

Long-Lived Colitogenic CD4⁺ Memory T Cells Residing Outside the Intestine Participate in the Perpetuation of Chronic Colitis¹

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To understand the perpetuation of inflammatory bowel disease (IBD), it is important to clarify whether the colitogenic CD4⁺ T cells are self-limited effector or long-lived memory T cells. We here investigate the latency of colitogenic CD4⁺ T cells in the remission stage of colitis under germfree (GF) conditions. We isolated splenic (SP) CD4⁺ T cells from colitic CD4⁺CD45RB^{high} T cell-injected SCID mice maintained under specific pathogen-free (SPF) conditions and transferred them into SPF or GF SCID mice. Donor colitic SP CD4⁺ T cells have a characteristic CD44^{high}CD62L⁻IL-7R α ^{high} effector-memory T-type phenotype. Six weeks after transfer of cells to GF SCID mice, one group of mice was continued in GF conditions (GF→GF), and the other was transferred into SPF conditions (GF→SPF). GF→SPF but not GF→GF SCID mice developed colitis with elevated production of Th1 and Th17 cytokines at 4 wk after transfer. Surprisingly, a large number of CD4⁺ effector-memory T cells and a small but substantial number of central-memory T cells remained resident in SP and bone marrow, but not in lamina propria, of the GF→GF SCID recipients. Consistent with this, GF→SPF but not GF→GF SCID mice rapidly developed colitis. Taken together, these findings suggest that long-lived colitogenic memory CD4⁺ cells can be established even in the presence of commensal Ags, reside outside the intestine in the absence of commensal bacteria, and participate in the perpetuation of colitis. Thus, blocking a stimulus of colitogenic memory CD4⁺ cells such as IL-7 may have therapeutic benefit for treatment of inflammatory bowel disease. *The Journal of Immunology*, 2009, 183: 5059–5068.

Studies of Ag exposure in murine models of acute virus infection have provided much information about the dynamics of naive, effector, and memory CD8⁺ T cell responses (1–3). Acute virus infection elicits massive proliferation of viral Ag-specific CD8⁺ T cells, which acquire effector functions (effector phase). After the peak of T cell proliferation, most of the effector CD8⁺ T cells are eliminated (contraction phase). Following virus (Ag) clearance, a small proportion of the remaining cells differentiate into memory CD8⁺ T cells that are maintained by cytokine (IL-7 and/or IL-15)-dependent homeostatic proliferation and survive in the absence of their corresponding Ags (memory phase). Thus, Ag clearance is essential for the emergence of memory CD8⁺ T cells.

In contrast to the generation of CD8⁺ memory T cells, it is controversial whether Ag clearance is needed for the generation of CD4⁺ memory T cells. Indeed, there is evidence that persistence of the Ag

itself is essential for the maintenance of CD4⁺ memory T cells (4–6). Given that Ag-specific effector T cells are thought to be terminally differentiated and short-lived cells, there must be cellular mechanisms by which memory T cells specific for persistent Ags are maintained in the host as “memory-stem cells.” In support of the idea that persistent Ag helps maintain CD4⁺ memory T cells, rats immunized with irradiated sporozoites are protected from malaria infection, but this protection is lost after drug treatment to remove the remaining parasites (5). A similar phenomenon is found in *Leishmania major* infection in mice where CD4⁺CD25⁺ regulatory T cells and IL-10 are thought to prevent a Th1 immune response from clearing the infection, thus allowing the host to retain CD4⁺ memory T cells specific to the organism (6). Furthermore, more recent data have shown that in the absence of MHC class II signals that are essential for Ag presentation to CD4⁺ T cells, surviving memory T cells are functionally impaired, suggesting that TCR signaling is involved in the maintenance of CD4⁺ memory T cells (7).

All complex metazoans, including humans and mice, are colonized with microbial organisms that comprise an indigenous microflora (8). Although the host evidently benefits from the resident microflora in the gut (9), the presence of commensal bacteria appears to be of crucial importance in the development of human inflammatory bowel disease (IBD)³ and in almost all animal models of IBD (10, 11). For instance, results from most IBD models showing that in germfree (GF) rodents intestinal inflammation is

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³ Abbreviations used in this paper: IBD, inflammatory bowel disease; BM, bone marrow; CD62L, L-selectin; LP, lamina propria; MLN, mesenteric lymph node; SP, spleen or splenic; T_{EM}, effector-memory T; TSLP, thymic stromal lymphopoietin; WT, wild type.

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absent indicate that commensal bacteria are indispensable contributors to the pathogenesis of chronic immune-mediated intestinal inflammation.

IBD is caused by excessive and tissue-damaging chronic inflammatory responses, which are thought to be due to inappropriate activation of the immune system in many cases and which commonly take a persistent, disabling course (10, 11). In some patients, disease progresses steadily, whereas in others, it follows a relapsing-remitting course. According to present understanding, the disease is caused and controlled by colitogenic effector and memory CD4⁺ T cells presumably reacting to commensal bacterial Ags. Importantly, however, it is not known whether different effector CD4⁺ T cells are recruited at each relapse or whether sequential memory CD4⁺ T cells are derived from members of the initial attack cohort throughout the entire course of disease. In other words, the nature and regulation of colitogenic effector and memory CD4⁺ T cells in the host-commensal interaction over time and the correlation between colitogenic effector and memory CD4⁺ T cells in chronic colitis remain largely unknown.

Given the possible importance of the microflora in intestinal inflammation, we conducted a series of experiments to test the *in vivo* effect of commensal bacteria using a GF system for the establishment and maintenance of colitogenic CD4⁺ T cells. We also attempted to assess whether colitogenic CD4⁺ T cells are self-limited effector cells or long-lived memory cells in a host-commensal bacteria mutualism to understand the perpetuation of IBD and to develop a strategy for IBD treatment.

Materials and Methods

Mice

C3H/HeN mice (6–8 wk old) and C57BL/6-Ly5.2 mice were purchased from CLEA Japan. C57BL/6-Ly5.1 and C57BL/6-Ly5.2 RAG-2^{-/-} mice were obtained from Taconic Laboratory and the Central Experimental Animal Institute. Specific pathogen-free (SPF) and GF breeding colonies of C3H-SCID mice (C3Smnc Prkdc scid/J; The Jackson Laboratory) were maintained at the Animal Facilities (SPF) and the Gnotobiotic Facilities (GF), respectively, of our institute. Sterility in the Gnotobiotic Facilities was tested monthly by culturing of feces and bedding as well as Gram staining. The Institutional Committees on Animal Research of both Tokyo Medical and Dental University and Yakult Central Institute approved the experiments.

Antibodies

The following mAbs other than biotin-conjugated anti-mouse IL-7R α (A7R34; eBioscience) and anti-CCR7 (EBI-1; eBioscience) were obtained from BD Pharmingen and used for purification of cell populations and flow cytometric analysis: Fc γ (CD16/CD32)-blocking mAb (2.4G2); PE-, PerCP-, and PECy5-conjugated anti-mouse CD4 (RM4-5); FITC-conjugated anti-mouse CD3 (145-2C11); PE- and allophycocyanin-conjugated anti-mouse CD44 (IM7); FITC- and PE-conjugated anti-mouse L-selectin (CD62L) (MEL-14); FITC-conjugated anti-mouse CD69 (H1.2F3); PE-conjugated anti-mouse $\alpha_4\beta_7$ (DATK32); FITC-conjugated anti-mouse CD45RB (16A); PE-conjugated anti-mouse Ly5.1 (CD45.1); PE-conjugated streptavidin; biotin-conjugated rat IgG2; PE-conjugated mouse IgG; and PE-conjugated rat IgG.

Adoptive transfer experiments

To assess the role of commensal bacteria in the persistence of T cell-mediated chronic colitis, CD4⁺ T cells (5×10^5 cells/mouse) isolated from spleen (SP) of colitic mice induced by an adoptive transfer of CD4⁺CD45RB^{high} T cells into C3H-SCID mice under SPF conditions were injected into new GF ($n = 16$) or SPF ($n = 8$) C3H-SCID mice. Six weeks after the transfer, one group of GF SCID recipients was maintained in GF conditions (GF \rightarrow GF group, $n = 8$), and the other was moved into SPF conditions (GF \rightarrow SPF group, $n = 8$). All groups (SPF, GF \rightarrow GF, and GF \rightarrow SPF) were kept for an additional 4 wk and sacrificed 10 wk after cell transfer. They were observed for clinical signs such as hunched posture, piloerection, diarrhea, and blood in the stool. After sacrifice, mice were given a clinical score defined as the sum of four parameters: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild

thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; 3, bloody stool) (12). To assess the histological scores, two parts of the colon were evaluated. The proximal section was defined as 1 cm anal to the cecum, and the distal section was defined as 1 cm oral from the anus. Both tissue samples were cut 5 mm long and fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μ m) were stained with H&E. The sections were analyzed without prior knowledge of the type of T cell reconstitution or recipient. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system as the sum of three parameters: crypt elongation, 0–3; mononuclear cell infiltration, 0–3; and frequency of crypt abscesses, 0–3 (13).

In vivo CFSE labeling

CD4⁺ T cells were isolated from lamina propria (LP) of colitic RAG2^{-/-} mice previously injected with Ly5.1⁺ CD4⁺CD45RB^{high} T cells and were labeled with CFSE (Invitrogen) at a concentration of 5 μ M according to the manufacturer's instructions; then, CFSE-labeled LP CD4⁺ T cells (1×10^7 /mouse) were injected into new Ly5.2⁺ wild-type (WT) C57BL/6J mice.

Antibiotic treatment

C57BL/6J mice in the antibiotic-treated group were given drinking water, including ampicillin (1 g/L), vancomycin (500 mg/L), neomycin sulfate (1 g/L), and metronidazole (1 g/L), 2 wk before beginning the adoptive transfer and during the course of the experiment. Control mice received drinking water without antibiotics.

Administration of anti-IL-7R α mAb to GF SCID mice

Anti-IL-7R α mAb (A7R34) was described previously (14). GF SCID mice were treated with rat anti-murine IL-7R α mAb by *i.p.* injection of a 1-mg dose once per week for 2 wk (0, 1, and 2 wk after transfer). Control mice were treated with the same amounts of rat control IgG (Sigma-Aldrich). All mice were killed on the day after the last treatment.

Flow cytometry

To detect the surface expression of a variety of molecules, isolated bone marrow (BM), SP, mesenteric lymph nodes (MLN), or colonic LP mononuclear cells were preincubated with an FcR-blocking mAb (CD16/32 and 2.4G2; BD Pharmingen) for 20 min before incubation with specific FITC-, PE-, PerCP-, and APC-labeled Abs for 30 min on ice. When biotin-conjugated Abs were used, cells were incubated with Abs for 30 min, then with PE-labeled streptavidin for 30 min on ice. Standard four-color flow cytometric analyses were performed using a FACSCalibur (BD Biosciences) and analyzed by CellQuest software (BD Biosciences). Background fluorescence was assessed by staining with irrelevant isotype-matched mAbs.

Cytokine ELISA

To measure cytokine production, 1×10^5 CD4⁺ T cells from LP were cultured in 200 μ l of 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 2 μ g/ml hamster anti-mouse CD28 mAb (37.51; BD Pharmingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA as per the manufacturer's recommendation (R&D Systems).

Immunohistochemistry

Consecutive cryostat BM and colon sections were used in all studies. Immunohistochemistry was performed using purified mAb against mouse CD4 (RM4-5; BD Pharmingen) or biotin-conjugated polyclonal IL-7 Ab (BAF407; R&D Systems). In brief, fresh frozen 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 incubated with the primary Ab at 4°C overnight, then stained for 60 min at room temperature with AlexaFluor 488 goat anti-rat IgG for CD4 detection or AlexaFluor 488 streptavidin (Molecular Probes) for IL-7 detection. All slides were counterstained with 4,6-diamidino-2-phenylindole (Vector Laboratories) and observed under a BioZERO BZ8000 microscope (Keyence).

Statistical analysis

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

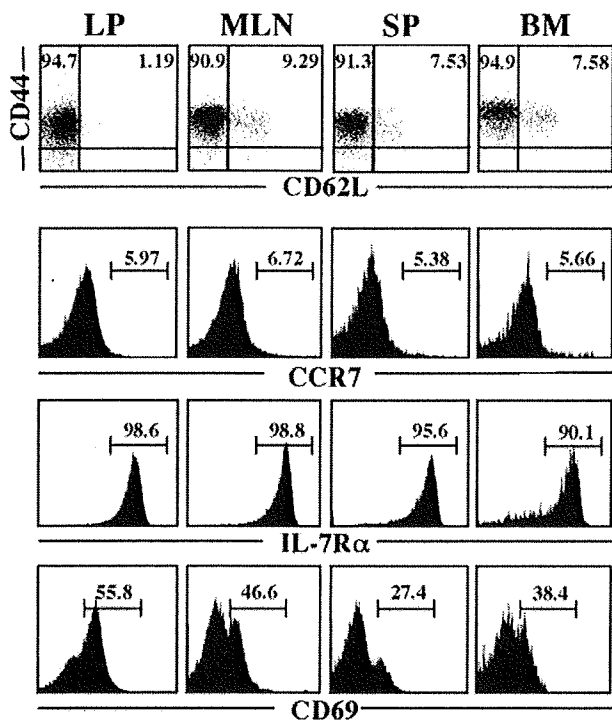


FIGURE 1. Colitic SP CD4⁺ donor T cells are CD44^{high}CD62L⁻CCR7⁻IL-7Rα^{high}T_{EM} cells. Expression of CD44, CD62L, IL-7Rα (CD127), and CD69 on CD4⁺ T cells obtained from SP, MLN, LP, and BM in colitic C3H-SCID mice induced by adoptive transfer of CD4⁺CD45RB^{high} T cells (6 wk after transfer). Freshly isolated cells from colitic mice were stained with FITC-labeled anti-CD4 and PE-labeled anti-CD44, anti-CD62L, anti-IL-7Rα, or anti-CD69. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and side scatter profiles. Data are displayed as dotted plot (four-decade log scale), and quadrant markers were positioned to include >98% of control Ig-stained cells in the *bottom left*. Percentages in each quadrant are indicated. Representatives of three mice in each group.

