

Figure 3. Colonic mucosal inflammation is milder in $\alpha\gamma^{-/-}$ mice lacking $\gamma\delta$ T cells than in $\alpha^{-/-}$ mice possessing $\gamma\delta$ T cells. (A) Representative histologic pictures of colon from WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ littermate mice. These mice were maintained in the same cages under specific pathogen-free conditions and examined at 28 weeks of age. (B) Body weight, wet weight of large intestine (LI weight), and ratio (%) of LI weight to body weight in WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ littermate mice at 20 to 32 weeks of age are shown. Values in parentheses are numbers of mice examined. $***P < .001$. (C) Disease scores (0–10) of colitis in 24- to 32-week-old WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice were assessed. Values in parentheses are numbers of mice examined. $*P < .05$. (D) Incidence of ARP in 24- to 60-week-old WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice. (E) The histologic findings (original magnification, $\times 20$) of colon of recipient $\alpha\gamma^{-/-}$ mouse groups that received transfer of $\gamma\delta$ T cells (left panel) and that received PBS (right panel).

$\times 10^5$, 32.3×10^5 , and 12.0×10^5 colonic LP cells, respectively. Based on the absolute numbers of infiltrated cells and the flow cytometry results shown in Figure 4A, it was evident that fewer Mac-1⁺Ly-6G⁻ cells and Mac-1⁺Ly-6G⁺ cells were present in the colonic LP cell population of $\alpha\gamma^{-/-}$ mice as compared with those of $\alpha^{-/-}$ mice. Monocytes express Mac-1 but not Ly-6G, whereas neutrophils express both Mac-1 and Ly-6G.²¹ Therefore, our results suggest that, in addition to monocyte infiltration (Figure 4A), there is a marked infiltration of neutrophils in the inflamed colonic LP of $\alpha^{-/-}$ mice. We also confirmed our previous finding¹⁵ that a remarkable increase in $\gamma\delta$ T cells was observed in the inflamed colonic LP of $\alpha^{-/-}$ mice (Figure 4A).

Immunohistochemical examination of inflamed colons from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice at approximately 28 weeks of age was performed to further confirm flow cytometric results. Consistent with the flow cytometric observations, significantly smaller numbers of Mac-1⁺ cells and Ly-6G⁺ cells were observed in the colonic LP of $\alpha\gamma^{-/-}$ mice as compared with those in the colonic LP of $\alpha^{-/-}$ mice (Figure 4B).

To investigate whether $\gamma\delta$ T cells contribute to the generation of colonic environment for enhancing the

migration of neutrophils and monocytes into the inflamed colon, we examined chemotactic activity of colonic extracts from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice to neutrophils and monocytes (Figure 5A). As a result, chemotactic activity to neutrophils of colonic extracts from $\alpha\gamma^{-/-}$ mice was significantly weaker than that from $\alpha^{-/-}$ mice, whereas the chemotactic activities to monocytes of both extracts were almost comparable (Figure 5B). The marked infiltration of neutrophils into the inflamed colonic LP of $\alpha^{-/-}$ mice is most likely mediated by some factors, such as MIP-2 (Figure 5A), that are enhanced in the presence of $\gamma\delta$ T cells.

Taking all of these results together, and in conjunction with our previous findings,²² colonic $\gamma\delta$ T cells of $\alpha^{-/-}$ mice exert aggravating effect on the UC-like colitis by increasing primarily the influx of neutrophils into the inflamed mucosa.

Attenuation of Colonic Proinflammatory Cascades by the Absence of $\gamma\delta$ T Cells

In view of the severe colitis, increased infiltration of Mac-1⁺Ly-6G⁺ and Mac-1⁺Ly-6G⁻ cells, and marked production of neutrophil chemotactic factor(s) in the inflamed colonic LP of $\alpha^{-/-}$ mice, quantitative real-time

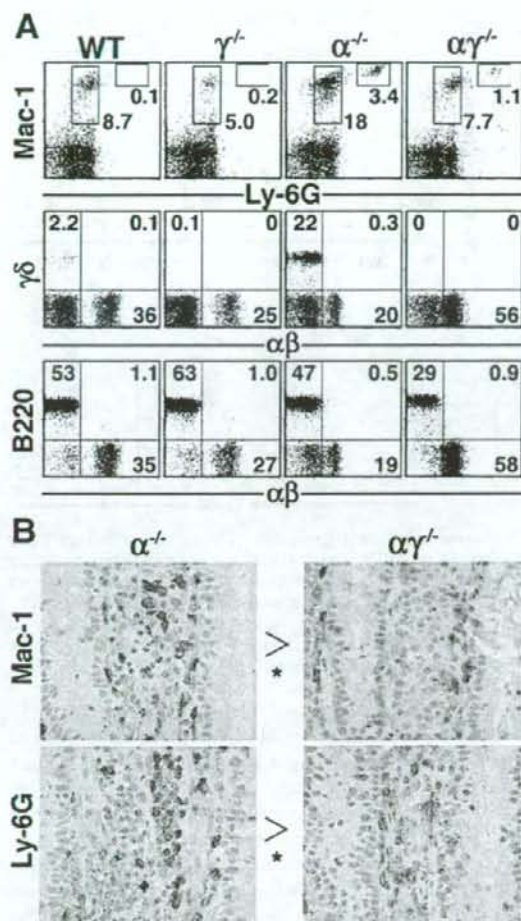


Figure 4. Decrease in colonic Mac-1⁺Ly-6G⁻ and Mac-1⁺Ly-6G⁺ cells in the absence of $\gamma\delta$ T cells. WT (n = 3), $\gamma\delta^{-/-}$ (n = 3), $\alpha^{-/-}$ (n = 4), and $\alpha\gamma^{-/-}$ (n = 4) mice from 28 weeks of age were examined. (A) Flow cytometric profiles of colonic LP cells. Absolute numbers of LP cells isolated from these WT, $\gamma\delta^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ individuals were 5.1×10^5 , 6.1×10^5 , 32.3×10^5 and 12.0×10^5 , respectively. (B) Representative immunohistochemical verification of the prominent infiltrations of Mac-1⁺ and Ly-6G⁻ cells into the inflamed colonic LP of $\alpha^{-/-}$ mice. Five tissue sections prepared from ascending colon to rectum per mouse, namely, 20 sections obtained from inflamed large intestines of $\alpha^{-/-}$ mice and those obtained from inflamed large intestines of $\alpha\gamma^{-/-}$ mice, were examined in a blinded fashion by 5 independent investigators, and the statistical difference in absolute numbers of Mac-1⁺ and Ly-6G⁻ cells between large intestinal mucosa from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice were determined by 2-sided Mann-Whitney U test. *P < .05.

RT-PCR analysis and measurement of the amounts of representative proinflammatory cytokines as well as chemokines were performed to dissect further the role of $\gamma\delta$ T cells in the UC-like colitis in $\alpha^{-/-}$ mice.

To this end, messenger RNA (mRNA) and proteins prepared from the large intestines of WT, $\gamma\delta^{-/-}$, $\alpha^{-/-}$, and

$\alpha\gamma^{-/-}$ mice were examined. Inflamed colonic tissues from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice contained at least 10 times higher levels of cytokine (Table 1)- and chemokine (Table 2)-specific mRNA than those of WT and $\gamma\delta^{-/-}$ mice except for IL-7 and IL-10 mRNA. In contrast to the mRNA from colonic tissues of $\alpha^{-/-}$ mice, those of $\alpha\gamma^{-/-}$ mice contained significantly smaller amounts of cytokine (TNF- α , IL-1 β , IL-6, and TGF- β)- and chemokine (KC and MIP-2)-specific mRNA. With these observations in mind, we measured the amounts of representative cytokines as well as chemokines that had exhibited the differences in mRNA levels between the colonic tissues of $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice. First, in situ production of TNF- α , IL-1 β , and IL-6 but not TGF- β proteins was significantly down-regulated in the inflamed colonic mucosa of $\alpha\gamma^{-/-}$ mice as compared with that of $\alpha^{-/-}$ mice (Table 1). Second, KC and MIP-2 chemokines that are involved in the chemoattract of neutrophils and/or monocytes²³ were significantly decreased in large intestines of $\alpha\gamma^{-/-}$ mice compared with those in large intestines of $\alpha^{-/-}$ mice (Table 2).

To investigate the cell types responsible for the increases in these proinflammatory cytokines and chemokines, real-time RT-PCR analysis of mRNA present in the purified cell subsets from the inflamed colonic LP of $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice was performed (see Supplementary Figure 1 online at www.gastrojournal.org). The IL-1 β and MIP-2 mRNA were expressed preferentially by Gr-1⁺ cells, F4/80⁺ cells, and CD11c⁺ cells in the colon, whereas IL-6 mRNA was mainly expressed by Gr-1⁻F4/80⁻CD11c⁻ cell populations. Expression levels of TNF- α - and KC-specific mRNA were comparable between all cell populations (Gr-1⁺ cells, F4/80⁺ cells, CD11c⁺ cells, and Gr-1⁻F4/80⁻CD11c⁻ cells) examined. Finally, the expression levels of these cytokine- and chemokine-specific mRNA in every cell subset were lower in cells from $\alpha\gamma^{-/-}$ mice than those in cells from $\alpha^{-/-}$ mice (see Supplementary Figure 1 online at www.gastrojournal.org).

Discussion

The $\alpha^{-/-}$ mice spontaneously develop colitis that shares many features with human UC.^{16,24} Commensal enteric flora is required for the development of this colitis as indicated by the absence of colitis in $\alpha^{-/-}$ mice that are maintained under germ-free conditions.^{22,25} The number of colonic $\gamma\delta$ T cells drastically decreases in the $\alpha^{-/-}$ mice under germ-free conditions.²⁴ However, the study to identify the role of $\gamma\delta$ T cells in the UC-like chronic colitis in $\alpha^{-/-}$ mice has been hampered by the difficulty in generating TCR $\alpha\delta$ double mutant ($\alpha\delta^{-/-}$) mice because of the genomic organization of these TCR genes.²⁰ In the present study, we overcame this problem by newly generating $\gamma\delta^{-/-}$ mice and subsequently crossing these mice with $\alpha^{-/-}$ mice to generate TCR α double mutant mice that lacked $\gamma\delta$ T cells. By using these $\alpha\gamma^{-/-}$ mice, we herein provide a novel insight into the role of $\gamma\delta$ T cells

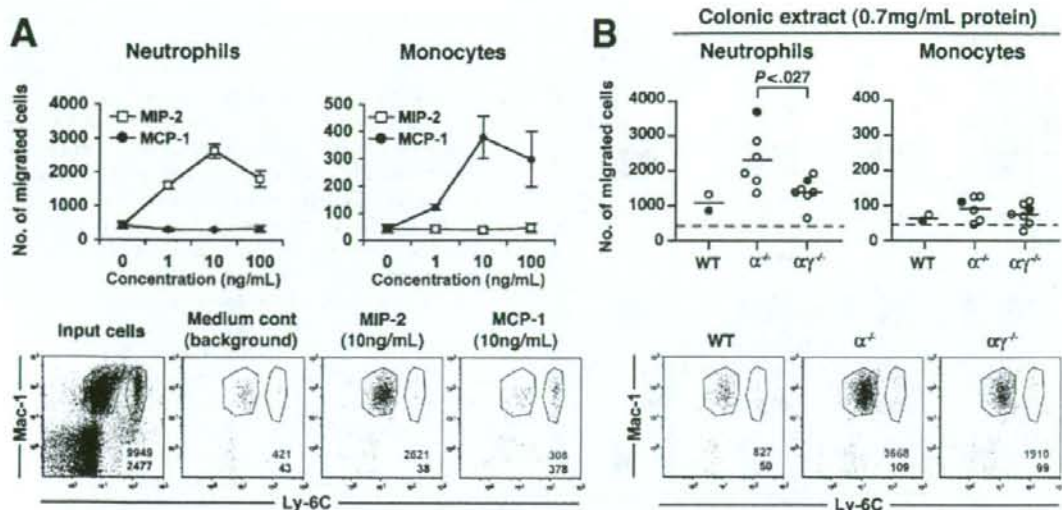


Figure 5. Chemotactic activity of colonic extracts from WT, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice to neutrophils and monocytes. (A) The number of neutrophils and monocytes migrated in response to the increasing concentration of MIP-2 (open square) and MCP-1 (solid circle). The representative flow cytometric profiles are shown in the lower panels, and red and blue gates indicate the neutrophils and monocytes, respectively. Colored numbers represent means of the number of cells in each gate. (B) Chemotactic responses of neutrophils and monocytes to colonic extracts (0.7 mg/ml protein) from WT, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice, and each circle represents an individual mouse. Horizontal bars show mean values, and dotted lines indicate the number of migrated cells in medium alone. The representative flow cytometric profiles of 3 individual animals indicated by the solid red and blue circles (upper panels) are shown in the lower panels, and red and blue gates indicate the neutrophils and monocytes, respectively.

that contributes to the exacerbation of UC-like colitis in $\alpha^{-/-}$ mice.

