Direct and Quantitative Single-Cell Analysis of Human Immunodeficiency Virus Type 1 Reactivation from Latency

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The ability of human immunodeficiency virus type 1 (HIV-1) to establish latent infections in cells has received renewed attention owing to the failure of highly active antiretroviral therapy to eradicate HIV-1 in vivo. Despite much study, the molecular bases of HIV-1 latency and reactivation are incompletely understood. Research on HIV-1 latency would benefit from a model system that is amenable to rapid and efficient analysis and through which compounds capable of regulating HIV-1 reactivation may be conveniently screened. We describe a novel reporter system that has several advantages over existing in vitro systems, which require elaborate, expensive, and time-consuming techniques to measure virus production. Two HIV-1 molecular clones (NL4-3 and 89.6) were engineered to express enhanced green fluorescent protein (EGFP) under the control of the viral long terminal repeat without removing any viral sequences. By using these replication-competent viruses, latently infected T-cell (Jurkat) and monocyte/macrophage (THP-1) lines in which EGFP fluorescence and virus expression are tightly coupled were generated. Following reactivation with agents such as tumor necrosis factor alpha, virus expression and EGFP fluorescence peaked after 4 days and over the next 3 weeks each declined in a synchronized manner, recapitulating the establishment of latency. Using fluorescence microscopy, flow cytometry, or plate-based fluorometry, this system allows immediate, direct, and quantitative real-time analysis of these processes within single cells or in bulk populations of cells. Exploiting the single-cell-analysis abilities of this system, we demonstrate that cellular activation and virus reactivation following stimulation with proinflammatory cytokines can be uncoupled.

The regulation of retrovirus expression within the infected host is controlled at many levels by both viral and host factors. For complex retroviruses such as human immunodeficiency virus type 1 (HIV-1) and HIV-2, several viral elements contribute cis and trans functions that regulate virus expression within host cells (23). The infected host cell, on the other hand, provides the transcription and translation machinery essential for the expression of viral proteins and viral replication. Following integration of the viral cDNA into the cellular genome, HIV-1 expression leads to the production of infectious virus, frequently resulting in the death of the host cell. In some instances viral expression can be down-modulated, leaving the provirus in a latent state characterized by low or absent viral mRNA and protein production (11, 48). This latent state may persist within the host cell for the natural life span of the cell or until external factors induce the virus to resume expression. A substantial reservoir of latently infected cells has recently been shown to be established early in HIV infection in vivo within macrophages and memory T cells (3, 9, 14, 16, 18, 26, 27, 37, 41, 52). This reservoir of latently infected cells is thought to be a contributing factor to the failure of highly active antiretroviral therapy to eradicate HIV-1 from the host (15, 16, 19). Thus, a better understanding of the underlying molecular mechanisms of HIV-1 latency and reactivation is needed in order to develop targeted therapies that could control or eradicate latently infected cells.

To date, it has been impossible to expand chronically infected primary cells; thus the most appropriate in vitro cell models for viral latency have been HIV-1-infected transformed cell lines such as ACH-2, J1.1, U1, and OM-10.1 (10, 22, 28, 29, 44). These cell lines contain one or two copies of integrated virus and constitutively display low levels of HIV-1 gene expression. Studies of these cells have revealed important roles for the site of viral integration (54), for cellular (33–35, 43, 49, 56) and viral proteins (30, 38, 39, 42, 47), and for histone acetylation and DNA methylation (4, 5, 51, 53) in the establishment and maintenance of latency. Nevertheless, the state of latency in these cells, on a population basis or at the single-cell level, can only be determined by indirect and time-consuming procedures (i.e., p24 enzyme-linked immunosorbent assay [ELISA], reverse transcriptase assay, and intracellular staining for viral proteins). As such, research on HIV-1 latency would benefit from a relevant model that is amenable to rapid and efficient analysis and through which useful pharmacological compounds capable of controlling HIV-1 reactivation may be efficiently screened.

