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Facts and Fiction: Cellular Models for High Throughput Screening for HIV-1 Reactivating Drugs

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Abstract

A curative therapy for HIV-1 infection will have to include measures to eliminate the reservoir of latently HIV-1 infected cells that allow the virus to persist despite otherwise successful therapy. To date, all efforts to deplete the latent reservoir by triggering viral reactivation have used preexisting drugs that are believed to potentially target molecular mechanisms controlling HIV-1 infection. These therapeutic attempts were not clinically successful. Only in the last few years have cellular models of latent HIV-1 infection suitable for high throughput screening been developed and concerted drug discovery efforts were initiated to discover new HIV-1 reactivating drugs. We here provide a historic overview about the development of cell models with latent HIV-1 infection that lend themselves to drug discovery. We provide an overview from the first reported latently infected cell lines to current *in vitro* models of latent HIV-1 infection in primary T cells, and compare their potential to be used in future large-scale drug screening efforts.

INTRODUCTION

Current research to identify HIV-1 reactivating drugs focuses on two major molecular mechanisms that are implicated in the control of latent HIV-1 infection. The first treatment design approach is based on multiple reports that support the idea that HIV-1 latency is a function of the relative absence of cellular activation factors in resting T cells. In particular low levels of active nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and nuclear factor of activated T cells (NFAT) seem to be important for the maintenance of the latent viral state (1, 2). Because NF- κ B is described as a major control factor of HIV-1 replication, early attempts to trigger HIV-1 reactivation to drive the elimination of latent reservoirs used IL-2 and the anti-CD3 monoclonal antibody, OKT3. Both stimuli had been reported to trigger an increase in NF- κ B activity in T cells (3-5); however, their application in HIV-1 infection did not impact the size of the latent viral reservoir in treated patients, but triggered detrimental side effects in some reported studies (4). Such treatment side effects were not necessarily a surprise, as high concentrations of either OKT3 or IL-2 were known to cause side effects that can ultimately result in a fatal “cytokine storm” syndrome (6). Only treatment approaches that trigger HIV-1 reactivation with minimal to no cytokine induction can be considered suitable for therapeutic use. The idea that dissociation of cell activation from HIV-1 reactivation is possible has been suggested by some findings revealed during recent drug screening efforts (7-9).

The second therapeutic approach that is currently being investigated is based on early reports that restrictive histone structures are established at the integrated viral promoter

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region (long terminal repeat or LTR) of latent HIV-1 infection events (10-13), causing inhibition of virus transcription similar to the mechanisms that control cellular gene expression. Histone deacetylase (HDAC) enzymes regulate histone molecular structure and function; as a result, pharmaceutical HDAC inhibitors (HDACi) may be capable of reversing this restriction to promote HIV-1 reactivation in the absence of classic T cell activation. A major advantage of such an approach is that HDAC inhibitors should not trigger cellular gene expression through NF- κ B activation, and therefore, are not expected to induce high cytokine expression levels. Reports described the ability of HDACi to reactivate latent HIV-1 infection efficiently in some T cell lines (10, 12), in patient-derived T cell populations (14) and with less or no potency in certain models of latency in primary T cells (7, 15) and more recently established latently HIV-1 infected T cell lines (16). A proof-of-principle study using the clinically approved HDAC inhibitor valproic acid, which is used to treat some forms of seizures and mania in people with bipolar disorder and epilepsy, seemed to suggest a modest decrease in the latent reservoir in 3 out of 4 patients (17). However, further studies by the same group and others failed to show a statistically significant decrease in the size of the latent reservoir in response to valproic acid treatment (18-22).

More recent studies have demonstrated that latent HIV-1 infection events, found both *in vivo* and *in vitro*, occur during preferential integration into actively expressed host-genes. Therefore, it is unclear how restrictive histone structures would be established in an otherwise actively transcribed DNA environment (16, 23, 24). The idea that transcriptional interference due to viral insertion could contribute to a latent infection state was brought forward to explain these observations (25, 26). On the other hand, there are multiple studies that implicate the involvement of a series of cellular factors at transcriptionally inactive HIV-1 proviral LTR sites that are also associated with gene control and epigenetic modifications (27-29). For example, a report by Williams *et al.* demonstrated that NF- κ B p50 could recruit HDAC1 to a silenced HIV-1 promoter region (30). Keedy *et al.* demonstrated that HDAC recruitment to the HIV-1 LTR is selective for specific HDAC classes, which can vary among different cell types and tissues (31). Using lentiviral vectors encoding an attenuated version of Tat, Pearson *et al.* presented data that suggest that restrictive chromatin structures at the viral LTR can drive the progressive entry of HIV-1 into a latent state (29). Studies by du Chene *et al.* indicated a role for the histone lysine N-methyltransferase Suv39H1 and Heterochromatin Protein-1 (HP1), a protein containing a chromo-domain that recognizes H3K9 methylation in chromatin-mediated silencing of HIV-1 transcription (32). Other transcription factors, such as CTIP2, have also been implicated in HIV-1 silencing through the recruitment of chromatin-modifying enzymes to the LTR (33). In addition, several studies have demonstrated that RNA polymerase II is paused on the HIV-1 LTR in latency and that this pause is controlled by the binding of Negative Elongation Factor (NELF) and DRB Sensitivity Inducing Factor (DSIF) proteins (34-37). These molecular findings should be reflected in a relevant drug screening system.

There are, however, practical concerns that must guide the development of any high throughput screening (HTS) assay, in particular, when an assay is designed to screen libraries of >100,000 small chemical molecules. Throughput criteria become even more important when compound libraries are screened at multiple drug concentrations, such as high-low screens or quantitative HTS (qHTS) (38). For identification of HIV-1 reactivating drugs, the importance of screening strategies that evaluate each test compound at two or more concentrations is exemplified by the phorbol ester PMA, which is a powerful HIV-1 reactivating agent in T cell lines and in some primary T cell models (7, 16, 39). PMA would likely not be discovered in a classic drug screen for HIV-1 reactivating agents performed at a single concentration. Using a standard screening concentration range of 3 - 10 μ M, PMA is highly cytotoxic; however, its activity for HIV-1 reactivation is evident at much reduced concentrations of 3 - 30 nM. The risk of obtaining false negative hits is thus substantial, and

has to be considered when deciding which HTS assay is most appropriate for an automated platform.

Currently, the way forward in identifying new HIV-1 reactivating drugs is being discussed arduously, and sometimes dogmatically. This review aims to discuss some of the facts and fiction of available models of HIV-1 latency and the extent to which the various models lend themselves to true high throughput efforts that will require the screening of millions of compounds in the quest to identify experimental drugs for the reactivation of HIV-1 from latency.

Early cell based models of HIV-1 latency

Initially and in short succession, a panel of latently HIV-1 infected cell lines was established, including the T cell lines ACH2, OM10.1 and J1.1, and the promonocytic cell line U1 (40-44). These cell lines were used in a series of early studies that established the foundation for our current understanding of HIV-1 latency. The relevance of NF- κ B activation to HIV-1 reactivation was first demonstrated through stimulation of these cell lines, using TNF- α or the phorbol ester PMA (40, 41, 43). The cell lines were also instrumental in the identification of HDAC inhibitors as reactivating agents of latent HIV-1 infection (10, 14).

