Review Article

Sphingosine 1-phosphate receptor type 1 as a novel target for the therapy of autoimmune diseases

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Sphingosine 1-phosphate (S1P), a lysophospholipid mediator, is generated from sphingosine by sphingosine kinases and binds 5 types of G protein-coupled S1P receptors. It has been well documented that S1P receptor type 1 ($S1P_1$) plays an essential role in lymphocyte egress from secondary lymphoid organs (SLO), because lymphocytes are unable to exit from SLO to periphery in mice lacking lymphocytic $S1P_1$ conditionally. FTY720 (fingolimod) is an orally active first-in-class S1P receptor modulator and is highly effective in various experimental autoimmune disease models including encephalomyelitis, adjuvant- or collagen-induced arthritis, and lupus nephritis. FTY720 is a structural analogue of sphingosine and is effectively converted to an active metabolite, FTY720 phosphate (FTY720-P) by sphingosine kinases. FTY720-P binds to four types of S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$) except for $S1P_2$ and acts as an agonist at these receptors. Particularly, FTY720-P strongly internalizes $S1P_1$ from the cell surface, almost completely inhibits S1P responsiveness of lymphocytes in SLO, and acts as a functional antagonist at lymphocytic $S1P_1$. Consequently, FTY720 inhibits $S1P_1$-dependent lymphocyte egress from SLO to decrease circulation of lymphocytes including autoreactive T cells and shows immunomodulating effects on autoimmune disease models. Recently, it has been reported that FTY720 has a superior efficacy in relapsing remitting multiple sclerosis patients compared to interferon-β. From these results, it is presumed that $S1P_1$ is a novel target for the therapy of autoimmune diseases including multiple sclerosis.

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Introduction

Circulation of mature lymphocytes among blood, lymph, and secondary lymphoid organs (SLO) plays a central role in the establishment of the immune response to foreign antigens. Homing of lymphocyte from blood into SLO beyond high endothelial venules is highly dependent on the interaction between the chemokines (CCL19, CCL21, CXCL12, and CXCL13), and their receptors (CCR7, CXCR4 and CXCR5) on lymphocytes. These

chemokines involved in lymphocyte homing are constitutively expressed in SLO and can induce migration of T cells, B cells and dendritic cells into there[1]. On the other hand, it has been clarified that a lysophospholipid mediator, sphingosine 1-phosphate (S1P), and its receptor type 1 ($S1P_1$) play an important role in lymphocyte egress from the SLO and thymus throughout the analyses of the mechanism of action of FTY720 (fingolimod)[1-3].

FTY720 is a first-in-class S1P receptor modulator with a chemical structure closely related to sphingosine[3,4]. FTY720 at an oral dose of 0.1 mg/kg or higher significantly prolongs allograft survival and shows a synergistic effect in combination with calcineurin inhibitors in experimental skin, cardiac, and renal allotransplanation models[3,5,6]. In addition, FTY720 is highly effective in various experimental autoimmune disease models including encephalomyelitis, adjuvant- or collagen-induced arthritis, and lupus nephritis[3]. FTY720[4,7] was found by chemical modification of an immunosuppressive natural product, myriocin[8], isolated from a culture broth of *Isaria sinclairii*, a kind of vegetative wasp; however FTY720, unlike myriocin, does not inhibit serine-palmitoyl-transferase, the first enzyme in sphingolipid biosynthesis[3]. Furthermore, unlike calcineurin inhibitors or other immunosuppressive drugs, FTY720 does not impair lymphocyte function including T cell activation and production of interleukin 2 (IL-2) and interferon-γ (IFN-γ) by type 1 helper T cells (Th1 cells)[9].

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 show immunomodulating effects[5,6]. The reduction of circulating lymphocytes by FTY720 is predominantly caused by sequestration of circulating mature lymphocytes into SLO and thereby decreasing T cell infiltration into inflammatory sites[6,10]. Reverse pharmacological approaches revealed that FTY720 is effectively converted to an active metabolite, FTY720 phosphate (FTY720-P) by sphingosine kinases and that FTY720-P binds to S1P receptors and acts as a high affinity agonist at these receptors[3,11-14]. Particularly, FTY720-P strongly internalizes $S1P_1$ from the cell surface, almost completely inhibits S1P responsiveness of lymphocytes in SLO, and acts as a functional antagonist at lymphocytic $S1P_1$[3,12]. Consequently, FTY720 inhibits $S1P_1$-dependent lymphocyte egress from SLO to decrease the number of circulating lymphocytes in peripheral blood. This paper summarizes the current understanding of the S1P receptor modulator, FTY720 and discusses about the feasibility of $S1P_1$ as a novel target for the therapy of autoimmune diseases.

