Investigating the Role of HIV-1 Nef During Reactivation of Latent Viral Reservoirs

by

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B.Sc, Simon Fraser University, 2011

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in the Department of Molecular Biology and Biochemistry Faculty of Science

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Abstract

A major barrier to development of a cure for HIV-1 is the ability of the virus to establish latent infection that reactivates to cause disease if antiretroviral therapy is discontinued. The HIV-1 protein Nef displays multiple in vitro and in vivo functions, including the ability to modulate T cell signaling events that may alter the activation status of infected cells. Nef is critical for viral pathogenesis, but its role during latency remains unclear. To investigate this, I generated a novel panel of latent T cell clones (C-Lat) harbouring a single integrated copy of HIV-1 encoding functional or defective nef genes. By assessing the location and genomic features of the proviral DNA integration sites in these clones, I observed that the presence of functional Nef variants was associated with a broader repertoire of inducible latent T cell lineages. By characterizing the reactivation phenotypes of these clones following stimulation with latency reversing agents (LRAs), I observed higher early and late viral protein expression in C-Lat clones encoding functional nef compared to those encoding defective nef. I confirmed these observations by disrupting the functional nef gene in C-Lat clones using CRISPR/Cas9 strategies. Variable viral reactivation phenotypes were observed in Nef knock-out (NefKO) clones following stimulation with LRAs, but the efficiency of early and late viral protein expression was consistently lower in NefKO clones compared to their corresponding parental isolates.

My research highlights the ability of Nef to modulate HIV-1 reactivation from latency. Results indicate that Nef may play an important role in determining the breadth and diversity of inducible viral reservoirs following infection. If so, a better mechanistic understanding of Nef’s impact may uncover new strategies to enhance viral reactivation from latency that are clinically beneficial.

**Keywords:** HIV-1; Nef; viral latency; shock and kill; viral integration; CRISPR/Cas9
Dedication

To my family and friends for their unconditional support.
To past and present members of my lab family for their friendship and support.
To my supervisors, Mark and Zabrina, for their continuous guidance and mentorship.
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<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation induced cell death</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
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<td>ASK1</td>
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<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter</td>
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<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<td>Bcl-xL</td>
<td>B-cell lymphoma-extra large</td>
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<td>cART</td>
<td>Combination antiretroviral therapy</td>
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<td>CEM-derived latent cell line</td>
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<td>COMMD1</td>
<td>Copper metabolism gene MURR1 (mouse U2af1-rs1 region 1) domain</td>
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<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeat</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
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<td>Green fluorescent protein</td>
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<td>gRNA</td>
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<td>Histone deacetylase inhibitor</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>IκB-α</td>
<td>Inhibitor of kappa B</td>
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<tr>
<td>IP3R</td>
<td>Inositol triphosphate receptor</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motifs</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>IRES</td>
<td>Internal ribosome entry site</td>
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<td>Lymphocyte-specific protein tyrosine kinase</td>
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<td>LRA</td>
<td>Latency reversing agent</td>
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<td>Long terminal repeat</td>
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<td>Mitogen-activated protein kinase</td>
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<td>Median fluorescence intensity</td>
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<td>Mesenger RNA</td>
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<td>NAKC</td>
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<td>Nef</td>
<td>Negative factor</td>
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<td>NHEJ</td>
<td>Non-homologous end joining</td>
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<td>N-TEF</td>
<td>Negative transcription elongation factor</td>
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<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<td>p21-activated kinase</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<td>PD-1</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>P-TEFb</td>
<td>Positive transcription elongation factor</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>Serine incorporator</td>
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<td>Specificity protein 1</td>
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<td>Transactivation-response</td>
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<td>Transcription activator-like effector nuclease</td>
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<td>Trans-Golgi network</td>
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<td>ZFN</td>
<td>Zinc finger nuclease</td>
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Chapter 1.

Implications for HIV-1 Nef in “Shock and Kill” Strategies to Eliminate Latent Viral Reservoirs

1.1. Current state of HIV-1 pandemic

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that causes acquired immunodeficiency disease syndrome (AIDS), a medical condition where a weakened immune system consequently leads to elevated risk of opportunistic infections (1-4). Since its discovery in 1983 (5, 6), HIV-1 has caused over 36 million AIDS-related deaths. According to the latest UNAIDS report, over 36 million people are currently living with HIV (7). While Sub-Saharan Africa accounts for the highest global burden of HIV-1 infection, ~75,000 cases are also reported in Canada (8, 9). Fortunately, with the introduction of combination antiretroviral therapy (cART) in the mid-1990s (10-12), it has become possible to suppress HIV-1 replication and prevent progression to AIDS in most treated individuals (13). Hence, HIV-1 infection is now considered to be a manageable chronic disease in Canada and other high-income countries (14, 15). Recent efforts to extend global cART access has resulted in over 20 million individuals on treatment (7), but availability of effective medications remains a challenge in some settings, particularly in lower-income countries (16, 17). Due to the ability of HIV-1 to establish stable cellular reservoirs of latent infection that are not targeted by existing drugs, cART must be maintained indefinitely to avoid rapid viral rebound (18-22). Furthermore, despite advances in cART potency, life-long antiretroviral treatment is associated with an elevated risk of cardiovascular and renal diseases that may be due to drug toxicities and the ongoing threat of developing drug resistance (23-28). Although the number of new HIV-1 infections has declined globally to less than 2 million per year, the total number of HIV-infected people continues to rise. Therefore, HIV-1 remains a global health crisis that urgently requires new interventions to prevent, treat and potentially cure infected individuals.
1.2. HIV-1 pathogenesis

The virion structure, genetics and replication mechanisms of HIV-1 are well-characterized. HIV-1 contains an ~10 kb positive-sense single-stranded RNA genome that encodes for 9 genes (Figure 1-1). Three genes that are common to all retroviruses, gag, pol, and env, produce major structural and enzymatic polyproteins that are essential for HIV-1 replication. Two regulatory genes, tat and rev, are crucial for viral gene transcription and protein expression. Finally, four accessory genes, nef, vif, vpr and vpu, which are often dispensable for viral replication *in vitro*, play significant roles to enhance HIV-1 pathogenesis and persistence *in vivo* (29).

Multiple features of HIV-1 contribute to the current challenge to eradicate (or cure) this viral infection. First, HIV-1 selectively targets and infects immune cells expressing the surface CD4 (cluster of differentiation 4) marker, a majority of which are CD4+ helper T cells (30). In the absence of cART, dramatic loss of CD4+ T cells ultimately leads to immunodeficiency, which increases morbidity and mortality associated with opportunistic infections and cancers (31). Second, HIV-1 is highly polymorphic, due to its error prone reverse transcriptase enzyme and rapid viral replication rate of up to $10^{10}$ virions/day (32-34). In fact, the ability of HIV-1 to evade immune recognition and to develop drug resistance can be attributed to its capacity to adapt to environmental pressure through the generation of mutations (35, 36). Finally, the HIV-1 proviral genome is integrated into the host cell genome as normal part of the viral replication cycle, allowing HIV-1 to persist in target cells in a largely silent state for the lifetime of the infected host (37).

**Figure 1-1**  Overview of HIV-1 genome
The ~10 kb HIV-1 genome contains 9 genes (*gag, pol, env, vif, vpr, vpu, tat, rev, and nef*) that are flanked by untranslated 5’ and 3’ long terminal repeats (LTRs). The gene of interest for this thesis is *nef*, which is highlighted in red.
1.2.1. Overview of HIV-1 life cycle

To initiate HIV-1 infection (Figure 1-2), the viral envelope (Env) glycoprotein binds to the CD4 receptor and a co-receptor (either CCR5 or CXCR4) present on the surface of target cells (38-40). Receptor engagement leads to fusion of the viral and cellular membranes at the attachment site, which allows the viral core particle to penetrate into the target cell and subsequently “uncoat” to discharge its viral contents and facilitate subsequent steps of the replication cycle (41). Once inside the cell, the viral reverse transcriptase enzyme converts the single-stranded RNA genome into double-stranded DNA (42). The viral DNA is transported to the nucleus as part of a pre-integration complex and subsequently introduced into the host cell genome through a process mediated by the viral integrase enzyme (37). Under optimal cellular conditions, the integrated form of HIV-1, also known as the “provirus”, immediately begins to synthesize new viral transcripts and proteins to support progeny virion assembly and budding at the cell plasma membrane. Following budding, virions undergo a maturation process (i.e. morphological change) mediated by the viral protease enzyme, resulting in fully infectious virion particles that can initiate new rounds of infection.

While the viral accessory genes (nef, vif, vpr and vpu) are often not essential for HIV-1 replication in vitro, they play important roles in enhancing viral pathogenesis, primarily through their abilities to modulate the status of infected cells and to evade host immune responses (reviewed in (43)). One of these genes, nef, is of particular interest because it encodes for Negative factor (Nef), a protein that has been shown to contribute to HIV-1 pathogenesis and persistence via multiple mechanisms.
1.2.2. HIV-1 Nef enhances viral pathogenesis

HIV-1 Nef is a 27-34 kDa myristoylated phosphoprotein. It is encoded by the highly variable \textit{nef} gene, which is located near the 3’ end of the viral genome. Nef is one of the earliest and most abundant viral proteins expressed by cells following infection (44-47). Although Nef is often not required for HIV-1 replication \textit{in vitro}, it has been clearly documented to be essential for viral pathogenesis \textit{in vivo}. For example, members of the Sydney Blood Bank Cohort, which consisted of eight individuals who were accidentally
infected with a nef-defective strain of HIV-1 following treatment for blood disorders, exhibited exceptionally slow disease progression (44, 48) and similar clinical outcomes, including long-term survival in the absence of cART, have been reported for other HIV-1 cases where nef is deleted or defective (49, 50). Furthermore, viral pathogenesis was significantly attenuated in animal models using rhesus macaques or SCID-hu mice infected with nef-deleted strains of SIV or HIV, respectively (51, 52).

As an essential protein for viral pathogenesis, Nef does not display any enzymatic activity; rather, it serves as a multi-functional adaptor protein that interacts with host proteins to interfere with a variety of processes in infected cells (53, 54). Selected Nef motifs and their corresponding functions and cellular binding partners are described in Table 1-1 and Figure 1-3. The full scope and impact of Nef during HIV-1 infection has not yet been determined. Indeed, new activities and new interactions continue to be uncovered, including the recent observation that Nef counteracts cellular host restriction factors SERINC3 and SERINC5 (55, 56). While critical Nef motifs have been described for many of its known functions, these tend to be highly conserved in natural HIV-1 isolates (57). In the absence of genetically separable Nef activities that can be linked to different clinical outcomes in human populations, it will remain difficult (if not impossible) to define which of Nef’s cellular interactions is most important to promote viral pathogenesis.

### Table 1-1  List of Nef domains and corresponding host protein interactions

<table>
<thead>
<tr>
<th>Nef variant</th>
<th>Function(s)</th>
<th>Cellular binding partner(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2</td>
<td>Membrane localization</td>
<td>N-myristoyltransferases</td>
<td>(58-60)</td>
</tr>
<tr>
<td>M20</td>
<td>HLA-I downregulation</td>
<td>AP-1</td>
<td>(61, 62)</td>
</tr>
<tr>
<td>EEEE65</td>
<td>Cell signaling and HLA-I downregulation</td>
<td>PACS-1, PACS-2</td>
<td>(63-66)</td>
</tr>
<tr>
<td>PxxP75</td>
<td>Cell signaling</td>
<td>SH3/Src kinases</td>
<td>(67, 68)</td>
</tr>
<tr>
<td>LL165</td>
<td>CD4 downregulation</td>
<td>AP-1, AP-2, AP-3</td>
<td>(69-71)</td>
</tr>
<tr>
<td>DD175</td>
<td>CD4 downregulation</td>
<td>AP-2</td>
<td>(72, 73)</td>
</tr>
</tbody>
</table>
Figure 1-3  Schematics of Nef protein structure.
Nef protein structure shown in two different views (rotated 90ºC), each highlights the six conserved motifs (in red) described in Table 1-1.

**CD4 downregulation**

One extensively studied Nef function is its ability to downregulate CD4 molecules on virus-infected cells (74). Nef has been shown to bind directly to the cytoplasmic tail of CD4 (75, 76), triggering its internalization via clathrin-mediated endocytosis (77, 78). In addition, Nef can retain CD4 inside the endosome, preventing it from recycling to the plasma membrane and accelerating its degradation in lysosomes (79, 80). CD4 is a membrane glycoprotein found on the surface of various immune cells such as T lymphocytes, macrophages, monocytes and dendritic cells. Because CD4 is the primary receptor for HIV-1 attachment and entry into target cells, interactions between CD4 on the infected cell surface and Env present on budding virions can inhibit the release of newly formed HIV-1 particles (81, 82). Reduced CD4 expression also appears to increase the incorporation of the functional HIV-1 Env glycoprotein trimers on viral particles, thereby enhancing virion infectivity (83). Furthermore, downregulation of CD4 has been shown to inhibit superinfection, which is the re-infection of a target cell that can lead to premature cell death (84). Finally, interaction between CD4 and Env glycoproteins within the same infected cell has been shown to change the conformation of Env to expose epitopes that
are recognized by antibodies with potent antibody-dependent cellular cytotoxicity (ADCC) activity (85-87). Hence, efficient downregulation of CD4 by Nef can also protect infected cells from elimination by ADCC (88).

**HLA class I downregulation**

Another well-documented function of Nef is its ability to evade the host immune response by selectively downregulating two human leukocyte antigen class I (HLA-I) molecules, HLA-A and HLA-B (89-91). HLA-I is an extremely polymorphic surface protein that presents foreign peptide antigens typically 8-11 amino acids in length to CD8+ cytotoxic T-lymphocytes (CTL). In the case of HIV, presentation of virus-derived peptides by HLA-I molecules can result in the recognition and elimination of infected cells by CTL (92). Consistent with this, greater breadth and magnitude of HLA-restricted CTL responses are associated with better control of viremia during primary HIV-1 infection (93, 94) as well as a slower rate of clinical disease progression (95, 96). Hence, reduced expression of HLA-A and HLA-B molecules on the surface of infected cells in the presence of functional Nef can protect these cells from immune surveillance (92). Additionally, the retention of other HLA molecules on the cell surface, such as HLA-C and HLA-E that inhibit the cytotoxic activity of natural killer (NK) cells, is thought to protect infected cells from elimination by this innate immune response (90, 91). The precise mechanisms of Nef-mediated HLA-I downregulation have not been elucidated fully, but this activity is distinct from that of CD4 downregulation (97, 98). That is, instead of initiating the formation of clathrin-coated vesicles, Nef interacts with a series of host proteins to induce signaling events that lead to clathrin-independent internalization of surface HLA-I molecules (98-100). Additionally, Nef disrupts HLA-I trafficking by redirecting newly synthesized HLA-I molecules through the trans-Golgi network (TGN) to endosomes (98, 100, 101). These two pathways are not mutually exclusive, and both ultimately result in the degradation of HLA-I molecules in lysosomal compartments, thereby reducing HLA-I expression at the cell surface. Altogether, the ability of Nef to selectively downregulate HLA-A and HLA-B prevents targeted killing of infected cells by CTL (and possibly NK cells), which likely contributes to the establishment of persistent HIV-1 infection.
**SERINC downregulation**

A novel strategy to explain the ability of Nef to enhance viral infectivity was elucidated by two groups of researchers in 2015. Specifically, it was shown that Nef can antagonize host restriction factors serine incorporator 3 and 5 (SERINC3/5) (55, 56). SERINC3 and SERINC5 are members of the SERINC family of transmembrane proteins, which consists of five proteins (102). While there are lots of unknowns with respect to the actual function of SERINC proteins, they seem to play conserved roles to facilitate the biosynthesis of sphingolipids and phosphatidylserine at the cell membrane (102). SERINC3 and SERINC 5 also display the additional ability to restrict viral infection (55, 56), which appears to be independent of their activities in lipid synthesis. While understanding the precise mechanisms responsible for SERINC3/5’s anti-HIV activity are currently areas of active investigation (103, 104), it has been shown that incorporation of SERINC3 or 5 onto the membrane of newly formed virions significantly reduces their ability to form fusion pores with target cells, resulting in lower HIV-1 infectivity (105). To counteract these host restriction factors, HIV-1 Nef can downregulate SERINC3/5 from the surface of infected cells, which ultimately leads to the production of more highly infectious progeny virions (106). While the precise mechanisms for Nef-mediated SERINC3/5 downregulation have not yet been defined, this process displays some similarities with that used by Nef to modulate CD4, namely a requirement for clathrin-mediated endocytosis and subsequent degradation of these proteins in lysosomes.

**Modulation of T cell signaling**

Another critical role of Nef during HIV-1 infection is its ability to modulate T cell signaling. The activation of CD4+ T cell is initiated upon engagement between its T cell receptor (TCR) complex and the peptide-bound HLA class II (HLA-II) molecule on antigen presenting cells such as B cells, dendritic cells and macrophages. Typically, efficient cellular activation also requires stabilization of the TCR-peptide-HLA-II complex by CD4 as well as co-stimulatory signals elicited by interactions between the CD28 receptor on the T cell with the B7 family ligands CD80 and CD86 present on the antigen presenting cell (107). These interactions promote the formation of an immunological synapse that further enhances signal transduction events leading from the TCR that
subsequently results in the translocation of transcription factors such as NFAT (nuclear factor of activated T cells), AP-1 (activator protein 1) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) into the nucleus to induce gene transcription (107, 108). In the CD4+ T cell, signaling begins when the Src family tyrosine kinase Lck (lymphocyte-specific protein tyrosine kinase) phosphorylates the ITAM (immunoreceptor tyrosine-based activation motifs) on the TCR complex, which is exposed following a conformational change in the TCR complex that occurs upon peptide recognition (109). By downregulating CD4 and CD28 molecules on virus-infected T cells (74, 110), Nef might reduce the efficiency of T cell activation through the TCR. To further suppress activation responsiveness in infected T cells, Nef binds Lck and redirects it to the TGN (away from the plasma membrane) where it can no longer participate in proximal TCR signal amplification events (111-113). Together, the reduced availability of CD4, CD28 and Lck signaling molecules also prevents the formation of an immunological synapase at the plasma membrane (111, 113, 114). Paradoxically, while altered trafficking of Lck interrupts TCR-mediated signaling, it permits the activation of Ras and downstream MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinases) signaling events at the intracellular TGN compartment by forming a protein complex that has been referred to as the Nef “signalosome” (112). Alternatively, Nef can induce Ras activity via formation of a Nef-associated kinase complex (NAKC), which is comprised of Nef, Lck, LAT (linker of activated T cells) and Ras proteins (112, 115). In synergy with activated Ras signaling, interaction between Nef and the ER (endoplasmic reticulum)-resident inositol triphosphate receptor (IP3R) can trigger calcium flux into the cytosol and induce TCR-independent NFAT activation (116, 117). Together, Nef’s uncoupled effects on T cell activation pathways can simultaneously suppress activation-induced cell death (AICD) triggered by extracellular antigen recognition and also increase viral gene transcription by enhancing distal signaling events leading to activation of critical transcription factors.

**Modulation of programmed cell death/apoptosis**

In additional to its abilities to evade immune-mediated killing and antigen-induced AICD, Nef has also been shown to modulate apoptotic pathways in order to protect infected
cells from undergoing programmed cell death. In particular, Nef inhibits the activity of apoptosis signal-regulating kinase 1 (ASK1) (118), which is a crucial mediator of apoptotic pathways triggered by Fas and tumor necrosis factor-alpha (TNF-α) (119, 120). Another anti-apoptotic activity of Nef requires association with PI3K (phosphatidylinositol 3-kinase) and PAK (p21-activated kinase) to mediate the phosphorylation and inhibition of pro-apoptotic protein BAD (Bcl-2-associated death promoter) (121). Contrary to its ability to inhibit apoptosis, several studies have reported a pro-apoptotic role for Nef, suggesting that selective induction of apoptosis could contribute to immune evasion and disease pathogenesis. For example, Nef-mediated upregulation of Fas ligand induced apoptosis of uninfected bystander CD4+ T cells and CTL (122-124), thereby dampening the local immune response against HIV-infected cells. During advanced stages of HIV infection, Nef could induce apoptosis by reducing the expression of cell death inhibitors Bcl-2 (B-cell lymphoma 2) and Bcl-xL (B-cell lymphoma-extra large) (125) and by upregulating the transcription of inhibitory receptor PD-1 (programmed cell death) (126), eventually leading to massive cell death. Current evidence thus indicates that Nef may protect virus-infected cells from apoptosis, while simultaneously eliciting the death of bystander immune cells that may be beneficial to enhance pathogenesis.

