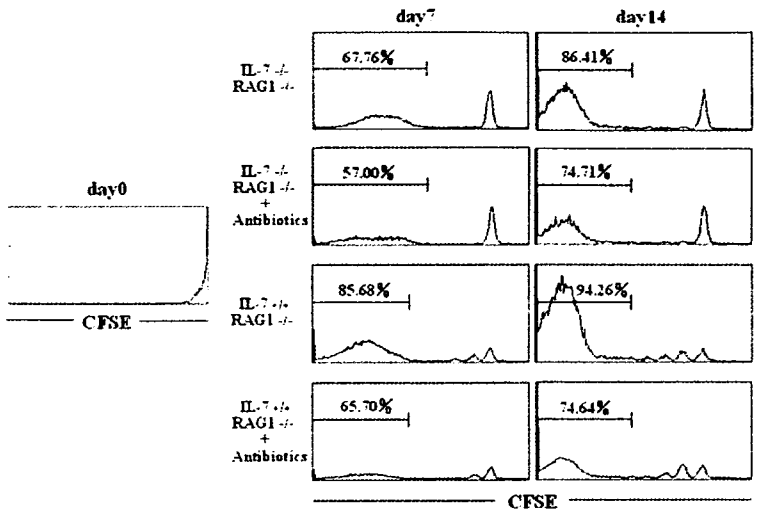

FIGURE 6. Commensal bacteria-partially dependent rapid proliferation and IL-7-dependent slow proliferation. Aliquots (3 10^6/mouse) of CFSE-labeled CD4 CD25 cells obtained from normal spleen were injected into IL-7 / RAG-1 / and IL-7 / RAG-1 / mice treated with or without a set of four antibiotics (ampicillin,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

cells (Fig. 4*D*) were 4.83 0.98 in IL-7 / 3 IL-7 / mice (Fig. 4*E*). In contrast, IL-7 / 3 IL-7 / recipients appeared healthy and did not exhibit any signs of colitis (Fig. 4*B*), with gradual increase of body weight and no apparent thickening of the colonic wall (Fig. 4*C*). No evident pathological changes were observed in the colon (Fig. 4*D*). This histological difference was also confirmed by histological scoring, which was 0 in IL-7 / 3 IL-7 / recipient mice (p 0.005 as compared with IL-7 / 3 IL-7 / mice) (Fig. 4*E*). The average recovered numbers of LP and splenic CD4 T cells from colitic IL-7 / 3 IL-7 / recipients were 173.7 11.2 10^5 cells/colon (Fig. 4*F*) and 51.5 11.3 10^5 cells/spleen (Fig. 4*G*), respectively, whereas those from IL-7 / 3 IL-7 / mice were 0.64 0.7 10^5 cells/colon (Fig. 4*F*) (p 0.01) and 0.54 0.29 10^5 cells/spleen (Fig. 4*G*) (p 0.05), respectively. As shown in Fig. 4*H*, LP CD4 T cells from IL-7 / 3 IL-7 / recipients produced significantly less IFN- , IL-2, and TNF- as compared with those from IL-7 / 3 IL-7 / mice upon in vitro stimulation (Fig. 4*H*). Furthermore, flow cytometry analysis showed that the LP CD4 T cells isolated from both IL-7 / 3 IL-7 / and IL-7 / 3 IL-7 / recipients were sustained by the phenotype of $CD44^{high}CD62L$ IL-7R ^{high} T_{EM} cells (Fig. 4*I*).

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

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

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

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

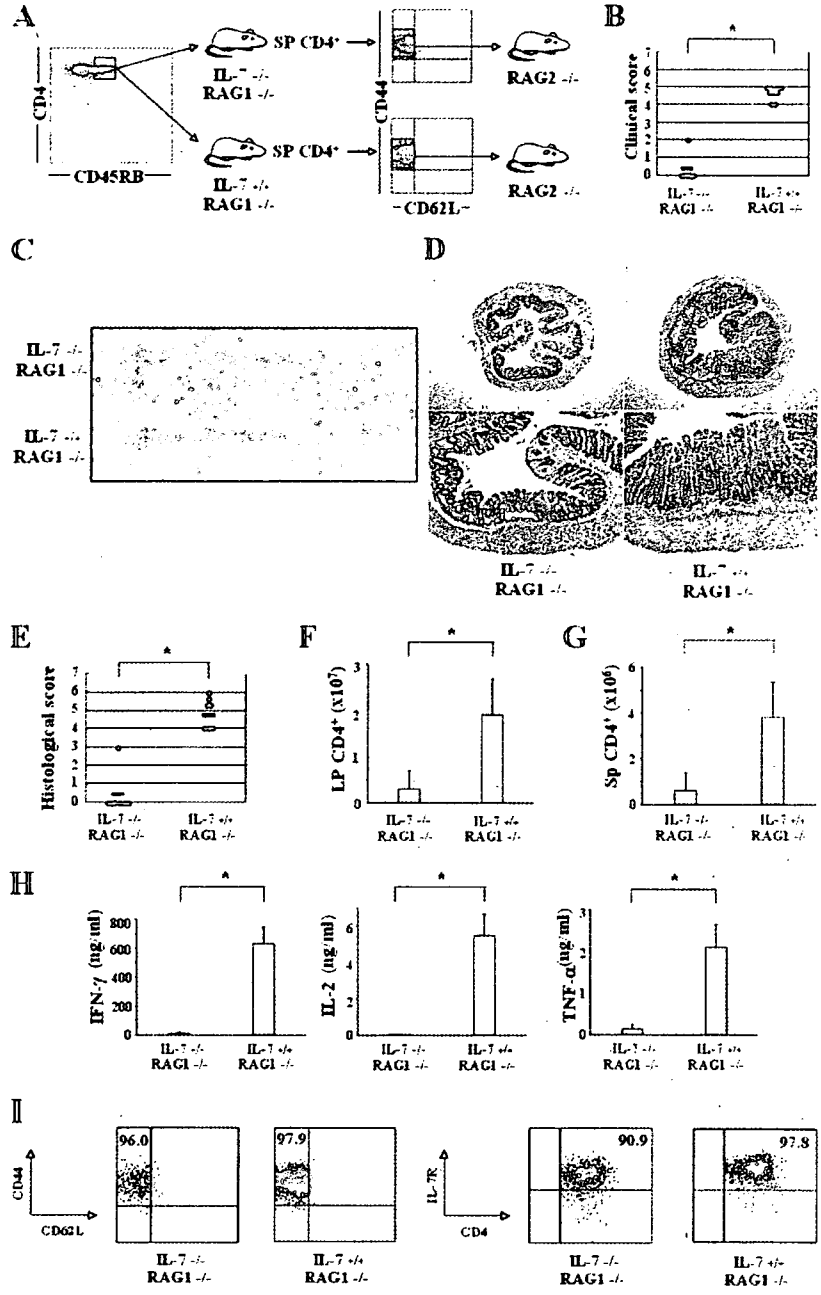

FIGURE 7. Sustained CD4 T cells in IL-7 / RAG-1 / recipients do not have a potential to induce colitis when transferred to IL-7 / RAG-1 / recipients. *A*, IL-7 / RAG-1 / mice were transferred with splenic CD4 T cells obtained from colitic CD4 CD45RB^{high} T cell-transferred IL-7 / RAG-1 / mice (IL-7 / 3 IL-7 / , n 7) and noncolitic CD4 CD45RB^{high} T cell-transferred IL-7 / RAG-1 / mice (IL-7 / 3 IL-7 / , n 7). *B*, Clinical scores were determined 4 wk after transfer as described in *Materials and Methods*. Data are indicated as the mean SEM of seven mice in each group. , p 0.0005. *C*, Gross appearance of the colon, spleen, and mesenteric lymph nodes from IL-7 / 3 IL-7 / (*top*) and IL-7 / 3 IL-7 / (*bottom*) mice 9 wk after transfer. *D*, Histological examination of the colon from IL-7 / 3 IL-7 / and IL-7 / 3 IL-7 / mice 9 wk after transfer. Original magnification: 40 (*upper*); 100 (*lower*). *E*, Histological scoring of IL-7 / 3 IL-7 / and IL-7 / 3 IL-7 / mice 4 wk after transfer. Data are indicated as the mean SEM of seven mice in each group. , p 0.001. LP (*F*) and spleen (*G*) CD4 T cells were isolated from IL-7 / 3 IL-7 / and IL-7 / 3 IL-7 / mice 4 wk after transfer, and the number of CD4 cells was determined by flow cytometry. Data are indicated as the mean SEM of seven mice in each group. LP, , p 0.005; spleen, , p 0.05. *H*, Cytokine production by LP CD4 T cells. CD4 LPL were isolated from each mouse at 4 wk after transfer and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. IFN- , IL-2, and TNF- concentrations in culture supernatants were measured by ELISA. Data are indicated as the mean SD of seven mice in each group. , p 0.005. *I*, Phenotypic characterization of LP CD4 T cells isolated from IL-7 / 3 IL-7 / and IL-7 / 3 IL-7 / mice 9 wk after transfer.

