LETTER

doi:10.1038/nature12274

HIV-1 causes CD4 cell death through DNA-dependent protein kinase during viral integration

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Human immunodeficiency virus-1 (HIV-1) has infected more than 60 million people and caused nearly 30 million deaths worldwide[1], ultimately the consequence of cytolytic infection of $CD4^+$ T cells. In humans and in macaque models, most of these cells contain viral DNA and are rapidly eliminated at the peak of viraemia[2–4], yet the mechanism by which HIV-1 induces helper T-cell death has not been defined. Here we show that virus-induced cell killing is triggered by viral integration. Infection by wild-type HIV-1, but not an integrase-deficient mutant, induced the death of activated primary CD4 lymphocytes. Similarly, raltegravir, a pharmacologic integrase inhibitor, abolished HIV-1-induced cell killing both in cell culture and in $CD4^+$ T cells from acutely infected subjects. The mechanism of killing during viral integration involved the activation of DNA-dependent protein kinase (DNA-PK), a central integrator of the DNA damage response, which caused phosphorylation of p53 and histone H2AX. Pharmacological inhibition of DNA-PK abolished cell death during HIV-1 infection *in vitro*, suggesting that processes which reduce DNA-PK activation in CD4 cells could facilitate the formation of latently infected cells that give rise to reservoirs *in vivo*. We propose that activation of DNA-PK during viral integration has a central role in $CD4^+$ T-cell depletion, raising the possibility that integrase inhibitors and interventions directed towards DNA-PK may improve T-cell survival and immune function in infected individuals.

To investigate the molecular mechanism underlying HIV-1-induced cell death during acute infection, we first infected CEMX174 T leukaemia cells with HIV-1_{NL4-3} and monitored viral replication and cell death (Fig. 1a). Cells expressing p24 ($p24^+$) remained viable throughout the experiment. By contrast, the majority of cells lacking p24 ($p24^-$) were nonviable by day 6. Similar results were obtained using a single cycle, green fluorescent protein (GFP) encoding vesicular stomatitis virus-G (VSVG)-pseudotyped lentiviral vector (Fig. 1b), indicating that death was independent of the viral envelope and syncytia formation and occurred during a single cycle of viral replication. Cell death induced by this vector was more rapid and correlated with the faster kinetics of infection conferred by VSVG (ref. 5). Primary human CD4 cells showed a similar effect, whether infected by laboratory or primary virus isolates (Fig. 1c and Supplementary Fig. 1).

To determine whether cells lacking viral gene expression had been productively infected before cell death, primary lymphocytes were infected with a replication-competent HIV-1 encoding GFP and sorted for expression and viability (Fig. 2a). Viral cDNA was detected by quantitative real-time PCR (qPCR) in nonviable GFP-negative cells, indicating that these cells had been infected (Fig. 2b). Time-course analysis of viable GFP-positive cells sorted from a culture infected with a GFP-encoding HIV-1 revealed that a very high proportion of these cells died within 2 days and lost GFP expression, yet they retained viral DNA (Fig. 2c and Supplementary Fig. 2a), indicating the dying cells had undergone productive infection that enabled transient reporter gene expression before causing cell death.

To determine which step in viral replication was responsible for cell death, primary lymphocytes were infected in the absence or presence of raltegravir, an integrase inhibitor. Indinavir, a viral protease inhibitor that blocks HIV-1 egress by preventing capsid maturation, was included to restrict viral infection to a single round. In the absence of raltegravir, $p24^-$ cells from the infected culture were largely nonviable (Fig. 3a), as shown above. Raltegravir diminished both cell death and proviral DNA integration. The level of two long terminal repeat (2 LTR) circles increased in the presence of raltegravir, probably because unintegrated reverse transcripts circularize in cells treated with this drug[6]. Efavirenz, a non-nucleoside reverse transcriptase inhibitor that acts earlier in the virus life cycle, also inhibited cell death (Fig. 3a), as expected. Similarly, both raltegravir and efavirenz diminished death of CEMX174 cells infected with a VSVG lentiviral vector (Supplementary Fig. 2b). AZT (3′-azido-3′-deoxythymidine), a late-stage reverse transcriptase inhibitor, and D-118-24, an integrase inhibitor with similar activity to that of raltegravir, also each prevented cell death (Supplementary Fig. 2b).

