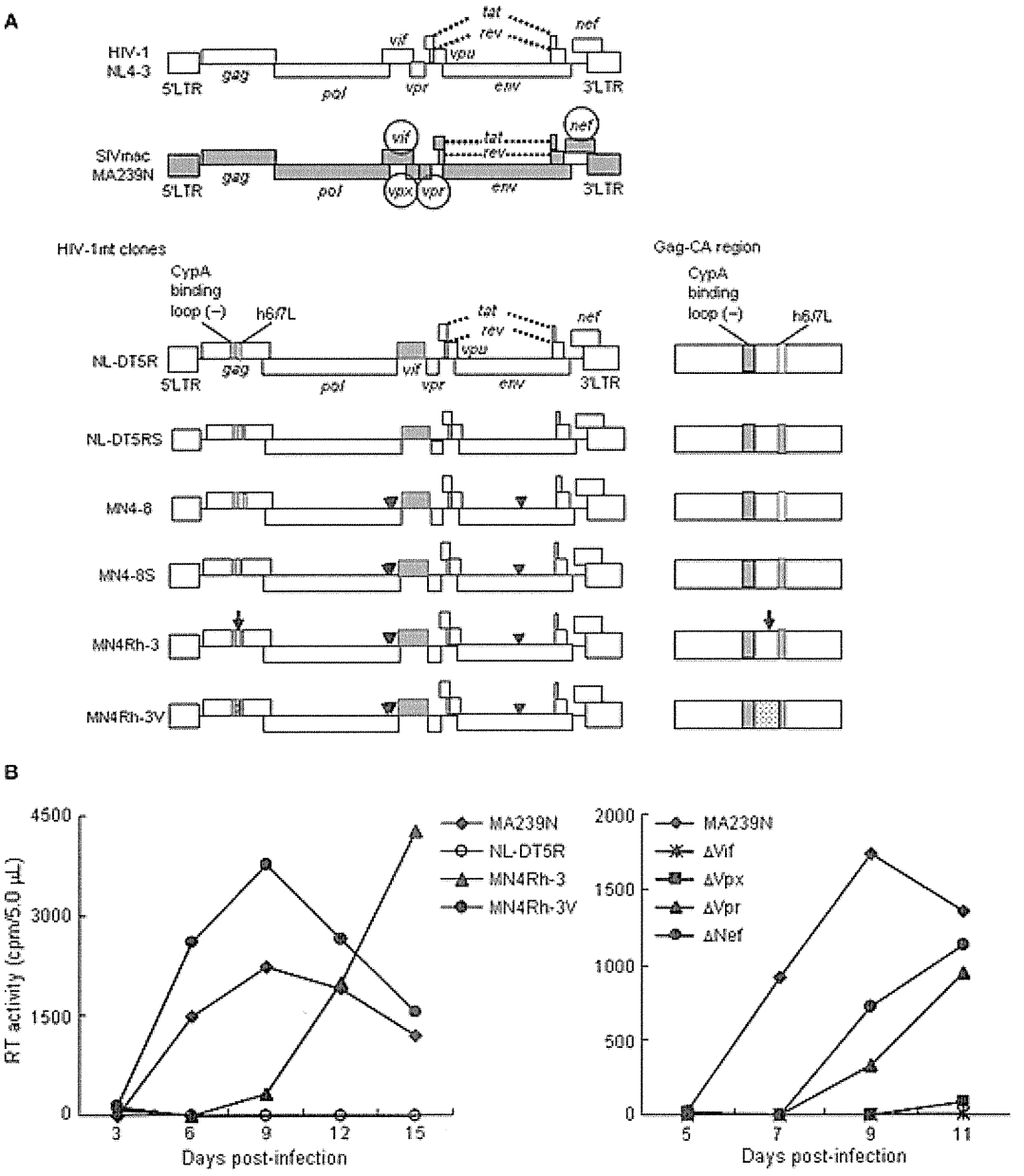

FIGURE 1 | Virological characteristics of HIV-1/SIVmac clones. (A) Proviral genome structure of various viruses used for infection of M1.3S. Genomes of various HIV-1mt clones generated in our laboratory are schematically illustrated. Parental clones HIV-1 NL4-3 (Adachi et al., 1986) and SIVmac MA239N (Doi et al., 2010) are shown at the top. Accessory gene-inactivated mutants of SIVmac MA239N (circled) were constructed by substituting an internal stop codon in the *nef* gene of MA239 mutants (Shibata et al., 1991) with a Glu codon as reported for construction of MA239N (Doi et al., 2010). White and gray areas indicate the genomic regions of NL4-3 and MA239N, respectively. An arrow and arrowheads indicate the site of the four mutations in *gag*, *pol*, and *env* genes, respectively. The dotted region contains additional single-nucleotide mutations relative to the other clones. Enlarged Gag-CA region is shown on the right. For details, see the text. **(B)** Replication kinetics of the viruses in M1.3S cells. Cell-free viruses were prepared from 293T cells transfected with proviral clones indicated, and inoculated into 1×10^6 cells of M1.3S. Viral replication was monitored at intervals by reverse transcriptase (RT) activity in the culture supernatants. The experiments were done as described previously (Kamada et al., 2006; Doi et al., 2010). For infection, 6.3×10^4 and 2.4×10^6 RT units of MA239N and HIV-1mt clones (left), respectively, and 5.0×10^4 RT units of MA239N clones (right) were used. The representative data in two independent experiments are shown. Replication of NL-DT5RS, MN4–8, and MN4–8S was not detected as observed for NL-DT5R.

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The fourth major restriction factor against HIV/SIV

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Human and simian immunodeficiency viruses (HIV/SIVs) carry a unique set of accessory proteins that enhance virus replication in an optimized manner. These viral proteins specific to HIV/SIVs are designated Vif, Vpx, Vpr, Vpu, and Nef, and are functional in certain cell types (Malim and Emerman, 2008; Fujita et al., 2010). While viruses of the HIV-1 group do not encode Vpx, the other HIV-2/SIVs are unable to replicate in cells of the myeloid lineage such as monocyte-derived dendritic cells (MDDCs) and macrophages (MDMs) in the absence of Vpx (Fujita et al., 2010). Vpx and its structural close relative Vpr are least well studied and understood with respect to their functional details (Khamsri et al., 2006; Fujita et al., 2010). Vpx had been long thought to be critical for the nuclear import of viral DNA in non-dividing cells until we and others have independently and clearly demonstrated that it is essential for the reverse transcription of viral RNA in MDDCs (Goujon et al., 2007) and MDMs (Fujita et al., 2008; Srivastava et al., 2008). Subsequently, these results have been well confirmed by the other studies (Goujon et al., 2008; Gramberg et al., 2010). We have also shown that the deletion of Vpr has only a modest (HIV-1) or almost no (HIV-2) effects on viral replication in MDMs (Fujita et al., 2010). On the basis of the new findings about HIV/SIV Vpx by us and others described above, it has been established that there is an innate factor against viral reverse transcription in cells of the myeloid lineage, and that Vpx counteract the factor responsible for the inhibition (Sharova et al., 2008; Bergamaschi et al., 2009; Kaushik et al., 2009). Of a particular note, the Vpx-mediated enhancement of viral replication is also applicable to HIV-1 infection (Goujon et al., 2008). Taken all together, it had been well predicted that an unidentified factor present in the myeloid cells is potently effective against a wide variety of retroviruses. Many active HIV/SIV researchers, therefore, had focused on its identification to better understand the life cycle of primate immunodeficiency viruses. Finally, Laguette et al. (2011) have identified SAMHD1 as the myeloid antiviral factor. If confirmed scientifically, SAMHD1 would represent the fourth (and most probably the last) major restriction factor against HIV/SIV (**Table 1**). SAMHD1 was initially identified in human dendritic cell cDNA library as an ortholog of a mouse interferon-γ-induced protein that is up-regulated in response to viral infections (Li et al., 2000; Prehaud et al., 2005; Hartman et al., 2007; Zhao et al., 2008). Mutations in the gene encoding SAMHD1 have been shown to be responsible for 5% of genetically inherited Aicardi–Goutières syndrome cases characterized by inappropriate activation of immune system and aberrant interferon-α secretion (Rice et al., 2009). This syndrome is a mimic of congenital infection and also shows an overlap with systemic lupus erythematosus (Rice et al., 2009). Collectively, SAMHD1 is considered to be a regulator of cellular intrinsic antiviral response (Rice et al., 2009). SAMHD1 has a sterile alpha motif (SAM) and an HD domain in tandem. SAMs are 65–70 residues in length and can serve as protein-interaction modules. The HD domain, which is characterized by a motif with a doublet of divalent-cation-coordinating histidine and aspartic acid residues, is found in a diverse superfamily of enzymes with a potential phosphohydrolase activity. In the article of Laguette et al. (2011) apart from the identification of a novel antiviral cellular factor, authors were mainly concerned about the restriction of Vpx-less HIV-1 replication in myeloid cells but not the replication of the other viruses with Vpx.