have divided over eight times (19) as is in a similar manner with IL-7 / RAG-1 / recipients, rapid-dividing area of IL-7 / RAG-1 / recipients was markedly decreased as compared with that of IL-7 / RAG-1 / recipients, indicating that rapid-proliferating cells in IL-7 / RAG-1 / recipients were subjected to undergo apoptosis or the rate of rapid-proliferating cells in IL-7 / RAG-1 / recipients were significantly faster as undetectable as for cells divided over eight times (19) by this CSFE method.

Sustained CD4 T cells in IL-7 / RAG-1 / recipients do not have a potential to induce colitis when transferred to IL-7 / RAG-1 / recipients

Finally, we address a question whether sustained CD4 CD44^{high} CD62L effector-memory type of T cells in IL-7 / RAG-1 / mice transferred with CD4 CD45RB^{high} T cells (Fig. 1) have a potential to induce colitis if they were transferred to new IL-7-competent IL-7 / RAG-1 / mice. Because it was very important to assess a possibility that a small but substantial number of CD4 T cells would be maintained by other factors, such as commensal bacterial Ag-driven TCR signaling and IL-15, as suggested by others (16, 23–25) in CD4 CD45RB^{high} T cell-transferred IL-7 / RAG-1 / mice, but not enough to expand to induce colitis due to the absence of IL-7, we isolated splenic CD4 T cells from colitic CD4 CD45RB^{high} T cell-transferred colitic IL-7 / RAG-1 / mice and noncolitic IL-7 / RAG-1 / mice at 8 wk after transfer (Fig. 7*A*). We next retransferred these splenic CD4 T cells into new IL-7 / RAG-1 / mice (Fig. 7*A*). Expectedly and similarly with the result from the adoptive transfer of colitic LP CD4 T cells (Fig. 4), IL-7 / RAG-1 /

recipients transferred with splenic CD4 T cells from colitic CD4 CD45RB^{high} T cell-transferred IL-7 / RAG-1 / (IL-7 / 3 IL-7 /) mice developed a severe colitis until 4–6 wk after the transfer, characterized by significant weight loss, diarrhea, and higher total clinical scores (Fig. 7*B*) and thickening of the colonic wall with inflammation (Fig. 7, *C* and *D*). In contrast, IL-7 / RAG-1 / recipient (IL-7 / 3 IL-7 /) mice appeared healthy and did not exhibit any signs of colitis until 9 wk after transfer (Fig. 7*B*), and no apparent thickening of the colonic wall (Fig. 7*C*). No evident pathological changes were observed in the colon (Fig. 7*D*). Average histological scores characterized by severe inflammation and epithelial hyperplasia (Fig.7*D*) were 4.90 0.87 in those IL-7 / 3 IL-7 / mice in contrast to 0.42 1.13 in those IL-7 / 3 IL-7 / mice (p 0.001) (Fig. 7*E*). The average recovered numbers of LP and splenic CD4 T cells from colitic IL-7 / 3 IL-7 / mice were 190.8 77.3 10^5 cells/colon (Fig. 7*F*) and 31.3 18.3 10^5 cells/spleen (Fig. 7*G*), respectively, whereas those from noncolitic IL-7 / 3 IL-7 / mice were 29.7 39.9 10^5 cells/colon (Fig. 7*F*) (p 0.005) and 6.23 13.68 10^5 cells/spleen (Fig. 7*G*) (p 0.05), respectively. As shown in Fig. 7*H*, LP CD4 T cells from noncolitic IL-7 / 3 IL-7 / mice produced significantly less IFN- , IL-2, and TNF- as compared with those from colitic IL-7 / 3 IL-7 / mice (Fig. 7*H*). Furthermore, flow cytometry analysis showed that the LP CD4 T cells isolated from both colitic IL-7 / 3 IL-7 / recipients and noncolitic IL-7 / 3 IL-7 / mice were sustained the phenotype of CD44^{high}CD62L IL-7R ^{high} T_{EM} cells (Fig. 7*I*).

Discussion

A central pursuit in the field of chronic immune-mediated diseases, such as IBD, has been to identify the specific factors that are responsible for the persistence of the diseases. In this study, we demonstrated that IL-7 is essential for the development and the persistence of chronic colitis by a series of adoptive transfer of normal CD4 CD45RB^{high} T cells and colitogenic LP CD4 CD44^{high}CD62L T_{EM} donor cells into IL-7 / RAG-2 / and IL-7 / RAG-2 / recipients. Although rapidly proliferative responses of donor colitogenic LP CD4 T_{EM} cells was observed in IL-7 / RAG-2 / recipients to a similar extent of those in recipient IL-7 / RAG-2 / mice after transfer, expression of Bcl-2 was significantly down-modulated in LP CD4 T cells, and the number of recovered LP CD4 T cells was markedly decreased in the IL-7 / RAG-2 / recipients as compared with IL-7 / RAG-2 / recipients. These results suggest that IL-7 is critical for the persistence of chronic colitis as a survival factor for colitogenic CD4 T_{EM} cells rather than proliferative factor to sustain the intestinal inflammation.

We have previously shown a potential role for IL-7/IL-7R-mediated immune responses in intestinal inflammation. First, IL-7 transgenic mice developed chronic colitis that mimicked histopathological characteristics of human IBD (12). As chronic colitis developed, IL-7 transgenic mice showed significant infiltration of IL-7R^{high}CD4 T cells in the colonic LP. Second, we clarified that mucosal IL-7R^{high}CD4 T cells in colitic TCR -deficient mice are the pathogenic T cells that can induce chronic colitis by the adoptive transfer of these cells into syngeneic immunodeficient RAG-2 / mice, and the selective elimination of IL-7R^{high}CD4 T cells by administrating toxin-conjugated anti-IL-7R mAb completely ameliorated colitis (13). Third, in vitro stimulation by IL-7 enhanced the significant proliferative responses and the survival of colitic LP CD4 , but not normal LP CD4 , T cells (26). These previous results suggest that IL-7 might be a crucial factor for the development of chronic colitis and prompted us to investigate to prove it directly using the adoptive transfer system in the completely IL-7-deficient condition. Because adult IL-7 / mice are highly lymphopenic in the peripheral blood and lymphoid organs due to the defected lymphopoiesis (27), it was impossible to compare wild-type mice and littermate IL-7 / mice in terms of disease susceptibility. To overcome this issue, we generated littermate IL-7 / RAG-1 / and IL-7 / RAG-1 / mice and used as recipients for the adoptive transfer of CD4 CD45RB^{high} T cells or the colitogenic LP CD4 CD44^{high}CD62L T_{EM} cells into these mice. Importantly, because IL-7 is not detected in lymphocytes, the present adoptive transfer system could provide a clue whether IL-7 is essential for the development and the persistence of chronic colitis.

