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CELL BIOLOGY

HIV-1 propagation is highly dependent on basal levels of the restriction factor BST2

Balaji Olety[†], Paul Peters, Yuanfei Wu, Yoshiko Usami, Heinrich Göttlinger*

BST2 is an interferon-inducible antiviral host protein antagonized by HIV-1 Vpu that entraps nascent HIV-1 virions on the cell surface. Unexpectedly, we find that HIV-1 lacking Nef can revert to full replication competence simply by losing the ability to antagonize BST2. Using gene editing together with cell sorting, we demonstrate that even the propagation of wild-type HIV-1 is strikingly dependent on BST2, including in primary human cells. HIV-1 propagation in BST2^{-/-} populations can be fully rescued by exogenous BST2 irrespective of its capacity to signal and even by an artificial BST2-like protein that shares its virion entrapment activity but lacks sequence homology. Counterintuitively, our results reveal that HIV-1 propagation is critically dependent on basal levels of virion tethering by a key component of innate antiviral immunity.

INTRODUCTION

HIV-1 encodes two membrane-associated accessory proteins, namely, Vpu and Nef, that counteract cellular antiviral transmembrane proteins. HIV-1 Vpu antagonizes the interferon-inducible host protein BST2 (or tetherin), which entraps nascent virions on the surface of infected cells (1, 2). In contrast to HIV-1, most simian immunodeficiency viruses use Nef to antagonize BST2, whereas HIV-1 Nef is inactive against BST2 (3–6).

BST2 is a dimeric type II transmembrane protein with an N-proximal transmembrane helix, a rod-like extracellular coiled coil, and a C-terminal glycosylphosphatidylinositol (GPI) anchor (7). Both membrane anchors are essential for the inhibition of virus release, suggesting that BST2 directly tethers virions to cells by embedding its membrane anchors simultaneously into host and viral lipid bilayers (8, 9).

In addition to inhibiting virus release, hominid BST2 proteins activate nuclear factor κ B (NF- κ B) and trigger proinflammatory gene expression when clustered by entrapped virions, implying that BST2 acts as an innate virus sensor (10). Furthermore, the BST2-mediated retention of virions increases the susceptibility of HIV-1-infected cells to antibody-dependent cell-mediated cytotoxicity, and the endocytic reuptake of BST2-tethered virions may also enhance cell-mediated immune responses (11).

Unexpectedly, BST2 deficiency had no or only a moderate impact on the spreading and pathogenesis of Moloney murine leukemia virus (Mo-MLV) in mice (12, 13). However, a different MLV that induced type I interferon and, thus, BST2 expression clearly replicated better in mice lacking BST2, indicating that BST2 is indeed an antiviral protein (12). Unexpectedly, the replication of other enveloped viruses was initially reduced in mice lacking BST2, suggesting that, in certain cases, BST2 facilitates virus spreading in vivo (13).

It has been argued that BST2 is a modulator of viral dissemination rather than a restriction factor, because in spreading infection assays, Vpu-deficient HIV-1 exhibited reduced levels of cell-free virus but not reduced replication kinetics, indicating efficient spreading by cell-to-cell transmission (14). We have now observed that loss of

Vpu mutations greatly accelerates the replication of Nef-deficient HIV-1 in cells in which it is normally highly restricted. Because this observation suggested that BST2 down-modulation can be detrimental to HIV-1 propagation, we examined HIV-1 replication in pure BST2^{+/+} and BST2^{-/-} cell populations obtained by CRISPR-Cas9-mediated gene editing and cell sorting. Our results reveal that HIV-1 propagation is remarkably dependent on BST2, both in cell lines and in primary cells, and irrespective of co-receptor usage. Together, our results indicate that the virion-tethering activity of BST2 is essential for its role in HIV-1 spreading, whereas BST2-mediated signaling is dispensable. Hence, while BST2 antagonism is likely critical for immune evasion, our findings imply that HIV-1 propagation depends on the maintenance of a basal level of this restriction factor.

RESULTS

Rescue of Nef-deficient HIV-1 through loss of Vpu

We recently reported that HIV-1 replication in MOLT-3 cells remains highly dependent on Nef even in the absence of the antiviral Nef targets SERINC3 and SERINC5 (15). To obtain a revertant, we passaged Nef⁻ HIV-1_{NL4-3} in MOLT-3 SERINC3/5 knockout cells until prominent cytopathic effects were observed. Progeny virus was then used to infect unmodified MOLT-3 cells. In parallel, MOLT-3 cells were infected with an equal amount of Nef⁺ or Nef⁻ HIV-1_{NL4-3} freshly produced by 293T cells transfected with HIV-1 proviral DNA. As expected, Nef robustly enhanced the spreading of 293T-derived HIV-1, as judged from Gag expression levels on day 8 after infection (Fig. 1A). While Gag remained undetectable in the culture infected with the 293T-derived Nef⁻ virus, the passaged Nef⁻ virus replicated even better than the 293T-derived wild-type (WT) virus (Fig. 1A). The passaged virus remained unable to express Nef (Fig. 1A), indicating that a compensatory mutation allowed efficient replication even in the absence of Nef.

To determine the molecular basis for the revertant phenotype, HIV-1 sequences amplified from cells infected with the passaged virus were used to replace the corresponding regions of Nef⁻ HIV-1_{NL4-3}. Two Nef-deficient recombinant proviral clones designated Nef⁻/R0-1 and Nef⁻/R0-2, whose *vpu* and *env* genes were entirely from the passaged virus, replicated vigorously in MOLT-3 cells (Fig. 1B). DNA sequencing revealed that both clones had the *vpu* gene disrupted by a mutation that changed the *vpu* initiation codon

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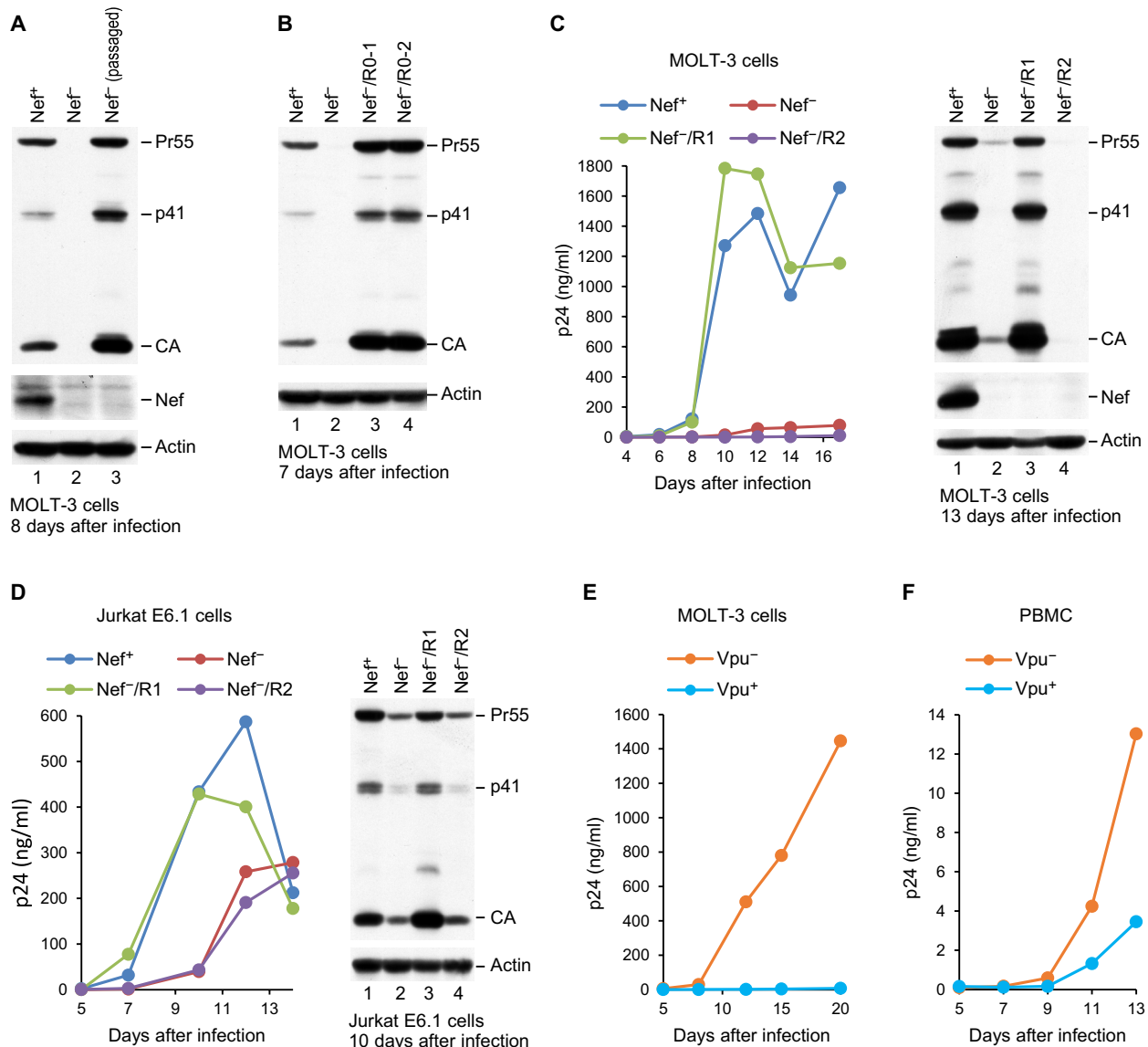


