Macrophages archive HIV-1 virions for dissemination *in trans*

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Viruses have evolved various strategies in order to persist within the host. To date, most information on mechanisms of HIV-1 persistence has been derived from studies with lymphocytes, but there is little information regarding mechanisms that govern HIV-1 persistence in macrophages. It has previously been demonstrated that virus assembly in macrophages occurs in cytoplasmic vesicles, which exhibit the characteristics of multivesicular bodies or late endosomes. The infectious stability of virions that assemble intracellularly in macrophages has not been evaluated. We demonstrate that virions assembling intracellularly in primary macrophages retain infectivity for extended intervals. Infectious virus was recovered directly from cytoplasmic lysates of macrophages and could be transmitted from macrophages to peripheral blood lymphocytes *in trans* 6 weeks after ongoing viral replication was blocked. Cell-associated virus decayed significantly from 1 to 2 weeks post infection, but decreased minimally thereafter. The persistence of intracellular virions did not require the viral accessory proteins Vpu or Nef. The stable sequestration of infectious virions within cytoplasmic compartments of macrophages may represent an additional mechanism for viral persistence in HIV-1-infected individuals.

Introduction

The ability of HIV-1 to persist within infected individuals reflects the ability of the virus to exist within different replicative states in different cellular reservoirs. In CD4^{+} T lymphocytes, the replicative state of the virus is dependent upon the cell cycle state of the host cell (reviewed in Stevenson, 2003). In activated lymphocytes, viral replication is efficient, and cytopathic effects of viral replication truncate the lifespan of the infected cell. In quiescent lymphocytes, the virus exists in a latent state and can resume replication if the

host cell is subsequently activated. The ability of the virus to exist within a latent state has been proposed to provide a mechanism for life-long persistence of the virus in infected individuals in the face of potent antiretroviral or immune pressure (Chun *et al*, 1997a, b; Finzi *et al*, 1997, 1999; Wong *et al*, 1997). In addition to CD4^{+} T lymphocytes, tissue macrophages and dendritic cells (DCs) are reservoirs for HIV-1 infection, but information on the replicative state of the virus within these non-T-cell compartments is limited. There are several unusual features of viral replication in these cell types that may favor viral persistence. For example, DCs capture and internalize extracellular virions via DC-SIGN and captured virions can subsequently be transmitted to T cells *in trans* (Geijtenbeek *et al*, 2000; Pohlmann *et al*, 2001; Kwon *et al*, 2002). However, once internalized via DC-SIGN, captured virions rapidly lose infectivity (within several hours) (Moris *et al*, 2004). In HIV-1-infected monocyte-derived macrophages, morphogenically mature viral particles can be observed within intracytoplasmic vesicles (Orenstein *et al*, 1988). These vesicles exhibit characteristics of multi-vesicular bodies otherwise known as major histocompatibility complex class II compartments or late endosomes (Raposo *et al*, 2002; Pelchen-Matthews *et al*, 2003). Whether these cytoplasmic compartments archive virions in an infectious form has not been determined. As such, the biological significance of cytoplasmic virion assembly is unclear. In this study, we demonstrate that virions assembling intracellularly within monocyte-derived macrophages persist and retain infectivity for extended intervals (weeks). The viral accessory protein Vpu, which is important for cell-to-cell spread of HIV-1 in macrophages, is relatively dispensable for transmission of cell-associated virions to T cells *in trans*. Similarly, the accessory protein Nef, which has been shown to induce intracellular accumulation of multivesicular bodies, appears dispensable for maintenance and transmission *in trans* of cell-associated virions. Our studies suggest that intracellular sequestration of HIV-1 virions may provide an additional mechanism for viral persistence.

Results

Cytoplasmic assembly of mature HIV-1 virions is maintained in long-term macrophage cultures

Cytoplasmic compartments into which HIV-1 virions assemble have characteristics of late endosomes (Raposo *et al*, 2002; Pelchen-Matthews *et al*, 2003). Since HIV-1 virions are inactivated in acid pH (Ongradi *et al*, 1990), one would expect that virions rapidly lose infectivity upon endosome acidification. Therefore, we set out to examine the infectious stability of intracellular virus particles in long-term cultures of monocyte-derived macrophages. Continued rounds of virus infection and virion production, even at low levels, would confound analysis of virus stability in long-term macrophages. Therefore, to minimize ongoing viral replication, macrophages were infected with a CXCR4-tropic HIV-1

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variant (HIV-1$_{LAI}$) that is unable to use CCR5 on macrophages for infection and, as a result, is poorly infectious for these cells. To promote infection by this X4-tropic HIV-1 variant, viruses were pseudotyped with vesicular stomatitis virus (VSV) envelope, which confers broad cell tropism. To further ensure that virus infection was restricted to a single cycle, macrophages were infected in parallel with an envelope-deleted variant of HIV-1 (HIV-1$_{\Delta ENV}$) (Figure 1A). At weekly intervals post infection, cell supernatants and cell lysates were analyzed for HIV-1 gag (Figure 1B) using an HIV gag p24 enzyme-linked immunosorbent assay (ELISA) assay, which selectively measures processed gag p24 but inefficiently measures the gag p55 precursor (see Supplementary Figure 1). At 2-week intervals over a 6-week period, cells were removed for analysis by transmission electron microscopy (Figure 1C). After single-cycle infection, levels of extracellular gag p24 decreased steadily over a 6-week period. In comparison, cell-associated gag p24 levels remained relatively constant (Figure 1B). Electron microscopic analysis confirmed the presence of morphogenically mature virus particles within cytoplasmic vesicles of these macrophages at 2, 4 and 6 weeks post infection (Figure 1C). Virus particles budding at the vesicular membrane were evident, suggesting that they were not extracellular virions that had been inter-

nalized by phagocytosis. In addition, cytoplasmic virions were not evident when macrophages were infected with nonpseudotyped viruses (data not shown). Since processing of precursor gag p55 to mature gag p24 protein predominantly occurs after viruses assemble and detach from cellular membranes (Kageyama *et al*, 1992; Kaplan *et al*, 1994), this experiment reveals the presence of a stable cellular compartment for assembly and maturation of HIV-1 virions.

