THE ROLE OF VIRAL RESERVOIRS IN HIV-1 INFECTION

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ABSTRACT

The major source of virus production during human immunodeficiency virus type 1 (HIV-1) infection is activated CD4 T-cells, although infection of some other cell types can also contribute to virus production. A viral reservoir is either a cell type or an anatomical site whose properties can result in the persistence of infectious virus for a longer time period than the primary source of virus production, and several different HIV-1 reservoirs are known to exist. The work presented in this thesis examines three different aspects of viral reservoirs in HIV-1 infection. The first part (Chapter 2) is an investigation of the role of long-lived virus-producing cells during antiretroviral therapy. Specifically, cell culture experiments were designed that have resulted in a further understanding of the inhibition of HIV-1 replication in viral reservoirs. The second and third parts of this thesis (Chapters 3 and 4) consider the role of latently infected CD4 T-cells in HIV-1 infection. Latently infected cells carry an HIV-1 genome that is integrated into the cellular chromatin and does not produce viruses, but that retains the capacity for infectious virus production in the future. These cells form the latent reservoir, which represents the major barrier to an HIV-1 cure and necessitates life-long antiretroviral therapy for infected individuals. The work presented in Chapter 3 demonstrates that it is possible to inhibit the establishment of latent infection *in vitro*, something that has not yet been achieved clinically. Chapter 4 considers the potential contribution of latent viruses to viral genetic diversity, and shows that latent viruses can contribute to the development of multidrug resistance. In summary, the work presented in this thesis provides for a greater understanding of the role of viral reservoirs in HIV-1 infection and of the ability of antiretroviral drugs to combat infection.

RÉSUMÉ

Les cellules T CD4 activées sont la principale source de virus pendant l'infection par le virus de l'immunodéficience humaine (VIH-1) même si d'autres cellules contribuent à la production virale. Un réservoir viral est un type de cellule ou un compartiment anatomique dont les propriétés permettent la persistance du virus infectieux pour plus longtemps que la source majeure de la production virale et le VIH-1 occupe plusieurs réservoirs. Dans cette thèse, nous examinons trois différents aspects des réservoirs du VIH-1. Dans la première partie (Chapitre 2), nous avons étudié le rôle des cellules à grande longévité qui produisent du virus dans la thérapie anti-rétrovirale. En particulier, nous avons étudié en culture cellulaire comment inhiber la réplication virale dans ces cellules. Dans la deuxième et troisième partie (Chapitres 3 et 4), nous avons étudié le rôle de la latence dans les cellules T CD4. Les cellules latentes contiennent le génome du VIH-1 intégré dans leur chromatine sans produire du virus. Néanmoins, ces cellules peuvent produire du virus infectieux dans le futur et sont un obstacle majeur contre la guérison des individus vivants avec le VIH, ce qui les oblige à prendre des médicaments pour toute leur vie. Dans le Chapitre 3, nous montrons qu'il est en théorie possible d'empêcher l'infection latente, ce qui n'a jamais été fait en clinique. Finalement, le Chapitre 4 étudie le rôle du virus latent dans la diversité génétique du VIH, et montre que les virus latents peuvent participés à l'émergence de la résistance contre plusieurs médicaments. En résumé, le travail de cette thèse contribue à une meilleure compréhension du rôle des réservoirs viraux dans l'infection au VIH-1.

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PREFACE

This thesis was written in accordance with McGill University's "Guidelines for preparation of a thesis." The format of this thesis conforms to the "Manuscript-based thesis" option.

Information detailing the manuscripts included in this thesis, as well as the contributions of co-authors, appears on the title page of each chapter.

All work described in this thesis is the result of original scholarship, and each chapter represents a distinct contribution to knowledge. Parts of Chapter 1 have been published in a review article; the work presented in Chapters 2 and 3 have been published; and the work presented in Chapter 4 in currently in preparation for submission.

Other manuscripts not included in this thesis, but to which a significant contribution was made by the candidate, include:

Kramer VG, Schader SM, Oliveira M, Colby-Germinario SP, Donahue DA, Singhroy DN, Tressler R, Sloan RD, Wainberg MA: Maraviroc and other HIV-1 entry inhibitors exhibit a class-specific redistribution effect that results in increased extracellular viral load. *Antimicrob. Agents Chemother.* 2012, 56:4154–4160.

Kuhl BD, Sloan RD, Donahue DA, Liang C, Wainberg MA: Vpu-mediated tetherin antagonism of ongoing HIV-1 infection in CD4(+) T-cells is not directly related to the extent of tetherin cell surface downmodulation. *Virology* 2011, 417:353–361.

Sloan RD, Kuhl BD, Donahue DA, Roland A, Bar-Magen T, Wainberg MA: Transcription of preintegrated HIV-1 cDNA modulates cell surface expression of major histocompatibility complex class I via Nef. *J. Virol.* 2011, 85:2828–2836.

Kuhl BD, Cheng V, Donahue DA, Sloan RD, Liang C, Wilkinson J, Wainberg MA:

The HIV-1 Vpu viroporin inhibitor BIT225 does not affect Vpu-mediated tetherin antagonism. *PLoS ONE* 2011, 6:e27660.

Kuhl BD, Sloan RD, Donahue DA, Bar-Magen T, Liang C, Wainberg MA: Tetherin restricts direct cell-to-cell infection of HIV-1. *Retrovirology* 2010, 7:115.

Bar-Magen T, Donahue DA, McDonough EI, Kuhl BD, Faltenbacher VH, Xu H, Michaud V, Sloan RD, Wainberg MA: HIV-1 subtype B and C integrase enzymes exhibit differential patterns of resistance to integrase inhibitors in biochemical assays. *AIDS* 2010, 24:2171–2179.

Bar-Magen T, Sloan RD, Donahue DA, Kuhl BD, Zabeida A, Xu H, Oliveira M, Hazuda DJ, Wainberg MA: Identification of novel mutations responsible for resistance to MK-2048, a second-generation HIV-1 integrase inhibitor. *J. Virol.* 2010, 84:9210–9216.

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Sloan RD, Donahue DA, Kuhl BD, Bar-Magen T, Wainberg MA: Expression of Nef from unintegrated HIV-1 DNA downregulates cell surface CXCR4 and CCR5 on T-lymphocytes. *Retrovirology* 2010, 7:44.

Bar-Magen T, Sloan RD, Faltenbacher VH, Donahue DA, Kuhl BD, Oliveira M, Xu H, Wainberg MA: Comparative biochemical analysis of HIV-1 subtype B and C integrase enzymes. *Retrovirology* 2009, 6:103.

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Chapter 1

Introduction

Sections of this chapter were adapted from the following review article:

Donahue DA and Wainberg MA: Cellular and molecular mechanisms involved in the establishment of HIV-1 latency. *Retrovirology* 2013, 10:11.

This review article was designed and written by myself under the supervision of Dr. Mark Wainberg, who offered suggestions for revisions to the manuscript.

1.1 HIV & AIDS

Human immunodeficiency virus type 1 (HIV-1) was discovered in 1983 [1], and was soon identified as the causative agent of acquired immunodeficiency syndrome (AIDS) [2-4]. The sequence of the HIV-1 genome was reported in 1985 [5-7], and its extraordinary genetic diversity was quickly realized [8-10].

HIV-1, and the closely related HIV-2, are retroviruses belonging to the *Lentivirus* genus. As a retrovirus, HIV-1 carries two copies of its positive-sense single-stranded RNA genome. HIV-1 is divided into groups M, N, O and P, each of which resulted from an independent cross-species transmission of a simian immunodeficiency virus (SIV) from its natural host. The worldwide HIV-1 pandemic is comprised of group M strains. Group M is subdivided into nine subtypes (A-D, F-H, and J-K) and >50 circulating recombinant forms (CRFs) (reviewed in [11]).

Globally there are approximately 34 million people living with HIV, and around this many AIDS-related deaths have occurred since the start of the pandemic. Worldwide, most infections are found in sub-Saharan Africa, and developing countries experience the highest rates of mortality due to AIDS. Although antiretroviral therapy is extremely effective if available, millions of infected individuals do not currently have access (reviewed in [12]).

1.2 HIV-1 GENOME AND VIRION STRUCTURE

The HIV-1 genome is approximately 9700 nucleotides in length, and encodes 9 genes which themselves encode a total of fifteen proteins (Figure 1.1). In its integrated proviral DNA form, the HIV-1 genome is flanked by two complete long-terminal repeats (LTRs). Each LTR includes a U3, R, and U5 region, with U3 of the 5' LTR serving as the viral promoter. The *gag* and *env* genes encode structural proteins. Proteolytic processing of the Gag polyprotein by viral protease results in the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins, while processing of the Envelope precursor gp160 by a cellular

protease yields gp120 and gp41. The *pol* gene encodes the three viral enzymes, protease (PR), reverse transcriptase (RT) and integrase (IN). Occasional frameshifting at the end of *gag* results in translation of a Gagpol polyprotein, which yields PR, RT and IN (in addition to Gag proteins) after proteolytic processing. The *tat* and *rev* genes encode proteins of the same names that are required for viral gene expression, while the accessory genes *vif*, *vpr*, *vpu* and *nef* encode proteins of the same names that carry out a diverse range of functions (reviewed in [13])

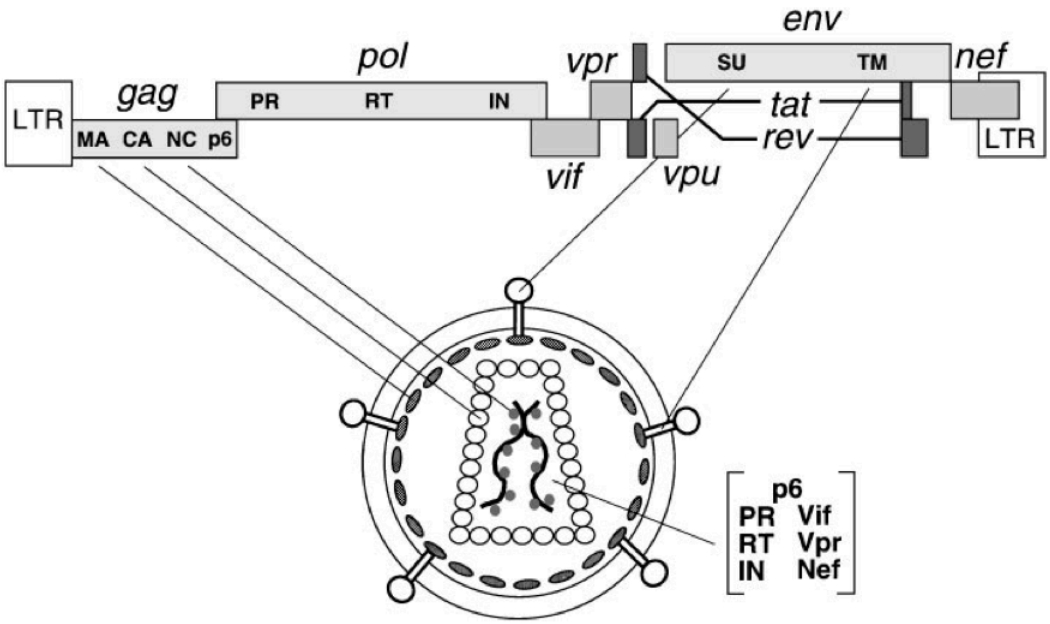

Figure 1.1. HIV-1 Genome and Virion Structure.

Schematic representation of the HIV-1 genome and virion structure, depicting the nine genes and fifteen proteins that these genes encode, as well as the overall structure of the mature virus. SU (surface) = gp120; TM (transmembrane) = gp41. Figure adapted from [13].

1.3 HIV-1 REPLICATION CYCLE

This section contains a basic description of the HIV-1 replication cycle. The emphasis is on the virological factors involved, and certain cellular factors are

discussed where appropriate. HIV-1 infects cells that express CD4, with CD4 T-cells being its major target. Other cell types that can be infected by HIV-1 include monocytes/macrophages, dendritic cells (*trans* infection), immature CD4+CD8+ thymocytes, and hematopoietic progenitor cells. The HIV-1 replication cycle is shown in Figure 1.2, which also highlights drug targets (discussed in section 1.4) and cellular restriction factors. While restriction factors are not discussed in detail here, they are briefly described below in the relevant sections of the viral replication cycle.

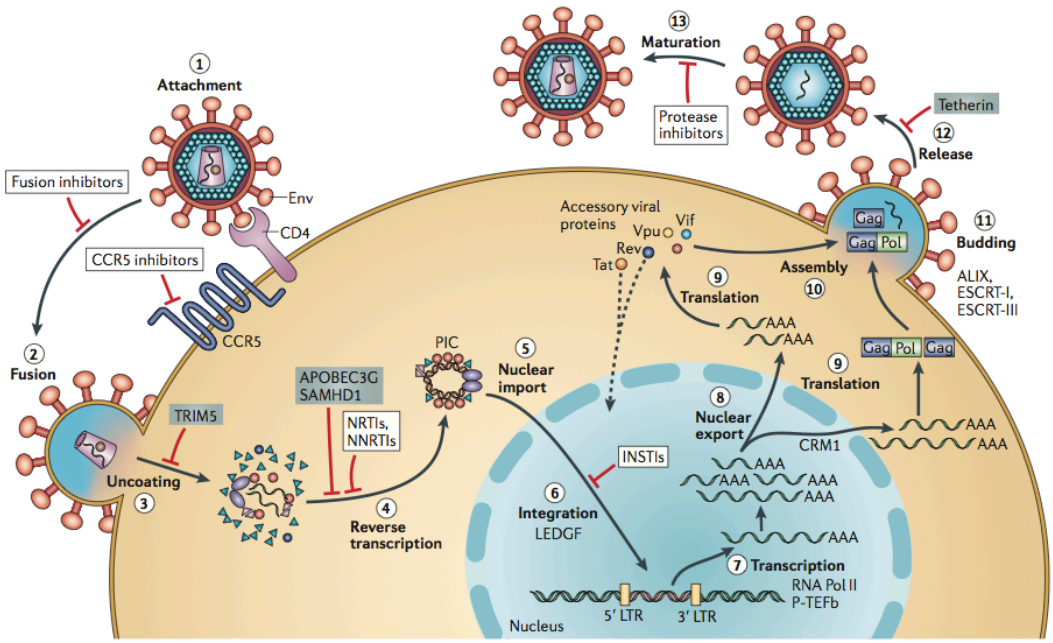

Figure 1.2. HIV-1 replication cycle, including drug targets and restriction factors.

Select host factors required for viral replication are shown (*e.g.* LEDGF), and are discussed in the relevant section of the replication cycle. Drug targets (white boxes) and restriction factors (dark boxes) are indicated. PIC, pre-integration complex. NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside

reverse transcriptase inhibitor; INSTI, integrase strand transfer inhibitor. The restriction factors TRIM5, APOBEC3G, SAMHD1 and tetherin are discussed below. Figure adapted from [14].

1.3.1 Entry to integration

While entry is classically depicted as occuring at the cell surface, it is also likely that entry can occur through endosomal pathways (that still require the processes described here). The HIV-1 replication cycle begins when gp120 of an Envelope trimer attaches to the cellular protein CD4, which functions as the viral receptor. gp120:CD4 interaction leads to conformational changes that expose the coreceptor binding site, permitting gp120 to interact with one of its two coreceptors, CCR5 or CXCR4. This triggers the gp41 “fusion peptide” to be inserted into the cellular plasma membrane. The viral and cellular membranes fuse, with formation of the gp41 six-helix bundle, and the viral core is released into the cytoplasm [15, 16].

Once the viral core has been released into the cytoplasm, the processes of uncoating and reverse transcription occur. While uncoating is traditionally depicted to occur shortly after cytoplasmic delivery of the viral core, followed by reverse transcription, there is mounting evidence for simultaneous temporal and/or physical relationships between these two processes. In fact, uncoating might accompany the transition from a reverse transcription complex (RTC) to a preintegration complex (PIC), at least for some virions [17]. Regardless of when and where uncoating occurs, this process involves the ordered removal of viral capsid proteins from the viral core, exposing the RTC and/or PIC. The host cell restriction factor TRIM5 acts by interfering with uncoating in a species-specific manner. Thus, human TRIM5 does not prevent infection by HIV-1 (since the HIV-1 capsid is largely “resistant” to recognition by human TRIM5), whereas TRIM5 from other primates does block HIV-1 infection. The precise mechanism of TRIM5 action is not yet fully defined [17].

Reverse transcription is orchestrated by the viral reverse transcriptase, and is a complex series of events that results in the conversion of the viral single-stranded positive-sense genomic RNA into a double-stranded DNA (dsDNA). Reverse transcription begins from a cellular tRNA annealed to the viral primer binding site. Two obligatory strand transfer events are required for the production of a complete viral LTR at each end of the resulting proviral DNA. As discussed in greater detail in Chapter 4, RT switches between the two genomic RNA templates several times during reverse transcription (in addition to the two obligatory strand transfer events). During this process, the RTC travels through the cytoplasm towards the nuclear pore [18]. Two host cell restriction factors can interfere with reverse transcription. APOBEC3G, which is packaged into assembling virions in the absence of Vif, can directly interfere with reverse transcription, and can cause cytidine deamination which ultimately hypermutates the resulting proviral DNA through the introduction of G to A mutations [19]. In addition, the restriction factor SAMHD1 depletes cellular dNTP pools, thus interfering with reverse transcription in myeloid-lineage cells (dendritic cells and monocytes/macrophages) and in resting CD4 T-cells. Only Vpx-encoding lentiviruses (*e.g.* many SIVs) can efficiently avoid the block induced by SAMHD1, by inducing degradation of the restriction factor [20].

Following uncoating and reverse transcription, the PIC enters the nucleus by transiting the nuclear pore. Nuclear import is a complex process that remains incompletely understood, and a number of cellular and viral components contribute to this process [21, 22]. Integration is carried out by the viral integrase, in concert with the cellular protein LEDGF/p75. Integration is composed of two enzymatic steps carried out by integrase: 3' processing removes two nucleotides from each end of the linear dsDNA, and strand transfer then covalently attaches the viral dsDNA into the host cell's chromosomal DNA. LEDGF targets integration into specific regions of the genome, and is responsible for the preferential integration of HIV-1 DNA into the introns of actively

expressed host genes [14, 23, 24]. When integration does not occur, the proviral DNA exists in the nucleus as linear unintegrated DNA, 1-LTR circles, or 2-LTR circles. These DNA forms are not capable of producing new viral particles, but can express some early viral gene products [25].

1.3.2 Gene expression

1.3.2.1 Transcriptional elongation control by Tat

Productive HIV-1 gene expression requires the viral protein Tat (transactivator of transcription). In the absence of Tat, transcription factors such as NF-kB, NFAT, and Sp1 bind the 5' LTR and transcription initiates normally. However, only short, abortive viral transcripts are produced due to RNA polymerase II (RNAPII) pausing shortly after promoter clearance. Rarely, full-length transcripts are expressed, which are then multiply spliced, and the first molecules of Tat are produced. Tat is a small, positively charged protein of approximately 101 amino acids, expressed from two exons. The first, 72 amino acid exon encodes all functions required for transcription, while the second exon is dispensable *in vitro* but carries out additional, poorly understood functions *in vivo* (Figure 1.3, and reviewed in [26-28]).

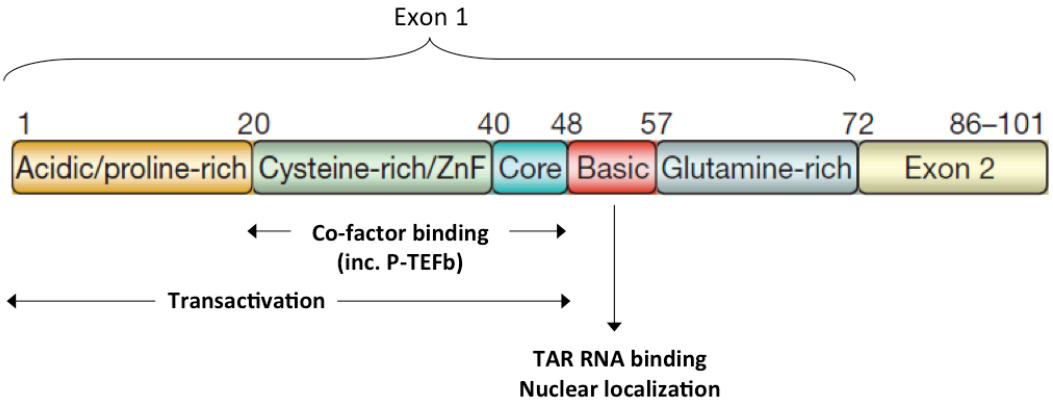

Figure 1.3. Schematic representation of HIV-1 Tat protein.

The various domains and functions of Tat are shown, and the corresponding amino acid numbers are indicated. Adapted from [29].

Once Tat is produced, it enters the nucleus and functions as a powerful activator of viral transcription. Tat controls transcription at the level of RNAPII elongation, as opposed to the vast majority of eukaryotic transcription factors that control the initiation of transcription by binding to DNA sequences. The first 59 nucleotides of each viral transcript form a stem-loop structure known as the TAR (transactivation responsive) RNA. Tat recruits a super-elongation complex (SEC) that includes many factors including the critically important positive transcription elongation factor b (P-TEFb), which itself is composed of Cyclin T1 (CycT1) and cyclin-dependent kinase 9 (CDK9). The SEC also includes the elongation factor ELL2, which increases the catalytic rate of RNAPII. Tat-P-TEFb-TAR interaction leads to several phosphorylation events mediated by the kinase CDK9, that together convert the paused RNAPII complex into a highly processive form. Phosphorylation of the negative elongation factors DSIF and NELF are key steps in this process; DSIF becomes a positive elongation factor and NELF dissociates from the complex. In addition, serine 2 of the RNAPII C-terminal domain heptapeptide repeat is phosphorylated by CDK9, thereby allowing additional factors to interact with the complex and contribute to productive elongation (reviewed in [26, 27]).

Tat activity is also regulated by a number of post-translational modifications of Tat itself, including phosphorylation, non-proteosome-associated polyubiquitination, methylation, and acetylation. Although many of these modifications are poorly understood, cycles of acetylation and deacetylation at lysines (K) 50 and 51 play an important role. Shortly after Tat interacts with P-TEFb/TAR, it is K50/K51-acetylated by the cellular acetylases p300/CBP and GCN5. This neutralizes the highly positively charged basic domain (Figure 1.3) of Tat, decreasing its electrostatic interaction with the TAR RNA and thus likely permitting Tat to leave TAR and travel with the elongating RNAPII complex. At the end of each round of transcription, Tat is deacetylated by the cellular

deacetylase SIRT1, which likely allows individual Tat molecules to engage in further rounds of transcription. The net result of these post-translational modifications to RNAPII complexes and to Tat itself, is efficient, high-level viral gene expression [30], as shown in Figure 1.4.

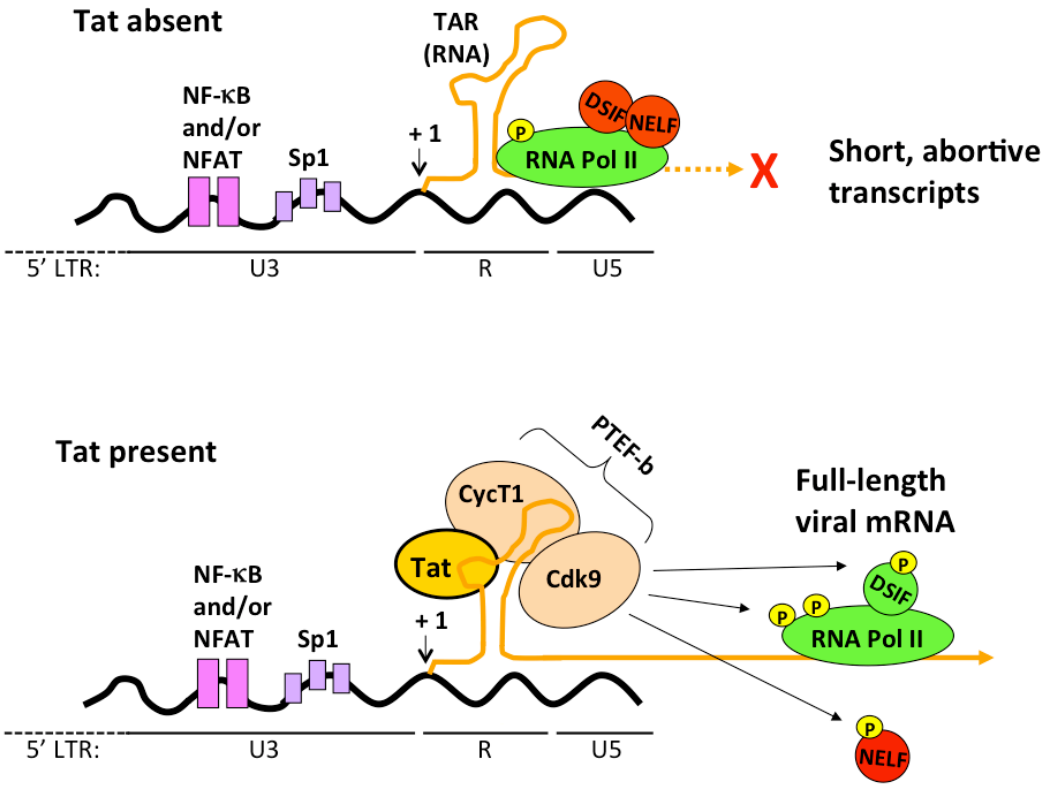

Figure 1.4. The role of Tat in the control of transcriptional elongation.

See text for details. NF-kB, NFAT and Sp1 = transcription factors; DSIF and NELF = negative elongation factors; P-TEFb = positive transcription elongation factor b; CycT1 = cyclin T1; Cdk9 = cyclin-dependent kinase 9.

1.3.2.2 mRNA splicing and nuclear export

All viral transcripts are initially full-length (9 kb) unspliced (US) viral mRNAs, originating from the 5' LTR and terminating at the 3' LTR polyadenylation site.

Due to the presence of various splice donor and splice acceptor sites, all unspliced transcripts are initially, by default, multiply spliced (MS) to yield a class of 1.8 kb viral mRNAs encoding Tat, Rev and Nef. These transcripts exit the nucleus through as regular cellular mRNAs and are then translated to produce Tat, Rev and Nef proteins. Tat and Rev then enter the nucleus, with Tat functioning as described in the section above. Multiple Rev molecules now in the nucleus bind the Rev-responsive element (RRE), a complex RNA secondary structure present in all US and singly spliced (SS) viral mRNAs. Through interaction with the cellular factor CRM1, as well as RanGTP, Rev (which contains both nuclear export and nuclear import signals) allows the nuclear export of SS and US viral mRNAs [31]. Alternative splicing thus allows temporal regulation of viral protein expression [32]. MS mRNA are produced first, and permit efficient viral gene expression (Tat and Rev), while Nef engages in numerous activities to facilitate viral replication including downregulation of CD4, coreceptors, and MHC molecules [33]. SS mRNA produces Env, as well as Vif, Vpr and Vpu. Vif functions to block incorporation of APOBEC3G into assembling virions; Vpr is packaged into virions and has numerous roles during additional rounds of replication including G2 cell-cycle arrest and PIC nuclear import in non-dividing cells; and Vpu functions to prevent CD4 association with Env before the latter reaches the cell surface, and also antagonizes the restriction factor tetherin [34]. US mRNA produces the Gag and Gagpol polyproteins, which are processed to yield structural and enzymatic viral components. In addition, US mRNA serves as the genomic RNA for assembling virions (reviewed in [35, 36]).

1.3.3 Assembly and release

Assembly occurs at cholesterol-rich regions of the plasma membrane, where Gag polyproteins form the “shells” of new virions. A number of viral and cellular components are specifically recruited to nascent virions, including two genomic RNA molecules. To orchestrate budding of assembling virions, HIV-1 usurps components of the cellular ESCRT pathway, which is normally involved in cell

membrane remodeling and fission events such as vesicle budding into endosomes. The p6 component of Gag encodes two late domains: PTAP, which interacts with the ESCRT-I component TSG101; and YPLTSL, which interacts with ALIX. ESCRT-III proteins orchestrate scission of the budding viral membrane from the host cell membrane through a mechanism that remains incompletely understood [35, 37, 38]. The restriction factor tetherin functions during viral budding/release, by tethering Vpu-deficient virions to the cell surface. Following release of immature virions, the viral protease within Gagpol polyproteins proteolytically cleaves both the Gag and Gagpol precursors to yield individual MA, CA, NC, p6, PR, RT and IN proteins (Figure 1.1), in a process known as virion maturation.

1.4 ANTIRETROVIRAL DRUGS

1.4.1 Drug targets

Clinically approved antiretroviral drugs target multiple stages of the viral replication cycle, and include ~30 compounds. The major classes of clinically approved drugs include entry inhibitors (sometimes considered as two separate classes: coreceptor antagonists and fusion inhibitors), reverse transcriptase inhibitors (both nucleoside and non-nucleoside inhibitors), integrase inhibitors, and protease inhibitors. Additional stages of the replication cycle, such as integrase:LEDGF/p75 interaction and virion maturation are the target of preclinical drugs.

Entry inhibitors include the CCR5 coreceptor antagonist maraviroc (MVC), and the fusion inhibitor enfuvirtide (T-20). The non-clinical CXCR4 antagonist AMD3100 acts in an analogous manner to MVC, and is of use experimentally (Chapter 2). Reverse transcriptase inhibitors are composed of nucleoside/nucleotide RT inhibitors (NRTIs), as well as non-nucleoside RT inhibitors (NNRTIs). NRTIs relevant to the work described in this thesis include lamivudine (3TC) and emtracitibine (FTC), while relevant NNRTIs include

nevirapine (NVP) and efavirenz (EFV). Integrase inhibitors function by blocking the strand transfer activity of integrase. Clinically approved integrase inhibitors include both raltegravir (RAL) and elvitegravir (EVG), and the non-clinical IN inhibitor MK-2048 is also relevant to the work described in Chapter 2. Protease inhibitors of relevance include lopinavir (LPV) and darunavir (DRV) (reviewed in [39]).

1.4.2 Highly active antiretroviral therapy (HAART)

Highly active antiretroviral therapy (HAART) became available in 1996 through the combination of three different antiretroviral drugs in one treatment regimen. The addition of a protease inhibitor to the RT inhibitors already available – and thus targeting multiple stages of viral replication simultaneously – substantially increased the effectiveness of therapy. There are currently more than thirty approved antiretroviral drugs targeting multiple classes of viral replication, as discussed above and shown in Figure 1.2. Although HIV-1 develops resistance against many if not all antiretroviral drugs given sufficient time, the large number of available treatment options today, at least in developed countries, ensures that HIV-1-infected individuals can remain relatively healthy for decades (reviewed in [39, 40]). The dynamics of viral load decay under HAART and what it can reveal about viral reservoirs is discussed in the following section.

1.5 PHASES OF VIRAL LOAD DECAY UNDER HAART

The major source of virus production during HIV-1 infection is activated CD4 T-cells. Infection of other cell types, including resting CD4 T-cells, monocytes/macrophages, dendritic cells, and hematopoietic progenitor cells, also contributes to virus production. A viral reservoir can be considered to be either a cell type or an anatomical site whose properties result in the persistence of infectious virus for a longer timeframe than for the major source of virus production [41]. Thus, all cell types infected by HIV-1, other than activated CD4 T-cells, can be considered viral reservoirs. In the era of HAART, which prevents

almost all ongoing viral replication, a clinically relevant viral reservoir is one in which replication-competent virus can persist for years [41]. The onset of HAART in 1996 led to some fundamental discoveries about HIV-1 reservoirs that are of central importance today, as discussed in the following sections.

1.5.1 First and second phases of viral load decay

The major clinical measure of HIV-1 infection, and of treatment efficacy, is measurement of viral RNA levels in the blood of patients, which is known as the viral load. HAART is very effective at stopping nearly all ongoing viral replication almost immediately upon treatment initiation, but does not affect the release of virus from cells that are already infected. Thus, the decline in viral load that occurs after treatment initiation reflects the deaths of cells that were already infected before treatment began. In 1997 it was reported that in patients receiving HAART, viral load decayed in a biphasic manner [42]; this is depicted schematically in Figure 1.5. The patients' viral loads dropped rapidly in the first two weeks following treatment initiation (to ~1% of initial levels), but then began to decay at a slower rate. The rapid first phase of decay was attributed to the loss of free plasma virions, and to the deaths of productively infected, activated CD4 T-cells. This is due to the short half lives of free virions (<6 hrs) and productively infected cells (~1 day), which are rapidly lost due to viral cytopathic effects, apoptosis, and immune-mediated clearance [43].

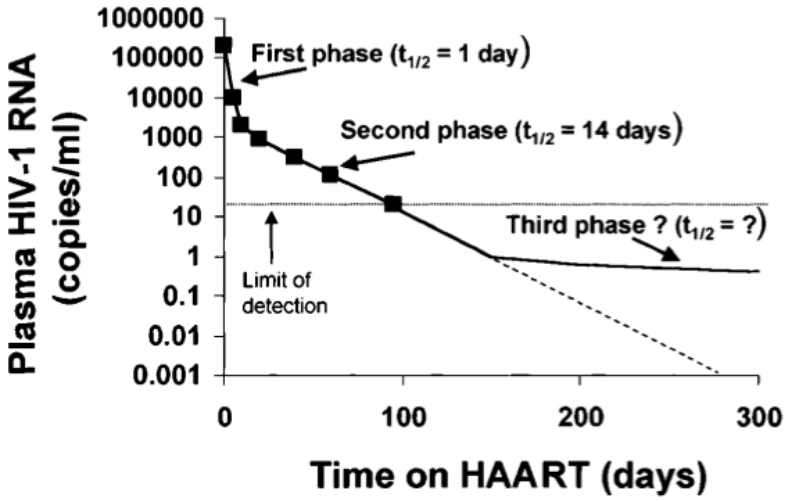

Figure 1.5. Viral load decay during HAART, as understood in 1998.

A biphasic decay of viral load during HAART was known to occur, while a hypothetical third phase is also depicted. Adapted from [44].

The slower second phase of viral load decay indicated virus production or release from a different cellular source. Mathematical analysis suggested that long-lived infected cells were releasing virus for several weeks during the second phase, and that these infected cells had a half-life of ~2 weeks. Although the cells responsible for virus release during the second phase were not known, they were presume to include one or more of the following: resting CD4 T-cells with unintegrated DNA (preintegration latency; discussed below), virions captured by follicular dendritic cells, or infected macrophages (Figure 1.6) [44]. The presence of second phase sources of viremia implied that, if no other reservoirs existed, eradication of HIV-1 from infected individuals might be possible after 2-3 years of continuous HAART [42]. However, between 1995 and 1997 it was already becoming apparent that an extremely long-lived latent reservoir of HIV-1 existed in resting memory CD4 T-cells [45-49].

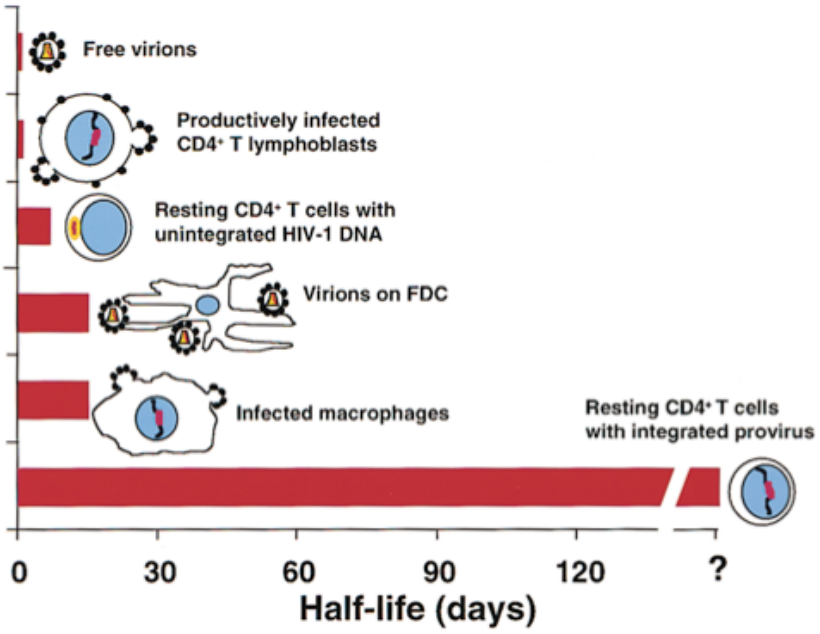

Figure 1.6. Potential sources of virus during HAART.

The half-lives of first, second and third phase sources of virus release are shown. FDC = follicular dendritic cell. Adapted from [44].

1.5.2 Third and fourth phases of viral load decay

By 1999 it was reported that, following the second phase of viral load decay, an extremely slow third phase of decay was detectable [50]. Using more sensitive viral load assays, viremia could be measured in all infected patients on long-term HAART, which either decayed slowly or not at all. The virus present in the third phase was due to post-integration latency (see next section), and specifically, to the activation of latently infected cells that went on to release infectious virus. The half-life of this reservoir was on the order of 44 months, which carried the massive implication that HAART would be required for the lifetime of infected individuals [50]. That is, even when all ongoing replication is halted, the latent reservoir would outlast a human lifetime (Figure 1.7).

