AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology

Kinetics of HIV-1 Latency Reversal Quantified on the Single-Cell Level Using a Novel Flow-Based Technique

G. Martrus,[a] A. Niehrs,[a] R. Cornelis,[a] A. Rechtien,[a,b] W. García-Beltran,[c] M. Lütgehetmann,[d] C. Hoffmann,[e,f] M. Altfeld[a]

Department of Virus Immunology, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany[a]; University Medical Center Hamburg-Eppendorf, 1st Department of Medicine, Hamburg, Germany[b]; Ragon Institute of MGH, MIT, and Harvard, Cambridge, Massachusetts, USA[c]; Department of Microbiology, Virology and Hygiene, University Medical Center Hamburg-Eppendorf (UKE), Hamburg, Germany[d]; ICH Study Center, Hamburg, Germany[e]; Department of Medicine II, University of Schleswig-Holstein, Campus Kiel, Kiel, Germany[f]

ABSTRACT

HIV-1 establishes a pool of latently infected cells early following infection. New therapeutic approaches aiming at diminishing this persisting reservoir by reactivation of latently infected cells are currently being developed and tested. However, the reactivation kinetics of viral mRNA and viral protein production, and their respective consequences for phenotypical changes in infected cells that might enable immune recognition, remain poorly understood. We adapted a novel approach to assess the dynamics of HIV-1 mRNA and protein expression in latently and newly infected cells on the single-cell level by flow cytometry. This technique allowed the simultaneous detection of *gagpol* mRNA, intracellular p24 Gag protein, and cell surface markers. Following stimulation of latently HIV-1-infected J89 cells with human tumor necrosis factor alpha (hTNF-α)/romidepsin (RMD) or HIV-1 infection of primary CD4$^+$ T cells, four cell populations were detected according to their expression levels of viral mRNA and protein. *gagpol* mRNA in J89 cells was quantifiable for the first time 3 h after stimulation with hTNF-α and 12 h after stimulation with RMD, while p24 Gag protein was detected for the first time after 18 h poststimulation. HIV-1-infected primary CD4$^+$ T cells downregulated CD4, BST-2, and HLA class I expression at early stages of infection, proceeding Gag protein detection. In conclusion, here we describe a novel approach allowing quantification of the kinetics of HIV-1 mRNA and protein synthesis on the single-cell level and phenotypic characterization of HIV-1-infected cells at different stages of the viral life cycle.

IMPORTANCE

Early after infection, HIV-1 establishes a pool of latently infected cells, which hide from the immune system. Latency reversal and immune-mediated elimination of these latently infected cells are some of the goals of current HIV-1 cure approaches; however, little is known about the HIV-1 reactivation kinetics following stimulation with latency-reversing agents. Here we describe a novel approach allowing for the first time quantification of the kinetics of HIV-1 mRNA and protein synthesis after latency reactivation or *de novo* infection on the single-cell level using flow cytometry. This new technique furthermore enabled the phenotypic characterization of latently infected and *de novo*-infected cells dependent on the presence of viral RNA or protein.

One major obstacle toward an effective human immunodeficiency virus type 1 (HIV-1) cure is the establishment of a pool of long-lived latently infected cells early after infection (1). Using the rhesus macaque model of simian immunodeficiency virus (SIV) infection, it was recently shown that latent reservoirs could be seeded as early as 3 days after SIV exposure and before the detection of viremia in blood (2). The vast majority of cells infected with HIV-1 will die as a consequence of the infection or will be eliminated by the immune system. A minority of infected cells, however, turns into latently infected resting cells. These cells can either be reactivated and release *de novo* HIV-1 particles (3) or persist and homeostatically proliferate as long-lived memory T cells (4, 5). While current antiretroviral treatments (ARTs) can efficiently suppress HIV-1 replication and have dramatically improved the life expectancy and life quality of infected individuals, ART cannot eradicate the latent viral reservoir.

Several different biological processes have been described to maintain latency in HIV-1-infected cells. Host transcription factors (TFs) such as nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) have multiple binding sites in the 5′ long terminal repeat (LTR) of the HIV-1 genome, and their binding has been demonstrated to be necessary to initiate HIV-1 transcription (6). Sequestration of these TFs in the cytoplasm is one of the mechanisms enabling viral latency (7). Another described HIV-1 latency mechanism involves histone deacetylase (HDAC)-mediated epigenetic silencing (8). During latency establishment, HDAC molecules are recruited toward the 5′ LTR of HIV-1 (9, 10) and therefore maintain the LTR in a repressed state (11). Several HDAC inhibitors (HDACis) targeting HDAC molecules have been tested for their ability to reactivate latently HIV-1-infected cells, including vorinostat, panobinostat, entinostat, and romidepsin (RMD). These HDACis proved to efficiently induce HIV-1 expression in latently infected resting CD4$^+$ T cells from HIV-1-infected individuals (8, 12, 13). RMD, a drug that has been used for the treatment of peripheral T-cell lymphoma,

Received 20 July 2016 **Accepted** 21 July 2016

Accepted manuscript posted online 27 July 2016

Citation Martrus G, Niehrs A, Cornelis R, Rechtien A, García-Beltran W, Lütgehetmann M, Hoffmann C, Altfeld M. 2016. Kinetics of HIV-1 latency reversal quantified on the single-cell level using a novel flow-based technique. J Virol 90:9018–9028. doi:10.1128/JVI.01448-16.

Editor: G. Silvestri, Emory University

Address correspondence to M. Altfeld, marcus.altfeld@hpi.uni-hamburg.de.

was also shown to be an effective HIV-1 latency reversal drug *in vivo* by mediating histone H3 acetylation through the inhibition of HDACs (14–16). Cytokines also play a role in HIV-1 latency reactivation. Specifically, human tumor necrosis factor alpha (hTNF-α), a proinflammatory cytokine, has been described as an activator of latently HIV-1-infected cells (17) by enhancing NF-κB translocation toward the nucleus and binding to the HIV-1 LTR, resulting in new rounds of HIV-1 replication (18–20).

Although HIV-1 latency reactivation has been extensively studied (1), several important aspects remain unknown, including the kinetics and extent of viral mRNA and viral protein production following reactivation on the single-cell level. Currently, several assays are being used to measure and quantify viral reservoirs and HIV-1 reactivation, including digital-droplet PCR (21), the Tat/Rev-induced limiting-dilution assay (TILDA) (22), and viral outgrowth assays (23). While these assays have shown good sensitivity, one limiting factor is the lack of phenotypic characterization of cells that reactivate HIV-1 replication. In contrast to those methods, flow cytometry allows staining for surface markers and intracellular molecules at the single-cell level. Here we describe a novel flow cytometry-based technique used to quantify and differentiate between HIV-1-infected cell populations producing only viral mRNA, viral mRNA and proteins, and viral proteins alone. This assay furthermore allowed the quantification of HIV-1 reactivation kinetics in latently infected cells and their consequences for surface molecule expression.

MATERIALS AND METHODS

Cell lines and reagents. MT-4 cells (a lymphocytic laboratory-stable cell line) and J-Lat full-length cells (clone 10.6) were obtained through the NIH AIDS Research and Reference Reagent Program (24–26). The J89 cell line (a Jurkat cell line infected with HIV-1) was a kind gift from David N. Levy (27). J89 and J-Lat cells (28) were maintained in RPMI 1640 L-glutamine medium (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated Superior fetal bovine serum (FBS) (Biochrom, Berlin, Germany). HEK 293T cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and were grown with Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, Munich, Germany) supplemented with 10% heat-inactivated FBS.

Recombinant hTNF-α (Peprotech, Rocky Hill, NJ, USA) was prepared at a final concentration of 10 μg/ml according to the manufacturer's instructions. RMD (Selleck Chemicals, USA) was diluted at a concentration of 1 mM, and the final concentration used in cell cultures was 5 nM. AMD3100 octahydrochloride hydrate (Sigma-Aldrich, Munich, Germany) was used at final concentrations of 20 μg/ml and 100 μg/ml, as indicated.

The following purified antibodies were used for cell line-based staining and/or blocking assays: anti-CD3 phycoerythrin (PE)-CF594 (clone UCHT1; BD Horizon), anti-CD4 BV711 (clone RPA-T4; BioLegend), anti-pan-HLA-I complex Pacific Blue (clone W6/32; BioLegend), anti-BST-2 PE (clone RS38E; BioLegend), and anti-HIV-1 core antigen in PE and fluorescein isothiocyanate (FITC) (clone KC57; Beckman Coulter).

Healthy cohort PBMCs and CD4$^+$ T cells. Peripheral blood mononuclear cells (PBMCs) were obtained from the blood of healthy donors from the Healthy Cohort Hansestadt Hamburg (HCHH), approved by the Ethical Committee of the Universitätsklinikum Hamburg-Eppendorf. PBMC purification was performed by using Biocoll (Biochrom, Berlin, Germany), and PBMCs were maintained in R10 medium (RPMI plus 10% heat-inactivated FBS).

