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Development of a Non-integrating Rev-dependent Lentiviral Vector Carrying Diphtheria Toxin A Chain and Human TRAF6 to Target HIV Reservoirs

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Abstract

Persistence of HIV despite highly active antiretroviral therapy (HAART) is a lasting challenge to virus eradication. To develop a strategy complementary to HAART, we constructed a series of Rev-dependent lentiviral vectors carrying diphtheria toxin A chain (DT-A) and its attenuated mutants, as well as human TRAF6. Expression of these suicide genes following delivery through viral particles is dependent on Rev, which exists only in infected cells. Among these toxins, DT-A has been known to trigger cell death with as little as a single molecule, whereas two of the attenuated mutants in this study, DT-A(176) and DT-A(ΔN), were well-tolerated by cells at low levels. TRAF6 induced apoptosis only with persistent overexpression. Thus, these suicide genes, which induce cell death at different expression levels, offer a balance between efficacy and safety. To minimize possible mutagenesis introduced by retroviral integration in non-target cells, we further developed a non-integrating Rev-dependent (NIRD) lentiviral vector to deliver these genes. In addition, we constructed a DT-A-resistant human cell line by introducing a human elongation factor 2 (EF-2) mutant into HEK293T cells. This allowed us to manufacture the first high-titer NIRD lentiviral particles carrying DT-A to target HIV-positive cells.

Keywords

HIV-1; lentiviral vector; Rev; integration; diphtheria toxin; TRAF6

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Conflict of interest

The authors declare that they have no competing interests.

Introduction

Despite the success of highly active antiretroviral therapy (HAART) in inhibiting HIV replication, viral latency and low-level replication in T cells and macrophages permit viral persistence 1–3. The identification and characterization of these viral reservoirs have highlighted the limitations of HAART, which is often incapable of eliminating the pool of persistently infected cells 4–12. HIV can be stably maintained in a variety of cells such as brain macrophages 13, blood monocytes and tissue macrophages 2,3,10,11,14,15,16, as well as resting CD4 T cells 6–8,17. These reservoirs are either less sensitive to antiviral drugs because of the presence of natural barriers 18, or do not respond to drug treatment because of the absence of viral activity 12. Furthermore, counter to the initial optimism that HAART would lead to immune system recovery 19,20, multiple clinical cohort studies have revealed that even though HAART can reduce the viral load to undetectable levels, this is not necessarily followed by full recovery of the immune functions (for a review, see 21). It appears that the immune system in HIV patients remains impaired and is therefore often unable to mount adequate anti-HIV immune responses, leading to frequent viral rebounds upon HAART discontinuation 22–24. A novel strategy to specifically target persistently infected cells is urgently needed to eradicate HIV-1.

In a previous report, we constructed a Rev-dependent lentiviral vector carrying anthrolysin O (AnlO) to target HIV-infected cells 25. We demonstrated that *anlO*-mediated cell killing is exclusively dependent on Rev, a unique HIV-1 protein present only in infected cells. Intracellular expression and oligomerization of AnlO result in membrane pore formation and cytolysis. In a proof-of-concept study, we demonstrated that the Rev-dependent AnlO lentivirus specifically diminishes HIV-1-positive macrophages and T cells. Nevertheless, numerous efficacy and safety issues limit the potential for *in vivo* application of this novel system. Firstly, AnlO is not very effective since 30 or more molecules are required in order to kill a cell 26. Secondly, AnlO kills cells by cytolysis and releases cellular contents into the environment, which may cause inflammation and bystander killing of healthy cells. Thirdly, permanent integration of a suicidal toxin gene into the human genome threatens to disrupt normal cellular function and cause mutagenesis 27,28, especially given that considerable amounts of viral particles may need to be injected into the body.

To improve this prototype Rev-dependent lentiviral vector, in this study we made numerous modifications to enhance both the efficacy and the safety of the vector. We selected diphtheria toxin A chain (DT-A) as the primary suicide gene to induce cell death. DT is a potent inhibitor of protein synthesis and catalyzes ADP ribosylation of human elongation factor 2 (EF-2), which triggers cell death by apoptosis without the leakage of cellular contents 29,30. It has been estimated that a single molecule of DT is sufficient to kill a cell 31, and the DT-A chain contains the catalytic domain of this enzymatic action. Another major advantage of using DT-A is that a wealth of information is available on this toxin 32–34. In particular, multiple human clinical trials have been conducted using DT-fusion proteins for cancer therapy. This provided much-needed information about its safe use in patients 35–37.

Given the ultimate potency of DT-A, tightly regulated expression, namely the expression of DT-A only in the presence of HIV Rev, is an essential regulatory strategy. However, low-level background expression of DT-A resulting from leakage in non-target cells is likely. Practical application of DT-A requires the attenuation of this toxin as previously demonstrated 38. As a complement to the use of extraneous toxins, we also chose to test an endogenous human protein, TRAF6 (tumor necrosis factor receptor-associated factor 6), based on a recent report that overexpression of TRAF6 induces apoptosis by activation of Caspase 8 39. As a self-protein, human TRAF6 has a unique advantage for *in vivo* application. While high-level expression of TRAF6 can trigger apoptosis, low-level expression would be tolerated by cells and the immune system, minimizing possible side effects from leakage and non-specific expression in non-target cells.

In this study, we also made another modification of the original vector system by incorporation of an integration defective vector. This non-integrating Rev-dependent (NIRD) lentiviral vector was created based on our studies of the transcriptional capacity of a non-integrating HIV-1 mutant, D116N 40. Previously, we demonstrated that low-level transcription occurs from non-integrating HIV DNA both in human T cells and in macrophages, two of the primary HIV targets 41–43. We also demonstrated recently that the templates for non-integrating transcription are a large population of viral DNA 44. These findings suggest that a non-integrating lentiviral vector can be as effective as an integrating vector in directing transient gene expression in non-dividing cells. Indeed, several recent studies have used non-integrating lentiviral vectors for the safer delivery of therapeutic genes for gene therapy 45–47. The efficacy of the system to express therapeutic genes has been clearly demonstrated.

Finally, in this report, we overcame a technical hurdle in producing the first DT-A NIRD vector. Unwanted killing of producer cells during viral production precludes the assembly of viral particles. This technical difficulty was resolved by the development of a DT-resistant human cell line through site-directed mutagenesis of the human EF-2 gene 29. The successful development of the DT-resistant cells allowed us to produce the first high-titer DT-A NIRD viral particles to target persistently infected cells.

Materials and Methods

Cloning of DT-A and TRAF6

Plasmid pNL-GFP-RRE-SA has been described previously 48,49. The codon-optimized DT-A chain carrying the start and stop codons was PCR amplified and cloned into pNL-GFP-REE-SA at the *BamH*I site. Specifically, DT-A was amplified from the plasmid template A-dmDT390biscFv (UCHT1) 50 using primers DT-BamHI-Start (5’CGCGGATCCATGGGTGCTGACGACGTCGTC3’) and DT-BamHI-Stop (5’CGCGGATCCTTAGGCTTGAGCCATATACTCATA3’). Cloning of DT-A was further confirmed by DNA sequence analysis. The packaging construct, pCMVΔ8.2, was kindly provided by Dr. Dider Trono. The TRAF6 expressing plasmid, YFP-hTRAF6, was kindly provided by Dr. Liusheng He 39. TRAF6 gene was cloned into pNL-GFP-REE-SA at the *Bam*HI site. Cloning of the TRAF6 gene was further confirmed by DNA sequence analysis.

DT-A mutagenesis

The DT-A mutants were generated using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) as recommended by the manufacturer. The PCR primers used for generating DT-A(E148D) were 5'E148D(5'GCTGAGGGT TCTTCTAGCGTTGATTATATTAATAACTGGGAACAG 3') and 3'E148D(5'CTGTTCCCAGTTATTAATATAATCAACGCTAGAAGAACCCTCAGC 3'); those for generating DT-A(E148S) were 5'E148S(5'GCTGAGGGTTCTTCTAGCGTTTCCTATATTAATAACTGGGAACAG3') and 3'E148S (5'CTGTTCCCAGTTATTAATATAGGAAACGCTAGAAGAACCCTCAGC3'); and those for generating DT-A(176) were 5'G128D(5'GAAGAGTTCATCAAGAGATTCGATGACGGTGCTTCCAGAGTCGTC3') and 3'G128D (5'GACGACTCTGGAAGCACCGTCATCGAATCTCTTGATGAACTCTTC3'). All mutants were confirmed by sequence analysis.

