

Article

FTY720, Sphingosine 1-Phosphate Receptor Modulator, Ameliorates Experimental Autoimmune Encephalomyelitis by Inhibition of T Cell Infiltration

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FTY720, a sphingosine 1-phosphate receptor modulator, induces a marked decrease in the number of peripheral blood lymphocytes and exerts immunomodulating activity in various experimental allograft and autoimmune disease models. In this study, we evaluated the effect of FTY720 and its active metabolite, (S)-enantiomer of FTY720-phosphate [(S)-FTY720-P] on experimental autoimmune encephalomyelitis (EAE) in rats and mice. Prophylactic administration of FTY720 at 0.1 to 1 mg/kg almost completely prevented the development of EAE, and therapeutic treatment with FTY720 significantly inhibited the progression of EAE and EAE-associated histological change in the spinal cords of LEW rats induced by immunization with myelin basic protein. Consistent with rat EAE, the development of proteolipid protein-induced EAE in SJL/J mice was almost completely prevented and infiltration of CD4⁺ T cells into spinal cord was decreased by prophylactic treatment with FTY720 and (S)-FTY720-P. When FTY720 or (S)-FTY720-P was given after establishment of EAE in SJL/J mice, the relapse of EAE was markedly inhibited as compared with interferon- β , and the area of demyelination and the infiltration of CD4⁺ T cells were decreased in spinal cords of EAE mice. Similar therapeutic effect by FTY720 was obtained in myelin oligodendrocyte glycoprotein-induced EAE in C57BL/6 mice. These results indicate that FTY720 exhibits not only a prophylactic but also a therapeutic effect on EAE in rats and mice, and that the effect of FTY720 on EAE appears to be due to a reduction of the infiltration of myelin antigen-specific CD4⁺ T cells into the inflammation site. *Cellular & Molecular Immunology*. 2005;2(6):439-448.

Key Words: FTY720, S1P receptor modulator, lymphopenia, EAE, T cell infiltration

Introduction

2-Amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride (FTY720), a new class of immunomodulator, sequesters circulating mature lymphocyte into secondary lymphoid

tissues and thymus by long-term down-regulation of S1P receptor type 1 (S1P₁) on lymphocytes, and exerts immunomodulating effect (1-6). It has been previously reported that a potent immunosuppressive natural product, ISP-I can be isolated from a culture broth of *Isaria scinclairii*, a kind of vegetative wasp (7-9). The chemical modification of ISP-I led to a novel synthetic compound, FTY720 that has more potent immunomodulating activity and less toxicity than ISP-I (10-13). FTY720 has been shown to be highly effective in prolonging allograft survival in various experimental allograft models (1-4, 14, 15). A striking feature of FTY720 is the induction of a marked decrease in the number of peripheral blood lymphocytes, especially T cells, at doses that prolong allograft survival (1-4). FTY720-induced

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Abbreviations: EAE, experimental autoimmune encephalomyelitis; FTY720, 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride; FTY720-P, FTY720-phosphate; IFN, interferon; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PLP, proteolipid protein; S1P, sphingosine 1-phosphate; S1P₁, sphingosine 1-phosphate receptor type 1; (S)-FTY720-P, (S)-enantiomer of FTY720-phosphate.

lymphopenia is mainly caused by sequestration of circulating mature lymphocytes into secondary lymphoid tissues such as lymph nodes and Peyer's patches (2). Recently, it has been reported that FTY720 is effectively phosphorylated to FTY720-phosphate (FTY720-P) by sphingosine kinase 2 and that FTY720-P is a high affinity agonist at S1P receptors (16, 17). Moreover it has been suggested that FTY720-P induces long-term down-regulation of S1P₁ on lymphocytes and inhibits S1P/S1P₁-dependent lymphocyte egress from secondary lymphoid tissues and thymus (5, 6).

Multiple sclerosis (MS) is a common and often disabling disease of the central nervous system (CNS). Early active MS lesions are characterized by the presence of infiltrated mononuclear cells around venules and small veins, followed by myelin breakdown and astrogliosis, resulting in irreversible disability (18, 19). The etiology of MS remains unknown, but is widely considered to involve the organ-specific autoimmune destruction of CNS myelin mediated by myelin-specific T cells (20, 21). Immunomodulating therapy using cyclophosphamide, interferon- β (IFN- β), or glatiramer acetate is widely used for the treatment of MS (22, 23). Experimental autoimmune encephalomyelitis (EAE), an animal model of human MS, is a demyelinating, inflammatory disease of the CNS and is induced by the immunization of susceptible strains of rats or mice with myelin antigens combined with adjuvant (24). Myelin antigen-specific, autoreactive Th1 cells can be found in the blood, lymphoid tissues and CNS in EAE and MS, and it is highly likely that trafficking and infiltration of myelin antigen-specific Th1 cells into CNS play an important role in the development and progression of EAE and MS (25-27).

As reported previously, immunohistochemical staining and flow cytometric analysis confirmed that FTY720 decreases T cell infiltration into the allograft at doses that show lymphopenia and a prolonging effect on allograft survival (3, 28, 29). These findings strongly suggest that FTY720 exerts immunomodulating activity by decreasing T cell infiltration into inflammatory sites. In this paper, we evaluate the prophylactic and therapeutic effect of FTY720 and its active metabolite, (S)-enantiomer of FTY720-P [(S)-FTY720-P] (30, 31) on EAE in rats and mice using myelin basic protein (MBP), myelin proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) as myelin antigens. We also demonstrate the relationship between the magnitude of improvement for EAE and the decrease in T cell infiltration into the CNS by FTY720 in these EAE models.