There is growing evidence supporting the fact that $\gamma\delta$ T cells are part of the innate immune system and play an active multifaceted immunoregulatory role in the coordinated innate and acquired immune responses that maintain the integrity of many organs containing epithelia.^{1,5,26,27} Nevertheless, the details of $\gamma\delta$ T-cell functions

are still not well understood as compared with those of $\alpha\beta$ T cells. $\gamma\delta$ T cells might play a defensive role against infections by various pathogenic microorganisms because exaggerated and severe infectious diseases occur in $\delta^{-/-}$ mice.²⁸⁻³³ However, the same $\delta^{-/-}$ mice have also been demonstrated to display an increased host resistance to infection.^{34,35} With regard to this, it is noteworthy that V γ 1⁺ $\gamma\delta$ T cells are reported to eliminate the

Table 1. Real-Time RT-PCR Analysis and ELISA Assay of Cytokines in the Colonic Tissues

Mice (n)	Cytokine							
	TNF- α	IL-1 β	IL-6	TGF- β	IFN- γ	IL-7	IL-10	IL-12
RT-PCR (copies per 10 ³ HPRT)								
WT (5)	11.4 \pm 0.53	6.86 \pm 0.55	2.45 \pm 0.64	123 \pm 14.6	ND	11.7 \pm 1.03	2.63 \pm 0.28	0.82 \pm 0.21
$\gamma^{-/-}$ (5)	9.20 \pm 0.76	6.01 \pm 0.61	2.14 \pm 0.88	106 \pm 10.7	ND	10.2 \pm 0.78	2.09 \pm 0.31	0.33 \pm 0.09
$\alpha^{-/-}$ (7)	457 \pm 35.7**	260 \pm 18.6***	7.06 \pm 1.04*	538 \pm 48.6*	64.4 \pm 8.90	11.0 \pm 0.89	4.99 \pm 0.72	9.58 \pm 1.67
$\alpha\gamma^{-/-}$ (6)	205 \pm 51.8**	98.5 \pm 21.4***	2.93 \pm 0.73*	319 \pm 57.4*	45.0 \pm 15.6	9.29 \pm 1.11	6.42 \pm 2.11	6.63 \pm 1.01
ELISA (pg/mg protein)								
$\alpha^{-/-}$ (7)	100 \pm 12.2**	975 \pm 70.1**	8.24 \pm 1.39**	2.09 \pm 0.11	8.34 \pm 1.50			
$\alpha\gamma^{-/-}$ (6)	27.0 \pm 8.28**	647 \pm 24.6**	2.92 \pm 0.52**	2.57 \pm 0.31	5.92 \pm 2.56			

NOTE. All results are expressed as mean \pm SE.

* $P < .05$.

** $P < .01$.

*** $P < .001$.

ND, not detected

Table 2. Real-Time RT-PCR Analysis and ELISA Assay of Chemokines in the Colonic Tissues

Mice (n)	Chemokine					
	KC	MIP-2	GCP-2	MCP-1	MIP-1 α	MIP-1 β
RT-PCR (copies per 10 ³ HPRT)						
WT (5)	6.05 \pm 1.38	0.23 \pm 0.01	12.3 \pm 4.11	1.67 \pm 0.17	0.78 \pm 0.07	1.36 \pm 0.22
$\gamma^{-/-}$ (5)	11.8 \pm 1.19	0.22 \pm 0.03	4.03 \pm 1.49	1.77 \pm 0.26	0.80 \pm 0.04	1.42 \pm 0.11
$\alpha^{-/-}$ (7)	382 \pm 88.6*	101 \pm 12.8**	721 \pm 136	23.3 \pm 4.37	35.0 \pm 3.01	19.8 \pm 0.88
$\alpha\gamma^{-/-}$ (6)	65.2 \pm 17.4*	28.0 \pm 9.80**	307 \pm 168	16.0 \pm 4.11	23.9 \pm 7.34	14.2 \pm 3.97
ELISA (pg/mg protein)						
$\alpha^{-/-}$ (7)	144 \pm 22.4**	113 \pm 21.2*	737 \pm 139			
$\alpha\gamma^{-/-}$ (6)	32.4 \pm 13.4**	44.9 \pm 14.0*	414 \pm 219			

NOTE. All results are expressed as mean \pm SE.* $P < .05$.** $P < .01$.

macrophages infected with *Listeria monocytogenes*, whereas $\gamma\delta$ T cells using V γ elements other than V γ 1 gene appear to lack the ability to control macrophages but possess the ability to protect hosts from the infection-induced tissue injury.^{36,37} In contrast to the beneficial function of $\gamma\delta$ T cells by virtue of the fact that they can maintain the homeostasis of different types of organs,^{1-5,8,27} a deleterious effect of $\gamma\delta$ T cells on the regulation of neutrophil-mediated tissue damage after thermal (postburn) injury has been reported.³⁸ In various chronic and/or autoimmune inflammatory diseases, such as collagen-induced arthritis in mice³⁹ and murine insulin-dependent diabetes,⁴⁰ $\gamma\delta$ T cells have been shown to exert a protective effect. Conversely, $\gamma\delta$ T cells may directly contribute to autoimmune pathology of murine experimental allergic encephalomyelitis⁴¹ as well as lupus in MRL/lpr mice.⁴² Overall, both the beneficial and detrimental roles of $\gamma\delta$ T cells in inflammatory process are evident.⁴³

In chemically induced acute intestinal inflammation models (2,4,6-trinitrobenzene sulfonic acid- or dextran sulfate sodium-induced colitis), $\gamma\delta$ T cells have been reported to play a protective role.¹¹⁻¹⁴ Depletion of $\gamma\delta$ T cells by administration of anti-TCR $\gamma\delta$ mAb into TNF Δ ARE mice with a high frequency of spontaneous ileitis⁴⁴ did not lead to any histologic changes of ileitis.⁴⁵ However, transfer of bone marrow-derived $\gamma\delta$ T cells has been shown to induce CD-like colitis in the bone marrow transplanted CD3 ϵ tg colitis model.⁴⁶ Although the role of $\gamma\delta$ T cells in spontaneous chronic colitis remains to be explored to date, the results of the present study demonstrate the exacerbating effect of $\gamma\delta$ T cells on the UC-like chronic colitis in $\alpha^{-/-}$ mice (Figures 3 and 4 and Tables 1 and 2). Interestingly, approximately 20% of $\alpha^{-/-}$ mice during 20 to 60 weeks of age suffered from ARP, whereas none of age-matched $\alpha\gamma^{-/-}$ mice showed ARP (Figure 3D). Of note, there were no differences in the age of onset of colitis and in the incidence of colitis (~80%) among 20- to 32-week-old $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice, but much more severe colitis was observed in $\alpha^{-/-}$ mice as compared with $\alpha\gamma^{-/-}$ mice. Therefore, it is possible that ARP may reflect

increased severity of colitis and that $\gamma\delta$ T cells may participate in the development of ARP.

Absence of $\gamma\delta$ T cells in $\alpha\gamma^{-/-}$ mice leads to a significantly reduced production of TNF- α , IL-1 β , and IL-6 proteins in the colonic tissues. These findings are consistent with our previous results³⁴ showing the involvement of TNF- α , IL-1 β , and IL-6 in the perpetuation of inflammatory process in $\alpha^{-/-}$ mice. These inflammatory mediators have been shown to be important for host defense and wound repair.⁴⁷ Both KC and MIP-2 attract neutrophils to inflamed sites, and, in certain microbial infection, the collection of neutrophils leads to suppuration reflecting an active and vigorous host response against microbes. We also confirmed that colonic extracts from $\alpha\gamma^{-/-}$ mice exhibited the significantly weaker chemotactic activity to neutrophils than those from $\alpha^{-/-}$ mice. KC and MIP-2 mRNA expressions were lower in all purified cell subsets (Gr-1⁺, F4/80⁺, CD11c⁺, and Gr-1⁻F4/80⁻CD11c⁻ cells) from $\alpha\gamma^{-/-}$ mice than those from $\alpha^{-/-}$ mice. Therefore, in the presence of $\gamma\delta$ T cells, many types of immune cells may be triggered to produce more chemokines, followed by infiltration of neutrophils into the colonic mucosa in $\alpha^{-/-}$ mice. $\gamma\delta$ T-cell responsiveness that is manifested by recruitment and activation of inflammatory cells in which neutrophils predominate has also been reported.^{1,31} In this context, it is of importance to note that the activity and severity of UC patients with increase in $\gamma\delta$ T cells in the inflamed mucosa^{9,10} (Figure 1) can be judged by the activation state of neutrophils in circulation⁴⁸ as well as by regional accumulation of neutrophils in the colonic crypt walls (cryptitis) or in the lumen of crypts (crypt abscess).⁴⁹

The suppressive role of B cells⁵⁰ and the aggravating role of TCR β ^{dim} T cells^{22,51} in the pathogenesis of colitis in $\alpha^{-/-}$ mice have been reported. Therefore, it is possible that $\gamma\delta$ T cells may contribute to the exacerbation of this colitis by dampening regulatory B-cell function or by cooperating the colitogenic TCR β ^{dim} T cells. The possible complicated mechanism remains to be explored in the future. Levels of TNF- α and IL-1 β mRNA in F4/80⁻ cells

are higher in $\alpha^{-/-}$ mice compared with $\alpha\gamma^{-/-}$ mice (see Supplementary Figure 1 online at www.gastrojournal.org), suggesting that $\gamma\delta$ T cells may activate macrophages to secrete large amounts of proinflammatory cytokines.

In conclusion, although $\gamma\delta$ T cells at the inflamed colonic LP of $\alpha^{-/-}$ mice may protect intestinal epithelial injury, proinflammatory cytokines and neutrophil- and/or monocyte-chemoattractant chemokines induced by $\gamma\delta$ T cells may directly and/or indirectly contribute to increased severity of UC-like chronic colitis in $\alpha^{-/-}$ mice. Further understanding of the molecular mechanisms of $\gamma\delta$ T cell-mediated exacerbation of colitis in $\alpha^{-/-}$ mice will lead us to work out better therapeutic strategies for human UC.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2007.11.056.