To document this point, we infected primary CD4 lymphocytes or CEMX174 cells with VSVG-pseudotyped HIV-1 containing a D64V point mutation that abolishes viral DNA integration[7]. Infection with this integrase mutant virus failed to trigger death in either cell type (Fig. 3b and Supplementary Fig. 2c). qPCR confirmed the near complete absence of provirus integration and the accumulation of 2 LTR circles in cells infected with this mutant (Fig. 3b). Together, these results demonstrated that integration was required for death during afferent HIV-1 infection.

Provirus integration requires nuclear import of fully reverse-transcribed viral genomes followed by strand transfer into the host chromosome. Notably, the DNA ends of reverse transcripts may promote apoptosis in cells with defective non-homologous DNA end joining[8]. Because nuclear reverse transcripts form 2 LTR circles in the absence of chromosomal integration[6], inhibition of cell death by a mutant integrase or by raltegravir could result either from cDNA circularization or from a block to strand transfer. To distinguish between these alternatives, we performed knockdown of DNA ligase 4, an enzyme required for 2 LTR circle formation[8]. This treatment substantially reduced 2 LTR circles and concomitantly increased unintegrated reverse transcripts in the nuclei of cells infected with an integrase mutant virus (Fig. 3c and Supplementary Fig. 2d). HIV-1-induced cell death was nonetheless blocked under these conditions (Fig. 3c), suggesting that DNA circularization is not involved in this pathway, and that death signalling requires strand transfer into the host chromosome.

To test whether proviral integration was sufficient to cause cell killing, we used a *tat*- and *rev*-deficient HIV-1 that eliminates viral gene expression without affecting the early steps including integration[9]. This mutant integrated similarly to a matching wild-type virus, and although no viral gene expression was detected, it caused substantial cell death (Fig. 3d). Integration was therefore both necessary and sufficient for triggering cell death by HIV-1.

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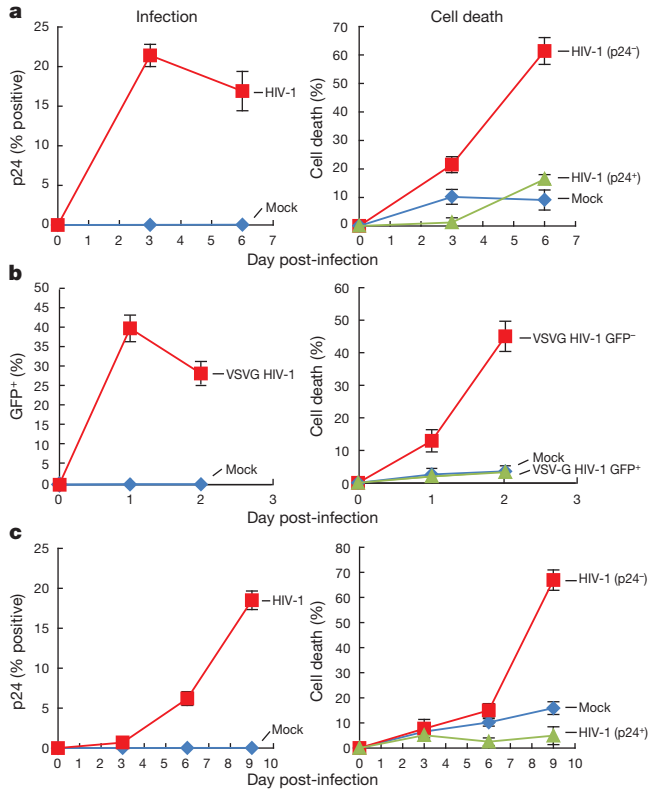

Figure 1 | CD4 lymphocytes killed during HIV-1 infection do not express viral gene products. Time-course analysis of viral replication and cell viability in CEMX174 cells infected with replication-competent HIV-1_{NL4-3} (**a**) or a single-cycle HIV-1 vector encoding GFP (VSVG HIV-1) (**b**) and in primary $CD4^+$ T cells infected with HIV-1_{NL4-3} (**c**). Viral replication was monitored using intracellular p24 Gag staining and cell viability analysis was simultaneously performed on gated $p24^-$ and $p24^+$ populations using Annexin V and the permeability dye Vivid. Time points are indicated at the bottom of each panel. Data are representative of four independent experiments and conducted in triplicate. Error bars represent the standard deviation of the mean for each time point.

