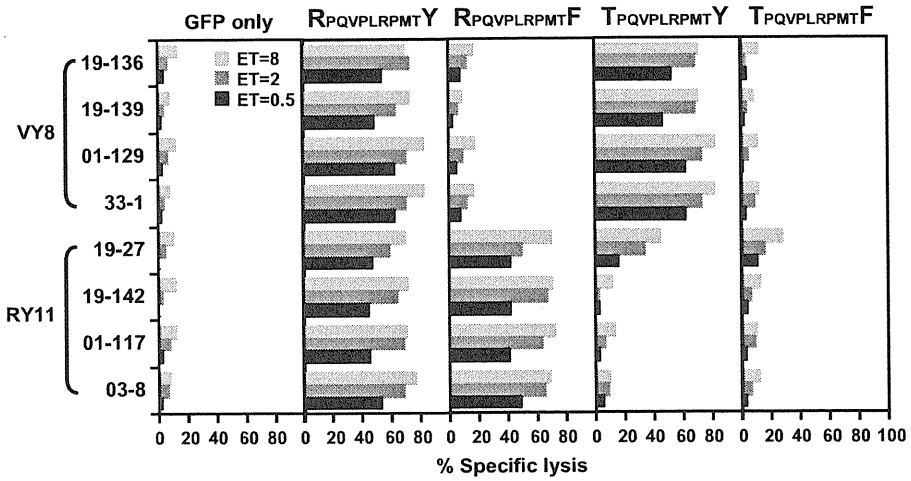

Fig. 2. Effects of the Nef variants on CTL recognition. The HLA-B*3501-restricted CTL clones specific for VY8 (VPLRPMTY) or RY11 (RPQVPLRPMTY) epitopes in Nef were analyzed for their cytolytic activity toward target cells at effector-to-target cell ratios (ET) of 0.5, 2, and 8, as indicated. The target cells were C1R-B3501 cells that had been transfected with DNAs encoding GFP alone or wild-type or various Nef variants. CTL clones were derived from four different donors: CTL19-136, 19-139, 19-27, and 19-142 were from patient No. 19; CTL01-117 and 01-129, from patient No. 1; and CTL 33-1 and 03-8, from patient Nos. 33 and 03, respectively. An additional experiment showed similar results.

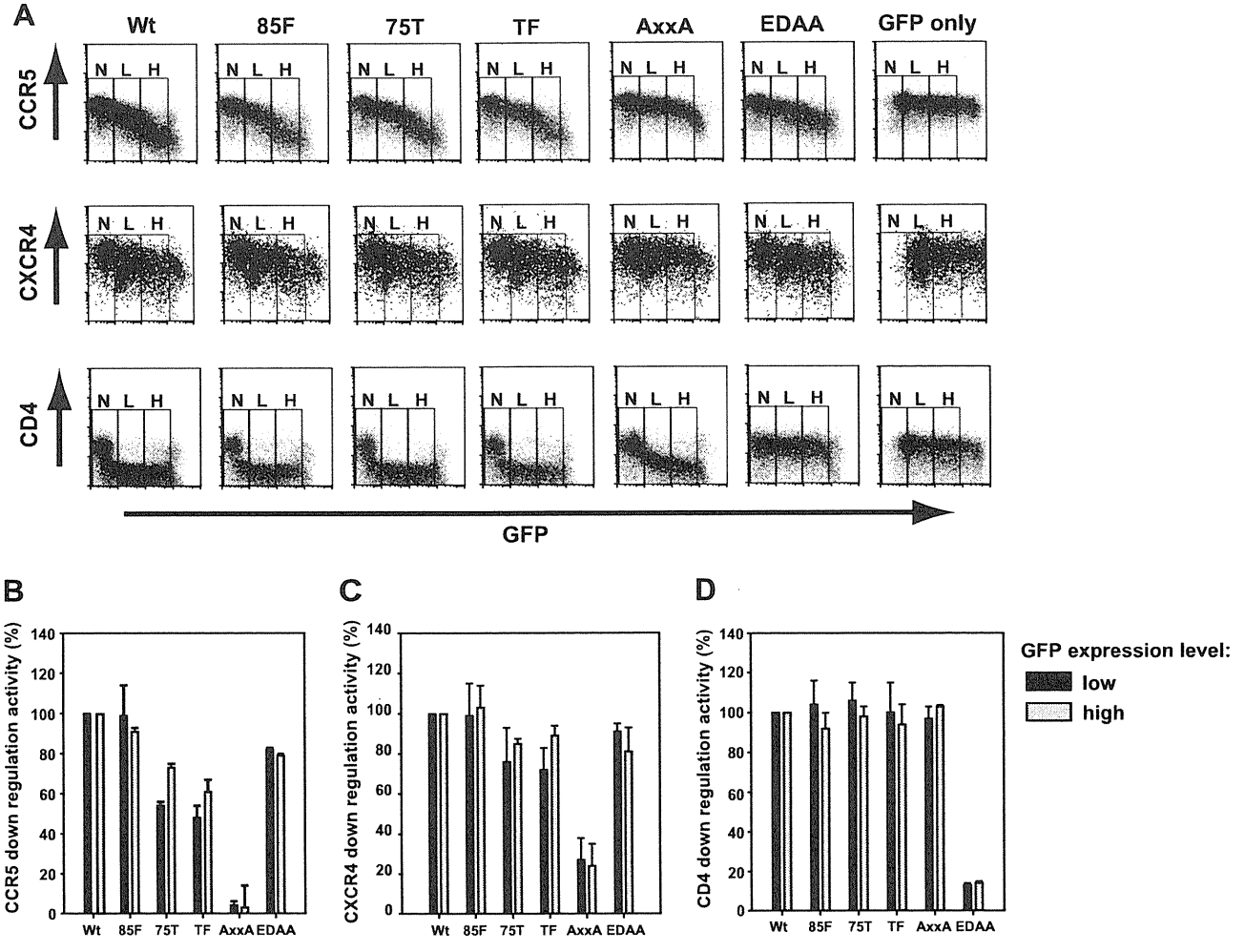

Fig. 3. Effects of the Nef variants on viral receptor down-regulation in TZM cells. (A) TZM cells were transfected with cDNA encoding GFP alone or Nef-GFP fusion proteins and analyzed for their expression levels of CCR5, CXCR4, and CD4 by flow cytometry. The Nef variants tested are indicated. N, L, and H indicate a negative, low, and high level of GFP expression, respectively. (B–D) The relative down-regulation activity of wild-type Nef and its various variants toward CCR5 (panel B), CXCR4 (panel C), and CD4 (panel D) is presented, with the wild-type Nef activity set to 100%. Data are presented as the mean ± SD of three independent experiments.

We then examined the HLA-B*3501-restricted CTLs for their recognition of these variants. Because we had previously identified two overlapping HLA-B*3501-restricted CTL epitopes, with the short epitope VY8 (Nef_{78-85}; VPLRPMTY) and the amino terminal-extended longer epitope RY11 (Nef_{75-85}; RPQVPLRPMTY) [15,21], we generated CTL clones specific for VY8 and RY11 from $CD8^+$ T cells isolated from four different HIV-infected patients and tested them for their cytotoxic activity toward target cells expressing wild-type or various variant Nef proteins (Fig. 2). Clearly, VY8-specific CTL clones failed to recognize the cells expressing the Nef variants having 85F or both 75T and 85F (TF) mutations (Fig. 2). Also, RY11-specific CTLs did not respond to cells expressing 75T or TF mutations (Fig. 2). These results indicate that the 75T and 85F were mutations that conferred escape from HLA-B*3501-restricted CTLs.

3.2. Effects of the Nef mutations on down-regulation of viral receptors

We then examined the effects of wild-type and variant Nef proteins in TZM cells on their down-regulation activity of CD4, CCR5, and CXCR4 receptors. Transfection of these cells with the gene encoding wild-type Nef or GFP fusion proteins resulted in a remarkable reduction in the expression of CD4, CCR5, and CXCR4 receptors on the cell surface (Fig. 3A), confirming the observation previously reported [1,13,23]. Interestingly, the down-regulation of CCR5 and CXCR4 by the wild-type Nef appeared to be proportional to the level of Nef-GFP expression; whereas the down-regulation of CD4 was substantial even at a low level of Nef-GFP expression (Fig. 3A). The CCR5 (Fig. 3B) and CXCR4 (Fig. 3C) down-regulation activity of the AxxA variant was impaired by >90% and ~70%, respectively, compared with the wild-type activity; whereas the CD4 down-regulation activities of the wild type and the AxxA variant were not substantially different (Fig. 3D). In contrast, the CCR5 and CXCR4 down-regulation activity of the EDAA variant was impaired by only <20% (Fig. 3B and C), whereas its CD4 down-regulation activity was much impaired, by ~90% (Fig. 3D). These results confirmed the previous findings showing that genetically separable regions in Nef mediate down-regulation of CD4 and chemokine receptors [13,23].

In CTL escape-conferring mutations, we found that the 75T and TF variants were markedly impaired in their CCR5 down-regulation activity by ~40% (Fig. 3B); whereas the CXCR4 down-regulation activity was not much impaired by the variants (Fig. 3C). In contrast, they had no diminishment of their CD4 down-regulation activity (Fig. 3D). Also, there was no substantial difference either in CCR5, CXCR4 or CD4 down-regulation activity between the wild type and the RF variant (Fig. 3B–D). These data demonstrate that the 75T and TF variants selectively diminished the CCR5 down-regulation activity by Nef. Considering that the 75T mutation alone did not show substantial impairment in the down-regulation of HLA class I or enhancement of viral infectivity [21], it is possible that different molecular pathways could be involved in CCR5 down-regulation and the other Nef functions associated with the proline-rich motif.

3.3. Effects of the Nef mutations on susceptibility to HIV superinfection

We postulated that a functional consequence of the down-modulation of viral entry receptors by Nef would be that HIV-infected cells would be protected from a deleterious HIV superinfection, as

