Bromodeoxyuridine Incorporation

Colitic mice and age-matched normal BALB/c mice were given 1 mg of bromodeoxyuridine (BrdU) in PBS by intraperitoneal injection. Twenty-four hours later, mice were killed and the lymphocytes were prepared from BM, MLN, and colonic LP. Cells were first stained with PE-conjugated anti-CD4 mAbs for 2-color flow-cytometric analysis, or peridinin chlorophyll protein-conjugated anti-CD4 mAbs, APC-conjugated anti-CD44 mAbs, and PE-conjugated anti-CD62L mAbs for 4-color flow-cytometric analysis, and fixed and permeabilized with Cytofix-Cytoperm (BD PharMingen) solution according to the manufacturer's instructions. Cells were stained with FITC-conjugated anti-mouse BrdU (BD PharMingen) diluted in perm/wash buffer.

Cell-Cycle Analysis

A total of 1×10^6 cells from colitic mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells were stained for PE-conjugated anti-CD4 mAbs, and fixed and permeabilized with Cytofix-Cytoperm (BD PharMingen) solution according to the manufacturer's instructions. 7-AAD (10 μg/mL) and RNase (200 μg/mL) were added, and cells were incubated for 20 minutes at room temperature. Cells were acquired on a FACSCalibur (BD PharMingen) in their staining solution. Cell-cycle analysis of DNA histograms was performed with Cell Quest Software (BD PharMingen).

Immunohistochemistry

Consecutive cryostat bone marrow sections (6 μm) were fixed and stained with the following rat antibodies: biotinylated CD4 (RM4-5) and polyclonal anti-IL-7 antibodies (R&D Laboratories). Alexa 594 goat anti-rat IgG, Alexa 488 goat anti-hamster IgG, and Alexa 488 rabbit anti-goat IgG (Molecular Probes, Eugene, OR) were used as second antibodies. All confocal microscopy was performed on a BioZERO BZ8000 (Keyence, Tokyo, Japan).

Adoptive Transfer Experiments

To assess the in vivo potential of the residual BM $CD4^+$ T cells in colitic SCID mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells to induce colitis, $CD4^+$ T cells (1×10^5 cells/mouse) isolated from the BM, MLN, and LP of colitic mice or BM of age-matched normal BALB/c mice were injected into new SCID mice. In another set of experiments, BM $CD4^+$ T cells (1×10^5 cells/mouse) isolated from colitic IL-$10^{-/-}$ mice (age, 20 wk) or age-matched normal C57BL/6 mice (1×10^5 cells/mouse) were injected into C57BL/6 RAG2$^{-/-}$ mice. To assess the role of commensal bacteria in the development of colitis and the retention of colitogenic BM $CD4^+$ effector-memory T (T_{EM}) cells, we used broad-spectrum antibiotics in another adoptive transfer experiment. CB-17 SCID mice were treated with or without ampicillin (1 g/L; Sigma, St. Louis, MO), vancomycin (500 mg/L; Abbott Labs, Abbott Park, Illinois), neomycin sulfate (1 g/L; Pharmacia/Upjohn, New York, NY), and metronidazole (1 g/L; Sidmak, Gujarat, India) in drinking water 4 weeks before beginning the adoptive transfer and during the course of the development of colitis based on a variation of the commensal depletion protocol of Fagarasan et al.[20] All recipient mice were weighed initially, then 3 times/wk after the transfer. They then were observed for clinical signs of illness as previously described.[18]

Adoptive Transfer Experiments Into IL-7$^{-/-}$ × Rag-1$^{-/-}$ Mice

To assess the role of IL-7 in the maintenance of BM $CD4^+$ T cells, we further transferred LP $CD4^+$ T cells (2×10^6 cells/mouse) isolated from colitic $CD4^+CD45RB^{high}$ T-cell-transferred mice into IL-$7^{-/-}$ × Rag-$1^{-/-}$ and IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice. Mice were killed 5 days after transfer, and the spleen and BM cells were isolated and stained with PE-conjugated rat anti-CD3ε mAbs and FITC-conjugated rat anti-CD69 mAbs or isotype FITC-conjugated control antibody. Before staining for intracellular Bcl-2, cells (2×10^6) were stained with PE-conjugated rat anti-CD3 mAbs as described earlier. After washing, cells were fixed and permeabilized with Cytofix-Cytoperm (BD PharMingen) solution according to the manufacturer's instructions. Cells were stained with either FITC-conjugated hamster anti-mouse Bcl-2 or a control antibody diluted in perm/wash buffer. To further assess the proliferative responses of $CD4^+$ T cells in IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients, LP $CD4^+$ T cells from SCID mice with colitis induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells were labeled with carboxyfluoroscein succinimidyl ester (CFSE) (Molecular Probes) by incubating at 5 μmol/L in PBS, quenching with fetal calf serum, and washing with PBS 3 times. Cells were resuspended in PBS, and 3×10^6 total cells were transferred by intravenous injection into IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice. In another set of experiments, we transferred with colitogenic BM $CD4^+$ T cells from colitic $CD4^+CD45RB^{high}$ T-cell-transferred Rag-$2^{-/-}$ mice into IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients to clarify whether these mice develop colitis. Mice were killed at 10 weeks after transfer.

Statistical Analysis

The results were expressed as the mean ± SD. Groups of data were compared by the Mann-Whitney *U* test. Differences were considered statistically significant when the *P* value was less than .05.

Results

Effector Memory T Cells Reside in the BM of Colitic Mice

To investigate the role of BM in consecutive immunopathology in immune-mediated diseases, we first compared the composition and phenotype of $CD4^+$ T

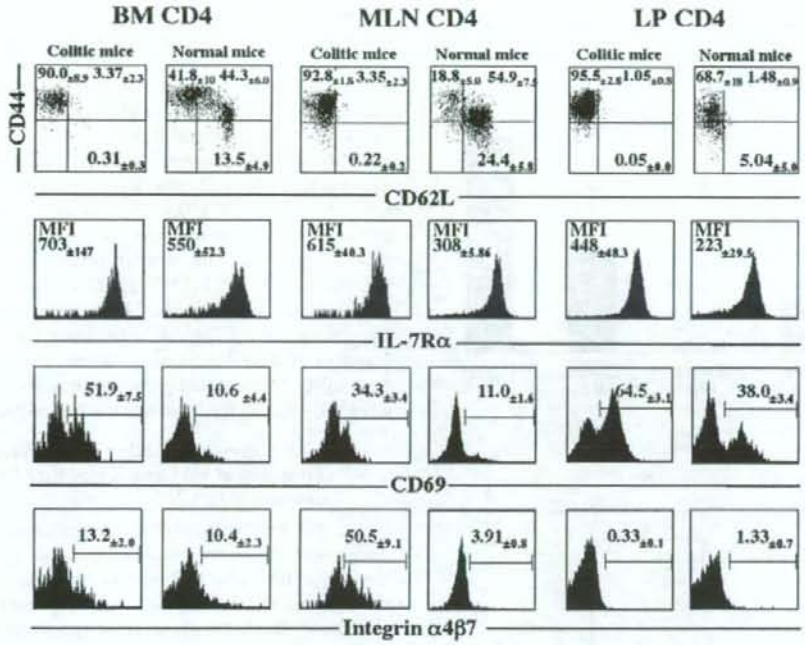


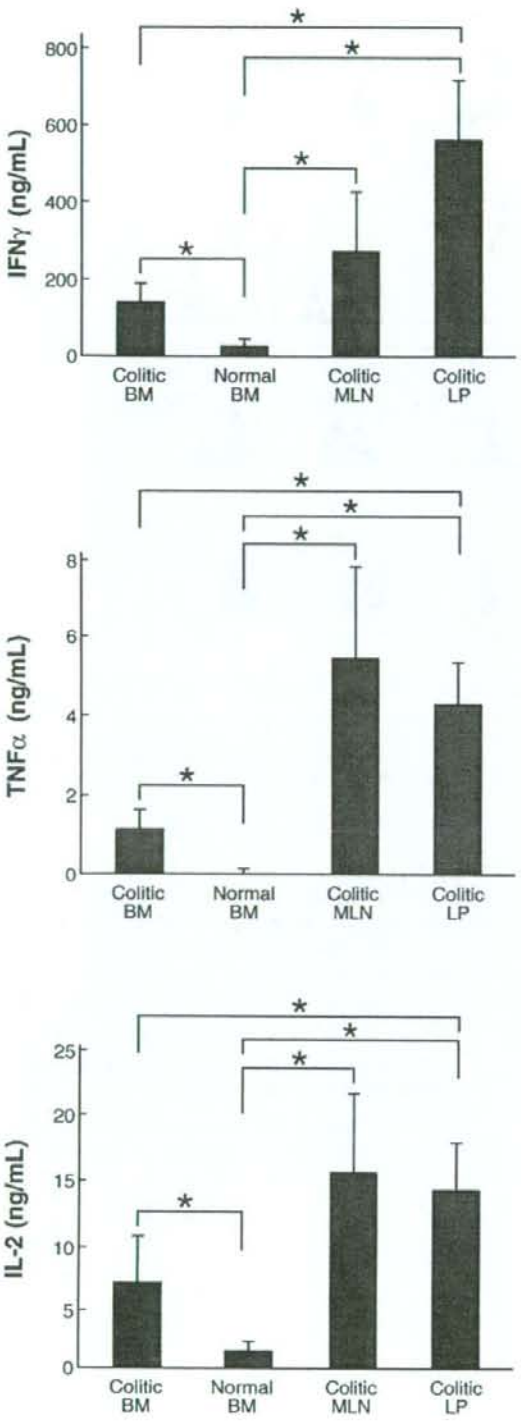
Figure 1. Colitic BM CD4+ T cells are CD44^{high}CD62L^{-} IL-7Rα^{high}. Expression of CD44, CD62L, IL-7Rα (CD127), CD69, and integrin $\alpha4\beta7$ on CD4+ T cells obtained from spleen, MLN, LP, and BM in colitic mice induced by adoptive transfer of CD4+CD45RB^{high} T cells into CB-17 SCID mice (6 weeks after transfer) and normal BALB/c mice (age, 8 wk). Freshly isolated cells from colitic mice and normal BALB/c mice were stained with FITC-labeled anti-CD4, and PE-labeled anti-CD44, anti-CD62L, anti-IL-7Rα, anti-CD69, or anti-integrin $\alpha4\beta7$ mAbs. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and side-scatter profiles. Data are displayed as a dotted plot (4-decade log scale) and quadrant markers were positioned to include more than 98% of control Ig-stained cells in the lower left. Percentages in each quadrant are indicated. Representative of 3 mice in each group.

cells in BM, MLN, and colonic LP of colitic mice induced by the adoptive transfer of CD4+CD45RB^{high} T cells into recipient CB-17 SCID mice and with those of age-matched normal BALB/c mice. CD3+CD4+ mature T cells were found to reside in BM, MLN, and LP (colitic mice: BM, 12.7 ± 4.4 × 10^5 per mouse; MLN, 7.01 ± 4.2 × 10^5; and LP, 187 ± 99 × 10^5; normal mice: BM, 16.6 ± 3.8 × 10^5; MLN, 99.6 ± 18 × 10^5; and LP, 4.17 ± 1.2 × 10^5). As shown in Figure 1, the BM CD4+ T cells, as well as MLN and LP CD4+ T cells, from the colitic mice, exclusively have a phenotype of CD44^{high}CD62L^{-} cells. Furthermore, these colitic BM CD4+ T cells expressed IL-7Rα highly, indicating that the colitic BM CD4+ T cells have a characteristic of T_{EM} cells. In contrast, the BM CD4+ T cells from normal mice are composed of 3 subpopulations: CD44^{low}CD62L+ naive cells, CD44^{high}CD62L+ central-memory T cells, and CD44^{high}CD62L^{-} T_{EM} cells (Figure 1). CD69, which is associated with cell activation, was expressed by a significantly higher proportion of CD4+ T cells from colitic mice than from normal mice. Interestingly, BM CD4+ T cells from colitic mice expressed relatively, but not significantly, high levels of integrin $\alpha4\beta7$, a homing receptor to the gut, as compared with BM CD4+ T cells from normal mice, but lower levels than did MLN CD4+ T cells from colitic mice. These data indicate that the integrin $\alpha4\beta7$-expressing CD4+ memory T cells, which are instructed to express the molecule in MLN or Peyer's patches,[21,22] migrate to the BM.

Colitic BM CD4+ Memory T Cells Produce a Large Amount of Th1 Cytokines

We next examined whether the colitic BM CD4+ T cells retained the ability to produce type-1 T helper (Th1) cytokines as well as the colitic CD4+ T cells in other sites. The production of IFN-γ, tumor necrosis factor-α, and IL-2 by anti-CD3/CD28 mAb-stimulated BM CD4+ T cells from colitic mice was significantly higher than that by normal BM CD4+ T cells, but lower than those by anti-CD3/CD28 mAb-stimulated LP CD4+ T cells (Figure 2), indicating that the colitic BM CD4+ T cells could be primed to Th1-type cells, and sustained in the BM.

