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The role of T cell specific factors and RNA Polymerase II pausing in HIV-1 replication in CD4+ T cells

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THE ROLE OF T CELL SPECIFIC FACTORS
AND RNA POLYMERASE II PAUSING
IN HIV-1 REPLICATION IN CD4+ T CELLS

by

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DEDICATION

This thesis is dedicated to my grandfather, Franciszek Kaczmarek, who has always encouraged me to learn new things, and to my godmother, Jolanta Dzieniaczyk Para, who has always supported me.
ACKNOWLEDGMENTS

I would like to thank the Department of Molecular and Translational Medicine faculty, staff, and fellow doctoral students for their friendship and support. I wish to express special thanks to my advisor, Dr. Andrew J. Henderson, who made my doctoral studies a pleasant experience and was always extremely patient, supportive and optimistic. I would like to thank the members of my Thesis Committee, Dr. Gregory A. Viglianti, Dr. Rahm Gummuluru, Dr. Kathrin H. Kirsch, and Dr. Manish Sagar, for all their feedback. I would like to thank the members of the Henderson Lab for all of their help over the years. I would also like to thank the members of the Sagar Lab, especially Dr. Behzad Etemad, for all the mental support.

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THE ROLE OF T CELL SPECIFIC FACTORS
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KATARZYNA KACZMAREK

Boston University School of Medicine, 2015

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ABSTRACT

In order to eradicate HIV-1 infection the virus needs to be specifically eliminated from latently infected memory CD4+ T cells. There does not seem to be a single mechanism that promotes HIV-1 latency. RNA Polymerase II (RNAP II) pausing, chromatin structure, tissue specific transcriptional repressors and transcriptional interference have been implicated in regulating HIV-1 transcription. The transcription factor B Lymphocyte-Induced Maturation Protein 1 (Blimp-1) is expressed in B and T cells and upregulated in patients chronically infected with HIV-1. I hypothesized that Blimp-1 is a T cell intrinsic factor that binds to HIV-1 LTR, inhibits HIV-1 transcription and contributes to HIV-1 latency. Blimp-1 is expressed in primary peripheral blood CD4+ T cells and is further induced by T cell activation. Importantly, Blimp-1 is highly expressed in memory CD4+ T cells compared to naïve CD4+ T cells. Ectopic expression of Blimp-1 in CD4+ T cells represses HIV-1 transcription, whereas decreasing Blimp-1 in memory CD4+ populations activates HIV-1 transcription. Reduction of Blimp-1 in infected primary T cells increases RNAP II processivity and histone H3 acetylation. Blimp-1 binds downstream of the HIV-1 5’-LTR to the interferon-stimulated response
element (ISRE) in resting primary CD4\(^+\) T cells and strongly represses Tat-dependent HIV-1 transcription. Upon T cell activation, Blimp-1 is released from the HIV-1 ISRE and this correlates with significant increase in HIV-1 transcription. These results demonstrate that Blimp-1 acts to limit HIV-1 transcription in memory CD4\(^+\) T cells and promotes the establishment and maintenance of latency. I also examined whether neighboring host promoters could impact HIV-1 transcription. Using a set of inducible cell lines I observed that neighboring promoters have minimal impact on HIV-1 transcription and that enabling release of paused RNAP II by diminishing negative elongation factor (NELF) is sufficient to reactivate transcriptionally repressed HIV-1 provirus. The implications of my results in the different mechanisms regulating HIV-1 latency are discussed.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AFF1</td>
<td>AF4/FMR2 Family Member 1</td>
</tr>
<tr>
<td>AFF4</td>
<td>AF4/FMR2 Family Member 4</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>APOBEC-3G</td>
<td>Apolipoprotein B mRNA-editing Enzyme Catalytic Polypeptide-like 3G</td>
</tr>
<tr>
<td>ASP</td>
<td>Antisense Protein</td>
</tr>
<tr>
<td>asRNA</td>
<td>Antisense RNA</td>
</tr>
<tr>
<td>BAF</td>
<td>BRG1-associated Factors</td>
</tr>
<tr>
<td>bNAb</td>
<td>Broadly Neutralizing Antibody</td>
</tr>
<tr>
<td>Brd4</td>
<td>Bromodomain-Containing Protein 4</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CA</td>
<td>Capsid</td>
</tr>
<tr>
<td>CBF-1</td>
<td>C-Promoter Binding Factor-1</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C Chemokine Receptor Type 5</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT-enhancer-binding proteins</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytidine and Guanine Separated by a Phosphate</td>
</tr>
<tr>
<td>CPNEI</td>
<td>Copine I</td>
</tr>
</tbody>
</table>
HSV-6 .......................................................... Herpes Simplex Virus type-6
HIV-1 .......................................................... Human Immunodeficiency Virus
HIV-1 LTR ............................... Human Immunodeficiency Virus-Long Terminal Repeat
HP1 .......................................................... Heterochromatin Protein 1
HTLV-1 ...................................... Human T-cell Leukemia Virus type-1
IFN .......................................................... Interferon
IL .......................................................... Interleukin
IN .......................................................... Integrase
INI1 .......................................................... Integrase Interactor 1
ITK .......................................................... Interleukin-2-Inducible T-Cell Kinase
LCK .......................................................... Lymphocyte-Specific Protein Tyrosine Kinase
LEDGF .............................................. Lens Epithelium-Derived Growth Factor
LTR .......................................................... Long Terminal Repeat
LPS .......................................................... Lipopolysaccharide
LRA .......................................................... Latency Reversing Agents
Luc .......................................................... Luciferase
MA .......................................................... Matrix
MBD2 .......................................................... Methyl-CpG Binding Domain Protein 2
MHC II .................................................. Major Histocompatibility Complex Class II
MMSET ................................................ Multiple Myeloma SET domain
mRNA .......................................................... Messenger RNA
NC .......................................................... Nucleocapsid
P-TEFb ........................................ Positive-Transcription Elongation Factor b
RBM12 ...................................................... RNA binding motif protein 12
Rev .......................................................... Regulator of Expression of Virion Proteins
RNA ........................................................... Ribonucleic acid
RNAP II ....................................................... RNA Polymerase II
RPMI ................................................................ Roswell Park Memorial Institute
RON ............................................................. Recepteur d'Origine Nantais
RT ............................................................... Reverse Transcriptase
RT-PCR ....................................................... Reverse Transcriptase Polymerase Chain Reaction
SDS ............................................................. Sodium Dodecyl Sulfate
SDS-PAGE .................................................. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC ........................................................... Super Elongation Complex
SH2 .................................................................... Src-Homology 2
SH3 .................................................................... Src-Homology 3
SKIP ............................................................ Splicing-associated c-Ski-interacting Protein
snRNA ............................................................. Small Nuclear RNA
Sp1 .............................................................. Specificity Protein 1
STAT .......................................................... Signal Transducer and Activator of Transcription
SWI/SNF ..................................................... SWItching-Defective-Sucrose Non-Fermenting
TAR ............................................................. Trans-Activation Response Element
Tat ............................................................... Trans-Activator of Transcription
TCR ............................................................ T Cell Receptor

xviii
T<sub>CM</sub> .................................................. Central Memory T Cell
T<sub>EM</sub> .................................................. Effector Memory T Cell
T<sub>FH</sub> .................................................. Follicular B Helper T Cell
TFI1H .................................................. Transcription Factor II Human
T<sub>H</sub>0 .................................................. Naïve T Cell
T<sub>H</sub>1 .................................................. Type 1 Helper T Cell
T<sub>H</sub>2 .................................................. Type 2 Helper T Cell
T<sub>H</sub>17 .................................................. Type 17 Helper T Cell
TIGD5 .................................................. Tigger Transposable Element Derived 5
TNFα .................................................. Tumor Necrosis Factor α
TSA .................................................. Trichostatin A
TSS .................................................. Transcription Start Site
T<sub>reg</sub> .................................................. Regulatory T Cell
T<sub>SCM</sub> .................................................. Stem Cell Memory T Cell
T<sub>TM</sub> .................................................. Transitional Memory T Cell
Vif .................................................. Viral Infectivity Factor
VLP .................................................. Virus-Like Particle
Vpr .................................................. Viral Protein R
Vpu .................................................. Viral Protein U
VSV-G .................................................. Vesicular Stomatitus Virus Glycoprotein
YY1 .................................................. Yin Yang 1
WHSC1 .................................................. Wolf–Hirschhorn syndrome candidate 1
I. Introduction

1. Epidemiology of Human Immunodeficiency Virus

Infection with human immunodeficiency virus type 1 (HIV-1) leads to the development of Acquired Immunodeficiency Syndrome (AIDS), a disease whose hallmark is a decline in CD4\(^+\) T cells and an increased susceptibility to opportunistic infections and cancer. In addition to directly impacting the number of CD4\(^+\) T cells, HIV-1 infection leads to indirect immune exhaustion by activating neighboring or bystander cells and results in a general imbalance of all T cell populations, facilitating the immune dysregulation associated with AIDS (Chevalier and Weiss, 2013; Moreno-Fernandez et al., 2012; Paiardini and Muller-Trutwin, 2013). HIV-1 is a zoonotic disease that resulted from cross-species transmission of simian immunodeficiency virus (SIV) naturally infecting African non-human primates (Sharp and Hahn, 2011). HIV-1 is transmitted via bodily fluids and the life expectancy without treatment is 9 to 11 years (Programme and (UNAIDS), 2014). HIV-1 was first identified as a causative agent of AIDS in 1983 (Barre-Sinoussi et al., 1983; Gallo et al., 1983) but the first HIV-1 infection dates back to the 1920s (Faria et al., 2014). In 2013 the number of people living with HIV-1 worldwide reached an estimated 35 million (Programme and (UNAIDS), 2014) (Fig. 1). There were approximately 2.1 million new HIV-1 infections and 1.5 million AIDS-related deaths in 2013 (Fig. 1) (Programme and (UNAIDS), 2014). Although the disease has plateaued in the recent years due to treatment options and public health efforts, there is still no cure for HIV-1/AIDS.
The current combination therapy, called Highly Active Antiretroviral Therapy (HAART), was available to only 12.9 million people in 2013 (36.9% of infected people) (Fig. 1) (Programme and (UNAIDS), 2014). Drugs used in HAART target viral fusion, reverse transcription, integration and maturation of the virus. HAART does not inhibit viral transcription or release and because it targets viral proteins (with the exception of entry inhibitors) the virus mutates to escape the drugs action and develops resistance. In addition, HAART has many side effects and does not reverse the inflammation and premature aging associated with HIV-1 infection even when viral load is undetectable.

Figure 1. HIV-1 Epidemic in Numbers (based on UNSAIDS data).
2. Human Immunodeficiency Virus

**Virion Structure**

HIV-1 is a retrovirus belonging to the Lentivirus genus. It is a single-stranded, positive-sense, enveloped RNA virus. The hexameric p24 capsid (CA) protein forms a cone-shaped core which encloses two copies of viral RNA genome in complex with the p7 nucleocapsid (NC) protein, as well as the accessory proteins Tat and Rev. The capsid is surrounded by the p17 matrix (MA) protein which assembles into trimers. The outer surface of the HIV-1 membrane is a lipid bilayer derived from host cell during viral budding that is studded with envelope spikes composed of gp120 and gp41 trimers. The HIV-1 virion also contains three enzymes, reverse transcriptase, integrase and protease, enclosed inside the capsid, as well as accessory proteins Nef, Vpr and Vif (Fig. 2).
The genome of integrated HIV-1 provirus is 9719 base pairs long and is flanked at each end by long terminal repeats (LTRs) (Fig. 3). The 5’-LTR serves as the viral promoter and transcriptional enhancer. The genes encoded by HIV-1 are as follow:

- *gag* — encodes the precursor polyprotein Pr55Gag which is cleaved by viral protease into MA, CA, NC, and p6, as well as two spacer peptides, SP1, and SP2. MA, CA and NC are structural proteins and the expression of Gag in the absence
of other viral proteins and RNA genome is sufficient for the production of non-infectious virus-like particles (VLPs). p6 is required for incorporation of Vpr into the HIV-1 virions and contains late domain motifs necessary for virus budding (Garrus et al., 2001).

- **pol** – encodes Pol polyprotein processed into HIV-1 protease, reverse transcriptase and integrase. HIV-1 protease cleaves viral polyproteins (Gag and Pol) and thus is required for viral maturation. HIV-1 reverse transcriptase is responsible for transcribing viral RNA into DNA, while HIV-1 integrase is required for integration of HIV-1 DNA into host genome (Sierra et al., 2005).

- **env** – encodes the viral envelope polyprotein gp160 which is spliced into gp120 and gp41. gp120 binds to CD4 receptor and CXCR4 or CCR5 co-receptors enabling insertion of gp41 into PM, which in turn leads to viral fusion with the target cell (Postler and Desrosiers, 2013). The cytoplasmic domain of gp41 also contains signaling motifs that can activate NFκB, which is important for HIV-1 transcription (Postler and Desrosiers, 2013).

- **tat** – encodes cell membrane-permeable Tat (trans-activator of transcription), which binds to trans-activation response (TAR) RNA stemloop and recruits p-TEFb to initiate HIV-1 transcription (Watson and Edwards, 1999). Tat is cell membrane permeable (Watson and Edwards, 1999).

- **rev** – encodes Rev (Regulator of Expression of Virion Proteins), a protein required for viral mRNA splicing and export from the nucleus (Pollard and Malim, 1998).
- **nef** – encodes Nef (negative regulatory protein) which is important in modulating host cell signaling and responses. The expression of Nef alone in animal models results in AIDS-like symptoms (Rahim et al., 2009).

- **vif** – encodes Vif (viral infectivity factor), a protein that increases viral infectivity by targeting APOBEC-3G, a cytidine deaminase (deaminates cytidine into uridine0, for proteasomal degradation (Goila-Gaur and Strebel, 2008).

- **vpr** – encodes Vpr (viral protein R), a protein that induces cell cycle arrest in CD4+ T cells and facilitates infection of macrophages by unknown mechanisms (Li et al., 2009).

- **vpu** – encodes Vpu (viral protein U) important for viral budding by targeting tetherin for proteasomal degradation (Kueck and Neil, 2012).

---

**Figure 3. Organization of Human Immunodeficiency-1 Virus Genome.**

HIV-1 genome has LTR at each of its ends that serve as viral promoter and enhancer. HIV--1 encodes gag, pol and env polyproteins, as well as accessory proteins: Tat, Vif, Vpr, Vpu, Rev and Nef.
3. Replication Cycle of HIV-1

HIV-1 replication cycle is composed of the following steps: virus binding, fusion, uncoating, reverse transcription, integration, transcription, translation, assembly and budding (Fig. 4). Upon the binding of the HIV-1 envelope to CD4 receptor and a chemokine co-receptor expressed at the surface of the target cell, the viral fusion peptide penetrates host plasma membrane (PM) and then the virion fuses with the PM or with endosomal compartment (Postler and Desrosiers, 2013). In the cytoplasm the viral core disassembles and viral RNA is reverse transcribed into double-stranded cDNA by the viral reverse transcriptase. The uncoating of the virion might be blocked by TRIM5α (Yan and Chen, 2012). Reverse transcription is blocked by such host-restriction factors as APOBEC-3G (Yan and Chen, 2012), a cytidine deaminase that deaminates cytidine to uridine in the viral DNA during the process of reverse transcription, SAMHD1, which hydrolyzes dNTPs and degrades viral RNA (Yang and Greene, 2014) and TREX, an exonuclease digesting excessive HIV-1 DNA (Yan et al., 2010). Recent studies show that RNA polymerase II-associated factor 1 (PAF1) inhibits early steps of viral replication leading to decreased reverse transcription and proviral integration (Liu et al., 2011). The viral cDNA is imported to the nucleus by preintegration complex (PIC) and is integrated into the host genome by viral integrase. Lens epithelium-derived growth factor (LEDGF) binds to chromosomal DNA and HIV-1 integrase and favors HIV-1 integration into LEDGF-regulated, A-T-rich genes, while integrase interactor 1 (INI1) facilitates HIV-1 integration by remodeling nucleosomes (Ciuffi et al., 2005; Lesbats et al., 2011). The provirus is next transcribed by host transcriptional machinery into RNA. While the
multiply spliced viral RNAs encoding for Tat, Rev and Nef are processed by the cellular splicing and nuclear export machinery, the unspliced viral RNA genome and singly-spliced viral mRNAs (encoding Gag, Env, Vif, Vpr and Vpu) are transported to cytoplasm, a process controlled by the viral protein Rev (Felber et al., 1989). The envelope is synthesized on the rough endoplasmic reticulum (ER) and is transported through the ER to Golgi and trans-Golgi network (TGN) (Saftig and Klumperman, 2009). Gag is synthesized by membrane-unattached ribosomes (Perlman and Resh, 2006). Newly synthesized Gag accumulates in the perinuclear region, and before reaching the PM it passes through a late endosome-like compartment (Klein et al., 2007; Perlman and Resh, 2006). Gag is responsible for targeting HIV-1 to lipid rafts in host plasma membrane (PM) by binding to phosphatidylinositol-4,5-bisphosphate (PIP2) (Klein et al., 2007). The MA domain targets Gag to the PM via the 14-carbon-long N-myristyl group at the glycine 2 position, and the basic region at the amino-terminal end (Klein et al., 2007). Gag binds to the genomic RNAs, and together with the envelope assembles into HIV-1 virions, which then bud off the cell (Klein et al., 2007; Postler and Desrosiers, 2013). The budding of HIV-1 is blocked by such host factors as tetherin, ISG15, miRNA, TIM-1, TIM-3 and TIM-4 (Chen et al., 2014; Li et al., 2014; Pincetic et al., 2010; Yan and Chen, 2012). HIV-1 matures by cleavage of the Gag precursor, Pr55Gag, by viral protease into MA, CA, NC, and p6, as well as two spacer peptides, SP1 and SP2.
HAART is a combination of several antiviral drugs that target viral fusion, reverse transcription, integration and maturation of HIV-1. A remaining challenge in efforts to cure HIV-1 infection is targeting cells harboring quiescent, yet replication-competent virus. HAART and host immune system do not affect latently-infected cells which are the source of viral rebound after cessation of antiretroviral treatments (Richman et al., 2009; Siliciano and Greene, 2011). Upon cessation of HAART, HIV-1 rapidly reemerges from latently infected cells to pretreatment viral loads (Fig. 5) (Richman et al., 2009; Siliciano and Greene, 2011). HIV-1 preferentially infects activated CD4+ T cells. HIV-1 infection either leads to productive HIV-1 replication and cell death or to the transition of effector cell into a memory CD4+ T cell and establishment of latency. Memory CD4+ T cells are considered the main reservoir of latent HIV-1. The latently infected cells persist for the life of an infected individual. In addition to memory CD4+ T cells, other latent reservoirs are thought to exist. Using dual-reporter virus
expressing one HIV-1 LTR-dependent marker to measure productive HIV-1 infection, and a second marker under a constitutive promoter independent of HIV-1 LTR it has been shown that HIV-1 establishes latency even in activated CD4+ T cells (Calvanese et al., 2013). Strategies to target latent reservoir requires characterizing the cell populations that harbor latent HIV-1 and understanding the biochemical mechanisms that regulate provirus expression in these cells.