Modulation of cytoskeleton reorganization

The rearrangement of the host cell cytoskeleton is necessary for a number of important cellular processes, including cell division, cell signaling and protein trafficking (127, 128). By manipulating cytoskeletal dynamics, HIV-1 Nef may produce a more permissive cellular environment to support viral replication or spread. Nef associates with the serine/threonine kinase PAK2 in a multiprotein complex and redirects its phosphorylation to a novel target, the actin depolymerization factor cofilin, (129, 130), which results in reduced F-actin turnover and actin cytoskeleton remodelling (131, 132). Consequently, this prevents F-actin accumulation at the immunological synapses upon TCR engagement (113), thereby contributing to the inhibition of AICD and prolonging the survival of infected cells (133). In the absence of actin cytoskeleton remodelling, the mobility and migration of HIV-infected cells is also inhibited. While the contribution of reduced cell mobility to viral replication remains unclear, two scenarios have been
proposed in the a review article by Stolp et al. (134). First, infected CD4+ T cells may be refrained from migrating towards the B cell follicles to induce B cell activation and HIV-specific antibody production (135), thus impairing the immune response (136). Second, infected CD4+ T cells may be retained inside the lymph tissues, where the majority of CD4+ T cells reside, thereby promoting cell-to-cell transmission and viral spread (137).

1.3. HIV-1 latency

An important feature of HIV-1 pathogenesis is its ability to establish reservoirs of latent virus-infected cells extremely early following infection (138, 139), which appear to result mainly from reversible inactivation of HIV-1 transcription from integrated proviral DNA genomes (140). The lack of viral gene expression and protein production shields these latent cells from current antiretroviral drugs as well as antiviral host immune mechanisms, thereby permitting them to cause rapid viral rebound upon cessation of cART (19). Indeed, elimination of latent viral reservoirs is currently one of the biggest challenges to developing an effective strategy to cure HIV-1.

1.3.1. Latent HIV-1 reservoirs

Latent viral reservoirs are best defined as HIV-infected cells harbouring an integrated copy of a replication-competent provirus that is transcriptionally silent. Correspondingly, these reservoirs are capable of producing infectious virus upon cellular activation (21) and initiating new rounds of infection. While latent reservoirs are comprised of various cell types distributed among many anatomical sites, resting CD4+ T cells represent the largest and most studied source of latent HIV-infected cells (141). It was shown 20 years ago that reservoirs of HIV-infected resting CD4+ T cells could be established within days of infection (21, 142, 143). While resting CD4+ T cells are comprised of both naïve and memory subsets, latent infection is more prevalent in the latter cell subset. This is consistent with activated T cells displaying higher permissiveness to HIV infection, and suggests that latency occurs preferentially in T cells that have recently encountered antigen stimulation but can no longer support productive viral replication. In particular, due to the long half-life (144 months) of central memory T cells (Tcm) and
sustained homeostatic proliferation of transitional memory T cells (T_{TM}), latent HIV infection has predominantly been identified in these two subsets of resting memory CD4^+ T cells ([144], [145]). Additionally, increasing evidence suggests that CD4^+ T memory stem cells (T_{SCM}), which have substantially slower decay rate (277 months) and more pronounced proliferative potential than T_{CM} or T_{TM} cells, could support extremely long-term viral persistence ([146]-[148]). Due to the extended survival and self-renewal properties of resting T cell reservoirs, these latently infected cells support rapid viral rebound despite their low frequency (estimated at 1 per 10^6 resting CD4^+ T cells) ([142]) and even after prolonged periods of suppressive antiretroviral treatment ([18], [19]).

1.3.2. Mechanisms of HIV-1 latency

During productive HIV-1 infection, insertion of proviral DNA into the host chromosome is followed by basal activation of the viral 5' LTR promoter. The initial phase of 5' LTR activation is mediated by host transcription factors, such as NF-kB and NFAT ([149], [150]), leading to inefficient expression of multiply-spliced mRNAs encoding early viral proteins Tat, Rev, and Nef ([45], [46], [151]). Sufficient accumulation of the viral transactivator protein Tat subsequently enhances transcription elongation at the 5' LTR, allowing unspliced/full-length or single-spliced viral mRNAs to be produced ([151], [152]). Simultaneously, accumulation of the viral Rev protein facilitates nuclear export of these longer mRNA species into the cytoplasm for translation of late viral proteins (including Gag) or encapsidation into progeny virions as the ssRNA genome ([153], [154]). During latent HIV-1 infection, this highly regulated process of viral gene transcription and protein expression is prohibited by multiple mechanisms involving both host and viral factors.

Genomic environment

The cellular chromatin environment is highly heterogeneous, and hence, the site and orientation of HIV-1 integration into the host cell genome is likely to contribute to the establishment of latency. While it was originally believed that HIV-1 preferentially integrated into the intragenic regions of actively transcribed genes to promote viral replication ([155]-[157]), a later study showed that a majority of proviruses from infected
resting CD4+ T cells also reside within active host genes (158). In fact, the distribution and pattern of integration are generally similar between actively replicating and latent proviruses (159-161). Nonetheless, the insertion of latent proviruses was slightly enriched in locations that disfavor cellular gene transcription, such as alphoid repeats, heterochromatin, and regions distal from enhancer motifs (155, 161, 162). Moreover, integration of latent provirus was also frequently observed to occur in genes associated with cell proliferation and cancer, which could enhance the ability of infected T cells to expand and persist (163, 164). Aside from genomic location, the orientation of HIV-1 provirus could also interfere with viral gene transcription; and two different mechanisms have been described. First, if the provirus integrates downstream of a highly active gene that shares the same transcriptional orientation, promoter occlusion could occur by preventing the assembly of a transcription initiation complex at the viral 5’ LTR (165, 166). Second, if the provirus and host gene are in a convergent orientation, RNA polymerase II from the respective promoters could collide, resulting in premature arrest of viral gene transcription (167). These mechanisms are not mutually exclusive, and both suggest that the strength of HIV-1 transcriptional activity relative to its genomic environment will be a crucial factor that contributes the fate of the integrated provirus.

**Epigenetics**

The expression of many host genes is regulated by epigenetic modifications of the chromatin structure. Likewise, chromatin changes at the HIV-1 5’ LTR promoter have also been shown to affect the transcriptional activation of provirus. With respect to the latent reservoirs, two types of chromatin modifications, namely histone acetylation and DNA methylation, have been studied extensively. For histone acetylation, an enriched number of acetyl groups on the histone proteins can displace a repressive nucleosome (i.e. nuc-1) that is preferentially located at the 5’ LTR (168, 169). This modification ultimately opens the chromatin structure and encourages gene transcription. The acetylation state of the HIV-1 promoter can be modulated by the recruitment of histone acetyltransferases (HATs) and histone deacetylases (HDACs), which catalyze the incorporation or removal of acetyl groups on the histone proteins, respectively (170, 171). Correspondingly, treating latent HIV-infected cells with HDAC inhibitors (HDACi) such as vorinostat and panobinostat
can increase histone acetylation and induce proviral transcription (172, 173). DNA methylation, on the other hand, suppresses gene expression by reducing the affinity of transcription factors at CpG-rich promoter regions (174, 175). One study demonstrated that, CpG islands near the HIV-1 promoter of latent proviruses are hyper-methylated; and that the use of a DNA methylation inhibitor reversed this inhibitory effect and promoted viral gene expression (176). Also, the inducibility of latent HIV-infected cells has been shown to correlate negatively with local CpG methylation (177).

**Cellular transcription factors**

Similar to cellular promoters, the HIV-1 5’ LTR promoter sequence encodes binding sites for numerous cellular transcription factors, including NF-κB, NFAT, AP-1, and Sp1 (specificity protein 1) (178, 179). Binding of these transcription factors at the 5’ LTR facilitates the recruitment of RNA polymerase II to the TATA box, and together, these proteins form a transcription-initiation complex that induces the early, Tat-independent phase of viral mRNA expression (180). As such, the basal viral gene expression is determined by the activation state of the infected cell as well as the abundance and activity of transcription factors. Resting CD4+ T cells display a quiescent phenotype consisting of reduced transcriptional activity, which is consistent with these cells being a more suitable cellular environment to establish and maintain latent HIV reservoirs. In particular, multiple cellular activation pathways are dampened, resulting in low nuclear translocation of transcription factors and hence lack of recruitment to the 5’ LTR. For instance, in the absence of PKC (protein kinase C) activation, IκB-α (inhibitor of kappa B) accumulates in the cytoplasm and consequently binds and sequesters NF-κB (181-183). Without PKC activation, calcium release is also inhibited, thereby preventing NFAT dephosphorylation and translocation into the nucleus (184, 185). Similarly, the lack of MAPK/ERK pathway activation prevents phosphorylation and nuclear localization of AP-1 (186). In addition to inhibition of nuclear translocation, resting cells also express the inactive form of NF-κB and Sp1 (187, 188). In particular for NF-κB, an abundance of inactive p50/p50 homodimers can compete for κB binding site on the HIV-1 promoter. Once bound to the promoter, the p50/p50 homodimer can recruit HDAC-1 for histone deactylation to further repress viral gene transcription (189). Furthermore, over expression of transcriptional
repressors in resting T cells could also inhibit 5’ LTR activation. For example, TRIM22 (tripartite motif-containing 22) prevents the binding of Sp1 to the HIV-1 promoter (190) whereas COMMD1 (copper metabolism gene MURR1 (mouse U2af1-rs1 region 1) domain) enhances IκB-α stability and promotes NF-κB degradation (191, 192).

**Host/viral protein interactions**

The HIV-1 Tat protein has been shown to influence the establishment and maintenance of viral latency. After the early phase of viral mRNA transcription, the expression of Tat protein is necessary to enhance transcription elongation and promote the generation of full-length HIV-1 transcripts (151). Mechanistically, Tat recruits the cellular P-TEFb (positive transcription elongation factor) complex to the 5’ LTR by binding to an RNA stem-loop motif present on the nascent viral transcript, referred to as the transactivation-response (TAR) element (193, 194). Subsequently, P-TEFb phosphorylates RNA polymerase II to increase its processivity, thereby preventing the premature termination of viral transcription (195, 196). P-TEFb also phosphorylates and dissociates negative transcription elongation factors (N-TEFs) from the 5’ LTR, relieving their inhibitory effects on transcription (197). Consistent with its role, tat sequence variants that display impaired transactivation activity have been observed more frequently in latent viral reservoirs (198). Mutations in the tat gene were also identified in a widely used latency model, the U1 myeloid cell line (199). Moreover, the latent phenotype of U1 cells can be reverted by restoration of functional Tat using a retroviral vector (200).

In contrast to Tat, the contributions of other viral accessory and regulatory proteins in post-integration latency have not been clearly defined. Two proteins of great interest are Nef, which will be discussed in greater detail below, and Vpr. The Vpr protein is known to facilitate the nuclear localization of the HIV-1 preintegration complex and to arrest the cell cycle in the G2/M phase (201-203). Vpr has also been shown to enhance HIV-1 transactivation and subsequent virus production (204, 205), which may be due its ability to promote targeted ubiquitination and proteasomal degradation of cellular proteins. In particular, Vpr induced the depletion of class I HDACs, which led to hyper-acetylation of the HIV-1 LTR and viral activation (206).
1.3.3. A role for Nef in HIV-1 latency

The contribution of Nef to HIV-1 latency has not been examined extensively; however, a role for Nef would be consistent with its abilities to enhance viral pathogenesis and infectivity through modulation of diverse cellular pathways. Indirectly, through its ability to enhance virion infectivity and entry by counteracting SERINC3/5 restriction factors (55, 56, 106), Nef may contribute to the establishment of latent infection. Indeed, the presence of Nef has been associated with increased proviral DNA synthesis (207). Additionally, through its ability to promote viral persistence by evading host CTL immunity, Nef may increase the seeding of latent viral reservoirs that accumulate over time in the absence of cART (208). More directly, through its ability to modulate the activation state of infected cells by redistributing critical signaling kinases, Nef may serve as an important viral regulator of productive versus latent infection.

It has been suggested that infection of both activated and resting CD4+ T cells could lead to HIV-1 latency (209), but the host and viral requirements may be different. For example, activated CD4+ T cells are highly sensitive to productive HIV-1 infection, but they tend to die rapidly due to cytopathic effects and immune-mediated clearance (210, 211). As such, mechanisms that extend T cell survival may need to be present to allow infected cells to revert back into a resting state where HIV-1 transcription becomes silent. In activated CD4+ T cells, multiple Nef activities could directly or indirectly delay cell death. For example, HLA-I downregulation inhibits CTL-mediated recognition and elimination of infected cells (92). Immune evasion is crucial for HIV persistence because the strength of CTL activity is associated with the control of virus spread during acute infection (212). Also, the ability of Nef to associate and interfere with ASK1- and PI3K-mediated pathways of apoptosis would prolong the life of infected cells (118, 121). Through its pro-apoptotic role, Nef also induces apoptosis of bystander cells such as CTLs, thereby further hinders the immune response against infected cells (122). Moreover, Nef-mediated downregulation of CD4 receptor and CXCR4 co-receptor inhibits superinfection and its consequent premature cell death (84, 213). Furthermore, Nef’s role in cytoskeleton remodeling and T cell signaling could indirectly suppress AICD and prolong cell survival.
On the other hand, despite being the major reservoir of latent HIV-infected cells, resting memory CD4\(^+\) T cells are relatively resistant to productive viral replication (214-216). Direct HIV-1 infection of these quiescent cells is possible (209, 217-219); however, major post-entry barriers exist, including inefficient nuclear import of the viral preintegration complex, which must occur prior to viral DNA integration into the host chromosome (215, 218, 220). One potential regulator of HIV-1 integration is JNK (c-Jun N-terminal kinase). Specifically, JNK catalyzes the phosphorylation HIV-1 integrase, which leads to conformational changes that increase its stability (220). Since Nef can activate the JNK signaling pathway via formation of NAKC (130), it is plausible that Nef could enhance the efficiency of proviral DNA integration in resting CD4\(^+\) T cells. Nef has been shown to interact with INI1 (integrase interactor 1) (221), which is a core subunit of the chromatin remodeling complexes that associates with HIV-1 integrase to promote viral DNA integration (222); however, the interaction between Nef and INI1 did not appear to enhance proviral DNA integration (221). Nonetheless, a recombinant Nef derived from SIVsm (sooty mangabey strain PBj1.9) and HIV-1, call HSIVnef, facilitated the nuclear transportation and integration of proviral DNA (221), leading to speculation that certain natural HIV-1 Nef strains may share the same capability.

Finally, interactions between microRNAs and the nef gene might contribute to latency regulation. MicroRNAs are short single-stranded noncoding RNAs of 19 to 25 nucleotides that can mediate post-transcriptional gene-silencing by binding to specific gene sequence (223). In resting CD4\(^+\) T cells, there’s an enrichment of cellular microRNAs targeting the 3’ end of HIV-1 mRNA (224). As a result, these microRNAs can potently inhibit virus production and induce viral latency. Two Nef-related microRNAs have also been identified. First, the human cellular microRNA hsa-miR29a targets a highly conserved region of Nef (225, 226). Second, the Nef transcript can get processed into miR-N367, which targets an overlapping region between Nef and 3’LTR (227, 228). Both hsa-miR29a and miR-N367 are capable of reducing Nef protein expression and repressing HIV-1 replication, implicating their role in the establishment and maintenance of viral latency.
1.4. Strategies to cure HIV

The presence of long-lived latent viral reservoirs is the major hurdle to achieving cART-free HIV-1 remission and a potential cure. To date, the only case of an apparently successful HIV cure is the “Berlin patient”, who received two hematopoietic stem cell transplants from separate CCR5∆32 homozygous donors to treat his leukemia (229, 230). He displays no evidence of HIV-1 infection despite remaining off therapy since 2007. However, such transplants are exceptionally high-risk procedures, and are thus not applicable to the global population of HIV-infected individuals. Furthermore, subsequent attempts to use similar transplantation strategies in HIV-infected individuals who were also undergoing cancer therapy have been unsuccessful, with viral rebound observed within weeks to months following cART discontinuation (231). Therefore, the development of safer and more effective methods to reduce or eliminate latent HIV-1 reservoirs in cART-treated individuals is a high priority for researchers and the community.

1.4.1. “Shock and kill”

The “shock and kill” strategies (Figure 1-4) are currently the most intensively investigated methods to eliminate HIV reservoirs in cART-treated individuals (232). In this approach, viral gene transcription is reactivated in latent HIV-infected cells using latency-reversing agents (LRAs) that can modulate chromatin structure or otherwise activate the 5’ LTR promoter. Subsequent protein expression and presentation of viral antigen is then expected to result in the elimination of these cells, mediated by viral cytopathic effects or by revealing these cells to the host immune system. While various “shock and kill” strategies have been tested in clinical studies, none have been reported to successfully reduce the latent viral reservoir in vivo (172, 233-235). The major hurdles encountered by these strategies include inefficient induction of viral protein expression and ineffective clearance of reactivated cells by the host immune system, both of which could be hindered by activities of HIV-1 Nef.

Different classes of LRAs have been identified and tested for their ability to “shock” the latent reservoirs. In particular, pan-HDAC inhibitors (HDACi), such as vorinostat (172, 234), romidepsin (235), and panobinostat (173), are currently one of the
most promising classes of LRAs. Through the inhibition of multiple HDAC enzymes, HDACi increases the overall level of acetylation on histone molecules. This ultimately reduces chromatin condensation and promotes nonspecific increases in both host and viral gene expression. Many HDACi are FDA-approved for cancer treatment, and their pharmacological and toxicological profiles are known. Hence, HDACi have quickly advanced to clinical trials in the context of HIV-1 latency, where they have demonstrated a range of abilities to induce latent viral reservoirs that broadly reflect their potency (233, 236). Several other classes of LRA have also been tested in clinical studies. For example, disulfiram modestly reverses HIV-1 latency by depleting PTEN (phosphatase and tensin homolog), which subsequently results in activation of the PI3K/Akt pathway (237). Moreover, PKC activators such as prostratin and bryostatin potently initiate viral transcription in ex vivo experiments (238, 239); however, treatment with tolerable doses of bryostatin showed minimal ability to reactivate latent HIV-1 in human studies (240). Additional LRAs such as TLR (Toll-like receptor) agonists (241) and cytokines (i.e. interleukin-7 and -15) (242) have also been examined. Overall, none of these clinically relevant LRAs can potently reverse HIV latency in HIV-infected participants. In fact, one in vitro study indicated that many latent reservoirs were not inducible despite maximal T cell activation using PHA (phytohemagglutinin) or PMA (phorbol 12-myristate 13-acetate) and ionomycin (143), suggesting that repeated stimulations using more potent LRAs may be necessary to achieve a clinically beneficial outcome.

Many factors that promote HIV-1 latency are likely to contribute to the inducibility of latent reservoirs upon treatment with an LRA. Of particular interest is the multifunctional Nef protein. Even though Nef’s role in the context of HIV latency is not well-characterized, several studies have highlighted its ability to induce viral reactivation. For example, Fujinaga et al. demonstrated that extracellular Nef was capable of activating virus production in latent cell lines (i.e. MOLT-20-2 and U1) as well as in peripheral blood mononuclear cells (PBMCs) of asymptomatic HIV-infected participants (243). Follow-up study by the same group suggested that this observation was driven by Nef’s ability to induce Ras-mediated MAPK/ERK signaling pathway (244). The effect of Nef on latency reversal was confirmed in a separate study using U1 cells (245). More recently, treatment using extracellular Nef alone was also found to be sufficient to activate the Akt pathway
and to increase HIV-1 reactivation in the Jurkat-derived 1G5 latent T cell line (246). In addition to Ras and Akt, Nef can also alter T cell signaling by interacting with other cellular proteins. Hence, it is not entirely surprising that Nef could activate latent HIV-infected cell lines. However, additional studies are required to examine the effects of Nef during viral reactivation following stimulation with LRAs.

In cART-suppressed participants, latent HIV-1 transcription can be induced by various LRAs, but to date, no significant reductions in viral reservoir size have been observed. This suggests that viral cytopathic effects and/or immune-mediated clearance of reactivated cells is inefficient. One possible explanation for this is immune dysfunction. For example, Shan et al. demonstrated that CTL isolated from most cART-treated patients were unable to efficiently eliminate HDACi-reactivated cells ex vivo without pre-stimulation using HIV-1 antigens (247). In addition, even though CTL activities appeared to be unaffected by the LRA doses used during clinical trials (235, 248), in vitro studies have suggested the opposite. In particular, HDACi’s suppressed the cytolytic function of HIV-specific CTL (249, 250). Apart from impairments in CTL function, the expression of Nef immediately following viral reactivation may also reduce the ability of these cells to recognize and eliminate latent reservoirs. Specifically, the ability of Nef to downregulate surface HLA-I molecules (89-91) may allow reactivated cells to evade immune surveillance. Consistent with this, the use of small molecules designed to inhibit Nef partially reversed this effect and promoted the elimination of reactivating reservoirs by HIV-specific CTL (251).

