Efficient magnetic bead-based separation of HIV-1-infected cells using an improved reporter virus system reveals that p53 up-regulation occurs exclusively in the virus-expressing cell population

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Abstract

HIV-1 infection in cell lines is very efﬁcient, since the target population is clonal and highly dividing. However, infection of primary cells such as CD4 T lymphocytes and monocyte-derived macrophages is much more difﬁcult, resulting in a very small percentage of infected cells. In order to study events occurring in productively infected primary cells, we determined that a way to isolate this population from bystander cells was needed. We engineered a novel HIV-1-based reporter virus called NL4-3-IRES-HSA that allows for the magnetic separation of cells infected with fully competent virions. This X4-using virus encodes for the heat-stable antigen (HSA/murine CD24) without the deletion of any viral genes by introducing an IRES sequence between HSA and the auxiliary gene Nef. Using commercial magnetic beads, we achieved efﬁcient puriﬁcation of HIV-1-infected cells (i.e. purity ~85% and recovery ~90%) from diverse primary cell types at early time points following infection. We used this system to accurately quantify p53 protein levels in both virus-infected and uninfected bystander primary CD4 + T cells. We show that p53 up-regulation occurs exclusively in the infected population. We devised a strategy that allows for an efﬁcient separation of HIV-1-infected cells from bystanders. We believe that this new reporter virus system will be of great help to study in depth how HIV-1 interacts with its host in a primary cells context.

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Introduction

A previous microarray study involving HIV-1 and primary CD4 + T cells pointed out the need to isolate HIV-1-infected cells from the total population for further analysis (Imbeault et al., 2009). Indeed, in vitro infection rates in human primary cells are very low, reaching about 1% in PHA-activated CD4 + T lymphocytes after 24 h of infection. This percentage can reach an average of 10% at 4 days post-infection. This situation is in sharp contrast to the very high infection rates routinely seen in the majority of CD4-expressing established cell lines (e.g. Jurkat). Such low infection rates make it hard to detect potential changes in gene and/or protein expression occurring exclusively in infected cells, the signal being diluted in the total cell population. Moreover, it complicates the discrimination between processes that occur exclusively in infected cells from those taking place in uninfected bystander cells using techniques such as Western blot or quantitative real-time PCR (qRT-PCR).

Reporter viruses are essential tools to follow the HIV-1 life cycle by making it possible to easily identify productively infected cells and quantify the total level of virus infection. Engineered from the basic HIV-1 genome, the encoded reporter protein can be measured or detected by biochemical means or flow cytometry. Examples of the most commonly used reporter genes include enhanced green fluorescence protein (eGFP) (Brown et al., 2005; Herbein et al., 1998; Kutsch et al., 2002; Rich et al., 2002), luciferase (Connor et al., 1995) and membrane-bound proteins that can be targeted by fluorescent-labelled antibodies such as murine heat-stable antigen (HSA) (Chiu et al., 2005; Jameson and Zack, 1998; Marodon et al., 1999) and human placental alkaline phosphatase (PLAP) (Chen et al., 1996; He and Landau, 1995). Some techniques even allow tracking of HIV-1 particles by tagging virus-encoded proteins that are incorporated into mature virions such as Gag (Muller et al., 2004) and Vpr (McDonald et al., 2002). However, given the complexity and compact size of the HIV-1 genome, insertion of reporter genes in different loci (such as Nef, Vpu, Vpr or Gag) often disrupts the expression or activity of viral proteins. In human primary cells, the introduced modifications can severely cripple viral replication, especially in non-dividing cells (reviewed in Li et al. (2005)). Moreover, results obtained with these viruses can sometimes lead to conclusions not relevant in a full-length virus context as every viral protein has a definite role to play in the virus replicative cycle.
Recently, Levy et al. (2004) generated NLENG1-IRES, a virus construct into which the reporter gene eGFP was inserted in front of an IRES sequence and the Nef gene, thus avoiding deletion or inactivation of a so-called accessory gene. Previous observations indicate that this strategy allows expression of Nef protein at levels comparable to what is seen in wild-type virus (Brown et al., 2005). Such eGFP-expressing reporter virus could in theory be used to isolate productively infected cells from the total cell population using a flow cytometry cell sorter apparatus. However, there are some technical problems associated with this approach. For example, a high speed cell sorter in a biosafety level 3 facility is required — getting the infected cells out of this protected environment requires virus inactivation through fixation with paraformaldehyde or other agents (e.g. methanol or ethanol), which have a negative impact on eGFP fluorescence (Kusser and Randall, 2003), membrane permeability (which causes cytosolic eGFP to leak out) (Kalejta et al., 1997) and/or integrity of both RNA and proteins (for crosslinking agents).