However in subsequent studies, some of these same cell lines were found to contain proviral mutations that contributed to the latent infection state. For example, ACH2 T cells were shown to have an integration event with a mutation in the TAR element that attenuated the viral transcription response to Tat (45). U1 cells were shown to harbor an attenuated form of the tat gene that favored progression to a transcriptionally inactive state of the virus (46). With more extensive investigation, it has become increasingly clear that some cell line models harbor viruses that are not truly latent, but rather impaired in their transcriptional ability. Because the reported mutations are hypomorphic, the transcriptionally inactive HIV-1 proviruses could be induced with powerful stimuli, such as PMA or over-expression of wild-type tat, (46). Although one recent report suggests that the latent reservoir *in vivo* accumulates Tat variants with reduced transactivation potential (47), the vast majority of prior studies *in vitro* (with cell lines or primary cells) or *ex vivo* (using patient cells) have demonstrated that the contribution of viral mutations to the latent state is minimal, at best (7, 15, 48). In fact, the ability of latent viruses to efficiently replicate upon *ex vivo* cell stimulation was a prerequisite for the initial studies that identified and quantified the latent HIV-1 reservoir in patients to be successful (3, 48-50). Also, latently infected T cell line models such as J89GFP, J-LAT or Ca5 T cells that have been established more recently are devoid of loss-of-function or hypomorphic mutations, which favor latency (16, 51, 52). Although the integration site for the latent provirus in the ACH2 cell model was described to favor transcriptional suppression (53), this has not been the case for the more recently described T cell lines that harbor inducible, latent HIV infection. In these cell lines, as in latently infected primary T cells, the majority of HIV-1 integration events are located in actively transcribed host-genes ((26, 52) and O.K., unpublished data).

In addition to the questionable viral molecular status of the early cell line models for latent HIV infection, such cells are not suitable for drug HTS purposes, due to their lack of an included reporter gene mechanism to function as a surrogate marker for activation of HIV-1 replication. In this setting, reactivation could only be determined by the use of time consuming and relatively costly means, such as RT assays or p24 quantification (ELISA or intracellular stains for flow cytometry), which do not readily lend themselves to an HTS effort.

Reporter systems to monitor latent HIV-1 infection

The use of surrogate markers to track active HIV-1 expression was first addressed through the generation of latently HIV-1 infected cell lines that contain GFP reporter virus constructs (J89GFP, JNLGFP and THP89GFP) (51). These reporter cell lines contain GFP reporter viruses with open reading frames for all the viral genes (51, 54). A similar concept was used later by Jordan *et al.* to establish a panel of J-LAT cell clones (52), except that the clones were constructed using a $\Delta env/\Delta nef$ HIV-1 GFP-reporter vector. No evidence has been presented to indicate that the lack of Env or Nef expression would affect the molecular state of HIV-1 latency in the utilized Jurkat T cell line.

Latently HIV-1 infected reporter cell lines (J89GFP/JNLGFP/THP89GFP or J-LAT) have been generated using two different approaches. Under the assumption that latent infection results from shut-down of initially active viral transcription, J89GFP/JNLGFP/THP89GFP cells were cloned from GFP-positive (“GFP-on”), and thus actively infected cells (single cell deposition cloning). Surviving cell clones that no longer expressed GFP were tested for their capacity to respond to TNF- α or PMA stimulation with the induction of GFP expression, representing reactivation of viral expression (51).

The J-LAT cell panel was generated by removing GFP-positive, and thus actively infected cells from the infection culture by fluorescence-activated cell sorting (FACS). The recovered “GFP-off” cells were then exposed to TNF- α , and the subset of cells that responded to stimulation by GFP expression was enriched by FACS selection. From this isolated subpopulation, a panel of clonal J-LAT cells was generated, in which GFP/virus expression was silenced under normal culture conditions, but still remained responsive to inductive stimulation with TNF- α (52).

Whether deriving latently infected T cell lines from a population of cells that early on are actively infected (JNLGFP/J89GFP) or that early on show no signs of productive infection (J-LAT) biases these cells to emphasize different molecular mechanisms of control has not been investigated. Latent infection events in all cell lines revert to a latent state following HIV-1 reactivation, suggesting that the molecular mechanism(s) controlling latent infection are similar in these cell line models.

Several reports indicate that integration of latent virus in T cell lines (16), primary T cells (24) and in latently infected cells from patients (23) is found primarily within actively-expressed host cell genes. We have recently screened a panel of latently HIV-1 infected, Jurkat-derived T cell lines, which was developed by one of our labs (16, 26) and found that the integration profile is very much in concordance with that observed in primary T cells taken from HIV-infected patients (23). Specifically, 14 of 15 latent integration events were located in actively expressed host cell genes. Among these 14 cell lines, 13 had provirus residing in intron sequences and one, in an exon sequence. None of the integrated viruses had mutations in either the LTR or the *tat* gene, suggesting that viral attenuation is not needed for the establishment and control of HIV-1 latency in this cell model (O.K.; unpublished data). Our T cell panel representing latent infection differs, therefore, from other established cell line models (such as ACH2), in which virus integration was seen within DNA regions that were not supportive of active transcription (53). With regard to integration site profiles, the recently developed reporter cell lines are representative of the latent infection state seen in primary T cells, in which the majority of virus integration occurs in actively expressed host cell genes (23) (52).

While the development of latently HIV-1 infected reporter cell lines has provided an important advancement over earlier cell line models (ACH2, U1, OM10.1, etc.), such reporter cell line systems remain sub-optimal for current HTS technology. At the time these

cell models were developed, the maximal achievable speed for flow cytometric analysis would have permitted the screening of 60 - 80 compounds per hour, an acquisition rate not truly considered to be high throughput. In addition, the application of microplate-based fluorometry analysis was not suitable due to the low levels of induced GFP expression per cell attained and high autofluorescence background common to the green fluorescence emission spectrum. For example, a minimum of 1×10^6 J89GFP reporter cells per well (96-well plate) would be required to produce an acceptable GFP signal-to-noise ratio for reliable detection. Such cell numbers would greatly exceed realistic input numbers for a HTS designed to screen large compound panels, in the range of 1×10^5 - 1×10^6 small molecules each (51). A potential solution to this problem, based on the use of alternate fluorochrome reporters, is discussed below.

Latent HIV-1 infected cell lines for drug discovery

The first latently HIV-1 infected reporter cell lines designed for true HTS application was reported by Micheva-Viteva *et al.* in 2005 (55). To establish this cell model, a lentiviral vector was constructed, in which LTR-Tat interaction controlled expression of GFP, as well as that of secreted alkaline phosphatase (SEAP). While GFP expression was used to identify the latently infected cell clones for selection, SEAP expression was used as a surrogate marker of HIV-1 transcriptional activity, which can readily be measured in a plate-based HTS format. The generated cell lines contained replication-defective virus, resulting from engineered insertion of the *SEAP* gene in the HIV-1 *env* position plus a 2.5 kbp deletion within the *pol* gene sequence. This design was used to ensure the safety of this reporter cell model for subsequent HTS assay development and to allow large-scale drug screening under less costly BSL-2 biosafety restrictions. The Z' -factors of assays based on this cell line in a 96-well format were found to range between 0.55 and 0.8, as a function of the stimulus used (TNF- α , PMA or VPA). The Z' -factor is a dimensionless characteristic that describes the quality of an HTS assay and takes into consideration reproducibility as well as the dynamic range (Equation 1) (56). A perfect assay would have a Z' -factor of 1.0, while very good to excellent assays range between >0.5 to 1.0. Of interest, the 24STNLSG reporter cell line developed by Micheva-Viteva *et al.* responds to TNF- α stimulation with a >140 -fold induction, but with only <10 -fold signal induction response to PMA, which is usually the strongest HIV-1 reactivating agent in T cell lines (57). Assays using the 24STNLSG reporter cells have been developed further for application to a 384-well format that was recently used for the identification of a novel HIV-1 reactivating compound (4-3,4-dichloroanilino-6-methoxyquinoline), short-named AV6. AV6 triggered HIV-1 reactivation alone or in combination with known HDAC inhibitors (57). The AV6 compound stimulated viral reactivation in an NFAT-dependent fashion, as evidenced by sensitivity to VIVIT and FK506 treatments. This was a somewhat surprising finding, given that prior studies indicated AV6 exerted its effect on HIV-1 reactivation via a NF- κ B dependent pathway.