The ability to monitor intracellular virus stability is confounded by the sustained *de novo* production of virions from stably integrated proviruses in infected macrophages. To monitor the decay characteristics of cell-associated virions in the absence of *de novo* virus production, the persistence of cell-associated viral gag protein was evaluated in macrophages infected with an HIV-1 variant that is under strict transcriptional control of doxycycline (DOX; Verhoef *et al*, 2001). Macrophages were infected with DOX-dependent virus (HIV-1$_{2\Delta teto}$) in the presence of DOX to permit infection and *de novo* virus production. At 4 days after infection, DOX was removed from half of the cultures to prevent *de novo* viral gene expression (Figure 2A). In the presence of DOX, cell-associated gag initially declined between days 4 and 9 and then remained relatively constant. In the absence of DOX, cell-associated gag levels dropped to barely detectable levels
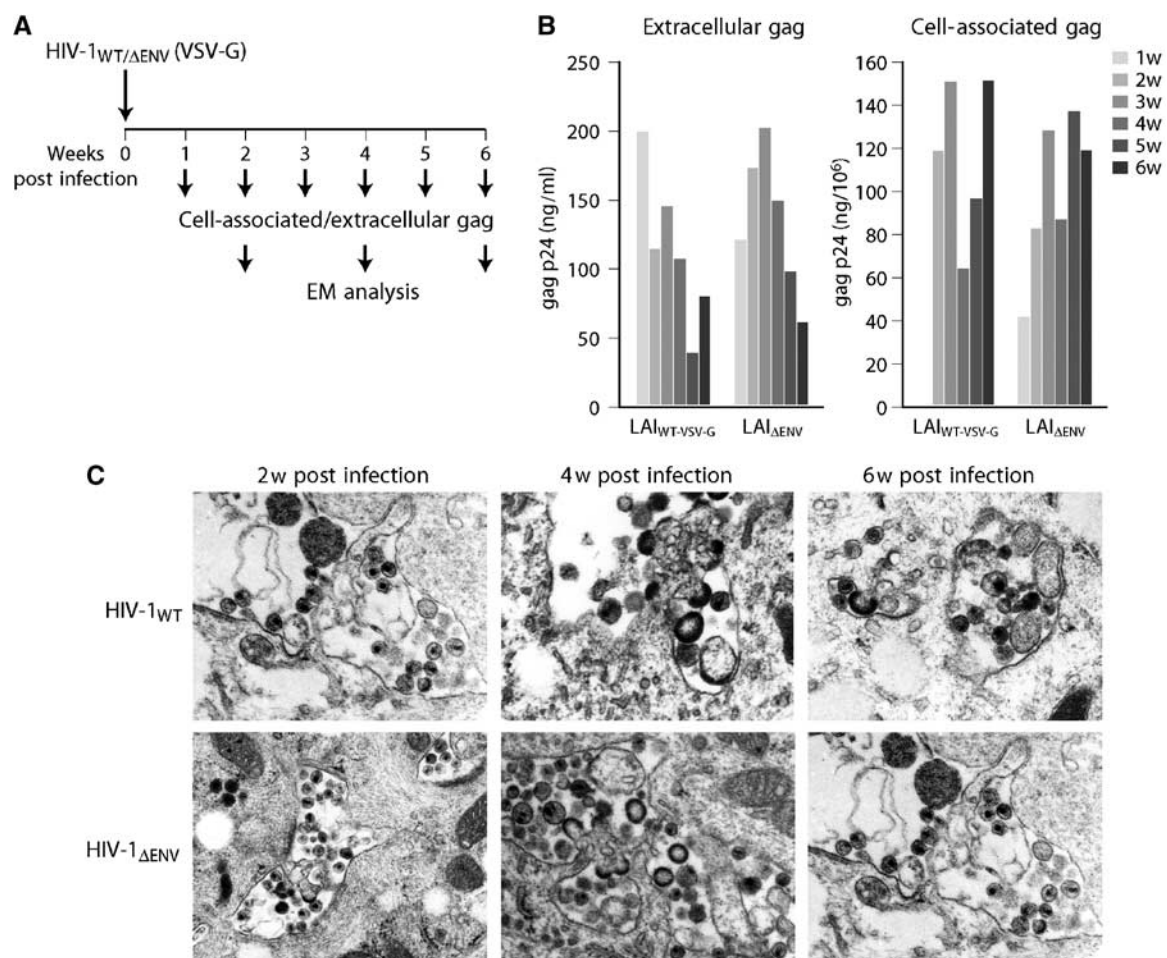

Figure 1 Cytoplasmic assembly of HIV-1 is maintained in macrophages in the absence of a spreading infection. (**A**) Experimental scheme. Monocyte-derived macrophages were infected with VSV-G-pseudotyped, X4-tropic HIV-1 variants containing an intact (HIV-1$_{WT}$) or a defective envelope gene (HIV-1$_{\Delta Env}$). At weekly intervals, cell-associated and extracellular gag p24 levels were determined (**B**) and at 2, 4 and 6 weeks post infection, the presence of morphogenically mature HIV-1 particles in cytoplasmic vesicles was determined by electron microscopy (**C**).

by day 13 post infection (Figure 2B). Therefore, in the absence of DOX, where transcriptional activity of the provirus is impaired, the majority of cell-associated gag declines within 2 weeks. This suggests that the maintenance of cell-associated gag in long-term macrophage cultures, as observed in Figure 1, is due predominantly to sustained *de novo* virus production.

Macrophages constitute a stable compartment for dissemination of viruses to lymphocytes in trans

