An HIV-1 Replication Pathway Utilizing Reverse Transcription Products That Fail To Integrate

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Integration is a central event in the replication of retroviruses, yet $\geq 90\%$ of HIV-1 reverse transcripts fail to integrate, resulting in accumulation of unintegrated viral DNA in cells. However, understanding what role, if any, unintegrated viral DNA plays in the natural history of HIV-1 has remained elusive. Unintegrated HIV-1 DNA is reported to possess a limited capacity for gene expression restricted to early gene products and is considered a replicative dead end. Although the majority of peripheral blood CD4⁺ T cells are refractory to infection, nonactivated CD4 T cells present in lymphoid and mucosal tissues are major targets for infection. Treatment with cytokine interleukin-2 (IL-2), IL-4, IL-7, or IL-15 renders CD4⁺ T cells permissive to HIV-1 infection in the absence of cell activation and proliferation and provides a useful model for infection of resting CD4⁺ T cells. We found that infection of cytokine-treated resting CD4⁺ T cells in the presence of raltegravir or with integrase active-site mutant HIV-1 yielded *de novo* virus production following subsequent T cell activation. Infection with integration-competent HIV-1 naturally generated a population of cells generating virus from unintegrated DNA. Latent infection persisted for several weeks and could be activated to virus production by a combination of a histone deacetylase inhibitor and a protein kinase C activator or by T cell activation. HIV-1 Vpr was essential for unintegrated HIV-1 gene expression and *de novo* virus production in this system. Bypassing integration by this mechanism may allow the preservation of genetic information that otherwise would be lost.

As for all retroviruses, integration of the newly reverse transcribed human immunodeficiency virus type 1 (HIV-1) cDNA genome into the host cell's DNA has been observed to be an essential replicative step, with the integrated provirus being the exclusive template for all *de novo* virus production (1, 2). Integration is mediated by the viral integrase enzyme, which is a product of the *pol* gene and the target of the recently developed and highly successful integrase inhibitor class of antiretrovirals (3). Since the integrated provirus will remain for the life of the infected cell and its descendants, integration is a major factor in HIV-1 persistence (4, 5).

Interestingly, irrespective of the activation status of the infected CD4⁺ T cell, $\geq 90\%$ of HIV-1 reverse transcripts fail to integrate *in vivo* and *in vitro* (6–10). *In vivo*, this inefficiency of integration results in an accumulation of unintegrated HIV-1 DNA (uDNA) in blood CD4⁺ T cells and monocytes (7, 8, 11, 12), as well as in lymphoid tissue (8–10), brain (13, 14), and untreated patients with high or low viral loads (15, 16). During untreated chronic infection, the majority of infected cells have been reported to contain only unintegrated HIV-1 DNA (7). However, it remains unclear whether unintegrated DNA plays any functional role in the natural history of HIV-1 (17, 18).

If the linear cDNA product of reverse transcription fails to integrate, it can be degraded or become circularized. Linear unintegrated HIV-1 DNA, one-long-terminal-repeat (1-LTR) circles, and 2-LTR circles are all capable of limited transcription (17, 19–21), which can enhance T cell activation (22). Intriguingly, it has been reported that similar numbers of integrated and unintegrated HIV-1 templates transcribe RNA (23). It is generally observed that uDNA gene expression is higher in nonproliferating cells than in proliferating cells, perhaps owing to a lack of dilution of uDNA templates and their RNA and protein products (17, 24–27). The virion-associated Vpr protein transactivates uDNA, en-

hancing expression of early viral proteins in infected activated CD4⁺ T cells (28, 29). Vpr has several other activities (30–33), including the ability to arrest cell proliferation (34) in the G₂/M phase of the cell cycle (35, 36), which is favorable to HIV-1 transcription (37, 38), as well as the capacity to enhance HIV-1 replication in nonproliferating cells (33, 39, 40), at least in part through assisting with viral nuclear import (30, 41, 42).

Primate immunodeficiency viruses replicate most efficiently in activated CD4⁺ T cells, which generate the majority of virions *in vivo* (43, 44). Resting CD4⁺ T cells derived from peripheral blood are refractory to productive infection (7, 45–48) but can be rendered permissive to productive infection by common gamma-chain cytokines, including interleukin-2 (IL-2), IL-4, IL-7, and IL-15, without inducing activation or activation-induced proliferation (49–51). During early HIV-1 infection in humans and acute simian immunodeficiency virus (SIV) infection of rhesus macaques, many viral RNA-positive cells lack activation and proliferation markers and thus resemble resting CD4⁺ T cells (52–58). Infected nonactivated, nonproliferating CD4⁺ T cells have been identified in high numbers near the sites of mucosal transmission (53, 57) and in lymphoid tissues (59) and are observed after *ex vivo* infection of lymphoid histocultures (55, 60–63). These findings indicate that local environmental factors,

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such as common gamma-chain cytokines, contribute to virus replication in these cells (55, 57, 60, 64–66). Common gamma-chain cytokines provide a useful and convenient system for studying HIV-1 replication in nonactivated, nonreplicating, permissive T cells.

We have previously examined gene expression in activated primary CD4⁺ T cells and in transformed CD4⁺ T cells coinfecting with integrase-wild-type (Int-WT) and integrase-defective viruses (67). We found that complementation of the integrase mutant virus by the WT virus allowed the mutant to complete its replication cycle (67). In the present study, we examined uDNA gene expression in primary resting CD4⁺ T cells rendered permissive to productive HIV-1 infection by cytokine treatment. We found that when infected cells were subsequently activated, uDNA HIV-1 functioned as a template for *de novo* virus production without the assistance of an integrated helper virus. Vpr was essential for gene expression and virus production in these cells. We also observed that integration-inhibited HIV-1 DNA established a latent reservoir in cytokine-treated resting CD4⁺ T cells from which virus production could be recruited several weeks after infection.

MATERIALS AND METHODS

Viruses. The viruses used are summarized in Fig. S1 in the supplemental material, and most have been described before, including those with mutations in the envelope, integrase, and *vpr* genes (67–69). All reporter viruses were constructed using the HIV-1 NL4-3 backbone (70). Virus names have been shortened from prior publication nomenclature (67, 69) (see Fig. S1 in the supplemental material for the full names). Infectious virions were generated by polyethylenimine (PEI; Sigma) transfection (71) of 293T cells as described previously (67). *env* gene-defective viruses were pseudotyped with the HIV-1 NL4-3 envelope by cotransfection of 293T cells with a plasmid expressing the NL4-3 envelope, as described previously (67, 69). Vpr complementation was achieved by coinfection with a Vpr-positive (Vpr⁺) virus containing an N136Y inactivating mutation in reverse transcriptase (72) (see Fig. 7C and D). Failure to express RNA from this virus *de novo* was documented by flow cytometry and quantitative reverse transcription-PCR (qRT-PCR) for viral RNA (unpublished data). Design of plasmid construction strategies was greatly facilitated by the Apple OS X program DNA Strider (73). When downstream quantitative PCR (qPCR) analysis for HIV-1 DNA was to be performed, virus stocks for infection were filtered through a 0.45- μ m-pore-size filter and then treated with Benzonase (Novagen), as per the manufacturer's instructions, at 25 units/ml for 30 min at 37°C, followed by inactivation at –80°C overnight.

Cells. CD4⁺ T cells were prepared by negative selection from the peripheral blood of healthy donors using a Dynabeads Untouched CD4 magnetic bead separation kit (Invitrogen), as previously described (67). Purified cells were routinely $\geq 99\%$ small, CD4⁺, CD25[–], CD38[–], CD69[–], and HLA-DR[–] quiescent T cells. Blood was purchased from the New York Blood Center or was collected after informed consent of the donors and with approval of the New York University Institutional Review Board. Purified CD4⁺ T cells were cultured at 2×10^6 cells per ml in Gibco Advanced RPMI 1640 with 10% fetal bovine serum (HyClone) plus penicillin and streptomycin (Gibco) and 50 μ M β -mercaptoethanol (Sigma). Where indicated, the following cytokines were added to the culture medium every 2 days: IL-4 (25 ng/ml; R&D Systems), IL-7 (2 ng/ml [see Fig. 3] or as indicated in Fig. 4; R&D Systems), IL-15 (25 ng/ml; BioVision), or IL-2 (50 U/ml; obtained from NIH). Where indicated, cells were activated with Dynabeads Human T-Activator CD3/CD28 (Invitrogen), as per the manufacturer's instructions, for the amount of time indicated below. IL-2 (50 U/ml) was added 24 h after bead stimulation, and beads were magnetically removed on the second day. Where indicated, prostratin (Pro; 330 nM; Santa Cruz Biotechnology) and trichostatin A (TSA; 130 nM; Fisher) were applied 2 days prior to analysis.