Materials and Methods

Animals and agents

Male LEW rats were purchased from Kyudo Co., Ltd. (Yoshitomi, Fukuoka, Japan), and female SJL/J and C57BL/6 mice were purchased from Charles River Japan Inc. (Yokohama, Japan). All animals were used at 8 to 12 weeks of age. PLP₁₃₉₋₁₅₁ (HSLGKWLGHDPDKF) and MOG₃₅₋₅₅ (MEVGWYRSPFSRVVHLYRNGK) were obtained from Bachem AG (Bubendorf, Switzerland) and Peptide Institute

(Osaka, Japan), respectively. FTY720 and (S)-FTY720-P were synthesized according to the methods described previously (11, 30). FTY720 dissolved in distilled water was given orally. (S)-FTY720-P was dissolved in 10% hydroxypropyl- β -cyclodextrin solution and administered intraperitoneally. Recombinant mouse IFN- β (rm-IFN- β) and prednisolone (Predonine[®] for injection) were purchased from Calbiochem (La Jolla, CA) and Shionogi & Co., Ltd. (Osaka, Japan), respectively. All monoclonal antibodies (mAbs) used in this paper were obtained from BD Biosciences (San Jose, CA).

Induction and scoring of EAE

MBP was extracted and purified from the spinal cord of guinea pigs according to the methods described previously (32). For the induction of EAE in LEW rats, guinea pig MBP saline solution (2 mg/ml) was emulsified with an equal volume of Freund's complete adjuvant containing *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). LEW rats were immunized by subcutaneous injection into the right and left hind footpads with the emulsion containing guinea pig MBP at 100 μ g/rat. PLP₁₃₉₋₁₅₁ and MOG₃₅₋₅₅ were used as antigens for the induction of EAE in SJL/J mice and C57BL/6 mice, respectively. A saline solution of PLP₁₃₉₋₁₅₁ at 0.5 mg/mL or MOG₃₅₋₅₅ at 2 mg/ml was emulsified with an equal volume of Freund's complete adjuvant containing *Mycobacterium tuberculosis* H37Ra. SJL/J mice and C57BL/6 mice were immunized by subcutaneous injection into the flank regions with the emulsion containing PLP₁₃₉₋₁₅₁ at 50 μ g/mouse and MOG₃₅₋₅₅ at 200 μ g/mouse, respectively. In addition, MOG₃₅₋₅₅-immunized C57BL/6 mice were given 200 ng of pertussis toxin by intravenous injection twice on the day of immunization and 2 days after. Individual animals were examined daily for clinical signs of neurological deficits scored on a 0 to 5 scale as follows: 0, no abnormality; 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; 5, death.

Histology of spinal cords in EAE

The spinal cords of EAE animals were obtained, fixed in 4% formalin in PBS, and embedded in paraffin. Six μ m-thickness sections were prepared and then stained with hematoxylin and eosin. In rat EAE, the histological score was evaluated as follows: 0, no lesions; 1, solitary lesions with cell infiltrates of low cellular density in the entire section; 2, a few lesions with moderate cell infiltration in each of a few fields; 3, many lesions in almost all fields with massive cell infiltration and accompanying edema (33).

Immunohistochemical staining of spinal cords in mouse EAE

The spinal cords in EAE mice were removed and frozen in isopentane prechilled in liquid nitrogen using embedding medium for frozen tissue specimens (Tissue-Tek OCT compound; Sakura Fine Technical, Tokyo, Japan). Six μ m-thickness frozen sections were immediately fixed in

cold-acetone and were incubated with rat anti-mouse CD3 mAb (clone: 17A2), rat anti-mouse CD4 mAb (clone: GK1.5), or rat anti-mouse CD8a mAb (clone: 53-6.7). The sections were then incubated with appropriate secondary antibodies conjugated with amino acid polymer and peroxidase (Histofine® Simple Stain MAX-PO kit; Nichirei, Tokyo, Japan), colorized with diaminobenzidine in the presence of hydrogen peroxide, and counterstained with hematoxylin.

Flow cytometry

Peripheral blood lymphocytes obtained from EAE mice were stained with FITC-conjugated rat anti-hamster CD3 ϵ mAb (clone: 145-2C11) and PE-conjugated rat anti-mouse CD45R/B220 mAb (clone: RA3-6B2), and the number of T cells and B cells was determined by 2-color flow cytometry using Cytomics™ FC500 (Beckman Coulter Inc., Fullerton, CA). In the analysis of T cell subsets, the numbers of CD4 $^{+}$ T cells and CD8 $^{+}$ T cells were determined by 3-color flow cytometry with FITC-conjugated hamster anti-mouse CD3 ϵ mAb (clone: 145-2C11), PE-conjugated rat anti-mouse CD4 mAb (clone: RM4-5), and PE-Cy5-conjugated rat anti-mouse CD8a mAb (clone: 53-6.7).

Measurement of IFN- γ and GAPDH mRNA levels by TaqMan® PCR

Total RNA was extracted from the spinal cords of EAE mice using RNA isolation reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol and then the concentration of total RNA was measured spectrophotometrically. An aliquot (0.5 μ g) of total RNA from the spinal cord was reverse-transcribed to cDNA in a 50 μ l volume at 25°C for 10 min, 48°C for 30 min and 95°C for 10 min with TaqMan® reverse transcription reagents using a thermal cycler, Gene Amp® PCR System 9700 (Applied Biosystems, Branchburg, NJ). The levels of mRNA for IFN- γ and GAPDH were determined using the TaqMan® polymerase chain reaction (PCR). Five microliters of cDNA were amplified with IFN- γ TaqMan® probe (6-carboxy-fluorescein label)/primer, GAPDH TaqMan® probe (VIC™ label) /primer, and TaqMan® Universal PCR Master Mix in an ABI PRISM™ 7900 Sequence Detection System (Applied Biosystems). The reaction was incubated for 2 min at 50°C, denatured for 10 min at 95°C and subjected to 40 two-step amplification cycles with annealing/extension at 60°C for 1 min followed by denaturation at 95°C for 15 sec. The detection of PCR product was monitored by measuring the increase in fluorescence caused by degradation of the probe. For every sample, the level of IFN- γ mRNA was normalized by calculating the ratio of IFN- γ /GAPDH levels.