Fig. 1. Rescue of Nef-deficient HIV-1 through loss of Vpu. (A) Western blots showing that Nef-deficient HIV-1 gained the ability to propagate efficiently in MOLT-3 cells after several passages. The cells were infected with equal amounts (0.2 ng/ml p24) of Nef⁺ or Nef⁻ HIV-1_{NL4-3} freshly produced in 293T cells or of Nef⁻ HIV-1_{NL4-3} that had been passaged in MOLT-3 SERINC3/5 knockout cells. Cell lysates were examined with anti-capsid (CA) monoclonal antibody (mAb) and anti-Nef antiserum 8 days after infection. (B) Western blots showing that *vpu* and *env* sequences from the passaged virus allow Nef⁻ HIV-1_{NL4-3} to replicate vigorously in MOLT-3 cells. (C and D) The Nef⁻/R1 recombinant, which harbors the disrupted *vpu* gene of the passaged virus, but not the Nef⁻/R2 recombinant, which harbors its mutant *env* gene, replicates, as well as Nef⁺ HIV-1_{NL4-3} in MOLT-3 (C) and Jurkat E6.1 cells (D). Virus propagation was monitored by measuring p24 in the culture supernatants and by Western blotting of cell lysates. (E) A disrupted *vpu* initiation codon allows the naturally Nef-deficient HXBH10 strain to propagate in MOLT-3 cells, which are nonpermissive for Vpu⁺ HXBH10. (F) The Vpu⁻ version of HXBH10 also replicates with moderately accelerated kinetics in primary human cells. PBMC, peripheral blood mononuclear cells.

to ATA and by a premature termination codon (TAA) in place of *vpu* codon 28. Since both clones also harbored identical mutations in *env*, we next examined versions of Nef⁻ HIV-1_{NL4-3} that harbored only the mutations in *vpu* (Nef⁻/R1) or in *env* (Nef⁻/R2). Despite being unable to express Nef, the Nef⁻/R1 mutant replicated at least as well as WT (Vpu⁺/Nef⁺) HIV-1_{NL4-3} in MOLT-3 cells (Fig. 1C), demonstrating that a disrupted *vpu* gene was sufficient to fully correct the pronounced replication defect of Nef⁻ HIV-1 in these cells.

Since it has been shown that Vpu-deficient HIV-1 exhibits hypersensitivity to interferon- α (IFN- α) (16), we examined whether

disabling Vpu also rescues Nef-deficient HIV-1 in the presence of IFN- α and found this to be the case (fig. S1). However, in agreement with previous observations (16), the release of p24 antigen over time after infection of MOLT-3 cells with the Vpu-deficient Nef⁻/R1 mutant proved to be more sensitive to IFN- α than the release of WT HIV-1_{NL4-3} (fig. S1).

Unlike in MOLT-3 cells, Nef enhances HIV-1 replication in Jurkat cells mainly by counteracting SERINC3 (15). Nevertheless, as in MOLT-3 cells, the Nef⁻/R1 mutant replicated with WT kinetics in Jurkat E6.1 cells, whereas the Nef⁻/R2 mutant replicated as poorly

as the parental Nef⁻ virus (Fig. 1D). To confirm that the accelerated replication in the absence of Nef was due to the disruption of *vpu*, we also used a different pair of Nef-deficient proviruses denoted HXBH10(*vpu*⁺) and HXBH10(*vpu*⁻) (17, 18). These proviruses are based on the naturally Nef-deficient HIV-1_{HXB2} and differ only at the position of the *vpu* initiation codon (ATG or ACG). As expected for a Nef⁻ strain (15), HXBH10(*vpu*⁺) failed to replicate in MOLT-3 cells (Fig. 1E). In contrast, the otherwise isogenic Vpu⁻ virus replicated vigorously in these cells (Fig. 1E). The Vpu⁻ virus also replicated with moderately accelerated kinetics in phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs) (Fig. 1F).

Vpu and Env are expressed from a bicistronic mRNA, and a point mutation in the *vpu* initiation codon can result in elevated Env expression levels (19), which could conceivably increase the infectivity of progeny virions. In contrast, disabling Vpu without altering the *vpu* initiation codon did not affect Env expression (19). Since the Nef⁻/R1 mutant and HXBH10(*vpu*⁻) both harbor a mutated *vpu* initiation codon, we additionally tested a 7-nucleotide (nt) deletion in *vpu* (*vpu*Δ7) that arose in an independently obtained revertant of Nef⁻ HIV-1_{NL4-3}. The *vpu*Δ7 deletion removes nucleotides 103 through 109 of HIV-1_{NL4-3} *vpu* and preserves the first 34 *vpu* codons, which are followed by a missense mutation and a premature termination codon. A Nef-deficient recombinant proviral clone that harbors the *vpu*Δ7 deletion but is otherwise identical to Nef⁻ HIV-1_{NL4-3} replicated even better than WT HIV-1_{NL4-3} in MOLT-3 cells, whereas Nef⁻ HIV-1_{NL4-3} with an intact *vpu* gene again failed to replicate (fig. S2A). Similarly, a single point mutation (termed *vpu*PTC) that replaced *vpu* codon 28 (GAA) with a premature termination codon (TAA) was sufficient to fully rescue the replication of Nef⁻ HIV-1_{NL4-3} in MOLT-3 cells (fig. S2B). Thus, disabling Vpu did rescue Nef⁻ HIV-1_{NL4-3} even when the *vpu* initiation codon was maintained. Overall, we conclude that Vpu can profoundly inhibit HIV-1 replication under conditions where replication is attenuated, such as in the absence of Nef.

HIV-1 propagation is highly dependent on the Vpu target BST2

The observation that the replication of an attenuated HIV-1 mutant was enhanced in the absence of the BST2 antagonist Vpu raised the possibility that BST2 may, at least under certain circumstance, facilitate HIV-1 spreading. To examine this possibility, MOLT-3 cells were nucleofected with Cas9 and a single guide RNA (sgRNA) targeting BST2 (sgRNA TS1), kept in culture until distinct BST2^{+/+} and BST2^{-/-} populations emerged, and subjected to fluorescence-activated cell sorting (FACS) to obtain the BST2^{+/+} (TS1) and BST2^{-/-} (TS1) subpopulations (Fig. 2A). Flow cytometry (Fig. 2A) and Western blotting (fig. S3) confirmed that the BST2^{+/+} (TS1) subpopulation and the parental unsorted cells expressed BST2 at comparable levels, whereas the BST2^{-/-} (TS1) subpopulation expressed very little BST2. Notably, the two subpopulations expressed identical levels of CD4 and CXCR4 and of the adhesion molecule CD11a [lymphocyte function-associated antigen-1 (LFA-1) alpha], which has been implicated in HIV-1 spreading (fig. S4A) (20). Nevertheless, in spreading infection experiments, the BST2^{-/-} (TS1) subpopulation reproducibly was markedly less permissive for WT (Vpu⁺/Nef⁺) HIV-1_{NL4-3} than the BST2^{+/+} (TS1) subpopulation (Fig. 2, B and C).

Since flow cytometry suggested a small amount of residual BST2 expression on the BST2^{-/-} (TS1) MOLT-3 subpopulation (Fig. 2A), it was subjected to a second round of gene editing with a different

sgRNA (sgRNA TS2) targeting BST2. Subsequent FACS yielded the BST2^{-/-} (TS1/2) subpopulation, which lacked residual BST2 detectable by flow cytometry (Fig. 2A), but expressed CD4, CXCR4, and CD11a at levels identical to those on the parental cells (fig. S4A). The BST2^{-/-} (TS1/2) subpopulation was even less permissive for WT HIV-1_{NL4-3} than the BST2^{-/-} (TS1) subpopulation (Fig. 2C). Furthermore, in contrast to the BST2^{-/-} (TS1) subpopulation, the twice-sorted subpopulation expressed essentially no HIV-1 Gag as late as 26 days after infection (Fig. 2D). These observations imply that even the minor amount of BST2 that remained on the surface of the BST2^{-/-} (TS1) subpopulation moderately enhanced HIV-1 spreading in long-term cultures.

In a separate experiment, we directly compared the abilities of Vpu⁺/Nef⁺, Vpu⁺/Nef⁻, Vpu⁻/Nef⁺, and Vpu⁻/Nef⁻ versions of HIV-1_{NL4-3} to replicate in parental (BST2^{+/+}) MOLT-3 cells and in the BST2^{-/-} (TS1) and BST2^{-/-} (TS1/2) subpopulations. As expected, Vpu⁺/Nef⁺ (WT) HIV-1_{NL4-3} replicated much faster than the Vpu⁺/Nef⁻ virus in the parental MOLT-3 cells, as judged from the release of p24 antigen over time (fig. S5A) and from Gag expression levels in the infected cells (fig. S5B). However, a very small amount of virus production became evident 2 weeks after infection of the parental BST2^{+/+} cells with the Vpu⁺/Nef⁻ virus when p24 antigen concentrations were plotted on a logarithmic scale (fig. S5A). In contrast, no p24 antigen release at all could be detected in the BST2^{-/-} subpopulations infected with the Vpu⁺/Nef⁻ virus (fig. S5A). On the other hand, Vpu⁻/Nef⁺ HIV-1_{NL4-3} replicated with clearly accelerated kinetics in the parental BST2^{+/+} cells, and even in the BST2^{-/-} subpopulations, compared to the Vpu⁺/Nef⁺ WT virus (fig. S5A). However, even the Vpu⁻/Nef⁺ virus replicated far less efficiently in the BST2^{-/-} (TS1) subpopulation than in the parental cells, based on both p24 antigen release (fig. S5A) and Gag protein expression levels in the infected cells (fig. S5B, compare lanes 7 and 8). Furthermore, the Vpu⁻/Nef⁺ virus replicated even slower in the twice-sorted BST2^{-/-} (TS1/2) subpopulation (fig. S5A), suggesting that residual BST2 expression on the sorted subpopulations played at least some role in its spreading. The Vpu⁻/Nef⁺ virus also exhibited accelerated replication kinetics in BST2^{+/+} MOLT-3 cells compared to the Vpu⁺/Nef⁺ WT virus but did not appear to replicate at all in the BST2^{-/-} subpopulations (fig. S5, A and B).