To determine whether the BM CD4+ T cells from colitic mice express their pathogenic potential on stim-



ulation with antigens derived from resident enteric bacteria, we examined in vitro IFN-γ secretion by normal and colitic BM, MLN, and LP $CD4^+$ T cells stimulated with various concentrations of CBA. The results show that significantly higher levels of IFN-γ were produced by colitic BM $CD4^+$ T cells in response to a high dose (1000 μg/mL) of CBA as compared with normal BM $CD4^+$ T cells, but significantly lower than those by colitic LP $CD4^+$ T cells, which responded to much lower concentrations (10, 100, 1000 μg/mL) of CBA (Figure 3). The similar result was obtained by paired samples of MLN (Figure 3) and splenic (data not shown) $CD4^+$ T cells. These results indicated that the colitic BM $CD4^+$ T cells have the potential to respond against bacterial antigens and thus have the possibility to be colitogenic similar to the colitic LP $CD4^+$ T cells as we have shown previously.[18]

IL-7–Expressing Cells are Scattered Throughout BM and Colocalized in Close Proximity to $CD4^+$ T Cells

We next examined the distribution of IL-7–producing cells[23] and their interaction with $CD4^+$ T cells in the colitic BM. The IL-7–expressing cells were scattered throughout the BM as has been reported previously[24] and most $CD4^+$ T cells were in close contact with the bodies of IL-7–expressing cells (Figure 4). In contrast, IL-7 was not expressed, and $CD4^+$ T cells did not reside in the BM of IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice used as a negative control (Figure 4).

BM Contains the Most Actively Dividing Pool of $CD4^+$ T Cells

To examine the homeostatic proliferation of the colitic BM $CD4^+$ T cells, 2 experimental approaches were used. First, we examined memory $CD4^+$ T cells from each tissue for evidence of active cell division by DNA staining using 7AAD (Figure 5*A*). Cells actively synthesizing DNA could be identified by their increased DNA content, allowing us to identify tissues where active cell division was occurring. A larger percentage of $CD4^+$ T cells was actively synthesizing DNA in both the colitic and normal BM than in any other tissues (Figure 5*A*). Although the difference was slight, it was reproducible over 3 independent experiments.

Second, colitic mice were injected with BrdU to provide evidence of recent DNA synthesis. To accurately examine the differences in cell proliferation in different tissues, it was necessary to give a short pulse of BrdU because

Figure 2. Colitic BM $CD4^+$ T cells produce Th1 cytokines. Cytokine production by $CD4^+$ T cells. Isolated $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs for 48 hours. The indicated cytokines in these supernatants were measured by ELISA. Data are indicated as the mean ± SD of 7 mice in each group.

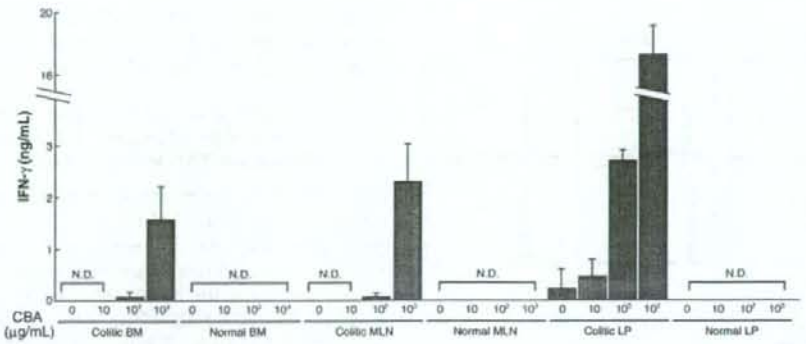


Figure 3. IFN-γ production by CD4+ T cells stimulated with APCs pulsed with CBA from colitic mice induced by adoptive transfer of CD4+CD45RB^{high} T cells. Supernatants collected on day 3 of culture were assayed for IFN-γ by ELISA. Data are indicated as the mean ± SD of 5 mice in each group. *$P < .05$. ND, not detected.

longer treatment with BrdU might obscure the differences among the various tissues, probably because of the migration of dividing cells among the tissues. Mice thus were killed 24 hours after the injection of BrdU, and BrdU incorporation was measured in the CD4+ T cells obtained from BM, MLN, and LP (Figure *5B*). Significantly higher percentages of memory T cells were synthesizing DNA in the colitic BM, MLN, and LP as compared

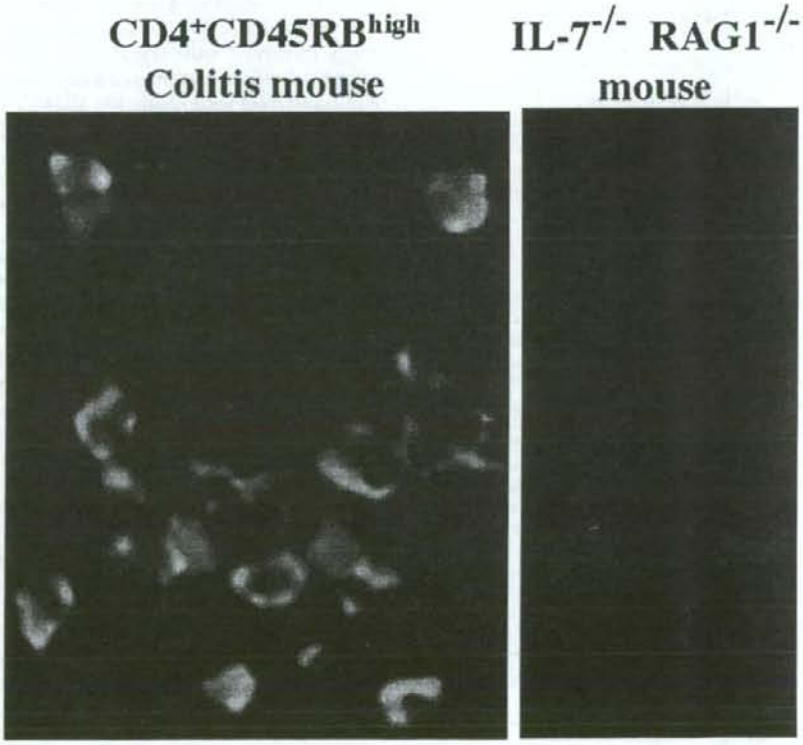


Figure 4. Cluster formation between CD4+ T cells and IL-7–expressing stromal cells within BM. Frozen sections of BM from colitic mice induced by adoptive transfer of CD4+CD45RB^{high} T cells (*left*) and untreated IL-7$^{-/-}$ × Rag-1$^{-/-}$ control mice (*right*) were stained with corresponding monoclonal antibodies. The IL-7–expressing cells (*green*) are scattered uniformly throughout the BM CD4+ T cells (*red*). CD4+ T cells lie close to IL-7–expressing stromal cells.

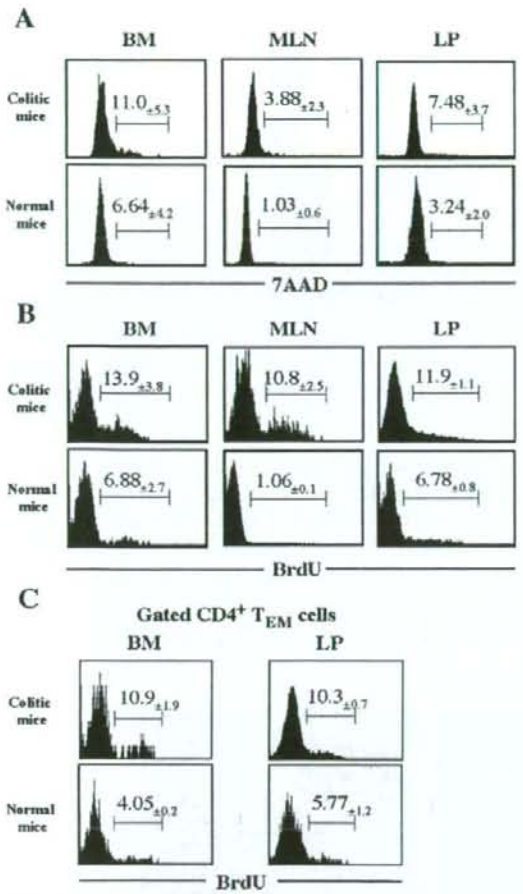


Figure 5. Colitic BM contains the actively dividing pool of memory $CD4^+$ T cells. (*A*) BM, MLN, and LP $CD4^+$ T cells from colitic mice or age-matched normal BALB/c mice were stained for DNA content using 7AAD. One representative mouse is shown of 5 mice analyzed. (*B*) Colitic mice and normal control mice were injected with BrdU for pulse-chase studies of BrdU incorporation. One representative mouse of 4 is shown. (*C*) Colitic mice and normal control mice were injected with BrdU as described in the Materials and Methods section. $CD4^+$ T cells were stained with CD4, CD44, and CD62L before intracellular staining for BrdU, and then the gated $CD4^+CD44^{high}CD62L^-$ T_{EM} cells in the BM and LP from colitic and normal mice were assessed by the BrdU incorporation. One representative mouse of 3 is shown.

with those in the paired normal BM, MLN, and LP. Because we compared dissimilar subsets in this setting because normal BM contains all subsets, such as naive, central memory, and T_{EM} $CD4^+$ T cells, yet in contrast colitic BM $CD4^+$ T cells are constituted of T_{EM} cells exclusively (Figure 1), we next compared colitic BM and LP $CD4^+CD44^{high}CD62L^-$ T_{EM} cells with the paired normal T_{EM} cells. As shown in Figure 5C, DNA synthesis in colitic BM and LP $CD4^+CD44^{high}CD62L^-$ T_{EM} cells was increased significantly as compared with that in the paired normal gated T_{EM} cells (Figure 5*C*).

Transfer of the BM Memory $CD4^+$ T Cells From Colitic Mice Into SCID Mice Reproduce Th1-Mediated Colitis

Based on the earlier-described results, we hypothesized that the colitic BM retaining $CD4^+$ T_{EM} cells is a pathogenic reservoir for persisting lifelong colitis. To prove this, we performed an adoptive transfer experiment by transferring colitic BM, MLN, and LP $CD4^+$ T_{EM} cells obtained from $CD4^+CD45RB^{high}$-transferred SCID mice and normal BM $CD4^+$ T cells into new SCID mice (Figure 6*A*). As shown in Figure 6*B*, mice transferred with the colitic BM, MLN, and LP $CD4^+$ T cells manifested progressive weight loss at 8 weeks after transfer. These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 4–6 weeks. In contrast, mice transferred with normal BM $CD4^+$ T cells appeared healthy, showing a gradual increase of body weight and no diarrhea during the period of observation (Figure 6*B* and *C*). At 8 weeks after transfer, colitic BM $CD4^+$ T-cell–transferred mice, but not mice transferred with normal BM $CD4^+$ T cells, had enlarged colons with greatly thickened walls (Figure 6*D*). The assessment of colitis by clinical scores showed a clear difference between mice transferred with colitic BM $CD4^+$ T cells and mice transferred with normal BM $CD4^+$ T cells (Figure 6*C*). In addition, the clinical scores of mice transferred with colitic BM $CD4^+$ T cells were comparable with those of mice transferred with colitic MLN or LP $CD4^+$ T cells. Histologic examination showed prominent epithelial hyperplasia with glandular elongation and massive infiltration of mononuclear cells in LP of the colon from colitic BM $CD4^+$ T-cell–transferred mice as well as colons from the colitic MLN or LP $CD4^+$ T-cell–transferred mice (Figure 6*E*). In contrast, pathologic findings were not observed in the LP of the colon from mice transferred with normal BM $CD4^+$ T cells (Figure 6*E*). This difference also was confirmed by histologic scoring of multiple colon sections (Figure 6*F*).

A further quantitative evaluation of $CD4^+$ T-cell accumulation was made by isolating $CD3^+CD4^+$ T cells. Few $CD3^+CD4^+$ T cells were recovered from the colonic LP in the normal BM $CD4^+$ T-cell–transferred mice as compared with the mice transferred with the colitic BM, MLN, or LP $CD4^+$ T cells (Figure 6*G*). Somewhat unexpectedly, the number of $CD4^+$ T cells recovered from the BM of normal BM $CD4^+$ T-cell–transferred mice was comparable with that from mice transferred with the colitic BM, MLN, or LP $CD4^+$ T cells (Figure 6*G*). Importantly, the number of $CD4^+$ cells recovered from the colitic BM $CD4^+$ T-cell–transferred mice far exceeded the number of cells originally injected (1×10^5), indicating extensive T-cell migration and/or proliferation in each

tissue. We also examined the cytokine production by isolated LP CD4+ T cells. As shown in Figure 6*H*, LP CD4+ T cells from colitic BM CD4+ T-cell-transferred mice produced significantly higher levels of IFN-γ and tumor necrosis factor-α than those from normal BM CD4+ T-cell-transferred mice on in vitro anti-CD3/anti-CD28 mAbs stimulation. In contrast, the production of IL-4 or IL-10 was not affected significantly (data not shown).