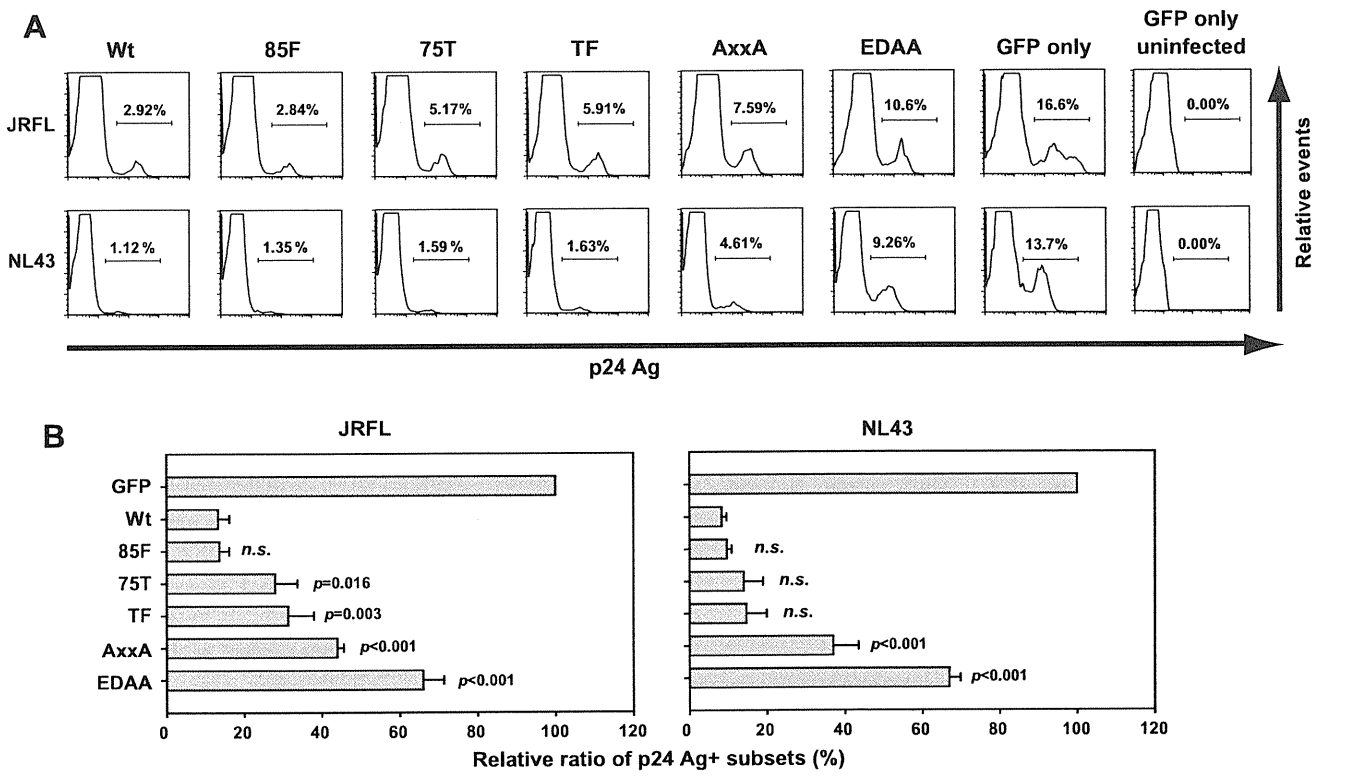

Fig. 4. Effects of the Nef variants on susceptibility to HIV-1 superinfection. (A) TZM cells that had been transfected with GFP alone or Nef-GFP constructs having various Nef mutations indicated in the figure were exposed to the wild-type HIV-1 (strain JRFL or NL43). Another fraction of GFP-expressing TZM cells remained uninfected as a negative control. The resultant cells were analyzed by flow cytometry. The live GFP^+ subset was gated and analyzed for its fluorescence intensity of p24 Gag. The frequency of p24 Gag^+ subsets within live GFP^+ subsets is indicated in the figure. (B) The relative ratio of p24 Gag^+ subsets is presented, with the value obtained for the TZM cells expressing GFP only set to 100%. Data are presented as the mean ± SD of at least three independent experiments, and statistical analysis was performed based on ANOVA with multiple comparisons *vs.* wt (Bonferroni *t*-test). *n.s.*, not significant.

described before [2,11,13,16]. In addition, the selective impairment of chemokine receptor down-regulation by the Nef variants shown above may differently influence the superinfection protection against the CCR5- or CXCR4-tropic viruses. To test this possibility, we examined the susceptibility of TZM cells expressing wild-type or various Nef variants to infection by the JRFL (CCR5-tropic) and NL43 (CXCR4-tropic) viruses. We quantified HIV-infected cells by flow cytometry, because this method enabled us to directly look at HIV-infected cells in terms of intracellular expression of p24 Gag proteins. The susceptibility of the cells to infection by JRFL or NL43 was much decreased when the target cells had been expressing the wild-type Nef, by ~90% (Fig. 4A and B), whereas this protective capacity effected by Nef was impaired by either the AxxA or EDAA mutation (Fig. 4A and B), in good agreement with a previous report [13]. These results suggest that the down-regulation of viral entry receptors by Nef played a substantial role in protection of HIV-infected cells from HIV superinfection. However, the EDAA mutation showed the strongest impairment of protection against superinfection (Fig. 4A and B), indicating that the CD4 down-regulation activity by Nef was the most influential factor for protection against superinfection by both JRFL and NL43 viruses. Of the CTL-escape variants, both 75T and TF variants gave impaired protective capacity against superinfection by JRFL, whereas the 85F variant had no effect on this activity (Fig. 4A and B). In contrast, we observed no substantial effects on superinfection protection by the NL43 virus in all CTL-escape variants tested (Fig. 4A and B). In addition, the conventional single-round infectivity assay based on β-galactosidase activity in HIV-infected TZM cells gave similar results (data not shown). These data demonstrate that the 75T and TF variants selectively diminished the protection activity against superinfection by the CCR5-tropic viruses, suggesting the disadvantageous property of these Nef variants in virus spread and persistence during an HIV-1 infection. However, it is conceivable that the functional impairment in Nef induced by CTL-escape variants could be compensated later by mutations at secondary sites in Nef, as recently observed in the case of Gag proteins [5]. Further studies using a large number of subjects with various disease status are needed to extend this observation, such as how mutational escape from Nef-specific CTL responses, altered Nef functions, and clinical outcome of HIV-infected individuals are related to each other at the population level.

4. Conclusions

We showed that some naturally arising amino acid variations in the well-conserved proline-rich region of Nef are associated with escape from HLA-B*3501-restricted CTLs. One of these mutations, Arg75Thr selectively impaired CCR5, but not CXCR4, down-regulation activity by Nef and decreased protection capacity against superinfection by CCR5-tropic HIV-1. Thus, certain Nef-specific CTL responses can constrain HIV-1 spread and persistence in the HIV-infected host, providing us with additional insight into the designing of vaccines against HIV-1.

Acknowledgments

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HIV-1 Nef impairs multiple T-cell functions in antigen-specific immune response in mice

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Abstract

The viral protein Nef is a key element for the progression of HIV disease. Previous *in vitro* studies suggested that Nef expression in T-cell lines enhanced TCR signaling pathways upon stimulation with TCR cross-linking, leading to the proposal that Nef lowers the threshold of T-cell activation, thus increasing susceptibility to viral replication in immune response. Likewise, the *in vivo* effects of Nef transgenic mouse models supported T-cell hyperresponse by Nef. However, the interpretation is complicated by Nef expression early in the development of T cells in these animal models. Here, we analyzed the consequence of Nef expression in ovalbumin-specific/CD4$^+$ peripheral T cells by using a novel mouse model and demonstrate that Nef inhibits antigen-specific T-cell proliferation and multiple functions required for immune response *in vivo*, which includes T-cell helper activity for the primary and memory B-cell response. However, Nef does not completely abrogate T-cell activity, as defined by low levels of cytokine production, which may afford the virus a replicative advantage. These results support a model, in which Nef expression does not cause T-cell hyperresponse in immune reaction, but instead reduces the T-cell activity, that may contribute to a low level of virus spread without viral cytopathic effects.

Keywords: AIDS, acquired immunity, humoral response

Introduction

The Nef protein of the primate lentiviruses HIV-1/2 and the simian immunodeficiency virus (SIV) is expressed from the earliest stage of viral gene expression (reviewed in ref. 1). Nef-defective viruses cause a slow progression of clinical disease with reduced viral loads in humans and rhesus macaques with HIV-1/2 and SIV infection, respectively, indicating that Nef plays a crucial role in viral pathogenesis in human and non-human primates (reviewed in ref. 1). Nef associates with host cell membranes through N-terminal myristoylation and functions as an adaptor bringing together a large number of proteins in host cells, mainly protein kinases and components of endocytic trafficking machinery (reviewed in ref. 1; refs 2–7).

Nef reduces surface level receptors, including CD4, the primary receptor for HIV and SIV and MHC class I and class II complex, facilitating HIV immune evasion and thus increases viral pathogenesis (reviewed in ref. 1). Additionally, extensive *in vitro* studies, mostly carried out by using human T-cell lines, have suggested that Nef expression enhances TCR-mediated signaling pathways and transcriptional activation (reviewed in ref. 1; refs 2–5). Such alterations in signaling events may lower the TCR activation threshold in CD4$^+$

T cells and help more responsive to T-cell activation signals, a process that could support higher virus production upon stimuli mediated via the TCR (reviewed in ref. 1; refs 2–5). Moreover, Nef may alter host cell death pathways to prevent apoptosis of infected cells, thereby fostering their longevity (reviewed in ref. 1) These observations have led to a model in which Nef reorganizes the host cell activity so as to optimize viral propagation and cell survival, thus facilitating immune evasion and participating in virus spread.

The consequence of Nef expression in primary cells has been examined by using Nef transgenic (Tg) mice, in which Nef was constitutively or transiently expressed under control of a T-cell-specific promoter–enhancer element (8, 9). In this model system, Nef promotes T-cell activation, however, interpretation of these findings is complicated by the fact that expression of Nef early in the development of T cells results in wholesale depletion of thymocytes and peripheral T cells. Moreover, it remains obscure whether the T-cell activation seen in Nef Tg mice is mediated by lymphopenia-induced mechanisms rather than by an intrinsic effect of Nef expression on T-cell activation and proliferation (9, 10).

In the present study, to examine the consequence of Nef expression in primary cells, we established a double transgenic mouse (dTg), which expresses human coxsackie/adenovirus receptor (CAR) (11) and an ovalbumin (OVA)-specific TCR that recognizes the OVA peptide on antigen-presenting cell (APC) with high affinity under MHC Class II I-A^d-restriction. This system allowed us to analyze the effect of Nef on antigen-specific peripheral T-cell function by transfer of the *nef* gene into peripheral T cells using an adenovirus vector. The present study demonstrates that Nef expression does not cause T-cell hyperresponse but instead impairs T-cell functions required for immune response.

Methods

Mice

BALB/c and CB17-scid mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and Clea Japan, Inc. (Tokyo, Japan), respectively. Tg mice expressing the CAR under the control of the Lck proximal promoter (CAR Tg mice) on the BALB/c background have been described previously (11). DO11.10 mice express a transgenic TCR with specificity for OVA peptide residues 323–339 ($OVA_{323-339}$) restricted by I-A^d on the BALB/c background (12). All mice used in this study were maintained under specific pathogen-free conditions and used at 6–12 weeks of age in accordance with the guidelines of the Institutional Animal Care and Use Committee, National Institute of Infectious Diseases.

Adenovirus vector

Recombinant adenovirus vectors were generated using the AdEasy Adenoviral Vector System (Stratagene) according to the manufacturer's instructions. In order to express the *nef* gene under the CAG promoter, the pShuttle vector was digested with *Kpn*I, blunt-ended with T4 polymerase and then, the CAG promoter DNA was ligated (pShuttle-CAG). Next, an *Xho*I–*Xba*I fragment of pIRES2-EGFP (Invitrogen) was inserted into the *Xho*I–*Xba*I site of pShuttle-CAG, which was designated as pShuttle-CAG-I2-EGFP. HIV-1 NL4-3 nef wild-type and a mutant ($^{57}W^{58}L$ to $^{57}A^{58}A$) were PCR amplified from pNL432 and pNL-n57/2A proviral DNA, respectively, using specific primers containing *Eco*RI sites at both ends and then subcloned into pBluscript KS^+ (Stratagene). The *Eco*RI fragment containing wild-type or mutant nef was inserted into the *Eco*RI site of pShuttle-CAG-I2-EGFP. These shuttle vectors were linearized and co-transformed into *Escherichia coli* strain BJ5183-AD-1, which contains the pAdEasy vector, to induce homologous recombination (Supplementary Figure 1 is available at *International Immunology* Online). Recombinant adenoviral plasmids were selected and transfected into 293 cells to produce recombinant adenovirus particles. Recombinant adenovirus were purified by two rounds of Cesium chloride density gradient centrifugation as described previously (13). The concentrated virus was dialyzed against PBS containing 10% glycerol. The titer of the virus stock was determined by a plaque formation assay using 293 cells.