Results

Effector-memory T (T_{EM}) type of CD4⁺ T cells reside in various sites in colitic mice

In research into antiviral CD8⁺ memory T cells, memory T cells are classically defined as cells that persist in the organism once the viral Ag has been cleared and that mediate a much quicker and stronger response when the Ag is met again (1–3). However, current evidence suggests that maintenance of a memory CD4⁺ T cell population responding to chronic infection is dependent on the persistent presence of Ag (4–6), and it is controversial whether CD4⁺ memory T cells can be maintained in the absence of Ag. In particular, to understand the persistence of IBD, it is important to know whether the colitogenic CD4⁺ T cells are self-limited effector cells or are long-lived memory cells within the host-commensal bacteria mutualism. We attempted to clarify this issue using a well-known chronic colitis model induced by adoptive transfer of CD4⁺CD45RB^{high} T cells into SCID mice, which characteristically involves the differential activation of Th1/Th17 cells (15, 16). We first checked the phenotype of CD4⁺ T cells in colonic LP, MLN, SP, and BM of colitic C3H-SCID mice that had previously been injected with syngeneic CD4⁺CD45RB^{high} T cells. Consistent with our previous reports using BALB/c/C.B.17 or C57BL/6J strains (12, 13), LP CD4⁺ T cells, as well as colitic SP, MLN, and BM CD4⁺ T cells, are exclusively CD44^{high}CD62L⁻CCR7⁻IL-7Rα^{high}T_{EM} cells (Fig. 1). CD69 was also expressed by a high

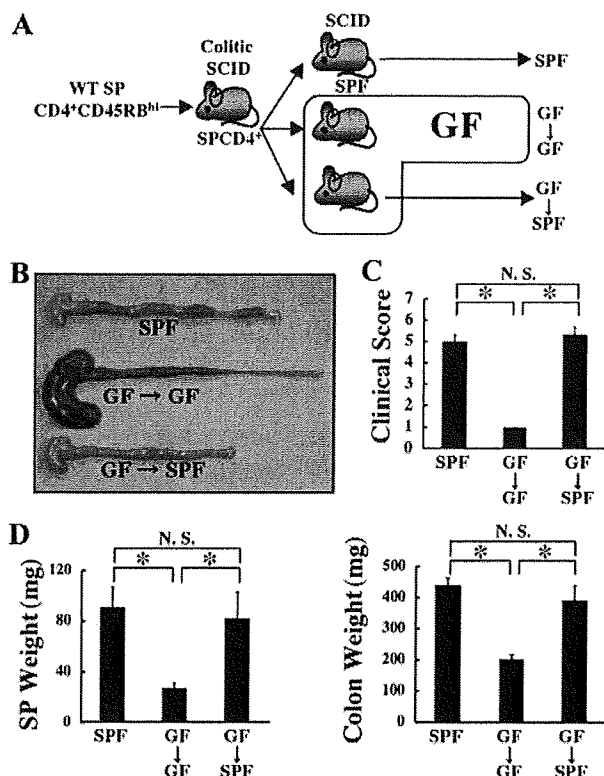


FIGURE 2. Commensal bacteria are not essential for survival of colitogenic memory CD4⁺ T cells. *A*, C3H-SCID mice were injected i.p. with normal splenic CD4⁺CD45RB^{high} T cells. Six weeks after transfer, mice developed chronic colitis, and then colitic CD4⁺ T cells were isolated from SP. Doses of 3×10^5 SP CD4⁺ T cells were injected into new SPF C3H-SCID mice (called SPF SCID mice) or GF C3H-SCID mice (called GF SCID mice). Six weeks after transfer, GF SCID mice were divided into two groups, one group was left in GF condition (called GF→GF SCID mice), and the other group was moved to SPF condition (GF→SPF SCID mice). Each group contained eight mice. All mice were sacrificed 10 wk after the retransfer. *B*, Gross appearance of the colon from SPF (*top*), GF→GF (*middle*), and GF→SPF (*bottom*) SCID mice. *C*, Clinical scores were determined at 10 wk after the retransfer as described in *Materials and Methods*. SPF and GF→SPF SCID mice showed the severe clinical signs of colitis, whereas GF→GF SCID mice showed no signs of colitis. Data are indicated as the mean \pm SEM of seven mice in each group. *, $p < 0.05$. *D*, Colon and SP weight of SPF and GF→SPF SCID mice were significantly larger than those of GF→GF SCID mice.

proportion of the CD4⁺ T cells in various site of colitic mice (Fig. 1). Consistent with this, we have previously shown that in sharp contrast to IL-7^{+/+} × RAG-1^{-/-} recipients, IL-7^{-/-} × RAG-1^{-/-} recipients given these colitic CD4⁺ T cells do not develop colitis (13), indicating that these CD4⁺ T cells could be categorized as T_{EM} cells.

Colitogenic CD4⁺ T_{EM} cells survive in the absence of commensal bacteria

Although it is well established that the existence of commensal bacteria is required for the initial development of most animal models of chronic colitis (10, 11, 17), it is still unclear whether commensal bacteria are also needed for the persistence of colitis and/or the maintenance of colitogenic CD4⁺ T cells after the T cell-priming process. To this end, we conducted an adoptive retransfer experiment under SPF or GF conditions (Fig. 2*A*). We used SP not LP CD4⁺ T cells isolated from colitic SCID mice as donor cells for two reasons: 1) we wanted to exclude possible contamination by commensal bacteria

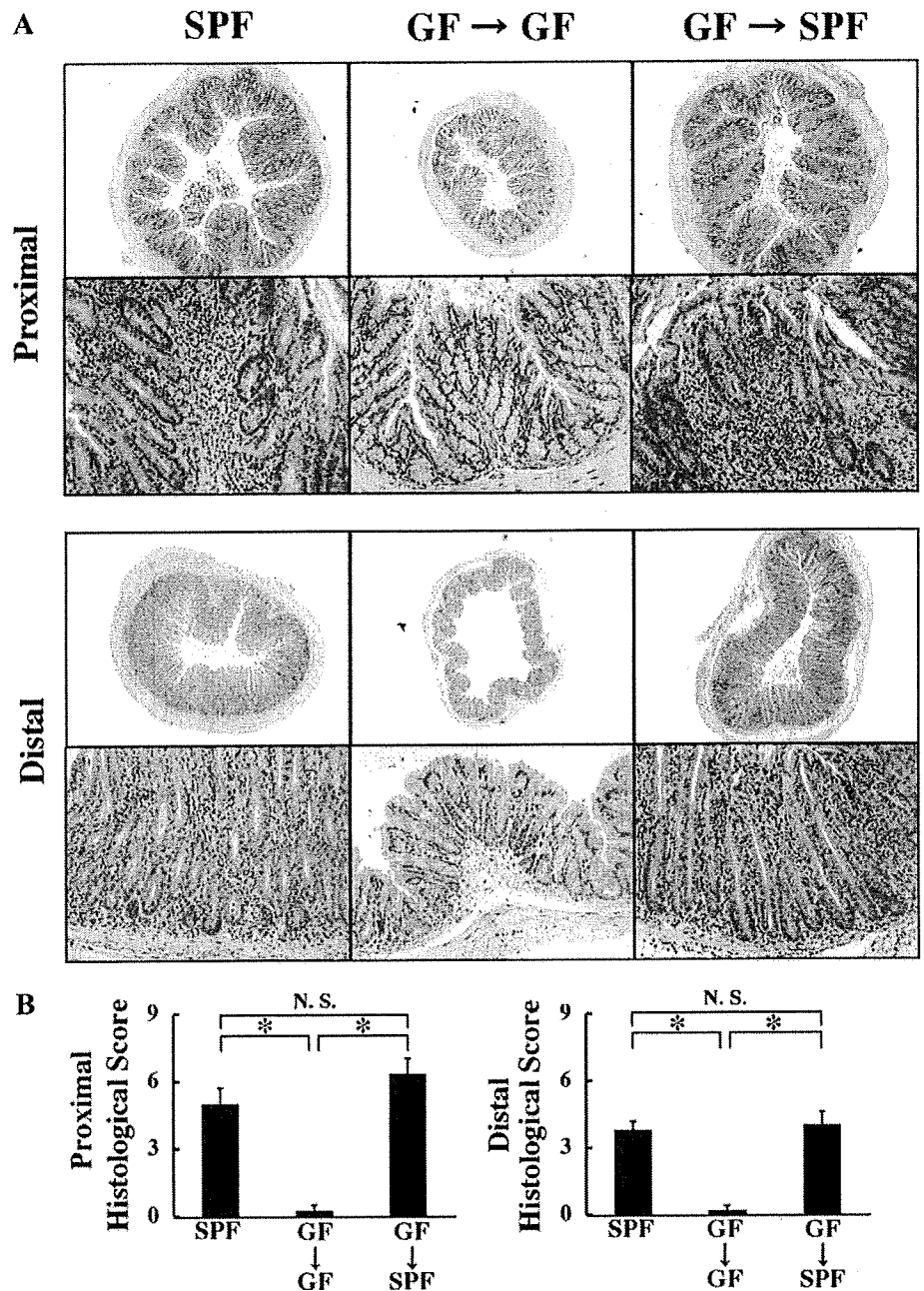


FIGURE 3. Commensal bacteria are needed to sustain the colitis. *A*, Proximal and distal colon from SPF, GF→GF, and GF→SPF SCID mice were stained with H&E. Original magnification: $\times 40$ (top) and $\times 100$ (bottom). *B*, Histological scores were determined at 10 wk after transfer as described in *Materials and Methods*. Data are indicated as the mean \pm SEM of five mice in each group. *, $p < 0.05$.

from colitic LP or MLN samples, and 2) we have previously shown that most of the SP CD4⁺ T cells in this mouse colitis model express the T_{EM} cell phenotype with a capacity to induce colitis similar to that of LP CD4⁺ T cells (12). Thus, we injected these SP cells into new SPF or GF C3H-SCID mice (hereafter called SPF or GF SCID mice, respectively) in a retransfer. Furthermore, to assess whether GF SCID recipients develop colitis when exposed to resident commensal bacteria, one group of the GF SCID recipients was moved into the SPF environment 6 wk after the retransfer and kept for an additional 4 wk (GF→SPF SCID mice), and the other group was kept in GF conditions for 10 wk (GF→GF SCID mice) (Fig. 2A). Consistent with previous reports (12, 13) and in sharp contrast to GF SCID mice, SPF SCID mice showed an enlarged colon with a greatly thickened wall (Fig. 2B) and manifested progressive weight loss from 3 wk after retransfer (data not shown). Consistent with this, the clinical score of SPF SCID mice at 10 wk after retransfer was significantly increased

compared with that of GF SCID mice, which showed no clinical signs of colitis or weight loss throughout the observation period (Fig. 2C). It was notable that GF→SPF SCID recipients also showed an enlarged and thickened colon, and their clinical scores were significantly increased compared with those of GF→GF SCID recipients, being comparable to those of SPF SCID recipients. As previously reported (18), a markedly enlarged cecum was also reproducibly observed in the GF→GF SCID-recipient mice (Fig. 2B). The integrity of the GF conditions was confirmed by repeated stool culture tests in the GF groups (data not shown). SP and colon weights of SPF SCID mice and GF→SPF SCID mice were significantly greater than those of GF→GF SCID mice (data not shown).

Histological examination showed a prominent epithelial hyperplasia with glandular elongation and massive infiltration of mononuclear cells in the LP of proximal and distal colon from SPF SCID and GF→SPF SCID mice (Fig. 3A). In contrast, the glandular

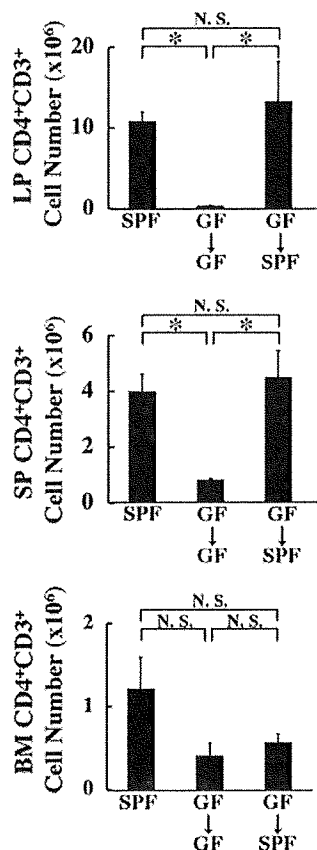


FIGURE 4. Commensal bacteria are needed for the expansion of CD4⁺ T cells. LP, SP, and BM CD4⁺ T cells were isolated from SPF, GF→GF, and GF→SPF SCID mice at 10 wk after retransfer, and the number of CD3⁺CD4⁺ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of seven mice in each group. *, *p* < 0.05.

elongation was mostly abrogated, and fewer mononuclear cells were observed in the LP of the colon from the recipient GF→GF SCID mice (Fig. 3A). This difference was also confirmed by histological scoring of multiple proximal and distal colon sections: 3.80 ± 0.37 and 4.40 ± 0.43, respectively, in SPF SCID mice; 4.00 ± 0.30 and 5.17 ± 0.056 in GF→SPF SCID mice; and 0.125 ± 0.001 and 0.125 ± 0.001 in GF→GF SCID mice (SPF SCID mice or GF→SPF SCID mice vs GF→GF SCID mice; *p* < 0.01) (Fig. 3B).

A further quantitative evaluation of CD4⁺ T cell infiltration was made by isolating LP, SP, and BM CD3⁺CD4⁺ T cells. As shown in Fig. 4, the number of CD4⁺ T cells recovered from LP and SP of SPF and GF→SPF SCID mice was significantly higher than that from GF→GF SCID mice. Surprisingly, however, a substantial number of CD4⁺ T cells remained resident in BM and SP in GF→GF SCID mice, and the number of CD4⁺ T cells recovered from BM of colitic SPF and GF→SPF SCID mice was not significantly higher than that recovered from non-colitic GF→GF SCID mice. This suggests that even in the absence of commensal bacteria, the colitogenic SP CD4⁺ T donor cells are not all short-lived effector cells but, in accordance with high expression of IL-7Rα, are long-lived T_{EM} cells or a mixture of effector cells and T_{EM} cells. Alternatively, it is possible that most persisting CD4⁺ T cells in GF SCID mice respond to environmental Ags derived from food and bedding.

We next examined the cytokine production by isolated LP CD4⁺ T cells from each group. As shown in Fig. 5, LP CD4⁺

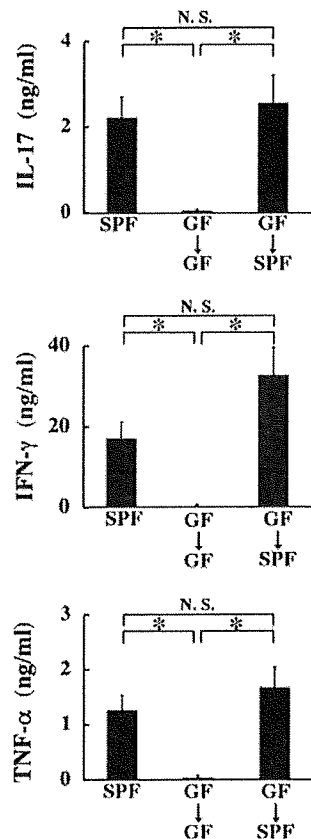


FIGURE 5. Commensal bacteria are needed for the expansion of Th1 and Th17 CD4⁺ T cells. Th1 and Th17 cytokine production by anti-CD3/CD28 mAbs-stimulated LP CD4⁺ T cells isolated from SPF, GF→GF, and GF→SPF SCID mice at 10 wk after retransfer was measured by ELISA. Data are indicated as the mean ± SD of six mice in each group. *, *p* < 0.05.

T cells from the GF SCID recipients produced significantly smaller amounts of IFN-γ, TNF-α, and IL-17 than those from the SPF SCID recipients after in vitro stimulation by anti-CD3/CD28 mAbs.

Colitogenic CD4⁺ central-memory T (T_{CM}) cells with down-modulated IL-7Rα expression are significantly increased in GF recipients

Flow cytometry analysis revealed that the cell-surface phenotype of LP, SP, and BM CD4⁺ T cells isolated from SPF or GF→SPF SCID recipients was very similar to that from the primarily injected colitic mice, that is, T_{EM} cell type with CD44^{high}CD62L⁻CD69⁺IL-7Rα^{high} cells (Figs. 1 and 6). Again, the expression level of CD69 on SP CD4⁺ T cells was significantly lower than that on LP and BM cells in the SPF or GF→SPF SCID recipients (percentage of positive cells in SPF: SP 18.9 ± 3.40, LP 60.4 ± 1.79, and BM 61.8 ± 3.37; SP vs LP or BM, *p* < 0.05; in GF→SPF, SP 9.83 ± 0.95, LP 55.8 ± 4.21, and BM 53.8 ± 6.28; SP vs LP or BM *p* < 0.05), suggesting the presence of activation mechanisms in LP and BM. Importantly, the expression pattern of all examined markers in all sites was not significantly different between SPF and GF→SPF SCID recipients (Fig. 6, right panels).