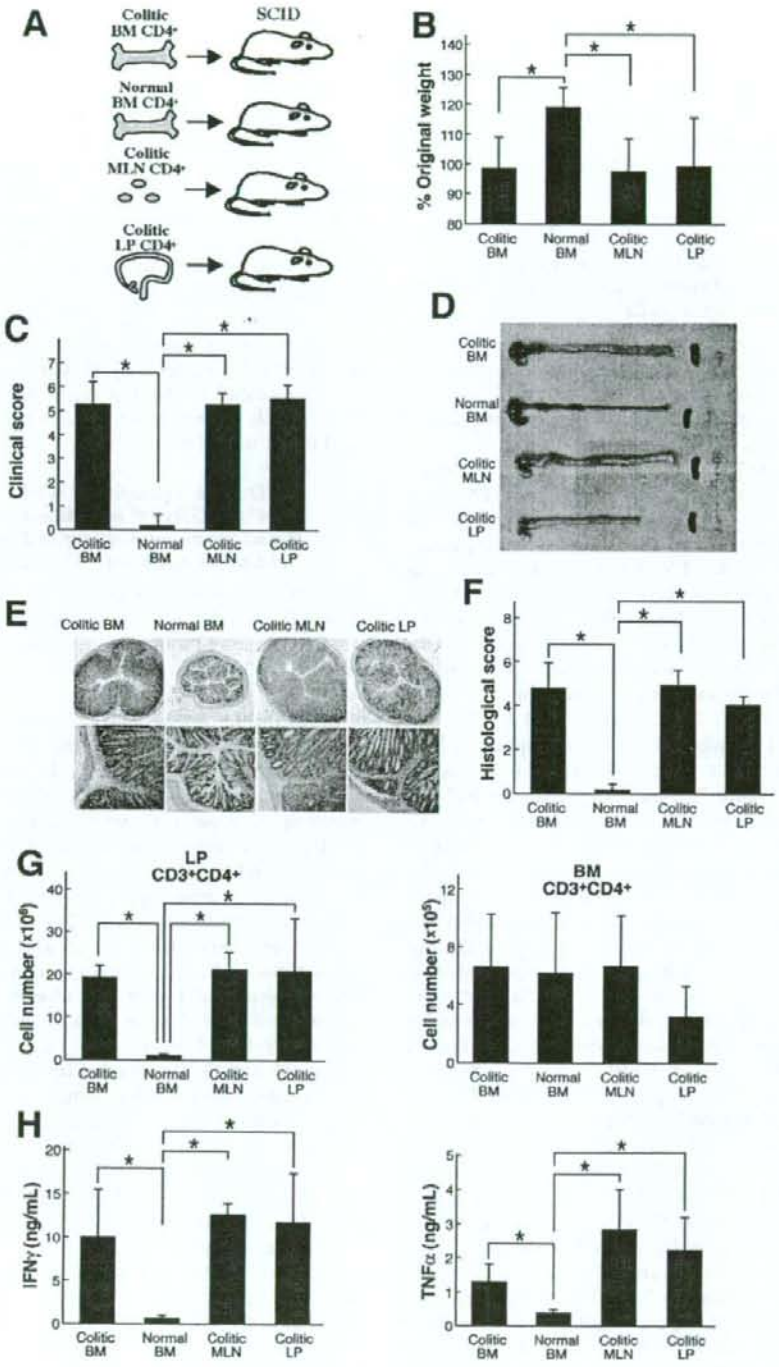
IL-7 Is Essential for the Survival and Homeostatic Proliferation of Colitogenic BM CD4+ Memory T Cells

To further analyze the role of IL-7 in the survival and homeostatic proliferation of the colitogenic BM CD4+ T cells, we retransferred CFSE-labeled LP CD4+ T cells obtained from CD4+CD45RB^{high} T-cell-transferred colitic mice into IL-7$^{+/+}$ × Rag-1$^{-/-}$ and IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice (Figure 7*A*). Rapid proliferation of donor colitic LP CD4+ T cells was observed in the BM from IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice 5 days after the transfer, although the relative size of the expanded T-cell populations in IL-7$^{-/-}$ × Rag-1$^{-/-}$ BM CD4+ T cells was approximately 80% of that observed in the control IL-7$^{+/+}$ × Rag-1$^{-/-}$ BM CD4+ T cells (Figure 7*B*). Somewhat unexpectedly, however, the recovered cell numbers of the BM and spleen CD4+ T cells from IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice were strikingly lower than those from IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice (BM: IL-7$^{-/-}$ × Rag-1$^{-/-}$ 2.3 ± 1.9 × 10^5; IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice, 45 ± 19 × 10^5; spleen: IL-7$^{-/-}$ × Rag-1$^{-/-}$ 3.8 ± .1 × 10^5; IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice, 32 ± 13 × 10^5) (Figure 7*C*), indicating that the IL-7 was essential for the survival rather than the homeostatic proliferation of the colitogenic CD4+ T cells in the BM. Consistent with this notion, we next assessed if regulation of Bcl-2 requires IL-7 at day 5 after the transfer, because induction of the anti-apoptotic protein, Bcl-2, is a hallmark of responses to IL-7.[14] As expected, the BM CD4+ T cells in IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice expressed lower levels of Bcl-2 than those in IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice (Figure 7*D*). Furthermore, the cell activation marker CD69 also was down-modulated significantly on the BM CD4+ T cells in IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice as compared with those in IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice (Figure 7*E*).

Finally, we asked whether adoptive transfer of colitogenic BM CD4+ T cells into IL-7$^{-/-}$ × Rag-1$^{-/-}$ or IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice induces colitis and results in the retention of BM CD4+ T cells (Figure 8*A*). Expectedly, transfer of colitogenic BM CD4+ T cells into the control IL-7$^{+/+}$ × Rag-1$^{-/-}$ mice led to a severe wasting disease 4–6 weeks after transfer, but IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice transferred with colitogenic BM CD4+ T cells appeared healthy and continued to gain weight during 10 weeks of observation (data not shown). The clinical score of IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients was almost zero, and significantly lower than that of IL-7$^{+/+}$ × Rag-1$^{-/-}$ recipients at 10 weeks after transfer (Figure 8*B*). The colon, the spleen, and the MLN from IL-7$^{+/+}$ × Rag-1$^{-/-}$ recipients, but not those from IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients, were enlarged and had a greatly thickened wall of colon (Figure 8*C*). Consistent with the lack of clinical signs in IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients, they displayed no histologic evidence of intestinal inflammation in contrast to IL-7$^{+/+}$ × Rag-1$^{-/-}$ recipients with severe inflammation (Figure 8*D*). Histologic analysis of colonic mucosa showed development of severe colitis in IL-7$^{+/+}$ × Rag-1$^{-/-}$, but not in IL-7$^{-/-}$ × Rag-1$^{-/-}$, recipients (Figure 8*E*). The total cell numbers of isolated BM, MLN, and LP CD3+CD4+ T cells from IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients were significantly lower than those from IL-7$^{+/+}$ × Rag-1$^{-/-}$ recipients (Figure 8*F*). Collectively, these results indicated that IL-7 is essential to develop colitis for colitogenic BM CD4+ T cells and to sustain these cells in the BM and in the LP and the MLN.

SCID Mice Transferred With CD4+CD45RB^{high} and Administered With Broad-Spectrum Antibiotics Did Not Develop Colitis, but Retained CD4+ T_{EM} in BM

It generally is accepted that colitis-inducing CD4+CD45RB^{high} T cells recognize bacterial and/or self-antigens that are induced by the presence of intestinal bacteria, and germ-free conditions prevent the development of intestinal inflammation in many animal models of colitis including the CD4+CD45RB^{high} T-cell–transfer model.[25] We therefore assessed whether SCID mice transferred with CD4+CD45RB^{high} T cells and treated with or without oral administration of a mixture of antibiotics (vancomycin, neomycin, metronidazole, and ampicillin) develop colitis and the persistence of BM CD4+ T cells (supplemental Figure 1*A*; supplementary material online at www.gastrojournal.org). As expected, we found that SCID mice transferred with CD4+CD45RB^{high} T cells without oral administration of antibiotics developed wasting disease (supplemental Figure 1*B*) and severe colitis (supplemental Figure 1*C*), whereas those with administration of antibiotics did not develop wasting disease and colitis 4 weeks after transfer (supplemental Figures 1*B* and *C*). The blinded histologic score of mice treated with antibiotics was almost zero in contrast to control recipient mice without administration of antibiotics (6.2 ± 1.3) (supplemental Figure 1*D*). The average number of CD3+CD4+ T cells recovered from recipient mice that transferred with CD4+CD45RB^{high} T cells and given drinking water without antibiotics was 11.0 ± 0.7 × 10^5 per mouse in BM, 52 ± 20 × 10^5 in MLN, and 240 ± 40 × 10^5 in LP (supplemental Figure 1*E*). In contrast, the cell number in mice transferred with CD4+CD45RB^{high} T cells and treated with antibiotics was decreased significantly compared with mice transferred with CD4+CD45RB^{high} T cells and given the antibiotics (BM, 2.2 ± 1.8 × 10^5 per mouse; spleen, 11 ± 11 × 10^5;


A
Colitic BM CD4+
SCID
Normal BM CD4+
Colitic MLN CD4+
Colitic LP CD4+
B
% Original weight
Colitic BM
Normal BM
Colitic MLN
Colitic LP
C
Clinical score
D
E
F
Histological score
G
LP CD3+CD4+
Cell number (x10⁶)
BM CD3+CD4+
Cell number (x10⁵)
H
IFNγ (ng/mL)
TNFα (ng/mL)

and LP, 28 ± 24 × 10^5) (supplemental Figure 1*E*). Therefore, the administration of antibiotics significantly suppressed colitis and resulted in the reduced expansion of BM $CD3^+CD4^+$ T cells and MLN and LP.

Transfer of BM CD4+ T Cells From Colitic IL-10–Deficient Mice, but not Normal Mice, Into Rag-2−/− Mice Reproduces Th1-Mediated Colitis

We finally addressed whether latent colitogenic $CD4^+$ T cells reside in the BM in a colitis model that develops colitis spontaneously, rather than the adoptive transfer model, in this case, IL-$10^{-/-}$ mice[26] (supplemental Figure 2*A*; supplementary material online at www.gastrojournal.org). We first isolated the BM $CD4^+$ T cells from diseased IL-$10^{-/-}$ mice and age-matched normal C57BL/6 mice, and analyzed the expression of CD44 and CD62L on $CD4^+$ T cells by flow cytometry. Similar to the BM $CD4^+$ T cells in colitic mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$, $CD4^+CD44^{high}CD62L^-$ T_{EM} cells preferentially resided in the BM of colitic IL-$10^{-/-}$ mice as compared with age-matched normal C57BL/6 mice (supplemental Figure 2*B*, upper). We next transferred the BM $CD4^+$ T cells from diseased IL-$10^{-/-}$ mice and age-matched normal C57BL/6 mice into recipient C57BL/6 Rag-$2^{-/-}$ mice (supplemental Figure 2*A*). Mice transferred with the colitic IL-$10^{-/-}$ BM $CD4^+$ T cells manifested progressive weight loss (wasting disease) at 10 weeks after transfer as compared with the mice transferred with normal C57BL/6 BM $CD4^+$ T cells (data not shown). These mice had significant clinical symptoms by 4–6 weeks after transfer, but mice transferred with normal BM $CD4^+$ T cells appeared healthy without diarrhea during the whole period of observation. The assessment of colitis by clinical scores showed a clear difference between the mice transferred with colitic IL-$10^{-/-}$ BM $CD4^+$ T cells and the mice transferred with normal BM $CD4^+$ T cells (supplemental Figure 2*C*). At 10 weeks after transfer, the colitic IL-$10^{-/-}$ BM $CD4^+$ T-cell-transferred mice, but not those transferred with normal BM $CD4^+$ T cells, had enlarged colons with greatly thickened walls (supplemental Figure 2*D*). Histologic examination showed severe signs of colitis, including epithelial hyperplasia and massive infiltration of mononuclear cells, in LP from the colitic IL-$10^{-/-}$ BM $CD4^+$ T-cell-transferred mice as compared with the colons from the normal BM $CD4^+$ T-cell-transferred mice (supplemental Figure 2*E*). This difference also was confirmed by histologic scoring of multiple colon sections (supplemental Figure 2*F*). Furthermore, few $CD4^+$ T cells were recovered from the colonic LP in the normal BM $CD4^+$ T-cell-transferred mice as compared with the mice transferred with the colitic IL-$10^{-/-}$ BM $CD4^+$ T cells (supplemental Figure 2*G*). As in the model of $CD4^+CD45RB^{high}$ T-cell-transferred colitis, the number of recovered BM $CD4^+$ T cells from the normal BM $CD4^+$ T-cell-transferred mice was comparable with that from mice transferred with the colitic IL-$10^{-/-}$ BM (supplemental Figure 2*G*). We finally examined the cytokine production by isolated LP $CD4^+$ T cells. LP $CD4^+$ T cells from the normal BM $CD4^+$ T-cell-transferred mice produced significantly less IFN-γ and tumor necrosis factor-α than those from the colitic IL-$10^{-/-}$ $CD4^+$ T-cell-transferred mice on in vitro stimulation (supplemental Figure 2*H*). These results suggested that the colitic IL-$10^{-/-}$ BM $CD4^+$ T cells have potent colitogenic $CD4^+$ T cells to reproduce Th1-mediated colitis in normal recipient SCID mice.