A second cell model, LWI-6, which was specifically designed for use in a HTS format, was published by Jones *et al.* in 2007 (58). This reporter T cell line utilized GFP as a surrogate marker for the HIV-1 transcriptional state. The LWI-6 cell line was obtained by infecting JLTRGR5 reporter T cells (59) with the WI70 primary HIV-1 clinical isolate and selecting cell clones with minimal GFP-background and maximal induced GFP expression (population level and mean channel fluorescence) following PMA or TNF- α stimulation. PMA stimulation of LWI-6 cells triggered a 40-fold increase in GFP signal over background after 48h, and the Z' -factor for the assay in a 384-well plate format was determined to be $Z' = 0.89$.

An advantage of LWI-6 T cell model (58) is that in contrast to luciferase or SEAP use, GFP detection does not require additional processing steps, and assay plates can be directly transferred onto a plate-reader for data acquisition. Thus, it provides a HTS assay that is

more cost-effective and less labor intensive. LWI-6 cells respond equally well to TNF- and PMA stimulation with achievable reactivation levels of 85 - 95% GFP-positive cells. Off-target drug effects can be simultaneously quantified in LWI-6 cells by changes in red fluorescence protein (RFP) expression, which is expressed under the control of the constitutively active murine stem cell virus LTR. A decrease in RFP fluorescence correlates with either decreased cell viability or inhibitory drug effects on other metabolic pathways. Changes in RFP fluorescence therefore indicate whether compounds may have been used at exceedingly high concentrations, which could potentially result in false-negative hits. This use of RFP further enables prioritization of compounds most likely to have a favorable therapeutic window. Because LWI-6 cells were generated using a primary clinical isolate (WI70), assay response artifacts due to virus mutations derived from *in vitro* virus passage are highly unlikely. On the other hand, drug screens need to be performed under >BSL2 laboratory conditions, as LWI-6 cells produce infectious viral particles.

Primary T cell based models of HIV-1 latency

It has been argued that latently HIV-1 infected T cell lines may not reflect all aspects of latent infection *in vivo*. In particular it was argued that *in vivo* latent HIV-1 infection is found in resting CD4+ memory T cells, a scenario that would not be reflected by the constantly proliferating immortalized T cell lines. Thus, great effort was spent to develop experimental models for HIV-1 latency based on primary T cells. Over the last years, several such models were successfully established. These models either utilize resting T cells for infection (60-62), or initially stimulate T cells by varying methods and infect these cultures at a time when cellular activation is decreasing or has returned to basal levels (7, 15).

Agosto *et al.* described a system in which resting memory T cells are infected by spinoculation in the absence of any particular stimulation (62). Saleh *et al.* demonstrated that stimulation with the CCR7 ligands CCL19 and CCL21 renders resting memory T cells susceptible to the establishment of latent HIV-1 infection events (60). However, these models produce rather limited amounts of latently HIV-1 infected cells and the cells have a short lifespan, rendering them not suitable for utilization in an HTS effort. These systems are more likely to be used as verification assays after large screening efforts.

Several models use initially stimulated T cells to produce latently infected T cells. Marini *et al.* used antigen-loaded dendritic cells to stimulate CD4+ T cells prior to HIV-1 infection (63). Sahu *et al.* established a system in which CD4+ T cells were stimulated with immobilized anti-CD3 mAb and IL-2 prior to infection with HIV-1 JRCSF. At later times these infected cultures were co-cultured with H80 feeder cells, which promote long-term survival of the now largely quiescent T cells. These T cells were known to harbor a 5-10% background of chronically (productively) infected cells. When these cells were stimulated with prostratin, the frequency of virus antigen-positive cells doubled, revealing the existence of a previously latent population (64). While the models from Sahu *et al.* and Marini *et al.* provide good verification assays during drug development efforts, neither of the two models is likely to produce sufficient amounts of cell material to be used in a HTS drug screening effort.

An extension of the model described by Sahu *et al.* was developed by Tyagi *et al.* (39). The Tyagi *et al.* model also uses the feeder cell approach for long-term cell maintenance, but the level of latent infection generated is much higher than that generated in the Sahu *et al.* model. The model is based on a lentiviral GFP vector that is devoid of several structural and accessory HIV-1 genes (gag- pol- vif- vpr- nef). The absence of these accessory and structural genes is unlikely a limitation for drug screening purposes and, in fact, it may constitute an advantage, as it permits screening in a BSL2 laboratory environment. This

system utilizes vectors containing an attenuated *tat*-mutant (H13L) that was found in U1 cells, and which strongly promotes latency establishment (46). The authors argue that this is consistent with one report that latently infected cells were found to harbor a higher frequency of *tat* variants with reduced trans-activation potential (47), a finding that has not been confirmed in other systems or studies (16, 23, 24, 52). Most recently, Marsden *et al.* also reported that accessory proteins influence latency establishment/reactivation in primary T cells (65). Thus, while vector systems are advantageous from the viewpoint of assay safety, they may suffer from shortcomings derived from the absence of viral genes and functions, which may regulate latency (65).

Yang *et al.* chose a unique approach to ensure high levels of cell viability in long-term cultures of latently HIV-1 infected primary T cells. In their protocol, primary resting CD4⁺ T cells are initially transduced to overexpress Bcl-2, a protein known to prevent apoptosis. The Bcl-2 overexpressing T cells are then restimulated and infected with the recombinant virus, NL4-3- 6-drEGFP. NL4-3- 6-drEGFP contains a truncated *nef* and premature stop codons in the ORFs encoding *gag*, *vif*, *vpr*, and *vpu*. Destabilized EGFP was introduced into the *env* ORF and the signal peptide of *env* was mutated to allow the destabilized EGFP to remain in the cytoplasm. Initial infection levels were described to range between 5 and 10%. Following removal of the GFP-positive cells by FACS the now GFP-negative population contains around 3% latently infected T cells, which can be identified by stimulation with either anti-CD3/CD28 mAb combinations or PMA (7, 8). This strategy has already been successfully used in a drug screening effort that identified 5-hydroxynaphthalene-1,4-dione, also known as juglone, and disulfiram (antabuse) as two agents that trigger HIV-1 reactivation in the absence of T cell activation (7, 8).

A third method to obtain large numbers of long-lived latently HIV-1 infected primary T cells was used by Bosque *et al.* (15, 66). In this system, naïve CD4⁺ T cells isolated from peripheral blood were stimulated with anti-CD3/CD28 mAbs in the presence of TGF- β , then cultured in IL-2 for three days, infected, and then allowed to return to quiescence in the presence of IL-2. This model reliably produces 30 - 60 % latently HIV-1 infected T cells with a central memory T cell phenotype and in combination with the utilization of an EGFP-reporter virus is amenable to medium HTS. The high frequency of latently infected T cells found in this system is an advantage for drug screening, as it allows to reduce the total number of input cells per well and to still reliably identify compounds that only have a weak HIV-1 reactivating effect, indicated by a small increase in the percentage of GFP-positive cells. Most recently, the assay has been adapted for utilization a fluorescence plate reader. For this assay, the EGFP in a reporter virus was replaced by tandem dimer Tomato Red fluorescent protein (tdTomato) (excitation and emission maxima occur at 554 nm and 581 nm, respectively). The assay exploits the exceptional brightness of tdTomato, which is almost 3-times brighter than EGFP. Combined with the high level of latently infected T cells that is generated in the Bosque *et al.* model of latent HIV-1 infection, these viruses upon reactivation produce a sufficiently high fluorescent signal to be readily detected in a fluorescence plate reader. Scanning a 96-well plate by fluorometry takes several seconds, compared with about the 30 minutes required for flow cytometric readouts. Inclusion of new technology will continue to improve available models and likely allow for further miniaturization and increased throughput in the near future.

