effect is attenuated by Vpu [4]. Recently, Neil and colleagues identified the inhibitor, hBST-2 (also called CD317 or HM1.24), in HeLa cells, and referred to this protein as "Tetherin" [5]. They also showed that the inhibitory action of hBST-2 on HIV-1 particle release was antagonized by Vpu, and they concluded that hBST-2 functions by tethering HIV-1 particles to the cell surface [5]. In addition, Van Damme and colleagues demonstrated that Vpu down-regulates hBST-2 from the surface of HeLa cells [6]. On the other hand, Miyagi and colleagues have recently reported that Vpu augments HIV-1 release without down-regulating surface hBST-2 in CEMx174 and H9 cells [7]. Therefore, the relevance of surface hBST-2 down-regulation and the antagonistic action of Vpu on the tethering ability of hBST-2 remain unclear.

We first set out to analyze the level of endogenous hBST-2 expression in a T cell line (MT-4 cells) and compared this level to that found for adherent cell lines (HeLa and HEK293 cells). Although flow cytometry indicated that the level of surface hBST-2 on MT-4 cells was comparable to that expressed on HeLa cells, Western blotting indicated that the total amount of endogenous hBST-2 protein in HeLa cells was much more than the level found in MT-4 cells (Figures 1A–C). These results indicate that endogenous hBST-2 in MT-4 cells is predominantly expressed on the plasma membrane.

To analyze the sensitivity of endogenous hBST-2 on the surface of MT-4 cells to Vpu antagonism, MT-4 cells were infected with either wild-type or Vpu-deficient HIV-1, and the level of surface hBST-2 was subsequently monitored. The amount of released virions in the culture supernatant of wild-type HIV-1-infected cells was significantly higher when compared to that of Vpu-deleted HIV-1-infected cells (Figure 1E), while the percentage of p24-positive cells in wild-type HIV-1-infected culture was similar to that in Vpu-deleted HIV-1-infected culture (Figure 1F). These results suggest that the liberation of Vpu-deficient HIV-1 virions was impaired by endogenous hBST-2 in MT-4 cells. In addition, we clearly found that the surface expression of hBST-2 on wild-type but not Vpu-deleted HIV-1-infected cells (i.e., p24-positive cells) was severely down-regulated (Figures 1D and 1H). Although it has remained ambiguous in the literature whether endogenous hBST-2 on the surface of human T cells is down-regulated by HIV-1 infection [6,7], this is the first demonstration of the significant down-regulation of endogenous hBST-2 in T cells by Vpu which resulted from HIV-1 infection and not from transfection with a Vpu-expressing plasmid [6,8].

Following the rapid down-regulation of surface hBST-2 by infection with wild-type HIV-1, the surface expression of hBST-2 was gradually but significantly replenished along with HIV-1 expansion (Figures 1D and 1H). It is unclear how and why the surface levels of hBST-2 increased; however, our finding indicates that the level of down-regulation of surface hBST-2 on HIV-1-infected T cells would vary depending on the time after infection.

Consistent with previous reports, our findings suggested that hBST-2 has the potential to attenuate HIV-1 release [5,6]. However, how hBST-2 acts against the release of HIV-1 particles remains unclear, and it is not known whether the hBST-2 function involves additional cellular co-factor(s). Since the potential of hBST-2 for the suppression of HIV-1 release has been reported only in primate cell lines [5-7], we hypothesized that hBST-2 may utilize co-factor(s) expressed uniquely in primate cells to tether virions. To investigate the role of hBST-2, we set forward to use various cell lines derived from 9 animal species including human, AGM, dog, cat, rabbit, pig, mink, potoroo, and quail. These cells were transfected with either wild-type or Vpu-deficient HIV-1-producing plasmid (pNL4-3 or pNL43-Udel). The amounts of released virions from HEK293, Vero, Cos-7, D-17, PK-15, RSC, Mv.1.Lu, and QT6 cells were quantified by TZM-bl titration assay [9], while those from CRFK and PtK2 cells were quantified by p24 ELISA because of their lower infectivity [10] (Figure 2). As previously described [4-6,11], HeLa cells were incompetent for the release of Vpu-deficient HIV-1 (Figure 2). In contrast, the other cell lines examined here were able to produce almost comparable amounts of Vpu-deficient HIV-1 when compared to the release of wild-type HIV-1 (Figure 2). These results indicate the absence in these examined cells of intrinsic factors which have the potential to be similar to hBST-2 and can be antagonized by Vpu.

Previous studies have shown that rhTRIM5α, a well-known restriction factor for HIV-1 replication [12,13], is able to efficiently elicit its suppressive ability for HIV-1 replication in feline CRFK cells, but not in canine D-17 cells [14,15]. These results suggest the species-specific ability of rhTRIM5α to suppress HIV-1 replication. To investigate the species-specific tethering ability of hBST-2, we next co-transfected an hBST-2-expressing plasmid (phBST-2) with either pNL4-3 or pNL43-Udel in the above examined cell lines and harvested released virions at 24 hours post-transfection. As shown in Figure 2, exogenous hBST-2 in these cell lines clearly suppressed the release of Vpu-deficient HIV-1 in a dose-dependent manner. This result strongly indicates that hBST-2 can tether released HIV-1 particles without any other unidentified co-factors that are expressed exclusively in primates. It remains conceivable that hBST-2 could employ certain elements ubiquitously expressed in many species for the tethering of released virions. Although it has been controversial whether wild-type HIV-1 release can be suppressed

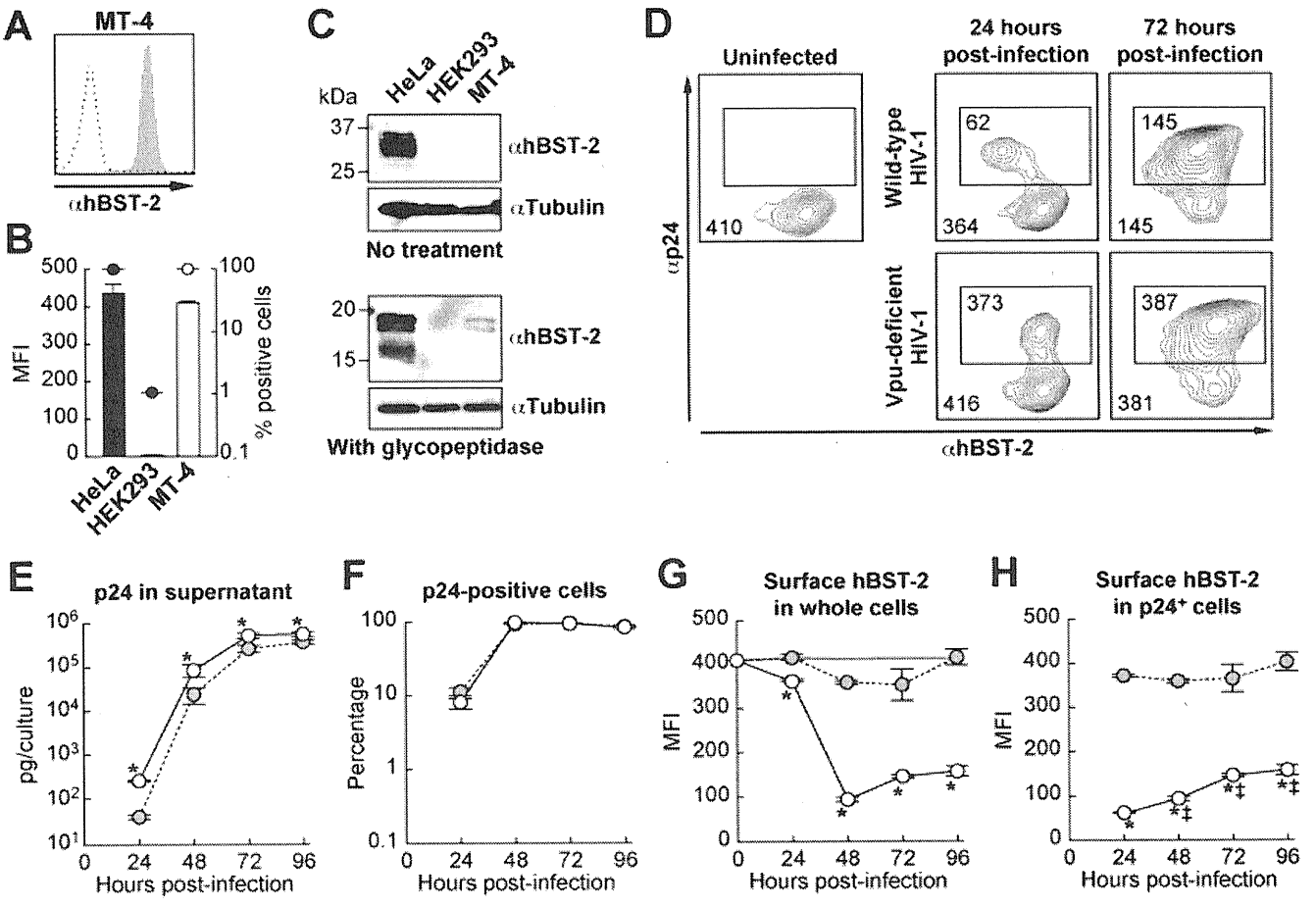

Figure 1

Sequential analysis on the level of endogenous hBST-2 on the surface of HIV-1-infected human T cells. (A and B) MT-4 cells were stained with a mouse anti-hBST-2 antibody, and the surface expression of endogenous hBST-2 (filled in gray) was analyzed by flow cytometry as described in the Materials and Methods. Isotype IgG was used as a negative control (broken line). A representative result (A) and summarized graph (B) are shown. The level of endogenous hBST-2 on the surface of MT-4 cells (opened bar and circle) is compared to that of HeLa and HEK293 cells (filled bars and circles). MFI is represented in bars (Y-axis on left), and the percentage of hBST-2-positive cells is represented in circles (Y-axis on right, log scale). (C) The level of endogenous hBST-2 expression in HeLa, HEK293, and MT-4 cells was analyzed by Western blotting (top panel). For clear detection of hBST-2, the cell lysates were treated with glycopeptidase as described in the Materials and Methods, and the level of deglycosylated hBST-2 was analysed by Western blotting (bottom panel). The input was standardized to Tubulin, and representative results are shown. kDa, kilodalton. (D-H) MT-4 cells were infected with either wild-type or Vpu-deficient HIV-1 (MOI 0.1). Endogenous hBST-2 on the cell surface and intracellular expression of p24 were sequentially analyzed by flow cytometry, and representative profiles are shown (D). The number in the corner of the plot indicates MFI of hBST-2 on the surface of whole cells, and that in the square in the plot indicates MFI of hBST-2 on the surface of p24-postive cells. The amount of p24 in the culture supernatant (E), the percentage of p24-positive cells (F), the level of hBST-2 on the surface of whole cells (G), and the level of hBST-2 on the surface of p24-positive cells (H) following infection with either wild-type (opened circles with line) or Vpu-deficient (filled circles in gray with broken line) HIV-1 were sequentially measured. The amount of p24 in the culture supernatant was quantified by p24 ELISA, and the other data were obtained by flow cytometry as described in the Materials and Methods. Gray line in panel G indicates MFI of surface hBST-2 on mock-infected cells. All experiments were performed in triplicate. Asterisks indicate statistical significance (Student's t test, $P < 0.05$) versus the values of Vpu-deficient HIV-1 at the same time point, and double daggers in panel H indicate statistical significance (Student's t test, $P < 0.05$) versus the values of wild-type HIV-1 at 24 hours post-infection. Error bars indicate standard deviations.