In this study, we found that IL-7 / RAG-1 / transferred with CD4 CD45RB^{high} T cells never developed chronic colitis 8 wk after the transfer (Fig. 1) and even 20 wk after the transfer (data not shown). The results showed that IL-7 is essentially needed to develop colitis in terms of disease susceptibility in this model, but it was still unclear whether IL-7 is critical for the initiation of T cell activation or the persistence of colitogenic CD4 T_{EM} cells. To clarify this issue in detail, we next conducted another adoptive transfer experiment using colitogenic LP CD4 CD44^{high}CD62L T_{EM} cells into IL-7 / RAG-1 / mice and IL-7 / RAG-1 / mice without the impact of T cell priming, activation, and differentiation of naive CD4 T cells. Again, we found that IL-7 / RAG-1 / transferred with the colitogenic LP CD4 T_{EM} cells never developed chronic colitis after transfer (Fig. 2) in contrast to the transferred IL-7 / RAG-1 / mice that developed severe colitis. The results showed that IL-7 is especially essential for the persistence of colitogenic CD4 T_{EM} cells.

Of note, however, de Latour et al. (28) very recently reported that IL-7 / RAG-1 / mice transferred with CD4 CD45RB^{high} T cells developed a wasting disease and colitis at 6 wk after transfer that was performed by very similar protocol of ours albeit to less inflammatory severity as compared with those in IL-7 / RAG-1 / recipients. However, the discrepancy between their result and ours was not surprising because they used recipients that were colonized by *Helicobacter hepaticus*, a bacteria known to be associated with colitis in immunodeficient mice, such as Rag-deficient and SCID mice, indicating that their result might be due to the activated innate immune responses induced by mucosal *H. hepaticus* infection, resulting in increasing production of signal 3 cytokines, such as IL-12, type I IFNs (IFN-), and type II IFN (IFN-), to promote expansion and survival of colitogenic effector and memory T cells (29). Because we demonstrated that the small but substantial number of memory-type of mucosal CD4 T cells were resided even in CD4 CD45RB^{high} T cell-transferred IL-7 / RAG-1 / , it is likely that *H. hepaticus*-induced activation of innate immunity in their setting might have accelerated the development of *H. hepaticus*-mediated T cell expansive colitis or just T cell-independent innate immune-mediated colitis by increasing activated macrophages and granulocytes. Although we performed the specific PCR for *Helicobacter* species, including *H. hepaticus* using stool samples from mice in our facility, all data were all negative for *Helicobacter* species (data not shown). Further studies will be needed this issue.

Somewhat at odds, however, we found that rapid proliferation of donor CD4 T_{EM} cells was observed after transfer of CFSE-labeled colitogenic LP CD4 T_{EM} cells into IL-7 / RAG-1 / mice as well as into IL-7 / RAG-1 / mice (Fig. 4), although the total number of recovered CD4 T cells from IL-7 / RAG-1 / mice was markedly decreased as compared with that from IL-7 / RAG-1 / mice (Fig. 3). Consistent with these results, we found that Bcl-2 expression was significantly decreased

and conversely the ratio of annexin V cells in rapidly proliferating CFSE CD4 cells was significantly increased in CD4 T cells from the transferred IL-7 / RAG-1 / mice as compared with those from the transferred IL-7 / RAG-1 / mice. These results suggest that IL-7 is not required for rapid proliferation of colitogenic LP CD4 T_{EM} cells in the lymphopenic condition but is critical for the survival of colitogenic CD4 T_{EM} cells, followed by the essential contribution for the persistent colitis. Furthermore, the kinetics study to assess an early effector phase showed no colonic inflammation at 1 and 2 wk after transfer of CD4 CD45RB^{high} T cells into the IL-7 / RAG-1 / recipients and thus suggests that chronic persistent colitis is not induced by only the expansion of effector cells, but what may be needed is the continuous conversion to T_{EM} and the equilibrium between effector cells and T_{EM} cells to maintain the diseases. Another possibility, which we do not favor, is that IL-7 might be essential for the maintenance of the colitogenic LP effector CD4 T cells to sustain the disease.

It should be discussed this colitis model induced by the adoptive transfer into lymphopenic mice from the standpoint of homeostatic regulation of T lymphocytes. Conditions present in congenital mutant mice have been exploited for many years as an animal model for chronic wasting IBD, which occur several weeks after adoptive transfer of syngeneic naive CD4 CD45RB^{high} T cells in the condition, which lacks regulatory T cells (14, 15). Chronic colitis results from secretion of large amounts of inflammatory cytokines, especially IFN- and TNF- , by infiltrated LP CD4 cells that are chronically activated presumably by the bacterial Ags in the colon (30). The essential role of enteric bacteria is affirmed by the fact that intestinal inflammation cannot be induced if the mutant mice are reared under germfree conditions (15), indicating that enteric bacterial Ags might be responsible for the expansion of colitogenic CD4 T cells. Apart from this model, it is now well accepted that the transfer of naive CD4 T cells into a lymphocyte-deficient environment initiates proliferative responses (5, 21, 22). Careful analysis reveals that some of the transferred cells proliferate rapidly and undergo robust differentiation to memory cells, a process designated "rapid proliferation" responding to external Ags, including enteric bacteria, and other cells proliferate relatively slowly, designated "slow proliferation" responding self-Ags (21, 22). Min et al. (19) recently demonstrated that rapid proliferation of T cells is IL-7 independent, whereas slow proliferation is IL-7 dependent. Although the mechanism of our colitis model induced by the adoptive transfer of CD4 CD45RB^{high} T cells would fit with enteric bacteria-inducing rapid proliferation model because rapid proliferation of donor LP CD4 T_{EM} cells was observed into IL-7 / RAG-1 / mice (Fig. 4), we found that IL-7 is critically required for the development and the persistence of colitis in mice transferred with CD4 CD45RB^{high} T cells or the colitogenic CD4 T_{EM} cells. The discrepancy may be due to the difference of IL-7 dependency between the rapid proliferation, which is IL-7 independent, and the following survival step of CD4 T_{EM} cells, which is IL-7 dependent. In other words, IL-7 may be critically needed to survive the colitogenic CD4 T_{EM} cells after their rapid proliferation in the lymphopenic condition. However, it should be also noted that rapid proliferation in antibiotics-treated mice, regardless of IL-7 / RAG-1 / and IL-7 / RAG-1 / mice, could be not fully abolished (Fig. 7). This indicates that other environmental Ags, such as food and bedding and self-Ags themselves, might be involved in rapid proliferation in the lymphopenic condition.

Such characteristics of our colitis model raise another important question whether the colitogenic CD4 CD44^{high}CD62L T cells can be defined as T_{EM} cells rather than effector T cells in the presence of Ags, in this case, intestinal bacteria. In general, immunological memory has evolved to warrant rapid and efficient elimination of microbial agents that repeatedly enter the organism. As a rule, immunological memory builds up, following successful elimination from the organism. In contrast, persistence of Ag, like in chronic infectious diseases, often leads to the exhaustion of the immune response (31). In immune responses in mice with chronic colitis, the target commensal bacteria are never eliminated but persist throughout life. Thus, would the colitogenic CD4 T cells in CD4 CD45RB^{high}-transferred colitis model build up memory against Ags? If so, do colitogenic memory CD4 T cells play a role in the course of chronic disease? First, we found that the colitogenic CD4 T cells highly expressed both CD44 and IL-7R . It is generally thought that highly expressed IL-7R is one of accepted memory, but not effector, T cell markers, and also IL-7R is down-regulated via TCR stimulation in the presence of Ags. Second, memory, but not effector, CD4 T cells are critically controlled the survival by IL-7 (3, 4). Consistent with this, we also found that the colitogenic LP CD4 T cells were markedly decreased in IL-7 / RAG-1 / mice transferred with the colitogenic CD4 T cells as compared with the transferred IL-7 / RAG-1 / mice. Collectively, these data indicate that the colitogenic CD4 T cells are T_{EM} cells rather than effector CD4 T cells. In fact, Zaph and colleagues (32) recently demonstrated that *Leishmania*-specific central memory T cells develop in the presence of parasites. Although it could be argued that IBD, including murine model of chronic colitis, are due to chronic infection (persistence) of intestinal bacteria, it is likely that host and intestinal bacteria must establish some form of long-term relationship in which the immunological rules may be somewhat different from those of a brief encounter. Thus, a hallmark of T cell-mediated immune reaction to persistently expressed commensal bacterial Ags in chronic colitis would be the continual generation of Ag-specific effector and memory T cells. Because effector T cells are short-lived cells, there must exist cellular mechanisms by which effector and memory T cells specific for persistent bacterial Ags are maintained in the colitic mice.

