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A combination HIV reporter virus system for measuring post-entry event efficiency and viral outcome in primary CD4+ T cell subsets

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Abstract

Fusion between the viral membrane of human immunodeficiency virus (HIV) and the host cell marks the end of the HIV entry process and the beginning of a series of post-entry events including uncoating, reverse transcription, integration, and viral gene expression. The efficiency of post-entry events can be modulated by cellular factors including viral restriction factors and can lead to several distinct outcomes: productive, latent, or abortive infection. Understanding host and viral proteins impacting post-entry event efficiency and viral outcome is critical for strategies to reduce HIV infectivity and to optimize transduction of HIV-based gene therapy vectors. Here, we report a combination reporter virus system measuring both membrane fusion and viral promoter-driven gene expression. This system enables precise determination of unstimulated primary CD4+ T cell subsets targeted by HIV, the efficiency of post-entry viral events, and viral outcome and is compatible with high-throughput screening and cell-sorting methods.

Keywords

HIV; reporter virus; fusion; LTR; gene expression; post-entry event efficiency; outcome

1. Introduction

Binding of human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp120 to CD4 and subsequently to CCR5 or CXCR4 coreceptors induces conformational changes in gp120 that culminate with the insertion of the gp41 fusion peptide into the host cell membrane, formation of an energetically favorable six-helix bundle, and fusion between the

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viral and host cell membranes (Reviewed in (Wilén et al., 2012)). Membrane fusion marks the end of the HIV entry process and the beginning of a series of post-entry events that must occur successfully for progeny viruses to be produced (Figure 1). Identifying host and viral proteins that modulate post-entry efficiency is critical to developing strategies to restrict HIV replication (Busschots et al., 2009) and improve transduction efficiency of HIV-based gene therapy vectors (Bobadilla et al., 2012; Durand et al., 2012). Not all cells that fuse with HIV progress to *productive* infection; many cells fail to express viral RNAs and proteins. A subset of these cells can produce infectious virus upon stimulation, referred to as *latent* infection (Karn, 2011). However, most cells undergo *abortive* infection and are unable to produce virions even when stimulated (Doitsh et al., 2010). Understanding the factors regulating these differential outcomes is important to HIV pathogenesis studies (Doitsh et al., 2010) and eradication efforts (Chun and Fauci, 2012; Siliciano, 2010).

Measuring post-entry event efficiency and viral outcome accurately depends on identifying cells that have fused with HIV and assessing their susceptibility to productive, latent, or abortive infection. Current methods for monitoring HIV involve detection of products from a single step of the life cycle, such as viral nucleic acids, enzymes, proteins, promoter activity, or cytopathic effects. Viral titers are calculated to standardize input concentrations; however, HIV nucleic acid and protein concentrations often fail to correlate with functional virus (Kutner et al., 2009; Sastry et al., 2002), likely due to the large percentage of defective virions (Piatak et al., 1993). Variation of functional virus levels between multiple *in vitro* preparations and of receptor expression levels on different cell types further complicate standardization of viral entry and confound attempts to measure post-entry event efficiency and outcome sensitively using traditional assays.

In this study, a combination reporter virus system is described that directly measures viral fusion and LTR-driven EGFP expression, enabling sensitive and reproducible calculation of early post-entry event efficiency and outcome in unstimulated primary immune cell subsets. This system is also compatible with high-throughput screening and cell sorting methods to facilitate identification of proteins regulating viral infection efficiency and outcome.

2. Methods

2.1 Production of combination reporter viruses

2.5×10^6 293T/17 cells were plated into 10 cm plates and transfected after 24 hours with 10 μ g pNL4-3-deltaE-EGFP core (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3-deltaE-EGFP (Cat# 11100) from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano), 7.5 μ g bla-Vpr plasmid (obtained from Dr. Robert Doms), and 6.0 μ g HIV Envs (obtained from Drs. Beatrice Hahn and George Shaw) using calcium phosphate methods. REJO.D12.1972 and JOTO.TA1.2247 are patient-derived subtype B CCR5- and CXCR4-tropic envelopes, respectively, that were cloned into pcDNA3.1 per the manufacturer's instructions (Invitrogen) as described previously (Keele et al., 2008; Wilén et al., 2011). Media was replaced 6 hours after transfection and supernatant was harvested at 72 hours. Virus-containing supernatants were filtered, concentrated by ultracentrifugation at 15,000 r.p.m. through a 20% sucrose cushion, and the viral pellet was resuspended in PBS and frozen according to published protocols (Kutner et al., 2009). Viral concentrations were determined by p24 ELISA (Cell Biolabs) and viruses were titrated on purified CD4+ T cells to ensure the dose utilized was within the linear range of the assay.

