The Journal of Experimental Medicine

CD226 (DNAM-1) Is Involved in Lymphocyte Function–associated Antigen 1 Costimulatory Signal for Naive T Cell Differentiation and Proliferation

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Abstract

Upon antigen recognition by the T cell receptor, lymphocyte function–associated antigen 1 (LFA-1) physically associates with the leukocyte adhesion molecule CD226 (DNAM-1) and the protein tyrosine kinase Fyn. We show that lentiviral vector-mediated mutant (Y-F^{322}) *CD226* transferred into naive $CD4^+$ helper T cells (Ths) inhibited interleukin (IL)-12–independent Th1 development initiated by CD3 and LFA-1 ligations. Moreover, proliferation induced by LFA-1 costimulatory signal was suppressed in mutant (Y-F^{322}) *CD226*-transduced naive $CD4^+$ and $CD8^+$ T cells in the absence of IL-2. These results suggest that CD226 is involved in LFA-1–mediated costimulatory signals for triggering naive T cell differentiation and proliferation. We also demonstrate that although LFA-1, CD226, and Fyn are polarized at the immunological synapse upon stimulation with anti-CD3 in $CD4^+$ and $CD8^+$ T cells, lipid rafts are polarized in $CD4^+$, but not $CD8^+$, T cells. Moreover, proliferation initiated by LFA-1 costimulatory signal is suppressed by lipid raft disruption in $CD4^+$, but not $CD8^+$, T cells, suggesting that the LFA-1 costimulatory signal is independent of lipid rafts in $CD8^+$ T cells.

Key words: LFA-1 • CD226 • costimulatory molecules • lentiviral vector • naive T cells

Introduction

The αLβ2 integrin, LFA-1 (CD11a/CD18), is expressed on most leukocytes and mediates cell–cell adhesion upon binding to its ligands, the intercellular adhesion molecule (ICAM)-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50; reference 1), or JAM-1 (2). Circulating peripheral blood (PB) leukocytes generally express an inactive form of LFA-1. Once leukocytes are activated, for instance through the TCR upon recognition of a peptide antigen or by PMA, intracellular signals (referred to as "inside-out" signals) cause a conformational change in LFA-1, resulting in intercellular binding and effector cell function (1, 3).

Antibody cross-linking of cell surface LFA-1 induces intracellular signals (referred to as "outside-in" signals; 4), suggesting that ligand binding may also affect cellular functions such as apoptosis, cytotoxicity, proliferation, cytokine production, and antigen presentation (1, 5). Studies using mice with disrupted *CD11a* or *CD18* genes have indicated a requirement for LFA-1 in T cell proliferation induced by the TCR–CD3 complex (6, 7). Moreover, recent reports have suggested that LFA-1 might be involved in Th differentiation (8, 9). These observations

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Abbreviations used in this paper: CB, cord blood; cPPT, central polypurine tract; CTS, central termination sequence; ICAM, intercellular adhesion molecule; IRES, internal ribosome entry site; MβCD, methyl-β-cyclodextrin; MOI, multiplicity of infection; PB, peripheral blood; SIN, self-inactivating; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2003/12/1829/11 $8.00
Volume 198, Number 12, December 15, 2003 1829–1839
http://www.jem.org/cgi/doi/10.1084/jem.20030958

indicate that LFA-1 not only mediates intercellular binding but may also deliver costimulatory signals in T lymphocytes (4). In contrast with inside-out signaling, however, little is known about the intracellular signals initiated by LFA-1 ligation.

The leukocyte adhesion molecule DNAM-1 (CD226) is constitutively expressed on the majority of T lymphocytes, NK cells, and monocytes (10, 11). We have previously reported that LFA-1 constitutively associates with CD226 in NK cells (12). Moreover, stimulation of PB T cells with either anti-CD3 or PMA induces the physical association of LFA-1 and CD226 (12). Once LFA-1 and CD226 associate, cross-linking LFA-1 with anti-CD18 mAb induced tyrosine phosphorylation of CD226, for which the Fyn protein tyrosine kinase is responsible (12), suggesting that CD226 plays an important role for costimulatory signals initiated by LFA-1 ligation.

Gene transfer techniques are important not only for clinical therapy of various diseases but also for studies of molecular mechanisms of immune responses. In particular, to strictly study gene functions in naive or resting lymphocyte differentiation, proliferation, or activation, an efficient method of gene transfer into these, rather than activated or stimulated, lymphocytes should be required. Although retroviral vectors, derived from Moloney murine leukemia virus, have been successful for gene transfer into various cell types, they are unable to transduce nondividing cells, including resting primary lymphocytes (13, 14). Previously, retroviral vectors have been used for studies of molecular mechanisms of Th differentiation from $CD4^+$ naive T cells, which required stimulation with antigen or anti-CD3 antibody and cytokines such as IL-2 for a few days before gene transduction (15, 16). These prestimulated T cells may lose the naive phenotype and may not be naive T cells anymore according to a rigorous definition, although they may remain in the uncommitted developmental stage. Recent studies have demonstrated that HIV-1–based lentiviral vectors are capable of transducing nondividing and terminally differentiated cells, including neurons, myoblasts, hepatocytes, and hematopoietic stem cells (17–20). Moreover, the infection and propagation of HIV-1 in resting $CD4^+$ T cells isolated from HIV-1–infected individuals have been described (21, 22). However, several reports also showed that resting primary blood cells, including T and B cells and monocytes, are refractory to lentiviral vector-mediated gene transfer, unless these cells are stimulated with exogenous cytokines and/or antibodies (23, 24).

In this study, we demonstrate the dramatically improved transduction efficiency of resting lymphocyte subsets, including naive T cells, by lentiviral vectors. Using the lentivirus-mediated gene transfer system, we investigated whether CD226 is involved in LFA-1 costimulatory function in naive T cell differentiation and proliferation.

Materials and Methods

Antibodies and Cytokines. Anti-Ki67, anti-phosphotyrosine mAb (4G10), and anti-Flag mAb were purchased from DakoCytomation, Upstate Biotechnology, and Sigma-Aldrich, respectively. Anti-CD226 (DX11) mAb and anti-Fyn polyclonal Ab were provided by J. Phillips and J. Bolen (DNAX, Palo Alto, CA). The other mAbs and cytokines used in this study were purchased from BD Biosciences. Anti-CD11a and anti-CD226 were labeled with Alexa 594 by Monoclonal Antibody Labeling Kit (Molecular Probes).

Isolation of Each Blood Cell Subset. $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, $CD3^-$ $CD56^+$ NK cells, and $CD14^+$ monocytes were separated from PB or cord blood (CB; provided by Y. Shiina, Shiina Hospital, Ibaraki, Japan) by positive selection using MACS. The purity of each cell was >95% as analyzed by flow cytometry. $CD4^+$ $CD45RA^+$ $CD45RO^-$ naive T cells were obtained by further purifications from PB or CB $CD4^+$ T cells using flow cytometry to achieve >99% purity.