Statistical analysis

All values are expressed as mean and standard error mean (SEM) in each group. The statistical differences of EAE scores and histology scores in FTY720- or FTY720-P-treated groups were calculated by Steel's test and compared with vehicle-treated control group. In the IFN- β - or prednisolone-

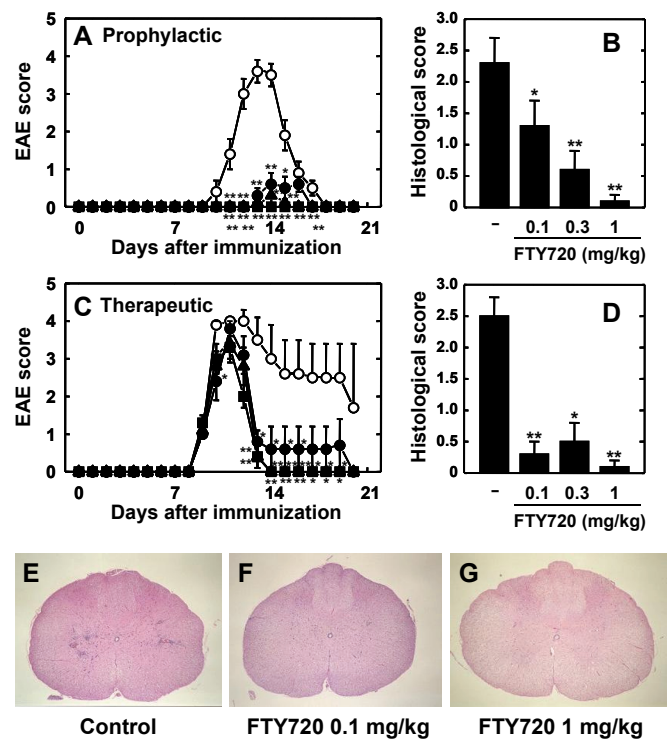


Figure 1. Effect of FTY720 on MBP-induced EAE in LEW rats. LEW rats were immunized with guinea pig MBP at 100 μ g/rat in the presence of Freund's complete adjuvant. FTY720 was administered orally to MBP-immunized LEW rats every day from the day of immunization (prophylactic treatment) or the day of onset (therapeutic treatment) until day 19. Rats in the control group were administered vehicle only. The spinal cords in EAE rats were obtained on day 20 after immunization, and hematoxylin and eosin (HE) staining and histological scoring were performed. ○, Control; ●, FTY720 0.1 mg/kg; ▲, FTY720 0.3 mg/kg; ■, FTY720 1 mg/kg. (A) EAE scores in prophylactic treatment; (B) Histological scores in prophylactic treatment; (C) EAE scores in therapeutic treatment; (D) Histological scores in therapeutic treatment; HE staining of a spinal cord in (E) control EAE rat, (F) FTY720 0.1 mg/kg therapeutically, (G) FTY720 1 mg/kg therapeutically. The results were expressed as the mean \pm SE of 8 animals and statistical differences were calculated by Steel's test (* p < 0.05; ** p < 0.01).

treated group, the statistical differences of EAE score were calculated by the Mann-Whitney U test. The number of lymphocytes (T cells, CD4 $^{+}$ T cells, CD8 $^{+}$ T cells, and B cells), body weight, and the ratio of IFN- γ /GAPDH mRNA levels were analyzed by Dunnett's multi-comparison test. Differences between groups were considered significant when p < 0.05.

Results

Prophylactic and therapeutic effects of FTY720 on MBP-induced EAE in rats

All of the LEW rats in the vehicle-treated control group developed EAE-associated clinical symptoms 10 days after

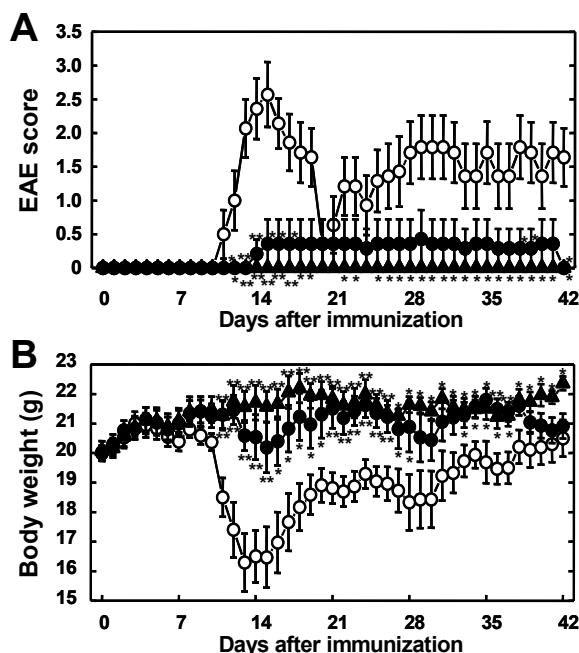


Figure 2. Prophylactic effect of FTY720 on PLP₁₃₉₋₁₅₁-induced EAE in SJL/J mice. SJL/J mice were immunized with PLP₁₃₉₋₁₅₁ at 50 µg/mouse in the presence of Freund's complete adjuvant. FTY720 was administered orally to PLP₁₃₉₋₁₅₁-immunized SJL/J mice every day from the day of immunization for 6 weeks. Mice in the control groups were administered vehicle only. ○, Control; ●, FTY720 0.1 mg/kg; ▲, FTY720 0.3 mg/kg. The results were expressed as the mean ± SE of 7 animals and statistical differences in EAE scores (A) and body weights (B) were calculated by Steel's test and Dunnett's multi-comparison (hyphenation) test, respectively (*, $p < 0.05$; **, $p < 0.01$).

immunization with guinea pig MBP, reaching a maximal level on day 13 followed by a gradual decline (Figure 1A). Elevation of EAE scores was significantly inhibited in groups given FTY720 prophylactically at oral doses of 0.1, 0.3 and 1 mg/kg for 20 days from the day of MBP immunization. Consistent with EAE-associated symptoms, the histological scores in FTY720-treated groups were decreased significantly and dose-dependently as compared with the vehicle-treated control group (Figure 1B). Significantly, there was no increase in the EAE and histological scores in the group treated with FTY720 at 1 mg/kg, indicating a complete prevention of EAE development in MBP-immunized LEW rats.

