UNIVERSITY OF
KWAZULU-NATAL

# The Effect of Cationic DNA-Binding Proteins on HIV-1 Latency

---

**Submitted by: Senzo Cebekhulu**

**Supervised by: Dr. Paradise Madlala**

**Co-Supervised by; Prof Jaclyn Mann**

Submitted in fulfilment of the requirements for the degree of Master of Medical Science in Virology in the Nelson R. Mandela School of Medicine, College of Health Sciences, University of Kwa-Zulu Natal, Durban.

**2025**

**Table of Contents**

**Preface**

This project represents original work done by the author and others whose contribution has been acknowledged in the text. All experimental work mentioned in this dissertation was accomplished at the HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal, Durban, South Africa, from August 2023 to November 2025, under the supervision of Dr Paradise Madlala and Prof Jaclyn Mann.

11 December 2025

Senzo Cebekhulu (Student) Date

Dr Paradise Madlala (Supervisor) Date

Dr. Jaclyn Mann (Co-Supervisor) Date

**Declaration**

I, Senzo Cebekhulu, declare that:

(i) The research reported in this study, except otherwise indicated, is my original work.

(ii) This study has not been submitted for any degree or examination at any other university.

(iii) This study does not contain other person's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.

(iv) This dissertation does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) Their words have been re-written, but the general information attributed to them has been referenced.

b) Where their exact words have been used, their writing has been placed inside quotation marks and referenced.

(v) This dissertation does not contain text, graphics or tables copied and pasted from the internet unless specifically acknowledged, and the source being detailed in the dissertation and in the references section.

Signed: 

Date: 11 December 2025

**Acknowledgements**

I thank God for granting me the strength and perseverance to complete this journey. I am deeply grateful to my family and friends for their unwavering support and encouragement. I extend my sincere appreciation to my supervisors, Dr Paradise Madlala and Prof Jaclyn Mann, for their guidance, patience, and steadfast mentorship throughout this project. I also acknowledge the dedicated staff and students at the HIV Pathogenesis Programme for their collegiality and assistance, and I thank the Poliomyelitis Research Foundation (PRF) and the Health and Welfare Sector Education and Training Authority (HWSETA) for the financial support that made this work possible.

**List of tables and figures**



**List of figures in appendix A**

**List of Abbreviations**

7SK snRNA - 7SK small nuclear RNA

AIDS – Acquired immune deficiency syndrome

AP-1 - Activator protein-1

ART - Antiretroviral therapy

ATP - Adenosine triphosphate

ATF2 - Activating transcription factor 2

Bax - B-cell lymphoma 2-associated X

Bcl-6 - B-cell lymphoma 6

BIRC2 - Baculoviral inhibitor of apoptosis repeat-containing 2

BIRC3 - Baculoviral inhibitor of apoptosis repeat-containing 3

BAF - Brahma-associated factor

CAK - CDK activating kinase

CCL - C-C motif chemokine ligand

CCK-8 – Cell counting kit-8

CCR5 - C-C chemokine receptor type 5

CD4 – Cluster of differentiation 4

CDK2 - cyclin-dependent kinase 2

CDKN1A - Cyclin-dependent kinase inhibitor 1A

ChIP-qPCR - Chromatin immunoprecipitation-quantitative polymerase chain reaction

CNS - Central nervous system

CoREST - Corepressor of RE1 silencing transcription factor

CRISPRi - Clustered regularly interspaced short palindromic repeats interference

CXCR4 – C-X-C Chemokine receptor type 4

CTD - C-terminal domain

DAG - Diacylglycerol

DC-SIGN - Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin

dCA - Didehydro-cortistatin A

DMSO - Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

DNMT1 - DNA methyltransferase -1

DNMT3B - DNA methyltransferase 3 beta

DNase I - Deoxyribonuclease I

E2F-1 - E2 promoter binding factor 1

EHMT2 - Euchromatic histone-lysine N-methyltransferase 2

ERK1/2 - Extracellular signal-regulated kinases 1 and 2

FACT - Facilitates chromatin transcription

FBS - Foetal bovine serum

Fe - Ferric iron

GATA-1 - GATA-binding factor 1

GFP - Green fluorescent protein

Gp120 - Glycoprotein 120

Gp41 - Glycoprotein 41

GADD45 - Growth arrest and DNA damage-inducible 45

H3K4me3 - Trimethylation of lysine 4 on the histone H3 protein

H3K9ac - Acetylation of histone H3 at residue K9

HBV - Hepatitis B virus

HCV - Hepatitis C virus

HDAC - Histone deacetylase

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HEXIM - Hexamethylene bisacetamide-induced protein

HEWL - Hen egg white lysozyme

HIV-1 - Human immunodeficiency virus type 1

HIF-1α - Hypoxia-inducible factor 1 alpha

HL9 - Nonapeptide (a peptide composed of nine amino acids) fragment of human lysozyme

HP1 - Heterochromatin protein 1

HPP – HIV pathogenesis programme

HSP90 - Heat shock protein 90

IAP - Inhibitor of apoptosis protein

ICAM1 - Intercellular adhesion molecule 1

IκBα - Inhibitor of nuclear factor kappa B alpha

IKK - Inhibitor of kappa B kinase

IL-8 – Interleukin 8

IL-12 – Interleukin 12

J-Lat – Latently infected Jurkat T-cell line

LEDGF/p75 – Lens epithelium-derived growth factor, 75 kDa variant

Lf - Lactoferrin

LPA -Latency promoting agent

LPS - Lipopolysaccharides

LPR1 - Lipoprotein receptor-related protein-1

LRA – Latency reversing agent

LTR - Long terminal repeat

Lz – Lysozyme

MAT1 - Ménage à trois 1

MDM2 - Murine double minute 2

mRNA – Messenger ribonucleic acid

NAM - N-acetylmuramic acid

NAG - N-acetylglucosamine

NF-κB - Nuclear factor-kappa B

NFAT - Nuclear factor of activated T-cells

ORC1 - Origin recognition complex subunit 1

P-TEFb - Positive transcription elongation factor

P53 - Tumor protein p53

PBMC - Peripheral blood mononuclear cells

PI3K/Akt - phosphoinositide 3-kinase / protein kinase B signalling pathway

PIC - Pre-integration complex

PFA - Paraformaldehyde

PKC - Protein kinase C

PLWH – People living with HIV

PMA - Phorbol 12-myristate 13-acetate

RPMI - Roswell Park Memorial Institute

RNA - Ribonucleic acid

RNAPII - RNA polymerase II

SAHA - Suberoylanilide hydroxamic acid

SARS-CoV - Severe acute respiratory syndrome coronavirus

Smad7 - Mothers against decapentaplegic homolog 7

Sp – Spironolactone

Sp1 - Specificity protein 1

STIs - Sexually transmitted infections

Suv39h1 - Suppressor of variegation 3-9 homolog 1

Tat - Trans-activator of transcription

TI – Transcriptional interference

TFIIH - Transcription factor II H

TGF-β - Transforming growth factor-beta

TLR4 - Toll-like receptor 4

TNF-α - Tumour necrosis factor alpha

Ts – Tanespimycin

URTIs - Upper respiratory tract infections

VISCONTI - Virological and immunological studies in controllers after treatment interruption

WHO – World health organization

XPB - Xeroderma pigmentosum group b protein

XPD - Xeroderma pigmentosum group D

YY1 - Yin yang 1

Zn - Zinc

**SI Units**

µM - Micromolar

µg – Microgram

kDa – Kilodalton

MFI - Mean fluorescent intensity

mg – Milligram

mL - Millilitre

nM – Nanomolar

nm – Nanometer

xg - Times gravitational force

**Abstract**

The latent reservoir remains the foremost obstacle to a HIV-1 cure development. This latent reservoir is composed of cells infected with replication-competent and yet transcriptionally silent proviruses, which are the source of viral rebound after antiretroviral therapy (ART) is interrupted. Therefore, people living with HIV-1 (PLWH) have to remain on treatment for their lifetime. However, ART is associated with cytotoxicity and comorbidities thus necessitating cure development. Despite extensive investigation, existing cure strategies, targeting HIV-1 latency such as the "shock and kill" approach, which uses latency-reversing agents (LRAs) to reactivate latent virus and expose infected cells to immune-mediated clearance, and the "block and lock" strategy, which employs latency-promoting agents (LPAs) to force the virus into deep latency, have shown limited long-term success. This underscores a need to continue the search for better and more effective agents for these HIV-1 cure strategies.

Lysozyme and lactoferrin are cationic antimicrobial proteins that play an important role in innate defence and have both been shown to block HIV-1 replication, bind DNA and RNA, and modulate gene expression. However, the effect of these cationic proteins on HIV-1 latency potential remains to be determined. Therefore, the aim of this study is to determine the effect of cationic proteins of the innate immune system on the propensity of HIV-1 latency reversal or enhancement.

The cytotoxicity of cationic proteins, chicken egg lysozyme (Lz), human lysozyme fragment (HL9), and/or lactoferrin (Lf), alone or in combination with established latency-reversing agents (LRAs; PMA and SAHA) and latency-promoting agents (LPAs; tanespimycin [Ts] and spironolactone [Sp]) was assessed in two HIV-1 latency cell lines, J-Lat A2 and J-Lat C, derived from a Jurkat E6 T-cell clone, using the Cell Counting Kit-8 assay. J-Lat A2 and J-Lat C cell lines harbour a latent minimal HIV-1-based retroviral vector reporter genome expressing subtype B (J-Lat A2) or subtype C (J-Lat C) consensus transactivator of transcription protein (Tat) together with green fluorescent protein (GFP), under the control of the corresponding subtype B or C consensus long terminal repeat (LTR), respectively. J-Lat A2 and J-Lat C were treated with individual cationic proteins Lz, HL9, or Lf, or in combination with PMA or SAHA and Ts or Sp. HIV-1 latency reactivation or enhancement was assessed after 24-48 hours by measuring GFP expression using flow cytometry.

Cytotoxicity assays revealed that all agents were non-toxic (≥85% viability) at tested concentrations and combinations. Our data from single-treatment latency-reversal experiments showed that only Lf significantly reactivated latent HIV-1 in J-Lat A2 (3.6 ± 1.4 % GFP, $p < 0.0009$) but not in J-Lat C (1.4

± 0.6 %, p = 0.997). Combinations Lz+Lf (4.9 ± 1.7 %, ≈25-fold, p < 0.0001) and Lz+HL9+Lf (6.6 ± 2.9 %, ≈34-fold, p < 0.0001) synergised and enhanced reactivation in J-Lat A2. Lactoferrin also enhanced classical LRAs, PMA-induced reactivation rose 2.3 ± 0.1-fold and SAHA 18.9 ± 2.3-fold (p < 0.0001) in J-Lat A2. The Lf+Lz combination raised these to 2.6- and 24.5-fold, respectively, versus LRA-only controls (p < 0.0001). The same pairs raised PMA 1.24 ± 0.2-fold (p = 0.02) and SAHA 9.5 ± 2.6-fold (p < 0.0001) in the J-Lat C cell line. The LPA Ts suppressed PMA-induced reactivation 9.0 ± 1.4-fold (p < 0.0001) in J-Lat A2. The inclusion of Lf restored the potent levels of reactivation, with combinations PMA+Ts+Lf and PMA+Ts+Lf+Lz resulting in 6.6 ± 0.5- and 7.8 ± 0.5-fold increases in GFP expression, respectively, relative to the PMA+Ts baseline (p < 0.0001). Although Ts did not block SAHA-induced reactivation, SAHA+Ts+Lf and SAHA+Ts+Lz+Lf combinations markedly potentiated SAHA, yielding 12.5 ± 1.4-fold and 15.5 ± 1.5-fold increases in GFP-expression compared to SAHA+Ts baseline (p < 0.0001). Data generated from the J-Lat C cell line followed a similar but less pronounced pattern where the combinations PMA+Ts+Lf, PMA+Ts+Lf+Lz, SAHA+Ts+Lf and SAHA+Ts+Lz+Lf significantly potentiated LRA-induced reactivation to levels that surpassed the LRA+LPA-only baseline. Sp reduced PMA-driven GFP expression 1.0 ± 0.07-fold compared to PMA alone (p < 0.0001); SAHA+Sp showed only marginal suppression. The Lz+Lf pair completely reversed Sp-induced suppression, PMA+Sp+Lz+Lf yielded 2.7 ± 0.7-fold and SAHA+Sp+Lz+Lf 26.4 ± 8-fold reactivation (both p < 0.0001). In J-Lat C, PMA+Sp+Lz+Lf (3.6-fold, p < 0.0001) and SAHA+Sp+Lz+Lf (2.2-fold, p = 0.0029) again overcame Sp-mediated deep latency. These findings suggest Lf, alone or in combination with Lz, can enhance latency reversal in permissive contexts and potentially counteracts LPA's blocking effects, which was more pronounced in J-Lat A2 compared to J-Lat C. This highlights Lf as a promising agent that can be further explored for utility in the "shock and kill" cure strategy, and underscores the importance of cellular and viral subtype context in therapeutic outcomes.

## 1. Introduction

It has been over four decades since human immunodeficiency virus type-1 (HIV-1) was identified as the causative agent of acquired immunodeficiency syndrome (AIDS), and to date, it remains a significant global public health challenge (WHO, 2025). As of the end of 2024, approximately 40.8 million people were living with HIV-1, 1.3 million people acquired HIV-1 infections, and HIV-related illnesses led to the death of about 630,000 individuals globally (WHO, 2025). Notably however, the 630,000 HIV-1-related deaths in 2024 underscore the progress that has been made in the fight against HIV-1 as this represents an approximate 70% drop from about 2.1 million people who succumbed to HIV-1 in 2004 (WHO, 2025). This significant reduction in the frequency of HIV-1-related deaths is mainly attributed to the rapid scale-up of access to effective HIV-1 prevention, diagnosis, antiretroviral therapy (ART), and care, including addressing opportunistic infections (WHO, 2025). This has transformed HIV-1 infection into a manageable chronic condition (WHO, 2025).

Although ART has been proven to be effective in ceasing HIV-1 replication and reducing HIV-related deaths, it is not curative due to the persistence of latent reservoirs that present a significant hurdle in achieving a cure for HIV-1 (Darcis et al., 2017). These reservoirs consist of cells harboring replication-competent yet transcriptionally silent HIV-1 proviruses, which serve as the source of viral resurgence when ART is interrupted (Hermankova et al., 2003). Several strategies aimed at curing HIV-1 are under investigation, including the "shock and kill" and "block and lock" approaches (Darcis et al., 2017). “Shock and kill” utilizes latency-reversing agents (LRAs) with the aim to purge the latent reservoir by forcing silent proviruses to resume transcription, translate viral proteins, and release new virions (Deeks, 2012, Darcis et al., 2017). This reactivation seeks to facilitate the clearance of these infected cells either through cytolysis or immune-mediated clearance in individuals on ART, thereby preventing new rounds of infection (Deeks, 2012, Darcis et al., 2017).

Despite remarkable efforts, expectations for the “shock and kill” strategy have not been met yet (reviewed in (Kim et al., 2018, Darcis et al., 2017)). The limitations of the “shock and kill” strategy are primarily attributed to the heterogeneity of the molecular mechanisms regulating HIV-1 latency, which contributes to inconsistent responses to LRAs and impedes the complete clearance of HIV-1 reservoirs (Darcis et al., 2017, Rodari et al., 2021). The limited success of the “shock and kill” strategy has led to a search for alternative approaches, such as a functional cure (Bailon et al., 2020). A functional cure aims to achieve long-term viral suppression or remission in the absence of ongoing ART (Bailon et al., 2020). This goal is pursued through the "block and lock" strategy, which seeks to permanently silence the virus by halting proviral transcription and locking the reservoirs in a deeply latent state (Bailon et

al., 2020). Various small molecules that target HIV-1 proteins, epigenetic proteins, and cell signalling pathways have been proposed as latency-promoting agents (LPAs) that can be utilised in the silencing of HIV-1 transcription (reviewed in (Bailon et al., 2020, Vansant et al., 2020)). However, the "block and lock" strategy still requires more scrutiny as it has been shown to be limited by the potential of non-specific off-target effects arising from the modulation of host cellular pathways, which may result in cytotoxicity (Vargas and Sluis-Cremer, 2022). Taken together, these reports suggest a need to continue to search for better and more effective agents for HIV-1 cure strategies.

Lysozyme (Lz) and lactoferrin (Lf) are cationic antimicrobial proteins that play an important role in innate immune defence and have both been shown to block HIV-1 replication (Lee-Huang et al., 1999, Lee-Huang et al., 2005, Berlutti et al., 2011). Both proteins exhibit anti-HIV-1 activity by disrupting viral interactions with host cell receptors. Lz competitively binds to cluster of differentiation 4 (CD4), while Lf strongly binds to dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), blocking the DC-SIGN–glycoprotein 120 (gp120) interaction and inhibiting viral adsorption (Lee-Huang et al., 1999, Lee-Huang et al., 2005, Berlutti et al., 2011). Moreover, experimental assays revealed that these proteins bind deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Steinrauf et al., 1999, Lin et al., 2009, Fleet, 1995) and modulate cellular gene expression (Lee-Huang et al., 2005, Bergamo et al., 2019, Mulligan et al., 2006). The gene expression pathways modulated by these proteins include nuclear factor kappa B (NF-κB) and protein kinase C pathways, both of which are implicated in HIV-1 transcription and latency reversal (Lee-Huang et al., 2005, Jiang and Dandekar, 2015). A previous study demonstrated that treatment of HIV-1-infected cells with HL9 or HL18, fragments of human Lz, upregulated the expression of several cellular genes, including activating transcription factor 2 (*ATF2*), BCL2-associated X (*Bax*), cyclin-dependent kinase inhibitor 1A (*CDKN1A*), baculoviral IAP repeat-containing 2 (*BIRC2*), and baculoviral IAP repeat-containing 3 (*BIRC3*) (Lee-Huang et al., 2005). Notably, *BIRC2* (IAP2) and *BIRC3* (IAP1), both inhibitors of apoptosis proteins (IAPs), play crucial roles in cell survival pathways (Lee-Huang et al., 2005). Detailed information on Lz and Lf's anti-HIV-1 traits, is provided in the literature review below.

Although the anti-HIV properties of these cationic proteins have been well-detailed in multiple studies, their effects on HIV-1 latency potential remain to be determined. Therefore, this study aims to determine whether cationic proteins of the human innate immune system can modulate HIV-1 latency. In this study we hypothesise that Lz, HL9, and Lf may affect the propensity of latency reversal and/or enhancement alone and/or in combination with already-known LRAs and LPAs. The objectives formulated for this study are as follows:

1. Test the potential of cationic proteins (Lz, HL9 and Lf), alone or in combination, to reverse or enhance HIV-1 latency in J-Lat cell lines.
2. Test the ability of these proteins to modulate (inhibit or enhance) latency reversal in the presence of known LRAs and LPAs using J-Lat cell lines.

## 2. Literature review

Although ART has had great success in reducing viral replication and plasma viremia to below detectable levels, it does not eliminate the HIV-1 latent reservoir, which very quickly reactivates systemic infection when treatment is stopped (Contreras et al., 2006, Blanco-Rodriguez et al., 2020). There is a general understanding in the field that resting memory CD4+ T-lymphocytes are the primary HIV-1 latent reservoir (Hakre et al., 2012, García et al., 2018). There are several reasons for this, including their long lifespan which allows HIV-1 persistence without activation over prolonged periods, while their resting state keeps very low and undetectable transcription levels of the integrated provirus (Hakre et al., 2012, García et al., 2018, Mbonye and Karn, 2024). Additionally, homeostatic proliferation of these resting memory CD4+ T-lymphocytes distributes latent virus among daughter cells during normal immune system maintenance (Hakre et al., 2012, García et al., 2018, Mbonye and Karn, 2024).

Although the concept of a persistent HIV-1 latent reservoir has been studied in great detail for decades, it remains not fully understood. This is primarily due to the complex processes involving numerous viral, cellular, and immunological factors that allow the virus to persist in a transcriptionally silent but replication-competent state (Contreras et al., 2006, Blanco-Rodriguez et al., 2020). Some of the major characteristics of the reservoir that are yet to be fully understood include the molecular mechanisms responsible for the establishment and maintenance of the reservoir. There is, however, a consensus on the idea that the establishment and maintenance of the HIV-1 reservoir are primarily modulated by mechanisms operating at a transcriptional and epigenetic level (Ruelas and Greene, 2013, Archin et al., 2014b).

### 2.1. Molecular mechanisms of HIV-1 latency establishment and maintenance

As aforementioned, an incomplete understanding of molecular mechanisms responsible for HIV-1 latency establishment and maintenance presents a principal challenge in cure research. Although transcriptional and epigenetic processes are established as the primary regulators, their multifaceted dynamics remain insufficiently understood (Abbas and Herbein, 2012, Van Lint et al., 2013, Chou et

al., 2024). This chapter therefore discusses (a) integration site selection and chromatin environment, (b) transcriptional interference, (c) epigenetic regulation, (d) sequestered cellular transcription factors, and (e) post-transcriptional regulation, all of which critically enable transcriptional silencing that underpins both the establishment and persistence of the latent reservoir (Abbas and Herbein, 2012, Van Lint et al., 2013, Chou et al., 2024).

### 2.1.1. Integration site selection and chromatin environment

Establishing HIV-1 latency begins with the integration of viral DNA into the host genome, a process far more strategic than once imagined. The virus doesn't merely drift into the nucleus, it actively hijacks cellular importin/karyopherin pathways to shuttle its pre-integration complex inside non-dividing cells (Bukrinsky et al., 1992, Gallay et al., 1997). What matters for latency, however, is where the virus lands after entering the cell. A recent study demonstrated that HIV-1 specifically targets the outer nuclear shell near nuclear pores, homing in on transcriptionally active chromatin regions, a preference that seems paradoxical for a virus seeking long-term silence (Marini et al., 2015). The host protein LEDGF/p75 (lens epithelium-derived growth factor, 75 kDa variant) acts as the molecular zip code that actively tethers viral DNA into these regions (Silvers et al., 2010). Rather than random misfortune, latency establishment involves deliberate insertion into genomic locations where the virus can exploit host factors to maintain transcriptional silence while preserving reactivation potential (Han et al., 2004, Mitchell et al., 2004, Lewinski et al., 2005, Abbas and Herbein, 2012).

Furthermore, chromatin accessibility sits at the heart of HIV-1's ability to integrate, persist, and remain latent for decades (Jones et al., 2025). Initial investigations into the chromatin landscape identified DNase I-hypersensitive sites at the LTR mark regions of active transcription, yet the virus strategically embeds itself in gene-dense chromatin where host machinery can repress it (Verdin, 1991). This repression is orchestrated through multiple mechanisms including a maintained state of hypoacetylation of nucleosomes by histone deacetylases (HDACs) (Van Lint et al., 1996), the occlusion of transcription factor binding by remodelling complexes like the Brahma-associated factor (BAF) and facilitates chromatin transcription (FACT) (Vanti et al., 2009, Rafati et al., 2011), and the inhibition of polymerase access to the provirus by local host promoters (Shan et al., 2011). Recent advances reveal that intact proviruses often integrate in non-genic regions or antisense orientations (Einkauf et al., 2019), while single-cell studies confirm that latency establishment, and by extension, latency-reversal, is dictated by integration site context and 3D nuclear architecture (Janssens et al., 2022, Jones et al., 2025). Understanding these mechanisms reveals that enhancing chromatin access and disrupting the virus's precise integration targeting could significantly modulate HIV-1 latency, underscoring the therapeutic potential of chromatin environment and viral integration in cure development.

### 2.1.2. Transcription interference

HIV-1 transcription is the process by which the integrated HIV-1 provirus uses its 5′ LTR promoter and host RNA polymerase II (RNAPII) to synthesize viral RNAs from proviral DNA (Roebuck and Saifuddin, 1999, Karn, 2011, Schiralli Lester and Henderson, 2012). This reaction is initiated by the binding of the specificity protein 1 (Sp1) and other cellular transcription factors to LTR cis-elements leading to basal transcription and the production of short, abortive viral mRNA transcripts (Sviderskaia and Meier-Stephenson, 2025).

Only a limited number of viral transcripts are fully elongated, which makes Tat one of the HIV-1 genes expressed early during infection (Easley et al., 2010). Tat first docks onto the TAR (trans-activation response element) stem-loop (nucleotides +1 – +59) of the nascent viral transcript, recruits the positive transcription elongation factor b (P-TEFb) to that RNA platform, and then triggers CDK9-mediated phosphorylation of RNAPII's C-terminal domain and the negative elongation factors DSIF/NELF, thereby releasing promoter-proximal pausing and committing the polymerase to processive elongation of full-length HIV-1 genomes. (Roebuck and Saifuddin, 1999, Karn, 2011, Schiralli Lester and Henderson, 2012). Efficient completion of the viral life cycle requires a threshold burst of productive transcription from the 5′ LTR; when this is restricted, the provirus can enter a latent state characterised by little or no HIV-1 RNA production (Roebuck and Saifuddin, 1999, Karn, 2011, Schiralli Lester and Henderson, 2012).

Transcriptional interference (TI) contributes to the establishment and maintenance of this latent state by suppressing LTR-driven transcription, even in the presence of Tat, when the provirus integrates within actively transcribed host genes (Greger et al., 1998, Lenasi et al., 2008, Ruelas and Greene, 2013). This process is achieved through two major mechanisms, namely promoter occlusion and convergent transcription. Promoter occlusion occurs when the HIV-1 provirus integrates downstream of a host gene in the same transcriptional orientation (Siliciano and Greene, 2011, Abbas and Herbein, 2012, Ruelas and Greene, 2013). In this configuration, transcription from the upstream host gene can elongate beyond its normal stop signal, a process known as **read-through transcription**, due to RNAPII failing to properly terminate (Greger et al., 1998, Lenasi et al., 2008, Ruelas and Greene, 2013). This read-through transcription can displace Sp1 and other transcription factors from the HIV-1 LTR, further reinforcing viral latency (Greger et al., 1998, Lenasi et al., 2008, Ruelas and Greene, 2013).

In contrast, convergent transcription is characterised by the integration of the provirus in an opposite orientation to the host gene (Lewinski et al., 2005, Siliciano and Greene, 2011, Ruelas and Greene, 2013). In this configuration, RNAPII complexes transcribing the host gene and the proviral DNA can collide during the elongation (Lewinski et al., 2005, Siliciano and Greene, 2011, Ruelas and Greene, 2013). These collisions can lead to premature termination at one or both promoters (Lewinski et al., 2005, Siliciano and Greene, 2011, Ruelas and Greene, 2013).