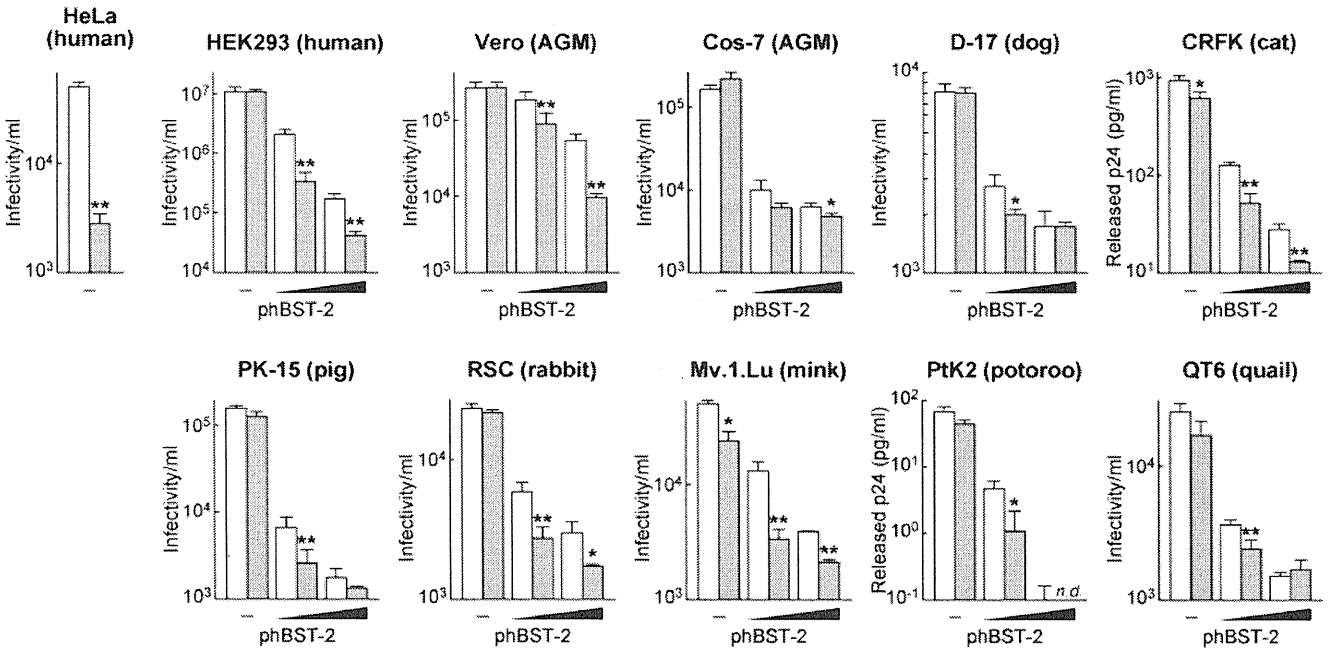

Figure 2

Suppression of HIV-1 release by exogenous hBST-2 in various cell lines. One microgram of pNL4-3 and pNL43-Udel was each co-transfected with (20 or 100 ng) or without (-) phBST-2 into several lines of cells as described in the Materials and Methods. The amount of wild-type (opened bars) or Vpu-deficient HIV-1 virion (bars filled in gray) released from HeLa, HEK293, Vero, Cos-7, D-17, PK-15, RSC, Mv.1.Lu, and QT6 was quantified by using TZM-bl cells, and the amount of HIV-1 released from CRFK and PtK2 cells was quantified by p24 ELISA. All experiments were performed in triplicate. Statistical significance (Student's *t* test) versus wild-type HIV-1 values is represented as follows: *, $P < 0.05$; **, $P < 0.01$. Error bars indicate standard deviations. *n.d.*, not detectable.

by ectopic hBST-2 or not [5,6], we observed here that the release of wild-type HIV-1 was attenuated by hBST-2 and that the efficiency of hBST-2 for the release of wild-type HIV-1 was significantly lower than that for the release of Vpu-deleted HIV-1 (Figure 2).

Ectopically expressed hBST-2 was detected on the surface of all cell lines used in this study (Figure 3A). Unexpectedly, we found the staining with this antibody in native AGM cell lines, Vero and Cos-7 cells (Figure 3A) that increased in intensity when treated with IFN-α (data not shown). It is known that hBST-2 expression is induced upon IFN-α treatment in HEK293 cells [5,6]. Therefore, the antibody-specific staining and its increased signal intensity that we observed in the AGM cells could be due to the cross-reactivity of the anti-BST-2 antibody with endogenous AGM BST-2.

As previously reported [6], we also found that endogenous hBST-2 on HeLa cells was significantly down-regulated by transfection with pNL4-3, but not with pNL43-Udel (Figure 3B). In contrast, at 24 hours post-transfection, the down-regulation of exogenous hBST-2 on the surface of the other cell lines was hardly observed except for Vero cells (Figure 3B). However, after 48 hours post-transfection, we could detect significant down-regulation of ectopically expressed hBST-2 on the surface of cells co-transfected with either pNL4-3 or a Vpu-expressing plasmid [8] (data not shown). These results suggest that the level of Vpu expression at 24 hours post-transfection is sufficient to antagonize the tethering ability of hBST-2, while not down-regulating surface hBST-2. In support of our data, a recent report showed that Vpu enhances HIV-1 release in the absence of surface down-regulation of hBST-2 [7]. Taken together, these results indicate that the down-regulation of surface hBST-2 may be dispensable for the antagonism of tethering ability of hBST-2 by Vpu.

We further assessed the results obtained from all the examined cell lines and focused on the correlation between the efficiency of particle release and the level of surface hBST-2 in these cells. All of the examined cell lines except for Vero cells showed significant suppression of virus release by exogenously expressed hBST-2 (Figure 4). In addition, a direct correlation between the suppression efficiency for virus release by hBST-2 and the level of surface hBST-2 was found in these cells with high correlation coefficients (Figure 4) and statistical significance ($P <$

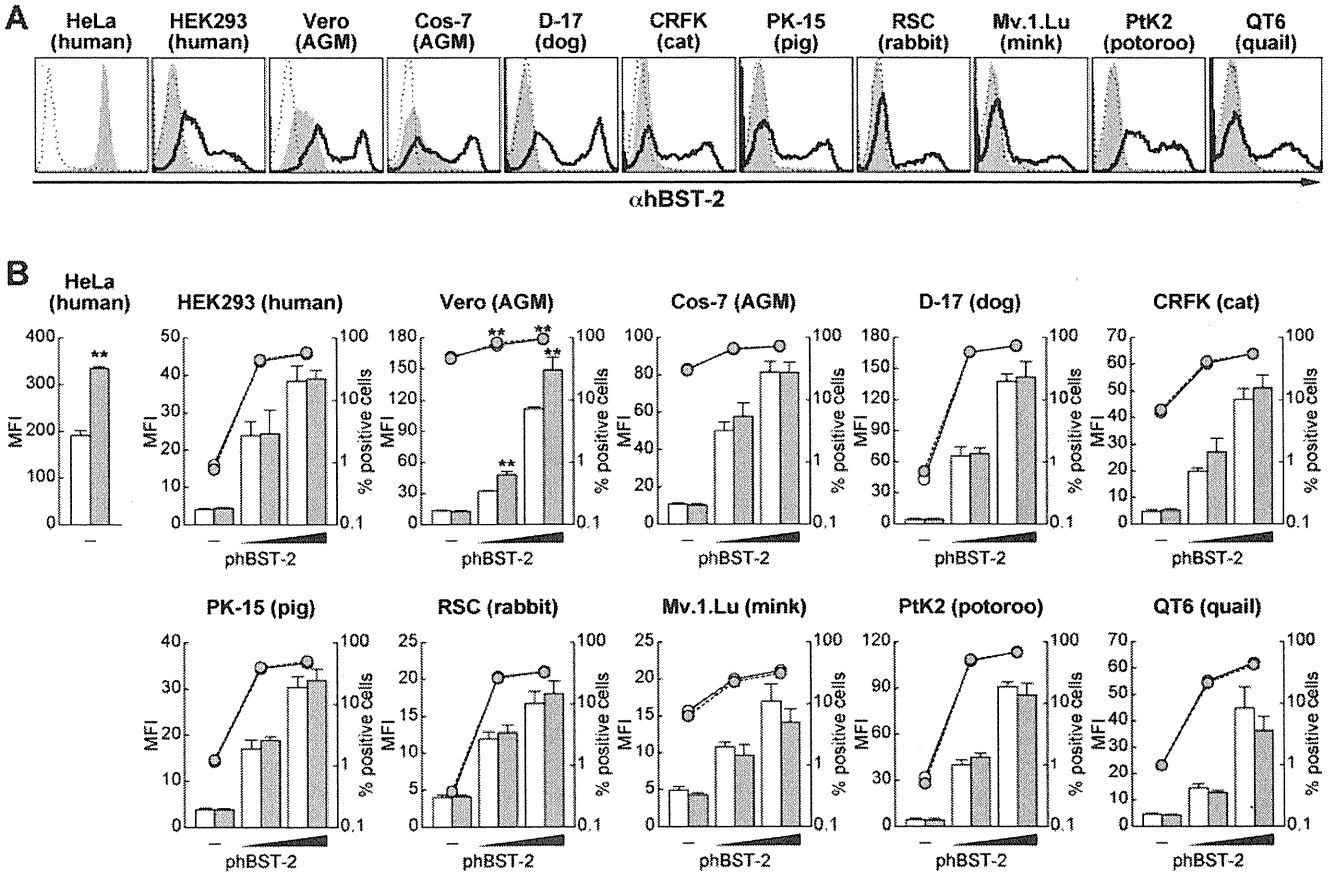

Figure 3

Surface expression of exogenous hBST-2 in various cell lines. (A) HEK293, Vero, Cos-7, D-17, CRFK, PK-15, RSC, Mv.1.Lu, PtK2, and QT6 cells were transiently transfected with 100 ng of phBST-2. phBST-2-transfected cells (black line) and mock-transfected cells (filled in gray) as well as HeLa cells (filled in gray) were stained with a mouse anti-hBST-2 monoclonal antibody, and the surface expression of hBST-2 was analyzed by flow cytometry as described in the Materials and Methods. Isotype IgG was used as a negative control (broken line). A representative result is shown. (B) One microgram of pNL4-3 and pNL43-Udel was each co-transfected with (20 or 100 ng) or without (-) phBST-2 into several lines of cells as described in Figure 2. The surface expression of hBST-2 on pNL4-3-co-transfected (opened bars and circles) and pNL43-Udel-co-transfected (gray bars and circles) cells was analyzed by flow cytometry. MFI is represented in bars (Y-axis on left), and the percentage of hBST-2-positive cells is represented in circles (Y-axis on right, log scale). All experiments were performed in triplicate. Statistical significance (Student's *t* test) versus wild-type HIV-1 values is represented as follows: *, $P < 0.05$; **, $P < 0.01$. Error bars indicate standard deviations.

0.01). On the other hand, the suppression efficiency for virus release by hBST-2 in Vero cells was relatively milder than in the other 9 cell lines even though Vero cells exhibited the highest levels of hBST-2 cell surface expression (Figure 4). Moreover, the result from Vero cells displayed a statistically different pattern than in the other cells (Figure 4, $P < 0.01$ by repeated measure ANOVA). These findings suggest that ectopic hBST-2 is unable to efficiently exert its inhibitory effect on virus release in Vero cells. One plausible explanation for this anomaly may be attributed to a defective IFN-α response. Although a previous study showed that the release of Vpu-deficient HIV-1 was suppressed upon IFN-α treatment [11], Vero cells are known to be genetically deficient in type I IFN genes, including IFN-α [16,17]. Therefore, it is conceivable that a signal cascade mediated by IFN-α may be needed to assist the tethering action of ectopic hBST-2, but that this cascade may not be operative in Vero cells because of its defects in type I IFN genes. Further studies in Vero cells will be needed to shed light on the unexplained aspects of the mechanism of suppression of virus release mediated by hBST-2.