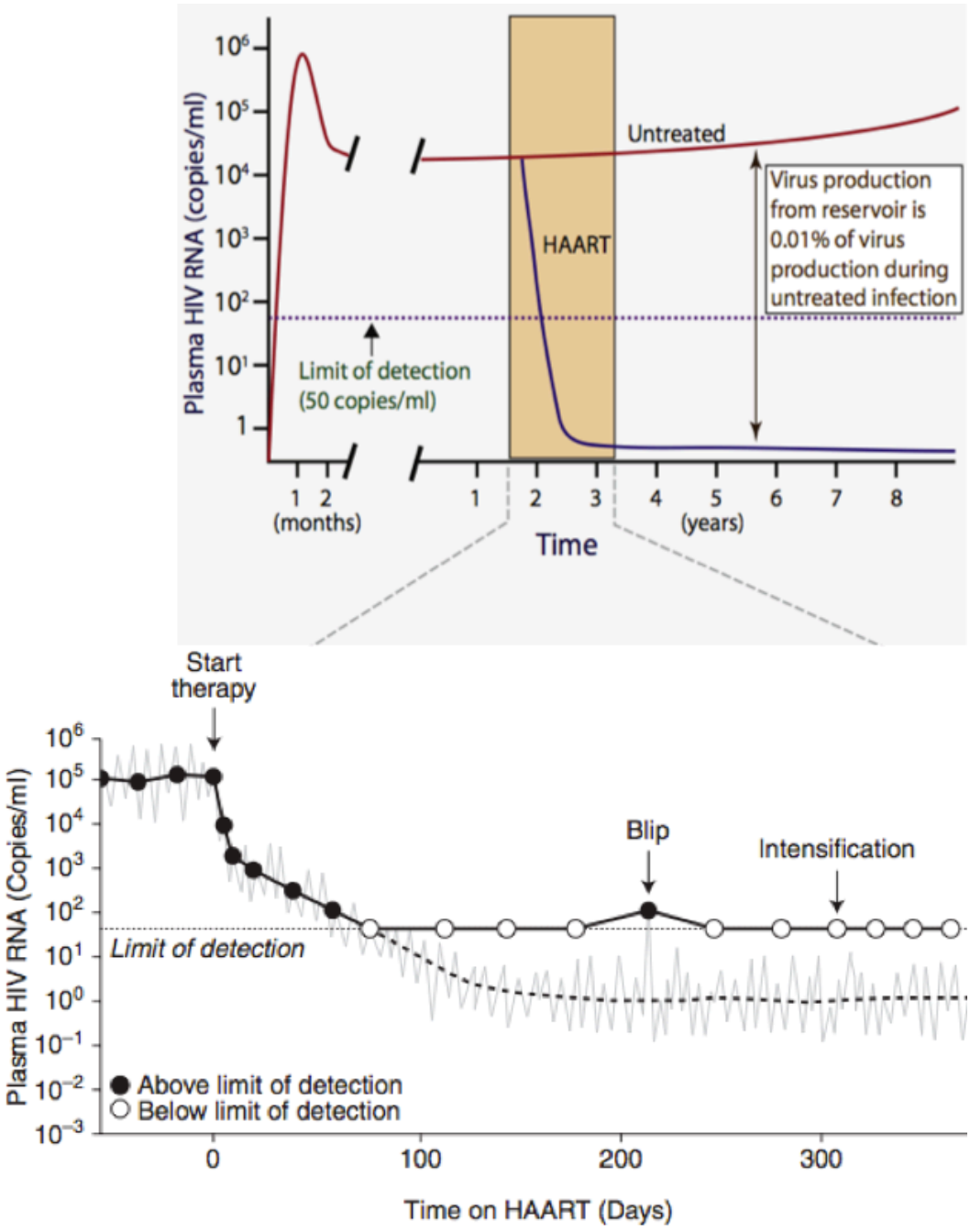

Figure 1.7. Viral load decay during HAART, as understood in 2013.

(Top) Levels of viral RNA over time in untreated or HAART-treated individuals. (Bottom) First, second, and third/fourth phases of decay during HAART as revealed with sensitive viral load assays. The limit of detection indicated (50 copies/ml) is for standard clinical assays. Blips indicate transient release of virus from third/fourth-phase sources. Treatment intensification studies indicate that

HAART has already reached its maximum potency, and that adding more drugs does not inhibit additional rounds of replication. In other words, third/fourth phase viremia is not due to ongoing replication, but to release of virus from latent reservoirs. Figure adapted from [41] (top) and [43] (bottom).

The latent reservoir is responsible for the viremia observed during the third phase of decay, and is composed primarily of resting memory CD4 T-cells that carry integrated proviruses that are not replicating, but that retain the capacity for replication given appropriate signals (discussed in detail in section 1.6). Recent data suggest that the third phase of viral load decay is in fact itself two distinct phases [51-53]. The third phase decays very slowly, whereas the fourth phase appears to be stable, *i.e.* it does not decay at all. Although both phases almost certainly represent latently infected resting CD4 T-cells, the differences between these two phases are poorly understood, and might represent different subsets of memory CD4 T-cells, or different mechanisms of latency (such as homeostatic proliferation of latently infected cells; see section 1.6) [54, 55].

It should now be clear that the dynamics of viral load decay following the initiation of HAART reveal critically important information about the cellular sources that enable viral persistence in the face of therapy. The work presented in Chapter 2 is focused on understanding second-phase sources of viremia during HAART, while Chapters 3 and 4 are focused on third/fourth phases of viremia. Given that the majority of the work presented in this thesis is focused on latently infected CD4 T-cells – the third/fourth phases sources of viremia, which represent the largest barrier to eradication of HIV from infected individuals – the remainder of this chapter contains a detailed discussion of HIV-1 latency.

1.5.3 Preintegration latency *vs* postintegration latency

As alluded to above, two forms of viral latency exist: pre- and post- integration latency. Preintegration latency results mainly from infection of resting CD4 T-

cells, which is much less efficient than infection of activated cells. Specifically, preintegration latency refers to a labile state after entry of the virus into the cell but prior to integration. The cell is not yet irreversibly infected, but the pre-integrated virus retains the capacity to integrate given sufficient time or activation signals. Because of the short half-life of preintegration complexes (several days), this form of latency is generally considered to be of relatively minor clinical significance [56-59].

In contrast, postintegration latency refers to an integrated provirus that exists in a latent state. Unless otherwise specified, the term "latency" always refers to postintegration latency with respect to HIV-1. Postintegration latency is discussed in detail in the following section, and is the focus of Chapters 3 and 4 of this thesis.

1.6 HIV-1 LATENCY

Latently infected cells represent the major obstacle to either a sterilizing or a functional HIV-1 cure. HIV-1 latency can be defined as a reversibly nonproductive infection of a cell [41], which is usually interpreted to refer to an integrated provirus that is replication-competent but transcriptionally silent. In light of recent evidence, this definition might be expanded to include proviruses that express some but not all gene products in the absence of virion production [51, 60-62]. The latent reservoir is established very early after infection [53, 63], and reactivation of latently infected cells serves as a major source of viral rebound upon treatment failure [64, 65]. As described above, recent studies of the dynamics of viral load decay have shown the presence of two kinetically distinct latent reservoirs, *i.e.* the sources of plasma viremia during the third and fourth phases of decay [52, 53, 66], potentially representing different memory CD4 T-cell subsets. Multiple approaches to reactivation and depletion of the latent reservoir have been attempted clinically (discussed below, and reviewed in [67, 68]), and these efforts aim to reactivate latently infected cells so as to render

them susceptible to viral cytopathic effects, an antiviral immune response, or other means of targeted cell killing [69, 70]. However, complete depletion of the latent reservoir remains a long-term goal.

1.6.1 Models to study HIV-1 latency

HIV-1 latency can be studied using a variety of models, from clonal cell lines to latently infected resting memory T-cells from patients on suppressive HAART isolated *ex vivo*. A state of virological latency was first characterized for HIV-1 using the clonal cell lines U1 and ACH-2. U1 cells contain two integrated proviruses, one of which has an attenuated *tat* mutation, and the other of which does not produce Tat. ACH-2 cells contain a single integrated provirus that carries a TAR mutation. Similarly, the JΔK cell line is derived from Jurkat cells, and carries an integrated provirus with an NF-kB site mutation. For all three of these clonal cell lines, treatment with various activating stimuli (*e.g.* TNF-α or PMA) induces viral gene expression [71]. J-LAT cells are a series of related clonal cell lines derived from Jurkat cells, each carrying an integrated reporter cassette (LTR-Tat-GFP-LTR) or an integrated, replication-defective reporter virus. These cells were isolated by sorting of single cells that were GFP-negative following infection but produced GFP upon cellular activation [72]. Clonal cell lines such as these have advantages in that they can be used to study the effects of specific integration sites, but at the same time, they are limited for that very reason.

Several Jurkat cell latency models have been reported in which latency is established at the population level for each experiment, as opposed to clonal cell lines with single integration sites. In these systems, integrated proviruses are present at hundreds or thousands of distinct chromosomal locations, allowing the study of latency across the full range of HIV-1 integration site preferences [73, 74]. A novel population-level model of HIV-1 latency establishment and reactivation in Jurkat cells was established during the course of this thesis work [75], and is described in Chapters 3 and 4.

Several primary cell models of HIV-1 latency have now been described. It is noteworthy that these have only been made available in the past five years or so. Although several major distinctions exist, these models can be grouped into two general categories. The first involve direct infection of resting CD4 T-cells, while the second involve activation of CD4 T-cells that are infected and allowed to return to a resting state (Table 1.1, and reviewed in [71, 76-80]). These models are discussed in greater detail below, with respect to pathways of latency establishment. HIV-1 latency can also be studied using both humanized mouse models of HIV-1 latency [81] and macaque models of SIV latency [82], as described in section 1.6.3.3.

Table 1.1. Primary cell models of HIV-1 latency.

Model	Description	Naïve	Central Memory	Effector Memory	*In vitro* stimulation	Enough cell for screening	Longevity
Burke/Zack	Thymoctyes	Yes[a]	Yes	Yes	Yes	No	No
Sahu/Cloyd & Tyagi/Karn	Stimulated na ve cells cultured on feeder cells	No	Yes[a]	No	Yes	No	Yes
Marini/Romerio	DC activated cells cultured in IL-7	No	Yes[a]	No	Yes	No	Yes
Bosque/Planelles	Na ve cells primed Cultured in IL-2	No	Yes[a]	Yes	Yes	Yes	Yes
Yang/Siliciano	BCL-2 transduced cells	No	No	Yes[a]	Yes	Yes	Yes
Swiggard/O'Doherty[b]	Direct infection resting cells	Yes	Yes	Yes	No	No	No
Saleh/Lewin[b]	CCR7 stimulation resting cells	Yes	Yes	Yes	Yes	Yes	No

a = majority of population.
b = All infections occurred after activation except Swiggard and Saleh.
Different *in vitro* models of latency are listed by the authors who developed them, followed by a description of the model and the types of cells present in the system (yes represents the presence of the cell in the system). Whether the system utilizes *in vitro* stimulation before infection is also listed. Finally, the amount of cells produced is compared followed by whether or not the system can be maintained over long periods of time (longevity).

The authors who described each model are indicated, as are the CD4 T-cell subsets included in each model (naïve, central memory and effector memory CD4 T-cells). DC = dendritic cells; IL = interleukin; BCL-2 = anti-apoptotic molecule; CCR7 = chemokine receptor 7. Table adapted from [76].

1.6.2 Establishment of HIV-1 latency

Although much attention is deservedly paid to defining how latency is maintained and how latent viruses can be reactivated, the mechanisms involved in the establishment of latency are incompletely understood. Given that the latent reservoir can be replenished during infection [83, 84], a deeper knowledge of how latency is established would be invaluable.

1.6.3 Establishment of HIV-1 latency at the cellular level

Although the pathways leading to latent virus reactivation can be studied *ex vivo*,

it is not possible to study the establishment of latency in this manner, since by definition latency has already been established in any latently infected cells that can be isolated from an infected individual. Nonetheless, studies that investigate which subsets of resting cells harbour integrated virus in patients can be instructive, since knowledge of cellular physiology can shed light on how latent infection might have been established in a given cell type. Latently infected resting memory CD4 T-cells form the largest reservoir and represent the reservoir of greatest clinical importance due to their long lifespan [41]. Although it is likely that latency can occur in other cell types (reviewed in [41, 85-87]), this section primarily focuses on the establishment of latency in CD4 T-cells.

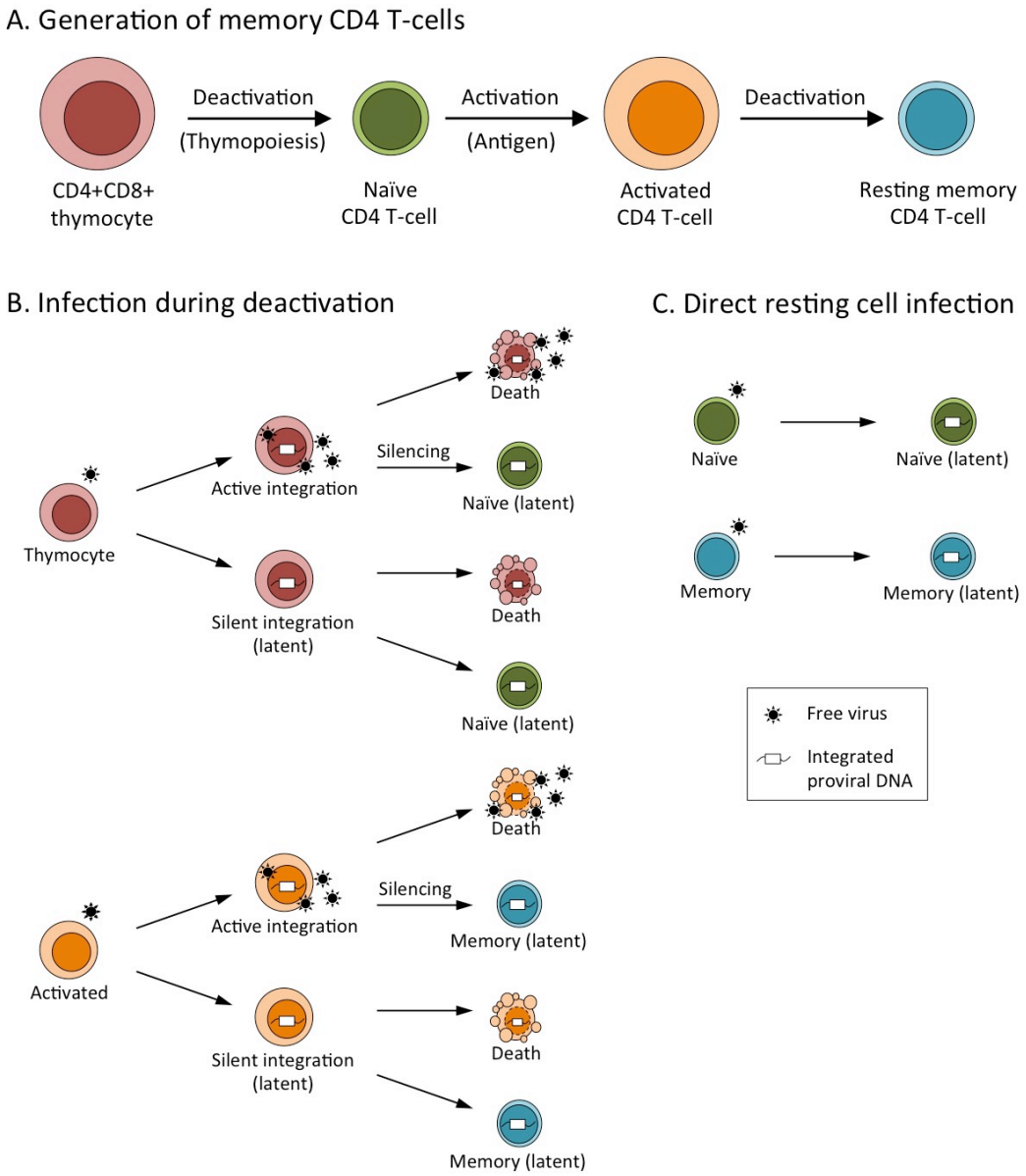

Figure 1.8. Cellular pathways of the establishment of HIV-1 latency in CD4 T-cells.

(A) Generation of memory CD4 T-cells. Transcriptionally active CD4+CD8+ (double positive) thymocytes transition to a resting state upon completion of thymopoiesis to become resting naïve CD4 T-cells. Naïve cells are activated upon encounter with antigen-bearing dendritic cells and undergo rapid clonal expansion. A small fraction of activated CD4 T-cells survive and transition to a

resting state, to become resting memory CD4 T-cells. (B) Infection during deactivation. Infection of an activated thymocyte can result in active or silent integration, and latency can be established upon the transition to a naïve CD4 T-cell. Infection of an activated CD4 T-cell can result in either active or silent integration, and latency can be established upon the transition to a resting memory CD4 T-cell. Note that for silent integration into an activated thymocyte or an activated CD4 T-cell, latency has already been established at the virological level. Due to the rapid deaths of activated cells, only cells which transition to a resting state represent clinically relevant latent infections. (C) Direct resting cell infection. Infection of a naïve CD4 T-cell, or of a resting memory CD4 T-cell, results in silent integration, *i.e.,* latency. Note that the relative contributions of the pathways shown here are not known.

1.6.3.1 Multiple CD4 T-cell subsets

Naïve CD4 T-cells are activated by interaction with dendritic cells (DC) that present an appropriate antigen. These activated T-cells then rapidly proliferate and differentiate into several subsets of effectors including Th1, Th2, Th17 and inducible regulatory T-cells [88]. While the majority of effector cells rapidly die, a small minority survive and undergo a transition to a resting state as memory CD4 T-cells. Memory CD4 T-cells, which provide for an enhanced immune response upon future encounter with the same antigen, are likely derived from all effector subsets [89]. In addition, memory CD4 T-cells are themselves composed of several subsets that probably represent a gradient of separate maturational stages [90]. Central memory cells (T_{CM}) migrate to secondary lymphoid organs where they can be activated by DCs to generate multiple waves of secondary effector cells. Effector memory cells (T_{EM}) are likely derived from T_{CM}, and are found in peripheral tissues, where they can act almost immediately as secondary effectors upon activation at sites of inflammation. Transitional memory cells (T_{TM}) represent an intermediate cell type that possess a phenotype intermediary between T_{CM} and T_{EM} [90-93]. Thus, the term “activated” CD4 T-cell can refer to

either a primary effector cell that resulted from activation of a naïve cell, or to a secondary effector cell that resulted from activation of a memory cell. Similarly, the term "resting" CD4 T-cell can either refer to a naïve cell or to a memory cell. Resting cells can be distinguished from activated cells by their small size, low RNA content, non-cycling status and lack of activation markers such as CD69, CD25 and HLA-DR [94].

1.6.3.2 Infection during deactivation vs. direct infection of resting cells

HIV-1 latency can arise in CD4 T-cells from infection of an activated effector cell that undergoes a reversion to a resting state during the process of memory cell generation (referred to herein as "infection during deactivation"), or from infection of a resting cell (direct resting cell infection), as illustrated in Figure 1.8. If latency is established during deactivation, then latent virus should be found mainly in memory cells. Conversely, direct infection of resting cells could result in latent virus being present in either naïve or memory cells. These pathways are not mutually exclusive. Latency can also be established during the deactivation process associated with thymopoiesis (discussed below), which would also result in latently infected naïve T-cells.

Infection of resting CD4 T-cells is inefficient due to many factors including low CCR5 expression [95], cytoskeletal barriers [96], limiting levels of deoxynucleoside triphosphates (dNTPs) [56, 97] due to SAMHD1 [98, 99], and inefficient nuclear import and integration [56, 100]. *In vitro*, direct infection of naïve CD4 T-cells is less efficient than direct infection of memory CD4 T-cells [101, 102]. This is because naïve cells have low to undetectable levels of CCR5 expression [95, 103, 104]; fusion is also less efficient in naïve cells [105], and cortical actin dynamics are lower compared to memory cells [106].

Several studies have examined the distribution of HIV-1 provirus in resting CD4 T-cells from peripheral blood and lymphoid tissues of patients. While some reports identified integrated DNA only in memory cells [46], most others have shown

that memory cells constitute the major reservoir but that naïve cells harbour lower provirus levels [101, 104, 107-111]. In one recent study of patients on suppressive therapy, 98% of all provirus-containing CD4 T-cells were memory cells (of these, 52% were T_{CM}, 34% were T_{TM} and 14% were T_{EM}), and only 2% were naïve cells [110]. In simian immunodeficiency virus (SIV)-infected rhesus macaques, most infected cells identified during early infection (*i.e.* the time of reservoir formation) were found to be resting CD4 T-cells [112]. Furthermore, cytokine/chemokine rich microenvironments in lymphoid tissues can aid infection of resting cells [113-116], and chemokine treatment of resting cells can lead to the establishment of latency *in vitro* [61, 117, 118]. It is therefore possible that the contribution of direct resting cell infection to the establishment of latency is greater than is commonly appreciated. Given that HIV-1 preferentially infects activated CD4 T-cells [56, 100], coupled with the ongoing generation of memory cells, the consensus is that infection prior to or during deactivation is the major route of establishment of latency, although this remains an unresolved issue.

1.6.3.3 Routes of latency establishment: in vivo models

SIV-infected macaques receiving suppressive antiretroviral therapy are now excellent models to better understand the role of tissue reservoirs, sanctuary sites, viral dynamics in response to therapy, and *in vivo* testing of eradication strategies (reviewed in [82]). Humanized mouse models of HIV-1 latency are also useful and include severe combined immunodeficient humanized thymus/liver (SCID-hu Thy/Liv) mice [119], NOD/SCID-gamma chain null (NSG) bone marrow-liver-thymus (BLT) mice [120, 121] and $Rag2^{-/-}\gamma_C^{-/-}$ mice [122]. In SCID-hu (Thy/Liv) mice, latent infection is established during thymopoiesis, leading to generation of latently infected naïve T-cells. Thymopoiesis mirrors the generation of memory T-cells, since transcriptionally active immature CD4+CD8+ thymocytes enter a quiescent state upon maturation to naïve T-cells (Figure 1.8A). Therefore, the establishment of latency during thymopoiesis [119] is an

example of latency arising from infection during deactivation. Latent virus was also identified in purified resting CD4 T-cells [121] and in naïve lymphocytes [120] of infected BLT mice, and in central memory CD4 T-cells of infected $Rag2^{-/-}$ $\gamma c^{-/-}$ mice [122]. Collectively, these studies suggest that both infection during deactivation and direct infection of resting cells likely contribute to the establishment of latency *in* vivo.

1.6.3.4 Routes of latency establishment: in vitro models

Several primary cell latency models have been established (for detailed comparisons see [76-80]). Some of these models involve infection of activated CD4 T-cells that are allowed to return to a resting state through various culture conditions [123-128], with latency established in 1% to 75% of cells depending on the system. Several other models involve direct infection of either untreated or chemokine-treated resting CD4 T-cells [117, 129, 130] and result in up to a few percent of cells becoming latently infected, reflecting the preferential infection of activated cells. Additionally, one model uses human thymocytes infected in cell culture that undergo a deactivation process during T-cell maturation [131]. Taken together, these models demonstrate that both pathways can give rise to latency under appropriate conditions.

One report described the establishment of latency in multiple subsets of CD34+ hematopoietic progenitor cells (HPCs) derived from either bone marrow or umbilical cord blood [132]. In this model, purified HPCs are infected shortly after isolation and latency is established within a few days, in a manner analogous to direct infection of resting CD4+ T-cells. Although the detection of HIV-1 DNA in HPCs from patients on suppressive highly active antiretroviral therapy (HAART) is controversial [133-136], it is clear that latency can be established in HPCs *in vitro* [132, 134] (reviewed in [137]). While a latently infected HPC could theoretically give rise to other types of latently infected cells *in vivo*, including CD4 T-cells, it is unlikely that the virus would remain in a latent state during HPC differentiation

[134].

Finally, a number of reports have described models of latency establishment at a population level in CD4 T-cell lines, including Jurkat [73, 75, 138-141], SupT1 [142, 143] and Molt-4 [73] cells. The establishment of latency in proliferating cell lines implies that latency might be established in some fraction of infected, activated CD4 T-cells, even *in vivo* (included schematically in Figure 1.8B). However, the short lifespan of activated cells *in vivo* [144] implies that any such latent infections would be clinically irrelevant. Having examined how latency is established in terms of cellular physiology, we now turn our focus to the molecular level.

1.6.4 Molecular mechanisms of the establishment of HIV-1 latency

The mechanisms associated with latency, particularly its maintenance and reactivation, have been extensively reviewed (for recent reviews see [43, 80, 145-147]). These mechanisms include transcriptional interference, insufficient levels of transcriptional activators, the presence of transcriptional repressors, epigenetics, nucleosome positioning, insufficient Tat activity, blocks to mRNA splicing or nuclear export, cellular microRNA (miRNA), and homeostatic proliferation of latently infected cells. While each of these is known to be involved in the maintenance of latency, here we discuss which of these mechanisms have been shown to promote viral entry into latency (summarized in Table 1.2). Homeostatic proliferation is an important mechanism of survival of resting CD4 T-cells that can be induced by homeostatic cytokines including IL-7 and IL-15 [148]. Since its role in maintaining latently infected cells occurs, by definition, after latency has been established, and in keeping with the focus of this review, homeostatic proliferation is not discussed here as a mechanism of establishment of latency.

Table 1.2. Mechanisms of latency establishment.

Mechanisms associated with latency	Evidence for a role in establishing latency in:	
	Cell line models[a]	Primary cell models[a]
Transcriptional interference	Yes[b] [127, 149, 150]	Yes [151]
Limiting transcription factors	Yes [73, 141, 152]	Yes [73]
Limiting P-TEFb	?[c]	Yes [128, 153]
Transcriptional repressors	?	?
Histone deacetylation	Yes [154] No[d] [73]	Yes [128]
Histone methylation	Yes [74, 155-157]	Yes [128]
DNA methylation	No [73]	?
Nucleosome positioning	Yes [158]	?
Insufficient Tat activity	Yes [74, 75, 140, 154, 159]	Yes [128]
Insufficient mRNA nuclear export	?	Yes [61]
Insufficient mRNA splicing	?	Yes [62]
miRNA	?	?
Homeostatic proliferation	?	?

[a] Only studies that explicitly examined the establishment of latency are included
[b] Yes: This mechanism has been shown to influence the establishment of latency
[c] ?: The effects of this mechanism on the establishment of latency have not been studied
[d] No: This mechanism has been shown to not influence the establishment of latency

1.6.4.1 Transcriptional interference

HIV-1 preferentially integrates into the introns of actively expressed genes in CD4 T-cell lines [24, 160], and both activated and resting primary CD4 T-cells that are infected *ex vivo* [151, 161, 162]. Initial studies in the Jurkat-based J-LAT system found that integration into both heterochromatin [138, 163] and highly expressed genes [163] was associated with latency. Proviruses in resting CD4 T-cells from patients on HAART were also shown to be integrated into highly expressed genes, with no preference for orientation relative to the host gene [164]. A consequence of integration into regions of high transcriptional activity is transcriptional interference, a process whereby transcription that originates at one promoter can interfere with transcription at another (reviewed in [165,

166]). One study found that convergently oriented integration resulted in transcriptional interference that silenced HIV-1 gene expression in a TNF-α-reversible manner [149], and similar findings were obtained in a Jurkat latency establishment model [127]. Another study found that transcriptional interference was responsible for latency in Jurkat and primary CD4 T-cells [167]. Transcriptional interference was also recently linked to the establishment of latency following viral integration into highly expressed genes in Jurkat cells, and the authors showed a role for chromatin reassembly factors in the maintenance of latency via transcriptional interference [150]. Finally, transcriptional interference contributed to the establishment of latency in a primary cell model, in which latent but not active proviruses had an orientation bias with respect to the host gene [151]. Although it is difficult to differentiate between a role for transcriptional interference in the establishment versus the maintenance of latency [73, 150], most evidence suggests that both can occur depending on the host cell chromosomal context.

1.6.4.2 Limited availability of transcription factors

A hallmark of quiescent lymphocytes is the low availability of transcriptional activators, either due to cytoplasmic sequestration, or regulation of protein levels or activity. This includes the transcription factors NF-κB and NFAT, which recruit histone acetyltransferases [80] and aid transcription initiation, and are critical for viral transcription. Both NF-κB and NFAT are sequestered in the cytoplasm in the absence of activation signals, in part due to the protein Murr1 in the case of NF-κB [168]. In one study, the establishment of latency in Jurkat cells was found to result from low levels of active NF-κB at the time of infection, and only cell lines with low basal levels of NF-κB activity supported the establishment of latency. Furthermore, the induction of NF-κB nuclear translocation by pre-treatment of Jurkat cells with phorbol myristate acetate (PMA) or prostratin, or of primary cells with phytohemagglutinin (PHA), strongly inhibited the establishment of latency [73]. Another group found that Sp1 or κB

site mutations (κB sites can be occupied by both NF-κB and NFAT) in the 5' long terminal repeat (LTR) led to higher levels of latency [141]. In a model of latency establishment in CD34+ HPCs, nuclear levels of NF-κB were low at the time of infection but were increased upon stimulation and subsequent reactivation of latent virus [132].

It has recently been reported that the establishment of latency in a polyclonal population of Jurkat reporter cells was regulated by an AP-1 binding site in the 5' LTR [152]. Deletion of this site severely limited the establishment of latency. Conversely, extension of this site from 4 to 7 nucleotides (as found in HIV-1 subtypes A and C) had no effect on initial latency levels but resulted in significantly greater levels of latency after several weeks of culture, likely due to lower rates of spontaneous reactivation of latent viruses carrying the 7 nucleotide sequence [152]. While this study does not necessarily provide evidence for a role of AP-1 in the establishment of latency, it suggests that variations in interactions involving transcription factors can have profound effects on the establishment of latency. Finally, it has been hypothesized that immunosuppressive cytokines including IL-10 and transforming growth factor beta (TGF-β) might indirectly aid the establishment of latency by reducing levels of T-cell activation [169], although this remains speculative.

1.6.4.3 Limited availability of elongation factors

The elongation factor P-TEFb is composed of Cyclin T1 and CDK9, and converts promoter-proximally paused RNA polymerase II complexes into efficient elongating complexes [146]. In many cell types P-TEFb is sequestered in the cytoplasm in a complex containing 7SK snRNA, Hexim1, and other components [170], and a study using a primary cell latency model found that low P-TEFb levels contributed to latency establishment [128]. However, a recent study found that P-TEFb availability in both naïve and memory CD4 T-cells is regulated by tight control of Cyclin T1 levels (by proteasome-mediated proteolysis and

microRNA regulation) and CDK9 T-loop phosphorylation (where only Thr-186-phosphorylated CDK9 is active), and not by the 7SK snRNA complex. The authors also showed that levels of Cyclin T1 and Thr-186-phosphorylated CDK9 decreased sharply during the transition of activated CD4 T-cells to central memory cells, during which time latency was established [153]. Thus, multiple mechanisms of transcriptional activator insufficiency can contribute to the establishment of latency.

1.6.4.4 Chromatin modifications

Epigenetic modifications dictate which proteins can interact with chromatin, and alter the physical structure of chromatin [171]. Proviral silencing after single-round infection of both Jurkat cells [156] and microglial cells [155] was shown to be mediated by the histone H3 lysine 9 (H3K9) methyltransferase Suv39H1 and its partner HP1γ. Entry into latency in Jurkat cells was associated with CBF-1-dependent histone deacetylase (HDAC)-1 recruitment to the 5' LTR [154], and H3K9/27 trimethylation [74]. Furthermore, CBF-1-dependent H3 deacetylation, followed by Suv39H1- and HP1α-dependent H3K9/27 trimethylation, led to the establishment of latency in primary cells [128]. Interestingly, CBF-1 is expressed in resting CD4 T-cells but is strongly downregulated upon T-cell activation [154]. Most recently, this group has demonstrated a role for the H3K27 methyltransferase EZH2, a component of the polycomb repressive complex 2, in establishing latency in Jurkat cells [157]. However, a different study found no evidence for histone deacetylation in the establishment of latency, since pre-treatment of Jurkat cells with the HDAC inhibitor valproic acid did not reduce the number of latently infected cells that were established [73].

DNA methylation at CpG islands is a repressive epigenetic modification that can inhibit transcription factor binding and can recruit HDAC-2. The available evidence suggests that DNA methylation is a later silencing event that is more important for the maintenance of HIV-1 latency than for its establishment [43,

172]. Additionally, one study showed that pre-treatment of Jurkat cells with the DNA methylation inhibitor 5-azacytidine did not inhibit the establishment of latency [73]. Finally, the SWI/SNF chromatin remodeling complex BAF, but not PBAF, was recently shown to facilitate the establishment of latency through repressive nucleosome positioning on the 5' LTR. BAF knockdown resulted in fewer latent infections in both Jurkat and SupT1 T-cell lines, without affecting levels of productively infected cells [158]. The evidence therefore supports a major role for epigenetic histone modifications and chromatin remodeling leading to provirus silencing and the establishment of latent infection.

1.6.4.5 Insufficient Tat activity

Since Tat is required for high-level viral transcription, due to recruitment of a super elongation complex to the 5' LTR [173, 174], it is perhaps unsurprising that insufficient Tat activity can lead to the establishment of latency. In one study, resting CD4 T-cells from treated patients were enriched for attenuated Tat variants [175]. Mutations that attenuated Tat activity led to higher levels of latency establishment in both Jurkat [74, 75, 154] and primary cell [128] models. Treatment of Jurkat cells with Tat at the time of infection led to a subsequent decrease in the frequency of latently infected cells [75]. Further, expression of Tat in *trans* prevented the silencing of actively infected cells [74] and strongly inhibited the establishment of latency in Jurkat cells [75]. Finally, random fluctuations in Tat concentrations at the single cell level were shown to influence the entry of HIV-1 into latency, as shown in mathematical models and experimentally [140, 159]. Based on these findings, proteins that modulate Tat activity might be expected to impact the establishment of latency, as has been suggested for Tat deacetylation *via* SirT1 [159].

1.6.4.6 Post-transcriptional mechanisms

Multiply spliced mRNA was found in the nucleus, but not in the cytoplasm, of resting CD4 T-cells from HAART-treated patients. This block was shown to be due

to low levels of polypyrimidine tract binding protein (PTB), the overexpression of which rescued multiply spliced mRNA nuclear export and virus production [60]. However, it was unclear whether limiting PTB levels contributed to the initial establishment of latency. In a primary cell model in which resting cells are directly infected after chemokine treatment [117], it was shown that multiply spliced mRNA accumulated in the nucleus but not the cytoplasm, in the absence of other transcripts or viral proteins [61]. In another resting cell model of latency establishment, [129] a block to mRNA splicing was recently identified, whereby latently infected cells produced Gag protein (at levels 1000-fold lower than in activated cells) but only barely detectable levels of Env. This result was reflected at the mRNA level, since unspliced transcripts were ~100-fold more abundant than singly spliced transcripts and ~10 000-fold more abundant than multiply spliced transcripts [62]. Together, these primary cell models highlight two post-transcriptional blocks that contribute to the establishment of latency. In addition, miRNA regulation of viral protein expression has been associated with latency, and several of the miRNAs that have been implicated in this process are expressed in resting cells but are downregulated upon T-cell activation. Although miRNAs can contribute to the maintenance of latency, as shown both *in vitro* and *ex vivo* [176, 177], the potential role of miRNAs in the establishment of latency remains unknown [178].

1.7 CLINICAL APPROACHES TO LATENT RESERVOIRS

Although HAART is extremely effective at limiting ongoing replication, it is not curative. Since curing an infected individual of HIV-1 requires eradication of latent reservoirs, this is now a major goal of the field, as highlighted by the "International AIDS Society Scientific Working Group on HIV Cure" [179]. Several clinical trials focused on eradication of latent reservoirs have already been carried out, as described in the following sections, although the field as a whole is in its infancy.

1.7.1 Immune activation therapy

The earliest attempts to deplete latent reservoirs were based on immune activation therapy, including several clinical trials going as far back as 1999. It should be noted that continued HAART is an implied component of any clinical trial aimed at eradication of latent reservoirs. As the name suggests, immune activation therapy is a general term to describe an intervention whose aim is the activation of immune cells, with the goal of reactivating latent viruses. It was assumed that latent virus reactivation would lead to the deaths of cells either by cytopathic effect, apoptosis, or immune clearance. The first immune activation therapy clinical trials used interleukin-2 (IL-2) [180-183], while subsequent trials combined IL-2 with interferon-gamma (IFN-γ) [184] or with direct antibody-mediated T-cell receptor activation [185-189]. None of these approaches were ultimately successful in part due to their non-specific nature, and some led to profound negative effects on patients [67] due to global T-cell activation and the induction of a "cytokine storm".