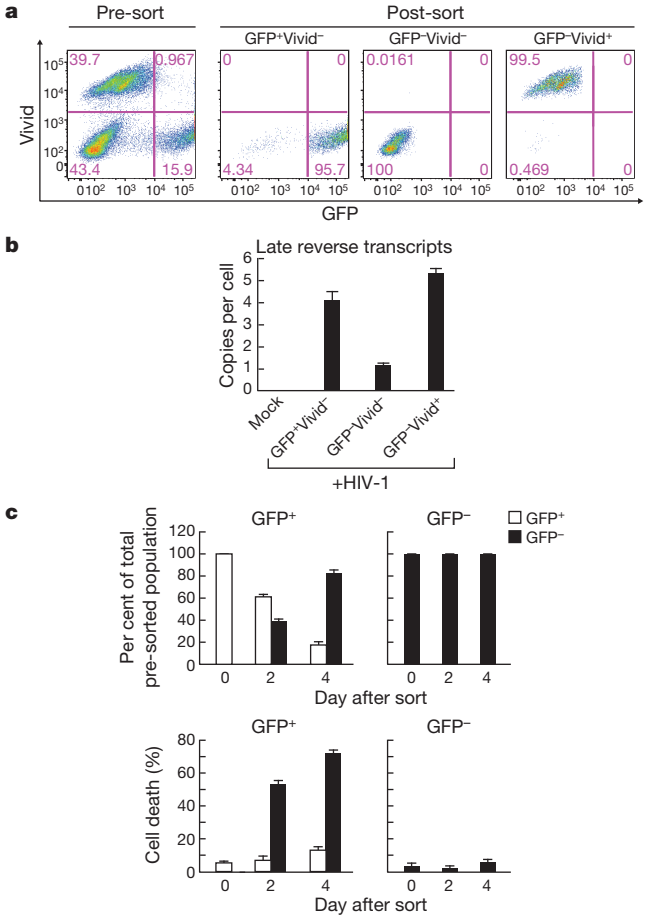

Figure 2 | Dying CD4 lymphocytes lacking viral gene expression had been productively infected prior to cell death. **a**, Flow cytometry analyses of Vivid-stained, primary CD4 lymphocytes infected for 8 days with a GFP-encoding, replication-competent HIV-1 before and after sorting of the indicated populations. **b**, qPCR analysis for late reverse transcripts in the indicated sorted populations shown in **a**. **c**, Time-course analysis of GFP fluorescence and viability in the indicated primary CD4 lymphocyte populations sorted 36 h after infection with VSVG-pseudotyped HIV-1 encoding GFP. Data are representative of two independent experiments from two different donors and conducted in triplicate. Error bars represent the standard deviation of the mean.

We examined this mechanism in natural infection using peripheral blood mononuclear cells from untreated infected subjects. These cells were activated *ex vivo* to induce viral replication, yielding $CD3^{hi}$ $CD8^-$ $p24^+$ cells (Fig. 3e, left and Supplementary Fig. 3a) that showed a statistically significantly reduction in death compared with $CD4^{hi}$ $p24^-$ cells (Fig. 3e, middle and Supplementary Fig. 3b), similar to the *in vitro* experiments. Furthermore, dying $p24^-$ CD4 lymphocytes contained viral DNA (Fig. 3e, right). Raltegravir substantially restored viability under these conditions (Supplementary Fig. 3c, d), indicating that viral integration significantly contributed to cell death. Together these findings suggest that cell death arises from a similar mechanism of signaling during natural infection.

We further explored the cell death mechanism by analysing markers of the double-stranded DNA damage response as they relate to the final steps of viral integration. Infection by both replication-competent HIV-1 and a single-cycle lentiviral vector stimulated DNA-PK activity and phosphorylation of p53 in primary CD4 lymphocytes and CEMX174 cells, as well as triggering H2AX phosphorylation (Fig. 4a). This response was observed with an integrase-competent VSVG HIV-1 but not with a matching integrase-mutant virus (Fig. 4a, right panel). DNA-PK localized to the nuclei of infected cells, confirming the presence of both the kinase and the viral genome in the relevant cellular compartment (Supplementary Fig. 4a). Together these results reveal that integration elicits a cellular double-stranded DNA damage response, suggesting a causal link between DNA-PK activation, p53 phosphorylation and virus-induced cell killing.

