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Some Human Immunodeficiency Virus Type 1 Vpu Proteins Are Able To Antagonize Macaque BST-2 *In Vitro* and *In Vivo*: Vpu-Negative Simian-Human Immunodeficiency Viruses Are Attenuated *In Vivo*▽

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Human immunodeficiency virus type 1 (HIV-1) Vpu enhances the release of viral particles from infected cells by targeting BST-2/tetherin, a cellular protein inhibiting virus release. The widely used HIV-1_{NL4-3} Vpu functionally inactivates human BST-2 but not murine or monkey BST-2, leading to the notion that Vpu antagonism is species specific. Here we investigated the properties of the CXCR4-tropic simian-human immunodeficiency virus DH12 ($SHIV_{DH12}$) and the CCR5-tropic $SHIV_{AD8}$, each of which carries *vpu* genes derived from different primary HIV-1 isolates. We found that virion release from infected rhesus peripheral blood mononuclear cells was enhanced to various degrees by the Vpu present in both SHIVs. Transfer of the $SHIV_{DH12}$ Vpu transmembrane domain to the HIV-1_{NL4-3} Vpu conferred antagonizing activity against macaque BST-2. Inactivation of the $SHIV_{DH12}$ and $SHIV_{AD8}$ *vpu* genes impaired virus replication in 6 of 8 inoculated rhesus macaques, resulting in lower plasma viral RNA loads, slower losses of $CD4^+$ T cells, and delayed disease progression. The expanded host range of the $SHIV_{DH12}$ Vpu was not due to adaptation during passage in macaques but was an intrinsic property of the parental HIV-1_{DH12} Vpu protein. These results demonstrate that the species-specific inhibition of BST-2 by HIV-1_{NL4-3} Vpu is not characteristic of all HIV-1 Vpu proteins; some HIV-1 isolates encode a Vpu with a broader host range.

The human immunodeficiency virus type 1 (HIV-1) Vpu protein enhances virus release from virus-infected cells (54, 55). This effect has been reported to be cell type dependent (44) and species specific (10, 14, 15, 24, 32, 41, 45, 58, 61, 62). Recently, BST-2 (also known as CD317, HM1.24, or tetherin) was identified to be the Vpu-sensitive cellular factor responsible for restricting HIV-1 virion release (36, 57). In this regard, Vpu and BST-2 are both integral membrane proteins, albeit with different membrane topologies. BST-2 has a short N-terminal cytoplasmic domain, with the bulk of the protein comprising the C-terminal ectodomain, while Vpu has virtually no ectodomain and essentially consists of an N-terminal transmembrane (TM) domain and a C-terminal cytoplasmic domain. It is generally accepted that the BST-2 TM domain is critical for interference by Vpu (9, 11, 15, 27, 32, 33, 40, 41, 62) and is consistent with our previous observation of the importance of the Vpu TM domain for regulating particle release (46). Indeed, the physical interaction of Vpu and human BST-2 and the critical importance of the BST-2 TM domain for this interaction were demonstrated in several coimmunoprecipitation studies (9, 10, 15, 23, 24, 32, 41) and bimolecular fluorescence complementation analyses (27). The inability of HIV-1 Vpu to target macaque BST-2 has been attributed to sequence differences in the TM domains of human and monkey BST-2 proteins (45, 62). However, virtually all of the published work assessing the interaction of HIV-1 Vpu with BST-2 has used the HIV-1_{NL4-3} molecular clone (1), a prototypical HIV-1 strain used in laboratories worldwide. These studies have led to the conclusion that Vpu function is species specific (*viz.*, it is directed against human BST-2) (14, 24, 32, 58, 61).

More recently, simian immunodeficiency virus (SIV) Nef and the Env glycoprotein of some SIV and HIV-2 isolates were found to have Vpu-like activity capable of antagonizing macaque and human BST-2s, respectively (16, 17, 24, 28, 45, 61, 63). The current study was inspired by a recent comprehensive evaluation of Vpu proteins from multiple strains of HIV-1 that included members of three different viral groups (HIV-1 groups M, N, and O) and suggested that certain HIV-1 Vpu proteins may have a broader anti-BST-2 activity than NL4-3 Vpu (45). Our study was further motivated by the observation that during the serial passaging of CXCR4 (X4)-tropic simian-human immunodeficiency virus DH12 ($SHIV_{DH12}$), the pathogenic virus which emerged ($SHIV_{DH12R}$) (20) and its molecularly cloned derivative $SHIV_{DH12\text{-}CL7}$ (43) each carried a *vpu* gene that differed genetically from the homologue present in HIV-1_{NL4-3}. This led us to investigate whether the Vpu encoded by $SHIV_{DH12\text{-}CL7}$, despite its presumed inability to target macaque BST-2 and despite encoding a SIV_{mac239} Nef protein with presumed Vpu-like function, might contribute to the augmented pathogenic potential observed in inoculated rhesus macaques. Surprisingly, we found that the $SHIV_{DH12\text{-}CL7}$ Vpu protein was able to functionally inactivate rhesus BST-2 in *ex vivo* assays while retaining its activity against human BST-2. We also observed that the expanded host range of $SHIV_{DH12\text{-}CL7}$ Vpu was not the result of serial passaging in