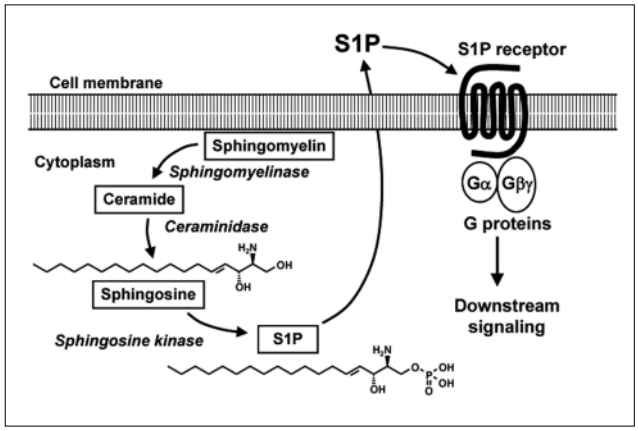

Fig.1 Generation of S1P and interaction with S1P receptor

S1P is generated primarily by the phosphorylation of intracellular sphingosine by sphingosine kinases and binds to five related G-protein-coupled S1P receptors ($S1P_{1-5}$).

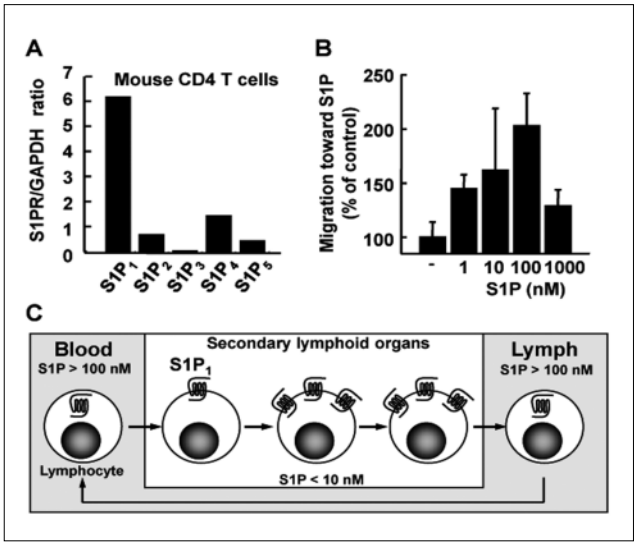

Fig.2 $S1P_1$ plays an essential role in lymphocyte egress from the SLO

(A): The mRNA expressions of S1P receptors in mouse CD4 T cells. (B): Migratory response of mouse CD4 T cells toward S1P. (C): $S1P_1$ on lymphocytes is down-regulated in the blood, up-regulated in SLO, and down-regulated again in the lymph, because $S1P_1$ surface expression on lymphocytes is highly dependent on the extracellular concentration of S1P. $S1P_1$-dependent chemotactic responsiveness plays an essential role in lymphocyte egress from the SLO.

S1P

S1P, a pleiotropic lysophospholipid mediator, is generated primarily by the phosphorylation of intracellular sphingosine by sphingosine kinases (Fig.1). S1P stimulates multiple signaling pathways resulting in calcium mobilization from intracellular stores, polymerization of actin, chemotaxis/migration, and escape from apoptosis. Significant amounts of S1P (100 to 400 nM) are found in blood and lymph whereas the S1P levels in the SLO are relatively lower (<10 nM), indicating a concentration gradient of S1P existing between blood-lymph and SLO (Fig.2)[15,16]. Plasma S1P is tightly associated with albumin and lipoproteins, particularly high-density lipoprotein and the major source of plasma S1P is red blood cells and platelets[17]. Excessive production of S1P can be induced at inflammatory sites as a result of cell activation by pro-inflammatory cytokines. The S1P gradient between blood-lymph and SLO, as well as over production of S1P at inflammatory sites, appears to play an important role in recirculation and trafficking of lymphocytes.

