SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Amino acid sequence alignment for three alleles of rhesus tetherin

The predicted amino acid sequences are shown for three alleles of rhesus macaque tetherin. Full-length tetherin cDNA sequences were amplified by RT-PCR from mRNA isolated from rhesus macaque lymphocytes using primers to sequences in the 5' and 3' untranslated regions of the gene, cloned into pGEM-T Easy (Promega) and sequenced.

Figure S2. The expression of ψNef *in trans* does not rescue virus release in the presence of rhesus tetherin

(A) The predicted amino acid sequences for the *nef* open-reading frames of SIV Δ*nef* (ΔNef) and SIV Δ*nefP* (ψNef) are shown in an alignment with the wild-type Nef protein of $SIV_{mac}239$. Positions of amino acid identity are indicated with periods, deletions are indicated by dashes and translational stop sites are indicated by asterisks. (B) ψNef was tested for the ability to antagonize rhesus tetherin by transfecting 293T cells with SIV Δ*env*Δ*nef* proviral DNA, an expression construct for rhesus tetherin (rBST-2.1), and increasing amounts of an expression construct for ψNef (75-200 ng). Virus release was measured by SIV p27 antigen-capture ELISA and is shown as a percentage of maximal release in the absence of tetherin. Error bars indicate the standard deviation of mean percent maximal virus release. (C) These results were corroborated by comparing virion-associated p27 in the cell culture supernatant to the levels of p55 Gag in cell lysates by western blot analysis. ψNef expression was confirmed by probing western blots of cell lysates with an antibody to an HA tag that was introduced at the C-terminus of the protein. (D and E) Jurkat cells were electroporated with bicistronic constructs expressing

Nef, ΔNef, ψNef Env or EnvITM, and enhanced green fluorescent protein (eGFP), and stained the next day with a PE- and PerCP-conjugated monoclonal antibodies to MHC class I (MHC-I) and CD4. The mean fluorescence intensities for MHC-I (D) and CD4 (E) staining on the cell surface are indicated for the transfected, $eGFP^+$ cell populations.

Figure S3. Subsets of the five amino acid changes at the C-terminus of gp41 afford partial resistance to rhesus tetherin

(A) The indicated amino acid substitutions were introduced into the gp41 cytoplasmic domain of SIV Δ*nef*. (B) These SIV Δ*nef* mutants were tested for virus release at increasing expression levels rhesus tetherin (rBST-2.1). Error bars indicate the standard deviation (+/-) of mean percent maximal virus release.

Figure S4. Phenotypic differences in the expression levels and distribution of Env and tetherin in infected rhesus macaque lymphocytes

Activated primary rhesus macaque lymphocytes were infected with (A) wild-type SIV, (B) SIV Δ*nef* and (C) SIV Δ*nefP*. Twenty-four hours after the addition of IFNα, the cells were fixed, permeabilized and stained with monoclonal antibodies to BST-2 (green), SIV Env (red), and with a nuclear dye (blue). The overlay images show at least 15 cells infected with each virus. The scale bar corresponds to 1 μm.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Plasmid DNA constructs

(a) BST 2 expression constructs. Human *BST-2* (*hBST-2*) was obtained from the Harvard Plasmid Database, and rhesus macaque *BST-2* alleles *rBST-2.1*, *rBST-2.2* and r*BST-2.8* were amplified by RT-PCR from mRNA isolated from peripheral blood lymphocytes of separate macaques. *BST-2* cDNAs were cloned into the *Kpn* I and *Xho* I sites of pcDNA3 (Invitrogen, Carlsbad, CA). The tetherin recombinants *rN/hBST-2*, *hN/rBST-2, rTM/hBST-2* and *hTM/rBST-2* were generated by exchanging sequences coding for the cytoplasmic (N) and transmembrane (TM) domains of hBST-2 and rBST-2 by PCR overlap extension (Jia et al., 2009). Additional amino acid substitutions were introduced into *rBST-2* and *hBST-2* by site-directed mutagenesis using QuickChange reagents (Stratagene, La Jolla, CA).