T-cell purification and recombinant adenovirus infection

For recombinant adenovirus infection, $CD4^+$ T cells were enriched by negative selection on a MACS column (Miltenyi Biotec GmbH, Gladbach, Germany) as previously described (14). Briefly, cells were blocked with anti-FcγRII/III (2.4G2; BD PharMingen, San Diego, CA, USA) and incubated with biotinylated mAbs against B220(RA3-6B2), IgM(II/41), IgD(11-26), Gr1(RB6-8C5), CD11c(N418), CD49b(DX5), CD11b(M1/70) and CD8(53–6.7) (eBioscience, San Diego, CA, USA), followed by incubation with streptavidin-coated microbeads (Miltenyi Biotec GmbH). Purified $CD4^+$ T cells (>95%) were infected with recombinant adenovirus vector at a multiplicity of infection of 10 (MOI 10) for 2 days in 24-well plates at a concentration of 2×10^6 per well in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 5×10^5 M 2-mercaptoethanol, L-glutamine, antibiotics and IL-7 (20 ng ml^{-1}; PeproTech, London, UK) at 37°C in an atmosphere of 5% CO_2.

Proliferation assays and ELISA

Sorted $CD4^+$ GFP^+ T cells were cultured in microtiter wells at a concentration of 4×10^4 cells per well in the presence of $OVA_{323-339}$ peptide and 5×10^5 irradiated T-depleted spleen cells. DNA synthesis of cultured cells in triplicate was estimated by the incorporation of [^{3}H] thymidine (0.5 μCi) added 12 h prior to cell harvest. The level of IFN-γ and IL-2 in the culture supernatants was measured by a Ready-Set-Go! ELISA assay kit (eBioscience), according to the manufacturer's instruction. In some experiments, $CD4^+$ GFP^+ T cells (2×10^6) were cultured for 2–3 days in 96-well plates immobilized with anti-TCR mAb (5 μg ml^{-1}) and anti-CD28 mAb (1 μg ml^{-1}) (BD PharMingen).

Chemotaxis assay

Chemotaxis assays were performed in Transwell (Corning Coster, Coring, NY, USA) with polycarbonate filters (5 μm pore size) as described previously (15). Briefly, purified $CD4^+$ GFP^+ T cells were suspended at 5×10^6 cells ml^{-1} in RPMI 1640 medium containing 1% FBS and 25 mM HEPES. One

hundred microliters of cell suspension was loaded onto the upper wells and placed in a 24-well plate containing 600 μl of media with the indicated doses of CXCL12 (SDF-1α) (PeproTech) or CCL19 (ELC) (R&D Systems, Minneapolis, MN, USA). Cells were incubated at 37°C for 90 min, and cells in the bottom wells were counted using a FACSCalibur.

Activation-induced cell death assay

Sorted $CD4^+$ GFP^+ T cells were cultured at a concentration of 1×10^6 cells ml^{-1} in 96-well plates immobilized with 5 μg ml^{-1} of anti-CD3ε mAb (2C11) (BioLegend, San Diego, CA, USA) in RPMI medium supplemented with 10% FBS. Cells were harvested 2 days later and then re-cultured for 3 days in 96-well plates containing immobilized with anti-CD3 mAb or medium containing 200 U ml^{-1} of human IL-2 (PeproTech). To detect apoptotic cells, a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay was performed using the ApopTag Red In Situ Apoptosis Detection Kit (CHEMICON International Inc., Temecula, CA, USA). Briefly, the cells were collected and deposited on glass slides by cytospin (Shandon, London, UK), fixed with PBS containing 1% PFA for 10 min and the DNA free 3′ OH were enzymatically labeled with digoxigenin-labeled nucleotides, which were detected using rhodamine-labeled anti-digoxigenin polyclonal antibodies according to the manufacturer's instructions. After applying 6 μg ml^{-1} of Hoechst33342 (Invitrogen) for nuclear staining, slides were processed for analysis using an LSM 510 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany). The proportion of apoptotic cells was determined by counting at least 100 cells in the captured images.

T-cell migration in vivo

BALB/c mice were intravenously injected with 2×10^6 of purified $CD4^+$ GFP^+ T cells uninfected or infected with a recombinant adenovirus vector. Twenty-four hours later, the recipient mice were subcutaneously immunized with 0.2 mg of LPS-free OVA in CFA on the back at three sites. The number of $CD4^+KJ1\text{-}26^+$ T cells in the draining lymph nodes was measured by flow cytometry at 5 days after immunization.

Adoptive cell transfer

Transfer of B cells and OVA-specific/$CD4^+$ T cells infected with a recombinant adenovirus vector in adoptive hosts was performed as described previously (14).

Briefly, $CD4^+$ GFP^+ T cells were prepared by FACS sorting from dTg T cells infected with a recombinant adenovirus vector *in vitro*. B cells were negatively selected from the pooled spleens of either naive mice or 4-hydroxyl-3-nitrophenylacetyl-conjugated chicken γ-globulin (NP-CGG)-primed mice using a MACS system and biotinylated anti-CD5 (53–7.3), anti-CD90.2 (53–2.1), anti-Gr1, anti-CD11b (eBioscience), anti-CD43 (57) and anti-CD138 (281-2) (BD PharMingen). The procedure consistently yielded >95% $B220^+$ cells. Purified B cells (5×10^6) together with $CD4^+$ GFP^+ T cells infected with recombinant adenovirus vector (3×10^4) were intravenously injected into CB17-scid mice. One day later, the recipient mice were intraperitoneally challenged with 25 μg of soluble NP-OVA, and the sera were collected from individual mice at day 7 after challenge. Anti-NP serum antibody titers were estimated by ELISA assays using NP_2-BSA and NP_{18}-BSA as coating antigens as described previously (14). The relative affinity of anti-NP antibodies was estimated by calculating the ratio of anti-NP_2/anti-NP_{18} antibody.

Statistics

The results were evaluated statistically by two-tailed Student's *t*-test ($n = 3$) or Mann–Whitney nonparametric test ($n > 4$), with $P < 0.05$ regarded as significant.

Results

Nef impairs T-cell proliferation upon antigen stimulation in vitro

In order to determine the effect of Nef expression in peripheral T cells, we crossed Tg mice that express an OVA-specific T-cell receptor (12) with mice expressing CAR on T cells (11). OVA-specific/$CD4^+$/CAR^+ T cells were purified from the pooled spleens of dTg mice and infected *in vitro* with an adenovirus vector encoding green fluorescence protein (GFP) driven by the CAG promoter with (Ad-nef) or its mutants [Ad-nef (mu)] or without the *nef* gene (Ad) in the presence of IL-7, which supports T-cell survival and promotes progression into the G_{1b} stage of the cell cycle (16, 17). Thereafter, GFP^+ cells were purified by FACS and provided for analysis as below.

Consistent with previous observations in human T-cell lines, Fig. 1(A) shows that CD4 expression on murine peripheral T cells was down-regulated by Nef but not by the Nef mutant carrying amino acids replacements of $^{57}W^{58}L$ to $^{57}A^{58}A$, abrogating the ability to down-regulate CD4 (18). Nef expression had no effect on the expression of CD25, CD28, CD44, CD62L, CD69, TCRβ and MHC class I (data not shown).

To examine the effect of Nef in T-cell response, GFP^+ cells were purified by FACS from $CD4^+$/CAR^+ T cells infected with Ad-nef, Ad-nef (mu) and cultured in the presence of irradiated splenocytes as APCs, which had been pulsed with OVA peptide ($OVA_{323–339}$). Expression of wild-type as well as mutant forms of Nef diminished T-cell proliferation upon stimulations with OVA peptide at a dose of 0.1 μM (Fig. 1B). These Nef proteins also reduced the level of cytokines produced by T cells in response to different doses of OVA peptide (Fig. 1C). These results suggest that Nef prominently affects T-cell proliferation, irrespective of Nef's ability to down-modulate CD4 but not completely abrogate T-cell activation.

Nef-expression diminishes T-cell migration activity in the primary immune response

Chemokines and their receptors play pivotal roles in the initial homing of lymphocytes and their subsequent trafficking during an immune response (6). It has been reported that Nef impairs the migratory capacity of human T-cell lines *in vitro* in response to the chemokine CXCL12, which binds to T-cell receptor, CXCR4, owing to alteration of the signal cascades downstream of chemokine receptors (7, 15). Consistently, the expression of Nef or its mutant in murine $CD4^+$ T cells reduced their migration in response to CXCL12 *in vitro*, without altering the surface receptor expressions (Fig. 2A and B).

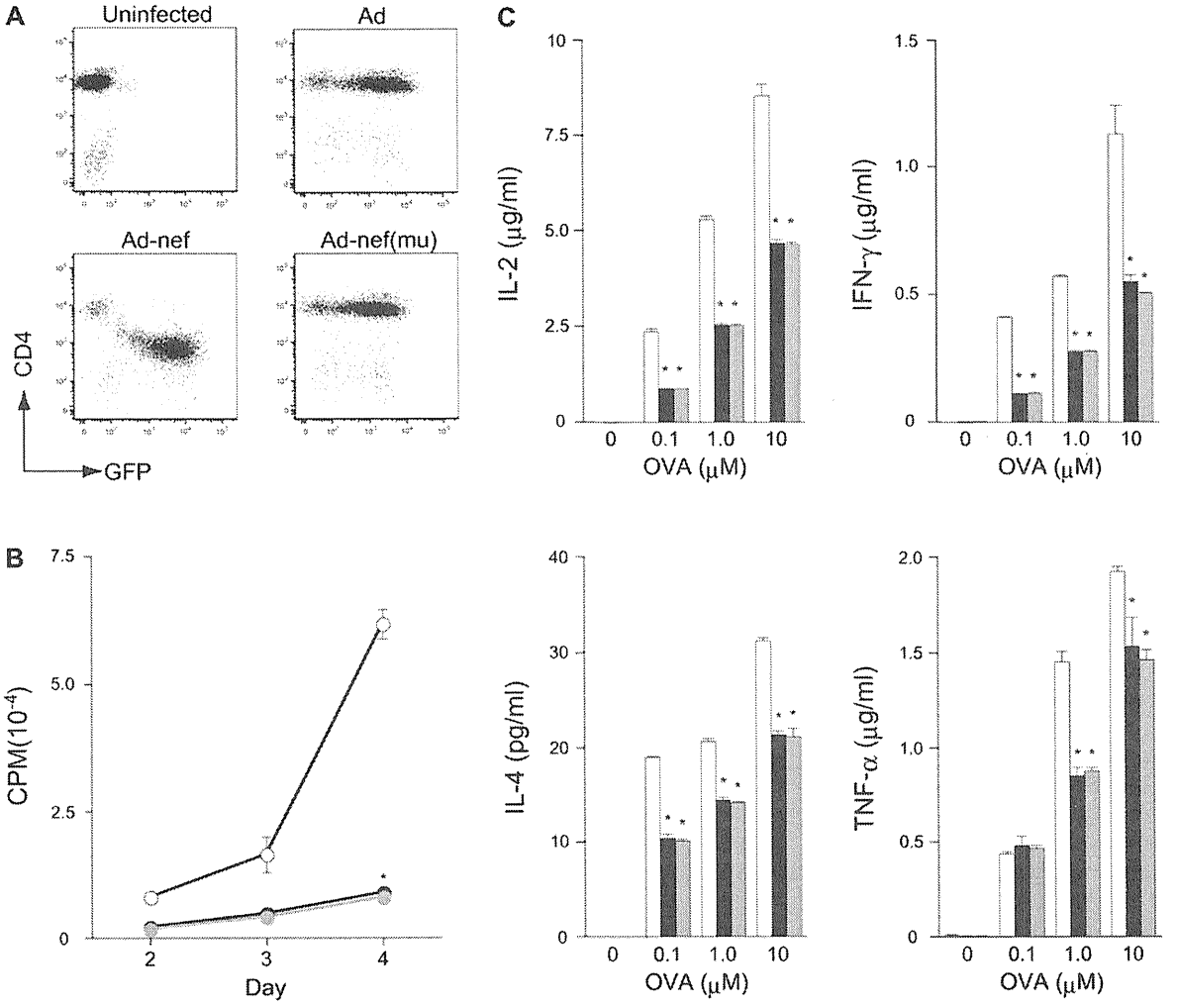

Fig. 1. (A) Characterization of Nef-expressing T cells. A *EGFP* gene-containing adenoviral vector was used to evaluate the efficiency of adenovirus (Ad) infection in DO11.10/CAR$^+$/CD4$^+$ T cells. Naive CD4$^+$ T cells from dTg mice were infected with Ad-nef, Ad-nef (mu) or Ad vector as a control. Two days later, GFP and CD4 expression were assessed by FACS. (B) Nef represses antigen-specific T-cell proliferation. Purified CD4$^+$/GFP$^+$ T cells (5×10^4) infected with Ad-nef (closed), Ad-nef (mu) (gray) and Ad (open) were cultured with T-cell depleted spleen cells as APCs (5×10^5) pulsed with 0.1 μM of $OVA_{323-339}$ peptide. Their DNA synthesis in the triplicate culture was estimated at the indicated periods by the incorporation of [^{3}H] thymidine added 12 h prior to cell harvest. *$P < 0.001$ versus Ad. (C) Purified CD4$^+$ GFP$^+$ T cells and APCs were co-cultured with various concentrations of $OVA_{323-339}$ peptide. Cytokine production in culture supernatant was measured by ELISA on day 3 of culture. *$P < 0.001$ versus Ad. Shown is the representative data from two independent experiments.