Discussion

In the present study, we showed that $CD4^+CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ T_{EM} cells, but not central-memory T cells and naive T cells, preferentially reside in the BM obtained from Th1-mediated colitic SCID/Rag-$2^{-/-}$ mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells. Importantly, these resident BM $CD4^+$ T_{EM} cells are attached closely to IL-7–producing stromal cells in the BM, and retain significant potential to induce colitis by the adoptive retransfer into new SCID/Rag-$2^{-/-}$ mice. Of particular importance, we showed here that IL-7 is essential for the development of colitis induced by the adoptive transfer of colitogenic BM $CD4^+$ T_{EM} cells using IL-$7^{-/-}$ × Rag-$1^{-/-}$ and the control IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice. Furthermore, the accumulation

Figure 6. SCID mice transferred with the BM $CD4^+$ T cells obtained from $CD4^+CD45RB^{high}$ T-cell–transferred colitis develop chronic colitis. (*A*) CB-17 SCID mice were injected intraperitoneally with normal splenic $CD4^+CD45RB^{high}$ T cells. Six weeks after transfer mice developed chronic colitis, and $CD4^+$ T cells were isolated from each organ. Doses of 2 × 10^5 BM, MLN, or LP $CD4^+$ T cells were injected into new CB-17 SCID mice. As a negative control, 2 × 10^5 BM $CD4^+$ T cells obtained from normal BALB/c mice also were injected into SCID mice. (*B*) Mice transferred with the colitic BM $CD4^+$ T cells did not gain weight. *$P < .05$. (*C*) Mice transferred with the colitic BM $CD4^+$ T cells showed severe clinical signs of colitis. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*D*) Gross appearance of the colon, spleen, and MLN from mice transferred with the colitic BM $CD4^+$ T cells (*first row*), the normal BM $CD4^+$ T cells (*second row*), the colitic MLN $CD4^+$ T cells (*third row*), or LP $CD4^+$ T cells (*fourth row*). (*E*) Histopathologic comparison of distal colon from mice injected with the colitic BM, the normal BM, the colitic MLN, or the colitic LP $CD4^+$ T cells. Original magnification: *upper*, 40×; *lower*, 100×. (*F*) Histologic scores were determined at 8 weeks after transfer as described in the Materials and Methods section. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*G*) LP and BM $CD4^+$ T cells were isolated from mice injected with colitic BM, normal BM, colitic MLN, or colitic LP $CD4^+$ T cells 8 weeks after transfer, and the number of $CD3^+CD4^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*H*) Cytokine production by LP $CD4^+$ T cells. IFN-γ and tumor necrosis factor-α concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SD of 6 mice in each group. *$P < .05$.

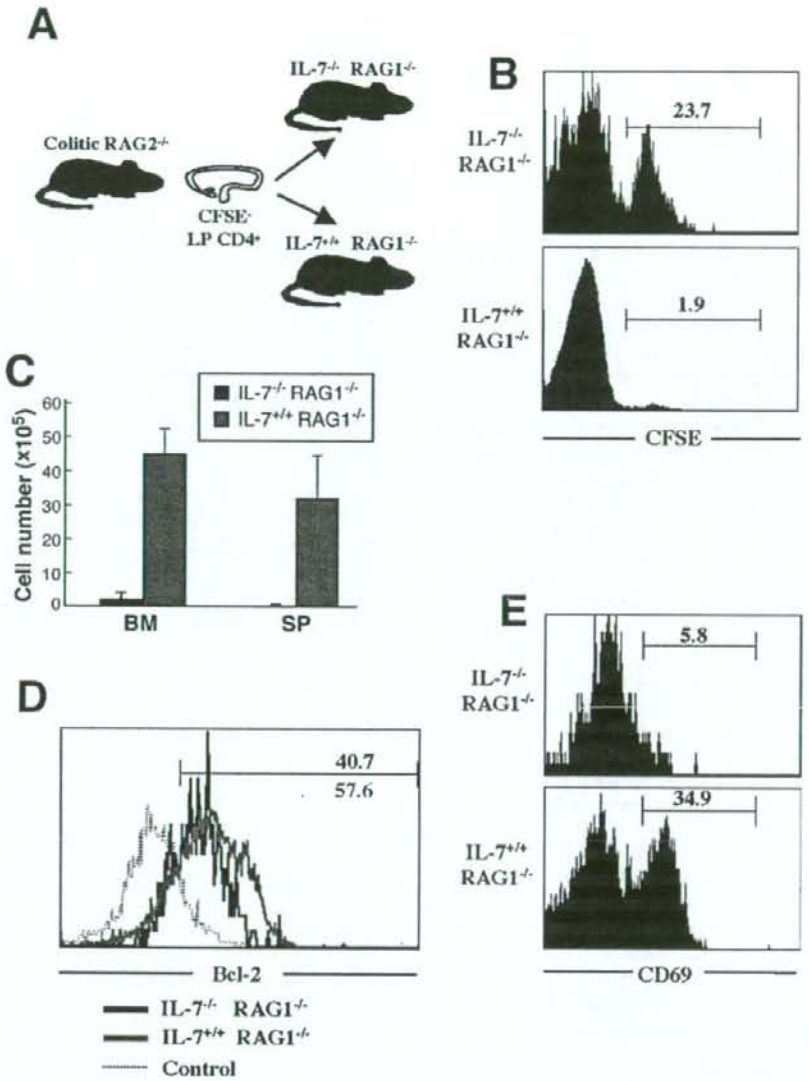


Figure 7. IL-7 is essential for the survival and in part for the cell turnover of colitogenic BM CD4+ T cells. (*A*) C57BL/6 Rag-2$^{-/-}$ mice were injected intraperitoneally with normal splenic CD4+CD45RB^{high} T cells. Six weeks after transfer, the LP CD4+ T cells were isolated. Colitogenic LP CD4+ T cells were labeled with CFSE and adoptively transferred into new IL-7$^{+/+}$ × Rag1$^{-/-}$ or IL-7$^{-/-}$ × Rag1$^{-/-}$ mice. Five days after transfer, CFSE incorporation was determined by flow cytometry. Histograms are gated on CD3+ T cells. (*C*) The BM and spleen (SP) CD4+ T cells were isolated from IL-7$^{+/+}$ × Rag1$^{-/-}$ or IL-7$^{-/-}$ × Rag1$^{-/-}$ mice injected with the colitic LP CD4+ T cells 5 days after transfer, and the number of CD4+ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .05$. (*D*) Representative flow-cytometric histograms showing the expression of Bcl-2 in BM CD4+ T cells from IL-7$^{+/+}$ × Rag1$^{-/-}$ or IL-7$^{-/-}$ × Rag1$^{-/-}$ mice injected with the colitogenic LP CD4+ T cells 5 days after transfer from 3 independent similar experiments. (*E*) Representative flow-cytometric histograms showing the expression of CD69 on BM CD4+ T cells from IL-7$^{+/+}$ × Rag1$^{-/-}$ or IL-7$^{-/-}$ × Rag1$^{-/-}$ mice injected with the colitogenic LP CD4+ T cells 5 days after transfer from 3 independent similar experiments.

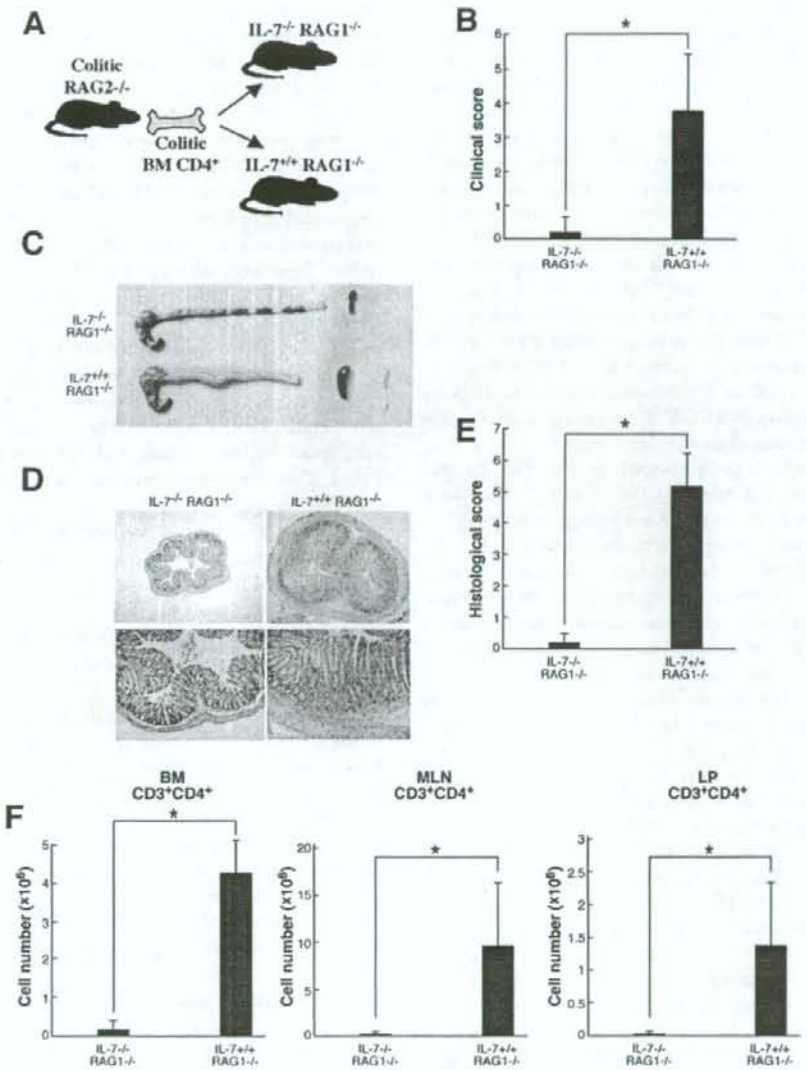


Figure 8. IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice transferred with colitogenic BM CD4$^+$CD44^{high}CD62L$^-$ T_{EM} cells did not develop colitis. (*A*) IL-7$^{+/+}$ × Rag-1$^{-/-}$ (n = 5) and IL-7$^{-/-}$ × Rag-1$^{-/-}$ (n = 5) mice were transferred with colitic BM CD4$^+$ T cells. (*B*) Clinical scores were determined 10 weeks after transfer. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .005$. (*C*) Gross appearance of the colon, spleen, and MLN from IL-7$^{-/-}$ × Rag-1$^{-/-}$ (*top*) and IL-7$^{+/+}$ × Rag-1$^{-/-}$ (*bottom*) recipients 10 weeks after transfer. (*D*) Histologic examination of the colon from IL-7$^{-/-}$ × RAG-1$^{-/-}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice transferred with colitogenic BM CD4$^+$ T cells 10 weeks after transfer. Original magnification: *upper*, 40×; lower, 100×. (*E*) Histologic scoring of IL-7$^{+/+}$ × Rag-1$^{-/-}$ and IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients 10 weeks after transfer. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .005$. (*F*) BM, LP, and spleen cells were isolated from IL-7$^{+/+}$ × Rag-1$^{-/-}$ and IL-7$^{-/-}$ × Rag-1$^{-/-}$ recipients 10 weeks after transfer, and the number of CD3$^+$CD4$^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. *$P < .0005$.

of BM CD4$^+$ T cells was decreased significantly in IL-7-deficient recipients reconstituted with the colitogenic LP CD4$^+$ T_{EM} cells. Collectively, these findings suggest that the BM CD4$^+$ T_{EM} cells residing in mice with chronic colitis play a critical role as a reservoir for persisting lifelong colitis in an IL-7-dependent manner.

The present data raise the most important question of whether the colitogenic BM CD4$^+$CD44^{high}CD62L$^-$ T

cells can be defined as T_{EM} cells rather than effector T cells in the presence of antigens (Ags), in this case, probably intestinal bacteria. First, we found that these colitogenic BM $CD4^+$ T cells highly expressed IL-7Rα in accordance with the evidence that IL-7Rα is one of memory, but not effector, T-cell markers. Second, it is well known that memory, but not effector, $CD4^+$ T cells are critically controlled by the homeostatic proliferation and the survival by IL-7.[14] Consistent with this, we found that the BM $CD4^+$ T cells were decreased markedly in IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice transferred with the colitogenic LP or BM $CD4^+$ T cells as compared with IL-$7^{+/+}$ × Rag-$1^{-/-}$ recipients. Further, we showed that IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice transferred with the colitogenic BM $CD4^+$ T cells did not develop colitis in contrast to IL-$7^{+/+}$ × Rag-$1^{-/-}$ recipients with colitis. Collectively, these data indicate that the colitogenic BM $CD4^+$ T cells in our colitis model are T_{EM} cells rather than effector T cells.

