

FIGURE 2. Changes in S1P₁ expression in splenic CD4⁺ T cells during the induction of allergic diarrhea. S1P₁ expression was measured by quantitative RT-PCR. Splenic CD4⁺ T cells were isolated from naive mice or mice treated with systemic priming only (s.c. only) or systemic priming plus oral challenge (s.c. + oral). Data represent the relative ratio of S1P₁ expression to GAPDH. Results are the mean ± SE from three separate experiments.

help control allergic diarrhea by inhibiting allergen-specific CD4⁺ T cell migration from the spleen into the large intestine. To test this hypothesis, we used FTY720 as an agonist for S1P receptors, thereby inhibiting lymphocyte egress from lymphoid organs (16–18). Mice were treated with FTY720 beginning the day before systemic priming and continuing throughout the period of OVA administration. The induction of allergic diarrhea was completely abolished in the FTY720-treated mice (Fig. 3, A and B). We next sought to examine whether the priming or challenge stage was more important for development of the disease by administering FTY720 either once before systemic priming or before every oral challenge, respectively (Fig. 3C). When administered before oral challenge, FTY720 successfully inhibited the development of allergic diarrhea (Fig. 3C), but the drug had only minimal beneficial effects when administered solely before systemic priming. These results indicate that FTY720 selectively affects cell trafficking from the spleen to the large intestine during oral challenge.

FTY720 inhibits migration of systemically primed CD4⁺ T cells from the spleen into the large intestine in orally challenged mice

To examine whether FTY720 actually affected the migration of systemically primed CD4⁺ T cells from the spleen into the large intestine, we examined the influence of FTY720 on activated CD4⁺ T cells in the spleen and large intestine. After in vivo treatment with FTY720, the percentage of activated CD69-expressing CD4⁺ cells in the spleen increased but the percentage in the large intestinal lamina propria declined (Fig. 4A), indicating that acti-

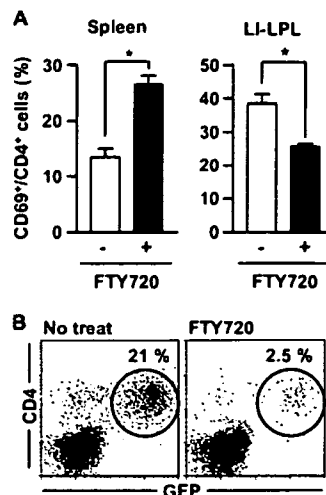


FIGURE 4. FTY720 inhibited pathogenic CD4⁺ T cells migration into the large intestine. **A**, Splenocytes and large intestinal lamina propria lymphocytes (LI-LPL) were isolated from mock-treated (–) and FTY720-treated (+) mice after 10 challenges with oral OVA. The percentage of activated (CD69⁺) cells among the CD4⁺ T cells was determined by FACS analysis. The data represent the mean ± SE from five separate experiments. *, *p* < 0.01. **B**, CD4⁺ cells were isolated from the spleen of GFP transgenic mice 1 wk after systemic priming with OVA and transferred into SCID mice. The reconstituted SCID mice were orally challenged with 50 mg of OVA. After three challenges with the oral OVA for 1 wk, the infiltration of GFP⁺ CD4⁺ cells into the large intestine was determined by flow cytometry. The percentage of gated area represents the infiltrated cells derived from adoptively transferred GFP⁺ CD4⁺ T cells. The results shown represent three independent experiments conducted.

vated T cells were prevented from migrating from the spleen to the large intestine. To determine whether FTY720 also could inhibit CD4⁺ T cell infiltration into the large intestine, we performed an adoptive transfer experiment in GFP transgenic mice (Fig. 4B). We prepared splenic CD4⁺ T cells from systemically primed GFP transgenic mice, adoptively transferred them via the i.v. route into SCID mice, and then repeatedly challenged those mice with OVA (Fig. 4B); 1 wk after the third oral OVA challenge, we isolated mononuclear cells from the large intestinal lamina propria and counted the number of infiltrated CD4⁺ T cells. We found that FTY720 treatment dramatically decreased the infiltration of GFP-positive systemically primed CD4⁺ T cells into the large intestine (Fig. 4B). These results demonstrate that FTY720 suppressed the preferential infiltration of systemically primed T cells into the large intestine, which was initiated by oral allergen challenge.

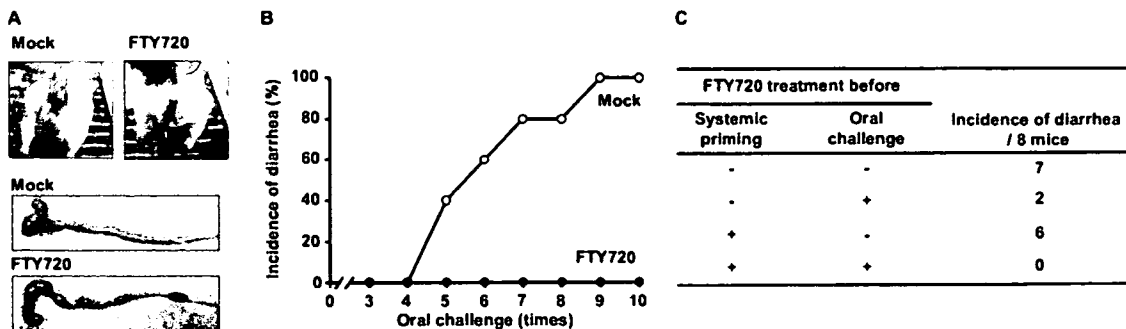


FIGURE 3. FTY720 suppressed the development of allergic diarrhea. Allergic diarrhea induction in mock- and FTY720-treated groups was compared. FTY720 (1 mg/kg) was i.p. administered before systemic priming with OVA/CFA and during induction of allergic diarrhea by OVA oral challenge as described in *Materials and Methods*. **A**, After 10 challenges with oral OVA, mock-treated mice showed severe diarrhea within 1 h, but FTY720-treated mice showed normal feces. **B**, The incidence of diarrhea in mock-treated (○) and FTY720-treated (●) groups (*n* = 5 mice per group) after each oral allergen challenge is shown. **C**, The incidence of diarrhea was examined in mice (*n* = 8) with a different experimental schedule of FTY720 treatments.

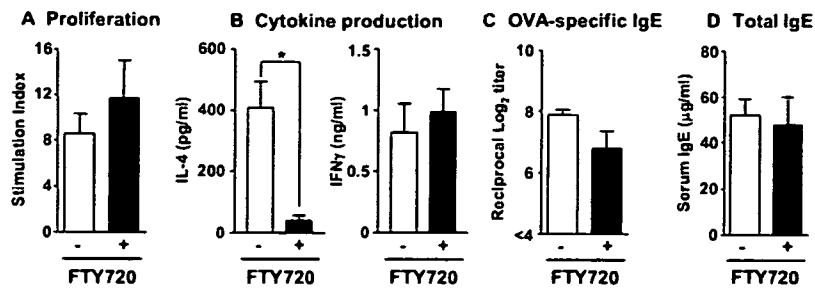


FIGURE 5. FTY720 treatment reduces IL-4 synthesis without affecting IgE production. *A*, CD4⁺ T cells were isolated from the spleen of mock-treated (–) or FTY720-treated (+) mice for the analysis of proliferative responses against OVA. The data represent the mean ± SE (*n* = 3). *B*, IL-4 and IFN-γ production from OVA-stimulated CD4⁺ T cells were measured. The data represent the mean ± SE (*n* = 4). *, *p* < 0.01. *C* and *D*, Serum was collected from mock- or FTY720-treated mice after challenge with oral OVA 10 times to measure OVA-specific IgE (*C*) or total IgE (*D*) production by ELISA. The data represent the mean ± SE (*n* = 6).

FTY720 treatment inhibits Th2 responses without affecting the induction of allergen-specific IgE production

We next elucidated the contribution of allergen-specific T cells in the spleen and their responses to FTY720 treatment during the process of allergic diarrhea development. An OVA-induced T cell proliferation assay showed that the degree of OVA-specific proliferative response by splenic CD4⁺ T cells isolated from FTY720-treated mice was similar to or slightly higher than the response from mock-treated mice (Fig. 5*A*). We also examined OVA-specific T cell proliferation in PPs and MLNs; these gut-associated lymphoid tissues are well-known contributors to the induction of immune responses against ingested Ag (1, 2). CD4⁺ T cells in PPs and MLNs showed less proliferative responses than did splenic T cells, and FTY720 did not affect these allergen-specific T cell responses (Fig. 6*A*). We then demonstrated a reduction of IL-4 production in the spleen, PPs, and MLNs of mice treated with FTY720 (Figs. 5*B* and 6*B*). These findings suggest that the FTY720 treatment inhibits IL-4 production by OVA-specific CD4⁺ T cells.

Because Th2-mediated IgE responses are involved in allergic diarrhea (11), we supposed that the reduction of IL-4 production in FTY720-treated mice led to a decrease in IgE production; this effect might be a key contributing factor for preventing allergic diarrhea. To test this hypothesis, we examined IgE production and found that, despite its complete inhibition of allergic diarrhea (Fig. 3, *A* and *B*), FTY720 treatment unexpectedly failed to affect either Ag-specific or total IgE production in serum (Fig. 5, *C* and *D*). These findings indicated that FTY720 effectively prevented allergic diarrhea development without altering IgE responses.

FTY720 treatment alters mast cell, but not eosinophil, infiltration into the large intestine

Eosinophils are inflammatory cells that are thought to be involved in allergic diseases in mice and humans, although this issue is a

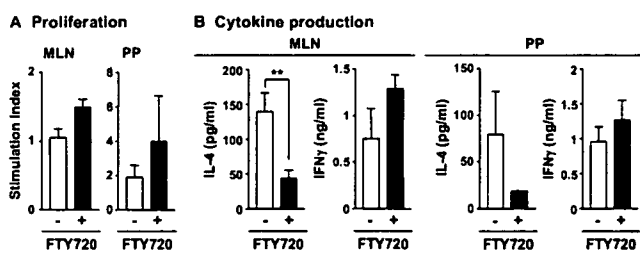


FIGURE 6. OVA-specific CD4⁺ T cells responses in the gut-associated lymphoid tissues of mock-treated (–) and FTY720-treated (+) mice. *A* and *B*, CD4⁺ T cells were isolated from the MLNs and PP of mock- or FTY720-treated mice and subjected to the analysis of OVA-specific T cell proliferative responses (*A*) and their cytokine production (*B*). The data represent the mean ± SE from three separate experiments. **, *p* < 0.05.

subject of debate (12, 39). We next investigated whether FTY720 inhibited intestinal eosinophilia. According to the criteria outlined in a previous study (40), we defined eosinophils as those cells in CD4⁺ populations that expressed CCR3 and the α₄β₇ integrin. Flow cytometric analysis revealed that FTY720 treatment did not reduce eosinophil numbers in the large intestine (Fig. 7*A*). The conclusion was further confirmed by H&E staining, which showed similar distributions of eosinophils in the large intestines of FTY720-treated and nontreated mice (Fig. 7*A*). These results suggest that eosinophils are not targets of FTY720.

