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

Takayuki Tomita, PhD, Takanori Kanai, MD, PhD, Yasuhiro Nemoto, MD, PhD, Toshimitsu Fujii, MD, Kengo Nozaki, MD, Ryuichi Okamoto, MD, PhD, Kiichiro Tsuchiya, MD, PhD, Tetsuya Nakamura, MD, PhD, Naoya Sakamoto, MD, PhD, Teruji Totsuka, MD, PhD, and Mamoru Watanabe, MD, PhD

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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From the Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, Tokyo.

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The first 2 authors contributed equally to this work.

Current address for T. Kanai: Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, T. 35 Sinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.

Reprints: Takanori Kanai, MD, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan (e-mail: taka.gast@tmd.ac.jp).

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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-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 α4β7. 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-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-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.[21] 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-

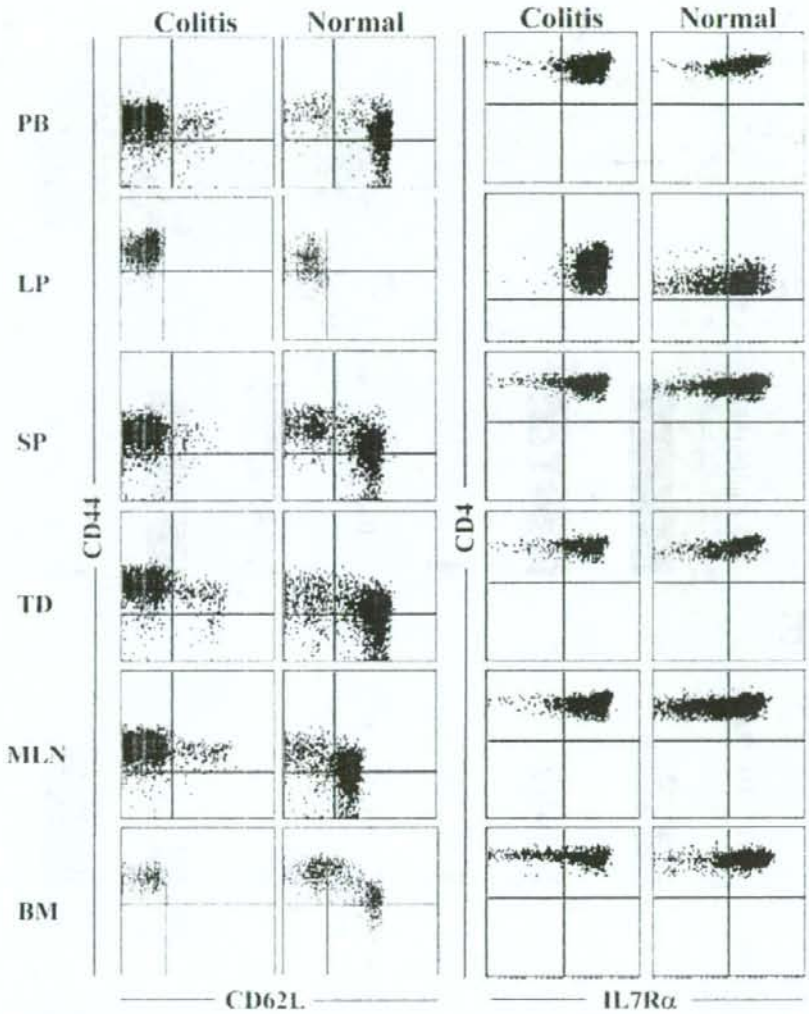


FIGURE 1. $CD4^+CD44^{high}CD62L^-$ T_{EM}-like cells reside in various sites including LP, MLN, TD, PB, SP, and BM, of established colitic SCID mice at 8 weeks after adoptive transfer of $CD4^+CD45RB^{high}$ T cells. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic 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.

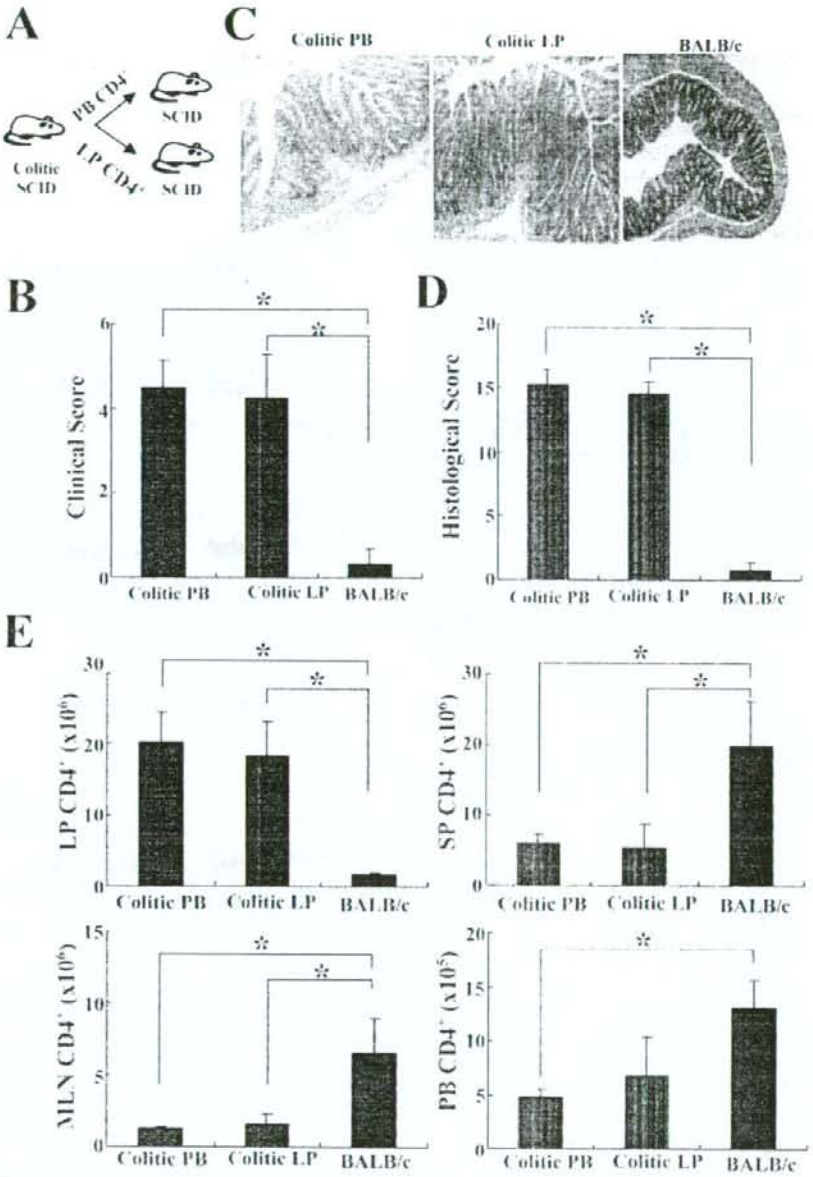


FIGURE 2. New SCID mice transferred with circulating PB CD4$^+$ T cells obtained from established colitic CD4$^+$CD45RB^{high} T cell-transferred SCID mice develop colitis. A: New SCID mice were transferred with colitic PB CD4$^+$ T cells ($n = 6$) or colitic LP CD4$^+$ T cells ($n = 6$). B: Clinical 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