IL-7 originally was discovered in the BM stromal cells.[23] However, the role for $CD4^+$ T cells in the BM is largely unknown, especially in pathologic conditions, although it has been recognized recently that a high number of antigen-specific $CD8^+$ memory T cells persist in the BM for several months after resolution of acute viral infection.[7,8] Furthermore, recent accumulating evidence suggests that IL-7 is a critical factor for the survival and homeostatic proliferation of memory $CD4^+$ T cells.[14] Thus, we hypothesized that IL-7–producing BM harbors the colitogenic memory $CD4^+$ T cells as a reservoir, causing persistent lifelong colitis. Consistent with this hypothesis, we found that IL-7–expressing cells were scattered throughout the BM and most $CD4^+$ T cells were in close contact with the bodies of IL-7–expressing BM cells in colitic SCID mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells (Figure 5). However, the possibility cannot be excluded of a recently described novel pathway for dendritic cell migration that allows dendritic cells to collect Ags in peripheral sites and traffic them to the BM to elicit recall responses by the resident BM T cells.[27] This, however, is unlikely in this case because the production of IFN-γ by anti-CD3/CD28- or CBA-stimulated colitic BM $CD4^+$ T cells was significantly lower than that of anti-CD3/CD28- or CBA-stimulated colitic LP $CD4^+$ T cells (Figures 2 and 3), indicating that the BM colitogenic T cells in colitic mice might be indicative of a recent encounter with Ags in the LP, and may migrate into the BM, which is abundant in IL-7, but not in Ags.

In this article we asked how $CD4^+$ memory T cells accumulate in the BM in mice with chronic colitis. Indeed, BM stromal cells can support lymphoid precursor cell differentiation into mature T cells in vitro[28] and in athymic mice in vivo.[29] Mature T cells in the BM are probably immigrants from the blood because T cells normally are produced in the thymus. However, the mechanisms by which in vivo–generated memory cell subsets are recruited to tissues have been difficult to study in the case of polyclonal and physiologic systems rather than the monoclonal T-cell receptor transgenic system because such studies require unattainable numbers of purified cells for in vivo assay. In this study, however, we were able to circumvent this obstacle by using the SCID/Rag-$2^{-/-}$-colitis model induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells because a large number of $CD4^+$ T cells infiltrated the colonic LP in this model, and they technically could be isolated in the order of approximately 1×10^7 cells per mouse. By using the present adoptive transfer system, we found that $CD4^+$ T cells resided in the BM from Rag-$1^{-/-}$ mice transferred with colitogenic LP $CD4^+$ T cells at the early time point of 5 days after transfer (Figure 7). We also found that the recovered cell number of BM $CD4^+$ T cells was parallel to that of LP $CD4^+$ T cells in mice given antibiotics without colitis and the control mice with colitis. These results indicate that colitogenic LP $CD4^+$ T cells exit from the gut, and directly migrate into the BM, (Supplemental Figure 1, see supplemental material online at www.gastrojournal.org although further studies will be needed to show direct evidence for this issue.

Although the Ags driving the T-cell immune response in the experimental system of T-cell–induced IBD have not yet been identified with certainty, and thus it is impossible to chase the biological behavior of antigen-specific T cells, overwhelming evidence supports the idea that the triggering factor in this experimental system is of bacterial origin. Furthermore, the present study significantly complements recent reports that BM harbors Ag-specific memory $CD8^+$ T cells.[2,30-31] A recent report has shown very efficient interactions between T cells and dendritic cells in the BM microenvironment.[11] It may be that the similar environment that promotes T-cell priming also triggers homeostatic proliferation and survival of the colitogenic BM T_{EM} cells by IL-7. Perhaps, as has been suggested for plasma cells and Ag-specific $CD8^+$ memory T cells, a unique combination of the cytokine milieu including IL-7 and contact-dependent interactions in the BM supports the colitogenic BM T_{EM} cells. Furthermore, the possibility that other sites, such as MLN and spleen, also might play a role as other reservoirs for colitogenic $CD4^+$ T_{EM} cells, as well as the BM in colitic mice, cannot be excluded. Further studies will be needed to address this issue.

In conclusion, our findings show that a proportion of colitogenic $CD4^+$ T cells in colitic mice may leave peripheral tissues, such as LP and MLN, and gain access to the IL-7–abundant BM via the bloodstream. By using adoptive transfer protocols, we have shown that these BM $CD4^+$ T_{EM} cells possess the ability to induce colitis, suggesting that the colitogenic BM $CD4^+$ T cells residing in colitic mice play a critical role as a reservoir for persisting lifelong colitis and participate in relapses after remissions in IBDs.[17]

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi;10.1053/j.gastro.2006.10.035.

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IL-7 Is Essential for the Development and the Persistence of Chronic Colitis[1]

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Although IL-7 has recently emerged as a key cytokine involved in controlling the homeostatic turnover and the survival of peripheral resting memory CD4$^+$ T cells, its potential to be sustained pathogenic CD4$^+$ T cells in chronic immune diseases, such as inflammatory bowel diseases, still remains unclear. In this study, we demonstrate that IL-7 is essential for the development and the persistence of chronic colitis induced by adoptive transfer of normal CD4$^+$CD45RB^{high} T cells or colitogenic lamina propria (LP) CD4$^+$ memory T cells into immunodeficient IL-7$^{+/+}$ × RAG-1$^{-/-}$ and IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice. Although IL-7$^{+/+}$ × RAG-1$^{-/-}$ recipients transferred with CD4$^+$CD45RB^{high} splenocytes developed massive inflammation of the large intestinal mucosa concurrent with massive expansion of Th1 cells, IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients did not. Furthermore, IL-7$^{-/-}$ × RAG-1$^{-/-}$, but not IL-7$^{+/+}$ × RAG-1$^{-/-}$, mice transferred with LP CD4$^+$CD44^{high}CD62L$^-$IL-7Rα^{high} effector-memory T cells (T_{EM}) isolated from colitic CD4$^+$CD45RB^{high}-transferred mice did not develop colitis. Although rapid proliferation of transferred colitogenic LP CD4$^+$ T_{EM} cells was observed in the in IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice to a similar extent of those in IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice, Bcl-2 expression was significantly down-modulated in the transferred CD4$^+$ T cells in IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice compared with those in IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice. Taken together, IL-7 is essential for the development and the persistence of chronic colitis as a critical survival factor for colitogenic CD4$^+$ T_{EM} cells, suggesting that therapeutic approaches targeting IL-7/IL-7R signaling pathway may be feasible in the treatment of inflammatory bowel diseases. *The Journal of Immunology,* **2007, 178: 4737–4748.**

Inflammatory bowel diseases (IBD)[3] is caused by excessive and tissue damaging chronic inflammatory responses in the gut wall and commonly take persistent, disabling courses (1). In some patients, disease progresses steadily, while in others, relapses alter with remissions. According to present understanding, disease is caused and controlled by pathogenic effector and memory CD4$^+$ T cells, which are accumulated in their target tissues and thus determine activity and clinical character. However, the nature of pathogenic memory CD4$^+$ T cells over time and the correlation between effector and memory CD4$^+$ T cells in chronic colitis in the presence of commensal bacteria remains largely unknown.

IL-7 is a stromal cell-derived cytokine that is secreted by fetal liver cells, stromal cells in the bone marrow and thymus, and other epithelial cells (2–4). Recently, IL-7 has emerged as a key cytokine involved in controlling the survival of peripheral resting CD4$^+$ T cells, including naive and memory cells and their homeostatic turnover (3–10). The effect of IL-7 on T cells is controlled by the expression of the specific receptor for IL-7, the state of differentiation of the T cell, the available concentration of the cytokine, and whether there is concomitant TCR signaling. IL-7R consists of the α-chain (CD127) and the common cytokine receptor γ-chain, which is shared by the common γ-chain family cytokines (IL-2, IL-4, IL-9, IL-15, and IL-21) (3, 4).

In contrast to the role of IL-7 in naive and memory CD4$^+$ T cells in the resting state, the pathological role of IL-7 in chronic immune-mediated diseases, such as autoimmune diseases and IBD, remains largely unclear. We have previously demonstrated that 1) IL-7 is constitutively produced by intestinal epithelial cells (11), 2) IL-7 transgenic mice developed chronic colitis that mimicked histopathological characteristics of human IBD (12), 3) mucosal CD4$^+$IL-7R^{high} T cells in mice with colitis are colitogenic (13), and 4) the selective elimination of CD4$^+$IL-7R^{high} T cells by administrating small amounts of toxin-conjugated anti-IL-7Rα mAb completely ameliorated ongoing colitis (13).

In this study, we attempt to clarify the link between the colitogenic CD4$^+$ T cells and IL-7 more extensively in terms of pathogenesis of chronic colitis using adoptive transfer system. The adoptive transfer of CD4$^+$CD45RB^{high} T cells into syngeneic immunodeficient mice, such as SCID mice and RAG-1 or RAG-2-deficient mice, induces human IBD-like diseases (14). The key factors for the development of colitis are an expanding CD4$^+$ T cell subset in lymphopenic condition, an intact gut flora of the host, and various cytokines (15). Dysregulation of mucosal CD4$^+$ T cell responses is supposed to play a key role in the pathogenesis of colitis in this model, with exaggerated IFN-γ and TNF-α responses as major mediators of these models (14, 15). These pathogenic CD4$^+$ T cell responses may be derived by Ags of the intestinal flora to which these CD4$^+$ T cells are normally tolerant in the presence of regulatory T cells. Because the use of IL-7$^{-/-}$ or IL-7Rα$^{-/-}$ mice in inflammatory models is not useful because of the

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; HPF, high power field; IEL, intraepithelial cell; LP, lamina propria; T_{EM}, effector-memory T.

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lymphoid organ aberrations and the lack of lymphocytes (3), we, in this study, used IL-$7^{-/-}$ × RAG-$1^{-/-}$ and IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice for adoptive transfer of $CD4^+CD45RB^{high}$ T cells and colitogenic lamina propria (LP) $CD4^+CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ effector-memory T (T_{EM}) cells obtained from colitic mice that have been transferred previously with $CD4^+CD45RB^{high}$ T cells to assess a role of IL-7 for the development and the persistence of chronic colitis. We studied this polyclonal system rather than system using monoclonal TCR transgenic memory $CD4^+$ T cells because they not only represent a diverse memory pool, but they also provide the most pathophysiolocally relevant setting to examine the role of IL-7 in the development of colitis.

Materials and Methods

Animals

Female C57BL/6 mice were purchased from Japan CLEA. C57BL/6 background RAG-$2^{-/-}$ mice were obtained from Taconic Farms. C57BL/6 background RAG-$1^{-/-}$ mice and C57BL/6 background IL-7-deficient (IL-$7^{-/-}$) mice were provided from Dr. R. Zamoyska (National Institute for Medical Research, London, U.K.) (16). IL-$7^{-/-}$ mice were intercrossed into RAG-$1^{-/-}$ mice to generate IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice in the Animal Care Facility of Tokyo Medical and Dental University. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Female donors and recipients were used at 6–12 wk of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Purification of T cell subsets

$CD4^+$ T cells were isolated from spleen cells from C57BL/6 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-CD45RB (16A; BD Pharmingen). Subpopulations of $CD4^+$ cells were generated by two-color sorting on a FACSVantage (BD Biosciences). All populations were >98.0% pure on reanalysis. To isolate LP $CD4^+$ T cells, colitis was induced in RAG-$2^{-/-}$ mice by adoptive transfer of $CD4^+$ $CD45RB^{high}$ T cells as described previously (17). The colitic $CD4^+$ $CD45RB^{high}$ T cell-transferred RAG-$2^{-/-}$ mice were sacrificed at 6–8 wk after transfer. The entire length of colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca^{2+}-, Mg^{2+}-free Hanks' BSS containing 1 mM DTT (Sigma-Aldrich) for 45 min to remove mucus and then treated with 2.0 mg/ml collagenase (Worthington Biomedical) and 0.01% DNase (Worthington Biomedical) 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.

In vivo experimental design

We performed a series of in vivo experiments below to investigate the role of IL-7 in the development and the persistence of murine chronic colitis. Experiment 1: To assess the necessity of IL-7 in the initial development of colitis, including the processes for T cell priming, activation, and persistence of pathogenic effector and memory $CD4^+$ T cells, we performed the cell transfer experiments using IL-$7^{+/+}$ × RAG-$1^{-/-}$ and IL-$7^{-/-}$ × RAG-$1^{-/-}$ littermate recipients. IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (n = 10) and IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice (n = 10) were injected i.p. with 3 × 10^5 splenic $CD4^+CD45RB^{high}$ T cells from normal C57BL/6 mice. Experiment 2: To assess the necessity of IL-7 for the persistence of colitogenic memory $CD4^+$ T cells, we performed the adoptive retransfer of colitogenic LP memory $CD4^+$ T cells. First, colitis was induced in RAG-$2^{-/-}$ mice by adoptive transfer of $CD4^+CD45RB^{high}$ T cells. The colitogenic memory $CD4^+$ LP T cells were obtained from the colitic mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells 6–8 wk after transfer and then were injected again into the control IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (IL-$7^{+/+}$→IL-$7^{+/+}$; n = 10) and IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice (IL-$7^{+/+}$→IL-$7^{-/-}$; n = 10). Experiment 3: To assess the initial role of IL-7 for T cell priming, differentiation, and sustainment of colitogenic effector and memory $CD4^+$ T cells, we performed another adoptive retransfer experiment. First, IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice were transferred with $CD4^+CD45RB^{high}$ T cells. Six weeks after transfer, splenic $CD4^+$ T cells were isolated from the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice and retransferred into new IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (IL-$7^{-/-}$→IL-$7^{+/+}$; n = 7). As a positive control, splenic $CD4^+$ T cells obtained from colitic IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice that have been initially transferred with $CD4^+CD45RB^{high}$ T cells were also transferred into new IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (IL-$7^{+/+}$→IL-$7^{+/+}$; n = 7). The recipient mice after transfer were weighed initially and then three times per week. They were also observed for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool. Mice were sacrificed 4–8 wk after transfer and assessed for a clinical score (17) 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) (17).