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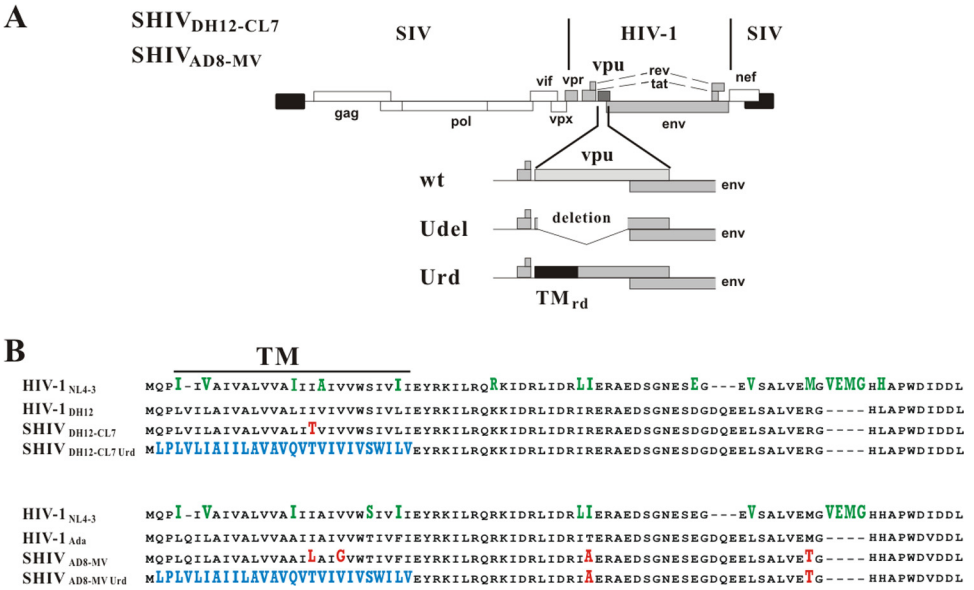

FIG. 1. Constructs used in the current study. (A) Schematic representation of the SHIV constructs. $SHIV_{DH12\text{-}CL7}$ and $SHIV_{AD8\text{-}MV}$ are chimeric viruses expressing HIV-1 Vpr, Tat, Rev, Vpu, and Env products (shaded areas) in a SIV_{mac239} genetic background. The region encompassing the *vpu* gene is expanded, and *vpu* mutants are shown schematically. TM_{rd}, randomized TM domain. (B) Amino acid alignment of Vpu variants employed in this study. Amino acid differences between parental HIV-1 Vpu proteins and those present in SHIV isolates are highlighted in red. The randomized TM sequences in the Urd mutants are shown in blue. Amino acid differences between $HIV\text{-}1_{NL4\text{-}3}$ Vpu and $HIV\text{-}1_{DH12}$ or $HIV\text{-}1_{Ada}$ Vpu are shown in green.

monkeys but was an intrinsic property of the Vpu protein encoded by the parental HIV_{DH12} isolate. The capacity of a second *vpu* gene, derived from CCR5 (R5)-tropic $HIV\text{-}1_{Ada}$ (13, 56) and also present in the recently described R5-tropic $SHIV_{AD8}$ (38), to antagonize macaque BST-2 was similarly evaluated. Compared to $SHIV_{DH12\text{-}CL7}$ Vpu, the $SHIV_{AD8}$ Vpu was less potent in abrogating monkey BST-2 in the same *in vitro* assays. Nonetheless, a majority of monkeys inoculated with Vpu-defective X4- or R5-tropic SHIVs generated lower set-point viremia, exhibited better maintenance of $CD4^+$ T lymphocyte levels, and experienced delayed disease onset compared to SHIVs carrying wild-type *vpu* genes. Taken together, these results suggest that HIV-1 Vpu proteins are functionally different and that the inability of the prototypic $HIV\text{-}1_{NL4\text{-}3}$ Vpu to inhibit macaque BST-2 may not be a typical property of Vpu proteins encoded by primary HIV-1 isolates.

MATERIALS AND METHODS

Plasmids. The construction of plasmids pNL4-3, pNL4-3 Udel, pNL4-3 Urd, and $pSHIV_{DH12\text{-}CL7}$ has been previously described (1, 26, 43, 46). $pSHIV_{AD8\text{-}MV}$ was constructed in two steps: (i) insertion of the 8,556-bp KasI to HindIII fragment (containing viral *gag* through *env* gene sequences) from a molecular clone of unintegrated DNA obtained from rhesus peripheral blood mononuclear cell (PBMC)-infected $SHIV_{AD8PBMC}$ (38) into the genetic background of $pSHIV_{AD8}$ (38) to generate $SHIV_{AD8\text{-}KH17}$ and (ii) insertion of the 3,029-bp EcoRI to HindIII fragment (containing *vpr* through *env* gene sequences) obtained by reverse transcriptase (RT) PCR cloning of the RNA present in the week 42 plasma of macaque CK15, previously inoculated with $SHIV_{AD8\#2}$ (38), into similarly digested $SHIV_{AD8\text{-}KH17}$. The *vpu*-defective $SHIV_{DH12\text{-}CL7}$/Udel and $SHIV_{AD8\text{-}MV}$/Udel mutants were constructed by first subcloning the respective EcoRI to KpnI fragments and then deleting the 5′ 163 bp of the *vpu* gene (up to the 5′ terminus of the *env* gene) by PCR-based mutagenesis. $SHIV_{DH12\text{-}CL7}$/Urd was constructed by randomizing the TM domain of the DH12-CL7 Vpu using oligonucleotide-based mutagenesis. The resulting sequence is shown in Fig. 1B. The $SHIV_{DH12\text{-}CL7}$/Urd TM domain sequence was subsequently transferred into $SHIV_{AD8\text{-}MV}$, resulting in $SHIV_{AD8\text{-}MV}$/Urd (Fig. 1B). A *nef*-deficient variant, $SHIV_{DH12\text{-}CL7}/Nef^-$, was constructed using PCR-based site-directed mutagenesis by mutating the Nef initiation codon (ATG) to TAG, followed by a second in-frame stop codon (TGA) at Nef position 3. A Vpu/Nef double mutant, $SHIV_{DH12\text{-}CL7}$/Udel/Nef^-, was created by transferring the mutated *nef* gene sequence into the backbone of $SHIV_{DH12\text{-}CL7}$/Udel. pNL4-3/TM-CL7 carries the Vpu TM domain of $SHIV_{DH12\text{-}CL7}$ and was constructed using standard PCR techniques. All constructs were verified by sequence analysis.

Cell culture. HEK293T (293T) is a human kidney cell line lacking expression of endogenous BST-2. 293T cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum. Rhesus monkey PBMCs were prepared and cultured as described previously (22).