CD4$^+$ T cells from healthy donors were isolated by using the EasySep human CD4$^+$ T-cell enrichment kit (Stemcell, Cologne, Germany) according to the manufacturer's instructions. CD4$^+$ T cells were counted and prepared in R10 medium at a final concentration of 1×10^6 cells/ml. CD4$^+$ T cells were stimulated with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific, Carlsbad, CA, USA) and 100 U/ml of interleukin-2 (IL-2) (Peprotech, Rocky Hill, NJ, USA) and maintained for 3 days at 37°C in 5% CO_2.

HIV-1 stock production. An HIV-1 NL4-3 plasmid (pNL4-3) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Malcolm Martin (29). HIV-1 NL4-3 strains were produced by transfecting HEK293T cells with 20 μg of pNL4-3 with Lipofectamine 2000 (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's recommendations. The first viral stock was collected at 72 h posttransfection and used to infect MT-4 cells. The final viral stock was recovered by centrifugation at 96 h postinfection from the supernatant of MT-4 cells, filtered, and aliquoted. Virus titration allowing the calculation of the tissue culture dose for 50% infectivity ($TCID_{50}$) per milliliter of virus stock was performed in MT-4 cells according to a previously described protocol (30).

CD4$^+$ T-cell infection. CD4$^+$ T cells were infected with the HIV-1 stock at a multiplicity of infection (MOI) of 0.02. Briefly, cell pellets were spinoculated at 1,200 × *g* for 2 h at 37°C together with the appropriate amount of the HIV-1 stock. After 2 h, cells were resuspended at a final concentration of 1×10^6 cells/ml in RPMI medium supplemented with 10% FBS plus 100 U/ml of IL-2. Infected CD4$^+$ T cells were left for 72 h in an incubator at 37°C. Uninfected controls were spinoculated with medium only and later treated identically to the infected samples.

J89/J-Lat cell reactivation. J89 and J-Lat cells were prepared at a final concentration of 1×10^6 cells/ml in RPMI medium supplemented with 10% FBS, and 2.5 ml of cells was seeded per well of a 6-well plate. At 1 h 30 min, 3 h, 6 h, 12 h, 18 h, 24 h, 36 h, and 48 h, cells were stimulated with hTNF-α or RMD at final concentrations of 10 ng/ml and 5 nM, respectively.

PrimeFlow RNA assay. The PrimeFlow RNA assay (Affymetrix Inc.) was performed according to the manufacturer's recommendations, including minor modifications, and used the branched-DNA technology to amplify the signal from the respective mRNA of interest. Cells of interest were centrifuged, and the respective supernatant was cryopreserved for subsequent analysis of virus production. As a modification from the original manufacturer's protocol, incubation of the PBMC samples with the RNA probes was done for 3 h instead of 2 h at 40°C, and subsequent incubations with Pre-Amp, Amp, and the label probes were done for 2 h instead of 1 h 30 min, as longer incubations increased the mRNA signal. Finally, cells were washed twice, resuspended in 200 μl of storage buffer, and analyzed by using a BD LSRFortessa instrument. A p24 Gag enzyme-linked immunosorbent assay (ELISA) to quantify HIV-1 p24 antigen levels in the cell culture supernatants was performed according to the manufacturer's recommendations (GeneScreen HIV-1 Ag assay; Bio-Rad). The cytotoxicity of RMD (5 nM) and hTNF-α (10 ng/ml) was assessed in Jurkat cells at 48 h and 36 h poststimulation. Staining for live/dead cells was performed by using ZombieAqua (BioLegend, Germany) and showed no toxicity for hTNF-α and minimal cytotoxicity for RMD (<7%).

Real-time PCR. In addition, HIV-1 cellular mRNA was quantified via real-time PCR. Briefly, HIV-1 mRNA was extracted from J89 cells by using the High Pure RNA isolation kit (Roche Diagnostics, Germany). HIV RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels were analyzed by using the LightCycler480 II system (Roche, Mannheim, Germany). For HIV-1 RNA detection, the Artus HI Virus-1 Rotor-Gene reverse transcription-PCR (RT-PCR) kit (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendations, with minor modifications. GAPDH expression was analyzed by using QuantiFast one-step RT-PCR for detection of viral RNA and an internal control (QuantiFast pathogen RT-PCR +IC kit; Qiagen) and the primer-and-probe set for GAPDH (TaqMan Gene Expression assay Hs99999905_m1; Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations, using 5 μl of a 1:1,000 dilution of "dilution buffer" eluant (Qiagen, Hilden, Germany). In parallel, the same samples

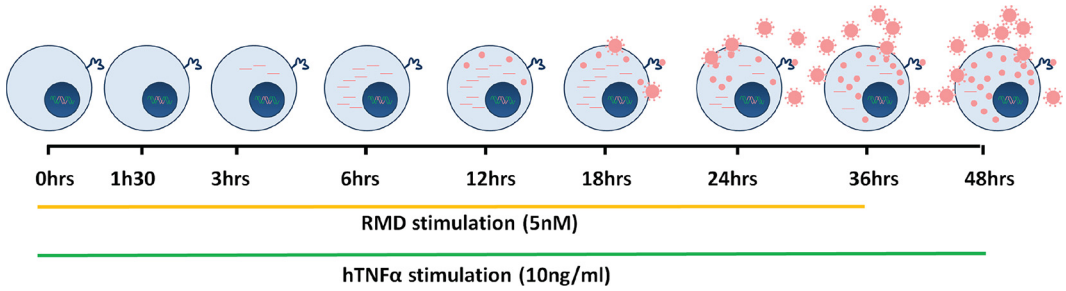


FIG 1 Outline of reactivation assay to determine HIV-1 latency reversal kinetics. J89 cells were stimulated either with hTNF-α (10 ng/ml) at time points ranging from 1 h 30 min to 48 h or with RMD (5 nM) at time points ranging from 1 h 30 min to 36 h. As a control, unstimulated cells were used. The expression levels of viral mRNA, viral protein, and cell surface markers were quantified by using the PrimeFlow RNA assay.

were processed, and a PrimeFlow assay was performed to correlate both measurements.

Blocking of reinfection of J89 cells. In order to test whether the single p24 protein-positive population observed by using the PrimeFlow RNA assay on reactivated J89 cells was due to *de novo* infection of J89 cells, blocking-of-reinfection experiments were performed. At the same time point at which J89 cells were reactivated with hTNF-α (10 ng/ml), AMD3100, a drug that inhibits the CXCR4-viral protein interaction and therefore blocks HIV-1 entry (31), was added to the cell culture at two different final concentrations: 20 μg/ml and 100 μg/ml. Subsequently, a PrimeFlow RNA assay was performed as described above.

H3K9 acetylation status. J89 cells were stimulated for 24 h, and a PrimeFlow RNA assay was performed as described above. For Western blot analysis, viral mRNA/p24 protein-double-negative and -double-positive J89 cells were sorted on a FACS-Fusion instrument. Proteins were extracted by using cell extraction buffer (Life Technologies, Germany). The samples were loaded onto an SDS-PAGE gel (4 to 12% gel) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% low-fat dried milk in Tris-buffered saline for 1 h and incubated overnight with primary antibodies, including anti-histone H3 (acetyl K9) antibody (Abcam, Germany) and histone 3 primary antibody (Cell Signaling Technologies, Germany). The corresponding secondary antibody was added, and the signal was developed by using the SuperSignal West Femto Maximum Sensitivity substrate (Life Technologies, Germany).

Analysis and statistics. All flow cytometry data were analyzed with FlowJo software (v10.1), and statistical analyses were performed by using GraphPad Prism (v5).

