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

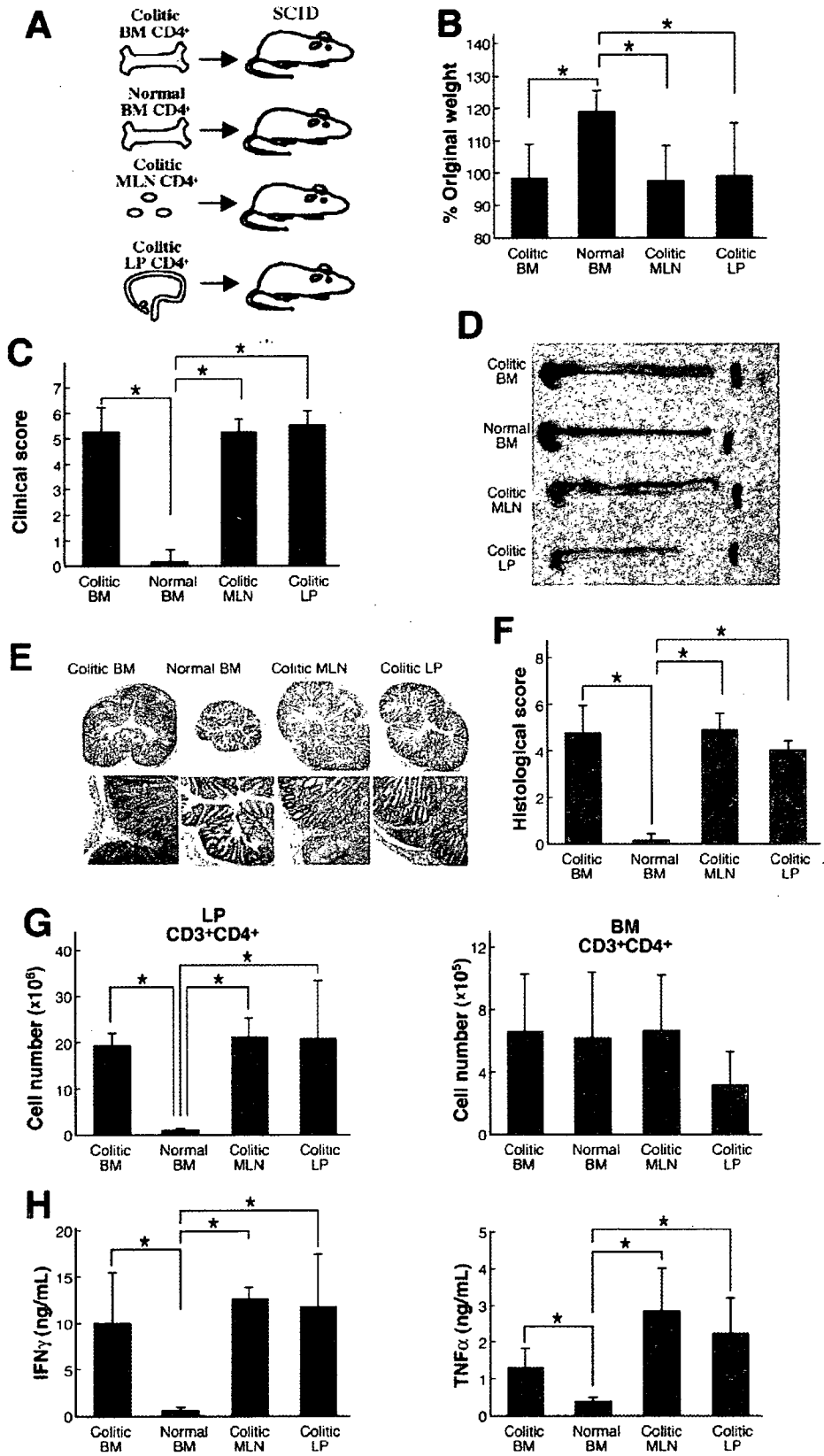
IL-7 Is Essential for the Survival and Homeostatic Proliferation of Colitogenic BM CD4+ Memory T Cells

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

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

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

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


A
Colitic BM CD4+
SCID
Normal BM CD4+
Colitic MLN CD4+
Colitic LP CD4+
B
% Original weight
Colitic BM
Normal BM
Colitic MLN
Colitic LP
C
Clinical score
D
E
F
Histological score
G
LP CD3+CD4+
Cell number (×10⁵)
BM CD3+CD4+
H
IFNγ (ng/mL)
TNFα (ng/mL)

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

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

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

Discussion

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

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

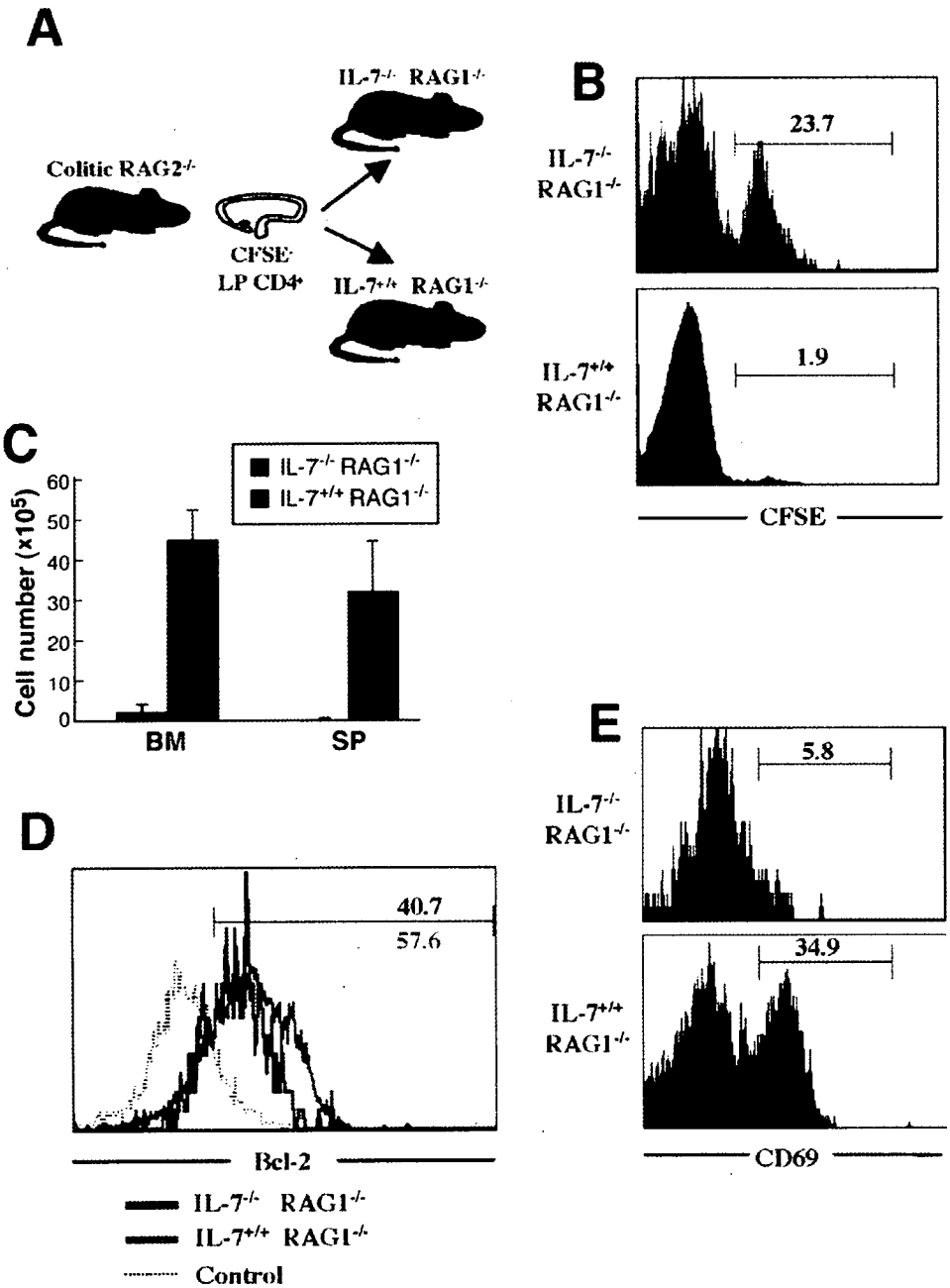


Figure 7. IL-7 is essential for the survival and in part for the cell turnover of colitogenic BM CD4+ T cells. (*A*) C57BL/6 Rag-2$^{-/-}$ mice were injected intraperitoneally with normal splenic CD4+CD45RB^{high} T cells. Six weeks after transfer, the LP CD4+ T cells were isolated. Colitogenic LP CD4+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

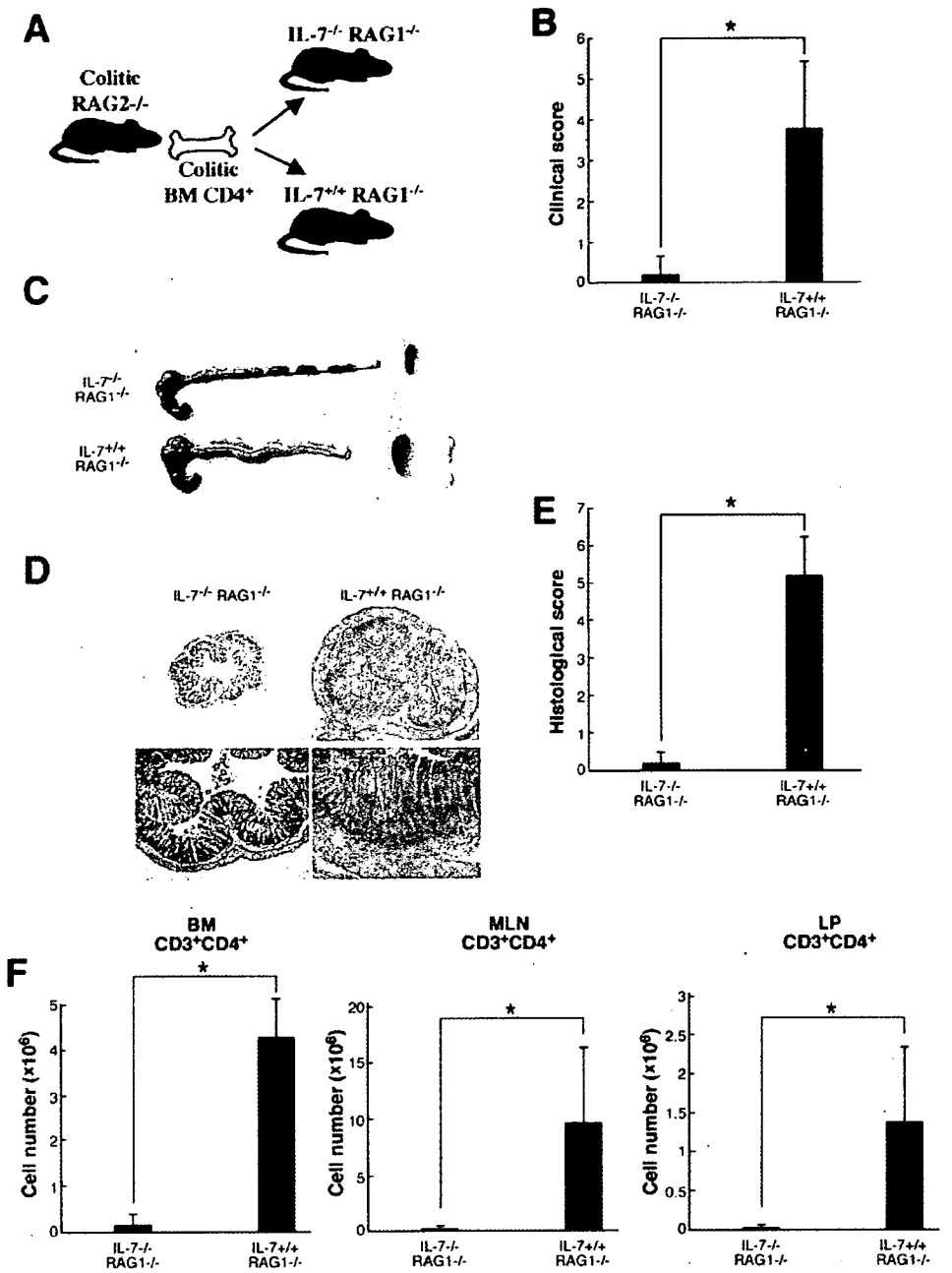


Figure 8. IL-7$^{-/-}$ × Rag-1$^{-/-}$ mice transferred with colitogenic BM CD4$^+$CD44^{high}CD62L$^-$ T_{EM} cells did not develop colitis. (*A*) IL-7$^{+/+}$ × Rag-1$^{-/-}$ (n = 5) and IL-7$^{-/-}$ × Rag-1$^{-/-}$ (n = 5) mice were transferred with colitic BM CD4$^+$ T cells. (*B*) Clinical scores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

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

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

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

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

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

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

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

Supplementary Data

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

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Address requests for reprints to: Takanori Kanai, MD, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. e-mail: taka.gast@tmd.ac.jp; fax: (81) 3-5803-0268.