(b) SIV proviral clones. Full-length proviral DNA clones for wild-type SIV, SIV Δ*nef*, SIV Δ*nefP*, SIV ITMΔ*nef*, SIV Δ*env*Δ*nef*, and SIV Δ*nef*ΔU3 were constructed from the following clones based on $SIV_{mac}239$; p239SpSp5', pSP72-239-3', pSP72-239-3'Δ*nef*, pSP72-239-3'ITMΔ*nef*ΔU3 (Alexander et al., 2003; Kestler et al., 1991; Regier and Desrosiers, 1990). Additional full-length clones containing subsets of the ITM changes in *env* were generated by site-directed mutagenesis of SIV Δ*nef*.

(c) Nef and Env expression constructs. The SIV Nef expression construct pCGCG-239-Nef was provided by Dr. Jacek Skowronski (Case Western Reserve University, Cleveland, OH). To express ψNef, the $SIV_{mac}239$ *nef* sequences in pCGCG-239-Nef were replaced with the *nef* open reading frame retained by SIV Δ*nefP* to generate pCGCG-ψNef. To verify protein expression, sequences coding for an HA tag were also

introduced in frame with the 3' end of ψ*nef*. Codon optimized versions of wild-type Env and EnvITM were also cloned into pCGCG.

(d) CD4-Env fusion constructs. A cDNA clone for human CD4 was obtained through the Harvard Plasmid Database. Constructs for the expression of CD4-Env fusion proteins were generated by cloning sequences for the transmembrane and/or cytoplasmic domain of Env and EnvITM downstream of sequences coding form the transmembrane and/or extracellular domain of CD4 into the *Kpn* I and *Xho* I sites of pcDNA3.

(e) GenBank accession numbers. Nucleotide sequences for novel alleles of rhesus tetherin and for pseudo-Nef were submitted to GenBank. The accession numbers for these sequences are as follows; rBST-2.2 (HM775182), rBST-2.8 (HQ596987) and pseudo-Nef (HM775183).

Virus release assays

293T cells were co-transfected with an SIV proviral DNA clone (100 ng) and one of the pcDNA3-based expression constructs for tetherin (0-200 ng). Differences in the amount of plasmid DNA in each transfection were offset by the addition of empty pcDNA3 vector (0-200 ng). To assess the ability of ψNef, EnvITM and the CD4-Env fusion proteins to rescue virus release in *trans*, 293T cells were co-transfected with proviral DNA for SIV Δ*nef* or SIV Δ*env*Δ*nef* (100 ng), pcDNA3 or pcDNA3-rBST-2 (50 ng), and either pCGCG, pCGCG-Env, pCGCG-EnvITM, pCGCG-ψNef, pCGCG-239-Nef, pcDNA3-CD4, pcDNA3-CD4-Env, pcDNA3-CD4-ITM, pcDNA3-CD4-TM-Env or pcDNA3-CD4-TM-ITM (100 ng). All transfections were performed in duplicate in 24-well plates seeded the day before at $5x10^4$ cells per well using GenJet Lipid Transfection

Reagents (SignaGen Laboratories, Gaithersburg, MD). Forty-eight hours after transfection, the amount of virus released into the cell culture supernatant was measured by SIV p27 antigen-capture ELISA (Advanced Bioscience Laboratories, Inc., Kensington, MD), and was expressed as the percentage of maximal particle release in the absence of tetherin for empty vector controls.

Western blots

293T cells were co-transfected with proviral DNA (100 ng) and pcDNA3-based expression constructs for either human or rhesus tetherin (0-200 ng) as described above. Forty-eight hours post-transfection, cell lysates were prepared by harvesting in 2x SDS sample buffer. Virions were recovered from the cell culture supernatant by centrifugation at 13,000 rpm for 2 hours at 4°C, and resuspended in 2x SDS sample buffer. Samples were boiled for 5 minutes, and separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to polyvinylidine fluoride (PVDF) membranes using a Trans-Blot SD transfer cell (BioRad, Hercules, CA). The membranes were then blocked with 5% non-fat dry-milk in PBS containing 0.05% Tween-20 for 1 hour, and probed overnight at 4°C with one of the following primary antibodies. Tetherin/BST-2 was detected with the monoclonal antibody HM1.24, generously provided by Chugai Pharmaceutical Co. (Kanagawa, Japan) at a dilution of 1:2000. The SIV Gag proteins p27 and p55 were detected with the monoclonal antibody 183-H12-5C (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) at a dilution of 1:1000. SIV Nef was detected using the mouse monoclonal antibody 17.2 (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) at a dilution 1:1000. The SIV Env