DCs capture extracellular virions via C-type lectin receptors such as DC-SIGN and transfer internalized virions upon subsequent contact with lymphocytes (referred to as infection *in trans*). Dissemination *in trans* can occur with very small amounts of captured virions (Pohlmann *et al*, 2001). Macrophages can also mediate efficient infection of lympho-cytes *in trans* (Carr *et al*, 1999; Swingler *et al*, 2003) and in this case, virions generated *de novo* in infected macrophages are able to initiate lymphocyte infection far more efficiently than cell-free virions (Carr *et al*, 1999). The data portrayed in Figure 2 indicate that when *de novo* gene expression was restricted, cell-associated gag had a half-life of several days. Taken at face value, this would predict that cell-associated virions also have a similar half-life. However, some gag is processed in the cytoplasm (Kaplan and Swanstrom, 1991). Therefore, not all cell-associated gag is likely to be virion associated and the physical stability of processed gag within the cytoplasm and virion-associated gag contained within intracytoplasmic vesicles may be different. Therefore, we used a strategy that would permit detection of infectious cell-associated virions even if present in low abundance. At the same time, *de novo* virus replication was prevented by the presence of suprainhibitory concentrations of the pro-tease inhibitor indinavir sulfate (IVS). In the presence of IVS, processing of the gag precursor is arrested and virus particles that are produced are immature and hence are noninfectious (Supplementary Figure 1). In spreading infections (macro-phages infected with an R5-tropic HIV-1 variant), $0.1\,\mu M$ IVS was completely inhibitory (Figure 3). A suprainhibitory con-centration of $10\,\mu M$ IVS was selected in order to ensure complete cessation of gag maturation and infectious virus production. Therefore, infectious viruses recovered from macrophages subsequent to addition of IVS should, by defini-tion, have been formed prior to addition of the inhibitor. We favored this approach over the use of a DOX-dependent virus because of the reported low basal level of virus production in the absence of DOX (Verhoef *et al*, 2001). The presence of infectious cell-associated virus in long-term macrophage cultures was determined by their ability to transmit virus to lymphocytes *in trans*. To maximize the sensitivity of the assay for infection of lymphocytes *in trans* in the presence of IVS, the synthesis of viral cDNAs (2-LTR (long terminal repeat) circles) in infected lymphocytes was determined by real-time PCR, which permits 2-LTR cDNA detection down to five copies (assay cutoff) (Sharkey *et al*, 2000) (Supplementary Figure 2). Macrophages were infected under single-cycle conditions (VSV-G-pseudotyped HIV-1$_{LAI}$) and IVS was added either 16 h or 7 days post infection (Figure 4A). Extracellular gag p24 levels were monitored weekly (Figure 4B), and at weeks 4 and 7 post infection, activated peripheral blood lymphocytes (PBLs) were added to the infected macrophage cultures for 2 h to allow infection
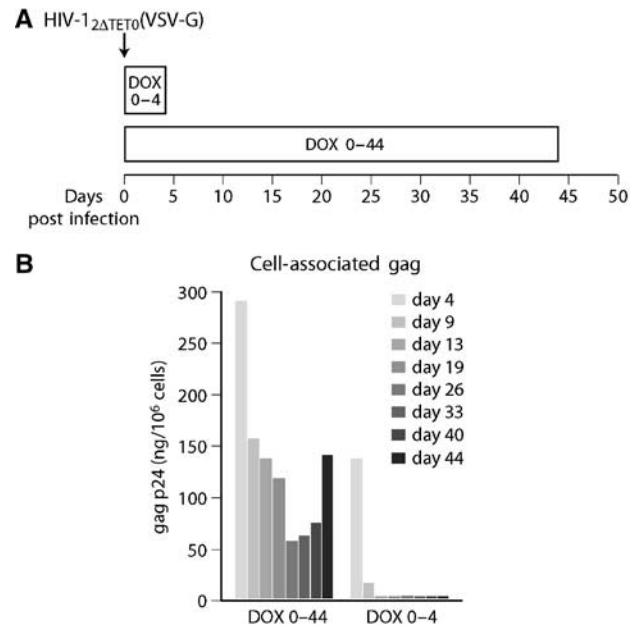

A HIV-1$_{2\Delta TET0}$(VSV-G)

Figure 2 Stability of cell-associated virus in the absence of *de novo* viral gene expression. (**A**) Experimental design. HIV-1$_{2\Delta teto}$ contains a bacterial tet promoter, and proviral gene expression is dependent upon the presence of DOX. Macrophages were infected with VSV-G-pseudotyped HIV-1$_{2\Delta teto}$ in the presence of DOX. After 4 days, *de novo* viral gene expression was terminated by removal of DOX. (**B**) Levels of cell-associated gag p24 in macrophages maintained in the presence of DOX (DOX 0–44) or in macrophages in which DOX was removed 4 days after infection (DOX 0–4).
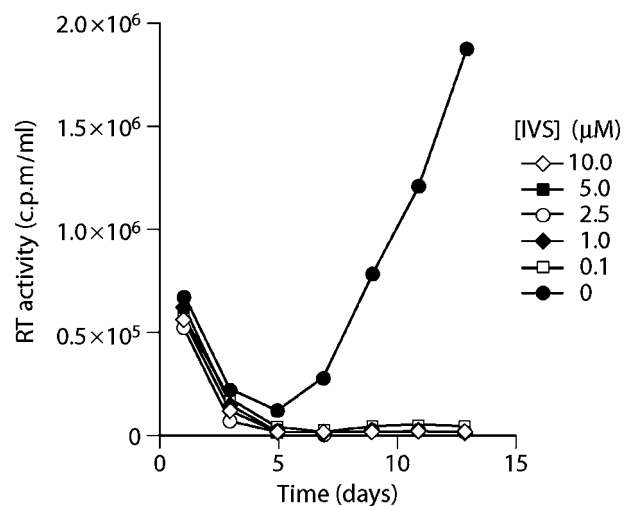

Figure 3 Potent suppression of spreading HIV-1 replication in macrophages by IVS. Macrophages were infected with an R5-tropic HIV-1 variant (HIV-1$_{ADA}$) and maintained in IVS at the indicated concentrations. Viral replication was monitored by measurement of RT activity in culture supernatants.