Establishment of the DT-A resistant cells

The human EF2 gene was cloned by RT-PCR amplification of total RNA extracted from HEK293T cells. The primers used for PCR were EF2-EcoRI (5'CCGGAATTCATGGTGAACTTCACGGTAGAC3') and EF2-XhoI (5'CCGCTCGAGCTACAATTTGTCCAGGAAGTTG3'). The PCR product was digested with *Eco*RI and *Xho*I and subsequently inserted into pET17b at the *Eco*RI and *Xho*I site. Mutagenesis of the human EF2 gene was achieved by site-directed mutagenesis of codon 717 (G717R, GGA to CGA) using a Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and the primers 5'CACGCCGACGCCATCCACCGC CGAGGGGGCCAGATCATCCCC3' and 5'GGGGATGATCTGGCCCCCTCGGCGGTGGATGGCGTCGGCGTG3'. The human EF2 mutant was subcloned into the retroviral vector pMSCVneo (Clontech, Mountain View, CA) at the *Eco*RI and *Xho*I sites and subsequently transfected into the RetroPackPT67 cells (Clontech, Mountain View, CA) to be assembled into infectious viral particles. HEK293T cells were transduced with the viral particles and grown in 1 mg/ml Geneticin (Invitrogen, Carlsbad, CA).

Virus production and infection

The Rev-dependent lentiviruses were produced by cotransfection of HEK293T or 5H7 cells with calcium phosphate (Promega, Madison, WI). Briefly, two million cells were cultured in a petri dish and cotransfected with 10 εg of either pNL-GFP-RRE-SA or pNL-DTΔN-GFP-RRE-SA or pNL-TRAF6-GFP-RRE-SA, plus 7.5 εg of pCMVΔ8.2 and 2.5 εg of the VSV-G envelope construct pHCMV-G. Transfected cells were cultured overnight, and then the supernatant was removed and replaced with 10 ml fresh DMEM plus 10% heat-inactivated fetal bovine serum (FBS). Viruses were harvested at 48 and 72 hours and then concentrated by multiple rounds of concentration through an anion exchange column Sartobind Q75 (Sartorius Stedium Biotech, Aubagne, France) and size-exclusion Vivaspin 20 and 500

columns (Sartorius Stedium Biotech, Aubagne, France) using conditions as recommended by the manufacturer. Concentrated virus was divided into 50 εl aliquots and stored at −80°C.

Viral p24 level was determined using a p24 ELISA assay (Beckman Coulter, Miami, FL). The titer of vNL-GFP-RRE-SA was measured directly on an HIV-1-positive cell line, J1.1 51 (provided by the NIH AIDS Research & Reference Reagent Program, NIAID, NIH), which was cultured in 50 ng/ml PMA (phorbol myristate acetate) to stimulate HIV-1 activity. GFP-positive J1.1 cells were enumerated on FACSCalibur (BD Biosciences, San Jose, CA). The titers of vNL-DTΔN-GFP-RRE-SA and vNL-TRAF6-GFP-RRE-SA cannot be measured directly due to their cytolytic activity, and thus were estimated based on the p24 levels, using the titer of vNL-GFP-RRE-SA as a reference.

The HIV-1 strains, NL4-3.HSA.R+E-(VSV-G) and the replication-competent NL4-3.HSA.R +E+ (“R” represents the Vpr gene and “E” represents the viral envelope gene) 52 were provided by the NIH AIDS Research & Reference Reagent Program, NIAID, NIH. In both viruses, the murine heat-stable antigen CD24 (HSA) gene was inserted into the *nef* region that allows HIV-1-positive cells to be monitored by surface staining of HSA. Viruses were produced by transfection of HEK293T cells, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. HIV-1 titer was determined using an indicator cell line, Rev-CEM, as previously described 48.

Human T cell lines CEM-SS 53 and J1.1 51 were acquired from the NIH AIDS Research & Reference Reagent Program, NIAID and were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 μg/ml) (Invitrogen, Carlsbad, CA). Human peripheral blood mononuclear cells (PBMC) were obtained from healthy donors at the Student Health Center, George Mason University, Fairfax, VA. Protocols involving human subjects were reviewed and approved by the George Mason University IRB. PBMC were purified by centrifugation of blood cells on lymphocyte separation medium (Mediatech, Inc., Manassas, VA) for 20 minutes at 400 × *g*. Cells were washed twice with PBS buffer and resuspended into fresh RPMI 1640 medium supplemented with 10% FBS and cultured at 1×10^6/ml. For infection, cells were infected with HIV-1 for two hours at 37°C, and then washed twice with medium to remove unbound virus. Infected cells were resuspended into fresh medium. Superinfection was carried out by adding lentiviral particles directly to HIV-infected cells, followed by continuous culturing.

Innunofluorescent staining and flow cytometry

One half to one million infected cells were removed from culture tubes and washed once with cold PBS, centrifuged for 5 minutes at 400 × *g* and resuspended in 400 εl cold staining buffer (PBS plus 1% BSA). Nonspecific binding was blocked by adding 5 μl Rat IgG (10 mg/ml) (Jackson Laboratories Inc., Westgrove, PA). HIV-positive cells were stained with 2 μl of PE-labeled Rat Anti-Mouse CD24 (BioLegend, San Diego, CA). For isotype control staining, PE-labeled Rat IgG_{2b} (BioLegend, San Diego, CA) was used. Stained cells were incubated on ice for 30 minutes and then washed with cold PBS plus 1% BSA and resuspended in 500 εl of 1% paraformaldehyde for flow cytometry analysis on a FACSCalibur (BD Biosciences, San Jose, CA). Normally 10,000 to 20,000 cells were

collected for analyses. Data analysis was performed using CellQuest (BD Biosciences, San Jose, CA) and FlowJo (Tree Star, San Carlos, CA).

Western blot detection of DT-A and luciferase

Proteins in cell lysates from cotransfection were resolved on 4–20% SDS-polyacrylamide gel and electroblotted onto 0.2 μm nitrocellulose membrane. A 1:1000 dilution of a monoclonal antibody against DT (Meridian Life Science, Inc., Saco, ME) or a goat polyclonal antibody against luciferase (Promega, Madison, WI) was incubated with the membrane, followed by a secondary goat antimouse antiserum (1:2000) or a rabbit polyclonal antigoat antibody (1:2000) conjugated with peroxidase (KPL, Gaithersburg, MD). Chemiluminescence was captured on a cooled CCD camera using chemiluminescent SuperSignal West Dura substrate (Pierce, Rockford, IL).

RESULTS

Specificity of the Rev-dependent Lentiviral Vectors in Mediating HIV-dependent Gene Expression

The Rev-dependent lentiviral vector was constructed based on the HIV-1 genome and has been described previously 48,49. As shown in Figure 1A, we placed a reporter gene, the green fluorescent protein (GFP) gene, under the control of Rev by introducing multiple splicing sites and a Rev responsive element (RRE). This arrangement regulates GFP as a late gene and renders its expression specific to Rev.

To further demonstrate the specificity of this vector, we measured the expression of GFP mediated through viral infection or by cotransfection with a HIV-based helper plasmid, pCMVΔR8.2, which carries all viral genes and sequences except the packaging signal and the viral envelope 54 (Figure 1A). When cotransfected with pCMVΔR8.2, pNL-GFP-RRE-SA expressed GFP in a dosage-dependent manner, generating GFP-positive cells from 13% (0.1 μg pNL-GFP-RRE-SA) to 52% (3 μg pNL-GFP-RRE-SA) (Supplemental Figure S1). In contrast, when identically cotransfected with a control empty vector, pMSCVneo, which does not express HIV genes, pNL-GPF-RRE-SA expressed almost no GFP (0.8% GFP-positive cells) at the lowest dosage (0.1 μg). However, with the increasing amounts of pNL-GFP-RRE-SA, HIV-independent GFP expression was observed, and reached 20% of cells when 3 μg pNL-GFP-RRE-SA was used (Supplemental Figure S1). Nevertheless, there was HIV-dependent enhancement of GFP expression in all dosages tested, and the enhancement was most dramatic at lower DNA dosages.