To evaluate the therapeutic potential of FTY720 in MBP-induced EAE in LEW rats, the administration was started from the day of EAE onset (Figure 1C). In the vehicle-treated control group, EAE had developed by day 9 after immunization and reached a maximal level on day 11 to 13. Thereafter, the mean of EAE scores remained within the 2 to 3 range until day 20, because 4 out of 8 EAE rats died in the control group. Therapeutic administration of FTY720 from the day of EAE onset significantly decreased post-peak EAE-associated clinical signs (Figure 1C). Moreover, the

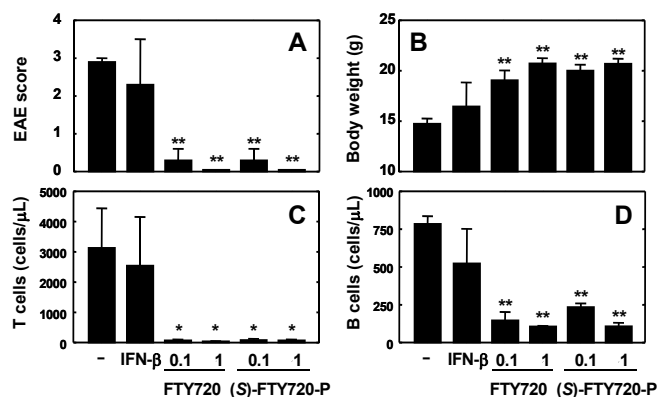


Figure 3. Prophylactic effects of FTY720, (S)-FTY720-P and rm-IFN-β on PLP₁₃₉₋₁₅₁-induced EAE in SJL/J mice. SJL/J mice were immunized with PLP₁₃₉₋₁₅₁ at 50 µg/mouse in the presence of Freund's complete adjuvant. FTY720 (0.1 and 1 mg/kg) was administered orally (*p.o.*) to PLP₁₃₉₋₁₅₁-immunized SJL/J mice every day. (S)-FTY720-P (0.1 and 1 mg/kg) was administered intraperitoneally (*i.p.*) every day and rm-IFN-β (10,000 IU/mouse) were given three times a week *i.p.*, respectively. Mice in the control groups were administered vehicle only. EAE scores, body weights, and the numbers of T and B cells in peripheral blood were determined at day 14 after immunization. The results were expressed as the mean ± SE of 6 animals. Statistical differences in EAE scores (A) were calculated by Steel's test, and those in body weights (B), number of T cells (C), and B cells (D) were done by Dunnett's multi-comparison test (* $p < 0.05$; ** $p < 0.01$).

therapeutic administration of FTY720 resulted in a significant decrease in the histological score of EAE (Figure 1D). The infiltration of inflammatory cells into the spinal cords of EAE rats was inhibited in the FTY720-treated group (Figures 1E, 1F, 1G). A marked decrease in the number of peripheral lymphocytes was observed in all of the FTY720 administration schedules (data not shown). These data indicate that FTY720 not only has a prophylactic but also a therapeutic potential in treating MBP-induced EAE in LEW rats.

Prophylactic and therapeutic effects of FTY720 on PLP-induced EAE in SJL/J mice

In human MS, neurological symptoms relapse over several years; however MBP-induced EAE in LEW rats was monophasic with no relapse. To clarify the therapeutic potential of FTY720 in human MS more precisely, we evaluated the effect of FTY720 and its active metabolite (S)-FTY720-P on relapsing EAE in SJL/J mice induced by PLP₁₃₉₋₁₅₁ immunization. SJL/J mice immunized with PLP₁₃₉₋₁₅₁ emulsified in Freund's complete adjuvant resulted in the development of EAE-associated clinical symptoms and a decrease in body weight 11 days after immunization. EAE scores were rapidly elevated and reached a maximal level on day 15. The first phase of EAE remitted with a low EAE score on day 20 and spontaneously relapsed thereafter (Figure 2A). The elevation of EAE-associated clinical score and the loss of body weight were prevented in groups given

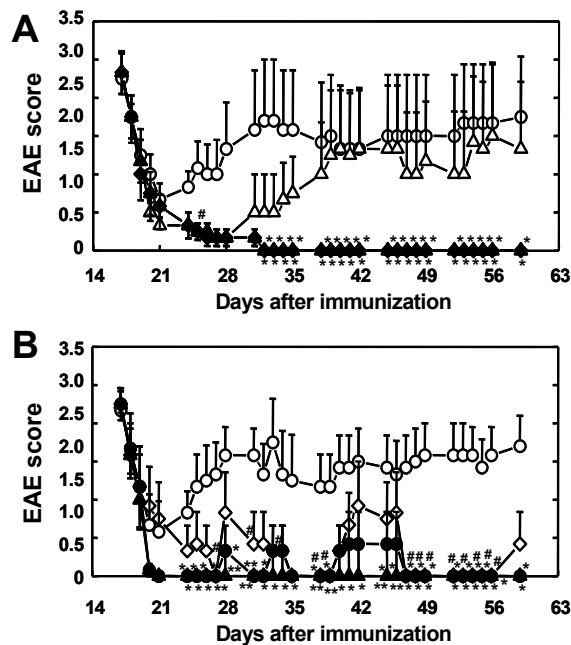


Figure 4. Therapeutic effect of FTY720 on PLP₁₃₉₋₁₅₁-induced EAE in SJL/J mice. SJL/J mice were immunized with PLP₁₃₉₋₁₅₁ at 50 µg/mouse in the presence of Freund's complete adjuvant. EAE-developed mice were pooled, divided into 4 groups and administrations of FTY720, rm-IFN-β and prednisolone were started from day 17 after immunization for 60 days. Mice in the control groups were administered vehicle only. (A) ○, Control; △, rm-IFN-β 10000 IU/mice *i.p.* 3 times a week; ▲, FTY720 0.3 mg/kg *p.o.* daily; ◆, FTY720 1 mg/kg *p.o.* daily; (B) ○, Control; ◇, prednisolone 1 mg/kg *p.o.* daily; ●, FTY720 0.1 mg/kg *p.o.* daily; ▲, FTY720 0.3 mg/kg *p.o.* daily. The results are expressed as the mean ± SE of 7 animals and statistical differences in EAE scores of FTY720 groups were calculated by Steel's test (**p* < 0.05; ***p* < 0.01), and those in rm-IFN-β or prednisolone were done by Mann Whitney U test (#*p* < 0.05).