protein was detected with the gp120-specific monoclonal antibody KK42 (AIDS Research and Reference Reagent Program) at a dilution of 1:1000. Endogenous β-actin was detected with the monoclonal antibody C4 (Chemicon, Billerica, MA) at dilution of 1:1000. HA-tagged ψNef was detected with the HA-specific monoclonal antibody HA.11 (Covance, Princeton, NJ) at a dilution of 1:1000. Human CD4 and CD4 fusion constructs were detected using a rabbit polyconal antibody (Sigma-Aldrich, St Louis, MO) at 1:200 dilution. After rinsing three times for 10 minutes in PBS 0.05% Tween-20, the blots were probed with an HRP-conjugated goat anti-mouse secondary antibody (Pierce, Rockford, IL) or a HRP-conjugated anti-rabbit secondary antibody (Sigma-Aldrich, St Louis, MO) at a 1:2000 dilution for 1 hour. The blots were then rinsed three more times in PBS 0.05% Tween-20, treated with SuperSignal West Femto Maximum Sensitivity substrate (Pierce, Rockford, IL), and imaged using a Fujifilm Image Reader LAS 3000 (Fujifilm Photo Film Co., Japan).

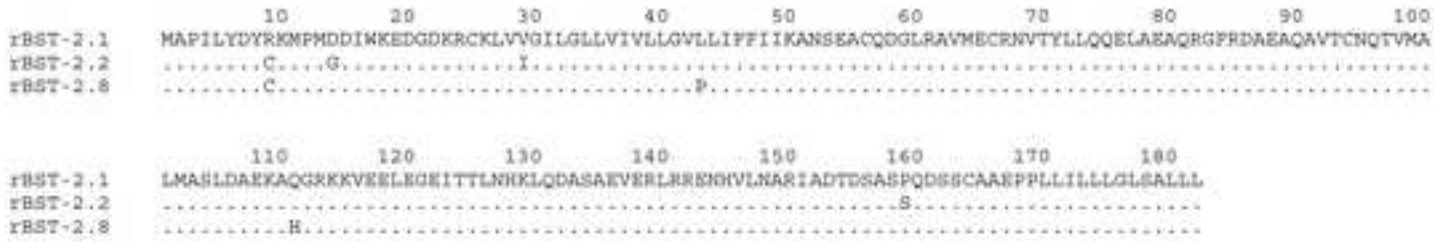
MHC class I and CD4 downregulation in transfected Jurkat cells

Ten million Jurkat cells were electroporated with bicistronic pCGCG constructs (40 μg) that express wild-type Nef, ΔNef, ψNef, Env or EnvITM and enhanced green fluorescent protein (eGFP) from a downstream internal ribosomal entry site. Twenty-four hours later, cells were stained with a PerCP-conjugated monoclonal antibody to CD4 (BD Pharmagen) and a PE-conjugated monoclonal antibody to MHC-I (HLA-ABC, Dako, Carpintería, CA). After gating on the $eGFP^+$ cells, the MFI of CD4 and MHC-I expression was determined. Data was collected using a FACSCalibur flow cytometer (Becton Dickenson) and analyzed using FlowJo 8.7 software (TreesStar).

