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Cell-to-cell transmission and intrinsic mechanisms that influence human immunodeficiency virus infection

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CELL-TO-CELL TRANSMISSION AND INTRINSIC MECHANISMS THAT INFLUENCE HUMAN IMMUNODEFICIENCY VIRUS INFECTION

by

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DEDICATION

I would like to dedicate this work to my parents Joe and Connie, my sisters Nicole and Shara, my grandmother Nancy, and my family and friends who have supported me.

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ABSTRACT

Early in the course of human immunodeficiency virus (HIV) infection a population of latently infected cells is established which persists despite long-term anti-retroviral treatment. This latent reservoir of HIV-infected cells, which reflects mechanisms of transcriptional repression, is the major barrier to cure. Efforts to target the latent reservoir have been inefficient, indicating a need for a more complete understanding of how HIV transcription is regulated.

The molecular networks involved in the regulation of HIV transcription remain incompletely defined. I hypothesized that utilization of a high throughput enhanced yeast one-hybrid assay would reveal novel host transcription factor-long terminal repeat (LTR) interactions and transcriptional networks that regulate HIV. The screen identified 42 human transcription factors and 85 total protein-DNA interactions with HIV LTRs. I investigated a subset of these factors for transcriptional activity in cell-based models of infection. Krüppel-like factors 2 and 3 (KLF2 and KLF3) are repressors of HIV-1 and HIV-2 transcription whereas PLAG1-like zinc finger 1 (PLAGL1) is an activator of HIV-2 transcription. These factors regulate HIV expression through direct protein-DNA interactions and correlate with epigenetic modifications of the HIV LTR.

Multiple signals converging from the cellular environment and cell-cell interactions converge at the HIV LTR to determine HIV replication and transcription. Previous work in our lab has shown that strong signaling through the T cell receptor (TCR) was required to support HIV expression and the establishment of an inducible latent infection, whereas weak TCR signaling was insufficient for these outcomes. I hypothesized that dendritic cells-CD4+ T cell interactions provide signals that compensate for weak TCR signaling, supporting HIV-1 expression and generation of inducible latent infection. I used CD4+ T cells that express chimeric antigen receptors in a dendritic cell coculture model to deliver differential signals to CD4+ T cells during cell-to-cell transmission of HIV. I found that signals from dendritic cells compensate for weak TCR signaling, facilitating cell activation, HIV expression and establishment of an inducible infection.

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LIST OF ABBREVIATIONS

7SKsnRNP ..... Human 7SK small nuclear RNA

AIDS ..... Acquired Immunodeficiency Syndrome

Akt ..... Protein kinase B

ANOVA ..... Analysis of variance

AP-1 ..... Activator protein 1

APC ..... Antigen-presenting cell

ART ..... Antiretroviral therapy

ATCC ..... American Type Culture Collection

AZT ..... Azidothymidine

BET ..... Bromodomain and extra-terminal motif

Blimp-1 ..... B lymphocyte-induced maturation protein 1

bnAbs ..... Broadly neutralizing antibody

bp ..... Base pair

BRD4 ..... Bromodomain-containing protein 4

BU ..... Boston University

BV421 ..... Brilliant violet 421

C2H2 ..... Cys2His2-like fold group

CAR ..... Chimeric antigen receptor

cART ..... Combination antiretroviral therapy

CBP ..... CREB-binding protein

CCR5 ..... C-C chemokine receptor type 5

CD .....Cluster of differentiation

CDC .....Centers for disease control

CDK9 .....Cyclin dependent protein kinase 9

cDNA ..... Complementary DNA

ChIP .....Chromatin immuno-precipitation

CO2 .....Carbon dioxide

CoREST ..... REST corepressor 1

CtBP .....C-terminal binding protein

CXCR4..... C-X-C chemokine receptor type 4

CycT1 ..... Cyclin T1

DC .....Dendritic cell

dCA ..... Didehydro-cortistatin A

DMEM .....Dulbecco's modified eagle medium

DNA ..... deoxyribonucleic acid

DSIF .....DRB sensitivity inducing factor

E2F1 ..... E2F transcription factor 1

ECL ..... Enhanced chemiluminescence

EDTA ..... Ethylenediaminetetraacetic acid

Efv .....Efavirenz

EGTA ..... Ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid

Env .....HIV envelope

ESCRT ..... Endosomal sorting complexes required for transport

eY1H ..... Enhanced yeast-one hybrid assay

FBS ..... Fetal bovine serum

FDA ..... Food & Drug Administration

FDR ..... False discovery rate

GABPA ..... GA binding protein

Gag ..... HIV gag

GAPDH ..... Glyceraldehyde 3-phosphate dehydrogenase

GFP ..... Green fluorescent protein

GMCSF ..... Granulocyte-macrophage colony-stimulating factor

gp120 ..... HIV envelope glycoprotein 120

gp41 ..... HIV envelope glycoprotein 41

HAT ..... Histone acetyltransferase

HCl ..... Hydrochloric acid

HDAC ..... Histone deacetylase

HDACi ..... Histone deacetylase Inhibitor

HEK293T ..... Human embryonic kidney cells

Her2 ..... Human epidermal growth factor receptor 2

HES5 ..... Hes family BHLH transcription factor 5

HEY1 ..... Hes related family BHLH rranscription factor with YRPW motif 1

HEY2 ..... Hes related family BHLH transcription factor with YRPW motif 2

HHEX ..... Hematopoietically expressed homeobox

HIV ..... human immunodeficiency Virus

HIV-1 .....Human Immunodeficiency Virus 1

HIV-2 .....Human Immunodeficiency Virus 2

HLA-DR ..... Human leukocyte antigen – DR isotype

HLSD .....histone lysine-specific demethylase

HMT.....Histone methyltransferase

HRP.....Horseradish peroxidase

hTERT.....Human telomerase reverse transcriptase

HuRI.....Human reference interactome

ICAM-1 ..... Intracellular adhesion molecule 1

IgG .....Immunoglobulin G

IL-2 .....Interleukin 2

IL-4 .....Interleukin 4

IL-7 .....Interleukin 7

INSTI ..... Integrase strand transfer inhibitor

IRF2 .....Interferon regulatory transcription factor 2

IS ..... Immunological synapse

ITAM ..... Immunoreceptor tyrosine-based activation motif

IU ..... Infectious unit

Jak/STAT .....Janus kinase-signal transducer and activator of transcription

kb..... Kilobase

KCl.....Potassium chloride

Kd..... Dissociation constant

KLF ..... Kru_ppel like factor

LacZ ..... _-galactosidase

LAT ..... Linker of activated T Cells

Lck ..... Lymphocyte-specific protein tyrosine kinase

LEDGF/p75 ..... Lens epithelium–derived growth factor p75 splice variant

LFA-1 ..... Lymphocyte function-associated antigen 1

LiCl ..... Lithium chloride

LPS ..... Lipopolysaccharide

LRA ..... Latency reversing agent

LTR ..... Long terminal repeat

Luc ..... Luciferase

MAPK ..... Mitogen-activated protein kinase

MDM ..... Monocyte-derived macrophage

MgCl2 ..... Magnesium chloride

MHC ..... Major histocompatibility complex

moDC ..... Monocyte-derived dendritic cell

MOI ..... Multiplicity of infection

mRNA ..... Messenger RNA

MSM ..... Men who have sex with men

NaCl ..... Sodium chloride

Nef ..... HIV negative regulatory factor

NELF ..... Negative elongation factor

NF-κB .....Nuclear factor kappa-light-chain-enhancer of activated B cells

NFAT ..... Nuclear actor of activated T cells

NIH .....National Institutes of Health

NNRTI .....Non-nucleoside reverse transcriptase inhibitor

NRTI .....Nucleoside reverse transcriptase inhibitor

NTP .....Nucleoside triphosphate

Opti-MEM.....Improved minimal essential medium

P-TEFb ..... Positive transcription elongation factor

p300..... Histone acetyltransferase 300

p50.....NF-_B subunit 50

p53..... Tumor protein p53

p65.....NF-_B subunit 65

PANTHER .....Protein analysis through evolutionary relationships

PBMCs .....Peripheral blood mononuclear cells

PBS .....Phosphate-buffered saline

PCAF .....P300/CBP-associated factor

Pcf11 ..... Pre-mRNA cleavage complex II protein Pcf11

PCR ..... Polymerase chain reaction

PEI..... Polyethylenimine

PEP..... Post exposure prophylaxis

pH.....Potential for hydrogen

PHA ..... Phytohaemagglutinin

PI ..... Protease inhibitor

PI3K ..... Phosphoinositide 3-kinase

PIC ..... Pre integration complex

PKC ..... Protein kinase C

PLAGL1 ..... PLAG1 like zinc finger 1

PLCγ1 ..... Phosphoinositide phospholipase C gamma 1

PLHIV ..... People living with hiv

PMA ..... Phorbol myristate acetate

pMHC ..... Peptide MHC complex

Pol ..... HIV DNA polymerase

PrEP ..... Pre-exposure prophylaxis

PtdIns(4,5)P2 ..... Phosphatidylinositol 4,5-Bisphosphate

RT-qPCR ..... Quantitative reverse transcriptase PCR

RNA ..... Ribonucleic acid

RNAPII ..... RNA polymerase II

RPL13A ..... Ribosomal protein L13a

RPMI ..... Roswell Park Memorial Institute Medium

SAHA ..... Suberanilohydroxamic acid (Vorinostat)

SAMHD1 ..... Sterile alpha motif and HD-domain containing protein 1

Saq ..... Saquinavir

SBHA ..... Suberoyl bis-hydroxamic acid

scFv ..... Single-chain variable fragment

SDS-PAGE ..... SDS polyacrylamide gel electrophoresis

Sh2 ..... Src homology 2

SIGLEC-1 ..... Sialic acid-binding immunoglobulin-type lectin 1

Sin3a ..... SIN3 transcription regulator family member A

siRNA ..... Small interfering RNA

SIV ..... Simian immunodeficiency virus

SNP ..... Single nucleotide polymorphism

SOX14 ..... SRY-box transcription factor 14

Sp ..... Specificity protein

Src ..... Proto-oncogene tyrosine-protein kinase Src

SWI/SNF ..... Switch / Sucrose Non-Fermentable

TAR ..... HIV trans-activation response element

Tat ..... HIV transactivator of transcription

TCR ..... T cell receptor

TE ..... Tris EDTA buffer

Tfh ..... T follicular helper cell

TGF-β ..... Transforming growth factor beta 1

TGIF2LX ..... TGFB induced factor homeobox 2 like X-linked

THR-β ..... Thyroid hormone receptor beta

TLR ..... Toll like receptor

TNFα ..... Tumor necrosis factor alpha

U=U ..... Undetectable equals untransmittable

UNAIDS ..... The Joint United Nations Programme on HIV/AIDS

URA3 ..... Orotidine-5-phosphate decarboxylase

USHHS ..... U.S. Department of Health and Human Services

Vif ..... HIV viral infectivity protein

VOA ..... Viral outgrowth assay

Vpr ..... HIV viral protein R

Vpu ..... HIV viral protein U

Vpx ..... HIV viral Protein X

VSV-G ..... Vesicular stomatitis virus envelope glycoprotein G

Wnt ..... Wnt family member

ZAP-70 ..... Zeta-chain-associated protein kinase 70

ZDHHC7 ..... Zinc finger DHHC-type palmitoyltransferase 7

ZIC1 ..... Zic family member 1

ZNF524 ..... Zinc finger protein 524

Portions of this dissertation were included in or adapted from:
Mechanisms of HIV-1 cell-to-cell transmission and the establishment of the T latent reservoir. Pedro KD, Henderson AJ, Agosto LM. *Virus Research* (2019) v256 p115-121

An unbiased functional screen identifies novel transcription factors and corresponding networks that regulate HIV-1 and HIV-2. Pedro KD, Agosto LM, Sewell JA, Eberenz KA, He X, Fuxman Bass JI, Henderson AJ (Submitted for review)

CHAPTER ONE: INTRODUCTION

Introduction

HIV infection results in the targeting and destruction of CD4+ T cells, eventually leading to immunodeficiency and death if left untreated. HIV/AIDS has claimed nearly 40 million lives and another 38 million people are living with HIV today. While effective treatments to manage infection have been developed, both cure and a vaccine have remained elusive. An end to the pandemic will require additional understanding of how HIV replication is regulated and how infection is disseminated.

History of the HIV/AIDS pandemic

Human immunodeficiency virus (HIV) arose from a number of zoonotic transmissions of simian immunodeficiency virus (SIV) from infected primates to humans and consists of two major evolutionary lineages: HIV type 1 and type 2. The origin of HIV-2 was described first, evolving from SIVsmm (sooty mangabey) in West Africa (Hirsch et al. 1989). Later, HIV-1 groups M and N were determined to have evolved from SIVcpz (chimpanzee), and groups O and P deriving from SIVgor (gorilla) in central Africa (Sharp and Hahn 2010). Exactly when and where these animal-to-human transmissions occurred

is unknowable, but likely occurred through bushmeat economy as SIV is prevalent in African primates and detection of SIV in bushmeat vendors has been reported (Sharp and Hahn 2011; Peeters et al. 2002).

From Africa, the virus spread across the globe unnoticed until 1981 when reports from Los Angeles, San Francisco, New York and elsewhere noted that primarily men who have sex with men (MSM) were dying due to *Pneumocystis* infections and Kaposi's sarcoma, previously rare disorders associated with immune dysfunction (Gottlieb et al. 1996; Friedman-Kien et al. 1981). As reported cases began to accumulate it was apparent that a novel infectious disease had emerged, which came to be known as acquired immunodeficiency syndrome (AIDS). At first, AIDS appeared to primarily affect MSM and people who inject drugs and was considered by many to be an affliction of sin, a misunderstanding of the disease that persists as stigma to this day. However, as the number of cases grew and were reported across the world, including among children and prominent celebrities, it became clear that AIDS was a concern for everyone. In 1983, the causative agent was discovered to be HIV, a novel retrovirus. The pandemic that followed, which has claimed 38 million lives, and sickened just as many, spurred a prolonged period of intense scientific and public health mobilization which was unmatched by anything that came before (Gallo et al. 1983; Barré-Sinoussi et al. 1983; Levy et al. 1984; Deeks et al. 2015). Despite four decades of intensive research and innovation, significant challenges remain.

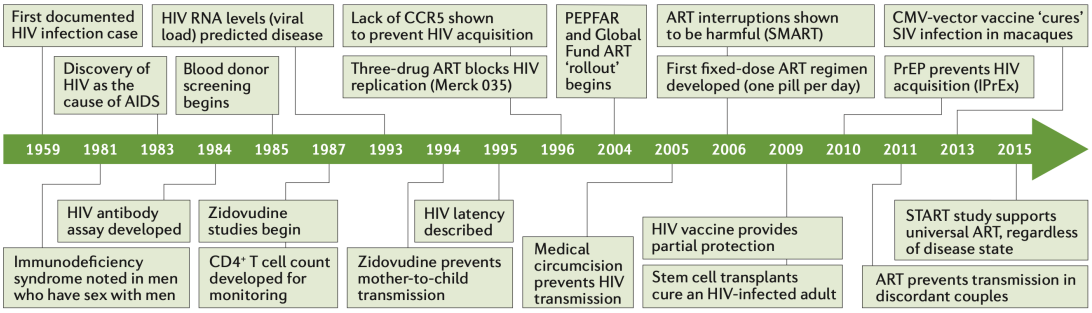

Figure 1.1: Key milestones in the HIV pandemic (Deeks et al. 2015).

The primary modes of HIV transmission are via sexual fluids, blood, and mother-to-child transmission (Patel et al. 2014). Untreated HIV infection proceeds through three general phases (NIH 2019). The acute phase is the earliest, occurring 2-4 weeks following infection and is characterized by high levels of virus in the blood as it replicates and disseminates throughout the body. Often, flu-like symptoms accompany the acute phase which can last a few weeks to months. Next follows the chronic phase of infection when symptoms of infection are mild or absent. Over the course of many years of infection, CD4+ T cell counts in circulation continue to drop as virus levels in the blood increase. In the final stage, AIDS, CD4+ T cells, a critical mediator of immune protection, decline below 200 cell/mm^3 and patients become susceptible to opportunistic infections associated with immunodeficiency as they lack the ability to fight them off.

By the end of 1984, 7,239 cases of AIDS and 5,596 AIDS-associated deaths had been reported in the United States alone (AmfAR 2011). Throughout most of the 1980s average survival at the time of AIDS diagnosis was 1-2 years. A major breakthrough came in 1987 with the clinical use of azidothymidine (AZT), the first FDA drug approved for the treatment of HIV/AIDS (Figure 1.1) (PHS 1987). This marked the beginning of a flurry of

significant advances in detection, monitoring and treatment, most notably the development of combination antiretroviral therapy (cART) in 1997 (Oy et al. 1997; Hammer et al. 1997). By utilizing a combination of multiple classes of antiretrovirals (ART) targeting different stages of the HIV replication cycle (Table 1), infected patients can be maintained in a chronic phase of infection with viral loads below the limit of detection while on treatment. This led to a shift in the field of HIV research, with some declaring the era of AIDS to be over, at least for those with access to treatment (Deeks, Lewin, and Havlir 2013). Indeed, AIDS-related deaths globally decreased from around 1.8 million per year at the peak in 2004 to roughly 770,000 in 2019, with the burden of mortality heavily concentrated in Africa and South Asia (UNAIDS 2019; CDC 2019). Patients on cART with sustained viral repression now have a life expectancy near that of an uninfected person, a significant achievement considering the prognosis only 20-30 years ago. However, strict adherence to a cART regimen is required to sustain repression of HIV. Early in the course of infection, HIV establishes a reservoir of infected cells that persist despite treatment and which contribute to a rebound in viral loads within a matter of weeks if treatment is interrupted (Figure 1.2) (Van Lint, Bouchat, and Marcello 2013). This poses a major challenge in treatment and eradication as the populations with the greatest burden of HIV infection are those with limited resources and least connected to a quality healthcare system (UNAIDS 2014).

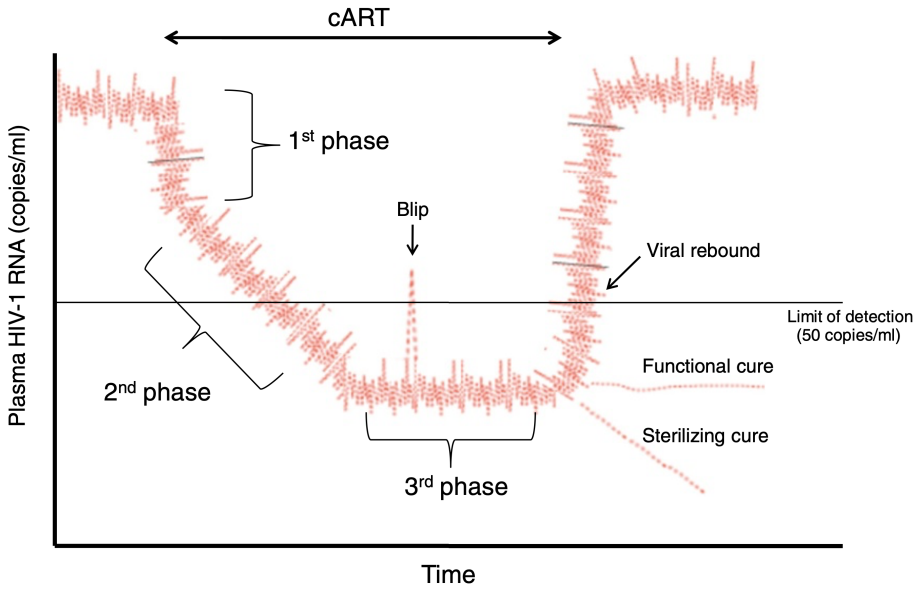

Figure 1.2: Dynamics of HIV infection under cART treatment and interruption (Van Lint, Bouchat, and Marcello 2013).

Recent advances and major challenges towards cure and eradication of HIV

Despite significant progress in management of infection, the ultimate goal of HIV research has been and continues to be cure. A broadly applicable cure strategy has remained elusive, with the primary barrier being the early establishment and long-term persistence of the viral reservoir. However, there have been some encouraging developments demonstrating that sterilizing cure is possible. In 2008, 25 years after the first report of HIV, a case of a potential cure was announced, known initially as the "Berlin Patient" and later revealed to be Timothy Ray Brown (Church 2009; Brown 2015). Brown was diagnosed with HIV in 1995 and was receiving cART therapy when in 2006 he was diagnosed with acute myeloid leukemia. After failing successive rounds of chemotherapy, he received two hematopoietic stem cell transplants from a donor who had a 32bp mutation in the CCR5 gene (CCR5Δ32), a mutation which had been shown to render cells resistant

to HIV infection and is commonly present in Northern European populations (Galvani and Novembre 2005; Y. Huang et al. 1996; Hoffman et al. 1997). Over the course of 20 months following the second transplant, a period during which Brown remained off cART, investigators were unable to retrieve viral DNA or RNA from tissue samples. To the present day, Brown remains off cART and has not experienced a viral rebound, leading many to declare Brown as the first instance of HIV cure. Variations on this procedure were attempted several times over the next decade without success (Saez-Cirion et al. 2013) until 2019 when the case of the "London Patient" was reported, whose viral loads remain undetectable by ultrasensitive assays more than two years after halting cART (Gupta et al. 2019; 2020). While these cases are important proofs-of-concept, this approach to cure is not foolproof and is not scalable to meet the need of the vast majority of people living with HIV (PLHIV). The London Patient is part of a 39 patient cohort who has received this therapy and at this time is the only patient who is thought to be cured (another patient is in sustained ART-free remission, but investigators continue to find HIV DNA) (Saez-Cirion et al. 2013). Furthermore, bone marrow stem cell transplants are only available to patients with hematological malignancies, carry significant risk, and are prohibitively expensive. Additionally, the CCR5Δ32 mutation is only prevalent in a subset of Europeans meaning that ethnic Africans and Asians, who bear the vast majority of the HIV burden, are unlikely to be HLA matched to donors and therefore are not eligible recipients (Galvani and Novembre 2005). A less invasive and more broadly applicable approach to cure is needed for the majority of PLHIV.

Complementing treatment and cure efforts, preventing the ongoing spread of new infections is critical to ending the pandemic. In the last decade, there have been two notable advancements in preventing new infections by repurposing existing HIV therapeutics. Pre-exposure prophylaxis (PrEP) is a dual ART regimen that HIV negative people can take to prevent HIV infection, a particularly important development for those in discordant couples or high-risk communities (Chou et al. 2019). In addition, the discovery of post-exposure prophylaxis (PEP), a multi ART regimen that is taken within 72 hours of a potential exposure, can effectively prevent establishment of HIV infection (Siedner, Tumarkin, and Bogoch 2018). Together these approaches, along with traditional non-biologic interventions, represent a path towards ending transmission of HIV infection.

Despite progress in treatment, surveillance, and prevention, significant challenges remain. PLHIV face significant quality of life issues related to societal stigma deriving from their status; despite campaigns such as U=U (Undetectable = Untransmittable), a public education campaign premised on the finding that infection is not transmissible if the infected person is virally suppressed below the limit of detection (Eisinger, Dieffenbach, and Fauci 2019). In addition, PLHIV face a long list of comorbidities associated with chronic HIV infection that include cardiovascular disease, neuropathy, anemia, osteoporosis, liver disease, and kidney disease (Deeks, Lewin, and Havlir 2013). Furthermore, even with current prophylactic and preventative treatment options, there were an estimated 1.7 million new HIV infections in 2018, with stigmatized or disadvantaged populations bearing the majority of the new infection burden, particularly in Sub-Saharan Africa (UNAIDS 2019; CDC 2019). The continuing spread of HIV is a multifactorial issue,

with challenges spanning sociology and medicine, including accessibility to treatment and connection to quality healthcare.

Indeed, the issues of accessible treatment, cure, and prevention of transmission are the focus of modern HIV research. An HIV vaccine is being pursued to end the spread of new infections, as are cures to eradicate the source of virus and to improve the lives of people currently living with HIV. To address these challenges, we need a better understanding of the basic virology, immunology, and regulation of the virus in different cellular targets. Exploring such mechanisms was a major goal of this thesis work.

Establishment of clinical infection, and replication of HIV

Transmission of HIV and the establishment of clinical infection

HIV virions are shed by infected people into blood, breast milk, and sexual fluids (Shaw and Hunter 2012). Sexual transmission is the major mode of transmission, though sharing of contaminated needles among people who inject drugs and mother-to-child transmission represent significant modes of transmission. There were also early reports of HIV transmission as a result of blood transfusions which led to the standardization of testing of the blood supply, making this path of infection uncommon today. All modes of transmission require HIV-contaminated fluids to contact mucous membranes, damaged tissue, or direct injection into the bloodstream.

In the case of sexual transmission, HIV is transmitted to an uninfected person via the sexual fluids of a non-virally suppressed HIV-positive individual. At the mucous membrane, for instance, the vaginal or rectal mucosa, HIV establishes infection of

epithelial CD4+ T cells via varied mechanisms, including capture and transfer of infectious virus by antigen-presenting cells such as dendritic cells (DCs) or Langerhans cells, or by transcytosis of the virus across the epithelium (L. Wu 2008). Alternatively, CD1a+ vaginal dendritic cells have been hypothesized to be the initial cell type infected by CCR5-tropic HIV that seeds subsequent spread to bystander CD4+ T cells (Peña-Cruz et al. 2018). Following transmission, HIV is disseminated throughout the body by infected cells or via virus-associated migratory cells (such as dendritic cells) to tissues with high numbers of susceptible CD4+ T cells, including lymph nodes and gut-associated lymphoid tissue (Veazey et al. 1998; Wong and Yukl 2016; Pope and Haase 2003).

Entry of HIV into the cell, establishment of provirus, and replication

HIV has a single-stranded, positive-sense, 10kb RNA genome. It contains nine genes that encode 15 primary protein products that can be broadly grouped into structural, regulatory, and accessory proteins. In a mature virion, a spherical structure measuring ~100nm in diameter, two RNA genomes are packaged within a conical capsid core which is itself enveloped by a lipid bilayer (Figure 1.3A). This lipid bilayer is studded with virally encoded Envelope (Env) glycoproteins in addition to a variety of host cell proteins as it's derived from the membrane of host cells.

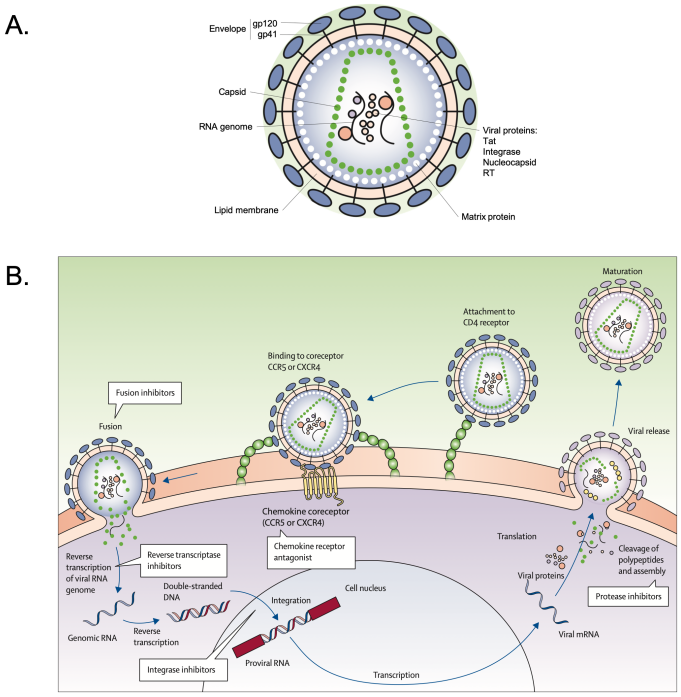

Figure 1.3: HIV virion and the HIV replication cycleA) Mature HIV virion with key components identified. B) The basic replication cycle of HIV with common antiretroviral targets noted in bubbles. Adapted from (Maartens, Celum, and Lewin 2014).

Following adsorption of the HIV virion by the surface of the host cell, the gp120 trimer peptide of Env binds its primary receptor CD4 on the surface cell (Figure 1.3B). This results in a conformational change of Env structure which facilitates binding of gp120 to the co-receptors CCR5 or CXCR4. This, in turn, exposes the gp41 trimer which inserts into the target cell membrane, resulting in its fusion of the membrane and the viral envelope and entry of the virion's contents into the host cell. HIV preferentially infects CD4+ T cells but can infect other cells including macrophages and dendritic cells (DCs).

