

The S1P receptor modulator FTY720 prevents the development of experimental colitis in mice

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Abstract. To evaluate the therapeutic effects of the new synthetic sphingosine-1-phosphate (S1P) receptor modulator, FTY720, we investigated how FTY720 affects the development of dextran sulfate sodium (DSS)-induced colitis and CD4⁺CD62L⁺ T cell transfer colitis. BALB/c mice were fed a chow containing 3.5% (wt/wt) DSS to induce colitis. The CD4⁺CD62L⁺ T cell transfer colitis was induced by an intra-peritoneal injection of CD4⁺CD62L⁺ spleen T cells into recipient CB17 SCID mice. The FTY720 was administered by lavage at a dose of 0.3 mg/kg/day. FTY720 was effective in preventing the body weight loss in the DSS-colitis model and the CD4⁺CD62L⁺ T cell transfer model. The disease activity index, histological colitis score, and MPO activity were all significantly lower in FTY720-treated mice than in the non-treated mice. Microscopically, mucosal edema, cellular infiltration and epithelial disruption were much more moderate in the FTY720-treated mice than in the non-treated mice. In both colitis models, FTY720 prevented the infiltration of CD4⁺ T cells into the inflamed colonic lamina propria. In conclusion, the development of DSS-colitis and CD4⁺CD62L⁺ T cell transfer colitis were significantly attenuated by FTY720. Since FTY720 is an immunosuppressive product that does not modulate T cell functions, it could be useful in the treatment of IBD patients.

Introduction

Inflammatory bowel disease (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), are associated with chronic relapsing inflammation of the intestinal tract of unknown etiology (1-4). Histologically, the mucosal accumulation of leukocytes is a characteristic feature of IBD, and the activation of T cells and monocytes/macrophages is regarded as a crucial

factor in its pathogenesis. The treatment of IBD depends on drugs such as 5-aminosalicylic acid, corticosteroids, azathioprine, mercaptopurines and cyclosporine (1). However, the use of these drugs is sometimes limited by drug-induced toxicity. Thus, there is an increasing need for alternative agents that may be equally or more effective but toxicity-free.

The new synthetic sphingosine-1-phosphate (S1P) receptor modulator FTY720 {2-amino-2-[2-(4-octyl-phenyl) ethyl]-1,3-propanediol hydrochloride} is a derivative of ISP-1 (myriocin) isolated from the fungus *Isaria Sinclairi* (5-11). FTY720 discloses S1P receptor signalling to be essential for lymphocytes to egress from lymph nodes and thymus (12), and the oral administration of FTY720 induces a rapid reduction of peripheral blood lymphocytes. FTY720 shifts the distribution of lymphocytes from the peripheral blood and spleen to secondary lymphoid tissues such as the Peyer's patches, peripheral lymph nodes and mesenteric lymph nodes (5-10). This cell redistribution is responsible for the immunosuppressive activity of FTY720, and the functions of the granulocytes, monocytes, and T and B cells remain unimpaired. Thus, the immunosuppressive mechanism of FTY720 differs markedly from other drugs currently used as immunosuppressive agents.

The therapeutic effects of FTY720 for spontaneous colitis developed in interleukin-10 gene-deficient (IL-10^{-/-}) mice have been reported (13). To further evaluate the therapeutic effects of FTY720 on other models of experimental colitis, we used the dextran sulfate sodium (DSS)-induced colitis and CD4⁺CD62L⁺ T cell transfer models of colitis (14,15). Since FTY720 does not cause any impairment in T cell functions, it is worthwhile to investigate the effects of FTY720 on experimental colitis as a novel candidate for a new therapeutic approach for IBD.

Materials and methods

Induction of experimental colitis. For the induction of DSS-colitis, six- to eight-week old male BALB/c mice were purchased from Charles River Japan (Kanagawa, Japan). They were acclimatized for one week before the experiment, and were housed individually in a room maintained at 22°C under a 12-h day/night cycle throughout the experiments. The mice were fed 3.5% (wt/wt) DSS (molecular weight 5000; Wako Pure Chemical Industries, Ltd., Osaka, Japan) mixed with normal chow (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water *ad libitum*. The study protocol was approved

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by the Animal Care and Use Committee of the Shiga University of Medical Science (Otsu, Japan).

