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Systemic, but Not Intestinal, IL-7 Is Essential for the Persistence of Chronic Colitis¹

Takayuki Tomita, Takanori Kanai,² Yasuhiro Nemoto, Teruji Totsuka, Ryuichi Okamoto, Kiichiro Tsuchiya, Naoya Sakamoto, and Mamoru Watanabe

We previously demonstrated that IL-7 is produced by intestinal goblet cells and is essential for the persistence of colitis. It is well known, however, that goblet cells are decreased or depleted in the chronically inflamed mucosa of animal colitis models or human inflammatory bowel diseases. Thus, in this study, we assess whether intestinal IL-7 is surely required for the persistence of colitis using a RAG-1/2^{-/-} colitis model induced by the adoptive transfer of CD4⁺CD45RB^{high} T cells in combination with parabiosis system. Surprisingly, both IL-7^{-/-} × RAG-1^{-/-} and IL-7^{+/+} × RAG-1^{-/-} host mice developed colitis 4 wk after parabiosis to a similar extent of colitic IL-7^{+/+} × RAG-1^{-/-} donor mice that were previously transferred with CD4⁺CD45RB^{high} T cells. Of note, although the number of CD4⁺ T cells recovered from the spleen or the bone marrow of IL-7^{-/-} × RAG-1^{-/-} host mice was significantly decreased compared with that of IL-7^{+/+} × RAG-1^{-/-} host mice, an equivalent number of CD4⁺ T cells was recovered from the lamina propria of both mice, indicating that the expansion of CD4⁺ T cells in the spleen or in the bone marrow is dependent on IL-7, but not in the lamina propria. Development of colitis was never observed in parabionts between IL-7^{+/+} × RAG-1^{-/-} host and noncolitic IL-7^{-/-} × RAG-1^{-/-} donor mice that were transferred with CD4⁺CD45RB^{high} T cells. Collectively, systemic, but not intestinal, IL-7 is essential for the persistence of colitis, suggesting that therapeutic approaches targeting the systemic IL-7/IL-7R signaling pathway may be feasible in the treatment of inflammatory bowel diseases. *The Journal of Immunology*, 2008, 180: 383–390.

Inflammatory bowel disease (IBD)³ are caused by chronic inflammatory responses in the gut wall, commonly take persistent courses, but in some patients relapse after remissions (1–6). Because the recurrent disease usually mimics the primary disease episode, it is possible that the disease is caused by the repeated activation and expansion of colitogenic effector CD4⁺ T cells arising from common long-lived colitogenic memory CD4⁺ T cells, which latently reside in their target tissues or in some reservoirs. Nevertheless, the nature of the colitogenic memory CD4⁺ T cells over time is not fully understood.

IL-7 is secreted by stromal cells in the bone marrow (BM) and thymus, and epithelial cells including the intestine (7–10). Recent findings revealed that IL-7 is an important cytokine supporting the survival of resting naive and memory CD4⁺ T cells, but not effector CD4⁺ T cells (9–16). We have previously demonstrated that, 1) IL-7 is constitutively produced by intestinal goblet epithelial cells (8), 2) IL-7 transgenic (Tg) mice, in which IL-7 overexpression was driven by SR α promoter, developed chronic colitis that mimicked histopathological characteristics of human IBD (17), 3) mucosal CD4⁺IL-7R α ^{high} T cells in CD4⁺CD45RB^{high} T cell-transferred colitic mice are colitogenic (18), and 4) IL-7^{-/-} × RAG-1^{-/-} mice transferred with colitogenic lamina propria (LP) CD4⁺ T cells isolated from colitic CD4⁺CD45RB^{high} T cell-transferred mice did not develop colitis (19).

Somewhat at odds, however, we also found that production of intestinal IL-7 was dramatically decreased in the inflamed mucosa of colitic IL-7 Tg mice in accordance with depletion of goblet cells (17). Because our IL-7 Tg mice were established by expressing IL-7 under regulation of the ubiquitous SR α promoter, it was possible that intestinal IL-7 is indeed decreased at the site of mucosal inflammation due to depletion of goblet cells, which is a feature often seen in the inflamed mucosa of human IBD, but systemic IL-7 of other tissue origin, such as BM (20) and thymus (21), is rather critical for the maintenance of colitogenic memory CD4⁺ T cells. Based on these complex backgrounds, in this study, we assess the distinct requirement of intestinal or systemic IL-7 in the development and persistence of colitis using a RAG-1/2^{-/-} colitis model (22, 23) induced by adoptive transfer of CD4⁺CD45RB^{high} T cells in combination with parabiosis system.

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Materials and Methods

Animals

C57BL/6-Ly5.2 mice were purchased from Japan CLEA. C57BL/6-Ly5.1 mice and C57BL/6-Ly5.2-RAG-2-deficient (RAG-2^{-/-}) mice were obtained from Taconic Farms and Central Laboratories for Experimental Animals. C57BL/6-Ly5.2-background RAG-1^{+/+} and IL-7^{+/+} mice were provided from Dr. Rosa Zamoyska (National Institute for Medical Research, London, U.K.) (24). These mice were intercrossed to generate IL-7^{-/-} × RAG-1^{-/-} and IL-7^{+/+} × RAG-1^{-/-} littermate mice in the Animal Care Facility of Tokyo Medical and Dental University (TMDU). Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of TMDU. Donors and recipients were used at 6–12 wk of

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³ Abbreviations used in this paper: IBD, inflammatory bowel disease; BM, bone marrow; LP, lamina propria; SP, spleen; Tg, transgenic; IEL, intraepithelial cell; HPF, high power field; DAPI, 4', 6'-diamidino-2-phenylindole; LN, lymph node.

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age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Parabiosis experimental design

To assess the specific requirement of mucosal or systemic IL-7 in the development of colitis, we performed adoptive transfer experiment in combination with a parabiosis system using IL-7^{+/+} × RAG-1^{-/-} and IL-7^{-/-} × RAG-1^{-/-} littermate recipients (Fig. 1A). 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 anti-mouse CD4 (RM4-5; BD Pharmingen) and FITC-conjugated anti-mouse (16A; BD Pharmingen). CD4⁺CD45RB^{high} cells were purified using a FACSaria (BD Biosciences). This population was >98.0% pure on reanalysis. IL-7^{+/+} × RAG-1^{-/-} mice (*n* = 18) and IL-7^{-/-} × RAG-1^{-/-} mice (*n* = 6) were then injected i.p. with 3 × 10⁵ splenic CD4⁺CD45RB^{high} T cells from normal C57BL/6-Ly5.2 mice. After 6 wk post transfer, IL-7^{+/+} × RAG-1^{-/-} mice, but not IL-7^{-/-} × RAG-1^{-/-} mice, transferred with CD4⁺CD45RB^{high} T cells developed a wasting disease and colitis as previously reported (19).