Likewise, the Nef proteins, including NL4-3 Nef, did not alter the expression of CXCR4 on human T cells (15, 19), however, there are controversial reports that HIV-1 Nef caused a modest decrease in expression of CXCR4 on human T cells, irrespective of Nef alleles, including NA7 and NL4-3 (7, 20). Further analysis is needed to resolve the discrepancy among these studies.

To examine whether Nef affects T-cell migration *in vivo*, OVA-specific CD4$^+$ T cells were purified from pooled splenocytes of dTg mice and infected with Ad-nef, Ad-nef(mu) or Ad. These cells were transferred into syngeneic recipients, followed by subcutaneous inoculation with OVA in CFA. Five days later, the frequency of OVA-specific (KJ1-26$^+$) CD4$^+$ T cells in the draining lymph node was estimated by FACS. As shown in Fig. 2(C), we observed that Nef impairs the physiological recruitment of T cells into the secondary lymphoid tissues in the immune response. A substantial number of GFP$^+$/OVA-specific/CD4$^+$ T cells infected with Ad accumulated in the draining lymph node after OVA stimulation, however, the number of cells was significantly reduced when the T cells expressed Nef or its mutant. T cells in the draining lymph nodes uniformly expressed high levels of CD44, a marker for activated T cells (21), irrespective of their expression of Nef or Nef mutant (Fig. 2D), suggesting that they were activated, but not involved in functional maturation. These results suggest that Nef affects trafficking of T cells to the regional lymph nodes during an immune response, independently of CD4 down-modulation.

As shown in Fig. 2(E), we examined the possibility that nef expression causes T cells to undergo AICD, which could reduce the number of cells migrating to the regional lymph nodes after stimulation. OVA-specific/CD4$^+$ or

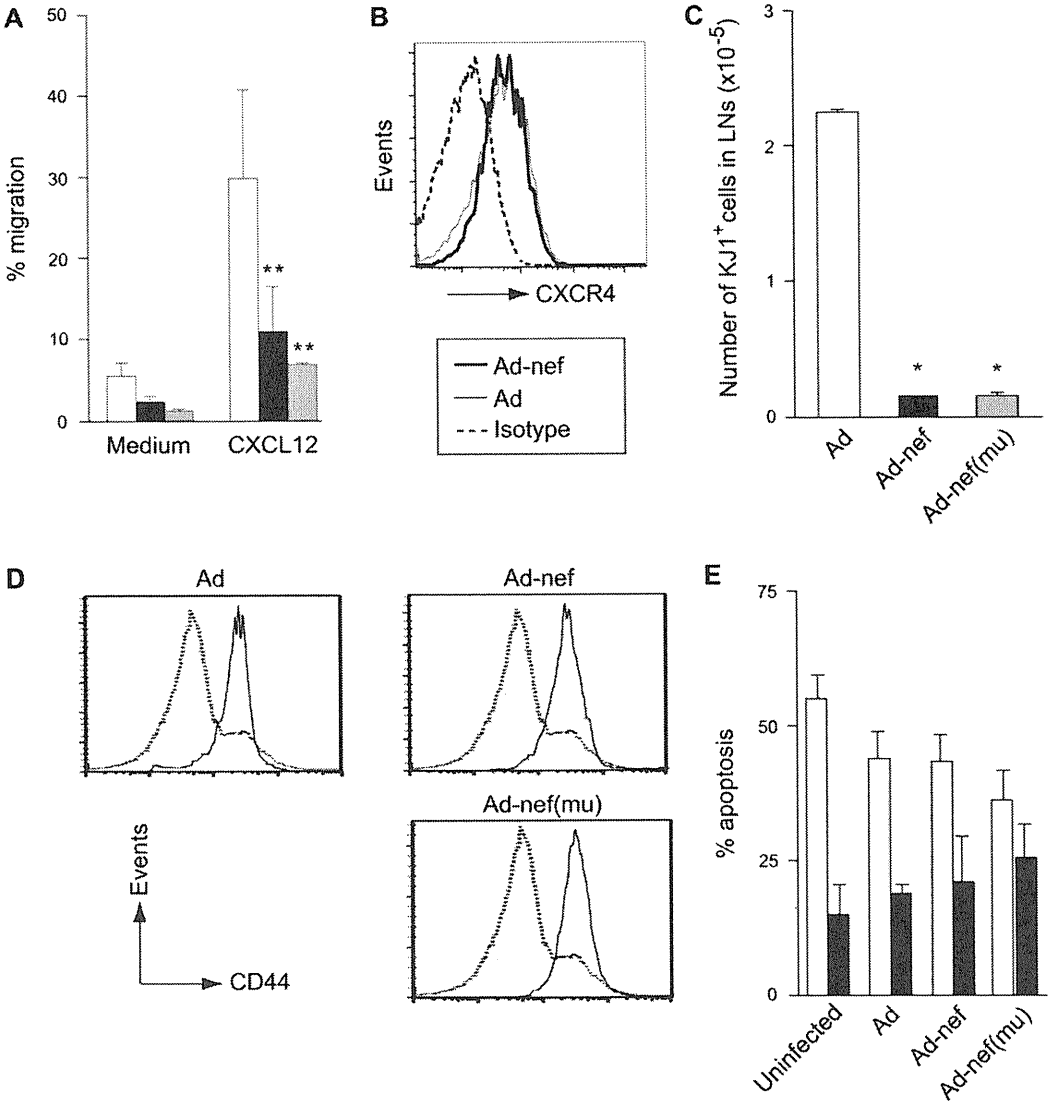

Fig. 2. Nef impairs T-cell migratory activity (A). $CD4^+$ GFP^+ T cells infected with Ad-nef (closed column), Ad-nef (mu) (gray column) and Ad (open column) were used in transwell chemotaxis assays in the presence of CXCL12 (PeproTech). Cells were allowed to migrate in the bottom wells for 90 min, and the proportion of cells that had migrated into the lower wells was determined by flow cytometry. The results are shown as mean ± SD ($n = 3$). *$P < 0.01$ versus Ad. (B) CXCR4 surface staining for $CD4^+$/GFP^+ T cells after infection with Ad-nef (solid line) or Ad (thin line), together with control IgG staining (broken lines). (C and D) $CD4^+$/GFP^+ T cells (2×10^6) infected with Ad-nef (closed column), Ad-nef (mu) (gray column) and Ad (open column) were transferred into BALB/c mice and 24 h later mice were injected subcutaneously with 0.2 mg of LPS-free OVA with CFA on the back in three sites. The cell number (±SD) of $CD4^+$/OVA-specific T cells in the draining lymph nodes (C) and the level of CD44 expression in Ad-infected donor (solid line) and recipient $CD4^+$ T cells (broken line) (D) were measured by flow cytometry using anti-CD4, anti-CD44 and KJ1-26 mAbs on day 5 after OVA injection. *$P < 0.001$ versus Ad. (E) $CD4^+$ T cells (1×10^6) or $CD4^+$/GFP^+ T cells (1×10^6) infected with Ad-nef, Ad-nef (mu) and Ad were stimulated with immobilized anti-CD3ε mAb for 2 days, followed by re-stimulation with anti-CD3 mAb/IL-2 (open column) or IL-2 alone (closed column) for 3 days. Apoptotic cells were analyzed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay. Representative data from two independent experiments in (A), (C) and (D) and from three independent experiments (B) is shown.

OVA-specific/$CD4^+$/GFP^+ T cells were hyperstimulated with immobilized anti-CD3ε mAb at 2-day intervals as previously described (22). The results show that Nef did not enhance the induction of AICD in T cells upon TCR-stimulation *in vitro* nor did it compromise the survival function mediated by IL-2. Therefore, it seems unlikely that Nef causes T-cell death, which could reduce the number of cells migrating to the regional lymph nodes.

Nef expression in T cells affects the primary and memory B-cell responses

To examine T-cell helper activity by Nef, OVA-specific/$CD4^+$ T cells were purified from the pooled spleens of dTg mice, followed by infection with or without Ad-nef, Ad-Nef (mu) or Ad. The GFP^+/$CD4^+$ T cells were purified by FACS (Fig. 3A) and transferred into CB17-scid mice, together with either naive or NP-primed B cells. The recipients were immunized