Convergent transcription can also result in the formation of double-stranded viral RNAs, which can trigger RNA interference, RNA-mediated methylation, and biologically active antisense RNAs (Siliciano and Greene, 2011, Ruelas and Greene, 2013). Collectively, these TI mechanisms, both promoter occlusion and convergent transcription, highlight that the orientation-dependent regulation of viral transcription is highly variable, depending on factors such as 5' LTR occupancy and the host gene transcription rate (Lewinski et al., 2005, Siliciano and Greene, 2011, Ruelas and Greene, 2013).

### 2.1.3. Epigenetic regulation

A key mechanism for maintaining HIV-1 latency involves epigenetic changes in chromatin that suppress viral gene expression, as is the case with host genes (Siliciano and Greene, 2011, Ruelas and Greene, 2013). Host gene expression is dynamically controlled through alterations in chromatin structure; condensed chromosomes during mitosis are highly compacted, whereas actively transcribed genes reside in "open" chromatin regions called *euchromatin*, and inactive genes are typically found in tightly packed heterochromatin (Siliciano and Greene, 2011, Ruelas and Greene, 2013, Mirabella et al., 2015). Heterochromatin formation inhibits gene expression by blocking transcription factors from accessing the DNA (Mirabella et al., 2015).

Given the role of chromatin in regulating transcription, HIV-1 integration is influenced by these epigenetic landscapes. Large-scale mapping of HIV-1 integration sites revealed a strong preference for insertion into actively transcribed regions of the genome (Schröder et al., 2002, Mitchell et al., 2004, Hakre et al., 2012). *In vitro* studies using Jurkat T-cell lines further characterised integration patterns, including transcription units, gene deserts, and heterochromatic regions, and latent viral populations enriched near centromeric alphoid repeats (Han et al., 2004, Mitchell et al., 2004, Lewinski et al., 2005). *In vivo* studies of resting CD4+ T-cells from PLWH on ART also showed that most integrated HIV-1 DNA resides within actively transcribed genes, challenging earlier assumptions about the mechanisms underlying HIV-1 latency (Han et al., 2004, Mitchell et al., 2004, Lewinski et al., 2005, Abbas and Herbein, 2012).

Integration into heterochromatin is not the primary mechanism of HIV-1 latency; rather, other features of chromatin biology play a more significant role (Siliciano and Greene, 2011, Ruelas and Greene, 2013). This led to further investigation of the chromatin environment, which revealed that histone modifications are key regulators of HIV-1 gene expression during latency (Imai and Ochiai, 2011). Specific histone marks are associated with transcriptional activity or repression. For example, trimethylation of lysine 4 on the histone H3 protein (H3K4me3) and acetylation of histone H3 at residue K9 (H3K9ac) are linked to active transcription, whereas H3K9me3 is a repressive mark associated with latency (Kim et al., 2017). H3K9me3 establishes repressive heterochromatin at the HIV-1 5′ LTR, silencing proviral transcription by recruiting heterochromatin protein 1 (HP1) and promoting nucleosome compaction (Turner and Margolis, 2017). This mark is deposited by the suppressor of variegation 3-9 homolog 1 (Suv39h1) and euchromatic histone-lysine N-methyltransferase 2 (EHMT2), and its repressive effect is reinforced by host factors such as the origin recognition complex subunit 1 (ORC1), creating a stable, heritable barrier to viral gene expression (Turner and Margolis, 2017).

DNA methylation is another mechanism of epigenetic regulation, in which a methyl group ($–CH_3$) is added to the 5-carbon of cytosine (Moore et al., 2013). This modification occurs most commonly at CpG sites (dinucleotide sequence in which a cytosine is directly followed by a guanine on the same DNA strand, connected by a phosphate group [5′-C-p-G-3′]) (Moore et al., 2013). This heritable mark compacts chromatin, limiting transcription factor access and silencing gene expression (Moore et al., 2013).

Studies of the 5′ LTR from aviremic individuals on ART, whose proviruses resist reactivation, revealed hypermethylation of CpG islands (Blazkova et al., 2009). This feature distinguishes aviremic individuals from viremic individuals, whose promoters are hypomethylated, transcriptionally permissive (Blazkova et al., 2009). Further investigations showed that this epigenetic blockade is catalyzed specifically by DNA methyltransferase (DNMT) -1 and DNA methyltransferase 3 beta (DNMT3B), which maintain repressive methylation patterns across cellular divisions, effectively locking the provirus in a dormant state that withstands immune and therapeutic pressure (Blazkova et al., 2009).

Notably, the role of DNA methylation extends beyond transcriptional silencing, it interacts with other epigenetic modifications to establish and maintain a repressive chromatin environment (Imai and Ochiai, 2011). DNA methylation often works jointly with histone methylation, particularly through the

recruitment of methyl-binding proteins and HDACs, thereby reinforcing transcriptional repression (Imai and Ochiai, 2011). This interplay between epigenetic mechanisms contributes to the stability of the latent state and serves as a barrier to spontaneous reactivation of viral transcription (Imai and Ochiai, 2011). Consequently, understanding the precise mechanisms by which DNA methylation interacts with histone modifications remains a critical area of research, with important implications for developing strategies aimed at either clearing the persistent viral reservoir or locking it in a deep state of latency.

### 2.1.4. Sequestered cellular transcription factors

A crucial component of HIV-1 latency is the limited availability of essential host transcription and elongation factors necessary for productive HIV-1 transcription (Siliciano and Greene, 2011, Ruelas and Greene, 2013). In latently infected memory CD4+ T-cells, many of these transcriptional activators are sequestered in inactive states, while transcriptional repressors accumulate at the HIV-1 promoter, further suppressing viral gene expression (Pace et al., 2011, Siliciano and Greene, 2011, Ruelas and Greene, 2013).

One prominent example is NF-κB, a key regulator of HIV transcription. Under resting conditions, NF-κB remains sequestered in the cytoplasm, while NF-κB homodimers occupy the HIV-1 promoter, 5′ LTR and recruit HDACs, thereby establishing a transcriptionally repressed chromatin environment (Coiras et al., 2007, Colin and Van Lint, 2009, Abbas and Herbein, 2012, Dutilleul et al., 2020).

As previously mentioned, P-TEFb is another cellular protein complex that plays a crucial role in host gene regulation and is hijacked by the HIV-1 to promote viral transcription (Quivy and Van Lint, 2004, Colin and Van Lint, 2009). When P-TEFb is bound to the inhibitory 7SK small nuclear RNA/hexamethylene bisacetamide-induced protein (7SK snRNA/HEXIM) complex, it is unable facilitate transcriptional elongation, thereby contributing to maintenance of HIV-1 transcriptional silence (Quivy and Van Lint, 2004, Colin and Van Lint, 2009). In addition to P-TEFb, other host factors, including activator protein-1 (AP-1), Sp1, cyclin-dependent kinase 2 (CDK2), and various chromatin-modifying enzymes, are also sequestered during latency, further contributing to transcriptional suppression (Coiras et al., 2007, Colin and Van Lint, 2009, Abbas and Herbein, 2012, Dutilleul et al., 2020). Understanding the mechanisms governing the sequestration and release of these factors may inform strategies aimed at manipulating latent HIV-1 transcription (Coiras et al., 2007, Colin and Van Lint, 2009, Abbas and Herbein, 2012, Dutilleul et al., 2020).

Overall, HIV-1 latency emerges as a tightly coordinated and multifactorial state governed by the dynamic interplay between epigenetic modification, chromatin architecture, and restricted access to host transcriptional control machinery (Abbas and Herbein, 2012). This multilayered regulatory network ensures durable silencing of the provirus while preserving the potential for rapid reactivation, underscoring the complexity of HIV-1 persistence and the challenges associated with targeting the latent reservoir.

### 2.2. Cure strategies: 'shock and kill' and 'block and lock'

Currently, ART is proving to be inefficient when it comes to clearing stable HIV-1 reservoirs (Abbas and Herbein, 2012). This has spurred the search for new HIV-1 treatment and cure strategies aimed at either preventing the establishment of latency, reactivating viral reservoirs in chronically infected individuals to enable viral clearance, or a combination of both approaches (Abbas and Herbein, 2012). There have been cases in the recent past that have fuelled hopes for an HIV-1 cure or durable control. First, it was the case of Timothy Brown who underwent stem cell transplantation using donor cells from a person with a rare genetic mutation (*CCR5-Δ32*) that provided resistance to HIV-1 (Hutter et al., 2009). Next, there were Boston patients who maintained undetectable viral loads for some time post-stem-cell transplant and cessation of ART (Henrich et al., 2014). This was followed by the case of the Mississippi child who was born with HIV-1 and maintained undetectable viral loads without ART for over two years after being put on therapy for eighteen months (Luzuriaga et al., 2015). Furthermore, there was a report of a VISCONTI cohort who began ART very early after infection. This cohort, after eventually discontinuing ART, maintained long-term control over their viral load without the need for continued treatment (Sáez-Cirión et al., 2013). Most recently, a perinatally infected child achieved viral remission despite a prolonged treatment interruption (Frange et al., 2016).

These findings have steered HIV-1 cure research towards more treatment interruption trials, which have yielded significant information regarding the nature of HIV-1 reservoirs. For example, Williams et al. (2014) revealed that the total HIV-1 DNA level is correlated with the timing of viral rebound after ART cessation. This finding established that the size of the HIV-1 reservoirs can predict the timing of viral rebound post-treatment interruption (Williams et al., 2014). These discoveries have further motivated strategies targeting the latent reservoirs in HIV-1-infected individuals, with the goal of inducing proviral expression to facilitate the elimination of the virus and infected cells (Deeks, 2012, Ruelas and Greene, 2013).

Early attempts to reactivate viral production through global T-cell activation resulted in harmful immune activation, prompting a shift toward strategies that reactivate the virus without inducing T-cell activation (Archin et al., 2009, Barton et al., 2013, Xing and Siliciano, 2013, Lewis et al., 2023). However, most of these approaches have primarily been tested in cell line models of HIV-1 latency, with limited validation in resting CD4+ T-cells from aviremic patients (Xing and Siliciano, 2013, Lewis et al., 2023). The development of primary cell models of latency has offered critical insights into the responses to various LRAs (Lewis et al., 2023). Nevertheless, translating these findings into clinical applications presents challenges (Archin et al., 2009, Barton et al., 2013, Xing and Siliciano, 2013, Lewis et al., 2023). Promising strategies include small-molecule inhibitors targeting HDACs to promote transcription at the HIV-1 LTR, with some HDAC inhibitors advancing to clinical trials (Archin et al., 2009, Archin et al., 2014a). However, the intricate nature of biological systems and the necessity for a deeper comprehension of the impacts of these interventions on host genes are crucial for designing effective and enduring strategies to induce latent HIV-1 genome expression and potentially achieve viral clearance (Archin et al., 2014a, Lewis et al., 2023).

### 2.3. Shock and kill

Despite the global roll-out of ART and its effectiveness in suppressing viral replication to levels below the limit of detection, ART alone is insufficient to clear the stable HIV-1 reservoir, necessitating lifelong treatment (Arts and Hazuda, 2012). This limitation has driven the development of novel cure strategies aimed at reactivating latent viral reservoirs to enable immune-mediated clearance, or combining both approaches to achieve durable viral control or eradication (Deeks, 2012, Acchioni et al., 2021). One of the leading strategies in HIV-1 cure research is the "shock and kill" approach, which aims to clear latent viral reservoirs by reactivating latent proviruses using LRAs. This reactivation forces previously latent proviruses to undergo viral gene transcription, proteins expression, and produce virions (Figure 1) (Deeks, 2012).

Once reactivated, these previously latently infected cells become susceptible to immune-mediated clearance or cytolysis, while ART prevents new rounds of infection (Figure 1) (Deeks, 2012, Cohn et al., 2020). To date, over 300 compounds (including epigenetic modulators, chromatin remodelers, signaling agonists, and transcriptional activators) have been tested for their ability to reactivate latent HIV-1 *in vitro* (Hashemi and Sadowski, 2020, Maina et al., 2021). Among these, HDAC inhibitors represent the most extensively studied class of LRAs in pre-clinical and clinical cure trials (Hashemi and Sadowski, 2020, Maina et al., 2021).

### i. Vorinostat/suberoylanilide hydroxamic acid, an HDAC-inhibitor

Vorinostat (suberoylanilide hydroxamic acid, SAHA) is a hydroxamate-class HDAC inhibitor that broadly targets class I and II HDAC enzymes, exerting diverse cellular effects through both transcriptional and non-transcriptional mechanisms (Bubna, 2015). Its inhibitory action occurs via chelation of the catalytic zinc ion in HDACs, with structural studies revealing how its phenyl ring extends onto the enzyme surface to block substrate access (Finnin et al., 1999, Bubna, 2015). This inhibition leads to accumulation of acetylated proteins, including histones, resulting in altered gene expression through both direct HDAC inhibition and indirect modulation of transcription factors such as E2 promoter binding factor 1 (E2F-1), yin yang 1 (YY-1), mothers against decapentaplegic homolog 7 (Smad7), tumor protein p53 (p53), B-cell lymphoma 6 (Bcl-6), and GATA-binding factor 1 (GATA-1) (Bereshchenko et al., 2002, Johnstone and Licht, 2003, Bubna, 2015).

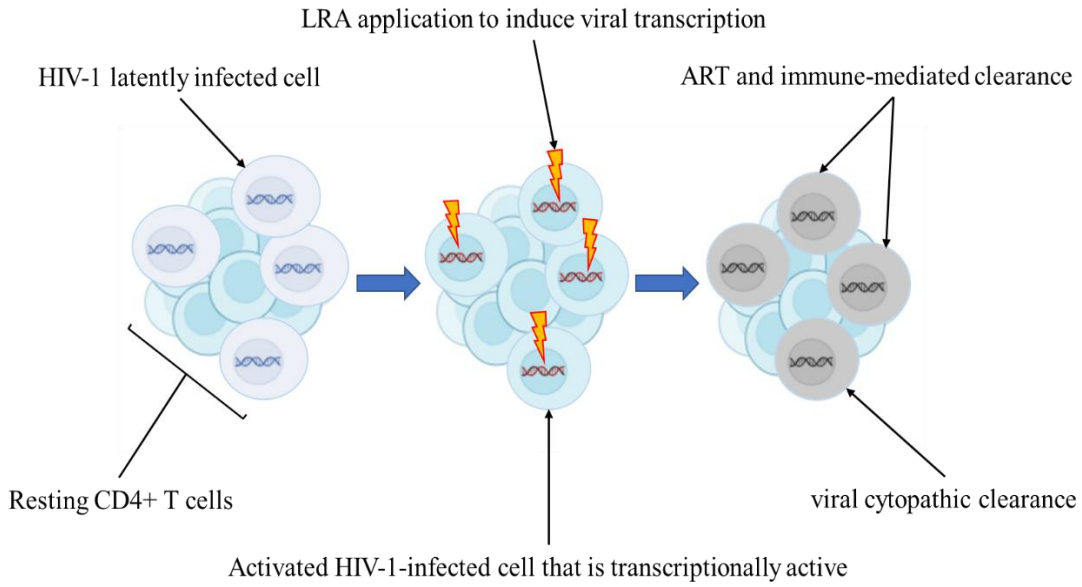


**Figure 1**: Conceptual overview of the "shock and kill" HIV-1 cure strategy. This figure shows that latency-reversing agents (LRAs) reactivate transcriptionally silent proviruses in resting CD4+ T-cells, inducing viral gene expression and exposing infected cells to immune-mediated clearance, viral cytopathic effects, and ART. The Figure was constructed using the online tool Biorender (https://app.biorender.com).

In HIV-1 cure research, vorinostat has emerged as a promising LRA for the "shock and kill" strategy. Proof-of-concept studies demonstrated its ability to significantly increase HIV-1 RNA expression in resting CD4+ T-cells from ART-suppressed individuals (Archin et al., 2009), while *in vivo* studies revealed additional cytoplasmic effects that enhance post-entry viral processes including reverse transcription, nuclear import, and integration in a dose- and time-dependent manner (Lucera et al., 2014). These dual mechanisms, both reactivating latent virus through LTR-driven transcription and

promoting viral replication steps, position vorinostat as a multifaceted agent in HIV-1 clearance strategies, capable of rendering infected cells more susceptible to immune clearance while preventing new infections through ongoing ART (Archin et al., 2014a, Lucera et al., 2014).

### ii. Phorbol 12-Myristate 13-Acetate, a protein kinase C agonist

T-cell activating agents that engage signal transduction pathways can effectively reverse HIV-1 latency in cell-based models (Ao et al., 2016). The protein kinase C (PKC) pathway plays a central role in this process by activating key transcription factors (NFAT, NF-κB, and AP-1) essential for T-cell activation and viral reactivation (Ao et al., 2016). PKC agonists, including phorbol esters (e.g., PMA), prostratin, bryostatin-1, and ingenol derivatives, have demonstrated potent latency-reversing activity in both cell lines and primary cells from PLWH (Ao et al., 2016). PMA (12-O-tetradecanoylphorbol-13-acetate), a diacylglycerol (DAG) mimetic, specifically activates PKC isoforms, triggering downstream phosphorylation events that regulate cellular activation and HIV-1 LTR-driven transcription (Brogdon et al., 2016, Rodari et al., 2021, Kazanietz and Cooke, 2024). Mechanistic studies reveal that PMA rapidly induces HIV-1 expression through PKC/NF-κB-dependent mechanisms, including enhanced formation of transcription factor complexes (Sp1-p53-c-Jun) at the viral promoter (Jabareen et al., 2018). PKC agonist-mediated HIV-1 reactivation was also reported in lymphoid cells (Kalvatchev et al., 1997). More recent work has shown that the next-generation DAG-lactone derivative YSE028 acts as a potent LRA in models of HIV-1 latency (Matsuda et al., 2021). *In vitro*, YSE028 not only reactivated latent HIV-1 in ACH-2 and J-Lat cell lines and in primary $CD4^+$ T-cells from PLWH on suppressive ART, but its latency reversal activity was synergistically enhanced when combined with the bromodomain and extra-terminal (BET) inhibitor JQ1 (Matsuda et al., 2021). Moreover, YSE028 selectively induced caspase-mediated apoptosis in latently infected cells while showing minimal toxicity *in vitro* and *in vivo*, supporting their potential as components of combination strategies to reduce the HIV-1 reservoir (Matsuda et al., 2021).

Despite remarkable efforts, expectations for this “shock and kill” strategy have not been met yet (Kim et al., 2018). Reasons behind the shortcomings of this strategy include transiency of the induced HIV-1 expression (Archin et al., 2014a). Clinical studies revealed that LRA treatment results in temporal increases in HIV-1 RNA expression, with viral transcription returning to baseline levels within 7-14 days following LRA cessation (Archin et al., 2014a). This makes achieving a sustained and robust reactivation of latent HIV-1 challenging and limits the efficacy of the subsequent "kill" phase (Archin et al., 2014a, Kim et al., 2018). These limitations have spurred interest in alternative approaches such

as the "block and lock" strategy, which aims for functional cure by permanently silencing residual proviruses rather than reactivating them (Maina et al., 2021).

### 2.4. Block and lock

The "block and lock" strategy represents a paradigm shift in HIV-1 cure research, aiming to establish permanently silenced viral reservoirs that are unable to initiate viral rebound after treatment interruption (Figure 2) (Vansant et al., 2020, Vargas and Sluis-Cremer, 2022). This approach utilizes LPAs to epigenetically suppress proviral transcription and enforce a deep latent state, effectively "locking" HIV-1 in place (Vansant et al., 2020, Vargas and Sluis-Cremer, 2022).

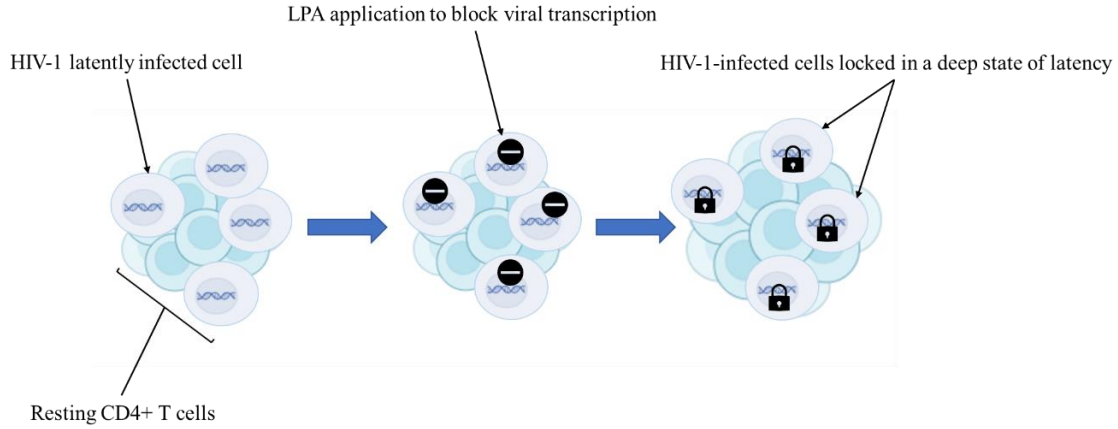


**Figure 2**: Conceptual overview of the "block and lock" HIV-1 cure strategy. Latency-promoting agents (LPAs) reinforce transcriptional silencing of integrated proviruses through epigenetic modifications, preventing reactivation and maintaining the virus in a deeply latent, non-inducible state; mechanistically opposing the "shock and kill" approach. The Figure was constructed using the online tool Biorender (https://app.biorender.com).

While integrated proviral DNA persists, transcriptional silencing blocks viral gene expression, thus locking the reservoir in a deep latent state, eliminating the need for continuous ART (Moranguinho and Valente, 2020). This method targets a functional cure, wherein viral replication is managed without the necessity of ART (Moranguinho and Valente, 2020). Functional cure approaches are anticipated to be more cost-effective, provide enduring effects, and be feasible in resource-limited settings compared to standard ART regimens (Moranguinho and Valente, 2020). Due to the intricate nature of HIV-1 transcription, which involves numerous factors, there are multiple compounds that target various steps in viral transcription that are being explored in the quest for a functional cure (Moranguinho and Valente, 2020).

### i. Tanespimycin, a HSP90i

Environmental temperature significantly influences HIV-1 replication dynamics, with clinical observations showing that fever episodes in PLWH can modulate viral activity (Modjarrad and Vermund, 2010). *In vitro* studies demonstrate that heat shock enhances HIV-1 production in both latently infected cell lines and peripheral blood mononuclear cells (PBMCs), revealing a temperature-dependent aspect of viral reactivation (Roesch et al., 2012). Central to this phenomenon is heat shock protein 90 (HSP90), an adenosine triphosphate (ATP)-dependent molecular chaperone that constitutes 1-2% of cellular proteins and plays a pivotal role in viral protein folding and stabilization (Csermely et al., 1998, Iyer et al., 2021). HSP90 ensures proper maturation of critical viral components, including polymerases and capsid proteins, thereby maintaining viral infectivity (Csermely et al., 1998, Iyer et al., 2021). The chaperone's involvement extends to HIV-1 transcriptional regulation, where it co-localises with active proviruses and enhances LTR-driven expression, particularly under hyperthermic conditions (Roesch et al., 2012, Anderson et al., 2014). Mechanistically, HSP90 intersects with key pathways in HIV-1 biology, i.e. it facilitates NF-κB-mediated T-cell activation and maintains p-TEFb functionality for transcriptional elongation (Roesch et al., 2012, Anderson et al., 2014, Pan et al., 2016). These dual roles make HSP90 inhibition an attractive therapeutic strategy, as demonstrated by studies showing that HSP90 knockdown suppresses viral reactivation while its overexpression enhances it (Pan et al., 2016, Trivedi et al., 2019).

The geldanamycin analog tanespimycin (17-AAG) exemplifies this therapeutic potential through its specific inhibition of HSP90's ATP-binding pocket (Prodromou et al., 1997, Banerji et al., 2005). *In vitro* studies reveal tanespimycin (Ts)' dose-dependent suppression of HIV-1 reactivation across J-Lat cell line models, with enhanced efficacy under hyperthermic conditions (Roesch et al., 2012, Anderson et al., 2014). By disrupting HSP90's chaperone functions, Ts impairs the stability of HIV-1 replication machinery, effectively blocking both basal and heat-induced viral transcription (Roesch et al., 2012). These findings position HSP90 inhibitors as promising candidates for therapeutic strategies targeting HIV-1 latency and replication, particularly in contexts of temperature-mediated viral activation.

### ii. Spironolactone, a degrader of the XPB subunit

Transcription factor II H (TFIIH) is a large multiprotein complex (~460 kDa) that forms part of the six transcriptional factors (TFII-A, -B, -D, -E, -F, and -H) required for basal transcription initiation (Orphanides et al., 1996, Schultz et al., 2000, Greber et al., 2019). These factors, in combination with RNAPII, interact with the integrated provirus LTR to form the transcription initiation complex (TIC)

(Orphanides et al., 1996, Schultz et al., 2000, Greber et al., 2019). The formation of the TIC is the first step in transcription initiation and is critical for promoter opening, synthesis of the initial phosphodiester bond, and promoter clearance (Orphanides et al., 1996, Schultz et al., 2000, Greber et al., 2019). TFIIH is unique among the aforementioned transcription factors due to its enzymatic activities and plays a role in both transcription and DNA repair (Compe and Egly, 2016, Rimel and Taatjes, 2018). TFIIH is composed of nine polypeptides and can be divided into two structural and functional complexes (Compe and Egly, 2016, Rimel and Taatjes, 2018). The CDK activating kinase (CAK) complex (comprising CDK7, cyclin H, and ménage à trois 1 [MAT1]) phosphorylates RNAPII's C-terminal domain (CTD) to facilitate transcription elongation (Schultz et al., 2000, Compe and Egly, 2016, Rimel and Taatjes, 2018). The core TFIIH complex contains the helicases xeroderma pigmentosum group b protein (XPB) and xeroderma pigmentosum group D (XPD) (with opposing polarities) and structural subunits (p62, p52, p44, p34), which collectively regulate transcription initiation (Greber et al., 2019).