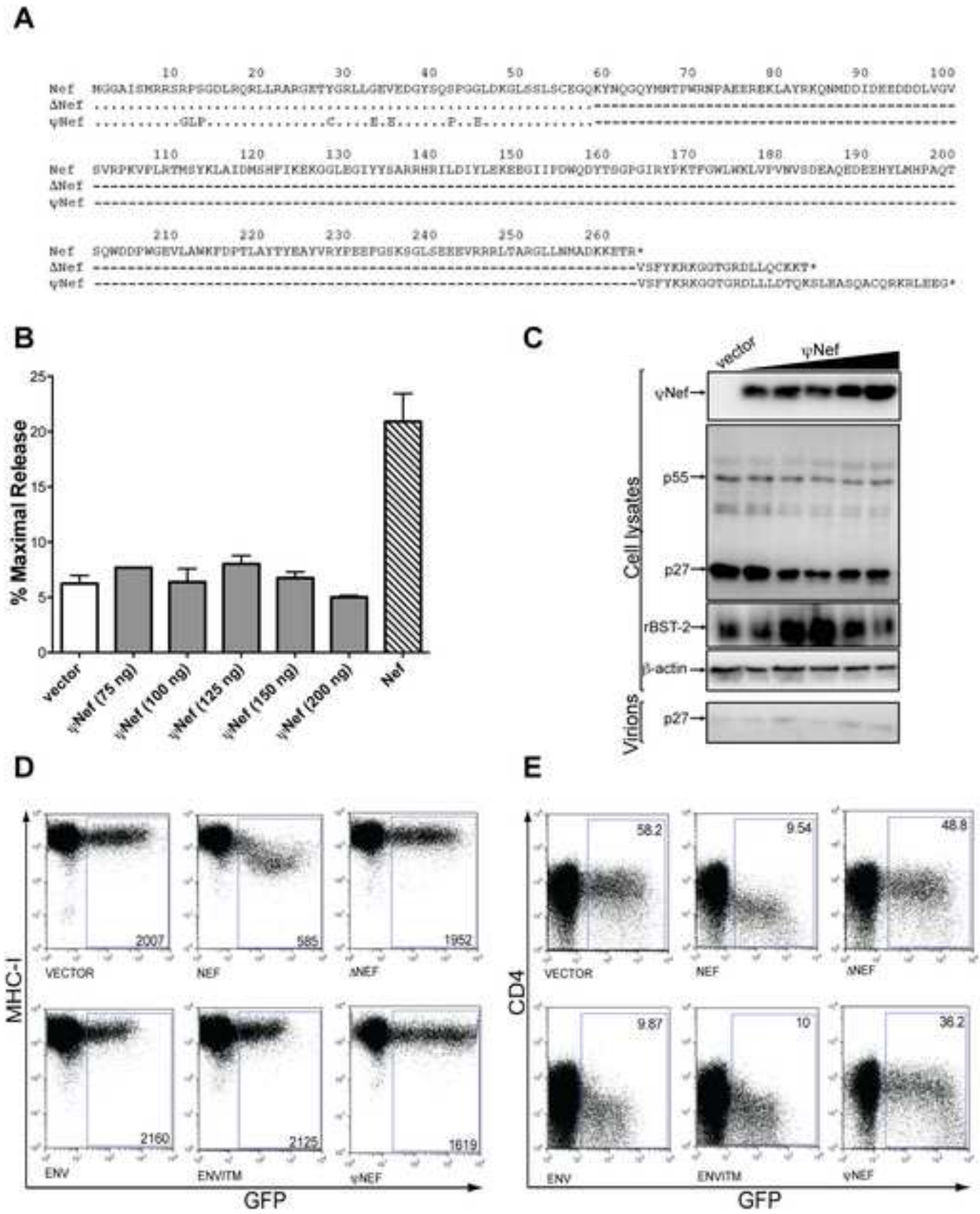
Tetherin downregulation in infected $CD4^+$ lymphocytes