To this end, we describe a reporter system to study HIV-1 latency and reactivation that combines the benefits of a latently infected immortal cell line with the convenience of using enhanced green fluorescent protein (EGFP) as a marker for HIV-1 expression. To establish this system, two recombinant

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HIV-1 viruses based on the dual-tropic 89.6 strain and the T-cell-tropic NL4-3 strain were engineered to express EGFP, while preserving all viral nucleotide sequences and potential cis elements. Following infection, three clonal, latently infected cell lines, representing both T-cell (Jurkat) and monocyte/macrophage (THP-1) lineages were developed. In the resulting cell lines, named JNLGFP, J89GFP, and THP89GFP, EGFP fluorescence is tightly linked to HIV-1 protein production and can be used as a quantitative marker for HIV-1 expression on a single-cell basis by fluorescence microscopy or flow cytometry and can be used on a population basis by fluorometry.

We find that different stimuli which are known to promote viral expression (tumor necrosis factor alpha [TNF-α], interleukin-1β [IL-1β], gamma interferon [IFN-γ], phorbol 12-myristate 13-acetate [PMA], and trichostatin A [TSA]) differ in both the percentage of cells which demonstrate viral reactivation and the extent of reactivation within individual cells. Differences between the T-cell and macrophage cell lines were seen as well, highlighting the apparent complexity of the processes involved in HIV-1 reactivation.

The ability of this system to quantify HIV-1 reactivation and subsequent replication on a single-cell level can be simultaneously combined with additional analyses (e.g., cell cycle analysis, apoptosis detection, and antibody staining techniques). Using this method we observe that virus activation and cellular activation by proinflammatory cytokines can be uncoupled. In THP89GFP cells IFN-γ induces cellular activation in the entire cell population while stimulating virus expression on a small subset of these cells. Likewise, low doses of TNF-α could induce expression of cellular activation markers in the complete population of cells while activating virus expression in only a subset.

Following TNF-α-induced virus reactivation in THP89GFP cells, a recapitulation of the latency induction process is observed over time, in which the population of cells shows a synchronous and progressive down-modulation of HIV-1 expression, reconstituting a fully latent and reactivatable state.

**MATERIALS AND METHODS**

**Cell culture and reagents.** Jurkat-derived and THP-1-derived cell lines were maintained in RPMI 1640 supplemented with 2 mM l-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% heat-inactivated fetal bovine serum. THP89GFP cells grow semiaherent; therefore all experiments used THP89GFP cells were performed in ultralow-attachment plates (Costar, Acton, Mass.). 293T cells were maintained in Dulbecco’s modified Eagle medium supplemented as for RPMI 1640. Cytokines (TNF-α, IL-1, IL-2, IL-6, IFN-γ, and lymphotixin alpha [LT-α]) were obtained from R & D Systems (Minneapolis, Minn.). PMA and TSA were purchased from Sigma (St. Louis, Mo.). HIV-1 p24, IL-1β, and IL-8 hemigenomic plasmids were purchased from R&D Systems (Minneapolis, Minn.).

**Construction of HIV-1 89ENG and HIV-1 NLENG1.** NL4-3 hemigenomic plasmids p83-5 and p83-10 were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program from Ronald Desrosiers. The 89.6 hemigenomic plasmids were a kind gift from Ronald Collman, University of Pennsylvania School of Medicine, Philadelphia, Pa. (2, 20). In brief, recombinant hemigenomic HIV-1 plasmids 3’89ENG and 3’NLENG1 were similarly constructed by using primer extension and sequence overlap extension (Deep Vent polymerase; New England Biolabs) to link the EGFP coding sequence to the HIV-1 genome and to introduce desired restriction sites and Kozak sequences into the DNA fragments to be ligated. The cloning was designed such that the EGFP open reading frame was placed directly between the env and nef genes within the HIV-1 sequence (Fig. 1).

For NLENG1, three PCR products were generated; these products represented (i) the NL4-3 genome from the unique BamHI site within env to the junction between the end of env and the start of the EGFP gene, (ii) the EGFP gene, and (iii) NL4-3 from the Kozak sequence within the junction with the EGFP gene to the unique BsrPI site within the 3’ LTR of NL4-3. Products i and ii were then linked by using PCR sequence overlap extension, and the resulting product was cut with BamHI and NcoI (within the Kozak sequence). Product iii was cut with NcoI and BsrBI, and the BamHI-BsrBI fragment was removed from p83-10. p83-10 plus the cut PCR products were ligated to create 3’NLENG1.