XPB, an adenosine triphosphate (ATP)-dependent helicase, is essential for promoter melting; mutations disrupting its ATP-binding site impair promoter opening and transcription (Compe and Egly, 2016, Rimel and Taatjes, 2018). Spironolactone (Sp) induces the XPB subunit in the TFIIH complex in a dose-dependent manner (Lacombe et al., 2016, Mori et al., 2021b). Cells treated with Sp exhibited a reduction in viral production, as evidenced by decreased levels of the HIV-1 capsid protein p24 and viral mRNA, without obvious cytotoxic effects (Lacombe et al., 2016, Mori et al., 2021b). While XPB is critical for transcription initiation, its degradation by Sp does not significantly affect global transcription but specifically inhibits genes that are highly dependent on NF-κB, including HIV-1 (Lacombe et al., 2016, Mori et al., 2021b). Furthermore, Sp inhibits HIV-1 replication beyond transcription initiation as it suppresses Tat-dependent HIV-1 transactivation, effectively blocking HIV-1 infections in permissive T-cells (Lacombe et al., 2016, Mori et al., 2021b). This positions Sp as a promising "block and lock" therapeutic agent that targets a host cellular protein rather than the virus itself.

### 2.5. Limitations of existing LRAs and LPAs

LRAs are being intensively investigated as a critical component of "shock and kill" HIV-1 cure regimens to activate the latent reservoirs of HIV-1 so that they can be eliminated. Although very potent in inducing viral transcription in the majority of *in vitro* and *ex vivo* models, their potential in depleting the reservoir in patients is restricted (Darcis et al., 2017, Rodari et al., 2021). Clinical studies of LRAs like HDAC inhibitors romidepsin and vorinostat have exhibited enhanced viral RNA expression without inducing significant depletion of latently infected cells or interrupting viral rebound after halting ART

(Kroon et al., 2020, McMahon et al., 2021). This muted clinical activity is a result of heterogeneity of HIV-1 latency reversal as well as complex silencing mechanisms utilised, rendering latent reservoirs refractory to homogeneous reactivation (Darcis et al., 2017, Rodari et al., 2021). Also, all but a few LRAs cause generalised T-cell activation or off-targeting, which again is not desirable from the safety aspect and limits their use *in vivo* (Darcis et al., 2017, Rodari et al., 2021). For instance, extremely potent LRAs that activate NF-κB or protein kinase C agonists cause generalised activation of the immune system and thus toxicity and inflammation (Darcis et al., 2017, Rodari et al., 2021). Furthermore, the abrogated cytotoxic T lymphocyte function in the chronic patient undermines their ability to kill reactivating cells, undermining the "kill" component of the strategy (Darcis et al., 2017, Rodari et al., 2021).

LPAs that augment the "block and lock" function by inducing long-term and stable viral latency are similarly severely handicapped by severe constraints largely because of the problem of specificity (Vansant et al., 2020, Vargas and Sluis-Cremer, 2022). Most LPAs function on host transcriptional machinery to knock down HIV-1 proviral expression at the risk of off-target activity against host gene expression (Vansant et al., 2020, Vargas and Sluis-Cremer, 2022). For example, certain CDK inhibitors and epigenetic modifiers suppress global transcriptional programs other than HIV-1, resulting in unwanted host cytotoxicity and interference with host gene expression (Janssens et al., 2024, Horvath et al., 2024) . Although drugs like didehydro-cortistatin A (dCA), a Tat inhibitor, have high specificity to suppress HIV-1 transcription with minimal host effect, most LPAs lack this to a great extent (Mediouni et al., 2019, Vansant et al., 2020, Vargas and Sluis-Cremer, 2022). Low specificity of such a nature limits their therapeutic applications because long-term delivery will create undesirable effects on cellular function in non-pathological cells (Mediouni et al., 2019, Vansant et al., 2020, Vargas and Sluis-Cremer, 2022). Therefore, LPAs and LRAs both presently encounter problems of potency, specificity, safety, and immune interaction, which need continued investigation for the construction of highly specific, potent agents for HIV-1 reservoir modulation safely in a cure (Mediouni et al., 2019, Vansant et al., 2020, Vargas and Sluis-Cremer, 2022).

### 2.6. Cationic proteins

The cationic proteins Lz and Lf are found in high concentrations in human mucosal secretions, such as those of the eye, respiratory tract, and reproductive system, as well as in the specific granules of polymorphonuclear leukocytes (Ellison and Giehl, 1991, Hanstock et al., 2019). Their presence at these sites suggests a role in host defense, and this is supported by their active secretion from the polymorphonuclear leukocyte into the external environment during inflammation (Wright and Gallin,

1979, Ellison and Giehl, 1991). Lactoferrin is an iron-binding glycoprotein that inhibits bacterial growth by sequestering iron and also has antiviral properties against pathogens like respiratory syncytial virus and influenza (Hong et al., 2024). Lysozyme is a bacteriolytic enzyme that is commonly known for fundamentally targeting bacterial cell walls (Ellison and Giehl, 1991). While Lz primarily targets bacteria, its role in immune regulation extends its protective function to a broader range of pathogens, including viruses that are responsible for upper respiratory tract infections (URTIs), and sexually transmitted infections (STIs) (Hanstock et al., 2019, Ferraboschi et al., 2021). These two proteins can work together to enhance their antimicrobial effects (Ellison and Giehl, 1991). In addition to directly combating pathogens, Lf and Lz are involved in modulating immune responses, including anti-inflammatory actions at mucosal surfaces (Ellison and Giehl, 1991, Ragland and Criss, 2017, Hong et al., 2024).

### 2.6.1. Lysozyme (Lz)

Lysozyme (muramidase, E.C. 3.2.1.17) is a highly conserved antimicrobial enzyme first discovered by Alexander Fleming through its bacteriolytic activity in nasal mucus (Ferraboschi et al., 2021). This remarkable enzyme specifically hydrolyzes β-1,4-glycosidic bonds between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in the peptidoglycan layer of Gram-positive bacterial cell walls (Ferraboschi et al., 2021). Notably, Lz holds the distinction of being the first enzyme to have its complete amino acid sequence and three-dimensional structure determined by X-ray crystallography (Blake et al., 1965, Phillips, 1966). Beyond its natural antimicrobial function (Bergamo and Sava, 2024), Lz has found widespread applications as a natural preservative in both the food and pharmaceutical industries due to its effective inhibition of bacterial growth (Khorshidian et al., 2022).

Lysozyme is classified into three major types, i.e. c-type (chicken), g-type (goose), and i-type (invertebrate), which maintain conserved structural characteristics despite its evolutionary diversity (Ferraboschi et al., 2021, Jiang et al., 2021, Bergamo and Sava, 2024). In mammalian systems, Lz demonstrates broad tissue distribution, occurring abundantly in hepatic tissue, circulatory systems, and various secretory fluids including tears, saliva, and breast milk (Bergamo and Sava, 2024). Notably, this enzyme is concentrated at mucosal interfaces and within professional phagocytic cells (macrophages, neutrophils, and dendritic cells), where it contributes to innate immune defense mechanisms (Ferraboschi et al., 2021, Jiang et al., 2021). Its presence in similar immune cells across non-mammalian species highlights its conserved role in defense mechanisms (Ferraboschi et al., 2021, Jiang et al., 2021, Bergamo and Sava, 2024). Chicken Lz (cLz) and human Lz (hLz) are both classified as c-type lysozymes (Ferraboschi et al., 2021, Jiang et al., 2021, Bergamo and Sava, 2024). Notably however, cLz consists of 129 amino acids with a molecular weight of 14.3 kDa, while hLz has 130 amino acids

and weighs 14.7 kDa (Ferraboschi et al., 2021, Khorshidian et al., 2022). Despite sharing 59% sequence identity, human Lz exhibits three times more antibacterial activity than its chicken counterpart (Ferraboschi et al., 2021, Khorshidian et al., 2022).

Originally valued for its antibacterial properties, Lz has gained recognition as a multifunctional therapeutic agent with diverse biological activities (Bergamo and Sava, 2024). The discovery of a 14.4 kDa plant Lz from mung beans demonstrated expanded antifungal and antibacterial capabilities (Wang et al., 2005). Recent research highlights Lz's antiviral potential, with both hen egg white (HEWL) and human forms showing charge-mediated inhibition of viral entry, independent of their muramidase activity (Cisani et al., 1984, Lee-Huang et al., 2005, Bergamo and Sava, 2024). Evidence supporting Lz's antiviral activity comes from studies showing its presence in human chorionic gonadotropin preparations (Lee-Huang et al., 2005). A human Lz nonapeptide, HL9, isolated from Lz shared sequence homology and muramidase activity with both human Lz and HEWL, exhibiting specific anti-HIV-1 effects while maintaining a non-cytotoxic profile in eukaryotic cells, though its precise antiviral mechanism was not fully elucidated (Lee-Huang et al., 2005).

### 2.6.2. Lactoferrin (Lf)

Lactoferrin was first identified in bovine milk in 1939 and was initially described as an iron-containing protein (Sorensen and Sorensen, 1939). However, its detailed structure and chemical properties were only described in 1960 and since then, it has become a focus of research due to its various functions (Groves, 1960, Hong et al., 2024). Lactoferrin is a highly cationic glycoprotein, with an isoelectric point (pI) between 8.4 and 9.0, and a molecular weight ranging from 76 to 80 kDa (Baker and Lindley, 1992, Britigan et al., 2001, Hong et al., 2024). It occurs in significant amounts [1–10 milligram per millilitre (mg/mL)] at mucosal surfaces and in milk, and it is a key component of neutrophil secondary granules (Baggiolini et al., 1970, Spitznagel et al., 1974, Britigan et al., 2001). Neutrophils secrete Lf in large quantities at sites of bacterial infection (Britigan et al., 2001, Baggiolini et al., 1970, Spitznagel et al., 1974). Additionally, Lf acts as an antioxidant by preventing iron from catalyzing the formation of harmful hydroxyl radicals (Britigan et al., 1986, Britigan et al., 2001).

Lactoferrin has two high-affinity binding sites for ferric iron (Fe) and is crucial for iron binding in milk, saliva, tears, and other exocrine secretions (Finkelstein et al., 1983, Weinberg, 1993, García-Montoya et al., 2012). This property of Lf contributes to host defense as sequestering iron from pathogens inhibits their growth (Finkelstein et al., 1983, Weinberg, 1993, García-Montoya et al., 2012). Initially, the unique ability to bind and retain Fe across a broad spectrum of pH values was regarded as the primary source

of Lf's antimicrobial properties (Bellamy et al., 1992, Bellamy et al., 1993, Kanyshkova et al., 1999). However, Lf interacts with the outer membrane of Gram-negative bacteria, altering their permeability and leading to cell damage and death (Bellamy et al., 1992, Bellamy et al., 1993). This activity is attributed to its N-terminal region, called lactoferricin, which contains many arginine residues responsible for its positive charge (Bellamy et al., 1992, Bellamy et al., 1993). Additionally, Lf interacts with cell surfaces through sulfated proteoglycans and specific membrane receptors, activating the extracellular signal-regulated kinases 1 and 2 (ERK1/2) and phosphoinositide 3-kinase / protein kinase B (PI3K/Akt) pathways (Gupta et al., 2015, Kanwar et al., 2015).

Three Lf isoforms have been identified i.e., Lf-α, Lf-β, and Lf-γ, each with varying abilities to bind iron and ribonuclease activity (Anderson et al., 1989, Lönnerdal and Iyer, 1995, Elzoghby et al., 2020). The protein can adopt different conformations depending on its iron saturation i.e., apo-Lf (iron-free), monoferric Lf, and holo-Lf (iron-saturated) (Anderson et al., 1989, Lönnerdal and Iyer, 1995, Elzoghby et al., 2020). These conformational changes affect its resistance to proteolysis (Anderson et al., 1989, Lönnerdal and Iyer, 1995, Elzoghby et al., 2020). Additionally, glycans attached to Lf enhance its stability against proteases and acidic environments (Anderson et al., 1989, Lönnerdal and Iyer, 1995, Elzoghby et al., 2020). Beyond its antibacterial activity, Lf also has bioactive effects against viruses such as HIV-1, hepatitis C virus (HCV), hepatitis B virus (HBV), and severe acute respiratory syndrome coronavirus (SARS-CoV) (Elzoghby et al., 2020, Hong et al., 2024). For example, Lf blocks the binding of SARS-CoV to host cells by interfering with viral attachment to heparan sulfate proteoglycans on the cell surface (Lang et al., 2011, Mann and Ndung'u, 2020). Moreover, Lf has been shown to prevent influenza virus-induced apoptosis and neutralise adenovirus infections, among other antiviral actions (Pietrantoni et al., 2010). Its potential as an anti-HIV agent has also been highlighted (Berkhout et al., 2002, Groot et al., 2005).

### 2.6.3. Antiviral Activity of lysozyme and lactoferrin, and their effect against HIV-1

As aforementioned, both Lz and Lf inhibit viral replication through various mechanisms (Fleet, 1995, Lee-Huang et al., 1999, Steinrauf et al., 1999, Groot et al., 2005). Specifically, Lf inhibits viral infection by binding directly to viruses, scavenging iron, or competing for host cell receptors (Elzoghby et al., 2020, Hong et al., 2024). The high pI and arginine-rich N-terminus enables Lf to bind various negatively charged molecules, such as lipopolysaccharides (LPS), heparin, heparan sulfates, and DNA (Elzoghby et al., 2020, Hong et al., 2024). The ability of Lf to bind DNA has sparked interest in its potential role in transcriptional regulation, with some evidence suggesting a sequence-specific interaction with eukaryotic DNA (He and Furmanski, 1995).

Bovine lactoferrin (bLf) has been proven to be a potent inhibitor of HIV-1, with biological activity against different HIV-1 strains that use C-X-C chemokine receptor type 4 (CXCR4) and C-C chemokine receptor type 5 (CCR5) coreceptors (Berkhout et al., 2002). Even though bLf is 69% identical to human lactoferrin (hLf) and 64% to murine lactoferrin (mLf), its superior anti-HIV properties have prompted studies to identify the precise amino acid sequences or structural domains responsible for this inhibitory effect on HIV-1 replication (Berkhout et al., 2002). Notably, lactoferricin, a basic N-terminal domain crucial for antibacterial activity in bLf, did not display strong anti-HIV-1 activity, suggesting that other regions of bLf may mediate its antiviral effects (Berkhout et al., 2002). The broad antiviral activity of bLf appears to result from its ability to interfere with viral entry into host cells, making it a relatively nonspecific inhibitor of a wide range of enveloped viruses (Berkhout et al., 2002).

Experimental bioassays provided supporting evidence to this hypothesis and illustrated that Lf's antiviral activity is exerted by disrupting the interaction between the virus and its cellular target (Harmsen et al., 1995, Puddu et al., 1998, Groot et al., 2005). Upon sexual transmission, intraepithelial and submucosal dendritic cells (DCs) are among the first cells to encounter HIV-1 (Sallusto et al., 1998). These DCs capture HIV-1 using C-type lectin receptors, particularly DC-SIGN, which facilitates the internalization of the virus (Sallusto et al., 1998, Manches et al., 2014). Notably, DCs can retain HIV-1 in an infectious state for several days and are capable of transmitting the it to CD4+ T-cells (Sallusto et al., 1998, Manches et al., 2014). Researchers investigated various proteins from milk and serum for their potential to inhibit DC-mediated HIV-1 transmission, identifying bLf as the most effective (Groot et al., 2005). Outperforming hLf, bLf expressed a high affinity for binding to DC-SIGN, thereby blocking the virus' capture and subsequent transmission (Groot et al., 2005). In another study, p24 release was completely halted in HIV-1-infected cells that were pre-incubated with iron-saturated bovine lactoferrin ($Fe^{3+}$bLf). This further supported that bLf prevents HIV-1 infection during the virus adsorption stage (Puddu et al., 1998).

Research indicates that Lz inhibits HIV-1 infection by interfering with viral attachment to host cells through CD4 receptor interactions (Lee-Huang et al., 1999, Behbahani et al., 2018). A different study reported that avian Lz from quail, turkey, and hen egg white demonstrated significant anti-HIV-1 activity [$EC_{50}$ values of 7.5 nanomolar (nM), 55 nM, and 10 nM, respectively], with time-of-addition experiments confirming inhibition occurs at the viral entry stage (Behbahani et al., 2018). Flow cytometry analyses revealed these avian Lzs reduce the frequency and mean fluorescent intensity (MFI) values of CD4+ T-cells in PBMCs without affecting CCR5/CXCR4 coreceptor levels, while molecular docking studies showed quail Lz specifically exhibits a stronger binding affinity for the CD4 receptor

compared to the rest of avian Lzs (Behbahani et al., 2018). These findings collectively suggest that avian Lz likely block HIV-1 infection by competitively binding to CD4 and modulating its cell surface availability, with potency variations reflecting species-specific structural differences in the enzymes (Behbahani et al., 2018).

### 2.6.4. Nucleic acid binding and the modulation of gene expression

In addition to restricting virus-target host cell interactions, both Lz and Lf have been reported to bind DNA/RNA (Fleet, 1995, Steinrauf et al., 1999, Lin et al., 2009). The N-terminal region of Lf is well-known for modulating interactions with cell protein receptors and iron-independent antibacterial activities (Mann et al., 1994, He and Furmanski, 1995). However, N-terminal amino acid residues, particularly clusters like GRRRRS and RKVR were discovered to exhibit a fundamental property of binding with polyanions such as DNA and RNA (Mann et al., 1994, He and Furmanski, 1995). Lactoferrin is understood to have the highest affinity to three specific DNA sequences: GGCACTT(G/A)C (ON1), TAGA(A/G)GATCAAA (ON2), and ACTACAGTCTACA (ON3) (He and Furmanski, 1995). Although Lf's ability to bind nucleic acids has been known for decades, there is still a gap in knowledge regarding the biological significance of this property (Kanyshkova et al., 1999, Kowalczyk et al., 2022). Kim et al. (2012) demonstrated that in endothelial cells, Lf translocates to the nucleus and competes with NF-κB for binding to the intercellular adhesion molecule (ICAM)-1 promoter at a specific sequence (GGAAATTCC) that resembles the canonical lactoferrin binding site (LBS). By physically occupying this proximal NF-κB site, Lf blocks recruitment of p65/p50 heterodimers to the promoter, thereby repressing tumour necrosis factor alpha (TNF-α)-induced transcription without affecting NF-κB nuclear translocation or mRNA stability (Kim et al., 2012). This finding aligns with earlier reports of Lf's nuclear localization and sequence-specific DNA binding, revealing a mechanistic basis for how this iron-binding glycoprotein can act as a transcriptional repressor at inflammatory gene loci (Kanyshkova et al., 1999, Kim et al., 2012, Kowalczyk et al., 2022).

There is evidence that suggests Lf binds DNA with varying stoichiometry depending on its concentration, indicating the presence of two nucleic acid-binding centers in the N-terminal region with distinct affinities (Kanyshkova et al., 1999). Notably, the two binding sites are hypothesised to overlap with the polyanion and antimicrobial domains of the protein that give rise to its innate defense characteristics (Kanyshkova et al., 1999). A study by Mulligan *et al*. provided affirmation to this hypothesis when they illustrated that Lf bound to bacterial DNA CpG motifs in extracellular compartments (Mulligan et al., 2006). The observed interaction significantly suppressed CpG motif-induced NF-κB activation and reduced transcriptional activity of interleukin (IL)-8 and IL-12 cytokine

genes, demonstrating potent immunomodulatory effects (Mulligan et al., 2006). Additionally, Lf has been shown to hydrolyze RNA, with ribonuclease activity targeting pyrimidine chains of specific substrates like poly(rA):poly(rU) (Furmanski et al., 1989). This activity is attributed to motifs in Lf that resemble those in RNase A proteins, although it has been suggested that these active sites may coincide with the N-terminal polyanion-binding region rather than forming a distinct RNase center (Furmanski et al., 1989, Sharada Devi et al., 1994).

Lysozyme contains multiple nucleic acid-binding sites, including one high-affinity site and several non-specific sites, suggesting potential roles in modulating DNA/RNA structure and function (Steinrauf et al., 1999, Zalar et al., 2023, Bergamo and Sava, 2024). While its ability to induce nucleic acid curvature remains unclear, its strong positive charge (+8e) and compact structure enable it to alter DNA electrophoretic mobility, potentially influencing transcription, translation, and viral replication (Steinrauf et al., 1999, Zalar et al., 2023, Bergamo and Sava, 2024). This is supported by its protective effects against HIV-1, where it may disrupt viral processes by interfering with nucleic acid dynamics (Lee-Huang et al., 2005).

A functionally critical nonapeptide (RAWVAWRNR, residues 107–115 of human Lz) was identified and reported to retain potent anti-HIV activity comparable to full-length Lz (Lee-Huang et al., 2005). Located in a non-catalytic α-helix, this fragment binds HIV glycoprotein 41 (gp41), inhibiting membrane fusion and modulating host gene expression in infected cells. Specifically, it reverses HIV-induced upregulation of stress/survival genes (e.g., *HSP27/90*, growth arrest and DNA damage-inducible 45 [*GADD45*], murine double minute 2 [*MDM2*]) while restoring anti-apoptotic proteins (baculoviral inhibitor of apoptosis repeat-containing [BIRC]2/BIRC3) (Lee-Huang et al., 2005). These effects implicate key pathways including NF-κB, p53, transforming growth factor-beta (TGF-β), and hedgehog signaling, highlighting Lz's broader role in regulating nucleic acid-driven processes during viral infection (Fleet, 1995, Lin et al., 2009).

## 3. Rationale of the study

The persistence of latent HIV-1 reservoirs represents the foremost challenge in developing a cure for HIV-1. In their transcriptionally silent state, integrated proviruses evade both antiretroviral therapy (ART) and immune surveillance, creating a critical barrier to HIV-1 cure. One fundamental hindrance to the search for an HIV-1 cure is the challenge that has faced both of the front-running therapeutic strategies. The "shock and kill" strategy, employing LRAs to induce the virus out of its latent state, has

shown significant disparity in clinical trials. For examples, drugs such as vorinostat/SAHA are capable of reactivating the virus, but to date, they have not yet shown a sustained reduction in the latent reservoir size or prevention of viral rebound upon treatment interruption. As discussed above, this stems from the sheer heterogeneity and complexity of the reservoir itself, insufficient specific immune targeting by most LRAs resulting in adverse systemic inflammation, and frequently-exhausted cytotoxic T-cells that cannot kill the reactivated cells.

Conversely, the "block and lock" strategy, seeking to silence the virus permanently with LPAs, is beleaguered by an inherent deficit of specificity. Most LPAs act by generally blocking host cell transcription, and this carries with it a concerning potential of off-target toxicity and disruption of fundamental cellular processes if used over long periods. Effectively, both avenues are now constrained by the same issues of accuracy, safety, and strength, underscoring the need for sophisticated, HIV-focused instruments to eventually achieve a cure. Current cure strategies necessitate deeper insights into the molecular regulation of viral latency and the development of innovative approaches to either *(i)* selectively reactivate latent proviruses for subsequent immune-mediated clearance or *(ii)* induce a state of deep latency in the reservoirs that will render them unsusceptible to reactivation and viral rebound even in the case of ART cessation.

Emerging evidence highlights the potential role of cationic DNA-binding proteins, including Lz and Lf, as modulators of gene expression. These multifunctional proteins *(a)* engage with cellular nucleic acids through electrostatic interactions, *(b)* influence critical signaling pathways (NF-κB, PKC) involved in HIV-1 transcription, and *(c)* demonstrate established immunomodulatory and antiviral properties.

Despite their known involvement in fundamental cellular processes, the specific impact of these cationic proteins on HIV-1 latency maintenance and reversal remains uncharacterised. This study systematically investigated their potential to modulate HIV-1 latency, by assessing their effect on viral reactivation alone or in combination with established latency-reversing agents and latency-promoting agents. The implications of elucidating these interactions, is that this study, may identify novel biological pathways governing viral persistence and reveal new therapeutic opportunities for targeted reservoir reduction. The impact of this study is that the unique properties of cationic proteins, particularly their nucleic acid-binding capacity and ability to modulate key cellular pathways, may make them promising candidates for developing affordable and broadly applicable LRAs or LPAs.

## 4. Materials and methods

### 4.1. Ethical considerations

Ethical approval was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal Nelson R Mandela School of Medicine (Ref: BREC/00006587/2024).

### 4.2. Culture of cell lines

J-Lat A2 (LTR-Tat-IRES-GFP) and J-Lat C (LTR C-Tat C-IRES-GFP), collectively referred to as J-Lat cells, were used in this study. Both cell lines have been derived from a clone of Jurkat T-cells that has been transduced with an HIV-1 clone. J-Lat A2 (Jordan et al., 2001) and J-Lat C (Maikoo et al, unpublished) carry the LTR-Tat region from HIV-1 subtype B and HIV-1 subtype C respectively. Activation of the LTR transcription activity by LRAs results in the expression viral proteins and green fluorescent protein (GFP) – thus GFP is a reporter of HIV-1 transcriptional activation. J-Lat A2 and J-Lat C cells were maintained in complete growth medium (R10), which was prepared by adding 10% foetal bovine serum (FBS) (Capricorn, Ebsdorfergrund, Germany), 1% L-glutamine, and 1% penicillin/streptomycin solution (Gibco, Grand Island, NY, USA) to Roswell Park Memorial Institute (RPMI)1640 medium (Gibco, Grand Island, NY, USA). J-Lat cells were retrieved from liquid nitrogen storage and quickly thawed in a 37°C water bath. Briefly, about $5x10^6$ thawed cells were transferred into a 15 mL conical tube containing 9 mL pre-warmed R10 medium to prevent cell damage. The cells were immediately centrifuged in a Heraeus Multifuge 35R+ centrifuge (Thermo Scientific, Waltham, Massachusetts, USA) at 508 times gravitational force (xg) for 10 minutes to pellet the cells, and the supernatant containing dimethyl sulfoxide (DMSO) was aspirated off. The cell pellet was resuspended in 10 mL of R10 medium. A TC 20 automated Cell Counter (Bio-Rad, Hercules, California, USA) was used to determine cell concentration and cell viability was calculated by trypan blue exclusion. J-Lat cells were seeded separately into tissue culture flasks at a density 2 x $10^5$-1.5 x $10^6$ cells/mL of R10 medium. Cells were split every 2-3 days and incubated in CellXpert C170i cell culture incubator (Eppendorf, Hamburg, Germany), at 37°C and 5% $CO_2$.