Taken together, IL-7 is essential for the development and the persistence of chronic colitis as a critical survival factor for colitogenic CD4 T_{EM} cells rather than proliferative factor to sustain the intestinal inflammation, suggesting that therapeutic approaches targeting IL-7/IL-7R signal pathway may be feasible in the treatment of IBD.

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Disclosures

The authors have no financial conflict of interest.

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Systemic, but Not Intestinal, IL-7 Is Essential for the Persistence of Chronic Colitis[1]

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

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

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

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

Materials and Methods

Animals

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

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

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

Parabiosis experimental design

To assess the specific requirement of mucosal or systemic IL-7 in the development of colitis, we performed adoptive transfer experiment in combination with a parabiosis system using IL-$7^{+/+}$ × RAG-$1^{-/-}$ and IL-$7^{-/-}$ × RAG-$1^{-/-}$ littermate recipients (Fig. 1*A*). For adoptive transfer, $CD4^+$ T cells were first isolated from SP cells of C57BL/6-Ly5.2 mice using the anti-CD4 (L3T4)-MACS system (Miltenyi Biotec) according to the manufacturer's instruction. Enriched $CD4^+$ T cells (96–97% pure, as estimated by FACSCalibur (BD Biosciences)) were then labeled with PE-conjugated anti-mouse CD4 (RM4–5; BD Pharmingen) and FITC-conjugated anti-mouse (16A; BD Pharmingen). $CD4^+CD45RB^{high}$ cells were purified using a FACSAria (BD Biosciences). This population was >98.0% pure on reanalysis. IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice (n = 18) and IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice (n = 6) were then injected i.p. with 3×10^5 splenic $CD4^+CD45RB^{high}$ T cells from normal C57BL/6-Ly5.2 mice. After 6 wk post transfer, IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice, but not IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice, transferred with $CD4^+CD45RB^{high}$ T cells developed a wasting disease and colitis as previously reported (19).

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

Histological examination

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

Tissue preparations

Single cell suspensions were prepared from SP, LP, and BM as previously described (18). To isolate LP $CD4^+$ T cells, the entire length of the colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca^{2+}, Mg^{2+}-free HBSS containing 1 mM DTT (Sigma-Aldrich) for 45 min to remove mucus and then treated with 3.0 mg/ml collagenase (Roche) and 0.01% DNase (Worthington Biochemical) for 2 h. The cells were pelleted two times through a 40% isotonic Percoll solution, and then subjected to Ficoll-Hypaque density gradient centrifugation (40/75%). Enriched LP $CD4^+$ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells when analyzed by FACSCalibur contained >95% $CD4^+$ cells. BM cells were obtained by flushing two femurs with cold RPMI 1640. For in vitro assay, only live cells were counted by using trypan blue staining method, and confirmed that the viability of cells was almost the same (>96% live) among the sample groups.

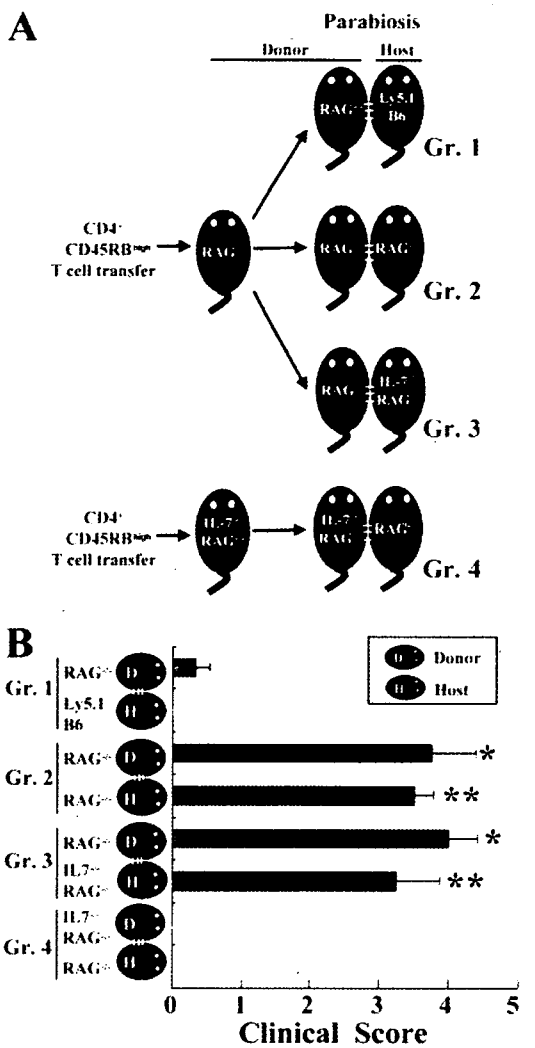

FIGURE 1. Host IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice in parabionts with diseased IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice show a wasting disease and clinical signs of colitis. *A*, Parabiosis experimental design. For an adoptive transfer, splenic $CD4^+CD45RB^{high}$ T cells were isolated from C57BL/6-Ly5.2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

Reverse transcription polymerase chain reaction

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

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

Immunohistochemistry

We used consecutive cryostat colon sections in all studies. Immunohistochemistry using purified mAb against mouse CD4 (RM4–5; BD Pharmingen) or biotin-conjugated polyclonal IL-7 Ab (BAF407; R&D Systems) was performed. In brief, O.C.T. compound-embedded tissue samples were cut into serial sections 6-μm thick, placed on coated slides, and fixed with 4% paraformaldehyde phosphate buffer solution for 10 min. Slides were then incubated with the primary Ab at 4°C overnight, followed by staining with AlexaFluor 488 goat anti-rat IgG for CD4 detection or AlexaFluor 488 streptavidin (Molecular Probes) for IL-7 detection at room temperature for 60 min. All slides were counterstained with 4′, 6′ -diamidino-2-phenylindole (DAPI; Vector Laboratories) and observed under a confocal microscopy (LSM510 Carl Zeiss).

Cytokine ELISA

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

Flow cytometry

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

Statistical analysis

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

Results

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

We have previously demonstrated that IL-7 is essential for the development and the persistence of colitis as a survival factor for colitogenic $CD4^+$ memory T cells (19). Furthermore, we have found that IL-7 Tg mice, in which IL-7 was systemically overproduced, develop colitis spontaneously, but production of intestinal IL-7 was conversely decreased in the inflamed mucosa because of depletion of the goblet cells. Based on such paradoxical findings, in this study, we assess whether intestinal or systemic IL-7 is essential for the perpetuation of colitis, by adoptive transfer experiment in combination with parabiosis system using IL-7$^{+/+}$ × RAG-1$^{-/-}$ and IL-7$^{-/-}$ × RAG-1$^{-/-}$ littermate recipients (Fig. 1*A*). To this end, we first induced chronic colitis by adoptive transfer of splenic $CD4^+CD45RB^{high}$ T cells from normal C57BL/6-Ly5.2 mice into IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice (Fig. 1*A*). Consistent with our previous report (19), the transferred IL-7$^{+/+}$ × RAG-1$^{-/-}$ mice manifested progressive weight loss from 3 wk after transfer and clinical symptoms of colitis 6 wk after transfer (data not shown). In contrast, the $CD4^+CD45RB^{high}$ T cell-transferred IL-7$^{-/-}$ × RAG-1$^{-/-}$ mice showed no clinical signs of colitis and weight loss (data not shown) (19), indicating that IL-7 is essential for the development of colitis.

