choic acid (LTA), PolyI:C and flagellin could significantly up-regulate IL-8 secretion (Fig. 1A). The result that lipopolysaccharide (LPS) and oligodeoxynucleotide (ODN) stimulation showed only a minimal response appeared to be due to the expression level of the corresponding receptor, as our PCR analysis of TLR expression showed only extremely low level of TLR4 and TLR9 in DLD-1 cells (data not shown). Under these conditions, we examined whether stimulation of TLRs may have effect on IL-7 secretion by DLD-1 cells. Surprisingly, ligands that were able to significantly up-regulate IL-8 secretion also significantly down-regulated IL-7 secretion, whereas ligands that were not able to up-regulate IL-8 secretion had no effect on IL-7 secretion (Fig. 1B). These results suggested that stimulation by TLR ligands, including flagellin, down-regulates secretion of IL-7 by IECs, presumably by a signaling downstream of TLRs.

3.2. Flagellin antagonizes up-regulation of IL-7 secretion induced by IFN-γ

According to previous reports describing the molecular regulation of IL-7 secretion, exposure of cells to IFN-γ represents the extracellular stimuli which could up-regulate IL-7 secretion [21,23]. Thus we next examined whether TLR ligands could modulate not only the basal secretion, but also the induced secretion of IL-7. For this purpose, we focused on flagellin, as it induced the most significant change in both IL-8 and IL-7 production, upon addition to DLD-1 cells (Fig. 1). Addition of flagellin to DLD-1 cells at various concentrations confirmed that both up-regulation of IL-8 secretion (Fig. 2A) and down-regulation of IL-7 secretion (Fig. 2B) could be induced in a dose-dependent manner. Also, stimulation by IFN-γ showed marked induction of IL-7 secretion, but had no effect on IL-8 secretion. However, when flagellin was added before IFN-γ stimulation, marked suppression of IL-7 induction was observed also in a dose-dependent manner (Fig. 2B). These results showed that flagellin stimulation on IECs could not only suppress basal IL-7 secretion, but also suppress induction of IL-7 secretion by IFN-γ, suggesting a possible interaction between the TLR pathway and IFN-γ pathway.

3.3. Flagellin down-regulates IL-7 mRNA expression in IECs

As we found that flagellin stimulation could down-regulate IL-7 secretion in DLD-1 cells, we sought to examine at which level IL-7 expression is suppressed. For this purpose, we examined whether mRNA expression of IL-7 could be suppressed by flagellin. Consistent with the former results, expression of IL-8 mRNA was significantly up-regulated by flagellin, suggesting activation of TLR5 (Fig. 3A). Conversely, expression of IL-7 mRNA was significantly down-regulated by flagellin (Fig. 3B). Such effect of flagellin was also observed upon co-stimulation by IFN-γ. These results indicated that flagellin stimulation on IECs could down-

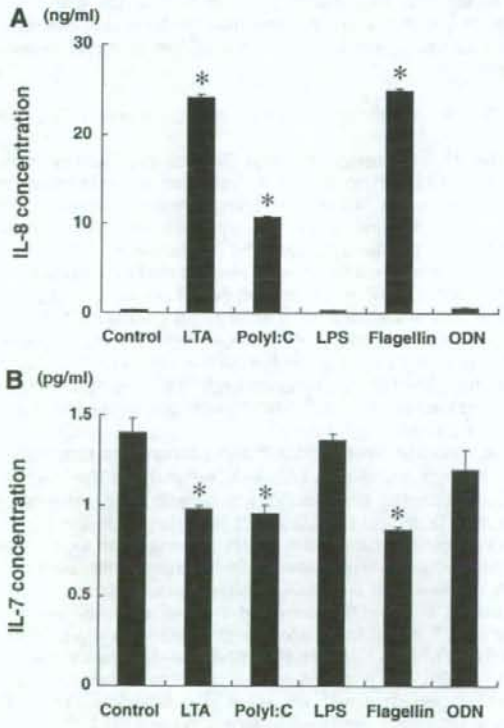

Fig. 1. TLR ligands up-regulate secretion of IL-8, but down-regulate secretion of IL-7 by IECs. DLD-1 cells were cultured either with LTA (10 μg/ml), PolyI:C (100 μg/ml), LPS (10 μg/ml), flagellin (10 ng/ml), or ODN2006 (5 μg/ml). After 12 h of stimulation, secretion of IL-8 (A) and IL-7 (B) was measured by ELISA. Cells cultured without any ligand stimulation served as control. Error bars represent SEM. * indicates $P < 0.05$ judged by paired Student's t-test.

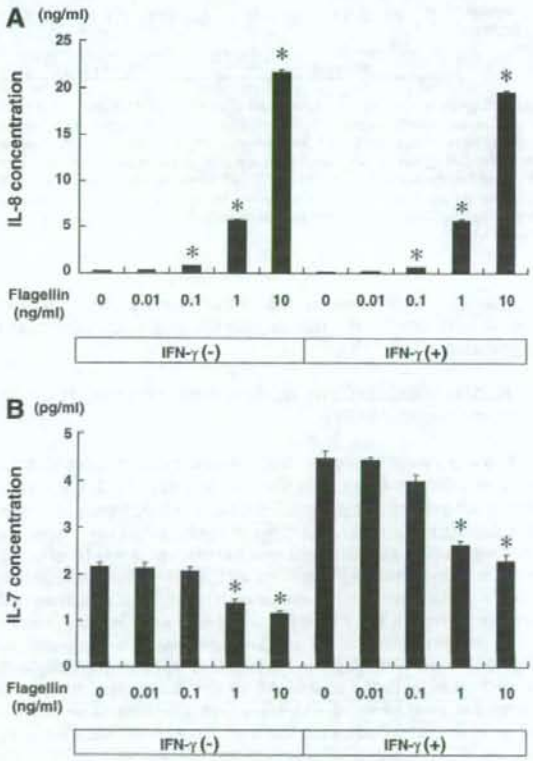

Fig. 2. Flagellin suppresses both basal and induced IL-7 secretion by IECs, in a dose-dependent manner. DLD-1 cells were cultured with or without IFN-γ (50 ng/ml), and additionally stimulated by various concentrations of flagellin. After 12 h of stimulation, secretion of IL-8 (A) and IL-7 (B) was measured by ELISA. Error bars represent SEM. * indicates $P < 0.05$ judged by paired Student's t-test.

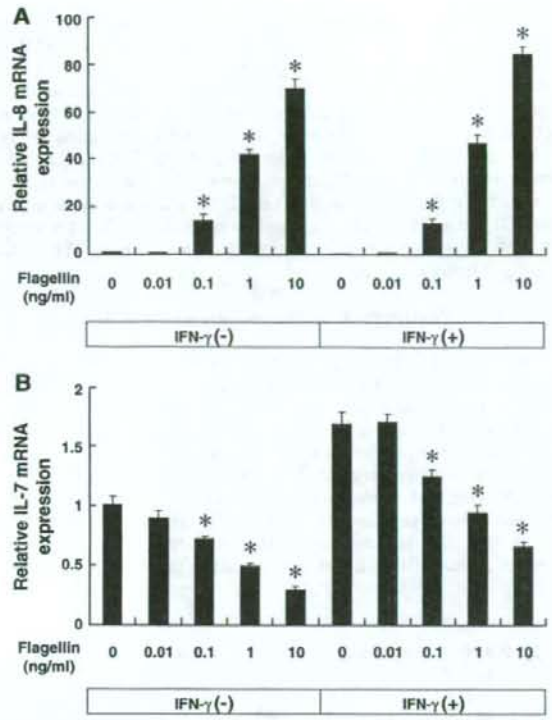

Fig. 3. Flagellin suppresses both basal and induced IL-7 mRNA expression by IECs, in a dose-dependent manner. DLD-1 cells were pre-incubated with various concentrations of flagellin for 2 h and subsequently co-stimulated with flagellin and IFN-γ (50 ng/ml) for another 3 h. At the end of the stimulation, cells were subjected for analysis of IL-8 (A) and IL-7 (B) mRNA expression by quantitative RT-PCR. Data are presented as relative expression level normalized by the expression level of β-actin. Error bars represent SEM. * indicates $P < 0.05$ judged by paired Student's *t*-test.

regulate IL-7 expression at the mRNA level, possibly through mechanisms such as transcriptional repression or mRNA degradation.

3.4. Flagellin stimulation down-regulates two distinct transcripts of IL-7 that are expressed in IECs

In our previous study, we have revealed that human IECs express two distinct transcripts from the IL-7 gene [21]. These transcripts are generated by the difference in transcription start site; both encode mature IL-7, but differ in the length of the 5′ untranslated region (Fig. 4A). To reveal which transcript is regulated by flagellin, we analyzed the expression of IL-7 mRNA by northern blot, using a probe covering the entire coding region. Consistent with our former report, the 1.8 kb transcript was induced, whereas the 2.4 kb transcript remained unchanged upon IFN-γ stimulation (Fig. 4B). However, flagellin stimulation appeared to suppress expression of both 2.4 kb and 1.8 kb transcript (Fig. 4B). As the expression level of the 2.4 kb transcript appeared to be too low for definite evaluation by Northern blot, we confirmed its expression by RT-PCR, using a transcript specific primer (Fig. 4C). The RT-PCR analysis not only confirmed down-regulation of total IL-7 mRNA expression (Fig. 4C, right), but also showed that the basally expressed 2.4 kb transcript is indeed down-regulated by flagellin (Fig. 4C, left).

3.5. Flagellin stimulation down-regulates IL-7 expression via a distinct pathway independent of IRF-E-dependent transcription

We have previously shown that the two transcripts of IL-7 are both regulated at the transcriptional level by the members of the IRF family, IRF-1 and IRF-2 [21]. According to the study, expression of the basal 2.4 kb transcript is dependent on IRF-2, whereas expression of the inducible 1.8 kb transcript is dependent on IRF-1. Such observations strongly prompted us to examine whether flagellin stimulation might down-regulate the transcriptional activity that is dependent on IRF-1 or IRF-2. First, we tested whether flagellin could change the nuclear distribution of IRF-1 or IRF-2 protein. However, immunoblot analysis using nuclear extracts of DLD-1 cells revealed no significant change in distribution of both IRF-1 and IRF-2 proteins (Fig. 5A). Furthermore, electrophoretic mobility shift assay (EMSA) using a probe containing interferon regulatory factor element (IRF-E) of IL-7 gene origin (locating at −268 to −258 of the promoter region, with respect to the translation start site) showed no distinct change in nuclear distribution of IRF-E-binding proteins by flagellin (Fig. 5B). To examine whether flagellin could suppress transcription of the human IL-7 gene through other *cis*-acting elements of the promoter region, we performed a reporter assay using plasmids containing various length of the previously identified promoter region, extending up to 3194 bps upstream from the translation start site (Fig. 5C). Surprisingly, results showed that flagellin stimulation has no significant effect upon transcriptional activity of the IL-7 gene that is regulated by the identified promoter region. These results suggested that flagellin stimulation suppresses IL-7 expression at the mRNA level, through a distinct pathway other than the previously determined transcriptional regulation via IRF-E of the proximal promoter region.

