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R88-APOBEC3Gm Inhibits the Replication of Both Drug-resistant Strains of HIV-1 and Viruses Produced From Latently Infected Cells

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Human immunodeficiency virus type 1 (HIV-1) drug resistance and the latent reservoir are the two major obstacles to effectively controlling and curing HIV-1 infection. Therefore, it is critical to develop therapeutic strategies specifically targeting these two obstacles. Recently, we described a novel anti-HIV approach based on a modified human intrinsic restriction factor, R88-APOBEC3G (R88-A3G). In this study, we further characterized the antiviral potential of R88-A3G$_{D128K}$ (R88-A3Gm) against drug-resistant strains of HIV-1 and viruses produced from latently infected cells. We delivered R88-A3Gm into target cells using a doxycycline (Dox)-inducible lentiviral vector and demonstrated that its expression and antiviral activity were highly regulated by Dox. In the presence of Dox, R88-A3Gm–transduced T cells were resistant to infection caused by wild-type and various drug-resistant strains of HIV-1. Moreover, when the R88-A3Gm–expressing vector was transduced into the HIV-1 latently infected ACH-2 cell line or human CD4$^+$ T cells, on activation by phorbol-12-myristate-13-acetate or phytohemaglutinin, R88-A3Gm was able to curtail the replication of progeny viruses. Altogether, these data clearly indicate that R88-A3Gm is a highly potent HIV-1 inhibitor, and R88-A3Gm–based anti-HIV gene therapy is capable of targeting both active and latent HIV-1–infected cells to prevent subsequent viral replication and dissemination.

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Introduction

The introduction of highly active antiretroviral therapy has significantly improved the quality of life and enhanced the life expectancy of human immunodeficiency virus type 1 (HIV-1) patients. However, problems associated with antiretroviral drugs have emerged. Among them, drug resistance is a major cause of treatment failure.[1] Drug-resistant strains of HIV-1 have been found in ~80% of patients experiencing treatment failure[2] and can spread to HIV-susceptible populations.[3] This transmitted resistance leads to fewer treatment options and reduced treatment efficacy.[4] Additionally, it is known that the currently available antiviral therapies fail to eradicate the virus entirely from infected people, and virus establishes a latent infection in cellular reservoirs, mainly in resting CD4$^+$ T cells and in tissue reservoirs, such as central nervous system and gut-associated lymphoid tissue.[5] These latent reservoirs, containing both wild-type and drug-resistant strains of HIV-1,[6] have a long life and are resistant to the host immune response and antiviral treatment.[7] Once highly active antiretroviral therapy is discontinued, viral replication resumes from reservoirs, leading to a rapid rebound of viremia in most patients.

Considering these obstacles for HIV-1 eradication, there is a growing need for novel antiviral therapeutic strategies. Gene therapy approaches, by targeting one or more steps of the viral life cycle, are able to suppress viral replication, or even provide life-long protection if hematopoietic progenitor cells are targeted.[8,9]

Host restriction factors are attractive candidates for gene therapy due to their potent antiviral activities.[10] Importantly, the introduction of host restriction factors *in vivo*, unlike foreign proteins, could avoid the immunogenicity associated with protein-based gene therapy approaches. The first identified restriction factor, human cytidine deaminase apolipoprotein B messenger RNA editing enzyme catalytic polypeptide 3G (APOBEC3G; hereafter referred to as A3G), inhibits HIV-1 replication by inducing lethal mutations on proviral DNA[11,12] and by disrupting viral reverse transcription and integration.[13,14] Compared with other proteins of the APOBEC3 family, A3G exhibits advanced antiviral activity in human primary target cells[15] and in a humanized mice model.[16] Therefore, A3G can be considered as an attractive and potent candidate for anti-HIV gene therapy.

Although A3G is a strong host restriction factor against HIV, the virus encodes the viral infectivity factor (Vif) to counteract A3G's antiviral activity. Vif acts as a physical barrier and directly blocks virion encapsidation of A3G.[17] More importantly, Vif binds to A3G and promotes its poly-ubiquitination and proteasomal degradation.[17] In our previous studies, to overcome Vif's blockage and maximize A3G antiviral activity, we fused A3G with the virion-targeting polypeptide R88.[18] R88 is derived from the HIV-1 Vpr protein, which efficiently delivers A3G into the Vif$^+$ virion through interacting with Gag-p6.[19] We then introduced Vif-binding mutants into the R88-A3G fusion protein,[20] which made the fusion protein resistant to Vif-mediated degradation. Of the R88-A3G mutants, R88-A3G$_{D128K}$ (R88-A3Gm) was the most resistant to Vif-induced degradation and exhibited most potent anti-HIV activity. Additionally, Voit *et al.* have shown that A3G$_{D128K}$ confers over 100-fold protection against HIV-1 infection, compared with the 3- to 64-fold protection provided by human–rhesus hybrid tripartite motif 5-α.[21] Therefore,

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in the present study, R88-A3Gm was chosen as the candidate for anti-HIV gene therapy.

Here, we delivered R88-A3Gm into target cells using a lentiviral vector system and studied its potential against the replication of both wild-type and drug-resistant strains of HIV-1, as well as the spread of viruses produced from latently infected cells.

Results

Generation of a lentiviral vector system for inducible expression of R88-A3Gm

To generate an A3G-based anti-HIV gene therapy approach, we used a lentiviral vector due to its long-term expression ability. However, because A3G has intrinsic antiretroviral effects,[11,12,14] the constitutive expression of R88-A3Gm during the packaging process leads to R88-A3Gm protein incorporation into the lentiviral vector, which could further impair the infectivity of produced vector. Therefore, to avoid this side effect and to increase the transduction efficiency, the doxycycline (Dox)-inducible lentiviral vector system pTRIPZ (pTZ)-red fluorescent protein (RFP) was used to temporarily suppress the expression of R88-A3Gm during vector packaging (**Figure 1a**). As shown in **Figure 1b**, the expression of RFP in pTZ-RFP vector–transduced C8166 T cells was activated by Dox (right).

We then replaced RFP with an R88-A3Gm transgene to generate pTZ-R88-A3Gm. A3Gm ($A3G_{D128K}$) is a Vif-resistant

