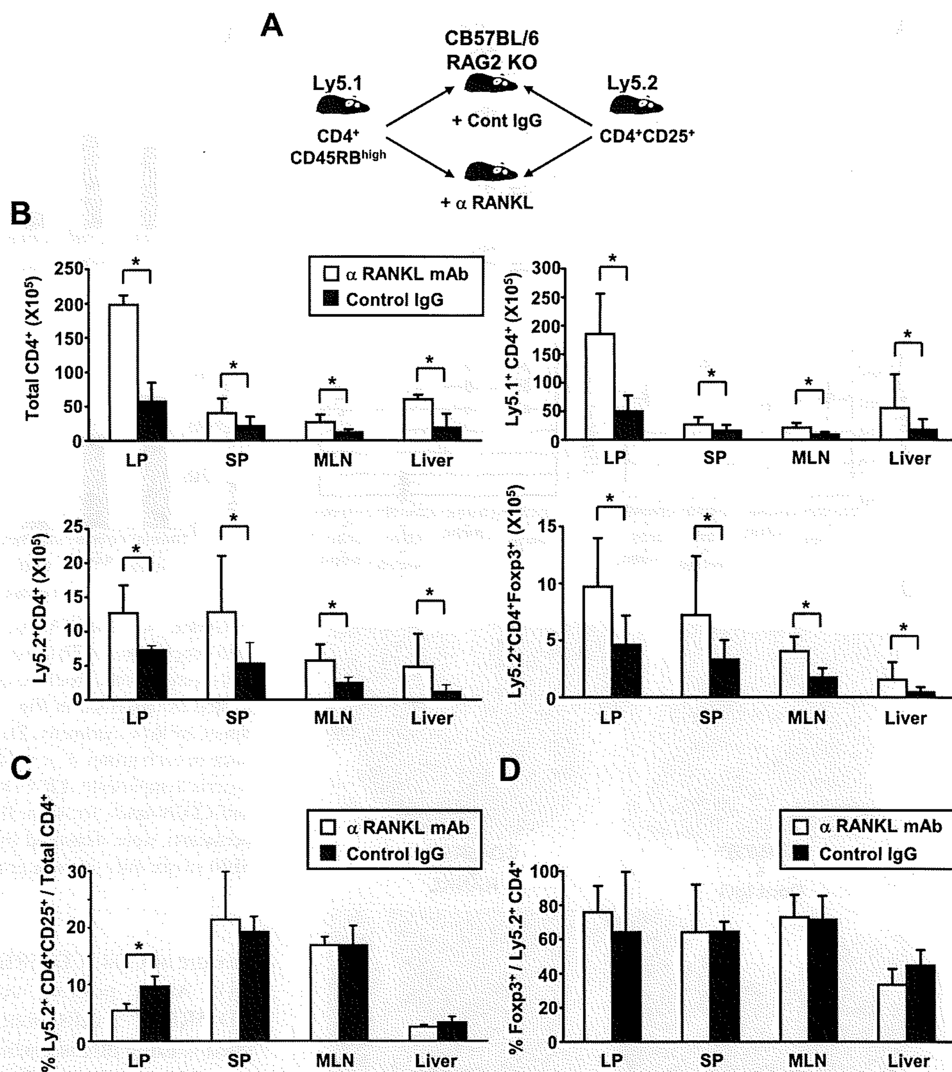

FIGURE 5. Blockade of the RANK/RANKL pathway induces the dysregulated cell balance between effector $CD4^+$ T cell and T_R cells in the inflamed mucosa of colitic mice. *A*, C57BL/6 RAG-$2^{-/-}$ mice were injected i.p. with $Ly5.1^+CD4^+CD45RB^{high}$ (3×10^5/mouse) alone or $Ly5.1^+CD4^+CD45RB^{high}$ (3×10^5) + $Ly5.2^+CD4^+CD25^+$ 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 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. 4*C*, 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. 5*A*). 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. 5*B*). Also, the absolute 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. 5*B*), 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. 5*C*). 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

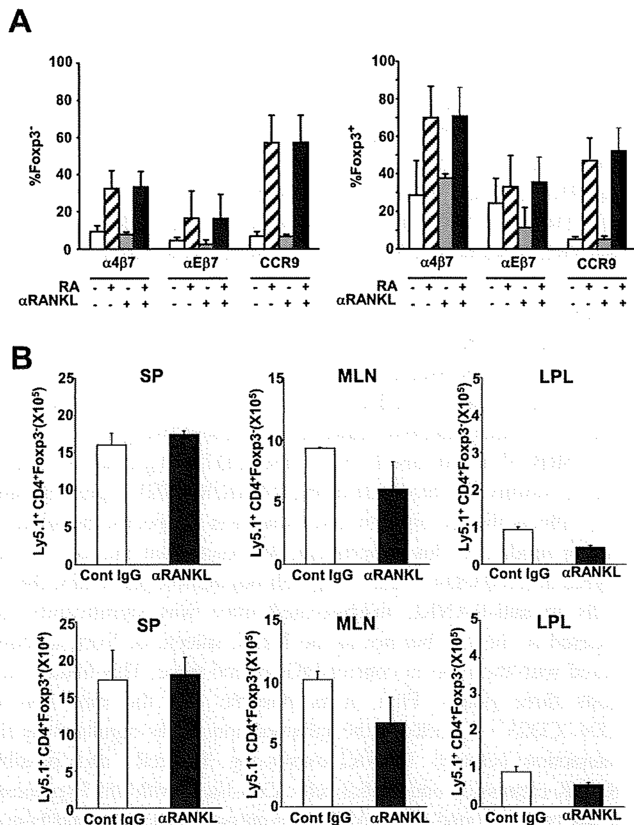

FIGURE 6. Blockade of the RANK/RANKL pathway does not affect the migration of $CD4^+CD25^+$ T_R cells to the inflamed mucosa of colitic mice. *A*, $CD4^+$ T cells (2×10^5) purified from normal BALB/c mice were cultured with purified SP $CD11c^+$ DC (2×10^3) from colitic mice in addition to soluble 1 μg/ml anti-CD3 mAb, 10 ng/ml human rTGF-β, all-*trans* RA, and 5 ng/ml rIL-2 with or without 1 μg/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 ± 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 μg 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.

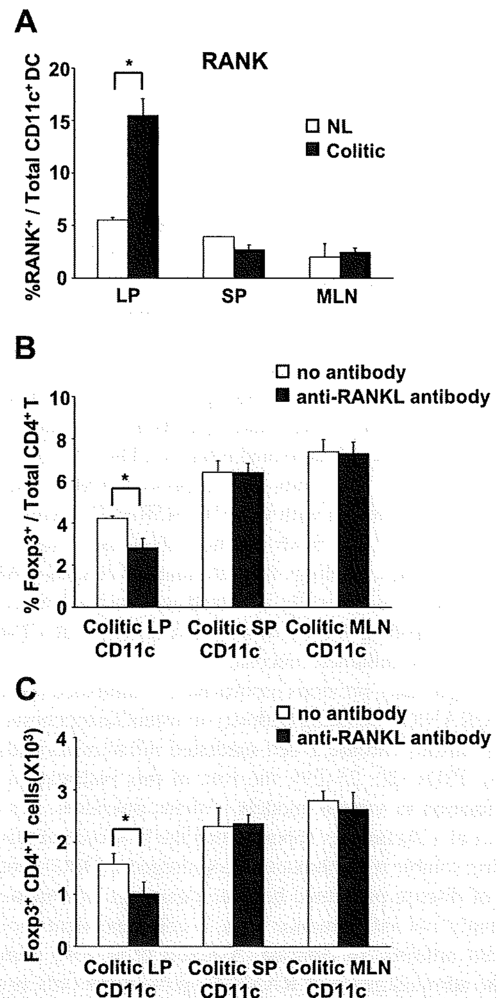

FIGURE 7. Blockade of the RANK/RANKL pathway suppresses the expansion of $CD4^+CD25^+$ T_R cells in the inflamed mucosa. *A*, Expression of RANK on LP, SP, or MLN DC cells obtained from colitic (□) or normal (■) mice. *B* and *C*, Splenic $CD4^+$ T cells from normal mice were cultured with colitic LP, 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 ± SEM of seven samples per group. *, $p < 0.05$.

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. 6*A*).

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. 6*B*). One day after the second transfer, the number of $Ly5.1^+CD4^+Foxp3^+$ or $Ly5.1^+CD4^+Foxp3^-$ T cells in SP, MLN, and LP was found not to be modified by anti-RANKL-treatment at all (Fig. 6*B*).

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. 7*A*, 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 upregulated 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. 7*B*) and the number of $CD4^+Foxp3^+$ T_R cells (Fig. 7*C*) 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 β_7-deficient ($\beta 7^{-/-}$) 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 $\alpha_4\beta_7$ and $\alpha_E\beta_7$ 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^{bright} 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$\alpha^{-/-}$ $\times$ 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$\alpha^{-/-}$ $\times$ 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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Long-Lived Colitogenic CD4+ Memory T Cells Residing Outside the Intestine Participate in the Perpetuation of Chronic Colitis[1]

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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 $CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ 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.**

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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-7Rα (A7R34; eBioscience) and anti-CCR7 (EBI-1; eBioscience) were obtained from BD Pharmingen and used for purification of cell populations and flow cytometric analysis: Fcγ (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→GF group, n = 8), and the other was moved into SPF conditions (GF→SPF group, n = 8). All groups (SPF, GF→GF, and GF→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 μm) 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 μM 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-7Rα mAb to GF SCID mice

Anti-IL-7Rα mAb (A7R34) was described previously (14). GF SCID mice were treated with rat anti-murine IL-7Rα 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 μl of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar) precoated with 5 μg/ml hamster anti-mouse CD3ε mAb (145-2C11; BD Pharmingen) and 2 μg/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 μm 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 ± SEM. Groups of data were compared by the Mann-Whitney U test. Differences were considered to be statistically significant when $p < 0.05$.

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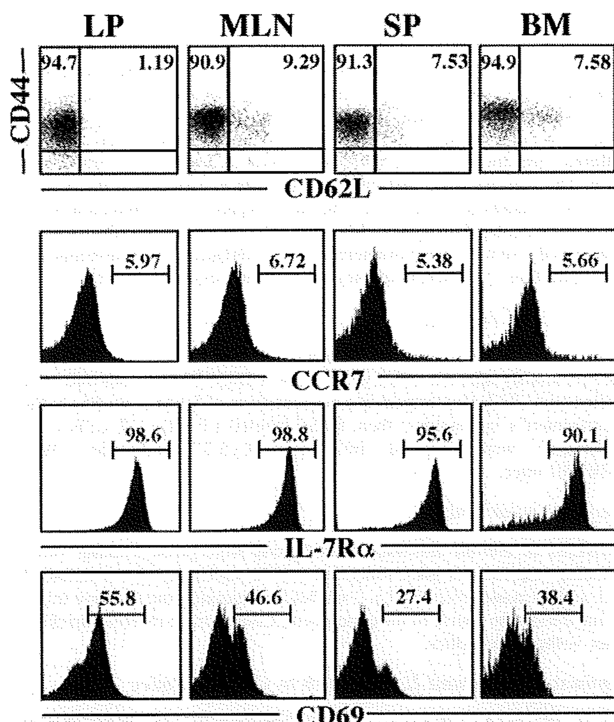

FIGURE 1. Colitic SP $CD4^+$ donor T cells are $CD44^{high}CD62L^-CCR7^-IL\text{-}7R\alpha^{high}$ T_{EM} cells. Expression of CD44, CD62L, IL-7Rα (CD127), and CD69 on $CD4^+$ T cells obtained from SP, MLN, LP, and BM in colitic C3H-SCID mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells (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.

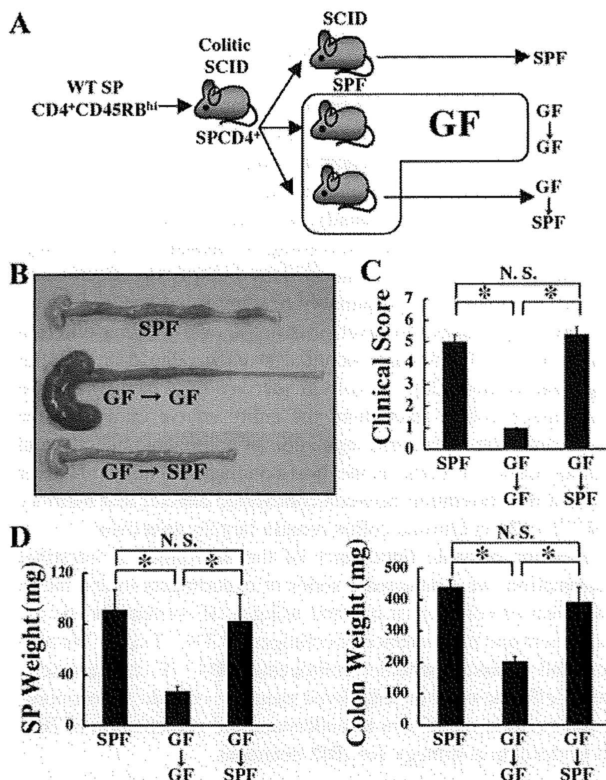

FIGURE 2. Commensal bacteria are not essential for survival of colitogenic memory $CD4^+$ T cells. *A*, C3H-SCID mice were injected i.p. with normal splenic $CD4^+CD45RB^{high}$ T cells. Six weeks after transfer, mice developed chronic colitis, and then colitic $CD4^+$ T cells were isolated from SP. 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 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 ± 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.

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 $CD44^{high}CD62L^-CCR7^-IL\text{-}7R\alpha^{high}$ T_{EM} cells (Fig. 1). CD69 was also expressed by a high 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 $IL\text{-}7^{+/+} \times RAG\text{-}1^{-/-}$ recipients, $IL\text{-}7^{-/-} \times RAG\text{-}1^{-/-}$ 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

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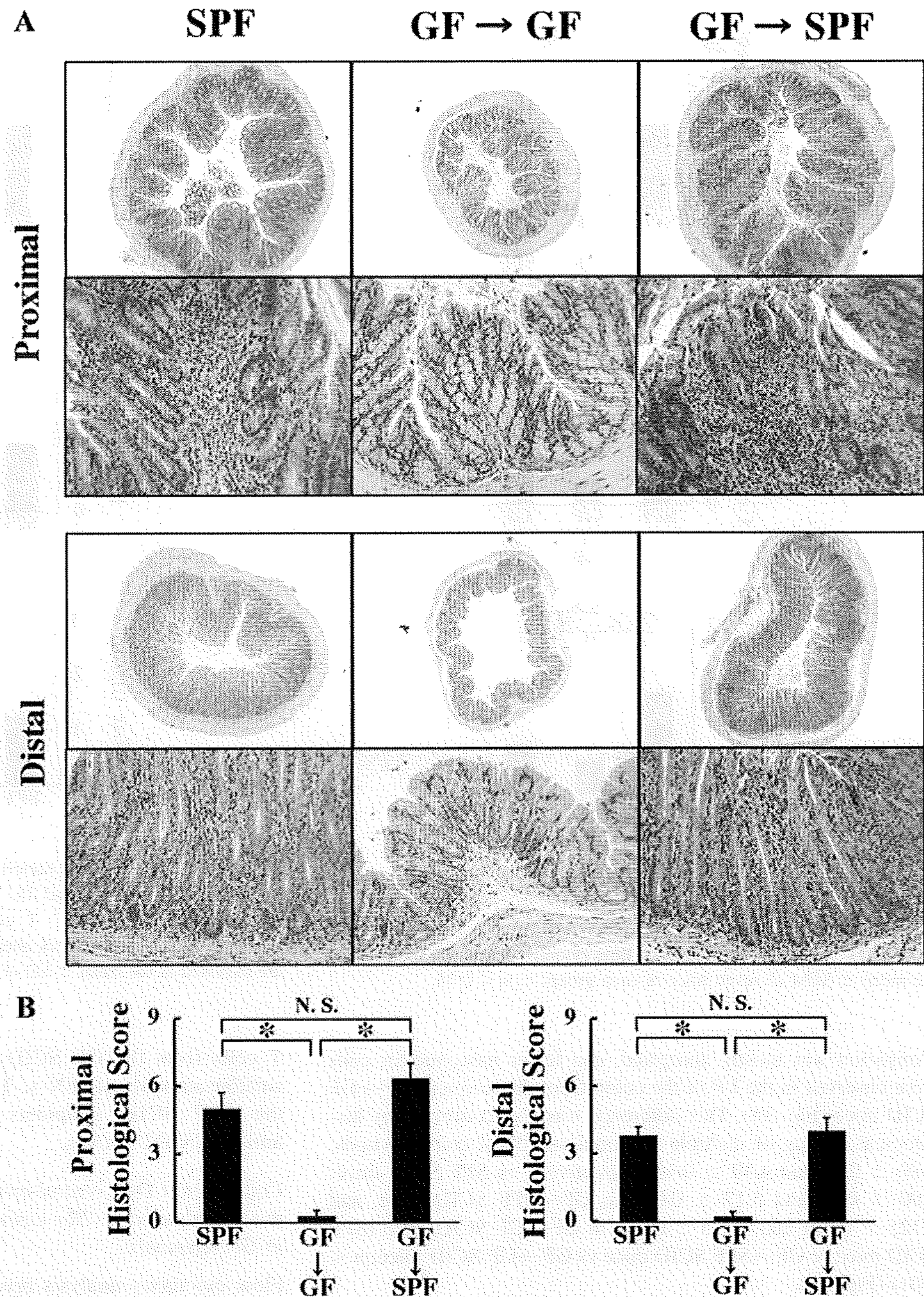

FIGURE 3. Commensal bacteria are needed to sustain the colitis. *A*, Proximal and distal colon from SPF, GF→GF, and GF→SPF SCID mice were stained with H&E. Original magnification: ×40 (*top*) and ×100 (*bottom*). *B*, Histological scores were determined at 10 wk after transfer as described in *Materials and Methods.* Data are indicated as the mean ± 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. 2*A*). 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. 2*B*) 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. 2*C*). 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. 2*B*). 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. 3*A*). In contrast, the glandular

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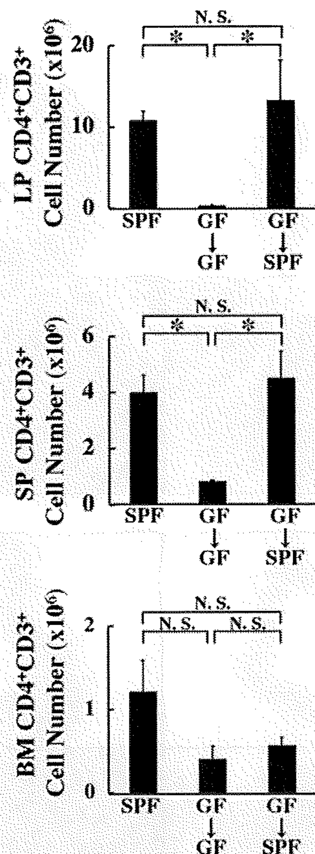

FIGURE 4. Commensal bacteria are needed for the expansion of $CD4^+$ T cells. LP, SP, and BM $CD4^+$ T cells were isolated from SPF, GF→GF, and GF→SPF SCID mice at 10 wk after retransfer, and the number of $CD3^+CD4^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of seven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

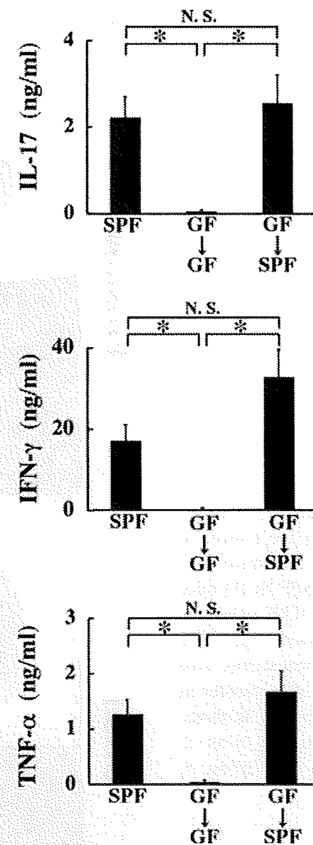

FIGURE 5. Commensal bacteria are needed for the expansion of Th1 and Th17 $CD4^+$ T cells. Th1 and Th17 cytokine production by anti-CD3/CD28 mAbs-stimulated LP $CD4^+$ T cells isolated from SPF, GF→GF, and GF→SPF SCID mice at 10 wk after retransfer was measured by ELISA. Data are indicated as the mean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

elongation was mostly abrogated, and fewer mononuclear cells were observed in the LP of the colon from the recipient GF→GF SCID mice (Fig. 3*A*). 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. 3*B*).

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 that that recovered from noncolitic 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 $CD4^+$ 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 $CD44^{high}CD62L^-CD69^+IL\text{-}7R\alpha^{high}$ 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

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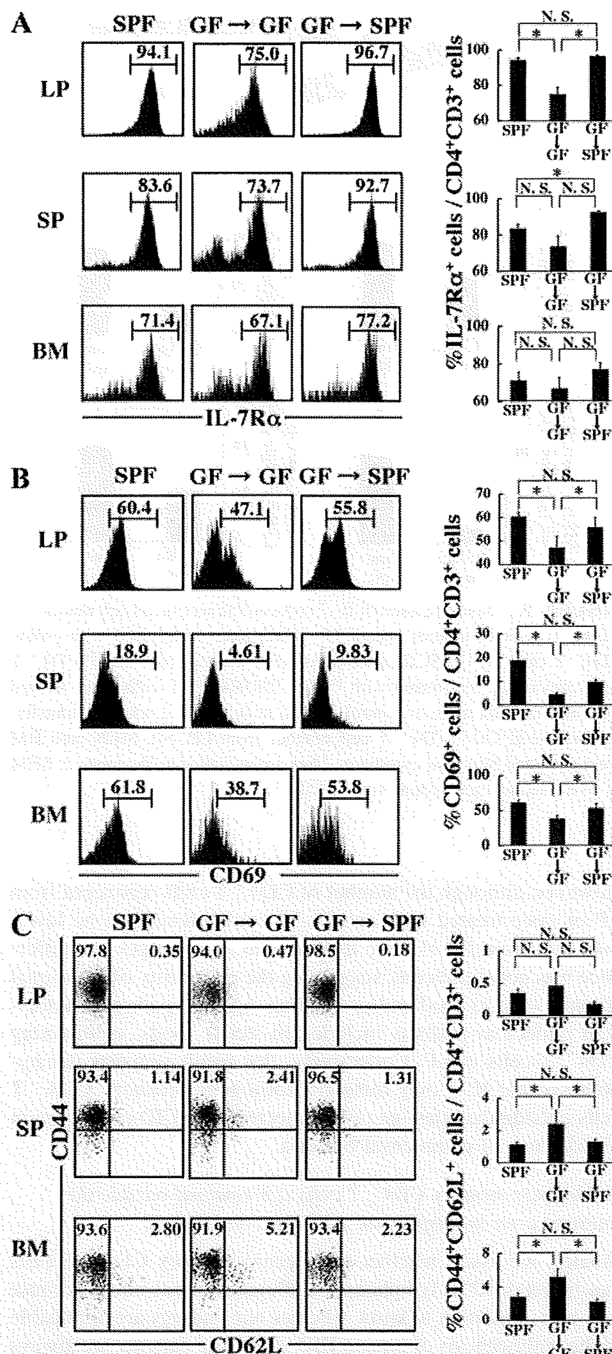

FIGURE 6. Colitogenic CD4^{+} T_{CM} cells with the down-modulated IL-7Rα expression were significantly increased in GF recipients. Expression of CD44 and CD62L (*C*), IL-7Rα (CD127) (*A*), and CD69 (*B*) on CD3^{+}CD4^{+} T cells obtained from LP, SP, and BM from SPF, GF→GF, and GF→SPF SCID mice at 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-7Rα, 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.

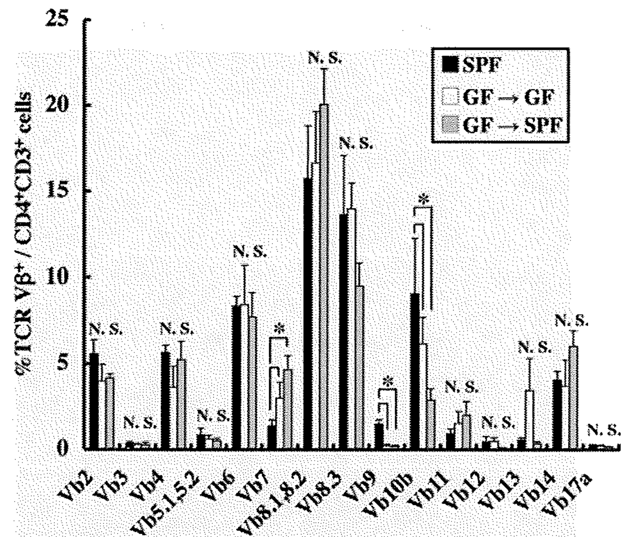

FIGURE 7. TCR Vβ repertoires show little difference in the presence or absence of commensal bacteria. Flow cytometric analysis of Vβ families on the surface of the splenic CD4^{+} T cells in SPF, GF→GF, and GF→SPF mice as described in Fig. 2. To analyze the TCR Vβ family repertoire, splenic cells were 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 Vβ mAbs. Each percentage value indicates the frequency of each Vβ ($n = 6$). Data are indicated as the mean ± SEM of five mice in each group. *, $p < 0.05$.

of SPF or GF→SPF SCID recipients (Fig. 6*A*). Second, and as expected, CD69 expression on cells from all sites was also reduced compared with the matching cell source from SPF or GF→SPF SCID recipients (Fig. 6*B*). 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→GF SCID recipients (SP 2.41 ± 0.85%; BM 5.21 ± 0.95%; LP 3.09 ± 0.95%) compared with SPF SCID recipients (SP 1.14 ± 0.1%, $p < 0.05$; BM 2.80 ± 0.48%, $p < 0.05$; LP 0.35 ± 0.059%, $p = 0.10$) and GF→SPF SCID recipients (SP 1.31 ± 0.17%, $p < 0.05$; BM 2.23 ± 0.33%, $p < 0.05$; LP 0.18 ± 0.040%, $p = 0.28$), suggesting the conversion of cells from T_{CM} to T_{EM} after movement of mice from GF→SPF (Fig. 6*C*).

TCR Vβ repertoires of CD4^{+} T cells do not change in the presence or absence of commensal bacteria

The TCR Vβ repertoires of SP CD4^{+} T cells from SPF, GF→GF, and GF→SPF SCID mice differed only slightly and only in such Vβ families as Vβ7, Vβ9, and Vβ11, 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

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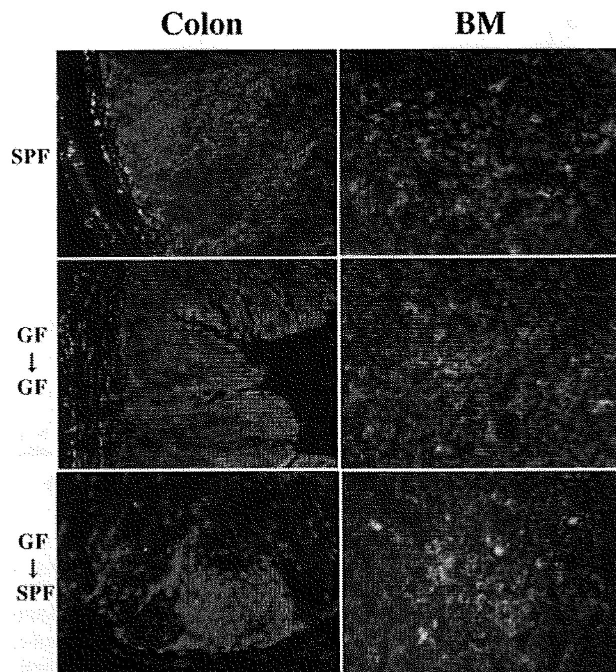

FIGURE 8. Substantial number of $CD4^+$ T cells was resident in BM of noncolitic SCID recipients in the absence of commensal bacteria. Frozen sections of colon from each group (SPF, GF→GF, and GF→SPF) were stained with polyclonal anti-IL-7 Abs (green) and anti-CD4 Abs (red). Representative of five separate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

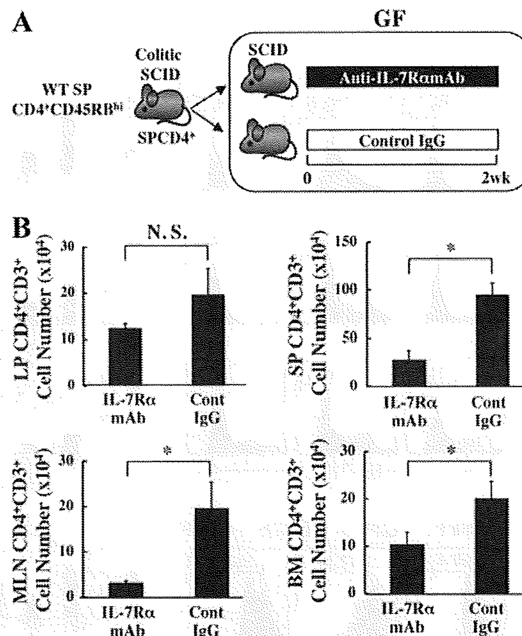

FIGURE 9. Neutralization of IL-7 reduced the recovered cell number of $CD4^+$ T cells in various site of GF SCID mice transferred with colitic $CD4^+$ T cells. *A*, GF SCID mice transferred with colitogenic SP $CD4^+$ T cells previously transferred with $CD4^+CD45RB^{high}$ T cells were treated with 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. *, $p < 0.05$.

(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 blockaded 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. 9*A*). 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. 9*B*). 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. 9*B*). 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. 10*A*). 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. 10*B*, 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

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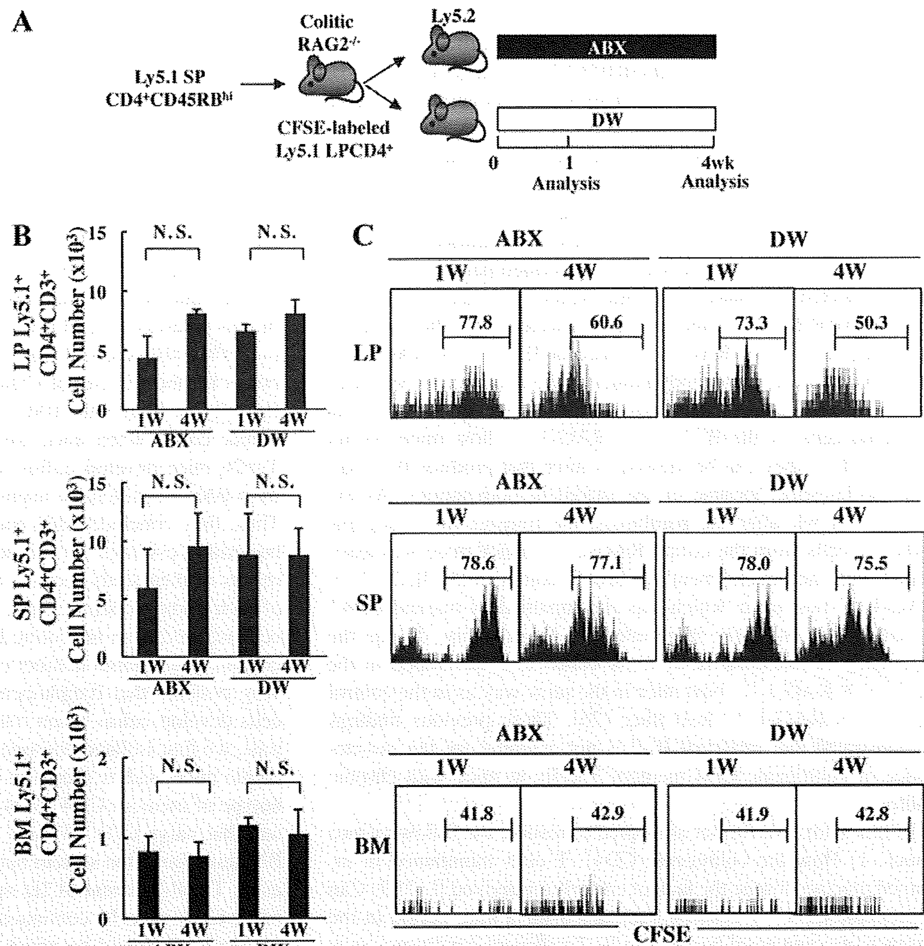

FIGURE 10. Colitogenic memory CD4$^+$ T cells were retained outside the intestine even in the normal immunosufficient condition. *A*, CFSE-labeled LP CD4$^+$ T cells obtained from colitic RAG2$^{-/-}$ mice previously transferred with Ly5.1$^+$CD4$^+$CD45RB^{high} T cells were transferred into Ly5.2$^+$ 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 ± 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$^{-/-}$ × RAG-1$^{-/-}$ mice injected with colitogenic CD4$^+$ T cells never develop colitis, whereas evidence of disease was seen in IL-7$^{+/+}$ × 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

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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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IL-7 is essential for lymphopenia-driven turnover of colitogenic $CD4^+$ memory T cells in chronic colitis

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We previously demonstrated that IL-7 is essential for the persistence of T-cell-mediated colitis, by showing that adoptive transfer of $CD4^+CD45RB^{high}$ T cells into IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice did not induce colitis; and that intestinal IL-7 is not essential for this colitis model, by showing that IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice parabiosed with colitic $CD4^+CD45RB^{high}$ T-cell-transferred RAG-$1^{-/-}$ mice developed colitis. Here, we investigated the role of IL-7 in the maintenance of colitogenic $CD4^+$ T cells by surgically separating these parabionts. Surprisingly, the separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice were consistently diseased after separation, although no IL-7 mRNA was detected in the tissues of separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ partners. $CD4^+$ T cells isolated from the separated RAG-$1^{-/-}$ or IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice were then transferred into new RAG-$1^{-/-}$ or IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice. Regardless of the source of donor cells, RAG-$1^{-/-}$ recipients developed colitis, whereas IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients did not. Collectively, these results demonstrate that IL-7 is essential for lymphopenia-driven turnover of colitogenic $CD4^+$ T cells rather than the maintenance of those cells in established colitic mice. They also provide a basis for the timing of IL-7/IL-7R blockade for the treatment of inflammatory bowel diseases.

Key words: Cytokines · Inflammation · Mucosa · Rodent · T cells

Introduction

Inflammatory bowel diseases (IBD) are caused by chronic inflammatory responses in the gut wall and commonly take persistent courses [1–6]. When IBD recurs after remission, it generally resembles previous episodes [7]; and thus the disease may be caused by the activation and expansion of colitogenic effector $CD4^+$ T cells generated from long-lived colitogenic memory $CD4^+$ T cells [8]. Nevertheless, it remains largely unknown how colitogenic memory $CD4^+$ T cells are maintained over time in the presence of commensal bacteria.

Accumulating evidence suggest that there are two types of memory T cells. Under circumstances where antigens are cleared, for instance during acute viral infection or vaccination, the presence of antigens is not required for the maintenance of memory T cells, especially for $CD8^+$ memory T cells [9–11]. In the absence of the corresponding antigens, memory T cells are maintained by signals transmitted *via* receptors for various homeostatic cytokines, such as IL-7 for $CD4^+$ and $CD8^+$ memory cells, and IL-15 for $CD8^+$ memory cells [12–14]. In contrast, under circumstances where antigens persist, as in chronic viral or parasitic infection or autoimmune diseases, antigens themselves

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appear to be essential for the maintenance of antigen-specific memory T cells [11, 15–17], as these cells cannot survive when adoptively transferred into an antigen-free environment. Also, it has recently been recognized that antigen stimulation itself often has substantial disadvantages for the maintenance of memory T cells through the mechanism of "deletion" or "exhaustion" [18, 19]. This may also be the case with the chronic colitis model of IBD, as the essential role of enteric bacteria is affirmed by the fact that intestinal inflammation cannot be induced if the model mice are reared under germ-free [3, 5, 20] or antibiotics-treated conditions, indicating that enteric bacterial antigens might be responsible for the expansion of colitogenic $CD4^+$ T cells. However, it remains unknown whether homeostatic cytokines are involved in the maintenance of these cells.

IL-7 is secreted by stromal cells in the BM and thymus and by epithelial cells, including intestinal epithelia [14, 21–23]. It is an important cytokine involved in supporting the survival of resting naïve and memory $CD4^+$ T cells, but not effector $CD4^+$ T cells [13, 14, 23]. We have previously demonstrated that (i) IL-7 is constitutively produced by intestinal goblet epithelial cells [22]; (ii) IL-7 transgenic mice in which strong promoters drove systemic overexpression developed chronic colitis that mimicked histopathological characteristics of human IBD [24]; (iii) mucosal $CD4^+$ IL-$7R^{high}$ T cells in $CD4^+CD45RB^{high}$ T-cell-transferred colitic mice are colitogenic [25]; and (iv) IL-7 is essential for the maintenance of colitogenic $CD4^+$ T cells, since IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with these colitogenic $CD4^+$ T cells isolated from inflamed lamina propria (LP) of $CD4^+CD45RB^{high}$ T-cell-transferred colitic mice did not develop colitis [26].

In this study, we further investigated the requirement of IL-7 for the maintenance of colitogenic $CD4^+$ T cells, focusing on the colitis stage (lymphopenic stage *versus* established stage) in terms of IL-7 requirement using an adoptive transfer model of colitis in RAG1/$2^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells [27] in conjunction with parabiosis [28] and subsequent separation.

Results

Separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice are consistently diseased after separation

We previously demonstrated that IL-7 is essential for the development of T-cell-mediated colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ naïve T cells or colitogenic $CD4^+$ memory T cells obtained from colitic $CD4^+CD45RB^{high}$ T-cell-transferred RAG-$2^{-/-}$ mice into RAG-$1^{-/-}$ or IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice [26]. Surprisingly, however, we also found that intestinal IL-7 is not essential for this colitis model, although the site of inflammation is solely within the intestine, because IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice parabiosed with colitic $CD4^+CD45RB^{high}$ T-cell-transferred RAG-$1^{-/-}$ mice developed colitis [28]. Thus, as the next logical step, we here investigated the role of IL-7 in the maintenance of colitogenic $CD4^+$ memory T cells by surgically separating the colitic parabionts at 6 wk after parabiosis surgery and observing the separated mice for a further 12 wk (Fig. 1).

As expected, the separated RAG-$1^{-/-}$ mice were consistently diseased during observation up to 12 wk after separation, in contrast to the age-matched C57BL/6 mice (Fig. 2A). Surprisingly, however, the separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ donor mice were also consistently diseased to a similar level to their RAG-$1^{-/-}$ partners, despite the absence of IL-7 (Fig. 2A). Twelve weeks after separation, the colons from RAG-$1^{-/-}$ and IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice were equally enlarged and had greatly a thickened wall (data not shown). Histological examination showed prominent epithelial hyperplasia with glandular elongation and a massive infiltration of mononuclear cells in all mice (Fig. 2B). The difference between the separated mice and normal mice was confirmed by histological scores of multiple colon sections (Fig. 2C). A further quantitative evaluation of $CD4^+$ T cell expansion was made by isolating LP $CD3^+CD4^+$ T cells. As shown in Fig. 2D, the number of $CD4^+$ T cells from LP of the separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice was comparable to that of RAG-$1^{-/-}$ partners, but was significantly higher than that of age-matched control C57BL/6 mice. Finally, the levels of cytokines IFN-γ and TNF-α produced by anti-CD3/anti-CD28-mAb-stimulated LP $CD4^+$ T cells obtained from the separated RAG-$1^{-/-}$ or IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice were equivalent, but significantly higher than those of age-matched control C57BL/6 mice (Fig. 2E).

IL-7 is not detected in any organs of separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice

The finding that colitis persisted in the separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice up to 12 wk after separation surgery seems to conflict with the fact that IL-7 is essential for the persistence of colitogenic $CD4^+$ memory T cells, even allowing for the mean half-life of IL-7 (9.3–13.9 h) in our previous adoptive transfer system [29]. Thus, we assessed the possibility that IL-7-producing cells or their progenitor cells had migrated from colitic RAG-$1^{-/-}$ mice into IL-$7^{-/-}$ × RAG-$1^{-/-}$ partners during parabiosis, resulting in the continuous production of IL-7 was continuously produced in colitic IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice even after separation. As shown in Fig. 3A, however, quantitative RT-PCR assay for IL-7 mRNA expression revealed that IL-7 mRNA was never detected in any organ (colon, SP, MLN, kidney, liver, and BM) of colitic IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice after separation, in sharp contrast to the paired RAG-$1^{-/-}$ partners. Furthermore, the absence of IL-7 mRNA expression in the separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice was confirmed by RT-PCR analysis using purified $CD45^+$ T cells and $CD45^+$ non-T cells of BM and spleen (Fig. 3B). In contrast, mRNA of IL-15, another cytokine involved in maintenance of $CD4^+$ memory T cells [30–32], was detected in all the examined organs with the same pattern in the two groups (Fig. 3A). Although this result suggests that IL-15 may be involved in the maintenance of colitis in the separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ partners with colitis in an IL-7-indendent manner, this is unlikely, because LP $CD4^+$ T cells obtained from both the separated RAG-$1^{-/-}$ and the IL-$7^{-/-}$ × RAG-$1^{-/-}$ partners expressed a high level of IL-7Rα, but

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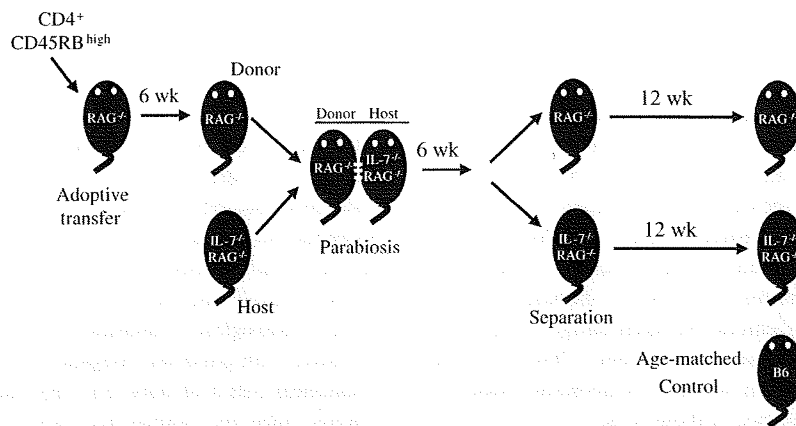

Figure 1. Experimental design of adoptive transfer, parabiosis, and separation. For adoptive transfer, splenic $CD4^+CD45RB^{high}$ T cells were isolated from C57BL/6-Ly5.2 mice and transferred into RAG-$1^{-/-}$ mice ($n = 12$). Six weeks after transfer, RAG-$1^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells developed colitis. Colitic RAG-$1^{-/-}$ donor mice were parabiosed with host IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice ($n = 12$ pairs). Joined animals were maintained for 6 wk, then surgically separated and observed for 12 wk after separation. Age-matched C57BL/6 mice ($n = 6$) were used as a negative control.

not IL-15Rβ (Fig. 4C). To further confirm that IL-15 plays no role in the maintenance of colitis in the separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice, we administered neutralizing anti-IL-15 mAb to these mice for 6 wk, and found that this did not ameliorate the colitis as compared with control antibodies by assessing the clinical (data not shown) and histological scores (Fig. 3D). Furthermore, IL-7 is known to be crucial for survival of $CD4^+$ memory T cells [12–14], we assessed apoptotic cells in spleen and LP of these mice using PI/Annexin V staining. As shown in Fig. 3E, the ratio of early apoptotic cells (PI^- Annexin V^+) and late apoptotic cells (PI^+ Annexin V^+) in spleen and LP of these mice was comparable to that of RAG-$1^{-/-}$ mice, suggesting that the survival of colitogenic $CD4^+$ T cells was not impaired by the lack of IL-7 in the separated IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice.

IL-7 is essential for the development of colitis in RAG-$1^{-/-}$ mice

To further evaluate the possibility that some colitogenic $CD4^+$ T cells in colitic RAG-$1^{-/-}$ or IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice during parabiosis or after separation acquire IL-7-independency for their maintenance, we conducted an adoptive re-transfer experiment in four groups (Fig. 4A). As expected, RAG-$1^{-/-}$ recipients transferred with LP $CD4^+$ T cells from the separated RAG-$1^{-/-}$ (Group 1) or IL-$7^{-/-}$ × RAG-$1^{-/-}$ (Group 3) mice developed severe colitis by 4–6 wk after transfer, characterized by significant weight loss, diarrhea, and higher total clinical scores (Fig. 4B), and thickening of the colonic wall with inflammation (Fig. 4C). In contrast, IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients transferred with LP $CD4^+$ T cells from the separated RAG-$1^{-/-}$ (Group 2) or IL-$7^{-/-}$ × RAG-$1^{-/-}$ (Group 4) mice appeared healthy, exhibiting no signs of colitis until 6 wk after transfer (Fig. 4B), and no apparent thickening of the colonic wall (Fig. 4C). Average histological score characterized by severe inflammation and epithelial hyperplasia of Group 1 (16.2±0.37) or Group 3 (15.4±0.51) was significantly higher than that of Group 2 (0.6±0.24) or Group 4 (1.6±0.81) (Fig. 4D). The average number of LP, SP, and BM $CD4^+$ T cells recovered from Group 1 or Group 3 was significantly higher than that from Group 2 or Group 4, respectively (Fig. 5A). As shown in Fig. 5B, LP $CD4^+$ T cells from Group 1 or Group 3 produced significantly higher amounts of IFN-γ and TNF-α than those from Group 2 or Group 4, respectively. Furthermore, flow cytometry analysis revealed that the expression of CD69 on LP and SP $CD4^+$ T cells from Group 1 or Group 3 was significantly higher than that on cells from Group 2 or Group 4, respectively (Fig. 5C). In addition, all the LP and SP $CD4^+$ T cells regardless of group sustained the phenotype of $CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ T_{EM} cells (data not shown).

To further assess the role of IL-7 in the maintenance of chronic colitis, we finally performed another adoptive transfer experiment using different donor cell numbers (3×10^5 and 3×10^6 cells) for a longer period of observation (12 wk after retransfer) (Fig. 6A). Again, we confirmed that IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipients irrespective of donor cell number had not developed colitis at 12 wk after transfer by assessing clinical scores (Fig. 6B), histological scores (Fig. 6C and D), and the recovered cell number of LP $CD4^+$ T cells (Fig. 6E), in sharp contrast to the control RAG-$2^{-/-}$ recipients. Further, LP $CD4^+$ T cells from Group 1 produced significantly higher amounts of IFN-γ and TNF-α than those from Group 2 or Group 3, respectively (Fig. 6F). Collectively, these data suggest that, unlike the parabiosis system, adoptive transfer using colitogenic $CD4^+$ memory T donor cells is strictly dependent on the presence of IL-7 in recipient mice.

Discussion

In this article, following by a series of our previous reports [22, 24–26, 28], we have demonstrated that IL-7 is not essential for

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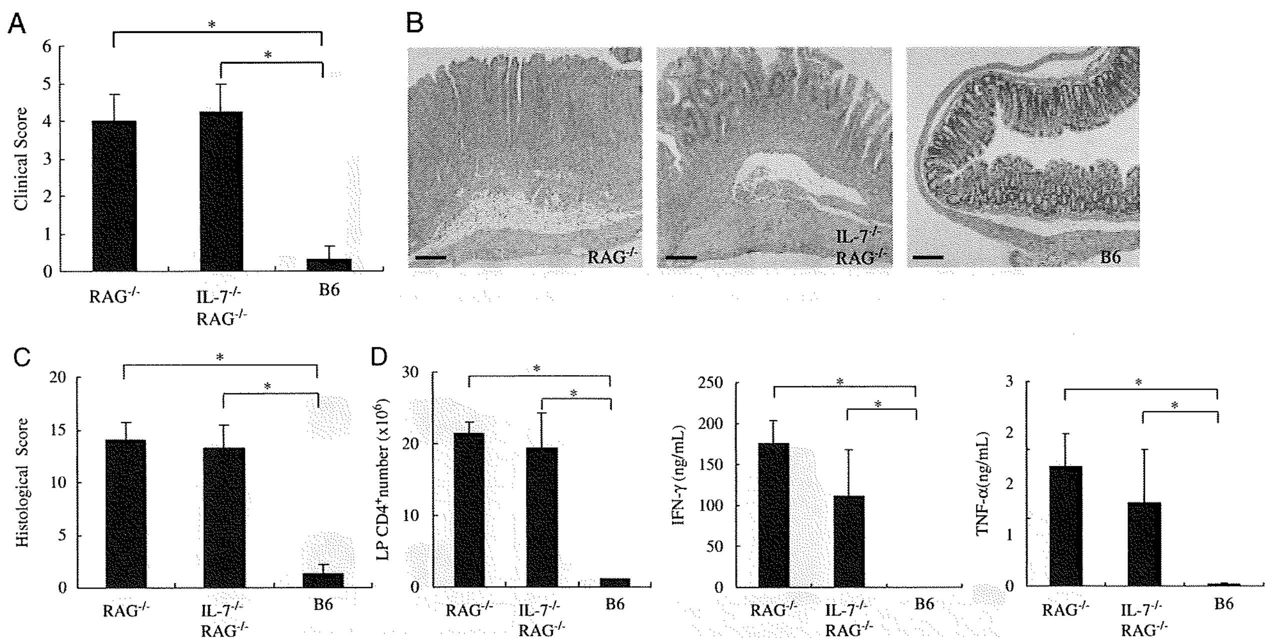

Figure 2. Separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice are consistently diseased after separation. (A) Clinical scores were determined at 12 wk after separation as described in *Materials and methods* and Fig. 1. Data show mean ± SEM ($n = 6$/group). *$p<0.05$ *versus* age-matched C57BL/6 mice. (B) Histological examination by H&E staining of the colon. Original magnification, × 100. Bars: 200 μm. (C) Histological scoring of colon. Data show mean ± SEM ($n = 6$/group). *$p<0.01$ *versus* control mice. (D) Cell number of CD4$^+$ T cells recovered from LP was determined by flow cytometry. Data show mean ± SEM ($n = 6$/group). *$p<0.01$ *versus* control mice. (E) Cytokine production. LP CD4$^+$ T cells were prepared from colons at 12 wk after surgery and stimulated with anti-CD3 and anti-CD28 mAb for 48 h. Concentrations of IFN-γ and TNF-α in culture supernatants were measured by ELISA. Data show mean ± SEM ($n = 6$/group). *$p<0.01$, *versus* control C57BL/6 mice.

the persistence of established colitis. Here, by showing that colitis continues in IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice after separation from parabiotic union with colitic RAG-1$^{-/-}$ mice that had previously been transferred with CD4$^+$CD45RB^{high} T cells. In sharp contrast, we found that IL-7 is essential for the development of on-going colitis under lymphopenic conditions by showing the absence of IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transferred with colitogenic LP CD4$^+$ T cells obtained from the separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice after separation surgery. These results clearly demonstrate that IL-7 is essential for the turnover of colitogenic CD4$^+$ T cells in the lymphopenic stage of the adoptive transfer system, but not for the turnover of those cells in the established stage of colitis in this commensal-dependent adoptive transfer model.

The explanation first considered for the finding that colitic IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice sustained severe colitis up to 12 wk after separation to a similar extent to the paired RAG-1$^{-/-}$ mice was the straightforward migration of IL-7-producing cells or their progenitor cells from RAG-1$^{-/-}$ to IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice in parabionts along with colitogenic CD4$^+$ T cells, since IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transferred with colitogenic LP CD4$^+$ T cells alone did not develop colitis [26]. However, this possibility was excluded, as RT-PCR analysis could not detect mRNA of IL-7 in the organs or purified CD45$^+$CD4$^+$ T cells and CD45$^+$ non-T cells of the IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice (Fig. 3A and B), although the possibility remains that IL-7 expressed in the separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice at below the level of detection by RT-PCR, contributed to the present result.

Interestingly, however, mRNA expression of IL-15, which has recently been recognized as a homeostatic cytokine for CD4$^+$ memory T cells as well as for the maintenance of CD8$^+$ memory T cells [30–32] was detected at similar levels in various organs of both the separated RAG-1$^{-/-}$ and the IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice (Fig. 3A). However, colitic LP CD4$^+$ T cells in both mice expressed very low levels of IL-15Rβ, an essential receptor for IL-15 signaling, in sharp contrast to the strong expression of IL-7Rα (Fig. 3C). Also, we demonstrated that administration of neutralizing anti-IL-15 mAb did not ameliorate the established colitis in the separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice (Fig. 3D). This result suggests that IL-15 is not essential for the sustaining colitis in the separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice. Thus, the colitic CD4$^+$ T cells in the parabionts in this study might have acquired a dependency on other cytokines, such as thymic stromal lymphopoietin, receptors of which are composed of IL-7Rα and thymic stromal lymphopoietin, and remained independent of IL-7 during parabiosis. However, this possibility is also unlikely because new IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients subsequently transferred with colitic LP CD4$^+$ T cells from the separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice did not develop colitis (Fig. 4), indicating that IL-7 is essential for the development of on-going colitis in the adoptive transfer system, but not essential for the sustainment of the established chronic colitis after the parabiosis and subsequent separation system.

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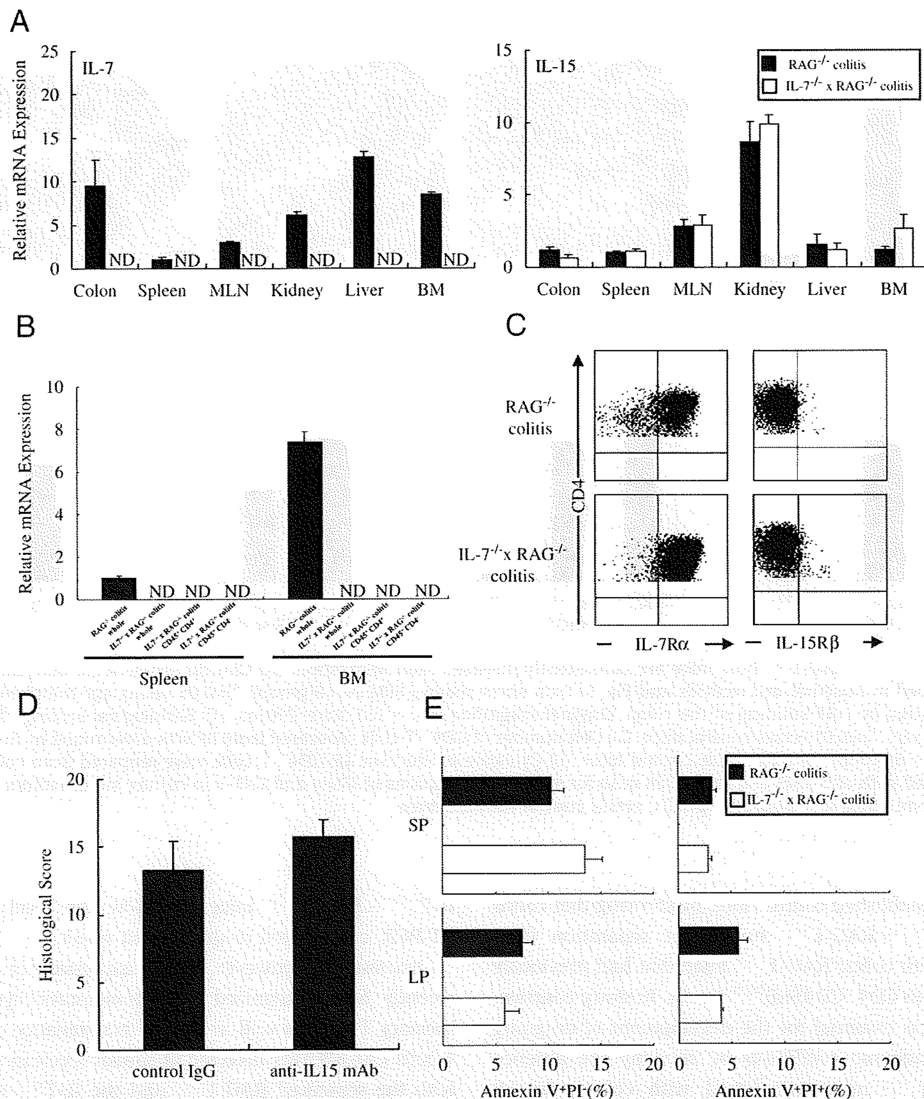

Figure 3. Separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice after parabiosis do not express mRNA for IL-7. (A) Expression of IL-7 and IL-15 in mRNA various tissues as determined by real-time RT-PCR. ND, not detected. (B) Expression of IL-7 in various isolated cell populations from spleen and BM. Whole mononuclear cells, CD45$^+$ CD4$^+$ T cells, and CD45$^+$ non-T cells (each 5×10^6 cells) were used for real-time RT-PCR. (C) IL-7Rα and IL-15Rβ expression on LP CD4$^+$ T cells was analyzed by flow cytometry. Data are representive of six mice *per* group. (D) Histological scoring. The separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice were administered with anti-IL-15 mAb or control IgG for 6 wk. Data show mean±SEM ($n = 5$/group). (E) The ratio of early (Annexin V$^+$PI$^-$) and late (Annexin V$^+$PI$^-$) apoptotic cells in freshly isolated spleen and LP CD4$^+$ T cells was determined by the Annexin V-FITC/PI. Data show mean±SEM ($n = 5$/group). NS, not significantly different.

How can this discrepancy in IL-7 requirement in this adoptive transfer model in combination with parabiosis system be explained? The most striking difference is in the *in vivo* experimental setting, parabiosis *versus* adoptive transfer. In the adoptive transfer system, only a small number of colitogenic CD4$^+$ T cells (approximately 3×10^5–3×10^6 cells/mouse) are transferred into immunodeficient RAG-1$^{-/-}$ mice, whereas in the parabiosis system, a large number of these cells can be continuously transferred into recipients. Thus, it is possible that IL-7 is required for the lymphopenia-driven expansion of these cells in the adoptive transfer system. In contrast, the continuous and abundant supply of these cells in the parabiosis system might overcome the requirement of IL-7 for the development of colitis, this being replaced presumably by other stimuli, such as commensal bacterial antigens and other cytokines. Consistent with this notion, we previously demonstrated that CFSE-labeled colitogenic CD4$^+$ T cells could expand by over eight cell divisions within 7 days after transfer in IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice, a similar extent to that in RAG-1$^{-/-}$ mice, whereas the number of donor-derived cells recovered in IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients was over 100 times less than that in RAG-1$^{-/-}$ recipients, in line with the decreased expression of Bcl-2 and conversely the increased

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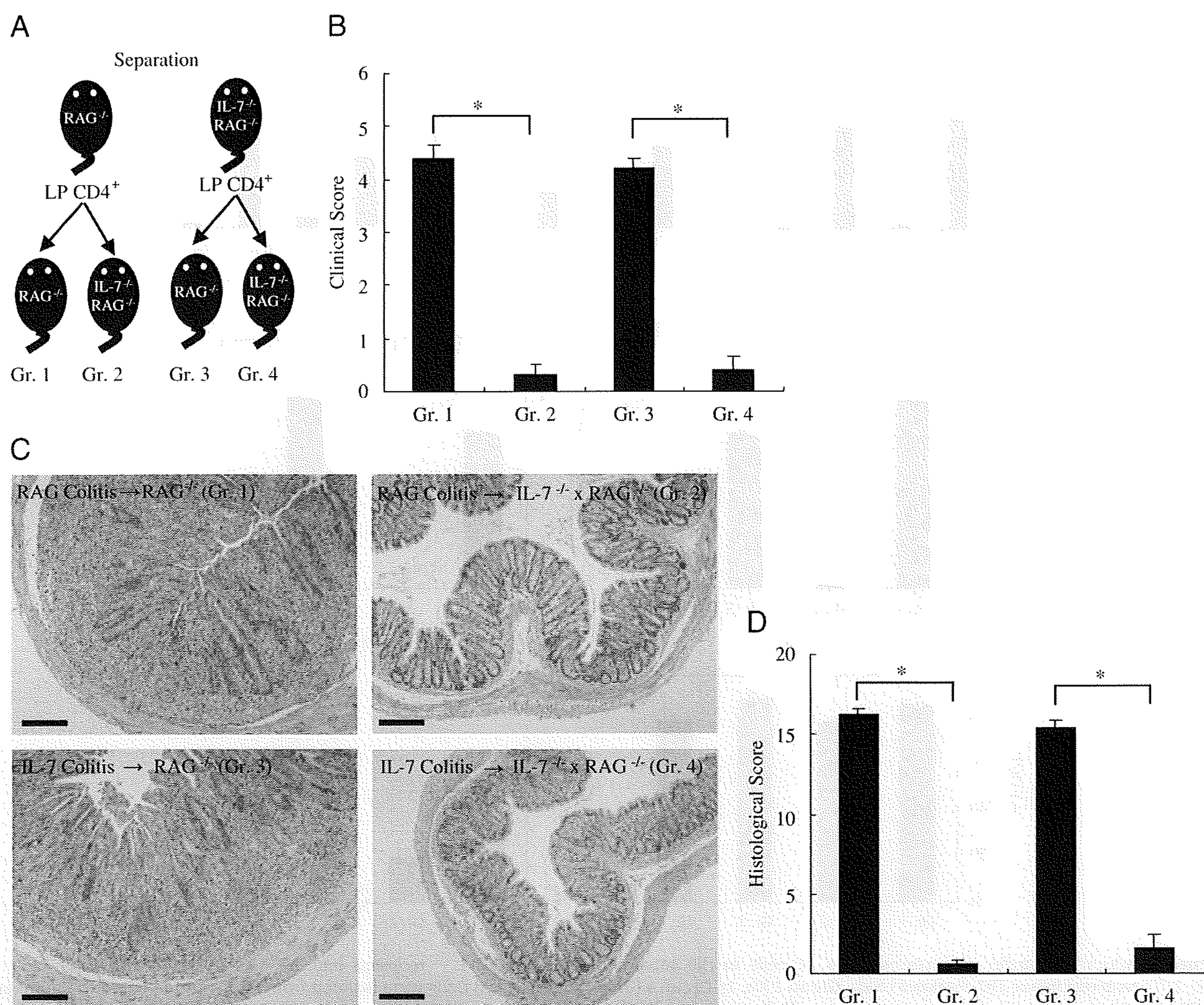

Figure 4. Sustained CD4$^+$ T cells in the separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ *recipients do not have the potential to induce colitis when transferred to new IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients.* (A) New RAG-1$^{-/-}$ or IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice were transferred with LP CD4$^+$ T cells obtained from previously parabiosed RAG-1$^{-/-}$ or IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice at 12 wk after separation (each group; $n = 7$). Group 1 (Gr. 1), new RAG-1$^{-/-}$ mice transferred with LP CD4$^+$ T cells obtained from the separated RAG-1$^{-/-}$ mice; Group 2 (Gr. 2), new RAG-1$^{-/-}$ mice transferred with LP CD4$^+$ T cells from the separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice; Group 3 (Gr. 3), new IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transferred with LP CD4$^+$ T cells from the separated RAG-1$^{-/-}$ mice; Group 4 (Gr. 4), new IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice transferred with LP CD4$^+$ T cells from the separated IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice. (B) Clinical scores were determined 6 wk after transfer. Data show mean ± SEM ($n = 7$/group). *$p<0.01$. (C) Histological examination of the colon. Original magnification, ×100. Bars: 200 μm. (D) Histological scoring. Data show mean ± SEM ($n = 7$/group). *$p<0.01$.

ratio of Annexin V$^+$ apoptotic cells in donor CD4$^+$ T cells of IL-7$^{-/-}$ × RAG-1$^{-/-}$ recipients [26]. Hence, these results suggest that IL-7 is indeed not required for rapid proliferation of colitogenic LP CD4$^+$ T cells in lymphopenic conditions, but is concurrently critical for the survival of those cells until the time that recipient mice become lympho-competent.

The current findings might also shed light on the clinical aspect of IBD. Most current treatments for IBD, such as corticosteroid, immunosuppresants (azathiopurine and cyclosporine), and biologics (infliximab), induce apoptosis of activated CD4$^+$ T cells [33], and thereby are often accompanied by lymphopenia. This is also the case with the recently developed therapy by autologous hematopoietic stem cell transplantation for patients with intractable Crohn's disease [34]. However, since most patients who achieve remission by these treatments inevitably relapse some time later, it is possible that subsequent blockade of the IL-7/IL-7R signal pathway to suppress IL-7-dependent lymphopenia-driven expansion of colitogenic CD4$^+$ T cells will be beneficial for the long-term remission of the diseases.

In summary, we here demonstrated that IL-7 is essential for the turnover of colitogenic CD4$^+$ T cells in the lymphopenic stage of colitis in the commensal-dependent adoptive transfer model, suggesting an effectual timing for therapeutic approaches that target systemic IL-7 using the biologics against IL-7 in the treatment of IBD.

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