In contrast, the expression pattern on cells from GF→GF SCID recipients was quite different. First, surprisingly, IL-7Rα expression on LP but not SP and BM CD4⁺ T cells was significantly down-modulated compared with the paired LP cells

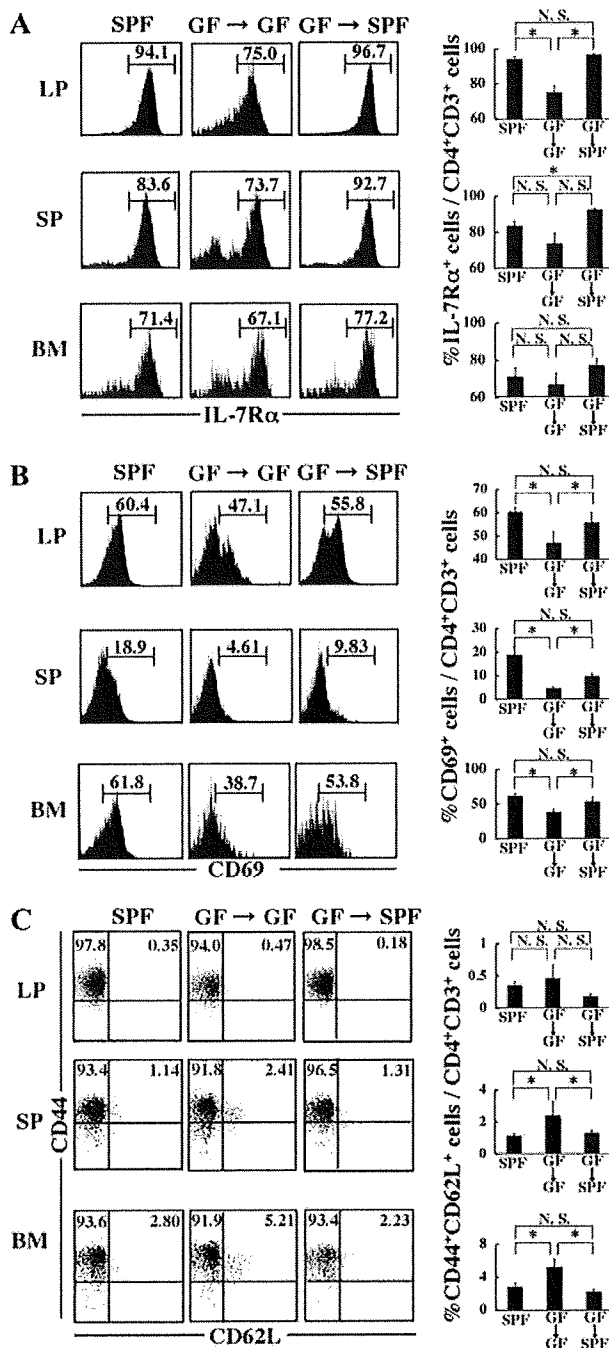


FIGURE 6. Colitogenic CD4⁺ T_{CM} cells with the down-modulated IL-7R α expression were significantly increased in GF recipients. Expression of CD44 and CD62L (C), IL-7R α (CD127) (A), and CD69 (B) on CD4⁺CD3⁺ T cells obtained from LP, SP, and BM from SPF, GF \rightarrow GF, and GF \rightarrow SPF SCID mice at 10 wk after retransfer. Freshly isolated cells were stained with allophycocyanin-labeled anti-CD4, PerCP-labeled anti-CD3, PE-labeled anti-CD44, FITC-labeled anti-CD62L, biotin-labeled anti-IL-7R α , FITC-labeled anti-CD69 mAb, or streptavidin PE. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and side scatter profiles. Data are displayed as dotted plot (four-decade log scale), and quadrant markers were positioned to include >98% of control Ig-stained cells in the bottom left. Percentages in each quadrant are indicated. Representatives of five mice in each group.

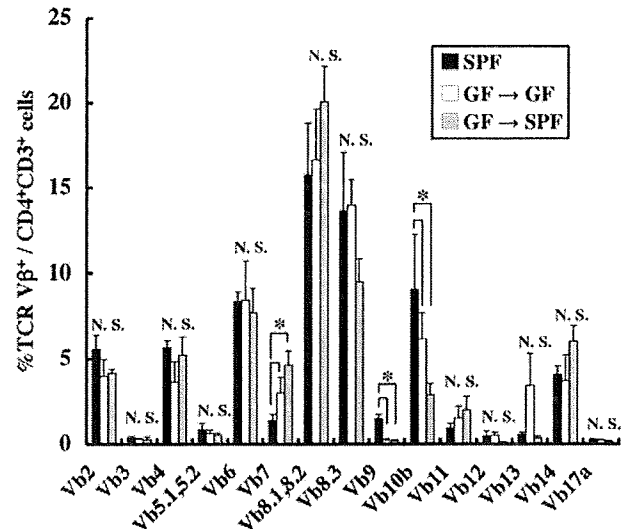


FIGURE 7. TCR V β repertoires show little difference in the presence or absence of commensal bacteria. Flow cytometric analysis of V β families on the surface of the splenic CD4⁺ T cells in SPF, GF \rightarrow GF, and GF \rightarrow SPF mice as described in Fig. 2. To analyze the TCR V β family repertoire, splenic cells were four-color-stained with PerCP-conjugated anti-CD3mAb, allophycocyanin-conjugated anti-CD4 mAb, PE-conjugated anti-Ly5.1 or Ly5.2 mAb, and a panel of 15 FITC-conjugated V β mAbs. Each percentage value indicates the frequency of each V β ($n = 6$). Data are indicated as the mean \pm SEM of five mice in each group. *, $p < 0.05$.

of SPF or GF \rightarrow SPF SCID recipients (Fig. 6A). Second, and as expected, CD69 expression on cells from all sites was also reduced compared with the matching cell source from SPF or GF \rightarrow SPF SCID recipients (Fig. 6B). Third, and quite interestingly, the proportion of CD3⁺CD4⁺CD44^{high}CD62L⁺ T_{CM} cells was significantly increased in SP and BM but not in LP of GF \rightarrow GF SCID recipients (SP 2.41 \pm 0.85%; BM 5.21 \pm 0.95%; LP 3.09 \pm 0.95%) compared with SPF SCID recipients (SP 1.14 \pm 0.1%, $p < 0.05$; BM 2.80 \pm 0.48%, $p < 0.05$; LP 0.35 \pm 0.059%, $p = 0.10$) and GF \rightarrow SPF SCID recipients (SP 1.31 \pm 0.17%, $p < 0.05$; BM 2.23 \pm 0.33%, $p < 0.05$; LP 0.18 \pm 0.040%, $p = 0.28$), suggesting the conversion of cells from T_{CM} to T_{EM} after movement of mice from GF \rightarrow SPF (Fig. 6C).

TCR V β repertoires of CD4⁺ T cells do not change in the presence or absence of commensal bacteria

The TCR V β repertoires of SP CD4⁺ T cells from SPF, GF \rightarrow GF, and GF \rightarrow SPF SCID mice differed only slightly and only in such V β families as V β 7, V β 9, and V β 11, which are not major groups in this model (Fig. 7). This suggested that long-lived colitogenic memory CD4⁺ T cells are broadly polyclonal rather than specifically oligoclonal. Furthermore, this finding suggests that in GF conditions, colitogenic memory CD4⁺ T cells may be maintained by homeostatic cytokines that would lead to polyclonal proliferation, rather than by response to a specific Ag such as food or bedding that may lead to a specific oligoclonal expansion.

BM IL-7 may support colitogenic memory CD4⁺ T cells in the absence of commensal bacteria

We have previously reported that colitogenic memory CD4⁺ T cells are retained in an IL-7-dependent manner in BM of SPF-conditioned CD4⁺CD45RB^{high} T cell-injected colitic mice

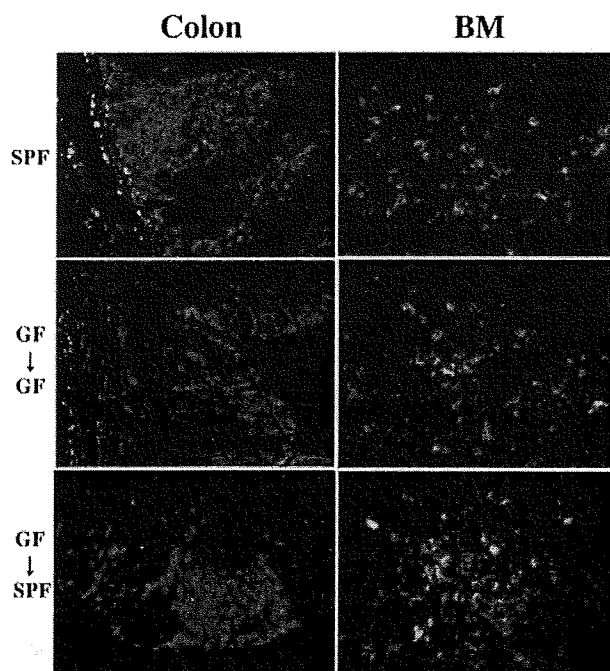


FIGURE 8. Substantial number of CD4⁺ T cells was resident in BM of noncolitic SCID recipients in the absence of commensal bacteria. Frozen sections of colon from each group (SPF, GF→GF, and GF→SPF) were stained with polyclonal anti-IL-7 Abs (green) and anti-CD4 Abs (red). Representative of five separate samples in each group. Original magnification: ×100.

(19). Thus, we hypothesized that BM IL-7 may maintain colitogenic memory CD4⁺ T cells in noncolitic GF→GF SCID mice even in the absence of commensal bacteria. To test this hypothesis, frozen sections of colon and BM from each group were stained with polyclonal anti-IL-7 Ab (green) and anti-CD4 mAb (red). First, Fig. 8 clearly demonstrates marked infiltration of CD4⁺ T cells in the colon of colitic SPF and GF→SPF SCID recipients, although the expression of IL-7 in epithelial cells was markedly decreased in these two groups (Fig. 8, *left top and bottom*). In sharp contrast, only a scattering of CD4⁺ T cells were found in the LP of noncolitic GF→GF SCID recipients despite the presence of epithelial IL-7 (Fig. 8, *left middle*). However, consistent with the constant expression of IL-7 in BM, substantial numbers of CD4⁺ T cells were resident in BM of all three groups, regardless of the presence or absence of commensal bacteria (noncolitic or colitic) (Fig. 8, *right panel*).

Neutralization of IL-7 reduces the number of CD4⁺ T cells recovered in various sites of GF SCID mice injected with colitic CD4⁺ T cells

To confirm the importance of IL-7 for the maintenance of colitogenic memory CD4⁺ T cells even in GF conditions, we next blocked IL-7. GF SCID mice injected with colitogenic SP CD4⁺ T cells were separated in two groups: one group was treated with anti-IL-7Rα mAb, and the other group was treated with control rat IgG (Fig. 9A). Two weeks after transfer, all mice were sacrificed, and LP, MLN, SP, and BM cells were analyzed by flow cytometry to determine the number of CD3⁺CD4⁺ T cells recovered (Fig. 9B). As expected, the numbers of CD4⁺ T cells recovered from sites other than LP of GF SCID mice treated with anti-IL-7Rα mAb were significantly reduced compared with the control IgG-treated group (Fig. 9B).

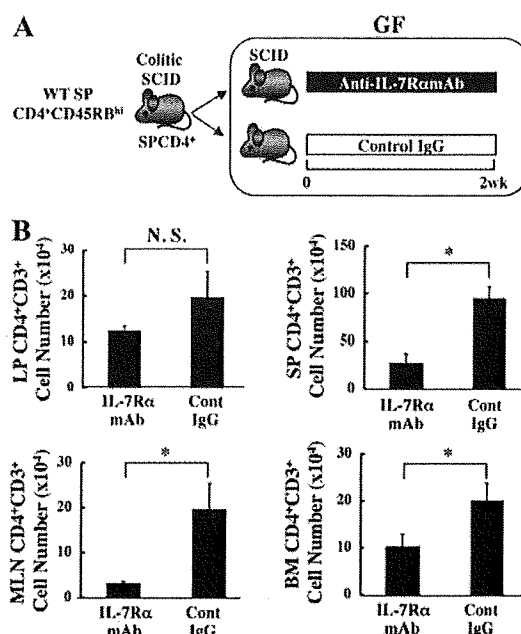


FIGURE 9. Neutralization of IL-7 reduced the recovered cell number of CD4⁺ T cells in various site of GF SCID mice transferred with colitic CD4⁺ T cells. *A*, GF SCID mice transferred with colitic CD4⁺ T cells previously transferred with CD4⁺CD45RB^{high} T cells were treated with anti-IL-7Rα mAb or control rat IgG at 0, 1, and 2 wk after transfer. *B*, Recovered CD3⁺CD4⁺ T cell number from LP, SP, MLN, and BM were analyzed by a flow cytometry. Data are indicated as the mean ± SEM of five mice in each group. *, $p < 0.05$.

However, although the number of CD4⁺ T cells recovered from LP of mice treated with anti-IL-7Rα mAb tended to be lower than that from LP of mice treated with control IgG, the difference was not significant, suggesting the possibility of additional stimuli, such as food and/or bedding Ags and pathogen-associated molecular patterns included in sterile foods, maintaining CD4⁺ T cells in LP. Collectively, this result supports our hypothesis that IL-7 may maintain colitogenic memory CD4⁺ T cells outside the intestine of the injected GF SCID mice even in the absence of commensal bacteria.

Colitogenic memory CD4⁺ T cells are retained outside the intestine even in lymphocyte-sufficient conditions

Finally, we asked whether colitogenic memory CD4⁺ T cells can be retained in lymphocyte-sufficient normal mice, because it is very important to know whether our findings are applicable to the pathogenesis of human IBD where the patients are always immunosufficient. To this end, we injected CFSE-labeled colitogenic LP Ly5.1⁺CD4⁺ T cells into Ly5.2⁺ WT mice treated with either a mixture of antibiotics in distilled water or distilled water alone (Fig. 10A). One week and 4 wk after transfer, cell numbers were recovered from LP, SP, and BM from injected WT mice, and their numbers of cell divisions were assessed by a flow cytometry (Fig. 10, *B and C*). As shown in Fig. 10B, a substantial number of Ly5.1⁺CD4⁺ T cells were recovered from LP, SP, and BM of lymphocyte-replete mice at 1 and 4 wk after cell transfer, regardless of antibiotic treatment. Notably, there is almost no difference between antibiotic-treated and control mice in the number of CD4⁺ T cells in various sites. Consistent with this, a CFSE dilution assay revealed that Ly5.1⁺CD4⁺ T cells divided well in LP, SP, and BM at the

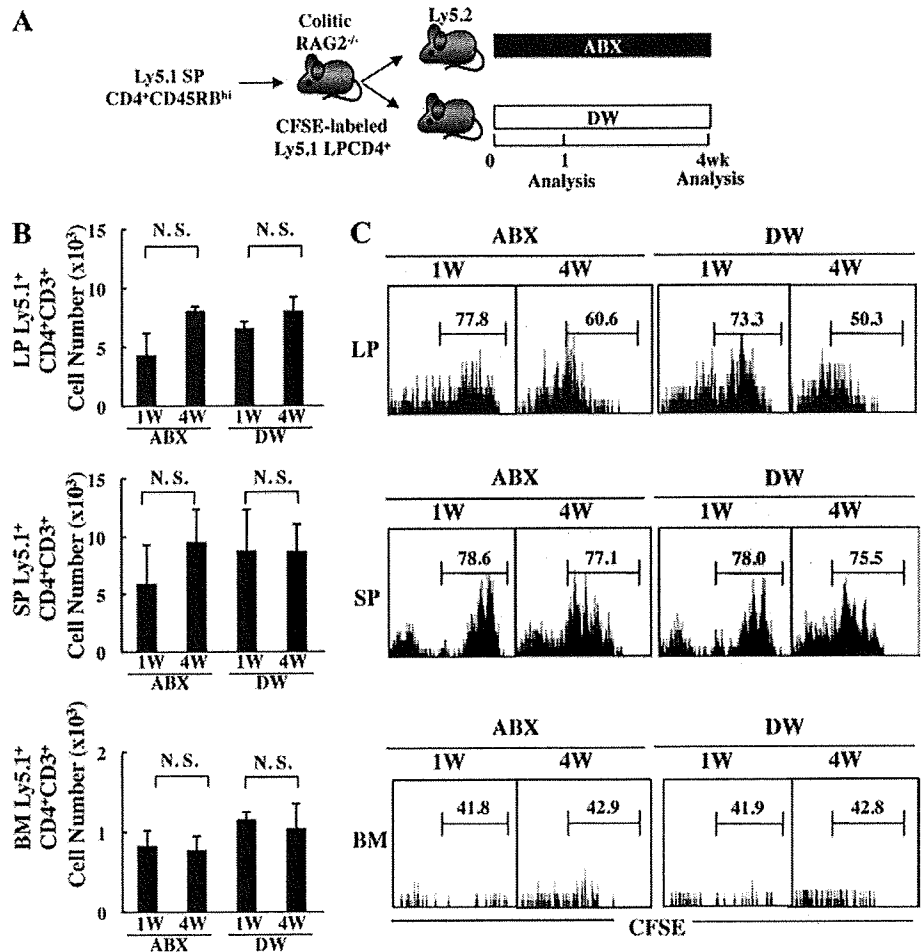


FIGURE 10. Colitogenic memory CD4⁺ T cells were retained outside the intestine even in the normal immunosufficient condition. *A*, CFSE-labeled LP CD4⁺ T cells obtained from colitic RAG2^{-/-} mice previously transferred with Ly5.1⁺CD4⁺CD45RB^{high} T cells were transferred into Ly5.2⁺ WT mice treated with antibiotics or DW. *B*, One week and 4 wk after transfer, LP, SP, and BM cells from transferred WT mice were analyzed by a flow cytometry, and the recovered cell number of CD3⁺CD4⁺ T cells in LP, SP and BM was calculated. Data are indicated as the mean \pm SEM of five mice in each group. *, $p < 0.05$. *C*, Cell division assay. At the indicated time points after transfer (1 and 4 wk after transfer), CFSE incorporation was determined by flow cytometry. Histograms are gated on CD4⁺ T cells. These data were representative from six experiments.

indicated time points regardless of antibiotic treatment. Collectively, these results suggest that in the noncolitic immunosufficient condition, the maintenance of the colitogenic memory CD4⁺ T cells is entirely independent of the presence of commensal bacterial Ag but is presumably dependent on homeostatic cytokines such as IL-7.