We next investigated whether FTY720 treatment affected mast cells, which are other effector cells involved in intestinal allergic

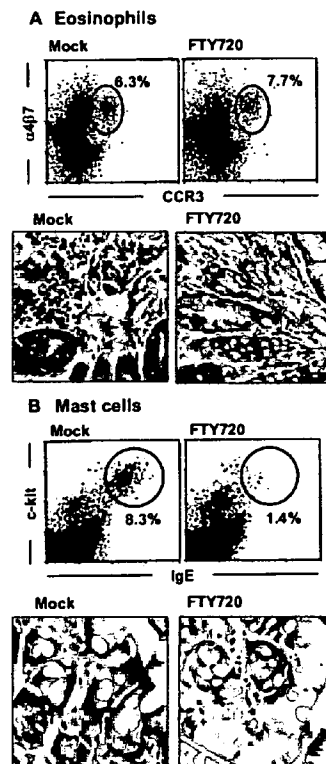


FIGURE 7. Infiltration of mast cells but not eosinophils was inhibited by FTY720 treatment. *A*, After challenge 10 times with oral OVA, CCR3⁺ α₄β₇ integrin⁺ CD4⁺ eosinophils in the large intestinal lamina propria were analyzed by flow cytometry analysis (*top*) or H&E staining (*bottom*; arrowheads). *B*, Similarly, IgE-bound c-kit⁺ mast cells were determined by flow cytometry (*top*) and naphthol AS-D chloroacetate esterase staining (*bottom*; arrowheads). Results shown are the representative data of five separate experiments. The percentage of gated area represents infiltrated eosinophils (*A*) and mast cells (*B*).

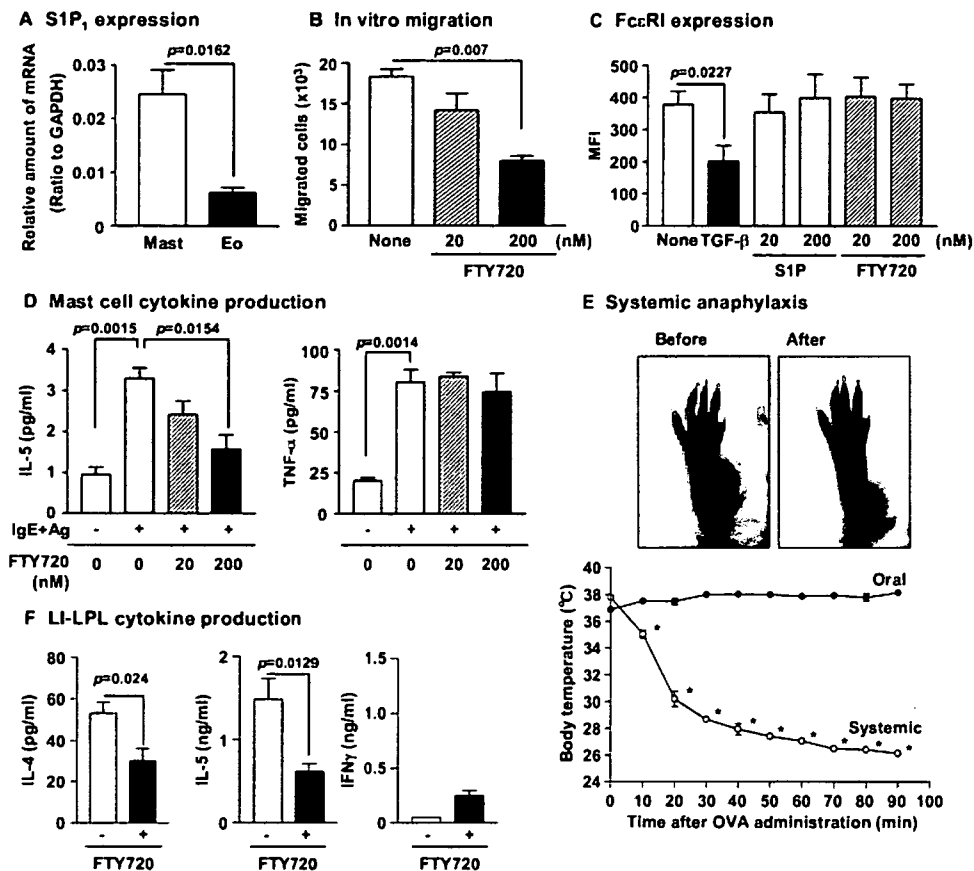


FIGURE 8. FTY720 mediates the migration and function of S1P₁-positive mast cells. **A**, Mast cells and eosinophils were isolated from the large intestine of mice with allergic diarrhea. S1P₁ expression was examined by quantitative RT-PCR. Data shown represents the relative ratio of S1P₁ expression to GAPDH. Results represent the mean \pm SE from three separate experiments. **B**, A transmigration assay was performed with bone marrow-derived mast cells. Mast cells were preincubated with DNP-IgE in the presence of 0, 20, or 200 nM FTY720 for 12 h before being added to the fibronectin-coated upper chamber of a Transwell plate with 20 ng/ml DNP-HSA in the lower chamber. Six hours later, the number of cells that had migrated into the lower chamber was counted. **C**, After 3 days of culture of peritoneal mast cells in 50% WEHI-3 conditioned culture medium containing TGF-β1 (10 ng/ml), S1P (20 or 200 nM), or FTY720 (20 or 200 nM), the expression of FcεRIα on the mast cells was determined by flow cytometry. Data are presented as the mean fluorescence intensity (MFI) of the experiment ($n = 6$). **D**, MC/9 cells were stimulated with DNP-IgE and 20 ng/ml DNP-HSA in the presence or absence of FTY720, and their production of IL-5 and TNF-α production was examined. Data represent the mean \pm SE ($n = 3$). **E**, Mice were i.p. administered with 50 mg of OVA after systemic priming and 10 challenges with oral OVA. The anaphylactic response in the foot was measured after 1 h and body temperature were also examined at various time points. Data represent the mean \pm SE ($n = 5$). *, $p < 0.01$. **F**, IL-4, IL-5, and IFN-γ production from OVA-stimulated large intestinal lamina propria lymphocytes was measured. Experiments were repeated twice with similar results. Data represent the mean \pm SE ($n = 6$).

hypersensitivity. Flow cytometric analysis indicated that c-kit⁺ and IgE-bound mast cells were markedly reduced in the large intestinal lamina propria after the treatment with FTY720 (Fig. 7B). A similar result was obtained in mast cells costained with anti-c-kit and anti-FcεRIα Abs (data not shown). Naphthol AS-D chloroacetate esterase staining further confirmed this observation (Fig. 7B). Therefore, treatment with FTY720 effectively inhibited the accumulation of mast cells at the disease site.

Two-layered effects of FTY720 on mast cell trafficking

Inasmuch as the FTY720 treatment specifically affected mast cells, we examined whether intestinal mast cells expressed S1P₁. Consistent with the results of a previous study (26), lamina propria mast cells expressed S1P₁ (Fig. 8A). In agreement with the effects of FTY720 on mast cells but not eosinophils (Fig. 7), the expression of S1P₁ on lamina propria mast cells was much higher than expression on eosinophils (Fig. 8A). We then performed an in vitro assay using bone marrow-derived mast cells to examine whether FTY720 directly inhibited mast cell migration. We revealed that mast cells treated with Ag-specific (DNP) IgE were triggered to

migrate by the corresponding Ag (DNP), but also that this migration was impaired by treatment with FTY720 (Fig. 8B). We measured FcεRIα on mast cells because some regulatory cytokines, including TGF-β and IL-10, directly inhibit FcεRI expression in mast cells and subsequently prevent their interaction with Ag (41, 42). In vitro culture of mast cells with physiological concentrations of S1P or FTY720 did not alter FcεRI expression, whereas TGF-β1 reduced FcεRIα expression (Fig. 8C) as previously reported (42). Additional analyses revealed that FTY720 treatment inhibited IL-5 production but not TNF-α production from allergen-stimulated mast cells (Fig. 8D). This finding is consistent with a previous report demonstrating that FTY720 inhibited PGD₂ production from mast cells without affecting their degranulation and TNF-α production (28). These findings suggest that FTY720 selectively inhibits specific biologic functions (e.g., IL-5 production) of intestinal mast cells.

Anaphylactic shock and hypothermia were induced when FTY720-treated mice received i.p. the same amounts of OVA as given orally, although the FTY720-treated mice did not show any allergic diarrhea after oral OVA challenge (Fig. 8E). These data

suggest that FTY720 exerts its influence by inhibiting the migration of mast cells into the large intestinal lamina propria and their synthesis of some biologically active molecules (e.g., inhibition of IL-5 and PGD₂), but mast cells retain their ability to capture IgE via FcεRIα and subsequently induce degranulation in response to a corresponding systemically administered allergen. As an additional layer in the inhibitory mechanism of FTY720, we found that lymphocytes in the large intestinal lamina propria of FTY720-treated mice showed impaired production of IL-4 and IL-5 (Fig. 8F), cytokines that activate and recruit mast cells (43, 44). Therefore, it is plausible that FTY720 hampers mast cell migration into the large intestine by both direct inhibition of S1P-mediated mast cell migration and indirect effects on Th2-associated cytokine production for mast cell migration or activation. Both of these effects may be critical factors in FTY720-associated inhibition of allergic diarrhea.

Discussion

Increased understanding of S1P and its receptor S1P₁ has opened an avenue of investigation into a previously unexplored facet of the immune system, i.e., the role played by lipid-associated biological metabolites in the cell trafficking between primary and secondary lymphoid tissues (13, 14). Because S1P and S1P₁ mediate the egress of lymphocytes from lymphoid tissues, molecular interruption of the interaction between ligand and receptor may lead to the inhibition of the effector and pathological lymphoid cell trafficking for the establishment of pathologic conditions. In acting as an agonist for S1P receptors and thereby inhibiting lymphocyte egress from lymphoid organs, FTY720 shows strong therapeutic potential for preventing the rejection of transplanted organs and for treating autoimmune diseases, such as autoimmune encephalomyelitis and systemic lupus erythematosus (20). In fact, some of these possible therapeutic uses are currently being clinically evaluated (20). Although S1P has been implicated in allergic responses in the lung (30) and although high enzymatic activity, required for S1P generation, exists (e.g., sphingosine kinase and neural ceramidases) in the intestine (45, 46), the involvement of S1P in the development of allergic responses, specifically food allergies, has remained obscure. In the current study, we have addressed this issue and demonstrated that inhibition of the S1P-mediated pathway leads to suppression of Ag-specific intestinal allergic hypersensitivity.

Our current results indicate that allergic responses are inhibited when activated CD4⁺ T cells are prevented from migrating from the spleen into the large intestine at the sensitization phase, because that migration is the pathway responsible for the induction of aberrant allergic responses. Therefore, removing the spleen during systemic sensitization impaired the development of allergic diarrhea, whereas splenectomy during oral challenge failed to prevent allergic diarrhea (Fig. 1). In this experiment, we noted that ~30% of splenectomized mice still showed allergic symptoms, a finding suggesting that other lymph nodes may compensate for the spleen as a site for priming allergen-specific CD4⁺ T cells (Fig. 1). However, FTY720 treatment completely inhibited the development of allergic diarrhea (Fig. 3, A and B). Therefore, it seems that FTY720 prevents the migration of pathogenic cells from the spleen and possibly other systemic lymphoid tissues into the large intestine.

There is now increased evidence that naive lymphocytes express high levels of S1P₁; that expression is down-regulated when lymphocytes are activated by T cell receptors and then up-regulated once again to promote egress from lymphoid organs (17, 47). Consistent with this fact, splenic CD4⁺ T cells showed a temporal reduction in S1P₁ expression after systemic priming (Fig. 2). The

reduction seemed to allow cells to remain in the spleen until they once again had an opportunity to be activated and subsequently begin their egress from the spleen. S1P₁ expression was restored by repeated oral challenge, which enabled the cells to migrate to the large intestine in an S1P-dependent manner.

In addition to CD4⁺ T cells, accumulating evidence demonstrates that dendritic cells express S1P₁ and that FTY720 treatment inhibits their migration to draining lymph nodes (48–50). A recent study revealed that local treatment with FTY720 inhibited the migration of dendritic cells from the lung to the mediastinal lymph nodes, leading to impaired formation of allergen-specific Th2 cells in lymph nodes (51). Therefore, it is also possible that FTY720 may inhibit the migration of dendritic cells from the intestine to the draining lymph nodes, subsequently preventing the activation of CD4⁺ T cells in the large intestine.