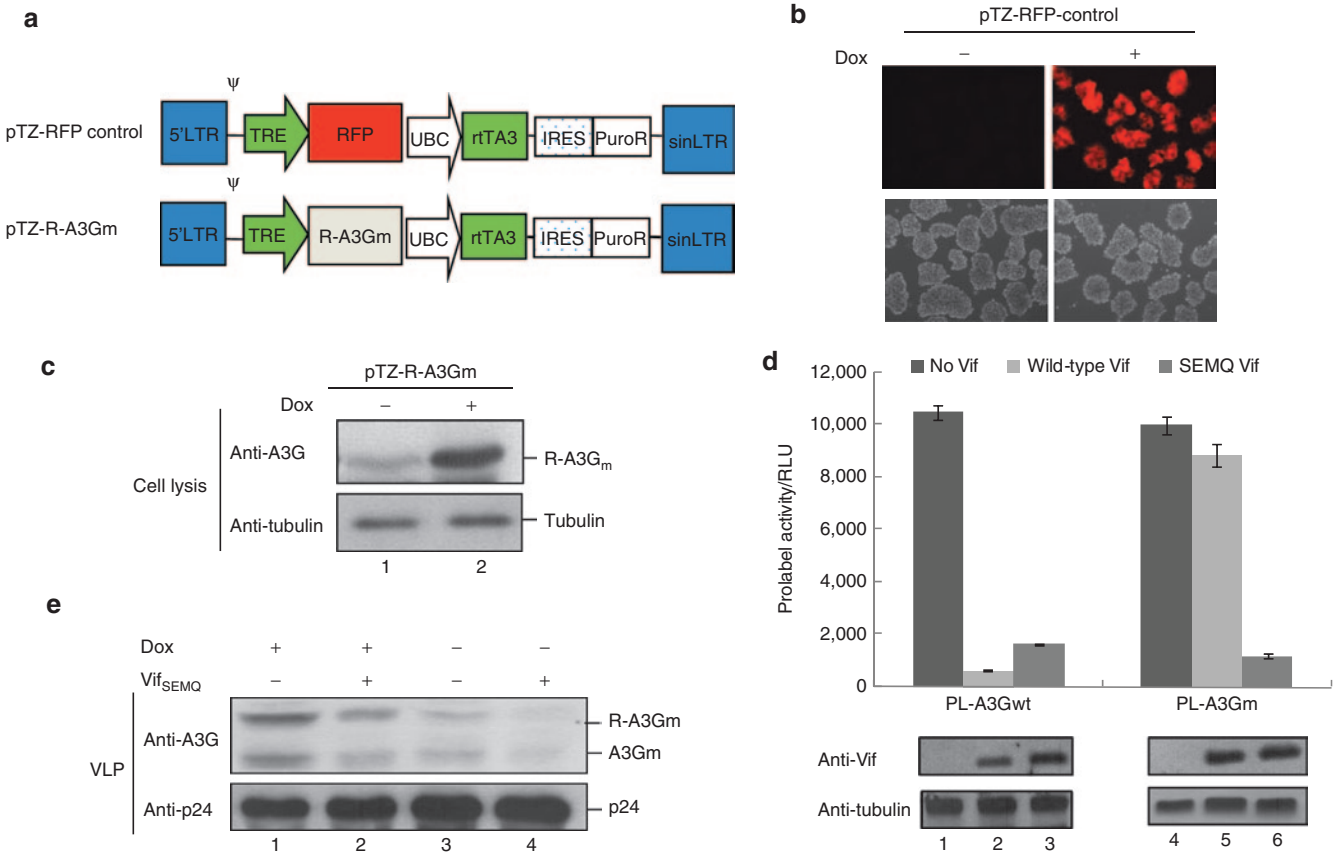

Figure 1 Regulated A3G expression in 293T cells. (**a**) Schematic representation of pTRIPZ (pTZ) vectors for doxycycline (Dox)-inducible transgene expression. In this vector, the expression of the transgenic red fluorescent protein (RFP) and R88-$A3G_{D128K}$ (R88-A3Gm) is under the control of the tetracycline response element (TRE) promoter, which can be activated by the reverse tetracycline transactivator 3 (rtTA3) in the presence of Dox. In the presence of Dox, rtTA3, whose expression is driven by the constitutively active ubiquitin C promoter (UBC), binds to and activates expression from TRE promoters. pTZ vectors contain the 5′ and 3′ long terminal repeats (LTRs) with a self-inactivating deletion in the U3 region, packaging signal(Ψ), internal ribosome entry site (IRES) and puromycin selectable marker (PuroR). (**b**) Inducible RFP expression in pTZ-RFP control transduced C8166 T cells. C8166 T cells were transduced with pTZ-RFP vectors, and cultured in the absence (left panel) or presence (right panel) of 2 μg/ml Dox. RFP expression was assessed at 72 hours by fluorescence microscopy. (**c**) Inducible expression of A3G in particle–producing 293T cells. 293T cells were transfected with pTZ-R88-A3Gm and cultured in the absence or presence of 2 μg/ml Dox. After 48 hours, cells were collected and the levels of A3G in transduced cells were measured by western blotting with anti-A3G antibody. As a control, cell lysates were probed with an anti-tubulin antibody. (**d**) Vif_{SEMQ} mutant promotes the degradation of A3Gm. 293T cells were transfected with either ProLabel (PL)-A3Gwt or PL-A3Gm plus the control vector pcDNA3.1 (lanes 1 and 4), Vif_{wt} (lanes 2 and 5), or Vif_{SEMQ} (lanes 3 and 6). Degradation of A3G in transfected cells was determined by measuring ProLabel activity. Expression of Vif_{wt} and Vif_{SEMQ} were confirmed in the cell lysates by western blotting with anti-Vif antibody. Tubulin was detected as loading control. (**e**) Regulated A3G expression in produced vector particles. 293T cells were cotransfected with pTZ-R88-A3Gm, the packaging plasmid Δ8.2 and a VSV-G expression plasmid with or without Vif_{SEMQ} and cultured in the absence or presence of 2 μg/ml Dox. After 72 hours, vector particles were collected from supernatant by ultracentrifugation through a 20% sucrose cushion. The level of A3G in produced vector particles was measured by western blotting with an anti-A3G antibody. As a control, vector particles were probed with an anti-p24 antibody. The data are representative of three independent experiments. A3G, human cytidine deaminase apolipoprotein B messenger RNA editing enzyme catalytic polypeptide 3; Vif, viral infectivity factor.

mutant that fails to interact with HIV-1 Vif; therefore, it can inhibit HIV-1 infection even in the presence of Vif.[22,23] The regulated expression of R88-A3Gm in vector particle–producing 293T cells was assessed. As expected, in the presence of Dox, the expression of R88-A3Gm was highly induced (**Figure 1c**, upper panel, comparing lane 2 with lane 1). This result not only confirms that the expression of R88-A3Gm in the vector particle–producing cells requires Dox induction but also indicates that the expression of R88-A3Gm can be minimized in the absence of Dox.

However, due to the incomplete suppression of the tetracycline response element promoter,[24] a very small amount of the R88-A3Gm protein was still expressed in the absence of Dox (**Figure 1c**, lane 1), which could also incorporate into vector particles and impair the vector's transduction efficiency. We thus attempted to promote R88-A3Gm degradation by cotransfecting vector particle–producing cells with the $A3G_{D128K}$-susceptible HIV-1 Vif_{SEMQ}-expressing plasmid.[25] Indeed, when Vif_{SEMQ} was coexpressed with ProLabel-A3Gm (PL-A3Gm) in 293T cells, the cellular level of the A3Gm protein was reduced by eight times, as measured by ProLabel assay, which is based on the chemiluminescent detection of PL-A3G fusion protein expression (**Figure 1d**).

Finally, we attempted to confirm that reduced R88-A3Gm expression in vector particle–producing cells could lead to reduced protein incorporation into vector particles. As shown in **Figure 1e**, incorporation of the R88-A3Gm protein was reduced when Vif_{SEMQ} was coexpressed (comparing lane 1 with lane 2) and minimized by Vif_{SEMQ} coexpression and non-Dox induction. Therefore, by combining the inducible promoter and expression of Vif_{SEMQ}, we could minimize the presence of R88-A3Gm in produced vector particles.

Transduction efficiency of pTZ-R88-A3Gm is greatly enhanced by minimizing the presence of the A3G protein

We then examined the transduction efficiency of the pTZ-R88-A3Gm vector produced in the presence of Vif_{SEMQ} from non-Dox–induced cells. The same panel of pTZ-R88-A3Gm vector particles as described in **Figure 1** and the control vector pTZ-RFP were quantified by measuring the amount of vector-associated Gag-p24 and used to transduce $CD4^+$ C8166 T cells. Cell viability was assessed after puromycin (puro) selection, where only the cells expressing the puro resistance gene from integrated pTZ-R88-A3Gm vectors could survive. The results showed that once C8166 T cells were transduced with vector particles produced in the absence of Dox, 78% of them were survived (**Figure 2a**, sample 4). When Vif_{SEMQ} was present in particle-producing cells, the percentage of survived cells was increased to 93% (**Figure 2a**, sample 5). This result was further confirmed by evaluating the transduction efficiency of these vector particles in HeLa cells, where more puro-resistant colonies were present in cell cultures transduced with the pTZ-R88-A3Gm vector produced in the presence of Vif_{SEMQ} from non-Dox–treated cells (**Figure 2b**).