RESULTS

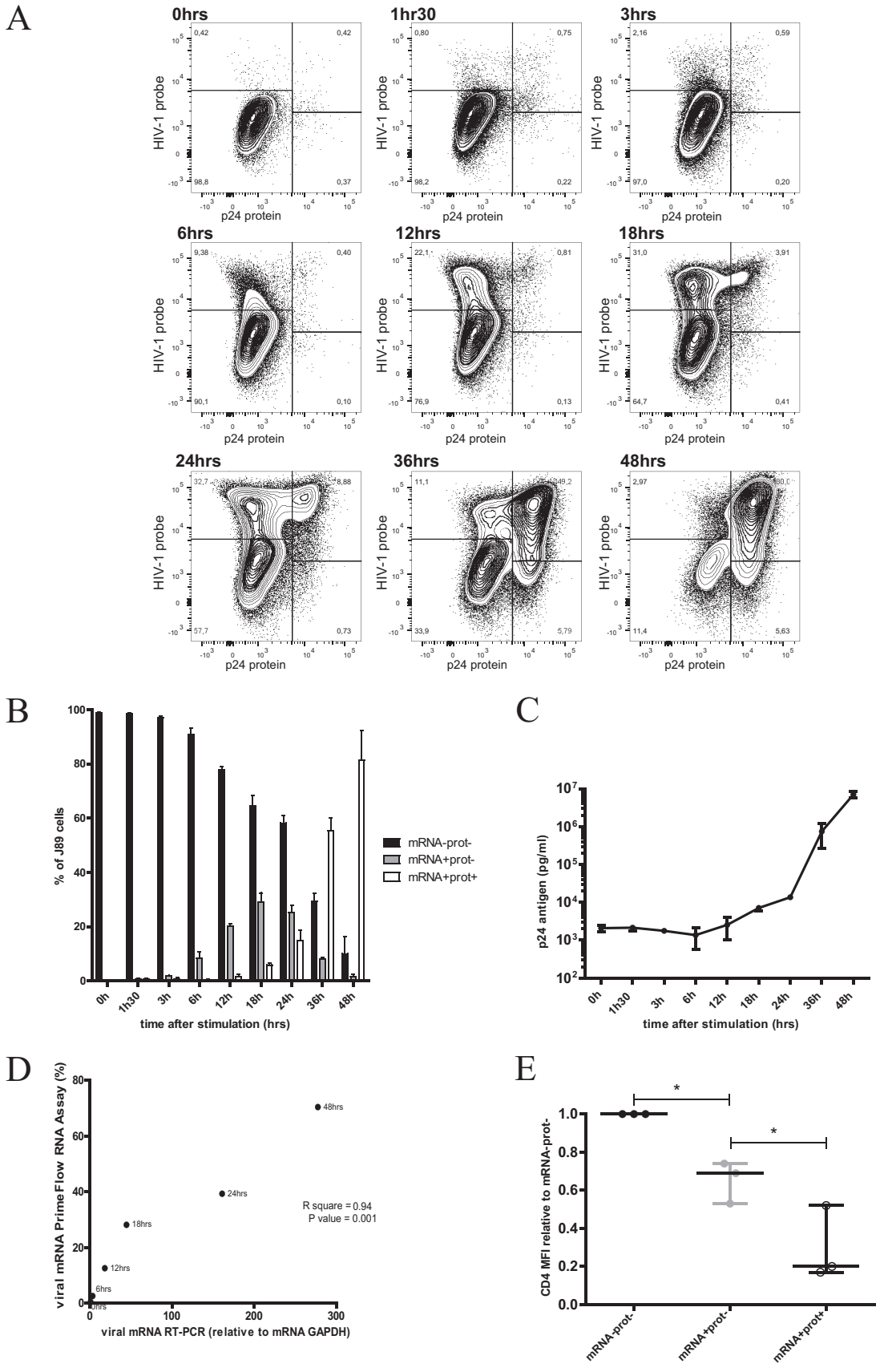
Simultaneous quantification of viral mRNA and proteins following HIV-1 latency reversal. In order to quantify viral reactivation of latently HIV-1-infected cells, we initially used the J89 cell line containing a single, integrated, transcriptionally silenced copy of the full HIV-1 genome (27). The integrated provirus contains the gene for enhanced green fluorescent protein (EGFP) cloned downstream of *nef* that can be used as a marker for HIV-1 expression upon stimulation of the LTR. The J89 cell line was selectively subcloned from a pool of HIV-1 89ENG-infected Jurkat cells to identify a cell line with a low basal level of EGFP expression (27).

In order to visualize different stages of HIV-1 reactivation from mRNA expression to virus production, viral replication in J89 cells was reactivated with two different stimuli, hTNF-α and the HDACi RMD. J89 cells were stimulated over time periods ranging from 1 h 30 min to 36 h (for RMD) and from 1 h 30 min to 48 h (for hTNF-α) (Fig. 1). Using a flow cytometry-based assay allowing combined staining of intracellular mRNAs and proteins (PrimeFlow RNA assay), HIV-1 p24 *gagpol* mRNA, intracellular p24 Gag protein, and cell surface protein levels were simultaneously quantified on the single-cell level. The mRNA of interest was targeted by a set of probes against *gag-pol* areas and was stained by using Alexa Fluor 750. Unstimulated cells (0 h) were used as a control for HIV-1 baseline replication in J89 cells.

Following 3 h of stimulation of J89 cells with 10 ng/ml hTNF-α, a small population of *gagpol* mRNA-single-positive cells (1.7%) was detectable by flow cytometry, which increased over time to up to 27.4% at 18 h. At 18 h, the viral mRNA-positive ($mRNA^+$) cell population shifted toward a population of cells that was simultaneously expressing *gagpol* mRNA and p24 Gag protein (5.2%) (Fig. 2A and B). This viral mRNA/protein-double-positive population further expanded over time. At the last time point, at 48 h poststimulation, the percentage of viral mRNA-single-positive cells decreased to <3% within the J89 population, indicating that the first stages of HIV-1 reactivation were completed. The viral mRNA/protein-double-negative population representing J89 cells that did not reactivate HIV-1 in response to hTNF-α was on average <15% of the total population at that time point (Fig. 2A and B).

Compared to hTNF-α treatment, stimulation of J89 cells with RMD resulted in a slow induction of HIV-1 mRNA production, and at 12 h poststimulation, only 4.4% of J89 cells were positive for *gagpol* mRNA (Fig. 3A and B). The proportion of viral mRNA-single-positive cells quickly increased subsequently to 25.2% at 18 h poststimulation. At this time point, as also observed for hTNF-α stimulation, the viral mRNA/protein-double-positive cell population became apparent (4.9%) (Fig. 3A and B). RMD induction of HIV-1 replication in J89 cells reached its peak at 36 h poststimulation, with 50% viral mRNA/protein-double-positive J89 cells; however, even at this time, ~40% of J89 cells did not reactivate HIV-1 (Fig. 3B). RMD stimulation resulted in significant cell toxicity at later time points, not allowing an assessment at later time points following stimulation (data not shown). RMD has been shown to affect the acetylation status of H3K9 in treated cells (32, 33). Indeed, RMD treatment resulted in acetylated H3K9 (H3K9ac) upregulation at 24 h poststimulation in double-negative cells compared to unstimulated J89 cells, which was increased in cells that reactivated HIV-1 (Fig. 4). These results suggested that RMD affected the H3K9 acetylation status of J89 cells and, to a greater extent, in cells that were producing HIV-1.

Both RMD and hTNF-α stimulation of J89 cells also resulted in the production of novel HIV-1 particles detectable in the cell culture supernatant by a p24 Gag ELISA. The amount of p24 Gag in the supernatant increased exponentially after 18 h of stimulation


A
0hrs
1hr30
3hrs
6hrs
12hrs
18hrs
24hrs
36hrs
48hrs
HIV-1 probe
p24 protein
B
% of J89 cells
time after stimulation (hrs)
mRNA-prot-
mRNA+prot-
mRNA+prot+
C
p24 antigen (pg/ml)
time after stimulation (hrs)
D
viral mRNA PrimeFlow RNA Assay (%)
viral mRNA RT-PCR (relative to mRNA GAPDH)
R square = 0.94
P value = 0.001
E
CD4 MFI relative to mRNA-prot-

until the experiment endpoint at 48 h (Fig. 2C and 3C), matching the presence of p24 Gag-positive cells observed with the PrimeFlow RNA assay and suggesting a fast release of virus. Furthermore, the amount of cellular viral mRNA quantified by real-time PCR strongly correlated with the amount of viral cellular mRNA measurable by PrimeFlow RNA assay (R^2 of 0.94 and P value of <0.005 for hTNF-α; R^2 of 0.99 and P value of <0.001 for RMD), demonstrating that both techniques were comparable in the measurement of viral mRNA levels (Fig. 2D and 3D).