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FTY720 suppresses the development of colitis in lymphoid-null mice by modulating the trafficking of colitogenic CD4⁺ T cells in bone marrow

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2-Amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride (FTY720) suppresses T-cell egress from LN, thereby preventing pathogenic T cells from migrating toward disease sites. However, little is known about whether FTY720 could control the trafficking of T cells without the presence of lymphoid tissues. Here we demonstrate that FTY720 treatment suppresses the recirculation of CD4⁺ T cells in splenectomized (SPX) lymphotoxin- $\alpha^{-/-}$ (LT- $\alpha^{-/-}$) mice that lack LN and spleen, as shown by peripheral blood (PB) lymphopenia in FTY720-treated SPX LT- $\alpha^{-/-}$ mice. In a short-term transfer experiment, the cell number of transferred Ly5.1⁺CD4⁺ T cells recovered from host FTY720-treated SPX LT- $\alpha^{-/-}$ mice (Ly5.2⁺) was markedly decreased in PB, but conversely increased in BM. Notably, FTY720 treatment prevented the development of colitis that is otherwise induced in untreated SPX LT- $\alpha^{-/-}$ × RAG-2^{-/-} mice upon transfer of colitic lamina propria CD4⁺ T cells. In such mice, the number of CD4⁺ T cells in PB or lamina propria of FTY720-treated SPX LT- $\alpha^{-/-}$ × RAG-2^{-/-} recipients was significantly reduced, but that in the BM was significantly increased as compared with untreated control mice. Altogether, the present results indicate that FTY720 treatment may offer an additional role to direct trafficking of CD4⁺ T cells in BM, resulting in the prevention of colitis.

Key words: Chronic colitis · Colitogenic CD4⁺ T cells · FTY720 · Inflammatory bowel disease · Mucosal immunity



Supporting Information available online

Introduction

2-Amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride (FTY720) is a sphingosine-1-phosphate (S1P) receptor modulator, which induces prolonged down-modulation of the surface expression of the S1P receptor and thereby inhibits the egress of lymphocytes from thymus, LN, and Peyer's patches

leading to peripheral blood (PB) lymphopenia [1–8]. From the view of clinical application, in animal models, FTY720 has been shown to prevent autoimmune diseases [9, 10], viral infection [11, 12], or graft rejection after allotransplantation [13]. Moreover, it was recently shown that FTY720 reduced the number of lesions and clinical disease activity of patients with multiple sclerosis in a phase II, placebo-controlled trial [14].

In inflammatory bowel diseases (IBD), it is believed that colitogenic memory CD4⁺ T cells are intermittently reactivated in

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regional lymphoid organs in response to antigen-loading activated dendritic cells and thereafter return to inflammatory tissues [15–18]. We recently reported that colitogenic memory T cells survive for a long period in IL-7-dependent manner, by using a model of colitis induced by adoptive transfer of CD4⁺CD45RB^{high} T cells into SCID/RAG-deficient mice [19]. Although little was known about how colitogenic memory CD4⁺ T cells in IBD are controlled by FTY720, we demonstrated that FTY720 suppresses the development of colitis induced by adoptive transfer of colitogenic lamina propria (LP) CD4⁺CD44^{high}CD62L⁻ effector-memory T (T_{EM}) cells [20, 21] that were obtained from colitic CD4⁺CD45RB^{high} T-cell-transferred SCID mice [22]. Furthermore, we found that FTY720 treatment induced marked lymphopenia of colitogenic CD4⁺ T_{EM} cells in the periphery [22]. In this previous study, however, it was curious but very interesting as to why the colitogenic CD4⁺ T_{EM} cells were controlled by FTY720 treatment, as they should preferentially reside in non-lymphoid tissues such as the gut [23, 24].

Thus, these previous results prompted us to investigate another possible effect of the FTY720 treatment, modulating a yet-known cell trafficking independent of the presence of LN and spleen. To this end, we here used splenectomized lymphotoxin- $\alpha^{-/-}$ (LT- $\alpha^{-/-}$) \times RAG-2 $^{-/-}$ mice lacking spleen, LN, and Payer's patches, as recipients for adoptive transfer of colitogenic CD4⁺ T_{EM} cells and assessed the lymphoid tissue-independent effect of FTY720 treatment upon accumulation of donor T cells in the BM and prevention of colitogenic CD4⁺ T_{EM} cell-mediated colitis.

Results

FTY720 induces lymphopenia in LT- $\alpha^{-/-}$ mice

To first assess the alterations of systemic T-cell number or its subset composition upon FTY720 treatment without an impact of lymphoid tissues including spleen, LN, and Peyer's patches, we used splenectomized (SPX) LT- $\alpha^{-/-}$ and the control SPX LT- $\alpha^{+/+}$ littermate mice in this study (Fig. 1A). Two weeks after splenectomy, mice were i.p. administered with a single dose of FTY720 (1.0 mg/kg) or control PBS, and then the tissue distribution of lymphocytes (CD3⁺CD4⁺, CD3⁺CD8⁺, and CD19⁺ cells) in the PB, LP, and BM at 24 h after administration was analyzed. The number of total CD3⁺CD4⁺ lymphocytes in the PB of FTY720-treated SPX LT- $\alpha^{+/+}$ mice was markedly decreased compared with that of PBS-treated SPX LT- $\alpha^{+/+}$ mice (Fig. 1B), suggesting a previously recognized LN-dependent mechanism of FTY720 that promotes the sequestration of lymphocytes and inhibits the egress of lymphocytes from LN [1–4]. Unlike the decreased number of CD3⁺CD4⁺ lymphocytes in the PB of FTY720-treated SPX LT- $\alpha^{+/+}$ mice, the difference in the number of those cells in LP or BM was not significant between FTY720- and PBS-treated SPX LT- $\alpha^{+/+}$ mice. In PBS-treated SPX LT- $\alpha^{-/-}$ mice, interestingly, the cell numbers of CD3⁺CD4⁺

lymphocytes in PB, LP, and BM were significantly increased compared with those in the paired PBS-treated SPX LT- $\alpha^{+/+}$ mice presumably due to the lack of LN serving as a reservoir of lymphocytes (Fig. 1B). Surprisingly, the cell number of CD3⁺CD4⁺ lymphocytes in the PB of FTY720-treated SPX LT- $\alpha^{-/-}$ mice was also significantly decreased compared with that of the paired PBS-treated SPX LT- $\alpha^{-/-}$ mice, suggesting an existence of as-yet-unknown reservoir for CD3⁺CD4⁺ lymphocytes other than LN or spleen (Fig. 1B). Moreover, although the number of CD3⁺CD4⁺ lymphocytes in the BM of SPX LT- $\alpha^{+/+}$ mice was not affected by FTY720 treatment, the number of such cells in the BM of FTY720-treated SPX LT- $\alpha^{-/-}$ mice was significantly increased compared with that of PBS-treated SPX LT- $\alpha^{-/-}$ mice. In contrast, the number of CD3⁺CD8⁺ T cells and CD19⁺ B cells in the BM or in the LP was not affected by FTY720 treatment in any group of mice (Fig. 1B), whereas the number of those cells in the PB of FTY720-treated SPX LT- $\alpha^{+/+}$ and SPX LT- $\alpha^{-/-}$ mice was significantly decreased compared with that of the paired PBS-treated mice.

To further assess the possible effect of FTY720 on accumulation of CD3⁺CD4⁺ T cells within the BM of SPX LT- $\alpha^{-/-}$ mice, we next performed a short-term adoptive transfer of splenic CD4⁺ T cells that were obtained from Ly5.1-derived C57BL/6J mice into Ly5.2-derived SPX LT- $\alpha^{-/-}$ mice, by treating them with or without FTY720 at 3 h before transfer. Twenty-four hours after the transfer, mice were sacrificed, and the recovered cell number of the total and Ly5.1⁺ CD4⁺ donor cells at various sites was analyzed (Fig. 2A). Consistent with the above-mentioned results (Fig. 1), we confirmed that the number of CD4⁺ T cells was significantly decreased in PB in FTY720-treated SPX LT- $\alpha^{-/-}$ mice regardless of the host (Ly5.2⁺) or donor (Ly5.1⁺), but conversely, the number of those cells in the BM was significantly increased (Fig. 2B). Interestingly, the decreased cell number of CD4⁺ T cells in the PB of FTY720-treated SPX LT- $\alpha^{-/-}$ mice ($1.33 \pm 0.80 \times 10^7$) was almost identical to the increased number of those cells in the BM ($1.45 \pm 1.47 \times 10^7$), according to the calculating formula estimating that (i) the total number of BM cells could be calculated as 7.9 times the number of cells in two femurs [25] and (ii) the total volume of PB is 2.4 mL [26].

FTY720 treatment suppresses the development of colitis in spleen and LN-null recipients

We previously reported that FTY720 treatment suppresses the development of colitis induced by adoptive transfer of CD4⁺CD45RB^{high} T cells [22]. This was legitimate because most CD4⁺CD45RB^{high} T cells were naive CD44^{low}CCR7⁺CD62L⁺ T cells that are accessible to mesenteric LN but at the same time their egress could be inhibited by FTY720 treatment. However, we also demonstrated that FTY720 treatment suppresses the development of colitis in SCID mice that were adoptively transferred with colitogenic LP CD4⁺CD44^{high}CD62L⁻IL-7R α^{high} T_{EM} cells obtained from colitic CD4⁺CD45RB^{high} T-cell-transferred

SCID mice [22]. This was wholly unexpected because colitogenic LP CD4⁺ T cells have the characteristics of T_{EM} (CD44^{high}CD62L⁻) cells that are believed to preferentially migrate to non-lymphoid

tissues such as the gut [23, 24], but do not migrate to LN including mesenteric LN. Thus, it remained possible that the migration of colitogenic LP CD4⁺ T cells is controlled by FTY720