3.6. Flagellin stimulation does not promote degradation of IL-7 mRNA

Former results suggested that flagellin might down-regulate IL-7 mRNA expression through a mechanism other than transcriptional repression. There are several reports suggesting that cytokine expression could be regulated by mRNA stability [24,25]. The p38 mitogen-activated protein kinase (MAPK) pathway and adenylate/uridylate-rich element (ARE)-mediated regulation of mRNA stability has been implicated in such gene regulation [26]. Our database search revealed a group V cluster of ARE within the 3′-UTR of the human IL-7 mRNA, along with group II cluster of ARE within the 3′-UTR of the human IL-8 mRNA [27]. As flagellin activates p38-MAPK pathway through TLR5, we next examined whether the stability of IL-7 mRNA might be regulated by flagellin through its ARE.

To elucidate whether flagellin down-regulates expression of IL-7 through degradation of mRNA, we analyzed the kinetics of IL-7 mRNA under the effect of actinomycin D or cycloheximide (Fig. 6A). Treatment of DLD-1 cells by actinomycin D completely blocked up-regulation of IL-8 mRNA in response to flagellin, suggesting successful termination of transcription. Under such condition, expression of IL-7 mRNA decreased over time up to 5 h of treatment, but no difference could be observed in the decreasing rate of IL-7 mRNA upon addition of flagellin. In sharp contrast, treatment of DLD-1 cells by cycloheximide significantly enhanced and stabilized IL-8 mRNA expression in response to flagellin, which is consistent with the effect of cycloheximide upon ARE-dependent mRNA described in former reports [28,29]. However, such treatment had no effect to the basal expression of IL-7 mRNA without flagellin, and also to the decrease of IL-7 mRNA in response to flagellin stimulation. These results collectively suggested that the decrease of IL-7 mRNA upon flagellin stimulation is predominantly mediated by transcriptional repression, rather

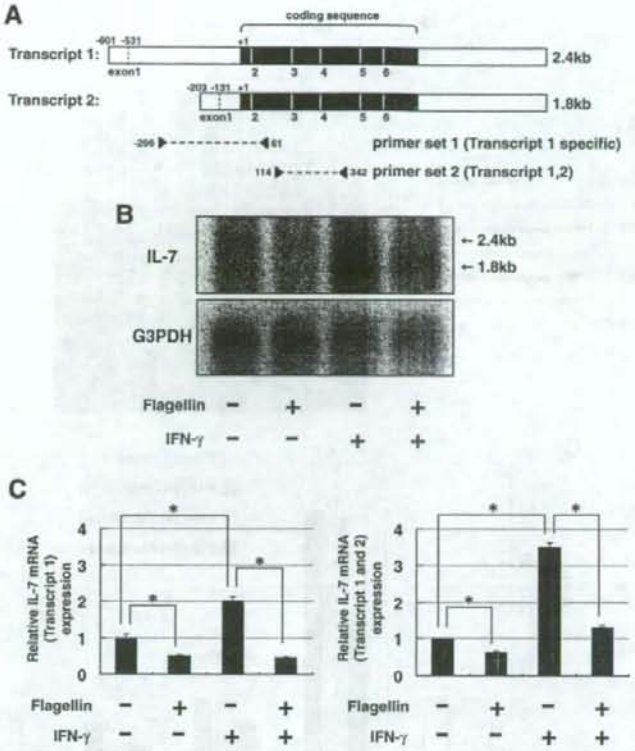

Fig. 4. Flagellin down-regulates expression of the two distinct transcripts encoding IL-7. (A) Schematic presentation of the two major transcripts of the human IL-7 gene, which are expressed in IECs. The nucleotide number is designated with respect to the translation start site (+1). The location of the primers used in RT-PCR assay of the present study is also shown. Primer set 1 was used in the RT-PCR assay shown in (C), whereas primer set 2 was used in the RT-PCR assay shown in Figs. 3 and 4C. (B) DLD-1 cells were stimulated by IFN-γ (50 ng/ml) or flagellin (10 ng/ml), and subjected to Northern blot to analyze expression of the IL-7 transcripts. The upper panel shows expression of 2.4 kb (Transcript 1 in A) and 1.8 kb (Transcript 2 in A) transcripts of the IL-7 gene, whereas the lower panel indicates equal expression of G3PDH serving as a control to confirm equal loading of the extracted mRNA. (C) DLD-1 cells were stimulated by IFN-γ (50 ng/ml) or flagellin (10 ng/ml) for 3 h and subjected for quantitative RT-PCR analysis using primer set 1 (transcript 1 specific) or primer set 2 (detecting both transcript 1 and 2) shown in (A). Error bars represent SEM. * indicates $P < 0.05$ judged by paired Student's *t*-test.

than mRNA degradation. To further confirm that the ARE existing in the 3′UTR of the IL-7 mRNA is not involved in its decrease upon flagellin stimulation, we constructed a luciferase reporter plasmid, which could express luciferase mRNA that is 3′ flanked with the 3′UTR of IL-7 mRNA (IL-7-3′-UTR-Luc, Fig. 6B). Reporter assays using such reporter plasmid showed that although the activity itself was slightly lower compared to Control-Luc, no difference in activity could be observed upon flagellin stimulation (Fig. 6C). Thus, these results confirmed that the ARE existing in the 3′UTR of the IL-7 mRNA is not involved in down-regulation of IL-7 mRNA by flagellin.

4. Discussion

In the present study, we have shown that stimulation of the bacterial components could down-regulate IL-7 secretion by IECs through suppression of mRNA expression, but via a distinct pathway other than the known transcriptional regulation or mRNA degradation. These results suggest a novel link between the bacterial stimuli and IL-7 secretion within IECs, and provide evidences that the bacterial load within the gut lumen might modulate secretion of IL-7, thereby contributing to modulate the local immune response mediated by T lymphocytes.

In organs such as thymus or bone marrow, IL-7 is said to be constitutively secreted and is rarely modulated by extrinsic stimuli. Thus consumption rather than production had been featured as the main mechanism regulating the local amount of IL-7 [30]. However, it has been shown that several cytokines such as IL-1β, TNF-α, and IFN-γ up-regulate IL-7 secretion, whereas TGF-β conversely down-regulates IL-7 secretion of cells such as stromal cells, osteoblasts, or IECs [21,31–33]. Thus our results add a novel category of extrinsic stimulus that can modulate IL-7 secretion. Moreover, as we have previously shown that TGF-β does not change IL-7 secretion of IECs, the present finding show for the first time, a stimulus that could down-regulate IL-7 secretion of IECs.

We also showed that flagellin down-regulates expression of the two distinct transcripts arising from the IL-7 gene, both of which could give rise to mature IL-7 protein. Our previous analyses have shown that these transcripts are either constitutively expressed by an IRF2-dependent manner or inducibly expressed by an IRF1-dependent manner [21]. However, the present analysis clearly showed that flagellin has no effect on both IRF-1 and IRF-2-dependent transcription, which regulates IL-7 gene expression through the identified IRF-E, locating at −268 to −258 of the promotor region. Thus, we looked for another possibility, whether flagellin might promote degradation of the IL-7 mRNA. However, our results

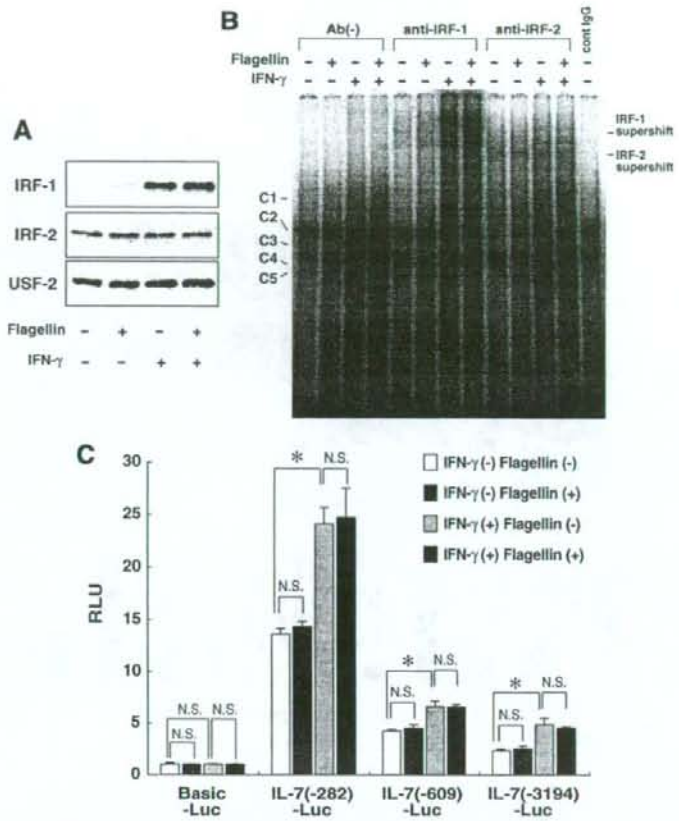

Fig. 5. Flagellin suppresses IL-7 expression through a pathway independent from IRF-mediated transcription. (A) DLD-1 cells were cultured with or without IFN-γ (50 ng/ml) or flagellin (10 ng/ml) for 3 h, and subjected to nuclear protein extraction. Twenty micrograms of each nuclear protein extract was analyzed by immunoblot. Expression of USF-2 shows equal loading of the nuclear protein extracts. (B) Ten micrograms of each nuclear extracts collected in (A) were further analyzed by EMSA using a ^{32}P-labeled oligonucleotide probe containing the IRF-E found at −268 to −258 (with respect to the translation start site) of the promotor region of the human IL-7 gene. Supershift assays shown in lanes 5–13 were performed by pre-incubating the nuclear protein extracts with antibodies for IRF-1, IRF-2, or mouse IgG (The left end lane is designated as lane 1). A total of five protein–DNA complexes were observed (designated as C1–C5). Supershift assay using anti-IRF-1 antibody confirmed that C3 represents the complex between IRF-1 and IRF-E probe, whereas the assay using anti-IRF-2 antibody confirmed that C2 represents the complex between IRF-2 and IRF-E probe. (C) DLD-1 cells were transiently transfected with luciferase reporter plasmids containing various length (from −3 to −282, −609 and −3194, with respect to the translation start site) of the promoter region of human IL-7 gene (IL-7-Luc), or a control reporter plasmid (Basic-Luc), and cultured with or without IFN-γ (50 ng/ml) or flagellin (10 ng/ml) for 6 h. Luciferase activity was examined at the end of the culture. Data were normalized by Renilla luciferase activity, and are shown as relative light units (RLU). Error bars represent SEM. * indicates $P < 0.05$, whereas N.S. indicates not significant, judged by paired Student's *t*-test.

showed that the major mechanism that down-regulates IL-7 mRNA expression upon flagellin stimulation is transcriptional repression, rather than mRNA degradation. From these collective findings, we suggest that the present effect of flagellin upon IL-7 expression is possibly mediated through transcriptional repression via a yet unknown promoter region. In our previous study, we have searched for the *cis*-acting elements within the 5′ flanking region of the human IL-7 gene extending up to 3194 bp from the translation start site, and found IRF-E as the only candidate [21]. However, a recent study of the IL-6 promoter using bioinformatic analysis has identified a novel *cis*-regulatory element at 5 kb upstream of the transcription start site [34]. Thus, a study searching for much distal regions of the IL-7 gene might identify potential *cis*-elements regulated by downstream signals of flagellin, presumably via TLR5. However, these issue remains to be determined. Also, our results showed that cycloheximide treatment has no effect upon present function of flagellin (Fig. 6A). As cycloheximide blocks protein synthesis, the results indicate that decrease of IL-7 mRNA is possibly a direct effect of flagellin, but not via a secondary signal that requires de novo protein synthesis.