Meanwhile, the number of vector DNA copies in transduced C8166 cells was detected by real-time–polymerase chain reaction (PCR) assay. As shown in **Figure 2c**, the greatest transduction efficiency was observed in cells transduced with vector particles produced in the presence of Vif_{SEMQ} and without Dox treatment (bar 5), which was similar to the efficiency of the pTZ-RFP vector (~1 copy/cell, bar 1). Overall, these results indicate that this Dox-inducible pTZ-R88-A3Gm vector, produced in the presence of Vif_{SEMQ} from non-Dox–induced cells, can be used to efficiently transduce $CD4^+$ C8166 T cells and HeLa cells.

The $CD4^+$ C8166 T cell line transduced with the Dox-inducible R88-A3Gm vector is highly resistant to HIV-1 infection in the presence of Dox

To study the anti-HIV effect mediated by R88-A3Gm, we first generated a stable C8166 T-cell line by pTZ-R88-A3Gm vector transduction and puro selection, and then tested whether this cell line expressing R88-A3Gm was resistant to HIV-1 infection. Data from **Figure 3a** show that viral replication was inhibited in pTZ-R88-A3Gm–transduced C8166 T cells pretreated with Dox at different concentrations (ranging from 0 to 2 μg/ml), and a maximal inhibition was achieved with 1–2 μg/ml of Dox treatment. Therefore, 1 μg/ml was used as the optimal concentration for Dox induction in the following studies.

To check whether this antiviral effect correlated with R88-A3Gm expression and virus incorporation, the presence of R88-A3Gm in virions released from infected R88-A3Gm–expressing cells was further measured. As shown in **Figure 3b**, on Dox treatment, viral replication and spread were inhibited, as indicated by the significantly reduced Gag-p24 levels in the supernatants at 5 days postinfection (lane 3, lower panel). Remarkably, a large amount of R88-A3Gm protein was incorporated into virions produced by Dox^+-infected cells (lane 3, upper panel). Additionally, a low background level of R88-A3Gm was observed in virions from non-Dox–treated cells, which would be responsible for the slightly impaired virus replication in these cells.

To further evaluate the effect of R88-A3Gm on viral replication and spread, we infected transduced C8166 T cells with HIV-1 pNL-4.3 virus at a lower multiplicity of infection (0.01) and monitored viral replication for a prolonged period of time. As shown in **Figure 3c** (left), HIV-1 replication in pTZ-R88-A3Gm–expressing cells cultured with Dox was completely abolished, whereas in non-Dox–treated cells, HIV-1 replication still occurred, even though viral replication kinetics was delayed compared with pTZ-RFP control cells. Furthermore, the HIV-1–induced cytopathic effect was blocked in the pTZ-R88-A3Gm–expressing C8166 T cells induced by Dox (**Figure 3c**, right, picture 3). Together, these results show that the induced expression of R88-A3Gm efficiently blocks HIV-1 replication and spread.

pTZ-R88-A3Gm inhibits the replication of virus drug-resistant strains of HIV-1

Transmission of drug resistance has increased the difficulty of HIV-1 drug treatment and the control of the HIV pandemic. Here, we chose viruses resistant to different classes of currently used antiretroviral drugs, including zidovudine, lamivudine, nevirapine, protease inhibitors (MK-639, XM323, A-80987, Ro31-8959, VX-478, and SC-52151), and raltegravir and examined whether the expression of R88-A3Gm could block their replication. As shown in **Figure 4**, control vector–transduced C8166 T cells were highly susceptible to all drug-resistant viruses, and viral replication peaked

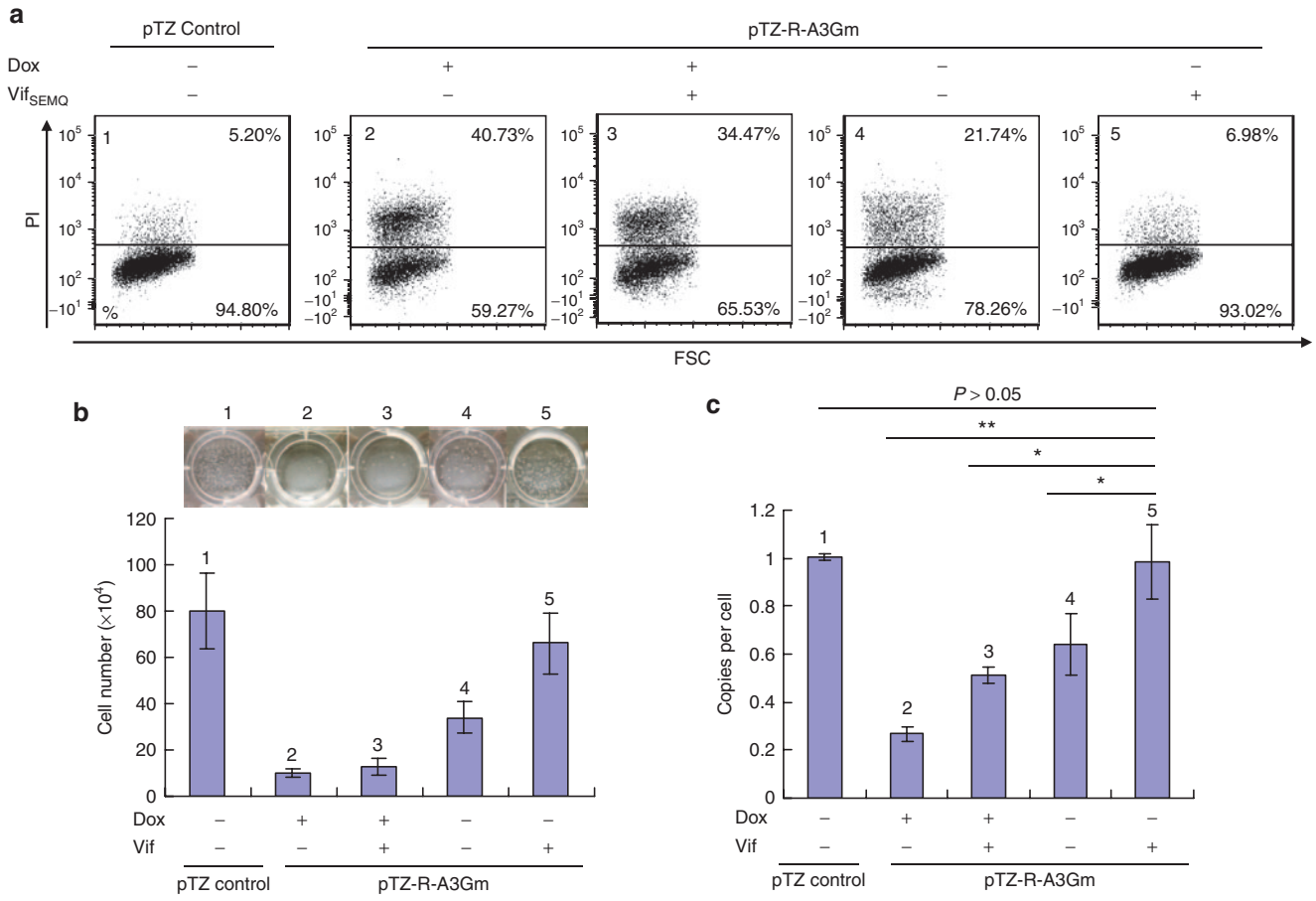

Figure 2 Evaluation of transduction efficiency of pTZ-R88-A3Gm vectors. vector particles containing pTZ-R88-A3Gm were produced in the presence or absence of doxycycline (Dox) (2μg/ml) and/or Vif$_{SEMQ}$ as indicated. Equal amounts of vector particles (normalized by p24 enzyme-linked immunosorbent assay) were used to transduce target cells. (**a**) Transduced C8166 T cells were selected with puro (0.5 μg/ml) for 7 days. After staining with propidium iodide, cells were analyzed by flow cytometry. (**b**) Transduced HeLa cells were selected with puro (2 μg/ml) for 10 days. Selective medium was replaced every 2 days to remove dead cells. The pictures showing puro-resistant colonies were captured by digital camera (upper panel). Total cell numbers from individual wells were counted with trypan blue staining (lower panel). (**c**) Real-time polymerase chain reaction detecting the U5-R sequence was performed on total DNA extracted from C8166 T cells at 2 days posttransduction. The values shown were the averages of triplicates with the indicated standard deviations. Statistical significance was calculated using the Student's *t*-test and denoted as * for $P \leq 0.05$, **for highly significant with $P \leq 0.01$. Results **a** and **b** were from one representative of two independent experiments. A3G, human cytidine deaminase apolipoprotein B messenger RNA editing enzyme catalytic polypeptide 3; pTZ, pTRIPZ; R88-A3Gm, R88-A3G$_{D128K}$; Vif, viral infectivity factor.

after 6–9 days of infection. In contrast, no viral replication was observed in C8166 T cells expressing R88-A3Gm over a period of 12 days. These data indicate that the presence of R88-A3Gm in highly susceptible C8166 T cells is able to completely block HIV-1 replication, including the replication of drug-resistant viruses.