Infections. The titers of the virus stocks were determined using TaqMan qRT-PCR for HIV-1 RNA (target in integrase) and normalized to a nominal 400 virion equivalents per cell for infection. Infections were performed by spinoculation (74) in the presence of 5 μ g/ml DEAE dextran (Sigma) for 2 h at $1,200 \times g$ and 37°C. T cells were incubated for 2 additional hours and then treated with trypsin-EDTA and extensively washed to remove residual virions and carry-over plasmid DNA. Control infections performed without spinoculation resulted in lower infection frequencies but qualitatively similar results as spinoculation (unpublished findings), consistent with prior reports (74). Where indicated, reverse transcriptase inhibitors zidovudine (AZT) and efavirenz (1 μ g/ml each) were applied at the time of infection and replenished in culture every 2 days. Where indicated, indinavir sulfate (2 μ M) was applied to cells within 12 h of infection and replenished in culture every 2 days. Where indicated, the integration strand transfer inhibitor (INSTI) raltegravir (RAL; 1 μ M or as indicated below) (75) was applied to the cells at the time of the infection and replenished in culture every 2 days. Unless specified otherwise, raltegravir was present during the entire time of the indicated experiments. All antiretrovirals were obtained from the NIH AIDS Research and Reference Reagent Program.

HIV-1 transmission assay. T cells were infected with the indicated virus in the presence or absence of raltegravir (1 μ M). Seven days after infection, eFluor670-positive (eFluor670⁺) yellow fluorescent protein (YFP)-positive (YFP⁺) T cells were sorted to 100% purity and then cocultured at the ratio indicated below with uninfected IL-4-treated CD4⁺ T cells labeled with CellTrace Violet (CTV; eBioscience). eFluor670 was from Invitrogen. During this mixing phase, the raltegravir was removed to allow integration of viruses infecting target cells. eFluor670-negative, CellTrace violet-positive target cells were gated on in order to measure YFP expression in postacquisition analysis. The coculture was performed in the presence of anti-CD3/CD28 activation beads (Invitrogen) plus IL-2 over 3 days, with or without the indicated antiretroviral controls. Indinavir (2 μ M) and T-20 (10 μ g/ml) were added after sorting to a subset of cells to block superinfection prior to DNA PCR analysis.

Flow cytometry. Flow cytometry was performed on a Becton, Dickinson FACSsort flow cytometer upgraded by Cytek Development, Fremont, CA, to contain 488-nm, 407-nm, and 637-nm lasers and 5 fluorescence detectors. Green fluorescent protein (GFP) and YFP were detected in FL-1 and FL-2 using 510/21-nm and 550/30-nm filters, respectively, and a 540-nm short-pass dichroic splitter. Cyan fluorescent protein (CFP) was detected using a 575/15-nm filter off the 407-nm laser. Compensation was applied during data collection on the basis of single-color controls. Data collection was with CellQuest Pro software for Mac OS X (BD Biosciences). Flow data were analyzed using FlowJo (version 9) software for Mac OS X (Tree Star). Proliferation analysis was performed using eFluor670 (eBioscience) or CellTrace Violet (Invitrogen). CD4 and CD45RO antibodies were purchased from BD Pharmingen. CD45RA and CCR7 antibodies were purchased from BioLegend. Intracellular staining for p24 was performed with a phycoerythrin (PE)-conjugated HIV-1 p24Gag antibody (KC57-RD1; Beckman Coulter) on cells treated with a Cytofix/Cytoperm kit (BD Bioscience). Antibody specificity was assessed with an isotype control IgG1 κ -PE monoclonal antibody from BD Pharmingen. Fixation and permeabilization of cells resulted in a 1-log-unit reduction in GFP/YFP fluorescence. Cell sorting was performed using a BD FACSaria flow cytometer at the New York University Medical Center Flow Cytometry Core Facility.

HIV-1 recombination assay. The HIV-1 recombination assay has been previously described (67, 69). In this system, heterozygous virions containing genomes from both YFP and CFP reporter viruses undergo recombination to generate a reporter gene that produced GFP fluorescence in target cells. Infection of cells with a mixture of homozygous YFP and CFP reporter viruses produces little or no GFP fluorescence (69); thus, the appearance of GFP fluorescence in target cells positively demonstrates both recombination and the occurrence of a second round of HIV-1 replication. Modifications for this study were as follows: 375,000

IL-4-treated resting CD4⁺ T cells were infected with single-color YFP or CFP reporter viruses or coinfecting with equal amounts of both. Raltegravir treatment of some cultures was maintained throughout the experiment. At 7 days after infection, 375,000 uninfected IL-4-treated resting CD4⁺ T cells were added to each sample to provide more targets for virus transmission. At 11 days after infection, T cells were activated with anti-CD3/CD28 beads plus IL-2 for 2 days, and then the cultures were analyzed by flow cytometry, as described previously (67).

Quantification of HIV DNA and RNA. DNA from cells infected with Benzonase-treated viruses was purified using a DNeasy Blood & Tissue minikit or an AllPrep minikit (Qiagen) with RNase A (Sigma) digestion. Quantitative real-time PCR for DNA analysis was performed using a QuantiTect Probe PCR kit (Qiagen). Primers and TaqMan probes were purchased from Integrated DNA Technologies. Amplification and detection were performed on a Chromo4 real-time PCR machine (Bio-Rad), and the data were analyzed using the manufacturer's Opticon Monitor 3 software.

Quantification of total HIV DNA was performed using forward primer ZXF F (5'-AAGTAGTGTGTGCCGTCTGT-3'), reverse primer ZXF R (5'-GCTTCAGCAAGCCGAGTC-3'), and probe ZXF (5'-56-FAM-TGTGACTCT-ZEN-GGTAAGTACAGATCCCTCAGACCC-3IABlk_FQ-3', where FAM is 6-carboxyfluorescein, ZEN is an internal quencher (Integrated DNA Technologies [IDT]), and 3IABlk_FQ is the 3' Iowa black FQ quencher) (76). Quantification of 2-LTR circles was performed using forward primer 2L-f2 (5'-TGTTGTGTGACTCTGGTAACTAGAGATCCC-3'), reverse primer 2L-r2 (5'-GATATCTGATCCCTGGCCCTGG-3'), and probe 2L-z2 (5'-56-FAM-CCACACACAAGGCTACTTCCCTGATTGGCA G-3IABlk_FQ-3').

Sequences for the 2-LTR primer were kindly provided by Dimitrios Vatakis and Jerome Zack, UCLA.

β -Globin was detected with the primers and probes described previously (76) and was used for normalization of the total number of HIV-1 DNA copies per cell and the number of 2-LTR HIV-1 DNA copies per cell. Standardization for quantification of total HIV-1 DNA was performed with 10-fold serial dilutions of genomic DNA extracted from Jurkat cells containing 2 integrated HIV-1 NL4-3 proviruses. Standardization for quantification of 2-LTR circles was performed using serial dilutions of a plasmid containing a single copy of the 2-LTR PCR target.

Quantification of integrated HIV DNA by *Alu* PCR. Detection of integrated HIV-1 DNA was performed using a modification of the previously described *Alu* PCR technique (67, 77, 78). The first PCR was performed using forward primer AluI (5'-TCCCAGCTACTGGGGAGGCTGAGG-3', 40 nM) and reverse primer gag2 (5'-GCTCTCGACCCATCTCTCTCC-3', 400 nM) (78), or a control PCR was performed using the gag2 reverse primer only.

The following thermal cycling program was used: step 1, 95°C for 15 min; step 2, 93°C for 30 s; step 3, 60°C for 60 s; step 4, 70°C for 1 min 40 s; steps 2 through 4 were repeated 24 times.

The PCR product from the first PCR was diluted 10 times in nuclease-free water before addition to a new 96-well plate for second-round nested kinetic PCR. The second PCR amplification employed the internal ZXF primers and probe described above.

The second PCR used the following thermal cycling program: step 1, 95°C for 15 min; step 2, 94°C for 15 s; step 3, 60°C for 30 s; step 4, fluorescence data collection; and step 5, return to step 2, which was run 49 times.

A standard curve representing integrated HIV sequences was generated from 10-fold dilutions of genomic DNA containing a known quantity of integrated HIV-1 DNA. To prepare the integration standard (IS), Jurkat cells were infected with envelope-defective, vesicular stomatitis virus glycoprotein G-pseudotyped HIV-1 conferring resistance to puromycin, pNLpuro Δ vpr (34), and selected in puromycin (2 μ g/ml; Sigma) for ≥ 2 weeks. The pNLpuro Δ vpr virus stock was filtered and Benzonase treated as described above. Genomic DNA was extracted using a Gentra PureGene kit (Qiagen). The HIV-1 DNA content (number of viral copies

per cell) was quantified by qPCR with ZXF primers, with the results normalized using standardization to the β -globin gene level. The standard ranged from 2,500 integration copies per 1,000 total cells to 2.5 copies per 1,000 cells. To each serial dilution of IS was added genomic DNA from uninfected Jurkat cells to bring the total DNA content to 1,000 cell equivalents per μ l.