It has recently been reported that hBST-2 has the potential to suppress the release of not only HIV-1 but also other retroviruses [18], Ebola virus [18], Lassa virus [19], and

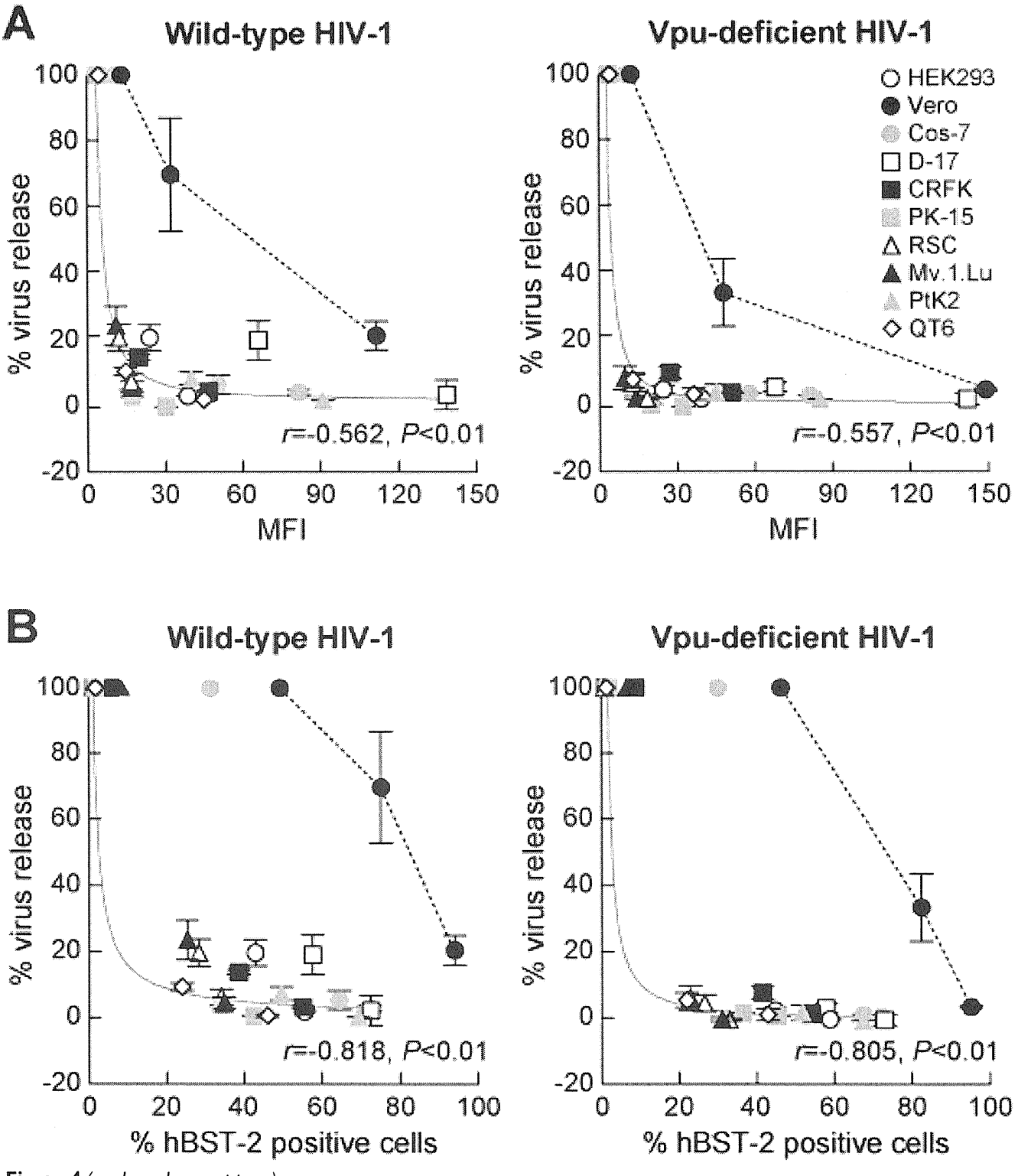

Figure 4 *(see legend on next page)*

Figure 4 *(see previous page)*
Comparison of the level of exogenous hBST-2 on plasma membrane with its inhibition efficiency for HIV-1 release in various cell lines. (A and B) The results shown in Figures 2 and 3 were summarized and rearranged as follows: the level of surface expression of hBST-2 is shown in MFI (A) and the percentage of surface hBST-2 positive cells (B) in the X-axis. To calculate % virus release (Y-axis), the infectivity of the culture supernatant of phBST-2-untransfected cells (for HEK293, Vero, Cos-7, D-17, RSC, Mv.1.Lu, and QT6 cells) or the amount of p24 in the culture supernatant of phBST-2-untransfected cells (for CRFK and PtK2) was defined as 100%. Statistical significance of the correlation between the level of surface hBST-2 (X-axis, shown in MFI or % positive cells) and % virus release (Y-axis) in the results from the 9 analyzed cells (HEK293, Cos-7, D-17, CRFK, PK-15, RSC, Mv.1.Lu, PtK2, and QT6 cells) was determined by Pearson's correlation test, and $P < 0.01$ was considered significant. Approximation curve of the result from the 9 analyzed cells is drawn in gray lines, and a representative result from Vero cells is drawn in broken line. *r*, Pearson's correlation coefficient.

Marburg virus [18,19]. Therefore, further studies on the mechanism of BST-2 function will provide beneficial information leading to novel therapeutic strategies against several virus-induced diseases including AIDS.

Methods

Cell culture

HEK293 cells (human kidney), Vero cells (AGM kidney), Cos-7 cells (AGM kidney), rabbit skin cells (RSC, kindly provided by Dr. B. Roizman), and TZM-bl cells (obtained from AIDS reagent program, National Institute of Health) were maintained in low-glucose DMEM (Nikken) containing 10% FCS and antibiotics. D-17 cells (canine osteosarcoma), CRFK cells (feline kidney), PK-15 cells (porcine kidney), Mv.1.Lu cells (*Mustela vison*, mink lung), and QT6 cells (*Coturnix coturnix japonica*, quail fibrosarcoma) were maintained in high-glucose DMEM (Sigma) containing 10% FCS, 2 mM GlutaMax (Invitrogen), and antibiotics. PtK2 cells (potoroo kidney) were maintained in Eagle's minimum essential medium (Sigma) supplemented with 1 mM sodium pyruvate, 2 mM GlutaMax, 10% FCS and antibiotics. MT-4 cells were maintained in RPMI1640 (Nikken) containing 10% FCS and antibiotics. Mv.1.Lu cells and QT6 cells were kindly donated by Dr. A. Koito.

Plasmid construction

To construct phBST-2, a *bst-2* cDNA (GenBank: NM_004335, bases 10-552) was amplified by polymerase chain reaction from a human leukocyte cDNA library (Invitrogen), and the resulting fragment was inserted into peGFP-C1 (Clontech). Sequence of the construct was confirmed with an ABI 3130xl genetic analyzer (Applied Biosystems).

Transfection and virus preparation

Cells were seeded in 6-well plate to appropriate densities 1-day prior to transfection and were transfected by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacture's protocol. Briefly, 1 μg of pNL4-3 [20] or pNL43-Udel (kindly donated by Dr. K. Strebel) [1] was cotransfected with 20 or 100 ng of phBST-2. The amount of plasmid DNA for transfection was normalized to 2 μg per well. Four hour after transfection, culture medium was replaced freshly. The culture supernatant was harvested, centrifuged, and then filtrated with 0.45-μm filter (Millipore) to produce virus solutions at 24 hours post-transfection. All experiments were performed in triplicate. To prepare wild-type or Vpu-deficient HIV-1 for its infection assay, pNL4-3 or pNL43-Udel was transfected into HEK293 cells by the calcium phosphate method as previously described [21]. The prepared viruses were titrated by using peripheral blood mononuclear cells, and the $TCID_{50}$ was calculated as previously described [22].

TZM-bl assay

Quantification of the amount of released HIV-1 virion was performed by using TZM-bl cells as previously described [5]. Briefly, appropriate virus solution was inoculated into 1×10^5 TZM-bl cells per 12-well plate. The cells were harvested at 48 hours post-infection, and β-galactosidase assay was performed by using Galacto-Star Mammalian Reporter Gene Assay System (Applied Biosystems) according to the manufacture's procedure. Activity was measured with a 1420 ALBOSX multilabel counter (Perkin Elmer).

p24 ELISA

The amount of HIV-1 virion released from CRFK, PtK2, and MT-4 cells was quantified by using HIV-1 p24 ELISA kit (ZeptoMetrix) according to the manufacture's instructions.

Flow cytometry

Flow cytometry was performed as previously described [21]. A mouse anti-hBST-2 monoclonal antibody (donated by Chugai Pharmaceutical Co., Japan) [6,23] and a Cy5-conjugated donkey anti-mouse IgG antisera (Chemicon) were used. For costaining of cell surface hBST-2 and intracellular p24, the anti-hBST-2 monoclonal antibody was pre-labelled with Zenon Alexa Fluor 647 mouse IgG2a labelling kit (Invitrogen) according to the manufacture's protocol. Cell surface hBST-2 was stained with the pre-labelled anti-hBST-2 antibody, and

the cells were permeabiliezed and fixed with BD Cytoperm/Cytofix solution (BD Pharmingen). Then, intracellular p24 was stained with a FITC-conjugated anti-HIV-1 p24 antibody (clone 2C2, kindly provided by Dr. Y. Tanaka) [24].

Western blotting

Western blotting was performed as previously described [21] with some modification. Briefly, the cells were lysed with lysis buffer (1% NP-40, 50 mM Tris-HCl [pH7.5], 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, and 1 mM PMSF). The lysates were separated by SDS-PAGE and transferred to Immobilon transfer membrane (Millipore). For detection, the mouse anti-hBST-2 monoclonal antibody, a mouse anti-Tubulin monoclonal antibody (clone DM1A; Sigma), and an HRP-conjugated horse anti-mouse IgG antibody (Cell Signalling) were used. It has been reported that hBST-2 is a highly glycosylated protein [25]. To remove the sugar chains in hBST-2 protein and detect hBST-2 more clearly, the lysates were treated with glycopeptidase F (TaKaRa) according to the manufacture's procedure.

Statistical analyses

Student's *t* test was used to determine statistical significance, and $P < 0.05$ and $P < 0.01$ were considered significant. The Pearson correlation coefficient was applied to determine statistical significance for the correlation between the suppression efficiency for particle release by hBST-2 and the level of surface hBST-2 in the 9 kinds of cells lines (Figure 4), and $P < 0.01$ was considered significant. Repeated measure ANOVA was applied to determine statistical significance between Vero cells and the other cell lines (Figure 4), and $P < 0.01$ was considered significant.

Abbreviations

h: human; BST-2: bone marrow stromal cell antigen-2; HIV-1: human immunodeficiency virus type 1; Vpu: viral protein U; AGM: African green monkey; ELISA: enzyme-linked immunosorbent assay; rhTRIM5α: rhesus macaque tripartite motif-containing 5 isoform α; phBST-2: hBST-2-expressing plasmid; IFN: interferon; AIDS: acquired immunodeficiency syndrome; DMEM: Dulbecco's modified Eagle medium; FCS: fatal calf serum; $TCID_{50}$: 50% tissue culture infectious dose; FITC: fluorescein isothiocyanate; EDTA: ethylenediaminetetraacetic acid; PMSF: phenylmethylsulfonyl fluoride; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HRP: horseradish peroxidase; MOI: multiplicity of infection; MFI: mean fluorescence intensity.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KS and YK designed the research; KS, SPY, NM, TM, and TY prepared the materials; KS, SPY, and NM performed the experiments and analyzed the obtained data; KS and SPY prepared the figures; KS, TM, and YK wrote the manuscript.

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References

1. Klimkait T, Strebel K, Hoggan MD, Martin MA, Orenstein JM: **The human immunodeficiency virus type 1-specific protein vpu is required for efficient virus maturation and release.** *J Virol* 1990, **64**:621-629.
2. Nomaguchi M, Fujita M, Adachi A: **Role of HIV-1 Vpu protein for virus spread and pathogenesis.** *Microbes Infect* 2008, **10**:960-967.
3. Strebel K, Klimkait T, Martin MA: **A novel gene of HIV-1, vpu, and its 16-kilodalton product.** *Science* 1988, **241**:1221-1223.
4. Varthakavi V, Smith RM, Bour SP, Strebel K, Spearman P: **Viral protein U counteracts a human host cell restriction that inhibits HIV-1 particle production.** *Proc Natl Acad Sci USA* 2003, **100**:15154-15159.
5. Neil SJ, Zang T, Bieniasz PD: **Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu.** *Nature* 2008, **451**:425-430.
6. Van Damme N, Goff D, Katsura C, Jorgenson RL, Mitchell R, Johnson MC, Stephens EB, Guatelli J: **The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein.** *Cell Host Microbe* 2008, **3**:245-252.
7. Miyagi E, Andrew AJ, Kao S, Strebel K: **Vpu enhances HIV-1 virus release in the absence of Bst-2 cell surface down-modulation and intracellular depletion.** *Proc Natl Acad Sci USA* 2009, **106**:2868-2873.
8. Nguyen KL, Ilano M, Akari H, Miyagi E, Poeschla EM, Strebel K, Bour S: **Codon optimization of the HIV-1 vpu and vif genes stabilizes their mRNA and allows for highly efficient Rev-independent expression.** *Virology* 2004, **319**:163-175.
9. Koito A, Shigekane H, Matsushita S: **Ability of small animal cells to support the postintegration phase of human immunodeficiency virus type-1 replication.** *Virology* 2003, **305**:181-191.
10. Munk C, Zielonka J, Constabel H, Kloke BP, Rengstl B, Battenberg M, Bonci F, Pistello M, Lochelt M, Cichutek K: **Multiple restrictions of human immunodeficiency virus type 1 in feline cells.** *J Virol* 2007, **81**:7048-7060.
11. Neil SJ, Sandrin V, Sundquist WI, Bieniasz PD: **An interferon-α-induced tethering mechanism inhibits HIV-1 and Ebola virus particle release but is counteracted by the HIV-1 Vpu protein.** *Cell Host Microbe* 2007, **2**:193-203.
12. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J: **The cytoplasmic body component TRIM5α restricts HIV-1 infection in Old World monkeys.** *Nature* 2004, **427**:848-853.

