

Role of Sphingosine 1-Phosphate (S1P) Receptor 1 in Experimental Autoimmune Encephalomyelitis

—I. S1P-S1P1 Axis Induces Migration of Th1 and Th17 Cells

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Received October 11th, 2013; revised November 2nd, 2013; accepted November 12th, 2013

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ABSTRACT

Infiltration of myelin-specific helper T (Th) cells into the central nervous system (CNS) plays a key role in pathogenesis of experimental autoimmune encephalomyelitis (EAE). In this study, we investigated the involvement of sphingosine 1-phosphate (S1P)-S1P receptor 1 (S1P1) axis in lymphocytes for EAE development when C57BL/6 mice were immunized with myelin oligodendrocyte glycoprotein (MOG). The expression of S1P1 mRNA and S1P responsiveness of lymphocytes in draining lymph nodes (DLN) were down-regulated markedly after MOG immunization until onset of EAE. Accompanying with reacquisition of down-regulated S1P1 transcript and S1P responsiveness in DLN lymphocytes, MOG-immunized mice developed EAE symptoms with significant infiltration of Th1 and Th17 cells into the CNS and a marked elevation of IFN- γ , T-bet, IL-17, and ROR γ t mRNA expressions. Prophylactic administration of an S1P1 functional antagonist, fingolimod hydrochloride (FTY720, 0.3 mg/kg, orally) significantly inhibited EAE development and almost completely prevented infiltration of Th1 and Th17 cells into the CNS with a marked reduction of IFN- γ , T-bet, IL-17, and ROR γ t mRNA expressions. Similar results were obtained by treatment with an S1P1-selective agonist, SEW2871 or an S1P lyase inhibitor, 2-acetyl-4-tetrahydroxybutylimidazole. Moreover, FTY720-phosphate and SEW2871 inhibited *in vitro* migration of Th1 and Th17 cells toward S1P but did not affect cytokine production or generation of Th1 or Th17 cells. These results suggest that reacquisition of S1P1 expression in DLN lymphocytes plays a major role in trafficking of myelin antigen-specific Th1/Th17 cells from DLN to the CNS in EAE and that prophylactic effect of FTY720 on EAE is predominantly caused by functional antagonism via lymphocytic S1P1.

Keywords: Sphingosine 1-Phosphosphate Receptor 1; Fingolimod Hydrochloride (FTY720); Experimental Autoimmune Encephalomyelitis; Th1 Cells; Th17 Cells

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) is a CD4 T cell-mediated inflammatory disease of the central nervous system (CNS) which clinically manifests as ascending paralysis [1,2]. It can be induced in susceptible animals by immunization with myelin proteins or peptides or by adoptive transfer of myelin-specific CD4 T cells. EAE shares many clinical and histopathological features with multiple sclerosis (MS) and is a commonly used animal model of this human autoimmune disease [3,4]. The generation of effector CD4 T cells is a critical event for the progression of EAE. EAE was believed to

be a Th1-induced autoimmune disease because of the increased expression of Th1 cytokines in the affected CNS and because injection of myelin specific CD4 Th1 but not Th2 cells into immune-competent mice is sufficient to induce EAE [5-9]. On the other hand, interleukin 17 (IL-17)-producing Th17 cells have been implicated in pathogenesis of EAE [10-12]. It is currently thought that IL-17 up-regulates the expression of pro-inflammatory cytokines/chemokines that mediate inflammation in the CNS to promote EAE [13,14]. In contrast, Th2 cells and regulatory T cells play a protective role in EAE [15-17].

A lipid mediator, sphingosine 1-phosphate (S1P) and its receptor 1 (S1P1) play an essential role in lymphocyte egress from the secondary lymphoid organs (SLO)

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and thymus [18-22]. Fingolimod hydrochloride (FTY720) is a first-in-class, orally active S1P1 receptor functional antagonist [23-28], and has been used as a first oral drug for relapsing MS in more than 70 countries, because of its superior efficacy compared with interferon (IFN)- β -1a injection in relapsing-remitting MS [29-33]. FTY720 is phosphorylated to FTY720-phosphate (FTY720-P) by sphingosine kinases and FTY720-P down-regulates S1P responsiveness of lymphocytes by internalization and degradation of S1P1 [27,34-36]. Consequently, FTY720-P converted from FTY720 acts as a functional antagonist at lymphocytic S1P1 and inhibits S1P-S1P1 axis-mediated lymphocyte egress from the SLO and thymus [19].