Another potential mechanism to explain why reservoir size remains unchanged following LRA stimulation is the ability of HIV-1 to counteract apoptosis pathways. Several HIV-1 proteins, including Nef, can impair apoptosis and promote cell survival (252). Specifically, Nef impedes apoptosis by blocking the ASK1-dependent death signaling pathway (118) and reducing the activity of pro-apoptotic protein BAD2 (121). Furthermore, broad reactivation of HIV-1 proteins using LRAs may lead to AICD among the proportion of reservoir that is HIV-specific (253). In this case, Nef’s ability to modulate T cell signaling may protect these cells from undergoing AICD.
Figure 1-4  Overview of “shock and kill” strategy
Illustration of the expected outcome when latent HIV-infected T cells are induced (“shock”) with LRAs. Upon reactivation, integrated HIV genome is transcribed into mRNA (1) followed by translation into viral proteins (2). While portion of the viral proteins are used to complete HIV replication cycle, others are being degraded into peptide antigens and are loaded onto HLA-I molecules (3) for presentation on the cell surface (4). Recognition of peptide-bound HLA-I complex by CTL (5) induces the production and excretion of cytotoxic molecules that kill the infected cell. Alternatively, the expression of viral protein may induce viral cytopathic effect that lysis the infected cell.

1.4.2. Other strategies

In addition to “shock and kill” strategies, other potentially curative approaches are also under development. For example, the “block and lock” pharmacological approach aims to keep HIV-1 reservoirs in a persistent state of “deep” latency and thus prevent viral reactivation following the discontinuation of cART. Inhibitors of the HIV-1 Tat protein, such as didehydro-Cortistatin A, have been used to successfully block latency reversal ex vivo (254) and also delayed the time to viral rebound in a humanized mouse model (255). Moreover, numerous immune-based therapies, including cytokines/chemokines (such as IL-15), cytokine receptor super-agonists, and checkpoint inhibiting antibodies (such as anti-PD-1), are being explored to boost the antiviral host immune response towards the reactivating reservoirs. Other strategies include therapeutic vaccines and infusion of broadly neutralizing and bispecific antibodies targeting HIV-1 Env (233). Even though these different approaches have shown some promise in blocking HIV-1 replication, since none of them can singly prevent viral rebound they are being incorporated into the “shock and kill” interventions to enhance clearance of the latent reservoirs (236).
Recent advancement in gene editing tools have also led to the development of gene therapies as a potential method to cure HIV. In particular, the main target for gene editing has been the CCR5 co-receptor, since individuals who are homozygous for the CCR5Δ32 mutant display natural resistance to HIV-1 infection (256). The CCR5 gene has been successfully disrupted in CD4+ T cells using various technologies, including zinc finger nucleases (ZFNs) (257), transcription activator-like effector nucleases (TALENs) (258), and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein nuclease-9 (CRISPR/Cas9) (259, 260), resulting in knockout of CCR5 expression in ex vivo cell culture and animal studies. However, infusion with CCR5-modified autologous CD4+ T cells was unable to induce HIV-1 remission in infected participants (261). These gene editing technologies were also used to target the HIV-1 provirus of latently infected cells directly (262-264), showing that targeted disruption of the highly conserved LTR promoter could prevent latency reversal. Overall, gene therapy approaches that may be suitable for use as an HIV cure are still in early stages of clinical development. Additional studies will be required to increase the efficiency and specificity of these tools.

1.5. Thesis objectives

The main objective of this thesis is to investigate the impact of HIV-1 Nef on viral latency. The overall hypothesis is that Nef is capable of modulating LRA-induced reactivation of latent HIV-infected cells. To examine this hypothesis, I generated a novel panel of inducible latent CD4+ T cell clones that encoded functional or defective nef genes and assessed their ability to be stimulated by various LRAs. In chapter 2, I described the creation and initial characterization of these latent T cell lines. By assessing the location and genomic features of the proviral DNA integration sites in these clones, I observed that the presence of functional Nef variants was associated with a broader repertoire of unique latent clonal cell lineages. These observations suggest that Nef can enhance the inducibility of latent proviruses that are integrated into less favorable genomic sites. In chapter 3, I examined the effect of Nef on viral reactivation phenotypes. Through analysis of bulk cultures and latent HIV-infected T cell clones that expressed functional or defective nef genes, I observed higher rates of HIV-1 reactivation in the presence of Nef, regardless of the LRA used for stimulation. Nef’s ability to enhance viral reactivation was further
confirmed by knocking out the functional *nef* gene in multiple latent T cell clones using a CRISPR/Cas9 approach. In **chapter 4**, I aimed to develop and optimize gene-editing methods that would allow unbiased comparison of different *nef* alleles in an identical proviral context. While I demonstrated that the CRISPR/Cas9 system could be used to knockout or replace the proviral *nef* gene in latent T cells, the resulting cell lines displayed variable molecular and viral reactivation phenotypes. These observations suggest that cellular repair of Cas9-mediated DNA breaks can vary and that use of this system should be accompanied by a more detailed molecular characterization of progeny cells. Taken together, the results from this thesis highlight the importance of Nef during HIV-1 reactivation, indicating that may be a relevant consideration for current “shock and kill” strategies.
1.6. References


Chapter 2.

HIV-1 Nef is Associated with a Broader Repertoire of Inducible Proviral Integration Sites

Contributions: Ms. Shayda Swann assisted with collection of proviral DNA sequences and analysis of HIV integration sites using BLAT, shown in Table 2-2.

2.1. Introduction

Combination antiretroviral therapy (cART) can efficiently suppress HIV-1 replication in infected individuals, but the treatment must be maintained indefinitely to prevent viral rebound (1-5). The presence of long-lived reservoirs of latent HIV-infected cells are responsible for viral rebound during treatment interruption (4, 6, 7). In an attempt to eradicate these viral reservoirs, various “shock and kill” strategies are being investigated wherein latently infected cells are stimulated with a latency reversing agent (LRA) to induce HIV-1 protein production (8). While the overarching goal of these approaches is to expose latent HIV-infected cells to elimination via either viral cytopathic effects or CTL-mediated clearance; this process is inefficient and encompasses multiple challenges. One major issue is the inefficient induction of viral protein expression using clinically relevant LRAs that are currently available (7, 9). In fact, the use of even a strong LRA such as phytohemagglutinin also failed to induce the entire latent reservoir (7). Consequently, none of the clinical studies reported successful reduction of latent reservoir in cART-suppressed patients after LRA treatment (9-14).

Viral proteins, in particular Tat, have been reported to modulate HIV-1 reactivation from latency. Of particular interest to our laboratory is the multifunctional viral Nef protein, which plays a critical role in the enhancement of HIV-1 pathogenesis. The ability of Nef to induce viral latency reversal has been shown in independent studies (15-17). Treatment of latently infected cells with extracellular Nef appears to mimic the activity of LRAs through a mechanism similar to (or perhaps requiring) TNF (18, 19). However, the use of extracellular Nef in these studies may not accurately capture the kinetics of Nef
protein expression during the earliest stages of viral reactivation in latent cellular reservoirs. In particular, expression of viral genes, including nef, is generally absent in latent cells prior to stimulation with LRAs (20, 21).

Many in vitro models of HIV-1 latency have been described, each of which has limitations that reduce their ability to directly evaluate the role of Nef during viral reactivation. For example, latent T cell lines such as ACH2 (22) and J1.1 (23) do not harbour a surrogate marker of reactivation (i.e. GFP), so tracking early viral protein expression (i.e. Nef) is challenging. The Jurkat-derived panel of J-Lat (full-length) latent cell lines generated by Jordan et al. are commonly used to study HIV-1 latency, but the nef gene in this system was deleted and replaced by a GFP reporter (24). Other latent cell lines such as J89GFP and JNLGFP contain a GFP reporter as well as open reading frame for all viral genes, including nef (25). However, the HIV-1 strains used to construct these cell lines are replication-competent, so spontaneous viral replication and accumulation of viral mutations may take place during extended cell culture. Many research groups have also developed primary cell models to study HIV-1 latency in which different viral constructs (i.e. presence or absence of env and/or nef) were used for infection (26-31). Unfortunately, these existing in vitro cell model systems display highly variable HIV-1 reactivation profiles when treated with LRAs (32), which may be due to intrinsic differences between the viral strains and cell types used as well as the diversity of proviral DNA integration sites (which may be clonal or genetically mixed). Thus, it is difficult to explore the impact of Nef on HIV-1 latency using current cell models.

To avoid potential bias associated with comparing different cell models generated in the presence or absence of Nef, I created a new panel of latent CEM-derived T cell clones to examine the role of Nef during viral reactivation. These cells incorporate an inducible latent HIV+ provirus harbouring either functional or defective nef. In this cell model, Nef expression was linked to a GFP reporter (as a fusion protein or IRES construct), which allows Nef/GFP and viral reactivation to be monitored simultaneously. For each latent clone, linked data on the HIV-1 integration site, basal GFP expression, and viral reactivation against three different LRA classes (i.e. TNF-α, panobinostat and prostratin) were obtained. Through analysis of HIV-1 proviral integration sites, I observed a larger repertoire of unique latent clones in the presence of nef. Further characterization of the
proviral integration sites revealed differences in genomic features between inducible clones expressing functional versus defective nef. While no significant associations were observed between genomic feature(s) and HIV-1 reactivation, a modest inverse correlation was found between distance of the proviral integration site to the centromere and viral reactivation induced by the LRA prostratin. Overall, these results indicate that Nef contributes to HIV-1 reactivation by broadening the repertoire of latent proviral reservoirs that are capable of reactivating following treatment with LRAs. Further investigations will be required to identify the mechanism(s) responsible for this observation.

2.2. Methods

2.2.1. Plasmids and cell lines

The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pBR43IeG-nef+ and pBR43IeG-nef- from Dr. Frank Kirchhoff (33-38); HIV Gag-iGFPΔEnv from Dr. Benjamin Chen (39-41); and pHEF-VSVG from Dr. Lung-Ji Chang (42). pFLAG-NefSF2:GFP, pFLAG-NefM20A:GFP, and pCR2.1-TOPO-NefG2A:GFP plasmids were constructed by Tristan M. Markle. HEK 293T cell line was purchased from Clontech and they were maintained in DMEM high glucose with L-glutamine (Lonza) supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (D10+). CEM-SS cells that stably express HLA-A*02:01 (abbreviated as CEM-A*02) was constructed as described previously (43, 44), and they were maintained in RPMI-1640 medium (Lonza) supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (R10+).
2.2.2. Oligo and primer list

Table 2-1 List of oligos and primers and their corresponding application

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCS linker (double-stranded)</td>
<td>5'-ATG CAA ACG CGT GGC GCG CCC RCC GGY GAT CGA TCC GCG GCC CGG GGG AAT AC-3'</td>
<td>Plasmid construction</td>
</tr>
<tr>
<td>NEF8746_Env_Hpal_F</td>
<td>5'-TGC TGT TAA CTT GCT CAA TGC CAC AGC CAT AGC AGT AGC TGA GGG GAC AGA TAG GGT TAT AGA AGT ATT ACA AGC AGC TTA TAG AGC TAT TCG CCA CAT ACC TAG AAG AAT MAG ACA RG-3')</td>
<td>Plasmid construction</td>
</tr>
<tr>
<td>Nef:GFP*_AscI_SacIR</td>
<td>5'-TAA TCC GGC GGG CGC GCC TCA CTT GTA CAG CTC ATC CAT TCC CAG-3'</td>
<td>Plasmid construction</td>
</tr>
<tr>
<td>GA889R</td>
<td>5'-TCT AGC TCC CTG CTT GCC CAT ACT A-3'</td>
<td>Integration site amplification</td>
</tr>
<tr>
<td>GA1074F</td>
<td>5'-GAK RTA AAA GAC ACC AAG GAA GCT TTA GA-3'</td>
<td>Integration site amplification</td>
</tr>
<tr>
<td>GA850R</td>
<td>5'-ACC GAA TTT TTT CCC ATC GAT-3'</td>
<td>Integration site amplification</td>
</tr>
<tr>
<td>Pan1dFx</td>
<td>5'-ACC TAG AAC TTT AAA TGC ATG GG-3’</td>
<td>Integration site amplification</td>
</tr>
<tr>
<td>5’LTR-REV</td>
<td>5’-CGA GTC YTG CGT YGA GAG A-3’</td>
<td>Integration site amplification</td>
</tr>
<tr>
<td>FNL43</td>
<td>5’-AAA AGA GAC CAT CAA TGA GGA AGC-3’</td>
<td>Integration site amplification</td>
</tr>
<tr>
<td>5’LTR_4R</td>
<td>5’-GGC GCC ACT GCT AGA GAT TTT-3’</td>
<td>Integration site sequencing</td>
</tr>
<tr>
<td>3’UTRi</td>
<td>5’-AGG CTT AAG CAG TGG GTT CCC TAG-3’</td>
<td>Integration site sequencing</td>
</tr>
</tbody>
</table>

Bolded letters: nucleotides corresponding to new restriction sites

2.2.3. HIV-1_{NL4.3}ΔEnv plasmid construction

The HIV-1_{NL4.3} plasmid backbones used for engineering were pBR43IeG-nef+ and pBR43IeG-nef-. The HIV Gag-iGFPΔEnv plasmid contains frame-shifted Env that impedes viral replication. To generate pBR43IeG-nef+ΔEnv and pBR43IeG-ΔnefΔEnv plasmids, wildtype Env in pBR43IeG-nef+ and pBR43IeG-nef- was replaced with frameshifted Env from HIV Gag-iGFPΔEnv using HpaI and SalI restricted digestion and ligation. Note that partial digestion was performed for pBR43IeG-nef plasmids due to presence of multiple SalI cut sites. For pBR43-Nef:GFP-ΔEnv, IRES-GFP (IeG) gene fragment
between the MluI and XmaI restriction sites was removed in pBR43IeG-nef+ΔEnv, and this gap was subsequently sealed using a double-stranded DNA fragment (MCS linker) encoding additional restriction sites (AscI/SgrAI/ClaI/SacII) indicated in bold. Nef_{SF2}:GFP, Nef_{M20A}:GFP, and Nef_{G2A}:GFP were amplified from pFLAG-Nef_{SF2}:GFP, pFLAG-Nef_{M20A}:GFP and pCR2.1-TOPO-Nef_{G2A}:GFP plasmids, respectively, using NEF8746_{Env_HpaI_F} and Nef:GFP*_{AscI_SacIIR} primers. Afterwards, Nef_{SF2/M20A/G2A}:GFP amplicon was inserted between the HpaI and AscI restriction sites in pBR43ΔIeG-Nef+ΔEnv. PCR (polymerase chain reaction) amplicons were generated using Expand™ High Fidelity PCR System (Roche). All restriction enzymes were obtained from New England BioLabs, and restriction digest was performed following manufacturer’s instruction. Ligation reactions were performed using T4 DNA ligase (Thermo Fisher Scientific). Restriction-digested plasmids and PCR amplicons were ran on agarose gel, and the correct sized products were extracted using GeneJET Gel Extraction Kit (Thermo Fisher Scientific). All engineered plasmids were transformed into Invitrogen™ Stbl3 Chemically Competent E. Coli (Fisher Scientific), and grown in Lysogeny broth containing 100 µg/mL ampicillin. Plasmid DNA used for virus production were purified using E.N.Z.A.® Plasmid Mini Kit I or FastFilter Plasmid Maxi Kit (Omega Bio-tek).

### 2.2.4. Virus production

VSV-G pseudotyped HIV-1_{NL4.3}ΔEnv viruses were generated using Lipofectamine 3000 (Life Technologies) according to manufacturer’s instructions. Briefly, 6.5 µg of pBR43IeG-nef+ΔEnv, pBR43IeG-ΔnefΔEnv or pBR43-Nef_{SF2/M20A/G2A}:GFPΔEnv, 1 µg of pHEF-VSVG, 16.5 µL of Lipofectamine 3000 and 13 µL of P3000 reagents were prepared in Opti-MEM (Life Technologies). This mixture (500 µL) was then topped with 4.5 mL of D10 (no penicillin and streptomycin) and added to 2.7×10^6 pre-seeded HEK 293T cells. After 48 h of incubation, virus-containing supernatant was harvested, and aliquots were stored at -80°C until use.
2.2.5. Viral infection

1.0×10^6 CEM-A*02 cells were exposed to 40 µL of VSV-G pseudotyped HIV-1 NL4.3ΔEnv virus in a total volume of 500 µL R20+ (20% FBS). After 16 h, 1 mL of R20+ was added to infected cell culture followed by an additional 24 h of incubation. Afterwards, infected CEM-A*02 cells were maintained in R10+ and HIV-1 infection (measured as GFP expression) was monitored using flow cytometer.

2.2.6. LRA treatment

TNF-α was reconstituted to 7.5 µg/mL in 0.1% bovine serum albumin. Panobinostat (Selleckchem) and prostratin (Sigma-Aldrich) were prepared as 25 µM and 1.5 mM working stocks in 0.004% and 1.5% dimethyl sulfoxide (DMSO), respectively. Cells (1.0×10^6 cells/mL) resuspended in R10+ were treated with 50 ng/mL TNF-α, 0.5 µM panobinostat and 30 µM prostratin, either in combination (for screening) or individually (for cell sorting or reactivation profiling). Afterwards, treated cells were incubated for 24 h prior to cell sorting and flow cytometry follow up.

2.2.7. Flow cytometry and cell sorting

GFP expression was measured using Guava® easyCyte 8HT flow cytometer (EMD Millipore). Cell sorting and post-sort analyses were performed using 1.0 drop pure mode on the FACSJazz™ flow cytometer (BD Biosciences). On day 5 post viral infection, GFP-negative CEM-A*02 cells (represents either uninfected or latently infected cells) were sorted and maintained in R10+. These cells were subsequently treated with individual LRA, and latently infected cells (GFP-negative cells that become GFP+ following LRA treatment) were isolated as single cells for expansion on 96-well flat bottom plate containing 100 µL of conditioned medium (1:1 volume of R40+ (40% FBS)-to-filtered supernatant from CEM-A*02 cells). Data analysis was performed using FlowJo (TreeStar).


2.2.8. Integration site amplification

A modified version of nested-inverse PCR (45, 46) was used to amplify proviral integration sites. Briefly, total nucleic acid was extracted from C-Lat clones (2.0×10^5 to 4.0×10^5 cells) using Invitrogen™ PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific), but omitting the addition of RNase A. Subsequently, 2 µL (~100-200 ng) of total nucleic acid was digested with 10 U of PstI (New England BioLabs) in a 10 µL reaction volume for 3 h and inactivated at 80°C for 20 min. Afterwards, 40 µL of ligation mixture containing 5 U of T4 DNA ligase (Thermo Fisher Scientific) and 5 µL of 10X T4 ligation buffer was added to each digestion reaction and incubated for 2 h at room temperature followed by 15 min inactivation at 65°C. Circularized DNA was amplified using 2X PCR Bestaq™ MasterMix with dye (Applied Biological Materials). First round of amplification was a touch-down inverse PCR with the following thermocycling conditions: 94°C for 2 min -> 35 cycles of: 94°C for 15 s, 62°C (with AutoX -0.2°C/cycle) for 30 s, and 72°C for 1 min -> 72°C for 7 min -> hold at 10°C. For second and third round nested PCR, DNA templates were diluted 100X in DEPC-treated water and the thermocycling conditions were similar to that of first round touch-down PCR except 62°C (with AutoX) was replaced with 55°C. Third round PCR was optional unless there was no amplification after second round PCR. All PCR reactions were done using 3 µL of DNA template as well as 0.5 µL each of forward and reverse primers (10 µM working stock) in a 25 µL reaction volume. Primers used were as following: first round, GA889R and GA1074F; second round, GA850R and Pan1dFx; third round, 5’LTR-REV and FNL43. Resulting amplicons were diagnosed on an agarose gel, and samples with visible band(s) were subjected to DNA sequencing.

2.2.9. DNA sequencing

Full-length HIV-1 genome, excluding 5’ and 3’ LTRs, was sequence-validated for both pBR43IeG-nef+ΔEnv and pBR43IeG-ΔnefΔEnv plasmids using 48 primers that span the HIV-1 genome. Since pBR43-NefSF2/M20A/G2A:GFPΔEnv plasmids were engineered from pBR43IeG-nef+ΔEnv, only the NefSF2/M20A/G2A:GFP portion was confirmed by DNA sequencing. DNA fragments from integration site amplification were sequenced using
primers 5’LTR_4R and 3’UTRi. All DNA samples were diluted 15 to 20 folds in DEPC-treated water for sequencing using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing data were collected on an 3130xl Genetic Analyzer (Applied Biosystems), and analyzed using Sequencher v5 (Gene Codes).

### 2.2.10. Integration site analysis

DNA sequence upstream of 5’ HIV-1 LTR nucleotides (TGGAAGG) represents host DNA and was used to identify proviral integration through Human BLAT Search (https://genome.ucsc.edu/cgi-bin/hgBlat) developed by UCSC Bioinformatics Human Genome database. At the integration site, chromosomal location, type of gene (coding, non-coding or pseudogene), presence of H3K27Ac histone modifications, percent GC content, and distance to centromere were determined. Within a 5 kb region of the integration site, the presence of transcription factor binding sites, CpG islands and DNase I hypersensitive sites were noted.

