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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.

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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 $Y\text{-}F^{322}$ CD226 in naive T cells, *Flag*-tagged WT or $Y\text{-}F^{322}$ (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 $Y\text{-}F^{322}$ *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

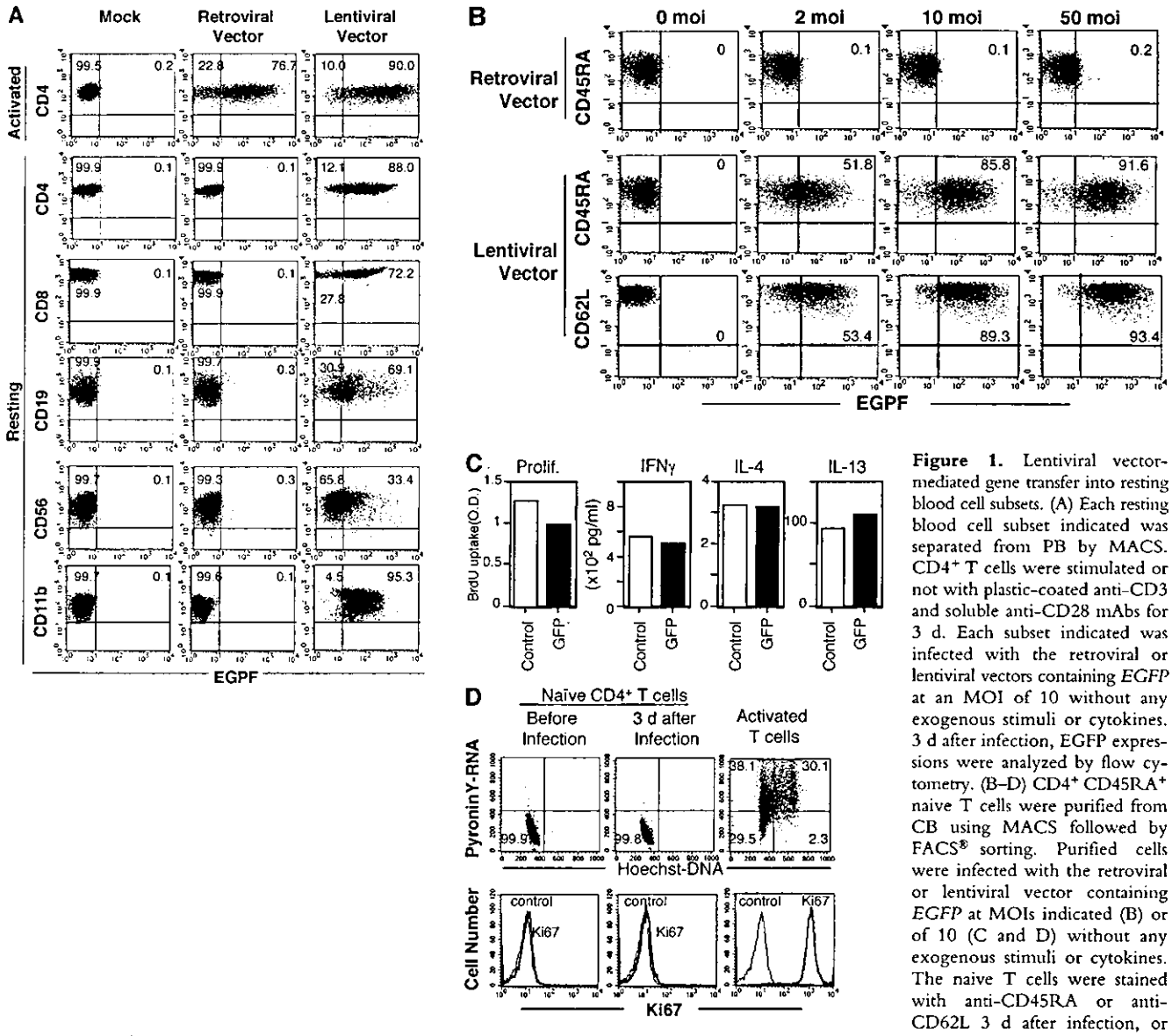

Figure 1. Lentiviral vector-mediated gene transfer into resting blood cell subsets. (A) Each resting blood cell subset indicated was separated from PB by MACS. $CD4^+$ T cells were stimulated or not with plastic-coated anti-CD3 and soluble anti-CD28 mAbs for 3 d. Each subset indicated was infected with 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-

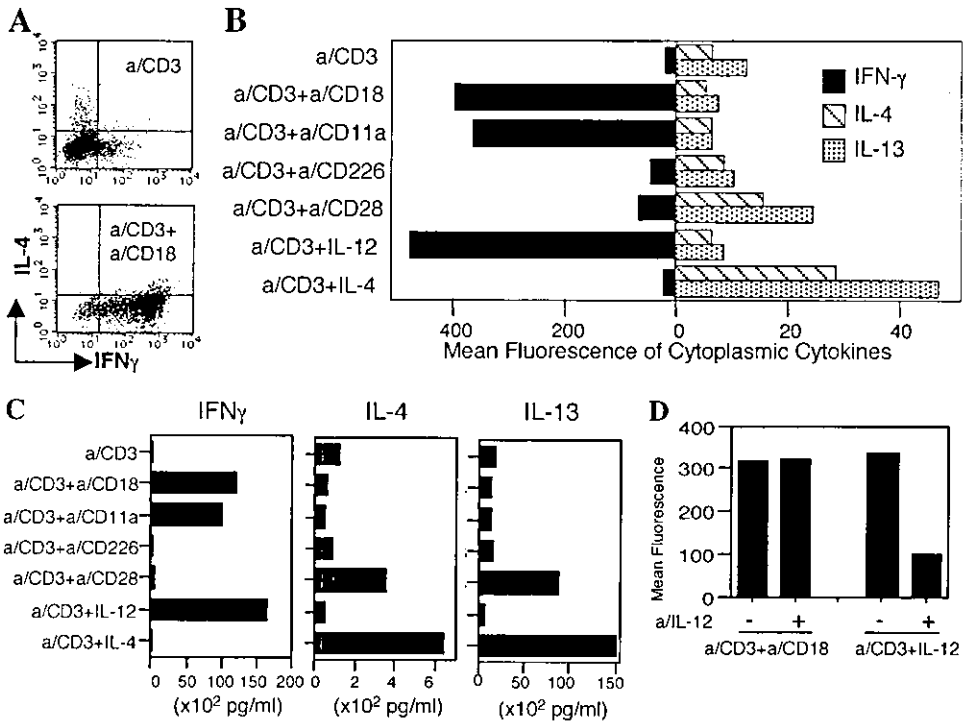

Figure 2. Stimulation of CD3 and LFA-1 induces Th1 development from naive CD4+ Ths. (A–C) CD4 naive T cells were stimulated with plate-coated anti-CD3 alone or anti-CD3 plus mAbs indicated on days 1 and 8 and cultured for 14 d in IL-2–containing medium. Intracellular IFN-γ, IL-4, and IL-13 syntheses 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