We examined the role of DNA-PK in cell death by using NU7026, a pharmacological inhibitor of this kinase. NU7026 abolished p53 and H2AX phosphorylation and blocked death of HIV-1 infected cells without substantially affecting viral replication (Fig. 4b–d and Supplementary Fig. 4b–d). A similar effect was observed with an independent DNA-PK inhibitor, NU7441 (Fig. 4c, d and Supplementary Fig. 4b–d). By contrast, both DNA-PK inhibitors enhanced etoposide-induced killing in primary CD4 lymphocytes (Supplementary Fig. 4e), ruling out non-specific protective effects of these drugs. We further examined the causal link between p53 phosphorylation and cell death using pifithrin, a specific pharmacologic p53 antagonist. This inhibitor substantially decreased cell death following infection of CEMX174 cells (Supplementary Fig. 4f). Finally, ATM, a kinase involved in repair and apoptosis following DNA damage, is also stimulated following infection, but its activation was independent of DNA-PK (Supplementary Fig. 4g). A specific inhibitor of this kinase, KU55933, did not substantially affect the HIV-1-induced damage response and cell death (Fig. 4b and Supplementary Fig. 4h), demonstrating that DNA-PK plays a non-redundant role in inducing cell death after infection.

We demonstrate here that integration triggers signal transduction that causes HIV-1-induced killing in activated CD4 lymphocytes. This cell death occurs in nearly all infected cells and is associated with