Histological examination

Tissue samples were fixed in PBS containing 6% 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 the type of T cell reconstitution or treatment. 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 (17), 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.

Cytokine ELISA

To measure cytokine production, 1 × 10^5 LP $CD4^+$ T cells were cultured in 200 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar) precoated with 5 μg/ml hamster anti-mouse CD3ε mAb (145-2C11; BD Pharmingen) and hamster 2 μg/ml anti-mouse CD28 mAb (37.51; BD Pharmingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA per manufacturer's recommendation (R&D Systems).

Flow cytometry

To detect the surface expression of a variety of molecules, isolated splenocytes or LP mononuclear cells were preincubated with a FcγR-blocking mAb (CD16/32, 2.4G2; BD Pharmingen) for 20 min followed by incubation with specific FITC-, PE-, PECy5-, or biotin-labeled Abs for 30 min on ice. The following mAbs other than biotin-conjugated anti-mouse IL-7Rα (A7R34; Immuno-Biological Laboratories) were obtained from BD Pharmingen: anti-CD4 mAb (RM4-5), anti-CD25 mAb (7D4), anti-CD45RB mAb (16A), anti-CD62L mAb (MEL-14), anti-CD44 mAb (IM7), anti-CD69 mAb (H1.2F3), and anti-Bcl-2 mAb (3F11). Biotinylated Abs were detected with PE- or CyChrome-streptavidin. Standard two- or three- color flow cytometric analyses were obtained using the FACSCalibur using CellQuest software. Background fluorescence was assessed by staining with control irrelevant isotype-matched mAbs.

To analyze Bcl-2 expression in LP $CD4^+$ T cells, cells were fixed and permeabilized with BD Cytofix/Cytoperm solution before intracellular cytokine staining with FITC-conjugated anti-Bcl-2 mAb (BD Pharmingen). To analyze the TCR Vβ family repertoire, splenic cells were double-stained with PE-conjugated anti-CD4 mAb (RM4-5), and the following FITC-conjugated mAbs: Vβ2; KJ25, Vβ3; KT4, Vβ4; MR9-4, Vβ5; RR4-7, Vβ6; TR310, Vβ7; MR5-2, Vβ8.1/2; B21.14, Vβ8.3; MR10-2, Vβ9; B21.5, Vβ10; RR3-15, Vβ11; MR11-1, Vβ12; IN12.3, Vβ13; 14.2, Vβ14; and KJ23, Vβ17. All Abs were purchased from BD Pharmingen.

CFSE labeling of T cells

T cell division in vivo was assessed by flow cytometry of CFSE-labeled cells. Isolated LP $CD4^+$ T cells were stained in vitro with the cytoplasmic dye CFSE (Molecular Probes) before reconstitution. Briefly, cells were incubated for 7 min at 37°C with 5 μM CFSE. The reaction was quenched by washing in ice-cold DMEM supplemented with 10% FCS. The recipient mice were injected i.p. with 3.0 × 10^6 CFSE-labeled $CD4^+$ T cells for

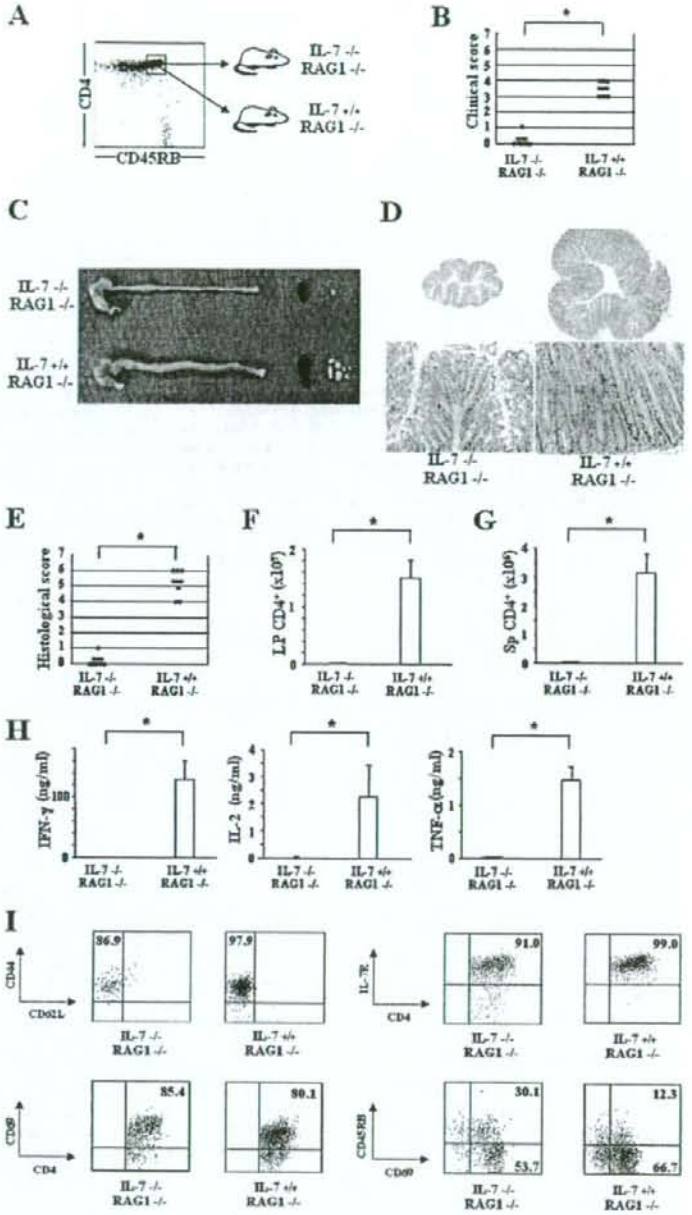

FIGURE 1. IL-7$^{-/-}$ × RAG-1$^{-/-}$ transferred with CD4$^+$CD45RB^{high} T cells did not develop chronic colitis. *A*, IL-7$^{-/-}$ × RAG-1$^{-/-}$ (n = 10) and IL-7$^{+/+}$ × RAG-1$^{-/-}$ (n = 10) mice were transferred with normal splenic CD4$^+$CD45RB^{high} T cells (3×10^5 cells/mouse). *B*, Clinical scores were determined at 8 wk after the transfer as described in *Materials and Methods*. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.001$. *C*, Gross appearance of the colon, spleen, and mesenteric lymph nodes from CD4$^+$CD45RB^{high} T cell-transferred IL-7$^{-/-}$ × RAG-1$^{-/-}$ (*top*) and IL-7$^{+/+}$ × RAG-1$^{-/-}$ (*bottom*) mice at 8 wk after the transfer. *D*, Histological examination of the colon from IL-7$^{-/-}$ × RAG-1$^{+/+}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ recipients at 6 wk after the transfer. Original magnification: ×40 (*upper*); ×100 (*lower*). *E*, Histological scoring of IL-7$^{-/-}$ × RAG-1$^{-/-}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells after 8 wk after transfer. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.005$. LP (*F*) and spleen (*G*) CD4$^+$ T cells were isolated from IL-7$^{-/-}$ × RAG-1$^{-/-}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells 8 wk after transfer, and the number of CD4$^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.01$. *H*, Cytokine production by LP CD4$^+$ T cells. LP CD4$^+$ T cells were isolated at 8 wk after transfer and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. IFN-γ, IL-2, and TNF-α concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SD of seven mice in each group. *, $p < 0.001$. *I*, Phenotypic characterization of LP CD4$^+$ T cells isolated from IL-7$^{-/-}$ × RAG-1$^{-/-}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice transferred with CD4$^+$ CD45RB^{high} T cells 8 wk after transfer.

5–10 days. At the indicated time points, isolated splenic cells were stained with allophycocyanin-labeled anti-CD4 mAb (L3T4), PerCP-labeled anti-CD3 mAb (2C11), and PE-labeled Annexin V (MBL), and the intensity of CFSE and annexin V was determined after gating on CD3$^+$CD4$^+$ T cells. To assess the role of commensal bacteria in cell division, a group of mice were treated with a set of four antibiotics, i.e., ampicillin (1g/L; Sigma-Aldrich), vancomycin (500 mg/L; Abbott Laboratories), neomycin sulfate (1 g/L; Pharmacia), and metronidazole (1 g/L; Sidmack) in drinking water 2 wk before beginning the adoptive transfer and during the course of experiment based on a variation of the commensal depletion protocol of Fagarasan et al. (18). At the indicated time points, isolated spleen cells were stained with APC-labeled anti-CD4 mAb and PerCP-labeled anti-CD3 mAb, and the intensity of CFSE was determined after gating on CD3$^+$ CD4$^+$ T cells.

Statistical analysis

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

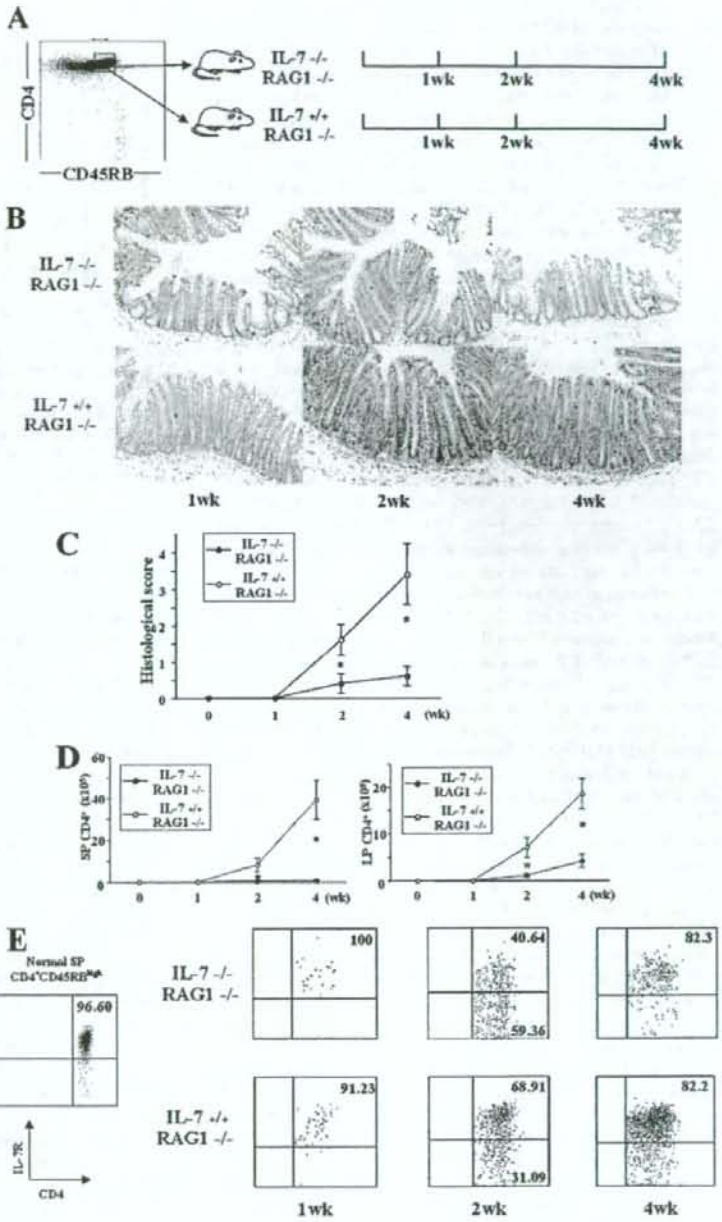

FIGURE 2. Kinetics of development of colitis and IL-7Rα expression. *A*, IL-$7^{-/-}$ × RAG-$1^{-/-}$ (n = 12) and IL-$7^{+/+}$ × RAG-$1^{-/-}$ (n = 10) mice were transferred with $CD4^+CD45RB^{high}$ T cells (3×10^5 cells/mouse) and assessed at the indicated time points (each n = 4). *B*, Histological examination of the colon from IL-$7^{-/-}$ × RAG-$1^{-/-}$ and IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice transferred with colitogenic LP $CD4^+CD44^{high}CD62L^-$ T_{EM} cells at 1, 2, and 4 wk after transfer. Colonic sections were stained with H&E and photographed at ×100 magnification. *C*, Histological scoring of IL-$7^{-/-}$ × RAG-$1^{-/-}$ and IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients at the indicated time points. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.05$. *D*, Cell number of splenic and LP $CD4^+$ T cells recovered from IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.05$. *E*, Cell surface IL-7Rα expression on $CD4^+CD45RB^{high}$ T cells before transfer and LP $CD4^+$ T cells from IL-$7^{+/+}$ × Rag-$1^{-/-}$ and IL-$7^{-/-}$ × Rag-$1^{-/-}$ recipients at the indicated time points.