To examine whether this effect of BST2 on HIV-1 spreading is cell line specific, we additionally subjected Jurkat E6.1 T lymphoid cells to gene editing with sgRNA TS1, followed by FACS. Again, this single sgRNA approach yielded a BST2^{-/-} subpopulation that was largely, albeit not completely, devoid of surface BST2 (Fig. 2E). Alternatively, we used a combination of two sgRNAs designed to target sites upstream (TS3) and downstream (TS4) of the BST2 gene to delete the entire gene. After FACS, the latter approach yielded pure BST2^{+/+} and BST2^{-/-} subpopulations (Fig. 2E). Notably, CD4, CXCR4, and CD11a levels on all subpopulations were comparable (fig. S4B). However, HIV-1 replication in these subpopulations was highly affected by their surface BST2 levels. Whereas WT (Vpu⁺/Nef⁺) HIV-1_{NL4-3} replicated efficiently in the BST2^{+/+} (TS1) and BST2^{+/+} (TS3+4) subpopulations as expected, virus replication was markedly impaired in the corresponding BST2^{-/-} subpopulations (Fig. 2, F and G). No virus replication at all was detected in the BST2^{-/-} (TS3+4) subpopulation during 3 weeks of monitoring. Furthermore, the BST2^{-/-} (TS3+4) subpopulation remained largely refractory to HIV-1 spreading when infections were started with a 10-fold higher amount of virus (Fig. 2, G and H). Overall, these

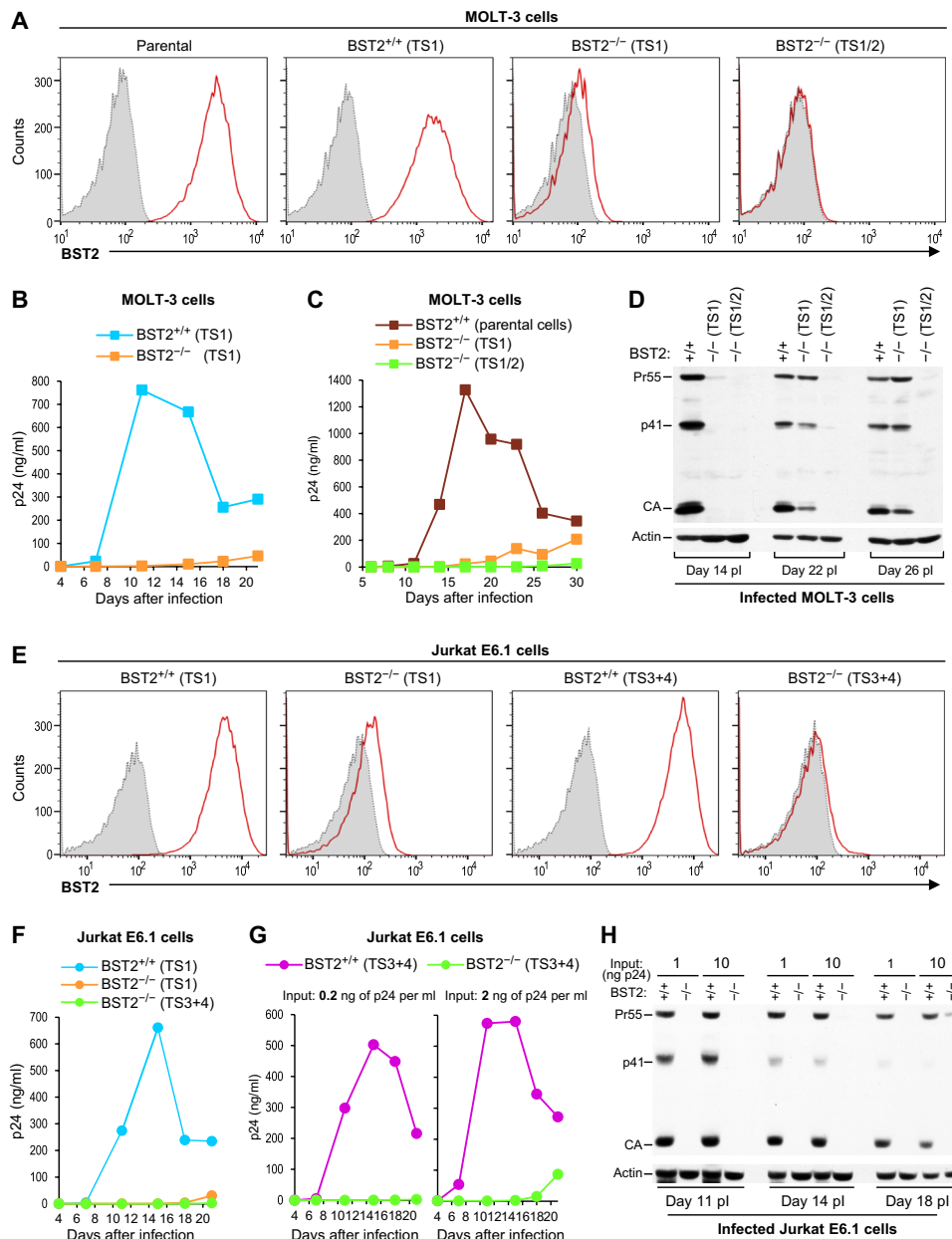


Fig. 2. HIV-1 propagation is highly dependent on the Vpu target BST2. (A) Expression of BST2 on parental MOLT-3 cells and on FACS-sorted subpopulations. The BST2^{+/+} (TS1) and BST2^{-/-} (TS1) subpopulations were obtained by sorting after one round of gene editing with sgRNA TS1, and the BST2^{-/-} (TS1/2) subpopulation was obtained by subjecting BST2^{-/-} (TS1) cells to a second round of editing with sgRNA TS2, followed by a second round of sorting. Open histograms represent staining with anti-BST2 mAb; gray-shaded histograms represent the isotype control. (B and C) Virus growth curves showing that the propagation of WT (Vpu⁺/Nef⁺) HIV-1_{NL4-3} in the BST2^{-/-} (TS1) subpopulation is highly impaired (B) and is even more impaired in the twice-sorted BST2^{-/-} (TS1/2) subpopulation (C), which lacks detectable surface BST2. All subpopulations were infected with 0.2 ng p24/ml, and virus release was monitored by p24 enzyme-linked immunosorbent assay (ELISA). (D) Virus propagation in the same cultures monitored by comparing Gag expression levels in the infected cells at different days after infection by Western blotting with anti-CA. pi, post infection. (E) Expression of BST2 on FACS-sorted Jurkat E6.1 subpopulations. The BST2^{+/+} (TS1) and BST2^{-/-} (TS1) subpopulations were obtained after gene editing with a sgRNA (TS1) that targets the BST2 gene, whereas two sgRNAs (TS3 and TS4) that target sites flanking the BST2 gene were used simultaneously to generate the BST2^{+/+} (TS3+4) and BST2^{-/-} (TS3+4) subpopulations. (F) Propagation of WT HIV-1_{NL4-3} in Jurkat E6.1 subpopulations monitored by p24 ELISA after infection with 0.2 ng p24/ml. (G and H) Propagation of WT HIV-1_{NL4-3} in the BST2^{+/+} (TS3+4) and BST2^{-/-} (TS3+4) subpopulations after infection with 0.2 or 2 ng p24/ml, monitored in parallel by p24 ELISA (G) and by Western blotting of cell lysates with anti-CA (H).

observations revealed that HIV-1 spreading in two different T lymphoid cell lines is highly dependent on BST2.

BST2 dependency is shared by R5-tropic HIV-1 but not by a gammaretrovirus

To determine whether the BST2 dependency of the X4-tropic HIV-1_{NL4-3} is shared by R5-tropic strains, we stably expressed CCR5 at comparable levels in parental MOLT-3 cells and in the BST2^{-/-} (TS1/2) subpopulation (Fig. 3A). The cells were then infected with variants of HIV-1_{NL4-3}, termed NL-JRFL and NL-ZM109 (both Vpu⁺/Nef⁺), that encode the Env proteins of primary subtype B (JRFL) and subtype C (ZM109) HIV-1 strains, respectively. Both R5-tropic viruses replicated to high levels in the parental (BST2-positive) cells but completely failed to spread in the BST2^{-/-} (TS1/2) subpopulation (Fig. 3, B and C).