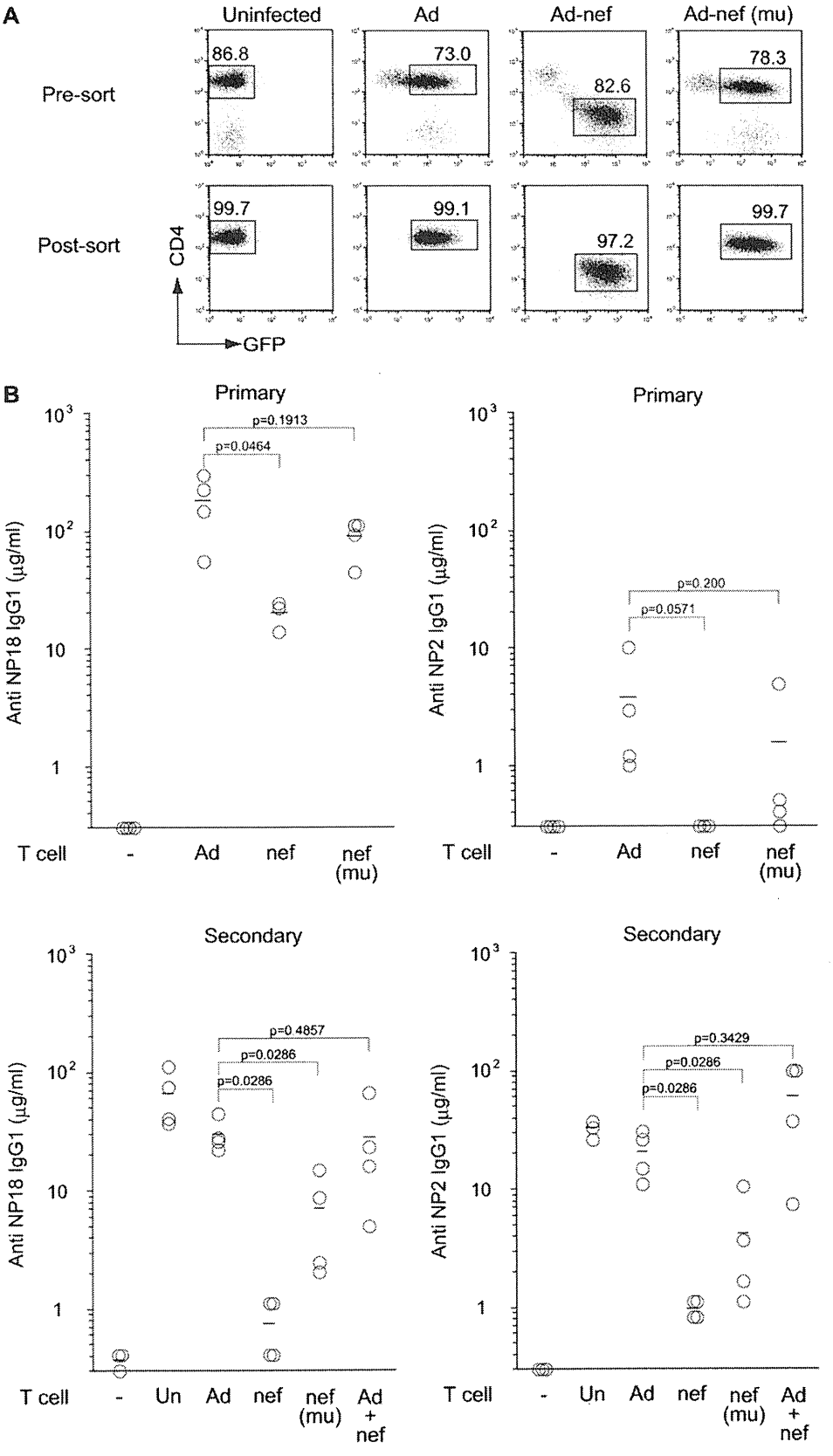

Fig. 3. Nef affects primary and memory B-cell response. (A) OVA-specific/CD4$^+$ T cells were purified from dTg mice and infected with Ad-nef, Ad-nef (mu) and Ad, followed by FACS purification (Post-sort). Numbers in plots indicate percent of GFP$^-$ uninfected cells and GFP$^+$ cells before (Pre-sort) and after purification (Post-sort). (B) Purified GFP$^+$ T cells (3×10^4) were transferred into CB17-scid mice, together with

with NP-OVA in alum for the primary response or soluble NP-OVA for the secondary response (Fig. 3B).

The results show that Nef expression in T cells reduced the level of anti-NP IgG1 serum antibodies by ~10-fold (NP_{18}; $P = 0.0464$, NP_2; $P = 0.0571$) in the primary response (Fig. 3B), whereas when the T cells were infected with Ad-Nef (mu), which does not down-regulate CD4 (Fig. 3A), the response was close to the control level (NP_{18}; $P = 0.1913$, NP_2; $P = 0.200$). As shown in Fig. 3(B), the impact of Nef on the secondary response was even more dramatic; there was a 30- to 40-fold reduction in both total and high-affinity anti-NP IgG1 antibodies (NP_{18}; $P = 0.0286$, NP_2; $P = 0.0286$). Reconstitution with equal numbers of non-infected and Nef-expressing OVA-specific $CD4^+$ T cells normalized the secondary adoptive response (NP_{18}; $P = 0.4857$, NP_2; $P = 0.3429$), excluding the possibility that Nef expression was generating suppressor T cells. Expression of the Nef mutant that was unable to down-modulate CD4 also reduced the secondary response (NP_{18}; $P = 0.0286$, NP_2; $P = 0.0286$), although the magnitude of the reduction was less than that induced by expression of wild-type Nef. These results demonstrate that Nef expression in peripheral T cells markedly diminishes their helper activity for the secondary IgG1 response and that this defect was only partially associated with the Nef-induced CD4 down-modulation. By contrast, this CD4 down-regulation appeared to be even more important for the reduced primary IgG1 response. These findings underscore the differential regulation in the primary and memory B-cell response. Thus, Nef affects helper T-cell activities in the primary and secondary response through different processes with different CD4 down-modulation susceptibility.

Discussion

In the present study, we have examined the consequence of Nef expression in primary splenic T cells. In order to avoid complications arising from expression of Nef early in T-cell development, e.g. lymphopenia, we established a double transgenic mouse (dTg), which expresses human CAR adenovirus receptor and an OVA-specific T-cell receptor that recognizes the OVA peptide on APC with high affinity under MHC Class II I-A^d-restriction. OVA-specific/$CD4^+$ T cells were purified from the spleen of dTg mice and infected with a recombinant adenovirus vector encoding Nef and GFP, followed by purification of GFP^+ cells using flow cytometry. To promote efficient introduction of the adenovirus vector into resting T cells, they were cultured for 2 days in the presence of the vector and IL-7, which is known to be important for survival of naive and memory T-cell populations (16). Neither naive nor memory $CD4^+$ T cells proliferate in response to IL-7, but they progress into the G_{1b} stage of the cell cycle (17). Thus, the present system allowed us to study the role of Nef in resting T cells in response to antigen-specific stimulation *in vitro* and *in vivo*.

During HIV-1 infection, the virus enters resting $CD4^+$ T cells and Nef is expressed even before the virus is integrated (1). It has been previously suggested that Nef expression in resting human T cells enhances IL-2 production upon activation by TCR cross-linking (1). This led to the proposal that Nef may enhance TCR signaling pathways that could help virus replication in partially stimulated T cells. In line with this viewpoint, it has been reported that Nef in human leukemic T cell lines and $CD4^+$ T-cell lines established from PBMC enhanced TCR signaling pathways and activated IL-2 production upon stimulation with TCR/CD28 or mitogens (2–5). In addition, Nef affects activation of murine T-cell hybridomas stimulated with anti-CD3 mAb (23), suggesting that the effect of Nef is not species specific.

In striking contrast, the present study demonstrates that Nef significantly reduces OVA-specific T-cell activation *in vitro* as defined by reduced proliferation and cytokine production, including IL-2 and IFNγ, but not completely. Furthermore, we demonstrate for the first time that Nef expression in OVA-specific resting T cells in the periphery reduced their ability to help anti-NP/$IgG1^+$ primary and secondary antibody responses in adoptive hosts after immunization with NP-OVA. In addition, in agreement with a previous *in vitro* analysis (7, 15), our *in vivo* results support the notion that Nef impairs the physiological recruitment of lymphocytes from the blood into the secondary lymphoid tissues after primary immunization, which promotes efficient antigen presentation and immune responses. Thus, Nef expressed in T cells at the early cell cycle stage impairs multiple functions in their subsequent antigen-specific response *in vivo*.

Why is the Nef-associated T-cell hyperresponse previously reported not detected in the present studies? The discrepancy does not reflect the differences in pathogenesis in *Nef* alleles (24) because the previous transgenic mouse models (8–10) and the present studies used the same NL4-3 Nef for characterization of the role of Nef protein in the immune system. Furthermore, the activation phenotype of T cells *in vitro* was induced by Nef proteins, irrespective of their alleles, including NL4-3 Nef (2–5). The discrepancy could be due to the cell state in the previous studies caused by transient over-expression of the protein in either the Jurkat T-cell line or in an activated human $CD4^+$ T-cell line established from PBMC (2–7). Another possible explanation is that previously reported assays utilized different TCR stimuli; the cells were stimulated by strong TCR ligation using immobilized antibodies (2–5). Such strong TCR ligation by antibodies forms stable TCR aggregates associated with the signaling complex (25). However, TCR stimulation with APC-presented antigen peptide forms an immunological synapse (IS) at the

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B cells (5×10^6) which were enriched from the pooled spleens of either naive or 4-hydroxyl-3-nitrophenylacetyl-conjugated chicken γ-globulin (NP-CGG)-primed mice using a MACS system, followed by challenge with 100 μg of NP-OVA in alum (primary) or 25 μg of soluble NP-OVA (secondary). Serum anti-NP antibody titers were estimated by ELISA assays at day 7 after challenge using NP_2-BSA and NP_{18}-BSA as coating antigens. The relative affinity of anti-NP antibodies was estimated by calculating the ratio of anti-NP_2/anti-NP_{18} antibody. Representative data from two independent experiments is shown. Bars represent the mean of each group.

T-cell APC interface, facilitating signaling through TCR recognizing the peptide-loaded MHC molecules (26). The formation of IS was impaired *in vitro* by HIV-1 infection in a Nef-dependent manner (27), providing an explanation for the present results that Nef lowers the cognate interaction strength between T cells and APCs in antigen-specific response, thereby denying complete progression and activation of the cell cycle.

Nef affects helper T-cell activities in the primary and secondary response through different processes with different CD4 down-modulation susceptibility. However, the underlying mechanism remains obscure. In the B-cell response, antigen-activated helper T cells form a complex with B cells by interacting with several co-stimulatory molecules as well as with the TCR and peptide-loaded MHC class II molecules on B cells. As a consequence, T cells and B cells are mutually stimulated and T cells produce cytokines promoting B-cell proliferation and differentiation into antibody-forming cell (28). Therefore, it is likely that Nef-induced repression of T-cell helper activity for an antigen-specific B-cell response may also reflect an inefficient cognate interaction between T cells and B cells in the primary and secondary response.

We observed that Nef in resting murine $CD4^+$ T cells down-regulates the expression of CD4 on the cell surface, concordant with the previous results using human and murine T-cell lines (reviewed in ref. 1). It has been previously suggested that CD4 plays an important role in the activation of T cells by increasing the avidity of TCR for the peptide/MHC class II molecule and by transducing signals through the associated tyrosine kinase p56Lck (29). CD4 down-modulation significantly affects T-cell helper activity for the primary antibody response; however, it only partially affects T-cell helper function for the secondary response. On the other hand, Nef-mediated repression of antigen-specific T-cell function for the migratory capacity in the primary immune response is not the result of CD4 down-regulation. Thus, Nef affects multiple antigen-specific T-cell activities in the primary and secondary response through different processes with different CD4 down-modulation susceptibility, probably reflecting the T-cell signature and/or B-cell signature involved in the primary or the secondary antibody response.

CD4 binds to the MHC and boosts the recognition of ligand by the TCR in early T-cell activation, afforded by the IS formation (30) and functions to deliver Lck to the T-cell APC interface (31). However, signaling and co-stimulation later result in the movement of CD4 toward the periphery of the IS (30), suggesting that once Lck has been recruited to the synapse, the function of CD4 may become dispensable, allowing CD4 to leave the synapse, compatible with the idea that initial signal strength for T-cell activation may be crucial for the primary B-cell response. Nef might affect T-cell activity to form the IS with B cells, although, it remains unknown whether primary and memory B-cell responses require the same co-receptor molecules for T-cell interaction or if they need help from the same subset of T cells. Further analysis is needed to clarify how memory and naive B-cell responses are differentially regulated.

In summary, the present results support a model in which Nef expressing HIV-1 infected $CD4^+$ T cells fail to attain multiple functions required for normal immune responses. Thus, these combined effects of Nef may not facilitate extensive HIV-1 productions by increasing the numbers of productively infected cells through T-cell activations in antigen-specific immune response.