Preparation of Lentiviral and Retroviral Vectors and Transduction of Resting Blood Cells. For preparation of lentiviral vectors, the packaging construct pMDLg/pRRE, in which all accessory genes (vif, vpr, vpu, and nef) and regulatory genes (tat and rev) have been deleted, was used in this study (25). For expression of EGFP, the self-inactivating (SIN) vector construct pCS-CDF-CG-PRE that contains the EGFP gene under the control of the CMV promoter, the central polypurine tract (cPPT), and the central termination sequence (CTS), and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE; 26, 27) was used. For expression of WT or Y-F^{322} CD226 in naive T cells, *Flag*-tagged WT or Y-F^{322} (12) *CD226* was inserted in the multiple cloning sites of the SIN vector pCSII-CMV-MCS-internal ribosome entry site (IRES)-hrGFP. Lentiviral vectors pseudotyped with the vesicular stomatitis virus G glycoprotein were generated as previously described (27). In brief, 293 T cells were transiently cotransfected with appropriate amounts of the SIN vector construct (pCS-CDF-CG-PRE or pCSII-CMV-[Flag-tagged WT or Y-F^{322} *CD226*]-IRES-*hrGFP*), the packaging construct (pMDLg/pRRE), the Rev-expressing construct (pRSV-Rev), and the vesicular stomatitis virus G glycoprotein–expressing construct (pMD.G). The viral supernatants were collected 72 h after transfection and concentrated ~1,000-fold by ultracentrifugation at 19,400 rpm (SW28; Beckman Coulter) for 2 h and virus precipitates were resuspended by pipetting extensively (usually more than 200 times). For preparation of a retroviral vector, we used the plasmid construct pGCsam-IRES-EGFP as previously described (28). Vector titers were determined by infection of HeLa cells with serial dilutions of the vector stocks, followed by flow cytometry analysis for EGFP or hrGFP expression. For infection, 10^5 cells isolated from CB or PB were cultured in RPMI medium in the presence or absence of 10% FBS containing lentivirus or retrovirus supernatants at a multiplicity of infection (MOI) of 2, 10, or 100 in 96-well U-bottom plates for 72 h at 37°C in 5% CO_2. Total volume of culture medium per well was <100 μl.

Stimulation of Naive $CD4^+$ T Cells. Naive $CD4^+$ T cells (2.5×10^5 per well) were stimulated with plate-coated anti-CD3 and anti-CD18 mAbs and 10 ng/ml IL-2 in a total volume of 2 ml in 24-well plates. T cells were expanded and maintained in the same culture conditions for 2 wk. For the control of Th1/Th2 differentiation, naive T cells were stimulated with plate-coated anti-CD3, 10 μg/ml soluble anti-CD28, and 10 ng/ml IL-2 in the presence of either 10 ng/ml IL-12 plus 10 μg/ml anti–IL-4 or 10 ng/ml IL-4 plus 10 μg/ml anti–IL-12. For neutralization of IL-12, 40 μg/ml anti–IL-12 mAbs were added in the cultures.

Analysis of Intracellular Cytokines and ELISA. Intracellular IFN-γ, IL-4, and IL-13 syntheses were analyzed by flow cytome-

try as previously described (29). Concentrations of IFN-γ, IL-4, IL-13, and IL-2 in culture supernatants were determined using an ELISA kit (Biosource International) according to the manufacturer's instructions.

Cell Cycle Analysis. Cells were stained with Hoechst 33342 and Pyronin Y (both from Sigma-Aldrich) as previously described (30). In brief, naive CD4 T cells were washed once in PBS, incubated in PBS containing 5 μg/ml Hoechst 33342 at 37°C for 75 min, and Pyronin Y was then added to give a final concentration of 5 μg/ml followed by an additional 15-min incubation. For Ki67 staining, cells were fixed with 1% paraformaldehyde in PBS, permeated with 0.1% Triton X-100 (Sigma-Aldrich) in IFA buffer (10 mmol/liter Hepes, pH 7.4, 150 mmol/liter NaCl, 4% FCS, 0.1% NaN_3), and then incubated with anti-Ki67 antibody for 30 min. Washes between each step were performed with IFA buffer containing 0.1% Triton X-100. Cells were analyzed on a FACS Vantage™ flow cytometer and were analyzed using CELLQuest™ software.

Biochemistry. To examine the tyrosine phosphorylation of CD226, cells were stimulated with plastic-coated mAbs for 2 min or 7 d at 37°C. Cells were lysed with 1% NP-40 lysis buffer, immunoprecipitated with control Ig, anti–DNAM-1 mAbs, or anti-FLAG, and analyzed by immunoblotting with anti-phosphotyrosine 4G10 as previously described (12). To examine association of CD226 with LFA-1, cells were stimulated with anti-CD3 for 5 min at 37°C and lysed with 1% digitonin lysis buffer. Lysates were immunoprecipitated with anti-CD18 or control Ig and analyzed by immunoblotting with anti-CD226 as previously described (12).

Immunofluorescence. Receptor clustering and lipid raft aggregation by beads have been described (31). In brief, 6-μm diameter latex beads (Polysciences Inc.) were coated with anti-CD3ε

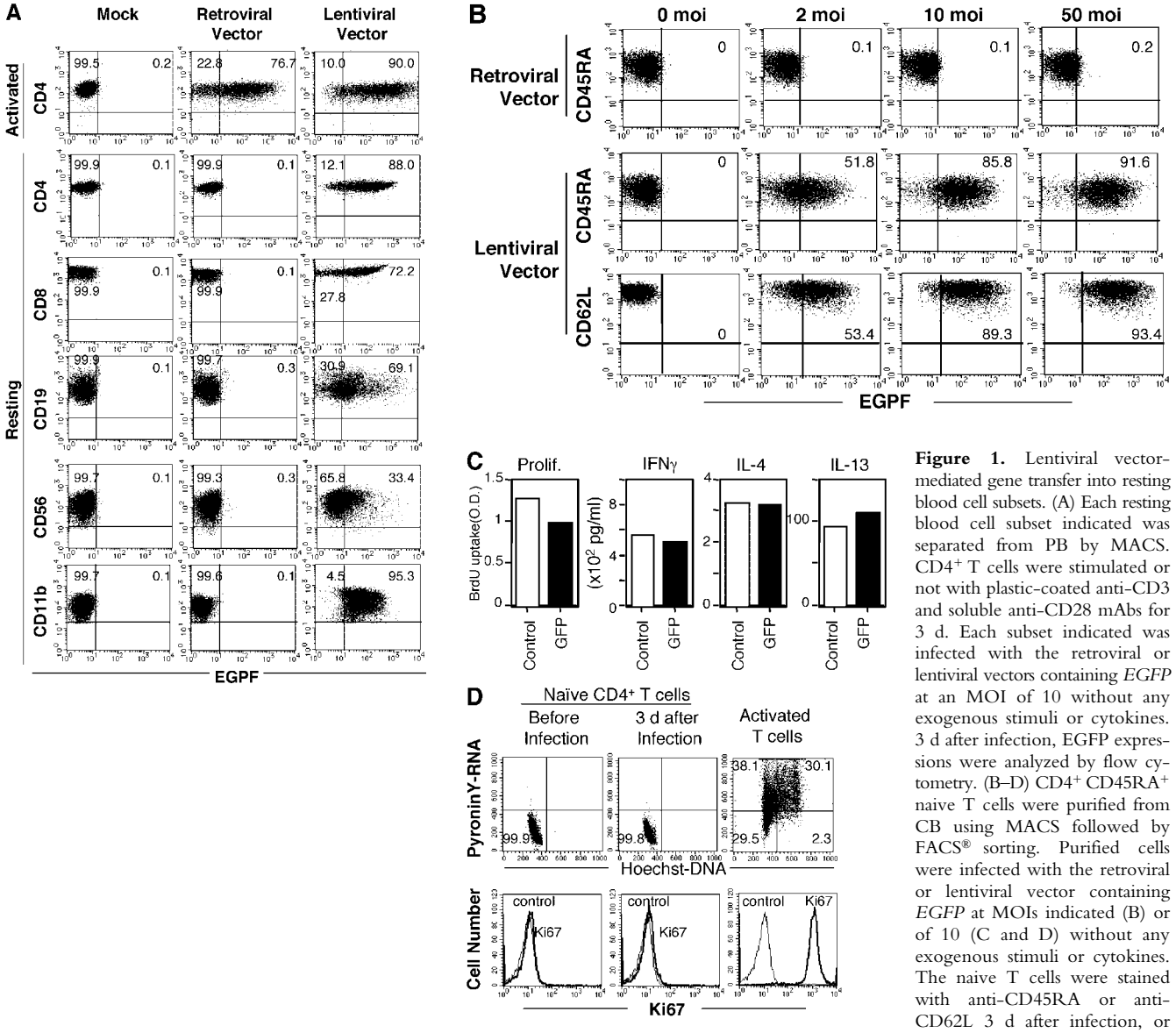

Figure 1. Lentiviral vector-mediated gene transfer into resting blood cell subsets. (A) Each resting blood cell subset indicated was separated from PB by MACS. $CD4^+$ T cells were stimulated or not with plastic-coated anti-CD3 and soluble anti-CD28 mAbs for 3 d. Each subset indicated was infected with the retroviral or lentiviral vectors containing *EGFP* at an MOI of 10 without any exogenous stimuli or cytokines. 3 d after infection, EGFP expressions were analyzed by flow cytometry. (B–D) $CD4^+$ $CD45RA^+$ naive T cells were purified from CB using MACS followed by FACS® sorting. Purified cells were infected with the retroviral or lentiviral vector containing *EGFP* at MOIs indicated (B) or of 10 (C and D) without any exogenous stimuli or cytokines. The naive T cells were stained with anti-CD45RA or anti-CD62L 3 d after infection, or were stained with Hoechst and Pyronin Y or anti-Ki67 before and 3 d after infection (D) and analyzed by flow cytometry. Staining of $CD4^+$ $CD45RA^+$ naive T cells activated with anti-CD3 and anti-CD28 were used as a positive control (D). The naive T cells 3 d after infection were also stimulated with plate-coated anti-CD3 and anti-CD28 mAbs and proliferation and cytokine production in culture supernatants were analyzed (C).

plus either anti-CD28, anti-CD11a, or anti-CD18. Purified human PB T cells were mixed with Ab-coated beads at ratios of 1:2 and 4:3, respectively, and incubated for 30 min at 37°C. T cell–bead complexes were transferred onto poly-L-lysine precoated coverslips to allow attachment for 5 min at 37°C, fixed with 2% formaldehyde, and permeabilized with 0.2% Triton X-100 in PBS. T cells were stained with FITC-conjugated cholera toxin subunit B (Sigma-Aldrich), Alexa 594–conjugated anti-CD11a, and anti-CD226. T cells were also stained with rabbit anti–Fyn polyclonal antibody, followed with Alexa 594–coupled anti–rabbit secondary antibodies (Molecular Probes). Coverslips were mounted with Slow Fade (Molecular Probes) and analyzed by Leica TCS SP2 confocal laser scanning microscopy with a 63X/1.32-0.6 oil objective lens.

Proliferation Assays. $CD4^+$ and $CD8^+$ naive T cells were stimulated with plastic-coated antibodies. 2 d after stimulation, T cell proliferations were measured by ELISA using BrdU Kit (Roche). For the study of involvement of raft in T cell proliferation, $CD4^+$ and $CD8^+$ T Cells were labeled with CFDA-SE (Molecular Probes) and incubated in medium containing methyl-β-cyclodextrin (MβCD; Sigma-Aldrich) at 10 mM for 30 min at 37°C. Cells were washed and then stimulated or not with plastic-coated antibodies in the dark. 3 d after culture, cells were harvested and analyzed by flow cytometry.

Results

Gene Transfer into Resting Blood Cell Subsets by Lentiviral Vectors. To examine whether lentiviral vectors can efficiently mediate gene transduction into resting blood cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells, and $CD11b^+$ monocytes sorted from PB were infected with a retroviral or lentiviral vector containing the *EGFP* gene and cultured for 72 h in RPMI medium containing 10% FBS without any exogenous stimuli and cytokines. Consistent with previous reports (32), each subset was refractory for transduction with the retroviral vector at an MOI of 10, although PB $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 mAbs were efficiently transduced at the same MOI (Fig. 1 A). In contrast, the lentiviral vector efficiently transduced the *EGFP* gene into all blood cell subsets tested at an MOI of 10 (Fig. 1 A).

Gene Transfer into $CD4^+$ Naive T Cells by Lentiviral Vector. We further examined whether lentiviral vectors are capable of gene transfer into naive $CD4^+$ Ths. $CD4^+$ $CD45RA^+$ $CD45RO^-$ cells were separated from CB or PB by sorting with MACS and then with flow cytometry. We infected these naive $CD4^+$ T cells with the retroviral or lentiviral vector containing the *EGFP* gene and cultured them for 72 h, as described above. The viability of purified naive $CD4^+$ T cells was >95% after 72 h of culture in the presence (95.9%) and absence (98.9%) of lentiviral vectors, as determined by staining with trypan blue, without any exogenous cytokines. Although the retroviral vector was refractory for transduction of naive $CD4^+$ T cells even at an MOI of 50, the lentiviral vector efficiently transduce these cells at an MOI of 2 and the transduction efficiency was increased in an MOI-dependent manner (Fig. 1 B). Importantly, $CD4^+$ naive T cells maintained the cell surface expressions of CD45RA and CD62L, which are believed to be markers for naive T cell phenotypes, after gene transfer (Fig. 1 B). Moreover, proliferation and cytokine production of $CD4^+$ naive T cells, in response to stimulation with anti-CD3 and anti-CD28 mAbs, were not affected by infection with the lentiviral vector containing the *EGFP* gene (Fig. 1 C). We also observed that these virus-infected cells could give rise to both polarized Th1 and Th2 cells after culture in the presence of the appropriate cytokines such as IL-12 and IL-4, respectively (unpublished data). Because CB contains a much larger number of naive $CD4^+$ Ths than PB, we basically used CB as a source of naive T cells. However, by using PB-derived naive T cells, we also obtained consistent results with those obtained using CB-derived naive T cells. These results suggest that lentiviral vector-mediated gene transfer does not affect functional properties of PB and CB naive $CD4^+$ Ths.

To examine whether $CD4^+$ naive T cells remain in the resting (i.e., nondividing) state after transduction with the lentiviral vector, DNA and RNA contents were analyzed by staining with Hoechst and Pyronin Y, respectively, before and 3 d after transduction. As demonstrated in Fig. 1 D, DNA and RNA contents in CB $CD4^+$ naive T cells did not change 3 d after transduction, indicating that lentiviral vector-mediated transduction does not stimulate nondividing $CD4^+$ naive T cells to enter the cell cycle and synthesize DNA. Moreover, the expression of Ki67, a nuclear antigen present in all cycling cells and absent in G_0 cells, did not increase in CB naive T cells after transduction (Fig. 1 D). Taken together, these results suggest that $CD4^+$ naive T cells preserve the functional and phenotypical characteristics after gene transfer by the lentiviral vector.

LFA-1–mediated Signal Induces IL-12–independent Th1 Differentiation from $CD4^+$ Naive T Cells. Th1 cells develop from naive $CD4^+$ T cells during activation by APC, for which IL-12 secreted from APC plays a crucial role. However, Th1 development is not completely abolished in mice deficient in IL-12 p40 gene (33), suggesting the presence of IL-12–independent signaling pathways for Th1 development. Because $CD4^+$ naive T cells express a variety of costimulatory or adhesion molecules, including CD28, LFA-1, and CD226, we examined whether intercellular binding between naive $CD4^+$ T cells and APC through these adhesion or costimulatory molecules are involved in IL-12–independent Th1 differentiation. CB $CD4^+$ naive T cells were stimulated either with plate-coated anti-CD3 alone or in combination with either mAb against CD28, CD11a (α chain of LFA-1), CD18 (β chain of LFA-1), or CD226, and cultured for 2 wk in IL-2–containing medium. Stimulation with anti-CD11a or anti-CD18 in combination with anti-CD3 mAb significantly drove Th1 development (Fig. 2). The amount of IFN-γ production from these cells was comparable to that in Th1 cells induced by IL-12 (Fig. 2, B and C). However, Th1 cells were not generated from naive $CD4^+$ T cells by stimulation with anti-CD3 alone (Fig. 2, A–C), suggesting that engagement of LFA-1 delivers a costimulatory signal that drives Th1 polarization. IL-12–pro-

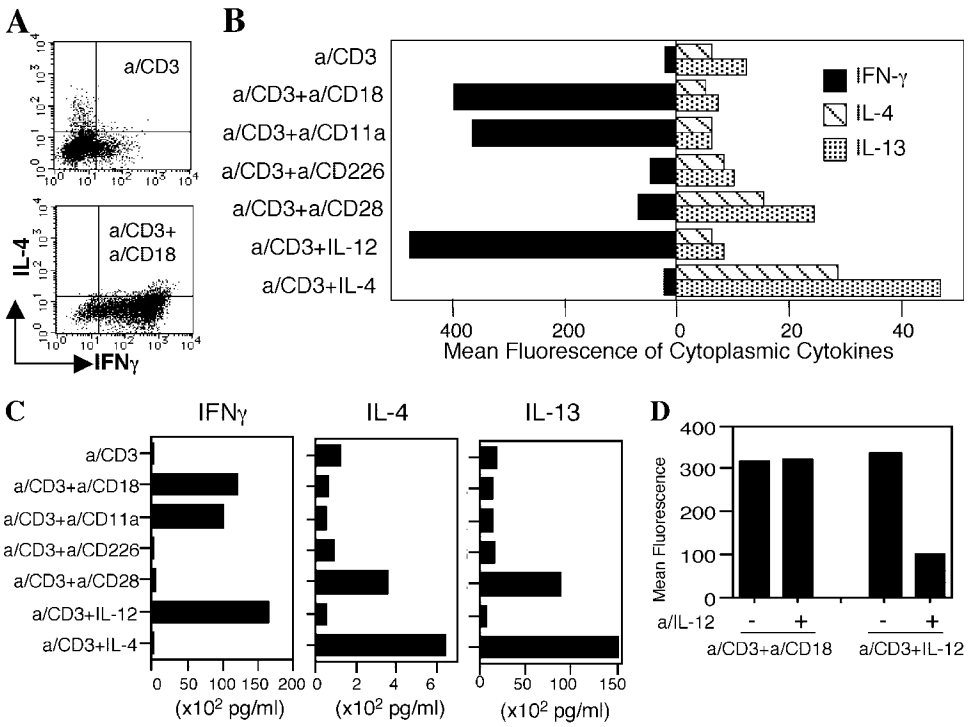

Figure 2. Stimulation of CD3 and LFA-1 induces Th1 development from naive $CD4^+$ Ths. (A–C) CD4 naive T cells were stimulated with plate-coated anti-CD3 alone or anti-CD3 plus mAbs indicated on days 1 and 8 and cultured for 14 d in IL-2–containing medium. Intracellular IFN-γ, IL-4, and IL-13 syntheses were analyzed in these cells by flow cytometry (A and B). Cytokine concentrations in culture supernatants were also analyzed by ELISA (C). (D) $CD4^+$ naive T cells were stimulated with plate-coated anti-CD3 plus either plate-coated anti-CD18 or 10 ng/ml soluble IL-12 on days 1 and 8 and cultured for 14 d in the presence or absence of 40 μg/ml anti–IL-12.

ducing cells such as APC did not exist in the culture and, moreover, the neutralizing antibody against IL-12 did not affect Th1 development induced by stimulation with anti-CD3 and anti-CD18 (Fig. 2 D). Thus, Th1 development by engagement of CD3 and LFA-1 was triggered by an IL-12–independent, novel signaling pathway.

CD226 Is Involved in the LFA-1 Signal for Th1 Development from $CD4^+$ Naive T Cells. We have previously reported that cross-linking CD3 and LFA-1 with anti-CD3 and anti-CD18 mAbs activated the Fyn protein tyrosine kinase, resulting in the tyrosine phosphorylation of CD226 at the residue 322 in its cytoplasmic region in Jurkat T cells (12). In this study, we examined whether this is also the case in CB naive $CD4^+$ T cells as well. As demonstrated in Fig. 3 A, coligation of both CD3 and CD18 with mAbs induced CD226 tyrosine phosphorylation in naive $CD4^+$ T cells. To determine whether the tyrosine residue 322 of CD226 is phosphorylated, we infected CB naive $CD4^+$ T cells with the lentiviral vectors containing Flag-tagged WT, mutated (Y-F^{322}) *CD226*, or a mock control vector under the control of the CMV promoter linked to the IRES-*hrGFP*. More than 80% of naive $CD4^+$ T cells were efficiently transduced as determined by the hrGFP expression 3 d after infection. These transduced cells were then stimulated with anti-CD3 and anti-CD18 mAbs for 2 min and examined for tyrosine phosphorylation of WT and mutant (Y-F^{322}) CD226. As demonstrated in Fig. 3 B, WT, but not mutant (Y-F^{322}), CD226 resulted in the phosphorylation, suggesting that LFA-1–mediated signals phosphorylate the tyrosine at residue 322 of CD226 in naive $CD4^+$ T cells.

Next, We purified $hrGFP^+$ naive $CD4^+$ T cells by flow cytometry and stimulated them with anti-CD3 and anti-CD18 mAbs for 7 d. The phosphotyrosine signal of CD226 induced by stimulation with anti-CD3 and anti-CD18 mAbs in T cells transduced with mutant (Y-F^{322}) CD226 was scarcely detected or significantly less than that in T cells transduced with WT CD226 (Fig. 3 C). These results suggest the following possible results induced by the lentivirus-mediated transfer of mutant (Y-F^{322}) *CD226*: (a) inhibition of tyrosine phosphorylation of endogenous CD226 induced by LFA-1–mediated signals, (b) suppression of endogenous CD226 protein synthesis itself in a dominant-negative fashion, or (c) nothing affected on the tyrosine phosphorylation of endogenous CD226. Nonetheless, IFN-γ production was significantly decreased in T cells transduced with mutant (Y-F^{322}) CD226 in response to stimulation with anti-CD3 and anti-CD18 mAbs compared with those in T cells infected with mock control vector or the vector containing WT *CD226* gene (Fig. 3, E and F). Moreover, we observed the similar amount of WT and mutant (Y-F^{322}) CD226 expressions on the T cells introduced by the lentiviral vectors, as determined by Flag protein expression (Fig. 3, D and E). Taken together, these results suggest that CD226 is involved in LFA-1–mediated costimulatory signal toward Th1 polarization from $CD4^+$ naive T cells.

CD226 Is Involved in LFA-1 Signal for T Cell Proliferation in the Absence of Exogenous IL-2. To examine whether CD226 is involved in the LFA-1 costimulatory signal for proliferation of naive T cells, CB naive $CD4^+$ and $CD8^+$ T cells were transduced with the lentiviral vector containing Flag-tagged WT, mutant (Y-F^{322}) *CD226*-IRES-*hrGFP*, or the mock control vector. After infection, >90% of the naive T cells infected with these lentiviruses expressed Flag

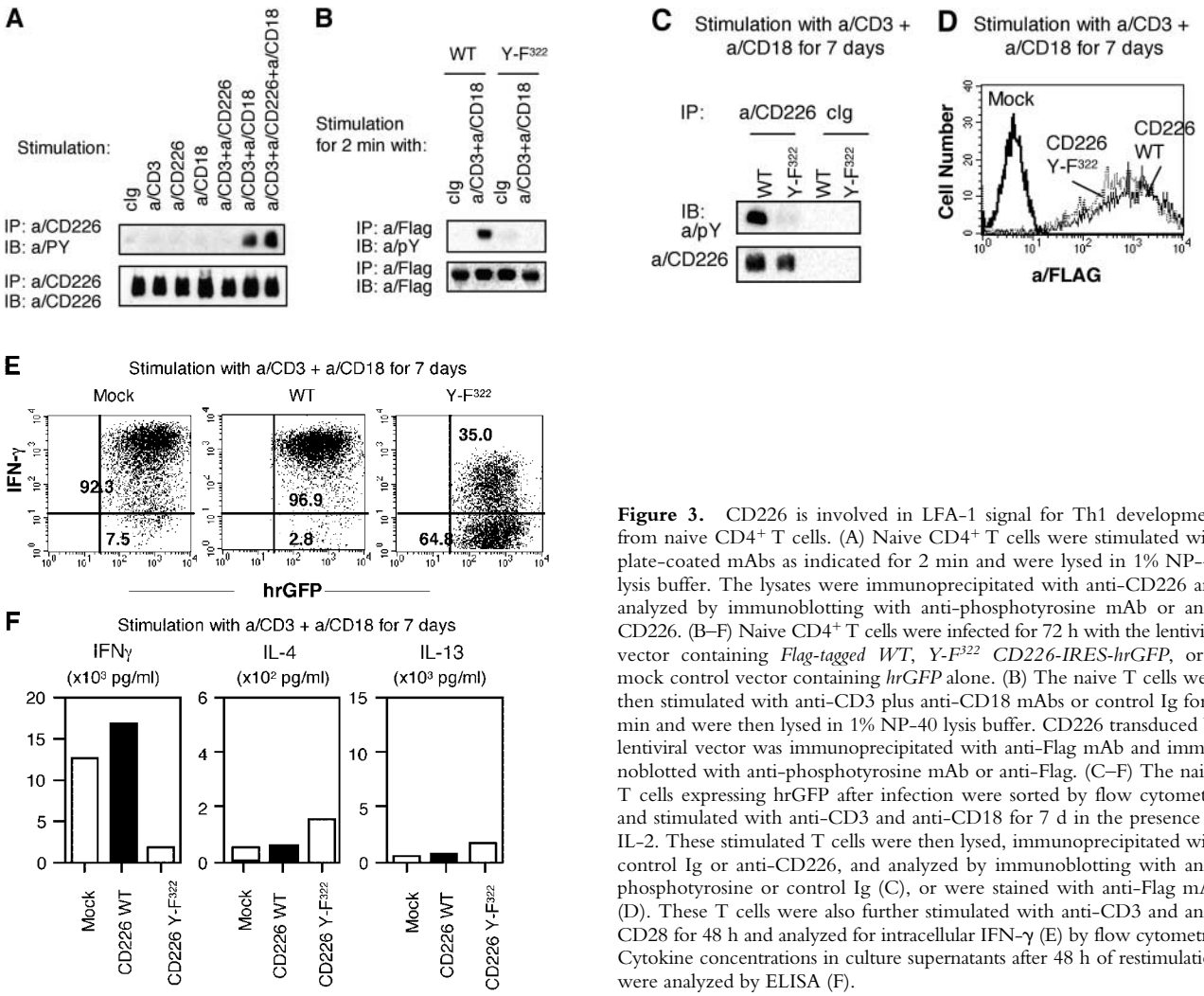

Figure 3. CD226 is involved in LFA-1 signal for Th1 development from naive CD4+ T cells. (A) Naive CD4+ T cells were stimulated with plate-coated mAbs as indicated for 2 min and were lysed in 1% NP-40 lysis buffer. The lysates were immunoprecipitated with anti-CD226 and analyzed by immunoblotting with anti-phosphotyrosine mAb or anti-CD226. (B–F) Naive CD4+ T cells were infected for 72 h with the lentiviral vector containing *Flag-tagged WT*, *Y-F322 CD226-IRES-hrGFP*, or a mock control vector containing *hrGFP* alone. (B) The naive T cells were then stimulated with anti-CD3 plus anti-CD18 mAbs or control Ig for 2 min and were then lysed in 1% NP-40 lysis buffer. CD226 transduced by lentiviral vector was immunoprecipitated with anti-Flag mAb and immunoblotted with anti-phosphotyrosine mAb or anti-Flag. (C–F) The naive T cells expressing hrGFP after infection were sorted by flow cytometry and stimulated with anti-CD3 and anti-CD18 for 7 d in the presence of IL-2. These stimulated T cells were then lysed, immunoprecipitated with control Ig or anti-CD226, and analyzed by immunoblotting with anti-phosphotyrosine or control Ig (C), or were stained with anti-Flag mAb (D). These T cells were also further stimulated with anti-CD3 and anti-CD28 for 48 h and analyzed for intracellular IFN-γ (E) by flow cytometry. Cytokine concentrations in culture supernatants after 48 h of restimulation were analyzed by ELISA (F).

on the cell surface and/or GFP, demonstrating the similar transduction efficiencies among these lentiviruses (Fig. 4 A). The naive T cells were stimulated with anti-CD3 mAb alone or in combination with either mAb against CD11a, CD18, CD226, or CD28 in the presence or absence of IL-2. As shown in Fig. 4 B, introduction of WT *CD226* did not affect the proliferation of both CD4+ and CD8+ naive T cells as compared with mock control vector. In contrast, in the absence, but not the presence, of IL-2, the proliferation of both naive T cells transduced with mutant (Y-F322) *CD226* was significantly suppressed as compared with those transduced with WT *CD226* after stimulation of CD11a or CD18 in combination with CD3 (Fig. 4 B). These results indicate that CD226 is responsible for naive CD4+ and CD8+ T cell proliferations initiated by CD3 and LFA-1 ligations in the absence of exogenous IL-2.

These results also suggested the hypothesis that introduction of mutant (Y-F322) CD226 affects the production of IL-2 that stimulates T cells themselves in an autocrine fashion. To examine this possibility, we measured IL-2 production from CD4+ and CD8+ naive T cells infected with mock control vector, or the vectors containing WT *CD226* or mutant (Y-F322) *CD226*, after stimulation with anti-CD3 plus anti-CD18. As demonstrated in Fig. 4 C, IL-2 production from CD4+ naive T cells infected with the lentivirus-containing mutant (Y-F322) *CD226* was significantly suppressed upon stimulation with anti-CD3 plus anti-CD18, but not with anti-CD3 plus anti-CD28. These results support the possibility that mutant (Y-F322) CD226 suppressed IL-2 production initiated from LFA-1 costimulatory signal, resulting in suppression of the IL-2–dependent T cell proliferation. However, we observed undetectable levels of IL-2 (<5 pg/ml) from naive CD8+ T cells infected with any lentivirus after stimulation with anti-CD3 plus anti-CD18 or anti-CD3 plus anti-CD28 (unpublished data), suggesting that at least in the case of naive CD8+ T cells, suppression of T cell proliferation infected with the lentiviral vector-containing mutant (Y-F322) *CD226* was not due to deficiency in IL-2 production.

Physical Association of LFA-1 with CD226 Is Independent on the Lipid Raft in CD8+ T Cells. LFA-1 physically associates with CD226 upon cross-linking of CD3 on PB T cells (12). Because CD3 stimulation on T cells polarizes the lipid raft compartment at the site of immunological syn-

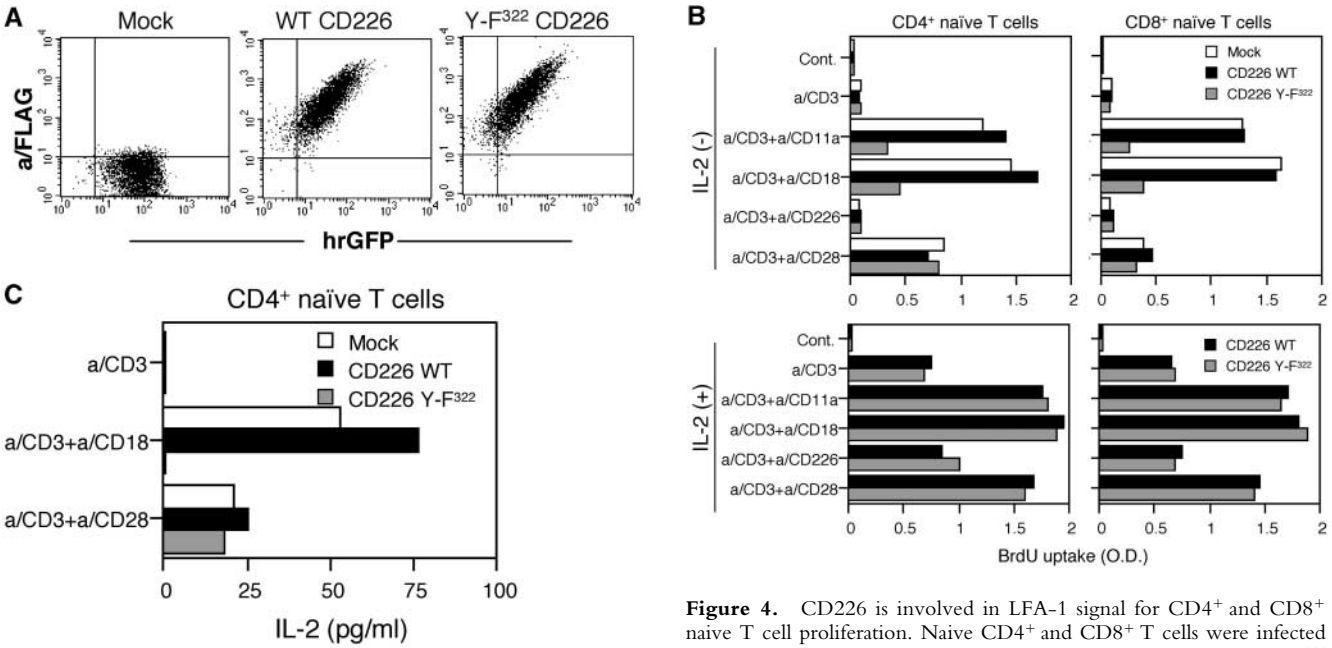

Figure 4. CD226 is involved in LFA-1 signal for $CD4^+$ and $CD8^+$ naive T cell proliferation. Naive $CD4^+$ and $CD8^+$ T cells were infected for 72 h with the lentiviral vector containing *Flag-tagged WT*, *Y-F322 CD226-IRES-hrGFP*, or a mock control vector containing *hrGFP* alone. The naive T cells were stained with anti-Flag mAb and analyzed by flow cytometry (A). The naive $CD4^+$ and $CD8^+$ T cells after infection were also stimulated with plate-coated mAbs indicated for 48 h in the presence (B) or absence (B and C) of IL-2. Cell proliferations (B) and IL-2 production in culture supernatants (C) were analyzed by BrdU uptake and ELISA, respectively. IL-2 was not detected in culture of $CD8^+$ naive T cells after stimulation with any mAbs (not depicted).