A Student *t* test was performed to compare the cycle threshold (C_T) values from *Alu*-gag and gag-only amplification replicates, and the average integration event values were determined according to the IS. If no statistically significant difference was observed in the samples, indicating that the number of integration events was below the sensitivity of the assay, we monitored individual PCR replicates for the occurrence of *Alu*-gag amplification that reached a C_T value lower than that for the gag-only replicates by at least 2 standard deviations of the gag-only amplifications. In all the experiments where integration was inhibited by RAL treatment or the D116N mutation, the level of integration was based on the IS, but the positive integration values were not statistically significant. On the basis of the IS and the number of cells analyzed in multiple (≥ 6) replicates, the sensitivity of our assay was minimally 1 integrated provirus per 300 (see Fig. 1G) and 140 (see Fig. 6D) cells, allowing an upper limit of integration to be assigned. In Fig. 6D, integration was detected with statistical significance when transmission was not inhibited.

qRT-PCR for quantification of virus stocks and virus output in supernatants from infected cells. RNA was isolated from virus-containing culture medium using an RNeasy minikit (Qiagen) with on-column DNase digestion (40 min). Quantitative real-time RT-PCR for RNA was performed with a QuantiTect Probe RT-PCR kit (Qiagen). qRT-PCR was performed using primers and TaqMan probes (Integrated DNA Technologies) specific for a shared region of integrase RNA sequence and a QuantiTect Probe RT-PCR kit (Qiagen). Quantification of HIV-1 RNA was performed against serial 10-fold dilutions of a synthetic RNA molecule containing the integrase target (67) using forward primer FL-U Sense (5'-CAATTTCACCAGTACTACAGTT-3'), reverse primer FL Sense (5'-GAATGCCAAATTCCTGCTTGA-3') (79), and probe FL-U (5'-56-FAM-AAGGCCGCC-ZEN-TGTTGGTGGGCG-3IABlk_FQ-3').

Control assays for DNA carryover were routinely performed and were either completely negative or contained $\leq 1/40,000$ RNA molecules.

qRT-PCR for quantification of fully spliced HIV RNA in infected cells. RNA was isolated from cells using an RNeasy minikit (Qiagen). Quantitative real-time RT-PCR for RNA was performed with a QuantiTect Probe RT-PCR kit (Qiagen). qRT-PCR was performed using primers and TaqMan probes detecting fully spliced RNA specific for NL4-3 HIV. Quantification of FS HIV RNA was performed against serial 10-fold dilutions of a synthetic RNA molecule containing the fully spliced (FS) target using forward primer DNL 287 (5'-GGAGACAGCGACGAAGAGC-3'), reverse primer DNL 288 (5'-CTCTCCACCTTCTTCTTCTATTCC-3'), and probe DNL 289 (5'-56-CATCAGAACAGTCAGACTCATCAAGCTTCTCTATCAAAG-3IABlk_FQ-3').

p24Gag enzyme-linked immunosorbent assay (ELISA) was performed by the Aaron Diamond AIDS Research Center using a PerkinElmer Alliance p24 HIV-1 antigen ELISA kit according to the manufacturer's instructions on triplicate serial dilutions of culture supernatants (see Fig. 2F).

RESULTS

This study employed a series of HIV-1 reporters containing a fluorescent reporter gene (GFP, YFP, or CFP) expressed coordinately with the early/late gene *nef* (see Fig. S1 in the supplemental material) (67, 69). Experiments in which it was desirable to limit infection to a single round (see Fig. 1 to 5 and 7) employed *env* gene-defective viruses that were pseudotyped with an HIV-1 envelope protein. When HIV-1 transmission was examined (see Fig. 6 and Fig. S5 in the supplemental material), the viruses expressed HIV-1 envelope protein from the native virally encoded *env* gene.

FIG 1 *De novo* virus production from unintegrated HIV-1 when T cell activation follows infection. (A) Schematic of the protocols for infection and analysis of CD4⁺ T cells either activated with anti-CD3/CD28 activation beads plus IL-2 prior to infection (preactivation) or treated for 3 days with IL-4 prior to infection (IL-4 infection) and every 3 days thereafter and then activated 7 days after infection (postactivation) or maintained unactivated in IL-4 throughout. Viruses were *env* gene defective and pseudotyped with the HIV-1 envelope for a single round of infection. (B) Flow cytometric analysis of infected cells. Dot plots display all

High-level gene expression and *de novo* virus production from integration-inhibited HIV-1 when T cell activation followed infection. Initial tests indicated that among the cytokines IL-2, IL-4, IL-7, and IL-15, IL-4 primed resting peripheral blood CD4⁺ T cells for HIV-1 infection as well as or better than the other cytokines (not shown; see Fig. 3) while consistently inducing the least cell proliferation over the widest range of concentrations for cells from multiple donors. IL-4 upregulates CXCR4 on CD4⁺ T cells (80, 81) and increases HIV-1 expression in CD38⁺ resting T cells (82, 83), and in tonsils, IL-4 has been shown to increase the permissiveness of resident CD4⁺ T cells to HIV-1 infection (61). Based on these properties, we chose IL-4 stimulation as the model system for the majority of the following studies.

We compared HIV-1 gene expression following infection of IL-4-treated primary peripheral blood CD4⁺ T cells with activated CD4⁺ T cells. CD4⁺ T cells from the peripheral blood mononuclear cells of healthy HIV-negative donors were negatively selected using Dynabeads Untouched magnetic beads (Invitrogen), yielding a population that was consistently $\geq 99\%$ small, CD4⁺, CD25⁻, CD28⁻, CD38⁻, CD69⁻, and HLA-DR⁻ resting T cells. One set of cells was activated 2 days prior to infection with anti-CD3/CD28 beads plus IL-2, a condition that we refer to as “preactivation” (Fig. 1A). These cells were phenotypically activated (CD25⁺, CD28⁺, CD69⁺, HLA-DR⁺) and proliferating at the time of infection (data not shown). In the second instance, we applied IL-4 to the resting T cells for 3 days prior to infection, a condition that we refer to as “IL-4 infection,” and then activated these cells 5 or more days after infection, a procedure that we refer to as “postactivation” (Fig. 1A). IL-4-treated T cells were $\geq 98\%$ small, CD25⁻, CD28⁻, CD38⁻, CD69⁻, and HLA-DR⁻ and showed a 1% increase in activation from the level for freshly isolated resting cells (not shown). The postactivation protocol was designed to simulate the *in vivo* situation in which an infected resting T cell is stimulated via its T cell receptor during an adaptive immune response. This sequence of events has been proposed to aid the spread of HIV-1 from sites of transmission (59, 64).

As we have previously observed using these reporter viruses (67), infection of preactivated CD4⁺ T cells with GFP reporter virus with the wild-type integrase (Int-WT) generated a majority GFP⁺ bright population of cells as well as a minority GFP⁺ dim population (Fig. 1B, left). Our prior study demonstrated that the GFP⁺ bright cells contain at least one integrated provirus per cell and are thus the virus-producing population, while most of the

GFP⁺ dim cells contain only unintegrated genomes (67). When integration was inhibited, either by applying the integrase inhibitor raltegravir (RAL) (75) during infection with the Int-WT virus or by infecting cells with a reporter virus containing a mutant integrase gene (Int-D116N), only GFP⁺ dim cells were generated in preactivated cells. Cells infected with Int-WT viruses in the presence of raltegravir (84) and cells infected with D116 active-site HIV-1 mutants (85, 86) display 2- to 3-log and 3- to 4-log reductions in integration, respectively. It is worth pointing out that these observations are enabled by the fact that the reporter gene (GFP) is coordinately expressed with *nef* during the early phase of HIV-1 gene expression. HIV-1 reporter systems in which the reporter gene is expressed as a late gene (87–90) do not permit the examination of reporter expression from unintegrated DNA following infection of preactivated T cells.

Infection of IL-4-treated T cells generated GFP expression in a pattern similar to that for preactivated cells, the salient feature being the absence of GFP⁺ bright cells produced by infection with RAL-treated or Int-D116N HIV-1 (Fig. 1B, middle). Surprisingly, when we induced T cell activation in these infected IL-4-treated cells ≥ 5 days subsequent to infection, a substantial population of GFP⁺ bright cells that we had never previously observed under any conditions was generated by integration-inhibited HIV-1 (Fig. 1B, right). The proportion of GFP⁺ bright cells generated by infection with RAL-treated and D116N mutant HIV-1 isolates was less than that produced by untreated Int-WT viruses (28% to 32% of the frequency for Int-WT virus), but they were still >10 -fold too frequent to be the result of integration, according to integration frequencies after raltegravir treatment or treatment with Int-D116N mutants published previously (84–86). Though they also had GFP fluorescence 2.1- to 2.2-fold lower than that of the Int-WT (no RAL treatment) GFP⁺ bright cells, their presence nevertheless suggested the possibility of *de novo* HIV-1 production from unintegrated HIV-1.

