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Migration of CD4 T Cells and Dendritic Cells toward Sphingosine 1-Phosphate (S1P) Is Mediated by Different Receptor Subtypes: S1P Regulates the Functions of Murine Mature Dendritic Cells via S1P Receptor Type 3

Yasuhiro Maeda, Hirofumi Matsuyuki, Kyoko Shimano, Hirotohi Kataoka, Kunio Sugahara, and Kenji Chiba¹

Dendritic cells (DCs) and lymphocytes are known to show a migratory response to the phospholipid mediator, sphingosine 1-phosphate (S1P). However, it is unclear whether the same S1P receptor subtype mediates the migration of lymphocytes and DCs toward S1P. In this study, we investigated the involvement of S1P receptor subtypes in S1P-induced migration of CD4 T cells and bone marrow-derived DCs in mice. A potent S1P receptor agonist, the (*S*)-enantiomer of FTY720-phosphate [(*S*)-FTY720-P], at 0.1 nM or higher and a selective S1P receptor type 1 (S1P₁) agonist, SEW2871, at 0.1 μM or higher induced a dose-dependent down-regulation of S1P₁. The pretreatment with these compounds resulted in a significant inhibition of mouse CD4 T cell migration toward S1P. Thus, it is revealed that CD4 T cell migration toward S1P is highly dependent on S1P₁. Mature DCs, when compared with CD4 T cells or immature DCs, expressed a relatively higher level of S1P₃ mRNA. S1P at 10–1000 nM induced a marked migration and significantly enhanced the endocytosis of FITC-dextran in mature but not immature DCs. Pretreatment with (*S*)-FTY720-P at 0.1 μM or higher resulted in a significant inhibition of S1P-induced migration and endocytosis in mature DCs, whereas SEW2871 up to 100 μM did not show any clear effect. Moreover, we found that S1P-induced migration and endocytosis were at an extremely low level in mature DCs prepared from S1P₃-knockout mice. These results indicate that S1P regulates migration and endocytosis of murine mature DCs via S1P₃ but not S1P₁. *The Journal of Immunology*, 2007, 178: 3437–3446.

Circulation of mature lymphocytes between blood and secondary lymphoid tissues plays a central role in the establishment of the immune response to foreign Ags (1, 2). Homing of lymphocytes from blood into secondary lymphoid tissues beyond the high endothelial venules is highly dependent on an interaction between the chemokines CCL19, CCL21, CXCL12, and CXCL13 and their receptors CCR7, CXCR4, and CXCR5 on lymphocytes (1). These chemokines involved in lymphocyte homing are constitutively expressed in secondary lymphoid tissues and can induce migration of T cells, B cells, and dendritic cells (DCs)² into lymph nodes and Peyer's patches. Recently, it has been clarified that sphingosine 1-phosphate (S1P) plays an important role in lymphocyte egress from secondary lymphoid tissues and the thymus (3, 4).

S1P, a pleiotropic lysophospholipid mediator, is converted primarily from sphingosine by sphingosine kinase (SPHK) and stimulates multiple signaling pathways that result in calcium mobilization from intracellular stores, polymerization of actin, chemotaxis/migration, and escape from apoptosis (5–7). S1P is formed in various cells, including mast cells, platelets, and macrophages, in response to diverse stimuli such as growth factors, cytokines, G-protein-coupled receptor agonists, and Ags (7–9). Significant amounts (100–300 nM) of S1P are found in human plasma (10), and S1P binds with nanomolar affinities to five related G-protein-coupled receptors termed S1P_{1–5} (formerly Edg-1, -5, -3, -6, and -8) (11, 12). In mice whose hemopoietic cells lack a single S1P receptor, S1P receptor type 1 (S1P₁), there are no T cells in the periphery because mature T cells are unable to exit secondary lymphoid tissues and the thymus (4). Because expression of S1P₁ on the surface of lymphocytes is highly dependent on the extracellular concentration of S1P, S1P₁ expression on lymphocytes is down-regulated in the blood, up-regulated in secondary lymphoid tissues, and down-regulated again in the lymph node (13). S1P₁ is clearly demonstrated as essential for lymphocyte recirculation, especially lymphocyte egress from secondary lymphoid tissues and the thymus.

FTY720 (fingolimod) is a new class of immunomodulator with a sphingosine-related chemical structure. Its most striking feature is the induction of a marked decrease in the number of peripheral blood lymphocytes at doses that display an immunomodulating activity in various experimental allograft and autoimmune disease models (14–20). Our previous studies suggested that FTY720 induces the sequestration of circulating mature lymphocytes into secondary lymphoid tissues such as lymph nodes and Peyer's

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² Abbreviations used in this paper: DC, dendritic cell; CHO, Chinese hamster ovary; FITC-Dx, FITC-dextran; HA, hemagglutinin; hS1P₁-CHO cell, CHO cells stably expressing human S1P₁; mS1P₁-CHO cell, CHO cells stably expressing mouse S1P₁; rm, recombinant mouse; (*S*)-FTY720-P, the (*S*)-enantiomer of FTY720-phosphate; S1P, sphingosine 1-phosphate; S1P₁, S1P receptor type 1; SPHK, sphingosine kinase.

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patches and thereby decreases the number of lymphocytes in peripheral blood, thoracic duct lymph, and the spleen (14). In addition, Yagi et al. (21) reported that FTY720 inhibits mature thymocyte emigration from the thymus to the periphery in mice. It has been demonstrated that FTY720 is phosphorylated to a (*S*)-enantiomer of FTY720-phosphate [(*S*)-FTY720-P] by SPHK1a and SPHK2 (22, 23). (*S*)-FTY720-P acts as an agonist at S1P receptors except for S1P₂, internalizes S1P₁ on lymphocytes, and thereby inhibits the migration of lymphocytes toward S1P (24). Consequently, treatment with FTY720 down-regulates S1P₁ and creates a temporary pharmacological S1P₁-null state in lymphocytes. This provides an explanation for the mechanism of FTY720-induced lymphocyte sequestration. It is likely that FTY720 reduces circulating lymphocytes by inhibiting S1P/S1P₁-dependent lymphocyte egress from secondary lymphoid tissues and the thymus.

DCs, as well as lymphocytes, are key constituents of the immune system. DCs play pivotal roles in the induction of Ag-specific immune responses as powerful APCs (25–27). In contrast to the widely accepted importance of S1P/S1P₁ interaction in lymphocyte recirculation, the involvement of S1P and its receptors in migration and trafficking of DCs is largely unknown. Idzko et al. (28) described that S1P stimulates pertussis toxin-sensitive Ca²⁺ increase, actin polymerization, and chemotaxis in only immature DCs, although similar amounts of mRNA for S1P₁, S1P₂, S1P₃, and S1P₄ are expressed in both immature and mature DCs derived from human blood monocytes. Moreover, S1P inhibits the secretion of TNF- α and IL-12, whereas it enhances the secretion of IL-10 from human immature DCs (28). In contrast, mouse bone marrow-derived mature, but not immature, DCs are reported to migrate toward S1P. This phenomenon seems to be correlated to the up-regulation of S1P₁ and S1P₃ (29). Renkl et al. (30) reported that S1P has chemotactic activity in human immature DCs, whereas S1P induces chemotaxis and actin polymerization in both immature and mature DCs in mice. As described above, there are several studies regarding the effect of S1P on DC migration (28–30). However, it is unclear whether the same S1P receptor subtype mediates the migration of lymphocytes and DCs toward S1P.