1.7.2 Shock and kill

A promising approach to the reactivation and depletion of latent reservoirs is a strategy known as "shock and kill". This refers to pharmacological interventions aimed at disrupting one or more of the mechanisms of latency (detailed in section 1.6) (the "shock") followed by any of several methods of cell death/clearance (the "kill").

1.7.2.1 The "shock" phase

The first clinical trials of this type made use of the nonspecific histone deacetylase inhibitor (HDACi) valproic acid, and were carried out beginning in 2005 [190-195]. Despite some indications of success, valproic acid was ultimately ineffective at reactivation of latent viruses *in vivo*. Clinical trials using the more potent and selective HDACi suberoylanilide hydroxamic acid (SAHA) are currently in progress, and initial findings have shown that a single dose of SAHA in several

patients resulted in increased histone acetylation and an increase in viral mRNA expression from these patients' resting CD4 T-cells (Archin *et al*, CROI 2012, abstract #157LB). However, there are conflicting reports as to whether or not *ex vivo*-administered SAHA can reactivate latent viruses from the resting CD4 T-cells of patients on suppressive HAART [196, 197]. Several HDACis with greater specificity and potency are also in various stages of experimental testing in terms of reactivation of latent viruses [198, 199].

A number of additional "shock" strategies are in various stages of investigation, which target other mechanisms of latency besides histone deacetylation. These include inhibitors of histone methylation or DNA methylation, and agonists of NF-kB or NFAT, or P-TEFb, as well as compounds that activate latent viruses through other, sometimes unknown, pathways (reviewed in [200-202]). One novel strategy involves the use of lipid nanoparticles targeted specifically to cells that express CD4. The nanoparticles package both the potent PKC activator bryostatin (which ultimately leads to NF-kB activation) and a protease inhibitor, to reactivate latent viruses and simultaneously render them non-infectious [203]. This approach has been validated in a humanized mouse model of HIV-1 latency, and promises greater specificity than simple oral drug dosing. Pharmacological approaches to the "shock" phase of latent virus reactivation are summarized in Figure 1.9.

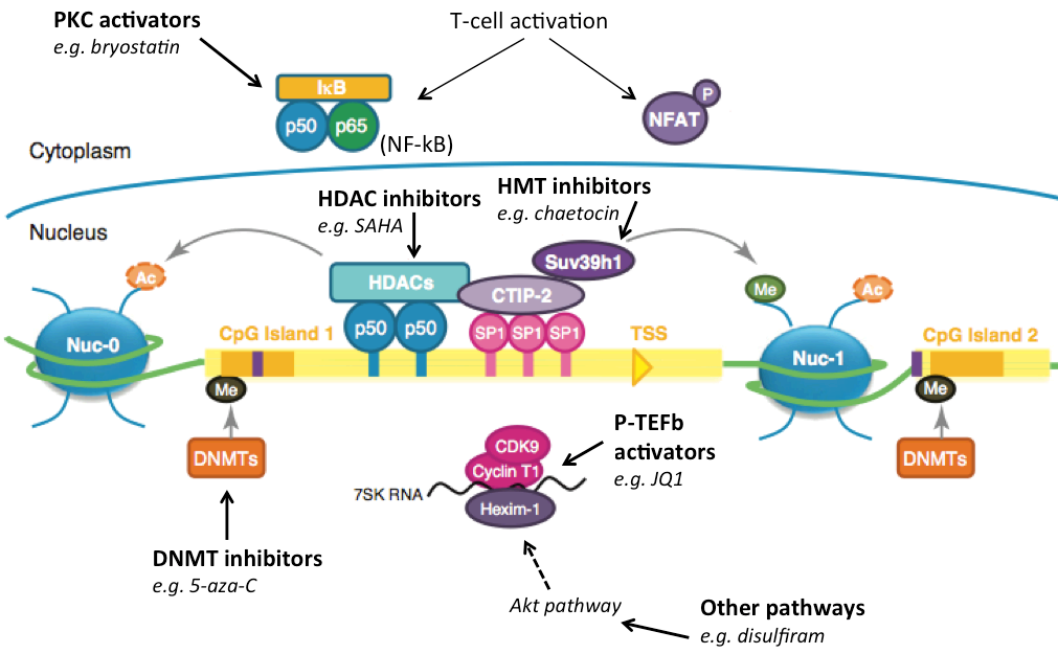

Figure 1.9. Pharmacological approaches to "shocking" HIV from latency.

Mechanisms of latency that can be targeted pharmacologically are shown, and representative compounds are highlighted. These pathways include activation of NF-kB by the PKC pathway; inhibition of histone deacetylation; inhibition of histone methyltransferation; inhibition of DNA methylation; activation of P-TEFb activity; and other undefined pathways known to reactivate latent viruses. PKC = protein kinase C; HDAC = histone deacetylase; HMT = histone methyltransferase; nuc = nucleosome; CpG island = sites of DNA methylation; DMNT = DNA methyltransferase; TSS = transcription start site, *i.e.* the first nucleotide of the TAR RNA. Figure adapted from [202].

1.7.2.2 The "kill" phase

It was originally thought that following reactivation of latent viruses, the now virus-producing cells would be cleared by default, through a combination of cytopathic effect, apoptosis and an immune response. However, recent evidence suggests that reactivation of latent viruses is often insufficient to lead to cell death, and thus, would not on its own deplete latent reservoirs [69]. A number of approaches have been proposed that would aid the killing of cells after latent

virus reactivation. These include enhancement of the immune response through the use of a prophylactic vaccine [68], and multiple methods of targeted cytotoxic therapy including antibodies conjugated to toxic drugs; radioimmunotherapy; immunotoxins; transplantation with genetically modified autologous T-cells capable of a greater immune response; targeted cytotoxic viruses; and liposome-mediated delivery of cytotoxic moieties (reviewed in [70]). One early approach to cytotoxic therapy was the use of cyclophosphamide to deplete latently infected as well as uninfected T-cells, although this non-specific method did not reduce latent reservoirs [204].

1.7.3 Immune modulation

The use of immune modulating compounds to deplete latent reservoirs has been proposed. Since homeostatic proliferation of latently infected resting memory CD4 T-cells is an important mechanism ensuring the maintenance and potentially the expansion latent reservoirs [110, 148], disruption of homeostatic proliferation might lead to smaller latent reservoirs. This might be accomplished by anti-IL-7 therapies, since IL-7 has a critical role in homeostatic proliferation, or by interfering with other factors involved in long-term T-cell survival such as FOXO3a [148, 169]. Alternatively, since latent viruses are preferentially found in central and transitional memory CD4 T-cells [110], the specific depletion of these T-cell subsets has been proposed [148, 205].

1.7.4 Genetic approaches

One approach to curing HIV-1-infected patients would involve transplantation of stem cells from a naturally occurring CCR5Δ32/CCR5Δ32 homozygous donor (this mutation results in a lack of cell-surface expression of the CCR5 coreceptor). This would be in a manner similar to the “Berlin patient”, the only reported case of a functional HIV-1 cure. This individual received an allogeneic CCR5Δ32/CCR5Δ32 bone marrow transplant as part of acute myeloid leukemia treatment. It should be noted, however, that Timothy Brown (the “Berlin patient”) received extensive

pre-transplant conditioning to deplete hematopoietic cells, as well as post-transplant immune therapeutics, and it is likely that a combination of all these factors contributed to his functional cure [179, 206]. Alternatively, several approaches based on the genetic modification of a patient's cells have been proposed. A patient's hematopoietic stem cells could be genetically modified with zinc finger nucleases to eliminate expression of CCR5 and/or CXCR4 [68], which are required for HIV-1 entry, and reintroduced following myeloablative treatment. Other proposed methods of genetic modification include the use of anti-HIV ribozymes, shRNAs, dominant negative proteins, intracellular antibodies, decoy RNAs, or broadly neutralizing antibodies (reviewed in [207]). These approaches are all designed to generate HIV-resistant cells, in all cell types normally able to be infected by HIV-1.

Despite the multitude of options for eradication of latent reservoirs that are envisioned, the development of a reproducible, functional HIV-1 cure remains an ambitious yet long-term goal.

1.8 OBJECTIVES

Detailed objectives are described in the Preface and Introduction to each chapter. The broad objectives of this thesis were to further understand the roles of viral reservoirs in HIV-1 infection. In Chapter 2, I focus on second phase viral reservoirs. As will be discussed, the clinical trials leading to the approval of HIV-1 integrase inhibitors in 2007 turned out to be very useful toward furthering our understanding of viral reservoirs that are composed of long-lived, productively infected cells. It is these cells that contribute the majority of virus production during the second phase of viral load decay, and due to the addition of integrase inhibitors to HAART, novel information concerning viral reservoirs was uncovered. Chapters 3 and 4 are focused on the latently infected CD4 T-cells that comprise third/fourth phase viral reservoirs. As discussed above, clinical approaches to HIV-1 latency are focused on eradication of already-established

latent reservoirs. In Chapter 3, I focus on a novel approach to limit the establishment of latent infection, providing proof that it is theoretically possible to inhibit the establishment of HIV-1 latency. In Chapter 4, I focus on the ability of latent viruses to contribute to viral genetic diversity and drug resistance in the face of selective pressure. I demonstrate that latent viruses can be reactivated when their host cells are superinfected by another HIV-1 virion, and that under appropriate conditions, the reactivated latent viruses can contribute to the generation of multidrug-resistant recombinant viruses.

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Chapter 2

Stage-Dependent Inhibition of HIV-1 Replication Can Help Explain Clinically Observed Second-Phase Viral Load Decay Dynamics

This chapter was adapted from the following published manuscript:

Donahue DA, Sloan RD, Kuhl BD, Bar-Magen T, Schader SM, Wainberg MA: Stage-dependent inhibition of HIV-1 replication by antiretroviral drugs in cell culture. *Antimicrob. Agents Chemother.* 2010, 54:1047–1054.

All experiments and data analysis included in this chapter were performed by myself under the supervision of Dr. Mark Wainberg. RD Sloan, BD Kuhl, T Bar-Magen and SM Schader assisted with some aspects of experimental design and offered suggestions for revisions to the manuscript.

2.1 PREFACE

Chapter 2 is based on viral infection and its inhibition in second phase reservoirs. In 2007, raltegravir became the first integrase inhibitor to be clinically approved. The clinical trials that led to its approval compared treatment with raltegravir to treatment with the NNRTI efavirenz, each as part of HAART with a backbone composed of two NRTIs. Efavirenz-based HAART was the "standard of care" against which other drugs were compared, and new drugs needed to be equivalent if not superior to efavirenz-based therapy for approval.

The efficacy of antiretroviral drugs and the progression of HIV-1 infection are measured by RT-PCR for levels of viral RNA in the blood, termed the viral load. Clinical trials showed that use of raltegravir as part of HAART was equal to efavirenz-based HAART in the long-term suppression of viral load, but viral loads in patients taking the integrase inhibitor reached undetectable levels much more quickly. This effect had not previously been observed for any antiretroviral drug from any drug class.

Analysis of the viral load decay dynamics in these trials indicated that first phase decay was the same in both treatment arms, but that there were ~70% lower viral loads at the onset of the second phase of decay in patients taking raltegravir [1]. Several hypotheses to explain these effects were suggested, although the reason for the enhanced antiviral effect of raltegravir was not known. Figure 2.0.1 depicts the decay of viral load that was observed with raltegravir treatment.

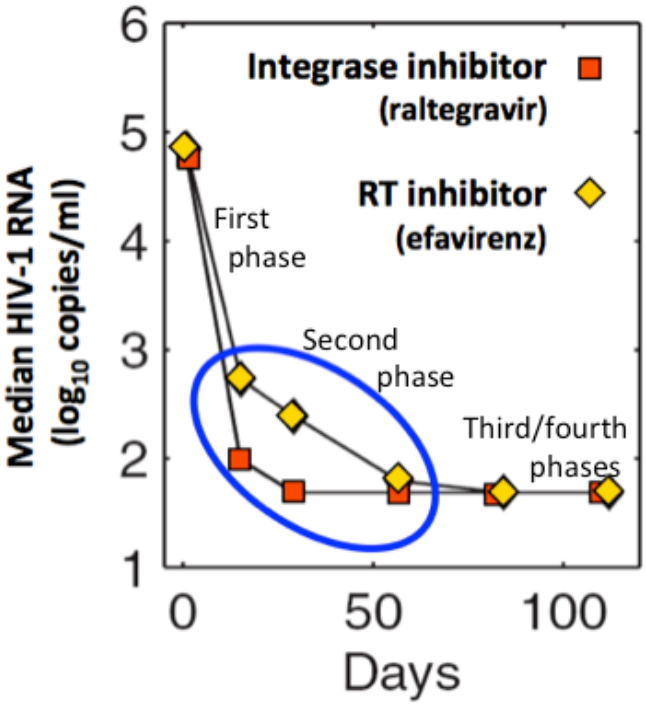

Figure 2.0.1. Viral load decay dynamics observed with the integrase inhibitor raltegravir, compared to the NNRTI efavirenz, in clinical trials.

Analysis of the viral load decay observed with raltegravir treatment showed viral loads were ~70% lower at the onset of second-phase decay, compared to patients taking efavirenz, as circled above. Figure adapted from [1].

When I began my graduate studies in 2008, my initial objective was to help explain these unique second-phase viral load decay dynamics. Not only could this contribute to a greater understanding of why integrase inhibitors appeared so efficacious – with potential implications for the design of future therapies – but these trials themselves also served as a useful tool to further understand virus production by second-phase cellular reservoirs.

The hypotheses put forth to explain the unique viral load decay dynamics with raltegravir included: (a) greater potency for raltegravir; (b) more rapid bioavailability for raltegravir; (c) access of raltegravir to efavirenz-impermeable

sanctuary sites; (d) the effects of raltegravir on preintegration latency; (e) the temporal stage of viral replication targeted by raltegravir compared to efavirenz; and (f) cellular responses to the unintegrated DNA that accumulates when integration is blocked. Some of these hypotheses were based on informed speculation, while others were based on mathematical models of viral load decay. Of note, the identity of the cells responsible for virus production during the second phase is not currently known, but at this time it was suspected that they were likely monocytes/macrophages. Although no consensus was reached, and several authors provided data supporting or refuting different hypotheses, we thought that mathematical models of viral load decay that took into account the stage of viral replication targeted by raltegravir compared to efavirenz were most consistent with the clinical observations. Chapter 2 represents work that I carried out to validate the findings of these mathematical models in a cell culture system, and provides the first experimental evidence to support the conclusions of these models. These data were published in 2010, and provide insight into the dynamics of viral replication and inhibition in second-phase viral reservoirs.

2.2 ABSTRACT

Recent clinical trials have shown that the use of the HIV-1 integrase (IN) inhibitor raltegravir (RAL) results in drops in the viral load that are more rapid than those achieved by use of the reverse transcriptase (RT) inhibitor efavirenz. Previously, mathematical modeling of viral load decay that takes into account the stage of viral replication targeted by a drug has yielded data that closely approximate the clinical trial results. This model predicts greater inhibition of viral replication by drugs that act later in the viral replication cycle. In the present study, we have added drugs that target entry, reverse transcription, integration, or proteolytic processing to acutely infected cells and have shown modest viral inhibition by entry inhibitors, intermediate levels of inhibition by RT and IN inhibitors, and high levels of inhibition by protease inhibitors relative to the levels of growth for the no-drug controls. When dual or triple combinations of these drugs were added to acutely infected cells, we found that the levels of inhibition achieved by any given combination were comparable to those achieved by the latest-acting drug in the combination. In single-round infections in which the kinetics of reverse transcription and integration had been determined by quantitative PCR, addition of IN inhibitors at various times post-infection resulted in levels of inhibition equal to or greater than those achieved by addition of RT inhibitors. Collectively, our data provide *in vitro* evidence of the stage-dependent inhibition of HIV-1 by clinically relevant drugs. We discuss how stage-dependent inhibition helps to explain the unique viral load decay dynamics observed clinically with RAL.

2.3 INTRODUCTION

Recent clinical trials with the first clinically approved HIV-1 IN inhibitor, RAL, have yielded promising results. The phase II Merck protocol 004 part II [2, 3] and the phase III STARTMRK [4] trials compared RAL to the non-nucleoside reverse transcriptase inhibitor (NNRTI) EFV, each as part of standard combination therapy in drug-naïve HIV-1-infected individuals. While both drugs showed equal

efficacy in long-term suppression of viral load, the limit of detection was reached more rapidly with RAL. This has been attributed to a 70% decrease in virus production from second-phase sources with RAL compared to EFV [1].

Several hypotheses may explain these unique viral load decay dynamics. They include differences in time until drug bioavailability; differences in drug potency; a role for preintegration latent cells; greater penetration of certain drugs into sanctuary sites; the stage of viral replication targeted; and IN inhibitor-induced accumulation of unintegrated viral DNA [1, 2, 5-7]. On the basis of mathematical modeling of viral load decay, it has been proposed that the clinical observations can be explained by the effect of RAL on preintegration latent cells [1]. These models also suggest that differences in drug potency could play a minor role, but are inconsistent with a role for sanctuary sites. Mathematical models of viral load decay by others suggests that the stage of viral replication targeted by RAL versus EFV can explain the clinical trial results, but is inconsistent with roles for preintegration latent cells, differences in drug potency or time until bioavailability, or sanctuary sites [6, 7]. Furthermore, the latter models involving preintegration latent cells used parameters based on experimentally determined kinetics of reverse transcription, integration and preintegration complex decay during infection of resting $CD4^+$ T cells [8-10], and strongly argue that the action of RAL in this cell type cannot account for the clinical trial observations. This contrasts with the parameter choices concerning preintegration latent cells used previously [1], which are inconsistent with the known properties of this viral reservoir. Others have suggested that the greater decay observed with RAL could be a result of gene expression from, or apoptosis triggered by, unintegrated DNA that accumulates following use of an IN inhibitor [5]. However, these effects were suggested to occur in primarily in CD4 T-cells [5], which are not expected to contribute substantially to second-phase virus production [6, 7]. Given that certain mathematical models of viral load decay [6, 7] have closely approximated the clinical trial results, it seems probable that the stage of viral replication

targeted by RAL versus EFV may explain the observed viral load decay dynamics.

The mathematical model of stage-dependent inhibition of viral replication is detailed elsewhere [6] but can be summarized as follows. Large numbers of virions are produced only by cells that were infected prior to the start of treatment (since the majority of active viral replication is immediately blocked following the initiation of HAART), and virus production from these cells lasts until their death, implying that the viral load decays according to the death of previously infected cells. Two factors that affect the decay of viremia can differ depending on the stage of viral replication targeted by a drug and the cell type in which the drug is acting. These factors are (i) the death rates of the virus-producing cells and (ii) the time of transition from early- to late-stage-infected (i.e., virus-producing) cells. This model predicts that the most rapid decay of virus production should result from the use of drugs targeting the latest stages of viral replication. This concept can also be presented in terms of the number of cells available to a given drug when treatment begins. At the time of drug addition, drugs that act at a later stage of replication, such as integration or proteolytic processing, will have more available target cells in which to prevent viral replication than drugs that act earlier, such as at entry or reverse transcription. This is because some viruses will have completed the earlier but not the later stages of replication during ongoing infection, such that a later-stage inhibitor can block most or all of these viruses, while the earlier-stage inhibitor cannot be effective beyond the early stage of replication that it targets. Importantly, this model applies to infection of any cell type, including those responsible for either first-phase decay (productively infected CD4 T-cells) or second-phase decay (e.g., macrophages) and can be used to analyze either phase individually or both phases together. Since productively infected CD4 T-cells are rapidly cleared (by cytopathic effect or CD8 T-cell-mediated killing) but long-lived infected cells such as macrophages can produce virus over the remainder of their lifetimes, the influence of the stage effect on viremia should have a greater duration in the

second phase. While this hypothesis is supported by mathematical modeling, it has not been examined experimentally. Our study was carried out to examine this hypothesis in cell culture in order to better understand the results obtained in clinical trials with RAL.

2.4 MATERIALS & METHODS

2.4.1 Cells and viruses

PM1 [11] and SupT1 [12] cells were obtained through the NIH AIDS Research and Reference Reagent Program. PM1 and SupT1 cells were maintained in RPMI 1640 medium (Invitrogen), and 293T cells were maintained in DMEM (Invitrogen), each supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. The viral constructs pNL4-3 [13], pNL4-3(AD8) [14] and pNL4-3-deltaE-EGFP [15] (courtesy of Dr. R Siliciano) were obtained through the NIH AIDS Research and Reference Reagent Program. pVPack-VSV-G (Stratagene), encoding the vesicular stomatitis virus (VSV) envelope glycoprotein, was used to produce VSVg-pseudotyped NL4-3-deltaE-EGFP. The envelope sequence of pNL4-3(AD8) (accession number AF004394) was cloned into pcDNA3.1 (Invitrogen) and used to produce AD8env-pseudotyped NL4-3-deltaE-EGFP. All transfections were performed in 293T cells with Lipofectamine 2000 (Invitrogen). Forty-eight hrs after transfection, viruses were treated with 100 U/ml DNase I (Invitrogen) in the presence of 10mM (added) $MgCl_2$ for 1 hour at 37°C to digest contaminating plasmid DNA, prior to 0.2 µm pore filtration and storage at -80°C.

2.4.2 Antiviral compounds

AMD3100 (a CXCR4 co-receptor antagonist), darunavir (DRV), enfuvirtide (T-20), and nevirapine (NVP) were obtained through the NIH AIDS Research and Reference Reagent Program. Efavirenz (EFV) was a gift from Bristol-Myers Squibb Inc. Elvitegravir (EVG; an integrase inhibitor), emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF) were gifts from Gilead Sciences, Inc. Lopinavir (LPV)

was a gift from Abbott Laboratories. MK-2048 (an integrase inhibitor) and raltegravir (RAL) were gifts from Merck-Frosst Canada, Inc.

2.4.3 Cell culture model to test stage-dependent inhibition

PM1 cells (0.125×10^6) were infected with 18 ng p24 NL4-3 in T-25 flasks in 10 ml RPMI 1640 medium by adding virus and gently mixing the components without washing off the virus. Four days later, duplicate flasks of infected cells were pooled and the cells were seeded into 96-well plates at 190 µl per well. Uninfected cells were similarly grown to the same density before they were seeded into 96-well plates. Twenty-four hours after seeding of the plates, 10 µl of drug was added to replicate wells (to concentrations that could block virus replication by close to 100%, as determined in preliminary experiments in which the cells were pretreated with drug 1 h prior to infection). At 24 h following addition of drug, supernatants were collected to quantify the p24 antigen level by enzyme-linked immunosorbent assay (ELISA) or to quantify the viral RNA load by quantitative reverse transcriptase PCR (qRT-PCR). At no point were the cells split or was the virus washed from the wells. The final drug concentrations were as follows: T-20, 250 nM; AMD3100 and DRV, 1 µM; FTC and NVP, 10 µM; TDF, 100 µM; EFV, RAL, MK-2048, and EVG, 500 nM; and LPV, 5 µM. When they were used in combinations, each drug was used at the same concentration indicated above.

2.4.4 Single-round infections and flow cytometry

PM1 cells were infected with 576 ng p24 AD8env-pseudotyped NL4-3-deltaE-EGFP per 10^6 cells, and SupT1 cells were infected with 12 ng p24 VSVg-pseudotyped NL4-3-deltaE-EGFP per 10^6 cells, each by spinoculation for 2 h at $1{,}200 \times g$ at 25°C, as described previously [16]. Pseudotyped viruses were used to ensure that all viral DNA quantified by quantitative PCR (qPCR) was derived from a single round of replication. The start of spinoculation denotes the start of infection. For determination of the viral replication kinetics by qPCR, infected PM1 cells were used. Cells were washed three times with RPMI 1640 medium

immediately following spinoculation to remove unbound virus and were resuspended in RPMI 1640 medium. The cells were collected at various times postinfection (p.i.) and stored at −80°C. Mock infections with heat-inactivated virus were carried out to quantify any residual plasmid DNA from transfection. For flow cytometry-based experiments, both infected PM1 cells and infected SupT1 cells were used (in separate experiments). Drug addition time courses were performed by addition of individual drugs to different wells of infected cells at different times. For drug addition at time zero (immediately prior to the start of spinoculation), RAL, MK-2048, EFV, or NVP was individually added at a concentration that was ≥99.5% the inhibitory concentration (as determined in preliminary experiments), while the remaining wells contained RPMI 1640 medium only. Spinoculation was then performed as described above. The cells were washed three times with RPMI 1640 medium immediately, following spinoculation to remove unbound virus, and were then resuspended in RPMI 1640 medium with drug (time zero wells) or without drug (all other wells). For other drug addition time points, RAL, MK-2048, EFV, or NVP was added to individual wells. At 48 h p.i., the cells were fixed in 1% paraformaldehyde for 20 min. Flow cytometry was performed with a FACSCalibur instrument (Becton Dickinson) by gating for live cells and quantifying the number of cells positive for the expression of the virally encoded green fluorescent protein (GFP), and the data were analyzed with CellQuest Pro software.

2.4.5 *qRT-PCR for v*iral RNA and qPCR for viral DNA

2.4.5.1 Viral RNA

Viral RNA was extracted from the supernatants of infected cells by using a QIAamp viral RNA minikit (Qiagen). RT-PCR was performed with a Superscript III Platinum one-step qRT-PCR kit (Invitrogen) on a Corbett Rotor-Gene 6000 thermocycler. Dually labeled probes for this and all reactions described below were obtained from Biosearch Technologies (Novato, CA). The cycling conditions were 50°C for 15 min, 95°C for 8 min, and 45 cycles at 95°C for 15 s and 60°C for

30 s. The primers and probe used were primer total F, primer total R, and total probe [17]. Reactions carried out in the absence of reverse transcriptase (Platinum *Taq* only) confirmed the absence of contaminating DNA. The samples were quantified against cloned standards.

2.4.5.2 Early and late reverse transcripts and 2-LTR circles

Cellular DNA was extracted with a DNeasy blood and tissue kit (Qiagen). PCR was performed with Platinum qPCR SuperMix-UDG (Invitrogen) on a Corbett Rotor-Gene 6000 thermocycler. The samples were normalized for their beta-globin contents and quantified against cloned standards that were diluted with DNA from uninfected cells. The cycling conditions were 50°C for 2 min, 95°C for 1 min, and 45 cycles at 95°C for 3 s and 60°C for 30 s, with 65 ng template being used per reaction mixture. The primers and probes used for the early reverse transcripts were primers ERT2F and ERT2R [18] and probe ERT (5'-6-carboxyfluorescein [FAM]-ACTAGAGATCCCTCAGACCCTTTT-BHQ1-3'. For the late reverse transcripts, primer total F, primer total R, and total probe were used [17]. For 2-long terminal repeat (2-LTR) circles, primer circle F, primer circle R, and circle probe were used [17]. For beta-globin, primer BetaGlo-F (5'-GGTACGGCTGTCATCACTTAGAC-3'), primer BetaGlo-R (5-AACGGCAGACTTCTCCTCAG-3'), and the BetaGlo-probe (5'-FAM-CTCACCCTGTGGAGCCACACC-BHQ1-3') were used.

2.4.5.3 Integrated DNA

DNA was extracted and normalized as described above. A previously described Alu-*gag* PCR [19] was used with the following modifications. The first-round reaction was performed with undiluted samples (65 ng template) and 1:10 dilutions of each sample (6.5 ng template diluted with uninfected DNA, 65 ng DNA total) in the presence of 2 mM $MgCl_2$ and 200 µM deoxynucleoside triphosphates (dNTPs). Nine microliters of the resulting first-round product was used as the template for the second round of the nested reaction in the presence of 5 mM $MgCl_2$ (final concentration, including the carryover from first

round) and 200 µM dNTPs was added; only the wild-type probe was used [19]. The second-round cycling conditions were as described above for the 2-LTR circles. To generate a standard curve for the relative quantification of integrated DNA, the Alu-*gag* PCR was first performed with a dilution series of DNA from infected PM1 cells (diluted with DNA from uninfected cells).

2.4.6 Statistical Analyses

Unpaired two-tailed t-tests were performed to test for statistically significant differences between each treatment group and the no drug control group in Figure 2.2. One-way analysis of variance (ANOVA) tests were performed to test for statistically significant differences within treatment groups in Figure 2.3. Where such differences were found ($p<0.05$), Dunnett's multiple comparison test was performed to test for statistically significant differences between the single drug and the drug combination treatments. All statistical analyses were performed with GraphPad Prism 4.0 software.

2.5 RESULTS

2.5.1 Establishment of a cell culture model of stage-dependent inhibition of HIV-1 replication

To test the concept of stage-dependent inhibition of virus replication *in vitro*, we established a cell culture model of ongoing infection using the PM1 cell line (Figure 2.1A). Initial experiments were performed to determine growth and infection conditions that would meet two criteria: (i) infection would be ongoing for several days, without washing off newly produced virus, to ensure the simultaneous presence of viruses at various stages of replication; and (ii) levels of virus production would be sufficient for quantification while remaining low enough after several days of infection to avoid excessive cytopathic effect. Thus, infection of PM1 cells with NL4-3 could be continued for 5 days prior to the addition of different drugs, and supernatants were collected 24 hrs after drug addition. Since the drug concentrations used were such that close to 100%

inhibition of replication could be achieved, any virus release between days 5 and 6 should result only from cells in which virus replication by day 5 had progressed beyond the stage of replication targeted by the particular drug that had been used. In our system, most virus production (in the absence of drug) occurred between days 5 and 6 (Figure 2.1B). Therefore, the amount of virus present on day 6 (24 hrs after drug addition) can be used to determine the stage-dependent effect that different inhibitors might have on virus production. Since high drug levels were present at the time of sample collection, we could not use either reverse transcriptase or infectivity assays to measure virus production. Similarly, measurement of viral RNA would be impractical since the latter can still be produced in the presence of PR inhibitors. Therefore, we assayed for the presence of processed, extracellular p24 antigen as a measurement of virus production.

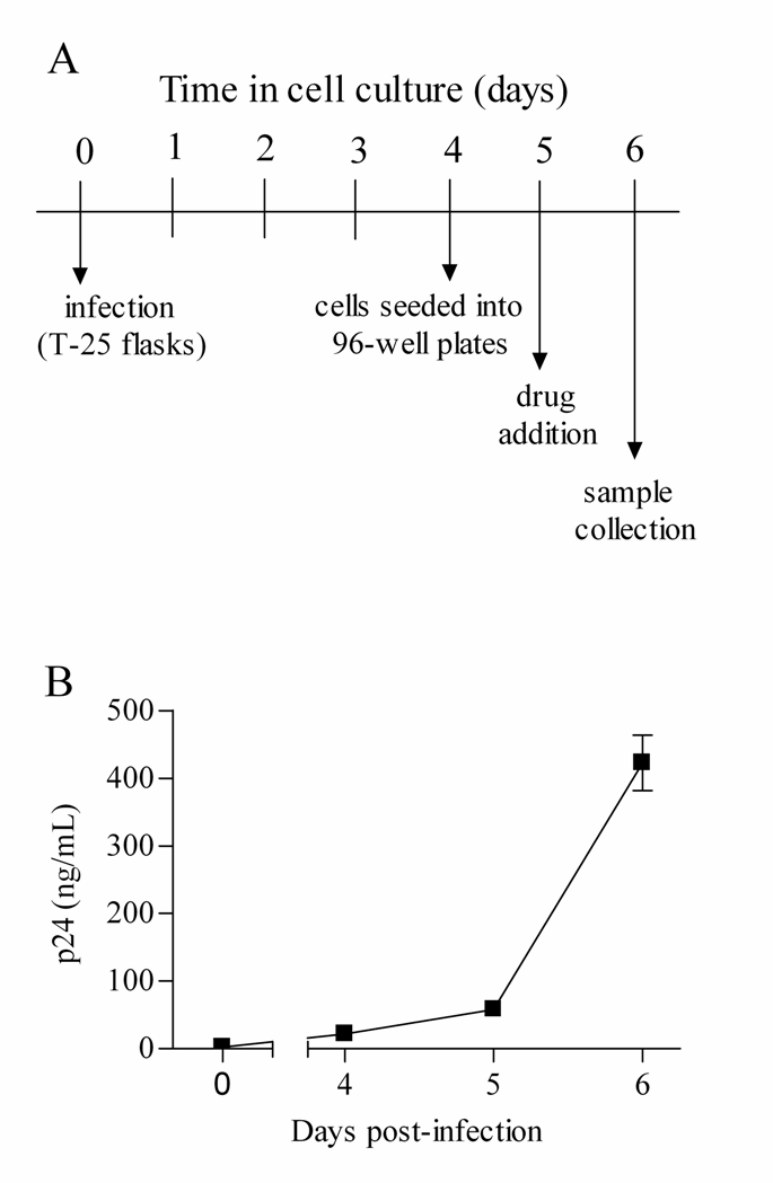

Figure 2.1. Cell culture model of ongoing infection to test stage-dependent inhibition of HIV-1 replication.

(A) Schematic representation of the experimental approach used to test stage-dependent inhibition of HIV-1 *in vitro*. PM1 cells were infected in bulk with NL4-3, and seeded into 96-wells 4 days later to ensure that all wells contained cells that produced approximately the same amount of replicating virus. (B) Progress of infection over the course of six days in the absence of drug. Results depict the mean +/- standard error of the mean (SEM), and are representative of three independent experiments.

2.5.2 Later-acting drugs inhibit virus production to a greater extent than earlier-acting drugs when added during ongoing infection

We investigated four different stages of viral replication, i.e. entry, reverse transcription, integration, and proteolytic processing, using two or more drugs that act at each of these stages, as well as various drug combinations (Table 2.1). Drugs were added individually after 5 days of infection, and the amount of p24 antigen present at day 6 was determined. The results of Figure 2.2 show that the drugs that target later steps of the viral replication cycle (i.e. PR inhibitors) inhibited virus production to a greater extent than the drugs that act at earlier stages (i.e. entry inhibitors), although both the NNRTIs EFV and NVP inhibited virus production at least as well as the other RT and IN inhibitors that were employed. The greater inhibition by EFV can likely be attributed to previously reported pharmacological slope parameters [20], as discussed below.

Table 2.1. Drugs used in Chapter 2

Individual Drugs Targeting:				Drug Combinations	
Entry	Reverse transcriptase	Integrase	Protease	Dual Combinations	Triple Combinations
AMD3100	TDF	RAL	LPV	EFV + AMD3100	EFV + TDF + FTC
T-20	FTC	MK-2048	DRV	RAL + AMD3100	RAL + TDF + FTC
	NVP	EVG		RAL + EFV	DRV + TDF + FTC
	EFV			DRV + AMD3100	
				DRV + EFV	
				DRV + RAL	

Drug names, abbreviations and concentrations used are given in Materials & Methods. All drugs listed except AMD3100 and MK-2048 are currently in clinical use as part of various HAART regimens.

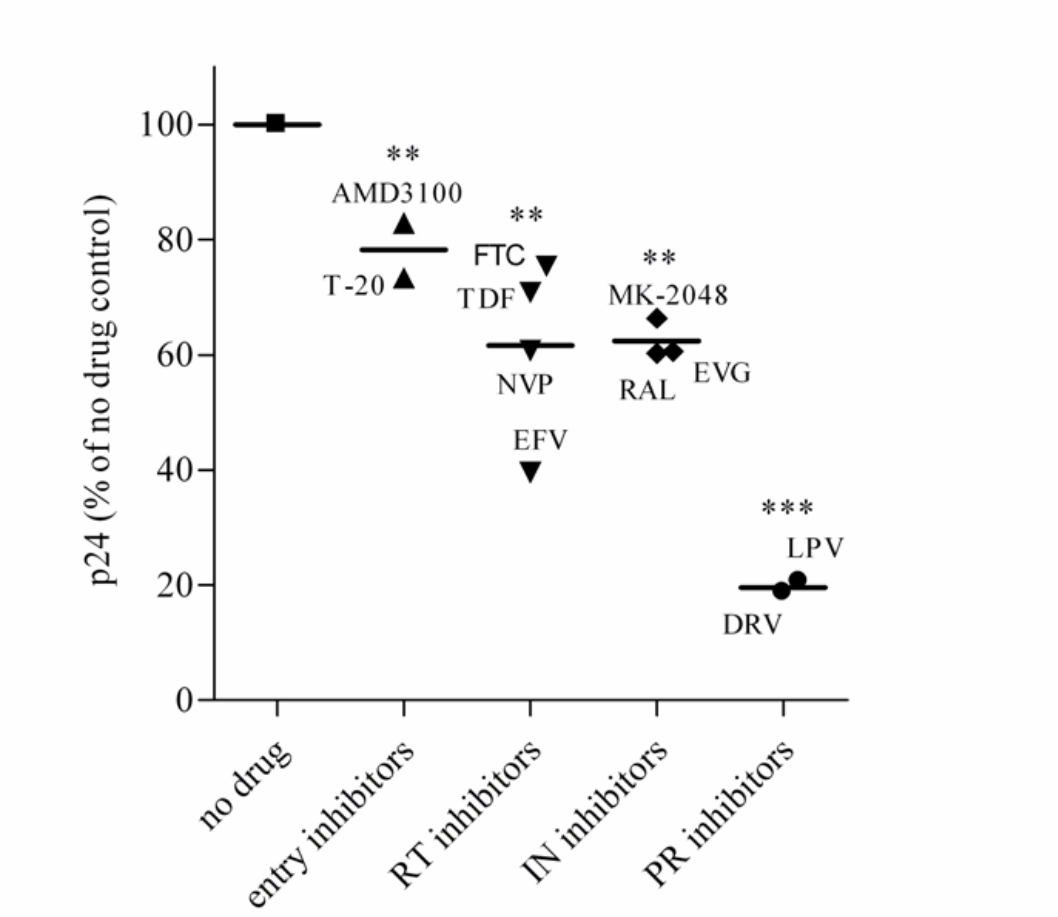

Figure 2.2. Stage-dependent inhibition of HIV-1 replication by individual drugs. Infection of PM1 cells with NL4-3, and drug treatments, were carried out as described in Figure 2.1. For each of four stages of viral replication, two or more drugs were individually added during ongoing replication at five days after infection. p24 measurements were performed on samples collected six days after infection (one day after drug addition). Each data point represents, for any given drug, the average of the means of three independent experiments, relative to no drug controls. Horizontal bars represent the mean of all drugs targeting that stage of replication. Unpaired two-tailed t-tests were used to test for statistically significant differences between the no drug controls and each treatment group. No drug vs. entry inhibitors, $p=0.0061$; vs. RT inhibitors, $p=0.0059$; vs IN inhibitors, $p=0.0021$; and vs. PR inhibitors, $p<0.0001$.