Transfection, infection, and reverse transcriptase assays. Virus stocks were prepared by transfecting 293T cells with each molecular clone using Lipofectamine 2000 (Invitrogen, Carlsbad, CA); culture supernatants were collected 48 h later and stored at −80°C until use. Virion-associated RT activity was measured as described previously (60). Concanavalin A (ConA)-stimulated rhesus PBMCs (5×10^6) were infected with the indicated viruses (normalized by particle-associated RT activity) by spinoculation (39) for 1 h and maintained for 12 days. Tissue culture medium was replaced daily; these supernatant samples were monitored for RT activity.

Virus release assay. Cells were transfected as described in the text with constant amounts of proviral vectors and increasing amounts of BST-2. Twenty-four hours later, cells were washed with phosphate-buffered saline (PBS), scraped, and resuspended in 3 ml labeling medium lacking methionine (Millipore Corp., Billerica, MA). Cells were then incubated for 10 min at 37°C to deplete the endogenous methionine pool. Cells were then suspended in 400 μl of labeling medium together with 150 μCi of Express$^{35}S^{35}S$ protein labeling mix (Perkin Elmer, Shelton, CT). Cells were labeled for 90 min at 37°C. Cells and virus-containing supernatants were then separated by centrifugation and processed separately for immunoprecipitation, as follows. To identify HIV-1-specific proteins, cells were lysed with 150 μl of Triton lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100) and incubated on ice for 5 min. To identify SHIV-specific proteins, cells were lysed in NP-40–deoxycholate (DOC) lysis buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 20 mM NaCl, 1% Igepal detergent, 0.5% sodium deoxycholate) and incubated on ice for 5 min. After lysis, the cells were pelleted at 13,000 × *g* for 2 min to remove insoluble material. The supernatants were used for immunoprecipitation. Virus-containing supernatants were

treated with 150 μl of Triton lysis buffer (HIV-1) and NP-40–DOC lysis buffer (SHIV) to disrupt viral membranes. Cell and virus lysates were adjusted to a 1.1-ml total volume with PBS containing bovine serum albumin (BSA; final concentration of BSA, 0.1%) and incubated on a rotating wheel for 1 h at 4°C with protein A-Sepharose coupled with an HIV-positive patient serum sample or plasma from SHIV-infected macaques. Beads were washed twice with wash buffer (50 mM Tris, pH 7.4, 300 mM NaCl, 0.1% Triton X-100). Bound proteins were eluted by heating in sample buffer for 10 min at 95°C, separated by SDS-PAGE, and visualized by fluorography. Virus release was quantified by PhosphoImage analysis using a Fujifilm FLA7000 system.

Animals. Rhesus macaques were maintained in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals (8) and were housed in a biosafety level 2 facility; biosafety level 3 practices were followed. Phlebotomies, intravenous virus inoculations, and euthanasias were performed as described previously (12, 19).

Plasma viral RNA quantitation. Viral RNA levels in plasma were determined by real-time RT-PCR (Prism 7700 sequence detection system; Applied Biosystems, Foster City, CA) as described previously (12).

Flow cytometric analysis. To detect BST-2 expression on the surface of rhesus macaque T cells, $CD4^+$ T cells were isolated from rhesus PBMCs and stimulated with concanavalin A (25 μg/ml) and interleukin-2 (20 units/ml) as described previously (22, 25). BST-2 expression was analyzed 72 h later using a cross-reactive rabbit polyclonal antibody against human BST-2 (34). Cells were washed twice in ice-cold PBS containing 1% BSA. Cells were blocked for 10 min with mouse IgG (1.25 μg/ml; Millipore Corp., Billerica, MA). Cells were incubated with primary antibody (anti-BST-2; 1:100) for 30 min at 4°C, washed twice with ice-cold PBS containing 1% BSA, followed by the addition of allophycocyanin (APC)-conjugated anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in PBS containing 1% BSA. Incubation was for 30 min at 4°C in the dark. Cells were then washed twice with ice-cold PBS containing 1% BSA and fixed with 1% paraformaldehyde in PBS. For *in vivo* lymphocyte immunophenotyping, EDTA-treated blood samples were stained for flow cytometric analysis as described previously (37) using combinations of the following fluorochrome-conjugated monoclonal antibodies (obtained from BD Biosciences Pharmingen, San Diego, CA): CD3 (phycoerythrin [PE]), CD4 (PE, peridinin chlorophyll protein [PerCP]-Cy5.5]), CD8 (APC), CD20 (fluorescein isothiocyanate [FITC]), CD28 (FITC), and CD95 (APC). Finally, cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed using CellQuest Pro (BD Biosciences) and Flow Jo (Tree Star Inc., Ashland, OR) software.

RESULTS

Vpu from HIV-1 DH12 can antagonize rhesus BST-2. SHIVs have been used as challenge viruses in vaccine experiments to assess the potency of candidate immunogens designed to elicit antiviral neutralizing antibodies because they carry the HIV-1 *env* gene. They are usually constructed by inserting a gene segment, which may encode a portion of Vpr and all of the Tat, Rev, Vpu, and Env proteins, from a particular HIV-1 isolate into the genetic background of SIV_{mac239} (20, 43). In this study, we have used X4-tropic $SHIV_{DH12}$ and R5-tropic $SHIV_{AD8}$, which are phenotypically distinct. The intravenous inoculation of rhesus macaques with large doses (>2,500 50% tissue culture infective doses [$TCID_{50}$s]) of the X4-tropic molecular clone $SHIV_{DH12\text{-}CL7}$ (43), derived from $SHIV_{DH12}$ (19, 20, 52), results in high sustained levels of plasma viremia; the rapid, unrelenting, and systemic depletion of naïve and memory $CD4^+$ T cells; and death from immunodeficiency in 3 to 6 months. In contrast, a new pathogenic R5-tropic SHIV clone ($SHIV_{AD8\text{-}MV}$), derived from the recently described $SHIV_{AD8}$ (38), maintains variable plasma viral loads (10^2 to 10^5 RNA copies/ml), exclusively targets memory $CD4^+$ T lymphocytes, and causes clinical symptoms of AIDS at 1 to 2 years postinfection (p.i.).