In this study, we investigated the involvement of S1P receptor subtypes in S1P-induced migration of CD4 T cells and bone marrow-derived DCs in mice. By real-time PCR analysis, mature DCs, as compared with CD4 T cells and immature DCs, expressed relatively high level of S1P₃ mRNA. S1P at 10–1000 nM induced a marked migration and enhanced the endocytosis in mature but not immature DCs. Moreover, we found that S1P-induced migration and endocytosis were at an extremely low level in mature DCs prepared from S1P₃-knockout mice. Based on these results, we, for the first time, demonstrate in this study that S1P regulates migration and endocytosis of murine mature DCs via S1P₃.

Materials and Methods

Mice

Inbred strains of male C57BL/6 mice were purchased from Charles River Japan. Male S1P₃-knockout mice on the C57BL/6 background were obtained from Deltagen. All mice were used at 6–12 wk of age. All animal experiments were performed under an experimental protocol approved by the ethics review committee for animal experimentation of Pharmaceuticals Research Division, Mitsubishi Pharma.

Reagents and Abs

An active metabolite of FTY720, (*S*)-FTY720-P, was synthesized with high enantioselectivity (>99.5% enantio excess) in Mitsubishi Pharma according to the method as previously described (24) and dissolved in ethanol. S1P₁-selective agonist, SEW2871 (31), was also synthesized in Mitsubishi Pharma. S1P, FITC-dextran (FITC-Dx), and LPS were purchased from Sigma-Aldrich. Recombinant mouse (rm)-GM-CSF, rm-CCL21, and anti-human S1P₁ mAb were purchased from R&D Systems. Anti-HA poly-

clonal Ab (Y-11) and Alexa 488-conjugated anti-rabbit IgG were obtained from Santa Cruz Biotechnology. FITC-conjugated mouse anti-human CD4 mAb (RPA-T4) and PE-Cy5-conjugated mouse anti-human CD3 mAb (UCHT1) were purchased from eBioscience. FITC-, PE-, CyChrome-, or biotin-conjugated mAbs recognizing the following mouse surface markers were obtained from BD Biosciences: CD4 (GK1.5), CD3 (RA3-6B2), CD8 (53-6.7), CD11c (HL3), CD86 (GL1), I-A^b (KH74), CD40 (3/23), and CD80 (16-10A1). PE-conjugated goat anti-mouse Ig-specific polyclonal Ab and streptavidin-CyChrome conjugate were also purchased from BD Biosciences.

Human S1P₁ expression in CHO cells

Chinese hamster ovary (CHO) cells purchased from Dainippon Pharmaceutical were transfected with DNAs encoding human S1P₁ using LipofectAMINE (Invitrogen Life Technologies). Then the cells stably expressing human S1P₁ (hS1P₁-CHO cells) were selected in Ham's F-12 medium (Sigma-Aldrich) supplemented with 10% FCS (Invitrogen Life Technologies) and 600 μ g/ml geneticin (Sigma-Aldrich) at 37°C. hS1P₁-CHO cells were grown on culture dishes, followed by serum starvation using RPMI 1640 medium supplemented with 10 mM HEPES, 100 U/ml penicillin, 60 μ g/ml kanamycin sulfonate, 50 μ M 2-ME, and 0.5% fatty acid-free BSA (Sigma-Aldrich) for 18 h at 37°C, and were incubated with S1P, (*S*)-FTY720-P, or SEW2871 at various concentrations for 1 h at 37°C. After the incubation, the cells were collected and stained with anti-human S1P₁ mAb or isotype-matched control IgG, followed by detection with PE-conjugated goat anti-mouse Ig specific polyclonal Ab. The expression of human S1P₁ on the cell surface was analyzed by flow cytometry with FACScan (BD Biosciences), and the results were expressed as the mean fluorescence intensity of triplicate determinations.

S1P₁ expression in human CD4 T cells

Peripheral blood anticoagulated with EDTA was obtained from volunteer healthy donors with approval of the ethics review committee of Pharmaceuticals Research Division, Mitsubishi Pharma. PBMCs were isolated by density gradient centrifugation using Lymphosepar I (IBL). After serum starvation for 18 h, human PBMCs were incubated with S1P, (*S*)-FTY720-P, or SEW2871 at various concentrations for 1 h at 37°C. Cell surface expression of S1P₁ in human CD4 T cells was analyzed by the method as described above. To identify human CD4 T cells, human PBMCs were stained with anti-human CD3 mAb and anti-human CD4 mAb.

Mouse S1P₁ expression in CHO cells

CHO cells stably expressing C-terminal hemagglutinin (HA)-tagged mouse S1P₁ (mS1P₁-CHO cells) were obtained by transfection as described above. mS1P₁-CHO cells were grown on Lab-Tek chamber slide (Nunc), followed by serum starvation for 18 h, and then incubated with S1P, (*S*)-FTY720-P, or SEW2871 at various concentrations. After incubation, the cells were washed and fixed with 3.7% formaldehyde in PBS at room temperature for 20 min. After permeabilization and blocking, the cells were stained with anti-HA polyclonal Ab, followed by detection with Alexa 488-conjugated anti-rabbit IgG. The slides were rinsed in water and mounted on to glass slides using Mowiol 4-88 (Calbiochem). The cell images were digitally captured under a confocal laser microscope (Carl Zeiss).

Preparation of mouse CD4 T cells from spleen

Spleens were removed from mice and single-cell suspensions were prepared by mincing and passing through stainless mesh. After removing RBCs by lysis with Tris-NH₄Cl solution, the spleen cells were suspended in RPMI 1640 medium with 0.5% fatty acid-free BSA. For real-time PCR analysis, the spleen cells were purified to >95% of CD4 T cells by passing through mouse CD4 subset enrichment columns (R&D Systems).

Preparation of DCs from bone marrow

DCs were prepared from mouse bone marrow cells according to the method as described Son et al. (32). Briefly, the bone marrow cells were flushed out from femurs and tibias using RPMI 1640 medium and were passed through cotton to remove bone marrow particles. After removing RBCs by Tris-NH₄Cl solution, the bone marrow cells were suspended in RPMI 1640 medium supplemented with 10 mM HEPES, 100 U/ml penicillin, 60 μ g/ml kanamycin sulfonate, 50 μ M 2-ME, 10% FCS, and 10 ng/ml rm-GM-CSF. The bone marrow cells at 2×10^5 cells/ml were cultured in 6-well plates at 37°C in 5% CO₂, and 10 ng/ml rm-GM-CSF was added on day 4 of the culture. After culturing for 6 days, both nonadherent and loosely adherent cells were collected and purified to >95% of CD11c⁺

immature DCs by incubation with anti-CD11c MACS beads (Miltenyi Biotec) for 15 min at 4°C, followed by two cycles of MACS-positive selection. In some experiments, immature DCs at 5×10^5 cells/ml in 10-cm culture dishes were cultured for an additional 24 h with LPS (1 $\mu\text{g}/\text{ml}$). More than 90% of I-A^b high/CD86^{high} mature DCs were obtained by LPS-stimulation of immature DCs as determined by flow cytometry with FACScan. For real-time PCR analysis, each of immature and mature DCs was further purified to >98% of I-A^b int/CD86^{low} immature and I-A^b high/CD86^{high} mature DCs by a MoFlo cell sorter (DakoCytomation).