apse, in which LFA-1 and the Fyn protein tyrosine kinase are recruited (34, 35), we examined whether CD226 is also recruited in the lipid raft compartment upon stimulation with anti-CD3 and anti-CD28 mAbs. $CD4^+$ and $CD8^+$ PB T cells were cocultured with beads precoated with anti-CD3 and anti-CD28 mAbs. As demonstrated in Fig. 5 A (top), CD226 as well as LFA-1 and the Fyn on PB $CD4^+$ T cells were aggregated in the polarized lipid raft at the contact site with beads. On the contrary, however, these beads did not polarize the lipid raft in $CD8^+$ T cells although all LFA-1, CD226, and the Fyn were aggregated in the contact site. Moreover, although LFA-1 was coimmunoprecipitated with CD226 in PB $CD8^+$ T cells as well as in $CD4^+$ T cells upon stimulation with anti-CD3 mAb (Fig. 5 B, top), lipid raft disruption with MβCD did not inhibit the coimmunoprecipitation of LFA-1 and CD226 in $CD8^+$, but not $CD4^+$, T cells (Fig. 5 B, bottom). Thus, physical association of LFA-1 with CD226 induced by CD3 stimulation is independent on polarization of the lipid raft in $CD8^+$ T cells.

LFA-1 Signal for T Cell Proliferation Is Independent on the Lipid Raft in $CD8^+$ T Cells. To investigate the involvement of the lipid raft in the LFA-1 signaling, $CD4^+$ and $CD8^+$ T cells were stimulated with anti–LFA-1 (CD11a or CD18) and anti-CD3–coated beads. As demonstrated in Fig. 5 A (middle and bottom), the stimulation also induced polarization of LFA-1, CD226, and the Fyn at the contact site of both T cell subsets with beads. However, as was observed when stimulated with anti-CD3 and anti-CD28 mAbs, the lipid raft compartment was polarized in $CD4^+$, but not $CD8^+$, T cells stimulated with anti-CD3 and anti–LFA-1 (Fig. 5 A). Furthermore, lipid raft disruption with MβCD suppressed the proliferation of $CD4^+$, but not $CD8^+$, T cells initiated by CD3 and LFA-1 ligations (Fig. 5, C and D). Taken together, these results indicate that although LFA-1 costimulatory signals require CD226 for proliferation of both $CD4^+$ and $CD8^+$ T cells in the absence of IL-2 (Fig. 4 B), $CD8^+$ T cells do not depend on polarization of the lipid raft for their proliferation induced by CD3 and LFA-1 ligations.

Discussion

In this study, we have demonstrated that costimulatory signals initiated by LFA-1 ligation induced IL-12–independent Th1 development from naive $CD4^+$ T cells. This is consistent with previous reports that blocking LFA-1–ICAM-1 or LFA-1–ICAM-2 interactions led to a significant increase of Th2 cytokines in mice (8, 36). Th1 polarization from naive $CD4^+$ T cells induced by LFA-1/ICAM-1 ligation has also been reported more recently (9). Several lines of evidence demonstrated that engagement of LFA-1 facilitates T cell activation by lowering the amounts of antigen necessary for T cell activation (37). In addition, exposure of high antigen doses can lead naive $CD4^+$ T cells to give rise to Th1 polarization (38, 39). These observations suggest that LFA-1 ligation may modulate the TCR signal leading Th1 development. We demonstrated here that CD226 physically associates with LFA-1 upon stimulation of CD3 and is involved in Th1 development induced