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

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

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

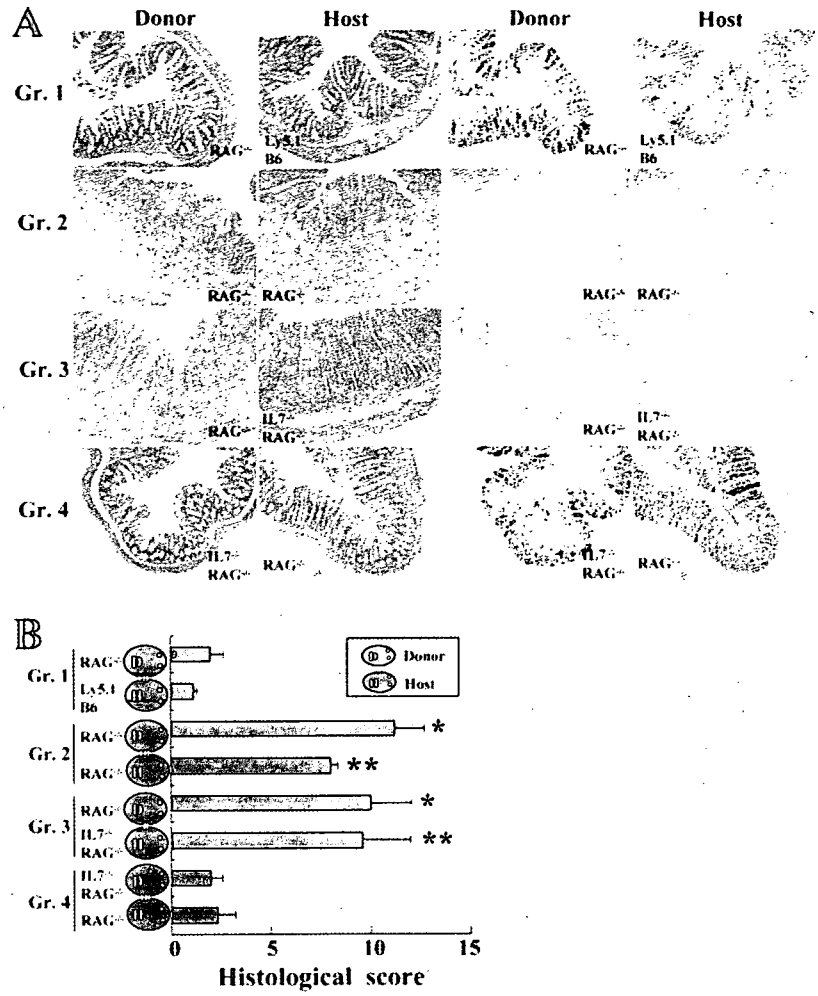

FIGURE 2. IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice in parabionts with diseased IL-7$^{+/+}$ × RAG-1$^{-/-}$ donor mice develop colitis. *A*, Histological examination by H&E staining (*left*) and Alcian blue staining (*right*) of the colon from each group at 4 wk after surgery. Representative of four separate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

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

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

We next examined the cytokine production by LP CD4$^+$ T cells from each mouse in Groups 1–4. As shown in Fig. 4, LP CD4$^+$ T cells from donor and host mice in Groups 2 and 3 produced significantly higher amounts of IFN-γ and TNF-α as compared with those from mice in Groups 1 and 4, indicating that colitic LP CD4$^+$ T cells in IL-7$^{-/-}$ × RAG-1$^{-/-}$ host mice or IL-7$^{+/+}$ × RAG-1$^{-/-}$ host mice of Groups 2 and 3 have functions of Th1-mediated immune responses. Importantly, the elevated production of these cytokines in Groups 2 and 3 was dependent on the presence of colitis, but not on the expression of IL-7 in the colon.

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

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

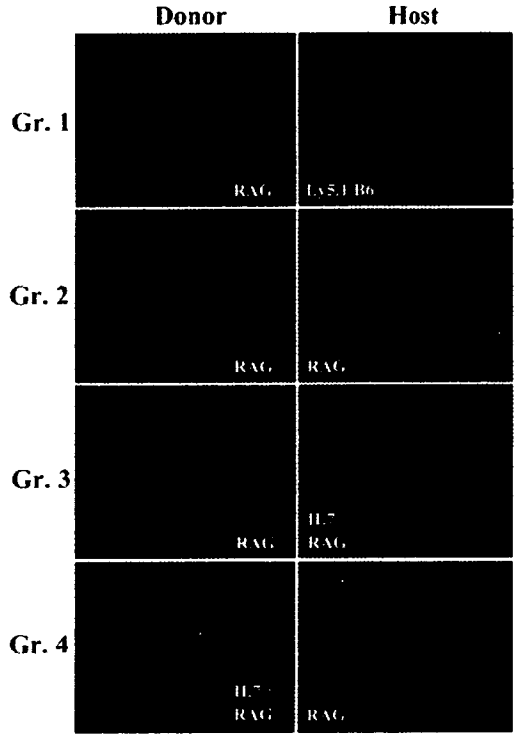

FIGURE 3. IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice in parabionts developed colitis with the marked infiltration of immigrant $CD4^+$ T cells from donor mice. CD4 immunostaining and DAPI counterstaining of the colon from Groups 1–4 at 4 wk after surgery. Frozen sections were fixed with 4% paraformaldehyde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

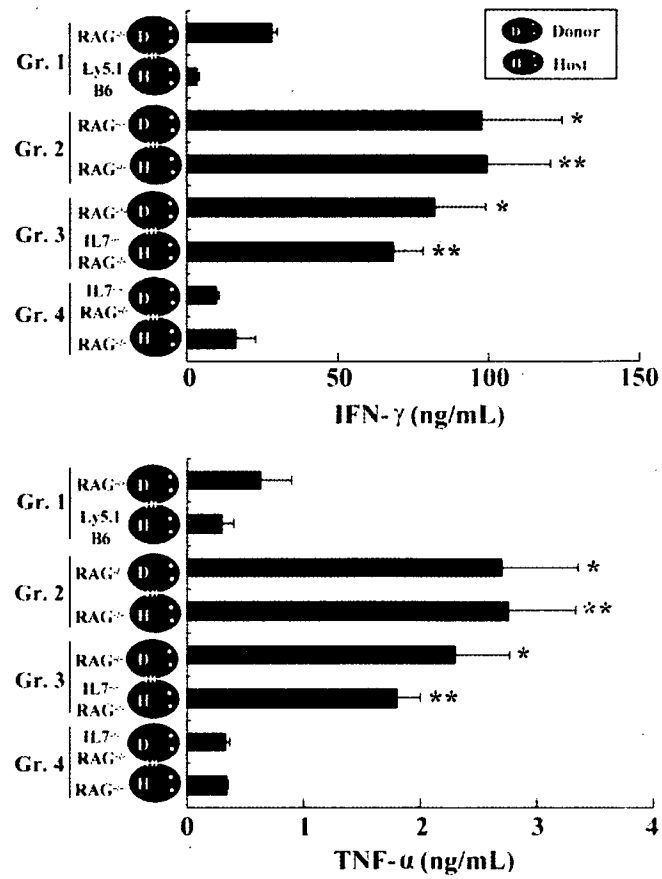

FIGURE 4. IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice in parabionts develop Th1-mediated colitis. LP $CD4^+$ T cells were prepared from colons at 4 wk after surgery and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. Concentrations of IFN-γ, and TNF-α in culture supernatants were measured by ELISA.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