After entering the cell, the capsid core is disassembled while reverse transcription of the single-stranded RNA genome takes place and is translocated to the cell nucleus via cytoskeleton (Fernandez et al. 2015; Jayappa, Ao, and Yao 2012; McDonald et al. 2002).

At the nuclear envelope, this pre-integration complex (PIC) of viral DNA and protein interacts with nuclear pore complexes and is extruded into the nucleus (Francis and Melikyan 2018; Jayappa, Ao, and Yao 2012). Through interaction with LEDGF/p75 and other host proteins the PIC is tethered to chromatin and integrated into the host genome (Pandey, Sinha, and Grandgenett 2007; Desimmie et al. 2015; Engelman and Cherepanov 2008). HIV is preferentially integrated into host DNA that is actively transcribed, near the nuclear pore, which has an open chromatin structure (Han et al. 2004a; Marini et al. 2015; Achuthan et al. 2018; Koh et al. 2013).

HIV transcriptional regulation

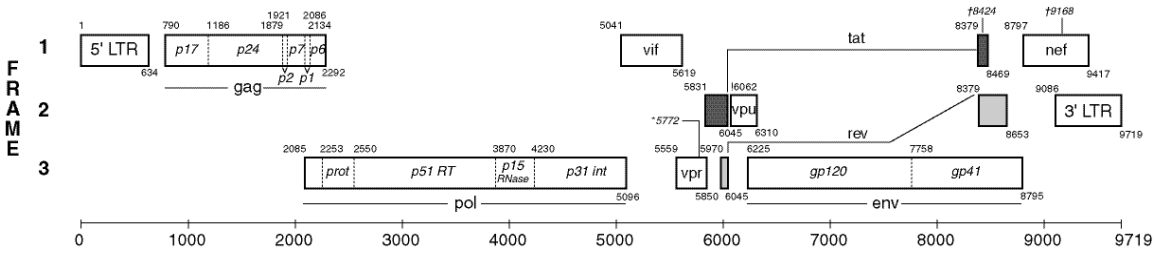

Figure 1.4: The HIV genome, with open reading frames and protein products noted. From Los Alamos National Labs.
https://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html

Once integrated the HIV DNA is referred to as provirus, a 10kb genome containing nine genes and flanked on either end by identical 5' and 3' long terminal repeats (LTRs) (Figure 1.4). The 5' LTR functions as the promoter and enhancer for the provirus and is divided into three regions, U3, R, and U5. Beginning at the 5' end of the LTR and terminating downstream at the transcription start site is the U3 region, which contains modulatory, enhancer, and core promoter subregions (Figure 1.5A). The transcriptional

start site marks the beginning of the R region and is followed by U5. The 5' LTR contains two nucleosomes, Nuc-0 positioned in the U3 region of the LTR and Nuc-1 straddling the R-U5 junction just downstream of the transcriptional start site (Verdin, Paras Jr., and Van Lint 1993; Van Lint 2000). The stretch between Nuc-0 and Nuc-1 is chromatin-free and contains the majority of the characterized transcription factor binding sites important for HIV expression, including NF-kB and Sp1 sites. RNA Polymerase II (RNAPII) which transcribes DNA into messenger RNA is recruited to the 5' LTR by host transcription factors (Figure 1.5B) (Daelemans, De Clercq, and Vandamme 2000; Cullen 1991; Pereira et al. 2000).

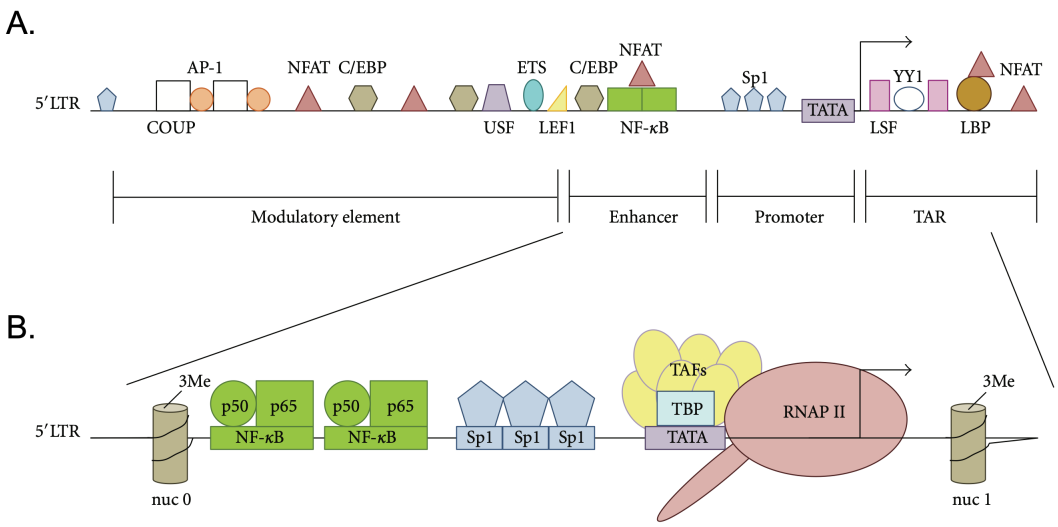

Figure 1.5: Regulation of HIV transcription and elongation. A) Organization of the HIV LTR with a subset of HIV transcription factors and their relative binding sites shown. B) Assembly of the host transcriptional machinery on a nucleosome-free region of the LTR. Figure adapted from (Schiralli Lester and Henderson 2012).

Although initial proviral expression is weak, the HIV protein *trans*-activator of transcription (Tat) is produced early in infection which results in a dramatic increase in

transcriptional efficiency (Karn and Stoltzfus 2012). Tat binds the regulatory stem-loop TAR (*trans*-activation response) element of nascent HIV RNA and recruits the positive transcription elongation factor (P-TEFb) complex, via the CycT1 subunit, to the LTR. The CDK9 subunit of P-TEFb phosphorylates the C-terminal domain of RNAPII which in turn allows for recruitment of histone modifying enzymes, in particular SWI/SNF and proteins with HAT activity (Figure 1.6A). At the same time, P-TEFb phosphorylates negative elongation factor (NELF) and DSIF enabling efficient processivity and elongation of transcription (Fujinaga et al. 2004; Kaczmarek Michaels, Wolschendorf, et al. 2015; Ivanov et al. 2000; Natarajan et al. 2013). Together, the recruitment of positive factors, inactivation of negative factors, and modification of local chromatin to an "open" structure results in increased RNAPII processivity and proviral RNA expression.

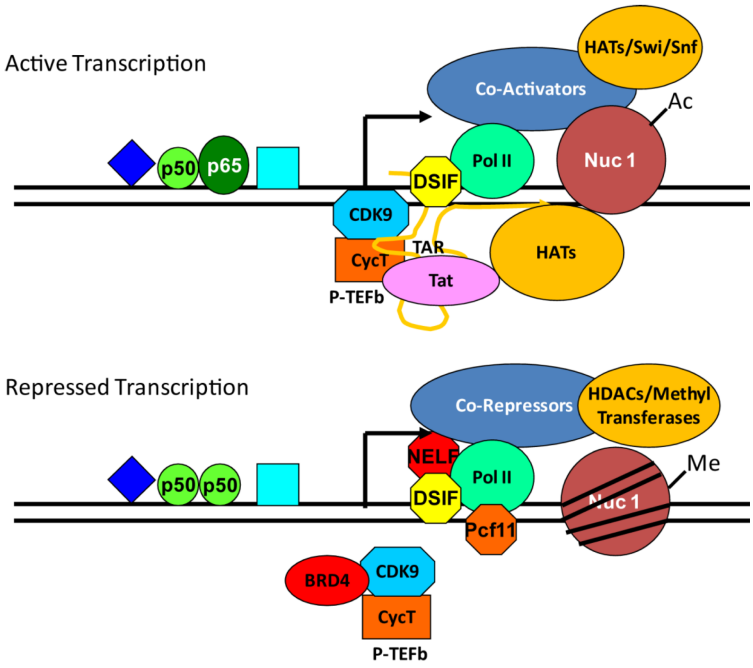

Figure 1.6: Key mechanisms in active and repressed HIV transcription. Adapted from (Agosto, Gagne, and Henderson 2015).

The presence of positive transcriptional regulatory complexes at the HIV LTR is correlated with T cell activation. For instance, in resting CD4+ T cells expression of the CycT1 subunit of P-TEFb is low, and much of the P-TEFb that is present is sequestered by 7SK small nuclear ribonucleoprotein (7SKsnRNP) which structurally resembles TAR. However, activating signal from the T cell receptor not only increases total levels of P-TEFb but it also releases it from sequestration, resulting in a rapid increase in P-TEFb available to the HIV LTR (Heusinger and Kirchhoff 2017; Cary, Fujinaga, and Peterlin 2016; Zhiyuan Yang et al. 2001; Z Yang et al. 2005). Overall, the increase and availability of key transcriptional modulators, as well as their activation creates an environment supportive of robust HIV expression (Nabel and Baltimore 1987; Perkins et al. 1993; Mbonye and Karn 2011; Schiralli Lester and Henderson 2012). If mature HIV virions are being produced, either by basal or a more robust expression, this is referred to as a productive infection.

Similarly, the absence of Tat or lack of activating signals from the T cell receptor (TCR) results in repression of HIV expression. Lacking Tat, RNAPII binds the LTR and initiates RNA transcription for approximately 45-50 bases before DSIF and NELF pause transcription allowing for the premature termination of transcription by Pcf11 (Z Zhang et al. 2007; Natarajan et al. 2013; Klatt et al. 2008; Z Zhang et al. 2007). Without sufficient activating signal from the T cell receptor, PTEF-b remains sequestered in the 7SKsnRNP complex, and levels of active transcription factors such as NF-κB and NFAT remain low. Additionally, repressive factors such as NF-κB p50 homodimers and NELF bind the LTR to recruit histone deacetylases (HDACs) that modify chromatin to a more “closed”

conformation, further preventing efficient transcription (Figure 1.6B). HIV-infected cells that harbor replication-competent virus but are not transcriptionally active are said to be latently infected. However, latent infection is not a permanent state. Proviral replication can be induced by various cellular signals such as T cell receptor signaling, or by so-called latency reversing agents (LRAs).

To better understand how latency is established and maintained, we will need a more comprehensive understanding of HIV transcription and the interplay of the multiple mechanisms of transcriptional control. This is the primary focus of this thesis and will be discussed in greater detail in the following chapters.

HIV protein expression and virion production

HIV utilizes a complex system of RNA splicing to generate nine proteins from its polycistronic genome (Figure 1.7) (Purcell and Martin 1993; Kuzembayeva et al. 2014). The 2kb multiply spliced RNAs encode Tat, Rev, and Nef. Tat and Rev (regulator of expression of virion proteins) are regulatory factors required for efficient HIV expression and replication. Nef is dispensable for infection, however it modifies cell biology to promote cell survival and replicative success (Mesner et al. 2020). The singly spliced 4.5kb RNAs are expressed later in infection and encode the Env, Vif, Vpr, Vpu (HIV-1) or Vpx (SIV, HIV-2). Vif, Vpr, Vpu, and Vpx are accessory proteins that aid virus replication by targeting HIV restriction factors (Le Rouzic and Benichou 2005; Frankel and Young 1998; Jacotot et al. 2000; Campbell and Hirsch 1997). These, as well as Env, are packaged into immature virions prior to budding. Finally, the unspliced 9.2kb RNAs encode the Gag and

Pol polyprotein precursors, as well as provide the genomic RNA packaged into budding virions. Gag and Pol are expressed later in infection and fulfill critical structural and enzymatic functions.

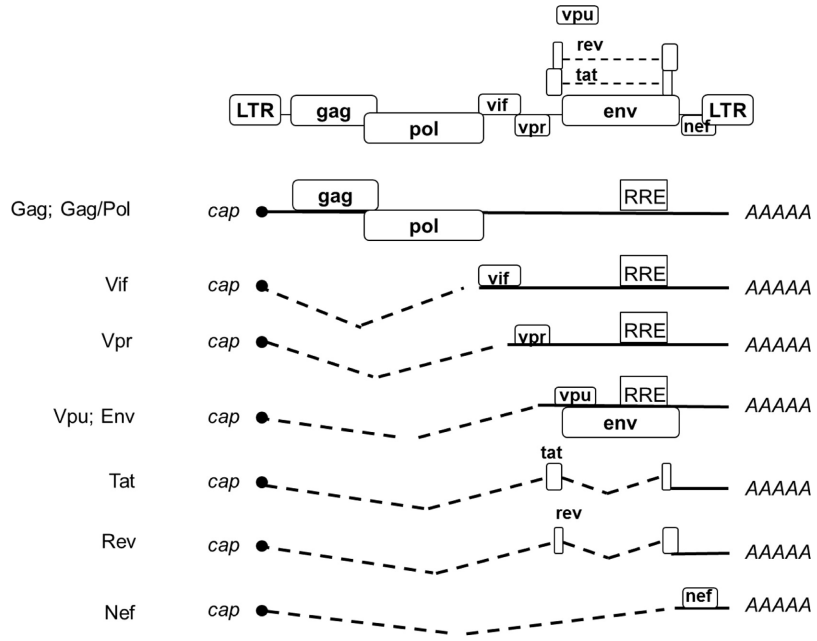

Figure 1.7: Schematic representation of the HIV genome and its spliced RNA products. (Kuzembayeva et al. 2014)

These different species of mRNA are translated into protein by host machinery. Env accumulates at the cell surface in lipid rafts and interacts with the MA domain of Gag for specific incorporation into assembling virions (M. Huang et al. 1995; Frankel and Young 1998; Freed et al. 1994; Tedbury and Freed 2014). The accessory protein Vpr as well as *de novo* RNA genome are packaged into budding immature virions (Sundquist and Kräusslich 2012). Gag expression alone is sufficient for formation of virus particles (Freed and Martin 1996). After the immature virions bud from the cell the PR peptide of Pol cleaves Gag into matrix, capsid, nucleocapsid, p1 and p2 subunits which assemble the

contents of the virion into the distinctive conical core containing the viral RNA (Wiegers et al. 1998; Freed 2015), resulting in a mature infectious virion which can then go on to infect new cells or shed into fluids that may transmit to a new host organism.

ART targets multiple stages of the HIV replication cycle

There exists a class of FDA-approved therapeutics used to interfere with nearly every stage of HIV replication (Table 1.1) (USHHS 2020). Fusion inhibitors, CCR5 antagonists, and post-attachment inhibitors prevent entry of HIV by interfering with HIV Env interactions with cellular receptors for fusion and entry (Qian, Morris-Natschke, and Lee 2009). Nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) block the reverse transcription of genomic RNA into cDNA (Hu and Hughes 2012). Integrase strand transfer inhibitors (INSTIs) bind HIV integrase to prevent integration of reverse-transcribed viral DNA into the host cell genome (Smith et al. 2018). Protease inhibitors (PIs) interfere with the proteolytic activity of the HIV protease activity critical for maturation of virions rendering them non-infectious (Lv, Chu, and Wang 2015). These drugs are highly effective at controlling HIV-1 infection when used in combination (Arts and Hazuda 2012; USHHS 2020). Notably, we lack an effective therapeutic that targets RNA expression or the provirus itself. Therefore, a transcriptionally repressed provirus will evade the antiviral effects of cART and will persist for the lifespan of the infected cell (R. F. Siliciano and Greene 2011; Margolis et al. 2020). HIV's capacity to persist in the face of cART, and our inability to effectively reverse latent

infection, is the major barrier to cure and underscores the need to understand how this state is established and maintained.

Drug Class (acronym) *Function*	Generic Name (acronym)	FDA Approval Year
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)		
NRTIs block reverse transcription	zidovudine	1987
	lamivudine	1995
	abacavir	1998
	tenofovir disoproxil fumarate	2001
	emtricitabine (FTC)	2003
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)		
NNRTIs block reverse transcription	nevirapine	1996
	efavirenz (EFV)	1998
	etravirine	2008
	rilpivirine	2011
	doravirine	2018
Protease Inhibitors (PIs)		
PIs inhibit virion maturation by HIV protease	saquinavir (SQV)	1995
	ritonavir	1996
	fosamprenavir	2003
	atazanavir	2003
	tipranavir	2005
	darunavir	2006
Fusion Inhibitors		
Fusion inhibitors block fusion of virion with cell membrane	enfuvirtide	2003
CCR5 Antagonists		
CCR5 antagonists prevent entry by blocking CCR5	maraviroc	2007
Integrase Inhibitors		
Integrase inhibitors block HIV integrase	raltegravir	2007
	dolutegravir (RAL)	2013
Post-Attachment Inhibitors		
Post-attachment inhibitors are monoclonal antibodies to CD4	ibalizumab-uiyk	2018
Combination HIV Medicines		
Two or more medicines from one or more drug class	22 combinations	1997-2019
Long acting combinations		
Combination medicines provided in a long acting form	carbotegravir / rilpivirine	Phase III

Table 1.1: FDA approved antiretroviral medications, drug class, HIV target, and the year approval was gained. Therapeutics and approval dates retrieved from NIH AIDSinfo.https://aidsinfo.nih.gov/understanding-hiv-aids/fact-sheets/21/58/fda-approved-hiv-medicines

Human Immunodeficiency Virus type 2

Human immunodeficiency virus type 2 (HIV-2) infection arose from zoonotic transmissions from SIVsmm that occurred sometime in the first half of the 20th century (Visseaux et al. 2016; Sharp and Hahn 2011). Up to 2 million people are infected with HIV-2, primarily in West Africa, but significant populations of HIV-2 infection also exist in Portugal, France and western India (World Health Organization 2009). Clinical presentation of HIV-2 infection is similar to HIV-1, though is distinguished by lower levels of viremia, lower levels of virus shedding, a delayed onset of AIDS and higher CD4+ T cell counts at AIDS onset (Alabi et al. 2003; Drylewicz et al. 2008). HIV-2 is intrinsically resistant to NNRTIs and the fusion inhibitor enfurvitide, and there is no rigorously established therapeutic regimen for HIV-2 infection (Campbell-Yesufu and Gandhi 2011). It was previously believed that HIV-2 represented a less pathogenic infection relative to HIV-1, but that notion has been recently challenged by studies showing that untreated HIV-2 infection results in AIDS and death at similar rates as HIV-1 infection (Esbjörnsson, Månsson, et al. 2019). These characteristics of HIV-2 infection have been attributed to a lower replicative potential of HIV-2 in CD4+ T cells compared to HIV-1, as well as a smaller population of cells harboring HIV-2 provirus, but the precise mechanisms remain unclear (Heigele et al. 2016; Krebs et al. 2001; Samri et al. 2019). One potential explanation is that HIV-2 is less well adapted to replication in, and transmission to, memory CD4+ T cells. For instance, the HIV-2 LTR is less responsive to T cell activation signals as it lacks an NFAT site and has only one NF-κB site (Krebs et al. 2001; Saleh et al. 2017). Furthermore, HIV-2 Nef downregulates CD3, an important source of activating

signal, from the surface of CD4+ T cells which has been shown to negatively impact HIV-2 expression and cell-to-cell transmission (Mesner et al. 2020; Johnson and McCarthy 2019). However, overall, transcriptional regulation of HIV-2, and whether differences in regulation compared to HIV-1 impact clinical presentation, are not well understood. Further investigation into factors that influence HIV-2 expression will benefit the 2 million people infected with HIV-2 and may provide insights into HIV-1 as well (Esbjörnsson, Jansson, et al. 2019; Saleh et al. 2017).

Establishment and maintenance of HIV latency

The latent reservoir

The latent reservoir is a long-lived population of HIV-infected cells harboring persistent and transcriptionally inactive or minimally active provirus. Latency is established in a small proportion of infected cells, about 1 in a million, and due to the lack of RNA or protein expression, these cells are not readily targeted by host defenses or any existing cART regimen (J. D. Siliciano and Siliciano 2005). Attempts to measure and characterize the reservoir have found the highest quantities of provirus to be in memory CD4+ T cells, in particular the central memory subset, though viral DNA and RNA can be recovered from other cells including macrophages, dendritic cells, microglia, and naïve CD4+ T cells (Chomont et al. 2009; Saez-Cirion et al. 2013; Zerbato et al. 2016; Wong and Yukl 2016; Ganor et al. 2019).

Although memory CD4+ T cells themselves are not particularly long-lived, they provide long-term immunity to pathogens through homeostatic proliferation, a process of

self-renewal (Liu, Simonetti, and Ho 2020; Chomont et al. 2009). Moreover, in response to antigen exposure, these cells can expand in number through antigen-driven proliferation. Infection and adoption of a latent transcriptional state in cells that are capable of both self-renewal and proliferation provides HIV with a mechanism for expansion and persistence that does not require proviral replication (Simonetti et al. 2016; Maldarelli et al. 2014; Strain et al. 2003; Maldarelli et al. 2007; Liu, Simonetti, and Ho 2020). Attempts to prevent the establishment of the reservoir by initiating cART early in the acute phase of infection fails to do so, though there is some evidence that early cART limits the size of the reservoir. This indicates that the founding of this population happens early in the course of infection, within a matter of days, and expands rapidly during acute infection (Whitney et al. 2014; Leyre et al. 2020; Persaud et al. 2013). Furthermore, patients maintained on a suppressive cART regimen do not effectively clear the latent reservoir, even after decades of treatment. Using a half-life of 44 months for the reservoir, the Siliciano group estimated that it would take 60 years to decay under sustained cART, longer than the lifespan following infection for most people (Pierson, McArthur, and Siliciano 2000; J. D. Siliciano et al. 2003; Bruner et al. 2019). The degree to which the conditions during cellular infection influence establishment of persistent latent infection is an active area of investigation but is not yet well understood.

Clinical approaches towards modulation of latency

Attempts to measure the latent reservoir by activating cells harboring provirus has led to our understanding that not all proviruses are similarly inducible (Y.-C. Ho et al.

2013). Agents such as phytohemagglutinin (PHA) that induce CD4+ T cell activation do not activate HIV expression in the vast majority of cells harboring provirus (Finzi et al. 1997; Eriksson et al. 2013; Y.-C. Ho et al. 2013). A large portion of these non-induced proviruses are defective (Figure 1.8, in dark blue), and lack the capacity to produce replication-competent virions and do not contribute to viral rebound. However, the remaining population of cells harboring intact, replication-competent provirus are latently infected and drive viral rebound if cART is interrupted for an extended period. The intact proviruses can be subdivided into those that are readily inducible by activation, those that are induced only after multiple rounds, and those that are refractory to induction despite multiple rounds of activation (Figure 1.8, yellow, red and pink, respectively). It's not clear what differentiates these proviruses, though interaction with APCs and the signaling environment at the time of infection influences whether latent provirus is readily inducible (Agosto et al. 2018; Gagne et al. 2019; Pedro, Henderson, and Agosto 2019), and proviruses established in different CD4+ T cell subsets may also be differentially responsive to induction (Baxter et al. 2016). However, because active transcription is necessary for targeting and clearance of the infected cell, the proviruses that are not induced even after multiple rounds of activation are particularly challenging for cure efforts to eliminate HIV provirus. Suggested explanations for their refractory nature include repressive CpG methylation marks of the HIV-LTR or silencing by integration of provirus into heterochromatin (Kauder et al. 2009; Schroder et al. 2002; Han et al. 2004b). However, an in-depth look at this population by Ho et al. found little CpG methylation of the LTR as well as integration into actively transcribed genes (Y.-C. Ho et al. 2013). This indicates

that the difficult-to-induce proviruses are similarly capable of supporting active transcription but may be being maintained in a latent state through additional mechanisms.

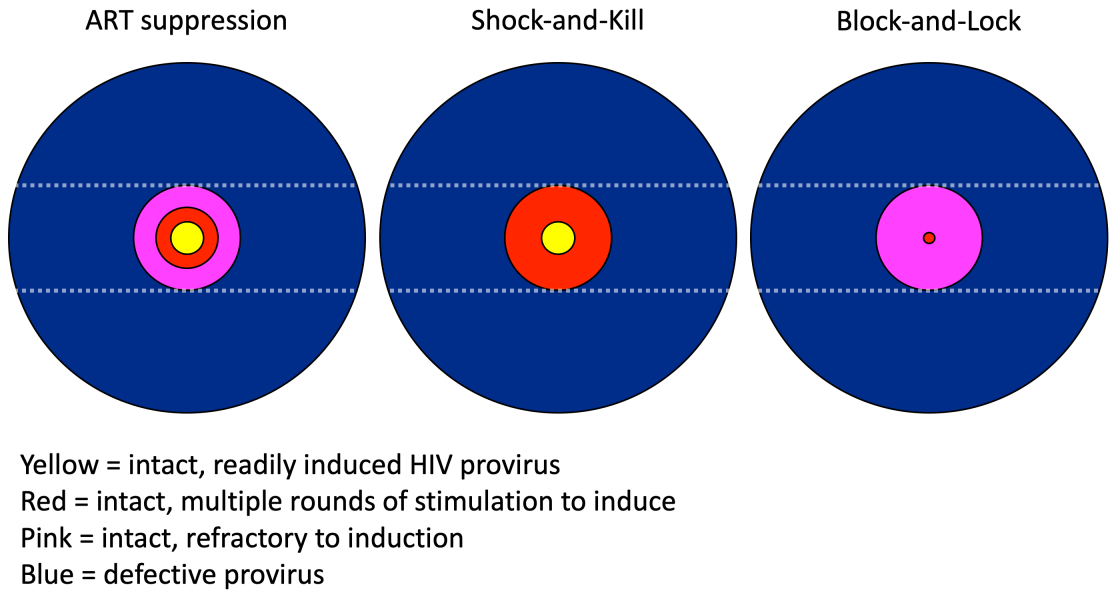

Figure 1.8: The latent HIV reservoir. The HIV reservoir consists of four broad populations. The largest, in dark blue, are defective proviruses that are unable to produce infectious particles. Yellow, red and, pink circles represent proviruses have intact genomes and are theoretically inducible by spontaneous reactivation or following perturbation. An estimation of the relative composition of populations A) during sustained ART suppression, B) following an ideal shock-and-kill strategy, and C) following an effective block-and-lock strategy. Figure inspired by (J. D. Siliciano and Siliciano 2014).

In the 'shock-and-kill' approach a latency-reversing agent (LRA) is administered to stimulate latently infected cells into a transcriptionally active state (the shock) (Figure 1.8B), which renders the infected cell susceptible to clearance by the immune system (the kill) and HIV-mediated apoptosis. There are several classes of molecules under investigation to provide the shock including epigenetic modifiers, TLR agonists, TCR activators, PKC agonists, NF-kB agonists, and molecules targeting the PI3K/Akt pathway (Kim, Anderson, and Lewin 2018; Margolis et al. 2020). To date, only the HDAC

inhibitors, PKC agonists, PI3K/Akt modulators, and TLR agonists have been used in human clinical trials. Although, many of these trials resulted in increased detection of HIV RNA none significantly altered the size of the DNA reservoir (Kim, Anderson, and Lewin 2018; Macedo, Novis, and Bosque 2019; Gay et al. 2020; Fidler et al. 2020; Archin et al. 2017; Harper et al. 2020; Margolis et al. 2020). The failure to decrease the size of the reservoir in these trials is thought to be primarily attributed to the LRAs inability to effectively activate transcription across a diverse population of latently infected cells. Secondarily, it's hypothesized that immune exhaustion due to chronic HIV infection limits the immune system to effectively clear infected cells once activated. Additional shock-and-kill strategies are under investigation that would provide successive rounds of reactivation, combining multiple classes of LRAs to provide a more effective shock, in addition to a kill phase that has greater potential (such as a therapeutic vaccine, TLR agonists, or bnAbs) (Burnett et al. 2010; Cary, Fujinaga, and Peterlin 2016; Laird et al. 2015; Ait-Ammar et al. 2020).

Conversely, the 'block-and-lock' approach seeks to permanently silence HIV expression through modification of the 5' LTR landscape. In this scenario, the intact inducible latent reservoir would be converted to non-inducible, as shown in Figure 1.8C. Essentially, patients would be chronically HIV infected/suppressed without the continued use of cART and would be unable to transmit infection. The strategies for this are varied and include Tat inhibition, BRD4 modulators, and integrase inhibition (Vansant et al. 2020). An example of the 'block and lock' approach is the use of didehydro-cortistatin A (dCA) which binds Tat directly and inhibits its association with the TAR element,

interrupting the feedback regulatory loop of Tat (Mediouni et al. 2015; 2019). dCA is given in conjunction with cART which together limits viral replication while at the same time imparting a repressive state on the ART-persistent reservoir of HIV (Mousseau et al. 2019). Over time, the lack of positive transcriptional events impart a repressive landscape on the 5' LTR including deacetylation of Nuc-1 and decreased RNAPII recruitment (C. Li, Mousseau, and Valente 2019) and in early trials delays viral rebound following treatment interruption compared to cART alone (Kessing et al. 2017).