We also tested the specificity of the Rev-dependent vector when assembled into virion particles. Plasmids pNL-GFP-RRE-SA, pCMVΔR8.2, and pHCMV-G expressing the glycoprotein of vesicular stomatitis virus (VSV-G) were cotransfected into HEK293T cells. Virion particles (vNL-GPF-RRE-SA) generated were harvested and concentrated to superinfect HIV-1-positive CEM-SS T cells. As shown in Figure 1B, we observed dosage-dependent GFP expression exclusively in the HIV-1-postive cell population, whereas we did not observe any GFP expression in HIV-1-uninfected cells even with the highest multiplicity of infection (m.o.i. = 10) by vNL-GFP-RRE-SA. This high stringency observed in viral

infection was in great contrast to the cotransfection experiments, in which cells were overdosed with plasmid DNA and membrane disrupting agents. In cotransfection, even with 0.1 μg pNL-GFP-RRE-SA, each cell was roughly transfected with 10,000 molecules of pNL-GFP-RRE-SA, a condition that is significantly different from infection. Given that the Rev-dependent lentiviral vector is designed for gene delivery via infection, the specificity of the vector to express genes only in HIV-positive cells is reasonably high in infection conditions.

Intracellular Cytotoxicity of DT-A and TRAF6 Expressed from the Rev-dependent Vectors

To clone the DT-A chain and the human TRAF6 gene into the Rev-dependent vector, we used pNL-GFP-RRE-SA as the backbone, which also contains an internal ribosome entry site (IRES) that allows the expression of two genes simultaneously (Figure 2A). We demonstrated previously that the IRES permitted the use of GFP as a convenient indicator for the measurement of cell killing, since the accumulation of GFP inside the cell is prevented by co-expressed toxins. Thus, diminished GFP expression directly correlates with the cytotoxicity of the toxins 25.

We also used cotransfected HeLa and HEK293 T cells as model systems to compare Rev-dependent killing of cells by DT-A and TRAF6. Cells were cotransfected with the HIV-1 helper construct, pCMVΔR8.2, and either pNL-DT-GFP-RRE-SA, pNL-TRAF6-GFP-RRE-SA, or the control GFP vector, pNL-GFP-RRE-SA (Figure 2B). The degree of cell killing from toxin expression was measured by comparing GFP expression in these parallel cotransfection experiments. As mentioned above, the reduction in the GFP-positive population was used as an indicator of toxin-mediated cell killing.

As shown in Figure 2B, HeLa cells cotransfected with pCMVΔ8.2 plus the GFP vector pNL-GFP-RRE-SA generated 53% GFP-positive cells, whereas HeLa cells cotransfected with pCMVΔR8.2 plus the DT-A vector pNL-DT-GFP-RRE-SA generated almost no GFP-positive cells (0.5%). Similar results were also observed in cotransfected HEK293T cells, among which approximately 39% were GFP-positive when cotransfected with pCMCΔR8.2 plus pNL-GFP-RRE-SA, but none were GFP-positive when cotransfected with pCMVΔR8.2 plus pNL-DT-GFP-RRE-SA. These results are consistent with the universal killing of cells by DT-A at a single-molecule level 31. In contrast, the TRAF6 vector generated 7.6% low-intensity GFP HeLa cells, suggesting that low-level expression of TRAF6 is tolerated to a certain extent (Figure 2B). Indeed, as much as 25% GFP-positive cells were obtained in TRAF6 cotransfected HEK293T cells. These results also indicate that TRAF6 killing is cell-type dependent. HeLa cells appear to be more subject to TRAF6-induced apoptosis than HEK293T cells.

We also compared DT-A and TRAF6 with Anthrolysin O (AlnO), the first bacterial toxin tested in the Rev-dependent vector 25. It was apparent that DT-A and TRAF6 were more potent than AlnO in HeLa cells, but the killing by TRAF6 and AlnO was comparable in HEK293T cells (Figure 2B).

These Rev-dependent vectors were also cotransfected with an empty vector, pMSCVneo, instead of pCMVΔR8.2. Similar results were observed from the background toxin

expression in the absence of HIV-1 proteins, demonstrating again that expression of these suicide genes can lead to the killing of GFP-positive cells.

Mutagenesis of DT-A and the Construction of Rev-dependent Lentiviral Vectors Carrying DT-A Mutants

When introduced into human cells, DT inactivates elongation factor 2 (EF-2) by ADP ribosylation and inhibits protein translation, which triggers apoptosis. DT is extremely toxic, and a single molecule can kill a cell 31. This extreme toxicity is attractive for killing target cells with minimal toxin expression. In the meantime, it poses a significant problem in that any unexpected, non-specific expression in non-target cells would not be tolerated. DT-A mutants with reduced toxicity ranging from 30% to 0.2% have been successfully used previously 55,56.

We took advantage of these previous findings and generated a panel of DT-A chain mutants and tested their toxicity in cotransfected HEK293T cells. DT-A(E148S) has a single substitution of glutamic acid at codon 148 with serine, whereas DT-A(E148D) has a single substitution of the same amino acid with aspartic acid. Both mutants have been described previously 55. DT-A(176) was the mutation originally described in a DT-A chain mutant, tox176 56. The mutation was identified as a replacement of the glycine at codon 128 with aspartic acid. DT-AΔN was a new mutant generated in our own laboratory for the first time by removing the first 14 amino acids at the N-terminus of the DT-A chain. All of these DT-A mutants were cloned into the Rev-dependent vector, pNL-GFP-RRE-SA. When cotransfected with the helper plasmid, pCMVΔR8.2, into HEK293T cells, the Rev-dependent vectors carrying these DT mutants generated different percentages of GFP-positive cells in comparison with the wild-type DT-A. As shown in Figure 3, while wild-type DT-A generated 0% GFP-positive cells, DT (E148S) generated 0.47%, DT (E148D) generated 0.82%, DT176 generated 4.19%, and DTΔN generated 5.72% of GFP-positive cells, respectively. The DTΔN-GFP is the least toxic of all the mutants and permitted the accumulation of low-level GFP in cells.

We also similarly cotransfected these plasmids into a DT-resistant, EF-2 mutant cell line, 5H7, which we constructed (see below). We observed a drastic increase in the GFP-positive cells, which were approximately 70–80% of those generated by the control vector pNL-GFP-RRE-SA (Figure 3B). These data confirmed that the reductions in GFP percentages observed in HEK293T cells were related to EF-2 cytotoxicities of DT-A.

Construction of DT-A-resistant Cell Lines with Mutated EF-2 Gene

The extreme toxicity of DT-A causes a problem in lentiviral production. Cotransfected HEK293T cells would be killed rapidly without being able to generate lentiviral particles carrying the DT-A gene. To solve this problem, we took advantage of a previous observation that a mutant hamster cell line carrying a single point mutation in the EF-2 gene confers resistance to the DT-A chain 57. The mutation was mapped to codon 717. We cloned the human EF-2 gene by PCR amplification and subsequently introduced a single point mutation (from G to A in the first nucleotide of codon 717) into EF-2. The mutant EF-2 was then introduced into human HEK293T cells by a retroviral vector for stable

