443 or IPI-3063 (1, 10 or 100 nM) for 24 hours. Four separate LRAs; 1uM vorinostat, 30nM panobinostat, 40nM romidepsin and 12.5nM bryostatins were then added to cell cultures for an additional 48 hours. Cells were harvested 48 hours after the addition of LRAs and stained with a Live/Dead Fixable Violet Dead Cell Stain and analysed using flow cytometry. Reactivation of viral GFP reporter expression (A) was determined with the percentage of GFP+ cells shown. Cells treated with PMA and Ionomycin were included as a positive control for latency reactivation. (B) The percentage of overall dead cells in the populations are shown. Each symbol denotes an experiment performed at separate times, n=3.

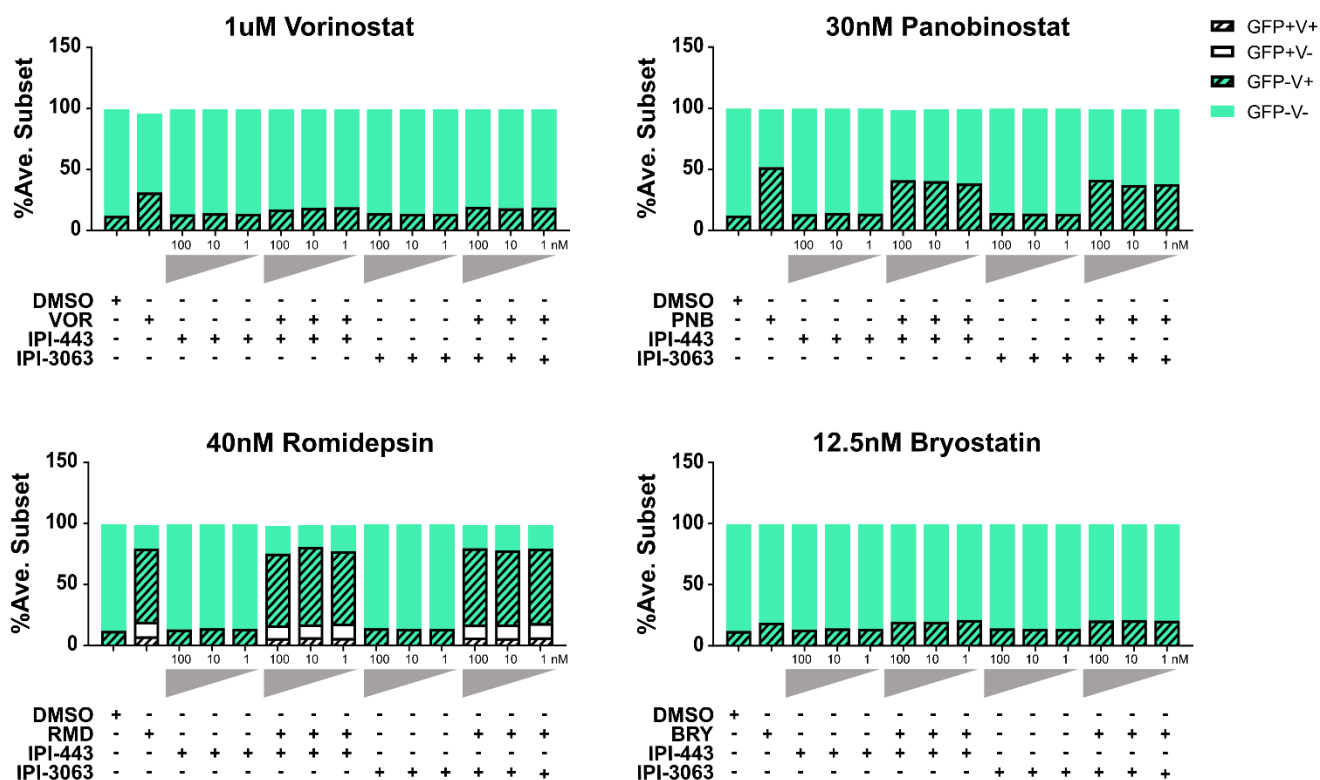


Figure S3.2. Pre-treatment of J-Lat6.3 T-cells with PI3K inhibitors together with LRAs resulted in the moderate levels of overall cell death of the reactivated GFP+ cells

J-Lat6.3 T-cells were pre-treated with doses of PI3K inhibitors IPI-443 and IPI-3063 for 24 hours. The LRAs: 1uM vorinostat, 30nM panobinostat, 40nM romidepsin and 12.5nM bryostatatin were added for an additional 24 hours. Cells were then harvested and stained with a Live/Dead Fixable Violet Cell Death Stain and analysed using flow cytometry. Reactivation of viral expression was determined using expression of the GFP viral reporter. The percentage of dead cells (violet+) in the GFP+ reactivated population (White dashed), live cells in the GFP+ (GFP+V-, white solid), dead cells in the GFP- (GFP-V+, green dashed) and the live cells in the GFP- (GFP-V-, green solid) are shown above, n=3.

Supplementary Table 3.1. Clinical information for PLWH on ART from Melbourne Leukapheresis Cohort

Participant ID	Age	Gender	Ethnicity	Years on ART	Current ART Regimen	Viral Load (copies/ml)	Nadir CD4 (cells/mm ³)	Current CD4 (cells/mm ³)
LKA11	50	Male	Caucasian	21.8	abacavir, dolutegravir, lamivudine	<20	189	594
LKA12	56	Male	Caucasian	21.0	raltegravir, darunavir, ritonavir	<20	624	744
LKA13	58	Male	Caucasian	11.3	lamivudine, darunavir, ritonavir, nevirapine	<20	27	520
LKA15	42	Male	Caucasian	10.2	tenofovir disoproxil fumarate, emtricitabine, nevirapine	<20	198	681
LKA16	67	Male	Caucasian	10.9	abacavir, dolutegravir, lamivudine	<20	315	534
PRA09	49	Male	Caucasian	> 3 years*	elvitegravir, cobicistat, emtricitabine, tenofovir alafenamide fumarate	<20	42	474

*Time on ART unknown. At study enrolment, ART information collected for previous 3 years only.

Supplementary Table 3.2. Treatment of CD4+ T-cells from PLWH on ART resulted in dual, triple or shifted peaks in the cell-associated unspliced HIV RNA

SAMPLE TYPE	DUAL / TRIPLE or SHIFTED PEAKS copies / 100 ng input RNA (# replicates in total replicates)					
	PRA9	LKA11	LKA12	LKA13	LKA15	LKA16
DMSO		1.34 (2/4)	16.87 (2/12)		1.42 (1/12)	
DMSO + IPI-4X				1.67 (2/4)	61.28 (1/12)	5.78 (2/8)
DMSO + IPI-3063		12.28 (1/4)		50.36 (4/4)	25.84 (1/12)	
DMSO + WN	9.31 (2/8)	2.91 (1/4)	57.84 (3/12)	120.75 (4/4)	15.71 (2/12)	
DMSO + 5nM VNX			42.00 (4/12)	0.76 (2/4)		11.47 (1/8)
				3.56 (2/4)		
DMSO + 1uM VNX		n/a		0.50 (1/4)	148.44 (1/8)	3.33 (1/4)
						0.50 (1/4)
RMD			9.28 (1/4)	2.53 (2/4)		
RMD + IPI-4X			177.15 (4/4)	0.50 (1/4)		
RMD + IPI-3063	541.83 (4/8)	0.50 (1/4)	n/a		58.42 (2/4)	
RMD + WN	101.92 (1/4)					
RMD + 5nM VNX	6.50 (1/4)	0.50 (1/4)				15.27 (1/4)
		1.80 (3/4)				
RMD + 1uM VNX		n/a	n/a	1.94 (1/4)	n/a	n/a
PNB	64.43 (1/4)	n/a		n/a		
PNB + IPI-4X		n/a		n/a		
PNB + IPI-3063	61.30 (2/4)	n/a		n/a		0.50 (1/4)
PNB + WN	57.33 (1/4)	n/a	35.66 (1/4)	n/a		
PNB + 5nM VNX		n/a		n/a		
PNB + 1uM VNX		n/a		n/a		9.25 (2/4)
BRY		n/a	25.09 (2/4)	n/a	12.36 (2/4)	
BRY + IPI-4X		n/a	9.80 (4/4)	n/a	1.28 (1/4)	
BRY + IPI-3063		n/a		n/a	23.79 (1/4)	
BRY + WN		n/a		n/a	77.30 (4/4)	
BRY + 5nM VNX		n/a	117.20 (4/4)	n/a		
BRY + 1uM VNX		n/a		n/a	11.68 (1/4)	
JQ1		n/a		n/a		
JQ1 + IPI-4X		n/a	4.55 (1/4)	n/a		
JQ1 + IPI-3063		n/a	29.51 (1/4)	n/a		
JQ1 + WN		n/a	117.10 (1/4)	n/a		22.91 (1/4)
JQ1 + 5nM VNX		n/a		n/a		
JQ1 + 1uM VNX		n/a		n/a		120.75 (4/4)
PMA/PHA	22.48 (4/8)		99.37 (5/12)			
			141.17 (3/12)			
PMA/PHA + IPI-4X	154.40 (1/8)		132.12 (6/12)	30.94 (4/4)	24.45 (1/12)	
					9.87 (1/12)	
PMA/PHA + IPI-3063		1.25 (1/4)	267.58 (4/8)			69.15 (2/8)
		0.50 (1/4)				
PMA/PHA + WN	60.69 (3/8)	3.08 (1/4)	196.8.8 (5/12)	33.48 (4/4)		58.25 (1/8)
			358.17 (3/12)			
PMA/PHA + 5nM VNX	34.27 (1/8)	53.22 (1/4)	123.21 (5/12)	28.99 (4/4)		60.11 (4/8)
						48.86 (1/8)
PMA/PHA + 1uM VNX		n/a	257.45 (4/8)	25.12 (1/4)		33.48 (4/4)
			92.94 (4/8)	4.69 (3/4)		

% replicates	Dual/Triple	Shifted	
	Peak	Peak	
0 – 25%			n/a = Not assessed
26 – 50%			
51 – 75%			
76 – 100%			

Chapter 4

Development of an assay that can co-detect the reactivation of latent HIV and cell death

Chapter 4

Development of an assay that can co-detect the reactivation of latent HIV and cell death

4.1 Abstract

Latently infected CD4⁺ T-cells present as a major hurdle in finding a cure for PLWH on ART. One strategy to eliminate latently infected cells is through the use of drug compounds such as LRAs combined with pro-apoptotic drugs that induce apoptosis. Recent work with pro-apoptotic drugs such as venetoclax has demonstrated the potential of pre-sensitisation of latent cells towards apoptosis prior to latency reversal to deplete latently infected cells. However, the discovery of novel LRA and pro-apoptotic drug compounds is hampered by the lack of methods to differentiate drugs that selectively reactivate and kill infected cells versus drugs that also non-selectively kill uninfected cells via drug toxicities. Here, we developed a novel assay that combines HIV RNA *in situ* hybridisation using probes and branched DNA-technology (PrimeFlow) with a cell death stain followed by flow cytometry to identify latently infected cells with reactivated HIV RNA that have died. Using this novel assay, we have found new pro-apoptotic and LRA drug combinations that result in selective death of the latently infected ACH2 T-cell line. The combination of PMA LRA with pro-apoptotic drugs IPI-443 and venetoclax resulted in an increase in HIV RNA expressing cells undergoing cell death. This newly described assay could successfully determine latently reactivated cells undergoing selective cell death.

4.2 Introduction

Despite the benefits of ART, HIV persists in infected individuals. The existence of a stable latent reservoir comprised of resting memory CD4⁺ T-cells [34] that have a very long half-life of approximately 44 months [457] demands lifelong antiretroviral therapy. Strategies to purge the latent reservoir are diverse and include the use of pharmacological agents to reactivate the latent virus (termed shock and kill) plus strategies that aim to illicit a strong host immune response [214]. Quantification of cell associated HIV RNA and total or integrated HIV DNA are commonly used as markers of HIV persistence and an increase in cell associated

HIV RNA is commonly used as evidence of activity of LRAs [242-244, 248]. Measuring HIV persistence using PCR-based assays will measure both intact and defective viruses as well as intact non-inducible virus [145] and therefore can lead to an overestimation of the frequency of cells infected with replication-competent virus [151, 152, 156, 164]. In addition, PCR-based quantification of RNA provides information on a population level and for cell associated HIV RNA, can't distinguish between a cell that produces many copies of RNA or many cells that produce low amounts of RNA. These assays are needed to better understand the frequency of cells that have the capacity to produce and potentially increase production of HIV RNA.

One approach to detect the frequency of infected cells producing HIV RNA, is to use TILDA. This assay measures the frequency of CD4+ T-cells that carry inducible provirus, distinguishing it from PCR-based assays. CD4+ T-cells are maximally stimulated with the mitogens PMA and ionomycin followed by RT-qPCR to measure the levels of *tat/rev* MS RNA [164]. The frequency of latently infected cells carrying inducible provirus was 24 cells per 10⁶ CD4+ T-cells [164]. This number was 48 times higher than the QVOA and 6-27 times lower than PCR-based methods.

Another approach is to use direct staining of cell associated RNA which also would allow phenotypic assessment of the RNA producing cell. RNA-flow cytometric *in situ* hybridisation (RNA-flow FISH) combined with branched-DNA technology to amplify signals from RNA-flow FISH - termed the PrimeFlow RNA assay (ThermoFisher) – is a technique that can detect HIV RNA. Using gene specific oligonucleotide HIV RNA probes that contain 20-40 probe pairs binding to the target HIV *gag-pol* (*gag*) and *env-nef* (*env*) sequences, the probe pairs can bind adjacent to each other, increasing the specificity of detection. Pre-amplification molecules hybridise to the target probes, followed by binding of the Amplifier molecules. Finally, oligonucleotides that have been conjugated to fluorophores hybridize to the Amplifier molecules. These amplify the target RNA signal by 8000 to 16000-fold. Stained cells are then detected using flow cytometry.

PrimeFlow for HIV RNA has been combined with an intracellular antibody stain for HIV p24^{Capsid} Gag protein and surface marker staining, to enhance specificity [166, 167]. In these

two published assays, probes were designed to detect *gag-pol*. Given that not all reactivated cells that produce RNA will produce protein, this assay detects both the transcriptionally and translationally competent viruses. Using this assay to detect RNA and Gag positive cells from PLWH on ART, a total of 3-6 cells per one million CD4+ T-cells were detected [166, 167]. These estimates are higher than previous studies identifying one per million CD4+ T-cells that harbour replication-competent virus determined using the gold standard QVOA [146] but lower than the approximately 300-1000/10⁶ latently infected cells determined using PCR-based methods for HIV total or integrated DNA [156, 166].

The goal of our work in this chapter was to optimise the PrimeFlow RNA assay to detect *gag* and *env* mRNA and to combine this with a marker of apoptosis, to identify cells that die following latency reversal. Using anti-caspase antibody as an early marker of apoptosis, we first optimised the assay using a latently infected cell line ACH2 cells. We then examined a combination of LRAs and pro-apoptotic drugs and were able to demonstrate that the combination of PMA with either the PI3K inhibitor IPI-443 or the Bcl-2 inhibitor venetoclax resulted in an increase in the number of latently infected ACH2 T-cells that were expressing HIV RNA and also undergoing selective cell death. Future work will evaluate this approach using CD4+ T-cells from PLWH on ART.

4.3 Methods

4.3.1 Culture of T-cell lines

ACH2 latently infected and uninfected Jurkat T-cell lines (NIH AIDS Reference Reagent Program) were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS (Interpath, Vic, AUS), 100U/ml penicillin (Life Technologies, Carlsband, CA), 100µg/ml streptomycin (Life Technologies, Carlsband, CA) and 29.2mg/ml L-glutamine (Life Technologies, Carlsband, CA) (RF10) at 37°C, 5% CO₂ atmosphere. Cells were passaged every 2-3 days and maintained for approximately 30 passages for experiments prior to thawing a new aliquot of cells at early passage number for subsequent experiments.

4.3.2 Compounds

The following pharmaceuticals were dissolved in DMSO to make stock concentrations stored at -80°C: 10mM romidepsin (Selleck Chemicals, Houston, TX), 10mM venetoclax (Selleck Chemicals, Houston, TX), 1mM PMA (Sigma-Aldrich, St. Louis, MO), 10mM PEP005 (Ingenol-3-angelate) (In Vitro Technologies, Vic, AUS). IPI-443 and IPI-3063 (Infinity Pharmaceuticals, Cambridge, MA) were provided by Infinity Pharmaceuticals, constituted in DMSO at a stock concentration of 10mM and stored at -20°C. The following compounds were reconstituted in DMSO and stored at -20°C: 1mg/ml PHA (Sigma-Aldrich, St. Louis, MO) and 1mM (S)-(+)-Camptothecin (CPT) (Sigma-Aldrich, St. Louis, MO).

4.3.3 Active Caspase-3 antibody staining using BD and PF buffers

4.3.3.1 Cell culture for active caspase-3 staining

ACH2 and Jurkat T-cells were resuspended in culture media and counted in trypan blue (Sigma-Aldrich, St. Louis, MO) using a haemocytometer. Cells were then pelleted at 350xg for 7 minutes at room temperature, resuspended in RF10 at a concentration of 1×10^6 cells/ml and transferred to a T75cm² flask. Cells were activated with PMA at a final 5nM concentration and cultured overnight at 37°C, 5% CO₂. The next day cells were separated into groups, pelleted, resuspended and 5×10^6 cell aliquots treated with either DMSO or 10µM venetoclax to induce apoptosis for a further 24 hours. Cells were also treated with 20µM camptothecin for 6 hours in some experiments as an apoptosis-inducing control.

4.3.3.2 Becton Dickson (BD) buffer active casapase-3 staining

Following drug treatment, cells were stained with active caspase-3 in BD Perm/Wash buffer. Cells were resuspended, transferred to 15ml falcon tubes and cells pelleted at 800xg for 5 minutes, 4°C. Cells were washed twice using cold PD with the above centrifuge conditions. After aspiration of the solution from the second wash, cell pellets were resuspended in Cytofix/Cytoperm (BD Biosciences, Franklin, NJ) solution at a final concentration of 5×10^6 cells/ml and incubated at 4°C for 30 minutes to fix the cells. Following fixation, cells were pelleted (800xg, 5 minutes, 4°C) and then washed twice using the same conditions in 2.5ml of Perm/Wash buffer with 1x PrimeFlow (PF) RNase inhibitors. Perm/Wash buffer with 100µl

per 5×10^6 cells of PE.Active Caspase-3 rabbit antibody (BD Biosciences, Franklin, NJ, #550821) was added to samples or Perm/Wash buffer and incubated at 4°C in the dark for 30 minutes. Antibody stained cells were washed twice in 1ml Perm/Wash buffer with PF RNase inhibitors and fixed in 1ml PF RNA Fixation buffer 2 overnight at 4°C in the dark. The next day PrimeFlow staining was completed (see section 4.3.5).

4.3.3.3 PrimeFlow (PF) buffer staining

After drug treatment, cells were resuspended and transferred to 15ml falcon tubes and pelleted at 800xg for 5 minutes at 4°C. Cells were washed twice with EBioscience Stain Solution (Thermo Fisher Scientific, Waltham, MA). After the second wash, cells were resuspended in PF fixation buffer 1 and incubated for 30 minutes at 4°C in the dark. Following fixation, cells were washed twice in PF RNA permeabilization buffer with RNase inhibitors using 800xg, 5 minutes, 4°C to pellet cells. Cell pellets were stained with PE.Active Caspase-3 rabbit antibody (100µl per 5×10^6 cells, BD Biosciences, Franklin, NJ, #550821) diluted in PF RNA permeabilization buffer with RNase inhibitors for 30 minutes at 4°C in the dark. Following antibody staining, cells were washed in PF RNA permeabilization buffer containing RNase inhibitors and stained overnight in PF RNA Fixation buffer 2 at 4°C in the dark. The next day PrimeFlow staining was completed (see section 4.3.5).

4.3.4 Live/Dead Near Infrared Dead Cell Stain

ACH2 T-cells were counted, activated with 5nM PMA for 24 hours, and cultured a further 24 hours in DMSO or 10µM venetoclax as described above. Cells were then stained with Live/Dead Near Infrared (NIR) Fixable Dead Cell stain (Invitrogen, Carlsband, CA) as per manufacturer's instructions. Briefly, cells were resuspended and transferred to 15ml tubes at 350xg for 7 minutes at room temperature. Cell pellets were washed in 1.5ml of PD and transferred to 1.5ml tubes provided in the PrimeFlow kit. Cells were washed twice and pelleted at room temperature for 7 minutes at 350xg. Pellets were stained with 1ml of 5x NIR stain at a final concentration of 5µl per 1×10^6 cells diluted in PD. 1ml of PD was added to cells that did not require NIR staining. All samples were stained for 30 minutes on ice in the dark. Following the staining, cells were washed in PD at 350xg for 7 minutes at room temperature. Importantly, the supernatant was aspirated to the 100µl line indicated on the 1.5ml PF tubes

as per the PrimeFlow staining instructions. Each sample was resuspended in 1ml PF Fixation Buffer 1 using the pipette tip. Samples were placed at 4°C in the dark for 30 minutes prior to centrifugation at 800xg for 5 minutes at 4°C. Staining solution was aspirated to the 100µl line on PF tubes and then cell pellets were washed twice in 1ml PF RNA permeabilization buffer with RNase inhibitors. Cells were fixed in PrimeFlow RNA Fixation buffer 2 overnight in the dark at 4°C. PrimeFlow staining was completed the next day.

4.3.5 PrimeFlow Staining

Samples stained with either PE.Active Caspase-3 antibody or NIR Live/Dead stain and incubated overnight at 4°C in PF Fixation buffer 2 were first brought up to room temperature for approximately 15 minutes. During this time PF RNA target probe diluent was warmed to 40°C and RNA wash buffer was warmed to room temperature. Once samples reached room temperature, they were pelleted at 800xg for 5 minutes at room temperature. Of note all proceeding centrifugation steps were completed at these conditions unless stated otherwise. Cell pellets were washed in 1ml RNA wash buffer containing PF RNase inhibitors twice and both times, the wash buffer was aspirated to the 100µl line indicated on the side of the tubes. 20x Target probe sets were thawed at room temperature and diluted into the RNA target probe diluent for a final 1x concentration in 100µl. 100µl of 1x diluted target probe set/s were directly added to the cell suspension and mixed using the pipette tip. Cells were incubated at 40°C for 2 hours in the dark, with the tubes inverted once at 1 hour of incubation. The Pre-Amp mix was also pre-warmed to 40°C after 1 hour of target probe incubation. Following incubation with the target probe set/s, cells were washed twice in RNA wash buffer with RNase inhibitors. 100µl of 1x PF RNA Pre-Amp mix was directly added to the cell suspension and gently mixed with the pipette tip. Samples were incubated at 40°C in the dark for 1.5 hours. During this incubation, the 1x PF RNA Amp mix was warmed to 40°C. 1ml of RNA wash buffer was added to each sample and tubes were inverted to mix. The wash step was repeated twice more and following the last aspiration, 100µl of 1x PF RNA Amp mix added to the cell suspension. Again, the cell suspension was mixed with a pipette tip and samples incubated for 1.5 hours in the dark at 40°C. Samples were washed twice more in RNA wash buffer and then resuspended in 1x PF RNA label probes in PrimeFlow RNA label probe diluent for 1 hour at 40°C in the dark. Samples were washed twice in RNA wash buffer. Cells were washed once

in 1ml of PrimeFlow RNA storage buffer, supernatant aspirated to the 100µl line on the 1.5ml tubes and finally mixed in 100µl of IC Fixation buffer, transferred to a 5ml labelled polystyrene FACS tubes and stored at 4°C in the dark for up to 3 days before analysis using flow cytometry.

4.3.6 ACH2 and Jurkat T-cells treated with pro-apoptotic drugs and LRAs

ACH2 and Jurkat T-cells were counted using trypan blue staining in a haemocytometer and treated at a concentration of 2×10^6 cells/ml in RF10 with either DMSO, 5nM PMA, 100nM IPI-443 or 1µM venetoclax for 24 hours at 37°C, 5% CO₂. All cells were cultured in a T75cm² flask. Twenty-four hours after pro-apoptotic drug treatment, each condition was counted and 5×10^6 cells in 2.5ml were aliquoted into 6-well plates for different pro-apoptotic drug + LRA combinations. 2x LRA drug solutions were prepared in RF10 and 2.5ml of media with DMSO or LRA were then added to cells in the 6-well plates for final concentrations of 40nM romidepsin, 50nM PEP005 or 10nM PMA or 0.001% matched DMSO diluent control. All plates were cultured at 37°C, 5% CO₂. Four hours following romidepsin treatment, cells were washed twice in RF10 at 350xg, 7 minutes at room temperature. Washed cells were put back into culture in the background of pro-apoptotic drug for 20 hours. All other conditions were cultured with their respective LRAs for 24 hours. At the end of the culture, cells were stained with both Live/Dead Near Infrared Dead Cell stain and PrimeFlow as outlined above.

4.3.7 Flow cytometry and data analysis

All samples were analysed using a BD LSR Fortessa running FACS Diva software. HIV *gag* RNA stained cells were detected using the red laser at a bandwidth of 670/10 while the HIV *env* RNA and PE.Active Caspase-3 stained cells were both detected using yellow laser at a bandwidth of 610/20. Cells stained with NIR Live/Dead Cell Stain were also detected using the red laser at a bandwidth of 780/60. An autofluorescence channel was also collected on the violet laser at 525/50 as a control for autofluorescence. Flow cytometry data was analysed using FlowJo v10. Cells were first gated using forward scatter versus side scatter. Single cells were then gated on forward scatter height versus width and then repeated using side scatter height and width. The single cells were then gated using the HIV *gag-pol* (*gag*) AF647 channel versus HIV *env-nef* (*env*) AF568 channel. A quadrant gate was used to divide the population into env-gag-, env-gag+, env+gag- and env+gag+. Each quadrant was then gated on an

autofluorescence channel versus the NIR dead cell stain. The average percentages of the four cell subsets HIV RNA- Live (NIR-), HIV RNA- Dead (NIR+), HIV RNA+ Live and HIV RNA+ Dead were back calculated off the percentages of HIV *gag* positive and HIV *env* positive cell populations that were also NIR live/dead positive and negative. Average cell subset percentages were calculated using Microsoft Excel and graphed using GraphPad Prism v8.

4.3.8 Statistical analysis

Flow cytometry results were graphed using GraphPad Prism (version 8, GraphPad Software, La Jolla, CA). Statistical testing was not appropriate as the small sample size ($n < 6$) precluded the use of non-parametric tests. Parametric t-tests were not performed due the small sample size and the inability to test for normality. Therefore, we have reported the mean, range and fold-difference between means of the samples.

4.4 Results

4.4.1 PrimeFlow detection of HIV *gag* and *env* RNA in reactivated ACH2 latently infected T-cell line

Using PrimeFlow RNA and probes to detect HIV *gag* and HIV *env* we first examined the latently infected ACH2 T-cell line stimulated with PMA. We reasoned that of RNA binding to two genes would enhance specificity (as demonstrated by co-staining with p24) and the combination of *gag* and *env* probes will be more likely to detect intact HIV from PLWH on ART [499]. The positive control was a probe to the human ribosomal protein L13A (RPL13A) gene and the negative control a probe to bacterial *dapB* gene, both conjugated to the same fluorophores as the HIV probes. Additionally, we included HIV uninfected Jurkat T-cells.

As shown in flow plots in Figure 4.1, we observed staining of both HIV RNA *gag* and *env* transcripts in ACH2 T-cells reactivated with 10nM PMA. Each HIV RNA probe tested in isolation detected an abundant population of cells expressing HIV *gag* or HIV *env* RNA (75.9% and 88.7% respectively). Moreover, when the two probes were combined, a total of 76.7% of cells were positive for both RNA transcripts (Figure 4.1). There no background staining with the HIV *gag* and *env* probes in the uninfected Jurkat cell line. Both the positive RPL13A and

negative dapB controls showed positive and negative populations as expected. Unstimulated ACH2 T-cells served as a control for the reactivation of latent HIV.