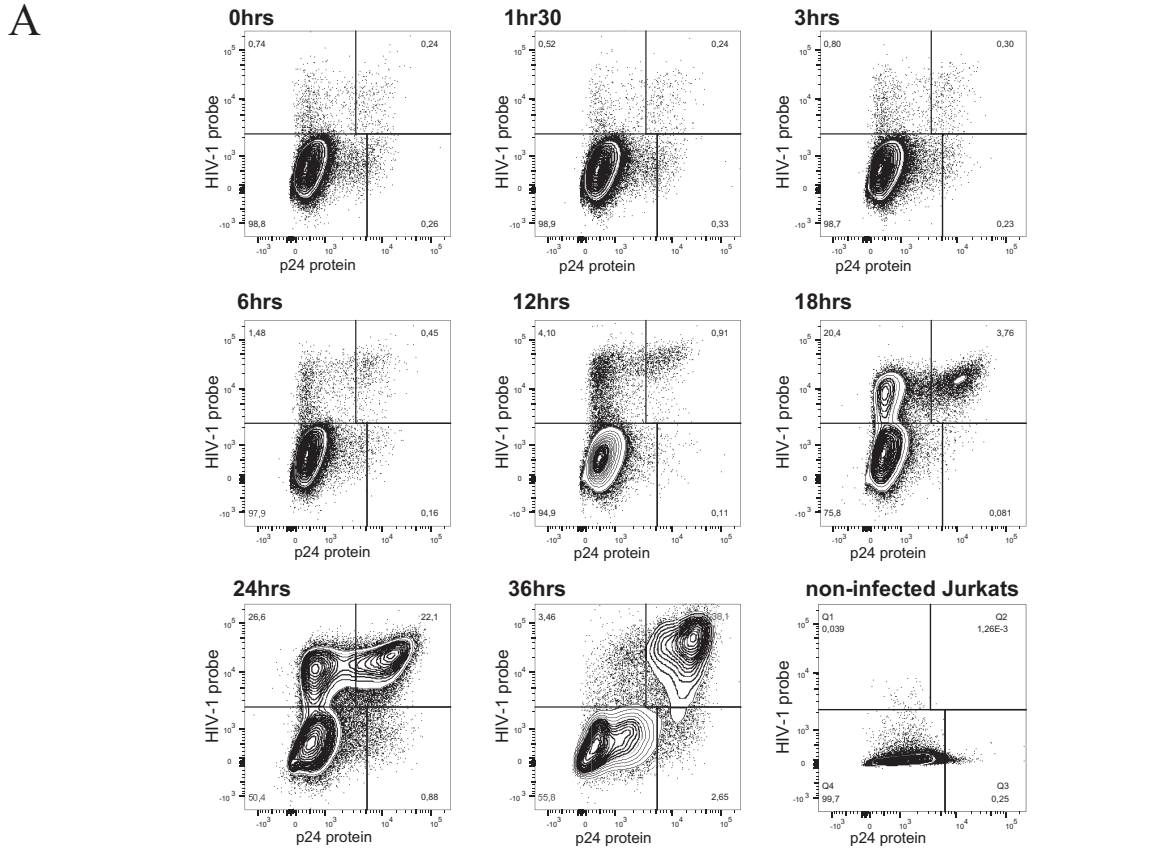
Of note, a fourth population of J89 cells expressing only p24 Gag protein was detectable at late stages (starting at 24 h) following reactivation with hTNF-α and RMD (Fig. 2A and 3A). To test whether the appearance of this fourth population that was positive only for HIV-1 p24 Gag protein was due to newly incoming p24 Gag resulting from reinfection of J89 cells that did not reactivate viral replication, or, rather, late stages of HIV-1 production without detectable viral mRNA, we performed a reinfection-blocking experiment. AMD3100, a CXCR4 antagonist that competes with HIV-1 for infection (31), was added to J89 cells simultaneously with hTNF-α stimulation during 24 h, and a PrimeFlow RNA assay was performed. The addition of AMD3100 did not reduce the proportion of the viral protein-single-positive population (Fig. 5A), suggesting that this population was not the result of incoming p24 Gag protein due to *de novo* infection. To confirm this result, a second set of experiments was performed by using J-Lat cells. J-Lat cells are Jurkat cells infected with a full-length HIV-1 genome bearing a frameshift in *env* and therefore producing incomplete viral particles that cannot infect new cells (28). These cells were stimulated for 24 h and 48 h as described above for J89 cells. As shown in Fig. 5B, stimulation with hTNF-α or RMD induced an accumulation of p24 Gag protein-single-positive J-Lat cells, further demonstrating that this population was not due to reinfection but rather was the result of late stages of viral reactivation when viral RNA had already disappeared.

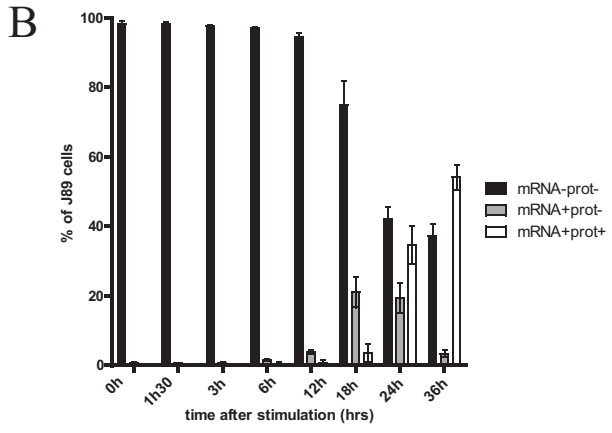
Assessment of the impact of HIV-1 latency reversal on cell surface marker expression. As the PrimeFlow RNA assay also allows the detection of cell surface markers, CD4 expression levels on J89 cells following HIV-1 reactivation using RMD and hTNF-α were measured. Twenty-four hours after stimulation with hTNF-α, CD4 expression was significantly downmodulated in the viral mRNA/protein-double-positive cell population compared to the double-negative population, as previously described ($P < 0.05$) (27) (Fig. 2E). Of note, this downmodulation was already quantifiable and significant in the *gagpol* mRNA single-positive population, as reflected by a 30% decrease of the CD4 mean fluorescence intensity (MFI) ($P < 0.05$) (Fig. 2E). RMD treatment, similarly to hTNF-α treatment, also led to a statistically significant decrease of CD4 expression levels on the cell surface of the single-positive *gagpol* mRNA population compared to nonactivated cells ($P < 0.005$), and this decrease was even more pronounced in the double-positive viral mRNA/protein population ($P < 0.05$) (Fig. 3E). Taken together, the PrimeFlow RNA assay enabled the simultaneous assessment of viral reactivation kinetics and cell surface expression in latently HIV-1-infected J89 cells on the single-cell level.

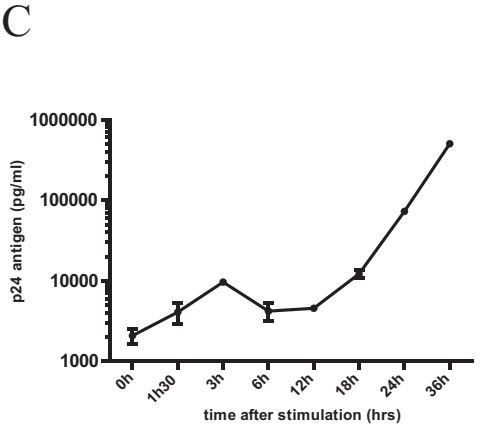
Detection of HIV-1 mRNA and protein production on the single-cell level in primary CD4$^+$ T cells following *in vitro* infection. To determine whether the PrimeFlow RNA assay also allowed the detection of HIV-1 infection in primary cells, CD4$^+$ T cells isolated from three HIV-1-negative individuals were activated with anti-CD3/28 beads and IL-2 over 3 days and infected by using an NL4-3 virus. To detect viral mRNA and/or viral protein, the PrimeFlow RNA assay was performed at 24 h, 48 h, and 72 h postinfection. As shown in Fig. 6A, viral *gagpol* mRNA and p24 Gag protein were detectable in *in vitro*-infected cells by as early as 24 h postinfection, indicating that HIV-1 infection can be traced in primary cells by using this method. In line with data from previous studies (34, 35), HIV-1$^+$ populations within CD4$^+$ T cells exhibited reduced expression levels of CD4, BST-2, and HLA class I molecules (Fig. 6B and C). In all donors analyzed, the effect was already present at an early stage of viral replication (single-positive *gagpol* mRNA$^+$ populations) and increased in viral mRNA/protein-double-positive populations (Fig. 6A). The analysis of CD4 expression levels at the surface of infected cells allowed the differentiation of three different populations of CD4$^+$ T cells (CD4^{neg}, CD4^{dim}, and CD4^{bright}) at day 3 postinfection, as depicted in Fig. 6B. As expected, the amount of CD4 surface expression declined as viral mRNA/protein production increased, and HIV-1 replication (measured by viral mRNA and protein production) was taking place almost exclusively within the CD4^{neg} population (Fig. 6C and D). In contrast, no viral RNA or protein was detectable in the CD4^{bright} population, and viral mRNA and protein expression patterns differed significantly between CD4^{neg}, CD4^{dim}, and CD4^{bright} T-cell populations (Fig. 6C and D). Similarly, assessment of HLA class I and BST-2 expression levels on the surface of HIV-1-infected CD4$^+$ T cells also revealed the presence of distinct populations (Fig. 6B and C). This resulted in statistically different viral mRNA/protein expression levels between the different populations based on either HLA class I expression or BST-2 expression (Fig. 6D).

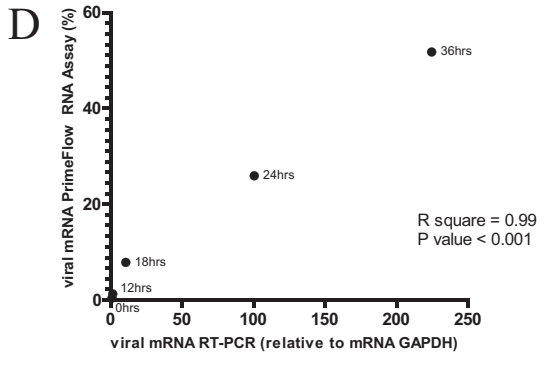
Taken together, these data demonstrate that the use of the PrimeFlow RNA assay enables the evaluation of HIV-1 mRNA and protein production on the single-cell level in primary CD4$^+$ T cells infected with HIV-1 and the differentiation of surface protein expression between cells expressing HIV-1 proteins and cells expressing only HIV-1 mRNA.