Several reports have been demonstrated that FTY720 shows marked prophylactic and therapeutic effects on EAE in mice and rats [37-41]. As reported previously, oral administration of FTY720 (0.3 to 1 mg/kg) after establishment of EAE showed almost complete preventing effects on relapsing of EAE induced by myelin proteolipid protein (PLP) in SJL/J mice [39]. In this EAE model, treatment with FTY720 markedly reduced infiltration of PLP-specific Th17 and Th1 cells into the spinal cord of EAE mice. On the contrary, FTY720 increased the frequency of PLP-specific Th17 and Th1 cells to approximately 3-fold in inguinal lymph nodes. These findings suggest that FTY720 shows preventing effects on relapsing of EAE by inhibiting egress of myelin antigen-specific Th17 and Th1 cells from draining lymph nodes (DLN).

In this study, we investigated the role of S1P-S1P1 axis in DLN lymphocytes for EAE development when C57BL/6 mice were immunized with myelin oligodendrocyte glycoprotein (MOG). Our findings suggest that S1P1 expression in DLN lymphocytes was down-regulated immediately after antigen immunization and that the reacquisition of S1P1 expression in DLN lymphocytes triggers the trafficking of Th1 and Th17 cells into the CNS in EAE. We also demonstrate that prophylactic effect of FTY720 on EAE is likely due to sequestration of myelin-specific Th1/Th17 cells into DLN by down-regulation of lymphocytic S1P1.

2. Materials and Methods

2.1. Mice

Inbred strains of female C57BL/6 mice were purchased from Charles River Japan and were used at 7 to 9 weeks of age. All animal experiments were performed under an experimental protocol approved the ethics review committee for animal experimentation of Research Division, Mitsubishi Tanabe Pharma Corporation.

2.2. Agents and Antibodies

FTY720 was synthesized according to the method de-

scribed previously [23] and was dissolved in distilled water for oral administration. The (*S*)-enantiomer of FTY720-P (>99.5% enantio excess) was synthesized according to the previous method [26] and was dissolved in ethanol for *in vitro* experiments. An S1P1-selective agonist, SEW2871 and an S1P lyase inhibitor, 2-acetyl-4-tetrahydroxybutylimidazole (THI) were synthesized and used according to the respective methods reported previously [42,43]. S1P was purchased from Sigma-Aldrich.

MOG₃₅₋₅₅ (MEVGWYRSPFSRVVHLYRNGK) was obtained from Peptide Institute. RPMI 1640 medium was supplemented with 10% fetal calf serum (FCS) which was pretreated with charcoal, 10 mM HEPES, 50 U/ml penicillin, 50 μ g/ml streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 μ M 2-mercaptoethanol. Cy-Chrome-conjugated rat anti-mouse CD4 monoclonal antibody (mAb) (GK1.5), fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IFN- γ mAb (XMG1.2), phycoerythrin (PE)-conjugated rat anti-mouse IL-17 mAb (TC11-18H10.1), PE-conjugated rat anti-mouse CD3 mAb (RA3-6B2), and Cy-Chrome-conjugated rat anti-mouse CD8 mAb (53 - 6.7) were purchased from BD Bioscience. Hamster anti-mouse CD3 mAb (145-2C11), FITC-conjugated rat anti-mouse CD4 mAb (RM4-5), hamster anti-mouse CD28 mAb (37.51), and rat anti-mouse CD16/32 mAb (93, FcR block) were purchased from eBiosciences. Rat anti-mouse IFN- γ mAb (RMMG-1) and rat anti-mouse IL-4 mAb (11B11) were obtained from Biosource. IL-2, IL-6, and transforming growth factor (TGF)- β 1 were purchased from Peprotech. IL-23 was obtained from R&D Systems.

2.3. EAE Induction

For the induction of EAE, C57BL/6 mice received a single immunization of MOG₃₅₋₅₅ in Freund's complete adjuvant containing killed *Mycobacterium tuberculosis* H37Ra subcutaneously on day 0, followed by intravenous injection with 200 ng of pertussis toxin (List Biological Laboratories) on day 0 and 2 [39]. Individual mice were scored for clinical signs of EAE using the following criteria: 0, no paralysis; 0.5, stiff tail; 1, limp tail; 1.5, limp tail with inability to right; 2, paralysis of one limb; 2.5, paralysis of one limb and weakness of one other limb; 3, complete paralysis of both hind limbs; 4, moribund state; 5, death.