From the results of Laguette et al. (2011) future directions in a short or medium range for biological and biochemical characterization of the interaction between HIV/SIV Vpx and SAMHD1 are quite evident and manifold. Most importantly, we should perform a systemic genetic analysis of non-HIV-1 viruses carrying the *vpx* gene in the presence of SAMHD1. Numerous studies on HIV/SIV Vpx thus far conducted, both *in vitro* and *in vivo*, have indicated that Vpx is critical for viruses containing the *vpx* gene (Fujita et al., 2010). In particular, monkey model studies have clearly demonstrated that Vpx is quite necessary for viral replication and pathogenesis in individuals. Whether SAMHD1 is a natural target of HIV/SIV Vpx and is a negative modulator of the viruses should be extensively and repeatedly verified. On the other hand, we empirically know that *vpx*-minus HIV-1 can replicate, spread, and persist, and survive in human populations. If SAMHD1 can determine or alter the outcome of HIV-1 infection in humans, how does HIV-1 overcome its serious negative effect? Can the activity of Vpx be replaced, at least in part, with structurally related HIV-1 Vpr? Alternatively, SAMHD1-mediated virus restriction does not have much impact on the survival of HIV-1 in humans. It is conceivable that

Table 1 | Restriction factors against HIV/SIV.

Cellular restriction factors	Antiviral activity	Counteracting or interacting viral proteins
*APOBEC3G/F	Induction of lethal mutations in viral genome	Vif
CypA, *TRIM5α, and TRIMCyp	Induction of disordered uncoating?	Gag-CA
*Tetherin	Inhibition of virion release	Vpu
*SAMHD1	Inhibition of reverse transcription	Vpx

**Major restriction factors against HIV/SIV so far reported.*

the Vpx-mediated enhancement of viral replication by counteracting SAMHD1 is important for the survival of primate immunodeficiency viruses in monkey species. After all, it is time now to re-start the Vpx study, focusing on SAMHD1 molecule.

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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).

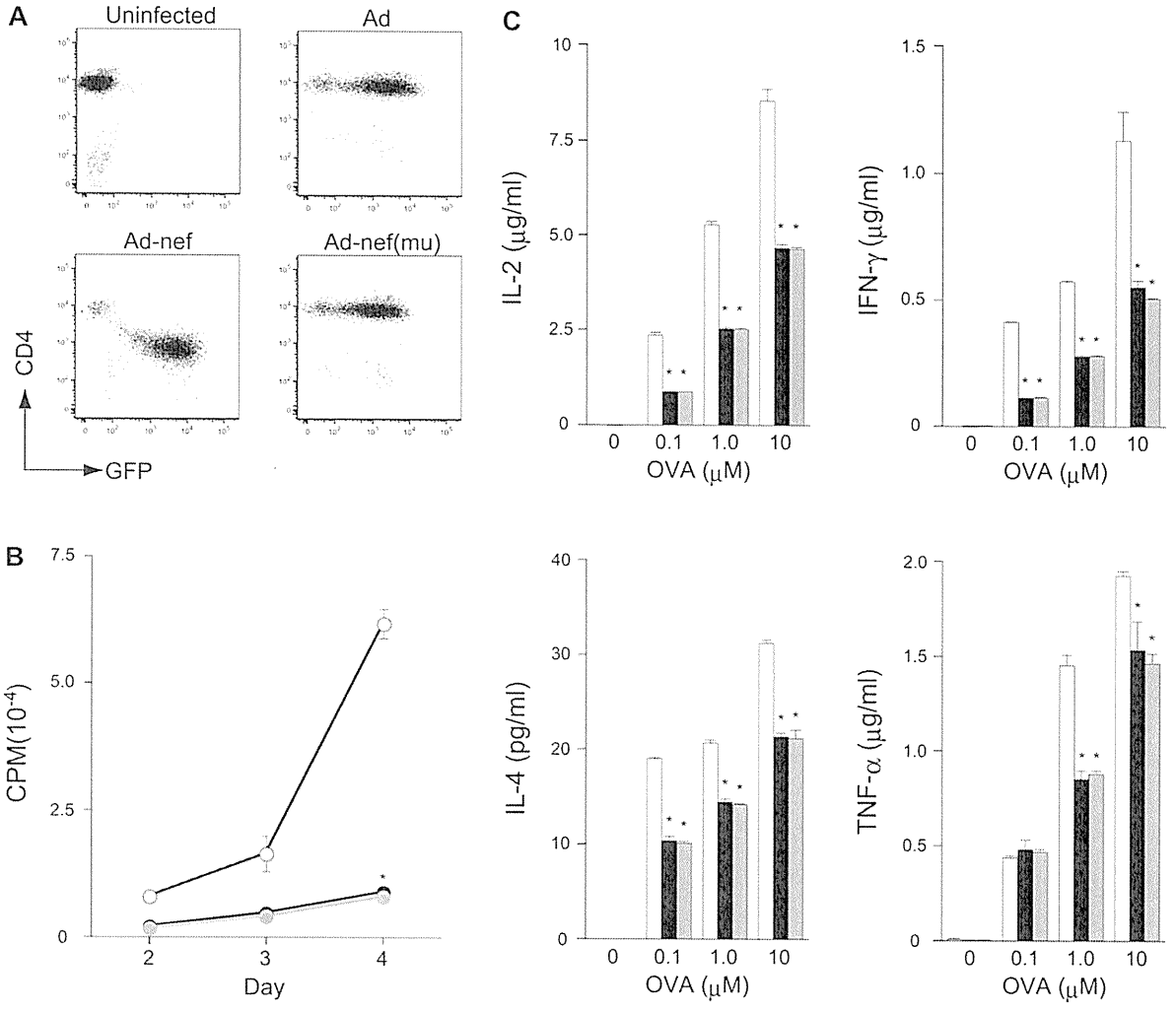

Fig. 1. (A) Characterization of Nef-expressing T cells. A *EGFP* gene-containing adenoviral vector was used to evaluate the efficiency of adenovirus (Ad) infection in DO11.10/CAR$^+$/CD4$^+$ T cells. Naive CD4$^+$ T cells from dTg mice were infected with Ad-nef, Ad-nef (mu) or Ad vector as a control. 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