Through our series of studies regarding the role of IL-7 in colitis, we have shown that IL-7 is required for the persistence of colonic inflammation [35–38], but at the same time, local production of IL-7 is decreased in the inflamed colonic mucosa [35]. The present observation may provide a possible mechanism by which local production of IL-7 by IECs is suppressed in the inflamed colonic mucosa, where IECs are exposed to increased load of bacterial components. Such response of IECs might contribute to regulate the local T-cell-mediated immune response against bacterial components, in concert with the innate immune response mediated by IL-8 or CCR20, but its precise role in the development and persistence of colitis also remains to be elucidated.

In conclusion, we have found a novel function of flagellin to down-regulate mRNA expression and secretion of IL-7 by IECs. The present results raise possible regulation of the local lymphocyte pool by the gut bacterial load, via control of IL-7 secretion.

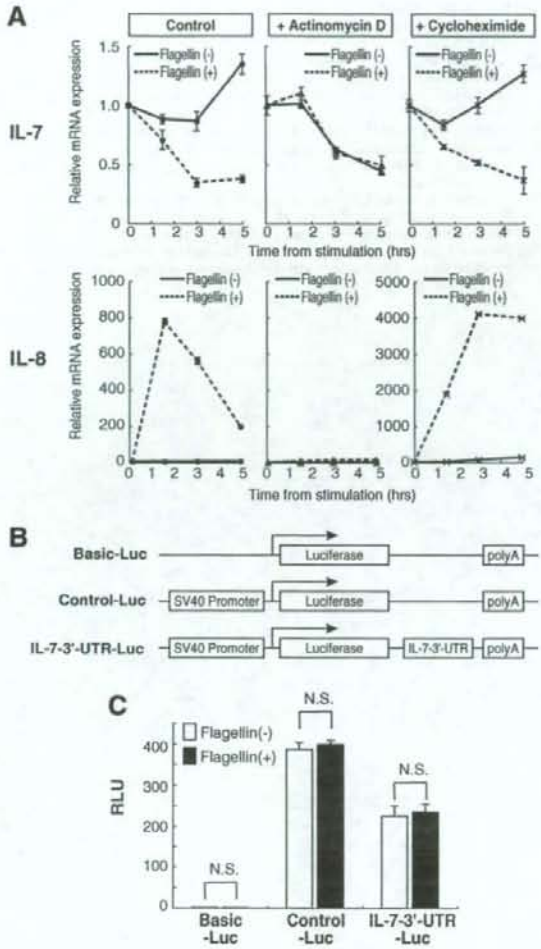

Fig. 6. Flagellin does not promote degradation of IL-7 mRNA. (A) DLD-1 cells were stimulated by flagellin (10 ng/ml) under the effect of actinomycin D (5 μg/ml) or cycloheximide (20 μg/ml), and subjected for analysis of IL-7 and IL-8 mRNA expression at various time points (0, 1.5, 3 and 5 h from flagellin addition) by quantitative RT-PCR. Actinomycin D or cycloheximide treatment was started at the time of flagellin addition. Cells cultured without treatment by either actinomycin D or cycloheximide served as control. Data are presented as relative expression level normalized by the expression level of β-actin. Expression level at 0 h is set to 1. Error bars represent SEM. (B) Schematic presentation of the reporter plasmids used in (C). "Basic-Luc" lacks the SV40 promoter element upstream the luciferase gene, which is present in "Control-Luc". "IL-7-3'-UTR-Luc" expresses luciferase mRNA that is 3' flanked by the 3'UTR sequence of the IL-7 mRNA, under the control of SV40 promoter. (C) DLD-1 cells were transfected with reporter plasmids shown in (B), and cultured for 6 h with or without flagellin (10 ng/ml). At the end of the culture, cells were subjected to analysis of luciferase activity. All the data were normalized by Renilla luciferase activity, and shown as relative light units (RLU). Error bars represent SEM. N.S. indicates not significant, judged by paired Student's *t*-test.

Acknowledgments

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Colitogenic CD4$^+$ Effector-memory T Cells Actively Recirculate in Chronic Colitic Mice

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Background: Although the clinical usefulness of leukocytapheresis for patients with inflammatory bowel disease (IBD) has been reported as a selective removal therapy targeting pathogenic immune cells in blood circulation, it remains unclear whether colitogenic CD4$^+$ T cells continuously recirculate in peripheral blood during the chronic phase of colitis.

Methods: To resolve this question we conducted a series of in vivo experiments using a murine chronic colitis model induced by adoptive transfer of CD4$^+$CD45RB^{high} cells into SCID mice in combination with a parabiosis system.

Results: In colitic SCID recipients, first, almost all CD4$^+$ CD45RB^{high} donor cells were converted to CD4$^+$CD44^{high}CD62L IL-7Rα^{high} effector-memory T (T_{EM}) cells at 8 weeks after transfer and were distributed throughout the whole body, including colonic lamina propria, mesenteric lymph nodes, thoracic duct, peripheral blood, spleen, and bone marrow. Second, SCID mice retransferred with the colitic peripheral blood CD4$^+$ T cells developed colitis that is identical to the original colitis. Third, CD4$^+$ cells in parabionts between established colitic RAG-2$^{-/-}$ mice induced by adoptive transfer of Ly5.1$^+$ or Ly5.2$^+$ CD4$^+$CD45RB^{high} T cells were well mixed in almost equal proportions at various sites 2 weeks after parabiosis surgery, and the redistribution of Ly5.1$^+$ and Ly5.2$^+$ CD4$^+$ T cells was significantly suppressed in FTY720-treated parabionts.

Conclusions: Together, these findings indicate that colitogenic CD4$^+$ T_{EM} cells continuously recirculate in established colitic mice, suggesting that therapeutic approaches targeting systemic CD4$^+$ T_{EM} cells, such as bone marrow transplantation, rather than those targeting only intestinal CD4$^+$ T cells, may be feasible for the treatment of IBD.

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Key Words: colitogenic memory T cells, FTY720, parabiosis, colitis, recirculation

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), are characterized by a wasting disease with chronic intestinal inflammation.[1–6] Importantly, it is well recognized that surgery never cures CD, as relapses are the rule after remissions,[7] and extraintestinal disorders often occur in UC patients even after total colectomy,[8] suggesting that IBD may not be a circumscribed disease, but rather a systemic disease that colitogenic memory lymphocytes, which might memorize the disease prototype, distribute throughout the body via the bloodstream as if they were 'benign leukemia cells,' and might hide in a reservoir other than the inflamed intestine. Consistent with this hypothesis, recent findings of the usefulness of leukocytapheresis for IBD patients[9–11] suggest that the recirculation of colitogenic memory lymphocytes between the gut and some reservoir may play a role in the perpetuation of IBD. Furthermore, we have recently demonstrated that FTY720, which is able to inhibit the circulation of lymphocytes,[12–14] prevents the development of SCID colitis induced by adoptive transfer of lamina propria (LP) colitogenic CD4$^+$ effector-memory T (T_{EM}) cells and suppresses IFN-γ, IL-2, and TNF-α production by LP CD4$^+$ T cells.[15] The findings reported in the literature just cited suggest that the hemodynamics of colitogenic CD4$^+$ T_{EM} cells in IBD is sufficiently active to cause colitogenic CD4$^+$ T_{EM} cells to circulate continuously in the peripheral blood.

Egress of immune cells from nonlymphoid tissues is an important step in lymphocyte migration as well as lympho-

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cyte homing to nonlymphoid tissues.[12] Draining lymphatics of tissues contains substantial numbers of lymphocytes, some of which are cells with memory phenotype.[16] Early studies demonstrated that sheep thoracic duct lymph (TDL), which drains from the intestine and empties into the blood, contains many lymphocytes,[16] although the exact source and fate of this population are of considerable controversy. Furthermore, in a landmark experiment Gowans and Knight[17] demonstrated that labeled lymphocytes in TDL that were intravenously transferred into syngenic recipients were detected in their TDL again. This indicates that lymphocytes recirculate continuously between blood and lymph. In the intestine, however, it is also thought that the altered phenotype of memory cells in intestinal LP and the lack of such cells elsewhere suggest that the memory T cells do not exit the tissue.[18,19]

Based on such complex backgrounds we conducted a series of adoptive transfer experiments in combination with parabiosis using mice to assess the hemodynamics of colitogenic $CD4^+$ T_{EM} cells in chronic colitis.

MATERIALS AND METHODS

Animals

BALB/c, C.B-17 SCID, and C57BL/6-Ly5.2 mice were purchased from Japan Clea (Tokyo, Japan). C57BL/6-Ly5.1 and C57BL/6-Ly5.2-RAG-2 deficient ($RAG\text{-}2^{-/-}$) mice were obtained from Taconic Farms (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). Mice were maintained under specific pathogen-free (SPF) conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 weeks of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Antibodies

The following mAbs other than biotin-conjugated antimouse IL-7Rα (A7R34; eBioscience, San Diego, CA) were obtained from BD PharMingen (San Diego, CA) for purification of cell populations and flow cytometry analysis: 145-2C11, FITC-conjugated antimouse CD3; RM4-5, PE-conjugated antimouse CD4; C363.16A, FITC-conjugated antimouse CD45RB; 104, FITC-conjugated antimouse Ly5.1 (CD45.1); A20, FITC-conjugated antimouse Ly5.2 (CD45.2); IM7, Allophycocyanine-conjugated antimouse CD44; MEL-14, PE-conjugated antimouse CD62L; H1.2F3, PE-conjugated antimouse CD69; DATK32, PE-conjugated antimouse $\alpha4\beta7$. Biotinylated antibodies were detected with PE-streptavidin (BD PharMingen).