To ascertain whether the SHIV *vpu* gene might contribute to its replication phenotype in macaque cells, wild-type (wt) X4- and R5-utilizing SHIVs and their Vpu mutant derivatives were used to infect rhesus monkey PBMCs. The X4-tropic molecular clone $SHIV_{DH12\text{-}CL7}$ (43), derived from $SHIV_{DH12}$ (20, 52), and a new pathogenic R5-tropic SHIV clone ($SHIV_{AD8\text{-}MV}$), derived from the recently described $SHIV_{AD8}$ (38), were used in these studies. Two types of Vpu mutants were constructed for each SHIV molecular clone (Fig. 1A). The Vpu Udel mutants retain the Vpu initiation codon but carry an out-of-frame deletion eliminating *vpu* gene sequences upstream of the *env* gene. These mutants were modeled on a natural deletion present in the *vpu* gene of the $HIV\text{-}1_{NY5}$ isolate (4, 26). The Urd mutants express a Vpu protein carrying a randomized Vpu TM domain. These mutants were constructed to specifically inactivate Vpu-mediated particle-releasing activity but not the CD4 downregulation function (46). As shown in Fig. 1B, the amino acid sequences of the parental $HIV\text{-}1_{DH12}$ and $HIV\text{-}1_{Ada}$ Vpu TM regions differ from one another as well as from the sequence of the $HIV\text{-}1_{NL4\text{-}3}$ Vpu prototype. With respect to the latter comparison, the $HIV\text{-}1_{DH12}$ Vpu differs from the $HIV\text{-}1_{NL4\text{-}3}$ Vpu at 20 amino acid (aa) positions, including 6 located in the TM region. $HIV\text{-}1_{AD8}$ Vpu differs from $HIV\text{-}1_{NL4\text{-}3}$ at 17 aa residues, 6 of which map to the TM domain. In addition, 1 and 2 amino acid changes in the Vpu TM region, associated with the animal passaging used to generate pathogenic SHIVs, were present in the $SHIV_{DH12\text{-}CL7}$ and $SHIV_{AD8\text{-}MV}$ Vpu proteins, respectively.

Prior to infecting macaque PBMCs, we assessed the BST-2 expression by fluorescence-activated cells sorter (FACS) analysis and found that $CD4^+$ T lymphocytes from four rhesus monkey donors expressed cell surface BST-2 (Fig. 2A). To determine whether the expression of Vpu might modulate virus production during spreading infections in macaque T cells, rhesus PBMCs were infected with wt $SHIV_{DH12\text{-}CL7}$ or Vpu mutant derivatives. The results of two of three independent experiments are shown in Fig. 2B (top panels). The results indicated that both of the Vpu mutant viruses (Udel, Urd) released less progeny virions into the supernatant medium than the wt SHIV, suggesting that the Vpu protein was functioning in monkey cells. Of note, the difference in peak RT activities of wt and Vpu-defective viruses was relatively small and in the range of 2-fold. These results are consistent with the reported small 2- to 3-fold effects of Vpu on HIV-1 release during virus spread in human PBMCs and likely reflect an inherently inefficient inhibitory effect of BST-2 on virus release in these cells (46, 48). A similar result was obtained with wt and Vpu mutants of the R5-tropic $SHIV_{AD8\text{-}MV}$, although the differences between the amounts of wt and Vpu-minus viruses in the medium were not as great (Fig. 2, bottom panels). To ascertain whether the *in vivo* passaging, which attended the generation of the pathogenic SHIVs and had resulted in minor alterations in their Vpu TM domains (shown by red letters in Fig. 1B), was responsible for the Vpu-mediated enhanced replication in rhesus PBMCs, SHIV derivatives containing the parental HIV-1 Vpu TM regions were constructed (TM HIV_{DH12} and TM HIV_{AD8}). Interestingly, these SHIV derivatives released similar amounts of progeny virions as the passaged wt SHIV, indicating that the *vpu* gene in the original HIV-1 isolates was also active in rhesus cells. Because each SHIV isolate encoded a distinct Vpu TM domain and the $SHIV_{DH12\text{-}CL7}$ Vpu appeared to have higher activity in macaque PBMCs, a