What could be the advantages for HIV-1 to inhibit proliferation and multiple T-cell functions required for immune response? Of note, Nef does not completely abrogate T-cell activation upon stimulation, as defined by expression of activated cell surface markers and a low level of cytokine production, which may allow a replicative advantage for the virus (reviewed in ref. 32). In activated $CD4^+$ T cells, viral replication is efficient and cytopathic (reviewed in ref. 32), though rapid death of infected cells may limit the production of the virus. By lowering the T-cell activity, Nef might facilitate a lowered level of viral spread and an increased infected T-cell life span by avoiding viral cytopathic effects. These cells may decay more slowly *in vivo* relative to activated cells, leading to vital consequences for the pathogenic outcome of infection in humans.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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Structural basis for the antiviral activity of BST-2/tetherin and its viral antagonism

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The interferon-inducible host restriction factor bone marrow stromal antigen 2 (BST-2/tetherin) blocks the release of HIV-1 and other enveloped viruses. In turn, these viruses have evolved specific antagonists to counteract this host antiviral molecule, such as the HIV-1 protein Vpu. BST-2 is a type II transmembrane protein with an unusual topology consisting of an N-terminal cytoplasmic tail (CT) followed by a single transmembrane (TM) domain, a coiled-coil extracellular (EC) domain, and a glycosylphosphatidylinositol (GPI) anchor at the C terminus. We and others showed that BST-2 restricts enveloped virus release by bridging the host and virion membranes with its two opposing membrane anchors and that deletion of either one completely abrogates antiviral activity. The EC domain also shows conserved structural properties that are required for antiviral function. It contains several destabilizing amino acids that confer the molecule with conformational flexibility to sustain the protein's function as a virion tether, and three conserved cysteine residues that mediate homodimerization of BST-2, as well as acting as a molecular ruler that separates the membrane anchors. Conversely, the efficient release of virions is promoted by the HIV-1 Vpu protein and other viral antagonists. Our group and others provided evidence from mutational analyses indicating that Vpu antagonism of BST-2-mediated viral restriction requires a highly specific interaction of their mutual TM domains. This interpretation is further supported and expanded by the findings of the latest structural modeling studies showing that critical amino acids in a conserved helical face of these TM domains are required for Vpu–BST-2 interaction and antagonism. In this review, we summarize the current advances in our understanding of the structural basis for BST-2 antiviral function as well as BST-2-specific viral antagonism.

Keywords: HIV-1, Vpu, BST-2, transmembrane, restriction factor, antagonist, interaction

INTRODUCTION

As a result of exposure to viral pathogens over millions of years, humans and other mammals evolved intrinsic immunity proteins that provide resistance to infection by directly interfering with different stages of the viral life cycle. These so-called host restriction factors are normally induced by interferon-α (IFN-α) during induction of the innate immune response by viral infection. A case in point is HIV-1, an extensively studied pathogen for which four major restriction factors have been identified: the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3) family of cytidine deaminases (Sheehy et al., 2002); the α-isoform of the tripartite motif-containing protein 5 (TRIM5α; Stremlau et al., 2004); the bone marrow stromal antigen 2 (Neil et al., 2008; Van Damme et al., 2008; BST-2, also known as tetherin or CD317, referred to hereafter as BST-2), which is the subject of this review article; and, more recently, SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011). HIV-1, in turn, evolved countermeasures to overcome the antiviral activity of their host restriction factors, mainly by acquiring a series of *trans*-acting viral accessory proteins, including Vif and Vpu. Vif blocks the above-described APOBEC3 proteins that mediate extensive deamination of cytosines in single-stranded viral DNA, thus halting HIV replication. Vpu is another viral antagonist of the transmembrane BST-2 protein that blocks the release of enveloped viruses by physically binding the budding viral particles to the membrane of infected cells. Likewise, in HIV-2 and related simian immunodeficiency viruses, Vpx acts as an antagonist of SAMHD1 that blocks HIV-1 replication in dendritic and myeloid cells. It should be noted that HIV-1 is not susceptible to human TRIM5α antiviral action (Stremlau et al., 2004). In this review, we focus on current advances in structure-based analyses of BST-2 and viral antagonists.

BST-2: MOLECULAR CHARACTERISTICS

BST-2 is an interferon-induced type II membrane glycoprotein of unusual topology (Ishikawa et al., 1995; Kupzig et al., 2003), which efficiently blocks the release of diverse mammalian enveloped viruses by directly tethering viral particles to the membranes of infected cells. Viruses restricted by BST-2 are found among diverse families, including filoviruses, arenaviruses, paramyxoviruses (Jouvenet et al., 2009; Kaletsky et al., 2009; Sakuma et al., 2009a; Radoshitzky et al., 2010), gamma-herpesviruses (Mansouri et al., 2009; Pardieu et al., 2010), rhabdoviruses (Weidner et al., 2010), and a wide array of retroviruses from several mammal host species (Arnaud et al., 2010; Dietrich et al., 2011; Xu et al., 2011).

A recent study characterizing a feline BST-2 ortholog reported the protein's strong activity against FIV particle release *in vitro* (Dietrich et al., 2011). BST-2 comprises a short, 21-amino-acid cytoplasmic N-terminal tail (CT), followed by an α-helical transmembrane (TM) domain, an extracellular domain (EC) that is predominantly helical and contains an extended parallel coiled-coil, and a C-terminal glycosylphosphatidylinositol (GPI) component that acts as a second anchor linking the protein back to the cell membrane (Kupzig et al., 2003; **Figure 1A**). This double-anchor topology is extremely unusual and is only shared by an isoform of the prion protein (Moore et al., 1999).

Accumulating evidence supports the view that the structural features of BST-2 are key to its antiviral activity, as discussed in detail in the following sections. In agreement with a direct tethering mechanism, a requirement for both the TM and GPI anchors has been found for BST-2's antiviral activity (Neil et al., 2008; Iwabu et al., 2009; Perez-Caballero et al., 2009). Additionally, the EC of BST-2 contains a series of important residues that are conserved throughout the protein's mammalian orthologs, and these residues are essential to the inhibition of viral release (Van Damme et al., 2008; Andrew et al., 2009; Sakuma et al., 2009b). Whereas the stability of BST-2 is maintained by disulfide-links (Hinz et al., 2010; Schubert et al., 2010), the EC forms an extended coiled-coil domain that contains several conserved destabilizing amino acid residues, providing the conformational flexibility necessary for the molecule to sustain its role as a physical tether, as described later. Salient BST-2 structural motifs important for antiviral function are summarized in **Table 1**.

Based on the identification of these structural features critical for BST-2's antiviral activity, Perez-Caballero et al. (2009) through domain replacement experiments, were able to show that BST-2's configuration rather than its primary sequence is critical for antiviral activity. In an elegant demonstration, the authors generated a completely artificial BST-2-like protein made of structurally similar domains from three unrelated heterologous proteins (the TM from the transferrin receptor, the coiled-coil from dystrophia myotonica protein kinase, and the GPI anchor from the urokinase plasminogen activator receptor). Despite its lack of sequence homology with native BST-2, this artificial protein reproduced the latter's antiviral activity as it was able to inhibit the release of HIV-1 and Ebola virus-like-particles.

BOTH TM AND GPI ANCHOR ARE IMPORTANT FOR THE RESTRICTION OF VIRUS RELEASE

The TM (amino acid positions 22–43) of BST-2 is a short single-pass α-helix that anchors the molecule to the plasma membrane, while the GPI anchor is located at the C-terminal region of the protein (Kupzig et al., 2003). These two membrane anchors in part determine the antiviral function of BST-2. This unusual topology suggests a model that BST-2 directly tethers budding virions to the membrane of infected cells. Indeed, unequivocal support for this model has come from immunoelectron microscopy studies demonstrating that BST-2 is associated with virions and located between the viral and cell membranes as well as between tethered virions (Neil et al., 2008; Fitzpatrick et al., 2010; Hammonds et al., 2010).

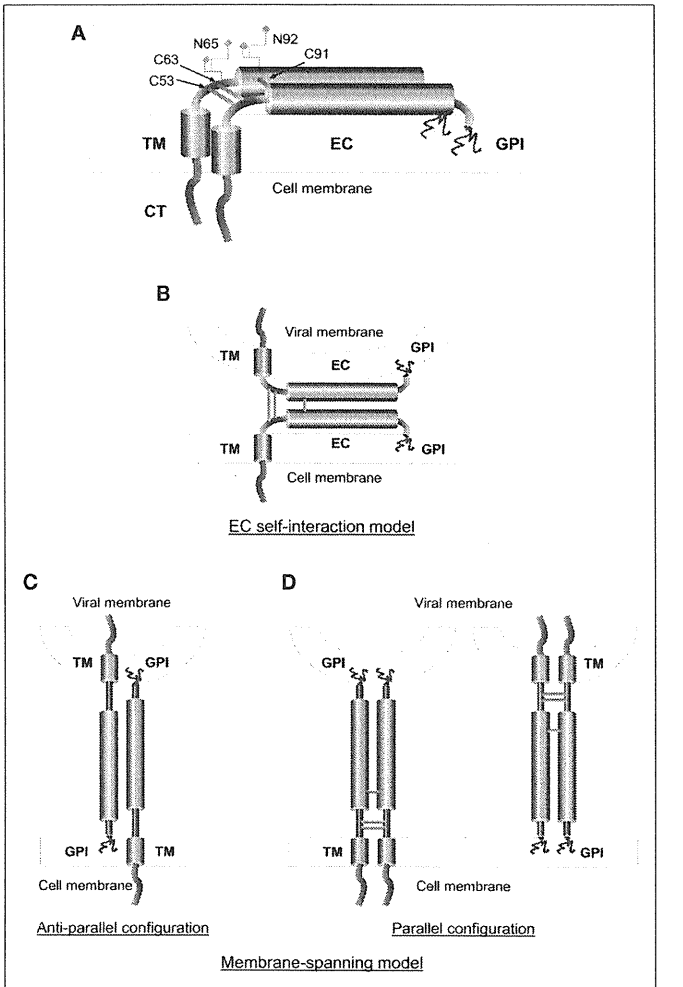

FIGURE 1 | Topological characteristics of human BST-2. (A) Schematic representation of the domain structure of BST-2, a type II transmembrane (TM) protein. BST-2 features a short cytoplasmic N-terminus followed by an α-helical single-pass TM domain and an extended coiled-coil extracellular domain that is linked back to the plasma membrane by a C-terminal GPI anchor. *N*-glycosylation sites and cysteine residues for disulfide-bond formation in the extracellular domain (EC) are noted. **(B–D)** Topological models of BST-2's functional state. **(B)** The EC self-interaction model, in which individual BST-2 monomers are anchored at both ends to the same membrane, with interaction between the ECs of cell-bound and virion-bound monomers. **(C)** Membrane-spanning anti-parallel model. Monomers are anchored in both membranes with opposing orientations. **(D)** Membrane-spanning parallel model. Monomers are anchored in both membranes with the same orientation.

As shown in **Table 1**, two structural elements are absolutely required for BST-2-mediated restriction of viral release; (1) the presence of both the TM and the GPI anchor (Neil et al., 2008; Van Damme et al., 2008; Iwabu et al., 2009; Perez-Caballero et al., 2009); and (2) homodimer formation through EC disulfide-bond interactions (Andrew et al., 2009; Perez-Caballero et al., 2009). The latter is discussed in greater detail in a later section of this review. These two elements form the basis of the two proposed topological models of BST-2. In the "EC self-interaction model (**Figure 1B**)," individual BST-2 monomers are anchored at

Table 1 | Salient structural features of human BST-2.

Domain	Structural motif	Function	Necessary for antiviral action?	Reference
CT (1–21)	YxY_{6-8}	Clathrin-dependent internalization	No	Masuyama et al. (2009), Rollason et al. (2007)
	$DDIWK_{14-18}$	Nef recognition sequence	No	Yang et al. (2010a), Sauter et al. (2009), Lim et al. (2010)
	K18	Putative ubiquitination site by K5	No	Mansouri et al. (2009), Pardieu et al. (2010)
TM (22–43)	Alpha-helix (22–43)	Membrane anchor	Yes	Neil et al. (2008), Perez-Caballero et al. (2009), Iwabu et al. (2009)
	I34, L37, L41	Vpu recognition face	No	Iwabu et al. (2009), Gupta et al. (2009a), Rong et al. (2009), McNatt et al. (2009), Kobayashi et al. (2011), Skasko et al. (2011b)
EC (44–160)	N65, N92	N-linked glycosylation	No	Sakuma et al. (2009a), Andrew et al. (2009), Ohtomo et al. (1999)
	C53, C63, C91	Putative disulfide-bond formation	Yes	Perez-Caballero et al. (2009), Andrew et al. (2009), Hinz et al. (2010)
	Coiled-coil (68–138)	Molecular ruler	Yes	Hinz et al. (2010), Yang et al. (2010a), Swiecki et al. (2011), Schubert et al. (2010)
	C91, V95, L98, L102, E105, V113, L116, I120, L123, L127, V134, L137	Destabilizing residues at core heptad positions	Yes	
GPI anchor	GPI signal peptide	Membrane anchor	Yes	Kupzig et al. (2003), Perez-Caballero et al. (2009), Iwabu et al. (2009)

both ends to the same membrane (cellular or viral), and interaction between the EC domains of cell-bound and virion-bound monomers is required for the restriction of virus release. The alternative is the "membrane-spanning model (**Figures 1C,D**)," in which both BST-2 end tails (TM and GPI anchor) are anchored in different membranes (i.e., cellular and viral). Theoretically, the BST-2 monomers in this model can be arranged in either an anti-parallel (**Figure 1C**) or parallel (**Figure 1D**) configuration.