### 2.2.11. Statistical analysis

Statistical analyses were conducted on Prism 5.0 (Graphpad Software). The Fisher’s exact test was used to compare the proportion of unique integration sites and genomic features between C-Lat cell lines encoding functional versus defective Nef. The Mann-Whitney U test was used to compare selected genomic features between HIV integrations sites from functional and defective Nef C-Lat cell lines. Spearman’s rank correlation was used to identify correlations between viral reactivation and genomic features. All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

### 2.3. Results

#### 2.3.1. Generation and characterization of C-Lat cell lines

To investigate the effect of Nef on HIV-1 latency, latent CEM-A*02-derived T cell lines (referred to as C-Lat cells) harbouring either functional or defective nef variants were generated as described in the experimental workflow shown in Figure 2-1. Briefly, parental
CEM-A*02 cells were infected using VSV-G pseudotyped replication-defective viral strains containing a full-length HIV-1_{NL4.3} genome that encodes a frameshifted \textit{env} gene. Two different Nef/GFP reactivation markers were used in this study, each of which included one WT and one defective Nef variant. In particular, \textit{nef}:GFP fusion constructs included WT \textit{Nef}_{SF2}, \textit{Nef}_{M20A} (which is impaired for HLA class I downregulation, but otherwise functional), and defective \textit{Nef}_{G2A}, while \textit{nef}-IRES-GFP (\textit{NefIeG}) constructs included WT \textit{Nef}_{NL4.3} and Δ\textit{Nef} (\textit{NefIeG} lacking the initiation codon and encompassing multiple premature stop codons). On day 5 post-infection, GFP-negative cells representing a mixture of uninfected and latently infected population were isolated using a cell sorter. Immediately after sorting, flow cytometry analysis was performed to ensure high purity (>98%) of GFP-negative cells. However, subsequent analysis on day 3 post-isolation revealed a subpopulation of isolated cells that spontaneously expressed GFP, presumably due to cellular stress associated with cell sorting. As a result, isolated cells were maintained in bulk culture until <0.5% GFP\textsuperscript{+} events were present prior to treatment using individual LRAs (TNF-\textalpha, panobinostat, or prostratin). Afterwards, GFP-negative cells that were induced to reactivate (i.e. produce GFP) in the presence of LRA were sorted as single cells and expanded for validation and further characterization, during which time the percentage of GFP expressing cells in cultures diminished to a low basal frequency.

Each clonal C-Lat cell line was characterized for viral reactivation and proviral integration. First, basal GFP expression and maximal viral reactivation were measured in the absence of drug and in the presence of a combination of LRAs (TNF-\textalpha, panobinostat and prostratin), respectively. C-Lat cell lines displaying <10% basal GFP expression and >50% maximal viral reactivation were further tested using individual LRAs. For C-Lat clones that expressed >0.5% GFP\textsuperscript{+} events before and/or after LRA treatment, total nucleic acid was isolated for amplification of proviral integration sites using nested-inverse PCR. Host cellular DNA sequence upstream of the HIV-1 5’LTR (5’-TGGAGGG-3’) was obtained through Sanger sequencing, and then used to identify the location and genomic features of integration sites using the BLAT analysis tool (UCSC Genome Browser).
Figure 2-1  Generation of CEM-A*02-derived latent (C-Lat) T cell lines using NL4.3∆Env virus

(A) Illustration of a modified full-length HIV-1 genome (NL4.3-Nef:GFP(IeG)∆Env) used for generating C-Lat T cells. Major modifications to the HIV-1 genome include replacement of env and nef genes with frameshifted, defective Env and Nef:GFP or NefIeG reactivation reporter, respectively. (B) Schematic illustrating the procedures for generating C-Lat cell lines. CEM-A*02 T cells were infected with VSV-G pseudotyped HIVNL4.3∆Env containing either Nef:GFP or NefIeG reactivation marker. On day 5 post infection, GFP-negative population representing a mixture of uninfected and latently infected cells were isolated and subsequently induced using TNF-α, panobinostat or prostratin. GFP-negative cells that express GFP after LRA stimulation were isolated and expanded as single cells for further validation and characterization.

2.3.2. Overview of C-Lat cell lines

A total of 138 clonal C-Lat cell lines were obtained for this study. Of these, 58% (N=80) were generated using HIV-1 strains encoding functional nef, including 17 NefSF2:GFP, 24 NefM20A:GFP, and 39 NefNL4.3IeG. The remaining 43% (N=58) of C-Lat cell lines were generated using HIV-1 strains encoding defective nef, including 45 NefG2A:GFP and 13 ΔNefIeG (Figure 2-2A). To validate latent infection, viral reactivation was assessed for each cell line following stimulation with a combination of three LRAs (TNF-α, panobinostat, and prostratin). Surprisingly, even though GFP-expressing cells were targeted for single cell isolation and expansion, 18 out of the 138 expanded cell lines (13%) failed to produce >0.5% GFP+ events upon LRA treatment (Figure 2-2B). A similar number of C-Lat clones encoding functional (N=10) and defective (N=8) nef alleles were
unresponsive to subsequent LRA stimulation, indicating that this result was unlikely to be related to Nef expression. To avoid potential confounding effects associated with major cellular defects, these 18 cell lines were excluded from further analyses. Hence, the proviral integration site was examined in 120 inducible C-Lat cell lines. Of these cell lines, 78% (N=93) were found to harbour a single copy of the integrated HIV provirus, while 2% (N=2) harboured two copies (Figure 2-2C). Integration sites of the remaining 20% (N=24) of C-Lat cell lines were not identifiable due to technical limitations, including unsuccessful host DNA amplification or sequencing. In total, 93 host DNA sequences were obtained from 52 functional Nef and 41 defective Nef C-Lat cell lines, which were then used to further characterize genomic locations and features.

2.3.3. Larger repertoire of unique latent clones was inducible by LRA in the presence of Nef

The genomic location of HIV-1 proviral integration sites were identified for all 93 C-Lat cell lines using the BLAT genome analysis tool. The query sequences (i.e. host DNA 5’ upstream of provirus) used for BLAT search ranged from 55-813 base pairs, with at least 98.7% identity with respect to human reference genome GRCh38/hg38. Despite the relatively large number of C-Lat clones analyzed, my results indicated that only 23 (24.7%) unique proviral integration sites were present in this panel. This is consistent with the notion that establishment of inducible latent HIV-1 reservoirs is a rare event and further

![Figure 2-2](Image)

**Figure 2-2**  Breakdown of all C-Lat clones generated for this study
(A) Following the schematic shown in Figure 2-1, a total of 138 C-Lat clones were generated for downstream validation and characterization. The proportion of clones corresponding functional and defective Nef are shown in the pie chart. (B) GFP expression before and after LRA treatment was measured for each clone, and the proportion of GFP-negative and GFP^+ve clones are shown. (C) HIV integration site was determined in each C-Lat clone using nested-inverse PCR, DNA sequencing, and BLAT genome analysis. The pie chart displays the proportional breakdown of C-Lat clones that encompass single or multiple HIV integration as well as clones that need to be determined (TBD).
suggests that my panel of C-Lat clones should be representative of the population of inducible latent cells generated in this cell model. A summary of these integration sites and their corresponding details can be found in Table 2-2. More than half (61%) of all integration sites were identified in at least two C-Lat cell lines (Figure 2-3A). Also, no proviral integration sites were shared by C-Lat cell lines generated using different Nef/GFP constructs. Together, these observations suggest that C-Lat cells underwent a high degree of clonal expansion following latency establishment prior to isolation of clonal lineages. This is an expected outcome because during their generation, the GFP-negative cell population was sorted on day 5 post-infection and then maintained in culture for an extended period of time to allow basal GFP expression levels to stabilize.

Next, I stratified these 23 unique insertion sites according to whether they were present within C-Lat cell lines that expressed functional or defective nef genes. Surprisingly, a disproportionate number of unique proviral integration sites was observed between the two groups. In particular, most of the unique sites (20 of 23; 87%) were identified in C-Lat clones that encoded functional nef (Figure 2-3B). While only 3 unique integration sites were observed in the Nef-defective group, 2 of these sites were identified in at least 5 independently isolated C-Lat lines, suggesting substantial clonal expansion. Proportionally, 20 of 52 (38%) C-Lat cell lines encoding functional nef harboured a unique integration site compared to 3 of 41 (7%) C-Lat cell lines that expressed defective Nef ($P = 0.006$, Figure 2-3C). Together, these data demonstrate that C-Lat clones encoding a functional nef variant displayed a larger repertoire of inducible proviral integration sites.
### Table 2-2  Summary of unique C-Lat integration sites

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<thead>
<tr>
<th>Nef/Defective</th>
<th>Nef variant</th>
<th>Chromosome locus</th>
<th>Nature of IS(^a)</th>
<th>Transcriptional orientation(^b)</th>
<th>Host nt at junction</th>
<th>Host gene(^c)</th>
<th># of clones</th>
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\(^a\) Nature of integration site: intron, exon, and intergenic; intron*, provirus integrated into the intron of a pseudogene

\(^b\) Transcriptional orientation: +, provirus and host gene shared the same orientation; −, provirus and host gene are in opposite orientation; N/A, provirus was not inserted into host gene

\(^c\) Host gene: description of host gene can be found in Table A1
Figure 2-3  Higher frequency and proportion of the unique integration sites were identified in C-Lat cells encoding functional Nef

(A) Pie chart displays the proportion of unique proviral integration site identified in 1, 2, 3, or ≥5 C-Lat cell lines. (B) Bar graph shows the distribution of unique integration sites (in absolute number) found in C-Lat cell lines encoding functional or defective Nef. Within each group, the integration sites were further categorized based on the number (1, 2, 3, or ≥5) of C-Lat clones sharing the same site. The percentage inside each region represents the proportion of corresponding category in the total number of unique integration sites (N=23). (C) Bar graph compares the proportion of unique integration sites in C-Lat cells expressing functional (N=52) versus defective (N=41) Nef variant. The Fisher’s exact test value is shown.

2.3.4. Genomic features of HIV-1 integration sites in C-Lat clones expressing functional and defective Nef

For further comparison between C-Lat cell lines that expressed functional and defective nef alleles, the genomic features of all integration sites were characterized and examined. Due to the limited number of unique proviral insertion sites observed for defective nef strains, these analyses are descriptive. I observed that the 23 unique proviral integration sites were distributed among a total 11 different cellular chromosomes, with the highest frequency (22%) located in chromosome 19 (Figure 2-4A). Specifically, 20 integration sites derived from C-Lat clones expressing functional nef were mapped to 11 chromosomes (Figure 2-4B); with a modestly higher proportion of sites found in chromosomes 3, 16, and 19. In comparison, the 3 unique integration sites identified for C-Lat clones expressing defective Nef were located in chromosomes 2 and 19 (Figure 2-4C).

In addition to the chromosomal location, I analyzed other genomic properties that may contribute to cellular gene expression. At the site of proviral DNA integration, the type of gene (coding, non-coding or pseudogene), the presence of H3K27Ac histone modifications, the percent GC content, and the distance to centromere were determined.
Consistent with previous studies (7, 47, 48), a majority (87%) of the identified proviruses were inserted into protein-coding genes (Figure 2-5A); with similar frequencies found for C-Lat cell lines encoding functional nef (85%) and defective nef (100%) (Figure 2-5B). I noted that proviruses in C-Lat cell lines harbouring defective nef alleles were integrated into genomic regions associated with a higher total median transcript expression compared to those of clones harbouring functional nef (Figure 2-5C); however, my study is underpowered to assess the significance of this difference. Moreover, 60% of the HIV-1 integration sites present in C-Lat clones from the functional nef group harboured H3K27Ac histone modifications, compared to 100% in the defective nef group (Figure 2-6A). On the other hand, the percent GC content and distance to centromere were comparable between the two groups of C-Lat cell lines (Figures 2-6B and 2-6C). Furthermore, the presence of transcription factor binding sites, CpG islands and DNase hypersensitive sites were analyzed within 5kb region of the HIV integration site. While the presence of DNase I hypersensitive sites were comparable between C-Lat clones encoding functional nef (45%) and defective nef (33%), both the transcription factor binding sites and CpG islands tended to distribute differently between the two groups; but these differences were not statistically significant (Figures 2-7).
Figure 2-4  Genome-wide distribution of HIV-1 integration differed between C-Lat clones expressing functional versus defective Nef

Pie charts display the distribution of chromosome location for unique HIV-1 integration sites identified in all C-Lat clones (N=23) (A), or in clones that express functional Nef (N=20) (B) or defective Nef (N=3) (C). Coloured and pop-up/detached region respectively represent chromosomes with the highest integration frequency in total and Nef-stratified C-Lat clones.
Figure 2-5  Distribution and expression of protein-coding genes in C-Lat clones expressing WT and defective Nef

(A) Horizontal chart depicts the proportion of all identified HIV integration sites (N=23) that are located in coding, non-coding and pseudogene. (B) Bar graph compares the proportion of gene types (coding, non-coding and pseudogene) in C-Lat cells expressing functional versus defective Nef. (C) For HIV-1 integrated into a gene-coding region, the total median expression (measured in reads per kilobase per million mapped reads [RPKM]) of the corresponding gene is plotted for C-Lat cells expressing functional versus defective Nef variant. Mann-Whitney U test value is shown.
Figure 2-6  Comparison between C-Lat clones expressing WT and defective Nef: genomic properties at the HIV-1 integration site

(A) Bar graph compares the proportion of HIV-1 integration sites that are located in regions with H3K27Ac modifications. (B) Graph compares percent GC contents at the HIV-1 integration sites. (C) Graph compares the distance (in bp) of HIV-1 integration sites to centromere. All comparisons were grouped by C-Lat cells expressing functional versus defective Nef variant.
DNase I Hypersensitive Regions

Transcription Factor Binding Site

CpG Islands

Figure 2-7  Comparison between C-Lat clones expressing WT and defective Nef: genomic properties within 5kb region of the HIV-1 integration site

(A to C) Graphs compare the proportion of HIV-1 integration sites that are located within 5kb of genomic features such as DNase I hypersensitive regions, transcription factor binding site, and CpG islands. All comparisons were grouped by C-Lat cells expressing functional versus defective Nef variant.

2.3.5. Minimal association between genomic features and viral reactivation

Finally, I examined potential associations between HIV-1 reactivation and genomic properties of the proviral integration site using my panel of C-Lat clones. For independent cell lines that shared the same proviral integration site (i.e. clonally expanded cells from a common progenitor), the mean value of viral reactivation was calculated and used for this analysis. Associations between viral reactivation and continuous genomic features, including total median gene expression, distance to centromere, and percent GC content, were tested using the Spearman’s rank test. Association between viral reactivation and dichotomous genomic features, including presence of H3K27Ac histone modifications, transcription factor binding sites, CpG islands and DNase hypersensitive sites, were compared using the Mann-Whitney U test. For each genomic feature, I assessed correlations with basal GFP expression, maximal viral reactivation as well as viral reactivation induced by individual LRA (TNF-α, panobinostat and prostratin). Overall, I only noted a modest inverse correlation between viral reactivation induced by prostratin and the distance of the proviral integration site to the centromere (Figure 2-8), but the significance of this finding is unclear.
2.4. Discussion

In this study, I generated a novel panel of inducible latent C-Lat T cell clones harbouring full-length, replication-incompetent proviruses that encode either functional or defective nef alleles linked to GFP as a marker of viral reactivation. A similar number of C-Lat clones containing functional and defective nef were isolated. Analysis of proviral insertion sites ensured that only clones harbouring a single copy of HIV were used for subsequent functional analyses. Overall, I observed a higher absolute number (and proportion) of unique integration sites among cell lines encoding functional nef compared to that of clones encoding defective nef. In other words, a larger repertoire of unique latent cells could be induced by LRAs in the presence of Nef, suggesting a potential role of Nef in enhancing the efficiency of viral reactivation from latency following stimulation with LRAs. The number of HIV-1 integration sites obtained in this study was small, but they resemble those described in prior, larger-scale analyses. For example, an in vitro analysis...
of HIV-1 integration sites showed that latent proviruses can be mapped to all human chromosomes (47). Nonetheless, an enrichment was observed in gene-rich regions, such as chromosomes 17 and 19. Consistent with those results, unique integration sites were mapped in this study to 11 chromosomes, with the highest density detected in chromosome 19. Also consistent with previous findings, the majority of proviral integration sites identified in my cell model were found to be in the intronic regions of active, protein-coding genes (7, 47, 48). Moreover, HIV-1 integration appeared to favour genomic regions associated with H3K27Ac histone acetylation and disfavoured CpG islands (49).

While important differences were potentially revealed by comparing the features of HIV-1 proviral integration sites between C-Lat clones harbouring functional and defective nef alleles, none of these comparisons reached statistical significance. This may be due, in part, to the small number of unique clones available lacking nef. First, the frequency of HIV-1 integration near a transcription factor binding site was higher in the presence of functional nef. In contrast, the presence of CpG islands were less frequently observed in cell lines expressing nef. While both of these characteristics may suggest the elevated expression of nearby host genes, their contribution to the inducible rate of latent HIV-1 provirus remains undetermined. In particular, proviral integration into a highly active genomic region may cause transcriptional interference and thus inhibit HIV-1 activation. Regardless, my data indicate that the transcriptional activity of protein-coding genes tends to be lower near proviral integration sites in C-Lat cell lines expressing functional nef. This leads us to speculate that Nef enhances viral reactivation following stimulation with LRAs, in particular when the provirus is integrated into less transcriptionally active genomic regions. However, additional studies will need to be performed to confirm this hypothesis. Specifically, deeper sequencing approaches to analyze a larger number inducible versus non-inducible integration sites may confirm the differences that I have observed for genomic features in the presence versus absence of functional nef.

Another limitation in this study is that I cannot rule out is potential bias associated with Nef’s implicated contribution to the establishment of viral latency, which could increase the size of the latent reservoir and contribute to a higher number of unique latent cell lines being obtained. For example, the ability of Nef to antagonize SERINC3/5 enhances virion infectivity (50-52), which could improve early events following infection
leading to proviral synthesis and integration. To diminish the impact of this in my assays, I used VSV-G-pseudotyped viruses that should bypass the antiviral effects of SERINC3/5. Also, I monitored the percent of viral infection to ensure that this was comparable between HIV-1 strains expressing functional versus defective nef alleles. Moreover, Nef has been described to display various anti-apoptotic mechanisms including interference with the ASK1 and PI3K pathways (53, 54), which could also enhance the establishment of latent HIV-1 following infection. Additional studies will be needed to address these issues in greater detail.

In conclusion, I generated a novel panel of inducible latent T cell clones that allow an unbiased investigation of Nef’s role during HIV-1 reactivation from latency. Characterization of these clones revealed a broader repertoire of inducible proviruses in the presence of nef. This observation supports a role for Nef to enhance HIV-1 reactivation, which may be relevant to the efficiency of “shock and kill” strategies. However, additional experiments are required to validate these findings and to investigate the mechanism(s) of Nef on viral reactivation.
2.5. References


Chapter 3.

HIV-1 Nef is required for efficient Gag protein production during viral reactivation

Contribution: I completed all data collection and analyses included in this chapter.

3.1. Introduction

Nef exhibits multiple functions that are believed to be critical to enhance HIV-1 pathogenesis. These include the ability to downregulate surface HLA class I (HLA-I) molecules (1, 2) required for viral epitope presentation and CTL-mediated recognition of infected cells. Indeed, recent studies suggest that small molecule inhibitors of Nef enhanced CTL-mediated cytolytic activity against reactivating HIV-1 reservoirs (3, 4). Nef is also able to interfere with T cell signaling events (reviewed by Markle et al.), which may modulate cellular activation status to enhance viral replication and persistence (5). The impact of Nef on cellular gene expression has not been examined carefully in the context of viral latency; however, since the “shock and kill” strategies to eradicate latent HIV-1 reservoirs utilize LRAs to alter cellular gene expression, it is plausible that Nef could affect the efficiency of HIV-1 reactivation and subsequent viral protein expression.

Using a panel of latent CEM-derived T cell clones, I observed a more diverse repertoire of inducible proviral integration sites in the presence of functional nef alleles (described in chapter 2). To investigate Nef’s ability to enhance HIV-1 reactivation from latency more directly, I used three different experimental approaches to examine early (i.e. Nef/GFP) and/or late (i.e. Gag) viral reactivation events following stimulation with LRAs targeting different cellular mechanisms: TNF-α, which activates NF-κB and MAPK pathways (6, 7); panobinostat, which inhibits HDAC (8); and prostratin, which induces the PKC pathway (9, 10). For the first approach, bulk latent HIV-infected cells were tested. In cells latently infected with HIV-1 strains encoding functional nef, higher viral reactivation was observed regardless of the LRA used for stimulation. Next, latent T cell clones were isolated and then used to assess viral reactivation. Varying HIV-1 reactivation phenotypes
were observed among these latent clones following stimulation with LRAs, but the production of late viral protein Gag was consistently reduced in clones lacking nef. To overcome the potential bias associated with differences in proviral DNA integration sites between clones encoding nef versus those lacking nef, which has been reported to affect viral reactivation efficiency (11-13), the nef gene was disrupted in multiple T cell clones using the CRISPR/Cas9 system. The absence of Nef expression by the resulting knock-out clones was associated with impaired HIV-1 reactivation. Overall, my results indicate that Nef is required for efficient reactivation of HIV-1 from latency in response to LRAs. My findings highlight the need to examine the impact of natural variation in the nef gene on HIV-1 latency phenotypes in HIV-infected individuals. Additional studies will be needed to characterize the molecular mechanism(s) associated with Nef’s ability to enhance latency reversal, which could lead to novel interventions that improve the efficiency of “shock and kill” strategies.