In Vivo Adoptive Transfer Experiments

We performed a series of in vivo experiments to assess the role of circulating colitogenic $CD4^+$ effector-memory T (T_{EM}) cells in the perpetuation of chronic colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells. For adoptive transfer, $CD4^+$ T cells were first isolated from SP cells of C57BL/6-Ly5.2 mice using the anti-CD4 (L3T4)-MACS system (Miltenyi Biotec) according to the manufacturer's instruction. Enriched $CD4^+$ T cells (96-97% pure, as estimated by FACSCalibur (BD Biosciences)) were then labeled with PE-conjugated antimouse CD4 (RM4-5; BD Pharmingen) and FITC-conjugated antimouse (16A; BD Pharmingen). $CD4^+$ $CD45RB^{high}$ cells were purified using a FACSAria (BD Biosciences). This population was 98.0% pure on reanalysis. Experiment 1: The distribution of $CD4^+$ T cells in LP, mesenteric lymph nodes (MLN), thoracic duct (TD), peripheral blood (PB), spleen (SP), and bone marrow (BM) after the establishment of colitis at 8 weeks after transfer was first assessed using flow cytometry. Experiment 2: To assess whether circulating PB $CD4^+$ T cells in established colitic SCID mice at the late stage of colitis are colitogenic, new SCID mice were transferred with colitic PB or LP $CD4^+$ T cells (3×10^5) obtained from the established colitic SCID mice. Mice were monitored during the course and sacrificed at 6 weeks after transfer. Experiment 3: To assess the hemodynamics of colitogenic $CD4^+$ T_{EM} cells in established colitic mice, an adoptive transfer experiment was performed in combination with a parabiosis system[20] between established $RAG\text{-}2^{-/-}$ mice transferred with C57BL/6-Ly5.1- or C57BL/6-Ly5.2-$CD4^+CD45RB^{high}$ T cells at 8 weeks after transfer. Briefly, sex-matched colitic $RAG\text{-}2^{-/-}$ mice were anesthetized prior to surgery and incisions were made in the skin on the opposing flanks of the donor and recipient animals. Surgical sutures were used to bring the body walls of the 2 mice into direct physical contact. The outer skin was then attached with surgical staples. In another set of experiments, colitic parabionts were treated with FTY720 (1.0 mg/kg) or phosphate-buffered saline (PBS) daily over a period of 4 weeks starting 1 day before parabiosis surgery and were monitored for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool. Mice were sacrificed 4 weeks after surgery and assessed for a clinical score as the sum of 4 parameters: 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).[21]

T-cell Preparation

For the preparation of TD cells, mice were fed 500 μL of olive oil by gavage. Then, 45 minutes later, mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg, Alexis Biochemicals, San Diego, CA) and xylazine (10

mg/kg, Sigma-Aldrich, St. Louis, MO) and subjected to laparotomy. A heparinized PE-10 polyethylene catheter (Natsume Seisakusho, Japan) was inserted into the cysterna chili and lymph was collected for 30 minutes. For isolation of PB, 600 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 minutes at room temperature. The lymphocytes were then isolated from the plasma-Ficoll interface. SP and MLN were mechanically disrupted into single-cell suspensions. BM was collected from the femur by flushing with sterile PBS. For the preparation of colonic LP cells, the colon was first flushed extensively to eliminate the lumen content, then longitudinally opened and cut into small pieces. The dissected mucosa was incubated with Ca^{++} Mg^{++}-free Hanks' BSS containing 1 mM DTT (Sigma-Aldrich) for 30 minutes to remove mucus, then treated with 3 mg/mL collagenase (Roche, Nutley, NJ) and 0.01% DNase (Worthington Biochemical, Freehold, NJ) for 2 hours. After filtering through gauze, cells were pelleted twice through a 40% isotonic Percoll solution, and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched $CD4^+$ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells contained >94% $CD4^+$ cells when analyzed by FACS Calibur ((BD Biosciences).

Histological Examination and Immunohistology

Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin (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.[22]

Flow Cytometry

To detect the surface expression of a variety of molecules, isolated splenocytes, MLN, PB, TD, PC, BM, or LP mononuclear cells (LPMCs) were preincubated with an FcγR-blocking mAb (CD16/32; 2.4G2, BD PharMingen) for 15 minutes, then incubated with specific FITC-, PE-, PerCP-, Allophycocyanine-, or biotin-labeled antibodies for 20 minutes on ice. Biotinylated antibodies were detected with PE-streptavidin. Standard 3- or 4-color flow cytometric analyses were obtained using the FACS Calibur with CellQuest software. Background fluorescence was assessed by staining with control-irrelevant isotype-matched mAbs.

Statistical Analysis

The results were expressed as the mean ± standard error of mean (SEM). Groups of data were compared by Mann–Whitney *U*-test. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

$CD4^+CD44^{high}CD62L^-$IL-$7R\alpha^{high}$ T_{EM} Cells Were Distributed Throughout Whole Body in Established Colitic Mice

Although the SCID transfer model of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells is characterized by a marked infiltration of colitogenic $CD4^+$ T cells in the colonic tissues, we previously showed that SP and BM $CD4^+$ T cells in the colitic mice were also colitogenic, because their adoptive transfer into new SCID mice induces a similar colitis to the original one.[23] This suggests a link between the inflamed colon and other reservoir sites such as BM through the continuous circulation of colitogenic $CD4^+$ T cells. To investigate this notion, we first checked the distribution of $CD4^+$ T cells in various sites of established colitic $CD4^+CD45RB^{high}$ T-cell-transferred SCID mice at the late stage of this model (8 weeks after transfer). As depicted in Figure 1, $CD4^+$ T cells resided in all sites of colitic mice examined including PB, LP, SP, TD (efferent lymphatics side), MLN, and BM, and had solely the character of $CD44^{high}CD62L^-$IL-$7R\alpha^{high}$ T_{EM} cells. Importantly, the fact that the detection of such cells in TD and PB indicates continuous recirculation of $CD4^+$ T cells even after the establishment of colitis. In contrast, CD4-gated T cells obtained from normal mice consisted of $CD44^{low}CD62L^+$ naïve, $CD44^{high}CD62L^+$ central-memory (T_{CM}), and $CD44^{high}CD62L^-$ T_{EM} cells in all the examined sites except LP, which contained solely $CD44^{high}CD62L^-$ T_{EM} cells (Fig. 1).

Adoptive Transfer of Colitic PB $CD4^+$ T Cells Induces Colitis

We next attempted to show whether continuously circulating PB $CD4^+$ T cells in the established colitic mice are also colitogenic, in the same way that colitic LP $CD4^+$ T cells were previously shown to be.[24] To this end, we transferred colitic PB or LP $CD4^+$ T cells obtained from colitic $CD4^+CD45RB^{high}$ T-cell-transferred SCID mice into new SCID mice as illustrated in Figure 2A. As expected, SCID mice transferred with colitic LP $CD4^+$ T cells (positive control) manifested progressive weight loss from 3 weeks after transfer (data not shown). These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 5–6 weeks after transfer, in sharp contrast to age-matched control BALB/c (Fig. 2B). Similarly, SCID mice transferred with colitic PB $CD4^+$ T cells also developed a wasting disease with symptoms of colitis to a similar extent to those with colitic LP $CD4^+$ T cells (Fig. 2B). At 6 weeks after transfer the colon from colitic PB or LP $CD4^+$ T cells was enlarged and had a greatly thickened wall, in sharp contrast to the control BALB/c mice (data not shown). Histo-

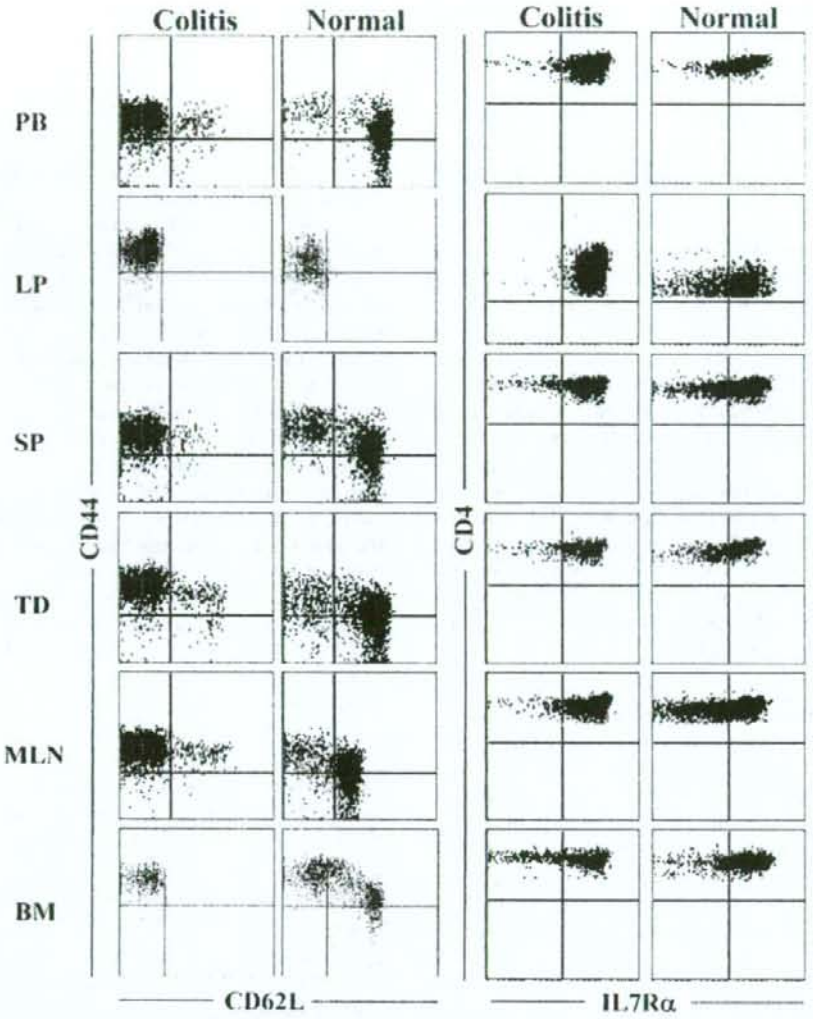


FIGURE 1. $CD4^+CD44^{high}CD62L^-$ T_{EM}-like cells reside in various sites including LP, MLN, TD, PB, SP, and BM, of established colitic SCID mice at 8 weeks after adoptive transfer of $CD4^+CD45RB^{high}$ T cells. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and sidescatter profiles. Data are displayed as dotted plots (4-decade log scale), and quadrant markers were positioned to include >98% of control IgG-stained cells in the lower left. Percentages in each quadrant are indicated. Representatives of 3 mice in each group.

logical examination showed prominent epithelial hyperplasia with massive infiltration of mononuclear cells in the colonic LP from mice transferred with colitic PB or LP $CD4^+$ T-cell-transferred SCID mice, but not from the control BALB/c mice (Fig. 2C). This difference was also confirmed by histological scoring of multiple colon sections, the scores being 14.5 ± 1.0 in mice transferred with colitic LP $CD4^+$ T cells, 15.3 ± 1.2 in mice transferred with colitic PB $CD4^+$ T cells, and 0.7 ± 0.7 in control BALB/c mice ($P < 0.005$) (Fig. 2D).