transduction. Cells were screened for mutant EF-2. Originally, we obtained about 100 clones, and 5 of them turned GFP-positive when cotransfected with the DT-A-containing lentiviral vector pNL-DT-GFP-RRE-SA plus the helper vector pCMVΔR8.2 (Figure 4A). These cell clones were named 5H7, CB2, AB1, 4H10 and 5E12 and further tested for DT-A resistance. As shown in Figure 4A, while the parental HEK293T cells generated 0% GFP-positive cells after cotransfection with pNL-DT-GFP-RRE-SA plus pCMVΔR8.2, these clones generated different percentages of GFP-positive cells: 46% in 5H7, 28% in CB2, 24% in AB1, 14% in 4H10, and 9% in 5E12, respectively. Clone 5H7 demonstrated the highest resistance to DT-A-mediated killing, and thus was selected as the DT-A resistant cell line for continuous culturing. To more accurately measure the degree of resistance of 5H7 to DT-A-mediated killing, both HEK293T cells and 5H7 cells were cotransfected identically with pCMVΔR8.2 plus pNL-DT-GFP-RRE-SA. As a control, cells were also cotransfected with pCMVΔR8.2 plus pNL-DT(R)-GFP-RRE-SA, a construct with DT-A cloned in the reverse orientation to prevent DT-A expression. As shown in Figure 4B, in HEK293T cells, cotransfection with pNL-DT-GFP-RRE-SA generated 0% GFP-positive cells, whereas cotransfection with pNL-DT(R)-GFP-RRE-SA generated 43% GFP-positive cells. These results demonstrated 100% killing of HEK293T cells by DT-A. In contrast, in 5H7 cells, cotransfection with pNL-DT-GFP-RRE-SA generated 54% GFP-positive cells, whereas cotransfection with pNL-DT(R)-GFP-RRE-SA generated 64% GFP-positive cells. These results suggest that over 80% of the 5H7 cells survived the toxin. Some 5H7 cells can even tolerate high levels of DT-A expression, judging from the high levels of GFP expression observed (Figure 4B). We further confirmed intracellular expression of DT-A in 5H7 cells by Western blot using a monoclonal antibody against DT-A, which only detected a protein with the size of DT-A in 5H7 cells but not in HEK293T cells (Figure 4C). The successful establishment of the DT-resistant 5H7 cells allowed us to assemble viral particles from the Rev-dependent DT-A vector.

Construction of Non-integrating Rev-dependent (NIRD) Lentiviral Vector Carrying DT-A Chain and Human TRAF6

HIV Rev-regulated expression from the Rev-dependent lentiviral vector permitted selective expression of DT-A and TRAF6 in HIV-positive cells. However, the vector can enter both HIV-positive and -negative cells, and subsequently becomes integrated. Although we did not observe reporter gene expression in uninfected cells using GFP as a marker 25, any permanent integration of a toxin gene into the human genome poses a threat. Especially if the integration occurs at a transcriptionally active site, high-level gene expression may ensue and eventually trigger the Rev-independent protein synthesis and the subsequent death of an uninfected cell. To alleviate this potential risk, we decided to construct a NIRD lentiviral vector to deliver DT-A and TRAF6 as an episomal vector. Previously, we observed that a non-integrating HIV mutant, D116N, can transcribe from a DNA population as large as an integrating wild-type virus 44. However, each non-integrating DNA template is less active and expresses genes at a level approximately 10% that of an integrated proviral DNA 41–43. Additionally, we and others have also shown that D116N can express Rev-dependent late genes in the presence of high levels of Rev 42 or the wild-type HIV-1 58. Thus, we constructed the NIRD vector based on D116N, and a single point mutation (from Asp to Asn) was introduced into pCMVΔR8.2 at the integrase amino acid 116 within the D(35)E

functional motif. This single point mutation has been shown to completely abolish viral integrase activity without affecting other known viral functions such as reverse transcription and nuclear targeting 40.

To demonstrate Rev-dependent expression of the NIRD vector in HIV-positive cells, we also cloned a luciferase reporter into the Rev-dependent vector and cotransfected this construct with pCMVΔR8.2(D116N) and the VSV-G envelope construct pHCMV-G, resulting in the production of a luciferase NIRD virion particle, vNL-Luc-RRE-SA(D116N). As a control, a similar integrating version of the viral particle, vNL-Luc-RRE-SA, was also assembled. These viruses were subsequently used to superinfect an HIV-1-positive Jurkat cell line, J1.1 51, or to infect the HIV-negative, parental Jurkat cells as a control. As shown in Figure 5B, we detected dosage-dependent luciferase expression in J1.1 cells superinfected with vNL-Luc-RRE-SA(D116N) or vNL-Luc-RRE-SA, indicating that the NIRD vector was capable of mediating HIV-dependent gene expression. However, the expression level was much lower than that of the integrating vector. The background luciferase readings in HIV-negative Jurkat cells were likely derived from residual luciferase that could be present in the viral preparation and be subsequently introduced into cells during infection. Indeed, a Western blot analysis of Jurkat cells immediately after infection (2 hours) detected the presence of the luciferase protein (Figure 5C). Nevertheless, this background luciferase activity can be drastically reduced by purification of virion particles through anion exchange and size-exclusion columns (Figure 5D).

Production of NIRD Viral Particles Carrying DT-A and Human TRAF6 to Target HIV-positive Cells

Given the demonstrated ability of the NIRD vectors described above, and the successful development of the DT-resistant 5H7 cell, we assembled the first NIRD viral particle carrying the DT-A gene. The NIRD particle carrying TRAF6 was also assembled in HEK293T cells due to the low toxicity of TRAF6 to this particular cell line (Figure 2B). As shown in Table 1, following cotransfection of components of the NIRD vectors into 5H7 and HEK293T cells, viral particles were harvested at day 2 and day 3 post transfection, and comparable levels of viral production were obtained from the NIRD vectors and their integrating counterparts. The TRAF6 NIRD vector produced a p24 level of 870 ng/ml at day 2, whereas the DTΔN NIRD vector reached a p24 level of 60 ng/ml in 3 days. These viruses were concentrated 1000- to 2000-fold through an anion exchange column and size-exclusion columns to approximately 100–3000 εg/ml, a dosage sufficient for studies to target HIV-positive cells.

To test whether the assembled NIRD particles are capable of killing HIV-1-positive cells, human peripheral blood mononuclear cells (PBMC) were purified and cultured, and the lymphocyte subpopulation was infected with a replication-competent T-tropic virus, NL4-3.HSA.R+ (Vpr $^{+}$, Env $^{+}$), a clone with the murine heat-stable antigen CD24 (HSA) gene inserted into the *nef* region to facilitate the identification of HIV-1-positive cells by surface murine CD24 staining 52. Following HIV-1 infection for 24 hours, cells were superinfected with the NIRD particle, vNL-TRAF6-GFP-RRE-SA(D116N), and then continuously cultured for three days. Two additional doses of the TRAF6 NIRD particles

were then added at days 4 and 7 post HIV-1 infection. The spread of HIV-1 was monitored by HSA staining. As shown in Figure 5E, HIV-1 replication resulted in the infection of 16.3% positive cells in 9 days. Superinfection with the TRAF6 NIRD particles reduced the HIV-positive cells to 5.5%, a 66% reduction in HIV-positive cells.

The selective reduction of HIV-positive cells did not result from possible non-specific killing by the TRAF6 NIRD particles. When HIV-uninfected cells were identically treated with the TRAF6 NIRD particles, we observed only a slight increase in cell death (from 4.4% to 6.6%, Figure 5F, g and h) in comparison with the untreated control, demonstrating that the TRAF6 NIRD particle resulted in about 2% non-specific killing of HIV-uninfected cells. This is significantly lower than the 66% reduction of HIV-positive cells. Based on these results, we calculated that approximately 97% of the killing mediated by TRAF6 NIRD particles was specific towards HIV-positive cells.

DISCUSSION

HIV establishes a persistent, lifelong infection which if left untreated almost invariably leads to acquired immune deficiency syndrome (AIDS) and death. The development of highly active anti-retroviral therapy (HAART) has allowed for effective control of HIV-1 replication and a reduction in mortality from AIDS 59. However, the success of HAART is associated with significant setbacks such as toxic side effects, high pill burden, and the development of viral resistance. More importantly, HAART does not completely eliminate HIV from the body, meaning that patients have to be on drugs for a lifetime: if treatment stops, residual viral reservoirs expand rapidly, allowing disease to progress 24,60. A novel strategy to specifically target persistently infected cells is urgently needed to eradicate HIV.