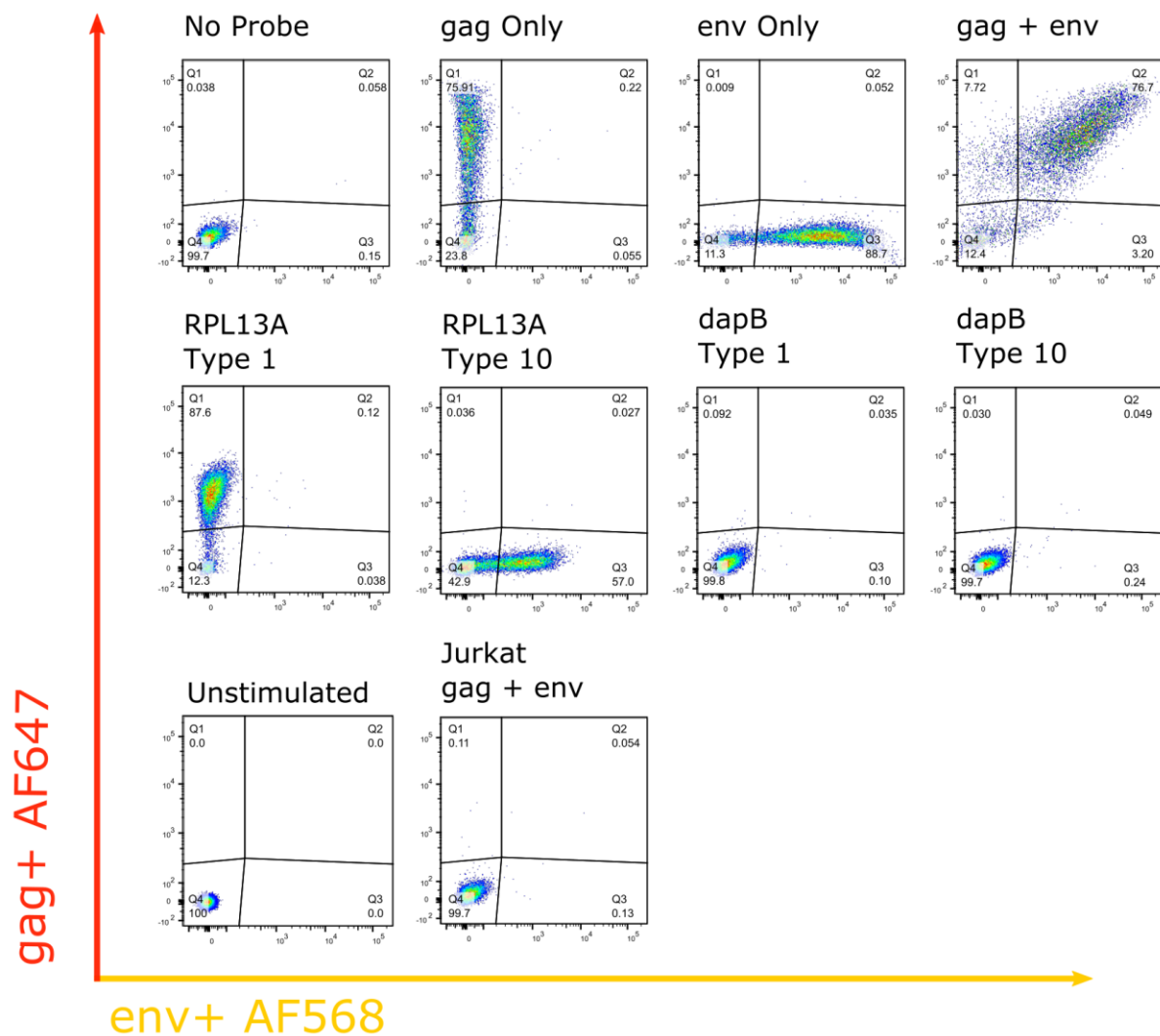


Figure 4.1. PrimeFlow HIV probes for the gag and env region successfully detect HIV RNA in ACH2 T-cells reactivated with PMA with some background staining in uninfected Jurkat cells

Latently infected ACH2 cells were reactivated with 10nM PMA for 24 hours. The reactivated cells were then stained using PrimeFlow using HIV gag and env RNA probes identified by AlexaFluor (AF) 647 (AF647) and AF568, respectively. Positive control probe sets, RPL13A Type 1 and RPL13A Type10 in the same fluorescent channels were included as well as bacterial dapB negative control probes. Jurkat T-cells that do not contain HIV RNA also served as a negative control to demonstrate the HIV probe set specificity. Lastly, unstimulated ACH2 T-cells were included as a control for the reactivation of latent virus. The flow plots represent a single representative experiment. Representative examples from one experiment, $n \geq 2$.

4.4.2 Detection of active caspase-3 staining is improved when using BD buffer

Next, we aimed to combine PrimeFlow with a stain for cell death, either active caspase-3 antibody staining to mark cell death by apoptosis, or a live/dead fixable dead cell stain to detect cells death by any mechanism including apoptosis, pyroptosis or necrosis. ACH2 were reactivated with 5nM PMA for 24 hours followed by a further 24 hours with either DMSO diluent negative control or 10 μ M venetoclax to induce apoptosis (Figure 4.2). Cells were then stained for intracellular active caspase-3 using a phycoerythrin (PE) conjugated anti-active caspase-3 antibody. Unstained cells that omitted the antibody in the appropriate staining solution were included for comparison.

During the staining process, we also tested two different buffers: either the BD buffers supplied with the caspase-3 antibody or the PrimeFlow (PF) buffers provided with the PrimeFlow kit for antibody staining. We found that using these two different buffers for the antibody staining procedure followed by PrimeFlow staining for RNA resulted in slightly different outcomes (Figure 4.2). Performing the caspase-3 staining using the recommended BD buffers resulted in a higher number of caspase-3 positive cells detected in the 0.1% DMSO drug diluent control and 10 μ M venetoclax treated cells compared to using the recommended PrimeFlow buffers. However, there was poor separation of caspase-3 antibody positive and negative cell populations when combined with subsequent PrimeFlow RNA staining steps (RNA staining not shown in Figure 4.2).

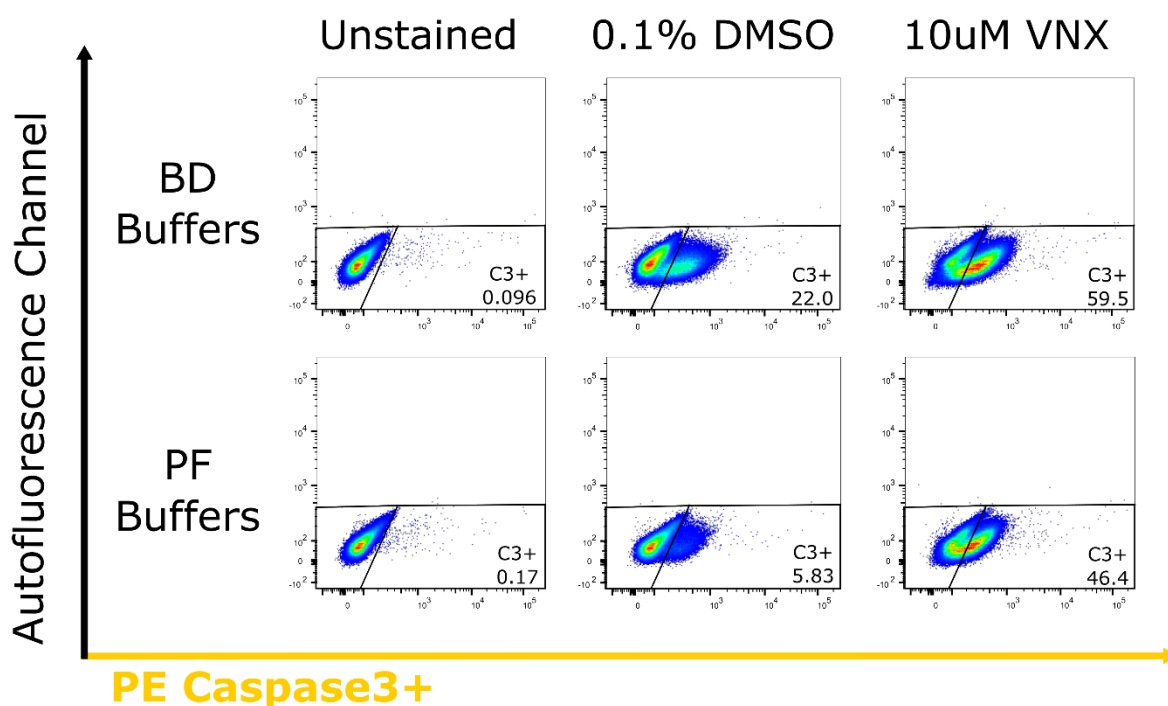


Figure 4.2. PrimeFlow staining buffers reduce the detection of PE active caspase-3 in ACH2 cells reactivated with PMA and treated without or with venetoclax.

ACH2 cells were reactivated with 5nM PMA for 24 hours, and 10 μ M venetoclax to induce apoptosis or DMSO added as a diluent negative control for a further 24 hours. Parallel samples were then stained with an intracellular PE conjugated anti-active caspase-3 antibody using either the recommended BD buffers supplied with the antibody or the PrimeFlow (PF) buffers provided for antibody staining in the PrimeFlow kit. Following caspase-3 staining, all samples were stained with the AlexaFluor 647 HIV gag RNA probe set using the PrimeFlow buffers provided (data not shown). The unstained control underwent the same staining procedure but omitted the PE.active caspase-3 antibody and PrimeFlow HIV gag target probe in the appropriate staining solutions. Flow cytometry dot plots showing PE.caspase-3 versus an autofluorescence violet 525/50 channel from one representative experiment is shown, n=3.

We further tested combining the PE active caspase-3 antibody staining using BD or PF buffers with subsequent PrimeFlow staining for HIV *gag* RNA. The use of PE conjugated active caspase-3 antibody precluded use of the HIV *env* probe set (Alexa Fluor 568), as both are detected using the same laser and filters on a flow cytometer. Therefore, only HIV *gag* mRNA staining (AlexaFluor 647 detection) was combined with the PE conjugated caspase-3 antibody staining.

The ACH2 latently infected T-cell line was reactivated 24 hours with PMA and then treated either with high dose 10 μ M venetoclax as a positive control for apoptosis or DMSO control. Cells were stained with the PE active caspase-3 antibody using either the BD or PF buffers and subsequently stained for HIV *gag* mRNA with the PrimeFlow kit. Cells were analysed by flow cytometry and single cells gated on the violet autofluorescence channel versus *gag* mRNA first to identify *gag*⁺ and – populations, and each population then gated on the violet autofluorescence channel versus caspase-3 staining to determine the percentage of caspase-3 positive cells in the *gag*⁺ and – populations. This gating strategy enabled the best separation of caspase positive and negative populations. Representative flow cytometry plots from one experiment is shown in Figure 4.3. The percentage of *gag*⁺ caspase-3⁺, *gag*⁺ caspase-3⁻, *gag*⁻ caspase-3⁺ and *gag*⁻ caspase-3⁻ was then calculated for the original single cell population and the results from 3-4 experiments combined as shown in Figure 4.4. We found that stimulation with PMA with DMSO, induced expression of *gag* and few of these cells expressed caspase-3. Stimulation with PMA and venetoclax significantly increased expression of caspase-3, in both *gag*⁻ and to a lesser extent *gag*⁺ cells.

We observed that cells expressing caspase-3 were difficult to separate from caspase-3 negative cells using both staining buffers (Figure 4.4). The overall percentages of *gag*⁺ caspase-3⁻ cells was on average higher using the PF versus BD buffers in both DMSO and venetoclax treated cells. Additionally, this high dose of venetoclax increased the *gag*⁺ caspase-3⁺ population by 2.57-fold and 3.03-fold compared to their matched DMSO control (BD and PF respectively, Figure 4.4). Venetoclax also increased the *gag*⁻ caspase-3⁺ population by 5.84- and 7.66-fold compared to their matched DMSO control (using BD and PF buffers respectively, Figure 4.4).

Given the poor separation of caspase-3 positive and negative cells, we investigated an alternate marker of cell death using live/dead cell staining.

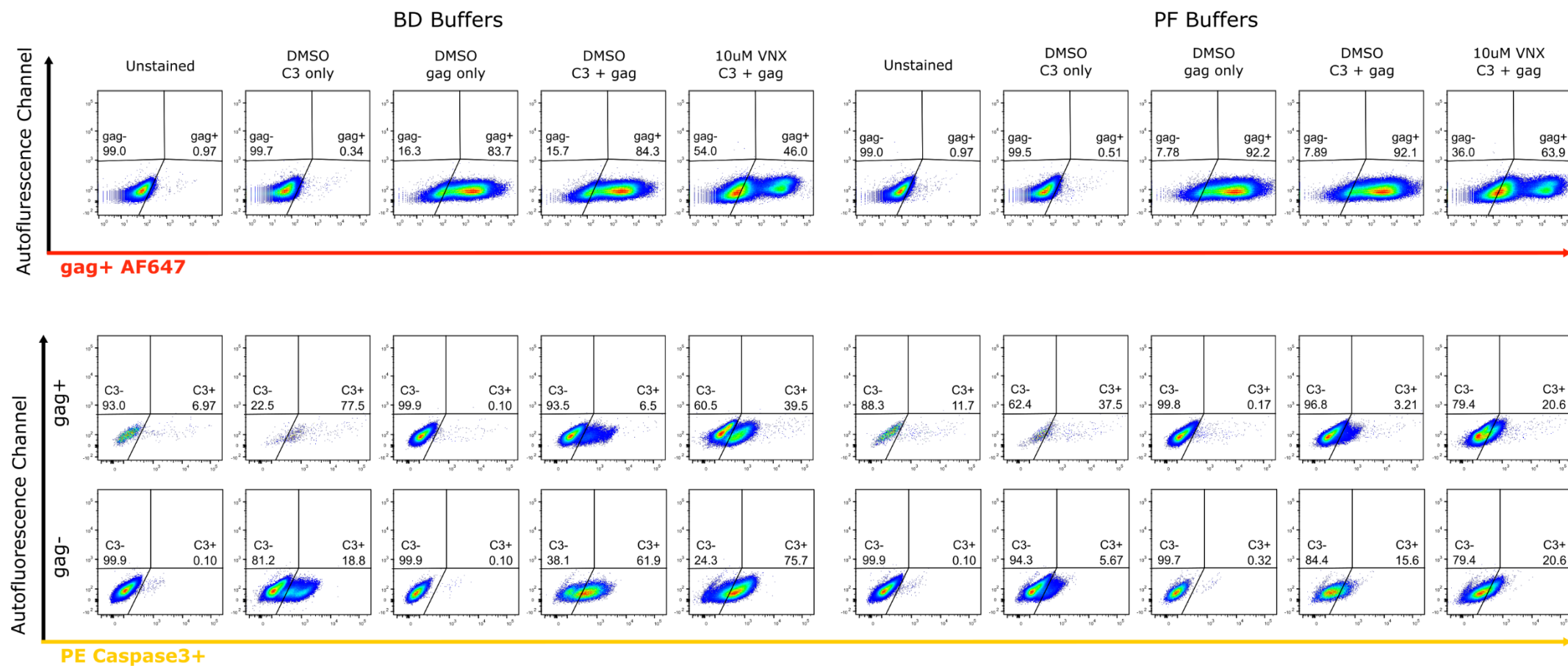


Figure 4.3. Combination of PrimeFlow with HIV gag RNA and staining for caspase-3 using both BD and PrimeFlow buffers

ACH2 cells were reactivated with 5nM PMA for 24 hours and then treated with 10μM venetoclax to induce apoptosis or DMSO as a negative control for a further 24 hours. Parallel samples were stained with PE conjugated anti-active caspase-3 antibody in either the recommended BD

buffers or the recommended PF buffers, and subsequently stained for HIV gag RNA using the PrimeFlow buffers. The negative unstained control cells were incubated with all of the buffers but lacked the caspase-3 antibody and HIV gag RNA target probe. The samples were analysed using flow cytometry. The flow plots from on representative experiment are shown. C3- values were calculated by adding together Q1 and Q4 while C3+ values were the combined numbers of Q2 and Q3.

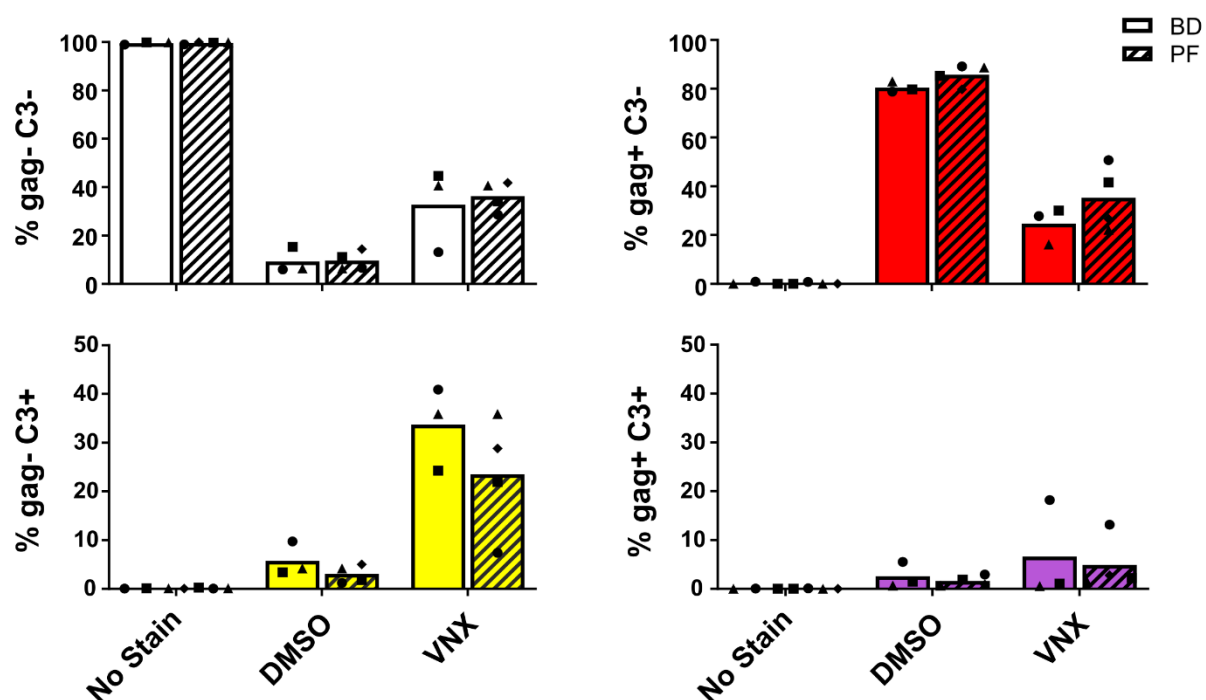


Figure 4.4. Detection of HIV gag RNA and caspase-3 positive cells in activated ACH2 cells using PrimeFlow and BD buffers

ACH2 T-cells reactivated with 5nM PMA for 24 hours and then treated with 10 μ M venetoclax or DMSO (negative diluent control) for an additional 24 hours were stained with PE conjugated anti-active caspase-3 antibody with the recommended BD buffers or with the recommended PF buffers. Cells were then stained for HIV gag-pol RNA using the PrimeFlow kit and PrimeFlow recommended buffers. Stained samples were then analysed using flow cytometry and the collective results (n=3) are shown with columns denoting the mean, each symbol representing an independent experiment, and open columns (BD buffers) and hatched columns (PF buffers). VNX- venetoclax.

4.4.3 Near infrared Live/Dead Staining of ACH2 T-cells results in clear separation of live and dead cell populations versus caspase-3 antibody staining

We next tested a NIR live/dead fixable cell death stain with PrimeFlow to detect cell death by any mechanism. As the NIR fluorescent probe is in a different channel to the HIV *gag* and *env* PrimeFlow probe sets, both the HIV *gag* and *env* mRNA probes were tested with the NIR live/dead stain.

ACH2 cells were activated with 5nM PMA for 24 hours and DMSO diluent or 10μM venetoclax added to induce apoptosis for a further 24 hours. The cells were then stained with a near infrared live/dead cell death stain followed by PrimeFlow for both HIV probes, *gag* AlexaFluor 647 and *env* AlexaFluor 568 (Figure 4.5). Additionally, parallel cells were stained with PE caspase-3 followed by HIV *gag* probe only using the PF buffers for comparison (Figure 4.5). Compared to the active caspase-3 staining where separation of caspase-3 positive and negative populations was poor (Figure 4.3), we observed two clearly distinct populations of live (NIR-) and dead (NIR+) cell populations using the NIR live/dead stain in the HIV RNA negative and positive (*gag* and/or *env* RNA+) populations (Figure 4.5). This suggests that the NIR live/dead stain was a superior option to use in conjunction with PrimeFlow staining for HIV RNA due to the clearer separation of live and dead cells.

Next, the percentage of HIV RNA- Live, HIV RNA- Dead, HIV RNA+ Live, and HIV RNA+ Dead cells was calculated (Figure 4.5). The percentage of HIV RNA- live cells and HIV RNA+ dead cells was comparable between using NIR and caspase-3 staining. Following treatment with venetoclax, there were fewer HIV RNA+ live cells and a higher percentage of HIV RNA- dead cells with the NIR staining versus the caspase-3 staining. This might reflect the clearer separation of live and dead cells with the near infrared stain resulting in less false positive and negative cells versus the caspase-3 stain (Figure 4.5), or the different types of cell death quantified (as caspase only measures apoptotic cells), and/or elimination of HIV RNA+ cells during the 30 minute NIR live/dead staining prior to fixation.

As there was clearer separation of live and dead cells using the NIR live/dead stain and two HIV probes could be used i.e. *gag* and *env*, NIR live/dead stain together with PrimeFlow staining for HIV *gag* and *env* RNA was used for future experiments.

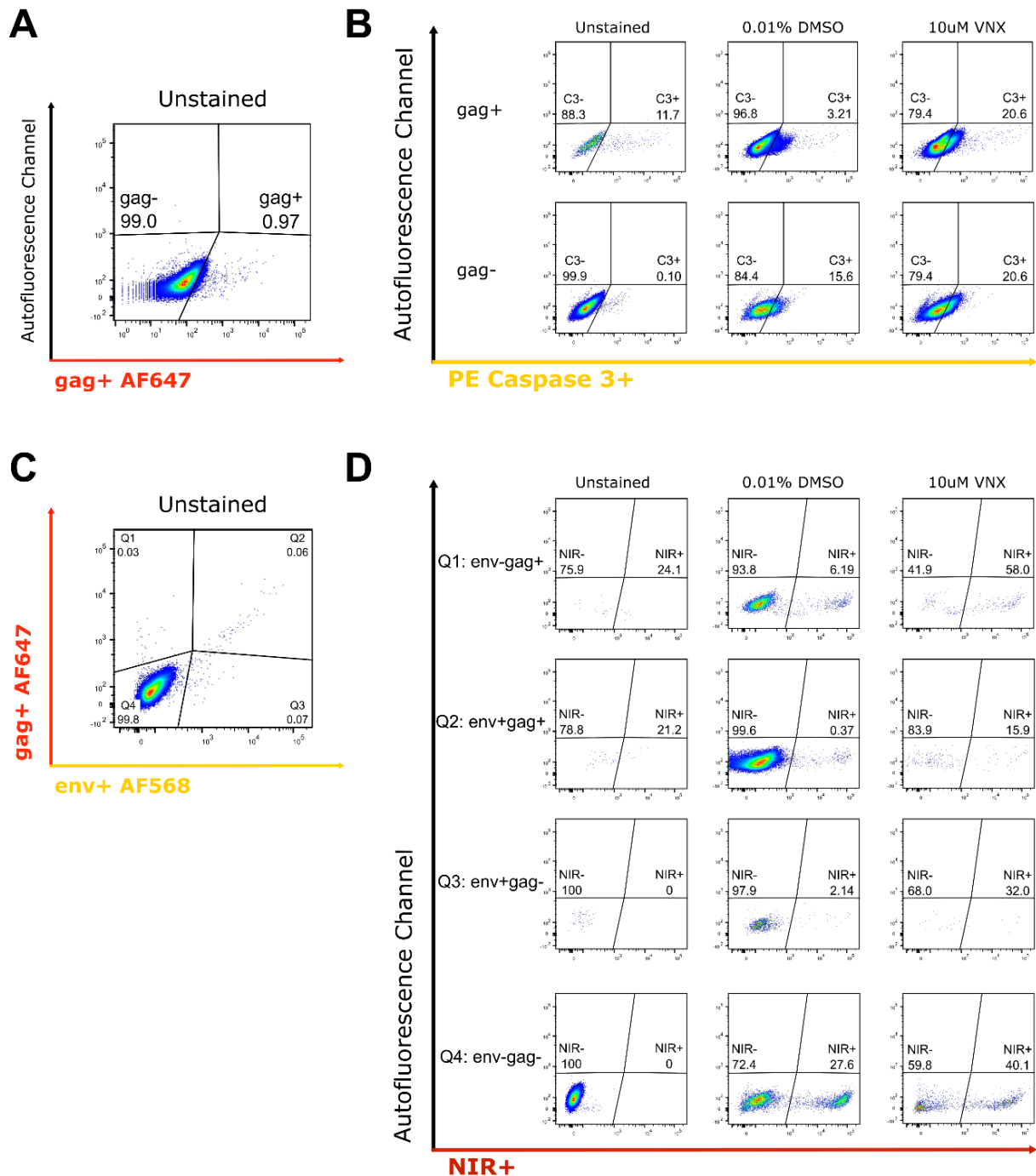


Figure 4.5. Staining with near infrared live/dead stain shows clear separation of live and dead cell populations compared to PE active caspase-3 staining

ACH2 cells were activated with PMA for 24 hours followed by 10 μ M venetoclax to enhance apoptosis or DMSO diluent control a further 24 hours. Cells were stained with either: PE active caspase-3 followed by PrimeFlow using the HIV gag RNA probe AlexaFluor 647 (A and B) or near infrared live/dead cell death stain followed by PrimeFlow using both the HIV gag

AlexaFluor 647 and HIV env AlexaFluor 568 RNA probes (C and D). Samples were analysed on a BD Fortessa flow cytometer, gated on single cells, then gated on HIV RNA positive and negative subsets (A HIV gag RNA; C. HIV gag and/or env RNA), and then finally gated for either caspase-3 antibody stain or near Infrared live/dead populations versus a violet 525/50 autofluorescence channel. Flow plots from a representative experiment is shown. Cells stained with all of the buffers but lacking the caspase-3 antibody or near Infrared stains and HIV RNA target probes were included as a negative unstained control. NIR negative percentages were calculated by combining the values from quadrant 1 and 4 (Q1 and Q4) from C while NIR positive percentages were calculated from the combined values of Q2 and Q3.