The size of a latently-infected viral reservoir is still a matter of debate (Stevenson, 2013). Originally, based on the ability to grow virus from blood samples in vitro (viral outgrowth assay), the size of latent reservoir was estimated to be 1 cell in a million. However, recent studies have suggested that the size of latent reservoir is at least 60-fold higher than previously thought (Ho et al., 2013). Plus, exactly what cells and tissues harbor latent infected cells has made it difficult to accurately measure the total size of the viral reservoir in vivo. It has been also estimated that the latent reservoir must be reduced by more that 10,000-fold to cure HIV-1 infection (Hill et al., 2014).
The establishment of latent reservoir occurs within the first 3 days of SIV infection (Whitney et al., 2014). Studies using dual fluorescent reporter virus that allows detection of infected cells independent of transcription from HIV-1 LTR confirm that the majority of virus becomes latent shortly after infection and that it can be reactivated (Dahabieh et al., 2013). Thus, initiation of HAART as early as possible increases the chances of not only stopping viremia but also decreasing the possibility of viral rebound (Whitney et al., 2014). The “Mississippi Baby” who was perinatally infected with HIV-1...
and who within 30 hours after birth was put on HAART for 18 months did not have a viral rebound for over 2 years after cessation of treatment (Ledford, 2014). In contrast, “Boston patients”, whose viremia was actively suppressed by HAART prior to receiving bone marrow transplants to repopulate hematopoietic cells had viral rebound within a few months of HAART interruption (Henrich et al., 2014) suggesting that early treatment might diminish but not completely suppress virus reservoir establishment and is associated with positive prognosis.

5. Molecular Mechanisms of HIV-1 Latency

The mechanisms that establish HIV-1 latency remain incompletely defined and research has focused on general events that control gene expression including transcription initiation, elongation and epigenetic regulation of chromatin (Richman et al., 2009; Schiralli Lester and Henderson, 2012; Siliciano and Greene, 2011). HIV-1 latency is driven by transcriptional repression; however there does not seem to be a single mechanism that promotes HIV-1 latency. RNA Polymerase II (RNAP II) pausing, chromatin structure and recruitment of ubiquitously expressed transcription factors as well as non-coding RNAs have been implicated in the regulation of HIV-1 transcription. Additionally, T cell specific factors may be repressing HIV-1 transcription in memory CD4+ T cells, the major reservoir of latent HIV-1. Transcriptional interference from neighboring host promoters is also thought to contribute to establishing and maintaining HIV-1 latency (Fig. 6).
Additionally, HIV-1 latency can be a stochastic process, where the virus enters latency and gets reactivated regardless of T cell activation level (Ho et al., 2013). The presence of Tat has been shown to inhibit the establishment of HIV-1 latency (Donahue et al., 2012). Superinfection with HIV-1 results in Tat-dependent reactivation of latent virus, suggesting that limiting amounts of Tat drive HIV-1 latency (Donahue et al., 2013). Strategies targeting signals which regulate recruitment of transcription factors, chromatin remodeling complexes or regulators of RNAP II have been devised to activate and purge HIV-1 latent reservoirs (Deeks et al., 2012; Lafeuillade, 2012; Margolis and Hazuda, 2013; Richman et al., 2009; Siliciano and Greene, 2011; Tyagi and Bukrinsky, 2012).
HIV-1 LTR controls provirus transcription by functioning as a promoter and enhancer recruiting host transcription factors and co-activators necessary to initiate transcription. HIV-1 LTR has three binding sites for the specificity protein 1 (Sp1) and two binding sites for the nuclear factor κB (NFκB). Binding of Sp1 and NFκB to HIV-1 LTR, as well as their physical interaction is required for robust HIV-1 transcription (Perkins et al., 1993). In addition to Sp1, NFκB acts in synergy with the activator protein 1 (AP1), a heterodimer of c-Fos and c-Jun, to induce HIV-1 transcription (Yang et al., 1999). HIV-1 LTR also contains three binding sites for CCAAT-enhancer-binding proteins (C/EBP) (Tesmer et al., 1993), which although not required, enhance basal and active HIV-1 transcription in macrophages (Henderson and Calame, 1997; Henderson et

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**Figure 6. Model for HIV-1 Transcriptional Regulation.**

During initiation of HIV-1 transcription RNAP II is recruited to HIV--1 LTR and transcribes nascent TAR RNA. However, transcriptional repressors bind to HIV--1 LTR and NELF forms a complex with RNAP II, DSIF and Pcf11 resulting in paused RNAP II at HIV--1 LTR. Additionally, methyltransferases and HDACs condense chromatin making it inaccessible to the transcriptional machinery. All these events are cooperative and combinatorial. Upon cell stimulation, transcriptional activators and enhancers are recruited to HIV--1 LTR, Tat binds to TAR RNA and recruits P-TEFb which activates RNAP II by phosphorylating its CTD. P-TEFb also phosphorylates NELF and DSIF. Phosphorylated NELF dissociates from RNAP II, while phosphorylation of DSIF changes it from transcriptional repressor to transcriptional activator. Histone modifying factors remodel and remove methyl groups from Nuc-1 making the chromatin more accessible to the transcriptional machinery. DSIF and Pcf11, which is required for transcript termination, travel down the provirus with RNAP II. For a more detailed description of HIV-1 transcription please see the text.

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5.1 Transcription Factors
al., 1995). Upon cell activation, the transcription factor II human (TFIIH) gets recruited to HIV-1 LTR where it phosphorylates CTD of RNAP II enabling HIV-1 reactivation (Kim et al., 2006). T cell stimulation also activates RBF2, a complex composed of USF-1, USF-2 and TFII-I to enhance HIV-1 transcription (Chen et al., 2005). Ets-1 stably binds to HIV-1 LTR 1 and physically interact with NFκB, NFAT and USF-1 to induce HIV-1 transcription (Bassuk et al., 1997; Gegonne et al., 1993; Seth et al., 1993; Sieweke et al., 1998). A subset of T cell transcription factors have been suggested to rapidly reactivate latent HIV-1 (Kaczmarek et al., 2013). NFAT was considered critical for overcoming latency and induction of HIV-1 transcription in different T cell subsets (Dahabieh et al., 2011; Malcolm et al., 2008), although recent studies have questioned its role in the reactivation of latent HIV-1 (Bosque and Planelles, 2009; Kinoshita et al., 1998; Pessler and Cron, 2004; Robichaud et al., 2002).

HIV-1 LTR also can be bound by transcriptional repressors and contains negative regulatory element (NRE) between -420 and -154 of HIV-1 LTR which mediates inhibition of HIV-1 transcription (Rosen et al., 1985; Siekevitz et al., 1987). p50 homodimers, a subunit of NFκB, bind to HIV-1 LTR and recruit histone deacetylase 1 (HDAC1), which in turn decreases histone acetylation and recruitment of RNAP II repressing proviral transcription (Williams et al., 2006). p50 can also form heterodimers with E2F1 protein which repress NFκB-induced HIV-1 transcription (Kundu et al., 1997). Yin Yang 1 (YY1) has been shown to recruit HDAC1 to a positioned Nucleosome-1 (Nuc-1) adjacent to the HIV-1 transcriptional start site, to decrease histone H4 acetylation at Nuc-1, and to co-operate with LSF to represses HIV-1 (He and Margolis, 2002;
Margolis et al., 1994; Romerio et al., 1997). However, recent studies showed that decreasing YY1 re-activates latent HIV-1 without affecting the recruitment of HDACs or the levels of acetylated histone H3 (Barton and Margolis, 2013). C-promoter binding factor-1 (CBF-1), a heterochromatin marker, represses HIV-1 transcription by inhibiting recruitment of RNAP II and acetylation of Nuc-1 (Tyagi and Karn, 2007). The T cell commitment factor B-cell lymphoma 11b (Bcl-11b, also known as CTIP-2) (Liu et al., 2010) binds HIV-1 LTR and limits transcription by binding Tat and redirecting it to heterochromatic regions (Le Douce et al., 2012; Marban et al., 2007; Rohr et al., 2003).

5.2 RNA Polymerase II Promoter Proximal Pausing

RNAP II pausing is defined by stalling of the RNAP II at a promoter and is a critical checkpoint in controlling gene transcription (Gaertner et al., 2012; Rahl et al., 2010). RNAP II pausing is characterized by the accumulation of short initiated mRNA transcripts but lack of transcriptional elongation. RNAP II pausing may be beneficial for the transcription of a gene, as deletion of eleven-nineteen lysine-rich leukemia (ELL), which facilitates RNAP II pause site entry, results in loss of P-TEFb recruitment to pre-initiation complex and disrupts elongation (Byun et al., 2012). Negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF) induce promoter-proximal pausing of RNAP II (Wada et al., 1998a; Yamaguchi et al., 1999). DSIF is a heterodimer composed of hSpt4 and hSpt5 that interacts with RNAP II and nascent RNA. NELF is
composed of four subunits (NELF-A, NELF-B, NELF-C/D and NELF-E) and binds to pre-formed DSIF-RNAP II complexes (Narita et al., 2003; Yamaguchi et al., 2002). P-TEFb, a heterodimer of Cdk9 and cyclin T1, phosphorylates C-terminal domain (CTD) of RNAP II activating it and releasing the pause induced by NELF and DSIF (Fujinaga et al., 2004; Peterlin and Price, 2006; Wada et al., 1998b; Yamaguchi et al., 1999). P-TEFb also phosphorylates NELF-E causing its release and hSpt5 subunit of DSIF, switching DSIF to a stimulator of RNAP II processivity (Fujinaga et al., 2004; Ivanov et al., 2000; Wada et al., 1998a; Yamada et al., 2006). P-TEFb can be brought to poised genes by bromodomain-containing protein 4 (Brd4), which recruits it from the repressive 7SK/HEXIM1 complex composed of 7SK small nuclear RNA (snRNA), HEXIM, LARP7 and MEPCE (Jang et al., 2005; Krueger et al., 2008; Yang et al., 2005). CDK7 is a subunit of TFIH that recruits DSIF to promoters inducing RNAP II pausing, and that is also required for P-TEFb-mediated phosphorylation enabling transcriptional elongation (Larochelle et al., 2012). In addition, Gdown-1 inhibits transcriptional initiation by TFIIF (Cheng et al., 2012). TRIM28 has been recently implicated in regulating RNAP II pausing at mammalian genes (Bunch et al., 2014). Nonphosphorylated TRIM28 at S824 stabilizes paused RNAP II, while S824-phosphorylated TRIM28 is required for release of paused RNAP II (Bunch et al., 2014). c-Myc is another factor required for the release of paused RNAP II (Rahl et al., 2010). While these cellular factors have been shown to be necessary for general transcription from host promoters, roles in HIV-1 LTR-mediated transcription have still not been confirmed for all.
In latently-infected cells, transcription of HIV-1 provirus is inhibited at the step of transcription elongation. NELF binds at HIV-1 LTR to RNAP II/DSIF complex inducing RNAP II promoter proximal pausing (Zhang et al., 2007). NELF also recruits Pcf11 and NCoR1-GPS2-HDAC3 complex to HIV-1 LTR which results in premature termination of transcription and condensed chromatin, respectively (Natarajan et al., 2013). Recently, the microprocessor complex composed of RNase III Drosha and the dsRNA-binding protein Dgcr8 has been shown not only to cleave the 5' stem loop RNA structure, TAR, but also to recruit Setx, Xrn2, and Rrp6 leading to RNAP II stalling at HIV-1 LTR (Wagschal et al., 2012). Setx and Xrn2 are transcription termination factors mediating premature termination of transcription, while Rpr6 processes cleaved TAR RNAs to repress HIV-1 transcription (Wagschal et al., 2012). To overcome RNAP II promoter proximal pausing, HIV-1 encodes a transcriptional activator, Tat, which via cyclin-dependent kinase substrate 1 (NUCKS1)-dependent mechanism binds TAR RNA (Kim et al., 2014) and gets activated by the lysine methyltransferase Set7/9-KMT7 (Pagans et al., 2010). Cyclin T1-associated Tat recruits AF4/FMR2 family member 1 (AFF1)/P-TEFb complexes (Lu et al., 2014a) to HIV-1 LTR that are normally sequestered away by HEXIM1–7SK snRNA regulatory complex (du Chene et al., 2007; Garber et al., 1998; Wei et al., 1998). P-TEFb alleviates transcriptional repression by phosphorylating components of the paused RNAP II complex including the CTD of RNAPII facilitating productive transcription elongation (Isel and Karn, 1999). Protein phosphatase 1A (PPM1A) is present at high levels in resting CD4+ T cells and inhibits P-TEFb activation by preventing CKD9 T-loop phosphorylation, which in turn leads to repression of HIV-1
transcription (Budhiraja et al., 2012). In addition, Brd4 competes with Tat for binding to P-TEFb (Bisgrove et al., 2007). The splicing-associated c-Ski-interacting protein, SKIP, binds to Tat/P-TEFb complexes, and together with c-Myc and Menin transactivates Tat, as well as promotes transcriptional elongation of HIV-1 (Bres et al., 2005; Bres et al., 2009). The Super Elongation Complex (SEC) composed of P-TEFb, Eleven-Nineteen Lysine-Rich Leukemia (ELL2), AFF1, AF4/FMR2 Family Member 4 (AFF4), eleven-nineteen-leukemia (ENL), and ALL1-fused gene from chromosome 9 protein (AF9) binds to RNAP II and promotes efficient elongation (He et al., 2011).

5.3 Chromatin Structure

Regardless of the integration site the 5’ end of HIV-1 provirus is associated with Nucleosome-0 (Nuc-0) and Nuc-1 (Verdin et al., 1993). Nuc-0 is positioned upstream of 5’-HIV-1 LTR, while Nuc-1 is located directly downstream of the HIV-1 LTR at approximately 100 nt after the transcription start site (TSS) and is better studied in regulation of HIV-1 transcription than Nuc-0 (Verdin et al., 1993). Histones can be post-translationally modified by acetylation, methylation, phosphorylation and ubiquitination. Factors associated with the HIV-1 LTR recruit histone acetyltransferases (HATs) and KDM demethylases that regulate chromatin organization of the integrated provirus (Schiralli Lester and Henderson, 2012). HATs such as p300/CBP and P/CAF, are recruited by Tat to acylate not only histones but also NF-κB (Deng et al., 2001; Furia et
al., 2002). However, transcriptional repressors are also recruited to HIV-1 LTR, such as methyltransferases including SUV39H1 (du Chene et al., 2007), G9a (Imai et al., 2010), GLP (Ding et al., 2013) and enhancer of Zeste 2 (EZH2) (Friedman et al., 2011), as well as HDACs (Keedy et al., 2009), which methylate and deacetylate histones, respectively, within positioned nucleosomes favoring condensation of chromatin and making the proviral LTR less accessible for efficient transcription. HDAC2 and HDAC3 have been shown to be present in the nuclei of latent cells and to repress HIV-1 transcription (Keedy et al., 2009). In microglial cells Bcl-11b recruits HDAC1, HDAC2, and SUV39H1 to HIV-1 LTR resulting in deacetylation and methylation of Nuc-1 (Marban et al., 2007). Furthermore, Bcl-11b facilitates binding of heterochromatin protein 1 (HP1) (Marban et al., 2007). In addition, CpG methylation of the 5′-HIV-1 LTR by methyl-CpG binding domain protein 2 (MBD2) is not required for the establishment of HIV-1 latency, but has been associated with increased resistance to reactivation (Blazkova et al., 2009; Kauder et al., 2009). BRG1-associated factors (BAF), a member of switching-defective-sucrose non-fermenting (SWI/SNF) family, represses HIV-1 transcription by re-positioning Nuc-1closer to the HIV-1 LTR (Rafati et al., 2011). PBAF, another member of (SWI/SNF family), gets recruited by Tat and enables remodeling of Nuc-1 and transcriptional elongation (Easley et al., 2010; Rafati et al., 2011). USF-1 co-operates with Sp1, NF-kB and LEF-1 to remove histone NH(2) tails from DNA (Angelov et al., 2000). Integration of HIV-1 close to nuclear bodies containing promyelocytic leukemia protein (PML) is associated with increased H3K9me2-methylation mediated by the methyltransferase G9a and thus increased latency (Lusic et al., 2013). Overall, these multiple posttranslational
changes of histones contribute to coordinated mechanism that can positively and negatively influence the epigenetic state of HIV-1.