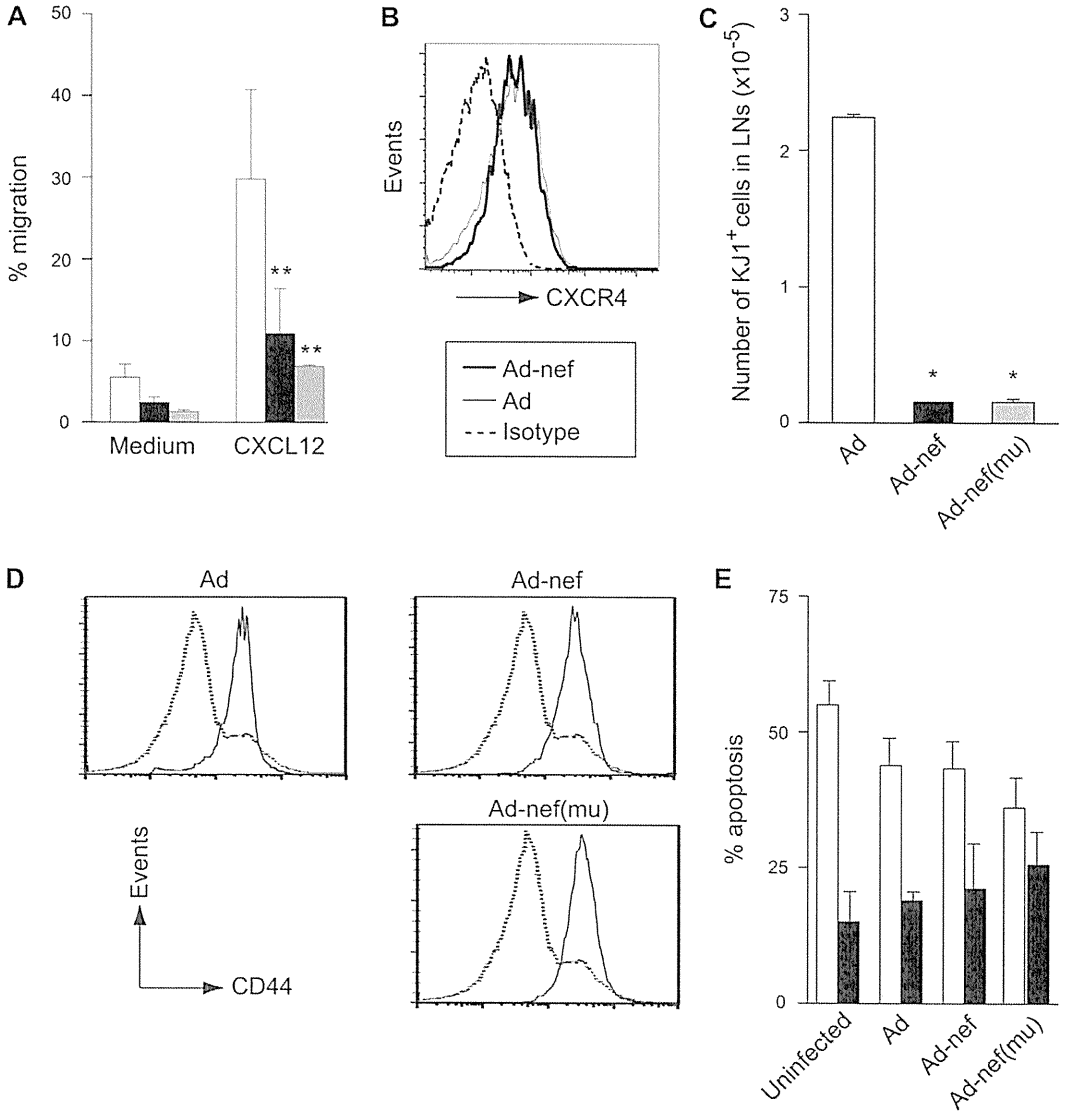

Fig. 2. Nef impairs T-cell migratory activity (A). $CD4^+$ GFP^+ T cells infected with Ad-nef (closed column), Ad-nef (mu) (gray column) and Ad (open column) were used in transwell chemotaxis assays in the presence of CXCL12 (PeproTech). Cells were allowed to migrate in the bottom wells for 90 min, and 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

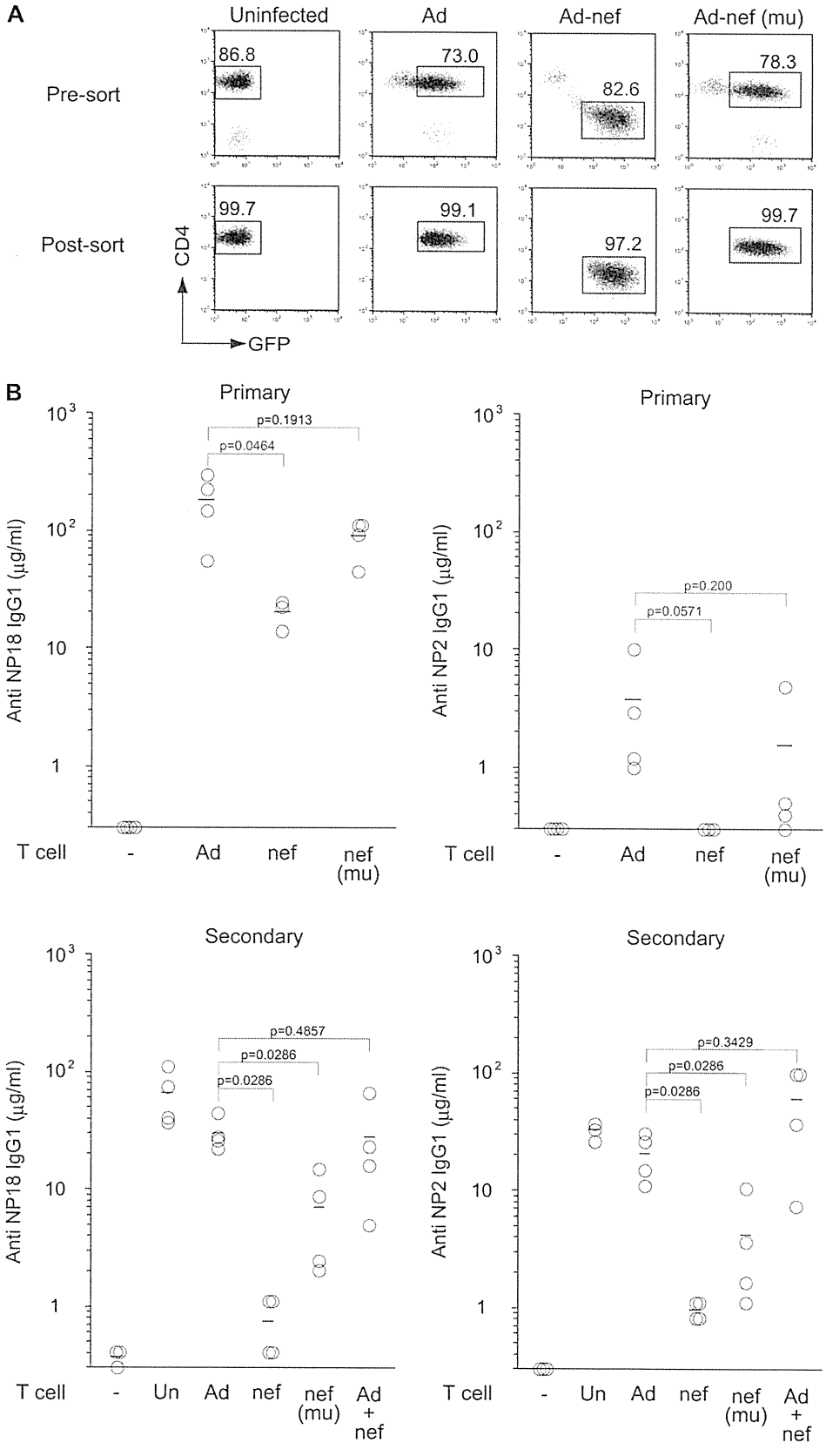

Fig. 3. Nef affects primary and memory B-cell response. (A) OVA-specific/CD4$^+$ T cells were purified from dTg mice and infected with Ad-nef, Ad-nef (mu) and Ad, followed by FACS purification (Post-sort). Numbers in plots indicate percent of GFP$^-$ uninfected cells and GFP$^+$ cells before (Pre-sort) 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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Commentary on a new era of investigating 3D structure-based human–virus protein network dynamics

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A commentary on

Structural principles within the human–virus protein-protein interaction network *by Franzosa, E. A., and Xia, Y. (2011). Proc. Natl. Acad. Sci. U.S.A. 108, 10538–10543.*

Virology is a fundamental research field for viruses that encompasses studies on minimum replication units, rapid evolution, and clinical applications. Similar to the other biological systems, structural study of viral system is based on three distinct phases of understanding: (I) modeling of steady-state network; (II) involvement of spatial confinements to the model; (III) involvement of timing asymmetry to the model. Most molecular biological understandings are limited to phase (I), rendering later phases to be investigated by future systems biology.