2.2 Cells

Peripheral blood mononuclear cells (PBMCs) and CD4⁺ T cells were obtained from leukopheresis from ALLCELLS, LLC on five healthy donors. All subjects for this study were de-identified healthy control patients negative for HIV, hepatitis B virus, and hepatitis C virus. CD4⁺ T cells were purified by adding additional red blood cells and Rosettesep CD4⁺ T cell enrichment kit antibodies (StemCells, Inc) prior to ficoll gradient separation. Cells were cryopreserved on liquid nitrogen and thawed prior to infection. This study was approved by the Case Western Reserve University Institutional Review Board.

2.3 Infection of CD4⁺ T cells

5×10^5 - 1×10^6 CD4⁺ T cells were infected in parallel with viruses bearing CCR5-tropic (REJO.D12.1972) or CXCR4-tropic (JOTO.TA1.2247) Envs. Viral concentrations were based upon titration results. 12.5-50 ng of virus was used for non-saturating conditions and over 250 ng for saturating conditions. Cells were spinoculated with virus at 1200×g for 2 hours at 24°C and then placed at 37°C for 1 hour. The fusion plate of the parallel infections was then washed with CO₂-independent media (Gibco), resuspended in CCF2-AM (Invitrogen) for 1 hour at room temperature in accordance with manufacturer instructions, washed, and resuspended overnight in CO₂-independent media containing human AB serum and probenidol. The EGFP plate was incubated for 72 hours at 37°C prior to staining for flow cytometry. For outcome determination experiments, cells were set up in three parallel plates. The fusion plate was analyzed as described above. SAHA-induced EGFP expression was monitored as described for the EGFP plate above, with the modification that raltegravir was added to a final concentration of 1 μM 46 hours following infection and SAHA added to a concentration of 2 μM 2 hours later. Cells were incubated in the presence of SAHA for 24 hours prior to staining for flow cytometry. Spontaneous EGFP conditions were identical to SAHA-induction with the exception that SAHA was not added.

2.4 Flow Cytometry

Cells were incubated with anti-human CCR7 IgM (Becton Dickinson) and live/dead fixable yellow viability dye (Invitrogen) for 30 minutes at 37°C, washed, and incubated with anti-human CD3 Brilliant Violet 650 (BioLegend), CD4 APC, and CD27 PE-Cy7 (eBioscience), CD45RO ECD (Beckman Coulter), and anti-IgM PE (Invitrogen) for 30 minutes at 4°C. Cells were washed and resuspended in 1% paraformaldehyde prior to data collection on an LSRII analytical flow cytometer (Becton Dickinson). At least 50,000 events were collected for fusion and 200,000 events for EGFP and were analyzed using FlowJo software (TreeStar).

2.5 Statistics

The data collected were from five independent healthy donors. Infections were performed in duplicate for both fusion and productive infection conditions and repeated 2-3 times using cells from 5 donors. Spinoculation and bla-Vpr +/- experiments were performed in triplicate on cells from 3 healthy donors. Data are presented as the mean ± standard error of the mean unless otherwise noted. Wilcoxon matched-pairs signed rank tests were used to generate p-values.

3. Results

3.1. Combination Reporter Virus Assay

A single reporter virus system that measures HIV fusion with cells and viral promoter-driven gene expression was developed so that the efficiency of early post-entry events could be accurately determined and investigated. This assay was designed to: (1) closely mimic

the physiological infection of primary cells, (2) identify subsets of immune cells susceptible to HIV, (3) reveal the outcome of virus following entry, (4) be amenable to high-throughput screening methods, and (5) be compatible with intact, living cells to allow cell sorting and further analysis of purified populations. Briefly, packaging cells were transfected with plasmids encoding β -lactamase-vpr (*bla-vpr*) (Cavrois et al., 2002), HIV *envs* with known coreceptor tropism, and an NL4-3 *env*⁻ HIV genome containing an *egfp-env* chimeric gene under control of the viral LTR promoter (Zhang et al., 2004). The resultant virions incorporate bla-Vpr protein and package the HIV core containing the *egfp-env* gene. Viral fusion was determined by loading cells with CCF2-AM, a dye consisting of two fluorescent moieties linked through a β -lactam ring. In the absence of β -lactamase, excitation of cells loaded with the dye with 409 nm light results in Förster resonance energy transfer (FRET) with resultant emission at 520 nm. In cells that have fused with combination reporter virions, bla-Vpr protein cleaves the β -lactam ring of CCF2, eliminating the FRET linkage and resulting in emission at 447 nm. If virus subsequently completes early post-entry viral events including uncoating, reverse transcription, integration, LTR-driven gene expression, and protein translation, EGFP accumulates in the cell. CCF2 fluorescence changes and EGFP accumulation can be detected by flow cytometry or fluorescence microscopy.