Although our current findings clearly suggest that FTY720 effectively targeted the S1P-mediated migration pathway between the systemic and large intestinal compartments and thereby helped to prevent allergic diarrhea, we still do not know the exact molecular mechanism for the S1P-dependent preferential migration of systemically primed pathogenic CD4⁺ T cells from the spleen to the large intestine. Indeed, our previous and current findings suggested that, after repeated oral challenge, pathogenic T cells were preferentially located in the large intestine but not in the small intestine, MLNs, and PPs (7) (Fig. 6). In relation to this issue, our group has recently demonstrated that PP provide a milieu for inducing regulatory T cells for the control of food allergy (32). In particular, PPs-null mice were more susceptible to developing allergic diarrhea than were PPs-intact mice. Therefore, it is plausible that these regulatory T cells in the PP might prevent the migration, proliferation, or activation of pathogenic T cells in the small intestinal compartment.

We also observed that mast cell migration into the large intestine was inhibited by the interruption of S1P- and S1P₁-mediated molecular events by FTY720 (Fig. 7B). There are a number of possible underlying mechanisms for the regulation of mast cell trafficking into the large intestine. First, FTY720 might directly inhibit the migration of mast cells into the large intestine. Mast cells express S1P₁ (26) and our data also confirmed that result (Fig. 8A). This idea is further supported by the data generated through our *in vitro* migration assay, which showed a direct suppressive effect of FTY720 on mast cell migration toward Ag (Fig. 8B). In agreement with this observation, mast cells capturing Ag-specific IgE preferentially migrated toward Ag in an S1P-dependent manner in previous studies (27, 52). Therefore, cross-linking of allergen-IgE complexes and FcεRI can activate sphingosine kinase 1, resulting in the generation of S1P (26, 27). Transactivation of the S1P₁-mediated G_i protein-signaling cascade is important in the rearrangement of the cytoskeleton and the migration of mast cells toward Ag (25–27).

In an alternative mechanism, pathogenic CD4⁺ T cells may guide mast cells to the large intestine via secretion of hypersensitivity-associated cytokines. To this end, IL-4 and IL-5, the major Th2 cytokines associated with allergic responses, have been shown to possess activity of mast cell activation and recruitment (43, 44) and promote switching of mast cell precursors to connective tissue- or mucosal-type mature mast cells in the presence of IL-3 (44). Our current results show that FTY720-induced inhibition of pathogenic T cell migration from the spleen to the large intestine led to simultaneous inhibition of IL-4 and IL-5 production (Fig. 8F), supporting the possibility that FTY720 treatment led to the inhibition of IL-4- and IL-5-mediated mast cell migration and activation in the large intestine. This idea is further supported by our recent and separate preliminary observations that repeated oral

challenge did not induce the infiltration of mast cells into the large intestine of SCID mice that were adoptively transferred with or without mast cells pretreated with serum derived from mice with diarrhea (our unpublished observations).

The fact that we did not detect mast cells in the large intestine without oral challenge suggests the third possibility that mast cells infiltrated the large intestine only in the presence of allergen (data not shown). FTY720 thus may inhibit Ag transport from the intestinal lumen into the large intestine. Previous studies revealed that allergen transport under allergic conditions was enhanced by CD23 (Fc ϵ RII) that was expressed on intestinal epithelial cells, and that enhancement protected allergen from degradation during the translocation (53). Perhaps, FTY720 negatively regulates CD23 expression on epithelial cells and reduces allergen transport into the large intestine, thus inhibiting mast cell migration. We also cannot disregard the possibility that interactions between mast cells and endothelial cells were disrupted by treatment with FTY720 because endothelial cells also express S1P receptors for the regulation of their barrier function (54). Continuous study will be required to shed further light on the molecular mechanisms of migration.

Although it showed remarkable effects on mast cell migration, FTY720 treatment did not affect eosinophil migration into the large intestine (Fig. 7A). Because human eosinophils express S1P receptors, including S1P₁, and because S1P promotes CCR3-dependent migration in human eosinophils (29), modification of the eosinophils trafficking through the S1P-mediated pathway may offer a novel therapeutic strategy (39). However, to date no study has been done on S1P receptor expression on murine eosinophils. Our current experiment indicated that S1P₁ expression was higher in mast cells than in eosinophils (Fig. 8A). These observations imply that the S1P-S1P₁ dependency of murine eosinophils is much lower than that of mast cells and of human eosinophils, accounting for our observing fewer inhibitory effects of FTY720 on eosinophils in our murine intestinal allergy model. These findings indicate that eosinophils are not involved in the development of food allergy as a previous report demonstrated (12). This evidence strengthens the hypothesis that mast cells play a key role in the development of allergic diarrhea and that the inhibition of mast cell migration into the large intestine is a critical step in the FTY720-mediated inhibition of allergic diarrhea. Our study sheds the light on a novel approach to the prevention and treatment of intestinal allergic disease; targeting S1P-mediated pathogenic cell migration.

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Disclosures

The authors have no financial conflict of interest.

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Sphingosine 1-phosphate dependence in the regulation of lymphocyte trafficking to the gut epithelium

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It is well established that intraepithelial T lymphocytes (IELs) are derived from conventional single-positive (SP) thymocytes, as well as unconventional double-negative (DN) thymocytes and CD103⁺CD8 $\alpha\beta$ recent thymic emigrants (RTEs). We show that IELs can be divided into two groups according to their dependency on sphingosine 1-phosphate (S1P) for trafficking into the intestines. CD4 or CD8 $\alpha\beta$ naive lymphocytes originating from SP thymocytes express high levels of type 1 S1P receptor (S1P₁), and their preferential migration into the large intestine is regulated by S1P. In contrast, RTEs migrate exclusively into the small intestine, whereas DN thymic IEL precursors expressing either TCR $\alpha\beta$ or TCR $\gamma\delta$ migrate into both the small and large intestines. S1P does not play a role in the migration pathways of these unconventional thymic IEL precursors. Thus, down-regulation of S1P₁ expression or disruption of the S1P gradient halted conventional CD4 or CD8 $\alpha\beta$ IEL trafficking into the intestines, but did not affect the trafficking of unconventional thymic IEL precursors. These data are the first to demonstrate that a lipid-mediated system discriminates IELs originating from conventional and unconventional thymic precursors.

The gastrointestinal tract harbors numerous luminal foreign antigens, including food products and commensal and pathogenic microorganisms. To maintain appropriate homeostasis in this harsh environment, both innate and acquired immunity are required (1–3). In the intestinal epithelium, innate and acquired mucosal immunity are bridged in part by intraepithelial T lymphocytes (IELs) located between epithelial cells (ECs) (4, 5). Previous studies of small intestinal IELs have shown them to possess several features that distinguish them from peripheral T cells. For instance, small intestinal IELs are composed of conventional CD4 and CD8 $\alpha\beta$ cells, as well as unique cells expressing CD8 α as a homodimer (CD8 $\alpha\alpha$) with either TCR $\alpha\beta$ or TCR $\gamma\delta$ (4, 5). Additionally, in contrast to the strict selection of CD4 and CD8 $\alpha\beta$ T cells in the thymus, CD8 $\alpha\alpha$ IELs possess several unique developmental pathways (6–12).

Several lines of evidence have demonstrated that the composition of IELs in the large intestine differs from that in the small intestine (4, 13–15), but the molecular mechanism underlying this distinction has remained obscure. Its identification would greatly improve our understanding of immune surveillance and homeostasis in the intestine, as the physiological function and surrounding microenvironment of these two portions of the digestive tract are different.

Recently, sphingosine 1-phosphate (S1P) has received considerable attention for its biological activity against different cell types, including lymphocytes (16, 17). To date, five S1P receptors have been identified, each of which associates with a different type of G protein, resulting in a distinct signal transduction (16, 17). Mounting evidence demonstrates that lymphocytes preferentially express type 1 S1P receptor (S1P₁) and S1P₄, and the former has been shown to regulate lymphocyte emigration from the thymus and secondary lymphoid organs (18, 19).

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Abbreviations used: CCR9, CC chemokine receptor 9; CP, colonic patch; DN, double-negative; DOP, deoxyribose; DP, double-positive; EC, epithelial cell; IEL, intraepithelial T lymphocyte; IBD, inflammatory bowel disease; MLN, mesenteric LN; PP, Peyer's patch; RTE, recent thymic emigrant; S1P, sphingosine 1-phosphate; S1P₁, type 1 S1P receptor; SLN, sacral LN; SP, single-positive; z TN, triple-negative; TP, triple-positive.

The online version of this article contains supplemental material.

FTY720 binds to four types of S1P receptor, including S1P₁, and induces down-regulation of their expression on thymocytes and lymphocytes (18, 20, 21). Thus, FTY720 induces lymphocyte sequestration in lymph and blood by inhibiting lymphocyte emigration from the secondary lymphoid organs and thymus (18, 20–22). In addition, a recent study demonstrated that oral administration of deoxypridoxine (DOP), which is a vitamin B6 antagonist, increases S1P concentration in the thymus and secondary lymphoid organs by inhibiting S1P degradation (23). This increased S1P concentration causes lymphocytes to accumulate in the thymus and simultaneously to be depleted from the blood and lymph (23). We recently revealed that S1P also plays an important role in the regulation of peritoneal B cell trafficking into intestinal compartments for intestinal secretory IgA production (24). Although these findings suggest that S1P plays an essential role in the regulation of lymphocyte trafficking in both systemic and mucosal immunity, its involvement in IEL trafficking remains largely unknown.

In this study, we aimed to elucidate the role of S1P in the trafficking of the different subsets of small and large intestinal IELs. We present evidence that the proportion of S1P₁⁺ and S1P₁⁻ IELs differs in the small and large intestines. We also show that S1P regulates the migration of S1P₁⁺ naive IELs into the intestinal compartments through secondary lymphoid organs. In addition, we demonstrate that recent thymic emigrants (RTEs) preferentially migrate into the small intestine, whereas double-negative (DN) thymocytes expressing either TCRαβ or TCRγδ migrate into both the small and large intestines. Thus, IEL trafficking from the thymus into the intestines was not regulated by S1P-mediated pathways.

RESULTS

FTY720 reduces CD8αβ and CD4 IELs in the large intestine and some populations of CD4 IELs in the small intestine

We initially tested whether IEL populations in the small and large intestines were affected by treatment with FTY720. Confirming the findings of a previous study (25), we showed that 5 d of treatment with FTY720 reduced the total cell numbers in the spleen without affecting the cell composition (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20062446/DC1>). We also observed increased numbers of single-positive (SP) thymocytes, whereas CD8⁺ or CD4⁺ cells were markedly reduced in the liver of mice receiving FTY720 (Fig. S1 A) (22). In these mice, a significant