3.2. Methods

3.2.1. Plasmid and cell lines

The pX330-U6-Chimeric_BB-CBh-hSpCas9 (abbreviated as pSpCas9(BB)) was a gift from Feng Zhang (Addgene plasmid #42230) (14, 15). The pBR43IeG-nef+ΔEnv, pBR43IeG-ΔnefΔEnv, and pBR43-NefSF2/G2A::GFP vectors were generated in chapter 2. Selected C-Lat latent T cell lines generated in chapter 2 were tested here. These cell lines include C-Lat_NefSF2::GFP (N=9), C-Lat_NefG2A::GFP (N=32), C-Lat_107 and C-Lat_121. All cell lines have been verified to harbour a single copy of integrated HIV-1, and they express <10% basal GFP expression and >50% maximal viral reactivation. HEK 293T cell line was purchased from Clontech and CEM-A*02 cell line was constructed as described previously (16, 17). HEK 293T cells were maintained in D10+ whereas C-Lat and CEM-A*02 cells were maintained in R10+.
# 3.2.2. Primer List

## Table 3-1 List of primers and their corresponding applications

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<td>5'-CCT TGT AAG TCA TTG GTC TTA AAG GTA CC-3'</td>
<td>Nef sequencing</td>
</tr>
<tr>
<td>NefGFP724R</td>
<td>5'-TGG TGA TGT GAA TGG CCA CA-3'</td>
<td>Nef sequencing</td>
</tr>
<tr>
<td>NefIeG807R</td>
<td>5'-CCC TGT CTT CTT GAC GAG CAT-3'</td>
<td>Nef sequencing</td>
</tr>
<tr>
<td>CLat_3LTR69_F</td>
<td>5'-TCG AGC TTG CTA CAA GGG AC-3'</td>
<td>5' LTR sequencing</td>
</tr>
<tr>
<td>CLat_3LTR316_R</td>
<td>5'-AGA GTC ACA CAA CAG ACG GG-3'</td>
<td>5' LTR sequencing</td>
</tr>
</tbody>
</table>

Italicized letters: nucleotide overhangs for cloning gRNA into the pSpCas9(BB) vector (15)
Lower-case letters: additional guanine (g) and its complementary cytosine (c) appended to 5’ end of target site to increase the efficiency of gRNA expression (15)

3.2.3. Virus production

VSV-G pseudotyped HIV-1_{NL4.3ΔEnv} viruses were generated using Lipofectamine 3000 (Life Technologies) according to manufacturer’s instructions. Briefly, 6.5 µg of pBR43IeG-nef+ΔEnv, pBR43IeG-ΔnefΔEnv or pBR43-NefSF2G2A:GFPΔEnv, 1 µg of pHEF-VSVG, 16.5 µL of Lipofectamine 3000 and 13 µL of P3000 reagents were prepared in Opti-MEM (Life Technologies). This mixture (500 µL) was then topped with 4.5 mL of D10 (no penicillin and streptomycin) and added to 2.7×10^6 pre-seeded HEK 293T cells. After 48 h of incubation, virus-containing supernatant was harvested, and aliquots were stored at -80°C until use.

3.2.4. Viral infection

1.0×10^6 CEM-A*02 cells were exposed to 40 µL of VSV-G pseudotyped HIV-1_{NL4.3ΔEnv} virus in a total volume of 500 µL R20+ (20% FBS). After 16 h, 1 mL of R20+ was added to infected cell culture followed by an additional 24 h of incubation. Afterwards, infected CEM-A*02 cells were maintained in R10+ and HIV-1 infection (measured as GFP expression) was monitored using flow cytometer.
3.2.5. Cloning of pSpCas9(BB)-gRNA

Four Nef-specific guide RNA (gRNA): 1. Nef\textsubscript{350-369} (CACAAGGCTACTTCCCTGAT), 2. Nef\textsubscript{606-658} (GTACTCCGGATGCAGCTCTC), 3. Nef\textsubscript{30-49} (GATTGGATGGCCTGCTGTAA) and 4. Nef\textsubscript{561-580} (CCGCCTAGCATTTTCATCACG) were independently cloned into pSpCas9(BB) vector following instructions described in Ran et al., 2013 (15). All pSpCas9(BB)-crRNA were confirmed by DNA sequencing.

3.2.6. Transfection of C-Lat cells

To knockout Nef allele, 1.0×10\textsuperscript{7} C-Lat cells were transfected with two pSpCas9(BB)-gRNA (3 µg each) targeting different Nef sequences and 0.5 µg of pMAX-GFP (Lonza) in 150 µL total volume of Opti-MEM, no phenol red (ThemoFisher Scientific). All transfections were done in a 96-well electroporation plate (Bio-Rad Gene Pulser MXcell\textsuperscript{TM}: square wave, 250 V, 2,000 µF, infinite Ω, 25 ms), and transfected cells were recovered (4.0×10\textsuperscript{6} cells/mL) and maintained in R10+ until use.

3.2.7. LRA treatment

TNF-α was reconstituted to 7.5 µg/mL in 0.1% bovine serum albumin. Panobinostat (Selleckchem) and prostratin (Sigma-Aldrich) were prepared as 25 µM and 1.5 mM working stocks in 0.004% and 1.5% dimethyl sulfoxide (DMSO), respectively. Cells (1.0×10\textsuperscript{6} cells/mL) resuspended in R10+ were treated with 2 ng/mL or 50 ng/mL TNF-α, 0.5 µM panobinostat and 30 µM prostratin, either in combination (for screening) or individually (for cell sorting or reactivation profiling). Afterwards, treated cells were incubated for 24 h prior to cell sorting and flow cytometry follow up.

3.2.8. Flow cytometry

During generation of Nefko cell lines, transient pMAX-GFP transfection was monitored as GFP expression. For the figures presented in this chapter, GFP is a marker for either viral infection or reactivation depending on the experiment. Surface CD4 and
HLA-A*02 molecules were stained with anti-human CD4-APC (BD BioSciences) as well as HLA-A*02-PerCP/Cy5.5 (BioLegend) or HLA-A*02-PE (BD BioSciences) antibodies, respectively. Intracellular Gag-p24 was stained with KC57-RD1 (Beckman Coulter) antibody (Miltenyl Biotec) according to the BD cytoperm/cytofix plus kit (BD Biosciences). All flow cytometry data were collected using Guava® easyCyte 8HT flow cytometer (EMD Millipore). Data analysis was performed using FlowJo (TreeStar).

3.2.9. Cell sorting

Bulk and single cell sorts were performed using 1.0 drop enrich mode and 1.0 drop pure mode, respectively on the FACSJazz™ flow cytometer (BD Biosciences). For the NefKO experiment, 150,000 transfected (based on GFP expression from pMAX-GFP) C-Lat cells were sorted into a 5 mL polypropylene tube containing 300 µL of R10+. Sorted cells were pelleted at 1500 rpm for 10 min, resuspended in 600 µL R10+, and transferred onto 24-well flat bottom plate for culture until transient GFP expression diminish. Prior to isolation of single NefKO cells, transfected cells were treated with TNF-α. The gating for NefKO cells were GFP’-ve or GFP-negative (depending on parental cell line) as well as high levels of surface CD4 and HLA-A*02. Single cells were sorted into 96-well flat bottom plate containing 100 µL of conditioned medium for expansion.

3.2.10. HIV-1 genome and 5’ LTR amplification

Total nucleic acids from C-Lat NefKO clones (1.0×10^6 to 1.5×10^6 cells) were extracted using Invitrogen™ PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific), but omitting the addition of RNase A. Using total nucleic acid as template, nested PCR was performed to amplify HIV genome and 5’ LTR using 2X PCR Bestaq™ MasterMix with dye (Applied Biological Materials) following manufacturer’s instruction. The HIV genome was amplified in two parts using nested PCR. The 5’ half of HIV (4.5 kb) was amplified using the following primers: first round, 623Fi and P17; second round, G1 and P16. The 3’ half of HIV (5.3 or 5.9 kb, depending on Nef:GFP construct) was amplified using the following primers: first round, IN4534F and FB12; second round, VIF4952F and FB13. For 5’ LTR amplification, the forward primers used were cell
line/integration site-specific whereas the reverse primers were universal for all cell lines. In particular, first round forward primers were CLat14IS_F1, CLat16IS_F1, CLat107IS_F1 and the reverse primer was t1bR. The forward primers for second round PCR were CLat14IS_F2, CLat16IS_F2, CLat107IS_F2 and the reverse primer was GA932R.

First round of HIV genome amplification was a touch-down PCR with the following thermocycling condition: 94°C for 3 min -> 35 cycles of: 94°C for 30 s, 62°C (with AutoX -0.2°C/cycle) for 30 s, and 72°C for 2 min -> 72°C for 7 min -> hold at 10°C. Thermocycling conditions for the second round nested PCR is similar to that of first round touch-down PCR except 62°C (with AutoX) was replaced with 55°C. The amplification for 5’ LTR was performed as described for that of HIV genome, except that the extension step at 72°C was reduced from 2 min to 30 s. All PCR reactions were done using 3 µL of diluted (100-fold in DEPC-treated water) DNA template as well as 0.5 µL each of forward and reverse primers (10 µM working stock) in a 25 µL reaction volume. Resulting products from second round PCR was ran on agarose gel followed by imaging on Gel Doc™ XR (Bio-Rad). Samples with successful amplification were subjected to DNA sequencing.

3.2.11. DNA sequencing

The gRNA in pSpCas9(BB) plasmid was validated using sequencing primers U6_F and pSpCas9_517_R (Ran et al.). Nef region in the 3’ half HIV was sequenced using 3 Nef-specific primers NEF8748F, NEF9112F, and EIR as well as a cell line-specific primer: NefGFP724R for C-Lat_14/16 or NefG807R for C-Lat_107/121. The 5’ LTR was sequenced using CLat_3LTR69_F and CLat_3LTR316_R. All DNA samples were diluted 15 to 20 folds in DEPC-treated water for sequencing using the BigDye v3.1 kit (ABI). Sequencing data were collected on an 3130xl Genetic Analyzer (Applied Biosystems), and analyzed using Sequencher v5 (Gene Codes).

3.2.12. Statistical analysis

Statistical analyses were conducted on Prism 5.0 (Graphpad Software). The Mann-Whitney U test was used to compare viral reactivation between NefSF2:GFP and
Nef\textsubscript{G2A}:GFP C-Lat clones. The paired sample t test and one sample t test were used to compare viral reactivation in bulk and clonal Nef\textsubscript{KO} cells, respectively. All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

3.3. Results

3.3.1. Reduced viral reactivation in CD4\textsuperscript{+} T cells infected with HIV-1 lacking functional Nef

In order to investigate the effect of Nef on HIV-1 reactivation, latent CEM-derived T cell lines (C-Lat) were generated using replication-incompetent HIV-1\textsubscript{NL4.3} strains encoding either functional or defective nef genes. Briefly, parental CEM-A\*02 cells were incubated with VSV-G pseudotyped viruses containing a full-length HIV-1\textsubscript{NL4.3} genome modified to express a frameshifted env gene and a Nef/GFP reporter gene. Two different Nef/GFP reporter constructs were used – resulting in the production of a Nef:GFP fusion protein or a linked Nef-IRES-GFP transcript. In each case, viruses were constructed encoding functional nef and defective nef alleles. In particular, the Nef:GFP fusion constructs included WT Nef\textsubscript{SF2} and defective Nef\textsubscript{G2A}, while the Nef-IRES-GFP (NefIeG) constructs included WT Nef\textsubscript{NL4.3} and ΔNef (a Nef mutant lacking the initiation codon and encompassing multiple premature stop codons). Following infection, GFP-negative cells representing either uninfected or latently infected cells were isolated by FACS. These cells were subsequently treated with individual LRAs (TNF-α, panobinostat or prostratin) and viral reactivation was assessed as the percentage of GFP expressing cells and as the intensity of GFP expression in GFP\textsuperscript{+}ve cells.

As shown in Figure 3-1A, the percentage of infected cells following treatment with VSV-g pseudotyped viruses was comparable between strains harbouring functional and defective nef genes; 8% and 5% differences were observed for Nef:GFP and NefIeG strains, respectively. Based on these results, I anticipated that the size of latent reservoirs would be similar between the groups. Following isolation of GFP-negative C-Lat cells by FACS, spontaneous GFP expression was slightly higher in CEM-A\*02 cells infected with viruses encoding functional nef (0.3-0.5%) compared to those infected with viruses encoding defective nef (0.04-0.2%, Figure 3-1B). This observation suggests that Nef may
promote viral reactivation from latency following cellular stress. In addition, following stimulation with LRAs, the proportion of reactivating (GFP^+ve) cells was 0.4-2.0% higher in the presence of functional nef, depending on the Nef/GFP construct and the LRA used. One exception was for cells expressing functional Nef_{SF2}:GFP versus defective Nef_{G2A}:GFP, which displayed the same percentage of GFP expression following stimulation with TNF-α. However, the reactivation intensity of these cells, measured as median fluorescence intensity (MFI) of GFP^+ve cells, was 4.3-fold lower in cells expressing Nef_{G2A}:GFP (MFI = 131) compared to those expressing Nef_{SF2}:GFP (GFP^+ve MFI = 564). Similar results were obtained for cells treated with panobinostat or prostratin as well as for cells infected with Nef{GFP viruses. That is, reactivation intensity was lower in latently infected cells lacking functional Nef. These results suggest that Nef can enhance HIV-1 latency reversal in the presence of LRAs.

Figure 3-1  The overall viral reactivation was higher CEM-A*02 cells latently infected with HIV-1_{NL4,3} encoding WT Nef

(A) Histograms show percent infection, measured as GFP expression, on day 5 post viral infection. Top and bottom panels represent data for HIV-1_{NL4,3ΔEnv} constructs encoding Nef-GFP and Nef{GFP, respectively.

(B) Flow plots depict basal GFP expression (no drug) and LRA-induced reactivation in bulk GFP-negative cell population (i.e. a mixture of uninfected and latently infected cells). A dashed line (---) indicates cut-off for GFP^+ve events, and the percent and MFI of GFP^+ve population are indicated on each flow plot.

(A) No Virus
- NL4.3-Nef_{SF2}:GFP
- NL4.3-Nef_{G2A}:GFP

(B) Flow plots:

- No Drug
- TNF-α
- Panobinostat
- Prostratin

GFP expression (Infection) and GFP expression (Reactivation)
3.3.2. C-Lat clones require functional Nef for efficient viral reactivation and Gag expression

To further examine Nef’s contribution during reactivation from viral latency, I isolated and characterized a total of 9 NefSF2:GFP and 32 NefG2A:GFP C-Lat clones. For each clone, I obtained linked data on its proviral integration site as well as its reactivation phenotypes following treatment with different LRAs (TNF-α, panobinostat and prostratin). In my analysis of proviral integration sites (as described in chapter 2), I unexpectedly observed significant expansion for one NefG2A:GFP C-Lat lineage, where 30 of 32 isolated clones (93.8%) displayed an identical integration site in the chr2(14.1) region. As a result of this and similar duplication of several NefSF2:GFP clones, I identified 5 NefSF2:GFP and 3 NefG2A:GFP C-Lat clones with unique integration sites for further analysis. To allow a proper statistical comparison between C-Lat clones encoding functional versus defective nef alleles, C-Lat clones that shared identical integration sites were grouped and average viral reactivation values were used to assess differences in reactivation phenotype following stimulation with LRAs. Due to the small sample size, none of the comparisons was found to be statistically significant (Figure 3-2A) with the exception of viral reactivation in the presence of panobinostat. In particular, functional NefSF2:GFP C-Lat clones stimulated with panobinostat displayed a higher percentage of reactivating cells (median [IQR] = 47.6% [22.2-53.8]) compared to clones encoding defective NefG2A:GFP (7.3% [5.5-10.2]) (P = 0.036). Moreover, clones encoding functional NefSF2:GFP displayed a trend towards enhanced reactivation intensity (Figure 3-2A, bottom).

Next, I assessed the expression of the HIV-1 Gag protein, which is encoded by a late viral gene, in C-Lat clones treated with TNF-α, panobinostat or prostratin. Consistent with the elevated intensity of viral reactivation as assessed using GFP, NefSF2:GFP C-Lat clones also expressed a higher proportion of Gag+ve cells compared to NefG2A:GFP C-Lat clones, regardless of the LRA used (Figure 3-2B). For example, following TNF-α stimulation, the majority (71%) of cells encoding NefSF2:GFP co-expressed GFP and Gag, and the resulting MFI for the Gag-p24+ve population was 408. In contrast, less than half (28%) of cells encoding NefG2A:GFP co-expressed GFP and Gag, and the MFI for the Gag-p24+ve population was 10-fold lower. Furthermore, the greatest difference in Gag
expression between C-Lat clones was observed in the presence of panobinostat, where none of the three clones encoding Nef\textsubscript{G2A}:GFP responded to stimulation.

In addition to C-Lat clones harbouring functional or defective Nef:GFP fusion proteins, I also generated latent clones where Nef and GFP were separated by the IRES element. Representative data for C-Lat clones encoding either functional Nef\textsubscript{NL4.3}IeG or defective ΔNefIeG are shown in Figure 3-3. In contrast to the Nef:GFP fusion proteins, IRES-mediated GFP expression in the NefIeG construct is transcriptionally linked to nef but may be less indicative of Nef protein expression. However, I confirmed Nef expression in C-Lat cells by assessing another well-characterized function of this protein, namely downregulation of HLA-I. Specifically, I compared the relative surface expression of HLA-A*02 on GFP\textsuperscript{+}ve cells following TNF-α stimulation compared to that of GFP-negative cells in the absence of LRA. As expected, HLA-A*02 expression was reduced on C-Lat cells encoding Nef\textsubscript{NL4.3}IeG, but not on cells encoding ΔNefIeG; relative HLA-A*02 expression levels were 5% and 95%, respectively (Figure 3-3A). Consistent with the differences in reactivation phenotypes observed in C-Lat clones expressing functional versus defective Nef:GFP fusion proteins, intracellular Gag expression was dramatically reduced in C-Lat cells encoding defective ΔNefIeG compared to those encoding functional Nef\textsubscript{NL4.3}IeG. Specifically, 79% of C-Lat cells encoding Nef\textsubscript{NL4.3} expressed Gag-p24 following stimulation with TNF-α (Figure 3-3B). In contrast, Gag-p24 production was absent in C-Lat cells encoding defective ΔNefIeG, despite nearly 100% reactivation of these cells based on expression of an early marker (GFP). Together, these results indicate that Nef is necessary to support efficient production of late viral proteins such as Gag following stimulation with LRAs.
(A) **TNF-α**

**Panobinostat**

**Prostratin**

(B) **TNF-α**

**Panobinostat**

**Prostratin**

**Intracellular Gag-p24 Expression**

**GFP expression (Reactivation)**

---

**C-Lat NefG2A:GFP**

**C-Lat NefG2A:GFP**

**C-Lat NefG2A:GFP**

---

**% p24=71**

**% p24=59**

**% p24=76**

**p24_MEF=408**

**p24_MEF=448**

**p24_MEF=371**

---

**% p24=78**

**% p24=75**

**% p24=84**

**GFP=391**

**GFP=468**

**GFP=440**

---

**% p24=28**

**% p24=4**

**% p24=59**

**p24_MEF=40**

**p24_MEF=56**

**p24_MEF=127**

---

**% GFP=62**

**% GFP=12**

**% GFP=75**

**GFP_MEF=95**

**GFP_MEF=96**

**GFP_MEF=195**
Figure 3-2  Reduced viral reactivation and Gag-p24 production in Nef:GFP C-Lat clones expressing defective G2A variant

(A) Graphs display viral reactivation (percent GFP+ve [top] and MFI of GFP+ve cells [bottom]) for NefSF2:GFP (N=9) and NefG2A:GFP (N=32) C-Lat clones, grouped by proviral DNA integration site. Open circle indicates that the mean values from multiple independent clones were used. (B) Flow plots illustrate HIV-1 reactivation phenotypes of representative C-Lat clones by assessing early (GFP) and late (Gag-p24) protein expression following stimulation with TNF-α, panobinostat or prostratin.
Figure 3-3  Reduced Gag protein expression in NefIeG C-Lat clones lacking Nef
(A) Flow plots display the reactivation (GFP⁺ve) phenotypes of representative C-Lat clones (NefNL4.3IeG or ∆NefIeG) in the absence (grey) or presence (green) of TNF-α. HLA class I downregulation was measured to confirm Nef function. In the merged plots, relative % HLA was calculated by dividing the MFI of HLA
expression in GFP+ve cells (TNF-α) by that of GFP-negative cells (no drug). (B) Flow plots display viral reactivation (GFP) and Gag-p24 protein expression in the same C-Lat clones. Percent and MFI for GFP+ve and Gag-p24+ve populations are indicated in the corresponding plots.