We then conducted parabiosis surgery according to institutional guidelines and Home Office regulations. In brief, sex-matched mice were anesthetized before 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 two mice into direct physical contact. The outer skin was then attached with surgical staples. For this parabiosis experiment, we divided colitic IL-7^{+/+} × RAG-1^{-/-} (*n* = 18) mice that were previously transferred with CD4⁺CD45RB^{high} T cells into three groups: Group 1, colitic IL-7^{+/+} × RAG-1^{-/-} mice joined with normal C57BL/6-Ly5.1 mice (*n* = 6); Group 2, colitic IL-7^{+/+} × RAG-1^{-/-} mice joined with new IL-7^{-/-} × RAG-1^{-/-} mice (*n* = 6); Group 3, colitic IL-7^{+/+} × RAG-1^{-/-} mice joined with new IL-7^{-/-} × RAG-1^{-/-} mice (*n* = 6). As Group 4, noncolitic IL-7^{-/-} × RAG-1^{-/-} mice previously transferred with CD4⁺CD45RB^{high} T cells were joined with new IL-7^{+/+} × RAG-1^{-/-} mice (*n* = 6). All mice were observed for clinical signs, such as hunched posture, piloerection, diarrhea, and blood in the stool. At autopsy, mice were assessed for a clinical score (25) that is the sum of three parameters as follows: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; 3, bloody stool) (25).

Histological examination

Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. Three tissue samples from the proximal, middle, and distal parts of the colon were prepared. The sections were analyzed without prior knowledge of each mouse. 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 (25) as follows: mucosa damage, 0; normal, 1; 3–10 intraepithelial cells (IEL)/high power field (HPF) and focal damage, 2; >10 IEL/HPF and rare crypt abscesses, 3; >10 IEL/HPF, multiple crypt abscesses and erosion/ulceration, submucosa damage, 0; normal or widely scattered leukocytes, 1; focal aggregates of leukocytes, 2; diffuse leukocyte infiltration with expansion of submucosa, 3; diffuse leukocyte infiltration, muscularis damage, 0; normal or widely scattered leukocytes, 1; widely scattered leukocyte aggregates between muscle layers, 2; leukocyte infiltration with focal effacement of the muscularis, 3; extensive leukocyte infiltration with transmural effacement of the muscularis.

Tissue preparations

Single cell suspensions were prepared from SP, LP, and BM as previously described (18). To isolate LP CD4⁺ T cells, the entire length of the colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca²⁺, Mg²⁺-free HBSS containing 1 mM DTT (Sigma-Aldrich) for 45 min to remove mucus and then treated with 3.0 mg/ml collagenase (Roche) and 0.01% DNase (Worthington Biochemical) for 2 h. The cells were pelleted two times through a 40% isotonic Percoll solution, and then subjected to Ficoll-Hypaque density gradient centrifugation (40/75%). Enriched LP CD4⁺ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells when analyzed by FACSCalibur contained >95% CD4⁺ cells. BM cells were obtained by flushing two femurs with cold RPMI 1640. For in vitro assay, only live cells were counted by using trypan

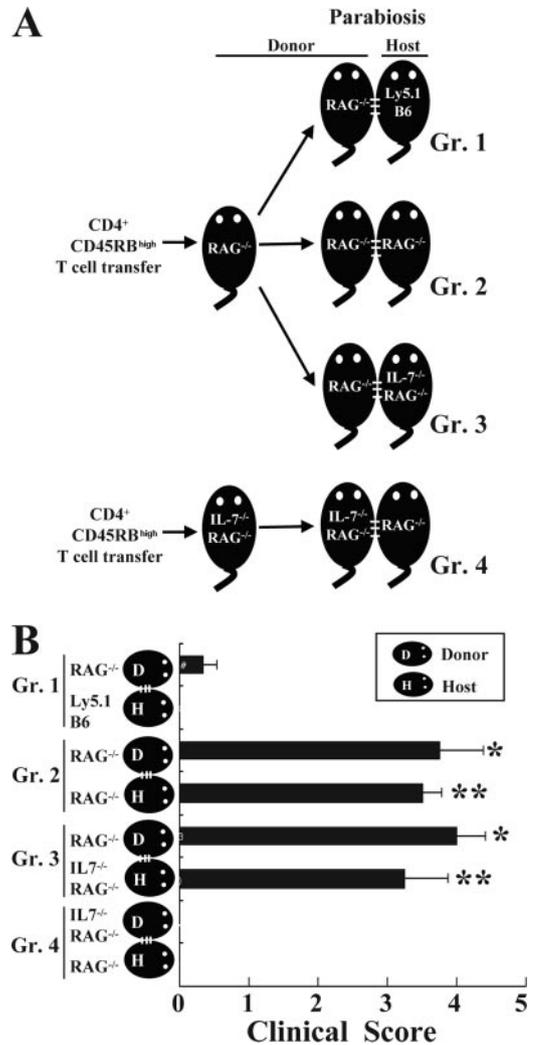


FIGURE 1. Host IL-7^{-/-} × RAG-1^{-/-} mice in parabionts with diseased IL-7^{+/+} × RAG-1^{-/-} donor mice show a wasting disease and clinical signs of colitis. **A**, Parabiosis experimental design. For an adoptive transfer, splenic CD4⁺CD45RB^{high} T cells were isolated from C57BL/6-Ly5.2 mice, and then transferred into female IL-7^{+/+} × RAG-1^{-/-} mice (*n* = 18) and IL-7^{-/-} × RAG-1^{-/-} mice (*n* = 6). Six wk after transfer, IL-7^{+/+} × RAG-1^{-/-}, but not IL-7^{-/-} × RAG-1^{-/-}, mice transferred with CD4⁺CD45RB^{high} T cells developed a wasting disease and colitis. As parabiosis pairs, Group 1 parabionts were joined between colitic donor IL-7^{+/+} × RAG-1^{-/-} mice and normal host C57BL/6-Ly5.1 mice (*n* = 6 pairs). Group 2 parabionts were joined between colitic donor IL-7^{+/+} × RAG-1^{-/-} mice and new host IL-7^{-/-} × RAG-1^{-/-} mice (*n* = 6 pairs). Group 3 parabionts were joined between colitic donor IL-7^{+/+} × RAG-1^{-/-} mice and new host IL-7^{-/-} × RAG-1^{-/-} mice (*n* = 6 pairs). Group 4 parabionts were joined between noncolitic donor IL-7^{-/-} × RAG-1^{-/-} mice and new host IL-7^{+/+} × RAG-1^{-/-} mice (*n* = 6 pairs). Jointed animals were maintained for 4 wk after surgery. Gr. 1, Group 1; Gr. 2, Group 2; Gr. 3, Group 3; and Gr. 4, Group 4. **B**, Clinical scores were determined at 4 wk after surgery as described in *Materials and Methods*. Data are indicated as mean ± SEM of six mice in each group. *, *p* < 0.01, vs Group 1 donor mice. **, *p* < 0.01, vs Group 1 host mice.

blue staining method, and confirmed that the viability of cells was almost the same (>96% live) among the sample groups.

