Universitat Autònoma de Barcelona

Identification of Novel Immunomodulatory Strategies to Tackle HIV-1 Persistence

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Thesis to obtain a PhD degree in Advanced Immunology

from the Universitat Autònoma de Barcelona, June 2022

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Certifiquen:

Que el treball experimental i la redacció de la memòria de la Tesi Doctoral titulada "Identification of novel immunomodulatory strategies to tackle HIV-1 persistence" han estat realitzades per Ifeanyi Jude Ezeonwumelu sota la seva direcció i considera que és apta per a ser presentada per optar al grau de Doctor en Immunologia Avançada per la Universitat Autònoma de Barcelona.

I per tal que en quedi constància, signen aquest document a Badalona,

20 de Juny de 2022

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Ifeanyi Jude Ezeonwumelu was supported by an INPhINIT PhD fellowship from the “la Caixa” Foundation (LCF/BQ/IN18/11660017) co-funded by the European Union’s Horizon 2020 research and innovation programme under Marie Sklodowska-Curie grant agreement No. 713673. The content of this thesis was partly funded by Instituto de Salud Carlos III, Fondo de Investigación Sanitaria (FIS) PI19/00194, PI17/00624 and CP14/00016 co-financed by FEDER, the Generalitat de Catalunya and Grifols.

The printing of this thesis was made possible with the financial aid of the Universitat Autònoma de Barcelona.

“So I realized that all we can do is to be happy and do the best we can while we are still alive”

~ Ecclesiastes 3 verse 12

To God be the Glory

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SUMMARY

Antiretroviral therapy (ART) has dramatically improved the life expectancy for people with HIV (PLWH), but it is not curative and must be taken for life. The persistence of HIV in transcriptionally silent but replication-competent latent reservoirs is a major barrier to HIV cure. Thus, strategies targeting the latent HIV reservoir are crucial in achieving HIV cure, as the "shock and kill" strategy using latency reversing agents (LRAs) to induce virus transcription, exposing latent HIV to immune clearance, or the permanent silencing of viral transcription using latency promoting agents (LPAs) in the "block and lock" strategy.

Considering the heterogeneity of the latent reservoir comprising cells from distinct lineages, we developed non-clonal cellular models of HIV-1 latency using cell lines from lymphoid or myeloid lineage for identifying new LRAs. We performed a high-throughput screening (HTS) of an anticancer compound library using these models of HIV-1 latency, allowing the identification of LRAs with potent activity across multiple cell lineages. Interestingly, we identified two classes of innate immunomodulating agents as novel LRAs in these models: IκB/IKK inhibitors (IKKis) and JAK inhibitors (JAKi). As previously described for acitretin and TLR agonists, modulation of innate immune stimulation could impact viral latency and contribute to the clearance of the HIV reservoir.

In the first chapter of the thesis, we focused on validating the LRA capacity of IKKis in both models of HIV latency. Our results show the latency-reversing activity of IKKis in HIV-1 latently infected lymphoid and myeloid compartments. In particular, selective inhibitors targeting TBK1 and IKKε showed relevant HIV reactivation capacities through a mechanism of NF-κB-induced HIV transcription *in vitro* and *ex vivo*. Since HIV-associated immune activation and hyperinflammatory responses persist in PLWH, we also evaluated the IKKis effect on immune cell function, observing that the LRA activity of IKKi did not alter the activation status in CD4+T cells, concomitant with significantly decreased proinflammatory responses.

Next, in the second chapter of the thesis, we characterized the latency reactivation capacity of JAKi as a potential novel therapeutic strategy for HIV-1 cure. We observed that contrary to the previously described role of JAKi as potent inhibitors of latency reactivation, a subclass of selective JAK2 inhibitors (JAK2i) showed a significant capacity for reversing HIV latency. Notably, the JAK2i fedratinib reversed HIV-1 latency in non-clonal lymphoid and myeloid *in vitro* models of HIV-1 latency and *ex vivo* in CD4+ T cells from ART+ PLWH, albeit its function was not dependent on JAK2

expression. To delineate the mechanism of action for JAK2i-induced latency reactivation, we performed whole transcriptomic analysis, identifying a significant upregulation of IRF7 expression despite the blockade of the JAK-STAT pathway and downregulation of proinflammatory cytokines and chemokines. Moreover, IRF7 expression levels positively correlated with HIV latency reactivation capacity of JAK2 inhibitors and other common LRAs.

Furthermore, we characterized the latency promoting capacity of the JAK2i pacritinib, demonstrating its potency in blocking LRA-induced reactivation *in vitro* and in *ex vivo*-treated primary cells from PLWH. These results were consistent with the relevance of JAK-STAT signalling blockade as a strategy to prevent HIV-1 latency reversal. Collectively, these results represent a promising step towards HIV eradication by demonstrating the potential of innate immune modulation for reducing the viral reservoir through a novel pathway driven by IRF7.

Overall, our studies highlight the diverse outcomes of modulating immune responses to target HIV persistence in latent reservoirs, either reactivating HIV-1 latency as the TBKi/IKKi and the selective JAK2i fedratinib or epigenetic silencing of the provirus as the JAK2i pacritinib, here described. In addition, the capacity for counteracting chronic immune activation and dysregulated inflammatory responses make immune-modulating agents interesting candidates for combination therapy with ART.

RESUMEN

La terapia antirretroviral (TAR) ha mejorado drásticamente la esperanza de vida de las personas con VIH (PLWH), pero no es curativa y debe tomarse de por vida. La persistencia del VIH en reservorios latentes transcripcionalmente silenciosos pero competentes para la replicación es una barrera importante para la cura del VIH. Por lo tanto, es necesario el desarrollo de nuevas estrategias dirigidas a eliminar el reservorio de VIH latente para avanzar hacia la cura del VIH. Entre ellas cabe destacar la estrategia conocida como "shock and kill" que utiliza agentes reversores de latencia (LRA) para inducir la transcripción viral, exponiendo las células latentemente infectadas a la eliminación por parte del sistema inmune o la estrategia conocida como “block and lock”, la cuál persigue el silenciamiento permanente de la transcripción viral utilizando agentes promotores de latencia (LPA).

Teniendo en cuenta la heterogeneidad del reservorio latente que comprende células de distintos linajes, hemos desarrollado modelos celulares de latencia del VIH-1 no clonales utilizando líneas celulares de origen linfoide o mieloide, con el objetivo de identificar nuevos LRA. Realizamos un cribado de alto rendimiento (HTS) de una biblioteca de compuestos utilizando estos modelos de latencia del VIH-1, lo que permitió la identificación de LRA con actividad en múltiples tipos celulares. Curiosamente, identificamos dos clases de agentes inmunomoduladores como nuevos LRA en estos modelos: inhibidores de IκB/IKK (IKKis) e inhibidores de JAK (JAKi). Como se describió anteriormente para la acitretina y los agonistas de TLR, la modulación de la estimulación inmunitaria innata podría afectar la latencia viral y contribuir a la eliminación del reservorio de VIH.

En el primer capítulo de la tesis, nos centramos en validar la capacidad como LRA de los IKKis en ambos modelos celulares de latencia del VIH. Nuestros resultados demuestran la capacidad de reversión de la latencia viral de los IKKis, tanto en células de origen linfoides como mieloides. En particular, los inhibidores selectivos dirigidos a TBK1 e IKKε fueron capaces de reactivar la latencia viral a través de un mecanismo dirigido a la inducción de la transcripción del VIH mediada por NF-κB, tanto *in vitro* y *ex vivo*. Dado que la activación inmunitaria asociada con el VIH y las respuestas hiperinflamatorias persisten en las PLWH, también evaluamos el efecto de IKKis en la función de las células inmunitarias y observamos que la actividad LRA de IKKi no alteró el estado de activación en las células T CD4+, a la vez que presentaba una disminución significativa de las respuestas proinflamatorias.

El segundo capítulo de la tesis se centró en el estudio de la capacidad de reactivación de latencia de los JAKi, como una potencial estrategia terapéutica novedosa para la cura del VIH-1. Contrariamente al papel inhibidor de la reactivación viral descrito anteriormente para determinados JAKi, identificamos una subclase de inhibidores selectivos de JAK2 (JAK2i), los cuáles fueron capaces de revertir significativamente la latencia del VIH. En particular, el JAK2i fedratinib, revirtió la latencia del VIH-1 *in vitro* en los modelos linfoides y mieloides no clonales desarrollados al inicio de la tesis doctoral y también *ex vivo* en células T CD4+ de PLWH, aunque su función no dependía de la expresión de JAK2. Para delinear el mecanismo de acción subyacente para la reactivación de la latencia inducida por JAK2i, realizamos un análisis transcriptómico completo, identificando una regulación positiva significativa de la expresión de IRF7 a pesar del bloqueo de la vía JAK-STAT y la regulación negativa de citocinas y quimiocinas proinflamatorias. Además, los niveles de expresión de IRF7 se correlacionaron positivamente con la capacidad de reactivación de la latencia del VIH de los inhibidores de JAK2 y otros LRA.

Además, caracterizamos la capacidad de promover la latencia viral del inhibidor de JAK2 pacritinib, demostrando su potencia para bloquear la reactivación inducida por LRA *in vitro* y *ex vivo* en células de PLWH. Además, estos compuestos presentaron un fueron bloqueo de la señalización JAK-STAT, lo cuál puede representar una nueva estrategia para prevenir la reversión de la latencia del VIH-1. En conjunto, estos resultados representan un paso prometedor hacia la erradicación del VIH al demostrar el potencial de la modulación inmunitaria innata para reducir el reservorio viral a través de una vía novedosa impulsada por IRF7.

Así, nuestros estudios destacan la capacidad de compuestos moduladores de la respuesta inmune para modular la persistencia del VIH en reservorios latentes, ya sea reactivando la latencia viral con TBKi/IKKi o inhibidores selectivos de JAK2, como fedratinib, o alternativamente, silenciando la expresión del provirus como es el caso de pacritinib, aquí descrito. Además, la capacidad de contrarrestar la activación inmunitaria crónica y las respuestas inflamatorias desreguladas hacen que los agentes inmunomoduladores sean candidatos interesantes para la terapia combinada con ART.

RESUM

La teràpia antiretroviral (TAR) ha millorat dràsticament l'esperança de vida de les persones amb VIH (PLWH), però no és curativa i s'ha de prendre tota la vida. La persistència del VIH en reservoris latents transcripcionalment silenciosos però competents per a la replicació és una barrera important per a la cura del VIH. Per tant, cal el desenvolupament de noves estratègies dirigides a eliminar el reservori viral latent per avançar cap a la cura del VIH. Entre elles cal destacar l'estratègia coneguda com a "shock and kill" que utilitza agents reversors de latència (LRA) per induir la transcripció viral, exposant les cèl·lules latentment infectades a l'eliminació per part del sistema immune o l'estratègia coneguda com a “block and lock ”, la qual persegueix el silenciament permanent de la transcripció viral utilitzant agents promotors de latència (LPA).

Tenint en compte l'heterogeneïtat del reservori latent que comprèn cèl·lules de diferents llinatges, hem desenvolupat models cel·lulars de latència del VIH-1 no clonals utilitzant línies cel·lulars d'origen limfoide o mieloide, amb l'objectiu d'identificar nous LRA. Hem realitzat un cribratge d'alt rendiment (HTS) d'una biblioteca de compostos utilitzant aquests models de latència del VIH-1, identificant una sèrie d’agents amb activitat com a reactivadores de la latència en múltiples tipus cel·lulars. Curiosament, s’han identificat dues classes d'agents immunomoduladors com a nous LRA en aquests models: els inhibidors de IκB/IKK (IKKis) i els inhibidors de JAK (JAKi). Com es va descriure anteriorment per a l'acitretina i els agonistes de TLR, la modulació de l'estimulació immunitària innata podria afectar la latència viral i contribuir a eliminar el reservori del VIH.

Al primer capítol de la tesi, ens hem centrat a validar la capacitat com a LRA dels IKKis en ambdós models cel·lulars de latència del VIH. Els nostres resultats demostren la capacitat de reversió de la latència viral dels IKKis, tant en cèl·lules d'origen limfoides com mieloides. En particular, els inhibidors selectius dirigits a TBK1 i IKKε van ser capaços de reactivar la latència viral a través d'un mecanisme dirigit a la inducció de la transcripció del VIH mitjançada per NF-κB, tant *in vitro* com *ex vivo*. Atès que l'activació immunitària associada amb el VIH i les respostes hiperinflamatòries persisteixen a les PLWH, també hem avaluat l'efecte dels IKKis en la funció de les cèl·lules immunitàries, observant que l'activitat com a LRA dels IKKi no va alterar l'estat d'activació a les cèl·lules T CD4+, alhora que presentava una disminució significativa de les respostes proinflamatòries.

El segon capítol de la tesi s’ha centrat en l’estudi de la capacitat de reactivació de la latència dels JAKi, com una potencial estratègia terapèutica per a la cura del VIH-1. Contràriament al paper

inhibidor de la reactivació viral descrit anteriorment per a determinats JAKi, hem identificat una subclasse d'inhibidors selectius de JAK2 (JAK2i), els quals van ser capaços de revertir significativament la latència del VIH. En particular, l'inhibidor de JAK2i fedratinib, va revertir la latència del VIH-1 *in vitro* en els models limfoides i mieloides no clonals desenvolupats a l'inici de la tesi doctoral i també *ex vivo* en cèl·lules T CD4+ de PLWH, encara que la seva funció no depenia de l'expressió de JAK2. Per delinear el mecanisme d'acció subjacent a la reactivació de la latència induïda per JAK2i, hem realitzat una estudi de l'expressió gènica global, identificant una regulació positiva significativa de l'expressió d'IRF7 malgrat el bloqueig de la via JAK-STAT i la regulació negativa de citocines i quimiocines proinflamatòries. A més, els nivells d'expressió de IRF7 es van correlacionar positivament amb la capacitat de reactivació de la latència del VIH dels inhibidors de JAK2 i d'altres LRA.

Per altra banda, hem caracteritzat la capacitat de promoure la latència viral de l'inhibidor de JAK2, pacritinib, demostrant-ne la potència per bloquejar la reactivació induïda per LRA *in vitro* i *ex vivo* en cèl·lules de PLWH. A més, aquests compostos van presentar un bloqueig de la senyalització JAK-STAT, la qual cosa pot representar una nova estratègia per prevenir la reversió de la latència del VIH-1. En conjunt, aquests resultats representen un pas prometedor cap a l'eradicació del VIH en demostrar el potencial de la modulació immunitària innata per reduir el reservori viral mitjançant una via nova impulsada per IRF7.

Així, els nostres estudis destaquen la capacitat de compostos moduladors de la resposta immune innata per modular la persistència del VIH en reservoris latents, ja sigui reactivant la latència viral amb TBKi/IKKi o els inhibidors selectius de JAK2 com fedratinib; o, alternativament, silenciant l'expressió del provirus com és el cas de pacritinib, aquí descrit. A més, la capacitat de contrarestar l'activació immunitària crònica i les respostes inflamatòries desregulades fan que els agents immunomoduladors siguin candidats interessants per a la teràpia combinada amb ART.

ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
Akt	Protein kinase B
APC	Antigen-presenting cell
ART	Antiretroviral therapy
ARV	Antiretroviral drug
AZT	Azidothymidine (Zidovudine)
Bcl-2	B-cell lymphoma 2
BET	Bromodomain and extraterminal
bNAb	Broadly neutralizing antibody
CA	Capsid
CC50	50% cytotoxic concentration, or the concentration needed to induce 50% cell death
CCR5	C-C chemokine receptor 5
CCR5Δ32	CCR5 with a 32-bp deletion
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
cGAS	Cyclic GMP-AMP synthase
CNS	Central nervous system
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/CRISP-associated protein nuclease-9
CTL	Cytotoxic T lymphocytes
CXCR4	C-X-C chemokine receptor type 4
DC	Dendritic cell
dCA	didehydro-Cortistatin A
DEG	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EC50	50% effective concentration, or the concentration needed to induce the half-maximal response
EFV	Efavirenz
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked ImmunoSpot assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FC	Fold-change
FDA	Food and drug Administration
FLT3	FMS-like tyrosine kinase 3

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GIT	Gastrointestinal tract
GSEA	Gene set enrichment analysis
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HSCT	Hematopoietic stem cell transplantation
HSP	Heat shock protein
HTS	High-throughput screening
IFN	Interferon
IKK	IκB kinase
IKKis	IκB/IKK inhibitors
IL	Interleukin
IN	Integrase
INF	Infected
INSTI	Integrase strand transfer inhibitor
IRF	IFN-regulatory factors
IRES	Internal ribosome entry site
ISGs	IFN-stimulated genes
IκBα	Nuclear factor κ-light-chain-enhancer of activated B cells inhibitor α
JAK	Janus-activated kinases
JAKi	JAK inhibitor
JAK2i	JAK2 selective inhibitor
LOD	Limit of detection
LPA	Latency promoting agent
LPS	Lipopolysaccharide
LRA	Latency promoting agent
LTR	Long terminal repeat
MA	Matrix
MAVS	Mitochondrial antiviral-signalling protein
MDA5	Melanoma Differentiation-Associated antigen 5
MDM	Monocyte-derived macrophages
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
ND	No drug
Nef	Negative regulatory factor
NF-κB	Nuclear factor-kB
NK	Natural killer cells
NNRTI	Non-nucleoside reverse transcriptase inhibitor
ORF	Open reading frame

PAI	Post-attachment inhibitor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHA	Phytohemaglutinin
PMA	Phorbol ester phorbol myristate acetate
PMAi	PMA plus ionomycin
PI	Protease inhibitor
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PNB	Panobinostat
PR	Protease
PRR	Pattern recognition receptor
P/S	Penicillin/Streptomycin
P-TEFb	Positive transcription elongation factor b
PVDF	Polyvinylidene difluoride
qPCR	quantitative real-time PCR
RAL	Raltegravir
Rev	Regulator of expression of virion proteins
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-like receptor
RNA	Ribonucleic acid
RNAi	RNA interference
RNAPII	RNA polymerase II
RNAseH	Ribonuclease H
RT	Reverse transcriptase
RT-PCR	Reversetranscriptase PCR
SAMHD1	Sterile alpha motif (SAM) histidine-aspartic (HD) domain protein 1
SD	Standard deviation
SEM	Standard error of mean
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
ssRNA	Single stranded RNA
STAT	Signal transducer and activator of transcription
T_{CM}	Central memory CD4+ T cell
T_{EM}	Effector memory CD4+ T cell
T_N	Naïve CD4+ T cell
T_{TM}	Transition memory CD4+ T cell
TAR	Trans-activation response
Tat	Trans-activator of transcription
Th	T helper (cell)
TBK1	TANK-binding kinase 1 (serine/threonine-protein kinase)
TCR	T-cell receptors

TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TRAF	TNF receptor-associated factor
TSS	Transcription start site
UN	Untreated
UNAIDS	Joint United Nations Programme on HIV/AIDS
Vif	Viral infectivity factor
VIP-SPOT	Viral protein spot assay
VL	Viral load
VLP	Viral-like particles
VOR	Vorinostat, SAHA
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV-G	Vesicular stomatitis virus
WHO	World health organization
ZFN	Zinc fingers nuclease

INTRODUCTION

1. HIV PANDEMIC: CURRENT STATUS

The human immunodeficiency virus (HIV) infection is one of the leading causes of mortality and morbidity worldwide[1]. HIV is the causative agent of the acquired immunodeficiency syndrome (AIDS), which continues to be a major global public health issue, with 36.3 (27.2-47.89) million lives lost due to AIDS-related illnesses since the start of the HIV pandemic four decades ago. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), there were 37.7 (30.2-45.1) million people living with HIV at the end of 2020. Since 2010, new HIV infections have steadily declined, from 2.1 (1.5-2.9) million to 1.5 (1.0–2.0) million in 2020, and AIDS-related deaths reduced by 47%, from 1.3 (0.91-1.9) million to 0.68 (0.48-1.0) million (**Figure 1**). These decreasing trends in new HIV infections and mortality coincide with a significant increase in the number of people living with HIV (PLWH) with access to antiretroviral therapy (ART). Globally, 27.5 (26.5-27.7) million PLWH were receiving ART in 2020, compared to 7.8 (6.9-7.9) million in 2010[2].

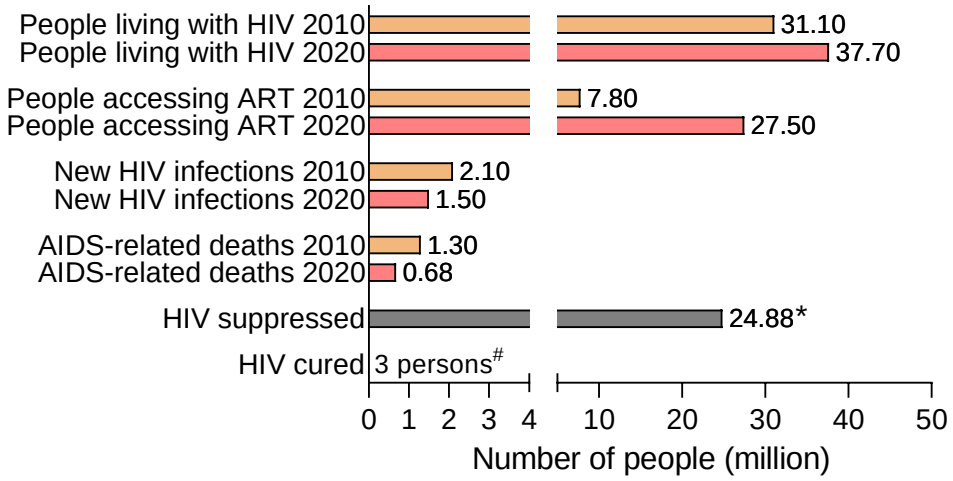


Figure 1. Evolution of global HIV epidemic in the last decade (2010-2020). Global roll-out of ART has saved many lives and decreased transmission of new infections. *In 2020, 66% of all people living with HIV in 2020 (37.7 million) were virally suppressed, but #only 3 persons have been cured of HIV till date. Source[2].

Although the rate of new infections has progressively waned over the years, prevalence of HIV infection has steadily increased because of the successful scale-up of ART, increasing the lifespan of PLWH. However, the financial burden of providing lifelong therapy, adherence and long-term toxicities due to ART warrant further research efforts to accelerate development and delivery of an HIV cure, in addition to existing HIV prevention strategies[3].

2. HUMAN IMMUNODEFICIENCY VIRUS

HIV is a lentivirus that belongs to the *Retroviridae* family. HIV isolates are broadly classified into two types based on its origin and genome organization: HIV-1 and HIV-2. Both HIVs evolved from multiple cross-species transmissions of simian immunodeficiency viruses (SIVs) naturally infecting non-human primates, HIV-1 from Central African chimpanzees (SIVcpz) and HIV-2 from West African sooty mangabeys (SIVsm)[4,5]. Although both HIVs are causative agents of AIDS, HIV-1 is more virulent and widely distributed worldwide whereas, the less virulent HIV-2 is mostly confined to sub-saharan Africa[4,6–8].

2.1. Viral particle and Genomic organization

The HIV genome is enclosed within the core of the virus particle and consists of two identical single-stranded RNA molecules. The HIV-1 genome is about 9.8 kb long consisting of overlapping open reading frames that encode several structural, regulatory and accessory proteins. Flanking both ends of the viral genome are the long terminal repeats (LTR) sequences. The 5´ LTR region codes for the promoter that initiates transcription of viral genes (**Figure 2**)[9–11].

Structural genes

Three genes code for the distinct structural proteins. Starting from the 5'LTR, the *gag* gene encodes p17 (outer core membrane, MA), p24 (capsid, CA), p7 (nucleocapsid, NC) and p6 proteins. Following *gag,* is the *pol* reading frame coding for the enzymes p12 (protease, prot), p51 (reverse transcriptase, RT), p15 (RNaseH) and p32 (integrase, int). Towards de 3' end is located the *env* reading frame coding for the two envelope glycoproteins gp120 (surface protein) and gp41 (transmembrane protein).

Regulatory genes

The HIV-1 genome encodes two essential regulatory proteins: *tat* (transactivator of HIV gene expression), that enhances viral transcription initiation and elongation, and *rev* (RNA splicing-regulator), that facilitates the export of unspliced viral mRNA from the nucleus to the cytoplasm.

Accessory proteins

In addition, the genome encodes several accessory genes whose gene products impact viral replication, virus budding and pathogenesis, respectively, *nef* (negative regulatory factor), *vif* (viral infectivity factor), *vpr* (virus protein r) and *vpu* (virus protein unique).

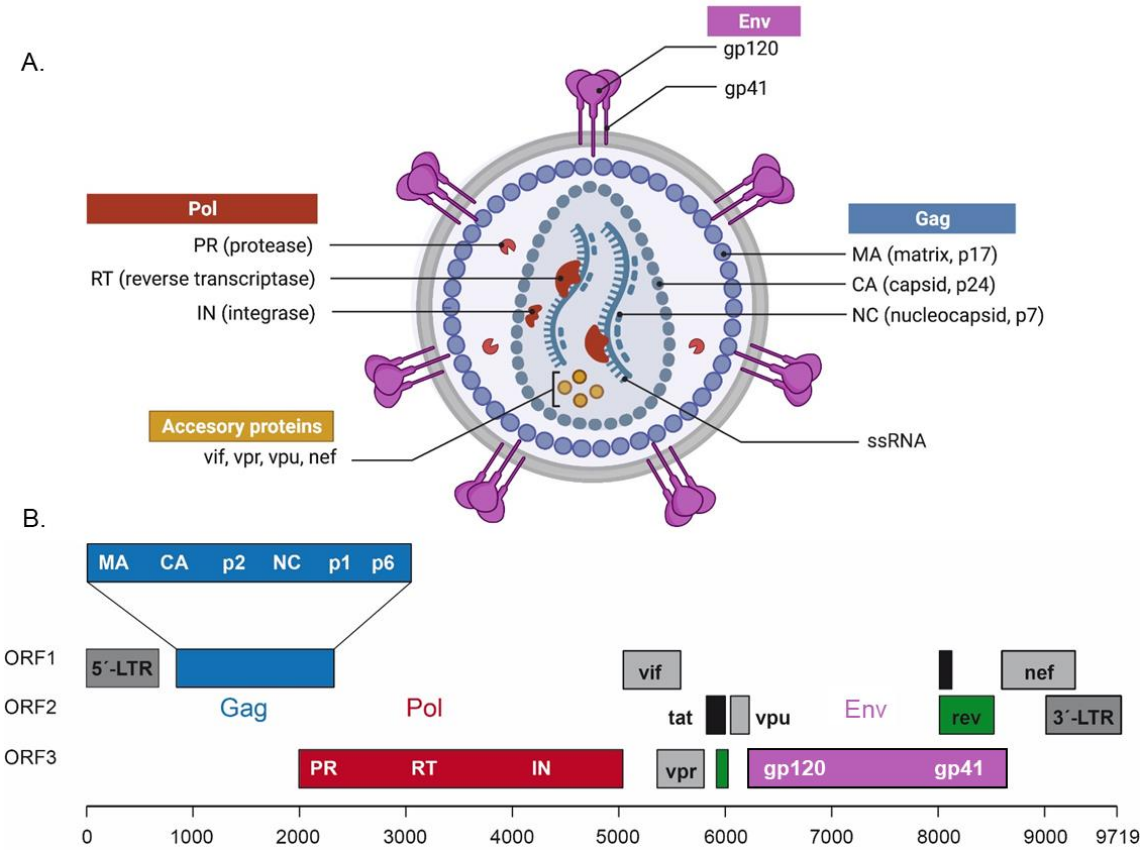


Figure 2. HIV-1 particle structure and genomic map. (**A**) Schematic structure of HIV-1 virion. (**B**) Genomic landmark of HIV-1. The genome consists of ~9,800 nucleotides coding for structural, regulatory and accessory proteins organized in three open reading frames (ORF1-3). The 5´ and 3´ LTR nucleic acid sequences flanking the internal unique sequences are not translated into proteins. Moving from left to right (5´LTR to 3´LTR direction), the genes coding for the structural proteins are organized as follows: *gag*, *pol* and *env*. Both regulatory genes *tat* and *rev* consists of two exons. Adapted from[11].

2.2. Replication cycle of HIV-1

The HIV-1 replication cycle can be divided into an early phase and a late replication phase, and although its duration is variable depending on the cell type, it lasts one to two days in highly activated CD4+ T cells. The early phase begins with the attachment of the viral Env glycoproteins (gp41 and gp120) to the cell surface CD4 receptor and a co-receptor (either CC-chemokine receptor 5 (CCR5) or CXC-chemokine receptor 4 (CXCR4)). Fusion of the viral and the host membranes allows the entry of the viral capsid into the cell. As the viral capsid containing the HIV-1 genome shuttles from the cytosol into the nucleus, the viral RNA is reverse transcribed to double-stranded DNA for integration into the host genome following uncoating of the viral capsid. The late phase of replication starts with the proviral transcription of the HIV DNA to mRNAs mediated by the host enzymes. Following nuclear export of these mRNAs into the cytoplasm, viral mRNAs are translated into viral proteins and assembled with the unspliced viral

RNA genome. Finally, immature virions bud from the cell and mature via viral proteolytic machinery, giving rise to mature virions capable of propagating new infections (**Figure 3**)[12–15].

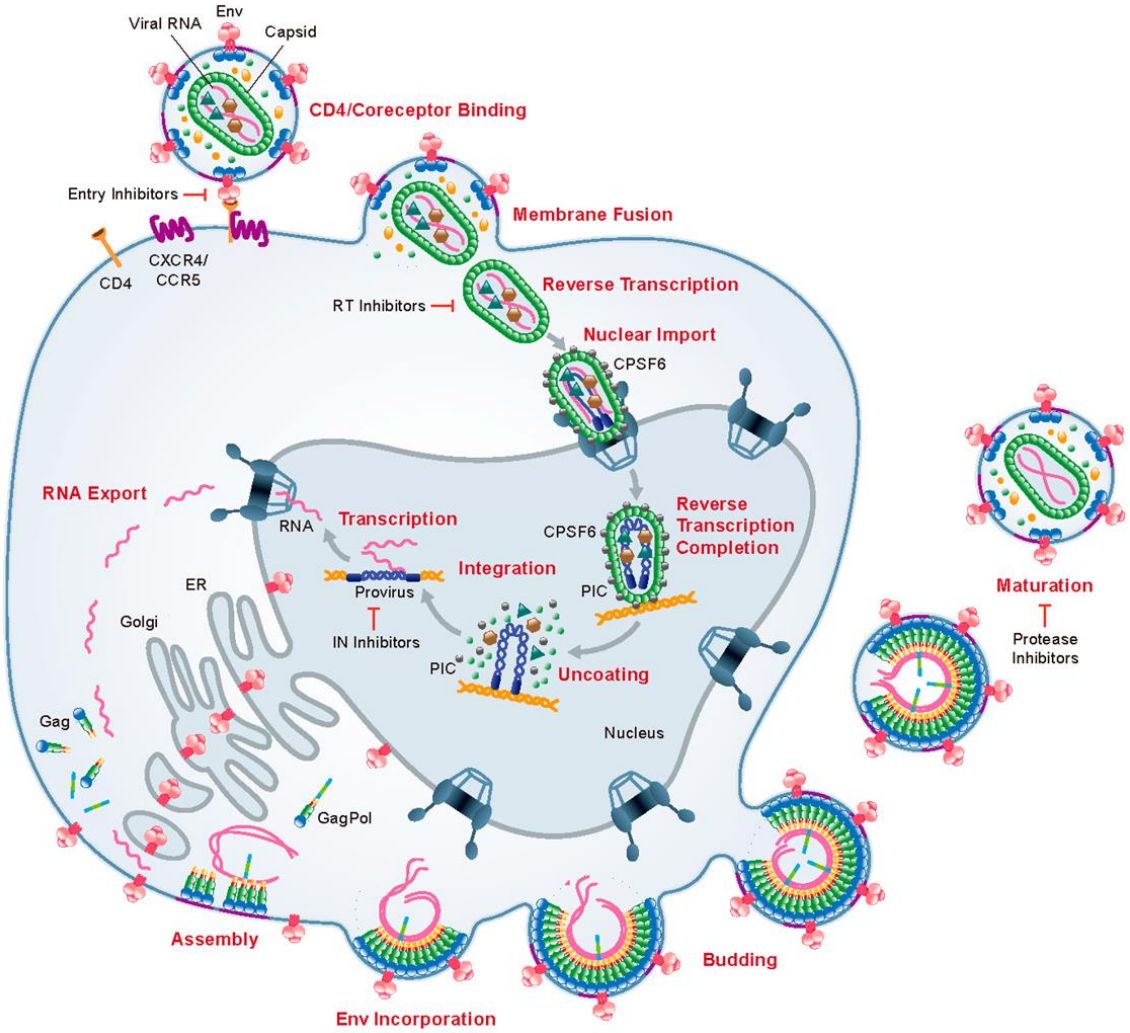


Figure 3. HIV-1 replication cycle. The life cycle of HIV consists of several steps initiated by the attachment of HIV virion to cell surface receptors of target cells. Following the fusion of the viral and cell membranes, the capsid is deposited into the cytosol. Reverse transcription of the viral RNA into viral DNA proceeds as the capsid traffics towards the nucleus, followed by uncoating of the capsid and integration of the viral DNA into the host genome. This is followed by transcription of the proviral DNA into viral mRNA. These mRNAs are eventually exported out of the nucleus into the cytoplasm, translated into viral proteins, assembled into virions before budding and maturation. Essentially, each step of the HIV life cycle are therapeutic targets for antiretroviral drugs as indicated in the figure: entry inhibitors, reverse transcription (RT) inhibitors, integrase (IN) inhibitors and protease inhibitors[15].

3. CLINICAL COURSE OF HIV-1 INFECTION

HIV-1 infection is a chronic disease with a fatal outcome resulting from AIDS-defining symptoms in treatment-naïve HIV+ individuals. HIV-1 preferentially targets CD4+T cells, but also infects other immune cells as monocytes, macrophages, and dendritic cells. The natural course of HIV-1 infection can be classified into 3 stages based on HIV-1 viremia and CD4 cell count: (i) acute phase; (ii) chronic phase and (iii) acquired immunodeficiency syndrome (AIDS) (**Figure 4**).

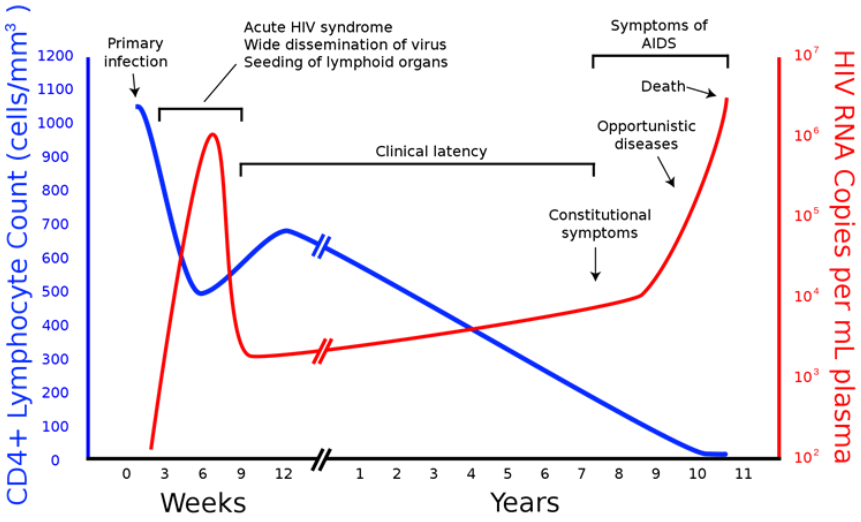


Figure 4. Typical course of HIV-1 infection. In treatment-naïve HIV-1 infected individuals, there is an accelerated loss of viremic control following a sustained low-level HIV-1 viremia and an initial uninhibited viral replication (HIV RNA copies in red). CD4 cell count (in blue) progressively declines despite repeated recovery periods over time. The time interval from infection with HIV-1 to the onset of AIDS may vary among PLWH without ART, ranging from 2 to 25 years[10]. Figure from Wikicommon, available under the Creative Commons CC0 1.0 Universal Public Domain Dedication.