Expression of R88-A3Gm in primary human cells inhibits active HIV-1 infection

CD4$^+$ T lymphocytes and macrophages are considered to be two major targets for HIV-1 infection, and thus, it is important to determine whether R88-A3Gm expression could inhibit viral replication in these cells. First, primary CD4$^+$ T cells and macrophages were transduced with pTZ-R88-A3Gm vector particles, and the copy number of the R88-A3Gm gene in transduced cells was measured by real-time PCR. As shown in **Figure 5a**, R88-A3Gm DNA was detected from transduced CD4$^+$ T cells and macrophages at 0.35 and 0.4 copies per cell, respectively, indicating the successful transduction of R88-A3Gm into primary cells.

To evaluate the antiviral effect of R88-A3Gm in primary CD4$^+$ T cells, pTZ-R88-A3Gm–transduced CD4$^+$ T cells were induced with Dox for 2 days followed by infection with nevirapine-resistant virus. The result from one representative donor showed that viral replication in R88-A3Gm–transduced primary CD4$^+$ T cells was inhibited by approximately two- to threefold when compared with pTZ-RFP control vector–transduced cells (**Figure 5b** left). Next, we assessed the antiviral effect of R88-A3Gm in macrophages. Transduced macrophages were induced with Dox and infected with pNL4.3-Bal virus. Data showed that viral replication in control vector–transduced cells was peaked at day 13, whereas in R88-A3Gm–transduced cells, viral replication was reduced by more than threefold and failed to reach a peak within 17 days of observation (**Figure 5b**, right). Taken together, these results indicate that the expression of R88-A3Gm is capable

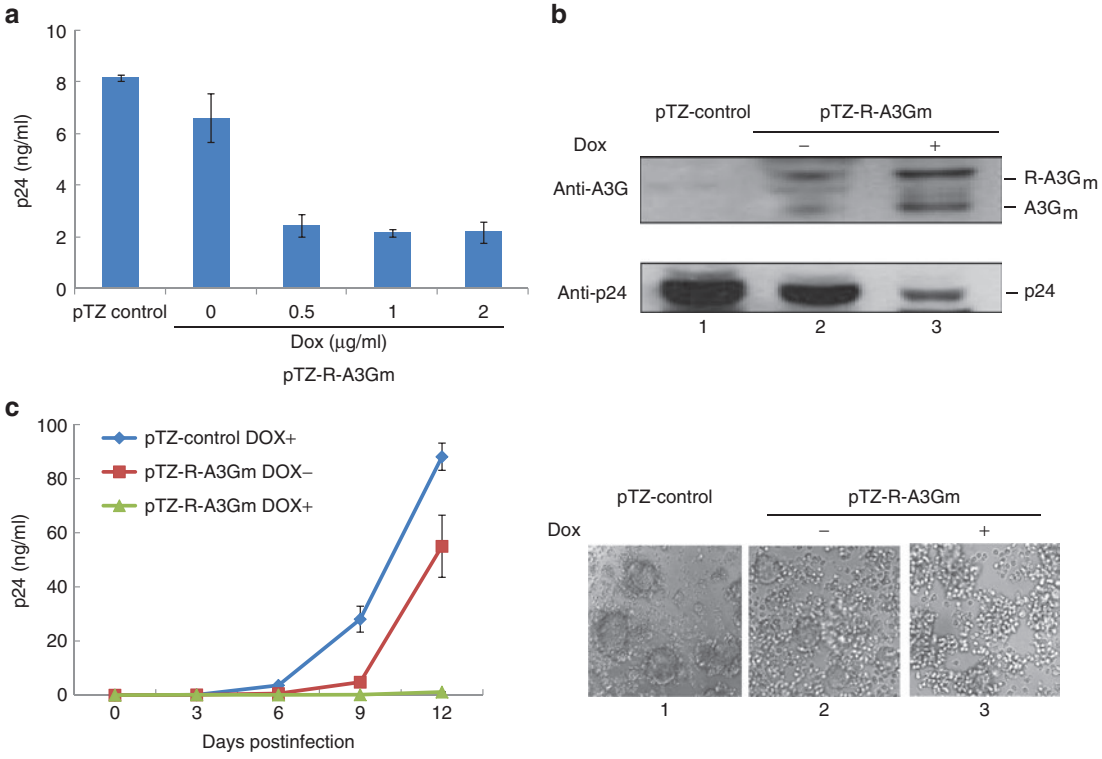

Figure 3 Doxycycline (Dox) regulated R88-A3G$_{D128K}$ (R88-A3Gm) expression and antiviral effect in pTZ-R88-A3Gm–transduced CD4$^+$ C8166 T cell line. pTZ-control and pTZ-R88-A3Gm–transduced C8166 T cells were induced with Dox (1 μg/ml or as indicated) for 2 days and infected with pNL-4.3 virus at (**a**,**b**) a multiplicity of infection (MOI) of 1 or (**c**) MOI of 0.01. (**a**) Levels of HIV-1 Gag-p24 in the supernatants were measured by p24 enzyme-linked immunosorbent (ELISA) assay at 5 days postinfection. (**b**) Viruses produced from infected C8166 T cells were concentrated from the supernatant by ultracentrifugation after 5 days of infection. The presence of R88-A3G was analyzed by western blotting with anti-A3G antibody, and HIV-1 Gag protein was detected by anti-p24 antibody. (**c**) Virus replication was measured by p24 ELISA from the supernatant at different time intervals (left). HIV-1–induced syncytium formation was visualized under microscopy (right). Results were from one representative of two independent experiments. A3G, human cytidine deaminase apolipoprotein B messenger RNA editing enzyme catalytic polypeptide 3; pTZ, pTRIPZ.

of inhibiting active HIV-1 replication in primary CD4$^+$ T cells and macrophages.

Introduction of pTZ-R88-A3Gm into HIV-1 latently infected cells disrupts the infectivity of progeny viruses

Given that A3G mainly targets newly produced viruses and disrupts their infectivity,[11,26] it would be of interest to investigate whether introducing R88-A3Gm into HIV-1 latently infected cells could block the infectivity of progeny virus produced after latency reactivation. First, pTZ-R88-A3Gm and pTZ-control vectors were used to transduce ACH-2 cells, a well-characterized HIV-1 latently infected cell line that contains a point mutation in HIV-1 trans-activation response element. This mutation disables the function of tat trans-activation response element axis in the transactivation of HIV gene transcription.[27] Following Dox induction and phorbol-12-myristate-13-acetate stimulation, the newly produced viruses were collected and quantified from supernatants by measuring Gag-p24 levels (**Figure 6a**). The results showed that 2 days postinfection, similar levels of progeny viruses were produced from pTZ-R88-A3Gm– and pTZ-control–transduced ACH-2 cells (**Figure 6b**), suggesting that R88-A3Gm does not affect viral production from infected cells when latency is reversed.