in trans (Figure 4C). IVS was maintained in cultures during infection *in trans* to avoid potential restoration of virus maturation. In the presence of IVS, there was, as expected, a marked inhibition of gag processing and virus maturation, as evidenced by the levels of extracellular gag p24 detectable by ELISA (Figure 4B). Surprisingly, the presence of infectious virus, capable of initiating lymphocyte infection *in trans*, was
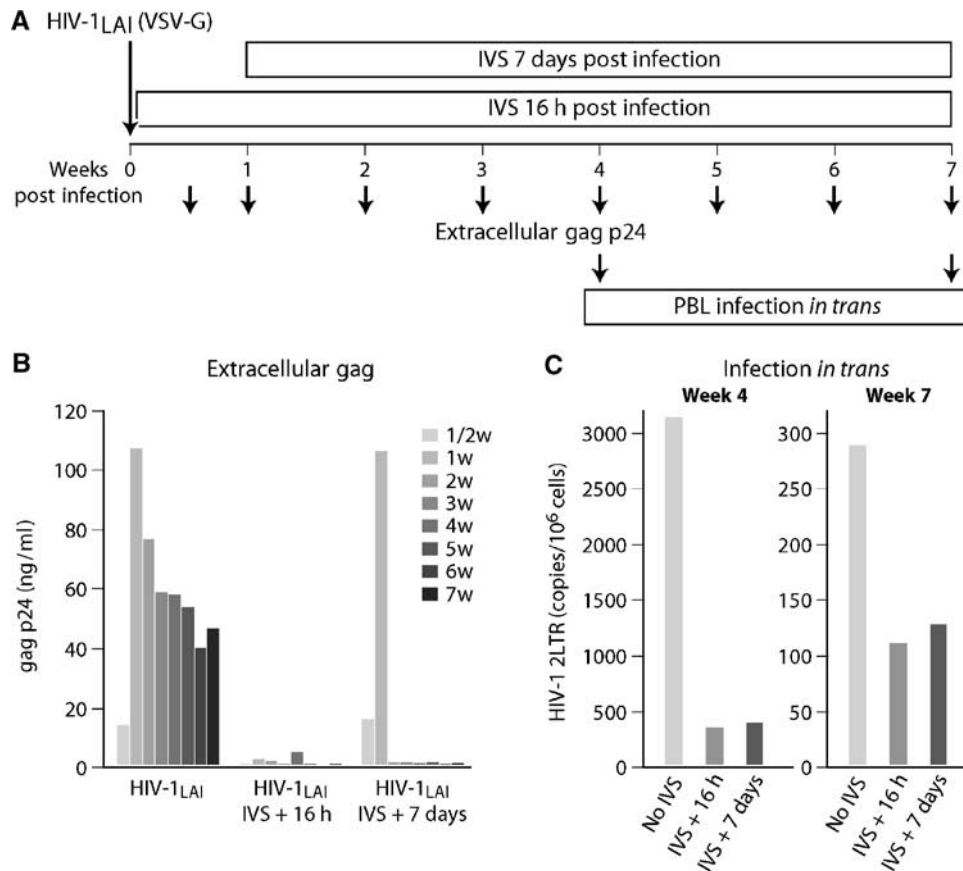
Figure 4 Intracellular virions capable of initiating T-cell infection *in trans* persist in macrophages. (**A**) Macrophages were infected with a VSV-G-pseudotyped X4-tropic HIV-1 variant (HIV-1$_{LAI}$). At 16 h and 7 days post infection, further *de novo* production of infectious virus was blocked by IVS. At the indicated intervals, culture supernatants were examined for the presence of mature virions containing processed gag p24 by ELISA (**B**). At 4 and 7 weeks post infection, the presence of infectious virus, able to initiate lymphocyte infection *in trans,* was determined after addition of activated PBLs. Infection *in trans* (**C**) was determined by PCR quantitation of 2-LTR circle cDNAs in PBLs. Data shown are representative of four independent experiments.

evident in these cultures at 4 and 7 weeks post infection even if IVS was added 16 h after infection (Figure 4C). Notably, levels of extracellular processed gag p24 were almost 2 logs lower in IVS-treated cultures at 7 weeks post infection (Figure 4B). However, there was no more than a three- to seven-fold difference in the efficiency of lymphocyte infection *in trans* by macrophages incubated in the presence and absence of IVS (Figure 4C). In the presence of 10 μM IVS, gag processing is blocked (see Supplementary Figure 1). Extracellular gag in IVS-treated macrophage cultures fluctuated around background levels and was due to inefficient detection of unprocessed gag p55 by the ELISA assay (see Supplementary Figure 1). In an independent experiment, when lymphocytes were added to infected macrophage cultures in the presence of the reverse transcriptase (RT) inhibitor nevirapine, 2-LTR circle formation was blocked (see Supplementary Figure 3). This demonstrated that infection of lymphocytes *in trans*, as evidenced by 2-LTR circle formation, was not due to carryover of infected macrophages upon removal of lymphocytes from the cocultures.

In order to evaluate the decay characteristics of the virus reservoir that was initiating infection *in trans*, macrophages were infected and 4 days after infection, IVS was added to block *de novo* infectious virus production (Figure 5A). At weekly intervals over a 6-week period, activated PBLs were

added and infection *in trans* was determined by PCR for viral cDNA. Between 1 and 2 weeks post infection, there was approximately a 1 log reduction in the amount of virus disseminated to lymphocytes *in trans* (Figure 5B). However, between weeks 2 and 6 post infection, the level of cell-associated virus, able to initiate lymphocyte infection *in trans,* declined by approximately three-fold overall (Figure 5B). Taken together with the data in Figure 2, these results suggest an initial rapid decay in cell-associated virus followed by a second more gradual decay. Infectious virus was evident 5 weeks after *de novo* infectious virus production was blocked by IVS, suggesting that some intracellular virions are relatively stable.

Stability and dissemination of intracellular virions does not require a functional Nef protein

Several studies have suggested that the HIV-1 accessory protein Nef may influence the biogenesis and function of cellular endosomes. For example, HIV-1 and SIV Nef, when expressed in lymphocytes, induce accumulation of late endosomes and lysosomes as well as the accumulation of multivesicular bodies in HeLa cells (Guatelli, 1997; Sanfridson *et al*, 1997; Stumptner-Cuvelette *et al*, 2003). This raises the possibility that the viral Nef protein may contribute to the retention and stability of cell-associated
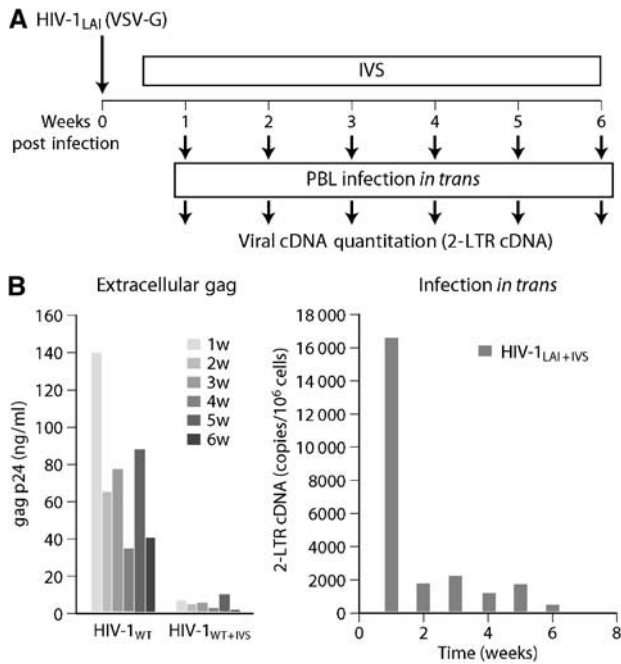
Figure 5 Decay characteristics of cell-associated virus in long-term macrophage cultures. (**A**) Macrophages were infected with VSV-G-pseudotyped HIV-1$_{LAI}$ and 4 days after infection, *de novo* virus production was blocked by addition of IVS. At the indicated time intervals, the presence of processed gag p24 in culture supernatants (**B**, left panel) was determined and the presence of infectious cell-associated virus was evaluated *in trans* following addition of activated PBLs (B, right panel). Data shown are representative of three independent experiments.