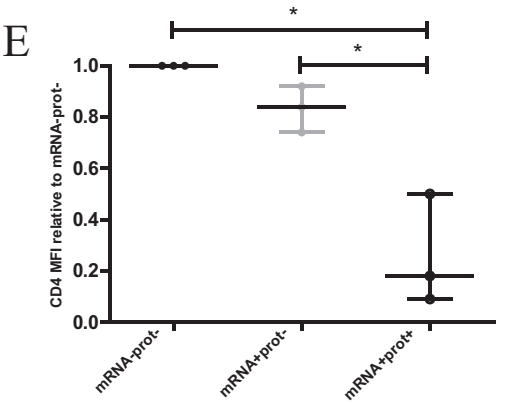
FIG 2 hTNF-α stimulation of J89 cells. J89 cells were activated with hTNF-α (10 ng/ml) at time points ranging from 1 h 30 min to 48 h. A PrimeFlow RNA assay was performed at the indicated time points following stimulation. (A) Representative data showing the kinetics of the viral mRNA-single-positive population, the viral mRNA/protein-double-positive population, and the viral protein-single-positive population. (B) Data from three independent replicates show that the double-negative population decreased over stimulation time, while at 3 h, the viral mRNA single-positive population started to be detectable, reaching its peak at 18 h poststimulation and decreasing to almost undetectable levels by 48 h poststimulation. The viral mRNA/protein-double-positive population was first detectable at 12 h poststimulation and increased up to maximum levels of 80% (as a median) at 48 h poststimulation. (C) Measurement of p24 antigen levels in stimulated J89 cell supernatants with a p24 ELISA (Bio-Rad). Unstimulated cells were used as a background control (0 h). Experiments were performed in triplicates, and the horizontal bars are representative of medians ± standard deviations. (D) Parallel measurement of viral mRNA levels by real-time PCR and a PrimeFlow RNA assay on stimulated J89 cells at the indicated time points showed a statistically significant correlation ($R^2 = 0.94$; $P < 0.005$). (E) CD4 expression in J89 cells was measured at 24 h poststimulation ($n = 3$), after hTNF-α treatment (10 ng/ml). Data evaluated with a Wilcoxon signed-rank test indicated that CD4 levels were already downregulated at the viral mRNA$^+$ stage ($P < 0.05$), and CD4 downregulation increased with increasing viral protein production ($P < 0.05$).


A
0hrs
1hr30
3hrs
6hrs
12hrs
18hrs
24hrs
36hrs
non-infected Jurkats
HIV-1 probe
p24 protein
0,74
0,24
98,8
0,26
0,52
0,24
98,9
0,33
0,80
0,30
98,7
0,23
1,48
0,45
97,9
0,16
4,10
0,91
94,9
0,11
20,4
3,76
75,8
0,081
26,6
22,1
50,4
0,88
3,46
55,8
2,65
Q1
0,039
Q2
1,26E-3
Q4
99,7
Q3
0,25


B
% of J89 cells
time after stimulation (hrs)
0h
1h30
3h
6h
12h
18h
24h
36h
mRNA-prot-
mRNA+prot-
mRNA+prot+


C
p24 antigen (pg/ml)
time after stimulation (hrs)
1000000
100000
10000
1000
0h
1h30
3h
6h
12h
18h
24h
36h


D
viral mRNA PrimeFlow RNA Assay (%)
viral mRNA RT-PCR (relative to mRNA GAPDH)
36hrs
24hrs
18hrs
12hrs
0hrs
R square = 0.99
P value < 0.001


E
CD4 MFI relative to mRNA-prot-
mRNA-prot-
mRNA+prot-
mRNA+prot+
*
*

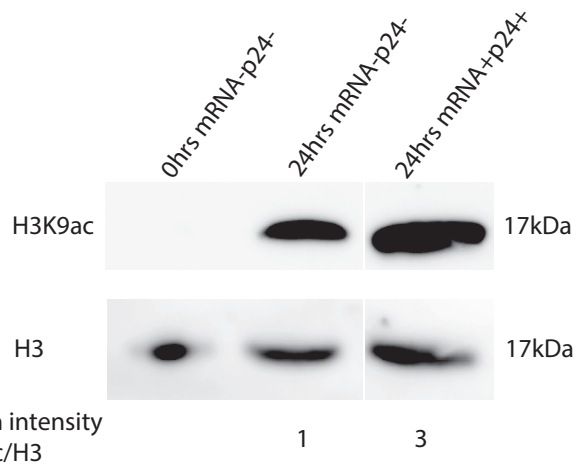


FIG 4 Western blot analysis of H3K9 acetylation status upon RMD treatment. J89 cells were stimulated for 24 h with RMD (5 nM). Viral mRNA/protein-double-negative and -double-positive populations were sorted, total protein was extracted, and Western blotting targeting H3K9ac and total H3 was performed. Quantification was performed by using ImageQuant TL software.

DISCUSSION

Current treatment approaches cannot eradicate HIV-1 infection, and a persistent pool of latently HIV-1-infected cells provides a permanent source of viremia, representing a major obstacle to HIV-1 cure attempts. Here we describe a novel approach based on fluorescence *in situ* hybridization to quantify HIV-1 reactivation kinetics on the single-cell level by flow cytometry, enabling the identification of different cell populations according to their expression of viral mRNA and viral proteins and characterization of their surface molecule expression depending on the stage of HIV-1 reactivation. By using an HIV-1 latency cell line model, faster kinetics of HIV-1 reactivation were observed for hTNF-α than for RMD, and hTNF-α also reactivated a larger subset of the latently infected J89 cells. Fluorescence *in situ* hybridization by flow cytometry furthermore enabled the simultaneous characterization of surface marker expression on latently infected cells depending on viral reactivation kinetics and showed downregulation of CD4 expression levels. Moreover, by using a *de novo* infection model of HIV-1 in primary $CD4^+$ T cells, CD4, BST-2, and HLA class I expressions in relation to viral replication on the single-cell level were visualized, demonstrating early and rapid downregulation of these surface molecules.

In recent years, new techniques have focused on quantifying HIV-1 reservoirs and characterizing virus-producing cells (21–23). Detailed studies of viral reactivation kinetics are essential for understanding both the mechanisms of viral suppression by antiviral drugs and the mechanisms leading to HIV-1 reactivation in latently infected cells (36). The major strength of the fluorescence *in situ* hybridization assay (PrimeFlow RNA assay) used here is the ability to simultaneously and specifically quantify intracellular viral mRNA and protein expression levels as well as the expression of cell surface molecules by flow cytometry on the single-cell level. This approach allowed the distinction of cells expressing only viral mRNA from cells producing viral proteins following HIV-1 latency reversal and the consequences for cell surface molecule expression. Similarly, by using an *in vitro* model in which primary $CD4^+$ T cells from healthy donors were infected with HIV-1, fluorescence *in situ* hybridization enabled the distinction and further characterization of viral mRNA- and protein-expressing cell populations.

By analyzing the HIV-1 reactivation kinetics in J89 cells, we observed that hTNF-α acted faster than RMD in inducing HIV-1 mRNA expression. Moreover, and in line with previously reported results, RMD exhibited higher toxicity than hTNF-α (37). Interestingly, hTNF-α induced HIV-1 latency reactivation in J89 cells more efficiently than did RMD. The distinct mechanisms of action between hTNF-α and RMD might account for the differences observed in the kinetics and extent of HIV-1 latency reactivation in J89 cells. hTNF-α acts by triggering intracellular signals that stabilize the NF-κB transcription factor, allowing nucleus translocation. Once in the nucleus, NF-κB can bind to the HIV-1 LTR region and promote HIV-1 transcription (38, 39). In contrast, the RMD mechanism of action is focused on inhibiting HDACs that repress the HIV-1 LTR and help to initiate viral transcription (15). The ability to measure and quantify HIV-1 reactivation kinetics on the single-cell level will be critical not only for the evaluation of latency-reversing drugs but also for understanding the mechanisms by which these drugs act.

Fluorescence *in situ* hybridization by flow cytometry provided the ability to quantify the levels of viral mRNA and protein production simultaneously with cell surface marker expression on latency cell models and infected primary cells. During HIV-1 infection, CD4, the main entry receptor for HIV-1, has been shown to be downregulated by the viral Nef, Vpu, and gp120 envelope proteins (40, 41). CD4 downregulation enhances viral replication, potentially by protecting virus-producing cells from cytotoxicity related to superinfection events (39). As described previously by Kutsch et al. (27), treatment of J89 cells with hTNF-α led to the downregulation of the CD4 receptor at the cell surface at the stage of viral protein production. Furthermore, hTNF-α and RMD treatment already induced CD4 downmodulation in the *gagpol* mRNA-single-positive population before the detection of p24 Gag

FIG 3 RMD stimulation of J89 cells. J89 cells were activated with RMD (5 nM) at time points ranging from 1 h 30 min to 36 h. A PrimeFlow RNA assay was performed at the indicated time points following stimulation. (A) Representative data showing the kinetics of the viral mRNA-single-positive population, the viral mRNA/protein-double-positive population, and the viral protein-single-positive population. (B) Analysis of viral mRNA and viral protein production over time in J89 cells stimulated with RMD (5 nM) in three independent replicates. The double-negative population decreased over time with stimulation, while at 6 h, the single-positive viral mRNA population already started to be detectable, reaching its peak at 18 h to 24 h poststimulation and decreasing by 36 h poststimulation. The viral mRNA/protein-double-positive population was first measurable at 12 h poststimulation and increased up to maximum levels of 56% (as a mean) at 36 h poststimulation. Uninfected Jurkat cells were included as a control. (C) Measurement of p24 antigen levels in supernatants of stimulated J89 cells with a p24 ELISA (Bio-Rad). Unstimulated cells were used as a background control (0 h). Experiments were performed in triplicates, and the horizontal bars are representative of medians ± standard deviations. (D) Parallel measurement of viral mRNA by real-time PCR and a PrimeFlow RNA assay on stimulated J89 cells at the indicated time points showed a statistically significant correlation ($R^2 = 0.99$; $P < 0.001$). (E) CD4 expression in J89 cells was measured at 24 h poststimulation ($n = 3$), after RMD treatment (5 nM). Data evaluated with a Wilcoxon signed-rank test indicated that CD4 levels were already downregulated at the viral $mRNA^+$ stage ($P < 0.005$), and CD4 downregulation increased with increasing viral protein production ($P < 0.05$).