'Shock-and-kill' and 'block-and-lock' provide two strategies to deliver functional cure via modification of proviral transcriptional activity in the reservoir. However, both approaches have failed to date in vivo and in vitro because they incompletely reactivate or repress HIV, indicating that we need a greater appreciation for the complexity of signals which are incorporated into transcriptional decision-making in HIV. My studies address these fundamental questions about how signals influence HIV infection.

The establishment and maintenance of latency in CD4+ T cells

T cell receptor signaling influences HIV infection and the establishment of latency

The T cell receptor (TCR) is a complex of proteins found on the cell surface which recognizes peptide-major histocompatibility complex (MHC) complexes presented by APCs. The TCR is a heterodimer of α and β chain proteins, each consisting of variable domains responsible for antigen recognition, proximal constant regions that bind the chains together by disulfide linkages, and transmembrane regions which anchor the complex in the lipid bilayer. The α/β chain molecule is expressed as a complex with three invariant

CD3 dimers, including the CD3ζ homodimer, which contain intracellular domains known as ITAMs responsible for transmitting signal from the TCR. CD4+ T cells require antigen presentation on Class II MHC molecules by professional APCs. Antigen presentation by APCs to CD4+ T cells occurs via the formation of a transient structure termed the immunological synapse (IS) (L. Wu and KewalRamani 2006; Mempel, Henrickson, and von Andrian 2004; Evans et al. 2013a). TCR recognition of its cognate antigen in this context results in polarization of the APC and CD4+ T cell towards each other and the recruitment of additional signaling molecules to the site of cell-cell contact, including those required to support TCR signaling (Friedl, Den Boer, and Gunzer 2005). Recognition of cognate antigen and MHCII is followed by the recruitment and clustering of TCR complexes and CD28 co-stimulatory molecules (Yokosuka et al. 2005; Bunnell et al. 2002). The recruitment of the Src kinase Lck to the TCR by CD4 results in the phosphorylation of the ITAMs of the TCR complex as well as the cytoplasmic tail of CD28. The subsequent binding and phosphorylation of ZAP-70 mediates a signaling cascade that results in CD4+ T cell activation, including the strengthening of cell-cell contact, reorganization of actin, and the downstream activation of transcription factors including NF-kB and AP-1 (Figure 1.9).

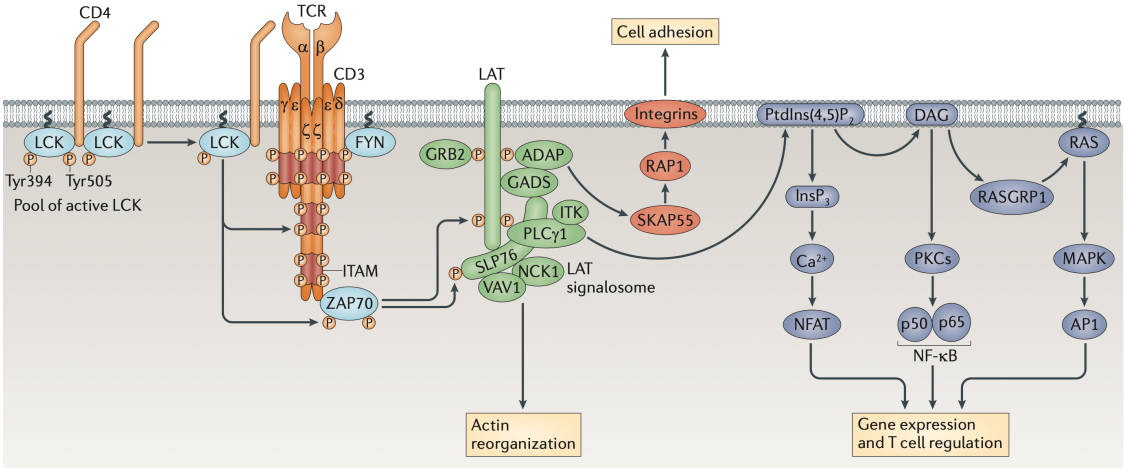

Figure 1.9: Overview of T cell receptor signaling. Recognition of cognate antigen by the TCR initiates signal transduction, activation of a number of signaling networks, actin reorganization, and regulation of gene expression. (Brownlie and Zamoyska 2013).

Activated and HIV-specific CD4+ T cells are the cells most supportive of HIV infection (Douek et al. 2002; R. F. Siliciano and Greene 2011). Restriction factors such as SAMHD1, a phosphohydrolase which depletes the cytoplasmic pool of dNTPs and inhibits reverse transcription, are downregulated with CD4+ T cell activation, promoting efficiency of reverse transcription (Descours et al. 2012; Ruffin et al. 2015). Furthermore, TCR signaling promotes an intracellular state conducive to HIV transcription. For instance, T cell activation results in the release of P-TEFb from sequestration in the 7SKsnRNP complex and translocation of the p65 NF-κB subunit to the nucleus (Nabel and Baltimore 1987; Brès, Yoh, and Jones 2008). Other transcription factors that promote HIV transcription are activated or in greater abundance following T cell activation (Figure 1.9) including AP-1 and NFAT (Cron et al. 2000; R. Chen, Yang, and Zhou 2004; Schiralli Lester and Henderson 2012). Counterintuitively, infection of activated CD4+ T cells is also supportive of establishment of latent infection. The majority of infected activated CD4+ T cells will die soon after infection due to cytopathic effects of the virus (D. D. Ho et al.

1995; X. Wei et al. 1995). However, some minority of these cells will survive and transition to a resting memory state (Sotirova-Kohli 2018; Pace et al. 2011; R. F. Siliciano and Greene 2011). There may be specific mechanisms that impart latency during this period of transition, but these remain unclear (Shan et al. 2017). Generally, CD4+ T cells in a resting state are not as supportive of efficient transcription due to the absence of significant levels of positive transcriptional regulators, the presence of transcriptional repressors and epigenetic modifications (Figure 1.6) (Pan et al. 2013; Schiralli Lester and Henderson 2012; Zack, Kim, and Vatakis 2013). Over time, additional repressive marks such as DNA methylation may accumulate, reinforcing the latent state (Trejbalová et al. 2016; Palacios et al. 2012; Blazkova et al. 2009). In this way, multiple layered mechanisms contribute to both the transcriptional activity of HIV and repression of provirus.

Direct infection of unstimulated or resting CD4+ T cells also results in the generation of latent infection and may play an important role for the generation of the latent reservoir *in vivo* (Agosto et al. 2007; Cameron et al. 2010; Lassen et al. 2012; Swiggard et al. 2005; Spina, Guatelli, and Richman 1995). Previous work from our lab found that unstimulated CD4+ T cells established latent infection on par with cell cultures receiving a range of TCR activating signal strengths during establishment of infection (Gagne et al. 2019). However, the same study found that the strength of activating signal provided was important for biasing infection towards either a readily inducible latent infection or one that was refractory to reversal. These findings and others have indicated that the context in which infection takes place may be critical in determining whether latency can be established or reversed (Agosto and Henderson 2018).

Cell-to-cell transmission and the establishment of latency

The bulk of HIV dissemination *in vivo* likely occurs via transmission of virus across direct cell-cell contacts. This mode of transmission is more efficient than cell-free infection in part because virus-donor cells concentrate viral particles at the site of contact, directing them towards target cells (Dimitrov et al. 1993; Iwami et al. 2015; Zhong, Agosto, Munro, et al. 2013; P. Chen et al. 2007). Additionally, virus-donor cells provide signals during contact and infection that may impact whether productive or latent infection is established (Kumar et al. 2015; Agosto et al. 2018; Len et al. 2017). These signals may include antigen-specific TCR signaling, which exclusively occurs as in the context of APC-T cell interaction *in vivo*, signaling via interactions of cell-surface signaling molecules, or through exchange of soluble factors such as chemokines and cytokines (Dustin and Choudhuri 2016; Smith-Garvin, Koretzky, and Jordan 2009; Schilthuis et al. 2018). These various signals and the networks they engage are integrated by CD4+ T cells and HIV to influence cell state and transcriptional programs.

Several modes of cell-to-cell transmission have been described for HIV (Bracq et al. 2018; B. K. Chen 2012; Sattentau 2008; Zhong, Agosto, Munro, et al. 2013) including transmission from APCs and infected CD4+ T cells. How signal transduction during cell-cell contact and transmission of HIV influence target cell transcriptional regulation and how changes in transcriptional regulation affect subsequent HIV replication remains unclear. A number of *in vitro* cell-to-cell transmission models have been developed to study the relative importance of exogenous signals during CD4+ T cell infection,

recapitulating to a degree the conditions present *in vivo*. These include infections of resting CD4+ T cells in the presence of cytokines/chemokines, different APC subsets, infected and activated CD4+ T cells, and endothelial cells. In a qualitative review of these models of cell-to-cell transmission, we found that, overall, these models were similarly capable of supporting establishment of productive infection in the target resting CD4+ T cells (Pedro, Henderson, and Agosto 2019). However, whether establishment of latent infection followed, and whether latency was readily inducible, differed greatly depending on conditions during infection. For instance, in cultures where infected and activated CD4+ T cells mediate transmission to uninfected resting cells, latent infection was established but not easily reversed (Agosto et al. 2018) whereas latency was more readily induced in monocyte and myeloid DC cultures (Kumar et al. 2018). One hypothesis for the differences in outcome of HIV infection observed in these models is that virus-donor cells are engaging multiple distinct and overlapping signaling pathways in target cells, including signals associated with T cell activation (Len et al. 2017; Kumar et al. 2015). How signals provided by virus-donors crosstalk with signals from the TCR signaling pathway to impact the outcome of infection following cell-to-cell transmission is not well understood.

Hypothesis

Due to the early formation of a reservoir of latently infected cells, which persists despite long-term treatment, a cure has remained elusive. Resting memory CD4+ T cells are the cell subset harboring the majority of replication competent, transcriptionally inactive provirus. Multiple signals deriving from the cellular environment and regulatory

mechanisms converge at the HIV LTR to determine its transcriptional state. Our understanding of HIV transcription has been primarily shaped by dominant transcription factors, transcriptional activators in particular. I utilized a high throughput yeast one-hybrid assay to reveal interactions between HIV LTRs and human transcription factors. I hypothesized that these interactions could be leveraged to describe a diverse set of transcriptional networks that converge on the HIV LTR, and that transcription factors identified by the screen would be novel factors that activate and repress HIV transcription. Additionally, cell-to-cell transmission is a major mode of dissemination of HIV *in vivo*. How signals from virus-donor cells impact HIV infection and latency, and how those interact with signals from the TCR, has not been thoroughly investigated. I hypothesized that DC-CD4+ T cell interactions provide signals that compensate for weak TCR signaling, supporting HIV-1 expression and generation of inducible infection.

CHAPTER TWO: MATERIALS & METHODS

Cells

Human embryonic kidney cells (HEK293T) and Jurkat CD4+ T cells (Clone E6-1) were obtained from American Type Culture Collection. CEM-GFP cells were obtained from the NIH AIDS Reagent Program. HEK293T cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 100 U/mL penicillin/streptomycin (Invitrogen), 2 mM of L-glutamine (Invitrogen), 10% fetal bovine serum (FBS) (Gemini Bio-Products). Jurkat and CEM-GFP cells were cultured in RPMI 1640 (Invitrogen) supplemented with 100 U/mL penicillin/streptomycin, 2 mM of L-glutamine, and 10% FBS. Cell cultures were maintained at 37 °C and 5% CO2 replacing medium every 2-3 days.

Primary CD4+ T cells, monocyte-derived macrophages (MDM), and monocyte-derived dendritic cells (DC or moDC) were derived from leukapheresis blood packs purchased from New York Biologics. Peripheral blood mononuclear cells (PBMC) were enriched by density gradient centrifugation over Lymphoprep (StemCell Technologies). CD4+ T cells were isolated from PBMCs by negative selection using EasySep Human CD4+ T Cell Isolation Kits (StemCell Technologies) and cultured in RPMI supplemented with 100 U/mL penicillin/streptomycin, 2 mM of L-glutamine, and 10% FBS. Cell cultures were maintained at 37°C and 5% CO_2 replacing medium twice per week.

MDMs were differentiated from PBMCs by resuspending PBMCs in RPMI lacking serum at a density of 5×10^6 cells/ml and plated 1 ml per well in 12-well plates. Cells were incubated at 37°C for 2 h to allow attachment of monocytes to the plates. The plates were

then swirled to resuspend unbound cells and these cells were discarded. The attached cells were cultured in RPMI supplemented with 10% Human AB Serum (Corning), 100 U/mL penicillin/streptomycin, 2 mM of L-glutamine, and 10% FBS. MDMs were differentiated for a week at 37°C and 5% CO_2, and spent medium containing unbound cells was removed and replaced with fresh medium every 2–3 days.

To obtain mature DCs, CD14+ monocytes were isolated from PBMCs by positive selection using EasySep Human CD14+ Positive Selection Kit II (StemCell Technologies) and then cultured at a concentration of 1.2 x 10^6 cells/ml in RPMI supplemented with 1000 U/mL interleukin-4 (IL-4) (Miltenyi Biotec), 0.5 µg/mL human granulocyte-macrophage colony-stimulating factor (GMCSF) (Miltenyi Biotec), 100 U/mL penicillin/streptomycin, 2 mM of L-glutamine, and 10% FBS. Cell cultures were maintained at 37°C and 5% CO_2. Every 2 days for 6 days, half of the culture medium was removed from the top layer of the culture and replaced with an equal volume of 2X supplemented medium. To mature DCs, 100 ng/mL of *E. coli* LPS (Sigma) was supplemented for 24-48 h.

Viruses, transfections, and transductions

Viruses were made by transfection of expression vectors into HEK293T cells by polyethyleneimine (PEI) Max 40,000 (Polysciences, Inc.). For a 50% confluent T-75 plate of HEK293T cells, 15 µg of total plasmid DNA was transfected in 45 µl of PEI and 1.2 ml of Opti-MEM (Invitrogen). Plates were incubated overnight and fresh culture media was added the following morning. 24 h later, culture supernatant was collected and syringe-filtered through a 0.20 µm membrane (Corning), followed by ultracentrifugation over a

20% sucrose density (in PBS) gradient for 1.5 hours, resuspended in 1/20th of the original volume of supernatant, and titered on CEM-GFP cells as follows. CEM-GFP cells are a T lymphoblastic cell line containing an HIV tat-sensitive GFP reporter gene. When infected, HIV Tat provided drives GFP expression that can be quantified by flow cytometry analysis. Virus titer (Janas and Wu 2009) was calculated using the following formula:

Infectious Units (IU)/ml = (cell number) × (% of GFP-positive cells) × (dilution factor)

To make full-length HIV viruses, transfections consisted of only PEI and viral plasmid. Single round ΔEnv viruses were co-transfected with VSV-G or NL4-3 Env at a 1:10 or 1:5 envelope-to-virus quantity ratio, respectively. CAR viruses were made by co-transfection of an equal quantity of CAR vectors with a Gag-Pol packaging construct (Derse et al. 2001) and VSV-G at 1:10 total DNA quantity. Because CAR viruses do not encode HIV Tat, successful virus production was confirmed by infection primary CD4+ T cells or T cell lines and subsequent flow cytometry to measure the encoded mCherry tag.

Transfections of HEK293T cells in Chapter Three for overexpressing transcription factors utilized the same approach, with quantities of PEI and plasmid DNA adjusted for the size of the plate. For instance, for a T-25 flask 5 µg of total plasmid DNA was used with 15 µl of PEI.

For transductions of primary CD4+ T cells with CAR vectors, cells were stimulated for 4 h with 10 µg/ml of PHA +10 U/ml IL-2 +10ng/ml IL-7, then washed with PBS and spinoculated in media containing lentivirus and 5 µg/ml polybrene (Millipore) at x1200 g

for 90 minutes. Cells were washed with PBS 2 h later and provided fresh culture media. Cells were then rested a minimum of 6 d before use in coculture assays. Successful transduction of the CAR vector was monitored by flow cytometry using the mCherry marker.

Cell-free infections

CD4+ T cell infections in Chapter Three were performed by spinoculation of HIV at an MOI 0.1 (as determined by titration in CEM-GFP cells), washed and incubated for 72 h at 37 °C in the presence of 1 μM of the protease inhibitor saquinavir (AIDS Reagents Program) to prevent any viral spread past the first round. Cells treated with 1 μM of efavirenz (AIDS Reagents Program) or 1 μM emtricitabine (AIDS Reagents Program) served as negative controls of infection.

To infect MDMs, confluent or near confluent wells of MDMs on a 12-well plate were provided approximately 15 μl of VSV-G pseudotyped HIV in fresh MDM culture media and incubated overnight. Virus media was removed the following morning and replaced with fresh culture media.

To infect HEK293T cells for chromatin immunoprecipitation experiments, a 50% confluent plate of cells were infected with approximately 15 μl of VSV-G pseudotyped HIV by addition to the culture media and incubated overnight. Virus media was removed the following morning and replaced with fresh culture media.

DC-CAR coculture and reactivation assays

LPS-matured DCs were pulsed with HIV-1 by incubating them in virus media at a concentration of 1 IU/cell at 37 °C for 4 hours followed by three washes in PBS +2% FBS to remove virus not bound to cells. Autologous CD4+ CAR T cells were cocultured with HIV-pulsed moDCs at a ratio of 2 CD4+ T cells to 1 moDC, at a concentration of about 2.5 x 10^6 cells/ml, for 48 h in culture media with 1μM saquinavir (NIH AIDS Reagent Program), ±1 μM efavirenz (NIH AIDS Reagent Program), ±1 μg/ml Her2 (ThermoFisher) (Akiyama et al. 2015). Following coculture, cells in suspension were removed from wells and moDCs were depleted by magnetic bead separation as moDCs retain some beads used during positive selection of monocytes from PBMCs.

For transwell assays, low affinity CAR T cells were resuspended in HIV-1 luciferase resuspended 500 μl of culture media and placed into a well of a 24 well plate a final concentration of ±1 μg / ml Her2. 0.4μm transwell chambers (Corning) were placed into appropriate wells and moDCs were added in a 200 μl volume into transwell chambers. 200μl culture media was added to wells that did have moDCs as a mock condition. Transwell cocultures were incubated for 48 h. Transwells containing the moDCs were removed and discarded. Remaining CAR T cells were lysed and analyzed by luciferase assay.

For reactivation assays, CD4+ CAR T cells were cultured in culture media supplemented with 1 μM saquinavir for a minimum of 6 days. To reactivate, culture media was supplemented with PHA, IL-2, and IL-7 and incubated overnight.

HIV and transcription factor mRNA expression analysis

For luciferase analysis cells were collected into tubes, washed in PBS, and resuspended in 40 µl of 5X lysis buffer (Promega) diluted to 1X in water. 20 µl samples were added to wells of a black walled, clear bottom 96-well plate. 80 µl of luciferin substrate (Promega) was added to each well and luciferase activity was measured using the BioTek Synergy HT Microplate reader at a sensitivity of 100 for 1 s.

For RNA analysis cells were collected into tubes and incubated in PBS supplemented with DNase 5 µg/ml, RNase (50 µg/ml) (Thermofisher), and MgCl2 for 15 minutes at 37 °C. Following this, cells were resuspended in 0.5 ml Trizol (ThermoFisher). Ethanol precipitation of RNA and preparation of cDNA followed. RNA expression was analyzed from 5 µl of 1:4 dilution of cDNA using GoTaq PCR master mixture (Promega) and the primers listed in Table 2.1. Reactions were performed on an Applied Biosystems QuantStudio 3 Real-Time PCR machine with the following program: 15 m hot start at 94 °C, 45 cycles of 15 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C and plate read. Relative levels of mRNA transcripts were calculated by the ΔΔCT method using RPL13a or GAPDH as a housekeeping gene (Livak and Schmittgen 2001; Ledderose et al. 2011).

Oligos used for RT-PCR analysis of mRNA		
Target	Primer	Primer sequence
Total HIV-1 RNA	Forward	5'-GGGTCTCTCTGGTTAGA-3'
	Reverse	5'-AGAGCTCCCAGGCTCA-3'
Total HIV-2 RNA	Forward	5'-GGTCGCTCTGCGGA-3'
	Reverse	5'-CTCCCAGGGCTCAATCT-3'
HIV-1 Tat, spliced	Forward	5'-TCCCTCAGACCCTTTTAGTCAG-3'
	Reverse	5'-CATCTGTCCTCTGTCAGTTTC-3'
HIV-2 Tat, spliced	Forward	5'-TTAGGACCCTTCTTGCTTTG-3'
	Reverse	5'-CATCTGTCCTCTGTCAG-3'
KLF2	Forward	5'-CCGTCCTTCTCCACTTTC-3'

	Reverse	5'-CCATGGACAGGATGAAGTC-3'
KLF3	Forward	5'-CTTTATTTCTCGTCGGCGGC-3'
	Reverse	5'-GGGTATGACACTGAGACAGGG-3'
PLAGL1	Forward	5'-ACCTCACTTAGAAAGTGGTTCTGA-3'
	Reverse	5'-CCTCCCAGAAGTTTGTCTGAAG-3'
GAPDH	Forward	5'-TGATGACATCAAGAAGGTGGTGAAG-3'
	Reverse	5'-TCCTTGGAGGCCATGTGGGCCAT-3'
RPL13a	Forward	5'-CAAGCGGATGAACACCAAC-3'
	Reverse	5'-CGCTTTTTCTTGTCGTAGGG-3'

Table 2.1: Oligos used for RT-qPCR analysis of transcription factor and HIV expression

Measuring HIV DNA

Integrated HIV-1 DNA within CD4+ T cells infected *in vitro* was measured as previously described (Agosto et al. 2016; 2007; Gagne et al. 2019). Total HIV-2 DNA was measured using the GoTaq PCR master mixture (Promega). Serially diluted plasmid encoding HIV-2_{ROD9} was used as a copy standard. A parallel reaction for cellular albumin was done as previously described to determine the number of cells within the DNA sample (Agosto et al. 2016; 2007).

Oligos used for *Alu*-PCR		
Target	Primer	Primer sequence
Alu (F) / Luc (R)	Forward	5'-CGTCGCCAGTCAAGTAAC-3′
	Reverse	5'-CTGTAATCCCAGCAGTTTGGGAGGC-3′
Albumin	Forward	5'-GCTGTCATCTCTTGTGGGCTGT-3′
	Reverse	5'-AAACTCATGGGAGCTGCTGGTT-3′
HIV-1 R / U5	Forward	5′-GCCTCAATAAAGCTTGCCTTGA-3′
	Reverse	5′-TCCACACTGACTAAAAGGGTCTGA-3′
	Probe	5′-FAM-CCAGAGTCACACAACAGACG-TAMRA-3′
HIV-2 R / U5	Forward	5'-CTAGACTCTCACCAGCACTT-3'
	Reverse	5'-GTTCCAAGACTTCTCAGTCTTCTTC-3'
	Probe	5'-FAM-CCCTAGCAGGTTGGCGCCTG-TAMRA-3'

Table 2.2: Oligos used for Alu-PCR analysis of proviral DNA

Signaling pathways associated with HIV transcriptional networks

To perform pathway enrichment analyses we included the transcription factors that interact with LTRs of HIV-1 or HIV-2 by eY1H assays as well as select HIV-transcription factor interactions reported in the literature. In addition, we included protein-protein interactions with these transcription factors reported in HuRI and the literature (interactome.baderlab.org) (Luck et al. 2020). Transcription factors and proteins expressed in at least one subset of CD4+ T cells with a TPM > 0 (Blueprint Epigenome) were included in the analysis. PANTHER Pathway enrichment analysis was performed using human proteins as a background, and using Fisher's exact test and false discovery rate correction for multiple hypothesis testing (Mi, Muruganujan, and Thomas 2013). Terms with at least three genes in our query set were included.

Enhanced yeast one-hybrid screen

We used enhanced yeast one-hybrid assays to evaluate the binding of transcription factors to HIV LTR sequences. The two components of the system are the human transcription factor "prey" library and the LTR "bait". The U3-R-U5 LTR bait sequences, which included 1 kb of sequence that span full proviral LTRs extending approximately 500 bp beyond the transcriptional start site, were cloned into the pDONR-P4P1R vector and then transferred to two vectors upstream of two reporter genes, *LacZ* and *HIS3,* using Gateway cloning. The reporter constructs were integrated into the HIS3 and URA3 *loci* in the *S. cerevisiae* genome to generate chromatinized LTR-bait strains. Transcription factors are expressed as fusion proteins that include the yeast Gal4 activation domain, and human

transcription factor DNA binding domains allowing the detection of DNA interactions of both activators and repressors (Fuxman Bass et al. 2015). An array of 1,086 yeast strains each expressing a different transcription factor, which represents greater than 66% of the known human transcription factors, was tested against LTR sequences in a pairwise manner using a robotic platform that mates LTR-bait strains with each transcription factor prey strain in a 1,536 colony format (Fuxman Bass et al. 2015; Reece-Hoyes et al. 2011). Matings were performed in quadruplicate to assure reproducibility of hits. Positive hits were identified as blue X-Gal-positive colonies that grew on His-minus plates and in the presence of the competitive His3p enzyme inhibitor 3-amino-1,2,4-triazole, and only interactions with reporter activity above background for at least two colonies were deemed positive. As observed in previous studies, more than 90% of interactions detected were positive in all four colonies tested (Fuxman Bass et al. 2015; Reece-Hoyes et al. 2011; Fuxman Bass et al. 2016).

Transcription factor expression in immune cell subsets

Expression of transcription factors identified by the eY1H screen in CD4+ T cell and myeloid lineage subsets was evaluated using the Blueprint Epigenomics (http://dcc.blueprint-epigenome.eu) online database. Values were plotted using heatmapper (http://www.heatmapper.ca/) (Babicki et al. 2016).

Microarray

Unstimulated CD4+ T cells were plated in a solution of 1 μg/mL CD28 (Mouse Anti-Human CD28, #555725, BD Biosciences) on previously coated wells of 1 μg/mL CD3 (Mouse Anti-Human CD3, #555329, BD Biosciences). Cells were then washed in PBS and lysed for RNA extraction using Qiagen miRNeasy Mini Kit (#217004). Microarrays and statistical support were provided by BU Microarray and Sequence Resource Core Facility, as previously described (Gagne et al. 2019).

Transfections of HEK293T cells for overexpression of transcription factors

To validate transcriptional activity of the selected transcription factors on HIV LTRs, HEK293T cells were co-transfected with firefly luciferase reporter plasmids under the control of HIV-1_{NL4-3} (Henderson, Zou, and Calame 1995) or HIV-2_{ROD9} (kindly donated by Suryaram Gummuluru, Boston University School of Medicine) LTRs. For luciferase assays, 10,000 cells were plated per well in black walled clear bottomed 96-well plates and incubated for 24 h at 37 °C prior to transfection. Each well was transfected with 10 μl of a transfection mix containing Opti-MEM (Invitrogen), 0.008 μg of HIV-LTR-Luciferase, 0.042 μg of plasmid expressing KLF2 (Origene - SC127849), KLF3 (Addgene - 49102), PLAGL1 (Origene - SC115928) or empty pcDNA3 vector and 0.03 μg/ml of PEI Max 40,000 (Polysciences, Inc.). Each transcription factor was tested in triplicate. Cells were incubated for 24 h and luciferase signal was read using the Luciferase Assay System (Promega) and a BioTek Synergy HT plate reader at 1 s per well.

For chromatin immunoprecipitation experiments where transcription factors were overexpressed, 0.5 x 10^6 cells were plated on a T-25 plate (Thermofisher) and incubated overnight. Plates were infected the next day with VSV-G pseudotyped HIV-1 $_{NL4-3}$ and incubated overnight. The following day plates were transfected with plasmids expressing KLF2, KLF3, or empty pCDNA3 vector using PEI Max.