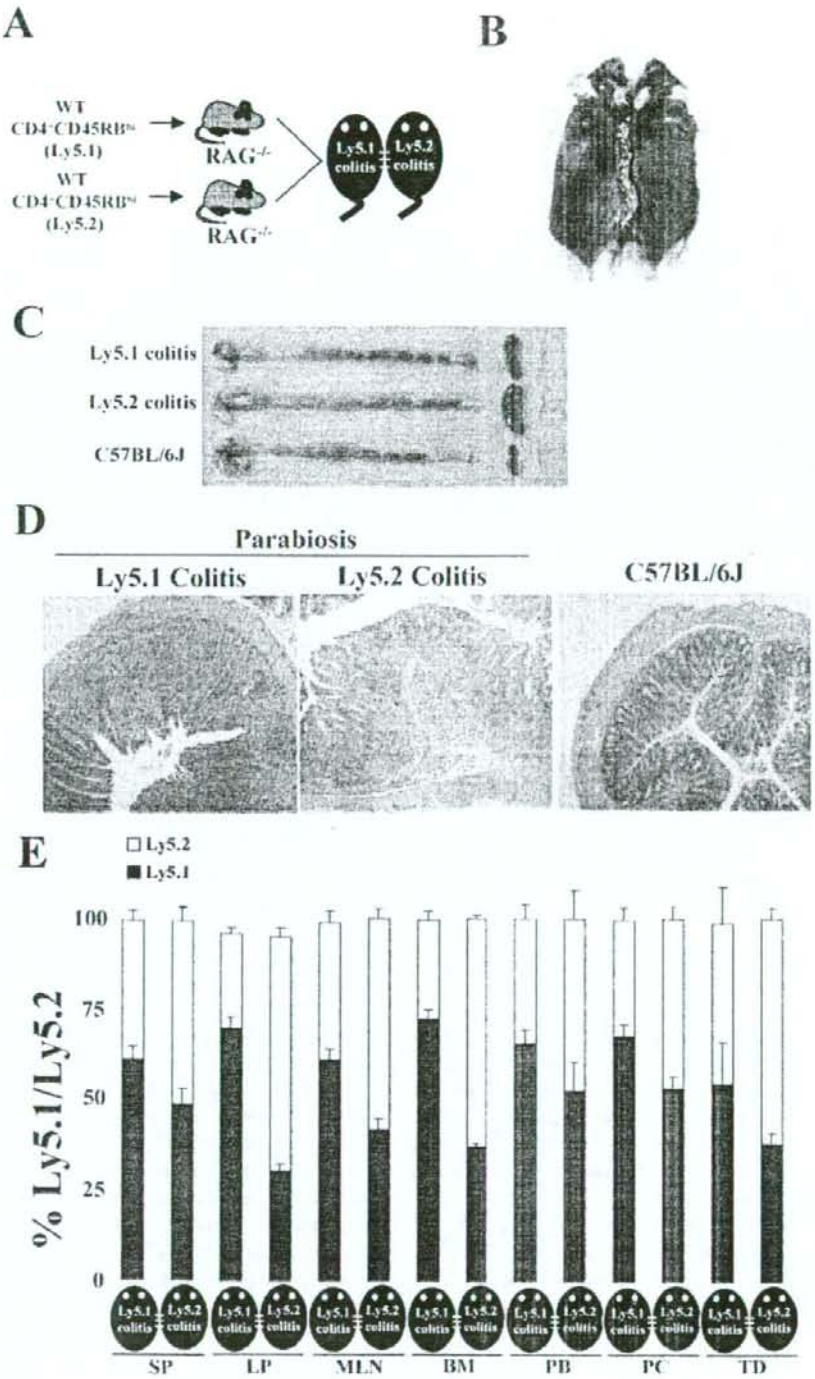
To finally assess whether FTY720, which has the ability to turn off the circulation of lymphocytes by promoting their sequestration and inhibiting their egress,[12,13] suppresses the continuous recirculation of colitogenic $CD4^+$ T cells and thereby ameliorates the established colitis in this parabiosis 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_{EM} 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.[15,24-25] 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
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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
25
0
SP
LP
MLN
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FIGURE 3.

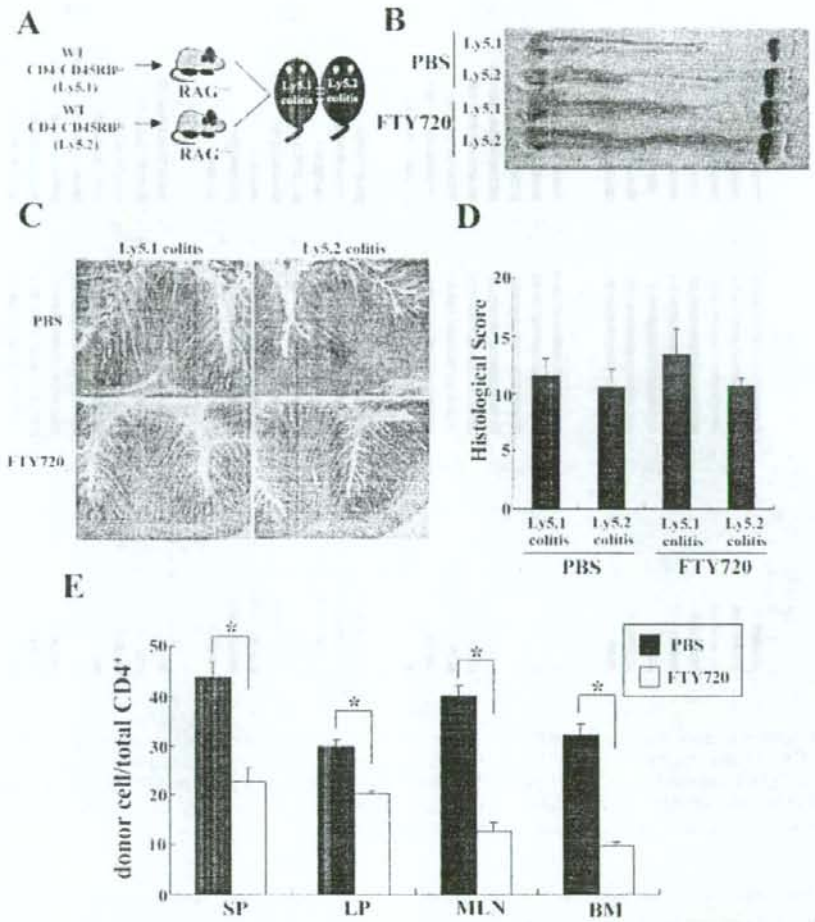

FIGURE 4. FTY720 treatment suppresses recirculation of colitogenic CD4+ T cells, but does not ameliorate the established colitis in parabionts. A: Parabionts between established colitic RAG-2$^{-/-}$ mice transferred with Ly5.1+ or Ly5.2+ CD4+ CD45RB^{high} T cells were treated with FTY720 (1.0 mg/kg) 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.

