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List of abbreviations:

AZT: 3'-Azydo-3'-Deoxythymydine.

HAART: highly active antiretroviral therapy

2-LTR: two-long terminal repeat

ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) persistently infected cell lines are characterized by the continuous viral production without cytopathic effect. However, it is not completely clear if this production is contributed only by viral transcription or also by new cycles of viral replication. We studied an HIV-1 persistently infected cell line, designated H61-D, providing evidence of new replication cycles as sustained by: i) a decrease in viral production, measured by p24 protein, after treatment of the culture with 3'-Azydo-3'-Deoxythymydine (AZT); ii) detection of new integration events in the course of cell culture, and iii) finding of two-long terminal repeat (2-LTR) circles in the cells. H61-D cells were not infected by cell free virus, but infection was possible by co-culture with another productive infected cell line. In conclusion, ongoing viral replication is taking place in H61-D persistent cells and new infections are mediated by a cell-to-cell spread mechanism.

INTRODUCTION

A wide range of cell types are susceptible to infection by human immunodeficiency virus type 1 (HIV-1). Most of the viral replication "in vivo" is taking place in actived CD4+ T lymphocytes, but other cell types like macrophages, monocytes, $CD8^+$ T lymphocytes, B lymphocytes, natural killer cells and follicular dendritic cells are also infected (Saksena et al., 2010). However, differential HIV-1 replicative patterns occur in these different cell types ranging from the massive replication in activated $CD4^+$ T lymphocytes to limited replication in macrophages or latency in resting memory $CD4^+$ T lymphocytes (Chun et al., 1997a, Haase, 1999). Moreover cells in distinct anatomical sites, like lymphoid tissue, gastrointestinal tract, central nervous systems, genital tract and lung, may act as reservoirs for HIV-1, reviewed in (Eisele & Siliciano, 2012, Saksena et al., 2010).

Natural HIV-1 infection is considered a persistent infection because of continuous viral production. "In vivo", a low level residual viremia persists in patients even after prolonged highly active antiretroviral therapy (HAART) (Dornadula et al., 1999, Palmer et al., 2008). However, the origin and mechanisms responsible for this residual viremia still remains unclear. Since HIV-1 persists as a latent provirus in resting memory $CD4^+$ T lymphocytes (Chun et al., 1997b, Wong et al., 1997), one possible explanation for the residual viremia is the reactivation of latently infected cells. But other authors question if these cells are the origin of the residual viremia (Bailey et al., 2006, Brennan et al., 2009). Residual viremia could also be explained by the existence of a low-level continuous viral replication in an unknown reservoir. Existence of viral replication is supported by the presence of two-long terminal repeat (2-LTR) circles in patients on long-term treatment (Buzon et al., 2010), and by the evolution of viral sequences during HAART treatment in HIV-1 infected patients (Gunthard et al., 1999).

Persistently infected cell lines have been widely used in many viruses to study persistent infections and to gain information on the mechanism of viral persistence, like in foot-and mouth disease virus (Martin Hernandez et al., 1994), hepatitis C virus (Zhong et al., 2006), lymphotropic minute virus (Ron & Tal, 1985), poliovirus (Gosselin et al., 2003), mouse hepatitis virus (Chen & Baric, 1996) or measles virus (Robinzon et al., 2009). HIV-1 latency and its underlying mechanisms have been extensively studied in chronically infected cell lines, like ACH-2 (Folks et al., 1989), U1(Folks et al., 1988), OM-10.1 (Butera et al., 1994) or J-Lat cells (Jordan et al., 2003). These cells have been

used as *"in vitro"* models for HIV-1 latency. These cell lines showed characteristics of latent infection, expressing virus only after cell activation, and they permitted the identification of cellular proteins controlling HIV-1 latency (Williams et al., 2007). These cells allowed the study of epigenetic regulators of HIV-1 latency, such as cytosine methylation (Kauder et al., 2009) or transcriptional interference (TI) by viral genome integration into actively transcribed host genes (Lenasi et al., 2008).

HIV-1 persistence with continuous viral production has not been much studied "in vivo" and "in vitro". In a previous work, three persistently infected cell lines were established using the same HIV-1 isolate (S61) and characterized in our laboratory (Sanchez-Merino et al., 2007). In this report, we describe the features of a cellular clone, named H61-D, derived from one of these HIV-1 persistently infected cell lines (H61). We provide several evidences of ongoing viral replication and new infectious cycles in H61-D cells.