13. Sakuma R, Noser JA, Ohmine S, Ikeda Y: **Rhesus monkey TRIM5α restricts HIV-1 production through rapid degradation of viral Gag polyproteins.** *Nat Med* 2007, **13**:631-635.
14. Saenz DT, Teo W, Olsen JC, Poeschla EM: **Restriction of feline immunodeficiency virus by Ref1, Lv1, and primate TRIM5α proteins.** *J Virol* 2005, **79**:15175-15188.
15. Berube J, Bouchard A, Berthoux L: **Both TRIM5α and TRIMCyp have only weak antiviral activity in canine D17 cells.** *Retrovirology* 2007, **4**:68.
16. Emeny JM, Morgan MJ: **Regulation of the interferon system: evidence that Vero cells have a genetic defect in interferon production.** *J Gen Virol* 1979, **43**:247-252.
17. Mosca JD, Pitha PM: **Transcriptional and posttranscriptional regulation of exogenous human beta interferon gene in simian cells defective in interferon synthesis.** *Mol Cell Biol* 1986, **6**:2279-2283.
18. Jouvenet N, Neil SJ, Zhadina M, Zang T, Kratovac Z, Lee Y, McNatt M, Hatziioannou T, Bieniasz PD: **Broad-spectrum inhibition of retroviral and filoviral particle release by tetherin.** *J Virol* 2009, **83**:1837-1844.
19. Sakuma T, Noda T, Urata S, Kawaoka Y, Yasuda J: **Inhibition of Lassa and Marburg virus production by tetherin.** *J Virol* 2009, **83**:2382-2385.
20. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA: **Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone.** *J Virol* 1986, **59**:284-291.
21. Sato K, Aoki J, Misawa N, Daikoku E, Sano K, Tanaka Y, Koyanagi Y: **Modulation of human immunodeficiency virus type 1 infectivity through incorporation of tetraspanin proteins.** *J Virol* 2008, **82**:1021-1033.
22. Koyanagi Y, Tanaka Y, Kira J, Ito M, Hioki K, Misawa N, Kawano Y, Yamasaki K, Tanaka R, Suzuki Y, *et al.*: **Primary human immunodeficiency virus type 1 viremia and central nervous system invasion in a novel hu-PBL-immunodeficient mouse strain.** *J Virol* 1997, **71**:2417-2424.
23. Ohtomo T, Sugamata Y, Ozaki Y, Ono K, Yoshimura Y, Kawai S, Koishihara Y, Ozaki S, Kosaka M, Hirano T, Tsuchiya M: **Molecular cloning and characterization of a surface antigen preferentially overexpressed on multiple myeloma cells.** *Biochem Biophys Res Commun* 1999, **258**:583-591.
24. Okuma K, Tanaka R, Ogura T, Ito M, Kumakura S, Yanaka M, Nishizawa M, Sugiura W, Yamamoto N, Tanaka Y: **Interleukin-4-transgenic hu-PBL-SCID mice: a model for the screening of antiviral drugs and immunotherapeutic agents against X4 HIV-1 viruses.** *J Infect Dis* 2008, **197**:134-141.
25. Rollason R, Korolchuk V, Hamilton C, Schu P, Banting G: **Clathrin-mediated endocytosis of a lipid-raft-associated protein is mediated through a dual tyrosine motif.** *J Cell Sci* 2007, **120**:3850-3858.

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Efficient transduction of cytotoxic and anti-HIV-1 genes by a gene-regulatable lentiviral vector

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Abstract Lentiviral vectors modified from human immunodeficiency virus type 1 (HIV-1) offer a promising approach for gene therapy, facilitating transduction of genes into non-dividing cells both in vitro and in vivo. When transducing cytotoxic or anti-HIV genes, however, the vector must avoid self-inhibition by the transgene that can lead to a disruption in production of infectious virions. In this study, we constructed two HIV-1-based lentiviral vectors harboring the mifepristone-inducible gene expression unit in either the forward or the reverse orientation with respect to the direction of viral genomic RNA. The ability of these vectors to transduce cytotoxic and anti-HIV genes was evaluated. When human CD14 was used as a transgene, infectious lentiviral vectors were produced by both forward and reverse vector systems. CD14 expression was efficiently induced in cells transduced by both lentiviral vectors following treatment with mifepristone. However, a higher level of basal transgene expression was observed in the forward vector system in the absence of mifepristone. In contrast, high titers of infectious lentiviral vector containing the cytotoxic *vesicular stomatitis virus M* gene were successfully generated using the reverse vector, but not the forward vector. In addition, when a VPS4B-dominant negative mutant against HIV-1 budding was cloned into the reverse vector, significant amounts of lentiviral vector were obtained. Subsequent transduction of cells with the VPS4B mutant resulted in approximately 50% inhibition of HIV-1 production only in the presence of mifepristone. Our study thus demonstrates that incorporation of a mifepristone-regulatable gene expression unit in the reverse orientation makes significant advances toward development of a lentiviral vector that allows transduction of harmful genes.

Keywords Lentiviral vector · Mifepristone-regulatable system · VSV M · VPS4B

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Introduction

A variety of gene-transfer vectors based on RNA and DNA viruses have been developed to deliver foreign genes to target cells in vitro and in vivo [1]. Retroviral vectors derived from gammaretroviruses and lentiviruses have the potential advantage of sustained expression of transgenes in transduced cells, because of their ability to stably integrate viral DNA into the host genome. While gammaretroviruses require cell division to establish infections, lentiviruses including human immunodeficiency virus type 1 (HIV-1) are capable of infecting both dividing and non-dividing cells [1]. Lentivirus-based vector systems thus potentiate long-term gene expression in non-dividing cells such as neurons and hematopoietic stem cells [2, 3].

Lentiviral vectors hold great promise for a gene therapy approach to inherited and acquired diseases such as cancer and acquired immunodeficiency syndrome (AIDS).

A potential application of lentiviral vectors would be the transduction of a gene cytotoxic to tumor cells or virus-infected cells resulting in the eradication of these unwanted cells from the body. For the treatment of AIDS, an alternative approach would be to deliver an anti-HIV gene to a population of cells rendering them resistant to HIV infection. However, insertion of toxic or anti-HIV genes into an HIV-based lentiviral vector can create problems for production of the vector itself. Expression of anti-HIV transgenes in vector packaging cells can interfere with production of lentivirus particles, blocking the ability to make lentiviral vector [4]. One strategy to solve this problem is the use of a regulatable system in which the target transgene is kept silent during vector production and expression is subsequently switched on following transduction in the context of a lentiviral vector.

The first generation of regulatable gene expression systems was based on naturally occurring inducible promoters [5]. However, this type of system had limitations due to high levels of "leaky" or basal expression driven by such promoters, modest induction of transgene expression, and pleiotropic activity of the inducer. For these reasons, the last two decades have seen development of chimeric regulatable systems engineered from a number of prokaryotic, eukaryotic, and viral elements designed to enhance specificity and activity of transgene expression [6]. Amongst the reported chimeric regulators is one based on a mutated human progesterone receptor which is unable to bind endogenous hormone but is activated by binding the progesterone antagonist, mifepristone (RU-486) [7, 8]. The chimeric transactivator (regulator) protein of this so-called GeneSwitch system comprises the GAL4 DNA-binding domain from *Saccharomyces cerevisiae* fused to the ligand-binding domain of a mutant progesterone receptor and the activation domain of the p65 subunit of human NF-κB [8]. In the presence of mifepristone, this transactivator binds to GAL4 activation sequences upstream of the inducible transgene, stimulating transcription of the target gene by more than 200-fold in cultured cells [9]. An advantage of the GeneSwitch system is that the majority of its components are modified human proteins having no impact on cell viability. In addition, although mifepristone has anti-progesterone and -glucocorticoid activities, the concentration needed for ligand-inducible transactivation of the target gene (10^{-8} to 10^{-11} M) is much lower than the concentration producing an anti-progesterone effect in humans [10]. Furthermore, use of a mifepristone-inducible (autoinducible) promoter to regulate expression of the chimeric transactivator dramatically reduced basal expression of the transgene in the absence of the inducer, thereby improving the dynamic range of in vivo transgene regulation [9].

Many types of regulatable gene expression systems have been incorporated into lentiviral vectors [11–21]. The most commonly used inducible system is based on the bacterial tetracycline-responsible gene expression system (Tet system) [22]. While representing an important tool for controlling target gene expression, Tet-regulatable systems in the context of lentiviral vectors have shown high basal levels of transgene expression without induction [22]. Such leakiness would be undesirable especially in the production of lentiviral vectors aimed at transducing toxic proteins into target cells.

To generate a viral vector in which transgene expression was tightly controlled, we combined an HIV-1-based lentiviral vector with the GeneSwitch system described above. The mifepristone-inducible lentiviral vector reported here minimized the interference of transgene expression during virus production and permitted efficient transduction of cytotoxic and anti-HIV genes into target cells.

Materials and methods

Cells

GeneSwitch-293 cells (a HEK293-derived human cell line expressing the GeneSwitch protein) were purchased from Invitrogen. GeneSwitch-293 and human 293T cells were maintained in Dulbecco's modified Eagle medium (D-MEM) containing 10% fetal calf serum (FCS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (D-MEM/10% FCS).

Construction of lentiviral vector plasmids

The mifepristone-inducible promoter sequence, GAL4/TATA, was amplified by polymerase chain reaction (PCR) from the vector pGene/V5-His5C (Invitrogen) and subcloned into LITMUS28 (New England Biolabs) to create pLITMUS28-GAL4/TATA. A GAL4/TATA fragment was then inserted into a Gateway-compatible lentiviral vector plasmid pYK005C [23] producing the plasmid for the forward vector (fragment positioned in the forward orientation).

To generate a plasmid for the reverse vector, a fragment containing the Gateway cloning cassette, internal ribosome entry site (IRES), and humanized *Renilla* green fluorescent protein (hrGFP) sequences together with a fragment containing bovine growth factor hormone poly(A) (BGH pA) sequence were isolated from pYK005C and pSwitch (Invitrogen), respectively. These were subcloned into LITMUS28. Then, the GAL4/TATA sequence derived from pLITMUS28-GAL4/TATA was inserted into the subcloning plasmid. To prepare a vector backbone, the internal human

elongation factor 1 α subunit promoter of a self-inactivating (SIN) lentiviral vector plasmid, CSII-EF-MCS [24], was replaced with a fragment containing GAL4/TATA, Gateway-cassette, IRES, hrGFP, and BGH pA sequences, thus generating the final plasmid.

Preparation of all Gateway plasmids containing the *ccdB* gene was carried out using *Escherichia Coli* (*E. Coli*) strain DB3.1.

Cloning of transgenes

All transgenes were cloned into the lentiviral vector plasmid via the Gateway cloning system [23]. Entry plasmid clones encoding transgenes were constructed as follows. Human CD14 was amplified by PCR using primers containing the *att*B1 tail (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′) at the 5′-end of the forward primer and the *att*B2 tail (5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-3′) at the 5′-end of the reverse primer [23]. PCR products were subcloned into the entry plasmid, pDONR201 (Invitrogen), using a Gateway BP reaction [23]. Vesicular stomatitis virus matrix protein (VSV M) cDNA fused with a FLAG epitope tag sequence (FLAG-VSV-M) was generated from a VSV M-expressing plasmid, pEGFPN3-M [25]. This involved use of a forward primer containing the *att*B1 tail and subsequent FLAG tag sequence (5′-ATGGATTACAAGGATGACGACGATAAG-3′) and a reverse primer containing the *att*B2 tail. The PCR fragment encoding FLAG-VSV-M was subcloned into entry plasmid, pDONR221 (Invitrogen), by Gateway BP reaction. Fragments encoding the VPS4B K180Q mutant (VPS4B-KQ) or firefly luciferase (Luc) were amplified by PCR from VPS4B K180Q-expressing plasmid [26] and pGL3-Basic (Promega), respectively. These fragments were inserted into *Eco*RI-*Mlu*I sites downstream of three FLAG epitope tags in pCMV-SPORT6-3xFLAG, a Gateway-compatible pCMV-SPORT6 (Invitrogen)-derived entry plasmid. After sequence confirmation, individual transgenes in entry plasmids were transferred to lentiviral vector plasmids by Gateway LR reaction.