### 4.3. Cytotoxicity assays

To confirm that the concentrations of cationic proteins, LRAs and LPAs do not harm cell viability, J-Lats (J-Lat A2 and J-Lat C) cells were cultured in the presence of dilutions or absence of these compounds. After the 24-hour treatment period, cell viability was assessed using the Cell Counting Kit-8 assay (CCK-8) (Dojindo, Kumamoto, Japan) (Figure 3).

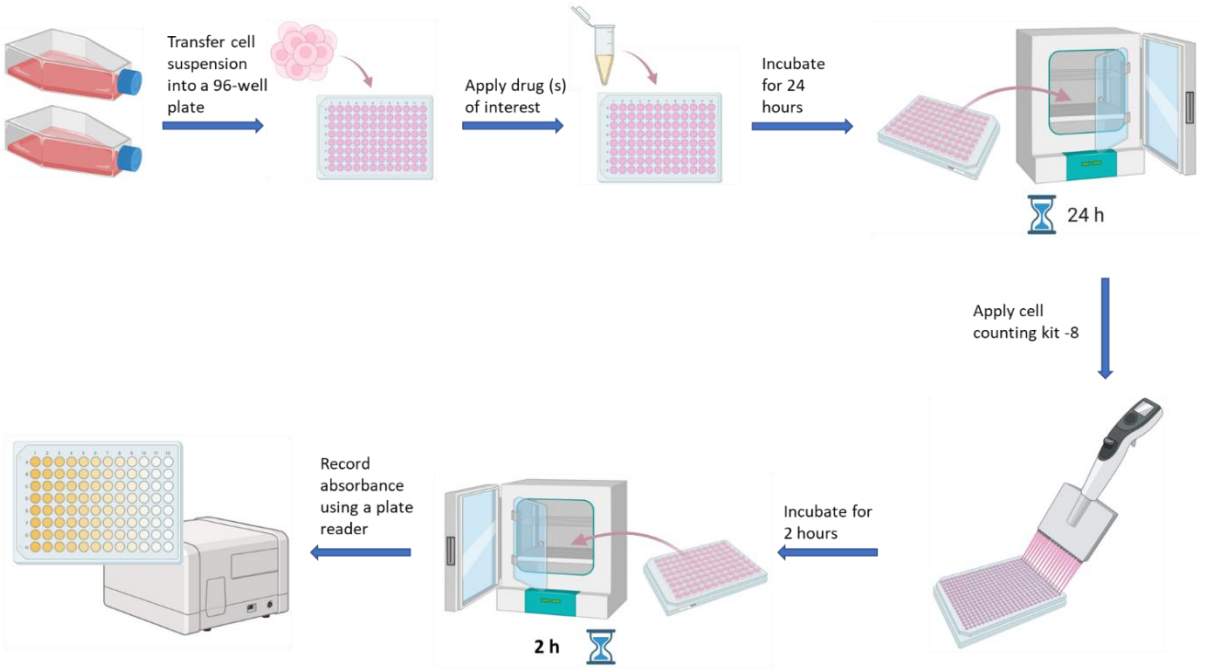


**Figure 3:** Assessing the cytotoxicity of cationic proteins, LRAs, and LPAs using a cell counting kit-8. Cell viability of J-Lat cells following exposure to cationic proteins, LRAs, and LPAs, alone or in combination, was quantified by CCK-8 assay and expressed as a percentage of the untreated control. We measured cell viability following treatment with combinations of cationic proteins, LRAs, and LPAs and expressed the results as a percentage relative to untreated controls (Matsuda et al., 2021).

### 4.4. Latency reversal experiments: LRAs, LPAs, and cationic proteins assays

Latency reversal experiments were performed using J-Lat cells, seeded at a density of 2 x $10^5$/well in 96-well tissue culture plates. Cells were exposed to 24-hour treatments of Lz [10 micromolar (µM), HL9 (10 µM), Lf (10 µM)], or various combinations of the cationic proteins, respectively, to assess the effect of these proteins on HIV-1 latency potential. Additionally, these cationic proteins were co-administered with LRAs (PMA [16 nM] or SAHA [900 nM]), where all compounds were added simultaneously to the cells to investigate the modulatory effects of cationic proteins on LRA potency. Determining whether cationic proteins potentiated LPA activity required cells to be co-cultured with LPAs (Ts [2 µM] or Sp [2 µM]) and cationic proteins (Lz, HL9, and Lf; 10 µM) for 24 hours before being stimulated with PMA or SAHA (Mori et al., 2021b). It was established in the course of these experiments that the blocking effects of Ts and Sp were more pronounced when cells are pre-exposed to these LPAs for at least 24 hours before being stimulated with PMA or SAHA. The various combinations tested are illustrated in Table 1. Untreated cells served as negative controls, while cells treated with PMA (16 nM) or SAHA (900 nM) alone served as LRA positive controls. Furthermore, LRA+Ts (2 µM) and LRA+Sp (15 µM) combinations were utilised as LPA positive controls.

**Table 2**: Experimental design illustrating single treatments and various combinations of cationic proteins, LRAs, and LPAs.

| Cationic proteins | Cationic protein combinations | Cationic protein + LRAs | | Cationic protein + LPAs | |
|---|---|---|---|---|---|
| | | PMA | SAHA | PMA/SAHA + Ts | PMA/SAHA + Sp |
| Lz | Lz + HL9 | Lz + PMA | Lz + SAHA | Lz + PMA/SAHA + Ts | Lz + PMA/SAHA + Sp |
| HL9 | Lf + HL9 | HL9 + PMA | HL9 + SAHA | HL9 + PMA/SAHA + Ts | HL9 + PMA/SAHA + Sp |
| Lf | Lz + Lf | LF + PMA | LF + SAHA | LF + PMA/SAHA + Ts | LF + PMA/SAHA + Sp |
| | Lz + Lf + HL9 | LZ + Lf + PMA | Lz + Lf + PMA | LZ + Lf + PMA/SAHA + Ts | LZ + Lf + PMA/SAHA + Sp |

*Foot notes: Lz, hen egg white lysozyme; HL9, human lysozyme nonapeptide; Lf, lactoferrin; PMA, phorbol 12-myristate 13-acetate; SAHA, suberoylanilide hydroxamic acid; Ts, tanespimycin; Sp, spironolactone; LRAs, latency reversing agents; LPAs, latency promoting agents.

### 4.5. Flow cytometry

The reactivation of HIV-1 from latently infected cells was determined by changes in GFP expression in J-Lat cell lines (Gavegnano et al., 2014, Besnard et al., 2016, Matsuda et al., 2021). At the end of the incubation period with either LRAs or LPAs or cationic proteins or different combinations, J-Lat cell lines were transferred into cluster tubes and fixed with 2% paraformaldehyde (PFA) and then analysed for GFP expression. Cells were acquired on a 4-laser BD LSRFortessa (BD Biosciences, Franklin Lakes, New Jersey, USA) using BD FACSDiva v10 software. A total of 20,000 events was collected for each sample. The flow cytometry data were analysed using FlowJo Africa software (FlowJo LLC, Ashland, Oregon, USA). After forward scatter/side scatter (FSC/SSC) gating on lymphocytes, doublets were excluded using FSC-A vs FSC-H, and $GFP^+$ events were selected with a 530/30 nanometer (nm) filter; 20 000 $GFP^+$ cells were recorded per sample.

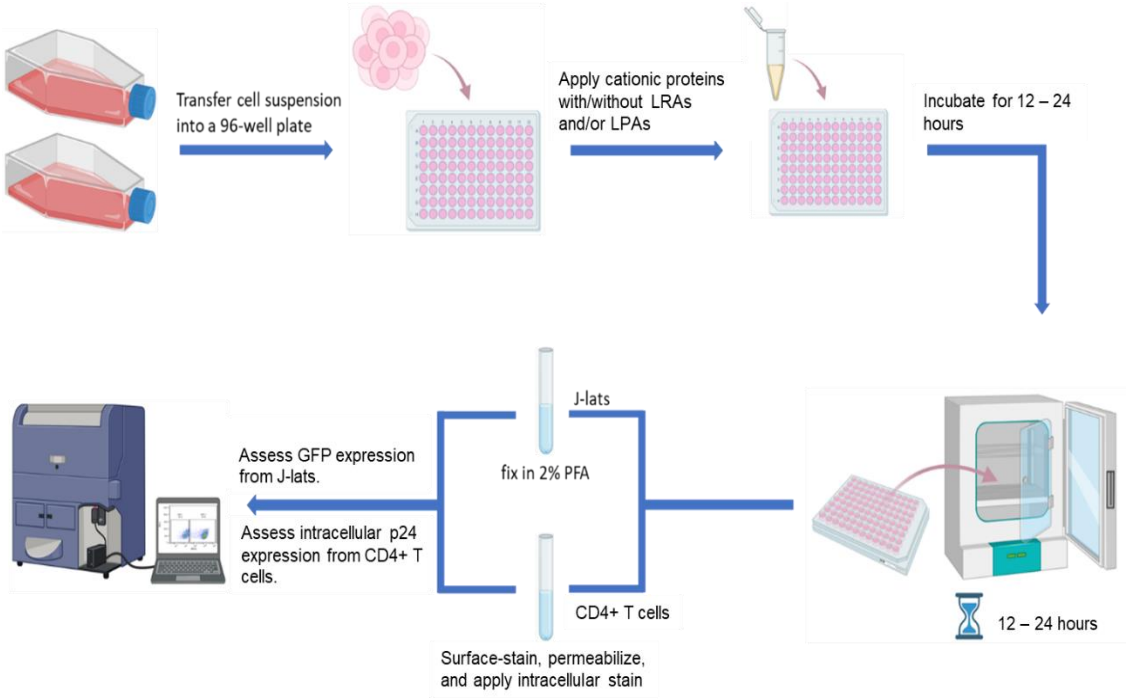


**Figure 4:** Reversal of HIV-1 latency with cationic proteins alone, and in combination with latency-reversing agents and/or latency-promoting agents. J-Lats cells were exposed to single or combinatory treatments of cationic proteins (Lz, HL9, and Lf; 10 µM) alone, or in combination with LRAs (PMA, SAHA) or LPAs (Ts and Sp). Appropriate fixing with 2% PFA was done post 24-hour incubation, and the latency reversal potential of the compounds was analysed using flow cytometry.

### 4.6. Statistical analyses

Data was statistically analysed using GraphPad Prism (v10.6.1). One-way analyses of variance (ANOVA) was used to determine if there were significant differences across the groups. In cases where the ANOVA p value was significant ($p<0.05$), Tukey's test (post-hoc tests [2-way comparisons]) were performed to identify the groups that differed significantly. Specifically, post-hoc tests were used to determine if there is a difference between (a) cells treated with cationic proteins and the negative controls (untreated cells), (b) cells treated with cationic proteins and the positive controls (LRAs only), (c) cells treated with cationic proteins and LRAs in combination and cells treated with LRAs only, (d) cells treated with cationic proteins and LRAs in combination and cells treated with LRAs and LPAs in combination, and (e) cells treated with cationic proteins, LRAs and LPAs in combination versus cells treated with just LRAs and LPAs in combination (see Table 1).

## 5. Results

### 5.1. Evaluation of cytotoxicity associated with cationic proteins, LRAs, and LPAs

Cytotoxicity analysis in J-Lat A2 and J-Lat C cell lines revealed that individual catanionic proteins Lz (10 µM; 117.1% ± 29.6%), HL9 (10 µM; 105.8% ± 6.4%), and (Lf; 10 µM; 108.3% ± 16.4%); as well as their combination Lz+Lf+HL9 (10 µM each; 106.7% ± 12.4%) maintained cell viability close to 100%, comparable to untreated controls. Additionally, when cationic proteins were combined with LRAs, cell viability remained high with PMA (16 nM) combinations with PMA+Lz (102.9% ± 12.2%), PMA+HL9 (100.9% ± 10.3%), PMA+Lf (107.3% ± 16.0%), and PMA+Lz+Lf (108.9% ± 9.1%) all averaging 101% viability and above. In contrast, SAHA (900 nM) combinations showed slightly reduced viability with SAHA+Lz (89.1% ± 2.9%), SAHA+HL9 (87.0% ± 1.6%), SAHA+Lf (88.8% ± 1.5%), and SAHA+Lz+Lf (86.1% ± 1.6%) all yielding average cell viability below 90%.

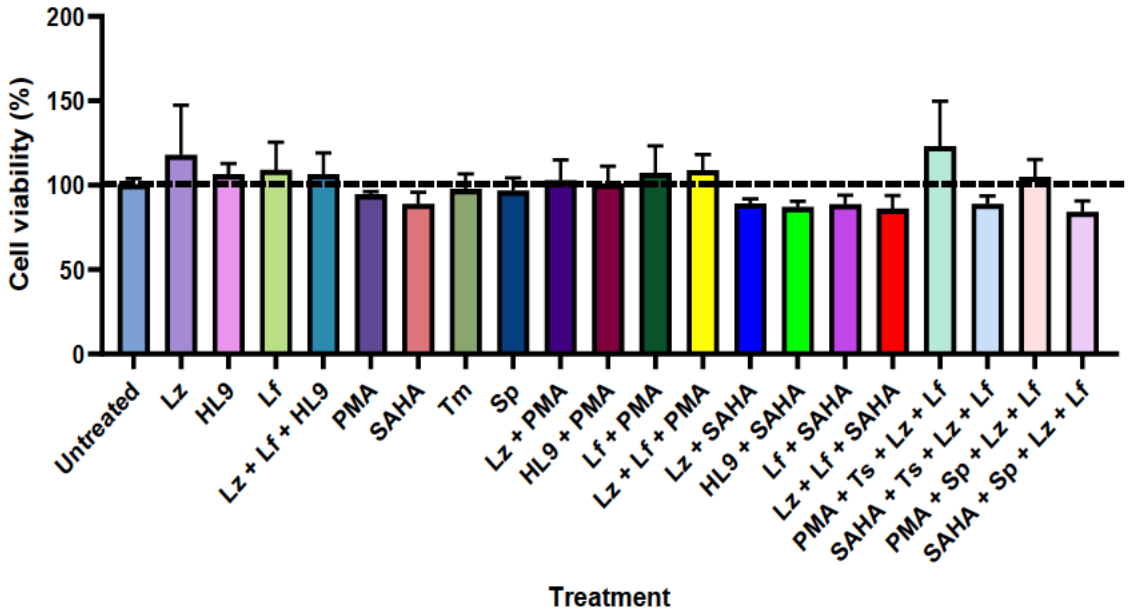


**Figure 5**: Cytotoxicity analysis of cationic proteins and their various combinations with latency-reversing agents and latency-promoting agents. Cell viability of J-Lat A2 cells following 24-hour exposure to 10 µM concentrations of cationic proteins (Lz, HL9, and Lf) alone, or in combination with LRAs (16 nM PMA/900 nM SAHA), and LPAs (2 µM Ts/ 15 µM Sp). Viability was quantified by CCK-8 assay and expressed as a percentage of the untreated control. Data shown are mean ± standard deviation (SD) from three independent experiments.

Combining cationic proteins with LRAs and LPAs yielded viabilities ranging from 85% to 122% of the untreated control. Combinations such as PMA + Ts (2 µM) + Lz + Lf showed 121.7% ± 25.8%, SAHA + Ts (2 µM) + Lz + Lf was 89.2% ± 4.6%, PMA + Sp (15 µM) + Lz + Lf was 104.6% ± 10.1%, and SAHA + Sp (15 µM) + Lz + Lf was 85.4% ± 3.9% (Figure 5). Overall, none of the tested combinations, including those with cationic proteins, LRAs, and LPAs, induced significant cytotoxicity, as defined by

a reduction in viability below 80% of control levels. These results support their suitability for further use in latency-reversal assays.

### 5.2. Lactoferrin exhibits latency-reversal potential

Results in the J-Lat A2 model indicated no HIV-1 reactivation with the individual use of Lz and HL9 (both ≤0.2%) (Figure 6A). However, Lf alone showed a modest viral reactivation effect (3.6% ± 1.4%). Notably, combination treatments, particularly the co-treatments- Lz+Lf (4.9% ± 1.7%), Lf+HL9 (5.2% ± 2.1%), and triple combination of Lz+HL9+Lf (6.6% ± 2.9%), modestly enhanced latency reversal, mediated by Lf, achieving 25-34-fold induction of GFP expression compared to the untreated control (0.2% ± 0.1). SAHA and PMA, as established LRAs, activated HIV-1 transcription and yielded 1.7% ± 0.5% and 26.8% ± 2.7% GFP-positive cells, respectively (Figure 6A). A one-way ANOVA revealed a significant difference among treatment groups ($p < 0.0001$). Lf induced a significantly higher GFP expression compared to untreated cells ($p < 0.0009$), Lz ($p < 0.0001$), and HL9 ($p < 0.0001$). Lysozyme- and HL9-mediated GFP expression did not differ significantly from untreated cells ($p > 0.9999$), with their combination (Lz+HL9) also exhibiting similar GFP expression levels to untreated cells ($p > 0.9999$) (Figure 6A).

The J-Lat C model also showed a significant difference in GFP expression among treatment groups ($p < 0.0001$) (Figure 6B). However, the overall levels of latency reversal were consistently lower across the cationic protein treatments compared to J-Lat A2 cells. Lactoferrin showed no difference in reactivation of latent virus compared to the untreated control (1.4% ± 0.6; $p = 0.9423$) compared with the untreated control. Consistent with the findings from J-Lat A2 cells, both SAHA and PMA induced significant viral reactivation compared to untreated cells in J-Lat C, with SAHA (1% ± 0.12%; $p = 0.0482$) exhibiting low potency compared to PMA (70.9 ± 8.6%; $p < 0.0001$) (Figure 6B).

Taken together, these findings suggest that Lf, either alone or in combination with other cationic proteins, can reactivate latent viruses, although at lower levels than PMA and with effects dependent on the cell line.

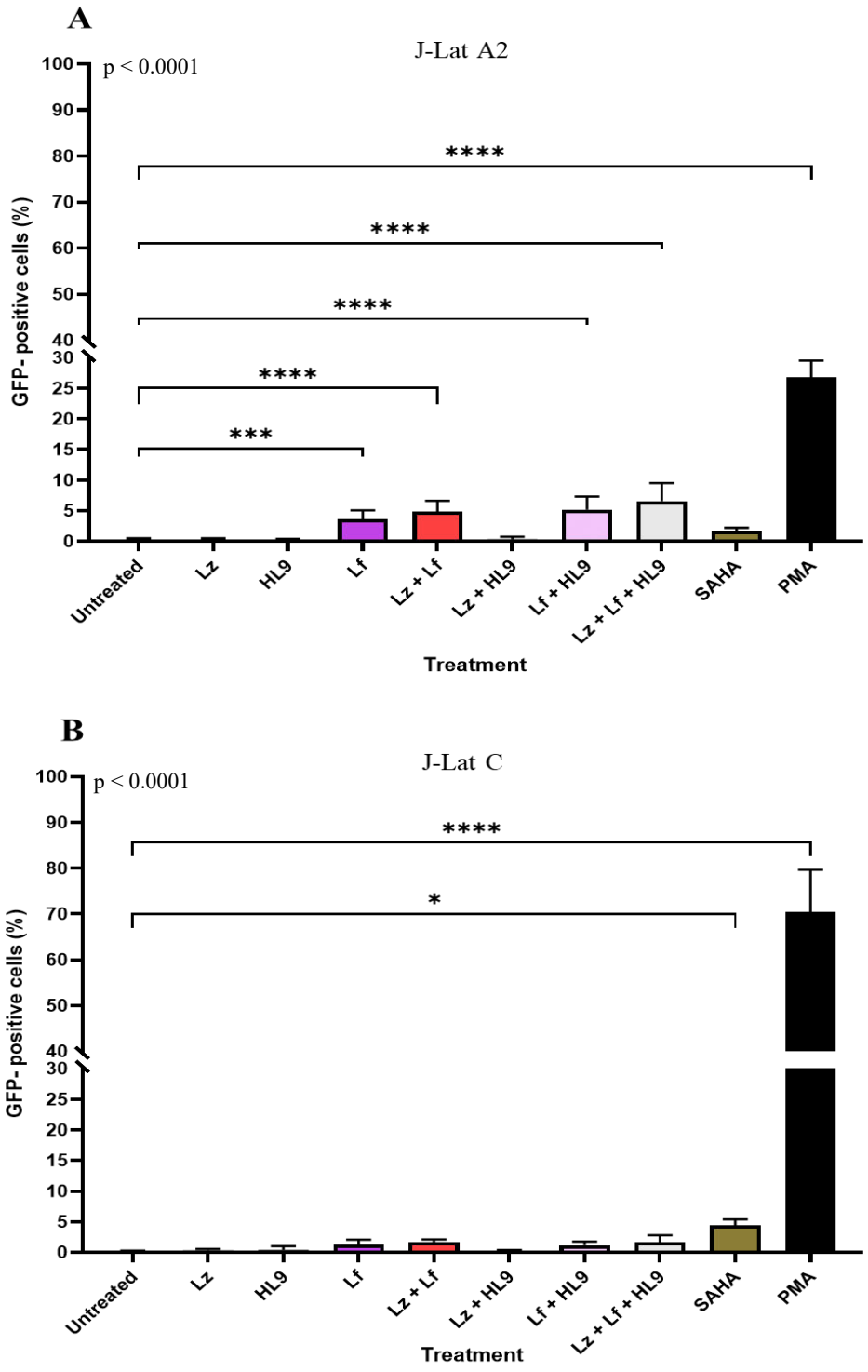


**Figure 6**: Reversal of HIV-1 with lysozyme, HL9, and lactoferrin in vitro. Latency reversal in (A) J-Lat A2 and (B) J-Lat C cells following 24-hour exposure to single, or combinatory treatments of cationic proteins (lysozyme [Lz], HL9, lactoferrin [Lf]; 10 µM), or LRA positive controls (PMA, 16 nM; SAHA, 900 nM). Data represent mean ± SD percentage of GFP-positive cells from three independent experiments. Untreated cells served as negative control. GFP, green fluorescent protein; p, overall ANOVA p-value. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ and ****, $p < 0.0001$ versus negative control.

### 5.3. Cationic proteins enhance LRA (PMA- and SAHA)-mediated HIV-1 reactivation in J-Lat models

The effect of cationic proteins Lz, HL9, and Lf on the ability of well-established LRAs to reactivate latent proviruses from in J-Lat A2 and J-Lat C cell models remained to be determined. Therefore, we next assessed the potential of these cationic proteins to enhance or reduce the latency-reversal activity of PMA and SAHA, and analysed fold changes in GFP expression relative to PMA or SAHA alone (Figure 7). Raw GFP percentages are shown in appendix 1.

In the J-lat A2 cell model, there was an overall significant difference in GFP fold change relative to PMA when comparing different treatment groups (one-way ANOVA, $p < 0.0001$) (Figure 7A). Specifically, co-treatment with PMA and each cationic protein (Lz, HL9, Lf) resulted in a consistent and statistically significant enhancement of provirus reactivation compared to PMA alone ($p<0.0001$). Among the combinations tested, PMA+Lz+Lf produced the greatest effect, inducing a 2.6 ± 0.2-fold increase in reactivation relative to PMA treatment only. Consistently, co-treatment with PMA and two cationic proteins, Lz and Lf, produced the highest level of reactivation in the J-Lat C cell model as well (1.2 ± 0.2-fold compared to PMA alone, $p < 0.0099$) (Figure 7B). Notably however, it was the only combination that significantly potentiated the reactivation potential of PMA in this model, significantly exceeding PMA+Lf ($p = 0.0003$) and PMA+Lz ($p = <0.0001$) (Figure 7B). These results suggest a synergistic relationship between Lz and Lf as the GFP expression produced by PMA alone did not differ significantly from that of PMA+Lf and PMA+Lz combinations ($p >0.9999$ for both) (Figure 7B). Additionally, the J-Lat C model also did not exhibit any significant response in the context of PMA enhancement via cotreatment with HL9, with both conditions (PMA-only and PMA+HL9) yielding non-statistically different GFP expression ($p = 0.9146$) (Figure 7B).

To validate that cationic protein-mediated modulation was consistent across LRA classes, SAHA was evaluated as a second, structurally distinct LRA. Contrary to PMA, the combination of SAHA with Lz or HL9 did not increase GFP expression compared with SAHA alone ($p > 0.9999$), making the SAHA+Lf the only double combination that significantly enhanced SAHA-mediated reactivation in the J-Lat A2 cell model (one-way ANOVA, $p < 0.0001$) (Figure 7C). SAHA in combination with Lf induced an 18.9 ± 2.3-fold increase relative to SAHA alone, while a triple combination of SAHA together with Lz and Lf further increased reactivation to 24.5 ± 5.0-fold, consistent with a pronounced synergistic effect (Figure 7C). A similar, though less pronounced, synergistic effect was observed in the J-Lat C cell model (Figure 7D). Specifically, co-treatment of SAHA with Lf (7.5 ± 1.1-fold compared to SAHA alone) and SAHA with both Lz and Lf (9.5 ± 2.6-fold compared to SAHA alone) significantly enhanced reactivation ($p < 0.0001$), while SAHA+Lz and SAHA+HL9 were unsuccessful

in enhancing SAHA's reactivation potential (Figure 7D). These results confirm that the potentiation of HDACi-induced reactivation by cationic proteins is a reproducible across models of different subtypes, although the magnitude of the effect varies with cellular context.