For the induction of the CD4⁺CD62L⁺ T cells transfer model of colitis, CD4⁺ T cells were purified from the spleen mononuclear cells of healthy BALB/c mice by the negative depletion of other cell types using anti-CD8, anti-MHC-II, anti-B220 and anti-11b antibodies (Pharmingen, Hamburg, Germany) (15,16), and anti-rat IgG immunomagnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The CD4⁺ T cells were further separated into CD62L⁺ and CD62L⁻ T cells. The CD4⁺CD62L⁺ T cells (0.25×10^6 cells) were resuspended in 200 μ l of sterile phosphate-buffered saline (PBS) and were injected intraperitoneally in recipient CB17 SCID mice (Charles River Japan) (15,16).

Each colitis model was divided into two subgroups, and one subgroup was treated with FTY720 (Novartis Pharma AG, Basel, Switzerland). The FTY720 was administered by lavage at a dose of 0.3 mg/kg/day for 14 days in the DSS colitis model and for 49 days in the CD4⁺CD62L⁺ T cell transfer model, respectively. The control mice received distilled water alone.

Assessment of inflammation in DSS-induced colitis. A daily clinical assessment of the colitis was performed, including a measurement of food intake and body weight, an evaluation of stool consistency, and the presence of blood in the stools by a guaiac paper test. The stool consistency was assessed using the following four point-scale: 0, normal; 1, soft; 2, very soft but formed; and 3, liquid. The intensity of the guaiac paper test was scored by the following scale: 0, negative; 1, faintly blue; 2, moderately blue; 3, dark blue; and 4, blood visible. A validated clinical disease activity index ranging from 0 to 4 was calculated using the following parameters: stool consistency, presence of fecal blood, and changes in body weight (17). The mice were sacrificed on the last day, and the length and weight of the colons were measured.

Histology. A histological examination was performed on three samples of the distal colon from each animal. The samples were fixed in 10% buffered formalin, dehydrated in ethanol, and then embedded in paraffin. Four micron-thick sections were then prepared and stained with hematoxylin and eosin. All histologic evaluation was performed in a blinded fashion using a validated scoring system.

For immunohistochemical staining, freshly isolated colonic tissue from the distal portion of the colon was frozen in dry ice using an OCT compound (Sakura Finetek, Tokyo, Japan). Acetone-fixed frozen sections (6- μ m thick) were blocked with Dako blocking reagent (#X0909, Dako Japan, Kyoto, Japan) followed by incubation with the primary antibodies [anti-mouse CD4 (BD Pharmingen, San Diego, CA)] diluted 1:100 in phosphate-buffered saline (PBS) containing 5% skim milk overnight at 4°C in a humidified chamber. After incubation with the primary antibody, the sections were reacted with 0.1% H₂O₂ in 0.1 M PBS, and treated with biotin-conjugated second antibodies (diluted 1:50 in PBS containing 1% skim milk; Vector, Burlingame, CA) for 60 min at room temperature.

Tissue myeloperoxidase activity. The samples were washed with cold PBS, blotted dry, and were immediately thawed for

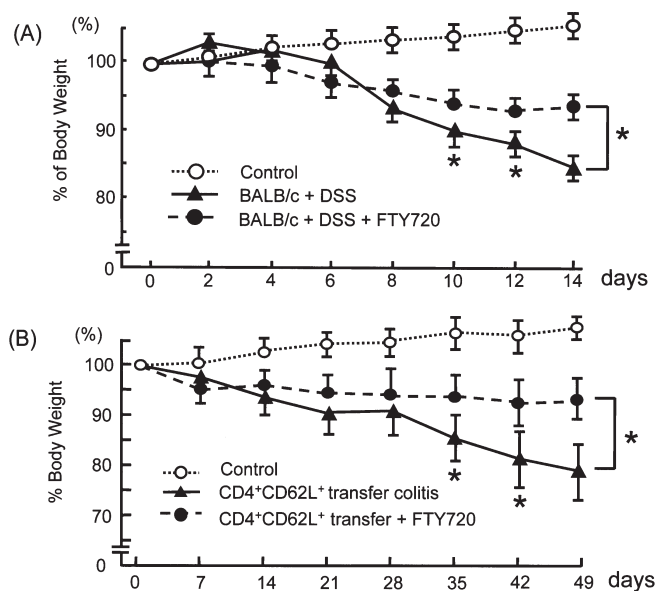


Figure 1. Changes in the body weight of DSS-colitis mice (A) and CD4⁺CD62L⁺ T cell transfer-colitis mice (B). The mice in the DSS-colitis model were fed DSS over 14 days, and the mice in the CD4⁺CD62L⁺ T cell transfer-colitis model were followed for 49 days. The weight of each individual mouse was assessed daily. The data represent means \pm SD (n=5 mice/group). *P<0.05.

the myeloperoxidase activity determination using the O-dianisidine method previously described. The activity was expressed as the amount of enzyme necessary to generate a change in absorbance of 1.0 per minute per gram wet weight of tissue.