Lentiviral vector production

293T cells were seeded to appropriate densities 20 h prior to transfection. Infectious lentiviral vectors pseudotyped with VSV G protein were produced by lentiviral vector plasmid, VSV G- and HIV-1 Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev), and HIV-1 Gag-Pol-expressing plasmid (pCAG-HIVgp) via calcium phosphate-mediated transfection, as described previously [27]. Conditioned medium was harvested 48-h post-transfection and concentrated 40-fold by ultracentrifugation at 4°C at 100,000×*g* for 90 min.

For titration of the lentiviral vectors, GeneSwitch-293 cells were infected with serial dilutions of vector stocks supplemented with 10 nM mifepristone (Invitrogen) 24-h post-infection. Vector titers (transduction units: TU) were determined 48 h after induction by quantitative flow cytometric analysis for hrGFP positive cells.

In the following text, the use of "p" and "v" to prefix nomenclature denotes vector plasmid and infectious lentiviral vector, respectively.

Transduction and induction of transgenes

GeneSwitch-293 cells were seeded in 6-well plates at a density of 1×10^5 cells/well with D-MEM/10% FCS 20 h prior to infection. Cells were then exposed to lentiviral vectors for 24 h at multiplicities of infection (MOI) of 0.1 (for vF-CD14 and vR-CD14) or 5 (for vR-VSV-M). To induce transgene expression, culture medium was replaced with D-MEM/10% FCS containing 10 nM mifepristone and cells were analyzed 48 h (vF-CD14 and vR-CD14 transductions) or 24 h (for vR-VSV-M transduction) later.

Flow cytometric analysis

Transduced cells were incubated with anti-human CD14 mouse monoclonal antibody (61D3, eBioscience) for 20 min at 4°C and then stained with Cy5-conjugated anti-mouse IgG donkey polyclonal antibody (Chemicon International Inc.) for a further 20 min at 4°C. Data were collected using the FACScalibur system (BD Bioscience) and analyzed with WinMDI software.

Western blotting analysis

Cells were lysed in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, 0.9% β-mercaptoethanol). Boiled samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilion Transfer Membranes (Millipore). Primary antibodies used were (i) anti-FLAG mouse monoclonal IgG (M2, Sigma) to detect FLAG-VSV-M; (ii) biotinylated anti-FLAG mouse monoclonal IgG (BioM2, Sigma) to detect FLAG-VPS4B-KQ and FLAG-Luc; and (iii) anti-α-tubulin mouse monoclonal IgG (DM1A, Sigma) to detect α-tubulin. Biotinylated anti-mouse IgG (BA-2000, Vector Laboratories) was then used for the detection of FLAG-VSV-M. Proteins were detected using horseradish peroxidase (HRP)-conjugated streptavidin (ZYMED Laboratories, for FLAG-tagged proteins) and HRP-conjugated anti-mouse IgG (Cell Signaling, for α-tubulin) using a Western Lightning Chemiluminescence Reagent Plus

(PerkinElmer). Signals were analyzed using a luminescence image analyzer, LAS-3000mini (Fujifilm).

Microscopy analysis

293T cells transfected with pF-VSV-M and pR-VSV-M were examined by light and fluorescent microscopy using an Eclipse TS100 microscope (Nikon) at the point of harvesting lentiviral vector. Images were obtained with a DFC480 digital camera and IM500 image manager software (Leica Microsystems).

To detect FLAG-VSV-M, transduced cells were fixed with 4% paraformaldehyde and incubated with PBS containing 5% normal goat serum and 0.05% Triton X-100 at room temperature for 1 h. This was followed by incubation with anti-FLAG-mouse monoclonal IgG (M2) at 4°C overnight. Samples were then incubated with Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen) at room temperature for 1 h and nuclei were stained using Hoechst 33342 (Invitrogen). Images were obtained using a CTR 6500 fluorescent microscope and FW4000 software (Leica Microsystems).

Assay for HIV-1 release from VPS4B mutant-transduced cells

GeneSwitch-293 cells were seeded at a density of 1×10^3 cells/well in a 96-well plate 20 h prior to infection and exposed to vR-VPS4B-KQ and vR-Luc at a MOI of 1 for 24 h. Transduced cells were then expanded for 4–5 weeks and reseeded at a density of 4×10^6 cells in a 15-cm diameter dish 20 h prior to induction. A portion of transduced cells was subjected to flow cytometric analysis to monitor hrGFP expression after induction with 10 nM mifepristone. To enrich transduced cells, single hrGFP positive cells were positively selected using the FACSAria cell sorting system (BD Bioscience). Sorted cells were cultured for a further 4–5 weeks and cell clones with the strongest hrGFP signals in respective transductions were used for an assay of HIV-1 release.

For the assay of HIV-1 release, 4 μg of pNL4-3 [28] was transfected to transduced cells with Lipofectamine 2000 (Invitrogen). Four hours post-transfection, culture medium was replaced with D-MEM/10% FCS containing 10 nM mifepristone. Culture supernatant was harvested 24 h later and virus production was monitored by HIV-1 $p24^{CA}$ enzyme-linked immunosorbent assay (ELISA, ZeptoMetrix).

Statistical analysis

Student's *t* test was used to determine statistical significance. A *P* value of <0.05 was considered significant.

Results

Generation of mifepristone-inducible lentiviral vectors

To establish a regulatable viral vector system, we cloned a mifepristone-inducible promoter sequence (as an internal promoter) into a self-inactivating (SIN) lentiviral vector plasmid in which the U3 region of the 5′-long terminal repeat (LTR) was replaced with the cytomegalovirus (CMV) promoter and the enhancer/promoter unit was deleted from the U3 region of the 3′-LTR [23, 27]. The inducible promoter was a hybrid consisting of the yeast GAL4 upstream activating sequences linked to the adenovirus major late E1b TATA box (GAL4/TATA) [7]. Gene expression from the GAL4/TATA promoter was controlled by a chimeric regulatory protein termed GeneSwitch. Binding of mifepristone to GeneSwitch induced a conformational change in the regulator to an active state, resulting in transcription of the gene of interest [7]. Besides an inducible promoter, our lentiviral vectors contained a Gateway cloning system reading frame cassette, facilitating cloning of genes of interest by site-specific recombination-based Gateway technology [23]. In addition to a conventional lentiviral vector containing the GAL4/TATA-Gateway component in the forward orientation (forward vector, Fig. 1a), we also constructed a version of the lentiviral vector plasmid in which the inducible gene expression unit was located in the reverse orientation, with the intention of reducing interference from the CMV promoter (reverse vector, Fig. 1a).

To evaluate our gene delivery system, the human *CD14* gene was cloned into both forward and reverse vector plasmids (designated pF-CD14 and pR-CD14, respectively). Infectious lentiviral vectors (vF-CD14 and vR-CD14) were produced following co-transfection of VSV G/Rev-expressing plasmid and Gag-Pol-expressing plasmid into 293T cells. Flow cytometric analysis of transfected cells showed that even in the absence of the GeneSwitch protein and mifepristone, expression of *CD14* and cistronic *hrGFP* genes occurred in pF-CD14-transfected producer cells (40.7 ± 3.1%), while such expression was repressed in pR-CD14-transfected cells (1.4 ± 0.4%) (Fig. 1b). This leaky expression of transgenes from the forward vector plasmid in virus producer cells might be due to transcriptional interference arising from the presence of a heterologous promoter in the same orientation (i.e., the CMV promoter), which was required for Tat-independent transcription of the viral RNA genome [27]. Culture supernatant containing the lentiviral vector was harvested and viral titer determined by quantification of the number of hrGFP-positive cells in viral vector-transduced GeneSwitch-293 cells, expressing the GeneSwitch regulatory protein, in the presence of mifepristone [8]. Lentiviral vectors produced by pF-CD14 showed 34.4-fold higher