To determine whether this dependence on BST2 is shared by other retroviruses, parental (BST2-positive) MOLT-3 cells and the BST2^{-/-} (TS1/2) subpopulation were infected with equal amounts of a xenotropic MLV (MLV-X) capable of replicating in human T cells. The presence or absence of BST2 had no effect on the steady-state levels of the MLV Gag precursor Pr65 in the infected cells or on the kinetics of MLV-X progeny virus release (fig. S6A). Furthermore, similar results were obtained when 25-fold less input virus was used (fig. S6B). In this case, the BST2^{-/-} (TS1/2) subpopulation clearly contained less cell-associated MLV capsid (CA) (fig. S6B), indicating that in the absence of BST2, fewer mature virions were tethered to the cell surface, as expected (1). However, the kinetics of Pr65

Gag expression and of progeny virus production in the presence and absence of BST2 were again comparable (fig. S6B), indicating that the propagation of MLV-X was largely unaffected by BST2.

BST2 is crucial for HIV-1 propagation in primary cells

The results described above revealed that basal levels of BST2 are required for HIV-1 propagation in MOLT-3 and Jurkat cells. However, HIV-1 can spread efficiently in SupT1 cells, which express little BST2, possibly because this T cell line is exceptionally susceptible to infection with cell-free virus due to a high CD4 surface density (21). It was therefore crucial to examine the role of BST2 in HIV-1 replication in primary cells.

To this end, PHA-activated PBMC subjected to gene editing with sgRNA TS1 (fig. S7A) were tightly sorted into pure BST2^{+/+} and BST2^{-/-} subpopulations (Fig. 4A) and infected with WT (Vpu⁺/Nef⁺) HIV-1_{NL4-3} at a relatively low concentration of input virus (0.2 ng p24/ml). HIV-1 replication could only be detected in the BST2^{+/+} subpopulation (Fig. 4B). However, because progeny virus production was relatively modest, pure BST2^{+/+} and BST2^{-/-} subpopulations from an additional three donors were infected with WT HIV-1_{NL4-3} at a higher input virus concentration (0.5 ng p24/ml) and kept in a fivefold smaller volume. Under these conditions, robust HIV-1 replication was observed in the BST2^{+/+} subpopulations from all donors examined (Fig. 4C). In notable contrast, no HIV-1 replication was detectable in the BST2^{-/-} subpopulations from donors B and C, and only relatively modest replication was observed in the BST2^{-/-} subpopulation from donor D (Fig. 4C).

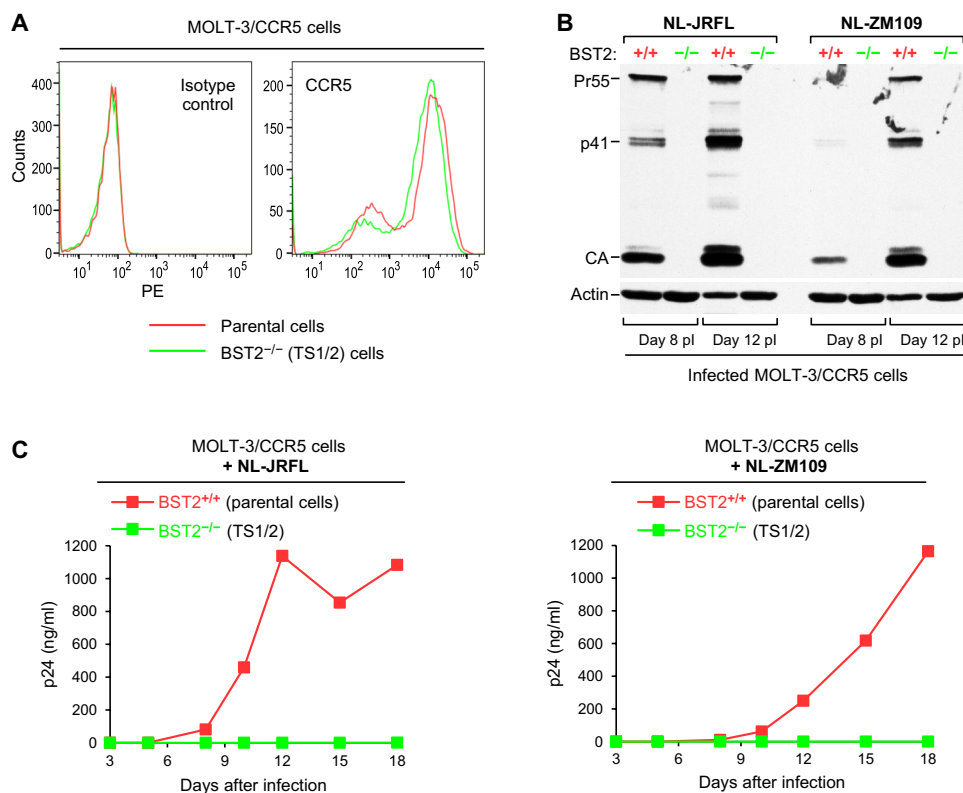


Fig. 3. BST2 dependency is shared by R5-tropic HIV-1. (A) CCR5 surface levels on parental MOLT-3 cells and on the twice-sorted BST2^{-/-} (TS1/2) subpopulation after stable transduction with a retroviral vector expressing CCR5. (B) Replication of R5-tropic HIV-1 viruses in these cells monitored by comparing Gag expression levels by Western blotting with anti-CA after infection with 0.1 ng (NL-JRFL) or 0.2 ng (NL-ZM109) p24/ml. (C) Virus replication monitored in parallel by p24 ELISA.

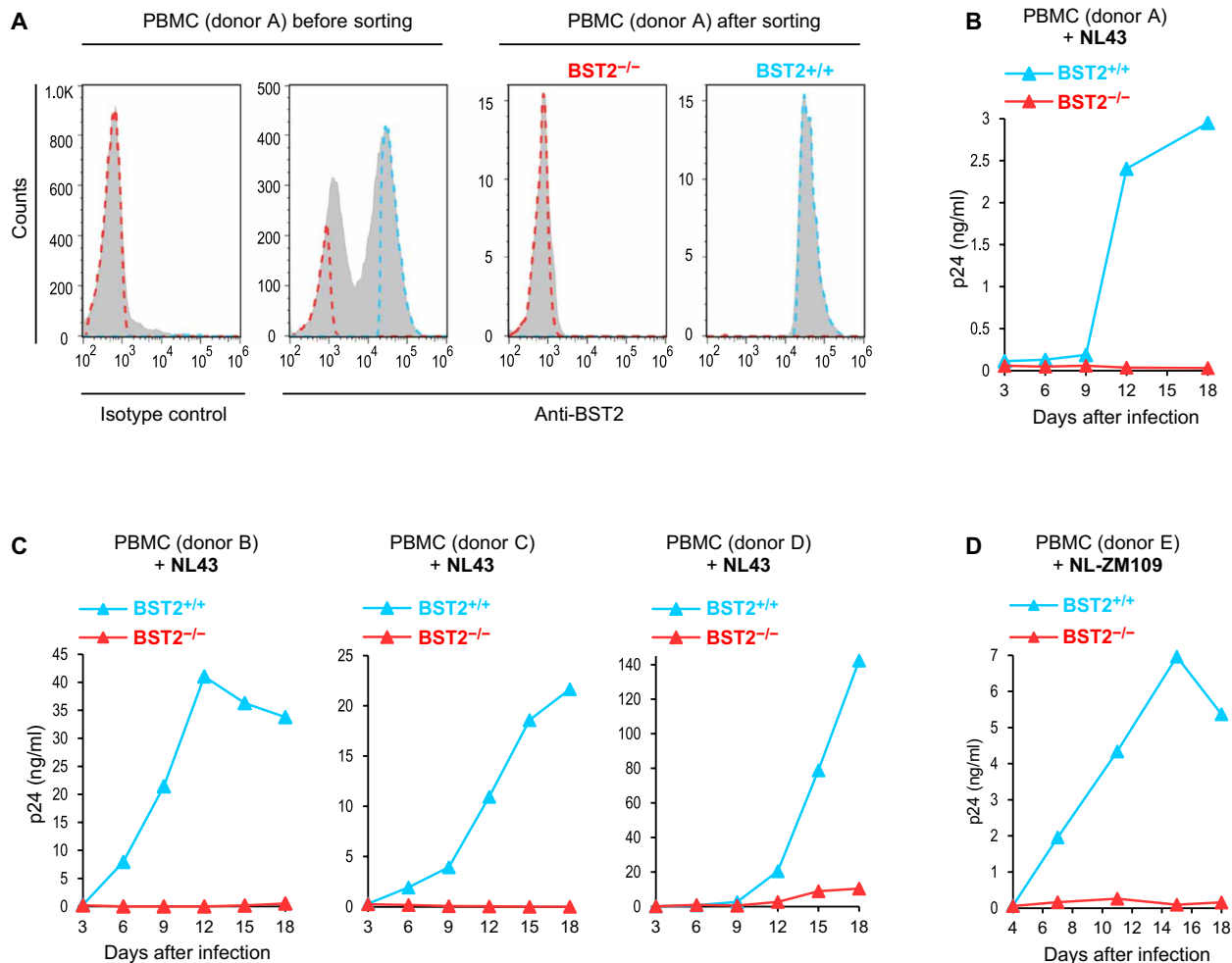


Fig. 4. BST2 is crucial for HIV-1 propagation in primary cells. (A) BST2 levels on bulk PBMC subjected to gene editing with sgRNA targeting BST2 and on sorted subpopulations. The sorted cells were immediately reanalyzed by flow cytometry. The fraction of cells that fell within the gates set for sorting BST2^{-/-} and BST2^{+/+} subpopulations is indicated by red and blue dashed lines, respectively. (B) Virus growth curves showing the replication of WT (Vpu⁺/Nef⁺) HIV-1_{NL4-3} in the sorted BST2^{+/+} and BST2^{-/-} subpopulations after infection with 0.2 ng p24/ml in 1 ml of medium. (C) Replication of WT HIV-1_{NL4-3} in sorted BST2^{+/+} and BST2^{-/-} PBMC subpopulations from additional three donors after infection with 0.5 ng p24/ml in 200 μ l of medium. (D) Replication of an R5-tropic variant of WT HIV-1_{NL4-3} with a primary *env* gene in sorted BST2^{+/+} and BST2^{-/-} PBMC subpopulations infected as in (C).