Discussion

The present study has demonstrated that latent long-lived colitogenic memory CD4⁺ T cells not only reside outside the intestine (for example in the IL-7-sufficient BM environment of noncolitic mice that had been previously injected with colitogenic CD4⁺ T cells under GF conditions) but also participate in the recurrence of colitis in the presence of commensal bacteria. In other words, these data clearly showed that colitogenic CD4⁺ memory T cells are established during the process of development of colitis even in the presence of commensal bacteria and can be maintained outside the intestine, possibly in an IL-7-dependent manner, in the remission stage of chronic colitis. Thus, our present results may explain why IBD is an intractable lifelong disease if it depends on the persistence of latent colitogenic CD4⁺ memory T cells.

Immunologically, "memory" is now generally believed to be a phenomenon that occurs after Ags have been eliminated (1–3). More specifically, Ags must be eliminated from the body in order for CD8⁺ memory T cells to form. For example, in the lymphocytic choriomeningitis virus model of acute viral infection, lymphocytes differentiate into CD8⁺ memory T cells for the first time after the virus has been eliminated (20, 21). In contrast, in a

chronic viral infection model using a mutant lymphocytic choriomeningitis virus, it is thought that no CD8⁺ memory T cells are produced and that the CD8⁺ effector T cells are ultimately destroyed as a result of "exhaustion" (22, 23). The fact that the Ags responsible for IBD are derived from commensal bacteria that can never be eliminated suggests the possibility that effector T cells continue to emerge and become exhausted without memory T cells ever being produced. However, a requirement for constant production of colitogenic CD4⁺ effector cells from naive CD4⁺ T cells that are continuously supplied by the thymus may be difficult to explain in view of the involution of the thymus in both mice and humans. We therefore hypothesized that even though commensal bacteria may permanently reside in the intestine, colitogenic CD4⁺ T cells could be retained as memory cells. Consistent with this hypothesis, we previously demonstrated that IL-7^{-/-} \times RAG-1^{-/-} mice injected with colitogenic CD4⁺ T cells never develop colitis, whereas evidence of disease was seen in IL-7^{+/-} \times RAG-1^{-/-} recipients (13). These findings demonstrated that IL-7 is essential for the development and maintenance of chronic colitis. Accordingly, it is now assumed that IL-7-dependent memory T cells make up at least part of the population of colitogenic CD4⁺ T cells and contribute to the overall maintenance of colitis even in the presence of enteric bacteria.

In contrast, our group has previously reported that IL-7 is produced by epithelial cells in the intestine, especially by intestinal goblet cells (24), and it is known that the pathology of IBD is characterized by a decrease in goblet cells when it becomes

chronic (25). There was also a marked decrease in strongly staining Alcian blue-positive goblet cells at the sites of the chronic colitis lesions in the CD4⁺CD45RB^{high} T cell transfer model that we used, and we discovered that there was a concomitant decrease in IL-7 production by the epithelial cells (Ref. 26; Fig. 8). Why then does IL-7 derived from inflamed intestine decrease when IL-7 is essential to the development of chronic colitis? We hypothesized that intestinal IL-7 is not required to maintain chronic colitis and that colitogenic CD4⁺ memory T cells are maintained by IL-7 outside the intestine. We previously performed parabiosis surgery that connected the flanks of colitic RAG-2^{-/-} mice into which CD4⁺CD45RB^{high} T cells had been injected with the flanks of untreated IL-7^{+/+} × RAG-1^{-/-} mice or IL-7^{-/-} × RAG-1^{-/-} mice. We allowed the hemodynamics of the two mice to be shared for several days after the parabiosis, and even though the intestinal epithelial cells of the IL-7^{-/-} × RAG-1^{-/-} host mice do not produce IL-7, they can be viewed as mice that produce IL-7 outside the intestine because of the shared hemodynamics. As expected, ~4 wk after the parabiosis, the transfer of colitogenic CD4⁺ T cells from the colitic RAG-2^{-/-} donor mice was associated with the development of chronic colitis in the IL-7^{+/+} × RAG-1^{-/-} host mice, which was accompanied by marked CD4⁺ T cell infiltration of the large intestine. Surprisingly, despite the deficiency of intestinal IL-7, frank colitis also developed in the IL-7^{-/-} × RAG-1^{-/-} host mice in the same way as in the control IL-7^{+/+} × RAG-1^{-/-} host mice (26). These previous findings demonstrated that intestinal IL-7 is not essential for the maintenance of colitogenic CD4 memory T cells in mice with chronic colitis.

In this study, we further attempted to resolve the following two issues. 1) How are colitogenic CD4⁺ T cells maintained in inflamed mucosa despite the lack of epithelium-derived IL-7? 2) Can colitogenic CD4⁺ T cells be maintained for a long period in the absence of commensal bacteria in IL-7-sufficient conditions, as is the case for memory CD8⁺ T cells? If so, are latent colitogenic CD4⁺ memory T cells involved in the recurrence of colitis after moving from a GF to an SPF environment?

First, we showed that almost all CD4⁺ T cells in LP of noncolitic GF→GF SCID mice disappeared despite the presence of epithelium-derived IL-7 (Fig. 8). In contrast, a substantial number of CD4⁺ T cells still resided outside the intestine of GF→GF SCID mice, showing that the number of CD4⁺ T cells recovered from these noncolitic mice was comparable with that from colitic SPF or GF→SPF SCID mice, especially in BM (Fig. 4). This result suggests that colitogenic CD4⁺ T cells may be reciprocally regulated in intestine and in the BM so that colitogenic LP and BM CD4⁺ T cells are stimulated via commensal bacterial Ags and via IL-7 signaling, respectively. Consistent with this idea, we previously showed that: 1) synchronous stimulation of human LP CD4⁺ T cells by anti-CD3 mAb and IL-7 induces apoptosis of those cells (16), and 2) colitogenic CD4⁺ T cells actively circulate in colitic mice and the blockade of the circulation by FYT720 treatment ameliorates the colitis in this model (27). This suggests that epithelial IL-7 plays a physiologically important role in intestinal immune homeostasis that prevents the triggering of IBD, whereas the absence of epithelium-derived IL-7 itself may be needed for the persistence of chronic colitis. Further study using IL-7-deficient recipient mice under GF conditions will be needed to test this hypothesis.

Second, and surprisingly, we showed that colitogenic SP CD4⁺ T cells survived for 6 wk in GF conditions and are involved in the recurrence of colitis after their movement to SPF conditions. Because effector T cells are believed to be short lived in contrast to long-lived memory T cells, this result indicates that the colitogenic

SP CD4⁺ T cells contained at least some fraction of colitogenic memory CD4⁺ T cells. As an additional explanation for the perpetuation of IBD, the ratio of T_{CM}/T_{EM} cells in GF→GF SCID mice may support the idea that colitogenic “memory stem”-like T_{CM} cells are generated in the process of development and/or persistence of chronic colitis.

The results of our current project show some differences from the findings published by Veltkamp et al. (17). Using their original model of chronic colitis induced by transplantation of BM from normal mice into CD3ε transgenic (BM→Tgε26) mice, they showed that BM→Tgε26 mice do not develop colitis in GF conditions whereas control BM→Tgε26 in SPF conditions do. However, they also showed that viable intestinal bacteria are not necessary for the survival of CD4⁺ T cells that retain their functional integrity in noncolitic BM→Tgε26 mice in GF conditions and induce colitis when mice are moved to SPF conditions and that Tgε26 mice develop colitis in SPF conditions when MLN cells from BM→Tgε26 mice maintained in GF conditions are injected. Thus, they concluded that continuous stimulation by commensal bacteria is essential for the development of colitis. Although the results of their study seem to be similar to ours, the interpretation of each study is distinct. First, it is very likely that peripheral MLN cells isolated from noncolitic BM→Tgε26 mice in GF conditions contain a substantial number of naive CD4⁺ T cells, and thus it is very possible that lymphopenic Tgε26 mice injected with these cells develop colitis when transferred to SPF conditions. In contrast, we first isolated colitogenic SP CD4⁺ memory T cells from colitic mice, and as shown in Fig. 1, these cells all have the phenotype of memory CD4⁺CD44^{high} T cells. Also, it is very important that naive CD4⁺ T cells are continuously generated in their BM transplantation system but not in our adoptive transfer system using T cell-deficient SCID mice that lack the ability to generate naive CD4⁺ T cells continuously from the thymus.

Finally, it should be noted that the expression of IL-7Rα was significantly reduced on CD4⁺ T cells from all sites of noncolitic GF→GF SCID mice compared with the paired sites in colitic SPF or GF→SPF SCID mice. This was very surprising, being inconsistent with the recent immunological dogma that memory CD4⁺ T cells express high levels of IL-7Rα. Although it makes sense that the high expression of IL-7Rα on CD4⁺ T cells of noncolitic GF→GF SCID mice would be maintained because the significant decrease in expression of the activation marker CD69 on those memory T cells suggests that they are in the resting state, we also obtained the same result using antibiotic-treated mice that had previously been injected with CD4⁺CD45RB^{high} T cells. Although further study will be needed to address this issue, colitogenic memory CD4⁺ T cells may be quite different from conventional memory CD4⁺ T cells.

Taken together, these findings suggest that long-lived colitogenic memory CD4⁺ cells can be established as CD4⁺ T_{EM} or T_{CM} cells during the process of development of colitis, even in the presence of commensal Ags, and that these cells participate in the perpetuation of colitis. Thus, blocking a stimulus of colitogenic memory CD4⁺ cells such as IL-7, or alternatively an immunological reset by BM transplantation to remove these colitogenic memory CD4⁺ T cells (28), may have therapeutic benefit for treatment of IBD.

Disclosures

The authors have no financial conflict of interest.

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RANK-RANKL Signaling Pathway Is Critically Involved in the Function of CD4⁺CD25⁺ Regulatory T Cells in Chronic Colitis¹

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It is now clear that functional CD4⁺CD25⁺ regulatory T (T_R) cells exist as part of the normal immune population and prevent the development of intestinal inflammation. We have recently shown that CD4⁺CD25⁺ T_R cells reside in the intestine and control intestinal homeostasis in humans and mice. In this study, we demonstrate that the TNF family molecule RANKL and its receptor RANK are critically involved in controlling the function of CD4⁺CD25⁺ T_R cells in the intestine. We first found that RANKL was preferentially expressed on both CD4⁺CD25⁺ T_R cells and colitogenic CD4⁺ T cells, whereas RANK was expressed on dendritic cells. Although neutralizing anti-RANKL mAb did not affect T_R activity of CD4⁺CD25⁺ T_R cells to suppress the proliferation of CD4⁺ responder cells in vitro, in vivo administration of anti-RANKL mAb abrogated CD4⁺CD25⁺ T_R cell-mediated suppression of colitis induced by adoptive transfer of CD4⁺CD45RB^{high} T cells into SCID mice. Interestingly, an adoptive transfer experiment using Ly5.1⁺CD4⁺CD45RB^{high} cells and Ly5.2⁺CD4⁺CD25⁺ T_R cells revealed that the ratio of CD4⁺CD25⁺ T_R cells in total CD4⁺ T cells in inflamed mucosa was significantly decreased by anti-RANKL mAb treatment. Consistent with this, the expression of RANK on lamina propria CD11c⁺ cells from colitic mice was significantly increased as compared with that from normal mice, and in vitro treatment with anti-RANKL mAb suppressed the expansion of CD4⁺Foxp3⁺ T_R cells in culture with colitic lamina propria CD11c⁺ cells. Together, these results suggest that the RANK-RANKL signaling pathway is critically involved in regulating the function of CD4⁺CD25⁺ T_R cells in colitis. *The Journal of Immunology*, 2009, 182: 6079–6087.

Intestinal mucosal surfaces are exposed to a large number of dietary and bacterial Ags (1–5). However, the gut-associated immune system fences off harmful Ags from systemic circulation and induces systemic tolerance against luminal Ags. In contrast, inflammatory bowel diseases (IBD)⁴ and animal models of T cell-mediated chronic colitis are associated with the activation of intestinal and systemic immune responses (2, 3). In this regard, CD4⁺CD25⁺ regulatory T (T_R) cells play a central role in the maintenance of immunological homeostasis (6, 7). CD4⁺CD25⁺ T_R cells have been detected mainly in lymphoid sites, including thymus, lymph nodes, and spleen. Because numerous studies have demonstrated the capacity of CD4⁺CD25⁺ T_R cells to prevent the

induction of immune responses and this suppression requires direct cell-cell contact with responder T cells or APCs, it is conceivable that CD4⁺CD25⁺ T_R cells act as a central regulator within lymphoid tissues (6–8).

The GALT can be divided into effector sites, which consist of lymphocytes scattered throughout the lamina propria (LP) of the intestinal mucosa and organized lymphoid tissues (inductive sites) such as mesenteric lymph nodes (MLNs) and Peyer's patches, which are responsible for the induction phase of immune responses (2, 9). It is thought that presentation of Ags to naive, effector, and memory T cells is concentrated at these inductive sites of organized mucosal lymphoid follicles, and thus APCs finely tune the balance between intestinal immune tolerance and inflammation.

In addition to the inductive sites, however, it remains unclear where CD4⁺CD25⁺ T_R cells suppress the development of colitis. Although it is reasonable to hypothesize that mechanisms for the induction, maintenance, and suppression of colitis would be centrally controlled in the inductive sites by CD4⁺CD25⁺ T_R cells, two-thirds of which constitutively express the lymph node-homing receptor CD62L (10), we previously demonstrated that human intestinal LP CD4⁺CD25^{high} T cells obtained from normal individuals possess T_R activity in vitro and therefore questioned whether these inductive sites alone were involved in the induction and suppression of intestinal inflammation (11). We also reported that peripheral CD4⁺CD25⁺ T_R cells actually migrated to the intestine and suppressed the development of colitis in the CD4⁺CD45RB^{high} cell transfer model of colitis without the involvement of lymph nodes in lymph node-null LTα^{-/-} × RAG-2^{-/-} recipient mice (12). Consistent with our previous reports, it has recently been reported that CD4⁺CD25⁺ T_R cells were detected in peripheral tissues and at sites of ongoing immune responses, such as synovial fluid from rheumatoid arthritis patients (13), tumors (14), transplants (15), skin lesions in mice infected

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⁴ Abbreviations used in this paper: IBD, inflammatory bowel disease; LP, lamina propria; MLN, mesenteric lymph node; RANK, receptor activator of NF-κB; SP, spleen; RANKL, receptor activator of NF-κB ligand; T_R, regulatory T; IEL, intraepithelial lymphocyte; HPF, high-power field; Fwd, forward; Rev, reverse; MMC, mitomycin C; DC, dendritic cell; RA, retinoic acid.