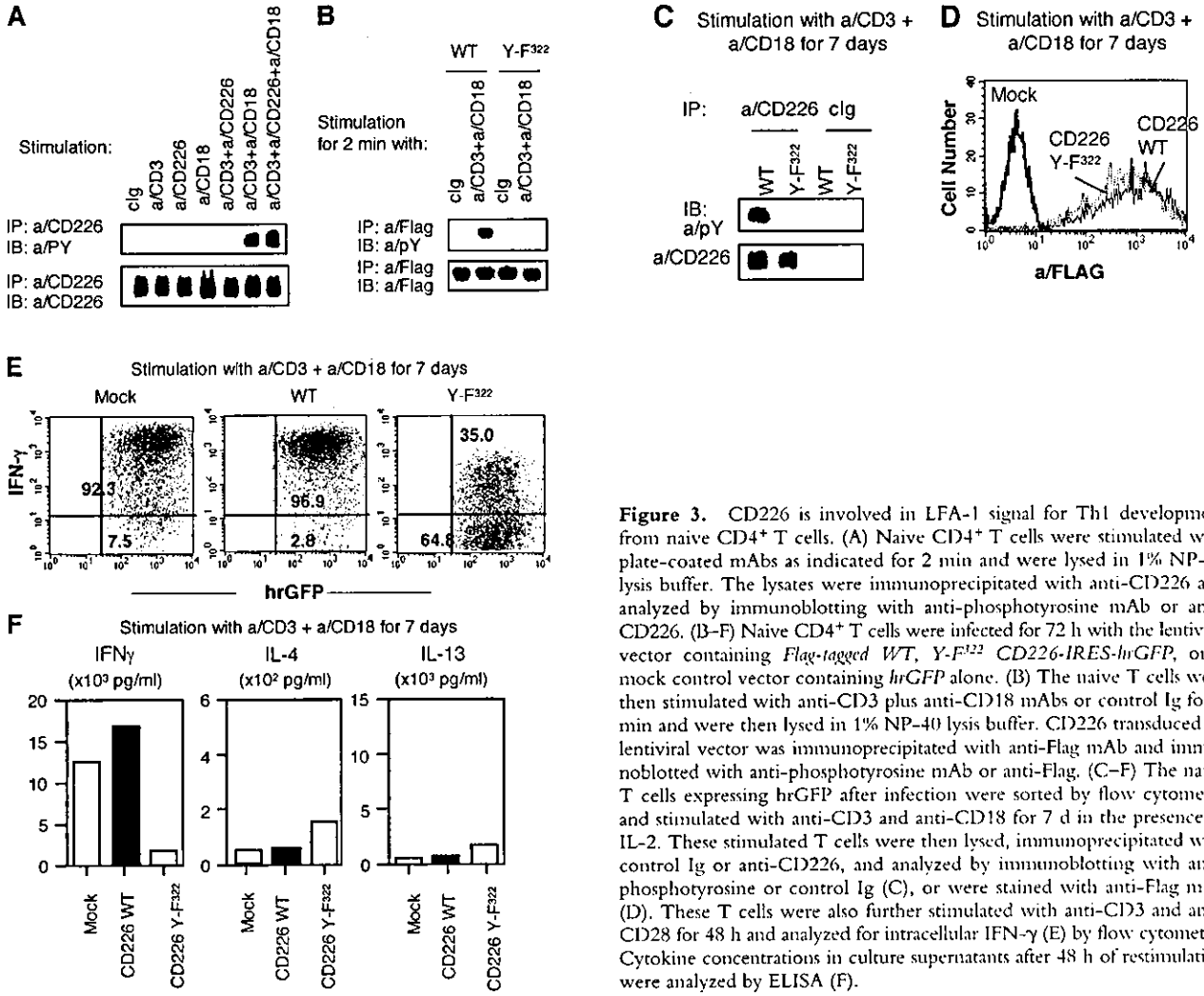

Figure 3. CD226 is involved in LFA-1 signal for Th1 development from naive CD4+ T cells. (A) Naive CD4+ T cells were stimulated with plate-coated mAbs as indicated for 2 min and were lysed in 1% NP-40 lysis buffer. The lysates were immunoprecipitated with anti-CD226 and analyzed by immunoblotting with 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-