Chemotaxis assays

Migration assays were conducted according to the method described previously (33). After serum starvation using RPMI 1640 medium with 0.5% fatty acid-free BSA for 3 h at 37°C, spleen cells or bone marrow-derived DCs (5×10^5 cells) were added to the upper wells of 5- μm pore, polycarbonate 24-well tissue culture inserts (Costar) in 100 μl , with 600 μl of S1P, (S)-FTY720-P, or rm-CCL21 dilution (or medium) in the bottom wells. Three chemotactic wells were set up for each chemoattractant. All migration assays were conducted in RPMI 1640 medium with 0.5% fatty acid-free BSA for 3 h at 37°C in 5% CO₂. In some experiments, the cells were pretreated with (S)-FTY720-P or SEW2871 for 5 min. The migrated cells recovered from each well were counted using comparison to a known number of beads as an internal standard of Flow-Count fluorosphere (Beckman Coulter) and were stained with appropriate mAbs to identify CD3⁺CD4⁺CD8⁻ T cells, CD11c⁺CD86^{low} I-A^b int immature DCs, and CD11c⁺CD86^{high}I-A^b high mature DCs. The number of the cells in the starting population and the migrated population was determined by flow cytometry with an Epics-XL (Beckman Coulter) and calculated for each phenotype, and the percentage migration was determined from these values.

Real-time chemotaxis assay

The microchemotaxis chamber (TAXIScan; Effector Cell Institute) was used to detect real-time horizontal chemotaxis according to the method described previously (34). After serum starvation for 3 h, mature DCs (1×10^5 cells/ μl) and S1P (10 μM) were put into the space in each side of the chamber and incubated for 2 h at 37°C. A charge-coupled device camera was used to record the migration of input cells toward S1P on the microchannel.

Endocytosis assay

Mouse immature and mature DCs (1×10^5 cells) were incubated in RPMI 1640 medium with 0.5% fatty acid-free BSA for 3 h at 37°C in 5% CO₂, and then FITC-Dx (1 mg/ml) and S1P (100 nM) were added concurrently before 1- to 60-min incubation. Endocytosis of FITC-Dx was halted at the indicated time points by rapid cooling of the cells on ice. The cells were then washed three times with ice-cold PBS containing 0.1% NaN₃ and stained with mAbs to identify CD11c⁺I-A^b int immature DCs or CD11c⁺I-A^b high mature DCs. The percentage of FITC-Dx-positive population in both types of DCs was analyzed by flow cytometry with FACScan. Incubation of the cells with FITC-Dx on ice was used as a background control. The results were plotted after the subtraction of background control from each experimental sample. S1P or (S)-FTY720-P was added to a final concentration of 1–1000 nM following incubation with FITC-Dx for 2 min. In some experiments, the cells were pretreated with (S)-FTY720-P or SEW2871 for 5 min. Each experiment was performed in triplicate determinations.

Real-time PCR for S1P receptor mRNA expression

Total RNA was isolated from splenic CD4 T cells and bone marrow-derived immature and mature DCs using the RNeasy kit (Qiagen). Complementary DNA was synthesized with TaqMan Reverse Transcription Reagents (Applied Biosystems) using random hexamers and 0.5 μg of total RNA. Real-time PCR was performed with an ABI PRISM 7700 sequence detector (Applied Biosystems), using SYBR Green PCR Master Mix (Applied Biosystems). The initial step was at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of a denaturing step for 15 s at 95°C and an annealing extension step for 1 min at 60°C. For normalization of data, determination of expression of a housekeeping gene, GAPDH, was performed using predeveloped primers (TaqMan rodent GAPDH control reagents; Applied Biosystems). Data were analyzed using Sequence Detector software (Applied Biosystems). Relative quantitation of mRNA levels was performed using the standard curve method and calculated as the ratio between each S1P receptor mRNA and GAPDH. Each experiment was performed in triplicate. The following primer pairs were used (5'→3'): S1P₁ forward, AAA TGC CCC AAC GGA GAC T; S1P₁ reverse, CTG ATT TGC TGC GGC TAA ATT C; S1P₂ forward, GCC ATC GTG GTG

GAG AAT CTT; S1P₂ reverse, AGG TAC ATT GCT GAG TGG AAC TTG; S1P₃ forward, GCG CAT CTA CTG CCT GGT CAA GTC C; S1P₃ reverse, AGC CAG CAT GAT GAA CCA CTG ACT C; S1P₄ forward, GGC TAT GCC CAT TGT CCA GTA; S1P₄ reverse, GGC TCT GAG CTA GAG AGC ATG AT; S1P₅ forward, CAT GGC TAA CTC GCT GCT GAA; and S1P₅ reverse, AGC TGT TGG AGG AGT CTT GGT T.

Determination of the number of peripheral blood CD4 T cells

(S)-FTY720-P was dissolved in 20% hydroxypropyl- β -cyclodextrin solution and was administered to mice i.v. Control mice received vehicle only. The peripheral blood was obtained 24 h after the administration, and the number of CD4 T cells in peripheral blood was determined by three-color flow cytometry using FITC-conjugated anti-CD4 mAb, PE-conjugated anti-CD3 mAb, and CyChrome-conjugated anti-CD8 mAb.

Results

S1P receptor agonists induce S1P₁ down-regulation in hS1P₁-CHO cells and human CD4 T cells

We first examined the effect of S1P, (S)-FTY720-P, and a selective S1P₁ agonist, SEW2871, on S1P₁ down-regulation using hS1P₁-CHO cells and mAb specific against human S1P₁ by flow cytometry. As shown in Fig. 1A, S1P (10–1000 nM), (S)-FTY720-P (0.1–10 nM), and SEW2871 (100–10,000 nM) induced a dose-dependent down-regulation of human S1P₁ on hS1P₁-CHO cells. In human CD4 T cells, these compounds also induced a dose-dependent down-regulation of S1P₁ (Fig. 1B). Particularly, S1P at 1,000 nM and (S)-FTY720-P at 1 nM or higher induced a marked down-regulation of S1P₁ in human CD4 T cells. Fig. 1C shows the dose-response relationships of (S)-FTY720-P, S1P, and SEW2871 on S1P₁ down-regulation in hS1P₁-CHO cells. (S)-FTY720-P at 0.1 nM or higher induced a significant and dose-dependent down-regulation of S1P₁ with IC₅₀ of 0.44 nM, and the cell surface expression of S1P₁ on hS1P₁-CHO cells was reduced almost completely at 10 nM or higher. S1P also induced a dose-dependent down-regulation of S1P₁ at 30–300 nM with IC₅₀ of 98 nM and a maximal down-regulation at 1,000 nM. Furthermore, SEW2871 induced S1P₁ down-regulation at 300–10,000 nM with IC₅₀ of 480 nM, and a marked down-regulation was observed only at high concentrations (>3,000 nM). Similar results were obtained when human CD4 T cells were treated with (S)-FTY720-P, S1P, and SEW2871 (data not shown).

S1P₁ receptor agonists induce S1P₁ down-regulation in mS1P₁-CHO cells and inhibit mouse CD4 T cell migration toward S1P

To examine the effect of S1P, (S)-FTY720-P, and SEW2871 on mouse S1P₁ down-regulation, we used HA-tagged mS1P₁-CHO cells and mAb specific against HA because there is no available anti-mouse S1P₁ mAb for flow cytometric analysis. Fig. 2 shows representative data. Both S1P and (S)-FTY720-P induced rapid S1P₁ down-regulation in mS1P₁-CHO cells. S1P₁ down-regulated by S1P recycled back to the plasma membrane at 3 h, whereas a long-term down-regulation of S1P₁ was induced by (S)-FTY720-P in mS1P₁-CHO cells.