The existence of multiple mechanisms of latency and models to study it could potentially polarize the field, so as to raise controversies about which models may be best or most physiologically relevant. Rather, we propose that different models be considered as complementary. Thus, the mechanistic differences can be exploited such that responsiveness in a particular system is likely an indication of a specific signaling pathway (see Table 1 for examples). However, from a drug screening point of view these differences cannot readily

be exploited, as the field remains unsure about the true molecular nature of latency establishment and maintenance *in vivo*. Tyagi *et al.* extensively compared mechanisms that control latency in their model in primary T cells with HIV-1 latency in Jurkat T cells, only to find that the molecular basis of latency in these two models was almost identical. The sole exception was the unresponsiveness of latent HIV-1 infection in their primary T cells to TNF- α stimulation (39). NF- κ B activation was found to be necessary, but insufficient in primary T cells that thus exhibit an additional layer of latency control. Even for the different available models of latent HIV-1 infection in primary T cells the situation is not straightforward. The various T cell activation/differentiation protocols seem to produce different memory T cell subsets as determined by cell surface marker analysis. Thus, there may be differences in the ability of the various models to respond to stimulation by compounds/drugs that target upstream signal transduction events, as these may stimulate pathways that are or are not active in the various T cell subpopulations. Responsiveness to certain stimuli in primary T cell models is likely also affected by donor-to-donor variation (V.P.; unpublished data).

As research in the field moves forward, more differences in the way latent HIV-1 infection is established and maintained as a function of the differentiation and activation state of the infected T cell subtypes will likely emerge. Unfortunately, at this time, we have no ability to translate these findings into the design of an optimal drug screening system, as we do not understand how findings in these *in vitro* models reflect the *in vivo* situation.

From a drug discovery standpoint, the HTS-suitable HIV-1 latency models should be used adapted to the goals and requirements of a particular drug screening effort. Today, drug repositioning efforts, or targeted compound libraries can easily be screened using the advanced primary T cell models. Large drug screens with >100,000 compounds may require the use of latently infected T cell lines. In either case, of particular importance will be the verification effort in which hit efficacy will need to be tested in several of the latency models using primary T cells. A successful drug discovery and development effort will have to identify drugs or therapeutic strategies that can reactivate latent HIV-1 infection reliably in all T cells sub-populations for which HIV-1 latency has been described.

Novel technology to advance current drug screening assays

At the present time, most primary T cell based models of HIV-1 latency that can be used for drug screening efforts incorporate GFP-reporter viruses/vectors. As a result of the low GFP signal that indicates drug-induced reactivation and the relative low frequency of latently infected cells that are generated in some of these models, data acquisition is limited to the use of flow cytometric analysis as the method of quantification. To move to an acceptable throughput level using flow cytometry some recent technology advances should greatly improve the quest to identify HIV-1 reactivating drugs, irrespective of the cell system used. In the last few years, mostly two systems have accelerated the ability to generate at least mid-throughput flow cytometry data. Cost-efficient plate-based throughput flow cytometry has been enabled with the availability of the GUAVA EasyCyte that allows to screen a 96 well plate in as little as 35 minutes. The GUAVA EasyCyte uses a sheath fluid free system that permits investigators to work with a small sample size of less than 50 μ l per well, and also allows for the determination of absolute cell counts during sample acquisition, a feature that can be exploited to directly determine off-target drug effects on cell proliferation or viability.

True high throughput capacity for flow cytometric analysis came with the introduction of the HyperCyt $\text{\textregistered}$ autosampler (67, 68). In conjunction with a flow cytometer that allows for digital acquisition of time resolved data, this instrument allows to screen for HIV-1 reactivating drugs in a 384-well format with sample sizes as little as 20 μ l. In our hands,

384-well plates can be analyzed with statistically significant event numbers in as little as 25 minutes, although faster acquisition has been reported. In particular for the utilization in drug screening efforts that utilize primary T cell based system that are struggling with low availability of experimental material, this system should greatly advance the ability to perform drug screens in the future. Another technology that could further the throughput of flow cytometry is fluorescent cell barcoding (FCB) (69). In FCB, individual cell populations are initially labeled with unique signatures of fluorescent dyes or fluorescent proteins (barcoded). These populations can represent different cell types or different treatment conditions. Prior to data acquisition on the flow cytometer, barcoded cell populations can be mixed together and analyzed as a single sample, thereby enhancing the speed of acquisition. In addition, FCB can be designed to improve data robustness through the combination of control and treated samples, which minimizes pipetting error and omits the need for normalization. An example from our laboratory demonstrates how we currently use an extension of this technology to screen for HIV-1 reactivating drug combinations by combining the HyperCyt autosampler technology with fluorescent cell barcoding (Figure 1).

Model suitability for drug discovery efforts

A good high throughput drug screen (HTS) reproduces the key elements that define a therapeutic target *in vivo* in an assay suitable for analysis in at least a 96-well assay format, but preferably in a higher format (384- or 1536-well plate). Currently, HIV-1 latency does not present a defined molecular drug target, in fact, there is not even a general consensus on how latent infection is established and maintained at the molecular level. As such, we are currently limited to performing phenotypic screens in which a surrogate marker of HIV-1 expression that is amenable to quantification in a HTS set-up is used.

Considerations for the design of high throughput drug screens involving large compound libraries (>100,000 compounds) are (i) cost, (ii) speed, (iii) reliability, and (iv) availability of material. By these considerations, latently HIV-1 infected reporter T cell lines present themselves superior to models of latent HIV-1 infection in primary T cells, which are costly and time consuming in their generation and limited in material availability.

However, given the advanced state of some of the primary T cell models, material availability may not be a dramatic limitation in small drug screen efforts comprising of <50,000 compounds. However, if such drug screens were to be performed as high-low screens or in a quantitative high throughput screening (qHTS) format, where each compound is evaluated at several concentrations (38), it would become difficult to even perform mid-size drug screening efforts using primary T cell models. The usefulness of high-low drug screens or qHTS for HIV-1 reactivating drugs is exemplified by the behavior PMA, likely the most potent HIV-1 reactivating compound for T cell lines and some primary T cell models (38). PMA is active at 10 - 30 nM, but highly cytotoxic at concentrations between 3 - 10 μ M, which is the standard compound concentration range used in single concentration drug screens. Performing drug screens at single concentrations thus bears a high risk of creating false negative hits (38).

The second aspect affecting the choice of a suitable drug screening assay is how well the assay represents the *in vivo* target. This choice determines the likelihood of whether a hit is indeed to be developed into a lead or possibly a drug. For drug screening assays that try to identify HIV-1 reactivating compounds this would be the answer to the question of whether primary T cell-based models solely reflect *in vivo* HIV-1 latency or whether well characterized latently HIV-1 infected T cell lines can serve as suitable substitutes that can serve as a compromise. In the following sections we revisit a few of the key arguments that should guide the choice of latency model for HTS.

a) The site of viral integration

The site of integration can influence viral expression and is likely an important feature to be considered. Using a Tat-deficient lentiviral GFP reporter construct Jordan *et al.* demonstrated that the site of integration would determine basal transcriptional activity (70). While these studies suggested that the viral LTR in the absence of the Tat feedback loop is influenced by the DNA environment they did not account for a possible role of Tat during establishment and maintenance of latent HIV-1 infection (71). As an early publication had already reported that latent HIV-1 integration in ACH2 cells had occurred as the result of a suppressive DNA environment (53), it was/is generally assumed that latency in T cell lines is found in areas of the host genome that are not conducive to viral transcription (72). A later publication clarified that latency in ACH2 was triggered by an attenuating mutation in the TAR element of the integrated virus and not as a function of the site of integration (70). Also, several recent reports now have demonstrated that latent integration in the more recently established reporter T cell lines, as in primary T cells, occurs in actively expressed host-genes (16, 26). A comprehensive analysis of the latently HIV-1 infected T cell lines in our laboratory confirmed that latent infection events in these T cell lines, with regard to their integration site profiles, reproduce findings in primary T cells from patients or in a latency model using primary T cells (O.K.; unpublished data).