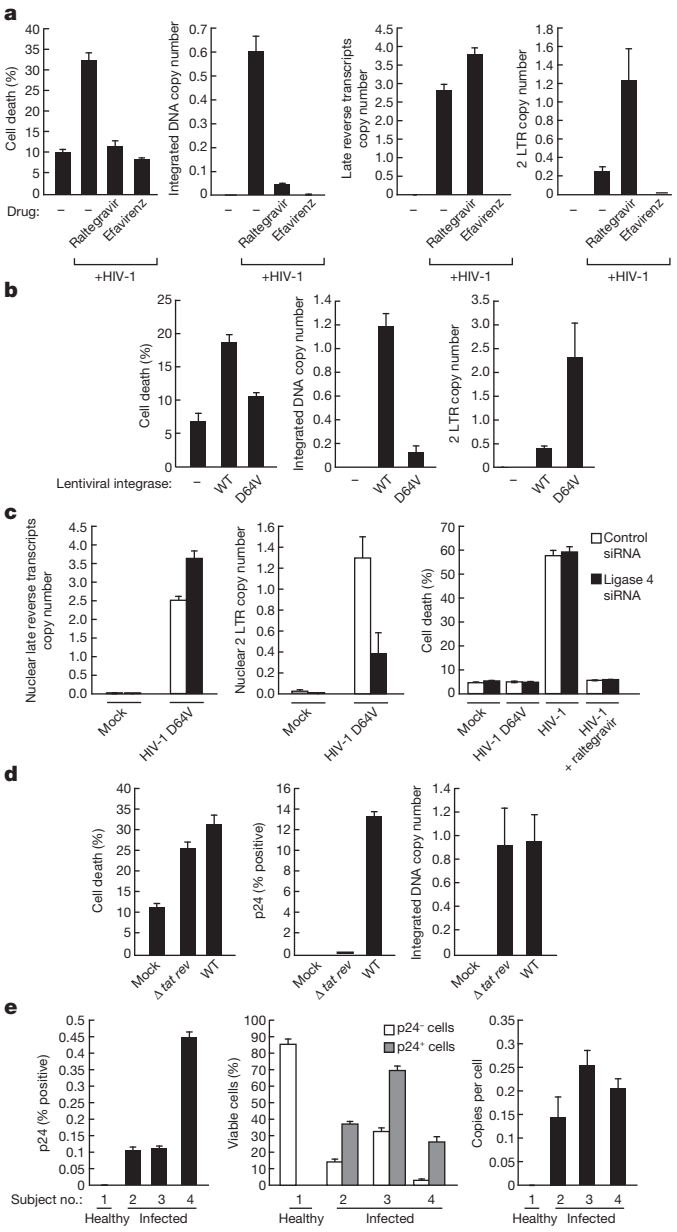

Figure 3 | Proviral DNA integration triggers cell death during HIV-1 infection. **a**, Cell death and the indicated viral DNA analyses in primary CD4 lymphocytes infected with HIV-1_{NL4-3} at a multiplicity of infection (m.o.i) = 1 for 3 days in the presence of indinavir, and in the absence or presence of the indicated inhibitors. **b**, Cell death and viral DNA analyses of primary lymphocytes infected with either a wild-type or a D64V integrase mutant HIV-1 GFP reporter virus. **c**, Cell death and the indicated nuclear viral DNA analyses of cells nucleofected with control or DNA ligase 4 siRNA and infected with the indicated viruses for 3 days. **d**, Cell death, intracellular p24 Gag staining and integrated proviral DNA analyses of primary CD4 lymphocytes either uninfected or infected with the indicated viruses for 3 days in the presence of indinavir. All data are representative of three different experiments done in triplicate. **e**, Death of CD4 lymphocytes lacking p24 expression freshly isolated from HAART-naive HIV-1-infected subjects, and presence of viral DNA in $p24^-$ cells. Viral replication was evaluated by intracellular p24 Gag staining in peripheral blood mononuclear cells from a healthy donor (subject 1) and three different viraemic, untreated patients (subjects 2, 3 and 4) that were activated for 3 days (left). Percentages represent the fraction of $CD3^{hi}$ $CD8^-$ cells. Cell death was determined using Vivid and Annexin V staining followed by flow cytometry analysis of $CD3^{hi}$ $CD8^-$ cells either expressing or lacking intracellular p24 staining in the same samples (middle), and qPCR analysis for HIV-1 late reverse transcripts was performed in dying, $p24^-$ $CD4^+$ T cells sorted from the same peripheral blood mononuclear cell samples (right). Data are representative of two experiments done in triplicate. Error bars represent the standard deviation of the mean.

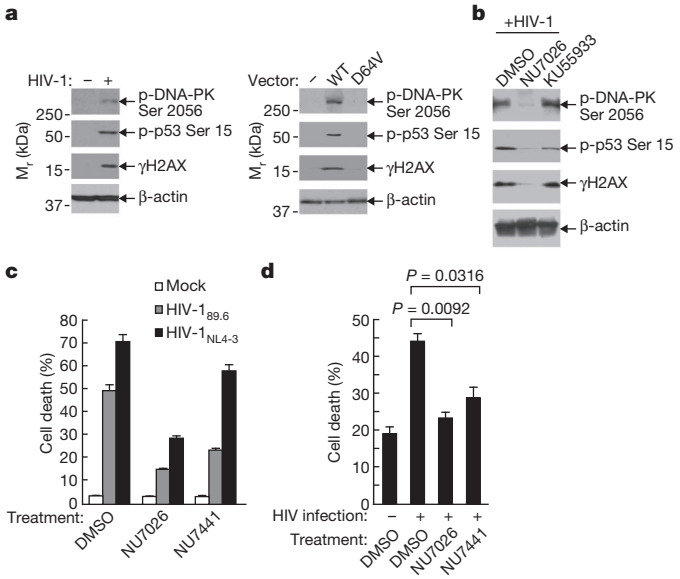

Figure 4 | DNA-PK orchestrates a DNA damage response and cell death following proviral DNA integration. **a**, Western blot analysis showing phosphorylation of DNA-PK, p53 and H2AX following high m.o.i. infections with HIV-1_{NL4-3} in primary CD4 lymphocytes (left panel) or following infection with either VSVG HIV-1 harbouring a wild type (WT) or a D64V integrase mutant (D64V). Extracts from the indicated cultures were analysed 48 h after infection, and cell death peaked after an additional day in the same cultures (data not shown). **b**, Western blot analysis showing phosphorylation of DNA-PK, p53 and H2AX following infection with HIV-1_{NL4-3} in the absence or presence of the indicated DNA-PK and ATM inhibitors. **c**, Cell death analysis of CEMX174 cultures, gated on $p24^-$ cells, that were either uninfected or infected with HIV-$1_{89.6}$ or HIV-1_{NL4-3} in the absence or presence of NU7026 or NU7441 for 4 days. **d**, Cell death analysis gated on $p24^-$ cells of primary CD4 lymphocyte cultures either uninfected or infected with HIV-$1_{89.6}$ in the absence or presence of NU7026 or NU7441 for 7 days. Data in panels **c** and **d** are representative of at least two independent experiments done in triplicate. Error bars represent the standard deviation of the mean of cell death.