Next, we examined the inhibitory effect of (S)-FTY720-P and SEW2871 on the migration of CD4 T cells toward S1P by Transwell assay. Consistent with our data previously reported (24), CD4 T cells showed a significant migratory response toward S1P in a dose-dependent manner, peaking at 10 nM (Fig. 3A, ■). In addition, we found that (S)-FTY720-P at 0.1–1 nM could also induce CD4 T cell migration, although its activity was about half that of S1P (Fig. 3A, □). Furthermore, SEW2871 at 1000 nM also induced CD4 T cell migration (data not shown). The migration of CD4 T cells toward 10 nM S1P was inhibited significantly and in a dose-dependent manner by pretreatment with (S)-FTY720-P at 0.1 nM or higher with IC₅₀ of 0.22 nM (Fig. 3B). Particularly,

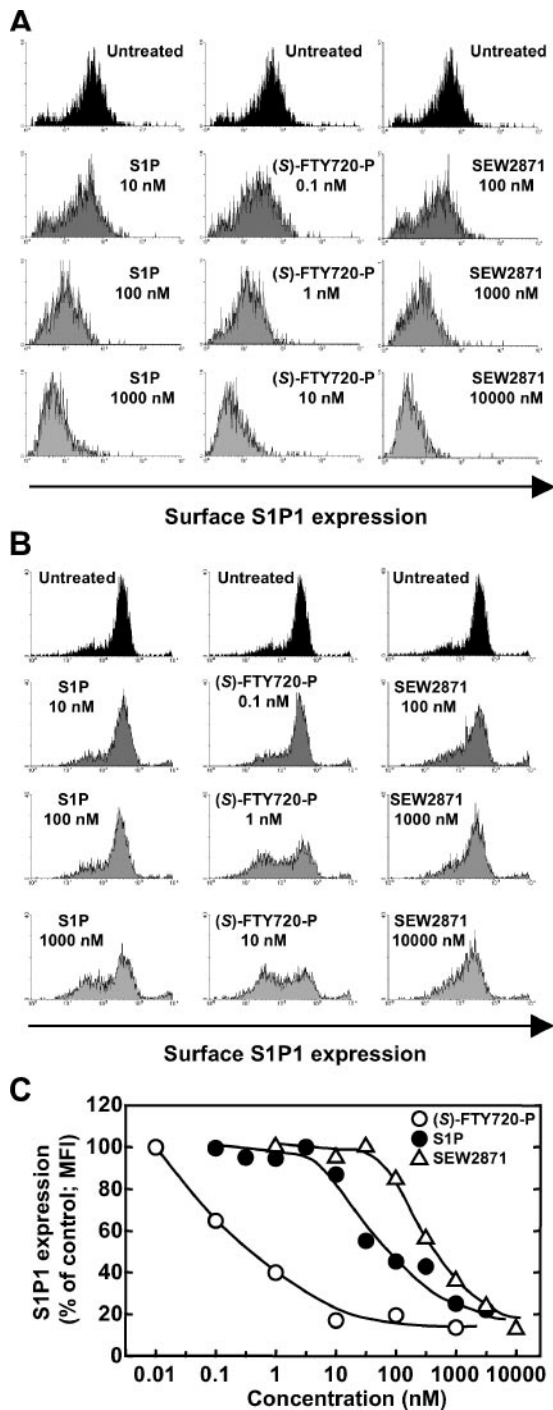


FIGURE 1. The effect of S1P receptor agonists on the expression of S1P₁ in hS1P₁-CHO cells and human CD4 T cells. After serum starvation for 18 h, hS1P₁-CHO cells or human CD4 T cells were incubated with (S)-FTY720-P (○), S1P (●), and SEW2871 (△) for 1 h at 37°C. Cell surface expression of S1P₁ was determined by flow cytometry. The typical results of S1P₁ down-regulation induced by S1P receptor agonists in hS1P₁-CHO cells (A) and human CD4 T cells (B). Human CD4 T cells were determined by staining with anti-CD3 and anti-CD4 mAbs. C, The results of hS1P₁-CHO cells were expressed as the mean fluorescence intensity in triplicate determinations.

pretreatment with (S)-FTY720-P at 1 nM or higher resulted in almost complete inhibition of S1P-induced CD4 T cell migration. SEW2871, like (S)-FTY720-P, almost completely inhibited S1P-induced CD4 T cell migration at concentrations of 300 nM or

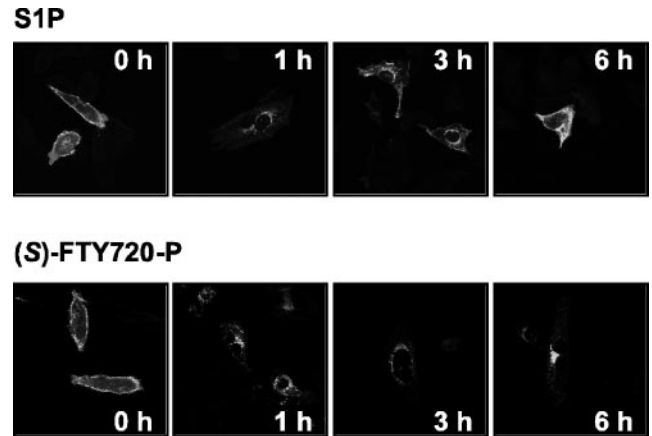


FIGURE 2. The effect of S1P and (S)-FTY720-P on the expression of S1P₁ in mS1P₁-CHO cells. After serum starvation for 18 h, mS1P₁-CHO cells were incubated with 100 nM S1P (upper panels) or (S)-FTY720-P (lower panels) at 37°C. At indicated times, the cells were fixed, permeabilized, and then stained with rabbit anti-HA polyclonal Ab and Alexa 488-conjugated anti-rabbit IgG.

higher with IC₅₀ of 220 nM (Fig. 3C). From these data, it is strongly suggested that inhibition of S1P-induced CD4 T cell migration by (S)-FTY720-P or SEW2871 is caused by the down-regulation of S1P₁. Thus, we confirmed that the migration of CD4 T cells toward S1P is highly dependent on S1P₁.

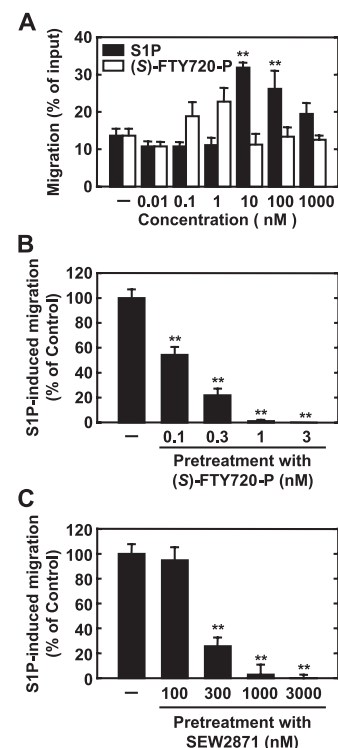


FIGURE 3. The effect of S1P receptor agonists on mouse CD4 T cell migration. A, After serum starvation for 3 h, mouse spleen cells were performed to migration assays toward 0.01–1000 nM S1P (■) or (S)-FTY720-P (□). The percent migration of CD4 T cells was determined by flow cytometry, and the results were expressed as the mean ± SEM of triplicate wells. **, $p < 0.01$, when compared with medium control (Dunnett's test). B and C, After serum starvation, the spleen cells were pretreated with 0.1–3 nM (S)-FTY720-P (B) or 100–3000 nM SEW2871 (C) for 5 min, and then migration assays toward 10 nM S1P were performed. The migration of CD4 T cells toward medium alone or S1P at 10 nM was designated as 0 or 100%, respectively. Data are expressed as the mean ± SEM of triplicate wells. **, $p < 0.01$, when compared with S1P-induced CD4 T cell migration (Dunnett's test).