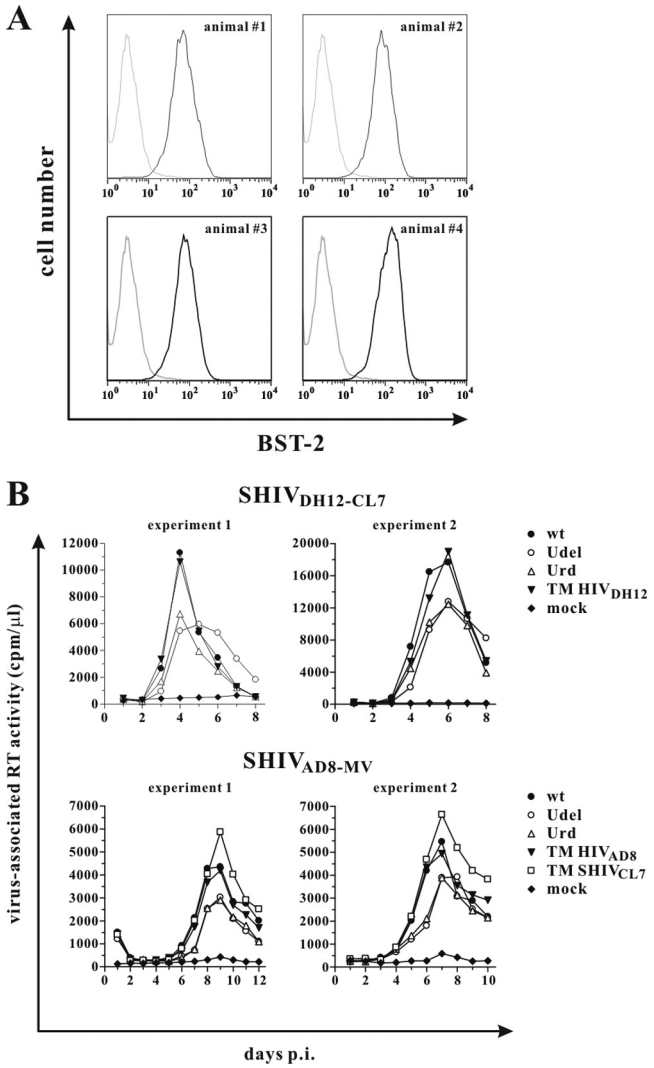

FIG. 2. Vpu-deficient SHIVs produce less cell-free virus in rhesus PBMCs than wt SHIVs. (A) CD4$^+$ T cells were isolated from PBMCs of four rhesus macaques and processed for FACS analysis as described in Materials and Methods. Black lines, BST-2 staining; gray lines, isotype controls. (B) ConA-activated rhesus PBMCs were infected with equal reverse transcriptase units of SHIV$_{DH12-CL7}$ variants (top) or SHIV$_{AD8-MV}$ variants (bottom). All virus stocks were produced in transfected 293T cells. Virus replication was monitored by measuring the virus-associated RT activity in the culture supernatants over time.

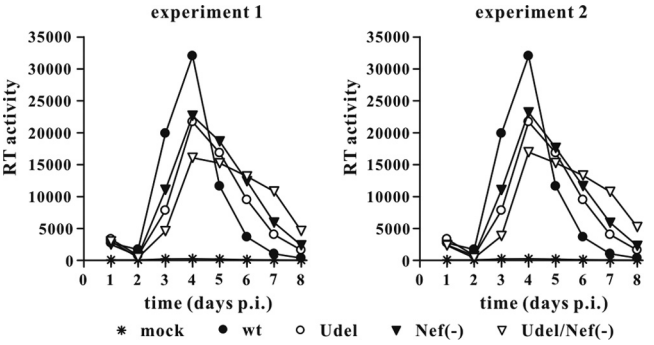

FIG. 3. SIV Nef and HIV-1 Vpu contribute equally to the antagonism of rhesus BST-2. ConA-activated rhesus PBMCs were infected with equal reverse transcriptase units of SHIV$_{DH12-CL7}$ wt or variants defective in Vpu (Udel) or Nef [Nef(−)] or lacking both Vpu and Nef [Udel/Nef(−)]. All virus stocks were produced in transfected 293T cells. Virus replication was monitored by measuring the virus-associated RT activity in the culture supernatants over time.

SHIV$_{AD8-MV}$ derivative bearing a chimeric *vpu* gene consisting of the SHIV$_{DH12-CL7}$ Vpu TM region and the SHIV$_{AD8-MV}$ Vpu cytoplasmic domain (TM SHIV$_{CL7}$) was constructed. The Vpu TM domain of SHIV$_{DH12-CL7}$ differs from that of AD8-MV in six positions (A_{16}L, L_{18}T, A_{19}V, G_{21}V, T_{24}S, F_{27}L; Fig. 1B). As shown in Fig. 2B, the SHIV isolate carrying the chimeric TM SHIV$_{CL7}$ *vpu* gene released higher levels of virus than wt SHIV$_{AD8-MV}$. Thus, the differences between SHIV$_{DH12-CL7}$ and SHIV$_{AD8-MV}$ with respect to their relative resistance to rhesus BST-2 could be due to differences in the TM domains of their Vpu proteins. Taken together, these results indicate that two SHIVs carrying *vpu* genes from primary HIV-1 isolates release larger amounts of progeny virions during spreading infections in rhesus PBMCs than their Vpu-minus derivatives. This augmented phenotype appears to map to the Vpu TM domain.

SIV Nef and DH12 CL7 Vpu contribute equally to functional inhibition of rhesus BST2. Several recent studies have reported that the Nef proteins of some SIV strains, including SIV$_{mac239}$, have Vpu-like activity because they can antagonize monkey BST-2 and facilitate virus release from BST-2-positive monkey cells (24, 45, 63). Our SHIV constructs are based on SIV$_{mac239}$ and are expected to express a functional Nef protein. To assess the relative contribution of SIV$_{mac239}$ Nef and HIV-1 Vpu to virus replication in rhesus PBMCs, we constructed two SHIV variants carrying a defective *nef* gene in the context of either SHIV$_{DH12-CL7}$ or SHIV$_{DH12-CL7}$/Udel, as described in Materials and Methods. Virus stocks of wt SHIV$_{DH12-CL7}$ and the Vpu-defective variant (Udel), as well as a Nef-deficient (Nef$^-$) variant and the Vpu/Nef double mutant (Udel/Nef$^-$), were produced in 293T cells and virus replication in rhesus PBMCs was monitored for 8 days (Fig. 3). Two independent infections by Nef$^-$ and Udel/Nef$^-$ viruses are shown (experiment 1 and experiment 2). Interestingly, inactivation of HIV-1 Vpu or SIV$_{mac239}$ Nef had very similar effects on virus replication and resulted in reduced peak RT values in both experiments. Of note, inactivation of both Vpu and Nef led to a further reduction in peak RT values, indicating that the antagonistic effects of Vpu and Nef on rhesus BST-2 are additive. The knockout of Nef did not affect viral replication kinetics, and the Nef/Vpu double mutant revealed only a slight delay in the replication profiles. This suggests that SIV Nef, like its HIV-1 Vpu counterpart, inhibits an activity of rhesus BST-2 that impairs the release of cell-free virions but does not inhibit cell-to-cell spread of the virus. We conclude that in the context of SHIV$_{DH12-CL7}$, Vpu and Nef contribute equally and in an additive manner to the release of particles from rhesus PBMCs.