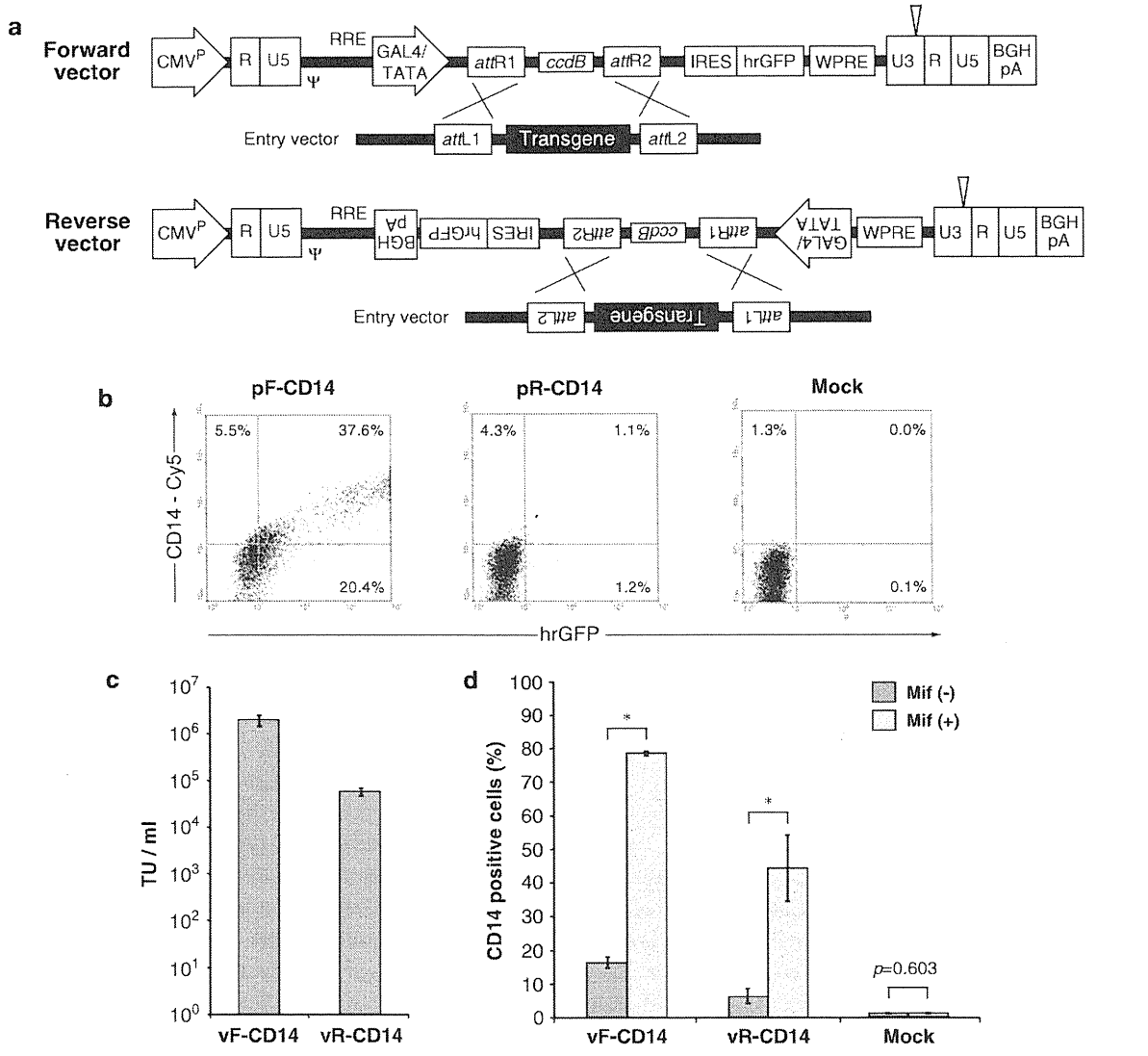


Fig. 1 Transduction of the *CD14* gene by mifepristone-inducible lentiviral vectors. **a** Schematic of forward and reverse vectors. In these SIN lentiviral vector plasmids, the U3 region of the 5′-LTR has been replaced with the CMV promoter (CMV^P) resulting in Tat-independent transcription. A portion of the U3 region containing the enhancer/promoter unit has been deleted from the 3′-LTR (represented as *triangles*). The gene of interest (transgene), flanked by *att*B sites, was subcloned into an entry plasmid and then transferred to the vector plasmid by a Gateway reaction. Note that site-specific recombination between *att*R sites on the vector plasmid and *att*L sites on the entry vector gave rise to new *att*B sites on the final lentiviral vector plasmid. The GAL4/TATA promoter regulated expression of the transgene and IRES-controlled hrGFP gene in the presence of the transactivator protein (GeneSwitch) and mifepristone. Ψ, packaging signal; *RRE* Rev responsive element; *WPRE* woodchuck post-regulatory element, *BGH pA* bovine growth factor hormone poly(A). **b** Expression of CD14 in vector producer cells. 293T cells were co-transfected with a VSV G/Rev-expressing plasmid, a Gag-Pol-expressing plasmid, and a forward or reverse vector plasmid coding *CD14* and *hrGFP* genes (pF-CD14 and pR-CD14, respectively). Expression of CD14 (*y*-axis) and hrGFP (*x*-axis) at the time of vector harvest (48 h after transfection) was analyzed by flow cytometric analysis. Untransfected cells (mock) served as a negative control. The representative results of three independent experiments are shown. **c** Infectious titers of lentiviral vectors. Vector titer (transducing units [TU]/ml) was determined by quantitative flow cytometric analysis for hrGFP positive cells on transduced GeneSwitch-293 cells in the presence of mifepristone. Values represent the mean ± standard deviation (SD) for triplicate determinations. **d** Transduction and induction activities of lentiviral vectors. GeneSwitch-293 cells were infected with vF-CD14 or R-CD14 at a MOI of 0.1 (or uninfected, mock) and cultivated in the presence or absence of 10 nM mifepristone (Mif (+) and Mif (−), respectively). Expression of CD14 protein 48 h after induction was determined by flow cytometric analysis. Values represent the mean ± SD of three independent experiments. The *P* value versus no mifepristone treatment is <0.05 by Student's *t* test (*)

viral titers (vF-CD14, 2.0 ± 0.5 × 10^6 TU/ml) than viral vectors obtained by pR-CD14 (vR-CD14, 5.8 ± 1.1 × 10^4 TU/ml) (Fig. 1c). The transduction and induction efficiencies of lentiviral vectors were then assessed following infection of GeneSwitch-293 cells at a MOI of 0.1. As shown in Fig. 1d, a low level of CD14 expression in

transduced cells was observed in both vF-CD14- and vR-CD14-infected cells in the absence of mifepristone. However, basal gene expression in vF-CD14-infected cells (16.5 ± 1.7%) was higher than that recorded in vR-CD14-infected cells (6.3 ± 2.2%), indicating that transgene expression from the reverse orientation vector was tightly controlled in both transduced cells and virus producer cells (Fig. 1b, d). In the presence of mifepristone, expression of CD14 increased significantly, with the percentage of CD14-positive cells rising to 78.6 ± 0.6% (vF-CD14) and 44.3 ± 9.9% (vR-CD14) (Fig. 1d). The regulation factor (ratio of maximal induced expression to basal expression levels in the absence of the inducer [29]) of vR-CD14 was higher than that of vF-CD14 (7.0-fold vs. 4.7-fold). These results demonstrated that our mifepristone-inducible lentiviral vectors functioned as an efficient gene-delivery system with a tight on–off switch for regulating transgene expression.

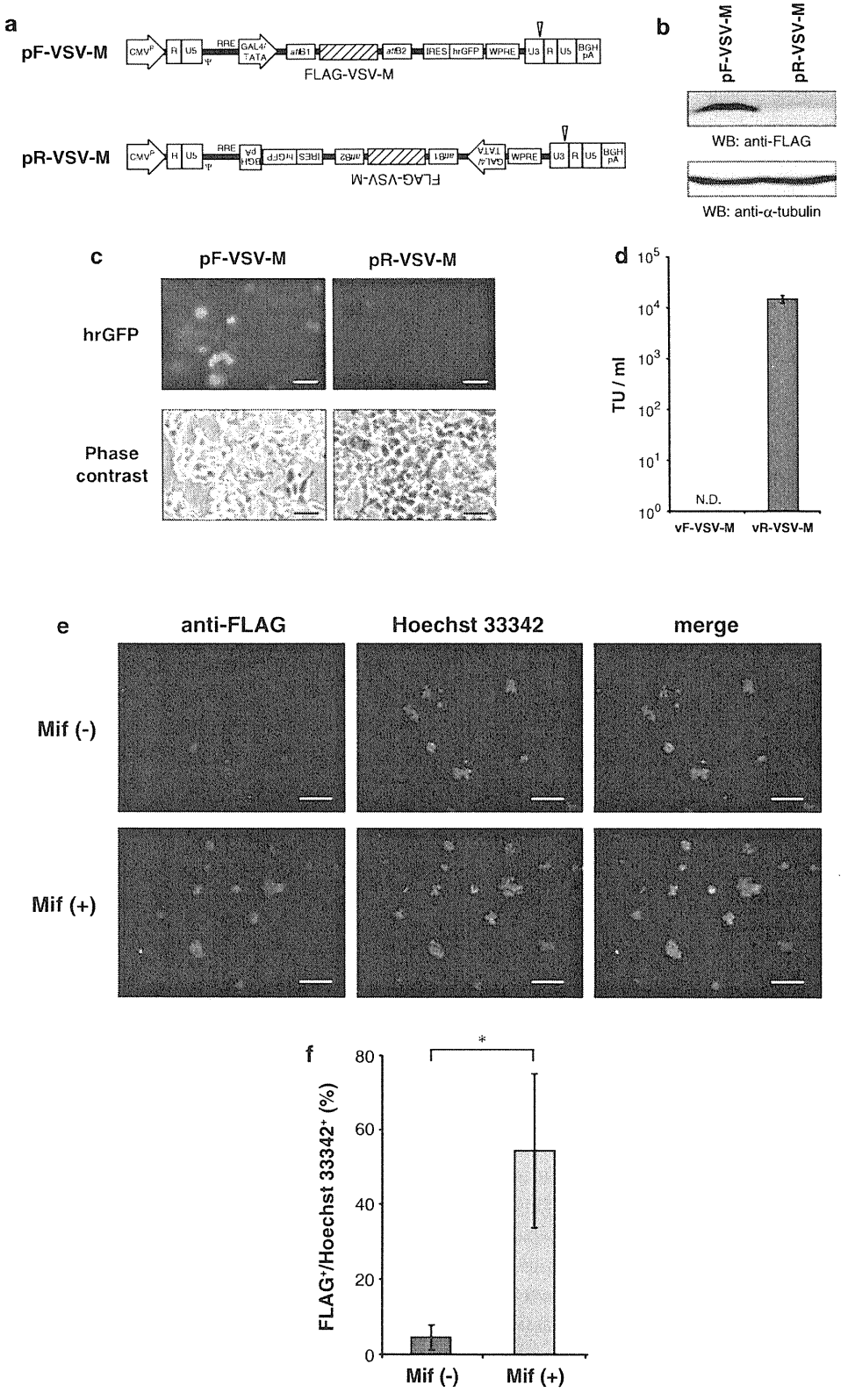
Transduction of a cytotoxic gene by the mifepristone-inducible lentiviral vector

In gene therapies for cancer patients, delivery of a cytotoxic gene by lentiviral vector is one strategy for destroying tumor cells. However, expression of a toxic gene in a lentiviral vector genome would hamper vector production resulting in a reduction in titer. Expression of such a cytotoxic gene thus needs to be blocked during the process of vector production. The lentiviral vector described here, containing a mifepristone-inducible gene expression unit in the reverse orientation, shows great promise as a carrier for a cytotoxic transgene with CD14 expression tightly repressed in pR-CD14-transfected producer cells (Fig. 1b). We examined this system further to ascertain if it could produce infectious lentiviral vector containing a cytotoxic gene and transduce that gene into target cells. Forward and reverse vector plasmids containing FLAG tag-fused VSV matrix protein (FLAG-VSV-M) were constructed (pF-VSV-M and pR-VSV-M, Fig. 2a) and transfected into 293T cells to produce VSV G-pseudotyped infectious vector. VSV M was chosen as it inhibits nuclear export of cellular RNA by interacting with nucleoporin. This protein is responsible for most of the cytopathic effects observed in VSV-infected cells [30]. Analysis of the level of FLAG-VSV-M in virus producer cells showed that, even in the absence of inducers, transgene expression was activated in pF-VSV-M-transfected cells, while expression was undetectable in pR-VSV-M-transfected cells (Fig. 2b). As predicted by Western blotting analysis, activated expression of the downstream *hrGFP* cistron, together with the rounded phenotype of infected cells (a hallmark of VSV infection in cell culture [30]), was observed in pF-VSV-M-transfected cells (Fig. 2c). In contrast, pR-VSV-M-transfected cells exhibited neither hrGFP expression nor cytopathic effect, demonstrating the ability of the reverse vector to suppress the cytotoxic gene in virus producer cells (Fig. 2c). Importantly, the ability of pR-VSV-M to suppress VSV M expression was reflected in the production of infectious lentiviral vector from transfected cells. Transfection with pR-VSV-M yielded lentiviral vector (vR-VSV-M) with titers of 1.5 ± 0.5 × 10^4 TU/ml, while no infectious vector could be obtained by transfection with pF-VSV-M (Fig. 2d). The ability of vR-VSV-M to transduce the transgene was then analyzed by infection of GeneSwitch-293 cells. Immunostaining analysis indicated that FLAG-VSV-M expression was induced in response to mifepristone in transduced cells (Fig. 2e) and the regulation factor (i.e., inducibility) was 11.8-fold (FLAG positive cells per Hoechst 33342 positive cells, 4.6 ± 3.2% without mifepristone treatment versus 54.5 ± 20.6% with mifepristone treatment (Fig. 2f). These data demonstrated that our reverse vector system is suitable for the transduction of a harmful gene.

Fig. 2 Effective transduction of VSV M by the reverse vector. ▶ **a** Forward and reverse vectors containing FLAG-tagged VSV M (FLAG-VSV-M) as a transgene (pF-VSV-M and pR-VSV-M, respectively). **b** Expression of FLAG-VSV-M in vector producer cells. 293T cells were co-transfected with a VSV G/Rev-expressing plasmid, a packaging plasmid, and pF-VSV-M or pR-VSV-M to produce lentiviral vectors. Cell extracts were prepared 48 h after transfection and subjected to Western blotting (WB) analysis using anti-FLAG (*upper panel*) and anti-α-tubulin (*lower panel*) antibodies. **c** Leaky expression of VSV M caused the death of producer cells. Fluorescent (*upper panels*) and light (*lower panels*) microscopic analysis of producer cells was undertaken at the time of vector harvest. Scale bar, 40 μm. **d** Titers of lentiviral vectors bearing the *VSV M* gene. TU was determined by hrGFP positive cells in GeneSwitch-293 cells in the presence of mifepristone. The expression of hrGFP in vF-VSV-M-infected cells was below the detection limit (not detected, N.D.). Value of vR-VSV-M represents the mean ± SD of three independent experiments. **e** Inducible expression of FLAG-VSV-M in vR-VSV-M-transduced cells. GeneSwitch-293 cells were infected with vR-VSV-M at a MOI of 5 and fixed with paraformaldehyde 24 h after induction. Expression of FLAG-VSV-M was examined as described in materials and methods. Hoechst 33342 indicates nucleus. Mif (−), without mifepristone treatment; Mif (+), with 10 nM mifepristone treatment; scale bar, 100 μm. **f** Quantification of inducibility. FLAG-VSV-M positive cells observed by immunofluorescence analysis were counted in a randomly selected visual field. Data are expressed as the percentage of $FLAG^+$ cells in Hoechst 33342^+ cells (mean ± SD of four independent transductions). The *P* value versus no mifepristone treatment is <0.05 by Student's *t* test (*)

Inhibition of HIV-1 release by mifepristone-inducible lentiviral vector

To evaluate whether the reverse vector could overcome self-inhibition caused by an anti-HIV transgene in producer cells, a dominant negative mutant of human VPS4B was



selected as a transgene. VPS4B is one of two known isoforms of AAA-ATPase VPS4 and is thought to catalytically remove endosomal sorting complex required for transport (ESCRT) complex III proteins from the plasma membrane, resulting in budding of HIV-1 from infected cells [26]. The ATPase activity of VPS4B is essential for

