7. Gambotto, A., Barratt-Boyes, S.M., de Jong, M.D., et al. (2008): Human infection with highly pathogenic H5N1 influenza virus. Lancet, 371, 1464-1475.
8. Abdel-Ghafar, A.N., Chotpitayasunondh, T., Gao, Z., et al. (2008): Update on avian influenza A (H5N1) virus infection in humans. N. Engl. J. Med., 358, 261-273.
9 de Jong, M.D., Simmons, C.P., Thanh, T.T., et al. (2006): Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat. Med., 12, 1203-1207.
10. Centers for Disease Control and Prevention: Seasonal Influenza (Flu). Influenza Diagnostic Testing Algorithm. Online at 〈http: // www.cdc.gov / flu / professionals / diagnosis / testing_ algorithm.htm〉.
11. Wright, P.F., Neumann, G. and Kawaoka, Y. (2007): Orthomyxoviridae. Lippincott Williams & Wilkins, Philadelphia.
12. World Health Organization: Influenza Continuing Progress towards a Unified Nomenclature System for the Highly Pathogenic H5N1 Avian Influenza Viruses. Online at 〈http://www.who.int/influenza/resources/documents/h5n1_nomenclature/en/〉.
13. Takahashi, Y., Hasegawa, H., Hara, Y., et al. (2009): Protective immunity afforded by inactivated H5N1 (nibrg-14) vaccine requires antibodies against both hemagglutinin and neuraminidase in mice. J. Infect. Dis., 199, 1629-1637.
14. Kozbor, D. and Roder, J.C. (1984): In vitro stimulated lymphocytes as a source of human hybridomas. Eur. J. Immunol., 14, 23-27.
15. Storch, G.A. (2001): Diagnostic virology. p. 493-531. Lippincott Williams & Wilkins, Philadelphia.
16. Ohnishi, K., Sakaguchi, M., Kaji, T., et al. (2005): Immunological detection of severe acute respiratory syndrome coronavirus by monoclonal antibodies. Jpn. J. Infect. Dis., 58, 88-94.
17. Graves, P.N., Schulman, J.L., Young, J.F., et al. (1983): Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: unmasking of cross-reactive HA2 determinants. Virology, 126, 106-116.
18. Russ, G., Polakova, K., Kostolansky, F., et al. (1987): Monoclonal antibodies to glycopolypeptides HA1 and HA2 of influenza virus haemagglutinin. Acta Virol., 31, 374-386.
19. Kashyap, A.K., Steel, J., Oner, A.F., et al. (2008): Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc. Natl. Acad. Sci. USA, 105, 5986-5991.
20. Throsby, M., van den Brink, E., Jongeneelen, M., et al. (2008): Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IGM$^+$ memory B cells. PLoS One, 3, e3942.
21. Sui, J., Hwang, W.C., Perez, S., et al. (2009): Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat. Struct. Mol. Biol., 16, 265-273.
22. Ekiert, D.C., Bhabha, G., Elsliger, M.A., et al. (2009): Antibody recognition of a highly conserved influenza virus epitope. Science, 324, 246-251.
23. Corti, D., Suguitan, A.L., Jr., Pinna, D., et al. (2010): Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J. Clin. Invest., 120, 1663-1673.
24. Wrammert, J., Koutsonanos, D., Li, G.M., et al. (2011): Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. J. Exp. Med., 208, 181-193.
25. Palese, P. and Shaw, M.L. (2007): Orthomyxoviridae: the viruses and their replication. p. 1647. Lippincot Williams & Wilkins, Philadelphia.
26. Imai, M., Ninomiya, A., Minekawa, H., et al. (2007): Rapid diagnosis of H5N1 avian influenza virus infection by newly developed influenza H5 hemagglutinin gene-specific loop-mediated isothermal amplification method. J. Virol. Methods, 141, 173-180.
27. Wei, H.L., Bai, G.R., Mweene, A.S., et al. (2006): Rapid detection of avian influenza virus a and subtype H5N1 by single step multiplex reverse transcription-polymerase chain reaction. Virus Genes, 32, 261-267.
28. He, Q., Velumani, S., Du, Q., et al. (2007): Detection of H5 avian influenza viruses by antigen-capture enzyme-linked immunosorbent assay using H5-specific monoclonal antibody. Clin. Vaccine Immunol., 14, 617-623.