A further quantitative evaluation of $CD4^+$ T-cell infiltration was made by isolating LP $CD4^+$ T cells. Significantly more LP $CD4^+$ T cells were recovered from SCID mice transferred with colitic LP or PB $CD4^+$ T cells than from the control BALB/c mice (Fig. 2E). The number of $CD4^+$ cells recovered from the colonic LP of mice transferred with colitic PB $CD4^+$ T cells ($202 \pm 42 \times 10^5$) far exceeded the number of originally injected cells (3×10^5), indicating an extensive T cell migration and/or proliferation in the inflamed colon.

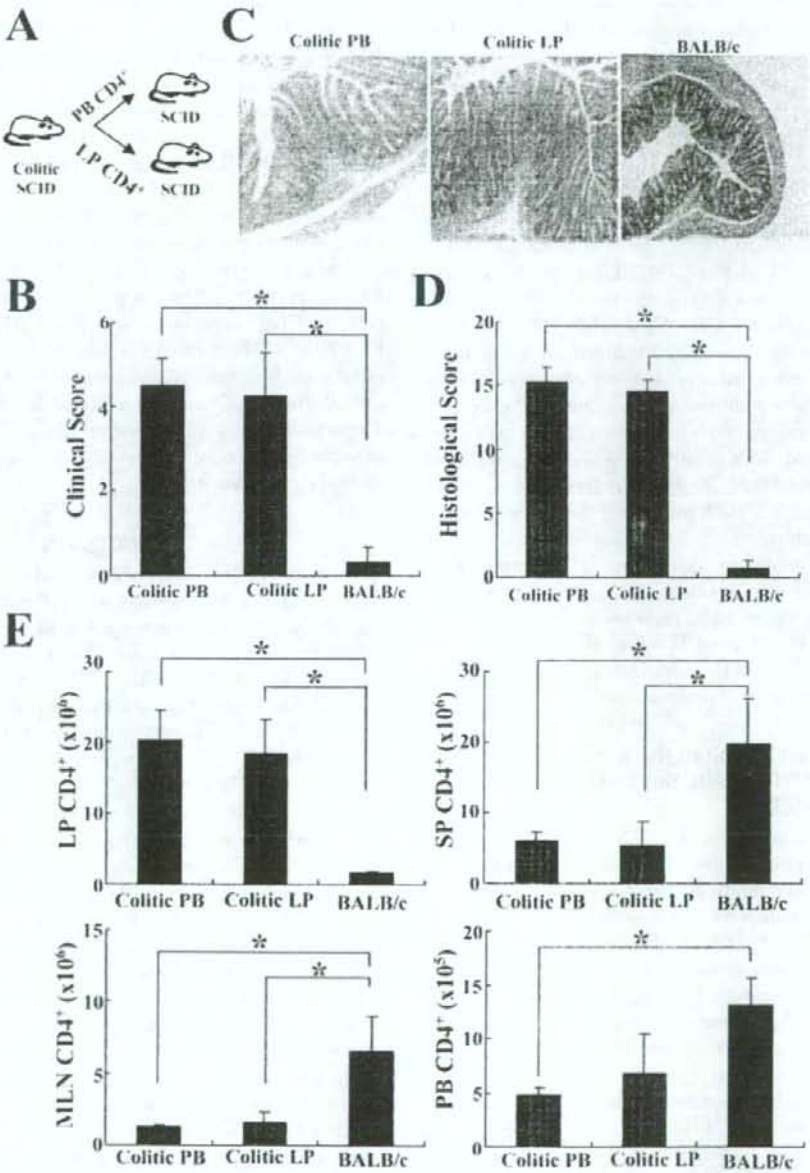


FIGURE 2. New SCID mice transferred with circulating PB $CD4^+$ T cells obtained from established colitic $CD4^+CD45RB^{high}$ T cell-transferred SCID mice develop colitis. A: New SCID mice were transferred with colitic PB $CD4^+$ T cells ($n = 6$) or colitic LP $CD4^+$ T cells ($n = 6$). B: Clinical scores were determined at 6 weeks after transfer. Data are indicated as mean ± SEM of 6 mice in each group. *Versus age-matched control BALB/c mice, $P < 0.01$. C: Histological examination of the colon from mice were transferred with colitic PB $CD4^+$ T cells (left) or colitic LP $CD4^+$ T cells at 6 weeks after transfer (middle). Original magnification, ×100. D: Histological scoring of mice transferred with colitic PB $CD4^+$ T cells or colitic LP $CD4^+$ T cells at 6 weeks after transfer. Data are indicated as mean ± SEM of 6 mice in each group. *$P < 0.01$. E: LP, SP, MLN, and PB cells were isolated from the colon at 6 weeks after transfer, and the number of $CD4^+$ cells was determined by flow cytometry. Data are indicated as mean ± SEM of 7 mice in each group. *$P < 0.05$.

Collectively, these results clearly showed that colitogenic $CD4^+$ T cells continuously circulate in PB of established colitic mice.

Colitogenic $CD4^+$ T Cells Dynamically Recirculate Even After the Establishment of Colitis

To further assess the hemodynamics of colitogenic $CD4^+$ T cells in colitic mice, we next conducted an in vivo experiment combining adoptive transfer and a parabiosis system. To this end, RAG-$2^{-/-}$ recipient mice and donor cells from C57BL/6-Ly5.1- or C57BL/6-Ly5.2-mice were used. First, RAG-$2^{-/-}$ mice were transferred with C57BL/6-Ly5.1- or C57BL/6-Ly5.2-$CD4^+CD45RB^{high}$ T cells (Fig. 3A). After confirming the establishment of colitis in these mice at 6 weeks after transfer, we performed parabiosis surgery between these colitic RAG-$2^{-/-}$ mice (Fig. 3A,B). At 2 weeks after surgery, both parabionts in each pair were consistently diseased, with greatly thickened colon wall and enlarged spleen and MLN, in sharp contrast to the control C57BL/6 mice (Fig. 3C). Both parabionts also showed severe colitis with crypt elongation, surface erosion, and a marked infiltration of mononuclear cells (Fig. 3D). Importantly, $Ly5.1^+$ and $Ly5.2^+$ cells in both the donor and recipient sides were well mixed in almost equal proportions in LP, as well as in SP, MLN, BM, PB, PC, and TD (Fig. 3E), suggesting that colitogenic $CD4^+$ T_{EM} cells continuously recirculate even after the establishment of chronic colitis.

FTY720 Treatment Inhibited the Recirculation of Colitogenic $CD4^+$ T_{EM} Cells, But Did Not Ameliorate the Established Colitis

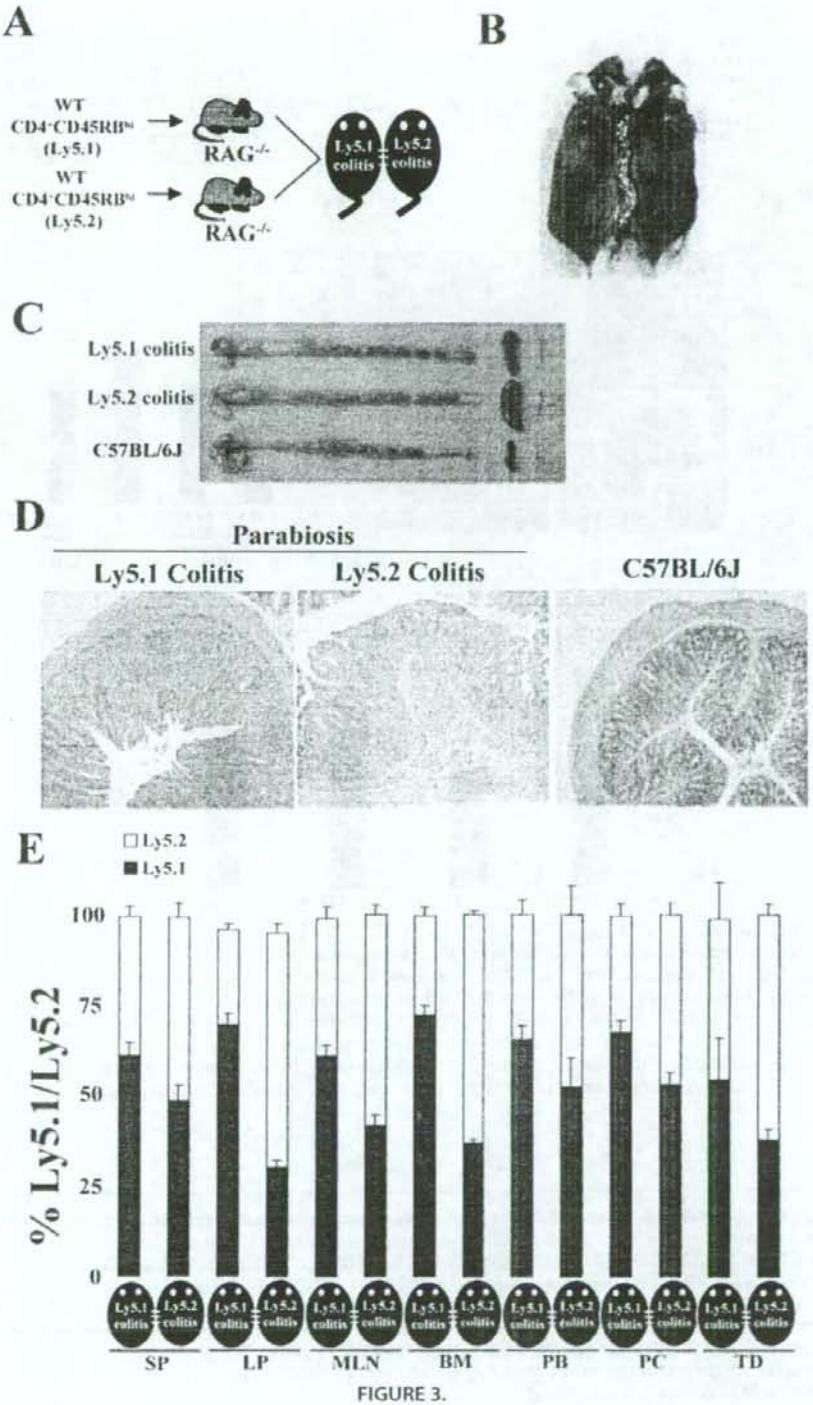
To finally assess whether FTY720, which has the ability to turn off the circulation of lymphocytes by promoting their sequestration and inhibiting their egress,[12,13] suppresses the continuous recirculation of colitogenic $CD4^+$ T cells and thereby ameliorates the established colitis in this parabiosis system, both parabionts in each pair of established colitic RAG-$2^{-/-}$ mice transferred with $Ly5.1^+$ or $Ly5.2^+$ $CD4^+CD45RB^{high}$ T cells were next treated intraperitoneally with FTY720 (1.0 mg/kg) or control PBS daily for 4 weeks starting 1 day before parabiosis surgery, and were monitored for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool (Fig. 4A). Unexpectedly, both parabionts treated with FTY720 were consistently diseased, with clinical symptoms of colitis to a similar extent to PBS-treated parabionts (data not shown). The colon, spleen, and MLN from all these mice, whether treated with FTY720 or PBS, were enlarged and had a greatly thickened wall due to severe colonic inflammation (Fig. 4B). Histological examination also showed a severe colitis with massive infiltration of mononuclear cells in LP of the colon from all the parabionts (Fig. 4C), and the scores of histology confirmed that FTY720 did not ameliorate the established colitis in parabionts (Fig. 4D). Interestingly, however, the ratio of donor cells in all the sites examined, namely, SP, LP, MLN, and BM, was significantly suppressed by FTY720 treatment (Fig. 4E).