productive, not abortive, infection as previously suggested[10], the differences possibly explained by alternative activation states of the cells and by our finding that HIV-1 gene expression is quickly diminished concomitant with death of productively infected cells. We further demonstrate that DNA-PK is activated by the integrating provirus and conveys the death signal. DNA-PK has been the subject of previous studies of HIV-1 infection, in which its role in the virus life cycle has generated controversy[11–14]. However, none of these studies analysed its role in primary human CD4 lymphocytes, the physiological target of HIV-1 infection. Although we observed no substantial change in viral replication when DNA-PK activity was inhibited in activated T cells, it nonetheless remains possible that DNA-PK plays a non-catalytic role in post-integration repair in some circumstances[11,15].

Lentiviral integration proceeds through a combination of cleavage and ligation such that double-stranded breaks in the host genome are avoided[16]. However, the integrase-catalysed reaction generates short gaps in the host genome at each newly formed junction that can potentially lead to DNA-PK activation directly[17], though cell death was not examined previously. Also, because these gaps are susceptible to breakage, a likely stimulus for DNA-PK activation is the presence of double-stranded DNA breaks which can arise secondarily at these junctions. Binding of DNA-PK subunits to the incoming viral pre-integration complex has been previously reported[8,18] and may prime DNA-PK as the sensor for integrating proviral DNA. There is increasing evidence that this kinase is involved in apoptotic signalling[15,19], and the mechanism controlling the relative DNA-PK activities in damage repair versus cell death involves differential autophosphorylation of this kinase and its subcellular localization during the cell cycle[20,21].

Notably, CD4 T-cell activation, which is strongly associated with HIV-induced depletion *in vivo*[2], promotes nuclear translocation and activation of DNA-PK[22], thus providing further support for its role in activated T cells. This observation further indicates that DNA-PK may respond differently during infection in other cell types, such as macrophages or even resting CD4 lymphocytes, a context in which HIV-1 replication may be less cytotoxic[23–25], potentially facilitating proviral integration and establishment of the latent reservoir.

Neither the role of viral integrase nor its effect on DNA-PK has been implicated previously in HIV-1-induced primary CD4 lymphocyte death, and thus this study defines a specific mechanism that significantly contributes to immunodeficiency caused by HIV-1. It may also provide an explanation for the short half-life and high turnover of productively infected CD4 lymphocytes documented in infected individuals[26,27]. Finally, it remains possible that antiviral therapy with integrase and/or DNA-PK inhibitors can help preserve T-cell function *in vivo*. This possibility has been raised recently by a study in patients switching to a raltegravir-containing regimen[28], and it therefore deserves further scrutiny.

METHODS SUMMARY

CD4 T cells were isolated from elutriated lymphocytes of healthy donors using magnetic bead purification, activated and infected as described in the Methods section. Samples from HIV-1-infected subjects were thawed and passed through a dead cell capture column (Miltenyi Biotech) before cell activation. Where indicated, antiretroviral drugs or DNA-PK inhibitors were used at the concentrations given in the Methods. Viability of infected cultures was determined using combined staining with Annexin V-APC (BD Pharmingen) and the amine reactive viability dye Vivid (Invitrogen). In some experiments, the cells were fixed, permeabilized and stained for intracellular p24 Gag (FITC (fluorescein isothiocyanate) or KC-57 PE; Coulter) before flow cytometry analysis with a LSR II cell analyser. Sorting of live, infected cells was done in a BSL-3 facility using a FACSAria sorter. For viral DNA analysis, the DNeasy Blood and Tissue Kit (Qiagen) was used according to the manufacturer's protocol, followed by qPCR using published primers and probes (see Methods). Western blotting for detection of phosphorylated DNA-PK, H2AX and p53 was done on total cell extracts made from uninfected or infected cells under non-denaturing conditions in the presence of phosphatase inhibitors.

Full Methods and any associated references are available in the online version of the paper.