Even in phase (I), complexities of protein–protein network system make the researchers puzzled over examining actual underlying principles in the observed data sets. One type of solution for such an abstraction of the principles is to model a protein–protein interaction network based on graph theory; a theory of pair-wise relations between objects of a certain collection. Graph is constituted of nodes (for protein of interests in this case) and edges (for a certain protein–protein interaction), sometimes accompanied by different evaluation weights for an expected dynamics. Scale-free network, in which a small number of nodes act as hubs and the other nodes possess extremely small numbers of links, is supposed to be a characteristic of actual protein–protein interaction network in biological system. This type of network is prominent when the network encounters adding-on system for growth and selection bias for linking nodes act as hubs. However, it is not clear whether an input and output of such an abstracted system represents actual dynamics of original biological system, and what biological significances of parameters characterizing the networks are, i.e., density, transitivity, reciprocity, centrality etc. Progressing of the type of study requires further information to analyze the network elegantly.

In the article that deals with underlying principles of human–virus protein interaction network (*Proc. Natl. Acad. Sci. U.S.A.* 108, 10538–10543), Drs Franzosa and Xia have reported the results from 3D surface structure-based protein interaction network system. Based on 3039 endogenous interactions among 2435 human proteins as well as 53 exogenous interactions among 50 viral proteins from 36 viral species and their 50 human target proteins, they developed combined method of BLAST homology evaluation algorithm and MSMS program based on solvent-accessible surface area (SASA). The unique properties of this model case study are (i) detection of convergent evolution (mimicking evolution of viral protein surfaces to non-homological host human protein surfaces, in contrast to host gene duplication resulted in overlapping protein surface interactions) and evolutionary "arms race" (fast evolving trait of exogenous interactions due to cutting-off and catching-up interactions between human target proteins and viral proteins) due to rapid evolution and highly antagonistic manner of viral proteins and their target proteins, in contrast to cooperative interaction of endogenous human protein interactions; (ii) higher resolution of data based on more information from 3D surface structure interface. The original idea of using 3D surface information to network analysis is from the yeast study by Kim et al. (2006). In contrast to Kim's work, the patterns of evolution became much clearer based on fast evolving antagonistic viral proteins of absolutely different origins from host human. Compared to endogenous interactions, exogenous interactions exhibit transient structure, increase in number of interacting proteins, higher tendency of regulatory function, and faster evolution. Addition of BLAST information elucidates co-evolutionary information such as convergent evolution and evolutionary arms race.

Although the researchers are still struggling in phase (I), the application of 3D structural information to a graph of protein–protein network is very useful for investigating the evolutionary dynamics of the network of interests applying huge sum of mechanical interactive information to a simple graph of the interaction network. Additional investigation by commonly used BLAST algorithm will be useful expecting clinical application of drug design to highly conserved protein–protein interaction, instead of fast evolving highly adaptive region for target viruses.

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The Identification of a Small Molecule Compound That Reduces HIV-1 Nef-Mediated Viral Infectivity Enhancement

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Abstract

Nef is a multifunctional HIV-1 protein that accelerates progression to AIDS, and enhances the infectivity of progeny viruses through a mechanism that is not yet understood. Here, we show that the small molecule compound 2c reduces Nef-mediated viral infectivity enhancement. When added to viral producer cells, 2c did not affect the efficiency of viral production itself. However, the infectivity of the viruses produced in the presence of 2c was significantly lower than that of control viruses. Importantly, an inhibitory effect was observed with Nef^+ wild-type viruses, but not with viruses produced in the absence of Nef or in the presence of proline-rich PxxP motif-disrupted Nef, both of which displayed significantly reduced intrinsic infectivity. Meanwhile, the overexpression of the SH3 domain of the tyrosine kinase Hck, which binds to a PxxP motif in Nef, also reduced viral infectivity. Importantly, 2c inhibited Hck SH3-Nef binding, which was more marked when Nef was pre-incubated with 2c prior to its incubation with Hck, indicating that both Hck SH3 and 2c directly bind to Nef and that their binding sites overlap. These results imply that both 2c and the Hck SH3 domain inhibit the interaction of Nef with an unidentified host protein and thereby reduce Nef-mediated infectivity enhancement. The first inhibitory compound 2c is therefore a valuable chemical probe for revealing the underlying molecular mechanism by which Nef enhances the infectivity of HIV-1.

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Introduction

Nef is a 25- to 30-kDa protein with no catalytic activity encoded by the HIV-1 genome [1–4]. Studies of HIV-1-infected patients have demonstrated Nef to be a critical determinant of the progression to AIDS: HIV-1 strains without an intact *nef* gene were frequently isolated from non-progressive long-term survivors [5,6]. A subsequent study of HIV-1 transgenic mice confirmed the pathogenetic activity of Nef: targeted expression of the entire coding sequence of HIV-1 in $CD4^+$ T cells and macrophages caused a severe AIDS-like disease in mice, which was completely abolished by disruption of the *nef* gene [7].

Nef is multifunctional. For instance, it accelerates the endocytosis of CD4 [8,9], the primary entry receptor for HIV-1, which allows efficient viral release from host cells [1–4]. Nef also reduces the surface expression of MHC I through multiple mechanisms [10–13], which diminishes the recognition of infected cells by CTL [1–4]. Nef is also known to activate the Src kinase Hck [14–16], which causes an impaired macrophage response to the cytokine M-CSF [17,18] or triggers cell fusion of HIV-1-infected macrophages [19]. Another hallmark function of Nef is the enhancement of the intrinsic infectivity of progeny viruses. This function of Nef is independent of CD4 downregulation and requires the presence of Nef in viral producer cells [20–23]. Moreover, this function appears to depend on an early step of the target cell infection process, as Nef is dispensable for the infectivity of HIV-1 pseudotyped with vesicular stomatitis virus glycoprotein VSV-G [24,25]. However, Nef does not affect viral assembly or maturation, and it is still unclear how Nef enhances viral infectivity [26].