Meanwhile, the infectivity of progeny viruses from ACH-2 cells was evaluated. Equal amounts of progeny viruses produced from pTZ-R88-A3Gm– and pTZ-control–transduced ACH-2 cells were used to infect normal C8166 T cells, and viral replication in these cells was monitored at days 2 and 3. The results showed that the infectivity of progeny viruses produced from R88-A3Gm–transduced ACH-2 cells was reduced by three- to sixfold compared with the viruses produced by the control vector–transduced cells (**Figure 6c**). Taken together, these results demonstrate that introducing R88-A3Gm into latently infected cells does not affect viral production from reactivated proviruses, but strongly disrupts the infectivity of newly produced viruses, thereby blocking additional rounds of infection.

To further assess the effect of R88-A3Gm on HIV-1 latent infection in primary cells, we used a recently established primary cell model of HIV latency.[28] In this model, latency can be established in resting CD4$^+$ T cells at the postintegration step, and activation of replication-competent proviruses can be achieved by stimulation with the appropriate inducing agents. As shown in **Figure 7a**, in the first 4 days following transduction, the difference in viral production between R88-A3Gm– and control vector–transduced cells was not significant. However, at days 6 and 8 posttransduction, viral replication was disrupted by R88-A3Gm, and about threefold reduced virus production was observed at day 8 in pTZ-R88-A3Gm–transduced CD4$^+$ T cells. In addition, we

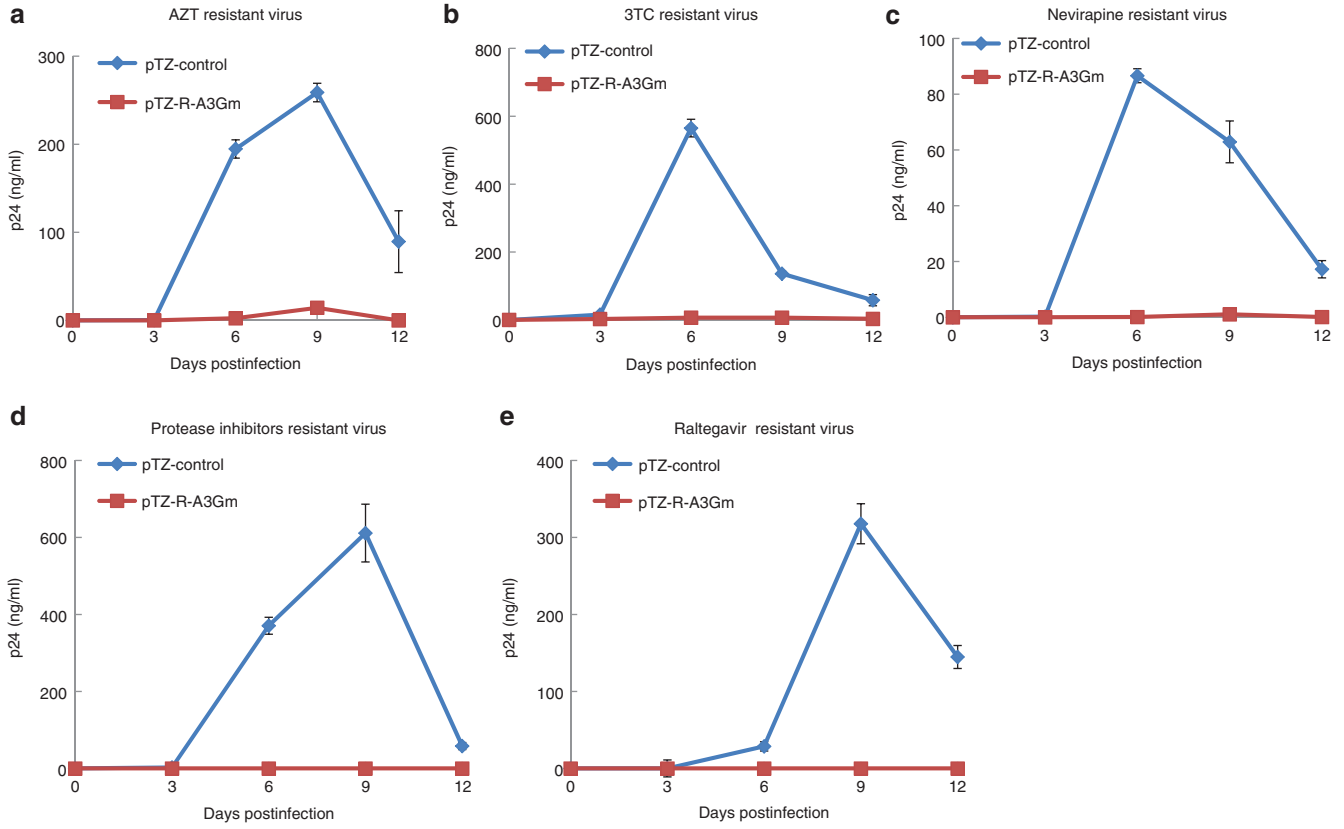

Figure 4 pTZ-R88-A3Gm blocked the replication of drug resistant strains of HIV-1. pTZ-R88-A3Gm or pTZ-control transduced C8166 T cell lines were induced with doxycycline (Dox) (1 µg/ml) and infected with five drug resistant strains (multiplicity of infection of 0.01) including AZT resistant virus (**a**), 3TC resistant virus (**b**), nevirapine resistant virus (**c**), protease inhibitors resistant virus (**d**), and raltegravir resistant virus (**e**). Viral replication was monitored by measuring supernatant Gag-p24 level for 12 days. Protease inhibitors-resistant virus is resistant to the following drugs: MK-639, XM323, A-80987, Ro31-8959, VX-478, and SC-52151. Results were from one representative of two independent experiments. A3G, human cytidine deaminase apolipoprotein B messenger RNA editing enzyme catalytic polypeptide 3; pTZ, pTRIPZ; R88-A3Gm, R88-A3G$_{D128K}$ AZT, zidovudine; 3TC, lamivudine.

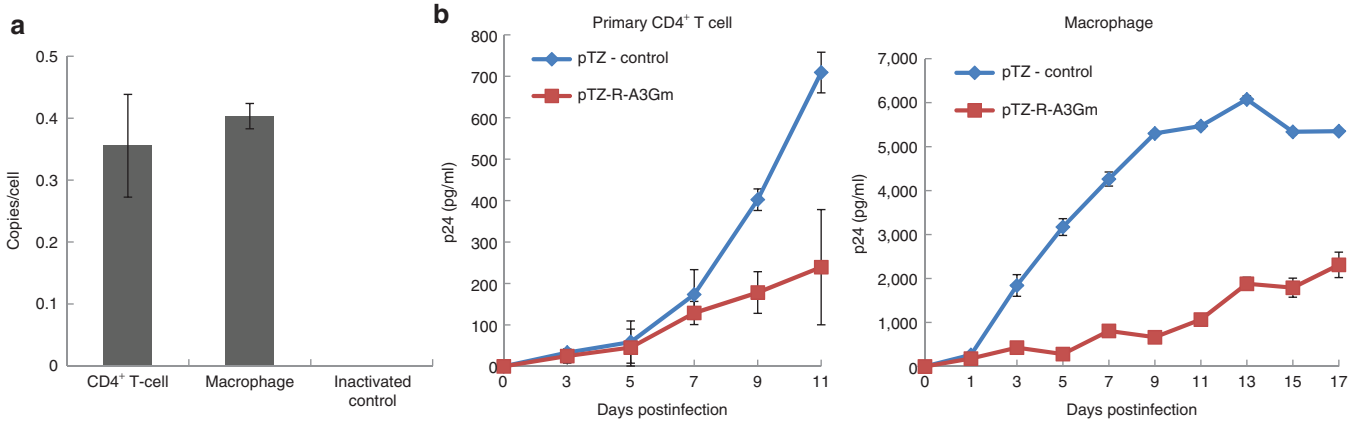

Figure 5 pTZ-R88-A3Gm–transduced primary cells were significantly resistant to HIV-1 replication. (**a**) Primary CD4+ T cells and macrophages were transduced with pTZ-R88-A3Gm or pTZ-control vectors for 3 days. Total DNA was extracted from transduced cells and subjected to real-time polymerase cgain reaction analysis to quantify the level of R88-A3G. (**b**) Transduced CD4+ T cells and macrophages were induced with doxycycline (Dox) (1 µg/ml) and infected with either nevirapine-resistant or pNL4.3-Bal strains of HIV-1. Viral replication was measured by p24 enzyme-linked immunosorbent assay. Result **a** was from one representative of two donors. Result **b** was from one representative of three donors. A3G, human cytidine deaminase apolipoprotein B messenger RNA editing enzyme catalytic polypeptide 3; pTZ, pTRIPZ; R88-A3Gm, R88-A3G$_{D128K}$.

