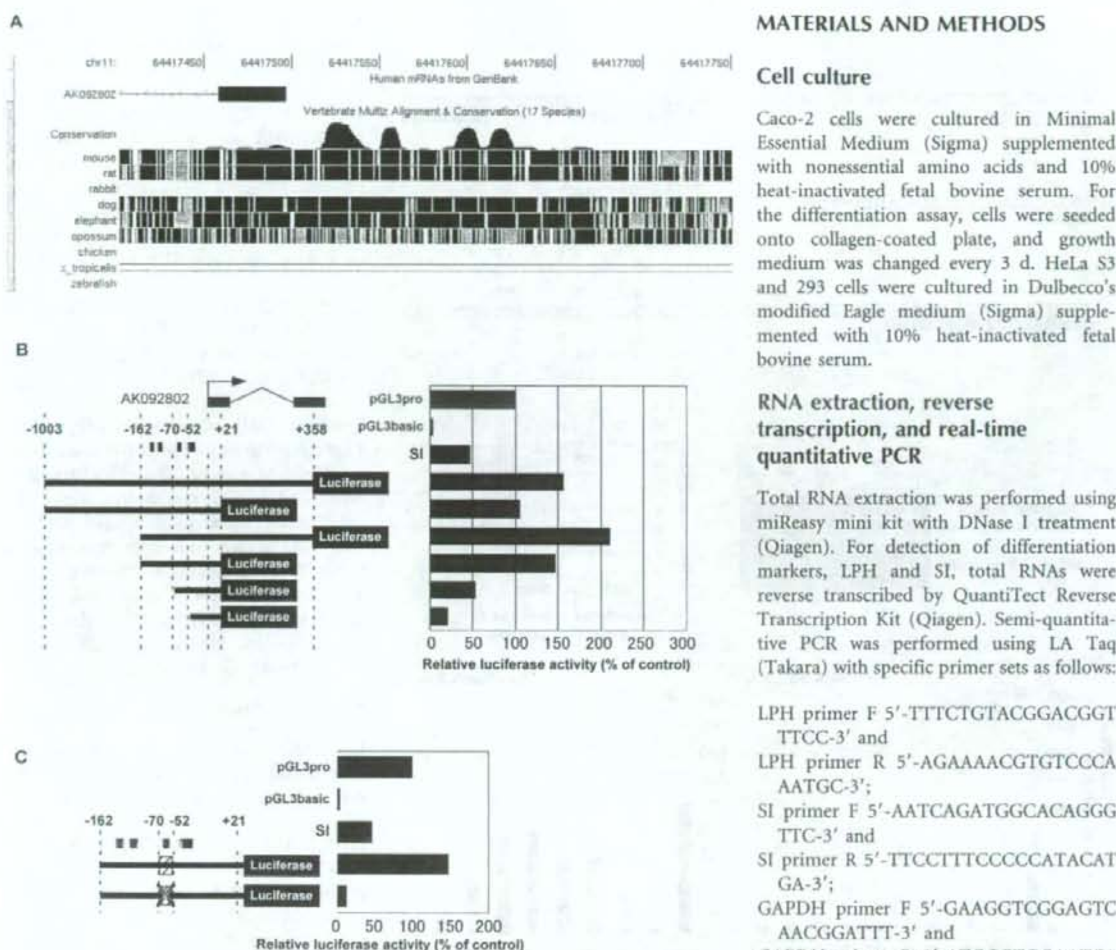

FIGURE 5. Potential HNF-1α binding element is located in the pri-miR-194-2 promoter. (*A*) Conserved sequences found within the pri-miR-194 promoter region. This figure is derived from the UCSC genome browser. (*B*) Schematic representation of human miR-194-2 promoter reporter constructs (*left*) and analysis of their promoter activity (*right*). Firefly luciferase reporter plasmids were constructed containing various regions of the putative pri-miR-194-2 promoter between positions −1003 to +358, designated with respect to the 5′end of AK092802. Arrow indicates transcription start site of pri-miR-194-2. pGL3 promoter (pGL3 pro) contains SV40 promoter, while pGL3basic has no promoter sequence upstream of the luciferase coding region. (*C*) Mutation of the region from −70 to −52 dramatically reduced pri-miR-194-2 promoter activity in Caco-2 cells. A scrambled sequence was introduced in the mutant pri-miR-194-2 promoter. Luciferase activities were normalized by *Renilla* luciferase activities.

is regulated by HNF-1α. As HNF-1α is one of the critical regulators of intestinal epithelial gene expression, control of miR-194 by this transcription factor adds miRNAs to the regulatory network of gene expression in intestinal epithelial cells. Therefore, the present work suggests that induced expression of miRNAs by tissue-specific transcription factors has an important role in intestinal epithelium maturation.

MATERIALS AND METHODS

Cell culture

Caco-2 cells were cultured in Minimal Essential Medium (Sigma) supplemented with nonessential amino acids and 10% heat-inactivated fetal bovine serum. For the differentiation assay, cells were seeded onto collagen-coated plate, and growth medium was changed every 3 d. HeLa S3 and 293 cells were cultured in Dulbecco's modified Eagle medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum.

RNA extraction, reverse transcription, and real-time quantitative PCR

Total RNA extraction was performed using miReasy mini kit with DNase I treatment (Qiagen). For detection of differentiation markers, LPH and SI, total RNAs were reverse transcribed by QuantiTect Reverse Transcription Kit (Qiagen). Semi-quantitative PCR was performed using LA Taq (Takara) with specific primer sets as follows:

LPH primer F 5′-TTTCTGTACGGACGGTTTCC-3′ and
LPH primer R 5′-AGAAAACGTGTCCCAAATGC-3′;
SI primer F 5′-AATCAGATGGCACAGGGTTC-3′ and
SI primer R 5′-TTCCTTTCCCCCATACATGA-3′;
GAPDH primer F 5′-GAAGGTCGGAGTCAACGGATTT-3′ and
GAPDH primer R 5′-ATGGGTGGAATCATATTGGAA-3′.

Quantification of mature miRNAs was performed by TaqMan miRNA assays Human Panel–Early Access Kit (Applied Biosystems) according to the manufacturer's instruction. As the kit did not contain probes for miR-192, data for miR-192 are not included in Figure 1B. Quantification of individual miRNAs (Fig. 2D) was performed by a TaqMan miRNA assay kit (Applied Biosystems).