To mimic physiological conditions as closely as possible, purified, unstimulated primary CD4⁺ T cells were infected. Due to temporal differences in the viral life cycle, equal populations of cells were infected in parallel: one microtiter plate was analyzed 24 hours post-infection to quantify HIV fusion and a second analyzed 72 hours post-infection to quantify LTR-driven EGFP expression. Analysis of fusion at time points after 24 hours resulted in a decrease in the percentage of cells undergoing bla-Vpr-mediated CCF2 cleavage without a corresponding increase in cellular death (data not shown), indicating that the bla-Vpr protein was likely being degraded in the cells. At 72 hours post-infection, the percentage of fusion-positive cells had decreased by ~75% from the maximum. Coupled with a viability stain and fluorescent antibodies directed against the T cell marker CD3, HIV fusion and productive infection of primary CD4⁺ T cells can be precisely determined (Figure 2A). CD4 was included to assess the purity of CD4⁺ T cells using uninfected samples (>97% for all donors), but was not included in the gating strategy as Nef-mediated CD4-downregulation was used in conjunction with EGFP to identify cells with LTR-driven EGFP expression. Including markers of T cell maturation (CCR7, CD45RO, and CD27) allows monitoring of CD4⁺ T cell subsets: naïve (T_N), central memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}), and terminal effector (T_{TE}) populations (Figure 2B). T_N CD4⁺ T cells express virtually no CCR5 and were highly refractory to fusion and productive infection with a primary CCR5-tropic HIV Env (REJO.D12.1972 (Keele et al., 2008)), providing internal validation of the assay. Since bla-Vpr-mediated CCF2 cleavage and LTR-driven EGFP expression do not require fixation or permeabilization, cells can be sorted to enrich for fused or productively infected subpopulations (Figure 2C). These purified cells can be analyzed further to identify genes and proteins that correlate with progression through the HIV life cycle.

3.2. Measuring efficiency of post-entry viral events

Having measured fusion and spontaneous EGFP expression, an indicator of active gene expression from the viral promoter and therefore a surrogate of productive infection, the fraction of cells that are productively infected following viral entry was calculated (PI/F). High PI/F values denote efficient progression from fusion to viral gene expression, while low PI/F values reflect poor infection efficiency as viruses fuse with many cells that fail to transcribe genes from the LTR promoter (Figure 3A). Titration of combination reporter viruses onto primary CD4⁺ T cells resulted in a linear increase in both fusion and productive infection as viral concentrations increased. Within a large linear range of viral input, PI/F

was stable (Figure 3B). PI/F increased at very high and low concentrations due to saturation of cells susceptible to HIV fusion and to background fluorescence in the EGFP channel, respectively.

Titration were also performed in the presence and absence of spinoculation to determine its effects upon viral fusion, productive infection, and PI/F values. As previously reported (O'Doherty et al., 2000), spinoculation dramatically increased the efficiency of HIV infection (Figure 3C). In three donors, the percentage of cells undergoing fusion when spinoculated was 76.7 ± 15.7 -fold higher than in the absence of spinoculation for a given viral concentration over the linear range of the assay ($p=0.03$). Productive infection was also significantly enhanced by spinoculation at equivalent concentrations of virus (46.7 ± 12.5 -fold higher over the linear range of the assay, $p=0.03$). Two of three donors demonstrated a slight increase in PI/F in the absence of spinoculation, suggesting more efficient completion of post-entry events, although this did not reach statistical significance (mean PI/F of 3 donors: $1.28 \pm 0.19\%$ with spinoculation v. $2.53 \pm 0.72\%$ without spinoculation, $p=0.5$). The effect of bla-Vpr on productive infection of CD4+ T cells was also investigated, since Vpr facilitates the infection of non-dividing cells (Heinzinger et al., 1994). Viruses were produced ($n=5$ for each group) bearing the REJO (R5-tropic) or JOTO (X4-tropic) Envs either with or without bla-Vpr. CD4+ T cells were infected with equivalent p24 concentrations of virus and productive infection assessed by EGFP expression (Figure 3D). Bla-Vpr had no effect on productive infection in three patients (R5-tropic HIV with bla-Vpr: $0.61 \pm 0.13\%$ EGFP+, without bla-Vpr: $0.67 \pm 0.11\%$, $p=0.19$; X4-tropic HIV with bla-Vpr: $1.39 \pm 0.20\%$ EGFP+, without bla-Vpr: $1.35 \pm 0.16\%$, $p=0.95$). Together, these results suggest that neither spinoculation nor bla-Vpr significantly affect the efficiency of post-entry viral events.