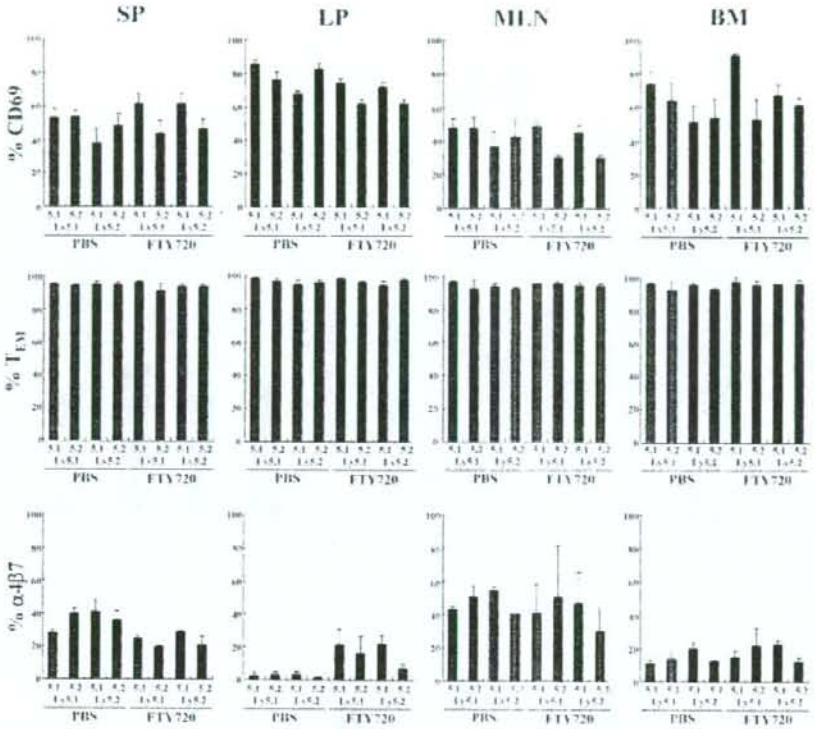

FIGURE 5. FTY720 treatment does not modulate the phenotype of $CD4^+$ T cells in parabionts. Expression of CD44, CD62L, CD69, and integrin $\alpha4\beta7$ on $CD4^+$ T cells obtained from SP, MLN, LP, and BM in colitic parabionts treated with FTY720 or PBS. Freshly isolated cells were stained with FITC-labeled 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-7Rα^{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-7Rα^{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,[37] 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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Immunosenescent colitogenic CD4$^+$ T cells convert to regulatory cells and suppress colitis

Teruji Totsuka, Takanori Kanai*, Yasuhiro Nemoto, Takayuki Tomita, Kiichiro Tsuchiya, Naoya Sakamoto, Ryuichi Okamoto and Mamoru Watanabe*

Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

Inflammatory bowel diseases progress steadily by the expansion of colitogenic CD4$^+$ cells. However, it remains unknown whether colitogenic CD4$^+$ cells are long-living like memory cells or exhausted like effector cells. To assess the longevity of colitogenic lamina propria (LP) CD4$^+$ cells, we performed sequential transfers of LP CD4$^+$ cells from colitic CD4$^+$CD45RB^{high} cell-transferred SCID mice into new SCID mice. Although SCID mice transferred with colitic LP CD4$^+$ cells stably developed colitis until at least the sixth transfer, the interval to the development of colitis gradually lengthened as the number of transfers increased. The incidence of colitis gradually decreased after the seventh transfer. Furthermore, non-colitic LP CD4$^+$ cells from mice transferred over seven times expressed significantly higher levels of PD-1 and produced significantly lower amounts of IFN-γ, TNF-α, and IL-17 than colitic LP CD4$^+$ cells recovered after the first transfer. Most notably, we found that re-transfer of non-colitic LP CD4$^+$ cells recovered after multiple transfers prevented the development of colitis in SCID mice co-transferred with CD4$^+$CD45RB^{high} cells. Thus, colitogenic LP CD4$^+$ cells may be exhausted over time, become non-functional, convert to regulatory cells, and finally suppress colitis in the process of immunosenescence.

Key words: Animal models · CD4$^+$ T cells · Intestinal immunity · Mucosal immunity

Introduction

Inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis, are thought to result from the inappropriate activation and expansion of colitogenic CD4$^+$ T cells, which are driven by activated macrophages and dendritic cells. Antigens derived from the persistently present commensal bacteria continuously stimulate such cells, and this is presumably required for the induction of colitis. In general, IBD progresses steadily or with transient remissions throughout life [1, 2]. Importantly, the recurrent disease shows similar clinical features to the previous disease episode, and it is extremely uncommon that a patient with Crohn's disease relapses with another form of disease, such as ulcerative colitis [3–6]. Thus, it is conceivable that the sequential disease episodes are driven by a group of disease-specific colitogenic CD4$^+$ memory T cells, which may be designated as 'memory stem cells' [7] of the disease. In this scenario, colitogenic CD4$^+$ effector T cells established in the initial attack seem to arise repeatedly from the colitogenic CD4$^+$ memory T cells, but are presumably suppressed by regulatory T (Treg) cells during remission [8].

Although it appears that memory T cells are generally long living [9, 10] as shown by a series of successful vaccine programs, the details of their longevity are still unknown. In fact, it has been reported that the number of memory CD4$^+$ T cells declines over time in mice infected with lymphocytic choriomeningitis virus (LCMV) [11]. Furthermore, decline of the immune function due to the immunosenescence may affect the maintenance of memory CD4$^+$ T cells [12–14]. To evaluate this unsolved issue, we assessed the longevity of colitogenic lamina propria (LP) CD4$^+$ T cells and

Correspondence: Takanori Kanai
e-mail: taka.gast@tmd.ac.jp

* These authors contributed equally to this work.

the characteristics of immunosenescent colitogenic LP CD4$^+$ T cells in a murine model of chronic colitis induced by adoptive transfer of CD4$^+$CD45RB^{high} T cells [15]. The present model is useful for this purpose, because the primarily transferred CD4$^+$CD45RB^{high} T cells in the recipient can be tracked over time, thereby allowing us to exclude the impact of new naive CD4$^+$ T cells that are continuously supplied from the thymus. Furthermore, we performed sequential adoptive transfers of the colitic LP CD4$^+$ T cells after developing CD4$^+$CD45RB^{high} T cell- or colitic LP CD4$^+$ T cell-transferred colitis in SCID mice. This model is also very useful to induce the extremely rapid proliferation of colitogenic LP CD4$^+$ T cells, which presumably respond to commensal bacterial-driven or autogenous antigens by lymphopenia-driven proliferation [9]. Using this unique sequential adoptive transfer model of colitogenic LP CD4$^+$ cells in SCID mice, we assessed the characteristics of immunosenescent colitogenic LP CD4$^+$ T cells that were generated by repeated transfers into lymphopenic host mice.

Results

Incidence of colitis is gradually decreased by repeated transfers of colitic LP CD4$^+$ T cells

We previously demonstrated that LP CD4$^+$ T cells obtained from colitic SCID mice that received adoptive transfer of CD4$^+$CD45RB^{high} T cells (the first transfer) are colitogenic CD44^{high}CD62L$^-$CD4$^+$IL-7Rα^{high} effector-memory (T_{EM})-like cells [16]. SCID mice transferred with such colitic LP CD4$^+$ T cells (the second transfer) develop colitis similar to the original CD4$^+$CD45RB^{high} T cell-transferred colitis in an IL-7-dependent manner [17]. This adoptive transfer model is also characterized by the rapid proliferation of donor CD4$^+$ T cells by lymphopenia-driven proliferation [9, 17], which provides an advantageous tool to assess the longevity and change in characteristics of these colitic LP CD4$^+$ T cells during repetitive transfer into SCID mice (Fig. 1A). As a rule of the current protocol, each mouse was killed when it reached over four points of the ongoing clinical score (see *Materials and methods*) within 40 weeks from transfer. Isolated LP CD4$^+$ T cells were then transferred into new SCID mice, and the procedure was repeated until the recipient mice failed to develop colitis within 40 weeks from transfer. Recipient mice that did not develop colitis within 40 wk from transfer were judged to be non-colitic, and were sacrificed for further assessment (Fig. 1A).

Although the interval between transfers gradually lengthened with the increase in number of transfers after the second (Fig. 1B), all the recipient mice examined until the sixth transfer stably developed wasting disease with colitis within 40 weeks from transfer. After the seventh transfer, however, some mice showed no sign of colitis up to 40 weeks from transfer as assessed by the ongoing clinical score (Fig. 1C), and the incidence of colitis development decreased (Fig. 1D). To further assess whether the cell viability of the transferred cells affected the present results, we performed Annexin V/PI staining of cells directly isolated from LP of mice by flow cytometry. As shown in Fig. 1E, there were no differences in the ratio of viable Annexin$^-$/PI$^-$ cells among LP CD4$^+$ T cells obtained from original CD4$^+$CD45RB^{high} T cell-transferred colitic mice (1^o colitic CD4$^+$), LP CD4$^+$ T cells obtained from colitic mice transferred with colitic LP CD4$^+$ T cells that were sequentially transferred over seven times (>7^o colitic CD4$^+$), and LP CD4$^+$ T cells from non-colitic mice transferred with colitic LP CD4$^+$ T cells that were sequentially transferred over seven times (>7^o non-colitic CD4$^+$).