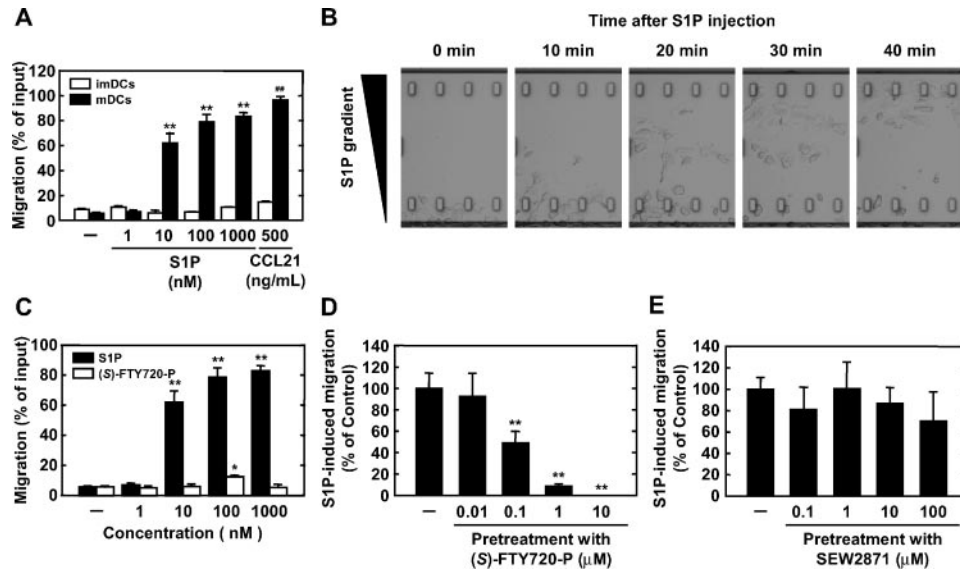


FIGURE 4. The effect of S1P receptor agonists on the migration of immature and mature DCs. *A*, After serum starvation for 3 h, immature (imDCs; □) and mature DCs (mDCs; ■) derived from bone marrow cells were performed to migration assays toward 1–1000 nM S1P or 500 ng/ml rm-CCL21. The percent migration of imDCs and mDCs was determined by flow cytometry, and the results were expressed as the mean ± SEM of triplicate wells. ##, $p < 0.01$, when compared with medium control (t test). **, $p < 0.01$, when compared with medium control (Dunnett's test). *B*, After serum starvation, 1 μ l of 1×10^6 mDCs was put into one hole of the microchemotaxis chamber, and 1 μ l of 10 μ M S1P was put into another hole. A charge-coupled device camera was used to record the migration of mDCs under the gradation of S1P. *C*, Migration assays of mDCs toward 1–1000 nM S1P (■) or (S)-FTY720-P (□) were performed. Data are expressed as the mean ± SEM of triplicate wells. *, $p < 0.05$; **, $p < 0.01$, when compared with medium control (Dunnett's test). *D* and *E*, After the pretreatment with 0.01–10 μ M (S)-FTY720-P (*D*) or 0.1–100 μ M SEW2871 (*E*) for 5 min, mDCs were performed to migration assays toward 100 nM S1P. The migration of mDCs toward medium alone or S1P at 100 nM was designated as 0 or 100%, respectively. Data are presented as the mean ± SEM of triplicate wells. **, $p < 0.01$, when compared with S1P-induced mDC migration (Dunnett's test).

S1P induces migration of mature, but not immature, DCs

We examined the effect of S1P, (S)-FTY720-P, and SEW2871 on mouse bone marrow-derived DCs. On day 6 of culture, CD11c⁺ cells were sorted by MACS column and used as immature DCs. Following treatment with LPS for 24 h, DCs exhibited a mature phenotype with increased expression of CD86 and MHC class II (I-A^b) molecules and were used as mature DCs. These immature and mature DCs were discriminated by their expression profile (immature DCs, CD86^{low} I-A^b intermediate; mature DCs, CD86^{high} I-A^b high) and were used in a chemotaxis assay to analyze their response to S1P, (S)-FTY720-P, and SEW2871.

S1P at 10–1000 nM induced a strong migratory response in mature DCs, with migration of ~70–90% of input cells (Fig. 4A, ■). To confirm a mature differentiation status of the tested DCs, migration was examined in response to the CCR7 ligand CCL21, known as a strong chemotactic inducer for mature DCs (27, 35–37). The treatment with rm-CCL21 at 500 ng/ml led to a potent migration comparable to that of S1P. In contrast, we did not observe any clear migration of mouse immature DCs toward S1P up to 1000 nM or rm-CCL21 (Fig. 4A, □). The addition of S1P to both sites of the filter revealed that both chemotaxis and chemokinesis contribute to the S1P-mediated cell motility (data not shown). By conducting the real-time chemotaxis assay using a microchemotaxis chamber, most of the mature DCs were found to start migrating toward the S1P gradient after 10 min (Fig. 4B). These migrating cells spread lamellipodium widely toward the high concentration of S1P. In contrast, no directional migration was observed when immature DCs were incubated with S1P in the chamber (data not shown).

As shown in Fig. 4C, S1P induced a marked migration, whereas (S)-FTY720-P at 100 nM produced only a slight, but significant, migratory response in mature DCs (Fig. 4C, □). However, SEW2871 did not induce the migration of mature DCs (data not

shown). When mature DCs were pretreated with (S)-FTY720-P for 5 min, the migration toward 10 nM S1P was inhibited, partially at 100 nM and completely at 1000 nM or higher concentrations, respectively, with IC₅₀ of 97 nM (Fig. 4D). However, the amount of (S)-FTY720-P necessary to inhibit the migration of mature DCs was ~1000-fold higher than that of CD4 T cells (Figs. 3B and 4D). In contrast, SEW2871, which inhibits S1P-induced CD4 T cell migration, did not show any clear effect on S1P-induced mature

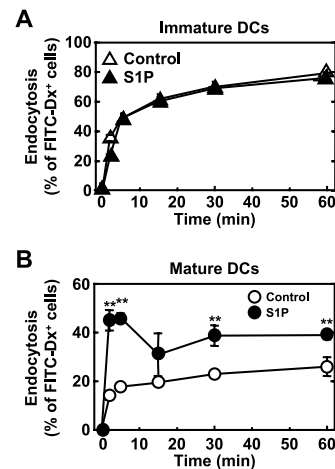


FIGURE 5. The effect of S1P on the endocytosis of FITC-Dx by immature and mature DCs. *A* and *B*, After serum starvation, immature (imDCs; *A*) and mature DCs (mDCs; *B*) were incubated with 1 mg/ml FITC-Dx in the presence or absence of 100 nM S1P for 2–60 min. The percentage of FITC⁺ DC population was determined by flow cytometry, and the results were expressed as the mean ± SEM of triplicate wells. **, $p < 0.01$, when compared with S1P-untreated control (t test). △, imDCs; ▲, imDCs incubated with S1P; ○, mDCs; ●, mDCs incubated with S1P.