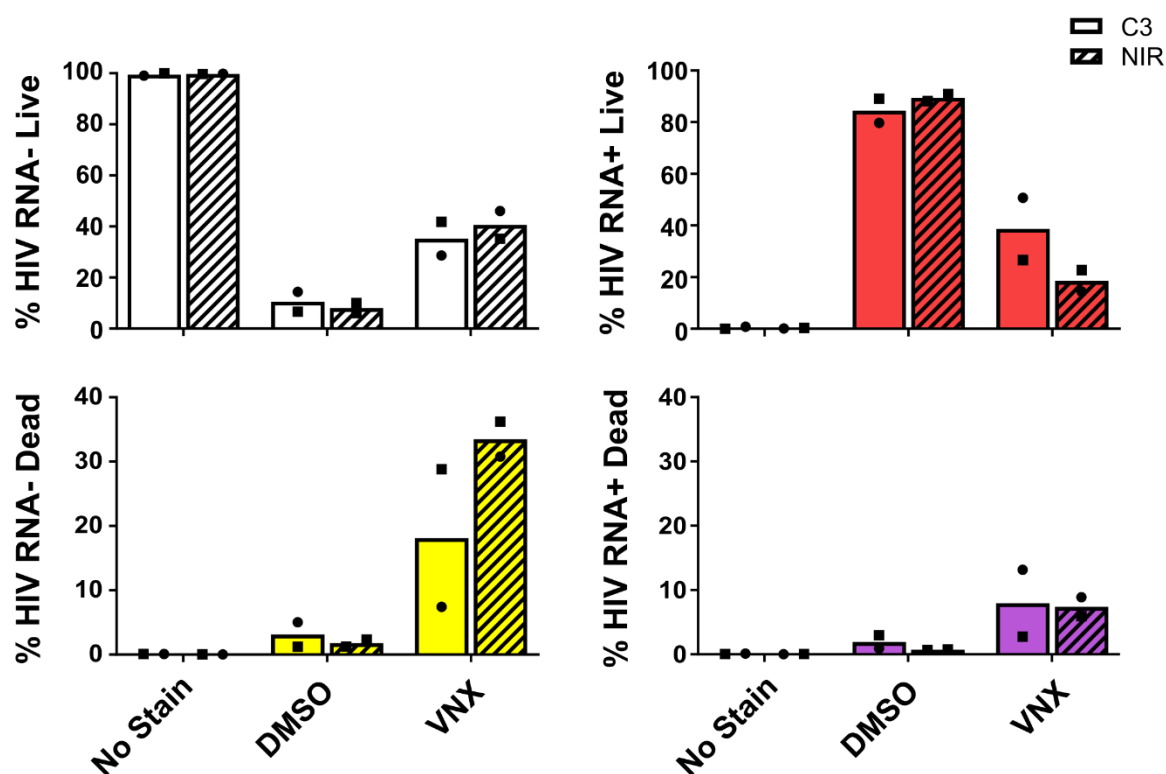


Figure 4.6. Co-staining of HIV RNA with either near infrared live/dead or PE active caspase-3 stain in stimulated ACH2 cells

ACH2 cells were activated with PMA for 24 hours followed by 10 μ M venetoclax to induce apoptosis or DMSO diluent control for an additional 24 hours. Cells were stained with either PE active caspase-3 or near Infrared Live/Dead Fixable Cell Death Stain, and then stained for HIV RNA using PrimeFlow staining; HIV gag RNA with caspase-3, or HIV gag plus env RNA with Near infrared live/dead). The no stain control lacks the caspase-3 antibody or near infrared live/dead stains and HIV RNA target probes as appropriate. Samples were analysed on a BD Fortessa flow cytometer, gated on single cells and then for HIV RNA+ (gag+ and/or env+) or HIV RNA- (gag- or gag-env-) cells. HIV RNA+ and RNA- cells were then gated for PE active caspase-3 positive and negative, or near infrared live negative or dead positive cells. The percentage of HIV RNA- live, HIV RNA- dead, HIV RNA+ live and HIV RNA+ dead cells was then calculated in the original single cell population and the results of 2 independent experiments graphed. Columns show the mean and symbols represent the values of each experiment n=2. Live denotes capsase-3- or near infrared- cells. Dead denotes caspase-3+ or near infrared+ cells. VNX; venetoclax, NIR; Near Infrared.

4.4.4 NIR live/dead and PrimeFlow HIV RNA staining of PMA and venetoclax treated ACH2 T-cell line captures cells that have reactivated and died

Further experiments were performed using PMA stimulated ACH2 cells with NIR live/dead stain and PrimeFlow for HIV *gag* and *env* RNA (Figure 4.7) to confirm the reproducibility of staining. In three independent experiments, ACH2 cells were reactivated for 24 hours with PMA followed by a further 24 hours with 10 μ M venetoclax to induce apoptosis or DMSO diluent as a negative control. Uninfected Jurkat T-cell line samples were included as uninfected controls. A representative gating strategy is shown in Figure 4.7. Cells were gated on single cells (not shown) and then gated based on their expression of HIV *gag* RNA (AF647) and HIV *env* RNA (AF568). A spider quadrant gate was used to determine *gag+env-*, *gag+env+*, *gag-env+* and *gag-env-* populations (Figure 4.7). Each quadrant was then further analysed for NIR- live and NIR+ dead cells using the NIR live/dead stain (Figure 4.7). The percentage of NIR- live and NIR+ dead cells in the four *gag/env* populations was used to calculate the percentage of the original single cell population that was HIV RNA- live, HIV RNA- dead, HIV RNA+ live and HIV RNA+ dead, with the results for the *gag+env-*, *gag+env+*, *gag-env+* quadrants combined for the collective HIV+ subset (Figure 4.7).

Treatment of PMA activated ACH2 cells with 10 μ M venetoclax resulted in a 3.78-fold decrease in HIV RNA+ live cells (red column) with a concurrent 6.43-fold increase in HIV RNA+ dead cells (purple column) compared to the DMSO treated cells (figure 4.8). This is consistent with venetoclax enhancing the death of latently infected ACH2 cells reactivated by PMA. Additionally, an 11.69-fold increase in the HIV RNA- dead cells (yellow column) was also observed, which could be explained by the loss of HIV RNA in cells that have died given that over 80% of PMA activated cells expressed HIV RNA in the DMSO control without venetoclax. The population of HIV RNA- live ACH2 cells (white column) also increased 3.48-fold in the venetoclax treated samples compared to the DMSO treated samples perhaps reflecting an enrichment in HIV RNA- live ACH2 cells proliferating in response to PMA. Uninfected Jurkat T-cell samples were included as an HIV RNA- control and were negative for HIV RNA as expected (Figure 4.8) confirming the specific detection of HIV RNA in the PMA reactivated ACH2 samples. Thus, these data demonstrate that using a NIR live/dead stain with PrimeFlow for HIV RNA can reproducibly detect latently infected T-cells that reactivate HIV RNA and selectively die in a cell line model.

In summary, we developed a new staining method using NIR live/dead staining together with PrimeFlow staining for HIV *gag* and *env* RNA to clearly detect reactivation of HIV RNA from latency and concurrent cell death induced by a high dose of venetoclax using the ACH2 T-cell line. This will now be used to test that ability of other LRA and pro-apoptotic drug combinations to drive the death of latently infected T-cells in primary cells from PLWH on ART.

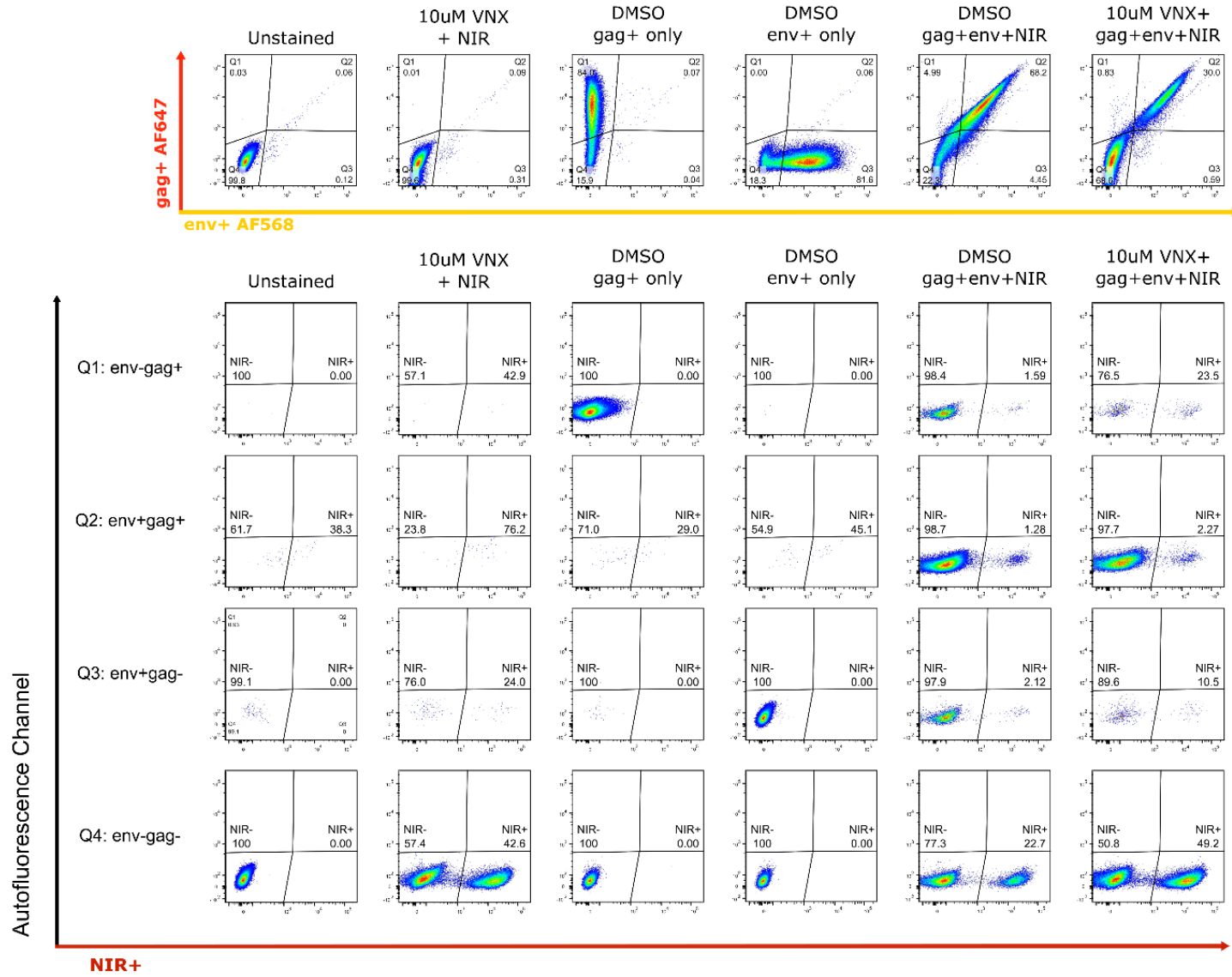


Figure 4.7. Detection of HIV gag and env RNA combined with NIR live/dead stain in reactivated ACH2 T-cells

Latently infected ACH2 cells were reactivated with PMA for 24 hours followed by 10 μ M venetoclax to induce apoptosis or DMSO diluent control for a further 24 hours. Cells were stained with near infrared (NIR) live/dead stain followed by PrimeFlow for HIV gag RNA (AlexaFluor 647) and env RNA (AlexaFluor 568). Samples were analysed for all three fluorochromes on a flow cytometer. Cells stained with all of the buffers but lacking the NIR stain and HIV RNA target probes were included as no stain negative controls. In flow analysis, samples were gated for single cells, then HIV RNA+ (gag+ and/or env+) and HIV RNA- (gag-env-) cells, and then NIR- live and NIR+ dead cells in the HIV RNA+ and – subsets. Example gating of samples from a representative experiment is shown. VNX- venetoclax; NIR- near infrared.

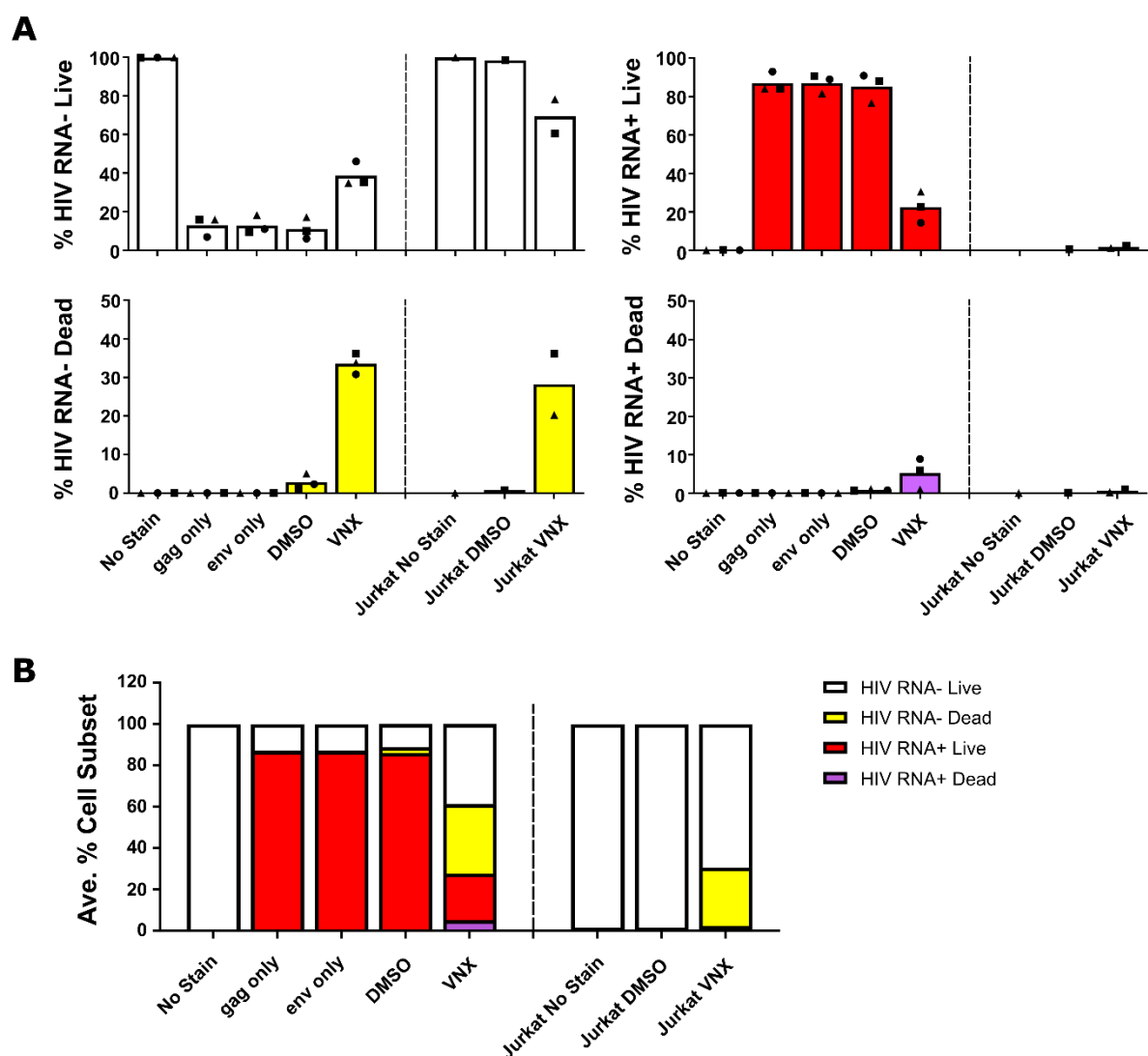


Figure 4.8. Venetoclax increases the number of dead RNA- and RNA+ cells following stimulation of ACH2 cells with PMA

ACH2 T-cells reactivated with 5nM PMA for 24 hours and then treated with 10 μ M venetoclax for an additional 24 hours. Cells were co-stained with NIR live/dead cell death stain and PrimeFlow HIV RNA gag and env probes. Additionally, uninfected Jurkat T-cell line samples treated without or with venetoclax were added as comparative controls. The percentage of HIV RNA-Live (NIR-), HIV RNA- Dead (NIR+), HIV RNA+ Live and HIV RNA+ Dead cells was then calculated in the original single cell population. Results from 3 independent experiments are shown either as: graphs showing columns for the mean with independent experiment values added as separate symbols (A); or a stacked graph with the mean of the four populations from the three independent experiments plotted (B). VNX, venetoclax. Each symbol represents an individual independent experiment.

4.4.5 Treatment of ACH2 T-cells with PEP005 and PMA together with pro-apoptotic drug IPI-443 leads to selective cell death

Previous work (Chapter 3) has shown that certain combinations of LRAs combined with pro-apoptotic drugs, such as romidepsin LRA with IPI-443 or venetoclax pro-apoptotic drug, can reactivate latently infected cells and also deplete these infected cells from PLWH on ART *ex vivo* by measuring HIV RNA and integrated DNA respectively. However, using qPCR for HIV integrated DNA to measure the reduction in the HIV viral reservoir does not indicate whether the decrease in infected cells is selective for HIV infected cells with inducible provirus that have reactivated viral RNA, or from cells that remained latent, or both. Therefore, we used our novel method combining a cell death stain with PrimeFlow detection of HIV *gag* and *env* RNA to detect the ability of LRA and pro-apoptotic drugs to reactivate and selectively kill latently infected T-cells.

We focussed on testing the PKC agonist PEP005, PMA and HDAC inhibitor romidepsin. These LRAs were chosen based on previous *ex vivo* work (Chapter 3). In the ACH2 T-cell lines, these LRAs were able to induce protein expression efficiently. The pro-apoptotic drugs IPI-443 and venetoclax were also chosen based on previous work that demonstrated some synergy with PMA and romidepsin.

ACH2 T-cell lines were first treated with the pro-apoptotic drugs: 100nM PI3K inhibitor IPI-443 or 1 μ M Bcl-2 inhibitor venetoclax, or DMSO drug diluent negative control for 24 hours. Following treatment with the pro-apoptotic drugs, cells were separated and treated with 3 different LRAs or DMSO as a drug diluent negative control. These LRAs included: 50nM PEP005 or 5nM PMA for a further 24 hours, or 40nM romidepsin for 4 hours (to minimise toxicity), which was washed out and returned to culture with the relevant pro-apoptotic drug for a further 20 hours. ACH2 T-cells were then stained with the NIR live/dead stain to mark dead cells followed by PrimeFlow staining for HIV *gag* and *env* RNA as previously described (Figure 4.9).

Using this novel method, we found that the treatment of ACH2 T-cell lines with the PKC agonist PEP005 alone resulted in a 1.66-fold increase in the HIV RNA+ live cells compared to the DMSO control suggesting reactivation of the ACH2 T-cells with PEP005 (Figure 4.9B).

There was also a 3.21-fold increase the percentages of HIV RNA+ dead cells suggesting that PEP005 alone can reactivate and lead to the death of these cells. The number of HIV RNA- dead and HIV RNA- live population of cells treated with PEP005 was comparable to cells treated with DMSO.

PEP005 together with IPI-443 lead to a 2.60-fold decrease in the number of HIV RNA+ live cells compared to cells treated with the DMSO negative diluent control, which was comparable to cells treated with PEP005 alone. Additionally, the increase in HIV RNA+ dead cells in ACH2 T-cells treated with PEP005 and IPI-443 was also comparable to cells treated with PEP005 alone. This suggests that the addition of IPI-443 to PEP005 did not result in an increase in HIV reactivation or cell death. Treatment with PEP005 combined with venetoclax also resulted in a 2.59-fold increase in HIV RNA+ dead cells compared to the DMSO negative control. The data here suggests successful detection of the reactivation and selective death of a small pool of latently infected ACH2 T-cells (<1% HIV RNA+ dead cells) following treatment with PEP005 alone. The addition of the pro-apoptotic drugs IPI-443 or venetoclax did not result in an increase in HIV reactivation or cell death detected by this assay.

Treatment of ACH2 T-cells with PMA combined with IPI-443 and venetoclax led to a decrease in the number of HIV RNA- live cells (3.50- and 3.75-fold, respectively) compared to the negative DMSO control (Figure 4.9C). We observed a 1.62- and 2.53-fold increase in the number of HIV RNA- dead cells and a 1.72- and 1.73-fold increase in the number of HIV RNA+ live cells. This indicates the combination of PMA with IPI-443 or venetoclax can reactivate latently infected cells and increase cell death. Importantly, PMA together with IPI-443 or venetoclax increased the number of HIV RNA+ dead cells 3.93- and 3.48-fold respectively above the DMSO negative diluent control (Figure 4.9C). PMA together with IPI-443 compared to PMA or IPI-443 alone led to a 2.58- and 3.03-fold greater increase in HIV RNA+ dead cells, respectively. PMA and venetoclax together compared to PMA or venetoclax alone resulted in a 2.29- and 3.83-fold increase in HIV RNA+ dead cells, respectively. This indicates that the combination of PMA with IPI-443 or venetoclax leads to the reactivation and selective death of a small pool of latently infected ACH2 cells (<1% HIV RNA+ dead cells).

Romidepsin treatment of ACH2 T-cells alone led to an average of 48.65% of cells expressing HIV in the live population of cells (HIV RNA+ live) and this was comparable to ACH2 T-cells treated with the DMSO negative diluent control (mean=48.50%). The addition of the two pro-apoptotic drugs IPI-443 and venetoclax did not result in large increases in the number of HIV RNA+ live or dead cells and were comparable cells treated with either romidepsin, IPI-443 and venetoclax alone (Figure 4.9A). This suggests that a 4 hour pulse of ACH2 cells with 40nM romidepsin and subsequent 20 hours of culture was not sufficient to effectively reactivate latent HIV in the ACH2 cells, preventing the analysis of romidepsin together with pro-apoptotic drugs to reactivate and selectively clear latently infected cells. This might be due to the romidepsin dose, incubation period, and/or reactivation properties of latently infected provirus in the clonal ACH2 T-cell line versus *ex vivo* CD4+ T-cells from PLWH on ART with diverse integrated provirus (Chapter 3).

Collectively, these data suggest that this novel assay combining a near infrared live/dead stain with PrimeFlow for HIV *gag* and *env* RNA can detect the reactivation and selective cell death of latently infected T-cells by drug combinations. Treatment of the ACH2 T-cell line with PMA together with IPI-443 PI3K inhibitor or venetoclax Bcl-2 inhibitor, or PEP005 alone or together with IPI-443 led to small increases in the pool of HIV RNA+ dead cells suggesting that these drug combinations can increase the preferential death of latently infected cells once reactivated. Further work is now needed to determine whether this assay can also be applied to primary CD4+ T-cells collected from PLWH on ART.

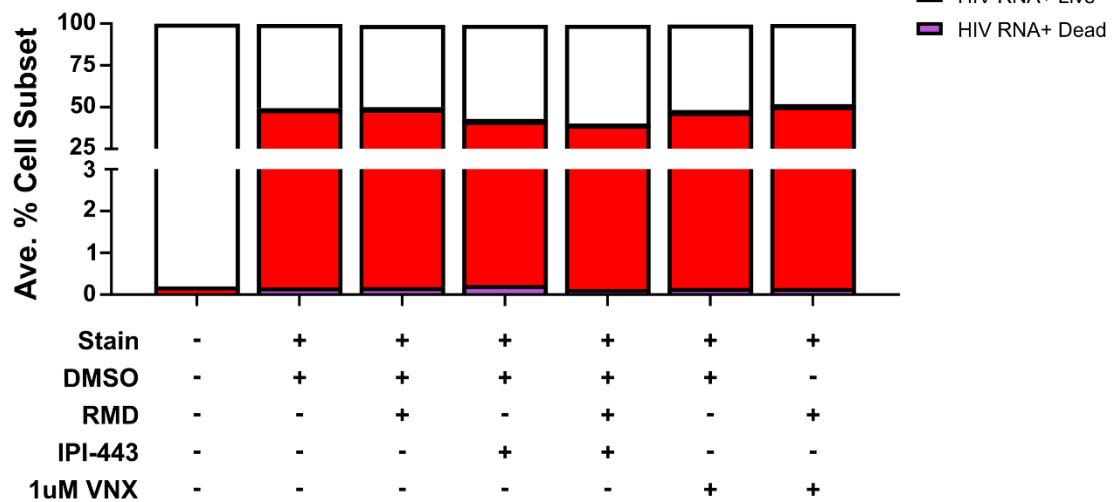
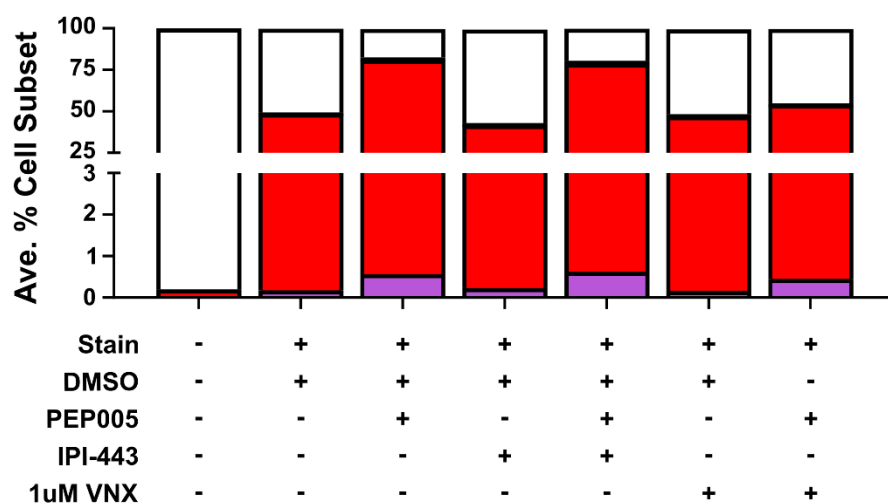
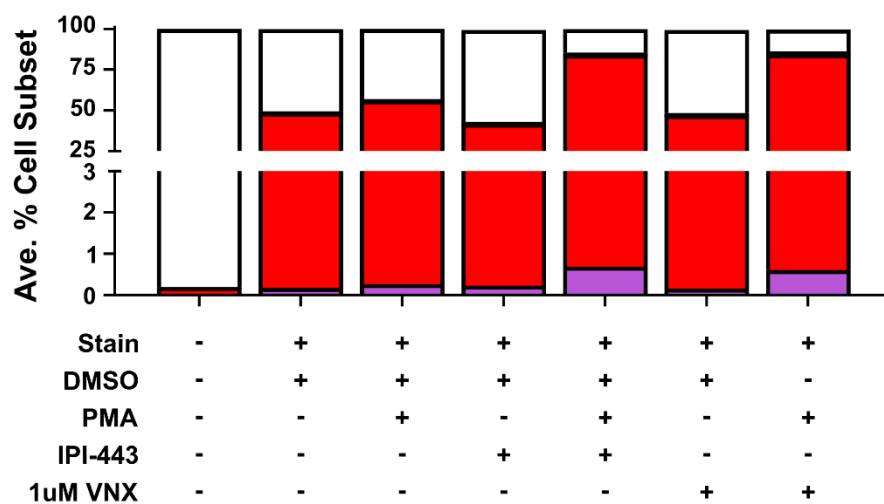
A**Romidepsin****B****PEP005****C****PMA**

Figure 4.9. Treatment with pro-apoptotic drugs IPI-443 and venetoclax combined with LRAs PEP005 or PMA in ACH2 T-cell lines led to an increase in selective cell death of cells expressing HIV RNA

ACH2 T-cell lines were treated with 100nM IPI-443 or 1 μ M venetoclax pro-apoptotic drug or DMSO diluent control for 24 hours, followed by latency reactivation by adding the LRA: 40nM romidepsin for 4 hours followed by 20 hours in the matched pro-apoptotic drug background (A), 50nM PEP005 for 24 hours (B) or 10nM PMA for 24 hours (C). A DMSO diluent only sample was also included as a no LRA control. Following latency reversal, cells were stained with NIR live/dead stain followed by PrimeFlow staining for HIV gag and env RNA. Control untreated ACH2 cells stained without the NIR live/dead and HIV RNA target probes. Control cells were not treated with LRAs or pro-apoptotic drugs and reactivated with 5nM PMA for 48 hours. Following staining, cells were analysed for all three fluorescent markers using flow cytometry. Percentages of HIV RNA- live, HIV RNA- dead, HIV RNA+ live and HIV RNA+ dead cells were calculated and the mean of three independent experiments (n=3) shown as a stacked graph. RMD; romidepsin, VNX; venetoclax, NIR; near infrared.

4.4.6 Staining with the HIV mRNA PrimeFlow assay results in an increase in cell loss

While early experiments demonstrated clear separation of NIR positive and negative ACH2 T-cells that were concurrently stained with PrimeFlow (Figure 4.7-4.9), it was noted that samples treated with the high 10 μ M dose of venetoclax in particular lost many cells during the staining process. This was apparent both by eye when pelleting cells during the elaborate staining process, and when running cells on the flow cytometer at the end of the experiment. We hypothesized that a proportion of dead cells were being lost during the staining process due to the excess number of washes involved in the PrimeFlow assay together with altered properties of the dead cells. To determine whether the dead cells were being washed away during the PrimeFlow staining, we examined the percentages of dead cells irrespective of HIV RNA staining in ACH2 T-cells treated with pro-apoptotic drugs and LRAs. A small aliquot of cells was taken from samples after NIR live/dead staining before and then after PrimeFlow staining and the percentage of NIR dead cells in samples analysed by flow cytometry.

Strikingly, it can be seen that the number of dead cells detected in all samples decreased more than 2-fold (Figure 4.10) at the end of PrimeFlow RNA staining prior to before PrimeFlow RNA staining. This demonstrates that dead cells were being preferentially lost versus live cells during the PrimeFlow staining.

The fold change in the percentage of dead cells detected before PrimeFlow staining (after NIR) compared to after PrimeFlow staining was greater in ACH2 T-cells treated with venetoclax and PEP005 (Table 4.1) and greater than cells treated with each drug alone. This trend was also seen in cells treated with romidepsin. The combination of pro-apoptotic drug and LRA resulted in a larger decline in detected dead cells than the drug alone samples. This suggests that a combination of potent drugs results in a greater loss of cells, leading to underestimations of dead cells with PrimeFlow staining. Additionally, the number of cell events measured in each condition varied, with some conditions having substantially less cells (Supp Table 4.1).