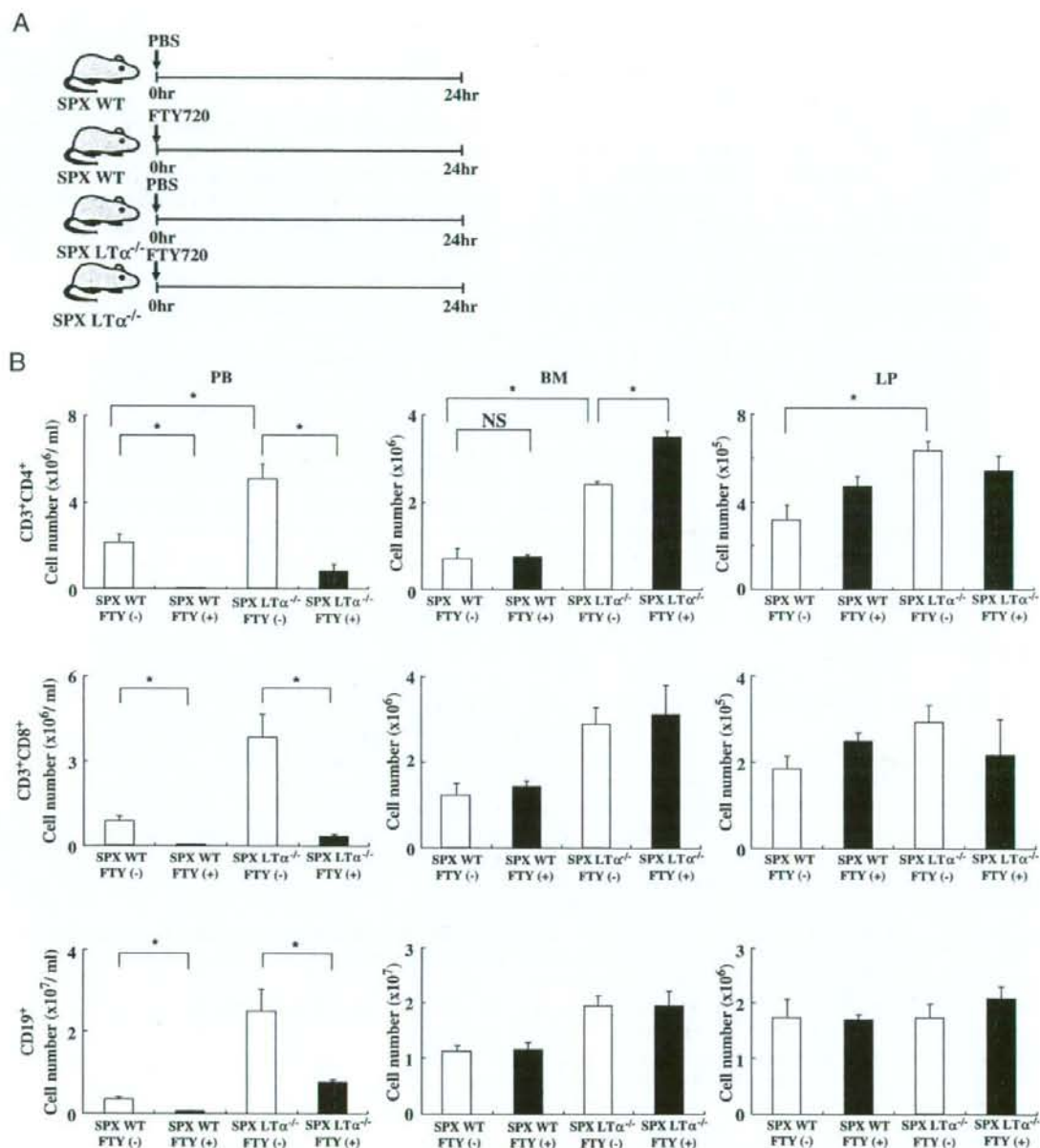


Figure 1. FTY720 treatment induces decrease in PB CD4⁺ T cells and increase in BM CD4⁺ T cells in LN/spleen-null mice. (A) FTY720 (1.0 mg/kg) or PBS was i.p. administered to C57BL/6 (WT) or LTα^{-/-} mice with SPX, and the changes in the absolute numbers of cells were determined at 24 h after treatment. (B) The absolute number of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cell subsets, and CD19⁺ B cells in PB, BM, and LP, was determined at 24 h after treatment using flow cytometry. Data are indicated as mean±SEM of six mice in each group. Groups of data were compared by Mann-Whitney U-test. *Indicates statistically significant at *p*<0.05. FTY, FTY720.

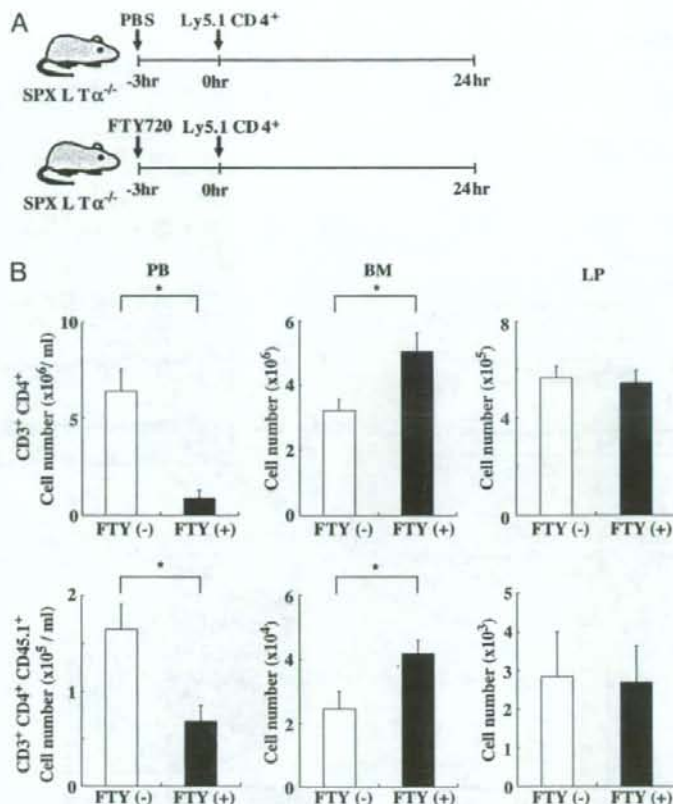


Figure 2. Transferred CD4⁺ T cells were preferentially accumulated in the BM of FTY720-treated LN/spleen-null mice. (A) SPX LT- $\alpha^{-/-}$ mice (Ly5.2⁺) were treated with FTY720 (1.0 mg/kg) or PBS at -3 h and then transferred with normal splenic CD4⁺ T cells (Ly5.1⁺) at 0 h. The changes in the absolute number of cells were determined at 24 h after the transfer. (B) The absolute number of total or transferred Ly5.1⁺ CD3⁺ CD4⁺ T cells in PB, BM, and LP was determined at 24 h after the transfer using flow cytometry. Data are indicated as mean \pm SEM of six mice in each group. * $p < 0.05$. FTY, FTY720.

treatment in an LN-independent manner. Furthermore, we previously demonstrated that SPX LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ mice transferred with colitogenic LP CD4⁺ T_{EM} cells develop chronic colitis to a similar extent with the control SPX LT- $\alpha^{+/+}$ \times RAG-2 $^{-/-}$ recipient mice [27]. Based on such a background, we next challenged to investigate the novel role of FTY720 in suppression of colitis by a series of *in vivo* system without an impact of LN and spleen.

To this end, SPX LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ or control littermate SPX LT- $\alpha^{+/+}$ \times RAG-2 $^{-/-}$ mice were transferred with colitogenic LP CD4⁺ T_{EM} cells obtained from colitic CD4⁺ CD45RB^{high} T-cell-transferred RAG-2 $^{-/-}$ mice and treated with daily PBS or FTY720 (0.3 or 1.0 mg/kg) i.p., which was started 1 day before transfer and continued up to 7 wk (Fig. 3A). The control PBS-administered SPX LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ and SPX LT- $\alpha^{+/+}$ \times RAG-2 $^{-/-}$ recipients manifested progressive weight loss after 2 wk from transfer (data not shown). These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 7 wk post transfer. The clinical score of both group

showed no significant difference at 7 wk post transfer (Fig. 3B). In contrast, the FTY720-treated mice appeared healthy with significantly low clinical scores, regardless of the doses (Fig. 3B). Altogether, the assessment of clinical scores showed a clear difference between control PBS-treated and FTY720-treated mice, regardless of the presence of LN (Fig. 3B). At 7 wk after the transfer, the colons from control PBS-treated mice, but not those from FTY720-treated mice, were enlarged and had a greatly thickened wall (Fig. 3C). Histological examination showed a marked inflammation in the colonic LP of control PBS-treated LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ or LT- $\alpha^{+/+}$ \times RAG-2 $^{-/-}$ mice (Fig. 3D). In contrast, it was mostly abrogated in the LP of FTY720-treated mice (Fig. 3D). This difference was confirmed by histological scores of multiple colon sections (Fig. 3E).

Furthermore, less number of CD4⁺ T cells was recovered from the PB of FTY720-treated mice compared with the paired control PBS-treated mice, regardless of LT- $\alpha^{-/-}$ or LT- $\alpha^{+/+}$ recipients (Fig. 4A). The number of CD4⁺ T cells recovered from the colon of control PBS-treated mice (SPX LT- $\alpha^{+/+}$ \times RAG-2 $^{-/-}$;

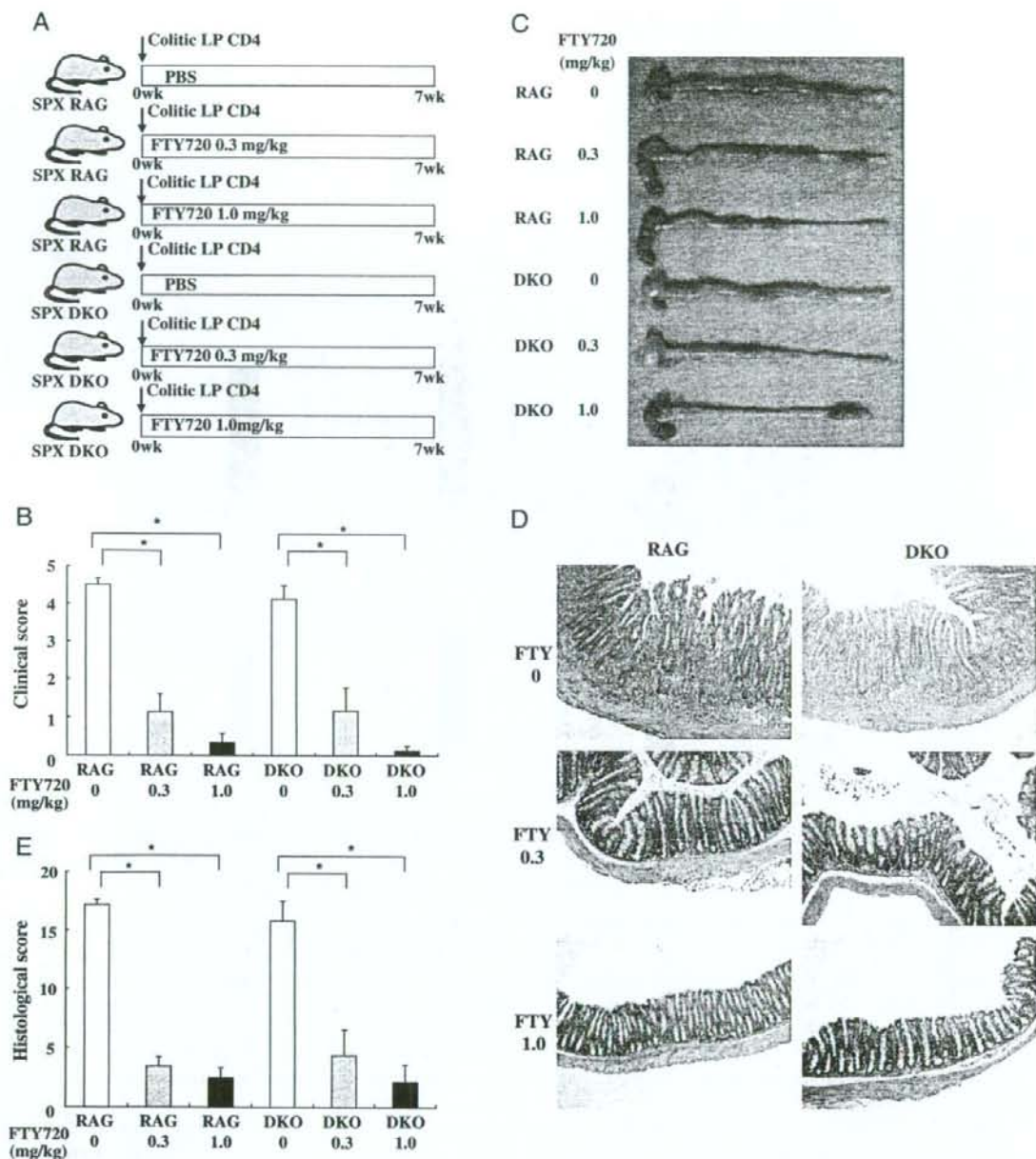


Figure 3. FTY720 prevents the development of colitogenic CD4⁺ T_{EM}-mediated colitis in LN/spleen-null mice. (A) Colitogenic CD4⁺ T_{EM} cells were isolated from the inflamed mucosa of colitic RAG-2^{-/-} mice transferred with CD4⁺CD45RB^{high} T cells. SPX LT- $\alpha^{-/-}$ × RAG-2^{-/-} and SPX LT- $\alpha^{-/-}$ × RAG-2^{-/-} mice were then injected i.p. with 4×10^5 colitic LP CD4⁺ T cells and were also treated i.p. with daily PBS (n = 9) or FTY720 (0.3 mg/kg; n = 9, 1.0 mg/kg; n = 9) starting 1 day before the transfer up to 7 wk. Mice were weighed initially upon transfer and then three times per week thereafter. (B) Clinical scores were determined at 7 wk after the transfer as described in *Materials and methods*. Data are indicated as mean ± SEM of nine mice in each group. *p < 0.05. (C) Gross appearance of the colon at 7 wk after the transfer. (D) Histological examination of the colon at 7 wk after the transfer. Original magnification: × 100. (E) Histological scoring of colitis at 7 wk after the transfer. Data are indicated as mean ± SEM of nine mice in each group. *p < 0.05 compared with the paired mice without FTY720 treatment.

