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Engineered lentiviral vectors pseudotyped with a CD4 receptor and a fusogenic protein can target cells expressing HIV-1 envelope proteins

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Abstract

Lentiviral vectors (LVs) derived from human immunodeficiency virus type 1 (HIV-1) are promising vehicles for gene delivery because they not only efficiently transduce both dividing and non-dividing cells, but also maintain long-term transgene expression. Development of an LV system capable of transducing cells in a cell type-specific manner can be beneficial for certain applications that rely on targeted gene delivery. Previously it was shown that an inverse fusion strategy that incorporated an HIV-1 receptor (CD4) and its co-receptor (CXCR4 or CCR5) onto vector surfaces could confer to LVs the ability to selectively deliver genes to HIV-1 envelope-expressing cells. To build upon this work, we aim to improve its relatively low transduction efficiency and circumvent its inability to target multiple tropisms of HIV-1 by a single vector. We investigated a method to create LVs co-enveloped with the HIV-1 cellular receptor CD4 and a fusogenic protein derived from the Sindbis virus glycoprotein and tested its efficiency to selectively deliver genes into cells expressing HIV-1 envelope proteins. The engineered LV system yields a higher level of transduction efficiency and a broader tropism towards cells displaying the HIV-1 envelope protein (Env) than the previously developed system. Furthermore, we demonstrated *in vitro* that this engineered LV can preferentially deliver suicide gene therapy to HIV-1 envelope-expressing cells. We conclude that it is potentially feasible to target LVs towards HIV-1-infected cells by functional co-incorporation of the CD4 and fusogenic proteins, and provide preliminary evidence for further investigation on a potential alternative treatment for eradicating HIV-1-infected cells that produce drug-resistant viruses after highly active antiretroviral therapy (HAART).

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Authors' contributions

CLL, JD and KIJ performed experiments. CLL and PW designed experiments, interpreted data, and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Keywords

Retrovirus; Lentivirus; Human immunodeficiency virus; Targeted gene delivery

1. Introduction

An important aspect of gene therapy is the delivery of genetic materials to target cells for therapeutic benefit. One of the most important and efficient methods for gene delivery is the use of viral vectors as transfer vehicles (Verma and Somia, 1997; Verma and Weitzman, 2005). Viral vectors are separated into two major groups—integrating viral vectors and non-integrating viral vectors. Integrating viral vectors are based on retroviruses, lentiviruses, and adeno-associated viruses while non-integrating viral vectors include adenoviruses (Somia and Verma, 2000). Of these viruses, HIV-based lentiviral vectors (LVs) are promising as a gene delivery system for certain applications because of their ability to induce stable transduction, maintain long-term transgene expression and transduce nondividing cells (Kohn, 2007; Naldini et al., 1996; Verma and Weitzman, 2005). Engineering LVs capable of delivering genes of interest to predetermined cells can reduce off-target effects and improve the safety profile, which will further enhance the promise of this vector system for gene therapy applications (Cronin et al., 2005; Waehler et al., 2007).

The entry of human immunodeficiency virus type 1 (HIV-1) into human host cells is mediated by interactions between the viral envelope glycoprotein (Env) and the receptor complex of target cells, which includes the CD4 receptor and the co-receptor (CCR5 or CXCR4) of the chemokine receptor family (Moore, 1997). The virus initially gains access to the host cells by interactions between CD4 and the surface unit (gp120) of Env, which triggers conformational changes in gp120 that allows for further binding with co-receptors. Subsequently the membrane unit (gp41) of Env evokes fusion to release the viral capsid into the cytoplasm of the host cells (Dimitrov, 1997). Based on this membrane fusion mechanism, the inverse fusion strategy has been developed to target HIV-1-infected cells (Fig. 1A, left panel). This strategy involves the incorporation of the CD4/co-receptor complex into the viral vector membrane for its specific entry into HIV-1 Env-expressing cells. Several reports have described their successes of utilizing this inverse fusion method to generate such targeting vectors (Bittner et al., 2002; Endres et al., 1997; Mebatsion et al., 1997; Peretti et al., 2006; Schnell et al., 1997; Somia et al., 2000; Ye et al., 2005).

Previously we showed that recombinant LVs can be engineered by a two-molecule method to possess a targeting ability for gene delivery to desired cells *in vitro* and *in vivo* (Yang et al., 2006). To accomplish this, the vector's binding and fusion functions are separated into two proteins. Antibodies or ligands are incorporated onto the vector surface to mediate binding, while a mutant Sindbis viral glycoprotein is co-displayed on the vector surface to execute its fusion activity. We further incorporated several different fusogenic molecules (FMs) that were engineered based on Kielian and co-workers' study (Lu et al., 1999) and showed that these FMs could significantly improve the transduction efficiency of targeting vectors (Yang et al., 2008). An entry study revealed that the engineered vector particles can be internalized through clathrin-dependent endocytosis upon binding to target cells and further transported into the endosomal compartment, where the FMs on the vector surface sense the low pH and undergo a conformation change to trigger fusion, releasing the viral core into the cytosol (Joo and Wang, 2008).

In this study, we investigated the application of this two-molecule method for generating LVs capable of specifically transducing HIV-1 Env-expressing cells. We demonstrated that LVs displaying the HIV-1 primary receptor CD4 and the FM derived from the mutant

Sindbis virus glycoprotein can achieve selective gene delivery to cells expressing HIV-1 Env *in vitro* with remarkable specificity and efficiency. Such an HIV-1 Env-specific LV system was shown to be able to deliver a suicide gene into a human T cell line that expresses HIV-1 Env and induce the specific killing of envelope-expressing cells cultivated with a prodrug.