RESULTS

Characterization of the HIV-1 persistently infected H61-D cells.

Persistently HIV-1 infected H61-D cells were obtained, as previously described (Sanchez-Jimenez et al., 2012), by limiting dilution cell cloning from a heterogeneous parental persistently infected cell line (H61). This cell line was obtained from infection of the human cutaneous T cell lymphoma highly permissive for HIV-1 replication (H9 cells) with the s61 viral isolate (Sanchez-Merino et al., 2007). H61-D cells showed continuous viral production, as measured by p24 in supernatant, without cytopathic effect (CPE). Viral expression was detected by immunofluorescence in 100% of H61-D cells (Fig.1), and the virus produced by the cells (named vH61-D) gave a titre of 5.1 ± 4.4 x 10^4 $TCID_5$/ml in TZM-bl cells. vH61-D was infectious and cytopathic for H9 cells (data not shown). Table 1 summarizes the characteristics of this virus, as well as viruses derived from parental H61 or acutely infected H9 cells. Provirus quantification determined by Alu-PCR, showed two copies of integrated proviral DNA per cell.

Expression of CD4 receptor and CCR5 and CXCR4 co-receptors in H61-D cells was measured by flow cytometry (FACS), and compared with expression in H61 and uninfected H9 cells (Table 2). In contrast with H9, expression of CD4 was undetectable in H61-D and H61 cells. Co-receptors expression was not altered in H61-D cells. To further characterize these cells, expression of the activation markers CD25, CD69 and

HLA-DR were measured by FACS (Table 2). Expression of CD69 and HLA-DR is higher in persistently infected cells that in H9 cell, indicating cellular activation. In spite of this activation state, doubling time is the same in the persistently infected than in the uninfected cells (Table 2).

Decrease of viral production in H61-D cells with AZT treatment.

To assess if new replication events are occurring in H61-D cells, cell cultures were treated with the reverse transcriptase inhibitor, 3'-Azydo-3'-Deoxythymydine (AZT). Previously, several concentrations of AZT were assayed in H9 cells infected with vH61-D and complete inhibition of viral replication was obtained at doses higher than 1μM (Fig. 2a). This treatment did not result in cell toxicity after 14 days, as measured by cell viability. Treatment of H61-D cells with 2 μM AZT caused a significant p24 level decrease of 18 ± 6%, when compared to a control culture without treatment (Fig. 2b). This result indicates the occurrence of new rounds of reverse transcription in H61-D cells.

Analysis of the proviral integration sites in H61-D cells.

Investigation of the proviral integration sites in H61-D cells was carried out in the initial stock (passage 0). To detect the HIV-1 integration sites, as explained in Methods, total cellular DNA from H61-D cells was digested with *Pst*I, re-ligated and amplified by an inverse PCR. Nested PCR produced two or three bands on agarose gels (Fig. 3) which after cloning and sequencing permitted the identification of the human sequences linked to the 5´-LTR. The first detected band corresponds to a sequence in the 2q11.2 locus in chromosome 2, more specifically in a non-coding region of the *RNF 149* gene (ring finger protein 149), also called DNA polymerase-transactivated protein 2. The second band identifies a provirus localized in locus 19q13.31 of chromosome 19, in a non-coding region between the *PLAUR* gene encoding the urokinase plasminogen activator receptor, and the gene encoding the immunity-related GTPase protein. The third band is an unspecific band because it did not contain cellular sequences but included viral sequences from a region spanning positions 8195 to 8755 in *env* gene. This amplification can be explained by hybridization of the LTR primer in the 3´-LTR and the unspecific hybridization of the *gag* primer in *env* gene. No other integrations sites were obtained at passage 0.

Cells were sub-cultured for 100 passages, corresponding to approximately eight months in culture, during which integrations sites were analysed. The same two characteristic bands were always observed (Fig. 3), but also new bands, identifying new integration events, appeared. When the inverse PCR was performed several times on the same DNA sample these new bands were not consistently detected. This result could indicate the low representation of these new integration events in the cell population. Table 3 shows the location and characteristics of the integration sites found at passages 0, 54 and 100. These results indicated that, during cell culture, new integration events were taking place in a small fraction of the cells; however as they did not reach a high representation, they were not fixed in the cell population.

2-LTR circles detection in H61-D cells.

Detection of 2-LTR circles has been associated with new events of reverse transcription (Sharkey et al., 2000). 2-LTR detection was performed on the DNA from H61-D cells at passage 0, 54 and 100, using a specific PCR (see Methods). An expected fragment of 453 bp was obtained in all samples, and its correspondence to the viral 2-LTR junction sequence was confirmed by cloning and sequencing. Fig. 4 shows some of these junction sequences. As expected, not all junction sequences were the canonical sequences produced by LTR ends ligation; most of the clones presented insertions or deletions at the junction site, as in previous works (Julias et al., 2002, Mandal et al., 2006, Randolph & Champoux, 1993, Svarovskaia et al., 2004).

To assess how many cells presented these 2-LTR circles, the sensitivity of the technique was evaluated. By this technique, four copies of the 2-LTR circles in DNA from 4×10^4 H9 cells could be detected (data not shown). In the DNA from H61-D cells between 4×10^2 and 4×10^3 cells were required to obtain a positive band (data not shown). This result means that less than 1% of the cells studied have 2-LTR circles, and the viral replication events are taking place only in a small fraction of the persistently infected H61-D cells.

Co-culture facilitates infection of H61-D cells.

All these experiments in H61-D cells provided evidence of new infection cycles. These cells did not express, however, the CD4 receptor at the membrane, so it is difficult to explain how new infection events are taking place. In order to study how the virus enters in H61-D cells, we used the recombinant NL4.3-GFP virus that constitutively

195 expresses the GFP protein (see Methods). To facilitate infection, NL4.3-GFP viruses
196 were added to the H61-D cells, in the presence of 8 μg DEAE-dextran ml^{-1} (Platt et al.,
197 2010), at a high m.o.i. of 1 $TCID_{50}$/cell. As control, H9 and the persistently infected
198 parental H61 cells were infected with the same virus. No evidence of infection in H61-
199 D cells was obtained, as monitored by cell viability, detection of GPF by fluorescence
200 microscopy (data not shown) and FACS. Fig. 5a shows the FACS analysis of H9, H61
201 and H61-D cells at day 7 post-infection. In H9 cell line, 21.7% cells were GFP positive,
202 whereas no positive cells were detected in H61 parental cells nor in H61-D.
203 Since HIV-1 transmission is more efficient by a cell-to-cell contact than by cell-free
204 virus (Chen et al., 2007, Dimitrov et al., 1993, Sattentau, 2008), to investigate the
205 infection of H61-D cells through a cell-to-cell contact a co-culture experiment was
206 designed. In this experiment, H4.3-G cells, an H9 cell line persistently infected with
207 NL4.3-GFP virus (see Methods), was used as donor and co-cultived with H61-D cells.
208 Since both H61-D and H4.3-G cells (target and donor cells, respectively) were derived
209 from the H9 cell line, target cells were stained before co-culture with the Celltracker
210 probe Red CMTPX for the differentiation between the two cell lines after co-culture. In
211 this setup, if H61-D cells labelled with CMTPX were infected with the NL4.3-GFP
212 virus, infected cells should become double-positive GFP/CMTPX. As positive control,
213 uninfected H9 cells were used as target cells, and H61 parental cells were also included
214 in the study. FACS analysis 48 hours after co-culture showed the presence of double-
215 positive GFP/CMTPX cells in H9, H61 and H61-D (Fig. 5b). To control for unspecific
216 cell aggregates, donor and target cells were mixed before fixation and submitted to the
217 same FACS analysis; the background of double-positive GFP/CMTPX cells ranged
218 from 1.09% to 1.61% (Fig.S2), values that were significantly minor (p values ≤ 0.05
219 using a nonparametric Mann-Whitney test) than the ones in the co-cultures.
220 Since in co-culture experiments percentages of double-positive GFP/CMTPX cells
221 depend on the final rate between target cells and H4.3-G cells, to calculate the average
222 of target cells that became GPF positives, we performed the analysis in the gated
223 positive CMTPX population (Table 4).
224 To exclude the role of free virus in infection, co-cultures were also performed
225 separating donor and target cells with a transwell insert allowing the diffusion of free
226 virus. In control H9 cells 1.92% of double-positive cells were found. No double-positive
227 cells were found in H61-D or H61 cells, indicating that H61-D cells in co-culture
228 experiments were infected by cell-to-cell contact (Fig. 5c).

In summary, all these experiments indicate that H61-D cells were re-infected with NL4.3-GFP virus through a cell-to-cell contact, and suggest that the new rounds of viral replication and integration detected in H61-D persistently infected cells could be explained by this mechanism.

To explore if infection occurs by the classical fusion entry via CXCR4, we treated the cells with the fusion inhibitor AMD3100 before and during co-culture. The dose of AMD3100 used was 1 μM, a concentration able to completely inhibit acute infection in H9 cells with the NL4.3-GFP virus (data not shown). When co-culture was performed in presence of AMD3100 with H9 cells, a decrease of double-positive cells was observed (Table 4). However, in co-cultures of H61-D or H61 cells the number of double-positive cells was not significantly different from the obtained in the absence of AMD3100. This result suggests that cell contact other than the classical CXCR4 fusion mechanism could be responsible for infection in co-cultures.

DISCUSSION

In the present study, we provide several lines of evidence for the existence of ongoing replication in an HIV-1 persistently infected cell line**.** First, viral production was decreased when cells were treated with the reverse transcriptase inhibitor AZT. Second, new integration events appeared in the course of cell culture; and third, 2-LTR circles were detected in the cells.

Decrease in level of p24 when cells were treated with AZT implies that production of virus is caused not only by transcription from initial integrated provirus, but also by new replication cycles. The same result was obtained with other reverse transcriptase inhibitors like Tenofovir and Emtricitabine (data not shown).

Since H61-D is a cloned cell line derived from a single cell, the two integration sites found in DNA from H61-D cells should be the same than in the original cell. In fact, these two integration sites were always detected in the cell cultures. Additional new sites were identified, however, in further passages, although its detection was sporadic. Since no other integration sites were found at passage 0 (Table 3 and Fig. 3), but found only in subsequent passages, this result showed the existence of new integration events, albeit at a low proportion, in the cell population.

The third evidence was the detection of 2-LTR circles in H61-D cells. 2-LTR circles are the result of the joining of the 5´and 3´ends of unintegrated viral DNA. Because these

forms of unintegrated DNA have a short half-life, the presence of these circles in HIV-1 patients, has been considered a marker of recent replication (Buzon et al., 2010, Sharkey et al., 2000). In latently infected cell lines ACH-2 or OM-10.1, presence of 2-LTR circles has been considered suggestive of reinfection (Besansky et al., 1991). The stability of 2-LTR circles is a controversial issue with results in favour to its rapid degradation (Sharkey et al., 2000) and others in favour of its stability (Butler et al., 2002, Pierson et al., 2002). In H61-D cells, the presence of 2-LTR circles could not originated from the initial cell cloning because after multiple cell passages they should have been diluted to undetectable levels. Moreover, in the analysis of the 2-LTR circle junction sequences, we did not find only the canonical sequence but also different variants, supporting the occurrence of new retrotranscription events.

Ongoing viral replication in H61-D cells implies super-infection. This is a difficult process in H61-D because the lack of the CD4 receptor at the cell membrane. Indeed, the most relevant mechanism of resistance to HIV-1 super-infection “in vitro” is the down-modulation of the CD4 receptor at the cell membrane, reviewed in (Nethe et al., 2005). Studies with HIV-1 chronically-infected cell lines demonstrated, however, that some of them can be superinfected with heterologous viral strains (Fernandez Larrosa et al., 2006, Iwabu et al., 2006, Kim et al., 1996, Marquina et al., 1997). These data led us to investigate how H61-D cells could be superinfected. For this purpose, we used a heterologous virus (derived from pNL4.3) expressing GPF protein. We demonstrated that infection was not achieved by free virus but through cell-to-cell contacts. This finding is consistent with recent reports suggesting that cell-to-cell contact makes possible the spread of virus in adverse conditions like presence of neutralizing antibodies or antiviral drugs (Chen et al., 2007, Hubner et al., 2009, Sigal et al., 2011). At present we do not know how the persistent cells contact each other for the transmission of virus. The classical entry of the virus by fusion via CXCR4 is not the mechanism in these persistently infected cells, because treatment of the cells with de fusion inhibitor AMD3100 did not prevent infection; this is in accordance with other works showing that cell-to-cell HIV transmission is co-receptor independent and non-inhibited by fusion antagonists (Blanco et al., 2004, Chen et al., 2007). Other distinct modes of cell-to-cell HIV transmission have been reported, like filopodial bridges (Sherer et al., 2007) or through nanotubes (Eugenin et al., 2009).

HIV-1 super-infection in patients is well documented (Altfeld et al., 2002, Chohan et al., , Gottlieb et al., 2004, Jost et al., 2002) and it is also supported by the existence of

multiple recombinant viruses (Pernas et al., 2006, Thomson et al., 2002). It remains unclear if recombination is caused by the simultaneous cell infection with two viruses, or if an already chronically infected cell is re-infected by another virus.

In the HIV-1 persistently infected cell line H61-D, super-infection with the same viral strain was supported by the evidence of retrotranscription, new proviral integrations and the presence of 2-LTRs. These results agree with other reports showing provirus accumulation in a chronically infected H9 cell line (Ott et al., 1995).

At present, the reservoirs responsible for the residual viremia in HIV-1 treated patients are not known. Residual viremia could be explained by the existence of a low-level of ongoing viral replication in an unknown reservoir (Bailey et al., 2006, Buzon et al., 2010, Gunthard et al., 1999). In this work we studied an HIV-1 persistently infected cell line with a low level of continuous viral replication. It is possible, that some cellular reservoir with similar characteristics as H61-D can exist “in vivo”, and H61-D can help as an “in vitro” model of this reservoir. Investigation of the mechanisms of viral replication in HIV-1 persistent infections could help in the design of new therapeutic approaches for the suppression of residual viral production in HIV-1 patients.

METHODS

Plasmids and viruses.

The pNL4.3-GFP molecular clone is a modification of HIV-1 pNL4.3-renila plasmid (Garcia-Perez et al., 2007), with the green fluorescent protein (GFP) gene inserted instead of *nef* gene. Plasmid was kindly provided by Drs. J. Alcamí and J. García-Pérez (Inmunopatología del SIDA, CNM).

The p2LTR was constructed by cloning a PCR fragment containing the junction sequence of 2-LTR circles using the TA cloning kit (Invitrogen). The cloned fragment was obtained from amplification of the HIV-1 persistently infected cell line H61 (Sanchez-Merino et al., 2007) using a PCR-based strategy to specifically amplify 2-LTR circles (described below).

Virus NL4.3-GFP was obtained by transfection of 293T cells with pNL4.3-GFP molecular clone using the calcium phosphate precipitation method. Virus used for acute infection of H9 cells (V61) was obtained from the infectious molecular clone 89ES061

(Olivares et al., 1998), from the HIV-1 Spanish isolate S61 (Sanchez-Palomino et al., 1993).

HIV-1 Persistently infected cell lines.

The H61-D cell line was established by limiting cell dilution from the persistently infected parental H61 cell line (Sanchez-Jimenez et al., 2012). H61 was obtained from an acute infection of the human cutaneous T cell lymphoma highly permissive for HIV-1 replication (H9 cells) with the s61viral isolate (Sanchez-Palomino et al., 1993), as described in (Sanchez-Merino et al., 2007). A cellular stock, named passage 0, was produced by expansion of the initial clone H61-D until 5 x 10^8 cells.

To establish the H4.3-G cell line, 5 x 10^5 lymphocytic H9 cells were infected with the NL4.3-GFP virus at a m.o.i of 0.1 $TCID_{50}$/cell. After the acute infection, with CPE in the cell culture, cells were maintained for four months. Sub-culture passages were performed three times a week and progress of the persistent infection was monitored by counting cell viability and measuring p24 protein concentration in the supernatant. After four months, no CPE was observed and cell viability remained above 90%. In addition, over 40% of cells expressed GFP. Cells were maintained in RPMI 1640 medium (Bio-Whittaker), supplemented with 10% FBS, 4 mM glutamine, 100 uds ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37ºC in 5% CO_2 atmosphere. Cellular viability was determined using trypan blue (Sigma) exclusion method.

Indirect Immunofluorescence

Cells were incubated on glass slides previously treated with 50 μg poly-L-lysine ml^{-1} (Sigma), in PBS for 1 hour at 37ºC, and then fixed for 10 minutes in 4% paraformaldehyde. Fixed cells were washed in PBS and permeabilized with 0.1% Triton X-100 for 5 minutes. After blocking cells with 20% FBS, they were incubated for 30 minutes with HIV-1 positive serum diluted 1:50 and DAPI diluted 1:2000 in PBS. After washing 3 times in PBS, slides were incubated in the dark for 30 minutes with 1:50 anti-human IgG antibody (Southern Biotechnology Associates, Inc) bound to FITC fluorocrome. Preparations were mounted in Mowioll 40-88 reagent (Sigma) and analysed in a Leica DMRD fluorescent microscope supported with IM1000 software.

Determination of viral markers.

Viral expression was monitored by p24 protein production in the cell culture

supernatant using the Elecsys HIV antigen detection assay (Roche). Viral titer $TCDI_{50}$ was determined in TZM-bl cells as in (Derdeyn et al., 2000).

Proviral quantification.

Quantification of provirus in H61-D cells was performed by quantitative Alu-PCR following a modification of the method previously described by (Chun et al., 1997b). Essentially, it consists in a first amplification, using a 5´primer from human Alu conserved sequences and a 3´primer from conserved HIV-LTR sequences, followed by a nested PCR to amplify a fragment of the LTR. The following modifications were introduced: a decrease in the number of cycles in the first amplification to 18 cycles, and the use of a real-time PCR for the nested PCR, using LightCycler 480 SYBR Green I Master Mix (Roche). Thermal cycler conditions were 10 minutes hot-star Taq activation at 95ºC and 33 cycles of amplification. Each amplification cycle was composed of 10 seconds denaturation at 95ºC, 5 seconds annealing at 62ºC and 14 seconds extension and acquisition at 72ºC. A standard curve was obtained using serially diluted DNA from ACH-2 cells that contain one copy of HIV provirus per cell.

AZT treatment

In order to obtain an adequate intracellular level of AZT and its phosphorylated derivative, H9 cells were pre-incubated for 2 hours with medium containing AZT at concentrations from 0.01 to 10 μM. Cells were infected with virus recovered in the supernatant of H61-D cells (vH61-D) at m.o.i of 0.1 $TCID_{50}$/cell. After viral adsorption for 2 hours at 37ºC, cells were washed in PBS and resuspended in medium containing the appropriate concentration of AZT. Cultures were maintained for 2 weeks and fresh medium containing the appropriate drug concentration was added every two days.

Analysis of the integration sites in H61-D cells.

HIV-1 integration sites were determined following the protocol described by Han et al. (Han et al., 2004). Genomic DNA was digested with *Pst* I and ligated with T4 DNA ligase in diluted conditions favouring intramolecular ligation. Circularized DNA was amplified by PCR using outward directed primers located in LTR (5'-GCTCAGATCTGGTCTAACAAGAGAGAC-3', complementary to positions 3 to 29) and *gag* (5'-GGTCAGCCAAAATTACCCTATAGTG-3', positions 713 to 738). A second nested PCR was carried out using an internal LTR primer (5'-

TAGCTTGAAGCACCATCCAAAGG-3' complementary to positions -330 to -308) and an internal *gag* primer (5'-TGTTAAAAGAGACCATCAATGAGGAAG-3', positions 931 to 958). Primers are numbered according to the sequence of HXB2 virus (Ratner et al., 1985). PCR products were cloned using TA cloning® Kit (Invitrogen) according to the manufacturer's protocol and sequenced in an automated ABI PRISM 3700. The human genomic sequence at the end of the 5´ LTR of HIV-1 was identified using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Bast.cgi).

Detection of 2-LTR circles in H61-D cells.

Total DNA from H61-D cells was extracted by a standard phenol-chloroform method. The PCR-based strategy, used to specifically detect 2-LTR circles described in (Bukrinsky et al., 1992), consists in a nested PCR using outward directed primers. Primers used for the first PCR were 198RU (5´-GTCTGTTGTGTGACTCTGGT-3´, positions 112 to 131) and 199RD (5´-GAGGCTTAAGCAGTGGGTTC-3´, complementary to positions 54 to 73). Primers for nested PCR were 366U (5'-GAGATCCCTCAGACCCTTTTAG-3´, positions 138 to 159) and 188D (5´-GCCACTCCCCTGTCCCGCCC-3´, complementary to positions -46 to -65). Primers are numbered according to the sequence of HXB2 (Ratner et al., 1985). PCR products were cloned using TA cloning® Kit and 20 positive clones were sequenced. The detection limit of the technique was evaluated by submitting serial dilutions of a plasmid containing the amplified region (p2LTR) in DNA from 4 x 10^4 H9 cells. Then, dilutions of DNA from H61-D cells in DNA from H9 cells were submitted to the same PCR amplification and analysed by agarose gel electrophoresis.

Infections.

Cells were infected with recombinant NL4.3-GFP virus, obtained from transfection of 293T cells with the pNL4.3-GPF molecular clone. Infections were carried out at m.o.i. of 1 $TCID_{50}$/ cell, in the presence of 8 μg DEAE-dextran ml^{-1} (Platt et al., 2010). Cultures were monitored at different times post-infection for 10 days by measuring cell viability using the trypan blue staining method, examination by fluorescence microscopy and FACS.analysis

Staining of cells and co-culture.

Targets cells (H9, H61 or H61-D) were stained with the Celltracker probe Red CMTPX (Invitrogen) at concentration of 20 μM during 30 minutes following manufacturer's instructions. After 24 hours, cells were extensively washed with PBS and co-cultures performed in a 24 well plate by mixing 1 x 10^6 of both donor H4.3-G cells and target cells. In Transwell experiments target cells were separated from donor H4.3-G cells by a polycarbonate membrane of 0.4-μm pore size (Costar).

When co-culture was performed in presence of AMD3100, target and donor cells were pre-treated during 1-2 hours with AMD3100 at concentration 1 μM before co-culture. Co-cultures were maintained for 48 hours with the same concentration of AMD3100 and analysed by FACS.

FACS.

5 x 10^5 cells were fixed during 15 minutes at room temperature using 50 μl of the fixation reactive A from the IntraStain Fixation and Permeabilization Kit (DAKO) and then examined in a FACSCalibur flow cytometer (BD Biosciences). A minimum of 10000 events were assessed for each analysis.

To measure expression of the membrane CD4 receptor and CXCR4 co-receptors, cells were fixed as above and then incubated with undiluted PE-conjugated mouse anti-human antibodies anti-CD4 or anti-CXCR4. CCR5 co-receptor was detected using FITC-conjugated anti CCR5. Antibodies used to measure expression of activation markers were: PE-conjugated anti CD25, PE-conjugated antiCD69 and FITC-conjugated HLA-DR. Corresponding isotype-matched controls were used. All these antibodies were from BD Pharmigen.

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Table 1.Virological markers of the persistently and acutely HIV-1 infected cells.

	H61-D	H61	Acute infection*
p24 (pg ml^{-1})	4.1 ± 0.7 x 10^4	2.5 ± 0.5 x 10^4	2.4 ± 0.69 x 10^5
Viral Titre ($TCID_{50}$/ml) †	5.1 ± 4.4 x 10^4	4.1 ± 2.4 x 10^4	6.6 ± 3.9 x 10^5
Proviral DNA (copies/cell) ‡	2	2	NA
CPE§	negative	negative	positive

*Acute infection was carried out with V61virus in H9 cells (see Methods).
†Determined in TZM-bl cells. Values represent mean (average) from 3 determinations ± SD.
‡Determined by quantitative Alu-PCR as described in Methods.
§Defined by the appearance of syncytia.
NA, not applicable.

Table 2. Cellular markers in persistently HIV-1 infected and parental cells.

	H61-D	H61	H9
Doubling time(days) *	1.07 ± 0.19	1.05 ± 0.14	1.04 ± 0.08
CD4+(%) †	u.d. §	u.d. §	55.92 ± 20.31
CXCR4+(%)†	90.6 ± 9.7	89.9 ± 8.72	92 ± 7.8
CCR5+(%)†	83.5 ± 0.7	33.29 ± 0.41	85.5 ± 6.36
CD25‡	4.97 ± 2.1	2.2 ± 1.6	7.3 ± 3.5
CD69‡	10.3 ± 10.1	17.6 ± 10.6	4.6 ± 2.3
HLA-DR‡	68.5 ± 30.5	77.9 ± 25.5	4.3 ± 3.6

*Doubling time was calculated during exponential cell growth.
†Determined by FACS. Values represent mean (average) from 3 or 4 determinations ± SD.
‡Determined by FACS. Values represent mean (average) from 2 determinations ± SD.
§ undetectable by FACS analysis.

Table 3. Characteristics of provirus integration sites appearing during H61-D cell culture.

Passage	Chromosome	Host Gene	Description
0	2*	RNF149	Ring finger protein 149
	19*	Intergenic región	12333 bp at 5´side: plaminogen activator, urokinase receptor isoform 1 36654 bp at 3´side: immunity-related GTPase family
54	19	PPP5C	Protein phosphatase 5, catalytic subunit
	11	LOC143913	Neural cell adhesion molecule isoform 2
	16	ACSF3	Acyl-CoA syntetase family member 3
	7	ANKIB1	Ankyrin repeat and IBR domain containing 1
	7	Intergenic region	94663 bp at 5' side: lipoma HMGIC fusion partner-like 3 39997 bp at 3' side: myeloid/lymphoid or mixed-lineage leukemia 5
100	5	NIPBL	Delangin isoform A
	4	ATP8A1	ATPase, aminophospholipid transporter (APLT)
	17	SMG6	Smg-6 homolog, nonsense mediated mRNA decay factor

*Integration sites detected in cells at all passages

Table 4. Percentage of target CMTPX cells GFP+ in co-culture experiments*.

	H61-D	H61	H9
Direct co-culture	3.12 ± 0.65	3.30 ± 2.38	30.59 ± 3.1
With transwell	0.03 ± 0.05	0.04 ± 0.07	1.2 ± 1.02
With AMD3100 (1μM)	2.33 ± 0.69	3.27 ± 2.77	6.3 ± 4.6
Background†	0.43 ± 0.34	0.62 ± 0.02	0.9 ± 0.32

*Obtained after analysis in gated positive CMTPX population. Values represent mean (average) from 3 determinations ± SD.

†Values obtained after mixing CMTPX stained cells with H43G before fix and analysis.

FIGURE LEGENDS

Figure 1. Images of the H61-D cell line.

Images of immunofluorescence with anti-HIV-1 (green) and 4'-6-Diamidino-2-phenylindole (DAPI) nuclear staining (blue) of H61-D cells.

Figure 2. Decrease of viral p24 production with AZT treatment.

Relative values of p24, represented as % viral production in (a) H9 cells after 14 days of infection with vH61-D in presence of the indicated concentrations of drug and (b) H61-D cells maintained for 14 days in presence of 2µM AZT. p24 values from cells without AZT treatment are considered 100% of viral production. Bars represent the standard deviation of three experiments.

Figure 3. Analysis of proviral integration sites by inverse PCRs.

DNA of H61-D cells at passages 0, 54 and 100 were submitted to inverse PCR (see Methods). Each gel shows the PCR products of three independent amplifications. Bands appearing in all amplifications correspond to the initial integration sites and are marked as 1 and 2. Band 3 corresponds to an unspecific amplification of *env* gene sequences. M: 1 Kb weight marker.

Figure 4. 2-LTR circle junction sequences.

Sequences obtained by amplification from DNA of H61-D cells at different times. 3´U5 and 5´U3 ends of viral DNA are indicated. The canonical ligation of the 2-LTRs are labelled as consensus and shown at the top. Boxes signal dinucleotide duplication at the DNA ends (Brown et al., 1989).

Figure 5. **FACS analysis of infection experiments.**

Representative dot plots of: a) H9, H61 and H61-D cells infected with cell-free NL4.3-GFP virus analysed 7 days post-infection. Plots show sideward scater (SSC) versus GFP fluorescence. b) Co-culture of H9, H61 and H61-D cells stained with Celltracker probe Red CMTPX, with H4.3-G cells 48 hours after co-culture and c) Co-culture separated by a transwell insert of the same donor and target cells as in b; only target H9, H61 and H61-D cells were collected and submitted to FACS analysis. Plots show GFP versus red

fluorescence. Numbers in the quadrants represent the percentage of gated cells in each quadrant. Figure is representative of three different experiments.