Reverse transcription polymerase chain reaction

Total RNA was isolated by using Isogen reagent (Nippon Gene). Aliquots of 5 μg total RNA were used for complementary DNA synthesis in a reaction volume of 20 μl using random primers. One microliter of reverse transcription product was amplified with 0.25 U of rTaq DNA polymerase

(Toyoba) in a 50 μ l reaction. Sense and antisense primers and the cycle numbers for the amplification of each gene were as follows: sense IL-7, 5'-GCCTGTACATCATCTGAGTGCC-3' and antisense IL-7, 5'-CAG GAGGCATCCAGGAACCTCTG-3' for IL-7 (35 cycles); and sense G3PDH, 5'-TGAAGGTCGGTGTGAACGGATTGGC-3' and antisense G3PDH, 5'-CATGTAGGCATGAGGTCCACCAC-3' for G3PDH (30 cycles). The amplification for each gene was logarithmic under these conditions. PCR products were separated on 1.8% agarose gels, stained with ethidium bromide, and visualized with a Lumi-Imager F1 (Roche).

Immunohistochemistry

We used consecutive cryostat colon sections in all studies. Immunohistochemistry using purified mAb against mouse CD4 (RM4-5; BD Pharmingen) or biotin-conjugated polyclonal IL-7 Ab (BAF407; R&D Systems) was performed. In brief, O.C.T. compound-embedded tissue samples were cut into serial sections 6- μ m thick, placed on coated slides, and fixed with 4% paraformaldehyde phosphate buffer solution for 10 min. Slides were then incubated with the primary Ab at 4°C overnight, followed by staining with AlexaFluor 488 goat anti-rat IgG for CD4 detection or AlexaFluor 488 streptavidin (Molecular Probes) for IL-7 detection at room temperature for 60 min. All slides were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI; Vector Laboratories) and observed under a confocal microscope (LSM510 Carl Zeiss).

Cytokine ELISA

To measure cytokine production, 1×10^5 LP CD4⁺ T cells were cultured in triplicate of 200 μ l culture medium at 37°C in a humidified atmosphere containing 5% CO₂ in 96-well plates (Costar) precoated with 5 μ g/ml hamster anti-mouse CD3 ϵ mAb (145-2C11, BD Pharmingen) and hamster 2 μ g/ml anti-mouse CD28 mAb (37.51, BD Pharmingen) in PBS overnight at 4°C. Culture supernatants were collected after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA following the manufacturer's recommendation (R&D Systems).

Flow cytometry

To detect the surface expression of a variety of molecules, isolated SP, BM, or LP mononuclear cells were preincubated with an Fc γ R-blocking mAb (CD16/32; 2.4G2, BD Pharmingen) for 20 min followed by incubation with specific FITC-, PE-, PerCP-, allophycocyanin-labeled Abs for 30 min on ice. The following mAbs were obtained from BD Pharmingen: anti-CD4 mAb (RM4-5), anti-CD45RB mAb (16A), anti-CD45.1 (Ly5.1; A20), and anti-CD45.2 (Ly5.2; 104). Standard four-color flow cytometric analyses were obtained using the FACSCalibur and analyzed by CellQuest software. Background fluorescence was assessed by staining with control irrelevant isotype-matched mAbs.

Statistical analysis

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

Results

IL-7^{-/-} \times RAG-1^{-/-} host mice joined with colitic IL-7^{+/+} \times RAG-1^{-/-} donor mice develop a wasting disease

We have previously demonstrated that IL-7 is essential for the development and the persistence of colitis as a survival factor for colitogenic CD4⁺ memory T cells (19). Furthermore, we have found that IL-7 Tg mice, in which IL-7 was systemically overproduced, develop colitis spontaneously, but production of intestinal IL-7 was conversely decreased in the inflamed mucosa because of depletion of the goblet cells. Based on such paradoxical findings, in this study, we assess whether intestinal or systemic IL-7 is essential for the perpetuation of colitis, by adoptive transfer experiment in combination with parabiosis system using IL-7^{+/+} \times RAG-1^{-/-} and IL-7^{-/-} \times RAG-1^{-/-} littermate recipients (Fig. 1A). To this end, we first induced chronic colitis by adoptive transfer of splenic CD4⁺CD45RB^{high} T cells from normal C57BL/6-Ly5.2 mice into IL-7^{+/+} \times RAG-1^{-/-} mice (Fig. 1A). Consistent with our previous report (19), the transferred IL-7^{+/+} \times RAG-1^{-/-} mice manifested progressive weight loss from 3 wk after transfer and clinical symptoms of colitis 6 wk after transfer (data

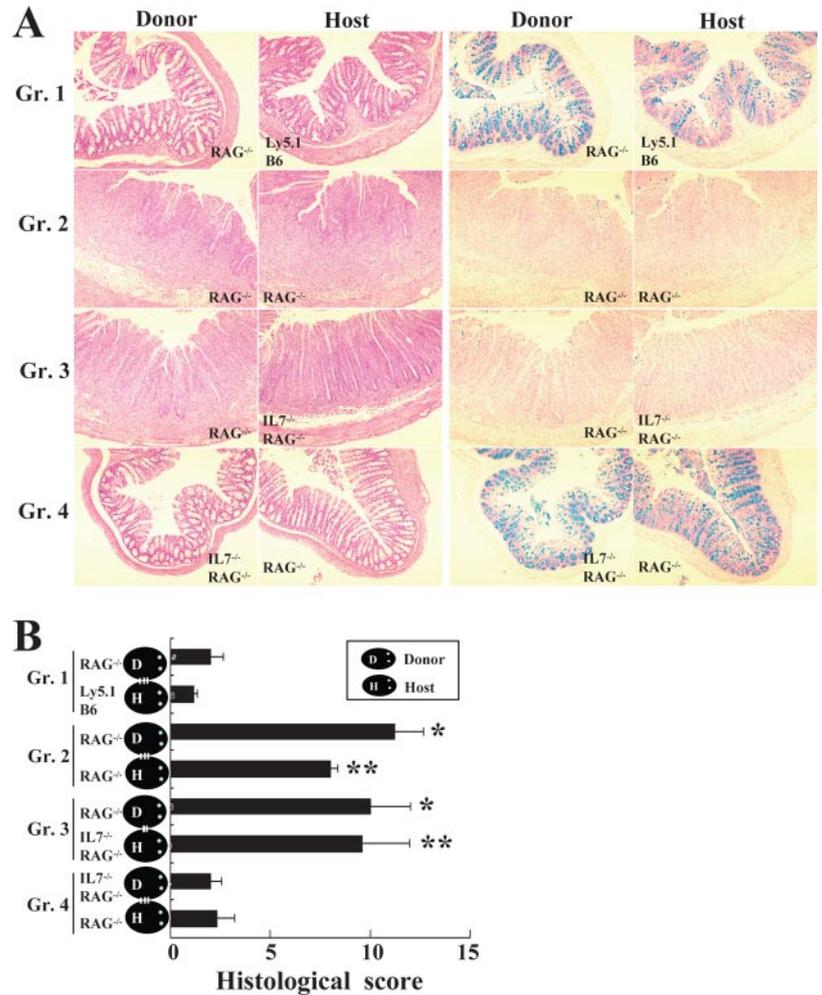
not shown). In contrast, the CD4⁺CD45RB^{high} T cell-transferred IL-7^{-/-} \times RAG-1^{-/-} mice showed no clinical signs of colitis and weight loss (data not shown) (19), indicating that IL-7 is essential for the development of colitis.