2. Materials and methods

2.1. Plasmids

The FM molecules derived from the Sindbis virus glycoprotein, AKN, AGM and SGN have previously been reported by our laboratory (Yang et al., 2008, 2006). Human CD4, CCR5, and CXCR4 cDNAs were cloned downstream of the CMV promoter in the pCDNA3 plasmid (Invitrogen, Carlsbad, CA) to create pCD4, pCCR5, and pCXCR4. The mouse stem cell virus-based retroviral transfer plasmid MIG (Yang and Baltimore, 2005) was kindly provided by Dr. David Baltimore's laboratory. The cDNA for the surface marker, human low-affinity nerve growth factor receptor (LNGFR), was extracted from the pMACS-LNGFR-IRES vector (Miltenyi Biotec, 51429 Bergisch Gladbach, Germany) using the NcoI and SalI sites and cloned into the MIG plasmid in place of the GFP gene. The resulting plasmid was referred to as MINFR. The cDNA of the CCR5-tropic HIV-1 Subtype C envelope glycoprotein was isolated from the plasmid pcDNA3-gp160C (Gao et al., 2003) (NIH AIDS Research and Reference Reagent Program, Germantown, MD, USA), and inserted into MINFR at a site upstream of IRES. The resulting plasmid was designated as MINFR-gp160R5. The cDNA of the CXCR4-tropic HIV-1 subtype B envelope glycoprotein was derived from pcDNA3-gp160HxBc2, generously provided by Dr. Pamela Bjorkman's laboratory at the California Institute of Technology, and cloned into the MINFR upstream of IRES. This plasmid was referred to as MINFR-gp160X4. The HIV-1-based lentiviral vector FUGW was reported by Dr. David Baltimore's Laboratory (Lois et al., 2002) and used in this study. The suicide gene, the mutant of Herpes Simplex Virus-1 thymidine kinase SR39Tk, was amplified from a reported construct (Black et al., 2001) and cloned downstream of the human ubiquitin-C promoter in the lentiviral vector plasmid FUW (Ziegler et al., 2008). The construct was referred to as FUWSR39TK.

The wild-type Rab5 and Rab7 cDNAs were PCR-amplified and cloned into the pDsRed-monomer-C1 (Clontech, Mountain View, CA, USA) to form the DsRed-Rab5^{WT} and DsRed-Rab7^{WT} constructs. The plasmid encoding the dominant-negative mutant of DsRed-Rab7^{DN} (Rab7T22N) was created by site-directed mutagenesis using the forward primer (5'-GTC GGG AAG AAC TCA CTC ATG AAC C-3') and the backward primer (5'-GGT TCA TGA GTG AGT TCT TCC CGA C-3'). The construct for the dominant-negative mutant of DsRed-Rab5^{DN} was obtained from Addgene (Cambridge, MA, USA).

The cDNAs for wild-type dynamin 2 and the dominant-negative dynamin 2 K44A mutant were derived from the pEGFP-Dyn2 and pEGFP-Dyn2 K44A vectors, respectively, which were kindly provided by Dr. Okamoto's laboratory, using the HindIII and EcoRI restriction sites and cloned into the pDsRed-monomer-C1 construct (Clontech). The resulting plasmids are designated as DsRed-Dyn2^{WT} and DsRed-Dyn2^{K44A}.

2.2. Cell lines

The 293T cells (human kidney embryonic cells with the Simian Virus 40 large T antigen) and Jurkat cells were obtained from the American Type Culture Collection. 293T, 293T.EnvR5 and 293T.EnvX4 cells were cultured in Dulbecco's modified Eagle's medium (Mediatech Inc., Manassas, VA, USA) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 2 mM L-glutamine. The human CD4 and CCR5 expressing cell line,

TZM-bl, was provided by the NIH AIDS Research & Reference Reagent Program. The TZM-bl cell line was cultured in the same medium described above with the addition of 50 µg/mL gentamycin (Sigma). All of the cells were maintained in a 5% CO₂, 37°C environment.

2.3. Cell–cell fusion assay

TZM-bl (0.2×10^6) or HeLa (0.2×10^6) cells were labeled with 20 µM of octadecyl rhodamine B chloride, R18 (Molecular Probes, Carlsbad, CA, USA), in serum-free medium for 30 min at 37 °C and washed with PBS three times. The cells were then seeded onto glass-bottom culture dishes (MatTek Corporation, Ashland, MA, USA) and grown at 37 °C overnight. 293T.EnvR5 cells (0.2×10^6) labeled with 10 µM of 5,6-carboxy-fluorescein diacetate succinimidyl ester, CFSE (Molecular Probes), were overlaid on TZM-bl or HeLa cells for 10 min at 37 °C. Fluorescent images were acquired on a Yokogawa spinning-disk confocal scanner system (Solamere Technology Group, Salt Lake City, UT) using a Nikon eclipse Ti-E microscope equipped with a 60×/1.49 Apo TIRF oil objective and a Cascade II: 512 EMCCD camera (Photometrics, Tucson, AZ, USA). An AOTF (acousto-optical tunable filter) controlled laser-merge system (Solamere Technology Group Inc., Salt Lake City, UT, USA) was used to provide illumination power at each of the following laser lines: 491 nm, 561 nm, and 640 nm solid state lasers (50 mW for each laser).

2.4. Vector production

The 293T cells were seeded in a 6-cm culture dish. After 18–20 h, the seeded cells were transfected with DNA plasmids using the standard calcium phosphate precipitation technique when confluency reached approximately 80%. 5 µg of the lentiviral vector plasmid FUGW, 2.5 µg each of the packaging plasmid pMGL and pRev (Tiscornia et al., 2006), 2.5 µg of the surface-expressing plasmid pCD4 and pCCR5/pCXCR4, and 2.5 µg of the plasmid pFM encoding AKN, AGM, or SGN, were mixed together with calcium chloride and added to 2×HBS buffer. The vector supernatant was harvested 48 h after transfection and filtered through a 0.45-µm pore size filter.

2.5. Vector transduction in vitro

Target cells (293T, 293T.EnvR5, Jurkat, and Jurkat infected with MINFR-gp160R5; 0.2×10^6) were plated in 24-well culture dishes with vector supernatant (2 mL per well) and spin-transduced for 90 min at 2500 rpm and 25 °C. After transduction, the vector supernatant was replaced with fresh media. The transduced cells were cultured for another 3–5 days at 37 °C and 5% CO₂. The transduction results were determined by flow cytometry analysis of GFP-positive cells. Vector titer was determined by counting GFP expression in the vector dilution range in which GFP-positive cells and vector volume exhibit a linear relationship.