Indeed, the postactivation protocol resulted in *de novo* HIV-1 synthesis from both Int-D116N and RAL-treated Int-WT HIV-1 at a rate about 5% of that from untreated Int-WT HIV-1 (Fig. 1C). *De novo* virus production from RAL-treated and D116N HIV-1 infections was statistically zero using the preactivation protocol. Across all experiments, the postactivation output from RAL-treated and D116N HIV-1 bulk cultures varied between 5% and 15% of that for untreated Int-WT virus (e.g., see Fig. 2D). The nearly identical results from the Int mutant and RAL-treated HIV-1 infections functioned to mutually confirm these results;

cells, while the contour plots present only GFP⁺ cells, in order to more clearly illustrate the appearance of the GFP⁺ bright populations. Data are representative of >5 independent experiments. SSC, side scatter; MCF, mean channel fluorescence. (C) *De novo* virus output measured by qRT-PCR for HIV-1 RNA in culture supernatants. Data represent averages and SDs from 3 independent experiments. The nonspecific release of virions from the initial inoculum was determined with a reverse transcriptase inhibitor control and was subtracted from the output obtained with no reverse transcriptase inhibitor. Reverse transcriptase inhibitor controls were $\leq 10\%$ of the WT virus output. Data are expressed as a percentage of the virus output from T cells infected with Int-WT virus without RAL treatment. (D) RAL titration under preactivation and postactivation conditions. Virus output is expressed as a percentage of the output from Int-WT (non-RAL-treated) HIV-1, as described for panel C. Data represent averages and SDs from 3 independent experiments. $C_{\min}$ and $C_{\max}$, minimum and maximum concentrations, respectively. (E) Virus production from FACS-purified GFP-infected T cells. IL-4-treated resting CD4⁺ T cells were infected with Int-WT GFP reporter HIV-1 with and without raltegravir treatment (1 μM) or with Int-D116N GFP reporter HIV-1. Viruses were *env* defective and pseudotyped with the HIV-1 envelope for a single round of infection. At 15 days after infection, GFP-positive cells were purified by FACS and then activated for 2 days with anti-CD3/CD28 activation beads plus IL-2. Raltegravir was removed prior to sorting. Virion production was analyzed by qRT-PCR for viral RNA. Data are from 2 independent experiments utilizing cells from 2 donors. (F) Comparison of two active-site integrase mutants. Cells were infected with Int-D116N or Int-D64 HIV-1 as described in panel A and the legends to panel C, and virus output was analyzed by qRT-PCR. (G) Analysis of HIV-1 DNA within GFP⁺ sorted cells before and after T cell activation. DNA from 3×10^4 to 10×10^4 T cells from the experiment whose results are shown in panel E (donor 1) was extracted before and after anti-CD3/CD28⁺ IL-2-induced T cell activation. Viral DNA was analyzed by qPCR for total HIV-1 DNA, 2-LTR circles, and integrated DNA (see Materials and Methods). ND, none detected.

FIG 2 *De novo* HIV-1 production from unintegrated templates is associated with a block in T cell proliferation. (A) Schematic of the protocols for infection and analysis of CD4⁺ T cells. The protocols are identical to those shown in Fig. 1A, except for the addition of eFluor670 staining. Viruses were *env* defective and pseudotyped with HIV-1 envelope for a single round of infection. (B) Simultaneous analysis of cells for proliferation and GFP and intracellular p24Gag expression by flow cytometry. (Top row) Cells were analyzed without permeabilization/fixation, as described in the legend to Fig. 1, for cell proliferation and GFP expression; (middle and bottom rows) cells were fixed and permeabilized for intracellular staining for p24Gag, which reduces GFP fluorescence but does not affect eFluor670; (middle row only) p24Gag⁺ cells are highlighted in light blue, with p24Gag-negative cells shown in red; (bottom row only) cells with viruses in early gene expression are GFP⁺ p24Gag negative (lower right quadrant), while cells with viruses in late gene expression that are producing HIV-1 *de novo* are GFP⁺ p24Gag⁺ (upper right quadrant). (C) Efficiency of transition into late gene expression measured as the percentage of the GFP⁺ cells that are also p24Gag⁺. Data represent the averages and SDs of two experiments. (D) Virus production under preactivation conditions measured by qRT-PCR. The output of control virus treated with reverse transcriptase inhibitor determined by qRT-PCR in Int-WT, RAL-treated, and D116N HIV-1 infections was 7.1% $\pm$ 0.3% of that from

however, in order to ensure that RAL was being used optimally, we performed a dose-response assay under preactivation and postactivation conditions, employing a 3,000-fold titration going from partial activity to the maximal dose achievable without significant cell toxicity (Fig. 1D). RAL was maximally effective at $>0.3 \mu\text{M}$, and no further inhibition of virion production or decrease in GFP-positive (GFP^+) cells (see Fig. S2 in the supplemental material) was observed at higher doses. RAL treatment of Int-D116N mutant-infected cells failed to reduce the appearance of GFP^+ cells further (see Fig. S2 in the supplemental material). In all cases, application of reverse transcriptase inhibitors prevented the appearance of GFP^+ cells, irrespective of virus or cell status (see Fig. S2 in the supplemental material).

In order to perform a direct cell-to-cell comparison between Int-WT, RAL-treated, and Int-D116N HIV-1, we sorted GFP^+ cells from the IL-4 infection condition and then activated these virus-expressing cells with anti-CD3/CD28 beads. Two days following activation, virus output from the sorted GFP^+ cells infected with RAL-treated Int-WT or Int-D116N HIV-1 was between 14.8% and 21.5% of the production from Int-WT HIV-1 on a per cell basis (Fig. 1E). As expected, a second integrase active-site mutant, the D64E mutant, yielded results essentially identical to those for the Int-D116N mutant (67, 91) (Fig. 1F).

We next performed DNA analysis of the GFP^+ sorted cells to directly test whether integrated proviruses could be the source of *de novo* virus production under conditions of RAL treatment and D116N mutation. We measured between 2 and 3.5 total copies of HIV-1 DNA per GFP^+ cell (Fig. 1G), and cells infected with Int-WT virus contained a single integrated provirus per cell. In contrast, the RAL-treated cells and the D116N mutant-infected cells contained at most 100 to 300 times fewer integrated genomes, a rate of integrase-independent integration consistent with that found in prior studies (84–86) and a level of integration too low to be a significant contributor to either the appearance of GFP^+ bright cells (or the p24Gag-positive [p24Gag^+] cells; see below) or the *de novo* level of virus production observed. Raltegravir was removed from raltegravir-treated cells on the day of cell sorting and activation, yet integration did not follow, consistent with the irreversibility of RAL binding to and inhibition of preintegration complexes (PICs) (3). Consistent with the findings of prior studies (92–95), 2-LTR circles were increased severalfold when integration was inhibited by RAL treatment or integrase mutation. Importantly, no increase in either total DNA, 2-LTR circles, or integration was observed following T cell activation (Fig. 1G, postactivation conditions), indicating that *de novo* virus production from unintegrated DNA did not result from completion of delayed reverse transcription, nuclear import, or integration occurring subsequent to T cell activation (preintegration latency) but, rather, was the result of gene expression from the unintegrated DNA itself. 2-LTR circles are formed exclusively following nuclear import of the preintegration complex (96–98); thus, their steady state before and after T cell activation indicates that this

process is completed prior to activation. We did not, however, parse the contribution of linear unintegrated DNA or 1-LTR and 2-LTR circles to virus production.

***De novo* HIV-1 production from unintegrated templates is associated with a block in T cell proliferation.** We next examined cell proliferation, GFP expression, and virion production simultaneously at the single-cell level by flow cytometry (Fig. 2A). Proliferation was measured by dilution of eFluor670 staining during cell division, and virion production was detected within cells by intracellular p24Gag staining (light blue dots in the middle row of Fig. 2B). Viruses in the early (Rev-independent) phase of HIV-1 gene expression express only early proteins, including GFP, while cells in the late, Rev-dependent productive phase express late proteins, including both GFP and p24Gag.

eFluor670 dilution (Fig. 2B, top 2 rows) revealed that infection of preactivated cells with Int-WT HIV-1 yielded GFP^+ cells that multiplied but did so fewer times than the GFP^- cells (Fig. 2B). This finding is consistent with the previous finding that Vpr inhibition allows at least one round of cell division after infection of preactivated T cells (36). On the other hand, at 7 days after infection, IL-4-treated cells showed little proliferation (Fig. 2B) and GFP^+ cells had not proliferated at all. Postactivation of these cells resulted in the proliferation of the GFP^- and the GFP^+ p24Gag-negative (p24Gag^-) cells but not of the p24Gag^+ cells, whether or not integration had been inhibited by raltegravir. Postactivated GFP^+ growth-arrested cells were enriched in the G_2/M phase, which is consistent with Vpr-induced growth arrest (see Fig. S3A in the supplemental material) (36). This failure to divide even one time occurred despite successful activation of the GFP^+ cells, as measured by an increase in cell size and increased expression of the activation markers CD25, CD38, and CD69 that was similar to that of activated GFP^- cells (see Fig. S3B in the supplemental material). Thus, inhibition of cell division by both integrated and unintegrated HIV-1 occurs through a known mechanism (Vpr-induced growth arrest; see below) that does not apparently interfere with T cell activation, at least by use of the criteria measured here.