Interestingly, treatment of ACH2 T-cells with romidepsin combined with IPI-443 and venetoclax pro-apoptotic drugs resulted in an increase in cell death above either drug alone before PrimeFlow staining but not after PrimeFlow (Figure 4.10A, red versus blue bars). Combination of PEP005 LRA with 1 μ M venetoclax also increased cell death compared to the single drug counterparts before PrimeFlow staining but not after PrimeFlow (Figure 4.10B-C, red versus blue bars). Taken together, this data indicates that the PrimeFlow procedure results in considerable loss of dead cells, contributing to the very low numbers of dead cells observed in Figure 4.9.

A**Romidepsin**

After NIR
After PF

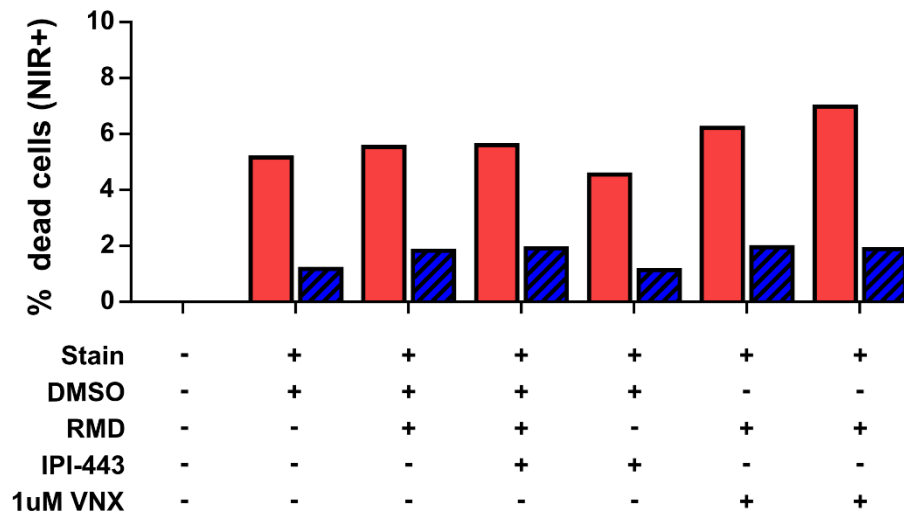
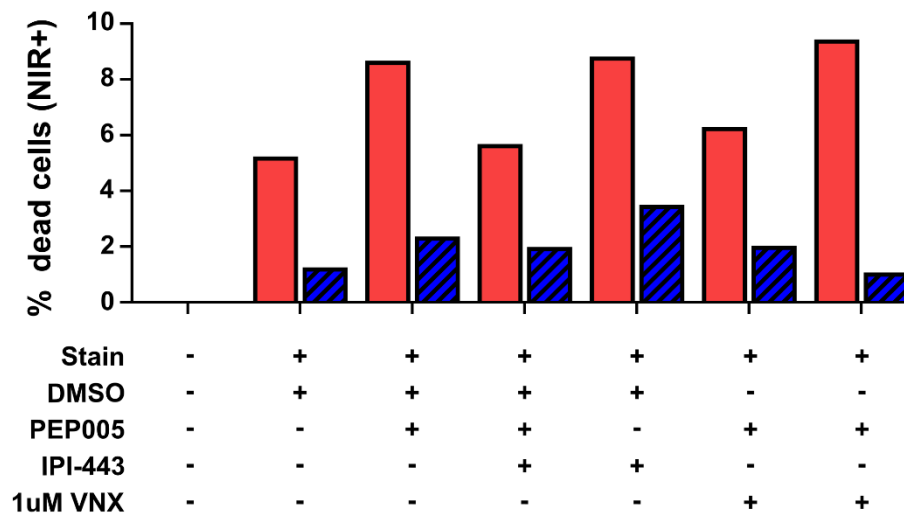
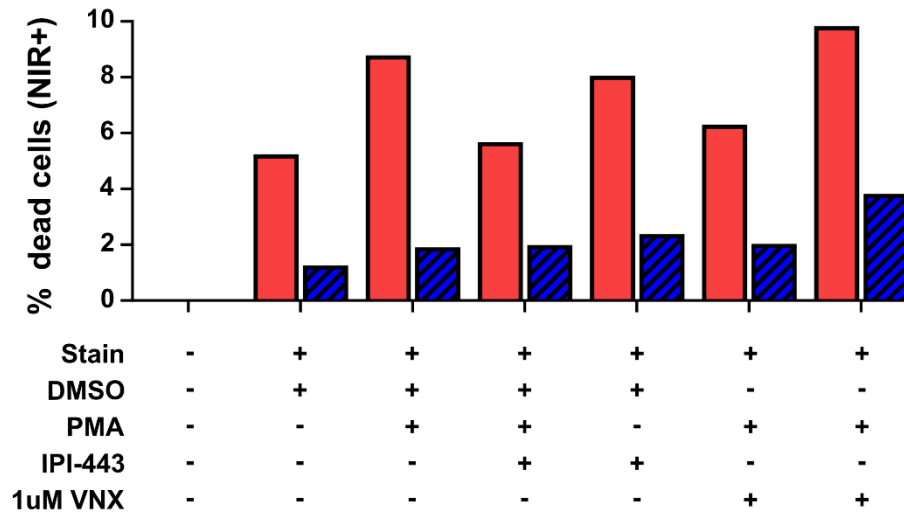
**B****PEP005****C****PMA**

Figure 4.10. Staining of ACH2 T-cells with PrimeFlow results in substantial loss of dead near infrared positive cells

ACH2 T-cells were treated with the pro-apoptotic drug IPI-443 or venetoclax, or DMSO diluent control for 24 hours. Following this treatment, cells were reactivated with the LRAs; 40nM romidepsin for 4 hours followed by 20 hours culture in the matched pro-apoptotic drug, or PEP005 or PMA for 24 hours. Cells were then stained with NIR live/dead stain. A small aliquot of cells was taken at this time and stored at 4°C, dark. The remaining cells were stained for HIV gag- and env mRNA using the PrimeFlow assay. All cells were analysed on a flow cytometer. The percentage of total dead cells (irrespective of HIV RNA) in samples are shown for 2 independent experiments (n=2). Columns show the mean of independent experiments. RMD; romidepsin, VNX; venetoclax, NIR; near infrared.

Table 4.1. Mean percentage of dead cells and fold change before and after PrimeFlow staining

Sample	Before PF	After PF	Fold Change (Before/After)
	Mean (% of dead cell)	Mean (% of dead cell)	
DMSO	5.24	1.25	4.19
RMD Alone	5.62	1.91	2.95
IPI-443 Alone	5.68	1.99	2.86
IPI-443 + RMD	4.63	1.21	3.82
VNX Alone	6.30	2.03	3.10
VNX + RMD	7.06	1.97	3.59
DMSO	5.24	1.25	4.19
PEP005 Alone	8.67	2.36	3.67
IPI-443 Alone	5.68	1.99	2.86
IPI-443 + PEP005	8.82	3.50	2.52
VNX Alone	6.30	2.03	3.10
VNX + PEP005	9.44	1.07	8.82
DMSO	5.24	1.25	4.19
PMA Alone	8.79	1.91	4.61
IPI-443 Alone	5.68	1.99	2.86
IPI-443 + PMA	8.06	2.38	3.38
VNX Alone	6.30	2.03	3.10
VNX + PMA	9.83	3.82	2.57

RMD- romidepsin; VNX- venetoclax

4.5 Discussion

We developed a method to detect the reactivation of HIV RNA from latency and selective cell death induced by LRA and pro-apoptotic drugs. We then identified LRA and pro-apoptotic drug combinations that induced the selective death of cells that were expressing HIV RNA. We demonstrated that this assay worked in ACH2 cells using PrimeFlow for gag and env RNA combined with near infrared staining. Finally, we found that PrimeFlow resulted in a loss of dead cells suggesting our assay may underestimate the proportion of cells that express both HIV RNA and markers of cell death.

In ACH2 T-cells reactivated with PMA and treated with either a high 10 μ M dose of venetoclax to induce cell death or DMSO negative diluent control for comparison, we observed several changes to HIV RNA and cell death subsets. First, the proportion of HIV RNA⁺ dead cells increased in the venetoclax treated cells versus the DMSO treated cells, as expected (Figure 4.6). The increase in HIV RNA⁺ dead cells by venetoclax was accompanied by a concurrent decrease in the HIV RNA⁺ live cell subset (Figure 4.6). As over 80% of PMA reactivated ACH2 treated with DMSO were HIV RNA⁺ live, this suggested that a subset of PMA reactivated cells shifted from a HIV RNA⁺ live phenotype in DMSO treated cells to a HIV RNA⁻ dead phenotype with venetoclax. Interestingly, we found that the drastic decrease in proportion of HIV RNA⁺ live cells did not correspond with the small increase in HIV RNA⁺ dead cells (Figure 4.6). This discrepancy could be due in part to the loss of dead cells from the PrimeFlow staining procedure (Figure 4.10). The NIR live/dead staining together with HIV RNA PrimeFlow likely detects a snapshot of cells with reactivated HIV RNA and undergoing cell death but not all events as HIV RNA has degraded in some of the dead cells.

We also found that the population of HIV RNA⁻ live cells increased when ACH2 T-cells were treated with venetoclax, which was perplexing given that the HIV RNA⁺ dead cells and HIV RNA⁻ dead cells also both increased. One possible reason is that the loss of dead cells overall through the extensive washing steps involved in PrimeFlow RNA staining (Figure 4.10) results in an enlarged population of HIV RNA⁻ live cells that are less prone to being washed away. Alternatively, the proliferation of this HIV RNA⁻ live cell population by the PMA mitogen [482] over the 48 hour culture period might also increase this population of cells.

Overall, PrimeFlow for HIV RNA combined with the NIR live/dead stain was able to successfully identify a subset of latently infected cells with reactivated HIV RNA and undergoing subsequent death due to PMA and venetoclax drug treatment. However, as we could not ascertain the cause of the increased frequency of HIV RNA- dead cells and HIV RNA- live cells, we thus propose that this novel method takes a “snapshot” of cellular phenotypes in cultures at the time of cell harvest. Different culture times in response to drug treatment may lead to different results than shown here. Thus, further investigation into the effects of cell harvest timing on the proportions of HIV RNA and dead cell subset observed in response to drug treatments is warranted.

This novel method combining the near infrared live/dead stain with PrimeFlow for HIV RNA was then used to measure the reactivation and selective cell death of the latently infected ACH2 T-cell line treated with other pro-apoptotic drug and LRA combinations. Interestingly, unlike the ACH2 T-cells treated with PMA and high 10 μ M dose venetoclax (Figure 4.6), an increase in the HIV RNA- live cell population was not seen in cells treated with these subsequent combinations of pro-apoptotic drug and LRAs (Figure 4.9) perhaps suggesting that not as many dead cells were lost in the latter experiment.

The combination of IPI-443 PI3K inhibitor or venetoclax Bcl-2 inhibitor together with PMA LRA led to a small <1% population of cells in which HIV RNA had reactivated from latency and cells died, versus the LRA or either pro-apoptotic drug alone (Figure 4.9). Treatment with PEP005 alone or combined with IPI-443 PI3K inhibitor also led to small <1% cell population that had successfully reactivated latent HIV and induced cell death (Figure 4.9). Thus, novel combinations of pro-apoptotic drugs such as IPI-443 together with LRAs like PMA and PEP005 can induce virus expression but there is minimal death of these cells. We anticipate that these findings might be quite different in primary cells, given the ACH2 cell line is an immortalised cell line which favours proliferation and cell survival.

To our knowledge, this is the first time that selective cell death of reactivated latently infected cells has been demonstrated in HIV-infected T-cell lines following a “shock and kill” drug

combination study. Several other groups have demonstrated the benefit of using PrimeFlow to study the impact of LRAs on reactivation of HIV *gag* RNA and p24^{Gag} protein expression (using antibody staining) at a single-cell level [166, 167, 509]. A live/dead cell death stain was included in the protocol of these however, these groups were focused on the induction of HIV RNA and protein in latently and newly infected cells by flow cytometry on a single-cell level and only used the cell death stain to exclude dead cells. More often than not, the “kill” part of the shock and kill approach is overlooked. In contrast, we used the live/dead stain to capture the impact of drug combinations to reactivate HIV RNA and subsequently induce cell death. This allows a more in-depth assessment of drugs upon the reactivation of HIV RNA as well as cell death, the ultimate goal of the “shock and kill” strategy.

Here we did not observe reactivation of ACH2 T-cells with romidepsin. The cells were pulsed for 4 hours with this LRA to reactivate the latent HIV. This pulse time and dose of romidepsin may not have been sufficient enough to reactivate the cells, which is in contrast to work from our laboratory that has shown 4-hour pulse with 40nM romidepsin is sufficient to induce HIV protein expression (Personal communication with Dr. Jennifer Zerbato). Moreover, previous work has also demonstrated reactivation of latent HIV in the ACH2 T-cells with a 4 hour pulse of romidepsin [419]. It is difficult to conclude whether or not the combination of romidepsin with PI3K inhibitor IPI-443 or Bcl-2 inhibitor venetoclax resulted in latent HIV reactivation and selective cell death. Increasing the pulse time of romidepsin or the dose could be done to improve the reactivation capabilities of romidepsin in the ACH2 T-cell lines.

An important limitation of our novel method using NIR live/dead staining with PrimeFlow staining for HIV RNA is the high level of cell loss following PrimeFlow staining in ACH2 T-cells treated with pro-apoptotic drug and LRAs (Figure 4.9). The large loss in cells using the PrimeFlow protocol has been observed by other groups in addition to our laboratory. Baxter *et al.* [166] observed an approximate 70% cell loss throughout the protocol while Zhang *et al.* [510] describes a similar percentage of cell loss between 70-82% due to the extensive washing steps. This substantial cell loss is an important caveat of the PrimeFlow and death stain assay and suggests that a greater number of starting cells might be needed for this assay than anticipated.

It is important to keep in mind that the ACH2 T-cell lines are an immortalised T-cell line derived from the parent A3.01 CEM T-cell line [178] and this might have limited the detection of dead cells. In this study, we used two different pro-apoptotic drugs to increase the sensitivity of cells to apoptosis: the PI3K inhibitor IPI-443 and the Bcl-2 inhibitor venetoclax. PI3K inhibitors have shown to increase apoptosis or sensitivity towards apoptosis in a wide variety of T-cells (reviewed in[511]) while treatment of CD4+ T-cells from PLWH on ART *ex vivo* with the Bcl-2 inhibitor venetoclax followed by reactivation with T-cell stimuli sensitises the cells for apoptosis [412]. Both of these drug compounds target different mechanisms and proteins of the apoptosis pathway. PI3K inhibitors can have indirect effects on the BH3-only proteins such as Bad [502] while venetoclax directly binds and immobilises Bcl-2 proteins [412]. Work from Berro *et al.* has demonstrated that the ACH2 T-cell line has upregulated expression of the anti-apoptotic protein, X-linked inhibitor of apoptosis (XIAP) [417]. Thus, unless the excessive levels of XIAP are inhibited, it could be difficult to induce cell death in ACH2 cells using PI3K inhibitors or a Bcl-2 inhibitor.

Further work is needed to delineate the level of detection using PrimeFlow combined with a cell death stain. Performing a limiting dilution using RNA+ in a background of RNA- cells will help determine if this assay can be used to detect reactivated latently infected CD4+ T-cells from PLWH on ART. Baxter *et al.* reported the detection of 3.56 HIV *gag* RNA and p24^{Gag} translation competent cells per million CD4+ T-cells [166] while Grau-Exposito *et al.* reported 6 HIV *gag-pol* RNA and p24^{Gag} protein positive cells per million CD4+ T-cells [167] induced *ex vivo* from the blood of PLWH on ART. These estimates are higher than the gold standard QVOA method but lower than PCR-based methods [156]. Recently, Pardons *et al.* describe a new assay termed HIV-Flow, that can successfully measure the frequency of infected cells using flow cytometry [168]. HIV-Flow utilises two HIV p24^{Gag} antibodies to detect cells that produce HIV p24^{Gag} protein following stimulation. Using this assay, it was demonstrated that a mean of 4.3 p24-producing cells per one million CD4+ T-cells from virally suppressed PLWH could be detected [168]. Our assay described here differs to these other reports as we only measure HIV RNA expression. Combining the PrimeFlow technique for HIV RNA with a HIV p24^{Gag} protein antibody stain and the near infrared stain for cell death could further enhance the

study of drug combinations upon the reactivation and death of HIV translationally competent cells from PLWH on ART *ex vivo*. Using this triple combination of stains warrants further investigation as it has the potential to detect and propel the discovery of drug compound combinations that lead to both a “shock” and “kill”.

4.6 Conclusion

We developed a PrimeFlow assay for HIV *gag-pol* and *env-nef* RNA combined with a near infrared live/dead fixable stain that can successfully differentiate HIV RNA expression and selective cell death using the latently infected ACH2 T-cell line. We used this assay to test the impact of combinations of pro-apoptotic drug and LRAs. We could detect low levels of selective cell death of cells expressing HIV RNA following treatment with a very strong activation signal, PMA, with IPI-443 PI3K inhibitor or venetoclax Bcl-2 inhibitor. The very low number of HIV RNA expressing dead cells may be due to the ACH2 cell line being immortalised and proliferating. Further development and optimisation of this assay is needed using CD4+ T-cells from PLWH on ART *ex vivo*. Once developed, novel combinations of LRAs and pro-apoptotic drugs can be evaluated.

Supplementary Table 4.1. Number of events collected from each sample condition in cells treated with pro-apoptotic drugs and latency reversing agents

Sample	Number of cells collected		
	Experiment 1	Experiment 2	Experiment 3
No Stain/Probe	41,157	19,211	77,330
Gag only	17,038	~9,435	73,429
Env only	77,020	23,540	92,949
5μM VNX, NIR only	10,647	13,723	100,000
Gag + Env	78,332	45,380	100,000
Gag + NIR	100,000	100,000	100,000
Env + NIR	23,465	100,000	65,798
dapB T1	39,840	69,052	100,000
RPL13A T1	24,564	13,790	74,017
dapB T10	96,380	5,615	33,296
RPL13A T10	100,000	8,071	85,962
DMSO + DMSO	100,000	100,000	42,596
DMSO + 40nM RMD	100,000	33,940	24,636
DMSO + 50nM PEP005	58,959	92,739	28,333
DMSO + 10nM PMA	12,318	100,000	58,288
100nM IPI-443 + DMSO	100,000	100,000	13,597
100nM IPI-443 + 40nM RMD	100,000	100,000	13,681
100nM IPI-443 + 50nM PEP005	52,538	15,309	48,741
100nM IPI-443 + 10nM PMA	35,386	63,289	43,553
1μM VNX + DMSO	100,000	100,000	21,803
1μM VNX + 40nM RMD	100,000	100,000	14,217
1μM VNX + 50nM PEP005	21,779	50,121	8,683
1μM VNX + 10nM PMA	86,134	64,806	13,447
Jurkat 1μM VNX + DMSO	100,000	100,000	17,106
Jurkat 1μM VNX + 40nM RMD	100,000	100,000	20,830
Jurkat 1μM VNX + 50nM PEP005	100,000	100,000	52,275
Jurkat 1μM VNX + 10nM PMA	100,000	100,000	70,815

T1- type 1; T10- type 10; RPL13A- ribosomal protein L13A; NIR- near infrared; RMD- romidepsin

Chapter 5

Integrated Discussion

Chapter 5

Integrated Discussion

5.1 Summary

The main barrier to finding a cure for PLWH on ART is the persistence of a reservoir of long-lived and proliferating latently infected cells that carry replication-competent provirus [26, 29, 457]. Following the cessation of ART, the virus rapidly rebounds [466]. In efforts to eradicate the reservoir of latently infected T-cells, multiple strategies are being investigated. One of these strategies is the “shock and kill” strategy. It involves the administration of LRAs to reactivate or “shock” the latent virus. Upon reactivation, the once latent cell begins to actively transcribe and translate HIV proteins. The “kill” was originally proposed to arise through immune-mediated recognition and clearance of viral protein expressing cells or viral-mediated cytopathic events [214]. Clinical trials with single LRAs have demonstrated transient increases in cell associated and/or plasma HIV RNA with several compounds as anticipated for latency reversal [242, 244, 248, 476]. However, these compounds alone have not led to declines in the number of latently infected cells in most individuals as measured by levels of HIV DNA. Additionally, these drugs did not result in the clearance of the reactivated CD4+ T-cells by autologous CD8+ T-cells in *ex vivo* [365, 419]. Thus, there is a need for new strategies and compounds that not only effectively reactivate latent HIV but can also lead to the clearance of the cells.

In this thesis, we developed two novel assays to enhance the ability to detect agents that reverse latency as well as induce cell death. We developed a dual reporter virus to screen for compounds that activate latent HIV and a flow cytometric method to detect cells that produce HIV RNA and are undergoing apoptosis. We evaluated the role of compounds that could specifically kill latently infected cells and focused on PI3K and Bcl-2 inhibitors and assessed their activity both alone and in combination with commonly used LRAs. In all aspects of the work, we first used latently infected cell lines, including the J-Lat cell line and ACH2 cells to initially test our novel assays and hypotheses and then evaluated primary CD4+ T-cells from PLWH on ART. A common theme in all our work was that although using cell lines is

convenient due to access and the incorporation of a fluorescent reporter and cheap, findings in cell lines were not always consistent with our finding in primary CD4⁺ T-cells, which we hypothesise is related to altered cell survival pathways in these immortalised cell lines.

Evaluation of LRAs together with pro-apoptotic drugs using CD4⁺ T-cells from PLWH on ART showed that the combination of romidepsin with the pro-apoptotic drugs IPI-443, IPI-3063, wortmannin and venetoclax reduced the levels of integrated HIV DNA. Interestingly, we also unexpectedly demonstrated that the PI3K inhibitors IPI-443, IPI-3063 and wortmannin and the Bcl-2 inhibitor venetoclax were all able to increase HIV RNA transcription and reduce HIV integrated DNA, when used alone. Our findings are highly encouraging and demonstrate that manipulation of cell survival pathways of latently infected primary CD4⁺ T-cells is a novel approach to get the 'kill' into shock and kill and should be pursued in further pre-clinical studies.

5.2 LRA and pro-apoptotic drug combinations can deplete latently infected T-cells

5.2.1 Combination of pro-apoptotic drugs and LRAs

In this thesis, we demonstrated that specific combinations of LRAs with pro-apoptotic drugs can not only reactivate the latent HIV provirus but also lead to declines in HIV DNA in cultured CD4⁺ T-cells from PLWH on ART *ex vivo*. In particular, the romidepsin HDACi LRA with any of the PI3K inhibitors tested or the venetoclax Bcl-2 inhibitor resulted in a synergistic decline of integrated HIV DNA versus the drugs alone. Additional combinations using panobinostat or JQ1 LRA with 1 μ M venetoclax also decreased the levels of integrated HIV DNA to a greater level than either drug alone (section 3.4.5). All LRAs were able to induce the expression of HIV RNA and interestingly, our data indicated that the pro-apoptotic drugs alone were also able to reactivate latent HIV and lead to the decline of integrated HIV DNA in T-cells from PLWH on ART *ex vivo* (section 3.4.5). To our knowledge, this is the first time PI3K inhibitors: IPI-443, IPI-3063, wortmannin and the Bcl-2 inhibitor venetoclax have demonstrated this capability.

The exact mechanism of latent HIV reactivation and reservoir clearance by pro-apoptotic drugs alone remains to be elucidated. The induction of cell stress by the pro-apoptotic drugs

may reactivate latent HIV. The induction of Hsp90 by proteasome inhibitors in both an *ex vivo* model of HIV latency and *in vivo* humanized mouse HIV latency model has demonstrated HIV reactivation capabilities [487-489]. The effects of the pro-apoptotic drugs used here may have a similar outcome leading to the increase in CA US HIV that was observed. Ultimately, the combinations of LRA and pro-apoptotic drugs reported in this thesis show hopeful potential in the advancement of shock and kill strategy to purge the latent HIV reservoir.

5.2.2 Further development of Romidepsin

The greatest reduction in integrated DNA was observed using romidepsin together with all the pro-apoptotic drugs. Romidepsin is a more potent HDAC inhibitor than panobinostat [246], a feature of romidepsin that may have contributed to the better effect of romidepsin on depleting the latently infected cells. Romidepsin is a depsipeptide that specifically targets class I HDACs [512] whereas panobinostat is a pan specific HDAC inhibitor [513]. The specific and potent inhibition of class I HDACs by romidepsin compared to panobinostat [246] that has broad inhibitory activity on all classes of HDACs may have contributed to the success of romidepsin to deplete latently infected cells. Class I HDACs have been implicated in the maintenance of HIV latency and thus the potent inhibition by romidepsin may explain why this drug led to an increase in HIV reactivation coupled with a decrease in HIV integrated DNA above panobinostat. Additionally, romidepsin can decrease mitochondria membrane permeability leading to an increase in the production of reactive oxygen species (ROS) in a cell line [477], which could potentially synergise with activation of other apoptotic pathways [514].

Inhibition of HDAC increases caspase-3 protein, BAK protein and can decrease XIAP, which can induce apoptosis and cell death [477, 515]. Importantly, it was shown that romidepsin inhibits the PI3K/Akt pathway in T-cell derived cell lines [477]. Romidepsin affected the levels of pan Akt and pAkt protein as well as directly inhibiting p85 catalytic subunit of PI3K. This provides a potential mechanistic explanation for the synergistic effect of romidepsin and PI3K inhibitors in decreasing infected T-cells *ex vivo*, the combination of romidepsin and PI3K inhibitors could inhibit multiple cell survival pathways, ultimately leading to the production of ROS and an increase in pro-apoptotic molecules. Our findings extend the utility of

romidepsin in the shock and kill strategy given that we found romidepsin combines with PI3K inhibitors and Bcl-2 inhibitor to enhance latently infected cell death, enhancing the “kill” part of this eradication approach.

Our data would suggest that combining romidepsin with PI3K inhibitors or Bcl-2 inhibitors like venetoclax might better deplete latently infected cells in PLWH on ART and is worthy of further investigation.

5.2.3 Timing of drug administration

Prior to a clinical trial with pro-apoptotic drugs and romidepsin, the timing of administration of the two drugs should be thoroughly investigated. In this thesis, the pro-apoptotic drug was first administered to pre-sensitize the latently infected T-cell towards apoptosis, followed by reactivation of HIV proteins with LRAs, based on a previous study by Cummins *et al.* [412]. Reversing the timing of the drug administration so that reactivation of HIV pro-apoptotic proteins occurs first followed by the pro-apoptotic drug should also be investigated. Adding romidepsin LRA first for the initial production and accumulation of HIV proteins that function as pro-apoptotic molecules such as viral protease, Env, Vpr, Nef and Tat [516] during the late stages of HIV replication may lower the threshold for cell death within the latently infected cell so that the subsequent addition of pro-apoptotic drug serves as a final blow, more effectively pushing the cell over the threshold to apoptosis and death. Testing the addition of the drugs together should also be tested *ex vivo* in case similar results are obtained versus when drugs are added separately. Administration of the drugs at the same time in one dose to treat participants would be more convenient than separate drug administration at a set timing and thus should also be further investigated.

5.2.4 The use of animal models in shock and kill strategies

Following studies in cell culture-based models of HIV latency and before testing in PLWH on ART novel combinations should first be evaluated in animal models, given that mucosal and secondary lymphoid organs play a key role in HIV persistence [62]. Experiments *in vitro* may not reflect the complex nature of interactions between different cell types that occur *in vivo*. Following the observation of a synergistic effect of pro-apoptotic drugs combined with

romidepsin in CD4+ T-cells from PLWH *ex vivo*, further studies in animal models may provide insightful knowledge on potency, as well as toxicity and tissue penetration.