FTY720 prophylactically at oral doses of 0.1 and 0.3 mg/kg for 42 days from the day of immunization (Figures 2A, 2B). Consistent with MBP-induced EAE in LEW rats, there was no increase in EAE score in the group treated with FTY720 at 1 mg/kg, indicating a complete prevention of PLP₁₃₉₋₁₅₁-induced EAE in SJL/J mice. The prophylactic effects of FTY720, (S)-FTY720-P and rm-IFN-β were compared in PLP₁₃₉₋₁₅₁-induced EAE in SJL/J mice, and the results on day 17 are shown in Figure 3. Oral administration of FTY720 at 0.1 and 1 mg/kg resulted in a markedly preventing effect on EAE score elevation and body weight loss, and induced a marked decrease in the number of T cells and B cells in peripheral blood. Almost the same prophylactic effect was observed when (S)-FTY720-P at 0.1 and 1 mg/kg was administered intraperitoneally to PLP₁₃₉₋₁₅₁-immunized SJL mice from the day of immunization. By contrast, prophylactic treatment with rm-IFN-β at 10,000 IU three times a week intraperitoneally showed no clear effect or no lymphopenia.

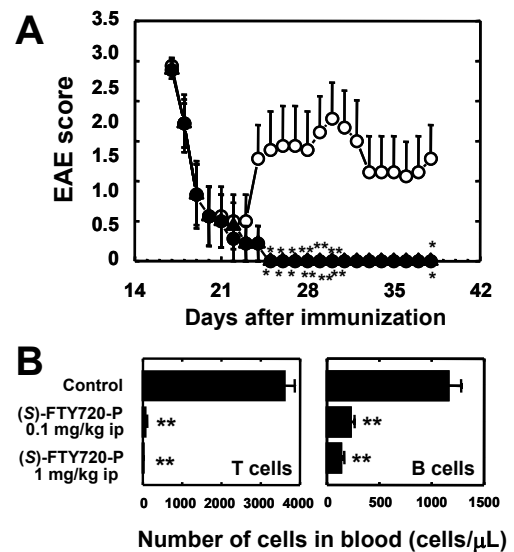


Figure 5. Therapeutic effect of (S)-FTY720-P on PLP₁₃₉₋₁₅₁-induced EAE in SJL/J mice. SJL/J mice were immunized with PLP₁₃₉₋₁₅₁ at 50 µg/mouse in the presence of Freund's complete adjuvant. EAE-developed mice were pooled, divided into 3 groups and therapeutic administration of (S)-FTY720-P was started from day 17 after the immunization for 20 days. Mice in the control groups were administered vehicle only. The numbers of T and B cells in peripheral blood were determined at day 38 after immunization. ○, Control; ●, (S)-FTY720-P 0.1 mg/kg *i.p.* daily; ▲, (S)-FTY720-P 0.3 mg/kg *i.p.* daily. The results are expressed as the mean ± SE of 7 animals. Statistical differences in EAE scores (A) were calculated by Steel's test, and those in numbers of T and B cells (B) were done by Dunnett's multi-comparison test. (**p* < 0.05, ***p* < 0.01).

To evaluate the therapeutic effect of FTY720 on PLP₁₃₉₋₁₅₁-induced, relapsing EAE in SJL/J mice, EAE-developed mice were pooled, divided into 4 groups consisting of six mice, and administration of FTY720 was started 17 days after immunization. EAE-associated clinical signs were decreased rapidly by day 21, and thereafter, relapse of EAE occurred in the vehicle-treated control group (Figure 4A). By contrast, the relapse of EAE was markedly inhibited and no EAE-associated clinical signs were observed from day 32 to 59 in groups given FTY720 at 0.3 and 1 mg/kg therapeutically, indicating complete inhibition of EAE relapse. In the group given rm-IFN-β at 10,000 IU three times a week intraperitoneally, the EAE score was significantly lowered at day 24, and relapse was delayed; however rm-IFN-β failed to inhibit the relapse of EAE. In another experiment, it was confirmed that the magnitude of the therapeutic effect of FTY720 is almost equal to that of prednisolone (Figure 4B). We also evaluated the therapeutic effect of (S)-FTY720-P on relapsing EAE in SJL/J mice induced by PLP₁₃₉₋₁₅₁. (S)-FTY720-P was administered intraperitoneally to mice developed EAE from day 17 after immunization. In results similar to those seen in FTY720 treatment, the relapse of EAE was markedly inhibited in

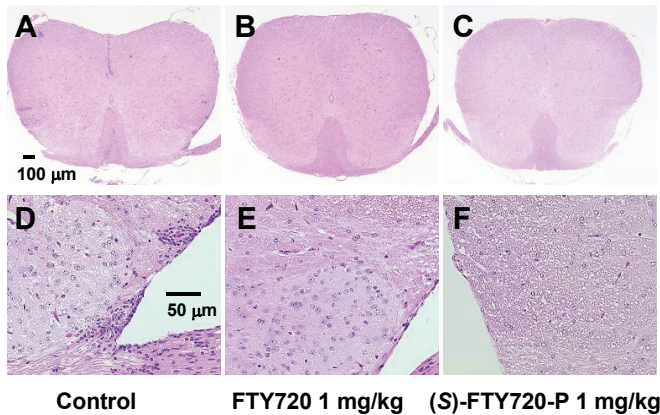


Figure 6. Prophylactic administration of FTY720 and (S)-FTY720-P decreased the infiltration of inflammatory cells in the spinal cord of PLP₁₃₉₋₁₅₁-induced EAE mice. SJL/J mice were immunized with PLP₁₃₉₋₁₅₁ at 50 μg/mouse in the presence of Freund's complete adjuvant and administered FTY720 at 1 mg/kg *p.o.* and (S)-FTY720-P at 1 mg/kg *i.p.* prophylactically. The spinal cords of EAE-developed mice at day 17 after immunization were obtained, and HE staining were performed. Control: (A) (× 100), (D) (× 400); FTY720 1 mg/kg *p.o.*: (B) (× 100), (E) (× 400); (S)-FTY720-P 1 mg/kg *i.p.*: (C) (× 100), (F) (× 400).

groups given (S)-FTY720-P at 0.1 and 1 mg/kg therapeutically (Figure 5A). All the (S)-FTY720-P-treated groups showed a significant decrease in the number of T cells and B cells in peripheral blood on the day following the final administration (Figure 5B).