Activated rhesus macaque PBMCs ($2x10^6$ cells) were infected with wild-type SIV, SIV Δ*nef*, SIV Δ*nefP* and SIV Δ*nefP* $Y_{721}A$ (50 ng p27 eq.). Five days post-infection, the cells were treated with IFNα (1000 U/ml). Forty-eight hours later, the cells were stained for BST-2 using the monoclonal antibody HM1.24 (Chugai Pharmaceutical Co.), followed by an APC-conjugated, goat anti-mouse secondary antibody (BD Pharmingen, San José, CA). The cells were then washed, and stained with a PerCP-conjugated antibody to CD4 (BD Pharmingen). After surface staining for BST-2 and CD4, the cells were permeabilized using Fix and Perm® reagents (Invitrogen), and stained for SIV Gag using the FITC-conjugated monoclonal antibody 2F12 (provided by the DAIDS/NIAID Reagents Resource Support Program for AIDS Vaccine Development, under contract of Quality Biological, Inc. and BioMolecular Technology, Inc; Principal Investigator, Ronald Brown; Program Officer, Jon Warren). The samples were then washed, fixed in 2% paraformaldehyde PBS, and data was collected using a FACSCalibur flow cytometer (Becton Dickenson, San José, CA). The data were analyzed using FlowJo 8.7 software (TreesStar, San Carlos, CA).

Supplemental Figure 1



Supplemental Figure 2



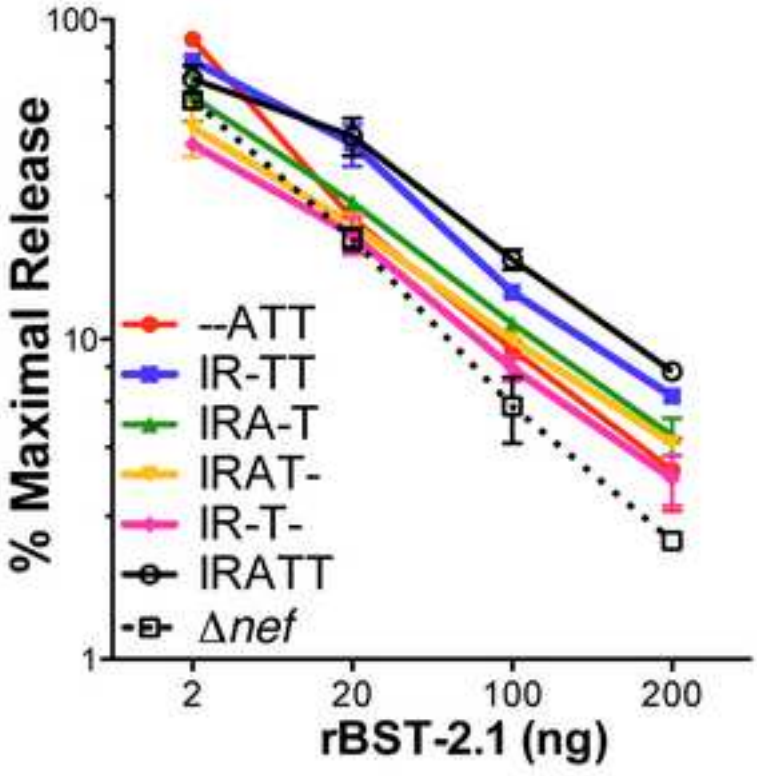
Supplemental Figure 3

A

```
                720       730       740       750       760       770       780       790       800
Env     KLRQGYRPVFSSPPSYFQQTHIQQDPALPTREGKERDGGEGGGNSSWPWQIEYIHFLIRQLIRLLTWLFSNCRTLLSRVYQILQP
IRATT   ..................................................................................
--ATT   ..................................................................................
IR-TT   ..................................................................................
IRA-T   ..................................................................................
IRAT-   ..................................................................................
IR-T-   ..................................................................................

                810       820       830       840       850       860       870       880
Env     ILQRLSATLQRIREVLRTELTYLQYGWSYFHEAVQAVWRSATETLAGAWGDLWETLRRGGRWILAIPRRIRQGLELTLL
IRATT   ..................................IR.A...T.........................T............
--ATT   ..................................A...T.........................T............
IR-TT   ..................................IR.....T.........................T............
IRA-T   ..................................IR.A.............................T............
IRAT-   ..................................IR.A...T.....................................
IR-T-   ..................................IR.....T.....................................
```

B



Supplemental Figure 4