Similarities in the viral structural, regulatory and most accessory proteins of the HIV and SIV viruses makes the NHP model an ideal candidate to test the effects of drug combinations prior to clinical trials in humans. It is important to note that there are differences between HIV and SIV, particularly the *vpx* gene in SIV that is important for overcoming the SAMHD1 restriction factor to enable replication in cells that is absent in HIV [517-519]. Additionally, the role of clonal expansion of infected cells, which can account for over 50% of infected cells in PLWH in ART [90], is far less frequent in models of SIV or SHIV [520]. In our studies, we were unable to determine if the decline in integrated HIV DNA *ex vivo* was due to clearance of expanded clones or other infected cells, but this would be an important question to answer in the future. Using tools such as combined integration site and proviral sequencing prior to a following treatment with these drug combinations will be of interest [521]

Humanised mice models present as an alternative animal model to NHP [522]. Humanised mice that are engrafted with human immune cells and/or human tissue can provide rapid and reproducible models of HIV that can be readily sampled and monitored. HIV viral load levels, immune activation and CD4+ T-cell exhaustion are just a number of parameters that humanized mice models can be used to investigate [523]. Longitudinal experiments can be performed as the engraftment of human CD34+ hematopoietic cells allow for continuous *de novo* production and importantly, humanized mice can be infected with laboratory adapted and primary HIV strains that support transmission and replication [523]. Using humanised mice to evaluate strategies to eliminate latently infected cells also has several limitations. Firstly, there can be inefficient spread of human cells to the periphery of mice leading to incomplete colonisation of the murine tissue, reconstitution of human cells following surgery can take a long time, emergence of graft versus host disease (GvHD), engraftment of tissue requires major surgery and a number of mice models do not illicit primary immune responses (reviewed in [524]). Importantly, assessments of the viral reservoir can be limited as tissue reservoirs can't be established in this artificial model [524] and there are varying levels of tissue penetration of antiretrovirals in animal models compared to humans [525]. However,

many groups have used mouse models to evaluate HIV cure interventions and have demonstrated long lived latently infected cells [526] specifically evaluated the time to rebound after stopping ART as a surrogate for an effect on the number of latently infected cells [527].

5.2.6 Future Development of PI3K inhibitors for HIV cure interventions

PI3K inhibitors can be subdivided into pan-PI3K inhibitors or isoform-specific inhibitors. Numerous pan-PI3K inhibitors and isoform-specific PI3K inhibitors are in different stages of clinical trials for targeted cancer therapy (summarised in [511]). In this thesis, an isoform specific PI3K inhibitor (IPI-443) and a pan-PI3K inhibitor (IPI-3063) were tested with and without latency reversing agents (section 3.2.3). There are two FDA-approved PI3K inhibitors, idelalisib and duvelisib. In future work, these two compounds should be evaluated to determine their effects on HIV latency. Idelalisib is an oral and specific PI3K δ isoform inhibitor approved to treat small lymphocytic lymphoma and follicular lymphoma [528]. Duvelisib is an oral dual inhibitor of PI3K δ/γ isoforms approved to treat relapsed or refractory CLL and small lymphocytic lymphoma (SLL) [529, 530]. Adverse effects using idelalisib and duvelisib include neutropenia, diarrhea, fatigue and nausea, however overall safety of the drugs was recorded [531, 532]. In a recent study, the combination of duvelisib and venetoclax in chronic lymphocytic leukemia cells demonstrated an enhanced apoptotic response [533]. The combination of two different pro-apoptotic drugs to reactivate and eliminate latent HIV should be further evaluated, as we have demonstrated that PI3K inhibitors and venetoclax alone led to an increase in latent HIV reactivation and a reduction in latently infected cells (section 3.4.6).

An increasing number of studies that aim to elucidate the role of PI3K in HIV infection have emerged. Recently, treatment of T-cell lines with the PI3K α isoform-specific inhibitor PIK-75 blocked HIV entry [534]. Additionally, dactolisib, a PI3K/mTOR dual inhibitor successfully suppressed HIV replication in primary human macrophages [535]. Although there are currently no clinical trials involving PI3K inhibitors to treat PLWH, the work presented here supports further investigation of the role of PI3K inhibitors in latency reversal.

5.2.7 Future development of Bcl-2 antagonists for HIV cure intervention

Antagonism of the anti-apoptotic Bcl-2 protein by compounds such as venetoclax have emerged as tools to eliminate latent HIV in the past few years [412, 428]. Venetoclax is an FDA-approved Bcl-2 antagonist used to treat relapsed or refractory CLL [427]. Common side effects of drug administration include fatigue, upper respiratory tract infection, neutropenia and diarrhea [536]. In addition to venetoclax, other Bcl-2 antagonists and inhibitors to different Bcl-2 family proteins are currently in development. Inhibitors to Bcl-X_L and Mcl-1 are being tested in T-cell and B-cell models for their effect on apoptosis [537]. Currently, a phase I dose-escalation study to assess the safety and tolerability of this drug in patients with relapsed or refractory CLL has been completed (NCT02920697). Latently HIV infected T-cells were shown to have increased Bcl-2 protein expression [412]. It is possible that latently infected T-cells may also overexpress other anti-apoptotic proteins such as Bcl-X_L and Mcl-1 and examination of the ability of Bcl-X_L and Mcl-1 inhibitors alone or in combination with and without LRAs to reactivate and/or kill latently HIV infected T-cells is warranted.

5.2.8 Induction of apoptosis with other drug compounds

5.2.8.1 Pro-caspase-8 inducers

HIV protease has the ability to cleave pro-caspase-8 into Casp8p41, a fragment that is generated exclusively in cells that are infected with HIV [412, 468]. Four FDA-approved drugs flurbiprofen, indomethacin, doxycycline and bezafibrate increased the levels of pro-caspase-8 substrate and resulted in a decrease in HIV DNA in primary CD4⁺ T-cells *in vitro* and *ex vivo* when combined with T-cell stimulation as the LRA [432]. These oral FDA-approved compounds are used for their non-steroidal anti-inflammatory properties (flurbiprofen, indomethacin), tetracycline antibiotic property (doxycycline) and interferon gamma-inducing property to treat chronic Hepatitis C (bezafibrate). The oral availability and relatively low toxicity of these drugs together with targeting a different step in the viral protease apoptosis-inducing pathway to venetoclax [432] suggest that it would be interesting to test the ability these compounds to clear latently infected cells together with romidepsin in place of venetoclax. Of note, Sampath *et al.* have demonstrated that these drugs decrease *ex vivo* latently infected CD4⁺ T-cells when combined with α -CD3/ α -CD28 stimulation as the LRA,

which will induce global T-cell activation [216, 217]. However, global activation of T-cells creates a toxic environment and is thus unwanted. As our findings indicate that the combination of the romidepsin LRA and a pro-apoptotic compound can purge the latent HIV reservoir, further examination into these drugs that increase pro-caspase-8 expression in combination with romidepsin and potentially other LRAs is warranted.

5.2.8.2 Smac mimetics

Additionally, other pro-apoptotic drugs such as Smac mimetics have been used to eliminate HIV infected T-cells [538] (section 1.7.4.4). Three Smac mimetics: birinapant, GDC-0152 and embelin showed a dose-dependent increase in latently CD4+ T-cells undergoing cell death [538]. These drugs were able to eliminate latently infected CD4+ T-cells without latency reversal. Recently, AZD5582 has also shown to be capable of reversing latent HIV infection in cell lines, primary CD4+ T-cell and both humanised mice and non-human primate models of HIV [287, 539]. It would be of interest to see whether a greater number of cells can be killed when the Smac mimetics are combined with LRAs as Smac mimetics can reverse latency when combined with panobinostat [286].

5.2.8.3 Bruton's tyrosine kinase inhibitors

Another class of pro-apoptotic drug that has shown an ability to increase apoptosis in HIV infected cells are inhibitors to Bruton's tyrosine kinase (BTK). BTK is upregulated on HIV infected T-cells [417] and can result from PI3K activation [540]. Ibrutinib is an FDA-approved inhibitor of BTK used in the treatment of CLL [541]. The treatment with ibrutinib resulted in a decrease in p24 expression and increased apoptosis selectively of HIV infected T-cells [541]. Although the exact mechanism of selective cell death in HIV infected T-cells is not clear, a strategy to clear these infected cells could be to combine BTK inhibitors with LRAs to increase the amount of BTK on the cell's membrane, which has been previously observed [417].

Further analysis into the understanding the underlying mechanisms that lead to cell death with these compounds is warranted. Elucidation into the combination of pro-apoptotic drugs with LRAs or other pro-apoptotic drugs to eliminate latent HIV will provide insightful knowledge that may allow us to exploit these pathways.

5.3 Assays to measure RNA production per cell

5.3.1 HIV RNA PrimeFlow combined with a cell death stain

PrimeFlow offers an alternative method in quantifying the inducible latent HIV reservoir on a single-cell level. The use of both HIV *gag* and *env* RNA probes and a HIV protein marker would allow for the identification of cells that are capable of transcription and translation. The addition of the HIV *env* RNA probe to the HIV *gag* RNA probe in our studies provides an extra benefit that may help to identify replication-competent provirus as this method would detect intact mRNAs with *gag* and *env* sequences. Moreover, the inclusion of other stains such as cell surface markers and a cell death stain would allow for T-cell phenotyping of cells that reactivate HIV and die following drug treatments, which is not available with the QVOA, TILDA or PCR assays. Thus, the PrimeFlow assay can offer a unique glimpse into the reactivation and death of latently infected T-cell subsets by novel LRA and pro-apoptotic drug combinations at a single cell level.

We optimised the PrimeFlow assay to assess cell death initially using activated ACH2 cells. However, this cell line is immortalised and has upregulated expression of the anti-apoptotic protein XIAP [417]. This upregulation of XIAP may ensure apoptotic resistance and thus, would be difficult to overcome unless the cells were treated with specific anti-XIAP compounds which were not used in these experiments. Thus, the use of ACH2 or other immortalised latently infected T-cell lines may mask the ability of pro-apoptotic drugs together with LRAs to clear primary CD4⁺ T-cells. Therefore, although technically challenging, examination of pro-apoptotic drug and LRA combinations to promote selective death would be preferable in primary CD4⁺ T-cells which would better mimic outcomes in vivo.

5.3.2 Limitations of the HIV PrimeFlow assay combined with a cell death stain

As shown in both chapter 2 and 3, latently infected cell lines are useful tools, but they do not always reflect what happens in primary cells. Therefore, our work on the PrimeFlow assay will need further evaluation in primary CD4⁺ T-cells from PLWH on ART. The first issue to optimise will be sensitivity of detection of infected cells. One approach could be to include an antibody stain for HIV p24^{Gag} protein as shown by others [166]. Another priority will be to reduce the amount of cell loss from the multiple cell washing steps.

Performing PrimeFlow alone without a cell death stain to measure the decline in cells that express RNA could be one approach. The exclusion of a cell death stain may improve cell recovery and thus increase sensitivity. Finally, because PrimeFlow staining was originally developed from RNAscope/viewRNA (section 1.6.2.2) used to detect RNA from tissue samples [163], adapting the PrimeFlow and cell death stain co-staining method for tissue samples that harbour more RNA positive cells [53] should also be examined. Moreover, in future experiments, single-cell RNA sequencing (scRNAseq) that can detect both HIV RNA [88] and expression of multiple other host cell genes could also be informative.

5.3.3 DuoAdvance: a new dual-fluorescent reporter virus

In this thesis we describe the development of DuoAdvance, a new dual-fluorescent HIV reporter virus in a model of HIV latency. We observed this virus to infect the Jurkat T-cell line and express the latency and productive infection reporter at similar frequencies to the parental Duo-Fluo I construct (section 2.4.1) showing the promise of DuoAdvance as a new dual-fluorescent reporter virus and potential utility of this construct in T-cell lines. While DuoAdvance could effectively infect the Jurkat T-cell line, unfortunately subsequent experiments testing the ability of this virus to track direct latent infection of resting CD4+ T-cells in the CCL19 chemokine model of HIV from our laboratory [103, 192] were less successful. Despite low constitutive expression of the GFP latent reporter, we were able to demonstrate that at day 6 post infection, the sorted E2 Crimson- (non-productively infected) cell pool carried inducible virus after 3 days of PMA/PHA reactivation. This demonstrated that latent infection was established in some CCL19-treated cells however expression of the GFP latency reporter was poor limiting the use of this reporter virus in a pre-activation model of HIV latency.

5.3.4 Improvements to DuoAdvance viral construct

Consequently, the current DuoAdvance dual-fluorescent HIV reporter construct is limited to studying the establishment and reactivation of HIV latency in T-cell lines due to the low constitutive expression of the latent reporter in primary CD4+ T-cells. Further improvements to the construct may be able to absolve this caveat to enable use in primary CD4+ T-cells. We

were unable to determine if the low constitutive expression of GFP in primary CD4⁺ T-cells was a result of ineffective EF1 α promoter function. Replacing the EF1 α promoter with an alternative promoter such as Ubiquitin C (UbC) or phosphoglycerate kinase (PGK) will help determine if the cause of low expression of GFP is from the EF1 α promoter. Of note, CMV promoter would not be an ideal promoter to use for the latency reporter as CMV promoter activity is subject to the cell cycle phase of the transduced cell [200]. Additionally, CMV can show variable activity in cells derived from hematopoietic stem cells whilst also requiring a degree of T-cell activation for activity [200, 542]. In addition to changing the promoter, separately the introduction of an IRES between the EF1 α promoter and the GFP latent reporter may improve translation initiation and thus expression of GFP in resting CD4⁺ T-cells [465] and it worthy of further investigation.

5.3.5 Applications of DuoAdvance

Despite the limited use of DuoAdvance in a pre-activation model of HIV latency, we have demonstrated successful infection of the Jurkat T-cell line (section 2.4.1). Therefore, DuoAdvance may have a variety of applications in drug screening to identify compounds for further testing in primary cells.

First, the effective discrimination between latently and productively infected Jurkat T-cells with DuoAdvance encourages the possibility of using this model for high-throughput drug screening of novel compounds for LRAs. After infection of Jurkat T-cells with DuoAdvance, selective sorting on the latent GFP⁺ population of cells would provide an ideal population of enriched latently infected T-cells for drug screening. Compounds that potentially reactivate latent HIV would result in the highest frequency and/or mean fluorescence intensity of E2 Crimson⁺ cells. The “hits” or “leads” that are found to potentially reactivate latently infected Jurkat T-cells could then be moved onto further analysis in primary CD4⁺ T-cells from PLWH *ex vivo*.

Secondly, DuoAdvance could also be used to screen for drugs that also block latent reactivation. An alternative approach to inactivating latently infected T-cells via the “shock and kill” strategy is the “block and lock” concept. The “block and lock” concept centralises on

latency promoting agents (LPAs) which target host-specific mechanisms or HIV to drive the latently infected cell into a state of deeper latency [543, 544]. While “shock and kill” aims to reactivate viral HIV RNA and protein and clear the latently infected cells, “block and lock” aims to further suppress latency and prevent the production of HIV viral RNA and proteins in the absence of ART to prevent viral rebound off ART. Through the use of DuoAdvance in a high-throughput system screening novel compounds, compound-mediated suppression of E2 Crimson+ cells from the pool of latent cells despite attempted reactivation would reveal novel LPAs.

Thirdly, DuoAdvance could be used to compare the reactivation dynamics of different LRAs from various classes in latently infected T-cell lines, including the time taken to reactivate viral RNA, the potency of reactivation (displayed by the mean fluorescence intensity of individual cells), how long reactivation lasts and if there is any pattern to reactivation of latency. Additionally, cells that are latently infected with HIV can spontaneously reactivate. There is evidence to support that residual virus replication in PLWH on ART [72, 80] can come from latently resting CD4+ T-cells that become activated in the body. Therefore, DuoAdvance could be used to elucidate the frequency of stochastic reactivation of the latent virus. Following infection of Jurkat or another T-cell line, the latent population of cells could be sorted using flow cytometry and kept in culture for long periods of time to discern the percentage of cells that will spontaneously reactivate and whether this occurs in a burst of transcription. RNA and protein transcription can occur in bursts followed by phases of inactivity [545, 546] and bursts of transcription of genes can influence the fate of cells in HIV [547, 548]. Utilising DuoAdvance, the timing of stochastic transcriptional bursts and burst size could be studied. PrimeFlow for HIV RNA could also be performed at fixed time points to determine the amount of HIV RNA that is produced in the spontaneous reactivation.

Overall, DuoAdvance is able to successfully differentiate latently and productively infected cells in a T-cell line model of HIV. Despite the limited success in a primary CD4+ T-cell model, DuoAdvance can still be utilised in an array of different assays in T-cell lines to study the establishment and reactivation of latent HIV infection, the dynamics involved and identify novel compounds in high-throughput drug screens to more effectively reactivate latency in a

“shock and kill” approach, or shut down viral transcription in a “block and lock” approach. There is still much to learn and elucidate about latently infected cells and this dual-fluorescent virus may be a stepping-stone to discovering new compounds that reactivate latent HIV or suppress reactivation. Each of these strategies warrant further investigation.

5.4 T-cell lines are not an ideal model of HIV latency

Models of HIV latency in T-cell lines are beneficial in that these cell lines are easily accessible, cheap and unlimited. The inclusion of fluorescent reporters also makes working with these cell lines high throughput. Despite this, in all aspects of the work described in this thesis, we found that findings in T-cell lines did not concur with results obtained in a primary CD4⁺ T-cells (Chapter 2 and 3). For instance, infection of the Jurkat T-cell line with DuoAdvance showed successful differentiation of latently and productively infected cells however these populations of cells, especially latently infected T-cells, were rarely detected in resting primary CD4⁺ T-cells. The activation status of the cells as well as the difference in sequestration of transcriptional factors may account for the differences between expression of the DuoAdvance latency infection reporter in the T-cell line versus the primary CD4⁺ T-cells. Additionally, we showed that the combination of PI3K inhibitors IPI-443 and IPI-3063 with romidepsin did not result in increased death of the reactivated latently infected J-Lat6.3 T-cell line. However, this combination among several others both reactivated HIV RNA and decreased the levels of integrated HIV DNA in CD4⁺ T-cell cultures from PLWH on ART *ex vivo*.

The lack of concordance of our findings in cell lines and primary cells appears to be very important when assessing apoptosis. For example, the J-Lat6.3 T-cell line is deficient PTEN resulting in upregulated PI3K signalling and cell survival by preventing apoptosis [174]. This upregulated, pro-survival state of the immortalised J-Lat6.3 T-cell line may prevent romidepsin and either IPI-443 or IPI-3063 PI3K inhibitors from depleting infected cells in contrast to CD4⁺ T-cells from PLWH on ART *ex vivo* where PTEN should be functional. Furthermore, the ACH2 T-cell line used in the PrimeFlow studies has been described to have upregulation of the anti-apoptotic protein XIAP [417]. In these studies, the use of compounds that inhibit PI3K and Bcl-2 and not XIAP could potentially explain why we did not observe

decreased HIV integrated DNA in CD4+ T-cells from PLWH on ART *ex vivo* resulting in selective cell death in the PrimeFlow studies.

These examples demonstrate that HIV latency and the response to LRA and pro-apoptotic drugs differ substantially between latently infected T-cell line models and CD4+ T-cells from PLWH on ART *ex vivo*. Therefore, T-cell lines are not an ideal model to study the ability of LRA and pro-apoptotic drug combinations to reactivate and kill HIV latently infected T-cells. While T-cell lines provide a convenient means of performing a high-throughput drug screen to assess activation of the LTR when looking for LRAs, the differing properties within T-cell lines to primary CD4+ T-cells questions the use of these cell lines for studies of HIV latency and novel strategies to clear HIV latently infected T-cells.

5.5 Limitations of the “shock and kill” strategy

In this thesis, we have demonstrated several novel pro-apoptotic drugs and LRAs combinations that synergistically worked together to reduce the latent HIV reservoir. Clinical trials with LRAs have demonstrated no effects on reducing the latent HIV reservoir [242, 244, 248, 270, 476]. In addition, several limitations to the shock and kill approach need to be addressed before a functional cure can be achieved. The inability of single LRAs to clear and reduce the latent HIV reservoir in clinical trials suggests that LRA monotherapy is not sufficient to reduce the latent reservoir. The number of HIV latently infected T-cells may be 60-fold higher than initially thought [145] and thus a single round of LRA monotherapy stimulation may not be sufficient to target every single replication-competent provirus hidden within the HIV reservoir [145]. Every cell may be targeted through increased rounds of LRA stimulation, but this can then potentially lead to increased side effects and unwanted toxicity. Thus, there is a trend towards the combination of different LRAs that reactivate latent HIV through different mechanistic pathways.

Combination LRA treatment has shown promise with several compounds working together to reactivate latent HIV. Laird *et al.* has shown that the bromodomain inhibitor JQ1 with PKC agonists reactivated latent HIV in CD4+ T-cells *ex vivo* [471], while Jiang *et al.* demonstrated potent reactivation using a combination of JQ1 with the Ingenol-3-

angelate compound PEP005 [268]. Although reactivation of latently infected T-cells may have been effective with combination LRA treatment, it remains to be elucidated as to whether these LRA combinations can also lead to the clearance of cells subsequent to reactivation. Alternatively, the use of LRA monotherapy or combinational therapy may only deplete a small fraction of latently infected T-cells that respond well and are easy to activate. Cells that have a decrease in responsiveness to LRAs might be in a state of deep latency, making them harder to reactivate. Siliciano *et al.* have demonstrated that provirus in a number of latent cells can remain silent following a round of maximal T-cell activation, but these cells ultimately responded after multiple rounds of activation [145]. These non-induced proviruses present as a caveat when studying latently infected T-cells. Non-induced proviruses will require multiple rounds of stimulation, while some may not reactivate at all. Considering these limitations to the shock and kill approach, other strategies that attempt to permanently silence the latent reservoir have developed.

5.6 Future directions

Having demonstrated the potential of the pro-apoptotic drugs IPI-443, IPI-3063, wortmannin and venetoclax combined with LRAs such as romidepsin in total CD4⁺ T-cells isolated from PLWH on ART, further studies that investigate the mechanisms of these drugs that cause synergism to deplete latently infected T-cells should be pursued. The recently described mass cytometry method, also known as cytometry time of flight (CyTOF), demonstrated an upregulation of anti-apoptotic protein Bcl-XL in cancerous B-cells [549]. This method could be adapted to detect which apoptotic proteins and/or HIV viral proteins are up- or down-regulated in HIV infected CD4⁺ T-cells from PLWH. scRNAseq is an additional method that would be utilised to investigate the mechanisms behind the synergistic combination of romidepsin and pro-apoptotic drugs that led to a decrease in HIV DNA (section 3.4.5). This method could allow for the characterisation of HIV viral proteins and host apoptotic proteins at the transcriptional level. Moreover, CyTOF and scRNAseq could be used to assess which subsets of T-cells preferentially die when treated with LRA and pro-apoptotic drugs and whether these drug combinations have proficiency in clearing certain T-cell subsets better than others.

Additional studies that explore other FDA-approved pro-apoptotic compounds such as: the Idelalisib and Duvelisib alternatives to the IPI-3063 and IPI-443 PI3K inhibitors respectively; or flurbiprofen, indomethacin, doxycycline or bezafibrate that act on pro-caspase-8 in the same pathway as venetoclax, is also warranted as these compounds could be tested in clinical trials in humans as their safety profile is well understood. Studying the timing and dose of adding LRAs and pro-apoptotic compounds should also be optimised for maximal reactivation and death of latently infected T-cells from PLWH on ART. The ability of the PrimeFlow and cell death stain to detect reactivated cells undergoing apoptosis simultaneously in primary CD4⁺ T-cells must be explored to determine the selective ability of pro-apoptotic drugs and LRAs to selectively clear latently infected T-cells and minimise impact on uninfected T-cells.

The first person to be cured of HIV, often referred to as the Berlin patient received two allogeneic stem-cell transplants using a donor with a mutation in the CCR5 co-receptor (CCR5 Δ 32/ Δ 32) [550]. This individual has been off ART for more than 10 years and no virus has been detected in either plasma or extensive analysis of PBMC and tissue sites. A second patient, the “London patient” has now been reported to have similar success following a stem cell transplant from a CCR5 Δ 32/ Δ 32 donor. This patient stopped ART and has also remained aviremic for more than 2 years and no intact virus identified in blood or tissue [551-553]. Despite these two success stories, allogeneic stem cell transplantation is not feasible to the millions of people living with HIV and thus studies that aim to discover alternative cure methods such as the work carried in this thesis are important and needed.

5.7 Concluding remarks

Based on these data, we propose further studies that examine the interplay between combinations of pro-apoptotic drugs and LRAs is warranted, but new assays are also required in order to observe whether the effects of the drugs are selective for infected cells and particular subsets of T-cells that harbour latent virus. Here we have shown that the PI3K inhibitors IPI-443 and IPI-3063 as well as the Bcl-2 inhibitor venetoclax can synergistically combine with romidepsin to increase HIV RNA transcription and decrease HIV DNA in CD4⁺ T-cells isolated from PLWH on ART. Moreover, we report for the first time that the pro-apoptotic drugs alone can also reactivate and decrease the latent HIV reservoir. Further

studies that explore the synergistic effects of pro-apoptotic drugs and LRAs is warranted so that clinical trials in humans with these combinations can be performed safely. The results presented here will be a starting point in the “shock and kill” approach that successfully combines the “shock” and the “kill” components. This may lead to the eradication of the latent HIV reservoir and remission from daily ART to ultimately achieve a functional cure to this disease.

Chapter 6

Bibliography

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Bibliography

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Chapter 7

Appendix

Chapter 7

Appendix

7.1 Sequence of DuoAdvance

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atgtactacaaacttagtagt

Red- E2 Crimson (E2 CRM)

Green- Green Fluorescent Protein (GFP)

Yellow- EF1 α promoter sequence

Getting the “Kill” into “Shock and Kill”: Strategies to Eliminate Latent HIV

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Despite the success of antiretroviral therapy (ART), there is currently no HIV cure and treatment is life long. HIV persists during ART due to long-lived and proliferating latently infected CD4⁺ T cells. One strategy to eliminate latency is to activate virus production using latency reversing agents (LRAs) with the goal of triggering cell death through virus-induced cytolysis or immune-mediated clearance. However, multiple studies have demonstrated that activation of viral transcription alone is insufficient to induce cell death and some LRAs may counteract cell death by promoting cell survival. Here, we review new approaches to induce death of latently infected cells through apoptosis and inhibition of pathways critical for cell survival, which are often hijacked by HIV proteins. Given advances in the commercial development of compounds that induce apoptosis in cancer chemotherapy, these agents could move rapidly into clinical trials, either alone or in combination with LRAs, to eliminate latent HIV infection.

Introduction

Antiretroviral therapy (ART) effectively suppresses HIV replication, but treatment is life long and there is no HIV cure (Chun et al., 1997a, 1999; Finzi et al., 1997; Rathbun et al., 2006). The major obstacle that impedes HIV eradication in ART-treated individuals is the establishment of long-lived and proliferating latently infected cells (Finzi et al., 1999; Pierson et al., 2000; Siliciano et al., 2003). HIV latency is defined as the persistence of integrated HIV DNA (Koup et al., 1994; Pedersen et al., 1989) that is transcriptionally silent but replication competent (Chun et al., 1995, 1997b). Because there is little or no expression of viral proteins, latently infected cells escape immune recognition and clearance. Latently infected cells may also have a potential survival advantage, but this mechanism remains unknown.

CD4⁺ T cells are the main target of HIV infection and are thought to be the predominant cell type harboring latent virus. Macrophages are also permissive to HIV infection *in vitro* and infected macrophages are clearly detected in HIV-infected individuals, but whether HIV-infected macrophages persist on ART *in vivo* remains controversial (Clayton et al., 2017). Additionally, other cell types have been shown to harbor latent provirus, although their contribution to the long-term persistence and viral rebound off ART remains less well understood. These include naive CD4⁺ T cells (Wightman et al., 2010), $\gamma\delta$ T cells (Soriano-Sarabia et al., 2015), and hematopoietic progenitor cells (Carter et al., 2010).

Within these infected cells, multiple mechanisms maintain HIV latency (recently reviewed in Mbonye and Karn, 2017). These include the site of proviral DNA integration, low levels of cellular transcription factors in a resting cell that are needed for virus production, epigenetic modifications such as histone acetylation/methylation and chromatin remodeling, impaired RNA splicing and nuclear export, and impaired translation (reviewed in Ruelas and Greene, 2013). One strategy to eliminate latently infected

cells is to activate virus transcription, protein expression, and virion production using latency reversing agents (LRAs), potentially triggering cytolysis or immune-mediated clearance. This approach is termed “shock and kill” and is performed in individuals on ART to prevent new rounds of infection (Figure 1).