2.5.3 The latest-acting drug in a combination largely determines the level of inhibition of viral replication when added during ongoing infection

Next, we looked at the effect of drug combinations to determine whether, as predicted [6], the latest-acting drug in a combination would dictate the level of inhibition of viral replication. In the same manner as for individual drugs, dual or triple drug combinations were added to cells that had been infected for 5 days. Dual combinations were selected such that each pair of different-stage inhibitors would be employed (Table 2.1), while triple combinations were selected taking into account both the clinical trials comparing RAL and EFV [2-4], as well as current first-line treatment recommendations in the United States [21]. The results of Figure 2.3 show that the addition of an earlier-acting to a later-acting drug resulted in similar levels of inhibition as that obtained using the later-acting drug alone. One-way ANOVA showed that when an RT or PR inhibitor was the latest-acting drug in a combination, there was no statistically significant difference in the amount of viral inhibition compared to the use of the RT or PR inhibitor alone. However, one-way ANOVA showed that there was a statistically significant difference ($p=0.021$) in the responses within the group where an integrase inhibitor was the latest-acting drug. A subsequent Dunnett's multiple comparison test (a post-test used after an ANOVA that looks for differences between control and treatment groups, in this case differences between the use of RAL alone or in combination as the latest-acting drug) showed that the addition of EFV as an earlier-acting drug resulted in greater inhibition ($p<0.05$) than the use of RAL alone, consistent with the results reported in Figure 2.2. In contrast, there were no significant differences to the addition of other earlier-acting drugs to RAL compared to the use of RAL alone.

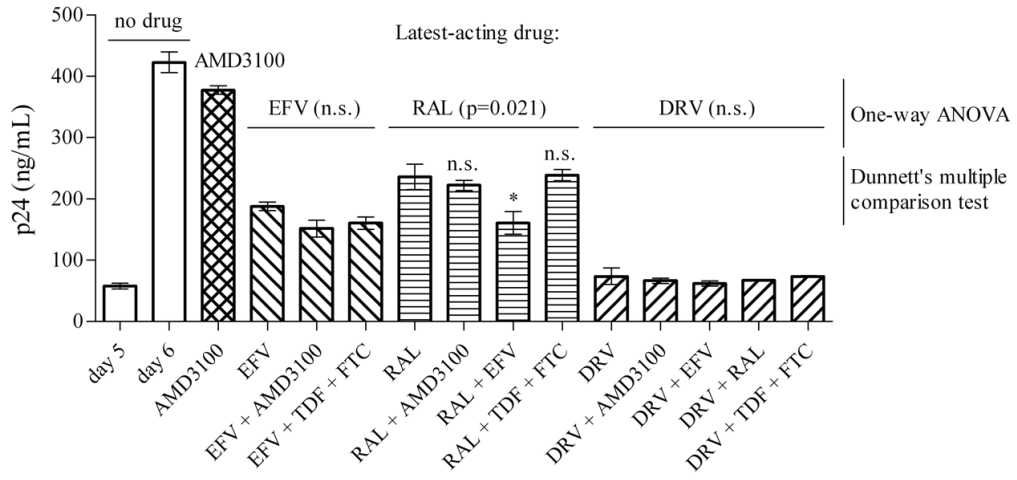

Figure 2.3. Stage-dependent inhibition of HIV-1 replication by drug combinations.

Infection of PM1 cells with NL4-3, and drug treatments, were carried out as described in Figure 2.1. Individual drugs or drug combinations were added during ongoing replication at five days after infection. p24 measurements were performed on samples collected six days after infection (one day after drug addition). Drug combinations are grouped according to the drug that acts latest in the viral replication cycle (for example, the combination of an IN inhibitor and an entry inhibitor is in the IN inhibitor group, while the combination of an IN inhibitor and a PR inhibitor is in the PR inhibitor group). Data are expressed as the mean +/- SEM and are representative of three independent experiments. For each of the three "latest-acting" drug combination groups (RT, IN or PR inhibitors), one-way ANOVA was performed to test for statistically significant differences in p24 levels within each group. Where one-way ANOVA indicated that $p<0.05$, which was only observed in the IN inhibitor (RAL) group, Dunnett's multiple comparison test was performed. This was used to test for statistically significant differences between the single drug (RAL) and all combinations in which RAL was the latest-acting drug to be used, to identify which combination(s) differed significantly from the RAL only treatment. n.s., not significant; * $p<0.05$.

2.5.4 Measurement of viral RNA masks the antiviral activity achieved by protease inhibitors following drug addition but not that achieved by other drug classes.

The model of stage-dependent inhibition of viral replication predicts that drugs acting the latest in the viral replication cycle will result in the greatest level of decay of viremia [6], and our results are consistent with that prediction (Figures 2.2 and 2.3). However, clinical trials with RAL showed more rapid drops in viral load than had previously been shown for other drugs, including PR inhibitors, which act at a later stage of replication than IN inhibitors. We therefore wished to determine whether this apparent discrepancy might be due to measurement of the viral load as a marker of drug efficacy, as opposed to measurement of a viral product whose production is blocked by PR inhibitors (such as processed p24). In experiments similar to those whose results are presented in Figures 2.1 to 2.3, all drugs listed in Table 2.1 were added individually to acutely infected PM1 cells at 5 days p.i., and the levels of p24 and viral RNA in supernatants collected at 24 h after drug addition were determined. The results presented in Figure 2.4 show that the measurements of p24 or viral RNA were comparable in tests with all drugs acting prior to proteolytic processing. Conversely, the use of PR inhibitors led to very low levels of processed p24 compared to the levels for the no-drug controls, although the levels of viral RNA present after the use of PR inhibitors were significantly higher than those for the other drugs used.

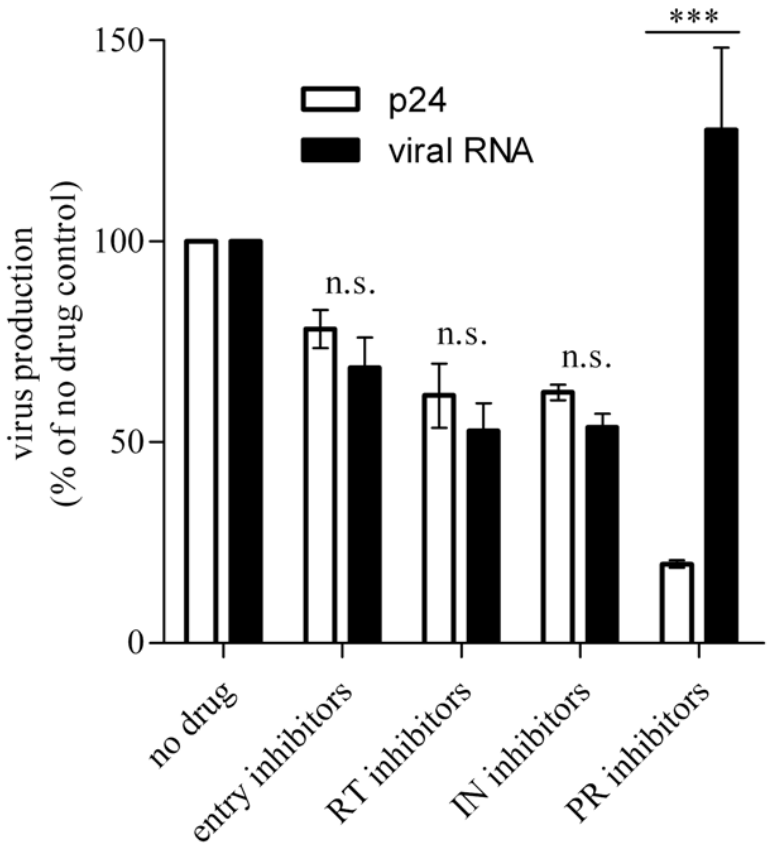

Figure 2.4. Comparison of p24 and viral RNA levels as markers for the antiviral activities of drugs acting at different stages of HIV-1 replication.

Infections were carried out as described Figure 2.1. For each of four stages of viral replication, two or more drugs were individually added during ongoing replication at 5 days after infection (all drugs listed in Table 2.1 were used individually). Measurements of p24 (by ELISA) or viral RNA (by qRT-PCR) were performed using samples collected 6 days after infection (1 day after drug addition). Each bar represents the mean inhibition of virus production relative to that for the no-drug controls, for all drugs acting at each stage of replication, from three independent experiments. Error bars represent SEM. Unpaired two-tailed *t* tests were used to test for statistically significant differences between measurements of p24 or viral RNA as an indicator of inhibition of virus replication for each class of drug. n.s., not significant; ***, $P < 0.0001$.

2.5.5 Stage-dependent inhibition during single-round infection

To complement the above results, we also wished to study pseudovirus capable of only a single round of infection. This would allow us to determine whether or not stage effects might occur in a more tightly controlled system, in which levels of inhibition might be correlated to the stage of viral replication that was underway at the time of drug addition.

First, qPCR was used to determine the replication kinetics of a GFP-encoding NL4-3-based pseudovirus following synchronous infection of PM1 cells. Early and late reverse transcription products, 2-LTR circles, and integrated DNA were quantified (Figure 2.5A-B), from which we determined the time points for drug addition in further experiments. Next, we added the RT inhibitors EFV or NVP, or the IN inhibitors RAL or MK-2048, at defined time points after infection of PM1 cells by the same virus. The percentage of GFP-positive cells was measured by flow cytometry at 48 hrs p.i. (Figure 2.5C). Our data show that IN inhibitors consistently resulted in equal or greater inhibition of viral replication over time than did RT inhibitors, when added at discrete time points following single-round infection. Similar results were obtained when these experiments were repeated with SupT1 cells (data not shown).

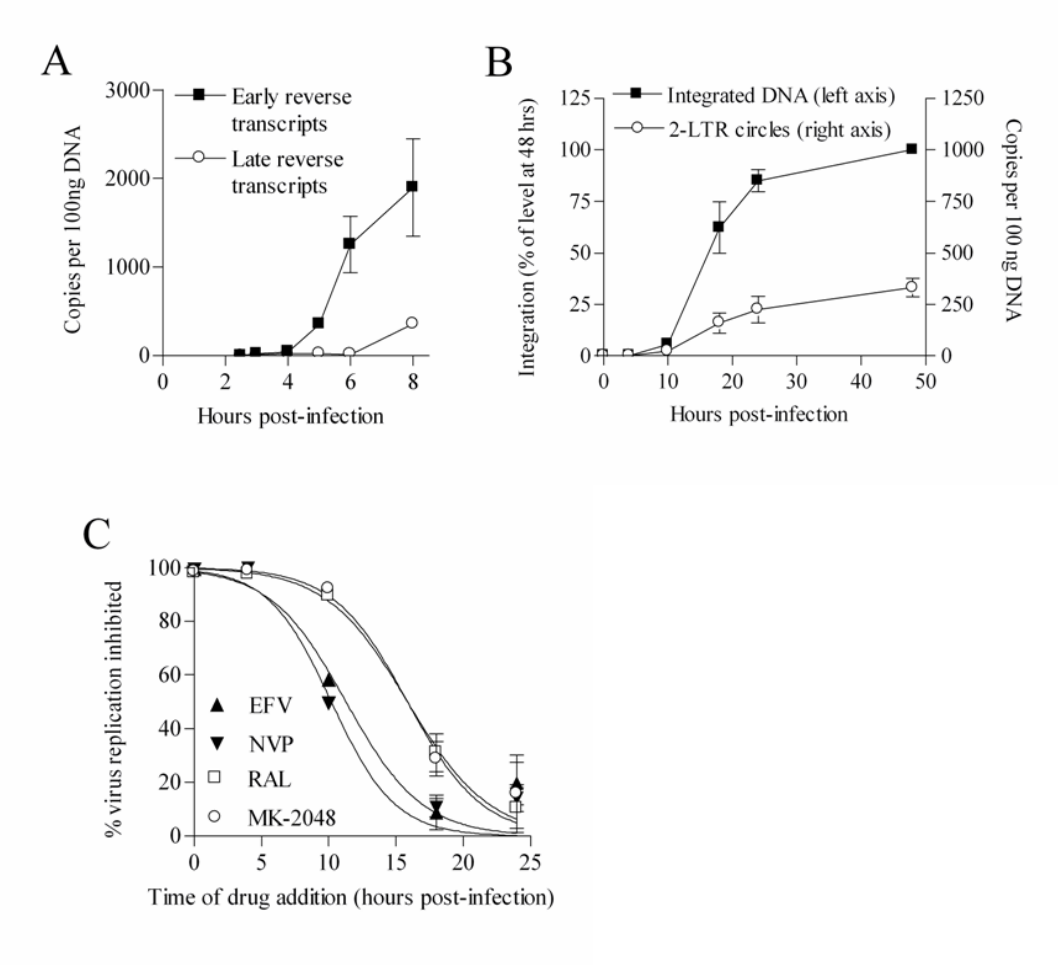

Figure 2.5. Kinetics of reverse transcription, integration, and stage-dependent inhibition of HIV-1 replication during single-round infections.

PM1 cells were infected by spinoculation with NL4-3-deltaE-EGFP pseudotyped with the AD8 envelope (capable of only a single round of infection), and washed extensively to remove unbound virus. (A) Early and late reverse transcription products and (B) integrated DNA and 2-LTR circles, were quantified by qPCR. (C) The RT inhibitors EFV or NVP, or the IN inhibitors RAL or MK-2048, were added individually at defined time points after infection of PM1 cells by the same virus. The percentage GFP-positive cells was determined at 48 hrs p.i. by flow cytometry, and is expressed relative to no drug controls. Data represent the means +/- SEM of three independent experiments, and were fitted to sigmoidal dose-response (variable slope) curves.

2.6 DISCUSSION

The continuous evolution of drug-resistant variants of HIV-1 and -2, combined with the adverse effects and toxicity associated with many available drugs, necessitates the ongoing development of novel antiretrovirals with non-overlapping resistance profiles and improved tolerance. RAL represents a major step forward in this regard since it is the first in a new class of drugs, has fewer reported adverse effects than EFV [2, 3], and appears to have achieved better initial results as part of first-line combination therapy than one of the most successful regimens currently available. The most plausible explanation reported in the literature to help interpret the unique viral load decay dynamics observed with RAL comes from mathematical modeling of stage-dependent inhibition [6, 7].

In the present study, we wished to establish a cell culture model of stage-dependent inhibition of viral replication that would provide *in vitro* evidence to support or counter the predictions made by mathematical modeling of the stage effect. Since this model applies to both first- and second-phase sources of virus and can be used to analyze either phase of decay separately but since the cells responsible for second-phase virus production remain to be conclusively identified [7, 22, 23], we decided to use a cell line that represents a first-phase source of virus production. Additionally, we reasoned that if our data provided evidence for stage-dependent inhibition in a first-phase source such as T cells, this would add strength to the application of this concept to additional (second-phase) sources.

We first described the establishment of a cell culture model of ongoing infection in which drug is added after several days of infection, such that viruses at various stages of replication are represented simultaneously (Figure 2.1). The infection conditions in this model permit the detection of p24 levels at one day after drug addition to determine whether stage effects occur *in vitro*. When drugs targeting

one of four different stages of viral replication were added individually to infected cells, we observed that the levels of inhibition achieved were, in general, strongly influenced by the stage of viral replication targeted (Figure 2.2) We next showed that the latest-acting drug in a combination largely determined the extent of inhibition achieved, since the addition of one or more earlier-acting drugs to a later-acting drug generally resulted in little additional effect (Figure 3.3).

We then showed that measurement of viral RNA rather than another marker of virus production (i.e., processed p24) masks the antiviral activity that is achieved by PR inhibitors but not by inhibitors of other stages of viral replication following drug addition (Figure 2.4). This is an expected result, since viral RNA is still produced in the presence of PR inhibitors and likely explains the apparent discrepancy between the greater antiviral effect that late-acting drugs are predicted to have, and clinical observations that the use of RAL achieves a decay in the level of viremia more rapid than that which had been observed with all other drugs. Finally, we performed infections with viruses capable of only a single round of replication; in those experiments, we had already determined the kinetics of reverse transcription and integration. Levels of inhibition by the later-acting drugs equal to or greater than the levels of inhibition by earlier-acting drugs were observed when drugs were added at any time point up to the time that the latest stage of replication targeted was mostly complete (Figure 2.5). Taken together, our data represent the first *in vitro* evidence that the stage of viral replication targeted by a class of drug during ongoing infection contributes to the level of viral inhibition initially achieved by that drug.

It is notable that EFV achieved a greater level of inhibition than other RT or IN inhibitors in our ongoing-infection experiments but not in the single-round-infection experiments, a deviation from the overall trend of the stage-dependent inhibition of viral replication. This highlights the fact that additional factors,

including pharmacological differences between individual drugs, likely also contribute to the levels of viral inhibition that are achieved. The results achieved with EFV in particular may be attributable to its reported ability to achieve very high levels of inhibition at each round of viral replication, as illustrated by pharmacological parameters related to the slope of a drug's dose-response curve [20]. Our experiments were designed to determine whether or not stage effects occur in general when the activities of drugs that target different stages of replication are compared and not to make direct comparisons of the activities of any two drugs. Furthermore, our data show that stage-dependent inhibition contributes to the level of viral inhibition achieved by a drug or drug combination following drug addition but that the stage at which a drug acts is not the sole determinant of its antiviral activity.

A limitation of our work is also that infections were performed by using a representative first-phase but not a second-phase cell type. Although the mathematical model can be used to analyze stage-dependent inhibition in first- or second-phase cell types separately, additional *in vitro* data obtained with a second-phase cell type, potentially a monocyte-derived macrophage culture, would prove valuable. A potential factor that might have affected our determination of the stage-dependent antiviral activity of entry inhibitors is that the direct cell-to-cell transfer of virus occurs with a much greater efficiency than cell-free infection in both cell lines and *in vivo*. Data as to whether direct cell-to-cell transfer might shield viral particles from the effects of entry inhibitors are inconclusive.

How exactly can stage-dependent inhibition account for the unique aspects of second-phase decay that were observed clinically with RAL? Notably, the rate of viral load decay during the second phase was observed to be the same with both EFV and RAL, but the level of viral RNA at the start of the second phase was 70% lower with RAL. It can be assumed that virus in this phase is likely produced by

cells of the monocyte/macrophage lineage [7, 24, 25] that were infected before treatment began. Since infected macrophages appear to be resistant to immune-mediated clearance and die at the same rate, regardless of infection status, the rate of viral load decay in the second phase would not be expected to differ, no matter which drugs are used. The lower viral load at the start of the second phase with RAL is likely a result of the delayed kinetics of reverse transcription and integration during infection of monocytes and macrophages [26-30]. The relatively long lag time between these two processes (up to 5 days in monocytes [26]) may give RAL a greater number of target cells in which to act compared to that for EFV when treatment begins. Thus, the additional viral load present at the start of the second phase with EFV but not RAL may arise from long-lived infected cells whose viruses had completed reverse transcription but not integration at the time that treatment began.

2.7 CONCLUSIONS

Our results provide the first experimental (*i.e.* not *in silico*) evidence in favour of a model whereby the temporal stage of HIV-1 replication that is targeted by an inhibitor can have a substantial impact on levels of virus production. As described above, this effect is observed following the initial addition of drugs to an ongoing infection. Furthermore, the duration of this stage-dependent effect is equal to the remaining lifespans of all cells that were already infected when the treatment was initiated. In a first-phase cell source such as activated CD4 T-cells or T-cell lines, stage-dependent inhibition occurs, but only lasts for ~1-2 days (*i.e.* until all infected cells die and new rounds of infection are prevented by the inhibitors used). In a longer-lived second-phase cell source, this effect would be much greater in duration, and in fact, our results strongly suggest that this contributes to the differential viral load decay dynamics observed clinically with raltegravir (Figure 2.0.1). A model of stage-dependent inhibition, as originally proposed in reference [6] and supported by the results of our study, is presented in Figure 2.6.

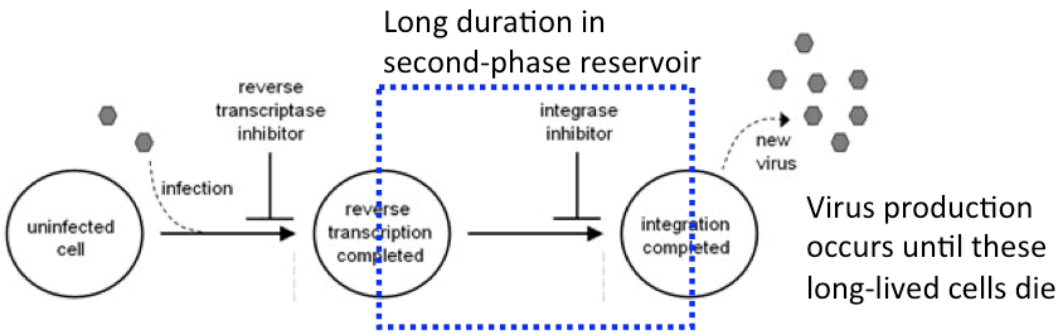

Figure 2.6. Model of stage-dependent inhibition of viral replication.

Integrase inhibitors, but not RT inhibitors, would block production of viruses from cells in which reverse transcription but not integration had already occurred, at the time of drug addition. In long-lived infected cells, and/or in cells where there is a long lag time between the completion of reverse transcription and integration, this effect could make a substantial contribution to viral load decay dynamics.

After our results were published, an additional report from the authors of the original stage-dependent mathematical model presented further support for the idea that the effectiveness of HAART can be influenced by viral life cycle kinetics [31]. Furthermore, after the publication of our data, a study appeared which suggested that macrophages are not likely to represent the major source of virus production during the second phase of viral load decay [32]. Thus, the identity of these second-phase cells remains unknown.

In conclusion, the work presented in this chapter provides insights into virus production by second-phase reservoirs and the associated viral load decay dynamics observed in clinical trials.

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Chapter 3

The Viral Protein Tat Can Inhibit the Establishment of HIV-1 Latency

This chapter was adapted from the following published manuscript:

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All experiments and data analysis included in this chapter were performed by myself under the supervision of Dr. Mark Wainberg. BD Kuhl and RD Sloan assisted with some aspects of experimental design and offered suggestions for revisions to the manuscript.

3.1 PREFACE

As discussed in Chapter 1, clinical approaches to HIV-1 latency are focused on eradication of already-established latent reservoirs, primarily through reactivation and depletion of latently infected resting CD4 T-cells. In Chapter 3, I focus on a novel approach to limit the establishment of latent infection. Section 1.6.4 provided a detailed discussion of the known mechanisms of latency establishment. As covered in greater detail in the introduction to Chapter 3, below, HIV-1 Tat protein can counteract many of the pathways involved in the establishment/maintenance of latency, in addition to its primary role in regulating transcriptional elongation. We therefore hypothesized that Tat protein might be useful, at least experimentally, as a "treatment" to limit the establishment of latency. It is worth highlighting that although the effects of Tat on these various pathways have been partially characterized, no previous studies have examined the ability of Tat to inhibit latency establishment in a system where levels of latency could be quantified. The work presented in this chapter provides evidence that it is theoretically possible to inhibit the establishment of HIV-1 latency.

3.2 ABSTRACT

The establishment of HIV-1 latency can result from limiting levels of transcription initiation or elongation factors; restrictive chromatin modifications; transcriptional interference; and insufficient Tat activity. Since the viral protein Tat can counteract many of these factors, we hypothesized that the presence of exogenous Tat during infection might inhibit the establishment of latency. This was explored using a Jurkat model of latency establishment and reactivation. PCR and RT-PCR confirmed the latent state in this model and showed evidence of transcriptional interference. To address our hypothesis, cells undergoing infection were first exposed to either purified recombinant Tat or a transactivation-negative mutant. Only the former resulted in a modest inhibition of the establishment of latency. Next, Jurkat cells stably expressing intracellular Tat were used in our latency model to avoid limitations of Tat delivery. Experiments confirmed that intracellular Tat expression did not affect the susceptibility of these cells to viral infection. Eight weeks after infection, Jurkat cells expressing Tat harboured up to 1700-fold fewer ($P<0.01$) latent viruses than Jurkat cells that did not express Tat. Additionally, Tat delivered by a second virus was sufficient to reactivate most of the latent population. Our results suggest that inhibition of the establishment of latent infection is theoretically possible. In a hypothetical scenario of therapy that induces viral gene expression during acute infection, activation of viruses which would otherwise have entered latency could occur while concurrent HAART would prevent further viral spread, potentially decreasing the size of the established latent reservoir.

3.3 INTRODUCTION

HIV-1 gene expression is dependent upon the viral protein Tat, which controls transcription at the level of RNAPII elongation through interaction with the TAR RNA and the positive transcription elongation factor b (P-TEFb, composed of Cyclin T1 (CycT1) and cyclin-dependent kinase (CDK) 9). The recruitment of P-TEFb by Tat leads to several phosphorylation events carried out by CDK9 that convert the paused elongation complex to a highly processive form (reviewed in [1]). The net result of these post-translational modifications is synthesis of high levels of full-length viral transcripts.

The establishment of HIV-1 latency primarily results from one or more blocks at the transcriptional level [2]. NF-κB and/or NFAT (depending on the cell type) are required for initiation of viral transcription through binding to κB sites on the 5' LTR. These transcription factors can have their target DNA sequences occupied by transcriptional repressors, or can be sequestered in the cytoplasm (especially in resting CD4+ T cells), limiting transcription initiation such that the virus enters latency [3]. Mutations at κB or Sp1 sites on the 5' LTR can also promote entry into latency [4]. Additional blocks at the level of transcription initiation are imposed by specific epigenetic chromatin modifications at nucleosomes on the 5' LTR, notably, deacetylation and methylation of histone N-terminal tails. These modifications both alter the physical conformation of chromatin and dictate which proteins can interact with chromatin [5], and are believed to be a driving force in the establishment of HIV-1 latency [6, 7]. DNA methylation has been associated with HIV-1 latency, but likely enhances silencing of already-latent viruses rather than contributing to entry into latency [8, 9]. P-TEFb is required for efficient elongation of viral transcripts. It is regulated by sequestration into the 7SK ribonucleoprotein (RNP) complex that includes 7SK small nuclear RNA (snRNA) and Hexim1; Hexim1 obstructs the ATP pocket of CDK9 (reviewed in [10]). Additionally, limited levels of active P-TEFb have been associated with the establishment of latency in primary CD4+ T cells [7]. Since most HIV-1 integration

occurs within introns of actively expressed genes [11-14], transcriptional interference can result. This phenomenon occurs when transcription from a cellular promoter antagonizes transcription initiation or elongation from the integrated viral promoter, and is an important mechanism contributing to the establishment and maintenance of latency [3, 14-17]. Additional blocks to elongation can occur if overall Tat activity is insufficient, which can result from subthreshold Tat levels (for example, due to random fluctuations [18-20]) or mutations that attenuate Tat activity [21]. Finally, post-transcriptional blocks to HIV-1 gene expression have been associated with latency, including insufficient nuclear export of unspliced viral mRNA [22] and silencing by cellular micro RNAs [23], although whether these contribute to the establishment of latency is unclear.

There are several mechanisms by which Tat, if present in sufficient quantities, might counteract the establishment of HIV-1 latency by promoting transcriptional initiation or elongation. The transcriptionally active form of NF-κB, p50/p65, can be sequestered in the cytoplasm by IκBα. Tat itself can induce nuclear translocation of NF-κB p50/p65 [24], probably via direct interaction with PKR (double-stranded RNA-dependent protein kinase) which can result in the degradation of IκBα [25]. In addition to promoting transcriptional initiation, NF-κB p50/p65 can displace HDAC1 (histone deacetylase 1)-bound p50/p50 homodimers from κB sites on the viral promoter. Restrictive chromatin modifications are also subject to regulation by Tat. Histone acetyltransferases (HATs) including p300, CBP and PCAF are recruited to the 5' LTR by Tat [26-28], where they can reverse the effects of histone deacetylation. Nucleosome remodeling is induced by Tat via recruitment of the Ini1, BRG-1 and Brm components of the SWI/SNF chromatin remodeling complex [29-32] and via recruitment of the histone chaperone hNAP-1 [33], relaxing chromatin structure and thereby permitting transcription. Tat can also overcome blocks to elongation by disruption of the 7SK RNP complex through direct displacement of Hexim1,

resulting in increased nuclear levels of enzymatically active P-TEFb [34-37]. Recent findings show that Tat also stimulates elongation through recruitment of the elongation factor ELL2 (which aids elongation by appropriately aligning nascent mRNA in the RNAPII active site) *via* interaction with AFF4, resulting in cooperative stimulation of elongation between ELL2 and P-TEFb [38]. Further, there is evidence that Tat can partially overcome transcriptional interference from some cellular genes, possibly by tipping the "balance of power" in favour of the 5'LTR rather than the cellular promoter [15, 17, 39]. The effects of Tat on mechanisms of latency are summarized in Table 3.1. Finally, it was reported that CD4+ T- cells from patients on suppressive HAART were enriched for viruses with attenuated transactivation activity resulting from mutations in Tat. This implies that decreased levels of Tat activity can contribute to the establishment of latency *in vivo* [21].

Table 3.1. Effects of Tat on pathways of latency

Latency mechanism	**Known to be influenced by Tat?**
Transcriptional interference	Yes
Limiting transcription factors	Yes
Limiting P-TEFb	Yes
Transcriptional repressors	Yes
Histone deacetylation	Yes
Histone methylation	No
DNA methylation	No
Nucleosome positioning	Yes
Insufficient Tat activity	Yes
Insufficient mRNA nuclear export	No
Insufficient mRNA splicing	No
miRNA	Yes
Homeostatic proliferation	No

Given the above, we hypothesized that activation of viral gene expression during infection, by exogenous Tat, would inhibit the establishment of latent infection. We found that purified recombinant Tat had only a limited impact on the number of latently infected cells, while Tat that was provided intracellularly strongly inhibited the entry of HIV-1 into latency.

3.4 MATERIALS AND METHODS

3.4.1 Cells and Viruses

Jurkat (clone E6-1), Jurkat-*tat* [40], JLTRG-R5 [41] and Tzm-bl cells were obtained through the NIH AIDS Research and Reference Reagent Program. Jurkat and JLTRG-R5 cells were maintained in RPMI 1640 medium (Invitrogen); Jurkat-*tat* cells were maintained in RPMI 1640 with 800 µg/ml G-418; and Tzm-bl and 293T cells were maintained in DMEM (Invitrogen). All cells were supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. The HIV-1 molecular clones pNL4-3 and pNL4-3-ΔE-EGFP [42] were obtained through the NIH AIDS Research and Reference Reagent Program. The following mutations were individually introduced into pNL4-3-ΔE-EGFP using the Stratagene QuikChange II XL site-directed mutagenesis kit: H13L (CAT to TTA), C22G (TGT to GGA) and T23N (ACC to AAC). Pseudovirus was produced by co-transfection of 9×10^6 293T cells with 6.25 µg pVPack-VSV-G (Stratagene) – a vesicular stomatitis virus G protein (VSV-G) envelope-encoding construct – in combination with 18.75 µg of pNL4-3-ΔEnv-EGFP (or *tat* mutant derivatives) using Lipofectamine 2000 (Invitrogen). Replication-competent virus was similarly produced, using 25 ug of pNL4-3 or pBR-NL4-3-IRES-dsRed. Supernatants were harvested at 48 h post-transfection, clarified by centrifugation for 5 min at 470 x *g*, and passed through a 0.45 µm filter. Virus was treated with 50 U/ml benzonase (Sigma) in the presence of added benzonase buffer (10X = 500mM Tris-HCl pH 8.0, 10mM $MgCl_2$ and 1 mg/ml bovine serum albumin (BSA)) at 37°C for 20 min to digest contaminating plasmid DNA [43].

3.4.1.1 Cell culture model of latency establishment and reactivation

3.75 x 10^5 Jurkat or Jurkat-*tat* cells were infected with 150 ng p24 of VSV-G-pseudotyped NL4-3-ΔE-EGFP (or the *tat* H13L derivative) in 24-well plates. At 16 hrs post-infection (p.i.), cells were centrifuged at 470 x g for 5 min, virus-containing media was removed, and cells were resuspended in 1 ml fresh media. Cells were cultured for up to 56 days p.i. and split with fresh media as needed. Beginning 2 days p.i. and on subsequent days, the percentage of active, total and silent infection was determined as follows. One third of each well was treated for 24 hrs with TNF-α (20 ng/ml) to reactivate silent/latent virus, one third of each well was subject to control treatment (RPMI only, since TNF-α stocks were made in RPMI), and medium was added to replenish the remaining one third of each well. 24 hrs after TNF-α or control treatment, cells were fixed in 1% paraformaldehyde for 20 min. Flow cytometry was performed using a BD FACSCalibur instrument (Becton Dickinson) and data were analyzed with FCS Express software, by gating for live cells by forward and side scatter and then quantifying the number of cells positive for expression of the virally-encoded EGFP. The percentage of EGFP positive cells after control treatment represents active infection, the percentage after TNF-α treatment represents total infection, and subtracting active infection from total infection represents latent infection.

3.4.2 PCR, RT-PCR and real-time PCR

3.4.2.1 PCR for integrated viral DNA

Cellular DNA was extracted with a DNeasy blood and tissue kit (Qiagen). A previously described nested Alu-gag real-time PCR [44] was modified as described below for use in endpoint PCR with Platinum Taq (Invitrogen). The first round reaction (performed in both the presence and absence of an Alu-specific primer) was performed using 26 ng DNA, 2 mM $MgCl_2$ and 200 µM dNTPs in a total volume of 20 µl, using the primers *Alu*-F (5'-GCCTCCCAAAGTGCTGGGATTACAG-3') and *gag*-R (5'-GTTCCTGCTATGTCACTTCC-3'). Cycling conditions were 95°C for 2 min, and 15 cycles of 95°C for 15 s, 50°C

for 15 s, and 72°C for 3.5 min. 4 µl of the resulting first round product was used as template for the second round nested reaction in the presence of 5 mM $MgCl_2$ (final concentration including carryover from first round) and 200 µM added dNTPs. Cycling conditions were 95°C for 2 min, and 25 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. Second-round primers were LTR-F (5'-TTAAGCCTCAATAAAGCTTGCC-3') and LTR-R (5'-GTTCGGGCGCCACTGCTAGA-3'). β-globin was amplified as an internal control using primers BetaGlo-F (5'-GGTACGGCTGTCATCACTTAGAC-3') and BetaGlo-R (5'-AACGGCAGACTTCTCCTCAG-3').

3.4.2.2 RT-PCR for transcriptional interference

Using a strategy similar to one previously reported [3], RT-PCR was performed to detect transcriptional interference arising from latent viruses integrated into actively transcribing host genes. The forward primer anneals to U3 sequence, *i.e.* upstream of the HIV-1 transcription start site, whereas the reverse primer anneals downstream of U5 near the viral primer binding site (PBS), implying that mRNA containing U3-PBS sequence originated from cellular promoters. Cellular RNA was extracted with an RNeasy mini kit (Qiagen) and treated with Turbo DNase (Ambion) to remove contaminating genomic DNA. RT-PCR was performed using a SuperScript III One-step RT-PCR kit (Invitrogen) and 50 ng RNA, with primers TI-F1 (5'-CACACACAAGGCTACTTCCCT-3') or TI-F2 (5'-GCGAGCCCTCAGATGCTAC-3'), and TI-R (5'-CTTTCGCTTTCAAGTCCCTGTTC-3'). Cycling conditions were 55°C for 15 min, 94°C for 2 min, and 32 cycles of 94°C for 20 s, 60°C for 20 s, and 68°C for 20 s. Reactions were also performed with Platinum Taq only without reverse transcriptase ("no RT" reactions), to ensure that amplified products were derived exclusively from mRNA. GAPDH mRNA was amplified as an internal control with primers GAPDH-F (5'-AGGTCGGAGTCAACGGATTTGG-3') and GAPDH-R (5'-GATGGCAACAATATCCACTTTACCA-3').