For 89ENG, three PCR products were generated similarly to those for the NLENG1 construct except that the 5’ and 3’ restriction sites within the 89.6 3’ hemigenomic plasmid were BsrBI and Nhel sites, respectively. Also, a Kozak site was introduced upstream of the EGFP gene. These three products were cut and cloned into the 3’ 89.6 hemigenomic plasmid to generate 3’89ENG.

**Generation of virus stocks.** 3’ and 5’ HIV-1 hemigenomic plasmids were linearized at the shared EcoRI site in each plasmid. The DNAs were extracted with phenol-chloroform, precipitated with isopropanol, and resuspended in water. 293T cells were transfected with the two plasmids by using CaPO4 (Stratagene), and 48 h later the supernatants were used to infect CEMx174 cells. At near-peak virus production, as measured visually by EGFP fluorescence and cell death, the medium was changed, and after 24 h this medium was collected, filtered, and stored in aliquots at −80°C until use.

**Flow-cytometric analysis.** Flow-cytometric analysis was performed with a FACStar Plus and CellQuest software (BD Biosciences). For analysis of surface antigen expression, cells were washed with phosphate-buffered saline (PBS) and then preincubated with 50 μl of PBS containing 0.01% azide and 10% rabbit serum to block nonspecific binding. The directly conjugated antibodies were added, incubated at 4°C for 30 min, and washed in 4 ml of PBS prior to flow-cytometric analysis.

**Photomicroscopy.** Cells were photographed in culture through a Nikon TE300 inverted microscope and Hoffman optics (Modulation Contrast, Inc.) at ×100 by using a SenSys:1401E B&W cooled charge-coupled device camera (Photometrics, Inc.). To detect EGFP fluorescence the Piston green fluorescent protein filter set was used (Chroma, Inc.).

**Fluorometric analysis of HIV-1 reactivation.** Cells were analyzed for cumulative EGFP fluorescence in flat-bottom 96-well tissue culture plates (Costar) in 200 μl of PBS by using a BIO-TEK FL600 fluorometer. Excitation was set at 435 nm, and emission was set at 530 nm. Ideal excitation for EGFP, as given by the manufacturer (Clontech, Palo Alto, Calif.) is at 488 nm, and ideal emission is at 588 nm.

**RESULTS**

Selection of latent and reactivatable HIV-1-infected clonal cell lines. Jurkat (T-lymphocytic) and THP-1 (promonocytic) cells were infected with the EGFP-containing recombinant...
viruses NL2 (T cell-tropic) and 89NG (dual-tropic) (Fig. 1). Four days following infection, EGFP-positive cells were cloned by using the automatic cell deposition unit of the FACStar Plus, and the surviving chronically infected clones were monitored for EGFP expression. Clones which lost fluorescence in the majority of cells were selected for further characterization. From this set, clones in which EGFP expression could be reactivated by stimulation with TNF-α, a powerful activator of the HIV-1 LTR (22, 32), were expanded. Finally, three cell lines representing each combination of virus and natural cellular target, J89GFP and THP89GFP (Fig. 2) and JNLGFP (data not shown), were chosen for further study because of low constitutive EGFP fluorescence and strong up-regulation of EGFP expression following TNF-α stimulation.

**Correlation of EGFP fluorescence, p24 Gag protein expression, and secretion of infectious viral particles in JNLGFP, J89GFP, and THP89GFP cells.** To examine whether EGFP fluorescence in JNLGFP, J89GFP, and THP89GFP cells can
be used as a quantitative marker for HIV-1 expression, we stimulated the cells with various concentrations of TNF-α and after 48 h analyzed EGFP expression, p24 secretion, and infectious-virus production. Cells were further found to express HIV-1 Env at levels similar to those seen in CEMx174 cells infected with wild-type HIV-1 89.6. Nef expression in HIV-1 Vpu at levels similar to those seen in CEMx174 cells indicated that latency in these cells is the result of low LTR activity and not simply the result of suboptimal late-gene expression.