5.4 Influence of Neighboring Promoters

Three-fourths of HIV-1 provirus preferentially integrates into introns of actively transcribed host genes (Ding et al., 2013; Lenasi et al., 2008; Lewinski et al., 2005; Shan et al., 2011; Sherrill-Mix et al., 2013). Transcriptional interference is thought to occur when HIV-1 integrates next to a gene with stronger promoter. HIV-1 can integrate in three orientations relative to the host gene: parallel or tandem, convergent and divergent orientation (Fig. 7). Transcriptional interference has been shown to suppress gene expression, especially when the genes are in a convergent or parallel orientation (Eszterhas et al., 2002). In the parallel orientation scenario, RNAP II from the upstream gene reads through HIV-1 LTR displacing its transcriptional machinery (Fig. 7) (Lenasi et al., 2008). The inhibition of transcription from the upstream promoter can induce HIV-1 transcription in the parallel orientation (Lenasi et al., 2008). In the convergent orientation the two transcriptional machineries from two promoters are thought to collide leading to transcriptional interruption of both genes (Fig. 7). In the divergent orientation scenario the two promoters may be competing for limited quantities of transcriptional activators and RNAP II (Fig. 7). The integration site of provirus has been shown to not only affect the transcriptional activity of provirus (Jordan et al., 2001), but also the
persistence and expansion of HIV-1-infected cells (Maldarelli et al., 2014). For example, integration of provirus into introns of BACH2 and MKL2 in the same transcriptional orientation correlates with clonal expansion and persistence of infected cells (Ikeda et al., 2007; Maldarelli et al., 2014). Early studies showed that transcriptional interference is strongest in the convergent orientation, while it has the weakest influence when promoters are in a divergent orientation (Eszterhas et al., 2002). The integration site played the strongest role on transcriptional activity in the parallel orientation (Eszterhas et al., 2002).
Additionally, numerous antisense RNA (asRNA) transcripts that may originate from transcriptional interference have been identified which might also regulate HIV-1 transcription and replication (Landry et al., 2007; Michael et al., 1994). In addition, HIV-1 LTR has been also implicated in the generation of asRNAs (Bentley et al., 2004; Peeters et al., 1996), which have been shown to halt viral transcription (Kobayashi-Ishihara et al., 2012; Tagieva and Vaquero, 1997). A recent study detected antisense protein (ASP) which is encoded by a region in the minus strand env gene, and which regulates HIV-1 replication by inducing autophagy (Torresilla et al., 2013).

5.5 T Cell Transcription Factors Implicated in Regulation of HIV-1 Transcription

CD4+ effector T cell subsets possess diverse specialized functions. CD4+ T helper cells (T_{H1}, T_{H2}, T_{H17}) are responsible for the production of cytokines stimulating specific immune responses, follicular B helper T cells (T_{FH}) support B cell activation, regulatory T cells (T_{reg}) suppress immune responses elicited by CD4+, CD8+ T cells and
B cells, while memory T cells are important for the recall of immune response (Murphy and Stockinger, 2010; van Leeuwen et al., 2009; Zhu et al., 2010). During infection antigen presenting cells (APC) display antigenic peptides in the context of MHC II to naïve CD4⁺ T cells (T₉₀), promoting their clonal expansion and polarization into effector T cells. A subset of activated CD4⁺ cells will generate memory T cells responsible for rapid recall of the adaptive immune response upon re-exposure. The differentiation of CD4⁺ T cells is driven in part by avidity of T cell receptor (TCR) engagement, strength of signaling, co-stimulatory signals and tissue microenvironments which include cytokine milieu and differential interactions with APC (Fig. 8) (Zhu et al., 2010). Additionally, CD4⁺ T cell development is controlled by a constellation of transcription factors that activate and repress batteries of genes that influence proliferation, differentiation and lineage commitment (Murphy and Stockinger, 2010; van Leeuwen et al., 2009; Yamane and Paul, 2013).
Although all CD4+ T cells are susceptible to HIV-1 infection due to their expression of CD4 and chemokine receptors, CXCR4 and CCR5, the ability of different T cell populations to support HIV-1 replication varies (Chevalier and Weiss, 2013; Figure 8. Model of Sequential CD4+ T Cell Differentiation. Modified from (Kaczmarek et al., 2013)

Upon activation by antigen presenting cell (APC), naïve T cell (T\textsubscript{H0}) undergoes differentiation into effector or memory populations. The different effector populations have capacity to mature into effector memory T cells (T\textsubscript{EM}). Stem cell memory T cells (T\textsubscript{SCM}), follicular helper T cells (T\textsubscript{FH}) and regulatory T cells (T\textsubscript{reg}) mature into central memory T cells (T\textsubscript{CM}), which then can become transitional memory T cells (T\textsubscript{TM}) and further convert into T\textsubscript{EM}. 

Although all CD4+ T cells are susceptible to HIV-1 infection due to their expression of CD4 and chemokine receptors, CXCR4 and CCR5, the ability of different T cell populations to support HIV-1 replication varies (Chevalier and Weiss, 2013;
Moreno-Fernandez et al., 2012; Paiardini and Muller-Trutwin, 2013), possibly reflecting differential expression of T cell-specific transcription factors that regulate HIV-1 expression. It is possible that these T cell factors, by promoting HIV-1 transcription, influence the dissemination of virus at different stages of AIDS or, by repressing proviral transcription contribute to the establishment of latently infected T cells. By targeting CD4\(^+\) effector T cells, HIV-1 has a dramatic impact on the depletion, expansion and function of the different polarized T cell subsets. The maturation of T cell lineages is in part driven by intrinsic transcription factors which potentially influence how efficiently HIV-1 replicates (Table 1). Quiescent memory CD4\(^+\) T cells have been implicated as the primary HIV-1 reservoir because they are susceptible to HIV-1 infection, are long-lived and, with their ability to self-renew, potentially maintain pools of latently infected cells. Whether there are T cell specific factors that predispose memory cells to latent HIV-1 infection has not been demonstrated.

The polarization of T\(_{H1}\) verses T\(_{H2}\) cells is mediated by two primary transcription factors, T-bet and GATA-3, respectively, whereas ROR\(\gamma\)t facilitates the differentiation of T\(_{H17}\) cells (Amsen et al., 2009; Muranski and Restifo, 2013; Zhu et al., 2010). T\(_{H1}\) cells have been reported to be limited in their ability to support HIV-1 replication, although there is no evidence that T-bet, the T\(_{H1}\) master transcription factor, directly influences HIV-1 transcription (Gosselin et al., 2010). T-bet antagonizes GATA-3 function, which is considered the key regulator of T\(_{H2}\) differentiation (Amsen et al., 2009; Kanhere et al., 2012; Zhu et al., 2010). GATA-3 binds to several sites in the HIV-1 LTR and induces HIV-1 transcription (Galio et al., 1997; Yang and Engel, 1993). Therefore, T-bet
potentially limits HIV-1 transcription by targeting GATA-3. In addition to GATA-3, c-Maf promotes T_{H2} differentiation (Ho et al., 1998) and, in regards to HIV-1 transcription, binds HIV-1 LTR and cooperates with NF-κB and NFAT to enhance HIV-1 transcription (Zhang et al., 2012). The ability of GATA-3 and c-Maf to activate HIV-1 transcription is consistent with findings that T_{H2} cells support HIV-1 replication (Gosselin et al., 2010; Zhang et al., 2012). T_{H17} cells also support robust HIV-1 replication (Rodriguez-Garcia et al., 2014), although RORγt and RORc, factors which are abundant in T_{H17} cells (Gosselin et al., 2010), have not been shown to directly impact HIV-1 transcription. Thus, induction of downstream T cell signaling pathways that culminate in activation of NF-κB, NFAT and STATs probably strongly influence HIV-1 transcription in T_{H17} cells.

There has been recent interest in characterizing the role of T_{reg} cells in HIV-1 (Chevalier and Weiss, 2013; Moreno-Fernandez et al., 2012). In general, changes in T_{reg} numbers and function have been documented in patients and include higher T_{reg} frequencies in untreated AIDS patients and diminished ability of these cells to suppress immune activation in HAART treated patients (Chevalier and Weiss, 2013; Moreno-Fernandez et al., 2012). Whether these changes are a direct result of HIV-1 infection or reflect more general immune dysfunction requires further investigation. T_{reg} development is in part driven by the transcription factor FoxP3 (Zhu et al., 2010). The data as to whether FoxP3 directly impacts HIV-1 transcription are conflicting. Ectopically expressed FoxP3 in primary CD4^{+} T cells inhibits activation and recruitment of transcriptional activators NF-κB, CREB and NFAT2 to the LTR (Grant et al., 2006; Selliah et al., 2008). FoxP3 may also facilitate HIV-1 transcription by inhibiting HDAC1
(Holmes et al., 2011; Holmes et al., 2007). However, overexpression of FoxP3 in different T cell subsets may result in different transcriptional outcomes. For example, forced expression of FoxP3 polarized naïve CD4\(^+\) T cells towards a T\(_{\text{reg}}\) phenotype and enhanced HIV-1 replication, but had no impact on T memory cell phenotypes or HIV-1 transcription (Oswald-Richter et al., 2004). These conflicting results may reflect intrinsic differences of T cell subsets or challenges associated with overexpressing factors, and underscore the need to further study HIV-1 transcription in primary T\(_{\text{reg}}\) cells.

A unique feature of the adaptive immune response is the generation of memory. For CD4\(^+\) T cells two distinct memory populations have been well characterized based on the expression of surface markers, homing capacity and function upon reactivation; T central memory (T\(_{\text{CM}}\)) and T effector memory (T\(_{\text{EM}}\)) cells (Pepper and Jenkins, 2011; Sallusto et al., 2004; van Leeuwen et al., 2009). Recently, stem cell memory T cells (T\(_{\text{SCM}}\)) have been described in humans (Gattinoni et al., 2011). These self-renewing memory CD4\(^+\) cells are reported to generate T memory and effector populations. The array of factors, including cytokines, signaling events and transcription factors, that influence the proliferation and generation of T\(_{\text{SCM}}\), T\(_{\text{CM}}\) and T\(_{\text{EM}}\) have not been fully elucidated. Critical transcription factors that regulate the generation and survival of T\(_{\text{CM}}\) include Schnurri-2, STAT3, STAT5a TBR2, FOXO3a, Bmi1 and LKLF (Catalfamo et al., 2008; Hirschhorn-Cymerman et al., 2012; Juffroy et al., 2010; Kimura et al., 2007; Kuo et al., 1997; van Grevenynghe et al., 2008; Yamashita et al., 2008). Furthermore, Bach-2, IRF-1 and p27 (Kip1) have been suggested to suppress T\(_{\text{EM}}\) differentiation (Jatzek et al., 2012; McElligott et al., 1997; Tsukumo et al., 2013b). Relevant to HIV-1,
memory cells, especially $T_{CM}$ and $T_{SCM}$, have been implicated as primary reservoirs harboring latent provirus because they are susceptible to HIV-1 infection, long-lived and, with their ability to self-renew, may maintain and/or renew the pool of cells harboring latent provirus (Bosque et al., 2011; Buzon et al., 2014). This homeostatic proliferation of infected $T_{CM}$ and $T_{SCM}$ in the absence of T cell activation and robust HIV-1 transcription presents a major barrier to eradicating persistent HIV-1 infection and underscores the need to characterize the tissue distribution and factors that regulate these different T cell memory subsets (Henrich and Gandhi, 2013). The predisposition for HIV-1 to establish latency in $T_{CM}$ may reflect the expression levels of transcription factors; $T_{CM}$ express T-bet (Marshall et al., 2011) and have lower levels of Cyclin T1 and phosphorylated CDK9, the two subunits of P-TEFb required for transcription elongation (Budhiraja et al., 2013), whereas, $T_{EM}$ have increased expression of factors shown to activate HIV-1 transcription including GATA-3, c-Maf and RORγt. (Dutta et al., 2013; Wang et al., 2010) The discrepancy of what key check points limit HIV-1 transcription in latently infected cells may reflect heterogeneity of memory cells, difference in cell culture conditions and isolation of cell subsets. The factors that repress transcription in T memory cells have not been defined but most likely include epigenetic factors such as HDACs or methyltransferases (Richman et al., 2009; Siliciano and Greene, 2011).
### Table 1. Factors Influencing CD4$^+$ T Cells Differentiation and HIV-1 Transcription.