3.4.2.3 RT-PCR for viral genomic RNA

Viral RNA was extracted from supernatants of infected cells using a QIAamp viral RNA mini kit (Qiagen). Cycling conditions were the same as for the transcriptional interference RT-PCR, above, but for 30 cycles, using primers total-F (5'-CCGTCTGTTGTGTGACTCTGG-3') and total-R (5'-GAGTCCTGCGTCGAGAGATCT-3') [45]. Products of all PCR and RT-PCR reactions were visualized on 1% agarose TAE gels.

3.4.2.4 Real-time PCR for total viral DNA

Cellular DNA was extracted from infected Jurkat or Jurkat-*tat* cells with a DNeasy blood and tissue kit (Qiagen). PCR was performed with Platinum qPCR SuperMix-UDG (Invitrogen) on a Corbett Rotor-Gene 6000 thermocycler. Cycling conditions were 50°C for 2 min, 95°C for 1 min, and 45 cycles of 95°C for 3 s and 60°C for 30 s, using 65 ng DNA per 20 µl reaction. Primers and probes were total-F, total-R and total-probe (5'-FAM-TCTAGCAGTGGCGCCCGAACAGG-BHQ1-3') [45].

3.4.3 Cloning, expression and purification of recombinant Tat

Tat86 derived from HIV-1_{IIIB} was amplified by RT-PCR from mRNA of Jurkat-*tat* cells using primers that introduced a 5' *NcoI* restriction site (with internal ATG start codon), as well as a 3' (C-terminal) 6xHis tag followed by two stop codons and an *AscI* restriction site. This PCR product was cloned into the *NcoI* and *AscI* sites of a previously used pDEST14 (Invitrogen) expression vector (*i.e.* the sequence between the vector's *attR* recombination sites had previously been removed). The resulting construct was confirmed by sequencing and by detection of expressed Tat86-6xHis by Western blot with an anti-Tat monoclonal antibody (NIH AIDS Research and Reference Reagent Program catalog # 1974). C22G and T23N Tat mutations were introduced separately, using a QuikChange II site-directed mutagenesis kit (Stratagene). Expression of recombinant Tat was induced with 1 mM IPTG (for 3 hrs beginning at OD 0.4) and pellets were frozen until further use. Purification was performed as previously reported [46], with several modifications as described below. All solutions were buffered with HEPES

at pH 8.0, and siliconized materials were not used until collection of eluted proteins. For each 0.5 L starting culture, 15 ml lysis buffer (with 20 mM imidazole) was added and sonication was performed on 2 x 7.5 ml samples. Sonication conditions proved to be critical to successful protein purification, and were optimized as suggested [47]; each 7.5 ml of lysate was subject to 10 rounds of sonication for 20 s at 20% amplitude, with 40 s rest between rounds. Ni-NTA agarose beads (Qiagen) were added to clarified lysates (2 ml of 50% slurry for each 0.5 L original culture) in the batch method, for 1 hr at 4°C. Using 5 ml columns, beads were washed with 40 ml of wash buffer containing triton X-100, followed by 20 ml of wash buffer without triton X-100, 5 ml of wash buffer containing 0.5 M NaCl and 100 mM imidazole, and 5 ml of wash buffer containing 0.15 M NaCl and 200 mM imidazole. Recombinant Tat was eluted with 5 ml of elution buffer containing 0.15 M NaCl and 500 mM imidazole. Eluted proteins were dialyzed against 2.5 L of dialysis buffer (20 mM HEPES pH 8.0, 0.15 M NaCl, 1 mM DTT) in 3.5K MWCO slide-a-lyzer dialysis cassettes (Thermo Scientific) for 18 hrs at 4°C. Protein concentration was calculated from absorbance at 280 nm using appropriate extinction coefficients (0.798 and 0.803 for the T23N and C22G variants, respectively, of Tat86-6xHis in reducing conditions). 6xHis tags were not removed since their presence does not affect Tat's biological activities [46].

3.4.4 Use of recombinant Tat in cell culture

The HIV-1 LTR/Tat-dependent reporter cell lines Tzm-bl and JLTRG-R5 were treated with recombinant Tat proteins to determine biological activity. Tzm-bl cells were treated in 96-well plates with 2.5 µg per well Tat (C22G or T23N) for 4 hrs in serum-free Opti-MEM reduced serum media (Invitrogen), in the presence or absence of a protein transfection reagent (Bioporter Quickease protein delivery kit, Genlantis), and then 1X volume DMEM containing 20% FBS was added. 24 hrs after treatment, luciferase activity was determined using a Bright-Glo luciferase assay system (Promega). JLTRG-R5 cells were treated in 24-well

plates with 12.5 ug Tat (C22G or T23N) as described above, in the presence or absence of a protein transfection reagent, and then 1X volume RPMI containing 20% FBS was added. JLTRG-R5 cells were additionally treated with 125 ug Tat (T23N only, due to insufficient yield of C22G) in the absence of protein transfection reagent. 24 hrs after treatment, JLTRG-R5 cells were fixed in 1% PFA and the percentage of GFP-positive cells was determined by flow cytometry as described above. To determine the effects of purified Tat on the establishment of latency, Jurkat cells were treated during infection as follows. Beginning 16 hrs p.i. (as described above for the Jurkat latency model) cells were spun at 470 x g for 5 min and resuspended in 0.5 ml serum-free media containing 12.5 µg Tat (C22G or T23N) in the presence of a protein transfection reagent. 4 hrs later, 0.5 ml RPMI containing 20% serum was added. This treatment was repeated after 24 and 48 hrs (*i.e.* on days 2 and 3 p.i.), for a total of three treatments. Cultures were maintained until 23 days p.i. Similar experiments were performed with a single, 24 hr treatment of 125 µg Tat (T23N only) beginning at 16 hrs p.i., in the absence of protein transfection reagent. All experiments with recombinant Tat were performed at least three times, using at least three different purifications of each protein.

3.4.5 Statistical Analyses

Unpaired two-tailed t-tests or one-way analysis of variance (ANOVA) were used to test for statistically significant differences as indicated in the figure legends. When significant differences were found by ANOVA, Bonferroni's multiple comparison post-test was used to determine where such differences exist. All statistical analyses were performed with GraphPad Prism 5.0 software.

3.5 RESULTS

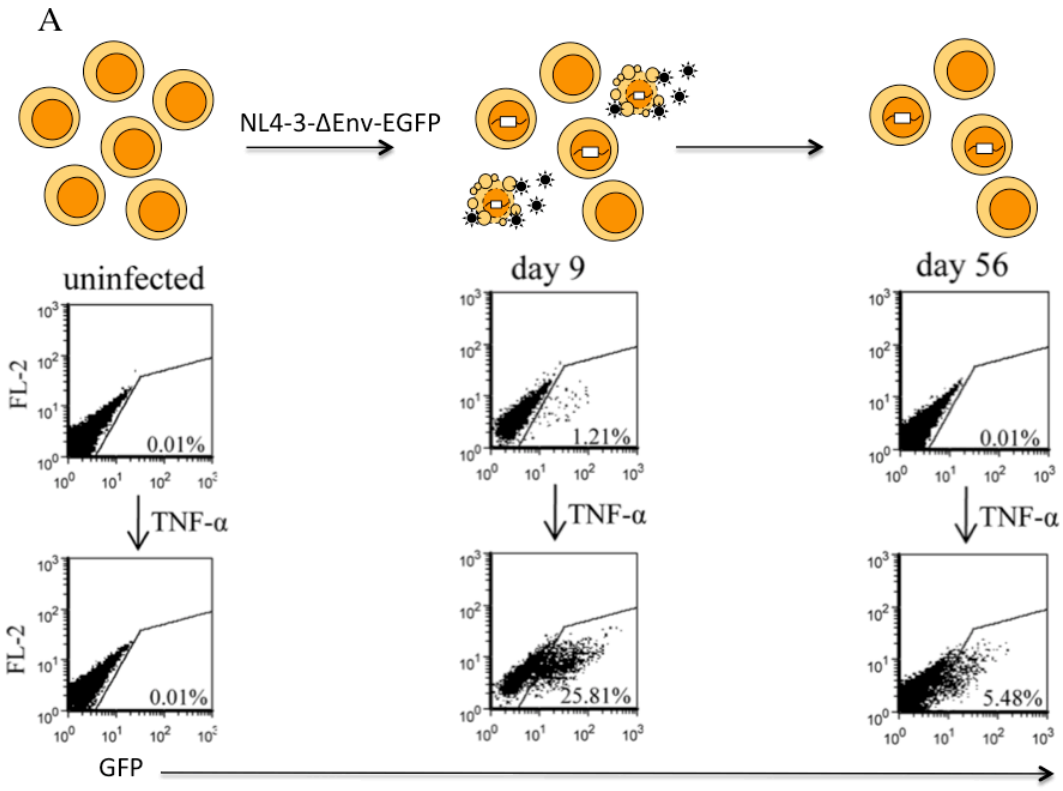
3.5.1 Characterization of a Jurkat-based model of HIV-1 latency establishment and reactivation

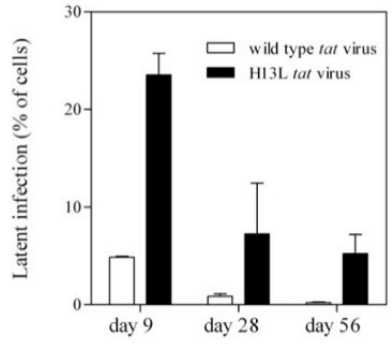
We describe a model of latency establishment and reactivation using CD4+

Jurkat T-cells, which generates heterogeneous populations of latently infected cells carrying full-length viral genomes and that does not select for or against initial patterns of viral gene expression (for example, by restricting the study to only GFP+ or GFP- populations *via* cell sorting). Jurkat cells were infected with NL4-3-Δ*env*-EGFP, and cultured in the absence of selection so as to include all integration events and initial viral gene expression profiles. Following an extended culture period, productively infected cells die by cytopathic effect, leaving only uninfected and latently infected cells. At various times post-infection (p.i.) silent/latent viruses were reactivated with TNF-α and identified by flow cytometry for viral-derived EGFP expression, and could be detected at frequencies as low as 0.002%. Levels of latent infection are calculated by subtracting levels of active infection (% EGFP+ cells following control treatment) from levels of total infection (% EGFP+ cells following TNF-α treatment). Since relatively low levels of latency were obtained with virus expressing wt *tat* (Figure 3.1B), experiments were also conducted with attenuated *tat* viruses carrying the H13L mutation [48] that decreases the affinity of Tat for CDK9 [49]. This mutation decreases transactivation activity by approximately 40% (data not shown), and has been previously used to generate higher levels of latency [6, 7, 50]. Latent infections were 10-20-fold more abundant when the attenuated *tat* virus was used (Figure 3.1A-B).

The latent state was further characterized at 28 days p.i. by PCR and RT-PCR (Figure 3.1C). First, equal levels of integrated viral DNA were present in latently infected cells lacking viral gene expression and TNF-α-treated cells showing EGFP expression. Second, mRNA species containing viral sequence but derived from cellular promoters were present in latently infected (and TNF-α-treated) cells. These transcripts contained U3-PBS sequence spanning nucleotides 407-665 and 57-665. Viral mRNA does not contain the U3 sequence since the transcription start site is located immediately downstream of U3 at nucleotide 454, implying that these U3-PBS mRNAs originated from host promoters. Such transcriptional

interference has previously been reported to be an important mechanism involved in the establishment and maintenance of HIV-1 latency [3, 14-17]. Finally, viral genomic RNA was absent from the supernatants of latently infected cells, but was detected in supernatants of these cells following treatment with TNF-α.


A
NL4-3-ΔEnv-EGFP
uninfected
day 9
day 56
FL-2
0.01%
1.21%
0.01%
TNF-α
TNF-α
TNF-α
FL-2
0.01%
25.81%
5.48%
GFP


B
Latent infection (% of cells)
wild type tat virus
H13L tat virus
day 9
day 28
day 56

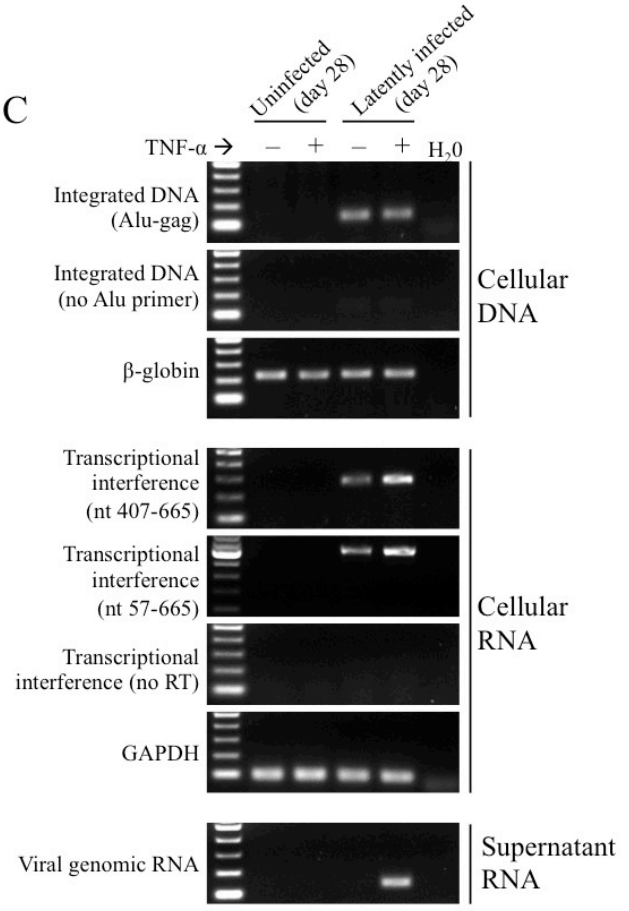

C
Uninfected (day 28)
Latently infected (day 28)
TNF-α →
H20
Integrated DNA (Alu-gag)
Integrated DNA (no Alu primer)
β-globin
Cellular DNA
Transcriptional interference (nt 407-665)
Transcriptional interference (nt 57-665)
Transcriptional interference (no RT)
GAPDH
Cellular RNA
Viral genomic RNA
Supernatant RNA

Figure 3.1. Model of HIV-1 latency establishment and reactivation.

Jurkat cells were infected with NL4-3-ΔE-EGFP (or the attenuated *tat* (H13L) derivative), and cultured for up to eight weeks. Productively infected cells die by cytopathic effect, leaving only uninfected and latently infected cells, which can be reactivated with TNF-α and quantified by flow cytometry for viral EGFP. (A) Schematic representation of our model of HIV-1 latency establishment and reactivation. Representative flow cytometry results for one of three independent experiments with attenuated *tat* virus are shown. GFP = virus reporter gene expression. FL-2 = empty parameter (red fluorescence, not used here). (B) Results of three independent experiments with attenuated or wild type *tat* viruses; results represent mean +/- SD. (C) PCR and RT-PCR characterization of uninfected or latently infected cells 28 days p.i. Integrated DNA was detected with Alu-gag primers; reactions with no Alu primer serve as a control to confirm that the Alu-gag band is derived from integrated DNA. Two different products of transcriptional interference were detected by RT-PCR, containing viral U3-PBS sequence that is derived from host cell promoters; "no RT" reactions confirm that U3-PBS products are derived from mRNA. Viral genomic RNA was absent in supernatants of control-treated latently infected cells, but was detected following reactivation of latent virus by treatment with TNF-α.

3.5.2 Characterization of recombinant Tat proteins in cell culture

We modified a previously reported protocol to produce Tat that retains a wide array of biological activities and contains low levels of endotoxin [46]. We took advantage of the T23N Tat variant which exhibits greater binding to CDK9 and therefore higher transactivation activity than wild type [51], since this should yield the greatest effect in terms of LTR transactivation. Additionally, we used the C22G Tat mutant which cannot interact with CDK9 [52, 53] and is therefore transactivation negative. Two Tat/LTR-dependent reporter cell lines – the HeLa-based Tzm-bl and the Jurkat-based JLTRG-R5 – were used to test the

transactivation activities of purified T23N or C22G Tat. Direct addition of Tat to JLTRG-R5 cells resulted in detectable activity only at high Tat concentration. However, T23N but not C22G Tat was active in these cells at moderate concentrations when a protein transfection reagent was used, with a maximum of <10% of cells showing detectable Tat activity (Figure 3.2A-B). T23N but not C22G Tat had detectable but low activity when added directly to Tzm-bl cells at moderate concentration, but exhibited high levels of activity when a protein transfection reagent was used (Figure 3.2C).

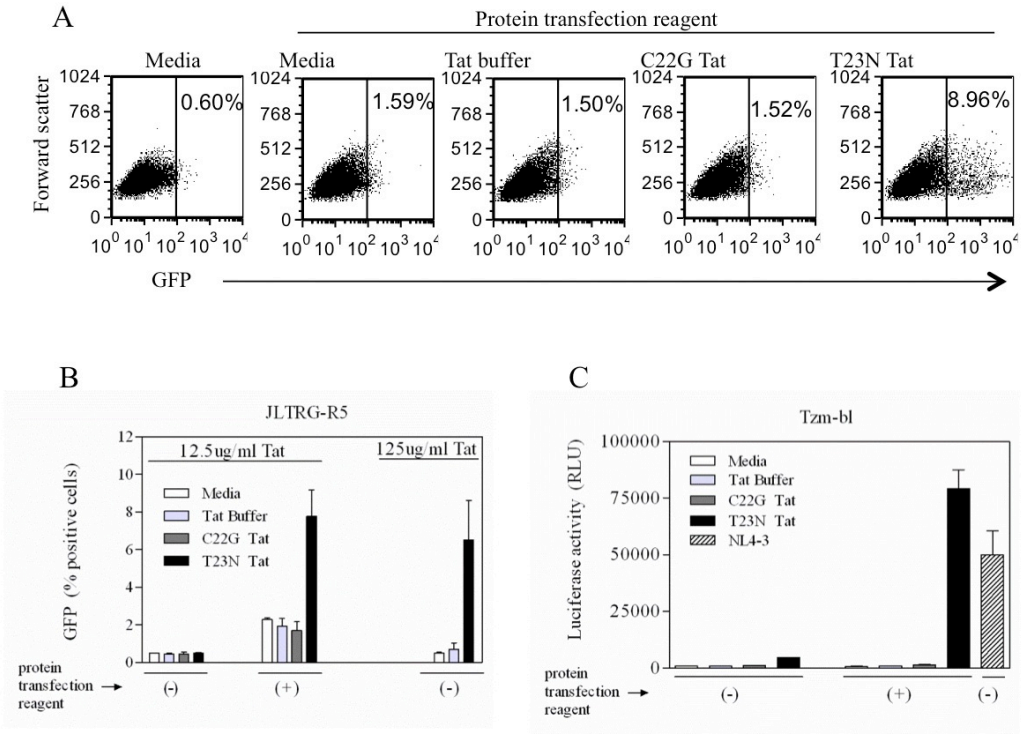


Figure 3.2. Biological activity of recombinant Tat proteins.

Purified T23N (increased transactivation variant) and C22G (transactivation-negative) Tat proteins were added to the LTR/Tat-dependent reporter cell lines JLTRG-R5 (A-B) or Tzm-bl (C), in the presence or absence of a protein transfection reagent. Reporter activity (GFP or luciferase) was determined 24 hrs after Tat addition. (A) Representative flow cytometry results from one of three independent experiments with JLTRG-R5 cells. Cells were treated with 12.5

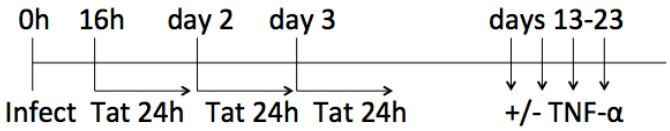
μg/ml recombinant Tat using a protein transfection reagent. (B) Results of three independent experiments in JLTRG-R5 cells treated with moderate (12.5 μg/ml) or high (125 μg/ml) concentration Tat. C22G Tat was not used for 125 μg/ml Tat treatments. (C) Results of three independent experiments in Tzm-bl cells treated with 12.5 μg/ml Tat. Infection with NL4-3 (50ng p24 per well; 24 hr infection) serves as a reference for levels of luciferase activity. Results in (B) and (C) represent mean +/- SD. A protein transfection reagent was used as indicated.

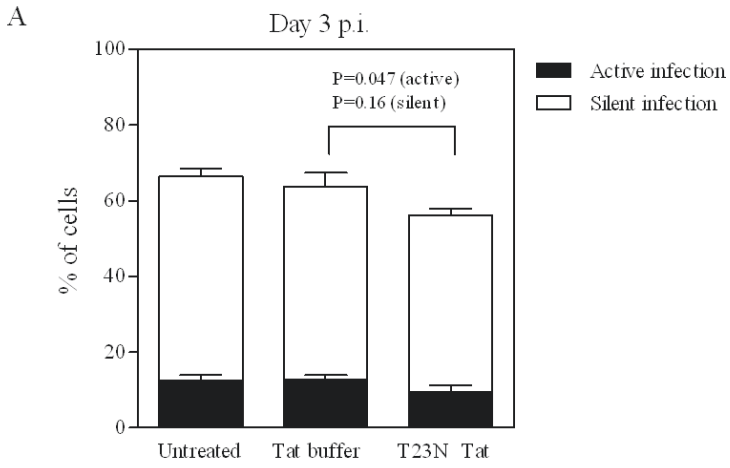
3.5.3 Purified Tat protein modestly inhibits the establishment of HIV-1 latency

We wished to determine whether purified Tat protein could inhibit the establishment of HIV-1 latency, when added during infection of CD4+ T cells. Initial experiments were carried out by treating Jurkat cells directly (no protein transfection reagent) with high concentration T23N Tat for 24 hrs, beginning 16 hours p.i. (since before this time most integration has not occurred [54]). Following Tat treatment, levels of active and silent infection were determined by control or TNF-α treatment followed by flow cytometry for virus-derived EGFP. As shown in Figure 3.3A, for cells treated with T23N Tat, a modest but significant reduction in active infection (26% decrease; P=0.047) was observed compared to control Tat buffer treated cells. An 8% decrease in silent infection levels was observed for these treatments, although this was not statistically significant (P=0.16). Analysis at later times or with longer treatment durations was not possible, since this high concentration Tat treatment resulted in cell death after several days.

Therefore, similar experiments were carried out with moderate Tat concentrations. A protein transfection reagent was used to introduce T23N or C22G Tat into Jurkat cells daily for each of three days p.i., and cultures were continued for up to 23 days to allow for death of productively infected cells and quantification of latent infections. At 13 days p.i., T23N-treated cells harboured

31% fewer latent infections compared to cells treated with control Tat buffer ($P<0.05$); however, this trend was not statistically significant at later times (Figure 3.3B).





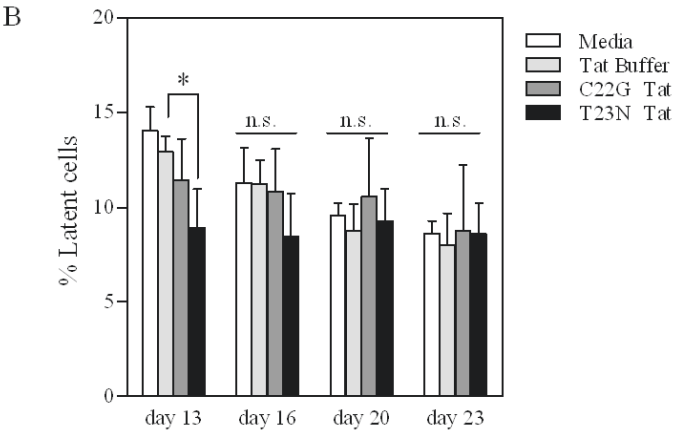


Figure 3.3. Modest inhibition of the establishment of latency by purified Tat.

A schematic representation of the experiments used for this figure is depicted at the top. (A) Jurkat cells were infected with attenuated *tat* virus as described for

Figure 3.1. A single 24 hr treatment with high concentration (125 μg/ml) T23N Tat, in the absence of a protein transfection reagent, was begun starting at 16 hrs p.i. At 48 hrs p.i., cells were treated with TNF-α (or control) to reactivate silently integrated virus. 72 hrs p.i., levels of total, active and silent virus were determined by flow cytometry for viral EGFP. Total infection = % GFP+ cells after TNF-α treatment; active infection = % GFP+ cells after control treatment; silent/latent infection = active infection subtracted from total infection. Unpaired two-tailed t-tests were performed on T23N-treated *vs.* control Tat buffer-treated cells, for both active infection and silent infection. (B) Jurkat cells were infected with attenuated *tat* virus as described for Figure 3.1. Beginning at 16 hrs p.i., cells were treated with 12.5 μg/ml C22G or T23N Tat in the presence of a protein transfection reagent, for 24 hrs. This treatment was repeated twice (*i.e.* starting 24 and 48 hrs after the first treatment began) for a total of three 24 hr treatments. Latent virus was reactivated with TNF-α and quantified by flow cytometry for viral EGFP on days 13, 16, 20 and 23 p.i. One-way ANOVA was performed on the results for each treatment day; Dunnett's multiple comparison post test was used to compare Tat-treated *vs.* buffer-treated cells, when significant differences were found by one-way ANOVA. *, $P<0.05$; n.s., not significant. All results represent mean +/- SD of three independent experiments.

3.5.4 Intracellular Tat expression does not alter the susceptibility of Jurkat cells to infection

Since our results indicated that addition of purified Tat might inhibit the establishment of latency (Figure 3.3), but fewer than 10% of CD4+ Jurkat-based reporter cells exhibited detectable Tat activity (Figure 3.2A-B), we hypothesized that a more efficient delivery of Tat might circumvent this issue. Therefore, we took advantage of Jurkat cells that stably express Tat86 [40]. To rule out any differences in susceptibility to infection between Jurkat and Jurkat-*tat* cells, we infected cells under identical conditions with viruses carrying wt or attenuated (H13L) *tat* genes and confirmed by real-time PCR that viral DNA levels at 18 hrs

p.i. in both cell lines were equivalent (P=0.55 and P=0.42 for wt and H13L *tat* viruses, respectively; Figure 3.4A). To confirm that there are no cell line-specific differences in the capacity for integration and subsequent viral gene expression between Jurkat and Jurkat-*tat* cells (with the obvious exception of intracellular Tat expression in the latter), we treated cells with TNF-α for 24 hrs, beginning at 18 hrs p.i., to induce expression of any silently integrated viral genomes. As expected, total (TNF-α treated) viral gene expression was slightly lower in Jurkat cells infected with attenuated *tat* virus (8% less than infection of Jurkat-*tat* cells; P=0.033) due to the absence of wild type Tat (Figure 3.4B). However, our results show that the capacity for total viral gene expression in Jurkat and Jurkat-*tat* cells is equivalent, when wild type Tat derived from either the cell or the virus is present (P=0.78). Collectively, these results imply that no Tat-independent differences exist between these two cell lines in terms of their abilities to support viral infection. Therefore, these cells were used to determine the effects of intracellularly expressed Tat on the establishment of HIV-1 latency.

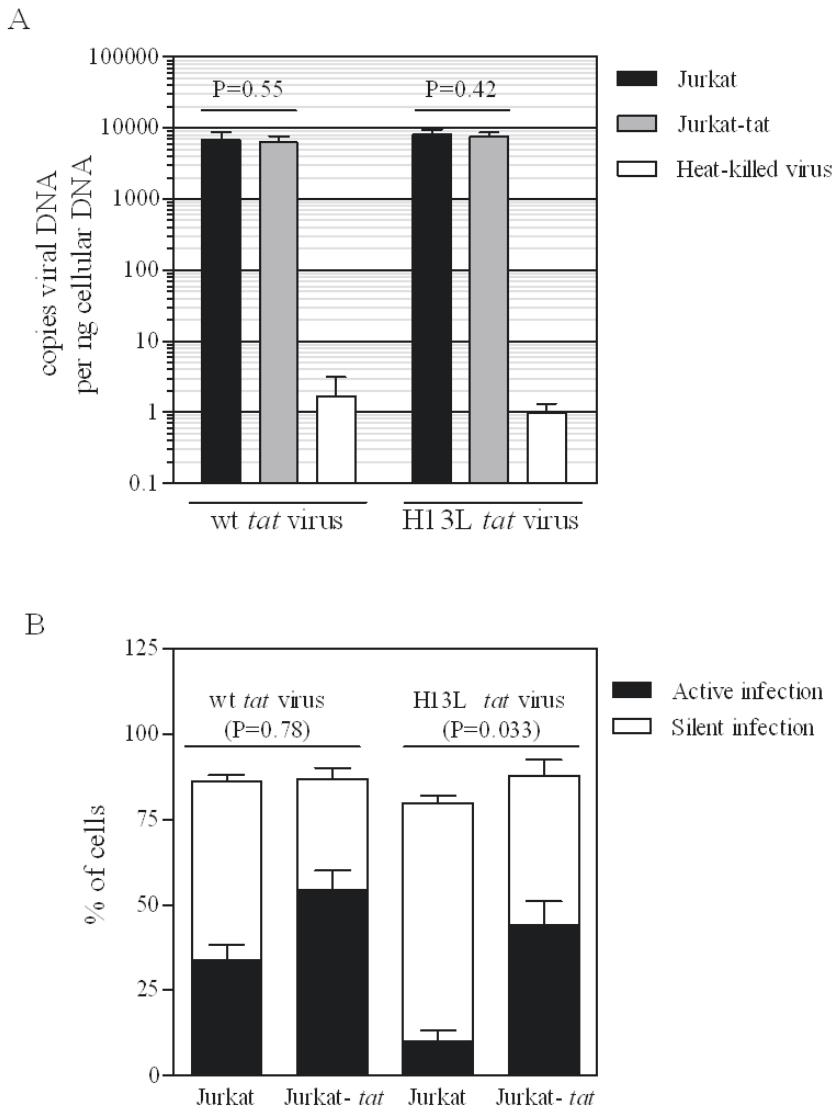


Figure 3.4. Jurkat and Jurkat-*tat* cells are equally susceptible to viral infection.

(A) Jurkat or Jurkat-*tat* cells were infected with VSV-G-pseudotyped NL4-3-ΔE-EGFP (or the H13L *tat* derivative; 400 ng p24 per 10^6 cells) for 18 hrs. Real-time PCR was performed on cells collected 18 hrs p.i. to determine levels of viral DNA. Heat-killed virus serves as a control for residual plasmid contamination from transfection; mock infections were carried out with heat-killed virus under identical conditions. t-tests were used to compare levels of viral DNA in Jurkat *vs.* Jurkat-*tat* cells. Results represent mean +/- SD of two independent experiments. (B) Infections were carried out as in (A). 18 hrs p.i., cells were treated with TNF-α

or control, and viral EGFP was measured by flow cytometry 24 hrs later. t-tests were used to compare total (active + silent) infection levels for Jurkat *vs.* Jurkat-*tat* cells (total infection = % GFP+ cells after TNF-α treatment; active infection = % GFP+ cells after control treatment; silent infection = active infection subtracted from total infection). Results represent mean +/- SD of four independent experiments.

3.5.5 Intracellular expression of Tat during infection strongly inhibits the establishment of HIV-1 latency

After infection in the presence or absence of intracellularly expressed Tat, levels of active, total and silent viral gene expression were monitored over the course of 56 days (as in Figure 3.1A-B). Actively expressing viral genomes led to death of infected cells, so that over time, only uninfected and latently infected cells were present. As shown in Figure 3.5A, the intracellular expression of Tat caused rapid declines in levels of active and total infection over the first 1-2 weeks of infection; this trend is especially pronounced in H13L *tat* virus-infected cells (Figure 3.5B). At 56 days p.i., levels of active infection (control-treated cells) were extremely low in all cases and were not significantly different whether or not intracellular Tat was expressed (P=0.47 and P=0.22 for infection with wt or H13L *tat* virus infection, respectively). However, levels of total infection (TNF-α-treated cells) were significantly lower at 56 days p.i. when intracellular Tat was expressed (P=0.0066 and P=0.0098 for wt or H13L *tat* virus infection, respectively). Levels of latent infection throughout the duration of the experiment are shown in Figure 3.5C. For infection with wt *tat* virus, Jurkat-*tat* cells harboured 13.5-fold fewer (P=0.0060) latent infections by day 56 compared to Jurkat cells. When H13L *tat* virus was used, Jurkat-*tat* cells harboured >1700-fold fewer (P=0.0096) latent infections by day 56 compared to Jurkat cells.

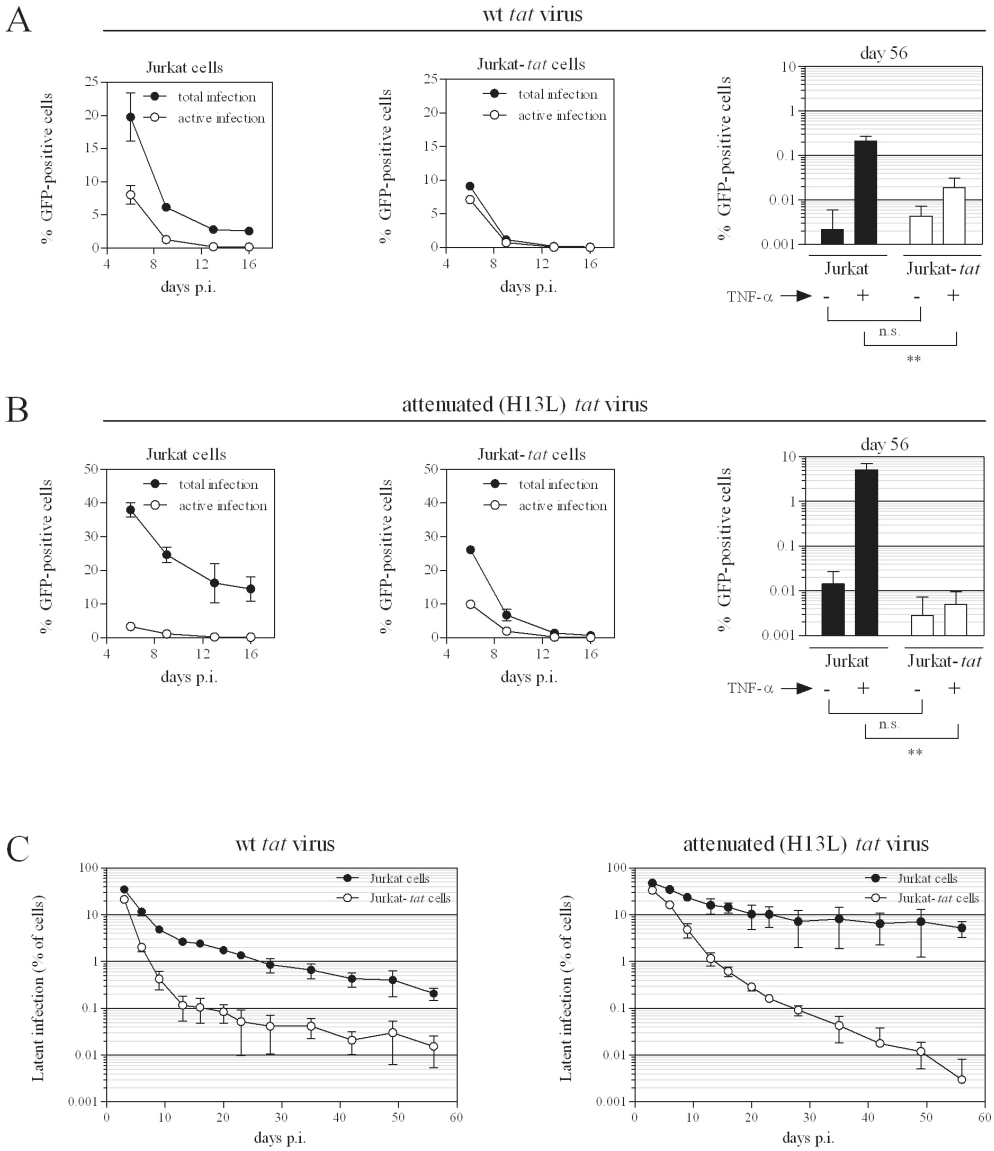


Figure 3.5. Potent inhibition of the establishment of latency by intracellularly expressed Tat.

(A-B) Jurkat or Jurkat-*tat* cells were infected with wt (A) or attenuated (B) *tat* virus as described for Figure 3.1. Levels of total and active infection over the first 16 days of infection are shown (left and middle panels), and on day 56 (right panels). Total infection = % GFP+ cells after TNF-α treatment; active infection = % GFP+ cells after control treatment; silent infection = active infection subtracted from total infection. t-tests were used to compare levels of GFP-positive cells at 56 days p.i. following TNF-α or control treatment, for Jurkat *vs* Jurkat-*tat* cells.