3.3.3. CRISPR/Cas9-mediated knockout of Nef reduced viral reactivation

In order to overcome the potential bias associated with differences in the proviral integration site between C-Lat clones encoding functional and defective nef genes, I used the CRISPR/Cas9 system to disrupt Nef expression. Specifically, two pairs of guide RNA (gRNA) were designed to target nef for knockout in four C-Lat clones, including two functional NefSF2:GFP clones (C-Lat_14 and C-Lat_16) and two functional NefNL4.3IeG clones (C-Lat_107 and C-Lat_121), that displayed unique proviral integration sites. The first gRNA pair targeted nucleotide sequences Nef350-369 and Nef606-587 (antisense), which are conserved between SF2 and NL4.3 strains. In contrast, the second gRNA pair targeted only NefNL4.3-specific sequences Nef30-49 and Nef561-580. To enrich for a population that contained Nef knockout (NefKO) cells, I co-transfected cells with pMAX-GFP and isolated transfected cells by FACS. Since I also use GFP as a marker for HIV-1 reactivation in C-Lat cells, the isolated cells were maintained in culture until transient pMAX-GFP expression had diminished (~2 weeks) prior to stimulation with LRAs and analysis of intracellular Gag-p24 expression. For each C-Lat clone tested, no DNA and empty vector (pSpCas9(BB)) transfection controls were included, both of which showed comparable level of Gag protein expression (<2% difference) after stimulation using TNF-α (Figure 3-4A and 3-4B). As expected for the Nef:GFP fusion protein in C-Lat_14 and C-Lat_16 clones, disruption of the nef gene also abolished GFP expression, resulting in a population of Gag+ve/GFP-negative cells following stimulation (Figure 3-4A). On the other hand, because nef and GFP are separated by an IRES element in C-Lat_107 and C-Lat_121 clones, the majority of Gag-expressing cells retained GFP expression following TNF-α treatment (Figure 3-4B). Moreover, both pairs of Nef gRNA reduced Gag-p24 expression comparably in the NefIeG C-Lat clones, with gRNA pair 2 displaying a marginally lower percentage of Gag+ve events (4-5%). After normalizing Gag expression to the corresponding no DNA control, all C-Lat clones transfected with Nef gRNA showed significantly lower percentage of Gag expressing cells (59-87%) following treatment with LRA, compared to empty vector control (99-102%, P = 0.0015) (Figure 3-4C). Similarly,
the relative MFI of Gag-p24<sup>+/ve</sup> cells was significantly reduced in C-Lat clones transfected with a Nef gRNA (73-85%) compared to empty vector controls (96-100%, \( P = 0.0002 \)) (Figure 3-4D). These results are highly consistent with my observations for C-Lat clones encoding defective nef alleles, and similarly indicate that Nef plays an important role to enhance Gag protein production during viral reactivation from latency.

![Figure 3-4](image)

**Figure 3-4** Knocking out WT Nef in C-Lat cells lowered Gag production
(A and B) Flow plots show TNF-α-induced viral reactivation and Gag expression in four independent C-Lat clones. No DNA and empty vector controls were included for each clone; Nef gRNA pair 1 targeted WT Nef for knockout in all four clones whereas Nef gRNA pair 2 only targeted Nef allele in NefIeG clones. Percent and MFI for Gag-p24<sup>+/ve</sup> population are indicated in the corresponding plots. (C and D) Graphs depict percent and MFI of Gag-p24 expression in bulk Nef<sub>ko</sub> clones relative to no DNA control of corresponding parental cell line. A dashed line (---) in each panel illustrates normalized Gag-p24 expression of 100; values below 100 indicate reduced Gag expression relative to no DNA control. The paired sample t-test value is shown.

**3.3.4. Viral reactivation and Gag protein production are reduced in Nef<sub>ko</sub> C-Lat clones**

There are two major limitations associated with analyzing Nef<sub>ko</sub> cells following CRISPR/Cas9 modification in mixed cultures. First, I could not confirm or calculate the
percentage of cells that were engineered successfully. Second, I could not rule out potential off-target effects of Cas9 nuclease that might affect viral reactivation. To verify that changes in HIV-1 reactivation and Gag protein expression phenotypes were associated with disruption of nef, I isolated single NefKO clones derived from three C-Lat cell lines based on the loss of two canonical Nef functions, namely the ability to downregulate HLA-I and CD4, following stimulation with LRAs; thus, high expression of these two markers indicated successful disruption of the nef gene. Briefly, I sorted single Nefko cells based on GFP expression (GFP-negative for Nef:GFP C-Lat clones or GFP+ve for NefIeG C-Lat clones) as well as high CD4 and HLA-A*02 expression following LRA treatment. Next, I sequenced the proviral DNA of each clone to confirm disruption of the nef region. By obtaining full-length nef gene sequences, I observed gene modification events near the gRNA target sites and ensured the absence of an intact Nef open reading frame (ORF). Since the HIV-1 5’ LTR shares sequence similarity with the nef gene and an overlapping portion of the 3’ LTR, it is an excellent candidate for CRISPR/Cas9 off-target effects. To address this concern, I used genomic integration site-specific primers to amplify and sequence the 5’ LTR of all NefKO clones, confirming the absence of mutations in this region.

A total of 12 unique Nefko clones were obtained and sequence-verified. For each NefKO clone, the expression of GFP and Gag-p24 expression was assessed after stimulation with LRAs (TNF-α, panobinostat or prostratin) or negative controls. As expected for C-Lat_14 and C-Lat_16 Nef:GFP cell lines, GFP was not expressed in any of the NefKO clones (N=4) prior to or after HIV-1 reactivation, regardless of the LRA used (Figure 3-5A). Compared to their corresponding parental C-Lat cell line, most NefKO clones displayed significant reductions in the percentage of Gag-p24 expressing cells as well as in the intensity of Gag-p24 expression following stimulation with all LRAs tested (Figures 3-5B and 3-5C). The only exceptions were two NefKO clones that showed a similar or higher percentage of Gag expressing cells following stimulation with TNF-α compared to their parental controls, but in both cases the relative MFI in Gag+ve cells was lower. The third parental cell line used for CRISPR/Cas9 studies, C-Lat_107, harboured a NefIeG reporter construct and, as expected, the resulting Nefko clones (N=8) retained their ability to express GFP following reactivation from latency (Figure 3-6A). This characteristic
allowed us to measure and compare the expression of both early (GFP) and late (Gag) viral proteins. Notably, I observed that following disruption of nef, C-Lat_107-derived cells became less capable of producing both GFP and Gag (Figures 3-6B to 3-6D). For example, compared to parental C-Lat 107 cells stimulated using TNF-α, the relative proportion of GFP expressing cells in the NefKO clones was reduced to a median of 77%, whereas the relative proportion of Gag-p24 expressing cells was reduced to a median 21% ($P < 0.001$). These results underscore Nef’s ability to modulate HIV-1 reactivation events, including late viral protein expression. While additional studies will be necessary to define cellular mechanisms to explain this phenotype, the severe impairment in Gag expression observed for NefKO cells may be due in part to reduced production of early viral gene products, such as Rev which is necessary to export unspliced Gag mRNA into the cytoplasm.

Figure 3-5   Less Gag protein was produced in Nef:GFP$_{KO}$ clones

A) Flow plots display GFP and Gag-p24 expression in parental and representative Nef$_{KO}$ clones for two Nef:GFP C-Lat cell lines (C-Lat_14 and C-Lat_16). GFP and Gag-p24 expression were measured at basal level (no drug) and after stimulation using TNF-α, panobinostat or prostratin. Percent and MFI for GFP$^{+}$ and Gag-p24$^{+}$ population are indicated in the corresponding plots. (B and C) Graphs depict percent and MFI of Gag-p24 expression in C-Lat_14 (closed circle) and C-Lat_16 (open circle) Nef$_{KO}$ clones relative to corresponding parental cell line. A dashed line (---) in each panel illustrates normalized Gag-p24 expression.
of 100; values below 100 indicate reduced Gag expression relative to corresponding LRA control. One sample t-test values are shown; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

![Figure 3-6](image)

**Figure 3-6** Efficiency of viral reactivation and Gag protein production was reduced in NefleGKO clones

(A) Flow plots display GFP and Gag-p24 expression in parental and representative NefKO C-Lat_107 (NefleG) clones. GFP and Gag-p24 expression were measured at basal level (no drug) and after stimulation using TNF-α, panobinostat or prostratin. Percent and MFI for GFP+ and Gag-p24+ populations are indicated in the corresponding plots. (B to D) Graphs depict percent and MFI of GFP (B and C) and Gag-p24 (D and E) expression in NefKO clones (N=8) relative to that of C-Lat_107 parental cell line. Triangles indicate representative NefKO clones displayed in panel A. A dashed line (---) in each panel illustrates normalized GFP/Gag-p24 expression of 100; values below 100 indicate reduced GFP/Gag expression relative to corresponding LRA control. One sample t-test values are shown; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.4. Discussion

In this comprehensive study, I examined the effect of HIV-1 Nef on viral protein expression during reactivation from latency. I used several replication-incompetent virus strains to generate a panel of latent T cell clones that encoded either functional or defective nef variants. By fusing Nef to GFP for use as a reactivation marker, I was able to monitor the expression of both early (Nef/GFP) and late (Gag) viral proteins. I isolated a total of 41 Nef:GFP C-Lat clones, and obtained linked data on proviral integration site and reactivation phenotypes for each clone. Even though only 8 out of 41 C-Lat clones displayed unique integration sites in the host genome (i.e. the others were progeny from shared precursor cells), those that expressed functional NefSF2 displayed a trend towards enhanced reactivation intensity (based on GFP expression). This observation is consistent with a prior study showing that extracellular Nef could activate latent HIV-1 in a dose-
dependent manner (18). Similar results were also observed for the late viral protein Gag. Indeed, I found that enhanced reactivation intensity (based on GFP) was accompanied by a higher frequency and intensity of Gag expression. This phenotype is consistent with Nef’s ability to increase the total amount of Gag proteins in virus-infected cells via an unknown mechanism (19). Finally, I confirmed that Nef expression was associated with viral reactivation phenotypes using C-Lat clones generated using independent HIV-1 strains encoding NefIeG, where Nef and GFP proteins are expressed from the same RNA transcript but the proteins are unlinked.

The proviral DNA integration site, and hence its nearby chromatin environment, was first described by Jordan et al. to be a determinant for HIV-1 induction by Tat transactivation (12, 13, 20). Multiple research studies have confirmed this observation using improved experimental methods and bioinformatics tools (21-23). For example, Chen et al. recently used barcoded HIV-1 ensembles to show that distinct repertoires of integrated proviruses can be induced by different LRAs (24). To rule out the potential bias associated with differences in proviral integration site between C-Lat clones, I performed targeted gene editing using the CRISPR/Cas9 system. In particular, two different sets of gRNA were used to disrupt nef in four independent C-Lat clones. Following knockout of Nef expression, I consistently observed lower Gag protein production in bulk Cas9-transfected cells as well as in sequence-validated NefKO clones. In contrast to most studies of HIV-1 latency, I generated latent clones containing a single copy of integrated HIV-1, and tested each clone against three different classes of LRA. While this approach is more labour-intensive, it may provide a clearer picture of viral reactivation compared to models where polyclonal latent cell populations are used. Together, my experimental design addressed the potential confounding effects of differences between LRAs and proviral integration sites, and still found a consistent effect of Nef to enhance HIV-1 reactivation from latency.

In addition to the proviral integration site, viral factors such as the Tat protein and the LTR promoter region have been reported to affect the inducibility of latent virus following LRA treatment (25, 26). Specifically, the ability of Tat to enhance HIV-1 transcription through interaction with the viral transactivation response (TAR) element and recruitment of the positive transcription elongation factor (p-TEF) complex has been well
documented (27-30). Consistent with Tat’s essential role in transcription activation, many studies have demonstrated Tat’s potential to reverse HIV-1 latency (31-34). For example, introduction of Tat protein in abundance can induce viral reactivation, whereas inhibition of Tat using the small molecule didehydro-cortistatin A prevents LRA-induced latency reversal (35, 36). Moreover, since Tat is a highly polymorphic protein, naturally occurring polymorphisms such as P10S and W11R have been shown to impair transactivation activity, which in turn may promote viral latency and reduce the frequency of viral reactivation. Aside from Tat, transcription factor binding sites within the LTR also influence viral gene expression and HIV-1 replication (37-41). Consequently, natural variation in these binding elements could alter viral latency or reactivation. Indeed, in latently infected cells lacking Sp1 and NF-κB binding motifs, basal gene expression (i.e. transcriptional “noise”) was increased and the variation in viral reactivation frequency was elevated (26).

On the other hand, despite its recognized role as a regulator of T cell signaling, few studies have explored the impact of Nef on viral latency. In fact, many cell line models of HIV-1 latency do not encode the nef gene in their minimal or full-length HIV-1 provirus since Nef is not essential for viral replication and thus a preferred site to incorporate reporter genes such as GFP (12, 42-45). Hence, the panel of C-Lat cells generated for this study represent the first direct comparison of Nef-expressing and Nef-defective proviruses in the same T cell model of latency. To maintain an optimal cellular environment for efficient production of progeny, Nef modulates T cell activation by altering the intracellular trafficking of various signaling proteins (5, 46). For example, Nef redirects the Lck protein away from the plasma membrane, preventing early TCR signaling events such as tyrosine phosphorylation that are required for the formation of immunological synapses and robust cellular activation (47-50). Nef has also been shown to induce calcineurin-dependent activation of NFAT through interaction with inositol triphosphate receptor (IP3R), resulting in calcium release from the ER (51). Additionally, the formation of a Nef-associated kinase complex comprised of Nef, Lck, LAT, and Ras has been shown to trigger Erk/MAPK (52, 53), which may enhance basal transcriptional activity in resting T cells.

During HIV-1 reactivation from latency, different cellular activation pathway(s) can be triggered depending on the class of LRA used. Hence, in this study, I tested the
effect of Nef in the context of three different LRAs - TNF-α, panobinostat and prostratin; which target distinct cellular pathways for latency reversal. TNF-α is capable of inducing two activation pathways, namely NF-κB and MAPK, reviewed in (7). Briefly, binding of TNF-α to TNF receptor type 1 enables adaptor protein TRADD (TNF receptor-associated DEATH domain) to recruit multicomponent protein kinase that: 1) phosphorylates the inhibitor of kB, allowing NF-κB to translocate into the nucleus and 2) induces a MAPK cascade, leading to activation of c-Jun NH2-terminal kinase (JNK). Activation of both pathways subsequently leads to initiation of gene transcription. Panobinostat is an inhibitor of HDACs, and thus remodels the chromatin structure to a less compact and more transcriptionally active conformation by inhibiting the removal of acetyl groups from lysine amino acids on histones (54, 55). Prostratin also induces translocation of NF-κB into the nucleus, but it achieves this through the activation of the PKC signaling cascade (56, 57). My results indicate that Nef enhances HIV-1 reactivation regardless of which LRA was used for stimulation, suggesting that Nef acts through an independent mechanism, perhaps by re-directing other protein kinases or signaling pathways to enhance T cell activation or viral gene expression. Additional studies will be necessary to identify the cellular factors involved in this phenotype; however, some clues may be found in the list of Nef-interacting proteins described by prior studies. Finally, because Nef is highly polymorphic (58, 59), its effects on viral reactivation may vary among HIV-infected individuals. An improved understanding of the cellular mechanism would allow the screening of primary Nef clones, potentially leading to the discovery of new viral determinants affecting HIV-1 latency.

Some limitations of this study merit discussion. First, majority of the viral reactivation data shown only comprised of a single replicate. However, I was able to observe consistent effect of Nef on viral reactivation through analysis of mix culture containing uninfected and latently infected cells, latent HIV-infected T cell clones expressing functional and defective nef as well as nef gene knockout experiments. Second, the discovery of one particular NefG2A:GFP C-Lat cell with substantial clonal expansion (30 of 32 independent clones) was surprising and unexpected. This result negatively impacted the statistical power to test for differences in viral reactivation in the presence versus absence of nef. I noted that the provirus of this particular NefG2A:GFP C-Lat clone
was integrated into the intronic region of coiled-coil domain containing protein 93 (CCDC93) at chr2(q14.1). CCDC93 is known to be involved in the trafficking of copper transporting ATPase (ATP7A) between the trans-Golgi network and vesicles in the cell periphery, which is an important process for regulating copper level in cells (60). Even though CCDC93 has no confirmed role in cancer development, its gene expression was altered significantly in hepatocellular carcinoma cells (61, 62), suggesting potential importance in cell growth and proliferation. Regardless, I was able to confirm the ability of Nef to enhance HIV-1 reactivation by generating an alternative panel of Nef-IRES-GFP cell lines and by knocking out nef using CRISPR/Cas9. Third, while I have re-sequenced the nef gene in Nefko clones following CRISPR/Cas9 modification, I have not re-sequenced the entire HIV-1 proviral genome to verify the absence of off-target effects. However, the specificity of gene modifications at the Nef target sites and the absence of mutations at 5’ LTR suggest that remainder of the viral genome is intact and uninterrupted. Last, to assess late viral protein expression, I only measured the Gag-p24 protein; however, since Gag is derived from a 160 kDa precursor GagPol fusion protein, it is highly likely that Nef affects the expression of other late proteins, including the essential enzymes reverse transcriptase and protease.

In this study, I provided evidence that Nef plays a novel role in the enhancement of HIV-1 reactivation and Gag production following stimulation with LRAs. My data suggested that functional Nef variants are required to induce robust viral protein expression in latent T cells. While the clinical implications of this observation are still unclear, I noted that Nef is highly polymorphic in nature and that a majority of latent HIV-1 reservoirs consist of proviruses encoding large deletions and/or hypermutations that may preclude expression of Nef. Thus, variable Nef sequences and/or Nef functionality could hinder the effectiveness of “shock and kill” strategies. Moreover, the latent T cell lines described here may be valuable tools for further studies to elucidate the molecular mechanisms underlying Nef’s role in latency reversal or screens to identify novel small molecules that can modulate viral reactivation from latency.
3.5. References


Chapter 4.

Development and characterization of Nef gene editing in latent T cells

Contribution: I completed all data collection and analyses included in this chapter.

4.1. Introduction

Previous studies have demonstrated the use of extracellular Nef protein to induce HIV-1 reactivation in latent cells (1, 2). In chapters 2 and 3 of this thesis, I confirmed Nef’s ability to enhance latency reversal by generating a panel of latent T cell lines that harboured either functional or defective nef genes. However, the induction of HIV-1 reservoirs from latency is often controlled by a combination of host and viral factors. For example, proviruses that are located in sites where the chromatin environment favours gene transcription are likely to be more susceptible to induction following stimulation with an LRA. Depending on the distance and orientation of integrated provirus with respect to the host gene, a chromatin environment that normally favours host gene expression may also interfere with viral gene transcription (3-5). Furthermore, variations in epigenetic modification and transcription factor binding sites at the HIV-1 LTR promoter (6-8) as well as polymorphisms that affect the function and expression of viral Tat (9) and Vpr (10) proteins could contribute to the inducibility of latent cells. Hence, to adequately assess the role of Nef during latency reversal adequately, other host and viral factors that influence viral reactivation should ideally be held constant.

A potential unbiased approach to test the role of Nef in latent T cells is through targeted gene editing using the RNA-guided CRISPR/Cas9 nuclease system (11-15). I chose this system because it is described to be highly specific, efficient and more versatile compared to other genome editing technologies, such as zinc finger nucleases (ZFNs) (16) and transcription activator-like effector nucleases (TALENs) (17). To briefly describe the CRISPR/Cas9 system, a sequence-specific gRNA is used to form a complex with the Cas9 protein and direct it to the target site (18). Upon recognition and interaction with the target
sequence, the Cas9 endonuclease creates a DNA double-stranded break (19, 20) that consequently gets repaired through two different cellular mechanisms: non-homologous end joining (NHEJ) or homology-directed repair (HDR) (20). Using this approach, gRNA have been designed to target different regions of the integrated HIV-1 genome with the eventual goal of achieving HIV-1 remission in the absence of cART (21-23). In these studies, CRISPR/Cas9 was shown to precisely and consistently cause insertion/deletion events at the target site, resulting in inactivation of proviral DNA. However, similar to other gene editing methods, a concern with CRISPR/Cas9 is the induction of off-target effects, which could be affected by gRNA design, target site selection, Cas9 activity and the delivery method used. Hence, detailed evaluation and characterization should be performed on cells that have undergone CRISPR/Cas9-mediated gene editing.