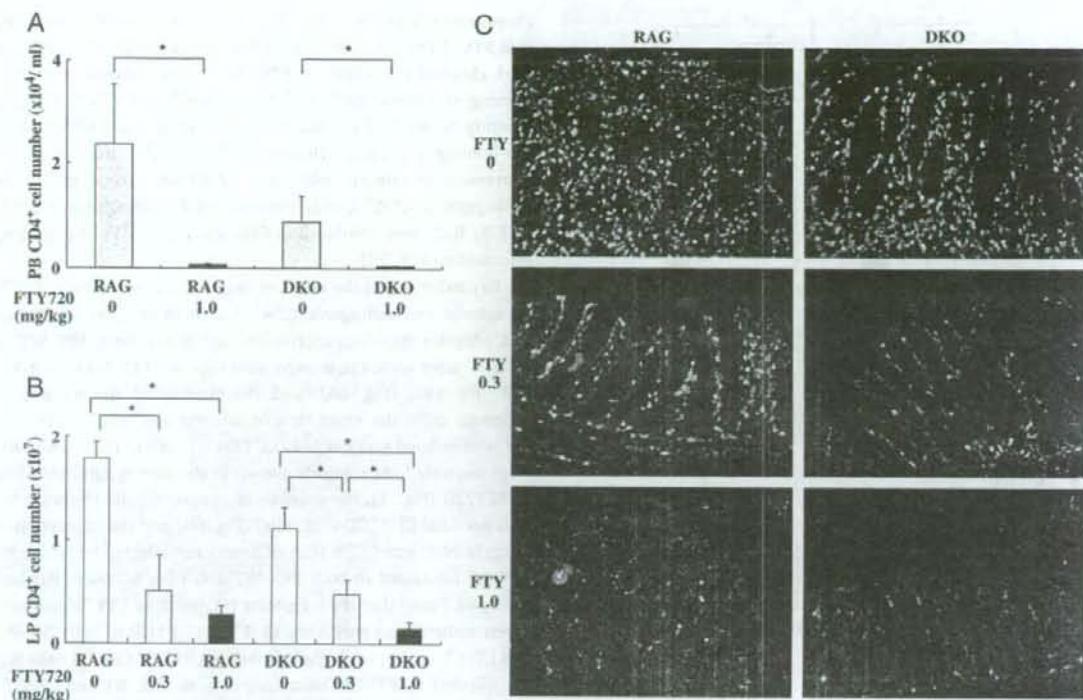


Figure 4. The number of LP CD4⁺ T cells was significantly decreased while PB lymphopenia was observed in FTY720-treated LN/spleen-null mice transferred with colitogenic CD4⁺ T_{EM} cells. (A–B) Number of CD3⁺CD4⁺ T cells in PB (A) or LP (B). Cells were stained with FITC-anti-CD3 and allophycocyanin-anti-CD4, and the total number of CD3⁺CD4⁺ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of nine mice in each group. **p* < 0.05. (C) CD4 immunostaining and DAPI counterstaining of the colon from frozen sections were fixed with acetone and stained with anti-mouse CD4 mAb, followed by Alexa Fluor[®] 488 goat anti-rat IgG as secondary antibody and DAPI counterstaining. Representative of four separate samples in each group. Original magnification: × 100.

$1.73 \pm 0.15 \times 10^7$, SPX LT- $\alpha^{-/-}$ × RAG-2 $^{-/-}$; $1.07 \pm 0.14 \times 10^7$) far exceeded the number of originally injected memory cells (3×10^6), indicating an extensive T-cell migration and/or proliferation in the inflamed colon, which did not apply to FTY720-treated mice (Fig. 4B). To clarify that the suppressed colitis in FTY720-treated mice was surely mediated by the decreased infiltration of CD4⁺ T cells into the LP, we next assessed the colonic infiltration of CD4⁺ T cells by immunohistochemistry. Figure 4C clearly demonstrated the markedly decreased infiltration of CD4⁺ T cells in the colon of FTY720-treated mice, regardless of LT- $\alpha^{-/-}$ or LT- $\alpha^{+/+}$ recipients. In contrast, a large number of CD4⁺ T cells were found in the inflamed mucosa of PBS-treated recipients (Fig. 4C).

FTY720 treatment suppresses the production of Th1 and Th2 cytokines

Production of cytokines such as IFN- γ , TNF- α , or IL-4 by LP CD4⁺ T cells of the control PBS- or FTY720-treated mice transferred with colitogenic LP CD4⁺ T_{EM} cells was assessed by ELISA. As shown in Fig. 5, LP CD4⁺ T cells obtained from FTY720-treated mice

produced significantly less IFN- γ , TNF- α , IL-4 upon *in vitro* stimulation regardless of LT- $\alpha^{-/-}$ or LT- $\alpha^{+/+}$ recipients compared with those from the paired control PBS-treated mice.

We further asked whether FTY720 affects the cell differentiation and activation of transferred CD4⁺CD62L^{hi}CD44^{hi} T_{EM} cell *in vivo*. However, almost all LP CD4⁺ T cells from PBS-treated or FTY720-treated mice retained the characteristics of CD69⁺CD62L^{hi}CD44^{hi}IL-7R α^{hi} activated CD4⁺ T_{EM} cell (Supporting Information Fig. 1). Furthermore, although Daniel *et al.* recently showed a significant increase in Foxp3 mRNA expression in isolated LP CD4⁺ T cells of FTY720-treated mice using Th1-mediated TNBS colitis model [28], we were not able to detect any increase in Foxp3 protein in LP CD4⁺ T cells of our FTY720-treated mice regardless of LT- $\alpha^{-/-}$ or LT- $\alpha^{+/+}$ recipients (Supporting Information Fig. 1).

FTY720 treatment does not induce apoptosis or modulate the expression of gut-homing receptors

Given the evidence that FTY720 prevents the development of colitis induced by adoptive transfer of colitogenic LP CD4⁺ T_{EM}

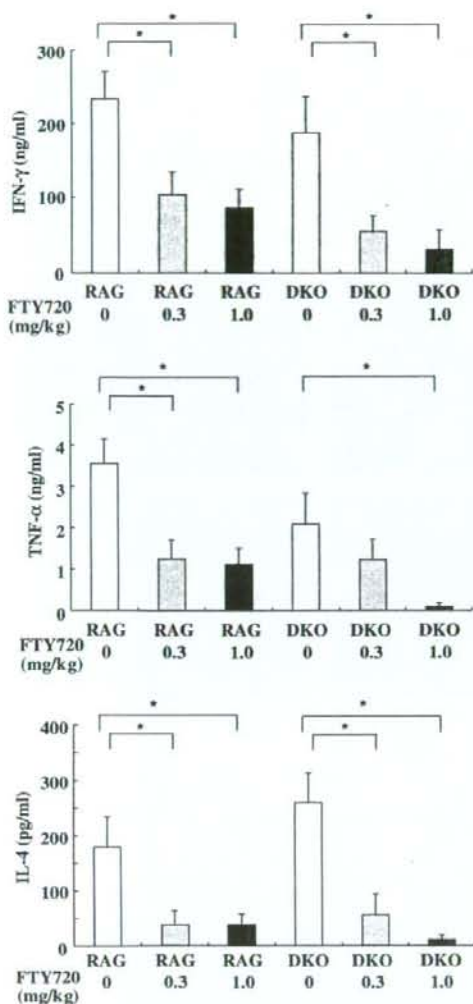


Figure 5. LP CD4⁺ T cells in FTY720-treated LN/spleen-null mice transferred with colitogenic CD4⁺ T_{EM} cells produce significantly less amount of Th1 and Th2 cytokines. LP CD4⁺ T cells were isolated from mice 7 wk after the transfer of colitogenic LP CD4⁺ T_{EM} cells and stimulated with anti-CD3 and anti-CD28 mAb for 48 h. IFN- γ , TNF- α , and IL-4 concentrations in culture supernatants were measured by specific ELISA. Data are indicated as mean \pm SEM of nine mice in each group. * $p < 0.05$.

cells even in the absence of LN and spleen, we next investigated the mechanism by which FTY720 suppresses the development of colitis independently from such lymphoid tissues. To this end, we first checked whether FTY720 could directly induce apoptosis of colitogenic LP CD4⁺ T_{EM} cells, contributing to the preventive effect, as suggested by other studies regarding the effect of FTY720 [29]. However, this possibility was excluded by showing that addition of various concentrations of FTY720 did not induce apoptosis of normal splenic or colitogenic LP CD4⁺ T cells *in vitro*

(Supporting Information Fig. 2A). To further assess the possibility that FTY720 might affect the gut-homing axis of CD4⁺ T cells, we next checked the effect of FTY720 on the expression of gut-homing receptors, such as integrin $\alpha 4\beta 7$ and CCR9, using a recently established *in vitro* assay of retinoic acid (RA)-induced gut-homing receptors. However, FTY720 did not skew the expression of integrin $\alpha 4\beta 7$ and CCR9 on normal splenic or colitogenic LP CD4⁺ T cells, even after 5 days of culture with RA, TGF- β , IL-2, and soluble anti-CD3 mAb [30, 31] (Supporting Information Fig. 2B).

To further assess the effect of long-term treatment of FTY720 on normal and colitogenic CD4⁺ T cells *in vivo*, we conducted two adoptive transfer experiments as follows. First, SPX WT or LT- $\alpha^{-/-}$ mice were i.p. treated with PBS or FTY720 (1.0 mg/kg) daily for 4 wk (Fig. 6A), and the changes in the number of apoptotic cells, the expression of integrin $\alpha 4\beta 7$ and CCR9, and the accumulated number of CD3⁺CD4⁺ T cells in PB, LP, and BM were measured. As similarly shown in short-term administration of FTY720 (Fig. 1), the number of apoptotic cells (Annexin V⁺ cells per total CD3⁺CD4⁺ T cells) (Fig. 6B) and the expression of integrin $\alpha 4\beta 7$ and CCR9 (Fig. 6C) were not affected by long-term FTY720 treatment in both SPX WT and LT- $\alpha^{-/-}$ mice. Furthermore, we found that the long-term treatment of FTY720 induced severe reduction in peripheral CD3⁺CD4⁺ T cells in both SPX WT and LT- $\alpha^{-/-}$ mice, while the number of LP CD3⁺CD4⁺ T cells was not affected by FTY720 treatment in both SPX WT and LT- $\alpha^{-/-}$ mice (Fig. 6D). Interestingly, the number of accumulated CD3⁺CD4⁺ T cells in FTY720-treated SPX WT LT- $\alpha^{-/-}$ mice was significantly increased compared with that in PBS-treated mice, while this significant change was not observed in SPX WT mice (Fig. 6D). Similar results were also obtained in another adoptive transfer experiment of RAG-2^{-/-} versus LT- $\alpha^{-/-}$ \times RAG-2^{-/-} mice transferred with colitogenic LP CD4⁺ T_{EM} cells with or without FTY720 treatment for 4 wk after the transfer (Fig. 7A). Namely, the expression of gut-homing receptors (Fig. 7B) and the frequency of apoptotic CD4⁺ T cells (Fig. 7C) were not changed by *in vivo* long-term FTY720 treatment. The number of PB and LP CD3⁺CD4⁺ T cells in FTY720-treated mice regardless of RAG-2^{-/-} or LT- $\alpha^{-/-}$ \times RAG-2^{-/-} recipients was significantly decreased compared with paired PBS-treated mice (Fig. 7D). Again, we found that the accumulated number of BM CD3⁺CD4⁺ T cells in FTY720-treated LN-null LT- $\alpha^{-/-}$ \times RAG-2^{-/-} mice was significantly increased compared with that in PBS-treated LT- $\alpha^{-/-}$ \times RAG-2^{-/-} mice (Fig. 7D).

FTY720 treatment promotes the accumulation of colitogenic CD4⁺ T cells in BM

As FTY720 treatment induced increase in CD3⁺CD4⁺ T cells in BM and also lymphopenia in the PB of non-colitic SPX LT- $\alpha^{-/-}$ mice in short-term FTY720 administration system (Figs. 1 and 2), those in long-term administration system (Fig. 6), and SPX LT- $\alpha^{-/-}$ \times RAG-2^{-/-} mice transferred with colitogenic LP CD4⁺ T_{EM} cells in long-term FTY720 administration system (Fig. 7), we

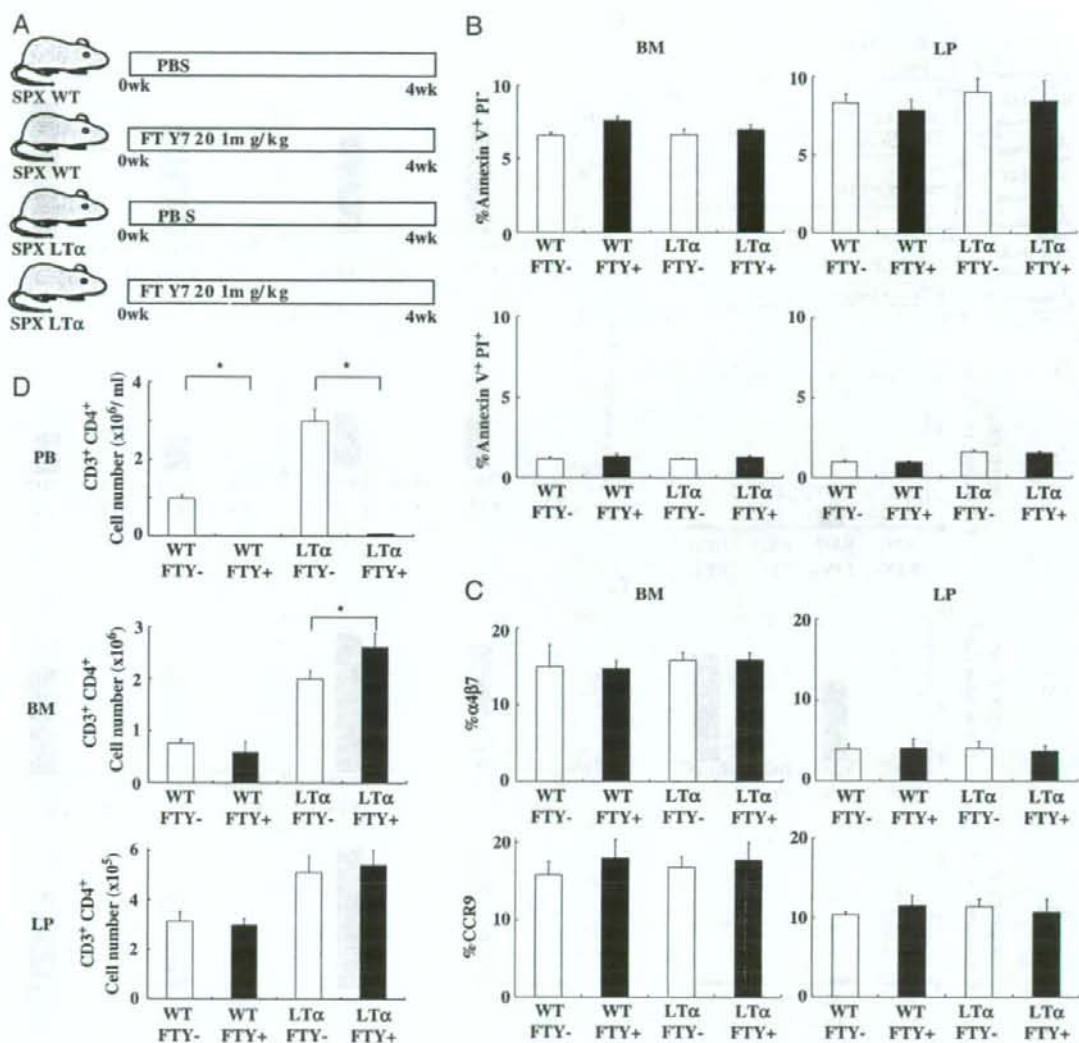


Figure 6. FTY720 treatment does not induce apoptosis or modulate the expression of gut-homing receptors of normal CD4⁺ T cells in vivo. (A) SPX WT or LT- $\alpha^{-/-}$ mice were treated with FTY720 (1.0 mg/kg) or PBS for 4 wk. (B) The number of dead or apoptotic cells in the isolated BM and LP CD3⁺CD4⁺ T cells from each group was determined by the Annexin V-FITC/PI. Data are represented as mean \pm SEM of %PI⁻ Annexin V⁺ (dead) cells and %PI⁺ Annexin V⁺ (apoptotic) cells in each group. Data are indicated as the mean \pm SEM of five mice in each group. * $p < 0.05$. (C) Long-term FTY720 treatment does not modulate the expression of gut-homing receptors. Isolated BM and LP cells were stained with FITC-labeled anti-CD3, and allophycocyanin-labeled anti-CD4 or PE-labeled anti- $\alpha 4\beta 7$ or PE-labeled anti-CCR9. The samples were analyzed by flow cytometry. Data are indicated as the mean \pm SEM of five mice in each group. (D) The absolute number of CD3⁺CD4⁺ T cells in PB, BM, and LP was determined at 4 wk after treatment. Freshly isolated PB, BM, and LP cells were stained with FITC-labeled anti-CD3 and allophycocyanin-labeled anti-CD4 mAb. The samples were analyzed by flow cytometry. Data are indicated as mean \pm SEM of five mice in each group.

finally addressed the possibility that FTY720 treatment might modulate the cell trafficking of colitogenic CD4⁺ T_{EM} cells in BM, thereby inducing lymphopenia in the PB of FTY720-treated SPX LT- $\alpha^{-/-}$ \times RAG-2^{-/-} recipients, leading to inhibition of colitogenic CD4⁺ T_{EM} cell circulation and subsequent suppression of the development of colitis. The preventive protocol of FTY720 in

the current colitis model (Figs. 3–5) was not accurate to assess this possibility, as it was impossible to discriminate whether FTY720 treatment itself or the development of colitis in mice without treatment induced the increased number of colitogenic CD4⁺ T_{EM} cells in the BM of SPX LT- $\alpha^{-/-}$ \times RAG-2^{-/-} recipients. Therefore, SPX LT- $\alpha^{-/-}$ \times RAG-2^{-/-} mice that were transferred

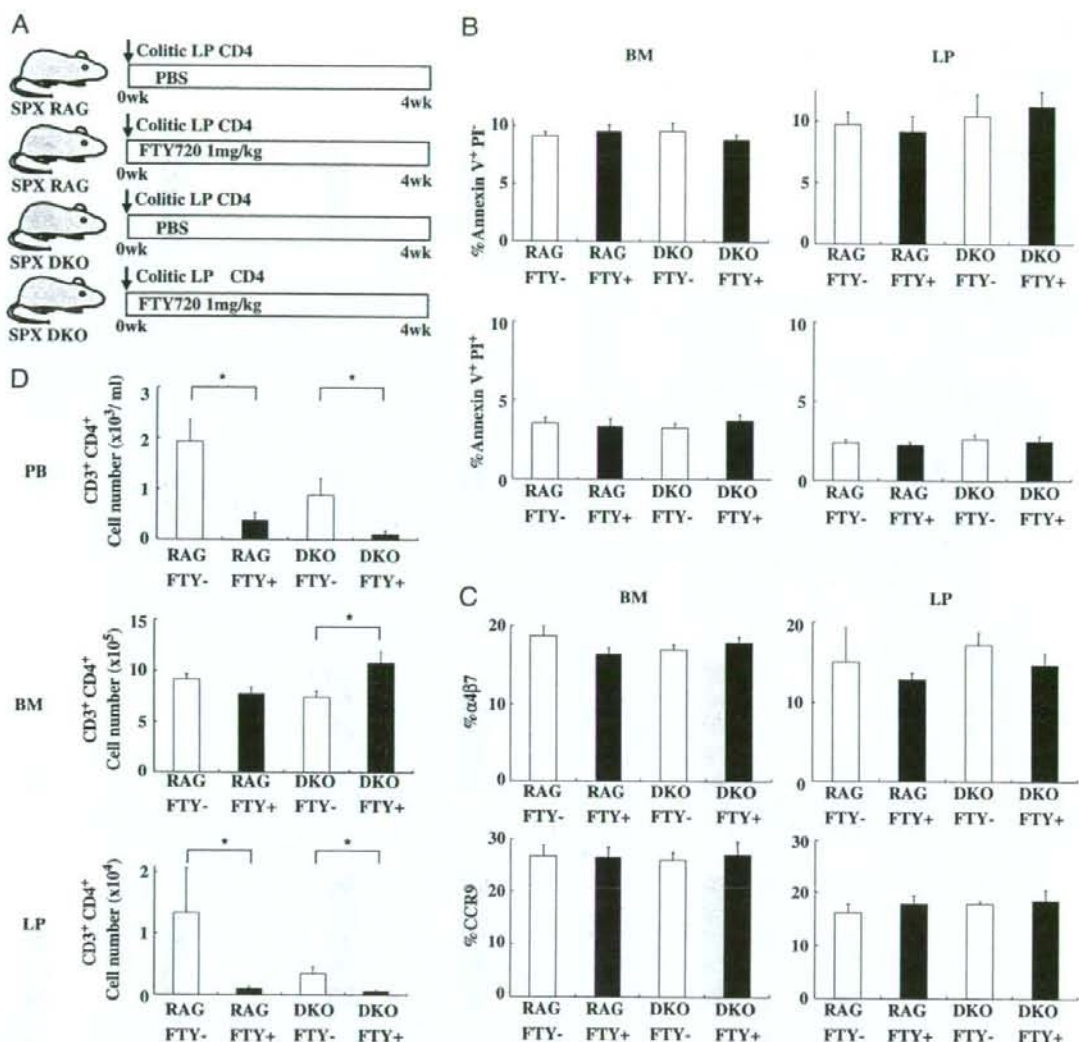


Figure 7. FTY720 treatment does not induce apoptosis or modulate expression of gut-homing receptors of CD4⁺ T cells in SPX RAG-2^{-/-} or LT-α^{-/-} mice transferred with colitogenic LP CD4⁺ T_{EM} cells *in vivo*. (A) SPX RAG-2^{-/-} or LT-α^{-/-} mice transferred with colitogenic LP CD4⁺ T_{EM} cells were treated with FTY720 (1.0 mg/kg) or PBS for 4 wk. (B) The number of dead or apoptotic cells in the isolated BM and LP CD3⁺CD4⁺ T cells from each group was determined by the Annexin V-FITC/PI. Data are represented as mean ± SEM of %PI⁺ Annexin V⁺ (dead) cells and %PI⁺ Annexin V⁺ (apoptotic) cells in each group. Data are indicated as the mean ± SEM of five mice in each group. **p* < 0.05. (C) Long-term FTY720 treatment does not modulate the expression of gut-homing receptors. Isolated BM and LP cells were stained with FITC-labeled anti-CD3, and allophycocyanin-labeled anti-CD4 or PE-labeled anti-αβ7 or PE-labeled anti-CCR9. The samples were analyzed by flow cytometry. Data are indicated as the mean ± SEM of five mice in each group. (D) The absolute number of CD3⁺CD4⁺ T cells in PB, BM, and LP were determined at 4 wk after treatment. Freshly isolated PB, BM, and LP cells were stained with FITC-labeled anti-CD3 and allophycocyanin-labeled anti-CD4 mAb. The samples were analyzed by flow cytometry. Data are indicated as mean ± SEM of five mice in each group.

with colitogenic LP CD4⁺ T_{EM} cells were treated by single injection of FTY720 (1.0 mg/kg) at 3 wk after the transfer, and the recovered cell number of BM CD3⁺CD4⁺ T cells was measured at 24 h after treatment (Fig. 8A). The recovered cell number of CD3⁺CD4⁺ T cells was indeed significantly increased in the BM but, conversely, significantly decreased in the PB of

FTY720-treated mice compared with that of PBS-treated mice (Fig. 8B), suggesting a similar mechanism with normal CD3⁺CD4⁺ T cells that FTY720 treatment induces a lymphopenia by trapping colitogenic CD4⁺ T_{EM} cells in BM. In addition, the number of LP CD3⁺CD4⁺ T cells was not affected by the treatment (Fig. 8B).