To date, clinical trials with LRAs have demonstrated that activation of viral gene expression is possible *in vivo*, but there is limited or no clearance of the reactivated cells (Archin et al., 2012, 2017; Elliott et al., 2014, 2015; Gutiérrez et al., 2016; Rasmussen et al., 2014; Søgaard et al., 2015). To promote the elimination of reactivated cells, immune-based strategies, including antibodies, T cell vaccines, and immunotherapeutics that enhance T cell function, are being investigated to kill reactivated cells following latency reversal (reviewed elsewhere: Brockman et al., 2015; Marsden and Zack, 2015; Wykes and Lewin, 2017). Alternatively, multiple lines of evidence reveal that HIV infection can impact both pro-apoptotic and anti-apoptotic pathways, potentially favoring cell death or cell survival, respectively. Consequently, manipulation of the pro-apoptotic functions of HIV to favor apoptosis and cell death may lead to the clearance of latently infected cells after reactivation. Therefore, strategies that could potentially eliminate latently infected cells after reactivation through the induction of apoptosis are reviewed here.

Latency Reversing Agents

Various LRAs can increase viral gene expression from latency *in vitro*, including histone deacetylase inhibitors (HDACis), histone methyltransferase (HMT) inhibitors, DNA methyltransferase inhibitors, bromodomain inhibitors, and protein kinase C (PKC) agonists (reviewed in Rasmussen and Lewin, 2016), as well as PI3K/Akt pathway inhibitors and agonists for the innate immune receptors TLR7 or TLR9 (Offersen et al., 2016; Tsai et al., 2017) (Figure 2). Only a limited number of these agents have been



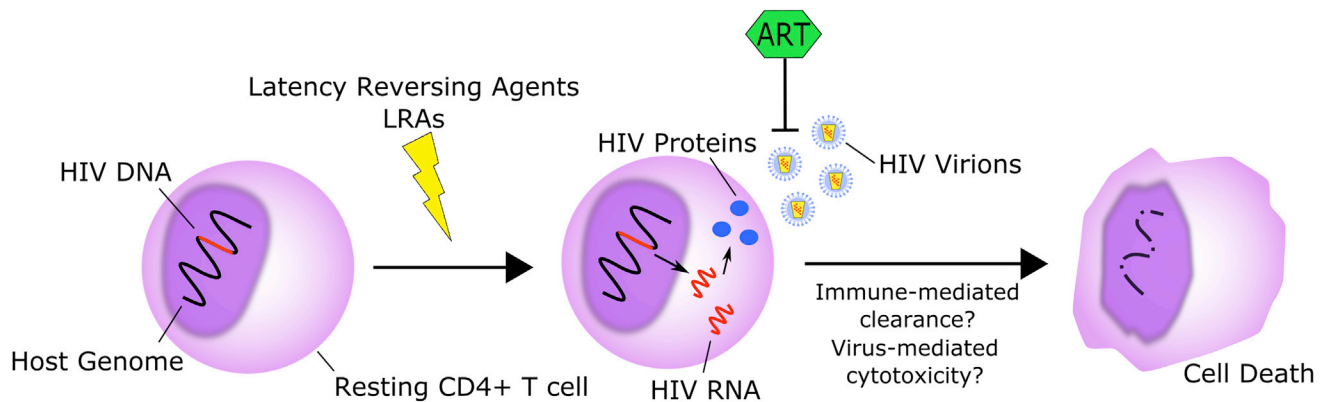


Figure 1. Shock and Kill Strategy to Eliminate HIV Latently Infected Cells

The “shock and kill” strategy uses LRAs to increase HIV transcription, protein expression, and virion production. The cell may potentially die through virus-mediated cytopathic events or immune-mediated clearance. LRAs, latency reversing agents; ART, antiretroviral therapy.

studied in clinical trials. These include the HDACis vorinostat (Archin et al., 2012, 2017; Elliott et al., 2014), panobinostat (Rasmussen et al., 2014), and romidepsin (Sogaard et al., 2015), the disulfiram drug shown to affect PI3K/Akt and more commonly used to treat alcoholism (Elliott et al., 2015; Spivak et al., 2014; Xing et al., 2011), and the TLR9 agonist MGN1703 (Vibholm et al., 2017). The PKC agonist bryostatins was also evaluated *in vivo* but had no effect on the transcription of latent HIV (Gutiérrez et al., 2016). In these clinical studies, all LRAs except bryostatins increased HIV RNA transcription when administered alone, but there was no decline in the number of infected cells (Archin et al., 2012; Elliott et al., 2014, 2015; Gutiérrez et al., 2016; Rasmussen et al., 2014; Sogaard et al., 2015). In one clinical trial, the combination of the HDACi romidepsin with a T cell vaccine led to a modest reduction in HIV DNA, although the mechanism for this reduction was not explored (Leth et al., 2016). More recently, in studies of simian immunodeficiency virus (SIV)-infected rhesus macaques on ART and HIV-infected individuals on ART, the administration of TLR7 and TLR9 agonists led to significant increases in plasma SIV and HIV RNA, respectively, consistent with latency reversal (Vibholm et al., 2017; J.B.L. Whitney et al., 2015, Conference for Retroviruses and Opportunistic Infections, Seattle, WA, conference; C.G. Whitney et al., 2016, Conference for Retroviruses and Opportunistic Infections, Boston, MA, conference).

There may be multiple reasons why HDACis alone are unable to induce clearance of infected cells. First, at least in an artificial *in vitro* model of HIV latency, where there is overexpression of the pro-survival protein Bcl-2, HDACis did not induce virus-mediated cytolysis (Shan et al., 2012). Second, the more potent HDACis have been shown to directly inhibit CD8+ T cell and natural killer (NK) cell function *in vitro* (Pace et al., 2016; Jones et al., 2014, 2016), although CD8+ T cell inhibition was not observed with clinical trials of HDACis (Elliott et al., 2014; Sogaard et al., 2015). Third, in HIV+ individuals treated with ART during chronic infection, immune escape mutations have been demonstrated in latently infected cells (Deng et al., 2015). Fourth, there is persisting immune dysfunction as demonstrated by increased expression of T cell exhaustion markers and this may impair T cell-mediated clearance of infected cells (Chew et al., 2016;

Day et al., 2006). Collectively, these data indicate that reactivation of latently infected cells alone with LRAs is unlikely to lead to elimination of the infected cells and an additional intervention for the “kill” will be needed.

Apoptosis and HIV Latency

Apoptosis is a cellular process that results in programmed cell death of unwanted or damaged cells (Bai et al., 2014). Apoptosis is tightly controlled and defective regulation of apoptosis is implicated in many human diseases such as cancer, inflammation, and autoimmune disease (Bai et al., 2014). There are two main apoptotic pathways, the intrinsic and extrinsic pathways.

The extrinsic pathway is mediated by death receptors on the surface of the cell, including members of the tumor necrosis factor (TNF) receptor family. Binding of ligands to their respective death receptors results in the recruitment and activation of members of the caspase protease family that induce apoptosis (Igney and Krammer, 2002). Well-characterized death receptors and their corresponding ligands include Fas/Fas ligand (FasL) and tumor necrosis factor receptor 1 (TNFR1)/TNF- α (Elmore, 2007). When Fas binds to Fas ligand, the receptors recruit Fas-associated protein with death domain (FADD). Dimerization of the death effector domain results in association of FADD with procaspase-8 and the formation of a death-induced signaling complex (DISC) that cleaves procaspase-8 into active caspase-8 (Kischkel et al., 1995). Active caspase-8 promotes activation of the caspase cascade, including cleavage and activation of caspase-3 leading to apoptosis (Green and Lymbi, 2015).

In contrast, the intrinsic pathway is often referred to as the mitochondrial pathway and is initiated by cellular stress, DNA damage, radiation, and other stress signals. These stress signals lead to the formation of a pore in the mitochondria membrane. This leads to permeabilization of the mitochondrial membrane and the release of cytochrome c and second mitochondrial-derived activator of caspases (Smac) and the protease Omi into the cytosol (Estornes and Bertrand, 2015). Once in the cytosol, these mediators trigger apoptosome formation and activate the caspase cascade to induce apoptosis (Green and Lymbi, 2015).

Apoptosis is regulated by members of the Bcl-2 protein family (Adams and Cory, 1998), which are summarized in Table 1.

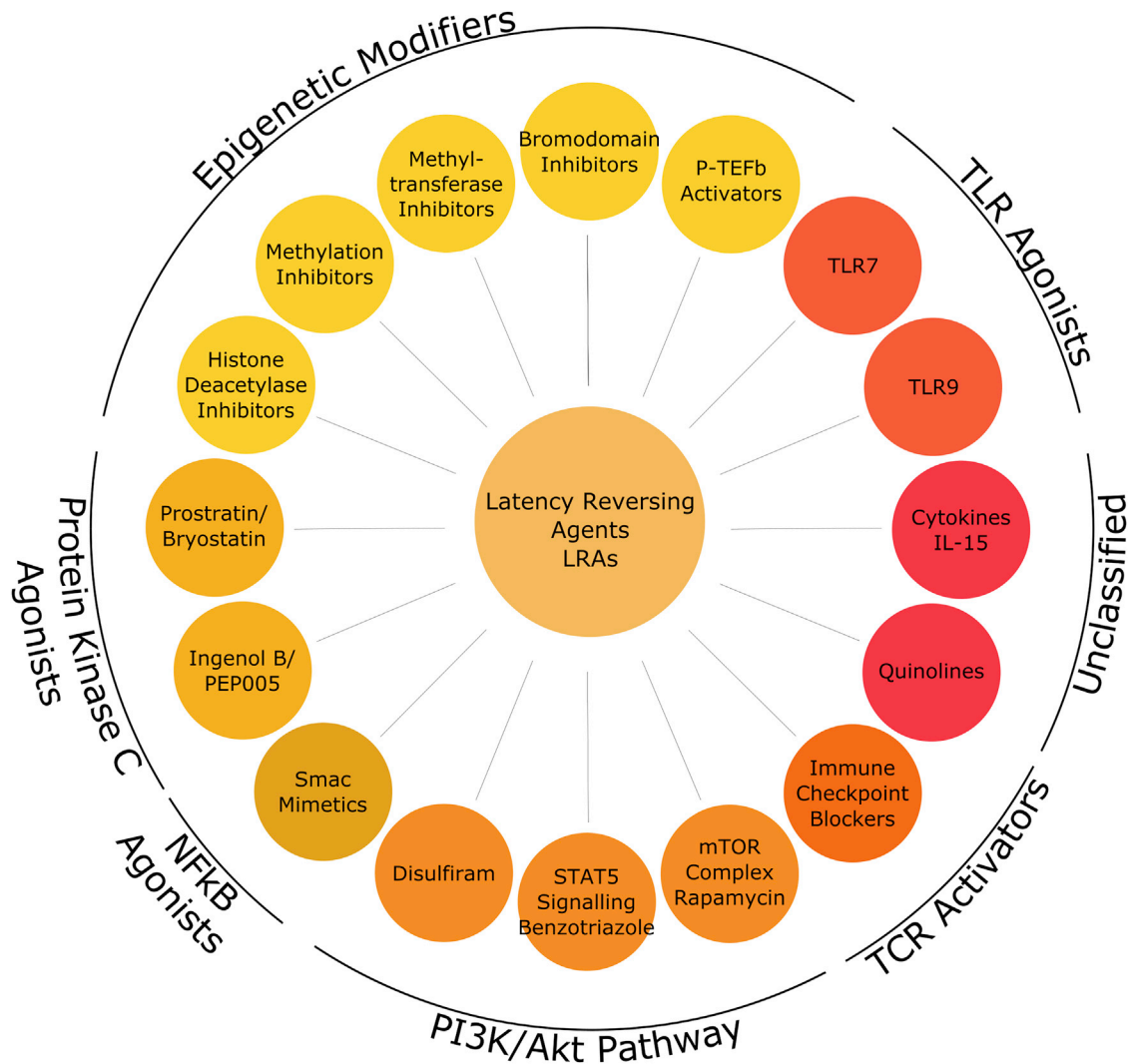


Figure 2. Classes of LRAs

LRAs can act on different pathways, resulting in an increase in HIV transcription and/or virion production. P-TEFb, positive transcription elongation factor b; TLR, Toll-like receptor; mTOR, mechanistic target of rapamycin; STAT5, signal transducer and activator of transcription 5; IL-15, interleukin-15.

Bcl-2 family members that tightly regulate apoptosis at the mitochondrial level include the pro-apoptotic molecules Bak and Bax; anti-apoptotic molecules Bcl-X_L, Bcl-2, Bcl-W, A1, and Mcl-1; and BH3-only proteins that act as sensors of apoptotic stimuli including Bad, Bid, Bim, Bik, Bmf, Hrk, Puma, and Noxa (Czabotar et al., 2014; Happon et al., 2012) (Table 1). Members of the inhibitor of apoptosis (IAP) protein family, including X-linked IAP (XIAP) and cIAP, can also regulate apoptosis by preventing caspase activation to suppress apoptosis (Czabotar et al., 2014).

Both the intrinsic and extrinsic pathways converge at the stage of caspase-8 activation. When the extrinsic apoptotic pathway is triggered, the formation of active caspase-8 can lead to apoptosis in a mitochondrial-independent pathway through the activation of the effector caspases (caspase-3 and -7) (Elmore, 2007). Additionally, active caspase-8 can cleave the BH3-only protein Bid, producing truncated Bid (tBid) (Li et al., 1998). tBid can induce mitochondrial outer membrane permeabilization, leading to the activation of mitochondrial-dependent apoptosis

(Luo et al., 1998). Therefore caspase-8 provides a link between the extrinsic “death-receptor” and intrinsic “mitochondrial” apoptotic pathways.

HIV proteins can interfere with apoptosis pathways, leading to a range of outcomes including activation, inhibition, or delay in cell death (Timilsina and Gaur, 2016) (summarized in Figure 3). During the early stages of the viral life cycle, the viral proteins Nef, Tat, and Vpr can impair apoptosis to promote cell survival, allowing for viral replication (Timilsina and Gaur, 2016). In contrast, in the late stages of the viral life cycle, the HIV envelope (Env) and Vpu proteins can promote apoptosis of infected cells by a variety of mechanisms (Timilsina and Gaur, 2016). Expression of the viral protease later in viral replication can also lead to the generation of a pro-apoptotic Casp8p41 peptide that promotes apoptosis (Cummins et al., 2016). HIV Tat, Vpr, and Nef also transition to pro-apoptotic roles later in the viral life cycle when Tat and Vpr expression increases (Timilsina and Gaur, 2016) (Figure 3). It is likely that the balance between

Table 1. Bcl-2 Family Proteins and Their Main Mechanism of Action

Pro-/Anti-apoptotic	Protein	Mechanism of Action	References
Anti-apoptotic	Bcl-2	binds to and sequesters pro-apoptotic proteins Bax, Bim, Puma, Bmf, and Bad	(Chen et al., 2005; Letai et al., 2002)
	Bcl-X _L	binds to and sequesters pro-apoptotic proteins Bax, Bak, Bim, Puma, Bmf, Bik, Hrk, and Bad	–
	Bcl-W		
	A1	binds to and sequesters pro-apoptotic proteins Bak, Bim, Puma, Noxa, and Hrk	(Dai et al., 2016)
	Mcl-1		
Pro-apoptotic	Bax/ Bak	once activated, Bax and Bak undergo conformational changes, oligomerize, and permeabilize the mitochondrial outer membrane, resulting in the release of cytochrome c and Smac/DIABLO	(Annis et al., 2005; Kim et al., 2009; Kuwana et al., 2002)
	activator BH3-only proteins		
	Bid	(1) high affinity for Bax/Bak, (2) binds directly to and	(Chen et al., 2005; Kim et al., 2006; Letai et al., 2002)
	Bim	activates Bax/Bak, and (3) sequestered by all	
	Puma	anti-apoptotic proteins	
	sensitizer BH3-only proteins		
	Bik	(1) binds to anti-apoptotic molecules, preventing these	(Certo et al., 2006; Kuwana et al., 2005; Letai et al., 2002)
	Bmf	molecules from sequestering Bax/Bak, and (2) displaces	
	Bad	activator BH3-only proteins from anti-apoptotic proteins,	
	Hrk	allowing the activator proteins to bind and directly	
	Noxa	activate Bax/Bak	

Bcl-2, B cell lymphoma-2; Bcl-X_L, B cell lymphoma-extra large; Bcl-W, B cell lymphoma-W; A1, Bcl-2-related gene A1; Mcl-1, myeloid cell leukemia 1; Bax, Bcl-2-associated X protein; Bak, Bcl-2 antagonist killer 1; Bid, Bcl-2 interacting domain death agonist; Bim, Bcl-2 interacting mediator of cell death; Puma, p53-upregulated modulator of apoptosis; Bik, Bcl-2 interacting killer; Bad, Bcl-2 antagonist of cell death; Bmf, Bcl-2 modifying factor; Hrk, Harakiri.

anti-apoptotic and pro-apoptotic viral and cellular proteins determines the fate of infected cells between survival and apoptosis.

While productive HIV infection typically leads to the death of infected cells (Petit et al., 2002), HIV latently infected CD4+ T cells are long lived (Finzi et al., 1999). These latently infected T cells can also proliferate (Maldarelli et al., 2014; Simonetti et al., 2016; Wagner et al., 2014), leading to clonally expanded cells that may contain identical and intact provirus (Bui et al., 2017; Simonetti et al., 2016). It is possible that latently infected cells avoid death from the virus integrating into genes like BACH2 encoding the transcriptional regulator that may promote cell survival (Maldarelli et al., 2014), or because there is no expression of pro-apoptotic viral proteins like HIV Env, protease, and Vpr (Abbas and Herbein, 2012), or there is an absence of viral RNA detection through the RIG-I pattern recognition receptor (PRR) (Li et al., 2016). Additionally, increases in the anti-apoptotic protein Bcl-2 result in elevated levels of antioxidant molecules like glutathione and thioredoxin, thereby protecting latently infected cells from oxidative stress-induced apoptosis (Aillet et al., 1998). The XIAP protein, which inhibits the protease activity of caspase-3, -7, and -9 (Berro et al., 2007; Neumann et al., 2015), is also increased in latently infected cells compared to uninfected cells (Berro et al., 2007) to favor cell survival. Finally, inhibition of other pro-apoptotic cellular proteins in latently infected cells may also be involved. For example, knock-down of pro-apoptotic proteins such as Bax and FADD has been shown to prolong cell survival in Jurkat T cells infected with HIV (Wang et al., 2011).

These data collectively suggest that in latently infected cells, the absence of pro-apoptotic viral proteins, together with elevated anti-apoptotic and decreased pro-apoptotic cellular proteins, promotes cell survival.

Non-apoptotic Cell Death Mediated by HIV

HIV infection of CD4+ T cells can lead to cell death through numerous mechanisms. The main proposed form of programmed cell death is apoptosis. However, other forms of programmed cell death may contribute. Pyroptosis is a highly inflammatory form of cell death that features cellular swelling, rupture of the plasma membrane, and the release of the cell's cytoplasmic contents and pro-inflammatory cytokines into the extracellular milieu (Doitsh et al., 2014). During active HIV infection, abortive reverse transcription of the viral RNA genome to DNA can result in incomplete viral DNA reverse transcripts that are sensed by protein recognition receptors such as interferon-gamma-inducible protein 16 (IFI16). This results in assembly of the inflammasome by caspase-1 activation and ultimately leads to pyroptosis (Monroe et al., 2014). Pyroptosis is mediated by caspase-1 and works independently of the other pro-apoptotic caspases. It is unclear if pyroptosis plays any role in a latently infected cell.

Another mechanism of cell death is necrosis, which is a non-apoptotic, passive, accidental, and unregulated form of cell death. Necrosis does not require activation of specific pathways and can occur as a consequence of extensive cell damage. However, necrosis is not always passive and can result from a signal cascade in a regulated manner, much like apoptosis, aptly

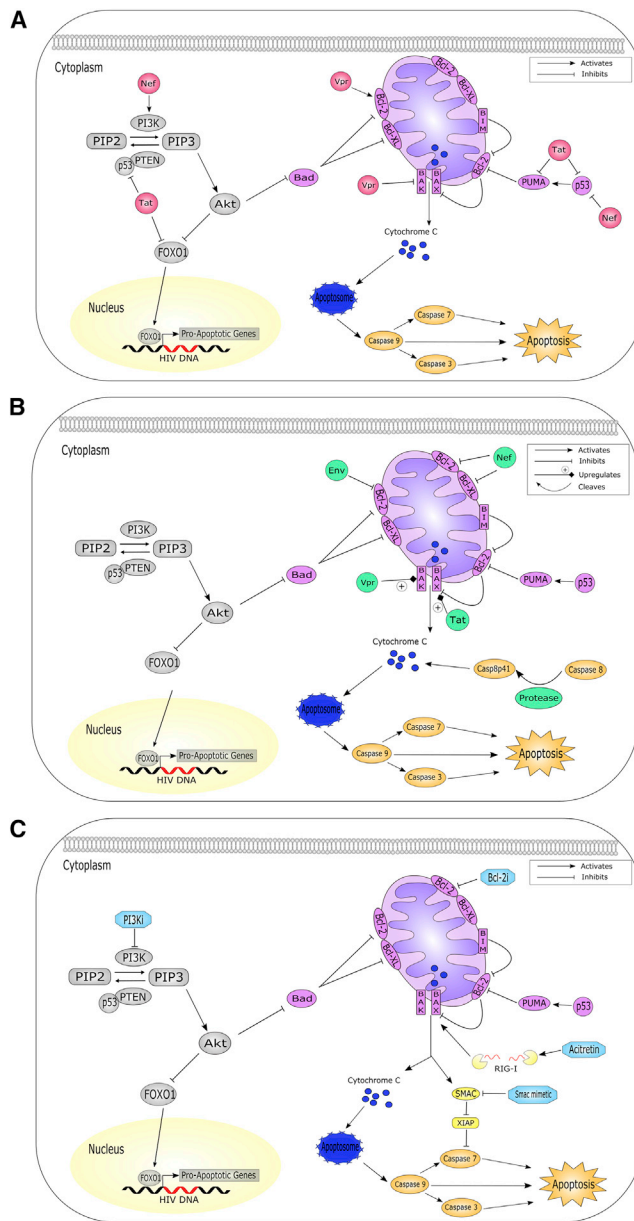


Figure 3. Effects of HIV Proteins on Apoptosis and Compounds to Foster Apoptosis of Cells

When stress stimuli induce the intrinsic mitochondrial pathway, the Bak or Bax members of the B cell lymphoma 2 (Bcl-2) family are activated to promote apoptosis. However, activation of the PI3K pathway promotes cell survival by preventing steps in the apoptosis pathway. PI3K activation leads to conversion of phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). This step is also negatively regulated by phosphatase and tensin homolog (PTEN). The conversion of PIP₂ to PIP₃ leads to the activation of Akt protein. Akt can inhibit the forkhead box protein O1 (FOXO1) transcription factor from translocating into the nucleus to induce pro-apoptotic target genes, preventing apoptosis. Additionally, Akt inhibits the pro-apoptotic protein Bad from inhibiting anti-apoptotic Bcl-2 members like Bcl-2 and Bcl-X_L, which in turn is inhibited from activating Bak or Bax, thereby also preventing apoptosis.

(A) Pathways leading to cell survival. Anti-apoptotic HIV proteins (shown in red) expressed early in the virus life cycle that can promote cell survival include Nef, Tat, and Vpr. Nef can bind PI3K proteins to increase the conversion of PIP₂ to PIP₃. This leads to an increase in Akt protein that subsequently leads to inhibition of pro-apoptotic molecules such as Bad from activating apoptosis, thereby promoting cell survival. Additionally, Nef can prevent p53 from activating the pro-apoptotic molecule PUMA, which directly inhibits Bcl-2 to prevent apoptosis. HIV Tat protein can inhibit PTEN, thereby promoting the PI3K/Akt pathway to promote cell survival. Tat can also directly inhibit FOXO1 transcription factors to prevent the transcription of pro-apoptotic genes, preventing apoptosis. Tat also prevents the pro-apoptotic molecule PUMA from inhibiting Bcl-2. HIV Vpr can lead to the increase of anti-apoptotic molecule Bcl-2 in addition to inhibiting the pro-apoptotic Bak molecule.

termed necroptosis (Degterev et al., 2005). Necroptosis can be triggered by death receptors tumor necrosis factor (TNF) and TNFR1, which requires the activity of receptor interacting protein kinase-1 (RIPK1) and -3 (RIPK3) (Holler et al., 2000). When TNF- α binds to TNFR1, TNF receptor-associated death domain (TRADD) recruits RIPK1. In the absence of caspase-8, RIPK1 assembles with RIPK3 to form a complex termed the necrosome, acting as the signal transducer for necroptosis (Vanden Berghe et al., 2014). Mixed lineage kinase domain-like (MLKL) protein is then activated through phosphorylation by the necrosome, resulting in the insertion of this protein into the plasma membrane and subsequent expulsion of intracellular contents (Wang et al., 2014). Alternatively, other death receptors such as the Toll-like receptors (TLRs) and the cytosolic DNA sensor DNA-dependent activator of IFN regulator genes (DAI) have also been shown to induce necroptosis (Kim et al., 2001; Upton et al., 2012). There is accumulating evidence that pyroptosis and necroptosis play a role in untreated, active HIV infection, leading to the loss of CD4⁺ T cells (Doitsh et al., 2014; Pan et al., 2014). However, there is little evidence that pyroptosis or necroptosis is activated following latency reversal. However, further work is needed to determine whether these pathways could be exploited to preferentially eliminate latently infected cells.

Pro-apoptotic Compounds to Clear HIV Latently Infected T Cells

Multiple pro-apoptotic compounds have been developed, typically for the cancer field (reviewed in Hassan et al., 2014; Kalimuthu and Se-Kwon, 2013). Compounds that may be useful in clearing HIV latently infected cells include Bcl-2 antagonists,

activating the pro-apoptotic molecule PUMA, which directly inhibits Bcl-2 to prevent apoptosis. HIV Tat protein can inhibit PTEN, thereby promoting the PI3K/Akt pathway to promote cell survival. Tat can also directly inhibit FOXO1 transcription factors to prevent the transcription of pro-apoptotic genes, preventing apoptosis. Tat also prevents the pro-apoptotic molecule PUMA from inhibiting Bcl-2. HIV Vpr can lead to the increase of anti-apoptotic molecule Bcl-2 in addition to inhibiting the pro-apoptotic Bak molecule.

(B) Pathways leading to cell death. When activated, Bax proteins form pores in the mitochondrial membrane, leading to the release of cytochrome c and Smac into the cytosol. Cytosolic cytochrome c leads to the formation of the apoptosome that activates caspase-9. Caspase-9 then cleaves pro-caspase-3 and -7 into their active caspase-3 and caspase-7 forms, which lead to apoptosis. Multiple HIV proteins (shown in green) can interact with the members of the apoptosis pathway later in the viral life cycle, leading to increased apoptosis. HIV Env protein can inhibit the anti-apoptotic protein Bcl-2 to favor apoptosis. The HIV protease protein also cleaves caspase-8 to the pro-apoptotic Casp8p41 peptide to promote apoptosis. It is likely that the balance of pro-apoptotic versus anti-apoptotic cellular and viral proteins decides the fate of infected cells.

(C) Modulation of apoptosis pathways using compounds. Compounds (in light blue) that act on different cellular proteins in these pathways could potentially be used to tip the balance toward apoptosis of HIV-infected cells. PI3K inhibitors block the conversion of PIP₂ to PIP₃, decreasing active Akt within a cell to stop Akt from inhibiting apoptosis. Akt inhibitors directly decrease active Akt to prevent Akt from inhibiting apoptosis to ultimately induce apoptosis. Bcl-2 inhibitors such as Venetoclax inhibit anti-apoptotic Bcl-2 to sensitize the cells toward apoptosis. Smac mimetics competitively bind inhibitors of apoptosis proteins like X-linked inhibitor of apoptosis (XIAP) to promote apoptosis. RIG-I detects viral RNA and activates pro-apoptotic Bak/Bax proteins, leading to apoptosis. In addition to viral RNA, RIG-I inducer compounds like the RA derivative acitretin can also trigger apoptosis.

PI3K/Akt inhibitors, Smac mimetics/XIAP inhibitors, and RIG-I inducers (summarized in [Figure 3C](#) and [Table 2](#)). The potential activity of these compounds in eliminating HIV latently infected cells is discussed below.