Next, the ability of HIV to infect maturation subsets of CD4+ T cells was analyzed. First, saturating levels of virus were utilized to maximize infection and measure the proportion of T_N , T_{CM} , T_{TM} , T_{EM} , and T_{TE} subsets that are potential targets for fusion with a primary X4-tropic HIV isolate (JOTO.TA1.2247 (Wilens et al., 2011)). Fusion with X4-tropic HIV was highest in T_N and T_{CM} subsets and lowest in the T_{TE} subset (Figure 4A), consistent with CXCR4 expression patterns. Next, concentrations of virus within the linear range of the assay were used to measure fusion and productive infection and accurately calculate PI/F within CD4+ T cell maturation subsets (Figure 4B). Strikingly, T_N and T_{CM} cells were approximately 5-10 times less likely to become productively infected following viral fusion than T_{EM} cells (PI/F mean: 0.94%, 1.37%, and 8.97%, respectively, $p=0.0625$ for each comparison). T_{TM} and T_{TE} subsets showed similar infection efficiencies to T_{EM} cells; however these cells were present at low levels and infected cells were only detected in 3 of 5 patients.

3.3. Determining outcome after viral fusion

A striking feature of these infections was the small number of cells productively infected following fusion. To determine if the majority of cells were becoming abortively or latently infected, cells were stimulated with the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA, vorinostat), which reactivates latent HIV expression (Archin et al., 2009). Combining spontaneous and SAHA-induced EGFP expression with fusion measurements allows calculation of the percentages of cells that were productively, latently, or abortively infected following HIV entry (Figure 5A). Importantly, the integrase inhibitor raltegravir was added 2 hours prior to stimulation with SAHA for the induced EGFP condition and at the same time for the spontaneous EGFP condition, blocking further integration events. Infection with CXCR4-tropic HIV overwhelmingly led to abortive infection, consistent with previously reported high levels of defective HIV particles (Piatak et al., 1993). T_{TE} cells were most likely to be latently infected (Figure 5B).

4. Discussion

The combination reporter virus system described here facilitates sensitive measurement of the efficiency of post-entry events. Host proteins modulating infection efficiency include cofactors used by the virus to promote replication, such as LEDGF/p75 (Maertens et al., 2003), and antiviral restriction factors such as SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011). There are undoubtedly many more host proteins that regulate the outcome of HIV infection (Jäger et al., 2012). Application of the combination reporter virus system to high-throughput screening using siRNA or cDNA libraries will help identify novel host proteins regulating post-entry HIV events. Equally important is the ability of this assay to monitor *outcome* of viral infection, including latent infection of cell subsets that are the primary barrier to eradication of the HIV virus *in vivo* (Chun and Fauci, 2012; Siliciano, 2010). Clarifying the subsets of cells most likely to result in latent HIV infection will guide efforts to measure and to eradicate the latent reservoir *in vivo*.

Two important caveats of measuring latency using the combination reporter virus system deserve mention. First, reactivation of latent cells was performed using the HDAC inhibitor SAHA, which does not induce HIV transcription in all latently infected cells. Compared to more powerful activation stimuli such as mitogens or anti-CD3/CD28, SAHA reactivates up to 50% of virus in primary cells and 80% in cell lines (Jonathan Karn, personal communication). However, in contrast to stronger stimuli, SAHA does not induce proliferation and maturation of CD4⁺ T cell subsets, facilitating attempts to measure viral outcome in different cell subsets. The use of SAHA is a strategic compromise to allow identification of a subset of latently infected cells without altering the naïve/memory phenotype. Second, several types of latency have been demonstrated for HIV infection. Pre-integration latency occurs in some cells, particularly resting cells, following fusion (Zack et al., 1990). This form of latency is believed to be due to a block in reverse transcription (Zack et al., 1990) and unintegrated, but integration-competant, reverse transcription products decay with a half-life of approximately 1 day (Zhou et al., 2005). Post-integration latency appears to be a much more stable form of latency due to extremely long half-lives of infected resting memory cells (Chun et al., 1995; Finzi et al., 1997), and represents the primary barrier to eradication efforts. The combination reporter virus system can measure both types of latency by including or excluding an integrase inhibitor to block further integration events. Addition of SAHA plus raltegravir allows for post-integration silent or latent infection to be monitored, while addition of SAHA in the absence of raltegravir can induce EGFP expression in cells with either pre- or post-integration latency.

