A Checkpoint in the Cell Cycle Progression as a Therapeutic Target to Inhibit HIV Replication

Andrea Foli, Maria Angela Maiocchi, Julianna Lisziewicz, and Franco Lori

1Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, Pavia, Italy; 2Research Institute for Genetic and Human Therapy, Pavia, Italy, and Bethesda, Maryland

Human immunodeficiency virus (HIV) expression is boosted after T lymphocyte stimulation. It is not known, however, in which phase(s) of the cell cycle HIV is maximally expressed. We demonstrate here that cell activation induces limited HIV expression and that progression to cell proliferation is required for optimal HIV replication. We also show that the G1/S cell cycle transition is a critical checkpoint in this process and that limiting progression at this step with antiproliferative drugs suppresses HIV replication. These results identify a specific phase of the cell cycle progression that is critical for HIV expression and suggest a new discrete target for anti-HIV treatment.

Chronic immune stimulation plays a similar pivotal role in the pathogenesis of simian immunodeficiency virus (SIV) infection in rhesus macaques [13, 14]. In contrast, the immune response to SIV infection in sooty mangabeys (SIV’s natural hosts) is contained. Subsequently, immune overreaction and exhaustion do not occur and infected animals do not progress to AIDS, despite high levels of viral replication [15, 16].

Immune stimulation induces a 2-step process: (1) T cell activation (a dynamic process resulting in cytokine production and expression of activation markers) followed by (2) cell proliferation [17, 18]. The latter step is the natural extension of the first, and often the 2 are inseparably linked. In few instances (e.g., cytokine-induced homeostatic proliferation in human naive T cells) [19–21], T cell receptor (TCR)–major histocompatibility complex–driven activation is bypassed. However, naive cells are not natural HIV targets [7, 22–25], and this exception may not be relevant for HIV replication. More frequent and relevant situations are (1) when memory cells are activated without progressing to division and proliferation (i.e., covert TCR signals are insufficient to induce entry into the cell cycle but are sufficient to keep the memory cell alive during the homeostatic process) and (2) antigen-driven activation in the absence of sufficient time to induce entry into the cell cycle [20, 26–28] or of proper costimulatory signals [29]. Importantly, a defect in cell cycle progression might be a trait of HIV infection [30].

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Reprints or correspondence: Dr. Franco Lori, RIGHT, 4400 East-West Hwy., Suite 1126, Bethesda, MD (rightpv@tinn.it).

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though it is recognized that immune stimulation of T cells promotes HIV replication, it is not known whether T cell activation is sufficient or whether cell proliferation is required to sustain HIV replication. This distinction has pathogenic relevance, because chronic immune stimulation induced by HIV plays an important role in the progression to AIDS. Moreover, if cell cycle progression is critical to HIV replication, cell cycle modulators could be developed as a new class of antiviral drugs.

In light of these considerations, it could be anticipated that limiting immune system overactivation would decrease HIV replication and delay disease progression. The key question becomes at which point after antigenic stimulation of a T lymphocyte \[17, 18\] can one intervene to prevent the cell machinery from massively expressing HIV. We demonstrate here that T cell activation alone allows limited HIV expression, whereas the bulk of HIV replication occurs during cell proliferation. We identify the transition from the G1 to the S phase of the cell cycle as being critical for optimal HIV replication and demonstrate that limiting cell cycle progression beyond this checkpoint can substantially reduce HIV replication.