Chromatin Immunoprecipitations

For CD4+ T cell chromatin immunoprecipitations 5 × 10^6 HIV infected resting cells or those activated for 24 h with anti-CD3/CD28 Dynabeads (Thermofisher) were collected. For HEK293T cell chromatin immunoprecipitations, cells were infected for 24 h with HIV and then transfected with transcription factor expression constructs for 48 hr, cells from confluent T-25 plates were lifted from the plate using 0.05% trypsin and washed in PBS +5% FBS to deactivate trypsin. Cells were washed with PBS and fixed with 1% paraformaldehyde (Electron Microscopy Sciences) in PBS for 10 min, followed by quenching with saturating amounts of glycine (MP Biomedicals) for 5 min. Samples were transferred to 4 °C and washed with PBS to remove paraformaldehyde. Pellets were resuspended in cell lysis buffer (5 mM Tris-HCl pH8, 90 mM KCl – Thermo Fisher Scientific, 1% NP-40 – Boston BioProducts, 1x Halt Protease and Phosphatase Inhibitor Cocktail – Thermo Fisher Scientific), centrifuged at 6,000rpm for 10 min at 4°C, lysed with nuclear lysis buffer (50 mM Tris-HCl pH8, 10mM EDTA - Thermo Fisher Scientific, 0.5% SDS – EMD Millipore, 25mM sodium butyrate - Acros Organics, 1x Halt Protease and Phosphatase Inhibitor Cocktail) and sonicated in nuclear lysis buffer using a Bioruptor

Pico (Diagenode) for 15 cycles of 1.5 min on, 30 s off for CD4+ T cells, and 10 cycles of 30 s on, 30 s off for HEK293T cells. Following sonication, the lysate was centrifuged at 12,000rpm for 10 min at 4°C, and the supernatant was transferred to a fresh tube. The pellet was lysed one last time with RIPA-like buffer (20 mM Tris-HCl pH8, 2 mM EDTA, 0.5mM EGTA pH8 - Thermo Fisher Scientific, 1% Triton X, 140mM NaCl – Thermo Fisher Scientific, 0.25% sodium deoxycholate – Acros Organics, 1x Halt Protease and Phosphatase Inhibitor Cocktail), centrifuged at 12,000rpm for 10 min at 4°C and the supernatant was combined with the rest of the sonicated chromatin. Chromatin was pre-cleared with Protein-A sepharose beads (Invitrogen), a small sample was removed to serve as the "input" control and the rest was divided into equal volume aliquots depending on the target protein for immuno-precipitation. Immuno-precipitation was conducted using the following antibodies: anti-KLF2 (clone 665333, R&D Systems), anti-KLF3 (clone B-12, Santa Cruz Biotechnology), anti-PLAGL1 (clone F-9, Santa Cruz Biotechnology), anti-acetyl-histone H3 (catalog 06-599, Millipore Sigma), anti-HDAC2 (clone 3T3, Santa Cruz Biotechnology), normal mouse IgG (catalog NI03, Millipore Sigma) or rabbit anti-GFP (catalog G10362, Invitrogen). After antibody binding, chromatin was bound to Protein-A sepharose beads. The beads were pelleted and washed with low salt buffer (20 mM Tris-HCl pH8, 0.1% SDS, 1% Triton X, 2mM EDTA, 150mM NaCl), high salt buffer (20 mM Tris-HCl pH8, 0.1% SDS, 1% Triton X, 2mM EDTA, 500 mM NaCl), lithium wash buffer (10 mM Tris-HCl pH8, 10mM, 0.25 M LiCl – Acros Organics, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA) and TE buffer. Chromatin was eluted in TE buffer containing 0.3% SDS, 0.47 mg/ml of proteinase K (Invitrogen) incubated overnight at 65 °C. DNA

was purified using the ChIP DNA Clean & Concentrator kit (Zymo Research). Purified DNA was analyzed by qPCR using GoTaq PCR master mixture (Promega) and the primers listed in Table 2.3 were used to detect HIV DNA immunoprecipitated with KLFs, PLAGL1, acetylated histone H3 and HDAC2 (Kaczmarek Michaels, Natarajan, et al. 2015). DNA purified from the "input" sample was serially diluted to serve as a standard.

Oligos used for chromatin immunoprecipitations		
Target	Primer	Primer sequence
HIV-1 TF binding site	Forward	5'-GAGCTTTCTACAAGGGACTTTC-3'
	Reverse	5'- AGACCCAGTACAGGCAAA-3'
HIV-1 Nuc1 site	Forward	5′-CTGGGAGCTCTCTGGCTAACTA-3'
	Reverse	5′-AGACCCAGTACAGGCAAAA-3′
HIV-2 TF binding site	Forward	5'-TAACCAAGGGAGGGACAT-3'
	Reverse	5'-GCAAGCTAGCGGGTATATTT-3'
HIV-2 Nuc1 site	Forward	5'-AGCCCTGGGAGGTTCT-3'
	Reverse	5'-GGCCAAGTGCTGGTGA-3'

Table 2.3: Oligos used for RT-qPCR analysis of chromatin immunoprecipitation assays

Knockdown of transcription factors

For testing the effect of KLF2, KLF3, and PLAGL1 on HIV transcription unstimulated CD4+ T cells were infected and factors were diminished with siRNA. 5 x 10^6 cells per condition were resuspended in 100 µl of buffer 1SM (Chicaybam et al. 2013), 10 µl of 20 µM siRNA stocks targeting KLF2, KLF3, PLAGL1 or non-targeting control were added (Dharmacon - L-006928-00-0005, L-006987-02-0005, L-006546-00-0005, D-001810-10-05) and electroporated using the Nucleofector I (Amaxa/Lonza) program U-14. Following electroporation, 400 µl of pre-warmed RPMI supplemented with 100 U/mL penicillin/streptomycin, 2 mM of L-glutamine and 20% FBS were added to cells and incubated for 10 min at room temperature before transferring to a well of a 24-well plate

containing 1 ml of medium with 20% FBS. Saquinavir was maintained in all culture conditions and efavirenz or emtricitabine was added to control wells. 24 h later, cells were spun down and resuspended in CD4+ T cell culture media. Cells were harvested for transcriptional analysis 24 h later.

For testing the effect of transcription factor knockdown on HIV transcription in MDMs, confluent or near confluent wells were infected with VSV-G pseudotyped HIV for overnight then washed twice with PBS +2% FBS. Wells treated with 1 μM of emtricitabine (NIH AIDS Reagents) served as negative controls of infection. 2.5 μl of 20 μM siRNA packaged in 5μl of Lipofectamine 2000 (ThermoFisher) added to each well overnight. The next day wells were washed with PBS +2% FBS and media was replaced with fresh MDM culture media. Cells were harvested for HIV transcriptional analysis 24-48 h later.

Western blotting for transcription factor expression

Cells collected at 24 h post-transfection with transcription factor over-expression plasmids (HEK293T) or 48 h post-electroporation with siRNA (CD4+ T cells) were resuspended in lysis buffer: 20 mM Tris-HCl (pH 7.4), 1% Triton X-100 – Thermo Fisher Scientific, 10% glycerol – Thermo Fisher Scientific, 137 mM NaCl – Thermo Fisher Scientific, 2 mM EDTA - Thermo Fisher Scientific, and 25 mM β-glycerophosphate – Sigma, protease inhibitor cocktail III – Millipore Sigma, and PMSF – Thermo Fisher Scientific. The cell lysates were mixed in Laemmli's SDS-Sample Buffer (Boston BioProducts, # BP-111R) and then heated at 95 °C for 5 min. 15-30 μg of protein were loaded onto a 12% SDS-PAGE Gel then subjected to electrophoresis for 1.5 h. The protein

samples then were transferred onto polyvinylidene difluoride membrane (Millipore). After blocking 1 h with 5% non-fat dry milk blocking buffer, the blots were incubated with primary antibodies: anti-KLF2 (clone 665333, R&D Systems), anti-FLAG (clone M2, Millipore Sigma), anti-PLAGL1 (clone F-9, Santa Cruz Biotechnology) overnight at 4°C and probed with secondary HRP-conjugated anti-mouse antibody (Santa Cruz Biotechnology). The membranes were developed with the ECL Prime Western Blotting System (GE Healthcare) and visualized on X-ray film. Membranes then were stripped and re-probed with anti-β-Actin (Cat# VMA00048, Bio-Rad) for loading control.

Flow cytometry

Flow cytometry data was collected using an LSR-II SORP (BD Biosciences) and analyzed using FlowJo software. All cells were washed and stained in PBS +2% FBS and 500 mM EDTA, then fixed by the addition of paraformaldehyde at a final concentration of 1%. Cells were stained with Zombie NIR (Biolegend) CD25 (clone 2A3, BD Biosciences), CD69 (clone FN50, Biolegend), and/or HLA-DR (clone L243, Biolegend).

Statistical analysis

Data are presented as mean values ± standard error of the mean. *p* values were calculated based on the Mann-Whitney U test using GraphPad Prism software. * denotes $p < 0.05$; ** denotes $p < 0.05$ and *** denotes $p < 0.005$.

CHAPTER THREE: AN UNBIASED FUNCTIONAL SCREEN IDENTIFIES NOVEL TRANSCRIPTION FACTORS AND CORRESPONDING NETWORKS THAT REGULATE HIV-1 AND HIV-2

Introduction

Experimental Rationale

A major checkpoint for HIV replication and latency is proviral gene transcription. Understanding how transcription is regulated is critical for developing strategies aimed at eradicating HIV infection. Proviral transcription is regulated by a combinatorial set of transcription factors and coregulatory complexes coupled with the viral encoded factor Tat. The balance between these factors regulates whether viral RNAs are expressed or repressed to establish and maintain latency. The promoter and enhancer elements for HIV are within the 5' long terminal repeat (LTR) of the integrated provirus and include sequences for the binding of numerous cellular transcription factors, such as NF-κB, NFAT, Sp1 and AP-1 (Colin and Van Lint 2009; Krebs et al. 2001). HIV replicates most efficiently in activated CD4+ T cells in which positive transcriptional regulators are not limiting, RNAP II is processive, and chromatin organization is favorable for transcription (Stevenson et al. 1990; Alcamí et al. 1995; Van Lint et al. 1996). If HIV-infected, activated CD4+ T cells transition to a long-lived resting memory state, proviral gene expression can be repressed due to the absence of positive transcriptional regulators and the accumulation of repressive chromatin modifications (Smith-Garvin, Koretzky, and Jordan 2009; Mbonye and Karn 2011; Ruelas and Greene 2013; Agosto, Gagne, and Henderson 2015). Importantly, these infected memory cells persist, forming a latently infected reservoir that is resistant to

antiretroviral therapies. Attempts to modulate HIV expression *in vitro* and *in vivo* have been inefficient thus far, potentially indicating additional regulatory mechanisms. A cure for HIV-1 infection will either require eliminating these latently infected cells or preventing the reactivation of latent provirus, underscoring the need to fully understand events that control HIV transcription.

Much of our current understanding of HIV transcription reflects classic molecular biology approaches used to define transcriptional elements and DNA binding proteins (Sen and Baltimore 1986; Alcamí et al. 1995; Cron et al. 2000; Duverger et al. 2013; Jones et al. 1986; Pereira et al. 2000). These approaches typically do not explore the full repertoire of transcription factors in an unbiased or functional manner and rely on predicting potential DNA binding sites and/or available antibodies for chromatin immunoprecipitation (ChIP) based assays. Furthermore, studies have focused on dominant actors in transcriptional regulation minimizing factors that fine-tune transcription or act cooperatively in larger transcriptional regulatory networks. Additionally, studies of HIV expression have focused largely on active transcription or induction of latent provirus which may bias our understanding of key transcriptional regulators.

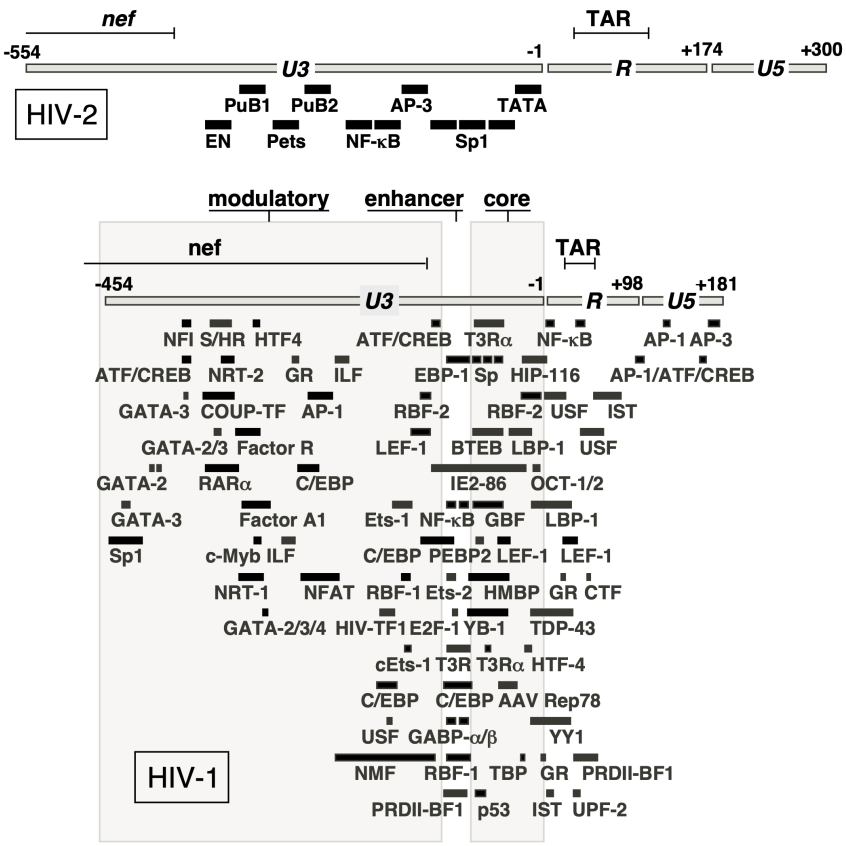

Figure 3.1: HIV LTR transcription factor binding sites described in a literature review by Los Alamos National Laboratories

Characterization of HIV transcription has thus far been primarily focused on HIV-1 (Figure 3.1). This has yielded descriptions of numerous transcription factor binding sites and regulatory mechanisms for the HIV-1 LTR. However, while HIV-2 shares some key regulatory landmarks, including Sp1 and NF-κB sites, the two viruses share only a 40% sequence similarity across the LTR (Krebs et al. 2001). While it is reasonable to expect HIV-1 and HIV-2 to utilize many of the same host factors to regulate expression, differences in their LTRs suggests HIV-1 and HIV-2 specific factors. Regardless, relative to HIV-1, the HIV-2 LTR is poorly characterized and warrants further investigation. To

test the hypothesis that unique transcription factor networks independently regulate HIV-1 and HIV-2 LTRs, I utilized a high throughput unbiased yeast one-hybrid assay to reveal novel transcription factor-LTR interactions. These interactions were subsequently leveraged to describe a set of transcriptional networks that regulate HIV-1 and HIV-2 using a computational approach. Furthermore, candidates derived by the assay were validated in cell models of infection in order to describe novel host transcription factor interactions with HIV-1 and HIV-2.

Results

A yeast one-hybrid screen reveals novel human transcription factor interactions with HIV-1 and HIV-2 LTRs that intersect with key cellular processes

To gain insights into intrinsic transcription factor networks that mediate HIV transcription, we used an unbiased functional enhanced yeast one-hybrid (eY1H) screen, consisting of a transcription factor "prey" library and an LTR "bait" (Figure 3.2A) (Fuxman Bass et al. 2015). The transcription factor array consists of 1,086 different yeast strains expressing human transcription factors fused to the yeast Gal4 activation domain. This library represents approximately 66% of the known repertoire of human transcription factors. The LTR bait sequences included 1 kb of sequence that spans the full proviral LTR extending approximately 500 bp beyond the transcriptional start site. LTRs were cloned upstream of two reporter genes, *LacZ* and *His3*, and integrated into the *S. cerevisiae* genome to generate chromatinized DNA-bait strains. Matings between the yeast prey and bait strains were performed in quadruplicate to assure specificity of hits. Positive hits were

identified as blue X-Gal-positive colonies that grew on histidine-deficient plates and only interactions with reporter activity above background for at least two colonies were deemed positive (Fuxman Bass et al. 2015; Reece-Hoyes et al. 2011; Fuxman Bass et al. 2016).

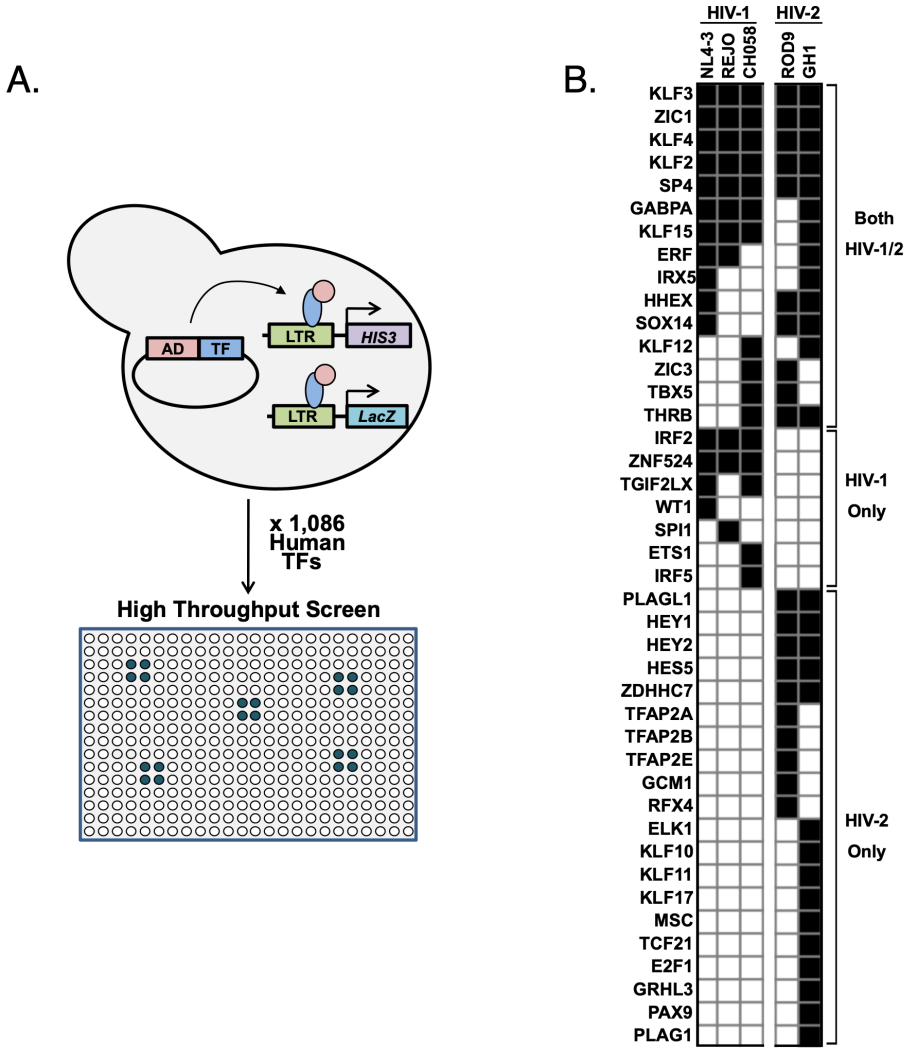

Figure 3.2: Enhanced yeast one-hybrid screen reveals human transcription factor interactions with HIV LTRs. (A) Visual representation of the eY1H assay. (B) TF-HIV LTR interactions identified by the eY1H assay. Columns indicate different HIV LTRs, rows indicate human transcription factors, and black boxes represent positive hits. Hits were arranged based on the pattern of interaction with HIV-1 and HIV-2 LTRs.

Binding was detected for 42 transcription factors to three HIV-1 clade B LTRs (NL4-3, REJO, and CH058), and two HIV-2 group A LTRs (ROD9 and GH1) totaling 85 interactions (Figure 3.2B). Transcription factors identified included several reported to influence HIV transcription such as Sp-related factors, Ets-related factors, and interferon responsive factors (IRFs) (Majello et al. 1994; Sieweke et al. 1998; Sgarbanti et al. 2002). In addition, several C2H2 zinc finger-containing proteins were identified. A subset of transcription factors, including KLF2, KLF3, KLF4, and Sp4 interact with both HIV-1 and HIV-2 LTRs, while other transcription factors interacted mostly with LTRs from HIV-1 (GABPA, KLF15, IRF2, ZNF524, and TGIF2LX) or HIV-2 (HHEX, THRB, SOX14, ZDHHC7, HEY1, HEY2, HES5, and PLAGL1) suggesting differential binding to HIV-1 and HIV-2 LTRs. As a control, a 1,500 bp sequence derived from the HIV-1_{NL4-3} gag/pol was screened and did not facilitate any transcription factor binding, indicating the specificity of the screen for transcriptional elements.

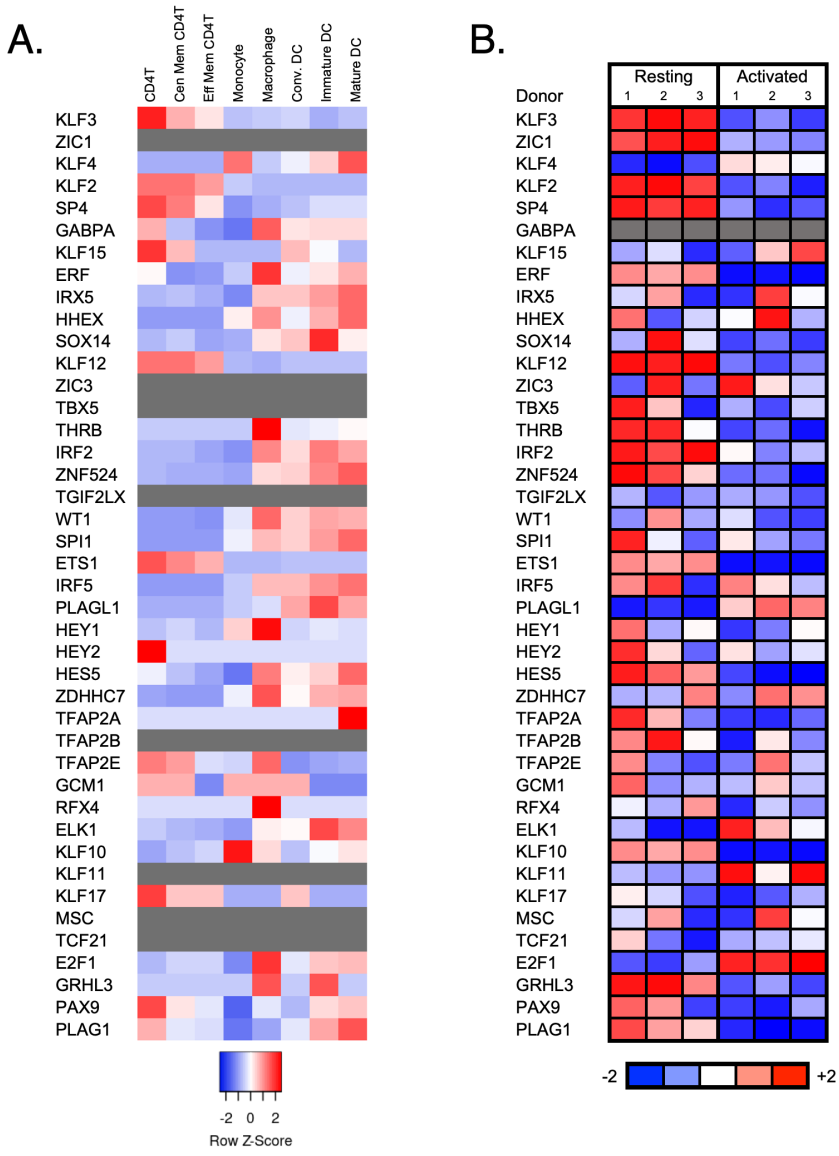

Figure 3.3: Transcription factors identified by the eY1H screen are differentially expressed in immune cell subsets. (A) RNA expression relative to median across cell types, derived from Blueprint Epigenomics database and selected for CD4+ T cell and myeloid subsets. Grey boxes indicate no expression in indicated cell type. Made with Heatmapper (Babicki et al. 2016). (B) CD4+ T cells isolated from human PBMCs were stimulated with anti-CD3/CD28 beads in culture. RNA was isolated 24 h later and converted to cDNA before being run on a Huan Clariom S array. Gene expression of CD3/28 stimulated CD4+ T cells were compared to unstimulated CD4+ T cells as well as sub-optimally activated CAR T cells (Appendix Figure 1). All genes with a one-way Anova FDR-corrected q value of < 0.01 were plotted in a heat map. These results were cross-referenced with hits from the eY1H assay and ranked similarly. Data from three human donors are shown.

Most of the transcription factors identified are expressed in CD4+ T cells, monocytes, macrophages, and/or dendritic cells based on expression profiles from Blueprint Epigenomics (http://dcc.blueprint-epigenome.eu) online database (Figure 3.3A). I confirmed the expression of the factors in CD4+ T cells by microarray analysis of resting CD4+ T cells and activated CD4+ T cells from three human donors (Gagne et al. 2019). All of the transcription factors display some degree of differential expression in CD4+ T cells. In particular, KLF3, ZIC1, KLF2, Sp4, KLF12, and IRF2 are highly expressed in unstimulated CD4+ T cells relative to activated cells, while KLF4, PLAGL1, and E2F1 show increased RNA expression in activated cells compared to unstimulated CD4+ T cells (Figure 3.3B), suggesting that these factors may be regulated in response to T cell activation.

To understand the dynamics of transcription factor expression in response to activation over time, RNA expression was measured in response to anti-CD3/CD28 bead stimulation as well as following bead removal (Figure 3.4A). KLF2 and KLF3 RNA expression decreased by 100- and 10-fold, respectively, and PLAGL1 increased by about 50-fold following 24 hours of CD3/CD28 stimulation. One week after removal of beads, however, factor RNA had returned to near original levels.

The tissue microenvironment in which HIV infection takes place *in vivo* provides a variety of signals to CD4+ T cells that may influence the outcome of HIV infection. In a previous study in CD8+ T cells, changes in KLF2 expression were shown to correlate with affinity of the TCR for its ligand as well as cytokine signaling (Preston et al. 2013). The

microarray presented in Figure 3.3B also included conditions in which suboptimal activating signal was provided to CD4+ T cells and, in those analyses, KLF2, KLF3, and PLAGL1 all display gradient changes in RNA expression based on signal strength (Appendix Figure 1). To understand if expression of these factors was responsive to other stimuli, CD4+ T cells were cultured in the presence of cytokine (Figure 3.4B) or autologous APCs (Figure 3.4C). Both KLF2 and KLF3 RNA expression decreased by 50% with the supplementation of IL-2 or IL-7 to the culture media, and KLF2 decreased by 75% when both were added. On the other hand, PLAGL1 RNA expression increased by 2-fold only when both IL-2 and IL-7 were added. IL-2 and IL-7 signaling are associated with T cell activation programs, and these data are consistent with the changes in expression observed in response to anti-CD3/CD28 bead stimulation, though the degree of change in RNA expression is substantially lower. On the other hand, coculture of CD4+ T cells with autologous APCs did not result in large changes in overall expression of these factors, with MDMs and DCs inducing 2-fold increases in CD4+ T cell expression of KLF2 and PLAGL1, respectively. These preliminary data shows that environmental signals can impact expression of these factors, and that this may influence expression of the genes that KLF2, KLF3 and PLAGL1 regulate as well.