Northern blot

To synthesize DIG-labeled RNA probe, vectors were constructed by inserting the following oligonucleotides into pcDNA3:

For mature miR-194 detection, 5′-TCCACATGGAGTTGCTGTTACA-3′;
For pre-miR-194-1 detection, 5′-AACTCCATGTGGACTGTGTACCAATTTCCAGTGGAGATGC-3′; and

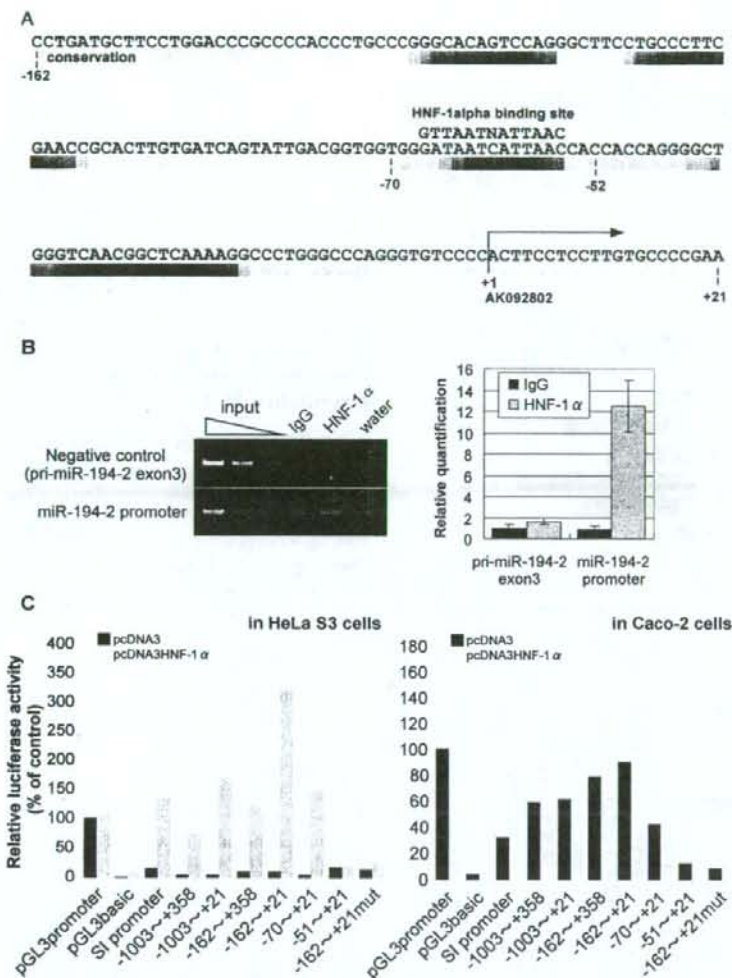

FIGURE 6. HNF-1α binds and regulates the miR-194-2 promoter in Caco-2 cells. (*A*) Sequence of pri-miR-194-2 promoter between −162 and +21. The region between −162 and +21 is partially conserved among human, mouse, and dog. Conserved sequences are indicated as density map (higher conservation becomes black). The transcription start site is indicated by an arrow. Consensus sequence of HNF-1α binding site is located at region between −67 and −55. (*B*) Chromatin immunoprecipitation (ChIP) analysis showing binding of HNF-1α to the miR-194-2 promoter in vivo in Caco-2 cells. Fixed chromatin from differentiated Caco-2 cells was prepared and immunoprecipitated either by anti HNF-1α antibody or normal goat IgG. ChIP primers were designed to amplify the region containing putative HNF-1α binding site in the pri-miR-194-2 promoter. Negative control primers were designed to amplify exon 3 of the pri-miR-194-2. PCR products were separated by acrylamide gel electrophoresis and stained by EtBr (*left*). Results of the quantitative PCR are shown as the relative amount of precipitated chromatin, in which chromatin precipitated by goat-IgG is set to 1. (*C*) Human pri-miR-194-2 promoter activity is up-regulated by forced expression of HNF-1α. Various luciferase reporter plasmids were cotransfected with either pcDNA3 mock vector (black bar) or HNF-1α expression vector (open bar) into HeLa S3 cells (*left*), or Caco-2 cells (*right*). SI-promoter contains promoter sequence of the sucrase-isomaltase (SI) gene, which is formerly reported to be up regulated by HNF-1α. Luciferase activities were normalized by *Renilla* luciferase activities.

For pre-miR-194-2 detection, 5′-AACTCCATGTGGAAGTGCCCACTGGTTCCAGTGGGGCTGC-3′.

DIG-labeled RNA probes were synthesized by DIG RNA labeling kit (Roche). For pre-miRNA detection, knockdown of Dicer was performed before RNA extraction, using siRNA. The Dicer siRNA was transfected into cells by LipofectAmine2000 (Invitrogen). The sequence of the Dicer siRNA was as follows:

Dicer siRNA sense, 5′-UGCUUGAAGCAGCUCUGGAdTdT; and
Dicer siRNA anti-sense, 5′-UCCAGAGCUGCUUCAAGCAdTdT.

The letters "dT" represent deoxythymidine. Thirty micrograms of total RNA was separated by 8 M urea PAGE and transferred to Nybond N+ (GE Healthcare). After UV cross-linking, the membrane was hybridized with DIG-labeled probes in hybridization buffer (50% formamide, 5× SSC, 0.1% SDS, 2× Denhardt's solution, and salmon sperm DNA). Detections were achieved by AP-conjugated anti-DIG antibody (Roche) and CDP-star (GE Healthcare).

RT-PCR for pri-miRNA

Pri-miRNA cluster detection PCR was performed using Quantitect SYBR PCR kit (Qiagen) with specific sets of primers as follows:

pri-miR-194-1 F, 5′-AGCGTTTCAAATCTACCAGT-3′;
pri-miR-194-1 R, 5′-TATCTTCTGTGTACCTGCCA-3′;
pri-miR-194-2 F, 5′-ATGATAAGAAGCCTCGGTGA-3′; and
pri-miR-194-2 R, 5′-GTGGGACCATGAGTGCTGCA-3′.

AK092802 and miRNA cluster RT-PCR (Fig. 3B) was performed with the following primers, and the sequence of the detected PCR products were confirmed by direct sequencing. Forward primer downstream of the transcription start site (DTSS F) was 5′-TTCCTCCTTGTGCCCCGAAG-3′, and pri-miR-194-2 R was used as the reverse primer (this primer is placed within the second intron). Template cDNA was prepared by reverse-transcription by SuperScript II (Invitrogen) using oligo dT primer.

RT-PCR for pri-miR-194-2 entire transcript (Fig. 4C) was performed with the following primers, Forward primer was DTSS F, and reverse primer was; 5′-CATCCCAGCCACAGAGCATC-3′.

RACE analysis

5′ RACE was performed using GeneRacer kit (Invitrogen) according to manufacturer's protocol, except for the use of random primer in reverse transcription. PCR amplification of 5′ end of pri-miR-194-2 was performed by touchdown PCR by LA Taq (Takara) using GeneRacer 5′ primer and reverse primer 5′-CAGCAGGCA TTTTGGGAGAC-3′. Amplified 5′ RACE fragments were cloned into pGEM T-Easy (Promega) for sequence analysis. 3′ RACE was also performed using GeneRacer kit (Invitrogen). PCR Amplification of the 3′ end of pri-miR-194-2 was also performed using the gene-specific forward primer 5′-TTCCTCCTTGTGCCCCGAAG-3′ and GeneRacer 3′ primer. Amplified 3′ RACE fragments were also cloned into pGEM T-Easy (Promega) for sequence analysis.