When p24Gag staining was plotted against GFP expression (Fig. 2B, bottom row), the division between early (GFP^+ p24Gag^-) and late (GFP^+ p24Gag^+) expression was evident. There was a 140-fold reduction in p24Gag^+ cells when integration was inhibited in preactivated cells (1.4% versus 0.01%), consistent with the ≥ 2 -log reduction of integrated proviruses measured in Fig. 1G as well as the repeated finding that *de novo* virus production in preactivated cells requires integration (Fig. 2D) (20, 22, 67; reviewed in references 17 and 21). On the other hand, in IL-4-treated cells and postactivated cells, there was an increase in late p24Gag expression compared with that in preactivated cells (Fig. 2B and C) whether or not integration was inhibited. The high number of p24Gag^+ cells generated when integration was inhibited by raltegravir is inconsistent with its published ability (85, 86) to block integration and our measurement of integration by PCR

Int-WT virus infections. Data represent the averages and SDs of 3 independent experiments. (E) Virus production under IL-4 infection and postactivation conditions measured by qRT-PCR. The output of control virus treated with reverse transcriptase inhibitor determined by qRT-PCR in Int-WT, RAL-treated, and D116N HIV-1 infections was $0.23\% \pm 0.09\%$ of that from Int-WT virus infections treated with IL-4 only and $0.94\% \pm 0.05\%$ of that from Int-WT virus infections postactivation. Data represent the averages and SDs of 3 independent experiments. (F) p24Gag ELISA of culture supernatants from one representative experiment whose results are shown in panel E. SDs represent multiple replicates of individual samples. (G) Virus production measured by qRT-PCR from RAL-treated Int-WT virus infections expressed as a percentage of the production from Int-WT virus infections not treated with RAL. Data are from 5 independent experiments.

FIG 3 Unintegrated HIV-1 generates *de novo* virus in resting CD4⁺ T cells rendered permissive to HIV-1 infection by cytokines IL-2, IL-4, IL-7, and IL-15. (A) The infection protocol was identical to that described in Fig. 1A (resting infection, postactivation) for each cytokine: IL-2 (50 U/ml), IL-4 (25 ng/ml), IL-7 (2 ng/ml), and IL-15 (25 ng/ml). Viruses were *env* defective and pseudotyped with the HIV-1 envelope for a single round infection. Data represent the averages and interexperimental SDs of 3 independent experiments. Virus outputs are expressed as a percentage of the virus production obtained with nonactivated, IL-4-treated T cells infected with Int-WT virus. (B) Fold induction of virus production following postactivation of infected cells calculated from the data in Fig. 3A. The null symbols represent no treatment.

in Fig. 1G. By sheer numbers, the vast majority of p24Gag expression and virus production in postactivated cells can be the result only of (late) gene expression from unintegrated templates. *De novo* virus production was again measured, but this time it was measured by both qRT-PCR (Fig. 2E and G) and p24Gag ELISA (Fig. 2F), reaffirming the findings shown in Fig. 1 and confirming the validity of our qRT-PCR method. qRT-PCR results from 5 independent experiments are summarized in Fig. 2G.

Most of the experiments described in this report utilize CXCR4-tropic (X4) HIV-1, as these viruses infect cells with a high efficiency *in vitro*. We treated resting CD4⁺ T cells with IL-15, which increases CCR5 expression (99, 100), and then infected them with a CCR5-tropic Int-D116N GFP reporter virus, obtaining similar results for GFP and p24Gag from Int-D116N virus as with CXCR4-tropic HIV-1, albeit at lower overall infection frequencies (not shown).

IL-2, IL-4, IL-7, and IL-15 all prime resting CD4⁺ T cells for *de novo* virus production from integrase mutant HIV-1. In our preliminary testing, we confirmed the findings presented in prior reports (49, 50, 81, 83) that exposure of resting peripheral blood CD4⁺ T cells to IL-2, IL-4, IL-7, or IL-15 enhances infection without inducing T cell activation. Prior studies have demonstrated that cytokines have multiple influences on resting T cells that increase the permissiveness of these cells to HIV-1 replication. Among these findings are that IL-2 and IL-15 in lymphoid tissues contribute to infection of naive T cells at least in part by relieving the block to infection imposed by APOBEC3G (66) and that IL-7 drives T cells into the G_{1b} cell cycle phase that is associated with permissiveness to infection (50). IL-7 enhances HIV-1 infection of cervicovaginal tissues *ex vivo* (101), and IL-7 levels *in vivo* correlate with and may contribute to the viral load (102). IL-2 and IL-7

can reactivate a subset of latent viruses (103, 104), and on the basis of this property, each has been tested in clinical trials for its potential usefulness in viral eradication therapies (105, 106).

We examined if these cytokines enhance *de novo* virus production from Int-D116N HIV-1 similarly to IL-4. In each case, using a concentration of cytokine that we predetermined to be optimal for virus production and following the protocol described in Fig. 1A, we observed enhanced *de novo* virion production following the postactivation protocol (Fig. 3A). Interestingly, for each cytokine the integrase mutant responded to T cell activation with a fold induction greater than that for the Int-WT virus (Fig. 3B). Thus, while IL-4 appears to be the most effective cytokine for postactivation-induced HIV-1 production from Int-D116N HIV-1, it is not unique in this regard. This may not be surprising, considering that each of these cytokines belongs to the common gamma-chain family of cytokines that share signaling pathways (107–111), aid the survival of both resting memory and naive T cells, and forestall activation-induced cell death (112–116).

T cell homeostatic proliferation during the early stage of viral expression is antagonistic to the generation of virus from integrase mutant HIV-1. A well-defined activity of the cytokines utilized in this study is induction of homeostatic proliferation in a subset of cells (109, 110, 117, 118). While seeking the optimal concentration of each cytokine, we observed that a subset of T cells proliferated in a dose-dependent manner in the days after treatment with each cytokine and prior to HIV-1 gene expression (not shown). IL-7 displayed a particularly well-defined and reproducible dose-response, and we obtained an interesting result regarding the ability of cells to generate *de novo* HIV-1 (Fig. 4). While cell proliferation prior to activation increased with each increasing dose of IL-7 (Fig. 4A and B), the

FIG 4 T cell homeostatic proliferation is antagonistic to the generation of virus-producing cells infected with Int-D116N HIV-1. The infection protocol was identical to that described in Fig. 1A, except that flow cytometry on cells under the IL-7-treated condition was performed on day 7 after infection, 2 days before postactivation analysis. Viruses were *env* defective and pseudotyped with the HIV-1 envelope for a single round of infection. Data are from one experiment representative of 3 independent experiments. (A) Flow cytometric analysis of GFP expression and cell proliferation before (IL-7, day 7) and after (day 9) postactivation. (B) Percentage of cells that divided at least once before activation (red lines) (calculated using the FlowJo, version 9, proliferation platform) and number of GFP⁺ bright cells obtained after postactivation in the experiment whose results are presented in A (bars). (C) Virus output from the cells for which the results are shown in panel A measured by qRT-PCR for HIV-1 RNA in culture supernatants.

production of GFP⁺ bright cells and nearly maximal *de novo* virus production (Fig. 4C) from Int-WT virus were obtained with almost no homeostatic proliferation induction. The number of GFP⁺ bright cells and high-level virus production from

Int-WT HIV-1 were maintained (20% drop in virus production) as T cell proliferation increased.

In stark contrast, the effect on GFP expression and virus production from Int-D116N HIV-1 dropped coordinately with in-

creasing IL-7-induced proliferation, strongly suggesting that cell proliferation soon after infection is antagonistic to *de novo* virus production from unintegrated HIV-1 but not from integrated HIV-1. This observation reinforces the notion that unintegrated HIV-1 expression is facilitated by the absence of host cell proliferation (17, 24–26). The divergence between the behavior of Int-WT and Int-D116N HIV in response to the various levels of IL-7-induced homeostatic proliferation emphasizes the distinctive nature of integrated and unintegrated templates.

Durable latency is established by unintegrated HIV-1 in resting CD4⁺ T cells. Understanding the various forms of HIV-1 latency as well as the mechanisms for the establishment, maintenance, and subsequent activation of latent viruses is necessary for the development of shock-and-kill therapies intended to clear latently infected cells (119–121). HIV-1 gene expression from latently integrated genomes is inducible by T cell activation (8, 122–124) and by the synergistic activities (125, 126) of protein kinase C (PKC) activators (127) and agents that promote chromatin remodeling, including histone deacetylase inhibitors (HDACi), such as trichostatin A (TSA) (128, 129). HDACi have recently been shown to increase expression from integrase mutant lentiviral vectors in both dividing and nondividing cells (130). Both HDACi and PKC activators are being tested for potential utility in HIV-1 eradication strategies, where avoiding generalized T cell activation is desirable (120, 131).