virions in macrophages by promoting the accumulation of cytoplasmic vesicles into which virions assemble. To investigate this, macrophages were infected with VSV-G-pseudotyped HIV-1 variants that were wild type or deleted with regard to the *nef* gene. At 4 days post infection, *de novo* virus production was arrested by addition of IVS (10 μM). Extracellular gag p24 and infectious virions were measured at weekly intervals over a 7-week period (Figure 6B and C). The presence of cell-associated infectious virus, as assayed by the ability to infect lymphocytes *in trans,* was further determined at 3, 5 and 7 weeks post infection (Figure 6D). Despite the sustained presence of IVS, infectious virus, capable of initiating lymphocyte infection *in trans,* was evident even at 7 weeks post infection (Figure 6D). For macrophages infected with a *nef*-deleted virus, the level of cell-associated infectious virus was comparable (Figure 6D). Although the levels of extracellular gag and extracellular virions were markedly reduced in the presence of IVS (Figure 6B and C), the presence of cell-associated virions, as evidenced by the ability to initiate lymphocyte infection *in trans* (Figure 6D), was comparable for long-term macrophage cultures maintained in the presence and absence of IVS. The efficiency of PBL infection *in trans* by wild-type and ΔNef-infected macrophages was comparable (Figure 6C). However, when assayed directly on MAGI cells (HeLa CD4-β-galactosidase indicator cell line), supernatants from HIV-1$_{Δnef}$-infected macrophages were approximately 1 log less infectious when compared to supernatants from wild-type-infected macrophages (Figure 6C). This is to be expected since previous

studies have demonstrated that ΔNef viruses are 1 log less infectious in MAGI cells (Guatelli, 1997). Therefore, the Nef infectivity defect in MAGI cells is apparent when virions are recovered from infected macrophages. However, Nef appears to be dispensable for maintenance and dissemination *in trans* of intracellular virions.

The accessory protein Vpu is required for a spreading infection in macrophages but is not required for dissemination in trans of cell-associated virus

The Vpu protein of HIV-1 has been shown to promote virus particle release from the plasma membrane although the replication of wild-type and Vpu-deleted viruses compares favorably in lymphocytes and T-cell lines (Bour and Strebel, 2003). In agreement with a previous study (Deora and Ratner, 2001), a ΔVpu virus containing an R5-tropic envelope replicated inefficiently in macrophages relative to its wild-type counterpart (Figure 7A). We next compared whether *Vpu* is required for stability or dissemination of cell-associated virus. Macrophages were infected with X4-tropic wild-type and ΔVpu HIV-1 variants (VSV-G-pseudotyped). At 4 days after infection, IVS was added to prevent virus maturation (Figure 7B). At 4 and 6 weeks post infection, cell-associated and extracellular gag p24 levels were determined (Figure 7C) and the presence of cell-associated virus was determined by titration of cytoplasmic lysates on MAGI cells and by lymphocyte infection *in trans*, as outlined in Figure 6. At 4 and 6 weeks, the levels of particle release (extracellular gag) were approximately three-fold lower in macrophages infected with a ΔVpu virus, while cell-associated gag p24 levels were equivalent (Figure 7C). To detect directly the presence of cell-associated infectious virions in these long-term cultures, macrophages were trypsinized to remove cell-surface-bound virions, and cytoplasmic lysates were prepared and titered on MAGI cells. Infection of MAGI was determined by 2-LTR cDNA quantitation rather than β-galactosidase production because of the far greater sensitivity of the 2-LTR assay in detecting low levels of MAGI cell infection. Despite the sustained presence of IVS, infectious virions could be recovered from lysates of macrophages infected with wild type or ΔVpu variants at 4 and 6 weeks post infection (Figure 7E). Production of viral cDNA in MAGI cells was blocked in the presence of an RT inhibitor (result not shown), indicating that this was due to *de novo* infection and cDNA synthesis and not due to macrophage carryover. Based on PBL infection *in trans* (Figure 7D) and titration of cell lysates (Figure 7E), there appeared to be higher levels of ΔVpu virions in these long-term cultures at 4 and 6 weeks post infection. However, in an independent experiment, sequestration of wild-type and ΔVpu virions in long-term macrophage cultures, as evidenced by lymphocyte infection *in trans*, was comparable (not shown). Therefore, despite a strong requirement for Vpu in viral replication in macrophages, Vpu appears dispensable for the maintenance and dissemination *in trans* of cell-associated virions in macrophages.