2.4. Real Time Polymerase Chain Reaction

Total RNA was extracted using TRIZOL (Invitrogen) and concentrations of total RNA were measured spectrophotometrically. A two-step quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to determine various molecules mRNA ex-

pression using the relative standard curve method, with cellular housekeeping enzyme, GAPDH as the normalization control. 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 7900 Sequence Detection System (Applied Biosystems). In the real-time PCR, following TaqMan probes (6-carboxy-fluorecein label)/primers were used: CD3 (Mm00599683_m1), IFN- γ (Mm00801778_m1), TNF- α (Mm00443258_m1), T-bet (Mm00450960_m1), IL-17 (Mm00439619_m1), TGF- β 1 (Mm00441724_m1), IL-6 (Mm00446190_m1), and IL-23 (Mm00518984_m1) in the presence of GAPDH TaqMan probe (VIC label)/primer. To measure the level of mRNA of S1P1 and ROR γ t, real time PCR was performed with an ABI PRISM 7900 sequence detector using SYBR Green PCR master mix (Applied Biosystems). 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 [34], ROR γ t forward, CCG CTG AGA GGG CTT CAC; ROR γ t reverse, TGC AGG AGT AGG CCA CAT TAC A [44]. The reaction was incubated for 2 min at 50°C denatured for 10 min at 95°C and subjected to 40 step amplification cycles with annealing/extension at 60°C for 1 min followed by denaturation at 95°C for 15 s. Data were analyzed using Sequence Detector software (Applied Biosystems). For quantification, standard curves were generated for various molecules using serially diluted cDNA samples from the spinal cord in EAE mice (day 14 after immunization). For every sample, the level of mRNA normalized by calculating the ratio of each target molecule/GAPDH level.

2.5. Intracellular Cytokines Staining

Lymphocytes prepared from the inguinal lymph nodes or spinal cords were stimulated with 50 ng/ml phorbol-12-myristate-13-acetate and 500 nM ionomycin in the presence of 2 µM monensin for 5 h in RPMI 1640 medium containing 10% FCS at 37°C in 5% CO₂. After blocking with rat anti-mouse CD16/32 mAb, the cells were stained with Cy-Chrome-conjugated anti-mouse CD4 mAb and permeabilized with 0.5% Triton X-100. The intracellular cytokine staining was carried out by using FITC-conjugated anti-mouse IFN- γ mAb and PE-conjugated anti-mouse IL-17 mAb. Flow cytometry analysis was conducted using FACScan with CellQuest software (Becton Dickinson).

2.6. Migration Assay

Migration assays were conducted according to the method described previously [34]. After serum starvation, mouse inguinal lymph node lymphocytes (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 10 nM S1P solution or medium in the bottom wells. All migration assays were conducted in RPMI 1640 medium containing 0.5% fatty acid free bovine serum albumin for 3 h at 37°C in 5% CO₂. 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 CD4 T cells, Th1 cells, and Th17 cells. The number of the cells in the starting population and the migrated population was determined by flow cytometry with an Epic-XL (Beckman Coulter), calculated for each phenotype, and the percent migration was determined from these values.

2.7. Generation of Th1 and Th17 Cells *in Vitro*

Mouse spleen cells were purified to >95% of CD4 T cell by passing through mouse CD4 subset enrichment columns (R&D Systems). CD4 T cells (10^6 cells) were stimulated with anti-CD3 mAb (10 µg/ml) and anti-CD28 mAb (1 µg/ml) in RPMI 1640 medium containing 10% FCS for 48 h under Th1 condition: anti-IL-4 mAb (20 µg/ml); or Th17 condition: IL-6 (20 ng/ml), TGF- β 1 (10 ng/ml), anti-IFN- γ mAb (10 µg/ml), and anti-IL-4 mAb (20 µg/ml) [11,45]. Then the cells under Th1 or Th17 condition were added IL-2 (10 ng/ml) or IL-23 (5 ng/ml), respectively and cultured for additional 96 h. Proportions of Th1/Th17 cells were analyzed by intracellular cytokine staining according to the method as described above. The recovered cells (2.5×10^4 cells) were re-stimulated with anti-CD3mAb plus anti-CD28 mAb for 24 h and amounts of IFN- γ and IL-17 in the culture supernatants were determined by ELISA.

2.8. Statistical Analyses

Results were express as the mean \pm standard error mean (SEM). Statistical differences of EAE clinical scores were analyzed by Mann-Whitney *U* test. In other experiments, statistical differences were calculated by Student's *t*-test. Differences between groups were considered significant when $p < 0.05$ (*: $p < 0.05$, **: $p < 0.01$).