International Immunology, Vol. 23, No. 7, pp. 433–441
doi:10.1093/intimm/dxr031
Advance Access publication 3 June 2011

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

Hideki Fujii[1,2,*], Manabu Ato[2,*], Yoshimasa Takahashi[2], Kaori Otake[3], Shu-ichi Hashimoto[4], Tomohiro Kaji[5], Yasuko Tsunetsugu-Yokota[2], Mikako Fujita[6], Akio Adachi[7], Toshinori Nakayama[8], Masaru Taniguchi[9], Shigeo Koyasu[1] and **Toshitada Takemori[2,5]**

[1]Department of Microbiology and Immunology, Keio University School of, Medicine, Tokyo 160-8582, Japan
[2]Department of Immunology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan
[3]Gifu Social Insurance Hospital, Gifu 509-0206, Japan
[4]Chiome Bioscience Inc., Saitama 351-0104, Japan
[5]Laboratory for Immunological Memory, Riken Research Center for Allergy and Immunology, Kanagawa 230-0045, Japan
[6]Department of Bioorganic Medicinal Chemistry, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan
[7]Department of Virology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8503, Japan
[8]Department of Immunology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan
[9]Laboratory for Immune Regulation, Riken Research Center for Allergy and Immunology, Kanagawa 230-0045, Japan

*These authors contributed equally to this study.

Correspondence to: T. Takemori; E-mail: mttoshi@rcai.riken.jp

Received 10 February 2011, *accepted* 2 May 2011

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).