Thus far, only a few chemical compounds that interfere with the functions of Nef have been identified. Among them, a series of guanidine alkaloid analogs were found to be too toxic for cell-based assays [27]. A unique diphenylfuropyrimidine and its analogs were identified to be strong inhibitors of the Nef-dependent activation of Hck, but their primary target seemed to be Hck not Nef [28]. In contrast, the chemical compounds D1 and 2c directly target Nef. Betzi et al. identified D1 and showed that it reduced Nef-mediated MHC I, but not CD4, downregulation in a dose-dependent manner [29]. Subsequently, we identified 2c, the structure of which is distinct from that of D1, and showed that it almost completely inhibited the Nef-dependent activation of Hck

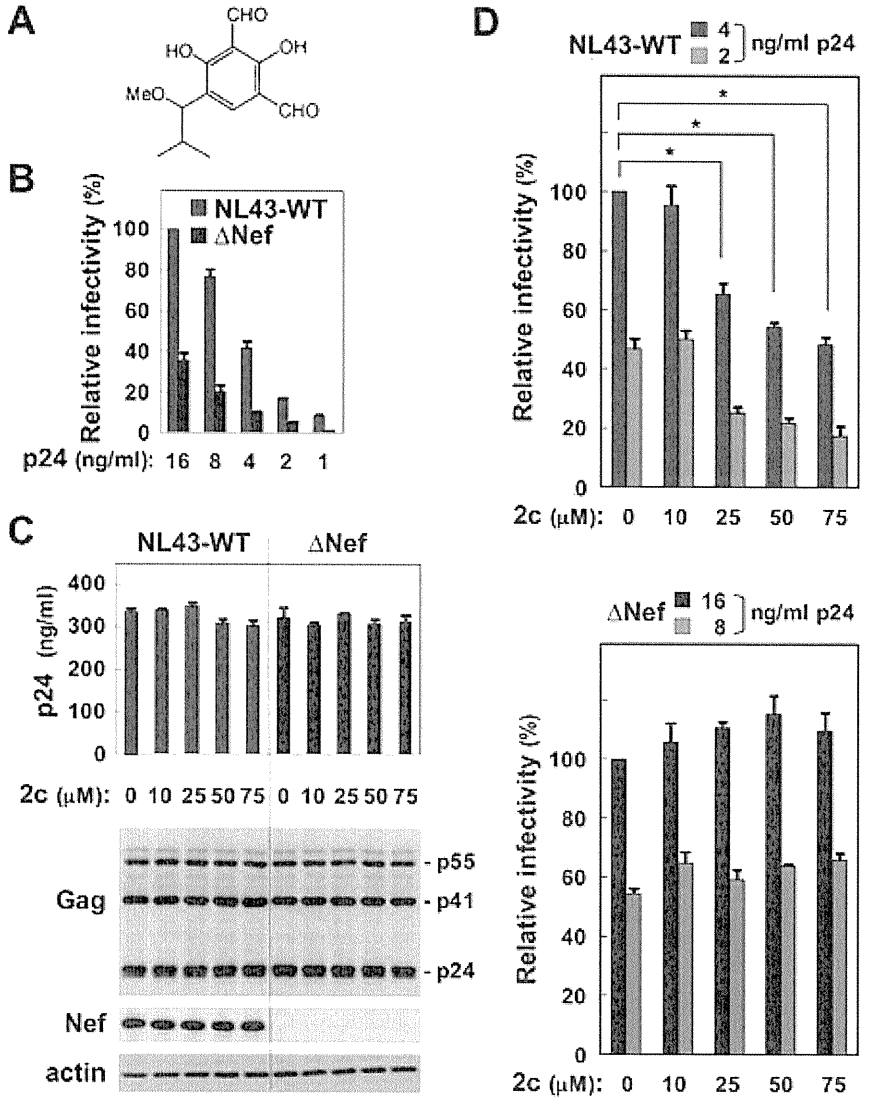

Figure 1. The effect of 2c on the infectivity of NL43 wild-type and Nef-defective mutant viruses. (A) The chemical structure of 2c. (B) The infectivity of the NL43 wild-type (WT) and Nef-defective mutant (ΔNef) viruses to the target TZM-bl cells was compared by varying the concentration of p24 Gag protein and is expressed as a percentage of the value for the sample on the far left. Data are shown as the mean±SD of triplicate assays and are representative of two independent experiments with similar results. (C) 2c was added to 293 cells producing NL43-WT or ΔNef viruses at the indicated concentrations for 2 days, and the concentration of p24 Gag protein in the cell supernatants was determined by ELISA (bar graph). Data are shown as the mean±SD of triplicate assays and are representative of two independent experiments with similar results. Alternatively, the producer cells were lysed and analyzed for the expression of Gag and Nef by Western blotting (lower blots). The actin blot was used as a loading control. (D) The infectivity of NL43-WT (upper) or ΔNef viruses (lower) produced by 293 cells in the absence or presence of the indicated concentrations of 2c was determined using TZM-bl cells as the target cells. The WT and ΔNef viruses were inoculated by changing the p24 concentration (2 or 4 ng/ml and 8 or 16 ng/ml for the WT and ΔNef viruses, respectively) so that the two viruses were similarly infective to the target cells. Infectivity is expressed as a percentage of the value for the sample on the far left. Data are shown as the mean±SD of triplicate assays and are representative of three independent experiments with similar results. *$p<0.05$.
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[30] and significantly reduced Nef-mediated MHC I, but not CD4, downregulation [31]. The fact that 2c has the inhibitory effect on MHC I downregulation and Hck activation, but not on CD4 downregulation, agrees with the finding that MHC I downregulation and Hck activation are mediated by overlapping motifs or amino acids of Nef, which are distinct from those required for CD4 downregulation [3,9,14,18]. However, none of these compounds have been tested for their ability to interfere with the enhancement of viral infectivity by Nef.

In contrast to its requirement for elevated *in vivo* viral load [5,6], Nef is not essential for viral replication in *ex vivo* cell cultures. Nonetheless, Nef significantly enhances viral replication in primary $CD4^+$ T cells and macrophages that have been exposed to HIV-1 prior to their stimulation with mitogens [32,33], a function of Nef that is likely determined by enhancement of the initial infection with cell-free HIV-1 [34]. In this regard, a compound that can reduce viral infectivity would be a valuable chemical probe for revealing the underlying mechanism of this function of Nef. In this study, we identified 2c as the first small compound that has an inhibitory effect on Nef-mediated HIV-1 infectivity enhancement and reported its inhibitory mechanism.

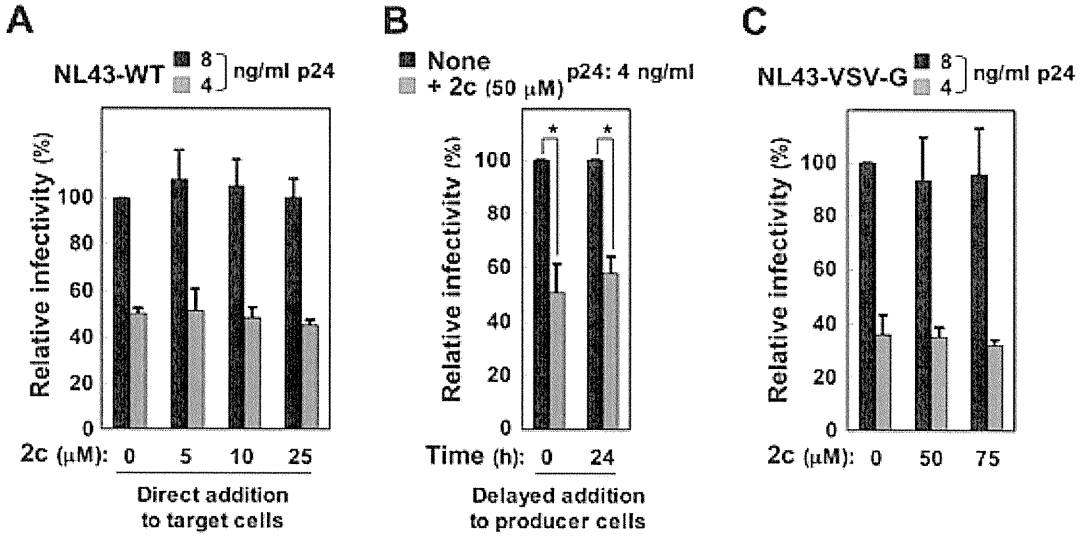

Figure 2. Several features of the activity of 2c on viral infectivity. (A) 2c was added to the target TZM-bl cells at the indicated concentrations together with the NL43 wild-type (WT) viruses produced in the absence of 2c. The amount of p24 inoculated was 4 or 8 ng/ml. The infectivity is expressed as a percentage of the value for the sample on the far left. (B) 2c (50 μM) or the control DMSO was added to the producer 293 cells immediately after transfection (0 h) or 24 h after transfection of the NL43 WT plasmid. The infectivity of the viruses was determined using TZM-bl cells and is expressed as a percentage of the value for the sample on the far left. The amount of p24 inoculated was 4 ng/ml. (C) 2c (50 or 75 μM) or the control DMSO was added to the producer 293 cells immediately after co-transfection of Env-defective NL43 plasmid and VSV-G expression plasmid. The infectivity of the pseudotyped viruses was determined using TZM-bl cells and is expressed as a percentage of the value for the sample on the far left. The amount of p24 inoculated was 4 or 8 ng/ml. (A–C) Data are shown as the mean±SD of triplicate assays and are representative of two independent experiments with similar results. *$p<0.05$.
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Results and Discussion