We postulated that a virus encoding for a membrane reporter protein along with all viral genes was needed in order to study HIV-1-induced gene expression in human primary HIV-1-infected and uninfected bystander cells. We therefore constructed a novel HIV-1-based reporter virus by replacing eGFP with HSA in the NLENG1-IRES vector. Such a viral construct expresses all viral proteins and can serve to separate infected cells using commercially available magnetic beads coated with an antibody specific for the membrane-bound HSA which is expressed along early virus genes upon productive infection. We named the new construct NL4-3-IRES-HSA and derived also R5-tropic (NL4-3-Bal-IRES-HSA) and Env-deficient variants.

In order to demonstrate the usefulness of NL4-3-IRES-HSA, we separated HIV-1-infected primary CD4+ T cells from bystander cells following infection and quantified p53 protein expression levels. p53 phosphorylation and subsequent accumulation are a hallmark of HIV-1-induced apoptosis (Castedo et al., 2005). However, there is still no information as to whether HIV-1 is able to induce p53-dependent

![Cloning strategy used to generate the NL4-3-IRES-HSA vector. See Materials and methods section for complete details.](image)
apoptosis in bystander cells, infected cells or both. In this manuscript we demonstrate that p53 up-regulation happens exclusively in the cell population productively infected with HIV-1, thus supporting the hypothesis that DNA damage induced by HIV-1 integration is required for p53 accumulation.

Results

Construction and in vitro replication of NL4-3-IRES-HSA

In order to generate a reporter virus that would express all viral genes along with a membrane-bound protein suitable for magnetic bead-based separation, we replaced eGFP for HSA in NLENG1-IRES. HSA is an ideal candidate for a reporter gene because of its very small size (about 200 nucleotides) which is important in a compact viral genome context as to minimize interference with viral replication kinetics. Natural selection over multiple infection cycles would tend to eliminate a large insert to optimize the virus replicative fitness. We devised a PCR-based cloning technique which can easily be adapted to insert any reporter gene in the HIV-1 genome, provided that it does not contain BamHI or XhoI restriction enzyme recognition site (Fig. 1). In addition, we constructed R5-tropic (Bal) and Env-deficient variants of NL4-3-IRES-HSA.

We first made comparative analyses of NLENG1-IRES and NL4-3-IRES-HSA in mitogen-stimulated CD4\(^+\) T cells. To this end, target cells were inoculated with similar amounts of reporter viruses that were standardized in terms of infectious units per ng of p24 through the use of the TZM-BL reporter cell line. Although virus production as monitored by measuring the p24 content in cell-free supernatants was comparable for the two tested reporter viruses (data not shown), the number of cells productively infected with HIV-1 we could detect was on average two-fold higher with the new construct compared to wild-type NL4-3 and NL4-3-IRES-HSA virions in human primary CD4\(^+\) T cells. This task was achieved by inoculating human primary CD4\(^+\) T cells with fully infectious NL4-3-IRES-HSA or NL4-3-Bal-IRES-HSA reporter virus, respectively, before separation with EasySep\textsuperscript® (LSM). The latter method is preferred for routine analysis of the isolated cell population as it can be achieved using much lower amounts of cells (i.e. 5\times10^5) compared to flow cytometry (i.e. 1\times10^5 cells). Nevertheless, we used flow cytometry studies to establish the initial purity and enrichment rates because it is a useful technique for quantitative assessment of a specific cell marker and is less labor-intensive than LSM. Subsequent characterization at pre and post immuno-magnetic cell separation by confocal microscopy confirmed that the isolated populations are highly enriched (Fig. 4).