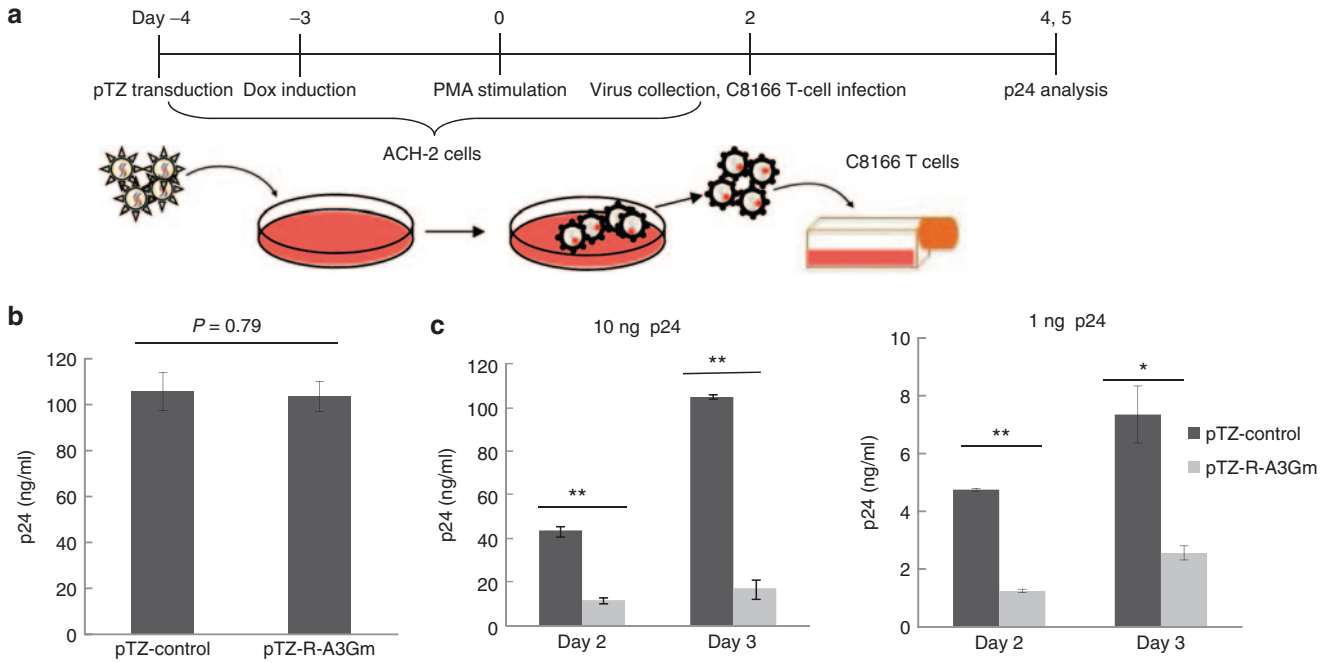

Figure 6 pTZ-R88-A3Gm impaired the infectivity of virus produced from latently infected ACH-2 cells. (**a**) Experimental setup. (**b**) Viral production from pTZ-R88-A3Gm– or pTZ-control–transduced ACH-2 cells was monitored at 48 hours post phorbol-12-myristate-13-acetate (PMA) stimulation by measuring p24 level in cell culture supernatants. (**c**) High volumes (100 µl, equals to 10 ng p24) or low volumes (10 µl, equals to 1 ng p24) of cell free supernatants collected from stimulated pTZ-R88-A3Gm– and pTZ-control–transduced ACH-2 cells were used to infect C8166 T cells. Viral replication was measured at 2 and 3 days postinfection. The values were the averages of triplicates with the indicated standard deviations. Statistical significance was calculated as described in **Figure 2c**. A3G, human cytidine deaminase apolipoprotein B messenger RNA editing enzyme catalytic polypeptide 3; pTZ, pTRIPZ; R88-A3Gm, R88-A3G$_{D128K}$.

collected the virus-containing supernatants from transduced cells at 3 days posttransduction and used them to infect normal C8166 T cells. Similar to progeny viruses from ACH-2 cells, the infectivity of the viruses produced by reactivated R88-A3Gm–transduced CD4$^+$ T cells was reduced by three- to sixfold (**Figure 7b**). The infected CD4$^+$ T cells were transduced with pTZ-R88-A3Gm or pTZ-control vectors prior to stimulation (**Figure 7c**). After stimulation, the viral replication in R88-A3Gm–transduced CD4$^+$ T cells was decreased by over threefold. Moreover, the infectivity of the progeny viruses from these cells was significantly reduced, as induced by C8166 T-cell infection experiments (**Figure 7d**). Taken together, data obtained from primary latency model indicate that R88-A3Gm could provide postinfection protection and inhibit the replication of reactivated virus.

Discussion

In the current study, we have established an anti-HIV gene therapy approach based on R88-tagged human A3G. The strategy to fuse R88 to A3G has several advantages: (i) to increase the copy of A3G incorporated into virus[29] and (ii) to increase the difficulty for the virus to develop resistance. Since once the virus develops resistance against R88, it also can not package viral protein Vpr into virus. Our study showed that the stable expression of R88-A3Gm in highly susceptible C8166 T cells significantly inhibited the replication of both laboratory-adapted and primary HIV-1 isolates that are resistant to several first-line antiviral drugs. Meanwhile, R88-A3Gm is effective in HIV-1 latently infected cells, which renders the progeny viruses from these cells noninfectious. Notably, R88-A3Gm exerted similar anti-HIV effects in human primary cell models.

Given that lentiviral vectors are capable of transducing both dividing and nondividing cells and providing sustained gene expression, in this study, we used a Dox-inducible lentiviral vector to deliver the anti-HIV gene R88-A3Gm. In this vector, the expression of transgenes is under the control of Dox, such that during vector particle production, we could keep the transgene silenced in order to avoid the inhibitory effect of R88-A3Gm following vector particle transduction. Once CD4$^+$ T cells were transduced with pTZ-R88-A3Gm, the transgene can be turned on or off depending on the administration of Dox. Thus, this inducible R88-A3Gm-lentiviral vector has obvious advantages than a previously described R88-A3Gm–expressing adeno-associated virus vector. The potential side effects of the continued expression of R88-A3Gm in transduced cells can be limited or avoided. However, we still detected a trace amount of A3G expression in the vector particle–producing cells in the absence of Dox, which led to a modest inhibitory effect on pTZ-R88-A3Gm vector transduction. This Dox-independent basal expression was also observed in other studies.[30] To further reduce the residual expression of R88-A3Gm, we coexpressed the HIV-1 Vif$_{SEMQ}$ protein in the vector particle–producing cells, which decreased the cellular level of the R88-A3Gm protein by eight times. These regulations make the R88-A3Gm vector capable of transducing target cells as efficiently as the control vector that does not contain R88-A3Gm.