b) Resting versus proliferating T cells

At present, latent HIV-1 infection *in vivo* has been described for several T cell populations (naïve; central, transitional and effector memory) (73). All of these cell types have a resting phenotype, which is in stark contrast to the continuously proliferating phenotype of immortalized T cell lines. While no scientific evidence has so far been presented on this subject, it is generally accepted that cell cycle status must regulate latency control at the molecular level (74). However, memory T cells are known to undergo low-level proliferation as part of T cell homeostasis, which is a prerequisite for the maintenance of the lifelong immunological memory (reported or reviewed in (75-77)). For central memory T cells data have been presented that these cells undergo multiple divisions per year in healthy individuals and in HIV-1 infected patients (78-80). More recently, a discussion has started that proliferation of latently infected memory T cells may contribute to the extreme stability of the latent viral reservoir (73, 81, 82). Experimental evidence that *in vitro* generated latently HIV-1 infected central memory T cells can be induced to proliferate by IL-7 without triggering reactivation of latent HIV-1 infection has recently been published (83). Therefore, the ability of immortalized cells to proliferate continuously may not, by itself, pose a problem in our mechanistic understanding of latency and reactivation, since *in vivo*, latently infected cells also display a certain ability to divide (73).

c) Responsiveness to stimuli

Our understanding of the latent viral state has been propelled by the development of multiple T cell models of HIV-1 latency (both, primary and immortalized) that can be implemented in the laboratory. However, no single experimental system of HIV-1 latency is likely to completely recapitulate the biological properties of the latent reservoir *in vivo*. This is due primarily to two reasons. First, the latent or persistent HIV reservoir *in vivo* is likely to reside in multiple cell types/sub-populations, which may confer upon the latent virus different modes of reactivation. Second, the molecular mechanisms that may lead to the establishment of latent infection are likely to be multiple as well. Potential HIV-1 reactivating agents could function by triggering cell surface receptors, activate signal transduction pathways at various levels or interact with the latent promoter at the DNA level. The diversity of signaling pathways that can be responsible for latent HIV-1 reactivation is illustrated in Table 1 with a small subset of known latency models. As can be appreciated in Table 1, J-Lat clone 6.3 cells (52) are responsive to stimuli that activate the

protein kinase C/NF- κ B axis, but they are unresponsive to agents that signal through the TCR complex, such as PHA and CD3/CD28 antibodies. This J-Lat clone is also responsive to viral reactivation by various HDAC inhibitors (52).

In contrast, cultured central memory T cells are highly responsive to TCR engagement, but are not at all responsive to protein kinase C agonists or to HDAC inhibitors (15). Unlike JLAT cells, latent HIV-1 infection in J89GFP T cells is responsive to antibody-mediated CD3/CD28 co-stimulation and PHA activation, but does not respond to HDAC inhibitors. While this is reminiscent of the reactivation pattern seen in cultured central memory T cells, latent HIV-1 infection in J89GFP also responds to PMA, prostratin or TNF- α (51). These stimuli are ineffective in cultured central memory T cells (15), but are effective in BCL-2 transformed latently HIV-1 infected T cells (7).

Primary T cells in the Yang model (7) are highly responsive to calcium influx inducers, such as ionomycin, whereas cultured central memory T cells (15) or J-Lat 6.3 are not (52). Responsiveness to TNF- α is also not conserved, being high in J-Lat 6.3 (52) and J89GFP T cells (51) and negative in cultured central memory T cells (15) and in primary cells of the Tyagi model (39) (Table 1). The transcription factors underlying the above patterns of responsiveness are being investigated, but in certain instances documentation is already available. For example, cultured central memory T cells are exquisitely responsive to signaling cascades that culminate in NFAT activation, while inhibitors of NF- κ B have little or no effect on TCR stimulation (15). Conversely, J-Lat cells and T cells in the Tyagi model primarily or entirely reactivate latent viruses via NF- κ B-associated pathways (39, 52). These differences have to be considered when choosing a latency model for a drug discovery effort, and during data interpretation. Combining complementary assays for primary and secondary screens and for verification assays is crucial in order to minimize false-negative or false-positive hits. Unfortunately, knowledge of which models provide the most clinically and physiologically relevant information is lacking at this point in time.

Table 1 also lists patient cell-based models (49, 84) for informative purposes, as their use in a primary HTS screen would be unrealistic due to limiting cell numbers. Where available, the information provided by patient cell-based assays is particularly valuable given that these cells are primary and have the desired properties (quiescent, latently infected) upon isolation, and are not generated by *in vitro* differentiation methods.

Care with regard to generalization of findings is also advised when experiments are performed using latently HIV-1 infected promonocytic cell lines such as U1 or THP89GFP cells. These cells respond to many stimulators with the secretion of TNF- α , which then reactivates latent HIV-1 infection in an autocrine manner (85, 86). However, such indirect TNF- α effects on latent HIV-1 infection are not seen in T cell lines or primary T cells, the latter seemingly being unresponsive to TNF- α in general.

Aside from limitations stemming from low cell numbers, primary cells present another potential disadvantage, which is that of donor-to-donor variation. It is expected that responsiveness to certain stimuli may vary across cell samples from different individuals, both quantitatively and qualitatively. The Planelles laboratory has observed both subtle and dramatic variations in the ability of cells from various human donors to respond to TCR activation and to IL-7 stimulation (V.P., unpublished observations). While it is conceivable that a secondary screen could be performed using primary cells from more than one donor, performing a medium or high throughput screen in that fashion could be a logistic nightmare. A possible solution that the Planelles laboratory is investigating would be to pre-test cells from individual donors with a broad battery of agonists (as shown in Table 1) and antagonists (15). Comparisons of the resulting patterns of viral reactivation and inhibition of

reactivation among cells from about one hundred donors has revealed, so far, that there are a small number of phenotypic subsets among the tested human samples. For example, cells from these subsets are clearly distinct from one another in terms of which signaling pathways will successfully reactivate latent viruses. Another conclusion that may be derived from these studies is that anti-latency drugs may not work uniformly from patient to patient due to these differences.

Conflicting results are also produced regarding the ability of HDAC inhibitors to trigger HIV-1 reactivation. Whereas earlier reports claim that latent infection can be reactivated by HDAC inhibitors (10, 14), these agents triggered at best low-level HIV-1 reactivation in the latency models published by the Siliciano group and the Planelles group, for which data have been published (7, 15). These data, however, would be consistent with publications on the inability of HDAC inhibitors to prevent the establishment of latent infection or to trigger HIV-1 reactivation in some of the recently published latently infected T cell lines (16).

Overall, the literature does not suggest that one particular model of HIV-1 latency generally exhibits superior qualities over another to be used in drug discovery efforts. For HTS, each model seems to present advantages and limitations and the decision in favor of a particular model will need to be made based on the particular circumstances and goals of the intended drug screen.

Conclusions

Small drug screens in the range of 10,000 - 50,000 compounds or drug repositioning screens run at a single drug concentration should likely use primary T cell models. A risk of false negative hits will arise mostly from screening at a single compound concentration, but such screens are more likely to identify drugs that interact with relevant signaling pathways or even cell surface molecules expressed on resting primary T cells. Large-scale drug screens are unlikely to be performed efficiently using primary T cell-based systems. Generation of sufficient amount of cell material is likely too costly and time-consuming, in particular when high-low HTS or qHTS is considered. However, with the continued introduction of novel technologies that allow for assay miniaturization, utilization of primary T cell-based models in HTS efforts becomes increasingly feasible. On the other hand, the more recently established, well characterized latently infected reporter T cell lines should reflect the basic molecular mechanism that control latent HIV-1 infection to an extent that utilization of these T cell lines in HTS efforts is very promising. False negative hits can eventually occur because some signal transduction pathways that are functional in memory T cells are affected. This disadvantage will be offset by the ability to screen large compound libraries with millions of compounds, eventually in a high-low or qHTS format that should minimize false negative hits due to compound concentration issues. Also, the increased numbers of false positive hits that may be identified in these screens can be quickly reduced using the established primary T cell models of latent HIV-1 infection as verification assays.