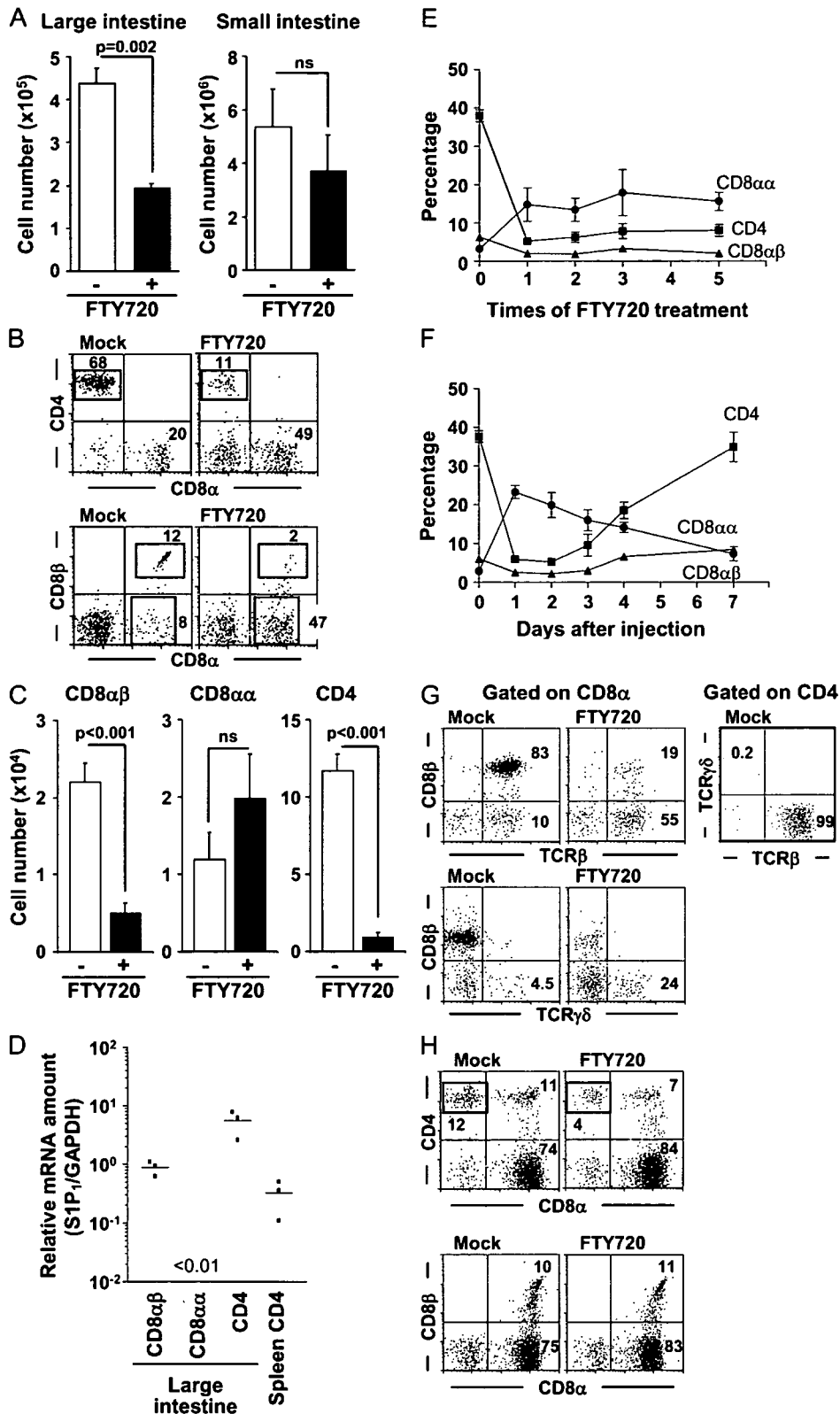
reduction in IEL numbers was observed in the large intestine, with a more modest reduction noted in small intestine (Fig. 1 A). Based on these findings, we focused our initial experiment on large intestinal IELs. Flow cytometric analysis revealed that FTY720 treatment almost completely suppressed CD4 IELs, which are a major population of large intestinal IELs, but increased the number of CD8α cells in the CD3⁺ T cell fraction (Fig. 1 B). Of the two subsets found in the large intestine CD8α IEL fraction, the CD8αβ IELs were dramatically decreased by FTY720 treatment, whereas the CD8αα IELs showed an increase (Fig. 1 B). Calculation of the absolute cell numbers indicated a marked decrease in cell numbers of CD4 IELs and CD8αβ IELs, but a slight increase in CD8αα IELs after FTY720 treatment (Fig. 1 C).

We next used quantitative RT-PCR to test whether the sensitivity of large intestinal IELs to FTY720 was attributable to the expression of S1P receptors. We found that FTY720-sensitive CD4 IELs and CD8αβ IELs expressed high levels of S1P₁, whereas FTY720-insensitive CD8αα IELs showed barely detectable levels of S1P₁ (Fig. 1 D). Despite their high expression of S1P₁, large intestinal CD4 IELs and CD8αβ IELs showed lower, and sometimes barely detectable, levels of other types of S1P receptor (S1P₃ and S1P₄; unpublished data).

We next sought to determine whether FTY720 treatment had to be continuous to block S1P-mediated signals, leading to decreased numbers of large intestinal CD4 and CD8αβ IELs. In this experiment, mice were injected with FTY720 once daily for several days, and the large intestinal IEL population was examined 12 h after each injection. Dramatic reductions in CD4 and CD8αβ IEL numbers were observed after a single injection of FTY720, but additional injections did not enhance this effect (Fig. 1 E). We next analyzed the kinetics of IEL recovery after a single FTY720 treatment. A partial recuperation was detected on day 3, with full recovery observed 7 d after the injection (Fig. 1 F). These data suggest that the effect of FTY720 on large intestinal CD4 IELs and CD8αβ IELs is rapid, but reversible.

As it is well established that intestinal IELs, especially CD8αα IELs, uniquely express either TCRαβ or TCRγδ (4, 5), we set out to determine whether FTY720 treatment influenced the pattern of TCR expression by various subsets of large intestinal IELs. We found that CD8αβ IELs and CD4 IELs in the large intestine expressed TCRαβ, but not TCRγδ, and that both populations of IELs were significantly reduced when mice received FTY720 (Fig. 1 G). In contrast,

Figure 1. FTY720 treatment dramatically reduces CD4 IEL and CD8αβ IEL numbers in the large intestine, and modestly reduces CD4 IELs in the small intestine. (A) BALB/c mice received 1 mg/kg FTY720 (shaded bar) or water (open bar) for 5 d. IELs were collected from large (left) and small (right) intestines 12 h after the final administration of FTY720. The data represent the mean ± the SEM of eight independent experiments. (B) Flow cytometric analysis was performed to determine the cell population in the CD3⁺ fraction of the large intestinal IELs affected by FTY720. The data are representative of five independent experiments. (C) Cell numbers of each population were calculated using the total cell number and flow cytometric data. The error bars represent the average ± the SEM (*n* = 5). (D) Quantitative RT-PCR analysis for S1P₁ was performed using RNA isolated from the sorted large intestinal IELs and splenocytes. The relative mRNA was expressed as a ratio to GAPDH. (E) BALB/c mice received a daily intraperitoneal injection of FTY720. At 12 h after each injection, the large intestinal IELs were analyzed by flow cytometry (square, CD4; triangle, CD8αβ; circle, CD8αα). The data represent the means ± the SD (*n* = 5). (F) Flow cytometry was used to evaluate the degree of IEL recovery in the large intestine after one administration of FTY720 (square, CD4; triangle, CD8αβ; circle, CD8αα). The data represent the means ± the SD (*n* = 5). (G) TCRαβ versus TCRγδ expression on large intestinal IELs of



mice receiving 5 doses of 1 mg/kg FTY720 or water (mock). The data for cells gated on CD8 α (left) and CD4 (right). The data are representative of four independent experiments. (H) Flow cytometric analysis was performed to identify the cell population in CD3⁺ cells in small intestinal IELs affected by FTY720. The data are representative of five independent experiments.

65% of CD8 $\alpha\alpha$ IELs expressed TCR $\alpha\beta$, whereas the remaining 35% expressed TCR $\gamma\delta$. The ratio between TCR $\alpha\beta$ (68%) and TCR $\gamma\delta$ (32%) in the CD8 $\alpha\alpha$ IELs was not changed by FTY720 treatment (Fig. 1 G), although a modest increase in the number of CD8 $\alpha\alpha$ IELs was observed (Fig. 1 C). These data suggest that the efficacy of FTY720 treatment does not depend on TCR expression.

As FTY720 induced a reduction in small intestinal IEL numbers, albeit a more modest one than in the large intestine (Fig. 1 A), we next focused on the small intestinal IELs. Flow cytometric analysis indicated that CD4 IELs were significantly decreased by FTY720 treatment (mock, $13.7 \pm 0.89\%$ vs. FTY720, $5.7 \pm 1.21\%$; $P = 0.006$), whereas CD4CD8 double-positive (DP) IEL numbers were unaltered and CD8 α -positive cells were increased in CD3 $^+$ fractions (Fig. 1 H). We found a modest enhancement of CD8 $\alpha\alpha$ IELs, but only a slight increase in CD8 $\alpha\beta$ IELs (Fig. 1 H). Although FTY720 was observed to affect intestinal IELs, it had no influence over Peyer's patches (PPs) in the small intestine or colonic patches (CPs) and small lymphoid aggregates in the large intestine (Fig. S1, A and B). We also confirmed that the epithelium was specifically removed during the separation process of IELs (Fig. S1 C), and found that similar results were obtained when saline perfusion was performed before tissue isolation (unpublished data). These findings suggest that the reduction of CD4 and CD8 $\alpha\beta$ lymphocytes occurred specifically in the IEL population, and was not caused by contamination from other tissues.

The specific expression pattern of adhesion molecules correlates with the sensitivity of small and large intestinal IELs to FTY720

Tissue-specific lymphocyte trafficking is regulated by a combination of adhesion molecules and chemokines. In the current study, we focused on two representative adhesion molecules expressed specifically on gut-associated lymphocytes: $\alpha 4\beta 7$ integrin and CD103 (αE integrin) (26, 27). Each population of small and large intestinal CD4 IELs has its own distinct expression pattern (Fig. 2). All CD4 IELs express $\alpha 4\beta 7$ integrin, but $\sim 80\%$ of CD4 IELs in the small intestine are CD103 $^-$ (Fig. 2 A). After FTY720 treatment, the small intestinal epithelia of mice showed a simultaneous decrease in the percentage of CD103 $^-$ CD4 IELs and an increase in the percentage of CD103 $^+$ cells (Fig. 2 A). Because CD4 IELs include CD4 SP cells and CD4CD8 DP cells (Fig. 1 H), we determined that the CD103 $^-$ CD4 $^+$ population contained a larger number of CD4 SP cells than DP cells, whereas DP cells comprised the majority of the CD103 $^+$ CD4 cell population (Fig. 2 A). FTY720 treatment preferentially reduced CD4 SP cells, but had little effect on DP cells, suggesting that CD103 $^-$ CD4 SP cells were the main target cell of FTY720 in the small intestine (Fig. 2 A). We further divided small intestinal CD4 IELs according to their expression of CD62L, finding that CD103 $^+$ CD4 IELs do not express CD62L, whereas CD103 $^-$ CD4 IELs comprise three populations expressing different levels of CD62L (CD62L high , CD62L int , and CD62L neg ; Fig. 2 A). Of these three populations, only CD62L high CD103 $^-$ CD4 IELs

were completely vanished from the small intestinal epithelium after FTY720 treatment (Fig. 2 A).

Although the various subsets of small intestinal CD4 IELs express variable levels of CD103, consistently high levels of CD103 are expressed by both CD8 $\alpha\alpha$ IELs and CD8 $\alpha\beta$ IELs (Fig. 2 A). Nonetheless, a few CD8 $\alpha\beta$ IELs did not express CD103, but exhibited high levels of CD62L (Fig. 2 A). As with CD62L high CD103 $^-$ CD4 IELs (Fig. 2 A), the CD62L high CD103 $^-$ CD8 $\alpha\beta$ IELs were effectively suppressed by FTY720 treatment (Fig. 2 A). Quantitative RT-PCR analysis demonstrated that the level of S1P $_1$ expression in CD62L high cells was greatly higher than in CD62L neg cells (Fig. 2 B), suggesting that the expression pattern of CD62L and CD103 in the small intestinal IELs correlates with the sensitivity to FTY720. Collectively, these findings show that S1P is primarily responsible for regulating the trafficking of CD62L high CD103 $^-$ IELs expressing either CD4 or CD8 $\alpha\beta$ in the small intestinal epithelium.