At 6 wk after transfer, we next generated four groups of parabionts (Fig. 1A). In parabionts between colitic IL-7^{+/+} \times RAG-1^{-/-} donor mice that has been previously transferred with Ly5.2⁺CD4⁺CD45RB^{high} T cells and normal C57BL/6-Ly5.1 host mice (Group 1) (Fig. 1A), clinical symptoms, such as diarrhea, anorectal prolapse, and hunched posture, gradually decreased over time in IL-7^{+/+} \times RAG-1^{-/-} donor mice as compared with the mice at the time of surgery, and completely disappeared at 4 wk after surgery by assessing the clinical score (Fig. 1B). C57BL/6-Ly5.1 host mice were consistently healthy during the observed period (Fig. 1B). In parabionts between colitic IL-7^{+/+} \times RAG-1^{-/-} donor mice and new IL-7^{+/+} \times RAG-1^{-/-} host mice (Group 2) (Fig. 1A), all the IL-7^{+/+} \times RAG-1^{-/-} donor mice were consistently diseased (Fig. 1B), and clinical symptoms of colitis gradually increased in new IL-7^{+/+} \times RAG-1^{-/-} host mice, which reached to the equal level of the paired IL-7^{+/+} \times RAG-1^{-/-} donor mice at 4 wk after surgery (Fig. 1B). In parabionts between colitic IL-7^{+/+} \times RAG-1^{-/-} donor mice and new IL-7^{-/-} \times RAG-1^{-/-} host mice (Group 3) (Fig. 1A), IL-7^{+/+} \times RAG-1^{-/-} donor mice remained diseased to a similar level of IL-7^{+/+} \times RAG-1^{-/-} donor mice in Group 2 (Fig. 1B), and notably, IL-7^{-/-} \times RAG-1^{-/-} host mice, albeit with the absence of intestinal IL-7, were gradually sick and clinical symptoms of colitis reached to the equal level of paired IL-7^{+/+} \times RAG-1^{-/-} donor mice and the IL-7^{+/+} \times RAG-1^{-/-} host mice in Group 2 at 4 wk after surgery (Fig. 1B). In sharp contrast, in parabionts between the nondiseased IL-7^{-/-} \times RAG-1^{-/-} donor mice that were transferred with CD4⁺CD45RB^{high} T cells and new IL-7^{+/+} \times RAG-1^{-/-} host mice (Group 4) (Fig. 1A), both IL-7^{-/-} \times RAG-1^{-/-} donor and IL-7^{+/+} \times RAG-1^{-/-} host mice were consistently healthy during the observed period (Fig. 1B), indicating that CD4⁺CD45RB^{high} T cell-transferred IL-7^{-/-} \times RAG-1^{-/-} mice never retained colitogenic CD4⁺ T cells.

IL-7^{-/-} \times RAG-1^{-/-} host mice parabiosed with colitic IL-7^{+/+} \times RAG-1^{-/-} donor mice develop Th1-mediated colitis

Four wk after surgery, the colons from parabionts between IL-7^{+/+} \times RAG-1^{-/-} donor mice and C57BL/6-Ly5.1 host mice in Group 1 and parabionts between IL-7^{-/-} \times RAG-1^{-/-} donor mice and IL-7^{+/+} \times RAG-1^{-/-} host mice in Group 4 were macroscopically normal (data not shown). In contrast, the colon from all mice in Groups 2 and 3, regardless of IL-7^{+/+} \times RAG-1^{-/-} or IL-7^{-/-} \times RAG-1^{-/-} mice and as donors or hosts, were equally enlarged and had a greatly thickened wall (data not shown). In addition, the enlargement of spleen was also present in donors and hosts of Groups 2 and 3 mice (data not shown). Histological examination showed that in colons from Group 1, donor IL-7^{+/+} \times RAG-1^{-/-} mice, which initially had clinical symptoms of colitis, exhibited no pathological change 4 wk after surgery, and were indistinguishable from the colons of C57BL/6-Ly5.1 host mice (Fig. 2A, left). In turn, we could not detect any pathological finding in Group 4 parabionts between IL-7^{-/-} \times RAG-1^{-/-} donor mice and IL-7^{+/+} \times RAG-1^{-/-} host mice. In contrast, all the donor and host mice in Groups 2 and 3 parabionts showed prominent epithelial hyperplasia with glandular elongation and a massive infiltration of mononuclear cells (Fig. 2A, left). This difference was also confirmed by histological scoring of colon sections (Fig. 2B), showing that the host mice in parabionts in Groups 2 and 3 developed colitis comparable to the paired diseased donor mice that had sustained colitis, while all the donor and host mice in Groups

FIGURE 2. IL-7^{-/-} × RAG-1^{-/-} host mice in parabionts with diseased IL-7^{+/+} × RAG-1^{-/-} donor mice develop colitis. **A**, Histological examination by H&E staining (*left*) and Alcian blue staining (*right*) of the colon from each group at 4 wk after surgery. Representative of four separate samples in each group. Original magnification, ×100. **B**, Histological scoring of the colon from Groups 1–4 at 4 wk after surgery. Data are indicated as the mean ± SEM of six mice in each group. *, *p* < 0.01, vs Group 1 donors. **, *p* < 0.01, vs Group 1 hosts. Gr., Group.