S1P receptors

S1P binds with subnano to nano-molar affinities to five related G-protein-coupled receptors, termed $S1P_{1\text{-}5}$. $S1P_1$, $S1P_2$, and $S1P_3$ receptors are widely expressed in the immune, cardiovascular, and central nervous systems. $S1P_4$ is selectively expressed in lymphoid tissues and lung whereas $S1P_5$ is expressed in spleen and white matter tracts of the central nervous systems[17]. The expression of $S1P_1$ mRNA in CD4 T cells is markedly higher than the other S1P receptors, suggesting that $S1P_1$ is the dominant receptor on lymphocytes (Fig.2).

It has been reported that $S1P_1$ is essential for lymphocyte recirculation and that $S1P_1$ regulates lymphocyte egress from SLO[2,3,18]. In mice whose hematopoietic cells lack a single S1P receptor, $S1P_1$, there are no T cells in the periphery because mature T cells are unable to exit SLO[2]. Moreover, $S1P_1$-dependent chemotactic responsiveness is suggested to be up-regulated in lymphocytes before exit from SLO, whereas $S1P_1$ is down-regulated during peripheral lymphocyte activation, and this is associated with retention of lymphocytes in SLO[15]. Because $S1P_1$ surface expression on lymphocytes is highly dependent on the extracellular concentration of S1P, $S1P_1$ on lymphocytes is down-regulated in the blood, up-regulated in SLO and down-regulated again in the lymph (Fig.2). Consequently, it is proposed that cyclical modulation of $S1P_1$ surface expression on circulating lymphocytes by S1P contributes to establishing their transit time in SLO[15].

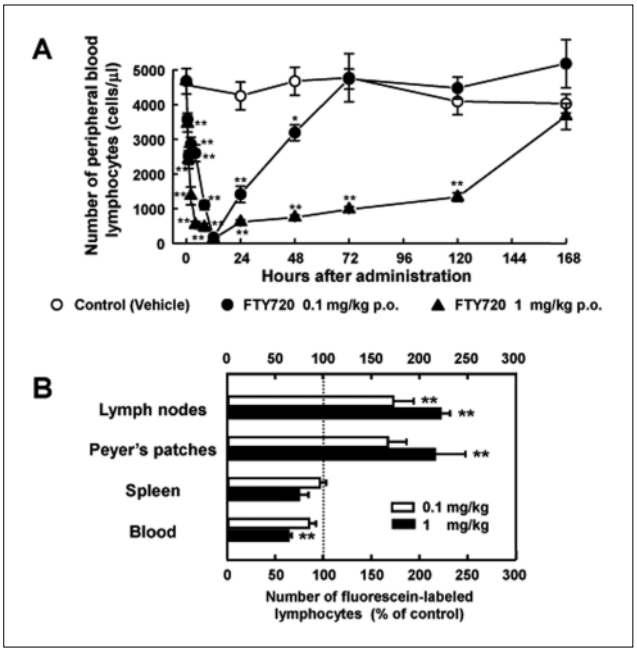

Fig.3 FTY720 sequesters circulating lymphocytes into the SLO

(A): The number of peripheral blood lymphocytes in rats was determined by flow cytometry. Each symbol represents the mean ± S.E.M. of six animals. Statistical significance was calculated by Dunnett's test (* $p<0.05$, ** $p<0.01$ vs vehicle-treated control group). (B): The fluorescein-labeled lymphocytes were transfused intravenously into rats 2.5 h after oral administration of FTY720. The numbers of fluorescein-labeled lymphocytes in blood, spleen, lymph nodes and Peyer's patches were determined by flow cytometry. Each column represents the mean ± S.E.M. of four animals. Statistical significance was calculated by Dunnett's test (** $p<0.01$ vs vehicle-treated control group). (Adapted from Chiba[3])

Functional antagonism of $S1P_1$ by S1P receptor modulator

A striking feature of FTY720 is the induction of a marked decrease in the number of peripheral blood lymphocytes at doses that show immunomodulating effects (Fig.3)[5,6]. When FTY720 at an oral dose of 0.1 mg/kg or higher is given to rats or mice, the number of lymphocytes is decreased markedly in the peripheral blood and thoracic duct lymph whereas that in SLO is increased significantly. Intravenous transfusion of fluorescein-labeled lymphocytes into rats revealed that the labeled lymphocytes are accumulated in SLO by FTY720 administration (Fig.3)[6]. These data suggest that FTY720 induces sequestration of circulating mature lymphocytes into SLO and decreases the number of lym-