Received 18 May 2012; accepted 9 May 2013.
Published online 5 June 2013.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank D. Ambrozak, R. Nguyen, and S. Perfetto for help with cell sorting, U. Olshevsky, J. Casazza, D. Bolton, A. Pegu and M. Louder for discussions and technical help, and A. Tislerics and B. Hartman for manuscript preparation. This research was supported by the Intramural Research Program of the Vaccine Research Center, NIAID, National Institutes of Health. The findings and conclusions in this report are those of the authors and do not necessarily reflect the views of the funding agency.

Author Contributions A.C. and G.J.N. designed the research studies; A.C., M.G, C.P. and T.Y. performed the research; A.C., M.G. and T.Y. contributed to development and generation of vectors; A.C., C.P., R.A.K. and G.J.N. analysed data; and A.C., R.A.K. and G.J.N. wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to G.J.N. (Gary.Nabel@sanofi.com).

METHODS

HIV-1 infected subjects. Samples from three HIV-1 infected, antiretroviral treatment-naive subjects with CD4 T-cell counts ranging from 24 to 609 cells per μl and viral loads ranging from 47,000 to 500,000 copies per ml were from the Vaccine Research Center study VRC200. To remove dead cells following the thawing process the samples were passed through a dead cell capture column (Pierce), resulting in >90% viability for all samples before activation. The cells were then activated by addition of PHA and IL-2 (40 U per ml) for the indicated times.

Plasmids and virus stocks. The 2 LTR circle construct used for a standard curve in the qPCR analysis was a gift from F. Bushman[6]. The HIV-$1_{89.6}$ construct was a gift from R. Collman and was previously described[29]. The HIV-1_{NL4-3} construct was obtained from M. Martin through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH[30]. The HIV-1 MC99IIIBΔTat-Rev virus was obtained from H. Chen, T. Boyle, M. Malim, B. Cullen, and H. K. Lyerly through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH[9]. The NL4-$3_{n\text{-}e\text{-}GFP}$ construct which contains a mutation in the viral envelope gene and harbours the GFP open reading frame instead of that of the viral Nef gene was a gift from M. Lenardo and was described previously[31]. NL4-$3_{n\text{-}e\text{-}GFP}$ was mutated by the Stratagene Quick Change Site-Directed Mutagenesis Kit to obtain the integrase D64V mutant used in this study. The replication-competent, GFP-encoding virus used in sorting experiments was previously described[32]. For generation of virus stocks, HIV-$1_{89.6}$ and HIV-1_{NL4-3} constructs were transfected into HEK293 T cells using the calcium phosphate method and the supernatants were collected 48 h after transfection. The viruses were then amplified for 7–10 days in CEMX174 cells and the supernatants were collected, clarified by low speed centrifugation, filtered and concentrated using Centricon Plus-70. For generation of VSVG-pseudotyped single-cycle lentiviral vectors, NL4-$3_{n\text{-}e\text{-}GFP}$ encoding either wild-type or a D64V mutant integrase were co-transfected with a plasmid encoding VSVG (pHCMV-VSVG) into 293T cells and viruses were collected and concentrated as above. Virus stocks were titred using TZM-bl cells and were frozen at −80 °C for storage.

Cell lines and primary CD4$^+$ T cell isolation. CEMX174 cells were cultured in RPMI with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). HEK293T cells were cultured in DMEM supplemented with 10% FBS and antibiotics. CD4$^+$ T lymphocytes were isolated from elutriated lymphocytes prepared from blood of healthy donors by negative selection with a CD4$^+$ T cell isolation kit II (Miltenyi Biotech) according to the manufacturer's instructions. The purity of the isolated T cells was assessed by fluorescence-activated cell sorting (FACS) analysis for CD4 (BD-Pharmingen) and 95% of the cells were CD4$^+$ upon isolation. The cells were stimulated with phytohemagglutinin (0.5 μg ml^{-1}) (Remel) and 40 U ml^{-1} IL-2 (Peprotech) for 2-3 days and maintained in 20 U ml^{-1} IL-2 thereafter. CD4$^+$ T cell activation was verified using CD25 staining (BD Pharmingen) and flow cytometry.