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

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

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

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

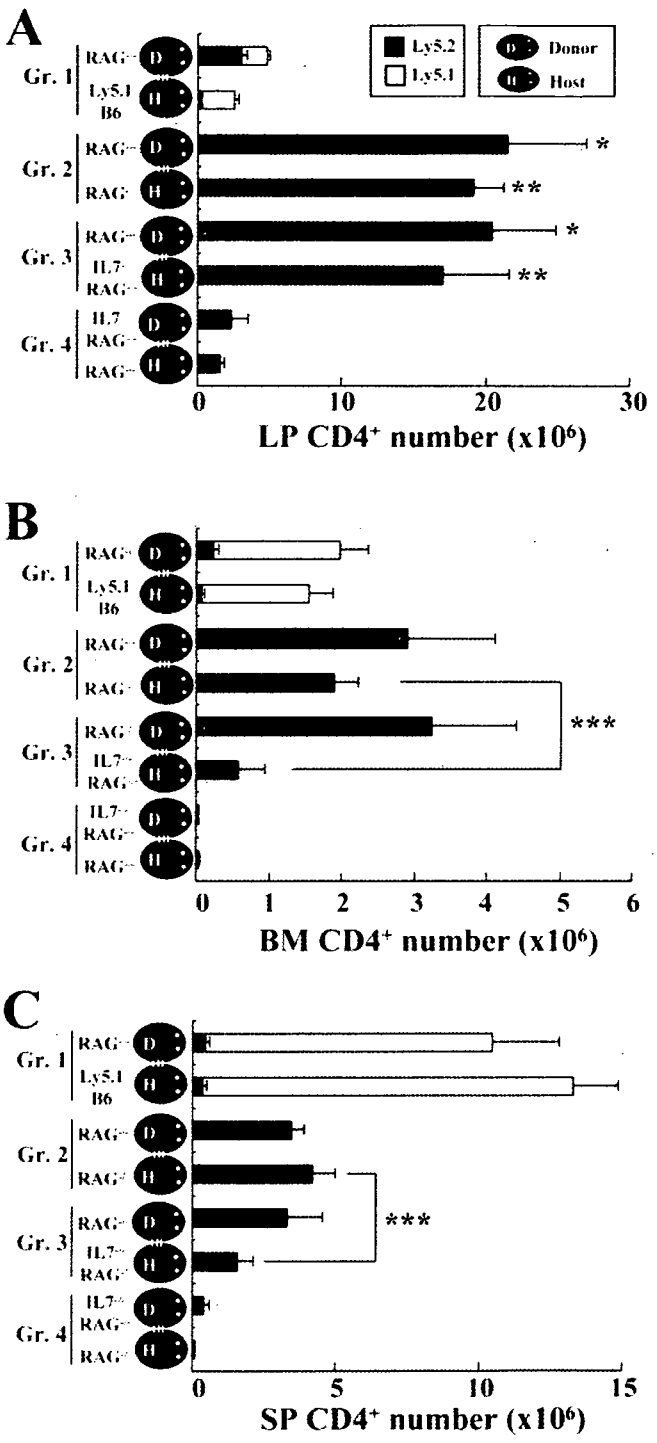

FIGURE 5. Expansion of BM and SP, but not of LP, $CD4^+$ T cells in IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice in parabionts is dependent on IL-7. LP (*A*), BM (*B*), and SP (*C*) $CD4^+$ T cells were isolated from each mouse of Groups 1–4 at 4 wk after surgery, and the number of $CD4^+$ cells were determined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

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

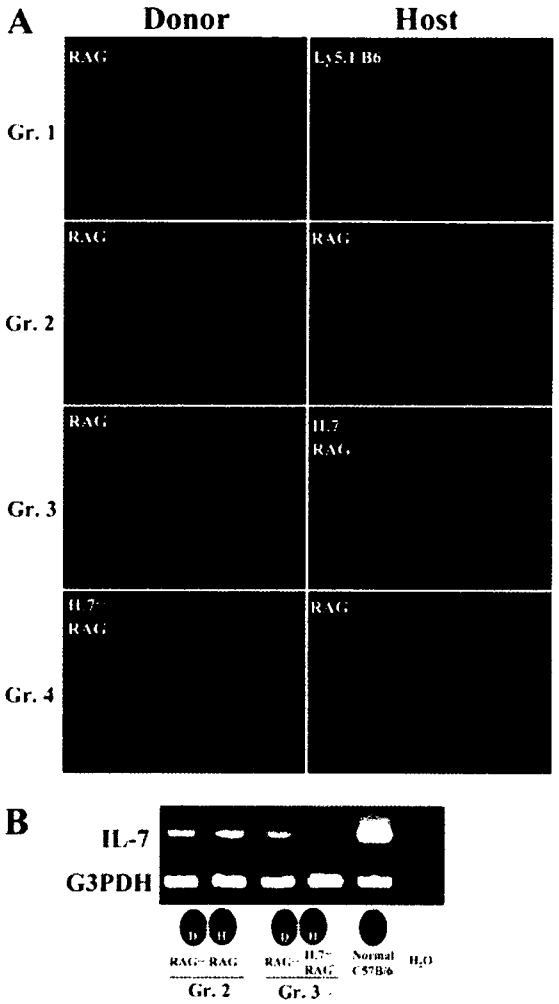

FIGURE 6. IL-7 is not detected in host IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice in parabionts with diseased IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice. *A*, Frozen sections of colon from each mouse in Groups 1–4 at 4 wk after surgery were stained with polyclonal anti-IL-7 Abs. Representative of five separate samples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

Discussion

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

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

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

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

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

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

BM is a major source of IL-7 in the body (26). In contrast to the LP, it is noteworthy that the number of $CD4^+$ T cells recovered from the BM and SP of the colitic IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice (Group 3) was significantly decreased compared with that of the IL-$7^{+/+}$ × RAG-$1^{-/-}$ host mice. Regarding this result, we recently demonstrated that $CD4^+$ effector-memory-like T (T_{EM}-like) cells reside in the BM of colitic SCID and RAG-$1/2^{-/-}$ mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells (20). Importantly, these resident BM $CD4^+$ T_{EM}-like cells are closely attached to IL-7-producing stromal cells in the colitic BM. Most importantly, the accumulation of BM $CD4^+$ T_{EM}-like cells was significantly decreased in IL-7-deficient recipients reconstituted with the colitogenic LP $CD4^+$ T_{EM}-like cells. Together with the present study, these findings suggest that the BM $CD4^+$ T_{EM}-like cells residing in mice with chronic colitis play a critical role as a reservoir for lifelong persisting colitis in an IL-7-dependent manner. However, it is still possible that IL-7 produced by sites other than intestine or BM, such as skin, liver, eye, lymph nodes (LN), and SP, also contribute to the development and perpetuation of colitis. In this regard, we very recently demonstrated that splenectomized LN-null lymphotoxin $\alpha^{-/-}$ × RAG-$2^{-/-}$ mice transferred with colitogenic LP $CD4^+$ T cells develop colitis (35), suggesting that IL-7 production at least by LN and SP does not appear to be essential. To further clarify the role of IL-7 produced by BM mesenchymal cells in the pathogenesis of chronic colitis, BM chimera of IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice, which are lethally irradiated and transplanted with the BM cells from IL-$7^{+/+}$ × RAG-$1^{-/-}$ mice, may be quite beneficial. Interestingly, however, it is also well known that extraintestinal complications of IBD patients such as skin, liver, and mucocutaneous manifestations (36) appears to be closely associated with sites of local IL-7 production by keratinocytes, hepatocytes, and uvea cells. Although no inflammation was not observed at least in liver and skin in the present model of colitis (data not shown), further studies will be needed to address this issue.

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

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

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Disclosures

The authors have no financial conflict of interest.