NL4-3-IRES-HSA allows for an efficient magnetic bead-based separation of infected cells

We next proceeded to isolate cells expressing early gene products of HIV-1. This task was achieved by inoculating human primary CD4\(^+\) T cells and MDMs with fully infectious NL4-3-IRES-HSA or NL4-3-Bal-IRES-HSA reporter virus, respectively, before separation with EasySep\textsuperscript® magnetic nanoparticles (Biotin Selection Kit, StemCell Technologies Inc.). We found that the purity of isolated cells ranged from 80 to 94% and recovery rates of 85 to 90% were obtained routinely with both cell types (Fig. 3). The technique is able to recover cells at early time points (24 h) even when few infected cells are present (less than 0.5%). Sufficient amounts of cells to perform Western blot analyses and qRT-PCR tests could consistently be harvested, provided that 5\times10^5 to 1\times10^6 HSA-expressing cells were available in the starting sample. The purity and recovery rates of the target population were assessed either by flow cytometry or laser scanning microscopy (LSM). The latter method is preferred for routine analysis of the isolated cell population as it can be achieved using much lower amounts of cells (i.e. 5\times10^5) compared to flow cytometry (i.e. 1\times10^5 cells). Nevertheless, we used flow cytometry studies to establish the initial purity and enrichment rates because it is a useful technique for quantitative assessment of a specific cell marker and is less labor-intensive than LSM. Subsequent characterization at pre and post immuno-magnetic cell separation by confocal microscopy confirmed that the isolated populations are highly enriched (Fig. 4).

Fig. 2. Infection kinetics of reporter constructs in human primary CD4\(^+\) T cells. (A) Target cells were infected with a similar number of infectious NL4-3-IRES-HSA and NLENG1-IRES viruses before quantifying the percentage of HSA- and eGFP-positive cells, respectively, at the indicated time points. The difference between HSA and eGFP signals for a same level of infection has been observed in multiple experiments. (B) At 3 days post-infection, cells were triple stained with anti-HSA (red), intracellular anti-p24 (green) and DNA dye DRAQ5 (blue). The central slice of a confocal microscopy stack is represented.

p53 is stabilized in HIV-1 infected cells but not in bystander cells

In order to demonstrate the utility of the viral reporter construct, we infected PHA-activated primary CD4\(^+\) T lymphocytes with NL4-3-IRES-HSA for 72 h. We then proceeded to magnetic bead-based separation using a biotinylated antibody against HSA as described previously. Whole protein extracts were obtained from the positive (infected) and negative (bystander) fractions as well as from unseparated cells and mock-treated cells. Next, we quantified p53 protein levels from these extracts by Western blot. The rationale behind this experiment was to verify whether virus-mediated p53 stabilization is occurring in infected and/or uninfected CD4\(^+\) T cells. Results illustrated in Fig. 5 demonstrate that a significant increase in the p53 protein level is seen at 72 h post-infection only in the CD4\(^+\) T cell population that is expressing HIV-1 early gene products as monitored by HSA staining and not in uninfected bystander cells. Similar findings were made when infection was allowed to proceed for 48 h (data not shown).
Fig. 3. Efficiency of magnetic bead-based infected cells separation. (A) Flow cytometry analysis of primary CD4+ T lymphocytes infected with NL4-3-IRES-HSA 2 days post-infection. (B) Flow cytometry analysis of the same cell population post-separation with magnetic beads. (C) Flow cytometry analysis of monocyte-derived macrophages infected with NL4-3-Bal-IRES-HSA, 9 days post-infection. (D) Flow cytometry analysis of the same cell population post-separation with magnetic beads (positive fraction).

Fig. 4. Confocal microscopy analysis of primary CD4+ T lymphocytes infected with NL4-3-IRES-HSA. Unseparated infected cells, positive (infected cells) and negative (bystander cells) fractions following the magnetic separation procedure are shown. A significant enrichment of infected cells is achieved in the positive fraction while simultaneously depleting the negative fraction, yielding bystander cells. HSA-positive cells are indicated with a star (⁎). Highly fluorescent aggregates in the positive fraction are magnetic beads coated with fluorescent-labelled antibodies against HSA.
Discussion

Reporter viruses are essential tools in the quest for a better understanding of HIV-1 biology, allowing scientists to efficiently track infection events within a population of cells exposed to the virus. Over the last few years, many variants of such tools have been developed, each with its own advantages and drawbacks geared toward a specific application.