2c reduces the infectivity of wild-type HIV-1

We assessed the effect of the compound 2c (Fig. 1A) on Nef-mediated infectivity enhancement using a standard single-round of replication assay [21–23]. HIV-1 viruses were prepared by transfecting HIV-1 proviral clones into 293 cells (producer cells), and infectivity was analyzed by inoculating TZM-bl cells (target cells) with defined amounts of p24 Gag protein of the resultant viruses. We first used the proviral clone NL43 and a Nef-defective mutant (ΔNef) and confirmed that the infectivity of the ΔNef viruses was lower than that of the NL43 wild-type (WT) viruses (Fig. 1B). When added to the producer 293 cells, 2c did not affect the production of WT or ΔNef viruses, even at a high concentration such as 75 μM (Fig. 1C): there was no significant difference in the supernatant p24 Gag protein concentration (upper graph) or the processing of the Gag polyprotein in the cells (lower blots) between the control and 2c-treated cells. However, we found that the infectivity of the WT viruses produced in the presence of 2c was significantly lower than that of the control viruses (Fig. 1D, upper). An inhibitory effect of 2c was detectable at a minimal concentration of 25 μM. Importantly, no such inhibition was observed for the ΔNef viruses, even at a high 2c concentration (75 μM) (Fig. 1D, lower). In the experiment shown in Fig. 1D, WT and ΔNef viruses were inoculated into TZM-bl cells, and the concentration of p24 was adjusted (2 or 4 ng/ml and 8 or 16 ng/ml for WT and ΔNef viruses, respectively) so that the two viruses were similarly infective to the target cells (see Fig. 1B). As the supernatant of proviral plasmid-transfected 293 cells was used as a viral stock, 2c was also present in the culture of target cells (<5 μM). However, 2c did not reduce the infectivity when added to the target cells at a high concentration (10 or 25 μM) together with WT viruses produced in the absence of 2c (Fig. 2A), suggesting that the presence of 2c in the producer cells was essential for its inhibitory effect. Although 2c was added to the producer cells immediately after transfection in Fig. 1D, the inhibitory effect was also observed when 2c was added 24 h after transfection (Fig. 2B). Importantly, 2c did not show any inhibitory effect on the infectivity of Nef^+ HIV-1 viruses pseudotyped with VSV-G (Fig. 2C), which was consistent with the finding that Nef was dispensable for the infectivity of VSV-G-pseudotyped HIV-1 [24,25]. Therefore, these results indicated that 2c specifically reduced the infectivity of the wild-type NL43 viruses produced in the presence of Nef.

We also assessed the effect of 2c on viral replication. 2c decreased by half in the number of viable peripheral blood mononuclear cells after 9 days when used at 50 μM (data not shown). On the other hand, 2c at the same concentration showed no detectable toxicity to 293, TZM-bl, Jurkat T cells and macrophages (data not shown). We therefore used Jurkat and macrophages as target cells. As previously reported [28], the replication of HIV-1 NL43 was independent of Nef in Jurkat T cells (Fig. 3A). Accordingly, 2c failed to inhibit viral replication in the cells (Fig. 3A). However, WT JRFL viruses replicated more efficiently than ΔNef viruses in monocyte-derived macrophages, and 2c significantly reduced the replication of WT viruses (Fig. 3B). The result further supported the idea that the primary target of 2c was Nef.

The inhibitory effect of 2c requires the proline-rich PxxP motif of Nef

Next, we tested the inhibitory activity of 2c on the infectivity of NL43 viruses with point mutations in Nef; i.e., R77A, K82A, D86A, F90A, or G119L [35]. As shown, 2c reduced the infectivity of all these viruses, although to a varying degree (Fig. 4A). Interestingly, the intrinsic infectivity of the NL43-G119L viruses was shown to be low [35] (also see Fig. 4A), but 2c further reduced the infectivity of the mutant viruses to the level of the ΔNef viruses