For transduction of drug-treated cells, 293T.EnvR5 cells were preincubated with drugs (chlorpromazine: 10 µg/mL; genistein: 50 µg/mL; nystatin: 25 µg/mL) for 30 min at 37 °C and then spin-transduced with 2 mL vector supernatant for 90 min at 2500 rpm and 25 °C. The drug-containing media were then replaced with flash D10 media 3 h after transduction. For vector transduction with dynamin or Rab protein-treated cells, 293T.EnvR5 cells were transfected with DsRed-Dyn2, DsRed-5, or DsRed-7 (either wild-type or dominant-negative mutant), seeded, and spin-transduced with 2 mL vector supernatant. GFP-positive cells were analyzed by flow cytometry three days post-transduction.

2.6. Cell–vector binding

293T or 293T.EnvR5 cells (0.2×10^6) were incubated with 1.5 mL of the LV supernatant at 4 °C for 5 min. The cell–vector complexes were washed with cold PBS and fixed by 4%

formaldehyde on ice for 10 min. To probe the cell–vector complexes, an anti-HA antibody (Miltenyi Biotec) was used to stain the HA-tag expressed on the FM, followed by Alexa594-conjugated streptavidin (Zymed Laboratories, South San Francisco, CA, USA), and analyzed by flow cytometry (FACSort, BD Bioscience, San Jose, CA, USA).

2.7. Soluble CD4 competition and NH_4Cl neutralizing

293T.EnvR4 cells (0.2×10^6) were co-incubated with the vector supernatant and various quantities of soluble CD4 or ammonium chloride (NH_4Cl) in a 24-well culture dish at 37°C and 5% CO_2 for 8 h. The media was then replaced with fresh media. After incubation for an additional 4 days, the GFP-positive cells were analyzed by flow cytometry (FACSort, BD Bioscience).

2.8. Targeted delivery of suicide gene therapy in vitro

Jurkat cells were transduced by a retroviral vector, MINFR-gp160R5, pseudotyped with VSVG to express HIV-1 Env proteins. Three days later, the transduced cells were incubated with the concentrated LVs encoding SR39Tk (FUWSR39tk/CD4+SGN) for transduction by the method previously described. Afterwards, the cells were cultured in media with or without prodrug ganciclovir (GCV; Sigma–Aldrich, St. Louis, MO, USA) for an additional seven days. The treated cells were stained with an Alexa594-conjugated CD271 (LNGFR) antibody (Miltenyi Biotec) and analyzed by flow cytometry (FACSort, BD Bioscience).

3. Results

3.1. Generation of the HIV-1 envelope protein-expressing cell line

We have previously reported that lentiviral vectors (LVs) pseudotyped with an antibody and a FM can transduce cells in a cell type-specific manner (Yang et al., 2006). The FM was derived from the altered Sindbis virus glycoprotein with mutations in the E2 binding domain. Because this method separates the two key functions of viral glycoproteins (binding and fusion) into two distinctive proteins, we can theoretically generate a new targeting vector by simply changing the binding protein on the vector surface. Therefore, we hypothesized that co-incorporation of a human CD4 protein and an engineered FM could confer upon an LV with the capability to specifically deliver genes into cells infected with HIV-1 (Fig. 1A, right panel).

To test this hypothesis, we created a cell line expressing HIV-1 Env. A mouse stem cell virus-based retroviral transfer plasmid was constructed to encode an HIV-1 clade C and CCR5-tropic Env gene (gp160R5, from the 96ZM651 strain) and a surface marker (LNGFR: truncated low affinity nerve growth factor receptor gene); the construct is designated as MINFR-gp160R5 (Fig. 2A). An internal ribosome entry site (IRES) was used to link gp160R5 and LNGFR to achieve co-expression that was driven by the 5' LTR promoter (Yang and Baltimore, 2005). We have tested several commercially available antibodies and could not identify one that could be reliably used for flow cytometry analysis of Env expression. Thus, this design could allow us to readily quantify the expression of HIV-1 Env by surface staining of the marker LNGFR. To generate the HIV-1 Env-expressing cell line, 293T cells were transduced with the MINFR-gp160R5 vector pseudotyped with the vesicular stomatitis virus glycoprotein (VSVG). After a few weeks of cell passaging, individual single cell clones were selected, stained with an anti-LNGFR antibody, and analyzed by flow cytometry. An LNGFR-positive clone (Fig. 2B) was subsequently tested by a cell–cell fusion assay. The fusion events between cells expressing HIV-1 Env and cells expressing CD4 and co-receptor can be monitored by the redistribution of dye molecules; this assay has been widely used in the observation of cell and viral membrane fusion (Blumenthal et al., 2002). We labeled HeLa or TZM-b1 (a HeLa cell line expressing CD4

and CCR5) cells with fluorescent membrane dye, octadecyl rhodamine B chloride (R-18), and overlaid them with the selected clonal cells labeled with an intracellular dye, 5,6-carboxy-fluorescein diacetate succinimidyl ester (CFSE). Redistribution of the red fluorescence dye (R-18) only from labeled TZM-b1 cells, but not from HeLa cells, to the target cells confirmed the functional expression of HIV-1 Env on the selected clonal cells (Fig. 2C). This clonal cell line was designated as 293T.EnvR5 and was used throughout this study.

3.2. Generation of recombinant LVs

To generate HIV-1 Env-specific LVs, 293T cells were co-transfected using the standard calcium phosphate protocol with the lentiviral transfer plasmid FUGW, the lentiviral vector packaging plasmids (pMGL and pRev) (Tiscornia et al., 2006), the plasmid encoding CD4 (pCD4), and the plasmid encoding the FM (pFM) (Fig. 1B). The resulting vector was designated as FUGW/CD4+FM. FUGW is a self-inactivating HIV-1-based transfer vector backbone plasmid with a GFP reporter gene encoded downstream of the human ubiquitin-C promoter (Lois et al., 2002). Three previously described FMs (AKN, AGM, and SGN) were used in this study (Yang et al., 2008). These FMs were derived from the Sindbis virus glycoprotein with different combinations of mutations to yield proteins with variations in fusion activity. We wanted to test engineered LVs incorporating various combinations of FMs with CD4 for their transduction efficiencies and targeting specificities.