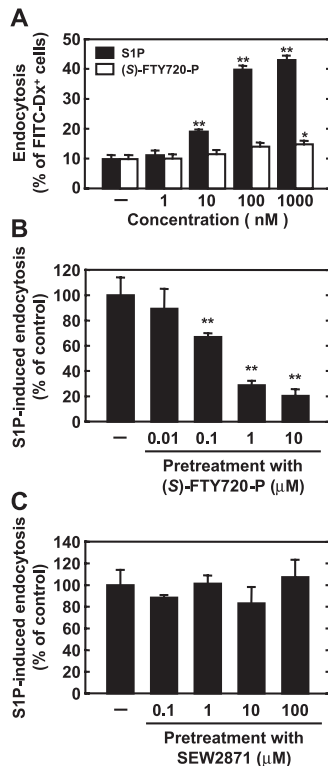


FIGURE 6. The effect of (S)-FTY720-P and SEW2871 on S1P-induced endocytosis of FITC-Dx by mature DCs. **A**, After serum starvation, mature DCs (mDCs) were incubated with 1 mg/ml FITC-Dx in the presence of 1–1000 nM S1P (■) or (S)-FTY720-P (□) for 2 min. The results were expressed as the mean \pm SEM of triplicate wells. *, $p < 0.05$; **, $p < 0.01$, when compared with medium control (Dunnett's test). **B** and **C**, After the pretreatment with 0.01–10 μ M (S)-FTY720-P (**B**) or 0.1–100 μ M SEW2871 (**C**) for 5 min, mDCs were performed to migration assays toward 100 nM S1P. The migration of mDCs toward medium alone or S1P at 100 nM was designated as 0 or 100%, respectively. Data are presented as the mean \pm SEM of triplicate wells. **, $p < 0.01$, when compared with S1P-induced mDC migration (Dunnett's test).

DC migration in the range of 0.1–100 μ M (Fig. 4E). These results suggest that different S1P receptor subtypes mediate the migration of CD4 T cells and mature DCs toward S1P.

S1P enhances endocytosis in mature DCs

We examined the effect of S1P on endocytosis of FITC-Dx in immature and mature DCs. Approximately 80% of immature DCs became FITC-Dx positive after 60 min of incubation and S1P at 100 nM did not influence FITC-Dx uptake by immature DCs (Fig. 5A). On the contrary, the proportion of FITC-Dx-positive mature DCs at 60 min was <26.0% of the total cells and was significantly increased to 45.0% after treatment with S1P for 2 min (Fig. 5B). As shown in Fig. 6A, treatment with S1P at 10–1000 nM significantly increased the endocytosis of FITC-Dx by mature DCs in a

dose-dependent manner and only a high concentration (1000 nM) of (S)-FTY720-P slightly, but significantly, enhanced the endocytosis of mature DCs. Moreover, pretreatment with (S)-FTY720-P resulted in an inhibitory effect on S1P-induced endocytosis of mature DCs at 100 nM or higher concentrations (Fig. 6B). In contrast, SEW2871 did not show any clear effect on the endocytosis of mature DCs, suggesting that S1P-induced endocytosis is independent on S1P₁ (Fig. 6C).

The mRNA expression patterns of S1P receptors in CD4 T cells and immature and mature DCs in mice

Because there are significant differences in the responsiveness of mouse mature DCs and CD4 T cells for S1P, (S)-FTY720-P, and SEW2871 (Figs. 3 and 4), we further characterized the involvement of specific receptors. To analyze the mRNA expression patterns of S1P receptors on CD4 T cells and immature and mature DCs, we performed real-time PCR analysis for S1P receptor mRNA. The mRNA expression of S1P₁ and S1P₄ was the most prominent on CD4 T cells (Table I). Furthermore, we found subtype-specific differential expression of S1P_{1–5} mRNA between immature and mature DCs (Table I). Especially, the expression level of S1P₃ mRNA was massively increased in mature DCs (>20-fold), whereas S1P₁ and S1P₄ mRNA levels showed approximately a 2-fold increase and a slight decrease (0.7-fold), respectively. In contrast, both differentiation stages showed similar expression patterns of S1P₂ and S1P₅. It showed clearly a lower level expression of S1P₁ mRNA in mature DCs than in CD4 T cells, whereas the expression level of S1P₃ mRNA in mature DCs was higher than that in CD4 T cells (Table I).

S1P₃ is essential for S1P-induced migration and endocytosis of mature DCs but not CD4 T cells

Because S1P₃ was expressed more abundantly in mature DCs than immature DCs, we used S1P₃-knockout mice to test whether this receptor was required in mature DC migration toward S1P. Mature DCs prepared from S1P₃-knockout mice had similar expression levels of S1P₁, S1P₂, S1P₄, and S1P₅ mRNA transcripts compared with those from littermate control mice; however these cells showed an extremely low level of migration toward S1P (Fig. 7, A and B). Furthermore, S1P at concentrations up to 1000 nM could not enhance the endocytosis of FITC-Dx in mature DCs prepared from S1P₃-knockout mice (Fig. 7C). To confirm a mature differentiation status of the LPS-treated DCs from S1P₃-knockout mice, we performed phenotypic analysis and chemotaxis assays. The expression levels of CD40, CD80, CD86, and MHC class II (I-A^b), and the chemotactic responsiveness to rm-CCL21 in mature DCs was indistinguishable between S1P₃-knockout mice and littermate control mice (Fig. 7, D and E). These results demonstrated that S1P induces not only migration but also endocytosis in mature DCs via S1P₃.

There was no significant difference in the mRNA levels of S1P receptors, except for S1P₃, in CD4 T cells between S1P₃-knockout

Table I. The mRNA expression patterns of S1P receptors in CD4 T cells and immature and mature DCs in mice^a

	S1P ₁	S1P ₂	S1P ₃	S1P ₄	S1P ₅
CD4 T cells	5.73 \pm 0.14	0.08 \pm 0.01	0.41 \pm 0.02	2.76 \pm 0.06	0.77 \pm 0.05
Immature DCs	0.14 \pm 0.01	0.05 \pm 0.00	0.07 \pm 0.00	0.49 \pm 0.02	0.08 \pm 0.00
Mature DCs	0.25 \pm 0.02	0.05 \pm 0.00	1.56 \pm 0.05	0.33 \pm 0.01	0.06 \pm 0.00

^a Real-time quantitative PCR analysis of the mouse S1P receptors in CD4 T cells and immature and mature DCs was performed as described in *Materials and Methods*. The amount of product for each PCR was calculated from the incorporation of SYBR green and normalized to GAPDH. (Mean \pm SEM of individual three experiments.)