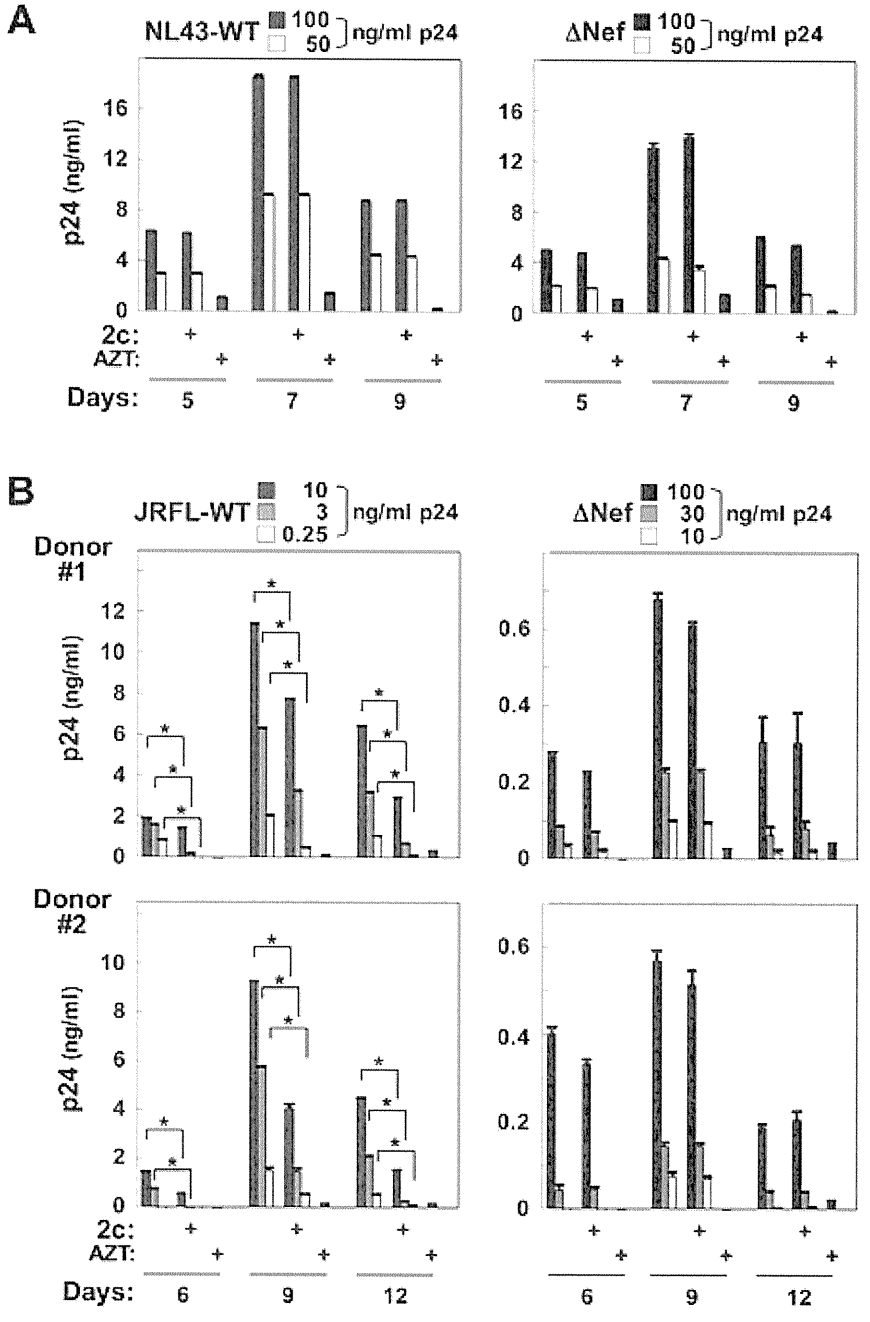

Figure 3. The effect of 2c on the replication of HIV-1. (A) Jurkat cells were infected with either the NL43 wild-type (WT) or Nef-defective (ΔNef) viruses at the indicated concentrations of p24, and cultured in the presence (50 μM) or absence of 2c. AZT was also used at 5 μM. The concentration of p24 in the supernatants (at day 5, 7 or 9) was determined by ELISA. Data are shown as the mean±SD of triplicate assays and are representative of two independent experiments with similar results. (B) Peripheral blood monocyte-derived macrophages were obtained from two different donors, infected with either the JRFL wild-type (WT) or Nef-defective (ΔNef) viruses at the indicated concentrations of p24, and cultured in the presence (50 μM) or absence of 2c. AZT was also used at 5 μM. The concentration of p24 in the supernatants (at day 6, 9 or 12) was determined by ELISA. Data are shown as the mean±SD of triplicate assays. *$p<0.05$.
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(Fig. 4B). This result supported the conclusion that 2c reduced the infectivity of the NL43 viruses in a Nef-dependent manner.

The dileucine motif of Nef (^{164}LL^{165}) that is required for CD4 downregulation is also required for the enhancement of infectivity [3,36]. However, it was unlikely that the inhibitory activity of 2c was mediated through the motif, as 2c did not inhibit CD4 downregulation [31]. On the other hand, Nef has another characteristic motif; i.e., a proline-rich PxxP motif, and the substitution of the proline residues for alanine residues (AxxA) is known to result in reduced viral infectivity [3]. Thus, we tested whether 2c further reduced the infectivity of Nef-AxxA viruses as it did with G119L mutant viruses (see Fig. 4B). To test whether 2c is also effective against Nef derived from an additional HIV-1 strain, we used an HIV-1 JRFL construct in which *nef* gene was replaced with that of the SF2 strain Nef or its AxxA mutant [30] in the subsequent experiments. First, as expected, the infectivity of the

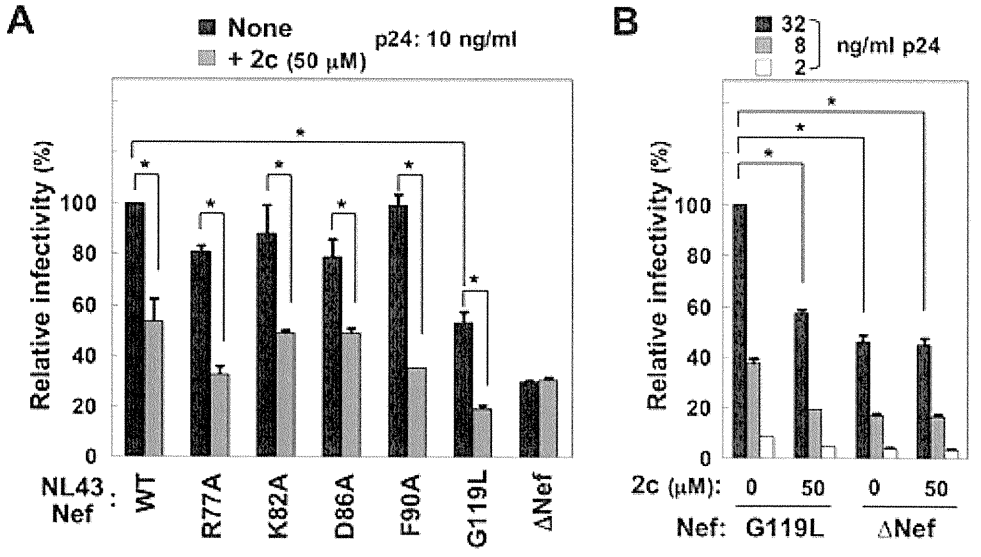

Figure 4. The effect of 2c on the infectivity of NL43 viruses with point amino acid mutations in Nef. (A) The infectivity of the indicated NL43 viruses produced by 293 cells in the absence or presence of 50 μM 2c was determined using TZM-bl cells as the target cells and is expressed as a percentage of the value for the sample on the far left. The amount of p24 inoculated was 10 ng/ml. Wild-type (WT), Nef-defective (ΔNef), or viruses with the indicated amino acid point mutations in Nef (R77A, K82A, D86A, F90A, or G119L) were used. (B) The infectivity of NL43 viruses with the G119L mutation in Nef or ΔNef viruses produced by 293 cells in the absence or presence of 50 μM 2c was determined using TZM-bl cells as the target cells and is expressed as a percentage of the value for the sample on the far left. The amount of p24 inoculated was 2, 8, or 32 ng/ml. (A, B) Data are shown as the mean±SD of triplicate assays and are representative of two independent experiments with similar results. *$p<0.05$.
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Nef- AxxA viruses was lower than that of the wild-type (WT) viruses, although it was still higher than that of the ΔNef viruses (Fig. 5A). As was the case with the NL43 viruses (see Fig. 1C), 2c did not affect viral production in the JRFL-SF2 Nef viruses (Fig. 5B): there was no change in the amount of p24 Gag protein in the supernatants (upper graph), the processing of the Gag polyprotein, or the expression of Nef or another viral protein, Vif, (lower blots) between the control and 2c-treated cells. Moreover, as was the case with the NL43 viruses (see Fig. 1D), 2c significantly reduced the infectivity of the produced JRFL-SF2 Nef WT viruses, but not that of the ΔNef viruses (Fig. 5C). However, we found that 2c minimally affected the infectivity of the Nef-AxxA mutant viruses (Fig. 5C, middle), which was in contrast with the finding that it further reduced the infectivity of the Nef-G119L mutant viruses (see Fig. 4B). These results suggested that the inhibitory activity of 2c is mediated, at least in part, through the proline-rich motif of Nef.

2c binds directly to Nef in a similar manner to the Hck SH3 domain

Although 2c was the first small molecule to be found to reduce the Nef-mediated infectivity of HIV-1, the overexpression of mutant forms of Hck in viral producer cells was also reported to result in reduced viral infectivity [37]. Hck is a cellular tyrosine kinase, and its SH3 domain has been shown to bind to Nef with high affinity [14–16], although its pathological significance is not yet understood. It is also known that the SH3 domain forms an intra-molecular interaction with the linker region of Hck [15,16] (also see Fig. 6A). Thus, the SH3 domain of mutant Hck, which lacks the linker region and the subsequent kinase domain (see Fig. 6A, HckN), is devoid of the intra-molecular interaction, and is thought to more efficiently bind to Nef and thereby reduce viral infectivity. Indeed, when co-expressed with the NL43 proviral clone, HckN and HckN-R151S, which carries a mutation in its SH2 domain, but not HckN-W93F, which carries a mutation in its SH3 domain, significantly reduced the infectivity of viruses produced from the cells (Fig. 6B).