**METHODS**

**HIV-1 replication, cell activation, and cell proliferation in infected resting CD4+ T cells.** CD4+ cells were separated from peripheral blood mononuclear cells (PBMCs) obtained from healthy donors by use of anti-CD4–coated magnetic beads (Miltenyi Biotec), and cell activation was immediately evaluated by CD69 staining (Immunotech-Coulter). CD4+ cells were infected with either HIV-1_{IIIb} or HIV-1_{NL4-3} strains and cultured for 5 days in the absence of stimulation. On day 5, cells were activated with either 5 μg/mL phytohemagglutinin-P (PHA-P; Sigma-Aldrich) or 10 ng/mL phorbol 12-myristate 13-acetate plus 100 ng/mL calcimycin A23287 (PMA/Ci; both from Sigma-Aldrich); after 2 days, 20 U/mL interleukin-2 (Boehringer-Mannheim) was added. In parallel, infected CD4+ cells were cultured in medium supplemented with an anti-CD28 antibody at 2 μg/mL (BD Biosciences Pharmingen) in a plate previously coated with anti-CD3 antibody (Zymed Laboratories). On day 10, supernatants were collected to measure viral replication by HIV-1 p24 ELISA (Beckman Coulter) or,
were infected with HIV-1 NL-r-HSAS as described above and cultured with or without 1 mmol/L HU or 25 μmol/L MPA, doses routinely used for cell cycle synchronization [33, 34]. The percentage of carboxyfluorescein diacate succinimidyl ester (CFSE)dim cells (a measure of cell proliferation) was calculated by staining CD4+ cells with CFSE (Molecular Probes; Invitrogen) on day 0. Cells were defined as CFSEdim if they had gone through at least 1 cell proliferation cycle. Cell proliferation was calculated by determining the percentage of CFSEdim cells in a given population (e.g., the mCD24+ or mCD24- population). HIV-1 replication was calculated by determining the percentage of mCD24+ cells in a given population (i.e., % of mCD24+ CFSEdim cells × 100% of CFSEdim cells). Treatment with 100 μmol/L hydroxyurea (HU; Sigma-Aldrich) or 10 μmol/L mycophenolic acid (MPA; Sigma-Aldrich), which represent pharmacologically relevant concentrations that correspond to peak concentrations achieved in HIV-1–infected subjects [31, 32], was started on day 0.

**Results**

**Optimal HIV-1 expression in CD4+ cells that proliferate after activation.** HIV-1 expression after stimulation of infected resting CD4+ cells was evaluated in relation to cell activation and subsequent proliferation. We used in our experiments the viral strain HIV-1NL-r-HSAS in which the murine heat-stable antigen (mCD24) has been cloned in place of the vpr gene and is expressed on the membranes of cells actively replicating HIV-1 [35]. Measurement of mCD24 expression enables simultaneous study of the percentage of infected cells actively replicating HIV-1 and their activation and proliferation status.

Activation markers presented different patterns of expression (figure 1A). CD69 expression progressively decreased during consecutive cycles of proliferation, whereas HLA-DR and CD28 expression did not. This is consistent with previous observations indicating that CD69 is an activation marker that progressively diminishes through repeated cycles of cell division after activation of T lymphocytes [19]. CD69 reveals whether the activation event has recently occurred or whether the cell has undergone repeated cell division cycles after activation, distinguishing recently activated cells from those that have progressed to proliferation. Because other activation markers that “permanently label” the cells are carried over during subsequent cycles of cell proliferation and cannot differentiate activation from proliferation, CD69 expression was the measurement chosen in the following experiments.

When HIV-1 replication was analyzed together with cell activation and cell proliferation, mCD24 (HIV-1) gene expression was sustained throughout subsequent rounds of cell proliferation (figure 1B), whereas CD69 expression progressively decreased (figure 1A). The mean fluorescence intensity (MFI) of the CD69+ cells consistently decreased on cell proliferation (MFI ± SD among the CFSEdim+ cells was 26.00 ± 6.30, compared with 8.97 ± 3.78 after 5 replication cycles), whereas the mCD24 MFI remained stable (MFI ± SD among the CFSEdim+ cells was 15.17 ± 13.20, compared with 11.83 ± 8.69 after 5 replication cycles); almost all mCD24+ cells were CD69− (figure 1B).

On the contrary, cell proliferation (measured as the percentage of proliferating cells in a population) was higher among productively infected (mCD24+) cells than among uninfected (mCD24−) cells. HIV-1 replication, measured as the percentage of mCD24+ cells, was >15-fold higher in proliferating cells than in cells that had not divided (table 1).