The infiltration of inflammatory cells was observed in the spinal cord of SJL/J mice 17 days after immunization with PLP₁₃₉₋₁₅₁. Prophylactic administration of FTY720 at 1 mg/kg orally and (S)-FTY720-P at 1 mg/kg intraperitoneally resulted in a marked reduction of the infiltration of inflammatory cells in the spinal cord of PLP₁₃₉₋₁₅₁-immunized SJL/J mice (Figure 6). Immunohistochemical staining analysis using anti-T cell subset mAbs revealed the infiltration of CD4⁺ T cells rather than CD8⁺ T cells into spinal cords, especially the perivascular area and funiculus dorsalis in white matter under pia mater, of PLP₁₃₉₋₁₅₁-immunized SJL/J mice with developed EAE (Figure 7). Prophylactic administration of FTY720 at 1 mg/kg orally and (S)-FTY720-P at 1 mg/kg intraperitoneally markedly decreased the infiltration of CD4⁺ T cells into the spinal cord as compared with the vehicle-treated control EAE group. In addition to the infiltration of CD4⁺ T cells, demyelination was confirmed predominantly in the funiculus dorsalis in white matter under pia mater in another experiment (Figure 8). In the group given FTY720 at 1 mg/kg therapeutically, the area of demyelination and the infiltration of CD4⁺ T cells into the spinal cord were decreased as compared with the vehicle-treated control group.

Therapeutic effect of FTY720 on MOG-induced EAE in C57BL/6 mice

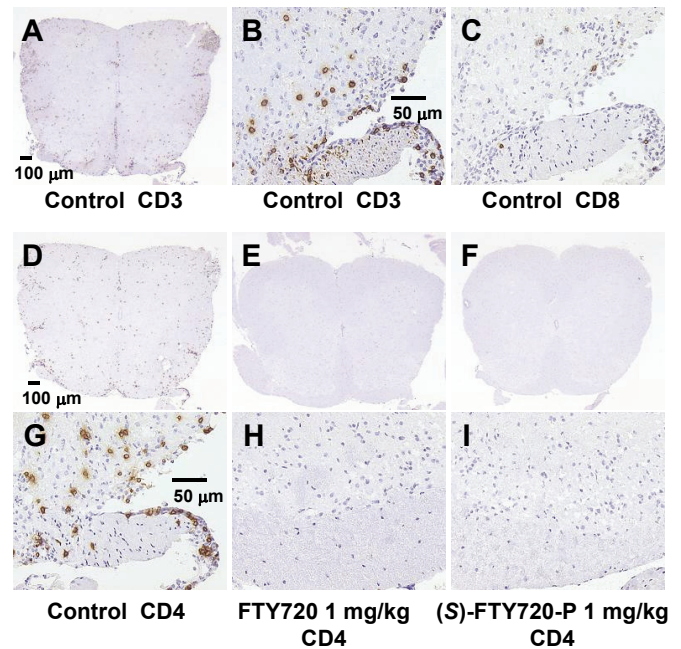


Figure 7. Prophylactic administration of FTY720 and (S)-FTY720-P decreased the infiltration of CD4⁺ T cells in the spinal cord of PLP₁₃₉₋₁₅₁-induced EAE mice. SJL/J mice were immunized with PLP₁₃₉₋₁₅₁ at 50 μg/mouse in the presence of Freund's complete adjuvant and administered FTY720 at 1 mg/kg *p.o.* and (S)-FTY720-P at 1 mg/kg *i.p.* prophylactically. The spinal cords of EAE-developed mice were obtained at day 17 after immunization and immunohistochemical staining were performed. Control: (A) CD3⁺ T cells (× 100), (B) CD3⁺ T cells (× 400), (C) CD8⁺ T cells (× 400), (D) CD4⁺ T cells (× 100), (G) CD4⁺ T cells (× 400); FTY720 1 mg/kg *p.o.*: (E) CD4⁺ T cells (× 100), (H) CD4⁺ T cells (× 400); (S)-FTY720 1 mg/kg *i.p.*: (F) CD4⁺ T cells (× 100), (I) CD4⁺ T cells (× 400).

EAE was developed 12 days after immunization of MOG₃₅₋₅₅ to C57BL/6 mice, and EAE-established mice were divided into 3 groups consisting of eleven mice 17 days after the immunization (Figure 9A). EAE-associated symptoms were maintained during administration period in the vehicle-treated control group of MOG₃₅₋₅₅-immunized C57BL/6 mice. The EAE score was significantly decreased when FTY720 was administered therapeutically at 0.1 and 0.3 mg/kg. The level of IFN-γ mRNA expression in the spinal cords of MOG₃₅₋₅₅-immunized EAE mice was markedly elevated compared to that of normal mice. The elevation of IFN-γ mRNA level was significantly inhibited in groups given FTY720 therapeutically at 28 days after immunization (Figure 9B). Demyelination and infiltration of lymphocytes were observed in the spinal cords in the control MOG₃₅₋₅₅-induced EAE mice at 42 days after immunization. The infiltration of CD4⁺ T cells was more markedly than that of CD8⁺ T cells in the spinal cord of MOG₃₅₋₅₅-induced EAE mice. In FTY720-treated groups, the area of demyelination and the infiltration of CD4⁺ T cells and CD8⁺ T cells in the spinal cord were decreased as compared with the vehicle-