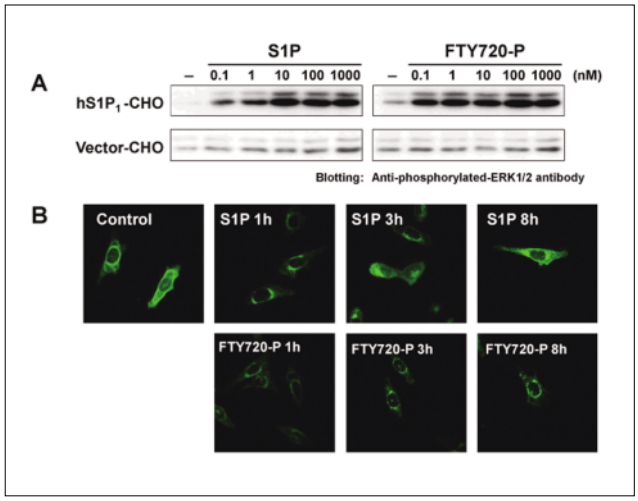

Fig.4 FTY720-P induces internalization of S1P1

(A): Agonist activity of S1P and FTY720-P at human S1P1 was determined by ERK1/2 phosphorylation in CHO cells stably expressing human S1P1 (hS1P1-CHO cells). (B): Confocal microscopy of hS1P1-CHO cells treated with S1P or FTY720-P (100 nM). (Adapted from Chiba et al.[20])

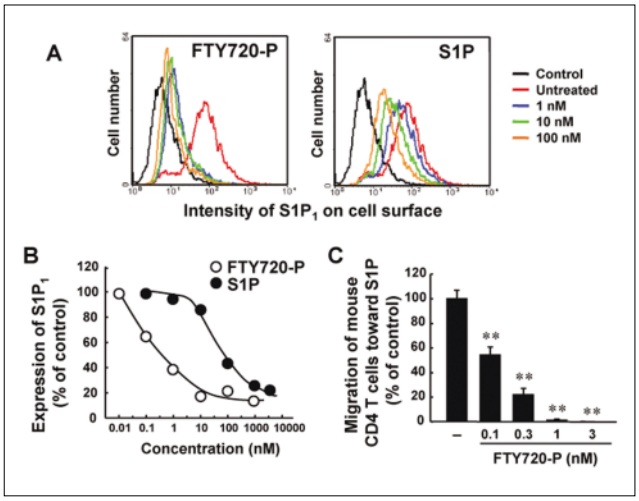

Fig.5 FTY720-P inhibits migration of CD4 T cells toward S1P

(A): hS1P1-CHO cells were stained with FITC-conjugated anti-human S1P1 monoclonal antibody and expression of S1P1 on cell surface was analyzed by flow cytometry. (B): S1P1 expression was represented as the mean fluorescence intensity. (C): Pretreatment with FTY720-P inhibits migration of mouse CD4 T cells toward 10 nM S1P. Each column represents the mean ± S.E.M of triplicate determination. Statistical significance was calculated by Dunnett's multiple comparison test (**p<0.01 vs control migration toward 10 nM S1P). (Adapted from Chiba et al.[20] and Maeda et al.[21])

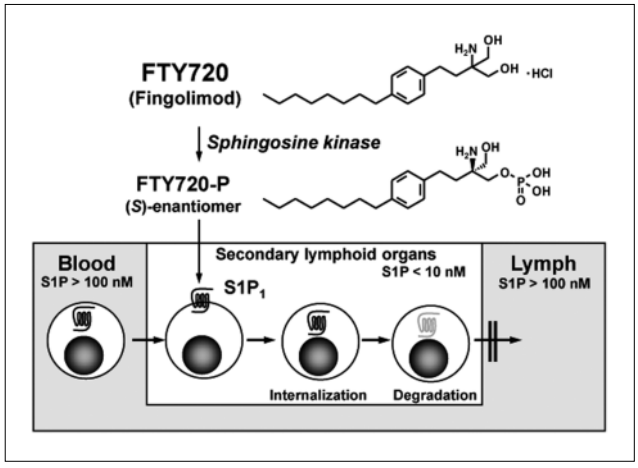

Fig.6 FTY720-P converted from FTY720 inhibits S1P1-dependent lymphocyte egress from the SLO by internalization of S1P1 on lymphocytes

phocytes in peripheral blood and lymph. Thus, sequestration of circulating mature lymphocytes is presumed to be the main mechanism of immunomodulating activity of FTY720.