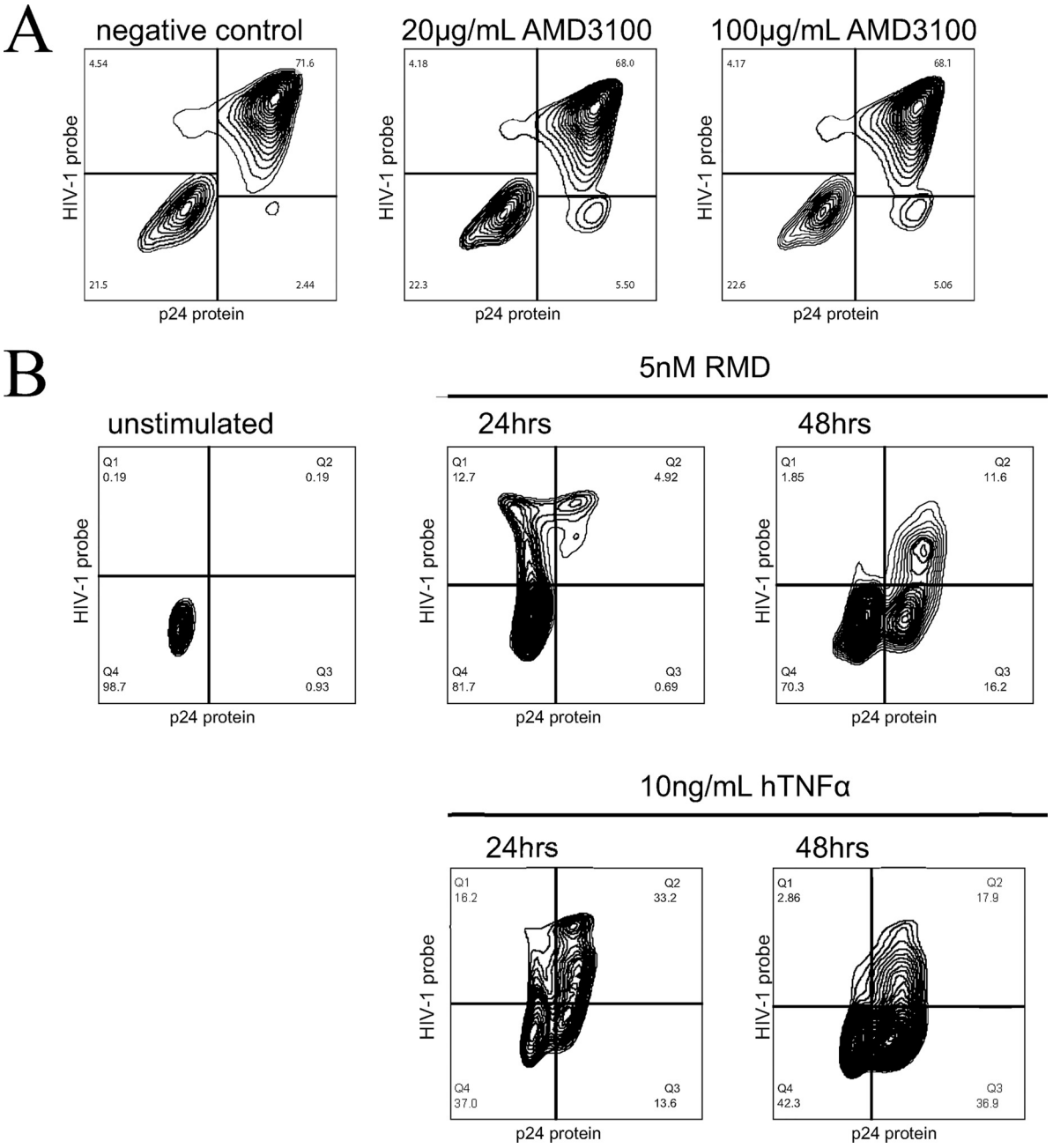


FIG 5 The single-positive protein population is not the result of new rounds of HIV-1 infection. (A) hTNF-α was used at a final concentration of 10 ng/ml to stimulate J89 cells for 24 h simultaneously with 20 μg/ml AMD3100 or 100 μg/ml AMD3100 or without AMD3100 (negative control). A PrimeFlow RNA assay was performed to distinguish the double-negative population, the viral mRNA-single-positive population, the viral mRNA/protein-double-positive population, and the protein-single-positive population. No blocking of the p24 protein-single-positive population was observed in the presence of the CXCR4 inhibitor AMD3100. (B) J-Lat latency reactivation. RMD at a final concentration of 5 nM and hTNF-α at a final concentration of 10 ng/ml were used to stimulate 2.5×10^6 J-Lat cells at two time points, 24 h and 48 h. Unstimulated cells were used as a control. A PrimeFlow RNA assay was performed as described in the text to distinguish four populations: the double-negative population, the viral mRNA-single-positive population, the viral mRNA/protein-double-positive population, and the protein-single-positive population.

protein, in line with the early production of Nef protein. Similarly, *in vitro* HIV-1 infection of primary $CD4^+$ T cells resulted in the downregulation of CD4, as well as BST-2 and HLA class I molecules, from the cell surface as soon as *gagpol* mRNA was detectable in infected cells. Overall, the combined quantification of levels of intracellular viral mRNA and proteins with surface markers allowed the assessment of the kinetics of changes in cell surface markers in relation to viral replication on the single-cell level.

Further studies will need to investigate whether fluorescence *in situ* hybridization by flow cytometry can be used to detect viral mRNA in primary samples from HIV-1-infected individuals and therefore measure latent reservoirs in different cell subsets after