We also wanted to compare our approach with the reported inverse fusion method (Fig. 1A, left panel) (Endres et al., 1997) involving co-incorporation of CD4 and a co-receptor (CCR5 or CXCR4) to generate targeting LVs (designated FUGW/CD4+CCR5 or FUGW/CD4+CXCR4). As a non-targeting control, we generated VSVG-pseudotyped LVs (FUGW/VSVG) with a broad tropism capable of transducing many different cell types (Cronin et al., 2005). For negative controls, we prepared LVs displaying either FM with a non-relevant protein CD20 (FUGW/CD20+FM) or CD4 alone (FUGW/CD4). Flow cytometry analysis of vector-producing cells showed that all of the transfected live cells expressed similar levels of GFP. This indicated that the vector backbone FUGW was compatible with the expression of these proteins (CD4, CCR5, CXCR4, and FMs) and was present in all of the vector-producing cells (Fig. 3A–D, upper panel). Gated on GFP-positive cells, around 40% of the transfected cells co-expressed CD4 and FM (Fig. 3A, lower panel). Approximately 17% of the cells co-expressed the non-relevant protein CD20 and FM (Fig. 3B, lower panel). This apparently low CD20 expression level can be partially attributed to the insensitivity of the anti-CD20 staining antibody, which was documented in a previous study (Ziegler et al., 2008). As expected from the previous inverse fusion study, the producing cells could co-express both CD4 and a co-receptor (CCR5 or CXCR4) on their surfaces (Fig. 3C). From this expression pattern analysis, we found that individual vector-producing cells could be transfected to co-express CD4 and FMs, a step that was necessary for them to be incorporated into the lentiviral surface.

3.3. Targeted transduction of HIV-1 Env-expressing cells

To test the ability of engineered LVs to specifically transduce cells expressing HIV-1 Env, the vector particles were harvested from the supernatant of transfected vector-producing cells and incubated with 293T.EnvR5; the parental 293T cell line was used as a negative control. Flow cytometry was used five days post-transduction to determine the transduction efficiency by calculating the percentage of GFP-positive cells. The transduction magnitude was obtained by measuring the mean fluorescence intensity (MFI) of the transduced cells. To compare the difference between the target viral vectors, we utilized integrated MFI (iMFI), which reflects the total intensity of the GFP signals from the transduced cells by multiplying the MFI with transduction efficiency to quantify the targeted transduction ability

(Lei et al., 2009) (Fig. 4). As expected, both 293T.EnvR5 and 293T cells could be transduced by the non-targeting vector FUGW/VSVG (Fig. 4B). The HIV-1 Env-expressing cells (293T.EnvR5) exhibited significant iMFI signal when exposed to the targeting vector FUGW/CD4+FM (Fig. 4A, left 3 columns). As a negative control, the LV displaying a non-relevant protein FUGW/CD20+FM showed only a background level transduction for 293T.Env (Fig. 4A, right 3 columns). The Env-negative cells (293T) yielded only background levels of iMFI signal when transduced with both relevant and non-relevant LVs (FUGW/CD4+FM and FUGW/CD20+FM). The LV displaying only CD4 on the surface (FUGW/CD4) could not transduce either cell line, confirming the requirement of FM to mediate the fusion of LVs to deliver genes (Fig. 4A).

As reported previously, we also observed targeted transduction with LVs engineered by the inverse fusion method to carry both CD4 and CCR5 (Fig. 4A, left 4th column). Because the Env expressed on 293T.EnvR5 is CCR5-tropic, only background transduction was seen when FUGW/CD4+CXCR4 was used. The transduction titers of various LVs for 293T.Env and 293T are summarized in Fig. 5. The titers of targeting vectors remained lower than that of the non-specific vector FUGW/VSVG. Because FUGW/CD4+SGN gave us the relatively higher transduction and reasonably low background (Fig. 5A), we decided to focus on this vector for the following investigations. As compared with the inverse fusion vector FUGW/CD4+CCR5, FUGW/CD4+SGN yielded a higher vector titer against HIV-1 Env-expressing cells.

3.4. Targeting a broader tropism of gp120 moiety

One disadvantage of using the inverse fusion method to target LVs is the need to switch between the two co-receptors CCR5 and CXCR4 on the vector surface in order to transduce cells expressing Env derived from different HIV-1 tropisms. Based on the inverse fusion method, CD4 plus CXCR4 pseudotyped vectors are required to transduce X4-tropic HIV-1 infected cells, while R5-tropic HIV-1 infected cells require a vector bearing a combination of CD4 and CCR5 (Peretti et al., 2006). Our experimental data confirmed this tropic-limited LV system. We found that the FUGW/CD4+CXCR4 vector could not effectively transduce 293T.EnvR5 cells expressing an Env derived from a R5-tropic HIV-1 subtype C virus strain 96ZM651 (Gao et al., 2003), but the same cells could be transduced by the CCR5-carrying vector (FUGW/CD4+CCR5) (Figs. 4 and 5). Since our targeting LV system does not involve co-receptors, their ability to transduce cells should be independent on the tropism of HIV-1 Env. To confirm this hypothesis, we used the same strategy to construct an X4-tropic Env-expressing cell line. The new cell line displayed an Env from an X4-tropic strain of HIV-1 subtype B virus (HXBc2 stain) and was designated as 293T.EnvX4 (Fig. 6A). This cell line was challenged with three different targeting LVs (FUGW/CD4+SGN, FUGW/CD4+CXCR4, or FUGW/CD4+CCR5). iMFI signals from 293T.EnvX4 cells exposed with both FUGW/CD4+SGN and FUGW/CD4+CXCR4 vectors were detected to be at a similar level (Fig. 6B). A lower iMFI signals was found in FUGW/CD4+CCR5-transduced cells, demonstrating that X4-tropic Env-expressing cells preferred transduction by the CXCR4-bearing LVs rather than the vector displaying CCR5. This higher than usual background transduction of FUGW/CD4+CCR5 towards 293T.EnvX4 cells is possibly due to non-tropism specific membrane fusion induced by X4-tropic Env (Pleskoff et al., 1998).

3.5. Specific binding between vectors and cells for targeted transduction

The study of binding between vector particles and target cells was performed to further investigate whether a specific interaction between the engineered LV and the Env-expressing cells was necessary to achieve targeted transduction. The LV particles (FUGW/CD4+SGN) were co-incubated with either 293T.EnvR5 or 293T at 4 °C for 5 min, after which the cell–vector complexes were immediately fixed by 4% formaldehyde to reduce the

binding-induced internalization of the LVs. The cell–vector complexes were then stained with an antibody against the FM. The FM signals were detected on 293T.EnvR5 surfaces, but not on 293T cell surfaces, indicating that the observed binding requires the presence of Env proteins (Fig. 7A).