3. Results

3.1. Reacquisition of S1P1 Expression in DLN Lymphocytes Triggers Infiltration of Th1/Th17 Cells into the CNS

When C57BL/6 mice were immunized with MOG₃₅₋₅₅ peptide on day 0, one mouse developed EAE on day 11 and more than 80% of mice showed EAE associated symptoms on day 14. To investigate the involvement of

S1P-S1P1 axis in EAE development, we analyzed S1P1 mRNA expression in the inguinal DLN by real time RT-PCR and S1P responsiveness of DLN lymphocytes by migration assay toward 10 nM S1P. As shown in **Figure 1(A)**, mean clinical scores were significantly elevated on day 14 to 28. The S1P1 mRNA expression in DLN down-regulated markedly on day 3, was maintained low until onset of EAE (day 9), and thereafter recovered gradually along with EAE development. Similarly, S1P responsiveness of DLN lymphocytes decreased markedly on day 3, was kept low until day 11, and thereafter returned to the level comparable with that before immunization. The mRNA expressions of IFN- γ and IL-17 in DLN increased significantly on day 3 to 6 and then decreased to the level before immunization (**Figure 1(B)**). On the other hand, the mRNA expressions of IFN- γ and IL-17 in the spinal cord elevated significantly on day 14 to 28 whereas no detectable level of IFN- γ /IL-17 mRNA was found before EAE onset (day 6) (**Figure 1(C)**).

Next, we analyzed infiltration of Th1 and Th17 cells into the spinal cord of EAE mice. As shown in **Figure 2(A)**, proportions of Th1 cells and Th17 cells in DLN began to increase on day 3, peaked on day 6 to 9, and decreased gradually along with EAE development. On the other hand, significant numbers of Th1 cells (22.4% to 52.3% of CD4 T cells) and Th17 cells (4.4% to 5.1% of CD4 T cells) were infiltrated into the spinal cord of EAE mice on day 14 to 28 (**Figure 2(B)**). We also confirmed no infiltration of Th1/Th17 cells in the spinal cord before the onset of EAE (data not shown). These results

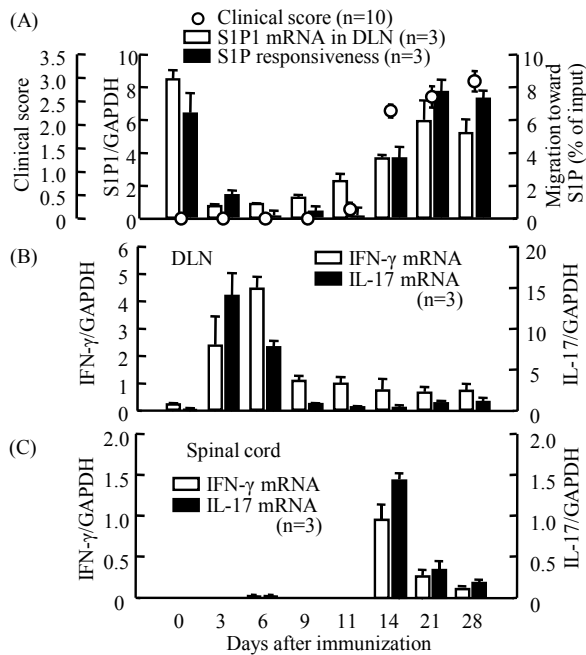


Figure 1. S1P1 transcript and S1P responsiveness in DLN, and IFN- γ /IL-17 mRNA expression in the DLN and spinal cord of EAE mice.

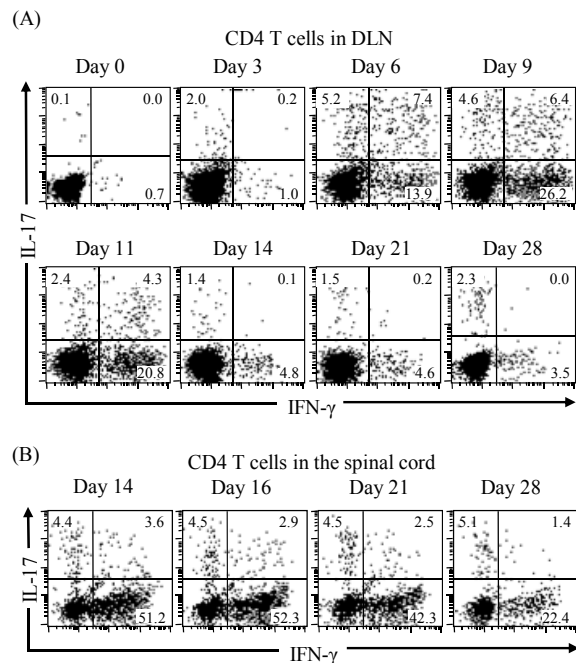


Figure 2. Frequency of Th1 and Th17 cells in the DLN and spinal cord of EAE mice.

suggest that reacquisition of S1P1 expression in DLN lymphocytes triggers infiltration of Th1/Th17 cells into the CNS and EAE development.