The first approach to resolve the topology of BST-2 involves cleavage of the GPI anchor by treatment with the hydrolytic enzyme phosphatidyl inositol-specific phospholipase C (Pi–PLC). However, the enzymatic treatment does not effectively release restricted virions from the cell membrane (Fitzpatrick et al., 2010), supporting either a membrane-spanning anti-parallel configuration (**Figure 1C**) or the EC self-interaction model (**Figure 1B**), in which monomers would be able to remain attached to the respective membrane by the TM domain even after cleavage of the GPI anchor.

The second approach is to evaluate the gap between the cellular and viral membranes in electron microscopy studies. If the BST-2 monomers are positioned parallel to the cellular and viral membranes (EC self-interaction model; **Figure 1B**), virions would be tethered very close to the membrane, less than 3–5 nm, as described in (Hinz et al., 2010). However, imaging studies show larger distances between virions and cells (Neil et al., 2008; Perez-Caballero et al., 2009; Hammonds et al., 2010), thus supporting a membrane-spanning model (**Figures 1C,D**).

The third approach to this problem has been the systematic determination of BST-2 function in mutational analyses. We have previously shown that the anchoring of BST-2 through both its N-terminal and C-terminal regions is required for antiviral activity (Iwabu et al., 2009). Briefly, mutagenesis studies using GPI-anchor-deleted and CD4 signal peptide chimeric versions of BST-2, in which the protein is linked to the cell membrane only through one of its ends, showed that removal of either end abrogated the antiviral effect of BST-2 on virus production. Therefore, we concluded that membrane binding through both the TM and GPI anchor of BST-2 is critical for its antiviral activity, supporting the model of the membrane-spanning parallel configuration (**Figure 1D**). Further evidence for this parallel-dimer model comes from the analysis of residual BST-2 found in virions released through proteolytic treatment with subtilisin (Perez-Caballero et al., 2009).

Finally and more importantly, four different groups have combined high-resolution crystallography (1.6–2.8Å), and small-angle X-ray scattering-based modeling to determine the structures of the entire human and murine BST-2 EC, and have shown that BST-2 forms parallel coiled-coil arrangements (Hinz et al., 2010; Schubert et al., 2010; Yang et al., 2010a; Swiecki et al., 2011). Taken together, these observations suggest that the antiviral state of BST-2 present at the cell membrane corresponds to the membrane-spanning parallel configuration model as shown in **Figure 1D**.

THE EC MEDIATES HOMODIMERIZATION

The BST-2 EC (amino acid positions 44–160) is predominantly an α-helical coiled-coil structure that contains a series of residues highly conserved among mammalian orthologs: two asparagines that are N-linked glycosylation sites (N65, N92), and three cysteines (C53, C63, C91) responsible for intermolecular disulfide-bonds that result in homodimerization (**Figure 1A**; Ohtomo et al., 1999; Andrew et al., 2009). Disulfide linkage through these cysteine residues is critical for the restriction of HIV production

(**Table 1**). Mutational analyses demonstrate that partial disulfide-bond formation through at least one such cysteine residue is necessary for the retention of antiviral activity, whereas mutations at all three positions result in the total loss of antiviral function even though expression of the protein at the cell membrane remains unaltered (Andrew et al., 2009; Perez-Caballero et al., 2009; Hinz et al., 2010), although this is not the case for filovirus or arenavirus (Lassa virus) particles (Perez-Caballero et al., 2009; Sakuma et al., 2009a).

Several conserved amino acids within the EC domain, which are also thought to stabilize the dimers through weak coiled-coil domain interactions, include two interhelical salt bridges (E105–K106, and E133–R138) and one interhelical hydrogen bond (N141), and contribute to stabilize the EC domain interface (Hinz et al., 2010). Glycosylation of residues N65 and N92 was shown to contribute to anterograde transport and correct protein folding, but mutations in these positions had no effect on BST-2 antiviral activity (**Table 1**; Andrew et al., 2009; Sakuma et al., 2009a). In summary, all evidence thus far suggests that BST-2 EC contains a dimeric coiled-coil that is stabilized by C53–C53, C63–C63, and C91–C91 disulfide-bonds, with the conservation of at least one of these, along with weak interactions within the coiled-coil domain, and is required for dimer stability and the antiviral activity of BST-2.

THE BST-2 EC EXHIBITS CONFORMATIONAL FLEXIBILITY

The most recent structural studies provide valuable clues to the biological function of the EC while at the same time reconciling the topological models of BST-2 dimer configuration with available electron microscopy data, as outlined above. Resolution of the crystal structure of human BST-2 EC (Hinz et al., 2010; Schubert et al., 2010; Yang et al., 2010a) together with small-angle X-ray scattering data suggest an elongated extracellular domain forming a long rod-like structure and a greatly extended EC separating the two membrane anchors, acting as a molecular ruler with a predicted distance of 170Å (**Table 1**). This distance would correspond to the predicted separation between membrane-tethered virions and the plasma membrane of the host cells, or between tethered viral particles, and is in agreement with the separation determined in published electron micrographic studies. This finding seems to be consistent with the aforementioned membrane-spanning model (**Figure 1D**).

The authors of those studies also described the presence of irregularities in the 90-Å coiled-coil motif. The irregularities arise from the introduction of destabilizing residues (see **Table 1**) that are arranged regularly in core heptad positions, i.e., amino acid residues located at the center of the α–helix. The destabilizing residues loosen regular coiled-coil packing increasing the pitch and radius of the α-helix, accounting for the low stability of BST-2's coiled-coil under reducing conditions in vitro. These positions are conserved throughout all available BST-2 sequences, and their mutations result in loss of the antiviral function of BST-2 (Hinz et al., 2010). Yet, despite this intrinsic instability, the disulfide-bonds are still able to be formed, restabilizing the EC domains in a dimeric form. These findings suggest that conformational flexibility allows adaptation to the dynamic events of virion budding, while disulfide-bond-mediated dimerization prevents major separation of the coiled-coils. Together, these two properties result in a dynamic structure that permits dimer dissociation and restabilization during the process of virion trapping (Hinz et al., 2010; Swiecki et al., 2011). A high-resolution crystal structure of the full-length mouse BST-2 EC confirmed the presence of an elongated EC characteristically unstable due to the insertion of destabilizing residues (Swiecki et al., 2011). In that study, structural and biophysical analyses of murine and human BST-2 EC domains revealed that an unstable coiled-coil motif is evolutionarily conserved. This evidence provides further support for the aforementioned model of conformational flexibility.

THE GPI ANCHOR MEDIATES SURFACE LOCALIZATION AND THE CT IS CRITICAL FOR BST-2 TRAFFICKING

BST-2 localizes both to the plasma membrane and internal compartments, particularly the trans-Golgi network (TGN) and recycling endosomes (Kupzig et al., 2003; Rollason et al., 2007; Dube et al., 2009; Masuyama et al., 2009; Habermann et al., 2010). At the cell surface, BST-2 localizes into cholesterol-enriched lipid rafts, due to its GPI anchor. This localization is implicated in the promotion of clathrin-mediated endocytosis (Rollason et al., 2007; Masuyama et al., 2009) and, importantly, it allows BST-2 to directly interfere with the virion-release process, as lipid rafts are the preferential site of budding of several enveloped viruses (Aloia et al., 1993; Panchal et al., 2003; Waheed and Freed, 2009). This also positions BST-2 at the virological synapse (VS; Casartelli et al., 2010; Jolly et al., 2010; Pais-Correia et al., 2010), but its potential to restrict cell-to-cell viral spread remains controversial. With respect to internalization and cell trafficking, it was previously shown that rodent BST-2 is internalized from the cell surface in a clathrin-dependent manner (Rollason et al., 2007; Masuyama et al., 2009). Internalization requires a non-canonical dual tyrosine motif at amino acid positions 6 and 8 of the protein's CT (YxY_{6-8}; **Table 1**). This motif is highly conserved through all mammalian orthologs and sequentially participates in the interaction of BST-2 with the clathrin adaptors AP-2, which mediates internalization by endocytosis, and AP-1, which retrieves BST-2 to the TGN. The CT domain of BST-2 indirectly interacts with the underlying actin cytoskeleton through a series of adaptor proteins (RICH2, EBP50, ezrin), although additional studies are required to understand the implications of these interactions for BST-2 function (Rollason et al., 2009).

VIRAL ANTAGONISM OF BST-2

Since BST-2 targets the lipid bilayer of the host cell, viruses cannot evade it simply by escape mutations. Therefore, enveloped viruses had been obliged to evolve trans-acting countermeasures specifically to overcome BST-2 restriction. Among primate lentiviruses, three different viral gene products are known to antagonize BST-2. In most SIV strains, the viral Nef protein antagonizes primate BST-2, while in HIV-1 and HIV-2, the Vpu protein and the Env glycoprotein, respectively, antagonize human BST-2. Other BST-2 antagonists include the Kaposi's sarcoma-associated herpesvirus (KSHV) K5 protein and the Ebola virus glycoprotein (GP). With the exception of Ebola GP, all of these viral proteins downregulate BST-2 at the plasma membrane, thus effectively removing it from viral budding sites.

HIV-1 Vpu

Just as the study of HIV-1 Vif led to the discovery of APOBEC3 as a host restriction factor (Sheehy et al., 2002), BST-2 was identified by searching for the host restriction factor antagonized by the accessory viral protein Vpu. This 16-kDa type I transmembrane viral protein is a BST-2 antagonist and as such promotes the release of HIV-1 virions (Cohen et al., 1988; Strebel et al., 1988; Malim and Emerman, 2008). Importantly, Vpu can directly mediate the removal of BST-2 away from its site of action on the cell surface, although the mechanisms remain hotly debated (Van Damme et al., 2008; Iwabu et al., 2009, 2010; Ruiz et al., 2010; Lau et al., 2011). Thus far, it appears that Vpu recruits cellular proteins to remove BST-2 from the surface (**Figure 2A**). As we and others have shown, BST-2 downregulation by Vpu involves a beta-transducin repeat-containing protein (β-TrCP)-dependent mechanism (Douglas et al., 2009; Iwabu et al., 2009; Mangeat et al., 2009; Mitchell et al., 2009; Dubé et al., 2010; Tokarev et al., 2011); however, this only partially explains the underlying mechanism, since mutations in the β-TrCP-binding motif of Vpu do not entirely abrogate its antagonism of BST-2 (Schubert and Strebel, 1994; Van Damme et al., 2008; Iwabu et al., 2009).