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with *Leishmania major* (16), lungs from mice infected with *Pneumocystis carinii* (17), and diseased lesions in delayed-type hypersensitivity models (18), as well as in inflamed mucosa of colitic mice (8, 19).

However, it remains largely unknown which molecular mechanisms actually control the function of CD4⁺CD25⁺ T_R cells in the intestine to suppress intestinal inflammation. In the present study, we show that both CD4⁺CD25⁺ T_R cells and colitogenic CD4⁺ T cells preferentially express a TNF family member, receptor activator of NF- κ B ligand (RANKL), and that blockade of the signaling pathway via RANKL and its receptor activator of NF- κ B (RANK) (20) by administering neutralizing anti-RANKL mAb abrogates the CD4⁺CD25⁺ T_R cell-mediated suppression of colitis induced by adoptive transfer of CD4⁺CD45RB^{high} T cells into SCID mice, indicating a critical role for the RANKL/RANK signaling pathway in the function of intestinal CD4⁺CD25⁺ T_R cells in attenuating colitis.

Materials and Methods

Animals

Female BALB/c, CB-17 SCID, and 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. Mice were maintained under specific pathogen-free conditions in the animal care facility of Tokyo Medical and Dental University. Mice were used at 7–12 wk of age. All experiments were approved by the regional animal study committees.

Antibodies

The following mAbs except anti-CCR9 mAb (R&D Systems) and reagents were purchased from BD Pharmingen: RM4-5-, PE-, or PerCP-conjugated anti-mouse CD4 (rat IgG2a); 7D4, FITC-conjugated anti-mouse CD25 (rat IgM); PC61, PE-conjugated anti-mouse CD25 (rat IgG1); H1.3F3, FITC-conjugated anti-CD69 (Ham IgG1); FJK-16s, allophycocyanin-conjugated anti-mouse Foxp3 (rat IgG2a); DATK32, PE-conjugated anti-integrin $\alpha_4\beta_7$ (rat IgG2a); M290, PE-conjugated anti-integrin $\alpha_n\beta_7$ (rat IgG2a); 242503, PE-conjugated anti-CCR9 (rat IgG2b); isotype control Abs, biotin-conjugated rat IgG2, FITC-conjugated rat IgM, PE-conjugated rat IgG2a, and PE-conjugated mouse IgG2a; PE-conjugated streptavidin; and CyChrome-conjugated streptavidin. The neutralizing anti-mouse RANKL mAb (IK22-5, rat IgG2a), and anti-mouse RANK mAb (12-31, rat IgG2a) were prepared as described previously (21).

Purification of T cell subsets

CD4⁺ T cells were isolated from spleen cells of BALB/c mice using the anti-CD4 (L3T4) MACS system (Miltenyi Biotec) according to the manufacturer's instructions. Enriched CD4⁺ T cells (96–97% pure, as estimated by FACSCalibur; BD Biosciences) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5), FITC-conjugated anti-CD45RB (16A), FITC-conjugated anti-CD25 (7D4), and streptavidin-PE. Subpopulations of CD4⁺ cells were isolated by two-color sorting on a FACS Vantage (BD Biosciences). All populations were >98.0% pure on reanalysis.

In vivo adoptive transfer experiments

A series of in vivo experiments was conducted to investigate the role of the RANK/RANKL pathway in the expansion and function of CD4⁺CD25⁺ T_R cells in the suppression of murine chronic colitis. In experiment 1, chronic colitis was induced by adoptive transfer of CD4⁺CD45RB^{high} T cells into SCID mice (22). CB-17 SCID mice were injected i.p. with one or two subpopulations of sorted CD4⁺ T cells in PBS and then administered 250 μ g of anti-RANKL mAb or control rat IgG in 250 μ l of PBS three times per week for 6 wk as follows: 1) CD4⁺CD45RB^{high} alone (3×10^5 /mouse) plus control IgG ($n = 8$), 2) CD4⁺CD45RB^{high} alone (3×10^5) plus anti-RANKL mAb ($n = 8$), 3) CD4⁺CD45RB^{high} (3×10^5) plus CD4⁺CD25⁺ (1×10^5) plus control IgG ($n = 8$), or 4) CD4⁺CD45RB^{high} (3×10^5) plus CD4⁺CD25⁺ (1×10^5) plus anti-RANKL mAb ($n = 8$). Mice were sacrificed at 6 wk after T cell transfer. For experiment 2, to further assess the localization of effector T cells and T_R cells in the recipients, we used Ly5.1⁺CD4⁺CD45RB^{high} cells and Ly5.2⁺CD4⁺CD25⁺ cells as donors and C57BL/6 RAG-2^{-/-} mice as recipients in the same treatment setting as experiment 1 (12). In experiment 3, to more properly assess the effect of anti-RANKL mAb on the trafficking of T_R cells to

inflamed mucosa of colitic mice, we conducted another in vivo setting. First, RAG-2^{-/-} mice were transferred with CD4⁺CD45RB^{high} T cells and, 4 wk after transfer, these colitic mice were treated with 250 μ g of anti-RANKL mAb or control IgG two times within 1 day. Then they were retransferred with splenic Ly5.1⁺CD4⁺ T cells from normal mice, and we evaluated the cell number of Ly5.1⁺CD4⁺Foxp3⁺ and Foxp3⁻ cells recovered from LP, spleen (SP), and MLNs at 24 h after the retransfer.

Disease monitoring and clinical scoring

The recipient SCID mice after T cell transfer were weighed initially, then three times per week thereafter. They were observed for clinical signs of illness: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. Mice were sacrificed and assessed for a clinical score as the sum of four parameters: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea; and an additional point was added if gross blood was noted) (12).

Histological examination

Tissue samples were fixed in PBS containing 6% neutral-buffered formalin. Paraffin-embedded sections (5 μ m) 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 (12), as follows: mucosal damage, 0; normal, 1; 3–10 intraepithelial lymphocytes (IEL)/high-power field (HPF) and focal damage, 2; >10 IEL/HPF and rare crypt abscesses, 3; and >10 IEL/HPF, multiple crypt abscesses and erosion/ulceration, submucosal 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, and 3; extensive leukocyte infiltration with transmural effacement of the muscularis.

Preparation of mucosal LP mononuclear cells

Colonic LP mononuclear cells were isolated using a method described previously (22). In brief, the entire length of intestine was opened longitudinally, washed with PBS, and cut into small (~5-mm) pieces. To remove epithelium including IEL, the dissected mucosa was incubated two times with Ca²⁺Mg²⁺-free HBSS containing 1 mM DTT (Sigma-Aldrich) for 30 min and then serially incubated two times in medium containing 0.75 mM EDTA (Sigma-Aldrich) for 60 min at 37°C under gentle shaking. The supernatants from these incubations, which included the epithelium and IEL, were desalted, and the residual fragments were pooled and treated with 2 mg/ml collagenase A (Worthington Biomedical) and 0.01% DNase (Worthington Biochemical) in 5% CO₂ humidified air at 37°C for 2 h. The cells were then pelleted two times through a 40% isotonic Percoll solution and further purified by Ficoll-Hypaque (Pharmacia) density gradient centrifugation (40%/75%). Enriched CD4⁺ LP T cells were obtained by positive selection using an anti-CD4 (L3T4) MACS magnetic separation system. The resultant cells when analyzed by FACSCalibur contained >96% CD4⁺ cells.

RT-PCR

Total cellular RNA was extracted from 7×10^5 cells using a RNeasy Mini Kit (Qiagen). Five micrograms of total RNA was reverse-transcribed using the Superscript II Reverse Transcriptase (Invitrogen). RANK and RANKL levels were measured with a QuantiTect SYBER green PCR kit using Applied Biosystems 17500 real-time PCR system and 7500 system SDS software with the following primers: RANK: forward (Fwd), 5'-GGT CTG CAG CTC TTC CAT GAC-3' and reverse (Rev) 5'-TGA GAC TGG GCA GGT AAG CC-3'; RANKL: Fwd, 5'-TTG CAC ACC TCA CCA TCA ATG-3' and Rev, 5'-TTA GAG ATC TTG GCC CAG CCT-3'; and G3PDH: Fwd, 5'-CTA CTG GCG CTG CCA AGG CAG T-3' and Rev, 5'-GCC ATG AGG TCC ACC ACC CTG-3'. PCR cycling conditions consisted of 95°C for 15 min, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 40 s. Data are expressed as the relative amount of indicated mRNA as normalized against G3PDH.

Flow cytometry

To detect the surface expression of various molecules, isolated splenocytes or LP mononuclear cells were preincubated with a Fc γ R-blocking mAb

(CD16/32, 2.4G2; BD Pharmingen) for 15 min, then incubated with specific FITC-, PE-, or biotin-labeled Abs for 20 min on ice. Biotinylated Abs were detected with PE- or CyChrome-streptavidin. Intracellular Foxp3 staining was performed with the allophycocyanin-anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions. Standard two- or three-color flow cytometric analyses were obtained using the FACSCalibur with CellQuest software. Background fluorescence was assessed by staining with control isotype-matched mAbs.

Cytokine ELISA

To measure cytokine production, 1×10^5 LP CD4⁺ T cells were cultured in 200 μ l of 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 2 μ g/ml hamster 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 the manufacturer's recommendation (R&D Systems).

In vitro regulatory function of CD4⁺CD25⁺ T cells

Spleen cells from BALB/c mice were separated into unfractionated whole CD4⁺ T cells and CD4⁺CD25⁺ T cells using the anti-CD4 (L3T4) MACS magnetic separation system and/or FACS Vantage as described above. Responder CD4⁺ cells (7×10^4) and mitomycin C (MMC)-treated CD4⁺ cells (5×10^4) as APCs, with or without CD4⁺CD25⁺ cells (1×10^4), were cultured in the presence or absence of neutralizing anti-RANKL mAb or anti-RANK mAb (1, 3, or 10 μ g/ml) for 72 h in round-bottom 96-well plates in RPMI 1640 medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μ M 2-ME. Incorporation of [³H]thymidine (1 μ Ci/well) by proliferating cells was measured during the last 9 h of culture. In some experiments, a CFSE dilution assay was performed. To this end, splenic CD4⁺ T cells were negatively obtained from whole spleen cells of normal C57BL/6 mice using a CD4 T Cell Isolation Kit (Miltenyi Biotec). CD4⁺CD25⁻ responder T and CD4⁺CD25⁺ T_R cells were separately isolated using anti-CD25 MACS beads (Miltenyi Biotec) from the CD4⁺ T cells. CD11c⁺ dendritic cells (DC) were isolated from SP and LP of normal C57BL/6 mice and colitic C57BL/6 background RAG-2^{-/-} mice previously transferred with CD4⁺CD45RB^{high} T cells. All populations were >92% pure on reanalysis. Then, CD4⁺CD25⁻ responder T cells were labeled with 1 μ M CFSE (Molecular Probes). CFSE-labeled CD4⁺CD25⁻ responder T cells (1×10^5) were cocultured with unlabeled Ly5.2⁺CD4⁺CD25⁺ T_R cells (0.33×10^5) with CD11c⁺ DC (2×10^4) and anti-CD3 (1 μ g/ml) in the presence of anti-RANKL mAb (1 μ g/ml) or control IgG (1 μ g/ml) in 96-well round-bottom plates for 120 h in triplicates. After incubation, cells were collected and analyzed by FACS. Propidium iodide was added to exclude dead cells. Proliferation analysis was based on division times of responder CFSE⁺CD4⁺ T cells at the condition that can discriminate the unlabeled CD4⁺CD25⁺ T_R cells and CD11c⁺ DC cells.

In vitro induction of gut-homing receptors and CD4⁺Foxp3⁺ T_R cells

For the isolation of DC from LP, MLN, and SP of colitic mice, total cells obtained by digestion with collagenase were passed through a 40- μ m cell strainer. CD11c⁺ DC were further purified using an anti-CD11c MACS magnetic separation system, resulting in >80% purity. In vitro assay for the induction of gut-homing receptors was performed by the modified protocol established by others (23). In short, 2×10^5 CD4⁺ T cells purified from normal BALB/c mice were cultured with 2×10^3 purified LP, MLN, or splenic CD11c⁺ DC from colitic mice in addition to a soluble 1 μ g/ml anti-CD3 mAb (BD Biosciences), human 10 ng/ml rTGF- β (PeproTech), all-trans retinoic acid (RA), and 5 ng/ml human rIL-2 (Shionogi Pharm) with or without 1 μ g/ml anti-RANKL mAb. On day 4, cells were stained with PE-conjugated anti-CD4 mAb and FITC-conjugated anti-integrin $\alpha_4\beta_7$, FITC-conjugated anti-integrin $\alpha_E\beta_7$, or FITC-conjugated anti-CCR9 mAb. In some experiments, 2×10^5 CD4⁺ T cells purified from normal BALB/c mice were cultured with 2×10^3 purified LP, MLN, or SP CD11c⁺ DC from colitic mice in addition to soluble 1 μ g/ml anti-CD3 mAb (BD Biosciences) with or without 1 μ g/ml anti-RANKL mAb. On day 4, intracellular Foxp3 staining was performed with the allophycocyanin-anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions.

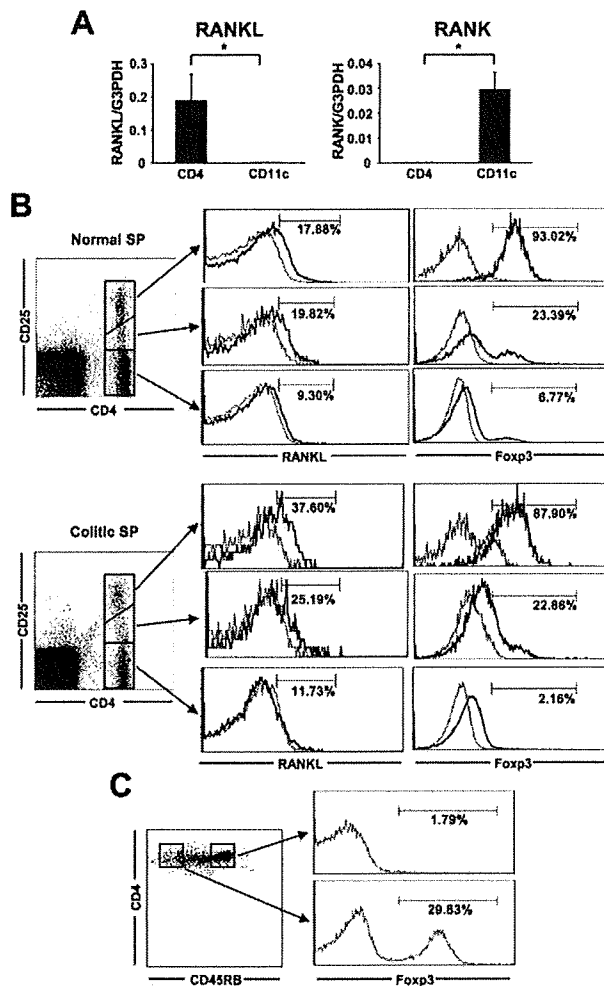


FIGURE 1. Expression of RANK/RANKL in spleen cells from normal and colitic mice. **A**, Expression of RANK, RANKL, and G3PDH mRNA was determined by RT-PCR using five SP samples each from normal and colitic mice and is shown as relative amount of the indicated mRNA normalized to G3PDH. Data are represented as the mean \pm SEM of five samples. *, $p < 0.005$. **B**, Phenotypic characterization of splenic CD4⁺ T cells expressing CD25 and Foxp3 by FACS. Correlation of CD25/Foxp3 and RANKL expression on/in splenic CD4⁺ T cells obtained from normal or colitic mice. Representatives of four separate samples in each group. **C**, Expression of Foxp3 in a population of CD4⁺CD45RB^{high} or CD45RB^{low} T cells purified from spleen of normal BALB/c mice.