Nucleofection and subcellular fractionation. For nucleofection of CEMX174 cells, 2×10^6 cells were mixed with 120 pmol of either non-targeting or human DNA ligase 4 SMARTpool siRNAs (Dharmacon) and electroporated using the Nucleofector II device (Lonza). The DNA Ligase 4 SMARTpool consisted of the following siRNA duplexes: siRNA Lig4-09 (5′-GCACAAGAUGGAGAUGUA-3′), siRNA Lig4-10 (5′-GGGAGUGUCUCAUGUAAUA-3′), siRNA Lig4-11 (5′-GGUAUGAGAUUCUUAGUAG-3′), siRNA Lig 4-12 (5′- GAAGAGGGAAUU AUGGUAA-3′). The cells were cultured for 2 days after nucleofection, and then infected. For subcellular fractionation and isolation of cell nuclei for western blot and qPCR analyses, NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) was used according to the manufacturer's instructions.

HIV-1 infection. Purified, activated CD4$^+$ T cells were infected 2–3 days after isolation. Typically, spreading infections were done by mixing 5×10^5 cells with viral stocks at an m.o.i. of 0.1 followed by washing the cells 16 h after mixing. For single round infection assays, infections were typically performed by spinoculation as previously described[33]. Briefly, 0.5×10^6 cells were mixed with viral stock at m.o.i. = 1 in a total volume of 200 μl in a well of a flat-bottom 24-well plate and centrifuged at 1,200 × *g* for 2 h at 25 °C. Subsequently the cells were repeatedly washed and cultured at a density of 10^6 cells per ml. Mock infections were done by mixing cells with media alone. Infection of CEMX174 cells was typically done by mixing, and cells were seeded at a density of 10^6 cells per ml, except in the experiment in which antiretroviral drugs were used (2×10^6 cells per ml). Where indicated, the following inhibitors were used: indinavir (1 μM), efavirenz (100 nM), AZT (10 μM), raltegravir (50 μM), D-118-24 (50 μM), NU7026(20 μM) (ref. 34) , NU7441(1 μM) (ref. 35), KU55933 (10 μM), and pifithrin[36]. Cells were incubated with the inhibitors 1 h before infection or spinoculation and were present throughout the culture time. For experiments conducted for a period longer than 3 days, cell cultures were replenished with fresh drugs every 2 days.

Flow cytometric analysis of HIV-1-infected cells. CD4$^+$ T lymphocytes were harvested for Annexin V, Vivid and intracellular p24 staining at the indicated times following exposure to virus. In brief, the cells were washed and stained with Annexin V-APC (BD Pharmingen) and Vivid (Invitrogen) for 20 min. Cells were washed once, fixed and permeabilized with Cytoperm/Cytofix (BD Pharmingen) containing 2.5 ml $CaCl_2$ for 20 min. The cells were then stained for p24 Gag (FITC or KC-57 PE; Coulter) for 30 min and washed once in 1× Perm/wash buffer (BD Pharmingen). Flow cytometry was performed with an LSR II cell analyser (Becton Dickinson), and data analysis was performed with FlowJo software (Tree Star).

Quantitative viral DNA analysis. Reverse transcription products of late cellular DNA intermediates of HIV-1 reverse transcription were quantified by TaqMan real-time qPCR at the indicated times post-infection. Cellular DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Published U5/gag primers (LG564/LG699; BioSource International) and a TAMRA-labelled probe (LG-FAM; Applied Biosystems) were used to detect full-length, double-stranded viral DNA[37]. The number of copies was determined using a YU-2 plasmid as standard and as controls for linear amplification in the reaction[38]. For integrated DNA determination, a two-step PCR amplification was performed as described[39]. Briefly, a genomic Alu forward primer and an HIV-1 *gag* reverse primer were used for non-kinetic pre-amplification, followed by a second-round PCR performed using forward and reverse LTR primers and 20 μl of the material from the pre-amplification reaction. These were run with an HIV-1 copy number standard prepared from graded doses of accurately counted ACH-2 cells, which harbour a known copy number of integrated progenomes. The signal potentially contributed from unintegrated DNA was subtracted from the total signal by including a one-way preamplification with the *gag* primer alone. The 2 LTR analysis was performed using published primers and probe as previously described[6]. Primers specific for the human *β*-globin gene were used to monitor the amount of cellular DNA loaded and for normalization. Thermal cycling conditions used during the experiments were 2 min at 95° C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

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