3.1. Acute phase

After the initial exposure and transmission, the incoming virus first infects target cells in mucosal tissues and then undergoes a systemic spread through the lymphoid system[13]. These infected cells can either actively replicate the virus, contributing to the dissemination of the infection, or establish a latent infection, constituting the first cells of the HIV-1 latent reservoir[16]. During this period of early infection, there is a gap of 10 to 12 days when the virus cannot be detected in the plasma, the eclipse phase[17], followed by a rapid increase of viral RNA in the blood (10^5 – 10^9 copies/mL). The detection of virus in the blood (viral RNA copies) is often associated with a short symptomatic phase which might last for 10 to 14 days[18], including fever, a non-specific rash, generalized lymphadenopathy, oral or genital ulcers, myalgias and/or malaise[13]. This increase in viremia is accompanied by a decrease of the CD4+ T cells, which is maintained until the body is able to generate an immune response to partially control the viremia.

3.2. Chronic phase

The onset of the chronic phase (also called asymptomatic HIV-1 infection or clinical latency) is characterized by a decline in the level of viral load (VL) driven by both humoral and cellular immune responses, including the generation of antibodies and CD8+ cytotoxic T lymphocytes (CTL)[19,20], respectively. The VL continues decreasing until it reaches a steady-state level, the viral set point, usually within 2 months after the primary exposure to HIV-1[21]. The establishment of this viral set point is also accompanied by an increase of the CD4+ T cells, although at lower levels compared to those found prior to infection. Regarding the clinical outcome and disease progression, individuals with high viral set point tend to progress more rapidly to AIDS than individuals with lower viral set point. For example, the so-called "HIV elite controllers", which are able to control HIV-1 infection, having a non-detectable viremia for years, typically have very low viral set points[13,22].

During asymptomatic infection, HIV-1 establishes a low but persistent infection, leading to a chronic HIV-associated systemic inflammation and immune activation. As the HIV-1 infection persists, viremia can be detected even without the presence of HIV-related symptoms. The persistent infection also leads to a steady decrease of the CD4+ T cell count, weakening the immune system, which characterizes the end of the clinical latency. At this point, viral replication and chronic activation of immune cells promote the destruction of the lymphoid tissue architecture, which leads to an increased viral replication and diffusion, and the subsequent depletion of CD4+ T cells.

3.3. Acquired Immunodeficiency Syndrome (AIDS)

The last stage of HIV-1 infection is characterized by extremely low levels of CD4+ T cells in blood (<200 cells/μL) and the dramatic increase of viremia. The CD4+T cell count can reach very low levels, being the lowest count the CD4+ nadir. As a result of this loss of immune competence, the asymptomatic phase turns into a more symptomatic one that eventually, in the absence of treatment, deteriorates into full-blown AIDS[23]. During this period, the individual becomes increasingly susceptible to opportunistic infections by other viruses, bacteria, fungi and parasites, and tumours. The overall time to reach the AIDS phase in typical progressors who are not on ART is 8-10 years after HIV-1 primary infection[24].

4. PATHOGENESIS OF HIV-1 INFECTION DURING ANTIRETROVIRAL THERAPY

Recent advances in ART have changed the natural course of HIV-1 infection from a lethal illness to a somewhat manageable chronic disease[25]. Nowadays, the use of combination therapy suppresses viral load below the limit of detection (LOD) (<50 copies of viral RNA/mL) following a four-phase decay of viremia, reaching stable level of viremia below LOD, that ideally should last for an unlimited period of time[26,27] (**Figure 5**). However, treatment failure might occur due to drug resistance causing virus replication to increase again and CD4+ T cell count plummet, reminiscent of HIV-1 acute phase in treatment naïve individuals[28].

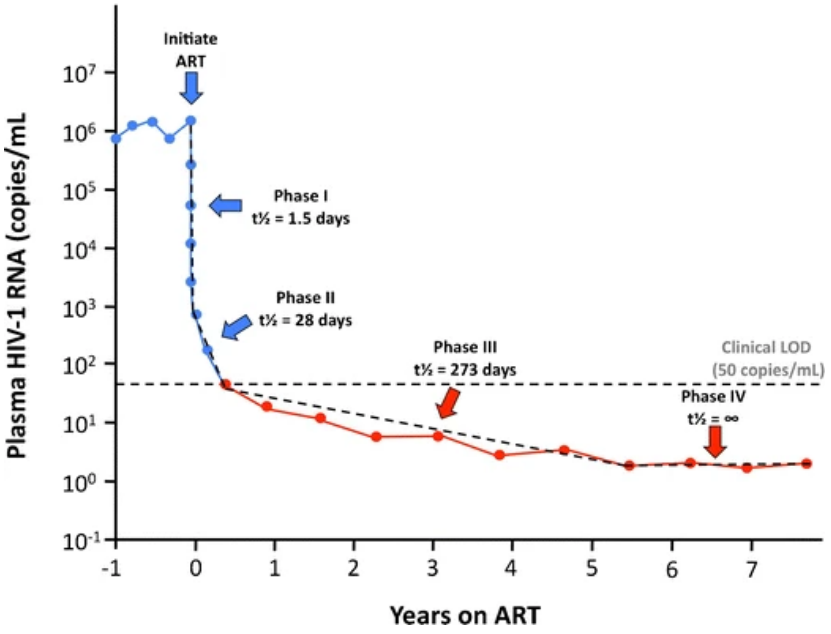


Figure 5. Decay dynamics of HIV-1 viremia during ART. The decrease in HIV-1 RNA (copies/ml) follows a four-phase decay characterized by the proportion of infected cells which can be cleared (half-lives) upon initiation of ART[26].

To prevent the emergence of acquired drug resistance, current combination therapy uses at least three antiretroviral (ARV) drugs targeting at least two different steps of the viral replication cycle, thus, the chances of a virus to evolve and become resistant to all three drugs are decreased[14,29]. The ARVs target different steps of the HIV-1 replication cycle including entry, reverse transcription, integration and protein maturation (**Figure 3**).

Current ART consists of combinations of at least three drugs from two different ARV classes that include:

- Nucleoside reverse transcriptase inhibitors (NRTIs) e.g. zidovudine (AZT) and emtricitabine;
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs) e.g. efavirenz (EFV);
- Integrase strand transfer inhibitors (INSTIs) as raltegravir (RAL) and dolutegravir;
- Protease inhibitors (PIs) e.g. atazanavir and dalunavir;
- Fusion inhibitors e.g. enfuvirtide;
- CCR5 antagonists e.g. maraviroc;
- Post-attachment inhibitors (PAIs) e.g. ibalizumab.

In addition, pharmacokinetic enhancers might be used in HIV treatment to increase the effectiveness of ARVs included in ART, and these include cobicistat and ritonavir (formerly used as a PI)[30].

ART has a proven and remarkable efficacy in preventing HIV-1 infection of new target cells, but it cannot eliminate infection once the HIV DNA is successfully integrated into the host genome. Although a majority of infected cells die quickly, a few revert to a metabolically inactive resting state in which HIV-1 gene expression is reversibly silenced[14]. The persistence of these long-lived latently infected CD4+ T cells represents a major barrier to HIV eradication, as they have long half-lives, harbour integrated HIV proviral DNA which is not transcribed and therefore are neither impacted by current ART regimens targeting viral proteins, nor restricted by host immune mechanisms which target HIV antigens[31]. Due to this incomplete decay of HIV-1 in the latent reservoir, HIV-1 viremia rebounds upon cessation of ART, independently of the time spent under treatment. Moreover, long-term viral suppression with ART is associated with toxicities and lower nadir CD4+ T cell counts due to persistent immune activation[32]. Therefore, it is imperative to develop novel strategies to achieve an effective HIV-1 cure – sustained remission of the virus after ART discontinuation.

5. THE LATENT RESERVOIR

It is not fully known as to when or how the latent reservoir is established, but increasing evidence points to the early seeding of the reservoir during acute HIV-1 infection. During long-term ART, most of the reservoir appears to be derived from cells that were infected just before treatment initiation[33–35]. These latently infected cells decay more rapidly during early ART compared with late ART[33,36,37], presumably because of the rapid clearance of a pool of relatively short-lived infected cells[38]. Following ART initiation, the significant reductions in HIV-1-associated inflammation and immune activation reduces the turnover of the reservoir, leading to generation of longer-lived cells harbouring intact viral genomes[39].

It remains unclear how the latent reservoir is established and maintained, although two major lines of evidence have been put forward. First, HIV residual replication during ART is known to occur despite ART intensification in patients, contributing to the replenishment of the latent reservoir. HIV persistence due to ongoing low-level viral replication during ART could be attributable to limited drug penetration within tissues, cell-to-cell viral transmission, and dysregulated immune activation and inflammation[40].

Second, the persistence of the reservoir may be sustained through clonal expansion of cells infected with both intact and defective proviruses. Several mechanisms might contribute to the clonal expansion of many latently infected cells during ART[39]:

(i) integration of HIV-1 DNA in or nearby genes associated with cell cycle control – actively transcribed host genes[41,42].

(ii) homeostatic proliferation in response to cytokines, such as interleukin-7 (IL-7)[43,44].

(iii) antigenic stimulation because of chronic exposure to microbial peptides, resulting in sequential episodes of reservoir expansion and contraction[45].

Proviral transcription is largely limited in latently infected cells through multiple cellular biochemical mechanisms: (1) absence of positive transcription factors to initiate proviral transcription, such as nuclear factor kappa-B (NF-κB)[46], (2) epigenetic changes to chromatin and proviral DNA, notably histone hypoacetylation by histone deacetylases (HDAC)[47], (3) presence of transcriptional repressors that prevent the processivity of the transcriptional machinery[48,49], and (4) the expression of microRNAs that hinder translation of viral RNA into protein[50]. Besides the cellular factors, HIV-1 transcription can also be modified by viral proteins like Tat, which is required for the elongation of viral transcripts (TAR) and whose inhibition can lead to viral latency in a repressive chromatin environment[51].

The best characterized reservoirs of HIV-1 infection are the latently infected resting memory CD4+ T cells. As infection of resting CD4+ T cells is significantly less efficient than activated cells, latency may primarily be established in infected activated CD4+ T cells as they transition to a resting memory state[52]. HIV-1 persists in all subsets of memory CD4+T cells, including memory stem cells T_{SCM}; central memory cells T_{CM}; transitional memory cells T_{TM} and effector memory cells T_{EM}[44,53]. Functional subsets of memory CD4+ T cells are equally important latent reservoirs particularly, T follicular helper cells, T regulatory cells, T helper 1 (Th1) cells, and Th17 cells[39]. The memory CD4+ T cell subsets display different HIV transcriptional activity, proviral inducibility, and contribution to the latent reservoir[54–57]. Specifically, HIV persistence is maintained by slow turnover of immature CD4+ T cells and clonal expansion of more differentiated cells[58]. For example, the more differentiated short-lived T_{EM} are highly enriched with intact proviral genomes in contrast to the less differentiated long-lived T_{CM} subset[59].

In addition to memory CD4+ T cells which constitute the bulk of the latent reservoir during ART, naïve CD4+ T cells can also contribute to HIV persistence[60,61]. Non-CD4+ T cells, as the cells from the monocyte-macrophage lineage which originate in the bone marrow (BM), are also of particular importance in HIV-1 persistence due to their ability to spread HIV-1 infection in immune-privileged sites, such as the central nervous system[62], genital tract[63], lungs[64] and liver[65]. Macrophages are long-lived cells, highly resistant to virally induced cytopathic effects and apoptosis and whose viral replication cycle is at least six times slower than in T cells, which makes them a long-lasting HIV-1 reservoir[66,67]. As most macrophages exist in anatomical sanctuaries with restricted penetration of ARV, HIV might persist for longer periods in these cells even during ART. Although the relevance of macrophages as replication competent latent reservoir remains largely undefined[68], macrophage-tropic virus which predate ART initiation can appear in rebounding viremia upon treatment cessation in patients[69].

Other immune cell types which might harbour intact HIV genomes are dendritic cells, brain astrocytes and microglia typically found in specific tissue compartments and can also contribute to HIV persistence during ART (reviewed in reference[70]), pointing towards substantial heterogeneity of the latent reservoir (**Figure 6**). Overall, there is a significant inter-host variability in the size and distribution of the latent reservoir[44], which complicates designing a "one-size-fits-all" curative strategy[39].

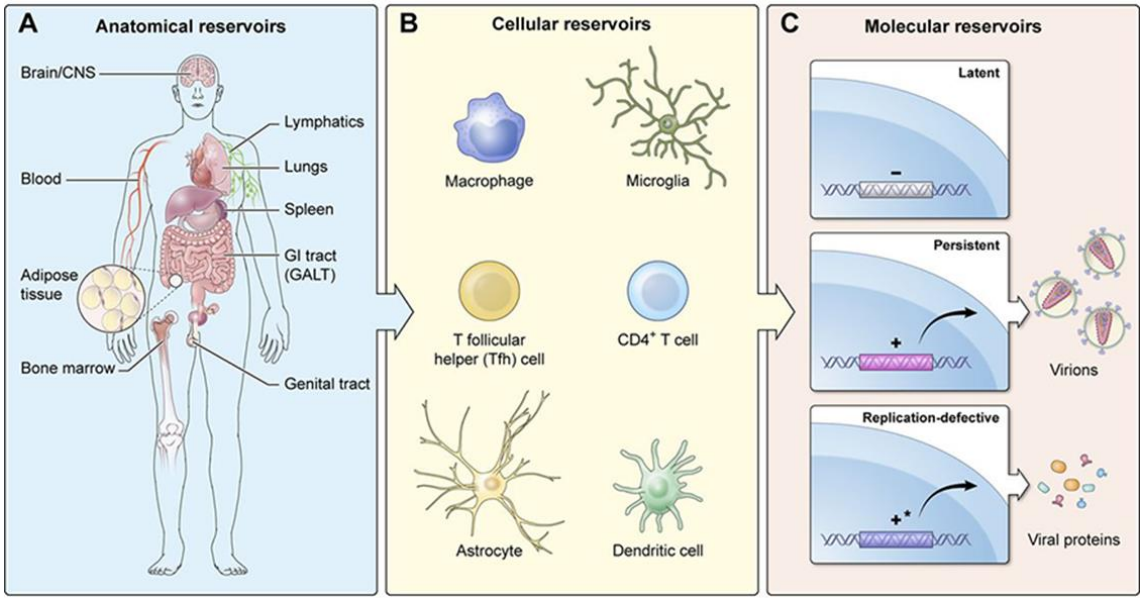


Figure 6. Landscape of HIV-1 reservoir. Latently infected cells persist in anatomical compartments (**A**) which are populated by distinct cell-types infected with HIV-1 (**B**). (**C**) The integrated provirus within infected cells may be transcriptionally silent but replication competent (latent), transcriptionally active and producing infectious virions (persistent), or transcriptionally active but replication defective due to mutations or deletions in the HIV genome[71].

5. HIV-1 CURE STRATEGIES

HIV-1 infection remains incurable due to the persistence of HIV in long-lived reservoirs that support viral rebounds upon treatment interruption. The current therapeutic option for HIV+ individuals is a life-long suppressive ART, albeit long-term side effects and development of treatment resistance hampering treatment efficacy, and cause immune dysfunction to persist indefinitely[72,73]. Hence, current efforts for achieving HIV-1 cure need to focus on either eliminating (sterilizing cure) or silencing (functional cure) the latent reservoir. In this section, we highlight several curative strategies that have been explored so far including those targeting HIV reservoirs, HIV-specific immune enhancement, and immune modulation.

5.1. Targeting HIV reservoirs

It has been 13 years since the first report of a cure for HIV following stem cell transplantation in Timothy Brown, the Berlin patient. This successful endeavour confirmed for the first time that a cure for HIV is possible, albeit not being a viable strategy for large-scale HIV cure in most PLWH. Nevertheless, it has emboldened efforts in advancing HIV cure strategies specifically, targeting latently infected cells as gene-editing technology. Further, approaches such as the so-called "shock and kill" and "block and lock" strategies focusing on either eradicating or silencing the latent reservoir, respectively, have gained considerable attention in recent years (**Figure 7**).

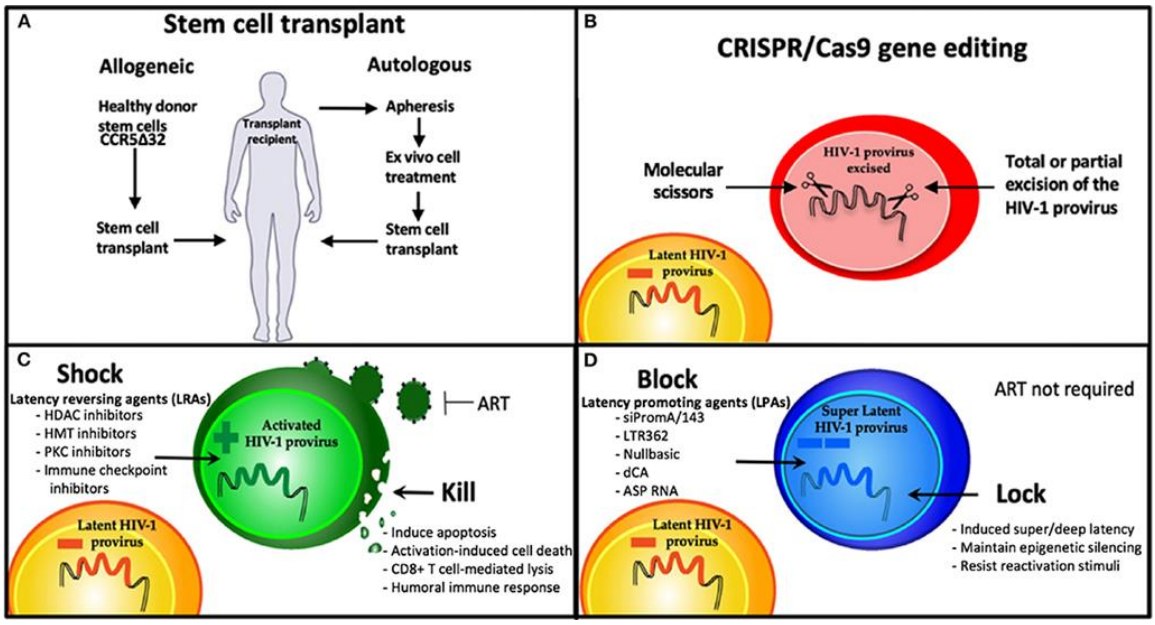


Figure 7. HIV cure strategies. (**A**) Haematopoietic stem cell transplantation and (**B**) CRISPR/cas9 gene editing approaches use modified cell therapies to target the latent reservoir and render cells resistant to HIV infection. The latent reservoir can be eradicated by the Shock and Kill strategy using LRAs (**C**) or transcriptionally silenced by the Block and Lock strategy using latency promoting agents to achieve sustained HIV remission (**D**)[74].

5.1.1 Stem cell transplant

Currently, only three people have been cured of HIV-1 infection, consistently testing negative for viral rebound without ART for at least 14 months namely, “Berlin”, “London” and “New York” patients[75–77]. A common denominator in all three cases is that all three patients received haematopoietic stem cell transplants (HSCTs) from donors homozygous for CCR5Δ32 as part of the management for haematological malignancies. Here, pretransplant conditioning significantly depletes the infected cell pool and, when replaced with donor cells having a large deletion in the CCR5 co-receptor, confers resistance to R5-tropic virus infection. Owing to difficulties in identifying potential CCR5Δ32 donors, the peculiarity of the medical condition that predetermines its use and high mortality rates preclude the use of HSCT as a feasible or routine curative strategy[50,78]. Nevertheless, it reiterates the relevance of silencing or eradicating the latent reservoir to achieve an HIV-1 cure.

5.1.2 Gene editing

An alternative approach to HSCTs is gene editing technology, such as zinc-finger nucleases and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas-9), targeting CCR5 or CXCR4 to induce HIV-1 resistance, or excising integrated provirus[79–81]. While this technology is highly versatile and could be adapted to target multiple steps in the viral replication cycle, its major limitation is its delivery, requiring enhanced delivery systems to access infected cells in

the blood and immune sanctuaries. Moreover, integration of some latent HIV proviruses in inaccessible chromatin regions poses a significant challenge for gene editing enzymes to successfully access and inactivate all latent proviruses - a challenge that needs to be overcome to achieve a sterilizing HIV cure using this strategy.

5.1.3 Shock and Kill

One of the most extensively investigated HIV cure strategies is the ¨Shock and Kill¨ strategy, which aims to reverse HIV latency by using latency-reversing agents (LRAs) that induce viral transcription and productive infection in latently infected cells, exposing these cells to immune clearance or virus-mediated cytopathic effect[82]. The earliest attempt to induce HIV-1 transcription in latent reservoirs using interleukin-2 (IL-2), produced a highly toxic "cytokine storm" and did not sufficiently reduce the size of the latent reservoir[83]. As opposed to the non-specific and broad-acting immune-activating cytokines, current research into "shock and kill" primarily focuses on developing or identifying small-molecule compounds that target HIV proviruses with considerable specificity but limited immune activation. High-throughput screening (HTS) of compound libraries, specifically approved drugs, has emerged as a gold standard for identifying compounds that exhibit HIV latency-reversing activity[84–86]. Several compounds acting as LRAs have been identified in this way, and their mechanisms of action further characterized using *in vitro* and *in vivo* models of HIV latency. Indeed, the LRAs identified to date encompasses a diverse group of compounds targeting different host-dependent mechanisms, including extracellular and cytosolic stimulators, histone post-translation and non-histone chromatin modulators, and undefined modulators (**Figure 8**)[87].

One of the most extensively studied LRAs are the protein kinase C (PKC) agonists, prostratin and phorbol 12-myristate 13-acetate (PMA), which induce HIV-1 gene expression by activating cellular transcription factors such as the NF-κB[88]. PKC agonists, notably PMA, are considered one of the most robust LRAs, but their capacity for inducing potentially life-threatening systemic inflammatory responses precludes their clinical use. Another well-known class of LRAs are the HDAC inhibitors (HDACi), including vorinostat and panobinostat, which induce HIV-1 gene expression by reversing epigenetic silencing[89]. Likewise, the Toll-like receptor (TLR) agonists, which stimulate immune signalling pathways leading to HIV-1 expression are currently being explored as "shock and kill" agents in ongoing clinical trials [90,91].

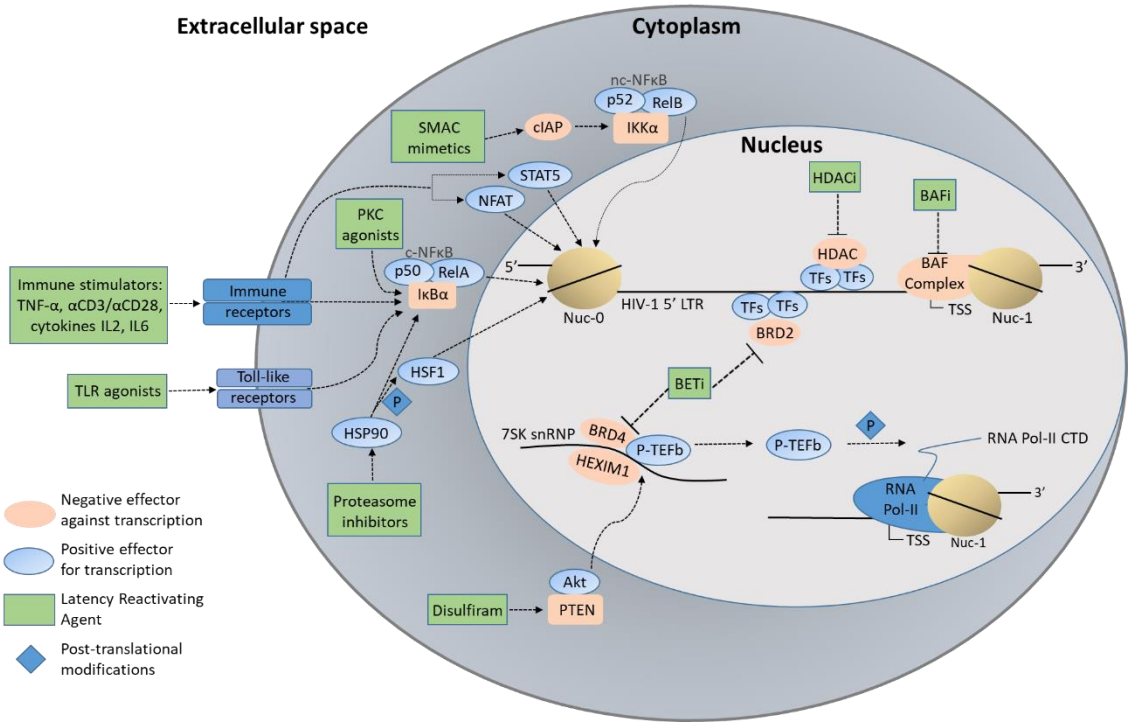


Figure 8. Cellular targets of latency revering agents (LRAs) used in ¨shock and kill¨ HIV cure strategy. Several LRAs reverse HIV-1 latency by targeting immune signalling pathways leading to the cytosolic activation and nuclear translocation of transcription factors NF-κB, STAT5 and NFAT. LRAs such as the PKC and TLR agonists, TNFα and cytokines signal through the canonical NFκB (c-NFκB), whereas the SMAC mimetics signal through the ubiquitin-dependent non-canonical NFκB (nc-NFκB). Proteasome inhibitors induce accumulation of HSP90 in the cytosol, which in turn activates other transcription factors. Inside the nucleus, histone modifications with the HDACi, BAF complex inhibition allowing release of Nuc-1, and BRD2 and BRD4 displacement by the BETi, all promote transcription elongation leading to HIV reactivation. Disulfiram reduces PTEN protein levels, allowing activated Akt kinase to localize to the nucleus, where it participates in releasing the positive elongation factor B (P-TEFb) from the repressive 7SK snRNP/HEXIM1 complex, also seen with the BETi, enabling the positive regulation of P-TEFb on RNA Pol-II-dependent elongation. Figure modified from[87].

Akt, protein kinase B; BAF, BRG1-or HBRM-associated factors; BETi, Bromodomain and extraterminal domain (BRD) inhibitor; CTD, C-terminal domain; HEXIM1, Hexamethylenebisacetamide inducible 1; HSF1, Heat shock factor 1; HSP90, Heat shock protein-90; NFAT, Nuclear factor of activated T-cells; P, phosphorylation; Pol-II, Polymerase II; PTEN, Phosphatase and tensin homolog; SMAC, Second mitochondrial-derived activator of caspases; STAT5, Signal transducer and activator of transcription 5; 7SK snRNP, 7SK small nuclear ribonucleoprotein complex; TSS, transcription start site.

Unfortunately, all LRAs studied in clinical studies that activated viral gene expression *in vivo* have failed to reduce the latent reservoir[89,92]. Therefore, compounds that selectively sensitize latently infected cells to immune-mediated clearance as the RIG-I inducer acitretin, could tip the balance towards depletion of the latent reservoir[93–95]. Besides low efficacy in the clinic, achieving broad and efficient latency reversal without eliciting toxic side effects or global immune activation remain elusive in current LRA research endeavours. Considering the limited capacity of LRAs in depleting the latent reservoir, combining these strategies with vaccination may have a synergistic effect in purging the latent reservoir. For example, the elimination of HIV-infected cells by CTLs can be impaired by certain LRAs such as the HDACi[96], and stimulating HIV-1 specific CTL response might remedy this undesired effect[97]. Recently, the combination of the TLR7 agonist vesatolimod and HIV-1 bNAbs in simian-HIV (SHIV)-infected non-human primate model delayed or prevented viral rebound following ART discontinuation[98,99], pointing towards its potential as a possible strategy for eradicating the viral reservoir in PLWH.

5.1.4 Block and Lock

Rather than activating latency, a feasible but far less explored curative approach than "shock and kill" is to reinforce latency by silencing HIV transcription permanently, termed "block and lock" (**Figure 9**). The rationale of this approach is that the induction of epigenetic or transcriptional silencing in the HIV-1 promoter over time will promote a state of "deep latency", blocking or limiting viral rebound upon treatment cessation[100]. This strategy utilizes latency promoting agents (LPA) to perpetually prevent the production of viral RNA and proteins and differs from conventional ART, which focuses on repressing the active viral cycle. Therapeutic interventions that have been explored under this novel curative strategy include targeted inhibition of the HIV-1 positive regulator, Tat by the small molecule inhibitor didehydro-Cortistatin A (dCA)[101]. As an LPA, dCA directly interferes with viral Tat-TAR-RNA interaction, blocking transcriptional elongation and hence viral production from latently infected cells. Importantly, inactivation of viral transcription was maintained upon dCA treatment following αCD3/αCD28 or prostratin stimulation of latently infected CD4+ T cells from ART-suppressed HIV-infected subjects.

Alternatively, rather than targeting the viral protein Tat, other approaches involve manipulating host-dependent pathways responsible for the maintenance of HIV latent reservoirs. Inhibitors of the mammalian target of rapamycin (mTOR) signalling pathway effectively induce Tat degradation through autophagy, in addition to suppressing PMA-mediated viral reactivation in both *in vitro* and *ex vivo* models of HIV-1 latency[102,103]. Similar to mTOR inhibitors, blockade of the HSP90-NF-κB-axis with HSP90 inhibitors is known to repress the HIV-1 latency reactivation

capacity of NF-κB stimulators *in vitro*[104]. Recent studies have also shown that targeting the latent reservoir through inhibiting the JAK-STAT signalling pathway, besides blocking pro-inflammatory signalling, reduced latency reactivation and productive infection[105,106]. However, there are fears that immunosuppressive therapies might be counterproductive in already immunocompromised patients, and closely associated with an increased susceptibility to other infections[107]. Ultimately, these therapies will require careful evaluation to establish their safety before clinical use.

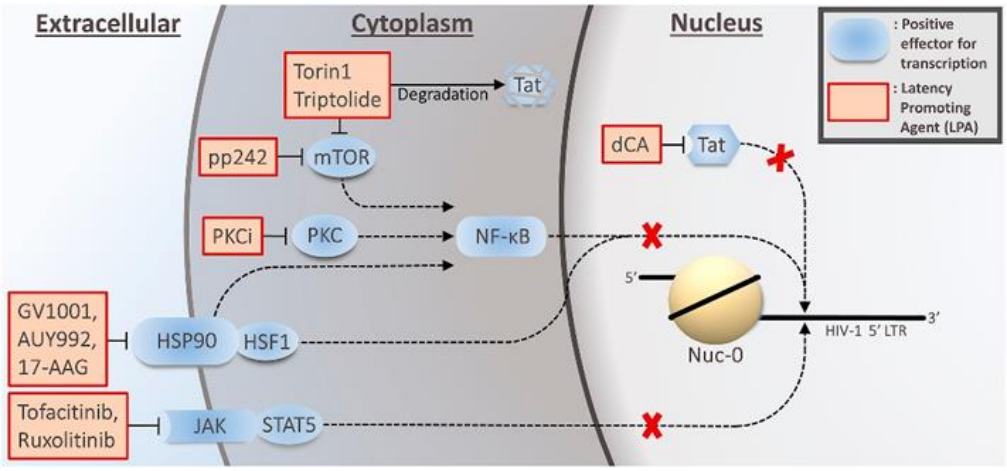


Figure 9. Cellular targets of latency promoting agents (LPAs) for ¨block and lock¨ strategy. LPAs primarily to limit viral transcription to achieve a state of deeper latency in latently infected cells that might allow for safe withdrawal of ART. Inhibitors of mTOR and PKC negatively impact on NF-κB-mediated transcriptional activity. The mTOR inhibitors Torin1 and Triptolide can further inhibit HIV transcription by directly targeting Tat for degradation. In the nucleus, dCA interacts directly with Tat, supressing its interaction with HIV TAR-RNA. Inhibition of HSP90 also restricts the transcription factors NF-κB and HSF1, while JAK-STAT signalling inhibition by the JAK inhibitors Tofacitinib or Ruxolitinib limits STAT5-dependent transcription[87].

Another method to induce a state of "deep latency" is RNA-directed epigenetic silencing using small interfering RNA (siRNA) as the siProm-A that targets the unique tandem NF-κB sites in the HIV-1 promoter[108]. As described above, the CRISPR/Cas9 system has also been exploited to disrupt HIV-1 genes or excise integrated proviruses. Although these new approaches have shown promising results *in vitro* and in animal models, they are yet to gain traction in the clinic compared to small molecule LPAs amid concerns regarding their safety, efficacy, specificity, and ethical considerations.

5.2. HIV-specific immune enhancement

The "shock and kill" as an eradication strategy against the latent reservoir is premised on the capacity of induced latency reactivation to facilitate HIV-mediated cytolysis or host immune clearance to purge the latent reservoir. Current studies utilizing LRAs as part of this strategy have shown promising results in reversing HIV-1 latency *in vivo* but have failed to reduce the latent reservoir[89,92]. This impaired capacity in mounting a "kill" response by LRAs may be due, in part, to the loss of adequate HIV-1 specific CTL responses in long-term ART-suppressed individuals, suggesting that natural CTLs seem incapable of clearing the latent reservoir despite reactivating antigen expression[109]. Therefore, HIV-specific immune enhancement using other complementary approaches, such as T-cell vaccines and bNAbs, may be needed in combination with the LRAs to induce selective killing of latently infected cells, a concept that is currently under investigation.

5.2.1 T-cell vaccines

Robust antiviral T-cell responses, especially those mediated by CTLs, play a vital role in controlling HIV infection, a common feature found in elite controllers[110,111]. Similarly, therapeutic vaccination aims to eliminate or significantly reduce viremia by inducing or enhancing the immune response against HIV-1 infection. Theoretically, these vaccines are expected to provide durable control of viremia, allowing for the safe discontinuation of ART. Common approaches in therapeutic vaccine development include inactivated envelope-depleted virus; viral vectors such as modified vaccinia Ankara (MVA), adenovirus and vesicular stomatitis virus (VSV); lentiviral vectors and dendritic cells as vaccine vehicles and delivery of viral DNA and RNA[111]. So far, there has been limited success in reducing viral loads with therapeutic vaccinations, as none has been able to induce long-term remission in patients without ART[112].

5.2.2 Broadly neutralising antibodies

Broadly neutralizing antibodies (bNAbs) targeting the HIV-1 envelope glycoprotein are also being assessed in clinical trials as a therapeutic strategy. These bNAbs are capable of neutralizing free viruses and can clear cells expressing viral antigen on their surfaces[113], although there are concerns about HIV-1 resistance to single bNAbs. Hence, antibodies with multiple specificities exhibiting great breadth and potency might be required either alone or in combination with other cure strategies[50]. Data from human HIV vaccine trials conducted to date, suggests that detectable bNAb responses are difficult to induce by vaccination (reviewed in[114]). Following the success of COVID-19 mRNA vaccines, a clinical trial exploring mRNA-based HIV vaccine is

currently underway (NCT05217641). The Moderna vaccine candidate uses prime and boost antigens to induce specific B-cell responses that ideally result in the development of HIV-specific bNAbs[115].