By reverse pharmacological approaches to clarify the mechanism of action of FTY720, it has been demonstrated that like sphingosine, FTY720 is a substrate for sphingosine kinases and that a phosphorylated form of FTY720 (FTY720-P) binds to four types of S1P receptors (S1P1, S1P3, S1P4, and S1P5) but not S1P2, and acts as a high affinity agonist at these receptors[11,12]. After oral or intravenous FTY720 administration, the plasma concentration of FTY720-P was 2 to 6 times higher than FTY720[11,12]. We have confirmed that only the (*S*)-enantiomer of FTY720-P can bind to four types of S1P receptors (S1P1,3,4,5) at nano-molar concentrations, but not S1P2, whereas FTY720 up to 10000 nM does not bind S1P receptors[19]. FTY720-P shows agonist activity for S1P1 at nano-molar concentrations using extracellular signal regulated kinase 1/2 (ERK1/2) phosphorylation assay and subsequently induces long-term internalization of S1P1 in Chinese hamster ovary (CHO) cells stably expressing human S1P1 (Figs.4 and 5)[20-22]. Consequently, FTY720 treatment down-regulates S1P1, creating a temporary pharmacological S1P1-null state in lymphocytes, providing an explanation for

the mechanism of FTY720-induced lymphocyte sequestration[2,3,18].

The internalization of $S1P_1$ by FTY720-P appears to be maintained longer than that by S1P and the difference between FTY720-P and S1P seems to be due to the distinct stability of FTY720-P and S1P for degradation by S1P lyase. S1P at concentrations of 10 to 100 nM induces migration of lymph node CD4 T cells in mice (Fig.2). The pretreatment with FTY720-P effectively inhibits the migration of CD4 T cells toward S1P (Fig.5)[20,21]. Based on these results, it is highly likely that FTY720-P converted from FTY720 acts as a functional antagonist at $S1P_1$ by long-term internalization of this receptor, reduces S1P responsiveness of lymphocytes in SLO, and inhibits $S1P_1$-dependent lymphocyte egress from SLO (Fig.6).

Effects of S1P receptor modulator on autoimmune disease models

Oral administration of FTY720 is highly effective in experimental autoimmune encephalomyelitis (EAE), a CD4 T cell-dependent model for multiple sclerosis (MS)[22-28]. The development of myelin proteolipid protein-induced EAE in SJL/J mice is almost completely prevented and infiltration of CD4 T cells into the spinal cord is decreased by prophylactic treatment with FTY720 and FTY720-P (Fig.7)[22]. When FTY720 or FTY720-P is given after establishment of EAE in SJL/J mice, the relapse of EAE is markedly inhibited as compared with recombinant mouse IFN-β, and the area of demyelination and the infiltration of CD4 T cells are decreased in the spinal cords of EAE mice (Fig.7)[22]. Similar therapeutic effects by FTY720 are obtained in the case of myelin oligodendrocyte glycoprotein-induced EAE in C57BL/6 mice[22].

It has been reported that infiltration of encephalitogenic CD4 T cells, particularly IL-17-expressing helper T cells (Th17 cells), into the central nervous system (CNS) plays a critical role in the development and progression of EAE in mice[29]. Oral administration of FTY720 at 0.1 mg/kg or higher significantly inhibits the development of EAE and markedly reduces the frequency of Th17 cells and mRNA expression of IL-17 in the spinal cords of EAE mice (Fig.8). On the contrary, the frequency of Th17 cells in draining inguinal lymph nodes is significantly increased by FTY720, suggesting sequestration of myelin antigen-specific Th17 cells into the draining lymph nodes. Moreover, Th17 cells can migrate toward 10 nM S1P and the pretreatment with 1 nM FTY720-P almost completely inhibits S1P-induced migration of Th17 cells (Fig.8). On the other hand, FTY720-P up to 100 nM shows no clear effect on generation of Th 17 cells or IL-17 production by them. Consequently, the ameliorating effects of FTY720 on EAE are likely due to reduction of infiltration of encephalitogenic Th17 cells into the CNS.