Statistical analysis

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

Results

Murine splenic CD4⁺CD25⁺ cells constitutively express RANKL

To assess the role of the RANK/RANKL axis in the pathogenesis of chronic colitis in terms of stimulatory and inhibitory effects, we first assessed the expression of RANK and RANKL molecules in normal splenic CD4⁺ T cells and CD11c⁺ DC by RT-PCR. As shown in Fig. 1A, CD4⁺ T cells preferentially expressed RANKL, but not RANK mRNA, while CD11c⁺ cells preferentially expressed RANK, but not RANKL mRNA. We further assessed the expression of RANKL on CD4⁺ T cells obtained from colitic SCID mice induced by adoptive transfer of CD4⁺CD45RB^{high} T

cells and age-matched normal BALB/c mice at a protein level by flow cytometry. As shown in Fig. 1B, splenic CD4⁺CD25^{high} and CD4⁺CD25^{low} T cells in normal mice slightly but substantially expressed RANKL, while CD4⁺CD25⁻ cells did not. We confirmed that almost all CD4⁺CD25^{high} T cells expressed Foxp3, while CD4⁺CD25^{low} T cells did not, indicating that CD4⁺CD25^{high} and CD4⁺CD25^{low} T cells are T_R and previously activated effector T cells, respectively (Fig. 1B). Interestingly, although colitic SCID mice were reconstituted with a splenic CD4⁺CD45RB^{high} T cell population that lacks CD4⁺CD25⁺ T_R cells, inducible CD4⁺CD25^{high}Foxp3⁺ T cells as well as activated CD4⁺CD25^{low}Foxp3⁻ T cells were found in the spleen and these cells expressed the RANKL molecule, in contrast to CD4⁺CD25⁻ T cells (Fig. 1B). To exclude the possibility that isolated CD4⁺CD45RB^{high} T cells were contaminated with CD4⁺Foxp3⁺ T_R cells, we examined the expression of Foxp3 in those cells by flow cytometry. Although almost one-third of the control CD4⁺CD45RB^{low} T cells expressed Foxp3, we confirmed that CD4⁺CD45RB^{high} T cells did not (Fig. 1C), indicating that a small part of CD4⁺CD45RB^{high}Foxp3⁻ T cells had differentiated into CD4⁺Foxp3⁺ T_R cells in LP or MLNs in the periphery and migrated to the spleen in colitic mice.

Blockade of the RANK/RANKL signaling pathway did not affect in vitro T_R activity of CD4⁺CD25⁺ cells

Having evidence that activated CD4⁺ T cells or CD4⁺CD25⁺ T_R cells express RANKL, we next assessed whether T_R activity of splenic CD4⁺CD25⁺ T cells is modulated by blocking the RANK/RANKL pathway using neutralizing anti-RANKL and anti-RANK mAbs in *in vitro* coculture assay. To this end, we used two approaches, [³H]thymidine uptake and CFSE dilution assay, to assess the proliferation of responder CD4⁺ T cells. As shown in Fig. 2A, splenic CD4⁺CD25⁺ T cells were able to suppress the proliferation of splenic CD4⁺ responder T cells when cocultured at a ratio of 1 T_R to 1 responder in the presence of MMC-treated CD4⁻ APCs, soluble anti-CD3 mAb, and control rat IgG. Similarly, neither anti-RANKL nor anti-RANK mAb at concentrations of 1–10 μg/ml affected the T_R activity of CD4⁺CD25⁺ T_R cells (Fig. 2A). To further precisely assess the role of the RANK/RANKL pathway in the suppressive activity of CD4⁺CD25⁺ T_R cells to block the proliferation of CD4⁺CD25⁻ responder T cells at the same ratio of the following *in vivo* adoptive transfer experiment, we next used CD4⁺CD25⁻ T cells as responder T cells and SP or LP CD11c⁺ DC from normal or colitic mice and adopted the CFSE dilution assay to assess the proliferation of responder CD4⁺CD25⁻ T cells without the impact of the proliferation of cocultured CD4⁺CD25⁺ T_R cells. As shown in Fig. 2B, the proliferation of CD4⁺CD25⁻ responder T cells is seen in the right part of the histogram, and they extensively proliferated in response to anti-CD3 mAb in the absence of CD4⁺CD25⁺ T_R cells with any CD11c⁺ DC regardless of SP or LP and normal or colitic DC. In contrast, the addition of CD4⁺CD25⁺ T_R cells prevented the proliferation of CD4⁺CD25⁻ responder T cells in the presence of anti-RANKL mAb to a similar extent in the presence of control IgG, indicating that the blockade of the RANK/RANKL pathway does not affect the suppressive activity of CD4⁺CD25⁺ T_R cells at least *in vitro*.

Blockade of the RANK/RANKL signaling pathway abolished CD4⁺CD25⁺ T_R cell-mediated suppression of colitis in vivo

Although blockade of the RANK/RANKL signaling pathway did not affect the T_R function of CD4⁺CD25⁺ cells in the coculture assay (Fig. 2), *in vitro* assays do not always reflect function *in vivo*. Thus, we next administered neutralizing anti-RANKL mAb to SCID mice transferred with CD4⁺CD45RB^{high} cells alone or a

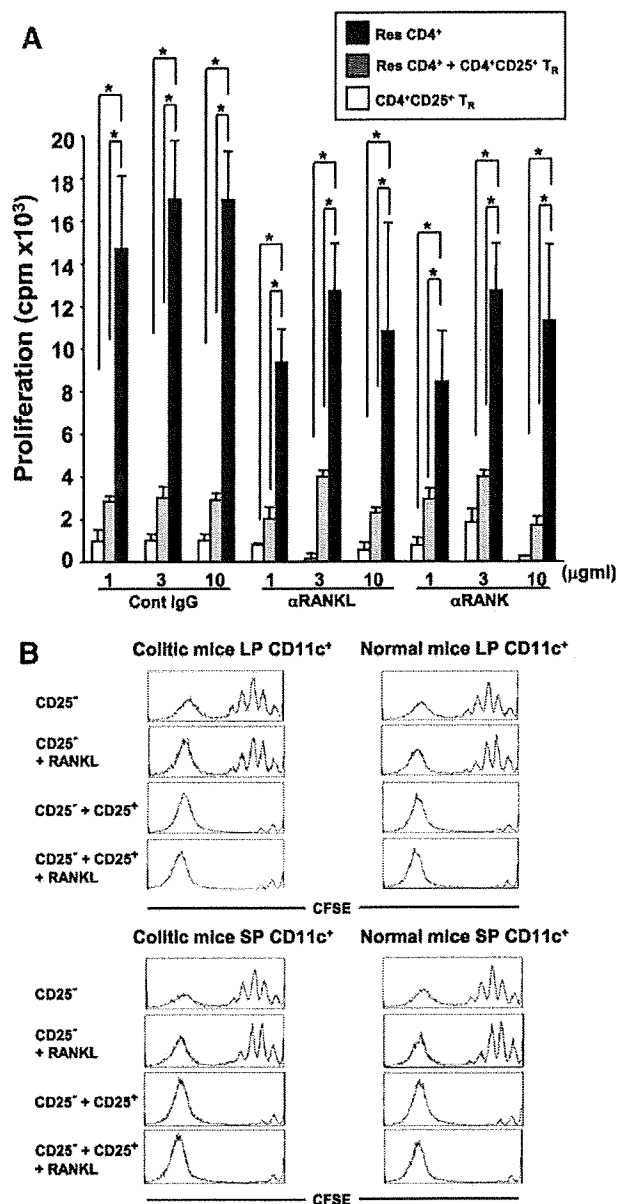


FIGURE 2. Blockade of the RANK/RANKL signaling pathway does not affect T_R activity in *in vitro* coculture assay. **A**, Splenic CD4⁺CD25⁺ T cells suppress the proliferation of responder (Res) CD4⁺ T cells *in vitro* in the presence of neutralizing anti-RANKL or anti-RANK mAb. Responder CD4⁺ T cells and sorted CD4⁺CD25⁺ T cells were cocultured for 72 h with anti-CD3 mAb at a 1:1 ratio in the presence of MMC-treated APCs and in the presence of various concentrations (1, 3, or 10 μg/ml) of control IgG, anti-RANKL, or anti-RANK mAb. [³H]Thymidine ([³H]TdT) uptake was determined for the last 9 h. Data are represented as the mean ± SEM of triplicate samples. *, *p* < 0.05. **B**, Splenic CD4⁺CD25⁺ T_R cells suppress the proliferation of CD4⁺CD25⁻ responder T cells *in vitro* in the presence of anti-RANKL mAb. CFSE-labeled CD4⁺CD25⁻ responder T cells (1 × 10⁵) and CD4⁺CD25⁺ T_R cells (0.33 × 10⁵) were cocultured for 120 h with CD11c⁺ DC obtained from SP or LP of normal or colitic mice (2 × 10⁴) in the presence of anti-RANK mAb (1 μg/ml) or control IgG (1 μg/ml) in addition to anti-CD3 mAb (1 μg/ml). Proliferation of the CD4⁺CD25⁻ responder T cells that is shown at the right part of the histogram at 120 h after culture. The unlabeled cells including CD4⁺CD25⁺ T_R cells and contaminated DC are seen at the left part of the histogram. Data are represented from four independent experiments.

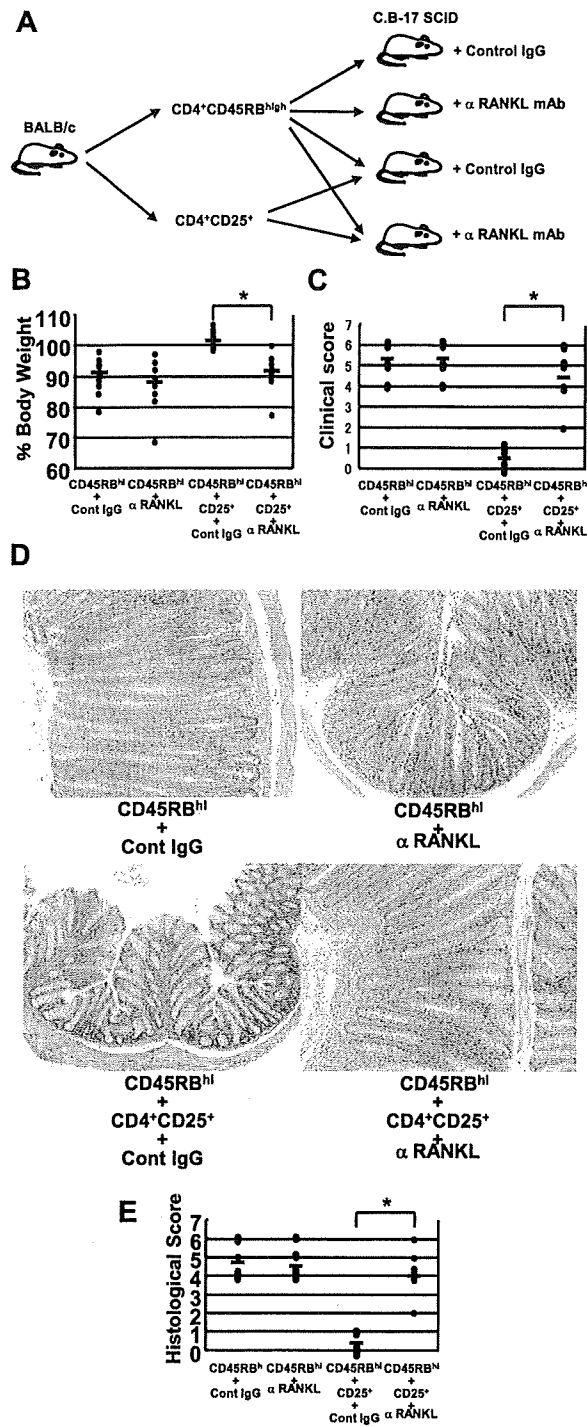


FIGURE 3. Anti-RANKL mAb abrogates CD4⁺CD25⁺ T_R cell-mediated suppression of colitis in vivo. *A*, C.B-17 SCID mice were injected i.p. with CD4⁺CD45RB^{high} (3×10^5 /mouse) alone or CD4⁺CD45RB^{high} (3×10^5) + CD4⁺CD25⁺ T cells (1×10^5) and treated with control IgG or anti-RANKL mAb by i.p. injection at a dose of 250 μg three times per week over 6 wk starting at the time of T cell transfer. *B*, Change in body weight over time is expressed as percentage of the original weight. Data are represented as the mean ± SEM of seven mice in each group. *, $p < 0.05$. *C*, Clinical scores were determined at 6 wk after transfer as described in *Materials and Methods*. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.05$. *D*, Histological examination of the colon at 6 wk after transfer. Original magnification, $\times 100$. *E*, Histological scores were determined at 6 wk after transfer as described in *Materials and Methods*. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.05$.

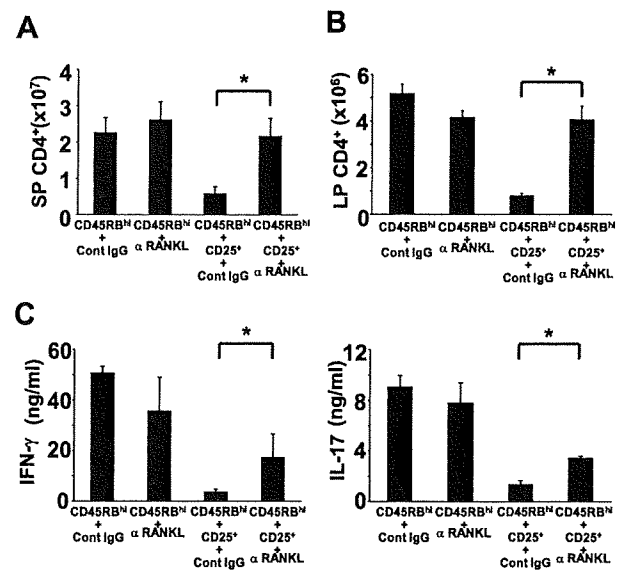
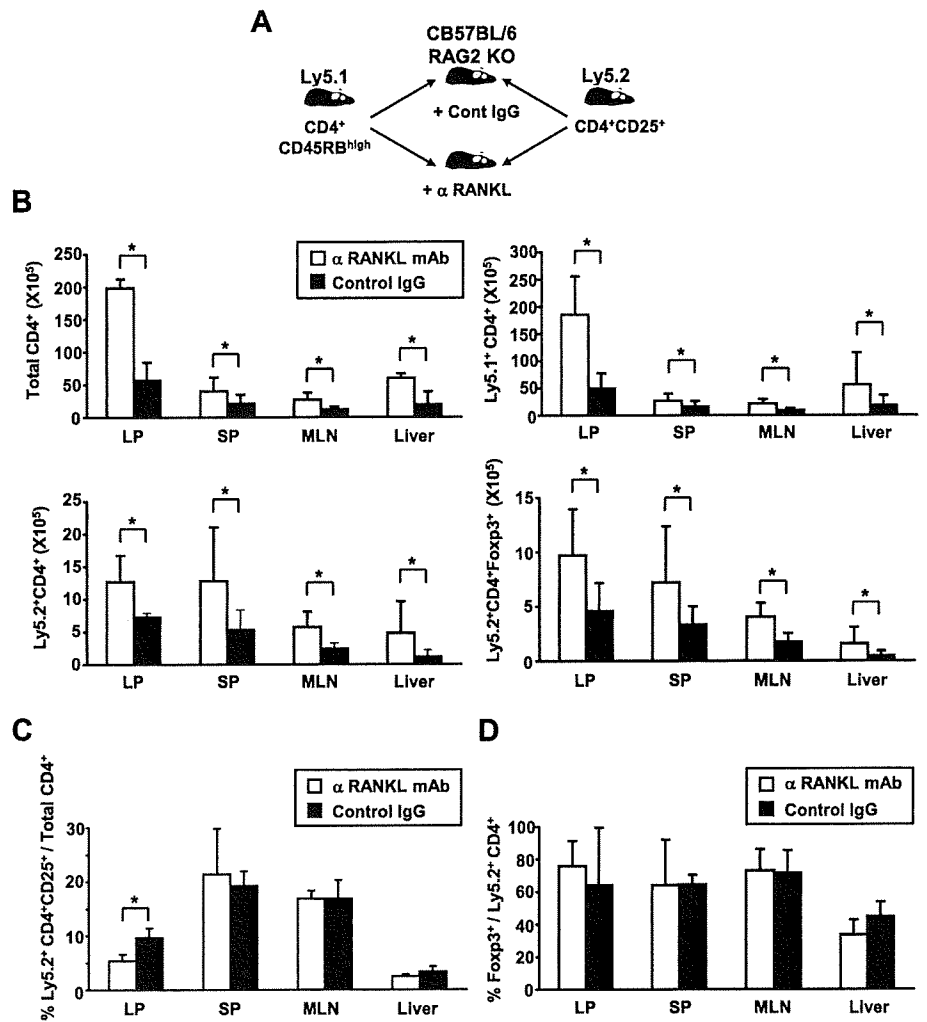