Statistical analysis. Statistical analyses were performed using a one-way ANOVA with Scheffe's post hoc test or the Kruskal-Wallis test when appropriate. A two-way ANOVA for repeated measures was used to test for group and time effects on clinical data (e.g., the disease activity index). A P-value <0.05 was considered to be statistically significant.

Results

As shown in Fig. 1A, on days 10, 12 and 14 after the initiation of DSS-induced colitis, the body weight loss was much more severe in the DSS-treated mice than in the DSS plus FTY720-treated mice. Similarly, in the CD4⁺CD62L⁺ T cell transfer model, FTY720 exerted significant preventive effects against the body weight loss on days 35, 42 and 49 after the induction of colitis (Fig. 1B). The disease activity index was significantly lower in the DSS plus FTY720-treated mice than in the DSS-treated mice (*P<0.05, Fig. 2A), and was significantly lower in the FTY720-treated CD4⁺CD62L⁺ T cell transfer models than in the non-treated colitis models (*P<0.05, Fig. 2B).

On day 14, the colon length shortening was much more severe in the DSS-treated mice as compared to the DSS plus FTY720-treated mice (*P<0.05, Fig. 3a). The colon weight/length ratio, a maker of tissue edema, was significantly higher in the DSS-treated mice than in the DSS plus FTY720-treated mice (*P<0.05, Fig. 3b). Similarly, in the CD4⁺CD62L⁺ T cell transfer model, FTY720 significantly ameliorated both the

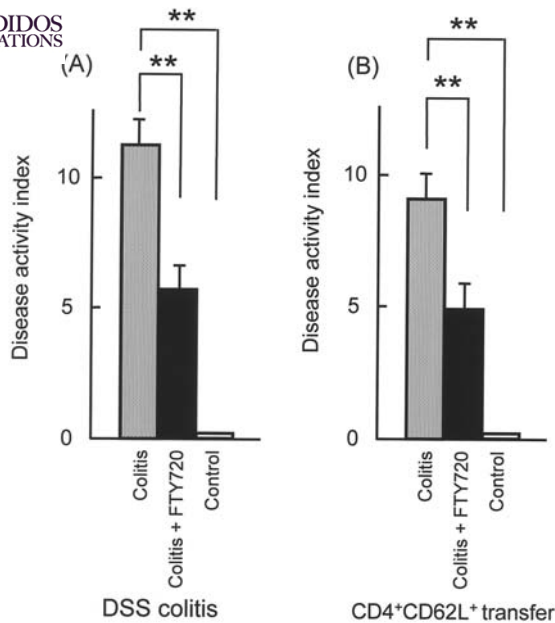


Figure 2. Disease activity index on day 14 in the DSS-colitis model (A) and on day 49 in the CD4⁺CD62L⁺ T cell transfer-colitis model. The data represent means \pm SD (n=5 mice/group). **P<0.01.

colon length shortening and the increase in the colon weight/length ratio (*P<0.05, Fig. 3c and d).

DSS-colitis is characterized by histological findings such as edema, the infiltration of inflammatory cells into both the mucosa and submucosa, the destruction of epithelial cells and mucosal thickening. As shown in Fig. 4A, the histological

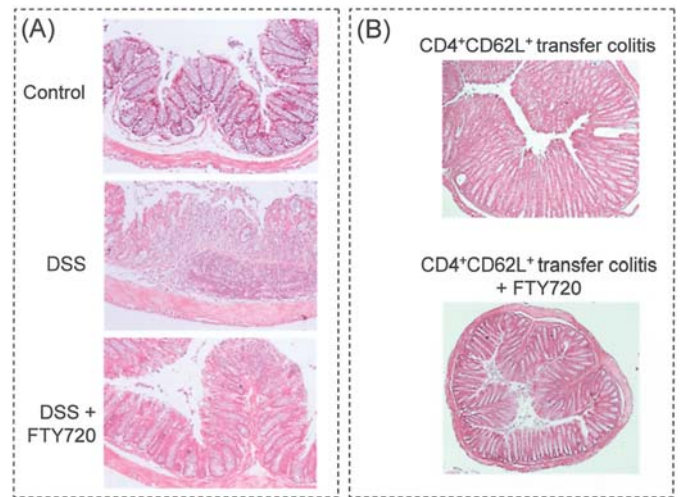


Figure 4. Histologic findings in the DSS-colitis model on day 14 (A) and in the CD4⁺CD62L⁺ T cell transfer-colitis model on day 49 (B). The colons were excised, and stained with hematoxylin and eosin. Magnification x20.