3.2. FTY720 Inhibits EAE by Reducing Infiltration of Th1/Th17 Cells into the CNS

To clarify the involvement of S1P1 on trafficking of Th1/Th17 cells from DLN to the CNS, we administered FTY720 (0.3 mg/kg, orally once a day) from day 0 to 16 to MOG₃₅₋₅₅-immunized mice. Consistent with previous studies, the prophylactic administration of FTY720 resulted in a marked delay in the onset of EAE with a significant decrease of mean clinical scores (**Figure 3(A)**). The mRNA expressions of CD3, IFN- γ , TNF- α , T-bet, IL-17, and ROR γ t in the spinal cords were significantly reduced to less than 10% of vehicle-treated EAE control on day 16 (**Figure 3(B)**). We also analyzed the infiltration of Th1 and Th17 cells into the spinal cords by intracellular cytokine staining on day 16. The number of lymphocytes prepared from the spinal cords was extremely decreased to less than 1% of control by treatment with FTY720 (lymphocytes in the spinal cords: EAE control, 9.4×10^5 cells/5mice; FTY720, 0.05×10^5 cells/5mice). Flow cytometry analyses revealed that FTY720 markedly decreased the numbers of Th1/Th17 cells in the spinal cord of EAE mice (**Figure 3(C)**).

There were no clear change in mRNA levels of CD3, IFN- γ , TNF- α , and T-bet whereas those of IL-17 and ROR γ t were increased to approximately 1.5-fold in DLN

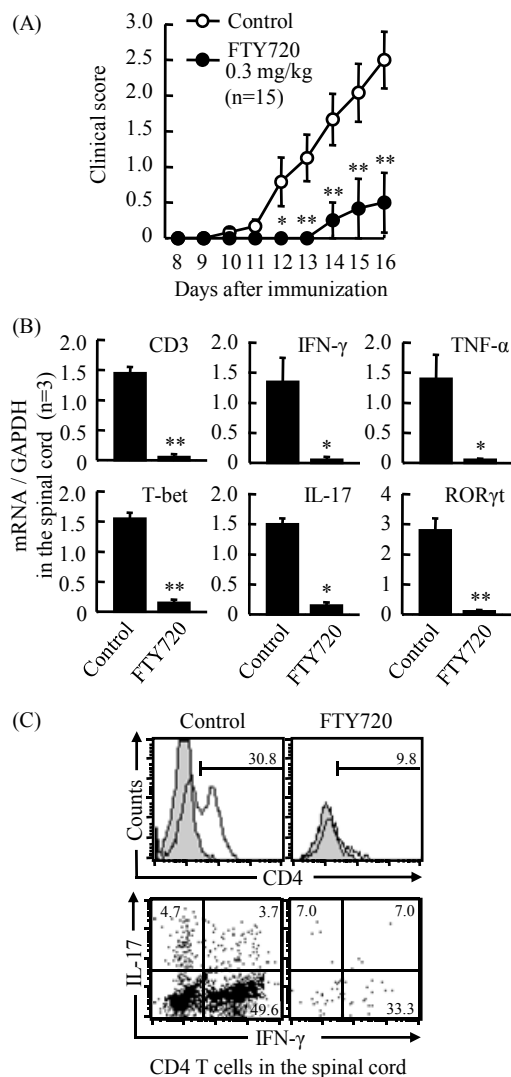


Figure 3. Prophylactic administration of FTY720 (0.3 mg/kg p.o.) inhibits EAE and decreases infiltration of Th1 and Th17 cells into the spinal cord of EAE mice.

of EAE mice given FTY720 (**Figure 4(A)**). In addition, no change was seen in mRNA level of IL-6, TGF- β 1, or IL-23 (data not shown). Flow cytometry analyses demonstrated that the proportions of Th1 and Th17 cells in DLN are increased to 2- to 4-fold by FTY720 treatment (**Figure 4(B)**). These findings suggest that prophylactic FTY720 inhibits EAE development by sequestering Th1/Th17 cells into the DLN and reducing infiltration of these Th cells to the CNS.