Vector constructions

miRNA expression vectors were constructed by cloning miRNA coding region into pcDNA3.1(−) (Invitrogen). miRNA coding regions were amplified by Phusion DNA polymerase (New England Biolab) from Caco-2 genomic DNA using primers as follows:

miR-194-1cluster F, 5′-ATACTCGAGTAGAACATGAATAAATC GAGAC-3′;
miR-194-1cluster R, 5′-TATGAATTCTTACTCAATACATTTA CATGGTAG-3′;
miR-194-2cluster F, 5′-ATACTCGAGCCTGGGGCCACGAAGAC TGG-3′; and
miR-194-2cluster R, 5′-ATAGGATCCGGGGAATGAGACAGAG GGAGG-3′.

miR-194-2 promoter deletion variants were amplified by the following primers and cloned into pGL3basic between XhoI-MluI sites. Cloning primers were as follows:

−1003 primer F, 5′-ATGCACGCGTATGTCACCACCAGGGGT CGC-3′;
−162 primer F, 5′-ATGCACGCGTCCTGATGCTTCCTGGACCCG-3′;
−70 primer F, 5′-ATGCACGCGTTGGGATAATCATTAACCACC-3′;
−51 primer F, 5′-ATGCACGCGTCACCAGGGGCTGGGTCAACG-3′;
+21 primer R, 5′-TCGACTCGAGTTCGGGGCACAAGGAGGAA G-3′; and
+358 primer R, 5′-TCGACTCGAGACTCAGCCTGGCGGCCCTTC-3′.

Scrambled mutation was induced using Scrambled F 5′-GATAC TAACGTAAGCCACCAGGGGCTGGGT-3′ and Scrambled R 5′-ACGTTAGTATCATAGCCACCGTCAATACTG-3′. HNF-1α expression vector was constructed by cloning HNF-1α cDNA into pcDNA3 (Invitrogen). HNF-1α primers were 5′-ATG CAAGCTTGCCACCATGGTTTCTAAACTGAGCCAGC-3′ and 5′-TAATGAATTCTTACTGGGAGGAAGAGGCCA-3′.

Transfection and luciferase assay

Transfections were performed using LipofectAmine2000 (Invitrogen) according to the manufacturer's protocol. For examination of miRNA generation, miRNA expression vector, luciferase miRNA-sensor vector, and pRL-TK were mixed (10:9:1), and cotransfected into cells were cultured in 96-well plate. For examination of miRNA promoter activity, the HNF-1α expression vector, luciferase miRNA promoter vector, and pRL-TK were mixed (20:19:1) and cotransfected into cells cultured in 96-well plate. Luciferase activity was measured by the Dual luciferase assay kit (Promega).

ChIP assay

To cross-link chromatin, differentiated Caco-2 cells were treated with 1% formaldehyde for 10 min at room temperature. Cross-linking was stopped by addition of 0.125 M glycine. After being washed twice with ice-cold PBS, cells were resuspended in NP-40 nuclear extraction buffer (10 mM HEPES at pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, and protease inhibitor) and centrifuged at 3000 rpm for 10 min. Crude nuclei were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl at pH 8.0, and protease inhibitor), and extensively sonicated by Bioruptor. Sonicated chromatin was centrifuged at 15,000 rpm, and the supernatant was collected (input control). The supernatant was mixed with 9 vol of ChIP dilution buffer (11 mM Tris/HCl at pH 8.0, 154 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1.1% Triton X-100, 0.11% sodium deoxycholate, and protease inhibitors) and precleared with preblocked Protein G–Sepharose (GE Healthcare). Precleared chromatin was immunoprecipitated with 5 μg of anti-HNF-1α antibody (Santa Cruz, sc-6547) or 5 μg of normal goat IgG (Vector Laboratory, I-5000), and the immuno complexes were collected by preblocked Protein G–Sepharose. The beads were washed sequentially by RIPA, RIPA containing 500 mM NaCl, LiCl wash buffer, and twice by TE. Collected chromatin were eluted in ChIP elution buffer (1% SDS, 100 mM $NaHCO_3$), adjusted to 200 mM NaCl, and incubated for at least 6 h at 65°C to reverse cross-link. After treatment with RNase A (Nippongene) and Proteinase K (Roche), DNA fragments were extracted by phenol/chloroform and ethanol precipitation. Quantitative PCR was performed with Quantitect SYBR PCR kit (Qiagen) using the following primers: miR-194-2 promoter ChIP primer F, 5′-TGATCAGTATTGACGGTGGTG-3′; primer R, 5′-AAGGAGGAAGTGGGGACAC-3′. Also used were negative control (exon3 of pri-miR-194-2) primer F, 5′-CCCACTGAC CTGTGTCCTTT-3′; primer R, 5′-AGAGGGGTTGGAGGTGAG AC-3′. Detected PCR products were sequenced after cloning into pGEM T-Easy vector (Promega).

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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-$\alpha^{-/-}$) × 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 naïve $CD44^{low}CCR7^+CD62L^+$ 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 $CD4^+CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ T_{EM} cells obtained from colitic $CD4^+CD45RB^{high}$ T-cell-transferred

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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