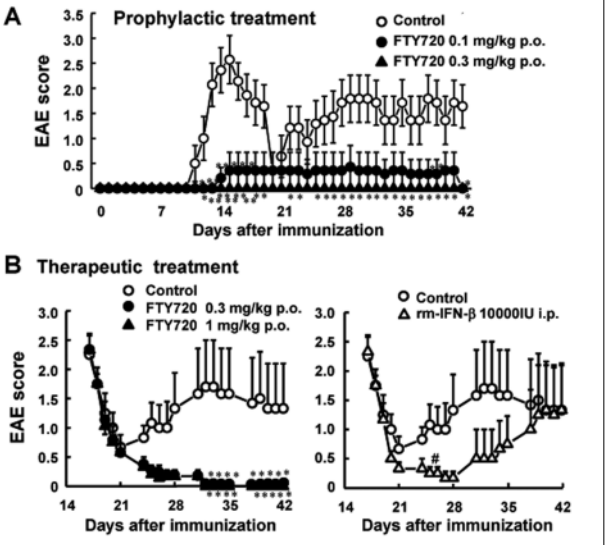

Fig.7 Prophylactic and therapeutic effects of FTY720 on mouse EAE

(A): SJL/J mice were immunized with myelin proteolipid protein and Freund's complete adjuvant. FTY720 (0.1 and 0.3 mg/kg) was administered orally (p.o.) to SJL/J mice every day from day 0 to 42. (B): EAE-developed mice were pooled, divided into 4 groups and administration of FTY720 (0.3 and 1 mg/kg p.o.) or recombinant mouse IFN-β (10000 IU/mouse, intraperitoneally) was started from day 17 to 42. Mice in the control group were administered vehicle only. The results are expressed as the mean ± S.E.M of 6 to 7 mice. Statistical differences in EAE scores of FTY720 groups were calculated by Steel's test ($*p<0.05$, $**p<0.01$), and those in IFN-β group were done by Mann Whitney *U* test ($^{\#}p<0.05$). (Adapted from Kataoka et al.[22])

In myelin basic protein-induced EAE in LEW rats, prophylactic administration of FTY720 at 0.1 to 1 mg/kg almost completely prevents the development of EAE symptoms, and therapeutic treatment with FTY720 significantly inhibits the progression of EAE and EAE-associated histological change in the spinal cords[22]. In myelin oligodendrocyte glycoprotein-induced EAE in DA rats, prophylactic therapy of FTY720 protects against the emergence of EAE symptoms, neuropathology, and disturbances to visual and somatosensory evoked potentials[27]. Moreover, therapeutic treatment of FTY720 markedly reverses paralysis in established EAE and normalizes the electrophysiological re-

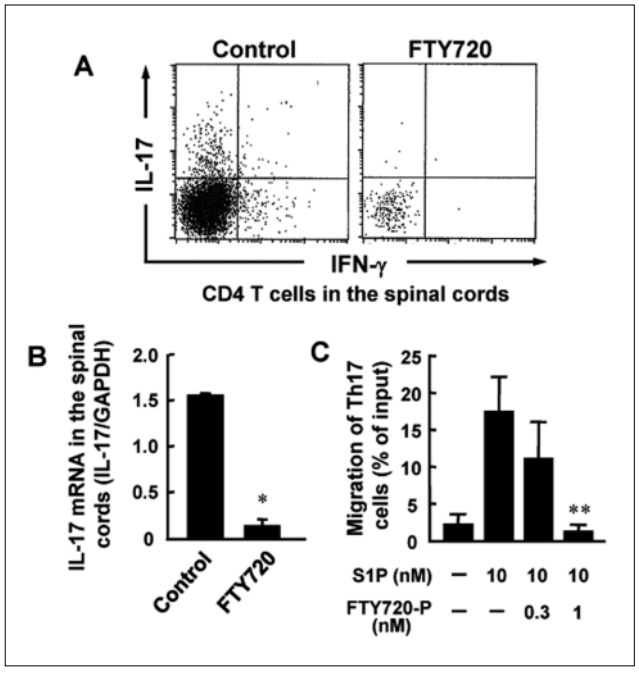

Fig.8 FTY720 decreases infiltration of Th17 cells into the spinal cords of EAE mice

(A): Th17 cells infiltrated into the spinal cords of EAE mice (day 16) were determined by flow cytometry using anti-mouse IL-17 and IFN-γ monoclonal antibodies. (B): The expression of IL-17 mRNA in spinal cords of EAE mice was determined by real time PCR. Statistical difference was calculated by Student's *t*-test (* $p<0.05$, n=3). (C): Pretreatment with FTY 720-P inhibits migration of Th17 cells toward S1P (10 nM). Statistical differences were calculated by Dunnett's multiple comparison test (** $p<0.01$, n=3).

sponses with decreased demyelination in the CNS[27]. 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 including Th17 cells into the CNS.