Based on these results, we hypothesized that 2c inhibits viral infectivity in a similar manner to mutant Hck. To this end, we examined whether 2c and Hck compete to bind to Nef using an *in vitro* pull-down assay. First, we performed a pull-down assay with various combinations of GST-Nef fusion proteins (Fig. 7A) and the Hck proteins described above. As a result, we found that the wild-type (WT) NL43 Nef bound to the wild-type (WT) Hck, HckN, and HckN-R151S, but not Hck-W93F, which had a mutation in its Nef-binding SH3 domain (Fig. 7B). In contrast, the PxxP motif-disrupted AxxA mutant did not bind to any of these Hck proteins (Fig. 7B), confirming that the pull-down system specifically detected Nef-Hck binding. As the affinity of the SF2 strain Nef for Hck was higher than that of NL43 strain Nef, which was due to the different amino acid present within the PxxP motif (Figs. 7A and B, NL43 Nef-TR mutant with a T71R substitution), we used SF2 Nef in the following experiments. Among three different competitive pull-down assays, the pre-incubation of Nef with 2c most effectively inhibited the binding of Hck to Nef (Fig. 7C, right). We therefore concluded that both the Hck SH3 domain and 2c directly bind to Nef and that their binding sites overlap.

To further confirm the above-mentioned conclusion, we used a GST fusion protein containing a 20-mer peptide derived from the PxxP motif of SF2 Nef (Fig. 8A, SF2-PxxP). As shown, the observed binding of the SF2-PxxP peptides to Hck was specific, albeit weak, in comparison with that of the full-length Nef, since it was detected with the wild-type Hck, HckN, and HckN-R151S, but not with the Nef binding-deficient HckN-W93F (Fig. 8A). Importantly, 2c inhibited the binding of Hck to the Nef-PxxP peptide, and its inhibitory effect was more marked when the Nef-PxxP peptide was pre-incubated with 2c prior to its incubation with Hck (Fig. 8B). This result suggests that 2c binds to Nef, at least in part, through the region including the PxxP motif, which is consistent with the finding that unlike the wild-type viruses, the

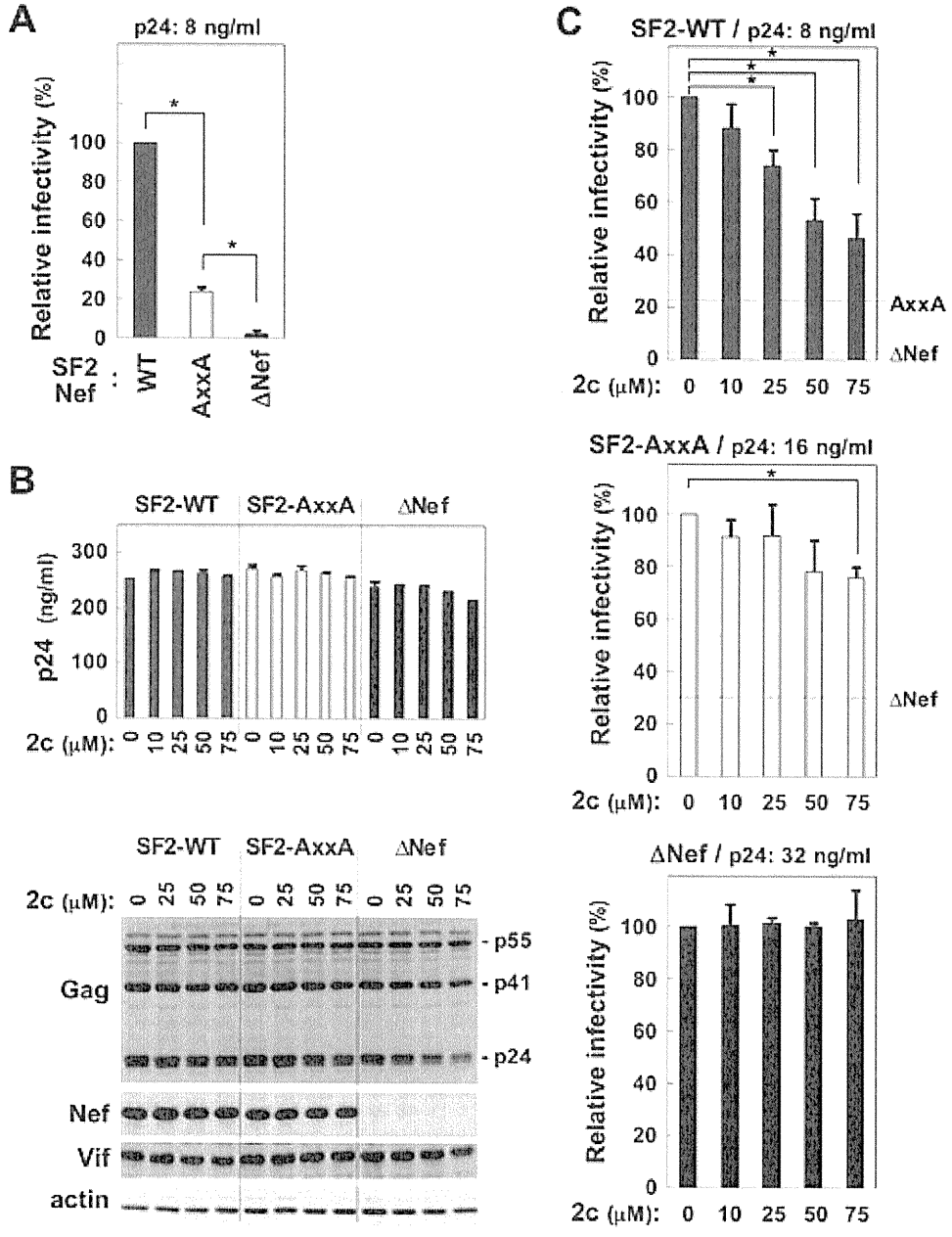

Figure 5. The effect of 2c on the infectivity of SF2 wild-type, Nef-defective, and Nef PxxP motif-disrupted viruses. (A) The infectivity of the SF2 wild-type (WT), Nef-defective (ΔNef), and Nef PxxP motif-disrupted viruses (AxxA) was compared by inoculating them into the target TZM-bl cells at a concentration of 8 ng/ml p24 and is expressed as a percentage of the value for the sample on the far left. Data are shown as the mean±SD of triplicate assays and are representative of two independent experiments with similar results. *$p<0.05$. (B) 2c was added to 293 cells producing SF2-WT, ΔNef, or AxxA viruses at the indicated concentrations for 2 days, and the concentration of p24 Gag protein in the supernatants was determined by ELISA (bar graph). Data are shown as the mean±SD of triplicate assays and are representative of two independent experiments with similar results. Alternatively, the producer cells were lysed and analyzed for the expression of Gag, Nef, and Vif by Western blotting (lower blots). The actin blot was used as a loading control. (C) The infectivity of SF2-WT (top), AxxA (middle), or ΔNef viruses (bottom) produced by 293 cells in the absence or presence of the indicated concentrations of 2c was determined using TZM-bl cells as the target cells. The WT, AxxA, and ΔNef viruses were inoculated by changing the concentration of p24 (8 ng/ml, 16 ng/ml, and 32 ng/ml for WT, AxxA and ΔNef viruses, respectively) so that these viruses were similarly infective to the target cells. Infectivity is expressed as a percentage of the value for the sample on the far left. In the top panel, the infectivity values of the AxxA and ΔNef viruses produced at the same concentration of p24 (i.e., 8 ng/ml) are also shown as a reference. In the middle panel, the infectivity values of the ΔNef viruses produced at the same concentration of p24 (i.e., 16 ng/ml) are also shown. Data are shown as the mean±SD of triplicate assays and are representative of three independent experiments with similar results. *$p<0.05$.
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infectivity of the PxxP motif-disrupted AxxA mutant viruses was minimally affected by 2c (see Fig. 5C).

Finally, a computer-assisted simulation of the 2c-Nef docking model supported the idea that 2c binds directly to Nef and suggested that R77, K82, A83, D86, I87, F90, Q118, and Y120 (positions are based on the sequence of NL43 strain Nef) may be responsible for this binding (Fig. 9). Among them, R77 lies within the PxxP motif (–PVTPQVPLR^{77}P–, the proline residues are