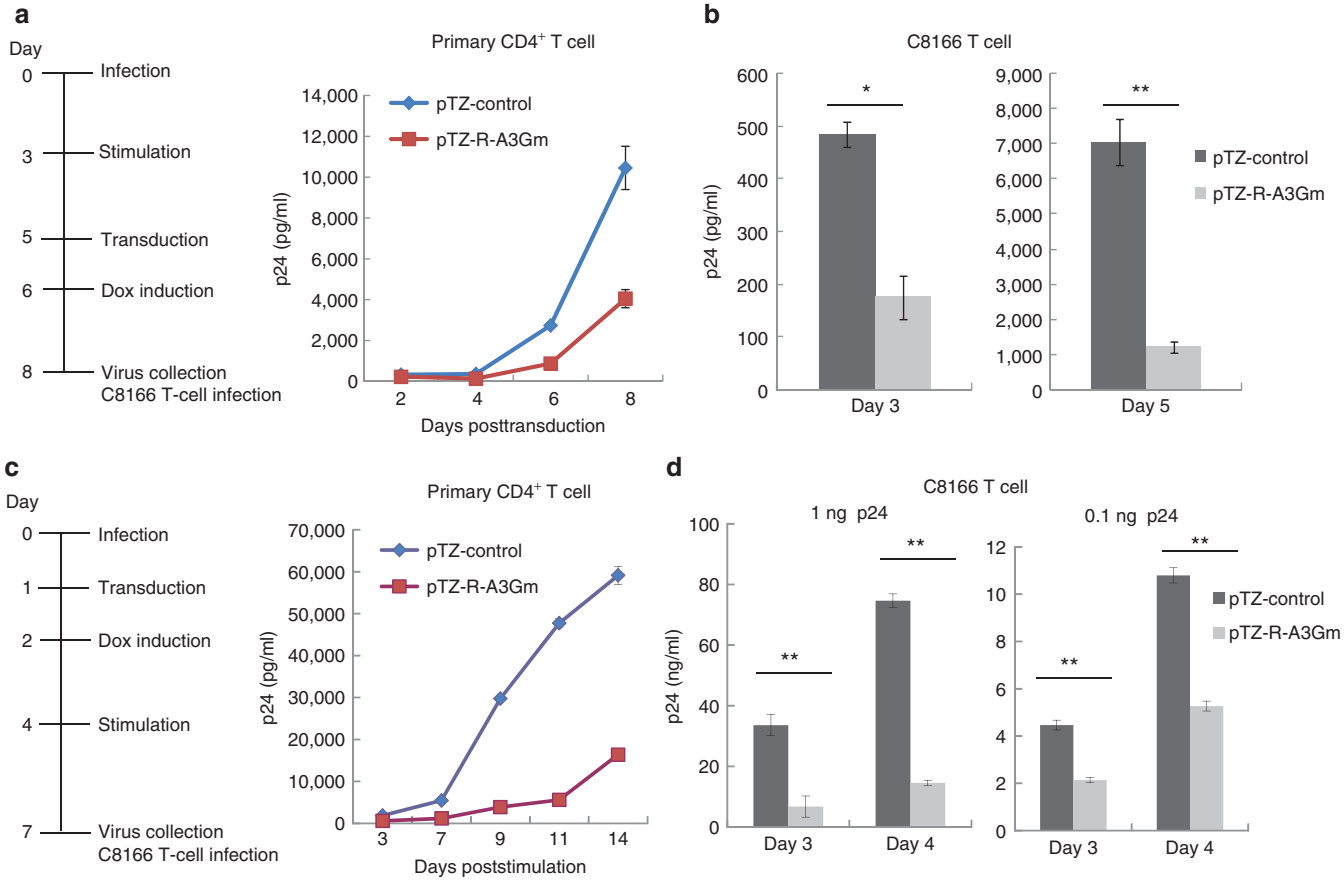

Figure 7 pTZ-R88-A3Gm–mediated inhibition of HIV-1 replication in primary latently infected cells. (**a**) Unstimulated CD4+ T cells were infected with nevirapine-resistant virus (multiplicity of infection of 2.5) by spinoculation (day 0). Three days later, the infected cells were activated by phytohemaglutinin (5 µg/ml) and interleukin-2 (IL-2) (100 U/ml) treatment (day 3), and then transduced with the pTZ-R88-A3Gm or pTZ-control vectors (day 5). Viral replication was monitored by Gag-p24 enzyme-linked immunosorbent assay (ELISA) at different time points after cells were induced with doxycycline (Dox) (1 µg/ml, day 6). (**b**) C8166 T cells were infected with cell free supernatants from pTZ-R88-A3Gm and pTZ-control transduced primary CD4+ T cells collected at day 8. Viral replication in C8166 T cells was measured by Gag-p24 ELISA at 3 and 5 days postinfection. The values were the averages of triplicates with the indicated standard deviations. (**c**) Unstimulated CD4+ T cells were infected with the same viruses as for (**a**) (day 0) and transduced with the pTZ-R88-A3Gm or pTZ-control vectors (day 1). Two days after Dox induction, the infected cells were activated by phytohemaglutinin (5 µg/ml) and interleukin-2 (100 U/ml) treatment (day 4), and viral replication was monitored by Gag-p24 ELISA at different time points. (**d**) C8166 T cells were infected with cell free supernatants from pTZ-R88-A3Gm and pTZ-control transduced primary CD4+ T cells collected at day 7. Viral replication in C8166 T cells was measured as for (**b**). The values were the averages of triplicates with the indicated standard deviations. Statistical significance was calculated as described in **Figure 2c**. Results were from one representative of two donors. A3G, human cytidine deaminase apolipoprotein B messenger RNA editing enzyme catalytic polypeptide 3.

In contrast to the drastic anti-HIV effect of R88-A3Gm in the CD4+ T-cell line, viral replication in R88-A3Gm–transduced CD4+ T lymphocytes and macrophages was not completely inhibited. This varied antiviral effect correlates with the transduction efficiency of R88-A3Gm in the different cell types. Each transduced C8166 T cell contained at least one copy of R88-A3Gm, whereas in transduced primary cells, less than 50% of the cells contained R88-A3Gm as measured by real-time PCR.[31] Therefore, partially impaired viral replication in primary cells is likely due to incomplete transduction with the pTZ-R88-A3Gm vector but not due to insufficient R88-A3Gm protein expression, because a single editing unit of incorporated A3G is capable of inactivating the virus rather than causing sublethal mutations in the viral genome.[32] Completely blocking HIV-1 infection in human primary cells is possible if we can enhance the transduction efficiency in these cells through further modifying the lentiviral vector or enriching the transduced cells by introducing a selectable marker.

The emergence of HIV-1 drug resistance occurs frequently after anti-HIV chemotherapy.[33] Notably, drug-resistant strains of HIV can be transmitted to susceptible individuals, resulting in a suboptimal response to first-line therapy and treatment failure.[34,35] Therefore, an ideal new therapeutic strategy should possess activity against the various existing drug-resistant strains of HIV. In this study, we chose representative viruses resistant to four classes of antiretroviral drugs and showed that R88-A3Gm–transduced CD4+ T cells were resistant to the replication of all of these drug-resistant viruses, highlighting the potential for R88-A3Gm to prevent the reinfection and spread of HIV-1 drug-resistant viruses. Moreover, because R88-A3Gm targets the single-stranded viral DNA but not particular viral proteins, it could be difficult for HIV-1

to develop resistance by introducing point mutations into the viral genome.