HIV-1 budding with dominant negative mutants of VPS4 (such as K180Q, where the residues required for ATP binding have been mutated), previously reported to block HIV-1 release and infectivity [26]. We inserted a FLAG tag-fused VPS4B K180Q mutant (FLAG-VPS4B-KQ) into both the forward and the reverse vector plasmids and constructed vector plasmids encoding FLAG tag-fused luciferase (FLAG-Luc) as control vectors (Fig. 3a). When these vector plasmids were used to produce lentiviral vector, expression of the transgenes (i.e., FLAG-VPS4B-KQ or FLAG-Luc) was observed in producer cells transfected with forward vector plasmids, but not in cells with reverse vector plasmids (Fig. 3b). While transfection with pR-VPS4B-KQ yielded infectious lentiviral vector, vR-VPS4B-KQ, transfection with pF-VPS4B-KQ did not generate infectious vector, indicating that leaky expression of the VPS4 dominant negative mutant from the forward vector plasmid self-inhibited lentiviral vector production (Fig. 3c). Titers of vR-VPS4B-KQ ($8.7 \pm 2.4 \times 10^4$ TU/ml) were comparable to those of lentiviral vector obtained by transfection with pR-Luc (vR-Luc, $5.9 \pm 4.1 \times 10^4$ TU/ml), indicating that titers of lentiviral vectors containing the gene

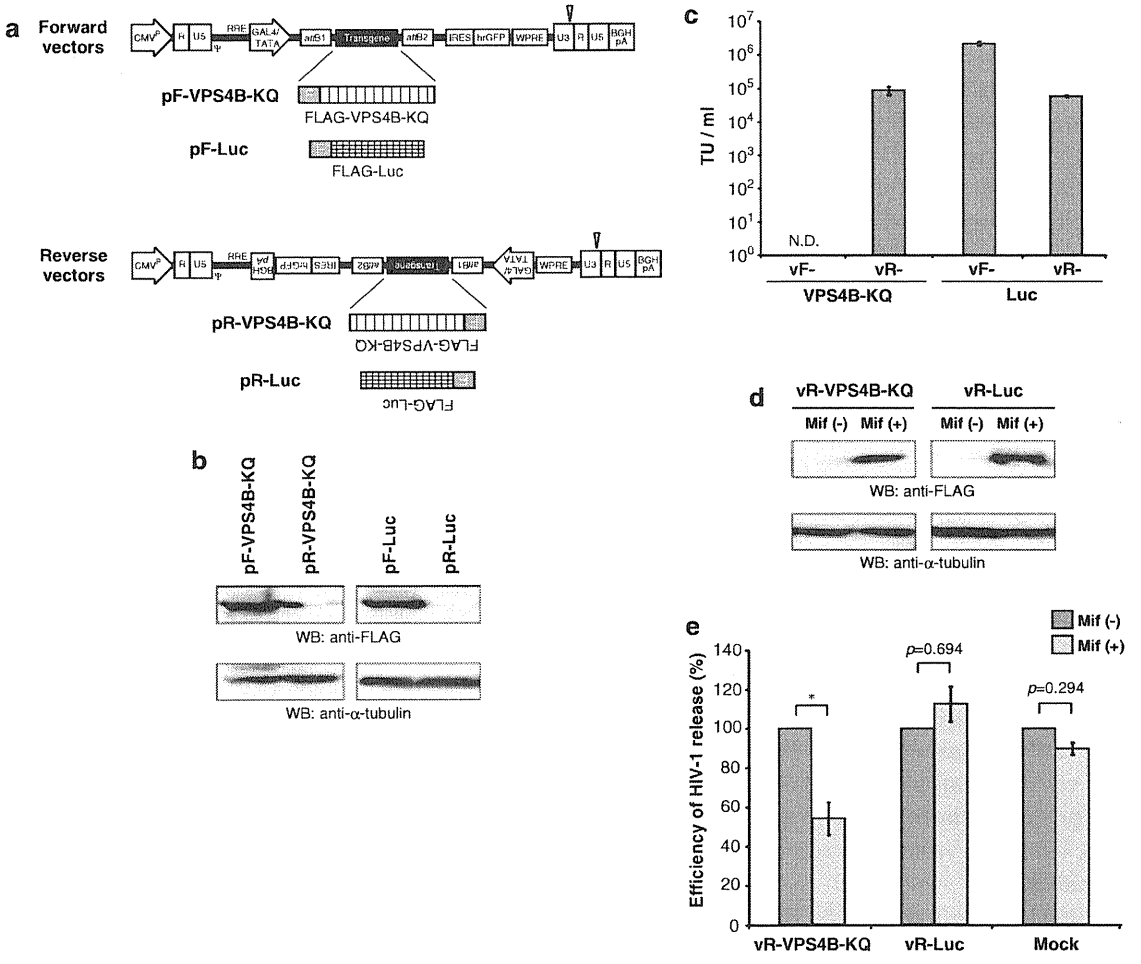


Fig. 3 Inhibition of HIV-1 release by a reverse vector encoding VPS4B dominant negative mutant. **a** Schematic representation of forward and reverse vectors bearing FLAG-tagged VPS4B K180Q (FLAG-VPS4B-KQ) or luciferase (FLAG-Luc). **b** Expression of transgenes in vector producer cells. The lentiviral vectors were produced as described in "Materials and methods". Producer cells were collected at the time of virus harvest and subjected to Western blotting (WB) analysis using anti-FLAG (*upper panels*) and anti-α-tubulin (*lower panels*) antibodies. **c** Titers of lentiviral vectors. N.D. means below the detection limit of flow cytometric analysis for hrGFP expression. **d** Induction of FLAG-VPS4B-KQ and FLAG-Luc expressions by reverse vectors. GeneSwitch-293 cells were infected with vR-VPS4B-KQ or vR-Luc at a MOI of 1 and cultivated in the absence of mifepristone. After sorting of cell clones that were capable of expressing hrGFP, the inducibility of FLAG-VPS4B-KQ and FLAG-luciferase expression was analyzed by western blotting. Mif (−) and Mif (+) indicate before and after induction with mifepristone, respectively. **e** Analysis of HIV-1 production in transduced cells. GeneSwitch-293 cell lines transduced with either vR-VPS4B-KQ or vR-Luc (and untransduced cells, mock) were transiently transfected with pNL4-3. The amount of HIV-1 virions present in the culture supernatant was measured by $p24^{CA}$ ELISA. The efficiency of HIV-1 release in the presence of 10 nM mifepristone is shown as a percentage of the value in the absence of mifepristone (mean ± SD of three independent transfections). The *P* value versus no mifepristone treatment is <0.05 by Student's *t* test (*)

expression unit in a reverse orientation were not affected by the anti-HIV transgene (Fig. 3c).

The ability of our lentiviral vectors to transduce the VPS4B dominant negative mutant for inhibition of HIV-1 release was then tested. GeneSwitch-293 cells were infected with vR-VPS4B-KQ and vR-Luc and sorted for hrGFP positive cells. When transduced cells were cultured with mifepristone, induction of transgene expression was seen (Fig. 3d). The inhibitory effect of transgenes on HIV-1 release from transduced cells was examined by transiently transfecting a plasmid DNA producing infectious HIV-1 virions (pNL4-3). In the presence of mifepristone, levels of HIV-1 production from vR-VPS4B-KQ-transduced cells dropped to 53.9 ± 8.5%, approximately half that seen in the absence of mifepristone treatment. However, no significant inhibitory effects on HIV-1 release were observed in vR-Luc or mock-transduced cells (112.3 ± 9.0% and 89.7 ± 3.1%, respectively). This indicated that induced expression of dominant negative mutant VPS4B proteins in transduced cells accounted for the observed inhibition of HIV-1 release. These results demonstrate the utility of our reverse vector to transduce an anti-HIV gene that functionally suppresses HIV-1 release in target cells.

Discussion

The basic principle of current gene therapy is to deliver genetic material to a population of cells in the body, thereby preventing a disease or improving the clinical status of a patient. Although, a key factor in successfully implementing gene therapy is the development of effective vector systems, a number of issues need to be addressed to apply them in a clinical setting. In terms of viral vector systems, one of the major problems is that insertion of cytotoxic or antiviral transgenes adversely affects viral titers during vector production. In this study, we incorporated a mifepristone-inducible gene expression unit into HIV-1-based lentiviral vectors to solve the problem of vector self-inhibition.

Previous studies have reported the delivery of various anti-HIV genes by HIV-1-based vectors in vitro and in vivo [31–36]. Some of the transgenes used in these studies target HIV-1 RNA sequences either directly or indirectly, aiming to inhibit transcription, nuclear translocation, or translation of viral RNA [32–36]. In these types of approach, the problem of self-inhibition can be solved by modifying the nucleotide sequence of a lentiviral vector such that the function of the vector RNA does not interfere with the anti-HIV transgene in producer cells. However, if the transgene targets a fundamental process of the HIV-1 life cycle, such as virion formation, another strategy to avoid self-inhibition is to express the transgene in a regulated manner such that its expression is blocked in producer cells and induced in target cells. This kind of approach would be of value in the transduction of a harmful gene into target cells. The data presented here demonstrate that a lentiviral vector bearing a regulatable gene expression unit is indeed capable of transducing cytotoxic (VSV M) and anti-HIV (VPS4B K180Q) genes into target cells without significant decrease in vector titer (Figs. 2, 3). In addition, induction of anti-HIV genes in transduced cells resulted in approximately 50% inhibition of HIV-1 release (Fig. 3e).

Expression of VPS4B-KQ mutant by transfection has been reported to inhibit HIV-1 release >100-fold [26]; although, the VPS4B-KQ expression induced by our mifepristone-regulatable system produced about 2-fold reduction in HIV-1 production (Fig. 3e). When we looked at the IRES-controlled hrGFP expression in mifepristone-induced cells that had been transduced by vR-VPS4B-KQ or vR-Luc and sorted, hrGFP expressions were only observed in 10.5% (vR-VPS4B-KQ) or 12.2% (vR-Luc) of the cells (data not shown). We speculate that uninducible population of cells was still permissive to HIV-1 production and thus lead to the observed 50% inhibition in the vR-VPS4B-KQ-transduced cells. During expansion of these transduced cells after cell sorting it is possible that some (e.g., gene shut-off) lost their ability to be induced by mifopristone. Besides, the cell sorting step might lead to this issue of lost inducibility. Therefore improving the way to enrich transduced cells should help to alleviate this problem.

To achieve tight regulation of transgene expression, enabling production of infectious vectors, it was necessary to place the mifepristone-inducible gene expression unit in the reverse orientation in the context of the lentiviral vector. Sirin and Park [12] tested the forward and the reverse orientations of a mifepristone-inducible gene expression unit in HIV-1-based lentiviral vectors and reported basal levels of transgene expression that were higher in lentiviral vectors bearing the expression cassette in the reverse orientation than those containing it in the forward orientation. This was in contrast to the findings presented here, where basal expression of the CD14 transgene in reverse vector-infected cells appeared to be lower than that in forward vector-infected cells (Fig. 1d). Similarly to the Sirin and Park [12] study, we used an HIV-1-based SIN vector in which the woodchuck post-regulatory element (WPRE) was inserted into the 3′-untranslated region of the viral genome (Fig. 1a). WPRE has been reported to increase the stability of RNA transcripts, thereby enhancing transgene expression from retroviral and lentiviral vectors [37]. Interestingly, WPRE functions only when placed in the sense orientation of a transgene and antisense WPRE actually shows an inhibitory effect on transgene expression [37]. In our reverse vector, WPRE

was positioned in the opposite orientation to the inducible gene expression unit (Fig. 1a), while the vector designed by Sirin and Park [12] contained WPRE in same orientation as the expression unit. Orientation-dependent elements such as WPRE can thus enhance basal expression of a transgene in both producer and transduced cells. In addition to WPRE, the SIN vector used in our study contained a hybrid 5′-LTR in which the U3 region was replaced with the CMV promoter [27]. We speculate that, in the context of our forward vector, these *cis*-acting sequences should increase background activity of the mifepristone-regulatable gene expression unit without induction, leading to leaky expression of cytotoxic/anti-HIV genes in producer cells and significant loss of vector titers (Figs. 2d, 3c).