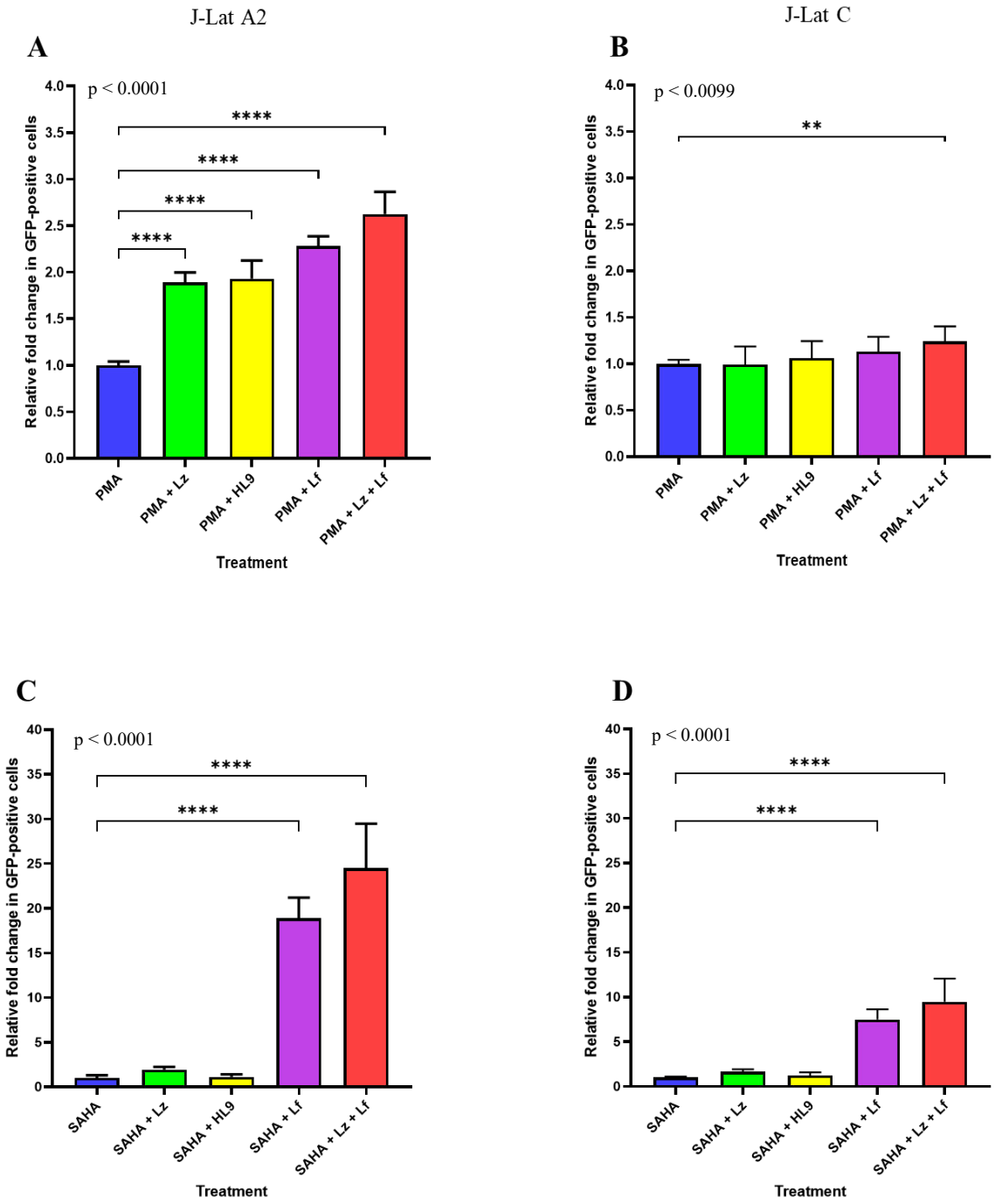


**Figure 7**: The modulatory effects of lysozyme, HL9, and lactoferrin on the potency of well-established latency-reversing agents in vitro. HIV-1 reactivation in J-Lats following 24-hour exposure to LRAs (PMA, 16 nM; SAHA, 900 nM), or LRA+cationic protein co-treatment (lysozyme [Lz], HL9, lactoferrin [Lf]; 10 µM). In J-Lat A2s (A, B), PMA and SAHA induced 26.8% ± 2.7% and 1.7% ± 0.5% GFP expression (appendix 1, Figure A1), respectively, whereas in J-Lat C (C, D), they induced 70.9 ±

8.6% and 1% ± 0.12% (appendix 1, Figure A1). Data was normalized and presented as fold-change in the frequency of GFP-positive cells over the LRA control (PMA/SAHA). GFP, green fluorescent protein; p, overall ANOVA p-value. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ and ****, $p < 0.0001$ versus negative control.

### 5.4. Cationic proteins modulate latency promoting agents

Co-treatment of both J-Lat A2 and J-Lat C cell models with combinations of PMA and Ts or Sp, as well as SAHA and Ts, or Sp served as the baseline conditions. In the J-Lat A2 model, a combination of PMA with Ts resulted in markedly lower GFP-expression (5.8 % ± 4.5%) compared to PMA alone treatment (52.5 % ± 8.3%, appendix 1, Figure A2), corresponding to a 9 ± 1.4-fold reduction in GFP-positive cells (Figure 8A). This illustrates a potent Ts-induced blockade of HIV-1 reactivation ($p < 0.0001$). The addition of Lz or HL9 to the PMA+Ts combination (PMA+Ts+Lz or PMA+Ts+HL9) did not significantly affect viral reactivation ($p > 0.9999$). However, the inclusion of Lf in combination with PMA+ Ts (PMA+Ts+Lf), and particularly the simultaneous addition of Lz and Lf (PMA+Ts+Lz+Lf), resulted in a substantial enhancement of latency reversal (Figure 8A). These conditions produced 6.6 ± 0.5-fold and 7.8 ± 0.5-fold increases in GFP expression, respectively, relative to the PMA+Ts baseline ($p < 0.0001$) (Figure 8A). A similar trend was observed in the J-Lat C model, where PMA+Ts+Lf and PMA+Ts+Lz+Lf were the only combinations to induced a statistically significant (1.2 - 1.3fold) increase in the frequency of GFP-positive cells compared to the PMA+Ts alone ($p < 0.0001$) (Figure 8B).

SAHA showed a trend mirroring that seen with PMA. In J-Lat A2 cells, the SAHA+Ts+Lf and SAHA+Ts+Lz+Lf combinations markedly overcame the blocking effect of Ts and increased reactivation ($p < 0.0001$), respectively yielding a 12.5 ± 1.4-fold and 15.5 ± 1.5-fold higher frequency of GFP-positive cells in comparison to the SAHA+Ts control (Figure 8C). Again, the presence of Lz and HL9 in LRA+LPA+protein triple combinations yielded non-significant results (Figure 8C). As the J-Lat C cell data corroborates the trend observed in J-Lat A2 cells, the significantly different groups were the same for both J-Lat cell lines as SAHA+Ts+Lz+Lf and SAHA+Ts+Lf remained the only combinations to significantly enhance reactivation relative to the SAHA+Ts alone baseline (Figure 8D).

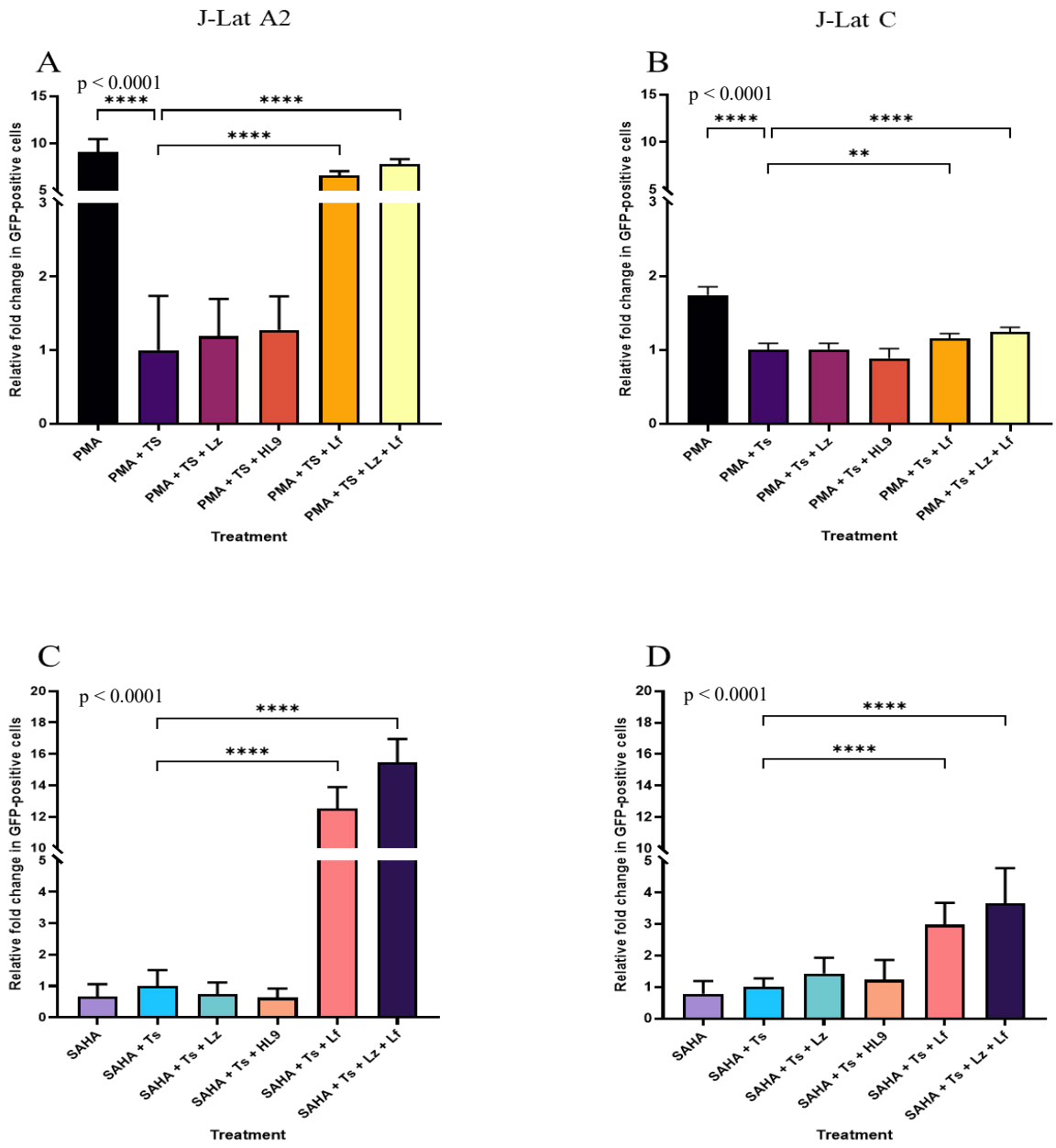


**Figure 8:** Investigating the modulatory effects of cationic proteins on the latency-promoting potential of tanespimycin in HIV-1 latently infected J-Lats. HIV-1 reactivation in J-Lats that were exposed to tanespimycin (Ts)+cationic protein co-treatments (lysozyme [Lz], HL9, lactoferrin [Lf]; 10 µM) for 24 hours, before being stimulated with LRAs (PMA, 16 nM; SAHA, 900 nM), and incubated for a further 24 hours. Ts exhibited strong latency-promoting activity by reducing PMA-induced reactivation in (A) J-Lat A2 (from 52.5 % ± 8.3%, PMA only; to 5.8 % ± 4.5%, PMA+Ts) and (C) J-Lat C (from 81.4 % ± 3.6% PMA only; to 46.9 % ± 6.3%; PMA+Ts) (raw GFP expression data shown in appendix 1, Figure A2). Conversely, Ts did not show any inhibitory effects on SAHA-induced HIV-1 reactivation, and was not potent enough to block the synergistic enhancement of GFP expression resulting from SAHA+Lf, and SAHA+Lf+Lz combinatory treatments in both J-Lat A2 (B) and J-Lat C (D). Actual GFP-expression data was normalized and presented as fold-change in the frequency of GFP-positive cells over the Ts+LRA control (PMA/SAHA). GFP, green fluorescent protein; p, overall ANOVA p-value. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ and ****, $p < 0.0001$ versus Ts+LRA (PMA/SAHA) control.

Sp was subsequently analyzed as a second, structurally distinct LPA positive control to confirm that the modulatory effects observed with cationic proteins were consistent across different LPA classes. In J-Lat A2 cells (Figure 9A), the combination of Sp with PMA (PMA+Sp, 1.0 ± 0.1-fold) resulted in markedly lower GFP expression compared to PMA alone ($p = 0.0001$), confirming the inhibitory role of Sp. The addition of Lz or HL9 to the PMA+Sp regimen (PMA+Sp+Lz, $p = 0.6735$; PMA+Sp+HL9, $p = 0.8638$) did not modulate the blocking effect of Sp on HIV-1 reactivation (Figure 9A). Notably, the inclusion of Lf completely reversed the Sp-mediated suppression. Specifically, the PMA+Sp+Lf combination significantly induced reactivation levels above the PMA+Sp control (2.3 ± 0.3- fold, $p < 0.0001$) (Figure 9A). The most potent effect was observed with the dual cationic protein combination, PMA+Sp+Lz+Lf, which yielded the highest frequency of GFP-positive cells across all PMA-based treatments in this experimental setup (2.7 ± 0.7- fold compared to the PMA+Sp control, $p < 0.0001$) (Figure 9A).

The modulatory effect of cationic proteins on Sp inhibition was also tested in the J-Lat C cell model. In the context of PMA-induced reactivation, the PMA+Sp baseline was again strongly suppressed (Figure 9B). The addition of Lz or HL9 or Lf individually to the PMA+Sp combination did not enhance PMA-driven reaction relative to the PMA+Sp baseline ($p > 0.9999$) (Figure 9B). However, the combined addition of the cationic proteins Lz and Lf to PMA+Sp resulted in a significant increase in reactivation compared with the PMA+Sp baseline (3.6 ± 1.2-fold $p < 0.0001$) (Figure 9B).

An even more profound effect was observed when Sp was combined with the HDACi SAHA. The SAHA+Sp baseline showed only modest, non-significant suppression of SAHA-driven reactivation, consistent with the weak intrinsic potency of SAHA in these models (Figure 9C-D). In the J-Lat A2 model specifically, the addition of Lf alone (SAHA+Sp+Lf, 22.7 ± 9-fold) or together with Lz (SAHA+Sp+Lz+Lf, 26.4 ± 8-fold) completely reversed Sp's block, boosting GFP frequencies an order of magnitude above the SAHA+Sp baseline and well beyond SAHA alone ($p < 0.0001$) (Figure 9C). Notably, this trend was conserved, but in a less pronounced manner in the J-Lat C model (Figure 9D). None of the individual cationic proteins Lz, HL9 or Lf, significantly enhanced SAHA-mediated reactivation relative to SAHA+Sp baseline (SAHA+Sp+Lz 1.1 ± 0.6-fold, $p < 0.9999$; SAHA+Sp+HL9 0.65 ± 0.25-fold, $p = 0.8309$; and SAHA+Sp+Lf 1.6 ± 0.8-fold, $p = 0.3218$) (Figure 9D).This suggests that cationic proteins can not only counter the inhibitory effect of Sp but also unlock a latent virus reactivation pathway.

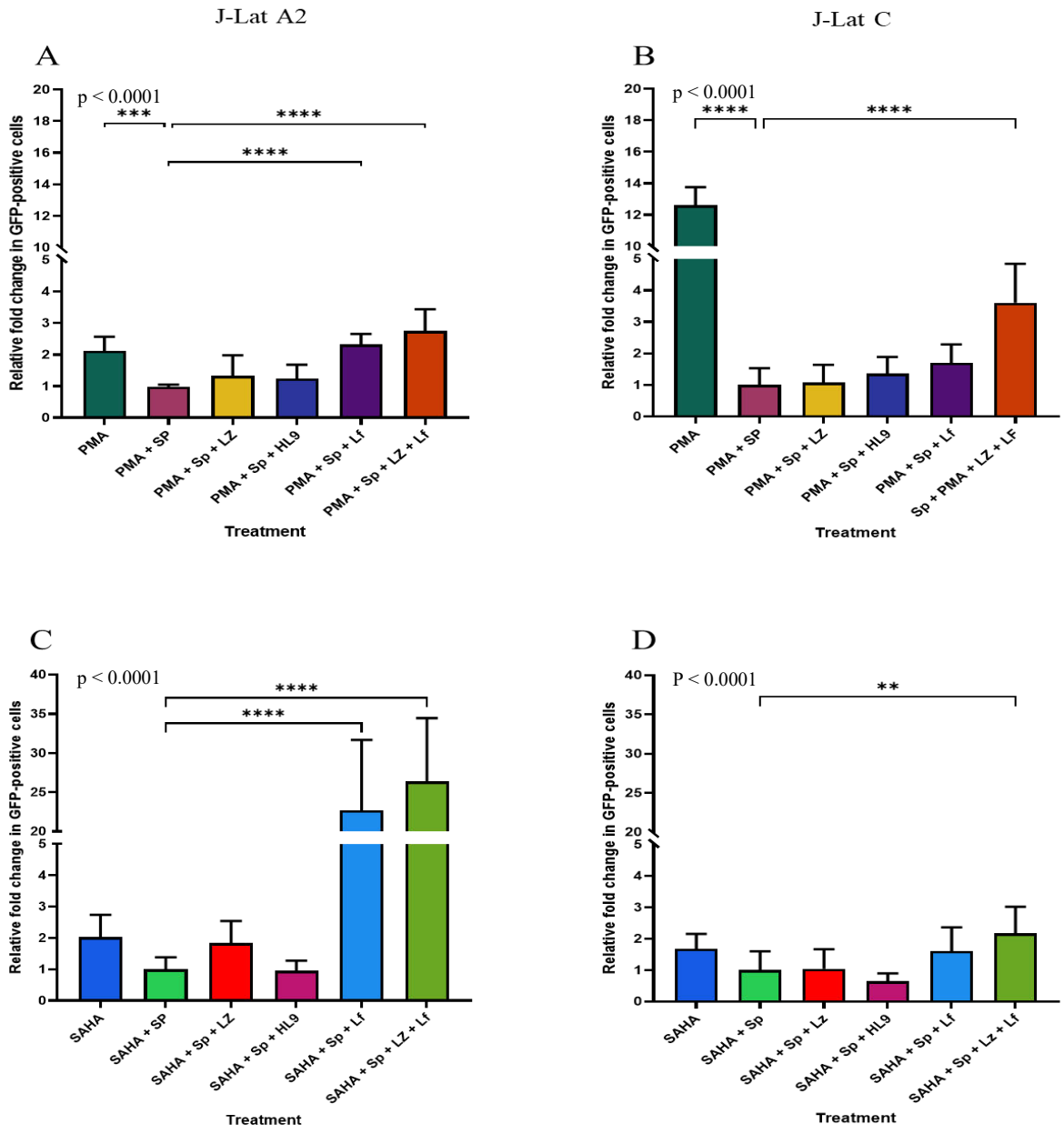


**Figure 9**: Investigating the modulatory effects of cationic proteins on the latency-promoting potential of spironolactone in HIV-1 latently infected J-Lats. HIV-1 reactivation in J-Lats that were exposed to spironolactone (Sp)+cationic protein co-treatments (lysozyme [Lz], HL9, lactoferrin [Lf]; 10 µM) for 24 hours, before being stimulated with LRAs (PMA, 16 nM; SAHA, 900 nM), and incubated for a further 24 hours. Sp exhibited strong latency-promoting activity by reducing PMA-induced reactivation in (A) J-Lat A2 (from 53.3 % ± 9.2%, PMA only; to 24.9 % ± 23%, PMA+Sp) and (B) J-Lat C (from 59.7 % ± 5.4% PMA only; to 4.7 % ± 2.5%; PMA+Ts) (raw GFP expression data not shown in appendix 1, Figure A3). Conversely, Ts did not show any inhibitory effects on SAHA-induced HIV-1 reactivation, and was not potent enough to block the synergistic enhancement of GFP expression resulting from SAHA+Lf, and SAHA+Lf+Lz combinatory treatments in both J-Lat A2 (C) and J-Lat C (D). Actual GFP-expression data was normalized and presented as fold-change in the frequency of GFP-positive cells over the Ts+LRA control (PMA/SAHA). GFP, green fluorescent protein; p, overall ANOVA p-value. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ and ****, $p < 0.0001$ versus Ts+LRA (PMA/SAHA) control.

# 6. Discussion

## 6.1. Introduction

The main aim of the present study was to investigate the potential of cationic proteins, specifically hen egg white lysozyme (Lz), a fractionated human lysozyme nonapeptide (HL9), and bovine lactoferrin (Lf), to modulate HIV-1 latency potential. Furthermore, the study determined whether these proteins had modulatory effects on the potency of well-established LRAs and LPAs, to explore their potential as combinatory modulators alongside established compounds under the "shock and kill", or "block and lock" cure strategies. We initially hypothesise that Lz, HL9, and Lf may affect the propensity of latency reversal and/or enhancement alone and/or in combination with already-known LRAs and LPAs. The generated data partially support this hypothesis. None of the tested conditions reduced cell viability below 85 % of untreated controls, confirming that all LRA, LPA and cationic-protein combinations used were essentially non-cytotoxic. Lactoferrin is the only cationic protein to induce detectable reactivation of latent HIV-1 in vitro, although, this was only observed in the J-Lat A2 model. The same model saw all three cationic proteins enhanced PMA-induced reactivation, with the Lz+Lf combination exhibiting the most potent effect. Contrary, Lz+Lf was the only condition that enhanced PMA-induced reactivation in the J-Lat C model. Lz and HL9 were mostly ineffective when added individually to SAHA, while Lf showed a robust boosting effect on the potency of the HDAC inhibitor. Moreover, Lz+Lf maintained their synergistic effects and so markedly potentiated PMA and SAHA that their combinations counteracted the latency-blocking effects of the Ts and Sp across both J-Lat models. Taken together, findings of the current study show that cationic proteins may enhance the propensity of well-established LRAs to reactivate HIV-1 transcription at non-cytotoxic concentrations. Our findings suggest that the modulatory characteristics these cationic proteins have been previously found to exhibit on multiple cellular processes (e.g., cell-signalling, chromatin remodelling, and gene expression) also extend to modulating HIV-1 latency, and positions them as promising agents that can be further explored for their utility in the "shock and kill" and "block and lock" cure strategies.

## 6.2. The role of lactoferrin in modulating HIV-1 latency: nuclear factor kappa-B signalling and epigenetic regulation

In the present study, we found that Lf was the only cationic protein that exhibited latency reversal activity when used alone. This activity was modest with low levels of GFP expression in the J-Lat A2 cell line only. Although this is the first study to investigate the effect of these cationic proteins on HIV-1 latency, previous studies have reported on their modulatory effects on epigenetic characteristics (Sharrouf and Suchkova, 2021), gene expression pathways (Bergamo et al., 2019, Yami et al., 2023), nucleic acid replication and transcription (Liu et al., 2023), all of which are implicated in HIV-1 latency. The latency reversal effect of Lf is perhaps not unexpected considering that Lf possesses the ability to

interact with two critical characteristics of HIV-1 latency establishment and maintenance, i.e., the NF-κB signalling pathway (Arain et al., 2024) and the epigenetic landscape, particularly DNA methylation (Sharrouf and Suchkova, 2021, Postnikova and Patkin, 2022). The interaction of Lf with these two mechanisms positions it as a unique immunomodulatory agent capable of influencing the state of HIV-1 proviral latency.

The first proposed possible mechanism underlying the modest reactivation effect of Lf in the current study is its interaction with the NF-κB pathway. In the presence of specific immunological cues, Lf can promote NF-κB activation (Kruzel et al., 2017). Bovine Lf has been shown to trigger phosphorylation and degradation of IκBα, the primary inhibitor of NF-κB, leading to the nuclear translocation of the p50/p65 heterodimer (Kruzel et al., 2017). This pro-inflammatory activity is linked to Lf's ability to bind to various cell surface receptors, including Toll-like receptor 4 (TLR4), low-density lipoprotein receptor-related protein-1 (LRP1), and intracellular nucleic acids (Grey et al., 2004, Ando et al., 2010). This interaction initiates downstream signalling cascades that converge on NF-κB (Actor et al., 2009, Kruzel et al., 2017, Arain et al., 2024). Notably however, in LPS-stimulated monocytes and other inflammatory models, Lf may also dampen NF-κB activation and reduce TNF-α and other cytokines, acting as an anti-inflammatory modulator (Håversen et al., 2002, Kruzel et al., 2017). In the context of HIV-1, Lf's NF-κB activation property suggests a potential "shock" function, where Lf could contribute to the reactivation of latent proviruses by providing a pro-transcriptional signal through the NF-κB-binding sites in the LTR.

An additional mechanism potentially underlying Lf-mediated HIV-1 reactivation in our experiments is related to histone modification. In addition to promoting NF-κB activation, Lf may indirectly influence histone modifications associated with transcriptional regulation (Guo et al., 2025). More specifically, Lf exposure was linked to modulation of histone marks like H3K9 acetylation and H3K4 methylation, both related to active transcription, in mouse models and cell lines (Guo et al., 2025). Moreover, Lf impacts gene expression via epigenetic changes involving hypoxia-inducible factor 1α (HIF-1α) signalling, which can recruit histone demethylases (Sharrouf and Suchkova, 2021, Postnikova and Patkin, 2022). Histone demethylases sit in large chromatin complexes, often with HDACs or the corepressor of RE1 silencing transcription factor (CoREST), and are targeted to specific loci by DNA-binding factors and histone-mark "reader" domains (Lan et al., 2008, Dimitrova et al., 2015). By erasing activating marks (for example H3K4me2/3) they help establish less accessible, repressive chromatin (Lan et al., 2008, Dimitrova et al., 2015). Structural and biophysical studies show that human Lf can reversibly bind not only $Fe^{3+}$ but also $Zn^{2+}$ (Rogowska et al., 2023), and zinc levels can modulate DNMT1 expression and activity (Zhang et al., 2012). Taking this into consideration, we speculate that

by sequestering zinc, Lf could potentially inhibit DNA methyltransferase (DNMT) activity. DNMT inhibition is understood to result in global or gene-specific hypomethylation (Besselink et al., 2023). A hypomethylated LTR would be more accessible to transcription factors and RNAPII, thereby lowering the threshold for reactivation. This hypothesised mechanism provides a plausible explanation for how Lf, as a single agent, could induce latency reversal, as observed in our J-Lat A2 data, by erasing a key epigenetic silencing mark.

### 6.3. The role of lysozyme in cell signalling, epigenetics, and HIV-1 latency

In the current study, both the full-length Lz and the fragmented HL9 showed minimal to no HIV-1 reactivation potential when applied alone and in combination with one another. This finding is not inconsistent with previous reports showing that Lz is a potent, dose-dependent inhibitor of nucleic acid replication and transcription, with a proposed mechanism being targeting of the polymerase enzyme (Liu et al., 2023). The authors of that study speculate that the cationic nature of Lz facilitates electrostatic interactions with the negatively charged active sites of polymerases such as the catalytic center of DNA Pol I/V or the α factor of RNA polymerase, thereby obstructing their enzymatic function (Liu et al., 2023). Based on that study, it is theoretically possible that Lz may inhibit the host RNAP II, which the integrated HIV-1 provirus relies on for transcription, and this could result in suppression of the production of new viral mRNAs and genomic RNA, thereby maintaining or even reinforcing a state of latency.