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>T Cell Subset Promoted</th>
<th>T Cell Subset Inhibited</th>
<th>Effect on HIV-1 Transcription</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Bcl-6</td>
<td>$T_{FH}$, $T_{CM}$</td>
<td>$T_{H2}$</td>
<td>Inhibition, Activation (?)</td>
<td>(Baron et al., 1997; Ichii et al., 2007; Pepper et al., 2011; Sawant et al., 2013; Yu et al., 2009)</td>
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<td>Bcl-11b (CTIP-2)</td>
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<td>Inhibition</td>
<td>(Le Douce et al., 2012; Liu et al., 2010; Marban et al., 2007; Rohr et al., 2003)</td>
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<td>Blimp-1</td>
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<td>Repression, Activation</td>
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<td>BRG1</td>
<td>$T_{H1}$, $T_{H2}$</td>
<td></td>
<td>Activation &amp; Inhibition</td>
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<tr>
<td>c-Maf</td>
<td>$T_{H2}$, $T_{FH}$, $T_{EUM}$</td>
<td>$T_{H1}$</td>
<td>Activation</td>
<td>(Ho et al., 1998; Kroenke et al., 2012; Sato et al., 2011;</td>
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<tr>
<td>Protein</td>
<td>Target Cell</td>
<td>Effect</td>
<td>References</td>
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<td>c-Myb</td>
<td>$T_H^2$, $T_{EM}$</td>
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<td>(Dasgupta et al., 1990; Kozuka et al., 2011; Nakata et al., 2010)</td>
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<td>CIITA</td>
<td>$T_H^2$ (?)</td>
<td>Activation &amp; Inhibition (?)</td>
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<td>$T_H^1$, $T_H^2$</td>
<td>Inhibition</td>
<td>(Li et al., 2008; Ribeiro de Almeida et al., 2009; Sekimata et al., 2009)</td>
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<tr>
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<td>Activation</td>
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<td>TH Subtypes</td>
<td>Activation/Inhibition</td>
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<td>GATA3</td>
<td>TH2, TEM, TH1</td>
<td>Activation</td>
<td>(Yang and Engel, 1993; Zheng and Flavell, 1997)</td>
<td></td>
</tr>
<tr>
<td>IRF-1</td>
<td>TH1, TEM</td>
<td>Activation</td>
<td>(Lohoff et al., 1997; Sgarbanti et al., 2002; Taki et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>IRF-8</td>
<td>TH17</td>
<td>Inhibition</td>
<td>(Ouyang et al., 2011; Sgarbanti et al., 2002)</td>
<td></td>
</tr>
<tr>
<td>JunB</td>
<td>TH2, Treg</td>
<td>Activation</td>
<td>(Blonska et al., 2012; Roebuck et al., 1996; Son et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>JunD</td>
<td>TH1, TH2</td>
<td>Activation</td>
<td>(Meixner et al., 2004; Roebuck et al., 1996)</td>
<td></td>
</tr>
<tr>
<td>Lef1</td>
<td>Treg</td>
<td>Activation</td>
<td>(Fu et al., 2012; Sheridan et al., 1995)</td>
<td></td>
</tr>
<tr>
<td>Menin</td>
<td>TH17</td>
<td>Activation</td>
<td>(Bres et al., 2009; Watanabe et al., 2014)</td>
<td></td>
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<tr>
<td>NFAT</td>
<td>TH1, Treg, TH2</td>
<td>Activation</td>
<td>(Cron et al., 2000; Ranger et al., 1998; Tone et al., 2008; Wu et al., 2006)</td>
<td></td>
</tr>
<tr>
<td>PU.1</td>
<td>TH9, TH2, Treg memory</td>
<td>Activation</td>
<td>(Chang et al., 2009; Chang et al., 2010; Hadjur et al.,</td>
<td></td>
</tr>
<tr>
<td>Cell Type</td>
<td>Treatment</td>
<td>Function</td>
<td>References</td>
<td></td>
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<td>------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;9</td>
<td></td>
<td></td>
<td>(Lodie et al., 1998)</td>
<td></td>
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<tr>
<td>SATB1</td>
<td>T&lt;sub&gt;H&lt;/sub&gt;2, T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Inhibition</td>
<td>(Fu et al., 2012; Kumar et al., 2005; Notani et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>SIRT1</td>
<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Activation &amp; Inhibition</td>
<td>(Pagans et al., 2005; van Loosdregt et al., 2010; Zhang and Wu, 2009)</td>
<td></td>
</tr>
<tr>
<td>SMAD3</td>
<td>T&lt;sub&gt;H&lt;/sub&gt;2, T&lt;sub&gt;reg&lt;/sub&gt;, T&lt;sub&gt;H&lt;/sub&gt;1, T&lt;sub&gt;EM&lt;/sub&gt;, T&lt;sub&gt;H&lt;/sub&gt;9, T&lt;sub&gt;H&lt;/sub&gt;17</td>
<td>Activation</td>
<td>(Blokszijl et al., 2002; Coyle-Rink et al., 2002; Elyaman et al., 2012; Giroux et al., 2011; Hu et al., 2012; Xiao et al., 2008; Zhang et al., 2013)</td>
<td></td>
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<tr>
<td>SMAD4</td>
<td>T&lt;sub&gt;H&lt;/sub&gt;1, T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Inhibition</td>
<td>(Coyle-Rink et al., 2002; Huss et al., 2011; Yang et al., 2008b)</td>
<td></td>
</tr>
<tr>
<td>STAT1</td>
<td>T&lt;sub&gt;H&lt;/sub&gt;1, T&lt;sub&gt;reg&lt;/sub&gt;, T&lt;sub&gt;FH&lt;/sub&gt;</td>
<td>Activation &amp; Inhibition</td>
<td>(Chang et al., 2002; Choi et al., 2013; Ouaked et al., 2009; Owaki et al., 2005)</td>
<td></td>
</tr>
<tr>
<td>STAT3</td>
<td>T&lt;sub&gt;FH&lt;/sub&gt;, T&lt;sub&gt;H&lt;/sub&gt;17, T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Unknown</td>
<td>(Ma et al., 2012; Owaki et al., 2008; Sawant et al., 2013; Siegel et al., 2011a;</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>T1, Treg, T1, Tfh, Activation &amp; Inhibition</td>
<td>(Burchill et al., 2007; Crotti et al., 2007; Della Chiara et al., 2011; Johnston et al., 2012; Laurence et al., 2007; Riou et al., 2007; Selliah et al., 2006; Takatori et al., 2005; Zhu et al., 2003)</td>
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<tr>
<td>STAT5</td>
<td>T1, Treg, T1, Tfh, Activation &amp; Inhibition</td>
<td>(Burchill et al., 2007; Crotti et al., 2007; Della Chiara et al., 2011; Johnston et al., 2012; Laurence et al., 2007; Riou et al., 2007; Selliah et al., 2006; Takatori et al., 2005; Zhu et al., 2003)</td>
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<td></td>
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<tr>
<td>T-bet</td>
<td>T1, Treg, T1, Tfh, Activation &amp; Inhibition</td>
<td>(Lazarevic et al., 2011; Lugo-Villarino et al., 2003; Mullen et al., 2001)</td>
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<td></td>
</tr>
<tr>
<td>TRAF-2</td>
<td>T1, Treg, T1, Tfh, Activation &amp; Inhibition</td>
<td>(Horie et al., 2007; Lieberson et al., 2001; Tsitsikov et al., 1997)</td>
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</table>
5.6 B Lymphocyte-Induced Maturation Protein 1

The *Prdm1* gene located on chromosome 6q21 encodes B lymphocyte-induced maturation protein-1 (Blimp-1), a 789 amino acid-long, weighting 87,990 Da, nuclear transcription factor (Martins and Calame, 2008). Blimp-1 is comprised of N-terminal acidic domain, PRDI-BF1-RIZ1 homologous (PR) domain, proline-rich domain (PRD), five Kruppel-like (C2H2) zinc-fingers and another acidic domain at C-terminus (Fig. 9). The PRD interacts with Grucho family proteins (Ren et al., 1999), the lysine demethylase LSD1 (Su et al., 2009) and HDAC2 (Yu et al., 2000). Full length Blimp-1 is called Blimp-1α, while Blimp-1 lacking PR domain is called Blimp-1β. Even though Blimp-1β binds the same genes as full length Blimp-1, it has attenuated ability to repress them (Gyory et al., 2003). The C-terminal C2H2 zinc-fingers contain a nuclear localization signal and mediate binding to DNA (Martins and Calame, 2008), as well as recruitments of the SET domain histone methyltransferase G9a (Gyory et al., 2004) and the arginine methyltransferase Prmt5 (Ancelin et al., 2006). The N-terminal PR domain and two acidic domains of Blimp-1 have been also shown to be critical in repression of gene transcription and can act independently of each other (Yu et al., 2000).
Blimp-1 is critical for the differentiation of mature B cells into long-lived, Ig-secreting plasma cells and has been recently demonstrated to be expressed in dendritic cells, macrophages, keratinocytes and T cells (Chan et al., 2009; Chang et al., 2000; Chiang et al., 2013; Kim et al., 2011; Lin et al., 2002; Lin et al., 1997; Magnusdottir et al., 2007; Shaffer et al., 2002; Shapiro-Shelef et al., 2003; Smith et al., 2011; Turner et al., 1994).

In T cells Blimp-1 regulates the activation and generation of CD4 and CD8 T cell effector populations (Hua et al., 2013; Kallies et al., 2006; Kallies et al., 2009; Martins et al., 2006) (Fig. 10). Blimp-1 inhibits both T cell proliferation and differentiation into T\textsubscript{FH} (Crotty et al., 2010). Murine CD4\textsuperscript{+} T cells express high Blimp-1 levels in effector and memory subsets and minimal Blimp-1 expression in T\textsubscript{H0} cells (Savitsky et al., 2007) (Table 2). In addition to its role in the differentiation of immune cells, Blimp-1 plays a crucial role in limb, pharynx and heart morphogenesis (Bikoff et al., 2009).

**Figure 9. Blimp-1 Structure. Modified from (Martins and Calame, 2008)**

Blimp-1 proteins is composed of two acidic regions, one PR domain, one proline-rich domain and five zinc-fingers. The zinc-fingers mediate the binding of Blimp-1 to DNA. The proline-rich region has been shown to interact with methyltransferases and histone deacetylases and to recruit them to Blimp-1 target-genes. The acidic and PR domains have also been shown to be involved in Blimp-1-mediated transcription.
Blimp-1 is an important regulator of differentiation and maturation of immune cells. Overexpression of Blimp-1 in B cells causes them to differentiate into long-lived, Ig-secreting plasma cells. Blimp-1 is expressed at different levels in CD4+ T cell subsets and represses the differentiation of T\textsubscript{FH}. In CT8+ T cells the expression of Blimp-1 causes them to differentiate into cytotoxic T cells.

**Table 2. Expression of Blimp-1 in Murine CD4\textsuperscript{+} T Cell Subsets.**

<table>
<thead>
<tr>
<th>Phase</th>
<th>T Cell Subset</th>
<th>Blimp-1 Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>T\textsubscript{H0}</td>
<td>+</td>
</tr>
<tr>
<td>Effector</td>
<td>T\textsubscript{H1}, T\textsubscript{H2}, T\textsubscript{H17}, T\textsubscript{reg}</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>T\textsubscript{FH}</td>
<td>+</td>
</tr>
<tr>
<td>Memory</td>
<td>T\textsubscript{CM}, T\textsubscript{T}, T\textsubscript{EM}</td>
<td>++</td>
</tr>
</tbody>
</table>

Blimp-1 represses the transcription of several regulatory factors including Bcl-6, T-bet, IL-2, IL-6, TNFα, IFN-γ, IFN-β and type-III IFNs while enhancing the expression of IL-10 and Mcl-1 (Chan et al., 2009; Cimmino et al., 2008; Iwasaki et al., 2013; Keller and Maniatis, 1991; Lin et al., 2007; Martins et al., 2008; Smith et al., 2010; Swider et
al., 2014). Blimp-1-deficient DCs secret significantly higher amounts of IL-6 and CCL2, and favor the differentiation of TFH (Chan et al., 2009; Kim et al., 2011). Blimp-1 also plays an important role in innate immunity by activating granzyme B, murine p202, a cytosolic DNA-sensor that inhibits AIM2 inflammasome formation, and repressing AIM2, and NLRP-12 (Gong and Malek, 2007; Lord et al., 2009; Panchanathan et al., 2012; Yin et al., 2013) (Fig. 11).

![Figure 11. Blimp-1 is a Global Transcriptional Regulator.](image)

Blimp-1 represses a constellation of genes in different cell types. However, Blimp-1 has been also shown to induce transcription of some genes.

Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, Sendai virus and LPS induce Blimp-1 expression in bone marrow–derived macrophages (Severa et al., 2014). Blimp-1 is regulated by several mechanisms in different cell types. It is induced by IL-2, IL-21, OX40, STAT3, STAT5, IRF5, IFN-α, PPARγ, RXRα and TLR9 (Boettler...
et al., 2013; Garcia-Bates et al., 2009; Genestier et al., 2007; Hodge et al., 2012; Lien et al., 2010; Nurieva et al., 2012; Panchanathan et al., 2012; Parlato et al., 2013) and inhibited by RIG-I (Sathe et al., 2014) and Bach2, a transcription factor suppressing CD4+ T cell differentiation (Tsukumo et al., 2013a) (Fig. 12).

Figure 12. Regulation of Blimp-1 Expression.

Blimp-1 is induced via various mechanisms in different immune cells.

In the context of HIV-1, Blimp-1 expression is increased in chronically infected patients but remains unaltered in long term non-progressors and correlates with enhanced expression of negative regulators of T cell activation including PD-1, LAG3 and CTLA-4, and with T cell exhaustion and apoptosis (Che et al., 2012; de Masson et al., 2014; Seddiki et al., 2013; Shankar et al., 2011). Interestingly, HIV-1 LTR contains binding
sites for both Bcl-6 and Blimp-1, suggesting that these factors directly regulate HIV-1 transcription (Baron et al., 1997; Kaczmarek et al., 2013).

$T_{FH}$ cells support productive HIV-1 infection and are reported to expand during the course of HIV-1 infection (Lindqvist et al., 2012; Perreau et al., 2013). Bcl-6 is the master transcription factor for the generation of $T_{FH}$ cells (Yu et al., 2009). In particular, Bcl6 and Blimp-1 have an antagonistic relationship (Crotty et al., 2010; Martins and Calame, 2008). Unlike Bcl-6, Blimp-1 is highly expressed in $T_{H2}$ cells compared to $T_{H1}$ cells and represses $T_{H1}$ differentiation by repressing interferon, T-bet and Bcl-6 expression (Cimmino et al., 2008).

6. Hypothesis

Memory CD4$^+$ T cells are the main cell reservoir harboring replication-competent, yet transcriptionally quiescent HIV-1. Though HIV-1 latency is a rare event, primarily because the majority of HIV-1 proviruses are preferentially integrated in actively transcribed host genes, establishment of this replication competent but transcriptionally quiescent provirus does occur and is a significant barrier to a functional cure of HIV-1. Blimp-1 is a DNA-binding transcription factor that recruits multiple chromatin-modifying complexes, such as HDACs and methyltransferases, to targeted promoters. Blimp-1 is a key maturation factor of T cells and is upregulated in chronically HIV-1-infected patients. Together, these findings lead us to hypothesize that Blimp-1
binds to the HIV-1 LTR, inhibits HIV-1 transcription and contributes to HIV-1 latency (Fig. 13). A second hypothesis that is explored in this dissertation is that host promoters proximal to the HIV-1 LTR in the integrated provirus are active but have minimal impact on HIV-1 transcription.

Figure 13. Model for Potential Role of Blimp-1 in HIV-1 Transcription.
Blimp-1 and Bcl-6 have potential binding sites in HIV-1 LTR. I hypothesize that Blimp-1, an antagonist of Bcl-6, contributes to paused RNAP II and thus to the establishment of HIV-1 latency. See text for details.
II. Materials and Methods

Cell Culture. Discarded deidentified tissues from otolaryngology surgeries performed at Boston Medical Center were mechanically separated and cultured on plastic plates for 2-3 d to eliminate adherent cells. Cells in suspension were positively selected for CD4\(^+\) using the Dynabeads CD4 Positive Isolation Kit (Invitrogen). Peripheral blood mononuclear cells were isolated from whole blood by centrifuging through Histopaque gradient (Sigma-Aldrich). CD4\(^+\) T cells were positively selected using the Dynabeads CD4 Positive Isolation Kit. Jurkat clone E6-1 was originally purchased from American Type Culture Collection (ATCC, Manassas, VA). Primary CD4\(^+\) T cells and Jurkat cells were propagated in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin (P/S), and 0.2 M L-glutamine. Human embryonic kidney 293T cells (HEK293T) were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium containing 10% FBS and P/S. Inducible Jurkat cell lines were a kind gift from Dr. Olaf Kutch and were previously described (Duverger et al., 2009). Cells were incubated in a 37°C humidified incubator with 5% CO\(_2\). Cells were either left untreated, or activated with 0.1 μg/ml anti-human CD3 (BD Biosciences) and 1.0 μg/ml anti-human CD28 (BD Biosciences) for 30 min. 5 μg/ml of goat anti-mouse antibody (Sigma) was added to crosslink the receptors. T cells were harvested 24 h post-stimulation.

Polychromatic flow cytometry. CD4\(^+\) T cells were isolated from whole blood by negative selection using RosetteSep\textsuperscript{TM} Human CD4\(^+\) T Cell Enrichment Cocktail (STEMCELL Technologies). To sort T cell subsets cells were stained with CD3-
PacificBlue (BD Biosciences), CCR7-PE-Cy7 (BioLegend), CD45RA-PE-Cy5.5 (Invitrogen), CD27-PE (BD Biosciences), CD4-APC (BioLegend). T cell subsets were separated with FACSARia.

Transfections, Virus Generation and Infections. HIV-1 LTR/GLS-luciferase reporter constructs with an intact or mutated (GAAAGCGAAAG mutated to GCCCGCGCCCG) ISRE or NL4-3ΔTat-luciferase, Blimp-1, Tat, RSV LTR-LUC (Yamamoto et al., 1980), FIP-LUC(Meiering et al., 2001) and DHFR-LUC (Gummuluru and Emerman, 1999) expression constructs were transiently transfected into HEK293T cells via calcium phosphate transfection as described previously (Natarajan et al., 2013). The RSV-LUC, FIP-LUC and DHFR-LUC were generously provided by Dr. S. Gummuluru, Boston University School of Medicine. Luciferase assays were performed 48 h post-transfection using Luciferase Assay System (Promega).

Lentiviral vectors pNL4-3-Luc(+)/Env(-)/Nef(-) (Henderson et al., 1995) (obtained from NIH AIDS Research and Reference Reagent Program), Blimp-1 shRNA (Dharmacon), FUGW Blimp-1 (kindly provided by Dr. Kathryn Calame, Columbia University, New York), NELF shRNA (Dharmacon) were packaged by cotransfecting Tat, RSV-Rev, Gag/Pol and VSV-G into HEK293T cells using calcium phosphate as previously described (Natarajan et al., 2013). HIV-1 titers were determined using a p24 ELISA (PerkinElmer). Viruses were collected 48 h post-transfection and filtered through a Puradisc 25 Syringe Filter with 0.45-μm Polyethersulfone membrane (Whatman). Jurkat cells were infected by culturing with supernatants containing HIV-1-LUC or lentiviral constructs for 12–16 h. CD4+ T cells were activated with 2μg/mL PHA and 10ng/mL
PMA for 16 h, rested for 12 h and infected by culturing with HIV-1-containing supernatant or spinoculated with HIV-1-LUC supernatant plus 1 µg/ml polybrene for 1.5 h at 1,200 ×g without prior activation described by O’Doherty et al (O’Doherty et al., 2000).

**Quantitative RT-PCR.** RNA was prepared by resuspending cells in TRIzol (Life Technologies), and cDNA was generated using SuperScript II Reverse Transcriptase (Invitrogen) and random primers (Promega). GoTaq qPCR Master Mix (Promega) was used for quantitative real-time PCR reaction. Blimp-1 transcripts (+2074 to +2372) were amplified using 5′-CAGCTCGCCCACCTGCAGAA-3’ and 5′-GCCGCAGCGCAGTTCCCTTT-3’ primers. Initiated HIV-1 transcripts (+1 to +40) were amplified using 5′-GGGTCTCTCTGGTTAGA-3′ and 5′-AGAGCTCCCAGGCTCA-3′ primers and elongated HIV-1 transcripts (+5396 to +5531) were amplified using 5′-GACTAGAGCCCTGGAAGCA-3′ and 5′-GCTTCTTCCTGCCATAGGAG-3′ primers as described previously (Natarajan et al., 2013). β-actin mRNA was amplified using a QuantiTect primer assay (Qiagen). PCR was carried out for 45 cycles, and the relative expression was calculated using the ΔΔCt method (Livak and Schmittgen, 2001), normalizing specific amplification of the transcripts of interest to the β-actin control for each specific sample. The product detected in the sh-Control was a calibrator, and the transcript levels in samples were calculated as fold changes in comparison to sh-Control.