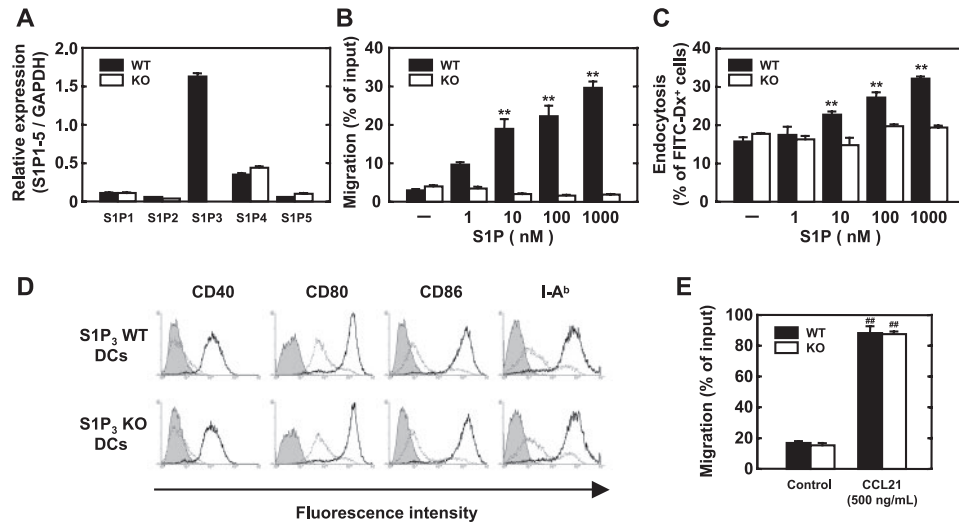


FIGURE 7. $S1P_3$ is essential for S1P-induced migration and endocytosis of mouse mature DCs. *A*, Quantitative PCR analysis of S1P receptors in mature DCs (mDCs) from $S1P_3$ wild-type (WT; ■) or $S1P_3$ -knockout ($S1P_3$ -KO; □) mice expressed as relative amount of mRNA normalized to GAPDH. *B* and *E*, After serum starvation for 3 h, mDCs from WT (■) or $S1P_3$ -KO (□) mice were performed to migration assays toward 1–1000 nM S1P (*B*) or 500 ng/ml rm-CCL21 (*E*). The percent migration of mDCs was determined by flow cytometry, and the results were expressed as the mean \pm SEM of triplicate wells. **, $p < 0.01$, when compared with medium control (Dunnett’s test). ##, $p < 0.01$, when compared with medium control (*t* test). *C*, After serum starvation, mDCs from WT (■) or $S1P_3$ -KO (□) mice were incubated with 1 mg/ml FITC-Dx in the presence of 1–1000 nM S1P for 2 min. The percentage of FITC⁺ mDC population was determined by flow cytometry and were expressed as the mean \pm SEM of triplicate wells. **, $p < 0.01$, when compared with medium control (Dunnett’s test). *D*, Immature (imDCs) and mature DCs from WT or $S1P_3$ -KO mice were analyzed for the expression of costimulatory molecules CD40, CD80, and CD86 as well as for MHC class II (I-A^b). Shaded area, isotype control; dotted line, imDCs; solid line, mDCs.

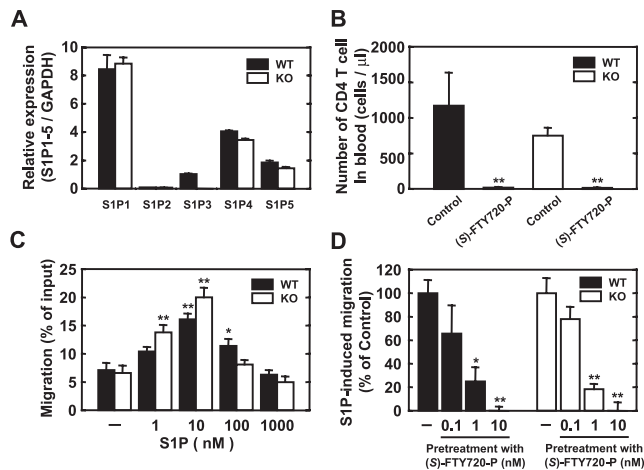


FIGURE 8. $S1P_3$ is not essential for S1P-induced migration of mouse CD4 T cells. *A*, Quantitative PCR analysis of S1P receptors in purified CD4 T cells from $S1P_3$ wild-type (WT; ■) or $S1P_3$ -knockout ($S1P_3$ -KO; □) mice expressed as relative amounts of mRNA normalized to GAPDH. *B*, (*S*)-FTY720-P at 1 mg/kg was i.v. administered to WT and $S1P_3$ -KO mice. Twenty-four hours after the administration, the number of CD4 T cells was determined by flow cytometry and expressed as the mean \pm SEM ($n = 3$). **, $p < 0.01$, when compared with vehicle-treated control mice (*t* test). There was no statistical difference between WT and $S1P_3$ -KO mice in the number of peripheral blood CD4 T cells by *t* test. *C*, After serum starvation, the spleen cells from WT (■) or $S1P_3$ -KO (□) mice were performed to migration assays toward 1–1000 nM S1P. The percent migration of CD4 T cells was determined by flow cytometry, and the results were expressed as the mean \pm SEM of triplicate wells. *, $p < 0.05$; **, $p < 0.01$, when compared with medium control (Dunnett’s test). *D*, After the pretreatment with 0.1–10 nM (*S*)-FTY720-P for 5 min, the spleen cells prepared from WT (■) or $S1P_3$ -KO (□) mice were performed to migration assays toward 10 nM S1P. The migration of CD4 T cells toward medium alone or S1P was designated as 0 or 100%, respectively. The results were expressed as the mean \pm SEM of triplicate wells. *, $p < 0.05$; **, $p < 0.01$, when compared with S1P-induced CD4 T cell migration (Dunnett’s test).

and wild-type mice (Fig. 8A). As shown in Fig. 8B, in vivo administration of (*S*)-FTY720-P at 1 mg/kg i.v. to $S1P_3$ -knockout and wild-type mice resulted in indistinguishable reduction of CD4 T cells in peripheral blood. Similar data were obtained in the number of total lymphocytes, CD3 T cells, and CD8 T cells in peripheral blood from both types of mice after treatment with (*S*)-FTY720-P (data not shown). Thus, we confirmed that S1P/ $S1P_3$ interaction is not essential to reduce circulating lymphocytes by (*S*)-FTY720-P administration. In addition, the migratory response toward S1P and the inhibitory effect of (*S*)-FTY720-P on S1P-induced migration in CD4 T cells showed almost the same patterns between $S1P_3$ -knockout and wild-type mice (Fig. 8, C and D). Based on these results, we concluded that $S1P_3$ and $S1P_1$ mediated S1P-induced migration of murine mature DCs and CD4 T cells, respectively.

Discussion

In the immune system, S1P is involved in immune cell migration, proliferation, differentiation, and cytokine production (5–7). Five types of specific receptors for S1P, termed $S1P_{1-5}$, are suggested to play several roles in the immune systems; however, their precise involvement in the function of immune cells remains unclear. Recently, results from gene-targeted mice have revealed that interaction of S1P and $S1P_1$ plays a critical role in lymphocyte egress from secondary lymphoid tissues and the thymus (38). When FTY720 is administered to animals, its active metabolite, (*S*)-FTY720-P, is rapidly converted from FTY720 by SPHK and acts as an agonist at four of five S1P receptors (11, 12). Moreover, it has been reported that (*S*)-FTY720-P can inhibit lymphocyte egress from secondary lymphoid tissues and the thymus by inducing long-term down-regulation of $S1P_1$ (6, 18). Indeed, T cells from $S1P_1$ -knockout mice show no migratory response toward S1P (4). However, there has been no report on a correlation between $S1P_1$ down-regulation and inhibition of T cell migration by (*S*)-FTY720-P.

In this study, we clarified the dose-response relationship of (*S*)-FTY720-P between S1P₁ down-regulation and the inhibition of CD4 T cell migration using (*S*)-FTY720-P with high enantioselectivity (>99.5%). We, for the first time, demonstrated that (*S*)-FTY720-P at a concentration of 1 nM or higher induced a marked down-regulation of S1P₁ in human CD4 T cells as well as hS1P₁-CHO cells (Fig. 1). At the same concentration range, S1P-induced migration of mouse CD4 T cells was almost completely inhibited by pretreatment with (*S*)-FTY720-P (Fig. 3B). In addition, we found that CD4 T cells can migrate toward lower concentrations of (*S*)-FTY720-P (Fig. 3A). Because (*S*)-FTY720-P at 0.1–1 nM partially induced S1P₁ down-regulation, it is presumed that residual S1P₁ on the surface of lymphocytes can respond to (*S*)-FTY720-P. Similar results were obtained when S1P and the S1P₁-selective agonist, SEW2871, were used (data not shown). Thus, it is likely that promotion or inhibition of CD4 T cell migration toward S1P depends on the magnitude of S1P₁ down-regulation by S1P, (*S*)-FTY720-P, or SEW2871. SEW2871 has been reported to induce S1P₁ internalization and recycling but not receptor degradation (31), and long-term, *in vivo* treatment with SEW2871 induces a marked decrease in peripheral blood lymphocytes in mice (39). Like FTY720, SEW2871 reduces the circulating lymphocytes by sequestering lymphocytes into secondary lymphoid tissues and the thymus (39). We showed here that SEW2871 inhibits S1P-induced migration of CD4 T cells at concentrations that induce internalization of S1P₁ (Figs. 1 and 3C). These results indicated that S1P-induced CD4 T cell migration is mediated by S1P₁.