**Synchronous reversion to latency in THP89GFP cells.** We next monitored EGFP expression in THP89GFP cells over a extended period of time after TNF-α stimulation in order to examine whether varying the dose of TNF-α resulted in prolonged or transient reactivation of virus expression. For this analysis we limited the range of TNF-α to 0.03 to 3 ng/ml, as higher levels led to substantial cell death after 4 days. At each dose of TNF-α tested, the maximum percentage of cells that were EGFP positive was achieved after 24 to 48 h. While the fluorescence intensity peaked during this time as well in the cultures receiving 0.03 to 0.3 ng/ml, peak fluorescence intensity was observed after 4 days in the cultures receiving the highest doses of TNF-α (1 and 3 ng/ml), consistent with the data from Fig. 4. Over the course of the following days and weeks, the expression of virus as measured by EGFP fluorescence from these cells steadily declined (Fig. 5B), while the percentage of cells which continued to produce at least some virus declined in a much more gradual manner (Fig. 5A). Interestingly, rather than individual cells spontaneously ceasing virus expression, a continual and synchronous decline in virus production from the population of cells was observed (Fig. 5C to F). Upon reexposure to TNF-α, virus expression, as measured by EGFP fluorescence (Fig. 5A to C; day 28), and infectious-virus production (data not shown) were once again reactivated in these cells, indicating that the process of induction of latency was recapitulated after the first reactivation.

**Reactivation of latent HIV-1 by various cytokines and chem-**
The replication of HIV-1 in vivo is influenced by the local cellular environment, including cytokines that regulate the immune response. Several of these are known to activate or inhibit HIV-1 replication under various circumstances, and some, such as IL-2, have been explored as part of anti-HIV-1 treatment regimens (17). We tested a panel of stances, and some, such as IL-2, have been explored as part of activate or inhibit HIV-1 replication under various circumstances. Several of these are known to activate or inhibit HIV-1 replication under various circumstances. One of these is the proinflammatory cytokine TNF-α, which activates macrophages and monocytes/macrophages (34). TNF-α can also reactivate HIV-1 infection in THP89GFP cells, while IFN-γ and IL-1β failed to do so. Interestingly, after 48 h, only a subpopulation of THP89GFP cells reactivated virus expression in response to IFN-γ and IL-1β (Table 1). PMA, a potent activator of HIV-1 LTR expression through protein kinase C and ultimately NF-κB activation (34), produced a strong increase in EGFP expression in J89GFP cells. Its effect on HIV-1 reactivation in THP89GFP cells was less pronounced, as HIV-1 expression was reactivated in only 57% of the viable cells. TSA, an inhibitor of histone deacetylases, said to reactivate HIV-1 infection (6, 53), also had a strong effect on HIV-1 reactivation in J89GFP cells. The effect of TSA on HIV-1 replication in THP89GFP cells again was less profound (34% reactivation). Although TNF-α, PMA, and TSA all produced virus reactivation in almost all J89GFP cells, the degree of reactivation within individual cells, as measured by mean channel fluorescence (MCF) intensity, was clearly lower following PMA and TSA exposure than following TNF-α stimulation at 48 h (Table 1) and all other time points tested (data not shown). Macrophage inhibitory protein 1α, MCP-1, SDF-1α, and IP-10 failed to reactivate HIV-1 expression in these cell lines (data not shown).