SHIV Vpu proteins can counteract human and rhesus BST-2. To examine whether Vpu-mediated SHIV production in macaque cells was associated with antagonism of rhesus BST-2, the BST-2-null human cell line 293T was cotransfected with HIV-1 or SHIV carrying wt, mutant, or chimeric *vpu*

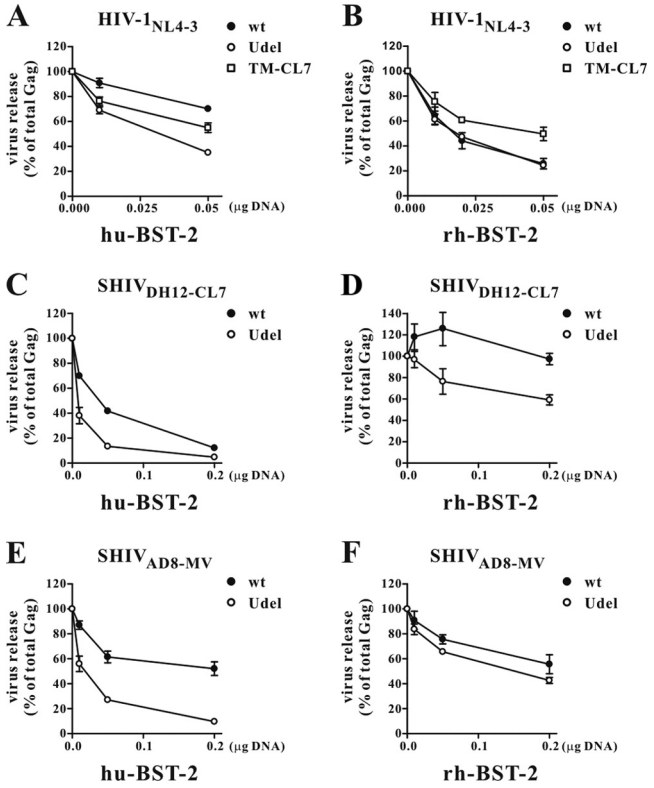

FIG. 4. SHIV Vpu proteins can counteract human (hu) and rhesus (rh) BST-2. (A and B) Analyses of HIV-1_{NL4-3} derivatives against human BST-2 (A) and rhesus BST-2 (B); (C and D) analyses of $SHIV_{DH12\ CL7}$ derivatives against human BST-2 (C) and rhesus BST-2 (D); (E and F) analyses of $SHIV_{AD8-MV}$ derivatives against human BST-2 (E) and rhesus BST-2 (F). Cells were metabolically labeled for 90 min with [^{35}S]methionine, and cell lysates and cell-free supernatants were subjected to immunoprecipitation by an HIV-positive patient serum sample or serum from an SHIV-infected monkey. Immunoprecipitates were subjected to SDS-PAGE, and virus release was quantified by PhosphoImage analysis using a Fujifilm FLA7000 system. Virus release was calculated independently for each sample by determining the percentage of cell-free CA protein relative to the total amount of intra- and extracellular Gag protein. Virus release in the absence of BST-2 (0 μg) was defined as 100% for each sample.

genes plus increasing amounts of plasmids expressing human or rhesus BST-2. Cells were labeled for 90 min with [^{35}S]methionine at 24 h posttransfection, and cell lysates or cell-free virus-containing supernatants were immunoprecipitated, using an HIV-1-positive patient serum sample or plasma from SHIV-infected macaques. Immunoprecipitated viral proteins were separated by SDS-PAGE and quantitated by PhosphoImage analysis, and virus release was calculated as the percentage of Gag protein in the supernatant medium compared to the total amount of intra- plus extracellular Gag protein.

Not unexpectedly, wt HIV-1_{NL4-3} Vpu was able to counteract human BST-2 but not rhesus BST-2 (Fig. 4A and B), as previously reported by several laboratories (10, 14, 15, 24, 32, 41, 45, 58, 61, 62). Interestingly, and consistent with the results of the PBMC infectivity assays, $SHIV_{DH12-CL7}$, bearing the wt *vpu* gene, released considerably more progeny virions than the Vpu mutant SHIV in the presence of large amounts of rhesus BST-2 (Fig. 4D). Since the Vpu TM domain confers virus-releasing activity, we wondered whether HIV-1_{NL4-3}, carrying a chimeric Vpu containing the TM region from $SHIV_{DH12-CL7}$, would better antagonize rhesus BST-2. As shown in Fig. 4B, $HIV_{NL4-3(TM-CL7)}$ did, in fact, generate augmented levels of supernatant particles compared to wt HIV-1_{NL4-3} in the presence of rhesus BST-2. The capacity of $SHIV_{AD8-MV}$ Vpu to counteract rhesus BST-2 was also evaluated. Although the wt *vpu* gene in $SHIV_{AD8-MV}$ consistently exhibited more activity against rhesus BST-2 than the mutant *vpu* gene, it was clearly not as potent as the $SHIV_{DH12-CL7}$ Vpu in counteracting rhesus BST-2 (compare Fig. 4D and F). This difference was also previously observed in rhesus PBMC infectivity assays. The higher basal particle-releasing activities observed with both SHIV Vpu Udel mutants in the presence of rhesus BST-2 are worth noting. This could reflect an SIV Nef (present in both SHIV plasmids)-mediated anti-rhesus BST-2 effect in this assay system.

Vpu-deficient SHIVs are less pathogenic in rhesus macaques than their wt counterparts. The attenuated replicative phenotype observed with $SHIV_{DH12-CL7}$ and $SHIV_{AD8-MV}$ mutants in rhesus PBMCs raised the possibility that these HIV-1 *vpu* genes might enhance infectivity and disease progression in macaques inoculated with these SHIVs. It should be noted that pathogenic X4-tropic SHIV infections of rhesus monkeys are typically dose dependent: (i) with large inoculum sizes (e.g., 1,000 to 5,000 $TCID_{50}$s), plasma viral loads are sustained (10^5 to 10^7 RNA copies/ml), CD4$^+$ T cell numbers rapidly and irreversibly fall to low levels, and animals succumb to immunodeficiency at 12 to 20 weeks p.i.; and (ii) following exposure to small inoculum sizes, peak levels of plasma viremia are durably controlled, CD4$^+$ T cell counts return to or remain at preinfection levels, and infected animals rarely develop symptomatic disease (21, 43). Normally progressing R5-tropic $SHIV_{AD8}$-infected macaques usually generate high peak virus loads, lower sustained levels of plasma viremia, and a gradual loss of CD4$^+$ T lymphocytes and disease development (38).