Pattern of TCR Vβ are equivalent irrespective of the number of transfer

One reason why repeated transfer of colitic LP CD4$^+$ T cells leads to delayed onset and decreased incidence of the murine colitis may be that extensively proliferating colitogenic CD4$^+$ T cell clones are selectively depleted over time. Thus, we next checked TCR Vβ repertoire patterns of the 1^o colitic CD4$^+$, >7^o colitic CD4$^+$, and >7^o non-colitic CD4$^+$ T cells by flow cytometry. As depicted in Fig. 2, although the patterns of TCR Vβ repertoire were actually skewed into some group of TCR Vβ repertoire after both single adoptive and multiple adoptive transfers compared to those before transfer, they never integrated into a single specific TCR Vβ repertoire.

Non-colitic LP CD4$^+$ T cells generated by repeated transfer are inactivated

We next compared the immunological phenotypes of the 1^o colitic, the >7^o colitic, and the >7^o non-colitic CD4$^+$ T cells. We first compared the phenotypic composition of these cells in the colonic LP and in the spleen (SP) of mice transferred with the corresponding cells. As shown in Fig. 3, the number of cells recovered from LP or SP was, as expected, significantly lower in mice transferred with the >7^o non-colitic CD4$^+$ T cells than in mice transferred with the 1^o colitic or >7^o colitic CD4$^+$ T cells.

Cell surface markers of the 1^o colitic, >7^o colitic, and >7^o non-colitic SP and LP CD4$^+$ T cells had a phenotype of CD44^{high}CD62L$^-$IL-7Rα^{high} T_{EM}-like cells (Fig. 4A). An activation marker, CD69, was expressed on approximately two thirds of the 1^o colitic, >7^o colitic, and >7^o non-colitic LP CD4$^+$ T cells and on one third of the 1^o colitic and >7^o colitic SP CD4$^+$ T cells, but was markedly down-modulated on the >7^o non-colitic SP CD4$^+$ T cells (Fig. 4A), indicating that >7^o non-colitic SP CD4$^+$ T cells were inactivated. Since it has recently been suggested that a costimulatory molecule, PD-1, might serve as a useful marker to indicate the degree of non-functional T cell exhaustion on virus-specific CD8$^+$ and CD4$^+$ T cells [18–20], we assessed the expression of this molecule on our cells. As expected, PD-1 expression was significantly up-regulated on the >7^o non-colitic CD4$^+$ T cells both in the LP and SP as compared with the paired 1^o colitic and >7^o colitic CD4$^+$ T cells (Fig. 4A). In contrast, no difference in CD28 expression was observed among the 1^o colitic, >7^o colitic, and >7^o non-colitic

$CD4^+$ T cells (Fig. 3A). These results were also confirmed by statistical analysis (Fig. 4B).

We next determined cytokine production by anti-CD3/CD28 mAb-stimulated 1^o colitic, $>7^o$ colitic, and $>7^o$ non-colitic $CD4^+$ T cells. As shown in Fig. 5, the $>7^o$ $CD4^+$ T cells, whether colitic or non-colitic, produced markedly less IFN-γ, IL-2, TNF-α, IL-10, IL-4, and IL-13 than 1^o colitic $CD4^+$ T cells. The production of IL-17 by the $>7^o$ colitic and $>7^o$ non-colitic $CD4^+$ T cells was significantly lower than that by the 1^o colitic $CD4^+$ T cells, although it was not completely abolished. Notably, the production of IL-17 by the $>7^o$ non-colitic $CD4^+$ T cells was significantly lower than that by the $>7^o$ colitic $CD4^+$ T cells. None of the cells produced TGF-β upon the present *in vitro* stimulation. These results suggested that the $>7^o$ non-colitic $CD4^+$ T cells have a functional defect in both activation and cytokine production, presumably due to immunosenescence or exhaustion induced by lymphopenia-induced proliferation for over 2 years.

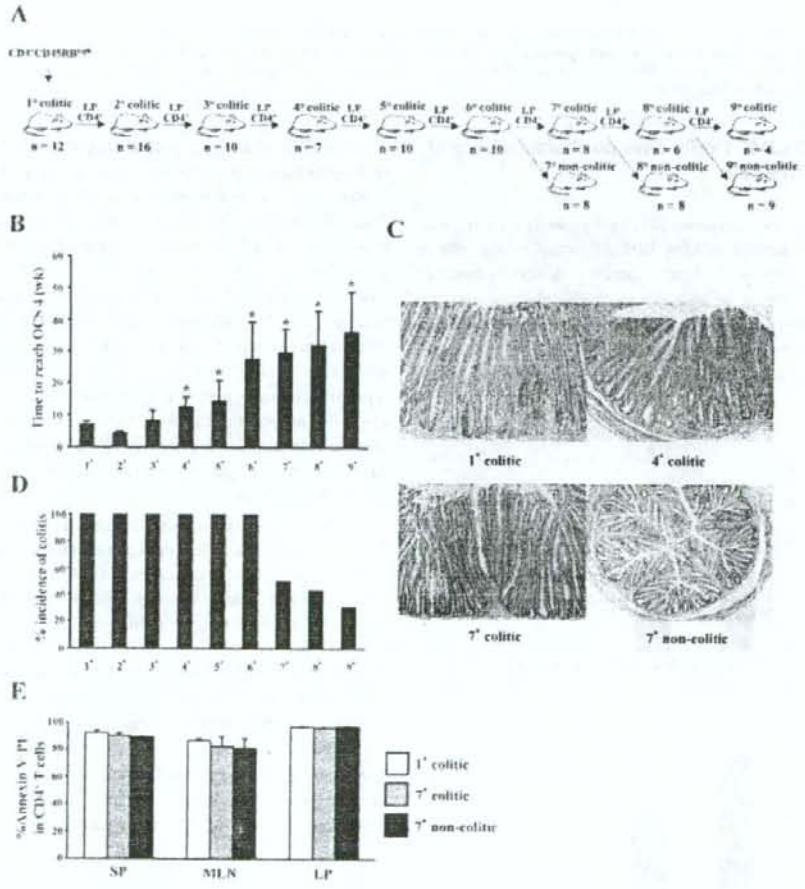

Figure 1. Incidence of colitis induced by adoptive transfer of colitic LP $CD4^+$ T cells gradually decreased as the transfers progress. (A) Schematic transfer protocol. C.B-17 SCID mice were transferred with BALB/c $CD4^+CD45RB^{high}$ T cells (1^o colitic). When they reached an ongoing clinical score of four (see *Materials and methods*), LP $CD4^+$ T cells were isolated, and transferred into new SCID mice. Transfer of LP $CD4^+$ T cells was repeated up to nine times (2^o–9^o colitic), but was terminated when colitis did not develop within 40 weeks from transfer (7^o–9^o non-colitic). (B) The mean interval between transfer and establishment of colitis with an ongoing clinical score of four. Mice that did not develop colitis within 40 weeks from transfer were excluded for this index and judged to be non-colitic. OCS, ongoing clinical score. *$p < 0.05$ *vs.* the 2^o transfer. (C) Histopathological findings of the colon. Original magnification, ×100. (D) The mean incidence rate of colitis in each transfer group. (E) Viability of cells directly isolated from LP of 1^o colitic, $>7^o$ colitic, or $>7^o$ non-colitic mice. The number of viable cells (Annexin V /PI) was determined by a flow cytometry. Data are presented as mean ± SEM of % PI^+ Annexin V /PI cells from four mice in each group. NS, not significantly different.