1 and 4 did not develop colitis. Furthermore, acid mucin production examined by Alcian blue staining revealed a marked decrease of mucin-producing goblet cells in all colitic mice in Groups 2 and 3 in contrast to mice in Groups 1 and 4 (Fig. 2A, right).

To clarify that newly developed colitis in host mice of Groups 2 and 3 was surely mediated by the infiltration of immigrant CD4⁺ T cells from donor mice, but not by innate immune cells such as granulocytes and macrophages, we next assessed colonic infiltration of CD4⁺ T cells by immunohistochemistry. Fig. 3 clearly demonstrated marked infiltration of CD4⁺ T cells in the colon of host mice as well as in donor mice in parabionts of Groups 2 and 3. In contrast, only a small population of CD4⁺ T cells was found in the host and donor mice in Groups 1 and 4 (Fig. 3). Especially, although the IL-7^{+/+} × RAG-1^{-/-} host mice in Group 1 had severe wasting disease with symptoms of colitis before surgery, there were only a few infiltrated CD4⁺ T cells observed in colonic LP, indicating that the previous colitis was suppressed and cured by certain immigrant suppressor cells derived from normal host mice.

We next examined the cytokine production by LP CD4⁺ T cells from each mouse in Groups 1–4. As shown in Fig. 4, LP CD4⁺ T cells from donor and host mice in Groups 2 and 3 produced significantly higher amounts of IFN- γ and TNF- α as compared with those from mice in Groups 1 and 4, indicating that colitic LP CD4⁺ T cells in IL-7^{-/-} × RAG-1^{-/-} host mice or IL-7^{+/+} × RAG-1^{-/-} host mice of Groups 2 and 3 have functions of Th1-mediated immune responses. Importantly, the elevated production of these cytokines in Groups 2 and 3 was

dependent on the presence of colitis, but not on the expression of IL-7 in the colon.

Expansion of CD4⁺ T cells is dependent on IL-7 in the SP or BM but is independent of IL-7 in the LP

We have previously reported that BM retaining colitogenic CD4⁺ T cells in colitic mice might play a critical role as a reservoir for persisting colitis (18). Furthermore, BM is physiologically a major source of IL-7, contributing to the development of B cells (24). To further investigate the role of intestinal and/or systemic IL-7 in consecutive immunopathology of the parabiosis model, we next compared the composition of CD4⁺ T cells in the LP, BM, and SP of donor and host mice in each parabiont using flow cytometry at 4 wk after surgery. The recovered cell numbers of CD3⁺CD4⁺ T cells from the donor and host LP in Groups 2 and 3 were significantly higher as compared with those of the paired donor and host colitic mice in Groups 1 and 4 parabionts, respectively (Fig. 5A). Furthermore, the recovered cell numbers of CD3⁺CD4⁺ T cells in the donor and host BM (Fig. 5B) and SP (Fig. 5C) in Groups 2 and 3 were significantly higher as compared with those of the paired donor and host colitic mice in Group 4, but not in Group 1, parabionts, respectively. In contrast, IL-7^{+/+} × RAG-1^{-/-} donor mice that were previously transferred with CD4⁺CD45RB^{high} T cells and C57BL/6-Ly5.1 host mice in Group 1 sustained a normal number of cells in the BM and SP (Fig. 5, data not shown). Most importantly, although the number of CD3⁺CD4⁺ T cells recovered from the SP or BM of the IL-7^{-/-} × RAG-1^{-/-} host mice in Group 3 was significantly decreased compared with that of the

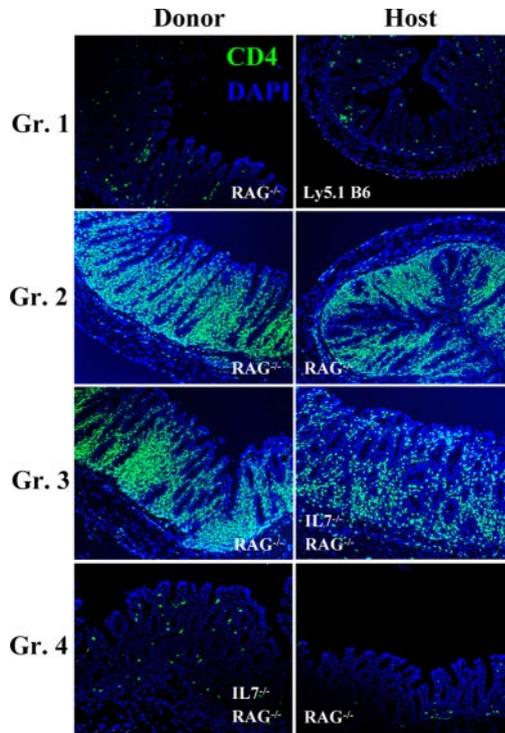


FIGURE 3. $IL-7^{-/-} \times RAG-1^{-/-}$ host mice in parabionts developed colitis with the marked infiltration of immigrant $CD4^{+}$ T cells from donor mice. $CD4$ immunostaining and DAPI counterstaining of the colon from Groups 1–4 at 4 wk after surgery. Frozen sections were fixed with 4% paraformaldehyde phosphate buffer solution and stained with anti-mouse $CD4$ mAb, followed by AlexaFluor 488 goat anti-rat IgG as secondary Ab and DAPI counterstaining. A large number of $CD4^{+}$ T cells were infiltrated in the colonic mucosa of $IL-7^{-/-} \times RAG-1^{-/-}$ host mice (Group 3) as well as in that of $IL-7^{+/+} \times RAG-1^{-/-}$ host mice (Group 2). Representative of four separate samples in each group. Original magnification: $\times 100$. Gr., Group.

$IL-7^{+/+} \times RAG-1^{-/-}$ host mice in Group 2, an equivalent number of $CD4^{+}$ T cells was recovered from the LP of both host mice in Groups 2 and 3, indicating that the expansion of $CD4^{+}$ T cells in the SP and BM is dependent on $IL-7$, but is independent in the LP.