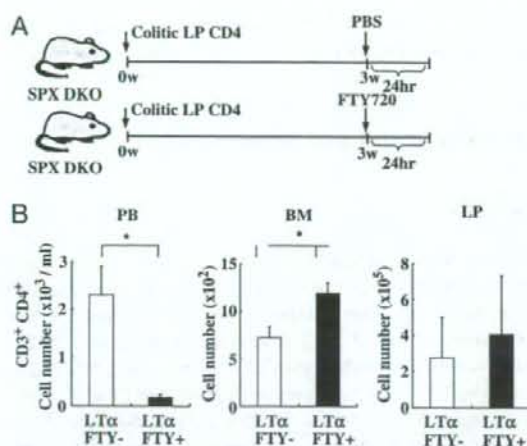


Figure 8. Colitogenic CD4⁺ T cells were preferentially accumulated in the BM of FTY720-treated LN/spleen-null recipients transferred with colitic LP CD4⁺ T cells. (A) SPX LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ mice were transferred with colitic LP CD4⁺ T cells and treated with FTY720 (1.0 mg/kg) or PBS at 3 wk after the transfer. The change in absolute numbers of cells was determined at 24 h after the transfer. (B) The absolute number of CD3⁺CD4⁺ T cells in PB, BM, and LP was determined at 24 h after treatment. Freshly isolated PB, BM, and LP cells were stained with FITC-labeled anti-CD3 and allophycocyanin-labeled anti-CD4 mAb. The samples were analyzed by flow cytometry. Data are indicated as mean \pm SEM of six mice in each group. * $p < 0.05$.

Discussion

In this study, we demonstrated that FTY720 is able to suppress the development of chronic colitis by modulating the trafficking of colitogenic CD4⁺ T_{EM} cells in BM in addition to the well-known effect to control the egress and sequestration of lymphocyte in LN. Apparently, altered T-cell trafficking, sequestration, and egress in LN and spleen by FTY720 cannot be held fully accountable for the reduced disease activity in FTY720-treated SPX LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ recipients that are transferred with colitogenic LP CD4⁺ T cells. Importantly, since we found that FTY720 treatment guides the significant decrease in both normal CD4⁺ T cells and colitogenic CD4⁺ T_{EM} cells in the PB, but conversely increase in those cells in the BM of SPX LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ recipients, it is strongly suggested that FTY720 may have an effect to control the trafficking of lymphocyte in BM, resulting in accumulation of CD4⁺ T cells in BM. Thus, the present study may provide a novel effect of FTY720 controlling the pathogenesis of IBD, by a mechanism other than the modification of trafficking of CD4⁺ T cells toward LN.

Based on the previous preventive protocols, since it had been largely accepted that the effect of FTY720 on autoimmune diseases is owing to the promotion of sequestration of naive T cells and the subsequent inhibition of egress of the generated effector T cells, but not those of memory T cells [1–12], it was unclear whether FTY720 could directly affect the migration of memory T cells in established autoimmune stages. To overcome this issue, we previously conducted adoptive transfer of colitogenic LP CD4⁺ T_{EM}

cells, excluded with naive cells, into SCID mice [22]. Although almost all colitogenic LP CD4⁺ T cells from colitic mice had the characteristics of CD44^{high}CD62L^{low}CD45RB^{low}IL-7R α ^{high} T_{EM} cells, FTY720 treatment suppressed the development of colitogenic memory T_{EM} cell-mediated colitis [22], indicating that FTY720 could surely affect the migration property of colitogenic LP CD4⁺ T_{EM} cells. Consistently, we demonstrated that the colitogenic LP T_{EM} cells as well as normal splenic CD4⁺CD45RB^{high} T cells did express several S1P receptors including S1P₁ [22]. However, we found that the number of CD4⁺ T cells in MLN was also significantly decreased in FTY720-treated mice transferred with colitogenic LP T_{EM} cells compared with that in the control mice [22]. Consistent with this finding, since colitogenic CD4⁺ memory T cells lack CD62L and CCR7, which naive T cells do express [22], it was suspicious to conclude that FTY720 solely controls trafficking of colitogenic CD4⁺ memory T cells toward LN.

We thus attempted to investigate a novel LN-independent mechanism of FTY720 using spleen/LN-null SPX LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ mice in this paper. Surprisingly, FTY720 treatment induces a severe PB lymphopenia in healthy SPX LT- $\alpha^{-/-}$ mice and colitogenic CD4⁺ memory T-cell-transferred SPX LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ mice to a similar extent with the paired SPX LT- $\alpha^{+/+}$ mice and colitogenic CD4⁺ memory T-cell-transferred SPX LT- $\alpha^{+/+}$ \times RAG-2 $^{-/-}$ recipients, respectively. This suggested the existence of as-yet-unknown reservoir other than LN and spleen, which could modulate the sequestration and/or the egress of colitogenic CD4⁺ memory T cells. Before focusing on this possibility, we assessed other possible mechanisms based on previous literatures. The first possibility that FTY720 might directly induce apoptosis of colitogenic CD4⁺ T cells was unlikely, because we showed that (i) the addition of various concentrations of FTY720 (10^{-6} – 10^{-8} mol/L) to colitogenic CD4⁺ memory T cells *in vitro* and *in vivo* FTY720 treatment did not induce apoptosis (Fig. 6A) and (ii) previous *in vivo* FTY720 (0.3–3.0 mg/kg) administration to normal mice did not promote apoptosis [22]. The second possibility that had been raised by others [28] was that FTY720 may induce conversion of colitogenic Th1 CD4⁺ memory T cells into CD4⁺CD25⁺Foxp3⁺ regulatory T cells. This was also unlikely, as we could not detect any increase in Foxp3 expression in LP CD4⁺ T cells of colitogenic CD4⁺ T-cell-transferred SPX LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ mice that were treated with FTY720 compared with those without FTY720 (Supporting Information Fig. 1). Although the previous paper demonstrated that FTY720 treatment could increase mRNA expression of CD25 and Foxp3 in LP CD4⁺ T cells of FTY720-treated TNBS-given mice along with no development of colitis in those mice [28], it appears to be possible that FTY720 could induce regulatory T cells from naive T cells as observed in their system, but not from established colitogenic CD4⁺ memory T cells in our system.

We next assessed the third possibility that FTY720 might skew the expression of gut-homing receptors on colitogenic CD4⁺ T cells, resulting in inhibition of the migration of these cells to the inflamed mucosa of the colon. To this end, we adopted an *in vitro* assay, in which addition of RA along with TGF- β , IL-2, and anti-CD3 mAb to colitogenic LP CD4⁺ T cells could induce the gut-homing receptors, such as integrin α 4 β 7 and CCR9. Although such a stimulation

up-regulated the expression of integrin $\alpha 4\beta 7$ and CCR9 on colitogenic LP CD4⁺ T cells indeed, FTY720 did not modify it.

Since recent works have suggested that BM is an important secondary lymphoid tissue [32] similar to LN and spleen, we finally focused on BM as a candidate reservoir to trap colitogenic CD4⁺ T cells, so as to explain the PB lymphopenia observed in FTY720-treated healthy SPX LT- $\alpha^{-/-}$ mice and SPX LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ mice transferred with colitogenic LP CD4⁺ T cells. Expectedly, we found that the number of BM CD4⁺ T cells was significantly increased in correlation with the decreased number of PB CD4⁺ T cells in FTY720-treated SPX LT- $\alpha^{-/-}$ mice. Interestingly, this was not the case with FTY720-treated SPX LT- $\alpha^{+/+}$ mice, indicating that the effect of FTY720 to direct CD4⁺ T cells toward BM may be cancelled by the presence of LN or spleen, which is the major site where lymphocytes are mainly trapped. Consistent with this hypothesis, it is interesting that the decreased number of PB CD4⁺ T cells was approximately equivalent to the increased number of BM CD4⁺ T cells in FTY720-treated SPX LT- $\alpha^{-/-}$ mice.

In a recent publication, Ledgerwood *et al.* reported that FTY720 inhibits the entry of T cells into afferent lymphatics under inflammatory condition in the skin [33]. Unlike their elegant system, injecting donor T cells subcutaneously into footpads of mice and monitoring the migration of cells into popliteal LN at different time points we could not detect any difference in the recovered cell number of colonic LP CD4⁺ T cells between FTY720-treated and FTY720-untreated SPX LT- $\alpha^{-/-}$ mice, even if we adopted the Ly5.1/Ly5.2 system to surely discriminate the transferred donor cells from host cells. However, further study will be needed to address whether BM alone is a target organ of FTY720 under LN/spleen-null condition, by assessing the precise concentration of S1P and FTY720-P in various tissues, and also by establishing a more precise method to monitor cell migration from LP to the afferent lymphatics, such as direct injection of donor cells into the LP of mice. Otherwise, it might be needed to use larger animals such as sheep for this issue, since it is very difficult to inject cells into very thin wall of intestine in mice system.

Finally, it should be addressed that LT- $\alpha^{-/-}$ mice have many other defects besides the lack of lymphoid organs, although we used SPX LT- $\alpha^{-/-}$ mice and LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ mice as spleen/LN-null mice to evaluate the role of FTY720 in this study. For example, (i) LT-1 $\alpha^{-/-}$ mice have been reported to have spontaneous infiltration of lymphocytes including CD4⁺ T cells in multiple peripheral tissues, possibly due to defective thymic negative selection [34], (ii) the total number of CD4⁺ T cells in the body of LT- $\alpha^{-/-}$ mice should be reduced, since spleen and LN harbor large number of lymphocytes as their reservoir, and (iii) LT- α itself, as one of the cytokines, which is critical for the cell migrations of lymphocytes, may be involved in the present results. Further studies will be needed for these points.

In conclusion, the present results indicated that FTY720 treatment could direct trafficking of CD4⁺ T cells not only toward LN but also toward BM, thereby contributing to the prevention of chronic colitis. Of clinical importance, this study also suggests that FTY720 treatment may be applicable to control the immune

response of patients who had undergone extended lymphadenectomy upon various surgeries such as intestinal resection.