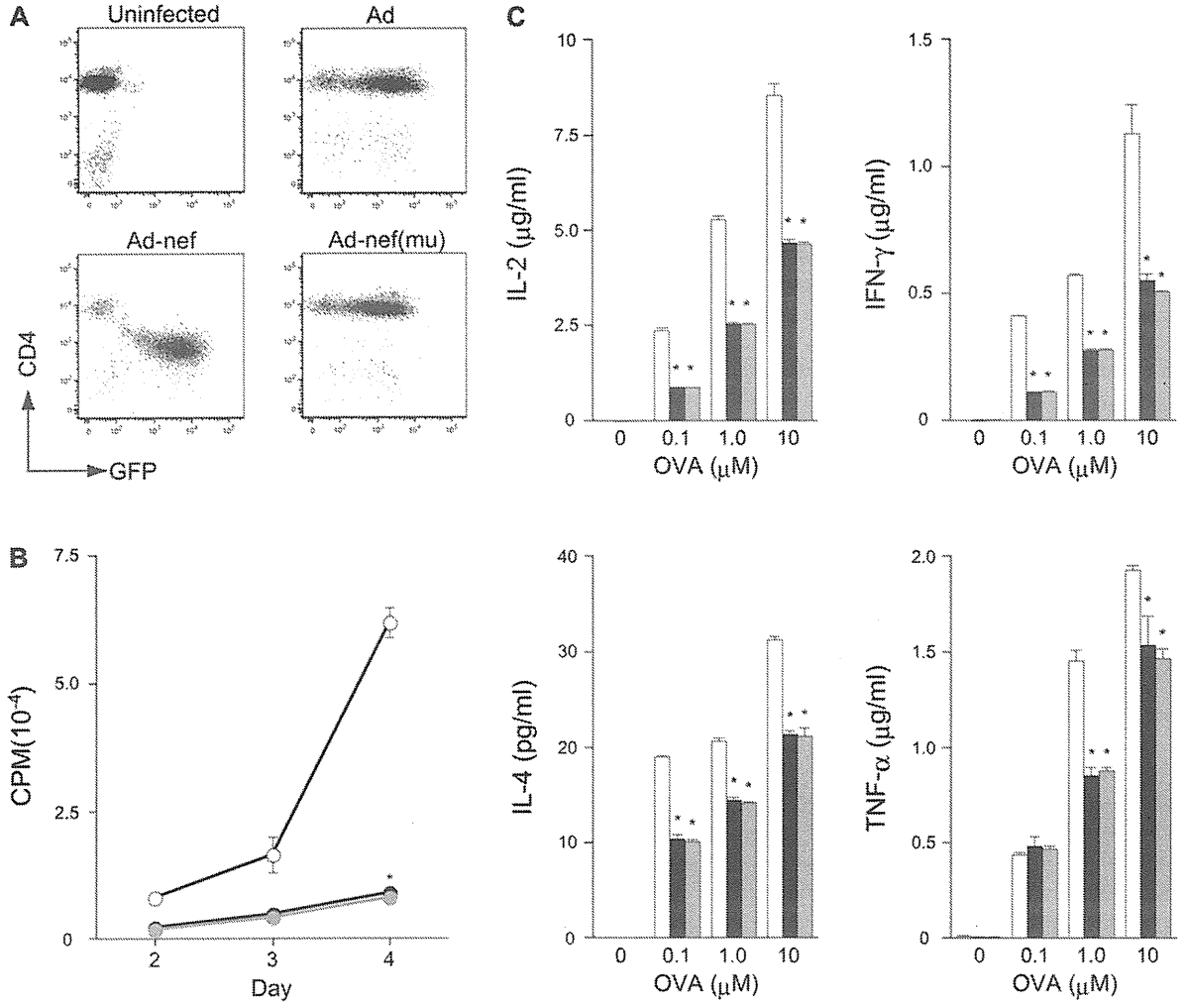

Fig. 1. (A) Characterization of Nef-expressing T cells. A *EGFP* gene-containing adenoviral vector was used to evaluate the efficiency of adenovirus (Ad) infection in DO11.10/CAR$^+$/CD4$^+$ T cells. Naive CD4$^+$ T cells from dTg mice were infected with Ad-nef, Ad-nef (mu) or Ad vector as a control.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