3.3. S1P-S1P1 Axis Regulates Trafficking of Th1/Th17 Cells from DLN to the CNS in EAE

An S1P1-selective agonist, SEW2871 and an S1P lyase inhibitor, THI have shown to reduce circulating lymphocytes in the blood by inhibiting S1P1-dependent lym-

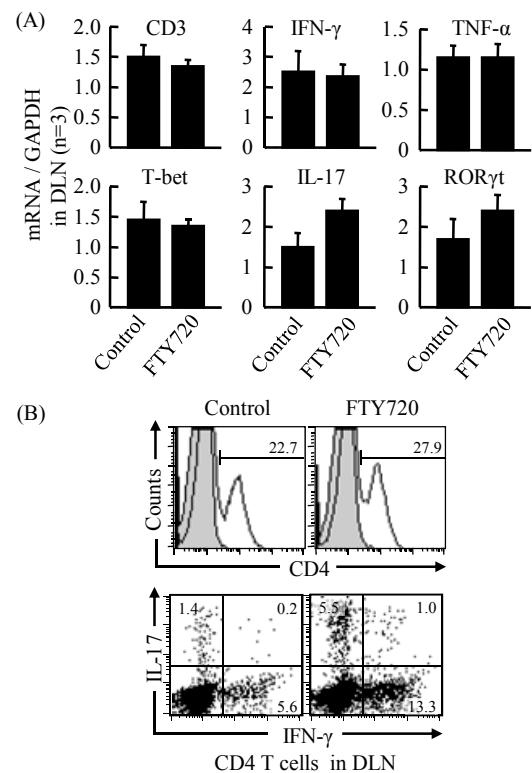


Figure 4. Prophylactic administration of FTY720 (0.3 mg/kg p.o.) increases proportions of Th1 and Th17 cells in DLN of EAE mice.

phocyte egress from the SLO [24,42,46,47]. Prophylactic administration of SEW2871 (30 mg/kg, orally twice a day) or THI (50 mg/kg, orally twice a day) significantly inhibited development of EAE (**Figure 5(A)**) with a significant reduction of mRNA expressions of CD3, IFN- γ , TNF- α , T-bet, IL-17, and ROR γ t (**Figure 5(B)**). Moreover, SEW2871 and THI markedly decreased the number of CD4 T cells and the proportions of Th1 and Th17 cells in the spinal cord of EAE mice (**Figure 5(C)**).

3.4. S1P-S1P1 Axis Induces Migration of Th1 and Th17 Cells

It has been well documented that S1P-S1P1 axis plays an essential role in lymphocyte egress from the SLO. Indeed, S1P at 10 to 100 nM induces migration of murine naive CD4 T cells *in vitro*; however it remains unclear whether S1P can induce migration of Th1 or Th17 cells.

In this study, we found that S1P (10 nM) significantly induces *in vitro* migration of both Th1 and Th17 cells from DLN lymphocytes of EAE-established mice (**Figure 6(A)**). Migration of Th1 cells or Th17 cells toward S1P was almost completely inhibited by pretreatment with FTY720-P at 1 nM or higher (**Figure 6(A)**). Similar results were obtained by pretreatment with 300 nM SEW2871 (data not shown). On the other hand, FTY720-P