As the infection efficiency of HIV-1 is known to be an order of magnitude lower in primary human cells compared to immortalized cell lines, a convenient/efficient/sensitive method to separate virus-infected cells from the bystander population is needed to study the infection process in fine detail at early time points. Some available reporter virus constructs expressing surface molecules such as HSA or PLAP could have been used for this purpose. However, all existing viral constructs expressing these membrane-bound proteins lack one or more viral genes such as Nef, Vpr and/or Env. One important caveat related with the use of these vectors is that the deleted proteins have important functions in the in vivo and in vitro replicative capacity and cytopathogenicity of HIV-1. For example, Nef protein has been shown to modulate expression of numerous cell surface molecules (e.g. CD4, CD8, CD28, MHC-I and MHC-II). This auxiliary virus-encoded protein can also interfere with cellular signal transduction pathways, enhance virion infectivity and regulate cholesterol trafficking in HIV-1-infected cells (reviewed in Li et al. (2005)).

In an attempt to overcome these serious limitations, we built the new reporter virus NL4-3-IRES-HSA by introducing the small reporter gene of murine origin HSA followed by an IRES sequence inserted in frame with the Nef locus in order to maintain the complete viral genome. Being located near the Nef sequence, HSA is expressed as an early gene and can be efficiently detected as soon as 24 h post-infection in both CD4+ T cells and MDMs, although the percentage of positive cells at such an early time point is extremely low (data not shown). The cloning strategy we devised is very flexible and allows for any other reporter gene to be cloned in place of HSA, provided that the selected gene does not contain recognition sites for the BamHI or XhoI restriction enzymes. Promising candidates for future consideration include variants of luciferase and the HSA-HA construct developed by Ali and Yang (2006). Both of these tools would be useful to study viral fitness in a complete virus backbone.

The higher frequency of HIV-1-infected CD4+ T cells we can detect when infection is performed with a similar amount of infectious NL4-3-IRES-HSA compared to the parental NLENG1-IRES can be explained by a combination of factors. First, eGFP is distributed throughout the entire cytoplasm while HSA is concentrated in lipid rafts on the plasma membrane because this cell surface molecule carries a glycosylphosphatidylinositol anchor. Therefore, a higher concentration threshold must be achieved to detect a signal with eGFP than with HSA. In this regard, intracellular p24 staining suffers from the same weakness, along with the fact that it is expressed later in the viral life cycle than HSA in our construct. Moreover, eGFP intensity is negatively affected at different degrees by fixation with various agents such as paraformaldehyde, ethanol, and methanol. In addition, eGFP is highly soluble in the cytoplasm and can leak from cells fixed with permeabilizing agents such as ethanol. Fixation is often a necessity to manipulate infected cells outside a level 3 biocontainment area. For all these reasons, we found that a reporter virus expressing a membrane-bound molecule such as HSA constitutes a better choice for microscopy and flow cytometry applications than eGFP-based viral constructs.

Our separation technique was optimized using the EasySep® Biotin Selectin kit from StemCell Technologies Inc. in combination with a biotinylated anti-HSA antibody. We routinely achieved a degree of purity greater than 85% and a recovery rate higher than 80% in both PHA-activated primary human CD4+ T lymphocytes and MDMs. Moreover, we have been able to successfully isolate HIV-1-infected cells from mitogen-activated peripheral blood mononuclear cells and peripheral blood lymphocytes as well as from human lymphoid tissues cultured ex vivo (i.e. small blocks of tonsils) (data not shown).

Depending on the cell type and the time frame between initial infection and analysis, the quantity of primary cells necessary to obtain a significant number of virus-infected cells is a limiting factor. For instance, to isolate 5 × 10^5 HIV-1-infected CD4+ T cells at 24 h post-infection, the starting material should be equal to a total population of at least 50 × 10^6 cells. Another limitation was seen when using phagocytic cells as targets. Indeed, the natural tendency of such cells to engulf magnetic particles results in a non-specific separation and thus a diminished purity. This technical problem was alleviated by performing magnetic separation at 4 °C, an experimental condition known to reduce phagocytosis. We found that viability of infected cells following the separation process was not optimal most likely due to the high level of viral replication they sustain. Therefore, the isolated cells should be used immediately after isolation for techniques that do not require live cells, such as Western blot, qRT-PCR or microarray studies.