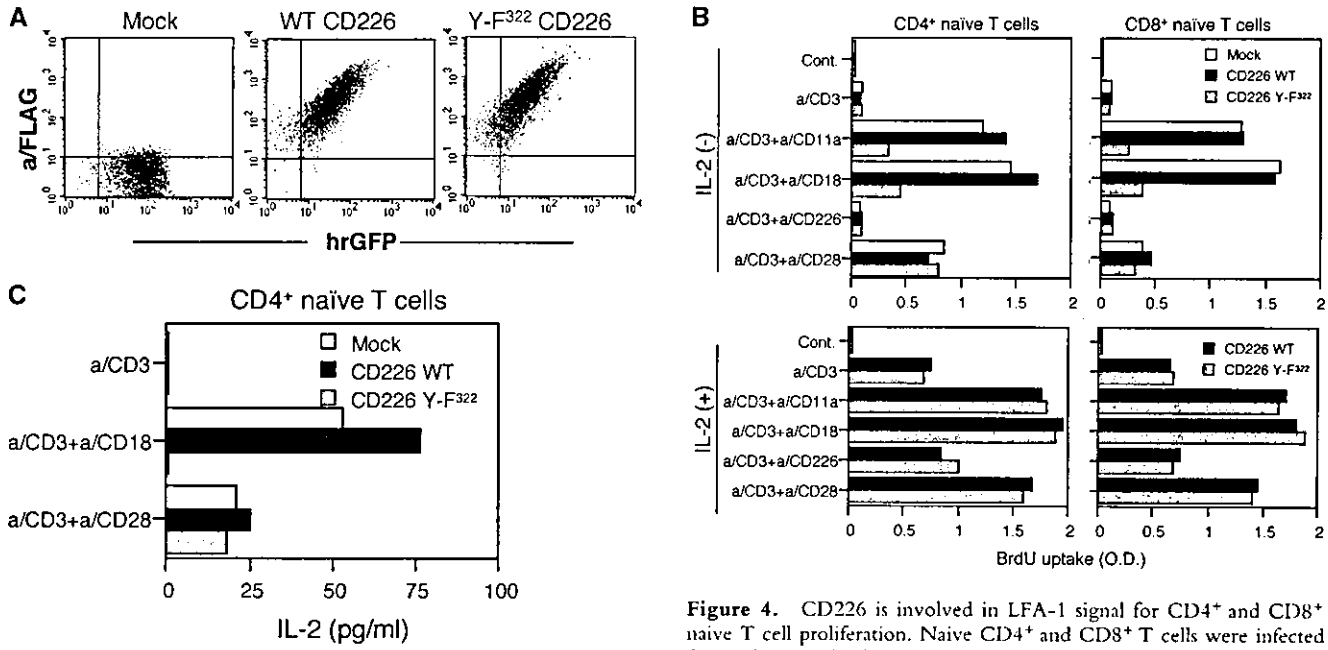

Figure 4. CD226 is involved in LFA-1 signal for CD4+ and CD8+ naive T cell proliferation. Naive CD4+ and CD8+ T cells were infected for 72 h with the lentiviral vector containing *Flag-tagged WT, Y-F322 CD226-IRES-hrGFP*, or a mock control vector containing *hrGFP* alone. The naive T cells were stained 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

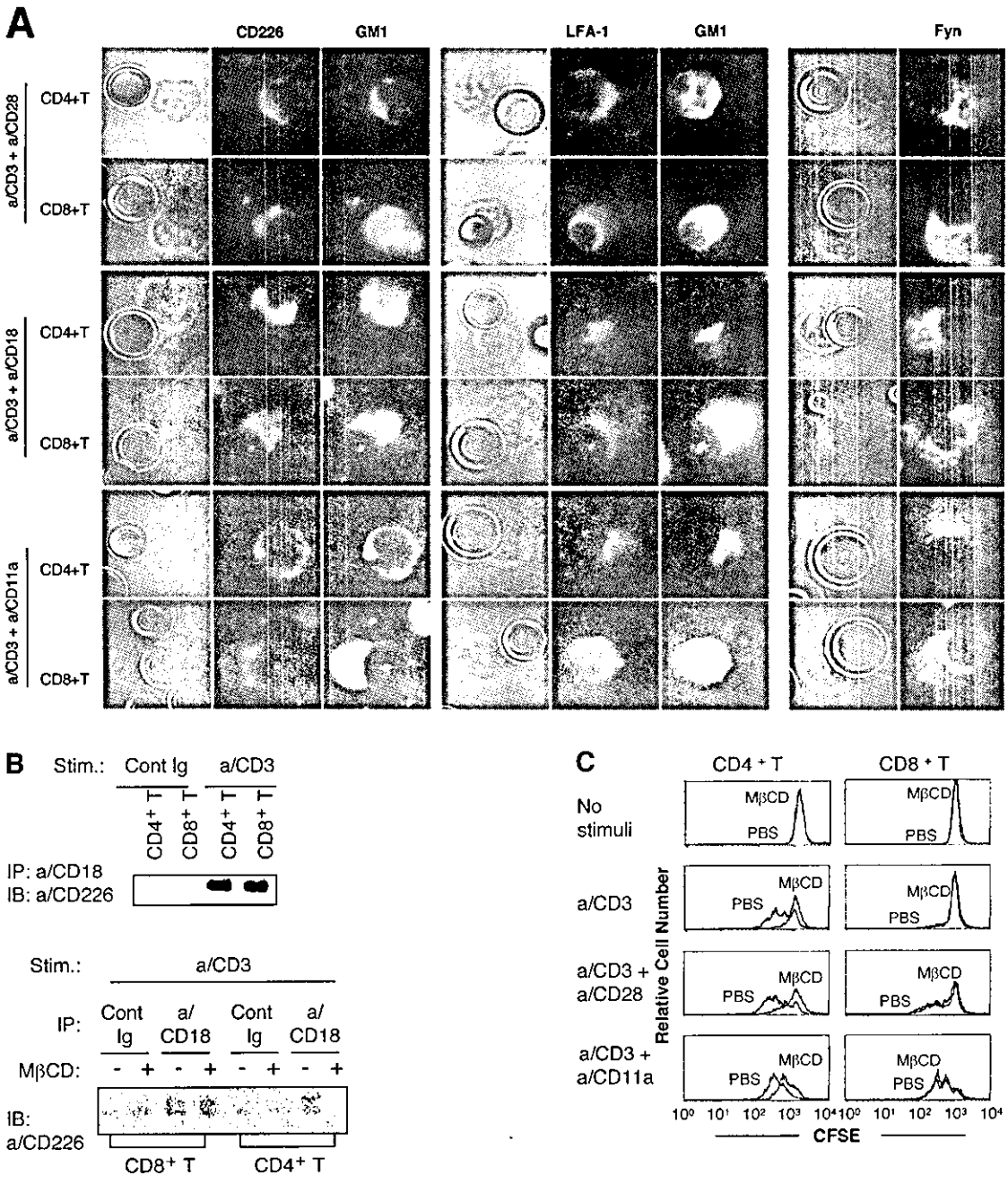

Figure 5. Relationship of LFA-1 signaling molecules with the lipid raft. (A) $CD4^+$ and $CD8^+$ T cells were incubated with beads precoated with anti-CD3 plus either anti-CD28, anti-CD11a, or anti-CD18 for 30 min at 37°C and then stained with FITC-conjugated cholela toxin subunit B (GM1), Alexa 594–conjugated 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.