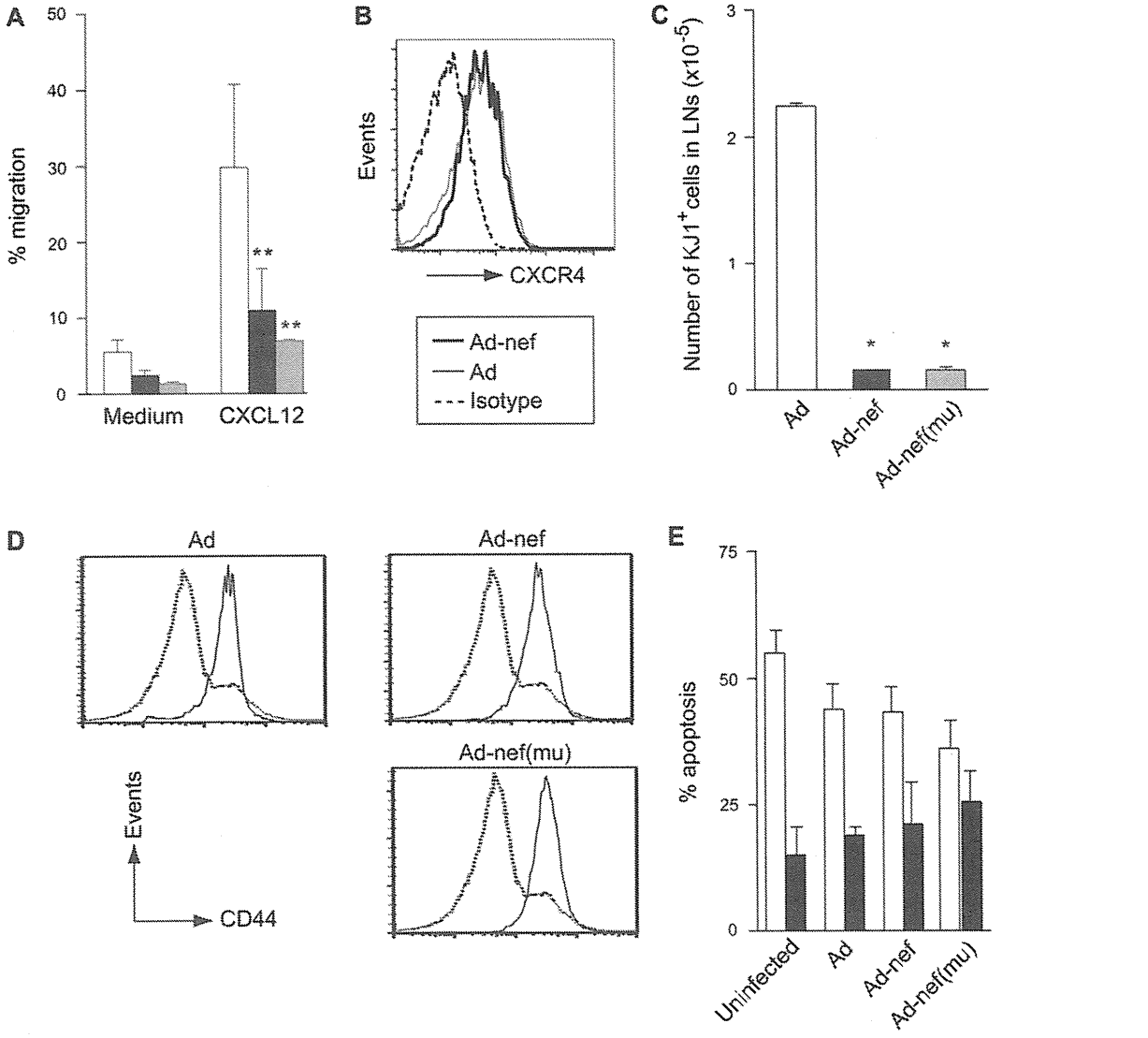

Fig. 2. Nef impairs T-cell migratory activity (A). $CD4^+$ GFP^+ T cells infected with Ad-nef (closed column), Ad-nef (mu) (gray column) and Ad (open column) were used in transwell chemotaxis assays in the presence of CXCL12 (PeproTech). Cells were allowed to migrate in the bottom wells for 90 min, and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