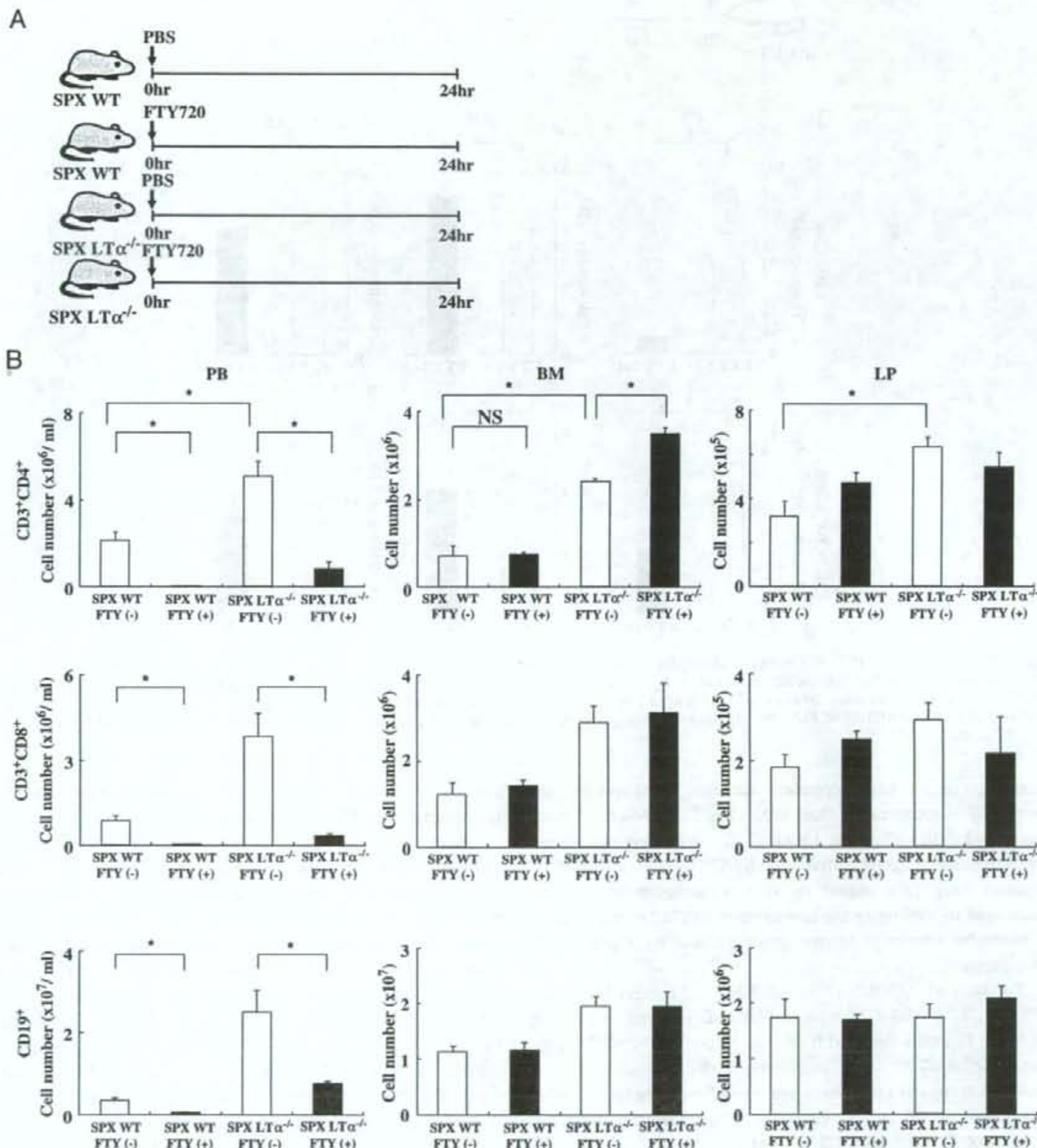

Figure 1. FTY720 treatment induces decrease in PB $CD4^+$ T cells and increase in BM $CD4^+$ T cells in LN/spleen-null mice. (A) FTY720 (1.0 mg/kg) or PBS was i.p. administered to C57BL/6 (WT) or $LT\text{-}\alpha^{-/-}$ mice with SPX, and the changes in the absolute numbers of cells were determined 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.

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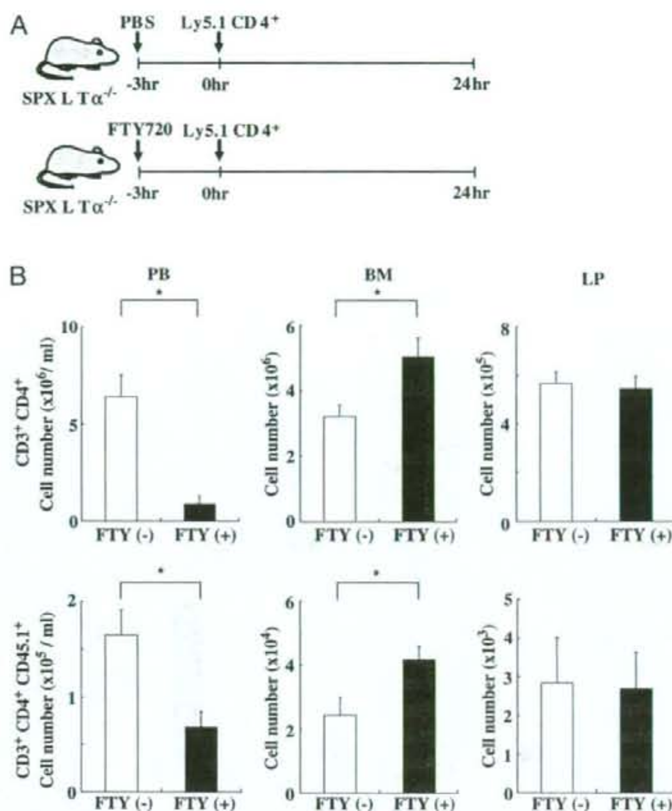

Figure 2. Transferred CD4$^+$ T cells were preferentially accumulated in the BM of FTY720-treated LN/spleen-null mice. (A) SPX LT-$\alpha^{-/-}$ mice (Ly5.2$^+$) were treated with FTY720 (1.0 mg/kg) or PBS at −3 h and then transferred with normal splenic CD4$^+$ T cells (Ly5.1$^+$) at 0 h. The changes 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 ± 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-$\alpha^{-/-}$ × RAG-2$^{-/-}$ mice transferred with colitogenic LP CD4$^+$ T_{EM} cells develop chronic colitis to a similar extent with the control SPX LT-$\alpha^{+/+}$ × 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-$\alpha^{-/-}$ × RAG-2$^{-/-}$ or control littermate SPX LT-$\alpha^{+/+}$ × 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-$\alpha^{-/-}$ × RAG-2$^{-/-}$ and SPX LT-$\alpha^{+/+}$ × 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-$\alpha^{-/-}$ × RAG-2$^{-/-}$ or LT-$\alpha^{+/+}$ × 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-$\alpha^{+/+}$ × RAG-2$^{-/-}$;

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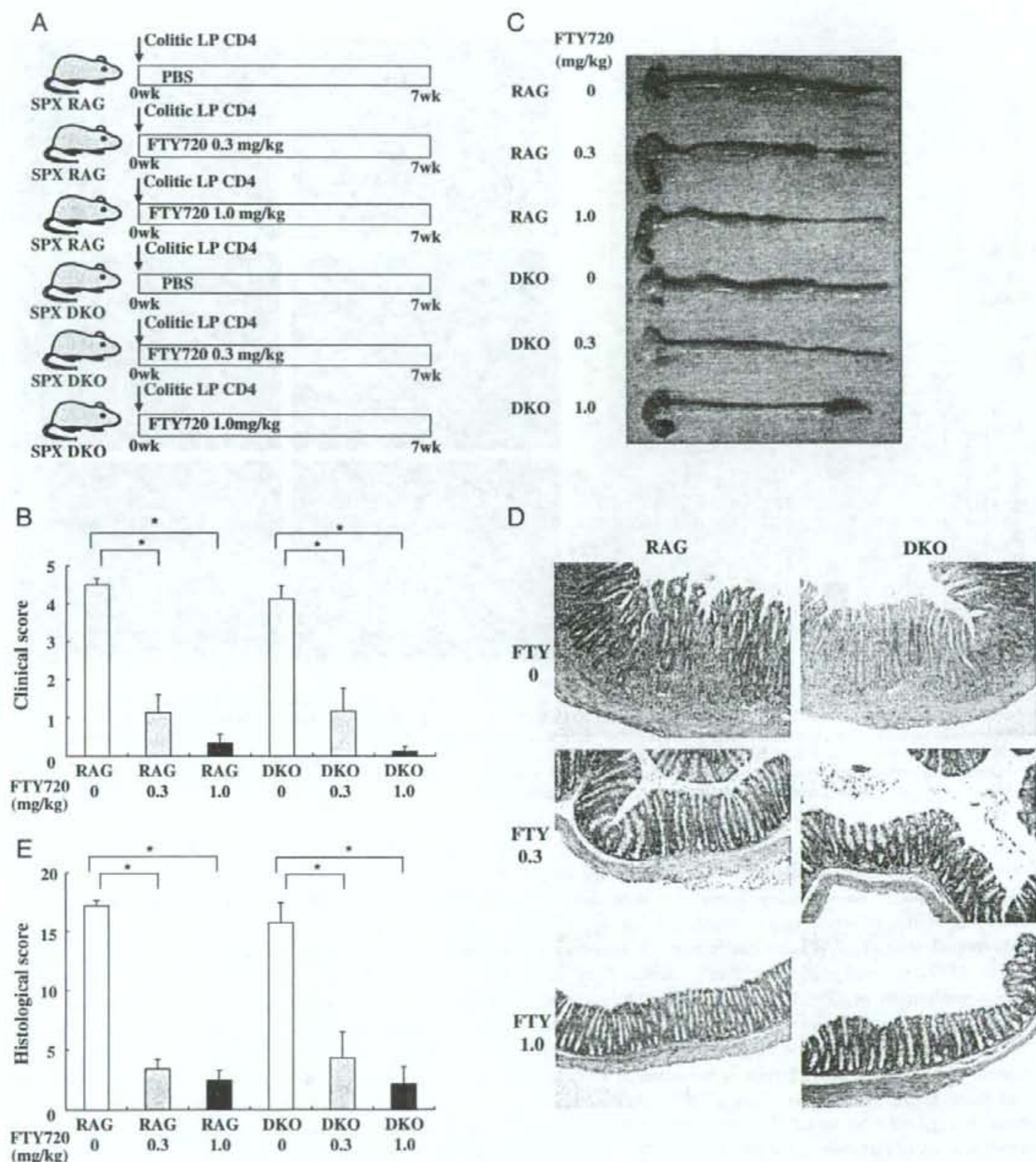