It has been demonstrated that DCs, as well as lymphocytes, migrate to S1P; however, several studies have observed differences in the responsiveness of immature and mature DCs to S1P (28). Idzko et al. (28) described that, by using DCs derived from human monocyte, both immature and mature DCs show similar mRNA expression patterns to S1P₁, S1P₂, S1P₃, and S1P₄ and that S1P stimulates a pertussis toxin-sensitive (Gi-sensitive) Ca²⁺ increase, actin polymerization, and chemotaxis of immature, but not mature, DCs. In contrast, it has been reported that, by using mouse DCs prepared from bone marrow cells, TNF- α /PGE₂-stimulated mature DCs express relatively higher mRNA levels of S1P₁ and S1P₃ and show a strong migratory response toward S1P, compared with immature DCs (29). The same pattern of S1P receptor expression was observed on DCs that reside in draining lymph nodes after FITC painting *in vivo*. Lan et al. (40) reported that immature DCs from blood, spleen, and bone marrow-derived DCs in mice express the mRNA of all five S1P receptors, and mRNA expression of S1P₁, S1P₂, and S1P₃ is elevated in bone marrow-derived mature DCs after LPS stimulation. LPS-matured DCs exhibit a modest, but significant, migratory response to S1P, corresponding to the higher expression of S1P₁₋₃ mRNA on these DCs (40). Radeke et al. (41) provided evidence that S1P₁ and S1P₃, but not S1P₄, are responsible for the S1P-induced migratory response in murine Langerhans cell line, XS52 cells.

Consistent with previous studies that used mouse bone marrow-derived DCs (29), our results in this study revealed that S1P, at 10–1000 nM, can induce the migration of LPS-matured but not immature DCs derived from mouse bone marrow cells (Fig. 4A). By using real-time PCR analysis, we have found that mature DCs, as compared with CD4 T cells or immature DCs, expressed relatively higher levels of S1P₃ mRNA (Table I). There are three differences in migratory responses between CD4 T cells and mature DCs: 1) the dose-response relationship between S1P concentrations and S1P-induced migration; 2) the inhibitory concentration of (*S*)-FTY720-P for S1P-induced migration; and 3) the inhibitory effect of SEW2871 on S1P-induced migration.

The migratory response of CD4 T cells was induced by S1P at 10–100 nM but was decreased at 1000 nM (Fig. 3A). On the contrary, the migration of mature DCs was increased by S1P at 10–1000 nM in a dose-dependent manner (Fig. 4A). Pretreatment with (*S*)-FTY720-P resulted in a marked inhibition of S1P-induced CD4 T cell migration at 1 nM or higher; however, the inhibition of mature DC migration by (*S*)-FTY720-P was observed at 100 nM or higher (Figs. 3B and 4D). (*S*)-FTY720-P strongly induces S1P₁ inactivation via receptor down-regulation, thereby inhibiting the migration of CD4 T cells toward S1P in a noncompetitive manner. Because it has been reported that S1P₃, unlike S1P₁, is not down-regulated by agonist stimulation (42), the higher concentration of (*S*)-FTY720-P may be required for the inhibition of S1P-induced migration of mature DCs. Furthermore, pretreatment of SEW2871 inhibited S1P-induced migration of CD4 T cells but not mature DCs. These observations strongly suggested that S1P-induced migration of CD4 T cells and mature DCs was regulated by a differential mechanism.

Because mRNA expression of S1P₃ was relatively high in mature DCs and SEW2871 did not affect S1P-induced mature DC migration, it is highly probable that the migration of mature DCs toward S1P is mediated by S1P₃ but not S1P₁. To confirm this hypothesis, we performed chemotaxis assays using S1P₃-knockout mice. As expected, mature DCs that lacked S1P₃ could not migrate toward S1P, whereas CD4 T cells prepared from S1P₃-knockout mice reacted to S1P as well as CD4 T cells from wild-type mice (Figs. 7B and 8, C and D). Consequently, we concluded that S1P-induced migration of mature DCs is mediated by S1P₃, but not S1P₁.

DCs developmentally regulate the activity of endocytosis: immature DCs exhibit vigorous acquisition of fluid-phase molecules, whereas mature DCs capture only low levels of exogenous Ags (43, 44). In this study, we found that a short exposure to S1P markedly enhanced endocytosis of FITC-Dx by mature but not immature DCs (Fig. 5). Endocytosis, as well as migration, of mature DCs induced by S1P was also at an extremely low level in S1P₃-knockout mice, suggesting that S1P induced dextran endocytosis in mature DCs via S1P₃. Yanagawa and Onoe (45) reported that the CCR7-ligand chemokines, CCL19 and CCL21, induce not only chemotaxis but also dextran endocytosis by mature DCs. Thus, it is likely that S1P and homing chemokines share certain characteristics of function, such as induction of migration and endocytosis. Recently, it has been reported that the CCR7-ligand chemokine-induced rapid uptake of Ag by mature DCs does not necessarily lead to efficient presentation of Ag on the cell surface (46). Consequently, it is presumed that the endocytosis of mature DCs plays different functional roles from immature DCs, such as rapid removal of the bacteria (46).

In circulating T cells, which require S1P₁ for egress from lymph nodes, FTY720 and other S1P₁ agonists cause S1P₁ inactivation via receptor down-regulation and induce sequestration of circulating lymphocytes into secondary lymphoid tissues and the thymus. This demonstrates their immunosuppressive effects against several types of autoimmune disorders (18, 20, 39, 47). DCs migrate to lymph nodes through afferent lymphatic vessels, whereas T cells do so via high endothelial venules (1, 2). Both processes for entry are dependent on CCR7. Our preliminary results suggested that the process for entry did not depend on S1P₃ because LPS-stimulated mature DCs prepared from S1P₃-knockout mice injected into the mouse footpad can migrate to draining lymph nodes (Y. Maeda, K. Sugahara, and K. Chiba, unpublished observation).

In contrast to T cell migration, it has been widely believed that after DCs migrate to a lymph node they die there and do not leave through efferent lymphatic vessels (48, 49). This idea was based

partly on the experimental difficulties in detecting the few DCs in the efferent lymph. Recently, Cavanagh et al. (50) reported that DCs are found not only in the blood but also in thoracic duct lymph in mice, suggesting that they can reenter the circulation from the periphery. Therefore, DCs released into the efferent lymph would reach the bloodstream and might have an effect on the induction of immune responses in distal organs. Indeed, injection of DCs into the skin can lead to priming of T cells in the spleen: suggesting that DCs can migrate into the bloodstream from the skin or skin-draining lymph nodes (51). Furthermore, Bonasio et al. (52) reported that circulating DCs can home to the thymus and induce clonal deletion of developing Ag-specific T cells in the thymus, suggesting that peripheral DCs may contribute to central tolerance. In conclusion, we provide evidence that SIP-induced migration of murine mature DCs and T cells is mediated by SIP₃ and SIP₁, respectively. Therefore, SIP₃ may be also involved in the egress of DCs from lymph nodes, as well as SIP₁, on T cells. To clarify the role of SIP₃ on DC migration in vivo, we are currently analyzing turnover of DCs under steady-state or inflammatory conditions in SIP₃-knockout mice.

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Disclosures

The authors have no financial conflict of interest.

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