It has been established that HIV-1 can induce apoptosis in CD4+ T lymphocytes by distinct pathways (Varbanov et al., 2006). However, there is still controversy about the precise mechanism(s) and whether apoptosis is occurring in infected cells (Gennini et al., 2001; Petit et al., 2002), uninfected bystander cells (Castedo et al., 2002), or both. As a simple demonstration of the utility of our reporter molecular
construct, we isolated both virus-infected and uninfected bystander CD4+ T cells after 3 days following acute virus infection and quantified the amount of p53, a protein playing a well-characterized role in HIV-1-induced apoptosis (Castedo et al., 2005; Corbeil et al., 2001; Komoto et al., 2002; Perfettini et al., 2005a, 2005b). It has been shown that p53 is controlled at a post-transcriptional level by HDM2, which binds to and ubiquitinylates p53, targeting it to the proteasome and preventing it from translocating to the nucleus to activate pro-apoptotic genes (Momand et al., 1992). Phosphorylation prevents HDM2 binding and allows for p53 accumulation. Our data clearly indicate that the virus-mediated p53 up-regulation is taking place in cells productively infected with HIV-1. This supports the hypothesis which proposed that DNA integration events and the subsequent DNA damage are necessary to lead to the p53-dependent apoptosis seen in the context of an HIV-1 infection (Genini et al., 2001). Interestingly, our results demonstrate that apoptosis in bystander CD4+ T lymphocytes does not appear to be dependent on p53 as no stabilization of the protein is detected in this cell population. Additional studies conducted in human primary CD4+ T cells are needed to define how HIV-1 can mediate apoptosis in a p53-independent manner in uninfected bystander cells. It should be noted that a weak 1.77-fold increase in p53 expression was detected in the total unseparated cell population. This small increment could have been dismissed if our work was focusing only on the total cell population. Therefore, infection studies performed with the presently described HIV-1-based reporter virus show that it fulfills its original goals that are to allow detection of HIV-1-dependent biological events occurring in a small number of cells and to determine whether HIV-1 can initiate metabolic changes in virus-infected versus uninfected bystander cells. In conclusion, we provide in this manuscript evidence that the NL4-3-IRES-HSA vector constitutes a worthy addition to the virologist’s toolbox, even if it can only be used in an in vitro setting. It has the potential for identifying HIV-1-induced proteomic and genomic changes in a small cell population and discriminating events generated in infected and/or uninfected cells. This knowledge is essential to obtain a more detailed understanding of early events in the process of HIV-1 infection and identify the gene/protein expression signature unique to this retrovirus.

Materials and methods

Construction of NL4-3-IRES-HSA

PCR sewing, also known as “fusion PCR”, was used to derive NL4-3-IRES-HSA from the original NLENG1-IRES vector (see Fig. 1 for more detailed information). Briefly, the NLENG1-IRES vector contains a full-length HIV-1 NL4-3-based genome with eGFP inserted in between the env and nef genes so that eGFP is expressed as an early and a late gene, as if it was Nef (kindly supplied by D.N. Levy, New York University College of Dentistry, New York, NY) (Levy et al., 2004). Nef expression is achieved by insertion of an IRES between eGFP and nef, yielding wild-type levels of Nef protein. HSA was amplified from a plasmid source with primers containing tails complementary to NL4-3 and flanking each side of eGFP in NLENG1-IRES (Table 1). Fragments flanking eGFP were amplified from NLENG1-IRES with primers containing tails complementary to HSA using Phusion polymerase (Finzymes, Finland). All three fragments were purified on gel using Qiagen Gel Extraction kits and quantified by spectrophotometry. Equivalent amounts of all three fragments were mixed in a primerless PCR reaction and allowed to anneal for 5 cycles of denaturation (94 °C for 30 s), annealing (50 °C for 30 s) and extension (72 °C for 45 s). Thereafter, inner primers were added to the mix and the reaction was allowed to proceed for 25 additional cycles. A 1200 basepair amplicon containing the three fused fragments was isolated on gel and then digested with BamHI and XhoI restriction enzymes. Purification was performed on a Qiagen column to get rid of small fragments, enzymes and buffer. The resulting fragment was ligated back in NLENG1-IRES to generate NL4-3-IRES-HSA or the Env-negative version of the same construct to generate NL4-3-IRES-HSA ENV-. The Sall–BamHI fragment that contains most of the env gene from NL4-3-Balenv (Dornadula et al., 1999) was swapped in place of the native NL4-3 env gene in NL4-3-IRES-HSA, generating a CCR5-tropic, replication competent HSA-encoding viral construct (called NL4-3-Bal-IRES-HSA).