Figure 3. FTY720 prevents the development of colitogenic CD4$^+$ T_{EM}-mediated colitis in LN/spleen-null mice. (A) Colitogenic CD4$^+$ T_{EM} cells were isolated from the inflamed mucosa of colitic RAG-2$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells. SPX LT-$\alpha^{+/+}$ × RAG-2$^{-/-}$ 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.

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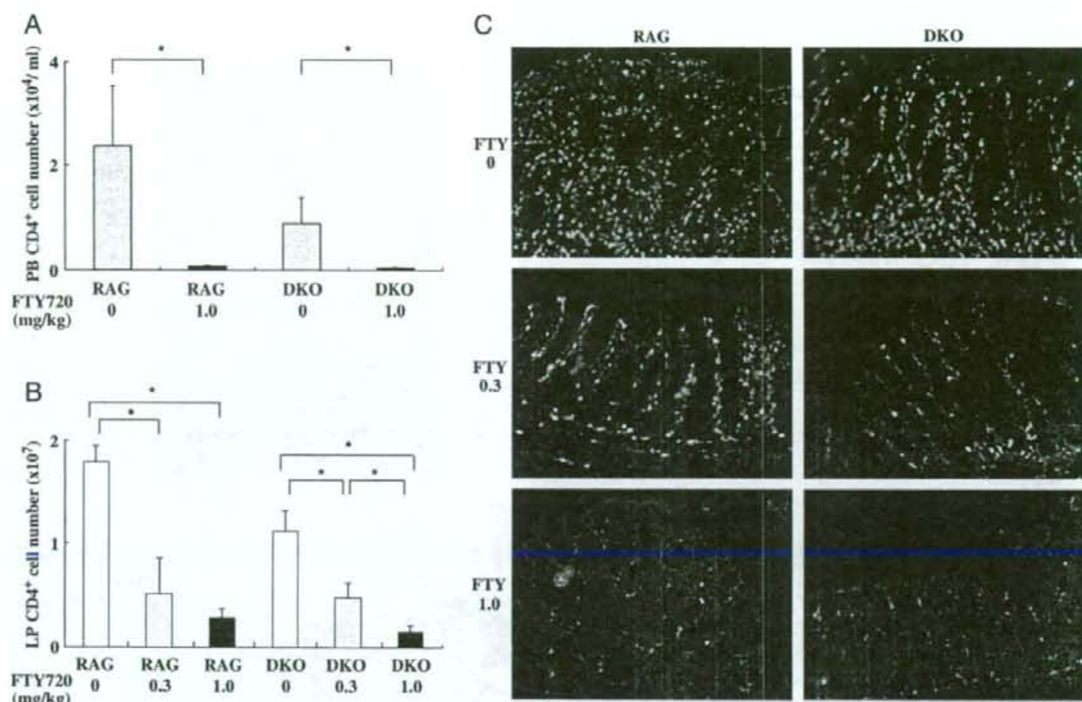

Figure 4. The number of LP $CD4^+$ T cells was significantly decreased while PB lymphopenia was observed in FTY720-treated LN/spleen-null mice transferred with colitogenic $CD4^+$ T_{EM} cells. (A–B) Number of $CD3^+CD4^+$ T cells in PB (A) or LP (B). Cells were stained with FITC-anti-CD3 and allophycocyanin-anti-CD4, 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.

$1.73\pm0.15\times10^7$, SPX LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$; $1.07\pm0.14\times10^7$) far exceeded the number of originally injected memory cells (3×10^5), 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 $CD4^+CD62L^-CD44^{high}$ T_{EM} cell *in vivo*. However, almost all LP $CD4^+$ T cells from PBS-treated or FTY720-treated mice retained the characteristics of $CD69^+CD62L^-CD44^{high}IL\text{-}7R\alpha^{high}$ 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}

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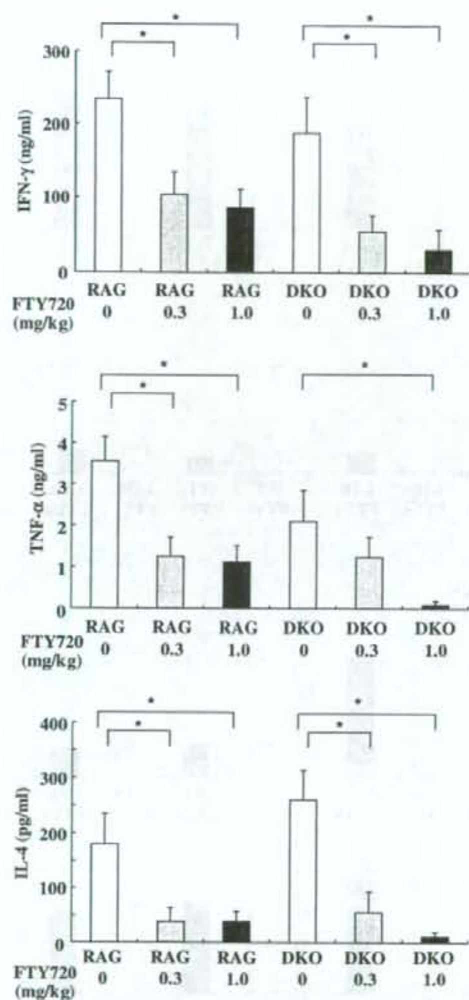

Figure 5. LP CD4+ T cells in FTY720-treated LN/spleen-null mice transferred with colitogenic CD4+ T_{EM} cells produce significantly less amount of Th1 and Th2 cytokines. LP CD4+ T cells were isolated from mice 7 wk after the transfer of colitogenic LP CD4+ T_{EM} cells and stimulated with anti-CD3 and 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+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 α4β7 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 α4β7 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 α4β7 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 α4β7 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-$\alpha^{-/-}$ × 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-$\alpha^{-/-}$ × 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-$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice was significantly increased compared with that in PBS-treated LT-$\alpha^{-/-}$ × 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-$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice transferred with colitogenic LP CD4+ T_{EM} cells in long-term FTY720 administration system (Fig. 7), we