Here, I aimed to optimize the use of CRISPR/Cas9 methods to examine and confirm the role of the HIV-1 nef gene in viral reactivation from latency. For the first part of this study, a functional nef allele encoded by a latent T cell line (C-Lat) was targeted for disruption, and a panel of NefKO clones were generated and characterized. Among these NefKO clones, gene modifications were observed to occur at the gRNA target site, confirming the precision of the CRISPR/Cas9 system. However, various unexpected genetic phenotypes were also observed in Nefko clones, which affected viral reactivation phenotypes differentially. In particular, large deletions in the HIV-1 provirus were associated with an increased frequency of early reactivation events compared to those that harboured intact HIV-1. For the second part of this study, I attempted to optimize the CRISPR/Cas9 system to replace the proviral nef gene using HDR. Nef is highly polymorphic and display tremendous functional variability (24-26), hence, the use of CRISPR/Cas9 gene replacement to incorporate patient-derived nef alleles into the setting of a latent provirus could be highly beneficial. With respect to this, I observed highly variable viral reactivation phenotypes among cells following nef gene replacement, suggesting that this method requires further optimization. Together, these results highlight the importance of performing comprehensive profiling to ensure the accuracy of gene modification prior to downstream usage of cells modified using CRISPR/Cas9 methods.
4.2. Methods

4.2.1. Plasmids and cell culture

The pX330-U6-Chimeric_BB-CBh-hSpCas9 (abbreviated as pSpCas9(BB)) plasmid was a gift from Feng Zhang (Addgene plasmid #42230) (27, 28). The pBR43-NefSF2/G2A/M20A::GFP plasmids were generated in chapter 2. CEM-A*02-derived C-Lat_14 and C-Lat_107 latent cell lines harbour a single copy of integrated HIV encoding NefSF2::GFP and NefIeG reactivation markers, respectively. All cells were cultured in R10+.

4.2.2. Primer List

Table 4-1 List of primers and their corresponding applications

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Application</th>
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<tr>
<td>gRNA_NEF350-369_top</td>
<td>5’-CACCgCACAAGGCTACTTCCCTGAT-3’</td>
<td>pSpCas9(BB)-gRNA cloning</td>
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<tr>
<td>gRNA_NEF350-369_bot</td>
<td>5’-AAACATCAGGGAAGTAGCCTTGTCGc-3’</td>
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</tr>
<tr>
<td>gRNA_NEF606-587_top</td>
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<td>pSpCas9(BB)-gRNA cloning</td>
</tr>
<tr>
<td>gRNA_NEF30-49_top</td>
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<td>pSpCas9(BB)-gRNA cloning</td>
</tr>
<tr>
<td>gRNA_NEF30-49_bot</td>
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</tr>
<tr>
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<tr>
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<td>Primer name</td>
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<td>DDNA construction</td>
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<td>DDNA amplification</td>
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<tr>
<td>DDNA_GFP656_R</td>
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<td>DDNA amplification</td>
</tr>
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</tr>
<tr>
<td>pSpCas9_517_R</td>
<td>5’-GGC GTT ACT ATT GAC GTC AAT GGG CG -3’</td>
<td>gRNA sequencing</td>
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<td>pCR2.1-TOPO-DDNA sequencing</td>
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<td>M13R</td>
<td>5’-CAG GAA ACA GCT ATG AC-3’</td>
<td>pCR2.1-TOPO-DDNA sequencing</td>
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Italicized letters: nucleotide overhangs for cloning gRNA into the pSpCas9(BB) vector (28)
Lower-case letters: additional guanine (g) and its complementary cytosine (c) appended to 5’ end of target site to increase the efficiency of gRNA expression (28)
Bolded letters: sites targeted for synonymous mutation.

### 4.2.3. Cloning of pSpCas9(BB)-gRNA

A total of 8 gRNA were independently cloned into the pSpCas9(BB) vector following instructions described in Ran et al., 2013 (28). Four Nef-specific guide RNA (gRNA): Nef350-369 (CACAAGGCTACTCCCTGAT), Nef606-587
(GTACTCCGGATGCAGCTCTC), Nef\textsubscript{30-49} (GATTGGATGGCCTGCTGTAA) and Nef\textsubscript{561-580} (CCGCCTAGCATTTCATCACG) were generated for Nef\textsubscript{KO}. Two Env-specific gRNA: Env\textsubscript{2252-2233} (GGATCCGTTCACTAATCGAA) and Env\textsubscript{2323-3402} (GGTGGAATCTCATGTA) and two GFP-specific gRNA: GFP\textsubscript{566-585} (CAGAACACCCCTATTGGTGA) and GFP\textsubscript{596-577} (GAACTGGGCCATCACCAATA) were generated for Nef\textsubscript{GR}. All pSpCas9(BB)-gRNA were confirmed by DNA sequencing.

4.2.4. Construction and preparation of donor DNA

Using pBR43-Nef\textsubscript{SF2/G2A/M20A}:GFP vectors as template, donor DNA (DDNA) containing synonymous mutations at the Env- and GFP-specific gRNA target sites was constructed via the PCR-based overlap extension method (29). Briefly, two DNA fragments Env\textsubscript{2143-2412} (270 bp) and Env\textsubscript{2383-GFP656} (1486 bp) that contain synonymous mutation at gRNA target site were generated using primer set 1 (Env2143\_SM\_F and Env2383\_SM\_R) and set 2 (Env2383\_SM\_F and GFP656\_SM\_R), respectively. Full-length DDNA (Env\textsubscript{2143-GFP656}, 1724 bp) was amplified using primers DDNA\_Env2143\_F and DDNA\_GFP656\_R, and subsequently cloned into pCR\textsuperscript{TM} 2.1-TOPO vector according to manufacturer’s instruction (TA Cloning® Kit, ThermoFisher Scientific). Prior to C-Lat cell transfection, full-length DDNA was amplified off the pCR2.1-TOPO-DDNA plasmid using DDNA\_Env2143\_F and DDNA\_GFP656\_R primers followed by DNA purification using E.Z.N.A® Cycle-Pure Kit (Omega Bio-tek). All PCR amplicons were generated using Expand\textsuperscript{TM} High Fidelity PCR System (Roche). The resulting pCR2.1-TOPO-DDNA plasmids were confirmed by DNA sequencing.

4.2.5. Transfection of C-Lat cells

To knockout Nef allele, 1.0\times10^7 C-Lat\_107 cells were transfected with two pSpCas9(BB)-gRNA (3 µg each) targeting different Nef sequences and 0.5 µg of pMAX-GFP (Lonza). For Nef\textsubscript{GR}, 1.0\times10^7 C-Lat\_14\_Nef\textsubscript{SF2}:GFP\textsubscript{KO} cells that lacked GFP expression were transfected with 3 µg each of four different pSpCas9(BB)-crRNA (two for Env and two for GFP) and 2 µg of purified DDNA. All samples were topped up with Opti-MEM, no phenol red (ThemoFisher Scientific) to a total transfection volume of 150
µL. Cell transfections were done in a 96-well electroporation plate (Bio-Rad Gene Pulser MXcell™: square wave, 250 V, 2,000 µF, infinite Ω, 25 ms), and transfected cells were recovered (4.0×10^6 cells/mL) and maintained in R10+ until use.

4.2.6. LRA treatment

TNF-α was reconstituted to 7.5 µg/mL in 0.1% bovine serum albumin. Panobinostat (Selleckchem) and prostratin (Sigma-Aldrich) were prepared as 25 µM and 1.5 mM working stocks in 0.004% and 1.5% dimethyl sulfoxide (DMSO), respectively. Cells (1.0×10^6 cells/mL) resuspended in R10+ were treated with 2 ng/mL or 50 ng/mL TNF-α, 0.5 µM panobinostat and 30 µM prostratin. Afterwards, treated cells were incubated for 24 h prior to cell sorting and flow cytometry follow up.

4.2.7. Flow cytometry

During generation of Nefko cell lines, transient pMAX-GFP transfection was monitored as GFP expression. For all the data presented in this chapter, GFP is a marker for viral reactivation. Surface CD4 and HLA-A*02 molecules were stained with antihuman CD4-APC (BD BioSciences) as well as HLA-A*02-PerCP/Cy5.5 (BioLegend) or HLA-A*02-PE (BD BioSciences) antibodies, respectively. Intracellular Gag-p24 was stained with KC57-RD1 (Beckman Coulter) antibody (Miltenyl Biotec) according to the BD cytoperm/cytofix plus kit (BD Biosciences). All flow cytometry data were collected using Guava® easyCyte 8HT flow cytometer (EMD Millipore). Data analysis was performed using FlowJo (TreeStar).

4.2.8. Cell sorting

Cell sorting was performed using 1.0 drop pure mode on the FACSJazz™ (BD Biosciences). Prior to isolation of cells, transfected cells were treated with TNF-α. The gating for single Nefko cells were GFP+ as well as high levels of surface CD4 and HLA-A*02. The gating for NefGR cells was based on successful rescue of GFP expression. NefGR cells were sorted and expanded in bulk (500 cells) or as single cells after TNF-α stimulation. Both bulk and single NefGR cells were sorted into 96-well flat bottom plate
containing 100 µL of conditioned medium (1:1 volume of R40+ (40% FBS)-to-filtered supernatant from C-Lat cells) for expansion.

4.2.9. DNA sequencing

The gRNA in pSpCas9(BB) plasmid was validated using sequencing primers U6_F and pSpCas9_517_R (Ran et al.). The DDNA in pCR2.1-TOPO plasmid was validated using sequencing primers NEF1F, NEF5F, NEF6R, NEF9112F, pSel116R, M13F, M13R, DDNA_Env2143_F, and DDNA_GFP656_R. All DNA samples were diluted 15 to 20 folds in DEPC-treated water for sequencing using the BigDye v3.1 kit (ABI). Sequencing data were collected on an 3130xl Genetic Analyzer (Applied Biosystems), and analyzed using Sequencher v5 (Gene Codes).

4.2.10. Statistical Analysis.

Statistical analyses were conducted on Prism 5.0 (GraphPad Software). The one sample t test was used to compare viral reactivation efficiency of NefKO cell lines relative to that of the parental cell line. The Mann-Whitney U test was used to compare GFP and Gag-p24 expression between NefKO cell lines containing different phenotypes of gene modification (i.e. large deletion versus Nef gene inversion). All tests were two-tailed, and \( P < 0.05 \) was considered statistically significant.

4.3. Results

4.3.1. NefKO C-Lat clones displayed variation in viral reactivation phenotype

Two pairs of Nef-specific gRNA were used to target the knockout of NefNL4.3 gene in C-Lat_107 parental cell line, which encodes a Nef-IRES-GFP reporter construct (described in chapter 3). The first gRNA pair recognized Nef350-369 and Nef606-587 (antisense) whereas the second gRNA pair recognized Nef30-49 and Nef561-580. A total of 90 C-Lat_107 NefKO clones (45 per gRNA pair) were isolated and expanded for validation and subsequent profiling of viral reactivation phenotypes.
Nef function was assessed in individual C-Lat_107 NefKO clones following stimulation with TNF-α by measuring surface expression of HLA-A*02 and CD4 using flow cytometry (Figure 4-1A). The relative expression of HLA-A*02 and CD4 molecules were also calculated for each NefKO clone by dividing the MFI of HLA-A*02 or CD4 in the reactivating (GFP<sup>+</sup>) cell population by that of the GFP-negative cell population. As reference, the parental C-Lat_107 displayed relative CD4 and HLA-A*02 expression of 33% and 80%, respectively. For a clone to be considered a Nef<sub>KO</sub>, I established cut-off values for relative expression of CD4 and HLA-A*02 molecules above 70% and 95%, respectively. A lower cut-off was selected for CD4 expression since the HIV-1 Vpu protein is also capable of downregulating this surface molecule (30). Based on these criteria, 39 Nef<sub>KO1</sub> and 21 Nef<sub>KO2</sub> clones were generated using gRNA pair 1 and pair 2, respectively. Subsequently, early (GFP) viral protein expression was measured by flow cytometry for all 60 Nef<sub>KO</sub> clones following stimulation with TNF-α, panobinostat or prostratin. Furthermore, 29 representative Nef<sub>KO</sub> clones (15 KO1 and 14 KO2) were examined for late (Gag-p24) viral protein expression (Figure 4-1B to 4-1D). Overall, these Nef<sub>KO</sub> clones showed significant reductions in basal viral protein expression (i.e. in the absence of LRA stimulation) and viral reactivation relative to that of the parental cell line (Table 4-2). Even though this observation is consistent with the 8 validated Nef<sub>KO</sub> clones presented in chapter 3, a high degree of variation in viral protein expression was observed among these Nef<sub>KO</sub> clones. Notably, the relative basal GFP expression for Nef<sub>KO2</sub> clones displayed a median of 53% with an interquartile range (IQR) of 6-233%. In addition, Nef<sub>KO1</sub> clones stimulated with TNF-α displayed median relative GFP expression of 63% with an IQR of 14-116%. Taken together, CRISPR/Cas9-mediated knockout of the nef gene appeared to reduce viral reactivation, but phenotypes varied greatly among individual clones.
Figure 4-1  Assessment of Nef function and viral reactivation in C-Lat_107 NefKO clones

The expression of HLA-A*02, CD4, GFP and intracellular Gag-p24 are measured in C-Lat cells following LRA (TNF-α, panobinostat or prostratin) stimulation. (A) Overlay flow plots compare surface HLA-A*02 (top) and CD4 (bottom) expression in C-Lat_107 parental versus bulk NefKO1 cells or representative NefKO clones. The green boxes represent cell population (GFP+/HLA-A*02 high/CD4 high) gated for single cell isolation and expansion of NefKO clones. NefKO clones generated using gRNA pair 1 and pair 2 are identified as KO1 and KO2, respectively. (B) Representative histograms depict Gag-p24 expression C-Lat_107 parental and NefKO cells treated with individual LRA. (C and D) Graphs display the relative percent (left) and MFI (right) of GFP (top) and Gag-p24 (bottom) expression for 15 KO1 clones. Each solid line represents linked results for one NefKO clone. Dashed line (---) at 100% indicates the same frequency or intensity of protein expression relative to the parental cell line.
Table 4-2  Statistical summary of all NefKO C-Lat cell lines

<table>
<thead>
<tr>
<th>Group</th>
<th>Rel. % GFP</th>
<th>Rel. GFP MFI</th>
<th>Rel. % Gag-p24</th>
<th>Rel. Gag-p24 MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Drug</td>
<td>KO1</td>
<td>23 [8-69]***</td>
<td>91 [61-143]***</td>
<td>5 [3-8]***</td>
</tr>
<tr>
<td></td>
<td>KO2</td>
<td>53 [6-233]</td>
<td>72 [56-92]</td>
<td>6 [4-22]***</td>
</tr>
<tr>
<td>TNF-α</td>
<td>KO1</td>
<td>63 [14-116]***</td>
<td>27 [21-39]***</td>
<td>23 [17-37]***</td>
</tr>
<tr>
<td>Pano.</td>
<td>KO1</td>
<td>65 [24-98]***</td>
<td>33 [25-49]***</td>
<td>4 [1-6]***</td>
</tr>
<tr>
<td></td>
<td>KO2</td>
<td>82 [40-122]***</td>
<td>36 [19-46]***</td>
<td>4 [2-16]***</td>
</tr>
<tr>
<td>Pros.</td>
<td>KO1</td>
<td>95 [75-100]***</td>
<td>36 [25-46]***</td>
<td>4 [2-10]***</td>
</tr>
<tr>
<td></td>
<td>KO2</td>
<td>100 [87-101]***</td>
<td>47 [19-65]***</td>
<td>6 [1-33]***</td>
</tr>
</tbody>
</table>

a GFP expression: statistical analyses performed on 39 C-Lat_107_KO1 and 21 C-Lat_107_KO2 cell lines
b Gag-p24 expression: statistical analyses performed on 15 C-Lat_107_KO1 and 14 C-Lat_107_KO2 cell lines

One sample t-test values: * = P < 0.05, ** = P < 0.01, and *** = P < 0.001.

4.3.2. Differential gene modification by CRISPR/Cas9-mediated NefKO was responsible for differences in viral reactivation

I investigated whether variation in HIV-1 reactivation phenotypes among NefKO clones could be linked to specific modifications of the nef gene during NHEJ repair. To ensure overall integrity of the proviral DNA after CRISPR/Cas9 treatment, I amplified the entire HIV-1 genome in two parts (5’ and 3’ halves) for 29 Nefko clones with linked data for GFP and Gag-p24 expression. As expected, the 5’ half of HIV genome (expected size, 4.5 kb) was present in all clones tested (Figure 4-2A). In contrast, the 3’ half of HIV genome (expected size, 5.9 kb) failed to amplify in 5 of 29 clones; for example, clones A, I and J of NefKO1 (Figure 4-2A). Since the two HIV-1 fragments overlap by approximately 200 bp, this lack of proviral amplification is likely due to deletion and/or mutation that prevented binding of the reverse primer(s) located in the 3’ LTR. Furthermore, despite successful amplification, substantial deletions were evident in the 3’ half of the proviral genome for several clones; for example, NefKO1 clone B and NefKO2 clone E.
To gain further insight regarding the precision of CRISPR/Cas9 gene editing, HIV-1 sequences spanning *n*ef and its flanking regions (i.e. *env* and *GFP*) were obtained and analyzed for clones with successful amplification of the 3’ half of the proviral genome. In all NefKO clones tested, gene modification was observed to occur at the expected target site(s), confirming the specificity of the gRNAs and Cas9 enzyme. Nonetheless, several different phenotypes of gene modification were observed, which are illustrated in Figure 4-2B. The expected phenotype for CRISPR/Cas9-mediated gene editing is a small deletion or insertion near the gRNA target sites; however, this was observed in only one NefKO clone. Unexpectedly, the most frequently observed modifications included inversion of the *n*ef gene between the two gRNA target sites (29%) and large (>200 bp) gene deletions (43%) (Figure 4-2C). In another case, a large fragment of host DNA derived from the region of provirus integration was incorporated into the HIV-1 genome of a NefKO clone. Notably, NefKO clones encoding a large gene deletion displayed significantly higher basal and LRA-induced GFP expression compared to clones encoding a *n*ef inversion (Figure 4-2D); however, Gag-p24 expression was comparable between the two groups (Figure 4-2E). These results suggest that variation in viral protein expression among NefKO clones is due, in part, to the different gene modification events following CRISPR/Cas9 editing.
**Figure 4-2** Various phenotypes of gene modification were observed in NefKO clones

(A) DNA gel displays 5' and 3' halves of the HIV-1 genome amplified from representative NefKO clones generated using gRNA pair 1 or 2. (B) Schematic illustrates the different phenotypes of HIV-1 modification identified in NefKO clones: wild-type HIV (top) is shown for comparison. Red, inverted triangles indicate gRNA target sites. (C) Pie chart displays the proportion of observed HIV-1 modification phenotypes among 28 NefKO clones. (D and E) Scatter plots compare relative GFP (D) and Gag-p24 (E) expression of NefKO clones that harbour large deletion in HIV-1 versus inversion within nef gene. Four different treatment conditions are shown: no drug, TNF-α, panobinostat and prostratin. Mann-Whitney U test values are shown; * P < 0.05, ** P < 0.01, *** P < 0.001.

**4.3.3. Development of CRISPR/Cas9 tool for Nef gene replacement in a Nef:GFPKO C-Lat cell line**

An ideal approach to study different nef variants without potential bias due to differences in HIV-1 integration sites would be to exchange the proviral nef gene in a latent cell line with a nef allele of interest. To facilitate this, a suitable Nef:GFPKO C-Lat clone was identified to design and optimize the CRISPR/Cas9 system for this purpose (Figure 4-3A). Specifically, the selected C-Lat clone encodes a NefSF2 allele where its sequence...
between nucleotides 379 and 602 were inverted. This inversion resulted in a premature stop codon in the *nef* gene, which also prevented expression of the Nef:GFP fusion protein following reactivation from latency. Furthermore, this Nef:GFP
KO clone expressed high levels of surface CD4 and HLA-A*02 molecules. Based on the above features, it was anticipated that restoration of GFP expression and Nef-mediated downregulation of surface CD4 and HLA-I could be used to screen progeny clones to identify those where *nef* gene replacement had been successful.

To develop this method, three double-stranded donor DNA (DDNA) were generated, each encoding a Nef variant sequence (namely SF2, M20A or G2A) in frame with the proviral Nef:GFP fusion protein. As expected, Cas9 transfection in the presence of DDNA was able to restore GFP expression in a small proportion (~1%) of Nef:GFP
KO C-Lat cells (Figure 4-3B). Moreover, downregulation of CD4 and HLA-A*02 molecules corresponding to each Nef variant could be detected in the GFP-expressing cell population, indicating successful *nef* gene replacement (Figure 4-3C, top and middle panels). For example, functional Nef
SF2 resulted in downregulation of both CD4 and HLA-A*02, whereas the Nef
M20A mutant was impaired for HLA-I downregulation and the Nef
G2A mutant was impaired for both downregulation functions. A significant number of reactivated cells with the Nef
G2A gene replacement were able to downregulate CD4, but less efficiently. This observation could be due to expression of Vpu protein, which also downregulates CD4 (30). Moreover, Gag-p24 was also detected in GFP
+'ve cells (Figure 4-3C, bottom panel), suggesting rescue of late viral protein expression.