Table 1 Primers used to build NL4-3-IRES-HSA.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer 3’</td>
<td>GAGCATACCAATCTGCAGTGG</td>
</tr>
<tr>
<td>Inner 5’</td>
<td>GCTGACTTTTCTAGGTAGACAGTA</td>
</tr>
<tr>
<td>Reverse 5’</td>
<td>CACCATGGCTGCCCATCACATGAG</td>
</tr>
<tr>
<td>HSA-F</td>
<td>GGCCTGGAAAGGATTGTTAGC</td>
</tr>
<tr>
<td>HSA-R</td>
<td>AACCCTGCCATGGAGGGTT</td>
</tr>
<tr>
<td>Forward 3’</td>
<td>CTTLATACACTGCTTGG</td>
</tr>
<tr>
<td>Inner 3’</td>
<td>TGCTGTCTGAGTTGCTCAGT</td>
</tr>
</tbody>
</table>

Cell separation

Virus-infected and uninfected bystander cells were isolated using the EasySep® Biotin Selection kit as suggested in the manufacturer’s protocol (StemCell Technologies Inc., Vancouver, BC) with slight modifications. Five rounds of separation of 7 min each were carried out. Only the fraction from the first round of separation was kept as a negative fraction. Experiments were performed with 0.5% BSA instead of 2% BSA. For some applications the protocol was carried out at 4 °C and incubation times were doubled. Isolation of adherent MDMs was achieved through the use of Versene (PBS supplemented with 5 mM EDTA). The biotinylated anti-HSA antibody (clone M1/69, BD Biosciences) was used at a final concentration of 3 μg/ml in all experiments.

Confocal microscopy

Purified populations of HIV-1-infected CD4+ T lymphocytes were seeded on 12 mm poly-l-lysine-coated coverslips (BD Biosciences) and fixed with 4% paraformaldehyde. Cells were then permeabilized using 0.1% Triton X-100, and non-specific binding was blocked with 1% BSA, 10% goat serum and 10% pooled human serum in PBS. In order to detect HIV-1-infected cells, the anti-HSA antibody was first used (1:300 dilution), followed by washes in PBS and staining with mouse anti-rat IgG conjugated to Alexa555. In some cases, cells were also stained for DNA using DRAQ5 (Biostatus), HIV-1 p24 capsid protein using FITC-conjugated anti-p24 (clone KC57, Coulter Clone), or F-actin using Alexa488-conjugated phalloidin (Molecular Probes). Cells were then mounted with Fluoromount (Southern Biotech, InterScience) and visualized with an Olympus Fluoview FV300 confocal microscope (Olympus). Digital images were produced using the Adobe Photoshop software (version 6.0; Adobe Systems).

Cell culture

Peripheral blood was obtained from normal healthy donors and peripheral blood mononuclear cells (PBMCs) were prepared by centrifugation on a Ficoll–Hypaque density gradient. Next, a cell population highly enriched in CD4+ T lymphocytes was isolated through the use of the human CD4+ T cell negative selection kit according to the manufacturer’s instructions (StemCell Technologies Inc.). The purity of the negatively selected cell population was estimated by quantifying the percentage of CD4+ expressing cells. Next, cells were cultured at a concentration of 2 × 10^5/ml in complete RPMI-1640 medium (Invitrogen, Burlington, ON), supplemented with 10% foetal bovine serum (FBS) (Wisent, St-Bruno, QC),...
l-glutamine (2 mM), penicillin G (100 U/ml), streptomycin (100 µg/ml), phytohemagglutinin-L (1 µg/ml) and recombinant human IL-2 (30 U/ml) for 3 days at 37 °C under a 5% CO2 atmosphere prior to virus infection. Human embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% FBS, glutamine (2 mM), penicillin G (100 U/ml) and streptomycin (100 µg/ml). MDMs were produced by incubating PBMCs at a concentration of 1.25 × 10^6 cells in a T-75 flask for 2 h at 37 °C, thus allowing adherence of monocytes to the bottom of the flask. Non-adherent cells were then removed and monocytes were washed twice with RPMI-1640 containing 5% autologous heat-inactivated plasma and cultured for 1 week in medium supplemented with 100 ng/ml macrophage-colony stimulating factor (M-CSF) to allow differentiation into MDMs.

**Preparation of virus stocks**

Virus particles were produced by calcium phosphate transfection in 293T cells using a commercial calcium phosphate coprecipitation kit according to the manufacturer’s instructions (CalPhos Mammalian Transfection Kit, Clontech Laboratories Inc., Palo Alto, CA). In brief, parental 293T cells were transiently transfected either with NLEN1-1RES, NL4-3-1RES-HSA, or NL4-3-Bal-1RES-HSA. Cell-free supernatants from such transfected 293T cells were filtered through a 0.22-µm pore-size cellulose acetate membrane (Millipore, Bedford, MA). To eliminate free p24, each supernatant was ultracentrifugated in Centricon® Plus-20 Biomax-100 filter devices (Millipore Corporation). Finally, samples were aliquoted before storage at −85 °C. Stocks of NLEN1-1RES and NL4-3-1RES-HSA reporter virus were normalized according to p24 and TCID50. Infectivity titration was determined through the use of the TZM-BL reporter cell line (Wei et al., 2002) obtained through the NIH AIDS Repository Reagent Program; TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Chard, R.I., Chen, B.K., Choe, S., Landau, N.R., 1995. Vpr is required for efficient virus infectivity in resting CD4+ T cells. Nature 378 (6578), 108–114.

**Flow cytometry**

Flow cytometry analyses were performed with 5 × 10^5 cells that were incubated with a monoclonal anti-HSA antibody for 30 min on ice. Following washes, cells were then labelled for 30 min on ice with 100 µl of a saturating amount of FITC-conjugated goat anti-rat immunoglobulin G (Caltag). Finally, cells were washed, fixed in 2% paraformaldehyde for 30 min and analyzed on a cytofluorometer (EPICS XL, Coulter Corp., Miami, FL).

**Western blots**

A cell population highly enriched in CD4+ T lymphocytes was exposed to NL4-3-1RES-HSA for 72 h at 37 °C. Thereafter, proteins were extracted from both virus-infected and uninfected bystander cells. Cell extracts were heated at 100 °C for 7 min in 1× sample buffer (62 mM Tris–HCl [pH 6.8], 2% SDS, 5% beta-mercaptoethanol, 9% glycerol and 0.002% bromophenol blue) containing PMSF (1 mM). The samples were then separated on a 7.5–20% gradient SDS-polyacrylamide gel and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Immunoblotting was performed using antibodies specific for p53 (clone DO-1, Santa Cruz) or β-actin. Membranes were labelled with horseradish peroxidase-conjugated secondary anti-rabbit and anti-mouse antibodies (Jackson Immunoresearch, Mississauga, ON) at 1:20,000 and 1:10,000 dilutions, respectively. Signals were revealed using the ECL™ Western blotting detection reagent (Amersham, Piscataway, NJ).

**Authors’ contribution**

M.O. designed NL4-3-1RES-HSA, carried out most of the experiments, and drafted the original manuscript. M.O. participated in the p53 Western blot and was involved in experimental design, result analysis and discussions throughout the study. R.L. subcloned the R5 and Env-deficient variants of NL4-3-1RES-HSA from the original plasmid and assisted for confocal and fluorescence microscopy. M.J.T. supervised and coordinated the study and finalized the manuscript. All authors read and approved the manuscript.

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