Bcl-2 Antagonists

The overexpression of Bcl-2 is common in many cancers and thus is a target of cancer therapy ([Reed and Pellecchia, 2005](#)). Many of the Bcl-2 antagonists currently being investigated are BH3 mimetics ([Lessene et al., 2008](#)). Compounds such as Venetoclax and Navitoclax mimic the BH3-binding domain present in pro-apoptotic molecules and bind with higher affinity to anti-apoptotic Bcl-2 family proteins ([Balakrishnan and Gandhi, 2013](#); [Cummins et al., 2016](#)), blocking their anti-apoptotic function and thereby favoring apoptosis. Venetoclax has recently been licensed for the treatment of chronic lymphocytic leukemia (CLL) and is in advanced clinical development for non-Hodgkin's lymphoma (NHL) ([Souers et al., 2013](#)).

In vitro, Bcl-2 antagonists have been shown to deplete latently infected T cells following T cell receptor activation with antibodies binding to CD3, a co-receptor for the T cell receptor (TCR), and CD28, a co-stimulation signal ([Cummins et al., 2016](#)). During active HIV replication, the HIV protease results in cleavage of pro-caspase-8 to generate the Casp8p41 fragment containing a BH3-like domain that subsequently binds to and activates Bak, triggering apoptosis ([Sainski et al., 2014](#)). However, during reactivation of HIV latency in resting CD4⁺ T cells, HIV protease-dependent apoptosis is prevented as the cells contain high levels of anti-apoptotic Bcl-2, which can sequester the pro-apoptotic Casp8p41 to prevent apoptosis ([Cummins et al., 2016](#)). Therefore, inhibiting Bcl-2 may prevent the Bcl-2-mediated sequestration of pro-apoptotic Casp8p41, liberating Casp8p41 generated by HIV protease to induce apoptosis of these activated latently infected cells.

Encouragingly, pre-treatment of latently infected cells from individuals on ART with the Bcl-2 antagonist Venetoclax and subsequent reactivation with anti-CD3 plus anti-CD28 T cell stimulation reduced the frequency of latently infected T cells in cultures from 8 of 11 individuals ([Cummins et al., 2016](#)). Whether combinations of Bcl-2 inhibitors like Venetoclax with LRAs that do not induce maximal T cell stimulation also lead to the death of latently infected cells remains unclear. Interestingly, Venetoclax also leads to the selective killing of HIV-infected primary T cells during productive infection *in vitro* ([Cummins et al., 2017](#)) and therefore Venetoclax could also potentially be used to reduce the establishment of latency.

Similar effects on latently infected cells were observed with the Bcl-2 inhibitor Navitoclax, but there was increased toxicity in uninfected cells. Dose-limiting thrombocytopenia from Navitoclax also makes this a less attractive drug for future clinical trials ([N. Cummins et al., 2016](#), International AIDS Conference, Durban, South Africa).

PI3K/Akt Inhibitors

PI3Ks are lipid kinases that produce secondary messengers that control a wide range of intracellular signaling pathways in leukocytes. Activation of the PI3K pathway has the cumulative effect of promoting cell survival ([Vanhaesebroeck et al., 2010](#)). PI3Ks also have roles in apoptosis and the survival of virus-infected cells ([Cooray, 2004](#)). Therefore, PI3K inhibitors could enhance the apoptosis of HIV-infected cells.

The major effector of the PI3K pathway is the serine/threonine kinase Akt (also referred to as protein kinase B or PKB) ([Chandrapaty et al., 2011](#)). Binding of the Pleckstrin homology (PH) domain of Akt to the phosphoinositide products of PI3K (PIP₃) results in the recruitment of Akt to the plasma membrane and Akt activation ([She et al., 2010](#)). Activated Akt then interferes with pro-apoptotic molecules either through direct inhibitory phosphorylation of the Bcl-2 family member Bad, or indirectly through the phosphorylation of transcription factors such as FOXO1 that subsequently translocate out of the nucleus, thereby preventing transcription of pro-apoptotic genes ([Rodrik-Outmezguine et al., 2011](#)). This prevents apoptosis and fosters cell survival.

Two HIV proteins expressed early in the virus life cycle interact with the PI3K pathway ([Figure 3](#)). The HIV protein Nef activates the PI3K signaling pathway through binding the p85 unit of PI3K ([Wolf et al., 2001](#)), which results in inhibitory phosphorylation of the pro-apoptotic factor Bad, blocking premature apoptosis in T cells ([Chugh et al., 2008](#)). HIV-1 Tat protein also interferes with cellular PTEN, a negative regulator of Akt ([Chugh et al., 2008](#); [Kim et al., 2010](#)). PTEN converts PIP₃ back to PIP₂, thereby inhibiting activation of Akt. PTEN is regulated by binding to host cell p53. p53 is a pro-apoptotic molecule that enhances PTEN expression, leading to the repression of the PI3K/Akt signaling pathway. However, HIV-1 Tat also binds p53, preventing p53 binding to PTEN and causing downregulation of PTEN. This leads to activation of PI3K/Akt signaling and impaired apoptosis ([Chugh et al., 2008](#); [Wolf et al., 2001](#)). Therefore, both HIV Nef and Tat expressed early during the virus life cycle can promote the pro-survival PI3K/Akt signaling pathway to prevent apoptosis. Given the role of PI3K/Akt signaling in preventing apoptosis and promoting survival, inhibition of key factors in the PI3K/Akt pathway may redirect the balance toward apoptosis and death of infected cells ([Lucas et al., 2010](#)).

Effects of PI3K Inhibition in HIV-Infected Macrophages. During HIV infection of macrophages, the HIV Tat protein promotes the stress-induced activation of the PI3K/Akt survival pathway ([Chugh et al., 2008](#)). Activation of this pathway limits cytotoxicity and may be important for long-term survival of infected macrophages. Inhibition of the downstream effector of the PI3K signaling pathway, Akt, prevented activation of the PI3K/Akt signaling pathway in infected macrophages ([Chugh et al., 2008](#)). Three Akt inhibitor compounds, Edelfosine, Perifosine, and Miltefosine, were able to inhibit Akt activation induced by HIV, ultimately leading to the death of macrophages upon cell stress ([Lucas et al., 2010](#)). Perifosine also antagonized the cytoprotective phenotype of HIV-infected macrophages and reduced HIV production. In a microglial cell line, three compounds, Lancemaside A, Compound K, and Arctigenin, induced cell death following cellular stress ([Kim et al., 2011](#)). All three compounds inhibit different steps in the PI3K/Akt pathway. Arctigenin binds to the PI3K enzyme, preventing conversion of PIP₂ to PIP₃, thus decreasing levels of Akt protein. Lancemaside A blocks the localization of Akt to the plasma membrane, thereby inhibiting Akt activation. Lastly, Compound K is thought to directly bind and inhibit Akt ([Kim et al., 2011](#)).

These studies show the potential of PI3K/Akt inhibitors to eliminate HIV-infected macrophages by sensitizing the cells to apoptosis. Whether these compounds can also sensitize latently infected T cells to apoptosis warrants further investigation.

Table 2. Summary of Compounds that Induce Apoptosis of HIV Latently Infected Cells

Inducers of Apoptosis	Pathway Description	Compounds to Induce Apoptosis	Effects on HIV Latency <i>In Vitro</i>	Clinical Development for Non-HIV Indications	References
Bcl-2 antagonists	Anti-apoptotic cellular Bcl-2 sequesters the pro-apoptotic Casp8p41 fragment generated by HIV protease. Inhibiting Bcl-2 liberates pro-apoptotic Casp8p41 generated by HIV protease to promote apoptosis.	Venetoclax	depletion of latently infected cells using CD4+ T cells from HIV-infected individuals on ART <i>ex vivo</i>	licensed—chronic lymphocytic leukemia	(N. Cummins et al., 2016, IAS, conference)
		Navitoclax	similar results to Venetoclax, but resulted in increased non-specific toxicity	phase II—chronic lymphocytic leukemia	
PI3K/Akt inhibitors	PI3K leads to activated Akt, which inhibits pro-apoptotic Bad and the transcription of pro-apoptotic genes. Inhibiting PI3K/Akt blocks this process, allowing pro-apoptotic Bad to function and transcription of pro-apoptotic genes, favoring apoptosis.	Edelfosine	inhibited Akt activation induced by HIV infection in macrophages, leading to death upon cellular stress	phase II—non-small-cell lung cancer	(Lucas et al., 2010)
		Perifosine		phase II—non-small-cell lung cancer	
		Miltefosine	eliminated cytoprotective phenotype of microglial cell lines infected with HIV upon cellular stress	licensed—visceral leishmaniasis	(Kim et al., 2011)
		Lancemaside A		N/A	
		Compound K		N/A	
Smac mimetics	Smac mimetics bind and block anti-apoptotic IAPs to promote apoptosis	Arctigenin	dose-dependent increase in death of HIV latently infected CD4+ T cells	phase I/II—hepatitis B	(G.R. Campbell et al., 2015, CROI, conference)
		Birinapant		phase I—locally advanced or metastatic malignancies	
		GDC-0152		phase IV—psoriasis	
RIG-I inducers	Induce RIG-I, which detects viral RNA and promotes apoptosis. Retinoic acid (RA) also activates p300 acetyltransferase cellular transcription factor, leading to HIV transcription.	Embelin	induced HIV expression, reduced HIV DNA, and induced preferential death in latently infected CD4+ T cells from HIV-infected individuals on suppressive ART	licensed—psoriasis	(Li et al., 2016)
		Acitretin			
			no induction in HIV expression or death in latently infected cells		(Garcia-Vidal et al., 2017)

Bcl2, B cell lymphoma 2; PI3K, phosphoinositide-3-kinase; Smac, second mitochondrial-derived activator of caspase; RIG-I, retinoic acid-inducible gene I.

Potentially priming and pre-sensitizing cells with PI3K/Akt inhibitors to decrease levels of phosphorylated Akt followed by LRA-induced expression of viral proteins from latent HIV may tip the balance of reactivated cells toward apoptosis, thus eliminating these cells.

Combining PI3K Inhibitors with LRAs. CUDC-907 is a small-molecule inhibitor currently in clinical trials for lymphoma or multiple myeloma (Ma et al., 2014). Interestingly, CUDC-907 has dual activities of potentially inhibiting class I PI3K (α , β , and δ) isoforms as well as inhibiting HDAC class I and II enzymes (A. Younes et al., 2013, ASH, conference). As HIV latency is regulated by HDACs (Sheridan et al., 1997; Verdin et al., 1993) and reactivated by HDACis (reviewed in Margolis, 2011; Mbonye and Karn, 2014), CUDC-907 could potentially both reactivate latent HIV via HDAC inhibition and also trigger death of reactivated cells through blocking the PI3K/Akt pathway.

Of note, some LRAs can have pro-survival effects on the PI3K/Akt pathway and thus act in a counterproductive manner to the PI3K inhibitors. For instance, the anti-alcohol drug disulfiram acts as an LRA via depletion of the PTEN regulator of the PI3K/Akt pathway, thus promoting pro-survival Akt activation and NF- κ B-driven HIV transcription (de Almagro and Vucic, 2012; Xing et al., 2011). Additionally, the HDACi vorinostat and the PI3K α isoform agonist 55704 also promote pro-survival PI3K/Akt signaling (Chen and Huerta, 2009). The activation of the PI3K/Akt pathway by these LRAs may enhance cell survival and might explain why clinical trials with vorinostat (Archin et al., 2012) and disulfiram (Elliott et al., 2015; Fulda, 2015) both failed to reduce the levels of HIV DNA in HIV-infected individuals on ART. Given that some LRAs potentially enhance survival of infected cells, this is a critical consideration when devising shock and kill strategies that include both PI3Ki and LRAs. LRAs with pro-survival effects on the PI3K-Akt pathway are expected to compete with the pro-apoptotic effects of PI3K/Akt inhibitors and may prevent apoptosis. Additionally, combining PI3K/Akt inhibitors with pro-survival LRAs like disulfiram, vorinostat, or 55704 may also reduce the potency of latency reversal. This is because HIV transcription depends on an increase in transcription factors like NF κ B that are induced by LRAs that activate the PI3K/Akt pathway, but will be impaired by PI3K/Akt inhibitors. In summary, combining PI3K inhibitors and LRAs is a potential strategy to clear the latent HIV reservoir. However, the choice of LRAs requires careful selection to avoid opposing effects of the LRAs on the PI3K/Akt pathway. Further pre-clinical evaluation is required to determine combinations of various PI3K/Akt inhibitors and LRAs that might effectively deplete latently infected cells.

Smac Mimetics and XIAP Inhibitors

IAPs are defined by the presence of one to three baculoviral IAP repeat (BIR) domains (Bai et al., 2014), and eight human IAPs have been identified: NAIP (BIR1), cellular-IAP1 (BIR2), cIAP2 (BIR3), XIAP (BIR4), survivin (BIR5), BRUCE/Apollon (BIR6), livin (ML-IAP1/BIR7), and ILP2 (BIR8) (de Almagro and Vucic, 2012). XIAP, cIAP1, and cIAP2 have major roles in both the intrinsic and extrinsic apoptosis pathways while the other IAPs regulate cell survival through cell-cycle control or inflammation (de Almagro and Vucic, 2012). IAPs regulate apoptosis either by direct or indirect inhibition. XIAPs directly bind and inhibit caspase-3, -7, and -9 activity, thus preventing the caspase cascade

and apoptosis. In contrast, cIAPs do not directly bind caspases but instead regulate caspase activation through their E3 ligase activity, modulating TNF-mediated apoptosis and NF- κ B pathways (Bai et al., 2014; Chen and Huerta, 2009; de Almagro and Vucic, 2012).

Upon activation of the intrinsic apoptosis pathway, mitochondria release both cytochrome c and Smac, also known as DIABLO (direct IAP-binding protein with low pI) (Fulda, 2015). Smac is a pro-apoptotic protein that binds to IAPs to inhibit their function. Most Smac proteins directly compete with caspases for XIAP binding, liberating caspases for apoptosis. Smac proteins can also activate the ubiquitin activity of cIAP1/2, leading to the degradation of these IAPs (Pache et al., 2015). Through their ability to bind and inhibit IAP activity, Smac mimetics can induce pro-apoptotic mechanisms and therefore are attractive compounds to induce the apoptosis of reactivated, latently infected cells.

Targeting XIAP may potentially selectively kill latently infected cells. The flavopiridol cyclin-dependent kinase 9 (CDK-9) inhibitor that also downregulates XIAP expression (Rosato et al., 2007) increased apoptosis of the latently infected ACH2 cell line compared to untreated cells (Berro et al., 2007). The XIAP antagonists GDC-0152 and embelin and the Smac mimetic Birinapant also resulted in a dose-dependent increase in apoptosis of latently infected primary memory CD4⁺ T cells. This was achieved without the addition of LRAs, providing intriguing evidence that HIV latently infected cells may be selectively killed without reactivation (G.R. Campbell et al., 2015, CROI, conference). Interestingly, Birinapant has been shown to clear hepatitis B virus-infected cells *in vitro* and in a mouse model, through a similar mechanism (Ebert et al., 2015a, 2015b). Finally, pretreatment of monocyte-derived macrophages (MDMs) with the AEG40730 Smac mimetic that depletes cIAP1, cIAP2, and XIAP also sensitizes MDMs to HIV Vpr-induced apoptosis (Busca et al., 2012). Whether this pathway is similar in latently infected T cells treated with an LRA warrants further examination.

Further evaluation of these compounds, especially those in clinical development, should be considered. The Smac mimetic Birinapant is undergoing clinical evaluation to treat refractory hematological and solid tumors, including lymphoma (Amara-vadi et al., 2015) and epithelial ovarian cancer (Noonan et al., 2016). Flavopiridol decreases XIAP and is under investigation for the treatment of chronic lymphocytic leukemia (Awan et al., 2016; Blachly et al., 2016). The impact of these inhibitors on uninfected cells also requires careful investigation to minimize undesirable toxicities on uninfected cells.

Aside from apoptosis, Smac mimetics can also overcome blocks in the death receptor pathway of necroptosis. In FADD- or caspase-8-deficient leukemia cells, the Smac mimetic BV6 primed the cells for necroptosis (Laukens et al., 2011) in a RIPK1-dependent and caspase-independent manner. In apoptotic-proficient cells, the Smac mimetic primed cells for TNF- α - and caspase-dependent death (Laukens et al., 2011). HIV-infected cells share similarities with leukemia cells such as prolonged survival and defective cell death pathways. This finding that Smac mimetics can prime cells for necroptosis proposes an alternative cell death mechanism that could be exploited to eliminate HIV latency. However, necroptosis also results in the release of intracellular contents and

damage-associated molecular patterns (DAMPs) into the extracellular matrix, which promotes inflammation (Pasparakis and Vandenabeele, 2015). The role of necroptosis induced by Smac mimetics in the context of HIV latency reversal is unclear but warrants further investigation.

RIG-I Inducers

The induction of the innate immune response plays a critical role in the host cell defense against invading viruses. Recognition of viral pathogens through the innate immune response PRRs, which include TLRs or retinoic acid-inducible gene I (RIG-I), can also drive the apoptosis of virus-infected cells (Mogensen, 2009). RIG-I is a DExD/H box RNA helicase that senses viral RNA within the cytoplasm of infected cells (Kato et al., 2005; Wang et al., 2008). During HIV infection, RIG-I can sense HIV RNA and induce anti-viral signaling and apoptosis (Wang et al., 2013). However, the HIV protease can also degrade or sequester RIG-I to abrogate RIG-I-mediated apoptosis (Solis et al., 2011) and circumvent this innate immune response.

Retinoic acid (RA) can induce RIG-I expression as well as activate the p300 acetyltransferase cellular transcription factor, thus leading to HIV transcription (Li et al., 2016). Therefore, treating HIV latently infected cells with drug compounds that mimic RA might similarly activate both HIV transcription and RIG-I-mediated apoptosis to both reactivate latent HIV and tip the balance toward apoptosis of these infected cells despite HIV protease expression impeding RIG-I.

Acitretin, an FDA-approved RA derivative used to treat psoriasis (Ortiz et al., 2013), was recently shown to reactivate latent HIV and induce the selective apoptosis of HIV latently infected cells *in vitro* (Li et al., 2016). In the study by Li et al., acitretin induced HIV expression in latently infected T cell lines and preferential death of HIV infected cells compared to uninfected samples. Moreover, this effect was enhanced when the LRA HDACi vorinostat was combined with acitretin. Cell death was driven by RIG-I-mediated increases in the pro-apoptotic protein Bax, which can trigger caspase-mediated apoptosis (Li et al., 2016). Unfortunately, these findings were not replicated in a subsequent study that also evaluated latently infected cells lines and patient-derived cells (Garcia-Vidal et al., 2017). Further work is needed to determine whether these agents can be used to eliminate latently infected cells.

Potential Challenges

Although many of the pro-apoptotic compounds discussed in this review are now in clinical development or licensed (Table 2), several issues require consideration when being evaluated in HIV infection. First, it is critical that there is minimal depletion of non-infected cells. This could be reduced if cell death is dependent on the expression of HIV proteins, and we believe that this will be a highly desirable strategy for drug development. To ensure only reactivated latently infected cells are eliminated, we propose first administering the “kill” compounds to sensitize latently infected cells toward apoptosis, followed by subsequent reactivation with LRAs to drive the expression of pro-apoptotic viral products and selectively clear the HIV-infected cells. Sensitizing the cell toward an apoptotic state followed by the production of pro-apoptotic HIV proteins such as protease and HIV RNA could tip the balance toward apoptosis

and specific killing of the cells that only produce these HIV proteins. This has been demonstrated with the pro-apoptotic drug Venetoclax (Cummins et al., 2016), which, when combined with LRAs, led to the selective apoptosis and clearance of HIV-infected cells. Additionally, the acitretin RIG inducer that acts as an LRA as well as RIG-I inducer to drive apoptosis also leads to selective death of HIV-infected cells (Li et al., 2016). A second issue for consideration involves examining interactions with LRAs to determine potential synergy, antagonism, or toxicity. Third, effects on non-T cell reservoirs should also be evaluated as cells such as infected macrophages may be particularly resistant to apoptosis. Fourth, the effect of these compounds on dividing and non-dividing cells should be evaluated to determine how effectively various HIV-infected cell types will be cleared. To this end, a recent study found that the Bcl-2 inhibitor Venetoclax induced apoptosis of the latently infected J-Lat10.6 T cell line model (Cummins et al., 2017), in which the J-Lat10.6 cells proliferate slowly in the absence of stimulation. While Venetoclax can induce apoptosis in both dividing and non-dividing HIV-infected T cells, it will be important to evaluate the impact of any kill compounds on diverse cell types to ascertain selective clearance of HIV-infected cells. Finally, penetration of these compounds into tissue sites such as the central nervous system and gut-associated lymphoid tissue (GALT) (Eisele and Siliciano, 2012) will be important to determine. Potency and specificity could be potentially enhanced using drug delivery systems such as nanoparticles coated with specific antibodies targeting CD4 or a latency marker, such as the recently described CD32a (Descours et al., 2017).

The timing of administration of the apoptosis-inducing compounds relative to the LRAs also requires careful examination to maximize the depletion of latently infected cells. Maximal depletion may be achieved by pre-sensitizing latently infected cells with the apoptosis-inducing compound before adding LRAs to induce pro-apoptotic viral products that drive apoptosis of HIV-infected cells. Alternatively, greater depletion of latently infected cells may be achieved by adding the apoptosis-inducing compound at the same time or potentially after LRA reactivation, in which pro-apoptotic viral products have been generated. The sequence of drug administration will require pre-clinical and *in vitro* assessment.

Ethical considerations must always be a top priority for all clinical trials but are especially challenging in this context. Individuals on ART have a near-normal life expectancy (Wandeler et al., 2016) and in early phase studies aimed at achieving HIV remission or cure, participants are unlikely to have a direct benefit from the intervention, while there are also potential risks. These issues have been reviewed elsewhere (Lo et al., 2013; Rennie et al., 2015). In contrast to interventions for malignancy, toxicity of any intervention must be low and the pre-clinical rationale for the study well justified in order to proceed to early phase clinical trials.

Conclusion and Future Perspectives

Latently infected resting CD4⁺ T cells remain a major barrier to curing HIV-infected patients on long-term ART. There is a critical need for novel compounds and therapies that not only potently reactivate latently infected cells, but also lead to the death of these reactivated cells. Identifying compounds that can be

combined to induce both reactivation and death of latently infected cells has great appeal as this strategy is not dependent on an effective immune response or understanding immune escape in latently infected cells. Additionally, this strategy is potentially scalable and cost effective to administer to individuals living in low-income countries. While the potential benefits of the administration of LRAs together with compounds that enhance HIV-induced death is yet to be investigated in HIV-infected individuals, more work is needed put the kill into “shock and kill.”

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DECLARATION OF INTERESTS

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SHORT REPORT

Limitations of dual-fluorescent HIV reporter viruses in a model of pre-activation latency

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Abstract

Introduction: HIV latency can be established *in vitro* following direct infection of a resting CD4⁺ T cell (pre-activation latency) or infection of an activated CD4⁺ T cell which then returns to a resting state (post-activation latency). We modified a previously published dual-fluorescent reporter virus seeking to track the establishment and reactivation of pre-activation latency in primary CD4⁺ T cells.

Methods: A previously published dual-fluorescent reporter virus was modified so that expression of enhanced green fluorescent protein (GFP) was under control of the elongation factor 1 alpha (EF1 α) promoter to detect latent infection, and E2 crimson (E2CRM) was under control of the *nef* promoter to detect productive infection. NL4.3 that expressed GFP in place of *nef* was used as a positive control. We infected the Jurkat T-cell line and primary CD4⁺ T cells that were either unstimulated or stimulated with either the chemokine CCL19 or phytohaemagglutinin (PHA)/IL-2 and quantified the expression of both fluorescent proteins by flow cytometry. The study was carried out over a period of two years from September 2016 to October 2018.

Results and Discussion: Expression of both fluorophores was detected following infection of the Jurkat T-cell line while only low levels of the latent reporter were observed following infection of primary CD4⁺ T cells. In unstimulated and CCL19-treated CD4⁺ T cells, expression of the GFP latent reporter, increased after further activation of the cells with PHA/phorbol 12-myristate 13-acetate (PMA).

Conclusions: Our findings demonstrate that the EF1 α promoter has poor constitutive expression in resting CD4⁺ T cells. Therefore, dual-fluorescent reporter viruses with the EF1 α promoter may underestimate the frequency of latent infection in resting CD4⁺ T cells.

Keywords: HIV; dual-fluorescent reporter; latency; pre-activation model of HIV; latency establishment; HIV cure; immunology; virology

Additional information may be found under the Supporting Information tab for this article.

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1 | INTRODUCTION

The main barrier to finding a cure for HIV infection is the persistence of latently infected cells in HIV-infected individuals on antiretroviral therapy (ART) [1–3]. *In vitro*, latent infection can be established through multiple pathways: direct infection of resting CD4⁺ T cells (pre-activation latency [4]), infection of an activated cell which survives and reverts to a resting state (post-activation latency) [5] or infection of a cell transitioning from an effector memory to central memory phenotype [6,7]. Given the low frequency of latently infected cells *in vivo* [8] insights into latency are often obtained through the use of *in vitro* models of latent infection established in primary T cells or T-cell lines (reviewed in [9]).

In recent years, dual-fluorescent reporter viruses have emerged as a common tool to study latency *in vitro* [10–12]

by including two fluorescent reporters – a latent reporter that is under the control of an HIV LTR-independent promoter that is constitutively expressed following integration and a productive reporter that is expressed following transcription from the HIV LTR. Duo-Fluo is a previously reported virus that contains mCherry under the control of the elongation factor 1 α (EF1 α) promoter as the latent reporter and enhanced green fluorescent protein (GFP) as the reporter for productive infection, both inserted into the *nef* gene [11]. One potential limitation of the Duo-Fluo virus is the possibility of recombination of the two reporters during reverse transcription due to the matching amino and carboxyl termini [13]. This limitation was recently addressed in a new virus HIV_{GKO}, whereby mCherry was replaced with Kusabira orange (mKO2) and GFP was switched to a codon-switched GFP protein (csGFP) [14].

In order to enhance infectivity in primary cells and to enhance the detection of latent virus established by direct infection (pre-activation latency), we designed a new dual-fluorescent reporter virus. We modified Duo-Fluo and restored *env* and *vpr* to wild type sequences and changed the reporter proteins to GFP (latent) and E2 crimson (productive infection), naming it DuoAdvance. Following direct infection of resting cells stimulated with CCL19 [4,15], we found low constitutive expression of GFP (the latent reporter), despite successfully establishing latent infection. We conclude that dual-fluorescent reporter viruses may underestimate the frequency of latently infected cells in resting CD4+ T cells.

2 | METHODS

2.1 | Cell line culture

The Jurkat T-cell line was cultured in RPMI-1640 (RF10) and human embryonic kidney (HEK) 293T cells and TZM-bl cells (cell lines: NIH AIDS Reference Reagent Program) were cultured in Dulbecco's modified eagle medium (DMEM10) at 37°C, 5% CO₂. All media was supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 29.2 mg/mL L-glutamine.

2.2 | Preparation of E2 crimson fluorescent protein plasmids, NL4.3-EGFP

E2 crimson fluorescent plasmid, NL4.3-EGFP and Duo-Fluo plasmids were transformed in Stbl-2 cells and plated on Luria broth (100 µg/mL ampicillin) or terrific broth (TB) (100 µg/mL kanamycin for Duo-Fluo). Single colonies were cultured for 16 hours and plasmids prepared using QIAprep Spin Miniprep Kits (QIAGEN, Hilden, Germany) as per the manufacturer's instructions.

2.3 | DuoAdvance plasmid generation

A 6668bp DNA plasmid insert containing the modified DuoAdvance sequence was synthesized by GenScript (GenScript, Piscataway, NJ). The DuoAdvance DNA plasmid insert was ligated into the Duo-Fluo plasmid using T4 DNA ligase. Plasmids were prepared using a Maxi Kit (QIAGEN, Hilden, Germany) and ligation confirmed using combined *NdeI* and *PacI* digestion for 2.5 hours at 37°C followed by 1 hour at 50°C with *BssHII*, resolved on a 1.2% gel.

2.4 | Preparation of virus stocks and determining infectivity

Viral stocks of DuoAdvance, Duo-Fluo and NL4.3-EGFP were prepared from transfection of HEK293T cells with each

respective plasmid, concentrated and used in all experiments as previously described [15].