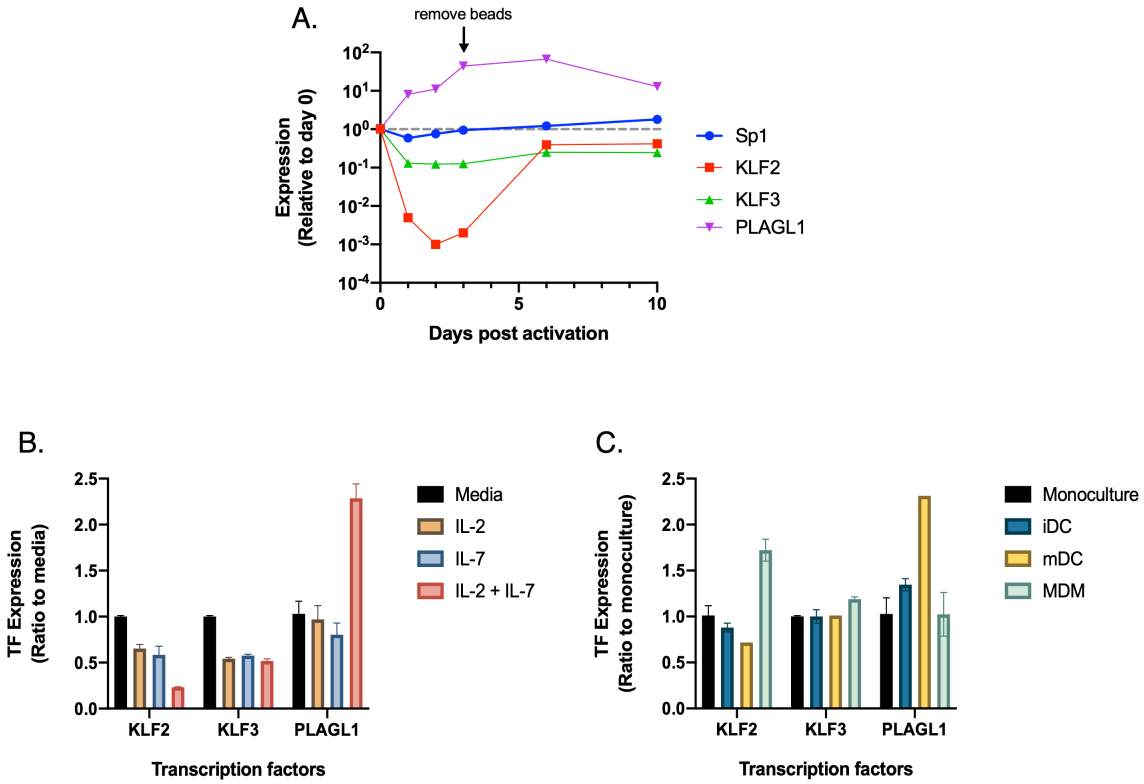

Figure 3.4: Impact of culture conditions on transcription factor expression. (A) Resting CD4+ T cells were cultured with anti-CD3/28 beads for 72 h. Beads were then removed by magnet and culture continued for 7 d in the absence of cytokine or additional stimulation. Cells were collected for measurement of factor transcript expression by RT-qPCR at T0 before stimulation, Day 1, 2, 3, 6, and 10. Sp1 RNA is not differentially expressed between unstimulated and activated CD4+ T cells so it was included as a control. Data are presented as fold change overexpression at T0 before bead stimulation. This assay was performed with three human donors, a representative experiment is shown. (B) CD4+ T cells were cultured in culture media supplemented with IL-2, IL-7, or IL-2 and IL-7, transcription factor RNA expression was measured by RT-qPCR. (C) CD4+ T cells were cultured alone or with immature DCs, LPS matured DCs, or MDMs. For MDM cultures, CD4+ T cell supernatants were collected from the wells for analysis. For DC cultures, CD4+ T cell supernatants were collected and DCs were further depleted by magnetic separation. RNA expression in remaining cells was measured by RT-PCR. For B and C results shown are from a single human donor assayed in duplicate for each condition, and data are presented as mean ± standard deviation.

Transcription factors act downstream of cellular signaling cascades and integrate multiple biological processes including cell division, cell stress, and DNA repair. I hypothesized that the array of transcription factors binding the LTR will provide a footprint

of the intrinsic cellular microenvironment that influences HIV transcription. Pathway enrichment analysis for transcription factors identified to bind the HIV-1 and HIV-2 LTRs by eY1H assays was performed and was combined with previous findings of LTR-interacting transcription factors reported in the literature (Figure 3.5A). Given that this is a small set of genes to perform enrichment analysis, interactors of these transcription factors (Luck et al. 2020) that are expressed in CD4+ T cells were also included (Blueprint Epigenome). Pathway analysis was performed using PANTHER (Mi, Muruganujan, and Thomas 2013) and 29 enriched pathways were identified, most of which are related to CD4+ T cell functions including Toll-like receptor signaling, Wnt, TGF-β, T cell activation, and p53 (Figure 3.5B). Several of these pathways, such as p53, Toll-like receptor signaling, and Jak/STAT signaling, have been linked to HIV replication and persistence providing proof of concept that this approach predicts upstream events that influence HIV transcription.

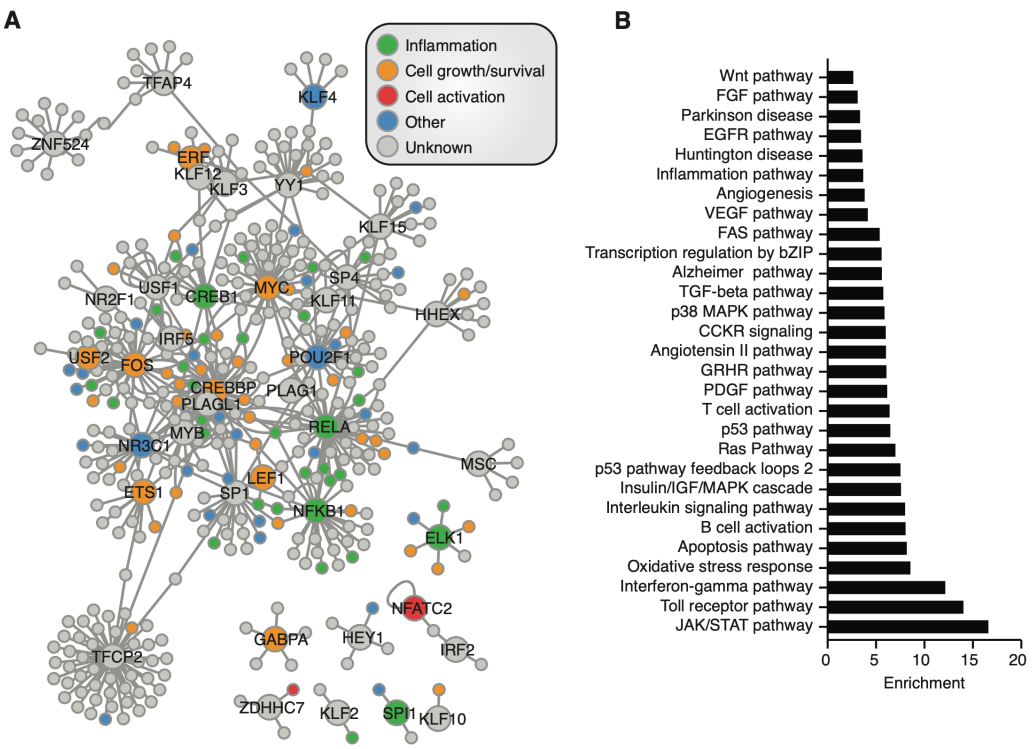

Figure 3.5: Transcription factor networks identify critical cascades. (A) Pathway enrichment analyses using transcription factors identified by eY1H assay as well as select TFs reported in the literature to regulate HIV expression. Interactors expressed in CD4+ T cells were also included. (B) PANTHER pathway enrichment analysis using the interactome from (A) was performed and identified 29 enriched pathways.

KLF2 and KLF3 repress HIV-1 transcription

Binding of eight of the Krüppel-like factor (KLF) family of proteins to HIV-1 or HIV-2 LTRs was detected in the eY1H assay. KLFs are a 17-member family of human transcription factors, sometimes expanded to include the specificity proteins (Sp) family, that are characterized by a highly conserved nuclear localization signal and three C-terminal C2H2 zinc finger domains (Turpaev 2020). These DNA binding domains primarily bind GC-box (CGCCC or CCCGC) or GT-box (CACCC or CCCAC) sequence motifs. The major differences in sequence and structure between proteins in the KLF

family are within the N-terminal domains, which interact with other proteins and determines their functional differences (Stubbs, Sun, and Caetano-Anolles 2011). The KLF family is subdivided into three groups based on their most well-known transcriptional activity, activating (group 1), inhibitory (group 2), and Sin3a interactors (group 3), though several are described to have dual roles (Pollak et al. 2018). Because their DNA-binding domains are highly homologous, KLF proteins often compete for transcription factor binding sites with transcriptional competency of the gene depending on whether activating or inhibitory KLF complexes are bound (Z. Wu and Wang 2013; Ilsley et al. 2017). The functions of KLFs in lymphocyte biology are partially defined, with KLF2 and KLF3 being implicated in lymphocyte trafficking, differentiation, and maintenance of quiescence (Hart, Hogquist, and Jameson 2012). I focused on KLF2 and KLF3, which bound all of the HIV-1 and HIV-2 LTRs in the eY1H screen.

KLF2 has been proposed to be a critical regulator of quiescence in T cells, the predominant subset associated with latent HIV infection (Chomont et al. 2009; M. Li et al. 2011). KLF2 also has described roles in regulating T cell differentiation, localization of T cells to lymph nodes, and in promoting expression of Blimp-1, a repressor of HIV transcription (Lee et al. 2015a; Bai et al. 2007; Kaczmarek Michaels, Natarajan, et al. 2015). Although KLF2 is primarily known as a group 2 activator of transcription, the N-terminus of KLF2 contains both *trans*-activating and *trans*-repression domains. (Cao et al. 2010; Kwon et al. 2014). Similarly, KLF3 has been proposed as a regulator of T cell quiescence. KLF3 is a group 1 repressor that acts through recruitment of transcriptional corepressors CtBP1/CtBP2, HDACs, HMTs, HLSDs, and/or CoREST corepressor

complexes to gene promoters (Knights et al. 2016; Pearson, Funnell, and Crossley 2011). In the context of erythroid cells, KLF3 has been shown to have a critical role in maintaining transcriptional silencing of an endogenous retrotransposon through binding of the LTR and recruitment of corepressor complexes (Mak et al. 2014). Based on the roles of these factors in lymphocyte biology and the results of the eY1H assay and microarray analysis, I hypothesized that KLF2 and KLF3 were repressors of HIV transcription in unstimulated CD4+ T cells.

To confirm binding of KLF2 and KLF3 to the HIV-1 LTR in primary cells, I performed chromatin immunoprecipitations (ChIP) for the factors in infected unstimulated CD4+ T cells. Additionally, since I previously observed changes in KLF2 and KLF3 RNA expression with anti-CD3/CD28 bead stimulation, I also measured factor binding in infected cells following activation. I detected binding of KLF2 and KLF3 at the HIV-1 LTR in unstimulated CD4+ T cells and a 4- to 5-fold decrease in LTR occupancy following 24 hours of CD3/CD28 stimulation, consistent with these transcription factors being downregulated during T cell activation (Figure 3.6). These data demonstrate that KLF2 and KLF3 differentially bind the HIV-1 LTR in resting and activated CD4+ T cells.

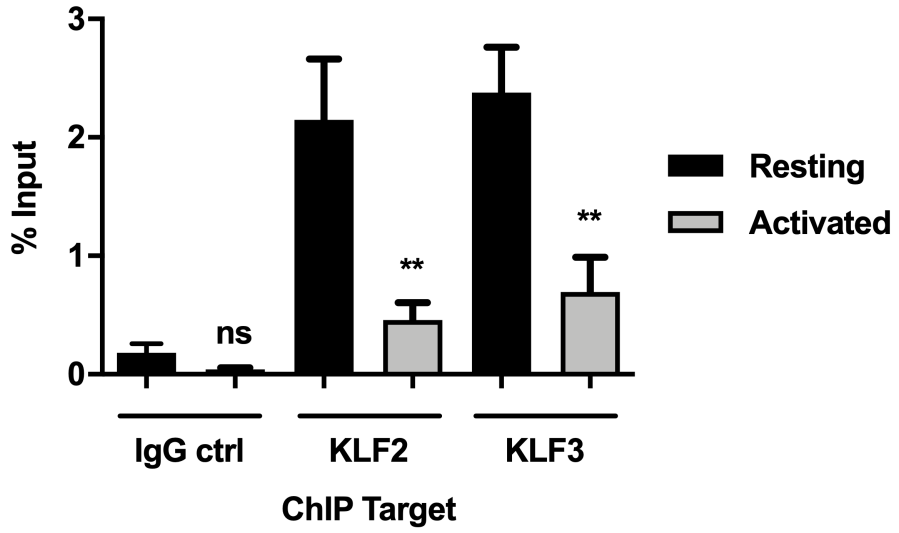

Figure 3.6: CD4+ T cell activation decreases presence of KLF2 and KLF3 at the HIV-1 LTR. Resting CD4+ T cells were infected with HIV-1_{NL4-3} by spinoculation. After 72 h half of the culture was activated using anti-CD3/CD28 beads for 24 h and fixed for chromatin immunoprecipitation. ChIP for presence of KLFs near the Sp1 sites, measured as percent input with input being total sonicated DNA for the corresponding condition. Data are presented as mean ± SEM. Primers used for occupancy are -85 and -8 relative to transcription start site. n=6 for resting cells, n=5 for activated cells. **p<0.005.

KLF2 and KLF3 function as either activators or repressors of gene expression (Cao et al. 2010). Since proviruses within resting CD4+ T cells tend to be transcriptionally silent, and I observed preferential binding of KLF2 and KLF3 in unstimulated cells, I hypothesized that these factors are transcriptional repressors of HIV. To determine the function of these factors for HIV-1 transcription, I infected unstimulated CD4+ T cells with HIV-1_{NL4-3} and knocked down KLF2 and KLF3 by nucleofection of siRNAs 72 h post-infection. Successful knockdowns of target factors were confirmed at the mRNA and protein levels and resulted in a 40-60% reduction in KLF2 and KLF3 expression (Figure 3.7A, 3.7B). Approximately 5% of cells contained integrated HIV-1 provirus measured by *Alu*-PCR (Figure 3.6C). siRNA nucleofection did not significantly affect cell viability, and

knockdown of KLF2 or KLF3 did not result in T cell activation as determined by flow cytometry analysis (Figure 3.7D). 50-60% of cells were estimated to take up siRNA as determined by nucleofection of a fluorescent oligonucleotide and measured by flow cytometry. Knockdown of KLF2 or KLF3 resulted in an about a 1.5- to 2.5-fold increase in total HIV-1 RNA expression (Figure 3.6E). Double knockdown of both KLF2 and KLF3 did not result in a significant additive induction of HIV-1 transcription, suggesting that they are either in the same biochemical pathway or are redundant in repressing HIV expression. Similar to HIV-1, knockdown of KLF2 or KLF3 in HIV-2 infected CD4+ T cells resulted in a roughly 2-fold increase of HIV-2 RNA expression (Figure 3.8A and 3.8B). These data are consistent with KLF2 and KLF3 functioning as direct repressors of HIV transcription in CD4+ T cells.

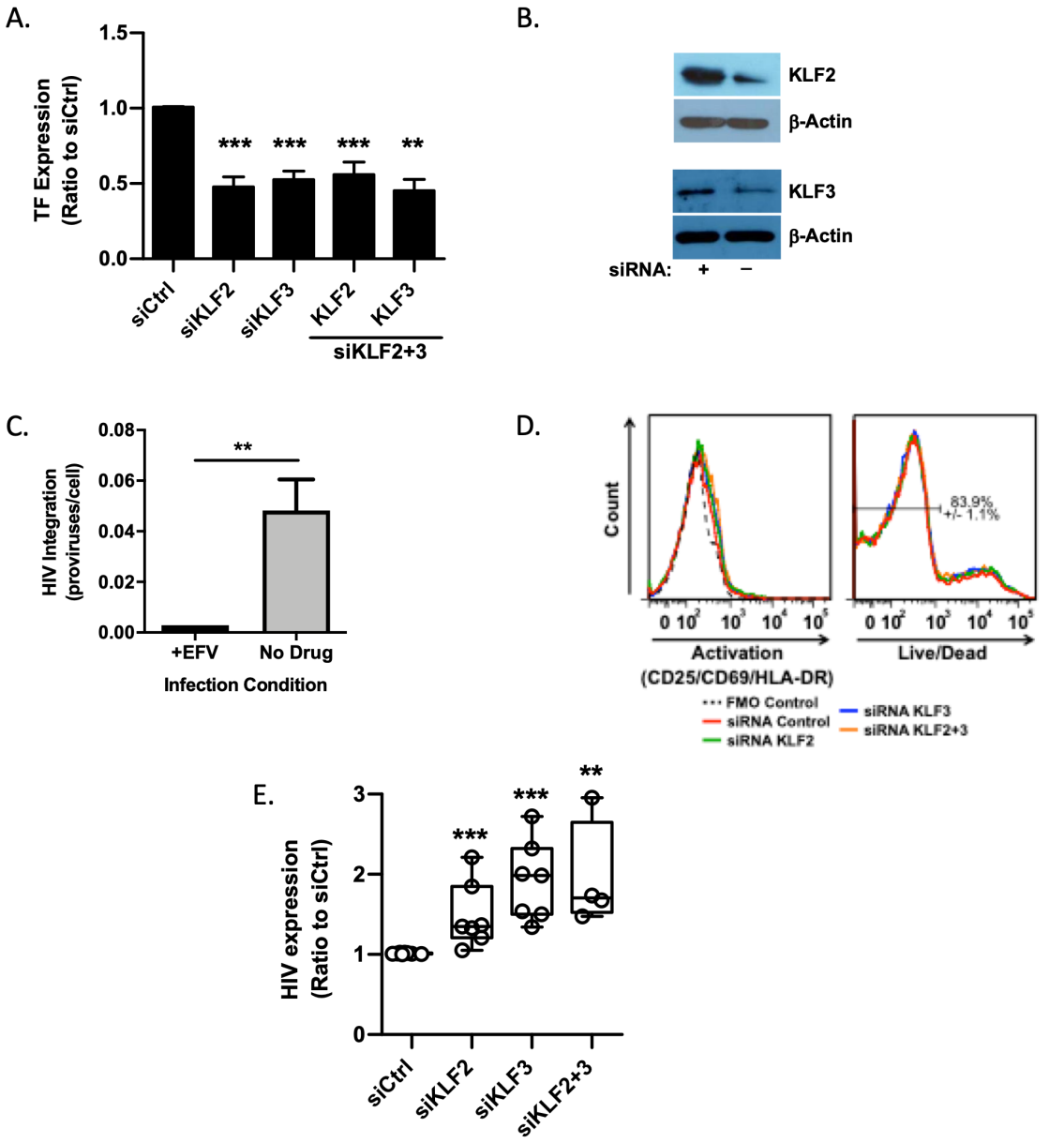

Figure 3.7: Knockdown of KLFs results in increased HIV-1 expression in infected resting CD4+ T cells. Resting CD4+ T cells were infected with HIV-1_{NL4-3} by spinoculation. After 72 h 5e6 cells were nucleofected with siRNA using the Amaxa system. 48 h later cells were collected for analysis. (A) Fold difference in KLF transcripts over siCtrl condition as an indication of efficiency of siRNA knockdown determined by RT-qPCR. (B) Change in KLF protein expression measured by immunoblot as an indication of knockdown efficiency. (C) Alu-PCR measuring average number of proviruses per cell in the population of cultured cells. Cells are unlikely to harbor more than one integrated provirus. (D) Cells were triple-stained with BV421 labeled antibodies for CD25, CD69, and HLA-DR to determine any changes in gross activation between experimental conditions. Cells were also live / dead stained to determine if nucleofections were

differentially tolerated. Analysis was done by flow cytometry. (E) Fold difference in total HIV transcripts over siCtrl condition. Primers used to measure total transcripts are +1 and +40 relative to the transcription start site. Representative experiments are shown for B and D. n=5 for Alu-PCR in C. n=6 for siCtrl, siKLF2, and siKLF3, n=4 for siKLF2+siKLF3 in A and E. Data are shown as mean ± SEM. **p<0.005, ***p<0.0005.

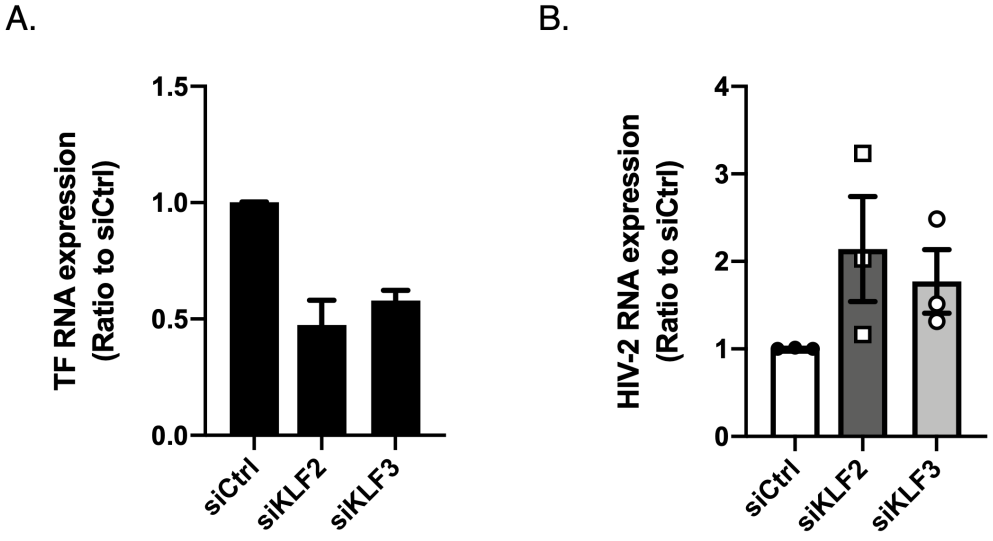

Figure 3.8: SiRNA knockdown of KLF2 and KLF3 results in increased HIV-2 expression in infected CD4+ T cells. Resting CD4+ T cells were infected with HIV-2_{ROD9} by spinoculation. After 72 h 5e6 cells were nucleofected with siRNA using the Amaxa system. 48 h later cells were collected for analysis by RT-qPCR. (A) Fold difference in KLF transcripts as an indication of efficiency of siRNA knockdown. (B) Fold difference in HIV-2 transcripts over siCtrl condition. Primers used to measure HIV-2 transcripts are +2 and +40 relative to the transcription start site. n=3 human donors. Data is shown as mean ± SEM.

HIV has been shown to infect cells of myeloid origin *in vivo* and *in vitro*, and these cells may contribute to the persistent viral reservoir (Wong and Yukl 2016; Ganor et al. 2019). Monocyte-derived macrophages (MDMs) are a common model for HIV infection that does not require isolation of macrophages from primary tissue. Monocytes were isolated from PBMCs by adhesion to tissue culture plates and subsequently differentiated to MDMs in media supplemented with GM-CSF and IL-4 for 10-14 days. Only KLF3 RNA

was reliably detectable in MDMs by RT-qPCR. I infected MDMs with HIV-1 or HIV-2 overnight and KLF3 was knocked down by siRNA transfection (Figure 3.9). Knockdown of KLF3 RNA was confirmed, resulting in a 50% decrease in KLF3 transcripts, as well as a 50% increase in both HIV-1 and HIV-2 RNA expression. This demonstrates that the KLF3 represses HIV transcription in MDMs as well as unstimulated CD4+ T cells.

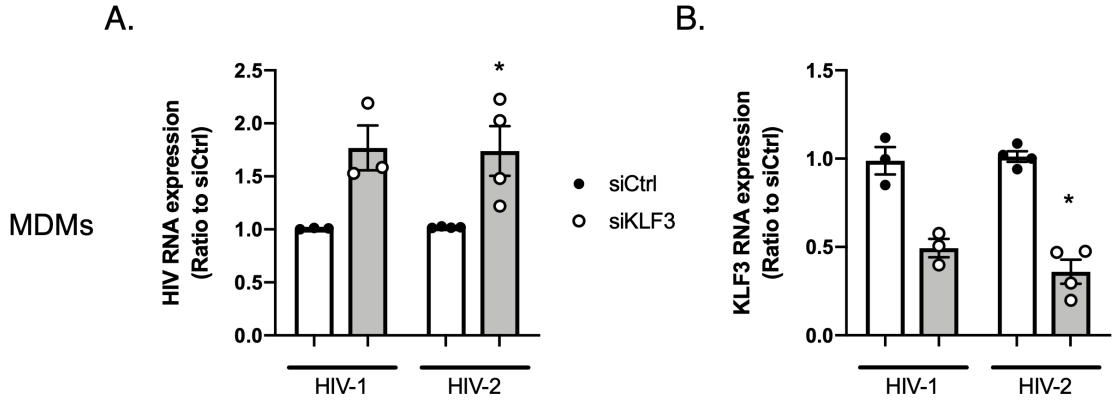

Figure 3.9: SiRNA knockdown of KLF3 results in increased HIV expression in infected MDMs. MDMs were infected with VSV-G pseudotyped HIV-1NL4-3ΔEnv.GFP or HIV-2ROD9ΔEnv.GFP overnight by addition to culture media. The next day siRNA was delivered by transfection using Lipofectamine 2000. 48 h after transfection cells were lysed in order to measure HIV and KLF3 transcripts by RT-qPCR. Fold difference of siKLF3 relative to corresponding siCtrl conditions are shown. (A) HIV-1 and HIV-2 expression and (B) KLF3 RNA expression. n=3 human donors for HIV-1 and n=4 for HIV-2. *$p<0.05$. Data are presented as mean ± SEM.

Overexpression of KLF2 and KLF3 also mediated repression of transcription mediated by the HIV LTR in HEK293T cells (Figure 3.10A and 3.10B). Both KLF2 and KLF3 are known to interact with and recruit histone modifying complexes to promoters of human genes. To assess histone post-translational modifications associated with KLF2 and KLF3 at the HIV-1 LTR, HEK293T cells were infected with HIV-1 and then transfected

with an expression construct for KLF2 or KLF3. Overexpression of KLF2 and KLF3 resulted in an increase in their occupancy at the HIV-1 LTR (Figure 3.11A and 3.11B) and correlated with a 50% or greater decrease in histone H3 acetylation (Figure 3.11C). HDACs have previously been described to contribute to repression of HIV in latency models (Keedy et al. 2009) and KLF3 complexes are known to recruit Class I HDACs (Shi et al. 2003). I specifically examined KLF3 and found that overexpression was associated with increased recruitment of the histone deacetylase complex HDAC2 to the HIV-1 LTR (Figure 3.11D). Together, these data suggest that KLF2 and KLF3 repress HIV-1, at least in part, by recruiting complexes that support histone H3 acetylation.

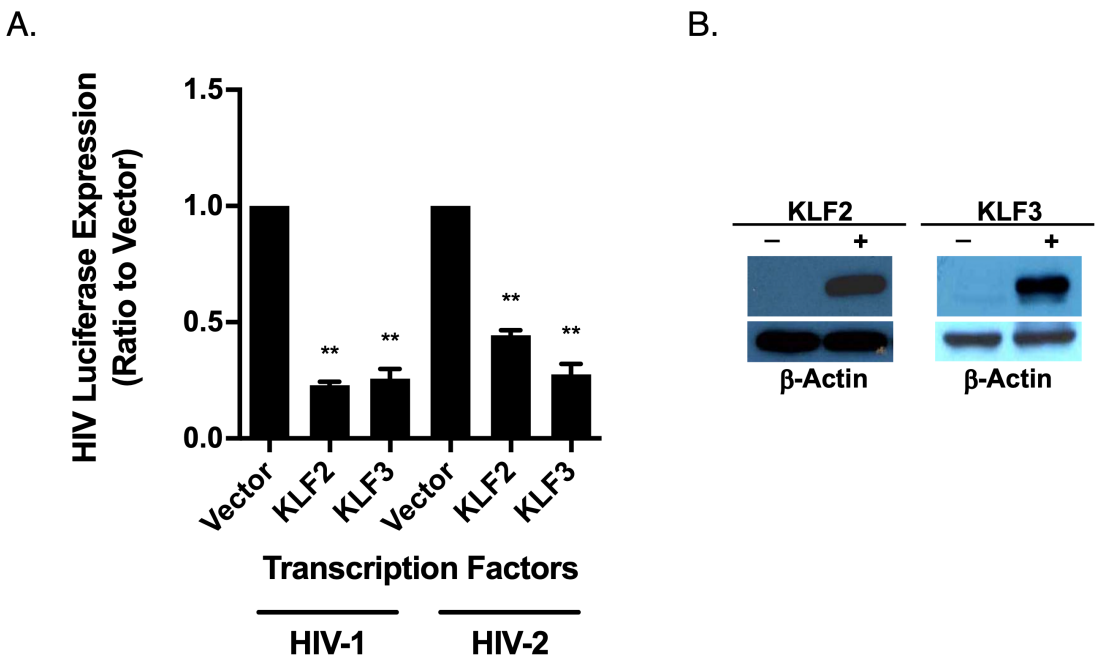

Figure 3.10: KLF2 and KLF3 repress HIV expression in HEK293T cells. (A) HEK293T cells were co-transfected with HIV-1 or HIV-2 LTR-luciferase and KLF2 or KLF3 expression constructs. Expression from the LTR was measured by luciferase assay. Data are presented as mean fold change relative to the corresponding empty vector control ± SEM. n=4 for each condition. **p<0.005. (B) Increased KLF protein expression following transfection with expression vectors was confirmed by immunoblot.