Materials and methods

Animals

C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). C57BL/6-RAG-2 $^{-/-}$ mice were obtained from Taconic Laboratory (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). LT-1 $\alpha^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). LT- $\alpha^{-/-}$ mice were intercrossed into RAG-2 $^{-/-}$ mice to generate the littermate LT- $\alpha^{+/+}$ \times RAG-2 $^{-/-}$ and LT- $\alpha^{-/-}$ \times RAG-2 $^{-/-}$ mice [27] in the Animal Care Facility of Tokyo Medical and Dental University. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 wk of age. All experiments were approved by the regional animal study committees (permission number: 2006-049) and were carried out according to the institutional guidelines and Home Office regulations.

Antibodies

The biotin-conjugated anti-mouse IL-7R α (A7R34) and PE-conjugated anti-mouse Foxp3 (FJK-16S) mAb were obtained from eBioscience (San Diego, CA). The Fc γ (CD16/CD32)-blocking mAb (2.4G2), PE-, PerCP-, and allophycocyanin-conjugated anti-mouse CD4 (RM4-5); FITC- and PerCP-conjugated anti-mouse CD3 (145-2C11); FITC-conjugated anti-mouse CD8 α (53-6.7); PE-conjugated anti-mouse CD19 (1D3); allophycocyanin-conjugated anti-mouse CD44 (IM7); FITC-conjugated anti-mouse CD62L (MEL-14); FITC-conjugated anti-mouse CD69 (H1.2F3); PE-conjugated anti-mouse $\alpha 4\beta 7$ (DATK32); FITC-conjugated anti-mouse CD45RB (16A); PE-conjugated anti-mouse Ly5.1 (A20); FITC-conjugated anti-mouse Ly5.2 (104) mAb and PE-conjugated streptavidin were obtained from BD Pharmingen (San Diego, CA). The PE-conjugated anti-mouse CCR9 (242503) mAb was obtained from R&D Systems (Minneapolis, MN).

Purification of T-cell subsets

CD4⁺ T cells were isolated from normal spleen and colon using the anti-CD4 (L3T4) MACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instruction. To isolate normal LP CD4⁺ T cells, the entire length of the colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca²⁺, Mg²⁺-free Hanks' balanced salt solution (BSS) containing 1 mM DTT (Sigma-Aldrich) for 45 min to remove mucus and then treated with 3.0 mg/mL collagenase (Roche Diagnostics GmbH, Germany) and 0.01% DNase (Worthington

Biomedical, Freehold, NJ) for 2 h. The cells were pelleted two times through a 40% isotonic Percoll solution and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched CD4⁺ T cells from the spleen and the colon (spleen; 94–97% pure, colon; 80–90%, as estimated by FACS Calibur (Becton Dickinson, Sunnyvale, CA)) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5) and FITC-conjugated anti-CD45RB (16A). For isolation of peripheral lymphocytes, 500 μ L of PB was collected from each mouse and diluted 1:1 with PBS. The diluted blood was layered over Lymphosepar II (IBL, Gunma, Japan) and centrifuged at 400g for 30 min at room temperature. The lymphocytes were then isolated from the plasma-Ficoll interface. BM was collected from the femur by flushing with sterile PBS. Subpopulations of CD4⁺ cells were generated by two-color sorting on a FACS Aria (Becton Dickinson). All populations were >98.0% pure on reanalysis.

FTY720 treatment

FTY720 (Novartis Pharma AG, Basel, Switzerland) was dissolved in sterile PBS. For *in vivo* treatment, FTY720 was administered *i.p.* at a dose of 0.3 or 1.0 mg/kg daily. All animal studies were performed according to the institutional guideline under specific pathogen-free conditions. Exp. 1: To assess the effect of FTY720 on colitogenic memory CD4⁺ T-cell-mediated colitis *in vivo*, we prepared colitogenic LP CD4⁺ T cells. First, colitis was induced in RAG-2^{-/-} mice by adoptive transfer of syngeneic CD4⁺CD45RB^{high} T cells as described previously [35]. Briefly, CD4⁺ T cells were isolated from splenocytes from normal C57BL/6 mice using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec). Enriched CD4⁺ T cells were labeled with PE-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-CD45RB mAb and isolated CD45RB^{high} (highest staining 30%) fraction on a FACS Aria. Each RAG-2^{-/-} mouse was injected *i.p.* with syngeneic 3×10^5 CD4⁺CD45RB^{high} T cells. Colitic mice were sacrificed at 5–7 wk after the transfer to isolate the colitogenic LP memory CD4⁺ T cells. SPX LT- $\alpha^{+/+} \times$ RAG-2^{-/-} mice and SPX LT- $\alpha^{-/-} \times$ RAG-2^{-/-} mice were then injected *i.p.* with 4×10^5 colitic LP CD4⁺ T cells and were treated with PBS ($n = 9$) or FTY720 (0.3 mg/kg; $n = 9$, 1.0 mg/kg; $n = 9$) daily starting 1 day before the transfer, over a period of 4 wk. Mice after the transfer were weighed initially and then three times *per week* thereafter. They were monitored for clinical signs of illness: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. The mice were sacrificed and assessed for a clinical score, which is the sum of four parameters as follows: hunched and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; and an additional point was added if gross blood was noted [35]). Exp. 2: To assess the *in vivo* effect of FTY720 on hemodynamics within lymphoid-null mice, FTY720 or PBS was administered to C57BL/6 mice or LT- $\alpha^{-/-}$ mice with or without splenectomy at 0 h, and the changes in absolute numbers of CD3⁺CD4⁺ and CD3⁺CD8⁺

T cell subsets, and CD19⁺ B cells at 24 h after treatment in PB, LP, and BM. Exp. 3: To exclude the influence of resident CD3⁺CD4⁺ T cells that are readily present before the treatment of FTY720, FTY720-treated splenectomized LT- $\alpha^{-/-}$ mice (Ly5.2⁺) were transferred with Ly5.1⁺ splenic CD4⁺ T cells at 0 h, and the changes in the absolute number of Ly5.1⁺ or Ly5.2⁺ CD3⁺CD4⁺ T cells at 24 h after the transfer were examined in PB, LP, and BM. Exp. 4: To specifically assess the effect of FTY720 on the trafficking of colitogenic CD4⁺ T_{EM} cell, SPX LT- $\alpha^{-/-}$ mice that were transferred with colitogenic CD4⁺ T_{EM} cells were *i.p.* treated with PBS or FTY720 (1.0 mg/kg) at 3 wk after the transfer, and the changes in the absolute number of CD3⁺CD4⁺ T cells at 24 h after treatment in PB, LP, and BM were measured. Exp. 5: To assess the effect of long-term FTY720 treatment *in vivo*, SPX WT or LT- $\alpha^{-/-}$ mice were *i.p.* treated with PBS or FTY720 (1.0 mg/kg) daily for 4 wk, and the changes in the expression of integrin $\alpha 4\beta 7$ and CCR9, the absolute number of CD3⁺CD4⁺ T cells in PB, BM, and LP, and the number of apoptotic cells (Annexin V⁺ cells *per total* CD3⁺CD4⁺ T cells) in BM and LP were measured. Exp. 6: To assess the effect of long-term FTY720 treatment *in vivo* specifically on colitogenic CD4⁺ T cells, SPX RAG-2^{-/-} or LT- $\alpha^{-/-} \times$ RAG-2^{-/-} mice that were transferred with colitogenic LP CD4⁺ T_{EM} cells were *i.p.* treated with PBS or FTY720 (1.0 mg/kg) daily for 4 wk after the transfer, and the changes in the expression of integrin $\alpha 4\beta 7$ and CCR9, the absolute number of CD3⁺CD4⁺ T cells in PB, LP, and BM, and the number of apoptotic cells were measured.

Disease monitoring and clinical scoring

The recipient mice were weighed initially upon T-cell transfer and then three times *per week* thereafter. They were observed for clinical signs of illness: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. Mice were sacrificed at the indicated time point and assessed for a clinical score, which is the sum (0–8 points) of four parameters as follows: hunched and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea); and an additional point was added if gross blood was noted [35]. To monitor the clinical sign during the observed period over time, the disease activity index is defined as the sum (0–5 points) of the above-mentioned parameters except colon thickening [35].

Histological examination and immunohistology

Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μ m) were stained with H&E. The sections were analyzed without prior knowledge of the type of T-cell reconstitution and recipients. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system [35].

Immunohistochemistry

Immunohistochemistry using purified mAb against mouse CD4 (RM4-5; BD PharMingen) was performed. Briefly, O.C.T. compound-embedded tissue samples were cut into serial sections 6 μm thick, placed on coated slides, and fixed with 4% paraformaldehyde phosphate buffer solution for 10 min. Slides were then incubated with the primary antibody at 4°C overnight, followed by staining with Alexa Fluor[®] 488 goat anti-rat IgG (Molecular Probes) for CD4 detection at room temperature for 60 min. All slides were counterstained with DAPI (Vector) and observed under a fluorescence microscope (BioZERO BZ8000 KEYENCE, Tokyo, Japan).

Flow cytometry

To detect the surface expression of a variety of molecules, isolated splenocytes, BM, or LP mononuclear cells were preincubated with an Fc γ R-blocking mAb (CD16/32; 2.4G2, BD PharMingen) for 15 min followed by incubation with specific FITC-, PE-, PerCP-, allophycocyanin- or biotin-labeled antibodies for 20 min on ice. Biotinylated antibodies were detected with PE-streptavidin. To detect the dead and apoptotic cells in the cells cultured with FTY720 (0, 10^{-8} , 10^{-7} , or 10^{-6} M) *in vitro* for 48 h, they were stained with Annexin V-FITC/PI (MBL, Nagoya, Japan). Standard three- or four-color flow cytometric analyses were obtained using the FACS Calibur using CellQuest software. Background fluorescence was assessed by staining with control irrelevant isotype-matched mAb.

Cytokine ELISA

To measure cytokine production, 1×10^5 LP CD4⁺ T cells were cultured in 200 μL culture medium at 37°C in a humidified atmosphere containing 5% CO₂ in 96 well plates (Costar, Cambridge, MA) pre-coated with 5 $\mu\text{g}/\text{mL}$ hamster anti-mouse CD3 ϵ mAb (145-2C11, BD PharMingen) and hamster 2 $\mu\text{g}/\text{mL}$ anti-mouse CD28 mAb (37.51, BD PharMingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA as per the manufacturer's recommendation R&D Systems.

In vitro induction of gut-homing receptors

Cells were cultured in RPMI 1640 media supplemented with 10% FBS, HEPES, 50 μM β -ME, and penicillin/streptomycin/l-glutamine. For 96-well plate cultures, 2×10^5 cells in round-bottom plates were cultured in 200 μL of media. WT SP CD4⁺ T cells or colitic LP CD4⁺ T cells were activated with 1 $\mu\text{g}/\text{mL}$ anti-CD3 and 2 $\mu\text{g}/\text{mL}$ anti-CD28 mAb in the presence of 10 ng/mL hTGF- β 1 (PeproTech), 100 U/mL hIL-2 (PeproTech), and with/without 100 nM all-trans RA (Sigma-Aldrich) [36]. The cells were cultured with FTY720 at a concentration of 0, 10^{-8} , 10^{-7} , or 10^{-6} M for 5 days.

Statistical analysis

The results are expressed as the mean \pm SEM. Groups of data were compared by Mann-Whitney *U*-test. Differences were considered to be statistically significant when $p < 0.05$.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: FTY720: 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride · IBD: inflammatory bowel disease · LP: lamina propria · LT- α : lymphotoxin alpha · PB: peripheral blood · RA: retinoic acid · S1P: sphingosine-1-phosphate · SPX: splenectomized · T_{EM}: effector-memory T

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