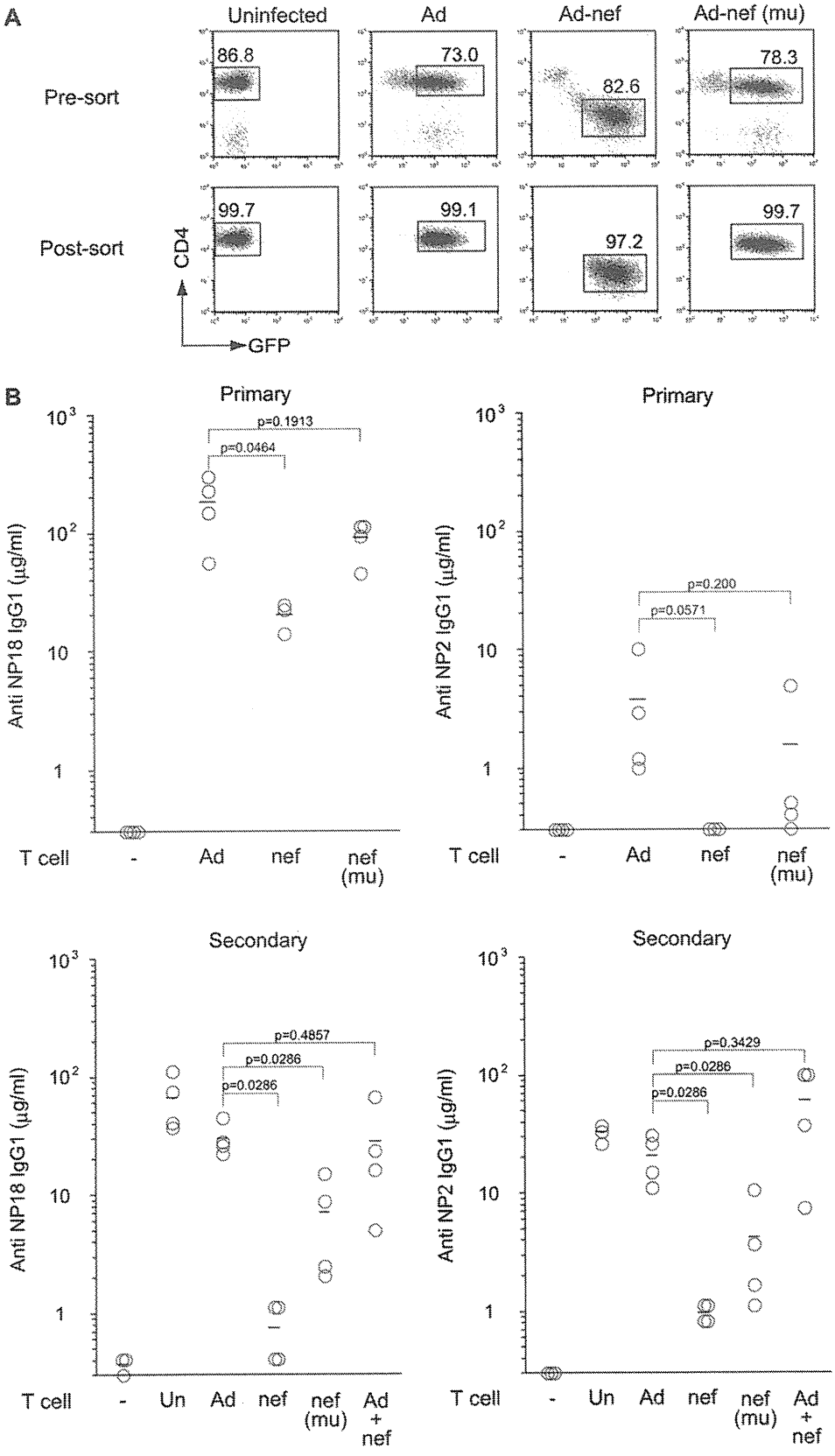

Fig. 3. Nef affects primary and memory B-cell response. (A) OVA-specific/CD4$^+$ T cells were purified from dTg mice and infected with Ad-nef, Ad-nef (mu) and Ad, followed by FACS purification (Post-sort). Numbers in plots indicate percent of GFP$^-$ uninfected cells and GFP$^+$ cells before (Pre-sort) 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 $I\text{-}A^d$-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

---

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.

Funding

This work was supported by a grant for Research on HIV/AIDS from the Ministry of Health, Labor and Welfare, Japan and in part by RIKEN (K94-34200 to T.T.).

Acknowledgements

We thank Drs Aya Isogai for constructing the nef (mu) plasmids and Peter Burrows for critically reviewing this manuscript and Ms Yoko Nakamura and Eri Watanabe for technical assistance.

References

1 Fackler, O. T. and Baur, A. S. 2002. Live and let die: Nef functions beyond HIV replication. *Immunity* 16:493.
2 Wang, J. K., Kiyokawa, E., Verdin, E. and Trono, D. 2000. The Nef protein of HIV-1 associates with rafts and primes T cells for activation. *Proc. Natl Acad. Sci. USA* 97:394.
3 Simmons, A., Aluvihare, V. and McMichael, A. 2001. Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. *Immunity* 14:763.
4 Manninen, A. and Saksela, K. 2002. HIV-1 Nef interacts with inositol trisphosphate receptor to activate calcium signaling in T cells. *J. Exp. Med.* 195:1023.
5 Fortin, J. F., Barat, C., Beausejour, Y., Barbeau, B. and Tremblay, M. J. 2004. Hyper-responsiveness to stimulation of human immunodeficiency virus-infected $CD4^+$ T cells requires Nef and Tat virus gene products and results from higher NFAT, NF-κB, and AP-1 induction. *J. Biol. Chem.* 279:39520.
6 Campbell, D. J., Kim, C. H. and Butcher, E. C. 2003. Chemokines in the systemic organization of immunity. *Immunol. Rev.* 195:58.
7 Janardhan, A., Swigut, T., Hill, B., Myers, M. P. and Skowronski, J. 2004. HIV-1 Nef binds the DOCK2-ELMO1 complex to activate rac and inhibit lymphocyte chemotaxis. *PLoS Biol.* 2:E6.
8 Hanna, Z., Kay, D. G., Rebai, N., Guimond, A., Jothy, S. and Jolicoeur, P. 1998. Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. *Cell* 95:163.
9 Rahim, M. M., Chrobak, P., Hu, C., Hanna, Z. and Jolicoeur, P. 2009. Adult AIDS-like disease in a novel inducible human immunodeficiency virus type 1 Nef transgenic mouse model:

$CD4^+$ T-cell activation is Nef dependent and can occur in the absence of lymphophenia. *J. Virol.* 83:11830.
10 Koenen, P. G., Hofhuis, F. M., Oosterwegel, M. A. and Tesselaar, K. 2007. T cell activation and proliferation characteristic for HIV-Nef transgenic mice is lymphopenia induced. *J. Immunol.* 178:5762.
11 Wan, Y. Y., Leon, R. P., Marks, R. *et al.* 2000. Transgenic expression of the coxsackie/adenovirus receptor enables adenoviral-mediated gene delivery in naive T cells. *Proc. Natl Acad. Sci. USA* 97:13784.
12 Murphy, K. M., Heimberger, A. B. and Loh, D. Y. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{lo}$ thymocytes *in vivo*. *Science* 250:1720.
13 Kanegae, Y., Makimura, M. and Saito, I. 1994. A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn. J. Med. Sci. Biol.* 47:157.
14 Takahashi, Y., Ohta, H. and Takemori, T. 2001. Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. *Immunity* 14:181.
15 Choe, E. Y., Schoenberger, E. S., Groopman, J. E. and Park, I. W. 2002. HIV Nef inhibits T cell migration. *J. Biol. Chem.* 277:46079.
16 Marrack, P. and Kappler, J. 2004. Control of T cell viability. *Annu. Rev. Immunol.* 22:765.
17 Dardalhon, V., Jaleco, S., Kinet, S. *et al.* 2001. IL-7 differentially regulates cell cycle progression and HIV-1-based vector infection in neonatal and adult $CD4^+$ T cells. *Proc. Natl Acad. Sci. USA* 98:9277.
18 Craig, H. M., Pandori, M. W. and Guatelli, J. C. 1998. Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity. *Proc. Natl Acad. Sci. USA* 95:11229.
19 Dubey, S., Khalid, M., Wesley, C., Khan, S. A., Wanchu, A. and Jameel, S. 2008. Downregulation of CCR5 on activated CD4 T cells in HIV-infected Indians. *J. Clin. Virol.* 43:25.
20 Venzke, S., Michel, N., Allespach, I., Fackler, O. T. and Keppler, O. T. 2006. Expression of Nef downregulates CXCR4, the major coreceptor of human immunodeficiency virus, from the surfaces of target cells and thereby enhances resistance to superinfection. *J. Virol.* 80:11141.
21 Zajac, A. J., Blattman, J. N., Murali-Krishna, K. *et al.* 1998. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med.* 188:2205.
22 Zhang, J. *et al.* 2000. Regulation of fas ligand expression during activation-induced cell death in T cells by p38 mitogen-activated protein kinase and c-Jun NH2-terminal kinase. *J. Exp. Med.* 191:1017.
23 Rhee, S. S. and Marsh, J. W. 1994. HIV-1 Nef activity in murine T cells. CD4 modulation and positive enhancement. *J. Immunol.* 152:5128.
24 Priceputu, E., Hanna, Z., Hu, C. *et al.* 2007. Primary human immunodeficiency virus type 1 nef alleles show major differences in pathogenicity in transgenic mice. *J. Virol.* 81:4677.
25 Bunnell, S. C., Hong, D. I., Kardon, J. R. *et al.* 2002. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J. Cell Biol.* 158:1263.
26 Dustin, M. L. 2009. The cellular context of T cell signaling. *Immunity.* 30:482.
27 Thoulouze, M. I., Sol-Foulon, N., Blanchet, F., Dautry-Varsat, A., Schwartz, O. and Alcover, A. 2006. Human immunodeficiency virus type-1 infection impairs the formation of the immunological synapse. *Immunity* 24:547.
28 Pereira, J. P., Kelly, L. M. and Cyster, J. G. 2010. Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses. *Int. Immunol.* 22:413.
29 Veillette, A., Bookman, M. A., Horak, E. M., Samelson, L. E. and Bolen, J. B. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. *Nature* 338:257.
30 Krummel, M. F., Sjaastad, M. D., Wulfing, C. and Davis, M. M. 2000. Differential clustering of CD4 and CD3ζ during T cell recognition. *Science* 289:1349.
31 Holdorf, A. D., Lee, K. H., Burack, W. R., Allen, P. M. and Shaw, A. S. 2002. Regulation of Lck activity by CD4 and CD28 in the immunological synapse. *Nat. Immunol.* 3:259.
32 Stevenson, M. 2003. HIV-1 pathogenesis. *Nat. Med.* 9:853.