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Bone Marrow Retaining Colitogenic CD4+ T Cells May Be a Pathogenic Reservoir for Chronic Colitis

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Background & Aims: **Although bone marrow (BM) is known as a primary lymphoid organ, it also is known to harbor memory T cells, suggesting that this compartment is a preferential site for migration and/or selective retention of memory T cells. We here report the existence and the potential ability to induce colitis of the colitogenic BM CD4+ memory T cells in murine colitis models.** ***Methods:*** **We isolated BM CD4+ T cells obtained from colitic severe combined immunodeficient mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells and colitic interleukin (IL)-$10^{-/-}$ mice that develop colitis spontaneously, and analyzed the surface phenotype, cytokine production, and potential activity to induce colitis. Furthermore, we assessed the role of IL-7 to maintain the colitogenic BM CD4+ T cells.** ***Results:*** **A high number of CD4+ T cells reside in the BM of colitic severe combined immunodeficient mice and diseased IL-$10^{-/-}$ mice, and they retain significant potential to induce type-1 T helper-mediated colitis in an IL-7–dependent manner. These resident BM CD4+ T cells have an effector memory (T_{EM}; $CD44^{high}CD62L^{-}IL\text{-}7R^{high}$) phenotype and preferentially are attached to IL-7–producing BM cells. Furthermore, the accumulation of BM CD4+ T_{EM} cells was decreased significantly in IL-7–deficient recipients reconstituted with the colitogenic lamina propria CD4+ T_{EM} cells.** ***Conclusions:*** **Collectively, these findings suggest that BM-retaining colitogenic CD4+ memory T cells in colitic mice play a critical role as a reservoir for persisting lifelong colitis.**

BASIC—ALIMENTARY TRACT

It has long been known that T-cell precursors generated in the bone marrow (BM) migrate to the thymus, where T-cell development occurs. However, a fact often neglected is that under physiologic conditions, mature CD4+ and CD8+ T cells undergo extensive migration from the blood to the BM and vice versa. In both human beings and mice, T-cell receptor $\alpha\beta^+$ cells constitute approximately 3%–8% of nucleated BM cells.[1,2] BM CD4+ and CD8+ T-cell populations contain a high proportion of cells displaying a memory phenotype, that is, expressing low levels of CD45RA in human beings[3] and high levels of CD44 in mice.[4,5]

As early as 1974 it was documented that mouse CD4+ T cells migrate to the BM after priming, and it was proposed that BM CD4+ T cells contributed to the development of a memory antibody response in this organ.[6] Recently, T cells persisting in extralymphoid organs such as the liver, lung, and skin have attracted increasing interest because it has been recognized that these T cells contribute considerably to the long-lived memory T-cell pool.[7,8] In this context, BM has been shown to harbor a high number of antigen-specific CD8+ T cells for several months after resolution of acute infection.[9] For instance, adoptive transfer of BM cells from lymphochoriomeningitis virus–immune mice (>90 days after acute infection) to immunodeficient recipients provides antiviral protection, and thus CD8+ memory T cells from the BM are able to mount an effective secondary response.[10]

Primary T-cell responses to blood-borne antigens also can be initiated in the BM. This was shown initially in conditions of altered lymphocyte trafficking in splenectomized mice and then in individuals with normal lymphoid organs, for both CD4+ and CD8+ T-cell responses.[11] Thus, the BM resembles a secondary lymphoid organ, although it lacks the organized T- and B-cell areas found in the spleen, lymph nodes, and Peyer's patches. Although accumulating evidence suggests that BM plays an important role in the communication with mature naive/memory T cells, there is no evidence for the role of BM memory CD4+ cells in chronic immune diseases, such as inflammatory bowel diseases (ulcerative colitis and Crohn's disease) and autoimmune diseases. Crohn's disease is characterized by chronic inflammation of the small and large intestine and structures apart from the

Abbreviations used in this paper: Ag, antigen; APC, antigen-presenting cell; BM, bone marrow; BrdU, bromodeoxyuridine; CBA, cecal bacterial antigen; CSFE, carboxyfluoroscein succinimidyl ester; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IFN, interferon; IL, interleukin; LP, lamina propria; mAb, monoclonal antibody; MLN, mesenteric lymph node; PE, phycoerythrin; SCID, severe combined immunodeficient; Th1, type-1 T helper.

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bowel. Surgery does not cure Crohn's disease, and recurrence after surgery is the rule rather than the exception.[12] There is also no correlation between recurrence of the disease and the dissection of regional lymph nodes and spleen.[13] The evidence suggests that other sites might play a critical role in the recurrence of diseases as reservoirs of colitogenic memory $CD4^+$ T cells.

Furthermore, it is well known that interleukin (IL)-7 is important as a critical factor for the survival and homeostatic proliferation of memory $CD4^+$ T cells, and that BM is a major site of IL-7 production.[14] We have shown previously that mucosal $CD4^+$ T cells in colitic mice express IL-7Rα highly, and they are pathogenic cells responsible for chronic colitis.[15] In vitro stimulation of these colitic lamina propria (LP) $CD4^+IL\text{-}7R^{high}$ T cells by IL-7, but not IL-15 and thymic stromal lymphopoietin, enhanced significant proliferative responses and survival of colitic $CD4^+$ T cells.[16] These backgrounds prompted us to investigate the role of the resident BM memory $CD4^+$ T cells in persisting lifelong colitis using a murine model of chronic colitis induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells.

Materials and Methods

Mice

Female BALB/c, CB-17 severe combined immunodeficient (SCID), and C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). Female C57BL/6 Rag-$2^{-/-}$ mice were provided by Central Laboratories for Experimental Animals (Kawasaki, Japan). C57BL/6 Rag-$1^{-/-}$ mice and IL-$7^{-/-}$ mice were kindly provided by Dr. Zamoyska (National Institute for Medical Research, London, UK).[17] IL-$7^{-/-}$ × Rag-$1^{-/-}$ mice and littermate IL-$7^{+/+}$ × Rag-$1^{-/-}$ mice were generated in our laboratory. All mice were maintained under specific-pathogen-free conditions in the Animal Care Facility of the Tokyo Medical and Dental University. The Institutional Committee on Animal Research approved the experiments.

Antibodies and Flow Cytometry

The following monoclonal antibodies (mAbs) other than biotin-conjugated anti-mouse IL-7Rα (A7R34; Immuno-Biological Laboratories, Takasaki, Japan) were obtained from BD PharMingen (San Diego, CA) and used for purification of cell populations and flow-cytometric analysis: Fcγ (CD16/CD32)-blocking mAb (2.4G2), phycoerythrin (PE)-, peridinin chlorophyll protein, and phycoerythrin-phycoerythrin- 5'- disulfonatoindodicarbocyanine conjugated anti-mouse CD4 (RM4-5); fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 (145-2C11); PE- and allophycocyanin-conjugated anti-mouse CD44 (IM7); FITC- and PE-conjugated anti-mouse CD62L (MEL-14); FITC-conjugated anti-mouse CD69 (H1.2F3); PE-conjugated anti-mouse integrin $\alpha_4\beta_7$ (DATK32); FITC-conjugated anti-mouse CD45RB (16A); FITC-conjugated hamster anti-mouse Bcl-2 (3F11); PE-conjugated streptavidin; biotin-conjugated rat IgG2; PE-conjugated mouse IgG; and PE-conjugated rat IgG. Flow cytometric 3-color analysis was performed as described.[18]

Induction of Colitis

Colitis was induced in SCID/Rag-$2^{-/-}$ mice by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells as described.[18] Colitic mice were killed at 6–8 weeks after transfer, and $CD4^+$ T cells were isolated from BM, mesenteric lymph nodes (MLNs), and colonic LP.

Cytokine Enzyme-Linked Immunosorbent Assay

To measure cytokine production, 3×10^4 $CD4^+$ T cells from MLN, LP, and BM were cultured in 200 μL of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar, Cambridge, MA) 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 phosphate-buffered saline (PBS) overnight at 4°C. Culture supernatants were removed after 48 hours and assayed for cytokine production. Cytokine concentrations were determined by specific enzyme-linked immunosorbent assay (ELISA) as per the manufacturer's recommendation (R&D, Minneapolis, MN).

Interferon-γ Production by $CD4^+$ T Cells Stimulated With APCs Pulsed With Cecal Extracts

Colitic SCID mice were killed and their cecums were removed. The cecums were opened and placed in 1 mL of PBS, and the cecal bacteria were expelled by mixing with a vortex, and residual cecal tissue was removed. After the addition of DNase (10 μg/mL), 1 mL of this bacterial suspension was added to 1 mL of glass beads.[19] The cells were disrupted at 5000 revolutions per minute in a Mini-Bead Beater (BioSpec Products, Bartlesville, OK) for 3 minutes and then iced. The glass beads and unlysed cells were removed by centrifuging at 5000 × g for 5 minutes. The lysates were filter-processed in a similar manner. For antigen-presenting cells (APCs), spleen cells from normal BALB/c mice were prepared and treated with the appropriate concentration of cecal bacterial antigens (CBAs) as indicated at 2×10^7 cells/5 mL in a 15-mL tube overnight at 37°C. After washing twice, these APCs were treated with mitomycin-c before being added to T-cell cultures. BM, MLN, and LP $CD4^+$ T cells obtained from normal mice and colitic $CD4^+CD45RB^{high}$ T-cell-transferred SCID mice were cultured in the presence of APCs pretreated with cecal extract antigens in complete media. The culture supernatants were collected on day 3 of culture for interferon (IFN)-γ assay by ELISA.