Further analysis of Group 1 mice using a four-colored $CD3/CD4/Ly5.1/Ly5.2$ FACS staining revealed that $>95\%$ of total $CD4^{+}$ T cells were derived from $Ly5.1^{+}$ cells and most resident $Ly5.2^{+}CD4^{+}$ T cells decreased to only 5–10% of total $CD4^{+}$ T cells in SP and BM in Group 1 $IL-7^{+/+} \times RAG-1^{-/-}$ donor mice (Fig. 5). Interestingly, although the absolute number of LP $CD4^{+}$ T cells was significantly decreased in Group 1 $IL-7^{+/+} \times RAG-1^{-/-}$ donor mice as compared with those of $IL-7^{+/+} \times RAG-1^{-/-}$ donor mice in Groups 2 and 3 colitic parabionts, $\sim 50\%$ of total LP $CD4^{+}$ T cells remained to be $Ly5.2^{+}$, suggesting that 1) colitogenic LP $Ly5.2^{+}CD3^{+}CD4^{+}$ T cells were resistant to the suppression by $Ly5.1$ -derived cells as compared with $Ly5.2^{+}CD3^{+}CD4^{+}$ T cells in other sites and/or 2) they remained in the intestine, and in other words could not exit, and redistribute outside the intestine. Furthermore, small but substantial percentages (1–5%) of total $CD4^{+}$ T cells in each tissue of host C57BL/6- $Ly5.1$ mice were donor-derived $Ly5.2^{+}$ cells, indicating that two-way recirculation of $CD4^{+}$ T cells from the donor to the host and vice versa had been established and most of $Ly5.2^{+}$ colitogenic $CD4^{+}$ T cells in both donor and host mice had undergone the contraction under a certain suppressive mechanism including suppression by $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells derived from host C57BL/6 mice.

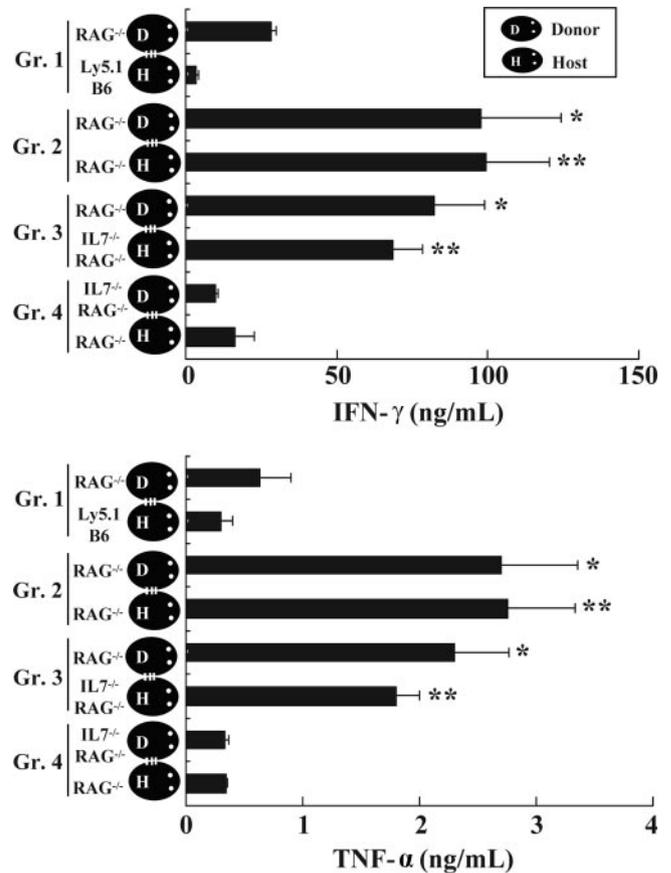


FIGURE 4. $IL-7^{-/-} \times RAG-1^{-/-}$ host mice in parabionts develop Th1-mediated colitis. LP $CD4^{+}$ T cells were prepared from colons at 4 wk after surgery and stimulated with anti- $CD3$ and anti- $CD28$ mAbs for 48 h. Concentrations of $IFN-\gamma$ and $TNF-\alpha$ in culture supernatants were measured by ELISA. Data are indicated as the mean \pm SEM of six mice in each group. *, $p < 0.01$, vs Group 1 donors. **, $p < 0.01$, vs Group 1 hosts. Gr., Group.

IL-7 is not detected in host $IL-7^{-/-} \times RAG-1^{-/-}$ host mice after parabiosis

Studies showing engraftment of BM-derived cells to various non-hemopoietic tissues including epithelial cells after BM transplantation are now on topic (26, 27), and we have previously demonstrated that human BM cells have a potential to repopulate the gastrointestinal epithelia by detecting Y-chromosomes in female cases that have undergone BM transplantation using male donor cells (28). It was thus needed to assess whether this was the case with our parabiosis setting, and if so, it was interesting to know whether $IL-7$ was produced by engrafted colonic epithelial cells derived from the BM of $IL-7^{+/+} \times RAG-1^{-/-}$ donor mice in $IL-7^{-/-} \times RAG-1^{-/-}$ host mice after surgery in Group 3. As shown in Fig. 6A, immunohistochemistry revealed that $IL-7$ is detected in uninfamed colonic epithelia of both $IL-7^{+/+} \times RAG-1^{-/-}$ donor and C57BL/6 host mice in Group 1 and $IL-7^{+/+} \times RAG-1^{-/-}$ host, but not in $IL-7^{-/-} \times RAG-1^{-/-}$ donor, mice in Group 4. Consistent with previous findings (17), $IL-7$ expression was detectable, but markedly decreased in inflamed colonic epithelia in Groups 2 and 3 of $IL-7^{+/+} \times RAG-1^{-/-}$ mice along with the decreased goblet cells, in both host and donor mice (Fig. 2A, right). In contrast, $IL-7$ was not detected in the inflamed colonic epithelia of Group 3 $IL-7^{-/-} \times RAG-1^{-/-}$ host mice (Fig. 6A). Consistent with these results, further RT-PCR analysis for $IL-7$ mRNA expression showed that $IL-7$ mRNA was not detected in