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Intestinal Lamina Propria Retaining CD4 CD25 Regulatory T Cells Is A Suppressive Site of Intestinal Inflammation[1]

Shin Makita,* Takanori Kanai,[2]* Yasuhiro Nemoto,* Teruji Totsuka,* Ryuichi Okamoto,* Kiichiro Tsuchiya,* Masafumi Yamamoto,† Hiroshi Kiyono,‡ and Mamoru Watanabe*

It is well known that immune responses in the intestine remain in a state of controlled inflammation, suggesting that not only does active suppression by regulatory T (T_{REG}) cells play an important role in the normal intestinal homeostasis, but also that its dysregulation of immune response leads to the development of inflammatory bowel disease. In this study, we demonstrate that murine CD4 CD25 T cells residing in the intestinal lamina propria (LP) constitutively express CTLA-4, glucocorticoid-induced TNFR, and Foxp3 and suppress proliferation of responder CD4 T cells in vitro. Furthermore, cotransfer of intestinal LP CD4 CD25 T cells prevents the development of chronic colitis induced by adoptive transfer of CD4 CD45RB^{high} T cells into SCID mice. When lymphotoxin (LT) -deficient intercrossed Rag2 double knockout mice (LT / Rag2 /), which lack mesenteric lymph nodes and Peyer's patches, are transferred with CD4 CD45RB^{high} T cells, they develop severe wasting disease and chronic colitis despite the delayed kinetics as compared with the control LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells. Of note, when a mixture of splenic CD4 CD25 T_{REG} cells and CD4 CD45RB^{high} T cells are transferred into LT / Rag2 / recipients, CD4 CD25 T_{REG} cells migrate into the colon and prevent the development of colitis in LT / Rag2 / recipients as well as in the control LT / Rag2 / recipients. These results suggest that the intestinal LP harboring CD4 CD25 T_{REG} cells contributes to the intestinal immune suppression. *The Journal of Immunology,* **2007, 178: 4937–4946.**

Intestinal mucosal surfaces are exposed to alimentary and bacterial Ags of the intestinal flora (1). The gut-associated immune system fences off potentially harmful intestinal Ags from systemic circulation and induces systemic tolerance against luminal Ags. In contrast, inflammatory bowel disease is associated with activation of the local intestinal and systemic immune responses (2, 3). CD4 CD25 regulatory T (T_{REG})[3] cells fulfill a central role in the maintenance of immunological homeostasis and self-tolerance (4, 5). CD4 CD25 T_{REG} cells have been detected mainly in lymphoid sites including thymus, lymph nodes, and spleen. Because numerous studies have demonstrated a capacity of T_{REG} cells to prevent the induction of immune responses and because suppression requires direct cell-cell contact with responder T cells or APCs, it is conceivable that T_{REG} cells act as central regulators within lymphoid tissues (6–8).

The gut-associated lymphoid tissue can be divided into effector sites, which consist of lymphocytes scattered throughout the epithelium and lamina propria (LP) of the mucosa and organized lymphoid tissues (inductive sites) that are responsible for the induction phase of the immune response (1, 9). These include Peyer's patches (PPs), mesenteric lymph nodes (MLNs), and isolated lymphoid follicles (ILFs). It is thought that presentation of Ags to immune naive and effector cells is concentrated at these inductive sites of organized mucosal lymphoid follicles, and thus APCs tune the delicate balance between intestinal immune tolerance and inflammation.

In addition to the inductive sites for the development of colitis, however, it also remains unclear where CD4 CD25 T_{REG} cells suppress the development of colitis. Although it is reasonable to hypothesize that mechanisms for the induction, maintenance, and suppression of colitis would be centrally controlled by CD4 CD25 T_{REG} cells in the inductive sites, we question in this study whether these inductive sites are solely involved in the induction and suppression of intestinal inflammation because we recently demonstrated that human intestinal LP CD4 CD25^{bright} T cells as well as peripheral CD4 CD25^{bright} T cells obtained from normal individuals possess T_{REG} activity in vitro (10). Consistent with our previous report, it has been recently reported that CD4 CD25 T_{REG} cells were detected in peripheral tissues and at sites of ongoing immune responses such as synovial fluid from rheumatoid arthritis patients (11), tumors (12), transplants (13), skin lesions in mice infected with *Leishmania major* (14), lungs from mice infected with *Pneumocystis carinii* (15), islets of Langerhans in diabetes models (16), and lesions in delayed-type hypersensitivity models (17) as well as in inflamed mucosa in colitic mice (8, 18). In the present study, we conducted a series of the adoptive transfer experiments focusing on intestinal

*Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan; †Department of Microbiology and Immunology, Nihon University School of Density at Matsudo, Matsudo, Japan; and ‡Department of Microbiology and Immunology, Division of Mucosal Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

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[2] Address correspondence and reprint requests to Dr. Takanori Kanai, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail address: taka.gast@tmd.ac.jp

[3] Abbreviations used in this paper: T_{REG}, regulatory T; LP, lamina propria; PP, Peyer's patch; MLN, mesenteric lymph node; ILF, isolated lymphoid follicle; GITR, glucocorticoid-induced TNFR; LT, lymphotoxin.

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LP CD4 CD25 T cells to understand where and how CD4 CD25 T_{REG} cells control the mucosal immune system in vivo.

Materials and Methods

Animals

Female BALB/c, C.B-17 SCID, and C57BL/6-Ly5.2 mice were purchased from Japan CLEA. C57BL/6-Ly5.1 and C57BL/6-Ly5.2 Rag2-deficient (Rag2 ′) mice were obtained from Taconic Farms. Ly5.2 background lymphotoxin (LT) -deficient (LT ′) mice were purchased from The Jackson Laboratory. LT ′ mice were intercrossed into Rag2 ′ mice to generate LT ′ Rag2 ′ and LT ′ Rag2 ′ mice in the Animal Care Facility of Tokyo Medical and Dental University. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 wk of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Abs and reagents

The following mAbs except DTA-1, biotinylated anti-mouse glucocorticoid-induced TNFR (GITR; eBioscience) and FJK-16s, PE-conjugated anti-mouse Foxp3 (eBioscience) were obtained from BD Pharmingen for purification of cell populations and flow cytometry analysis: PE-conjugated anti-mouse CD4 (RM4-5), PE-Cy5- and allophycocyanin-conjugated anti-mouse CD4 (L3T4), FITC-conjugated anti-mouse CD25 (7D4), PE-conjugated anti-mouse CD25 (PC61), PE-conjugated anti-mouse CD103 ($_E$ integrin) (M290), PE-conjugated anti-mouse $_{4\ 7}$ integrin (DATK32), PE-conjugated anti-CTLA-4 (UC10-4F10-11), FITC-conjugated anti-mouse CD45RB (16A), FITC-conjugated anti-mouse Ly5.1 (CD45.1, A20), FITC-conjugated anti-mouse Ly5.2 (CD45.2, 104), and FITC- and PerCP-conjugated anti-mouse CD3 (145-2C11). Biotinylated Abs were detected with PE- or CyChrome-streptavidin (BD Pharmingen).

Purification of T cell subsets

CD4 T cells were isolated from normal spleen and colon using the anti-CD4 (L3T4) MACS system (Miltenyi Biotec) according to the manufacturer's instructions. To isolate normal LP CD4 T cells, the entire length of colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca^2 , Mg^2 -free HBSS containing 1 mM DTT (Sigma-Aldrich) for 45 min to remove mucus and then treated with 2.0 mg/ml collagenase and 0.01% DNase (both Worthington Biomedical) for 2 h. The cells were pelleted two times through a 40% isotonic Percoll solution, and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched CD4 T cells from the spleen and the colon (spleen, 94–97% pure; colon, 80–90%, as estimated by FACSCalibur (BD Biosciences)) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5), FITC-conjugated anti-CD45RB (16A), and FITC-conjugated anti-CD25 (7D4). Subpopulations of CD4 cells were generated by two-color sorting on FACSVantage (BD Biosciences). All populations were 98.0% pure on reanalysis.

In vivo experimental design

A series of in vivo experiments was conducted to investigate the role of intestinal LP CD4 CD25 T cells in the suppression of murine-chronic colitis. In Experiment 1, to assess the role of intestinal LP CD4 CD25 T cells obtained from normal mice in the protection of colitis, we transferred 3 10^5 splenic CD4 CD45RB^{high} T cells from normal BALB/c mice with or without 1 10^5 intestinal LP CD4 CD25 T cells into syngeneic C.B-17 SCID mice. All recipient SCID mice were sacrificed at 7 wk after transfer. In Experiment 2, to assess the necessity of gut-associated lymphoid tissues including MLNs in the development and the protection of colitis, we transferred 3 10^5 splenic CD4 CD45RB^{high} T cells from normal C57BL/6-Ly5.2 mice with or without 1 10^5 splenic CD4 CD25 T_{REG} cells from C57BL/6-Ly5.1 mice into LT ′ Rag2 ′ mice and the control LT ′ Rag2 ′ mice. In Experiment 3, to exclude a possible role of spleen in the suppression of colitis in addition to MLNs, we transferred 3 10^5 colitogenic LP CD4 T cells (Ly5.2) obtained from established CD4 CD45RB^{high} T cell-transferred mice (19) with or without 3 10^5 splenic CD4 CD25 T_{REG} cells from C57BL/6-Ly5.1 mice into splenectomized LT ′ Rag2 ′ and LT ′ Rag2 ′ mice.

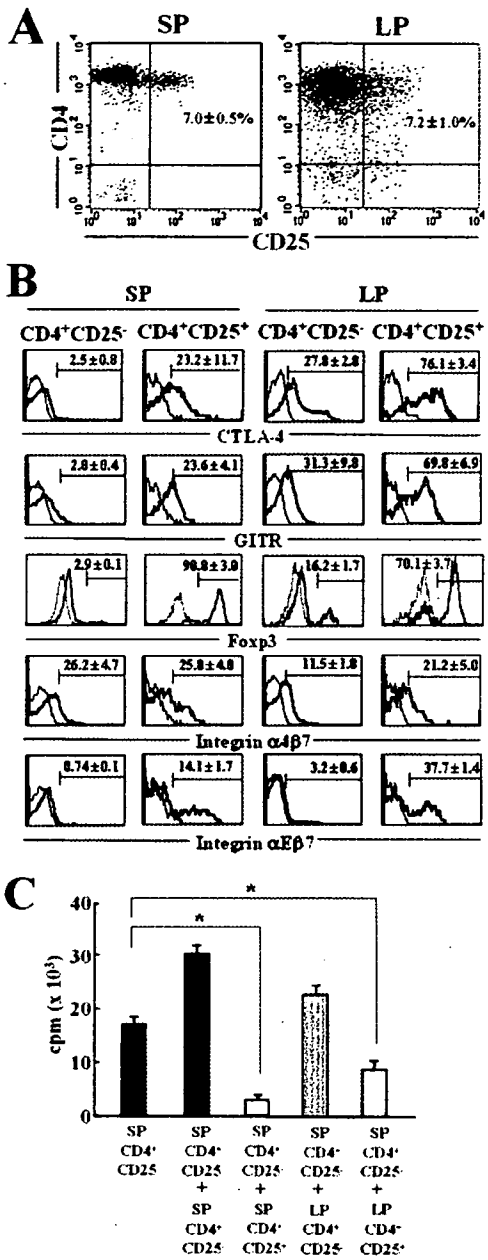

FIGURE 1. Identification and characterization of murine intestinal LP CD4 CD25 T cells in terms of T_{REG} cells in vitro. *A*, Freshly isolated murine spleen (SP) and LP mononuclear cells were assessed by a FACSCalibur. Representative sorting gates of the two cell populations, CD4 CD25 and CD4 CD25 , are shown. Percentages in the *upper right quadrant* represent CD25 cells at indicated site. *B*, Murine intestinal CD4 CD25 constitutively express CTLA-4, GITR, and Foxp3 and partially express $_{4\ 7}$ and $_{E\ 7}$ integrins on or in LP CD4 CD25 T cells. Thick line histogram represents staining with mAbs against the indicated markers. Thin line histogram represents staining with isotype-matched control IgG. *C*, Murine LP CD4 CD25 subsets suppress the proliferation of CD4 responder T cells in vitro. Splenic CD4 CD25 /CD4 CD25 and LP CD4 CD25 /CD4 CD25 populations were isolated from MACS-purified CD4 T cells by FACS sorting. The suppressive activity of the indicated subpopulations was determined by coculturing with splenic CD4 CD25 responder T cells at a 1:1 ratio of responder to T_{REG} cells in the presence of anti-CD3 mAb and mitomycin C-treated APCs for 72 h. [^{3}H]Thymidine uptake was determined for the last 9 h. Data are represented as the mean SD of triplicate samples. , p 0.05 compared with culture in splenic CD4 CD25 responder cells alone.

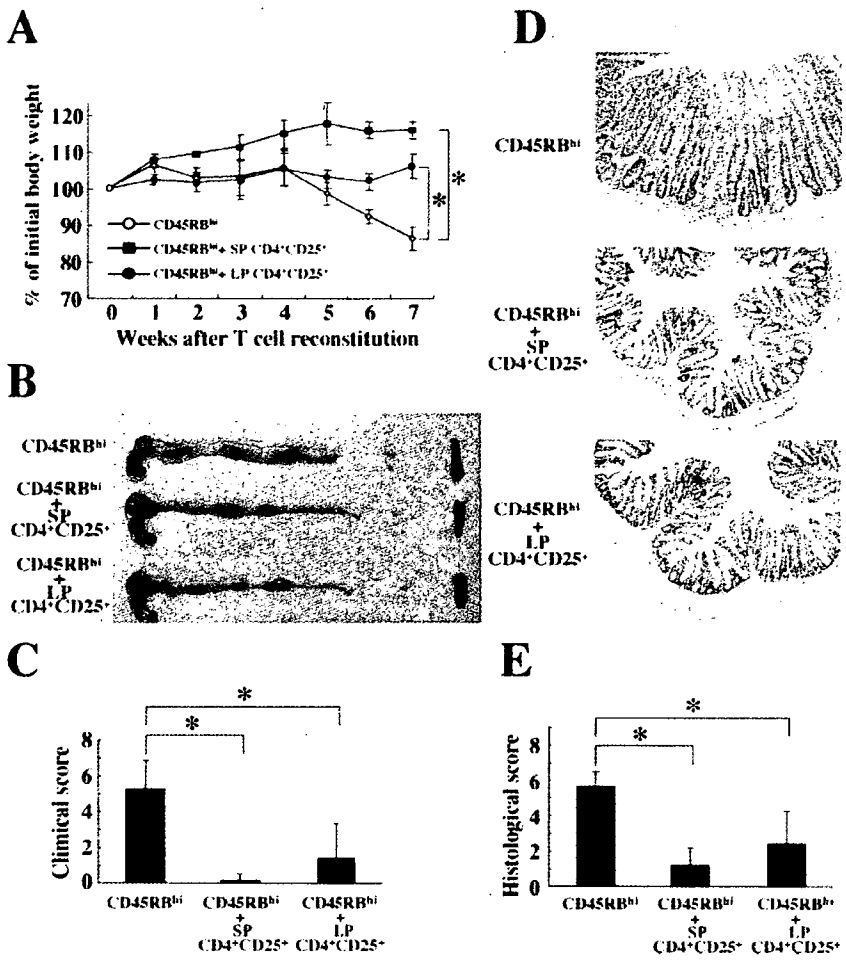

FIGURE 2. Murine intestinal LP CD4 CD25 T_{REG} cells as well as splenic CD4 CD25 T_{REG} cells inhibit the development of colitis induced by adoptive transfer of CD4 CD45RB^{high} T cells into SCID mice. Seven SCID mice in each group were injected i.p. with the following T cell subpopulations: 1) splenic CD4 CD45RB^{high} T cells alone (3 10^5 cells); 2) splenic CD4 CD45RB^{high} T cells (3 10^5 cells) splenic CD4 CD25 T cells (1 10^5 cells); or 3) splenic CD4 CD45RB^{high} T cells (3 10^5 cells) LP CD4 CD25 T cells (1 10^5 cells). *A*, Body weight during 7 wk after transfer. , p 0.05 compared with mice transferred with CD45RB^{high} T cells alone at 7 wk after transfer. *B*, Gross appearance of the colon, MLNs, and spleen from SCID mice transferred with splenic CD4 CD45RB^{high} T cells alone (*upper*), splenic CD4 CD45RB^{high} T cells splenic CD4 CD25 T cells (*middle*), or splenic CD4 CD45RB^{high} T cells LP CD4 CD25 T cells (*lower*) at 7 wk after transfer. *C*, Clinical score at 7 wk after transfer. , p 0.05 compared with mice transferred with CD45RB^{high} T cells alone. *D*, Histopathology of distal colon at 7 wk after transfer. Original magnification, 40. *E*, Histological score at 7 wk after transfer. , p 0.05 compared with mice transferred with CD45RB^{high} T cells alone.

Disease monitoring and clinical scoring

The recipient mice, after T cell transfer, were weighed initially and then three times per week thereafter. They were observed for clinical signs of illness: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. Mice were sacrificed at the indicated time point and assessed for a clinical score that is the sum (0–8 points) of four parameters as follows: 0 or 1, hunching and wasting; 0–3, colon thickening (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); 0–3, stool consistency (0, normal beaded stool; 1, soft stool; 2, diarrhea; 3, bloody stool); and an additional point was added if gross blood was noted (19). To monitor the clinical sign during the observed period over time, the disease activity index is defined as the sum (0–5 points) of the described parameters except colon thickening.

Histological examination and immunohistology

Tissue samples were fixed in PBS containing 6% neutral-buffered formalin. Paraffin-embedded sections (5 m) were stained with H&E. The sections were analyzed without prior knowledge of the type of T cell reconstitution and recipients. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system (19). To detect CD11c dendritic cells and CD4 T cells in the LP, consecutive cryostat sections (6 m) were fixed and stained with the following rat Abs: purified CD4 (L3T4) and biotinylated anti-CD11c (HL3) (BD Pharmingen). Alexa Fluor 594 goat anti-rat IgG and streptavidin-Alexa Fluor 488 (Molecular Probes) were used as second Abs. All confocal microscopy was conducted on a BioZERO BZ8000 (Keyence).

Flow cytometry

To detect the surface expression of a variety of molecules, isolated splenocytes or LP mononuclear cells were preincubated with an Fc R-blocking mAb (CD16/32, 2.4G2; BD Pharmingen) for 20 min followed by incubation with specific FITC-, PE-, PE-Cy5-, or biotin-labeled Abs for 30 min on ice. Biotinylated Abs were detected with PE- or CyChrome-streptavidin. Intracellular Foxp3 staining was performed with the PE anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions. Standard two- or three-color flow cytometric analyses were obtained using the FACSCalibur method and CellQuest software. Background fluorescence was assessed by staining with control irrelevant isotype-matched mAbs.

Cytokine ELISA

To measure cytokine production, 1 10^5 LP CD4 T cells were cultured in 200 l of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar) precoated with 5 g/ml hamster anti-mouse CD3 mAb (145-2C11; BD Pharmingen) and 2 g/ml hamster anti-mouse CD28 mAb (37.51; BD Pharmingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA per the manufacturer's recommendation (R&D Systems).

In vitro T_{REG} cell activity

LP mononuclear cells and splenocytes from normal BALB/c mice were separated into unfractioned CD4 T cells, CD4 CD25 and CD4 CD25 T cells using the anti-CD4 (L3T4) MACS magnetic separation system and/or FACSVantage as described. Cells (5 10^4) and mitomycin C-treated BALB/c CD4 cells (2 10^5) as APCs were cultured for 72 h in round-bottom 96-well plates in RPMI 1640 supplemented with 10% FCS, 100 IU/ml penicillin, 100 g/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 50 M 2-ME. Cells were stimulated with 1 g/ml anti-mouse CD3 mAb. In coculture experiments, the same number of splenic CD4 CD25 or CD4 CD25 cells, or LP CD4 CD25 or CD4 CD25 cells (5 10^4), were added into wells with the fixed dose of splenic CD4 CD25 responder cells (5 10^4) and mitomycin C-treated

CD4 cells (2 10^5), as APCs. Incorporation of [^{3}H]thymidine (1 Ci/well) by proliferating cells was measured during the last 9 h of culture.

Statistical analysis

The results were expressed as the mean SD. Groups of data were compared by Mann-Whitney *U* test. Differences were considered to be statistically significant for a value of p 0.05.

Results

Characterization of intestinal LP CD4 CD25 in terms of T_{REG} cell in vitro

Paired samples of spleen and colon obtained from normal BALB/c mice were analyzed by flow cytometry for the presence of the CD4 CD25 T cells. Consistent with previous reports described, in naturally occurring CD4 CD25 T_{REG} cells (4–6), 7.0 0.5% of the splenic CD4 T cells were CD25 (Fig. 1*A*). Similarly, 7.2 1.0% of the colonic LP CD4 T cells were also CD25 (Fig. 1*A*). Because we previously demonstrated that human intestinal LP CD4 CD25^{bright} T cells obtained from healthy individuals function as T_{REG} cells in vitro (10), we postulated that intestinal LP as well as MLNs is another important site of regulation of immune responses for intestinal homeostasis in vivo. To prove it, we first assessed whether murine intestinal LP CD4 CD25 T cells also express well-known T_{REG} markers, such as CTLA-4, GITR, and Foxp3. Like the control splenic CD4 CD25 T cells, the expression of CTLA-4, GITR, and Foxp3 was markedly up-regulated in or on the intestinal LP CD4 CD25 T cells (Fig. 1*B*) compared with the paired CD4 CD25 T cells. Unexpectedly, but consistent with our human study (10), colonic LP CD4 CD25 T cells expressed CTLA-4, albeit to lesser extent compared with the paired colonic CD4 CD25 T cells (Fig. 1*B*). To further investigate the migration property of these CD4 CD25 T cells, we assessed the expression of $_{4\ 7}$/ $_{E\ 7}$ integrins, which are gut-homing receptors essential to migrate into the colon. As shown in Fig. 1*B*, 10–30% of cells in each subpopulation expressed $_{4\ 7}$ integrin. In contrast, $_{E\ 7}$ integrin was predominantly expressed on the splenic and LP CD4 CD25 T cells, but not on the paired CD4 CD25 T cells, indicating that a part of splenic and LP CD4 CD25 T cells can directly migrate into the gut.

We next investigated the T_{REG} activity of the murine intestinal LP CD4 CD25 T cells by testing their ability to suppress the proliferative responses of the splenic CD4 CD25 responder T cells. As shown in Fig. 1*C*, both the splenic and LP CD4 CD25 T cells were able to suppress the proliferation of the splenic CD4 CD25 responder cells when cocultured at a ratio of 1:1 T_{REG} to responder in the presence of mitomycin C-treated CD4 APCs and soluble anti-CD3 mAb (Fig. 1*C*), indicating that the LP CD4 CD25 T cells were T_{REG} cells as well as the splenic CD4 CD25 T cells at least in vitro. As a control, it was shown that titration of the same dose of the splenic or LP CD4 CD25 cells with the splenic CD4 CD25 responder cells into the cultures did not affect the degree of proliferation, thereby excluding the possibility that an increase in total responder cell number was responsible for the suppressive effect (Fig. 1*C*).

Murine intestinal LP CD4 CD25 T cells suppress the development of the CD4 CD45RB^{high} T cell-transferred colitis

To next analyze the functional role of murine intestinal LP CD4 CD25 T cell subset in vivo, we tested the T_{REG} activity of the intestinal LP CD4 CD25 T cells using the classical SCID-transferred colitis model induced by the adoptive transfer of CD4 CD45RB^{high} T cells (19). C.B-17 SCID mice were injected i.p. with one or two subpopulations of sorted CD4 T cell in PBS: 1) splenic CD4 CD45RB^{high} T cells alone (3 10^5 per mouse) as a positive control, 2) splenic CD4 CD45RB^{high} (3 10^5 per mouse) with splenic CD4 CD25 T cells (1 10^5) as a negative control, and 3) splenic CD4 CD45RB^{high} (3 10^5) with LP CD4 CD25 T cells (1 10^5). The results clearly demonstrated that control of intestinal inflammation resided predominantly within the intestinal LP CD4 CD25 subpopulation as well as the splenic CD4 CD25 T cells, as these cells significantly inhibited the development of wasting disease (Fig. 2*A*) and colitis (Fig. 2, *B–E*). Colons from mice reconstituted with a mixture of

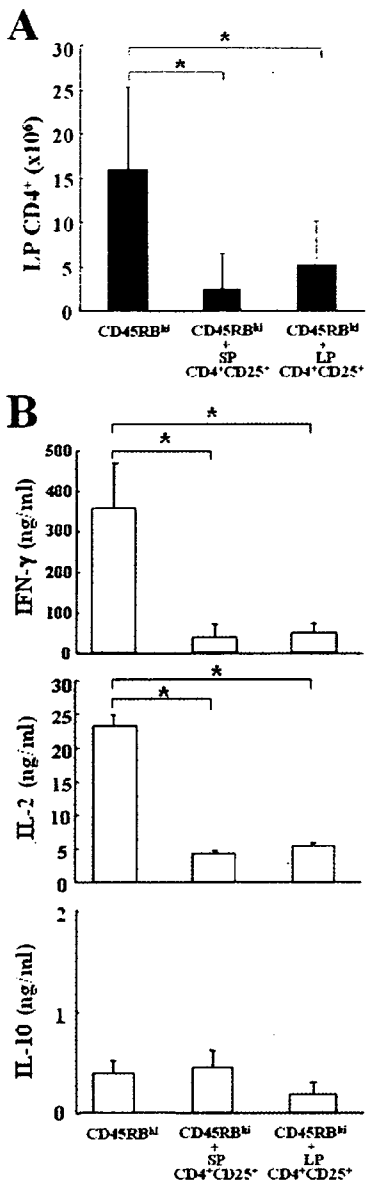

FIGURE 3. Cotransfer of intestinal LP CD4 CD25 T_{REG} cells inhibits the expansion of LP CD4 T cells and Th1 cytokine production in SCID mice transferred with CD4 CD45RB^{high} T cells. Transfer protocol is described in Fig. 2. *A*, Recovered LP CD4 T cells at 7 wk after transfer. Data are indicated as the mean SD of seven mice in each group. , p 0.05 compared with mice transferred with CD45RB^{high} T cells alone. *B*, Cytokine production by LP CD4 T cells. LP CD4 T cells were stimulated with plate-coated anti-CD3 mAb and soluble anti-CD28 mAb for 72 h. Cytokines in the supernatants were measured by ELISA. Data are indicated as the mean SD of seven mice in each group. , p 0.05 compared with mice transferred with CD45RB^{high} T cells alone.

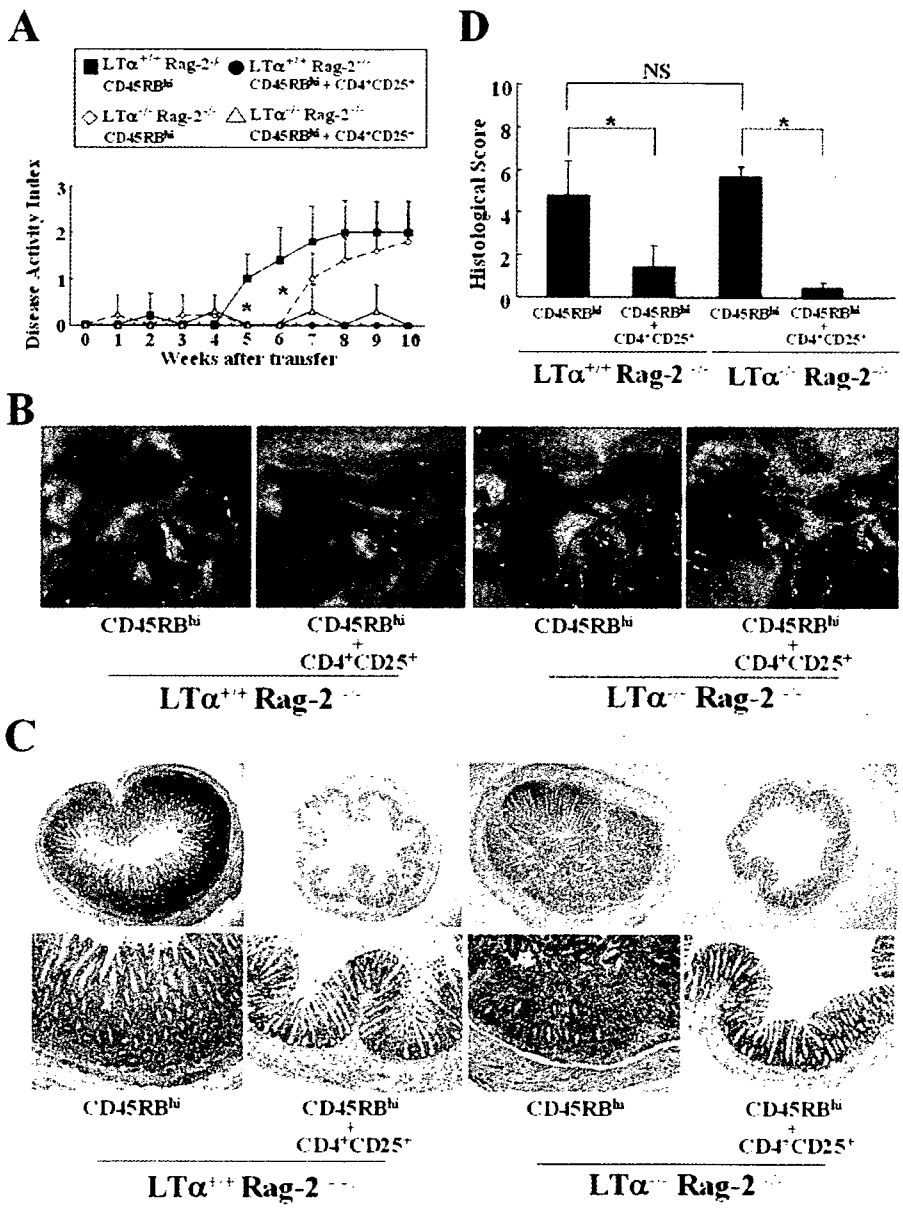

FIGURE 4. Splenic CD4 CD25 T_{REG} cells suppress the development of colitis in LT / Rag2 / mice transferred with CD45RB^{high} T cells. CD4 CD45RB^{high} T cells (3 10^5 cells) from Ly5.2-C57BL/6 congenic mice were injected into Ly5.2 background LT / Rag2 / and LT / Rag2 / mice with or without the cotransfer of 1 10^5 splenic CD4 CD25 T_{REG} cells derived from Ly5.1-C57BL/6 mice (n 7 mice per each group). *A*, Disease activity index during 10 wk after transfer. , p 0.05, LT / Rag2 / mice vs LT / Rag2 / mice, transferred with splenic CD4 CD45RB^{high} T cells and splenic CD4 CD25 T_{REG} cells. *B*, The lack of MLNs in LT / Rag2 / mice. The abdominal MLN area was dissected and examined for the presence or absence of MLNs in LT / Rag2 / mice and LT / Rag2 / mice after adoptive transfer. *C*, Histopathology of distal colon at 10 wk after transfer. Original magnification, 20 (*top*) and 100 (*bottom*). *D*, Histological score at 10 wk after transfer. , p 0.05 compared with the paired LT / Rag2 / or LT / Rag2 / mice transferred with splenic CD4 CD45RB^{high} T cells alone. NS, Not significant.

CD4 CD45RB^{high} and LP CD4 CD25 T cells exhibited no detectable pathological changes and were indistinguishable from colons from mice reconstituted with a mixture of CD4 CD45RB^{high} plus splenic CD4 CD25 T cells (Fig. 2*B*). In contrast, mice reconstituted with CD4 CD45RB^{high} cells alone developed wasting disease and severe colitis (Fig. 2). Totally, the assessment of colitis by clinical scores showed a clear difference among three groups (Fig. 2*C*). Clinical score for mice transferred with a mixture of CD4 CD45RB^{high} and LP CD4 CD25 T cells was significantly decreased as compared with that for mice transferred with CD4 CD45RB^{high} T cells alone. Histological examination showed prominent epithelial hyperplasia with glandular elongation with a massive infiltration of mononuclear cells in the LP of the colon from the control mice transferred with CD4 CD45RB^{high} T cells alone (Fig. 2*D*). In contrast, the glandular elongation was mostly abrogated and only a few mononuclear cells were observed in the colonic LP from mice reconstituted with a mixture of CD4 CD45RB^{high} plus splenic or LP CD4 CD25 T cells (Fig. 2*D*). This difference was also confirmed by histological scores of multiple colon sections, which were 5.63 0.89 in control mice transferred with CD4 CD45RB^{high} T cells alone, 1.21 0.97 in mice transferred with CD4 CD45RB^{high} T cells plus splenic CD4 CD25 T cells, and 2.40 1.83 in mice transferred with CD4 CD45RB^{high} T cells plus LP CD4 CD25 T cells (p 0.05, mice transferred with CD4 CD45RB^{high} T cells alone vs mice transferred with CD4 CD45RB^{high} T cells plus splenic or LP CD4 CD25 T cells) (Fig. 2*E*).

A further quantitative evaluation of CD4 T cell infiltration was made by isolating LP mononuclear cells from the resected colons. A significantly less number of CD4 T cells was recovered from the colonic tissue of mice reconstituted with CD4 CD45RB^{high} and with LP or splenic CD4 CD25 T cells as compared with mice reconstituted with CD4 CD45RB^{high} alone (Fig. 3*A*). To next determine the effect of cotransfer of LP CD4 CD25 T cells on Th1/Th2 development, we measured IFN- , IL-2, and IL-10 production by anti-CD3/CD28 mAb-stimulated LP CD4 T cells.

As shown in Fig. 3*B*, production of Th1 cytokines (IFN- , IL-2) was significantly reduced in LP CD4 T cells from the mice transferred with CD4 CD45RB^{high} plus LP or splenic CD4 CD25 T cells as compared with those transferred with CD4 CD45RB^{high} T cells alone (p 0.05). In contrast, production of IL-10 was not significantly affected among the groups (Fig. 3*B*).

Splenic CD4 CD25 T cells suppress the development of colitis in LT- / Rag2 / mice transferred with CD4 CD45RB^{high} T cells

To further investigate the origin of LP CD4 CD25 T_{REG} cells and their role in suppressing the development of colitis, we generated LT / Rag2 / mice, which lack conventional lymphoid tissues (inductive sites) including MLNs, PPs, and ILFs, as recipients for the adoptive transfer experiments. We excluded the impact of these inductive sites because it was possible that it is essential for LP CD4 CD25 T_{REG} cells to be instructed to differentiate to gut-homing LP T_{REG} cells in these inductive sites. Before addressing this issue, we first transferred splenic CD4 CD45RB^{high} T cells from normal C57BL/6 mice into LT / Rag2 / mice and the littermate control LT / Rag2 / mice to assess the role of MLNs as inductive sites in inducing colitis. When CD4 CD45RB^{high} T cells were transferred into the control LT / Rag2 / mice, expectedly, the recipients rapidly developed severe wasting disease associated with clinical signs of severe colitis, in particular, weight loss, persistent diarrhea and occasionally also bloody stool and anal prolapses (Fig. 4*A*). When CD4 CD45RB^{high} T cells were transferred into the LT / Rag2 / mice, however, the recipients also developed severe wasting chronic colitis despite the delayed onset and kinetics (Fig. 4*A*). Clinical scores in these mice eventually reached almost the same with those in LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells 10 wk after transfer (Fig. 4*A*). The rapid onset of colitis in the recipient LT / Rag2 / mice could easily be explained by the existence of MLNs in these mice and migration of effector CD4 cells primed in the these sites into the colon, but the evidence that the recipient LT / Rag2 / mice, albeit delayed, developed colitis indicates that there must be other sites where CD4 T cells could be primed besides the MLNs. These LT / Rag2 / and LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells had an enlarged colon with a significantly thickened wall 10 wk after the transfer (data not shown). At the autopsy of mice, we confirmed that our established LT / Rag2 / mice macroscopically lacked MLNs (Fig. 4*B*) and other peripheral LNs (data not shown) in contrast to control LT / Rag2 / mice (Fig. 4*B*). Tissue sections from LT / Rag2 / and LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells were characterized by inflammatory infiltrate, epithelial hyperplasia, crypt cell damage, and goblet cell depletion (Fig. 4*C*).

Having evidence that LN-null mice developed chronic colitis induced by the adoptive transfer of CD4 CD45RB^{high} T cells, we next asked whether splenic CD4 CD25 T_{REG} cells can migrate into the LP, and suppress the development of colitis in the absence of MLNs. Expectedly, LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells and splenic CD4 CD25 T_{REG} cells did not show weight loss and clinical signs of colitis throughout the entire observation period (Fig. 4*A*). Of note, LT / Rag2 / mice transferred with a mixture of CD4 CD45RB^{high} T cells and splenic CD4 CD25 T cells also did not manifest clinical signs of colitis (Fig. 4*A*). Consistent with the lack of clinical signs of colitis, LT / Rag2 / or LT / Rag2 / recipients cotransferred with CD4 CD45RB^{high} T cells and splenic CD4 CD25 T cells displayed no histological evidence of intestinal inflammation (Fig. 4*C*). The difference among each group was also confirmed by histological scoring of multiple colon sections, which was 4.85 1.58 in LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells alone, and 1.40 0.96 in LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells plus splenic CD4 CD25 T cells (p 0.05), and 5.60 0.40 in LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells alone, and 0.43 0.23 in LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells plus splenic CD4 CD25 T cells (p 0.05) (Fig. 4*D*).

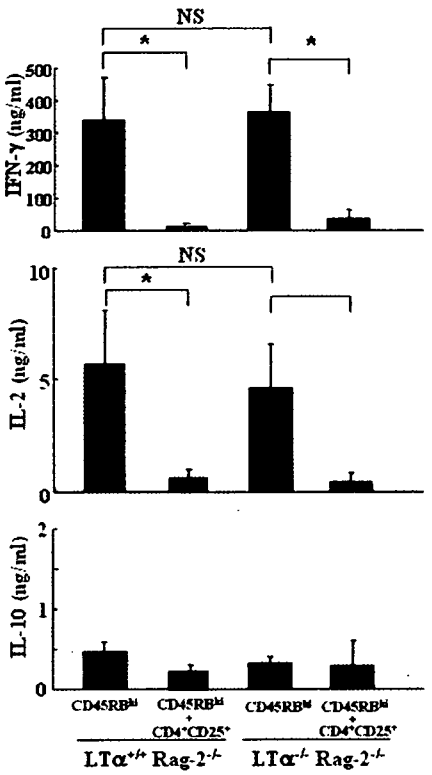

FIGURE 5. Splenic CD4 CD25 T_{REG} cells suppress the production of Th1 cytokines in LT / Rag2 / mice transferred with CD45RB^{high} T cells. CD4 CD45RB^{high} T cells (3 10^5 cells) from Ly5.2-C57BL/6 congenic mice were injected into Ly5.2 background LT / Rag2 / and LT / Rag2 / mice with or without the cotransfer of 1 10^5 splenic CD4 CD25 T_{REG} cells derived from Ly5.1-C57BL/6 mice (n 7 mice per each group) as described in Fig. 4. Cytokine production by LP CD4 T cells was measured by specific ELISA. LP CD4 T cells were stimulated with plate-coated anti-CD3 mAb and soluble anti-CD28 mAb for 72 h. Cytokines in the supernatants were measured by ELISA. Data are indicated as the mean SD of seven mice in each group. , p 0.05 compared with the paired LT / Rag2 / or LT / Rag2 / mice transferred with splenic CD4 CD45RB^{high} T cells alone. NS, Not significant.

We also examined the cytokine production by LP CD4 T cells from each group of mice. As shown in Fig. 5, LP CD4 cells from the LT / Rag2 / and LT / Rag2 / recipients transferred with CD4 CD45RB^{high} T cells alone produced significantly higher amount of IFN- and IL-2 as compared with those transferred with CD4 CD45RB^{high} T cells and splenic CD4 CD25 T cells upon in vitro anti-CD3/CD28 mAbs stimulation. In contrast, the production of IL-10 was not significantly affected.

Consistent with the reduction in the histological scores by the cotransfer of splenic CD4 CD25 T cells, there was also a striking reduction in the recovered number of LP CD4 T cells both in

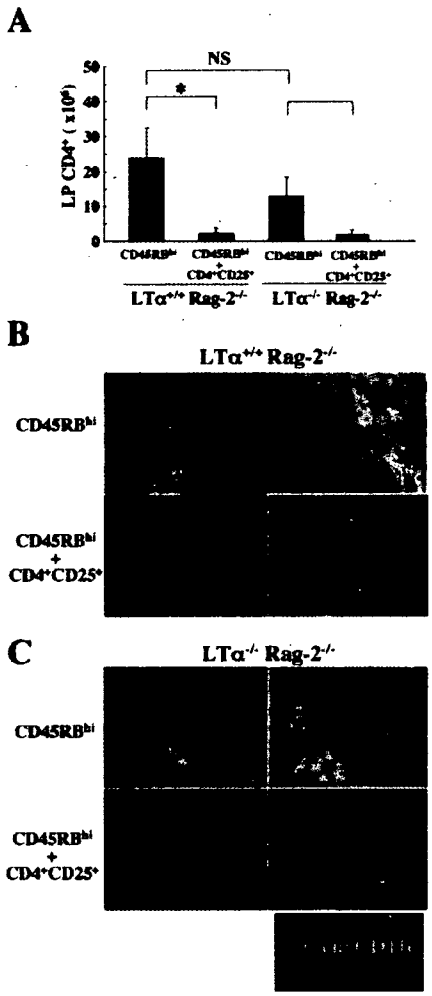

FIGURE 6. Splenic CD4 CD25 T_{REG} cells suppress the expansion of pathogenic LP CD4 T cells in LT / Rag2 / mice transferred with CD45RB^{high} T cells. CD4 CD45RB^{high} T cells (3 10^5 cells) from Ly5.2-C57BL/6 congenic mice were injected into Ly5.2 background LT / Rag2 / and LT / Rag2 / mice with or without the cotransfer of 1 10^5 splenic CD4 CD25 T_{REG} cells derived from Ly5.1-C57BL/6 mice (n 7 mice per each group) as described in Fig. 4. *A*, Recovered LP CD4 T cells at 10 wk after transfer. Data are indicated as the mean SD of seven mice in each group. , p 0.05 compared with the paired LT / Rag2 / or LT / Rag2 / mice transferred with splenic CD4 CD45RB^{high} T cells alone. *B*, Distribution of CD11c dendritic cells (green) and CD4 T cells (red) in the colon after adoptive transfer. Original magnification, 100 (*left*) and 400 (*right*).

LT / Rag2 / and LT / Rag2 / mice transferred with a mixture of CD4 CD45RB^{high} T cells and splenic CD4 CD25 T cells 10 wk after transfer (Fig. 6*A*) compared with those in the paired recipients transferred with CD4 CD45RB^{high} T cells alone. To further assess the role of LP as inductive and/or suppressive site, the expression of CD11c in the colon was investigated by immunohistochemistry (Fig. 6*B*). Immunohistochemical analysis of the colons revealed that significant numbers of CD11c dendritic cells were surrounded by many CD4 T cells in both in LT / Rag2 / and LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells alone, but with few CD4 T cells and CD11c cells in LT / Rag2 / and LT / Rag2 / mice transferred with a mixture of CD4 CD45RB^{high} T cells and splenic CD4 CD25 T cells (Fig. 6*B*), suggesting a possible role of LP as a site for actively interacting between CD11c dendritic cells and CD4 T cells.

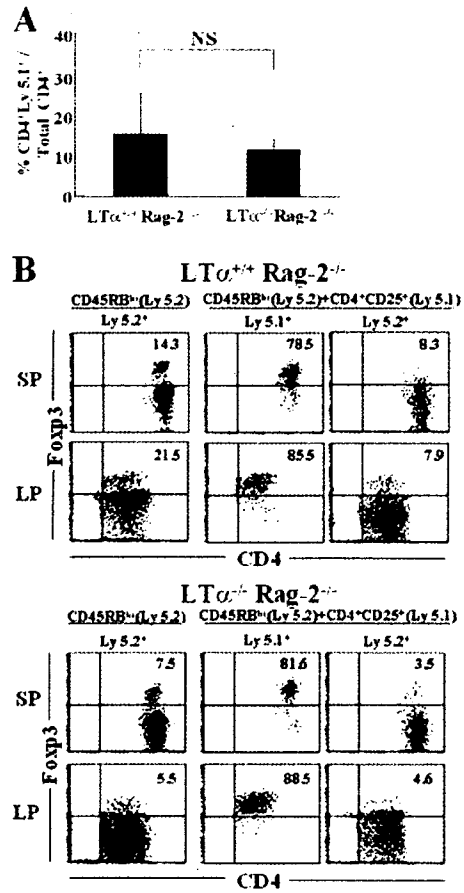

FIGURE 7. Splenic CD4 CD25 T_{REG} cells migrate into the colonic LP and are sustained in the LP in LT / Rag2 / mice transferred with CD45RB^{high} T cells. *A*, The ratio of CD4 CD25 T_{REG} (Ly5.1) cells to total CD4 T cells (Ly5.1 Ly5.2) at 10 wk after transfer was analyzed by gating Ly5.1 or Ly5.2 on CD4 cells. Results shown are from seven mice per group. NS, Not significant. *B*, Spleen (SP) and LP cells were collected and labeled for Ly5.1, Ly5.2, CD4, and intracellular Foxp3. Ly5.2 and Ly5.1 CD4 cells were gated and analyzed for the presence of converted Ly5.2 CD4 Foxp3 cells and Ly5.1 CD4 Foxp3 cells, respectively. Number in *upper right quadrant* represents the percentage of inducible CD4 Foxp3 cells per CD4 cells.

Splenic CD4 CD25 T cells migrate into the intestinal LP in LT / Rag2 / mice

To next assess the in vivo expansion of CD4 CD45RB^{high} T cells and CD4 CD25 T_{REG} cells after adoptive transfer at a 3:1 ratio (CD4 CD45RB^{high} (Ly5. 2) to CD4 CD25 T cells (Ly5.1)), colonic LP CD4 cells were analyzed for the ratio of Ly5.2- to Ly5.1-derived cells. As shown in Fig. 7*A*, 10–15% of total LP CD4 T cells both in LT / Rag2 / and LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells plus splenic CD4 CD25 T cells were derived from Ly5.1 CD4 CD25 T cells in the colon. These results suggested that splenic CD4 CD25 T cells migrated to the colon, and prevented the development of colitis primarily by inhibiting the expansion and/or infiltration of pathogenic CD4 T cells in the colon and secondarily by inhibiting the development of pathogenic Th1 cells producing IFN- and IL-2.

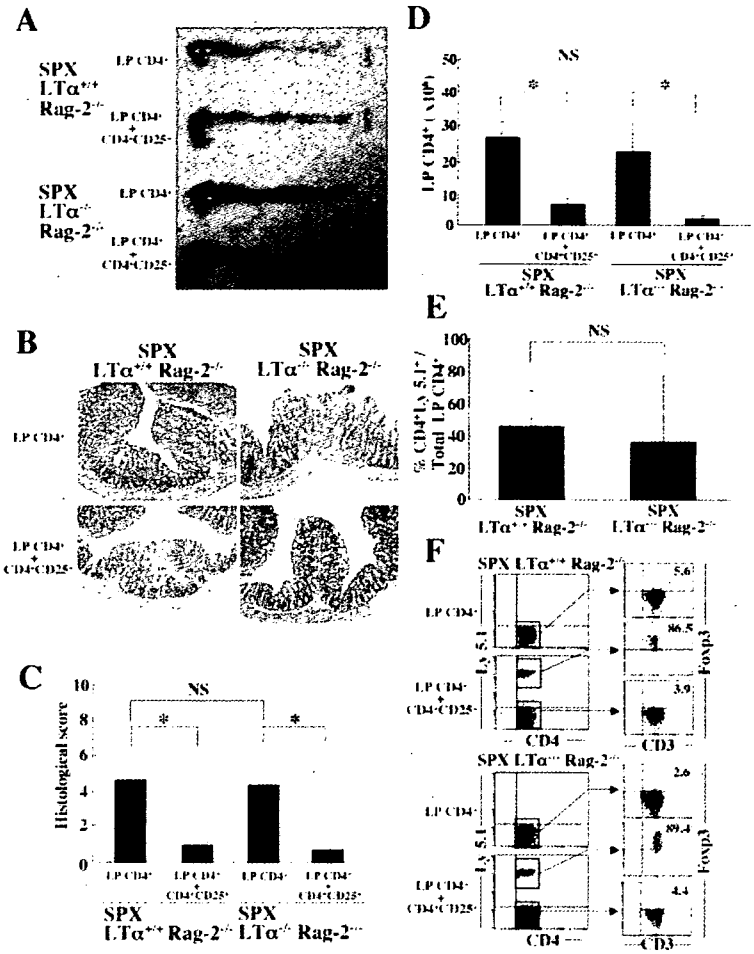

FIGURE 8. Splenic CD4 CD25 T_{REG} cells migrate into the gut and inhibit the development of colitis induced by adoptive transfer of colitogenic LP CD4 T cells into splenectomized (SPX) LT / Rag2 / mice. Seven Rag2 / mice in each group were injected i.p. with the following T cell subpopulations: 1) colitogenic LP Ly5.2 CD4 T cells (3 10^5 cells) into splenectomized LT / Rag2 / mice; 2) colitogenic LP Ly5.2 CD4 T cells (3 10^5 cells) splenic Ly5.1 CD4 CD25 T cells (3 10^5 cells) into splenectomized LT / Rag2 / mice; 3) colitogenic LP Ly5.2 CD4 T cells (3 10^5 cells) into splenectomized LT / Rag2 / mice; or 4) colitogenic LP Ly5.2 CD4 T cells (3 10^5 cells) splenic Ly5.1 CD4 CD25 T cells (3 10^5 cells) into splenectomized LT / Rag2 / mice. *A*, Gross appearance of the colon and MLN at 7 wk after transfer. *B*, Histopathology of distal colon at 7 wk after transfer. Original magnification, 40. *C*, Histological score at 7 wk after transfer. , p 0.05. *D*, Number of recovered LP CD4 T cells at 7 wk after transfer. Data are indicated as the mean SD of seven mice in each group. , p 0.05. *E*, Percentage of CD4 CD25 T_{REG} (Ly5.1) cells to total CD4 T cells (Ly5.1 Ly5.2) at 7 wk after transfer was analyzed by gating Ly5.1 or Ly5.2 on CD4 cells. Results shown are from seven mice per group. NS, Not significant. *F*, LP cells were collected and labeled for Ly5.1, Ly5.2, CD4, and Foxp3. Ly5.2 and Ly5.1 CD4 cells were gated and analyzed for the presence of Foxp3 cells. The percentage of induced Foxp3 cells per CD3 cells is indicated in *upper right quadrant* of enlarged gate.

However, it was also possible that a part of Ly5.2-derived CD4 CD45RB^{high} T cells was converted into inducible CD4 CD25 T_{REG} cells rather than pathogenic CD4 T cells in the gut. Thus, to evaluate this possibility that the LP CD4 CD25 T_{REG} cells are composed of naturally arising CD4 CD25 T cells (Ly5.1), inducible CD4 CD25 T cells (Ly5.2) and pathogenic CD4 T cells (Ly5.2), we performed three-color flow cytometry analysis (Fig. 7*B*). In this setting, we stained intracellular Foxp3 because it was difficult to distinguish between activated/pathogenic CD4 CD25 T cells and CD4 CD25 T_{REG} cells by staining CD25 molecule. Indeed, only 3–16% of splenic and LP Ly.5.2 cells were converted into inducible CD4 Foxp3 T_{REG} cells in both LT / Rag2 / and LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells alone or with a mixture of CD4 CD45RB^{high} T cells and splenic CD4 CD25 T cells, but most Ly.5.1-derived CD4 CD25 T_{REG} cells (78–88%) retained Foxp3 in both LT / Rag2 / and LT / Rag2 / mice transferred with a mixture of CD4 CD45RB^{high} T cells and splenic CD4 CD25 T cells (Fig. 7*B*).

Splenic CD4 CD25 T cells suppressed the expansion of colitogenic LP CD4 T cells in the gut

With respect to the site for suppression of effector and memory CD4 T cells, it was also possible that naturally arising CD4 CD25 T cells suppress the activation of CD4 CD45RB^{high} T cells and the expansion of the differentiated effector CD4 T cells in the spleen rather than in the gut. To clarify that CD4 CD25 T cells suppress the expansion of colitogenic effector and memory CD4 T cells in the gut, we finally transferred colitogenic LP CD4 T cells obtained from colitic mice transferred with CD4 CD45RB^{high} T cells (Ly5.2) (19) with or without splenic CD4 CD25 T cells (Ly5.1) into splenectomized LT / Rag2 / and LT / Rag2 / mice to exclude the impact of spleen. Both splenectomized LT / Rag2 / and LT / Rag2 / mice transferred with colitic LP CD4 T cells (Ly5.2) developed wasting disease (data not shown) and colitis by assessing histological findings (Fig. 8, *A–C*) and the recovered CD4 cell numbers from the LP (Fig. 8*D*). In contrast, splenectomized LT / Rag2 / and LT / Rag2 / mice transferred with a mixture of colitic LP CD4 T cells (Ly5.2) and splenic CD4 CD25 T cells (Ly5.1) at a 1:1 ratio did not develop wasting disease (data not shown) and colitis (Fig. 8, *A–D*). Of note, we found that 30–40% of LP CD4 T cells in splenectomized LT / Rag2 / and LT / Rag2 / mice cotransferred with a mixture were derived from Ly5.1 cells (Fig. 8*E*). Furthermore, we confirmed that CD4 Foxp3 T cells residing in the LP were mostly derived from Ly5.1 population in both splenectomized LT / Rag2 / and LT / Rag2 / mice transferred with a mixture of colitic LP CD4 T cells (Ly5.2) and CD4 CD25 T cells (Ly5.1) (Fig. 8*F*), indicating that LP acts as a suppressive site, and spleen is not solely essential to act as a suppressive site to inhibit the expansion of effector CD4 T cells.

Discussion

In this study, we demonstrate that intestinal LP CD4 CD25 T cells residing in normal mice constitutively express CTLA-4, GITR, and Foxp3 and suppress the proliferation of responder CD4 T cells in vitro. Furthermore, cotransfer of intestinal LP CD4 CD25 T cells prevents the development of CD4 CD45RB^{high} T cell-transferred colitis. Surprisingly, when LT / Rag2 / mice, which lack MLNs, ILFs, and PPs, were transferred with CD4 CD45RB^{high} T cells, they did develop severe wasting disease and colitis despite the delayed onset and kinetics as compared with the control LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells. Of note, splenic CD4 CD25 T cells can migrate into the LP, and prevent the development of CD4 CD45RB^{high} T cell-transferred colitis in MLN-null LT / Rag2 / recipient mice. These results suggest that at least in part intestinal LP CD4 CD25 T cells without the instruction by an MLN environment directly migrate into the gut and act as T_{REG} cells, and therefore may contribute to the intestinal immune homeostasis in vivo.

We have recently demonstrated that human CD4 CD25^{bright} T cells resided in the intestinal LP, expressed CTLA-4, GITR, and Foxp3, and possessed T_{REG} activity in vitro (10). Although the results indicate that these cells might serve as mucosal (nonlymphoid) T_{REG} cells to maintain intestinal homeostasis against many luminal Ags, it was impossible to determine whether they actually suppress the development of colitis in vivo using any human studies. To answer the question, it was necessary to translate into the mouse experimental system. To address this issue, we proceeded with two approaches using the different adoptive transfer experiments in this study. We first directly assessed whether the cotransfer of murine intestinal LP CD4 CD25 T cells isolated from normal mice suppress the development of colitis induced by the adoptive transfer of CD4 CD45RB^{high} T cells into SCID mice. As shown in Fig. 2, we found 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, indicating that the murine intestinal LP CD4 CD25 T cells maintain intestinal homeostasis to suppress the development of colitis in vivo. Consistent with this, murine intestinal LP CD4 CD25 T cells expressed constitutively CTLA-4, GITR, and Foxp3 and suppressed the proliferation of responder cells in vitro, such as human LP CD4 CD25^{bright} T cells (10). Furthermore, because we also found that LP CD4 CD25 T cells did partially express $_{4\ 7}$ and $_{E\ 7}$ integrins, it is conceivable that these gut-homing receptor-expressing LP CD4 CD25 T cells might migrate into the colon from outside of the gut. Although it has been reported that CD4 CD25 T_{REG} cells reside in nonlymphoid tissues (10–18), our current data now provide the first experimental evidence that intestinal LP CD4 CD25 T cells prevent the development of colitis in vivo.

Having the 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 next asked whether MLNs are not fully essential for the suppression of colitis by splenic CD4 CD25 T cells because it was still possible that 1) a part of the LP CD4 CD25 T cells was needed to be instructed in MLNs to differentiate to gut-homing receptor-expressing T_{REG} cells (17) to migrate to the gut, and also possible that 2) the transferred LP CD4 CD25 T cells acted as T_{REG} cells in MLNs rather than in the intestine in the first adoptive transfer experiment (Figs. 2 and 3). To address these issues, it was important to assess the CD4 CD25 T_{REG} cells without the impact of MLNs, which are thought to be representative inductive and suppressive sites for classical splenic CD4 CD25 T_{REG} cells because high expression levels of CD62 ligand enable both naive CD4 T cells and splenic CD4 CD25 T_{REG} cells to efficiently enter the Ag-draining lymph nodes from the bloodstream. As the second approach to address this issue, thus, we generated LT / Rag2 / mice as recipients for the following adoptive transfer experiment. Before starting the experiment, it was unclear whether the LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells alone develop colitis, or rather it was likely to envisage that these mice did not develop colitis because MLNs are thought to be very important as inductive sites for the development of colitis. However, it was noteworthy that these mice did develop wasting disease and colitis to a similar extent of the transferred LT / Rag2 / mice 10 wk after transfer, although it took a longer period to establish colitis as compared with the LT / Rag2 / recipients (Fig. 4A). Although this fact is actually not a main focus in this study, it is possible that spleen and/or LP are complimentary inductive sites to develop colitis under the absence of MLNs. Consistent with this hypothesis, it has been reported that naive T cells can recruit to the inflamed intestinal mucosa, although these cells are usually excluded from uninflamed nonlymphoid tissues (20). However, the delayed kinetics of the development of colitis in the LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells indicates that MLNs are involved in the induction of colitis by their functioning as a professional inductive site. Further study will be needed to address this initial immune response for the development of colitis.

As our focus in this study, we also found that the cotransfer of splenic CD4 CD25 T cells obtained from normal mice prevent the development of colitis in LT / Rag2 / mice transferred with CD4 CD45RB^{high} T cells as well as in LT / Rag2 / recipients, indicating that splenic CD4 CD25 T cells can suppress the development of colitis in the absence of MLNs. Moreover, we demonstrated that Ly5.1-CD4 CD25 T cells resided in the colon in MLN-null LT / Rag2 / 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_{REG} cells to suppress intestinal inflammation probably as a second line of suppression (17). It was also possible, however, that CD4 CD25 T_{REG} cells prevented the expansion of pathogenic effector CD4 T cells and the migration to the gut in the recipient's spleen rather in the gut. With respect to this issue, we also demonstrated that cotransfer of splenic CD4 CD25 T cells prevented the development of colitis induced by adoptive transfer of colitogenic LP CD4 T cells in splenectomized LT / Rag2 / recipients (Fig. 8). Because colitogenic LP CD4 T cells that have a phenotype of effector/memory CD4 CD44^{high} CD62L cells (21) should have migrated to the gut and expanded in the gut, it is very likely that splenic CD4 CD25 T cells can directly migrate to the gut and suppress the expansion of these colitogenic CD4 T cells in the gut. With respect to the equilibrium of pathogenic CD4 T cells and T_{REG} cells, however, further studies will be needed because we found that effector to T_{REG} cell ratio varied by different experimental settings (Figs. 6 and 8).

Finally, it should be discussed the protective mechanism by CD4 CD25 T_{REG} cells of this SCID/Rag2 / colitis model induced by the adoptive transfer of CD4 CD45RB^{high} T cells from the standpoint of the sites of active suppression. Indeed, Mottet et al. (18) previously demonstrated that not only effector CD4 T cells but also CD4 CD25 T_{REG} cells accumulate in the intestinal LP in addition to the MLNs in the cured SCID mice by retransferring splenic CD4 CD25 T cells 3–4 wk after the first transfer

of CD4 CD45RB^{high} T cells, it remains to be determined whether intestinal inflammation can be suppressed solely by LP or MLN CD4 CD25 T_{REG} cells in this setting. In contrast, Denning et al. (8) recently demonstrated that $_7$ integrin-deficient ($_7$ ′) CD4 CD25 T_{REG} 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 $_4$ $_7$/ $_E$ $_7$ integrin molecules, are capable of preventing intestinal inflammation, suggesting T_{REG} accumulation in the intestine is dispensable for the protection of this colitis model. In this protection protocol, indeed, it is possible that $_7$ ′ CD4 CD25 T_{REG} cells are not needed to suppress the development of colitis because $_7$ ′ CD4 CD25 T_{REG} cells directly migrate to MLNs and can inhibit naive CD4 CD45RB^{high} T cell activation and proliferation within Ag-draining MLNs, resulting in suppressing the development of the gut-seeking activated effector CD4 T cells instructed to express the gut-homing receptors such as $_4$ $_7$/ $_E$ $_7$ intergrin. However, it still remains unknown whether mucosal CD4 CD25 T_{REG} cells are necessary for the suppression of mucosal pathogenic effector CD4 T cell ex vivo especially in the therapeutic protocol that can be assessed and whether LP CD4 CD25 T_{REG} cells as effector T_{REG} cells can suppress the surrounding LP effector CD4 T cells ex vivo. In our adoptive transfer experiment using splenectomized MLN-null LT ′ Rag2 ′ mice, however, we clearly demonstrated that cotransfer of splenic CD4 CD25 T_{REG} cells suppressed the development of colitis despite the lack of spleen and MLNs and found that these T_{REG} cells migrated to the effector sites, in this case, the intestine, suggesting that intestinal LP CD4 CD25 T_{REG} cells play an important role at least in part for the suppression of intestinal inflammation in the gut.

In conclusion, our findings showed that intestinal LP functions not only as a critical effector site for inflammatory responses but also as a regulatory (suppressive) site that CD4 CD25 T_{REG} cells directly control the pathogenic effector CD4 cells as a second line of suppression (effector T_{REG}) site together with the MLNs as a first line of suppression (naive T_{REG}) site.

Disclosures

The authors have no financial conflict of interest.

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Exacerbating Role of γδ T Cells in Chronic Colitis of T-Cell Receptor α Mutant Mice

MASANOBU NANNO,[*] YASUYOSHI KANARI,[‡] TOMOAKI NAITO,[§,‖] NAGAMU INOUE,[¶] TADAKAZU HISAMATSU,[¶] HIROSHI CHINEN,[¶] KEN SUGIMOTO,[#,**] YASUYO SHIMOMURA,[#,**] HIDEO YAMAGISHI,[‡] TETSUO SHIOHARA,[‡‡] SATOSHI UEHA,[§§] KOUJI MATSUSHIMA,[§§] MAKOTO SUEMATSU,[‖] ATSUSHI MIZOGUCHI,[#,**] TOSHIFUMI HIBI,[¶] ATUL K. BHAN,[#,**] and HIROMICHI ISHIKAWA[§]

[*]Yakult Central Institute for Microbiological Research, Tokyo; [‡]Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto; [§]Department of Microbiology and Immunology, Keio University School of Medicine, Tokyo; [‖]Department of Biochemistry and Integrative Medical Biology, Keio University School of Medicine, Tokyo; [¶]Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan; [#]Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital, Boston; [**]Department of Pathology, Massachusetts General Hospital, Boston, Massachusetts; [‡‡]Department of Dermatology, Kyorin University School of Medicine, Tokyo, Japan; and [§§]Department of Molecular Preventive Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Background & Aims: **T-cell receptor (TCR) γδ T cells are an important component of the mucosal immune system and regulate intestinal epithelial homeostasis. Interestingly, there is a significant increase in γδ T cells in the inflamed mucosa of patients with ulcerative colitis (UC). However, the role of γδ T cells in chronic colitis has not been fully identified.** ***Methods:*** **TCRα-deficient mice, which spontaneously develop chronic colitis with many features of human UC including an increase in γδ T-cell population, represent an excellent model to investigate the role of γδ T cells in UC-like colitis. To identify the role of γδ T cells in this colitis, we herein have generated TCRγ-deficient mice through deletion of all TCR Cγ genes (Cγ1, Cγ2, Cγ3, and Cγ4) using the Cre/*loxP* site-specific recombination system and subsequently crossing these mice with TCRα-deficient mice.** ***Results:*** **An increase in colonic γδ T cells was associated with the development of human UC as well as UC-like disease seen in TCRα-deficient mice. Interestingly, the newly established TCRα$^{-/-}$ × TCRγ$^{-/-}$ double mutant mice developed significantly less severe colitis as compared with TCRα-deficient mice. The suppression of colitis in TCRα$^{-/-}$ × TCRγ$^{-/-}$ double mutant mice was associated with a significant reduction of proinflammatory cytokine and chemokine productions and a decrease in neutrophil infiltration.** ***Conclusions:*** **γδ T cells are involved in the exacerbation of UC-like chronic disease. Therefore, γδ T cells may represent a promising therapeutic target for the treatment of human UC.**

T cell receptor (TCR) γδ T cells are an evolutionary conserved T-cell subset with characteristic properties.[1] TCRγδ-bearing murine dendritic epidermal T cells are involved in the regulation of epidermal integrity and promote wound repair of the skin,[2] whereas intestinal intraepithelial γδ T cells (γδ-IEL) regulate intestinal epithelial homeostasis.[3,4] Recent evidence suggests that γδ T cells are also important in immune surveillance of the epithelium by providing a first line of defense against infectious pathogens attacking the surfaces of the body and in the regulation of linking of innate and acquired immunity.[1,5] Furthermore, γδ T cells appear to down-regulate αβ T cell-driven robust immune responses that often result in severe immunopathology.[1]

The incidence of inflammatory bowel diseases (IBD), namely ulcerative colitis (UC) and Crohn's disease (CD), has increased markedly in recent years. The factors including genetic predisposition, environmental conditions, and aberrant immune response driven by normal intestinal flora are vital for the development and persistence of the inflammatory process.[6,7] In the present study, we aimed at elucidating the role of γδ T cells in the pathogenesis of IBD because there is growing evidence supporting that γδ T cells play an active multifaceted immunoregulatory role in the coordinated innate and acquired immune responses that maintain the integrity of epithelial tissues[1,2,4,5,8] and an increase in γδ T cells in the diseased mucosa has been documented in UC patients.[9,10] In acute colitis induced by administration of either 2,4,6-trinitrobenzene sulfonic acid[11,12] or dextran sulfate sodium,[13,14] a protective role of γδ T cells has been demonstrated. However, the role of γδ T cells in chronic intestinal inflammation resembling UC has not yet been investigated. TCRα$^{-/-}$ (α$^{-/-}$) mice spontaneously develop chronic colitis with several features of human UC including a significant increase in γδ T cells.[15] To illuminate the role of γδ T cells in the pathogenesis of UC-like colitis in α$^{-/-}$ mice, we generated TCRγ$^{-/-}$

Abbreviations used in this paper: α$^{-/-}$, TCRα$^{-/-}$; ARP, anorectal prolapse; γ$^{-/-}$, TCRγ$^{-/-}$; γδ T cells, TCR γδ T cells; IBD, inflammatory bowel disease; IEL, intestinal intraepithelial T lymphocytes; LP, lamina propria; TCR, T-cell receptor; UC, ulcerative colitis.

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($\gamma^{-/-}$) mice and examined the severity of colitis in $\alpha^{-/-}$ mice that are genetically engineered to lack γδ T cells.

Materials and Methods

Mice

We newly generated $\gamma^{-/-}$ mice and crossed with $\alpha^{-/-}$ mice[16] to develop double mutant ($\alpha^{-/-} \times \gamma^{-/-}$) mice. The generations of these mice are described in Supplementary Materials (see Supplemental Materials online at www.gastrojournal.org). All mice used were of C57BL/6 (B6) background. The mice were maintained under specific pathogen-free conditions, and all animal procedures described in this study were performed in accordance with the guidelines for animal experiments of Keio University School of Medicine, Yakult Central Institute for Microbiological Research, Kinki University School of Medicine, and Massachusetts General Hospital.

Flow Cytometry and Immunohistochemical Procedures

Methods for isolation of intestinal intraepithelial T cells (IEL) from mouse small intestines and lamina propria (LP) cells from mouse and human large intestines are described in Supplementary Materials. Procedures of cell staining for flow cytometric and immunohistochemical analyses are also described in Supplementary Materials (see Supplemental Materials online at www.gastrojournal.org).

Histologic Evaluation of Colitis

The disease score of colitis (0–10) was estimated in a blind fashion using previously described criteria, namely, a combination of both gross and histologic findings.[17] The gross score was rated as 0, presence of normal beaded appearance; 1, absence of beaded appearance of colon; 2, focally thickened colon; and 3, marked thickness of entire colon. The histologic score was based on the extent of intestinal wall thickening (0–3), inflammatory cell infiltration into LP (0–3), and presence (0 or 1) of ulceration.

Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted from half of the frozen colonic tissue obtained from each one of wild-type (WT), $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ littermate mice, and complementary DNA (cDNA) was prepared. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) was conducted to assess the expression level of TNF-α, IL-1β, IL-6, TGF-β, IFN-γ, IL-7, IL-10, IL-12, KC, MIP-2, GCP-2, MCP-1, MIP-1α, MIP-1β, and HPRT genes using TaqMan probes (Applied Biosystems, Foster City, CA). The relative expression level of genes of interest was normalized to the HPRT gene expression. The detailed procedures are described in Supplementary Materials (see Supplemental Materials online at www.gastrojournal.org).

Measurement of Cytokines and Chemokines by Enzyme-Linked Immunosorbent Assay

Proteins were extracted from the above-described half of the frozen colonic tissue obtained from each one of WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ littermate mice. In brief, frozen colonic tissue was homogenized with a sonicator (Ultrasonic Disruptor UD-201, TOMY, Tokyo, Japan) in 5 mL lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, and complete, Mini EDTA-free proteinase inhibitor [Roche Applied Science, Mannheim, Germany]), the homogenate was clarified by centrifugation at 14,000 rpm for 10 minutes, and the supernatant was subjected to OptEIA ELISA (BD Biosciences, San Diego, CA) for detection of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 and to DuoSet ELISA (R&D Systems, Minneapolis, MN) for detection of transforming growth factor (TGF)-β, interferon (IFN)-γ, keratinocyte-derived chemokine (KC), macrophage inflammatory protein (MIP)-2 and granulocyte chemotactic protein (GCP)-2. Levels in the supernatants were standardized to the total amount of protein in the same supernatants assessed by RC DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Chemotaxis Assay

The assays were performed using the ChemoTx 96-well plate No. 101-3 (NeuroProbe, Gaithersburg, MD). Briefly, bone marrow cells collected from femurs, tibias, and humerus of WT mice were recovered by centrifugation at the interphase of 44% and 70% Percoll solutions. Subsequently, 2.5×10^5 bone marrow cells were loaded onto the membrane plate and placed on a flat-bottomed, 96-well microtiter plate containing the colon extracts (0.7 mg protein/mL) from WT, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice in addition to serially diluted MIP-2 and MCP-1 (R&D Systems). To identify the neutrophils and monocytes, bone marrow cells were labeled with fluorescent dye conjugated monoclonal antibodies (mAb) to Mac-1 and Ly-6C before the assay. After incubation at 37°C for 2 hours, the number of Mac-1^+Ly-$6C^{low}$ neutrophils[18] and Mac-1^+Ly-$6C^{high}$ monocytes[18] that migrated into the lower wells was determined by a flow cytometry.

Cell Transfer

γδ T cells were purified from the mesenteric lymph nodes (MLNs) and colon of $\alpha^{-/-}$ mice through MACS system, and 2×10^6 purified cells were intravenously transferred twice into $\alpha\gamma^{-/-}$ mice (n = 16) at 4 and 5 months of age. As control group, phosphate-buffered saline (PBS) was intravenously administrated into $\alpha\gamma^{-/-}$ mice (n = 15). The recipient mice were then killed at 6 months of age.

Statistical Analysis

The statistical difference was determined by 2-sided Student *t* test. For the statistical analysis of cell infiltration into the large intestine, 2-sided Mann-Whitney *U* test was used. Difference with $P < .05$ was considered significant.

Results

Generation of TCRγ-Deficient Mice

To begin with, we initially confirmed that γδ T cells were increased in the lymphoid cells isolated from the inflamed colonic mucosa of UC patients as compared with those from the unaffected colonic mucosa of patients with colon cancer (Figure 1*A* and *B*) and also in the lymphoid cells isolated from the inflamed colonic LP of $\alpha^{-/-}$ mice as compared with those from normal colonic LP of age-matched WT littermate mice (Figure 1*C* and *D*).

Precise appreciation of the role of γδ T cells in pathogenesis of colitis in $\alpha^{-/-}$ mice requires the generation of $\alpha^{-/-}$ mice deficient in γδ T cells. However, the previously generated TCRδ$^{-/-}$ ($\delta^{-/-}$) mice[19] lacking γδ T cells could not be used for this purpose because of the genomic localization of TCRδ coding segments within the *V* and *J* segments of TCRα gene.[20] To overcome this difficulty, we newly generated TCRγ$^{-/-}$ mice by disrupting the genes encoding TCR Cγ1, 2, 3, and 4 (CγΔ) using the Cre/*loxP* site-specific recombination system shown in Figure 2. The targeting vector pCγ4ΔNL carrying a *loxP*-flanked *pgk-neo* gene cassette in place of exon 1 of the Cγ4 gene (Figure 2*A*) was introduced into the embryonic stem (ES) clone Vγ6ΔL carrying the allele in which the Vγ6 region was replaced by a single *loxP* site (Figure 2*B*). Transfected cells were cultured in the presence of G418, and G418-resistant recombinant clones showing the joint transmission of Vγ6ΔL and Cγ4ΔNL genes were selected. These ES clones, including the clones carrying both transgenes on the same chromosome, Vγ6ΔL-Cγ4ΔNL (Figure 2*C*), were injected into B6 blastocysts. The chimeric mice obtained were crossed to the CAG-*cre* transgenic B6 mice to generate the Cγ1-, 2-, 3-, and 4-depleted TCRγ-deficient (CγΔ) allele (Figure 2*C*) by *cre*-mediated recombination in F_1 mice during embryonic development.

Subsequently, these F_1 mice were intercrossed to produce homozygous ($\gamma^{-/-}$) mice (Figure 2*D*), and these mutant $\gamma^{-/-}$ mice were backcrossed 8 times to B6 mice to obtain $\gamma^{-/-}$ mice carrying the B6 background. WT ($\alpha^{+/-} \times \gamma^{+/-}$), $\gamma^{-/-}$ ($\alpha^{+/-} \times \gamma^{-/-}$), $\alpha^{-/-}$ ($\alpha^{-/-} \times \gamma^{+/-}$), and $\alpha\gamma^{-/-}$ ($\alpha^{-/-} \times \gamma^{-/-}$) littermate F_2 mice were then produced by intercrossing $\alpha^{-/-}$ mice[16] with $\gamma^{-/-}$ mice. Flow cytometric analysis of IEL from the small intestine confirmed that γδ T cells were absent in $\gamma^{-/-}$ and $\alpha\gamma^{-/-}$ mice (Figure 2*E*).

Pathogenic Role of γδ T Cells in UC-Like Colitis

Histologic examination of the colons from 20- to 32-week-old $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice revealed that inflam-

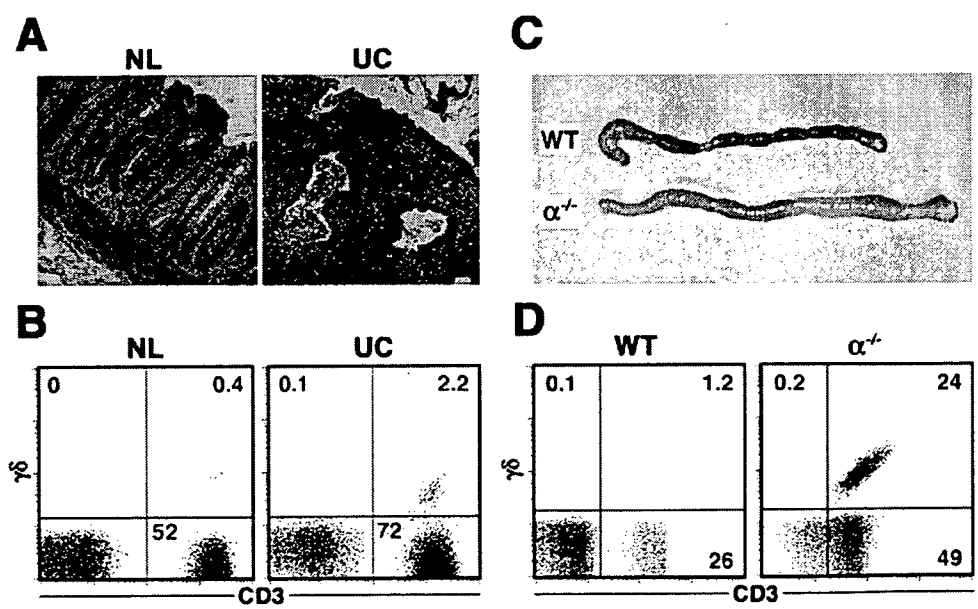

Figure 1. γδ T cells concentrate in the inflamed colonic mucosa of UC patients and colonic LP of $\alpha^{-/-}$ mice suffering from spontaneous chronic colitis. (*A*) A representative colonic tissue section from an ulcerative colitis (*UC*) patient shows a marked infiltration of lymphomyeloid cells, mucosal distortion, crypt abscess, and depletion of goblet cells compared with a normal colonic tissue section (*NL*) (original magnification, ×100). (*B*) A flow cytometry shows increased γδ T-cell population in a diseased colonic LP of UC patient compared with that in a normal colonic LP. This is a representative result of 3 UC patients. (*C*) Large intestines from wild-type (*WT*) mice and $\alpha^{-/-}$ mice suffering from spontaneous chronic colitis are shown. (*D*) A flow cytometry shows increased γδ T-cell population in a diseased colonic LP of $\alpha^{-/-}$ mice compared with that in colonic LP of WT littermate.