Though unintegrated HIV-1 DNA is rapidly lost in dividing cell populations (132, 133), circular forms of uDNA are resistant to degradation and persist indefinitely in nonproliferating cells, such as resting primary CD4⁺ T cells (26, 85, 134–137). The experiments described above demonstrated a reproducible increase in the number of GFP⁺ cells following postactivation of cells infected with RAL-treated and Int-D116N HIV-1, suggesting the presence of silent unintegrated genomes. To directly test this notion, we infected IL-4-treated resting CD4⁺ T cells with HIV-1 envelope-pseudotyped Int-WT, RAL-treated, and D116N mutant GFP reporter viruses for a single round, cultured these cells for 15 days, and then removed the GFP⁺ cells by fluorescence-activated cell sorting (FACS) (Fig. 5A). We placed the GFP-negative cells back in culture to test the responsiveness of putative latent genomes to activation stimuli. Two days later there was a low level of spontaneous GFP, p24Gag, and *de novo* virus expression from Int-WT HIV-1-infected cells (Fig. 5B to D) but only low-level GFP expression without virus production from RAL-treated and Int-D116N HIV-1-infected cells. Both memory and naive CD4⁺ T cells were latently infected by these X4 viruses (see Fig. S4 in the supplemental material). Postactivation of the cells in 3 independent experiments induced 6.6- to 25.8-fold increases in virus production from Int-WT HIV-1-infected cells and 5.1- to 23.8-fold increases in *de novo* HIV-1 production from RAL-treated and D116N HIV-1-infected cells. *De novo* virus production was again reflected in an increase in p24Gag⁺ cells (Fig. 5C). Following postactivation with anti-CD3/CD28 beads, the level of virion production from RAL-treated and Int-D116N HIV-1-infected cells on a per cell basis was 4.6- to 10.6-fold lower than that from Int-WT HIV-1-infected cells. Interestingly, Pro-TSA was proportionately stronger on RAL-treated and Int-D116N HIV-1 cells and weaker on Int-WT (non-RAL-treated) HIV-1-infected cells, inducing *de novo* the production of HIV-1 from RAL-treated and Int-D116N HIV-1-infected templates that was only 1.6- to 3.1-fold lower than that from Int-WT HIV-1-infected templates on a per cell basis. A

sorting performed 33 days after infection yielded similar results (not shown).

CD4⁺ T cells activated after infection generate infectious HIV-1, despite RAL treatment. We next measured the infectivity of viruses generated from Int-WT and RAL-treated HIV-1 through both cell-cell contact (138) and cell-free infection (Fig. 6). As diagrammed in Fig. 6A, we infected eFluor670-labeled resting cells with an *env*-positive YFP reporter virus and applied raltegravir to half of the cells for 7 days, reported to be a sufficient interval for dissolution of preintegration complexes (PICs) (8). We purified YFP⁺ eFluor670⁺ double-positive cells by FACS and then mixed them with CellTrace Violet-labeled target cells and anti-CD3/CD28 activation beads (Fig. 6B). Raltegravir was removed after sorting so that transmitted viruses could integrate in newly infected target cells and generate YFP⁺ bright cells, thus improving the sensitivity of the assay. At this point, RAL inhibition of PIC-mediated integration would be irreversible (3). PCR analysis performed on sorted YFP⁺ producer cells both before and after activation confirmed the results shown in Fig. 1G that RAL treatment resulted in less than 1 integration per 100 cells (Fig. 6D). When virus transmission was not prevented by antiviral treatment, increased integration was detected in producer cells after 3 days of activation, indicating that these cells could be superinfected.

The differential staining of producer and target cells allowed positive discrimination of these two populations (Fig. 6B). To control for nonspecific transfer of viruses or YFP, identical cultures included reverse transcriptase inhibitors to block *de novo* infection of targets. As a further control, we pretreated a set of producer cells with the protease inhibitor indinavir. This would stop the *de novo* production of virions from producer cells but not block infection of target cells by any virions remaining from the initial inoculum. In each case, virus transmission was completely blocked (Fig. 6B); thus, all transferred viruses were the result of *de novo* virus production from the producer cells. Cell-cell transfer of infectious HIV-1 from raltegravir-treated cells to primary CD4⁺ T cells was 9 to 18% as efficient as that from an equal number of cells infected with Int-WT HIV-1 across multiple experiments. Transmission was also observed using D116N HIV-1 (not shown). In a further independent experiment, a constant number of target cells was mixed with various numbers of producer cells to control for any effect of the different producer-target cell ratios (Fig. 6E and F). Each ratio resulted in an approximately 1-order-of-magnitude lower level of transfer of infectious virus from RAL-treated cells. In another test, supernatants from a separate set of postactivated producer cells from the experiment whose results are shown in Fig. 6B were applied to Jurkat-Tat cells to test for cell-free transmission (Fig. 6C). These supernatants transmitted infectious HIV-1 at a similar relative efficiency as cell-cell transmission to primary T cells. As with cell-cell transmission, reverse transcriptase inhibitors or pretreatment with protease inhibitors prevented transmission of infection to target cells.

Recombination between viruses generated from unintegrated HIV-1. Recombination in retroviruses is a form of sexual reproduction that combines the genetic information of two parental viruses, allowing rapid evolutionary jumps that would be difficult or impossible to achieve through single nucleotide incorporation errors (mutation) alone. We have previously described a system to measure recombination by flow cytometry (67, 69) and demonstrated that when integrated and uninte-

FIG 5 Durable latency is established by unintegrated HIV-1 in resting CD4⁺ T cells. (A) Schematic protocol for analyzing HIV-1 latency. eFluor670-stained CD4⁺ T cells were treated with IL-4 for 3 days (IL-4 infection protocol) and then infected with Int-WT (with or without raltegravir) or D116N virus and maintained in culture for 15 days. Viruses were *env* defective and pseudotyped with the HIV-1 envelope for a single round of infection. At 15 days after infection, GFP-negative (GFP⁻) cells were purified by FACS and then placed back in culture. Cells were cultured with IL-4 or stimulated for 2 days with either anti-CD3/CD28 activation beads plus IL-2 (postactivation protocol) or prostratin plus TSA (Pro/TSA). Data are representative of >5 independent experiments. (B) GFP expression and cell proliferation analyzed by flow cytometry on the day of sorting (day 15) and 2 days after sorting (day 17) on unfixed cells. (C) GFP expression versus intracellular p24Gag staining 2 days after sorting (day 17). (D) Virus output measured by qRT-PCR on the culture supernatants of 65,000 cells per condition 2 days after sorting (day 17). Virus output is expressed as a percentage of the virus production obtained after activation with T cells infected with Int-WT virus. Data are from 3 independent experiments. The SDs represent interexperimental variability.

egrated HIV-1 isolates are present in the same cell, recombination can occur between their progeny genomes (67). To test the ability of unintegrated HIV-1 to generate recombinant viruses without the assistance of an integrated helper virus, we performed this assay comparing cells infected with Int-WT, RAL-treated, and D116N HIV-1 and observed levels of recombination that reflected the efficiencies of virus production observed in the experiments described above (see Fig. S5 in the supplemental material).

Vpr is required for gene expression and virus production from Int mutant HIV-1 in resting T cells. The Vpr protein is incorporated into virions and delivered into cells by infecting virions, where it functions as an immediate early protein that can bind to nucleoporins and importins (139–142), participate in viral DNA remodeling prior to integration (143), facilitate nuclear import (30, 41, 42), transactivate unintegrated HIV-1 DNA (28, 29), and induce cell cycle arrest (144). Vpr that is generated *de novo* in cells during late gene expression can also cause cell growth arrest

FIG 6 Transmission of infectious virus generated from integration-inhibited HIV-1. (A) Schematic protocols for infection, transmission, and analysis of cells. eFluor670-labeled CD4⁺ T cells were treated with IL-4 for 3 days and then infected with Int-WT, Env⁺, YFP reporter HIV-1 in the presence or absence of raltegravir (1 μ M). At 7 days after infection, raltegravir was removed and then GFP⁺ eFluor670⁺ cells were purified by FACS and mixed at a 1:5 ratio with IL-4-treated, CellTrace Violet (CTV)-labeled autologous target CD4⁺ T cells. This coculture was activated for 3 days with anti-CD3 anti-CD28 activation beads and IL-2 prior to analysis. (B) Flow cytometric analysis of the producer cells after isolation (left, in red), the mixed producer and target cells after activation (middle, in black), and the expression of YFP from the eFluor670-negative CTV-positive target T cells (rightmost 3 panels, in blue). Reverse transcriptase inhibitor (RTI) and protease inhibitor (PI) controls demonstrate specificity for *de novo* virus production. Reverse transcriptase inhibitors were added at the time of cell mixing, and protease inhibitors were added at the time of infection of producer cells and on subsequent days. Data are representative of 2 independent experiments. FSC, forward scatter. (C) Transmission by cell-free virus generated from postactivated producer cells. Culture supernatants from activated producer cells from the experiment whose results are shown in panel B were applied to Jurkat-Tat cells, which were analyzed 3 days later. Assays with reverse transcriptase inhibitor and protease inhibitor controls were performed as described for panel B. (D) qPCR analysis of total and integrated DNA in FACS-purified producer cells. No PCR amplification for integrated DNA was detected in samples prior to activation, with a detection limit of ≤ 0.0075 copy/cell (samples labeled ND [none detected]). (E) Independent experiment similar to that described for panel B but with the ratios of producer to target cells varied. The percentage of YFP⁺ target cells was determined as described in the legend to panel B. (F) Transmission averages and SDs from panels B and D are expressed as a percentage of the value for untreated Int-WT HIV-1.

and transactivate HIV-1 (145–150). Vpr is important for HIV-1 replication in postmitotic macrophages and growth-arrested cell lines (37, 41; reviewed in reference 32). These reports and the negative correlation between cell proliferation and virus production from integration-inhibited HIV-1 production that we observed suggested a possible role for Vpr in this process.