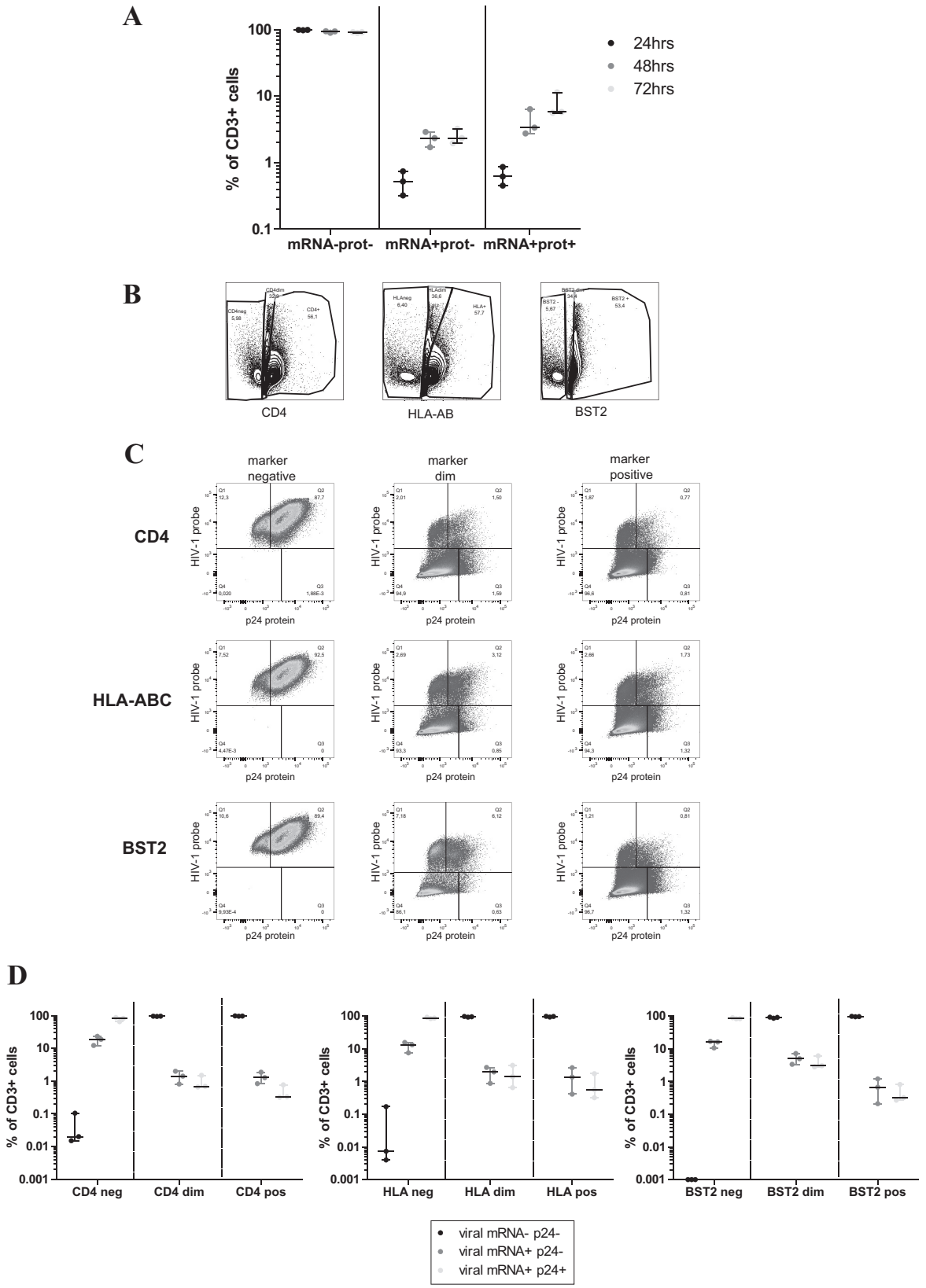


FIG 6 Cell surface levels of BST-2, HLA class I, and CD4 in the mRNA-negative and protein-negative, the mRNA$^+$ protein-negative, and mRNA$^+$ protein-positive CD4$^+$ T-cell populations. CD4$^+$ T cells isolated from three healthy donors were activated for 3 days with anti-CD3/28 beads and IL-2 (100 U/ml) and then infected at an MOI of 0.02 with NL4-3 virus. (A) Fluorescence *in situ* hybridization was performed at 24 h, 48 h, and 72 h postinfection to quantify levels of viral mRNA and viral protein production (B) The expression levels of BST-2, HLA-ABC, and CD4 at 72 h post-HIV-1 infection showed the presence of three populations for each marker: negative, dim, and positive. (C) Representative plot from one donor displaying the expression levels of viral mRNA and protein in the negative, dim, and positive populations for HLA-ABC, CD4, and BST-2 at 72 h postinfection. HIV-1 replication was almost exclusively restricted to the negative populations. (D) Viral mRNA and protein expression from three independent donors at 72 h postinfection according to the above-described gating strategy (shown in Fig. 6B). All the differences calculated with a Wilcoxon signed-rank test were statistically significant ($P < 0.001$).

reactivation. Our data indicated that the current technique will not be sufficiently sensitive to detect the frequency of HIV-1-infected cells in primary samples from HIV-1-infected individuals on highly active ART (HAART), in whom the frequency of infected cells is much lower. However, the PrimeFlow RNA assay allows tracing of the kinetics of HIV-1 replication using cell models and primary HIV-1-infected $CD4^+$ T cells and can be used as a tool to characterize the cell surface modifications occurring during HIV-1 replication. One limitation of the PrimeFlow RNA assay is that viral mRNA is measured by a combination of primers targeting the *gagpol* mRNA, and distinguishing between spliced and unspliced viral mRNA is therefore not possible. To detect spliced viral mRNA, probes would need to be designed to specifically bind to the short Tat-Rev junction, such as the ones used for TILDAs (22). However, the use of only two sets of primers for the PrimeFlow RNA assay is currently not yet specific enough to detect mRNA populations and therefore limits the technique to the detection of unspliced HIV-1 RNA at this point. In conclusion, simultaneous quantification of viral reactivation and replication dynamics and their effects on cell surface markers on the single-cell level is possible by using fluorescence *in situ* hybridization by flow cytometry and represents an important novel tool to study HIV-1 latency reversal kinetics and drug efficiency.

ACKNOWLEDGMENTS

J89 cells were kindly provided by David N. Levy (College of Dentistry, New York University, New York, NY). We acknowledge the Healthy Cohort Hansestadt Hamburg (HCHH) from the UKE for their contribution to this study.

G.M. is supported by a GILEAD Förderprogramm Infektiologie grant. M.A. receives funding from the Deutsche Forschungsgemeinschaft (DFG), the German Center for Infection Research (DZIF), and the Leibniz Gemeinschaft.

We have no conflicts of interest.

FUNDING INFORMATION

This work, including the efforts of G. Martrus, was funded by Gilead Förderprogramm Infektiologie. This work, including the efforts of M. Altfeld, was funded by Leibniz Gemeinschaft. This work, including the efforts of M. Altfeld, was funded by Deutsche Forschungsgemeinschaft (DFG). This work, including the efforts of M. Altfeld, was funded by Deutsches Zentrum für Infektionsforschung (DZIF).

REFERENCES

1. **Deeks SG, Autran B, Berkhout B, Benkirane M, Cairns S, Chomont N, Chun T-W, Churchill M, Di Mascio M, Katlama C, Lafeuillade A, Landay A, Lederman M, Lewin SR, Maldarelli F, Margolis D, Markowitz M, Martinez-Picado J, Mullins JI, Mellors J, Moreno S, O'Doherty U, Palmer S, Penicaud M-C, Peterlin M, Poli G, Routy J-P, Rouzioux C, Silvestri G, Stevenson M, Telenti A, Van Lint C, Verdin E, Woolfrey A, Zaia J, Barré-Sinoussi F.** 2012. Towards an HIV cure: a global scientific strategy. Nat Rev Immunol **12:**607–614. http://dx.doi.org/10.1038/nri3262.
2. **Whitney JB, Hill AL, Sanisetty S, Penaloza-MacMaster P, Liu J, Shetty M, Parenteau L, Cabral C, Shields J, Blackmore S, Smith JY, Brinkman AL, Peter LE, Mathew SI, Smith KM, Borducchi EN, Rosenbloom DIS, Lewis MG, Hattersley J, Li B, Hesselgesser J, Geleziunas R, Robb ML, Kim JH, Michael NL, Barouch DH.** 2014. Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. Nature **512:**74–77. http://dx.doi.org/10.1038/nature13594.
3. **Sigal A, Kim JT, Balazs AB, Dekel E, Mayo A, Milo R, Baltimore D.** 2011. Cell-to-cell spread of HIV permits ongoing replication despite antiretroviral therapy. Nature **477:**95–98. http://dx.doi.org/10.1038/nature10347.
4. **Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, Boucher G, Boulassel M-R, Ghattas G, Brenchley JM, Schacker TW, Hill BJ, Douek DC, Routy J-P, Haddad EK, Sékaly R-P.** 2009. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat Med **15:**893–900. http://dx.doi.org/10.1038/nm.1972.
5. **Bosque A, Famiglietti M, Weyrich AS, Goulston C, Planelles V.** 2011. Homeostatic proliferation fails to efficiently reactivate HIV-1 latently infected central memory $CD4^+$ T cells. PLoS Pathog **7:**e1002288. http://dx.doi.org/10.1371/journal.ppat.1002288.
6. **Pearson R, Kim YK, Hokello J, Lassen K, Friedman J, Tyagi M, Karn J.** 2008. 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 **82:**12291–12303. http://dx.doi.org/10.1128/JVI.01383-08.
7. **Archin NM, Sung JM, Garrido C, Soriano-Sarabia N, Margolis DM.** 2014. Eradicating HIV-1 infection: seeking to clear a persistent pathogen. Nat Rev Microbiol **12:**750–764. http://dx.doi.org/10.1038/nrmicro3352.
8. **Archin NM, Bateson R, Tripathy MK, Crooks AM, Yang K-H, Dahl NP, Kearney MF, Anderson EM, Coffin JM, Strain MC, Richman DD, Robertson KR, Kashuba AD, Bosch RJ, Hazuda DJ, Kuruc JD, Eron JJ, Margolis DM.** 2014. HIV-1 expression within resting $CD4^+$ T cells after multiple doses of vorinostat. J Infect Dis **210:**728–735. http://dx.doi.org/10.1093/infdis/jiu155.
9. **Williams SA, Chen L-F, Kwon H, Ruiz-Jarabo CM, Verdin E, Greene WC.** 2006. NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. EMBO J **25:**139–149. http://dx.doi.org/10.1038/sj.emboj.7600900.
10. **Jiang G, Espeseth A, Hazuda DJ, Margolis DM.** 2007. c-Myc and Sp1 contribute to proviral latency by recruiting histone deacetylase 1 to the human immunodeficiency virus type 1 promoter. J Virol **81:**10914–10923. http://dx.doi.org/10.1128/JVI.01208-07.
11. **Keedy KS, Archin NM, Gates AT, Espeseth A, Hazuda DJ, Margolis DM.** 2009. A limited group of class I histone deacetylases acts to repress human immunodeficiency virus type 1 expression. J Virol **83:**4749–4756. http://dx.doi.org/10.1128/JVI.02585-08.
12. **Spivak AM, Bosque A, Balch AH, Smyth D, Martins L, Planelles V.** 2015. *Ex vivo* bioactivity and HIV-1 latency reversal by ingenol dibenzoate and panobinostat in resting $CD4^+$ T cells from aviremic patients. Antimicrob Agents Chemother **59:**5984–5991. http://dx.doi.org/10.1128/AAC.01077-15.
13. **Wightman F, Lu H, Solomon A, Saleh S, Harman A, Cunningham A, Gray L, Churchill M, Cameron P, Dear A, Lewin SR.** 2013. Entinostat is a histone deacetylase inhibitor selective for class 1 histone deacetylases and activates HIV production from latently infected primary T cells. AIDS **27:**2853–2862. http://dx.doi.org/10.1097/QAD.0000000000000067.
14. **Søgaard OS, Graversen ME, Leth S, Olesen R, Brinkmann CR, Nissen SK, Kjaer AS, Schleimann MH, Denton PW, Hey-Cunningham WJ, Koelsch KK, Pantaleo G, Krogsgaard K, Sommerfelt M, Fromentin R, Chomont N, Rasmussen TA, Østergaard L, Tolstrup M.** 2015. The depsipeptide romidepsin reverses HIV-1 latency in vivo. PLoS Pathog **11:**e1005142. http://dx.doi.org/10.1371/journal.ppat.1005142.
15. **Wei DG, Chiang V, Fyne E, Balakrishnan M, Barnes T, Graupe M, Hesselgesser J, Irrinki A, Murry JP, Stepan G, Stray KM, Tsai A, Yu H, Spindler J, Kearney M, Spina CA, McMahon D, Lalezari J, Sloan D, Mellors J, Geleziunas R, Cihlar T.** 2014. Histone deacetylase inhibitor romidepsin induces HIV expression in CD4 T cells from patients on suppressive antiretroviral therapy at concentrations achieved by clinical dosing. PLoS Pathog **10:**e1004071. http://dx.doi.org/10.1371/journal.ppat.1004071.
16. **Saleh S, Wightman F, Ramanayake S, Alexander M, Kumar N, Khoury G, Pereira C, Purcell D, Cameron PU, Lewin SR.** 2011. Expression and reactivation of HIV in a chemokine induced model of HIV latency in primary resting $CD4^+$ T cells. Retrovirology **8:**80. http://dx.doi.org/10.1186/1742-4690-8-80.
17. **Duh EJ, Maury WJ, Folks TM, Fauci AS, Rabson AB.** 1989. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. Proc Natl Acad Sci U S A **86:**5974–5978. http://dx.doi.org/10.1073/pnas.86.15.5974.
18. **Griffin GE, Leung K, Folks TM, Kunkel S, Nabel GJ.** 1989. Activation of HIV gene expression during monocyte differentiation by induction of NF-kB. Nature **339:**70–73. http://dx.doi.org/10.1038/339070a0.
19. **Okamoto T, Matsuyama T, Mori S, Hamamoto Y, Kobayashi N, Yamamoto N, Josephs SF, Wong-Staal F, Shimotohno K.** 1989. Augmentation of human immunodeficiency virus type 1 gene expression by