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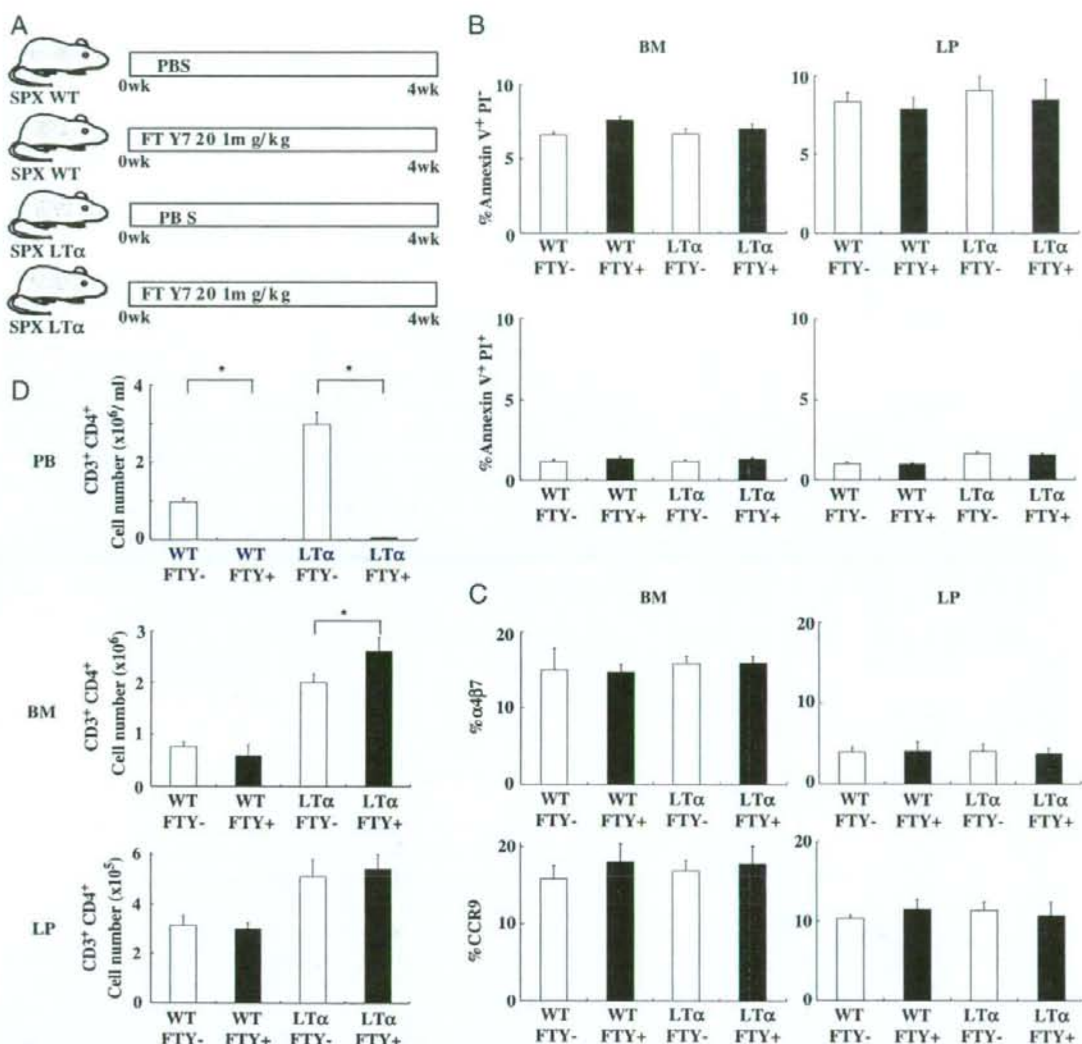

Figure 6. FTY720 treatment does not induce apoptosis or modulate the expression of gut-homing receptors of normal CD4+ T cells *in vivo*. (A) SPX WT or LT-$\alpha^{-/-}$ mice were treated with FTY720 (1.0 mg/kg) or PBS for 4 wk. (B) The number of dead or apoptotic cells in the isolated BM and LP CD3+CD4+ T cells from each group was determined by the Annexin V-FITC/PI. Data are represented as mean ± 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-α4β7 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 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 + 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-$\alpha^{-/-}$ × 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-$\alpha^{-/-}$ × RAG-$2^{-/-}$ recipients. Therefore, SPX LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice that were transferred

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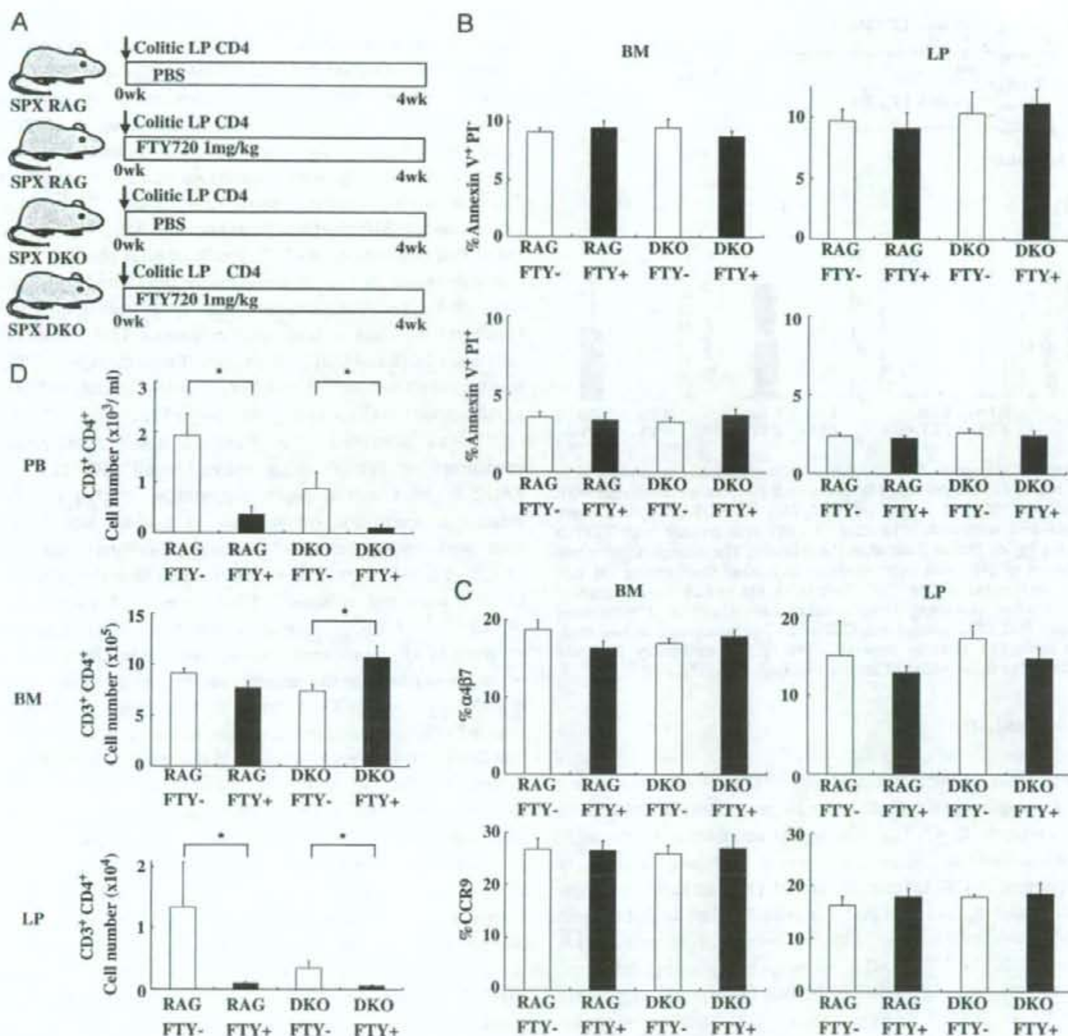

Figure 7. FTY720 treatment does not induce apoptosis or modulate expression of gut-homing receptors of CD4⁺ T cells in SPX RAG-$2^{-/-}$ or LT-$\alpha^{-/-}$ mice transferred with colitogenic LP CD4⁺T_{EM} cells *in vivo*. (A) SPX RAG-$2^{-/-}$ or LT-$\alpha^{-/-}$ mice transferred with colitogenic LP 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-α4β7 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).