We observed that Int-WT virus lacking the *vpr* gene was capable of generating GFP⁺ p24Gag⁺ cells, and in the absence of Vpr these cells proliferated after activation (Fig. 7A). This conclusively demonstrated that, as expected, Vpr was responsible for the block in activation-induced proliferation observed in this system (Fig. 2B; see Fig. S3B in the supplemental material). Int-D116N HIV-1,

FIG 7 Vpr is required for gene expression and *de novo* virus production from unintegrated HIV-1. Viruses were *env* defective and pseudotyped with the HIV-1 envelope for a single round of infection. (A) The postactivation protocol was performed, with the exception that the insets show the results of the preactivation protocol. Low and high are used to discriminate the two virus-producing p24Gag⁺ populations contained within the GFP⁺ bright population (Fig. 1B). (A) GFP, intracellular p24Gag, and proliferation in cells infected with Int-WT and Int-D116N HIV-1, with or without an intact *vpr* gene. Yellow lines indicate the level of eFluor670 intensity in cells that did not divide. (B) Virus output from cells infected with or without an intact *vpr* gene measured as the amount of HIV-1 RNA in culture supernatants determined by qRT-PCR. Data are from one experiment representative of five independent experiments. (C) Complementation of the *vpr* mutant HIV-1 with virion Vpr. Cells were coinfecting with *vpr* mutant GFP reporter HIV-1 from the experiments whose results are shown in panels A and B and reverse transcriptase mutant YFP reporter HIV with an intact *vpr* gene that packaged Vpr for delivery into cells but could not undergo reverse transcription in target cells (see Materials and Methods). In control experiments, no reverse transcripts and no YFP fluorescence was generated in cells infected with the reverse transcriptase mutant alone (not shown). Coinfection with *vpr*-negative reverse transcriptase-negative YFP⁺ HIV failed to restore GFP fluorescence (not shown). (D) Virus production from activated cells whose results are presented in panel C. Supernatants were collected 3 days after T cell activation with anti-CD3/CD29 beads. (E) Contour plot of GFP⁺ p24Gag⁺ T cells whose results are presented in panel A, lower row, for Int-WT (i, upper right quadrant of Int-WT Vpr⁺) and D116N (ii, upper right quadrant of Int-D116N Vpr⁺) virus infections, to illustrate the point that the dim and bright populations are distinct populations of cells. (F) qPCR analysis of HIV-1 DNA in GFP⁺ low and high sorted cells infected with Int-WT Vpr⁺ virus.

on the other hand, completely failed to express GFP fluorescence and p24Gag protein even following postactivation or after treatment with Pro-TSA (Fig. 7A; see Fig. S6A in the supplemental material). No cell proliferation was observed prior to activation, so dilution of unintegrated templates or their RNA and protein products cannot account for the absence of GFP expression. Early-phase, fully spliced RNA transcripts from Int-D116N HIV-1 were reduced about 19-fold in the absence of Vpr (see Fig. S6C in the supplemental material), consistent with the previously de-

scribed transactivating function of Vpr for unintegrated HIV-1 (28, 29), even though comparable numbers of reverse transcripts were generated (see Fig. S6D in the supplemental material). Importantly, a comparable increase in 2-LTR circles was observed in the absence of integration. Since 2-LTR circles are generated following nuclear import of the viral linear cDNA (97, 98), this suggests that nuclear import of Vpr-negative HIV-1 was not greatly affected.

Consistent with the absence of GFP and p24Gag expression, no

virus production was observed from Int-D116N HIV-1 without Vpr, even after postactivation or treatment with Pro-TSA (Fig. 7B). This is the first time, to our knowledge, that a condition under which Vpr is essential for virus expression and replication has been observed. Similar results were observed with raltegravir-treated T cells (see Fig. S6B in the supplemental material). This was not the case in preactivated T cells, where Int-D116N Vpr-negative HIV-1 yielded GFP expression, consistent with the findings of prior studies (29) (Fig. 7A, inset, middle right). Virion Vpr *trans*-complementation of the *vpr* mutant virus did allow the recovery of low-level GFP expression in resting T cells (Fig. 7C), but all of the cells proliferated after activation, GFP-high cells were not generated, and *de novo* virion production remained very low (Fig. 7D). Therefore, both virion Vpr and *de novo* Vpr synthesis are required for *de novo* virus production from Int-D116N HIV-1 in these cells. Virion Vpr is clearly required for initial gene expression, while *de novo* Vpr production is necessary for the generation of virions and inhibition of cell proliferation in this system. Providing Vpr within the Int-D116N virions or by coinfection with Vpr⁺ virions lacking reverse transcriptase activity that generated no reverse transcripts produced identical results (not shown). Finally, given that Int-WT HIV-1 expression was only modestly reduced by the absence of Vpr (both virion Vpr and Vpr produced *de novo*), the total absence of gene expression and virus output from the Vpr-negative integrase mutant and RAL-treated Int-WT virus confirms that rare integration events do not explain the generation of virions by Int-D116N HIV-1.

***De novo* virus production from unintegrated genomes arises naturally following infection with Int-WT HIV-1.** We unexpectedly observed an interesting result demonstrating that infection with Int-WT HIV-1 naturally generates unintegrated DNA that can produce virus *de novo*. Among the GFP⁺ bright cells generated following treatment of cells with Pro-TSA, Vpr⁺ Int-WT HIV-1 generated two distinct subpopulations of cells: a GFP⁺ p24Gag⁺ bright-low population and a GFP⁺ p24Gag⁺ bright-high population (Fig. 7A). We hypothesized that the bright-low population generates virus from mostly unintegrated templates, while the bright-high population generates virus from integrated templates. In the absence of Vpr, the bright-low population disappeared, while the bright-high population was diminished in brightness, both consistent with a role for Vpr. Vpr⁺ Int-D116N HIV-1 generated only this bright-low population, again consistent with our hypothesis.

To positively confirm that virus production from the naturally generated bright-low population was occurring from unintegrated templates, we sorted GFP⁺ bright-low and -high cells by FACS (Fig. 7E) and performed qPCR on the viral DNA (Fig. 7F). We observed that the bright-high population had at least one integrated provirus per cell but the bright-low population contained only one integrated provirus for every 31% of the cells, leaving 69% of the GFP⁺ p24Gag⁺ cells containing only uDNA. Therefore, during infection with Int-WT HIV-1, a population of cells can be generated from resting T cells that produce HIV-1 *de novo* from naturally generated unintegrated templates.

DISCUSSION

In this study, we describe an alternative, or salvage, pathway for HIV-1 replication in CD4⁺ T cells that takes advantage of one of the considerable inefficiencies of retroviral replication: the failure of the majority of reverse transcripts to successfully integrate into

the cellular chromosomal DNA. The novel pathway for HIV-1 production that we describe may function to allow some proportion of viruses that fail to integrate a second chance to contribute their genetic information to the next viral generation. The essential condition for high levels of gene expression and virus production from unintegrated HIV-1 was to infect cells several days before activating them, as infection of cells that were already activated did not allow *de novo* virus production from unintegrated HIV-1. To make resting CD4⁺ T cells permissive to infection, we utilized cytokines (IL-2, IL-4, IL-7, or IL-15) that reproduce important aspects of HIV-1 replication in mucosal or lymphoid tissues, where HIV and SIV replication occurs not only in activated CD4⁺ T cells but also in CD4⁺ T cells that display a nonactivated and nonproliferating phenotype, especially during early infection. Virus production from these cells by the alternative nonintegrating pathway could enhance the number of productively infected cells and the odds of successfully establishing infection.