Another challenge for an HIV cure is that HIV-1 can establish a stable latent infection in patients, even in those who are under going highly active antiretroviral therapy.[36] To deplete these latent HIV reservoirs, extensive efforts have being made to reactivate the reservoirs with the hope that latently infected cells will be eliminated by the host immune system and/or virus-mediated cell lysis.[36] Although R88-A3Gm itself is not able to eliminate the viral reservoirs, it can combine with latency reactivation compounds to target latent viruses. Here, we showed that R88-A3Gm can be introduced into latently infected ACH-2 cells and primary $CD4^+$ T cells via lentiviral vector. On reactivation, the R88-A3Gm protein can actively incorporate into produced viruses and render the progeny viruses noninfectious. Thus, introducing R88-A3Gm into HIV-1 latently infected cells inhibits the dissemination of the progeny virus, which further prevents viral rebound after latency reactivation. Instead, these viruses would just present as antigenic particles to elicit host immune responses. Therefore, this A3G-based therapeutic approach is capable of targeting not only actively infected cells but also latently infected cells, contributing to the elimination of HIV-1 latent reservoirs.

Overall, this study demonstrates the profound antiviral effect of R88-A3Gm in both acute and chronic HIV-1 infection. If delivered during acute infection, R88-A3Gm could block productive viral replication. Once delivered into latent reservoirs, R88-A3Gm does not preclude HIV reactivation but prevents viral dissemination, which would further postpone the onset of acquired immunodeficiency syndrome (AIDS). More importantly, R88-A3Gm has shown to be effective against drug-resistant viruses that not only productively replicate in active cells but also enter and persist in latent reservoirs.[37,38] In contrast to antiretroviral drugs, our approach has the potential to target the reactivated resistant viruses and prevent viral rebound. This approach will be further optimized by inserting R88-A3Gm into HIV-1–dependent lentiviral vector.[39] In that case, by sharing the promoter with HIV-1, R88-A3Gm can be expressed at the same time as viral particles are produced and further be incorporated into budding viruses, blocking subsequent viral spread. Meanwhile, because R88-A3Gm is expressed only during active viral replication, unpredicted side effect and/or toxicity associated with its overexpression can be avoided. Taken together, our finding encourages further *in vivo* studies to characterize the action of R88-A3Gm in appropriate animal models.

Materials and methods

Plasmids and lentiviral vector production. To construct the plasmid pTZ-R88-A3Gm, R88-A3Gm was amplified from the previously constructed plasmid CMVin-R88-A3Gm[20] with the following primers: 5′-R_{14}-*Age*I: 5′-ATCGCACCGGTCG CCACCATGCCACACAATGAAT-3′, 3′-A3G-*Cla*I: 5′-ATAGATC TATCGATTCAGTTTTCCTGATT-3′. The PCR fragment was digested with *Age*I and *Cla*I and cloned into the pTRIPZ-empty vector (Open Biosystems, Pittsburgh, PA), replacing the original RFP-expressing region between *Age*I and *Cla*I. The ProLabel-$A3G_{wt}$ plasmid was constructed previously.[40] ProLabel-A3Gm was generated using a PCR-based mutagenesis method with the following primers: 5′-$A3G_{D128K}$: 5′-ACTTCTGGAAACCAGATTAC-3′, 3′-$A3G_{D128K}$: 5′-GTAATCTGGTTTCCAGAAGTA-3′. pcDNA-hVif was obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program.[41] pCDNA-Vif_{SEMQ} was generated by PCR-based mutagenesis with the following primers: 5′- Vif_{SEMQ}: 5′-TGTGGCAGGTGA GCGAGATGCAGATTAACACCTG-3′, 3′-Vif_{SEMQ}: 5′-CAGGTG TTAATCTGCATCTCGCTCACCTGCCACA-3′.

Lentiviral vectors containing pTZ-R88-A3Gm or pTZ-control were produced by cotransfecting 293T cells with the corresponding pTZ plasmid, the HIV-packaging plasmid pCMVΔ8.2 and the VSV-G envelope expression plasmid, with or without pcDNA-Vif_{SEMQ}, as described previously.[18] Viral-like particles were concentrated from virus-containing supernatants by ultracentrifugation (35,000 rpm for 1.5 hours at 4 °C). Quantification of vector stocks was determined by Gag-p24 measurement using an HIV-1 p24 ELISA Kit (purchased from the AIDS Vaccine Program of the Frederick Cancer Research and Development Center; Ft. Detrick, MD).

Cell culture and virus. Human embryonic kidney 293T cells and HeLa cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. ACH-2 cells, obtained through the NIH AIDS Research and Reference Reagent Program,[42] and $CD4^+$ C8166 T cells were maintained in RPMI-1640 medium containing 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Peripheral blood mononuclear cells were isolated from the blood of healthy adult volunteers by sedimentation in Ficoll-Hypaque (Sigma-Aldrich St Louis, MO). $CD4^+$ T lymphocytes were isolated from peripheral blood mononuclear cells by negative selection with EasySep Human $CD4^+CD25^+$ T Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada), activated with 3 µg/ml phytohemaglutinin and maintained in RPMI-1640 supplemented with 10 U interleukin-2. Macrophages were generated by culturing peripheral blood mononuclear cells for 7 days in Dulbecco's Modified Eagle's Medium containing 10 ng/ml macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN).

Zidovudine resistant HIV-1,[43] lamivudine resistant HIV-1 ($HIV\text{-}1_{LAI\text{-}M184V}$),[44] nevirapine-resistant HIV-1,[45] protease inhibitor–resistant HIV-1 (L10R/M46I/L63P/V82T/I84V),[46] and raltegavir-resistant HIV-1(1556/W1235426-1)[47] were obtained through the NIH AIDS Research and Reference Reagent Program. pNL4.3-GFP was described previously.[18]

Antibodies and chemicals. Purified rabbit anti-hA3G (catalog no. 10201) and mouse anti-Vif (catalog no. 2221) antibodies were obtained through the NIH AIDS Research and Reference Reagent Program. Mouse anti-α-tubulin is from Sigma. Horseradish peroxidase–conjugated donkey anti-rabbit immunoglobulin G and sheep anti-mouse immunoglobulin G secondary antibodies were purchased from Amersham Biosciences, Piscataway, NJ. Puromycin was obtained from Calbiochem, Darmstadt, Germany. Doxycycline and propidium iodide were obtained from Sigma. ProLabel Detection Kit II was purchased from Clontech, Mountain View, CA.

Real-time PCR analysis. To determine the transduction efficiency of the lentiviral vectors, total DNA from C8166 T cells was isolated at 12 hours posttransduction using a QIAamp blood DNA Mini kit (Qiagen, Valencia, CA). To avoid carry over plasmid DNA contamination, vectors were pretreated with 340 U/ml DNase (Roche, Mannheim, Germany) for 60 minutes at 37 °C. DNA samples were processed for detecting total viral DNA synthesis with an Mx3000P real-time PCR system as described earlier.[48] The following primers were used. R-Fr: 5′-GGCTAACTAGGGAACCCACTGC-3′, U5-Rv: 5′-CTGCTAGAGATTTTCCACACTGAC- 3′. Similarly, to quantitatively determine the levels of R88-A3G DNA in primary $CD4^+$ T cells and macrophages, total DNA was isolated from the transduced cells at 3 days posttransduction. Real-time PCR analysis was performed as previously described.[20] The following primers were used. Vpr-5: 5′-GATACTTGGGCAGGAGTGG-3′ and A3G-3: 5′-CACCTGGCCTCGAAAGAT-3′.

Cell viability assay. Following harvesting, cells were washed and resuspended in 0.5% bovine serum albumin/phosphate-buffered saline (%w/v) at a concentration of 10^6 cells per ml. Propidium iodide was added at a final concentration of 5 µg/ml and incubated for 5 minutes at room temperature in dark. Cell viability was analyzed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) with excitation at 488 nm. Data were analyzed with fetal calf serum Express software (Denovo software, Los Angeles, CA).

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Author contributions. X.Y. designed the study, analyzed the data, and wrote the manuscript; X.W. and Z.A. participated in study design, performed the experiments, and wrote the manuscript; and K.D.J., B.S., and G. K contributed to the designing and discussion of the study and helped to edit the manuscript.

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