$$Z' = 1 - 3 (\sigma_{\text{act}} + \sigma_{\text{back}}) / |\mu_{\text{act}} - \mu_{\text{back}}|$$

$Z' = Z'$ factor as a dimensionless characteristic describing the quality of a HTS assay

μ_{act} = mean of samples with reactivated HIV-1 infection

μ_{back} = mean untreated samples (background)

σ_{act} = standard deviation of samples with reactivated HIV-1 infection

back = standard deviation of untreated samples (background)

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REFERENCES

1. Nabel G, Baltimore D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature*. Apr 16-22; 1987 326(6114):711-3. [PubMed: 3031512]
2. Alcamí J, Lain de Lera T, Folgueira L, Pedraza MA, Jacque JM, Bachelier F, et al. Absolute dependence on kappa B responsive elements for initiation and Tat-mediated amplification of HIV transcription in blood CD4 T lymphocytes. *Embo J*. Apr 3; 1995 14(7):1552-60. [PubMed: 7729429]
3. Chun TW, Engel D, Mizell SB, Hallahan CW, Fischette M, Park S, et al. Effect of interleukin-2 on the pool of latently infected, resting CD4+ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy. *Nat Med*. 1999; 5(6):651-5. [PubMed: 10371503]
4. van Praag RM, Prins JM, Roos MT, Schellekens PT, Ten Berge IJ, Yong SL, et al. OKT3 and IL-2 treatment for purging of the latent HIV-1 reservoir in vivo results in selective long-lasting CD4+ T cell depletion. *J Clin Immunol*. May; 2001 21(3):218-26. [PubMed: 11403229]
5. Kulkosky J, Nunnari G, Otero M, Calarota S, Dornadula G, Zhang H, et al. Intensification and stimulation therapy for human immunodeficiency virus type 1 reservoirs in infected persons receiving virally suppressive highly active antiretroviral therapy. *J Infect Dis*. 2002; 186(10):1403-11. [PubMed: 12404155]
6. Abramowicz D, Crusiaux A, Goldman M. Anaphylactic shock after retreatment with OKT3 monoclonal antibody. *N Engl J Med*. Sep 3.1992 327(10):736. [PubMed: 1495540]
7. Yang HC, Xing S, Shan L, O'Connell K, Dinoso J, Shen A, et al. Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. *J Clin Invest*. Nov; 2009 119(11):3473-86. [PubMed: 19805909]
8. Xing S, Bullen CK, Shroff NS, Shan L, Yang HC, Manucci JL, et al. Disulfiram Reactivates Latent HIV-1 in a Bcl-2-Transduced Primary CD4+ T Cell Model without Inducing Global T Cell Activation. *J Virol*. Jun; 85(12):6060-4. [PubMed: 21471244]
9. Wolschendorf F, Duverger A, Jones J, Wagner FH, Huff J, Benjamin WH, et al. Hit-and-run stimulation: a novel concept to reactivate latent HIV-1 infection without cytokine gene induction. *J Virol*. Sep; 2010 84(17):8712-20. [PubMed: 20538859]
10. Van Lint C, Emiliani S, Ott M, Verdin E. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. *EMBO J*. 1996; 15(5):1112-20. [PubMed: 8605881]
11. Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expr*. 1996; 5(4-5):245-53. [PubMed: 8723390]
12. Sheridan PL, Mayall TP, Verdin E, Jones KA. Histone acetyltransferases regulate HIV-1 enhancer activity in vitro. *Genes Dev*. 1997; 11(24):3327-40. [PubMed: 9407026]
13. He G, Ylisastigui L, Margolis DM. The regulation of HIV-1 gene expression: the emerging role of chromatin. *DNA Cell Biol*. Oct; 2002 21(10):697-705. [PubMed: 12443539]
14. Ylisastigui L, Archin NM, Lehrman G, Bosch RJ, Margolis DM. Coaxing HIV-1 from resting CD4 T cells: histone deacetylase inhibition allows latent viral expression. *Aids*. May 21; 2004 18(8):1101-8. [PubMed: 15166525]
15. Bosque A, Planelles V. Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells. *Blood*. Jan 1; 2009 113(1):58-65. [PubMed: 18849485]
16. Duverger A, Jones J, May J, Bibollet-Ruche F, Wagner FA, Cron RQ, et al. Determinants of the establishment of human immunodeficiency virus type 1 latency. *J Virol*. Apr; 2009 83(7):3078-93. [PubMed: 19144703]

17. Lehrman G, Hogue IB, Palmer S, Jennings C, Spina CA, Wiegand A, et al. Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. *Lancet*. Aug 13-19; 2005 366(9485):549–55. [PubMed: 16099290]
18. Archin NM, Cheema M, Parker D, Wiegand A, Bosch RJ, Coffin JM, et al. Antiretroviral intensification and valproic acid lack sustained effect on residual HIV-1 viremia or resting CD4+ cell infection. *PLoS One*. 5(2):e9390. [PubMed: 20186346]
19. Siliciano JM, Siliciano RF. Targeting HIV reservoirs with valproic acid. *Hopkins HIV Rep*. Sep; 2005 17(5):8–9. [PubMed: 16419304]
20. Smith SM. Valproic acid and HIV-1 latency: beyond the sound bite. *Retrovirology*. Sep 19; 2005 2:56. [PubMed: 16168066]
21. Siliciano JD, Lai J, Callender M, Pitt E, Zhang H, Margolick JB, et al. Stability of the latent reservoir for HIV-1 in patients receiving valproic acid. *J Infect Dis*. Mar 15; 2007 195(6):833–6. [PubMed: 17299713]
22. Archin NM, Eron JJ, Palmer S, Hartmann-Duff A, Martinson JA, Wiegand A, et al. Valproic acid without intensified antiviral therapy has limited impact on persistent HIV infection of resting CD4+ T cells. *Aids*. Jun 19; 2008 22(10):1131–5. [PubMed: 18525258]
23. Han Y, Lassen K, Monie D, Sedaghat AR, Shimoji S, Liu X, et al. Resting CD4+ T cells from human immunodeficiency virus type 1 (HIV-1)-infected individuals carry integrated HIV-1 genomes within actively transcribed host genes. *J Virol*. Jun; 2004 78(12):6122–33. [PubMed: 15163705]
24. Shan L, Yang HC, Rabi SA, Bravo HC, Shroff NS, Irizarry RA, et al. Influence of Host Gene Transcription Level and Orientation on HIV-1 Latency in a Primary-Cell Model. *J Virol*. Jun; 2008 82(11):5384–93. [PubMed: 21430059]
25. Han Y, Lin YB, An W, Xu J, Yang HC, O'Connell K, et al. Orientation-dependent regulation of integrated HIV-1 expression by host gene transcriptional readthrough. *Cell Host Microbe*. Aug 14; 2008 4(2):134–46. [PubMed: 18692773]
26. Lenasi T, Contreras X, Peterlin BM. Transcriptional interference antagonizes proviral gene expression to promote HIV latency. *Cell Host Microbe*. Aug 14; 2008 4(2):123–33. [PubMed: 18692772]
27. Tyagi M, Karn J. CBF-1 promotes transcriptional silencing during the establishment of HIV-1 latency. *Embo J*. Dec 12; 2007 26(24):4985–95. [PubMed: 18007589]
28. Jiang G, Espeseth A, Hazuda DJ, Margolis DM. c-Myc and Sp1 contribute to proviral latency by recruiting histone deacetylase 1 to the human immunodeficiency virus type 1 promoter. *J Virol*. Oct; 2007 81(20):10914–23. [PubMed: 17670825]
29. Pearson R, Kim YK, Hokello J, Lassen K, Friedman J, Tyagi M, et al. Epigenetic silencing of human immunodeficiency virus (HIV) transcription by formation of restrictive chromatin structures at the viral long terminal repeat drives the progressive entry of HIV into latency. *J Virol*. Dec; 2008 82(24):12291–303. [PubMed: 18829756]
30. Williams SA, Chen LF, Kwon H, Ruiz-Jarabo CM, Verdin E, Greene WC. NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. *EMBO J*. Jan 11; 2006 25(1):139–49. [PubMed: 16319923]
31. Keedy KS, Archin NM, Gates AT, Espeseth A, Hazuda DJ, Margolis DM. A limited group of class I histone deacetylases acts to repress human immunodeficiency virus type 1 expression. *J Virol*. May; 2009 83(10):4749–56. [PubMed: 19279091]
32. du Chene I, Basyuk E, Lin YL, Triboulet R, Knezevich A, Chable-Bessia C, et al. Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. *Embo J*. Jan 24; 2007 26(2):424–35. [PubMed: 17245432]
33. Marban C, Suzanne S, Dequiedt F, de Walque S, Redel L, Van Lint C, et al. Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing. *Embo J*. Jan 24; 2007 26(2):412–23. [PubMed: 17245431]
34. Kim YK, Bourgeois CF, Pearson R, Tyagi M, West MJ, Wong J, et al. Recruitment of TFIID to the HIV LTR is a rate-limiting step in the emergence of HIV from latency. *Embo J*. Aug 9; 2006 25(15):3596–604. [PubMed: 16874302]