After Cas9 and DDNA co-transfection, GFP
+'ve cell populations representing successful replacement of Nef were isolated either in bulk (500 cells) or as single cells for expansion and subsequent characterization. Surprisingly, contradictory viral reactivation phenotypes were observed in bulk cultures versus single cells. In particular, Nef
SF2 showed the lowest reactivation intensity amongst tested variants (Figure 4-4A). This observation contradicted my prior results suggesting that Nef enhanced viral reactivation. On the other hand, clonal cells replaced with Nef
SF2 tended to reactivate better compared to those replaced with defective Nef
G2A (Figure 4-4B and 4-4C). Nonetheless, viral reactivation was highly variable amongst independent clones replaced with the same *nef* allele, suggesting that additional gene modification events may have occurred in the HIV-1 genome during
Cas9 editing. Together, these results indicated that replacement of nef genes in latent proviral clone is possible, but more detailed functional and molecular characterization of single cells may be required to fully validate the efficiency of CRISPR/Cas9 methods and to rule out artefacts due to off-target effects.

Figure 4-3  CRISPR/Cas9-mediated Nef replacement rescued GFP expression in Nef:GFPKO C-Lat clone

(A) Schematic illustrates the use of Nef-containing double-stranded DDNA to replace the inverted Nef gene in a Nef:GFPKO C-Lat clones via homology directed repair. Red, inverted triangles indicate relative location of gRNA target sites that were independently cloned into the Cas9 plasmid. Yellow diamonds indicate synonymous mutations at gRNA sites that prevent cleavage of DDNA by the Cas9 enzyme. (B) Representative flow plots compare GFP expression in Nef:GFPKO cells electroporated with Cas9 plasmids alone (top) or in conjunction with DDNA (bottom). (C) GFP expression was rescued in Nef:GFPKO clone using DDNA encoding different Nef variants (indicated at the top). After stimulating with TNF-α, cells co-electroporated with Cas9 plasmids and DDNA were assessed for GFP, CD4, HLA-A*02 and intracellular Gag-p24 expression.
Differential viral reactivation in bulk and clonal C-Lat cells after Nef replacement

Figure 4-4

After Cas9/DDNA electroporation, Nef:GFPKO cells that are capable of expressing GFP upon TNF-α stimulation were sorted out for expansion either in bulk (500 cells) or as single cells. (A) Flow plots depict viral reactivation of bulk-sorted cells in the absence or presence of TNF-α. Reactivation intensity (measured as the MFI of GFP+ population) is indicated in corresponding plot for each Nef strain tested. (B) The basal GFP expression (i.e., no drug) and percent viral reactivation induced by TNF-α are plotted for NefSF2:GFP (N=6) and NefG2A:GFP (N=6) C-Lat clones isolated after Nef replacement. (C) Scatter plot displays reactivation intensity of the same C-Lat clones from (B).

4.4. Discussion

I previously generated latent T cell lines and showed that the presence of functional Nef protein was essential for robust HIV-1 reactivation and production of late viral proteins. However, the ability of latent reservoirs to reactivate in response to LRAs can be affected by various host and viral factors, and hence, it is difficult to fully assess the contribution of Nef during viral reactivation without potential biases due to intrinsic differences among latent clones. To address this issue, I attempted to develop and optimize the CRISPR/Cas9 technology for targeted editing of the nef gene in latent proviruses. Specifically, I tested the NHEJ- and HDR-based DNA repair mechanisms for gene knockout and gene replacement, respectively. Overall, both mechanisms produced latent T cells with on-target gene modifications and corresponding phenotypic changes that were...
consistent with altered Nef function. However, I observed that viral reactivation phenotypes were highly variable among individual clones. Through genotyping of selected NefKO clones, I identified various unexpected gene modifications that were not reported in previous CRISPR/Cas9 studies. Furthermore, a specific genotype consisting of large (>200 bp) deletions in the proviral genome of NefKO clones was associated with elevated expression of early viral proteins (GFP).

There are couple of valuable features in my CRISPR/Cas9-mediated NefKO experiments. First, I used two different sets of gRNAs to target the nef gene, ensuring that the resulting reduction in viral reactivation were likely to be Nef-specific. Second, I generated a large panel of NefKO clones, which allowed us to identify and link specific on-target mutations to HIV-1 reactivation. To quantify gRNA on-target efficiency, the most commonly used methods are Surveyor and T7 endonuclease I cleavage assays (31, 32). As follow ups, more sophisticated methods such as next generation sequencing and Sanger sequencing are frequently used to identify the precise editing mutations at the target sites (33-35). Furthermore, DNA sequencing is often performed on PCR products of a targeted genomic region, which are generally amplified using bulk samples. As such, mutational variants that contain large insertions and deletion (indels) or sequence inversion events are unlikely to be amplified or captured by these methods. By generating clonal cell lines in this study, I was able to analyze each genome individually, potentially allowing us to identify a wider variety of CRISPR/Cas9-associated mutations. Specifically, I used a combination of full-length genome amplification and Sanger sequencing to perform detailed evaluation of HIV genome integrity and on-target mutations, respectively. Based on my analyses, the Cas9 activity was highly precise and, in most cases, induced sequence mutations at the gRNA target sites. Nonetheless, I captured various gene modifications that have not been described previously.

In the absence of a DDNA template, Cas9-generated DNA breaks can be repaired via NHEJ. Correspondingly, the most frequently observed gene modification phenotype around the cleavage site is small indels (36). Also, to a smaller extent, nucleotide substitutions have been observed. When two or more gRNA are used for Cas9-mediated gene editing, excision of the DNA fragment between target sites can also occur (37, 38). Surprisingly, these commonly observed gene modifications were not present in the NefKO
clones tested in this study, except for one case where small indels were identified at both target sites. Instead, sequence inversion between the target sites and large (>200 bp) deletions near the target site were observed frequently. Other phenotypes included disruption of primer binding site(s) at the 3’ LTR region and incorporation of host DNA at the target site.

These unexpected genotypes could have resulted from various features of my study that are somewhat unique. First, they could pertain to the method I used for genotyping. That is, by amplifying the full-length HIV-1 genome from clonal cell populations, I could identify large or rare indels that would normally be missed if the targeted region used for PCR is small or if bulk samples were used. Second, single cells were isolated by gating on a cell population that was HLA-A*02\(^{\text{high}}\) and CD4\(^{\text{high}}\). Since the HIV-1 Vpu protein is also capable of downregulating CD4 molecules, such gating strategy could perhaps enrich for cells with extreme gene modifications (i.e. those lacking Nef and Vpu functions). Furthermore, it is also possible that the differences in modification outcomes are driven by defects in the NHEJ pathway. In particular, NHEJ is a crucial mechanism for maintaining genome stability such that defects in components involved in this pathway could elevate the risk of cancer development \((39, 40)\). Since the parental CEM-SS T cell line used in this study was originally obtained from an individual with acute lymphoblastic leukemia \((41)\), I cannot rule out potential deficiencies in NHEJ components. Additional studies will be required to discern the underlying mechanism responsible for these unusual DNA repairs.

By linking the phenotypes of gene modification to HIV-1 reactivation, an association was observed between higher induction of early viral protein (i.e. GFP) expression and large sequence deletion in the 3’ half of HIV genome. Interestingly, this association was mainly driven by elevated frequency of basal GFP. Using a bioinformatic approach, a previous study showed that highly expressed genes are significantly smaller \((42)\). Thus, I speculated that a shorter mRNA transcript encoding GFP may be advantageous in this context. I did not observe any correlation between the size of proviral DNA deletions and frequency of GFP expression; however, I have not examined the size or quantity of GFP transcripts directly. Regardless, the expression of late viral protein such as Gag-p24 was reduced similarly in cell lines harbouring different nef gene modifications.
For the gene replacement experiments described here, rescue of GFP expression and Nef-mediated downregulation functions were expected to be good indicators of successful HDR using DDNA template. However, the presence of a defective nef variant was not associated with a significant reduction in viral protein expression. This outcome is inconsistent with my observations from chapter 3 and other NefKO experiments. Considering that a variety of gene modifications were observed in NefKO clones, a similar situation may be occurring during nef gene replacement. More importantly, the DDNA constructs used in these studies encoded a nearly full-length Nef:GFP fusion protein, so it is also possible that random insertion of this sequence into the host genome could lead to GFP expression. Additional optimization and characterization will be required to improve the accuracy of CRISPR/Cas9-mediated gene replacement. In particular, this may involve the use of gRNA that target DNA sequence closer to the 5’ end of GFP and correspondingly, a shorter DDNA template that encodes minimal GFP sequence. Also, I could substitute the Cas9 endonuclease with a mutated form called Cas9 nickase. Cas9 protein contains two catalytic domains, HNH and RuvC-like, each of which is responsible for cleaving one strand of DNA (43). Cas9 nickase was made by mutating one of the two catalytic domains, and hence, it can only produce single-stranded nicks (11, 27, 44). The advantages of using Cas9 nickase include improved HDR efficiency and reduced off-target effects (27, 45).

Off-target effects remain the biggest challenge when using the CRISPR/Cas9 technology. In fact, it is important to acknowledge several limitations in my experimental design that could increase the rate of off-target effects. First, 2 gRNA were used to target each region of interest, which means a total of 4 gRNA were used for gene replacement. While the additional gRNAs were meant to enhance the efficiency of gene editing, they also increase the likelihood of off-target effects. Second, I used a plasmid-mediated delivery method that encodes both Cas9 endonuclease and gRNA. In comparison to mRNA- and protein-based approaches, plasmid DNA can persist for a longer time in transfected cells and exacerbate the chance of off-target effects (46). While I did not address the issue of off-target mutations experimentally, I took other measures to minimize potential off-target effects. For example, each gRNA was designed using the CRISPR Design tool (http://crispr.mit.edu), which uses a published algorithm to assess for potential
off-target effects in human genome (h38) (47). While the tool has a cut-off score of 50% for candidate gRNA, where higher score indicated lower off-target effects, all the gRNA tested in this study scored 71% or higher. Additionally, for the NefKO experiments, I tested 2 different sets of gRNAs to target Nef and showed highly comparable results on gene modification and viral reactivation. Furthermore, consistent results were also obtained among clonally expanded cells after gene editing.

In conclusion, I showed the feasibility of using the CRISPR/Cas9 system to study the role of HIV-1 Nef during reactivation of latent T cells. However, the unexpected variation in gene editing and viral reactivation phenotypes emphasize the need for further optimization and the importance of more detailed evaluation of gene edited populations in future studies. Nonetheless, initial results are promising and additional modifications to the CRISPR/Cas9 experimental design may greatly improve the efficiency and accuracy of gene editing, especially for use in gene replacement.
4.5. References


Chapter 5.

Concluding Remarks

Various “shock and kill” strategies (1) are being investigated as potential methods to eradicate long-lived latent HIV-1 reservoirs that are responsible for viral rebound in individuals who discontinue cART (2-4). These approaches hypothesize that latently infected cells can be induced to reactivate following stimulation with LRAs in the presence of cART, promoting viral mRNA expression and viral protein translation. Expression of these viral proteins will subsequently expose newly reactivated cells to elimination through immune-mediated clearance or viral cytopathic effects. However, this process is inefficient and as a result, clinical studies have been unable to reduce the size of latent reservoirs or achieve HIV-1 remission (5-8). A better understanding of host and viral factors that contribute to the establishment, maintenance and reactivation from latency could lead to new interventions that improve the efficiency of current “shock and kill” strategies.

In this dissertation, I used various cell-based approaches to examine the role of HIV-1 Nef during viral reactivation from latency. In chapter 2, I created a novel panel of inducible latent HIV+ T cell lines encoding functional and defective nef alleles and observed differences in their proviral integration site distribution and genomic features. Specifically, I found a larger number (and greater proportion) of unique proviral integration sites in latent cell lines in the presence of functional nef. Also, the integration sites of proviruses encoding functional nef were modestly enriched in protein-coding genes that are expected to be less transcriptionally active. Together, these results suggest that Nef may contribute to viral reactivation by broadening the repertoire of inducible proviral integration sites following establishment of latency.

In chapter 3, I tested large panels of latent T cell lines and showed that the presence of functional nef was associated with increased responsiveness of latent cells to stimulation by LRAs, leading to increased production of the late viral Gag protein. By using the same HIV-1 reporter strains to generate C-Lat clones and examining their linked data on viral reactivation and proviral integration site, I ensured that the effect of Nef was independent of other known regulators of HIV-1 latency, including Tat, the LTR promoter, and the
proviral integration site. These observations were confirmed through targeted knockout of the proviral nef gene using CRISPR/Cas9 methods. These results indicate that Nef is required for efficient latency reversal following stimulation with LRAs, suggesting that Nef should be considered as a novel modulator of latency when designing future “shock and kill” strategies to eradicate viral reservoir.

In chapter 4, I described the use of CRISPR/Cas9-based gene editing methods to manipulate proviral nef sequences, an approach that may allow an unbiased comparison of viral reactivation phenotypes in the context of otherwise identical latent HIV-1 genomes. I was able to successfully knockout and replace the proviral nef gene in latent T cell lines; however, detailed molecular characterization of the resulting cell lines suggested that there was substantial variability in the repair of DNA breaks in proviruses following cleavage with Cas9. These observations suggest that the CRISPR/Cas9 system can be used to study nef variants, but substantial molecular follow-up may be necessary to validate results, which could be labour-intensive. Hence, optimized strategies should be developed and considered for future studies to examine the role of Nef during HIV-1 reactivation from latency.

The efficiency of “shock and kill” strategies is determined by the degree to which latent HIV-1 reservoirs are reactivated and subsequently eliminated in the host. My results indicate that Nef plays a “dual” role in modulating both of these important factors (illustrated in Figure 5-1). While I have demonstrated in this thesis that Nef can increase viral protein production during latency reversal, Nef’s ability to mediate immune evasion and to enhance cell survival through inhibition of apoptosis are well established. Nef leads to downregulation of HLA-I molecules on the cell surface (9-11), which reduces presentation of viral peptide antigens on the cell surface and impairs CTL-mediated recognition and cytolytic activity against reactivating reservoirs (12). Additionally, Nef’s ability to modulate apoptotic pathways may prevent reactivated cells from dying due to viral cytopathic effects (13, 14). In contrast, latent cells that lack functional Nef may be unable to produce viral proteins efficiently. As a result, presentation of viral peptides may be limited despite retaining high levels of HLA-I expression on the cell surface. Hence, these different roles of Nef could create double-edged effects in the setting of a “shock and kill” strategy.
While molecular mechanisms to explain Nef’s role in viral reactivation have not been elucidated in my studies, these observations could be driven by Nef’s ability to regulate cellular activation status. For instance, the presence of Nef can trigger formation of a Nef associated kinase complex (NAKC) and induce downstream Ras/MAPK activity (15, 16). Through its interaction with IP3R, Nef can trigger calcium flux into the cytosol and induce NFAT activation (17, 18). In both cases, early production of Nef during viral reactivation might enhance latent T cell activation. Moreover, previous studies reported that Nef can be released into the extracellular space either in soluble form (19, 20) or within exosomes (21, 22). Both soluble and exosome-associated Nef have been shown to induce HIV-1 reactivation in latently infected cells (23, 24), but their proposed molecular mechanisms are different. In particular, soluble Nef may bind non-specifically to the surface of latent HIV+ cells and get internalized via endocytosis (25, 26). After entering the cell, Nef can induce Ras/MAPK (27) and PI3K/Akt (28) signaling pathways that ultimately activate viral gene transcription. On the other hand, Nef increases the production of exosomes containing activated ADAM17 (a disintegrin and metalloprotease domain 17) (29), an enzyme that converts pro-TNF-α into its active form. Uptake of ADAM17-containing exosomes by target cells can induce the release TNF-α (30), which subsequently binds to TNF receptor type 1 and activates NF-κB and JNK pathways (31). Additionally, Nef has been shown to increase exosome release, which presumably leads to transfer of Nef-associated signaling activities to nearby cells (32). Nef-mediated effects on signaling pathways are complex and their potential impacts on viral reactivation are not mutually exclusive. In fact, based on these previous findings, I speculate that Nef’s ability to enhance viral reactivation may be attributed to a positive feedback loop of cellular activation. Specifically, upon stimulation with LRAs, early Nef expression may increase viral gene expression. Subsequent secretion of soluble Nef and Nef/ADAM17-containing exosomes could further increase the activation of latent cells through direct effects of Nef or TNF-mediated signaling pathways.

Multiple molecular events are likely to contribute to the activation of latent HIV-1, including those that may inadvertently trigger the downregulation of HLA-I molecules. For instance, activation of PI3K can induce viral reactivation (28), but it was also shown to be essential for internalized of surface HLA-I molecules (33). Hence, it is crucial to elucidate
the precise underlying mechanisms responsible for the Nef-mediated viral reactivation in order to induce latently infected cells most efficiently without impairing host immune responses that are important to mediate viral clearance. With respect to this point, the latent cell lines and preliminary gene editing methods developed in this thesis could serve as excellent tools for further exploration of molecular mechanisms. For example, CRISPR/Cas9-mediated gene replacement can be used to examine *nef* genes of interest. Particularly, by testing a panel of Nef mutants with known functional differences (34, 35) it may be possible to identify host protein interactions that are critical for HIV-1 reactivation. A potential candidate is the PxxP75 motif, an indispensable site that associates with PAK2 (36, 37) and SH3 domain-containing Src family tyrosine kinases (38, 39) to regulate T cell signaling. Additional candidates include the EEEE65 acidic cluster domain and LL165 dileucine motif, which interacts with PACS-1 (phosphofurin acidic cluster sorting protein 1) and AP-1, respectively (40-44). The ability of EEEE65 and LL165 to recruit adaptor proteins lead to alteration of host protein trafficking, which could affect viral reactivation. For example, the PACS-1-mediated trafficking of the furin protease (45) could be involved in the conversion of pro-ADAM17 into its active form (29), which eventually promotes TNF-α release. Furthermore, Nef sequences (and thus functions) are highly variable among naturally occurring variants (46-48). Correspondingly, the CRISPR/Cas9 approach may be useful to examine primary *nef* variants and to identify novel sites that are critical for viral reactivation.

Even though *nef* gene replacement would be an ideal way to investigate Nef-mediated viral reactivation, it is labour-intensive and may require an extensive amount of genotyping and validation. As an alternative method, latent cell lines generated in this thesis may also be used to study Nef. For example, latent cells that express functional Nef can be reactivated in the presence of chemical inhibitors to block different signaling pathways (i.e. Ras/MAPK and PI3K/Akt) or exosome release (49-52). To test the effect of Nef variants on viral reactivation, Nef-containing exosomes might be generated and used *in trans* to treat latent cells that lack Nef in the presence or absence of LRAs.

In summary, this thesis has demonstrated that Nef plays a crucial role in the enhancement of HIV-1 reactivation from latency. In particular, Nef appeared to broaden
the repertoire of viable proviral integration sites to include less transcriptionally active genomic locations. Thus, in order to eradicate latent HIV-1 reservoirs, it may be important to overcome Nef’s dual-role as an enhancer of viral reactivation and mediator of immune evasion. In the course of this work, I have generated a large panel of novel latent/inducible HIV-infected GFP reporter T cell lines and developed CRISPR/Cas9 gene editing methods to study Nef’s contributions during viral reactivation. While further investigation of this Nef phenotype is clearly necessary, the materials and methods described here will be very useful for future studies to explore the mechanism(s) of Nef’s impact on viral reactivation, which should improve our understanding of host and viral factors that contribute to HIV-1 latency and may lead to enhanced clinical interventions, including more effective “shock and kill” strategies.

Figure 5-1  Overview of Nef’s contribution during “shock and kill” of latent reservoirs
Illustration outlining the contribution of Nef to clearance of reactivated latent HIV-infected T cells during “shock and kill”. In the presence of Nef, viral protein expression is robust, but HLA-1 molecules are downregulated, and apoptosis is inhibited. In the absence of Nef, HLA-1 molecules are expressed, but viral protein expression is reduced, and limited peptide antigens are available for presentation. In both scenarios, lack of peptide presentation results in inhibition of CTL-mediated recognition and elimination.
5.1. References


22. Raymond AD, Campbell-Sims TC, Khan M, Lang M, Huang MB, Bond VC, Powell MD. 2011. HIV Type 1 Nef is released from infected cells in CD45(+) microvesicles and is present in the plasma of HIV-infected individuals. AIDS Res Hum Retroviruses 27:167-78.


Appendix A.

Additional data for chapter 2

Table A1  Description of integrated host genes

<table>
<thead>
<tr>
<th>Nef</th>
<th>Nef variant</th>
<th>Host gene</th>
<th>Host gene description</th>
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<tr>
<td>SF2</td>
<td>IFT88</td>
<td>Intraflagellar transport protein 88 homolog</td>
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<td>SF2</td>
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<td>ANKRD11</td>
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<tr>
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<td>SAFB</td>
<td>Scaffold attachment factor B1</td>
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<td>Centrosome-associated protein 350</td>
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<td>Peptidyl-prolyl cis-trans isomerase-like 3</td>
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<td>UBR3</td>
<td>E3 ubiquitin-protein ligase</td>
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<td>Inositol hexakisphosphate kinase 2</td>
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<td>Ubiquitin-conjugating enzyme E2 G1</td>
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Functional