To further assess the change of surface markers of the donor and recipient cells in various sites (SP, LP, MLN, and BM) by FTY720 treatment, we performed flow cytometry analysis (Fig. 5). First, $Ly5.1^+$ and $Ly5.2^+$ cells, whether donor or recipient cells, and irrespective of FTY720 treatment, showed the character of $CD44^{high}CD62L^-$ T_{LM} cells. Second, the expression pattern of cell activation marker CD69 was dependent on the site, but not on donor or recipient cells, as CD69 upregulated in LP and BM $CD4^+$ T cells of FTY720- or PBS-treated parabionts as compared to that in other sites. Furthermore, the expression of gut-homing receptor integrin $\alpha4\beta7$ was not affected by FTY720 treatment. These results indicate that the effect of FTY720 treatment is to suppress the recirculation of lymphocytes, but not to modulate the cell phenotype.

DISCUSSION

In the present study we demonstrated that colitogenic $CD4^+$ T_{EM} cells continuously circulate in whole body through the blood vessels even after the establishment of colitis by showing that colitis can be induced by adoptive retransfer of colitic PB $CD4^+$ T_{EM} cells, and that $CD4^+$ T cells in parabionts between established colitic RAG-$2^{-/-}$ mice induced by adoptive transfer of $Ly5.1^+$ or $Ly5.2^+$ $CD4^+CD45RB^{high}$ T cells were well mixed in various sites including PB, TD, and LP.

Since colitogenic effector or memory T cells are thought to be generated in regional LN and from there migrate into the inflamed mucosa, it is logical to conclude that they probably circulate in the peripheral blood. Curiously, however, the question of whether they actively recirculate in PB has not hitherto been experimentally solved, although we and others previously showed that SP, MLN, and BM $CD4^+$ T cells obtained from colitic mice are colitogenic, as adoptive transfer of those cells induces colitis that is similar to the original colitis of donor mice.[15,23–25] The present model of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells is useful for this purpose, because 1) primarily transferred $CD4^+CD45RB^{high}$ T cells in recipient mice can be tracked over time under conditions that allow us to exclude the recruitment of naïve $CD4^+$ T cells that are continuously supplied from thymus; and 2) this model induces the extremely rapid proliferation of colitogenic LP $CD4^+$ T cells, which presumably respond to commensal bacterial antigens or self-antigens by lymphopenia-driven proliferation, and thereby accounts for almost all $CD4^+$ T cells in the body.[26,27] Using this adoptive transfer model, we here found that 1) $CD4^+CD44^{high}CD62L^-$ T_{EM} cells do indeed reside in PB of colitic mice; and 2) adoptive transfer of those cells success-


A
WT
CD4+CD45RBhi
(Ly5.1)
WT
CD4+CD45RBhi
(Ly5.2)
RAG-/-
RAG-/-
Ly5.1 colitis
Ly5.2 colitis
B
C
Ly5.1 colitis
Ly5.2 colitis
C57BL/6J
D
Parabiosis
Ly5.1 Colitis
Ly5.2 Colitis
C57BL/6J
E
Ly5.2
Ly5.1
% Ly5.1/Ly5.2
100
75
50
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SP
LP
MLN
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FIGURE 3.

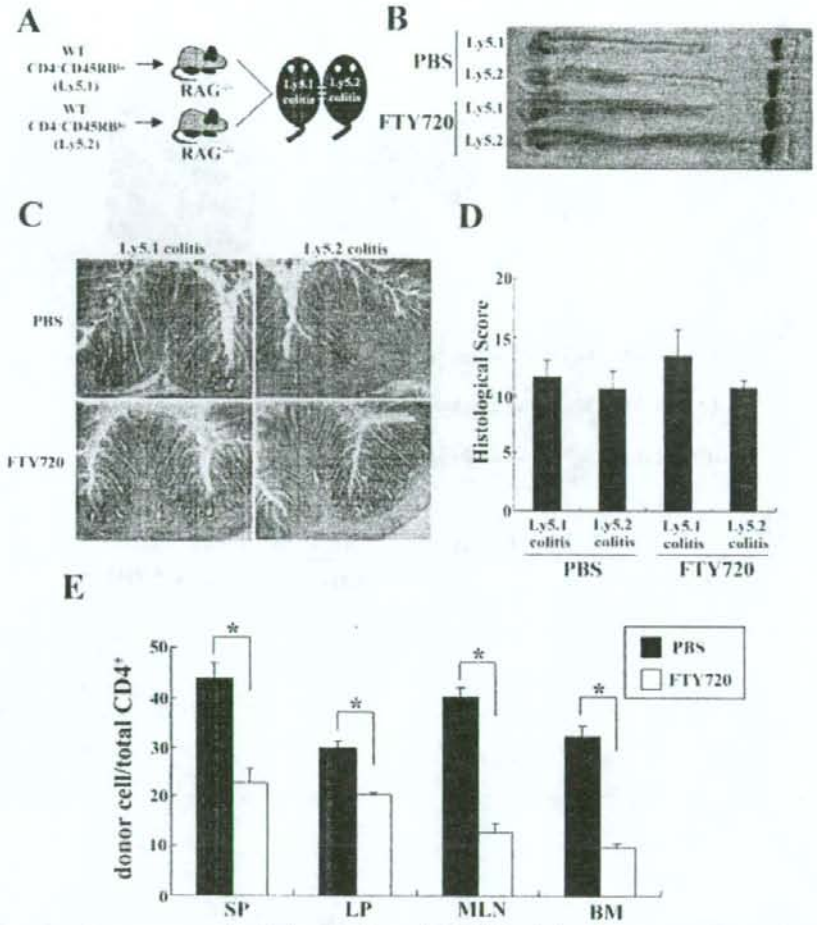

FIGURE 4. FTY720 treatment suppresses recirculation of colitogenic $CD4^+$ T cells, but does not ameliorate the established colitis in parabionts. A: Parabionts between established colitic RAG-$2^{-/-}$ mice transferred with Ly5.1^+ or Ly5.2^+ $CD4^+CD45RB^{high}$ T cells were treated with FTY720 (1.0 mg/kg) or control PBS daily for up to 6 weeks starting 1 day before parabiosis surgery, and were monitored for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool. B: Gross appearance of the colon, spleen, and MLNs obtained from parabionts treated with PBS (top) or FTY720 (bottom) after 6 weeks of treatment. C: Histological examination of the colon from PBS-treated (upper) or FTY720-treated (lower) mice. Original magnification; ×100. D: Histological scoring of colitis in PBS-treated and FTY720-treated mice. Data are indicated as mean ± SEM of 6 mice in each group. NS, not significant. E: Ratio of donor-derived $CD4^+$ T cells per total $CD4^+$ T cells of each parabiont. $^*P < 0.05$. Data are indicated as mean ± SEM of 6 parabionts in each group.

FIGURE 3. Colitogenic $CD4^+$ T cells dynamically recirculate even after the establishment of colitis. A: Parabiosis experimental design. For adoptive transfer, splenic $CD4^+CD45RB^{high}$ T cells were isolated from C57BL/6-Ly5.1 or -Ly5.2 mice and transferred into RAG-$2^{-/-}$ mice ($n = 6$ per group). Six weeks after transfer, when both groups of recipient mice had developed wasting disease and colitis, intergroup pairs were joined by parabiosis surgery. B: Photo of parabionts. C: Gross appearance of colon, spleen, and MLN obtained from parabionts between RAG-$2^{-/-}$ mice transferred with C57BL/6-Ly5.1- or C57BL/6-Ly5.2-$CD4^+CD45RB^{high}$ T cells at 4 weeks after surgery or age-matched control C57BL/6 mice. D: Histological examination of the colon from parabiont pairs at 6 weeks after transfer or control C57BL/6 mice. Original magnification, ×100. E: LP, MLN, TD, PB, SP, and BM cells were isolated from each mouse at 4 weeks after surgery, and the number of Ly5.1^+ and Ly5.2^+ $CD4^+$ cells was determined by flow cytometry. Data are indicated as mean ± SEM of 6 mice in each group.

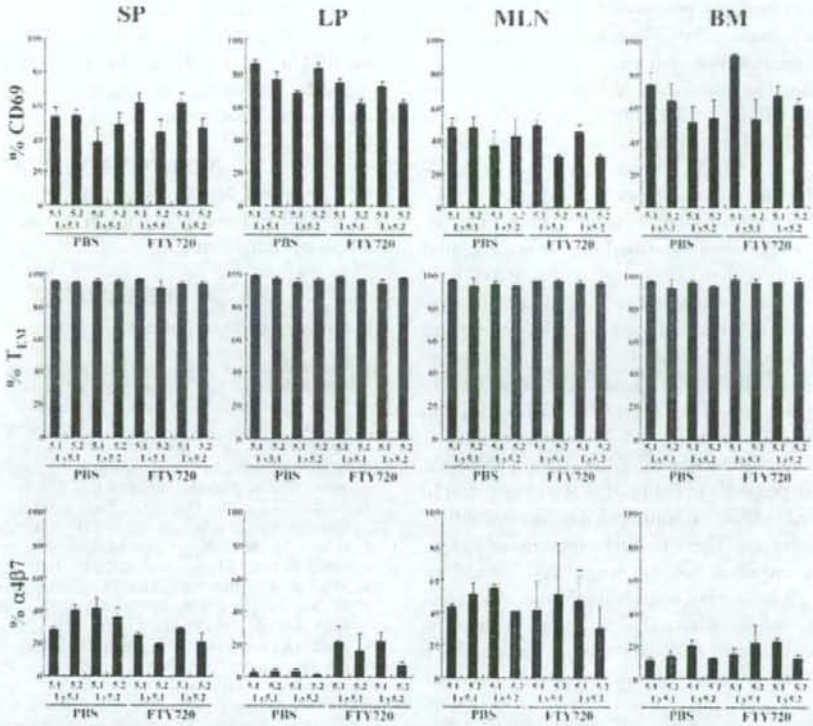

FIGURE 5. FTY720 treatment does not modulate the phenotype of $CD4^+$ T cells in parabionts. Expression of CD44, CD62L, CD69, and integrin $\alpha4\beta7$ on $CD4^+$ T cells obtained from SP, MLN, LP, and BM in colitic parabionts treated with FTY720 or PBS. Freshly isolated cells were stained with FITC-labeled anti-CD45.1 or CD45.2, and PerCP-labeled anti-CD4, and PE-labeled anti-CD62L, anti-CD69, or anti-integrin $\alpha4\beta7$ mAb and allophycocyanin-labeled anti-CD44. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and sidescatter profiles.

fully induces chronic colitis, even after the full establishment of colitis, indicating that colitogenic $CD4^+$ T_{EM} cells actively and continuously recirculate in chronic colitic mice.

Although the parabiosis system seems to be somewhat artificial and problematic on some level, as 2 mice are under the stress of surgery and behavioral limitation, the surgical joining of mice harboring colitogenic $CD4^+$ T cells results in prompt development of anastomoses of blood vessels within a few days. This model is also useful to assess the hemodynamics of circulating or resident $CD4^+$ T cells in colitic parabionts, these cells being distinguishable by using the Ly5.1/Ly5.2 system.[28] We showed that $Ly5.1^+$ and $Ly5.2^+$ cells in both parabionts had the character of $CD44^{high}CD62L^-$ T_{EM} cells and were well mixed and in almost equal proportions in LP, as well as in SP, MLN, BM, PB, PC, and TD 4 weeks after parabiosis surgery. This result also indicated that colitogenic $CD4^+$ T cells continuously egress from the inflamed mucosa, migrate into afferent lymphatics→MLN→efferent lymphatics→PB in sequence, and return to the intestine in the established colitic mice.

Hence, the present results support the notion that circulating colitogenic $CD4^+$ T cells might be appropriate targets for IBD therapy. In this regard, leukocytapheresis has been applied to the treatment of various autoimmune diseases including, in recent years, UC.[29] It is thought that leukocytapheresis removes pathogenic cells that contribute to the pathogenesis of IBD from the body. Interestingly, we recently demonstrated that leukocytapheresis treatment for patients with UC selectively removed $CD4^+CD45RO^+CD62L^-$ T_{EM} cells rather than $CD4^+CD45RO^+CD62L^+$ T_{CM} cells or $CD4^+CD25^{bright}$ regulatory T (T_{REG}) cells,[30] suggesting that the removal of circulating $CD4^+$ T_{EM} cells in PB of patients with IBD has logical advantages for the treatment of IBD.

As an essential factor in the persistence of T-cell-mediated colitis, we have focused on IL-7 in a series of studies.[23,27,32,35-37] IL-7 is secreted by stromal cells in BM and thymus, and epithelial cells including intestinal epithelia.[31-33] Also, accumulating evidence shows that IL-7 is an important cytokine involved in supporting the survival of memory $CD4^+$ T cells.[34] We have previously demonstrated

that: 1) IL-7 is constitutively produced by intestinal goblet epithelial cells in human[32]; 2) IL-7 transgenic (Tg) mice, where strong promoters drive systemic overexpression, develop chronic colitis that mimics the histopathological characteristics of human IBD[35]; 3) LP $CD4^+$ IL-$7R\alpha^{high}$ T cells in $CD4^+CD45RB^{high}$ T cell-transferred colitic mice are colitogenic[36]; 4) IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with colitogenic $CD4^+$ T cells isolated from the inflamed LP of $CD4^+CD45RB^{high}$ T cell-transferred colitic mice do not develop colitis[37]; 5) nevertheless, intestinal IL-7 is not essential for the perpetuation of colitis, since IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice parabiosed with colitic $CD4^+CD45RB^{high}$ T-cell-transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice develop severe colitis despite lacking intestinal IL-7[27]; 6) colitogenic $CD4^+CD44^{high}CD62L^-$ IL-$7R\alpha^{high}$ T_{EM} cells preferentially reside in the BM of colitic $CD4^+CD45RB^{high}$ T cell-transferred SCID/RAG-1/2$^{-/-}$ mice[23]; and 7) paradoxically, IL-7-producing goblet cells are easily decreased or depleted (so-called 'goblet depletion') in colitic IL-7 Tg mice[35] and in the present model of colitis,[17] resulting in the decreased IL-7 production in the intestine. These findings might have a close bearing on the present data, because it appears that colitogenic LP $CD4^+$ T_{EM} cells may intermittently have to egress from the inflamed mucosa, where IL-7 is absent or decreasing, and actively circulate to IL-7-producing reservoirs such as BM in order to survive and continue to cause the chronic inflammation.

Finally, the reason should be discussed why FTY720 could not ameliorate the colitis in parabionts between 2 established RAG-$2^{-/-}$ mice transferred with $Ly5.1^+$ or $Ly5.2^+$ $CD4^+CD45RB^{high}$ T cells. One possibility is that FTY720 treatment is not appropriate for therapeutic use, as almost all previous reports show the preventive, but not therapeutic, effect of FTY720 on the development of various diseases including autoimmune diseases and allo-transplantation.[38–42] It is also possible that more time is needed for the therapeutic effect of FTY720 to become evident in our parabiosis model, as we observed FTY720-treated parabionts for only 6 weeks because of the limitation of the parabiosis system. Consistent with this possibility, we previously demonstrated that colitogenic LP $CD4^+$ T cells are quite long-lived, because sequential adoptive transfers of colitogenic LP $CD4^+$ T cells into new SCID mice could stably induce colitis at least 6 times totally in experiments extending over 3 years. Thus, although FTY720 might inhibit the recruitment of colitogenic LP $CD4^+$ T cells into the inflamed mucosa in this parabiosis system (Fig. 4), the resident colitogenic LP $CD4^+$ T cells might to be able to maintain intestinal inflammation for an extended period, as FTY720 is unable to induce apoptosis of those cells (unpubl. data).[15]

In summary, we demonstrated that colitogenic $CD4^+$ T_{EM} cells actively recirculate in fully established colitic mice. Although IBD have hitherto been classified solely as intestinal disorders, unlike other autoimmune diseases such as connective tissue diseases, the present study suggests not only that IBD are not curable by local resection, but also that they should be reclassified as systemic diseases that pathogenic lymphocytes disseminate outside the intestine.

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Exacerbating Role of γδ T Cells in Chronic Colitis of T-Cell Receptor α Mutant Mice

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Background & Aims: **T-cell receptor (TCR) γδ T cells are an important component of the mucosal immune system and regulate intestinal epithelial homeostasis. Interestingly, there is a significant increase in γδ T cells in the inflamed mucosa of patients with ulcerative colitis (UC). However, the role of γδ T cells in chronic colitis has not been fully identified.** ***Methods:*** **TCRα-deficient mice, which spontaneously develop chronic colitis with many features of human UC including an increase in γδ T-cell population, represent an excellent model to investigate the role of γδ T cells in UC-like colitis. To identify the role of γδ T cells in this colitis, we herein have generated TCRγ-deficient mice through deletion of all TCR Cγ genes (Cγ1, Cγ2, Cγ3, and Cγ4) using the Cre/*loxP* site-specific recombination system and subsequently crossing these mice with TCRα-deficient mice.** ***Results:*** **An increase in colonic γδ T cells was associated with the development of human UC as well as UC-like disease seen in TCRα-deficient mice. Interestingly, the newly established TCRα$^{-/-}$ × TCRγ$^{-/-}$ double mutant mice developed significantly less severe colitis as compared with TCRα-deficient mice. The suppression of colitis in TCRα$^{-/-}$ × TCRγ$^{-/-}$ double mutant mice was associated with a significant reduction of proinflammatory cytokine and chemokine productions and a decrease in neutrophil infiltration.** ***Conclusions:*** **γδ T cells are involved in the exacerbation of UC-like chronic disease. Therefore, γδ T cells may represent a promising therapeutic target for the treatment of human UC.**

T cell receptor (TCR) γδ T cells are an evolutionary conserved T-cell subset with characteristic properties.[1] TCRγδ-bearing murine dendritic epidermal T cells are involved in the regulation of epidermal integrity and promote wound repair of the skin,[2] whereas intestinal intraepithelial γδ T cells (γδ-IEL) regulate intestinal epithelial homeostasis.[3,4] Recent evidence suggests that γδ T cells are also important in immune surveillance of the epithelium by providing a first line of defense against infectious pathogens attacking the surfaces of the body and in the regulation of linking of innate and acquired immunity.[1,5] Furthermore, γδ T cells appear to down-regulate αβ T cell-driven robust immune responses that often result in severe immunopathology.[1]

The incidence of inflammatory bowel diseases (IBD), namely ulcerative colitis (UC) and Crohn's disease (CD), has increased markedly in recent years. The factors including genetic predisposition, environmental conditions, and aberrant immune response driven by normal intestinal flora are vital for the development and persistence of the inflammatory process.[6,7] In the present study, we aimed at elucidating the role of γδ T cells in the pathogenesis of IBD because there is growing evidence supporting that γδ T cells play an active multifaceted immunoregulatory role in the coordinated innate and acquired immune responses that maintain the integrity of epithelial tissues[1,2,4,5,8] and an increase in γδ T cells in the diseased mucosa has been documented in UC patients.[9,10] In acute colitis induced by administration of either 2,4,6-trinitrobenzene sulfonic acid[11,12] or dextran sulfate sodium,[13,14] a protective role of γδ T cells has been demonstrated. However, the role of γδ T cells in chronic intestinal inflammation resembling UC has not yet been investigated. TCRα$^{-/-}$ (α$^{-/-}$) mice spontaneously develop chronic colitis with several features of human UC including a significant increase in γδ T cells.[15] To illuminate the role of γδ T cells in the pathogenesis of UC-like colitis in α$^{-/-}$ mice, we generated TCRγ$^{-/-}$

Abbreviations used in this paper: α$^{-/-}$, TCRα$^{-/-}$; ARP, anorectal prolapse; γ$^{-/-}$, TCRγ$^{-/-}$; γδ T cells, TCR γδ T cells; IBD, inflammatory bowel disease; IEL, intestinal intraepithelial T lymphocytes; LP, lamina propria; TCR, T-cell receptor; UC, ulcerative colitis.