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Low Expression Levels of Soluble CD1d Gene in Patients with Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To examine whether the expression of intact CD1d, a critical molecule for the presentation of glycolipid antigens to natural killer T (NKT) cells, and its variants differs between patients with autoimmune diseases including rheumatoid arthritis (RA) and healthy subjects. Recently, we identified 8 different CD1d variants, generated by alternative splicing. V1 lacking exon 4 (CD1d without $ß_2$ microglobulin, $ß_2$m) and V2 lacking exons 4 and 5 (soluble CD1d) may be functional molecules, because the antigen binding sites (exons 2 and 3) are intact.

Methods. Peripheral blood mononuclear cells (PBMC) from 44 patients with autoimmune disease (RA 19, systemic lupus erythematosus, SLE 10, Sjögren's syndrome, SS 15) and 15 healthy controls were separated and complementary (c)DNA was prepared. The expression of intact CD1d on PBMC was detected by flow cytometry. Alternatively spliced CD1d variants were quantified by TaqMan PCR using polymerase chain reaction with confronting 2-pair primers (PCR-CTPP) based amplification.

Results. The mean (± SEM) transmembrane and $ß_2$m binding site deleted CD1d mRNA level in 19 patients with RA (2.0 ± 0.33) was significantly lower than in 15 controls (6.9 ± 2.08; $p < 0.05$), whereas there were no differences in $ß_2$m deleted variants and intact CD1d mRNA.

Conclusion. Our findings suggest that low expression of soluble CD1d variants might play a role in the formation of symptoms or pathogenesis of RA. (J Rheumatol 2003;30:2524–8)

Key Indexing Terms:

ALTERNATIVE SPLICING VARIANT
AUTOIMMUNE DISEASE CD1D NATURAL KILLER T CELLS

Natural killer T (NKT) cells are a unique subset of T lymphocytes that express invariant T cell receptor (TCR) and NKR-P1A. A key feature of NKT cells is the expression of a heavily biased TCR, bearing AV24AJ18 and BV11 chains[1,2]. NKT cells recognize glycolipid antigen presented on CD1d molecules[3-5]. CD1d is a nonpolymorphic major histocompatibility complex (MHC) class I-like molecule[6]. We reported the expression of 8 alternatively spliced CD1d mRNA in peripheral blood mononuclear cells (PBMC)[7]. Two of these CD1d variants (V1 and V2) were considered functional because the antigen binding site was completely conserved. V1 lacks exon 4 [$ß_2$-microglobulin ($ß_2$m) binding site] of the gene, resulting in unstable antigen presentation. The other variant (V2) lacks both exons 4 and 5 (transmembrane domain), resulting in the soluble form of CD1d (sCD1d).

The number of NKT cells is selectively reduced in various human autoimmune diseases, such as systemic sclerosis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SS), and insulin-dependent diabetes mellitus (IDDM)[8,9]. Several mechanisms responsible for the low number of NKT cells in these patients have already been identified. These include inadequate presentation of the antigen, dysfunction of NKT cells, and abnormality of antigen presentation[9]. Our study was designed to examine whether there is a difference of intact CD1d, V1, and V2 CD1d variants between patients with autoimmune diseases, including RA, and healthy subjects, and thus elucidate the cause of abnormal antigen presentation and decreased NKT cells in patients. We analyzed the relative amounts of intact CD1d and isoforms, V1 and V2, using flow cytometry and real-time TaqMan polymerase chain reaction (PCR).

MATERIAL AND METHODS

Patients. The study group included 19 patients with RA diagnosed according to the American College of Rheumatology (ACR, formerly American Rheumatism Association) criteria[10], 10 patients with SLE, who fulfilled ACR diagnostic criteria[11], and 15 patients with SS diagnosed according to the criteria[12]. All patients and 15 disease-free healthy subjects were referred to the University of Tsukuba Hospital.

Preparation of cells and complementary DNA (cDNA). PBMC were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech Inc., Piscataway, NJ, USA). Total RNA was prepared from fresh PBMC with Isogen (Nippon Gene, Co., Tokyo, Japan), and reverse transcribed into cDNA using the method described by Sumida, *et al*[13]. Briefly, first-strand cDNA was synthesized in a 20 µl reaction mixture containing oligo (dT) primer from 1 µg of total RNA. A 0.1 µl aliquot of the reaction mixture encoding the cDNA was used for TaqMan PCR analysis.

Antibodies and flow cytometry. FITC-conjugated anti-human CD1d mono-

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clonal antibody (mAb) 42 was purchased from PharMingen (San Diego, CA, USA). The cells were resuspended in propidium iodide and then analyzed by FACSCalibur™ (Becton Dickinson, San Jose, CA, USA). A live gate was set by forward and side scatter and propidium iodide staining. Over 50,000 cells with high forward and side scatter were acquired from each sample, and data were analyzed using Cellquest™ software (Becton Dickinson).

TaqMan probe and primer design. One forward primer, 2 reverse primers, and a FAM-labeled probe were designed within the mRNA sequence of CD1d. The sequences of the primers and TaqMan probe used in this study were as follows: common CD1d forward: 5'-TGGGAGAT-ACTCAGCAACTCTGG-3'; CD1d E4 del.reverse (E4): 5'-GGAGGTG-TAGCTCCCACCTT-3'; CD1d E4E5 del.reverse (E45): 5'-GGACGC-CCTGATAGGAACTT-3'; and common CD1d PROBE FAM: 5'-FAM-CCTGACTCAAGGAGGCCACTGACAAATT-TAMRA-3'. The antisense primers were specific for the 5' edge of exon 5 (E4 del.) or exon 6 (E4E5 del.) of the CD1d gene, and the 3' end of these primers (3 bp) annealed with the end of exon 3.

Quantitative PCR. cDNA were obtained from PBMC. The real-time PCR consisted of one cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in an ABI Prism 7700 Sequence Detector (Applied Biosystems Japan K.K.). All experiments were carried out in triplicate. Quantitative analysis of gene expression was performed by the comparative CT (DCt) method[14]. To standardize the quantification of target genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from each sample was quantified on the same plate with the target genes by using TaqMan GAPDH control reagents kit (Applied Biosystems).

Statistical analysis. All data are expressed as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) and Scheffe's post-hoc test. P values less than 0.05 were considered statistically significant.