As shown in Fig. 5A and B, four historic wt $SHIV_{DH12-CL7}$-infected control animals (30) inoculated intravenously with 5,000 $TCID_{50}$s of virus experienced the expected high levels of plasma viremia and rapid depletion of their CD4$^+$ T cells. Four other macaques were newly inoculated with 10,000 $TCID_{50}$s of Vpu-defective $SHIV_{DH12-CL7}$ (Udel and Urd, shown in red in Fig. 5A and B). One of these monkeys (RHDBCK) exhibited a clinical course indistinguishable from that of the four animals inoculated with the wt $SHIV_{DH12-CL7}$ (shown in black in Fig. 5A and B). Two of the three remaining macaques (RHA4E029 and RHDBAI) experienced somewhat lower levels of set-point viremia, more gradual losses of CD4$^+$ T lymphocytes, and delayed death from AIDS at weeks 40 and 44 p.i., respectively. The third monkey (RHBEGA), also infected with Vpu-deficient X4 $SHIV_{DH12-CL7}$, is asymptomatic at week 74 p.i. with undetectable plasma viral RNA loads and little change in the number of CD4$^+$ T cells measured prior to inoculation. Of the four macaques inoculated with the Vpu-defective R5-tropic $SHIV_{AD8-MV}$, three currently have undetectable levels of plasma viremia and one (RHDBGC) has viral RNA loads similar to those of animals infected with wt virus (Fig. 5C). CD4$^+$ T cell numbers in macaque RHDBGC are lower than preinoculation values (data not shown). Taken together, these results indicate that X4- and R5-tropic SHIVs

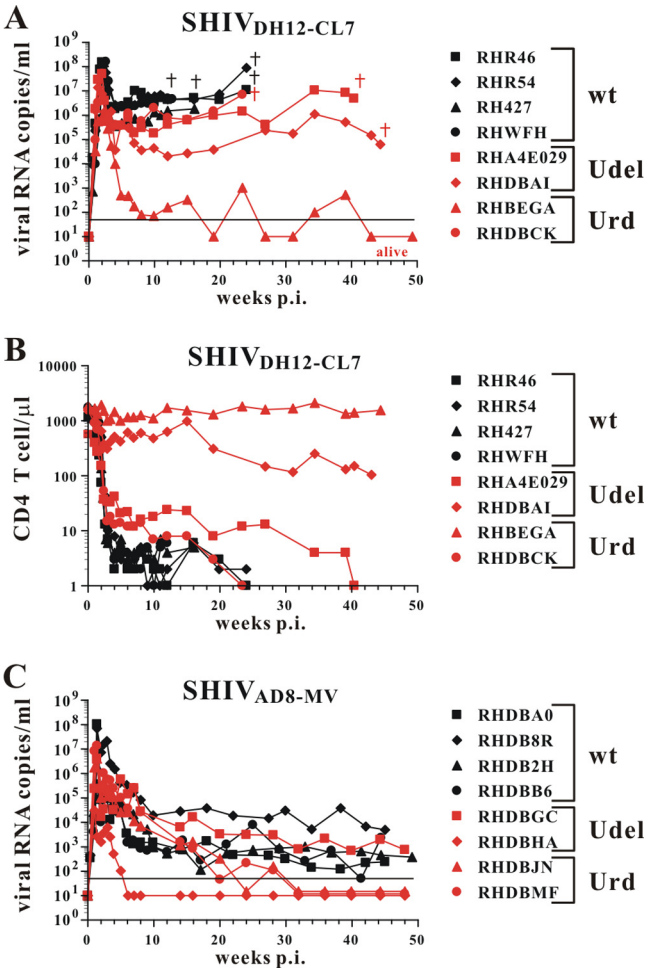

FIG. 5. Vpu-deficient SHIVs are less pathogenic in rhesus macaques than their wt counterparts. A group of four rhesus macaques each was infected with the X4-tropic wt or Udel $SHIV_{DH12-CL7}$ (A and B) or the R5-tropic $SHIV_{AD8-MV}$ (C). The levels of plasma viremia were monitored over a period of 45 weeks (A and C), and absolute numbers of peripheral $CD4^+$ T cells in animals infected by the X4-tropic viruses are shown (B). Euthanized monkeys are indicated (†).

carrying mutant *vpu* genes exhibit an attenuated replication phenotype *in vivo* compared to wt viruses.

DISCUSSION

Like other lentiviral accessory proteins, HIV-1 Vpu is multifunctional and has evolved to regulate several independent activities important for HIV replication and disease induction. Initially, Vpu was shown to enhance virion release from infected cells, a property that likely facilitates the dissemination of virus systemically (54, 55). It was subsequently noted that Vpu was also able to induce the degradation of the receptor for HIV-1 (CD4), a property that may augment the production of infectious virus particles by preventing the formation of intracellular, trafficking-incompetent, CD4-Env complexes (59). Finally, Vpu was found to induce host cell apoptosis (2). The role of Vpu-mediated host cell apoptosis for optimal HIV-1 replication is not immediately obvious, and host cell apoptosis may represent an unintended side effect of Vpu-induced CD4 degradation since both mechanisms involve binding to and sequestration of β-TrCP (6, 31). While there is still an ongoing debate about which of the reported Vpu functions is most relevant to virus replication and disease induction *in vivo*, the recent identification of BST-2 as a Vpu substrate has refocused attention on the regulation of virion release.