In this study, we constructed a series of Rev-dependent lentiviral vectors carrying DT-A or TRAF6 to target HIV-infected cells. We demonstrated that intracellular expression of DT-A or TRAF6 resulted in the killing of HIV-positive cells. To enhance the safety of this vector system, we further developed the vector into a non-integrating form. After overcoming a major technical hurdle to establishing a viable producer cell, we assembled the first DT-A and TRAF6 NIRD particles that allowed us to test for the killing of HIV-positive cells. In a proof-of-concept study, the TRAF6 NIRD particles were shown to moderately reduce the population of HIV-positive cells (a 66% reduction) with three doses of superinfection. Given the transient nature of gene transcription in the absence of integration, it became apparent that the TRAF6 NIRD vector was not as effective as a previously tested, integrating Rev-dependent vector carrying anthrolysin O 25. Nevertheless, the NIRD vector offers a safety advantage by reducing the possible risk of integration-mediated mutagenesis. Insertional transformation by retroviral vectors has been known to result in malignancy 27,28, which raises concerns for the safe application of lentiviral vectors for clinical gene therapy, especially given that large quantities of viral particles may need to be injected. The use of integrase mutants, although unable to completely eliminate integration 61, does provide a significant reduction (10^3- to 10^4-fold) in viral integration 62.

The utilization of lentiviral vector to deliver toxin genes faces potential problems of non-specific killing, one of which could be originated directly from toxin contamination of virion

particles. It is well-known that lentiviral particles are normally contaminated with materials such as plasmid DNAs and non-viral proteins from producer cells during viral assembly. Nevertheless, we found that these non-viral proteins, such as the luciferase protein detected in the viral preparation (Figure 5C and 5D), can be drastically reduced through column purification (Figure 5D). The problem of toxin contamination resembles the situation of plasmid DNA contamination of lentiviral particles. The plasmid DNA that contains cytopathic viral genes can usually be reduced by Benzonase treatment of virion particles to a minimal DNA level acceptable for clinical applications. In the case of toxin contamination, extensive virion purifications are likely required for reducing toxins to a low level that would not trigger non-specific killing of non-target cells.

In the human body, cells that need to be targeted include infected macrophages and resting CD4 T cells, two of the major reservoirs of HIV-1 3,6,10,11,15–17. Macrophages are potentially the prime targets of the NIRD vector because these cells are long-lived and resistant to HIV-1-induced apoptosis. In addition, macrophages are relatively insensitive to antiretroviral drugs, and compartmentalized macrophages such as tissue and brain macrophages are hard to reach with drugs 63. These difficulties may potentially be compensated by the intracellular delivery of therapeutic genes through the NIRD particles. The non-integrated DNA delivered through the NIRD vector is known to persist for weeks and months in macrophages 43. This would permit low levels of HIV-dependent transcription to occur until a sufficient amount of toxins accumulated in macrophages to induce cell death. On the other hand, the lack of viral activity in resting CD4 T cells may pose a significant problem for the NIRD vector. It may have to rely on transient stimulation of T cells with cytokines such as IL-2 and IFN-γ 64,65 to permit transient gene expression to induce cell death.

In this study, we selected human TRAF6 as a suicide gene for testing in the NIRD vector. The human TRAFs are intracellular proteins associated with the tumor necrosis factor receptor (TNF-R) 66. There are six mammalian TRAF family members (TRAF1-6) that are involved in signal transduction by TNF-R family members as well as some members of the Toll-like receptor (TLR) family and IL-1R. Unlike other TRAFs, which largely mediate signaling from the TNF-R superfamily, TRAF6 also participates in the signaling pathway from the IL-1R/TLR superfamily 67,68. TRAF6 also directly induces apoptosis, which results from the capacity of human TRAF6 to interact with and activate caspase 8. Both the C-terminal TRAF domain of human TRAF6, which directly interacts with the death effector domain of pro-caspase 8, and the N-terminal RING domain, which is required for activation of caspase 8, are necessary for apoptotic induction 39. The different sensitivity of the two transformed cell lines, HeLa and HEK293T cells, to TRAF6-induction of apoptosis, as we observed in this study (Figure 2B), is not currently understood. It may be related to the different levels of Rev expressed in these cells. Alternatively, it is also possible that certain cellular cofactors involved in the TRAF6-mediated apoptosis are differently expressed in these two cells. Nevertheless, these variations may not be an issue for *in vivo* targeting since most of the HIV-infected cells are non-transformed primary cells that should be vulnerable to apoptosis induction.

In comparison with TRAF6, DT is certainly the most extensively studied and well-understood bacterial toxin used for therapeutics. Ever since its discovery in the late 1800s, it has been a central focus in the field of toxicology. Several properties of DT, such as its high potency, high specificity, and well-defined mechanism of inhibition 69,70, have placed DT at the top of the list of therapeutic toxins 71,72. DT has been widely used in vaccine and therapeutic clinical trials, which have provided much-needed information about its safe use 73. These valuable attributes prompted us to select DT as one of the candidates to be tested in our NIRD vector.

Although the NIRD vector has demonstrated high specificity *in vitro*, future studies in animal models need to address several safety issues such as possible non-specific expression of toxin genes *in vivo*, and possible lentiviral vector recombination into competent viruses. In this regard, the endogenous human TRAF6 gene is probably a better choice than DT-A, since unexpected presence of low levels of TRAF6 in non-target cells could be tolerated. However, a good balance between efficacy and safety needs to be determined experimentally in future animal trials.

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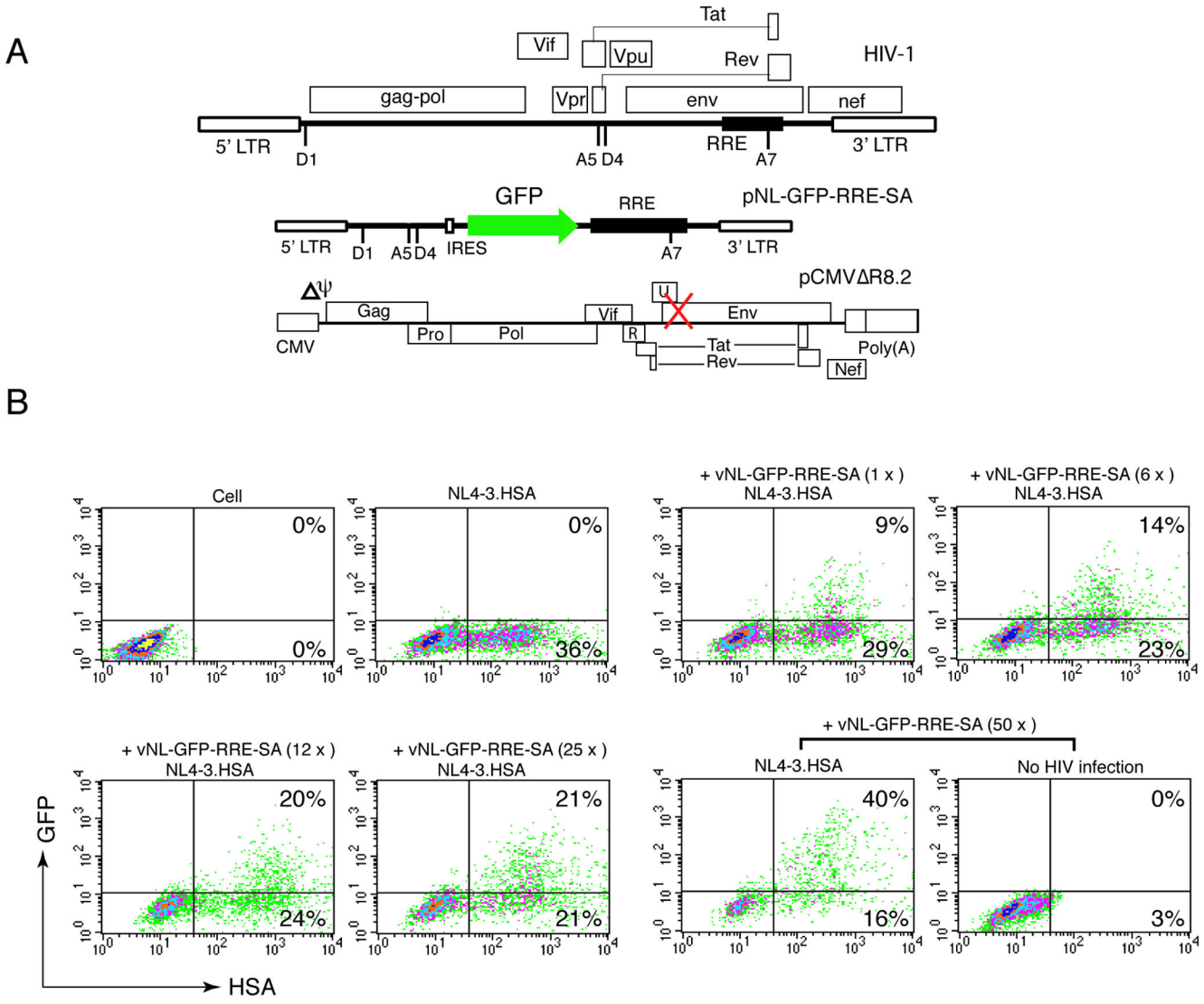

Figure 1. Specificity of the Rev-dependent lentiviral vectors in mediating HIV-dependent gene expression

(A) Schematic representation of the HIV-1 genome, a Rev-dependent lentiviral construct (pNL-GFP-RRE-SA), and the HIV-1 helper construct, pCMVΔR8.2, in which both the viral package signal (Δψ) and the envelope gene (Env) were deleted. Shown are the HIV-1 5' LTR, packaging signal (ψ), splice donors (D1, D4) and acceptors (A5, A7), IRES, RRE, and 3' LTR. (B) Specificity of the Rev-dependent lentiviral vector in HIV-1-positive T cells. CEM-SS cells were not infected (Cell) or infected with NL4-3.HSA.R+E-(VSV-G) (NL4-3.HSA, 1 μg p24 per million cells), a VSV-G pseudotyped HIV-1 strain with the murine heat-stable antigen CD24 (HSA) gene inserted into the *nef* region that allows HIV-1-positive cells to be monitored by surface staining of HSA. At 24 hours, cells were superinfected with lentivirus vNL-GFP-RRE-SA (1x, m.o.i. 0.2). For comparison, cells were also singly infected with either vNL-GFP-RRE-SA (No HIV infection) or NL4-3.HSA.R+E-(VSV-G) (NL4-3.HSA). At 72 hours, cells were harvested, stained with a PE-labeled rat

monoclonal antibody against mouse CD24 (HSA), and then analyzed on a flow cytometer for both HSA and GFP expression. Isotype staining is not shown.

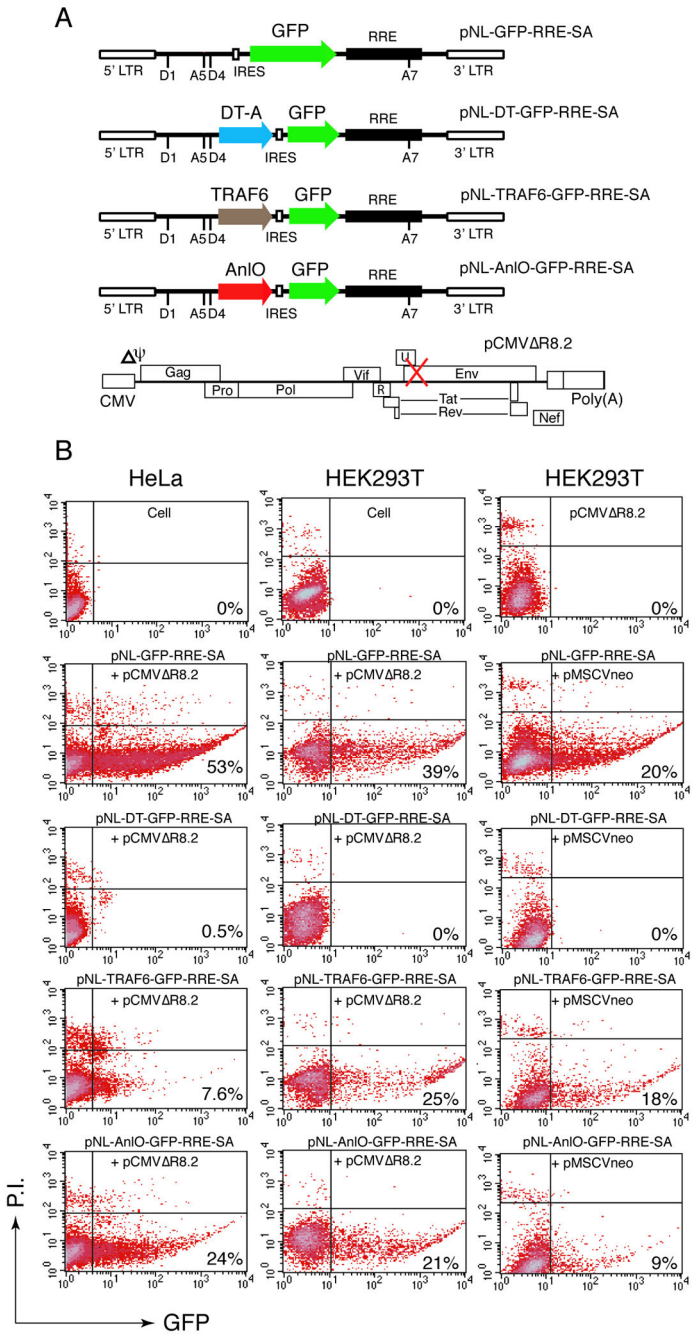

Figure 2. Rev-dependent killing of HIV-positive cells by DT-A, TRAF6, and AnlO

(A) Schematic representation of the Rev-dependent vectors carrying DT-A, TRAF6, and AnlO, and the helper construct, pCMVΔR8.2, that were used to cotransfect HEK293T or HeLa cells. (B) HeLa or HEK293T cells (1 million) were cotransfected with pCMVΔR8.2 (1 μg) plus pNL-DT-GFP-RRE-SA, pNL-TRAF6-GFP-RRE-SA, pNL-AlnO-GFP-RRE-SA, or pNL-GFP-RRE-SA (3 μg). As controls, these Rev-dependent vectors were identically cotransfected with an empty vector, pMSCVneo (1 μg). Cells were also cotransfected with pCMVΔR8.2 without the Rev-dependent vectors (1 μg pCMVΔR8.2 plus 3 μg pMSCVneo).

GFP expression was measured at 48 hours post cotransfection by flow cytometry. Propidium Iodide (P.I.) was added to identify viable GFP-expressing cells.

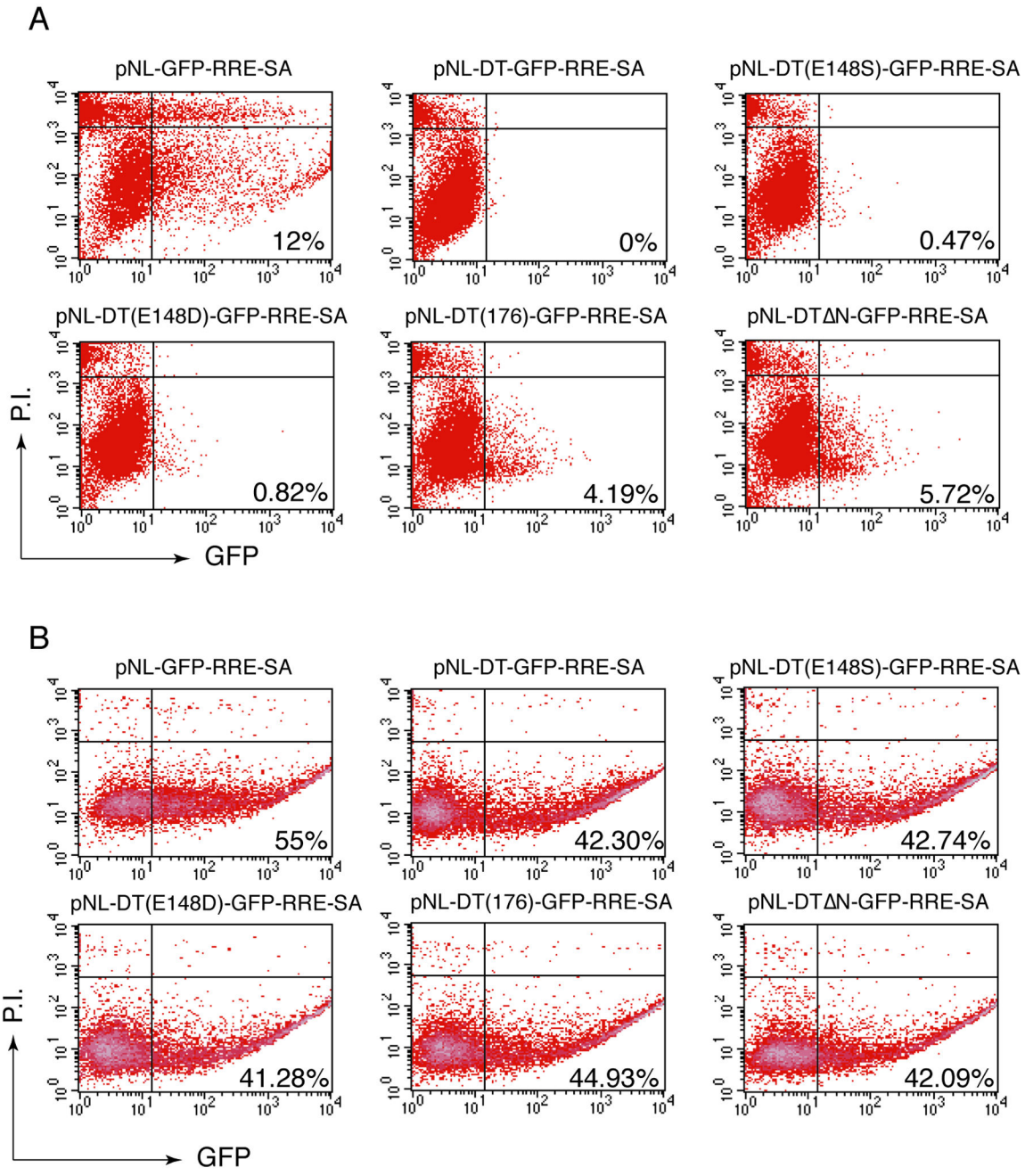

Figure 3. Rev-dependent killing of HIV-positive cells by DT-A mutants

(A) DT-A mutagenesis and the construction of the Rev-dependent vectors carrying these mutants were described in Materials and Methods. HEK293T cells (1 million) were cotransfected with pCMVΔR8.2 (1 μg) and one of the Rev-dependent constructs carrying the DT-A mutants, pNL-DT(E148S)-GFP-RRE-SA, pNL-DT(E148D)-GFP-RRE-(SA), pNL-DT(176)-GFP-RRE-SA, or pNL-DTΔN-GFP-RRE-(SA) (3 μg). Cell killing was monitored by GFP expression at 48 hours post infection using flow cytometry. (B) The same cotransfection experiments were repeated in a DT-A resistant cell line, 5H7.

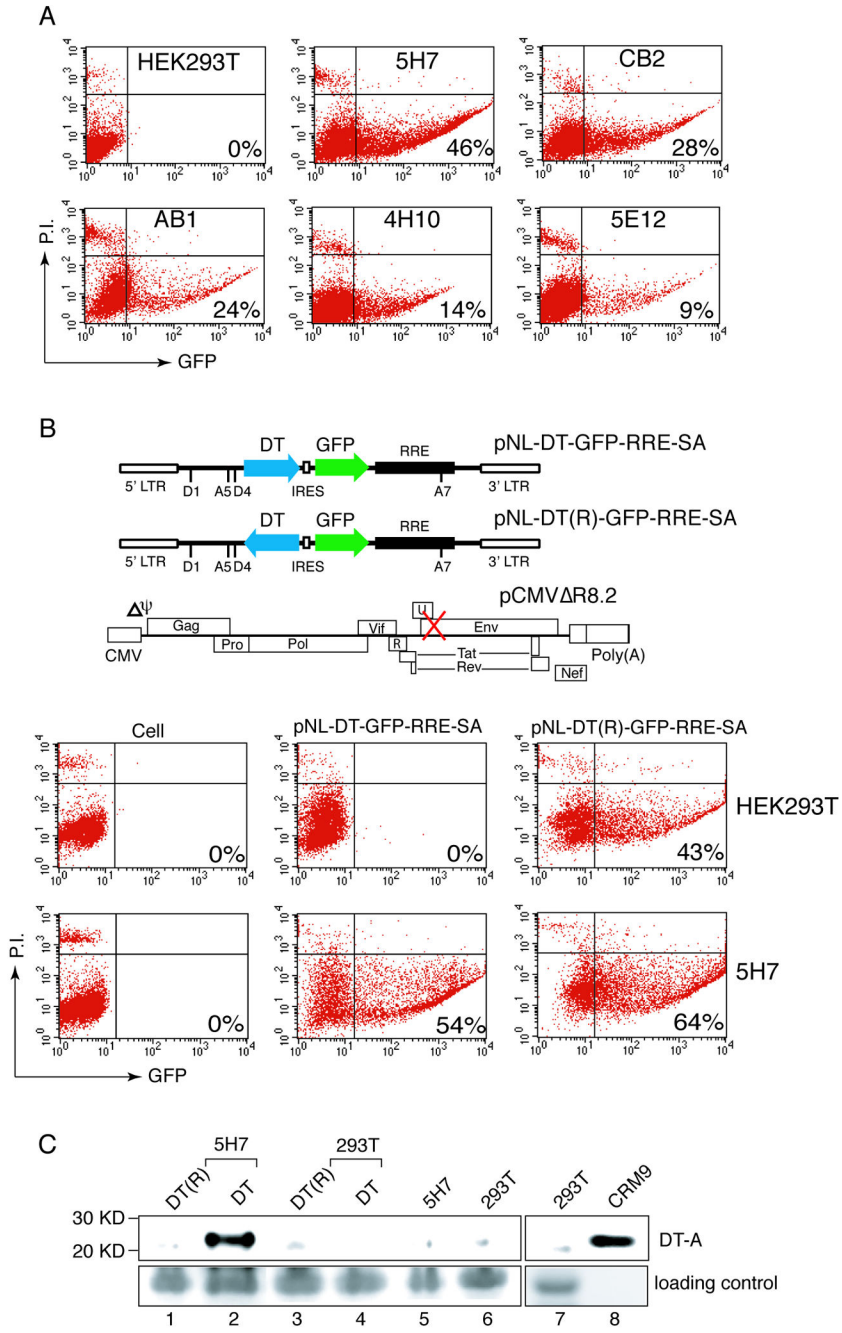

Figure 4. Testing of the DT-A-resistant HEK293T cells

(A) The human EF-2 mutant (G717R) was introduced into HEK293T cells by retroviral vector transduction. Cells were screened for the EF-2 mutation. Originally, 100 clones were selected, and 5 of them turned GFP-positive when cotransfected with pCMVΔR8.2 (1 μg for 1 million cells) plus the DT-A containing Rev-dependent vector, pNL-DT-GFP-RRE-SA (3 μg for 1 million cells). While the parental HEK293T cells generate 0% GFP-positive cells after the cotransfection, the DT-A resistant clones generate GFP-positive cells at different percentages: 46% in 5H7, 28% in CB2, 24% in AB1, 14% in 4H10, and 9% in 5E12,

respectively. (B) To further measure the degree of DT-A resistance, one of the clones, 5H7, was cotransfected with pCMVΔR8.2 plus pNL-DT-GFP-RRE-SA. As a control, the cells were also identically cotransfected with pCMVΔR8.2 plus pNL-DT(R)-GFP-RRE-SA in which the DT-A gene was placed in a reverse orientation to prevent protein expression. The parental HEK293T cells were also identically cotransfected with these constructs. (C) Western blot analysis of both 5H7 and HEK393T cells cotransfected with either pCMVΔR8.2 plus pNL-DT-GFP-RRE-SA (lanes 2 and 4, DT) or pCMVΔR8.2 plus pNL-DT(R)-GFP-RRE-SA [lanes 1 and 3, DT(R)]. Untansfected cells (lanes 5 to 7) and a purified, recombinant DT-A protein (CRM9) (lane 8) 74 were used as controls. A monoclonal antibody against DT was used for Western blot, and this antibody was also reactivated with a cellular protein (10–15KD) that was used as the loading control.

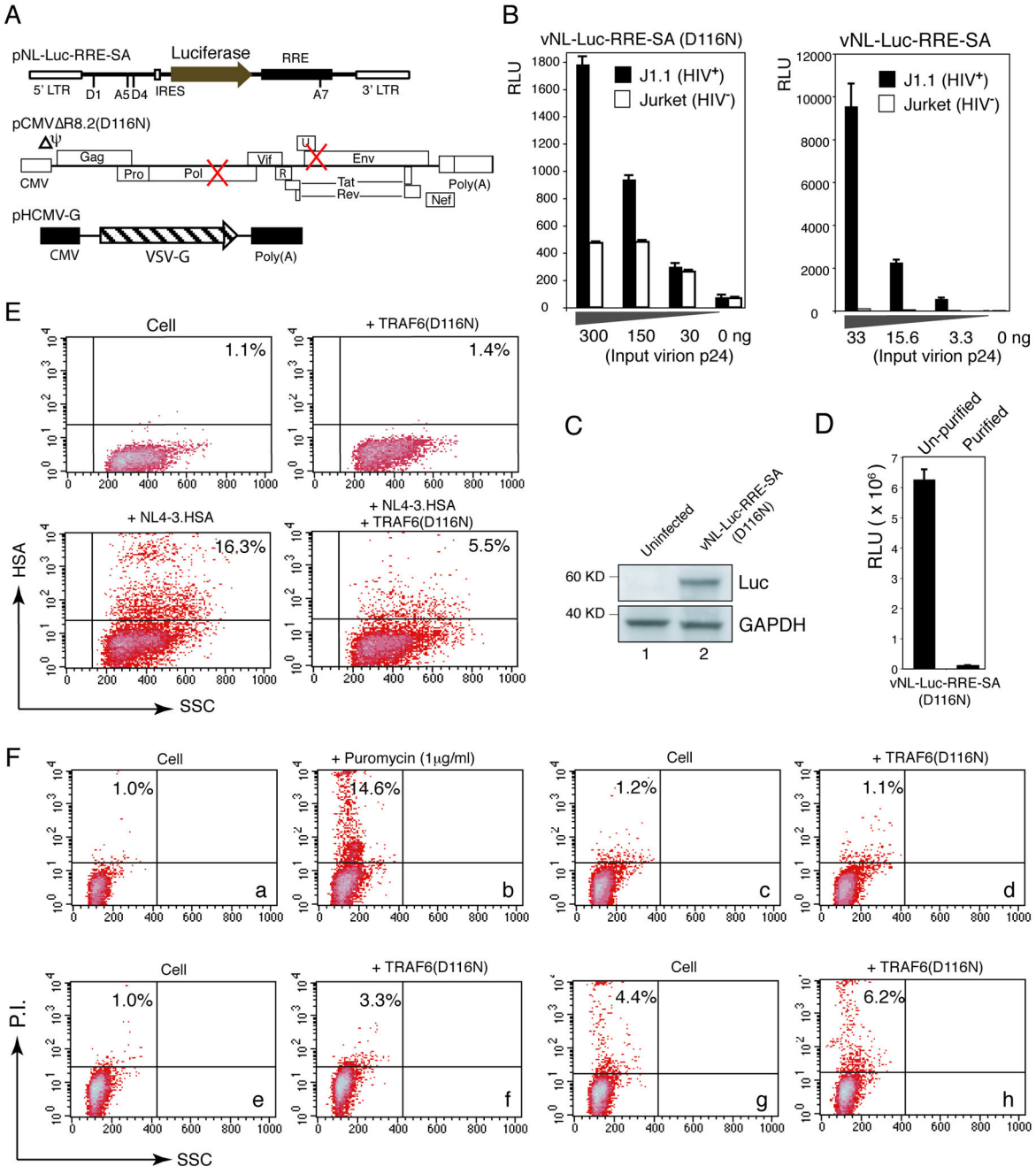

Figure 5. Development of the NIRD vector carrying DT-A and TRAF6

(A) Schematic representation of the Rev-dependent vector carrying luciferase, the non-integrating helper construct, pCMVΔR8.2(D116N), and pHCMV-G expressing VSV-G. (B) To demonstrate HIV-dependent expression of reporter genes from the NIRD vector, viral particles vNL-Luc-RRE-SA(D116N) and vNL-Luc-RRE-SA were generated by cotransfection of HEK293T cells with pCMVΔR8.2(D116N) or pCMVΔR8.2 plus pNL-Luc-RRE-SA plus pHCMV-G, and then used to superinfect an HIV-1-positive T cell line, J1.1 or the uninfected, parental Jurkat T cells (0.2 million cells). Luciferase was measured at

48 hours in J1.1 and Jurkat cells following infection. Both J1.1 and Jurkat were stimulated with 50 ng/ml PMA before infection. (C) To determine the background luciferase present in the HIV-negative Jurkat cells during infection with vNL-Luc-RRE-SA(D116N), cells (0.5 million cells) were pre-treated with azidothymidine (AZT) (50 μM) overnight, and then uninfected (lane 1) or infected with vNL-Luc-RRE-SA(D116N) for 2 hours (lane 2). Cells were washed and immediately lysed for Western blot analysis using a goat polyclonal anti-luciferase antibody. The blot was also probed with a goat polyclonal antibody to GAPDH for loading controls. (D) The background luciferase activity present in the vNL-Luc-RRE-SA(D116N) viral preparation was reduced by purifying the virion through anion exchange (Sartobind Q75) and size-exclusion (Vivaspin 20 and 500) columns. The relative luciferase activities (RLU) present in virion before and after purification were measured (normalized by virion p24). (E) Specific targeting of HIV-1-infected lymphocytes by TRAF6 NIRD vector. Human PBMC (1 million cells) were infected with a replication-competent virus NL4-3.HSA.R+ (10^4 $TCID_{50/Rev\text{-}CEM}$). Aliquots of the infected cells were then superinfected at days 1, 4 and 7 post HIV infection with vNL-TRAF6-GFP-RRE-SA(D116N) (5 μg p24). HIV-1-positive cells were measured by surface staining of mouse HSA followed by flow cytometry at day 9 post HIV-1 infection. (F) To measure non-specific killing of cells by TRAF6 NIRD vector, HIV-uninfected cells were infected with only vNL-TRAF6-GFP-RRE-SA(D116N) as described in (E) (panel d, f, h). Following infection at day 0, 3 and 6, cells were analyzed one day later by propidium iodide (P.I.) staining and flow cytometry (panel d, f, h, respectively). As controls, cells were also mock infected with medium (panel a, c, e, and g), or treated with puromycin to induce non-specific killing (panel b).

Table 1

Production of Integrating and NIRD Viral Particles carrying DT-A and TRAF6*

DNA construct	Producer cell	p24 level at 48 hours post cotransfection	p24 level at 72 hours post cotransfection
pNL-TRAF6-GFP-RRE-SA pCMVΔR8.2 pHCMV-G	HEK293T	1095 ng/ml	1659 ng/ml
pNL-TRAF6-GFP-RRE-SA pCMVΔR8.2(D116N) pHCMV-G	HEK293T	870 ng/ml	2059 ng/ml
pNL-DTΔN-GFP-RRE-SA pCMVΔR8.2 pHCMV-G	5H7	109 ng/ml	102 ng/ml
pNL-DTΔN-GFP-RRE-SA pCMVδR8.2(D116N) pHCMV-G	5H7	38 ng/ml	60 ng/ml

* DNA constructs were cotransfected into producer cells cultured in 10 cm petridish as described in Materials and Methods. Viral supernatant was harvested at 48 and 72 hours post infection, and levels of p24 were measured by ELISA.