Simultaneous analysis of cell activation, CD4 down-modulation and virus reactivation following TNF-α exposure. The importance of latency to the life cycle of HIV in vivo arises in part from the linkage between the status of immune system activation and the level of virus replication within those cells. It is commonly accepted that the latent state generally exists within the resting pool of CD4-positive T cells and monocytes/macrophages, and, importantly, the activation of those latently infected cells is considered the likely impulse that reactivates virus replication. Using a salient feature of this system, the ability to coordinately monitor virus expression and other cellular events, we simultaneously examined virus expression and the activation state of THP89GFP cells following TNF-α stimulation (Fig. 6). As an indication of cellular activation, we stained the cells for intercellular adhesion molecule 1 (ICAM-1) expression, which increases in cells of the monocyte/macrophage lineage following TNF-α exposure (Fig. 6A). As an indication of cellular activation, we stained the cells for intercellular adhesion molecule 1 (ICAM-1) expression, which increases in cells of the monocyte/macrophage lineage following TNF-α exposure (Fig. 6B). ICAM-1 expression was reactivated in only 57% of the viable cells. TSA, an inhibitor of histone deacetylases, said to reactivate HIV-1 infection (6, 53), also had a strong effect on HIV-1 reactivation in J89GFP cells, while IFN-γ and IL-1β failed to do so. Interestingly, after 48 h, only a subpopulation of THP89GFP cells reactivated virus expression in response to IFN-γ and IL-1β (Table 1). PMA, a potent activator of HIV-1 LTR expression through protein kinase C and ultimately NF-κB activation (34), produced a strong increase in EGFP expression in J89GFP cells. Its effect on HIV-1 reactivation in THP89GFP cells was less pronounced, as HIV-1 expression was reactivated in only 57% of the viable cells. TSA, an inhibitor of histone deacetylases, said to reactivate HIV-1 infection (6, 53), also had a strong effect on HIV-1 reactivation in J89GFP cells. The effect of TSA on HIV-1 replication in THP89GFP cells again was less profound (34% reactivation). Although TNF-α, PMA, and TSA all produced virus reactivation in almost all J89GFP cells, the degree of reactivation within individual cells, as measured by mean channel fluorescence (MCF) intensity, was clearly lower following PMA and TSA exposure than following TNF-α stimulation at 48 h (Table 1) and all other time points tested (data not shown). Macrophage inhibitory protein 1α, MCP-1, SDF-1α, and IP-10 failed to reactivate HIV-1 expression in these cell lines (data not shown).
fails to induce expression of MHC-II (Fig. 7C), at concentrations above 0.1 ng/ml is a powerful inducer of both ICAM-1 and virus expression in the whole cell population. Thus, as also seen in Fig. 6B, in which a low concentration of TNF-α/H9251 is used, in THP89GFP cells cellular activation and virus reactivation can be dissociated.

Plate-based fluorometric analysis of viral reactivation. We next investigated whether viral reactivation could be conveniently monitored by a 96-well-plate-based fluorometric assay. This type of analysis would be easily scalable for the analysis of many samples in a short period of time using relatively small numbers of cells and reagents. We compared fluorometric analysis using a 96-well format to flow cytometry for sensitivity of EGFP detection in J89GFP cells. Cells were stimulated with various concentrations of TNF-α for 24 h, and EGFP expression was then measured as cumulative fluorescence with the fluorometer or as MCF intensity by flow cytometry. Flow cytometry here revealed a 25-fold increase in MCF intensity in J89GFP cells stimulated with 100 ng of TNF-α/ml, compared to that in untreated control cells. A 26-fold increase...
in cumulative fluorescence for cells from the same experiment was measured with the fluorometer, indicating a very close correlation between the two methods. Similar results were obtained for THP89GFP cells (Fig. 8B).

**DISCUSSION**

The need for in vitro models of latency has been partially filled over the past years by HIV-1-infected transformed cell lines such as ACH-2, J1.1, U1, and OM-10.1 (9, 21, 27, 28). Virus expression in these cell lines can be induced by cellular (7, 10) or viral factors (12, 30, 38, 39) and chemical agents (29, 36) or inhibited by pharmacological agents (31, 55). Establishment of latency in these cell lines has been linked to mutations in viral genes such as tat (24) and the TAR region (25), the site of viral integration (54), and to certain cellular (34, 35) and viral proteins (30, 38, 39, 47). A particularly important insight into the regulation of HIV-1 expression comes from the observation that histone acetylation and DNA methylation patterns within and downstream of the viral promoter/enhancer elements can be critical to the suppression of HIV-1 expression or its release (4, 5, 50, 51, 53). HIV-1 Tat participates in these processes in part by recruiting p300 and CREB-binding protein, a protein with histone acetyltransferase activity, to the viral promoter (6, 21, 40). How these DNA acetylation and methylation patterns are established in the first place and how they may be influenced by cellular events are areas of intensive ongoing research.

Studies on the mechanisms governing HIV-1 latency would benefit from an in vitro system where the level and timing of HIV-1 expression can be quantified easily and directly at the single-cell level. The reporter cell lines described here have several features that make them especially useful in this context. The incorporation of the EGFP gene into the HIV-1 genome resulted in control of cellular fluorescence that is strictly coordinated with the expression of viral proteins, permitting immediate and quantitative measurement of the extent of viral expression in cells by using flow cytometry, fluorescence microscopy, or plate-based fluorometry. Flow-cytometric analysis allows both population and single-cell quantification of viral reactivation without any fixation, staining, or other manipulation of the cells that might affect the results or the ability to further manipulate and analyze the relevant cell populations. Multicolor flow-cytometric analysis permits a variety of cellular and viral events to be correlated.

Two cell lines were constructed by using Jurkat cells, which

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**TABLE 1. Reactivation of latent HIV-1 infection in THP89GFP and J89GFP cells by cytokines and chemical agents**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>MCF intensity (% positive cells) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>J89GFP</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>13 (4)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1,221 (92)</td>
</tr>
<tr>
<td>LT-α</td>
<td>1,881 (94)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>21 (6)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>24 (6)</td>
</tr>
<tr>
<td>IL-2</td>
<td>14 (4)</td>
</tr>
<tr>
<td>IL-6</td>
<td>14 (4)</td>
</tr>
<tr>
<td>PMA</td>
<td>691 (93)</td>
</tr>
<tr>
<td>TSA</td>
<td>272 (82)</td>
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</table>

Reactivation of latent HIV-1 infection is indicated by MCF intensity and the percentage of EGFP-positive cells. J89GFP and THP89GFP cells were stimulated for 48 h and then analyzed for EGFP expression by flow cytometry. For all tested cytokines the receptors were shown to be present on either J89GFP or THP89GFP cells by other relevant functional assays. TNF-α and LT-α were added at 10 ng/ml, IL-2 and IFN-γ were added at 200 U/ml, IL-1β was added at 2 ng/ml, and IL-6 was added at 50 ng/ml. The optimal concentration of PMA was 30 ng/ml. For TSA maximal subcytotoxic concentrations were 1 μM for THP89GFP and 300 nM for J89GFP cells. Addition of higher concentrations of PMA or TSA resulted in greatly increased cell death in the respective cell lines. Results are representative of two independent experiments.
have been widely used as models for T-cell studies. The third cell line is based on THP-1 cells, which constitute the most widely employed transformed cell line for studies of monocytes/macrophages. The THP-1-based latent cell line (THP89GFP) and one of the Jurkat-based cell lines (J89GFP) were infected with 89ENG, whose parental virus, 89.6, is considered a near-primary dual CXCR4- and CCR5-tropic molecular clone (20). The other Jurkat cell line was infected with a recombinant virus (NLENG1) whose parental virus, CXCR40-tropic NL4-3, is the best-characterized HIV-1 strain. We have observed identical patterns in response to different stimuli (TNF-α/H9251, LT-α/H9251, PMA, and TSA) in the two Jurkat-based cell lines infected with the divergent viruses (Table 1 and data not shown). On the other hand, differences in the responses to activating agents between the Jurkat- and THP-1-based cell lines are apparent (Table 1). THP89GFP cells, but not J89GFP cells, responded to IFN-γ and IL-1β by reactivating HIV-1 expression (Table 1), despite the fact that J89ENG cells responded to IFN-γ and IL-1β treatment by increasing surface ICAM-1 expression (data not shown). Differences between J89GFP and THP89GFP cells in the responses to PMA and to deacetylase inhibitor TSA were also apparent.

The importance of postintegration viral latency to the natural history of HIV-1 infection in vivo has perhaps been underestimated until fairly recently. The failure of highly active antiretroviral therapy to allow complete removal of virus from the body is due, at least in part, to the reemergence of viral replication from the pool of latently infected cells (15). In the past several years, latent HIV-1 has been identified in resting memory (14, 18, 19, 37) and naive (45) T cells, from which virus may be rescued long after infection of these cells.

The prevailing model of postintegration HIV-1 T-cell latency in vivo requires infection of an activated cell just before the cell enters a quiescent state (46). The reservoir of virus identified within the memory T-cell compartment is thought to be generated by infection of antigen-activated T cells (18, 19), while naive latently infected cells may arise through infection of CD4-positive thymic precursors (8). In either case, it is believed that in vivo viral latency is the direct result of an intracellular environment lacking the necessary factors for efficient transcription of the viral promoter. Following induction of the latent state, maintenance of latency may be assisted through cellular processes directed at the viral promoter region, such as histone deacetylation or DNA methylation (4, 5, 50, 51, 53). Because of the extremely long half-lives of these latent viral reservoirs, therapeutic reactivation is thought to be essential to achieve eradication of HIV-1 infection from patients. Administration of IL-2 as part of the treatment schedule has thus far failed to achieve clinical efficacy, emphasizing the difficulty in reaching the latent compartments with the proper signals for virus reactivation.