Whereas several reports suggest that BST-2 downregulation in the presence of Vpu is accomplished at least in part through proteasomal degradation (Goffinet et al., 2009; Gupta et al., 2009a; Mangeat et al., 2009), evidence obtained by our group and others supports a model of BST-2 downregulation through lysosomal degradation (Douglas et al., 2009; Iwabu et al., 2009; Mitchell et al., 2009; Janvier et al., 2011). It is proposed that Vpu causes the retention of BST-2 within endosomes by blocking its recycling after endocytosis (Mitchell et al., 2009; Dubé et al., 2010; Lau et al., 2011). Alternatively, it is hypothesized that Vpu inhibits the membrane transport of BST-2 by causing its intracellular sequestration within the TGN (Dube et al., 2010; Andrew et al., 2011; Lau et al., 2011). We and others suggested that Vpu directly internalizes BST-2 from the cell surface through TM interactions leading to lysosomes (Iwabu et al., 2009, 2010; Janvier et al., 2011; Skasko et al., 2011a). An additional level of complexity in the BST-2 downregulation mechanism stems from a report that in certain cell lines (CEMx174, H9), Vpu overexpression results in the enhancement of virion production, but without effectively reducing the surface levels of BST-2 (Miyagi et al., 2009). Thus, it is not yet clear how Vpu affects the internalization, recycling, or membrane transport, of BST-2.

Regardless of the mechanisms of Vpu-induced BST-2 downregulation, the ability of Vpu to bind to BST-2 is crucial for the antagonism of BST-2-mediated restriction (**Figure 2A**), as evidenced by data showing that the anti-BST-2 activity of Vpu is abrogated by mutations that disrupt TM-TM interaction. (Gupta et al., 2009a; Iwabu et al., 2009; McNatt et al., 2009; Rong et al., 2009; Skasko et al., 2011a). This interaction is highly specific since single point mutations in either BST-2 (I34, L37, L41; **Table 1**; Kobayashi et al., 2011) or Vpu (A14, A18, and W22; Vigan and Neil, 2010) render BST-2 resistant to Vpu antagonism. Their structural analyses showed that these residues form both hydrophobic faces of the helices, and therefore presumably contribute to their interacting surfaces. Recently, the aforementioned residues have been shown by NMR spectroscopy to interact directly in a membrane-embedded TM–TM interface (Skasko et al., 2011b).

Importantly, a high degree of species-specificity characterizes this interaction. Even though all primate BST-2 proteins are able to block HIV-1 virion-release, non-human BST-2 proteins are mostly insensitive to Vpu antagonism (Goffinet et al., 2009; Gupta et al., 2009a; Jia et al., 2009; Zhang et al., 2009). Analyses of codon-specific positive selection in the primate lineage showed that a mutation of residue T45 in human BST-2 is sufficient to reduce its sensitivity to Vpu (Gupta et al., 2009a). Likewise, the transfer of amino acid positions 30–45 of the human BST-2 TM domain into rhesus BST-2 was sufficient to render it Vpu-sensitive, while a single I48T mutation in rhesus BST-2 conferred partial Vpu sensitivity (Yoshida et al., 2011). These results suggest that this specificity of HIV-1 Vpu for BST-2 depends on conserved amino acids in the latter's TM domain (as described above) that are divergent between the human protein and its simian counterparts.

OTHER BST-2 ANTAGONISTS

Most of the primate lentiviruses that do not encode a Vpu protein instead use Nef to counteract BST-2's antiviral function (Jia et al., 2009; Sauter et al., 2009; Zhang et al., 2009). It should be noted that even though the primate ancestors of HIV-1, SIVcpz, and SIVgor from chimpanzees and gorillas encode Vpu, they also use Nef to antagonize BST-2 (Sauter et al., 2009; Yang et al., 2010b). Analogous to HIV-1 Vpu antagonism of human and chimpanzee, but not other primate BST-2 proteins (Goffinet et al., 2009; McNatt et al., 2009; Hauser et al., 2010), SIV Nef counteracts primate but not human BST-2 orthologs. This selectivity resides in the CT of non-human primate BST-2, which contains a discreet $DDIWK_{14-18}$ sequence (**Table 1**) that is required for the response to SIV Nef but is deleted in the protein's human counterparts (Sauter et al., 2009; Lim et al., 2010; Yang et al., 2010b). Furthermore, antagonism of non-human primate BST-2 is abrogated by mutations in the myristoylation site of SIV Nef (**Figure 2B**; Jia et al., 2009; Zhang et al., 2009). In addition, SIV Nef mutations that impair CD4 and CD28 downregulation also abrogate BST-2 antagonism, suggesting a similar mechanism of interaction (Zhang et al., 2009). By contrast, BST-2 antagonism by some strains of HIV-2 (as well as SIVtan from Tantalus monkeys) is mediated by the Env glycoprotein (**Figure 2C**; Bour and Strebel, 1996; Ritter et al., 1996; Abada et al., 2005; Gupta et al., 2009b). Although the exact determinants of interaction are not well understood, an endocytic motif (GYxxϕ) in the cytoplasmic region of gp41 (Boge et al., 1998) is known to be required to bind to AP-2, triggering BST-2 downregulation (Le Tortorec and Neil, 2009), while extracellular domains of HIV-2 Env apparently bind to the EC of BST-2. It was recently reported that an A100D point mutation of BST-2's EC abrogates the HIV-2 Env-mediated block of BST-2 restriction (Gupta et al., 2009b), supporting a model of interaction between HIV-2 Env and the EC of BST-2.

Other BST-2 antagonists include KSHV K5 protein, which ubiquitinates K18 residue in the CT domain of BST-2 (**Table 1**), leading to reduced surface and intracellular levels of BST-2, presumably through an endolysosomal process (**Figure 2D**; Mansouri et al., 2009; Pardieu et al., 2010). The Ebola virus GP2 appears to use a novel non-sequence-specific mechanism, overcoming

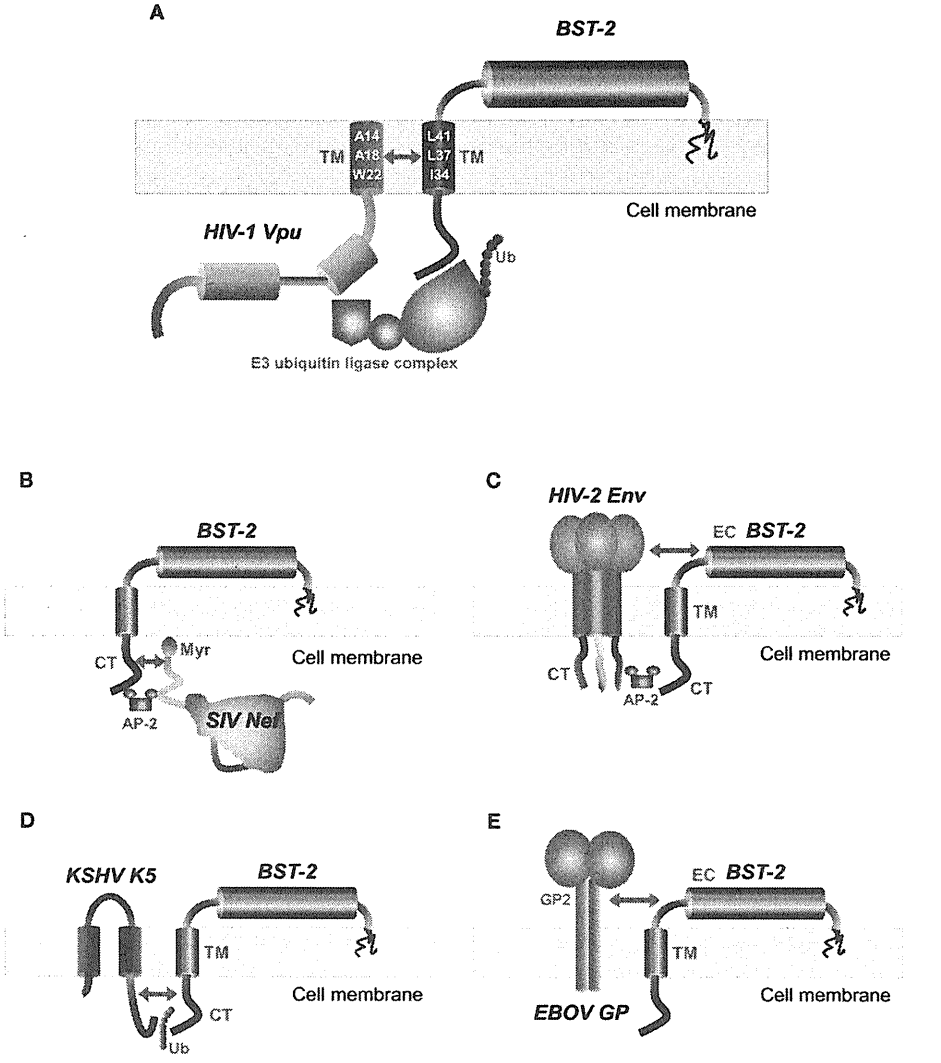

FIGURE 2 | Viral antagonists of BST-2 and their domains of interaction. Schematic representation of BST-2 and its known antagonists. The structural domains of interaction are indicated by red arrows. **(A)** HIV-1 Vpu and BST-2 interact through their mutual transmembrane (TM) domains. Key amino acid residues involved in the interaction are depicted in the TM helices. Also shown is the E3 ubiquitin (Ub) ligase complex required for BST-2 internalization. **(B)** SIV Nef recognizes the cytoplasmic (CT) domain of BST-2. The AP-2 clathrin adaptor recruited for BST-2 internalization is also shown. Myr, myristoylation site. **(C)** The envelope glycoprotein (Env) of HIV-2 and SIVtan binds to BST-2 through their mutual ectodomains (EC), and recruitment of AP-2 by the CT domain of Env required for internalization is also shown. **(D)** Kaposi's sarcoma-associated herpesvirus (KSHV) K5 protein that is an ubiquitin ligase ubiquitinates a target lysine motif in the CT domain of BST-2, resulting in its internalization. **(E)** The antagonistic mechanisms of the Ebola virus (EBOV) glycoprotein (GP) are unclear, but require interaction between GP2 subunit of EBOV–GP and BST-2 EC.

BST-2's restriction without significant removal of the protein from the cell surface (**Figure 2E**; Kaletsky et al., 2009; Lopez et al., 2010; Kühl et al., 2011). Influenza virus is suspected of harboring an unidentified viral antagonist against BST-2, since BST-2 expression was unable to block replication-competent influenza virus production but inhibited the release of influenza virus-like-particles (Watanabe et al., 2011).

CONCLUSION

Considerable progress was made recently in understanding the structure and function of BST-2, as well as the mechanisms by which viral antagonists counteract its activity. Through a combination of biological studies and structural analyses, the functional state of BST-2 is characterized as that of a parallel dimeric coiled-coil that, via its double-membrane anchors, physically binds budding virions to the infected cell. More importantly, current evidence shows that the unusual structural features of BST-2 determine its antiviral function independently of sequence homology. The EC has a prime role acting as a molecular ruler that separates the membrane anchors, in addition to allowing dimerization of BST-2 and providing conformational flexibility to sustain the protein's function as a viral particle tether. Likewise, loss of BST-2's double-membrane anchoring leads to the complete abrogation of the antiviral activity.

Although most of the evidence presented here was obtained from *in vitro* systems, a recent study using BST-2 knockout mice has shown that BST-2 inhibited the replication and release of a murine retrovirus *in vivo*, in a manner completely dependent on IFN-α production. Additionally, BST-2 restricted viral pathogenesis and delayed disease progression, suggesting that it has verifiable antiviral activity not only *in vitro* but also *in vivo*. (Liberatore and Bieniasz, 2011). Another study using rhesus macaques has confirmed the importance of the antagonism of BST-2 antiviral activity by Vpu *in vivo* (Shingai et al., 2011). Further investigation of the antiviral mechanisms exerted by host restriction factors, as well as the evolution of viral countermeasures, will not only advance our understanding of AIDS pathogenesis but also lead to the development of therapeutic alternatives.

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