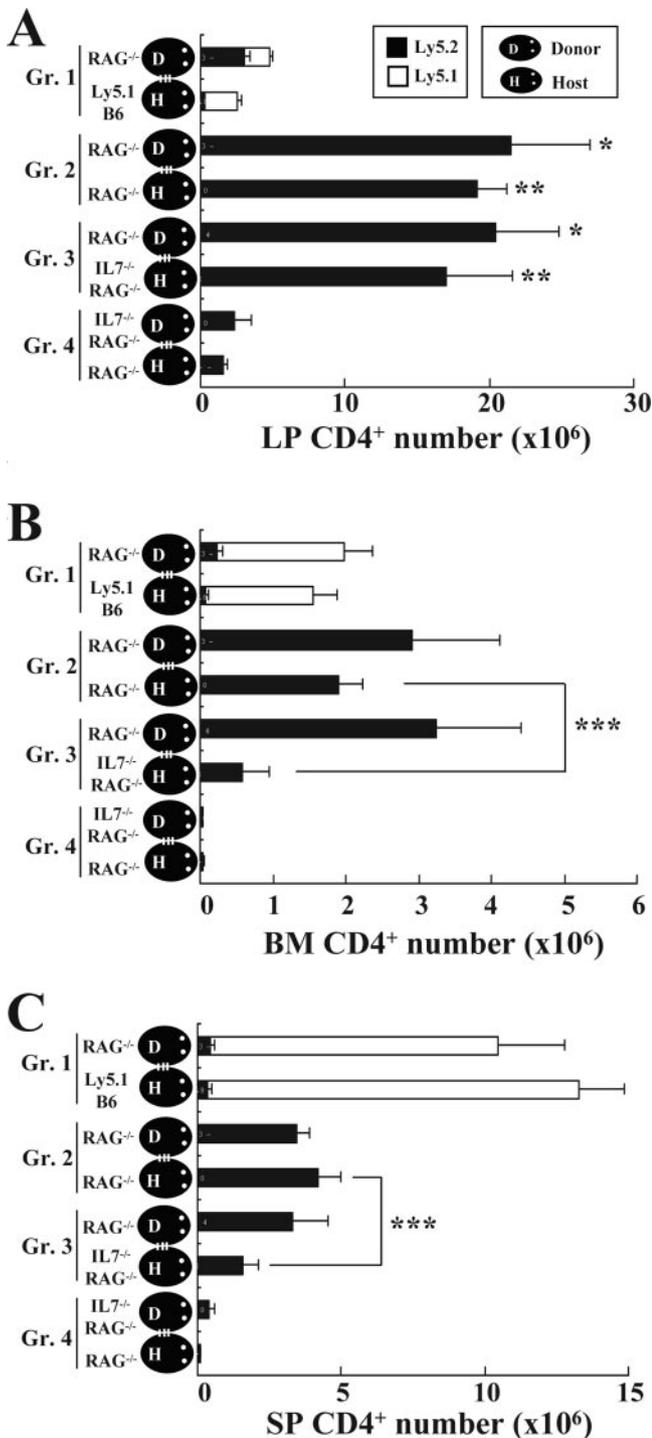


FIGURE 5. Expansion of BM and SP, but not of LP, CD4⁺ T cells in IL-7^{-/-} × RAG-1^{-/-} host mice in parabionts is dependent on IL-7. LP (A), BM (B), and SP (C) CD4⁺ T cells were isolated from each mouse of Groups 1–4 at 4 wk after surgery, and the number of CD4⁺ cells were determined by flow cytometry. Data are indicated as the mean ± SEM of six mice in each group. *, *p* < 0.01, vs Group 1 donors. **, *p* < 0.01, vs Group 1 hosts. ***, *p* < 0.01, vs Group 2 hosts. For cells in Group 1 parabionts, cells were stained with anti-CD45.1 mAb and anti-CD45.2 mAb to discriminate between donor or host origin. Gr., Group.

colitic IL-7^{-/-} × RAG-1^{-/-} host mice in Group 3, and was markedly decreased in colitic IL-7^{+/-} × RAG-1^{-/-} donor and host mice in Groups 2 and 3 in clear contrast to that of control C57BL/6 mice (Fig. 6B).

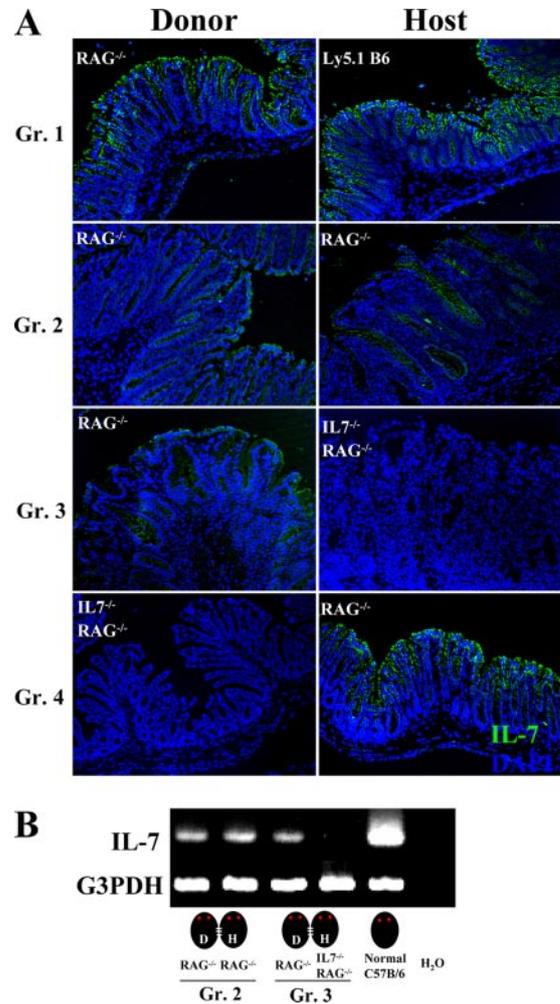


FIGURE 6. IL-7 is not detected in host IL-7^{-/-} × RAG-1^{-/-} mice in parabionts with diseased IL-7^{+/-} × RAG-1^{-/-} donor mice. A, Frozen sections of colon from each mouse in Groups 1–4 at 4 wk after surgery were stained with polyclonal anti-IL-7 Abs. Representative of five separate samples in each group. Original magnification: ×100. B, Expression of IL-7 mRNA in the whole colon was determined by RT-PCR. Representative of five separate samples in each group. Gr., Group.

Discussion

In this study, we demonstrated that intestinal IL-7 is not essential for the development and perpetuation of colitis by showing that IL-7^{-/-} × RAG-1^{-/-} host mice parabiosed with colitic IL-7^{+/-} × RAG-1^{-/-} donor mice develop a wasting disease and severe colitis. Because we previously demonstrated that IL-7 is needed to develop and sustain colitis by showing a lack of colitis development in IL-7^{-/-} × RAG-1^{-/-} mice transferred with CD4⁺CD45RB^{high} T cells or colitogenic LP CD4⁺ T cells (19), in this study, we suggest that IL-7 production from tissues other than the intestine, such as BM, is sufficient, or rather may be essential to develop and sustain the chronic colitis.

Before starting this study, we confronted a paradox between two facts. The first fact is that IL-7-producing goblet cells are easily decreased or depleted in patients with severe ulcerative colitis (29), colitic IL-7 Tg mice (17) and in the present model of colitis (Fig. 2A, right) resulting in the decreased IL-7 production in the intestine, and the second fact is that IL-7 appeared to be indispensable for the development and persistence of chronic colitis by adoptive transfer experiment using IL-7^{-/-} × RAG-1^{-/-} mice (19). Based on these backgrounds, we hypothesized that intestinal IL-7 is

indeed important to establish GALT, such as Payer's patches and cryptopatches, and also to maintain IELs (30), but not needed to develop and sustain colitis, since many Ags, such as intestinal bacterial Ags, may be sufficient to stimulate colitogenic CD4⁺ T cells in the intestinal LP without stimuli from IL-7. To prove it, we performed a combinational experiment using adoptive transfer and parabiosis systems in the present study. Although the parabiosis system seems to be somewhat artificial and problematic on some level as two mice, host and donor, are forced to have a surgical stress and behavioral limitation (Groups 3 and 4), mice laboring colitogenic CD4⁺ T cells are surgically joined, resulting in prompt development of anastomoses of blood vessels within a few days. Even in the present setting, it is noteworthy that IL-7^{-/-} × RAG-1^{-/-} host mice joined with colitic IL-7^{+/+} × RAG-1^{-/-} donor mice developed a wasting disease and colitis to the similar level of colitic IL-7^{+/+} × RAG-1^{-/-} donor mice over time.