analysis indicated that these changes were more severe in the DSS-treated mice as compared to the DSS plus FTY720-treated mice. Similarly, CD4⁺CD62L⁺ T cell transfer colitis is also characterized by marked elongation of the intestinal crypts, mucosal thickening, edema, severe infiltration of inflammatory cells, and epithelial destruction. These histological changes were much milder in the FTY720-treated mice than in the non-treated mice (Fig. 4B). The histological score was significantly lower in the FTY720-treated colitis models than in the non-treated colitis models (*P<0.05, Fig. 5A).

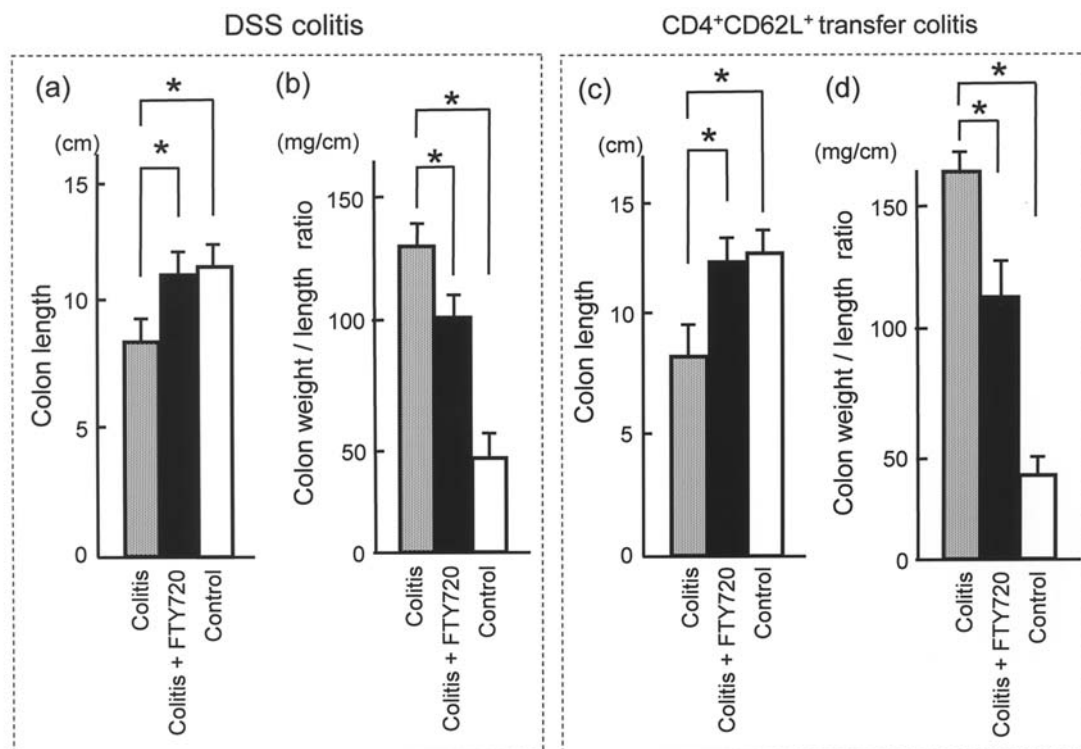


Figure 3. Colon length and colon weight/length ratio on day 14 in the DSS-colitis model and on day 49 in the CD4⁺CD62L⁺ T cell transfer-colitis model. The data represent means \pm SD (n=7 mice/group). *P<0.05.