The precise mechanism responsible for the Vpu antagonism of BST-2, thereby enhancing virus release, is still unresolved. However, there is increasing evidence indicating that Vpu functions intracellularly to prevent the surface transport of BST-2 (3, 10, 11, 17, 33, 53). It is interesting to note that Vpu is not ubiquitously expressed by all primate lentiviruses but is unique to HIV-1 and some SIVs (SIV_{cpz}, SIV_{gor}, and SIV_{gsn}). HIV-2 and most other SIVs do not encode a Vpu protein to counter BST-2. Instead, these viruses express Vpu-like activities in their *env* and *nef* genes, respectively (5, 7, 16, 17, 24, 45, 47, 49, 63). This suggests that regulation of virus release is a conserved property of lentiviruses and as such must be critical for virus replication. This is supported by the observation that compensatory changes in the cytoplasmic tail of gp41 confer resistance to BST-2 in a pathogenic SIV strain from which *nef* is deleted (49). Up to now it had been generally presumed that HIV-1 Vpu could antagonize only human and great ape BST-2 variants, a premise that was primarily based on studies conducted with the Vpu encoded by the prototypic $HIV\text{-}1_{NL4-3}$ isolate (14, 24, 32, 45, 58, 61, 62). In this regard, $HIV\text{-}1_{NL4-3}$ was originally constructed by ligating the 5′ half (long terminal repeat [LTR]-*gag-pol-vif-vpr* sequences) of the $HIV\text{-}1_{NY5}$ isolate to the 3′ half (*vpr-tat-rev-vpu-env-nef*-LTR sequences) of $HIV\text{-}1_{LAV}$ at the single EcoRI site located in the *vpr* gene (1). Thus, the source of $HIV\text{-}1_{NL4-3}$ Vpu is $HIV\text{-}1_{LAV}$. Interestingly, Ruiz et al. recently reported that $SHIV_{KU-2MC4}$, encoding the *vpu* gene from $HIV\text{-}1_{HXB2}$, also a derivative of $HIV\text{-}1_{LAV}$, failed to antagonize pig-tailed macaque BST-2 in virion release assays or TZM-bl infectivity assays (42). This result is consistent with our failure to show any inhibition of rhesus macaque BST-2 using the Vpu from $HIV\text{-}1_{NL4-3}$ (derived from the $HIV\text{-}1_{LAV}$ Vpu) (Fig. 3B). Taken together, these results reiterate the failure of the $HIV\text{-}1_{NL4-3}$ Vpu to antagonize monkey BST-2, whether it is evaluated as a component of HIV-1 or SHIV.

It is remarkable that Vpu encoded by $SHIV_{DH12-CL7}$ enhanced virus release, despite the presence of a functional *nef* gene. Indeed, the results shown in Fig. 3 indicate that neither SIV_{mac239} Nef nor DH12 Vpu expressed in the context of $SHIV_{DH12-CL7}$ is capable of fully antagonizing rhesus BST-2 in primary rhesus PBMCs. Instead, the two proteins appear to function additively to provide maximal virus release. It should be pointed out that the potent antagonism of the $SHIV_{DH12-CL7}$ Vpu against both simian and human BST-2 may reflect the way in which the parental $HIV\text{-}1_{DH12}$ was identified and selected for use in animal studies. In an attempt to generate an HIV-1 chimpanzee disease model in 1995, 23 primary isolates from AIDS patients were screened for their capacity to rapidly spread and release high levels of progeny virions from chimpanzee PBMCs (51). $HIV\text{-}1_{DH12}$ was unique among the three chimpanzee-tropic isolates obtained because of its ability to infect cultured PBMCs from 25 of 25 chimpanzee donors tested. Although $HIV\text{-}1_{DH12}$ failed to maintain detectable levels of plasma viremia or induce disease in inoculated chimpan-

zees, it was the source of the HIV-1 *vpu* gene sequences present in $SHIV_{DH12}$ (52) and its molecularly cloned and pathogenic derivative $SHIV_{DH12-CL7}$ (43). The relationship between replication capacity in chimpanzee cells and inhibition of macaque BST-2 is not presently understood.

There have been very few studies evaluating Vpu function during nonhuman primate lentivirus infections *in vivo*. The earliest, using first-generation nonpathogenic SHIVs carrying wt Vpu (from $HIV\text{-}1_{BH10}$) or mutant Vpu (from $SHIV_{HXBc2}$) to inoculate cynomolgus monkeys, reported somewhat lower levels of circulating virus in animals infected with Vpu-defective SHIVs (29). Nearly a decade later, Hout et al. observed profoundly attenuated replication for a SHIV Vpu mutant (with a scrambled TM region) compared to the wt $SHIV_{KU-1}$ (derived from $SHIV_{HXBc2}$) both *in vitro* (human C8166 cells) and *in vivo* (pig-tailed macaques) (18). In monkeys inoculated with the Vpu-defective SHIV, peak levels of plasma viremia at week 2 p.i. were reduced 50-fold and viral set points were 2 to 3 log units lower at week 24 p.i. than those measured for wt $SHIV_{KU-1}$-infected macaques. In a recent follow-up study, Ruiz et al. reported that the wt Vpu present in $SHIV_{KU-2MC4}$ failed to counteract pig-tailed macaque BST-2 (42). In addition, deletion of Vpu in $SHIV_{KU-2MC4}$ did not affect virus release due to the presence of SIV Nef, which antagonizes monkey BST-2 (24, 45, 61, 63). On the basis of the profound replication defect previously observed with the Vpu-minus $SHIV_{KU-2MC4}$ (18), Ruiz et al. proposed that the Vpu TM domain may possess functional activities unrelated to BST-2 antagonism, which facilitates virus replication *in vivo* (42). Because full-length sequencing of the wt and Vpu mutant $SHIV_{DH12-CL7}$ and $SHIV_{AD8-MV}$ genomes confirmed that each virus was isogenic except for the TM regions of Vpu, we believe that the attenuated phenotype observed in 6 of 8 animals inoculated with Vpu TM mutants in the present study, despite the presence of an intact SIV *nef* gene, is physiologically relevant. Given that replication and disease induction by primate lentiviruses are multigenic and Nef antagonism of simian BST-2 continues in the absence of a functional Vpu in the inoculated monkeys, the subtle defects in maintaining plasma viremia, depleting $CD4^+$ T cells, and disease induction observed could represent the loss of auxiliary particle release activity mediated by Vpu or recently described immunomodulatory functions attributed to Vpu, such as downregulation of Cd1d or NK T- and B-cell antigen (35, 50). While a contribution of Vpu-induced degradation of rhesus CD4 cannot formally be ruled out, the fact that our experiments involving Urd—a Vpu mutant capable of degrading human CD4—yielded results very similar to those observed with a variant lacking Vpu entirely (i.e., Udel) argues against a significant contribution of Vpu-induced CD4 degradation to the observed phenotypic differences.

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