Results

Lack of colitis in IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells

We used a chronic colitis model induced by an adoptive transfer of splenic $CD4^+CD45RB^{high}$ T cells from normal C57BL/6 mice into IL-$7^{-/-}$ × RAG-$1^{-/-}$ and IL-$7^{+/+}$ × RAG-$1^{-/-}$ littermate recipients to assess a requirement of IL-7 for the development of chronic colitis (Fig. 1*A*). Consistent with previous reports (14), the control IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice manifested progressive weight loss from 3 wk after transfer (data not shown) and clinical symptoms of colitis as shown as clinical scores estimated by diarrhea with increased mucus in the stool, anorectal prolapse, hunched posture, and weight loss by 8 wk after transfer (Fig. 1*B*). In contrast, the IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with

$CD4^+CD45RB^{high}$ T cells showed no clinical signs of colitis and weight loss throughout the entire observation periods (Fig. 1*B*). At 8 wk after transfer, the colon from the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice, but not that from the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice, was enlarged and had a greatly thickened wall (Fig. 1*C*). In addition, the enlargement of the spleen and mesenteric lymph nodes was also present in the control IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells as compared with the IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells (Fig. 1*C*). Totally, the assessment of colitis by clinical scores showed a clear difference between the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice and the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice (Fig. 1*B*). Histological examination showed prominent epithelial hyperplasia with glandular elongation with a massive infiltration of mononuclear cells in the LP of the colon from the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (Fig. 1*D*). In contrast, the glandular elongation was mostly abrogated, and only a few mononuclear cells were observed in the LP of the colon from the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice (Fig. 1*D*). This difference was also confirmed by histological scoring of multiple colon sections, which was 0.16 ± 0.04 in the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice vs 5.16 ± 0.19 in the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice ($p < 0.01$) (Fig. 1*E*). We confirmed that the IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells did not develop intestinal inflammation cells until 20 wk of observation after transfer (data not shown).

A further quantitative evaluation of $CD4^+$ T cell infiltration was made by isolating LP and splenic $CD4^+$ T cells. Only a few $CD4^+$ T cells were recovered from the colonic tissue of the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice as compared with the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (Fig. 1*F*). The number of $CD4^+$ cells recovered from the colon of the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice ($150.5 \pm 3.23 \times 10^5$) far exceeded the number of originally injected cells (3.0×10^5), indicating an extensive T cell proliferation and survival in the inflamed colon, which was mostly abrogated in the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice ($0.60 \pm 0.65 \times 10^5$) (Fig. 1*F*). Furthermore, the number of $CD4^+$ splenocytes from the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice was also significantly increased as comparable to that from the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients (Fig. 1*G*). We also examined the cytokine production by LP $CD4^+$ T cells from the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice and the transferred IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice. As shown in Fig. 1*H*, LP $CD4^+$ T cells from the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice produced significantly less IFN-γ, IL-2, and TNF-α as compared with those from the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice upon in vitro stimulation. Importantly, flow cytometry analysis revealed that the LP $CD4^+$ T cells isolated from both IL-$7^{+/+}$ × RAG-$1^{-/-}$ and IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients 8 wk after an adoptive transfer of $CD4^+CD45RB^{high}$ T cells were $CD44^{high}CD62L^-CD69^+CD45RB^{low}$IL-7Rα^{high} (Fig. 1*I*), indicating that the transferred $CD4^+CD45RB^{high}$ T cells could differentiate to activated T_{EM} cells even in the absence of IL-7. These results suggest that the lack of IL-7 prevented the development of colitis primarily by inhibiting the expansion and/or survival of colitigenic $CD4^+$ T_{EM} cells in the colon and secondarily by inhibiting the development of the Th1 T_{EM} cells.

Kinetics of development of colitis and IL-7Rα expression

When the immune system is first challenged by exposure to Ags, naive $CD4^+$ T cells become activated and undergo many rounds of expansion as they differentiate into effector and memory $CD4^+$ T cells. Because it is believed that IL-7 is not involved in the initial expansion of the effector cells but its role in maintaining pool size may influence either the onset or maintenance of colitis, we assessed the kinetics of the development of colitis and IL-7Rα expression to determine whether IL-7 is crucial for the onset of colitis in our model (Fig. 2*A*). At 1 wk after an adoptive transfer of $CD4^+CD45RB^{high}$ T cells into IL-$7^{+/+}$ × RAG-$1^{-/-}$ and IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice, both mice did not develop colitis (Fig. 2*B*), and the average histological scores in both mice were 0 (Fig. 2*C*). At 2 wk after the transfer, however, the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients develop mild colitis, characterized with the infiltration of a small number of mononuclear cells in the LP, but the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients did not (Fig. 2*B*). The average histology scores revealed that the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients had significantly higher colitis scores as compared with the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients (Fig. 2*C*). Four weeks after the transfer, the difference between two groups was more apparent, as the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice developed more severe colitis, but the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice did not (Fig. 2, *B* and *C*). To examine the effects of IL-7 on the expansion of $CD4^+$ T cells in the recipients, we compared the infiltration of $CD4^+$ T cells by determining the number of $CD4^+$ T cells in the spleen and the LP by FACS analysis. In accordance with the histological scores, the average number of LP and splenic $CD4^+$ T cells recovered from the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients were significantly increased from 2 wk after the transfer as compared with those from the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients (Fig. 2*D*).

To obtain a more comprehensive understanding of the development of effector and memory $CD4^+$ T cells over time in this setting, we carefully examined the cell surface expression of IL-7Rα on LP $CD4^+$ T cells obtained from the recipients at the indicated time points. As shown in Fig. 2*E*, the splenic $CD4^+$ T cells (donor $CD4^+CD45RB^{high}$ T cells) before the transfer (0 wk) and the LP $CD4^+$ T cells after 1 wk of transfer highly expressed IL-7Rα, but approximately half of $CD4^+$ T cells from both the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ and IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients had down-regulated IL-7Rα at 2 wk after transfer. However, a large portion of LP $CD4^+$ T cells from both the transferred mice at 4 wk after the transfer had regained higher expression of IL-7Rα to >80% per total $CD4^+$ T cells (Fig. 2*E*). These data indicate that IL-7Rα expression was regulated during T cell differentiation from

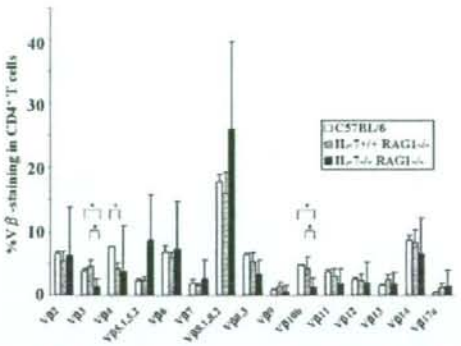

FIGURE 3. Flow cytometric analysis of Vβ families on the surface of the splenic $CD4^+$ T cells. To analyze the TCR Vβ family repertoire, splenic cells were double-stained with PE-conjugated anti-CD4 mAb (RM4-5), and the following splenic cells were double-stained with a panel of 15 FITC-conjugated Vβ mAbs. Each percentage value indicates the frequency of each Vβ pooled from three independent experiments ($n = 12$). *, $p < 0.05$.

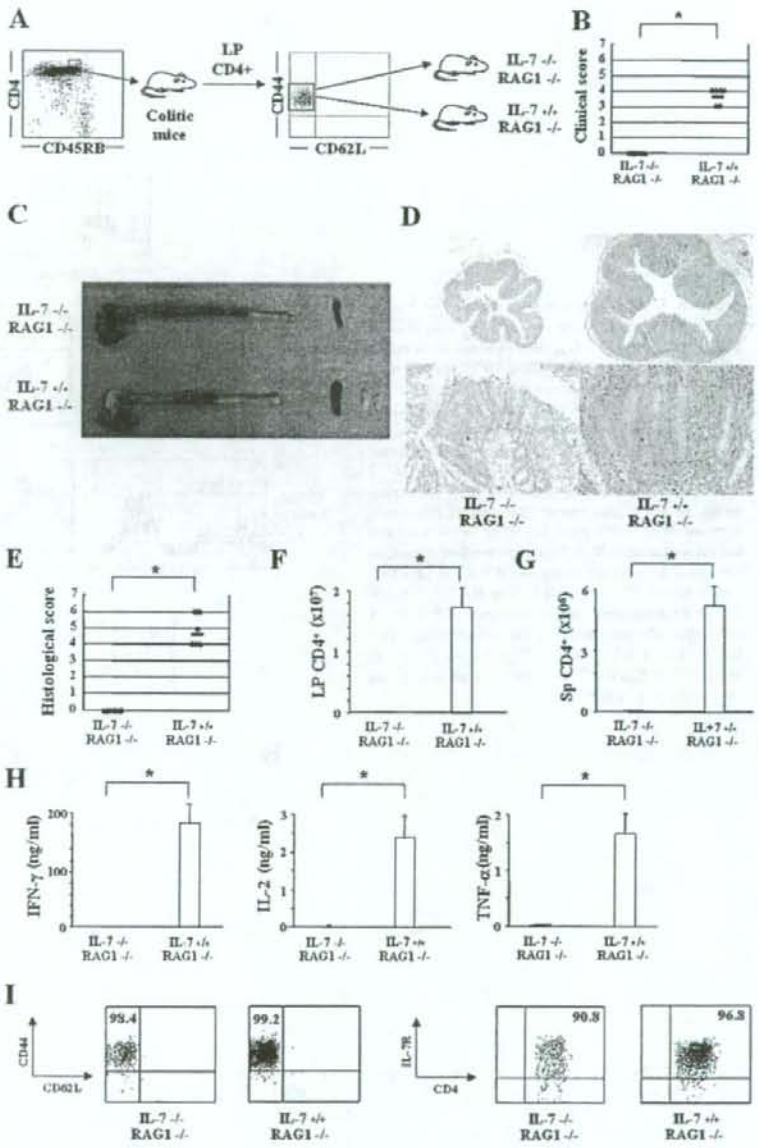

FIGURE 4. IL-7$^{-/-}$ × RAG-1$^{-/-}$ transferred with colitogenic LP CD4$^+$ CD44^{high}CD62L$^-$ T_{EM} cells did not develop chronic colitis. *A*, Colitogenic LP CD4$^+$CD44^{high}CD62L$^-$ T_{EM} cells obtained from colitic mice transferred with CD4$^+$CD45RB^{high} T cells were transferred into new IL-7$^{+/+}$ × RAG-1$^{-/-}$ (IL-7$^{+/+}$→IL-7$^{+/+}$; $n = 10$) and IL-7$^{-/-}$ × RAG-1$^{-/-}$ (IL-7$^{+/+}$→IL-7$^{-/-}$; $n = 10$) mice. *B*, Clinical scores were determined 4 wk after transfer as described in *Materials and Methods*. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.005$. *C*, Gross appearance of the colon, spleen, and mesenteric lymph nodes from IL-7$^{+/+}$→IL-7$^{-/-}$ (*top*) and IL-7$^{+/+}$→IL-7$^{+/+}$ (*bottom*) mice 4 wk after transfer. *D*, Histological examination of the colon from IL-7$^{+/+}$→IL-7$^{-/-}$ and IL-7$^{+/+}$→IL-7$^{+/+}$ mice 4 wk after transfer. Original magnification: ×40 (*upper*); ×100 (*lower*). *E*, Histological scoring of IL-7$^{+/+}$→IL-7$^{-/-}$ and IL-7$^{+/+}$→IL-7$^{+/+}$ mice 4 wk after transfer. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.005$. LP (*F*) and spleen (*G*) CD4$^+$ T cells were isolated from IL-7$^{+/+}$→IL-7$^{-/-}$ and IL-7$^{+/+}$→IL-7$^{+/+}$ mice 4 wk after transfer, and the number of CD4$^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.01$. *H*, Cytokine production by LP CD4$^+$ T cells. CD4$^+$ LPL were isolated from each mouse at 4 wk after transfer and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. IFN-γ, IL-2, and TNF-α concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SD of seven mice in each group. *, $p < 0.005$. *I*, Phenotypic characterization of LP CD4$^+$ T cells isolated from IL-7$^{+/+}$→IL-7$^{-/-}$ and IL-7$^{+/+}$→IL-7$^{+/+}$ mice 4 wk after transfer.

CD4$^+$CD45RB^{high}IL-7Rα^{high} naive T cells (0 wk before the transfer), CD4$^+$IL-7Rα^{low} effector T cells (1 and 2 wk after the transfer), to CD4$^+$IL-7Rα^{high} memory T cells (4 wk after the transfer), suggesting that naive and memory CD4$^+$ T cells may be more responsive to IL-7-mediated signaling.