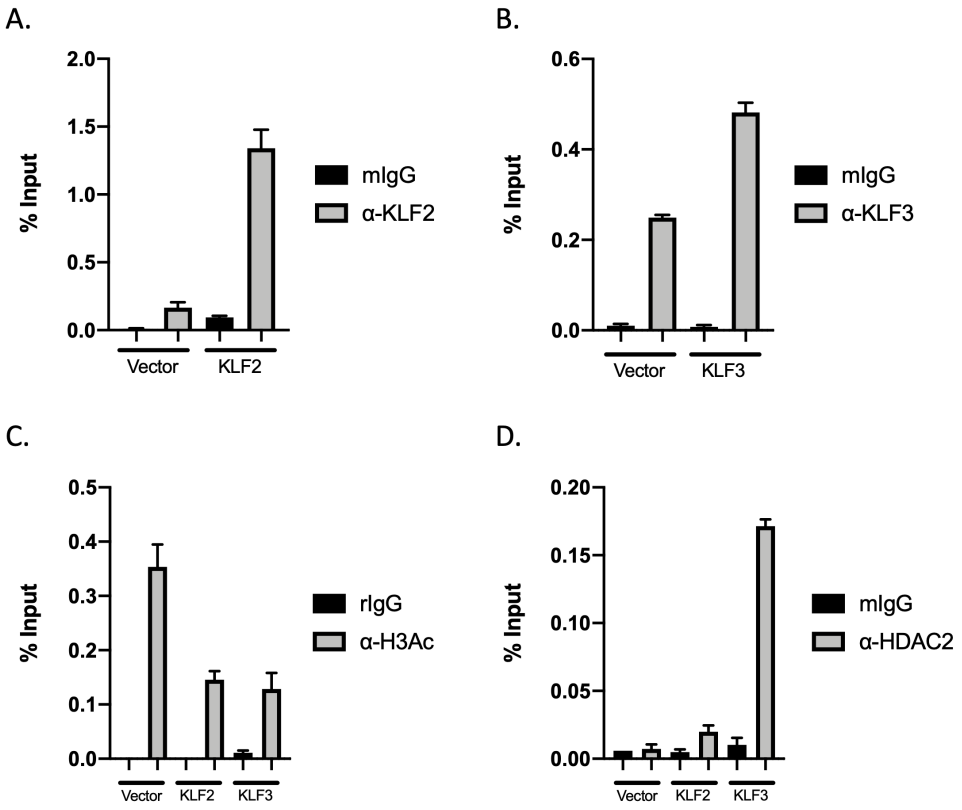

Figure 3.11: KLF2 and KLF3 occupancy at the HIV-1 LTR correlates with chromatin modification and recruitment of HDAC by KLF3. HEK293T cells were infected with VSV-G pseudotyped HIV-1_{NL4-3} followed by transfection of KLF2 or KLF3 expression constructs. Chromatin immunoprecipitation for presence of KLFs near the Sp1 sites, and modification of histone H3 or recruitment of HDAC2 near the Nuc1 site, was measured as percent input with input being total sonicated DNA for the corresponding condition. ChIPs were done with antibodies for (A) KLF2, (B) KLF3, (C) acetylated histone H3, or (D) histone deacetylase-2. Presence at the HIV-1 LTR was measured by RT-qPCR. Primers used for the Sp1 site were -8 and -85, primers used for Nuc1 were +30 and +110. Each ChIP was performed at least twice, a single representative experiment is shown, error bars show standard deviation of the technical replicates.

PLAGL1 binds to the LTR and promotes HIV-2 transcription

There has been limited investigation into HIV-2-specific mechanisms of transcriptional regulation. Using eY1H assays I identified several transcription factors that bound only HIV-2 LTRs (Figure 3.2B). PLAGL1 was an interesting candidate to explore

since it bound both HIV-2 LTRs in our screen, is widely expressed in immune cells, and is known to interact with other transcriptional activators of HIV, namely Sp1, AP-1, and PCAF/CBP/P300 (Vega-Benedetti et al. 2017). Furthermore, PLAGL1 is upregulated upon T cell activation (Figure 3.3C). PLAGL1 is a C2H2 zinc-finger transcription factor that recognizes GC-rich DNA regions via its C-terminal zinc finger array (Kas et al. 1998; Varrault et al. 2017). The N-terminal domain of PLAGL1 contains the *trans*-activation and coactivator binding domains (Vega-Benedetti et al. 2017). This factor is best understood in the context of cell cycle regulation and oncogenesis as it activates expression of p21 via interaction with p53 (Vega-Benedetti et al. 2017; S.-M. Huang, Schönthal, and Stallcup 2001); dysregulation of PLAGL1 is associated with a number of human neoplasms and cell line transformations (Resnicoff et al. 1995; Kas et al. 1998; Abdollahi et al. 2003; Kowalczyk et al. 2015; Vega-Benedetti et al. 2018). PLAGL1 has not been reported to directly regulate HIV transcription. Based on the results of the eY1H assay and the role of PLAGL1 described in the literature, I hypothesized that PLAGL1 was an activator of HIV-2 transcription.

For initial experiments, luciferase reporter constructs under the control of an HIV-1 or HIV-2 LTR were co-transfected into HEK293T cells along with a PLAGL1 expression construct or vector control. Overexpression of PLAGL1 resulted in a two-fold induction of luciferase from both LTRs, suggesting that PLAGL1 was a transcriptional activator for HIV (Figure 3.12A, 3.12B). To validate that PLAGL1 bound the LTRs in the context of human cells, chromatin was prepared from HEK293T cells infected with HIV-1 or HIV-2, then transfected with a PLAGL1 expression construct. Chromatin immunoprecipitation

using PLAGL1 antibodies showed increased occupancy of PLAGL1 at the HIV LTRs correlated with overexpression (Figure 3.12C, 3.12D). In HEK293Ts, PLAGL1 binds the LTR and promotes HIV expression.

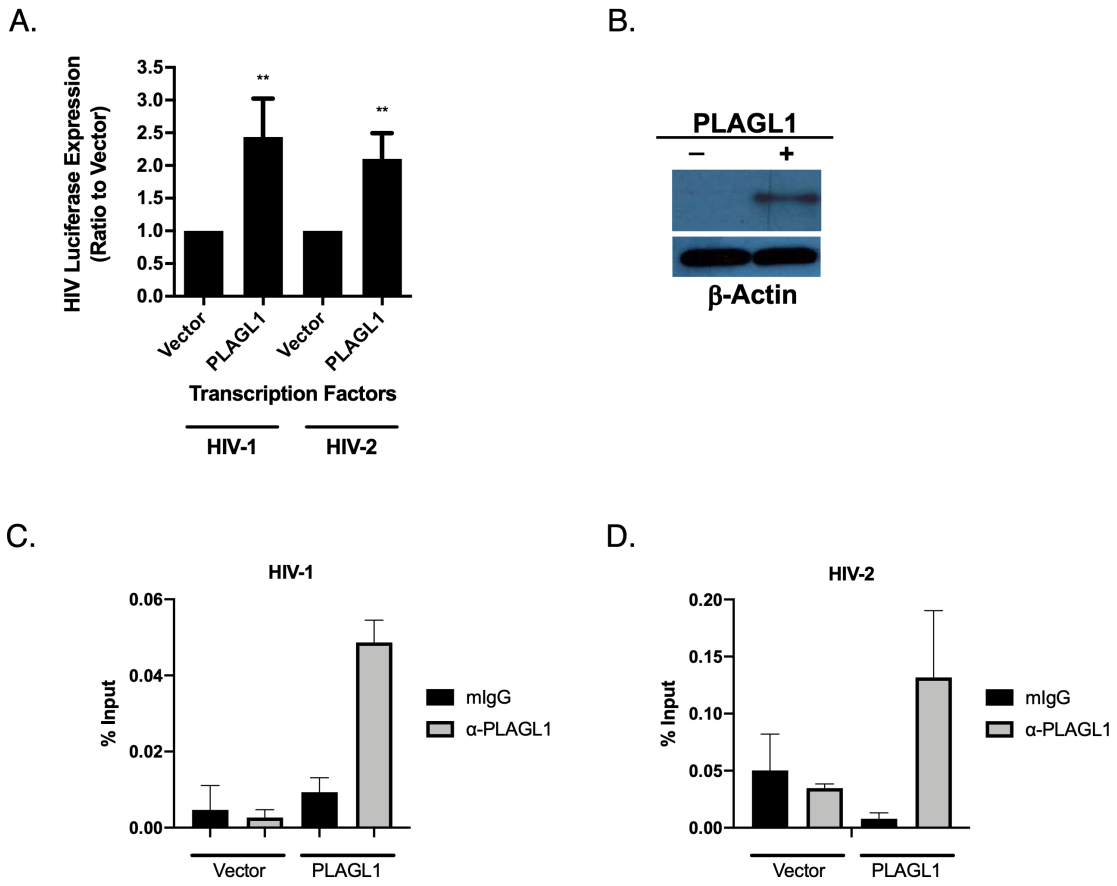

Figure 3.12: PLAGL1 activates HIV expression in cell lines. (A) HEK293T cells were co-transfected with HIV-1 $_{NL4-3}$ or HIV-2 $_{ROD9}$ LTR-luciferase and PLAGL1 expression constructs. Cells were lysed and LTR-mediated expression of HIV was measured by luciferase assay. Data are shown as mean ±SEM. n=4 **p<0.005 (B) Transfection of PLAGL1 expression constructs resulted in increased protein expression as measured by immunoblot. (C, D) HEK293T cells were infected with VSV-G pseudotyped HIV-1 $_{NL4-3}$ or HIV-2 $_{ROD9}$, followed by transfection of PLAGL1 expression construct. Chromatin immunoprecipitation for presence of PLAGL1 near the Sp1 sites was measured as percent input with input being total sonicated DNA for the corresponding condition. Presence at the HIV LTRs was measured by RT-qPCR. Primers used for the Sp1 site were -93 to -10 for HIV-1 and -85 to -8 for HIV-2. ChIPs were performed twice, with a single representative experiments shown. Data presented as mean ± standard deviation.

To determine if PLAGL1 binds the HIV LTR in primary CD4+ T cells, cells were infected by spinoculation followed by chromatin immunoprecipitation. PLAGL1 binding was detected to the LTR in both HIV-1 and HIV-2 infected cells from three human donors (Figure 3.13A, 3.13B), recapitulating what was observed in HEK293T cells. To understand if PLAGL1 influenced HIV expression in infected CD4+ T cells, PLAGL1 was knocked down by nucleofection of siRNA. Successful knockdown was confirmed at both the RNA (Figure 3.13C) and protein (Figure 3.13D) levels, achieving approximately 50% reduction of PLAGL1 expression. Despite binding to the HIV-1 LTR, knocking down PLAGL1 had no effect on HIV-1 transcription, while reduction of PLAGL1 in HIV-2 infected CD4+ T cells resulted in 4 out of 5 experiments in a 50%-80% decrease in HIV-2 RNA expression (Figure 3.13). These data show that, while PLAGL1 binds to both HIV-1 and HIV-2 LTRs, it acts as a specific activator of HIV-2 transcription in the context of CD4+ T cells.

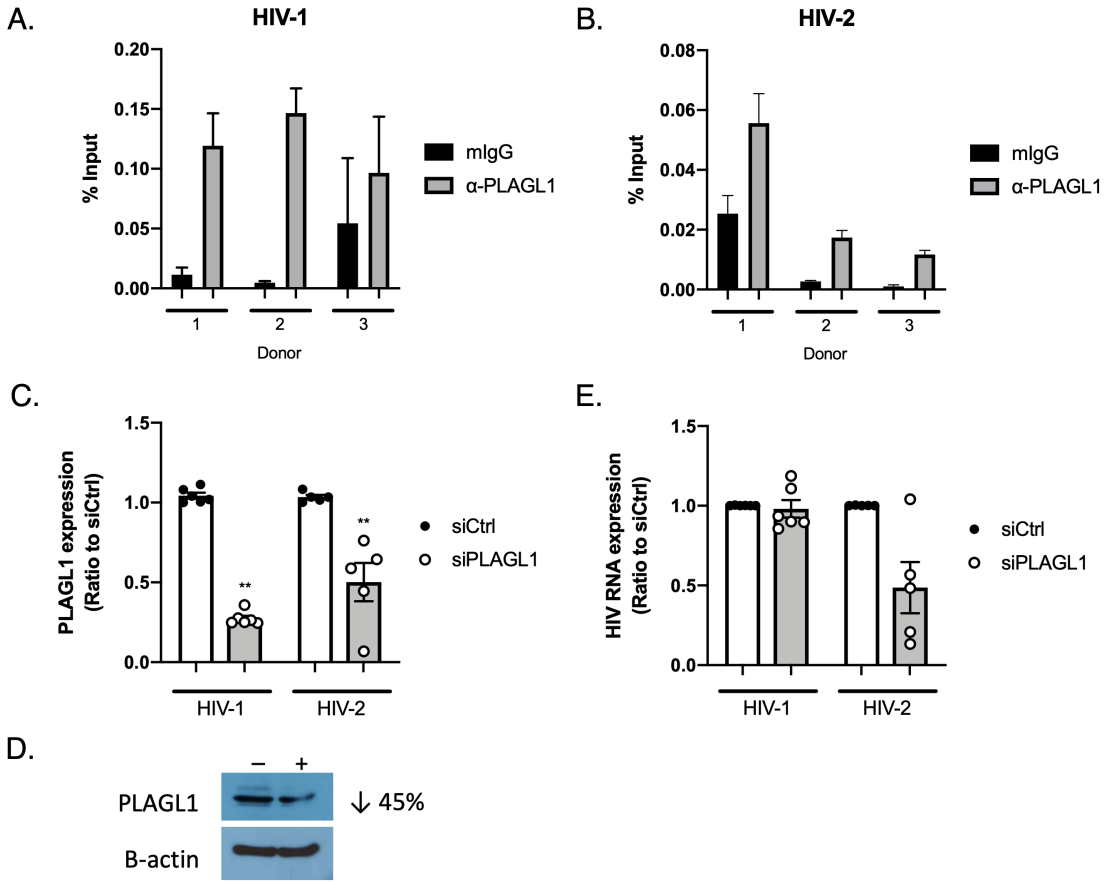

Figure 3.13: PLAGL1 occupies the HIV LTRs in resting CD4+ T cells, but selectively promotes HIV-2 transcription. CD4+ T cells from three human donors were infected with HIV-1_{NL4-3} or HIV-2_{ROD9} by spinoculation. (A, B) Chromatin immunoprecipitation for presence of PLAGL1 near the Sp1 sites was measured as percent input with input being total sonicated DNA for the corresponding condition. Presence at the HIV LTRs was measured by RT-qPCR. Primers used for the Sp1 site were -93 to -10 for HIV-1 and -85 to -8 for HIV-2. Experiments from three human donors are shown, and data are represented as the mean ± standard deviation. ChIP using PLAGL1 antibody was performed and occupancy was measured by RT-qPCR. (C, E) 72 h after infection PLAGL1 was knocked down by nucleofection of siRNA. Fold differences in fully spliced HIV or PLAGL1 transcripts compared to corresponding siCtrl conditions are shown. Spliced *tat* HIV RNAs are measured. n=6 for HIV-1 and n=5 for HIV-2. Data are shown as mean ± SEM. **p<0.005.

We also examined the role of PLAGL1 in regulating HIV-2 expression in MDMs. MDMs were infected with VSV-G pseudotyped HIV-1 or HIV-2 overnight, and then PLAGL1 was knocked down by transfection of siRNA. We achieved about a 70%

knockdown in PLAGL1 RNA (Figure 3.14A). Knockdown of PLAGL1 did not result in a significant change in HIV-1 RNA, however, HIV-2 RNA expression was reduced by approximately 60% (Figure 3.14B). These data recapitulate my findings in CD4+ T cells and demonstrates that PLAGL1 promotes HIV-2 transcription in multiple primary cells, including macrophages.

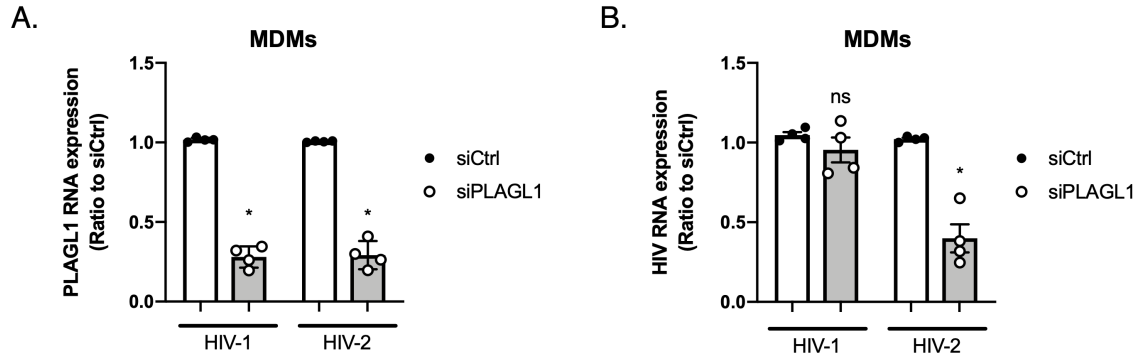

Figure 3.14: PLAGL1 knockdown promotes HIV-2 transcription in MDMs. MDMs were infected with VSV-G pseudotyped HIV-$1_{NL4\text{-}3}$ΔEnv.GFP or HIV-2_{ROD9}ΔEnv.GFP by addition of virus to culture media overnight. The next day siRNA was delivered by transfection using Lipofectamine 2000. 48 h after transfection cells were lysed in order to measure HIV and PLAGL1 transcripts by RT-qPCR. Fold differences in HIV or PLAGL1 transcripts compared to corresponding siCtrl conditions are shown. (A) PLAGL1 transcripts (B) HIV-1 and HIV-2 fully spliced Tat transcripts. n=4 human donors. *$p<0.05$. Data are presented as mean ± SEM.

Discussion

Our current understanding of HIV-1 and HIV-2 transcription is largely based on strategies that depend on LTR transcription factor binding site sequences, DNA affinity capture methods, and ChIP-seq which are performed with some combination of short segments of DNA, nuclear extracts, and antibodies often not well suited for capturing low abundance or cell type-specific protein-DNA interactions. The eY1H assay offers several advantages over these approaches. It is unbiased in terms of protein abundance, represents

two-thirds of the known human transcription factor repertoire, utilizes large DNA-baits containing multiple cis-elements, and generates high-confidence positive hits that can be validated using traditional biochemical methods. The eY1H assay has been used to characterize transcription factor binding to human enhancers and promoters, as well as identification of loss and gain of protein-DNA interactions for disease-associated variants, some of which only differed by a single base pair (Fuxman Bass et al. 2016; 2015; Shrestha et al. 2019). This approach was used to characterize transcription factor binding to the HIV-1 and HIV-2 LTRs to identify unique transcription factor interactions in order to gain insights into the networks that control HIV-1 and HIV-2 transcription. It is important to stress that the eY1H screen is a discovery assay and, although hits are likely to regulate HIV, failure to detect transcription factor binding cannot be interpreted as factors not binding to the LTR in infected cells or that they are dispensable for HIV transcription. Limitations to eY1H assays include chromatinization of the LTR, which might not be fully recapitulated in yeast, prey transcription factors which might not have similar post-translational modifications, and a screen biased against identification of hetero-multimeric complexes binding HIV LTRs. However, previous studies examining other transcriptional elements have indicated that the 30-60% validation rate of functional factors identified by eY1H approach is comparable to other methods including ChIP-seq and much higher than methodologies based on sequence motif prediction (Whiteld et al. 2012; Pro et al. 2018; Shrestha et al. 2019; Fuxman Bass et al. 2016).

LTRs from three HIV-1 and two HIV-2 molecular clones were screened using the eY1H assay, which showed both overlapping binding as well as unique binding of

transcription factors, especially between HIV-1 and HIV-2. It would be interesting to expand on this approach to include isolates from a variety of clades, or LTRs from different tissue sites and/or cell subsets, to provide insights into pathogenesis and disease progression. eY1H assays allow for comparison of protein-DNA interactions in standardized conditions and have detected differences in interactions between sequences that differ in just a single SNP, indicating that it is a sufficiently sensitive method to identify regulatory differences between cis-elements (Shrestha et al. 2019; Fuxman Bass et al. 2015).

A large subset of the transcription factors identified are preferentially expressed in unstimulated CD4+ T cells and have been described as transcriptional repressors of human genes. Studies of HIV expression have largely focused on active transcription or induction of latent provirus, which may bias our understanding of key transcriptional regulators. However, it is important to consider that most genes in cells are not expressed but are actively repressed. I speculate that a subset of the factors identified in our screen repress HIV transcription in resting CD4+ T cell subsets, and that by usurping repressive transcriptional programs HIV avoids immune recognition until CD4+ T cells are activated and provide a more favorable intrinsic program that supports HIV transcription.

Zinc-finger proteins are highly represented in the screen, making up 17 of 42 transcription factors identified, including eight out of 17 members of the KLF family of proteins that bound HIV LTRs. The KLF family has been implicated in lymphocyte development, maturation, and exhaustion (McConnell and Yang 2010). KLF2 and KLF3 bound all of the HIV LTRs we screened and repressed HIV transcription in unstimulated

cells. Our data suggest that KLF2 and KLF3 may be acting through multiple mechanisms that coordinate Nuc-1 positioning through histone acetylation. Additionally, KLF2 expression prevents the differentiation of activated T cells into Tfh cells and leads to increased expression of Blimp-1, which our lab has shown previously to repress HIV-1 transcription in T cell memory subsets (Lee et al. 2015b; Kaczmarek Michaels, Natarajan, et al. 2015). The differential regulation of KLF2 and KLF3 in unstimulated and activated CD4+ T cells suggests a role for these repressors in the establishment of latency in quiescent T cell populations. Additional studies to describe in more detail the repressive mechanisms through which KLF2 and KLF3 are acting, as well as understanding their role in the establishment of proviral transcriptional state are ongoing.

The results of the eY1H screen suggest that different LTRs bind unique constellations of transcription factors that regulate their proviral transcription. For example, my data suggest that PLAGL1 is a specific transcriptional activator for HIV-2; to my knowledge, there have not previously been any HIV-2 specific transcription factors described. It is interesting to note that although PLAGL1 did not bind HIV-1 in the eY1H assay, and selectively mediated expression of HIV-2 in primary cells, it was found associated with HIV-1 LTRs in human cells. The reasons for the observed differential transcriptional activity with HIV-1 and HIV-2 LTRs upon PLAGL1 overexpression despite binding to both subtype LTRs remain unclear. I speculate that PLAGL1 binds with higher affinity to the HIV-2 LTR or that binding to HIV-1 LTR is facilitated through interactions with neighboring transcription factors that are not present in the yeast screen. These data suggest that the HIV-2 LTR is differentially regulated compared to HIV-1 and a more

detailed transcriptional analysis of mechanisms that control HIV-2 proviral expression is warranted.

In summary, I used an eY1H screen as an unbiased approach to understand transcriptional regulation of HIV-1 and HIV-2. My findings not only have led to the discovery of novel factors that regulate HIV-1 and HIV-2 but provide insights into intrinsic networks that influence transcriptional activation and repression of HIV proviruses.

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CHAPTER FOUR: THE ESTABLISHMENT AND MAINTENANCE OF HIV-1 INFECTION FOLLOWING DC-T CELL TRANSMISSION

Introduction

Dendritic cell to CD4+ T cell transmission of HIV-1

HIV-1 infection can disseminate through the production and release of cell-free particles. However, cell-free spread exposes viral particles to the challenges of surviving the extracellular environment. To circumvent this, HIV-1 has evolved mechanisms of spread via cell-to-cell transmission. Several modes of cell-to-cell transmission have been described for HIV-1 including transmission from dendritic cells (DC) to CD4+ T cells through the formation of a structure known as infectious synapses (Bracq et al. 2018; B. K. Chen 2012; Sattentau 2008; Zhong, Agosto, Munro, et al. 2013). Similar in composition to immunological synapses used for antigen presentation and recognition, infectious synapse formation results in signal transduction in the virus-donor and target cells which influences viral spread and pathogenesis (Pinchuk et al. 1994; 1995; Dustin and Choudhuri 2016)

HIV-1 is captured by DC surface molecules, such as the C-type lectin SIGLEC-1, and retained within non-lysosomal compartments where it avoids degradation and antibody neutralization (Gummuluru, Pina Ramirez, and Akiyama 2014; Izquierdo-Useros et al. 2012; Puryear et al. 2012; 2013; Akiyama et al. 2015). Formation of the infectious synapse, following initial interactions between virus-bearing DCs and target CD4+ T cells, is mediated by engagement of attachment proteins ICAM-1 and LFA-1 (Rodriguez-Plata et al. 2013; Sanders et al. 2002). Upon initiation of cell contacts, DCs recruit a number of

molecules to the site of contact including MHC, B7 proteins, as well HIV-1 bound to SIGLEC-1 (Pinchuk et al. 1994; Akiyama et al. 2015; Huppa and Davis 2003). As a consequence of this interaction, a complementary process occurs in CD4+ T cells which includes reorganization of local cytoskeleton and recruitment of the TCR complex, costimulatory molecules including CD28, as well as CD4, CXCR4 and CCR5, the receptors required by HIV-1 for entry (L. Wu and KewalRamani 2006; Huppa and Davis 2003; McDonald et al. 2003). This process is proposed to benefit HIV-1 by concentrating particles and receptors in a discrete area of close contact (Dimitrov et al. 1993; Iwami et al. 2015; Zhong, Agosto, Ilinskaya, et al. 2013; P. Chen et al. 2007). Additionally, it has been hypothesized that infectious synapses facilitate HIV-1 infection by activating cell signaling cascades similar to the those activated by immunological synapse, but in an antigen-independent manner (Dustin and Choudhuri 2016; Hioe et al. 2011; Len et al. 2017; Contento et al. 2008; Benvenuti et al. 2004; Kondo et al. 2001; Revy et al. 2001). This includes activation of AP-1, NF-κB, and NFAT that are known to facilitate HIV-1 transcription and replication (Kaczmarek, Morales, and Henderson 2013; Karn and Stoltzfus 2012). Given that infectious synapse formation activates signaling cascades and transcriptional networks in target cells, it is probable that cell-cell contact contributes to transcriptional changes in the target cells that increase susceptibility to infection, replication, and establishment of latency.

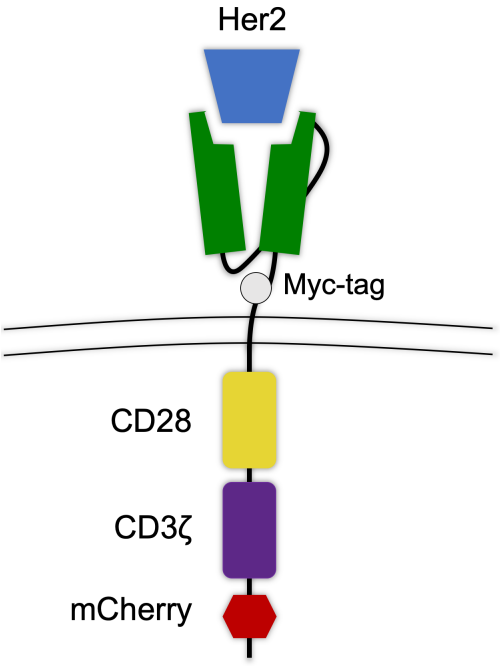
A number of studies have demonstrated a role for cell-to-cell transmission in establishing reversible latent infection from various virus donor cell types, summarized in our recent review (Pedro, Henderson, and Agosto 2019). In addition, different virus donor

cells show different abilities to support establishment of readily inducible latent infection in CD4+ T cells. As opposed to latency established upon endothelial cell-mediated HIV-1 transmission to T cells, cell-to-cell transmission from activated infected CD4+ T cells results in latent HIV-1 infection in target cells that is not easily reversed (Agosto et al. 2018; Choi et al. 2005; Shen et al. 2013; Schilthuis et al. 2018). Additional evidence for the generation of latent infection through cell-cell contact came from APC-T cell cocultures which found that CD14+ monocytes and myeloid DC subsets were able to generate latent infection in resting cells, while B cells, CD16+ monocytes, and plasmacytoid DCs were less efficient (Evans et al. 2013b; Kumar et al. 2015). These studies identified several mechanisms that were associated with support of latency formation, including signaling from attachment proteins, co-stimulatory molecules, and proteins associated with antigen presentation; APC subsets that did not engage with one or more of these pathways were less likely to establish latent infection, indicating that target CD4+ T cells integrate multiple signals that influence the outcome of HIV-1 infection.

Previous work done in our lab suggests that the strength of activating signal through the TCR plays a major role in biasing cell-free HIV-1 infection towards either a transcriptionally latent or transcriptionally active state (Gagne et al. 2019). I hypothesized that DC-CD4+ T cell interactions provide signals that compensate for weak TCR signaling in supporting HIV-1 expression and the generation of a population of infected cells conducive to reactivation. To investigate this, I utilized a panel of chimeric antigen receptors that deliver different strengths of TCR signaling in a DC coculture model.

Chimeric Antigen Receptors

Chimeric antigen receptors (CARs) are engineered proteins containing an extracellular receptor domain linked to intracellular signaling domains. Our lab has a panel of CARs that contain an extracellular receptor domain consisting of a single chain variable fragment that recognizes the receptor tyrosine-protein kinase Her2 (erbB-2) (Schier et al. 1996; Chmielewski et al. 2004). This is fused to a transmembrane linker derived from CD8 which connects the receptor to intracellular signaling domains for CD3ζ and CD28 that transduce T cell signaling upon receptor engagement (Figure 4.1A). Additionally, these proteins contain a terminal mCherry tag which allows for CD4+ T cells expressing the CAR to be identified. CARs in this panel differ in their affinities of the receptor domains for the Her2 ligand; provision of Her2 to cells expressing CARs with high affinity for the ligand results in transduction of strong activating signal while signaling through low affinity receptors results in a comparatively weak signal. For this study, I utilized a high and a low affinity CAR from our library that have a K_d for Her2 spanning three logs in order to differentially signal CD4+ T cells during infection (Figure 4.1B).



Receptor	K_d (M)	
C6-***B1D2***	1.5×10^{-11}	High Affinity
C6.5	1.6×10^{-8}	Low Affinity

Figure 4.1: Chimeric antigen receptors (CARS) used in this study. A) Composition of the CARs. A Her2-specific extracellular receptor domain is connected by a transmembrane linker to intracellular signaling domains for CD28 and CD3ζ. The receptor also contains a terminal mCherry tag for identification of cells expressing the receptor. B) The dissociation constants for the CARs and the Her2 ligand, with their relative affinities marked on the right. CARs and dissociation constants provided courtesy of the Wong Lab.

In cell-free infection, strength of T cell signaling biases HIV-1 replication and latency

Previous work in our lab found that strength of CAR signaling (phenocopy of TCR-associated CD3ζ / CD28 signaling) at the time of infection influenced active transcription of HIV-1 as well as the establishment of different populations of latently infected cells (Gagne et al. 2019). In summary, low and high affinity CAR+ T cells were infected with HIV-1 in the presence or absence of Her2. We found that cells transduced with low affinity CAR were less supportive of HIV-1 expression compared to cells transduced with high affinity CAR, despite both conditions supporting similar levels of proviral integration. Similarly, weaker signaling through the CAR resulted in fewer cells becoming activated as

measured by the T cell activation marker CD69. When latency reversal was attempted, cells receiving weaker activating signal at the time of infection were refractory to transcriptional reactivation relative to cells receiving stronger activating signal at the time of infection establishment. These studies demonstrated that HIV-1 infection could be biased towards establishment of a readily inducible or more difficult-to-induce transcriptional state depending on the signaling environment coincident at the time of establishment of infection.

However, the majority of infections *in vivo* results from cell-to-cell transmission of virus, implicating signaling cascades induced upon contact with virus-donor cells as a potentially important variable that determines the infection outcome in target CD4+ T cells. Whether DC-provided signals crosstalk with activating signals from the TCR, and how this impacts the outcome of HIV-1 infection, has not been thoroughly investigated and is the focus of this study.

Results

Her2 activates low and high affinity CAR+T cells in DC coculture

To evaluate the impact of TCR signaling during DC-mediated HIV-1 infection of CD4+ T cells, I adapted a previously described DC-CD4+ T cell coculture model (Figure 4.2) (Akiyama et al. 2015). Briefly, CD14+ monocytes were isolated from PBMCs by positive selection and from the flow-through autologous CD4+ T cells were enriched by negative selection. To generate CAR+ T cells, CD4+ T cells were briefly activated before transduction with lentiviral constructs for low or high affinity CARs. Cells were then

allowed to return to a resting state over the course of about 6 days. The resulting CD4+ T cell culture contained a mixture of CAR-positive and CAR-negative cells which were monitored by mCherry expression. Simultaneously, CD14+ cells were differentiated into monocyte-derived DCs by culturing in media supplemented with GM-CSF and IL-4 and matured by addition of LPS. To allow for capture of HIV, LPS-matured DCs were incubated in a small volume of virus-containing media for 4 hours and then washed to dilute unbound virus from the system. HIV-1 pulsed DCs were then cocultured with autologous low or high affinity CAR+T cells at a ratio of 1:2 in the presence or absence of the Her2 ligand. After 48 hours cocultures were collected for analysis of HIV-1 DNA, or expression of HIV, CD69 or chimeric antigen receptors.

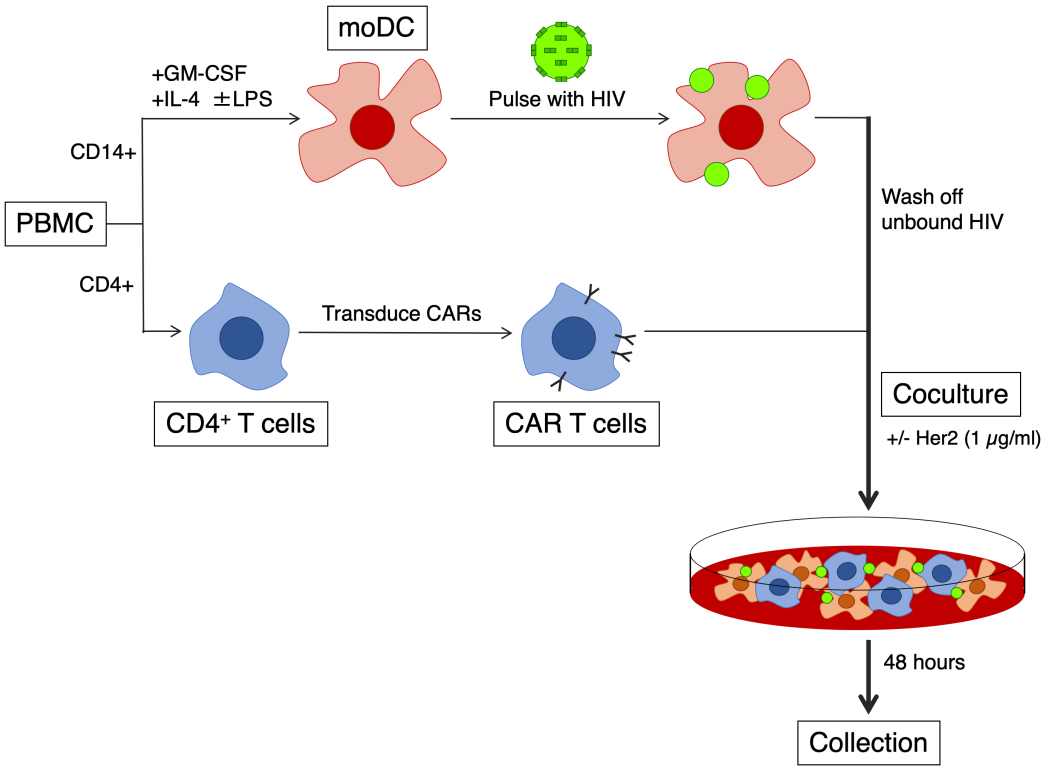


Figure 4.2: Strategy for DC-CAR+T cell coculture assay. PBMCs were isolated from a leukapheresis pack by density centrifugation. CD4+ T cells were enriched by negative selection and transduced with CAR viruses to generate CAR+T cells. Autologous CD14+ monocytes were isolated by positive selection using magnetic beads and differentiated into DCs, then matured by adding LPS. LPS-matured DCs were pulsed with HIV-1 and then cocultured with CAR+T cells ±1 µg / ml Her2 for 48 hours, then collected for analysis.

Our lab has previously shown that during cell-free infection, provision of Her2 to low or high affinity CAR+T cell cultures resulted in differentially activated T cell populations (Gagne et al. 2019). To determine if DC coculture impacts T cell activation, I measured expression of the activation marker CD69 by flow cytometry following coculture. Her2 stimulation resulted in the specific activation of cells expressing CARs but had little or no effect on CD69 expression of the CAR-negative populations, indicating that Her2 is selectively acting on CAR+T cells and that there is no significant bystander activation of CAR-negative cells. However, addition of Her2 induced CD69 expression in both the low and high affinity CAR+T cells to a similar degree (Figure 4.3A,B), suggesting that in this coculture system DCs are compensating for weak activating signal.

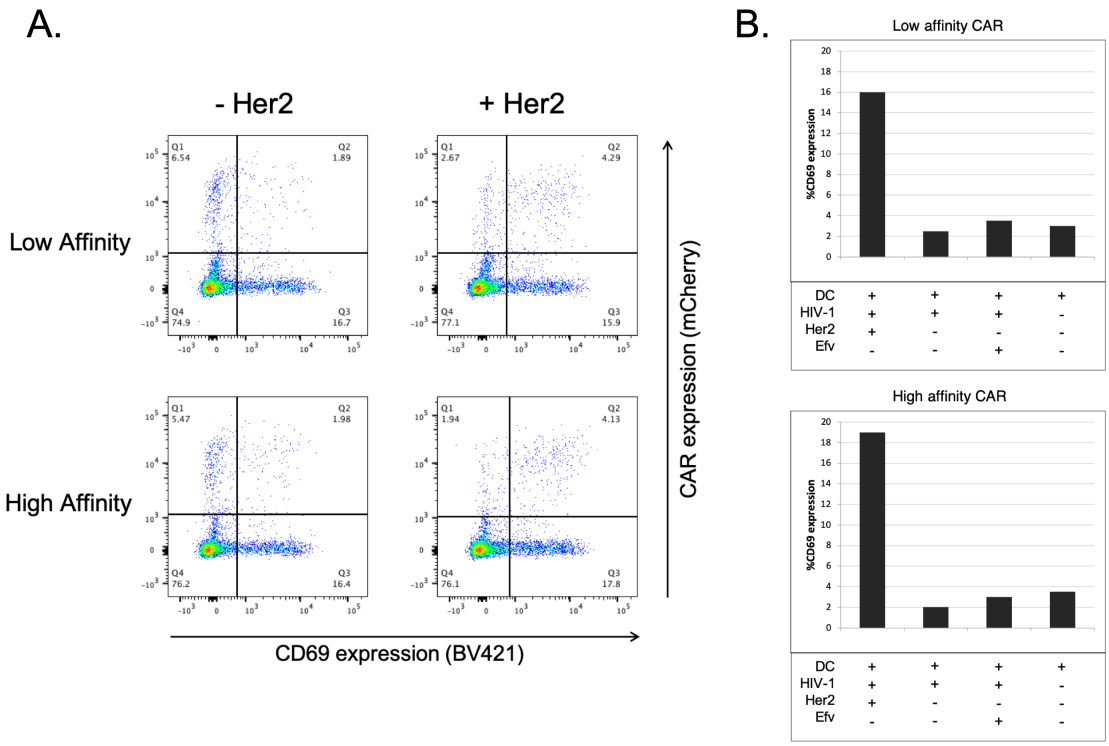


Figure 4.3: Her2 activates CAR+T cells in DC cocultures. Low and high affinity CAR+T cells were cocultured with DC pulsed with HIV-1 in the presence or absence of Her2. Cells were stained and analyzed by flow cytometry by first gating for the T cell-specific marker CD3 and then measured for expression of the activation marker CD69. Two different experiments are shown which represent the spectrum of CAR+T cell populations observed in these cocultures (CAR transduction efficiency and baseline CD69 expression of CD4+ T cells). A) Low and high affinity CAR expression measured by mCherry fluorescence shown on the y-axis and CD69 expression on the x-axis. B) Total CD69 expression among CD3+ cells in cocultures ±HIV-1 ±Efavirenz ±Her2, conditions noted by the plus or minus below each column.

To determine if cell-associated HIV-1 transmission or establishment of infection induced activation of CD4+ T cells, cocultures were initiated in the absence of Her2 or in the presence of the non-nucleoside reverse transcriptase inhibitor efavirenz (Efv). Changes in cell surface expression of CD69 was determined by flow cytometry. CD69 expression was only induced in conditions where Her2 was present demonstrating that HIV-1 transmission or establishment of infection in the absence of exogenous Her2 signaling in cocultures does not activate CD4+ T cells (Figure 4.3B). Together, these results show that

DC cocultures facilitate CD4+ T cell activation and support my hypothesis that DCs compensate for weak T cell receptor signaling.

Her2 stimulation of CAR+T cell cocultures results in increased HIV-1 expression

To understand if cell-cell contact compensates for weak activating signal to support HIV-1 expression, low and high affinity CAR+T cells were cocultured with DCs in the presence or absence of Her2. I collected DNA from the cocultures for *Alu*-PCR analysis, a nested PCR assay, to measure integrated provirus. Similar levels of integrated HIV-1 provirus were detected in both the low and high affinity CAR+T cell infection conditions in the presence or absence of Her2, suggesting that Her2 signaling does not have a significant impact on integration efficiency in DC-meditated infection and that different CAR signal strengths did not affect integration efficiency (Figure 4.4A). To measure HIV-1 expression following coculture, DCs were pulsed with viruses expressing a luciferase reporter and cocultured with CAR+ T cells in the presence or absence of Her2. Cell lysates from the cocultures were analyzed for luciferase activity as a quantitative measure of establishment of viral infection. In both low and high affinity CAR+T cell cocultures, Her2 activation resulted in an average 4-5 fold increase in luciferase expression relative to the corresponding conditions lacking Her2, indicating that DCs in coculture provide compensatory signals to overcome weak activating signals sufficient to support robust HIV-1 expression (Figure 4.4B). The luciferase assay measures HIV-luciferase expression in all cells in the culture, whether or not they express CARs. Note that LPS-matured DCs are highly refractory to HIV-1 infection, and luciferase activity in coculture lysates can be

primarily attributed to productively infected T cells (L. Wu and KewalRamani 2006). To better understand the induction of HIV-1 expression specifically in CAR+ T cells, I used a virus containing a GFP reporter in the coculture assays and analyzed cultures by flow cytometry. Among CD3+ / CAR+ cells, I observed a 4-fold increase in the number of HIV-GFP positive cells in cells receiving weak or strong activating signal relative to cocultures where Her2 was absent (Figure 4.4C). Taken together, these data show that although DC-mediated HIV-1 transmission can result in establishment of infection in the absence of CAR signaling, weak signaling at the time of infection is required to support robust HIV-1 expression from proviruses. Additionally, there was no difference by these measures between cells receiving weak or strong activating signal at the time of infection indicating that, in contrast to cell-free infection, weak CAR signaling is sufficient when DCs are present in the coculture.

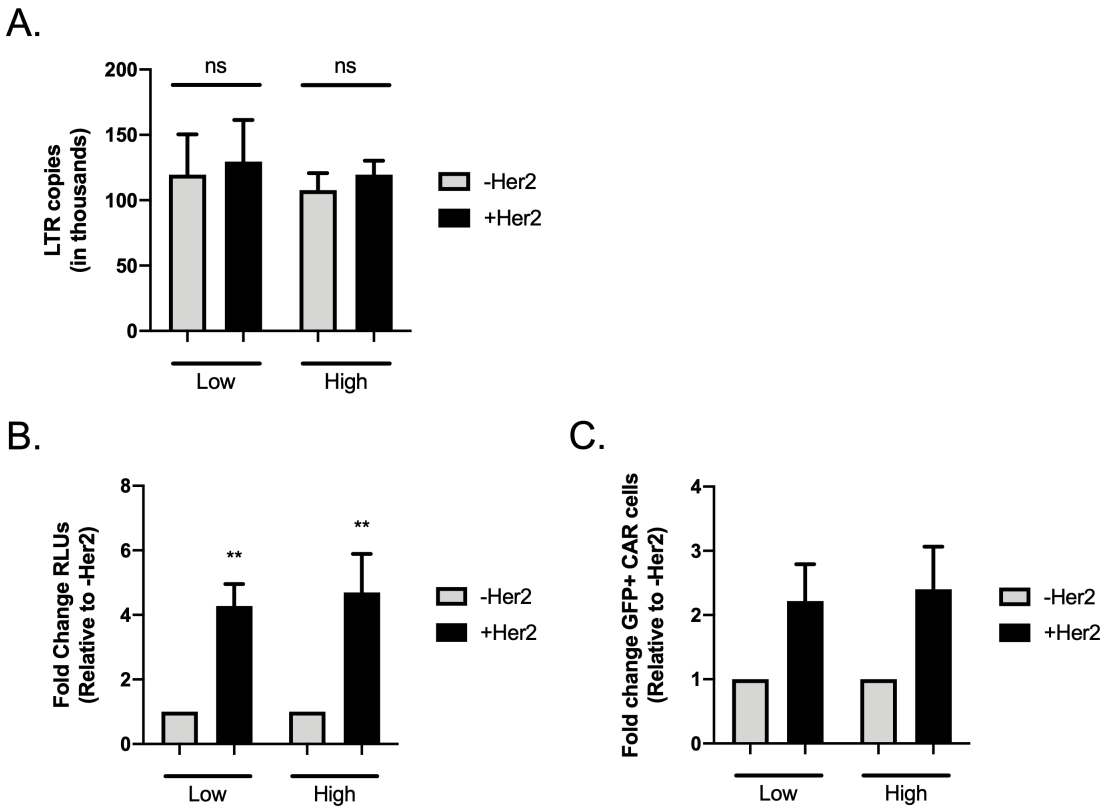


Figure 4.4: DCs facilitate HIV-1 expression in cocultures by compensating for weak activating signal. DCs were pulsed with HIV-luciferase or HIV-GFP and cocultured with low or high affinity CAR+T cells ±Her2. A) Integrated HIV-1 DNA was measured by Alu-PCR. n=3. B) Luciferase expression was measured by luciferase assay, values are set relative to the corresponding -Her2 condition. n=8. C) GFP expression in CAR+T cells was measured by flow cytometry, values are set relative to the corresponding -Her2 condition. n=3. Data are presented as mean ±SEM. **p<0.005.

During cell-cell contact, signals can be transmitted between cells via direct interaction of molecules expressed on cell surfaces or through secretion of soluble factors. To understand if direct contact is required for DCs to facilitate HIV-1 expression I utilized a transwell system to prevent direct interaction between DCs and T cells and compared to conditions where DCs were not added. Low affinity CAR+ T cells were infected by cell-free infection and plated in a 24-well plate ±1 μg / ml Her2. Then, DCs were added to the upper wells inside transwell chambers. After 48 hours of transwell coculture, the transwell

chamber containing the DCs was discarded and the CAR+ T cell fraction was assayed for HIV-1 expression by luciferase assay (Figure 4.5). As expected, Her2 stimulation of low affinity CAR+ T cell monocultures did not result in a significant increase in luciferase activity. However, when low affinity CAR+ T cells were cultured in transwells that contained DCs in the upper chambers and stimulated with Her2 there was a 4-fold increase in luciferase expression. These results suggest that DCs facilitate HIV-1 expression in CD4+ T cells at least in part through the exchange of soluble factors.

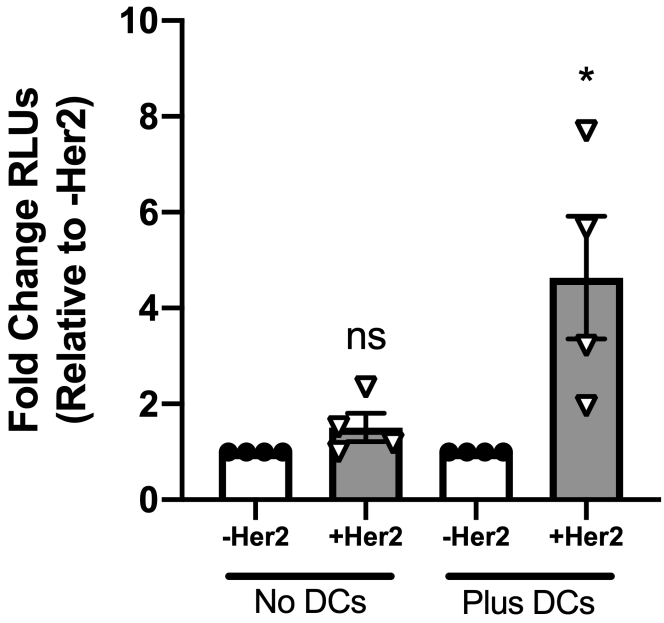


Figure 4.5: DCs facilitate HIV-1 expression in a transwell coculture system. Low affinity CAR+ T cells were resuspended in HIV-luciferase virus media and plated into wells. Transwell chambers were set into each well and DCs were added into the upper chambers of the transwells. Her2 was added to appropriate wells so that final concentration would equal 1 µg / ml. After 48 hours of culture transwells containing DCs were removed and discarded, and HIV-luciferase expression was assayed in remaining CAR+ T cell cultures. Luciferase values are set relative to conditions lacking Her2. Four individual experiments are shown, with columns representing the mean values ±SEM. *$p<0.05$.

Activating signal at the time of DC coculture does not determine potential for reactivation.

DCs and APCs have previously been shown to support establishment of latent infection in CD4+ T cells (Evans et al. 2013b; Kumar et al. 2015; 2018). To understand whether DC-T cell interaction and TCR signaling cooperate to influence the establishment of inducible latent infection, I performed a reactivation assay as depicted in Figure 4.6A. Briefly, DC-CAR+ T cell cocultures were carried out as described above. Following coculture, DCs were depleted and remaining CAR+ T cells were cultured in cytokine-free media for at least 7 days. I then activated cells by addition of phytohemagglutinin (PHA), IL-2, and IL-7 overnight and harvested cells for analysis the following day. To compare potential for induction between coculture conditions, I set HIV-1 expression in reactivated conditions relative to viral gene expression in corresponding conditions in the absence of reactivating stimuli. I did not observe any difference in induction of HIV-1 expression between conditions that were provided Her2 during coculture infection compared to conditions that were not (Figure 4.6B, 4.6C). These findings indicate that cell-to-cell transmission of HIV-1 by DCs is sufficient for establishment of an inducible infection and that T cell signaling through CARs does not influence potential for reactivation. So, although CAR signaling is required for robust HIV-1 expression, in this coculture context, Her2-dependent activation does not bias proviral transcriptional state, and the probability of establishment of a latent provirus that is either refractory to reactivation or more readily inducible remains the same.

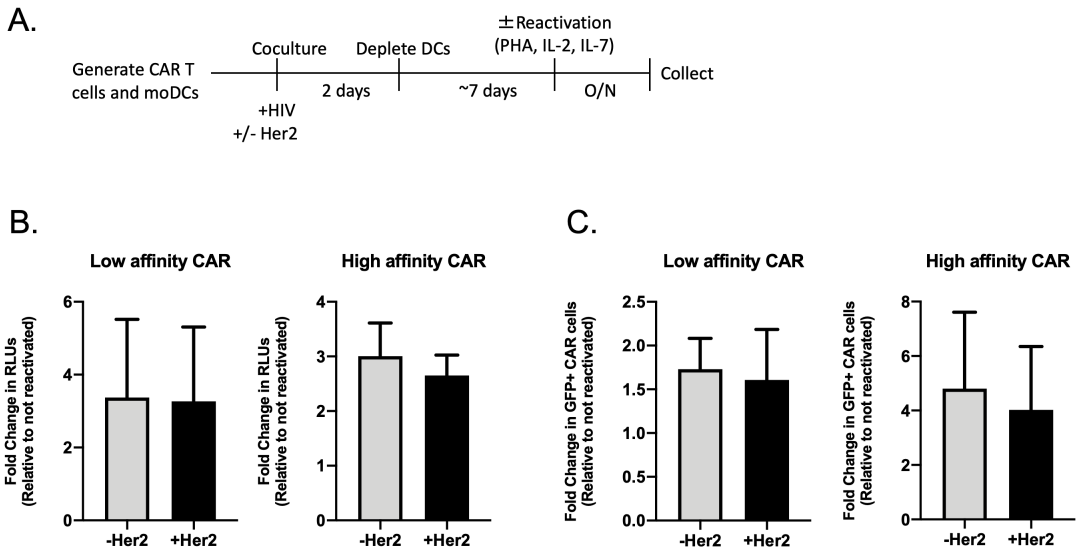


Figure 4.6: DCs are sufficient for establishment of inducible HIV-1 infection. A) Experiment strategy for experiments shown in B and C. Following coculture with DCs ±Her2, DCs were depleted and CAR+ T cell cultures were allowed to rest for 7 d, then reactivated with PHA, IL-2, and IL-7 and compared to corresponding conditions that were not reactivated. B) Relative luciferase expression was determined by comparison of cells that were reactivated to those that were not. n=3 for low affinity and n=2 for high affinity conditions. C) Relative GFP expression in CAR+ T cells was determined by comparison to cells that were not reactivated. n=3 for each. Data are presented as mean values ± SD.

Discussion

In vivo, T cell receptor signaling occurs in the context of additional signals transmitted during cell-cell contacts. Additionally, HIV-1 disseminates throughout the body at least in part through cell-to-cell transmission. The contribution of signals during cell-to-cell transmission of HIV-1, and how those signals crosstalk with T cell receptor signaling to impact HIV-1 infection, is not fully appreciated.

Our understanding of the role of TCR signaling during DC-CD4+ T cell transmission of HIV-1 is primarily based on studies using strong TCR signaling, such as those induced by superantigen-mediated crosslinking of TCR and MHC (Rodriguez-Plata

et al. 2013; Kumar et al. 2015, 2018). However, high affinity antigen-specific TCR-pMHC interactions are rare in vivo and even these interactions may not generate signals as strong as those observed with TCR crosslinking (Corse, Gottschalk, and Allison 2011; Germain and Stefanová 1999). Therefore, studying infection in these conditions is unlikely to capture the signaling environment for the majority of cells during infection in vivo. Most DC-T cell interactions will be antigen-nonspecific or low affinity pMHC-TCR interactions which still generate TCR signaling, in addition to signals transmitted during cell-cell contact (Dustin and Choudhuri 2016; Deng et al. 2016; Hioe et al. 2011; Len et al. 2017; Readinger et al. 2008; Vasiliver-Shamis et al. 2009). How different strengths of TCR signaling impact HIV-1 infection and how those intersect with other signals provided by the DC is not well understood. In this study, I developed a DC-CAR T cell coculture infection system to model signaling events during cell-associated HIV-1 transmission. Utilizing CAR+ T cells in this system allowed me to deliver different signaling strengths during DC-T cell transmission of HIV-1. CD4+ T cells in the coculture that did not express CARs were still susceptible to infection by DCs, but because Her2 specifically signals CAR-expressing cells, observed changes in HIV-1 expression were primarily due to changes specifically in the CAR+ population.

Previous work in our lab by Gagne et al. utilized these CARs to study the impact signal strengths on cell-free HIV-1 infection (Gagne et al. 2019). They found that cells receiving strong signals at the time of infection supported robust HIV-1 expression and established latent infections that were conducive to reactivation. Conversely, cells receiving weak or no signal during infection were comparatively less supportive of HIV-1

expression and established latent infections which were about 5-fold more refractory to expression following reactivation. These findings indicated that the strength of signals at the time of infection acted as a bifurcating event, biasing establishment of infection to a more readily inducible state or a form of latency that was more deep-seated. In this study, I sought to understand whether different strengths of activating signal had a similar impact on the establishment and maintenance of HIV-1 infection and latency during DC-T cell transmission.

I first assayed changes in CD69 expression in response to Her2 stimulation in DC cocultures. In low and high affinity DC-CAR+ T cell cocultures, provision of Her2 resulted in a similar induction of CD69 expression in the population. Importantly, an increase in CD69 expression was only observed in the CAR-expressing subset of CD4+ T cells and only in response to Her2. This differed from the response observed in monocultures in which Her2 resulted in twice as many CAR+ T cells becoming activated in response to strong signaling compared to weak signaling activity (Gagne et al. 2019). These findings indicate that DCs are providing compensatory or complementary signals to TCR signaling, thus facilitating efficient T cell activation even in conditions with sub-optimal TCR signals. Considering that in vivo CD4+ T cell activation occurs through recognition of antigen presented by an APC, it is logical that CD4+ T cells would be capable of integrating multiple signals. Since we are currently using the weakest signaling CAR from our panel in these experiments we do not know the lowest extent of signal strength that can support T cell activation in coculture, but it would be interesting to further investigate the minimal activating signals required. If a minimal signal strength for activation could be determined

in DC cocultures, comparing this to cocultures with other APCs could further elucidate the nature of signals that facilitate CD4+ T cell activation.

Transcriptional activity of HIV-1 is downstream of multiple signaling pathways that derive from the T cell receptor (Brownlie and Zamoyska 2013; Schiralli Lester and Henderson 2012). I utilized this coculture assay to determine if differences in signaling conditions resulted in different capacities for T cells to support integration of provirus or productive HIV-1 infection. Across all coculture conditions tested there was no appreciable difference in integration of provirus as measured by Alu-PCR (Agosto et al. 2007; 2009; Gagne et al. 2019) suggesting that DC-mediated infection is sufficient for establishment of infection and that the addition of activating signal did not increase efficiency of this process. This was somewhat surprising as it is well established that TCR-pMHC engagement stabilizes DC-T cell conjugates, which likely supports transmission of HIV-1 across the infectious synapse (Benvenuti et al. 2004; Benvenuti 2016; Ménager and Littman 2016; DeLucia, Rinaldo, and Rappocciolo 2018; Huppa and Davis 2003; Friedl, Den Boer, and Gunzer 2005). However, in this model system I provide activating signal via Her2 in soluble form rather than from the site of cell contact, which may impact the ability for DCs and T cells to reinforce their interaction or more efficiently transmit virus. In any case, in this model system the addition of activating signal did not improve efficiency of infection. However, when measuring establishment of productive infection, provision of activating signal via Her2 during coculture resulted in increased HIV-1 expression relative to conditions lacking Her2. Similar to what was observed with CD69 expression, signaling through the low and high affinity receptors resulted in equal support

for HIV-1 expression. Given that it is well established that activated cells are more supportive of HIV-1 replication (Pan et al. 2013; Oswald-Richter et al. 2004; Schiralli Lester and Henderson 2012; Stevenson et al. 1990; Zack, Kim, and Vatakis 2013), this result was expected, although I did not know for sure if the coculture conditions that were supportive of T cell activation would necessarily correlate to conditions supportive of HIV-1 expression. These data show that although DCs are sufficient to support HIV-1 transmission and infection, they alone cannot support robust HIV-1 expression. However, DCs facilitate HIV-1 expression by compensating for weak TCR signaling, therefore potentially enhancing HIV-1 replication in a tissue environment where there would be an abundance of weak TCR interactions.

A reservoir of latently infected cells is established early in the course of HIV-1 infection and is the major barrier to cure (Margolis et al. 2016; Mbonye and Karn 2017; R. F. Siliciano and Greene 2011). Understanding the conditions that are required or which favor formation of latency over productive infection is an active area of research and multiple models have been proposed to study this phenomenon (Pedro, Henderson, and Agosto 2019). Additionally, different DC and APC subsets have been shown to differentially support establishment of latency in CD4+ T cells (Evans et al. 2013b; Kumar et al. 2015; 2018). Whether additional TCR signaling in DC cocultures would further bias cells towards latency or productive infection is not known. In this study I began to probe this question using the DC-CAR T cell coculture system. I found that across all conditions, there was no impact on the potential for reactivation of HIV-1 expression associated with provision of activating signal via the CAR at the time of infection. This shows that although

activating signal is required for induction of robust HIV-1 expression, it does not bias infection towards a more refractory or inducible infection in DC cocultures. I should caution however that due to constraints of cell numbers in these experiments, I could not sort out cells that continued to express HIV-1 even after a week removed from coculture, so this is not a measure of true latency but rather inducible expression. Taken at face value, this suggests an intriguing model in which, in the context of DC-T cell transmission, activated CD4+ T cells are equally likely to establish an inducible infection as those that are not activated.

These data show that DCs facilitate CD4+ T cell activation and productive HIV-1 infection when paired with weak signals from the TCR. However, the addition of activating signal is dispensable for integration of provirus and does not impact the potential for reactivation. This study provides additional insight into a biologically important cell-cell interaction and mode of HIV-1 dissemination and suggests additional studies are warranted to better characterize the signaling environments that can facilitate the outcomes of HIV-1 expression and T cell activation.

CHAPTER 5: DISCUSSION AND CONCLUSIONS

Summary of Findings

Eradication of HIV will require permanently silencing or purging latent infection from the persistent reservoir. However, efforts to reactivate or silence viral expression have so far been inefficient, indicating a need for a more complete understanding of HIV transcription and expression. My project examines mechanisms of transcriptional regulation by looking at both transcription factors and networks, as well as signaling events upstream that are initiated by cell-to-cell transmission.

The molecular networks involved in the regulation of HIV replication and latency, especially proviral gene transcription, remain incompletely defined. To expand our understanding of these networks I utilized an unbiased high throughput yeast one-hybrid screen, which identified 42 human transcription factors and 85 total protein-DNA interactions with HIV-1 and HIV-2 long terminal repeats (Figure 3.2B). I investigated a subset of these transcription factors for their ability to regulate HIV transcription. KLF2 and KLF3, which bound all five HIV-1 and HIV-2 LTRs in the assay, were preferentially expressed in resting CD4+ T cells, and have established roles in transcriptional repression, which led me to hypothesize that KLF2 and KLF3 were repressors of HIV transcription. In infected unstimulated CD4+ T cells, KLF2 and KLF3 bind the HIV LTR and knockdown of these factors results in increased HIV RNA expression (Figure 3.7). In a cell line model of infection I show that increased occupancy of KLF2 and KLF3 at the HIV LTR correlates with decreased HIV expression, decreased acetylation of histone H3 and, in the case of KLF3, recruitment of HDAC2 to the LTR, suggesting that these factors repress HIV at

least in part by facilitating modification of local chromatin (Figure 3.10, 3.11). These data demonstrate that KLF2 and KLF3 are repressors of HIV expression in multiple models of infection. Another factor, PLAGL1, bound only HIV-2 LTRs in the eY1H assay and is a transcriptional activator, leading to the hypothesis that PLAGL1 was an activator of HIV-2 transcription. In unstimulated CD4+ T cells, PLAGL1 was found to occupy both HIV-1 and HIV-2 LTRs, however, knockdown resulted in a decrease of only HIV-2 RNA expression but not HIV-1 (Figure 3.13). A similar HIV-2 specific function was seen with PLAGL1 knockdown in infected MDMs (Figure 3.14). Overall, I used a high throughput functional screen and biochemical assays to identify and confirm processes and three novel candidate transcription factors that regulate HIV-1 and HIV-2 transcription.

To understand how DC-mediated signals crosstalk with TCR signaling to impact the outcome of cell-to-cell transmission of HIV-1, I employed a DC coculture model to deliver different strengths of activating signal to CAR T cells. I found that in cocultures DCs facilitate CD4+ T cell activation and HIV-1 expression by compensating or complimenting weak T cell signals (Figure 4.3, 4.4). DCs were able to influence both the active replication of HIV-1 in the presence of TCR-associated signaling and, in the absence of additional signals, DC-CD4+ T cell interactions supported establishment of inducible infection (Figure 4.6). These data suggest the existence of multiple signaling thresholds for establishment of HIV expression and establishment of inducible infection, with DC-provided signals compensating for TCR signaling to meet these thresholds.

These findings support my central hypothesis that multiple mechanisms including cell-to-cell transmission and intrinsic cell factors contribute to the establishment and maintenance of HIV transcriptional states including latency.

Remaining mechanistic questions

I have described regulatory roles for three novel HIV transcription factors, as well as ways in which DCs may contribute to functional outcomes in HIV infection and CD4+ T cell activation. However, these studies are not exhaustive and provoke several questions regarding mechanisms that might be the focus of future investigation. One question remaining about the transcription factors themselves relates to how they mediate changes in transcription and their discrete binding sites in the HIV LTRs. A second is whether LTRs from different HIV subtypes engage with or rely on common transcriptional networks for their expression. Third is the nature of signals provided by DCs that facilitate HIV expression and establishment of inducible infection.

KLF2 is a repressor of HIV RNA expression, and binding to the LTR is associated with a decrease in histone H3 acetylation. However, in the literature, KLF2 is primarily described as a transcriptional activator through recruitment of histone acetyltransferases CBP, p300, and PCAF to gene promoters (Turpaev 2020). How KLF2 might mediate repression and histone deacetylation at the HIV-1 LTR is not clear. Studies of KLF2 at other promoters have shown that KLF2 can repress through multiple mechanisms including, sequestration of HATs, interaction with HDAC5, and repression of hTERT in resting CD4+ T cells (Kwon et al. 2014; Hara et al. 2015; Turpaev 2020). Given that

deacetylation of histone H3 was associated with binding of KLF2 to HIV, I speculate that KLF2 binds and recruits class IIA HDAC complexes to the LTR to mediate repression of transcription.

On the other hand, KLF3 has a well-established mechanism of transcriptional repression. Group 1 KLFs, of which KLF3 is a member, are defined by their roles as transcriptional repressors through association with the adaptors C-terminal binding proteins (CtBP1/2) and recruiting histone modifying enzymes to gene promoters, including class I HDACs (Knights et al. 2016; Pearson, Funnell, and Crossley 2011). Given that this mechanism is well defined, and I detect changes in histone acetylation and presence of HDAC2 at the HIV-1 LTR, I expect that KLF3 is affecting histone deacetylation through CtBP1/2 and that this could be demonstrated by ChIP assay. However, KLF3 may be facilitating repression through additional mechanisms as well. KLF3/CtBP are also known to recruit histone methyltransferases, the lysine-specific demethylase LSD1, as well as the CoREST corepressor, which itself interacts with a vast network of histone and DNA modifying enzymes. Future investigations of KLF3-mediated repression of HIV should determine if changes in histone or DNA methylation patterns are associated with KLF3 binding to the LTR.

I present data showing PLAGL1 as an activator of HIV-2 transcription in primary cells. PLAGL1 is described as a transcriptional co-activator via interactions with several other factors including Sp1, AP-1, and CBP/p300, which also have known regulatory functions in HIV transcription (Vega-Benedetti et al. 2017; Krebs et al. 2001; Karn and Stoltzfus 2012; Schiralli Lester and Henderson 2012). Whether PLAGL1 is directly

cooperating with one or more of these factors to promote HIV expression is an outstanding question and could be addressed by ChIP assays. There was also my finding that PLAGL1 bound both HIV-1 and HIV-2 LTRs in cells, but knockdown of PLAGL1 only resulted in a change in HIV-2 RNA expression. I do not know why I observe differential transcriptional activity despite binding to both subtype LTRs, but I speculate that PLAGL1 may bind with higher affinity to the HIV-2 LTR, or that PLAGL1 binds HIV-1 but is dispensable for transcription. Although HIV-1 and HIV-2 share a number of regulatory elements, across the LTRs they only share a 40% sequence similarity, and a 50% similarity within the Sp1 sites specifically, so differences in factor binding affinities are not unlikely (Krebs et al. 2001). DNA binding assays using cell lysates and purified PLAGL1 protein could help determine if PLAGL1 has a higher affinity for binding the HIV-2 LTR.

Discrete binding sites for KLF2, KLF3, and PLAGL1 in the HIV LTRs would also be useful for further characterization of these factors. KLF and PLAG family proteins both have C2H2 zinc finger DNA-binding domains and preference for binding GC-rich regions of DNA (Cao et al. 2010; Varrault et al. 2017). Additionally, these domains are homologous to the DNA binding domains of Sp proteins which have known binding sites in both HIV-1 and HIV-2 LTRs and are GC-rich sites. KLF/Sp family proteins have previously been described to regulate gene expression in part through competition with other family members for access to DNA binding sites, and PLAGL1 has been shown to co-activate transcription of genes with Sp1 (Vega-Benedetti et al. 2017; Ilsley et al. 2017). In ChIP-PCRs using primers flanking the Sp1 sites I typically detected the greatest signal for these factors and detected weak or no signal when using primers specific for other

regions of the LTR. Taken together, I expect that KLF2, KLF3, and PLAGL1 are binding and mediating transcriptional regulation from or near the Sp1 sites in HIV LTRs.

In Chapter Four I present data demonstrating that DC-T cell infections establish inducible HIV-1 infection and compensate for weak signaling in CD4+ T cells, facilitating T cell activation and HIV-1 expression. However, which DC-provided signals are responsible for this are not yet clear. Antigen-independent DC-T cell interactions have been shown to engage some of the same signaling pathways as are activated by antigen-specific TCR-pMHC interaction, which could indicate that DCs are compensating for weak signaling through similar mechanisms. However, other signals from DCs are also likely to impact T cell activation and HIV expression. In a study comparing APC subsets that differed in their capacity to support HIV infection and latency in CD4+ T cells, Kumar et al. found that expression of certain cell surface signaling molecules was associated with latency establishment and engaged T cell signaling pathways including cell adhesion, co-stimulation, and antigen presentation, indicating that signaling through molecules not associated with the TCR may play an important role in the outcome of infection (Kumar et al. 2015). Additionally, in transwell experiments I show that DCs remain capable of supporting HIV expression even when physically separated from CD4+ T cells suggesting that soluble factors may contribute. It's likely that there is not a single signal or pathway responsible for determining the outcome of HIV infection, but rather that CD4+ T cells and HIV provirus are integrating multiple different signaling inputs to determine whether HIV transcription is supported or an inducible infection is established. To elucidate signaling pathways associated with establishment of latent infection, computational approaches

could be employed to compare the changes in CD4+ T cell gene expression associated with DC coculture to changes our lab observed following different strengths of T cell receptor signaling. Targeting common pathways using small molecule inhibitors would allow us to understand whether common mechanisms determine establishment of latent infection, or if DCs are facilitating latency though a parallel pathway.

I was not able to qualitatively describe a minimal signaling threshold required to support robust HIV expression in DC cocultures, as had previously been demonstrated by our lab in a cell-free infection model. In future work, cocultures with APCs that have different T cell engagement properties such as different DC subsets or macrophages, CARs with lower affinities for Her2, or CARs with alternative arrangements of signaling domains would help describe the nature of signals that promote HIV expression and establishment of inducible infection.

Strengths, limitations of experimental approaches and findings

As with all studies, there are strengths and limitations of the approaches employed in this dissertation that impact how these findings are interpreted or may be extrapolated to other settings. I will discuss some of those in this section.

In Chapter Three I utilize an eY1H assay to generate candidate transcription factors that bind to HIV and regulate transcription; the strengths and limitations of the assay itself were discussed in the chapter as well as in previous publications (Whiteld et al. 2012; Pro et al. 2018; Shrestha et al. 2019; Fuxman Bass et al. 2016), so here I will discuss how it was employed. Although over half of the transcription factors identified by the screen

bound to two or more LTRs, the five LTRs we screened do not capture the full diversity of HIV found in the human population. All three HIV-1 LTRs were derived from molecular clones of Group M subtype B viruses and both HIV-2 LTRs were derived from Group A viruses, together comprising about 12% of infections globally (Ingole et al. 2013; Taylor et al. 2008). There is significant diversity in the LTRs of HIV-1 Group M subtypes, moreover, the LTRs from a single infected individual can also differ depending on the tissue or cell type it was collected from (Krebs et al. 2001). eY1H assays have previously been shown to detect differences in binding to sequences that differ in just a single nucleotide polymorphism (Shrestha et al. 2019; Fuxman Bass et al. 2015), so differences in transcription factor binding patterns between subtypes or tissue-specific HIV-1 and HIV-2 viruses could be elucidated with further screens.

Additionally, because transcription factors act downstream of complex regulatory networks, results from eY1H assays can be leveraged to provide insights into signaling cascades critical for HIV expression. In our pathway enrichment analyses we were able to identify some common pathways utilized between the HIV-1 and HIV-2 LTRs we screened (Figure 3.5). In the future it would also be interesting to determine if different HIV LTRs engage with unique signaling networks to regulate their expression. Due to the relatively small number of transcription factors identified by our screen we were not sufficiently powered to determine differences by pathway enrichment analyses but screening additional LTRs or combining our results with other screens from the literature would permit evaluation of this question.

The knockdowns of KLF2 and KLF3 in HIV-infected CD4+ T cells resulted in predictable but fairly modest changes in HIV RNA expression. This was expected, as knockdown or inhibition of single repressive mechanisms in other studies has resulted in a relatively small increase in HIV expression (Panagoulias et al. 2018; Kaczmarek Michaels, Wolschendorf, et al. 2015; D. G. Wei et al. 2014; Archin et al. 2009). This is likely due to the existence of cooperative or redundant mechanisms that regulate expression in cells. In studies where inhibition of repression is combined with activation of pathways that promote transcription the effect on HIV expression is often synergistic, indicating that to an extent transcriptional repression and activation are distinct mechanisms and both need to be targeted for larger changes to be observed (Kim, Anderson, and Lewin 2018; Gutiérrez et al. 2016). To investigate this, knockdown of KLF2 and KLF3 could be paired with addition of latency reversing agents such as the protein kinase C agonist Bryostatin.

Although I describe a role in regulation of HIV by KLF2, KLF3 and PLAGL1, our eY1H screen generated many more transcription factor candidates to evaluate. One factor of particular interest is KLF4. KLF4 is a transcriptional repressor but is only expressed in CD4+ T cells following activation, has been implicated in imparting and maintaining T cell quiescence, has a homologous DNA-binding domain to KLF2, and was the strongest repressor of HIV expression I observed in cell line assays (Figure 3.3, Appendix Figure 2) (Yamada et al. 2009; Cao et al. 2010). I did not pursue KLF4 in these experiments primarily because nucleofection is not well-tolerated or efficient in activated CD4+ T cells, but future studies could employ tools such as shRNA lentiviral vectors for knockdowns. Several other

factors including KLF-, Sp- and Ets-family members also showed potential HIV regulatory activity in preliminary experiments and should be investigated in future studies.

Finally, as in all culture models, there are limitations as to the degree these findings can be extrapolated to the complex and diverse environments found *in vivo*. In these experiments I isolate CD4+ T cells and monocytes from PBMCs in blood, but cells in circulation differ in a number of ways from cells residing in tissues, including in terms of transcription factor expression and the nature of signals they are subject to. Though primary cell models do not perfectly recapitulate cells *in vivo,* I felt it was important to use them whenever possible. However, primary cells pose their own challenges such as donor-to-donor variability, limitations on cell numbers, and inefficient infection compared to cell lines. This was a limitation in my ability to do mechanistic studies of transcriptional regulation in primary cells, for instance, as ChIP-based assays are best suited to detect large changes in signal which often requires large cell populations. Using cell line models I was able to correlate changes in the landscape of the LTR to results from primary cell infections.

Significance and Implications

Of all the stages of HIV replication, transcription is unique in that it almost entirely relies on host cell factors for its replicative success. Once integrated as provirus, HIV is not fundamentally different from most other human genes, meaning that it is subject to the same basic principles of regulation. Therefore, the availability and activity of positive transcriptional regulators are a major determinant for HIV expression.

Transcriptional regulation of HIV occurs at multiple levels including epigenetic modifications, blocks to transcription initiation, and negative regulation of transcription elongation. Many, if not all, of these mechanisms are influenced by signals originating upstream from inputs in the cellular environment. For instance, in CD4+ T cells, signaling through the TCR signaling pathway simultaneously alleviates a number of blocks to efficient transcription and activates mechanisms that promote expression. However, the quantity and quality of TCR signaling itself is influenced by a number of factors including the potency of TCR-pMHC interaction, the density of pMHC presented on an APC, and the duration of APC-CD4+ T cell contact (Huppa and Davis 2003; Corse, Gottschalk, and Allison 2011). Additional layers of modulation of TCR signal strength come from costimulatory molecules, CD5, and adaptors such as Lck (Chakraborty and Weiss 2014; Gaud, Lesourne, and Love 2018). In this way, multiple mechanisms of repression occurring at multiple layers must be cleared to enable efficient HIV expression (Schiralli Lester and Henderson 2012). Additionally, although it's common practice to study the impact of TCR signaling on HIV expression and cellular processes using anti-CD3/CD28 beads or antibodies for stimulation, TCR signaling *in vivo* occurs only in the context of APC-T cell interaction. These cell-cell contacts include an exchange of a variety of additional signals provided by cell surface molecules and secretion of soluble factors (Molon et al. 2005; Grakoui et al. 1999; Griffiths, Tsun, and Stinchcombe 2010). It has been demonstrated that different virus-donor cells have differing capacities to support productive and latent HIV infection, but the signals they provide and the response in target CD4+ T cells that impact that process have not been thoroughly investigated. Furthermore, how signals from a virus-

donor cell crosstalk with TCR signaling to bias the outcome of HIV infection has not been the subject of detailed investigation.

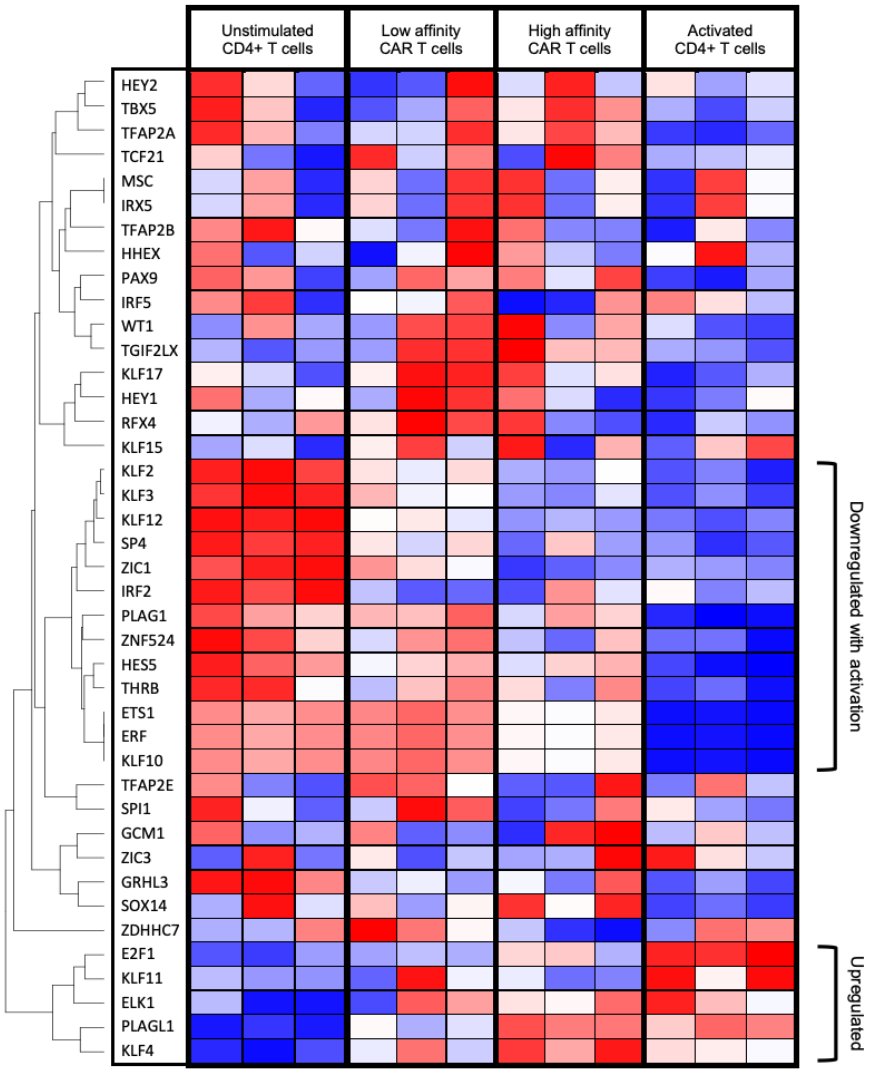
Computational approaches could leverage existing datasets in the literature including DC and CD4+ T cell gene expression analyses, the results of various HIV screens, and/or interactome tools to identify signaling cascades critical for HIV expression or latency. Enriched cascades could then be targeted by small molecule inhibitors in cell line models of infection to determine impact on HIV. Whether this approach identifies pathways that are already appreciated to regulate HIV, or whether novel pathways are identified, pinpointing critical cascades could help target therapeutic efforts. Additionally, targeting pathways upstream of transcription factors will have a broader impact on transcriptional regulation, modulating multiple transcriptional activating or repressive pathways simultaneously.

Finally, I describe what may be the first factor shown to have HIV-2 specific transcriptional activity in PLAGL1, though additional work and outside validation will be required. HIV-2 has a somewhat distinct clinical presentation compared to HIV-1 in that it is less pathogenic, immunogenic and has a delayed onset of immunodeficiency (Esbjörnsson, Månsson, et al. 2019; Marchant et al. 2006). Additionally, a number of observations have found that there is a lower viral load in HIV-2 infected patients (Buggert et al. 2016; Popper et al. 1999). It might be expected then, as some have proposed, that HIV-2 is transcriptionally repressed or less active compared to HIV-1 (Le Hingrat et al. 2020; Fenrick et al. 1989; Arya and Mohr 1994; Saleh et al. 2017). This is in part due to findings showing that the HIV-2 LTR is less responsive to stimulation, lacks an NFAT site,

and has only one conserved NF-κB site (Krebs et al. 2001). Additionally, recent work has shown that the HIV-2 Nef protein downregulated CD3 expression in infected cells, limiting a source of activating signals it might otherwise use to drive active transcription (Mesner et al. 2020; Hirao et al. 2020; Johnson and McCarthy 2019). It was therefore surprising to discover a transcriptional activator for HIV-2. I hypothesize that in order to support efficient replication in the absence of the key signals and transcriptional elements that are important for HIV-1 expression in CD4+ T cells, HIV-2 utilizes additional specific transcriptional activators to support expression, of which one factor is PLAGL1. The differences observed in in viral load then may be due to HIV-2 being less sensitive to activating signals, rather than it being an inherently repressed virus. Up to 2 million people are infected with HIV-2, approximately 5% of HIV infection globally, but receives a disproportionately small amount of attention in terms of treatment and cure efforts. This study and others suggest it is a functionally distinct virus and warrants more focused investigation.

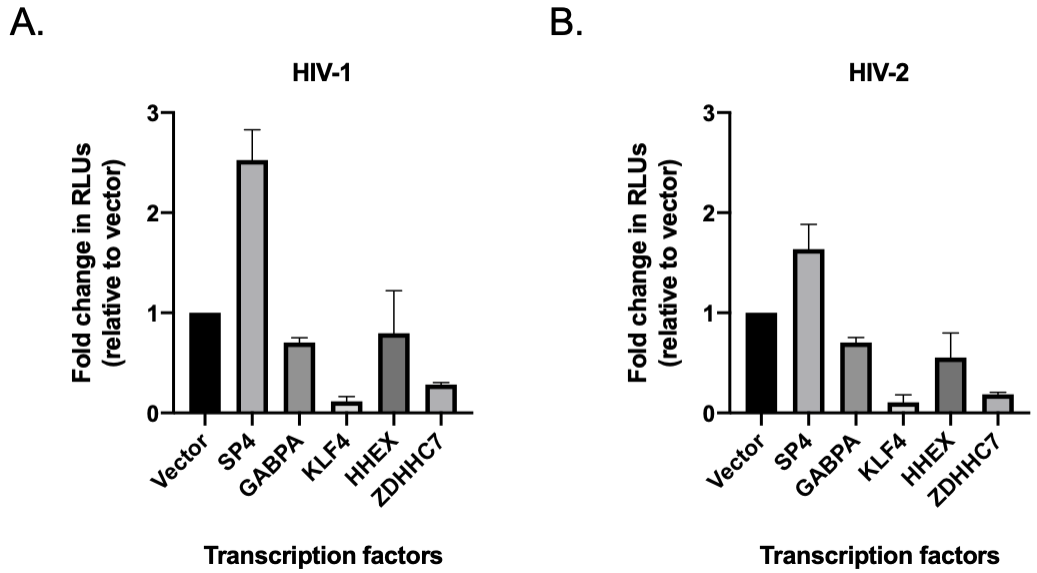
Overall these data presented here fit into and provide greater context to an established paradigm in which regulation of HIV transcription is an active process that is regulated at multiple levels and through numerous pathways.

APPENDIX 1: TRANSCRIPTION FACTOR EXPRESSION IN RESPONSE TO DIFFERENT STRENGTHS OF ACTIVATING SIGNAL



Appendix Figure 1: Gene expression of transcription factor hits from the eY1H assay in response to different strengths of activating signal. Unstimulated CD4+ T cells, anti-CD3/CD28 bead activated T cells, and Her2-stimulated low and high affinity CAR T cells were cultured for 24 h and gene expression was analyzed by microarray. This is an expanded figure of Figure 3.3B to include CAR+ T cells. Transcription factors that cluster together and are either upregulated or downregulated with activation are noted on the right. Many factors display a gradual change in gene expression associated with increasing strengths of activating signal. Three human donors were used in this assay. Clustering analysis was done using Heatmapper.ca (Babicki et al. 2016).

APPENDIX 2: CHANGES IN HIV EXPRESSION WITH TRANSCRIPTION FACTOR OVEREXPRESSION



Appendix Figure 2: Changes in HIV-luciferase expression in HEK293T cells with transcription factor overexpression. HEK293T cells were co-transfected with HIV-LTR constructs with luciferase reporters, and transcription factor expression constructs. Fold change in luciferase expression relative to vector control is shown. Mean values ±standard deviation is shown. n=2 for each condition.

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