Discussion

The experiments outlined in this study present evidence for an intracellular compartment in macrophages into which HIV-1 virions assemble and in which virions retain infectivity for extended intervals. Although infectious virions were
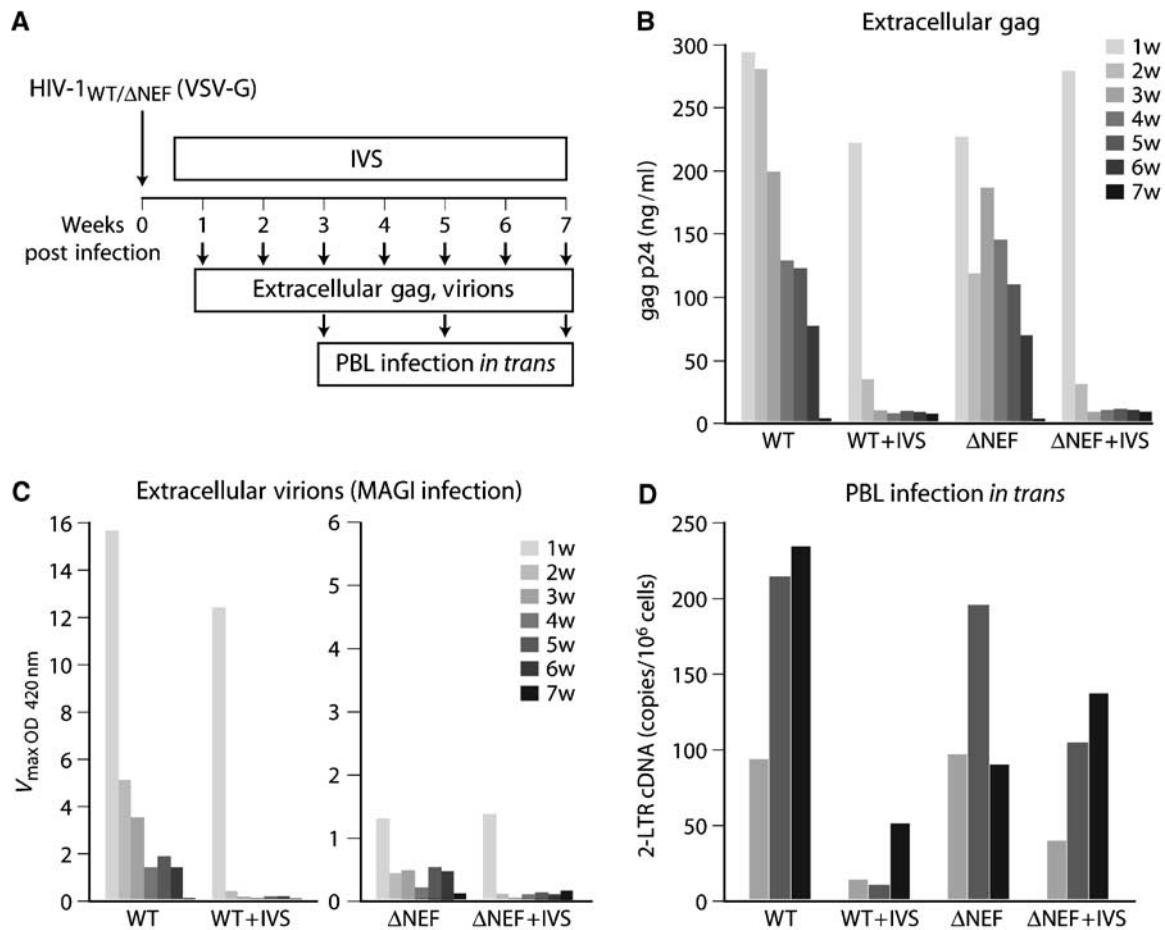
Figure 6 Role of the accessory protein Nef in the maintenance of cell-associated virus in long-term macrophage cultures. (**A**) Experimental scheme. Macrophages were infected with VSV-G-pseudotyped HIV-1 variants harboring intact (HIV-1$_{WT}$) or mutated (HIV-1$_{\Delta nef}$) *nef* genes. At 4 days after infection, cells were maintained in the presence or absence of IVS. At the indicated intervals, extracellular gag p24 was determined by ELISA (**B**), and extracellular infectious virions were titered on MAGI cells (**C**). The presence of infectious cell-associated virus was determined following addition of PBLs and measurement of infectivity *in trans* at 3, 5 and 7 weeks post-infection (**D**). Data shown are representative of three independent experiments.

sequestered intracellularly at low levels, these results have important implications regarding reservoirs for viral persistence. Because of the efficiency of viral dissemination *in trans* and the replicative capacity of the virus, low levels of intracellular infectious virus may ignite viral replication upon transmission to lymphocytes (Carr *et al*, 1999). By analogy, DCs can capture extracellular virions and, even though at undetectable levels, captured virions can initiate a vigorous infection upon dissemination to lymphocytes (Cameron *et al*, 1992). Furthermore, rare latent proviruses ignite efficient replication upon reactivation from latency (Kieffer *et al*, 2004). While such mechanisms may have a minor contributing role to cumulative virus burden in highly viremic individuals, they have central significance in aviremic patients on highly active antiretroviral therapy.

In long-term macrophage cultures, infectious virus could be recovered over 6 weeks after virus maturation was blocked. In addition, there appeared to be two decay characteristics in the infectivity of cell-associated virus. Between 1 and 2 weeks following infection, the level of cell-associated infectious virus fell by approximately 1 log and then declined minimally over the next 4–5 weeks. In macrophages infected with a DOX-dependent virus, the level of cell-associated gag declined by 2 logs within 2 weeks of DOX removal. Since gag processing can occur in the cytoplasm (Kaplan and Swanstrom, 1991; Kaplan *et al*, 1994), it is possible that cytoplasmic gag decays more rapidly compared with virion-associated gag, which is sequestered in cytoplasmic vesicles. Therefore, direct measurement of virus infectivity *in trans* rather than cell-associated gag may provide the most accurate assessment of the decay characteristics of intracellular virions. The initial rapid decline in viral infectivity may be due to degradation of virions or release of intracellular virions into the extracellular space. Indeed, late endosomes into which HIV-1 particles assemble in macrophages can be transported to the cell surface where they would normally release MHC class II-peptide complexes (Geuze, 1998). Furthermore, there are distinct intermediates in the multivesicular elements that constitute the endosomal degradation pathway (Gruenberg and Stenmark, 2004). Therefore, it is also possible that viruses are sequestered within different vesicular compartments with distinct recycling characteristics.

Several reports have characterized the cytoplasmic compartments into which HIV-1 assembles as late endosomes (Raposo *et al*, 2002; Pelchen-Matthews *et al*, 2003).
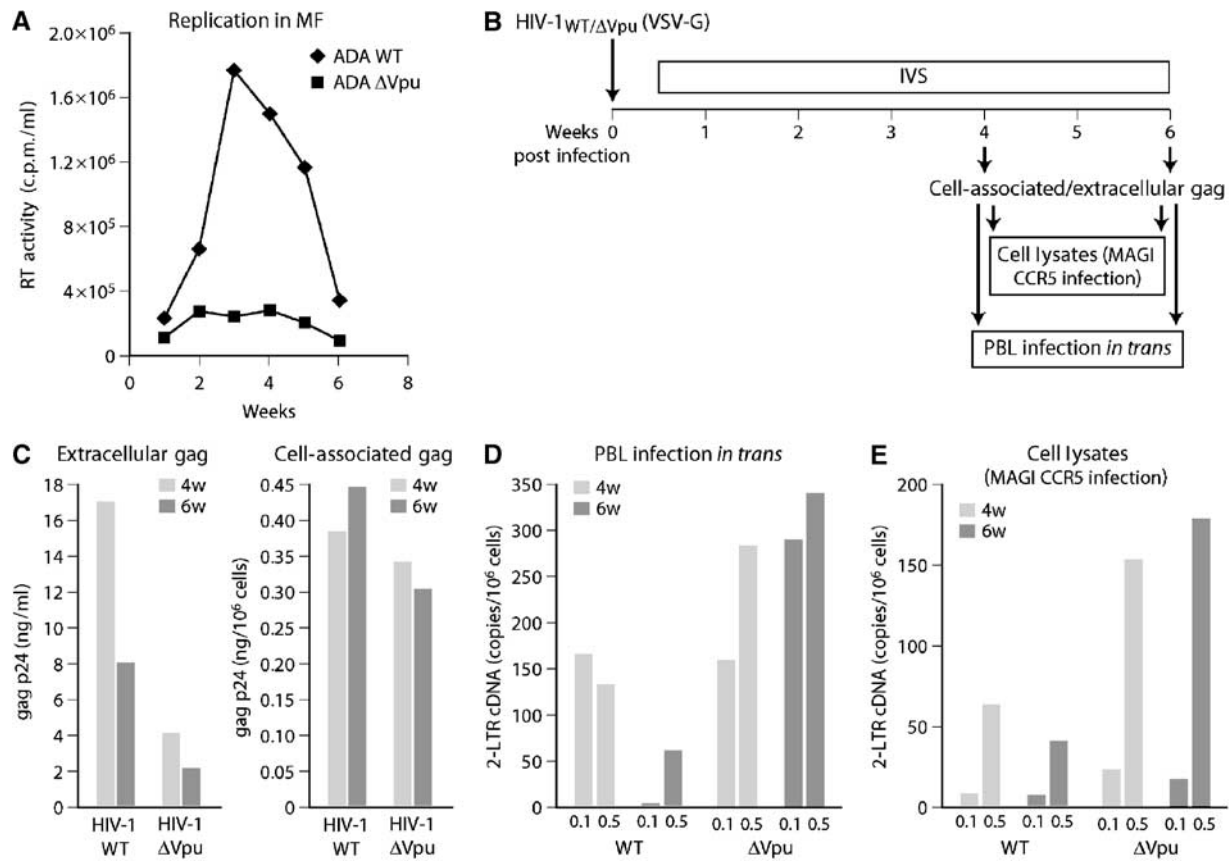
Figure 7 The accessory protein Vpu is required for an efficient spreading infection in macrophages but not for maintenance and viral dissemination *in trans* of cell-associated virus. (**A**) Requirement for Vpu in a spreading viral infection of macrophages. Macrophages were infected with R5-tropic HIV-1$_{ADA}$ variants comprising intact (ADA$_{WT}$) or mutated (ADA$_{\Delta Vpu}$) *vpu* genes. Viral replication in macrophages was assessed by measurement of RT activity in culture supernatants. (**B**) Role of Vpu in the maintenance of cell-associated virus was examined using the same strategy as in Figure 6. Macrophages were infected with VSV-pseudotyped X4-tropic HIV-1 variants containing functional or inactive *vpu* genes. IVS was then added 4 days post infection to inhibit virus production. At 4 and 6 weeks post infection, the presence of processed gag p24 in culture supernatants and in cell lysates was determined by ELISA (**C**). (**D**) Infectivity of cell-associated virus at 4 and 6 weeks post infection was determined following addition of PBLs and measurement of infectivity *in trans*. 2-LTR cDNAs were quantitated in two aliquots (0.1 and 0.5) of infected PBL lysates. (**E**) The presence of infectious virus in cytoplasmic macrophage lysates was directly assessed by titration on MAGI cells. 2-LTR cDNA products were quantitated in two aliquots (0.1 and 0.5) of MAGI cell extracts. Data shown are representative of two independent experiments.

Therefore, the apparent stability of cytoplasmic virions, as described in this report, would argue that endosomes, into which HIV-1 virions assemble, do not undergo acidification. Intriguingly, Nef proteins of primate lentiviruses have been shown to interact with vacuolar ATPase (Lu *et al*, 1998; Mandic *et al*, 2001) and this vacuolar proton pump plays a central role in endosome acidification. While it is possible that Nef, upon interaction with the vacuolar ATPase, might interfere with endosomal acidification, our experiments indicate that Nef does not contribute to the stability of cytoplasmic virions within primary macrophages. The long-term stability of cytoplasmic virions in macrophages, as reported here, contrasts with the reported instability of virions internalized by DCs via DC-SIGN (Moris *et al*, 2004). In this case, viral infectivity is retained for a few hours after capture by DCs. It is likely that DCs internalize virions into endosomes, which subsequently undergo acidification, thereby inactivating viral infectivity.

At present, it is unclear whether intracellular budding of virions in macrophages is a consequence of a leaky viral assembly process or whether it is a mechanism that HIV-1 has evolved because it is advantageous to viral replication. In cell lines, overexpression of gag proteins results in distribution and processing of the gag precursor in the cytoplasm (Kaplan and Swanstrom, 1991). Virus assembly in U1 promonocytic cells can be redirected to intracytoplasmic vesicles following interferon γ treatment (Biswas *et al*, 1992). In addition, HIV-1 gag contains a motif that promotes interaction with multivesicular bodies (Lindwasser and Resh, 2004). HIV-1 gag MA mutants, which inefficiently assemble at the cell surface of HeLa and T cells, assemble predominantly in the endosome of primary macrophages and are efficiently released as extracellular virions (Ono and Freed, 2004). Furthermore, this cell type-specific cytoplasmic assembly is regulated by phosphatidylinositol bisphosphate (PI(4,5)P$_2$) such that depleting cellular PI(4,5)P$_2$ redirects virus assembly from the plasma membrane to late endosomes (Ono and Freed, 2004). Therefore, endosomal assembly of HIV-1 appears to be a regulated process that the virus may have adopted in macrophages. One might envision that cytoplasmic assembly of virus particles may be advantageous to viral replication *in vivo*. During cell-to-cell transfer between an infected

macrophage and a substrate CD4$^+$ T lymphocyte, cytoplasmic vesicles harboring virus particles may be directed to the vicinity of contact between the macrophage and the T cell, thereby favoring viral dissemination. The ability of viruses to persist within cytoplasmic compartments for extended intervals further supports the notion that macrophages may archive virions in an infectious state, thereby contributing to viral persistence.