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($\gamma^{-/-}$) mice and examined the severity of colitis in $\alpha^{-/-}$ mice that are genetically engineered to lack γδ T cells.

Materials and Methods

Mice

We newly generated $\gamma^{-/-}$ mice and crossed with $\alpha^{-/-}$ mice[16] to develop double mutant ($\alpha^{-/-} \times \gamma^{-/-}$) mice. The generations of these mice are described in Supplementary Materials (see Supplemental Materials online at www.gastrojournal.org). All mice used were of C57BL/6 (B6) background. The mice were maintained under specific pathogen-free conditions, and all animal procedures described in this study were performed in accordance with the guidelines for animal experiments of Keio University School of Medicine, Yakult Central Institute for Microbiological Research, Kinki University School of Medicine, and Massachusetts General Hospital.

Flow Cytometry and Immunohistochemical Procedures

Methods for isolation of intestinal intraepithelial T cells (IEL) from mouse small intestines and lamina propria (LP) cells from mouse and human large intestines are described in Supplementary Materials. Procedures of cell staining for flow cytometric and immunohistochemical analyses are also described in Supplementary Materials (see Supplemental Materials online at www.gastrojournal.org).

Histologic Evaluation of Colitis

The disease score of colitis (0–10) was estimated in a blind fashion using previously described criteria, namely, a combination of both gross and histologic findings.[17] The gross score was rated as 0, presence of normal beaded appearance; 1, absence of beaded appearance of colon; 2, focally thickened colon; and 3, marked thickness of entire colon. The histologic score was based on the extent of intestinal wall thickening (0–3), inflammatory cell infiltration into LP (0–3), and presence (0 or 1) of ulceration.

Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted from half of the frozen colonic tissue obtained from each one of wild-type (WT), $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ littermate mice, and complementary DNA (cDNA) was prepared. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) was conducted to assess the expression level of TNF-α, IL-1β, IL-6, TGF-β, IFN-γ, IL-7, IL-10, IL-12, KC, MIP-2, GCP-2, MCP-1, MIP-1α, MIP-1β, and HPRT genes using TaqMan probes (Applied Biosystems, Foster City, CA). The relative expression level of genes of interest was normalized to the HPRT gene expression. The detailed procedures are described in Supplementary Materials (see Supplemental Materials online at www.gastrojournal.org).

Measurement of Cytokines and Chemokines by Enzyme-Linked Immunosorbent Assay

Proteins were extracted from the above-described half of the frozen colonic tissue obtained from each one of WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ littermate mice. In brief, frozen colonic tissue was homogenized with a sonicator (Ultrasonic Disruptor UD-201, TOMY, Tokyo, Japan) in 5 mL lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, and complete, Mini EDTA-free proteinase inhibitor [Roche Applied Science, Mannheim, Germany]), the homogenate was clarified by centrifugation at 14,000 rpm for 10 minutes, and the supernatant was subjected to OptEIA ELISA (BD Biosciences, San Diego, CA) for detection of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 and to DuoSet ELISA (R&D Systems, Minneapolis, MN) for detection of transforming growth factor (TGF)-β, interferon (IFN)-γ, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2 and granulocyte chemotactic protein (GCP)-2. Levels in the supernatants were standardized to the total amount of protein in the same supernatants assessed by RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Chemotaxis Assay

The assays were performed using the ChemoTx 96-well plate No. 101-3 (NeuroProbe, Gaithersburg, MD). Briefly, bone marrow cells collected from femurs, tibias, and humerus of WT mice were recovered by centrifugation at the interphase of 44% and 70% Percoll solutions. Subsequently, 2.5×10^5 bone marrow cells were loaded onto the membrane plate and placed on a flat-bottomed, 96-well microtiter plate containing the colon extracts (0.7 mg protein/mL) from WT, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice in addition to serially diluted MIP-2 and MCP-1 (R&D Systems). To identify the neutrophils and monocytes, bone marrow cells were labeled with fluorescent dye conjugated monoclonal antibodies (mAb) to Mac-1 and Ly-6C before the assay. After incubation at 37°C for 2 hours, the number of Mac-1$^+$Ly-6C^{low} neutrophils[18] and Mac-1$^+$Ly-6C^{high} monocytes[18] that migrated into the lower wells was determined by a flow cytometry.

Cell Transfer

γδ T cells were purified from the mesenteric lymph nodes (MLNs) and colon of $\alpha^{-/-}$ mice through MACS system, and 2×10^6 purified cells were intravenously transferred twice into $\alpha\gamma^{-/-}$ mice (n = 16) at 4 and 5 months of age. As control group, phosphate-buffered saline (PBS) was intravenously administrated into $\alpha\gamma^{-/-}$ mice (n = 15). The recipient mice were then killed at 6 months of age.

Statistical Analysis

The statistical difference was determined by 2-sided Student *t* test. For the statistical analysis of cell infiltration into the large intestine, 2-sided Mann-Whitney *U* test was used. Difference with $P < .05$ was considered significant.

Results

Generation of TCRγ-Deficient Mice

To begin with, we initially confirmed that γδ T cells were increased in the lymphoid cells isolated from the inflamed colonic mucosa of UC patients as compared with those from the unaffected colonic mucosa of patients with colon cancer (Figure 1*A* and *B*) and also in the lymphoid cells isolated from the inflamed colonic LP of $\alpha^{-/-}$ mice as compared with those from normal colonic LP of age-matched WT littermate mice (Figure 1*C* and *D*).

Precise appreciation of the role of γδ T cells in pathogenesis of colitis in $\alpha^{-/-}$ mice requires the generation of $\alpha^{-/-}$ mice deficient in γδ T cells. However, the previously generated TCRδ$^{-/-}$ ($\delta^{-/-}$) mice[19] lacking γδ T cells could not be used for this purpose because of the genomic localization of TCRδ coding segments within the *V* and *J* segments of TCRα gene.[20] To overcome this difficulty, we newly generated TCRγ$^{-/-}$ mice by disrupting the genes encoding TCR Cγ1, 2, 3, and 4 (CγΔ) using the Cre/*loxP* site-specific recombination system shown in Figure 2. The targeting vector pCγ4ΔNL carrying a *loxP*-flanked *pgk-neo* gene cassette in place of exon 1 of the Cγ4 gene (Figure 2*A*) was introduced into the embryonic stem (ES) clone Vγ6ΔL carrying the allele in which the Vγ6 region was replaced by a single *loxP* site (Figure 2*B*). Transfected cells were cultured in the presence of G418, and G418-resistant recombinant clones showing the joint transmission of Vγ6ΔL and Cγ4ΔNL genes were selected. These ES clones, including the clones carrying both transgenes on the same chromosome, Vγ6ΔL-Cγ4ΔNL (Figure 2*C*), were injected into B6 blastocysts. The chimeric mice obtained were crossed to the CAG-*cre* transgenic B6 mice to generate the Cγ1-, 2-, 3-, and 4-depleted TCRγ-deficient (CγΔ) allele (Figure 2*C*) by *cre*-mediated recombination in F_1 mice during embryonic development.

Subsequently, these F_1 mice were intercrossed to produce homozygous (γ$^{-/-}$) mice (Figure 2*D*), and these mutant γ$^{-/-}$ mice were backcrossed 8 times to B6 mice to obtain γ$^{-/-}$ mice carrying the B6 background. WT ($\alpha^{+/-} \times \gamma^{+/-}$), γ$^{-/-}$ ($\alpha^{+/-} \times \gamma^{-/-}$), $\alpha^{-/-}$ ($\alpha^{-/-} \times \gamma^{+/-}$), and αγ$^{-/-}$ ($\alpha^{-/-} \times \gamma^{-/-}$) littermate F_2 mice were then produced by intercrossing $\alpha^{-/-}$ mice[16] with γ$^{-/-}$ mice. Flow cytometric analysis of IEL from the small intestine confirmed that γδ T cells were absent in γ$^{-/-}$ and αγ$^{-/-}$ mice (Figure 2*E*).

Pathogenic Role of γδ T Cells in UC-Like Colitis

Histologic examination of the colons from 20- to 32-week-old $\alpha^{-/-}$ and αγ$^{-/-}$ mice revealed that inflam-

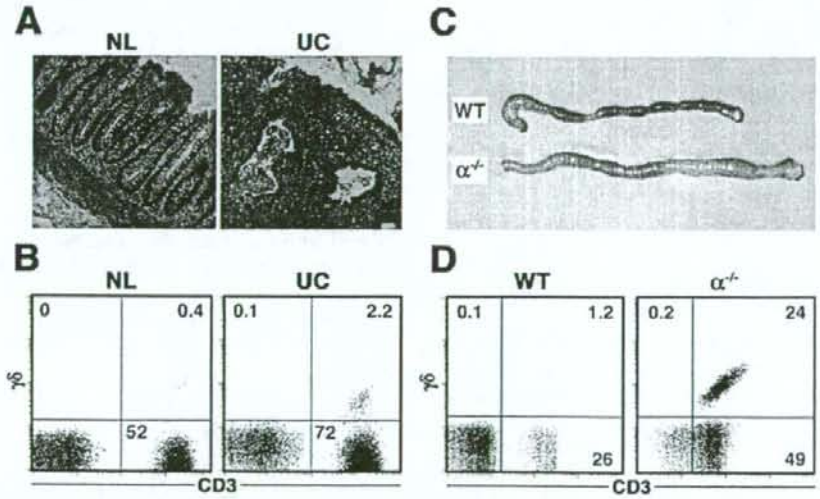

Figure 1. γδ T cells concentrate in the inflamed colonic mucosa of UC patients and colonic LP of $\alpha^{-/-}$ mice suffering from spontaneous chronic colitis. (*A*) A representative colonic tissue section from an ulcerative colitis (*UC*) patient shows a marked infiltration of lymphomyeloid cells, mucosal distortion, crypt abscess, and depletion of goblet cells compared with a normal colonic tissue section (*NL*) (original magnification, ×100). (*B*) A flow cytometry shows increased γδ T-cell population in a diseased colonic LP of UC patient compared with that in a normal colonic LP. This is a representative result of 3 UC patients. (*C*) Large intestines from wild-type (*WT*) mice and $\alpha^{-/-}$ mice suffering from spontaneous chronic colitis are shown. (*D*) A flow cytometry shows increased γδ T-cell population in a diseased colonic LP of $\alpha^{-/-}$ mice compared with that in colonic LP of WT littermate.