In this parabiosis system, however, it was also possible that certain stem cells that are committed to differentiate into IL-7-producing mesenchymal cells or epithelial cells homed to the intestine, and might have been involved in the development and persistence of colitis in IL-7^{-/-} × RAG-1^{-/-} host mice joined with colitic IL-7^{+/+} × RAG-1^{-/-} donor mice (Group 3). To rule out this possibility, we also demonstrated that IL-7 expression was not detected in the colon of the IL-7^{-/-} × RAG-1^{-/-} host mice both at the protein and mRNA levels (Fig. 6). Consistent with the present result, another group demonstrated that restoring intestinal IL-7 expression to IL-7^{-/-} mice did not result in the development of colitis (31). Collectively, the current results clearly indicate that intestinal IL-7 is not essential, but systemic IL-7 from extraintestinal sites is essential, for the development and sustainment of colitis.

It is also very important to know why IL-7 is decreased in the inflamed mucosa of colitis in terms of pathogenesis of chronic colitis. In other words, it is possible that the lack or decrease of IL-7 production in inflamed mucosa of colitis is pathologically needed to maintain chronic colitis. Consistent with this hypothesis, we previously demonstrated that although IL-7 promoted proliferation of human LP IL-7Rα-expressing CD4⁺ T cells, double stimuli by IL-7 and anti-CD3 mAb conversely suppressed it (21). In addition, Fluor and colleagues (32) very recently reported that IL-7 induces Fas-mediated T cell apoptosis by inducing Fas expression on CD4⁺ T cells. Thus, it appears that intestinal IL-7 physiologically plays a key role in the elimination of pathological LP CD4⁺ T cells activated by intestinal bacteria. Further studies will be needed to address this issue.

Interestingly, the recovered cell number of LP CD4⁺ T cells was equivalent between host and donor mice both in Group 2 and 3, although it was likely that total production of IL-7 in Group 3 parabionts between one IL-7^{+/+} mouse and one IL-7^{-/-} mouse was approximately half compared with that in Group 2 parabionts between two IL-7^{+/+} mice. Because it seems that the production of IL-7 is maintained at a constant rate and is uninfluenced by extrinsic stimuli (33, 34), this result indicates that factors other than IL-7, such as stimulation by commensal bacteria might control the homeostasis of cell number in the LP, but not in the BM and SP. Further studies will be needed to address this issue.

BM is a major source of IL-7 in the body (26). In contrast to the LP, it is noteworthy that the number of CD4⁺ T cells recovered from the BM and SP of the colitic IL-7^{-/-} × RAG-1^{-/-} host mice (Group 3) was significantly decreased compared with that of the IL-7^{+/+} × RAG-1^{-/-} host mice. Regarding this result, we recently demonstrated that CD4⁺ effector-memory-like T (T_{EM}-like) cells reside in the BM of colitic SCID and RAG-1/2^{-/-} mice induced by adoptive transfer of CD4⁺CD45RB^{high} T cells (20).

Importantly, these resident BM CD4⁺ T_{EM}-like cells are closely attached to IL-7-producing stromal cells in the colitic BM. Most importantly, the accumulation of BM CD4⁺ T_{EM}-like cells was significantly decreased in IL-7-deficient recipients reconstituted with the colitogenic LP CD4⁺ T_{EM}-like cells. Together with the present study, these findings suggest that the BM CD4⁺ T_{EM}-like cells residing in mice with chronic colitis play a critical role as a reservoir for lifelong persisting colitis in an IL-7-dependent manner. However, it is still possible that IL-7 produced by sites other than intestine or BM, such as skin, liver, eye, lymph nodes (LN), and SP, also contribute to the development and perpetuation of colitis. In this regard, we very recently demonstrated that splenectomized LN-null lymphotoxin α^{-/-} × RAG-2^{-/-} mice transferred with colitogenic LP CD4⁺ T cells develop colitis (35), suggesting that IL-7 production at least by LN and SP does not appear to be essential. To further clarify the role of IL-7 produced by BM mesenchymal cells in the pathogenesis of chronic colitis, BM chimera of IL-7^{-/-} × RAG-1^{-/-} mice, which are lethally irradiated and transplanted with the BM cells from IL-7^{+/+} × RAG-1^{-/-} mice, may be quite beneficial. Interestingly, however, it is also well known that extraintestinal complications of IBD patients such as skin, liver, and mucocutaneous manifestations (36) appears to be closely associated with sites of local IL-7 production by keratinocytes, hepatocytes, and uvea cells. Although no inflammation was not observed at least in liver and skin in the present model of colitis (data not shown), further studies will be needed to address this issue.

Clinicopathologically, IBD is characterized by chronic intestinal inflammation. Surgery does not cure IBD, especially Crohn's disease, as relapse is a rule after remission, suggesting that IBD is not a circumscribed disease, but rather a systemic disease mediated by colitogenic memory CD4⁺ T cells distributing throughout the body via the bloodstream, which may hide in their reservoir, such as BM. Consistent with this hypothesis, recent findings showing usefulness of leukocytapheresis, which removes peripheral blood cells for the treatment of refractory IBD patients (37, 38), suggests that recirculation of colitogenic memory CD4⁺ T cells from the gut to some reservoir and vice versa, may play a role in the perpetuation of chronic colitis. Furthermore, we have recently demonstrated that FTY720 that has an ability to inhibit circulation of lymphocytes prevents the development of SCID/RAG-1/2^{-/-} colitis induced by adoptive transfer of LP colitogenic CD4⁺ T_{EM}-like cells (39). Together with the current results, it would be possible that the circulation of colitogenic CD4⁺ T_{EM}-like cells is quite active in IBD, making them continue to circulate in the blood and migrate to IL-7-producing reservoir from the IL-7-depleted LP.

In summary, in this study, we demonstrated that systemic IL-7, but not intestinal IL-7, is essential for the development and perpetuation of colitis, suggesting that therapeutic approaches targeting systemic IL-7 using the biologics against IL-7 may be feasible in the treatment of IBD.

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Disclosures

The authors have no financial conflict of interest.

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