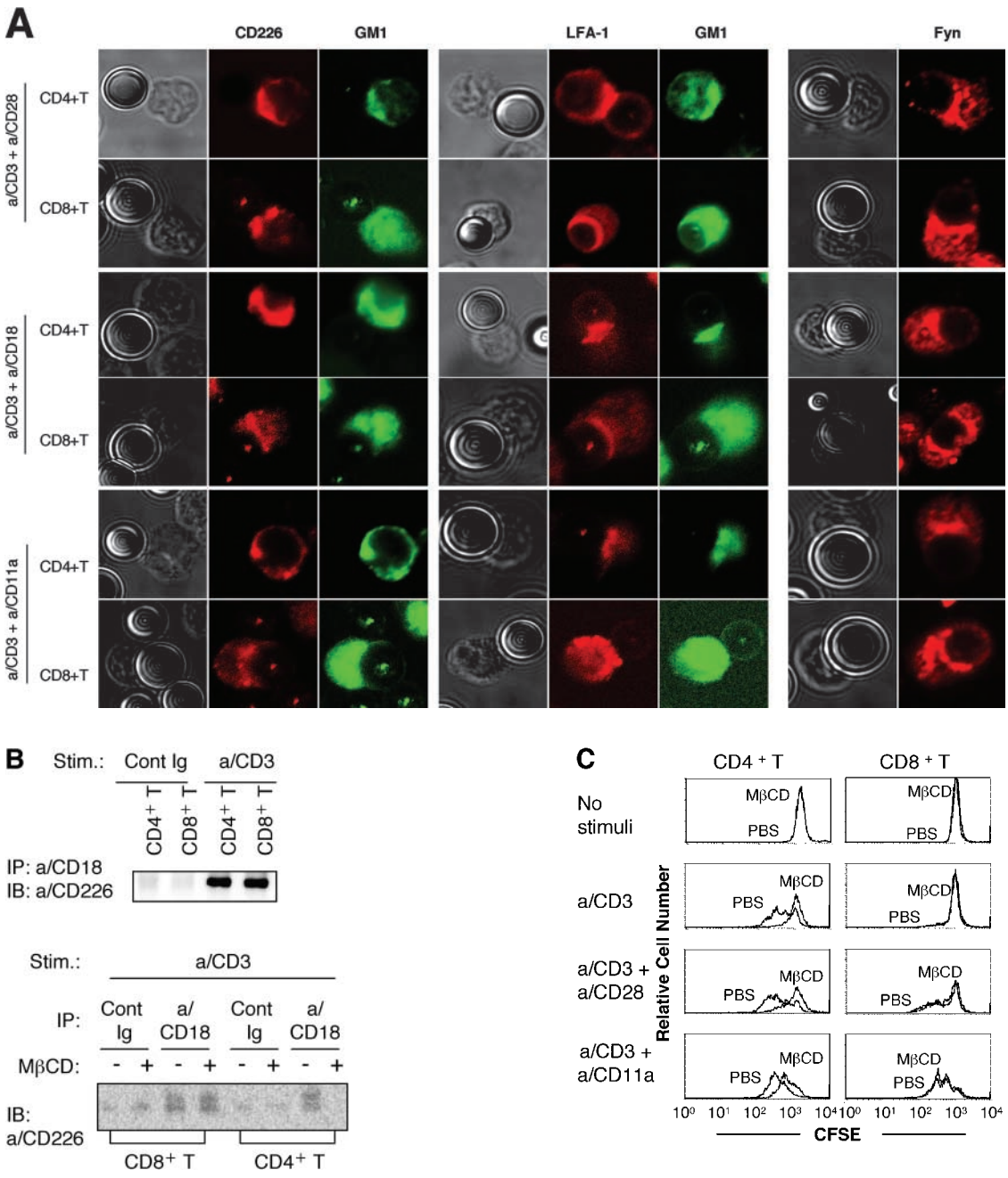

Figure 5. Relationship of LFA-1 signaling molecules with the lipid raft. (A) $CD4^+$ and $CD8^+$ T cells were incubated with beads precoated with anti-CD3 plus either anti-CD28, anti-CD11a, or anti-CD18 for 30 min at 37°C and then stained with FITC-conjugated cholela toxin subunit B (GM1), Alexa 594–conjugated anti-CD11a (LFA-1), and anti-CD226 mAbs. T cells were also stained with rabbit anti–Fyn polyclonal antibody, followed with Alexa 594–coupled anti–rabbit secondary antibodies and analyzed by confocal laser scanning microscopy. (B) $CD4^+$ and $CD8^+$ PB T cells were treated or not with MβCD at 10 mM for 30 min at 37°C. Cells were then stimulated with control Ig or anti-CD3 for 5 min and lysed in 1% digitonin buffer. The lysates were immunoprecipitated with anti-CD18 or control Ig and analyzed by immunoblotting with anti-CD226. (C and D) $CD4^+$ and $CD8^+$ PB T cells were labeled (C) or not (D) with CFSE and treated or not with MβCD at 10 mM for 30 min at 37°C. Cells were then stimulated or not with plastic-coated mAbs as indicated for 3 d and analyzed for proliferation by flow cytometry (C) or BrdU uptake (D).

by LFA-1 engagement. It is unclear at present, however, whether CD226 modulates the TCR signal or delivers the TCR-independent signal leading to Th1 development. It is of note that LFA-1 ligation induces aggregation and activation of focal adhesion kinase at sites of LFA-1–ICAM interaction and activated focal adhesion kinase then binds to the SH2 domain of the Srk family protein tyrosine kinase Fyn (40). Because the Fyn is responsible for CD226 tyrosine phosphorylation induced by LFA-1 signal (12), this kinase may also play an important role for Th1 polarization from $CD4^+$ naive T cells. This idea might be supported by the previous findings that Th2 clones express a low level of Fyn protein in mice (41) and $CD4^+$ naive T cells from the *fyn*-deficient mice polarize toward the Th2 cells even in the absence of IL-4 and IL-13 (42).

In this study, we have also shown that introduction of mutant ($Y\text{-}F^{322}$) *CD226* into naive $CD4^+$ and $CD8^+$ T cells suppressed their growth initiated by CD3 and LFA-1 ligations in the absence, but not presence, of IL-2. In contrast, their growth was not affected upon stimulation of CD3 and CD28 by the mutant ($Y\text{-}F^{322}$) *CD226* introduction. These results suggested that CD226 functions as a signal transducer of LFA-1 upon triggering T cell activation, in which T cells secrete no or a very low amount of IL-2. Once T cells are activated and produce IL-2 that stimulates themselves in an autocrine fashion, CD226 may not be required for LFA-1 signal for T cell proliferation. Geginat et al. (43) have recently described that both LFA-1– and CD28-mediated costimulations induce IL-2 mRNA stabilization and IL-2 production, resulting in T cell proliferation. However, LFA-1–, but not CD28-, induced IL-2 mRNA stabilization requires the integrity of the actin-based cytoskeleton. In addition, LFA-1 engagement is followed by an increase of the nuclear pool of a transcriptional coactivator Jun activation domain-binding protein 1 that modulates AP-1 activity (44). Future studies should be required for determining the physical and functional relationship between CD226 and these LFA-1 signaling molecules.

Recent evidence supports a model in which lipid rafts play an essential role in immune cell activation (45). Although several studies have shown the involvement of lipid rafts in the regulation of LFA-1 function (46, 47), we demonstrated here that lipid rafts are not involved in $CD8^+$ T cell proliferation induced by costimulatory signals from LFA-1, although LFA-1, CD226, and the Fyn were clustered and seemed to deliver intact costimulatory signals for T cell proliferation. The mechanisms by which these LFA-1 signaling molecules aggregated in $CD8^+$ T cells at the contact area with beads are uncertain at present. However, this is consistent with a recent report that $CD8^+$ T cells do not require the polarization of lipid rafts for activation and proliferation induced by CD3 and CD28 ligations by precoated antibody beads or a specific antigen on APCs (48). In contrast, lipid raft disruption with MβCD suppressed physical association of LFA-1 with CD226 and LFA-1–mediated costimulatory signal for proliferation in $CD4^+$ T cells. It should be noted that these results do not always lead to the conclusion that physical association of LFA-1 with CD226 and LFA-1 costimulatory signaling directly depends on lipid rafts in $CD4^+$ T cells because MβCD also disrupts the CD3-mediated signal (45) that is essentially required for the association of LFA-1 with CD226 (12) and LFA-1 costimulatory signal. Further studies should be required to clarify lipid raft structure and function in both $CD4^+$ and $CD8^+$ T cells. Nonetheless, coclustering of LFA-1, CD226, and the Fyn upon CD3 stimulation might be important for LFA-1 costimulatory function in both T cell subsets.

It has been reported that resting primary T cells appear highly resistant to transduction with lentiviral vectors and efficient transduction requires prestimulation of T cells (23, 24, 49–51). In contrast, we achieved efficient transduction of both resting and activated T cells with lentiviral vectors in this study. The lentiviral vector we used contains cPPT and the CTS, which promote the nuclear import of the viral DNA and improve the transduction efficiency (52, 53), and the WPRE, which enhances the expression of the transgene (54). The cPPT, CTS, and WPRE integrated in our lentivirus vector should be advantageous for efficient gene transduction and expression in resting primary T cells, although there are reports describing controversial results (49, 50, 55). We also consider our modified experimental procedures for lentivirus preparation (i.e., [a] high titer of lentivirus by a concentration $>$1,000-fold, [b] followed by extensive resuspension of lentivirus by pipetting $>$200 times, and [c] final volume of medium containing lentivirus for infection $<$100 μl/well) to be important to improve gene transduction efficiency because we observed much lower transduction efficiency before we developed these procedures. It is of note that our lentiviral vectors can also effectively transduce resting primary B cells, NK cells, and monocytes. Thus, lentiviral vector-mediated gene transfer should be a powerful tool for studies of gene functions in a wide range of immune responses.

We thank Yoshihiro Shiina for providing CBs, Lewis Lanier for critical reading of this manuscript, Satoshi Yamazaki, Kensuke Yamazaki, and Yohei Morita for technical assistance, and Yurika Soeda for secretarial assistance.

This research was supported in part by grants provided by the Ministry of Education, Science and Culture of Japan (to K. Shibuya and A. Shibuya), Special Coordination Funds of the Science and Technology Agency of the Japanese Government (to A. Shibuya), the Uehara Memorial Foundation (to K. Shibuya and A. Shibuya), and Yamashita Taro Memorial Foundation (to K. Shibuya).

Submitted: 12 June 2003
Accepted: 16 October 2003

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