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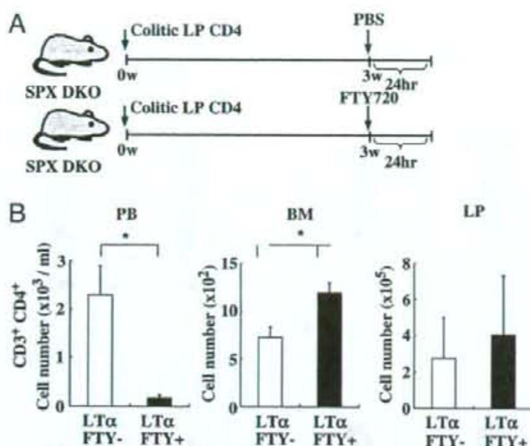

Figure 8. Colitogenic $CD4^+$ T cells were preferentially accumulated in the BM of FTY720-treated LN/spleen-null recipients transferred with colitic LP $CD4^+$ T cells. (A) SPX $LT\text{-}\alpha^{-/-} \times RAG\text{-}2^{-/-}$ mice were transferred with colitic LP $CD4^+$ T cells and treated with FTY720 (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+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 FTY-720-treated SPX $LT\text{-}\alpha^{-/-} \times RAG\text{-}2^{-/-}$ 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 $LT\text{-}\alpha^{-/-} \times RAG\text{-}2^{-/-}$ 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 naïve 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 naïve cells, into SCID mice [22]. Although almost all colitogenic LP $CD4^+$ T cells from colitic mice had the characteristics of $CD44^{high}CD62L^-CD45RB^{low}IL\text{-}7R\alpha^{high}$ 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 $S1P_1$ [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 naïve 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 $LT\text{-}\alpha^{-/-} \times RAG\text{-}2^{-/-}$ mice in this paper. Surprisingly, FTY720 treatment induces a severe PB lymphopenia in healthy SPX $LT\text{-}\alpha^{-/-}$ mice and colitogenic $CD4^+$ memory T-cell-transferred SPX $LT\text{-}\alpha^{-/-} \times RAG\text{-}2^{-/-}$ mice to a similar extent with the paired SPX $LT\text{-}\alpha^{+/+}$ mice and colitogenic $CD4^+$ memory T-cell-transferred SPX $LT\text{-}\alpha^{+/+} \times RAG\text{-}2^{-/-}$ 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 $LT\text{-}\alpha^{-/-} \times RAG\text{-}2^{-/-}$ 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 naïve 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 α4β7 and CCR9. Although such a stimulation

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up-regulated the expression of integrin α4β7 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-$\alpha^{-/-}$ × 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-$\alpha^{-/-}$ × 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-$\alpha^{+/+}$ × RAG-$2^{-/-}$ and LT-$\alpha^{-/-}$ × 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-7Rα (A7R34) and PE-conjugated anti-mouse Foxp3 (FJK-16S) mAb were obtained from eBioscience (San Diego, CA). The Fcγ (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 CD8α (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 α4β7 (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 Ca^{2+}, Mg^{2+}-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

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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 μL 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 400*g* 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: hunching 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 $\alpha4\beta7$ 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 $\alpha4\beta7$ 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: hunching 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 μm) 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].

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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 FcγR-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% CO_2 in 96 well plates (Costar, Cambridge, MA) pre-coated with 5 μg/mL hamster anti-mouse CD3ε mAb (145-2C11, BD PharMingen) and hamster 2 μg/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 μg/mL anti-CD3 and 2 μg/mL anti-CD28 mAb in the presence of 10 ng/mL hTGF- β1 (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 ± 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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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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Flagellin stimulation suppresses IL-7 secretion of intestinal epithelial cells

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ABSTRACT

IL-7 is a cytokine, which regulates development, maintenance and proliferation of T lymphocytes within the human immune system. Production of IL-7 is observed in a sterile environment such as thymus or bone marrow. However, it is also known that intestinal epithelial cells (IECs) residing in close contact with numerous bacterial stimuli also produce IL-7. Here we show that secretion of IL-7 by IECs is significantly suppressed upon stimulation by various bacterial components, including flagellin. Analysis of the intracellular mechanism by which flagellin regulates IL-7 production revealed that flagellin down-regulates expression of the two major transcripts encoding IL-7. Surprisingly, such function of flagellin was independent from the known transcriptional regulation of the IL-7 gene, as no significant change was observed in the transcriptional activity regulated by the previously identified promoter region. As the stability of IL-7 mRNA also remained unchanged upon flagellin stimulation, results suggested the possible involvement of a yet unknown transcriptional regulation of the IL-7 gene. These results describe a novel regulation of IL-7 production by bacterial stimuli, presumably mediated via Toll-like receptors. The present system might contribute to regulate the local lymphocyte pool, in response to the gut luminal or submucosal bacterial abundance.

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1. Introduction

IL-7 is a cytokine, well known for its non-redundant role upon development of T cells in the human immune system [1]. Recent studies have expanded its role to the maintenance and proliferation of peripheral T cells, including naïve T cells or memory T cells [2]. As one of the major roles of IL-7 is regulation of lymphoid precursor development, its production is observed in organs such as thymus or bone marrow, where immature lymphoid cells reside. However, in contrast to such central organs of the immune system, IL-7 is also produced in peripheral organs such as skin or intestine [3]. In such organs, IL-7 is produced by a distinct population of cells, such as stromal cells in the thymus, keratinocytes in the skin [4,5], and epithelial cells in the intestine [6]. Among these IL-7-producing cells, intestinal epithelial cells (IECs) reside in an extremely unusual environment, as it is continuously exposed to the bacterial stimuli arising from the commensal bacterial flora. The bacterial flora is well known to modulate various states of the host [7] and exhibit a central role in development of the host immune system. It regulates development and homeostasis of the gut lymphoid tissues [8,9], in which IL-7 is also known to play a crucial role [10–12]. However, the molecular mechanism by which the gut flora regulates IL-7 secretion has never been described.

Toll-like receptor (TLR) is one of the molecular system utilized to recognize pathogen-associated molecular patterns (PAMPs), including bacteria-associated fragments [13]. Its functional role has also been implicated in various gastrointestinal diseases, such as inflammatory bowel diseases [14] and colon cancer formation [15]. TLR consists of a number of receptors, which recognize specific PAMP as their ligand [13]. In mammals, 10 members have been identified so far, and all of them except TLR10 have been shown to be expressed within the human intestine [16]. Amongst them, TLR2, TLR4 and TLR5 are expressed by IECs, and are reported to play crucial roles in homeostasis of the intestinal mucosa [17]. In contrast to the low expression of TLR2 and TLR4, constitutive expression of TLR5 have been clearly detected at both apical and basolateral surface of human colonic IECs [18,19]. TLR5 is a receptor which recognize flagellin, a component of bacterial flagella, derived from species such as *Salmonella* [19]. Stimulation of TLR5 by flagellin initiates downstream molecular signaling via the adaptor molecule MyD88, and results in pro-inflammatory responses such as secretion of IL-8 or CCL20 [9,20]. However, involvement of flagellin in IL-7 secretion has never been described.