**Boosting of HIV-1 expression when the cell cycle progresses beyond G1/S and suppression of HIV replication by drugs targeting this step.** The above results demonstrate that HIV-1 replication is driven by cell stimulation and that cell proliferation is required for maximal viral gene expression. Because cell proliferation requires the transition from the G1 to the S
Figure 2. Effects of antiproliferative drugs on HIV-1 and T cell proliferation. A–F, Cell distribution within the cell cycle among uninfected and infected (mCD24+) cells, in untreated samples (A and B) and in samples treated with 1 mmol/L hydroxyurea (HU; C and D) or with 25 μmol/L mycophenolic acid (MPA; E and F). The no. of dots was lower in the infected samples than in the uninfected samples because there were considerably fewer infected cells than uninfected cells. Dot plots are representative single experiments, and pie charts show the average results from 6 experiments. Cells in the lower left gate are in the G0/G1 phase, those in the upper gate are in the S phase, and those in the lower right gate are in the G2/M phase. G, HIV-1 expression rate (mean ± SD) in G0/G1 (black bars) and S/G2/M (gray bars). H, Mean fold changes in the percentage of cells in the G0/G1, S, and G2/M phases and in the percentage of mCD24+ cells per total cells (HIV), after HU (left) and MPA (right) treatment. Each dot represents the value obtained from single experiments. Fold changes <1 indicate a decrease. 7-AAD, 7-amino-actinomycin D; BrdU, 5-bromo-2′-deoxyuridine.
and G2/M phases, progression to cell division during the cell cycle should be essential to maximize HIV-1 replication. To demonstrate this, we analyzed the correlation between HIV-1 replication and the different phases of the cell cycle.

We used the viral strain HIV-1NL-r-HSAS to simultaneously study the percentage of infected cells and their cell cycle distribution. The HIV-1NL-r-HSAS strain was also chosen because, unlike the p24 antigen, the mCD24 protein is not carried by the incoming virion and thus reflects bona fide novel HIV-1 gene expression. Although the HIV-1NL-r-HSAS is defective for vpr and lack of the corresponding protein might interfere with the later phases of the cell cycle [36], vpr expression occurs beyond the S phase and was unlikely to interfere with the experiments.

The majority of uninfected T cells (75.4% ± 12.8%) were found to be in the G0/G1 phase (figure 2A), whereas infected cells (figure 2B), representing 2.0% ± 1.6% of the cells in the untreated samples, were predominantly (65.7% ± 11.1%) distributed in the proliferating (S and G2/M) phases. To further prove that progression of the cell cycle beyond the G1/S checkpoint is critical for optimal HIV-1 expression, cells were treated with HU and MPA. As expected, these drugs discretely prevented progression of the cell cycle in uninfected cells from the G1 (activated) to the S (proliferating) phase (cells in G0/G1 were 95.55% ± 3.00% and 87.13% ± 6.14%, respectively) (figure 2C and 2E). Although the infected T cells were still predominately in the proliferating phases of the cell cycle (cells in G0/G1 were 54.9% ± 20.66% and 49.98% ± 13.24% in the HU- and MPA-treated samples, respectively) (figure 2D and 2F), the HIV-1 expression rate, calculated by determining the percentage of mCD24+ cells in a given cell cycle phase, was 0.053% ± 0.038% in S/G2/M, compared with 0.007% ± 0.005% in G0/G1 (figure 2G). This resulted in an 8-fold increase in the expression rate during the division phases of the cell cycle, compared with that during the phases preceding the commitment to division (occurring at the end of the G1 phase [37]). The decrease in the percentage of mCD24+ cells was not due to a decrease in the HIV-1 expression rate in either proliferating (S/M) or nonproliferating cells (G0/G1) (figure 2G) but was due solely to the reduction of the percentage of cells in the proliferating phases (figure 2H). In fact, the reduction in the percentage of cells in the S phase paralleled the reduction in the percentage of mCD24+ cells in S phase and per total cells (S and HIV, respectively, in figure 2H), both with HU (40% decrease in both S and HIV) and MPA (50% and 20% decrease in S and HIV, respectively) treatment.

HU and MPA treatment increased the percentage of mCD24+ cells in the G0/G1 phase because of the accumulation of synchronized cells that were not able to progress to S phase. This only marginally affected total HIV-1 gene expression (figure 2G). Because HU and MPA do not block progression of cells from S to G2/M, the percentage of cells in the G2/M phase (figure 2A, 2G, and 2E) and the percentage of mCD24+ cells in the G2/M phase (figure 2B, 2D, and 2F) were not affected. Therefore, if cells are unable to enter the S phase because of drug treatment, replication detected in the G2/M phase represents residual replication from cells that were already in the S phase when treatment was initiated and subsequently progressed to G2/M.