**, P<0.01; n.s., not significant. (C) Levels of latent infection throughout 56 days are shown for wt (left panel) or attenuated (right panel) *tat* virus infection of Jurkat and Jurkat-*tat* cells. All results represent mean +/- SD from three independent experiments. t-tests were used to compare latent infection levels at 56 days p.i. for Jurkat *vs.* Jurkat-*tat* cells; P=0.0060 for wt *tat* virus; P=0.0096 for attenuated *tat* virus.

3.6 DISCUSSION

Latently infected resting memory CD4+ T-cells are of critical importance given that the latent reservoir is established early during acute infection [55], is not susceptible to antiretroviral therapy or host immune attack, and serves as the major source of viral rebound upon treatment interruption or failure [56]. Although several approaches to reactivate latent reservoirs have been used in clinical trials and further trials are underway [57-60], additional strategies to combat HIV-1 latency would be invaluable. The viral Tat protein might, when present, counteract many of the mechanisms involved in the establishment of HIV-1 latency. Tat might do so by (i) inducing nuclear translocation of active NF-κB [24, 25]; (ii) recruiting HATs [26-28], members of the SWI/SNF chromatin remodeling complex [29-32], and histone chaperones [33]; (iii) directly displacing Hexim1 from the 7SK RNP complex thereby increasing levels of active P-TEFb [34-37]; (iv) recruiting the elongation factor ELL2 [38]; and (v) overcoming transcriptional interference from some cellular promoters [15, 17, 39]. Therefore we wished to determine whether exogenous Tat, if present during infection, could inhibit the establishment of HIV-1 latency.

We first characterized a model of latency establishment and reactivation that generates heterogeneous populations of latently infected cells (Figure 3.1). In this model productively infected cells die by cytopathic effect, similar to *in vivo* infection, while only uninfected and latently infected cells propagate. Potential integration site biases were excluded by the absence of any selection, such that

all potential latent infection events can be represented in the population. Since TNF-α has been shown to reactivate latent viruses in all Jurkat models of HIV-1 latency [61], this was used in our model to quantify numbers of latent infections. Gating 50 000 live cells by flow cytometry permitted the detection of latent viruses to a frequency of 0.002%. The use of viruses with the attenuated H13L *tat* mutation increased the number of latent infections by 10-20 fold (Figure 3.1B).

A more detailed characterization of our model by PCR and RT-PCR included detection of integrated DNA, products of transcriptional interference, and viral genomic RNA (Figure 3.1C). Unexpectedly, transcriptional interference appeared to increase rather than decrease upon reactivation of latent viruses by TNF-α treatment. While the reasons for this observation are unknown, it is possible that TNF-α induction of a large number of cellular genes results in a global increase in transcriptional interference, despite viral reactivation. Likewise one might hypothesize that after induction by TNF-α treatment, viral transcription could initiate at additional, upstream transcription start sites prior to the classic "+1" site. This is reasonable to speculate given that dispersed transcription initiation from multiple sites spanning up to 100 nucleotides occurs at many vertebrate promoters (reviewed in [62]), although we are unaware of any evidence for this at the HIV-1 5' LTR. Therefore, RT-PCR was repeated using a forward primer annealing ~400, rather than ~50, nucleotides upstream of the transcription start site and again we observed an increase in transcriptional interference following TNF-α treatment. This likely rules out transcription initiation from dispersed upstream sites. These observations highlight the idea that transcriptional interference – a series of related mechanisms operating at the transcriptional level (reviewed in [63, 64]) – plays a complex role in the establishment and maintenance of latency, as reported in more detailed studies of this phenomenon [14-17].

To determine whether exogenous Tat might inhibit the establishment of HIV-1 latency, purified Tat proteins were first assayed for biological activity on two reporter cell lines. While a Tat variant with increased transactivation activity (T23N) resulted in high-level LTR-driven gene expression when introduced into Tzm-bl cells (Figure 3.2C), treatment of more biologically relevant CD4+ JLTRG-R5 T-cells indicated that a maximum of <10% of treated cells displayed Tat-mediated transactivation activity (Figure 3.2A-B). This indicated that any impact purified Tat might have on the establishment of latent infections could be limited to a sub-population of cells, *i.e.*, those cells which contained biologically active exogenous Tat. This appears to have been the case, as the results of Figure 3.3 suggest that treatment of Jurkat cells with purified Tat protein during infection had only moderate effects at the population level. Only T23N Tat-treated cells, but not C22G Tat-treated cells, exhibited a decrease in latent infection levels, a likely reflection of the affinity of each Tat variant studied for P-TEFb. Unfortunately it was not possible to determine whether this limited effect was due to insufficient overall Tat activity as opposed to the absence of Tat in ~90% of cells undergoing viral infection. This is because when latent infection levels were measured at various days p.i., it was not possible to determine which individual cells might have contained exogenous Tat during the prior treatment period. It might be that the establishment of latency was inhibited to a greater extent in cells which contained exogenous Tat, but that this effect was diluted by the majority of cells that were not affected.

To avoid the concerns associated with delivery of purified Tat, Jurkat-*tat* cells were used to provide Tat intracellularly during infection and throughout the subsequent period of culture. After confirming that the expression of intracellular Tat did not alter the susceptibility of Jurkat-*tat* cells to infection (Figure 3.4), we looked for effects of intracellular Tat on the establishment of latency. During the first days following infection, intracellular Tat led to rapidly reduced levels of active and total viral infection (Figure 3.5A-B, Jurkat-*tat vs.*

Jurkat), due to greater initial viral gene expression and the resulting deaths of productively infected cells (data not shown). The full impact of intracellular Tat expression on the establishment of HIV-1 latency can best be appreciated in the results of Figure 3.5C. While a 13.5-fold reduction in latency levels was observed for wt *tat* virus infections by day 56 p.i., a >3-log decrease in the number of latent infections was observed for attenuated *tat* virus infections. The substantially greater effect observed for attenuated *tat* virus infection is likely due to the higher number of potential latent infections associated with this virus (Figure 3.1B) that were able to be inhibited by the Tat provided intracellularly.

A limitation of our work is that while our experiments used CD4+ Jurkat T-cells, important differences might exist in primary cells. For example, induction of NF-κB is sufficient to reactivate virus in Jurkat models of latency, but evidence suggests that induction of NFAT and/or P-TEFb are required to reactivate virus in primary cell models of latency [7, 61, 65].

Current reactivation strategies focus on already-established latent reservoirs, typically in patients past the stage of acute infection. These existing approaches could be complemented by strategies which attempt to limit the initial size of the latent reservoir. This idea has been discussed in recent studies, which have suggested that early treatment initiation during acute infection might decrease the size of established latent reservoirs [66-69]. Resolving whether this actually does occur has been highlighted as a question of importance [59]. Our results suggest that inhibition of the establishment of latent infection events is theoretically possible. A hypothetical therapy option during acute infection could include treatment with compounds that aim to counteract the factors involved in the establishment of latency. These compounds could induce PKC activity or increase the available P-TEFb pool, or they could inhibit restrictive chromatin modifications. In such a scenario, activation of viruses which would otherwise have entered latency could occur while concurrent HAART would prevent further

viral spread, potentially decreasing the size of the established latent reservoir.

3.7 CONCLUSION

The work presented in this chapter provides the first evidence using a biological system where latency levels could be measured, which suggests the inhibition of the establishment of latency is possible. Of course, the work in this chapter is far removed from a clinically useful intervention, but it provides validation for the concept of inhibiting the establishment of latency.

As this thesis was being prepared, a remarkable case of an infant being functionally cured of HIV-1 was presented at CROI 2013 (Persaud *et al, "*Functional HIV Cure after Very Early ART of an Infected Infant", abstract #48LB. Presented March 4, 2013 at the Conference on Retroviruses and Opportunistic Infections in Atlanta, GA). This claim is controversial, since it is debatable as to whether the infant was genuinely infected to begin with. For the sake of argument we will assume the infant was infected, but will return to this issue below.

The infant's mother was untreated, and did not receive treatment (*e.g.* single dose nevirapine) to prevent mother-to-child-transmission of HIV, so the infant was put on HAART at only 30 hours of age. Subsequent tests confirmed that both the mother and the baby were infected with HIV-1. After 18 months the mother discontinued the child's HAART, and the child is now 26 months old. Subsequent intensive testing with multiple methods on several occasions have failed to show evidence for replicating virus or for replication-competent inducible proviruses, in the 8 months since treatment discontinuation. This case provides unique and important evidence that extremely early treatment appears to have prevented the establishment of latent reservoirs in this infant. It is, of course, possible that latent viruses are present at extremely low frequency, although none have been recovered after screening 22 million resting CD4 T-cells (the usual frequency is ~1 latent virus per million resting CD4 T-cells). If confirmed, this would represent

the first ever case of HIV-1 infection without the subsequent establishment of latency.

The validity of this claim depends on whether the infant was actually infected with replication-competent virus. It is possible that the mother is an elite controller and that maternal (and infant?) genetics and/or viral characteristics render the virus only weakly infectious, although this is speculative. The main question is whether or not the baby was infected to begin with, since if the baby was infected, then a functional cure appears to have been achieved. The viral RNA that was detected in the baby at 30 and 31 hours of life could have been derived from some combination of free plasma virions or infected maternal cells acquired during delivery, and/or from genuine infection of the baby's cells. Regardless of the source, a viral load of ~19,000 copies/ml was detected in the baby at 30 and 31 hours of age. Subsequent viral load tests were also positive at days 7, 12 and 20, and were undetectable by day 29. These decay kinetics would suggest that infected cells were present, since free plasma virions have a maximum half-life of <6 hours (refer to Chapter 1, section 1.5). At this rate, if only free virions were present, then the viral load should have reached <50 copies/ml ~50 hours after initial blood collection (30 hours of age + 50 hours = 80 hours, or 3.5 days of life), and should have reached <1 copy/ml by 5 days of life. Infected CD4 T-cells have a half-life of 1-2 days. With a 2-day half-life, the viral load should have reached <50 copies/ml by day 19 of life, while in reality this level was reached between days 20 and 29. Thus, the evidence seems to indicate that infected cells were present in the infant for close to one month.

The issue is thus whether this case represents a cure, or whether these infected cells were only derived from the mother and in which case no infant cells were ever infected. If the latter were true, then HAART initiation at 30 hours of life would have prevented infant infection, as opposed to having cured an existing infection. This remains unanswerable at present, and may never be resolved.

What matters from a health care perspective is that very early HAART initiation resulted in a child who had measureable viral load, and now has no detectable virus at the age of 26 months following an eight-month treatment interruption. This additionally suggests that if any long-term viral reservoirs are present in this child, they are present at extremely low frequency.

An alternative hypothetical explanation for this case can be envisioned. Many untreated, HIV positive mothers give birth without preventive treatment (usually since their HIV status is unknown at the time of birth) and the child is often born HIV-negative, based on testing later in life. Thus it is possible that in these cases, maternal HIV-infected cells enter the infant's body but never establish infection. If this were true, the above case would simply represent a unique example whereby the infant had viral load testing at such an early age. However, whether infants' immune systems might routinely prevent infection following transmission of infected maternal cells is unknown, and would represent a major finding if confirmed. Regardless, none of the possible explanations for this case of an apparent infant cure can be validated at present with the data currently available.

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Chapter 4

Latent HIV-1 can be reactivated by cellular superinfection in a Tat-dependent manner, which can lead to the emergence of multidrug-resistant recombinant viruses

This chapter was adapted from the following manuscript:

Donahue DA, Bastarache SM, Sloan RD, Wainberg MA: Latent HIV-1 can be reactivated by cellular superinfection in a Tat-dependent manner, which can lead to the emergence of multidrug-resistant recombinant viruses (manuscript in preparation).

All experiments and data analysis included in this chapter were performed by myself under the supervision of Dr. Mark Wainberg. SM Bastarache and RD Sloan assisted with some aspects of experimental design and offered suggestions for revisions to the manuscript.

4.1 PREFACE

The focus of Chapter 3 was the inhibition of the establishment of latency, which was shown to be theoretically possible. Inhibiting the establishment of latent reservoirs would require intervention extremely soon after infection, as highlighted by the case of the infant who may have been functionally cured, discussed in Chapter 3. This case notwithstanding, a latent reservoir is already established in every HIV-1-infected individual, of which there are ~30 million worldwide. Thus even if a magic bullet were universally available tomorrow and latent reservoirs could be completely prevented from forming, already-established latent reservoirs are likely to be a major medical and scientific obstacle for several decades.

Chapter 4 is based on an examination of these already-existing latent reservoirs, and what impact they might have on an individual's infection. Latent viruses include a representation of all quasispecies that were present in a patient at any time there was ongoing replication. These quasispecies can include drug-resistant viruses, as well as other viral sequences that might provide resistance to CTL attack or neutralizing antibodies. These latent viruses are "resurrected" and can contribute to future infection and pathogenesis, if and when patients experience treatment failure [1]. Latent viruses can also be reactivated when their host cell is activated following antigen encounter. An additional mechanism of latent virus reactivation might include superinfection of latently infected cells. As discussed in greater detail below, superinfection of resting cells might not be as rare as thought, due to high *in vivo* multiplicities of infection as well as the ability of HIV-1 to infect resting or sub-optimally activated CD4 T-cells *in vivo*.

The work presented in this chapter demonstrates that superinfection of latently infected cells efficiently reactivates latent viruses. Using antiviral compounds and genetic approaches, the mechanism responsible for this reactivation is identified. Furthermore, by using different drug-resistant viruses I show that latent virus

reactivation by superinfection can ultimately lead to the development of drug-resistant recombinant viruses, the implications of which are discussed below.

4.2 ABSTRACT

The HIV-1 latent reservoir represents an important source of genetic diversity that could contribute to viral evolution, immune evasion and multidrug resistance following latent virus reactivation. This could occur by superinfection of a latently infected cell. We asked whether latent viruses might be reactivated when their host cells are superinfected, and if so, whether they could contribute to the generation of recombinant viruses. Using populations of latently infected Jurkat cells, we found that latent viruses were efficiently reactivated upon superinfection. Pathways leading to latent virus reactivation *via* superinfection might include gp120:CD4/CXCR4-induced signalling, modulation of the cellular environment by Nef, and/or the activity of Tat produced upon superinfection. Using a range of antiviral compounds and genetic approaches, we show that gp120 and Nef are not required for latent virus reactivation by superinfection, but that this process depends on production of functional Tat by the superinfecting virus. Drug-resistant latent viruses were also reactivated following superinfection, and were able to undergo recombination with superinfecting viruses. We show that, under drug selective pressure, reactivated latent viruses can undergo recombination with superinfecting viruses and can generate multidrug-resistant recombinants, which were identified by unique restriction digestion band patterns and by population-level sequencing. In a primary cell model of latency that involves infection of resting CD4 T-cells, superinfection also led to latent virus reactivation. Under conditions of poor drug adherence, treatment interruption or failure, or in drug-impermeable sanctuary sites, reactivation of latent viruses by superinfection or other means could provide for the emergence or spread of replicatively fit viruses in the face of strong selective pressures.

4.3 INTRODUCTION

Treatment failure occurs when a patient's viral quasispecies develops resistance to one or more of the drugs in a treatment regimen. Since viruses can be continually deposited into the latent reservoir during periods of low-level viremia or during treatment failure, and can exit the latent reservoir when their host cell is activated [2], latent viruses provide a means for the archival and re-emergence of sequences representing the history of a patients' quasispecies. When drug-resistant viruses are present, they are also archived in the latent reservoir [3-7]. Since viral rebound from latently infected cells occurs upon treatment interruption or treatment failure [1], previously existing drug-resistant viruses that are present in the latent reservoir would preclude patients from being treated with that drug or drug combination in the future.

In vivo, HIV-1-infected cells are often multiply infected. This is especially true in secondary lymphoid tissues [8] where the majority of lymphocytes reside, and splenocytes have been reported to harbour 3-4 proviruses on average with some cells containing up to 8 proviruses [9]. In addition, 5-25% of infected lymphocytes in peripheral blood were reported to carry multiple viruses [10]. Multiply infected cells can arise from one of two general mechanisms, namely, simultaneous infection by several viruses, or by sequential infection. Cell-to-cell transmission has been shown to lead to simultaneous transfer of multiple virions across virological synapses in a process referred to as multiploid inheritance [11]. In addition, the formation of polysynapses can lead to simultaneous transmission of virions from one infected cell to multiple target cells [12]. By locally increasing the multiplicity of infection, polysynapses might contribute to the generation of multiply infected cells by both cell-to-cell transmission as well as by superinfection. Superinfection, whether by cell-to-cell transmission or cell-free infection, leads to the generation of multiply infected cells *via* sequential infection [13].

The extreme genetic diversity of HIV-1 is a result of the high rate of nucleotide misincorporation and the propensity for template switching by the viral reverse transcriptase (RT). Retroviruses package two genomic RNA molecules into each viral particle, and RT switches between these two templates several times during each cycle of reverse transcription (reviewed in [14, 15]). During infection of Jurkat T-cells an average of 7-8 strand transfers per virus was reported to occur at essentially random locations, whereas an average of 30 strand transfers per virus were reported in macrophages [16]. When a cell is multiply infected, some of the resulting virions are heterozygous due to the copackaging of two unique genomes. These heterozygous virions form the templates for the generation of recombinant viruses, which arise when RT switches between non-identical templates during reverse transcription. The large number (>50) and high prevalence of circulating recombinant forms (CRFs) clearly demonstrate the evolutionary success of HIV-1 recombinants on a global scale [17], and recombination within individual patients has been documented in numerous studies (reviewed in [15]). Recombination involving drug-resistant viruses provides a mechanism for the spread of drug resistance throughout a patient's quasispecies [18-20].

Since the latent reservoir represents an archive of the history of a patient's quasispecies, including viruses with any previously existing drug-resistance mutations [3-7], this compartment represents an important source for the further generation of genetic diversity under selective pressure. This could occur following superinfection of a latently infected cell. Superinfection of latently infected cells could occur either during treatment interruption or failure, during periods of low-level viremia, or in compartmentalized sites of viral replication such as sanctuary sites that might result from poor drug penetration. Although this process is likely to be rare, the combination of the high multiplicity of infection that is common *in vivo* coupled with the potentially strong selective advantage of any resulting recombinant viruses renders this an important

process [21, 22]. In fact, it has been suggested that superinfection might modulate levels of latency for many viruses including HIV [23], and several studies have also suggested that latent viruses likely contribute to HIV-1 recombination *in vivo* [15, 19, 24].

In this study, we asked whether latent viruses would be reactivated upon superinfection of their host cells, and if so, whether they could contribute to the generation of recombinant viruses. Using cell line and primary cell models of HIV-1 latency establishment and reactivation, we found that superinfection efficiently reactivated latent viruses and that this process required Tat production from the superinfecting virus. We also found that drug-resistant latent viruses contributed to the development of multidrug-resistance *via* recombination with superinfecting viruses.

4.4 MATERIALS AND METHODS

4.4.1 Cell lines, viruses and antiviral compounds

Jurkat (clone E6-1) and HeLa-*tat*-III (referred to herein as "HeLa-tat") cells were obtained through the NIH AIDS Research and Reference Reagent Program. Jurkat cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin. HeLa-tat and 293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. pNL4-3-ΔE-EGFP was obtained through the NIH AIDS Research and Reference Reagent Program, while pBR-NL4-3-IRES-eGFP, pBR-NL4-3-IRES-dsRed and pBR-NL4-3-IRES-dsRed-*nef*-stop were kinds gifts of Drs. J. Münch and F. Kirchhoff [25]. The following constructs were created by either site-directed mutagenesis or cloning: pNL4-3-ΔE-EGFP-*tat*(H13L); pNL4-3-ΔE-EGFP-*tat*(H13L)-RT(Δ*SbfI*/K103N); pBR-NL4-3-IRES-dsRed-RT(M184V/Δ*MboI*); pBR-NL4-3-IRES-dsRed-*tat*(H13L); and pBR-NL4-3-IRES-dsRed-*tat*(C22G). Nucleotide changes introduced are as follows: *tat* H13L = CAT to TTA; *tat* C22G = TGT to GGA; Δ*SbfI* = CCTGCAGG to CCTGCTGG; reverse

transcriptase (RT) K103N = AAA to AAC; RT M184V = ATG to GTG; Δ*MboI* = GATC to GTTC. Replication-competent reporter viruses were produced by transfection of ~9 x 10^6 293T cells with 25 µg of plasmid DNA using Lipofectamine 2000 (Invitrogen). The Tat mutant reporter viruses used in Figure 4.3 (pBR-NL4-3-IRES-dsRed-*tat*(wt/H13L/C22G)) were produced under the same conditions except by transfection of HeLa-tat cells. Pseudoviruses were produced by cotransfection of 293T cells with 6.25 µg pVPack-VSV-G (Stratagene) – a vesicular stomatitis virus G protein (VSV-G) envelope-encoding construct – in combination with 18.75 µg of pNL4-3-ΔEnv-EGFP derivatives, as above. All transfections were carried out using Opti-MEM medium (Invitrogen) supplemented with 2.5 % FBS. Virus-containing supernatants were harvested at 48 h post-transfection, clarified by centrifugation for 5 min at 470 x g, and passed through a 0.45 µm filter. All viruses were then treated with 50 U/ml benzonase (Sigma) in the presence of added benzonase buffer (10X = 500mM Tris-HCl pH 8.0, 10mM $MgCl_2$ and 1 mg/ml bovine serum albumin (BSA)) at 37°C for 20 min to digest remaining plasmid DNA. Viral titers were determined by ELISA for viral capsid (p24), using a Vironostika HIV-1 Ag kit (bioMérieux). The RT inhibitor efavirenz (EFV), the integrase inhibitor raltegravir (RAL), and the protease inhibitor darunavir (DRV) were obtained through the NIH AIDS Research and Reference Reagent Program.

4.4.2 Jurkat cell latency model

Populations of latent viruses were established as described in Chapter 3 and published previously [26]. Briefly, Jurkat cells were infected with either VSV-G-pseudotyped NL4-3-ΔE-EGFP-*tat*(H13L) or NL4-3-ΔE-EGFP-*tat*(H13L)-RT(Δ*SbfI*/K103N) and were cultured for up to two months. At various time points samples were treated for 24 hrs with TNF-α (20 ng/ml) to reactivate latent viruses, before being fixed in 2% paraformaldehyde for 20 min. Flow cytometry was performed using a FACSCalibur or LSRFortessa (Becton Dickinson), and data were analyzed with either FCS Express or FlowJo software. Live cells were gated by forward and side scatter area, and single cells were then gated based on

forward and side scatter width and height (for samples acquired on the LSRFortessa), and levels of EGFP were then measured.

4.4.3 Primary cell latency model

A previously described primary cell latency model [27] was used with minor modifications. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of HIV-negative donors by Ficoll-Hypaque density gradient centrifugation. PBMCs were immediately processed to isolate CD4 T-cells using a Dynabeads Untouched Human CD4 T-cell isolation kit (Invitrogen). Isolated CD4 T-cells were stained with CD3-PE, CD4-e450 and CD69-FITC, and acquired on an LSRFortessa to determine isolation purity and cellular activation status. CD4 T-cells were cultured overnight in RPMI supplemented with 10 % FBS, 1% L-glutamine and 1% penicillin/streptomycin, in the absence of IL-2. The following day, CD4 T-cells (~0.5 million) were infected with NL4-3-IRES-dsRed (wt or H13L *tat*) by spinoculation in 5 mL polystyrene tubes at 1200 x g for 2 hrs at 25°C, using 200 ng p24 per million cells. Immediately after spinoculation, cells were resuspended in supplemented RPMI in the presence of 1 µM DRV to prevent spreading infection, and cultured in 96-well round-bottom plates for 3 days. On day 3 p.i., samples of uninfected or latently infected CD4 T-cells were incubated with anti-CD3/anti-CD28 magnetic beads (Invitrogen) at a 1:1 bead:cell ratio, in the presence of 10 µM raltegravir, to reactivate post-integration latent viruses. Two days later, cells were fixed as above and used for flow cytometry.

4.4.4 Superinfection

4.4.4.1 Jurkat cells

$2x10^5$ Jurkat cells latently infected with NL4-3-ΔE-EGFP-*tat*(H13L), or uninfected Jurkat cells, were infected with NL4-3-IRES-dsRed (or its *tat*/*nef* mutant derivatives; 120 ng p24 was used for each virus, except that 90ng p24 was used for *tat* mutant viruses). Similarly, $2x10^5$ Jurkat cells latently infected with NL4-3-ΔE-EGFP-*tat*(H13L)-RT(Δ*SbfI*/K103N), or uninfected Jurkat cells, were infected

with 300ng p24 of NL4-3-IRES-dsRed-RT(M184V/Δ*MboI*). Infection was by spinoculation in a total volume of 0.6 ml at 1 500 x g for 2 hrs at 37°C, in the presence of EFV, RAL, or DRV as required. Following spinoculation, cells were allowed to rest for 1 hr at 37°C. Virus-containing supernatants were then removed and fresh medium was added, supplemented with 1 µM EFV, RAL or DRV as required. At 72 hrs p.i. cells were fixed and analyzed by flow cytometry as described above.

4.4.4.2 Primary cells

Latently infected CD4 T-cells (3 days p.i.) were superinfected by spinoculation with NL4-3-IRES-GFP (or RPMI only for controls), as above, using 200 ng p24 per million cells. Cells were cultured in supplemented RPMI (in the absence of IL-2), plus 1 µM DRV, for 3 days, before fixation and measuring viral reporter gene expression by flow cytometry as above.

4.4.5 PCR

4.4.5.1 Integrated HIV-1 DNA

$2x10^5$ Jurkat cells were infected with 90 ng p24 of pBR-NL4-3-IRES-dsRed (or its *tat* mutant derivatives) by spinoculation as described above, and 1 µM DRV was added to prevent reinfection. Cellular DNA was extracted 48 hrs p.i. using a DNeasy blood and tissue kit (Qiagen). A previously described nested *Alu*-gag PCR [28] was used with the following modifications. The first round reaction (performed in both the presence and the absence of an *Alu*-specific primer) was performed using undiluted samples (65 ng DNA) and 1:4 dilutions of each sample (16.25 ng DNA from infected Jurkat cells diluted with DNA from uninfected Jurkat cells; 65 ng DNA total) in the presence of 2 mM $MgCl_2$ and 200 µM dNTPs in a total volume of 20 µl, using the primers *Alu*-F (5'-GCCTCCCAAAGTGCTGGGATTACAG-3') and gag-R (5'-GTTCCTGCTATGTCACTTCC-3'). Cycling conditions were 95°C for 2 min, and 20 cycles of 95°C for 15 s, 50°C for 15 s, and 72°C for 3.5 min. 9 µl of the resulting first round product was used

as template for the second round nested reaction in the presence of 5 mM $MgCl_2$ (final concentration including carryover from first round) and 200 µM added dNTPs, in a total volume of 20 µl. Second-round primers were LTR-F (5'-TTAAGCCTCAATAAAGCTTGCC-3') and LTR-R (5'-GTTCGGGCGCCACTGCTAGA-3'), and only the "wild-type" probe [28] was used. Second round cycling conditions were 50°C for 2 min, 95°C for 1 min, and 45 cycles of 95°C for 15 sec and 60°C for 30 sec, using Platinum qPCR SuperMix-UDG (Invitrogen) on a Corbett Rotor-Gene 6000 thermocycler. To generate a standard curve for relative quantification of integrated DNA, *Alu*-gag PCR was first performed on a 2-fold dilution series of DNA from infected Jurkat cells (diluted with DNA from uninfected Jurkat cells). Samples were normalized to their β-globin contents as described [29].

4.4.5.2 RT-PCR for viral genomic RNA

Viral RNA was extracted from supernatants of infected cells using a QIAamp viral RNA mini kit (Qiagen). RT-PCR was performed using a SuperScript III One-step RT-PCR kit (Invitrogen) and 6 µl viral RNA template, with primers Recomb-F (5'-AATGGATGGCCCAAAAGTTAAACA-3') and Recomb-R (5'-CTGTTAATTGTTTCACATCATTAGTGTGGG-3'), in a total volume of 30 µl. Cycling conditions were 55°C for 15 min, 94°C for 2 min, and 40 cycles of 94°C for 20 s, 60°C for 20 s, and 68°C for 1 min. Products were visualized on 1 % agarose TAE gels.

4.4.6 Identification of recombinant viruses

Recombinant viruses were identified by restriction enzyme digestion and by sequencing. To analyze recombination by restriction digestion, 5 µl of each RT-PCR product (containing amplified viral genomic RNA) were double digested with both *SbfI* and *MboI* (New England Biolabs) in a total volume of 15 µl for 15 min at 37°C. Products were then visualized on 1 % agarose TAE gels (1 hr at 125 V), and band patterns were compared to digests of plasmids representing wt, latent or superinfecting viruses. To analyze recombination by sequencing, RT-PCR

products containing amplified viral genomic RNA were sequenced by standard methods using primers Recomb-F and Recomb-R. All chromatograms were visually inspected, and chromatogram peak intensities at relevant nucleotide positions were manually compared to determine the relative proportion of each virus in the population. For example, the K to N mutation at RT position 103 is AAA to AAC. If band intensities were 60% A and 40% C at the third nucleotide position, the population was considered to be 40% K103N. Results from forward and reverse sequence reads were averaged for each position. As described in further detail in the Results section, estimates for the percentage of recombinant viruses in each population are conservative.

4.4.7 Statistical Analyses

Unpaired two-tailed t-tests and linear regression analysis were used as indicated in the figure legends. All statistical analyses were performed with GraphPad Prism 5.0 software.

4.5 RESULTS

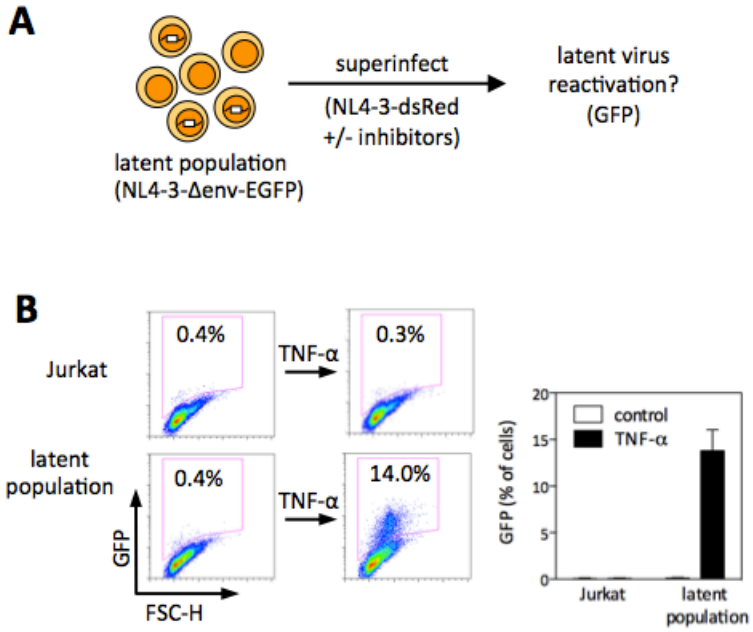
4.5.1 Superinfection of latently infected cells reactivates latent HIV-1

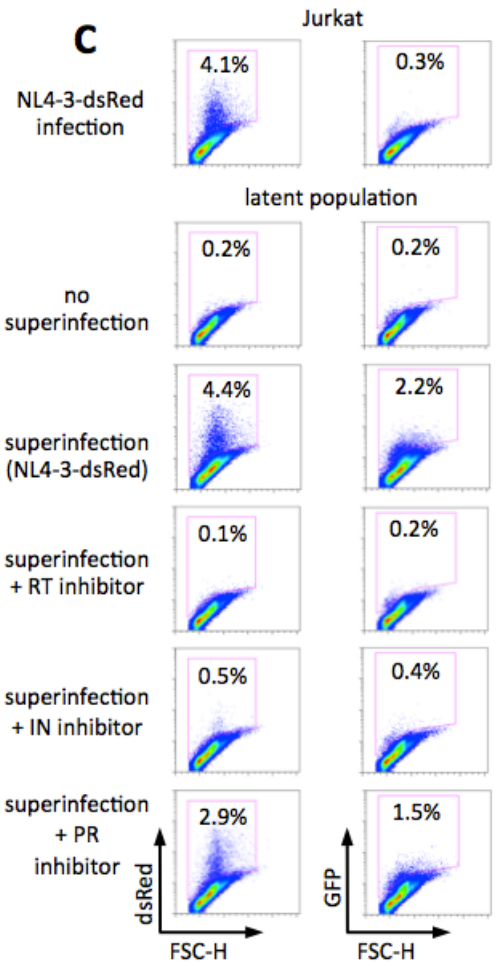
To determine whether superinfection of latently infected cells would reactivate latent viruses, we first used a Jurkat-based model of HIV-1 latency establishment and reactivation that we have previously described [26]. In this model a heterogeneous population of latent viruses representing thousands of unique integration sites is established, whereby the latent viruses encode a fluorescent reporter gene. Culturing these cells for several weeks gives rise to populations of cells harbouring TNF-α-inducible integrated proviruses, with no actively replicating viruses present. In the latent populations used here, approximately 14 % of cells harboured latent viruses (Figure 4.1A-B). Uninfected Jurkat cells, or latently infected Jurkat cells that encode viral EGFP, were then infected with the replication-competent reporter virus NL4-3-dsRed (where dsRed is expressed from an internal ribosome entry site (IRES) from *nef* transcripts [25]). We found

that superinfection of latently infected cells led to reactivation of latent virus, as demonstrated by the increase in the percentage of EGFP-positive cells upon superinfection of latently infected cells (Figure 4.1C-D).

4.5.2 Interaction of gp120 with CD4 and CXCR4 is not required for latent virus reactivation

We next wished to characterize the pathway(s) that lead to latent virus reactivation by superinfection. T-cell activation involves signal cascades that ultimately lead to nuclear translocation of the transcription factors NF-κB and NFAT. In resting or sub-optimally activated CD4 T-cells, interaction of gp120 with CD4 and either CCR5 or CXCR4 can lead to induction of Ca^{2+} and NFAT – an important transcription factor involved in HIV-1 transcription – in the absence of full cellular activation [30-34]. Additionally, HIV-1 envelope was reported to induce viral replication from resting cells of HIV-1-infected patients [35]. Thus, we wished to determine whether gp120 from our CXCR4-using superinfecting virus was responsible for some of the latent virus reactivation that was observed. Latently infected cells were superinfected in the presence of inhibitory levels (1 μM) of the RT inhibitor efavirenz (EFV). Blocking superinfection at reverse transcription, which is downstream of gp120:CD4/CXCR4 interaction, resulted in no increase in latent virus gene expression compared to latently infected cells that were not superinfected ($p=0.75$) (Figure 4.1C-D).


A
superinfect
(NL4-3-dsRed
+/- inhibitors)
latent virus
reactivation?
(GFP)
latent population
(NL4-3-Δenv-EGFP)
B
Jurkat
0.4%
TNF-α
0.3%
latent
population
0.4%
TNF-α
14.0%
GFP
FSC-H
GFP (% of cells)
control
TNF-α
20
15
10
5
0
Jurkat
latent
population


C
Jurkat
NL4-3-dsRed infection
4.1%
0.3%
latent population
no superinfection
0.2%
0.2%
superinfection (NL4-3-dsRed)
4.4%
2.2%
superinfection + RT inhibitor
0.1%
0.2%
superinfection + IN inhibitor
0.5%
0.4%
superinfection + PR inhibitor
2.9%
1.5%
dsRed
FSC-H
GFP
FSC-H

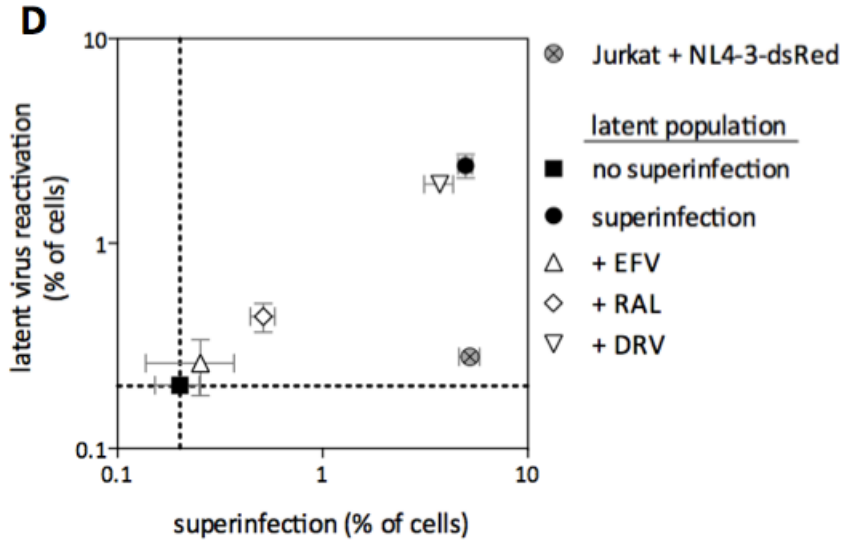

D
latent virus reactivation (% of cells)
10
1
0.1
0.1
1
10
superinfection (% of cells)
Jurkat + NL4-3-dsRed
latent population
no superinfection
superinfection
+ EFV
+ RAL
+ DRV

Figure 4.1. Superinfection of latently infected cells reactivates latent HIV-1 and requires gene expression but not gp120:CD4/CXCR4 signalling from the superinfecting virus.