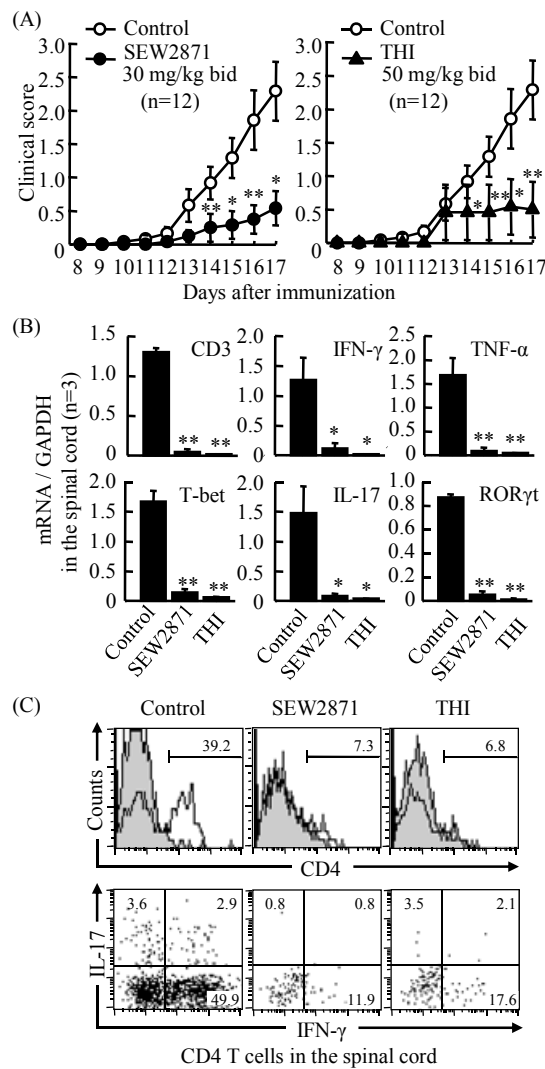


Figure 5. SEW2871 (30 mg/kg p.o. bid) and THI (50 mg/kg p.o. bid) inhibit EAE development and decrease infiltration of Th1 and Th17 cells into the spinal cord of EAE mice.

up to 100 nM did not affect either IFN-γ production by Th1 cells or IL-17 production by Th17 cells (Figure 6(B)). Furthermore, FTY720-P showed no effect on generation of either Th1 or Th17 cells *in vitro* (Figure 6(C)).

4. Discussion

EAE is a CD4 T cell-dependent animal model for human MS and development of EAE is likely due to infiltration of encephalitogenic, myelin antigen-specific Th1 cells and/or Th17 cells into the CNS [3,4,6]. Since the pathogenic Th1/Th17 cells are originally generated from naïve CD4 T cells after myelin antigen presentation in the DLN [10-12,48-50], trafficking of these Th1/Th17 cells from DLN to CNS is thought to play a key role in EAE development. On the other hand, it is strongly suggested that S1P-S1P1 axis plays an important role in lymphocyte

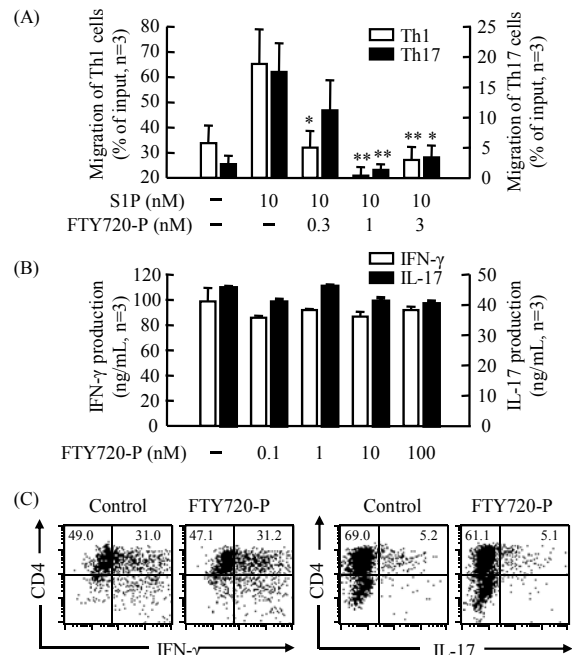


Figure 6. FTY720-P inhibits migration of Th1 and Th17 cells toward S1P but not cytokine production or generation of Th1/Th17 cells.

trafficking between DLN and inflammatory sites because lymphocyte egress from the SLO depends on lymphocytic S1P1 [19]. However it remains unclear relation between S1P-S1P1 axis in DLN lymphocytes including pathogenic Th1/Th17 cells and EAE development.

In the present study, we demonstrated that level of S1P1 mRNA expression in DLN was markedly down-regulated after immunization with MOG antigen and was maintained low for several days until EAE development. Similarly, S1P responsiveness of DLN lymphocytes strikingly reduced after antigen exposure and was kept low until the onset of EAE. Our flow cytometry data revealed that the numbers of Th1 cells and Th17 cells increased in DLN before EAE development, suggesting generation of Th1/Th17 cells by antigen stimulation. On the contrary, the number of Th1/Th17 cells decreased in DLN accompanying with the recovery from down-regulated S1P1 transcript and reacquisition of S1P responsiveness in DLN lymphocytes. In parallel, significant numbers of infiltrated Th1/Th17 cells were found in the CNS and most of MOG-immunized mice showed EAE associated symptoms. Namely, our results imply that encephalitogenic Th1/Th17 cells begin to infiltrate into the CNS when down-regulated lymphocytic S1P1 expression is recovered to a homeostatic level in DLN.