To examine the role of co-receptor usage, pure BST2^{+/+} and BST2^{-/-} PBMC subpopulations from a fifth donor were infected with the R5-tropic NL-ZM109. We observed that the ability of NL-ZM109 to spread in PBMC was also notably dependent on BST2 (Fig. 4D).

Adenosine 5'-triphosphate (ATP) measurements on day 18 after infection indicated that BST2^{+/+} and BST2^{-/-} PBMC subpopulations remained similarly viable (fig. S7B). Furthermore, surface CD4, CXCR4, and CCR5 levels on sorted BST2^{+/+} and BST2^{-/-} PBMC subpopulations were comparable (fig. S7C). Together, these results demonstrate that BST2 is required for the efficient spreading of both X4- and R5-tropic HIV-1 strains in primary cell cultures.

BST2 is dispensable for infectability but required for HIV-1 spreading

To visualize the effect of BST2 on the spreading of HIV-1, we introduced a Tat-inducible ZsGreen reporter into the Jurkat E6.1-derived BST2^{+/+} (TS3+4) and BST2^{-/-} (TS3+4) subpopulations. To compare their susceptibilities to infection under single-cycle conditions,

the BST2^{+/+} (TS3+4)/ZsGreen and BST2^{-/-} (TS3+4)/ZsGreen subpopulations were infected for 12 hours with a high amount of WT (Vpu⁺/Nef⁺) HIV-1_{NL4-3}, and the entry inhibitor AMD3100 was then added to prevent further rounds of HIV-1 replication. Flow cytometry after another 2 days of culture indicated that the BST2^{+/+} and BST2^{-/-} subpopulations were similarly infectable with WT HIV-1_{NL4-3} (Fig. 5A). ZsGreen fluorescence was negligible when AMD3100 was added at the time of infection (Fig. 5A).

To monitor virus propagation over multiple rounds, the BST2^{+/+} (TS3+4)/ZsGreen and BST2^{-/-} (TS3+4)/ZsGreen subpopulations were infected with WT HIV-1_{NL4-3} at a low input virus concentration. By day 10 after infection, numerous ZsGreen-positive cells were visible in the BST2^{+/+} culture, indicating that substantial spreading within and between aggregates of BST2^{+/+} Jurkat cells had occurred (Fig. 5B). In marked contrast, only isolated ZsGreen-positive cells could occasionally be detected within aggregates of BST2^{-/-} Jurkat cells (Fig. 5B). Overall, these observations indicate that while the BST2^{-/-} subpopulation was normally infectable with HIV-1, it did not support a spreading infection.

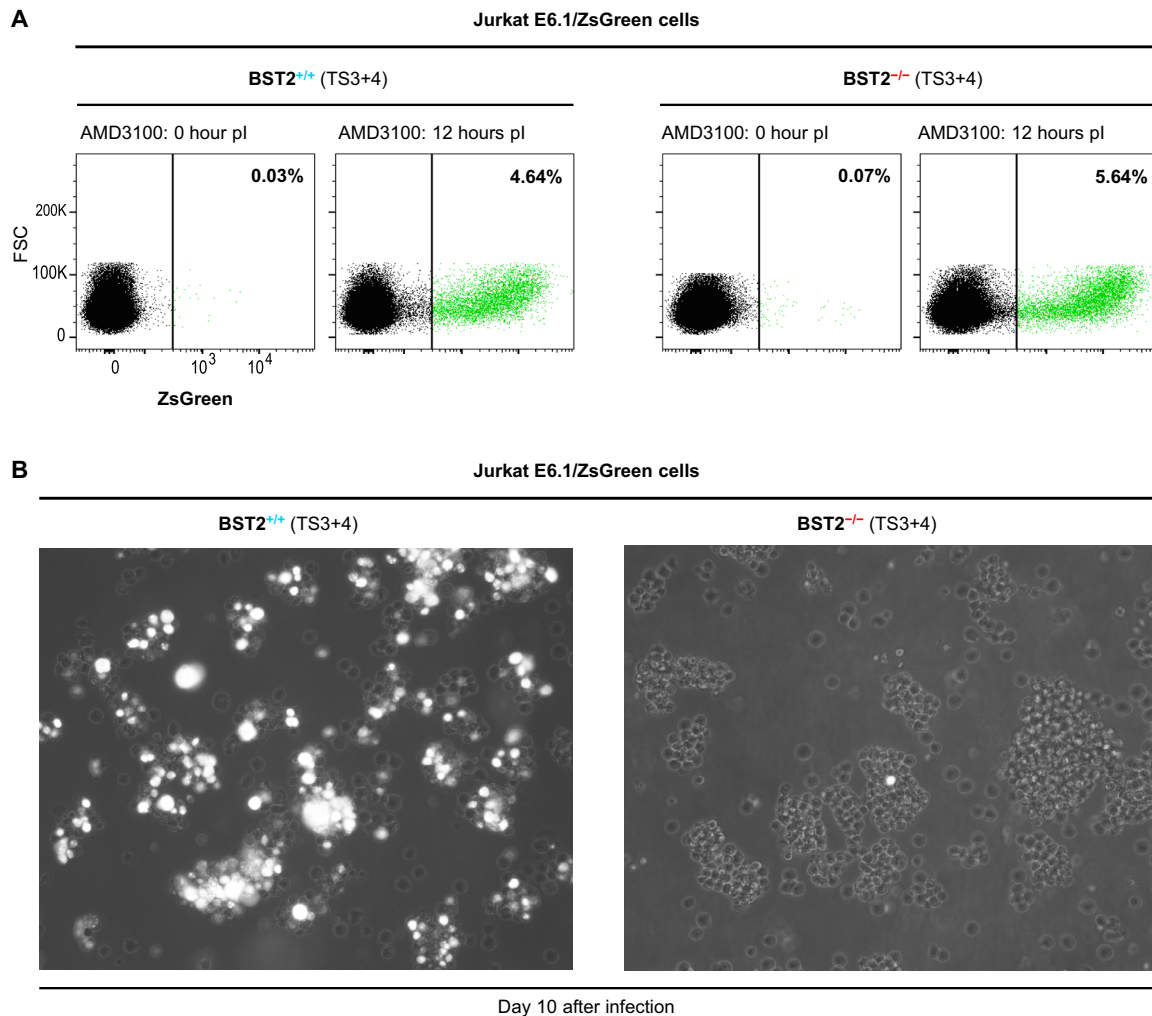


Fig. 5. BST2 is dispensable for infectability but required for HIV-1 spreading. (A) Dot plots showing that $BST2^{+/+}$ and $BST2^{-/-}$ Jurkat E6.1/ZsGreen subpopulations are equally susceptible to single-cycle infection with cell-free WT (Vpu^+/Nef^+) HIV-1_{NL4-3}. The entry inhibitor AMD3100 was added at 0 or 12 hours after infection with WT HIV-1_{NL4-3} at a high multiplicity of infection (MOI). Infected (ZsGreen⁺) cells were quantified by flow cytometry. (B) In the same cells, BST2 is essential for spreading infections. $BST2^{+/+}$ and $BST2^{-/-}$ Jurkat E6.1/ZsGreen subpopulations were infected with a low amount of cell-free WT HIV-1_{NL4-3} (2 ng p24/ml), and after 10 days infected (ZsGreen⁺) cells were detected by fluorescence microscopy. FSC, forward scatterer.

Innate sensing by BST2 is dispensable for its role in HIV-1 spreading

Two isoforms of human BST2 that are produced by alternative translation initiation have been identified (22, 23). The shorter isoform (here called S-BST2) lacks the 12 N-terminal cytoplasmic tail residues of the longer isoform (here called BST2), including a tyrosine motif that is crucial for the induction of NF- κ B in response to Vpu -deficient HIV-1 (22, 24, 25). Thus, BST2 but not S-BST2 can act as a virus sensor that activates an innate immune response, whereas both isoforms restrict virus release (24, 25).

To examine whether BST2-mediated signaling plays a role in HIV-1 spreading, the Jurkat E6.1-derived $BST2^{-/-}$ (TS3/4) subpopulation was stably transduced with retroviral vectors expressing BST2 or S-BST2. In addition, we stably expressed $BST2_{Y6,8A}$, whose ability to mediate NF- κ B activation is strongly impaired (24, 25). The cell surface expression of these three versions of BST2 was similar

(Fig. 6A), and all fully rescued the replication of WT (Vpu^+/Nef^+) HIV-1_{NL4-3} in the BST2 knockout cells (Fig. 6, B and C). Furthermore, similar results were obtained with MOLT-3-derived BST2 knockout cells (fig. S8, A to C).

To confirm these results, we stably transduced Jurkat- and MOLT-3-derived BST2 knockout subpopulations with a retroviral vector expressing mouse BST2, which has a negligible ability to activate NF- κ B (24). However, despite limited sequence homology with its human counterpart, mouse BST2 strongly restricts HIV-1 release (24). Although the surface expression of mouse BST2 in the stably transduced cells was quite heterogeneous and relatively modest (Fig. 6D and fig. S8D), WT HIV-1_{NL4-3} replication in the BST2 knockout cultures was restored by mouse BST2, albeit at a somewhat lower level than in the parental cells (Fig. 6, E and F, and fig. S8, E and F). We concluded that the ability to activate NF- κ B is dispensable for the role of BST2 in HIV-1 spreading.



Fig. 6. Innate sensing by BST2 is dispensable for its role in HIV-1 spreading. (A) BST2 surface levels on Jurkat E6.1 $BST2^{-/-}$ knockout cells reconstituted with variants of human BST2. (B and C) Both signaling-competent and signaling-defective versions of human BST2 fully rescue WT (Vpu^{+}/Nef^{+}) HIV-1_{NL4-3} replication in the knockout cells, as examined by Western blotting of cell lysates with anti-CA on day 10 after infection with 0.2 ng p24/ml (B) and in parallel by p24 ELISA of culture supernatants (C). (D) BST2 surface levels on Jurkat E6.1 $BST2^{-/-}$ knockout cells reconstituted with mouse BST2. (E and F) Mouse BST2 partially restores WT HIV-1_{NL4-3} replication in the knockout cells, as examined by monitoring cell-associated Gag levels (E) and p24 antigen release (F). PE, phycoerythrin.

Rescue of HIV-1 propagation by an artificial BST2 depends on its virus-tethering activity

It has been shown that an artificial BST2-like molecule named art-tetherin (here called art-BST2) mimics the virion retention activity of human BST2, although it lacks evident sequence homology (8). As previously described (8), art-BST2 is entirely composed of fragments from heterologous proteins that together are predicted to result in a BST2-like topology by providing an N-proximal transmembrane domain, an extracellular domain capable of forming homotypic disulfide bonds and a coiled coil, and a C-terminal GPI anchor (Fig. 7A). In addition, art-BST2 harbors a hemagglutinin (HA) epitope in its extracellular domain (Fig. 7A).

To examine whether art-BST2 can enhance HIV-1 spreading, it was stably expressed in the Jurkat E6.1-derived $BST2^{-/-}$ (TS3/4) subpopulation. As expected (8), art-BST2 was well expressed on the cell surface (Fig. 7B). art-BST2 markedly enhanced the replication of WT (Vpu^{+}/Nef^{+}) HIV-1_{NL4-3} in the $BST2^{-/-}$ (TS3/4) subpopulation (Fig. 7, C and D). While HIV-1 replication could not be detected at all in the $BST2$ knockout cells without art-BST2, the kinetics of HIV-1 replication in the presence of art-BST2 were very similar to

those in the $BST2^{+/+}$ (TS3/4) subpopulation (Fig. 7, C and D), which closely resembles the parental unsorted Jurkat E6.1 cells in terms of BST2 expression (fig. S3).

The potent effect of art-BST2 suggested a model in which virus tethering was directly responsible for the rescue of HIV-1 replication. To test this model, we examined the roles of the two membrane anchors of art-BST2, which are both essential for its tethering activity (8). In addition, we examined the role of the extracellular coiled-coil domain because the corresponding domain of native BST2 is also critical for its tethering activity (8). Notably, versions of art-BST2 that lacked any of these elements (Fig. 8A) completely lacked the ability to restore the replication of WT HIV-1_{NL4-3} in the $BST2^{-/-}$ cells (Fig. 8, B and C). No virus replication at all was observed in any of these cultures, whereas HIV-1 replication in the $BST2^{-/-}$ cells expressing unmodified art-BST2 was vigorous (Fig. 8, B and C). All art-BST2 mutants were expressed at least as well on the cell surface as unmodified art-BST2 (Fig. 8D). We conclude that the ability of art-BST2 to rescue HIV-1 replication in the absence of native BST2 strictly depends on its virus-tethering activity.

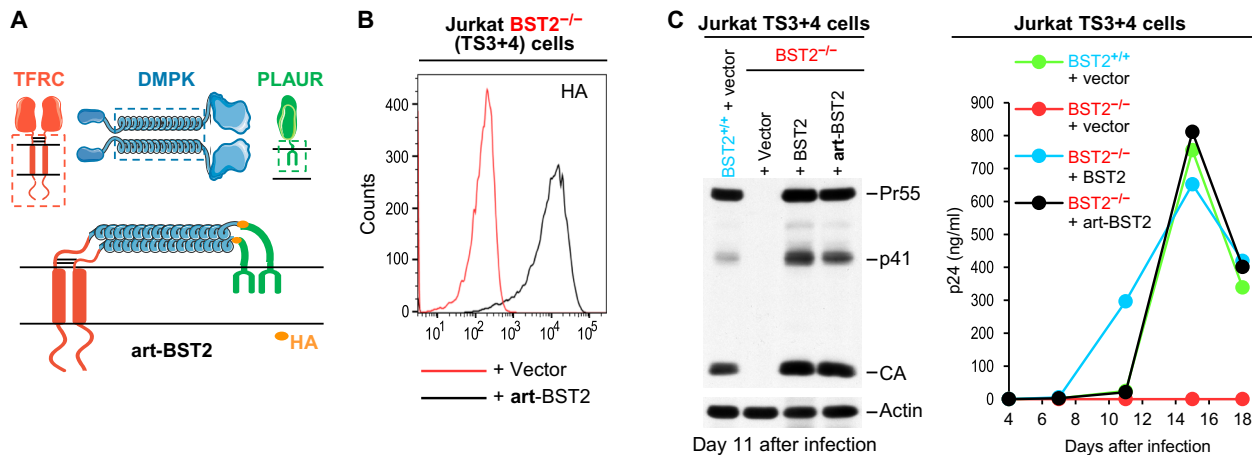


Fig. 7. An artificial BST2-like molecule fully rescues HIV-1 propagation in BST2 knockout cells. (A) Schematic illustration of an artificial BST2-like molecule (art-BST2) composed of sequences derived from the human transferrin receptor (TFRC), human dystrophin myotonia protein kinase (DMPK), and human urokinase-type plasminogen activator receptor (PLAUR) (8). In addition, art-BST2 has an HA tag within its predicted ectodomain. (B) Surface expression of art-BST2 on stably transduced Jurkat E6.1 $BST2^{-/-}$ knockout cells examined by flow cytometry using an anti-HA antibody. (C and D) Replication of WT (Vpu⁺/Nef⁺) HIV-1_{NL4-3} in $BST2^{+/+}$ and $BST2^{-/-}$ Jurkat E6.1 subpopulations stably transduced with empty vector or vectors encoding human BST2 or art-BST2, as indicated. The cultures were infected with 0.2 ng p24/ml, and virus replication was examined by comparing cell-associated Gag levels on day 11 after infection (C) and by monitoring p24 antigen in the culture supernatants (D).

DISCUSSION

BST2 is considered a restriction factor because it entraps nascent HIV-1 virions on the cell surface unless antagonized by Vpu (10, 26). However, the results reported here demonstrate that at least a basal level of BST2-mediated tethering of virions to the cell surface is critical for the spreading of HIV-1 in different T cell lines and, crucially, in primary cells.

HIV-1 spreading in BST2 knockout cultures could be fully restored by a previously published completely artificial protein (art-BST2) that has a comparable ability to tether HIV-1 virions to the cell surface (8). Because art-BST2 shares the major structural features of BST2 but has no appreciable sequence homology (8), this observation indicates that only the configuration of BST2 is important for its role in HIV-1 spreading. Notably, the two membrane anchors and the extracellular coiled coil of art-BST2 were each essential to support HIV-1 spreading. Since these elements are also critical for virion tethering (8), our results imply that the tethering function of BST2 is both essential and sufficient for its role in HIV-1 spreading.

The lack of HIV-1 spreading in BST2 knockout cultures, the complete rescue by both native and artificial BST2 proteins, and the strict dependency of this rescue on tethering activity together strongly suggest that the entrapment of virions on infected cells plays a major role in HIV-1 propagation. Since tethered virions appear fully infectious (27), their accumulation on the cell surface would be expected to facilitate virus transmission through cell-cell contacts. In support of this notion, the loss of Vpu can provide a selective advantage by increasing the cell-to-cell spread of HIV-1 (28). Furthermore, there is evidence that knocking down BST2 reduces the cell-to-cell spread of Vpu-deficient HIV-1 (27). Note that over-expressed BST2 in donor cells imposes a strong barrier to the cell-free but not the cell-to-cell transmission of Vpu-deficient HIV-1 (29). The crucial role of BST2 documented in the present study, including in primary cells, suggests that HIV-1 spreading after infection at a low multiplicity of infection (MOI) may initially depend primarily on cell-to-cell transmission and that cell-free virus

transmission only plays a substantial role once extracellular virus becomes sufficiently concentrated.

Virus replication studies in knockout mice have shown that BST2 is not required for the spreading of murine gammaretroviruses in vivo (12, 13). In the present study, a murine gammaretrovirus spread efficiently in MOLT-3 cells lacking BST2, whereas HIV-1 spreading in the same cells was markedly delayed, indicating that the reliance of the lentivirus HIV-1 on BST2 is not shared by simpler retroviruses.

Although we observed that mutations that disrupt Vpu markedly accelerated the replication of Nef-deficient HIV-1, it remains possible that the loss of Vpu did not compensate specifically for the lack of Nef because mutations that inactivate Vpu can also alleviate the replication defect of ALIX binding site mutants in Jurkat cells (30). Similarly, Vpu-truncated mutants with increased replication fitness occurred upon long-term HIV-1 propagation in another T cell line (31). Thus, losing Vpu can be broadly beneficial for HIV-1 propagation in vitro, possibly because higher BST2 levels on cells infected with Vpu-deficient virus promote the cell-to-cell spreading of HIV-1 and thus compensate for certain replication defects.

While defective *vpu* genes are unexpectedly common in primary HIV-1 isolates (32, 33), there is compelling evidence that the capacity to antagonize BST2 provides a selective advantage to HIV-1 and other primate lentiviruses in vivo (5, 34–37). It is also well documented that BST2 increases the susceptibility of HIV-infected cells to antibody-dependent cell-mediated cytotoxicity (38–42). In addition, BST2 can enhance antiretroviral immune responses through its capacity to internalize virions (43). These effects of BST2 likely exert substantial selective pressure on the evolution and maintenance of viral BST2 antagonists to facilitate immune evasion.

HIV-1 group M Vpu targets the long BST2 isoform, but homodimers of the shorter isoform are relatively Vpu resistant (23). Since the shorter isoform does not induce NF- κ B-dependent proinflammatory gene expression, the selective down-regulation of the long isoform may represent an evolutionary strategy to prevent BST2 from acting as an innate sensor of HIV-1 assembly (24) while ensuring

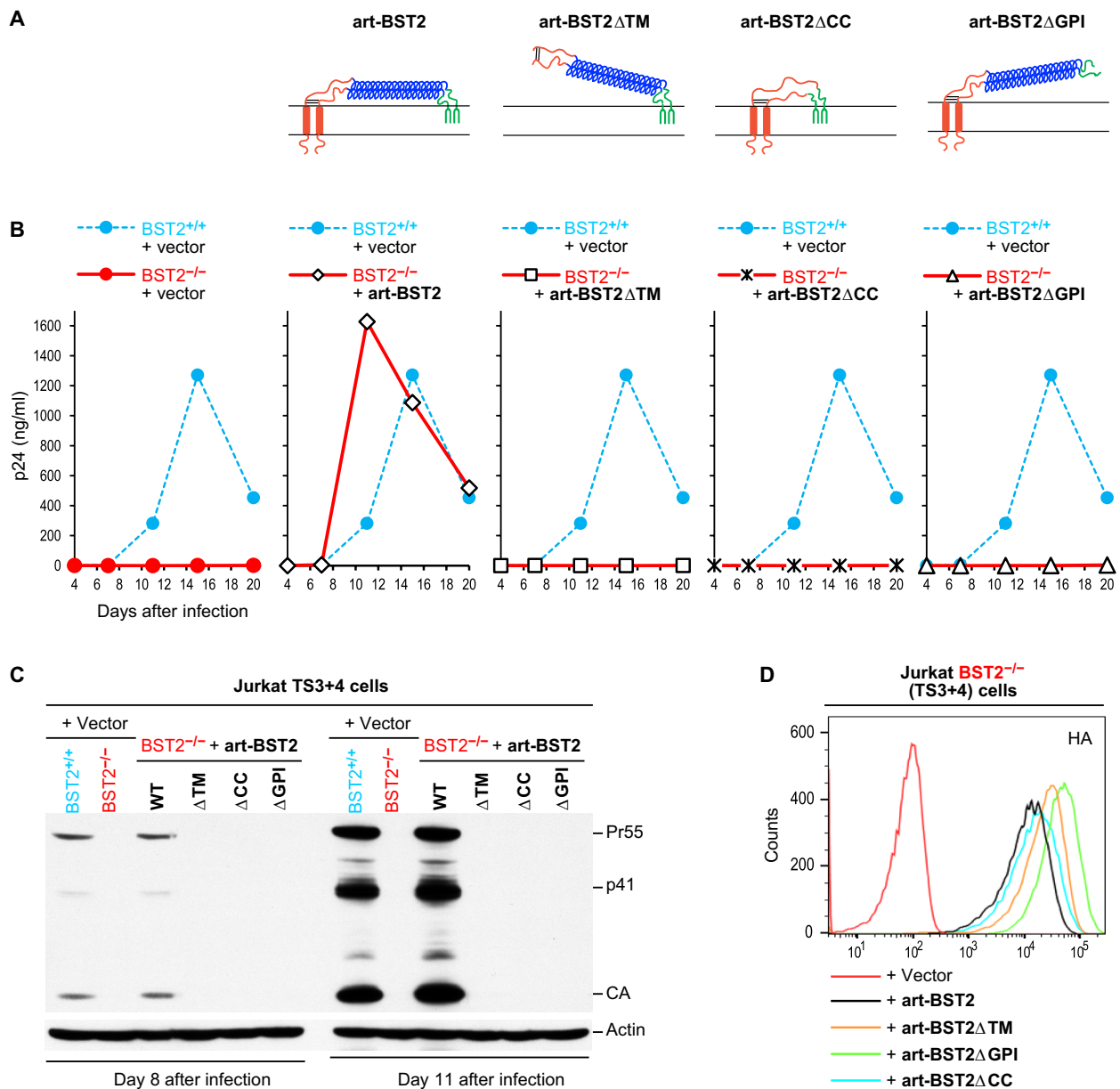


Fig. 8. The ability of art-BST2 to support HIV-1 propagation depends on its virus-tethering activity. (A) Schematic illustration of art-BST2 mutants that lack the N-proximal transmembrane domain (art-BST2 Δ TM), the extracellular coiled coil (art-BST2 Δ CC), or the C-terminal GPI anchor (art-BST2 Δ GPI). All mutants retain the HA tag in the art-BST2 ectodomain. (B) Virus growth curves showing that the two membrane anchors and the coiled coil of art-BST2 are all essential for its ability to rescue WT (Vpu⁺/Nef⁺) HIV-1_{NL4-3} replication in BST2^{-/-} knockout cells. BST2^{+/+} (TS3+4) and BST2^{-/-} (TS3+4) Jurkat E6.1 subpopulations stably transduced with empty vector or vectors encoding the indicated versions of art-BST2 were infected with 0.2 ng p24/ml, and virus replication was examined by monitoring p24 antigen release. (C) Comparison of cell-associated Gag levels in the same cultures by Western blotting with anti-CA. (D) Surface expression of WT and mutant versions of art-BST2 on stably transduced Jurkat E6.1 BST2^{-/-} knockout cells examined by flow cytometry with anti-HA.

that a basal level of BST2 capable of promoting virus spreading remains on the surface of cells expressing Vpu. It has also been reported that the Nef proteins of some HIV-1 group M strains have substantial activity against human BST2 (44). The Vpu proteins of these strains lacked anti-BST2 activity (44), suggesting that the ability to down-regulate BST2 below a certain level is selected against in vivo. In light of the results presented here, we thus propose that HIV-1 balances the immunological costs imposed by cell surface

BST2 against the benefits of maintaining a basal level for efficient virus propagation.

MATERIALS AND METHODS

Plasmid construction

NL4-3/Nefstop is a Nef-deficient variant of the prototypic HIV-1 proviral clone pNL4-3 (GenBank, M19921) that has *nef* codons 31 to 33

replaced by three consecutive premature termination codons (45). The infectious HIV-1 proviral clone NL-ZM109 was generated by replacing a Kpn I–Bam HI fragment of pNL4-3 with the corresponding fragment from ZM109F.PB4 (46). Sequences encoding human CCR5 (GenBank, BC038398), human BST2 (GenBank, BC033873), human S-BST2, human BST2_{Y6,8A}, and mouse BST2 (GenBank, BC027328) preceded by an optimal Kozak sequence were amplified from plasmids obtained from Open Biosystems and cloned into the retroviral vector pMSCVpuro (Clontech). Human S-BST2 lacks the first 12 codons present in the longer BST2 isoform (22), and human BST2_{Y6,8A} has the codons for Tyr⁶ and Tyr⁸ mutated to codons specifying Ala. Synthetic genes encoding art-BST2, art-BST2ΔTM, art-BST2ΔACC, and art-BST2ΔGPI preceded by a Kozak sequence were purchased from Integrated DNA Technologies and cloned into pMSCVpuro. As previously described (8), art-BST2 contains a methionine initiator codon followed by codons 43 to 121 of the human TFRC gene (GenBank, NM_003234) providing an N-terminal cytoplasmic tail, a transmembrane domain, and an extracellular stalk; codons 463 to 538 of human dystrophin myotonia protein kinase (DMPK; GenBank, BC062553) providing a coiled coil; 9 codons providing an HA tag; and codons 303 to 335 of human urokinase-type plasminogen activator receptor (PLAUR; GenBank, NM_002659) providing a GPI anchor. The art-BST2ΔTM mutant, which lacks the N-proximal transmembrane domain and instead has a secretory leader peptide, was obtained by replacing codons 1 to 45 of art-BST2 by codons 1 to 25 of human PLAT (GenBank, NM_000930). The art-BST2ΔACC mutant lacks DMPK codons 463 to 438 and, thus, an extracellular coiled coil. The art-BST2ΔGPI mutant lacks PLAUR codons 303 to 335 and, thus, a GPI anchor. Notably, all art-BST2 mutants retain an HA epitope in their predicted extracellular domains.

Molecular cloning of revertant sequences

MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in these cells, and genomic DNA was extracted at the peak of infection with DNazol (Invitrogen). An HIV-1 proviral segment containing *vpu* and *env* sequences was amplified with primers NL4-3 5795 5' (5'-CAGAATTGGGTGTCGACATAGC-3') and NL4-3 8469 3' (5'-CGTCCCAGATAAGTGCTAAGG-3'), which flank unique Sal I and Bam HI sites. The Nef⁻/R0-1 and Nef⁻/R0-2 recombinants were obtained by inserting a Sal I–Bam HI fragment into the Nef-deficient pNL4-3/Nefstop provirus in place of the corresponding WT fragment (nucleotides 5786 to 8465 of pNL4-3). DNA sequencing revealed that the Nef⁻/R0-1 and Nef⁻/R0-2 recombinants are identical. Both have the *vpu* initiation codon changed to ATA and harbor a premature termination codon (TAA) in place of *vpu* codon 28. In addition, both have changes in *env* that result in P204S and I593F mutations. To obtain proviral clones that harbor only the mutations in *vpu* or in *env*, a Sal I–Kpn I fragment (nucleotides 5786 to 6347) or a Kpn I–Bam HI fragment (nucleotides 6348 to 8465) was transferred from Nef⁻/R0-2 to pNL4-3/Nefstop, yielding the Nef⁻/R1 and Nef⁻/R2 recombinants, respectively. In addition, the Sal I–Kpn I fragment (nucleotides 5786 to 6347) containing the mutant *vpu* gene of the Nef⁻/R1 recombinant (which is Vpu⁻/Nef⁻) was transferred to WT pNL4-3, yielding the Nef⁺/R1 recombinant (which is Vpu⁻/Nef⁺). The Nef⁻/vpuPTC proviral clone, which differs from pNL4-3/Nefstop only by the presence of a premature termination codon (TAA) in place of *vpu* codon 28, was obtained by

replacing nucleotides 5786 to 6347 of pNL4-3/Nefstop with a gBlocks gene fragment (Integrated DNA Technologies) harboring the premature termination codon. Similarly, a proviral clone harboring the *vpu*Δ7 deletion, which arose in an independently obtained revertant of pNL4-3/Nefstop and removes 7 nt from *vpu* (nucleotides 6163 to 6169 of pNL4-3), was obtained by replacing nucleotides 5786 to 6347 of pNL4-3/Nefstop with DNA amplified from cells infected with the revertant.

Generation of knockout cell populations

Preassembled ribonucleoprotein complexes consisting of purified Cas9 (Synthego) and chemically modified synthetic sgRNAs TS1 (5'-AAGCGCUGUAAGCUUCUGCU-3') or TS2 (5'-CCUGCAACAAGAGCUGACCG-3') (Synthego), both of which target exon 1 of human BST2 within the region coding for the transmembrane domain, were delivered into MOLT-3 cells, Jurkat E6.1 cells, or PHA-stimulated PBMC via nucleofection using the Cell Line Nucleofector Kit V with the Nucleofector II device (Lonza). To delete the entire BST2 gene, Cas9 was complexed with sgRNAs TS3 (5'-CUCUUGCAUGAGGCACCUAC-3') and TS4 (5'-ACUC-CAGGAACUCGAGAGUC-3') to simultaneously target sites upstream and downstream of the BST2 gene. One week after nucleofection, the cells were stained with mouse anti-BST2 (BioLegend, 348402) or isotype control monoclonal antibody (mAb; BioLegend, 400102) and phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulin G (IgG; Jackson ImmunoResearch, 115-116-146) and sorted into BST2^{+/+} and BST2^{-/-} subpopulations by flow cytometry with a Sony SH800 cell sorter. To set gates for sorting, dot blots of forward scatter versus fluorescence intensity were used. Antibodies used for flow cytometry to characterize the sorted subpopulations included anti-CD4 (BioLegend, 300502), allophycocyanin (APC)-conjugated anti-CD4 (BioLegend, 300552), anti-CXCR4 (BD Pharmingen, 555972), APC-conjugated anti-CXCR4 (BioLegend, 306509), anti-CCR5 (BD Pharmingen, 555991), and anti-CD11a (BioLegend, 301202). Total BST2 expression levels were compared by Western blotting of cell lysates with anti-BST2 antibody E-4 (Santa Cruz Biotechnology, sc-390719) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson ImmunoResearch, 115-035-003).

BST2^{+/+} and BST2^{-/-} Jurkat E6.1/ZsGreen reporter cells were obtained by transducing FACS-sorted BST2^{+/+} (TS3/4) and BST2^{-/-} (TS3/4) Jurkat E6.1 subpopulations at a high MOI with a subviral construct based on HIV-1_{NL4-3} that harbors a large deletion (nucleotides 1968 to 7645 of M19921) and encodes an HIV-1 Tat-inducible ZsGreen-NLS in place of *nef* (47). The transduced subpopulations were then sorted by flow cytometry for negligible ZsGreen expression in the absence of HIV-1. Aliquots of the sorted transduced subpopulations were infected with VSV G-pseudotyped single-cycle HIV-1 pseudovirions at a high MOI to ensure that the vast majority of cells expressed ZsGreen upon infection with HIV-1.

Reconstitution of BST2 expression

Ectopic human BST2, mouse BST2, or art-BST2 expression cassettes were introduced into FACS-sorted BST2^{-/-} subpopulations by retroviral transduction with MSCVpuro-based vectors, followed by selection with puromycin (1 μg/ml). The ectopic expression of human or mouse BST2 was confirmed by flow cytometry after staining with anti-human (BioLegend, 348402) or anti-mouse (BioLegend, 127102) BST2 mAbs and PE-conjugated secondary antibodies (Jackson ImmunoResearch, 115-116-146 and 112-116-143). An anti-HA mAb

(BioLegend, 901513) was used to compare the surface expression of art-BST2 and of its derivatives by flow cytometry.

Virus replication studies

Replication-competent HIV-1 was produced by transfecting 293T cells with plasmids containing full-length proviruses. These included the prototypic molecular clone pNL4-3 (48), which encodes a CXCR4-tropic HIV-1 that replicates exclusively in T cells, the Nef-deficient mutant pNL4-3/Nefstop (45), variants of pNL4-3/Nefstop that contain revertant sequences, pHXBH10(vpu⁺) and pHXBH10(vpu⁻), which are derived from the CXCR4-tropic HXB2 provirus and differ in the presence or absence of a vpu initiation codon but are otherwise isogenic (17), and pNL-JRFL (15) and pNL-ZM109, which encode CCR5-tropic primary Env proteins. Virus containing supernatants were clarified by low-speed centrifugation, passed through 0.45- μ m filters, normalized for HIV-1 CA (p24) antigen with an HIV-1 p24 ELISA kit (XpressBio), and used to infect target cells. T lymphoid cell lines and FACS-sorted subpopulations (2×10^5) were infected in T25 flasks in 5 ml of medium at a p24 concentration of 0.2 ng/ml, unless indicated otherwise. FACS-sorted PBMC subpopulations were seeded into a 48-well plate in 1 ml of interleukin 2 (IL2)-containing medium at 1.4×10^5 cells per well (donor A) or into 96-well plates in 0.2 ml of IL2-containing medium at 2.3×10^5 (donor B), 3.0×10^5 (donor C), 4.5×10^5 (donor D), or 0.74×10^5 (donor E) cells per well. The PBMC subpopulations were infected at a p24 concentration of 0.2 ng/ml (donor A) or 0.5 ng/ml (donors B to E). Virus replication was monitored by comparing Gag protein expression levels in infected cells by Western blotting using anti-CA antibody 183-H12-5C (National Institutes of Health AIDS Reagent Program, 1513), anti-actin (Santa Cruz Biotechnology, sc-47778), and HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch, 115-035-003) and/or by measuring the accumulation of p24 antigen in the culture supernatants over time with an HIV-1 p24 ELISA kit (XpressBio). Cell viability was compared by quantitating the ATP present in the cultures with the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

MLV capable of infecting human cells was produced by transfecting 293T cells with pMLV-X, which encodes a version of Mo-MLV with a xenotropic env gene derived from NZB-9-1 (49). At 16 hours after transfection, the culture medium was replaced, and virus-containing supernatant was harvested 14 hours later, clarified by low-speed centrifugation, and passed through 0.45- μ m filters. FACS-sorted MOLT-3 subpopulations (2×10^5) that had been seeded into T25 flasks in 5 ml of medium were then infected overnight with 2 or 50 μ l of the 293T-derived supernatant. MLV replication kinetics in BST2^{+/+} and BST2^{-/-} subpopulations were compared by monitoring Gag expression levels in the infected cells and the accumulation of MLV CA protein (p30) in the culture supernatants by Western blotting using anti-MLV p30 antiserum (Quality Biotech).

Analysis of cellular infectability

To determine whether the presence or absence of surface BST2 on target cells affects their infectability, BST2^{+/+} and BST2^{-/-} Jurkat E6.1/ZsGreen reporter cells (2×10^6) were acutely infected with HIV-1_{NL4-3} at a high p24 concentration (666 ng p24/ml). As a negative control, infections were carried out in the presence of the entry inhibitor AMD3100 (at 2.5 μ M). After 12 hours of incubation, the input virus was removed, and the cells were subsequently cultured in the presence of 2.5 μ M AMD3100 to limit virus replication to a

single cycle. After further incubation to allow ZsGreen expression in infected cells, the cultures were fixed with 4% paraformaldehyde and analyzed on a Becton Dickinson LSR II flow cytometer.

Statistical analysis

Error bars represent the SD and were calculated with the Excel software (Microsoft). Arithmetic mean values \pm SD were calculated from three measurements.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <https://science.org/doi/10.1126/sciadv.abj7398>

[View/request a protocol for this paper from Bio-protocol.](#)

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