Next, we determined whether the specific interaction between the CD4 displayed on the vector surface and the HIV-1 Env on the surface of target cells is critical for mediating the specific transduction. To do this, 293T.EnvR5 cells were incubated with the LVs (FUGW/CD4+SGN) in the presence of various concentrations of soluble CD4. We found that the transduction efficiency decreased as the concentration of the soluble CD4 increased (Fig. 7B), suggesting that the presence of soluble CD4 could compete with the targeting LVs to bind to 293T.EnvR5 and thus block the vector transduction. These results confirmed that the binding between the HIV-1 Env on the cell surface and the CD4 on the vector was necessary for the specific transduction.

3.6. Dependence of targeted transduction on the endosomal pH

The conformation change of the FM derived from the Sindbis virus glycoprotein can be triggered within the lumen of the endosomes by acidic pH to allow for fusion to occur between the viral particles and the host cell's endosomal membrane (Glomb-Reinmund and Kielian, 1998). This fusion is a critical step for the viral vector to release its genetic content into the cell's cytoplasm. In order to verify the acidic pH requirement for our targeting LV, we incubated FUGW/CD4+SGN with 293T.EnvR5 cells in the presence of graded concentrations of ammonium chloride (NH₄Cl), which is known to neutralize the pH in the endosomal compartments (Mellman et al., 1986). The results showed that the transduction efficiency dramatically decreased with increasing NH₄Cl concentrations (Fig. 7C), thus confirming the low pH dependency of the incorporated FM in mediating gene delivery by engineered LV to target cells.

3.7. Dependence of clathrin and dynamin for internalization of engineered LVs

Clathrin and caveolin-mediated endocytosis have been well characterized for the internalization of many viruses into cells (Blanchard et al., 2006; DeTulleo and Kirchhausen, 1998; Doxsey et al., 1987; Joo et al., 2008; Kirchhausen, 2000; Nabi and Le, 2003; Nichols and Lippincott-Schwartz, 2001; Rust et al., 2004). To determine whether our engineered LVs entered cells through the clathrin or caveolin-mediated endocytosis pathway, we investigated the effects of different inhibitory drugs on the transduction efficiency (Fig. 8A). Chlorpromazine is a drug known to inactivate clathrin polymerization and thereby inhibit internalization mediated by clathrin-coated vesicles (CCV) (Wanget al., 1993), while genistein, a tyrosine phosphatase inhibitor, and nystatin, a cholesterol-binding reagent, have been shown to disrupt caveolar-mediated endocytosis (Alkhatib et al., 1996; Rothberg et al., 1992). The transduction results showed that the entry of FUGW/CD4+SGN was markedly inhibited by the chlorpromazine treatment (Fig. 8A), although no inhibitory effect was clearly seen for the treatments by genistein or nystatin. This indicates that the targeting LV system utilizes the clathrin-mediated endocytosis to enter the cells.

To further confirm that the clathrin pathway was involved in LV entry, we used a dominant-negative mutant of dynamin 2 K44A to block the dynamin-dependent endocytosis pathway (Vidricaire and Tremblay, 2007). 293T.EnvR5 cells were transiently transfected with the inhibitory plasmid DsRed-Dyn2^{K44A} and then exposed to FUGW/CD4+SGN. At the same time, the wild type dynamin plasmid, DsRed-Dyn2^{WT}, was used as a control. The Dyn2^{K44A}-treated cells showed a reduction in transduction efficiency when compared with Dyn2^{WT}-treated cells (Fig. 8B). The data revealed that the entry of the targeting LVs is mediated through a clathrin and dynamin dependent endocytosis pathway.

3.8. Transduction inhibition by dominant-negative Rab5 and Rab7

Fusion of many viruses is believed to occur either at the early or late stage of maturing endosomes. In order to examine which stage of the endosomal compartments was essential for the successful transduction of the targeting LV, 293T.EnvR5 cells were transfected with either the wild type (Rab5WT and Rab7WT) or dominant-negative mutants (Rab5DN and Rab7DN) of Rab5 and Rab7 to disable the early endosome (Stenmark et al., 1994) or late endosome (Press et al., 1998) functions, respectively. Cells with the dominant-negative mutant Rab5 showed a significant reduction in transduction rate, by almost 30%, as compared with the wild type Rab5-expressing cells (Fig. 8C), suggesting that successful transduction is at least in part associated with the early stage of LV-containing endosomes. However, expression of the dominant-negative Rab7 in 293T.EnvR5 did not markedly alter the transduction by FUGW/CD4+SGN (Fig. 8C), indicating that late endosome trafficking is unlikely to be involved in successful LV-infection of target cells.

3.9. Targeted transduction by engineered LVs towards a human T cells

HIV-1 naturally infects human T cells; therefore we investigated the selective transduction of a human T cell line expressing R5-tropic HIV-1 Env by the LV (FUGW/CD4+SGN). We generated Jurkat cells that expressed Env by infecting parent Jurkat cells, a CD4-positive T cell leukemia cell line, with the VSVG-pseudotyped retroviral vector MINFR-gp160R5. After four days, these treated Jurkat cells were analyzed by surface staining of LNGFR as an indication of Env expression. It was found that approximately 46% of the cells expressed the surface marker LNGFR (Fig. 9A). We intentionally maintained this mixed population of cells for the subsequent experiment because we were interested in testing the specificity of FUGW/CD4+SGN in targeting Env-expressing T cells. FUGW/CD4+SGN was then used to transduce this T cell population and the resulting GFP expression was analyzed after culturing the cells for an additional four days. There were approximately 51% T cells that were GFP-positive, out of which ~95% (47.7% of the total cell population) expressed both GFP and LNGFR (Fig. 9B), indicating a strong correlation between LV transduction and the presence of Env in the T cells. Only a background level of GFP was seen when the Env-negative Jurkat cells were exposed to FUGW/CD4+SGN (Fig. 9B), confirming the indispensability of Env expression for efficient targeting. We further assessed the ability of this engineered LV to target human primary T cells. The *in vitro* activated human peripheral blood mononuclear cells (PBMC) were engineered to express R5-tropic HIV-1 Env by MINFR-gp160R5/VSVG-mediated retroviral transduction. These treated PBMC were then exposed to the engineered FUGW/CD4+SGN vector; transduction to non-treated PBMC was included as a control. As shown in Fig. 9C, treated PBMC transduced with the targeting vector resulted in about 8% GFP⁺ cells, whereas only a background level of the GFP signal was detected in the non-treated cells. This result is in agreement with the T cell line data and confirms that the engineered LV could selectively modify Env-expressing cells in a mixed population, omitting the conventional step of purifying target cells.