During immune response, antigen-specific T cells are transiently retained within DLN, undergoing activation, and clonal expansion and then exiting as effector cells. This retention mechanism is believed to involve transient

loss of S1P responsiveness by down-regulation of S1P1 receptor on activated T cells. Matloubian *et al.* have been reported using OVA-specific DO11.10 TCR transgenic T cells that at one day after antigen exposure, the activated antigen-specific T cells in DLN had lost their responsiveness to S1P and they had down-regulated S1P1 expression in mRNA level markedly [19]. On the contrary, 3 days after antigen immunization, antigen-specific T cells appeared in circulation, and lymphocytes in DLN restored S1P responsiveness and increased S1P1 mRNA expression. Therefore, antigen-induced down-regulation of S1P1 transcript and S1P responsiveness is associated with the initial retention of antigen-specific activated T cells in DLN, and reacquisition of S1P responsiveness is associated with their exit. However, there is no report on lymphocyte retention into DLN by down-regulation of S1P1 transcript after antigen exposure in mouse EAE induced by MOG.

Our findings in MOG-induced mouse EAE clearly demonstrated that S1P1 mRNA expression and S1P responsiveness in DLN were markedly down-regulated after MOG immunization until the onset of EAE whereas the reacquisition of S1P1 mRNA expression and S1P responsiveness in DLN was observed in parallel with the development of EAE. On the other hand, the mRNA expressions of both IFN- γ and IL-17 and the frequency of Th1 and Th17 cells in DLN were increased markedly after MOG antigen exposure, suggesting retention of antigen-activated CD4 T cells in DLN for their maturation to Th1 and Th17 cells. Indeed, CD4 T cells in DLN on day 3 to 9 after MOG antigen immunization showed no detectable migratory response toward S1P.

Prophylactic administration of FTY720, SEW2871, or THI inhibited the development of EAE and decreased the infiltration of Th1 and Th17 cells into the CNS markedly. As reported previously, FTY720-P (0.1 nM or higher) and SEW2871 (300 nM or higher) can induce down-regulation of S1P1 in S1P1 stably-expressing Chinese hamster ovary cells and CD4 T cells [34]. On the other hand, THI inhibits S1P lyase activity *in vivo* and generated high S1P condition down-regulates S1P1 expression [42]. From these results, it is presumed that prophylactic administration of these compounds *in vivo* induces down-regulation of S1P1 and loss of S1P responsiveness in lymphocytes and inhibits egress of pathogenic Th1 and Th17 cells from DLN in EAE mice.

Although it has been reported that naïve murine CD4 T cells migrate 10 to 100 nM S1P [19], it remained unclear whether Th1 or Th17 cells can migrate toward S1P. Our results clearly demonstrated that S1P (10 nM) can induce migratory response of both Th1 and Th17 cells prepared from EAE mice. Furthermore, pretreatment with FTY720-P or SEW2871 almost completely inhibited the migration of Th1 and Th17 cells toward S1P.

These results suggest that S1P at physiological concentration induces migration of encephalitogenic Th1 and Th17 cells via S1P1 and that FTY720-P leads to loss of S1P responsiveness of these Th cells by internalization and subsequent degradation of S1P1.

Liao *et al.* have been reported that like IL-23, S1P at 1000 nM can enhance generation of Th17 cells when CD4 T cells were stimulated with anti-CD3 mAb plus anti-CD28 mAb and a mixture of TGF- β 1, IL-1, and IL-6 [51]. Since FTY720-P at 100 nM inhibits IL-17 production from CD4 T cells by stimulation with 1000 nM S1P, it is proposed that immunosuppression by FTY720 may be attributable partly to inhibition of Th17-mediated inflammation. On other hand, our results indicate that FTY720-P up to 100 nM showed no effect on generation or cytokine production of Th1 and Th17 cells without adding exogenous S1P. Furthermore, when THI is administered to mice, S1P concentrations increased to approximately 10 to 100-fold in the blood, lymph, and SLO [42,46]; however no enhancement of frequency of Th17 cells in DLN lymphocytes was seen in THI-treated EAE mice. Because S1P concentrations are kept low in the SLO [42,46], it is likely that low S1P condition in DLN does not affect the generation of Th17 cells.

In conclusion, our results in this study imply that S1P-S1P1 axis induces migration of Th1 and Th17 cells and plays a regulatory role in the trafficking of pathogenic Th1 and Th17 cells from DLN to the CNS in EAE development.

5. Acknowledgements

We thank Dr. Kunitomo Adachi in Medicinal Chemistry Laboratories I, Mitsubishi Tanabe Parma Corporation for synthesizing (*S*)-enantiomer of FTY720-P, SEW2871 and THI.

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