Materials and methods

Lymphocytes and macrophages
PBLs and monocytes were obtained by leukapheresis from normal donors sero-negative for HIV-1 and hepatitis B. Monocytes were further separated by counter-current centrifugal elutriation as detailed elsewhere (Gendelman et al, 1988). Elutriated monocytes were cultured for 2 days in medium containing monocyte colony stimulating factor (MCSF) (RD Systems) and for a further 5 days in medium lacking MCSF prior to use in experiments. Elutriated PBLs were activated with PHA (4 μg/ml) and were cultured in RPMI containing 10% human serum and 64 U/ml of interleukin-2 (ICN). MAGI cells (CD4$^+$ HeLa cells) (Kimpton and Emerman, 1992) and 293T cells were maintained in DMEM containing 10% FBS.

Viruses
HIV-1 stocks were obtained after transfection of 293T cells using a modified calcium phosphate/DNA precipitation method (GIBCO BRL, Gaithersburg, MD). To obtain pseudotyped viruses, 293T cells were transfected with an HIV-1 molecular clone (25 μg) and a VSV-G envelope expression vector (15 μg). Viruses in culture supernatants were harvested after 24 h, passed through a 0.45 μm filter and quantitated by measurement of RT activity.

Infections
For cell-free infections, 1×10^6 monocytes in 12-well plates were directly infected with VSV-G-pseudotyped viruses (1×10^6 c.p.m. RT/ml or 1 μg p24/ml). This inoculum typically results in 0.1 full-length cDNA copies/cell and 0.01–0.001 2-LTR circle copies/cell (Triques and Stevenson, 2004). After 3 h, cells were washed in fresh medium and incubated at 37°C. For infection in trans, 2×10^6 PBLs (PHA activated, 4 μg/ml) were added to 1×10^6 macrophages. After 2 h, PBLs were removed and cultured in 24-well plates and viral cDNA synthesis was determined by PCR after 48 h. For infections with DOX-dependent HIV-1 (HIV-1$_{2\Delta\text{teto}}$), virus was produced in the presence of 1 μg/ml DOX and infected cells maintained in the presence of DOX. For titration of low levels of virions in cytoplasmic lysates from long-term macrophage cultures, macrophages were washed 2× in PBS and then incubated with 200 μl of PBS containing 0.001% Triton X-100 and aliquots of the lysates were used for infection of MAGI cells in 96-well microtiter plates (2×10^4 cells/well). After 2 h, 150 μl DMEM/10% FBS was added. After 48 h, cells were harvested for analysis of 2-LTR cDNA by PCR in order to boost the sensitivity of detection. For titration of virions in culture supernatants, doubling dilutions of culture supernatants were added to MAGI cells for 2 h. After 48 h, 125 μl lysis buffer (Promega) was added and cells were freeze–thawed once to ensure complete lysis. β-Galactosidase reporter gene activity was assayed by combining cell lysates (50 μl) with 2× β-gal assay buffer (Promega). The V_{max} of the enzyme assay was determined at 420 nm over a 2 h period in a Spectramax 340 kinetic plate reader (Molecular Devices).

Viral assays
Viral gag p24 (capsid) was measured by ELISA according to the manufacturer's protocol (Beckman-Coulter). This assay inefficiently detects the gag p55 precursor protein (see Supplementary Figure 1). Viral RT activity was determined as outlined previously (Brichacek and Stevenson, 1997).

Electron microscopy analysis
Macrophages in 24-well culture plates were washed twice with PBS and incubated at room temperature in 1.25% glutaraldehyde/PBS for 10 min and then in 2.5% glutaraldehyde/PBS for 30 min. Fixed macrophages were detached by scraping and postfixed in 0.1 M sodium cacodylate buffer–1% osmium tetroxide for 1 h. Cells were dehydrated in an ethanol series to propylene oxide and then embedded in Epon 812/propylene oxide. Cell sections (60–70 nm thick) were stained with lead citrate for 6 min and uranyl acetate for 8 min and sections examined in a Philips CM 10 or CM 12 microscope (80 kV accelerating voltage).

Analysis of viral infection by PCR
Macrophages were washed with 0.1% trypsin in PBS to remove cell surface-associated virions. Cells were then incubated with 900 μl DNAzol (Invitrogen) and dispersed by repeated pipetting. A 500 μl portion of 100% ethanol was added and cell lysates were centrifuged at 14 000 r.p.m. for 8 min and the cell pellets washed with 75% ethanol. Lysates were centrifuged (14 000 r.p.m., 5 min) and pellets were resuspended in 50 μl of 8 mM NaOH. DNA lysates were neutralized with Tris (10 mM, pH 8.5) containing 2 μl polyacryl carrier. Real-time PCR quantitation of episomal 2-LTR cDNAs was performed as described previously (Sharkey et al, 2000; Jacque et al, 2002). Amplifications were performed in 50 μl reactions containing 1 × Hot Start Taq buffer (Qiagen), 200 nM dNTPs, 1.5 U Hot Start Taq and 400 nM primers (forward primer 5′-GTA GTT CTG CCA ATC AGG GAAG; reverse primer, 5′-TAG ACC AGA TCT GAG CCT GGGA). PCR reactions were performed on an ABI prism 7700 sequence detection system using 200 nM fluorogenic probe (5′-AGC CTC AAT AAA GCT TGC CTT GAG TGC) that was modified with 6-FAM (6-carboxyfluorescein) on the 5′ end and 6-TAMRA (6-carboxytetramethylrhodamine) quencher dye on the 3′ end. Copy number estimates of 2-LTR circles were determined using the ABI prism 7700 quantification software. The number of cell equivalents in DNA lysates was determined by PCR using primers directed to the CCR5 gene, as described previously (Kostrikis et al, 2002). 2-LTR circle quantitation by this method is sensitive down to five copies and linear over a 4–5 logs range (see Supplementary Figure 2).

Supplementary data
Supplementary data are available at The EMBO Journal Online.

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