Recently it has been suggested that the efficacy of FTY720 in EAE is due to additional direct effects in the CNS because neural cells constitutively express S1P receptors and relatively higher concentrations of FTY720-P are found in the CNS than blood[28]. Consequently, it is likely that the therapeutic effects of FTY720 on EAE is due to a culmination of mechanisms involving reduction of myelin antigen-specific T cells, neuroprotective influence of FTY720-P in the CNS, and inhibition of inflammatory mediators in the brain.

FTY720 at 0.1 mg/kg or higher almost completely inhibits joint destruction as well as paw edema in adjuvant- or type II collagen-induced arthritis in LEW rats[30], and shows a marked therapeutic effect on lupus nephritis in autoimmune MRL/*lpr* mice[3]. It has been reported that $S1P_1$ mRNA and protein were detected in synoviocytes of rheumatoid arthritis patients. S1P increases the proliferation of synoviocytes and enhances inflammatory cytokine-induced cyclooxygenase-2 expression and prostaglandin E_2 production by synoviocytes in rheumatoid arthritis[31]. In addition, Osteoclast precursors express functional $S1P_1$ and S1P induces chemotaxis and regulates the migration of osteoclast precursors *in vitro* and *in vivo*. Treatment with FTY720 relieved ovariectomy-induced osteoporosis in mice by reducing the number of mature osteoclasts attached to the bone surface[32]. Based on these findings, it is suggested that $S1P_1$ would be a novel target for the therapy of autoimmune diseases including MS, rheumatoid arthritis, and systemic lupus erythematosus and that functional antagonism at $S1P_1$ by S1P receptor modulator can provide a new approach for therapy in these autoimmune diseases.

Clinical trials of S1P receptor modulator in MS

Clinical trials of FTY720 have been performed in relapsing remitting MS patients[33-36]. FTY720 at an oral dose of 1.25 mg or 5.0 mg, or placebo is administered daily for 6 months to 281 patients with relapsing remitting MS and total of 255 patients has completed the clinical study. The median total number of gadolinium-enhanced lesions on magnetic resonance imaging (MRI) is significantly lower with 1.25 mg and 5.0 mg of FTY720 than with placebo. The annualized relapse rates in groups given 1.25 mg and 5.0 mg of FTY720 are 0.35 and 0.36, respectively and are significantly lower than that in the placebo group (0.77). By extension study for additional 6 months, the number of gadolinium-enhanced lesions and relapse rates remains low in groups given FTY720 and both measures decrease in patients who switched from placebo to FTY720. From these results, it is demonstrated that oral FTY720 reduces the number of lesion detected on MRI and clinical disease activity in relapsing remitting MS patients.

It has been shown in human studies that FTY720 decreases the number of peripheral blood lymphocytes[3,37]. Administration of single oral doses of FTY720, ranging from 0.25 to 3.5 mg causes a dose-dependent reduction in peripheral blood T cells and B cells. In MS patients, FTY720 primarily reduced the numbers of $CCR7^+$ $CD45RA^+$ naïve T cells and $CCR7^+$ $CD45RA^-$ central memory T cells but not $CCR7^-$ effector memory T cells in peripheral blood[17]. These findings suggest that FTY720 seques-

ters circulating naïve and central memory T cells into the SLO from peripheral blood because these T cells express the homing receptor, CCR7 that is essential for the recirculation of lymphocytes through the SLO. Moreover, FTY720 markedly reduced the number of circulating $CD4^+$ IL-17-producing T cells by >95% in peripheral blood of MS patients, suggesting the sequestration of Th17 cells into the SLO[17]. Because large numbers of Th17 cells are found in brain tissues from MS patients[38], it is highly probable that Th17 cells contribute to the pathogenesis of MS and that FTY720 inhibits trafficking and infiltration of Th17 cells into the CNS and thereby showing a superior efficacy in MS patients. More recently, it has been reported that FTY720 at oral dose of 0.5 and 1.25 mg shows a superior efficacy compared with a standard care, the injectable IFN-β 1a in relapsing remitting MS patients[34]. Based on these evidences, it is presumed that functional antagonism at $S1P_1$ by FTY720 provides a new therapeutic approach for autoimmune diseases including MS.

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