The infectivity of viral stocks was performed using a TZM-bl assay. TZM-bl cells were plated and infected with 10-fold dilutions of viral stocks in triplicate. Negative and positive control wells using media and a reference virus with known infectivity were also included respectively. 50% tissue culture infectious dose (TCID₅₀) was measured using luciferase readout and calculated using the Reed-Muench method [16].

2.5 | Infection of Jurkat T-cell line

Jurkat T cells were infected with concentrated DuoAdvance virus, Duo-Fluo virus, NL4.3-EGFP virus at a TCID₅₀ of 0.5 in an infection volume of 100 µL. The integrase inhibitor Raltegravir (1 µmol/L) or non-nucleoside reverse transcriptase inhibitor Nevirapine (10 µmol/L) (Selleck Chemicals, Houston, TX) were added to cells 30 minutes before infection and then maintained in cultures throughout the remaining experiment. Cells were infected for 2 hours, washed, cultured for three days and then fixed in 1% formaldehyde for flow cytometry. Samples were analysed on a BD LSR Fortessa using FACSDIVA software (BD Biosciences, San Jose, CA). Data were analysed using FlowJo v10 (FlowJo LLC, Ashland, OR).

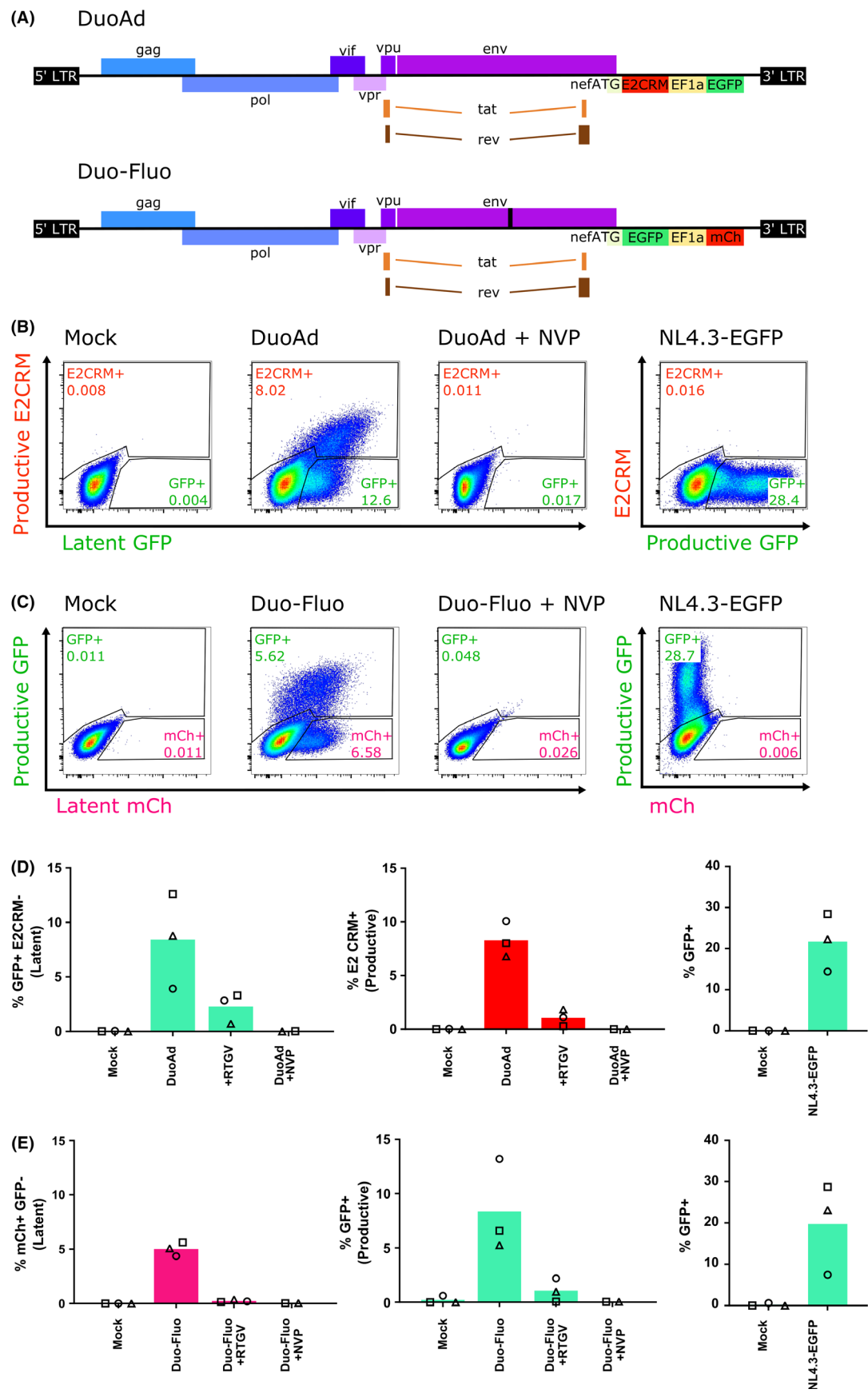
2.6 | Isolation and infection of resting CD4+ T cells

Peripheral blood mononuclear cells (PBMCs) and resting CD4+ T cells were isolated from whole blood from healthy donors as described previously [4] and cultured in three different conditions; unactivated, 100 nmol/L recombinant human CCL19/MIP-3β (In Vitro Technologies, Vic, AUS) treated and PHA/IL-2 activated. Untreated and treated primary CD4+ T cells were typically infected with 100 µL of DuoAdvance or NL4.3-EGFP (both at TCID₅₀ 0.5-1) per 1 × 10⁶ cells for 2 hours. Cells were also spinoculated (1200×g, 90 minutes, room temperature) followed by a further 30 minutes incubation. Cells were then washed and resuspended in RF10 plus 1 U/mL IL-2 and cultured for six days.

For reactivation studies, the E2CRM- fraction of cells were sorted out from CCL19-treated CD4+ T cells infected with DuoAdvance using a BD FACSAria Fusion (BD, Franklin Lakes, NJ) and then reactivated by the addition of 10 µg/mL PHA+10 nmol/L PMA, or DMSO diluent negative control, for three days in the presence of 1 µmol/L raltegravir. After reactivation, cells were fixed in 1% formaldehyde and run on a BD LSR Fortessa using bandpass filters for E2CRM (670/30 Yellow) and GFP (530/30 Blue) plus FACSDIVA software. Results were analysed using FlowJo v10.

Figure 1. DuoAdvance infection of the Jurkat T-cell line generates latent and productively infected cells similar to Duo-Fluo.

(A) Schematic of the DuoAdvance (DuoAd) dual-fluorescent reporter construct virus (top) and Duo-Fluo dual-fluorescent reporter construct (bottom). DuoAd contains an EF1α promoter driven enhanced green fluorescent protein (EGFP) marker for latent cells and an E2 Crimson (E2CRM) marker under the control of the HIV LTR that serves as the productive marker. This construct also contains wildtype HIV *env* and *vpr* sequence. Jurkat T cells were infected with either DuoAd (B, D) or Duo-Fluo (C, E) for three days in the presence or absence of nevirapine (NVP) or raltegravir (RTGV) and expression of EGFP (GFP), E2CRM or mCherry (mCh) quantified by flow cytometry. NL4.3-EGFP virus was used as a positive control for productive infection and expression of GFP (B-E). Representative flow plots and gates are shown in b and c. In D-F, each symbol represents an independent experiment and columns represent the mean.



2.7 | Data analysis

FlowJo results were graphed using GraphPad Prism (version 7, GraphPad Software, La Jolla, CA). Statistical testing was not appropriate as the small sample size ($n < 6$) precluded the use of non-parametric tests. Due to the small sample size, testing normality cannot be performed, thus parametric t-tests were also not used. Additionally, as the percentage fluorescence data points appeared skewed, this further suggests that a parametric t-test was not appropriate. Therefore, we have only reported the mean, range and fold-difference between means.

3 | RESULTS AND DISCUSSION

3.1 | DuoAdvance HIV reporter virus can establish latent and productive infection in a T-cell line

We first compared infection of the Jurkat T-cell line using DuoAdvance, the original Duo-Fluo (Figure 1A) and NL4.3-GFP as a comparison for GFP productive infection reporter expression. Cells were infected at 0.5 TCID₅₀ per cell as titration studies found comparable levels of latent infection of CCL19-treated CD4⁺ T cells using DuoAdvance at 0.5-2 TCID₅₀ after 72 hours (data not shown). Following infection of the Jurkat T-cell line with DuoAdvance, latently infected (GFP+E2CRM⁻) and productively infected cells (E2CRM⁺) were detected (Figure 1). All E2CRM⁺ cells were considered productively infected irrespective of GFP expression. The mean fluorescence intensity of GFP as a latency reporter following DuoAdvance infection (mean (range) = 8.43 (3.92 to 12.60)%) was below the intensity observed after infection with NL4.3-EGFP where GFP is a productive reporter (mean (range) = 21.70 (14.40 to 28.40)%) (Figure 1B,D). Infection with DuoAdvance and Duo-Fluo (Figure 1A) resulted in a mean frequency of 8.43% (range = 3.93 to 12.60%) and 5.02% (range = 4.36 to 5.62%) latently infected cells respectively (Figure 1B-E). The number of productively infected cells was also comparable between both viruses (Figure D,E). Nevirapine or raltegravir successfully inhibited infection of Jurkat T cells by both viruses (Figure 1B-E). As DuoAdvance and Duo-Fluo were comparable in their infection of the Jurkat T-cell line, DuoAdvance therefore showed promise as a new dual-fluorescent reporter virus.

3.2 | Minimal constitutive expression of EF1 α following infection of resting cells

We next used DuoAdvance to infect CCL19-treated and PHA/IL-2-activated CD4⁺ T cells. E2CRM was detected in

both models and this declined when nevirapine was added to the cell culture prior to, during and after infection. As expected, the frequency of E2CRM⁺ cells was higher in PHA/IL-2 activated (mean (range) = 2.55 (0.83 to 5.14)%) compared to CCL19-treated cells (mean (range) = 0.11 (0.01 to 0.28)%) (Figure 2A,C and D). Low frequencies of GFP (latent infection) were detected in both CCL19-treated (mean (range) = 0.01 (0.00 to 0.01)%) and PHA/IL-2-activated CD4⁺ T cells (mean (range) = 0.034 (0.02 to 0.05)%) (Figure 2A-B). These GFP frequencies were higher than mock infection and when infection was performed in the presence of nevirapine. Spinoculation of the DuoAdvance virus at the time of infection of primary T cells was also tested to enhance viral binding and infection [17]; however, we found little change in the number of infected cells following spinoculation (Figure 2B,C, closed symbols) or when we increased the TCID₅₀ from 0.5 to 1 (data not shown) to enhance infection.

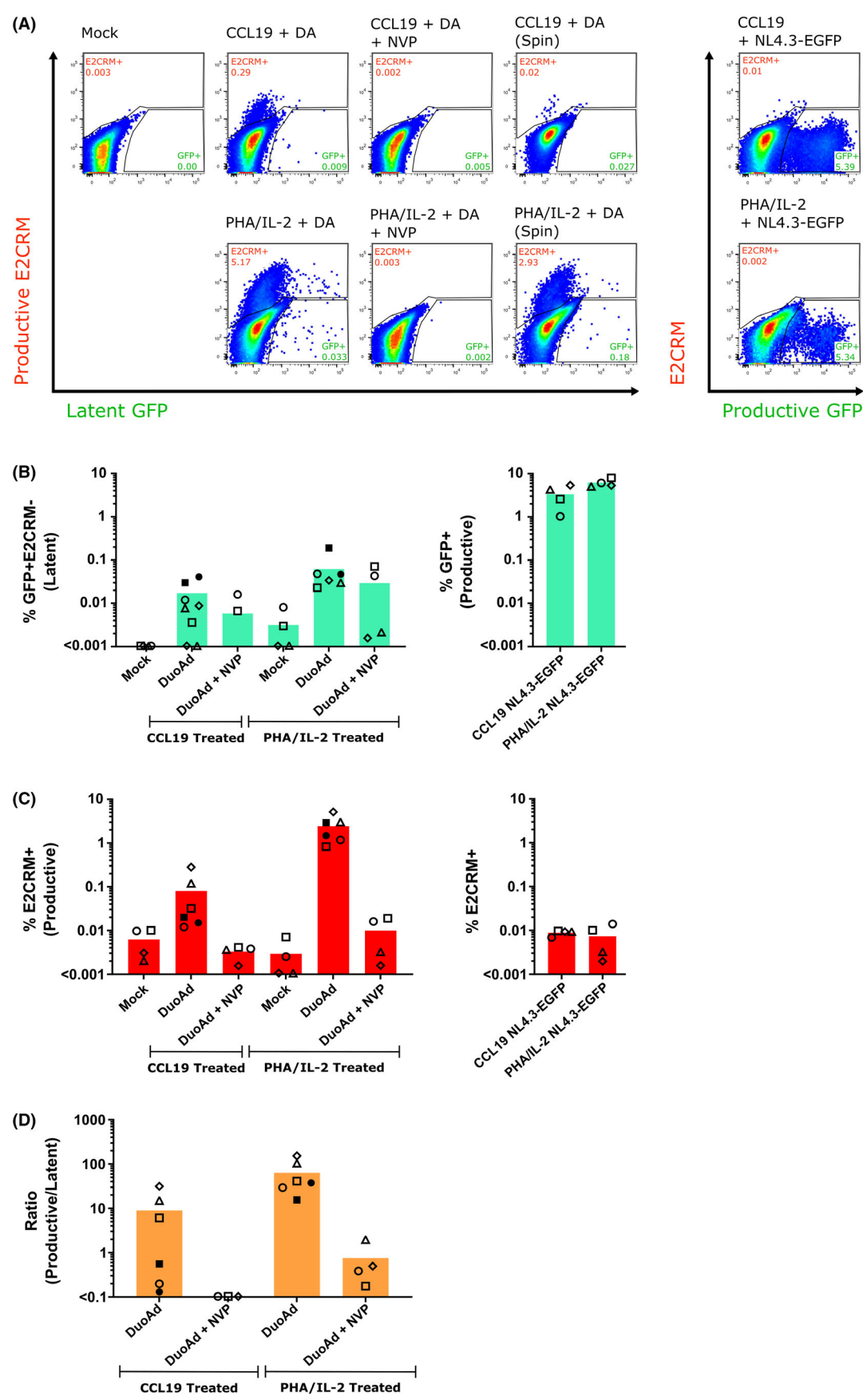
The low frequency of latently infected cells observed following infection of primary resting CD4⁺ T cells with DuoAdvance may be explained by inefficient expression of the EF1 α -driven GFP latent reporter in primary resting CD4⁺ T cells, in contrast to expression in the Jurkat T-cell line (Figure 1). Promoter silencing may account for the low expression levels in resting CD4⁺ T cells compared to the actively dividing Jurkat T-cell line. A key feature of resting CD4⁺ T cells is sequestration of transcription factors NF- κ B and nuclear factor of activated T cell (NFAT) [18]. These transcription factors are important for the initiation of transcription of HIV and may also be important for initiation of transcription of the latent GFP reporter from the EF1 α promoter. The downregulation of antiretroviral defence genes such as SAM domain and HD domain-containing protein 1 (SAMHD1) and guanylate-binding protein 5 (GBP5) in Jurkat T cells could further contribute to the success of viral infection in cell lines with these virus strains compared to primary CD4⁺ T cells, where these genes are not downregulated [19]. Additionally, the recognition of CG dinucleotides in viral RNA by the cellular zinc-finger antiviral protein (ZAP) could have contributed to depleted cytoplasmic viral RNA and impaired viral expression in primary resting CD4⁺ T cells [20].

3.3 | Inducible virus is detected in E2CRM negative cells

We next performed a reactivation experiment (Figure 3A) to test whether the E2CRM cells at six days post-infection with DuoAdvance contained inducible virus (consistent with latent infection), even if there was poor constitutive expression of GFP. Using cells from different donors, at six days post-

Figure 2. DuoAdvance can infect activated and resting primary CD4⁺ T cells.

DuoAdvance (DuoAd, DA) was added to CCL19-treated or PHA/IL-2-activated primary CD4⁺ T cells at a TCID₅₀ of 0.5 for 2 hours. The cells were washed and then cultured for a further six days. Uninfected cells (mock), cells infected with DuoAd in the presence of Nevirapine (NVP) to block infection, and cells infected with 0.5 TCID₅₀ NL4.3-EGFP as a comparative control were included. In some experiments, spinoculation (spin, closed symbols) was included. (A) FACS dot plots from a representative experiment is shown and includes the gating used to determine GFP+E2CRM⁻ (latent) and E2CRM⁺ (Productive) cells. (B, C) Resulting levels of GFP+E2CRM⁻ latent and E2CRM⁺ productive cells from $n \leq 6$ experiments are shown. On a separate graph, the levels of GFP⁺ (productive infection reporter) and E2CRM⁺ (background) from the NL4.3-EGFP infection positive control are also shown. (D) The ratio of productive to latent infection was calculated using the data from B and C, with the fluorescence background in the mock sample subtracted from the DuoAd-infected samples before calculating the productive to latent infection ratio. Each symbol represents a different donor and the height of the column represents the mean of the experiments.



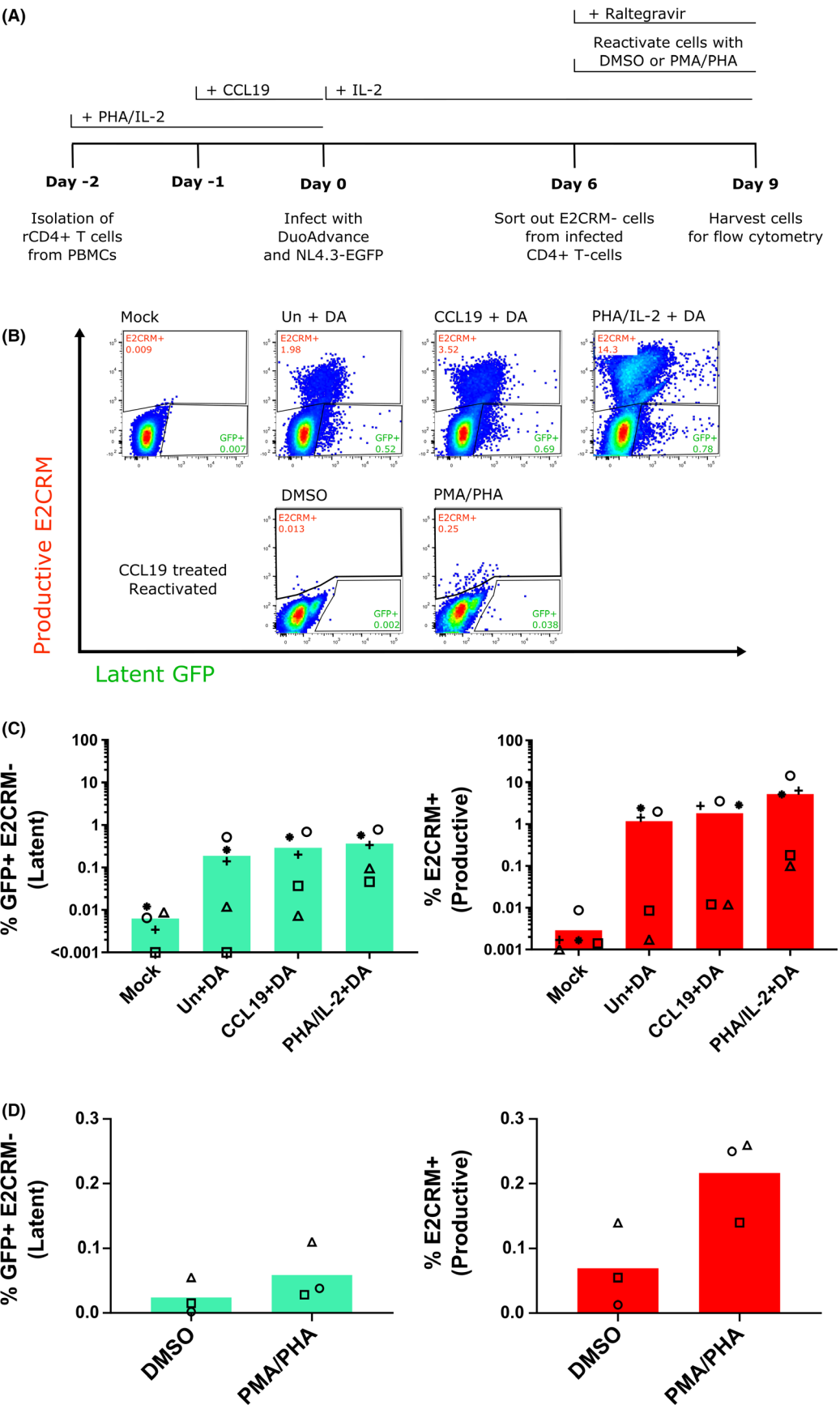


Figure 3. E2 Crimson negative cells harbour inducible latent virus.

CD4⁺ T cells were isolated from PBMC and left resting (unstimulated, Un) or stimulated with either CCL19 or PHA/IL-2 and then infected with either DuoAdvance (DA) virus or media only (mock) (A). After six days, expression of both fluorescent proteins was quantified by flow cytometry (B-D). The E2 Crimson negative cell population (cells that were not present in the E2CRM⁺ gate in B top panel) was sorted from the CCL19-treated samples six days after infection and then stimulated with either DMSO or PMA/PHA in the presence of 1 μ M raltegravir and expression of both fluorescent proteins quantified by flow cytometry (D). Each symbol represents a different donor and the column represents the mean.

infection with DuoAdvance, low levels of GFP expression were again detected in all conditions (unactivated cells: mean (range) = 0.19 (0.00 to 0.52)%; CCL19 cells: mean (range) = 0.29 (0.01 to 0.69)%; PHA/IL-2 cells: mean (range) = 0.37 (0.05 to 0.78)%) that was higher than mock infection (mean (range) = 0.01 (0.00 to 0.01)%) (Figure 3B and C left panel). There was an increase in E2CRM productive infection in all conditions compared to mock infection (unactivated cells: mean (range) = 1.17 (0.00 to 2.44)%; CCL19 cells: mean (range) = 1.83 (0.01 to 3.52)%; PHA/IL-2 cells: mean (range) = 5.21 (0.10 to 14.30)%) (Figure 3B and C right panel). Infection of PHA/IL-2 activated cells resulted in the highest levels of latent and productive infection (Figure 3B-C).

To test for inducible virus, the E2CRM⁻ population was isolated on day 6 from CCL19-treated infected cells and reactivated with PMA/PHA for three days. PMA/PHA led to an increase in the number of E2CRM⁺ cells **3.1-fold** versus the DMSO diluent control (Figure 3D). This revealed that the E2CRM cells sorted six days post-infection harboured inducible virus, even though these cells had low to absent GFP expression prior to stimulation. Interestingly, the level of GFP+E2CRM⁻ latent cells also increased **2.4-fold** with PMA/PHA reactivation compared to the DMSO diluent control (Figure 3D), suggesting that the latent reporter may need the cell to be in an activated state to effectively express GFP protein. We do not believe that this increase in GFP was a result of integration of unintegrated DNA following T cell activation as reactivation experiments were performed in the presence of an integrase inhibitor.

In our experiments, the expression of the EF1 α -driven GFP clearly increased following T cell activation. It is worth noting that we were unable to determine if the inefficient expression of GFP in resting T cells was a feature of the EF1 α promoter itself or a consequence of the EF1 α promoter in the background of the DuoAdvance construct. However, we propose that the use of the EF1 α promoter in a dual reporter virus might significantly underestimate the frequency of latently infected cells, particularly when using a pre-activation model of resting CD4⁺ T cell latency.

In activated primary CD4⁺ T cells, low expression of the latent reporter GFP was observed despite efficient expression of the HIV LTR-driven E2CRM (Figure 2) might be explained by promoter competition. This can occur when a promoter that has a higher affinity for a limiting factor competitively inhibits transcription from an adjacent promoter [21]. For example, once the HIV LTR becomes activated by Tat, there may be preferential recruitment of transcription factors to the HIV LTR promoter driving E2CRM expression at the expense of the EF1 α promoter and GFP expression, thereby suppressing GFP and resulting in a higher proportion of E2CRM⁺ cells. Similar promoter competition has been reported in other model systems such as human embryonic stem cells transduced with a dual-promoter vector [22] and HeLa cells co-

transfected with HIV and HIV Rev binding aptamer under the control of the human cytomegalovirus (CMV) promoter [23].

Collectively, these experiments demonstrate that while DuoAdvance can successfully infect the Jurkat T-cell lines and establish latent and productive infection, following infection of primary CD4⁺ T cells, only E2 Crimson expression was detected and there was poor constitutive expression of the EF1 α promoter as detected by GFP expression. Expression of E2 Crimson and GFP could be induced following activation of sorted E2CRM-CCL19 CD4⁺ T cells suggesting integration did occur in these cells, but GFP had poor constitutive expression and was not expressed in all cells that contained inducible virus.

Interestingly, Chavez and colleagues noted using Duo-Fluo, that in activated T cells returning to a resting state, the expression of both latent and productive infection fluorescent markers were progressively lost over time [12]. The second generation HIV_{GKO} variant of Duo-Fluo has only been used to infect activated T cells to date and expression of the new EF1 α -driven mKO2 latency reporter has not yet been examined in resting CD4⁺ T cells [14]. Another dual-fluorescent reporter virus (Hlfate-E) has some similarities to DuoAdvance [24]. In this construct the latency reporter was changed to GFP and the productive reporter to E2 Crimson and a composite EF1 α -HTLV-1 promoter was used in place of the EF1 α promoter to drive the GFP latency reporter. Following infection of activated CD4⁺ T cells with Hlfate-E, insufficient latently infected cells were generated to assess the response to further activation [24], suggesting a very low yield of latently infected cells despite successful infection of activated CD4⁺ T cells similar to this study. Further investigation with these two new viruses, HIV_{GKO} or Hlfate-E, in direct infection of resting CD4⁺ T cells will elucidate whether these viruses are suited to studying HIV latency in pre-activation models.

Developing a sensitive and specific marker of latently infected cells using an in vitro model of a dual reporter virus to infect primary CD4⁺ T cells is of high interest as this would allow for examination of host gene changes following latency using techniques such as scRNAseq as these studies cannot be performed with immortalized cell lines, and activation of the cell to express viral proteins changes the host transcriptome, as recently reported [25]. Using DuoAdvance, if latency is defined by GFP expression but not all latently infected cells express GFP, this would underestimate the frequency of latently infected cells. If these cells were being used to screen for LRAs, this would potentially overestimate the efficiency of a particular LRA. In addition, screening would miss latently infected cells that do not react to an LRA.

Adding an internal ribosome entry site (IRES) between the EF1 α promoter and the latency reporter might lead to the improved expression of the latency reporter in resting CD4⁺ T cells by enhancing the reporter gene activity as previously

described [26]. This approach could be pursued to drive better expression of the latency reporter in resting CD4+ T cells to study HIV latency and reactivation in resting CD4+ T cells [27].

Finally, a limitation of this study was the number of donors analysed. We examined six or fewer donors for each experiment and therefore could not determine whether the data was normally distributed. In preference to using parametric tests, as is often used for small samples, we have only reported the mean fold changes in different experimental conditions and not reported *p* values.

4 | CONCLUSIONS

We generated a modified dual-fluorescent reporter virus that was able to successfully infect a Jurkat T-cell line and generate latent and productively infected cell populations. However, DuoAdvance resulted in low levels of expression of the latency reporter in resting CD4+ T cells, using a pre-activation model of HIV latency. As current dual-fluorescent reporters are usually studied by infecting activated CD4+ T cells [11,12,14], and expression of latent and productive infection reporters can be progressively lost as activated cells return to a resting state [12], dual-fluorescent reporter viruses may significantly underestimate the frequency of latently infected cells *in vitro*.

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COMPETING INTERESTS

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AUTHORS' CONTRIBUTIONS

Y.K., S.R.L. and J.L.A. designed the research study. Y.K. and J.L.A. performed the research. Y.K., P.U.C. and J.L.A. analysed the data. Y.K., S.R.L. and J.L.A. wrote and edited the paper. All co-authors have read and approved the paper.

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SUPPORTING INFORMATION

Additional information may be found under the Supporting Information tab for this article.

Data S1. Sequence of DuoAdvance (6668bp) Plasmid Insert.