RESULTS

Percentages of CD1d+ cells in high forward and side scatter PBMC from patients with autoimmune diseases were not significantly different from controls (Figure 1B). Cell surface expression of CD1d molecules using fluorescence intensity showed no significant difference between controls and several autoimmune diseases (Figure 1C).

To specifically detect each variant, we used confronting 2-pair primers (PCR-CTPP) based PCR amplification, a simple method designed for detection of polymorphism. In this method, allele-specific DNA products are amplified by applying appropriately designed primers. We set the primer-pair and TaqMan probe site for the specific detection of V1 and V2 variant mRNA (Figure 2A). It was confirmed that these primer pairs amplified only a specific target by the specificity of the 3' end of each antisense primer (Figure 2B). We quantified the alternatively spliced CD1d mRNA with this system. As shown in Figures 3A and B, the results indicated that the mean level of mRNA encoding the V2 variant was significantly lower in patients with RA (2.0 ± 0.33) than controls (6.9 ± 2.08; $p < 0.05$). In contrast, there were no significant differences in V1 mRNA between patients with autoimmune diseases and controls. The results are summarized in Table 1.

DISCUSSION

We concluded from our results that the numerical change in

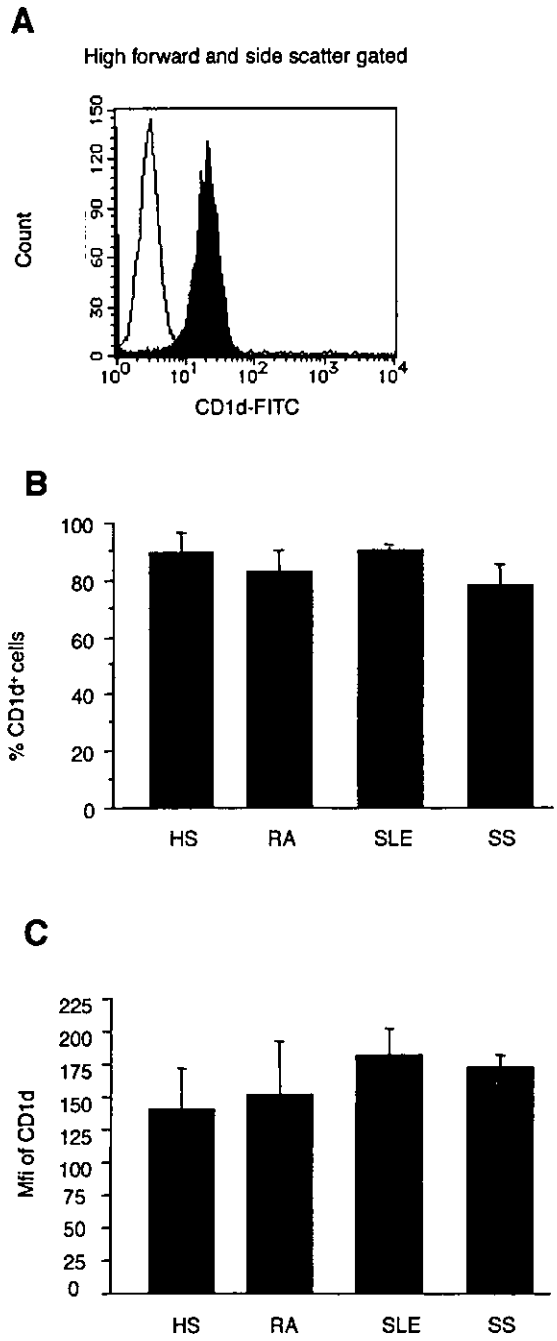
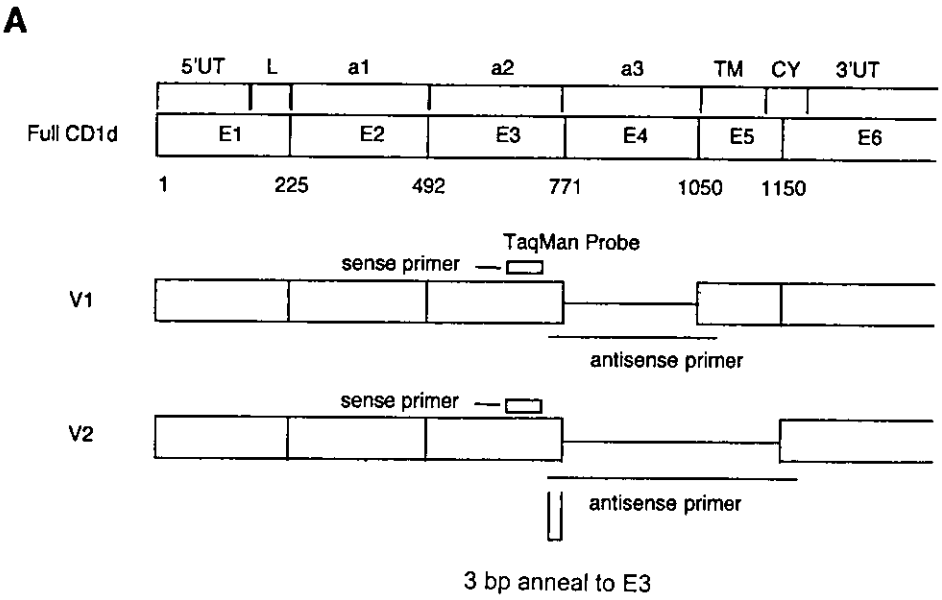


Figure 1. Relative expression of cell surface CD1d. PBMC from 15 patients with autoimmune diseases (7 RA, 6 SLE, 2 SS) and 4 healthy controls (HS) were stained with anti-CD1d mAb. (A) Typical staining pattern of CD1d in high forward and side scatter cells (black histogram: anti-CD1d mAb; white histogram: control mAb). (B) Percentage of CD1d positive cells. (C) Mean fluorescence intensity of CD1d. Data are mean ± SEM.