Previous studies indicate that Lz's modulatory capabilities extend beyond direct inhibition of viruses. Transcriptomic and proteomic profiling in the monocyte lineage demonstrated that Lz modulates the expression of genes within the TNF-α and IL-1 pathways, with prominent anti-inflammatory effects (Bergamo et al., 2019). Cells exposed to Lz exhibited rapid downregulation of proinflammatory cytokines (TNF-α, IL-1β) and chemokines (C-C motif chemokine ligands [CCL]1, CCL3, CCL7), inhibited NF-κB-driven genes, and suppressed pathways involved in cell adhesion, extracellular matrix regulation, and immune cell migration (Bergamo et al., 2019). In the context of HIV-1 latency, these properties suggest that Lz could potentially help maintain viral latency by reducing the inflammatory milieu necessary for robust HIV-1 transcriptional reactivation. However, it is important to note that the current study data do not provide evidence that Lz actively reinforces or maintains latency, only that it does not reactivate latent HIV-1 on its own.

### 6.4. Synergistic interactions between cationic proteins and latency-reversing agents

The synergistic interactions between Lz, HL9, Lf, and classical LRAs (PMA and SAHA) represent one of the most significant findings of this study. In J-Lat A2s, Lz/HL9 alone did not reactivate latent HIV, but both Lz and HL9 modestly enhanced latency reactivation by PMA. This appears contradictory to the reports showing that Lz potentially inhibits transcription via the suppression of NF-κB, TNF-α, and IL-1β (Bergamo et al., 2019). However, this discrepancy could reflect cell-type-specific and concentration-dependent bimodal activity of the cationic protein. The robust anti-inflammatory effects of Lz were reported to occur at a 1 μM concentration on monocytes (Bergamo et al., 2019), whereas this study employed a 10-fold higher concentration on T-lymphocytic cell lines. At this higher concentration, it is plausible that Lz may saturate inhibitory pathways, unmasking alternative effector functions in ways that sensitise cells to LRA stimulation, though the precise mechanisms remain to be determined. The biphasic dose-dependent effect of Lz has been described before, but in the context of *Candida albicans* biofilms (Sebaa et al., 2017). The study showed that at physiological concentrations [<30 microgram per millilitre (μg/mL)], Lz inhibits biofilm formation and reduces attached biomass, whereas at higher, non-physiological concentrations (≥300–1,000 μg/mL) it instead enhances biofilm growth and acts as a biofilm promoter in many strains (Sebaa et al., 2017).

The significant enhancement of the potency of PMA as a result of being combined with Lf in J-lat A2 cells is consistent with Lf's latency reversal effect when used alone (Figure 5A-B), and could be mediated by Lf's effect on NF-κB signalling and epigenetic modification as previously discussed. PMA, a phorbol ester, activates PKC signalling, leading to NF-κB nuclear translocation and RelA (RELA proto-oncogene, an NF-κB subunit) binding to HIV-1 LTR κB sites (Sánchez-Duffhues et al., 2011, Jiang and Dandekar, 2015). This phenomenon induces viral transcription in HIV-1 latently infected cells (Sánchez-Duffhues et al., 2011, Jiang and Dandekar, 2015). Taken together, the observed synergy between PMA and Lf may be mediated through complementary signalling pathways, including the overlapping modulation of the NF-κB/IκBα regulatory complex (Brogdon et al., 2016, Yami et al., 2023, Arain et al., 2024), although further experiments would be required to confirm this.

In addition to modulating the NF-κB signalling pathway, we propose that epigenetic targeting explains the synergistic effect observed between Lf and the HDACi, SAHA in reactivating latent HIV-1. SAHA is one of the best-characterised pharmacological inhibitors, directly blocking the enzymatic activities of HDACs by binding to their active sites (Lucera et al., 2014, Bubna, 2015). The result of this action is increased acetylation of histones, chromatin relaxation, and transcriptional activation of latent proviruses (Lucera et al., 2014, Bubna, 2015). Presumably, Lf may have an indirect epigenetic effect, through mechanisms previously discussed in section 6.2. Moreover, Lf is able to bind zinc (Actor et

al., 2009, Kruzel et al., 2017), and zinc is an essential cofactor that is required for the catalytic function of Class I and IIb HDACs (Yusuf et al., 2021). Sequestration of zinc ions disrupts the structural integrity and enzymatic activity of HDACs, thereby functionally inhibiting these key chromatin-modifying enzymes (Yusuf et al., 2021, Thompson, 2022). It is conceivable that the synergy between SAHA and Lf is a two-pronged mechanism: SAHA competitively inhibits HDAC active sites, while Lf depletes the essential zinc cofactor required to maintain HDAC functional conformation. Together, these complementary mechanisms could result in enhanced histone hyperacetylation at the integrated HIV-1 promoter, relaxed chromatin and a lowered energy barrier for transcriptional initiation, rendering the latent HIV-1 provirus more sensitive to endogenous transcription signals. Importantly, the current study findings highlight the potential of Lf as an enhancer of HDACis in latency-reversing strategies; however, validation in primary $CD4^+$ T-cells from PLWH will be essential. Such studies will determine whether Lf could be considered for inclusion in HIV-1 cure approaches targeting epigenetic regulation, bridging these *in vitro* observations to potential translational applications.

### 6.5. Counteraction of latency-promoting agents by lactoferrin

The combination of Lf with classical LRAs counteracted the blocking effects of LPAs. The addition of Ts and Sp in J-Lat A2s reduced the PMA-mediated GFP expression. However, the introduction of Lf significantly weakened the inhibitory effect of Ts and Sp on PMA. The SAHA+LPA+cationic protein experiments yielded results that followed a similar pattern, where Lf completely overcame the inhibitory effect of the LPAs on SAHA, and enhanced reactivation of latency.

HSP90 inhibitors such as Ts destabilize the Inhibitor of kappa B kinase (IKK) complex, leading to its degradation and blocking phosphorylation and subsequent degradation of IκBα (Lee et al., 2010). This confines NF-κB in the cytoplasm and inhibits it from initiating transcription (Lee et al., 2010). Sp, however, inhibits HIV-1 latency reversal by inducing degradation of XPB, a crucial subunit of the TFIIH transcription factor complex, thereby blocking RNAPII recruitment and the subsequent recruitment of other transcriptional factors to the HIV-1 LTR (Doggrell and Brown, 2001, Lacombe et al., 2016, Mori et al., 2021a). Both mechanisms could result in the suppression of transcription that prevents reactivation of the HIV-1 latent virus even after stimulation with latency-reversing agents (Lee et al., 2010, Lacombe et al., 2016, Mori et al., 2021a). Our findings may suggest that Lf activates alternate NF-κB signalling pathways to counteract this, by for example inducing IκBα degradation or NF-κB nuclear translocation, thus maintaining or restoring NF-κB activity even in the presence of HSP90 inhibition and impaired XPB function. However, further experiments would be required to test this hypothesis.

### 6.6. Subtype-specific divergence: implications for global cure strategies

Although the subtype B and C J-Lat models yielded similar patterns, modulatory effects of the cationic proteins on latency-reversal were more pronounced in the subtype B model. This finding appears counterintuitive, given that subtype C LTRs contain 1-2 more NF-κB binding sites (De Baar et al., 2000, Hunt and Tiemessen, 2000), which would be expected to enhance transcriptional responsiveness (Bachu et al., 2012, Verma et al., 2013). However, differences in integration sites, local chromatin environments, and promoter context may outweigh LTR sequence effects in these clonal models. Furthermore, a recent study showed that a subtype B J-Lat model exhibited higher sensitivity to LRA-induced HIV-1 reactivation than a -C, and highlighted how the presence of an extra NF-κB motif does not automatically translate to better "shock" response to LRAs in a subtype C J-Lat model (Maikoo et al., 2024). Given that NF-κB p50 homodimers recruit HDAC1 to the LTR, de-acetylating local histones and blocking RNAPII recruitment (Williams et al., 2006); the presence of only two κB motifs in subtype B while subtype C possesses three (and often four) could mean that the C promoter offers more docking sites for these repressor complexes. Our finding that subtype C responded weakly to stimulation with cationic proteins alone, and in combination with NF-κB- and HDAC-centred LRAs, therefore aligns with reports that additional κB sites stabilise the latent state by increasing p50–HDAC1 occupancy and reduces transcriptional noise (Pal et al., 2023). This suggests that further studies in primary cells and additional clones will be required to clarify the subtype-dependent effects observed in the current study.

### 6.7. Implications for HIV-1 cure strategies and therapeutic development

Our findings suggest that a combination of Lf and Lz with LRAs has therapeutic potential under this strategy and offers several advantages. Under the "shock and kill" strategy, current LRAs suffer from limited potency and systemic toxicity (Deeks, 2012, Darcis et al., 2017, Hashemi and Sadowski, 2020, Acchioni et al., 2021). Both Lz and Lf are natural dietary components with established safety profiles (Lönnerdal, 2003, Małaczewska et al., 2019). Our findings suggest a complementary role where Lf alone or in combination with Lz enhances reactivation by LRAs, hinting towards the potential to reduce required doses of cytotoxic LRAs in combinatory treatments. Notably, Lf also possesses the ability to cross the blood-brain barrier and may enable targeting of CNS reservoirs, a major sanctuary site (Fillebeen et al., 1999, Ash et al., 2021).

Lysozyme alone may have utility in latency maintenance strategies, although, our data did not directly show that Lz reinforces latency but rather that Lz alone did not reactivate latency. Further tests would be required to discriminate between these possibilities. However, assuming that Lz could maintain

latency, we hypothesise that by suppressing NF-κB and inducing metabolic quiescence, Lz, when used alone, could stabilise latent reservoirs during antiretroviral therapy, reducing the "leaky" transcription that contributes to chronic inflammation and reservoir replenishment (Kilroy et al., 2024).

### 6.8. Limitations and future directions

Several caveats must be acknowledged when interpreting these results. Firstly, the J-Lat model, while invaluable for high-throughput screening, represents a clonal cell line with an integrated Nef-defective provirus that lacks the heterogeneity of primary reservoirs and does not recapitulate the complex interplay between cell types *in vivo* (Planelles et al., 2011). Therefore, confirming the subtype-B-biased cationic protein boost in polyclonal, primary-cell and ex-vivo resting $CD4^+$ T-cell models from aviremic individuals is imperative to verify that our clonal J-Lat observations hold across the genetically and epigenetically diverse proviral landscape of the in-vivo reservoir.

While our work identifies Lf as a latency-reversing and LRA-boosting agent, the actual molecular mechanisms by which Lf reactivates latent HIV, and by which Lf and Lz can enhance LRAs, are not shown in the current study. Therefore, it is still not known whether these cationic proteins modulate latency and the effect of LRAs on HIV-1 reactivation through NF-κB mobilisation, HDAC zinc-chelation, chromatin remodelling, or a combination of these mechanisms. Possible mechanistic experiments that can be utilised to shed light on this include the clustered regularly interspaced short palindromic repeats interference (CRISPRi) knock-down of RELA, zinc-depletion/rescue assays, chromatin immunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR) for H3K9ac at the LTR, or phospho-proteomic mapping.

### 6.9. Conclusion

This study shows that when cationic proteins were tested individually, only Lf induced detectable latency reversal, and this effect was modest compared with established LRAs. However, combining these proteins, particularly Lz+Lf and Lz+HL9+Lf, produced a synergistic enhancement of latency reversal, which was more pronounced in the J-Lat A2 model. Classical LRAs such (SAHA and PMA) triggered expected levels of reactivation, and Lf consistently boosted their activity, especially the Lf+Lz+LRA combinations. In the LPA interaction experiments, Ts and Sp robustly suppressed PMA-induced reactivation, but this suppression was significantly reversed by Lf, the Lz+Lf combination, or both. Although the suppressive effects of LPAs were not observed in SAHA+LPA treatments, likely due to the weak intrinsic latency reversal activity of SAHA, the addition of Lf or Lf+Lz combination

significantly enhanced SAHA-induced reactivation and overrode the blocking effects these LPAs might have presented. Collectively, these findings demonstrate that although cationic proteins alone – in particular Lf - have limited capacity to reactivate latent HIV-1, they significantly enhance the activity of classical LRAs, suggesting a modulatory role in latency regulation that is strongly context- and cell-model dependent. This work supports further exploration into the potential for cationic proteins to contribute to combination strategies for latency reversal. Further studies are needed to clarify the underlying mechanisms and to evaluate translational relevance.

## 7. References


ABBAS, W. & HERBEIN, G. 2012. Molecular Understanding of HIV-1 Latency. *Adv Virol,* 2012**,** 574967.

ACCHIONI, C., PALERMO, E., SANDINI, S., ACCHIONI, M., HISCOTT, J. & SGARBANTI, M. 2021. Fighting HIV-1 Persistence: At the Crossroads of "Shoc-K and B-Lock". *Pathogens,* 10.

ACTOR, J. K., HWANG, S. A. & KRUZEL, M. L. 2009. Lactoferrin as a natural immune modulator. *Curr Pharm Des,* 15**,** 1956–73.

ANDERSON, B. F., BAKER, H. M., NORRIS, G. E., RICE, D. W. & BAKER, E. N. 1989. Structure of human lactoferrin: Crystallographic structure analysis and refinement at 2·8 Å resolution. *Journal of Molecular Biology,* 209**,** 711–734.

ANDERSON, I., LOW, J. S., WESTON, S., WEINBERGER, M., ZHYVOLOUP, A., LABOKHA, A. A., CORAZZA, G., KITSON, R. A., MOODY, C. J., MARCELLO, A. & FASSATI, A. 2014. Heat shock protein 90 controls HIV-1 reactivation from latency. *Proc Natl Acad Sci U S A,* 111**,** E1528–37.

ANDO, K., HASEGAWA, K., SHINDO, K., FURUSAWA, T., FUJINO, T., KIKUGAWA, K., NAKANO, H., TAKEUCHI, O., AKIRA, S., AKIYAMA, T., GOHDA, J., INOUE, J. & HAYAKAWA, M. 2010. Human lactoferrin activates NF-kappaB through the Toll-like receptor 4 pathway while it interferes with the lipopolysaccharide-stimulated TLR4 signaling. *Febs j,* 277**,** 2051–66.

AO, Z., ZHU, R., TAN, X., LIU, L., CHEN, L., LIU, S. & YAO, X. 2016. Activation of HIV-1 expression in latently infected CD4+ T cells by the small molecule PKC412. *Virol J,* 13**,** 177.

ARAIN, M. A., KHASKHELI, G. B., BARHAM, G. S. & MARGHAZANI, I. B. 2024. Lactoferrin's role in modulating NF-κB pathway to alleviate diabetes-associated inflammation: A novel in-silico study. *Heliyon,* 10**,** e34051.

ARCHIN, N. M., BATESON, R., TRIPATHY, M. K., CROOKS, A. M., YANG, K. H., DAHL, N. P., KEARNEY, M. F., ANDERSON, E. M., COFFIN, J. M., STRAIN, M. C., RICHMAN, D. D., ROBERTSON, K. R., KASHUBA, A. D., BOSCH, R. J., HAZUDA, D. J., KURUC, J. D., ERON, J. J. & MARGOLIS, D. M. 2014a. HIV-1 expression within resting CD4+ T cells after multiple doses of vorinostat. *J Infect Dis,* 210**,** 728–35.

ARCHIN, N. M., ESPESETH, A., PARKER, D., CHEEMA, M., HAZUDA, D. & MARGOLIS, D. M. 2009. Expression of latent HIV induced by the potent HDAC inhibitor suberoylanilide hydroxamic acid. *AIDS Res Hum Retroviruses,* 25**,** 207–12.

ARCHIN, N. M., SUNG, J. M., GARRIDO, C., SORIANO-SARABIA, N. & MARGOLIS, D. M. 2014b. Eradicating HIV-1 infection: seeking to clear a persistent pathogen. *Nat Rev Microbiol,* 12**,** 750–64.

ARTS, E. J. & HAZUDA, D. J. 2012. HIV-1 antiretroviral drug therapy. *Cold Spring Harb Perspect Med,* 2**,** a007161.

ASH, M. K., AL-HARTHI, L. & SCHNEIDER, J. R. 2021. HIV in the Brain: Identifying Viral Reservoirs and Addressing the Challenges of an HIV Cure. *Vaccines (Basel),* 9.

BACHU, M., YALLA, S., ASOKAN, M., VERMA, A., NEOGI, U., SHARMA, S., MURALI, R. V., MUKTHEY, A. B., BHATT, R., CHATTERJEE, S., RAJAN, R. E., CHEEDARLA, N., YADAVALLI, V. S., MAHADEVAN, A., SHANKAR, S. K., RAJAGOPALAN, N., SHET, A., SARAVANAN, S., BALAKRISHNAN, P., SOLOMON, S., VAJPAYEE, M., SATISH, K. S., KUNDU, T. K., JEANG, K. T. & RANGA, U. 2012. Multiple NF-κB sites in HIV-1 subtype C long terminal repeat confer superior magnitude of transcription and thereby the enhanced viral predominance. *J Biol Chem,* 287**,** 44714–35.

BAGGIOLINI, M., DE DUVE, C., MASSON, P. L. & HEREMANS, J. F. 1970. Association of lactoferrin with specific granules in rabbit heterophil leukocytes. *J Exp Med,* 131**,** 559–70.

BAILON, L., MOTHE, B., BERMAN, L. & BRANDER, C. 2020. Novel Approaches Towards a Functional Cure of HIV/AIDS. *Drugs,* 80**,** 859–868.

BAKER, E. N. & LINDLEY, P. F. 1992. New perspectives on the structure and function of transferrins. *Journal of Inorganic Biochemistry,* 47**,** 147–160.

BANERJI, U., O'DONNELL, A., SCURR, M., PACEY, S., STAPLETON, S., ASAD, Y., SIMMONS, L., MALONEY, A., RAYNAUD, F., CAMPBELL, M., WALTON, M., LAKHANI, S., KAYE, S., WORKMAN, P. & JUDSON, I. 2005. Phase I pharmacokinetic and pharmacodynamic study of

17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. *J Clin Oncol,* 23**,** 4152–61.
BARTON, K. M., BURCH, B. D., SORIANO-SARABIA, N. & MARGOLIS, D. M. 2013. Prospects for treatment of latent HIV. *Clin Pharmacol Ther,* 93**,** 46–56.
BEHBAHANI, M., NOSRATI, M. & MOHABATKAR, H. 2018. Inhibition of Human Immunodeficiency Type 1 Virus (HIV-1) Life Cycle by Different Egg White Lysozymes. *Appl Biochem Biotechnol,* 185**,** 786–798.
BELLAMY, W., TAKASE, M., WAKABAYASHI, H., KAWASE, K. & TOMITA, M. 1992. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *J Appl Bacteriol,* 73**,** 472–9.
BELLAMY, W. R., WAKABAYASHI, H., TAKASE, M., KAWASE, K., SHIMAMURA, S. & TOMITA, M. 1993. Role of cell-binding in the antibacterial mechanism of lactoferricin B. *J Appl Bacteriol,* 75**,** 478–84.
BERESHCHENKO, O. R., GU, W. & DALLA-FAVERA, R. 2002. Acetylation inactivates the transcriptional repressor BCL6. *Nat Genet,* 32**,** 606–13.
BERGAMO, A., GERDOL, M., PALLAVICINI, A., GRECO, S., SCHEPENS, I., HAMELIN, R., ARMAND, F., DYSON, P. J. & SAVA, G. 2019. Lysozyme-Induced Transcriptional Regulation of TNF-α Pathway Genes in Cells of the Monocyte Lineage. *Int J Mol Sci,* 20.
BERGAMO, A. & SAVA, G. 2024. Lysozyme: A Natural Product with Multiple and Useful Antiviral Properties. *Molecules,* 29.
BERKHOUT, B., VAN WAMEL, J. L., BELJAARS, L., MEIJER, D. K., VISSER, S. & FLORIS, R. 2002. Characterization of the anti-HIV effects of native lactoferrin and other milk proteins and protein-derived peptides. *Antiviral research,* 55**,** 341–355.
BERLUTTI, F., PANTANELLA, F., NATALIZI, T., FRIONI, A., PAESANO, R., POLIMENI, A. & VALENTI, P. 2011. Antiviral properties of lactoferrin--a natural immunity molecule. *Molecules,* 16**,** 6992–7018.
BESNARD, E., HAKRE, S., KAMPMANN, M., LIM, H. W., HOSMANE, N. N., MARTIN, A., BASSIK, M. C., VERSCHUEREN, E., BATTIVELLI, E., CHAN, J., SVENSSON, J. P., GRAMATICA, A., CONRAD, R. J., OTT, M., GREENE, W. C., KROGAN, N. J., SILICIANO, R. F., WEISSMAN, J. S. & VERDIN, E. 2016. The mTOR Complex Controls HIV Latency. *Cell Host Microbe,* 20**,** 785–797.
BESSELINK, N., KEIJER, J., VERMEULEN, C., BOYMANS, S., DE RIDDER, J., VAN HOECK, A., CUPPEN, E. & KUIJK, E. 2023. The genome-wide mutational consequences of DNA hypomethylation. *Scientific Reports,* 13**,** 6874.
BLAKE, C. C. F., KOENIG, D. F., MAIR, G. A., NORTH, A. C. T., PHILLIPS, D. C. & SARMA, V. R. 1965. Structure of Hen Egg-White Lysozyme: A Three-dimensional Fourier Synthesis at 2 Å Resolution. *Nature,* 206**,** 757–761.
BLANCO-RODRIGUEZ, G., GAZI, A., MONEL, B., FRABETTI, S., SCOCA, V., MUELLER, F., SCHWARTZ, O., KRIJNSE-LOCKER, J., CHARNEAU, P. & DI NUNZIO, F. 2020. Remodeling of the Core Leads HIV-1 Preintegration Complex into the Nucleus of Human Lymphocytes. *J Virol,* 94.
BLAZKOVA, J., TREJBALOVA, K., GONDOIS-REY, F., HALFON, P., PHILIBERT, P., GUIGUEN, A., VERDIN, E., OLIVE, D., VAN LINT, C., HEJNAR, J. & HIRSCH, I. 2009. CpG methylation controls reactivation of HIV from latency. *PLoS Pathog,* 5**,** e1000554.
BRITIGAN, B. E., LEWIS, T. S., WALDSCHMIDT, M., MCCORMICK, M. L. & KRIEG, A. M. 2001. Lactoferrin binds CpG-containing oligonucleotides and inhibits their immunostimulatory effects on human B cells. *J Immunol,* 167**,** 2921–8.
BRITIGAN, B. E., ROSEN, G. M., THOMPSON, B. Y., CHAI, Y. & COHEN, M. S. 1986. Stimulated human neutrophils limit iron-catalyzed hydroxyl radical formation as detected by spin-trapping techniques. *J Biol Chem,* 261**,** 17026–32.
BROGDON, J., ZIANI, W., WANG, X., VEAZEY, R. S. & XU, H. 2016. In vitro effects of the small-molecule protein kinase C agonists on HIV latency reactivation. *Sci Rep,* 6**,** 39032.
BUBNA, A. K. 2015. Vorinostat-An Overview. *Indian J Dermatol,* 60**,** 419.

BUKRINSKY, M. I., SHAROVA, N., DEMPSEY, M. P., STANWICK, T. L., BUKRINSKAYA, A. G., HAGGERTY, S. & STEVENSON, M. 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc Natl Acad Sci U S A,* 89**,** 6580–4.

CHOU, T. C., MAGGIRWAR, N. S. & MARSDEN, M. D. 2024. HIV Persistence, Latency, and Cure Approaches: Where Are We Now? *Viruses,* 16**,** 1163.

CISANI, G., VARALDO, P. E., INGIANNI, A., POMPEI, R. & SATTA, G. 1984. Inhibition of herpes simplex virus-induced cytopathic effect by modified hen egg-white lysozymes. *Current Microbiology,* 10**,** 35–40.

COHN, L. B., CHOMONT, N. & DEEKS, S. G. 2020. The Biology of the HIV-1 Latent Reservoir and Implications for Cure Strategies. *Cell Host Microbe,* 27**,** 519–530.

COIRAS, M., LÓPEZ-HUERTAS, M. R., RULLAS, J., MITTELBRUNN, M. & ALCAMÍ, J. 2007. Basal shuttle of NF-κB/IκBα in resting T lymphocytes regulates HIV-1 LTR dependent expression. *Retrovirology,* 4**,** 56.

COLIN, L. & VAN LINT, C. 2009. Molecular control of HIV-1 postintegration latency: implications for the development of new therapeutic strategies. *Retrovirology,* 6**,** 111.

COMPE, E. & EGLY, J. M. 2016. Nucleotide Excision Repair and Transcriptional Regulation: TFIIH and Beyond. *Annu Rev Biochem,* 85**,** 265–90.

CONTRERAS, X., LENASI, T. & PETERLIN, B. M. 2006. HIV latency: present knowledge, future directions. *Future Virol,* 1**,** 733–745.

CSERMELY, P., SCHNAIDER, T., SO"TI, C., PROHÁSZKA, Z. & NARDAI, G. 1998. The 90-kDa Molecular Chaperone Family: Structure, Function, and Clinical Applications. A Comprehensive Review. *Pharmacology & Therapeutics,* 79**,** 129–168.

DARCIS, G., VAN DRIESSCHE, B. & VAN LINT, C. 2017. HIV Latency: Should We Shock or Lock? *Trends Immunol,* 38**,** 217–228.

DE BAAR, M. P., DE RONDE, A., BERKHOUT, B., CORNELISSEN, M., VAN DER HORN, K. H., VAN DER SCHOOT, A. M., DE WOLF, F., LUKASHOV, V. V. & GOUDSMIT, J. 2000. Subtype-specific sequence variation of the HIV type 1 long terminal repeat and primer-binding site. *AIDS Res Hum Retroviruses,* 16**,** 499–504.

DEEKS, S. G. 2012. HIV: Shock and kill. *Nature,* 487**,** 439–40.

DIMITROVA, E., TURBERFIELD, A. H. & KLOSE, R. J. 2015. Histone demethylases in chromatin biology and beyond. *EMBO Rep,* 16**,** 1620–39.

DOGGRELL, S. A. & BROWN, L. 2001. The spironolactone renaissance. *Expert Opin Investig Drugs,* 10**,** 943–54.

DUTILLEUL, A., RODARI, A. & VAN LINT, C. 2020. Depicting HIV-1 Transcriptional Mechanisms: A Summary of What We Know. *Viruses,* 12**,** 1385.