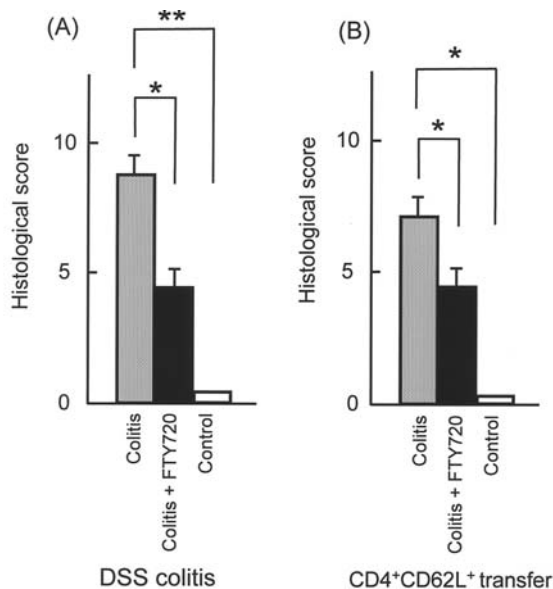


Figure 5. Histological score in the DSS-colitis model on day 14 (A) and in the CD4⁺CD62L⁺ T cell transfer-colitis model on day 49 (B). The data represent means \pm SD (n=5 mice/group). *P<0.05, **P<0.01.

As shown in Fig. 6A, the tissue myeloperoxidase activity, a marker of granulocyte infiltration, as well as the number of infiltrated CD4⁺ T cells were all significantly lower in the DSS plus FTY720-treated mice than in the DSS-treated mice (*P<0.05, Fig. 6A). Similarly, these markers were significantly lower in the FTY720-treated CD4⁺CD62L⁺ T cell transfer models than in the non-treated colitis models (*P<0.05, Fig. 6B).

Discussion

FTY720 has emerged as a potent S1P receptor immuno-modulator with usefulness in the control of organ transplant rejection and for the treatment of autoimmune diseases. In animal models, FTY720 is efficacious in prolonging graft survival, as well as in models of multiple sclerosis, acute lung injury, autoimmune diabetes, atherosclerosis and renal ischemia-reperfusion injury (18). Promising results have also been obtained from human trials on FTY720 for indications in renal transplantation and multiple sclerosis (19,20). In contrast to conventional immunosuppressants such as cyclosporine A or FK506, FTY720 selectively and reversibly sequesters lymphocytes but not monocytes or granulocytes from the blood and spleen into secondary lymphoid organs, thereby preventing their migration toward sites of inflammation and allograft rejection (5-11). Moreover, FTY720 does not impair T cell activation, expansion, and memory functions, and does not induce T cell apoptosis (11). Recent studies also demonstrated that FTY720 enhances functions of the CD4⁺CD25⁺-regulatory T cells (11), and that FTY720 alters IL-12 secreting dendritic cells to IL-10 producing cells (8). These mechanisms represent the overall immunosuppressive actions of FTY720.

Mizushima *et al* demonstrated that FTY720 prevented the development of spontaneous colitis in IL-10^{-/-} mice (13). Colitis in IL-10^{-/-} mice is characterized by the infiltration of activated CD4⁺ T cells, which may play a central role in the development of chronic colitis via the secretion of Th1 proinflammatory cytokines (13). FTY720 induced a reduction of the CD4⁺ T lymphocytes subpopulation, in