CD1d positive cells does not affect the NKT cell population commonly observed in RA, SLE, and SS. In comparison, Takahashi, *et al*[15] reported that the percentage of CD1a-positive cells was lower in monocyte-derived dendritic cells (Mo-DC) from patients with IDDM. CD1a molecule is a



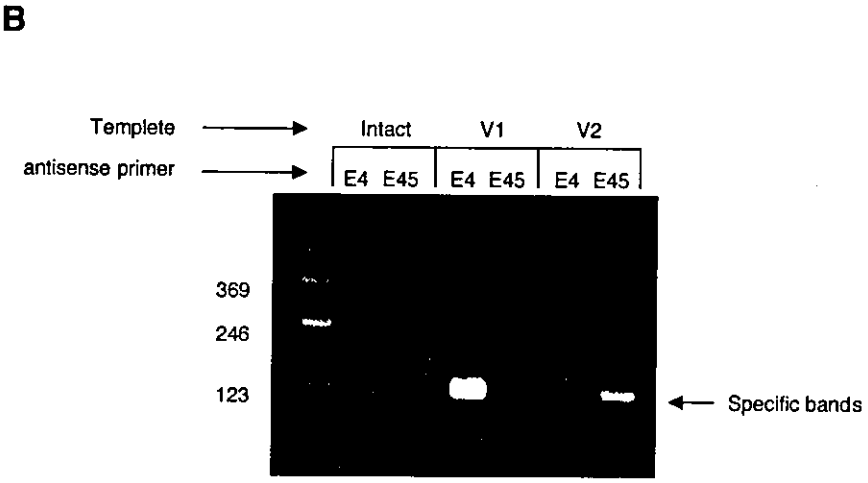


Figure 2. The human alternative splicing forms of CD1d transcript and the primer and probe sites for the TaqMan PCR. (A) The primer and probe sites. The CD1D transcript comprises 6 exons (E1–E6) encoding the leader (L), α1, α2, α3 domains, transmembrane (TM), and cytoplasmic (CY) domains. (B) Specificity of these primer sets was confirmed by PCR using previously cloned intact and variant CD1d cDNA as PCR templates.

member of the human CD1 gene family, and a marker for human immature Mo-DC[16]. Dendritic cells are antigen-presenting cells that efficiently activate NKT cells[17,18], therefore a reduction in dendritic cell population may result in reduction of NKT cells. However, we found no significant differences in percentage of CD1d-positive cells among patients with various autoimmune diseases including RA, SLE, and SS and controls.

We have reported the presence of alternatively spliced isoform mRNA on CD1d molecule[7]. Although the function of these alternatively spliced forms remains unclear, we assumed that these variants alter the antigen presentation of antigen-presenting cells to NKT cells. Therefore, we quantified the alternatively spliced CD1d mRNA by the TaqMan PCR method[19,20]. Our results indicated that mRNA encoding the V2 variant was significantly lower in patients with RA than controls; in contrast, there were no significant differences in V1 mRNA between patients with autoimmune diseases and controls.

Previous studies reported the presence of high serum levels of soluble MHC class I molecules in patients with active SLE, RA, and multiple sclerosis[21,22]. Our results

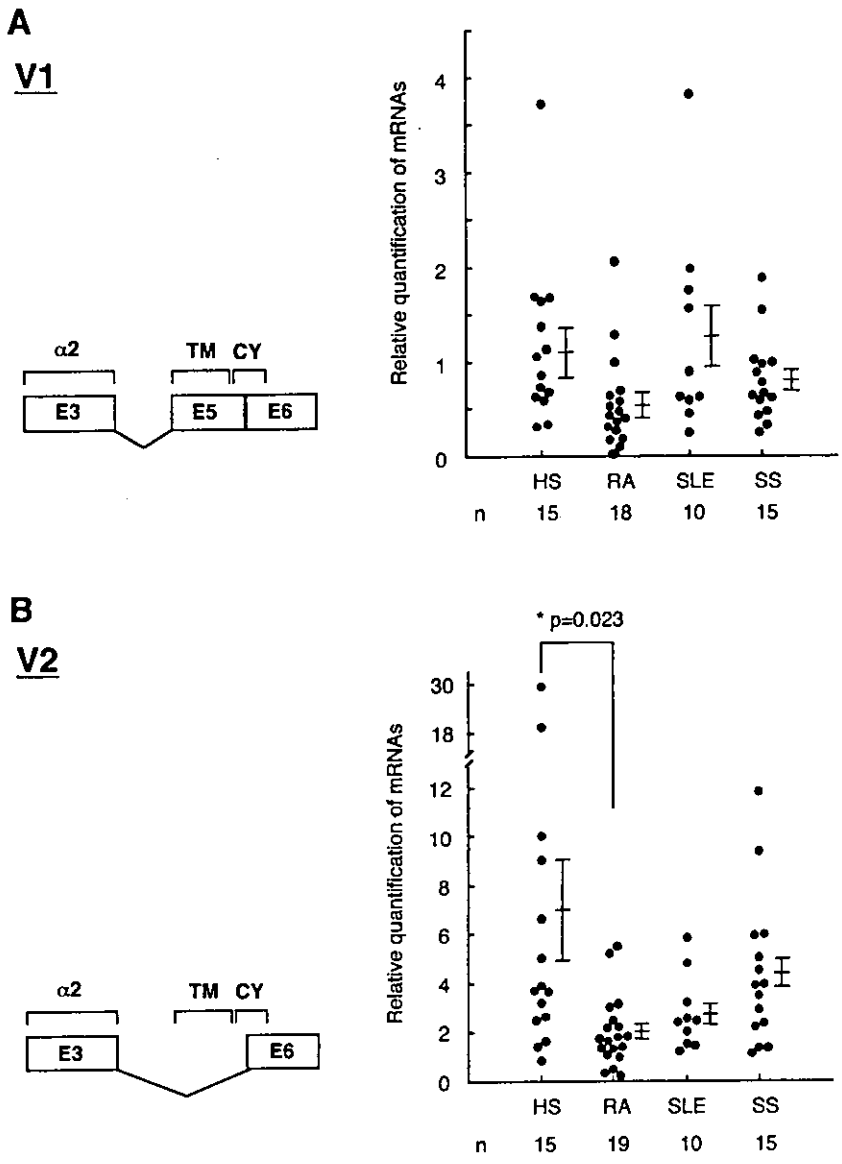


Figure 3. Reduction of sCD1d (V2) mRNA in patients with RA. The relative amounts of V1 (A) and V2 (B) were measured using the real-time TaqMan PCR method. V2 mRNA were significantly reduced in patients with RA compared with healthy controls (HS). Data represent mRNA levels of individuals, together with the mean ± SEM values of the indicated number of subjects (n). E3: exon 3; E5: exon 5; E6: exon 6; α2: α2 domain; TM: transmembrane domain; CY: cytoplasmic tail.

suggest that soluble MHC molecules may be useful as a marker of activity of rheumatic disease, and may function as immunoregulators of the autoimmune response. On the other hand, we found a reduction of sCD1d at the mRNA level in patients with RA. Here, we speculate the following 2 functions for the sCD1d molecule. First, sCD1d could directly stimulate and activate NKT cells. Several studies have reported that soluble MHC molecule binds to TCR and stimulates alloreactive T cells[23]. Hence, reduction of sCD1d might consequently lead to a reduction of NKT cells in RA patients. Second, sCD1d could inhibit hyperactivation of NKT cells by occupying T cell receptors. NKT cells are highly sensitive to activation-induced cell death (AICD) (e.g., induced by stimulation with anti-CD3e antibody), compared with conventional T cells[24]. Thus, low levels of sCD1d molecules cannot produce a sufficient inhibition of AICD of NKT cells, resulting in a reduction of NKT cells.

Our findings indicated significantly low expression of V2 variants lacking both exon 4 and 5 genes in patients with RA and the reduction of V2 molecules might be associated with decreased numbers of NKT cells in patients with RA. However, little is known about the mechanism of secretion

Table 1. Summary of V1 and V2 (sCD1d) mRNA expression in patients and healthy subjects (HS). Results are expressed as mean ± SEM.

	n	Relative Expression of mRNAs	p
V1			
HS	15	1.2 ± 0.23	
RA	18	0.5 ± 0.12	0.110
SLE	10	1.3 ± 0.34	0.994
SS	15	0.8 ± 0.11	0.595
V2			
HS	15	6.9 ± 2.08	
RA	19	2.0 ± 0.33	0.023
SLE	10	2.7 ± 0.48	0.162
SS	15	4.3 ± 0.78	0.473

or the immunoregulatory role of sCD1d. Further examination of the sCD1d molecule (V2) at a protein level is necessary.

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ELSEVIER

Mannose Binding Lectin Gene Polymorphism in Patients With Type I Diabetes

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ABSTRACT: Our purpose was to investigate a possible relationship between occurrence of type I diabetes and polymorphism of the mannose binding lectin gene. Polymorphism of codon 54 of the mannose binding lectin (MBL) gene, whose presence of the minority allele leads to significant reduction of serum MBL concentration, was investigated in 128 Japanese patients with type I diabetes and 78 healthy volunteers by restriction fragment length polymorphism method. Frequencies of the minority allele were compared between the patient group and the control group. Frequency of the minority allele was 24.2% in the patient group and 19.9% in the control group. The probability of being heterozygous or homozygous for the minority allele was 41.4% in the patient group and 33.3% in the control group. Patients with DRB1*0405-DQB1*0401 and/or DRB1*0901-DQB1*0303 haplotypes, the two major type I diabetes-prone human leukocyte antigen haplotypes, showed a slightly higher probability of being heterozygous or homozygous for allele B of the MBL gene. Possession of the minority allele of the MBL gene may be a minor risk factor for having type I diabetes. *Human Immunology 64, 621–624 (2003).* © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Inc.

KEYWORDS: mannose binding lectin, polymorphism, innate immunity, disease susceptibility, type I diabetes

ABBREVIATION
MBL mannose binding lectin

INTRODUCTION

Mannose binding lectin (MBL), a serum protein with a structure similar to complement C1q, is an important factor of innate immunity. MBL binds to various microorganisms and helps phagocytosis of those microorganisms by phagocytes. MBL also mediates lectin-dependent activation of the complement pathway [1]. The importance of MBL in host defense is shown by the observation that people lacking this protein are prone to severe episodes of bacterial infection from early life [2].

Serum MBL levels are greatly affected by the polymorphisms of the MBL gene. Several polymorphisms have been reported for the MBL gene. Codon 52, 54, and 57 polymorphisms are all on exon 1, a region coding the collagenous domain of the MBL protein. Presence of any of the minority allele, when heterozygous, results in significant reduction of the serum MBL concentration, and, when homozygous, in almost complete absence of the protein [3]. These conditions are suggested to be associated with recurrent infections [2, 4] and progression of chronic viral diseases [5–7]. Interestingly, recent studies show that individuals with the minority alleles of the MBL gene are at risk of having autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis [8–14]. The pathogenesis of these autoimmune diseases is not clearly understood, but is generally considered to be multifactorial, the onset being influenced by both genetic and environmental factors.

These previous studies prompted us to wonder

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TABLE 1 Relationship between mannose binding lectin polymorphism and occurrence of type I diabetes

	Type I DM		Controls		*p* Value	OR (95% CI)
Genotypes						
AA	75	58.6%	52	66.7%		
AB	44	34.4%	21	26.9%	0.4984	
BB	9	7.0%	5	6.4%		
Total	128		78			
AA/AB	119	93.0%	73	93.6%	0.8636	1.104 (0.356–3.423)
BB	9	7.0%	5	7.4%		
Total	128		78			
AA	75	58.6%	52	66.7%	0.2477	1.413 (0.785–2.543)
AB/BB	53	41.4%	26	33.3%		
Total	128		78			
Allele frequency						
A	194	75.8%	125	80.1%	0.3060	
B	62	24.2%	31	19.9%		
Total	256		156			

Abbreviations: DM = diabetes mellitus; A = wild-type allele; B = codon 54 minority allele; OR = odds ratio; CI = confidence interval.

whether occurrence of type I diabetes, which is also considered an autoimmune disease affected by genetic factors such as major histocompatibility complex (MHC) class II [15, 16], and environmental factors such as viral infection [16, 17], is influenced by polymorphisms of the MBL gene. In this study, MBL gene polymorphism was studied in Japanese type I diabetes patients and healthy individuals residing in the same district. In addition, results were stratified by MHC class II haplotypes, which is a major genetic factor affecting susceptibility to type I diabetes [15].