FIGURE 4. Anti-RANKL mAb abrogates CD4⁺CD25⁺ T_R cell-mediated suppression of Th1 and Th17 cells in vivo. *A*, SP and LP CD4⁺ T cells were isolated from the colon at 6 wk after adoptive T cell transfer and treated as described in Fig. 3, and the number of CD4⁺ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of seven mice in each group. *, $p < 0.01$. *C*, Cytokine production by CD4⁺ lamina propria lymphocyte. LP CD4⁺ cells were stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. IFN-γ and IL-17 concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean ± SEM of six mice in each group. *, $p < 0.01$.

mixture of CD4⁺CD45RB^{high} cells and CD4⁺CD25⁺ T cells (Fig. 3A). As a positive control, mice transferred with CD4⁺CD45RB^{high} T cells alone and treated with control rat IgG developed wasting disease, which became evident 3–5 wk after transfer (Fig. 3B), and clinical (Fig. 3C) and histological evidence of severe chronic colitis as estimated at 6 wk after transfer (Fig. 3, D and E). As a negative control, mice transferred with both CD4⁺CD45RB^{high} and CD4⁺CD25⁺ T_R cell populations and administered with the control rat IgG did not develop wasting disease or colitis (Fig. 3, A–E). Although we showed that activated CD4⁺CD25^{low}Foxp3⁻ effector T cells express RANKL (Fig. 1), the administration of anti-RANKL mAb to mice transferred with CD4⁺CD45RB^{high} cells alone did not affect the severity of colitis as compared with the control IgG-treated mice transferred with CD4⁺CD45RB^{high} cells alone (Fig. 3, A–E), suggesting that the RANK/RANKL pathway between RANKL-expressing activated effector CD4⁺ T cells and RANK-expressing APCs is not critically involved in the development of colitis. Surprisingly, however, the administration of anti-RANKL mAb did induce severe colitis in mice transferred with CD4⁺CD45RB^{high} cells and CD4⁺CD25⁺ T_R cell populations, indicating that the anti-RANKL mAb treatment abolished the CD4⁺CD25⁺ T_R cell-mediated in vivo suppression of colitis induced by adoptive transfer of CD4⁺CD45RB^{high} T cells.

A further quantitative evaluation of CD4⁺ T cell infiltration was made by isolating spleen cells and LP cells from the resected bowels. Significantly higher numbers of CD4⁺ T cells were recovered from the spleen (Fig. 4A) and LP (Fig. 4B) of the mice transferred with both CD4⁺CD45RB^{high} and CD4⁺CD25⁺ T_R cell populations and administered with anti-RANKL mAb as compared with control IgG, being comparable to those from mice transferred with CD4⁺CD45RB^{high} T cells alone and treated with anti-RANKL mAb or the control rat IgG. We also examined the cytokine production by CD4⁺

FIGURE 5. Blockade of the RANK/RANKL pathway induces the dysregulated cell balance between effector CD4⁺ T cell and T_R cells in the inflamed mucosa of colitic mice. **A**, C57BL/6 RAG-2^{-/-} mice were injected i.p. with Ly5.1⁺CD4⁺CD45RB^{high} (3 × 10⁵/mouse) alone or Ly5.1⁺CD4⁺CD45RB^{high} (3 × 10⁵) + Ly5.2⁺CD4⁺CD25⁺ T cells (1 × 10⁵) and treated with control IgG or anti-RANKL mAb by i.p. injection at a dose of 250 μg three times per week over 6 wk starting at the time of transfer. **B**, Absolute number of total CD4⁺ T cells (Ly5.1⁺ + Ly5.2⁺ cells), Ly5.1⁺CD4⁺ T cells, Ly5.2⁺CD4⁺ T cells, Ly5.2⁺CD4⁺Foxp3⁺ T_R cells in LP, SP, MLN, and liver of control IgG- or anti-RANKL mAb-treated mice. Data are indicated as the mean ± SEM of 12 mice per group for LP, SP, and MLN and six mice per group for liver. *, *p* < 0.01. **C**, Ratio of CD4⁺CD25⁺ T_R (Ly5.2⁺) cells per total CD4⁺ T cells (Ly5.1⁺ + Ly5.2⁺) at 6 wk after transfer was analyzed by flow cytometry. Data are indicated as the mean ± SEM of six mice per group. *, *p* < 0.01. **D**, Ratio of Foxp3⁺ cells to total Ly5.2⁺CD4⁺ T cells at 6 wk after transfer was analyzed by flow cytometry. Data are indicated as the mean ± SEM of six mice per group. *, *p* < 0.01.



lamina propria lymphocyte from the four groups of mice. As shown in Fig. 4C, IFN- γ and IL-17 production by LP CD4⁺ T cells was markedly suppressed by cotransfer of CD4⁺CD25⁺ T_R cells with CD4⁺CD45RB^{high} T cells, but this suppression was partially but significantly abrogated by the treatment with anti-RANKL mAb to the level of LP CD4⁺ T cells from the mice transferred with CD4⁺CD45RB^{high} T cells alone and treated with anti-RANKL mAb or control IgG.

Balance between effector CD4⁺ T cells and CD4⁺CD25⁺ T_R cells in the inflamed mucosa was dysregulated by treatment with anti-RANKL mAb

To further assess the balance in cell numbers between effector CD4⁺ T cells and CD4⁺CD25⁺ T_R cells in recipient SCID mice, we transferred Ly5.1⁺CD4⁺CD45RB^{high} cells and Ly5.2⁺CD4⁺CD25⁺ T_R cells into C57BL/6 RAG-2^{-/-} mice to distinguish between effector CD4⁺ T cells and T_R cells (Fig. 5A). We first confirmed that C57BL/6 RAG-2^{-/-} mice transferred with CD4⁺CD45RB^{high} T cells and CD4⁺CD25⁺ T_R cells did develop colitis with the marked expansion of CD4⁺ T cells when treated with anti-RANKL mAb, but not with control IgG (data not shown). As expected, the absolute number of total CD4⁺ T cells (Ly5.1⁺ plus Ly5.2⁺ cells) and Ly5.1⁺ effector CD4⁺ T cells in LP of anti-RANKL mAb-treated mice was significantly increased due to the presence of colitis as compared with that in control IgG-treated mice with no colitis (Fig. 5B). Also, the abso-

lute number of Ly5.2⁺ T cells and Ly5.2⁺CD4⁺Foxp3⁺ T_R cells in LP of colitic anti-RANKL mAb-treated mice was significantly higher than that in non-colitic control IgG-treated mice (Fig. 5B), suggesting that both effector CD4⁺ T cells and T_R cells extensively proliferated in LP of anti-RANKL mAb-treated colitic mice. Interestingly, however, the ratio of Ly5.2⁺CD4⁺CD25⁺ T_R cells to total CD4⁺ T cells in the recipient mice treated with anti-RANKL mAb was markedly decreased in the intestine as compared with that in the recipient mice treated with control IgG, although there were no significant differences in the spleen, MLN, or liver between the two groups (Fig. 5C). These results suggested that the blockade of the RANK/RANKL signaling pathway affected the expansion and/or migration of CD4⁺CD25⁺ T_R cells, resulting in dysregulated cell balance between effectors and T_R cells in the inflamed mucosa.

Blockade of the RANK/RANKL pathway does not affect migration of CD4⁺CD25⁺ T_R cells to the inflamed mucosa, but suppresses their expansion in the inflamed mucosa

To assess why blockade of the RANK/RANKL pathway abolished T_R function in mice transferred with CD4⁺CD45RB^{high} T cells and CD4⁺CD25⁺ T_R cells, we conducted several in vitro and in vivo experiments. First, to assess the possibility that blockade of the RANK/RANKL pathway skews the expression of gut-homing receptors on T_R cells, splenic CD4⁺ T cells were cultured for 72 h in the presence or absence of anti-RANKL mAb with a stimulating

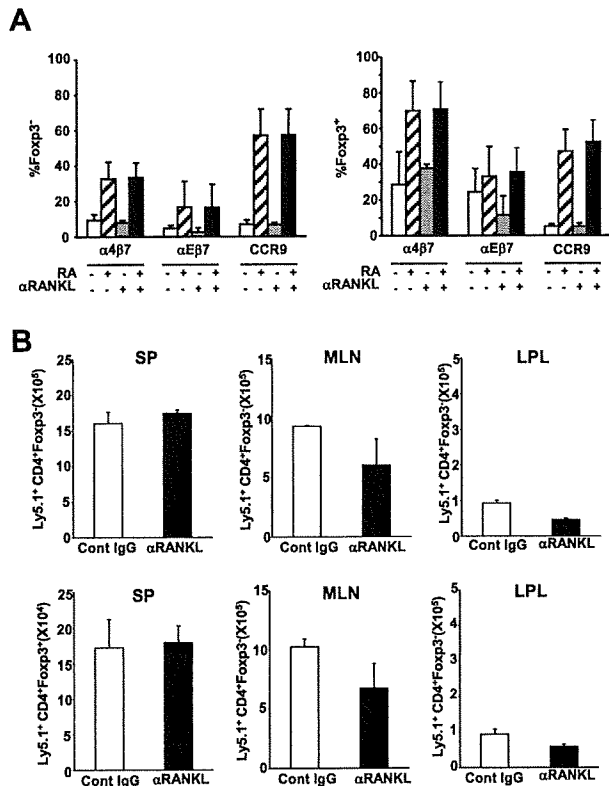


FIGURE 6. Blockade of the RANK/RANKL pathway does not affect the migration of CD4⁺CD25⁺ T_R cells to the inflamed mucosa of colitic mice. *A*, CD4⁺ T cells (2×10^5) purified from normal BALB/c mice were cultured with purified SP CD11c⁺ DC (2×10^3) from colitic mice in addition to soluble 1 μ g/ml anti-CD3 mAb, 10 ng/ml human rTGF- β , all-*trans* RA, and 5 ng/ml rIL-2 with or without 1 μ g/ml anti-RANKL mAb. On day 4, cells were stained with PerCP-conjugated anti-CD4 mAb and PE-conjugated anti-integrin $\alpha_4\beta_7$, PE-conjugated anti-integrin $\alpha_E\beta_7$, or PE-conjugated anti-CCR9 mAb, followed by intracellular staining by allophycocyanin-conjugated Foxp3 mAb. Data are indicated as the mean \pm SEM of six samples per group. *, $p < 0.01$. *B*, Anti-RANKL mAb treatment does not affect the migration of CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ T cells in vivo. To assess the effect of anti-RANKL mAb on the trafficking of T_R cells to inflamed mucosa of colitic mice, RAG-2^{-/-} mice were transferred with CD4⁺CD45RB^{high} T cells and, 4 wk after transfer, they were treated with 250 μ g of anti-RANKL mAb or control IgG two times in 1 day (or on 2 consecutive days?). They were then transferred with splenic Ly5.1⁺CD4⁺ T cells from normal mice and, 24 h after the second transfer, the cell number of Ly5.1⁺CD4⁺Foxp3⁺ (lower) or Foxp3⁻ (upper) T cells recovered from LP, SP, and MLN was evaluated by flow cytometry.

mixture including anti-CD3mAb, soluble IL-2, TGF- β , and RA, which is known to induce the gut-homing receptors (integrin $\alpha_4\beta_7$, $\alpha_E\beta_7$, and CCR9) (23). As expected, the mixture strongly induced integrin $\alpha_4\beta_7$, $\alpha_E\beta_7$, and CCR9, but the addition of anti-RANKL mAb did not affect the expression of these molecules (Fig. 6A).

To further assess the possibility that blockade of the RANK/RANKL pathway affected the T_R cell migration to the inflamed mucosa, we performed an additional in vivo adoptive transfer experiment, since it has been reported that RANK is expressed on endothelial cells (24). To this end, RAG-2^{-/-} mice were transferred with Ly5.2⁺CD4⁺CD45RB^{high} T cells and 4 wk after the transfer they were treated with control IgG or anti-RANKL mAb twice in 1 day. On the following day, they were transferred with splenic CD4⁺ T cells obtained from normal C57BL/6-Ly5.1 mice (Fig. 6B). One day after the second transfer, the number of Ly5.1⁺CD4⁺Foxp3⁺ or

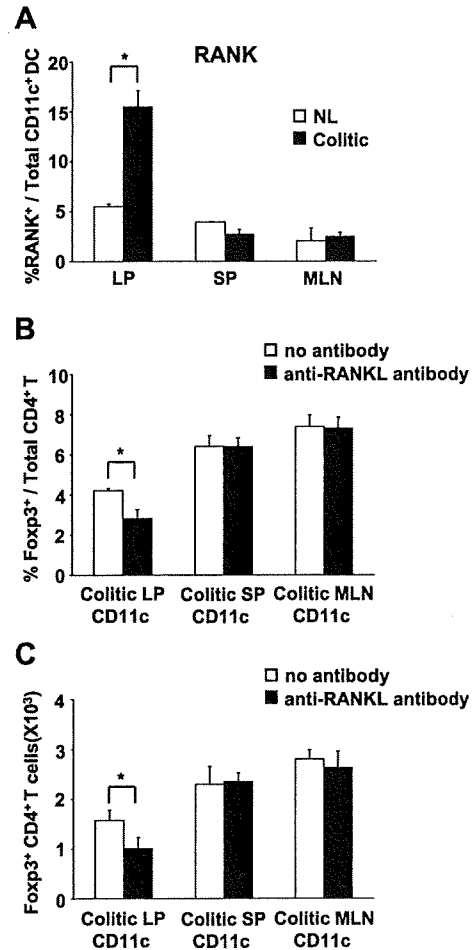


FIGURE 7. Blockade of the RANK/RANKL pathway suppresses the expansion of CD4⁺CD25⁺ T_R cells in the inflamed mucosa. *A*, Expression of RANK on LP, SP, or MLN DC cells obtained from colitic (■) or normal (□) mice. *B* and *C*, Splenic CD4⁺ T cells from normal mice were cultured with colitic LP, MLN, or SP CD11c⁺ DC in the presence of anti-CD3 mAb with control IgG (□) or anti-RANKL mAb (■) for 72 h, and the ratio of CD4⁺Foxp3⁺ T_R cells per total CD4⁺ T cells (*B*) and the number of CD4⁺Foxp3⁺ T_R cells (*C*) recovered from culture with colitic LP, MLN, or SP CD11c⁺ DC in the presence or absence of anti-RANKL mAb were evaluated by flow cytometry. Data are indicated as the mean \pm SEM of seven samples per group. *, $p < 0.05$.

Ly5.1⁺CD4⁺Foxp3⁻ T cells in SP, MLN, and LP was found not to be modified by anti-RANKL-treatment at all (Fig. 6B).