5.3. Immune modulation

HIV persistence promotes a sustained inflammatory environment resulting in the accumulation of “activated/exhausted" T cell phenotypes with diminished effector function. Immune exhaustion is actively controlled by the inhibitory immune checkpoint proteins, programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte associated protein (CTLA-4) which are highly expressed in HIV infected T cells. Several studies have demonstrated that selective blockade of these immune checkpoints can reinvigorate antigen-specific CTL function, induce latency reversal, and deplete the latent reservoir[116–118]. Anti-inflammatory agents are also gaining renewed interest as a strategy to tackle HIV persistence. For example, blockade of IFN-α/β receptor (IFNAR) in HIV-1-infected hu-mice using a monoclonal antibody induced an enhanced T cell recovery and reduced HIV-1 reservoirs. Likewise, inhibitors targeting the Janus kinases have shown promising results as potent anti-HIV and anti-inflammatory agents[119,120].

Overall, lessons learnt from elite and post-treatment controllers point to a more plausible strategy: reduce the reservoir to manageable levels and concurrently enhance immune control. Moreover, given the complexities of the mechanisms governing HIV latency, it is more likely that combination approaches will be required to achieve an HIV cure.

6. IMMUNE RESPONSES IN HIV-1 INFECTION

Similar to other viral infections, HIV-1 infection results in the initial activation of innate immunity which is non-specific, followed by the development of more specific and long-lasting adaptive immune responses (**Figure 10**). Given that the activation of robust innate immune induction positively correlates with stronger adaptive immune responses, the cross-talk between innate and adaptive immune responses is crucial in determining the outcome of HIV-1 infection and immune control[121,122]. Thus, modulating antiviral innate immune response is gaining prominence as a putative cellular target for developing novel pan-viral therapeutic strategies[107] and also as a relevant asset in HIV cure strategies. Thus, in this PhD thesis, we will focus on interrogating further the basis of innate immune modulation as a strategy for tackling viral infections and possibly achieving HIV cure.

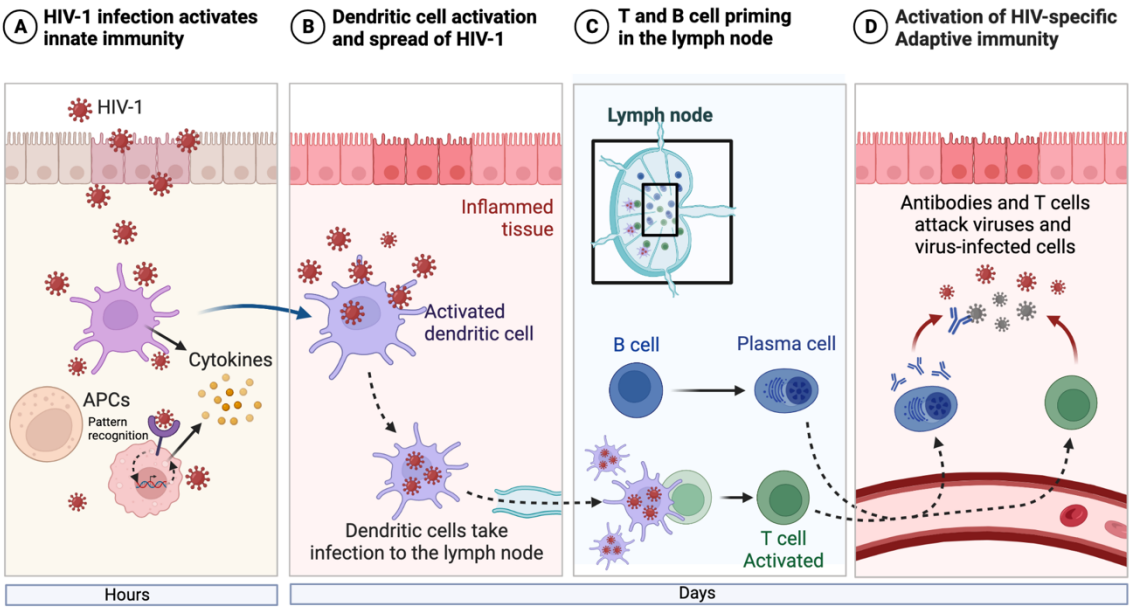


Figure 10. Innate immunity and adaptive immunity during HIV-1 infection. Sensing of HIV-1 infection by pathogen recognition receptors on antigen presenting cells (APCs) triggers the induction of innate immunity (**A**), resulting in the activation of dendritic cells and dissemination of HIV-1 through the lymphatic system (**B**). T and B cell priming in the lymph nodes (**C**), generate HIV-specific antibodies and activated T cells to attack infected cells (**D**). Image created with Biorender.

6.1. Innate immune response against viral infections

Innate immunity acts as our first line of defence for the detection and clearance of viral infections. Immediately after infection, all viruses trigger an antiviral response that relies on elements of innate immunity, such as physical barriers and the production of interferons (IFN) and groups of cytokines, a process orchestrated by innate immune cells—in particular, monocytes/macrophages, dendritic cells (DC), and natural killer (NK) cells[123,124]. Upon viral entry into target cells, pattern-recognition receptors (PRR) recognize the viral components and prompt IFN production. Activated PRRs signal through the effector proteins mitochondrial antiviral-signalling protein (MAVS), TNF receptor-associated factor (TRAF), serine/threonine-protein kinase (TBK1), and inhibitor of NF-kB (IκB) kinase complexes (IKK -γ, -α, -β). MAVS is found in the outer membrane of mitochondria, peroxisomes or endoplasmic reticulum and is triggered after the binding of cytosolic proteins, which activate TRAF proteins[125]. TRAF proteins are present in the cytoplasm and act as signalling adaptor proteins and E3 ubiquitin ligase to activate downstream signalling events. TRAF recruits TBK1 and IKK complexes, triggering the expression and phosphorylation of the transcription factors, NF-κB and interferon regulatory factors (IRFs) in the nucleus that in turn induce the expression of IFNs.

The secreted IFNs bind to their respective receptors and activate the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway[126], resulting in the production of hundreds of downstream antiviral IFN-stimulated genes (ISGs) and the secretion of proinflammatory cytokines (**Figure 11**). This process establishes an antiviral state that inhibits viral replication, stimulates the adaptive immune response, and recruits other immune cells to the site of infection[127,128]. However, secreted proinflammatory cytokines can cause local and systemic inflammation[123,124], resulting in the overactivation of innate immunity. Such overactivation may induce the robust, hyperproduction, and excessive secretion of IFNs, proinflammatory and anti-inflammatory cytokines, and chemokines, leading to cytokine storms in acute HIV-1 infection without therapy[129]. Indeed, in current COVID-19 pandemic, the cytokine storm is an important factor leading to the death of many patients[130,131].

The molecular basis of antiviral innate immune signalling is complex, multi-waved, interconnected, and may not always be antiviral. Understanding the complex mechanisms underlying the interactions between viruses and the host innate immune system is key to help develop rational treatment strategies against viral infections.

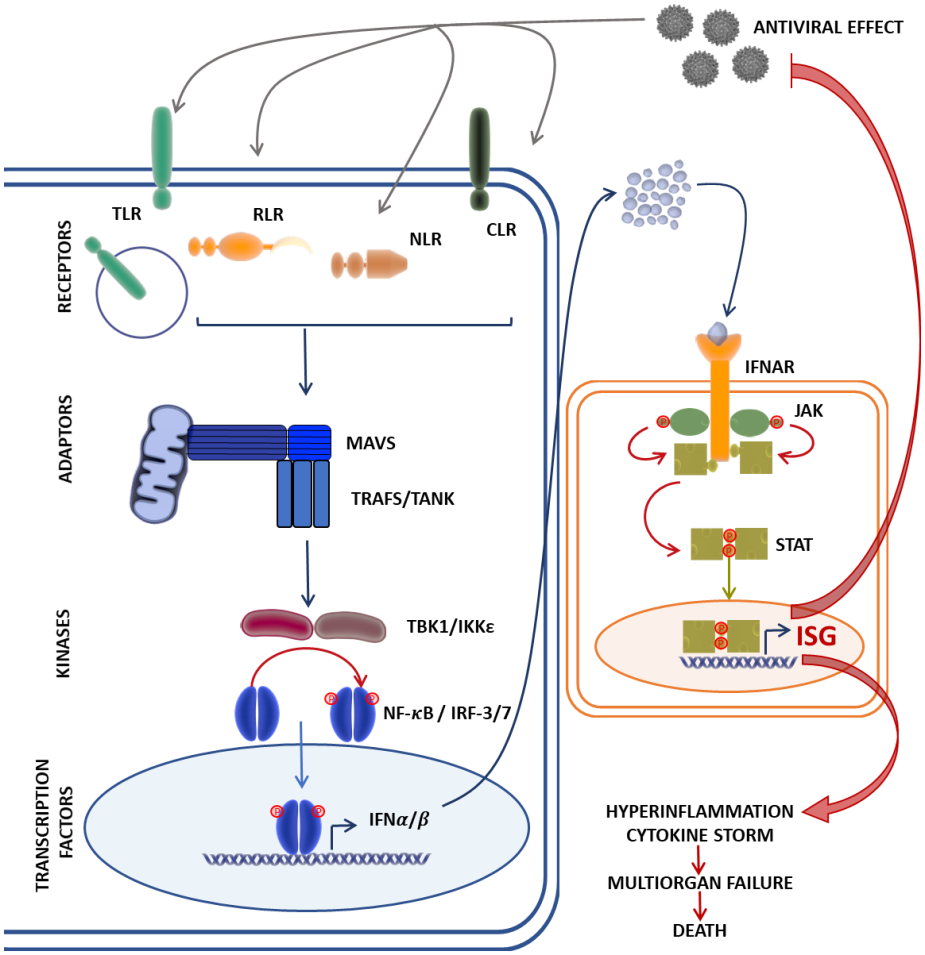


Figure 11. The Janus kinase–signal transducer and activator of transcription (JAK-STAT) signalling pathway mediates the innate immune response against viral infection. Cytosolic viral RNA recognition by pathogen recognition receptors such as the Toll-like receptors (TLR), RIG-I-like receptors (RLR), NOD-like receptors (NLR), and the C-type lectin receptors (CLR) induce interactions with the mitochondrial antiviral signalling protein (MAVS) and its co-adaptor molecules, TRAFs, which, acting through the TBK1/IKKε axis, activate nuclear factor-κB (NF-κB) and interferon regulatory factor (IRF-3/IRF-7) expression of type I interferons (IFNα/β). Type I IFN recognition and binding to IFNα receptors (IFNAR) activates JAKs, leading to the phosphorylation and translocation of STATs to the nucleus, resulting in the target gene expression of antiviral ISGs, and proinflammatory cytokines by transcriptional factors NF-κB and IRFs. A dysregulated immune response in rare cases might result in fatal outcomes due to hyperinflammation and the cytokine storm.
ISG, interferon-stimulated gene; RIG-I, retinoic acid-inducible gene I; TRAF, tumor necrosis factor receptor-related factor; TBK1, TANK-binding kinase 1; IKKε, IκB kinase ε.

6.2. TBK1/ IKKε-mediated antiviral immunity

The TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) is vital for the induction of antiviral innate immune responses against both RNA and DNA viral infection. TBK1 is a serine/threonine-protein kinase that is crucial for the activation of NF-κB and IRF signalling pathways and type I IFN production. TBK1 is regarded as a non-canonical IKK-related kinase which mediates NF-κB and IRF activation by phosphorylating the canonical IKKs (IKKα/β). TBK1 and IKK complexes are highly homologous, specially TBK1 and IKKε, the adaptor proteins that induce IκB degradation and activate the transcription factors[132,133]. Despite sharing similar homology, TBK1 unlike IKKε is indispensable in antiviral innate immunity and is ubiquitously expressed, whereas IKKε expression is limited to specific tissue compartments.

As a key kinase involved in antiviral immunity, TBK1 activity is tightly regulated via several mechanisms, such as phosphorylation, ubiquitination, and modulation of kinase activity and functional TBK1-containing complexes formation (reviewed in[134]). Therefore, several viruses have developed elaborate immune evasion strategies specifically targeting modulation of TBK1 activity to circumvent IFN responses and facilitate viral replication. For example, HIV-1 accessory proteins Vpr and Vif can directly bind to TBK1 resulting in abortive TBK1 autophosphorylation and blockade of type I and III IFN induction in DCs and macrophages[135]. Also, HIV-1 protease mediates cleavage of TBK1, blocks IRF3 phosphorylation and suppresses IFN production, although a protease inhibitor counteracted these effects *in vitro*. Conversely, other viruses, such as the human T-cell leukaemia virus type 1 (HTLV-1), may interact with TBK1/IKKε complex without circumventing IFN response since they might encode proteins recognized by the host immune system to induce antiviral IFN response. In this case, the HTLV-1 encoded Tax-1 protein may positively regulate IFN-β activation by enabling the interaction between TRAF3 and TBK1/IKKε complex and promoting IRF factors phosphorylation[136]. Thus, pointing towards the relevance of TBK1 modulation as an interesting therapeutic target against viral infections.

Development of therapeutic agents targeting TBK1 and IKK complexes is currently underway, with none approved yet for clinical use. Nevertheless, small-molecule inhibitors of TBK1 and IKK complexes have found increasing utility as a crucial tool in virus research to promote replication of IFN-sensitive viruses in cell cultures[137,138], and as an adjuvant for live-attenuated vaccines[139]. Moreover, selective disruption of TBK1/IKKε signalling complexes has shown promising prospects as a novel therapeutic intervention to counter SARS-CoV-2 hyper-inflammation in an animal model [140], suggesting a protective potential of related therapies against severe COVID-19 and other viral-induced interferonopathies.

6.3. The JAK-STAT signalling pathway in viral infections

The JAK-STAT signalling pathway is a transport hub for cytokine secretion and is used by many proinflammatory molecules to mediate the downstream effects and activate gene transcription. The JAK-STAT signalling pathway is mainly composed of three members: tyrosine kinase-related receptors, JAKs, and STATs [141]. Tyrosine kinase-related receptors are transmembrane cytokine receptors that are divided into class I and class II cytokine receptors depending on the specific cytokine families they recognize (the IL-2, IL-3, IL-6, and IL-12/IL-23 cytokine family for type I and IL-10 and the interferon family for type II, respectively)[142]. When cytokines bind to specific receptors, the molecular conformation of JAKs (including JAK1, JAK2, JAK3, and Tyk2) located in the cytoplasm changes, triggering its autophosphorylation or transphosphorylation [143]. Phospho-JAKs result in secondary phosphorylation of the receptors and subsequent docking and phosphorylation of STATs (including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6). STAT phosphorylation is a key step in JAK-STAT signalling and the downstream induction of antiviral ISGs. Phospho-STATs form homodimers or heterodimers that enter the nucleus, bind to DNA, and participate in the regulation of gene transcription. The heterodimer STAT1–STAT2 binds to a third partner, IFN-regulatory factor 9 (IRF9), forming the ISGF3 complex, which, once in the nucleus, binds to specific regulatory sequences, the IFN-stimulated response elements (ISRE), to activate the expression of many ISGs. ISGF3 induces most, if not all, of the ISGs encoding effector molecules of cell-intrinsic antiviral defences such as OAS or MX1. Alternative JAK-STAT pathways include the formation of STAT1 or STAT4 homodimers, which may drive different functional responses to IFN-I[141]. The diversity of these complexes may, in part, explain the broad effects and cell-type specificity of IFN-I-mediated signalling, as it allows the transcription of a wide range of innate and adaptive immune-related genes dedicated to providing protection against viral infection.

Overall, the induction of the JAK-STAT signalling pathway by IFNs leads to the upregulation of hundreds of ISGs, many of which have the ability to rapidly kill viruses within infected cells[127,128]. Since the induction of the antiviral response by IFN is a major threat to virus survival and the JAK-STAT pathway represents a common point governed by a limited group of highly similar proteins, viruses have evolved a myriad of mechanisms to target JAK-STAT signalling in an attempt to counteract host innate immunity (**Figure 12**).

Noteworthy, although it has become increasingly clear that the modulation of the JAK-STAT pathway is critical for those viruses that establish chronic or persistent infections, many viruses that cause acute infections also target the JAK-STAT pathway[144]. In the case of HIV-1, accessory

proteins Vpu and Nef block STAT1 phosphorylation following IFN-α stimulation, potentially enabling more effective replication in an IFN-α-rich environment[145]. Moreover, ubiquitination and proteasomal degradation and/or the mislocalization of essential components of the IFN-JAK-STAT pathway is another common strategy shared by several viruses to evade the induction of antiviral ISGs[146,147]. In the case of HIV, the accessory proteins Vif, Vpu, and Vpr are known substrate adaptors for the recruitment of ubiquitin ligase adaptors to cellular target proteins for ubiquitination and proteasomal degradation[148]. In this regard, HIV-1 Vif interferes with effective IFN-α signaling via degradation of the essential constituents of the JAK-STAT pathway, STAT1 and STAT3[149].

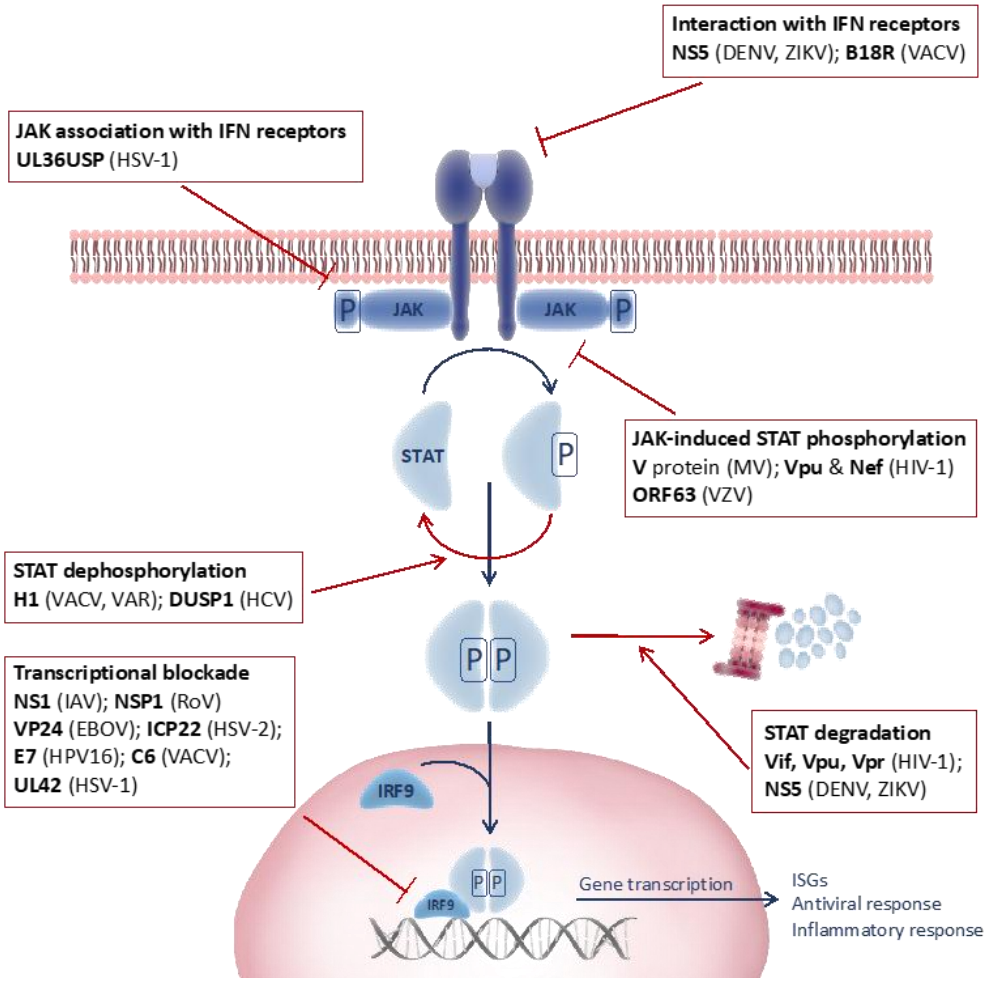


Figure 12. Virus-induced modulation of the Janus kinase–signal transducer and transcriptional activator (JAK-STAT) signalling pathway. Viruses encode several factors that target specific steps in the JAK-STAT signalling pathway through diverse evasion mechanisms to subvert the host immune response. Viral proteins are highlighted in bold and the viruses in parentheses. EBOV, Ebola virus; DENV, dengue virus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; HPV16, human papillomavirus type 16; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; IAV, Influenza A virus; MV, measles virus; RoV, rotavirus; VACV, vaccinia virus; VAR, variola "smallpox" virus; VZV, varicella-zoster virus; ZIKV, Zika virus.

In summary, the extraordinary diversity of strategies that viruses have evolved to interfere with JAK-STAT signaling stress the relevance of this pathway as a putative antiviral target for the design of new antiviral drugs, alternative therapeutic strategies, or as adjuvants for live attenuated vaccines.

6.4. Therapeutic strategies targeting JAK-STAT signaling pathway as modulators of viral infections

Although activation of the JAK-STAT pathway primarily promotes the upregulation of immune-related genes against infections, dysregulated immunity and interferonopathies could lead to several immune disorders, ranging from autoimmunity or allergic diseases to autoinflammatory diseases and cancer[142]. Therefore, soon after their discovery, JAKs were quickly identified as relevant therapeutic targets. The first JAK inhibitors (JAKi) were approved about a decade ago, and now, there are eight JAKi in use for the treatment of rheumatologic, dermatologic, hematologic, and gastrointestinal indications, along with an emergency authorization for COVID-19, the latter being the first JAKi used for the treatment of a viral infection[150].

JAKi are generally safe and effective. The group of inhibitory molecules were initially developed to exert their effects by blocking the ATP-binding pocket of the JAK catalytic domain. Although these compounds are relatively selective, with limited off-target effects compared to other kinases, these JAKi block the activity of multiple JAKs both *in vitro* and *in vivo* (reviewed in Reference[151]). Thus, several more selective JAKi, along with agents that target kinase families beyond JAKs, are being developed, opening the door to more specific treatments that might significantly impact the treatment of viral infections.

As described above, JAK-STAT modulation by a plethora of distinct viruses underlines the importance of this pathway as a putative antiviral strategy. In this regard, multiple associations between JAK inhibitors and viruses have been described (**Table 1**).

Table 1. Reported effects of JAKi on viral infections

JAK Inhibitor		Target	Modulation of Viral Infection		Indication/s *	Ref
			Negative/Antiviral	Positive/Proviral		
Approved	Ruxolitinib	JAK2/1	HIV, SARS-CoV-2	VZV, HCMV, HBV, EBV, HHV-6, BKV, HSV, HPyV2	Myelofibrosis Polycythaemia vera Graft-versus-host disease Atopic dermatitis	105,106,159,119,152–158
	Tofacitinib	JAK3/2	HIV, SARS-CoV-2	VZV, HCMV, BKV, HBV	Rheumatoid arthritis Ulcerative colitis	119,160–164
	Baricitinib	JAK1/2/Tyk2	HIV, SARS-CoV-2	VZV, HCMV, HBV, EBV, HEV	Rheumatoid arthritis Atopic dermatitis COVID-19 #	150,159,164–171
	Fedratinib	JAK2/FLT3/RET/BRD4	SARS-CoV-2	-	Myelofibrosis	159,172,173
	Filgotinib	JAK1/2/Tyk2	HIV	VZV	Rheumatoid arthritis	164,174
	Upadacitinib	JAK1/2	-	VZV	Rheumatoid and psoriatic arthritis Ankylosing spondylitis Atopic dermatitis	163
	Peficitinib	panJAK	-	VZV	Rheumatoid arthritis	163
	Delgocitinib	panJAK	-	-	Atopic dermatitis	
Phase III	Pacritinib	JAK2/FLT3/Tyk2	SARS-CoV-2	-	Myelofibrosis	170,175
	Lestaurtinib	JAK2/FLT3/TrkA	SARS-CoV-2	-	Acute lymphoblastic leukemia	176
	Decernotinib	JAK3	-	VZV	Rheumatoid arthritis	163
	Jaktinib	JAK1/2	SARS-CoV-2	-	Myelofibrosis Alopecia areata	177

HIV, human immunodeficiency virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; HSV, herpes simplex virus; VZV, varicella zoster virus; HCMV, human cytomegalovirus; HBV, hepatitis B virus; EBV, Epstein–Barr virus; HHV-6, human herpesvirus 6; BKV, BK virus/polyomavirus hominis 1; HPyV2, human polyomavirus 2; HEV, hepatitis E virus. * According to FDA authorization or the latest ongoing clinical trials. # Emergency use authorization.

Recently, the FDA-approved compounds ruxolitinib, tofacitinib, baricitinib, and filgotinib have been proposed as antiviral agents against human immunodeficiency virus (HIV) independently of their original clinical indications[105,106,119]. Using high-throughput drug screening, ruxolitinib and filgotinib were identified as putative inhibitors of HIV-1 transcription through the blockade of splicing, reducing the proliferation of HIV-1-infected cells and blocking HIV-1 latency reactivation through the suppression of T-cell activation pathways[174]. Similar effects on T-cell activation were proposed for ruxolitinib and baricitinib in a HIV latency model[105], along with the induction of proapoptotic protein BCL-2, therefore suggesting the ability of these JAKi to block HIV reactivation and reduce the latent reservoir. Moreover, other authors have also claimed that ruxolitinib and tofacitinib exert their anti-HIV activity by inhibiting both HIV-1 replication and HIV-1 latency reversal[106,119]. The suggested mechanisms for these observations include the inhibition of IL-6 and TNFα production, which, in turn, blocks viral gene transcription and HIV-1 replication, the blockade of STAT phosphorylation and the subsequent binding to HIV LTR, inhibiting viral gene transcription and the inhibition of T-cell activation and proliferation.

Given the implication of the JAK-STAT pathway in the production of proinflammatory cytokines, the repurposing of several JAK inhibitors has been proposed to ameliorate COVID-19 symptomatology (reviewed elsewhere in References[178–180]). Among them, baricitinib was authorized for emergency use to treat hospitalized COVID-19 patients due to its capacity to block the JAK-STAT signalling pathway and the subsequent overproduction of cytokines in severe patients but also affecting viral endocytosis[167]. A similar effect has been proposed for ruxolitinib and fedratinib[159]. In addition to these findings, JAK inhibitor pacritinib has also been proposed as a putative inhibitor of ACE2 and TMPRSS2 in an *in silico* study[170]. The hypothetical role of JAK inhibitors as both modulators of the cytokine storm and inhibitors of SARS-CoV-2 entry has prompted their study in clinical trials with COVID-19 patients. In fact, clinical trials have already started for JAK inhibitors tofacitinib (NCT04469114)[160], nezulcitinib (NCT04402866)[181], jaktinib (ChiCTR2000030170)[177], and baricitinib (NCT04421027). Clinical trials for JAK inhibitors ruxolitinib (NCT04362137) and pacritinib (NCT04404361) have also been reported; however, both have been terminated, as no clinical benefit was observed in comparison to the standard of care[175,182].

In contrast to the proposed antiviral role, treatment with JAK inhibitors for immune-mediated inflammatory diseases, such as rheumatoid arthritis, has also been associated with an increased risk of infections, including those of viral origin, due to their immuno-suppressive effects[153–157,161–163,165,166]. In this regard, JAK inhibition have been implicated in the reactivation of chronic-latent infections of distinct origin including incidence of herpes zoster infection caused by the reactivation of the latent varicella-zoster virus (VZV) as reported in several clinical trials and reviewed elsewhere[163]. Other viral infections reported in clinical trials include HCMV during baricitinib and tofacitinib treatment[162,165] and also in a case report from a myelofibrosis patient receiving ruxolitinib[157]. Reactivation of latent hepatitis B virus (HBV) has been observed in immunosuppressive treatments, and therefore, several studies have evaluated the incidence of this event in clinical trials and/or patients using JAK inhibitors. As expected, a positive correlation for HBV reactivation and JAK inhibitor treatment was found in baricitinib[166] and tofacitinib[161] and, also, in a prospective study with myeloproliferative neoplasm patients treated with ruxolitinib[156]. Although not common, infection with the opportunistic pathogen EBV, EBV reactivation, and EBV-driven suspected lymphoproliferative disorder have also been described, being associated with baricitinib[171] and ruxolitinib treatment[154], respectively.

Overall, the fact that JAKi can provide antiviral effects at the approved therapeutic dose is an undeniable advantage over other potential inhibitors of viral infections targeting host cellular pathways. However, some concerns could arise from the best-known aspects of the mechanisms of action of these drugs, mainly derived from the impairment of IFN-mediated antiviral responses, potentially facilitating the susceptibility and evolution of certain uncommon and chronic viral infections.

HYPOTHESIS AND OBJECTIVES

The persistence of latent HIV reservoirs allows for viral rebound upon antiretroviral therapy interruption, hindering effective HIV-1 cure. For this reason, people with HIV (PLWH) must be on a life-long suppressive antiretroviral therapy (ART). Unfortunately, immune dysfunction persists indefinitely in PLWH on ART. Also, increased risks of long-term side effects and treatment resistance may occur in these individuals. Thus, it is imperative to develop new therapeutic strategies to eliminate or silence the latent reservoir to achieve a sterilizing or functional HIV cure.

HIV cure strategies have focused primarily on purging HIV-1 latency from the major contributor to HIV latent reservoir, CD4+ T cells while overlooking the relevance of cells of myeloid origin, particularly macrophages, major orchestrators of HIV spread to immune-privileged compartments. Although several promising HIV cure strategies have emerged over the years, none has successfully eradicated HIV-1 from all possible latent reservoir compartments. Moreover, with many strategies targeting HIV-1 latency, induction of chronic immune activation and hyperinflammatory response persists.

Recently, immunomodulation by pharmacological agents was shown to both reactivate and selectively induced the elimination of HIV-infected cells. This line of evidence made us **hypothesize** that specific modulation of innate immune stimulation could impact viral latency and may contribute to the clearance of the HIV reservoir.

Thus, the **main objective** of the thesis is to identify and characterize novel innate immune modulators of HIV-1 latency for eradicating or silencing the latent reservoirs concomitantly inhibiting global immune activation and inflammation.

The **specific objectives** of the thesis are:

1. Development of robust and versatile non-clonal models of HIV-1 latency from different immune cell lineages for identifying novel modulators of HIV-1 latency.
   1.1. To determine the role of innate immune modulation as LRA
   1.2. To investigate the potential of TBK1/IKKε inhibitors as a novel class of LRAs
2. Identification and characterization of selective JAK2 inhibitors as modulators of HIV-1 latency
   2.1. To delineate the mechanism driving JAK2i-induced effects on HIV-1 latency reversal: the role of IRF7
   2.2. To demonstrate the potential of JAK-STAT signalling blockade as a strategy to prevent HIV-1 latency reversal

MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

Cells

The main cell lines and culture conditions used in the present work can be found in **Table 2**. If not otherwise specified, cells were cultured in DMEM or RPMI (Gibco, Madrid, Spain) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Madrid, Spain) and penicillin/streptomycin (P/S) (Life Technologies).

Table 2. Cell lines used in the thesis and culture conditions

Cell line	Provider	Culture method
TZM-bl cells	AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA	DMEM +10% FBS +(P/S)
HEK293T cells	AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA	DMEM +10% FBS +P/S
***A549-Dual™ hACE2 TMPRSS2 cells**	Invivogen	DMEM +10% FBS +P/S + 10 µg/mL of Blasticidin +100 µg/mL of Hygromycin +0.5 µg/mL of Puromycin +100 µg/mL of Zeocin (Invivogen)
MT-4 cells	AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA	RPMI 1640 L-Glutamine medium +10% FBS +P/S
HL60 cells	AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA	RPMI 1640 L-Glutamine medium +10% FBS +P/S
Jurkat cells	AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA	RPMI 1640 L-Glutamine medium +10% FBS +P/S
ACH2 cells	AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA	RPMI 1640 L-Glutamine medium +10% FBS +P/S

*A549-Dual™ cells are adherent epithelial cells derived from the human A549 lung carcinoma cell line with a stable integration of two inducible reporter constructs for studying the expression of NFκB and interferon regulatory factor (IRF) pathways.

All cell cultures were maintained at 37°C in a 5% CO2 incubator.

PBMCs and isolation

Peripheral blood mononuclear cells (PBMC) from buffy coats of healthy donors were obtained by Ficoll-Paque density gradient centrifugation and used for fresh purification of CD4+ T lymphocytes (StemCell Technologies). Purity of the CD4+ T lymphocyte population was confirmed by flow cytometry. CD4+ T lymphocytes were kept in complete RPMI culture medium hIL-2 (6.5 IU/mL, Roche) and stimulated (donor) or not (HIV+ study participants) for 3 days with PHA (4 µg/mL; Sigma-Aldrich). Buffy coats from healthy donors were purchased from the Catalan Banc de Sang i Teixits (http://www.bancsang.net/en/index.html). The buffy coats received were totally anonymous and untraceable and the only information given was whether or not they have been tested for diseases. All study participants provided informed consent at the time of blood collection. All methods were carried out in accordance with relevant guidelines and regulations and to the ethical principles suggested in the Declaration of Helsinki.

RNA interference

The latently infected HL-HIG cells were transfected using Amaxa Cell line Nucleofector Kit V (VCA-1003, Lot F-13862, Lonza) in a 6-well plate following manufacturer instructions for RNAi transfections. Briefly, 20 pmol of the corresponding siRNA was nucleofected in 3 x 10^6 of the corresponding cell line. RNA and protein lysates were collected 24 h post-transfection. The siRNAs used for transfection were ON-TARGETplus Non-targeting siRNA Pool (D-001810-10-05), human JAK2 siRNA-SMARTpool (L-003146-00- 0005), and human IRF7 siRNA-SMARTpool (L-011810-00-0005) all from Dharmacon, Waltham, USA.

Cell viability

For evaluation of cell death, cells were stained for 30 min with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Thermo Fischer Scientific) in PBS according to manufacturer's instructions. Alternatively, viable cells were identified according to forward and side laser light scatter flow cytometry analysis. Cells were washed and fixed in 1% formaldehyde before the analysis. Flow cytometry assays were performed in a FACS LSR II or a FACSCanto II flow cytometer (BD Biosciences). The data was analyzed using the FlowJo software (BD Biosciences).

VIRUS AND ANTIVIRAL ACTIVITY

Viral strains.

The envelope-deficient HIV-1 NL4-3 clone (HIG) encoding internal ribosome entry site (IRES)-green fluorescent protein (GFP) (NL4-3-GFP)[183] was pseudotyped with vesicular stomatitis virus G protein (VSV-G) by cotransfection of HEK293T cells using polyethylenimine (Polysciences) as previously described [184,185]. HIV-1 stock of the fully replicative NL4-3 clone (NL4-3) was grown in lymphoid MT-4 cells. Three days after transfection, supernatants were harvested, filtered and stored at -80°C. Viral stocks were concentrated using Lenti-X concentrator (Clontech). Viruses were titrated by infection of TZM-bl cells followed by GFP quantification by flow cytometry or qPCR for HIG or NL4-3, respectively.

Viral Infection and Antiviral Activity

Primary activated CD4 + T cells from healthy donors were infected with the envelope-deficient HIV-1 NL4-3 clone (HIG) encoding internal ribosome entry site (IRES)-green fluorescent protein (GFP) (NL4-3-GFP) by spinoculation (1200×g, 1 h 30 min at 37 °C). The anti-HIV activity of the different compounds was determined by the infection of cells in the presence of different concentrations of drugs, and 50% effective concentrations (EC_{50}) were calculated, as previously described[186]. Viral replication was measured 48 h later by quantification of GFP + expression by flow cytometry.

Generation of HIV latently infected cells and viral reactivation assays

Latently infected cells (J-HIG and HL-HIG) were generated by infecting lymphoid (Jurkat) or myeloid (HL60) cells with HIG viral strain and maintained in culture for 10 days to allow for the attrition of productively infected cells as previously described[93]. HIV-1 reactivation was measured as the percentage of GFP + cells by flow cytometry 20 h post incubation with compounds (**Figure 13**).

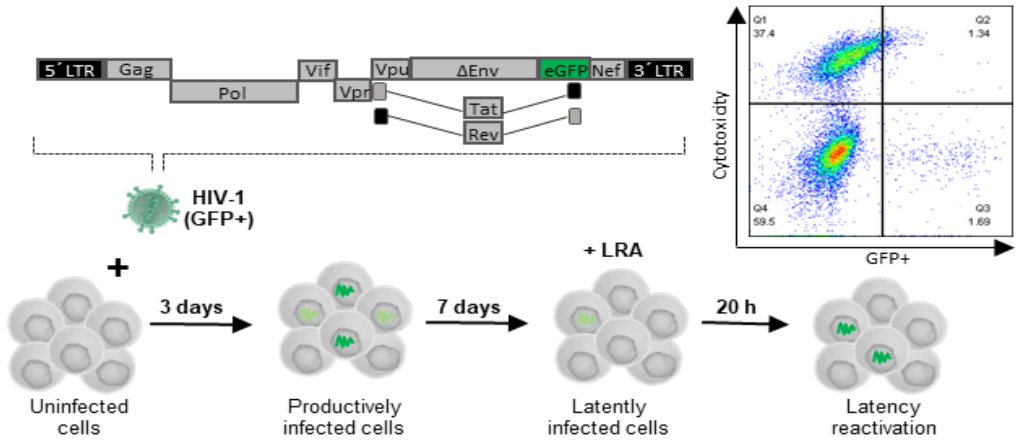


Figure 13. Generation of non-clonal models of HIV-1 latency for identifying new latency reversal agents (LRA) by flow cytometry. Latently infected models of HIV-1 latency were generated by infecting Jurkat (J-HIG) and HL-60 (HL-HIG) cell lines with the envelope-deficient HIV-1 NL4-3 clone (HIG) encoding GFP. Viral reactivation was measured as the proportion of GFP+ cells 20 h post incubation with compounds.

Similarly, ACH-2 cells were cultured in the presence or absence of compounds for 48 h at 37ºC and 5% CO_2. HIV reactivation was measured by the production of HIV CA p24 antigen using Genscreen HIV-1 Ag ELISA (BioRad) according to manufacturer's instructions.

Measurement of HIV production in latently infected cells by VIP-SPOT assay

ELISpot plates (Immobilon-P polyvinylidene difluoride membrane, Cat#MSIPS4W10; Millipore) were coated in advance with 10mg/mL capture antibody (mouse monoclonal antibody to HIV p24; clone 39/5.4A, Cat#ab9071; Abcam). On the day of cell culture, the wells were washed with sterile PBS and incubated with 100mL of a blocking solution containing 5% bovine serum albumin (BSA) (MACS BSA stock solution, Cat#130-091-376; Miltenyi) in PBS, for at least 30 min at room temperature. This was followed by additional washing of wells and replacement with culture medium containing HL-HIG or J-HIG cells at 9 x 10^3 cells per well and compounds. After 2 days of incubation at 37°C in a 5% CO2-humified atmosphere, the plates were developed as described elsewhere [187]. After drying, the spots were counted using an automated ELISpot reader unit (Cellular Technology Limited, Shaker Heights, OH).

Tat-dependent viral expression of HIV-1 LTR promoter

HeLa TZM-bl cells harbouring an integrated copy of HIV-1 LTR, controlling luciferase reporter gene expression was used for Tat-dependent viral transactivation assay. Cells were transfected or not with 10 – 50 ng of Tat-expressing plasmid[188] using Lipofectamine 3000 reagent (Invitrogen, Cat#L3000015) in a 24-well plate following manufacturer instructions. Cells were

treated with compounds 24 h post transfection. HIV-1 Tat expression was then measured by a luciferase-based assay 20 h post drug treatment.

COMPOUNDS AND DRUG TREATMENTS

PMA (Sigma-Aldrich) was used at 50ng/ml in combination with ionomycin (Sigma-Aldrich) at 100 ng/ml as positive control of HIV-1 reactivation in primary cells. ARV used in this study includes 3-azido-3-deoxythymidine (zidovudine; AZT) (Sigma-Aldrich) at 10 µM, raltegravir at 5 µM and efavirenz at 0.32 µM which were all obtained from the NIH AIDS Research and Reference Reagent Program. Already described LRA that were used for further validation of reactivation capacity and signaling pathways include TNFα, Lipopolysaccharide (LPS) (Merck), JQ1 (Selleckchem), Vorinostat (SAHA, VOR) (Prochifar srl, Italy) and panobinostat (PNB) (LC Laboratories). Compounds (innate modulating agents) included in the HIV reactivation HTS for novel LRAs, or LPAs are indicated in **Table 3**.

Table 3. Compounds included in the screening for latency reversing activity.

Innate Immune modulating agents		Target selectivity	Provider
TBKi/IKKi	TPCA1	IKKβ	Selleckchem
	SC514	IKKβ	Selleckchem
	MRT67307	TBK-1/IKKε	Invivogen
	BX795	TBK-1/IKKε	Invivogen
	INH1	TBK-1/IKKε	Selleckchem
	INH2	TBK-1/IKKε	Selleckchem
	GSK8612	TBK-1/IKKε	Selleckchem
JAKi	Ruxolitinib	JAK1/2 > TYK2 > JAK3	Selleckchem
	Fedratinib	JAK2 > JAK1 > TYK2 > JAK3	Selleckchem
	TG101209	JAK2 > JAK3	Selleckchem
	AZD-1480	JAK2 > JAK3	Selleckchem
	AZ960	JAK2 > JAK3	Selleckchem
	Pacritinib	JAK2 > TYK2 > JAK3 > JAK1	Selleckchem
	Baricitinib	JAK1/2 > TYK2	Selleckchem
	Gandolitinib	JAK2 > JAK1 > JAK3	Selleckchem
	XL019	JAK2 > JAK1 > JAK3	Selleckchem
	WP1066	JAK2	Selleckchem

RNA-SEQ AND ANALYSIS

RNA-Sequencing and library preparation

Cellular RNA was extracted from the latent model of HIV-1 infection, HL-HIG for RNA sequencing using Maxwell® HT simplyRNA Kit (Promega) on a KingFisher™ Flex Purification System (Thermofisher Scientific). RNA-sequencing samples were prepared in biological triplicates. Determination of RNA integrity (RIN) was performed using Agilent RNA 6000 Nano Kit (cat: 5067-1511) and Agilent 2100 Bioanalyzer System. After quality control check, RNA library was constructed using Illumina TruSeq Stranded mRNA LT Sample Prep Kit and sequencing was performed using NovaSeq 6000 System with 150 bp paired-ends reads.

Transcriptomic analysis

Transcriptomic analysis was performed as implemented in the computational workflow for the detection of differentially expressed genes and pathways from RNA-seq data [189]. Reads were aligned to the human GRCh37/hg19 (annotation NCBI_105.20190906) using HISAT2. Low-expression genes with at least one zero counts were filtered out and the remaining reads normalized with Relative Log Expression (RLE) method as implemented in DESeq2 R library. Differential gene expression between the control and treatment groups was estimated with DESeq2 Wald test [190]. Sequencing files can be accessed on Gene Expression Omnibus repository (GSE195855).

Gene Set Enrichment Analysis (GSEA) was performed on a pre-ranked GSEA list based on Log 2FC values of differentially expressed genes (DEGs: Log2FC > 1, p-value < 0.05), against Molecular Signatures Database (MsigDB v7.4) “Hallmark” and “Reactome” gene-sets. Weighted enrichment statistics were based on 1000 permutations. Significantly enriched gene-sets with FDR adjusted q-value <0.1 were selected for Enrichment map visualization as previously described [191,192]. Briefly, Enrichment files were inputted into the Enrichment Map app within the Cytoscape program for visualization [193]. Parameters were set at default values (node cutoff FDR Q value 0.1; Jaccard Overlap combined coefficient cutoff 0.375, k-constant 0.5). Nodes were manually laid out and combined into common biological process for clarity using the AutoAnnotate app.

MOLECULAR BIOLOGY

Quantitative RT-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using NucleoSpin RNA II kit (740955, Macherey-Nagel) or the Maxwell® HT simplyRNA Kit (REF: AX2420, LOT: 0000410025, Promega) and Total DNA was extracted using Magmax DNA multi-sample ultra 2.0 kit (A45721, appliedbiosystems) on a KingFisher™ Flex Purification System (Thermofisher Scientific) as recommended by the manufacturer. Reverse transcription was performed using the PrimeScript™ RT-PCR Kit (RR036A, Takara) following manufacturer instructions. mRNA levels of all genes were measured by two-step quantitative RT-PCR and normalized to GAPDH mRNA expression using the DDCt method. Primers and DNA probes were obtained from TaqMan Gene expression assays (Thermofisher Scientific) as indicated in **Table 4**.

Table 4. List of TaqMan gene expression assays

Gene	Reference
GAPDH	Hs00266705_g1
IRF1	Hs00233698_m1
IRF7	Hs01014809_g1
CXCL10	Hs00171042_m1
IL-1β	Hs01555410_m1
IL6	Hs00174131_m1
IL8	Hs00174103_m1
IL10	Hs00961622_m1
TNFα	Hs00174128_m1
CDKN1A	Hs00355782_m1
CIITA	Hs00931699_m1

Western blot

Treated cells were rinsed, lysed, subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane as previously described [94]. The following antibodies were used for immunoblotting: antirabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000; Pierce); anti-GAPDH (1:2500; ab9485; Abcam); anti-human Hsp90 (1:1000, 610418, BD Biosciences); anti-pJAK2 (1:1000; 3774), anti-STAT3 (1:1000; 3743), anti-phosphoSTAT1 (1:1000; 9167), anti-IRF7 (1:1000; 4920), anti-IRF3 (1:1000; 11904) anti-IRF1

(1:1000; 8478) all from Cell Signaling and anti-JAK2 (1;5000; 108596; Abcam). Blots were immersed in chemiluminescent substrate (SuperSignal West Pico Plus or Femto, Thermo Fisher Scientific), and signal was visualized using ChemiDoc MP imaging system (BIORAD).

Interferon signalling

To study the modulation of the NF-κB and interferon regulatory factor (IRF) signaling pathways by the JAK inhibitors, 5 x 10^4 A549-Dual™ hACE2-TMPRSS2 cells (InvivoGen, Toulouse, France) were seeded per well in flat 96-well plates and incubated for 24 h with the compounds. The activities of the NF-κB and IRF signaling pathways were assessed by incubating the cell supernatants with QUANTI-Blue™ Solution and QUANTI-Luc™ respectively (InvivoGen, Toulouse, France) according to manufacturer's instructions. SEAP and luciferase were read in an EnSight™ multimode plate reader (PerkinElmer, Waltham, MA, USA).

PRIMARY CELLS

***Ex vivo* reactivation of primary CD4+ T Cells from HIV+ subjects**

Purified CD4+ T lymphocytes from HIV-1+ participants were kept in complete RPMI culture medium and preincubated with the pan-caspase inhibitor Q-VD-Oph (10 µM, Sigma-Aldrich) for 2 h. To evaluate the latency reactivation capacity, 5 x 10^5 CD4+ T lymphocytes were cultured with PMA or test compounds at the indicated concentrations (5 µM fedratinib, and 50 ng/mL PMA + 1 µg/mL ionomycin). Cells were cultured in the presence of antiretroviral agents (efavirenz, zidovudine and raltegravir) and maintained in 10 µM Q-VD-Oph for 72 h.

Freshly collected cell-culture supernatant were centrifuged for 1 hour at 25 000 *g* to pellet HIV particles. HIV-1 latency reactivation was determined by quantification of viral RNA in the supernatant as previously described [43]. Briefly, viral RNAs were extracted using Viral RNA/DNA Mini kit (Invitrogen) and quantified using a nested real-time reverse transcription-polymerase chain reaction (RT-PCR). A Superscript III One-Step RT-PCR system (Invitrogen) was used to generate and pre-amplify viral RNA with the following primers: ULF1 (forward) 5'- ATG CCA CGT AAG CGA AAC TCT GGG TCT CTC TDG TTA GAC - 3'; UR1 (reverse) 5'- CCA TCT CTC TCC TTC TAG C -3'. The following cycling conditions were used: reverse transcription at 50°C for 30 min, denaturation at 94°C for 2 min, 16 cycles of amplification (94°C 15 s, 55°C 30 s, 68°C 1 min) and final elongation at 68°C for 5 min. Preamplified products were subjected to nested real-time PCR with the following primers and probe: LambdaT (forward) 5'- ATG CCA CGT AAG CGA AAC T - 3'; UR2 (reverse) 5' - CTG AGG GAT CTC TAG TTA CC - 3'; UHIV Taqman 5'- 56-FAM/CAC TCA AGG /ZEN/CAA GCT TTA TTG AGG C/3IbkFQ/ - 3' on a QuantStudio 5 PCR system (Applied

Biosystems). Serial dilutions of the HIV-1 NL43 strain were run in parallel with each experiment for the quantification of viral RNA.

Immunophenotypic characterization of CD4+ T lymphocytes by flow cytometry

Purified CD4+ T lymphocytes from freshly processed PBMCs isolated from healthy donors and HIV+ subjects were stained with CD4+T cell phenotype defining markers for flow cytometry 72 h post treatment with compounds: CD4-BV786, CD45RA-PE, CCR7-BV510 (Biolegend); CD3-FITC, CD27-400 (BD Biosciences). Immune activation was determined using CD69-BV650, CD25-APC (Biolegend) and HLA-DR-PeCy7 (BD Biosciences). Immunophenotyping of CD4+ T cell population identification was performed based on the following gating strategy (**Figure 14**): CD4 + T cell populations were defined based on the following expression combinations gated on the live singlet CD3+CD4+ lymphocytes: T naïve TN (CD45RA+CCR7+CD27+), T central memory TCM (CD45RA-CCR7+CD27+), T transitional memory TTM (CD45RA-CCR7-CD27+) and T effector memory TEM (CD45RA-CCR7+CD27-) [57]. Immune activation markers HLA-DR+, CD25+ and CD69+ CD4+ lymphocytes were also gated on the live singlet CD3+CD4+ lymphocytes.

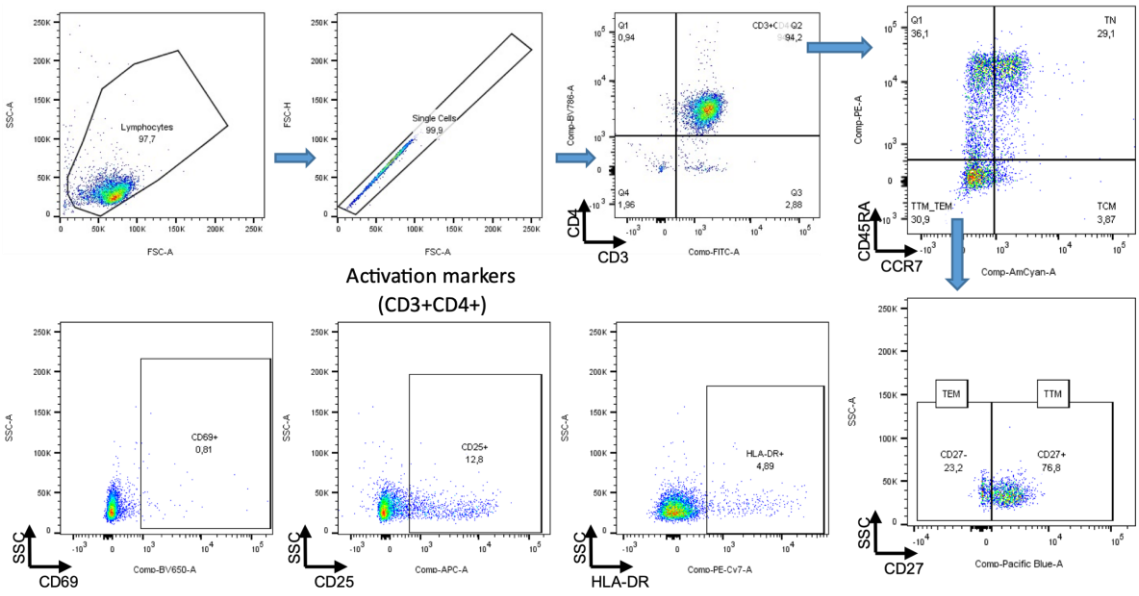


Figure 14. Gating strategy for the immunophenotypic characterization of PBMCs by flow cytometry. CD4 + T cell populations were gated on the live singlet CD3+CD4+ lymphocytes: T naïve T_N (CD45RA+CCR7+CD27+), T central memory T_{CM} (CD45RA-CCR7+CD27+), T transitional memory T_{TM} (CD45RA-CCR7-CD27+) and T effector memory T_{EM} (CD45RA-CCR7+CD27-). Immune activation markers HLA-DR+, CD25+ and CD69+ CD4+ lymphocytes were also gated on the live singlet CD3+CD4+ lymphocytes.

Statistical analysis

Statistical significance for *in vitro* and *ex vivo* experiments was calculated using appropriate t test in Graphpad Prism (v9.3.0). All experiments were performed in at least three independent replicates and n values are provided in the figure legends. Plots were drawn using GraphPad Prism and R software.

Quantitative analysis of drug combination interactions

The Bliss independence model for predicting the expected combined effects of multiple drug combinations for LPA blockade of LRA-induced latency reactivation is defined by the following equation[194]:

$$\boldsymbol{f_{axy,P} = f_{ax} + f_{ay} - (f_{ax}\, f_{ay})}$$

$$f_{ax} = fraction\ affected, drug\ x$$

$$f_{ay} = fraction\ affected, drug\ y$$

$$f_{axy,P} = predicted\ fraction\ affected, drug\ x + y$$

$$f_{axy,O} = observed\ fraction\ affected, drug\ x + y$$

$$\Delta \boldsymbol{f_{axy}} = \boldsymbol{f_{axy,O} - f_{axy,P}} \begin{cases} > 0, synergy \\ = 0, \text{Bliss independence} \\ < 0, antagonism \end{cases}$$

Assuming the drugs act through independent mechanisms, if the $\Delta f_{axy} < 0$, then the drug combination displays antagonism, as should be expected for inhibition of LRA-induced reactivation in the presence of an LPA.

RESULTS

CHAPTER 1. HIGH THROUGHPUT SCREENING IN NON-CLONAL MODELS OF HIV-1 LATENCY IDENTIFIES NOVEL IMMUNOMODULATORS OF HIV-1 LATENCY

Summary

To partially overcome the limitations of widely used HIV-1 latency models based in cell lines, we developed a series of non-clonal cellular models of HIV-1 latency using cell lines from lymphoid or myeloid lineage. The developed models were subsequently tested for robustness and versatility and used for identifying new LRAs from high-throughput screening (HTS) of small molecules. In this first chapter of the thesis, we revisit our previous data on HTS of anticancer compound libraries for LRA activity in the lymphoid HIV-1 latency model J-HIG cells and extend this screening to the myeloid-based HIV-1 latency model HL-HIG cells. Interestingly, two classes of novel immunomodulating agents were identified as LRAs in these models: IκB/IKK inhibitors and JAK inhibitors.

The use of IKK kinase inhibitors (IKKis) targeting different IKK complex subunits and IKK related kinases (TBK1 and IKKε) was evaluated as an alternative pathway to reverse HIV-1 latency. Our results show latency reversing activity of IKK inhibitors in HIV-1 latently infected lymphoid and myeloid compartments. In particular, selective inhibitors targeting TBK1 and IKKε, showed relevant HIV reactivation capacities through a mechanism of NF-κB-induced HIV transcription *in vitro* and *ex vivo*.

1.1 Identification of novel immuno modulating agents acting as LRAs in distinct non-clonal models of HIV latency

Non-clonal GFP-expressing cellular models of HIV latency J-HIG and HL-HIG were generated by infecting lymphoid (Jurkat) or myeloid (HL60) cells with HIG viral strain encoding GFP. After 10 days in culture, attrition of productively infected cells was confirmed by flow cytometry, with basal GFP expression between 1.0 – 2.5% for the latently HIV-infected J-HIG and HL-HIG models, respectively. Following incubation of latently infected cells with the HDACi panobinostat (PNB) at 0.16µM for 24 h, we observed at least 2-foldchange increase ($p < 0.001$) in GFP expression in both models, relative to the untreated condition. Comparing between the two models, the relative reactivation value of PNB 0.16µM was slightly lower in the lymphoid J-HiG model than in the myeloid HL-HIG model (2.16 vs 2.31 fold-change increase).

Next, we performed drug screening of a library of anti-cancer compounds in the two non-clonal models of HIV latency, J-HIG (lymphoid) and HL-HIG (myeloid). The library of anticancer compounds (n=426, Selleckchem ref., L3000-2017) includes small molecules with identified targets within the cell, directed towards a wide range of cell signalling pathways involved in distinct cell processes such as apoptosis, cell cycle progression, among others (**Figure 15**).

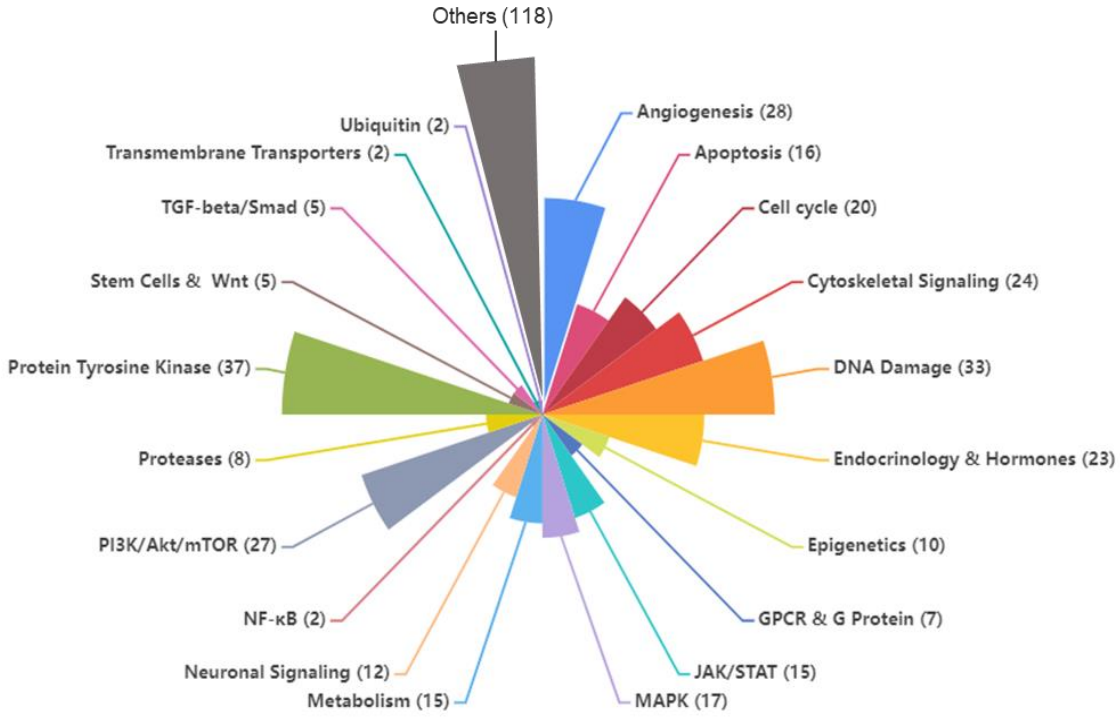


Figure 15. Signalling pathways targeted by library of anticancer compounds included in the drug screening. Signalling pathways are highlighted in different colours and the slice sizes corresponding to the number of anticancer compounds targeting specific pathways are also highlighted in parenthesis. The classification "Others" include anticancer compounds without well-defined signalling pathways per manufacturer´s designation, such as inhibitors of the HDAC, HSP and aurora kinases. Image made with Datamatic.io.

All anti-cancer compounds were used at 5µM for 24 h for the high throughput screening (HTS), and latency reactivation for individual compounds were measured as GFP expression by flow cytometry, then relativized to the untreated condition. Relative reactivation values were compared to that of the positive control, PNB. Compounds with relative reactivation values equal to or higher than that of PNB 0.16µM were classified as "hit" compounds for further exploration (**Figure 16A**).

Model specificity is an important determinant of the reactivation capacity of known and putative LRAs, being therefore highly relevant to test the compound potential in several models from distinct origin, as we did in the present screening. These models were robust in identifying potent LRAs such as the extensively studied HDACi[89,92] with seven compounds identified as LRAs in J-HIG and and a single compound in HL-HIG model (**Figures 16B-C**), respectively. In contrast to the screening in J-HIG, HDACi persistently induced cytotoxicity in HL-HIG although with potent capacity for HIV latency reversal in this model, making us to exclude all but one HDACi as hits (n = 8/9; Data not shown). This underscores the importance of using multiple comparative models in LRA-targeted HTS as no single model can reliably recapitulate HIV latency *in vivo* in PLWH[195].

Overall, we identified 16 and 27 compounds as hits in the J-HIG and HL-HIG models, respectively (**Table 5**). Despite differences in the constitution of identified hits because of model specificity, we identified three compounds common to both models, the JAK inhibitor fedratinib, the HSP inhibitor geldanamycin and the protein kinase inhibitor midostaurin (**Figure 16D).** We have previously described the dual role for the multi-kinase inhibitor midostaurin in HIV-1 infection, as an antiviral agent in acute HIV infection and as LRA in latently infected cells, depending on SAMHD1 activation[186]. The identification of JAKi and IκB/IKK inhibitors as putative LRAs in models of HIV latency supports our hypothesis that innate modulation might play a role in HIV latency and reactivation as we will demonstrate in subsequent sections of the thesis. Moreover, innate immunity because of its immune modulatory properties can also impact chronic immune activation, a problem of chronic HIV infection. Therefore, we focus on further characterizing the HIV latency reversal capacity of the innate immune modulating agents, including the Janus kinase inhibitors and the IκB/IKK inhibitors, as novel LRAs or LPAs in both non-clonal models of HIV latency.

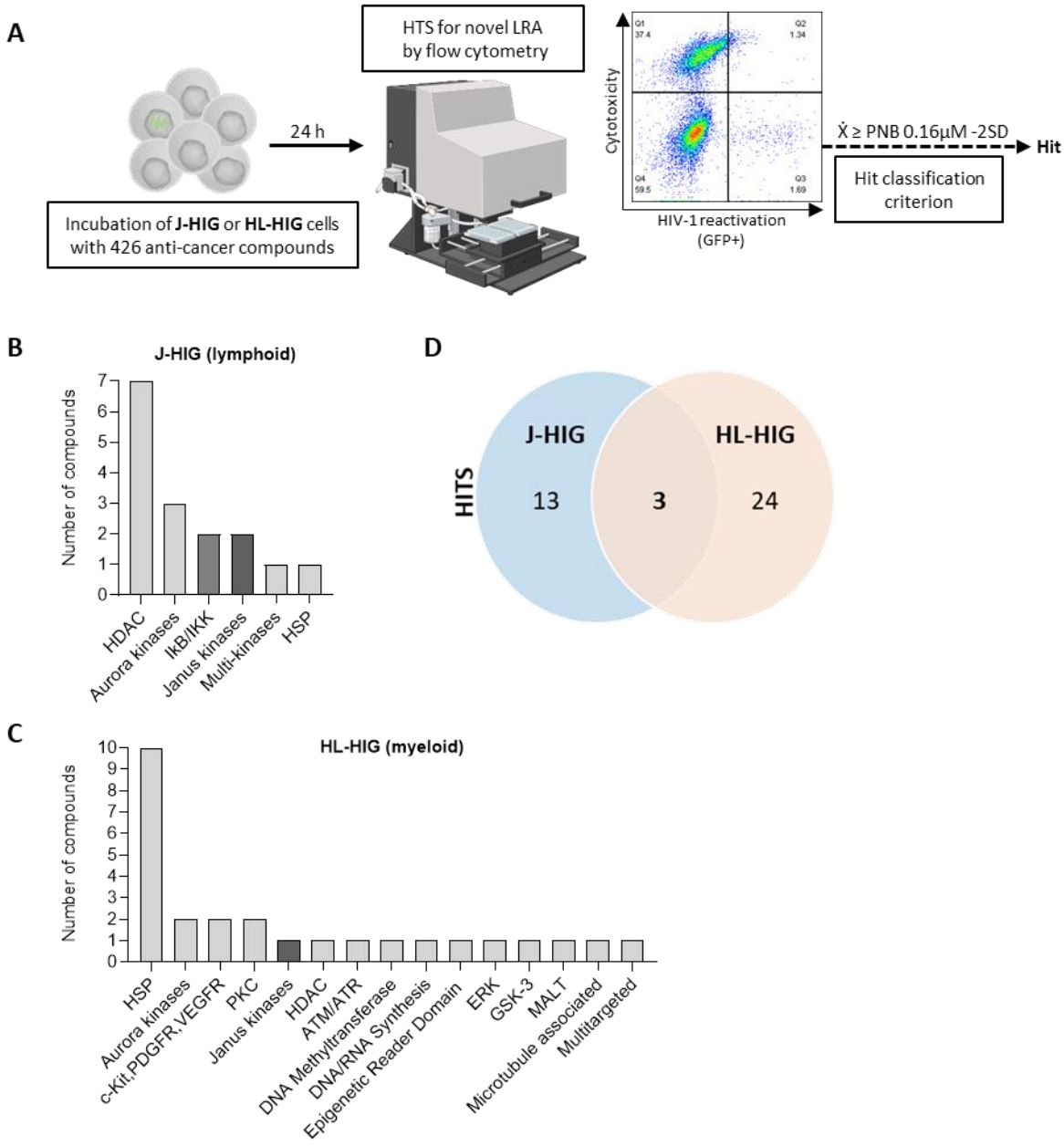


Figure 16. Identification of novel LRAs by High-throughput screening (HTS) of anticancer compounds in non-clonal models of HIV latency. (**A**) Schematic workflow used for LRA HTS and hit classification. Lymphoid (J-HIG) and myeloid (HL-HIG) cells were incubated with anti-cancer compounds at 5µM for 24h. Relative HIV-1 reactivation of each condition, measured as percentage of GFP+ cells by flow cytometer and normalized to untreated condition, was compared to that of the control LRA panobinostat (PNB). Tested compounds with reactivation values (Ẋ) ≥ PNB 0.16µM -2SD were classified as "hits". Number of compounds classified as hits by their cellular targets in J-HIG (**B**) and HL-HIG (**C**) models, and the uniquely or commonly identified hits in these models (**D**). The drug screening was assessed in three independent experiments. Classes of immune modulating agents identified in this screening are highlighted: IκB/IKK (light-grey) and Janus kinases (dark-grey) inhibitors.

Table 5. List of compounds tested in the LRA HTS that classified as hits, their reactivation values and their main cellular target

Model	Compound	Mean relative reactivation*	SD	Main target**
J-HIG	AMG-900	1.89	0.30	Aurora Kinase
	Barasertib (AZD1152-HQPA)	1.80	0.06	Aurora Kinase
	Tozasertib (VX-680, MK-0457)	1.70	0.03	Aurora Kinase
	Trichostatin A (TSA)	2.06	0.16	HDAC
	Abexinostat (PCI-24781)	2.01	0.25	HDAC
	Belinostat (PXD101)	1.98	0.20	HDAC
	Vorinostat (SAHA, MK0683)	1.97	0.15	HDAC
	Quisinostat (JNJ-26481585) 2HCL	1.90	0.19	HDAC
	Pracinostat (SB939)	1.82	0.13	HDAC
	CUDC-101	1.76	0.32	HDAC
	Geldanamycin	1.64	0.25	HSP
	TPCA-1	1.84	0.16	IκB/IKK
	BX-795	1.80	0.06	IκB/IKK
	AZD1480	1.86	0.13	JAK
	Fedratinib (SAR302503, TG101348)	1.80	0.27	JAK
	Midostaurin	1.80	0.16	Protein kinase
HL-HIG	KU-60019	1.62	0.30	ATM/ATR
	Alisertib (MLN8237)	1.69	0.09	Aurora Kinase
	SNS-314 Mesylate	1.60	0.10	Aurora Kinase
	Axitinib	1.53	0.20	c-Kit,PDGFR,VEGFR
	Tivozanib (AV-951)	1.53	0.11	c-Kit,PDGFR,VEGFR
	Azacitidine	1.63	0.26	DNA Methyltransferase
	Floxuridine	1.60	0.29	DNA/RNA Synthesis
	CPI-203	2.02	0.31	Epigenetic Reader Domain
	XMD8-92	1.91	0.27	ERK
	CHIR-99021 (CT99021) HCl	1.60	0.17	GSK-3
	Entinostat (MS-275)	2.15	0.08	HDAC
	PU-H71	2.03	0.16	HSP

Model	Compound	Mean relative reactivation*	SD	Main target**
HL-HIG (continued)	HSP990 (NVP-HSP990)	1.86	0.08	HSP
	VER-50589	1.79	0.07	HSP
	CH5138303	1.78	0.15	HSP
	BIIB021	1.75	0.14	HSP
	Geldanamycin	1.71	0.16	HSP
	Ganetespib (STA-9090)	1.69	0.11	HSP
	Tanespimycin (17-AAG)	1.63	0.13	HSP
	Alvespimycin (17-DMAG) HCl	1.58	0.07	HSP
	Luminespib (AUY-922, NVP-AUY922)	1.57	0.08	HSP
	Fedratinib (SAR302503, TG101348)	2.28	0.11	JAK
	MI-2 (MALT1 inhibitor)	1.73	1.51	MALT
	Cucurbitacin B	3.48	0.44	Microtubule Associated
	Enzastaurin (LY317615)	1.58	0.43	PKC
	Bisindolylmaleimide I (GF109203X)	1.54	0.22	PKC
	Midostaurin	1.77	0.23	Protein kinase

*Relative reactivation relativized to the untreated conditions. Mean reactivation values and standard deviation (SD) for PNB at 0.16µM were 2.16 ± 0.26 (J-HIG) and 2.31 ± 0.39 (HL-HIG). **According to the manufacturer. Common hits identified in both models are highlighted in bold.

HDAC, histone deacetylase; IκB/IKK, inhibitor of NF-kB (IκB) kinases; JAK, Janus kinases; HSP, heat-shock protein; ERK, extracellular signal-regulated kinases; MALT, mucosa-associated lymphoid tissue; ATM/ATR, Ataxia telangiectasia mutated/ATM and RAD3-related kinases; GSK-3, glycogen synthase kinase-3; PKC, protein kinase C; c-Kit, KIT proto-oncogene; PDGFR, platelet-derived growth factor receptors; VEGFR, vascular endothelial growth factor.

1.2 Pharmacological inhibition of IKK induces reactivation of latent HIV-1 infection

From the initial LRA HTS screening, the TBK-1/IKKε inhibitor BX795 and the IKKβ inhibitor TPCA1 showed capacity for latency reversal in the lymphoid model J-HIG, prompting us to expand this screening to other IKK kinase inhibitors (IKKis) targeting different IKK complex subunits. As most LRAs described to date are particularly restricted as therapeutics because of their tendency for inducing immune activation and/or inflammatory response, we further hypothesized that immune modulators as the IKKis might be a safer alternative, since they were canonically developed as anti-inflammatory agents.

1.2.1. IKK Inhibitors induce HIV reactivation in distinct clonal and non-clonal models of HIV latency in vitro

To identify other IKKis that may be acting as LRAs, we expanded the screening to include additional compounds, including the TBK-1/IKKε inhibitors MRT67307, Inh1 and Inh2 and GSK8612, and the IKKβ inhibitor SC514. IKKis ability to promote HIV transcription was first evaluated in lymphoid latently infected Jurkat cells (J-HIG). All TBK-1/IKKε inhibitors including, MRT67307, BX795, Inh1 and Inh2 and GSK8612, and the IKKβ inhibitor TPCA1 induced a dose-dependent *in vitro* HIV reactivation at micromolar concentrations compared to the non-treated control (1.6 and 1.8 fold increase at the highest concentrations tested, respectively) (**Figure 17A**). In contrast, weak LRA activity was observed only with the IKKβ inhibitor SC514. Since toxicity is a major concern for the use of HDACis, we used them at subtoxic concentrations as previously described[196]. Compared to HDACis, IKKis showed low cytotoxicity at micromolar level in the J-HIG latency model.

Next, we confirmed LRA activity of IKKis in myeloid latently infected cells (HL-HIG) which showed increased HIV-1 transcription compared to the untreated control (**Figure 17B**). Amongst IKKis, all compounds targeting TBK1/IKKε showed up to 1.5 fold increase in viral transcription at their highest concentrations without compromising cell viability. Accordingly to the J-HIG model, HIV-1 reversing activity of IKKis in myeloid HL-HIG, was higher than VOR (**Figure 17B**). Further, the *in vitro* capacity of IKKis to induce HIV-1 expression was confirmed in latently infected ACH-2 cells by measuring HIV p24 Ag in the supernatant (**Figure 17C**). Taken together, IKKis showed consistent HIV-1 reactivation capacity at similar or even higher levels compared to HDACi but with less impact on cell viability.

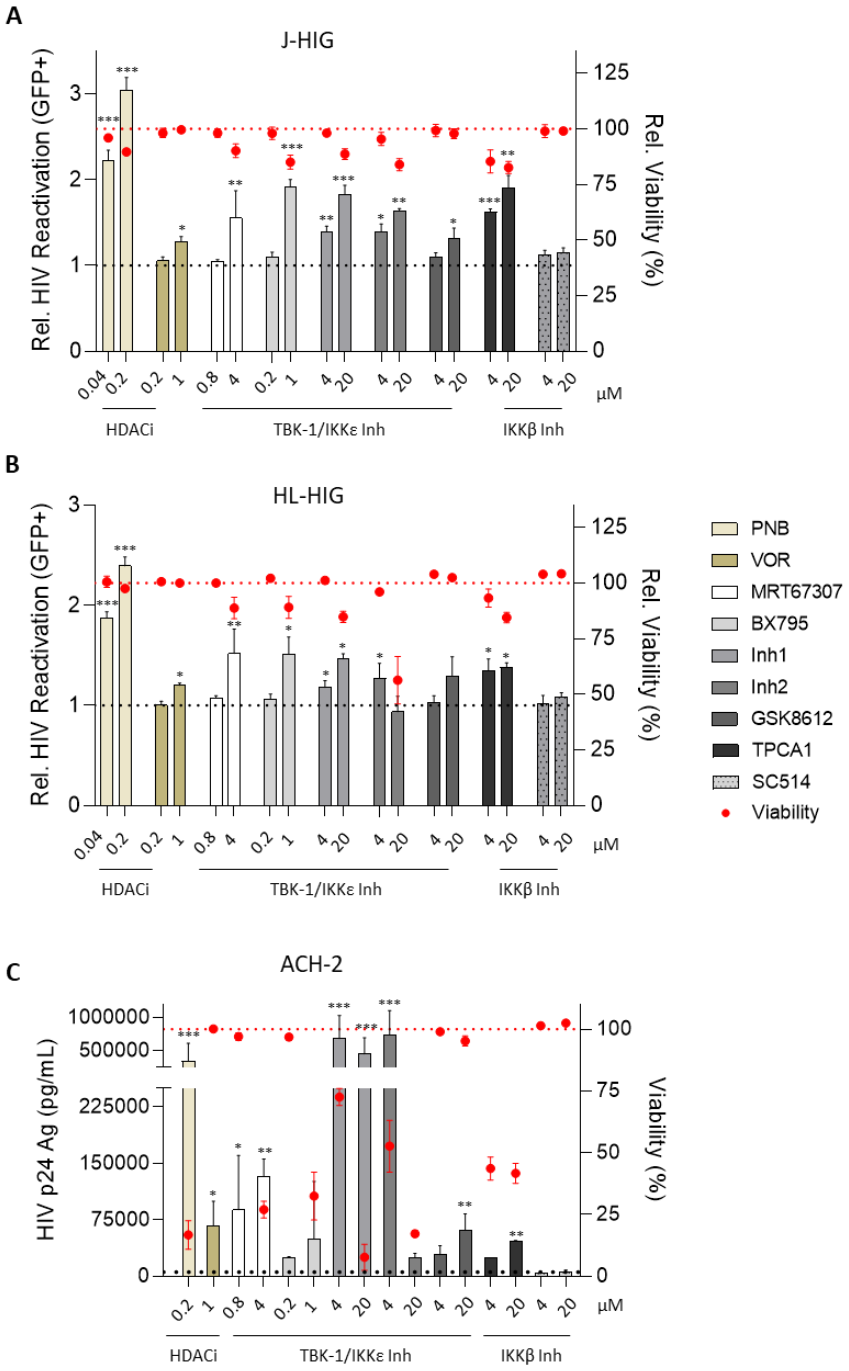


Figure 17. IKK Inhibitors induce HIV reactivation in *in vitro* models of latency. (**A**) HIV reactivation induced by IKKis in latently infected lymphoid Jurkat (J-HIG) and (**B**) myeloid HL-60 (HL-HIG) cells. Activity of IKKis MRT67307, TPCA-1, BX795, Inh1, Inh2, GSK8612 and SC514 was determined by the quantification of GFP+ cells (%) after culturing J-HIG or HL-HIG cells at their highest subtoxic concentrations for 24 h. HDCAi panobinostat (PNB) and vorinostat (VOR) were used as controls for HIV-1 reactivation. Basal reactivation (grey-dashed line) in J-HIG and HL-HIG was established according to the non-drug condition (ND). Toxicity of IKKis (red-dashed line) in J-HIG and HL-HIG cells was measured as percentage of viable cells by flow cytometry related to the ND condition. (**C**) IKK Inhibitors induce HIV reactivation in *in vitro* lymphoid ACH-2 model of latency. HIV reactivation was determined by the intracellular expression of HIV-1 p24 antigen as measured by flow cytometry in ACH-2 cultured for 48 h in the presence of subtoxic concentrations of IKKis. HDCAi panobinostat (PNB) and vorinostat (VOR) were used as controls for HIV-1 reactivation. Basal reactivation (grey-dashed line) in ACH-2 was established according to the non-drug condition (ND). Mean ± SD of three independent experiments is shown. *p < 0.05; **p < 0.01, ***p < 0.01.

1.2.2. TBK1/IKKε and IKKβ inhibitors induce HIV reactivation *ex vivo* in HIV+ CD4+ T cells without modifying cell activation in *ex vivo* treated primary CD4+ T cells

The latency reactivation capacities of MRT67307 and TPCA-1 were evaluated in CD4+ T cells from HIV+ individuals (n=3, **Table 6**). CD4+ T-cells from ART-suppressed PLWH were treated with either IKKis or PMAi for 72 h in the presence of ART. Latency-reversal was measured by determining HIV-1 RNA in the supernatant using nested PCR. Both IKKis induced HIV-1 reactivation in all patient samples similar to the positive control PMAi. Depending on the IKKi used, we observed 2.1-2.3-fold induction and 1.3-3.6-fold induction of HIV-1 reactivation compared to ND for MRT67307 and TPCA1, respectively (**Figure 18A**).

Although PMAi is a robust HIV reversing agent, it induces global immune activation, making it imperative to identify "safer" LRAs that should ideally reactivate latently infected cells without complete T cell activation to avoid cytotoxicity. To determine the impact of IKKis on immune cell activation, CD4+ T cells from healthy donors (n=8) were *ex vivo* treated with either MRT67307 or TPCA-1, and PMAi as positive control for latency reactivation and immune activation. Immunophenotypic characterization of CD4+ T cell populations revealed that both IKKis do not induce changes in immune cell activation, as cell surface markers CD25, CD69 and HLA-DR remained similar to resting CD4+ T cells, in contrast to the marked changes observed upon treatment with PMAi (**Figures 18B and 18C**). Moreover, there was evident PMA-induced CD4 down-regulation in the PMAi treatment condition[197], an effect that was not seen with either MRT67307 or TPCA-1 (**Figure 18B**). Thus, IKKis might represent a novel approach for eradicating the latent reservoir, moreso as current research efforts on LRAs pivot towards identifying agents capable of inducing HIV reactivation with minimal or no immune activation to mitigate immune cell exhaustion in PLWH.

Table 6. Immunological and virological characteristics of study participants at the time of cell sample collection

ID	Age	Sex	Ethnicity	Estimated min. length of HIV infection (years)	Estimated min. length of viral suppression (years)	CD4 Nadir (cells/µl)	CD4 count (cells/µl)	Viral Load (copies/ml)	ART Regimen*
1	38	M	Caucasian	4	4	460	932	<40	DRV/COBI
2	39	M	Caucasian	8	4,9	603	902	<40	DTG/ABC/3TC /DOVATO
3	36	F	Caucasian	7	6,4	504	1396	<40	ABC/3TC, DOVATO, RAL

*ABC, abacavir; COBI, cobicistat; DRV, darunavir; DTG, dolutegravir; FTC, emtricitabine; RAL, raltegravir; 3TC, lamivudine.

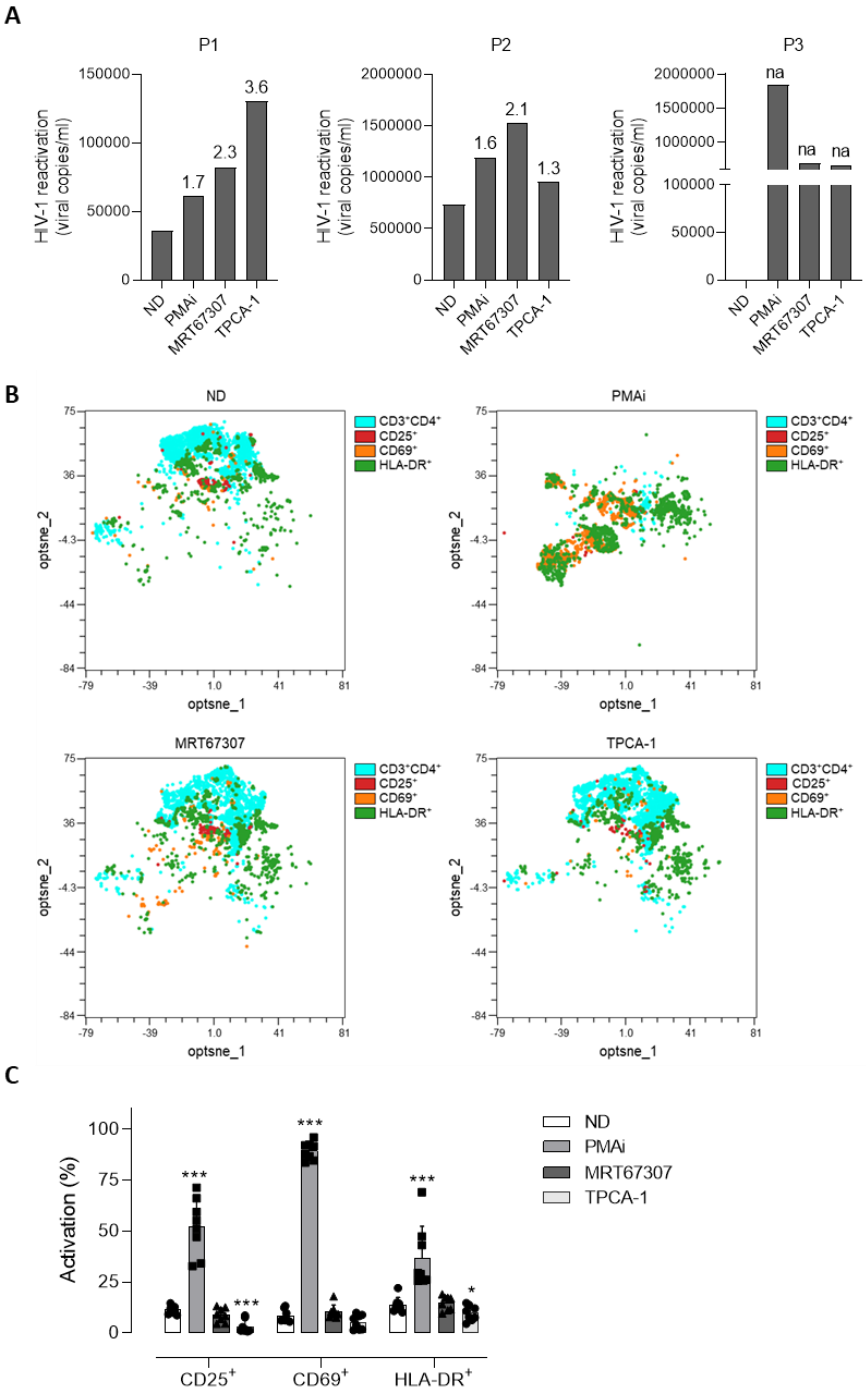


Figure 18. IKK Inhibitors induce HIV reactivation ex vivo in CD4+ T cells from HIV+ individuals without modifying immune cell activation in primary CD4+ T cells from healthy donors. (**A**) *Ex vivo* response to IKKis in CD4+ T cells from PLWH (N=3). HIV-RNA copies were determined in the cell culture supernatant from HIV+ CD4+ T cells incubated for 72 h with 4 µM of MRT67307 and TPCA-1. PMA (50 ng/mL) and ionomycin (1µM) (PMAi) were used as positive controls of HIV-1 reactivation. (**B**) Distribution of cell surface markers in CD4+ T cells from healthy donors treated with subtoxic concentrations of MRT67307 (4 µM) and TPCA-1 (4 µM) as measured by flow cytometry. Distribution was determined by Opt-tSNE-guided manual gating analysis of the signal strength of key phenotypic defining cell activation markers CD25, CD69 and HLA-DR from eight healthy donor samples. Opt-tSNE analysis was performed using 1,000 iterations, a perplexity of 30, a trade-off θ of 0.5. (**C**) Quantification of the cell activation markers compared to resting and PMAi activated CD4+ T cells.

1.2.3. IKK inhibitors do not limit HIV-1 infection

It remains to be seen whether immune mediated clearance or virus-induced cytopathy are sufficient to prevent viremic rebound following LRA-induced reactivation without ART, and LRAs with anti-HIV activity might also contribute to viremic control, allowing safe ART withdrawal in PLWH. Therefore, we determined anti-HIV activity of IKKis in acute infection of lymphoid Jurkat or myeloid HL-60 cells infected with pseudotyped HIV-1 (VSV-HIG). In contrast to the potent anti-HIV activity of the antiretroviral agents EFV and RAL used as positive control, none of the IKKis showed anti-HIV activity in both *in vitro* models of acute HIV-1 infection (**Figures 19A and 19B**). Indeed, the majority of LRAs discovered to date were originally not recognized for their antiviral properties, with many of these LRAs formerly described instead as anti-cancer agents[87].

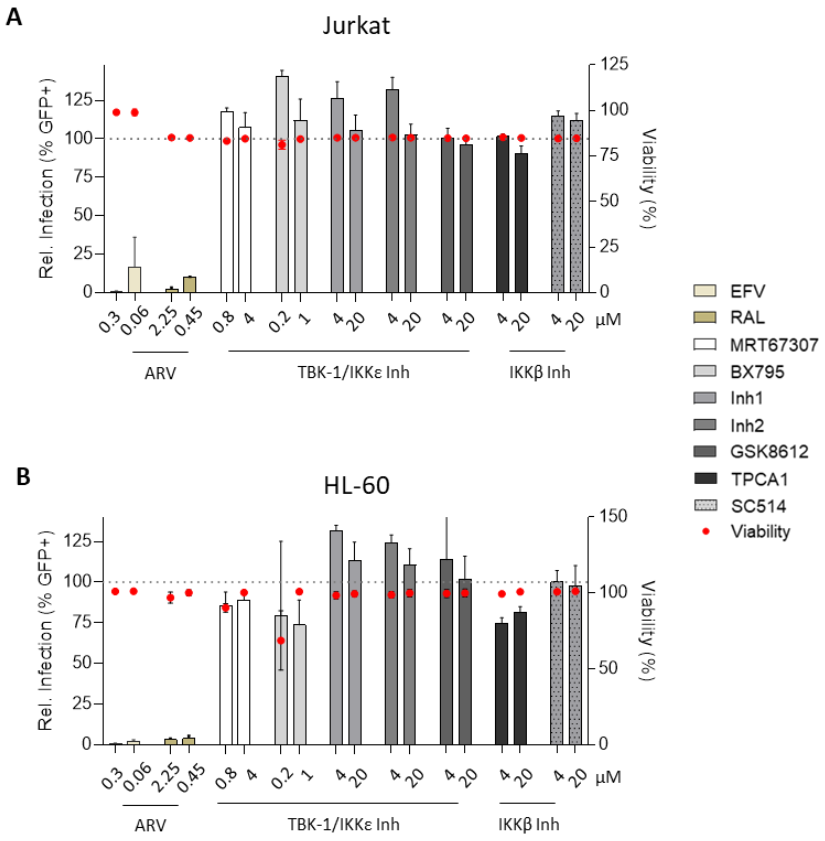


Figure 19. Antiviral activity of IKK inhibitors in acute HIV-1 infection cell models. IKKis do not exert anti-HIV activity on *in vitro* acute infection in lymphoid Jurkat **(A)** and myeloid HL-60 cells **(B)** as measured by the generation of GFP+ cells 72h after infection with VSV-HIG. Anti-HIV compounds efavirenz (EFV) and raltegravir (RAL) were used as controls. Grey-dashed line indicates infection level in ND control. Red dots indicate relative cell viability. Mean ± SD of three independent experiments is shown.

1.2.4. Anti-HIV efficacy of Antiretroviral drugs is not modified by IKK inhibitors

Given that IKKis do not exert anti-HIV activity, it follows that combinations with ART will be required to sustain viremic suppression in PLWH. Therefore, we evaluated possible interactions of IKKis with anti-HIV compounds in acute HIV-1 infection models. Similarly, we observed that the IKKis MRT67307 and TPCA-1 do not modify the activity of anti-HIV compounds in *in vitro* acute HIV-1 infection (**Figures 20A and 20B**). Altogether, these findings suggest a curative modality for reducing the viral reservoir with the IKKis that is less likely to compromise viremic control in patients undergoing ART.

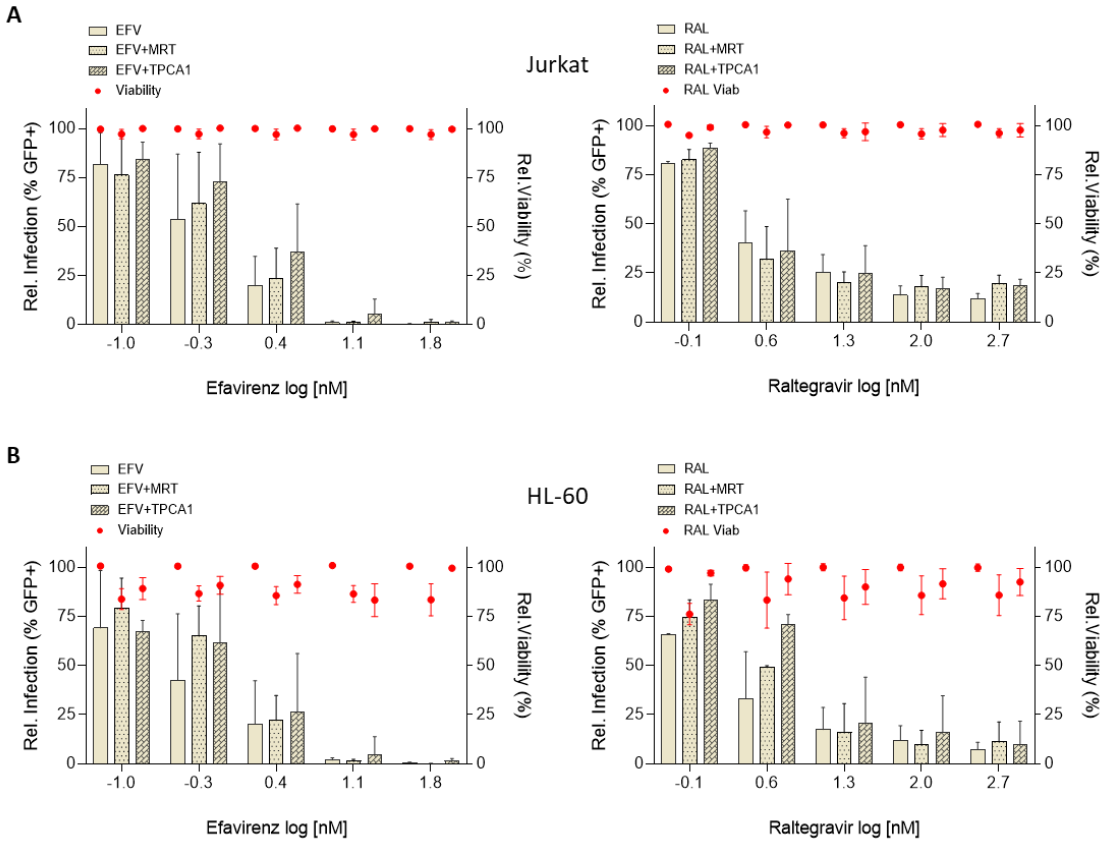


Figure 20. Antiviral activity of anti-HIV agents in combination with IKK inhibitors. Anti-HIV activity of EFV and RAL in Jurkat cells **(A)** coincubated with 4 µM of MRT67307 or TPCA-1 and infected with pseudotyped HIV-1 (VSV-HIG). Infection was measured as the percentage of GFP+ cells 48h after infection. Anti-HIV activity of EFV and RAL in HL-60 **(B)** incubated with 4 µM of MRT67307 or TPCA-1. Red dots indicate cell toxicity of each IKKi alone. Mean ± SD of three independent experiments is shown.

1.2.4. Antiretroviral drugs do not interfere with HIV reactivation activity induced by IKK inhibitors

It is equally important that the presence of ART would not negatively impact the potency of latency reactivation, making us to evaluate the potential interactions between the IKKis and commonly used anti-HIV compounds, MRT67307 and TPCA-1 activity in J-HIG and HL-HIG cells incubated with NNRTI (EFV) and integrase inhibitor (RAL). Neither EFV nor RAL modifies the activity of MRT67307 and TPCA-1 as a reversal agent of HIV latency in J-HIG (**Figure 21A**) and HL-HIG (**Figure 21B**). HIV-1 reactivation level was similar to the induction achieved by each IKKi alone.

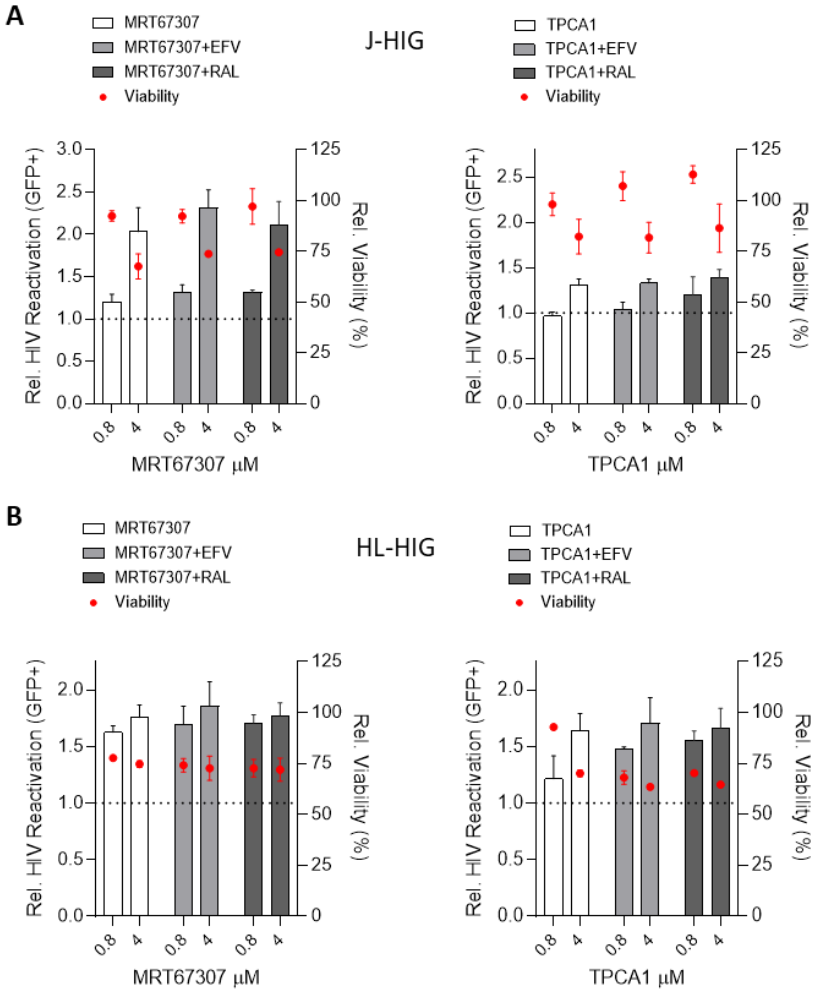


Figure 21. HIV-1 reactivation capacity of IKKis in the presence of antiretroviral drugs. Reactivation ability of MRT67307 (4 µM) and TPCA-1 (4 µM) is not affected by the co-treatment of latently infected lymphoid J-HIG (A) or myeloid HL-HIG (B) cells with fixed concentrations of anti-HIV compounds efavirenz (EFV) (0.3 µM) or raltegravir (RAL) (2.2 µM) determined by the quantification of GFP+ cells (%) after 48 h of co-culture. Grey-dashed line indicates basal reactivation in ND control. Red dots indicate relative cell viability. Mean ± SD of three independent experiments is shown. *p < 0.05; **p < 0.01, ***p < 0.001.

1.2.5. IKKis potently block innate immune signalling pathways

To confirm the capacity of IKKis to modulate innate immune responses, we analyzed protein and gene expression levels in myeloid HL60 treated with either MRT67307 or TPCA-1. Evaluation of innate immune signalling activation identified a partial block characterized by decreased phosphorylation of STAT1 and subsequent signalling pathway (**Figure 22A**). Importantly, either treatment with MRT67307 or TPCA-1 decreased the gene expression levels of cytokines IL1β, IL8, chemokine CXCL10 and TNFα in HL60, in contrast to the upregulation of these genes observed in PMA treatment condition (**Figure 22B**). Next, we evaluated the expression of proinflammatory cytokines by intracellular staining of PBMCs from healthy donors 24 h post-treatment with IKKis using flow cytometry. Treatment with MRT67307 or TPCA-1 did not trigger pro-inflammatory cytokines (IL-1β, IL-6, TNFα) compared to either PMAi or LPS treatment conditions in PBMCs (**Figure 22C**). Overall, these results support the role of IKKis as immune modulators which might be useful in tackling hyperinflammatory responses in chronic HIV-1 infection.

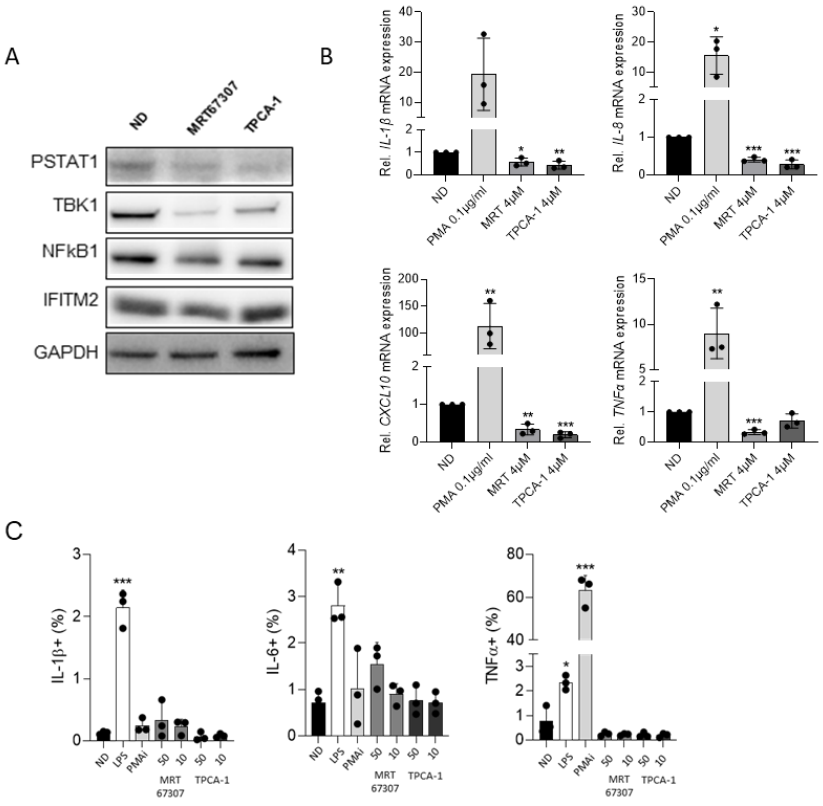


Figure 22. IKK inhibitors block innate immune signalling (A) Characterization of innate response signalling by Western blot analysis of lysates of untreated HL-60 (no drug, ND) or treated with 4 µM MRT67307 or and TPCA-1. A representative blot is shown. **(B)** Gene expression of proinflammatory cytokines IL-1β, IL-8, CXCL10 and TNFα as measured by RTqPCR after 16 h of treatment. **(C)** Expression of proinflammatory cytokines IL-1β, IL-6 and TNFα as measured by intracellular labelling by flow cytometry after 24 h of treatment. Mean ± SD of three independent experiments is shown. *p < 0.05; **p < 0.01.

CHAPTER 2. IDENTIFICATION AND CHARACTERIZATION OF SELECTIVE JAK2 INHIBITORS AS MODULATORS OF HIV-1 LATENCY

Summary

The latency reactivation capacity of a subclass of selective JAK2 inhibitors was characterized as a potential novel therapeutic strategy for HIV-1 cure. Notably, the JAK2i fedratinib reversed HIV-1 latency in non-clonal lymphoid and myeloid *in vitro* models of HIV-1 latency and also *ex vivo* in CD4+ T cells from ART+ PLWH, albeit its function was not dependent on JAK2 expression.

We performed whole transcriptomic analysis to delineate the mechanism of action for JAK2i-induced latency reactivation. Interestingly, in depth evaluation of differentially expressed genes, identified a significant upregulation of IRF7 expression despite the blockade of the JAK-STAT pathway and downregulation of proinflammatory cytokines and chemokines. Moreover, IRF7 expression levels positively correlated with HIV latency reactivation capacity of JAK2 inhibitors and also other common LRAs. Collectively, these results represent a promising step towards HIV eradication by demonstrating the potential of innate immune modulation for reducing the viral reservoir through a novel pathway driven by IRF7.

Furthermore, we characterized the latency promoting capacity of the JAK2i pacritinib, demonstrating its potency in blocking LRA-induced reactivation *in vitro* and in *ex vivo*-treated primary cells from PLWH. These results were consistent with the relevance of JAK-STAT signalling blockade as a strategy to prevent HIV-1 latency reversal.

2.1.1. Selective JAK2 inhibitors are potent novel LRAs in non-clonal lymphoid and myeloid models of HIV latency

We evaluated the capacity of JAK2 inhibitors to reverse HIV-1 latency in non-clonal lymphoid (J-HIG) and myeloid (HL-HIG) models of HIV-1 latency. A specific subclass of selective JAK2i, fedratinib and its structural analogue TG101209, showed potent latency reactivation capacity in both models of HIV-1 latency (2-fold increase, $p<0.01$), although two other JAKi, AZD-1480 and AZ-960, were able to reverse latent HIV-1 only in the lymphocyte model (**Figure 23A**). Conversely, the JAK2i pacritinib significantly blocked HIV latency reversal in both models (30% decrease, $p <0.01$), and the pan-JAKi ruxolitinib blocked HIV-1 latency reversal only in lymphoid cells, suggesting a putative role of pacritinib as a latency promoting agent (LPA). Expanded dose-response testing of selective JAK2i confirmed screening results, showing significant viral latency reactivation or blockade in a dose-dependent manner (**Figure 23B**). Results were confirmed by measuring the production of p24 capsid viral protein (CAp24) through viral protein spot (VIP-SPOT) assay, an enzyme-linked ImmunoSpot (ELISpot) approach [187] (**Figure 23C and 23D**), further suggesting that the use of specific subclass of JAK2i may represent a novel strategy for purging the HIV reservoir.

To determine the contribution of JAK2 as the target cellular factor affecting viral latency, we evaluated the latency reversal capacity of fedratinib in JAK2 depleted cells. Knockdown of JAK2 by siRNA did not affect HIV latency reversal capacity neither in fedratinib nor in phorbol 12-myristate 13-acetate (PMA) treated cells (**Figure 23E**), suggesting that JAK2 function is not affecting HIV-1 latency and thus, pointing to a distinct mechanism of action.

2.1.2. Fedratinib induces HIV-1 reactivation in the absence of immune activation in CD4+ T lymphocytes *ex vivo*

To evaluate the LRA capacity of JAK2i *ex vivo*, purified resting CD4+ T cells from infected individuals on ART were treated with fedratinib, the best performing JAK2i and viral RNA production in culture supernatant was quantified using an ultra-sensitive nested qPCR [43]. Characteristics of study participants are summarized in **Table 7**. In concordance with *in vitro* data, fedratinib was able to *ex vivo* reverse HIV-1 latency to a similar extent than in non-clonal *in vitro* cell line models (**Figure 24A**).

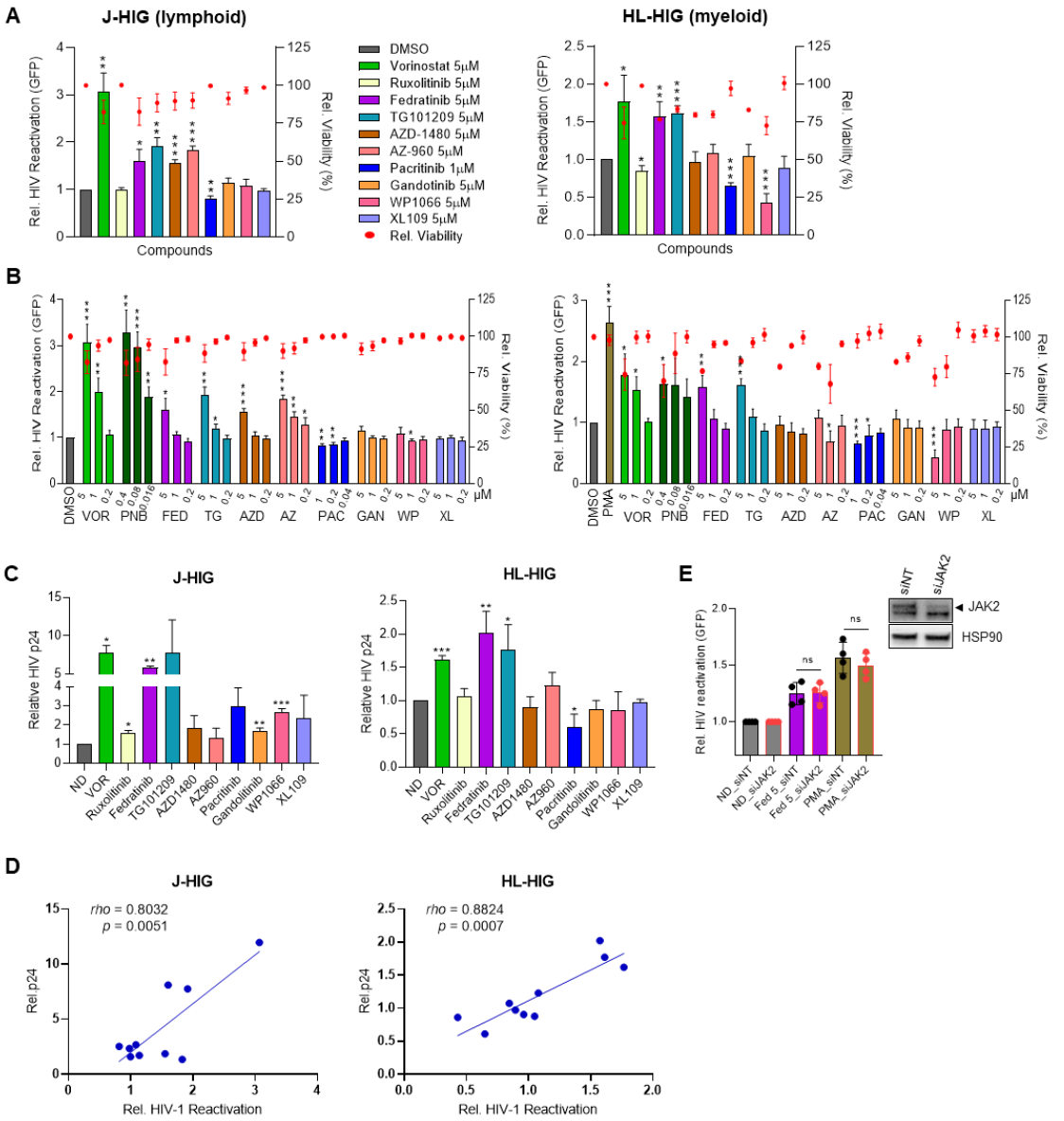


Figure 23. Identification of specific JAK2 inhibitors as latency reversing or latency promoting agents in non-clonal cellular models of HIV-1 latency. (**A**) HIV-1 latency reactivation capacity of JAKi in J-HIG (left panel) and HL-HIG (right panel) models. Viral reactivation was measured as the proportion of GFP+ cells 20 hours post incubation with compounds. Bar plots represent relative HIV-1 reactivation and red dots represent cell viability of treatment conditions normalized to the untreated control (DMSO). (**B**) Expanded screening of JAK2 specific inhibitors in J-HIG (left panel) and HL-HIG (right panel) models. Cells were treated with compounds at the indicated concentrations, as in (A). (**C**) Viral protein production in the J-HIG (left panel) and HL-HIG (right panel) models measured by quantifying HIV-1 CAp24 antigen production using VIP-SPOT assay. (**D**) Pearson correlation plots of CAp24 antigen capture (C) versus relative HIV-1 reactivation capacity as measured by GFP detection (flow cytometry) of indicated compounds (B) in J-HIG and HL-HIG models of HIV latency. (**E**) Effect of JAK2 siRNA knockdown on latency reactivation capacity of LRAs. JAK2 knockdown was confirmed by western blot. Bar plots represent relative HIV-1 reactivation of siRNA JAK2 (siJAK2) and non-targeting (siNT) cells, treated or not with fedratinib at 5 µM or PMA at 100 ng/ml. Data are expressed as mean ± SD of at least three independent experiments. ND, no drug; VOR, vorinostat. ∗p<0.05; ∗∗p<0.01; ∗∗∗p<0.001.

Global T-cell activation is generally detrimental for *in vivo* use, generating interest in LRAs that do not activate T cells [97,198]. To evaluate JAK2i impact on T-cell activation, primary CD4+T cells from uninfected donors were treated *ex vivo* with the selective JAK2i, fedratinib and pacritinib, the pan-JAKi ruxolitinib or PMA+ionomycin as a positive control of immune activation, prior to immunophenotypic characterization by flow cytometry. *Ex vivo*, all JAKi potently blocked CD4+T cells immune activation with significant reductions in the frequency of activation markers HLA-DR and CD25 (**Figure 24B**), which have been linked to increased susceptibility of CD4+T cells to productive HIV-1 infection [199]. However, in contrast to the other JAKi, treatment with fedratinib slightly induced the early activation marker CD69 but markedly less than the immune activation observed upon treatment with PMA+ionomycin (Fold change FC=2, p=0.002 vs FC=89, p<0.001).

Next, we characterized the relative abundance of the distinct CD4+ T cell subsets treated with JAKi, as distinct CD4+ T cell populations differentially contribute to HIV-1 reservoir [200]. Phenotypic characterization of the various CD4+ T-cell populations showed no significant changes in the quiescent naïve (T_N) and T_{CM} CD4+ T cell phenotype upon treatment with all JAKi relative to the untreated control (**Figures 24C** and **24D**). However, we observed significantly higher enrichment of T_{EM} CD4+ T cell phenotype in fedratinib treated lymphocytes (FC=2.3 vs. untreated control, p<0.01) compared to either pacritinib or ruxolitinib (FC=1.4 vs. untreated control, p<0.01), recapitulating previous observation that T_{EM} phenotype potentiates HIV-1 latency reversal in CD4+ T cells [56]. Notably, fedratinib favoured T_{EM} enrichment over T_{TM} CD4+ T cell enrichment, with a concomitant reduction in the T_{TM} phenotype showing a similar trend than PMA+ionomycin, although to a much lesser extent.

Table 7. Immunological and virological characteristics of study participants at the time of cell sample collection

ID	Age	Sex	Ethnicity	Estimated min. length of HIV infection (years)	Estimated min. length of viral suppression (years)	CD4 Nadir (cells/μl)	CD4 count (cells/μl)	Viral Load (copies/ml)	ART Regimen*
p1	56	M	Caucasian	13	10	535	1075	<40	RAL/FTC/TAF
p2	44	M	Caucasian	23	11	455	908	<40	DTG/ABC/3TC
p3	38	M	Caucasian	4	4	460	932	<40	DRV/COBI
p4	41	M	Caucasian	8	6	413	1002	<40	DTG/3TC

* ABC, abacavir; COBI, cobicistat; DRV, darunavir; DTG, dolutegravir; FTC, emtricitabine; RAL, raltegravir; TAF, tenofovir alafenamide; 3TC, lamivudine

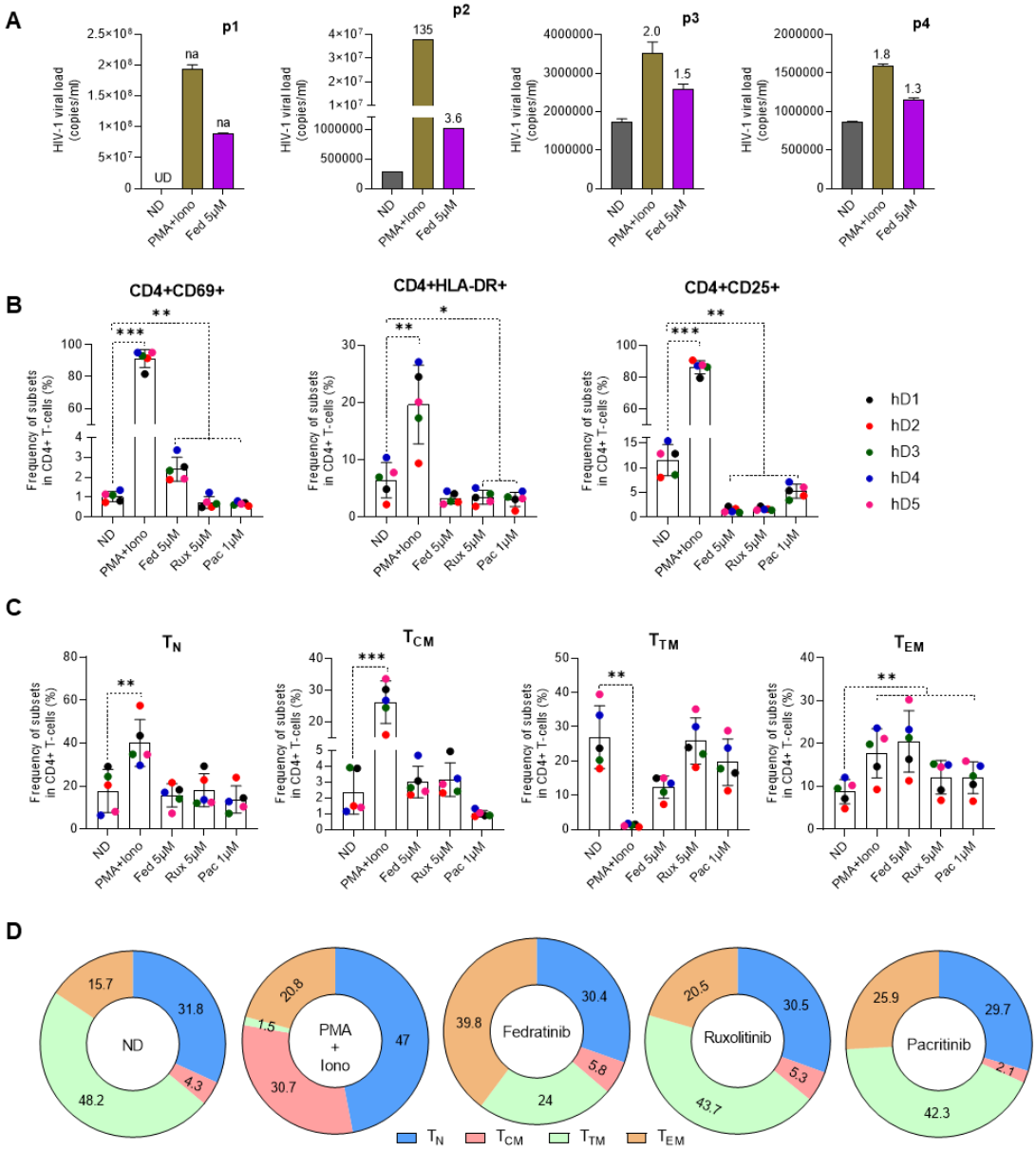


Figure 24. Fedratinib induces HIV-1 reactivation in the absence of immune activation in primary CD4+ T lymphocytes *ex vivo*. (**A**) Quantification of HIV-1 RNA in the cell culture supernatant of isolated CD4+ T cell from ART suppressed HIV-1+ individuals treated with fedratinib. PMA+Iono was used as a positive control for HIV-1 latency reactivation. Absolute HIV-1 copy number (copies/ml) for each HIV-1+ individual are represented as bar plots, and fold changes (FC) relative to the corresponding untreated replicate (ND) are indicated above treatment bars. Undetermined ct values (UD), and FC values if not available (na) are highlighted in the chart. (**B**) Immune cell activation of JAKi-treated CD4+ T cells from healthy donors (n = 5). CD4+ T cells were stained with early (CD69) and late activation (HLA-DR and CD25) markers for flow cytometry 72 h post treatment with compounds. (**C**) Frequency of major CD4+ T cell subsets from JAKi-treated CD4+ T cells from healthy donors. T_N, naïve T cells; T_{CM}, central memory T cells; T_{TM}, transitional memory T cells; T_{EM}, effector memory T cells. (**D**) Corresponding contribution of individual CD4+ T cell subsets to the major CD4+ T cell pool analysed in (C). Numbers inside sections indicate percentages. Data are pooled from the five healthy donors from (C). Bars show mean ± SD ∗p<0.05; ∗∗p<0.01; ∗∗∗p<0.001.

2.1.3. Gene expression changes following JAK2i treatment are comparable to other LRAs

To delineate the mechanism driving JAK2i-induced effects on HIV-1 latency, whole transcriptome profiling was performed on our myeloid latency *in vitro* model, treated with the LRA fedratinib, the LPA pacritinib, the pan-JAKi ruxolitinib and PMA as a positive control of latency reactivation and immune activation. Hierarchical clustering of all samples using the union of all differentially expressed genes (DEGs) comparing each condition to the control revealed distinct genetic signatures amongst the JAKi [201] (**Figure 25A**). Interestingly, within the JAKi cluster, the LRA fedratinib showed the highest similarity with PMA, the condition with the highest genetic perturbations in contrast to the LPA pacritinib that was identified as the condition with the lowest gene expression changes, being similar to the untreated control (**Figure 25B**). A closer study of overlapping DEGs between the LRAs fedratinib and PMA revealed co-upregulation of several host genes associated with HIV-1 replication cycle, including TNF-α, TNF-associated immediate early response gene 3 (*IER3*) and *CDKN1A* (encoding the cyclin-dependent kinase p21) (**Figure 25C** and **Figure 26**) [202].

Next, we performed gene-set enrichment analysis (GSEA) using the hallmark gene-sets to elucidate signaling pathways involved in HIV-1 latency modulation by these compounds [203]. Enriched gene-sets for each treatment condition relative to the untreated control were annotated and hierarchically clustered with respect to their normalized enrichment scores. Overall, JAKi induced global downregulation of signalling processes, including several pathways involved in cellular metabolism (**Figure 25D**). Unexpectedly, the pathways positively enriched upon fedratinib and PMA treatment (the majority of the genes in these pathways are upregulated), were mainly downregulated in the other JAKi tested. These include gene-sets up-regulated in response to ultraviolet light (UV_RESPONSE_UP), apoptosis (APOPTOSIS) and a group of pathways related to innate immune signaling as TNF-alpha and TGF-beta signalling and IL6-JAK-STAT3 pathway, being contrariwise to its reported function.

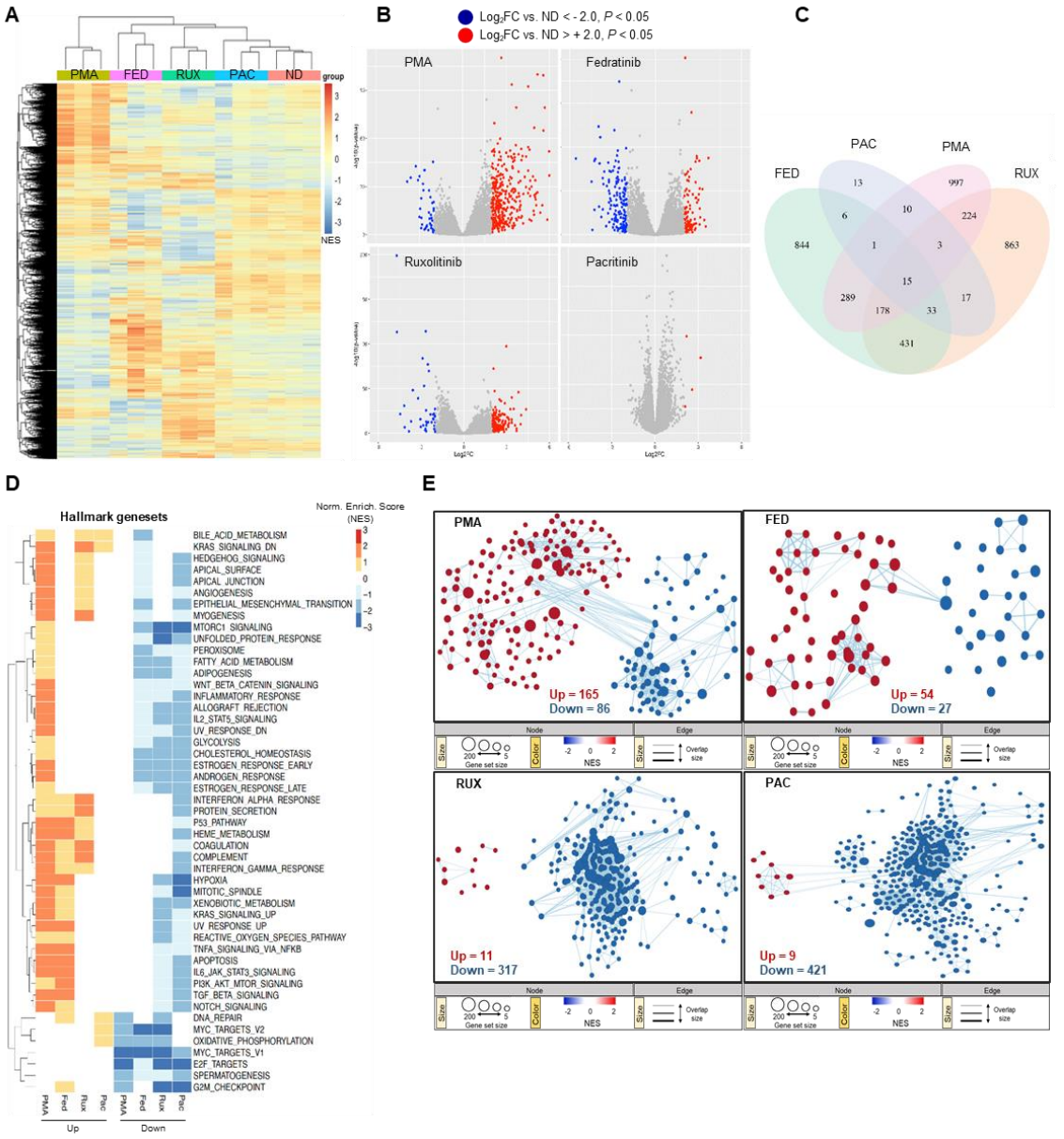


Figure 25. Gene expression changes following fedratinib treatment are comparable to common LRAs. (**A**) Heat map representation of gene expression changes after treatment of HL-HIG cells with JAKi and PMA and untreated control (ND). Drugs were used at concentrations previously shown to be effective at reversing or promoting latency in our model. Heat map for the different treatment conditions was generated by unsupervised hierarchical clustering of significantly expressed genes ($p < 0.05$). (**B**) Volcano plots of differentially expressed genes (DEG) for each treatment condition relative to the untreated control (ND). DEGs were selected based on Log2 gene expression (Log2FC) and $p < 0.05$. (**C**) Venn diagram of overlapping DEGs among treatment conditions shown in (B). (**D**) Gene set enrichment analysis (GSEA) for the different treatment conditions using Hallmark genesets. Heatmap visualization of the transcriptional signatures in the distinct treatment conditions was generated using an unsupervised hierarchical clustering of normalized enrichment scores (NES). (**E**) Reactome gene set enrichment map of significantly enriched pathways. The enrichment patterns for the Reactome gene set are highlighted and nodes manually laid out for clarity (full annotation of gene set enrichment map for fedratinib treatment condition is shown in Figure S4). Node size represents number of genes, node colour represents significance (NES), and edge thickness represents number of shared genes. Significantly down- or up-regulated genes (A and B) and gene sets (D and E) are highlighted in blue or red, respectively. Transcriptomic data represents RNA sequencing analysis from three independent experiments.

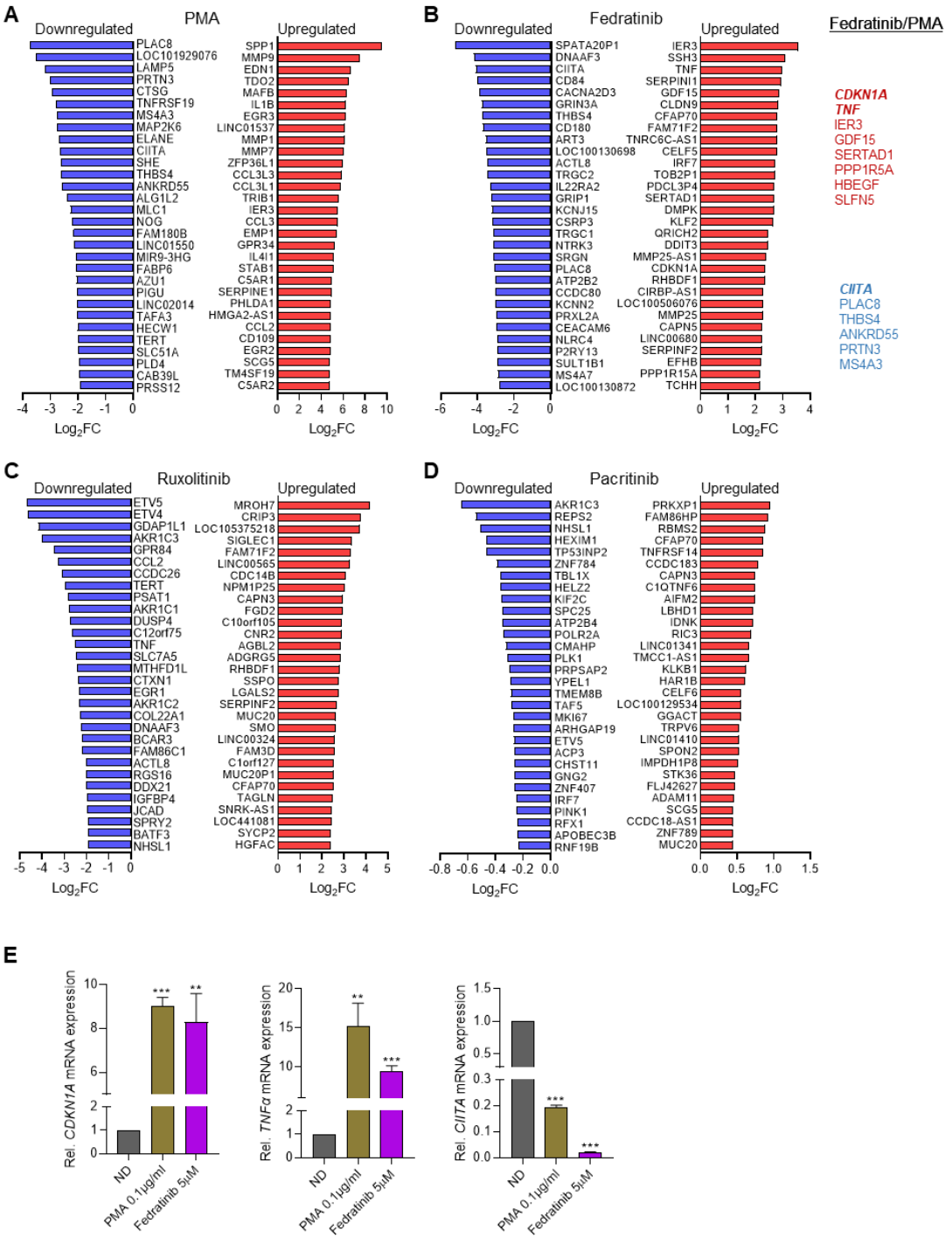


Figure 26. Top 30 downregulated and upregulated genes following JAKi treatment in a model of HIV-1 latency (HL-HIG). Bar plots of top 30 differentially enriched genes (DEG) for each treatment condition (**A, PMA; B, Fedratinib; C, Ruxolitinib and D, pacritinib**) relative to the untreated control (ND), based on Log2 gene expression (Log2FC) and $p < 0.05$. Significantly down- or up-regulated DEG are highlighted in blue or red, respectively. **(E)** Confirmation of significantly top upregulated and downregulated genes in additional samples. Bars show mean ± SD ∗p<0.05; ∗∗p<0.01; ∗∗∗p<0.001.

Reactome GSEA revealed similar gene-set enrichment patterns between PMA and fedratinib as the hallmark GSEA, further demonstrating the difference between fedratinib and the other JAKi tested (**Figure 25E**). Notably, fedratinib treatment resulted in the least modulation of signalling pathways compared to other JAKi and PMA. In addition, the gene-set enrichment profile of fedratinib mirrored that observed upon PMA treatment with twice as much tendency for up-regulating signalling pathways as PMA (**Figure 25E**, top panel). Moreover, out of all the treatment conditions, only fedratinib significantly enriched the Reactome HIV budding and maturation gene-set (**Figure 27**), confirming its LRA capacity to induce viral protein production (**Figure 23C**).

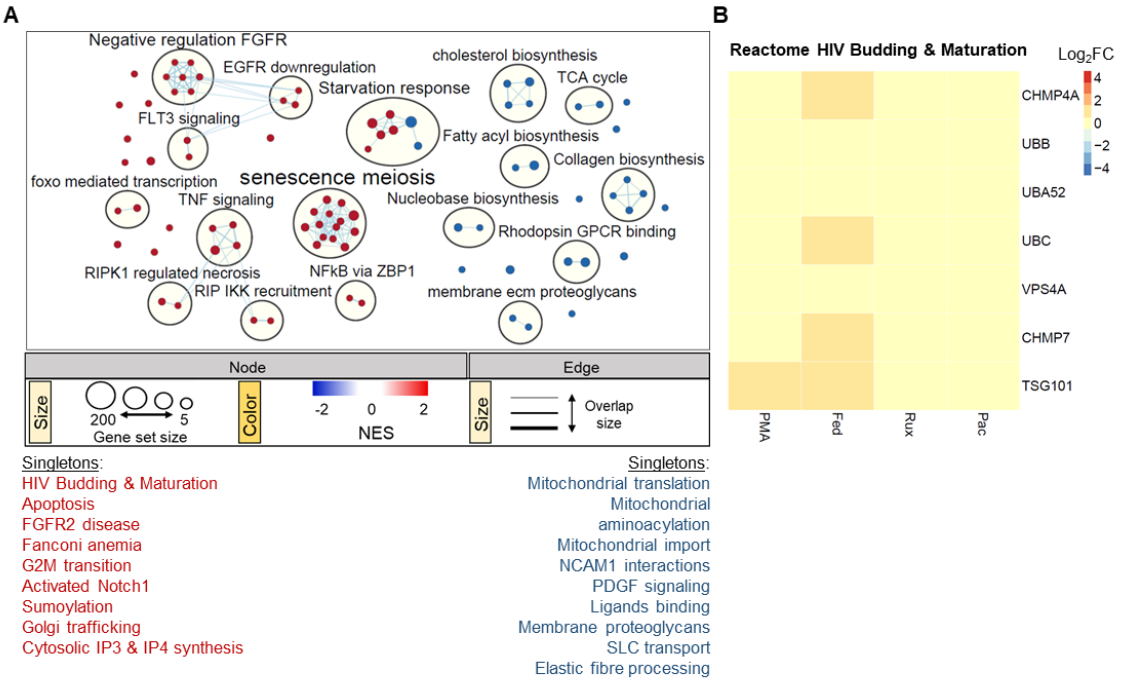


Figure 27. Reactome HIV budding, and maturation signalling is significantly enriched by fedratinib treatment in latently infected model (**A**) Reactome Gene set enrichment map of significantly enriched pathways for fedratinib treatment condition. Reactome Gene set clusters are annotated, and nodes manually laid out for clarity. Single gene sets are highlighted and annotated in-text, underneath the cytoscape enrichment map. Node size represents number of genes, node colour represents significance (NES), and edge thickness represents number of shared genes. (**B**) Gene expression of leading-edge genes from the Reactome HIV Budding and Maturation signalling gene set. Fedratinib treatment condition was used as a reference set for leading-edge genes selection and ranking. Significantly down- or up-regulated gene sets (A) and genes (B) are highlighted in blue or red, respectively.

2.1.4. Fedratinib upregulates IRF7 expression despite blockade of JAK-STAT signalling and cytokine production

Taking into account the differences observed between fedratinib and other JAKi, and the distinct observed effects on JAK-STAT signalling pathway, we conducted a more detailed evaluation of the pathway. Examination of leading-edge genes of the JAK-STAT hallmark gene-set indicated that none of the JAKs was enriched, but instead a significant upregulation of STAT3 and IRF1 was observed (**Figure 28A**). To confirm the RNA-Seq data, the modulation of JAK-STAT signalling pathway was validated in cells treated with either fedratinib or ruxolitinib. As expected, all JAKi induced a potent downregulation of pJAK2, pSTAT1 (**Figure 28B**) and IFN-stimulated genes as IL6, CXCL10 and IL8 in multiple cell lines (**Figures 28C and 28D**), indicating efficient blockade of the JAK-STAT signalling pathway. Similar results were obtained in A549 dual cells, where fedratinib blocked IFN-sensitive response element (ISRE) and NF-κB induced transcription, measured by corresponding reporter genes, despite enrichment of the TNFα signalling pathway (**Figures 28E-G**).

However, fedratinib upregulated the expression of the interferon regulatory factors IRF1 and IRF7 (**Figure 28B**), although only IRF7 expression was significantly modulated, depending on the JAKi used, ranging between no change to 8 FC from pacritinib to fedratinib, respectively (**Figure 29A**). We also observed that the expression of IRF7 positively correlated with other transcriptional activating IRFs, i. e., IRF1, IRF3, IRF5 and IRF9, but not with the transcriptional repressor IRF8 (**Figure 29B**) [204].

The transcriptional activity of the IRFs is induced by the activation of the JAK-STAT signalling pathway in response to cytokine and IFN-induced phosphorylation of the JAKs and STATs, leading to the transcription of ISGs and other target genes. Hence, we further evaluated the dependency of fedratinib-induced IRF7 expression and classical JAK-STAT signalling over time, using lipopolysaccharide (LPS) as a positive control for activation of the JAK-STAT signalling pathway. Interestingly, LPS failed to induce IRF7 expression despite clearly inducing activation of the JAK-STAT pathway (phospho-JAK2 induction). On the contrary, fedratinib treatment significantly upregulated IRF7 expression despite blocking JAK2 activation in a time-dependent manner (**Figure 29C**). Overall, our results suggest that IRF7 may play a role in HIV-1 reactivation induced by fedratinib.

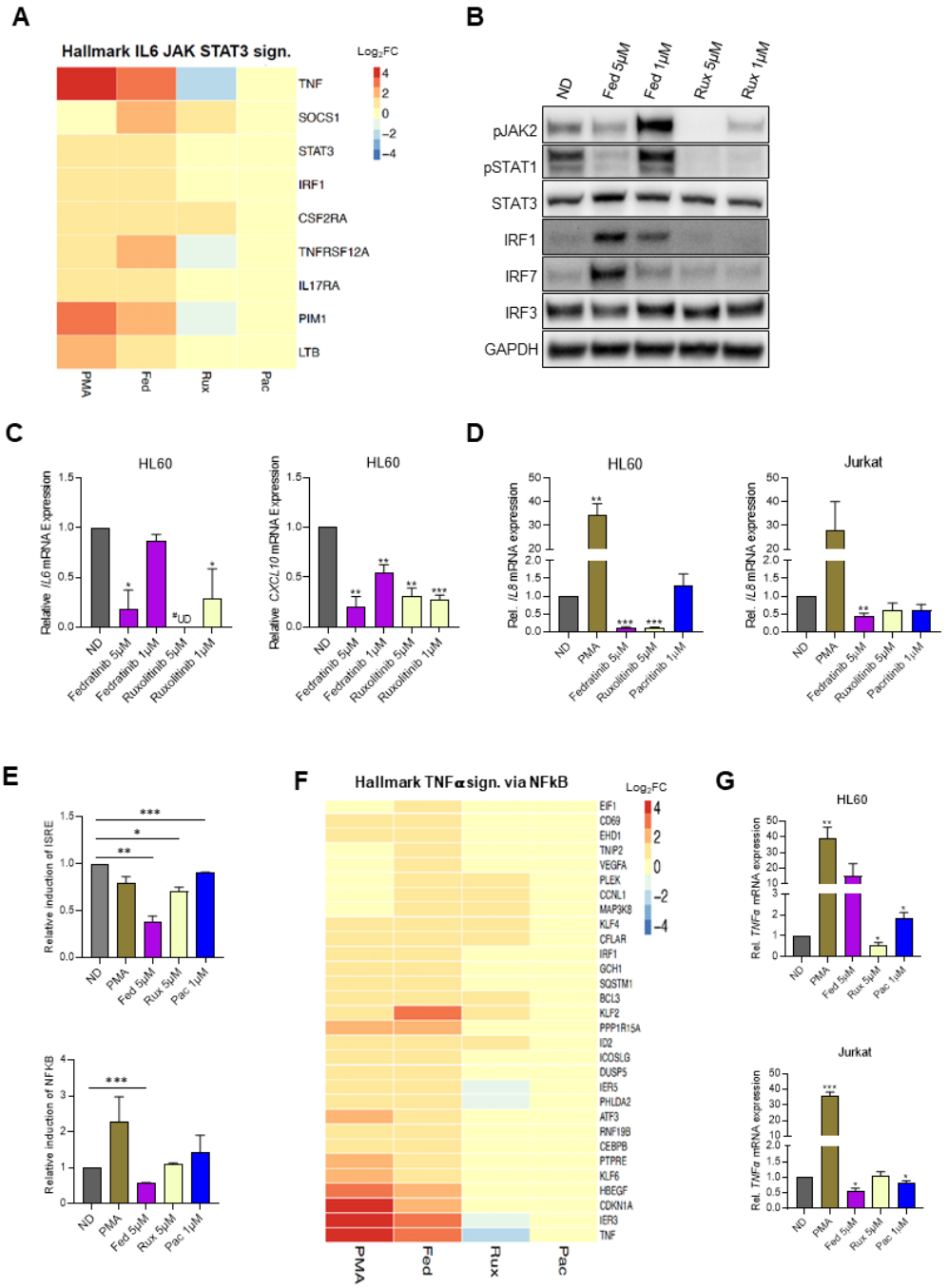


Figure 28. Fedratinib upregulates IRF7 expression despite blockade of JAK-STAT signalling and cytokine production. (**A**) Gene expression of leading-edge genes from the Hallmark IL6-JAK-STAT3 signalling gene set. Fedratinib treatment condition was used as a reference set for leading-edge genes selection and ranking. (**B**) Evaluation of JAK-STAT signalling pathway at protein level in JAKi-treated HL60 cells using western blot analysis. GAPDH was used as a loading control. (**C**) Gene expression of *IL6*, *CXCL10* and (**D**) *IL8* following treatment with JAKi or PMA. Relative mRNA expression was measured by quantitative RT-PCR and normalized to GAPDH. (**E**) Induction of promoters for interferon stimulatory response element (ISRE) and NFκB by JAKi in A549-Dual™ hACE2-TMPRSS2 cells. (**F**) Gene expression of leading-edge genes from the Hallmark TNFα via NFκB signalling gene set. Fedratinib treatment condition was used as a reference set for leading-edge genes selection and ranking. Significantly down- or up-regulated genes are highlighted in blue or red, respectively. (**G**) TNFα gene expression of JAKi-treated HL-60 (Top panel) and Jurkat (bottom panel) cells. Data are expressed as mean ± SEM of at least three independent experiments. ∗p<0.05; ∗∗p<0.01; ∗∗∗p<0.001.

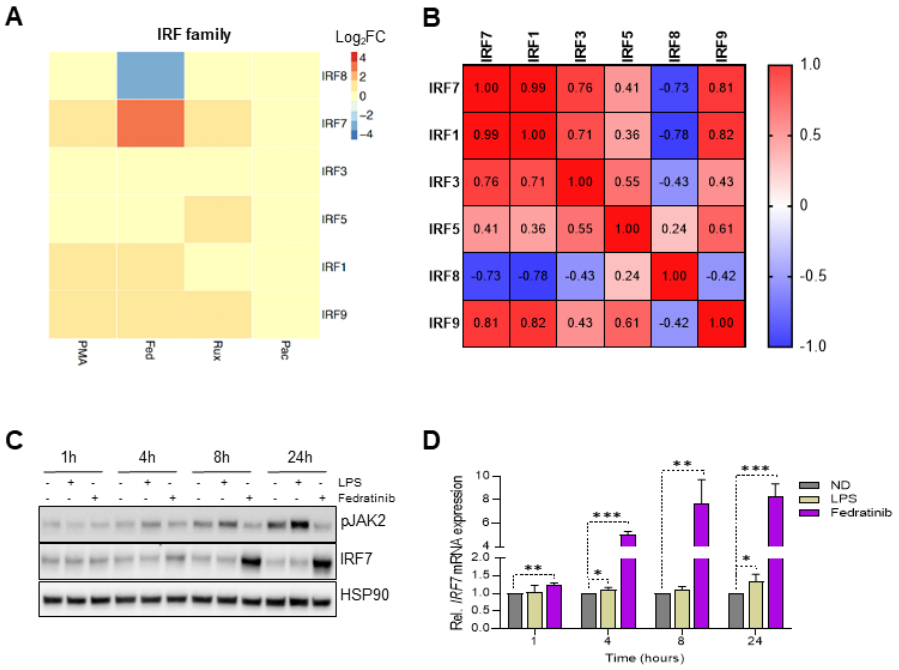


Figure 29. JAKi differentially-induced IRF7 expression is time-dependent. (**A**) Heatmap showing all differentially expressed interferon regulatory factors (IRF) from whole transcriptome analysis. (**B**) Spearman correlation matrix for differentially expressed IRF. Negatively and positively correlated gene expression of IRF in the transcriptomic data are shown in blue and red, respectively. (**C**) Western blot analysis showing time dependence of IRF7 induction by fedratinib despite consistent blockade of JAK2 phosphorylation. LPS treatment was used as a positive control for the activation of the JAK-STAT pathway (induction of pJAK2), and HSP90 as a loading control. (**D**) Induction of IRF7 gene expression was confirmed by qRT-PCR. Data are expressed as mean ± SEM of at least three independent experiments. ∗p<0.05; ∗∗p<0.01; ∗∗∗p<0.001.

2.1.5. Induction of IRF7 expression positively correlates with latency reversal modulation

To further investigate IRF7 role in latency reversal, IRF7 expression was knocked-down by siRNA in myeloid HIV-1 latently infected cells. IRF7 knockdown significantly impaired the HIV-1 latency reversal capacity of fedratinib (p = 0.005, **Figure 30A**), confirming the key role of IRF7 expression. Then, modulation of IRF7 expression was evaluated in the extended panel of JAK2i, showing significant upregulation only in cells treated with the other JAK2i acting as LRAs, in contrast to the significant downregulation of IRF7 by the LPA Pacritinib, at both protein (**Figure 30B**) and mRNA expression level (**Figure 30C**). Indeed, the latency-reversing capacity of the distinct JAK2i tested, directly correlated with IRF7 expression (rho=0.86, p=0.0056, **Figure 30D**). In contrast, treatment with JAK2i-LRAs significantly blocked CXCL10 production (**Figure 30E**), providing additional proof of the independent role of anti-inflammatory signalling and IRF7 induction and indicating an inverse correlation with latency reactivation in our model (rho=-0.715, p=0.046, **Figure 30F**). Overall, these results suggest a direct effect of IRF7 expression on HIV-1 latency reversal, putatively through LTR transcriptional induction.

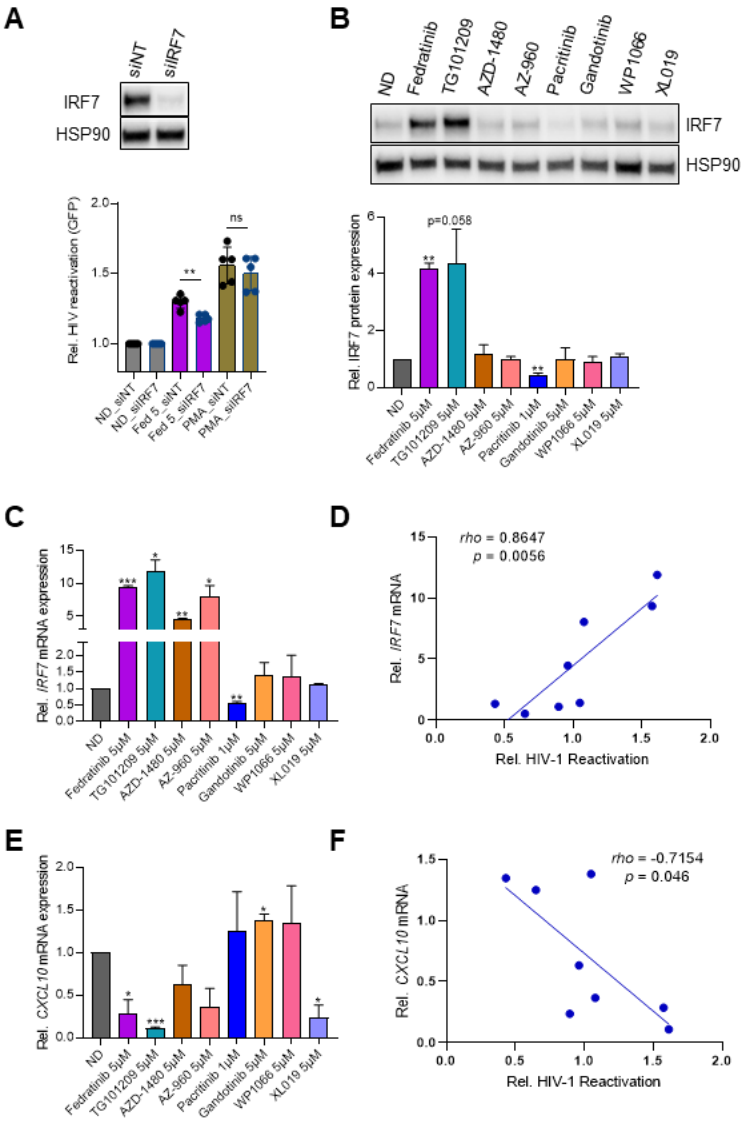


Figure 30. Modulation of IRF7 expression correlates with latency reversal capacity. (**A**) Effect of IRF7 siRNA knockdown on latency reactivation capacity of LRAs. IRF7 knockdown was confirmed by western blot (pictograph). Bar plots represent relative HIV-1 reactivation of IRF7 knockdown (siIRF7) and non-targeting (siNT) control cells, treated or not with fedratinib at 5 µM or PMA at 0.1µg/ml. (**B**) IRF7 protein expression in HL60 cells treated with the distinct JAK2i. A representative experiment is shown. Bar plots represent the mean quantification of bands obtained by densitometry analysis of 3 independent experiments. Values were normalized to that of Hsp90 used as a loading control and relativized to the untreated condition (ND). (**C** and **E**) Gene expression of *IRF7* and *CXCL10* in JAK2i -treated HL-60 cells. Relative mRNA expressions are expressed as fold-change differences in expression of gene-of-interest relative to GAPDH gene. (**D** and **F**) Correlation plots of *IRF7* and *CXCL10* gene expression (C and D) versus HIV-1 latency reversal capacity of JAK2i-treated cells. Values were normalized to the untreated control. Data are expressed as mean ± SD of at least three independent experiments. ∗p<0.05; ∗∗p<0.01; ∗∗∗p<0.001.

Regarding the viral proteins studied till date, HIV-1 Tat has attracted more attention in viral latency because it potently plays a crucial role in viral transcription regulation. Thus, to further investigate the role of IRF7 in HIV-1 viral transcription, an HIV-1 Tat transactivation assay was

performed in HeLa TZM-bl cells. TZM-bl cells harbour an integrated copy of HIV-1 LTR, controlling luciferase reporter gene expression, which mimics a latent integrated provirus. First, TZM-bl cells were validated as an appropriate model for measuring Tat-dependent viral reactivation by dose-dependent transfection of a Tat expression plasmid in the presence of PMA, as a positive control of transcription induction (**Figure 31A**). Then, Tat expressing TZM-bl cells were treated with JAK2i to determine the role of fedratinib-induced IRF7 expression on Tat-dependent viral reactivation. IRF7-dependent latency reactivation was observed upon treatment with JAK2i, with significant induction in Tat-mediated HIV-1 transcription by fedratinib in contrast to pacritinib (p=0.003 vs p=0.079, **Figures 31B** and **31C**). More importantly, IRF7 expression levels significantly correlated with Tat-mediated transactivation (**Figure 31D**, right panel), further demonstrating the role of IRF7 in Tat-mediated HIV-1 transcription.

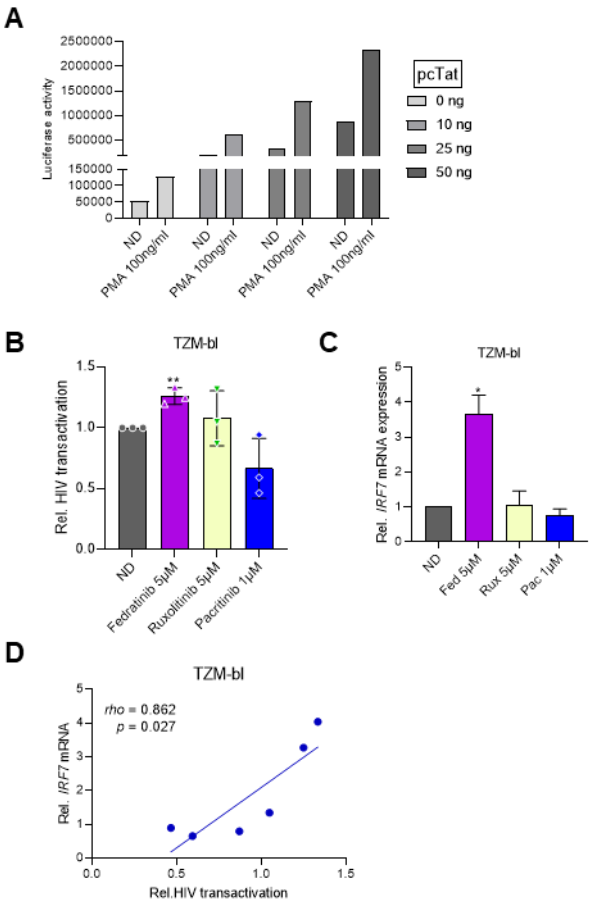


Figure 31. Fedratinib induces Tat-dependent viral expression of HIV-1 LTR promoter and correlated with IRF7 expression. (**A**) Luciferase activity of mock and HIV-1 Tat transfected HeLa TZM-bl cells. HeLa TZM-bl cells were transfected or not with Tat-expressing plasmid for 24h and HIV-1 Tat expression was measured by a luciferase-based assay 20 h post incubation with PMA. (**B**) Relative Tat-induced HIV-1 transactivation upon JAK2i treatment. (**C**) Relative *IRF7* mRNA expression of non-transfected TZM-bl cells treated with JAK2i for 20h. (**D**) Correlation between HIV-1 transactivation and IRF7 expression in TZM-bl cells. ∗p<0.05; ∗∗p<0.01; ∗∗∗p<0.001. Data are expressed as mean ± SD of at least three independent experiments.

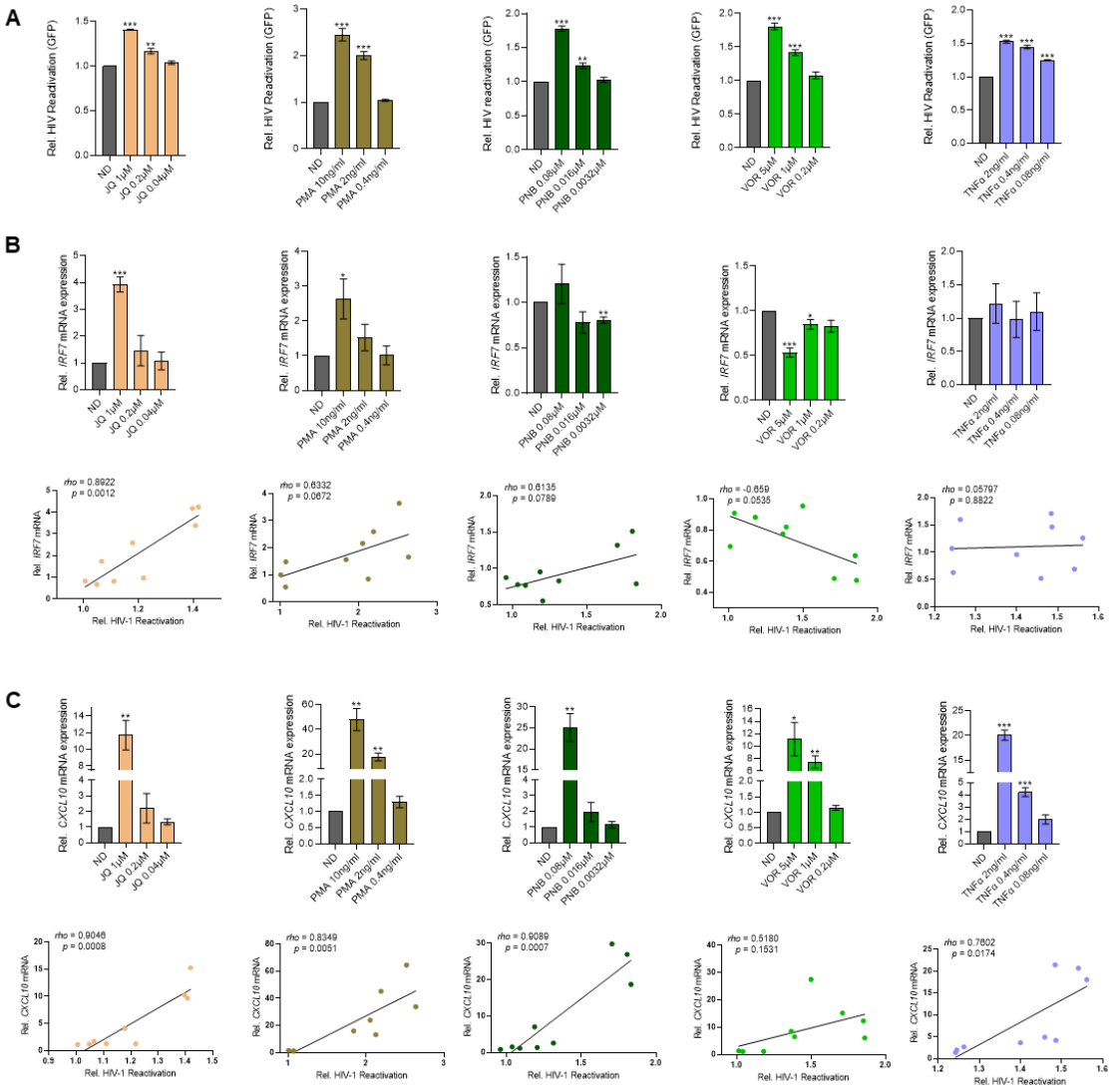


Figure 32. Latency reactivation by common LRAs positively correlates with the induction of ISG. (**A**) Dose-response latency reversal activity by distinct well-described LRAs in a model of HIV-1 latency (HL-HIG). Bar plots represent relative HIV-1 reactivation normalized to the untreated control (ND). (**B**) Top panels, *IRF7* gene expression upon treatment with the indicated LRAs. Relative mRNA expressions are expressed as fold-change differences in expression of gene-of-interest relative to GAPDH gene. Bottom panels, pearson correlation plots of HIV latency reversal activity versus *IRF7* gene expression. (**C**) Top panels, *CXCL10* gene expression upon treatment with the indicated LRAs. Relative mRNA expressions are expressed as fold-change differences in expression of gene-of-interest relative to GAPDH gene. Bottom panels, pearson correlation plots of HIV latency reversal activity versus *CXCL10* gene expression. All statistical comparisons were performed with Student's t tests. ∗p<0.05; ∗∗p<0.01; ∗∗∗p<0.001. Data are expressed as mean ± SD of at least three independent experiments.

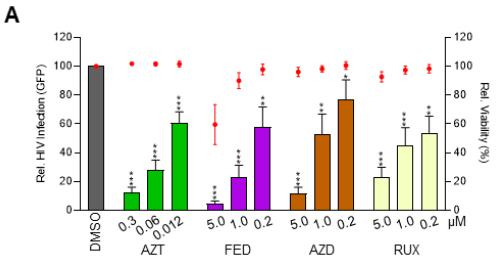
2.1.6. IRF7 is a general regulator of HIV-1 latency

To investigate whether IRF7 plays a more general role in HIV-1 latency, we screened a panel of common LRAs for IRF7 dependency, including the HDACi vorinostat (VOR) and Panobinostat (PNB), the BET bromodomain inhibitor JQ1, TNF-α, and PMA. As expected, all tested LRAs

showed significant latency reactivation capacity in the HL-HIG model (p<0.001, **Figure 32A**). JQ1 and PMA treatment significantly upregulated IRF7 expression which positively correlated with its latency reactivation capacity (rho=0.892, p=0.001 for JQ1; rho=0.633, p=0.067 for PMA, **Figure 32B**). Conversely, no effect was observed for TNF-α or PNB, and the HDACi VOR significantly blocked IRF7 induction in the HL60 model, being negatively correlated with its latency reactivation capacity. Importantly, all common LRAs significantly induced CXCL10 production with a strong positive correlation, in contrast to the JAK2i-LRA (**Figure 32C**). Altogether, these data suggest that although IRF7 role in latency reversal is not common to all LRAs, it is not limited to JAK2i, indicating a novel but yet unrecognized mechanism that might be relevant for the development of novel therapeutic strategies directed towards HIV cure.

2.1.7. Fedratinib is a potent antiviral agent in acute HIV infection

Several JAKi, including ruxolitinib and tofacitinib, are potent inhibitors of HIV-1 replication [105,106,119]. Thus, we evaluated the antiviral activity of selective JAK2i fedratinib and AZD1480, and the pan-JAKi ruxolitinib, in acute HIV-1 infection in primary CD4+T cells from healthy donors (n=3). Fedratinib and other JAKi potently blocked HIV-1 replication in acute HIV-1 infection (**Figure 33A**). Interestingly, fedratinib was the most potent antiviral agent of all the JAKi tested (EC_{50}=0.285µM, **Figure 33B**). Finally, this observation raises the possibility that fedratinib, besides reversing HIV-1 latency, might also block new HIV-1 infections, a highly desired effect in a clinical setting.



B

Drugs	EC_{50} (µM)	CC_{50} (µM)
AZT	0.02	>7.5
Fedratinib	0.285	6.661
AZD1480	1.12	>25
Ruxolitinib	1.863	>25

Figure 33. Fedratinib is a potent antiviral agent in acute HIV infection, specifically targeting multispliced HIV RNA transcripts. (**A**) Antiviral activity of JAKi in acute HIV infection of activated CD4+T cells from healthy donors (n = 3). HIV-1 infection was measured as proportion of GFP+ CD4+ T cells after infection with GFP-encoding NL4-3 env-deficient HIV-1 clone, 48 h post treatment with compounds at the indicated concentrations. Bar plots represent relative HIV infection and dot plots cell viability of corresponding treatment conditions normalized to the untreated control (DMSO). (**B**) EC_{50} and CC_{50} values of JAKi in acute HIV-1 infection model. Values were calculated from (A). EC_{50}, effective concentration required to block HIV-1 replication by 50% and CC_{50}, concentration required to induce 50% of cell death in cell culture. Values are expressed as mean ± SD. ***p<0.001.

2.2.1. JAK2i pacritinib and baricitinib are potent latency promoting agents

In the previous section, we identified the JAK2i pacritinib as a putative latency promoting agent (LPA) in non-clonal *in vitro* models of HIV-1 latency J-HIG and HL-HIG. This observation is consistent with already existing evidences supporting the role of specific JAKi as selective inhibitors of virus reactivation in cellular models of HIV latency[105,106,119], although baricitinib showed higher potency for blocking seeding of productive and latent infection than ruxolitinib[105], making us to include it as a positive LPA control in our screening. Spontaneous reactivation in HIV-1 latency models is not uncommon, for this reason, we first determined the reactivation block by either pacritinib or baricitiinib alone, observing that while both compounds did not significantly block reactivation in the J-Hig model, they modestly did so in the HL-HIG model (p-value < 0.05). To further evaluate the capacity of JAKi in blocking HIV latency reactivation in the presence of potent reactivation inducing stimuli, we first preincubated lymphoid (J-HIG) and myeloid (HL-HIG) models of HIV latency with either pacritinib or the dual JAK1/2i baricitinib for 2 h before induction of latency reactivation with the LRAs PMA or VOR for 20 h. Compounds were used at concentrations previously shown to be effective in promoting or reversing HIV latency *in vitro*.

There was variability in the extent of latency reactivation observed in both models of HIV latency depending on the different LPA+LRA combinations. In the lymphoid model, only pacritinib was able to significantly reduce PMA and VOR-induced latency reactivation (20% - 34%-fold reduction p-value < 0.05), whereas, baricitinib did not significantly impact LRA-induced reactivation (**Figure 34A**). In myeloid cells, both JAKi were able to limit LRA-induced viral reactivation, with pacritinib presenting similar inhibitory activities (32% - 40%-fold reduction in PMA, p-value = 0.08 and VOR-induced reactivation, p-value < 0.05) and baricitinib presenting higher reduction in PMA than VOR-induced reactivation (52%-fold vs 13%-fold reduction, p-value < 0.05 and 0.30 respectively; **Figure 34B**).

To quantitatively assess interactions between the LPA and LRA combinations, we applied the Bliss independence model for drug combinations. The modified Bliss independence model measure assumes that compounds act through different mechanisms or share similar target, such that their effects multiply (synergy) or diminish (antagonism) when administered in combination[194]. Thus, applying this model to the LPA + LRA combinations, we observed that pretreatment with pacritinib antagonized both LRA-induced reactivation while baricitinib blocked PMA but not VOR-induced reactivation in J-HIG (**Figure 34C**). In the HL-HIG model,

pacritinib and baricitinib exerted antagonistic effects on PMA and VOR-induced reactivation (**Figure 34D**).

Altogether, pacritinib consistently demonstrated a potent inhibitory effect against LRA-induced reactivation in both models of HIV latency, showing promising potential as LPA.

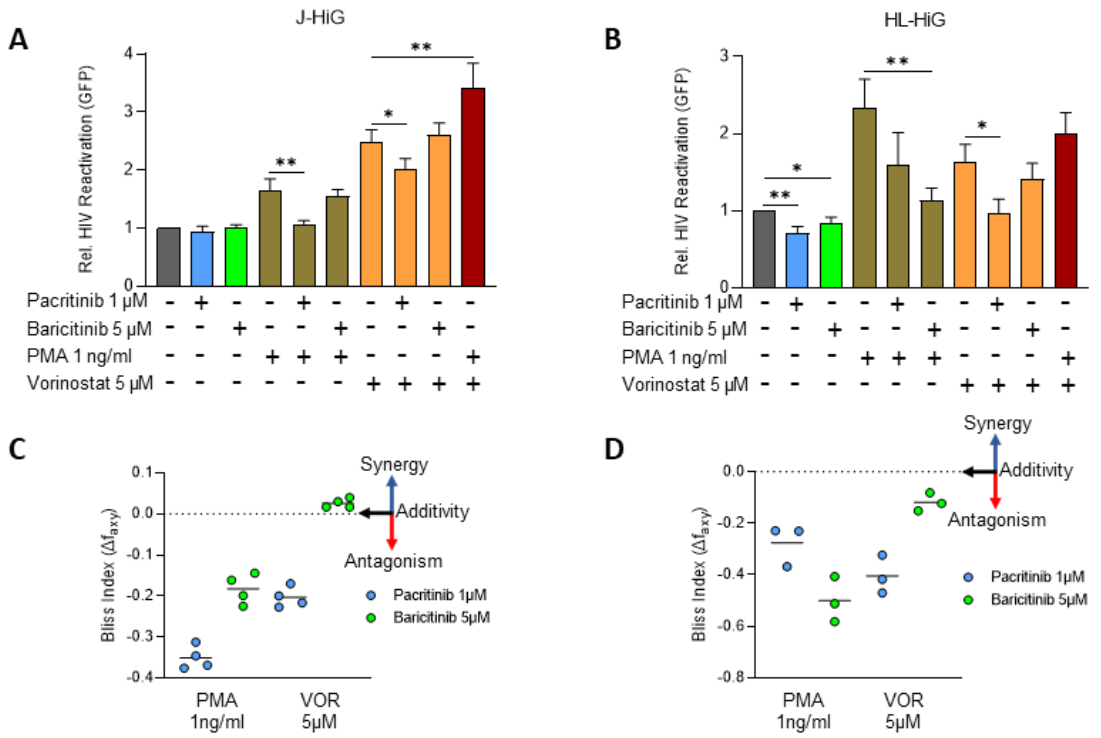


Figure 34. JAK2i antagonize LRA-induced HIV-1 latency reactivation *in vitro*. HIV-1 latency reactivation blocking capacity of either pacritinib or baricitinib in LRA stimulated non-clonal models of HIV-1 latency, lymphoid J-HIG (**A**) and myeloid HL-HIG (**B**). Cell cultures were preincubated with the LPAs pacritinib or baricitinib 2h prior to addition of the LRAs PMA or VOR. Viral reactivation was measured as the proportion of GFP+ cells 20 hours post incubation with compounds. Bar plots represent relative HIV-1 reactivation of treatment conditions normalized to the untreated control (grey bar). (**C**) and (**D**) Corresponding calculations of the Bliss independence index (Δf_{axy})[194] for the LPA+LRA combinations in (A) and (B) respectively. Data are expressed as mean ± SD of at least three independent experiments. ∗p<0.05; ∗∗p<0.01; ∗∗∗p<0.001.

2.2.2. Pacritinib inhibits HIV-1 reactivation and immune cell activation in primary CD4+ T cells *ex vivo*

Having demonstrated the latency promoting effect of pacritinib in LRA-induced reactivation *in vitro*, we extended these findings further by evaluating this effect in primary CD4+ T cells from PLWH (n = 3). CD4+ T cells from ART-suppressed PLWH were pre-treated with pacritinib for 4 h before incubation of cell cultures with PMA. Determination of HIV-RNA copies in the culture

supernatant 72 h post-treatment with compounds revealed a marked reduction in HIV reactivation in pacritinib-treated cells. As expected, PMA induced latency reversal *ex vivo* in CD4+ T cells. However, pre-treatment with pacritinib markedly blocked PMA-induced reactivation in this *ex vivo* model of HIV latency showing antagonism as calculated by the Bliss independence model (**Figure 35A**).

To further explore the impact of pacritinib on CD4+ T cells, we immunophenotyped the major CD4+ T cell populations and immune cell activation markers in pacritinib or PMAi treated primary CD4+ T cells from PLWH (**Table 8**) by flow cytometry. Receptor expression distribution of CD4+T cells as visualized by opt-SNE dimensionality reduction showed no changes between pacritinib and ND treatment conditions, in contrast to PMAi (**Figure 35B**). We observed major changes in the immune activation markers distinguishing PMAi from either ND or the LPA pacritinib. Treatment with pacritinib resulted in reduced expression of CD25 ($p < 0.05$) but did not modify the expression levels of the activation markers CD69 and HLA-DR relative to the ND condition (**Figure 35C**).

Interestingly, we did not observe major changes in enrichment of CD4+ T cell populations, as neither pacritinib nor PMAi significantly modified the frequency of T_N, T_{CM}, T_{TM} and T_{EM} in *ex vivo* treated CD4+ T cells from ART-suppressed PLWH (**Figure 35D**). Overall, this data suggests that pacritinib inhibition of latency reactivation induces a deeper state of latency that does not modify the immunophenotype of primary cells that would otherwise compromise immune cell recovery in PLWH undergoing ART.

Table 8. Immunological and virological characteristics of study participants at the time of cell sample collection

ID	Age	Sex	Ethnicity	Estimated min. length of HIV infection (years)	Estimated min. length of viral suppression (years)	CD4 Nadir (cells/μl)	CD4 count (cells/μl)	Viral Load (copies/ml)	ART Regimen*
p1	56	M	Caucasian	13	10	535	1075	<40	RAL/FTC/TAF
p2	44	M	Caucasian	23	11	455	908	<40	DTG/ABC/3TC
p3	38	M	Caucasian	4	4	460	932	<40	DRV/COBI
p4	41	M	Caucasian	8	6	413	1002	<40	DTG/3TC
P5	48	M	Hispanic	14	12	445	754	<40	DRV/COBI
P6	39	M	Caucasian	8	5	603	902	<40	DTG/ABC/3TC /DOVATO
P7	36	F	Caucasian	7	6	504	1396	<40	RAL/FTC/TAF

* ABC, abacavir; COBI, cobicistat; DRV, darunavir; DTG, dolutegravir; FTC, emtricitabine; RAL, raltegravir; TAF, tenofovir alafenamide; 3TC, lamivudine

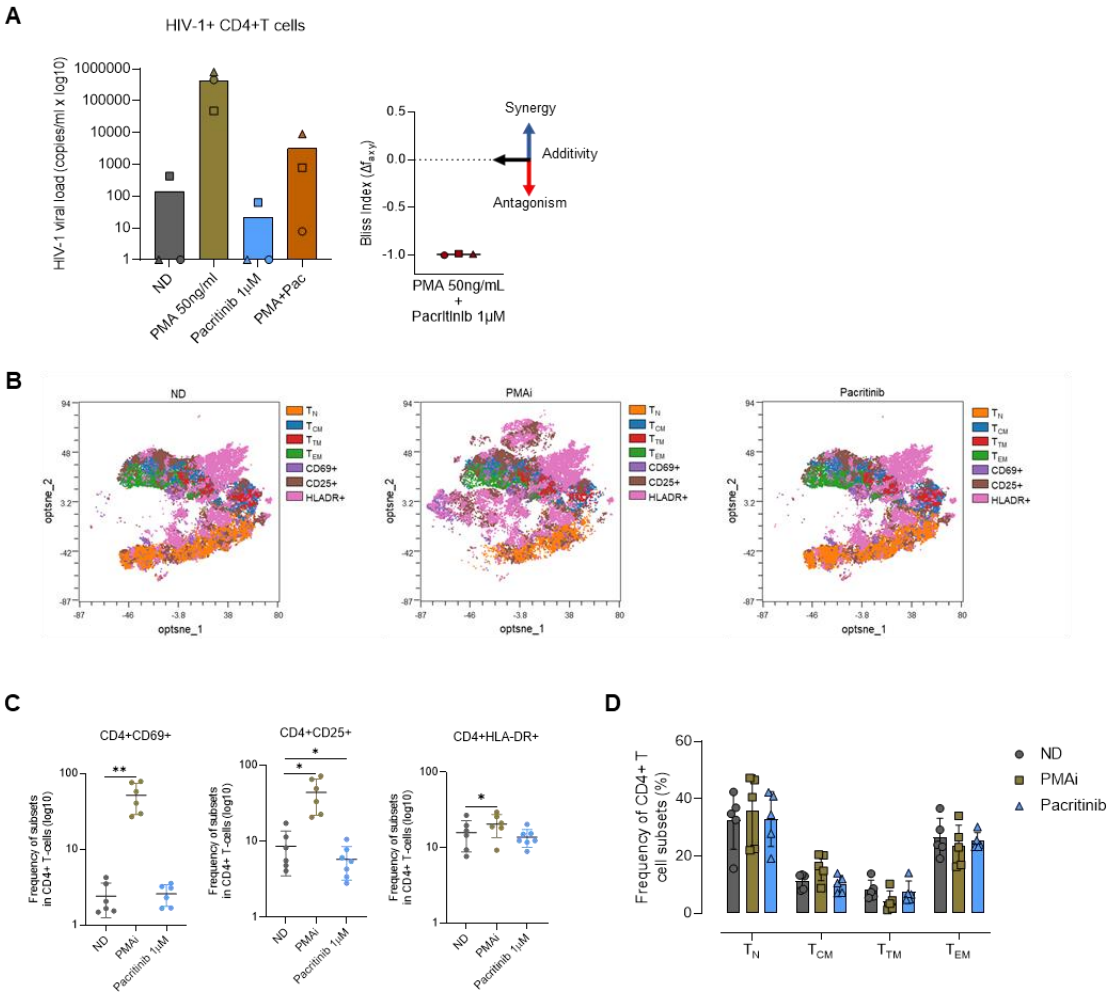


Figure 35. Pacritinib potently blocks LRA-induced *ex vivo* HIV-1 latency reactivation in CD4+ T cells. (**A**) Pacritinib blocks PMA induced latency reactivation in *ex vivo* treated CD4+ T cells from HIV+ individuals on ART**.** Isolated CD4+ T cells were preincubated with Pacritinib for 4h prior to PMA stimulation. HIV-RNA copies were determined in the cell culture supernatant 72 h post stimulation with PMA in the presence of ART. (**B**) Distribution of cell surface markers in CD4+ T cells from ART-treated HIV+ individuals treated with PMA and ionomycin (PMAi) or pacritinib as measured by flow cytometry. Distribution was determined by Opt-tSNE-guided manual gating analysis of the signal strength of key phenotypic defining cell activation markers CD25, CD69 and HLA-DR and CD4+ T subset defining markers for T_N, T_{CM}, T_{TM} and T_{EM}. (**C**) Quantification of the cell activation markers and (**D**) CD4+ T cell subsets compared to resting and PMAi activated CD4+ T cells presented in (B).

DISCUSSION AND PERSPECTIVES

Progress towards achieving HIV-1 cure

The human immunodeficiency virus (HIV) has been a major global public health issue since its emergence about four decades ago. The advent and successful scale-up of antiretroviral therapy (ART) have dramatically decreased the acquisition of new infections globally, increasing the life expectancy of people living with HIV (PLWH), which is comparable to uninfected individuals[3]. However, HIV-1 establishes a low but persistent infection, leading to chronic HIV-associated systemic inflammation and immune activation in virally-suppressed ART-treated PLWH. Although ART is effective in preventing new HIV-1 infections, it cannot eliminate infection once the HIV DNA has successfully integrated into the host genome.

The persistence of HIV-1 in latent reservoirs, primarily in resting memory CD4+ T cells, is a major barrier to HIV cure. Only three cases of HIV-1 cure have been reported to date, through haematopoietic stem cell transplantation (HSCT), albeit originally intended for the management of haematological malignancies. However, the inherent characteristics of HSCT, i. e., the requirement of suitable donors or the *ex vivo* engineering of autologous cells used in HSCT, the invasiveness of the intervention and the overall cost, converting HSCT to an unfeasible curative strategy. Alternatively, the "shock and kill"[82] represents an interesting approach, as it is less invasive or complex, more affordable and easily scalable for global rollout. The "shock and kill" strategy is based on the use of pharmacological agents as LRAs to induce latency reactivation, which ultimately will predispose infected cells to immune clearance. Other strategies such as the HIV transcription-inhibiting "block and lock" might represent also a feasible approach to tackle viral persistence by permanently silencing the latent reservoir, although it has been less studied than the "shock and kill".

Until date, the "shock and kill" strategy remains the most extensively studied HIV cure strategy, showing promising results *in vitro* and *in vivo* in animal models. However, the various LRAs that have been explored as putative cure strategies for the elimination or reduction of latent reservoirs, in clinical trials during the last decade, including several PKC activators and HDAC inhibitors, have shown mostly unsatisfactory curative effects [89,92,95]. Hence, there is still an urgent need to identify new LRAs to tackle HIV persistence. In this regard, and based on *in vitro* and clinical data, we propose that the ideal LRA should (i) maximally reverse latency in all infected cells irrespective of cell lineage, targeting the vast HIV-1 integration site repertoire without causing a broad and robust T-cell activation, (ii) promote cytopathicity and immune clearance specific to infected cells, (iii) express minimal side-effects alone or in combination therapy and possibly, (iv) induce antiviral effect comparable to current ART to avoid reinfections.

Consequently, the present PhD thesis focused on identifying novel LRAs bearing in mind these desired attributes of an ideal LRA.

Eradicating the latent reservoir should focus on targeting multiple HIV reservoir compartments

Over the years, considerable efforts in tackling HIV persistence have solely focused on eradicating lymphoid-derived latent reservoirs. However, it is now clear that other cell types, including the monocyte-macrophage lineage, also play a crucial role in the persistence of HIV-1 due to their ability to spread HIV-1 infection in immune-privileged sites[68]. Although, the extent and relevance of the monocyte and macrophage HIV reservoir in humans has been a subject of debate, the resistance of macrophages to HIV-1 mediated killing and the presence of infected macrophages in immune-privileged regions, including the central nervous system, may pose a barrier to eliminating infected cells by current "shock and kill" strategies[200]. Therefore, curative strategies need to take into account the heterogeneity of the latent reservoir to achieve a complete decay of all HIV-1 latently infected cells, irrespective of cell lineage.

Current LRAs are mainly designed to reactivate the HIV-1 provirus in CD4+ T cells, but their ability to abolish viral latency in myeloid-derived cells is largely unknown. Notable differences in sensitivity to HIV reactivation exist among *in vitro* cell model systems such that compounds shown to induce HIV latency reactivation in a particular model system fail to do so uniformly across different models[195]. Even with the same model system as the *ex vivo* CD4+T cells from ART-treated PLWH, LRAs do not impact equally the latent reservoir in the distinct CD4+ T subpopulations[54], which adds an additional layer of complexity in recapitulating the heterogeneity of latent HIV infection in *in vitro* models and in identifying LRAs with broad and potent activity. To our knowledge, most studies identifying novel LRAs often use *in vitro* "only T cell" models without parallel screening of putative LRAs in myeloid cells. Moreover, previous studies on LRA reactivation of latently infected cells have demonstrated that no single *in vitro* cell model adequately captures the characteristics of the viral reservoir *in vivo* in PLWH[41,42,195,205]. Therefore, we opted to use *in vitro* non-clonal cellular models of HIV-1 latency from both lymphoid (J-HIG) and myeloid (HL-HIG) lineages for the LRA-HTS, allowing for robust identification of putative LRAs in the present study.

The *in vitro* clonal models such as the lymphoid-derived J-Lat and ACH-2 cell lines, expanded from a single cell clone, have indeed facilitated a better understanding of HIV latency and reactivation mechanisms[206,207]. However, clonal cell models such as J-Lat harbour only one or two proviruses integrated into specific sites of the host genome in contrast to the high frequency of proviruses that abound in different integration sites observed *in vivo*[41,42]. Consequently, using

these clonal models for identifying novel LRAs might skew HIV-1 reactivation sensitivity toward or against specific drug classes, resulting in incongruence when applied to other *in vitro* models or to *ex vivo*-treated cells from PLWH. Although, we have not determined the provirus integration sites in the *in vitro* non-clonal models used in the present study, other studies lend support to our non-clonal models harbouring in each of them multiple HIV-1 latently infected cell clones similar to that observed *in vivo*[93,208,209]. Using barcoded viruses in a similar non-clonal latently infected Jurkat model as ours, Chen et al. not only confirmed that provirus integration sites abound in the non-clonal model, but also that these sites influence HIV latency reversal by distinct classes of LRAs[209], raising the possibility that cocktail of LRAs might be needed to meaningfully impact the latent reservoir.

To counter this impediment in identifying novel LRAs by clonal models, screening assays using non-clonal models could be a suitable alternative since they are highly robust and sensitive, as shown in our screening of compounds for HIV-1 latency reactivation activity.

Drug repurposing strategy permits identification of novel LRAs with limited side effects

Drug repurposing represents a potential novel strategy to address the urgent need for more effective antiviral treatments. In fact, the first FDA-approved antiretroviral agent, zidovudine (AZT), was developed as an anticancer agent[210]. Similarly, epigenetic modifying drugs developed for anticancer therapy, such as the HDACi, are now being evaluated in HIV cure research as a mechanism to reverse HIV latency, as are apoptosis promoting agents, targeting the depletion of HIV infected cells[211]. Moreover, as opposed to *de novo* synthesis and development of novel therapeutic agents, drug repurposing of already approved drugs is highly efficient, time-saving, cost-effective and minimizes the risk of failure while maximizing the therapeutic value of a drug[212]. Thus, we performed HTS of a library of anti-cancer compounds encompassing already approved drugs and drug candidates with known targets still under clinical development for the identification of novel LRAs. Overall, the compounds tested spanned a wide range of clinically safe drugs targeting distinct signalling pathways and often paralleled pathways, increasingly indicated in HIV persistence, such as epigenetic modification (HDAC), NF-κB and apoptosis signalling pathways[87,92,95,211].

Using the non-clonal models of HIV latency, we identified several compounds with latency reversal capacity whose activities as LRAs have not yet been described, including inhibitors of the IκB/IKK, Janus kinases, HSP90, protein kinases, and the well-known LRAs (HDACi). Previously, we demonstrated the latency-reversing capacity of the multi-kinase inhibitor midostaurin[186], as are the aurora kinase inhibitors (patent: PCT/ES2019/070596). Here, we focused on further

characterization of the IκB/IKK inhibitors (IKKis) and JAK inhibitors (JAKi) as LRAs, both type of agents sharing immunomodulatory properties.

Achieving immune clearance of infected cells without global cell activation is an unmet need in current cure strategies: innate immunomodulatory agents to the rescue

The limited transcription of the integrated HIV-1 proviruses into the host genome makes it particularly challenging to target latently infected cells for clearance by immune effector mechanisms. Therefore, strategies aimed at stimulating innate immunity have gained considerable interest in current HIV-1 cure research, notably acitretin, which increased HIV transcription and enhanced RIG-I mediated apoptosis specifically in HIV-infected cells, *ex vivo*[93]. Similarly, the TLR agonists which are excellent immune-modulating agents and have recently shown good prospects as LRAs because of their capacity to induce immune activation, causing reactivation of latently infected cells and priming the adaptive antiviral immune response (reviewed in[213]). TLR activation of DCs induces the secretion of proinflammatory cytokines and IFNs, leading to the activation of immune cells including macrophages and T lymphocytes. Recent clinical evidences suggest that immune cell stimulation with TLR7 and TLR9 induce HIV-1 reactivation and immune clearance through IFNα-dependent CD4+ T cell activation[214–216]. Although the activation of immune effector cells in this case could help boost the clearance of infected cells, there is still need for caution because if left unchecked, immune cell activation might compromise recovery of immune cell exhaustion in PLWH.

Achieving broad and efficient latency reactivation without eliciting toxic side effects or global immune activation remain elusive in current LRA research endeavours. In one of the earliest studies investigating the potency of TLR agonists as LRAs, Novis et al. reported that the TLR1/2 agonist Pam3CSK4 reactivates HIV-1 transcription via NF-κB and NFAT-dependent pathways, but without induction of IFNs or T cell activation[91]. Although Pam3CSK4 showed less potency compared to the HDACi panobinostat, this finding nevertheless lends credence to the possibility of harnessing immune modulation as a strategy to reverse HIV-1 latency and circumvent immune cell activation. Hence, the identification of new LRAs capable of reversing HIV-1 latency without causing global T-cell activation and reducing inflammatory signalling as the TBK1/IKKε inhibitors (IKKis) and specific subclass of JAK2 inhibitors (JAK2i) here characterized, are of key importance to achieve a functional cure.

Modulating innate immunity as a tool to overcome viral persistence and dysregulated immune responses in chronic HIV-1 infection

The IKK-related kinases TBK1 and IKKε play prominent roles in mediating IFN production upon pathogen recognition, leading to activation of relevant innate immune mediators, including transcription factors, ISGs, and induction of antiviral IFN-JAK-STAT signalling pathway, being, therefore, putative therapeutic targets for modulating innate immunity. Taking advantage of our previous experience in the screening of anticancer chemotherapeutic agent libraries, we identified IKKis as potential LRAs. Here, we show that IKKis have a demonstrable capacity to reverse HIV-1 latency in both non-clonal models of HIV-1 latency and the clonal ACH-2 latently infected cells. NF-κB modulation, through siRNA silencing of IkBα and NF-κB binding to the kB sites of the HIV-LTR, was previously described to activate HIV in latently infected monocytic U1 and lymphoid J-Lat 10.6[217,218]. Thus, we hypothesize that the pharmacological modulation of the NF-κB cascade by targeting the upstream IKK complex level could similarly induce HIV reactivation. Moreover, cross-talk between IKKα/IKKβ and TBK1/IKKε complexes was already described: TBK1/IKKε inhibits the canonical pathway that modulates IkBα/IkBβ and the NF-κB release[219]. Therefore, selective TBK1/IKKε inactivation, might render IKKα/IKKβ active to, in turn, allowing the release of NF-κB, binding to HIV-LTR and viral transcription initiation. Counterintuitively, inhibition of IKKβ by TPCA-1 induced HIV-1 reactivation in our study, although TPCA-1 was already reported to inhibit the NF-κB pathway[220]. Compensatory IKK regulatory mechanisms cannot be excluded and should be elucidated in future studies.

Furthermore, LRA activity of IKKis had minimal impact on immune cell phenotype and did not affect cell activation markers in primary CD4+ T cells. Importantly, IKKi-induced HIV reactivation was accompanied with decreased induction of proinflammatory cytokines such as IL8, IL1β and the chemokine CXCL10. Positive modulation of NF-κB by cytokines/chemokines or PKC agonists is discouraged due to drug tolerability concerns resulting from hyperinflammatory responses[217,221–223]. In fact, immune activation is closely associated with cell death through activation of NF-κB which regulates expression of many regulatory genes involved in inflammation, apoptosis and viral replication[224], suggesting a connection between immune activation and inflammation with viral replication and cell death that is mediated by regulatory transcriptional factors, such as NF-κB and others.

Although JAKi have been previously described as potent inhibitors of HIV infection and latency[105,106,119], we now demonstrate that not all JAKi block latency reactivation, particularly fedratinib that consistently reversed HIV latency in all *in vitro* models that we tested. Moreover,

none of the previous studies evaluating the impact of JAKi on HIV infection and latency suggested the inclusion of fedratinib in their screening panel, possibly because of the later approval of fedratinib as a therapeutic agent[225]. Another consideration could be that compounds are usually perceived to exert singular but not dual and opposing modulating effects, making it plausible that expectation for one effect over the other might result in overlooking several potential candidates, which might be the case for fedratinib. Additionally, distinct selectivities and/or off-target effects might play an influential role in distinguishing compounds of the same target class. Subsequently, when we expanded the screening to include additional compounds targeting the JAK and IκB/IKK proteins, we observed varying capacities for latency reactivation by these compounds, but also confirmed the inhibitory effects of the JAKi ruxolitinib and pacritinib on latency reactivation, consistent with previous reports[106,119].

Our initial *in vitro* screening performed in parallel in non-clonal myeloid and lymphoid models of HIV-1 latency, also allowed the identification of a specific subclass of JAK2 inhibitors with broad and potent latency reactivation activity. JAKi are FDA-approved drugs for the treatment of myelofibrosis and rheumatoid arthritis. The distinct JAKi differ in their selectivity for the JAKs, JAK1-3 and TYK2, with demonstrable phenotypic differences in their different modes of action[201]. Interestingly, reactivation potential was not common to all JAK inhibitors tested, putatively due to their distinct selectivities and more relevant, to the effects exerted over other host signalling pathways[201,226]. Previous studies have identified JAK1/2i as antiviral agents against HIV infection, blocking both latent and productive infection, in line with our findings for the pan-JAK inhibitor ruxolitinib and JAK2i pacritinib[105,119]. In contrast, the JAK2i fedratinib and its analogue TG101209 significantly induced HIV-1 reactivation both *in vitro* and *ex vivo*, an effect that was independent of their inhibitory activity on the JAK-STAT pathway and subsequent anti-inflammatory properties. Indeed, we found that *in vitro* treatment of primary lymphocytes with fedratinib did not induce major changes in immune cell activation or CD4+ T cell populations, except for enrichment in the proportion of CD4+ T effector memory cells. Emerging evidence suggests that agents impairing differentiation into T effector memory phenotype *in vivo* block latency reactivation[227], highlighting the importance of effector memory cells as an important latent reservoir for eradication strategies within the context of “shock and kill”.

The identification of FDA-approved compounds such as fedratinib that can purge HIV-1 reservoir in distinct cell types but also harbour potent immunomodulatory properties, specifically counteracting hyperinflammatory responses and restoring normal immune signalling, represents a valuable asset for HIV cure strategies[73]. These desirable attributes prompted us to characterize its mode of action to identify the cellular pathways underlying its latency

reactivation potential. Indeed, whole transcriptome profiling and pathway analysis confirmed the singularity of fedratinib compared to other JAKi. Interestingly, fedratinib and PMA, but not the JAKi non-LRAs ruxolitinib and pacritinib, similarly upregulated pathways associated with apoptosis and several immune-related signatures. More importantly, fedratinib was the inhibitor presenting the lowest number of altered gene sets in the pathway analysis, suggesting the existence of fewer undesired off-target interactions. Small molecule drugs interact with unintended, often unknown, biological targets, and these off-target effects may lead to both preclinical and clinical toxic events, thus, the lesser off-targets, the better for its clinical applications.

The mechanisms for the establishment, maintenance and reactivation of HIV-1 latency mainly operate at the transcriptional level by both viral[228–231] and host machinery, usually through chromatin modification and epigenetic regulations[230,232–234]. Thus, based on pathway analyses, in depth evaluation of specific candidate genes responsible for fedratinib latency reactivation activity focused on pathways controlling host cell transcription, leading to the identification of IRFs as putative targets, and among them IRF7, showing the most significant change in gene expression. IRFs are a family of transcription factors that play pivotal roles in many aspects of the immune response, including immune cell development and differentiation and regulating responses to pathogens. Among them, IRF3, IRF5, and IRF7 are critical to the production of type I interferons downstream of pathogen recognition receptors that detect viral RNA and DNA. IRF7 is a multifunctional transcription factor[235] that exhibits broader DNA binding specificity than other IRFs[236], and its expression and activation is also more tightly regulated[235].

At first glance, the upregulation of IRF7 expression despite the effective blockade of the JAK-STAT signalling pathway may seem counterintuitive. However, our data clearly pointed towards a new, as yet unrecognized role of IRF7, not directly linked to its canonical and well-described role as a master regulator of IFN expression[237]. We provide several compelling evidences demonstrating IRF7 role in distinct experimental models, including (i) specific impairment of fedratinib latency reactivation activity upon IRF7 knockdown, (ii) direct correlation of IRF7 expression levels and HIV latency reactivation and (iii) correlation between Tat-mediated HIV-1 transcription induced by JAK2i and IRF7 upregulation. Indeed, upregulation of IRF7 expression has been previously identified in cells treated with BRD4 inhibitors acting as LRA in J-Lat latently infected HIV-1 model[238]. In line with this data, we also observed that IRF7 expression directly correlated with HIV-1 reactivation in response to distinct LRAs, including PMA, the BRD4 inhibitor JQ1 and the HDACi panobinostat, in addition to specific JAK2i. Moreover, upregulation of IRF7 expression was independent of IFN-related gene expression as all LRAs except those

blocking JAK function significantly up-regulated ISG expression, providing further proof of the independent role of IRF7 in HIV-1 latency reactivation. Furthermore, through genome-wide quantitative trait locus analysis in chronic HIV-1 infected individuals, IRF7 has been identified as a host genetic factor affecting the size and transcriptional activity of HIV-1 reservoirs, further supporting the role of IRF7 in HIV-1 latency[239].

Overall, we have identified and characterized the JAK2i, fedratinib, as a novel LRA. Several attributes distinguish fedratinib as an excellent candidate for HIV cure strategy, including the demonstrated capacity in reversing HIV-1 latency without causing global T-cell activation, reducing inflammatory signalling, and high specificity in the modulation of a limited number of signalling pathways. Moreover, functional characterization of fedratinib latency reactivation capacity revealed new molecular determinants of HIV-1 latency maintenance and reactivation, specifically IRF7 (**Figure 36**). Therefore, mechanistic insights into the molecular basis of the divergent roles played by IRF7 in host protection against viruses warrant further exploration in future studies.

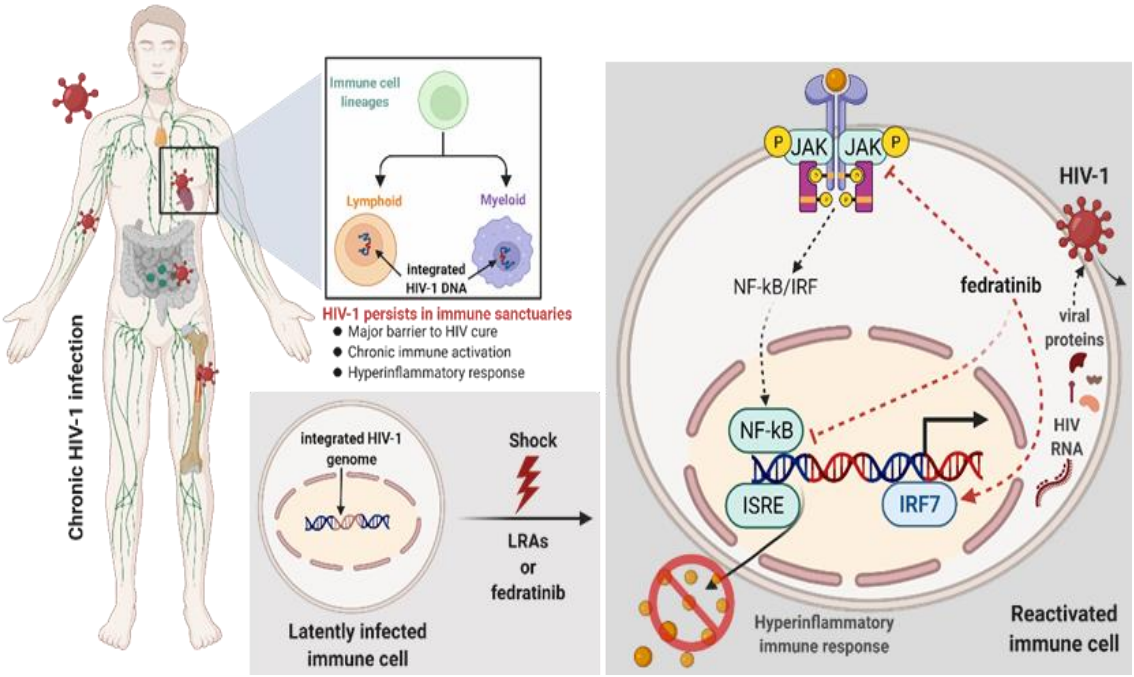


Figure 36. Targeting IRF7 is a novel strategy to modulate HIV-1 latency and circumvent chronic immune activation. During HIV-1 infection, HIV DNA integrates into the host genome and persists indefinitely in immune-privileged compartments, including lymphoid and myeloid cells, despite ART. HIV cure strategies such as the "shock and kill" using LRAs (or fedratinib) aim to induce viral transcription and virion production, exposing infected cells to immune clearance. In contrast to most LRAs, fedratinib blocks JAK/STAT signalling and NFκB-mediated immune activation and hyperinflammatory responses but allows IRF7-driven latency reactivation.

Is immunomodulation the strategy to durably suppress spontaneous viral rebound?

Despite decades of intensive research on eradicating the latent reservoir via the "shock and kill" strategy, none of the LRAs has successfully eliminated the latent reservoir or achieved ART-free HIV suppression for extended periods in PLWH[92,240,241]. Perhaps, a more realistic alternative may be to durably block transcription of the latent reservoir using latency promoting agents (LPA) to drive the provirus into a state of deep latency – termed "block and lock"[100]. Prominent amongst the LPAs is the didehydrocortistatin A (dCA), which selectively interferes with the interaction between HIV Tat and TAR-RNA[101]. Similarly, the JAK2i pacritinib identified as a potential LPA in our HTS of anticancer compounds showed inhibitory activity against Tat-dependent expression of HIV-1 LTR promoter, suggesting the capacity to target HIV transactivation and block viral transcription. Another promising approach for suppressing viral reactivation is by manipulating specific host-dependent pathways relevant to the maintenance of HIV latency. As previously described, blocking the JAK-STAT signalling pathway by JAKi, potently inhibits HIV-1 reactivation[105,119], albeit contradictory to our findings supporting contrasting effects of JAKi on HIV latency depending on IRF7 expression, which allowed us to distinguish the LRA fedratinib from the LPA pacritinib. It is important to reiterate that while inhibition of JAK kinases, including JAK2 is known to block HIV reactivation and productive HIV-1 infection, we found that modulation of IRF7 and not JAK2 was the key factor responsible for the observed differences between the JAKi.

Although ART is highly effective in suppressing HIV viremia to undetectable viral loads (VL < 50 copies/ml) in a majority of PLWH[26], intermittent spontaneous increase in VL, viral blips, occasionally occur in some individuals[242,243]. While there is no consensus regarding its causality, viral blips could be related to the immune activation-driven increase in HIV-1 replication from the latent reservoir[244], which could impair the complete decay of the latent reservoir. In this regard, JAKi-LPA might play a prominent role in reducing the frequency of viral reactivation and spontaneous blips as we observed by the abrogation of LRA-induced HIV reactivation with either pacritinib or baricitinib in *in vitro* models of HIV latency. Moreover, pacritinib besides inhibiting PMA-induced reactivation in *ex vivo*-treated primary CD4+T cells from ART-suppressed PLWH also blocked immune activation in these cells, which underscores its prospects in future therapeutic strategies addressing chronic immune activation in ART-treated PLWH.

Mitigating persistent HIV-1 induced immune dysregulation during ART

A major limitation for most LRAs studied in clinical trials is the failure to sustain viral-suppression without ART, making it unsafe to withdraw ART in PLWH. Considering the increased risks of ART-

related toxicities, alternative approaches, including LRAs with dual roles, one as a shock agent and the other as a kill agent with potent antiretroviral activity, might be pivotal in delivering a safer and effective HIV-1 cure.

Since inhibition of TBK1/IKKε or other IKK subunits have been described to impair replication of HSV-1 and HSV2, HCV or RVFV[245–247], we further evaluated the role of IKKis on acute HIV-1 infection, observing that IKKis do not exert antiviral effect in neither lymphoid nor myeloid *in vitro* models of acute HIV-1 infection. Conversely to present data, our previous findings showed a partial inhibitory capacity of MRT67307 on HIV replication in primary macrophages[248]. These discrepancies might be explained by the distinct model systems used in this present study, cell lines but not primary cells. No curative strategy described so far has achieved complete depletion of the latent reservoir or durable virus suppression without ART, making it imperative to determine possible drug interactions of new therapies which must be combined with ART to prevent viral rebound. Thus, we also evaluated the LRA activity of IKKis in combination with either EFV or RAL, observing that IKKis reactivation capacity were not modified by ARVs. Similarly, in acute HIV-1 infection models, combining IKKis with ARV did not diminish the antiviral efficacy of either EFV or RAL, suggesting the absence of IKKi-ARV drug interactions. Altogether, combinations of IKKis with ART did not induce toxicity in cell cultures, further supporting the safety of IKKis as alternative potential LRA in new HIV therapeutic approaches.

Indeed, the JAKi here tested are considered to be clinically well-tolerated, and their capacity to counteract hyperinflammatory responses makes them attractive candidates for targeting inflammatory-driven viral infections. In contrast to the IKKis which showed no antiretroviral activity in our screening, JAKi are potent antiviral agents as we and others have reported, supporting their use in anti-HIV therapy without need for combination therapy with ART. Recently, the JAKi baricitinib received emergency use authorization to treat COVID-19 cytokine storm[249], due to their potent efficacy in reducing type I IFN-driven inflammation *in vivo*, paving the way towards their potential use as treatment strategies against other viral infections, including HIV-1[120]. Moreover, blocking the JAK-STAT pathway reversed cognitive deficits and curtailed inflammatory markers in HIV-associated neurocognitive disorders (HAND) mice model[250], which could be beneficial to PLWH, since HAND occur in a significant proportion of PLWH, even with ART[251].

In summary, combinations of immune-modulating agents with ART are generally safe, being well-adapted to counteract HIV-associated immune activation and dysregulated inflammatory responses that persist in chronic HIV-1 infection despite ART.

Future perspectives

The low inducibility of latent proviruses is a major problem for achieving a sterilizing HIV cure with the "shock and kill", and until more effective ways of doing so emerge, combining LRAs with other strategies could be pivotal in delivering potential HIV cure. A possible combination strategy could entail depleting a significant proportion of the inducible latent reservoir using LRAs and immune enhancing therapy and subsequently use LPAs to reinforce a deeper state of latency (**Figure 37**). The expectation is that durable transcriptional suppression of residual non-inducible latent reservoir may eventually lead to lifelong viral control, allowing safe interruption of ART. Finally, although there is no effective HIV cure yet, the emergence of new therapies and combination therapies showing promising results in preclinical studies and early clinical trials gives us hope that we are moving in the right trajectory.

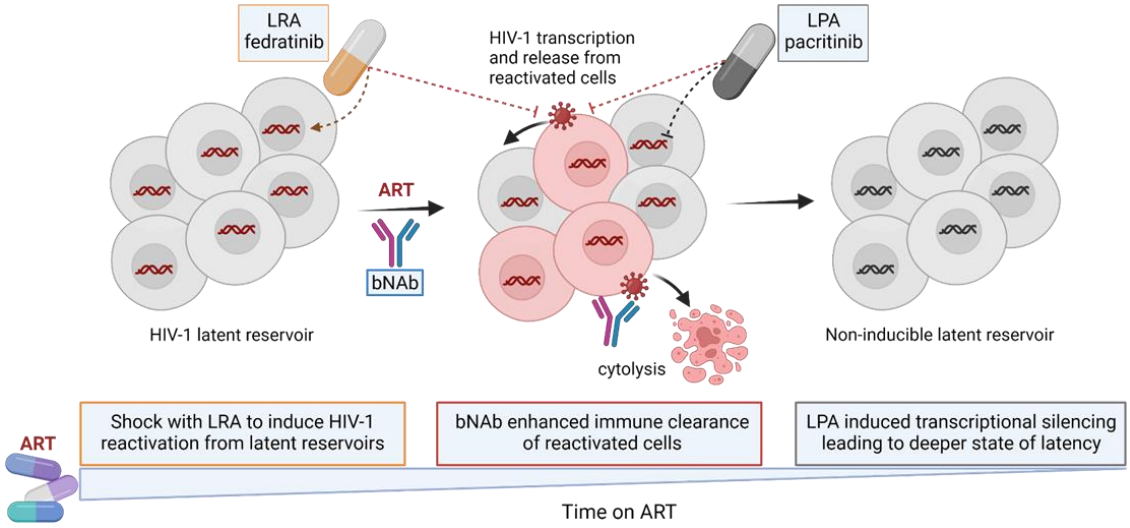


Figure 37. Combination strategy for tackling HIV persistence. LRAs can effectively reverse HIV-1 latency but are limited in their capacity to eliminate latently infected cells. The addition of bNAbs could stimulate robust immune clearance of a significant proportion of reactivated cells, leaving a small fraction of non-inducible latently infected cells, which upon treatment with an LPA, reinforces a state of deep latency, possibly allowing the safe withdrawal of ART overtime.

CONCLUSIONS

1. Non-clonal models of HIV-1 latency from myeloid or lymphoid origin are robust, versatile and sensitive *in vitro* tools for identifying novel broad-acting LRAs from high throughput screenings.

2. Modulating innate immune signalling pathways is a potential strategy for targeting HIV latency and circumventing debilitating broad T-cell immune activation in PLWH.

3. Inhibiting TBK1/IKKε signalling induces reactivation of latently HIV-infected cells and is compatible with combination therapy with ART.

4. Selective JAK2i reverses HIV latency *in vitro* and *ex vivo* in CD4+ T cells from ART-suppressed PLWH and reduces HIV-1 chronic induced inflammation concomitant to HIV-1 latency reversal.

5. Selective JAK2i show different capacities for inducing HIV-1 latency through mechanisms independent of JAK-STAT signalling in lymphoid and myeloid models of HIV-1 latency.

6. IRF7 is a novel key factor controlling HIV latency reversal by JAK2i and other LRAs.

7. Targeted inhibition of the JAK-STAT pathway provides a selective, effective, and novel mechanism for purging (shock and kill) and silencing (block and lock) the HIV-1 reservoir in lymphocytes and macrophages.

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LIST OF PUBLICATIONS

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ACKNOWLEDGEMENTS

It still feels like a couple of days ago since I first arrived in the beautiful and very vibrant city of Barcelona and at the fortress-like Can Ruti campus that would later become my research home for the next four years of the PhD thesis. During this time, I received tremendous support from my colleagues at the Institut Germans Trias I Pujol (IGTP) and Institut de recerca de la SIDA (IRSICAIXA), which has undoubtedly transformed the rigours and high demands of the PhD thesis into a memorable and fulfilling endeavour. Be rest assured that I will ever remain grateful to you.

First and foremost, I appreciate the support and mentorship that I have received from my thesis supervisor, **Ester**, which was beyond measure. Your optimism, when faced with awry situations, is an attribute that I have come to appreciate the most, especially when I have had to deal with seemingly disappointing experimental results. You are professional par excellence but humane nonetheless, always eager to assist me with navigating through medical appointments and other bureaucracy that otherwise would have been challenging for a foreigner living miles away from home.

This thesis wouldn´t have been possible without the contribution of the Virus-Host interaction team (ViHIT). To Eva for excellently managing the housekeeping of all the consumables in the group, and ensuring the smooth running of the day-to-day activities in the lab. I am still amazed at your efficiency in the lab that it is irresistible to count on your guidance when troubleshooting experimental protocols. To **Roger**, your good sense of humour is something to look forward to, much needed on not very good days, because you will never acknowledge that some days can be bad. Oh **Edurne**, when you handed over the baton of latency reactivation assays to me, you kindly followed that up with a prayer, “may God help Ify”, and I make bold to claim that sure He did. Thank you for laying the foundation for this PhD thesis and for your guidance with the documentation process. To my office neighbour **Lucia**, you have always inspired me with your attention to detail, particularly with presentations. Thank you for inspiring me to take up running as a sport, although I still don´t know if I will ever get to run at least a half-marathon anytime soon. To the only medical doctor in the team, **Eudald**, who has shown me the value of efficient time management, concurrently juggling hospital visitations, studies and PhD with so much ease – if that is not superpowers, I don’t know what is. To **Ignasi C.**, who joined us recently, I wish you the best as you progress through your PhD thesis.

To former members of our group, **Marc** and **Maria P**., the techniques I learned from you during the first few months of my thesis have been invaluable to the development of my thesis. At that

time, I recall how much I struggled with western blots, but Maria, “Queen in the West”, you made it look so easy and enjoyable, leading to my love story with this technique.

A **Ventura**, jefe de IRSI, merci per tot, també per molt bem ambient aquí. My Catalan is still a work in progress. To **Lourdes**, thanks for your support in transitioning to work here in IRSICAIXA. Thank you **Lidia** for being patient with me and always willing to clearly explain the intricate details of good practices for working in BSL2 and BSL3 laboratories.

To the RRHH at both IGTP and IRSICAIXA, **Montserrat**, **Maria O**., and **Laura,** your efficiency right from the onset of the PhD programme has never been in doubt and is well appreciated, readily providing supporting documentation to facilitate my visa applications, residence permits etc. A **Penelope**, **Cris** y **Arnau**, gracias a todos, tambien.

To my “Isla” family, you made the workplace so lively. Sorry, but I don´t know who or what I miss more, you or **Mace**´s “caixa de la felicitat” that **Sandra,** “la Reina de la chocolate”, kept ever flowing with assortments of chocolates and candies. I must confess how much I enjoyed the hearty banter from **Rita** and **Elena**, and the local scoops - the perks of sharing the same office with the communication department. To **Chiara**, my fashion plaudit, pardon me for becoming non-fashionable of late although there is a good reason for that, perhaps I should have asked **Ferran**, “Harry Potter”, for a reverse spell, knowing how selfless and generous he could be at all times. To **Ana** de la Canarias and **Isma**, you made HIBIC so much fun, and I will always cherish our friendship.

Over time, I have benefitted a lot from tapping into the experiences and the support network of my fellow predocs here at IRSICAIXA, work-related or otherwise. To **Xabi**, **Miguel**, **Angel**, **Silvia**, **Carlos A**, **Luis**, **Clara**, **Raquel**, **Ana**, **Anna**, **Carlos B**, **Irene**, **Fran** and **Fernandito**, keep up with the good work! To **Oscar**, un chico con mucho talentos, you have been of so much help to me that I could only wish that you will never lack help in your time of need. **Bruna**, I still don´t know how to express my gratitude to you for all you have done for me, muchisima gracias mi amiga. And to all the amazing people of IRSI and Albajuna who I have not mentioned herein, I am sure you know how dear you all are to me. You have been so welcoming, made my stay here worthwhile, and if you ever wondered why I am always cheerful, it is only because of **YOU**.

Gracias a tots!

My beloved "**la Caixitos**" (Barcelona), **Ivan M**, **Silvia**, **Aru**, **Ignasi**, **Loic** and **Andrea**, **Bradley**, **Poonam**, **Yasmin**, **Enrico**, **Luca**, **Ivan FA**, and **Paloma**, when "la Caixa" foundation proposed networking amongst fellows, little did I know that I would gain amiable companions and strong support. My story here in Barcelona would never be complete without reminiscing on our many adventures and good memories, from the merry house parties, Karaokes, sunsets at the beach, BBQ, Casa Rural, picnics, hiking to my favourite card game Bang-Bang. It is sad acknowledging that we are all completing our different programmes, returning to our countries or next destinations, but I am hopeful that the future will be favourable to all of us and look forward to meeting you elsewhere be it in Argentina, Italy, Switzerland, Brazil, India or the UK.

Ines M, **Catarina** and **Tomide**, you have made Lisbon feel like a home that I always feel a strong pull to visit Lisbon at the slightest opportunity, likewise **Rute**, **Jose**, **Ines B**, **Susanna**, **Pedro**, **Rui** and **Diogo**. Teaching in the pathogen omics courses at FFUL with **João** during these past few years, and continuously learning and improving my phylogenetic analytical skills under your guidance have been so impactful in my academic formation. I feel so honoured and grateful to answer positively to your invitations. And to my former mentor **Nuno**, you have been that light at the end of a dark tunnel, always encouraging me to realize my maximum potential. It has been a privilege collaborating with you, **Claudia** and **Veronica** and I look forward to many more opportunities in the future. Also, to **Shamsudeen** for the many research collaborations in Nigeria.

And to my homies **Adrian**, **Azedine** and **GG**, you have been amazing housemates and I can count on you as my family here already. **André**, my best buddy, thanks for always being there for me through thick and thin. You have inspired me so much, more than you can ever know. To my friends, **Cherechi** and **Nnamdi**, thank you for your steadfast support over the years.

Finally, to my family, you have sacrificed so much on my account over the years, yet, you have never wavered in supporting my life aspirations. Not being with you these past four years have been my most difficult undertaking in life, and can´t wait to be reunited with you once again. Daalu **Ifeabunike** and **Mkpurumma**! And to you my darling sisters **Oge**, **Chidi**, **Kelechi** and your families, **Chioma** and **Chiemelie,** my brother. See you soon!

Moltes gràcies a tots.

Muchas gracias a todos.

Thank you all.

Ifeanyi Jude Ezeonwumelu