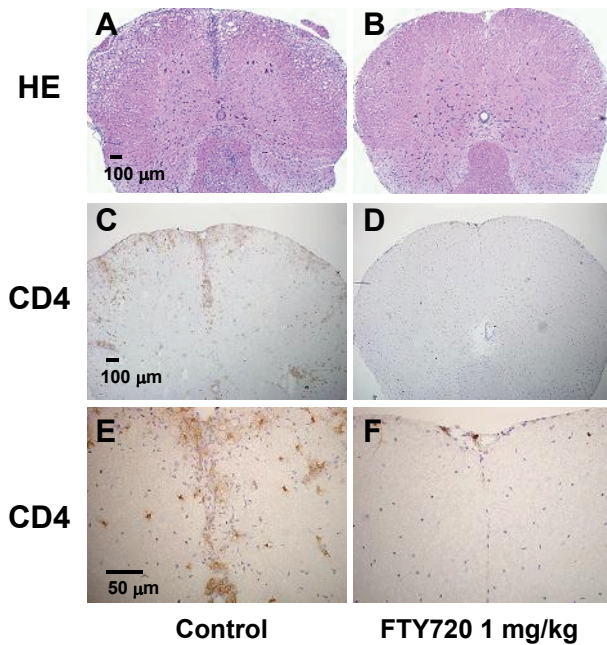


Figure 8. Therapeutic administration of FTY720 decreased the infiltration of CD4⁺ T cells into the spinal cords of PLP₁₃₉₋₁₅₁-induced EAE mice. SJL/J mice were immunized with PLP₁₃₉₋₁₅₁ at 50 μg/mouse in the presence of Freund's complete adjuvant and administered FTY720 at 1 mg/kg *p.o.* therapeutically from day 17. The spinal cords of EAE-developed mice were obtained at day 28, and HE and immunohistochemical staining were performed. Control: (A) HE (× 100), (C) CD4⁺ T cells (× 100), (E) CD4⁺ T cells (× 400); FTY720 1 mg/kg: (B) HE (× 100), (D) CD4⁺ T cells (× 100), (F) CD4⁺ T cells (× 400).

treated control group (Figure 10).

Discussion

MS is a chronic inflammatory and autoimmune disease characterized by discrete areas of inflammation and demyelination that can occur in multiple anatomical locations in the CNS (18, 21-23). Th1 CD4⁺ T cells reactive to myelin produce the proinflammatory cytokines such as IFN-γ and tumor necrosis factor-α, and these cytokines play an important role in orchestrating an immunopathological cascade and mediate the damage to the myelin sheath and eventually the axon (21, 34, 35). In this study, we demonstrate that FTY720, a new class of immunomodulator, has beneficial effects on myelin antigen-induced EAE diseases in rats and mice. Treatment with FTY720 or its active metabolite, (S)-FTY720-P during the course of EAE induced significant attenuation in EAE-associated clinical symptoms, as well as the pathology of disease. Analysis of the mechanism of amelioration by FTY720 or (S)-FTY720-P suggests that treatment with FTY720 or (S)-FTY720-P markedly decreases the infiltration of IFN-γ-producing, CD4⁺ T cells into CNS in myelin antigen-induced EAE. Thus, it is

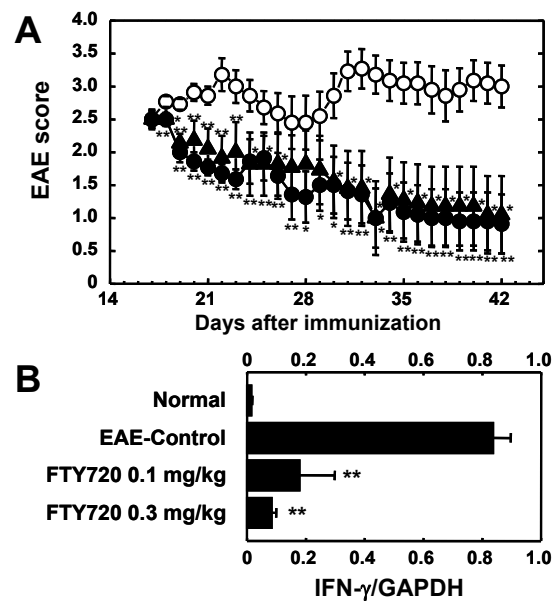


Figure 9. Therapeutic effect of FTY720-P on MOG₃₅₋₅₅-induced EAE in C57BL/6 mice. C57BL/6 mice were immunized with MOG₃₅₋₅₅ at 200 μg/mouse in the presence of Freund's complete adjuvant. In addition, MOG₃₅₋₅₅-immunized C57BL/6 mice were given 200 ng of pertussis toxin by intravenous injection twice on the day of immunization and 2 days after. EAE-developed mice were pooled, divided into 3 groups and therapeutic administration of FTY720 was started from day 17 after the immunization for 25 days. Mice in the control groups were administered vehicle only. (A) EAE scores were expressed as the mean ± SE of 11 animals. ○, Control; ●, FTY720 0.1 mg/kg *p.o.* daily; ▲, FTY720 0.3 mg/kg *p.o.* daily. (B) On day 28, the spinal cords from EAE mice were obtained and the level of IFN-γ mRNA expression was examined by TaqMan PCR. Statistical difference in EAE scores (A) was calculated by Steel's test, and that in the level of IFN-γ mRNA (B) was done by Dunnett's multi-comparison test (**p* < 0.05, ***p* < 0.01).

presumed that FTY720 provides a useful tool for therapy in MS.

Previously FTY720 has been reported to be effective in ameliorating several autoimmune disease models, including EAE (4, 6, 17, 36). The prophylactic effect of FTY720 at 0.3 mg/kg on the development of EAE in Wistar rats when administered from the day of immunization was reported (17). A similar prophylactic effect was observed in acute monophasic EAE using LEW rats. Administration of FTY720 at 1 mg/kg from the day of administration prevented mortality and almost abolished the clinical signs of EAE (37). In this study, we demonstrate that FTY720 shows a therapeutic effect as well as prophylactic effect on MBP-induced EAE. EAE-associated symptoms and histological changes were decreased significantly even when FTY720 was administered after the onset of EAE in MBP-immunized LEW rats. The most important point in the present study is the significant decrease in the histological score of EAE that results from the therapeutic administration of FTY720. The

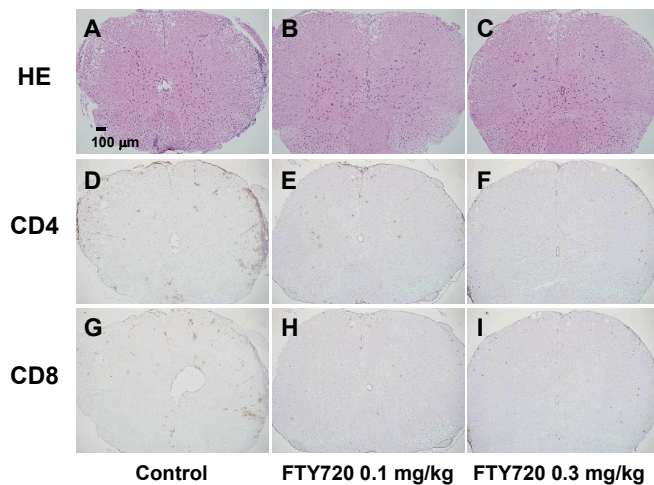


Figure 10. Therapeutic administration of FTY720 decreased infiltration of CD4⁺ T cells and CD8⁺ T cells into spinal cords of MOG₃₅₋₅₅-induced EAE mice. The spinal cords of EAE-developed mice were obtained at day 43, and HE and immunohistochemical staining were performed. Control: (A) HE ($\times 100$), (D) CD4⁺ T cells ($\times 100$), (G) CD8⁺ T cells ($\times 100$); FTY720 0.1 mg/kg: (B) HE ($\times 100$), (E) CD4⁺ T cells ($\times 100$), (H) CD8⁺ T cells ($\times 100$); FTY720 0.3 mg/kg: (C) HE ($\times 100$), (F) CD4⁺ T cells ($\times 100$), (I) CD8⁺ T cells ($\times 100$).

lower histological score in FTY720-treated groups means the reduction of inflammatory cells infiltrated into the CNS. As previously reported, oral administration of FTY720 at 0.03 mg/kg or higher induces a marked decrease in the number of peripheral blood lymphocytes in rats, mice, dogs, and monkeys (4, 6, 36). Thus, it is likely that the reduction of inflammatory cells infiltrated into the CNS in FTY720-treated groups is correlated to a marked decrease in the number of circulating lymphocytes induced by FTY720 treatment in MBP-induced EAE in LEW rats.

SJL/J mice with PLP-induced EAE and C57BL/6 mice with MOG-induced EAE, unlike LEW rats with MBP-induced monophasic EAE, display chronic diseases with a spontaneous pattern of relapses and remission (38-40). This disease pattern in these mouse EAE models is similar to the predominant form of human MS. Moreover, EAE-developed mice exhibit histopathology in lesioned areas similar to that shown in active plaques of MS patients. The major hallmarks of this histopathology are infiltration of inflammatory cells including T cells into CNS, demyelination, reactive gliosis, substantial axon loss and neurodegradation (41). It has been reported by Webb M, et al that administration of either FTY720 or FTY720-P to SJL mice with established relapsing EAE results in a rapid and sustained improvement (42); however they did not analyze the relationship between the therapeutic effect of FTY720 and the infiltration of T cells into the CNS.

In the present study, we demonstrate that FTY720 and its active metabolite, (S)-FTY720-P ameliorate chronic diseases with a spontaneous pattern of relapses and remission in

PLP-induced EAE in SJL/J mice and MOG-induced EAE in C57BL/6 mice. In addition, our data clearly show that FTY720 and (S)-FTY720-P decrease the demyelination and the infiltration of inflammatory cells, predominantly CD4⁺ T cells, in the spinal cords of these EAE mice. Consistent with the results obtained in MBP-induced EAE in LEW rats, FTY720 exhibited a marked prophylactic effect on EAE in PLP-immunized SJL/J mice. Moreover, therapeutic administration of FTY720 after the establishment of EAE resulted in a marked inhibition of relapse of EAE in PLP-immunized SJL/J mice and MOG-immunized C57BL/6 mice. Conversely, IFN- β widely used for therapy of MS showed only a marginal effect and could not inhibit the relapse of EAE. In FTY720-treated EAE mice, a marked decrease in the number of peripheral blood lymphocytes including pan-T cells, CD4⁺ T cells, CD8⁺ T cells and B cells was observed, and the area of demyelination and the number of CD4⁺ T cells infiltrated into the spinal cord were decreased as compared with those in control group. Since the infiltration of CD4⁺ T cells and the level of IFN- γ mRNA expression were increased in the spinal cord of EAE mice, it is highly likely that trafficking and infiltration of myelin antigen-specific Th1 cells into the CNS play an important role in the development and progression in our EAE models.

FTY720 is metabolized by omega-oxidation of the octyl side chain, and subsequent beta-oxidation, or phosphorylated to (S)-FTY720-P by sphingosine kinase 2 (6, 16, 17, 31). Recently, we successfully synthesized (S)-FTY720-P enantioselectively with an optical purity of > 99.5% and demonstrated that (S)-FTY720-P acts as a potent agonist at S1P₁, induces long-term down-regulation of S1P₁ on lymphocytes, and inhibits lymphocyte migration toward S1P (6, 30). We also reported that (S)-FTY720-P can induce lymphopenia and immunomodulating activity *in vivo* and that only (S)-FTY720-P was detected in blood from rats administered FTY720 (31). Since lymphocyte egress from secondary lymphoid tissues and thymus is known to be dependent on S1P/S1P₁ interaction, long-term down-regulation of S1P₁ on lymphocytes by (S)-FTY720-P causes the inhibition of lymphocyte egress and the sequestration of circulating lymphocytes (5, 6). In this study, we demonstrate that (S)-FTY720-P as well as FTY720 ameliorates EAE-associated symptoms at doses that show lymphopenia and reduction of CD4⁺ T cell infiltration into CNS in PLP-immunized SJL/J mice. Based on these observations, it is presumed that when FTY720 is administered, its active metabolite, (S)-FTY720-P induces long-term down-regulation of S1P₁ on lymphocytes, reduces the trafficking and infiltration of myelin-antigen specific Th1 cells into the CNS by sequestration of circulating mature lymphocytes from blood into secondary lymphoid tissues, and thereby shows a therapeutic effect as well as a prophylactic effect on spontaneously relapsing EAE in mice.

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