One general drawback of regulatable gene expression systems, including the Tet and mifepristone systems, is that they necessitate delivery of two expression units into a target cell; one to express the transactivator and the other to express the transgene in response to the activator. To exclude differences in experimental conditions due to differing levels of transactivator expression, a cell line stably expressing the GeneSwitch transactivator was used as a target cell in this study. While Sirin and Park [12] also described a two-lentiviral vector system in which GeneSwitch and inducible gene expression units were cloned into separate vectors, this type of binary approach would produce populations of singly transduced cells with either transactivator or transgene, resulting in low inducibility. Single-lentiviral vectors bearing the entire regulatable unit have been developed in Tet systems [11, 38, 39]. This single-vector approach would be an attractive option for the mifepristone-regulatable system, bypassing the need for co-transduction of target cells with high amounts of virus. However, RNA virus-based vectors, such as lentiviral vectors, are limited in their cloning capacity for larger genes. Theoretically, lentiviral vectors can accommodate 7–7.5 kb of foreign DNA [1], yet this packaging capacity will be decreased by the insertion of additional regulatory sequences. Improvements to the mifepristone system that would allow incorporation of both transactivator and inducible units into a single-lentiviral vector would be necessary to design a more versatile vector.

The mifepristone-regulatable gene expression system reported here has a number of potential advantages that suit it to gene therapy applications in humans. First, the majority of the system consists of modified human proteins with no impact on cell viability. Second, the induction response is specific and rapid. Third, mifepristone is orally effective and the dose required for induction is within the range acceptable for clinical use [10]. Importantly, mifepristone has been approved by the Food and Drug Administration (FDA) for use in humans. Although, no gene regulatory system has yet been approved by the FDA for clinical use, lentiviral vectors in conjunction with a mifepristone-regulatable gene expression system are a promising step toward achieving successful gene therapy.

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References

1. I.M. Verma, N. Somia, Nature **389**, 239–242 (1997)
2. L. Naldini, U. Blomer, P. Gallay, D. Ory, R. Mulligan, F.H. Gage, I.M. Verma, D. Trono, Science **272**, 263–267 (1996)
3. H. Miyoshi, K.A. Smith, D.E. Mosier, I.M. Verma, B.E. Torbett, Science **283**, 682–686 (1999)
4. J.A. Taylor, L. Vojtech, I. Bahner, D.B. Kohn, D.V. Laer, D.W. Russell, R.E. Richard, Mol. Ther. **16**, 46–51 (2008)
5. M. Fussenegger, Biotechnol. Prog. **17**, 1–51 (2001)
6. S. Agha-Mohammadi, M.T. Lotze, J. Clin. Invest. **105**, 1177–1183 (2000)
7. Y. Wang, B.W. O'Malley Jr., S.Y. Tsai, B.W. O'Malley, Proc. Natl Acad. Sci. USA **91**, 8180–8184 (1994)
8. M.M. Burcin, G. Schiedner, S. Kochanek, S.Y. Tsai, B.W. O'Malley, Proc. Natl Acad. Sci. USA **96**, 355–360 (1999)
9. R.V. Abruzzese, D. Godin, V. Mehta, J.L. Perrard, M. French, W. Nelson, G. Howell, M. Coleman, B.W. O'Malley, J.L. Nordstrom, Mol. Ther. **2**, 276–287 (2000)
10. J.L. Nordstrom, Steroids **68**, 1085–1094 (2003)
11. T. Kafri, H. van Praag, F.H. Gage, I.M. Verma, Mol. Ther. **1**, 516–521 (2000)
12. O. Sirin, F. Park, Gene **323**, 67–77 (2003)
13. B. Mitta, C.C. Weber, M. Rimann, M. Fussenegger, Nucleic Acids Res. **32**, e106 (2004)
14. S.C. Beutelspacher, N. Ardjomand, P.H. Tan, G.S. Patton, D.F. Larkin, A.J. George, M.O. McClure, Exp. Eye Res. **80**, 787–794 (2005)
15. F. Galimi, E. Saez, J. Gall, N. Hoong, G. Cho, R.M. Evans, I.M. Verma, Mol. Ther. **11**, 142–148 (2005)
16. S. Hartenbach, M. Fussenegger, J. Biotechnol. **120**, 83–98 (2005)
17. H.L. Heine, H.S. Leong, F.M. Rossi, B.M. McManus, T.J. Podor, Methods Mol. Med. **112**, 109–154 (2005)
18. B. Mitta, C.C. Weber, M. Fussenegger, J. Gene Med. **7**, 1400–1408 (2005)
19. K. Okamoto, J. Fujisawa, M. Reth, S. Yonehara, Genes Cells **11**, 177–191 (2006)
20. W. Weber, W. Bacchus, F. Gruber, M. Hamberger, M. Fussenegger, J. Biotechnol. **131**, 150–158 (2007)
21. H. Hurttila, J.K. Koponen, E. Kansanen, H.K. Jyrkkanen, A. Kivela, R. Kylatie, S. Yla-Herttuala, A.L. Levonen, Gene Ther. **15**, 1271–1279 (2008)
22. S. Goverdhana, M. Puntel, W. Xiong, J.M. Zirger, C. Barcia, J.F. Curtin, E.B. Soffer, S. Mondkar, G.D. King, J. Hu, S.A. Sciascia, M. Candolfi, D.S. Greengold, P.R. Lowenstein, M.G. Castro, Mol. Ther. **12**, 189–211 (2005)

23. Y. Kawano, T. Yoshida, K. Hieda, J. Aoki, H. Miyoshi, Y. Koyanagi, J. Virol. **78**, 11352–11359 (2004)
24. H. Kuwata, Y. Watanabe, H. Miyoshi, M. Yamamoto, T. Kaisho, K. Takeda, S. Akira, Blood **102**, 4123–4129 (2003)
25. H. Ebina, J. Aoki, S. Hatta, T. Yoshida, Y. Koyanagi, Microbes Infect. **6**, 715–724 (2004)
26. U.K. von Schwedler, M. Stuchell, B. Muller, D.M. Ward, H.Y. Chung, E. Morita, H.E. Wang, T. Davis, G.P. He, D.M. Cimbora, A. Scott, H.G. Krausslich, J. Kaplan, S.G. Morham, W.I. Sundquist, Cell **114**, 701–713 (2003)
27. H. Miyoshi, U. Blomer, M. Takahashi, F.H. Gage, I.M. Verma, J. Virol. **72**, 8150–8157 (1998)
28. A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, J. Virol. **59**, 284–291 (1986)
29. Z.L. Xu, H. Mizuguchi, T. Mayumi, T. Hayakawa, Gene **309**, 145–151 (2003)
30. H.R. Jayakar, M.A. Whitt, J. Virol. **76**, 8011–8018 (2002)
31. M.R. Mautino, R.A. Morgan, Aids Patient Care STDS **16**, 11–26 (2002)
32. M. Mukhtar, H. Duke, M. BouHamdan, R.J. Pomerantz, Hum. Gene Ther. **11**, 347–359 (2000)
33. M.R. Mautino, R.A. Morgan, Gene Ther. **9**, 421–431 (2002)
34. A. Banerjea, M.J. Li, G. Bauer, L. Remling, N.S. Lee, J. Rossi, R. Akkina, Mol. Ther. **8**, 62–71 (2003)
35. M.J. Li, G. Bauer, A. Michienzi, J.K. Yee, N.S. Lee, J. Kim, S. Li, D. Castanotto, J. Zaia, J.J. Rossi, Mol. Ther. **8**, 196–206 (2003)
36. H. Nishitsuji, T. Ikeda, H. Miyoshi, T. Ohashi, M. Kannagi, T. Masuda, Microbes Infect. **6**, 76–85 (2004)
37. R. Zufferey, J.E. Donello, D. Trono, T.J. Hope, J. Virol. **73**, 2886–2892 (1999)
38. E. Vigna, S. Cavalieri, L. Ailles, M. Geuna, R. Loew, H. Bujard, L. Naldini, Mol. Ther. **5**, 252–261 (2002)
39. R. Vogel, L. Amar, A.D. Thi, P. Saillour, J. Mallet, Hum. Gene Ther. **15**, 157–165 (2004)

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Selective infection of CD4$^+$ effector memory T lymphocytes leads to preferential depletion of memory T lymphocytes in R5 HIV-1-infected humanized NOD/SCID/IL-2Rγ^{null} mice

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ABSTRACT

To investigate the events leading to the depletion of CD4$^+$ T lymphocytes during long-term infection of human immunodeficiency virus type 1 (HIV-1), we infected human CD34$^+$ cells-transplanted NOD/SCID/IL-2Rγ^{null} mice with CXCR4-tropic and CCR5-tropic HIV-1. CXCR4-tropic HIV-1-infected mice were quickly depleted of CD4$^+$ thymocytes and both CD45RA$^+$ naïve and CD45RA$^-$ memory CD4$^+$ T lymphocytes, while CCR5-tropic HIV-1-infected mice were preferentially depleted of CD45RA$^-$ memory CD4$^+$ T lymphocytes. Staining of HIV-1 p24 antigen revealed that CCR5-tropic HIV-1 preferentially infected effector memory T lymphocytes (T_{EM}) rather than central memory T lymphocytes. In addition, the majority of p24$^+$ cells in CCR5-tropic HIV-1-infected mice were activated and in cycling phase. Taken together, our findings indicate that productive infection mainly takes place in the activated T_{EM} in cycling phase and further suggest that the predominant infection in T_{EM} would lead to the depletion of memory CD4$^+$ T lymphocytes in CCR5-tropic HIV-1-infected mice.

Introduction

While it is evident that human immunodeficiency virus type 1 (HIV-1) causes acquired immunodeficiency syndrome (AIDS) in humans, the mechanism by which HIV-1 accomplishes this remains unclear. The gradual loss of peripheral blood (PB) CD4$^+$ T lymphocytes during the asymptomatic phase of HIV-1 infection is one of the best prognostic predictors for the onset of AIDS (O'Brien et al., 1996), and CD4$^+$ T lymphocyte depletion is thought to be a serious pathological change in AIDS (McCune, 2001).

To define the mechanisms behind CD4$^+$ T lymphocyte depletion, a large number of studies have been conducted in humans, primates, and humanized mice by using HIV-1, simian immunodeficiency virus (SIV), and SIV/HIV-1 chimeric virus (SHIV) (Centlivre et al., 2007; Koyanagi et al., 2008; McCune, 2001). One of the important findings from previous studies was the dependence of pathogenesis on the co-receptor preference, CXCR4, and/or CCR5 (Berkowitz et al., 1998; Moore et al., 2004). CXCR4-tropic (X4) SHIV caused rapid and complete depletion of all subsets of CD4$^+$ T lymphocytes in rhesus macaques, which led to death from immunodeficiency (Nishimura et al., 2004). On the other hand, CCR5-tropic (R5) HIV-1 is the dominant type of HIV-1 found in patients, and clinical manifestation of HIV-1 infection resembles CCR5-tropic SIV infection (Berger, Murphy, and Farber, 1999). In both HIV-1-infected patients and SIV-infected rhesus macaques, the drastic onset of immunodeficiency is rare (Ambrose et al., 2007; McCune, 2001), and CD4$^+$ T lymphocytes in PB slowly decrease in number, eventually leading to immunodeficiency.

X4 virus uses CXCR4 as the co-receptor and R5 virus uses CCR5 as the co-receptor for viral infection into target cells (Berger, Murphy, and Farber, 1999; Lusso, 2006). CXCR4 is expressed on naïve T lymphocytes and thymocytes, thus X4 HIV-1 can infect naïve T lymphocytes and thymocytes (Pedroza-Martins et al., 1998). It is well known that faster depletion of immature thymocytes and T lymphocytes is observed after the appearance of X4 HIV-1 (Berkowitz et al., 1998; Pedroza-Martins et al., 1998; Schnittman et al., 1990). On the contrary, CCR5 is primarily expressed on CD4$^+$ effector memory T lymphocytes (T_{EM}) and macrophages but not on naïve and central memory CD4$^+$ T lymphocytes (T_{CM}) (Sallusto, Geginat, and Lanzavecchia, 2004). Therefore, the selective infection of T_{EM} is thought to leave naïve T lymphocytes and T_{CM} intact. Depletion of T_{EM} by R5 virus has been studied in SIV-infected rhesus macaques (Brenchley et al., 2004; Li et al., 2005). In 14–28 days following infection, the population of extra-lymphoid CCR5$^+$ T_{EM} was depleted up to 90% (Centlivre et al., 2007; Mattapallil et al., 2005; Okoye et al., 2007). At

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