EASLEY, R., VAN DUYNE, R., COLEY, W., GUENDEL, I., DADGAR, S., KEHN-HALL, K. & KASHANCHI, F. 2010. Chromatin dynamics associated with HIV-1 Tat-activated transcription. *Biochim Biophys Acta,* 1799**,** 275–85.

EINKAUF, K. B., LEE, G. Q., GAO, C., SHARAF, R., SUN, X., HUA, S., CHEN, S. M., JIANG, C., LIAN, X., CHOWDHURY, F. Z., ROSENBERG, E. S., CHUN, T. W., LI, J. Z., YU, X. G. & LICHTERFELD, M. 2019. Intact HIV-1 proviruses accumulate at distinct chromosomal positions during prolonged antiretroviral therapy. *J Clin Invest,* 129**,** 988–998.

ELLISON, R. T., 3RD & GIEHL, T. J. 1991. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J Clin Invest,* 88**,** 1080–91.

ELZOGHBY, A. O., ABDELMONEEM, M. A., HASSANIN, I. A., ABD ELWAKIL, M. M., ELNAGGAR, M. A., MOKHTAR, S., FANG, J. Y. & ELKHODAIRY, K. A. 2020. Lactoferrin, a multi-functional glycoprotein: Active therapeutic, drug nanocarrier & targeting ligand. *Biomaterials,* 263**,** 120355.

FERRABOSCHI, P., CICERI, S. & GRISENTI, P. 2021. Applications of Lysozyme, an Innate Immune Defense Factor, as an Alternative Antibiotic. *Antibiotics (Basel),* 10.

FILLEBEEN, C., DESCAMPS, L., DEHOUCK, M. P., FENART, L., BENAÏSSA, M., SPIK, G., CECCHELLI, R. & PIERCE, A. 1999. Receptor-mediated transcytosis of lactoferrin through the blood-brain barrier. *J Biol Chem,* 274**,** 7011–7.

FINKELSTEIN, R. A., SCIORTINO, C. V. & MCINTOSH, M. A. 1983. Role of iron in microbe-host interactions. *Rev Infect Dis,* 5 Suppl 4**,** S759–77.

FINNIN, M. S., DONIGIAN, J. R., COHEN, A., RICHON, V. M., RIFKIND, R. A., MARKS, P. A., BRESLOW, R. & PAVLETICH, N. P. 1999. Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature,* 401**,** 188–93.

FLEET, J. C. 1995. A new role for lactoferrin: DNA binding and transcription activation. *Nutr Rev,* 53**,** 226–7.

FRANGE, P., FAYE, A., AVETTAND-FENOËL, V., BELLATON, E., DESCAMPS, D., ANGIN, M., DAVID, A., CAILLAT-ZUCMAN, S., PEYTAVIN, G., DOLLFUS, C., LE CHENADEC, J., WARSZAWSKI, J., ROUZIOUX, C. & SÁEZ-CIRIÓN, A. 2016. HIV-1 virological remission lasting more than 12 years after interruption of early antiretroviral therapy in a perinatally infected teenager enrolled in the French ANRS EPF-CO10 paediatric cohort: a case report. *Lancet HIV,* 3**,** e49–54.

FURMANSKI, P., LI, Z. P., FORTUNA, M. B., SWAMY, C. V. & DAS, M. R. 1989. Multiple molecular forms of human lactoferrin. Identification of a class of lactoferrins that possess ribonuclease activity and lack iron-binding capacity. *J Exp Med,* 170**,** 415–29.

GALLAY, P., HOPE, T., CHIN, D. & TRONO, D. 1997. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proceedings of the National Academy of Sciences,* 94**,** 9825–9830.

GARCÍA-MONTOYA, I. A., CENDÓN, T. S., ARÉVALO-GALLEGOS, S. & RASCÓN-CRUZ, Q. 2012. Lactoferrin a multiple bioactive protein: an overview. *Biochim Biophys Acta,* 1820**,** 226–36.

GARCÍA, M., BUZÓN, M. J., BENITO, J. M. & RALLÓN, N. 2018. Peering into the HIV reservoir. *Rev Med Virol,* 28**,** e1981.

GAVEGNANO, C., DETORIO, M., MONTERO, C., BOSQUE, A., PLANELLES, V. & SCHINAZI, R. F. 2014. Ruxolitinib and tofacitinib are potent and selective inhibitors of HIV-1 replication and virus reactivation in vitro. *Antimicrob Agents Chemother,* 58**,** 1977–86.

GREBER, B. J., TOSO, D. B., FANG, J. & NOGALES, E. 2019. The complete structure of the human TFIIH core complex. *Elife,* 8**,** e44771.

GREGER, I. H., DEMARCHI, F., GIACCA, M. & PROUDFOOT, N. J. 1998. Transcriptional interference perturbs the binding of Sp1 to the HIV-1 promoter. *Nucleic Acids Res,* 26**,** 1294–301.

GREY, A., BANOVIC, T., ZHU, Q., WATSON, M., CALLON, K., PALMANO, K., ROSS, J., NAOT, D., REID, I. R. & CORNISH, J. 2004. The low-density lipoprotein receptor-related protein 1 is a mitogenic receptor for lactoferrin in osteoblastic cells. *Mol Endocrinol,* 18**,** 2268–78.

GROOT, F., GEIJTENBEEK, T. B., SANDERS, R. W., BALDWIN, C. E., SANCHEZ-HERNANDEZ, M., FLORIS, R., VAN KOOYK, Y., DE JONG, E. C. & BERKHOUT, B. 2005. Lactoferrin prevents dendritic cell-mediated human immunodeficiency virus type 1 transmission by blocking the DC-SIGN--gp120 interaction. *J Virol,* 79**,** 3009–15.

GROVES, M. L. 1960. The Isolation of a Red Protein from Milk2. *Journal of the American Chemical Society,* 82**,** 3345–3350.

GUO, X., YUAN, X., XU, Z., LIU, J., LV, R., GAO, Y., XU, W., JI, D. & GUO, Y. 2025. Lactoferrin supplementation modulates the oxidative and metabolic genes by NR5A2-mediated histone modifications in deoxynivalenol-induced ileum injury. *Stress Biol,* 5**,** 47.

GUPTA, I., SEHGAL, R., KANWAR, R. K., PUNJ, V. & KANWAR, J. R. 2015. Nanocapsules Loaded with Iron-Saturated Bovine Lactoferrin have Antimicrobial Therapeutic Potential and Maintain Calcium, Zinc and Iron Metabolism. *Nanomedicine,* 10**,** 1289–1314.

HAKRE, S., CHAVEZ, L., SHIRAKAWA, K. & VERDIN, E. 2012. HIV latency: experimental systems and molecular models. *FEMS Microbiol Rev,* 36**,** 706–16.

HAN, Y., LASSEN, K., MONIE, D., SEDAGHAT, A. R., SHIMOJI, S., LIU, X., PIERSON, T. C., MARGOLICK, J. B., SILICIANO, R. F. & SILICIANO, J. D. 2004. Resting CD4+ T cells from human immunodeficiency virus type 1 (HIV-1)-infected individuals carry integrated HIV-1 genomes within actively transcribed host genes. *J Virol,* 78**,** 6122–33.

HANSTOCK, H. G., EDWARDS, J. P. & WALSH, N. P. 2019. Tear Lactoferrin and Lysozyme as Clinically Relevant Biomarkers of Mucosal Immune Competence. *Frontiers in Immunology,* 10.

HARMSEN, M. C., SWART, P. J., DE BÉTHUNE, M. P., PAUWELS, R., DE CLERCQ, E., THE, T. H. & MEIJER, D. K. 1995. Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication in vitro. *J Infect Dis,* 172**,** 380–8.

HASHEMI, P. & SADOWSKI, I. 2020. Diversity of small molecule HIV-1 latency reversing agents identified in low- and high-throughput small molecule screens. *Med Res Rev,* 40**,** 881–908.

HÅVERSEN, L., OHLSSON, B. G., HAHN-ZORIC, M., HANSON, L. A. & MATTSBY-BALTZER, I. 2002. Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B. *Cell Immunol,* 220**,** 83–95.

HE, J. & FURMANSKI, P. 1995. Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. *Nature,* 373**,** 721–4.

HENRICH, T. J., HANHAUSER, E., MARTY, F. M., SIRIGNANO, M. N., KEATING, S., LEE, T. H., ROBLES, Y. P., DAVIS, B. T., LI, J. Z., HEISEY, A., HILL, A. L., BUSCH, M. P., ARMAND, P., SOIFFER, R. J., ALTFELD, M. & KURITZKES, D. R. 2014. Antiretroviral-free HIV-1 remission and viral rebound after allogeneic stem cell transplantation: report of 2 cases. *Ann Intern Med,* 161**,** 319–27.

HERMANKOVA, M., SILICIANO, J. D., ZHOU, Y., MONIE, D., CHADWICK, K., MARGOLICK, J. B., QUINN, T. C. & SILICIANO, R. F. 2003. Analysis of human immunodeficiency virus type 1 gene expression in latently infected resting CD4+ T lymphocytes in vivo. *J Virol,* 77**,** 7383–92.

HONG, R., XIE, A., JIANG, C., GUO, Y., ZHANG, Y., CHEN, J., SHEN, X., LI, M. & YUE, X. 2024. A Review of the Biological Activities of Lactoferrin: Mechanisms and Potential Applications. *Food & Function*.

HORVATH, R. M., BRUMME, Z. L. & SADOWSKI, I. 2024. Small molecule inhibitors of transcriptional cyclin-dependent kinases impose HIV-1 latency, presenting "block and lock" treatment strategies. *Antimicrob Agents Chemother***,** e0107223.

HUNT, G. & TIEMESSEN, C. 2000. Occurrence of additional NF-kappaB-binding motifs in the long terminal repeat region of South African HIV type 1 subtype C isolates. *AIDS Research and Human Retroviruses,* 16**,** 305–306.

HUTTER, G., NOWAK, D., MOSSNER, M., GANEPOLA, S., MÜß, A., ALLERS, K., SCHNEIDER, T., HOFMANN, J., KÜLCHERER, C., BLAU, O., BLAU, I. W., HOFMANN, W. K. & THIEL, E. 2009. Long-term control of HIV by CCR5 delta32/delta32 stem-cell transplantation. *New England Journal of Medicine,* 360**,** 692–698.

IMAI, K. & OCHIAI, K. 2011. Role of histone modification on transcriptional regulation and HIV-1 gene expression: possible mechanisms of periodontal diseases in AIDS progression. *J Oral Sci,* 53**,** 1–13.

IYER, K., CHAND, K., MITRA, A., TRIVEDI, J. & MITRA, D. 2021. Diversity in heat shock protein families: functional implications in virus infection with a comprehensive insight of their role in the HIV-1 life cycle. *Cell Stress and Chaperones,* 26**,** 743–768.

JABAREEN, A., SULEMAN, M., ABU-JAAFAR, A. & HULEIHEL, M. 2018. Different molecular mechanisms of HTLV-1 and HIV LTR activation by TPA. *Biochem Biophys Res Commun,* 500**,** 538–543.

JANSSENS, J., DE WIT, F., PARVEEN, N. & DEBYSER, Z. 2022. Single-Cell Imaging Shows That the Transcriptional State of the HIV-1 Provirus and Its Reactivation Potential Depend on the Integration Site. *mBio,* 13**,** e0000722.

JANSSENS, J., KIM, P., KIM, S. J., WEDRYCHOWSKI, A., KADIYALA, G. N., HUNT, P. W., DEEKS, S. G., WONG, J. K. & YUKL, S. A. 2024. Mechanisms and efficacy of small molecule latency-promoting agents to inhibit HIV reactivation ex vivo. *JCI Insight,* 9.

JIANG, G. & DANDEKAR, S. 2015. Targeting NF-κB signaling with protein kinase C agonists as an emerging strategy for combating HIV latency. *AIDS Res Hum Retroviruses,* 31**,** 4–12.

JIANG, L., LI, Y., WANG, L., GUO, J., LIU, W., MENG, G., ZHANG, L., LI, M., CONG, L. & SUN, M. 2021. Recent Insights Into the Prognostic and Therapeutic Applications of Lysozymes. *Frontiers in Pharmacology,* 12.

JOHNSTONE, R. W. & LICHT, J. D. 2003. Histone deacetylase inhibitors in cancer therapy: is transcription the primary target? *Cancer Cell,* 4**,** 13–8.

JONES, J. E., GUNDERSON, C. E., WIGDAHL, B. & NONNEMACHER, M. R. 2025. Impact of chromatin on HIV-1 latency: a multi-dimensional perspective. *Epigenetics Chromatin,* 18**,** 9.

JORDAN, A., DEFECHEREUX, P. & VERDIN, E. 2001. The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. *The EMBO Journal,* 20**,** 1726–1738.

KALVATCHEV, Z., WALDER, R. & GARZARO, D. 1997. Different effects of phorbol ester derivates on human immunodeficiency virus 1 replication in lymphocytic and monocytic human cells. *Acta Virol,* 41**,** 289–92.

KANWAR, J. R., MAHIDHARA, G., ROY, K., SASIDHARAN, S., KRISHNAKUMAR, S., PRASAD, N., SEHGAL, R. & KANWAR, R. K. 2015. Fe-Blf Nanoformulation Targets Survivin to Kill Colon Cancer Stem Cells and Maintains Absorption of Iron, Calcium and Zinc. *Nanomedicine,* 10**,** 35–55.

KANYSHKOVA, T. Y. G., SEMENOV, D. V., BUNEVA, V. N. & NEVINSKY, G. A. 1999. Human milk lactoferrin binds two DNA molecules with different affinities. *FEBS Letters,* 451**,** 235–237.

KARN, J. 2011. The molecular biology of HIV latency: breaking and restoring the Tat-dependent transcriptional circuit. *Curr Opin HIV AIDS,* 6**,** 4–11.

KAZANIETZ, M. G. & COOKE, M. 2024. Protein kinase C signaling “in” and “to” the nucleus: master kinases in transcriptional regulation. *Journal of Biological Chemistry***,** 105692.

KHORSHIDIAN, N., KHANNIRI, E., KOUSHKI, M. R., SOHRABVANDI, S. & YOUSEFI, M. 2022. An Overview of Antimicrobial Activity of Lysozyme and Its Functionality in Cheese. *Frontiers in Nutrition,* 9.

KILROY, J. M., LEAL, A. A. & HENDERSON, A. J. 2024. Chronic HIV Transcription, Translation, and Persistent Inflammation. *Viruses,* 16.

KIM, C. W., LEE, T. H., PARK, K. H., CHOI, S.-Y. & KIM, J. 2012. Human lactoferrin suppresses TNF-α-induced intercellular adhesion molecule-1 expression via competition with NF-κB in endothelial cells. *FEBS Letters,* 586**,** 229–234.

KIM, K. C., LEE, S., SON, J., SHIN, Y., YOON, C. H., KANG, C. & CHOI, B. S. 2017. Identification of novel genes associated with HIV-1 latency by analysis of histone modifications. *Hum Genomics,* 11**,** 9.

KIM, Y., ANDERSON, J. L. & LEWIN, S. R. 2018. Getting the "Kill" into "Shock and Kill": Strategies to Eliminate Latent HIV. *Cell Host Microbe,* 23**,** 14–26.

KOWALCZYK, P., KACZYŃSKA, K., KLECZKOWSKA, P., BUKOWSKA-OŚKO, I., KRAMKOWSKI, K. & SULEJCZAK, D. 2022. The Lactoferrin Phenomenon-A Miracle Molecule. *Molecules,* 27.

KROON, E. D. M. B., ANANWORANICH, J., PAGLIUZZA, A., RHODES, A., PHANUPHAK, N., TRAUTMANN, L., MITCHELL, J. L., CHINTANAPHOL, M., INTASAN, J., PINYAKORN, S., BENJAPORNPONG, K., CHANG, J. J., COLBY, D. J., CHOMCHEY, N., FLETCHER, J. L. K., EUBANKS, K., YANG, H., KAPSON, J., DANTANARAYANA, A., TENNAKOON, S., GORELICK, R. J., MALDARELLI, F., ROBB, M. L., KIM, J. H., SPUDICH, S., CHOMONT, N., PHANUPHAK, P., LEWIN, S. R. & DE SOUZA, M. S. 2020. A randomized trial of vorinostat with treatment interruption after initiating antiretroviral therapy during acute HIV-1 infection. *Journal of Virus Eradication,* 6**,** 100004.

KRUZEL, M. L., ZIMECKI, M. & ACTOR, J. K. 2017. Lactoferrin in a Context of Inflammation-Induced Pathology. *Front Immunol,* 8**,** 1438.
LACOMBE, B., MOREL, M., MARGOTTIN-GOGUET, F. & RAMIREZ, B. C. 2016. Specific Inhibition of HIV Infection by the Action of Spironolactone in T Cells. *J Virol,* 90**,** 10972–10980.
LAN, F., NOTTKE, A. C. & SHI, Y. 2008. Mechanisms involved in the regulation of histone lysine demethylases. *Curr Opin Cell Biol,* 20**,** 316–25.
LANG, J., YANG, N., DENG, J., LIU, K., YANG, P., ZHANG, G. & JIANG, C. 2011. Inhibition of SARS pseudovirus cell entry by lactoferrin binding to heparan sulfate proteoglycans. *PloS one,* 6**,** e23710.
LEE-HUANG, S., HUANG, P. L., SUN, Y., HUANG, P. L., KUNG, H. F., BLITHE, D. L. & CHEN, H. C. 1999. Lysozyme and RNases as anti-HIV components in beta-core preparations of human chorionic gonadotropin. *Proc Natl Acad Sci U S A,* 96**,** 2678–81.
LEE-HUANG, S., MAIOROV, V., HUANG, P. L., NG, A., LEE, H. C., CHANG, Y. T., KALLENBACH, N., HUANG, P. L. & CHEN, H. C. 2005. Structural and functional modeling of human lysozyme reveals a unique nonapeptide, HL9, with anti-HIV activity. *Biochemistry,* 44**,** 4648–55.
LEE, K. H., JANG, Y. & CHUNG, J. H. 2010. Heat shock protein 90 regulates IκB kinase complex and NF-κB activation in angiotensin II-induced cardiac cell hypertrophy. *Exp Mol Med,* 42**,** 703–11.
LENASI, T., CONTRERAS, X. & PETERLIN, B. M. 2008. Transcriptional interference antagonizes proviral gene expression to promote HIV latency. *Cell Host Microbe,* 4**,** 123–33.
LEWINSKI, M. K., BISGROVE, D., SHINN, P., CHEN, H., HOFFMANN, C., HANNENHALLI, S., VERDIN, E., BERRY, C. C., ECKER, J. R. & BUSHMAN, F. D. 2005. Genome-wide analysis of chromosomal features repressing human immunodeficiency virus transcription. *J Virol,* 79**,** 6610–9.
LEWIS, C. A., MARGOLIS, D. M. & BROWNE, E. P. 2023. New Concepts in Therapeutic Manipulation of HIV-1 Transcription and Latency: Latency Reversal versus Latency Prevention. *Viruses,* 15.
LIN, K. C., WEY, M. T., KAN, L. S. & SHIUAN, D. 2009. Characterization of the interactions of lysozyme with DNA by surface plasmon resonance and circular dichroism spectroscopy. *Appl Biochem Biotechnol,* 158**,** 631–41.
LIU, L., JIA, X., ZHAO, X., LI, T., LUO, Z., DENG, R., PENG, B., MAO, D., LIU, H. & ZHENG, Q. 2023. In vitro PCR verification that lysozyme inhibits nucleic acid replication and transcription. *Scientific Reports,* 13.
LÖNNERDAL, B. 2003. Nutritional and physiologic significance of human milk proteins. *Am J Clin Nutr,* 77**,** 1537s–1543s.
LÖNNERDAL, B. & IYER, S. 1995. Lactoferrin: Molecular Structure and Biological Function. *Annual Review of Nutrition,* 15**,** 93–110.
LUCERA, M. B., TILTON, C. A., MAO, H., DOBROWOLSKI, C., TABLER, C. O., HAQQANI, A. A., KARN, J. & TILTON, J. C. 2014. The histone deacetylase inhibitor vorinostat (SAHA) increases the susceptibility of uninfected CD4+ T cells to HIV by increasing the kinetics and efficiency of postentry viral events. *J Virol,* 88**,** 10803–12.
LUZURIAGA, K., GAY, H., ZIEMNIAK, C., SANBORN, K. B., SOMASUNDARAN, M., RAINWATER-LOVETT, K., MELLORS, J. W., ROSENBLOOM, D. & PERSAUD, D. 2015. Viremic relapse after HIV-1 remission in a perinatally infected child. *N Engl J Med,* 372**,** 786–8.
MAIKOO, S., PALSTRA, R.-J., DONG, K. L., MAHMOUDI, T., NDUNG'U, T. & MADLALA, P. 2024. Development of a latency model for HIV-1 subtype C and the impact of long terminal repeat element genetic variation on latency reversal. *Journal of Virus Eradication,* 10**,** 100575.
MAINA, E. K., ADAN, A. A., MUREITHI, H., MURIUKI, J. & LWEMBE, R. M. 2021. A Review of Current Strategies Towards the Elimination of Latent HIV-1 and Subsequent HIV-1 Cure. *Curr HIV Res,* 19**,** 14–26.
MAŁACZEWSKA, J., KACZOREK-ŁUKOWSKA, E., WÓJCIK, R. & SIWICKI, A. K. 2019. Antiviral effects of nisin, lysozyme, lactoferrin and their mixtures against bovine viral diarrhoea virus. *BMC Vet Res,* 15**,** 318.

MANCHES, O., FRLETA, D. & BHARDWAJ, N. 2014. Dendritic cells in progression and pathology of HIV infection. *Trends Immunol,* 35**,** 114–22.
MANN, D. M., ROMM, E. & MIGLIORINI, M. 1994. Delineation of the glycosaminoglycan-binding site in the human inflammatory response protein lactoferrin. *J Biol Chem,* 269**,** 23661–7.
MANN, J. K. & NDUNG'U, T. 2020. The Potential of lactoferrin, Ovotransferrin and Lysozyme As Antiviral and immune-modulating Agents in COVID-19. *Future Virology,* 15**,** 609–624.
MARINI, B., KERTESZ-FARKAS, A., ALI, H., LUCIC, B., LISEK, K., MANGANARO, L., PONGOR, S., LUZZATI, R., RECCHIA, A. & MAVILIO, F. 2015. Nuclear architecture dictates HIV-1 integration site selection. *Nature,* 521**,** 227–231.
MATSUDA, K., KOBAYAKAWA, T., KARIYA, R., TSUCHIYA, K., RYU, S., TSUJI, K., ISHII, T., GATANAGA, H., YOSHIMURA, K., OKADA, S., HAMADA, A., MITSUYA, H., TAMAMURA, H. & MAEDA, K. 2021. A Therapeutic Strategy to Combat HIV-1 Latently Infected Cells With a Combination of Latency-Reversing Agents Containing DAG-Lactone PKC Activators. *Frontiers in Microbiology,* 12.
MBONYE, U. & KARN, J. 2024. The cell biology of HIV-1 latency and rebound. *Retrovirology,* 21**,** 6.
MCMAHON, D. K., ZHENG, L., CYKTOR, J. C., AGA, E., MACATANGAY, B. J., GODFREY, C., PARA, M., MITSUYASU, R. T., HESSELGESSER, J., DRAGAVON, J., DOBROWOLSKI, C., KARN, J., ACOSTA, E. P., GANDHI, R. T. & MELLORS, J. W. 2021. A Phase 1/2 Randomized, Placebo-Controlled Trial of Romidespin in Persons With HIV-1 on Suppressive Antiretroviral Therapy. *J Infect Dis,* 224**,** 648–656.
MEDIOUNI, S., CHINTHALAPUDI, K., EKKA, M. K., USUI, I., JABLONSKI, J. A., CLEMENTZ, M. A., MOUSSEAU, G., NOWAK, J., MACHERLA, V. R., BEVERAGE, J. N., ESQUENAZI, E., BARAN, P., DE VERA, I. M. S., KOJETIN, D., LORET, E. P., NETTLES, K., MAITI, S., IZARD, T. & VALENTE, S. T. 2019. Didehydro-Cortistatin A Inhibits HIV-1 by Specifically Binding to the Unstructured Basic Region of Tat. *mBio,* 10.
MIRABELLA, A., FOSTER, B. & BARTKE, T. 2015. Chromatin deregulation in disease. *Chromosoma,* 125.
MITCHELL, R. S., BEITZEL, B. F., SCHRODER, A. R., SHINN, P., CHEN, H., BERRY, C. C., ECKER, J. R. & BUSHMAN, F. D. 2004. Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol,* 2**,** E234.
MODJARRAD, K. & VERMUND, S. H. 2010. Effect of treating co-infections on HIV-1 viral load: a systematic review. *Lancet Infect Dis,* 10**,** 455–63.
MOORE, L. D., LE, T. & FAN, G. 2013. DNA Methylation and Its Basic Function. *Neuropsychopharmacology,* 38**,** 23–38.
MORANGUINHO, I. & VALENTE, S. T. 2020. Block-And-Lock: New Horizons for a Cure for HIV-1. *Viruses,* 12**,** 1443.
MORI, L., JENIKE, K., YEH, Y.-H. J., LACOMBE, B., LI, C., GETZLER, A. J., MEDIOUNI, S., CAMERON, M. E., PIPKIN, M. E., HO, Y.-C., RAMIREZ, B. C. & VALENTE, S. T. 2021a. The XPB Subunit of the TFIIH Complex Plays a Critical Role in HIV-1 Transcription, and XPB Inhibition by Spironolactone Prevents HIV-1 Reactivation from Latency. *Journal of Virology,* 95**,** 10.1128/jvi.01247–20.
MORI, L., JENIKE, K., YEH, Y. J., LACOMBE, B., LI, C., GETZLER, A., MEDIOUNI, S., CAMERON, M., PIPKIN, M., HO, Y. C., RAMIREZ, B. C. & VALENTE, S. 2021b. The XPB Subunit of the TFIIH Complex Plays a Critical Role in HIV-1 Transcription and XPB Inhibition by Spironolactone Prevents HIV-1 Reactivation from Latency. *J Virol,* 95.
MULLIGAN, P., WHITE, N. R. J., MONTELEONE, G., WANG, P., WILSON, J. W., OHTSUKA, Y. & SANDERSON, I. R. 2006. Breast Milk Lactoferrin Regulates Gene Expression by Binding Bacterial DNA CpG Motifs But Not Genomic DNA Promoters in Model Intestinal Cells. *Pediatric Research,* 59**,** 656–661.
ORPHANIDES, G., LAGRANGE, T. & REINBERG, D. 1996. The general transcription factors of RNA polymerase II. *Genes Dev,* 10**,** 2657–83.

PACE, M. J., AGOSTO, L., GRAF, E. H. & O'DOHERTY, U. 2011. HIV reservoirs and latency models. *Virology,* 411**,** 344–54.
PAL, S., JAISWAL, V., NALA, N. & RANGA, U. 2023. Enhanced Transcriptional Strength of HIV-1 Subtype C Minimizes Gene Expression Noise and Confers Stability to the Viral Latent State. *J Virol,* 97**,** e0137622.
PAN, X. Y., ZHAO, W., WANG, C. Y., LIN, J., ZENG, X. Y., REN, R. X., WANG, K., XUN, T. R., SHAI, Y. & LIU, S. W. 2016. Heat Shock Protein 90 Facilitates Latent HIV Reactivation through Maintaining the Function of Positive Transcriptional Elongation Factor b (p-TEFb) under Proteasome Inhibition. *J Biol Chem,* 291**,** 26177–26187.
PHILLIPS, D. C. 1966. The three-dimensional structure of an enzyme molecule. *Sci Am,* 215**,** 78–90.
PIETRANTONI, A., DOFRELLI, E., TINARI, A., AMMENDOLIA, M. G., PUZELLI, S., FABIANI, C., DONATELLI, I. & SUPERTI, F. 2010. Bovine lactoferrin inhibits Influenza A virus induced programmed cell death in vitro. *BioMetals,* 23**,** 465–475.
PLANELLES, V., WOLSCHENDORF, F. & KUTSCH, O. 2011. Facts and fiction: cellular models for high throughput screening for HIV-1 reactivating drugs. *Curr HIV Res,* 9**,** 568–78.
POSTNIKOVA, L. A. & PATKIN, E. L. 2022. The possible effect of lactoferrin on the epigenetic characteristics of early mammalian embryos exposed to bisphenol A. *Birth Defects Res,* 114**,** 1199–1209.
PRODROMOU, C., ROE, S. M., O'BRIEN, R., LADBURY, J. E., PIPER, P. W. & PEARL, L. H. 1997. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell,* 90**,** 65–75.
PUDDU, P., BORGHI, P., GESSANI, S., VALENTI, P., BELARDELLI, F. & SEGANTI, L. 1998. Antiviral effect of bovine lactoferrin saturated with metal ions on early steps of human immunodeficiency virus type 1 infection. *Int J Biochem Cell Biol,* 30**,** 1055–62.
QUIVY, V. & VAN LINT, C. 2004. Regulation at multiple levels of NF-κB-mediated transactivation by protein acetylation. *Biochemical pharmacology,* 68**,** 1221–1229.
RAFATI, H., PARRA, M., HAKRE, S., MOSHKIN, Y., VERDIN, E. & MAHMOUDI, T. 2011. Repressive LTR nucleosome positioning by the BAF complex is required for HIV latency. *PLoS Biol,* 9**,** e1001206.
RAGLAND, S. A. & CRISS, A. K. 2017. From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS Pathog,* 13**,** e1006512.
RIMEL, J. K. & TAATJES, D. J. 2018. The essential and multifunctional TFIIH complex. *Protein Sci,* 27**,** 1018–1037.
RODARI, A., DARCIS, G. & VAN LINT, C. M. 2021. The Current Status of Latency Reversing Agents for HIV-1 Remission. *Annu Rev Virol,* 8**,** 491–514.
ROEBUCK, K. A. & SAIFUDDIN, M. 1999. Regulation of HIV-1 transcription. *Gene Expr,* 8**,** 67–84.
ROESCH, F., MEZIANE, O., KULA, A., NISOLE, S., PORROT, F., ANDERSON, I., MAMMANO, F., FASSATI, A., MARCELLO, A., BENKIRANE, M. & SCHWARTZ, O. 2012. Hyperthermia stimulates HIV-1 replication. *PLoS Pathog,* 8**,** e1002792.
ROGOWSKA, A., PRYSHCHEPA, O., SOM, N. N., ŚPIEWAK, P., GOŁĘBIOWSKI, A., RAFIŃSKA, K., DOBRUCKA, R., KURZYDŁOWSKI, K., BUSZEWSKI, B. & POMASTOWSKI, P. 2023. Study on the zinc ions binding to human lactoferrin. *Journal of Molecular Structure,* 1282**,** 135149.
RUELAS, D. S. & GREENE, W. C. 2013. An integrated overview of HIV-1 latency. *Cell,* 155**,** 519–29.
SÁEZ-CIRIÓN, A., BACCHUS, C., HOCQUELOUX, L., AVETTAND-FENOEL, V., GIRAULT, I., LECUROUX, C., POTARD, V., VERSMISSE, P., MELARD, A., PRAZUCK, T., DESCOURS, B., GUERGNON, J., VIARD, J. P., BOUFASSA, F., LAMBOTTE, O., GOUJARD, C., MEYER, L., COSTAGLIOLA, D., VENET, A., PANCINO, G., AUTRAN, B. & ROUZIOUX, C. 2013. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. *PLoS Pathog,* 9**,** e1003211.

SALLUSTO, F., SCHAERLI, P., LOETSCHER, P., SCHANIEL, C., LENIG, D., MACKAY, C. R., QIN, S. & LANZAVECCHIA, A. 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol,* 28**,** 2760–9.

SÁNCHEZ-DUFFHUES, G., VO, M. Q., PÉREZ, M., CALZADO, M. A., MORENO, S., APPENDINO, G. & MUÑOZ, E. 2011. Activation of latent HIV-1 expression by protein kinase C agonists. A novel therapeutic approach to eradicate HIV-1 reservoirs. *Curr Drug Targets,* 12**,** 348–56.

SCHIRALLI LESTER, G. M. & HENDERSON, A. J. 2012. Mechanisms of HIV Transcriptional Regulation and Their Contribution to Latency. *Mol Biol Int,* 2012**,** 614120.

SCHRÖDER, A. R., SHINN, P., CHEN, H., BERRY, C., ECKER, J. R. & BUSHMAN, F. 2002. HIV-1 integration in the human genome favors active genes and local hotspots. *Cell,* 110**,** 521–529.

SCHULTZ, P., FRIBOURG, S., POTERSZMAN, A., MALLOUH, V., MORAS, D. & EGLY, J. M. 2000. Molecular structure of human TFIIH. *Cell,* 102**,** 599–607.

SEBAA, S., HIZETTE, N., BOUCHERIT-OTMANI, Z. & COURTOIS, P. 2017. Dose-dependent effect of lysozyme upon Candida albicans biofilm. *Mol Med Rep,* 15**,** 1135–1142.

SHAN, L., YANG, H. C., RABI, S. A., BRAVO, H. C., SHROFF, N. S., IRIZARRY, R. A., ZHANG, H., MARGOLICK, J. B., SILICIANO, J. D. & SILICIANO, R. F. 2011. Influence of host gene transcription level and orientation on HIV-1 latency in a primary-cell model. *J Virol,* 85**,** 5384–93.

SHARADA DEVI, A., DAS, M. R. & PANDIT, M. W. 1994. Lactoferrin contains structural motifs of ribonuclease. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology,* 1205**,** 275–281.

SHARROUF, K. & SUCHKOVA, I. 2021. The influence of lactoferrin on the epigenetic characteristics of mammalian cells of different types. *Medical academic journal,* 21**,** 85–95.

SILICIANO, R. F. & GREENE, W. C. 2011. HIV Latency. *Cold Spring Harbor Perspectives in Medicine,* 1.

SILVERS, R. M., SMITH, J. A., SCHOWALTER, M., LITWIN, S., LIANG, Z., GEARY, K. & DANIEL, R. 2010. Modification of integration site preferences of an HIV-1-based vector by expression of a novel synthetic protein. *Hum Gene Ther,* 21**,** 337–49.

SORENSEN, M. & SORENSEN, S. 1939. The proteins in whey.

SPITZNAGEL, J. K., DALLDORF, F., LEFFELL, M., FOLDS, J., WELSH, I., COONEY, M. & MARTIN, L. 1974. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. *Laboratory Investigation,* 30**,** 774–785.

STEINRAUF, L. K., SHIUAN, D., YANG, W. J. & CHIANG, M. Y. 1999. Lysozyme association with nucleic acids. *Biochem Biophys Res Commun,* 266**,** 366–70.

SVIDERSKAIA, K. & MEIER-STEPHENSON, V. 2025. Viral Appropriation of Specificity Protein 1 (Sp1): The Role of Sp1 in Human Retro- and DNA Viruses in Promoter Activation and Beyond. *Viruses,* 17.

THOMPSON, M. W. 2022. Regulation of zinc-dependent enzymes by metal carrier proteins. *BioMetals,* 35**,** 187–213.

TRIVEDI, J., PARVEEN, A., ROZY, F., MITRA, A., BAL, C., MITRA, D. & SHARON, A. 2019. Discovery of 2-isoxazol-3-yl-acetamide analogues as heat shock protein 90 (HSP90) inhibitors with significant anti-HIV activity. *Eur J Med Chem,* 183**,** 111699.

TURNER, A. W. & MARGOLIS, D. M. 2017. Chromatin Regulation and the Histone Code in HIV Latency *Yale J Biol Med,* 90**,** 229–243.

VAN LINT, C., BOUCHAT, S. & MARCELLO, A. 2013. HIV-1 transcription and latency: an update. *Retrovirology,* 10**,** 67.

VAN LINT, C., EMILIANI, S., OTT, M. & VERDIN, E. 1996. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. *Embo j,* 15**,** 1112–20.

VANSANT, G., BRUGGEMANS, A., JANSSENS, J. & DEBYSER, Z. 2020. Block-And-Lock Strategies to Cure HIV Infection. *Viruses,* 12.

VANTI, M., GALLASTEGUI, E., RESPALDIZA, I., RODRÍGUEZ-GIL, A., GÓMEZ-HERREROS, F., JIMENO-GONZÁLEZ, S., JORDAN, A. & CHÁVEZ, S. 2009. Yeast Genetic Analysis Reveals the

Involvement of Chromatin Reassembly Factors in Repressing HIV-1 Basal Transcription. *PLOS Genetics,* 5**,** e1000339.
VARGAS, B. & SLUIS-CREMER, N. 2022. Toward a Functional Cure for HIV-1 Infection: The Block and Lock Therapeutic Approach. *Frontiers in Virology,* 2**,** 917941.
VERDIN, E. 1991. DNase I-hypersensitive sites are associated with both long terminal repeats and with the intragenic enhancer of integrated human immunodeficiency virus type 1. *J Virol,* 65**,** 6790–9.
VERMA, A., RAJGOPALAN, P., LOTKE, R., VEERAPANENI, S., BACHU, M. & RANGA, U. 2013. Unique molecular features of the HIV-1 subtype C enhancer and core promoter and their influence on the viral gene expression. *Retrovirology,* 10**,** P94.
WANG, S., NG, T. B., CHEN, T., LIN, D., WU, J., RAO, P. & YE, X. 2005. First report of a novel plant lysozyme with both antifungal and antibacterial activities. *Biochem Biophys Res Commun,* 327**,** 820–7.
WEINBERG, E. D. 1993. The development of awareness of iron-withholding defense. *Perspect Biol Med,* 36**,** 215–21.
WHO. 2025. *HIV statistics, globally and by WHO region, 2025* [Online]. Available: https://www.who.int/teams/global-hiv-hepatitis-and-stis-programmes/hiv/strategic-information/hiv-data-and-statistics [Accessed 28 November 2025].
WILLIAMS, J. P., HURST, J., STÖHR, W., ROBINSON, N., BROWN, H., FISHER, M., KINLOCH, S., COOPER, D., SCHECHTER, M., TAMBUSSI, G., FIDLER, S., CARRINGTON, M., BABIKER, A., WEBER, J., KOELSCH, K. K., KELLEHER, A. D., PHILLIPS, R. E. & FRATER, J. 2014. HIV-1 DNA predicts disease progression and post-treatment virological control. *Elife,* 3**,** e03821.
WILLIAMS, S. A., CHEN, L. F., KWON, H., RUIZ-JARABO, C. M., VERDIN, E. & GREENE, W. C. 2006. NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. *Embo j,* 25**,** 139–49.
WRIGHT, D. G. & GALLIN, J. I. 1979. Secretory responses of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. *J Immunol,* 123**,** 285–94.
XING, S. & SILICIANO, R. F. 2013. Targeting HIV latency: pharmacologic strategies toward eradication. *Drug Discovery Today,* 18**,** 541–551.
YAMI, H. A., TAHMOORESPUR, M., JAVADMANESH, A., TAZARGHI, A. & SEKHAVATI, M. H. 2023. The immunomodulatory effects of lactoferrin and its derived peptides on NF-κB signaling pathway: A systematic review and meta-analysis. *Immun Inflamm Dis,* 11**,** e972.
YUSUF, A. P., ABUBAKAR, M. B., MALAMI, I., IBRAHIM, K. G., ABUBAKAR, B., BELLO, M. B., QUSTY, N., ELAZAB, S. T., IMAM, M. U., ALEXIOU, A. & BATIHA, G. E. 2021. Zinc Metalloproteins in Epigenetics and Their Crosstalk. *Life (Basel),* 11.
ZALAR, M., BYE, J. & CURTIS, R. 2023. Nonspecific Binding of Adenosine Tripolyphosphate and Tripolyphosphate Modulates the Phase Behavior of Lysozyme. *J Am Chem Soc,* 145**,** 929–943.
ZHANG, Y., ANDREWS, G. K. & WANG, L. 2012. Zinc-induced Dnmt1 expression involves antagonism between MTF-1 and nuclear receptor SHP. *Nucleic Acids Res,* 40**,** 4850–60.

## 8. Appendix 1

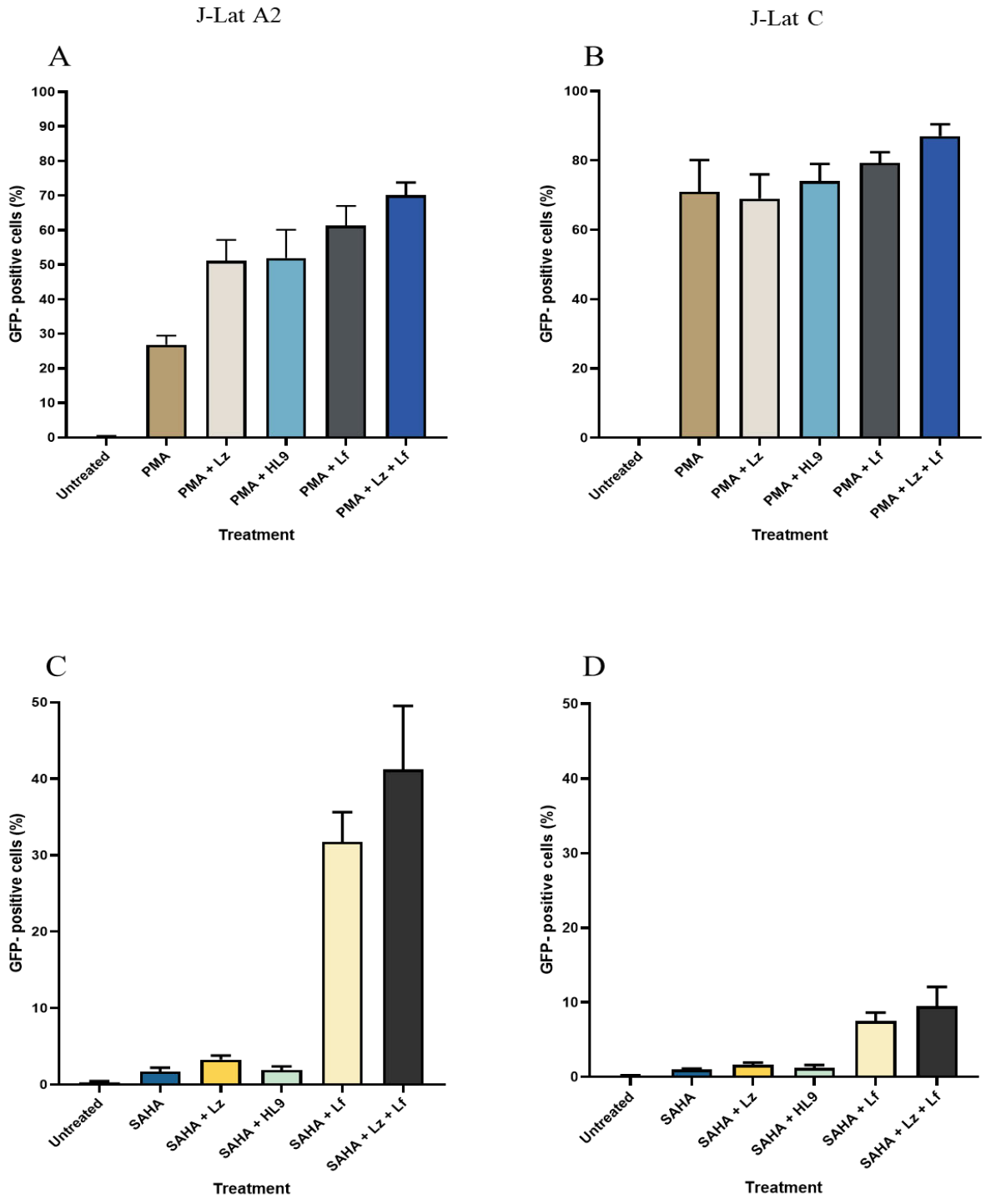


Figure A 4: The frequency of GFP-positive cells after a 24-hour co-treatment with cationic proteins (Lz, HL9, Lf [10 µM]) and LRAs (PMA [16 nM], SAHA [900 nM]).

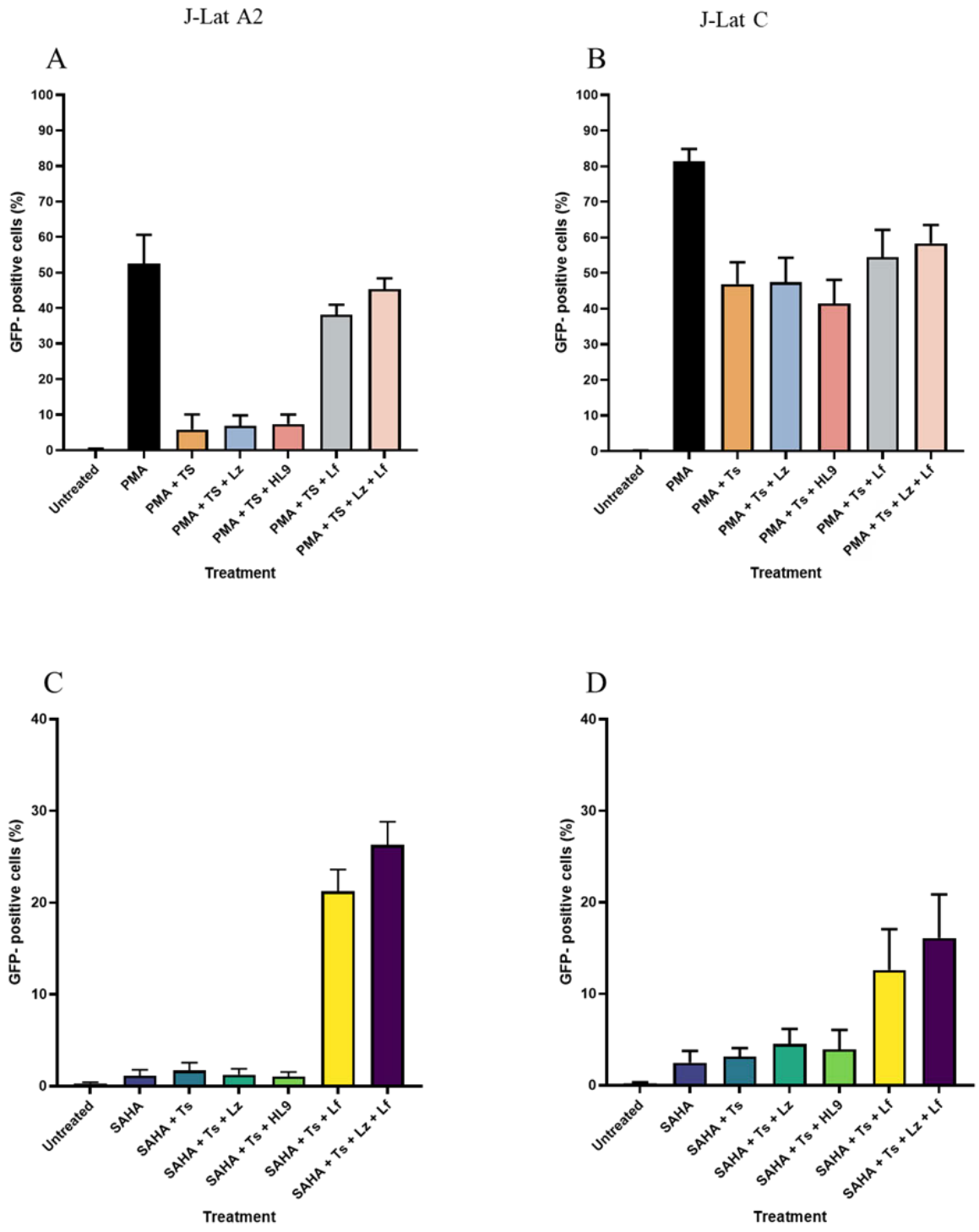


Figure A 5: The frequency of GFP-expression from J-Lat cells that were preincubated with Ts (2 µM) prior to being stimulated with a co-treatment of cationic proteins (Lz, HL9, Lf [10 µM]) and LRAs (PMA [16 nM], SAHA [900 nM]) for a further 24 hours.

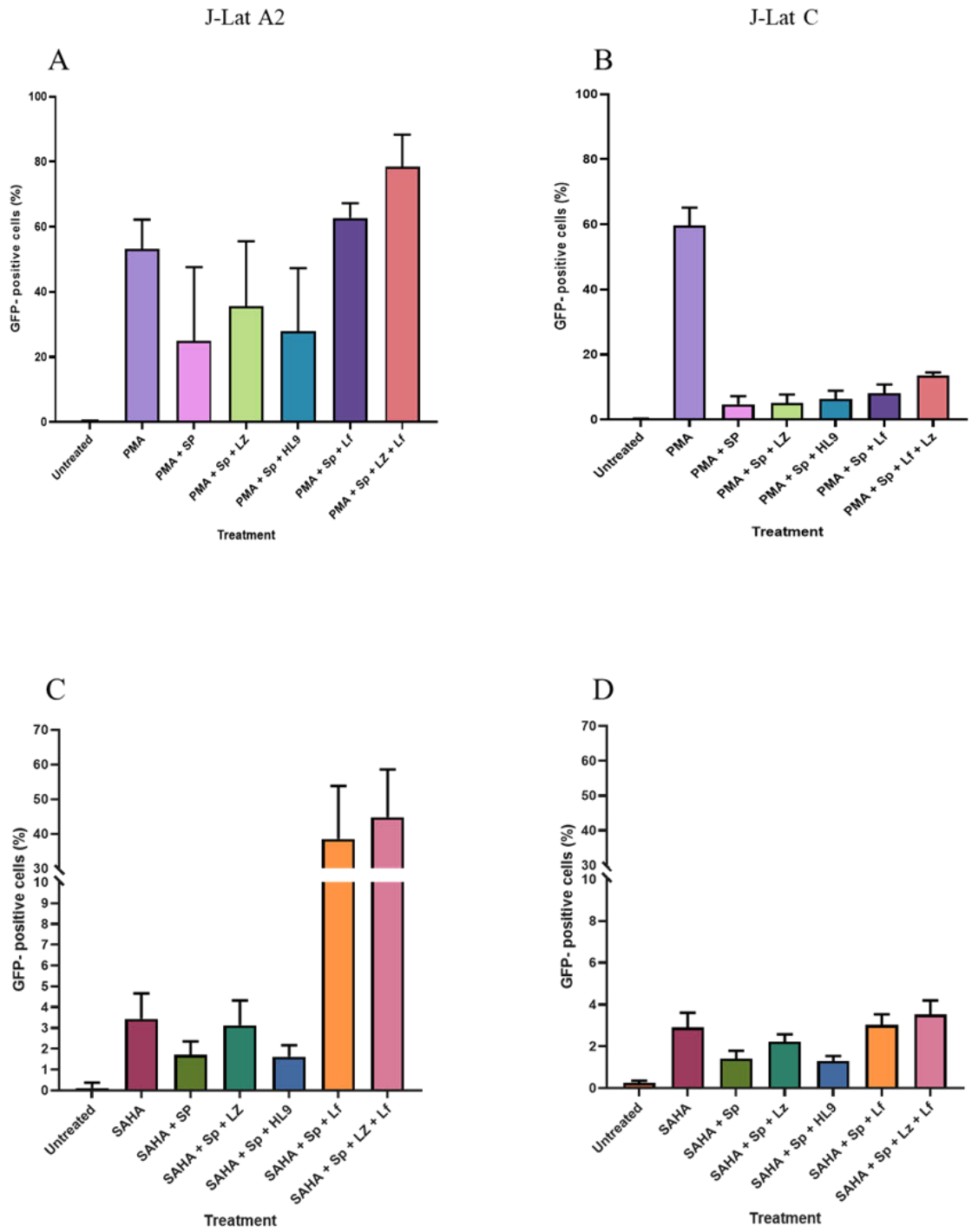


Figure A 6: The frequency of GFP-expression from J-Lat cells that were preincubated with Sp (15 µM) prior to being stimulated with a co-treatment of cationic proteins (Lz, HL9, Lf [10 µM]) and LRAs (PMA [16 nM], SAHA [900 nM]) for a further 24 hours.

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

16 March 2024

Mr Senzo Cebekhulu (214548216)
School of Laboratory Medicine & Medical Science
Medical School

Dear Mr Cebekhulu,

Protocol reference number: BREC/00006587/2024
Project title: The effect of cationic DNA-binding proteins on HIV-1 latency
Degree: Masters

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 16 March 2024. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from 16 March 2024. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on RIG on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 09 April 2024.

Yours sincerely,

[redacted]

Prof S Singh
Chair: Biomedical Research Ethics Committee