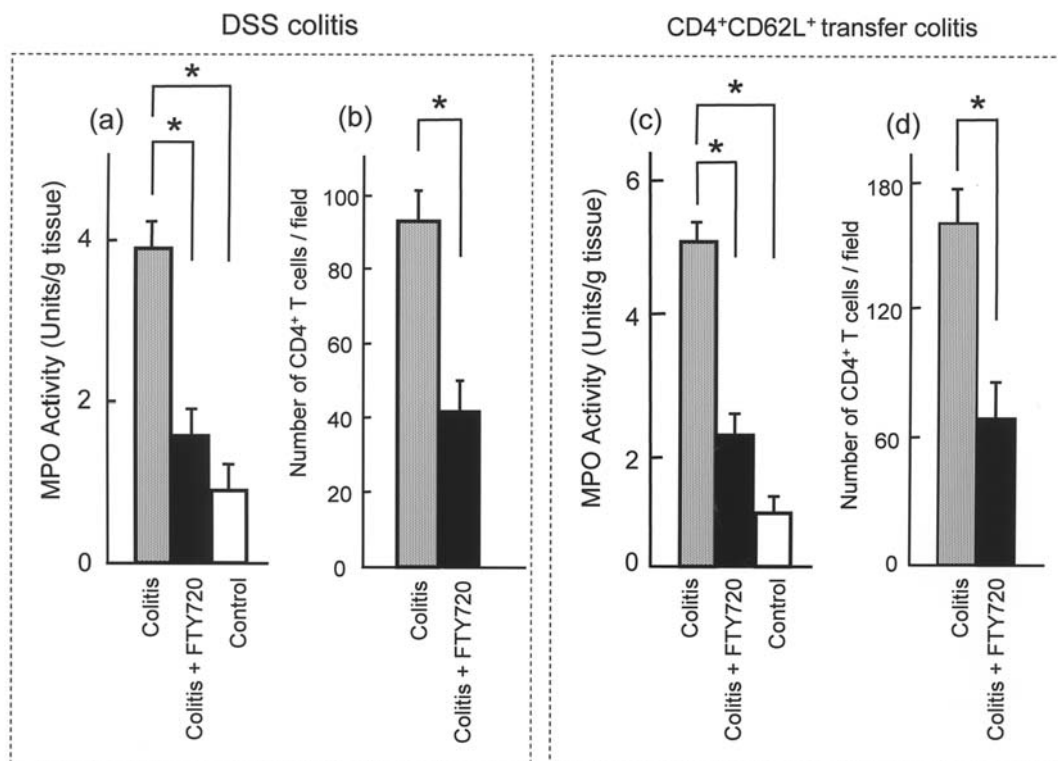


Figure 6. Tissue myeloperoxidase (MPO) activity and number of infiltrated CD4⁺ T cells in the colonic lamina propria. The tissue MPO activity was measured by the method described in Materials and methods. The number of infiltrated CD4⁺ T cells was determined by high magnification (x400) observation of immunostained samples. *P<0.05.



Th1 cells, in the colonic lamina propria, accompanied a decrease in the severity of the colitis (13). It was suggested that FTY720 ameliorated chronic colitis in IL-10^{-/-} mice through a depletion of the peripheral lymphocytes by promoting their sequestration in the secondary lymphoid tissues and the consequent reduction of the homing of lymphocytes into the lamina propria.

To further investigate the effects of FTY720 on the development of experimental colitis we used two different models, DSS-colitis and CD4⁺CD62L⁺ T cell transfer colitis. DSS and the transfer of CD4⁺CD62L⁺ T cell into SCID mice caused a substantial degree of inflammation and tissue injury in the mouse colon, which was characterized by the infiltration of inflammatory cells, epithelial disruption, edema and hemorrhage. The degree of these changes was substantially reduced in mice treated with FTY720. We have shown that the anti-inflammatory effects of FTY720 involve a reduction in myeloperoxidase activity and a reduction in the number of infiltrating CD4⁺ T cells. Thus, FTY720 ameliorated the development of both DSS-colitis and CD4⁺CD62L⁺ T cell transfer colitis, and these findings in different colitis models suggest the potential application of FTY720 as a novel therapeutic agent for IBD patients.

The development of both DSS-colitis and CD4⁺CD62L⁺ T cell transfer colitis have been reported to be associated with T cell activation. DSS is a sulfated polymer that is thought to induce mucosal injury and inflammation through a direct toxic effect on the epithelium, with the subsequent recruitment and activation of both macrophages and T cells, resulting in the induction of Th1 and Th2 cytokines and other inflammatory mediators which promote the development of colitis (14,17,21). On the other hand, CD4⁺CD62L⁺ T cell transfer colitis is widely used to investigate the role of specific T cells in the induction of intestinal inflammation, because colitis is mediated by the transfer of 'pathogenic' CD4⁺ lymphocytes into immune deficient mice (15,16). Since the infiltration of CD4⁺ T cells into the colonic lamina propria was significantly reduced by FTY720 in two colitis models, it is possible to explain that the molecular mechanisms underlying the therapeutic effects of FTY720 against DSS-colitis and CD4⁺CD62L⁺ T cell transfer colitis are associated with its actions to the promote sequestration of T cells in secondary lymphoid tissues.

In conclusion, we have demonstrated that a novel lymphocyte homing reagent, FTY720, effectively prevented the development of DSS- and CD4⁺CD62L⁺ T cell transfer-colitis. The most likely mechanism responsible for these effects is an accelerated sequestration of circulating lymphocytes into secondary lymphoid tissues, resulting in a reduction of circulating lymphocytes and an inhibition of their migration to inflammatory sites. Since FTY720 exerts potent immunosuppressive effects without any down-regulation of T cell functions, FTY720 may be a useful for the treatment of IBD patients.

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