(A) Schematic representation of the experimental design. (B) Characterization of the latently infected Jurkat cell population used in Figures 1-3. FSC-H, forward scatter height. Results of three independent experiments performed in duplicate are shown. (C-D) Uninfected or latently infected Jurkat cells were superinfected with NL4-3-dsRed in the presence or absence of 1 µM EFV, RAL or DRV. Representative results are shown in (C) and the results of three independent experiments, each performed in triplicate, are shown in (D). dsRed = superinfecting virus; GFP = latent virus. All error bars represent standard error of the mean (SEM; n=3 for all error bar calculations).

4.5.3 Reactivation of latent virus by superinfection requires gene expression of the superinfecting virus

We next wished to determine whether gene expression of the superinfecting virus is required for latent virus reactivation. Latently infected cells were superinfected in the presence of the integrase inhibitor raltegravir (RAL), which prevents integration and thus productive viral gene expression, or in the presence of the protease inhibitor darunavir (DRV), which acts after integration and viral gene expression. As shown in Figure 4.1C-D, latent virus reactivation required gene expression of the superinfecting virus. It is noteworthy that superinfection in the presence of an integrase inhibitor led to a slight and borderline statistically significant ($p=0.0496$) increase in latent virus reactivation. This could be due to incomplete inhibition of viral replication in the presence of 1 µM RAL, which can be explained by the comparatively poor inhibitory capacity of this drug during a single round of viral replication [36]. Alternatively, low-level gene expression from unintegrated viral DNA might explain this observation [37].

4.5.4 Nef is not required for latent virus reactivation *via* superinfection

HIV-1 Nef modulates numerous cellular pathways, including several related to T-cell activation. Recent studies suggest that Nef lowers the activation threshold for CD4 T-cells. This implies that when cells encounter activation signals in the presence of Nef, greater induction of transcription factors including NF-κB and NFAT, as well as greater Ca^{2+} release and IL-2 production, can result (reviewed in [38, 39]). Although Nef does not by itself induce T-cell activation, it has been reported that Nef alone is sufficient to upregulate numerous cellular genes involved in LTR-driven transcription [40]. Nef-upregulated genes include NFAT and many other transcription factors, as well as CDK9 and other factors involved in the elongation of viral transcripts [40]. In addition to Nef produced after integration, expression of Nef from unintegrated DNA is can modulate T-cell activation pathways [41]. Therefore we wished to determine whether production of Nef upon superinfection might contribute to the reactivation of latent virus that we observed in Figure 4.1. Latently infected cells were superinfected with a replication-competent reporter virus containing two stop codons near the start of *nef* (referred to herein as "ΔNef" virus). Consistent with the enhancement of infectivity associated with Nef, superinfection with ΔNef virus resulted in fewer infected cells compared to superinfection with Nef-encoding virus. However, latent viruses were reactivated at least as efficiently in the absence of Nef as with wt virus (Figure 4.2). This excludes a requirement for the modulation of cellular activation pathways by Nef in the reactivation of latent viruses by superinfection.

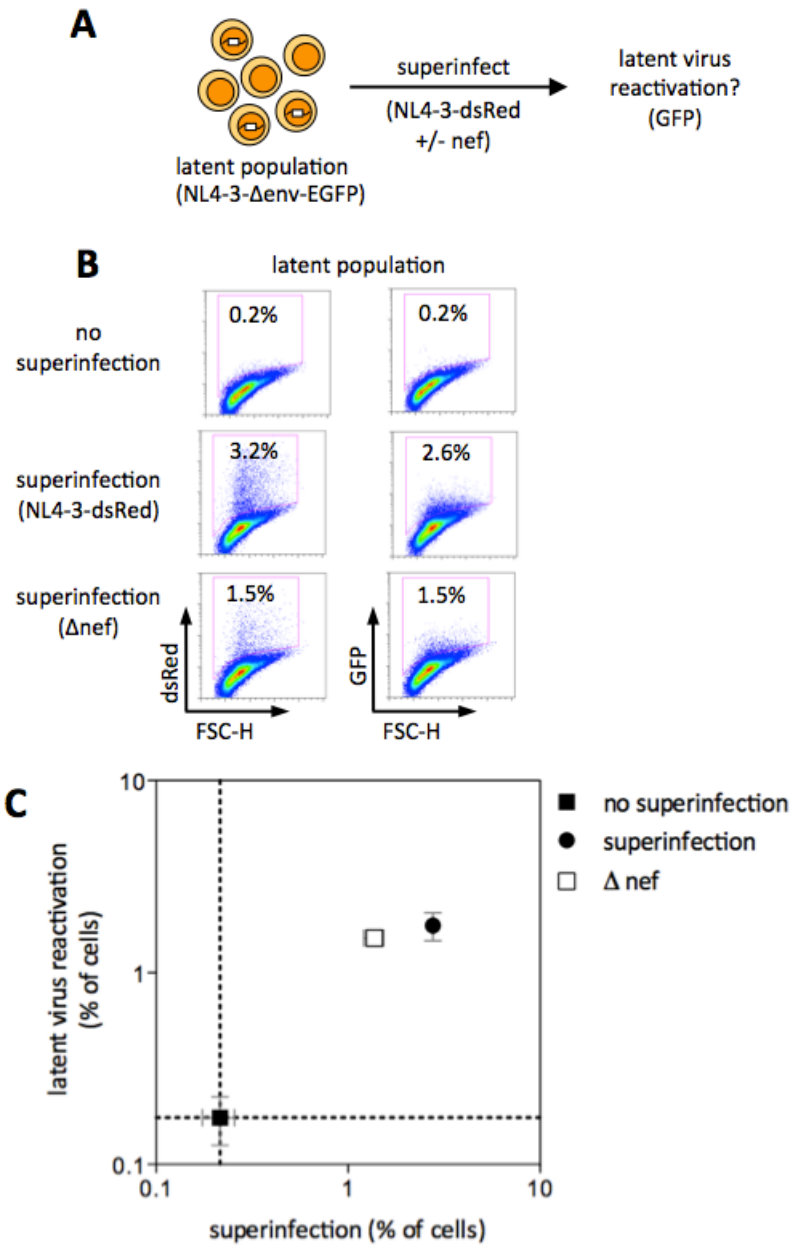


Figure 4.2. Superinfecting virus Nef is not required for latent virus reactivation. (A) Schematic representation of the experimental design. (B-C) Uninfected or latently infected Jurkat cells were superinfected with NL4-3-dsRed or NL4-3-dsRed-Δ*nef*. Representative results are shown in (C) and the results of three independent experiments, each performed in triplicate, are shown in (D). dsRed

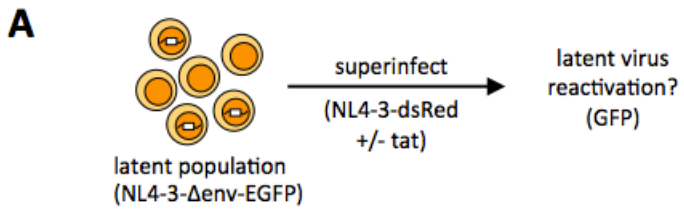
= superinfecting virus; GFP = latent virus. FSC-H, forward scatter height. All error bars represent SEM (n=3 for all error bar calculations).

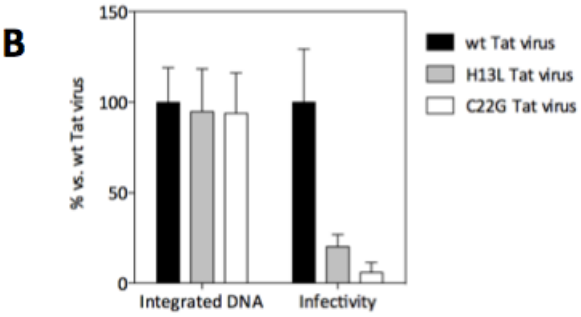
4.5.5 Latent virus reactivation *via* superinfection requires expression of functional Tat by the superinfecting virus

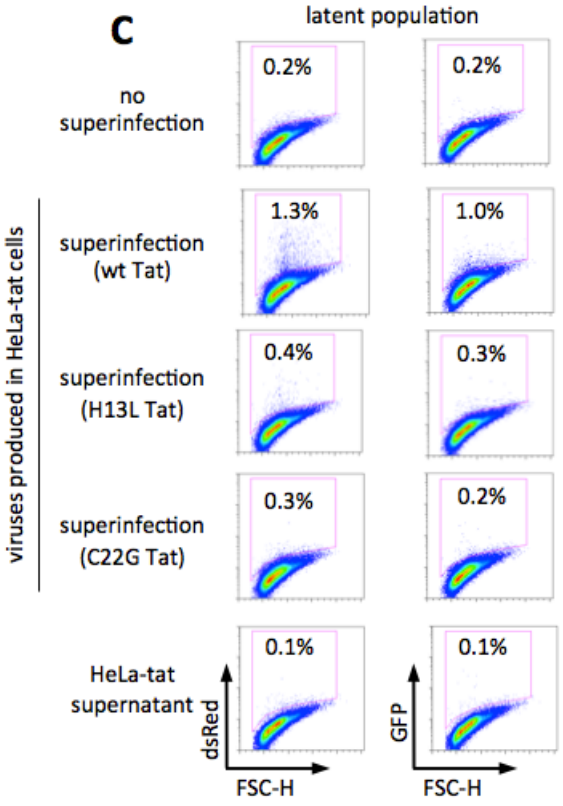
It is reasonable to hypothesize that production of Tat by superinfecting viruses might be required for latent virus reactivation. Accordingly, we produced replication-competent reporter viruses that encode functional (wt), attenuated (H13L), or transactivation-negative (C22G) *tat*. Since Tat is required for the production of HIV-1 virions, these viruses were first produced by transfection of HeLa cells that stably express Tat (HeLa-tat). Following infection of Jurkat cells we found that each of these viruses gave rise to equivalent integrated DNA levels, and that infectivity as defined by the percentage of cells positive for Tat-dependent viral reporter gene expression followed the expected pattern of wt > H13L > C22G (Figure 4.3B). These results demonstrate that the different viruses used here are equally functional for all steps from entry to integration, and so any differences in latent virus reactivation following superinfection would be due to their differential Tat activities. Next, these viruses were used to superinfect latently infected cells (Figure 4.3A-D). While superinfection with wt *tat* virus efficiently reactivated latent viruses, superinfection with attenuated *tat* virus resulted in a detectable but statistically insignificant reactivation of latent virus. Latent virus reactivation was not detectable when transactivation-negative *tat* virus was used for superinfection. Of note, the level of superinfection achieved with wt *tat* virus here is much lower than in comparable infections shown in Figures 4.1 and 4.2. This is due to the use of HeLa-tat cells for production of the viruses used here, as opposed to virus production in 293T cells used elsewhere in this study. Since HeLa-tat transfection produced relatively low viral titers, lower viral inputs were used for the subsequent infections. As a control for any secreted Tat that might result from use of HeLa-tat cells for virus production, latently infected cells were incubated directly with HeLa-tat supernatant; no

latent virus reactivation was observed in this case (Figure 4.3C-D).

Together, the results of Figures 4.1-4.3 show that reactivation of latent viruses by superinfection requires gene expression of the superinfecting virus, and specifically, production of functional Tat. Linear regression analysis demonstrates a strong positive correlation ($r^2 = 0.97$) between the extent of superinfection and the extent of latent virus reactivation (Figure 4.3E).


A
superinfect
(NL4-3-dsRed
+/- tat)
latent virus
reactivation?
(GFP)
latent population
(NL4-3-Δenv-EGFP)


B
150
100
50
0
% vs. wt Tat virus
wt Tat virus
H13L Tat virus
C22G Tat virus
Integrated DNA
Infectivity


C
latent population
no
superinfection
0.2%
0.2%
viruses produced in HeLa-tat cells
superinfection
(wt Tat)
1.3%
1.0%
superinfection
(H13L Tat)
0.4%
0.3%
superinfection
(C22G Tat)
0.3%
0.2%
HeLa-tat
supernatant
0.1%
0.1%
dsRed
FSC-H
GFP
FSC-H

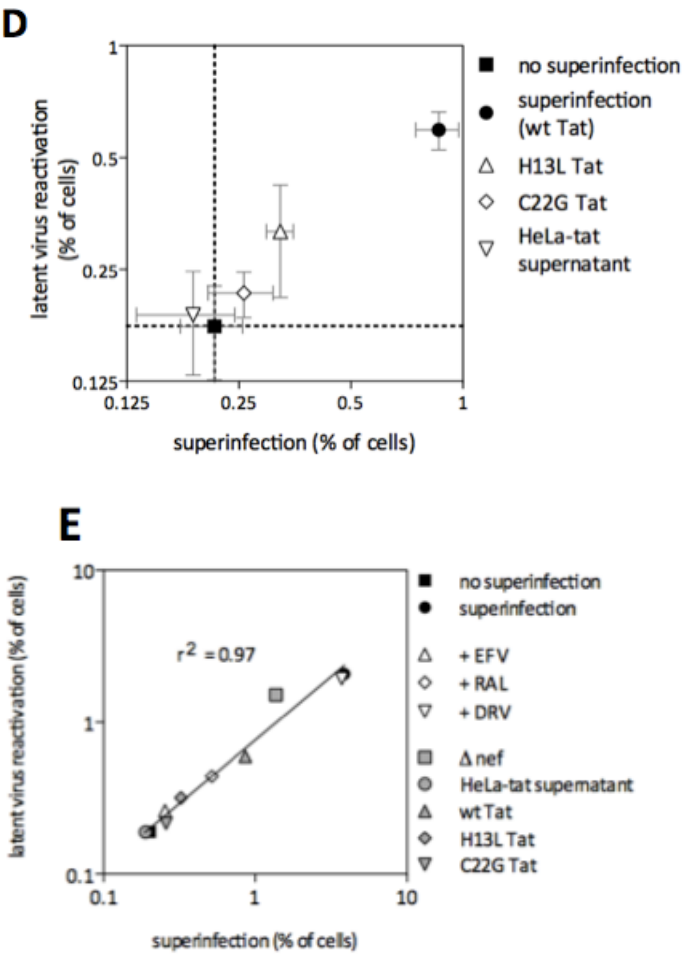


Figure 4.3. Latent virus reactivation *via* superinfection requires expression of functional Tat by the superinfecting virus.

(A) Schematic representation of the experimental design. (B) wt, attenuated (H13L) or inactivated (C22G) *tat* viruses were produced by transfection of HeLa-tat cells, and were then used to infect Jurkat cells in the presence of 1 µM DRV to prevent reinfection. Levels of integrated viral DNA were measured by *Alu*-gag qPCR, while infectivity was determined by flow cytometry for viral-encoded dsRed. (C-D) Latently infected Jurkat cells were superinfected with NL4-3-dsRed (wt, H13L or C22G *tat*), or were treated with HeLa-tat supernatant as a control for secreted Tat present in the superinfecting virus inoculum. FSC-H, forward scatter height. Representative results are shown in (C) and the results of three independent experiments, each performed in triplicate, are shown in (D). The

axes in (D) are $\log_2$ rather than $\log_{10}$. dsRed = superinfecting virus; GFP = latent virus. (E) Summary of the superinfection experiments shown in Figures 1-3. Linear regression analysis was used to look for any correlation between superinfection and latent virus reactivation. All error bars represent SEM (n=3 for all error bar calculations).

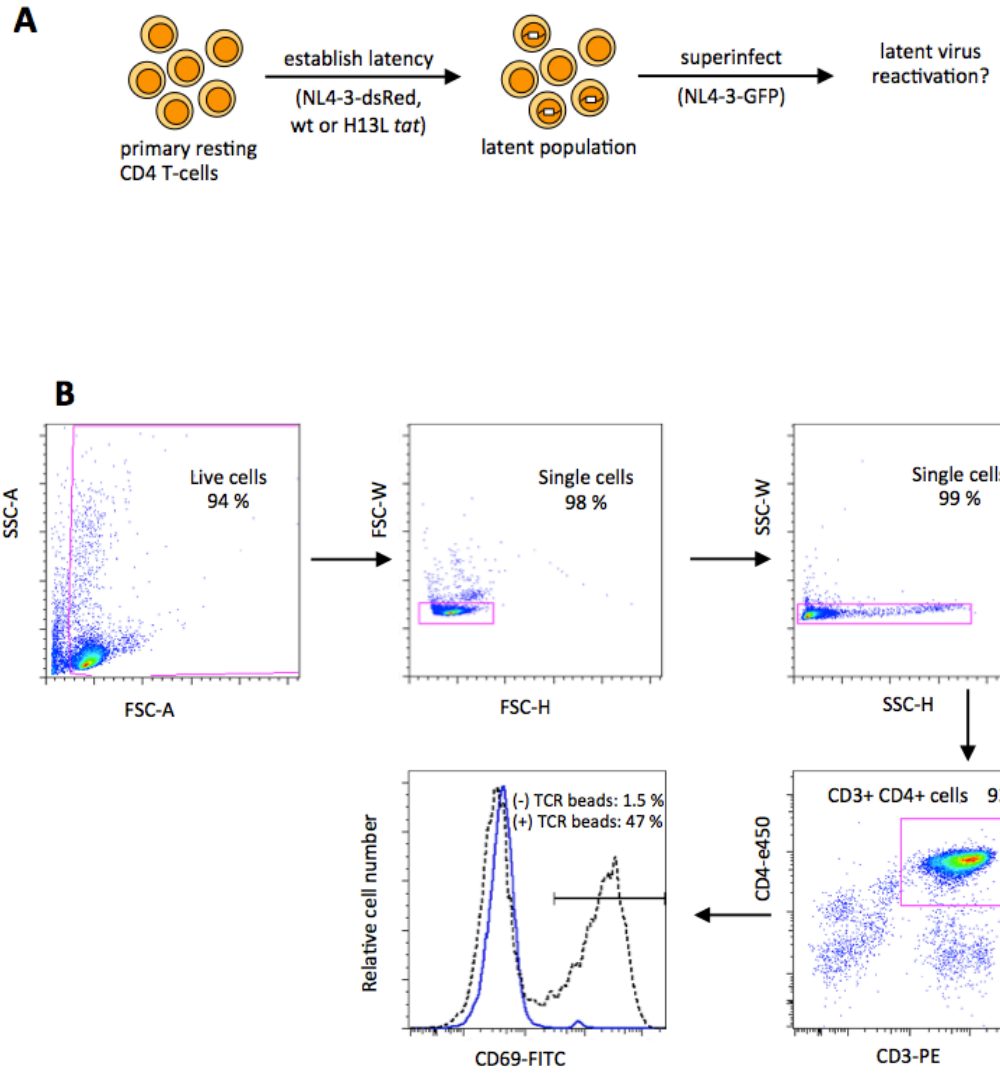
4.5.6 Latent HIV-1 can be reactivated by superinfection in primary resting CD4 T-cells

To confirm our findings in a more physiologically relevant system, we next used a primary cell model of HIV-1 latency that involves direct resting cell infection. In this model, latency is established in multiple CD4 T-cell subsets, including naïve, central memory and transitional memory cells [27]. Furthermore, CD4 T-cells are cultured in the absence of cytokines such as IL-2 and are infected shortly after isolation, preserving the *in vivo* distribution of CD4 T-cell subsets. The authors of this model also showed that isolation of whole CD4 T-cells gave near-identical results compared to use of more extensive resting memory cell purification steps, which is likely because activated CD4 T-cells in peripheral blood *in vivo* are present at only low frequency. Lastly, the latent viruses generated in this model respond to reactivation compounds with the same patterns observed for *ex vivo*-treated patient samples [27].

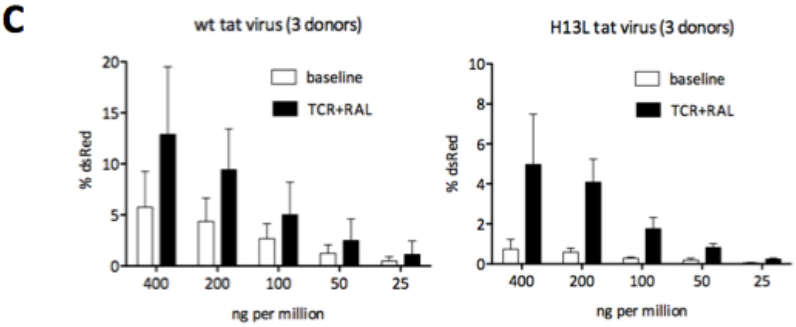
We first confirmed the purity and resting state of isolated CD4 T-cells from multiple donors. The vast majority of cells expressed both CD3 and CD4, but did not express the activation marker CD69 (Figure 4.4B). Isolated CD4 T-cells from individual donors were then infected with replication-competent dsRed-encoding reporter viruses, in the presence of 1 μM DRV to prevent spreading infection. As expected [27], infection with a wide range of wt *tat* virus inocula gave a baseline level of gene expression that results from viruses which are not silenced, but incubation with αCD3/αCD28 beads in the presence of 10 μM RAL led to reactivation of postintegration latent viruses (Figure 4.4C). We also used

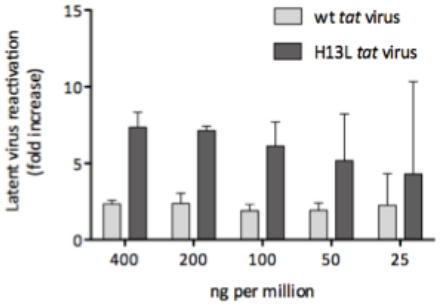
an attenuated (H13L) *tat* virus and performed the same infections, which led to a substantial increase in the percentage of silenced viruses (Figure 4.4C). Incubation with αCD3/αCD28 beads in the presence of 10 μM RAL led to reactivation of postintegration latent H13L *tat* viruses. As shown in Figure 4.4C, despite the lower overall percentages of latently infected cells that result from the use of attenuated *tat* virus, the fold reactivation of latent viruses over baseline was much greater due to higher levels of viral silencing.

Finally, we wished to determine whether superinfection of latently infected primary resting CD4 T-cells would lead to reactivation of latent viruses. Latently infected cells were superinfected with a GFP-expressing reporter virus by spinoculation (or were spinoculated in RPMI alone), and 3 days later the percentage of cells expressing dsRed was determined. As shown in Figure 4.4D, superinfection led to a modest but reproducible reactivation of latent viruses. Due to the low overall infection rates achievable in primary resting CD4 T-cells, higher rates of superinfection and latent virus reactivation would not be expected.


A
primary resting
CD4 T-cells
establish latency
(NL4-3-dsRed,
wt or H13L tat)
latent population
superinfect
(NL4-3-GFP)
latent virus
reactivation?
B
SSC-A
FSC-A
Live cells
94 %
FSC-W
FSC-H
Single cells
98 %
SSC-W
SSC-H
Single cells
99 %
CD4-e450
CD3-PE
CD3+ CD4+ cells 93 %
Relative cell number
CD69-FITC
(-) TCR beads: 1.5 %
(+) TCR beads: 47 %

C


wt tat virus (3 donors)
H13L tat virus (3 donors)
baseline
TCR+RAL
% dsRed
ng per million
400
200
100
50
25


wt tat virus
H13L tat virus
Latent virus reactivation (fold increase)
ng per million

D

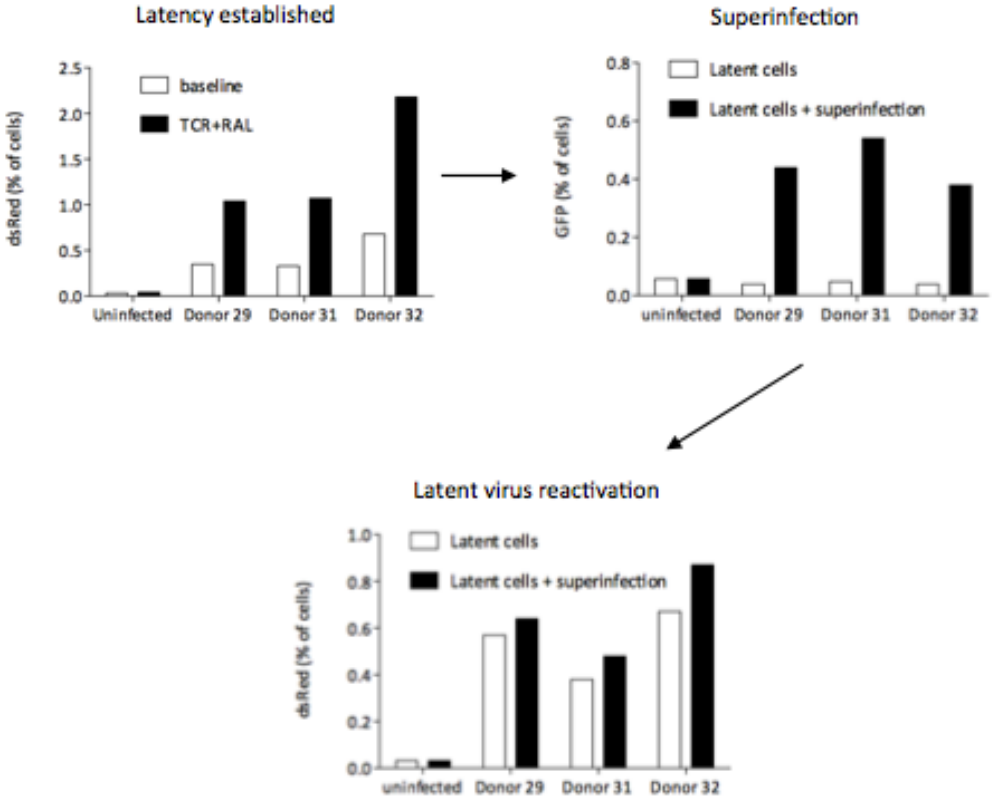

Latency established
Superinfection
Latent virus reactivation
baseline
TCR+RAL
Latent cells
Latent cells + superinfection
dsRed (% of cells)
GFP (% of cells)
Uninfected
uninfected
Donor 29
Donor 31
Donor 32

Figure 4.4. Superinfection of latently infected primary resting CD4 T-cells leads to reactivation of latent virus.

(A) Schematic representation of the experimental approach. (B) Isolated CD4 T-cells were stained with CD3-PE, CD4-e450 and CD69-FITC to determine purity and activation status. One representative donor is shown. In the histogram, solid blue depicts freshly isolated CD4 T-cells and the dashed black line depicts CD4 T-cells incubated for 24 hrs with αCD3/αCD28 beads (1:1 ratio) to induce T-cell activation, as a positive control. (C) A wide range of viral inocula (25 to 400 ng p24 per million cells) was used to infect CD4 T-cells and establish latency; both wt and attenuated *tat* viruses were used. The third panel represents the results of six individual donors (three each for wt and attenuated *tat* viruses), in terms of fold reactivation above baseline. (D) (First panel) Latency was established in three individual donors, using attenuated *tat* virus at an infection rate of 200 ng per million cells. Three days after infection, samples were incubated with αCD3/αCD28 beads (1:1 ratio) for 2 additional days to quantify the levels of latency. (Second panel) 3 days after the initial infection, latently infected cells were superinfected by spinoculation with GFP-expressing virus (or spinoculated with RPMI only as a control), using 200 ng p24 per million cells. (Third panel) The rate of superinfection was determined 3 days after superinfection.

4.5.7 Drug-resistant latent viruses are reactivated by superinfection with other drug-resistant viruses

Having demonstrated that latent viruses are efficiently reactivated by superinfection in a Tat-dependent manner, and can also be reactivated by superinfection in primary cells, we next wished to determine whether these previously latent viruses could contribute to the generation of recombinants. Therefore, we established populations of Jurkat cells where ~6 % of cells harboured TNF-α-inducible drug-resistant latent viruses (Figure 4.5A-B). The latent reporter viruses encode the RT drug-resistance mutation K103N, which confers resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs)

including EFV. To facilitate the identification of potential recombinants, these latent viruses also contain a non-coding restriction site change that removes an *SbfI* site (Δ*SbfI*) located ~20 nucleotides from the K103N mutation. K103N latent populations were superinfected with a dsRed-encoding drug-resistant reporter virus that encodes the RT mutation M184V, which provides for resistance to nucleoside RT inhibitors (NRTIs) including emtricitabine (FTC). As with the latent virus, the superinfecting virus contained a non-coding restriction site change that removed an *MboI* site (Δ*MboI*) located ~20 nucleotides from the M184V mutation. As demonstrated in Figure 4.5C-D, superinfection of K103N latent populations with M184V virus led to reactivation of drug-resistant latent viruses.

4.5.8 Reactivated latent viruses can recombine with superinfecting viruses, which can contribute to the development of multidrug-resistant recombinants

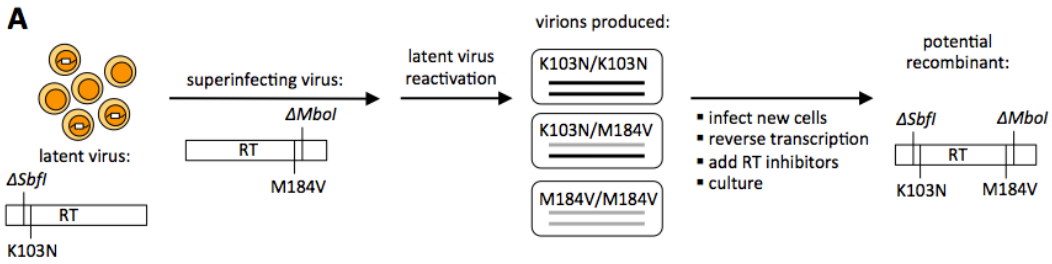
To determine whether recombination would occur between reactivated latent viruses and superinfecting viruses, supernatants of superinfected latent cells were harvested three days after superinfection (depicted schematically in Figure 4.5A). These supernatants, which are expected to include some heterozygous virions (*ΔSbfI*+K103N / M184V+Δ*MboI*), were used to infect new Jurkat cells. After 20 hrs (a time sufficient for the completion of reverse transcription but prior to the next round of viral replication [29]), EFV and FTC were added together at a range of concentrations to select for any recombination events that might have occurred during reverse transcription. This will select for only a small fraction of recombinant viruses, *i.e.* only those in which recombination occurred between amino acid positions 103 and 184 of RT, and in the correct orientation to maintain both resistance mutations. Following addition of RT inhibitors, cultures were maintained for six days, and ~1 kb of RT was then amplified by RT-PCR from supernatant viral genomic RNA.

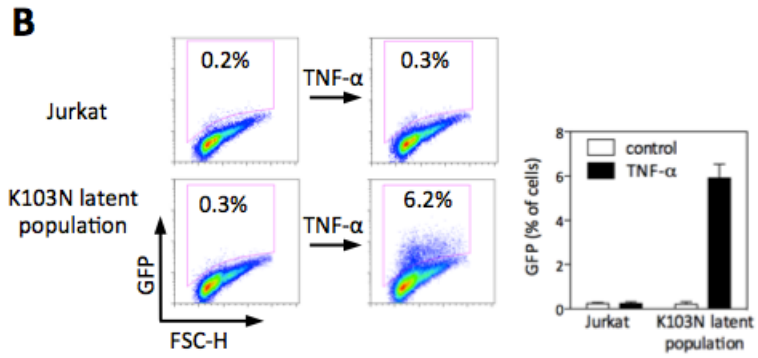
Two approaches were used for the identification of recombinant viruses. First, RT amplicons were subject to double restriction enzyme digestion with *SbfI* and

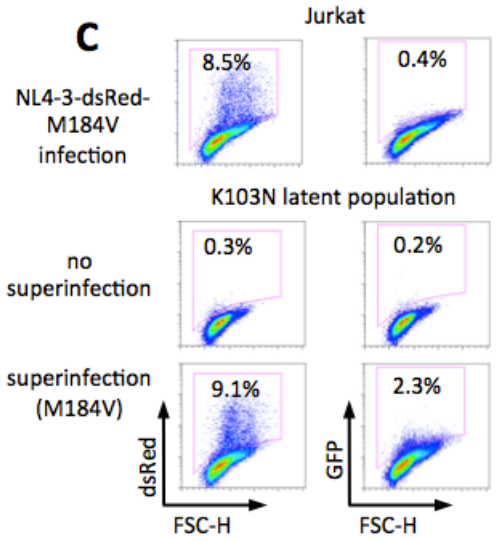
MboI. The noncoding restriction site changes introduced into each virus permitted the identification of recombinants by the presence of a unique band pattern. As shown in Figure 4.5E (left), only digestion of recombinant viruses is expected to produce both bands "a" and "c". Recombinant viruses were detected in many but not all biological replicates across a range of drug selective pressure, with representative results shown in Figure 4.5E (right). Based on the observed banding pattern it can be concluded that lanes 11, 13 and 14 (showing bands a, c, d and e) represent a mixture of recombinant and superinfecting viruses. In contrast, only superinfecting virus was present following infection of Jurkat cells (lanes 7-8) or of latent populations with no drug selective pressure (lane 10). Comparing the intensity of band "a" across different lanes gives an approximation of the overall level of virus present, since both superinfecting and recombinant viruses contribute to band "a" (*e.g.* lane 12 represents a lower level of virus than lanes 11, 13 or 14, consistent with the absence of recombinant viruses despite drug selective pressure in that sample).

Second, bulk sequencing was used to estimate the proportion of recombinants in the total virus population for each biological replicate. A population was considered to contain recombinant viruses only when mathematically necessary. For example, if a population was 40 % Δ*SbfI*+K103N, and 90 % M184V+Δ*MboI*, then at least 30 % of the population must be recombinant viruses (where Δ*SbfI*, K103N, M184V and Δ*MboI* are on the same genomic RNA). If all mutations were present at < 50 %, the population was not considered to contain recombinants. The presence of the Δ*SbfI* and Δ*MboI* mutations additionally confirms that recombinants are genuine, as opposed to the *de novo* acquisition of resistance mutations by either parental virus. As shown in Figure 4.5F, multidrug-resistant recombinant viruses resulted following superinfection of latently infected cells across a range of drug concentrations, in many but not all biological replicates. No recombinants were detected following infection of Jurkat cells, or following superinfection of latently infected cells in the absence of drug selective pressure.

Together, these results demonstrate that latent viruses can serve as a source for recombination, and can contribute to the emergence of multidrug-resistant recombinants.


A
latent virus:
ΔSbfI
RT
K103N
superinfecting virus:
ΔMboI
RT
M184V
latent virus reactivation
virions produced:
K103N/K103N
K103N/M184V
M184V/M184V
▪ infect new cells
▪ reverse transcription
▪ add RT inhibitors
▪ culture
potential recombinant:
ΔSbfI
ΔMboI
RT
K103N
M184V


B
Jurkat
0.2%
TNF-α
0.3%
K103N latent population
0.3%
TNF-α
6.2%
GFP
FSC-H
control
TNF-α
GFP (% of cells)
Jurkat
K103N latent population


C
Jurkat
NL4-3-dsRed-M184V infection
8.5%
0.4%
K103N latent population
no superinfection
0.3%
0.2%
superinfection (M184V)
9.1%
2.3%
dsRed
GFP
FSC-H
FSC-H

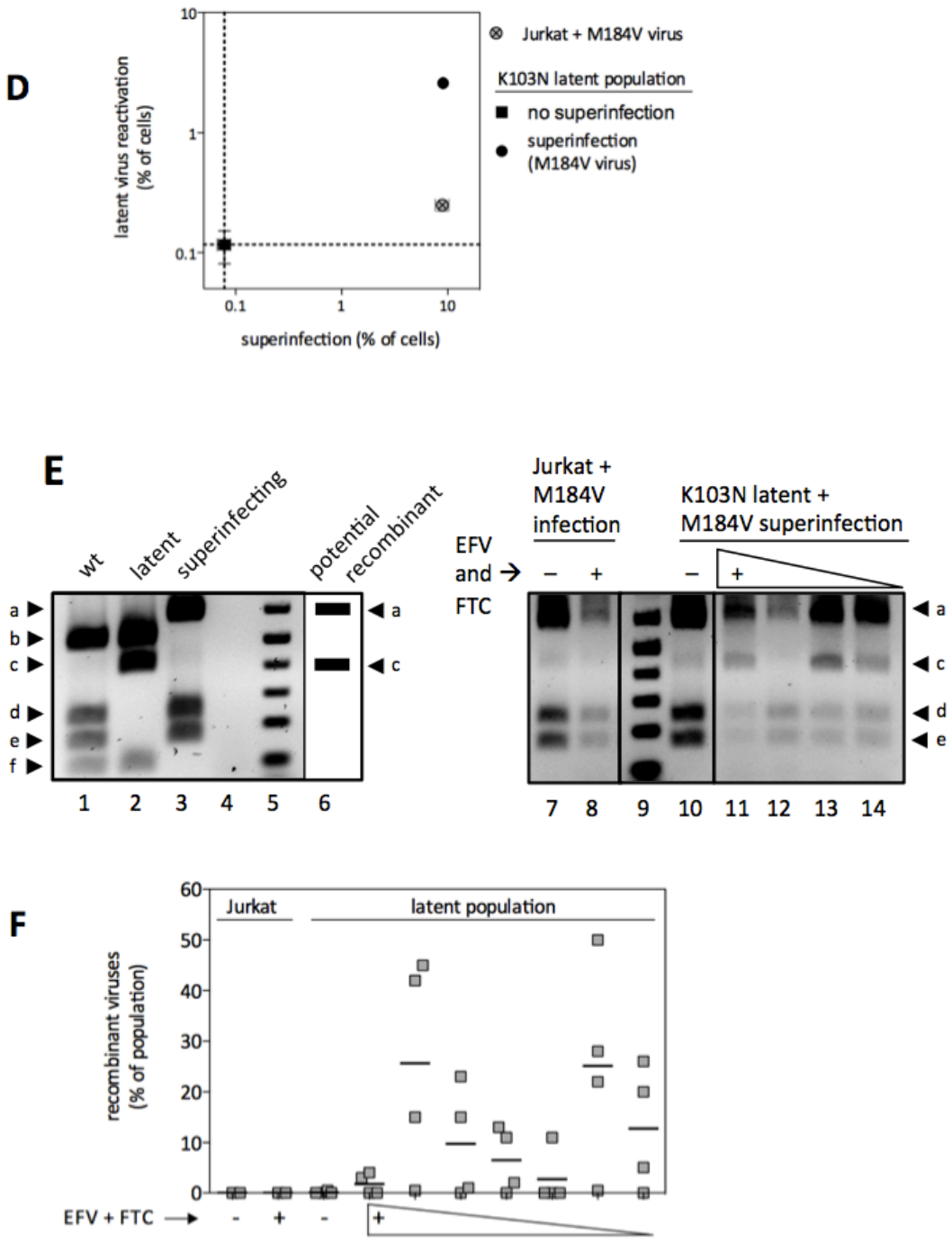


Figure 4.5. Drug-resistant latent viruses are reactivated by superinfection, can recombine with superinfecting viruses, and can contribute to the development of multidrug-resistant recombinants.