35. Klatt A, Zhang Z, Kalantari P, Hankey PA, Gilmour DS, Henderson AJ. The receptor tyrosine kinase RON represses HIV-1 transcription by targeting RNA polymerase II processivity. *J Immunol.* Feb 1; 2008 180(3):1670–7. [PubMed: 18209063]
36. Zhang Z, Klatt A, Gilmour DS, Henderson AJ. Negative elongation factor NELF represses human immunodeficiency virus transcription by pausing the RNA polymerase II complex. *J Biol Chem.* Jun 8; 2007 282(23):16981–8. [PubMed: 17442680]
37. Ping YH, Rana TM. DSIF and NELF interact with RNA polymerase II elongation complex and HIV-1 Tat stimulates P-TEFb-mediated phosphorylation of RNA polymerase II and DSIF during transcription elongation. *J Biol Chem.* Apr 20; 2001 276(16):12951–8. [PubMed: 11112772]
38. Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, et al. Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc Natl Acad Sci U S A.* Aug 1; 2006 103(31):11473–8. [PubMed: 16864780]
39. Tyagi M, Pearson RJ, Karn J. Establishment of HIV latency in primary CD4+ cells is due to epigenetic transcriptional silencing and P-TEFb restriction. *J Virol.* Jul; 2010 84(13):6425–37. [PubMed: 20410271]
40. Folks TM, Clouse KA, Justement J, Rabson A, Duh E, Kehrl JH, et al. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc Natl Acad Sci USA.* 1989; 86(7):2365–8. [PubMed: 2784570]
41. Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science.* 1987; 238(4828):800–2. [PubMed: 3313729]
42. Folks TM, Powell D, Lightfoote M, Koenig S, Fauci AS, Benn S, et al. Biological and biochemical characterization of a cloned Leu-3- cell surviving infection with the acquired immune deficiency syndrome retrovirus. *J Exp Med.* Jul 1; 1986 164(1):280–90. [PubMed: 3014036]
43. Perez VL, Rowe T, Justement JS, Butera ST, June CH, Folks TM. An HIV-1-infected T cell clone defective in IL-2 production and Ca2+ mobilization after CD3 stimulation. *J Immunol.* Nov 1; 1991 147(9):3145–8. [PubMed: 1833465]
44. Butera ST, Perez VL, Wu BY, Nabel GJ, Folks TM. Oscillation of the human immunodeficiency virus surface receptor is regulated by the state of viral activation in a CD4+ cell model of chronic infection. *J Virol.* Sep; 1991 65(9):4645–53. [PubMed: 1678437]
45. Emiliani S, Van Lint C, Fischle W, Paras P Jr, Ott M, Brady J, et al. A point mutation in the HIV-1 Tat responsive element is associated with postintegration latency. *Proc Natl Acad Sci U S A.* Jun 25; 1996 93(13):6377–81. [PubMed: 8692823]
46. Emiliani S, Fischle W, Ott M, Van Lint C, Amella CA, Verdin E. Mutations in the tat gene are responsible for human immunodeficiency virus type 1 postintegration latency in the U1 cell line. *J Virol.* Feb; 1998 72(2):1666–70. [PubMed: 9445075]
47. Yukl S, Pillai S, Li P, Chang K, Pasutti W, Ahlgren C, et al. Latently-infected CD4+ T cells are enriched for HIV-1 Tat variants with impaired transactivation activity. *Virology.* Apr 25; 2009 387(1):98–108. [PubMed: 19268337]
48. Chun TW, Finzi D, Margolick J, Chadwick K, Schwartz D, Siliciano RF. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat Med.* 1995; 1(12):1284–90. [PubMed: 7489410]
49. Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature.* 1997; 387(6629):183–8. [PubMed: 9144289]
50. Chun TW, Engel D, Mizell SB, Ehler LA, Fauci AS. Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. *J Exp Med.* Jul 6; 1998 188(1):83–91. [PubMed: 9653086]
51. Kutsch O, Benveniste EN, Shaw GM, Levy DN. Direct and quantitative single-cell analysis of human immunodeficiency virus type 1 reactivation from latency. *J Virol.* 2002; 76(17):8776–86. [PubMed: 12163598]
52. Jordan A, Bisgrove D, Verdin E. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. *Embo J.* Apr 15; 2003 22(8):1868–77. [PubMed: 12682019]

53. Winslow BJ, Pomerantz RJ, Bagasra O, Trono D. HIV-1 latency due to the site of proviral integration. *Virology*. Oct; 1993 196(2):849–54. [PubMed: 8372452]
54. Levy DN, Aldrovandi GM, Kutsch O, Shaw GM. Dynamics of HIV-1 recombination in its natural target cells. *Proc Nat Acad Sci*. 2004; 101(12):4204–9. [PubMed: 15010526]
55. Micheva-Viteva S, Pacchia AL, Ron Y, Peltz SW, Dougherty JP. Human immunodeficiency virus type 1 latency model for high-throughput screening. *Antimicrob Agents Chemother*. Dec; 2005 49(12):5185–8. [PubMed: 16304201]
56. Zhang JH, Chung TD, Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen*. 1999; 4(2):67–73. [PubMed: 10838414]
57. Micheva-Viteva S, Kobayashi Y, Edelstein LC, Pacchia AL, Lee HL, Graci JD, et al. High-throughput Screening Uncovers a Compound That Activates Latent HIV-1 and Acts Cooperatively with a Histone Deacetylase (HDAC) Inhibitor. *J Biol Chem*. Jun 17; 286(24):21083–91. [PubMed: 21498519]
58. Jones J, Rodgers J, Heil M, May J, White L, Maddry JA, et al. High throughput drug screening for human immunodeficiency virus type 1 reactivating compounds. *Assay Drug Dev Technol*. Apr; 2007 5(2):181–89. [PubMed: 17477827]
59. Ochsenbauer-Jambor C, Jones J, Heil M, Zammit KP, Kutsch O. T-cell line for HIV drug screening using EGFP as a quantitative marker of HIV-1 replication. *Biotechniques*. Jan; 2006 40(1):91–100. [PubMed: 16454046]
60. Saleh S, Solomon A, Wightman F, Xhila M, Cameron PU, Lewin SR. CCR7 ligands CCL19 and CCL21 increase permissiveness of resting memory CD4+ T cells to HIV-1 infection: a novel model of HIV-1 latency. *Blood*. Dec 15; 2007 110(13):4161–4. [PubMed: 17881634]
61. Brady T, Agosto LM, Malani N, Berry CC, O'Doherty U, Bushman F. HIV integration site distributions in resting and activated CD4+ T cells infected in culture. *Aids*. Jul 31; 2009 23(12):1461–71. [PubMed: 19550285]
62. Agosto LM, Yu JJ, Dai J, Kaletsky R, Monie D, O'Doherty U. HIV-1 integrates into resting CD4+ T cells even at low inoculums as demonstrated with an improved assay for HIV-1 integration. *Virology*. Nov 10; 2007 368(1):60–72. [PubMed: 17631931]
63. Marini A, Harper JM, Romero F. An in vitro system to model the establishment and reactivation of HIV-1 latency. *J Immunol*. Dec 1; 2008 181(11):7713–20. [PubMed: 19017960]
64. Sahu GK, Lee K, Ji J, Braciale V, Baron S, Cloyd MW. A novel in vitro system to generate and study latently HIV-infected long-lived normal CD4+ T-lymphocytes. *Virology*. Nov 25; 2006 355(2):127–37. [PubMed: 16919704]
65. Marsden MD, Burke BP, Zack JA. HIV latency is influenced by regions of the viral genome outside of the long terminal repeats and regulatory genes. *Virology*. Jul 19; 2006 355(2):127–37. [PubMed: 16919704]
66. Bosque A, Planelles V. Studies of HIV-1 latency in an ex vivo model that uses primary central memory T cells. *Methods*. Jan; 2006 38(1):54–61. [PubMed: 20970502]
67. Edwards BS, Young SM, Ivnitisky-Steele I, Ye RD, Prossnitz ER, Sklar LA. High-content screening: flow cytometry analysis. *Methods Mol Biol*. 2009; 486:151–65. [PubMed: 19347622]
68. Kuckuck FW, Edwards BS, Sklar LA. High throughput flow cytometry. *Cytometry*. May 1; 2001 44(1):83–90. [PubMed: 11309812]
69. Krutzik PO, Nolan GP. Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling. *Nat Methods*. May; 2006 3(5):361–8. [PubMed: 16628206]
70. Jordan A, Defechereux P, Verdin E. The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. *Embo J*. 2001; 20(7):1726–38. [PubMed: 11285236]
71. Weinberger LS, Burnett JC, Toettcher JE, Arkin AP, Schaffer DV. Stochastic gene expression in a lentiviral positive-feedback loop: HIV-1 Tat fluctuations drive phenotypic diversity. *Cell*. Jul 29; 2005 122(2):169–82. [PubMed: 16051143]
72. Choudhary SK, Margolis DM. Curing HIV: Pharmacologic approaches to target HIV-1 latency. *Annu Rev Pharmacol Toxicol*. Feb 10; 2011 51:397–418. [PubMed: 21210747]

73. Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, et al. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. *Nat Med.* Aug; 2009 15(8):893–900. [PubMed: 19543283]
74. Pace MJ, Agosto L, Graf EH, O'Doherty U. HIV reservoirs and latency models. *Virology.* Mar 15; 411(2):344–54. [PubMed: 21284992]
75. Homann D, Teyton L, Oldstone MB. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat Med.* Aug; 2001 7(8):913–9. [PubMed: 11479623]
76. Tokoyoda K, Zehentmeier S, Hegazy AN, Albrecht I, Grun JR, Lohning M, et al. Professional memory CD4+ T lymphocytes preferentially reside and rest in the bone marrow. *Immunity.* May; 2009 30(5):721–30. [PubMed: 19427242]
77. Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. *Nat Immunol.* Sep; 2003 4(9):835–42. [PubMed: 12942084]
78. Hellerstein MK, Hoh RA, Hanley MB, Cesar D, Lee D, Neese RA, et al. Subpopulations of long-lived and short-lived T cells in advanced HIV-1 infection. *J Clin Invest.* Sep; 2003 112(6):956–66. [PubMed: 12975480]
79. Michie CA, McLean A, Alcock C, Beverley PC. Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature.* Nov 19; 1992 360(6401):264–5. [PubMed: 1436108]
80. McLean AR, Michie CA. In vivo estimates of division and death rates of human T lymphocytes. *Proc Natl Acad Sci U S A.* Apr 25; 1995 92(9):3707–11. [PubMed: 7731969]
81. Shen L, Siliciano RF. Viral reservoirs, residual viremia, and the potential of highly active antiretroviral therapy to eradicate HIV infection. *J Allergy Clin Immunol.* Jul; 2008 122(1):22–8. [PubMed: 18602567]
82. Dahl V, Palmer S. Establishment of drug-resistant HIV-1 in latent reservoirs. *J Infect Dis.* May 1; 2009 199(9):1258–60. [PubMed: 19338473]
83. Bosque A, Famiglietti M, Weyrich A, Goulston C, Planelles V. Homeostatic Proliferation Fails to Efficiently Reactivate HIV-1 Latently Infected Central Memory CD4+ T Cells. *PLoS Pathog.* 2011 In Press.
84. Archin NM, Espeseth A, Parker D, Cheema M, Hazuda D, Margolis DM. Expression of latent HIV induced by the potent HDAC inhibitor suberoylanilide hydroxamic acid. *AIDS Res Hum Retroviruses.* Feb; 2009 25(2):207–12. [PubMed: 19239360]
85. Jones J, Kosloff BR, Benveniste EN, Shaw GM, Kutsch O. Amphotericin-B-mediated reactivation of latent HIV-1 infection. *Virology.* Jan 5; 2005 331(1):106–16. [PubMed: 15582657]
86. Kutsch O, Levy DN, Kosloff BR, Shaw GM, Benveniste EN. CD154-CD40-induced reactivation of latent HIV-1 infection. *Virology.* Sep 15; 2003 314(1):261–70. [PubMed: 14517079]

Figure 1. HTS using flow cytometry

HTS using flow cytometry can be enhanced by combining time resolved sample acquisition with fluorescence barcoding. (A) Time resolved acquisition of individual wells of a 96 well plate-based screening assay. Each peak represents cell counts from one well over a time axis. (B) Back gating on individual peaks allows us to obtain all acquired information (here RFP/GFP). (C) Analysis of the information derived from the primary screen of one 96 well plate screening for modulator compounds. The following activators were used: blue: HRF; red: PMA; yellow: CD3 mAb; black and white symbols indicate the cell viability in the respective wells.

Table 1
Comparison of stimuli and signaling pathways that result in HIV-1 induction across cellular models of latency

Stimulus	Physiologic Activity	Signaling Axis	Level of HIV Reactivation (-, +/-, +, ++, +++)						Patient T cells <i>ex vivo</i>
			J-Lat 6.3 [Jordan]	J89GFP [Kutsch]	Cultured T _{CM} [Bosque]	Cultured CD4 cells [Yang]	Cultured CD4 cells [Tyagi]	CCR7 ligand stim. CD4 cells [Saleh]	
CD3+ CD28 PHA	TCR engagement	Lck/Calcineurin/NFAT & PKC/NF B	-	++	+++	+++	+++	+++	+++
			-	++	+++	+++	?	?	+
PMA	PKC activation	PKC/NF B & PKC/MAPK	+	+++	-	+++	?	?	+
Prostratin			++	+++	-	++	?	?	?
Ionomycin	Ca ⁺⁺ influx	Calcineurin	-	-	-	++	?	?	?
PMA+ Ionomycin	PKC activation & Ca ⁺⁺ influx	PKC/NF B; PKC/MAPK & calcineurin	+++	+++	+/-	+++	?	?	++
TNF	TRAFs recruitment	NF B/AP-1	++	+++	-	+/-	-	?	++
IL-7 + IL-2	cR engagement	JAK/STAT & PI3K/AKT/NF B	?	?	+	++	?	?	++
VPA	HDAC inhibition	Chromatin remodeling & activation of transcription (not gene specific)	++	-	-	+/-	?	?	++
SAHA			++	?	-	++	?	?	++
Trichostatin A			++	-	-	+	?	?	+/-
Na butyrate			++	-	-	?	?	?	+
aza-CdR	DNA-me inhibition	methyltransferases	+	-	-	?	?	?	+/-
aza-CdR + TNF	DNA-me inhibition & TRAFs recruitment	Methyltransferases & NF B/AP-1	+++	+++	?	?	?	?	?