Could the virus production that results from the postactivation protocol be the result of integration events occurring despite the application of raltegravir or mutation of the integrase active site? Prior studies have reported a 3- to 4-log-unit reduction in HIV-1 integration from integrase active-site mutants, such as those with the D116N and D64E mutations that we employed (84), and a 2- to 3-log-unit reduction in the presence of raltegravir (85, 86). Either we did not detect integration under RAL-treated or D116N mutation conditions with an assay sensitivity of at least 2 log units lower than that for integration by WT virus or we positively detected low-level integration that was ≥ 2 log units lower than WT integration. Thus, the infections utilized in this study are consistent with those utilized in prior studies. The quantities of GFP⁺ p24Gag⁺ cells that were generated under the conditions of raltegravir treatment and integrase mutation were at least 1 order of magnitude too high to be the result of integration. The GFP⁺ p24Gag⁺ cells generated by RAL-treated and D116N mutant virus were also half as fluorescent as those generated by uninhibited Int-WT virus. In addition, the response of integration-inhibited viruses to Vpr was qualitatively different from that of uninhibited Int-WT virus, being entirely dependent upon Vpr for gene expression. This stands in contrast to the findings for Int-WT virus, which did not require Vpr for gene expression and high-level virus production. Finally, we observed that infection with Int-WT HIV-1 generated a substantial population of p24Gag⁺ cells containing only unintegrated viral DNA. These observations allow us to definitely conclude that low-level integration is not contributing significantly to the virus output observed under the postactivation protocol with integration-inhibited HIV-1. As this alternative method for virus production requires a particular set of circumstances that is clearly not the majority set of circumstances *in vivo* and because the level of virus production by unintegrated HIV-1 is about 1 order of magnitude lower than that by uninhibited viruses, our results are entirely consistent with the documented efficacy of raltegravir *in vivo*.

We found that high-level gene expression and *de novo* virus production from unintegrated HIV-1 were antagonized by cell proliferation. Critical threshold levels of Rev (151) and Gag (152) protein must accumulate in cells to accomplish virus assembly, and cell division would be expected to dilute both the uDNA templates and their RNA and protein products, potentially reducing the capacity of uDNA to act as a template for *de novo* virus pro-

duction. We found that when T cell activation was induced several days after infection, Vpr blocked activation-induced T cell proliferation and virus was produced only from the nonproliferating activated cells. Our unpublished findings indicate that at least 5 days between infection and activation is required for optimal virus production. By preventing activation-induced T cell proliferation but not T cell activation itself, Vpr may help create an environment that facilitates *de novo* virus production from unintegrated HIV-1 DNA. These results are consistent with those of prior studies linking cell proliferation inhibition to higher uDNA gene expression (17, 24–26) but do not formally demonstrate that the Vpr blockade of T cell proliferation after activation functions to enhance uDNA gene expression.

Surprisingly, Vpr was essential for early HIV-1 gene expression of unintegrated HIV-1 DNA in cytokine-treated resting CD4⁺ T cells. This is, to our knowledge, the first circumstance described under which Vpr is essential for HIV-1 gene expression and would seem to unify two important activities of Vpr (transactivation and cell growth inhibition) toward one purpose: to allow HIV-1 replication by the alternative pathway utilizing unintegrated genomes. Virion Vpr was sufficient for initial gene expression from Int-D116N HIV-1, but both virion Vpr and *de novo* Vpr production from an intact *vpr* gene were required for high-level gene expression, inhibition of cell proliferation, and *de novo* virus production following T cell activation. Integrase-defective lentiviral vector systems, so-called nonintegrating lentiviral vectors, targeting nonproliferating cells (24, 153–157) might benefit from inclusion of virion Vpr. Why is the transactivation function of Vpr essential for gene expression in resting T cells? One possibility is that Vpr might overcome an intrinsic antiviral mechanism in these cells through direct or indirect influences on viral transcription (28, 29) or cDNA structure (143). Protection from parasitic nucleic acid molecules is a well-understood priority in cellular self-protection (158). It is plausible that by quickly enabling viral transcription from unintegrated DNA, virion Vpr prevents cellular inhibition of these parasitic genomes.

According to our findings, T cell activation can be induced several weeks after infection and still elicit virus production from unintegrated HIV-1, suggesting the possibility that these templates could contribute to viral reservoirs *in vivo*. Pioneering studies by the Siliciano group demonstrated that latent HIV-1 exists predominantly within quiescent memory CD4⁺ T cells, referred to as “postintegration latency” (8). The assumption of prior studies characterizing HIV-1 latency in quiescent peripheral blood CD4⁺ T cells from HIV-1-infected individuals was that any residual unintegrated DNA was incapable of *de novo* virus production (8, 122–124). However, by activating infected resting CD4⁺ T cells after a cultivation period of several days, these studies performed a procedure that we find elicits *de novo* virus production from unintegrated HIV-1. A short-lived preintegration latency occurs when reverse transcription and integration are delayed following infection of quiescent cells, where subsequent activation of the cells within hours to a few days after infection allows productive infection to proceed via newly integrated proviral DNA (7, 159, 160). The replication pathway described in the present work, in contrast, bypasses integration altogether. We therefore refer to this form of viral persistence as “nonintegration latency” to distinguish it from postintegration latency and preintegration latency, both of which ultimately depend upon integration for *de novo* virus production.

The persistence or lability of unintegrated HIV-1 DNA remains a subject of contention. Though linear DNA is rapidly degraded (8, 159), it is well established that circularized HIV-1 can persist indefinitely within nonproliferating cells in tissue culture, including, importantly, within CD4⁺ T cells (26, 85, 135, 136, 161, 162). In fact, we detect little or no degradation in 2-LTR circles during this time by qPCR (unpublished findings). On the basis of this direct evidence, there seems to be no *a priori* reason to assume that the cell population containing transcriptionally silent circularized HIV-1 would be lost *in vivo* at a rate greater than that for the uninfected cell population. It is provocative that a recent study observed that 2-LTR circles represent the genetic pattern of viruses that reemerge following cessation of highly active antiretroviral therapy (HAART) (163). We did not parse the respective contributions of linear, 1-LTR, and 2-LTR HIV-1 DNA to *de novo* virus production, so it remains to be determined which forms are competent for *de novo* virus production in our system. On the other hand, cells exhibiting gene expression from uDNA might become targets for HIV-1-specific cytotoxic T lymphocyte killing, a possible mechanism for bystander CD4⁺ T cell loss. *In vitro* findings notwithstanding, the question of whether unintegrated HIV-1 persists *in vivo* remains controversial (164, 165). Studies of patients on HAART have variously found the persistence or the loss of measurable unintegrated HIV-1 DNA (17, 18, 164–175). Since 2-LTR circles are detected by a simple PCR assay, they are often utilized as a surrogate for total unintegrated HIV-1 DNA (12, 18), but in the absence of integration inhibition, 2-LTR circles are only about 1/30th of the total amount of unintegrated DNA initially produced (75, 132), and this scarcity can make determination of unintegrated HIV-1 DNA *in vivo* somewhat problematic when viral loads are low. Recent advances in PCR methods for the detection of 1-LTR circles will be instrumental for better understanding unintegrated HIV DNA *in vivo* (96).

The HIV-1 latency that we observed in our system was responsive to a combination of a histone deacetylase inhibitor and an NF- κ B activator. Pelascini et al. have recently demonstrated that unintegrated lentiviral vectors are responsive to HDACi, consistent with our findings (130). It is possible that histones assemble upon the uDNA transcriptional template and influence virus expression. There is a precedent for this in the hepatitis B virus (HBV), a small DNA virus with an episomal DNA genome that is densely covered in histones, forming a minichromosome from which *de novo* virus production is generated (176). HIV-1 integration preferentially targets decondensed chromatin favorable to gene expression (177), and integration sites which are less favorable to transcription bias toward viral latency (178, 179). Gene expression by unintegrated HIV-1 DNA, however, cannot be subject to variable positional effects (180, 181), so unintegrated HIV-1 latency may possess a more uniform and predictable response to activating agents than integrated proviruses.

A prevailing model for the establishment of HIV-1 latency in resting CD4⁺ T cells posits that an activated T cell becomes infected just prior to reentering a quiescent phase that withdraws necessary transcription factors and other intracellular requirements for virus expression (8). This process can be recapitulated *in vitro* to establish useful and relevant models of HIV-1 latency (124, 182). Alternatively, infection of resting T cells *in vitro* can result in a direct establishment of a latent state, provided that the cells have been stimulated out of G₀ stage of the cell cycle (50, 183–186). Our system utilizing IL-4 (or other common gamma-

chain cytokines, IL-2, IL-7, or IL-15) is further confirmation of the direct establishment of latency in CD4⁺ T cells (184, 185, 187, 188) and should be quite useful and relevant to the *in vivo* situation (61, 189). Since a single latent virus is theoretically sufficient to initiate viral rebound, curative therapies utilizing the shock-and-kill strategy will need to activate all latent HIV-1 genomes, despite possible various sensitivities to activating agents. Understanding the particulars of virus expression from uDNA in comparison to that from integrated DNA may be helpful for the development of strategies to elicit expression from and elimination of each genome in the latent reservoir (190).

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