tumor necrosis factor alpha. AIDS Res Hum Retroviruses **5:**131–138. http://dx.doi.org/10.1089/aid.1989.5.131.
20. **Cummins NW, Badley AD.** 2010. Mechanisms of HIV-associated lymphocyte apoptosis: 2010. Cell Death Dis **1:**e99. http://dx.doi.org/10.1038/cddis.2010.77.
21. **Henrich TJ, Gallien S, Li JZ, Pereyra F, Kuritzkes DR.** 2012. Low-level detection and quantitation of cellular HIV-1 DNA and 2-LTR circles using droplet digital PCR. J Virol Methods **186:**68–72. http://dx.doi.org/10.1016/j.jviromet.2012.08.019.
22. **Procopio FA, Fromentin R, Kulpa DA, Brehm JH, Bebin A-G, Strain MC, Richman DD, O'Doherty U, Palmer S, Hecht FM, Hoh R, Barnard RJO, Miller MD, Hazuda DJ, Deeks SG, Sékaly R-P, Chomont N.** 2015. A novel assay to measure the magnitude of the inducible viral reservoir in HIV-infected individuals. EBioMedicine **2:**874–883. http://dx.doi.org/10.1016/j.ebiom.2015.06.019.
23. **Chun TW, Carruth L, Finzi D, Shen X, DiGiuseppe JA, Taylor H, Hermankova M, Chadwick K, Margolick J, Quinn TC, Kuo YH, Brookmeyer R, Zeiger MA, Barditch-Crovo P, Siliciano RF.** 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature **387:**183–188. http://dx.doi.org/10.1038/387183a0.
24. **Harada S, Koyanagi Y, Yamamoto N.** 1985. Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. Science **229:**563–566. http://dx.doi.org/10.1126/science.2992081.
25. **Larder BA, Darby G, Richman DD.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonger therapy. Science **243:**1731–1734.
26. **Pauwels R, De Clercq E, Desmyter J, Balzarini J, Goubau P, Herdewijn P, Vanderhaeghe H, Vandeputte M.** 1987. Sensitive and rapid assay on MT-4 cells for detection of antiviral compounds against the AIDS virus. J Virol Methods **16:**171–185. http://dx.doi.org/10.1016/0166-0934(87)90002-4.
27. **Kutsch O, Benveniste EN, Shaw GM, Levy N, Levy DN.** 2002. Direct and quantitative single-cell analysis of human immunodeficiency virus type 1 reactivation from latency. J Virol **76:**8776–8786. http://dx.doi.org/10.1128/JVI.76.17.8776-8786.2002.
28. **Jordan A, Bisgrove D, Verdin E.** 2003. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J **22:**1868–1877. http://dx.doi.org/10.1093/emboj/cdg188.
29. **Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol **59:**284–291.
30. **Pannecouque C, Daelemans D, De Clercq E.** 2008. Tetrazolium-based colorimetric assay for the detection of HIV replication inhibitors: revisited 20 years later. Nat Protoc **3:**427–434. http://dx.doi.org/10.1038/nprot.2007.517.
31. **Hendrix CW, Collier AC, Lederman MM, Schols D, Pollard RB, Brown S, Jackson JB, Coombs RW, Glesby MJ, Flexner CW, Bridger GJ, Badel K, MacFarland RT, Henson GW, Calandra G.** 2004. Safety, pharmacokinetics, and antiviral activity of AMD3100, a selective CXCR4 receptor inhibitor, in HIV-1 infection. J Acquir Immune Defic Syndr **37:**1253–1262. http://dx.doi.org/10.1097/01.qai.0000137371.80695.ef.
32. **Luchenko VL, Litman T, Chakraborty AR, Heffner A, Devor C, Wilkerson J, Stein W, Robey RW, Bangiolo L, Levens D, Bates SE.** 2014. Histone deacetylase inhibitor-mediated cell death is distinct from its global effect on chromatin. Mol Oncol **8:**1379–1392. http://dx.doi.org/10.1016/j.molonc.2014.05.001.
33. **Khromov T, Pantakani DVK, Nolte J, Wolf M, Dressel R, Engel W, Zechner U.** 2011. Global and gene-specific histone modification profiles of mouse multipotent adult germline stem cells. Mol Hum Reprod **17:**166–174. http://dx.doi.org/10.1093/molehr/gaq085.
34. **Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, Strominger JL, Baltimore D.** 1999. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. Immunity **10:**661–671. http://dx.doi.org/10.1016/S1074-7613(00)80065-5.
35. **Neil SJD, Zang T, Bieniasz PD.** 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature **451:**425–430. http://dx.doi.org/10.1038/nature06553.
36. **Petravic J, Ellenberg P, Chan M-L, Paukovics G, Smyth RP, Mak J, Davenport MP.** 2014. Intracellular dynamics of HIV infection. J Virol **88:**1113–1124. http://dx.doi.org/10.1128/JVI.02038-13.
37. **Jønsson KL, Tolstrup M, Vad-Nielsen J, Kjær K, Laustsen A, Andersen MN, Rasmussen TA, Søgaard OS, Østergaard L, Denton PW, Jakobsen MR.** 2015. Histone deacetylase inhibitor romidepsin inhibits de novo HIV-1 infections. Antimicrob Agents Chemother **59:**3984–3994. http://dx.doi.org/10.1128/AAC.00574-15.
38. **Beans EJ, Fournogerakis D, Gauntlett C, Heumann LV, Kramer R, Marsden MD, Murray D, Chun T-W, Zack JA, Wender PA.** 2013. Highly potent, synthetically accessible prostratin analogs induce latent HIV expression in vitro and ex vivo. Proc Natl Acad Sci U S A **110:**11698–11703. http://dx.doi.org/10.1073/pnas.1302634110.
39. **Jiang G, Dandekar S.** 2015. Targeting NF-κB signaling with protein kinase C agonists as an emerging strategy for combating HIV latency. AIDS Res Hum Retroviruses **31:**4–12. http://dx.doi.org/10.1089/aid.2014.0199.
40. **Chen BK, Gandhi RT, Baltimore D.** 1996. CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of vpu, env, and nef. J Virol **70:**6044–6053.
41. **El-Far M, Ancuta P, Routy J-P, Zhang Y, Bakeman W, Bordi R, DaFonseca S, Said EA, Gosselin A, Tep T-S, Eichbaum Q, van Grevenynghe J, Schwartz O, Freeman GJ, Haddad EK, Chomont N, Sekaly R-P.** 2015. Nef promotes evasion of HIV-1 infected cells from the CTLA-4-mediated inhibition of T cell activation. J Gen Virol **96:**1463–1477. http://dx.doi.org/10.1099/vir.0.000065.