Herein, we show that secretion of IL-7 by human IECs is significantly down-regulated upon stimulation by TLR ligands, including flagellin. Flagellin appeared to down-regulate expression of the

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two major IL-7 transcripts expressed in IECs. The present effect of flagellin appeared to be independent from the previously identified transcriptional regulation or mRNA stability, suggesting an existence of a novel molecular pathway linking TLR signaling and IL-7 expression. Thus the present study shows an unexpected role of flagellin in regulation of IL-7 secretion, presumably leading to regulation of the development or survival of lymphoid cells within the intestinal mucosa.

2. Materials and methods

2.1. Cell culture and reagents

Human colon carcinoma-derived DLD-1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin, as previously described [21]. For analysis of RNA stability, cells were cultured in medium additionally supplemented with either actinomycin D (5 μg/ml, Calbiochem Biochemicals, San Diego, CA, USA) or cycloheximide (20 μg/ml, Sigma–Aldrich, Japan).

2.2. Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded onto 60-mm culture dishes at a density of 3.5×10^4 cells per cm^2. Subsequently, cells were cultured for 12 h in culture medium alone, or medium supplemented with either recombinant human IFN-γ, recombinant human IL-1β (each purchased from Peprotech, London, UK), lipoteichoic acid (LTA) from *Staphylococcus aureus*, lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4strain, Poly I:C, ODN2006 (each purchased from InvivoGen, San Diego, CA, USA), or flagellin from *Salmonella muenchen* (Calbiochem Biochemicals). The protein levels of IL-7 or IL-8 in the culture supernatants were determined by human IL-7 ELISA kit (R&D Systems, Minneapolis, MN, USA) or by human IL-8 ELISA Kit II (BD Biosciences, Tokyo, Japan), respectively.

2.3. Quantitative reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was isolated using RNA-Bee (AMS biotechnology, Oxon, UK) according to the manufacturer's instructions. Aliquots of 1 μg of total RNA were used per 20 μl of reaction volume for cDNA synthesis by QuantiTect® reverse transcription kit (QIAGEN, Tokyo, Japan). Real-time PCR was performed using the ABI Prism 7500 (Applied Biosystems, Darmstadt, Germany). One microliter of the RT product was used as a template for each 25 μl PCR using SYBR Green I dye (QIAGEN) under the following amplification conditions; 50 °C for 2 min and 95 °C for 10 min, followed by 94 °C for 15 s, 58 °C for 30 s and 72 °C for 40 s repeated for 45 cycles. Primers used were as follows: IL-7 (primer set 1): 5′-ATTGAACCT GCAGACCAAGC-3′ (sense), and 5′-GCAACAGAACAAGGATCAGG-3′ (anti-sense); IL-7 (primer set 2): 5′-GAGTGTTCTAATGGTCAGCA-3′ (sense), and 5′-CAGTATTGTTGTGCCTTCTG-3′ (anti-sense); IL-8: 5′-GACATACTCCAAACCTTTCCAC-3′ (sense), 5′-TTATGAATTCTCAGC CCTCTTC-3′ (anti-sense); β-actin: 5′-GGATGCAGAAGGAGATCAC TG-3′ (sense), 5′-CGATCCACACGGAGTACTTG-3′ (anti-sense). The results of quantitative RT-PCR were normalized by the expression level of β-actin within each sample.

2.4. Northern blot

Poly (A)$^+$ mRNA was isolated using a FastTrack 2.0 kit (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Five micrograms of Poly (A)$^+$ RNA was subjected to 1% agarose/formaldehyde gel electrophoresis and subsequently transferred onto a Hybond-N nylon membrane filter (GE Healthcare Biosciences, Tokyo, Japan). The cDNA probe corresponding to mRNA sequences of IL-7 or G3PDH were generated from total RNA extract of DLD-1 cells by RT-PCR, using the following primers: IL-7: 5′-AGCTTGCTCCTGCTCCAGTT-3′ (sense), and 5′-TGCATTTCTC AAATGCCCTAATCCG-3′ (anti-sense); G3PDH: 5′-TGAAGGTCGGA GTCAACGGATTTGGT-3′ (sense), 5′-CATGTGGGCCATGAGGTCCACC AC-3′ (anti-sense). Probes were labeled with [a-^{32}P] dCTP by RediPrime II (GE Healthcare Biosciences) according to the manufacturer's instructions. Hybridization was carried out at 42 °C overnight.

2.5. Plasmids

The reporter plasmids containing various length of the human IL-7 gene promoter region have been previously described [21]. For the present study, we subcloned the corresponding sequences into BglII–SacI site of pGL4.10(luc2) vector (Promega, Madison, WI, USA). For construction of IL-7-3′-UTR-Luc, the sequence corresponding to the entire 3′UTR of human IL-7 mRNA was inserted into XbaI site of the pGL3-Control vector (Promega).

2.6. Transient transfection and reporter assays

DLD-1 cells were seeded onto a 60-mm culture dish, and transfected with 3 μg of reporter plasmid along with 10 ng of pGL4.74(hRluc/TK) plasmid (Promega) as previously described [21]. Cells were harvested 6–12 h after transfection, lysed by three cycles of freezing and thawing, and then luciferase activities were measured by a luminometer, each in triplicate (Turner Designs, Sunnyvale, CA, USA). Luciferase activities are shown as arbitrary units normalized by Renilla luciferase activities of each sample.

2.7. Electrophoretic mobility shift assays (EMSA)

Preparation of nuclear extracts and EMSA were performed as previously described [21]. Ten micrograms of protein from each sample was subjected to EMSA. In supershift experiments, antibodies for either IRF-1 (sc-497, Santa Cruz Biotechnology, CA, USA) or IRF-2 (sc-498, Santa Cruz Biotechnology) were used as previously described [21].

2.8. Immunoblot

Immunoblot was performed as previously described [22]. Briefly, 20 μg of nuclear protein extract was separated in 12% SDS–polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes. Primary antibodies used were as follows: anti-IRF-1 (1:1000, sc-497, Santa Cruz biotechnology), anti-IRF-2 (1:1000, sc-498, Santa Cruz biotechnology), anti-upstream factor (USF)-2 (1:1000, sc-861, Santa Cruz biotechnology). Primary antibodies were detected by the appropriate HRP-conjugated secondary antibodies, and visualized by ECL detection system (GE Healthcare Biosciences), according to the manufacturer's instructions.

3. Results

3.1. TLR ligands down-regulate IL-7 secretion by IECs

To elucidate the possible relation between the gut bacterial flora and IL-7 secretion in IECs, we first examined whether any bacterial component can function as TLR ligand, and initiate downstream signals of TLRs in IECs. Addition of various TLR ligands to a human colonic epithelial cell line, DLD-1, revealed that lipotei-