The observation that the G1-S checkpoint is a suitable target for antiretroviral therapy was confirmed by additional experiments in which the cell cycle of the infected resting CD4 cells was blocked in G1 by either HU or MPA. In the HU-treated samples, the mean ± SD level of HIV-1 p24 was 0.41 ± 0.46 ng/mL, compared with 132.0 ± 138.8 ng/mL in the untreated samples (P < .0001). Proliferation was similarly inhibited, with 8.1% ± 8.6% CFSE™ cells in the HU-treated samples and 62.7% ± 61.4% in the untreated samples (P < .0001). Conversely, samples treated with HU showed an increase in the percentage of CD69+ cells: 50.5% ± 50.5%, compared with 29.6% ± 29.5% in the untreated samples (P = .002). We found a positive correlation between HIV-1 replication and cell proliferation (figure 3A) but not between HIV-1 replication and cell activation (figure 3B). When cell proliferation was suppressed by HU treatment, between day 5 (before cell stimulation) and day 10 there was no increase in HIV replication, despite increased cell activation. The above results were obtained using the viral strain HIV-1NL-r, which is defective for the vpr and nef genes; however, similar results were obtained in a second set of experiments using the viral strain HIV-1NL-r, which is not defective for any gene (data not shown).

Because cells can be stimulated through different pathways...
in vivo, stimulation with PMA/CI and with immobilized anti-CD3 plus soluble anti-CD28 was performed to validate the results obtained with PHA. HU inhibited HIV replication regardless of the stimulation method: the mean ± SD level of HIV-1 p24 with PMA/CI was 0.83 ± 0.64 ng/mL in the HU-treated samples, compared with 49.96 ± 59.25 ng/mL in the untreated samples, and the mean ± SD level of HIV-1 p24 with CD3 plus CD28 was 6.46 ± 2.32 ng/mL in the HU-treated samples, compared with 44.83 ± 21.85 ng/mL in the untreated samples. HU inhibited cell proliferation without affecting CD69 expression (data not shown).

Results obtained with MPA mirrored those obtained with HU. MPA treatment inhibited both HIV-1 replication and cellular proliferation. The mean ± SD level of HIV-1 p24 was 0.4 ± 0.4 ng/mL in the MPA-treated samples, compared with 37.1 ± 50.5 ng/mL in the untreated samples (P = .0008). CFSEdim cells were 3.0% ± 3.6% in the MPA-treated samples, compared with 41.5% ± 29.6% in the untreated samples (P = .0004). Cell activation analysis showed no significant differences among samples (P = .21). A positive correlation between HIV-1 replication and cell proliferation (figure 3C) and a weak negative correlation between HIV-1 replication and mере cell activation (figure 3D) were documented.

**DISCUSSION**

In the present study, we have confirmed that HIV-1 expression is stimulated by cell activation, as has been previously shown by others [38, 39]; however, we provide original evidence that HIV-1 gene expression is markedly boosted by progression of the cell cycle beyond the G1-S phase checkpoint. Consequently, the commitment of the cell to division is critical to achieving optimal HIV-1 replication.

Determination of the exact checkpoint of cell cycle transition that is crucial for HIV-1 replication provides a new target for drug development. HU and MPA were useful to validate the model. These drugs have been previously used for the treatment of HIV-1–infected patients because they potentiate the activity of some antiretrovirals [40]. Here, we provide the first experimental evidence that their antiproliferative effects mediate antiviral activity. The in vitro model described here can be used to screen for new agents that affect cell proliferation and inhibit HIV-1 replication. These drugs, targeting cellular instead of viral proteins, are likely to be less prone to inducing drug resistance; consequently, their antiviral activity should be long-lasting. Consistent with these results, the HU-didanosine combination has demonstrated continued viral load suppression in several studies for as long as 8 years [41–43]. The observation that the antiproliferative effects of HU and MPA were associated with the prevention of viral load rebound after withdrawal of highly active antiretroviral therapy [44–46] is also consistent with the results described here and supports the concept of introducing antiproliferative drugs as part of antiretroviral regimens to prevent viral load rebound during treatment failure.

It is plausible that hindering cell cycle progression could be exploited to control replication of other viruses that have pathogenic importance in humans. Several viruses, such as *Papillomaviridae*, *Polyomaviridae*, *Adenoviridae*, *Herpesviridae*, *Hepadnaviridae*, and *Flaviviridae* species, depend on cell cycle progression for their replication and/or to promote their pathogenic mechanism [47]. Therefore, further applications for cell cycle modulators in virology fields other than HIV-1 infection might be explored.

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**References**