(A) Schematic representation of the experimental design. (B) Characterization of

the drug-resistant latent virus population used in these experiments. FSC-H, forward scatter height. Results of three independent experiments, each performed in duplicate, are shown. (C-D) Uninfected or latently infected Jurkat cells were superinfected with NL4-3-dsRed-RT(M184V/Δ*MboI*). Representative results are shown in (C) and the results of three independent experiments, each performed in triplicate, are shown in (D). dsRed = superinfecting virus; GFP = latent virus. All error bars represent standard error of the mean (SEM; n=3 for all error bar calculations). (E) (Left) Plasmids representing wt, latent or superinfecting viruses (lanes 1-3) were double digested with *MboI* and *SbfI* and run on agarose gels. Lane 4 = empty, lane 5 = DNA ladder. The presence of bands "a" and "c" in the same lane would indicate a recombinant virus derived from both the latent and superinfecting viruses (lane 6). (Right) RT-PCR products from supernatants of one representative experiment, as depicted in (A), were double digested with *MboI* and *SbfI*. Lanes 7-8 represent infection of Jurkat cells, lane 9 = DNA ladder, while lanes 10-14 represent superinfection of latently infected Jurkat cells. As described in the results, virus populations containing recombinant viruses are represented by lanes 11, 13 and 14, but not lane 12. Note that lanes 7-14 are from one representative experiment and were run on the same agarose gel. (F) Results of sequence analysis from two independent experiments, each performed in duplicate, are shown. Recombinant virus = Δ*SbfI*+K103N+M184V+Δ*MboI* on the same genomic RNA. The highest drug concentrations applied were 20 nM EFV + 16 µM FTC, while the lowest drug concentrations were 2.5 nM EFV + 250 nM FTC.

4.6 DISCUSSION

The latent reservoir represents an important source of viral genetic diversity that could contribute to viral evolution, immune evasion and multidrug resistance. Latent virus reactivation might occur by superinfection of latently infected cells [23]. This would give rise to heterozygous virions, which are a prerequisite for the generation of recombinants, and could contribute to the emergence of

multidrug resistance [18-20]. Although several previous studies have provided evidence that latent viruses likely contribute to recombination *in vivo* [15, 19, 24], this process has not been experimentally characterized.

In this study, we asked whether latent viruses would be reactivated when their host cells are superinfected, and if so, whether they could contribute to the generation of recombinants. We first showed that superinfection of latently infected cells led to efficient reactivation of latent viruses (Figure 4.1). Pathways that might contribute to latent virus reactivation upon superinfection include the modulation of the cellular activation status by either gp120-induced signalling [30-35] or by Nef [38-40]. However, we found no evidence for gp120 (Figure 4.1) or Nef (Figure 4.2) in the reactivation of latent viruses by superinfection in our Jurkat latency model. Experiments with inhibitors targeting different stages of viral replication demonstrated that latent virus reactivation required gene expression of the superinfecting virus (Figure 4.1). The use of functional, attenuated, or inactivated *tat* viruses demonstrated that latent virus reactivation required the activity of newly expressed Tat by the superinfecting virus (Figure 4.3). Superinfection of latently infected primary resting CD4 T-cells also led to reactivation of latent viruses (Figure 4.4). As expected, populations of drug-resistant latent viruses were also subject to reactivation by superinfection in Jurkat cells (Figure 4.5A-D). Finally, restriction enzyme digestion and population-level sequencing demonstrated that reactivated latent viruses recombined with superinfecting viruses to produce multidrug-resistant recombinants (Figure 4.5E-F).

Lentiviruses including HIV-1 have evolved various strategies to downregulate cell-surface CD4, which might serve to impair immune recognition of infected cells and/or to limit cellular superinfection in a phenomenon referred to as superinfection immunity [25, 42, 43]. Although CD4 downregulation can decrease superinfection rates [25], this effect is not absolute and others have

observed minimal interference to superinfection [16]. Regardless of the magnitude of superinfection immunity, we were interested in superinfection of latently infected cells. Since latent viruses express little or no viral gene products, neither CD4 downregulation nor superinfection immunity would be expected.

Most clinically relevant latent viruses are found in resting CD4 T-cells. This implies that superinfection of latently infected cells *in vivo* would require infection of resting cells, which is much less efficient than infection of activated cells. Nonetheless, infection of resting cells does occur both *in vitro* and *in vivo* (reviewed in [44, 45]). Furthermore, infection of phenotypically resting CD4 T-cells is enhanced in chemokine/cytokine-rich environments such as secondary lymphoid tissues [12, 46-50] where the majority of lymphocytes reside – including multiply infected cells – and several studies have reported that pre-treatment of resting CD4 T-cells with various chemokines increases subsequent infection rates [51-53].

Reactivation of latent viruses by superinfection (Figures 4.1-4.3) results in cells expressing two genetically distinct viral genomes. Notably, HIV-1 has a much higher effective rate of recombination than some other retroviruses such as murine leukemia virus (MLV). This is not due to higher rates of RT template switching, but rather to higher rates of heterozygous genomic RNA dimerization and packaging [15, 54, 55]. The segregation of HIV-1 but not MLV genomic RNA molecules into assembling virions is effectively a random process, and there is now direct physical evidence that heterozygous HIV-1 virions are produced according to a Hardy-Weinberg equilibrium [56]. Furthermore, it has been estimated that nearly all HIV-1 virions undergo recombination during reverse transcription, as opposed to only a subpopulation of viruses [16, 55, 57]. As discussed above, multiply infected cells can result from either simultaneous infection by cell-to-cell transmission, or by sequential infection due to superinfection. It has been estimated that superinfection contributes to

recombination to a much greater extent than does cell-to-cell transmission, on the assumption that multiple infection by cell-to-cell transmission involves genetically identical virions [13]. Regardless of the pathways of infection through which recombinant viruses arise, their evolutionary success is apparent given the global abundance of CRFs. More direct examples of the success of recombinants in the face of selective pressure are shown by studies in which rhesus macaques were inoculated with two simian immunodeficiency virus (SIV) strains, each deleted in one or more accessory genes. In these studies, recombinants emerged as the dominant quasispecies in most macaques [21, 22]. In settings of highly active antiretroviral therapy (HAART), the selective advantages of multidrug-resistant viruses might be even greater than for these accessory gene-deleted lentiviruses.

Previous studies have examined superinfection of cell lines harbouring defective or latent viruses, although not in the same context as explored here. The authors of one study infected U1 and ACH-2 cell lines (which harbour latent or defective proviruses), or their parental cells lines, with HIV-1 that was pseudotyped with an amphotropic MLV envelope [58]. In this study superinfection was used as a tool to uncover cellular determinants of viral latency in U1 and ACH-2 cells, and provided useful insights into HIV-1 latency at a time when little was known in that regard. However, latent virus reactivation and subsequent recombination were not addressed. A second study demonstrated recombination when a cell line chronically infected with a Vpr-deleted provirus was superinfected with other accessory gene-deleted viruses [59]. More recently, our group has demonstrated recombination following superinfection of a cell line chronically infected with a multidrug-resistant virus, although the cell line carried an envelope-defective virus rather than a latent virus [60]. In the present study we have used populations of cells representing a true state of virological latency across thousands of integration sites (Figure 4.1 and [26]), as well as primary resting CD4 T-cells. Of note, the latent viruses used here express H13L Tat that

attenuates its activity by decreasing Tat-P-TEFb interactions. This is reminiscent of the enrichment of attenuated *tat* viruses that was identified in resting CD4 T-cells of patients on suppressive therapy, where these *tat* mutations also caused decreased affinity for P-TEFb [61].

While many of the same mechanisms appear to govern the establishment and maintenance of latency in Jurkat cells and primary cells [44, 62], their intracellular environments exhibit important differences. Thus, our examination of the effect of gp120 on latent virus reactivation (Figure 4.1) might not be applicable to latency in primary resting cells, whose activation state is unlikely to be represented by Jurkat cells. Similarly, this issue might apply to our examination of the role of Nef in latent virus reactivation (Figure 4.2). If anything, however, our results might suggest a modest inhibitory effect of Nef on latent virus reactivation in Jurkat cells (Figure 4.2C and 4.3E; compare the ΔNef data point to the linear regression line), although this is purely speculative. It is worth discussing our use of an NRTI-resistant virus in the recombination experiments presented in Figure 4.5, since some NRTI resistance mutations alter recombination rates. However, the M184V mutation used here has only a minor effect on RT template switching rates [63]. Additionally, it has been shown that recombination occurs at similar frequencies in Jurkat cells, used in our study, and in primary cells [16].

Recombination is expected to occur whenever there is ongoing replication. While two recent studies have provided evidence for ongoing HIV-1 replication during suppressive HAART [64, 65], the general consensus is that ongoing replication does not occur in most HAART-treated patients [66-69]. Nonetheless residual viremia is present in most HAART-treated individuals, which likely arises from reactivation of latent viruses, and a recent study demonstrated that residual viremia during long-term suppressive HAART was infectious [70]. This suggests that that new rounds of replication could occur during periods of low drug

adherence or treatment interruption, or even during adherent treatment if the residual virus was drug-resistant. Superinfection of latently infected cells might be expected to occur regularly in untreated patients, and could also occur during HAART as a result of infectious residual viremia, regardless of whether the residual viremia originated from activation of individual latent viruses, low-level ongoing replication, or viral rebound following treatment failure. As demonstrated here, reactivated latent viruses are capable of undergoing recombination. Recombination is widely acknowledged to increase viral evolution in individual patients [14, 24, 71, 72], often though not always accelerating the emergence of multidrug resistance [73-75]. Since all viral quasispecies including drug-resistant viruses can be latently archived [3-7], reactivation of latent viruses by superinfection or other means could provide for the emergence or spread of replicatively fit viruses in the face of strong selective pressures.

4.7 CONCLUSION

The work presented in this chapter contains two main findings. The first is that superinfection of latently infected cells can reactivate latent viruses, and the second is that reactivated latent viruses can recombine with superinfecting viruses and thereby contribute to the generation of recombinants, including multidrug-resistant recombinant viruses. These findings are summarized in Figure 4.6.

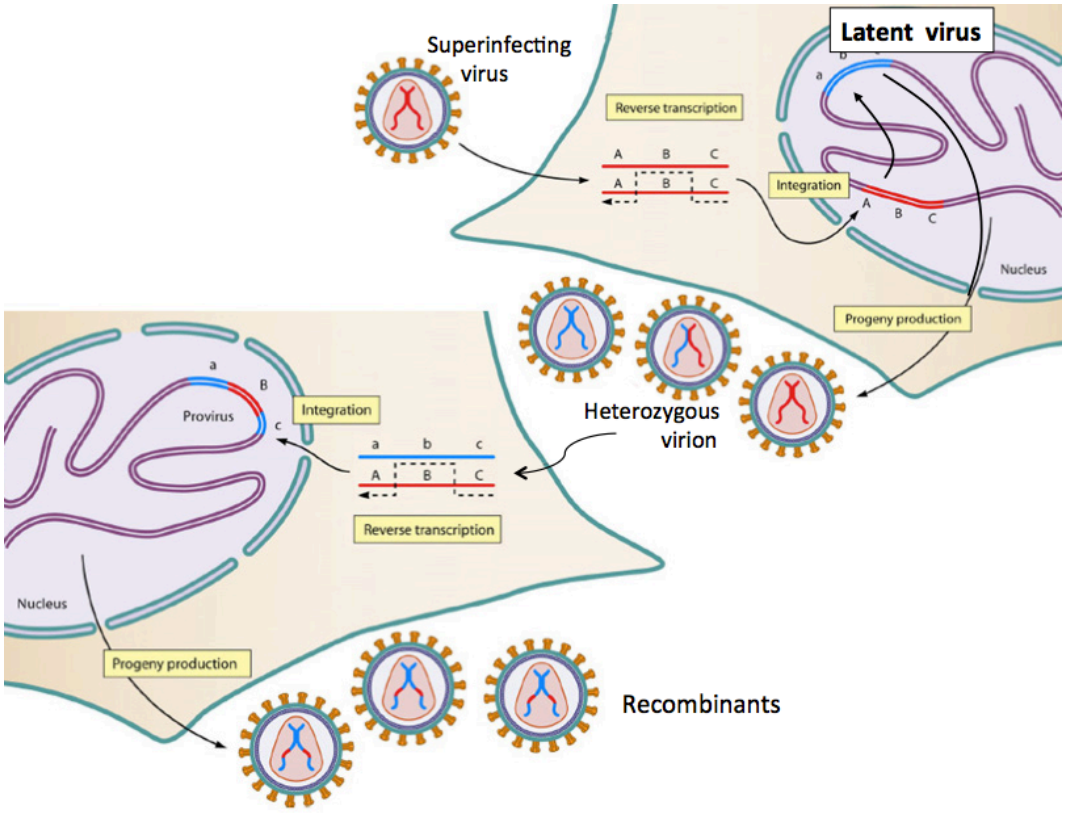


Figure 4.6. Summary of the main findings presented in this chapter.

(Top right) Superinfection of a latently infected cell. During reverse transcription, the superinfecting virus' RT switches templates, but for a virion containing identical RNA genomes, this has no effect on the resulting proviral DNA. Upon production of Tat by the superinfecting virus, latent viruses can be reactivated. The previously latently infected cell is now a coinfected cell expressing two genetically distinct proviruses, and some of the resulting virions will be heterozygous. (Bottom left) Heterozygous virions, containing one viral genomic RNA from the superinfecting virus and one from the reactivated latent virus, could infect a new cell under conditions of poor drug adherence, in sanctuary sites, during treatment failure, during low-level ongoing replication, or if the virus was a drug-resistant minority species. During reverse transcription, template switching by RT generates recombinant viruses. When both parental viruses contain different drug resistance mutations, this can lead to the formation of multidrug-resistant recombinants. Figure adapted from [15].

It is worth discussing why recombination involving latently archived viruses is so important in terms of HIV-1 infection under HAART. In terms of drug resistance, multiple mutations are usually required to provide high-level resistance without a large fitness cost. For example, resistance to protease inhibitors often requires several mutations before substantial resistance is possible, while for RT or integrase inhibitors, a primary mutation will often provide resistance but with a substantial fitness cost, while one or more secondary mutations will restore fitness to the drug-resistant virus. Normally, this requires the sequential acquisition of resistance mutations. Depending on the number of mutations and the fitness level of the intermediate (partially resistant / partially fit) viruses, this can take a long time to occur. This pathway is depicted in Figure 4.7A. In contrast, drug-resistant latent viruses can already contain several linked mutations. When recombination occurs between a virus with resistance to one drug, and a latent virus resistant to another drug, one result can be the rapid acquisition of high-level drug resistance in a replicatively fit virus. This pathway is depicted in Figure 4.7B.

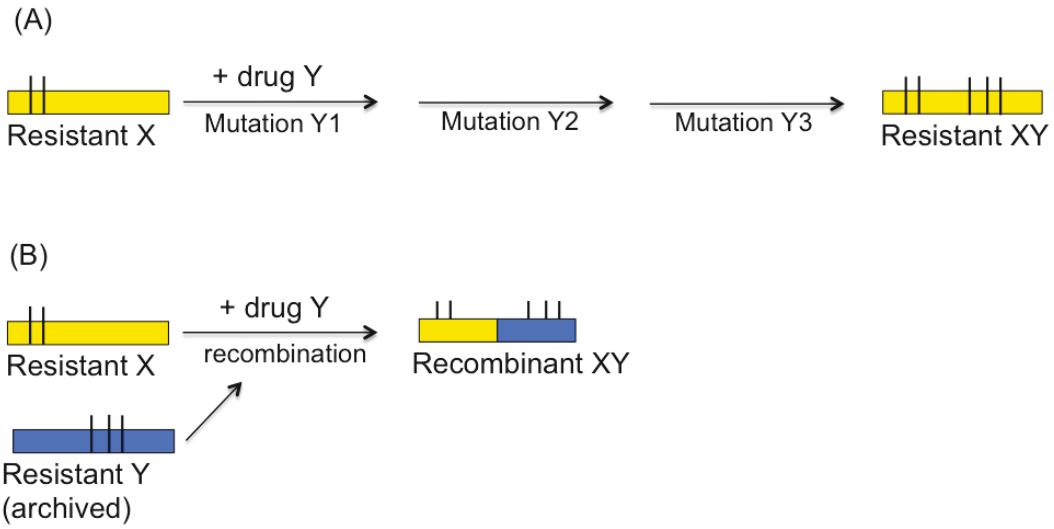


Figure 4.7. Development of multidrug resistance by *de novo* acquisition of resistance mutations, or by recombination.

(A) Sequential acquisition of drug resistance mutations. Each vertical line represents a mutation, and several mutations are required for resistance to a given drug. (B) Recombination involving previously archived drug-resistant viruses can lead to more rapid development of multidrug-resistant viruses.

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Chapter 5

Discussion

5.1 GENERAL DISCUSSION

Chapter 2 represents an investigation of the role of second phase viral reservoirs in HIV-1 infection. The work presented both provides for a greater understanding of the enhanced efficacy observed clinically with integrase inhibitors, and uses the results of clinical trials to further understand the dynamics of viral replication and inhibition. Chapters 3 and 4 represent an investigation into the role of third/fourth phase viral reservoirs in HIV-1 infection. The work presented in Chapter 3 examined the concept of inhibiting the establishment of HIV-1 latency in CD4 T-cells. The data presented demonstrate that this is theoretically possible to achieve, providing important proof-of-concept for further investigations of the clinical relevance of such an approach. The work presented in Chapter 4 examined the role of latent viruses in HIV-1 infection. Those data demonstrated that superinfection of latently infected CD4 T-cells can lead to reactivation of latent viruses that can contribute to further viral replication and the generation of multidrug-resistant recombinant viruses. Since each chapter already contains a detailed discussion of these specific findings, Chapter 5 is a more broad discussion of viral reservoirs in terms of both HIV-1 virology and clinical impact.

5.2 SECOND PHASE RESERVOIRS

Although the identity of second phase cells still remains unknown, it is possible that these cells might include macrophages or dendritic cells, or other cell types that can survive for several weeks while infected. In this regard, antigen-presenting cells (including macrophages and dendritic cells) might form part of the second phase reservoir. Thus, infection of these cells would be of great importance in terms of the ensuing adaptive immune response driven by these cytokine-producing immune sentinels, and the effects on subsequent pathogenesis could be substantial. In contrast, latently infected CD4 T-cells can survive for decades as opposed to weeks, due to the very nature of immunological memory. Since it is these third/fourth phase reservoirs that form the major barrier to HIV-1 eradication and provide the major source of viral

rebound during treatment failure [1], it is clear that these cells are of greater clinical relevance than second phase cells in their roles as viral reservoirs. For this reason, the remainder of the Discussion will focus on long-lived, latently infected CD4 T-cells.

5.3 ESTABLISHMENT OF THIRD/FOURTH PHASE RESERVOIRS

At present, it is not clear how latency is established in terms of initial silencing events. Conceptually, latency might be established by one or more pathways. First, infection of a cell might lead to actively expressing viruses that become silenced by one or several of the mechanisms discussed in Chapter 1. Second, infection of a cell might lead to immediate silent integration, where the virus is latent upon integration and is maintained in a silenced state. These two alternatives are not mutually exclusive, and both could occur depending on the cellular context.

5.3.1 Latency from silencing of active infections

In a small number of studies, one or both of these pathways have been directly addressed. Furthermore, close analysis of the data obtained from various cell culture models of latency establishment can help shed light on these issues, even from studies that did not directly look at these options. In some Jurkat [2] and primary cell [3] models of latency establishment, cell sorting was used to initially select for actively expressing fluorescent reporter viruses shortly after infection. Sorted, virus-expressing cells were then cultured and some of the active viruses became silenced. These studies demonstrate that the establishment of latency can result from the silencing of initially active infections, as evidenced by the title of one such report, “Epigenetic silencing of HIV transcription by formation of restrictive chromatin structures at the viral LTR drives the progressive entry of HIV into latency” [2]. It is noteworthy that in these two studies [2, 3] the viruses used did not express Gag (MA, CA, NC, p6), Pol (PR, RT, IN), Vif, Vpr or Nef. While these modifications are useful from a biosafety perspective and result in less cell

death due to viral cytopathic effects, it is possible that the near absence of viral protein expression allowed infected cells to survive for a much greater amount of time than would normally occur. This could have had the unintentional effect of making this pathway of latency establishment more likely to occur than with replication-competent viruses. However, this is only a concern if the authors' goal was to study the contributions of these two pathways to latency establishment; for other aspects of studying latency, the absence of cytopathic effect can be helpful. In addition, one study established latency in the CEM T-cell line and in primary cells [4]. Here, the authors used replication-competent virus and did not use cell sorting, and concluded that "Gradual Shutdown of Virus Production Resulting in Latency Is the Norm during the Chronic Phase of Human Immunodeficiency Virus Replication" [4]. Additionally, one model involves infection of transcriptionally active, immature (CD4+CD8+) thymocytes that are infected and then undergo a deactivation process, during which time latency is established [5].

5.3.2 Latency from immediate silent integration

The other possible pathway of latency establishment, in addition to silencing of active infections, is immediate silent integration. Several studies have provided evidence that latency can result from initially silent infection. A prime example of this, although often overlooked, is the popular J-Lat ("Jurkat-Latent") series of cell lines. These are latently infected Jurkat cell clones that contain either minimal viral reporter constructs, or full-length (replication-defective) reporter viruses [6]. These cells were first derived by infecting Jurkat cells, followed by sorting GFP-negative cells, which would contain uninfected cells and cells with silent integrated viruses. TNF-a was used to reactivate latent viruses, and the resulting GFP-positive cells were sorted and then cloned, resulting in the establishment of a number of J-Lat cell lines. This experimental outline is depicted in Figure 5.1.

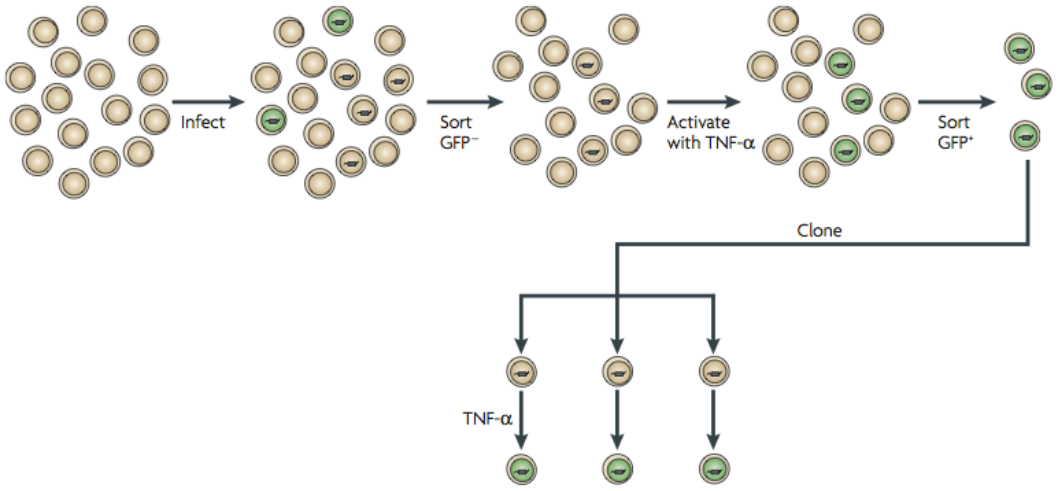

Figure 5.1. The origin of the J-Lat series of latent cell lines provides evidence for the establishment of latency by immediate silent integration.

See text, above, for details. Figure adapted from [7].

A recently published paper provides strong support for the idea that most HIV-1 infections are immediately silent. In this study [8], the authors used a double-fluorescent reporter virus where one marker is under the control of the HIV-1 LTR (to track viral gene expression) while the other marker is under the control of a CMV promoter (to provide a positive marker for integrated viruses, regardless of viral gene expression). In cell lines and in primary cells, infection with this double reporter virus yields mostly latent (LTR-reporter-negative) viruses shortly after infection. The authors additionally provided evidence to suggest that the level of NF-kB at the time of infection was responsible for the initial latency "decision". Interestingly, in our recently published review article that formed the basis for parts of Chapter 1, we speculated that even in primary activated CD4 T-cells, immediate silent integration was possible, but that it is usually ignored due to its clinical irrelevance ("The establishment of latency in proliferating cell lines implies that latency might be established in some fraction of infected, activated CD4 T-cells, even *in vivo*. However, the short lifespan of

activated cells *in vivo* implies that any such latent infections would be clinically irrelevant" [9]). When the double-fluorescent study [8] was published only a month after the publication of our review, those authors validated our speculation by showing that silent infections were more prevalent than active infections, in the activated CD4 T-cells of multiple individual donors.

Several additional studies have used CD4 T-cell lines to study latency, and either directly or indirectly provided evidence for latency resulting from immediate silent integration. Often, this was done by showing reactivation of latent viruses as early as one day after infection [10-14]. In fact, the work described in Chapter 3 demonstrates that immediate silent integration can lead to latency. Specifically, in Figure 3.3A, Jurkat cells were treated with TNF-a only 48 hrs after infection and latent virus was quantified 24 hrs later. We found that latent viruses were 4-5X more abundant than actively expressing viruses at this early time point. This might be a result of the attenuated *tat* (H13L) virus used, which might lead to higher than normal levels of silencing. However, in Figure 3.4, attenuated *tat* virus infection was compared directly to wt *tat* virus infection, except that cells were treated with TNF-a at only 18 hrs after infection. Silent (TNF-a-inducible) viruses, as measured 42 hrs after infection, were present at >5X the level of active viruses for attenuated *tat* infection. When wt *tat* virus was used for infection, immediate silent (latent) viruses accounted for more than half of all infections.

Lastly, several primary cell latency models provide evidence for latency that results from immediate silent integration. This includes one model where infection takes place during the transition of activated cells to a resting state [15], as well as all published latency models that involve the direct infection of resting cells [16-21]. Furthermore, the primary cell work presented in Chapter 4 provides further support for the establishment of latency by immediate silent integration, since levels of latency were determined only a few days after

infection. Based on the above discussion, it can be appreciated that both of these pathways of latency establishment are possible *in vitro*. That is, latency is likely to result from both initially active infections that are subsequently silenced, and from immediate silent integration. *In vivo,* the pathway of latency establishment probably relates to the activation / resting status of the cell undergoing infection. By this hypothesis, resting cells would be more likely to support immediate silent integration, whereas activated cells would support both immediate silent integration and actively expressing integration events, of which some become silenced and contribute to latency.

5.4 CAN THE ESTABLISHMENT OF LATENCY BE PREVENTED?

A major goal in the field of HIV/AIDS is now eradication of latently infected cells [22]. This approach might be complemented by strategies that aim to limit the establishment of latent reservoirs. The importance of this concept is highlighted by a 2010 review on the topic of achieving a functional cure, authored by several of the leading researchers in this field ("HIV Persistence and the Prospect of Long-Term Drug-Free Remissions for HIV-Infected Individuals") [23]. Among a list of important questions to be answered, under the heading "Some of what we want to know and should dare to ask," is the issue of whether early HAART initiation can limit the pool of latently infected cells. In fact there is now evidence from a number of clinical studies which suggests that very early initiation of therapy can result in smaller latent reservoirs being established, compared to when treatment is started later [24-29]. As discussed in Chapter 3, the very recent report of an infant who appears to have been functionally cured provides important validation of this concept. However, this infant was put on HAART at the age of 30 hours, something that is clearly not realistic for the vast majority of adult infections. It should also be mentioned that this was a unique case where the mother's HIV status was not known until the time of birth, and as such, she was not given single-dose nevirapine to prevent infection of the child. Had her status been known, the child would likely have been born HIV-negative.

Nonetheless, this case shows that it may be possible, at least under exceptionally ideal clinical circumstances, to fully inhibit the establishment of latent reservoirs.

Additional approaches to inhibition of the establishment of latency could include early treatment with compounds that antagonize one or more mechanisms of latency establishment. This is precisely the question that was examined *in vitro* in Chapter 3 and published last year [11], where either exogenously administered or intracellularly expressed Tat protein was provided shortly after cell culture infection. Although far removed from the clinic, the data presented in Chapter 3 provide proof-of-concept that approaches other than early HAART can limit the establishment of latent reservoirs. Our date showed a greater than 3-log reduction in the number of latently infected cells observed under certain conditions. Interestingly, in a plenary presentation at CROI 2013 by Dr. Siliciano – a leading voice in the field of HIV-1 latency – data were presented in which eradication was mathematically modeled (Abstract 16, "HIV-1 Eradication Strategies: Design, Assessment, and Clinical Consequences", CROI 2013, March 4, 2013 in Atlanta, GA). Various log reductions in the number of latently infected cells were used to project times until viral rebound after stopping HAART. A 3-log reduction was suggested to be the minimum requirement for a clinically significant delay in the time to viral rebound (years, as opposed to days/weeks as per the *status quo*). However, a 4-log reduction would be required for some patients to never experience viral rebound within their lifetimes, and a 5 to 6-log reduction would be needed for most or all patients to never experience viral rebound. Of course, this modeling is based on clinical parameters, and the 3-log reduction achieved under certain conditions in the data presented in Chapter 3 would probably not apply to all assumptions in the modeling.

Similarly, compounds currently under investigation for a "shock and kill" approach to depletion of latently infected cells might also achieve a similar effect as what we observed with Tat protein. That is, compounds aimed at reactivation

of latent reservoirs might also be useful with first-line therapy, although this is, of course, a large extrapolation from cell culture data that require validation before being applied to clinical practice. Nonetheless, a large number of compounds targeting a number of different pathways (discussed in Chapter 1) are now available that could be tested in this type of approach. It remains to be determined whether these approaches deserve clinical consideration.

An additional, novel approach to the inhibition of the establishment of latency has recently been proposed, which would act in resting CD4 T-cells [30]. As discussed in Chapter 1, the establishment of latency in resting cells has been observed following chemokine treatment of resting cells [18-20, 30]. Here, the chemokines act by inducing cytoskeletal changes that permit infection of resting CD4 T-cells. Thus, although the chemokines' effect is on trafficking of the viral core to the nucleus, the net result is that they can permit latent infection of resting CD4 T-cells. The proposed method to inhibiting latency establishment would make use of chemokine receptor antagonists, which would prevent chemokines from being able to signal through their receptors. Additionally, engineered dominant negative chemokines were proposed to be used, which could directly out-compete existing chemokines. These dominant negative chemokines could be administered as a drug, or they could be expressed following a genetic modification approach. These remain proposals that have not yet been experimentally tested. In addition to serving as a method to inhibit the establishment of latent infection in resting cells, these chemokine-mediated approaches could serve as a useful tool to help understand the relative contributions of the different cellular pathways of latency establishment. As discussed in Chapter 1, it is not yet clear whether most latent infections arise from infection during the transition of an activated CD4 T-cell to a resting state, or from direct infection of resting CD4 T-cells. The use of chemokine receptor antagonists or dominant negatives could potentially be used in a humanized mouse model of HIV-1 latency [31-34], with one outcome being to determine

what fraction of latent infection events arise from direct resting cell infection. This would be contingent upon the effectiveness of the proposed chemokine-mediated methods.

5.5 OUTSTANDING QUESTIONS CONCERNING VIRAL RESERVOIRS

As discussed in detail in Chapter 1, the establishment of HIV-1 latency is a multifaceted process that likely results from multiple simultaneous mechanisms. It is not currently known whether some of these mechanisms might be more important than others, or whether latency can result from a single mechanism or if multiple mechanisms are always required. This is complicated by the fact that many of these mechanisms are intricately linked. For example, sub-threshold levels of transcription factors such as NF-kB can result in the establishment of latency in some models, due to sequestration of p50/p65 (the "active" form of NF-kB) in the cytoplasm. In this case, p50/p50 homodimers bind the NF-kB binding sites in the LTR and recruit histone deacetylases, resulting in repressive epigenetic modifications. This, in turn, might favour transcriptional interference from nearby cellular promoters, due to a lack of LTR-based transcription. This example serves to highlight the complex nature of the mechanisms that drive the establishment of latency. Is the relative importance of these mechanisms of latency establishment dependent upon the activation status of the cell undergoing infection (for example, infection of a CD4 T-cell transitioning to a memory state, or infection of a phenotypically resting cell in the presence of chemokines in secondary lymphoid tissues)?

What we know about the mechanisms of latency establishment is derived from a wide range of cell types, including clonal T-cell lines, population-level latency models in cell lines, primary cell models, and resting cells of HAART-treated patients studied *ex vivo*. How accurately do these different systems reflect *in vivo* latency mechanisms? Of course patients' cells provide the most natural cellular environment, but the extremely low yield of latently infected cells *in vivo*

makes these impractical for many experimental questions. Do cell line models of latency accurately reproduce the mechanisms that are at play in primary cell models? Perhaps surprisingly, many of the same mechanisms appear to be active in both cell line and primary cell models. Nonetheless, it is likely that not all mechanisms of latency are functional in actively dividing cell lines. For example, P-TEFb was recently reported to be regulated differently in primary cells undergoing "deactivation" into memory cells, compared to the findings of a large body of work based on cell lines [35] (this study reported P-TEFb regulation by phosphorylation of one of its subunits, compared to cytoplasmic sequestration that had been observed previously by many groups studying cell lines). Additionally, it has been proposed that some of the mechanisms of latency are more specifically associated with quiescent cells, and therefore latency in cell lines might be more dependent on epigenetic silencing [21]. It is interesting, however, that a number of studies including the data presented in Chapter 3 have shown roughly similar ratios of active infection to immediate silent integration, using a range of different cell lines and primary cells.

Also unknown is whether different mechanisms of provirus silencing are required, depending if a cell is infected during deactivation or when it is already in a resting state, or for silencing of active infection compared to immediate silent integration. Perhaps the only conclusion that can be made in this regard is that all of these different pathways of infection can lead to the establishment of latency. Furthermore, it is not known whether latency establishment differs upon infection of different subsets of CD4 T-cells. This includes not only the "major" subsets like naïve, Th1, Th2, Th17, Treg or Tfh (follicular helper) cells, but also subsets of memory CD4 T-cells (central, transitional and effector memory cells, as well as the recently identified "stem cell memory" CD4 T-cells [36] which were very recently reported to serve as a long-term viral reservoir (M. Buzon, Abstract 44, CROI 2013)). Compared to latency in different CD4 T-cell subsets, less is known about the establishment and maintenance of latency in

other cell types (such as hematopoietic progenitor stem cells or second-phase cells). The mechanisms of latency in these cells could differ from those in CD4 T-cells.

An important goal in the field should be to determine which models of latency most accurately reflect the mechanisms active *in vivo*. This will remain an essentially unanswerable question for the time being, awaiting a more complete understanding of the routes of infection and mechanisms of latency in patients. Lastly, the potential use of pharmacological or other interventions to inhibit the establishment of latent reservoirs in patients, and what clinical benefits this might involve, are important outstanding questions in the field of HIV-1 latency.

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