3.10. Evaluation of the potential of LV-mediated suicide gene therapy in vitro

To demonstrate the potential utility of the Env-specific LV described above, we constructed the targeting vector encoding a thymidine kinase gene derived from Herpes Simplex Virus type 1 (HSV1-TK), a widely used suicide gene, and assessed its efficacy in eradicating HIV-1 Env-expressing cells. The working mechanism of this suicide gene approach is that the otherwise non-toxic prodrug ganciclovir (GCV), when supplied to the target cells, is transformed into a toxic metabolite by the HSV1-TK expressed in the cells to mediate specific killing (Blumenthal et al., 2007). The HSV1-TK-encoding lentiviral backbone plasmid FUWSR39tk, constructed in our laboratory and reported previously (Ziegler et al., 2008), was used in this experiment. The mixed population of Env-expressing Jurkat cells described above was challenged with the FUWSR39tk/CD4+SGN vector three times,

followed by further culture in media with or without the supplement of the prodrug GCV for an additional seven days. It was observed that more than 30% of the Env-positive T cells were eradicated, while the control groups (cells treated with GCV but without the vector, or cells treated with the vector but without GCV) maintained a normal presence of Env-expressing cells (Fig. 9D, left). The 7-aminoactinomycin D (7-AAD) staining of various treated Jurkat cell groups confirmed that the Env-expressing cells exposed to FUWSR39tk/CD4+SGN and GCV had the highest ongoing apoptosis (Fig. 9D, right). Our data demonstrated that it is possible to utilize this targeting LV carrying the suicide gene in combination with the prodrug treatment to specifically eliminate HIV-1-infected cells.

4. Discussion

We have developed a novel LV that displays the HIV-1 receptor (CD4) and the FM molecule. This LV/CD4+FM vector is inefficient in transducing normal cells, but can enter cells expressing HIV-1 Env proteins and mediate gene delivery with reasonable efficiency. The vector can be produced by transient transfection of 293T cells with an appropriate combination of plasmids harboring the lentiviral backbone, packaging proteins, CD4 and FM. The flow cytometry analysis confirmed the amazing ability of the transfected 293T cells to co-express all of necessary proteins for making this designed LV. The producer cells then bud vector particles containing CD4 and FM in the vector membrane and release them into the medium supernatant. An *in vitro* transduction assay revealed that the harvested supernatant containing LV/CD4+FM could efficiently transduce HIV-1 Env-expressing cells and only a background level of transduction was seen when this same vector was exposed to cells lacking HIV-1 Env. Various control experiments verified that both CD4 and FM are necessary for the Env-targeted transduction, and only background transduction was observed when vectors bearing either CD4 alone or CD4 along with a non-relevant protein were used. This specific transduction can be inhibited by the soluble CD4 protein or an endosome neutralization reagent (ammonium chloride) added to the cells. In a mixed population of cells of which half were Env-positive and half were Env-negative, LV/CD4+FM could selectively modify Env-expressing cells and no transduction was detected towards cells lacking Env.

Several groups have reported various vector systems targeting Env-expressing cells using a strategy termed inverse fusion, in which the HIV-1 receptor and co-receptor molecules, CD4 and CCR5/CXCR4, respectively, are introduced into the surface of individual vector particles, directing them to cells positive for HIV-1 Env (Bittner et al., 2002; Endres et al., 1997; Mebatsion et al., 1997; Peretti et al., 2006; Schnell et al., 1997; Somia et al., 2000; Ye et al., 2005). Coordinated interactions between incorporated CD4 and CCR5/CXCR4 on the vector surface with cell surface Env are sufficient to trigger Env-mediated fusion and achieve targeted gene delivery. This mimics the natural infection of HIV-1 to target cells, but with a swapped configuration. Success of this inverse fusion method has been demonstrated in vector systems derived from HIV-1 (Endres et al., 1997), rhabdovirus (Mebatsion et al., 1997), vesicular stomatitis virus (Schnell et al., 1997), and murine leukemia virus (Somia et al., 2000). The designer vectors based on this method, however, are limited to target cells infected with one tropism of HIV-1. Vectors pseudotyped with CD4 and CCR5 can only target cells expressing Env derived from the macrophage-tropic HIV-1 strain (R5 strain), while vectors enveloped with CD4 and CXCR4 are restricted to target cells expressing the T-cell tropic Env derived from the X4 strain of HIV-1. As a control group in the current study, we confirmed this tropism-limited targeting using the inverse fusion method (Figs. 4 and 5). In contrast, our system does not involve the HIV-1 co-receptor and thus can overcome this tropism restriction. We showed that the single LV/CD4+FM vector could target both the R5- and X4-tropism of HIV-1 Env and mediate gene delivery to cells expressing either type of Env with similar efficiency.

We hypothesized that the entry of the targeting LV is initiated when the vector carrying CD4 binds to the HIV-1 Env. Our vector–cell binding assay confirmed that vector-incorporated CD4 remained functional and was able to direct LVs to bind to 293T.EnvR5 cells. When soluble CD4 protein was added to the cells at the initial stage of transduction, a dose-dependent reduction of overall transduction was obtained. These data suggest that the interaction between vector CD4 and cell surface Env play a pivotal role in initiating the targeting. We further postulated that the binding should induce receptor-mediated endocytosis to uptake LVs into endosomal compartments, where the low pH environment can prompt the conformation change of the vector-carrying FM molecule and induce fusion between endosome and vector membranes (Joo and Wang, 2008). Using an endosomal neutralization assay, we found that the transduction efficiencies of the targeting vectors decreased with increasing concentrations of NH₄Cl. This trend indicates that the drop in pH is important for vector transduction. When the three FM molecules (AKN, AGM, and SGN) were employed to test their ability to mediate targeted transduction by LV, we obtained different transduction efficiencies. Among them, the SGN vector (FUGW/CD4+SGN) was the most efficient vector. Similar variations in targeting efficiencies were also observed in our previous studies (Yang et al., 2008). These FM molecules are based on a binding-deficient and fusion-competent version of the Sindbis virus glycoprotein with additional mutations in the E1 glycoprotein domain. Although these additional mutations (AGM and SGN) were originally identified from Sindbis viruses propagated in cholesterol-depleted cells and viruses carrying these mutations were believed to be less dependent on cholesterol for fusion, we speculate that these FM molecules might also have different responsiveness to the endosomal pH, with SGN being the most responsive and fusion-active. Our reported vector–liposome fusion assay actually supports this speculation, and SGN was found to be the most pH-sensitive fusogen (Lei et al., 2010). Nevertheless, the reduction in transduction efficiency by the endosomal neutralization assay and the variations in transduction efficiencies among the different FM molecules provide sufficient evidence that endocytosis and endosomal trafficking must also be involved in the transduction process. The inhibition study by various endocytosis-inhibitory drugs revealed that the targeting LVs enter HIV-1 Env-expressing cells through clathrin-mediated endocytosis. Cells transfected to express the dominant-negative dynamin mutant were more resistant to vector transduction, indicating that the dynamin-dependent endocytosis pathway plays a crucial role in the internalization of the engineered vector as well. When the dominant mutants of Rab5 and Rab7 were introduced into target cells to regulate the maturation of endosomes at early and late stages, respectively, the transduction efficiency was only affected by the Rab5 mutant, suggesting that the success of vector transduction is largely associated with the trafficking in the early endosomes.

The therapeutic relevance of our HIV-1 Env-specific LV system was demonstrated in a mixed population of T cells with some of them infected to express Env proteins. We showed that Env-positive Jurkat cells could be targeted by LV/CD4+FM to express the suicide gene HSV1-TK, which caused them to be susceptible to the prodrug treatment. Our preliminary *in vitro* study showed that the targeting vector FUWSR39tk/CD4+FM could significantly reduce the surface marker (LNGFR)-positive subpopulation of human T cells cultivated in the presence of the prodrug GCV, indicative of the elimination of HIV-1 Env-expressing cells because of the bicistronic nature of the IRES-linked Env and LNGFR. Experiments are under way to further investigate the reason as to why the targeting vector, in conjunction with GCV treatment, was unable to completely eliminate LNGFR/Env-expressing cells under our current experimental condition. This also raises an interesting question that if this method fails to eradicate infected HIV-1 virus completely, what effects might the imposed selective pressure have on the remaining virus reservoir? One logical possibility is that the selective pressure may cause the virus to become a CD4-independent strain. Hoffman et al. has actually identified such a strain capable of directly interact with the chemokine receptor

CXCR4 to achieve infection (Hoffman et al., 1999); this CD4-independent strain was further found to be more sensitive to neutralizing antibody treatment. It will be interesting to investigate whether such CD4-independent strains can emerge from our suicide gene therapy.

In conclusion, we have shown that functional incorporation of CD4 and FM into the envelope of LVs can be accomplished with resulting vectors having a rather selective ability to target HIV-1 Env-expressing cells. Compared to the inverse fusion method, the LVs bearing CD4 and FM exhibited higher transduction efficiencies and were able to target a broader range of HIV-1 Env proteins irrespective of their CCR5 or CXCR4 tropism. Although our preliminary experiment *in vitro* showed the feasibility of such a vector to selectively eliminate Env-expressing T cells through suicide gene therapy, further investigations will reveal whether this gene delivery vector system has a practical utility to specifically eradicate HIV-1-infected cells *in vivo*, thereby reducing the virus load of HIV-1-infected patients.

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Fig. 1. Schematic representations of the strategies for targeting LVs to HIV-1 Env-expressing cells and key constructs for making the recombinant LVs in this study. (A) Schematics for the inverse fusion method (left), which utilizes CD4 and a co-receptor (CCR5 or CXCR4) complex, and our method of displaying CD4 and a fusogenic molecule (FM) on the vector surface (right), to achieve LV targeting. (B) Key constructs for making LVs. These constructs include the lentiviral backbone plasmid FUGW, binding molecule CD4, and fusion protein FM. CMV: enhancer/promoter derived from human cytomegalovirus; Ubi: the human ubiquitin-C promoter; GFP: green fluorescence protein; WRE: woodchuck responsive element; E3: the leading peptide of the Sindbis virus glycoprotein; E2: the E2

protein of the Sindbis virus glycoprotein that binds to cellular receptors; E1: the E1 protein of the Sindbis virus glycoprotein that mediates fusion. Several alterations, including the deletion of amino acids 61–64 of wild-type E3, mutations of 157KE158 into 157AA158 of wild-type E2, and an HA tag (MYPYDVPDYA) insertion into E2 between amino acids 71 and 74, were introduced to yield a binding-deficient and fusion-competent FM AKN (previously denoted SINmu; Yang et al., 2006). Additional mutations in the fusion loop region of E1 (amino acids 226 and 228) were conducted to generate two FMs, AGM and SGN.

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Fig. 2.

Construction of a cell line stably co-expressing an HIV-1 Env protein and a surface marker LNGFR. (A) Schematic representation of a retroviral backbone plasmid, MINFR-Env, that encodes an HIV-1 Env gene. MINFR-gp160R5 and MINFR-gp160X4 are designated for this construct encoding a CCR5- or CXCR4-tropic Env gene, respectively. The bicistronic configuration of this construct with an IRES linker can allow for co-expression of Env and a surface staining marker LNGFR. MSCV: murine stem cell virus; LTR: long terminal repeat; Env: HIV-1 envelope protein; IRES: internal ribosome entry site; LNGFR: human low-affinity nerve growth factor receptor. (B and C) The stable cell line 293T.EnvR5 was constructed by transducing parent 293T cells with MINFG-gp160R5 pseudotyped with VSVG and passaging the cells for a few weeks. (B) Flow cytometry analysis of LNGFR expression in a selected single cell clone. (C) TZM-b1 and HeLa cells were labeled with octadecyl rhodamine B chloride, R18, and seeded onto glass-bottom culture dishes. 293T.EnvR5 cells were labeled by 5,6-carboxy-fluorescein diacetate succinimidyl ester, CFSE, and co-cultured with TZM-b1 or HeLa cells. Redistribution of the R18 fluorescence dye was imaged by a laser scanning confocal microscope.

Fig. 3.

Co-expression of binding and FM molecules on the surface of vector-producing cells. 293T cells were transiently transfected with the plasmids FUGW, pCD4, pCD20, pFM, pCCR5, and pCXCR4, along with other standard packaging plasmids to produce targeting vectors, FUGW/CD4+FM, or inverse fusion vectors, FUGW/CD4+CCR5 and FUGW/CD4+CXCR4. 293T cells were transfected to produce the vector FUGW/CD4 displaying human CD4 and FUGW/VSVG pseudotyped with VSVG to serve as controls. (A and B) Transfected cells were stained by anti-human CD4 (A) or CD20 (B) and anti-HA tag antibodies to detect the CD4 and FM expression, respectively (Yang et al., 2006). The upper row shows GFP-expression. The shaded area represents cells without vector backbone transfection, while the solid lines show cells transfected with FUGW. The lower panel shows co-expression of the CD4 (A) or CD20 (B) and FM on the cells gated with GFP-positive populations. (C) The upper panel shows GFP-expression. Gated on GFP-positive cells, the lower panel shows co-expression of CD4 and CCR5 on the left and CD4 and CXCR4 on the right. (D) The GFP signal of cells producing FUGW/VSVG (left) and co-expression of CD4 and GFP for cells making FUGW/CD4 (right).

Fig. 4.

In vitro targeted transduction of HIV-1 Env-expressing cells by engineered LVs.

293T.EnvR5 and 293T cells were either transduced with 2 mL of fresh, unconcentrated LVs (FUGW/CD4+FM, FUGW/CD20+FM, FUGW/CD4, or FUGW/VSVG) or with no vector as a control. The LVs generated by the inverse fusion method (FUGW/CD4+CCR5 or FUGW/CD4+CXCR4) were included for comparison. GFP expression was analyzed using flow cytometry. (A) Integrated mean fluorescence intensity (iMFI) on 293T.EnvR5 or 293T cells transduced by the indicated LVs. (B) iMFI on the non-specific FUGW/VSVG vector on 293T and 293T.EnvR5 cells.

Fig. 5.

Specific vector titers for various engineered LVs. (A) Titers of the unconcentrated vectors on 293T and 293T.EnvR5 cells. (B) Titers of the non-specific FUGW/VSVG vector on 293T and 293T.EnvR5 cells.

Fig. 6.

Engineered LVs transducing cells expressing different tropisms of HIV-1 Env. We constructed a new cell line expressing an X4-tropic HIV-1 subtype B Env protein and designated it as 293T.EnvX4. (A) 293T.EnvX4 cells were analyzed by flow cytometry for the surface marker LNGFR as an indication of HIV-1 Env expression. (B) iMFI on 293T.EnvX4 cells transduced with various unconcentrated LVs (FUGW/CD4+CCR5, FUGW/CD4+CCR4, and FUGW/CD4+SGN).

Fig. 7.

Engineered LVs are capable of binding to HIV-1 Env-expressing cells and are critical for specific transduction. (A) 293T.EnvR5 and 293T cells were incubated with the FUGW/CD4+SGN vector and then fixed by formaldehyde immediately following. The resulting vector–cell complexes were stained with an antibody against the FM (Yang et al., 2006) and detected by flow cytometry. (B) Various concentrations of soluble human CD4 were added to 293T.EnvR5 cells along with engineered LVs. Four days later, we used flow cytometry to detect GFP expression as an indication of inhibited transduction. (C) Effect of the neutralizing agent (NH_4Cl) on the pH-dependent transduction of targeting LVs. Various concentrations of NH_4Cl were added into the vector supernatant and incubated together with 293T.EnvR5 cells for 8 h. The media was replaced with fresh media and cells were cultured for 4 more days. The GFP expression was detected by flow cytometry.

Fig. 8.

Clathrin and dynamin-dependent entry of the engineered LV. (A–C) Relative transduction efficiency measured by flow cytometry. (A) 293T.EnvR5 cells were incubated with three different drugs – chlorpromazine to test for clathrin dependent entry, and genistein and nystatin to test for caveolin dependent internalization – for 30 min at 37 °C (chlorpromazine: 10 µg/mL; genistein: 50 µg/mL; nystatin: 25 µg/mL). The drug-treated cells were transduced with FUGW/CD4+SGN. (B and C) 293T.EnvR5 cells were transiently transfected either with wild-type or dominant-negative mutant dynamin2 (B), or with wild-type or dominant-negative mutants of Rab5 and Rab7 (C), and then transduced with FUGW/CD4+SGN.

Fig. 9.

Targeted transduction to a T cell line and suicide gene therapy delivered by LVs *in vitro*. A CD4-positive T cell leukemia cell line (Jurkat) was infected with an R5-tropic envelope-encoding retroviral vector (MINFR-gp160R5) pseudotyped with VSVG and analyzed by flow cytometry for LNGFR expression. (A) Flow cytometry analysis of Jurkat cells infected with the MINFR-gp160R5 vector. (B) The pretreated and non-treated Jurkat cell mixture was challenged with an engineered LV (FUGW/CD4+SGN). The GFP and surface marker (LNGFR) expressions were analyzed by flow cytometry. (C) The pretreated and non-treated PBMC were challenged with an engineered LV (FUGW/CD4+SGN). The GFP expression was analyzed by flow cytometry. (D, left) Mixed Jurkat cells pre-treated to express HIV-1 Env were challenged with the concentrated LV (FUWSR39tk/CD4+SGN) vector 3 times and then cultured in media containing GCV. The treated cells were then analyzed for the expression of the surface marker LNGFR using flow cytometry. The expression of LNGFR is indicative of Jurkat cells expressing HIV-1 Env. (D, right) Mixed Jurkat cells pre-treated (MINFR-gp160R5) to express HIV-1 Env were challenged with the LV (FUWSR39tk/CD4+SGN) vector and cultured in media containing GCV. Jurkat cells without the vector treatment, or only pretreated to express HIV-1 Env but no LV treatment were included as controls. The treated cells were analyzed for apoptosis via 7-AAD staining one week post-GCV treatment.