TCR Vβ repertoires in IL-7$^{+/+}$ × RAG-1$^{-/-}$ and IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells

Analysis of the TCR repertoire of the immunodeficient SCID/Rag-1/2$^{-/-}$ recipients in the CD4$^+$CD45RB^{high} T cell transfer model may provide an opportunity to characterize the unique T cell population present in the diseased individuals in a manner not possible in clinical studies on human patients. Furthermore, it was also possible that the adoptive transfer of CD4$^+$CD45RB^{high} T cells to IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients gains more skewed and restricted clonality of CD4$^+$ T cells as compared with that in IL-7$^{+/+}$ × RAG-1$^{-/-}$ recipients. To assess this possibility, we next made a comparison between TCR Vβ repertoires of splenic CD4$^+$ T cells from colitic CD4$^+$CD45RB^{high} T cell-transferred and IL-7$^{-/-}$ × RAG-1$^{-/-}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ and normal age-matched wild-type mice. Flow cytometric analysis of these splenic CD4$^+$ cells using a panel of 15 anti-Vβ mAbs showed that most major

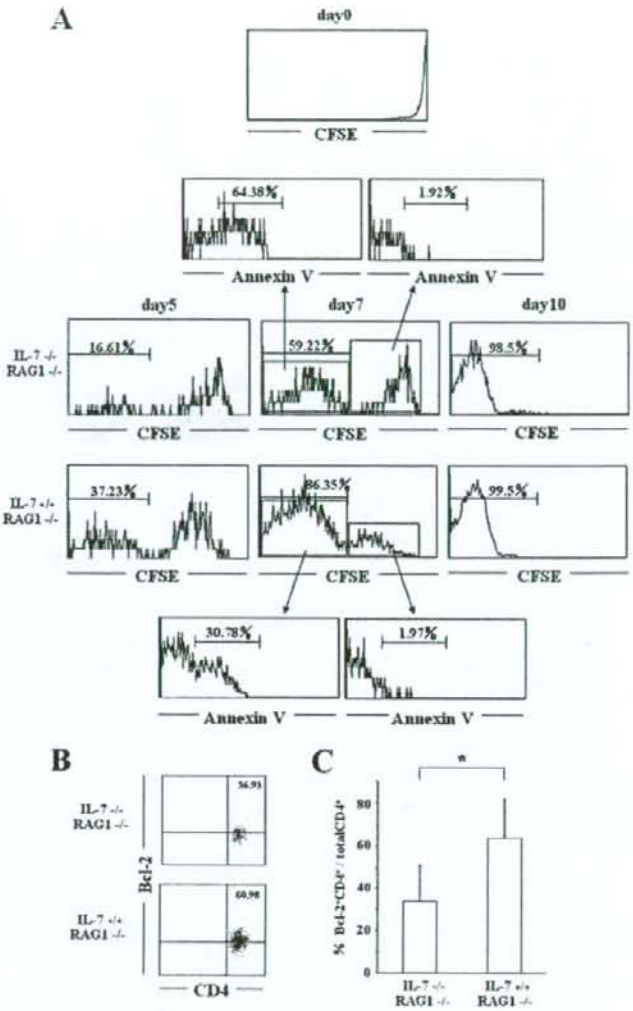

FIGURE 5. IL-7 is essential for the survival, but not the cell turnover, of colitogenic LP CD4$^+$ T_{EM} cells. *A*, Colitogenic LP CD4$^+$ T_{EM} cells obtained from colitic mice transferred with CD4$^+$CD45RB^{high} T cells were labeled with CFSE and adoptively transferred into IL-7$^{+/+}$ × RAG-1$^{-/-}$ (n = 16) or IL-7$^{-/-}$ × RAG-1$^{-/-}$ (n = 16) mice. At the indicated time points after transfer (days 4, 8, and 12), CFSE incorporation was determined by flow cytometry. Histograms are gated on CD4$^+$ T cells. These data were representative from six experiments. At day 8 after transfer, the gated CFSE$^-$ cells represent rapid proliferation, and CFSE$^+$ cells were also stained with annexin V. *B*, Representative flow cytometric histograms showing the expression of Bcl-2 in LP CD4$^+$ T cells from IL-7$^{-/-}$ × RAG-1$^{-/-}$ or IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice transferred with the colitogenic LP CD4$^+$ T cells 7 days after the transfer. *C*, Average percentage of LP Bcl-2$^+$CD4$^+$ T cells from IL-7$^{-/-}$ × RAG-1$^{-/-}$ (n = 6) or IL-7$^{+/+}$ × RAG-1$^{-/-}$ (n = 6) recipients 7 days after the transfer. *, $p < 0.05$.

Vβ population was Vβ8.1/8.2 in all three groups, i.e., age-matched C57BL/6J and IL-7$^{-/-}$ × RAG-1$^{-/-}$ and IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells. Although only a Vβ4 ratio in colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ recipients was significantly decreased as compared with that in normal C57BL/6J mice, there were no significant differences of other Vβ repertoires between these two groups (Fig. 3). In noncolitic IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients, however, the repertoires of Vβ3 and Vβ10b were significantly decreased as compared with those in normal C57BL/6J mice and colitic IL-7$^{+/+}$ × RAG-1$^{-/-}$ recipients (Fig. 3), indicating that the lack of IL-7 suppressed the expansion of certain type of Vβ populations as compared with other repertoires.

Lack of colitis in IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transferred with colitogenic LP CD4$^+$ T_{EM} cells

To next assess the role of IL-7 in the persistent colitis without the impact of naive T cell priming, activation, and differentiation, we first isolated LP CD4$^+$ T cells as colitogenic CD4$^+$ T_{EM} cells from CD4$^+$CD45RB^{high} T cell-transferred colitic RAG-2$^{-/-}$ mice at 4–6 wk after transfer because flow cytometry analysis revealed that the colitic LP CD4$^+$ T cells were CD44^{high} CD62L$^-$IL-7Rα^{high} T_{EM} cells (Fig. 1*I*), and we previously demonstrated that the adoptive transfer of these cells into new IL-7-competent RAG-2$^{-/-}$ mice induces chronic colitis (17). We then transferred these LP CD4$^+$ T_{EM} cells into IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice (IL-7$^{+/+}$→IL-7$^{+/+}$) and the IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice (IL-7$^{+/+}$→IL-7$^{-/-}$) to focus on the persistence of colitogenic CD4$^+$ T_{EM} cells (Fig. 4*A*). IL-7$^{+/+}$→IL-7$^{+/+}$ recipients developed a severe colitis 4 wk after the transfer, characterized by significant weight loss, diarrhea, and higher total clinical scores (Fig. 4*B*) and thickening of the colonic wall with inflammation (Fig. 4*C*). Average histological scores characterized by transmural inflammation with high numbers of lymphocytes in the LP and submucosa, and prominent epithelial hyperplasia with loss of goblet

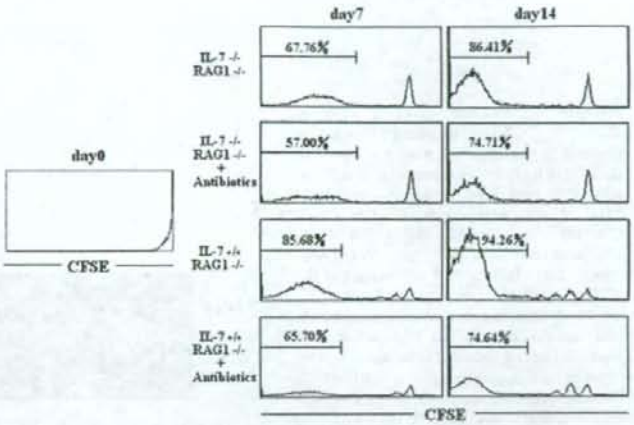

FIGURE 6. Commensal bacteria-partially dependent rapid proliferation and IL-7-dependent slow proliferation. Aliquots (3×10^6/mouse) of CFSE-labeled $CD4^+$ $CD25^-$ cells obtained from normal spleen were injected into IL-$7^{-/-}$ × RAG-$1^{-/-}$ and IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice treated with or without a set of four antibiotics (ampicillin, vancomycin, neomycin sulfate, and metronidazole) and analyzed at the indicated time points. Data are representative of five independent experiments.

cells (Fig. 4*D*) were 4.83 ± 0.98 in IL-$7^{+/+}$→IL-$7^{+/+}$ mice (Fig. 4*E*). In contrast, IL-$7^{+/+}$→IL-$7^{-/-}$ recipients appeared healthy and did not exhibit any signs of colitis (Fig. 4*B*), with gradual increase of body weight and no apparent thickening of the colonic wall (Fig. 4*C*). No evident pathological changes were observed in the colon (Fig. 4*D*). This histological difference was also confirmed by histological scoring, which was 0 in IL-$7^{+/+}$→IL-$7^{-/-}$ recipient mice ($p < 0.005$ as compared with IL-$7^{+/+}$→IL-$7^{+/+}$ mice) (Fig. 4*E*). The average recovered numbers of LP and splenic $CD4^+$ T cells from colitic IL-$7^{+/+}$→IL-$7^{+/+}$ recipients were 173.7 ± 11.2 × 10^5 cells/colon (Fig. 4*F*) and 51.5 ± 11.3 × 10^5 cells/spleen (Fig. 4*G*), respectively, whereas those from IL-$7^{+/+}$ → IL-$7^{-/-}$ mice were 0.64 ± 0.7 × 10^5 cells/colon (Fig. 4*F*) ($p < 0.01$) and 0.54 ± 0.29 × 10^5 cells/spleen (Fig. 4*G*) ($p < 0.05$), respectively. As shown in Fig. 4*H*, LP $CD4^+$ T cells from IL-$7^{+/+}$→IL-$7^{-/-}$ recipients produced significantly less IFN-γ, IL-2, and TNF-α as compared with those from IL-$7^{+/+}$→IL-$7^{+/+}$ mice upon in vitro stimulation (Fig. 4*H*). Furthermore, flow cytometry analysis showed that the LP $CD4^+$ T cells isolated from both IL-$7^{+/+}$→IL-$7^{+/+}$ and IL-$7^{+/+}$→IL-$7^{-/-}$ recipients were sustained by the phenotype of $CD44^{high}CD62L^-$IL-$7R\alpha^{high}$ T_{EM} cells (Fig. 4*I*).

IL-7 is essential for the survival of colitogenic $CD4^+$ T_{EM} cells

To further examine the effect of IL-7 on the proliferation and the survival of the colitogenic LP $CD4^+$ T_{EM} cells in vivo, we used the CFSE dilution method since dilution of CFSE provided a division history of the cells, allowing us to examine cells undergoing proliferation. First, the LP $CD4^+$ T_{EM} cells obtained from $CD4^+$ $CD45RB^{high}$-transferred colitic mice were labeled with CFSE and adoptively transferred into IL-$7^{+/+}$ × RAG-$1^{-/-}$ or IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice. Although the relatively delayed division of expanded $CD4^+$ T cells in IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice at the indicated time points, the LP $CD4^+$ T cells had markedly divided until 10 days after transfer both in the IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice and the IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients, indicating that it appears IL-7 is not essential for colitogenic $CD4^+$ T_{EM} cells to undergo lymphopenia-driven rapid proliferation (19). Interestingly, consistent with the markedly and significantly decreased cell numbers of splenic and LP $CD4^+$ T cells in $CD4^+CD45RB^{high}$ T cell (Fig. 1, *F* and *G*)- or colitogenic LP $CD4^+$ T cell (Fig. 4, *F* and *G*)-transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice as compared with those in the paired transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice, $CFSE^-$ cells that have divided more than eight times (19) in IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice were stained with annexin V with higher percentages at day 7 after transfer as compared with those in IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (Fig. 5*A*), indicating that rapidly dividing cells in IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice could not survive to maintain their cell number. To further address the survival checkpoint, we next assessed whether regulation of Bcl-2 requires IL-7 at 7 days after the transfer since induction of the anti-apoptotic protein, Bcl-2, is a hallmark of responses to IL-7 (20). As expected, the splenic $CD4^+$ T cells in the transferred IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice expressed significantly less level of Bcl-2 as compared with those in the transferred IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (Fig. 5, *B* and *C*).

Commensal bacteria-partially dependent rapid proliferation and IL-7-dependent slow proliferation

Because we found that IL-7 is essential for the development and persistence of colitis, and it has been previously demonstrated that the presence of commensal bacteria are needed to develop and sustain chronic colitis in various models of colitis (1), we finally addressed this point in the adoptive transfer setting using IL-$7^{-/-}$ × RAG-$1^{-/-}$ and IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients treated with or without antibiotics treatment by CFSE dilution assay at 7–21 days after transfer. In this experiment, we used normal splenic $CD4^+$ $CD25^-$ T cells, including $CD4^+CD45RB^{high}$ naive T cells and $CD4^+CD45RB^{low}$ T_{EM} cells, but not $CD4^+CD25^+$ regulatory T cells, rather than colitogenic LP $CD4^+$ T cells as donor cells (Fig. 5) to assess two types of cell division, rapid (spontaneous, endogenous) proliferation and slow (homeostatic) proliferation (19, 21, 22). As shown in Fig. 6, slow proliferation in IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients at days 7–14 after transfer was observed as two to three peaks of dividing cells regardless of the antibiotic treatment, whereas none of slowly dividing cells was observed in IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients, indicating that slow proliferation is dependent on the presence of IL-7. In contrast, rapid proliferation in IL-$7^{-/-}$ × RAG-$1^{-/-}$ and IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients treated with antibiotics at days 7 and 14 after transfer was partially but not completely impaired as compared with that in both IL-$7^{-/-}$ × RAG-$1^{-/-}$ and IL-$7^{+/+}$ × RAG-$1^{-/-}$ recipients without antibiotics treatment (Fig. 6), indicating that rapid proliferation in these recipients is driven not only by the presence of commensal bacterial Ags, but also presumably by other environmental Ags, such as food and bedding, and/or self-Ags. Furthermore, although rapid-proliferating cells in IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients should