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

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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 kB (NF-kB) 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-1NL4-3 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-1NL4-3 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-1NL4-3. 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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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 SERINC5s (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
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 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 nucleasefsed 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-1NL4-3 than the BST2+/− (TS1) subpopulation (Fig. 2B, 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-1NL4-3 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-1NL4-3 to replicate in parental (BST2+/+) MOLT-3 cells and in the BST2−/− (TS1) and BST2−/− (TS1/2) subpopulations. As expected, Vpu−/Nef− (WT) HIV-1NL4-3 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-1NL4-3 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−1NL4-3 replicated efficiently in the BST2+/+ (TS1) and BST2+/− (TS3+4) subpopulations as expected, virus replication was markedly impaired in the 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-1NL4-3 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 an 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-1NL4-3 in Jurkat E6.1 subpopulations monitored by p24 ELISA after infection with 0.2 or 2 ng p24/ml. (G and H) Propagation of WT HIV-1NL4-3 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<sub>NL4-3</sub> is shared by R5-tropic strains, we stably expressed CCR5 at comparable levels in parental MOLT-3 cells and in the BST2<sup>−/−</sup> (TS1/2) subpopulation (Fig. 3A). The cells were then infected with variants of HIV-1<sub>NL4-3</sub>, termed NL-JRFL and NL-ZM109 (both Vpu<sup>+</sup>/Nef<sup>+</sup>), 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<sup>−/−</sup> (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<sup>−/−</sup> (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<sup>−/−</sup> (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 (I). 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<sup>+/+</sup> and BST2<sup>−/−</sup> subpopulations (Fig. 4A) and infected with WT (Vpu<sup>+/Nef</sup>) HIV-1<sub>NL4-3</sub> at a relatively low concentration of input virus (0.2 ng p24/ml). HIV-1 replication could only be detected in the BST2<sup>+/+</sup> subpopulation (Fig. 4B). However, because progeny virus production was relatively modest, pure BST2<sup>+/+</sup> and BST2<sup>−/−</sup> subpopulations from an additional three donors were infected with WT HIV-1<sub>NL4-3</sub> 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<sup>+/+</sup> subpopulations from all donors examined (Fig. 4C). In notable contrast, no HIV-1 replication was detectable in the BST2<sup>−/−</sup> subpopulations from donors B and C, and only relatively modest replication was observed in the BST2<sup>−/−</sup> subpopulation from donor D (Fig. 4C).

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**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<sup>−/−</sup> (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.
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+/+ 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.

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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+/+ and BST2−/− PBMC 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-1NL4,3, 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 infectible with WT HIV-1NL4,3 (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-1NL4,3 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 infectible with HIV-1, it did not support a spreading infection.
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-κB 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-κB 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-1NL4-3 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-κB (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-1NL4-3 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-κB is dispensable for the role of BST2 in HIV-1 spreading.
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-1NL4-3 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). Notably, versions of art-BST2 that lacked any of these elements (Fig. 8A) completely lacked the ability to restore the replication of WT HIV-1NL4-3 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.
Jurkat BST2−/−
(TS3+4) cells

Jurkat TS3+4 cells

Jurkat TS3+4 cells

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 dystrophia myotonica 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 overexpressed 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-κB–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...
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
Molecular cloning of revertant sequences

MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells were infected with multiply passaged NL4-3/Nefstop virus that exhibited markedly accelerated replication kinetics in MOLT-3 cells.
(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 CXC4-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, pHXB10(vpu-) and pHXB10(vpu+), which are derived from the CXC4-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-μm 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 x 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 x 10^5 cells per well (donor A) or into 96-well plates in 0.2 ml of IL2-containing medium at 2.3 x 10^5 (donor B), 3.0 x 10^5 (donor C), 4.5 x 10^5 (donor D), or 0.74 x 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, 1513), anti-MLV p30 antiserum (Quality Biotech). 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 x 10^5) were acutely infected with HIV-1NL4-3 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 (2.5 μM). After 12 hours of incubation, the input virus was removed, and the cells were subsequently cultured in the presence of 2.5 μM 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 ± SD were calculated from three measurements.

**SUPPLEMENTARY MATERIALS**

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

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