To finally assess the possibility that colitic LP DC cells modulate the expansion of T_R cells in the inflamed mucosa in a RANK/RANKL-dependent manner, we evaluated the expression of RANK on CD11c⁺ DC cells obtained from the spleen, MLN, and LP of colitic and normal mice. As shown in Fig. 7A, the expression of RANK on colitic LP DC was significantly increased as compared with that on normal LP DC. In contrast, the expression of RANK in SP and MLN was similar in normal and colitic mice. Given the up-regulated expression of RANK on colitic LP DC cells, we next assessed the possibility that the RANK/RANKL pathway is involved in the expansion of T_R cells. To this end, splenic CD4⁺ T cells obtained from normal mice were cultured with colitic LP, MLN, or SP CD11c⁺ DC in the presence of anti-CD3 mAb with or without anti-RANKL mAb for 72 h. The ratio of CD4⁺Foxp3⁺ T_R cells per the total of CD4⁺ T cells (Fig. 7B) and the number of CD4⁺Foxp3⁺ T_R cells (Fig. 7C) recovered from culture with colitic LP, but not MLN

or SP, CD11c⁺ DC in the presence of anti-RANKL mAb was significantly decreased as compared with that in the presence of control IgG, suggesting that the RANK/RANKL pathway is critically involved in the expansion of LP T_R cells through the direct interaction with colitic RANK-expressing LP DC and T_R cells.

Discussion

In the present study, we demonstrated that 1) CD4⁺CD25⁺ T cells including CD4⁺CD25^{high} T_R cells and activated CD4⁺CD25^{low} effector cells rather than CD4⁺CD25⁻ T cells preferentially express the RANKL molecule and 2) blockade of the RANK/RANKL signaling pathway suppresses the expansion of CD4⁺CD25⁺ T_R cells and subsequently abolishes the T_R cell-mediated suppression of colitis due to dysregulation of the cell balance between effector CD4⁺ T cells and T_R cells in the inflamed intestine. Interestingly, although activated effector CD4⁺ T cells and inducible CD4⁺CD25⁺ T_R cells also express RANKL molecules in SCID mice transferred with CD4⁺CD45RB^{high} T cells alone, the administration of this mAb did not affect the course of colitis. Collectively, these findings indicate that the RANK-RANKL signaling pathway is critically involved in intestinal mucosal tolerance by controlling the expansion and function of CD4⁺CD25⁺ T_R cells in the inflamed mucosa.

Although many previous reports have established the role of the RANK/RANKL signaling pathway in osteoclastogenesis and bone loss in various chronic T cell-mediated inflammatory diseases including IBDs (20, 25–29), the role of this pathway in the local inflammation in various models remains unknown. For example, Kong et al. (26) initially reported that the blockade of this pathway by using soluble recombinant osteoprotegerin (OPG) protein at the onset of disease prevented bone and cartilage destruction, but interestingly not inflammation in a T cell-dependent model of rat adjuvant arthritis. In contrast, Ashcroft et al. (30) demonstrated that the administration of RANK-Fc protein not only reverses the bone loss in IL-2^{-/-} mice, which is a spontaneous model of osteoporosis and colitis, but also reduces the development of colitis by blocking the interaction between RANK-expressing DC and RANKL-expressing activated CD4⁺ T cells in the inflamed mucosa of the colon. In our colitis model induced by adoptive transfer of CD4⁺CD45RB^{high} T cells into SCID mice, however, administration of neutralizing anti-RANKL mAb did not prevent the development of colitis, although inducible CD4⁺CD25^{high} T_R cells and previously activated CD4⁺CD25^{low} T cells expressed RANKL. Consistent with this finding, Byrne et al. (31) previously demonstrated that administration of human osteoprotegerin-Fc increased bone density in this model, but had no effects on the intestinal inflammation. Several explanations have been advanced for the discrepancy, including differences in the species, the type of animal model, the type of blocking agents, and dosing regimens used. Furthermore, it has recently been demonstrated that in vivo administration of neutralizing anti-cytokine mAbs, such as anti-IL-2 mAb, enhances the corresponding cytokine activity due to the formation of cytokine/anti-cytokine mAb complexes, which are more stable and stimulatory (32). Although we previously demonstrated that our anti-RANKL mAb used in vivo successfully worked as a blocking mAb in a model of collagen-induced arthritis (21), further studies will be needed to address this issue.

Although we could not detect a suppressive effect of neutralizing anti-RANKL mAb on the development of colitis in SCID mice transferred with CD4⁺CD45RB^{high} T cells alone, we found that this treatment induced colitis in mice transferred with CD4⁺CD45RB^{high} T cells and CD4⁺CD25⁺ T_R cells at a ratio of 3:1, while mice transferred with CD4⁺CD45RB^{high} T cells and CD4⁺CD25⁺ T_R cells at the same ratio and given control IgG did not

develop colitis. This strongly suggested that the target cells for anti-RANKL mAb are CD4⁺CD25⁺ T_R cells rather than CD4⁺CD45RB^{high} T cells or the differentiated effector CD4⁺ T cells. Consistent with this notion, we found that RANKL was expressed on CD4⁺CD25^{high} T_R cells, but not on CD4⁺CD25⁻ cells (Fig. 1). In an in vitro coculture assay to further evaluate the role of RANKL on CD4⁺CD25⁺ T_R cells in modulating the T_R activity of CD4⁺CD25^{high} cells in vitro, however, the addition of anti-RANKL mAb or anti-RANK mAb produced no detectable reduction of T_R activity, suggesting that the direct interaction between RANKL-expressing CD4⁺CD25⁺ T_R cells or activated CD4⁺ T cells and RANK-expressing APCs including DC is not essentially important for abolishing T_R activity at least in vitro. Since in vitro assays do not always reflect the T_R function in vivo, we next performed another adoptive transfer experiment using Ly5.1⁺CD4⁺CD45RB^{high} T cells and Ly5.2⁺CD4⁺CD25⁺ T_R cells to evaluate the possibility that blockade of the RANK/RANKL signaling pathway affects the recruitment and expansion of specific populations in our model. In this experiment, we found that the ratio of the Ly5.2-derived CD4⁺CD25⁺ T_R cell population per total CD4⁺ T cells in anti-RANKL mAb-treated mice was significantly decreased in the LP, but not in the MLN, spleen, or liver, as compared with the ratio in control-IgG-treated mice. This finding suggests three points. First, it is possible that the migration of CD4⁺CD25⁺ T_R cells to the inflamed mucosa is regulated by the interaction between RANKL-expressing T_R cells and possibly RANK-expressing endothelial cells. Consistent with this hypothesis, it has been reported that RANK is expressed on murine and human endothelial cells (24). However, this is unlikely because our short-term in vivo adoptive transfer experiment (Fig. 7) demonstrated that treatment of anti-RANKL mAb did not affect the recovered cell number of CD4⁺CD25⁺ T_R cells in the inflamed mucosa. Second, it is possible that the in vivo expansion of RANKL-expressing CD4⁺CD25⁺ T_R cells or colitogenic CD4⁺ effector/memory T cells is modulated by RANK-expressing DC in the inflamed mucosa. Consistent with this hypothesis, we found that 1) the expression of RANK on colitic LP DC is significantly increased as compared with that on normal LP DC and 2) the ratio of CD4⁺Foxp3⁺ T_R cells to total CD4⁺ T cells after stimulation with colitic LP, but not MLN or splenic, CD11c⁺ DC was significantly suppressed by the addition of anti-RANKL mAb. Thus, it is possible that the interaction of RANKL-expressing CD4⁺CD25⁺ T_R cells and RANK-expressing activated DC in the inflamed mucosa plays an important role in the maintenance of T_R cells. Third, not only the first suppression of T cell priming in draining lymph nodes by CD4⁺CD25⁺ T_R cells, but also the second line of suppression in the inflamed mucosa by these cells is critically involved in the intestinal homeostasis to suppress the development of colitis. Consistent with our finding, Green et al. (33) previously reported that the blockade of the RANK/RANKL pathway resulted in a decreased frequency of CD4⁺CD25⁺ T_R cells in the draining lymph nodes and pancreas in the NOD mouse (33). However, it remains unknown why the ratio of CD4⁺CD25⁺ T_R cells in MLNs was unchanged in our adoptive transfer model. The exact mechanism of the function of CD4⁺CD25⁺ T_R cells in the inflamed mucosa warrants further investigation.

Finally, the suppressive site of colitis should be discussed. Denning et al. (34) previously demonstrated that integrin β₇-deficient (β7^{-/-}) CD4⁺CD25⁺ T_R cells that preferentially migrate to MLNs, but are impaired in their ability to migrate to the intestine because of the lack of the gut-homing integrin α₄β₇ and α_Eβ₇ molecules, are capable of preventing intestinal inflammation, suggesting that T_R accumulation in the intestine is dispensable for the protection of this colitis model. In their protection protocol, indeed, it is possible

that $\beta_7^{+/+}$ CD4⁺CD25⁺ T_R cells are not needed to suppress the development of colitis, because $\beta_7^{-/-}$ CD4⁺CD25⁺ T_R cells directly migrate to MLNs and can inhibit naive CD4⁺CD45RB^{high} T cell activation and proliferation within Ag-draining MLNs, resulting in suppression of the development of the gut-seeking activated effector CD4⁺ T cells instructed to express the gut-homing receptors such as integrins $\alpha_4\beta_7$ and $\alpha_E\beta_7$. However, it remains unknown whether mucosal CD4⁺CD25⁺ T_R cells are necessary for the suppression of mucosal pathogenic effector CD4⁺ T cells ex vivo, especially in an ongoing colitis system in which it can be assessed whether LP CD4⁺CD25⁺ T_R cells as effector T_R cells can suppress the surrounding LP effector CD4⁺ T cells ex vivo.

In this regard, we have previously demonstrated that human CD4⁺CD25^{brigh} and mouse CD4⁺CD25⁺ T cells reside in the intestinal LP, express CTLA-4, GITR, and Foxp3 and possess T_R activity in vitro (11, 12). We also found that the clinical score in SCID mice transferred with CD4⁺CD45RB^{high} T cells and intestinal LP CD4⁺CD25⁺ T cells at a ratio of 3:1 was significantly decreased as compared with that in SCID mice transferred with CD4⁺CD45RB^{high} T cells alone (12), indicating that the murine intestinal LP CD4⁺CD25⁺ T cells maintain intestinal homeostasis to suppress the development of colitis. Having evidence that the murine intestinal LP CD4⁺CD25⁺ T cells suppressed the development of colitis induced by the adoptive transfer of CD4⁺CD45RB^{high} T cells, we further asked whether MLNs are fully essential for the suppression of colitis by splenic CD4⁺CD25⁺ T cells. As a second approach to this issue, we also found that the cotransfer of splenic CD4⁺CD25⁺ T_R cells prevented the development of colitis in the lymph node-null $LT\alpha^{-/-}$ × $RAG-2^{-/-}$ mice transferred with CD4⁺CD45RB^{high} T cells, indicating that splenic CD4⁺CD25⁺ T cells can suppress the development of colitis in the absence of MLNs (12). Moreover, we demonstrated that CD4⁺CD25⁺ T_R cells actually migrated and resided in the colon in $LT\alpha^{-/-}$ × $RAG-2^{-/-}$ mice cotransferred with Ly5.2-derived CD4⁺CD45RB^{high} T cells and Ly5.1-derived splenic CD4⁺CD25⁺ T cells, suggesting that the LP might be a regulatory site between colitogenic effector/memory cells and T_R cells to suppress intestinal inflammation, probably as a second line of suppression (yy). Along with the present findings that the RANK/RANKL interaction is critically involved in the function of CD4⁺CD25⁺ T_R cells in the intestine, our research suggests that therapeutic approaches enhancing the migration of CD4⁺CD25⁺ T_R cells, such as the specific induction of RANKL on CD4⁺CD25⁺ T_R cells, may be feasible in the treatment of IBD.

Disclosures

The authors have no financial conflict of interest.

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MFG-E8 Attenuates Intestinal Inflammation in Murine Experimental Colitis by Modulating Osteopontin-Dependent $\alpha_v\beta_3$ Integrin Signaling¹

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MFG-E8 (milk fat globule-epidermal growth factor 8) deficiency is strongly associated with acquisition of immune-mediated disorders due to the loss of tissue homeostasis. However, comparatively little is known regarding its functions in gastrointestinal tract disorders, in which immune homeostasis is a major concern. Herein, we report altered MFG-E8 expression in inflamed colons during the acute phase of murine experimental colitis and found that treatment with recombinant MFG-E8, but not its arginine-glycine-aspartate mutant counterpart, ameliorated colitis by reducing inflammation and improving disease parameters. To reveal the MFG-E8-mediated anti-inflammatory mechanism, we employed an *in vitro* system, which showed the down-regulation of NF- κ B in an LPS-dependent manner. Additionally, MFG-E8 altered $\alpha_v\beta_3$ integrin-mediated focal adhesion kinase phosphorylation by impeding the binding of one of its potent ligands osteopontin, which becomes activated during colitis. Taken together, our results indicated that MFG-E8 has a novel therapeutic potential for treatment of colitis. *The Journal of Immunology*, 2009, 182: 7222–7232.

Ulcerative colitis (UC)³ and Crohn's disease (CD) are two major forms of inflammatory bowel disease (IBD), which are characterized by an elevated production of inflammatory mediators to induce inflammation, as well as tissue injury as a result of the migration and infiltration of innate immune cells (1, 2). Activated proinflammatory genes in the intestinal mucosa are in turn under the control of NF- κ B, which becomes up-regulated and induces a variety of inflammatory events during colitis (3, 4). Active intestinal inflammation is also accompanied by the up-regulation of certain integrins, including $\alpha_v\beta_3$ and $\alpha_v\beta_5$, while blockade of the murine angiogenic endothelial marker $\alpha_v\beta_3$ effectively decreased both neoangiogenesis and inflammation in a murine model of colitis (5–7). Considering these inflammatory consequences, several conventional therapeutic approaches for IBD, mainly based on suppression and control of inflammation, have been proposed (8–10). Also, recently developed novel cytokine antagonist therapies have been found to be effective in

certain IBD patients (11–13). Such molecular targeted inhibition of the inflammatory process may provide better therapeutic options for IBD, and various studies have been conducted to evaluate new innovative approaches.

Milk fat globule-epidermal growth factor 8 (MFG-E8), a glycoprotein secreted from different cell types, participates in phagocytosis of apoptotic cells by forming a link between phosphatidylserine on apoptotic cells and $\alpha_v\beta_3$ integrin on phagocytes (14–17). Several lines of evidence show that severe inflammatory and autoimmune consequences in MFG-E8-null mice are due to the infiltration of apoptotic cells, which subsequently causes dysregulated immune functions with abnormal homeostasis (18, 19). An MFG-E8-mediated potential therapeutic benefit is evident in sepsis or GM-CSF-deficient conditions, as it ameliorates the detrimental effects of accumulated apoptotic cells in organ systems (20, 21). In addition to this familiar scavenging function, MFG-E8 was also found to be effective in accelerating mucosal healing during intestinal injury in a phosphatidylserine-dependent manner (22). Although MFG-E8 is involved in several cell surface-mediated regulatory events and modulates immune responses in numerous conditions, the mechanism underlying these features has not been clearly defined. Furthermore, its expression and role in intestinal homeostasis are not fully elucidated.

The aim of the present study was to analyze the expression of MFG-E8 in normal and inflamed intestinal mucosa, and to investigate whether it has a protective role in colitis. We decided to use recombinant MFG-E8 in a model of acute phase of colitis and elucidate its effects by evaluating colitis parameters. Our findings indicate that MFG-E8-mediated novel antiinflammatory effects are generated by NF- κ B inhibition through the modulation of $\alpha_v\beta_3$ integrin signaling.

Materials and Methods

Reagents and Abs

Dextran sodium sulfate (DSS, 5 kDa; Wako Pure Chemicals), Lipofectamine 2000 (Invitrogen), Ni-NTA columns (Qiagen), ultrapure

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³ Abbreviations used in this paper: UC, ulcerative colitis; CD, Crohn's disease; IBD, inflammatory bowel disease; MFG-E8, milk fat globule-epidermal growth factor 8; DSS, dextran sodium sulfate; OPN, osteopontin; MPO, myeloperoxidase; FAK, focal adhesion kinase; RGD, arginine-glycine-aspartate; siRNA, small interfering RNA; EIA, enzyme immune assay; ECM, extracellular matrix protein; VN, vitronectin; FN, fibronectin.

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