**Immunoblot Analysis and Antibodies.** Whole-cell lysates were prepared by washing cells with cold PBS and lysing them with buffer containing 10 mM Tris-Cl (pH 7.4), 150
mM NaCl, 1.0 mM EDTA (pH 8.0), 2.0 mM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 1% Triton X-100, 1.0 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture III (Calbiochem). Protein was measured using the BSA assay (Pierce). Samples were heated for 5 min at 100 °C before loading onto a 10% SDS-PAGE gel. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting. Antibodies used were as follows: anti-Blimp-1 serum (kindly provided by Dr. Kathryn Calame, Columbia University, New York), anti-β-actin (Sigma-Aldrich), anti-Tat (4138; NIH AIDS Research and Reference Reagent Program), anti-Sp1 (Upstate), anti-IRF-1 (Santa Cruz Biotechnology), anti-IRF-8 (Santa Cruz Biotechnology), anti-RNAP II (Santa Cruz Biotechnology), anti-AcH3 (Upstate Biotechnology) and rabbit IgG (Upstate Biotechnology).

**ChIP-qPCR.** Chromatin immunoprecipitations were performed as previously described (Natarajan et al., 2013). Quantitative real-time PCR analysis was carried out using SYBR green reagents and the primers 5′-GACCTTCCGCTGGGGACTTTC-3′ and 5′-CTAACCAGAGAGACCCCAGTAC-3’, which amplify the −102 to +16 region of HIV-1 LTR, 5′-CTGGGAGCTCTCTGGCTAACTA-3’and 5’-TTACCAGAGTCACACACGACG-3’, which amplify the +30 to +134 region of HIV-1, 5′-TCCCTCAGACCCCTTTTAGTCAG-3′ and 5’-GTCGAGAGAGCTCCTCTGGTTT-3′, which amplify the +142 to +237 region of provirus, and 5′-ACAGTACTGGATGTGGGTGATG-3’ and 5’-AATCCCTGGTGCTCATTGTTT-3’, which amplify the +2415 to +2522 region of provirus.
Statistical analysis. Statistical analysis was carried out using Student t test. A two-tailed distribution was performed on paired samples. Values of <0.01 were considered significant.

III. Blimp-1 Represses HIV-1 Transcription in Memory CD4⁺ T Cells

1. Introduction

Blimp-1 is a key regulator of T cell differentiation and reciprocal inhibitor of Bcl-6. Bcl-6 supports differentiation of T_{FH}, a cell subset that supports robust HIV-1 replication (Lindqvist et al., 2012; Perreau et al., 2013). In CD4⁺ T cells Blimp-1 represses transcription of IL-2 gene (Martins et al., 2008) whose promoter share common cis-elements with HIV-1 LTR. Recent studies have shown that Blimp-1 is expressed at high levels in CD4⁺ T cells from HIV-1-infected patients and that it is induced in T cells by stimulation with HIV-1-pulsed DCs (Che et al., 2012; de Masson et al., 2014; Seddiki et al., 2013; Shankar et al., 2011). Therefore, it is important to decipher the role that Blimp-1 plays in HIV-1 infection.

In this chapter I show that Blimp-1 binds downstream of HIV-1 LTR to a region called ISRE and represses Tat-dependent and Tat-independent HIV-1 transcription. Blimp-1 favors closed chromatin structure at Nuc-1 by inhibiting its acetylation. Blimp-1 contributes to the accumulation of short HIV-1 mRNA transcripts and decreases RNAP II
processivity. I also show that Blimp-1 contributes to the development and maintenance of HIV-1 latency in memory CD4+ T cells, the main reservoir of latent HIV-1.

2. Results

2.1. Blimp-1 is Expressed in Primary Human CD4+ Cells Including Memory CD4+ T Cells.

Although Blimp-1 expression has been characterized in murine T cells I wanted to confirm that it shared a similar expression pattern in human primary CD4+ T cells. CD4+ T cells were isolated from tonsils, a rich source of follicular helper T cells (Tfh), and from peripheral blood. Blimp-1 mRNA was detected by qRT-PCR and protein with immunoblots. Consistent with previous reports (Johnston et al., 2009) tonsillar CD4+ T cells expressed low levels of Blimp-1 compared to the CD4+ T cells isolated from whole blood (Fig. 1A). Activation with anti-CD3 and anti-CD28 antibodies resulted in 3-fold increase in Blimp-1 mRNA and protein levels (Fig. 1B and C).

I also examined Blimp-1 expression in different memory CD4+ T cell populations obtained from peripheral blood. Flow cytometry based on CD45RA, CD27 and CCR7 expression was used to enrich for naïve T cells (T_N; CD4+CD3+CD45RA+), central memory T cells (T_CM; CD4+CD3+CD45RA–CCR7+CD27+), transitional memory T cells (T_TM; CD4+CD3+CD45RA–CCR7–CD27+) and effector memory T cells (T_EM; CD4+CD3+CD45RA–CCR7–CD27–) (Fig. 1D). Although T_CM have been implicated as
the primary cell type that is latently infected, recent reports suggest that $T_{EM}$ and $T_{TM}$ contribute to the latent HIV-1 reservoir (Bacchus et al., 2013; Chomont et al., 2009; Lassen et al., 2012). Blimp-1 mRNA was measured in memory cells by qRT-PCR and protein levels were determined by immunoblots. Blimp-1 expression was 10-fold higher in long-lived $T_{CM}$ compared to naïve T cells, whereas, even higher levels of Blimp-1 was observed in $T_{TM}$ and $T_{EM}$ (Fig. 14E, F). Overall, Blimp-1 is present in multiple T cell populations but is expressed at significantly higher levels in $CD4^+T$ memory cells found within the latent reservoir.
2.2. Blimp-1 Binds to the ISRE Element Downstream of the 5' HIV-1 LTR in Primary CD4+ Cells

Based on the transcriptional repressor function of Blimp-1 and my data showing its high expression in CD4+ memory T cells I hypothesized that Blimp-1 limits HIV-1 expression in these cells. To determine if Blimp-1 regulates HIV-1 LTR activity I performed co-transfection experiments with Blimp-1 and an HIV-1 LTR reporter. Blimp-1 inhibited HIV-1 LTR-driven luciferase reporter gene (HIV-1 LTR-LUC) by 67% when overexpressed in HEK293T cells (Fig. 15A). Overexpression of the HIV-1 transcriptional activator, Tat, was unable to rescue HIV-1 LTR-mediated transcription in the presence of Blimp-1 (Fig. 15A). In addition, Blimp-1 inhibited an HIV-1 clone that lacked Tat (ΔTat-
HIV-1-LUC) by only 30% but repressed transcription by greater than 70% when Tat was added back by co-transfection (Fig. 15B). Blimp-1 is not acting as a general repressor in these experiments since it failed to repress expression driven by Rous sarcoma virus LTR (RSV LTR) (Yamamoto et al., 1980) and foamy virus internal promoter (FIP) (Meiering et al., 2001) and even activated the dihydrofolate reductase (DHFR) (Gummuluru and Emerman, 1999) promoter (Fig. 15C). These data indicate that Blimp-1 inhibits Tat-dependent HIV-1 transcription.
I explored whether Blimp-1 regulates HIV-1 expression in CD4+ T cells. I established a Jurkat T cell line that stably overexpressed Blimp-1 and infected it with a single cycle env-minus HIV-1-luciferase virus (HIV-1-LUC). HIV-1 transcription, as measured by the luciferase assay, was decreased by 55% in cells overexpressing Blimp-1 (Fig. 15D). In addition, primary human CD4+ T cells were infected with a HIV-1-LUC virus (Henderson et al., 1995) and Blimp-1 was overexpressed using a lentiviral vector. Transduction of cells with Blimp-1 lentivirus resulted in over 13-fold increase in Blimp-1 mRNA (Fig. 15E). Cells overexpressing Blimp-1 had 65% decrease in HIV-1 transcription measured by HIV-1 mRNA levels (Fig. 15E) and over 90% inhibition of HIV-1 replication measured by HIV-1 p24 ELISA (Fig. 15E). The data from Jurkat T cells and primary human CD4+ T cells confirmed that Blimp-1 is a repressor of HIV-1 transcription.
HIV-1 provirus has four putative Blimp-1 binding sites, GAAAG, with two sites upstream of the transcription start site that overlap the NF-κB sites (-104 to – 80 bp) and a second set of sites located downstream of the transcriptional start site in an interferon sensitive response element (ISRE; +200 to +218 bp) (Liang et al., 1997) (Fig. 16A). Both regions have been reported to modulate HIV-1 transcription (Liang et al., 1997; Nabel and Baltimore, 1987). Mutating the HIV-1-ISRE ameliorated Blimp-1-mediated repression of HIV-1 following co-transfection into HEK293T cells (Fig. 16B).

Furthermore, chromatin immunoprecipitations (ChIPs) demonstrate that Blimp-1 directly binds HIV-1 provirus. Chromatin was prepared from CD4+ T cells infected with HIV-1 and protein-DNA complexes were enriched with Blimp-1-specific antibody. ChIPs show modest Blimp-1 binding at the -104/-80 bp site and 9-fold higher Blimp-1 binding at the +200/+218 bp ISRE site (Fig. 16C). Following T cell activation, Blimp-1 binding was not detected at the HIV-1 ISRE element despite increases in Blimp-1 mRNA and protein (Fig. 16C, Fig. 14B and C). Blimp-1 did not exclude binding of interferon regulatory factors (IRFs) IRF-1 and IRF-8 to the HIV-1 ISRE (Fig. 16D). These results suggest that Blimp-1 represses HIV-1 transcription by directly binding the proviral ISRE element.
The above gain of function experiments demonstrated that Blimp-1 represses HIV-1 expression. To determine if Blimp-1 limits HIV-1 expression in the context of primary cells I reduced Blimp-1 expression in primary CD4+ T cells using shRNA. I infected CD4+ T cells with HIV-1-LUC, transduced the infected cells with sh-Blimp-1 lentivirus and monitored HIV-1 expression. The efficacy of Blimp-1 knock-down was confirmed 96 h post-transduction by qRT-PCR (Fig. 17A) and immunoblots (Fig. 17B). Diminishing Blimp-1 increased basal HIV-1 transcription and replication as measured by qRT-PCR (Fig. 17C), luciferase assay (Fig. 17D) and p24 ELISA (Fig. 17E) indicating that Blimp-1 limits HIV-1 transcription in primary CD4+ T cells. Activating T cells through CD3+CD28 following Blimp-1 knockdown did not further induce HIV-1 transcription and actually decreased HIV-1 transcription by 80% suggesting that Blimp-1 was required for optimal induction of HIV-1 transcription (Fig. 17C). These data indicate that Blimp-1 acts as both a repressor and transcriptional activator in the context of HIV-1-infected primary T cells. However, the ability of Blimp-1 to activate HIV-1

Figure 16. Blimp-1 Binds HIV-1 Provirus.

(A) The location of HIV--1 LTR, four putative Blimp-1-binding sites and ISRE mutations in provirus. (B) HEK293T cells were transfected with HIV--1 LTR-LUC or mISRE-HIV--1 LTR-LUC and control vector or Blimp-1 in the absence or presence of Tat. Luciferase assays and western blot analyses were performed 48 h post-transfection. (C, D) 96 h post-HIV-1 infection primary human CD4+ cells were activated with anti-CD3 and anti-CD28 antibodies for 24 h and ChIPs were performed using anti-Blimp-1, anti-IRF-1, anti-IRF-8 or anti-rabbit antibody. Binding was detected with -102F/+16R and +142F/+237R HIV-1 primer sets. These data are performed in triplicate and represent at three independent experiments. Bars show average values ±SD, n=3. *p < 0.05, **p < 0.01 and ***p < 0.001 (Student’s t test).
transcription is independent of Blimp-1 binding to provirus since ChIPs show it is displaced following T cell activation (Fig. 17C).

To gain insight into how Blimp-1 limits HIV-1 transcription I examined with ChIPs the distribution of RNA Polymerase II (RNAP II) on the HIV-1 genome. In cells expressing Blimp-1 there was an accumulation of RNAP II at the transcriptional start site and modest amounts of RNAP II downstream suggesting that Blimp-1 leads to RNAP II promoter proximal pausing (Fig. 17F). Diminishing Blimp-1 reduced RNAP II at the promoter and increased RNAP II downstream in the provirus by 3-fold consistent with RNAP II release and greater processivity (Fig. 17F). I also used qRT-PCR to measure initiated versus elongated transcripts. In control cells there was an accumulation of initiated short HIV-1 mRNA but low expression of full length mRNA (Fig. 17G) as would be expected with RNAP II pausing (Zhang et al., 2007). Decreasing Blimp-1 with shRNA altered the ratio of initiated to elongated HIV-1 mRNA so that the ratio was approximately one indicating processive transcriptional elongation (Fig 17G).

Furthermore, knocking down Blimp-1 led to an increase in histone H3-acetylation (AcH3) at the positioned nucleosome (nuc-1) (Fig. 17H). These data suggest that Blimp-1 targets multiple steps of transcription regulation to limit HIV-1 transcriptional elongation.
Blimp-1 was most highly expressed in memory CD4⁺ T cells which do not support efficient HIV-1 transcription. To examine whether Blimp-1 was limiting HIV-1 transcription in these cells I infected isolated memory CD4⁺ T cells with HIV-1 by spinoculation (O'Doherty et al., 2000) which has been shown to facilitate infection of cells without using mediators that activate T cells and then decreased Blimp-1 with sh-Blimp-1 lentivirus. Spinoculation enhances viral binding which increases infection of cells without activating stimuli and results in higher integration rates than infection of cells in the absence of spinoculation. Decreasing Blimp-1 levels in all three CD4⁺ T cell
memory populations resulted in significant increases, 2 to 12-fold, in HIV-1 transcription (Fig. 18B). The levels of induction correlated with the efficiency of Blimp-1 knockdowns observed with more modest induction in T_CM and T_EM cells in which Blimp-1 knockdowns were inefficient compared to robust induction in T_TM where Blimp-1 expression was decreased by greater than 80% (Fig. 18A). These findings support a model in which Blimp-1 expression correlates with limited basal HIV-1 transcription in T memory cells.

Figure 18. Blimp-1 Represses Basal HIV-1 Transcription in Primary Memory CD4+ T Cells.

T_CM, T_TM and T_EM sorted as described above were infected with NL4-3 HIV-1 by spinoculation. 16 h post infection cells were transduced with sh-Ctrl and sh-Blimp-1. 72 h post-knockdown mRNA was collected. Expression of (A) Blimp-1 and (B) HIV-1 was measured by qRT-PCR using β-actin as a reference gene. These experiments were performed in triplicate and are representative of three separate infections from T cells obtained from three patients. Bars show average values ±SD, n=3. *p < 0.05, **p < 0.01 and ***p < 0.001 (Student’s t test).
2.3. T Cell Activation and Treatment with G9a and HDACs Inhibitors Do Not Abolish Blimp-1-Mediated HIV-1 Repression in Jurkat Cells

Posttranslational modifications of histones have been shown to regulate transcription of HIV-1. Trichostatin A (TSA), an inhibitor of class I and II HDACs, has been shown to increase HIV-1 transcription by favoring open chromatin structure (Van Lint et al., 1996). BIX01294, an inhibitor of the methyltransferase G9a, was also demonstrated to increase HIV-1 transcription and to reactivate latent HIV-1 (Imai et al., 2010). To gain insight into the mechanism by which Blimp-1 halts HIV-1 transcription I have treated HIV-1-infected Jurkat cells stably overexpressing Blimp-1 with TSA and BIX01294 and activated them with anti-CD3+28 Abs in the presence of the inhibitors. TSA induced HIV-1 transcription, however Blimp-1 was still able to repress it (Fig. 19). Treatment with BIX01294 did not induce HIV-1 transcription and Blimp-1 was able to repress HIV-1 transcription in not activated cells at the highest concentration (Fig. 19). In contrast to primary CD4+ cells, the CD3+28 activation of Jurkat cell lines did not abolish Blimp-1 mediated repression of HIV-1 transcription highlighting the differences between the two cell types (Fig. 19). These results suggest that Blimp-1-mediated repression of HIV-1 transcription does not depend on HDACs and G9a. However, these experiments lack the appropriate controls and should be repeated in primary CD4+ T cells in which Blimp-1 affects HIV-1 transcription in different manner than in Jurkat cell lines upon T cell activation.
Figure 19. G9a and HDACs Inhibitors do not Inhibit Blimp-1-mediated Repression of HIV-1 Transcription.

Jurkat T cells stably over-expressing Blimp-1 were infected with HIV-1 LUC. 12 h post infection cells were pre-treated with different concentrations of TSA, BIX01294 or DMSO for 1 h. Cells were activated with anti-CD3+28 antibodies in the presence of inhibitors for 16 h. Cells were lysed and luciferase activity was measured. Bars show average values ±SD, n=3. *p < 0.05 and ***p < 0.001 (Student’s t test).
3. Conclusions and Discussion

Memory CD4$^+$ T cells are a major reservoir of latent HIV-1 and their longevity and homeostatic proliferation prevents virus clearance and supports HIV-1 persistence. Although several mechanisms potentially inhibit HIV-1 transcription, T cell specific transcription factors that intrinsically program the maturation of CD4$^+$ memory T cells could contribute to the propensity of HIV-1 to become repressed in these cells. I present evidence that the lymphoid differentiation factor Blimp-1 limits HIV-1 transcription in CD4$^+$ memory T cell subsets.

Blimp-1 is expressed in a range of immune cells, such as B cells, macrophages, dendritic cells and T cells. I confirmed that Blimp-1 is differentially expressed in CD4$^+$ T cells, with low levels in T_N and CD4$^+$ tonsillar cells but elevated expression in cells that have been shown to contribute to the latent reservoir, T_{CM}, T_{TM} and T_{EM}. I also showed that Blimp-1 is induced upon T cell activation. Blimp-1 in part influences T cell maturation and function by regulating the expression of key lineage restricting transcription factors including PAX5, STAT6, Bcl-6 and T-bet (Cimmino et al., 2008; Lin et al., 2002; Shaffer et al., 2002). Furthermore, Blimp-1 is required for robust CD4 and CD8 T cells antiviral responses against influenza and lymphocytic choriomeningitis virus (LCMV) and mediates murine gamma herpesvirus latency in splenocytes (Hua et al., 2013; Kallies et al., 2009; Rutishauser et al., 2009; Shin et al., 2009; Siegel et al., 2010). Blimp-1 is associated with increased expression of inhibitory receptors and decreased polyfunctionality of exhausted CD4$^+$ T cells during LCMV infection.
(Crawford et al., 2014). In $T_{H1}$ Blimp-1 induces the expression of IL-10, a cytokine that suppresses anti-viral T cell responses, while in CD8$^+$ T cells Blimp-1-mediated repression of CD25 and CD27 decreases their survival and differentiation into memory cells during LCMV infection (Parish et al., 2014; Shin et al., 2013). During *Toxoplasma gondii* infection Blimp-1 suppresses inflammation which increases survival rate (Neumann et al., 2014). In chronically infected HIV-1 patients Blimp-1 is elevated in CD4$^+$ T cells and correlates with an increase in T cell exhaustion markers (de Masson et al., 2014; Seddiki et al., 2013). This increase in Blimp-1 may be a direct result of infection since HIV-1-pulsed dendritic cells enhance Blimp-1 expression (Che et al., 2012; Shankar et al., 2011). My results demonstrate that Blimp-1 limits HIV-1 transcription in T memory cells consistent with its function as a transcriptional repressor in different T cell subsets. Blimp-1 is expressed in macrophages and dendritic cells and it may limit HIV expression and promote latency in them although this possibility has not been explored.

Blimp-1 binds a GAAAG consensus sequence which is also found in ISREs present in Blimp-1 regulated genes CIITA, IDO1 and IFN-λ1 (Barnes et al., 2009; Piskurich et al., 2000; Siegel et al., 2011b). Although HIV-1 provirus contains four putative Blimp-1-binding sequences (Kaczmarek et al., 2013), I detected Blimp-1 binding only at the HIV-1 ISRE element. This cis-element has been implicated in regulating HIV-1 transcription by recruiting IRF family proteins IRF-1 and IRF-8 (Sgarbanti et al., 2002). Blimp-1 binds this element in resting cells and is displaced following T cell activation. It is possible that Blimp-1 antagonizes or competes with the transcriptional activator IRF-1, although, I do not observe significant changes in IRF-1 or IRF-8 binding in the absence
or presence of Blimp-1 or in response to T cell activation. There is also a possibility that T cell activation results in post-translational modifications of Blimp-1, such as sumoylation, phosphorylation or ubiquitination.

Blimp-1 mediates repression of genes by recruiting epigenetic factors such as methyltransferases G9a, Prmt5 and LDS1, Groucho-proteins and histone deacetylases to promoters (Ancelin et al., 2006; Gyory et al., 2004; Ren et al., 1999; Su et al., 2009; Yu et al., 2000). LSD-1 and G9a limit HIV-1 transcription and are candidates for the repression observed in CD4+ memory T cells (Imai et al., 2010; Le Douce et al., 2012). I observed that decreasing Blimp-1 increases acetylation at nuc-1 and releases RNAP II pausing. Blimp-1 limiting transcription elongation is also consistent with the observation that Tat cannot rescue HIV-1 expression in the presence of Blimp-1 and suggests that it is targeting a step prior to transcription elongation and recruitment of P-TEFb which is mediated by Tat.

Blimp-1 can act as a transcriptional activator and is necessary for the induction of IL-10 and XBP-1 (Cretney et al., 2011; Iwasaki et al., 2013; Lin et al., 2002). I also observe that Blimp-1 can act as a repressor and trans-activator in co-transfections with select promoters and in primary CD4+ T cells. Intriguingly, Blimp-1 is required for efficient induction of HIV-1 transcription upon T cell activation. However, Blimp-1 does not occupy either set of binding sites following T cell activation despite increased Blimp-1 expression following CD3 + CD28 activation suggesting that Blimp-1 is not directly inducing HIV-1 transcription. The mechanism by which Blimp-1 induces HIV-1 transcription following activation is not clear and may reflect Blimp-1 interacting with
other transcription factors, or its ability to influence the expression of other host genes, cytokines and restriction factors that regulate HIV-1 transcription. Another transcription factor that functions in a similar manner is DSIF. Unphosphorylated DSIF functions as a transcriptional repressor promoting RNAP II pausing at promoters (Yamaguchi et al., 2002). Phosphorylation of DSIF by P-TEFb changes it to a transcriptional activator (Peterlin and Price, 2006).

I propose a model in which Blimp-1 is highly expressed in memory CD4+ T cells which do not support robust HIV-1 replication. In resting cells Blimp-1 binds the HIV-1 ISRE and represses HIV-1 transcription elongation whereas upon T cell activation Blimp-1 is released from HIV-1 provirus derepressing proviral transcription (Fig. 20). I show that Blimp-1 is a transcriptional repressor of HIV-1 and its expression in memory CD4+ T cells makes them prone to HIV-1 latency. Understanding how Blimp-1 is regulated and the transcriptional processes it coordinates to silence HIV-1 expression will provide insights into the establishment and maintenance of the HIV-1 reservoir.
Blimp-1 is highly expressed in memory CD4⁺ T cells, binds the HIV-1 ISRE and inhibits Tat-dependent HIV-1 transcription. Following T cell activation Blimp-1 is released from HIV-1 provirus which correlates with increased RNAP II processivity, histone H3 acetylation and enhanced HIV-1 transcription.

Figure 20. Model for the Role of Blimp-1 in HIV-1 Transcription.
IV. Neighboring Promoters and Their Influence on HIV-1 Transcription

1. Introduction

HIV-1 latency is regulated by binding of factors to HIV-1 LTR, chromatin structure, intrinsic T cell factors, as well as NELF-mediated stalling of RNAP II at the viral promoter. Transcription from host neighboring genes in which HIV-1 is integrated has been suggested to influence HIV-1 transcription and establishment of latency. However, although HIV-1 has been shown to integrate into transcriptionally active host genes, proviral latency is established. Therefore, I hypothesized that active host genes have minimal impact on HIV-1 transcription. To investigate the role of proximal host promoters on HIV-1 transcription I have used five different cell lines with inducible expression of HIV-1; one with parallel promoters, three with convergent promoters and one with provirus integrated between two host genes (Fig. 21). My results demonstrate that these cells are differentially responsive to reactivation induced by T cell activation. Four out of five inducible cell lines were minimally responsive to treatment with HDAC inhibitor indicating that chromatin remodeling agents are not a general mechanism of repression. Additionally, I show that transcription of host genes does not exclude transcription from a proximally integrated HIV-1 LTR, and that HIV-1 integration may lead to an increase in transcription of the neighboring host gene. The majority of the cells had accumulation of short HIV-1 mRNA transcripts and bound RNAP II and NELF at the repressed HIV-1 LTR. Decreasing NELF levels led to reactivation of provirus suggesting
that NELF-mediated RNAP II pausing is a general mechanism of proviral repression in these cells.

2. Results

2.1. Characterization of Inducible Cell Lines

The inducible Jurkat cell lines, CA5, BA1, 11B10, EF7 and CG3 were a kind gift from Dr. Olaf Kutsch and the mapping of integration and orientation of HIV-1 provirus in these cells was performed by Dr. Frank Wolschendorf. The inducible cell lines were generated by infecting Jurkat T cells with HIV-1-GFP, activating GFP-non-expressors with PMA+PHA and establishing clonal cell lines from cells in which PMA+PHA treatment induced GFP expression (Duverger et al., 2009). CA5 cells have provirus integrated in the exon of RNA binding motif protein 12 (RBM12) in a parallel orientation (Fig. 21). RBM12 has been implicated in meibomian cell carcinoma (Kumar et al., 2007). RBM12 shares promoter and 5’ exons with copine I (CPNE1) (Yang et al., 2008a). The function of RBM12 remains unidentified, while CPNE1 plays role in cell-cycle and proliferation (Skawran et al., 2008). BA1 cells have HIV-1 integrated in the intron of PDZ domain containing 8 (PDZD8) in a convergent orientation (Fig. 21). PDZD8 is a cytoskeleton-regulating protein that binds HIV-1 Gag and stabilizes HIV-1 capsid contributing to enhanced reverse transcription (Guth and Sodroski, 2014; Henning et al.,
PDZD8 is also a suppressor of Herpes Simplex Virus type-1 (HSV-1) replication (Henning et al., 2011). 11B10 cell line has HIV-1 inserted in the intron of HELZ in a convergent orientation (Fig. 21). HELZ is a zinc-finger containing RNA-helicase important for global translational initiation (Hasgall et al., 2011). HELZ interacts with RNAP II and histone methyltransferases, Smyd2 and Smyd3 (Diehl et al., 2010; Hamamoto et al., 2004). In EF7 cells HIV-1 is integrated in the intron of WHSC1 in a convergent orientation (Fig. 21). Wolf–Hirschhorn syndrome candidate 1 (WHSC1), also known as Multiple Myeloma SET domain (MMSET), is a histone H3 lysine 36 (H3K36) trimethyltransferase which together with the histone chaperone HIRA is implicated in incorporation of histone H3.3 into actively transcribed genes and which is required for IFN-induced transcription (Sarai et al., 2013). WHSC1 also interacts with Brd4 and P-TEFb facilitating transcriptional elongation (Sarai et al., 2013). In CG3 cell line provirus is integrated between two genes, tigger transposable element derived 5 (TIGD5) and PYCRL, with a parallel orientation to TIGD5 and a convergent orientation to PYCRL (Fig. 21). TIGD5 is a member of the tigger subfamily of the pogo superfamily of DNA-mediated transposons. PYCRL is cytosolic pyrroline-5-carboxylate reductase that catalyzes the reduction of ornithine to proline (De Ingeniis et al., 2012).
Figure 21. Proviral Integration Sites in CA5, BA1, 11B10, EF7 and CG3.

Schematic of HIV-1 provirus integration sites relative to neighboring host promoter for latently infected Jurkat cells (obtained from the Kutsh lab UAB).
2.2. Repressed HIV-1 is Differentially Inducible

Signals that activate T cells, such as signaling downstream of the CD3 and CD28 receptors as well as treatment with Phorbol 12-myristate 13-acetate (PMA), a phorbol ester and phytohemagglutinin (PHA), a plant lectin present in red kidney beans, lead to induction of HIV-1 transcription and effective replication (Peterlin and Price, 2006). Activation with PHA requires intact CD3/TCR and increases intracellular free Ca\(^{2+}\) (Weiss and Imboden, 1987) whereas PMA binds to and activates PKC (Manger et al., 1987). To see if cells harboring repressed proviruses with divergent integration sites are responding in the same manner to T cell activation stimuli, CA5, BA1, 11B10, EF7 and CG3 cell lines were treated with PMA+PHA. Results indicate that provirus induction levels in response to PMA+PHA treatment range from 90-30% among T cell lines with the CA5 cells being the most inducible and the CG3 cells (harboring intragenic HIV-1 provirus) being the least inducible (Fig. 22 A).

HDACs have been shown to contribute to the repression of HIV-1 transcription by removing acetyl groups from histones, making the chromatin more condensed (Keedy et al., 2009). To explore the contribution of chromatin structure in limiting HIV-1 expression I have treated cells with Trichostatin A (TSA), an inhibitor of the class I and II HDACs, which has been shown to be a potent inducer of HIV-1 transcription. The treatment with TSA was not as effective as treatment with PMA+PHA at reactivating latent provirus (Fig. 22 B). TSA induced most HIV-1 expression in 11B10 and EF7 cells in which HIV-1 provirus and the host genes have convergent orientation (Fig. 22 B).
However, BA1 cells, which have both the host promoter and the HIV-1 LTR in the same orientation were minimally responsive to the TSA treatment (Fig. 22 B), implying that chromatin structure does not contribute to HIV-1 latency in all cell lines and that the promoter orientation does not impose a specific mode of transcriptional regulation of HIV-1 provirus. My data indicate that class I and II HDACs are not general regulators of HIV-1 transcription in these cell lines. This is consistent with the hypothesis that neighboring host genes being active and having open chromatin structure can influence transcription from the proximal HIV-1 LTR.
Figure 22. Provirus Integration Site Affects Reactivation Rate of Latent HIV-1.

Cells were treated with 2μg/mL PHA and 10ng/mL PMA or 0.5μM TSA for 24 h. GFP expression was measured by flow cytometry. Data represent fold induction over background HIV-1-GFP expression.
2.3. Correlation between the Expression of Host Genes and HIV-1 Induction

Because HIV-1 preferentially integrates into active host genes one may expect that HIV-1 transcription would interfere with transcription of the host gene (Lenasi et al., 2008). Thus, the reactivation of HIV-1 would be at the expense of the host, leading to a decreased expression of the endogenous gene. However, treatment with PMA+PHA resulted in decreased host gene expression only in 11B10 and CG3 (PYCRL gene) (Fig. 23). The m-RNA levels of the majority of neighboring host genes increased (BA1, EF7, CG3 (TIGD5 gene)) or remained constant (CA5 cells) upon T cell activation with PMA+PHA (Fig. 23). In addition, the integration of HIV-1 led to an increase in host gene expression in BA1, 11B10, EF7 and CG3 (TIGD5 gene). It is possible that host gene activity is crucial for the reactivation of provirus. These data indicated that host gene expression does not necessarily exclude HIV-1 expression.
To determine if HIV-1 transcription in the inducible Jurkat cell line is inhibited at the step of transcriptional initiation or elongation I looked at the accumulation of short HIV-1 mRNA transcripts. Using qRT-PCR I have calculated the ratio of initiated to elongated HIV-1 mRNAs (Fig. 24). The ratio of initiated to elongated HIV-1 mRNA transcripts decreased upon PMA+PHA treatment in CA5, BA1 and 11B10 cells, indicating productive RNAP II transcription. CG3 cells also had accumulated short HIV-1 transcripts, but to a much smaller degree (Fig. 24). However, EF7 cells did not contain accumulated initiated HIV-1 mRNAs (Fig. 24). The accumulation of short HIV-1 mRNAs is consistent with the paused RNAP II at HIV-1 LTR which decreases upon T cell activation. Effective HIV-1 transcription is initiated and elongated HIV-1 mRNA is produced shifting the ration of initiated to elongated transcripts to “1”.

Figure 23. Activation of Latent Cells Results in Altered Host Gene Expression.

Uninfected Jurkat T cells as well as CA5, BA1, 11B10, EF7 and CG3 cells were treated with 2μg/mL PHA and 10ng/mL PMA for 24 h. Expression of the neighboring host gene mRNA was measured by qRT-PCR and normalized to β-actin.
RNAP II pausing is characterized by accumulation of RNAP II and NELF at the transcriptionally repressed promoter. The RNAP II promoter proximal pausing induced by NELF was confirmed in ChIP experiments looking at the binding of RNAP II and NELF to HIV-1 LTR. Using a ChIP assay, I detected high binding of RNAP II at HIV-1 LTR in uninduced cells and this binding decreased upon activation with PMA+PHA representing release of a paused RNAP II (Fig. 25). Upon T cell activation, the levels of NELF, an inducer of RNAP II pausing at HIV-1 LTR, also decreased at the viral promoter (Fig. 25). These data suggest that HIV-1 induction correlates with a decrease in RNAP II pausing at HIV-1 LTR.
Our laboratory identified the negative elongation factor (NELF) as a key player in mediating RNAP II promoter proximal pausing and repressing HIV-1 transcription (Natarajan et al., 2013). Data obtained by Dr. Malini Natarajan and Dr. Gillian Schiralli Lester indicate that knockdown of NELF in these inducible Jurkat cell lines reactivates provirus, although to varying degree (Fig. 26). Thus, HIV-1 provirus in the inducible cell lines is repressed by NELF-induced RNAP II pausing, a widespread mechanism of gene regulation (Gaertner et al., 2012; Rahl et al., 2010), which I propose as a general mechanism of HIV-1 repression and latency.

**Figure 25. T Cell Activation Results in Altered Recruitment of RNAP II.**

Cells were treated with 2μg/mL PHA and 10ng/mL PMA for 24 h. ChIP assays were performed to examine RNAP II and NELF binding to HIV-1 LTR.
3. Conclusions and Discussion

I explored the effect of neighboring host promoters on HIV-1 expression in different inducible Jurkat cell lines. Previous reports showed that transcription from the proximal host promoters may lead to repression of HIV-1 transcription. However, my data demonstrate that genes in the vicinity of the HIV-1 provirus and chromatin structure have minimal impact on the maintenance of HIV-1 latency in the inducible Jurkat cells. The fact that CG3 cells, which harbor intergenic provirus, are the least inducible suggests that HIV-1 integration into transcriptionally active genes helps reactivate HIV-1. Thus
integration of the HIV-1 provirus into transcriptionally active genes instead of non-coding DNA may actually help in its reactivation by assisting with promoter clearance of repressive factors or open chromatin. In fact, HIV-1 integration increased expression of the neighboring host gene by an unknown mechanism. It is possible that HIV-1 induces transcription of the host gene by disrupting repressive chromatin structure, obstructing binding of transcriptional repressors to the host gene or recruiting them away from the host neighboring promoter to HIV-1 LTR. The high transcriptional activity of host neighboring promoter was decreased upon HIV-1 induction only in 11B10 cells, implying that in this particular cell line high transcriptional activity of the host gene may lead to repression of HIV-1 LTR. It remains to be determined if the decrease in the HELZ gene expression in 11B10 is a result of interruption of transcriptional initiation or elongation of this gene.

Recently RNAP II pausing has been shown to be one of the mechanisms of transcriptional interference (Palmer et al., 2009). My data show that all the inducible cell lines have high levels of RNAP II at HIV-1 LTR, as well as accumulation of short HIV-1 mRNA transcripts, implying that proviruses are inhibited at the step of transcriptional elongation rather than initiation. The data showing HIV-1 induction upon NELF knock-down are supportive of RNAP II promoter proximal as the primary mechanism of HIV-1 transcriptional repression.

Upon cell activation, BA1, EF7 and 11B10 have differential expression of the neighboring host gene despite having HIV-1 integrated in convergent orientation. HIV-1 induction decreased HELZ levels, did not affect PDZD8 expression, while it induced
WHSC1 levels. These differences may be possibly explained by the different distances of HIV-1 LTR from the proximal host gene promoter as well as the host gene promoter strength. In 11B10 cells, provirus is integrated 96,217 bp of HELZ compared to 36,311 bp of PDZD8 in BA1 cells and 1,129 bp of WHSC1 in EF7 cells. These data suggest that the inhibition of neighboring host gene expression correlates positively with the distance between the host gene and viral promoter. One can also assume that the promoter of HELZ is weaker, while the promoters of WHSC1 and PDZD8 have the same or greater strength than HIV-1 LTR.

My data demonstrate that T cell activation, inhibition of HDACs, as well as NELF-mediated RNAP II pausing play a differential role in maintaining the repression of provirus in inducible Jurkat cell lines. My data also suggests that although HIV-1 insertion and induction differentially affect neighboring host genes, the expression of a host gene does not necessarily prevent the reactivation of the HIV-1 provirus. I propose a model in which HIV-1 transcription is repressed by a combination of mechanisms rather than by a single one. In this model NELF causes RNAP II pausing at HIV-1 LTR characterized by accumulation of short viral transcripts, while the closed chromatin structure at Nuc-1 may additionally prevent RNAP II from processing downstream provirus after its release from the pause. T cell activation leads to recruitment of transcriptional activators to HIV-1 LTR as well as the displacement of transcriptional repressors, such as Blimp-1, from provirus allowing efficient HIV-1 transcription.
V. Conclusions

The inability to target and eliminate latently infected memory CD4+ T cells remains a barrier to eradicating HIV-1 infection. In an effort to cure HIV-1 infection, strategies to purge transcriptionally repressed HIV-1 provirus from latent reservoirs have been employed to complement current antiretroviral therapies (Margolis and Hazuda, 2013). Recent therapeutic approaches have focused on overcoming the repressive effects of chromatin, which has been implicated as a key regulator of HIV-1 transcription (Richman et al., 2009; Siliciano and Greene, 2011). For example, clinical trials have used HDAC inhibitors valproic acid (Archin et al., 2010; Routy et al., 2012) and vorinostat (Archin et al., 2012), which, despite modest ability to induce HIV-1 transcription in peripheral blood of HAART patients, did not decrease the HIV-1 reservoir. The limited success of these initial trials probably reflect the complexity of the latent reservoir in regards to the cells that are included in this compartment as well as the multiple mechanisms that establish and maintain latency (Richman et al., 2009; Schiralli Lester and Henderson, 2012; Siliciano and Greene, 2011). An additional confounder is that many of the factors that limit HIV-1 transcription are general transcriptional regulators which are necessary for normal gene expression. Targeting RNAP II, P-TEFb, and chromatin remodeling factors will likely be toxic, lack specificity, and have an impact on global gene expression.

I wanted to gain insight into the mechanisms that might contribute to HIV-1 latency in the context of T cells. My work explored two possible mechanisms of HIV-1
latency: tissue specific factors and host neighboring promoters. I show that Blimp-1 is an important transcriptional repressor of HIV-1 in memory CD4$^+$ T cells and that neighboring genes have minimal impact on HIV-1 transcription. I also demonstrate that RNAP II pausing appears to be necessary for establishment of proviral latency in both scenarios.

**Blimp-1-mediated repression of HIV-1 transcription in memory CD4$^+$ T cells**

CD4$^+$ T cell subsets differentially support HIV-1 replication. For example, quiescent CD4$^+$ memory T cells are susceptible to HIV-1 infection but do not support robust HIV-1 transcription and have been implicated as the primary reservoir of latent HIV-1. T cell transcription factors that regulate maturation potentially limit HIV-1 transcription and mediate the establishment and maintenance of HIV-1 latency. Attempts to purge HIV-1 from the latent reservoir by targeting general biochemical pathways have had modest success; however, events regulated by T cell specific factors may provide a more cell specific targeting strategy that would minimize potential off-target gene activation. I suggest that T cell restricted transcription factors strongly influence HIV-1 proviral transcription and that these factors may provide specific targets for eliminating latent HIV-1. I report that Blimp-1, a critical regulator of B and T cell differentiation, is highly expressed in memory CD4$^+$ T cells compared to naïve CD4$^+$ T cells and is induced following T cell activation. Furthermore, I show that Blimp-1 binds sequences downstream of HIV-1 LTR, called HIV-1 ISRE, and is displaced following T cell activation. Blimp-1 inhibits Tat-dependent HIV-1 transcription. Reduction of Blimp-1 in
infected primary T cells, including CD4+ memory T cells, increases RNAP II processivity, histone H3 acetylation and baseline HIV-1 transcription. Treatment with protease inhibitors (Pacenti et al., 2006) as well as HIV-1 Tat have been shown to increase Blimp-1 expression in T cells (Sforza et al., 2014), thus promoting HIV-1 latency. Therefore, the transcriptional repressor, Blimp-1, is an intrinsic factor that predisposes CD4+ memory T cells to latent HIV-1 infection. I predict that modulating the expression of Blimp-1 or targeting its cofactors would reactivate and potentially purge latent virus in specific T cell subpopulations without global T cell activation. However, further research is required to understand the Blimp-1-mediated mechanisms of repression of HIV-1 transcription in different T cell subsets and to assess which Blimp-1-interacting factors are the best candidates for the development of novel treatment strategies. NELF-induced RNAP II promoter proximal pausing is a common mechanism of repressing proviral expression. Further studies are required to elucidate whether RNAP II pausing at HIV-1 LTR can be disturbed by modulating intrinsic T cell factors.

Blimp-1 inhibits Tat-dependent and Tat-independent HIV-1 transcription. Based on the latter finding, I would speculate that that Blimp-1 represses the generation of TAR RNA, thus inhibiting the recruitment of Tat and P-TEFb to HIV-1 LTR. Blimp-1 has been shown to inhibit transcription of c-Myc, a factor implicated in the release of paused RNAP II (Lin et al., 1997; Rahl et al., 2010). It remains to be determined if targeting of c-Myc by Blimp-1 is another mechanism via which Blimp-1 inhibits HIV-1 transcriptional elongation and generation of TAR RNA stem loop. Blimp-1 is a transcription factor that regulates its target genes by recruiting histone-modifying complexes, such as
methyltransferases and HDACs. Decreasing Blimp-1 levels increased acetylation of histone H3 at Nuc-1 positioned next to HIV-1 LTR. It remains to be seen if Blimp-1 recruits histone-modifying enzymes to Nuc-1. Preliminary data shows that treatment with TSA, an inhibitor of HDACs I and II, and BIX02194, a G9a inhibitor, did not abolish Blimp-1 mediated repression of HIV-1 transcription. However, the experiments should contain proper controls verifying that the inhibitors are effective, such as ChIPs looking at the acetylation and methylation of Nuc-1. It will be critical to fully characterize the mechanism by which Blimp-1 leads to increase in HIV-1 transcription upon T cell activation. It may be informative in future studies to look if Blimp-1 recruits LDS1 (Su et al., 2009), a histone lysine demethylase, which has been shown to repress HIV-1 transcription by inducing trimethylation of H3K4 and H3K9 (Le Douce et al., 2012) as well as to activate Tat (Sakane et al., 2011). The interaction of Blimp-1 with LSD1 could provide a mechanism for Blimp-1-mediated repression of HIV-1 in resting T cells where Blimp-1 would recruit LSD1 to methylate histones, and for Blimp-1 mediated induction of HIV-1 transcription upon T cell activation where Blimp-1 may recruit LSD1 to Tat to activate it.

I also speculate that Blimp-1 mediated enhancement of HIV-1 transcription upon T cell activation may be a result of Blimp-1 inhibiting another transcriptional repressor of HIV-1, since upon T cell activation Blimp-1 is no longer bound to provirus. Further studies to investigate the induction of HIV-1 transcription by Blimp-1 in activated T cells could also investigate the interaction of Blimp-1 with the viral transcriptional activator Tat or P-TEFb complex. It remains to be determined why Blimp-1 dissociates from the
HIV-1 ISRE upon T cell activation. I show that Blimp-1 co-occupies the HIV-1 ISRE with IRF-1 and IRF-8 and that T cell activation leads to a decrease in the binding to this site of all of these factors. Thus IRF-1 and IRF-8 do not replace Blimp-1 at the ISRE in the HIV-1 LTR upon activation. Future studies investigating the post-translational modification of Blimp-1 may provide useful information as to why Blimp-1 is released from the HIV-1 ISRE upon T cell activation. Future experiments should look at the change in Blimp-1 phosphorylation, ubiquitination and SUMOylation upon T cell activation and identify the protein responsible for these modifications. Co-culture of HIV-1-pulsed DC with T cells induces the expression of Blimp-1, as well as markers of exhaustion, including PD-1, Tim-3, LAG-3 and CTLA-4 (Shankar et al., 2011). However, Blimp-1 has been recently shown to repress the expression of PD-1 and NFATc1, its activator, in CD8^+ T cells (Lu et al., 2014b). It is also possible that by inhibiting NFAT, a transcriptional activator of HIV-1, Blimp-1 contributes to repression of HIV-1 transcription. It remains to be determined if Blimp-1 directly regulates the expression of other exhaustion molecules in HIV-1 infection. Bach-2 is a repressor of Blimp-1 and T cell differentiation (Tsukumo et al., 2013a) that has been shown to be enriched for integrated provirus site over time (Ikeda et al., 2007). It is possible that the disruption of BACH2 gene by provirus is in part responsible for the induction of Blimp-1 levels in HIV-1-infected patients.
Role of neighboring host genes in HIV-1 transcription

I also examined the influence of host neighboring promoters on HIV-1 transcription to identify common mechanisms controlling HIV-1 latency. About 40% of human transcripts overlap with each other (Birney et al., 2007) suggesting that neighboring promoters affect each other’s expression. HIV-1 integrates into transcriptionally active host genes and its transcription is carried out and controlled by the host transcriptional machinery. I have shown that inducible cell lines are differentially responsive to T cell activation and treatment with HDACs inhibitor. In addition, integration of provirus in the convergent orientation resulted in the induction of the host gene, while HIV-1 transcription did not correlate with the neighboring host gene expression levels. Furthermore, my data indicates that expression of the host gene allows HIV-1 transcriptional activation. My research has demonstrated that the majority of repressed provirus integrated into host genes in the parallel and convergent orientation has NELF and stalled RNAP II at the LTR. Decreasing NELF was able to induce HIV-1 transcription. My data supports a model in which HIV-1 latency is established and maintained by multiple mechanisms, including RNAP II promoter proximal pausing, condensed chromatin structure, T cell activation and intrinsic T cell transcription factors but is not dependent on proximity or orientation to the neighboring host promoters.

My data suggests that neighboring promoters have minimal impact on HIV-1 transcription; however I was not able to modulate the transcription of the host genes to definitely rule out their contribution to the maintenance of HIV-1 latency as there is not sufficient data looking at regulation of transcription of these host genes. The orientation
of the host genes and HIV-1 LTR does not seem to correlate with a specific transcriptional repression mechanism. However, in the cell lines with the convergent orientation of promoters the increasing distance between the two promoters seems to decrease the level of host gene transcription upon cell activation. It would be intriguing to investigate whether increasing the distance between HIV-1 LTR and a convergent promoter would increase their transcriptional interference by constructing a plasmid with the HIV-1 promoter integrated at different positions in the same gene. Because there was a range of responses to TSA treatment among CA5, BA1, 11B10, EF7 and CG3 cells one should try another HDAC inhibitor, such as valporic acid or vorinostat. It may be informative in future studies to look at the influence of activation with CD3+28 to see if signaling downstream of the TCR has different effect on host genes and HIV-1 expression compared to treatment with PMA+PHA. Future studies should be also aimed at measuring the strength of the neighboring host promoter to see if it plays a role in repressing HIV-1 transcription. Additional experiments verifying that the host genes are transcriptionally active need to be performed. Future studies would benefit from looking at the presence of active RNAP II at the host genes by using an antibody specific to phosphorylated Ser-2 of RNAP II. Future studies would ideally look at the presence of chimeric mRNA messages spanning the upstream host gene and the viral promoter to see if the elongating RNAP II disrupts transcriptional machinery at HIV-1 LTR. Because HIV-1 latency in inducible cell lines is regulated by a combination of mechanisms it will be critical to see if NELF cooperates with Blimp-1 to repress HIV-1 transcription by inhibiting RNAP II processivity.
Significance and Implications

My data show that there are common mechanisms mediating HIV-1 latency. Blimp-1 represses RNAP II processivity to sequences downstream of the ISRE, while proviruses integrated into active neighboring host genes have RNAP II paused at their promoters. Both, the inducible cell lines as well as cells expressing high levels of Blimp-1 have accumulation of short HIV-1 mRNA transcripts showing that RNAP II pausing is a common checkpoint of HIV-1 transcription. Previous work from our laboratory showed that NELF recruits NCoR1-GPS2-HDAC3 complex to HIV-1 LTR to remodel chromatin and induce premature termination of transcripts in primary CD4+ T cells (Natarajan et al., 2013). Thus, RNAP II pausing is coordinating multiple repressive pathways and targeting it may help in reactivating latent provirus.

To eradicate HIV-1 infection it would be preferable to use “shock & kill” approach, where latency reversing agents (LRA) would be used to reactivate latent virus while the patient is on HAART (Fig. 27). This strategy would result in elimination of latent reservoir and cell death of infected cells. Recent studies showed that broadly neutralizing antibodies (bNAbs) prevent the establishment of new infections and in combination with LRA greatly reduce the chance of viral rebound after cessation of treatment (Halper-Stromberg et al., 2014). HIV-1 gene therapy is another promising venue, where by using zinc-finger endonucleases (ZFNs) one can disrupt either the genes encoding HIV-1 co-receptors or provirus itself (Manjunath et al., 2013). The “shock & kill” strategy could benefit from including bNAbs to block new infections and gene
therapy to further interfere with HIV-1 replication (Fig. 27). In addition drugs targeting NELF or Blimp-1 may aid in the release of paused RNAP II, increase RNAP II processivity and open chromatin structure.

Figure 27. "Shock & Kill" Strategy to Cure HIV-1.

Eradication of HIV-1 infection will require the disruption of HIV-1 latency by using LRA. The disruption of latency should co-occur with HAART administration. The addition of bNAbs may prevent new infections, while gene therapy may aid in disruption of latent provirus that is not responsive to LRA. The goal of the “shock & kill” strategy would be reactivation of all latent provirus concurrent with depletion of all infected cells.
APPENDIX

RATIONALE

The Src kinase leukocyte-specific protein tyrosine kinase (Lck) is essential for initiating T cell receptor (TCR) signaling by activating downstream signaling proteins, such as phosphoinositide 3-kinase (PI3K) which phosphorylates PIP2 to phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Cannons and Schwartzberg, 2004). The Tec kinase interleukin-2-inducible T cell kinase (Itk) is a critical mediator of cytoskeleton rearrangements induced by TCR signaling. Itk is recruited to the PM by binding to PIP3, where it is phosphorylated by Lck (Cannons and Schwartzberg, 2004).

Previously, our lab has identified Lck and Itk as being crucial in efficient HIV-1 replication. I have shown that Lck positively regulates HIV-1 release, and physically interacts with Gag (Strasner et al., 2008). In T cells Gag assembly takes place at the PM, but in Lck deficient T cells Gag accumulates in intracellular compartments (Finzi et al., 2007; Strasner et al., 2008). Itk is required for efficient viral entry, transcription, and release (Readinger et al., 2008). I wanted to confirm that Lck increases HIV-1 release by regulating multiple stages of Gag intracellular transport and processing. I was especially interested in determining whether Lck is involved in transporting Gag to the PM, or preventing endocytosis of assembling Gag from the PM.
METHODS

For fluorescence microscopy experiments I have generated GFP-tagged WT Lck expression construct by using CT-GFP Fusion TOPO TA Expression Kit (Invitrogen). 293T and HeLa cells were plated on glass coverslips (Fisher). At 16 h post-transfection, cells were fixed in 2% paraformaldehyde, incubated with DAPI (Invitrogen), and mounted on glass slides. Samples were imaged using a 60x objective on Nikon deconvolution wide-field Epifluorescence microscope. Stack of 31 images were collected at 0.3-m spacing. Images were deconvolved using 10 iterations with NIS elements software. The data analysis was done with NIS elements software. To look at Lck role in HIV-1 replication I have generated Jurkat T cell lines with stable Lck knockdown using sh-RNA (Dharmacon). For experiments looking at the viral release VLPs were purified by pelleting collected supernatants on 20% sucrose gradient at 100,000 xg for 1.5 h at 4°C. The HIV-1 concentration in the supernatants was measured using an Alliance HIV-1 p24 Elisa Kit (Perkin-Elmer). I have used cell fractionation to look at Lck, Itk and Gag co-localization in different cellular compartments. Whole cell lysates were run on SDS-PAGE (12% gel) and western blots were used to detect protein expression.

RESULTS

To test the role of Lck in HIV-1 release I have generated stable, Lck-deficient Jurkat cell lines using Lck shRNA. Although in the past our lab had used JCaM cells,
which do not express functional Lck, the use of cells in which Lck was knockdown represents a more plausible approach to study the effect of Lck on HIV-1 release. Examination of virus-infected sh-Lck Jurkat cells confirmed Lck’s role in viral replication as the Lck knockdown decreased HIV-1 release by approximately 75% (Fig. 28).

To determine if Lck and Itk are the primary non-receptor tyrosine kinases mediating HIV-1 replication I have also examined the role of Fyn. Fyn and Lck are the two Src kinases expressed in T cells. I have decreased Fyn expression in Jurkat cells using si-Fyn. The knockdown was successful as determined by immunoblots; however,
with or without activation with anti-CD3 and -CD28 Abs, there was no significant
difference in HIV-1 release between si-CTRL and si-Fyn transfected cells as measured by
p24 release (Fig. 29). Thus, Lck is the primary Src kinase required for efficient HIV-1
dependent particle release from infected T cells.
Figure 29. Fyn has Minimal Impact on HIV-1 Replication in T Cells.

Jurkat cells were electroporated with si-CTRL or si-Fyn, and infected 24 h post transfection with HIV-1-PLAP-Nef(+). Cells were activated with 0.1 µg/ml anti-CD3 and 1 µg/ml anti-CD28 Abs for 24 h before collection. Release of p24gag was monitored at days 3 and 5 post-infection. Whole cell lysates were probed with anti-Fyn and anti-actin Abs.
Previously our lab has shown that Lck promotes Gag assembly at the PM (Strasner et al., 2008). I have now demonstrated that in 293T (Fig. 30) and in HeLa cells (Fig. 31) Lck co-localizes with Gag at the PM and in intracellular compartments. Mutation of the basic region in the MA domain leads to redistribution of Gag and its intracellular accumulation in late endosomes (Chukkapalli et al., 2008; Ono and Freed, 2004; Zhou et al., 1994). I speculated that expression of Lck may redirect the mutant Gag to the PM through a direct interaction with it. To test this I used Δ-MA-Gag, which has a deletion of residues 15 through 99 in the basic region of MA domain. However, using fluorescent imaging I observed that Δ-MA-Gag was located in intracellular compartments, even in the presence of Lck. This data suggest that Δ-MA-Gag has altered trafficking and thus does not interact with Lck, because it is not present in the same subcellular compartments as Lck, or that Lck binds to the MA domain of Gag.
**Figure 30. Lck Co-localizes with Gag at the PM and in Intracellular Compartments.**

293T cells were plated onto glass coverslips and transfected (A) or cotransfected (B) with Lck, and Gag-cherry as indicated. 16H post-transfection cells were washed with PBS and then fixed with 2% paraformaldehyde and stained with antibody to Lck. The glass coverslips were mounted onto glass slides and viewed using a Nikon fluorescence microscope at 60x immersion and deconvoluted using 10 iterations with NIS elements software.
Figure 31. Lck Alters the Localization of Gag in HeLa Cells.

HeLa cells were plated onto glass coverslips and transfected (A) or cotransfected (B) with Lck-GFP and Gag-Cherry, as indicated. 72H post-transfection cells were washed with PBS then fixed with 2% paraformaldehyde. The glass coverslips were mounted onto glass slides and viewed using a Nikon fluorescence microscope at 60x oil immersion and deconvoluted using 10 iterations with NIS elements software.
A. Gag-AMA-Cherry

B. Gag-AMA-Cherry Lck

Figure 32. Lck Does Not Alter the Intracellular Accumulation of ΔMA-Gag.

293T cells were plated onto glass coverslips and transfected with ΔMA(15-99)-Gag-Cherry (A), or cotransfected with Lck and ΔMA-Gag-Cherry (B). 16H post-transfection cells were washed with PBS and then fixed with 2% paraformaldehyde and stained with anti-Lck Ab. The glass coverslips were mounted onto glass slides and viewed using a Nikon fluorescence microscope at 60x oil immersion and deconvolved using 10 iterations with NIS elements software.
The assembly of HIV-1 occurs at the PM in T cells and macrophages, though some studies have suggested that intracellular compartments in macrophages can support virus assembly (Ono and Freed, 2004). However, as a result of frequent endocytosis Gag is also found in late endosomes in cells with preferential Gag assembly at the PM (Finzi et al., 2007; Klein et al., 2007; Ono and Freed, 2004). Clathrin- and cholesterol-dependent endocytosis of Gag along microtubules is mediated by dynamin (Cannons and Schwartzberg, 2004; Klein et al., 2007). To identify the function of Lck in preventing intracellular Gag accumulation I treated cells with Dynasore, a dynamin inhibitor, to block dynamin-dependent endocytosis. The treatment of 293T cells transfected with Gag, as well as Jurkat cell lines infected with HIV-1 with Dynasore did not change the fold induction of released p24 mediated by Lck (Fig. 33 and 34). These data suggests that Lck does not prevent endocytosis of Gag, or that HIV-1 is not endocytosed via a dynamin dependent pathway. Clathrin- and dynamin-dependent endocytosis are the two major endocytic pathways. To assess whether Lck prevents nascent virus endocytosis future studies would benefit from including Chlorpromazine to inhibit the clathrin-dependent endocytosis. Further studies investigating whether Lck inhibits the endocytosis of Gag should also include live cell microscopy of cells transfected with Gag-Cherry and Lck-GFP. The movement of Gag at the PM may be visualized by fluorescence recovery after photobleaching (FRAP), where regions beneath the PM associated with Gag-Cherry would be photobleached and monitored for subsequent recovery of Gag-Cherry.
The free diffusion of Gag from cytosol to the PM is prevented by densely packed cytoplasm and HIV-1 has to use the cellular transport system to ensure successful exit from the cell (Naghavi and Goff, 2007). Transport of Gag RNA, as well as Pr55Gag to the PM is microtubule dependent (Mouland et al., 2001). To test whether Lck-mediated VLPs release is actin and microtubule dependent, I co-transfected 293T cells with Gag and Lck or infected stable Jurkat cell lines with HIV-1 and blocked cytoskeleton reorganization with different inhibitors. Latrunculin B- and Cytocholasin-D-mediated inhibition of actin polymerization interfered with viral release, but did not block Lck-mediated enhancement of VLPs and HIV-1 release (**Fig. 34 and 35**). The treatment of

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**Figure 33. Lck Mediated Virus Like Particles Release is Dynamin Independent.**

293T cells were transfected with Gag along with Lck or pcDNA3.1 vector control via calcium phosphate. 3 h after transfection new media with 2 and 10 µM Dynasore or DMSO was added to cells. 72 h after transfection supernatants were collected, spun on 20% sucrose cushion and analyzed for p24 via ELISA.
cells with Nocodazole, a microtubules polymerization inhibitor, did not impede the
release of virus like particles, both in the presence and absence of Lck (Fig. 34). The 7
day treatment of Jurkat cells with Nocodazole was toxic to the cells (Fig. 35). These data
suggest that Lck mediated enhancement of HIV-1 release does not depend on
cytoskeleton reorganization. Additional experiments should be performed using a
microtubule depolymerization inhibitor (taxol) to rule-out the potential contribution of
microtubules in Lck-mediated HIV-1 release.
Figure 34. Lck Mediated Virus Like Particles Release Does Not Require Cytoskeleton Reorganization.

293T cells were transfected with Gag along with Lck or pcDNA3.1 vector control via calcium phosphate. 3 h after transfection new media with 2.5µM Cytocholasin D, 2.5µM Latrunculin B and 10 µg/ml Nocodazole or DMSO was added to cells. 72 h after transfection supernatants were collected and analyzed for p24 via ELISA.
Rab proteins are small GTPases controlling vesicular trafficking (Saftig and Klumperman, 2009). One such protein, Rab11, is located on the cytosolic face of perinuclear recycling endosomes and controls the trafficking of vesicles from TGN and recycling endosomes to the PM (Saftig and Klumperman, 2009). Src kinases are present on clathrin-independent vesicles that recycle back out to the PM dependent on Rab11 and actin (Donaldson et al., 2009). The exit of Lck from Rab11 positive endosomes requires the activation of Rab11 by the adaptor protein Uncoordinated 119 (Unc119), which is present in Rab-11 positive endosomes (Gorska et al., 2009). Rab11 enhances HIV-1 release, and its deficiency results in an intracellular accumulation of Gag, and in a decreased HIV-1 release (Amet et al., 2008; Saftig and Klumperman, 2009). Because Lck

Figure 35. Lck Mediated HIV-1 Replication Is Not Abolished by Cytoskeleton and Dynamin Inhibitors.

sh-Control and sh-Lck Jurkat stable cell lines were infected with HIV-1-PLAP-Nef(+). 8 h post-infection new media with inhibitors were added to the cells. 7 days post-infection the supernatants were collected and analyzed for p24 via ELISA.
and Gag require Rab11 to be conveyed to the PM, it is conceivable that they both follow similar trafficking route (Amet et al., 2008; Gorska et al., 2009). To examine the effect of altering Lck trafficking on Gag release I have transfected 293T cells with Gag, Lck, Rab11 (Gorska et al., 2009) and Unc119 (Gorska et al., 2009) via calcium phosphate. Supernatants were collected 72 h post-transfection, VLPs purified and analyzed for p24 via ELISA. The overexpression of Rab11 alone did not affect VLPs release (Fig. 36), even though it has been previously shown to inhibit perinuclear accumulation of Gag (Amet et al., 2008). Additionally, over-expression of Rab11 slightly decreased Lck-mediated VLPs release (Fig. 36). One might expect that Unc119 increases VLPs release by inducing Lck to activate Abl leading to actin reorganization, as well as inhibiting dynamin-dependent endocytosis (Gu et al., 2009; Karim et al., 2010). However, overexpression of Unc119 also did not increase viral release and decreased Lck-mediated enhancement (Fig. 36). One possible explanation for these negative data may be the fact that in 293T cells Gag is transported to the PM even in the absence of Lck (Fig. 30). Future experiments should be performed in HeLa or preferably in T cells and use siRNAs to deplete Rab11 and Unc119 or dominant-negative forms of Rab11 and Unc119. Alternatively, Lck trafficking might be also altered by manipulating the expression of MAL protein (Anton et al., 2008).
Lck orchestrates T cell signaling by phosphorylating downstream proteins. To determine if Lck needs to be active to enhance HIV-1 replication I have treated Jurkat cells with Damnacanthal, a specific Lck autophosphorylation inhibitor, and PP2, a Src family inhibitor. There was no significant difference between the unstimulated cells treated with Damnacanthal and PP2 compared to the control cells (Fig. 37). However, stimulated cells treated with Damnacanthal and PP2 showed even greater amounts of HIV-1 being released than the control cells (Fig. 37). This suggests that the activation of

Figure 36. Rab11 and Unc119 do not Increase VLPs Release.

293T cells were transfected with Gag along with Lck, Rab11, Unc119 or pcDNA3.1 vector control via calcium phosphate. 72 h after transfection, supernatants were collected, spun on 20% sucrose cushion and analyzed for p24 via ELISA.
Lck is dispensable for efficient HIV-1 replication. These results support the previous data showing that the kinase dead Lck induces virus like particles release more efficiently than the wild type Lck (Strasner et al., 2008), suggesting that Lck may be acting as an adapter protein for Gag assembly.

**Figure 37. Activation of Lck is Not Required for Efficient HIV-1 Replication.**

Jurkat cells were pre-treated with kinase inhibitors for 1 hour, and infected with HIV-1-PLAP-Nef(+) in the presence of inhibitors. 12 h post-infection the new media with inhibitors was added to the cells. The cells were left unactivated (A) or activated with anti-CD3 and 28 Abs for 24h (B). At 7 days post-infection the supernatant was collected and analyzed for p-24 via ELISA. Whole cell lysates were probed with anti-pLck (Tyr505) Ab.

Lck and Itk are both T cell specific non-receptor tyrosine kinases that participate in T cell signaling, and enhance HIV-1 release (Readinger et al., 2008; Strasner et al.,
Consistent with previous reports the non-receptor tyrosine kinases Lck and Itk increased VLPs release (Fig. 38). Additionally, the co-transfection of Gag with Lck and Itk showed that Lck and Itk cooperatively interact to enhance VLPs release (Fig. 38). These data suggest that Lck and Itk functionally interact with each other to achieve optimal HIV-1 release. The synergistic effect between Lck and Itk on HIV-1 release should be confirmed in T cells by performing double knockdown of Lck and Itk.

To identify intracellular compartments in which Gag and Lck interact, and the route that they follow to the PM I used subcellular fractionation. sh-Lck and sh-CTRL Jurkat cells were infected for 24 h with HIV-1. Cells were homogenized 5 days post-infection, and separated using subcellular fractionation into 13 fractions, with fractions 1-

![Figure 38. Lck and Itk Co-operate to Enhance Gag Release.](image)

293T cells were transfected with Gag along with Lck, Itk or pEGFP vector control via calcium phosphate. 72 h after transfection, supernatants were collected, spun on 20% sucrose cushion and analyzed for p24 via ELISA.
5 predicted to represent lipid rafts (Triantafilou et al., 2002). Gag co-localized with Lck in fractions 2-5 (Fig. 40). As expected, cells stably expressing sh-Lck had overall reduced levels of Lck and Lck was present only in fractions 2-4 instead of 1-5 (Fig. 40). The reduction of Lck levels also resulted in decreased presence of Itk in fractions 2-5 (Fig. 39). These results suggest that the subcellular localization of Itk is Lck dependent. In the subcellular fraction experiment the different fractions should be probed with Abs for markers of lipid rafts (GM1), non-raft associated proteins (CD45), early endosomes (EEA1 and Rab5), late endosomes (Lamp1 and TGN), as well as Rab11 and Unc119, proteins controlling the trafficking and activation of Lck.

The above experiments do not provide definitive mechanism by which Lck enhances Gag release. All experiments with inhibitors should be better controlled, account for possible cell death and preferentially the cytoskeleton should be visualized using microscopy.
Figure 39. Lck Co-Fractionates with Gag in Lipid Rafts.

A) The experimental workflow used for subcellular fractionation of HIV-1-infected cell lines. B) Jurkat T cells stably transduced with sh-Lck or sh-Ctrl lentivirus were infected with HIV-1 for 7 days. Cells were lysed in buffer A containing 0.25% Triton X-100 at 4°C and the lysate was mechanistically disrupted. Lysates were mixed with one volume of 50% OptiPrep and centrifuged at 52,000 xg for 16 h at 4°C. Thirteen fractions were collected from the top of the gradient, and analyzed by western blot. Fractions 1-5 are predicted to contain lipid rafts (Triantafilou et al., 2002).
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**Foreign Languages**

- Polish
- French

**Hobbies**

Kokikai Aikido (black belt), outdoor activities such as hiking, rock climbing, skiing and sailing