In the next series of experiments, we sought to characterize the association between the expression of these adhesion molecules and FTY720 sensitivity in large intestinal IELs. FTY720-sensitive large intestinal CD4 IELs and CD8 $\alpha\beta$ IELs expressed the $\alpha 4\beta 7$ integrin and high levels of CD62L, with negative or intermediate levels of CD103 (CD103 $^{int/neg}$; Fig. 2 C), which is similar to the FTY720-sensitive CD62L high CD103 $^-$ population in the small intestinal IELs (Fig. 2 A). In contrast, the FTY720-insensitive CD8 $\alpha\alpha$ population expressed high levels of CD103, but did not express the $\alpha 4\beta 7$ integrin and CD62L (Fig. 2 C). Confocal microscopic analysis confirmed the presence of CD62L $^+$ CD4 cells in the large intestinal epithelium and FTY720 treatment removed them, providing convincing evidence of a correlation between CD62L high CD103 $^{int/neg}$ cells and the sensitivity of small and large intestinal IELs to FTY720 (Fig. 2 D). Because sacral LNs (SLNs) act as draining LNs for the large intestine, we next set out to examine FTY720-induced alterations in SLN cell populations, demonstrating that FTY720 increased the number of CD62L high cells in the SLN (Fig. 2 E).

Lymphocyte migration is regulated not only by integrins, but also by chemokines. S1P is thought to be a modulator of cellular responses to some chemokines (17), and CC chemokine receptor 9 (CCR9) is believed to be involved in the migration of lymphocytes into the intestinal compartment, especially the small intestine (28). Thus, we next investigated whether distinct CCR9 expression patterns were observed for FTY720-sensitive and -insensitive IELs. We found that CD8 $\alpha\beta$ and CD8 $\alpha\alpha$ IELs in the small and large intestines expressed varying levels of CCR9 (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20062446/DC1>). The CD8 $\alpha\beta$ population in the large intestinal epithelium was reduced after FTY720 treatment, regardless of CCR9 expression (Fig. S2). Additionally, no change in the ratio of CCR9 $^+$ and CCR9 $^-$ populations in CD8 $\alpha\alpha$ IELs was observed after FTY720 treatment (Fig. S2). Hence, the CCR9 expression level does not seem to be linked to the S1P-mediated migration of small and large intestinal IELs.

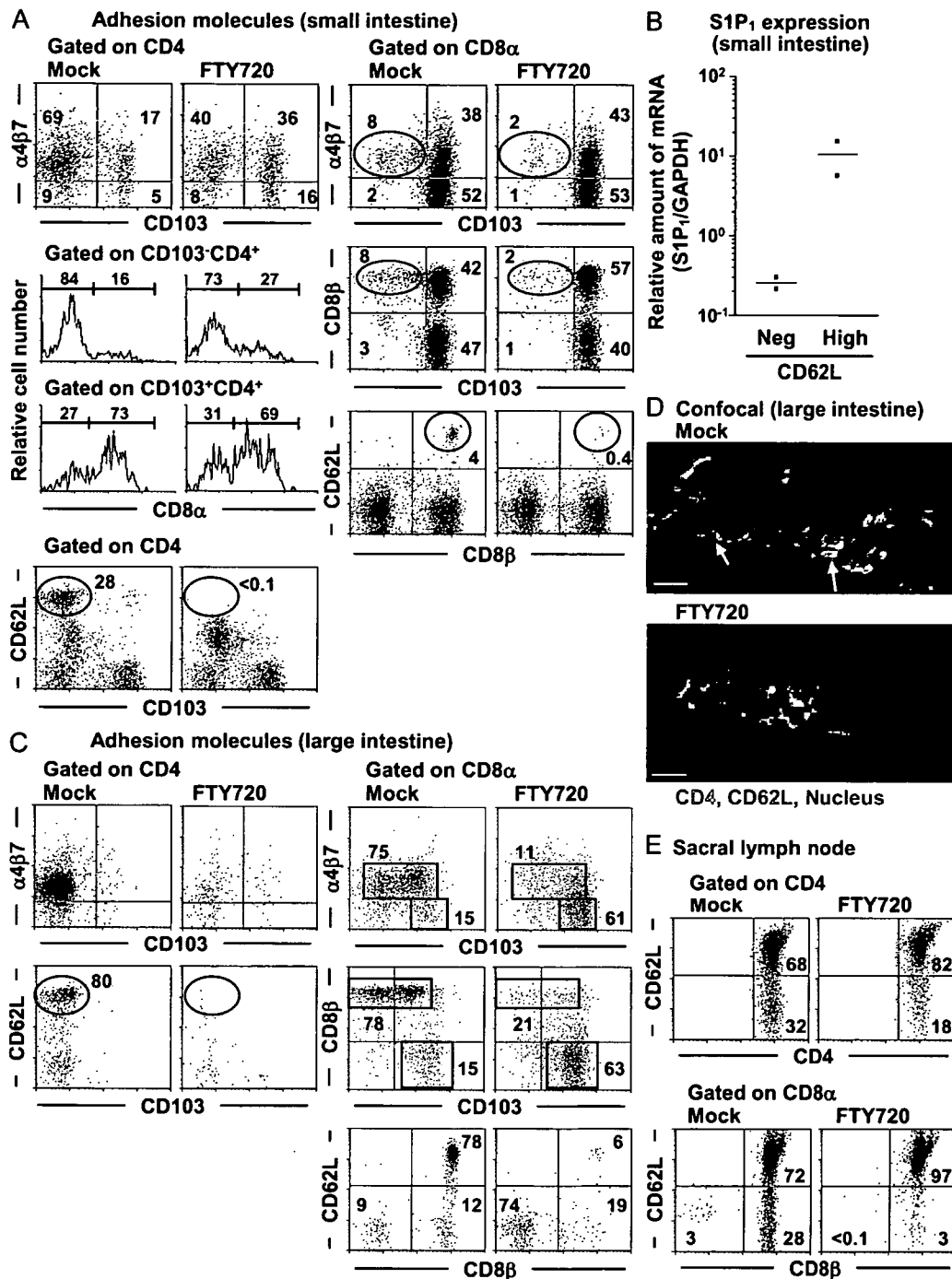


Figure 2. Unique expression of adhesion molecules determines the sensitivity of small and large intestinal IELs to FTY720. (A) Small intestines of BALB/c mice that were treated with FTY720 or water (mock) isolated for flow cytometric analysis of the adhesion molecule expression patterns. Flow cytometric profiles of cells gated on CD4 (left), CD103⁻CD4⁺ (left), CD103⁺CD4⁺ (left), and CD8 α (right) were shown. The data are representative of five independent experiments. (B) Quantitative RT-PCR analysis for S1P₁ on RNA isolated from sorted small intestinal CD4 IELs expressing CD62L^{high} or CD62L⁻. The relative mRNA is expressed as a ratio to GAPDH. (C) Experiments similar to those shown in A were performed using large intestinal IELs. Similar results were obtained from five independent experiments. (D) Confocal microscopic analysis of the large intestine was performed using antibodies for CD4 (green) and CD62L (red), and DAPI (blue) for counterstaining. Bars, 20 μ m. (E) Lymphocytes were isolated from SLN after five doses of FTY720 as shown in A and C, and were examined for the indicated populations by flow cytometry. Experiments were repeated three times.

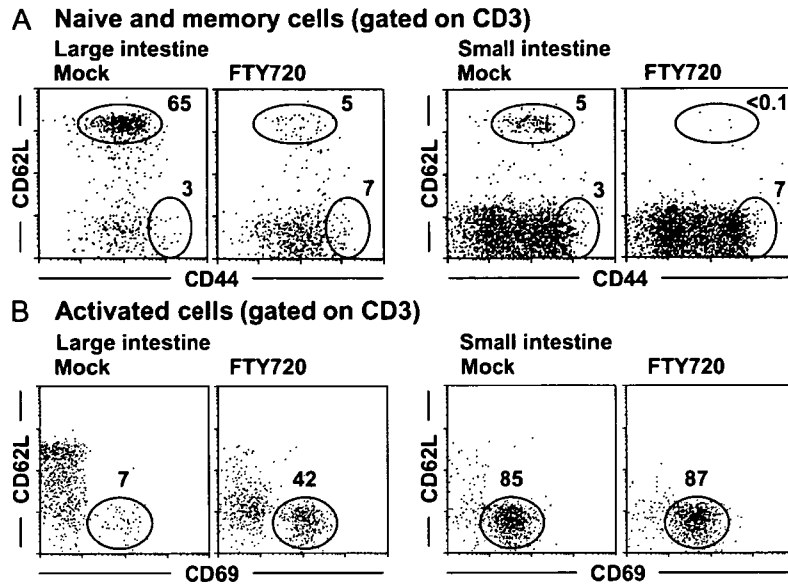


Figure 3. FTY720 selectively suppresses naive-type IELs in both the small and large intestines. IELs were isolated from the small and large intestines of mice receiving FTY720, as shown in Fig. 1. Flow cytometry was used to analyze the effects of FTY720 on naive (CD62L^{high}CD44^{int}; A) and activated (CD62L^{neg}CD69⁺; B) IELs in the CD3⁺ population. The data are representative of four independent experiments.

Naive IELs are the main targets of FTY720 in the intestinal epithelium

The high levels of CD62L expression by FTY720-sensitive cell populations (Fig. 2) led us to hypothesize that FTY720 influences naive IELs. To test this, we examined CD44, CD69, and CD62L expression patterns to determine their qualification for naive and activated cells (Fig. 3). We found that large intestinal epithelia contained greater numbers of naive cells expressing CD62L^{high}CD44^{int} than did small intestinal epithelia; however, despite this difference, naive IELs in both the small and large intestines were almost completely suppressed after FTY720 treatment (Fig. 3 A). In contrast, mice receiving FTY720 showed comparable numbers of CD62L^{neg}CD69⁺-activated IELs in the small intestine and an increased percentage in the large intestine (Fig. 3 B). Although FTY720 significantly affected naive IELs, it had almost no influence on naive cells in the PPs (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20062446/DC1A>). These findings suggest that the trafficking of naive IELs is solely regulated by S1P in the small and large intestines, and that the differing sensitivities of the small and large intestines to FTY720 can be attributed to differences in the composition of these naive cells.

FTY720 affects IEL migration and retention, but not cell activation

To determine whether FTY720 reduced naive IELs by inhibiting cell migration into the intestine, we intravenously transferred CFSE-labeled T cells isolated from mesenteric LNs (MLNs) and SLNs into mice and examined their migration into the intestinal compartments. As previously documented (18, 20–22), the number of CFSE⁺ T cells decreased in the

blood of mice treated with FTY720 both 1 and 7 d after the transfer (Fig. 4 A). In these mice, migration of CFSE⁺ cells into the MLN and SLN was reduced by FTY720 treatment, whereas that into the PPs was increased (Fig. 4, B–D), which was consistent with a previous study (29). Flow cytometric analysis of CFSE⁺ cells in these tissues revealed that their naive cell phenotype persisted after FTY720 treatment (Fig. 4, B–D). We also found CFSE⁺ cells in the large intestinal epithelium 1 d after the transfer, but the numbers of these cells were significantly decreased in mice treated with FTY720 (Fig. 4 E). Consistent with a previous work (30), fewer cells migrated into the small intestine than the large intestine under these experimental conditions (<0.01% cells were CFSE⁺ 1 d after the transfer; unpublished data). However, similar results were obtained in both the small and large intestines 7 d after transfer, demonstrating that the migration of CFSE⁺ cells was significantly curtailed by FTY720 in both the small and large intestines (Fig. 4 E). We obtained similar results when T cells isolated from GFP-transgenic mice were used instead of the CFSE-labeled system, ruling out the possibility that the CFSE⁺ cell numbers in FTY720-treated mice were reduced because of cell division during these 7 d (unpublished data). These findings suggest that prevention of ongoing naive cell homing into the gut from the systemic immune compartments is one mechanism of FTY720-induced naive IEL reduction.

As shown in Fig. 1 H, FTY720-mediated reduction of naive IELs was rapid (within 12 h). If this is attributable only to the inhibition of naive cell entry into the intestine, the turnover rate in the intestinal epithelium should be <12 h. To examine this, we performed a BrdU time course study of IELs.

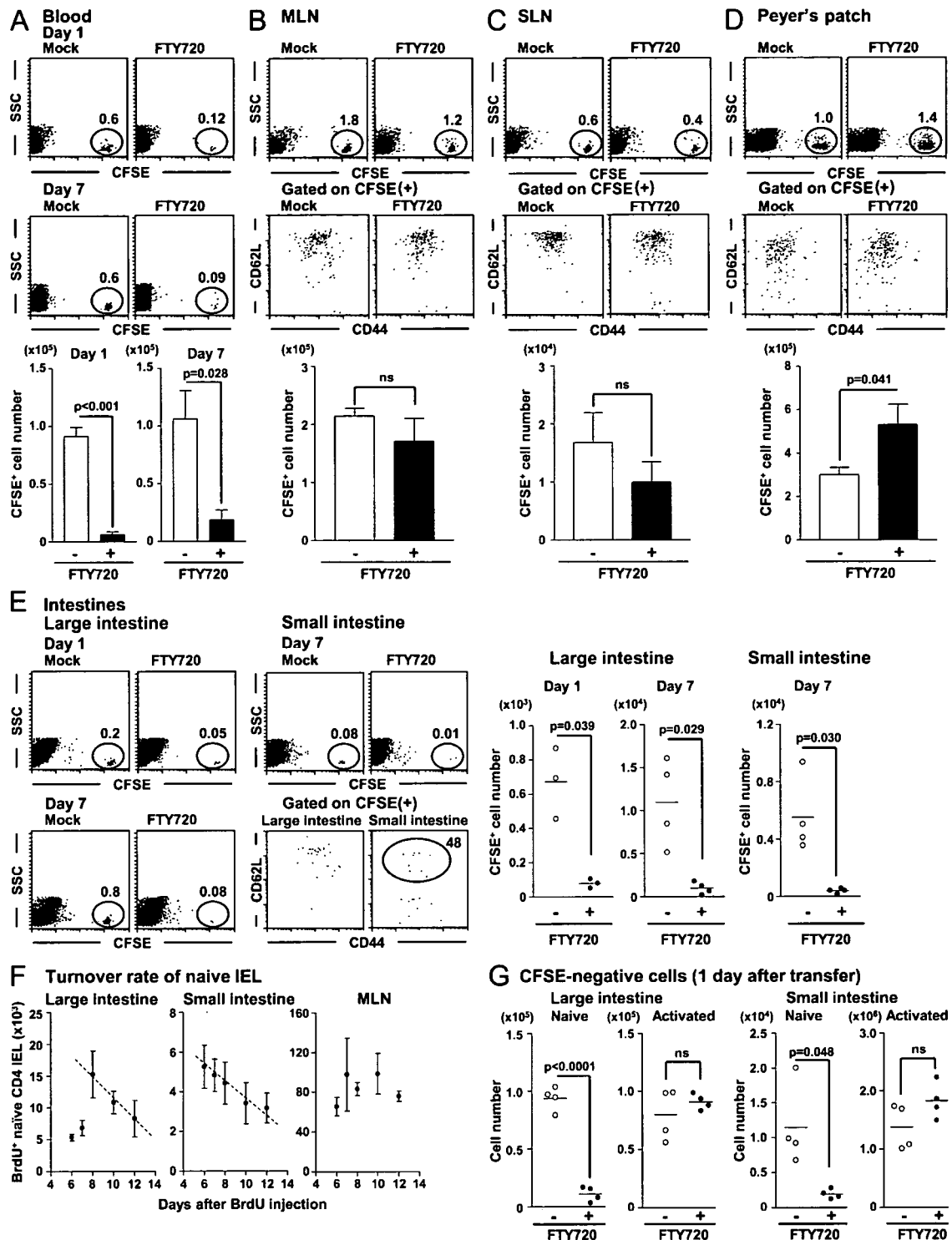


Figure 4. FTY720 inhibits T cell migration to the intestine and retention in the intestinal epithelium without affecting cell activation. (A–E) On day 1 (blood and large intestine) or day 7 (all tissues) after the adoptive transfer of CFSE-labeled T cells, cells were isolated from the blood (A), MLN (B), SLN (C), PP (D), and intestinal epithelium (E) of mice treated with water (mock, open) or FTY720 (filled), and the total number of CFSE⁺ cells was examined. The data are representative of four independent experiments, and graph data represent means \pm SEM ($n = 4$). (F) Cell numbers of BrdU⁺ naive (CD62L^{high}) CD4 IELs measured in large (left) and small (center) intestinal epithelia and MLN (right) of mice from day 6 to 12 after a single BrdU injection. The data represent the means \pm the SEM ($n = 5$). (G) Cell numbers of naive (CD62L^{high} and CD44^{int}) and activated (CD62L^{low} and CD69⁺) cells in CFSE⁻ populations were measured in the small and large intestinal epithelia of mice receiving mock (open circle) or FTY720 (filled circle) treatment (right) 24 h after transfer.

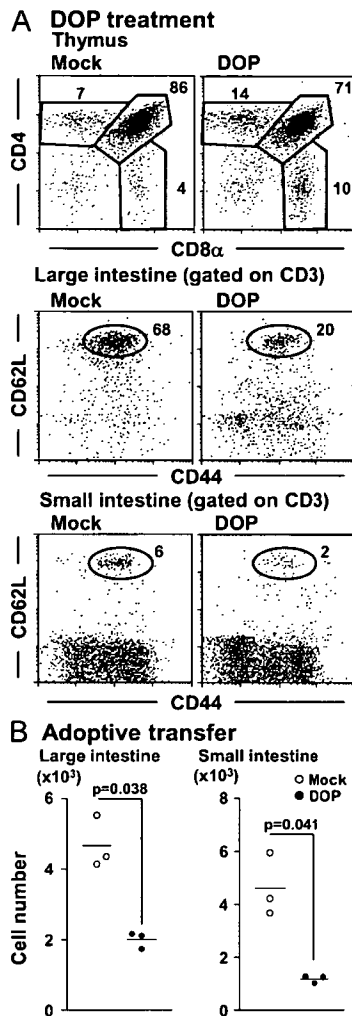


Figure 5. Disruption of S1P gradient halts naive IEL migration. (A) Lymphocytes were collected from the thymus (top) and large (middle) and small (bottom) intestines of mice treated with DOP (right) or water (left) for 3 d, and were analyzed for naive cells in the CD3⁺ population. The data are representative of three independent experiments. (B) At 7 d after the adoptive transfer of CFSE-labeled T cells, cells were isolated from the large (left) or small (right) intestinal epithelia of mice treated with mock (open circle) or DOP (filled circle), and the total number of CFSE⁺ cells was examined.

By 6 d after the single BrdU injection, BrdU-labeled cells were barely detected in the thymus (unpublished data), indicating that newly developed naive T cells were BrdU negative. The number of BrdU⁺ naive CD4 IELs started to fall on day 8 in the large intestine and day 6 in the small intestine, after a linear regression (Fig. 4 F). The estimated 50% turnover

rates of naive CD4 IELs were >3 d in both small and large intestines (Fig. 4 F). In contrast, the cell number of BrdU⁺ cells in the MLN was maintained at a similar level during the experiment, excluding the possibility that the decay curves reflected the survival time of BrdU⁺ cells. These findings led us to suggest that FTY720 affects naive IEL retention in the intestinal epithelium, which would lead to a reduction in the number of CFSE⁻ IELs. To test this hypothesis, we examined CFSE⁻ cells in the intestines 1 d after the transfer because few cells migrated into the intestinal epithelium after this time under this experimental condition (Fig. 4 E). We observed a significant decrease in naive (CD62L^{high}CD44^{int}), but not in activated (CD62L^{low}CD69⁺), CFSE-negative cells residing in the small and large intestines of FTY720-treated mice (Fig. 4 G). To examine the possibility that FTY720 treatment could lead to the disappearance of resident naive IELs by triggering either the activation of naive IELs or their programmed cell death, we next cultured naive lymphocytes with FTY720 for 2 d and examined their phenotype and viability and noted no changes in either cell activation or viability (Fig. S3 B and not depicted). These findings suggest that FTY720 inhibits not only naive lymphocyte migration into the intestine from the systemic immune compartments but also their retention in the intestinal epithelium.

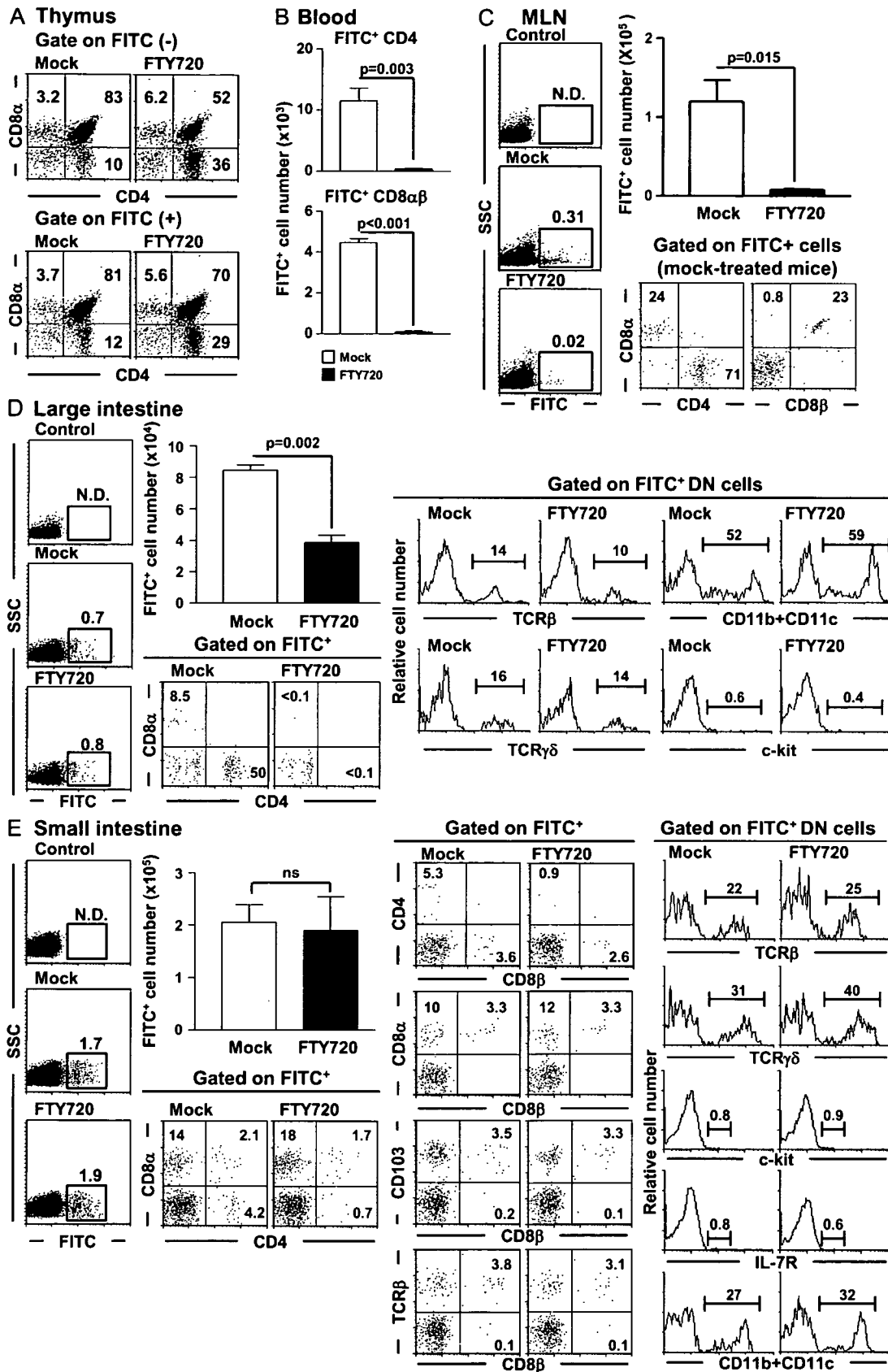
Inhibiting S1P lyase activity disrupts the S1P gradient and hampers cell trafficking into the intestine

To further confirm the involvement of S1P in the regulation of IEL trafficking, we next used DOP, which was reported to disrupt the S1P gradient by inhibiting S1P lyase (23). Our results confirmed this, and demonstrated that disruption of the S1P gradient by oral feeding with DOP for 3 d resulted in the accumulation of SP thymocytes (Fig. 5 A). DOP treatment also reduced the number of naive IELs in both the small and large intestines (Fig. 5 A). An adoptive-transfer experiment using CFSE-labeled naive T cells revealed that DOP treatment inhibited their trafficking into the intestines (Fig. 5 B). These findings convincingly demonstrated that an S1P-mediated pathway regulates the naive IEL trafficking.

FTY720-insensitive trafficking of unconventional thymic IEL precursors into the intestinal epithelium

Our focus was next shifted to FTY720-insensitive IEL populations, such as CD8α IELs. Recent studies have revealed several distinctive developmental pathways for IELs, including unconventional thymic IEL precursors (6–11). Thus, we examined whether S1P mediated the migration of these thymus-originated IEL precursors into the small and large intestines using the intrathymic FITC-labeling system. By 24 h

Figure 6. FTY720-insensitive migration of RTEs and thymic DN IEL precursors expressing TCRαβ or TCRγδ into the intestinal epithelium. By 24 h after intrathymic FITC injection, the cell populations in FITC⁺ and FITC⁻ thymocytes (A) in mock-treated (left) or FTY720-treated (right) mice were examined by flow cytometry. Cells were isolated from the blood (B), MLN (C), large intestine (D), and small intestine (E) and examined by flow cytometry. Absolute cell numbers of FITC⁺ cells were calculated using the total cell numbers and flow cytometric data. Expression of CD4, CD8α, CD8β, CD11b, CD11c, CD103, c-kit, IL-7R, TCRαβ, and TCRγδ was examined in FITC⁺ cells. The graph data depicts the means ± the SEM (n = 4). Other data are representative of at least four independent experiments.



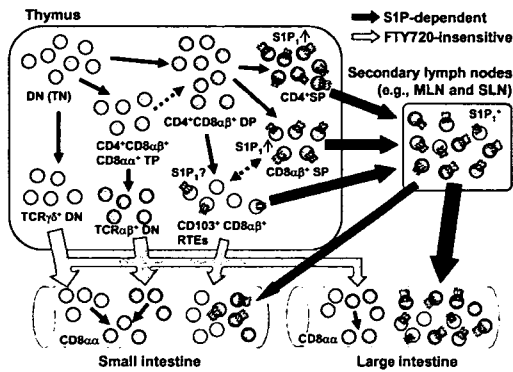


Figure 7. Dependency of lymphocyte migration into the small and large intestines on S1P. Conventional CD4 (green) and CD8 (orange) SP thymocytes emigrate from the thymus into secondary lymphoid organs, and, subsequently, into the small and large intestines. These SP cells express high levels of S1P₁ and pathways regulated by S1P (blue lines), and can be inhibited by FTY720. Unconventional thymic IEL precursors, including mature post-selected TCRαβ DN cells (red) derived from TP (CD4⁺CD8αβ⁺CD8αα⁺) thymocytes (pink), TCRγδ DN thymocytes (purple), and CD103⁺ CD8αβ RTEs (yellow), migrate into the intestinal epithelium through FTY720-insensitive pathways (red lines).

after the intrathymic FITC injection, we found that each population of thymocytes was equally stained with FITC. Thus, comparable percentages of CD4 and CD8 SP, DP, and DN thymocytes were found in FITC⁺ and FITC⁻ thymocytes in mock-treated control mice (Fig. 6 A). Additionally, FTY720 treatment resulted in similar accumulation levels of CD4 and CD8 SP thymocytes in both FITC⁺ and FITC⁻ fractions, suggesting that FTY720 inhibited the emigration of SP thymocytes regardless of FITC labeling (Fig. 6 A). These effects coincided with the barely detectable levels of CD4 and CD8 SP FITC⁺ cells in the blood circulation of FTY720-treated mice (Fig. 6 B).

Consistent with previous results (8), FITC⁺ cells were detected in the secondary lymphoid organs (such as the MLN) in mock-treated control mice (Fig. 6 C). The positive fraction mainly consisted of cells expressing CD4 or CD8αβ, but not DN or DP cells (Fig. 6 C). The group of mice treated with FTY720 possessed barely detectable levels of FITC⁺ cells (Fig. 6 C), which was plausibly caused by the inhibition of SP thymocyte emigration by FTY720. These findings led us to conclude that the pathway from the thymus into the MLN is regulated by S1P.

FITC⁺ cells were found in the large intestine of mock-treated control mice (Fig. 6 D). In FTY720-treated mice, a similar percentage of FITC⁺ cells was observed, but the absolute FITC⁺ cell number was decreased because of the reduction in total cell numbers in large intestinal IELs (Figs. 1 A and 6 D). Flow cytometric analysis revealed that FITC⁺ cells were comprised of DN cells in addition to cells expressing either CD8α or CD4, and that they did not contain any DP cells (Fig. 6 D). As with FITC⁺ CD4 and CD8α cells in the MLN (Fig. 6 C), FTY720 treatment inhibited the trafficking of

FITC⁺ cells expressing CD4 or CD8α into the large intestine, but did not influence the migration of DN cells (Fig. 6 D). As recent studies have revealed unconventional CD8α IEL precursors in the DN thymocytes (6, 7, 12), we analyzed TCR expression to reveal that FITC⁺ DN cells expressed TCRαβ (10–20%) or TCRγδ (10–20%; Fig. 6 D). We also found that FITC⁺ DN cells barely expressed c-kit, but 50–60% of FITC⁺ DN cells expressed CD11b and/or CD11c, suggesting that they were phagocytic cells taking up leaked FITC or FITC-labeled apoptotic cells (Fig. 6 D). In addition, FITC⁺ TCR⁺ DN cells were not detected when the same amount of FITC was injected intravenously (unpublished data), excluding a possibility that the FITC⁺ TCR⁺ DN cells observed in this experiment were labeled in the extrathymic compartments. These findings indicate that some FITC⁺ DN cells are derived from phagocytic cells, whereas others are composed exclusively of cells expressing TCRαβ or TCRγδ. In agreement with these findings, considerable numbers of DN cells expressing TCRαβ or TCRγδ were detected in the blood of FTY720-treated mice, although FTY720 significantly reduced CD4 and CD8 SP cells in the blood (Fig. S3 C). These data suggest that thymic CD4 and CD8 SP cells migrate into the large intestine via an S1P-mediated pathway, and DN cells expressing TCRαβ or TCRγδ migrate via FTY720-insensitive manner.

As one might expect based on the data summarized in Fig. 1, the number of FITC⁺ cells in the small intestine remained similar after FTY720 treatment (Fig. 6 E), and the FITC⁺ cells in the small intestine of mock-treated control mice were comprised of DP and DN cells, as well as cells expressing either CD4 or CD8α (Fig. 6 E). Because CD4⁺ cells exclusively expressed TCRαβ and no cells expressed CD4 together with CD8β in the FITC⁺ fraction, it seems that DP IELs are TCRαβ⁺CD8αα⁺ CD4 cells uniquely observed in the intestinal epithelium, and are not thymic DP cells (Fig. 6 E). Small intestinal FITC⁺ DN IELs included cells expressing CD11b and/or CD11c (~30%), TCRαβ (20–30%), or TCRγδ (30–40%) and few cells expressing c-kit or IL-7R (Fig. 6 E). Like the large intestine, FITC⁺ TCR⁺ DN cells were not detected when the same amount of FITC was injected intravenously (unpublished data). These findings suggest that, similar to the large intestine, some small intestinal FITC⁺ DN cells are derived from phagocytic cells, whereas others are composed exclusively of cells expressing TCRαβ or TCRγδ, and not cells derived from TCR-negative DN (triple-negative [TN]) cells.

Intriguingly, the ratio of CD8α to CD4 cells was higher in the small intestine than in MLN or the large intestine (Fig. 6, C and D). Those CD8α FITC⁺ IELs were composed of CD8αα IELs (75%) and CD8αβ IELs (25%; Fig. 6 E), the latter of which expressed CD103, which is a recently reported identifying characteristic of RTEs (Fig. 6 E) (8, 31). FTY720 significantly inhibited the migration of CD4 cells into the small intestine (mock, 4.3 ± 0.35%; FTY720, 1.1 ± 0.23%; P = 0.009), whereas other populations including TCRαβ DN cells, TCRγδ DN cells, and CD103⁺ CD8αβ RTEs were unaffected (Fig. 6 E).

Collectively, our current findings demonstrate that thymic DN IEL precursors expressing TCR $\alpha\beta$ or TCR $\gamma\delta$ are likely to migrate into the large or small intestine, whereas RTEs preferentially migrate into the small intestine (Fig. 7). Unlike S1P-dependent SP thymocytes' migration into the intestine through secondary lymphoid organs, the migration pathway of unconventional IEL precursors is totally insensitive to FTY720 treatment (Fig. 7).

DISCUSSION

In this study, we demonstrate that the lipid mediator S1P determines whether a given type of IEL goes into the small or the large intestine, resulting in varying proportions of naive cells, RTEs, and thymic DN IEL precursors in the two compartments. Naive IELs, which primarily express S1P₁, are more abundant in the large intestine than in the small intestine (Fig. 3 A), explaining why large intestinal IELs are more sensitive to FTY720 or DOP (Figs. 1, 3, and 5). These findings confirm previous reports that identify CD62L⁺ cells as the primary targets of FTY720 in systemic immunity, and show that S1P₁ is preferentially expressed on naive and central memory T cells rather than activated T cells for efficient S1P-mediated migration (18, 32, 33). In contrast, activated IELs, which were abundant in the small intestine but barely detectable in the large intestine, did not respond to FTY720 (Figs. 3 and 4). In this context, a previous study demonstrated that the activation marker CD69 itself induced down-regulation of S1P₁, thereby rendering activated cells unresponsive to S1P (34). These findings offer a plausible explanation for why activated IELs expressing CD69 in the intestinal compartments are less reactive to FTY720 (Fig. 3 B).

Our results imply that FTY720 inhibits not only the trafficking of naive IELs into the intestine, but also their retention in the intestinal epithelium (Fig. 4 and Fig. S3). This might explain why naive T cells accumulated in the SLN of FTY720-treated mice, despite their reduced migration from the blood into the SLN (Figs. 2 E and 4 C). A previous work has demonstrated that lymphocyte exit from the skin is not a random process, but is regulated by a chemokine-mediated pathway (35). Additionally, we recently reported that S1P regulates B cell retention in the peritoneal cavity (24). Our current study therefore not only confirms that lymphocyte trafficking in nonlymphoid tissues is regulated biologically, but also shows that S1P plays an important role in that pathway.

In contrast to the S1P-dependent trafficking of naive cells into the intestines through secondary lymphoid organs, thymic DN IEL precursors migrate into both the small and large intestines in an FTY720-insensitive manner (Fig. 6). Recently, several lines of evidence have proposed the presence of IEL precursors in DN thymocytes, including TCR $\alpha\beta$ ⁺ DN thymocytes, TCR $\gamma\delta$ ⁺ DN thymocytes, and TCR $\alpha\beta$ ⁻ CD25⁺ TN thymocytes (6, 7, 12). TCR $\alpha\beta$ ⁺ DN thymocytes are known as mature post-selected DN thymocytes, as they arise from CD8 $\alpha\alpha$ ⁺ CD4⁺ CD8 $\alpha\beta$ ⁺ triple-positive (TP) thymocytes after agonist selection (6). TCR $\alpha\beta$ ⁺ DN thymocytes then migrate into the intestine, where they reinduce

CD8 $\alpha\alpha$ under the influence of IL-15 (6). It has been shown that TN thymocytes emigrate from the thymus into the intestinal epithelium, where they characteristically express c-kit and IL-7R on the cell surface and mRNA encoding CD3 ϵ (7). This study demonstrates that FITC⁺ thymic IEL precursors in the intestine of FTY720-treated mice express either TCR $\alpha\beta$ or TCR $\gamma\delta$, but not c-kit and IL-7R (Fig. 6, D and E). Thus, although it remains to be determined whether other CD8 $\alpha\alpha$ IEL-generating pathways (such as the cryptopatch-dependent route) are dependent on S1P (11) and whether FTY720-resistant S1P-mediated pathways (e.g., S1P₂-mediated pathway) are involved in the trafficking of DN thymocytes into the intestines (21), our findings suggest that mature post-selected TCR $\alpha\beta$ ⁺ DN thymocytes and TCR $\gamma\delta$ ⁺ DN thymocytes, but not TN thymocytes, migrate into the gut in a FTY720-insensitive manner.

In addition to thymic DN IEL precursors, the migration of RTEs from the thymus into the small intestine was not inhibited by FTY720 treatment (Fig. 6 E). It has been shown that RTEs uniquely express $\alpha 4\beta 7$ integrin and CCR9 in the thymus, which enables them to migrate directly into the small intestinal epithelium without undergoing activation in the secondary lymphoid organs (8). Their preferential migration into the small intestine rather than the large intestine can be explained by their CCR9 expression in the thymus, as small intestinal ECs, unlike those of the large intestine, produce an abundance of the CCR9 ligand CCL25 (28). Our current data indicate that FTY720 does not affect the direct migration of RTEs from the thymus into the small intestine. Although FTY720 had no effect on RTEs, which were known to express CD62L (8), naive CD62L^{high} cells were barely detected in the small intestine of FTY720-treated mice (<0.1%; Fig. 3 A). This discrepancy is caused by the fact that the RTEs are very minor population in the small intestinal IELs. In this issue, it was previously demonstrated that ~2% of cells were RTEs in CD62L^{high}CD44^{int} CD8 $\alpha\beta$ cells, which consisted of 5% small intestinal CD8 $\alpha\beta$ IELs (8). Therefore, an estimated percentage of RTEs in the total small intestinal IELs, including CD4, CD8 $\alpha\alpha$, CD8 $\alpha\beta$, and DN cells, is <0.1%. Thus, it is likely that the RTEs are present, but difficult to detect, in the small intestine of FTY720-treated mice. Additionally, there is still a minor possibility of the involvement of other cells that express CD103, CD62L, and CCR9 (e.g., circulating naive CD8 T cells and SP CD8 thymocytes). However, together with well-established effects of FTY720 on circulating naive CD8 T cells and SP CD8 thymocytes on their retention in the secondary lymphoid organs and thymus (16, 17) and our data on FITC-labeling experiments, the most plausible interpretation is that RTEs use the unique FTY720-insensitive trafficking pathway from the thymus into the small intestinal epithelium.

In addition to activated platelets, mast cells, and monocytes, dietary sphingolipids are another source of S1P (36). Interestingly, enzymes involved in the generation of sphingosine, which is a precursor of S1P, are principally expressed in the intestinal tracts, where their expression patterns differ

according to the intestinal compartment (37, 38). For instance, alkaline sphingomyelinase and neutral ceramidase, which are both important enzymes in the sequential degradation of sphingomyelin and ceramide to generate sphingosine, were predominantly expressed at the luminal side of the brush border in the distal jejunum and ileum, indicating that the amount of sphingosine was much lower in the upper and middle sections than in the lower sections of the small and large intestines (37, 38). The varying expression patterns of these enzymes, which might determine to what degree IELs migrate into a given region of the small or large intestine, depend on S1P under natural conditions. Although FTY720 inhibits the naive cell trafficking into the intestinal epithelium by preventing their emigration from the secondary lymphoid organs in our experimental condition (Fig. 2 E), it is interesting to examine whether luminal sphingosine-derived S1P regulates intestinal immunity, including T cell trafficking. Studies are currently underway in our group to further investigate this issue.

As S1P mediates the migration of mucosal T cells, such as IELs, it might also regulate other groups of immunocompetent and/or pathological cells in the mucosal immune system. Most irritations of the gastrointestinal tract, including inflammatory bowel diseases (IBDs) and food allergies, are caused by the influx of pathological cells into the intestine from systemic compartments (39, 40). If the migration of these pathological cells to the gastrointestinal tract is indeed S1P-dependent, it should be susceptible to FTY720, perhaps opening a new avenue for the treatment of IBDs and intestinal allergic diseases. Indeed, our previous study demonstrated that FTY720 effectively inhibited the development of IBDs in IL-10-deficient mice (41). Moreover, our recent separate studies show that FTY720 is also effective at preventing the development of allergic diarrhea by inhibiting pathogenic cell migration into the large intestine (42). In addition to regulating IEL trafficking under natural conditions, these findings suggest that the S1P-mediated pathway is involved in the development of immunological diseases of the intestine, such as IBDs and food allergies.

MATERIALS AND METHODS

Mice and experimental treatment. Normal female BALB/c mice (7–9 wk of age) were purchased from Japan Clea or Japan SLC. All mice were provided with sterile food and water *ad libitum*. Mice were injected intraperitoneally with 1 mg/kg FTY720 (Novartis Pharma) to assess reactivity (18, 24). To inhibit S1P lyase activity, the mice received drinking water containing 10 g/liter glucose and 30 mg/liter 4-deoxyypyridoxine-HCl (Sigma-Aldrich) for 3 d (23). All animals were maintained in the experimental animal facility at the University of Tokyo, and the experiments were conducted in accordance with the guidelines provided by the Animal Care and Use Committee of the University of Tokyo.

Lymphocyte isolation. Single IEL cells were isolated from the small and large intestinal epithelium as previously described (43). In brief, after removing the PPs, the small and large intestines were dissected into short segments and stirred at 37°C in prewarmed RPMI 1640 containing 2% FCS and 0.5 mM EDTA for 15 min, followed by vigorous shaking for 15 s. This process was repeated twice. A discontinuous Percoll density gradient centrifugation

was performed to purify the lymphocytes. IELs were collected from the layer between the 40% and 75% fractions. The spleen, thymus, MLN, SLN, PPs, and CPs were removed, and single-cell suspensions were prepared by passing them through a 70- μ m mesh filter, as previously described (24, 44). Lymphocytes were prepared from the liver according to a previously established method (45). In brief, the liver was passed through a 200-gauge stainless mesh to obtain single cells. These cells were treated with erythrocyte-lysing solution (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA, and 170 mM Tris-HCl, pH 7.3) to remove the erythrocytes, and were then fractionated by centrifugation in 35% Percoll.

Flow cytometry and cell sorting. Flow cytometry and cell sorting were performed as previously described (24, 44). Cells were preincubated with anti-CD16/32 antibody, and then stained with fluorescent antibodies specific for CD4, CD8 α , CD8 β , CD11b, CD11c, CD44, CD62L, CD69, CD103, c-kit, IL-7R, TCR β , TCR $\gamma\delta$, α 4 β 7 integrin (BD PharMingen), and CCR9 (R&D Systems). A Viaprobe (BD PharMingen) was used to discriminate between dead and living cells. Flow-cytometric analysis and cell sorting were performed using FACSCalibur and FACSAria (BD Biosciences), respectively.

Histological analysis. Immunohistochemical analysis was performed as previously described (40). In brief, large intestines were fixed in 4% paraformaldehyde (Wako) and treated with a sucrose gradient after extensive washing. The tissue was embedded in Tissue-Tek OCT compound (Sakura Fine-technical). For confocal microscopy analysis, TSA-direct kit (Perkin Elmer) was used according to the manufacturer's instructions. In brief, 6- μ m cryostat sections were treated with 3% H₂O₂ in PBS for 15 min to quench endogenous peroxidase activity. Sections were preblocked with anti-CD16/CD32 antibody in PBS containing 2% FCS for 15 min at room temperature, and stained with biotin-conjugated antibodies specific for CD4 or CD62L for 15 h at 4°C. After washing with TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween20), sections were treated with horseradish peroxidase-conjugated streptavidin in TNT buffer for 30 min at room temperature. After washing with TNT buffer, amplification of the fluorescent signal with FITC or Cy5-tyramide was performed. The specimens were analyzed using a confocal laser-scanning microscope (TCS SP2; Leica). Hematoxylin and eosin staining was used to confirm the dissociation of the epithelial region, as previously described (40).

Quantitative RT-PCR. To measure mRNA expression for S1P receptors, quantitative RT-PCR using LightCycler (Roche) was performed as previously described (24). Total RNA was prepared using TRIzol reagent (Invitrogen), and cDNA was synthesized using Powerscript reverse transcriptase (BD Biosciences). The oligonucleotide primers and probes specific for S1P₁ (forward primer, TACACTCTGACCAACAAGGA; reverse primer, ATAATGGTCTCTGGGTTGTC; FITC-probe, TGCTGGCAATTCAAGAGGCCCATCATC; and LCRed 640-probe, CAGGCATGGAATTTAGCCGCAGCAAATC) and GAPDH (forward primer, TGAACGGGAAGCTCACTGG; reverse primer, TCCACCACCCTGTTGCTGTA; FITC-probe, CTGAGGAC-CAGGTTGTCTCCTGCGA; and LCRed 640-probe, TTCAACAGCA-ACTCCCACTCTTCCACC) were designed and produced by Nihon Gene Research Laboratory.

Adoptive transfer of CFSE-labeled lymphocytes. CD3⁺ T cells were isolated from the MLN and SLN using anti-mouse CD3-coupled microbeads and a MACS column (Miltenyi Biotec) as previously described (44). For CFSE labeling, 10⁷ cells were incubated in the dark with 10 μ M CFSE (Invitrogen) for 10 min at 37°C, before being washed twice with PBS (8, 24). Labeled cells (2 \times 10⁷) were adoptively transferred via the tail vein into naive mice, which were either treated with FTY720 5 min after the cell transfer or left untreated. Lymphocytes were isolated from the MLN, SLN, blood, PPs, and small and large intestinal epithelium 24 h and 7 d after the transfer for flow-cytometric analysis.

Intrathymic labeling of thymocytes with FITC. The intrathymic injection of FITC was performed as previously described (8, 22, 31). In brief, 10 μ l FITC solution (1 mg/ml; Sigma-Aldrich) was injected into the thymus of anesthetized mice and the skin was closed with silk sutures. For FTY720 treatment, the mice were treated with FTY720 5 min before the FITC injection. Lymphocytes were isolated from the thymus, blood, MLN, and small and large intestinal epithelium 24 h after the FITC injection for flow-cytometric analysis.

BrdU incorporation and measurement. Mice were injected intraperitoneally with 1 mg BrdU (Sigma-Aldrich) in PBS as previously described (46). At the indicated times, the IELs were isolated from the small and large intestines and stained with fluorescent antibodies specific for CD4 and CD62L (BD Pharmingen). BrdU incorporation was detected by flow cytometry with a BrdU Flow kit according to the manufacturer's instructions (BD Biosciences).

Statistics. Results were compared using the Student's or Welch's *t* test. Statistical significance was established at $P < 0.05$.

Online supplemental material. Fig. S1 shows the data on CD4 and CD8 cells in the thymus, spleen, liver, PPs, CPs, and lymphocyte aggregates of mice treated with FTY720. Fig. S2 provides the data on the CCR9 expression on small and large intestinal CD8 IELs. Fig. S3 shows data on FACS profile in the PPs of FTY720-treated mice, naive T cells cultured with FTY720, and cell numbers of blood lymphocytes of FTY720-treated mice. The online version of this article is available at <http://www.jem.org/cgi/content/full/jem.20062446/DC1>.

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