SUBJECTS AND METHODS

Genomic DNA was obtained from 128 Japanese patients with type I diabetes and 78 healthy volunteers. The study was approved by the local ethical committee, and informed consent was obtained from all subjects. All diabetes patients were hospital-based, ketosis-prone, lacked endogenous insulin secretion as judged by urinary C-peptide levels of <3.3 nmol/day, and needed more than four insulin injections per day. All subjects were unrelated Japanese individuals residing in the Osaka district in western Japan.

Typing of the MBL gene alleles was done by polymerase chain reaction (PCR)-restriction fragment length polymorphism method, according to the methods of Madsen et al [3]. The wild-type allele is designated allele A, and codon 54 substitution (glycine to aspartic acid) is designated allele B. Codon 57 substitution (glycine to glutamic acid) and codon 52 substitution (arginine to cysteine) were not analyzed in this study, because these substitutions were not found in our previous studies covering 447 Japanese subjects [5, 13], and thus seem to be absent or extremely rare in this population.

In brief, genomic DNA was amplified by PCR using primers AGTCGACCCAGATTGTAGGACAGAGA and AGGATCCAGGCAGTTTCCTCTGGAAGG, producing a product of 349 base pairs. The amplified product was digested with restriction enzyme *BanI*. Wild-type (allele A) PCR products would be cut to two segments of 260 and 89 base pairs, whereas codon 54 substitution (allele B) products remain uncut. The validity of this assay was previously confirmed by sequencing several randomly selected samples [5, 13]. Statistical analyses were done by chi-square analysis, or Fisher's exact test where appropriate. Human leukocyte antigen (HLA) haplotypes were determined as described previously [15].

RESULTS

Frequency of the MBL Genotypes or Alleles

Individuals with type I diabetes had a higher probability of having allele B of the MBL gene, but statistical significance was not achieved. Frequency of allele B was higher in patients with type I diabetes, but also without statistical significance (Table 1).

MBL Polymorphism and Age at Onset of Type I Diabetes

Patients homozygous for allele B of the MBL gene tended to develop diabetes at a younger age than other patients, although without statistical significance (Table 2).

MBL Polymorphism and HLA Haplotypes in Patients with Type I Diabetes

Patients with DRB1*0405-DQB1*0401 and/or DRB1*0901-DQB1*0303 haplotypes, the two major type I diabetes–prone HLA haplotypes, showed a slightly

TABLE 2 Ages of patients at onset of type I diabetes

MBL genotype (*n*)	Age at onset (mean + SD)
AA (75)	15.7 + 12.3
AB (44)	15.7 + 12.1
BB (9)	13.8 + 6.3

Abbreviations: MBL = mannose binding lectin; A = wild-type allele; B = codon 54 minority allele.

higher probability of being heterozygous or homozygous for allele B of the MBL gene, but without statistical significance (Table 3).

DISCUSSION

In this study, to test the hypothesis that MBL has some protective effects against emergence of autoimmunity, we investigated the allele and genotype frequencies of the MBL gene in patients with type I diabetes and compared them with healthy controls. The results obtained are in accord with our hypothesis, but statistical significance was not reached in any of the comparisons made. The frequency of the B allele in Japanese patients with type I diabetes was 24.2% in this study, which is higher than that observed in Japanese patients with systemic lupus erythematosus (22.3%) in our previous study [13]. Because the frequency of the B allele in healthy individuals was 19.9% in this study, a rather large number of samples would be necessary to achieve statistical significance if MBL indeed has some protective role against occurrence of autoimmune disorders.

The ages of patients at onset of type I diabetes were studied because we anticipated that, if impaired innate immunity caused by the polymorphism of the MBL gene has a role in the pathogenesis of type I diabetes, age at onset of diabetes may be younger in patients with the minority allele. Results indicated that patients homozygous for allele B of the MBL gene, and are thus almost devoid of serum MBL protein, may develop diabetes at a younger age than other patients, although also without statistical significance (Table 2).

Finally, the results were stratified by the MHC class II haplotypes *of patients and controls*. Patients with DRB1*0405-DQB1*0401 and/or DRB1*0901-DQB1*0303 haplotypes, the two major type I diabetes–prone HLA haplotypes, showed a slightly higher probability of being heterozygous or homozygous for allele B of the MBL gene than did controls. In other words, the relationship between type I diabetes–prone HLA haplotypes and type I diabetes seemed stronger in individuals with AB or BB genotypes of the MBL gene, although without statistical significance (Table 3).

Slightly higher frequency of AB or BB genotypes in type I diabetes, together with younger age at onset and higher frequency of susceptible HLA haplotypes in patients with AB or BB genotypes, suggest the contribution of the MBL polymorphisms to susceptibility to typical type I diabetes with young onset and susceptible HLA haplotypes. Given the effect of the polymorphisms on serum MBL concentration [3], the results suggest that reduction or deficiency of serum MBL may have some effect on the occurrence of type I diabetes, but at this stage, do not give us a definitive conclusion. The occurrence of type I diabetes is probably determined by a number of genetic and environmental factors. It seems that MBL polymorphism is not a major factor in the pathogenesis of type I diabetes, but may possibly be one of the minor factors. To get a more definitive conclusion, it would be necessary to study a much larger number of subjects, or to study the incidences of type I diabetes in large groups of individuals with AB or BB genotypes of the MBL gene, and compare them with that of the general population. If MBL polymorphisms do prove to be one of the genetic factors for determining the disease susceptibility for type I diabetes, it may be independent of MHC class II haplotypes, one of the major reported genetic factors.

Mannose binding lectin may have protective effects

TABLE 3 Association between type I diabetes and HLA haplotypes stratified by mannose binding lectine gene genotypes

	MBL genotype							
	AA		AB		BB		AB or BB	
HLA hyplotype	Type I DM	Controls	Type I DM	Controls	Type I DM	Controls	Type I DM	Controls
DRB1*0405/x	32	13	20	7	3	0	23	7
DRB1*0901/y	17	8	11	4	2	1	13	5
DRB1*0405/*0901	7	1	5	1	0	0	5	1
DRB1*0405 and/or *0901	56	22	36	12	5	1	41	13
Others	17	23	7	8	3	4	10	12

Abbreviations: DRB1*0405 = DRB1*0405-DQB1*0401 haplotype; DRB1*0901 = DRB1*0901-DQB1*0303 haplotype; x = non-DRB1*0901; y = non-DRB1*0405; MBL = mannose binding lectin; A = wild-type allele; B = codon 54 minority allele.