Unlike latently infected cells in the body, which can be identified ex vivo within the resting population of T lymphocytes, the cell lines described here are constantly proliferating, performing DNA synthesis and mitosis, and expressing genes at high levels. The finding by Brooks et al. (8) that reactivation of HIV-1 gene expression within thymocytes can be achieved in the absence of cellular DNA synthesis indicates that events more closely linked to cell activation than to increased cell proliferation are key to HIV-1 reactivation. NF-κB is a crucial factor for promoting transcriptional initiation and elongation from the HIV-1 LTR, and its activity may be induced in the

FIG. 7. Differential regulation of MHC-II and HIV-1 expression following stimulation with TNF-α or IFN-γ. THP89GFP cells (10^6 cells/ml) were stimulated with IFN-γ (300 U/ml) (B and E) or TNF-α (1 ng/ml) (C and F). Forty-eight hours after exposure, cells were stained for the expression MHC-II (A to C) or ICAM-1 (D to F). Levels of EGFP, ICAM-1, and MHC-II expression were then quantified by flow-cytometric analysis. Numbers represent the percentages of cells in the respective quadrants. Results are representative of two independent experiments.
absence of cellular proliferation by agents such as PMA and TNF-α (8, 37).

By exploiting the single-cell analysis capabilities of this system, we found that at least under certain circumstances cellular activation may be induced in the absence of virus reexpression. Using ICAM-1 as a cell activation marker, we observed that HIV-1 reactivation and cell activation can be uncoupled when low concentrations of TNF-α are applied (Fig. 6B). Low doses of TNF-α (0.1 ng/ml) apparently resulted in cellular activation of nearly the complete population of cells, as indicated by the uniform up-regulation of ICAM-1 expression, but HIV-1 was reactivated in only a subset of these cells. Also, IFN-γ stimulation, which led to a strong activation of the THP89GFP cells, did not result in substantial reactivation of the latent HIV-1 infection. Each of these patterns persisted for at least 96 h, the longest interval tested, indicating that the uncoupling of cellular and viral activation was not the result of a simple kinetic difference between the two. Therapies which seek to activate HIV-1 expression for the purpose of making these cells vulnerable to antiviral therapy or immune responses may need to consider that not all agents which promote cellular activation may in fact reactivate virus from all cells.

We have also found evidence for stochastic events in creating and maintaining the latent state in cells. The subcloning of unstimulated cells from each of the cell lines described here occasionally generates a cell line that is a constitutive producer of virus. Other subclones differ from the parental cell lines in exhibiting no spontaneously fluorescent cells or detectable p24 Gag protein, and these are uniformly unresponsive to TNF-α induction of virus expression (data not shown). Thus, while an important role for the site of viral integration has been found for many latently infected cells (54), clearly the outcome of HIV-1 infection is governed by complex and mutable processes. By observing recently infected cells sorted for EGFP fluorescence, we observed loss of EGFP in some cells in as little as 2 days, indicating that down-modulation of HIV-1 gene expression can occur rapidly following infection (data not shown). The defined latent reservoirs in vivo are composed of quiescent nonproliferating cells, but it may be possible for HIV-1 to enter latency in some cells which have yet to transition back to a nonproliferating state. Whether such cells exist in vivo is unknown, but since existing methodologies do not permit their detection, the relevant experiments have not been performed.

Another interesting and useful aspect of the system described herein is the ability of HIV-1 to return to a latent state following reactivation (Fig. 5). Interestingly, following reentry into the latent state, the virus could be fully reactivated once again, indicating that the original latent state has been reconstituted. As such, this system not only allows for the detailed study of HIV-1 reactivation, but also enables the controlled study of processes involved in the achievement of latency. It is most likely, we believe, that both this recapitulation of latency induction and the stochastic events described above are controlled by cellular modifications at the viral promoter, including histone acetylation and deacetylation events and DNA methylation patterns. Examination of these patterns in clonally related cell lines which exhibit divergent HIV-1 expression properties should be illuminating, and these patterns are the subject of current investigation in our laboratories.

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**REFERENCES**