The implications of this system extend beyond studies of pathogenic HIV infection. HIV-1 is used as a backbone for many viral-based gene therapy vectors due to its ability to infect non-dividing cells. Identifying and counteracting cell type specific restriction factors, such as SAMHD1, will be essential to future gene therapy efforts. Combination reporter viruses pseudotyped with VSV-G Env enable evaluation of transduction efficiency in a wide range of primary cells. Furthermore, the reporter virus system described here can be extended to other human pathogens and viruses studied in animal models. For example, HIV-2 and simian immunodeficiency viruses (SIVs) also package Vpr protein into their respective virions, while analogous β -lactamase CCF2 fusion assays have been developed for Nipah and Hendra virus (Wolf et al., 2009). Adapting these viruses to the combination reporter system would require minimal effort. This system can facilitate study of the host and viral proteins regulating infection efficiency and viral outcome for a potentially wide range of viruses.

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Highlights

- Combination HIV reporter viruses measure fusion and LTR-driven gene expression.
- This system allows for calculation of HIV post-entry event efficiency.
- We determine viral outcome after entry: productive, latent, or abortive infection.
- We measure infection of primary CD4+ T cell subsets.
- This system is compatible with high-throughput screening and cell-sorting techniques.

Figure 1. The viral life cycle of HIV-1

Combination reporter viruses contain β -lactamase-Vpr (bla-Vpr) protein that cleaves the fluorescent substrate CCF2 following viral fusion with target cells and an LTR-driven EGFP reporter gene that is expressed following successful completion of early post-entry events (dashed lines).

Figure 2. Combination reporter viruses allow sensitive detection of HIV fusion and LTR-driven EGFP expression in primary unstimulated CD4+ T cell subsets

(A) Gating strategy for flow cytometric detection of combination reporter virus fusion and EGFP production. Purified CD4+ T cells are gated on singlets, lymphocyte-size, viability, and CD3+ expression. Greater than 95% of viable cells were CD4+ T cells. Viral fusion is observed in HIV-infected cells by a conversion of uncleaved (520nm) CCF2 to cleaved (447nm) CCF2. LTR-driven EGFP expression is determined by a combination of EGFP expression and CD4 down-regulation. (B) Fusion and LTR-driven EGFP expression of the CCR5-tropic Env REJO.D12.1972 in naïve (T_N), memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}), and terminal effector (T_{TE}) populations. Numbers underneath the subset names refer to the percent of fusion(+) or EGFP(+) cells of that phenotype. (C) Sorting of CD4+ T cells into fusion(-) or fusion(+) and EGFP(-) or EGFP(+) subsets results in substantial enrichment of these populations for downstream analysis.

Figure 3. Determination of viral post-entry event efficiency using combination reporter viruses (A) Fusion (F) and spontaneous LTR-driven gene expression (a surrogate for productive infection, PI) in primary CD4⁺ T cells infected with combination reporter viruses bearing the CCR5-tropic Env REJO.D12.1972. PI/F reflects the proportion of cells progressing to productive infection following a fusion event. Data represent mean of 2-3 replicates for each of 5 healthy donors. Box and whiskers plot shows median, 25th-75th percentile, and minimum/maximum of 5 donors. (B) Titration of combination reporter viruses bearing the CXCR4-tropic Env JOTO.TA1.2247 on primary CD4⁺ T cells. Each concentration was performed in triplicate and data is representative from three replicates on different donors. (C) Effect of spinoculation on fusion and productive infection. Each concentration was performed in triplicate and data is representative from three replicates on different donors. (D) Effect of bla-Vpr on productive infection of cells by viruses bearing REJO.D12.1972 or JOTO.TA1.2247 Envs. Box and whiskers plots show median, 25th-75th percentile, and minimum/maximum of EGFP⁺ cells from 3 donors infected in triplicate with 5 separate viruses in each group.

Figure 4. HIV fusion and post-entry event efficiency in CD4+ T cell subsets

Maximal fusion and PI/F values of CD4+ T cell maturation subsets were measured using saturating and linear range concentrations virus, respectively. Box and whiskers plots show median, 25th-75th percentile, and minimum/maximum of EGFP+ cells from 2-3 replicate experiments from 5 donors.

Figure 5. Calculation of viral outcome using combination reporter viruses

(A) The percentage of abortively, latently, and productively infected cells can be calculated from spontaneous EGFP, SAHA-induced EGFP, and fusion measurements. (B) Productive, latent, and abortive infection of CD4+ T cell maturation subsets infected with JOTO.TA1.2247-bearing combination reporter viruses. Data shown are the median values from 2-3 replicate experiments using cells from 5 donors.