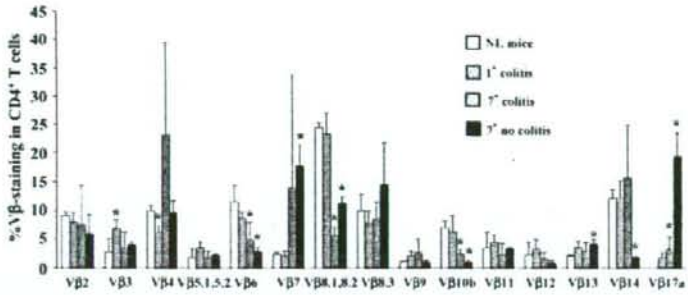

Figure 2. Flow cytometric analysis of the expression of Vβ families on the surface of the splenic normal, 1° colitic, >7° colitic, or >7° non-colitic CD4+ T cells. To analyze the TCR Vβ family repertoire, splenic cells were triple-stained with PECy5-conjugated anti-CD4 mAb and the following a panel of 15 FITC-conjugated Vβ mAb. Each percentage value indicates the frequency of each Vβ pooled from three independent experiments (each transfer; n=6). *$p<0.05$ *vs.* normal BALB/c mice.

Non-colitic LP CD4+ T cells have no characteristics of CD4+Foxp3+ Treg cells

Vukmanvic-Stejic and colleagues [21] have recently reported that a proportion of peripheral $CD4^+CD25^{high}Foxp3^+$ Treg cells in humans are generated from rapidly dividing memory $CD4^+CD45RO^+$ T cells in addition to thymus-derived classical $CD4^+CD25^{high}Foxp3^+$ Treg cells. Furthermore, colitogenic $CD4^+$ T cells in this colitis model proliferate and expand in response to foreign antigens more rapidly in immunodeficient SCID mice than do slow-dividing antigen-specific 'true' memory T cells [17]. We thus hypothesized that the colitogenic LP $CD4^+$ T cells gradually convert after multiple transfers to cytokine-non-producing $CD4^+$ Treg cells that have not only lost the ability to induce colitis but, conversely, gained the ability to suppress colitis. To assess this possibility, we next explored whether the >7° non-colitic $CD4^+$ T cells retain Treg cell activity *in vitro*. Since it has been shown that resting $CD4^+$ Treg cells express Foxp3 [22], we first analyzed the expression of Foxp3 in the 1° colitic, >7° colitic, and >7° non-colitic $CD4^+$ T cells, with splenic $CD4^+CD25^+$ Treg cells serving as a positive control. Unexpectedly, intracellular Foxp3 expression in the >7° non-colitic $CD4^+$ T cells was slight, and was not significantly higher than that in the 1° colitic and >7° colitic $CD4^+$ T cells, while the splenic $CD4^+CD25^+$ Treg cells expressed Foxp3 at a high level (Fig. 6A).

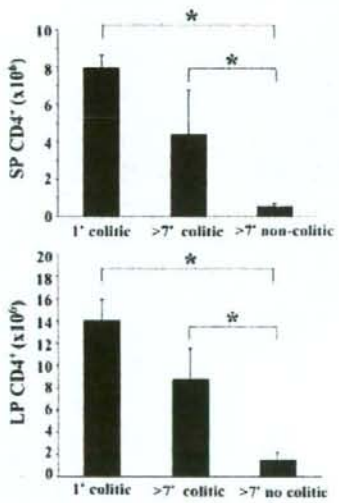

Figure 3. Expansion of $CD4^+$ T cells in the >7° non-colitic mice was significantly decreased. SP and LP $CD4^+$ T cells were isolated from colons when the colitic mice reached an ongoing clinical score of four, or the non-colitic mice lived up to 40 weeks post transfer. The number of $CD4^+$ T cells was determined by flow cytometry. Data are indicated as mean ± SEM of six mice in each group. *$p<0.05$ *vs.* the 1° coltic mice.

To further assess the possibility that the >7° non-colitic $CD4^+$ T cells may function as Treg cells, we examined whether these cells could suppress the proliferation of $CD4^+$ responder T cells in *in vitro* co-culture assay. Although splenic $CD4^+CD25^+$ Treg cells were able to suppress the proliferation of splenic $CD4^+CD25^-$ responder cells at a ratio of 1:1 to 1:0.125 of responder/Treg cells in the presence of mitomycin-C (MMC)-treated $CD4^-$ APC and soluble anti-CD3 mAb, the >7° non-colitic $CD4^+$ T cells and 1° colitic $CD4^+$ T cells could not suppress the proliferation at any ratio (Fig. 6B). Thus, at least in *in vitro* analysis, the >7° non-colitic $CD4^+$ T cells were a completely distinct cell population from peripherally induced $CD4^+Foxp3^+$ Treg cells, IL-10-producing Tr1 cells (Fig. 5) [23], and TGF-β-producing Th3 cells (Fig. 5) [24].

Co-transfer of non-colitic LP CD4+ T cells suppresses the development of colitis

Although the >7° non-colitic LP $CD4^+$ T cells did not show a regulatory function in the *in vitro* co-culture assay, such assays do not always represent *in vivo* function. To assess whether the >7° non-colitic LP $CD4^+$ T cells have characteristics of Treg cells and

can suppress colitis *in vivo*, we performed an *in vivo* adoptive transfer experiment with four groups of SCID mice: group 1, new SCID mice transferred with $CD4^+CD45RB^{high}$ T cells alone (3×10^5) as a positive control; group 2, SCID mice transferred with $CD4^+CD45RB^{high}$ T cells (3×10^5) and $CD4^+CD25^+$ Treg cells (1×10^5) as a negative control; group 3, SCID mice

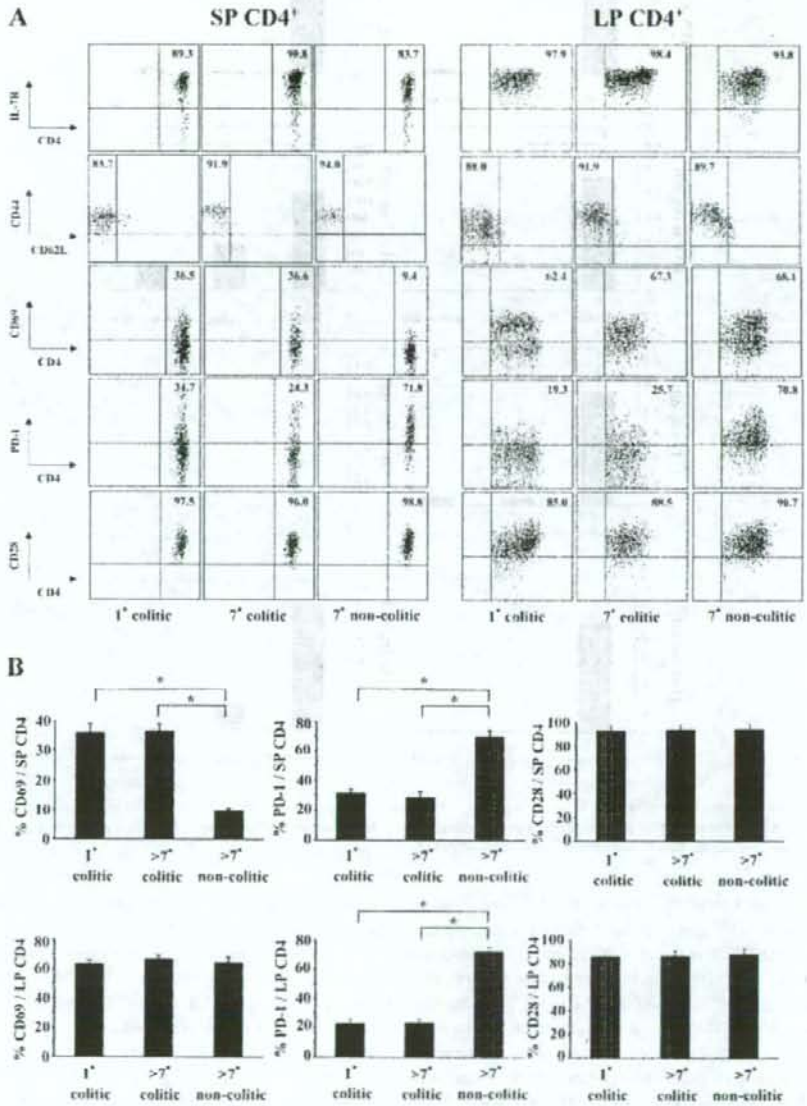

Figure 4. Expression of various cell surface markers on freshly isolated SP and LP $CD4^+$ T cells from 1° colitic, >7°colitic, and >7° non-colitic mice. (A) Representative analysis of IL-7Rα, CD62L, CD69, PD-1, and CD28 expression on SP or LP $CD4^+$ cells from 1° colitic, >7° colitic, and >7° non-colitic mice. Cells were stained with either FITC-conjugated anti-CD4, and the indicated biotinylated mAb, followed by PE-conjugated streptavidin or with fluorochrome-conjugated control Ig (not shown). (B) Percent positive cells of CD69, PD-1, and CD28 expression among SP or LP $CD4^+$ cells from 1° colitic, >7° colitic, and >7° non-colitic mice were determined by flow cytometry. Data are indicated as mean + SEM of seven mice in each group. *$p < 0.05$.

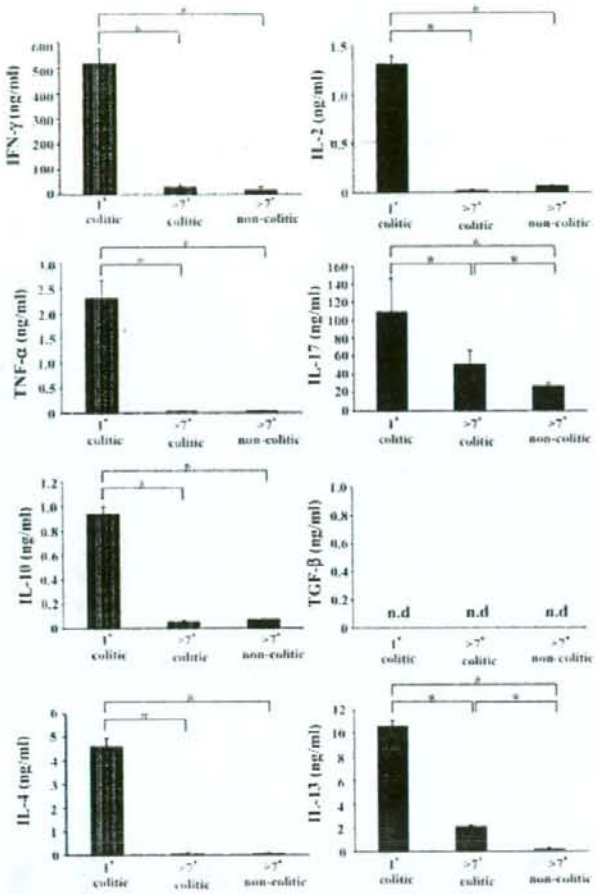

Figure 5. Cytokine production by LP CD4$^+$ T cells from 1° colitic, >7° colitic, and >7° non-colitic mice. LP CD4$^+$ T cells were isolated, and stimulated with anti-CD3/CD28 mAb for 48 h. The indicated cytokines in these supernatants were measured by ELISA. Data are shown as mean ± SD of seven mice in each group. *$p < 0.05$ vs. the 1° colitic mice. n.d., not detected.

transferred with CD4$^+$CD45RB^{high} T cells (3×10^5) and 1° colitic LP CD4$^+$ T cells (1×10^5); and group 4, SCID mice transferred with CD4$^+$CD45RB^{high} T cells (3×10^5) and >7° non-colitic CD4$^+$ LP T cells (1×10^5) (Fig. 7A). Mice were killed 6 weeks after transfer. Surprisingly, the >7° non-colitic LP CD4$^+$ T cell fraction, like the control CD4$^+$CD25$^+$ Treg fraction, clearly showed a regulatory function toward intestinal inflammation, as these cell types both significantly inhibited the development of both wasting disease and colitis, when co-transfer with CD4$^+$CD45RB^{high} T cells (Fig. 7B–E). Colons of group 4 mice exhibited no pathological changes and were indistinguishable from the colons of group 2 mice (negative control) (Fig. 7B and D). In contrast, group 1 mice (positive control) and group 3 mice both developed wasting disease with severe colitis (Fig. 7B and D). The clinical and histological scorings also statistically confirmed these results (Fig. 7C and E).

A further quantitative evaluation of CD4$^+$ T cell infiltration was made by isolating the SP and LP CD4$^+$ T cells. As depicted in Fig. 7F, significantly fewer CD4$^+$ T cells were recovered from the SP and LP of mice reconstituted with CD4$^+$CD45RB^{high} and >7° non-colitic CD4$^+$ T cells (group 4) or CD4$^+$CD45RB^{high} + CD4$^+$CD25$^+$ T_R cells (group 2) as compared with mice reconstituted with CD4$^+$CD45RB^{high} cells alone (group 1) or CD4$^+$CD45RB^{high} cells + 1° colitic CD4$^+$ T cells (group 3).

To determine the effect of >7° non-colitic CD4$^+$ T cells on Th1 development, we measured IFN-γ, IL-2, and TNF-α production by anti-CD3/CD28-stimulated CD4$^+$ LP T cells. As shown in Fig. 7G, the production of IFN-γ, IL-2 and TNF-α was significantly reduced

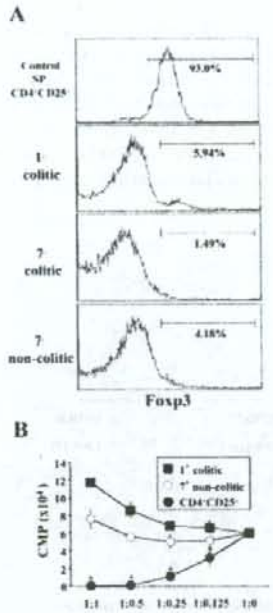

Figure 6. $CD4^+$ T cells obtained from non-colitic mice after seven or more transfers did not have a regulatory character in *vitro*. (A) Expression of Foxp3 in the indicated subpopulations was determined by flow cytometry as described in *Materials and methods*. (B) Suppressive activity of the indicated subpopulations was determined at a responder/Treg ratio of 1:0, 1:0.125, 1: 0.25, 1:0.5, or 1:1. $^*p<0.05$.

by the co-transfer of $CD4^+CD45RB^{high}$ and >7° non-colitic $CD4^+$ T cells (group 4) or $CD4^+CD45RB^{high}$ and $CD4^+CD25^+$ T_R cells (group 2) as compared with that of $CD4^+CD45RB^{high}$ T cells alone (group 1) or $CD4^+CD45RB^{high}$ and 1° non-colitic $CD4^+$ T cells (group 3). Collectively, these results indicated that, at least *in vivo*, the >7° non-colitic $CD4^+$ T cells act as Treg cells to suppress the development of Th1-mediated colitis in a comparable manner to the control $CD4^+CD25^+$ Treg cells.

Discussion

In the present study, we performed seven or more sequential adoptive transfers of colitogenic LP $CD4^+$ T cells obtained from colitic SCID mice into new SCID mice. The SCID mice transferred with colitic LP $CD4^+$ T cells stably developed colitis, but interestingly the interval between transfer and development of colitis gradually lengthened as the number of transfers increased. Furthermore, the incidence of colitis gradually decreased after seven sequential transfers, accompanied by markedly increased expression of PD-1 but decreased production of various cytokines by the LP $CD4^+$ T cells. Importantly, transfer of non-colitic LP $CD4^+$ T cells that were recovered after seven or more transfers suppressed the development of colitis in SCID mice, which should have been induced by the transfer of $CD4^+CD45RB^{high}$ T cells. Collectively, LP $CD4^+$ T cells that are colitogenic in origin may differentiate into $CD4^+$ Treg cells through the process of immunological exhaustion caused by lymphopenia-driven rapid proliferation [9], and gain the ability to suppress colitis. These findings have important implications for our understanding of the nature of colitogenic $CD4^+$ T cells as well as the natural course of IBD.

To exclude the possibility that changes of the bacterial flora in the examined mice affected the incidence of colitis in the present study, we routinely checked whether the examined mice might have been infected by pathological bacteria such as *Helicobacter hepaticus*, but found no evidence of contaminating bacteria throughout the experimental period for over 3 years (data not shown). In addition, we confirmed that colitis could be stably induced in SCID mice by transfer of $CD4^+CD45RB^{high}$ T cells throughout this period, including the time when colitis did not develop in some SCID mice that were co-transferred with immunosenescent LP $CD4^+$ cells. Nevertheless, further study will be needed to address this issue, since we could not evaluate the components of non-pathological commensal bacteria using a comparative 16S-rRNA-gene-sequence survey in the examined mice. Also it has recently been reported that the bacterial flora of diseased and non-diseased animals are apparently distinct, even if they appear to be in the same environment [25].

Although accumulating evidence from the models of acute virus infection suggests that memory T cells, especially $CD8^+$ memory T cells, are long living [9, 10], it remains controversial whether this is also the case with $CD4^+$ memory T cells [26]. Furthermore, it is believed that "true" memory T cells, especially $CD8^+$ memory T cells, are established after the first clearance of the corresponding antigens [27], but this is also doubtful in the case of $CD4^+$ memory T cells. Conversely, recent reports suggest that both homeostatic stimulation by IL-7 and antigenic stimulation are needed for the full maintenance of $CD4^+$ memory T cells [28]. In fact, we showed here that the colitic $CD4^+IL\text{-}7R\alpha^{high}$ T cells were stably transferable to new SCID mice, and that they continued to induce colitis in the presence of commensal bacteria through at least six transfers over a period of more than 2 years without additional supply of naive $CD4^+$ T cells.

We believe that the immunological memory of antigens is not related to the requirement of antigen clearance from the host body, because antigen-specific effector or memory T cells are inevitably separated from antigen-loading dendritic cells residing at draining lymph nodes, which leave there regardless of the presence of a corresponding antigen in the body [29]. It seems, however, that the interval before antigen-specific effector or memory T cells re-encounter the same antigen will be shorter. In fact, we previously demonstrated that colitogenic $CD4^+CD44^{high}IL\text{-}7R\alpha^{high}$ T cells reside within the SP and bone marrow [30], which lack commensal bacterial antigens. Thus, it is possible and also reasonable that separation from sites where the corresponding antigen resides, rather than complete removal of antigens, is important for the generation of memory T cells. Furthermore,

accumulating evidence suggests that IL-7 dependency is a reliable assumption for $CD4^+$ memory T cells *in vivo* [31]. Consistently, we previously demonstrated that colitic LP $CD4^+$ T cells from colitic $CD4^+CD45RB^{high}$ T cell-transferred RAG-$2^{-/-}$ mice express representative cell surface markers of memory T cells such as IL-7Rα and CD44 at a high level, and that the IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipient mice transferred with $CD4^+CD45RB^{high}$ T cells or colitic LP $CD4^+$ T cells never develop colitis [17].

However, even if the colitogenic $CD4^+$ T cells found in the $CD4^+CD45RB^{high}$ T cell-transferred colitic mice can be called memory T cells, or 'persistent antigen-specific T cells' [27], their longevity and how their characteristics change with immunosenescence through multiple rounds of cell division. To evaluate this issue, we conducted sequential adoptive transfers of colitic LP $CD4^+$ T cells into new SCID mice. Although this method may be artificial, it is quite useful to examine colitic LP $CD4^+$ T cells that

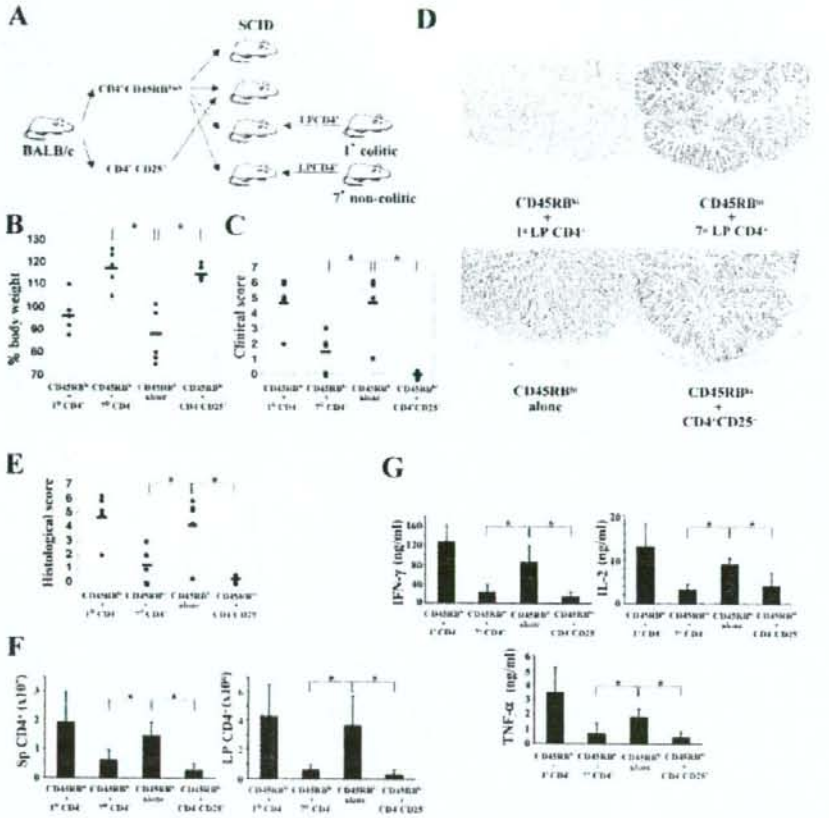

Figure 7. Cotransfer of LP $CD4^+$ T cells from 7° non-colitic mice prevents the development of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID mice. (A) New SCID mice were divided into four groups: mice transferred with $CD4^+CD45RB^{high}$ cells (3×10^5 per mouse) alone as a positive control, mice transferred with $CD4^+CD45RB^{high}$ cells (3×10^5) and $CD4^+CD25^+$ cells (1×10^5) as a negative control, mice transferred with $CD4^+CD45RB^{high}$ cells (3×10^5) and 1° colitic LP $CD4^+$ cells (1×10^5), and mice transferred with $CD4^+CD45RB^{high}$ cells (3×10^5) and 7° non-colitic LP $CD4^+$ cells (1×10^5). Mice were killed six weeks after transfer. Each experiment was performed with groups of three mice each. The data are the sum of three independent experiments (n=9). (B) Change in body weight over time is expressed as percent of the original weight. Data are represented as mean ± SEM of nine mice in each group. *$p < 0.05$ compared to mice transferred with $CD4^+CD45RB^{high}$ cells alone. (C) Clinical scores were determined at 6 weeks after transfer. Data are indicated as mean + SEM of nine mice in each group. *$p < 0.05$ compared to mice transferred with $CD4^+CD45RB^{high}$ cells alone. (D) Histological examination of the colon from each group of mice at 6 weeks after transfer. Original magnification, ×100. (E) Histological scores were determined at 6 weeks after transfer. Data are indicated as the mean ± SEM of nine mice in each group. *$p < 0.05$ compared to mice transferred with $CD4^+CD45RB^{high}$ cells alone. (F) SP and LP mononuclear 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 nine mice in each group. *$p < 0.05$ compared to mice transferred with $CD4^+CD45RB^{high}$ cells alone. (G) Cytokine production by LP $CD4^+$ T cells. LP $CD4^+$ T cells were prepared from the colons at 6 weeks after transfer and stimulated with anti-CD3/CD28 mAbs for 48 h. The indicated cytokines in these supernatants were measured by ELISA. Data are indicated as mean + SEM of six mice in each group, which was selected from the first two *in vivo* experiments. *$p < 0.05$ compared to mice transferred with $CD4^+CD45RB^{high}$ cells alone.

have undergone multiple rounds of cell division. In this study, we found that (i) the interval between transfer and development of colitis gradually lengthened as the number of transfers increased, (ii) a certain population of the recipient SCID mice did not develop colitis after seven or more transfers, and (iii) the production of IFN-γ, TNF-α and IL-17 by LP CD4$^+$ T cells recovered after seven or more transfers was significantly impaired. These data clearly indicate that colitogenic CD4$^+$ T cells are gradually exhausted over time and finally lose the ability to induce colitis. However, it remains unclear whether LP CD4$^+$ T cells recovered from recipient mice and maintained for over 40 weeks post transfer are able to induce colitis, since the designation of mice that did not develop colitis within 40 weeks post transfer as non-colitic was made arbitrarily. Further study will be needed to address this issue.

Most notably, however, we also found that new SCID mice transferred with CD4$^+$CD45RB^{high} T cells and LP CD4$^+$ T cells obtained from non-colitic SCID mice after seven transfers did not develop colitis. This finding further indicates that colitogenic CD4$^+$ T cells not only lose their colitogenicity over time, but also gain a regulatory function like CD4$^+$ Treg cells through the process of immunosenescence, and suppress colitis. It is also possible that LP CD4$^+$ T cells obtained from non-colitic SCID recipient mice may simply delay, but not completely suppress, the development of colitis through competition for cytokines (cytokine deprivation) [32] between newly recruited effector cells. However, our findings may also correlate with the clinical nature of IBD, as the majority of patients actually run a chronic or relapsing course, whereas patients with severe symptoms show diminishing severity of symptoms over time, presumably through the immunosenescence of colitogenic CD4$^+$ T cells along with the decrease of new naive T cell supply from the thymus [33].

At the moment, it is largely unknown which type of Treg cells is closely associated with the immunosenescent LP CD4$^+$ T cells described in the current study, which were colitogenic in origin, but acquired regulatory activity to suppress the development of colitis. A recent publication by Vukmanovic-Stejic and colleagues [21] demonstrated that a substantial proportion of peripheral human CD4$^+$CD25^{high}Foxp3$^+$ Treg cells is generated from rapidly dividing, highly differentiated CD4$^+$ memory T cells in addition to the cells of same phenotype derived from the thymus. Moreover, Liu and colleagues [34] reported that the interaction between neurons and CD4$^+$ T cells results in the conversion of encephalogenic CD4$^+$ T cells to CD4$^+$CD25$^+$Foxp3$^+$ T_R cells in a murine model of experimental autoimmune encephalomyelitis (EAE). Although peripherally inducible CD4$^+$CD25$^+$Foxp3$^+$ Treg cells appear quite similar to our immunosenescent LP CD4$^+$ T cells in that they are generated by continuous stimulation of antigens in the periphery, we could not detect the up-regulation of Foxp3 in the immunosenescent LP CD4$^+$ T cells.

It has recently been reported that PD-1 is a marker for exhausted CD8$^+$ and CD4$^+$ T cells in chronic lymphocytic choriomeningitis virus (LCMV) and HIV infections [18–20]. Consistent with this, we demonstrated that LP CD4$^+$ T cells obtained from non-colitic mice after over seven or more transfers expressed significantly higher levels of PD-1. In addition, we previously showed that peripheral CD4$^+$PD-1$^+$ T cells in normal mice possess a regulatory function both *in vivo* and *in vitro*, regardless of the expression of CD25 [35]. Thus, it is possible that the PD-1/PD-L1 pathway is required for immunosenescent LP CD4$^+$ T cells to function *in vivo* as Treg cells, although further study using mAb to block the PD-1/PD-L signal pathway is needed to address this issue. Apart from Treg cells, it is also noteworthy that dying exhausted cells (apoptotic cells) are frequently associated with an immunosuppressive activity against other immune cells including CD4$^+$ T cells [36, 37]. This mechanism involves anti-inflammatory TGF-β released by macrophages, which phagocyte the apoptotic cells. Although we could not detect decreased viability (Fig. 1) or increased TGF-β activity (Fig. 5) of the non-colitic LP CD4$^+$ T cells after seven transfers, it is conceivable that exhausted LP CD4$^+$ T cells are phagocytosed by surrounding macrophages, and the production of anti-inflammatory cytokines by such cells may be involved in the induction of immunosuppression. Further studies will be needed to address the regulatory mechanism of the immunosenescent LP CD4$^+$ T cells.

Finally, our results should be discussed in connection with a recent publication by Abadia-Molina and colleagues [38], which reported serial adoptive transfer of colitic CD4$^+$ T cells residing in the mesenteric lymph nodes (MLN) of their original model of colitis induced by the transplantation of wild-type bone marrow into adult tgε26 mice (called BM→tgε). They demonstrated that the isolated MLN CD4$^+$ T cells in colitic BM→tgε mice not only maintained colitogenicity with a dominant Th1 phenotype after over eight or more transfers, but also converged into a single TCR Vβ usage (Vβ8.1/2, Vβ8.3, Vβ10b or Vβ14) of up to 90% in a certain line of colitic mice, leading to a novel method for cloning colitogenic CD4$^+$ cells through serial adoptive transfers. In our system using LP cells as donor cells, however, we could not detect any convergence of TCR Vβ usage in Vβ8.1/2, Vβ8.3, Vβ10b and Vβ14 (Fig. 2). Furthermore, we found that the ability to reproduce colitis upon sequential transfer gradually decreased in terms of the interval between transfers and the incidence of colitis. These discrepancies would be explained by differences in the model of colitis; differences in the presence of NK and B cells, and differences in the type of donor cells, those from MLN being rich in central memory T (T_{CM}) cells, and those from LP being rich in T_{EM} cells [39]. Further study will be needed to address this issue.

In summary, we demonstrated through our unique model of sequential adoptive transfers into lymphopenic SCID mice that colitogenic CD4$^+$ T_{EM}-like cells in colitic mice are exhausted over time and are finally converted into cytokine-non-producing Treg cells that suppress the development of colitis. Thus, our current study may provide a new approach for the treatment of IBD by transfer of immunosenescent CD4$^+$ T cells generated from colitogenic CD4$^+$ T cells by *in vitro* acceleration of cell divisions to promote their regulatory function.