Bromodeoxyuridine Incorporation

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

Cell-Cycle Analysis

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

Immunohistochemistry

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

Adoptive Transfer Experiments

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

Adoptive Transfer Experiments Into $IL\text{-}7^{-/-} \times Rag\text{-}1^{-/-}$ Mice

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

Statistical Analysis

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

Results

Effector Memory T Cells Reside in the BM of Colitic Mice

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

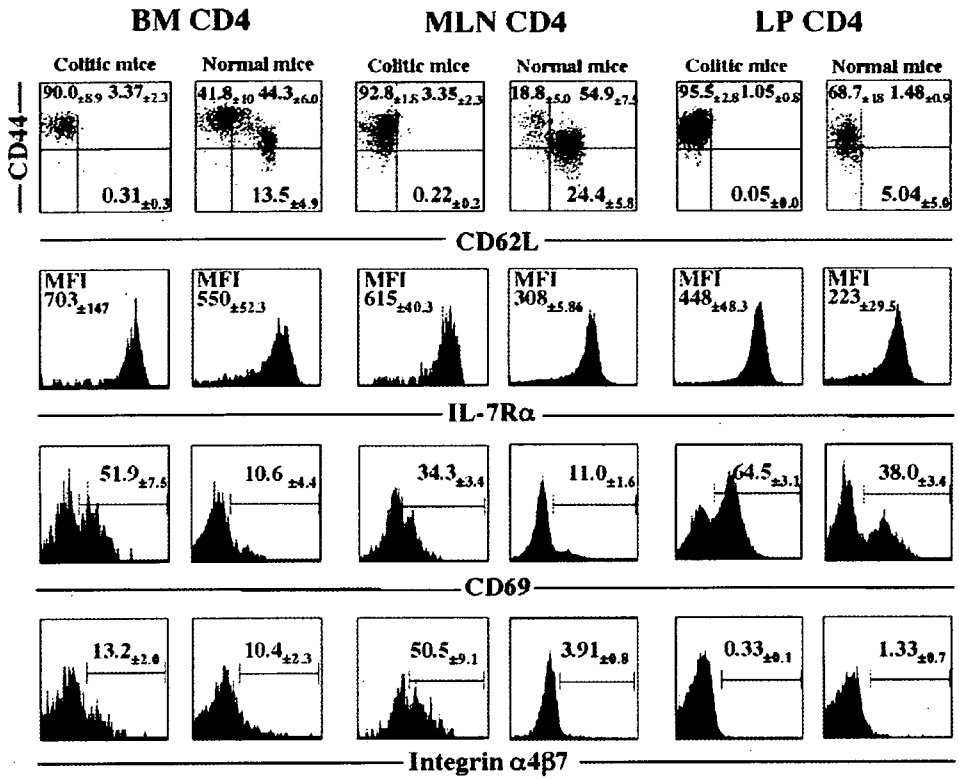


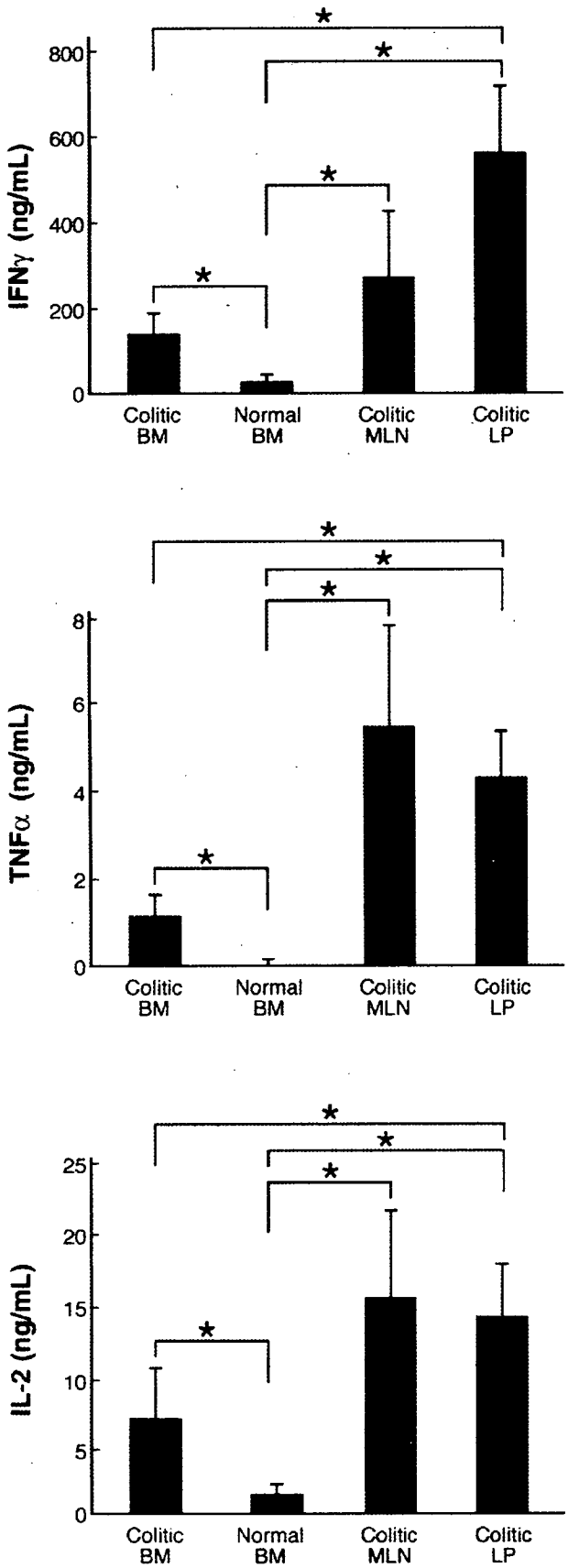
Figure 1. Colitic BM CD4+ T cells are CD44^{high}CD62L^{-}IL-7Rα^{high}. Expression of CD44, CD62L, IL-7Rα (CD127), CD69, and integrin α4β7 on CD4+ T cells obtained from spleen, MLN, LP, and BM in colitic mice induced by adoptive transfer of CD4+CD45RB^{high} T cells into CB-17 SCID mice (6 weeks after transfer) and normal BALB/c mice (age, 8 wk). Freshly isolated cells from colitic mice and normal BALB/c mice were stained with FITC-labeled anti-CD4, and PE-labeled anti-CD44, anti-CD62L, anti-IL-7Rα, anti-CD69, or anti-integrin α4β7 mAbs. Samples were analyzed by flow cytometry. Lymphocytes were identified by characteristic forward angle and side-scatter profiles. Data are displayed as a dotted plot (4-decade log scale) and quadrant markers were positioned to include more than 98% of control Ig-stained cells in the lower left. Percentages in each quadrant are indicated. Representative of 3 mice in each group.

cells in BM, MLN, and colonic LP of colitic mice induced by the adoptive transfer of CD4+CD45RB^{high} T cells into recipient CB-17 SCID mice and with those of age-matched normal BALB/c mice. CD3+CD4+ mature T cells were found to reside in BM, MLN, and LP (colitic mice: BM, 12.7 ± 4.4 × 10^5 per mouse; MLN, 7.01 ± 4.2 × 10^5; and LP, 187 ± 99 × 10^5; normal mice: BM, 16.6 ± 3.8 × 10^5; MLN, 99.6 ± 18 × 10^5; and LP, 4.17 ± 1.2 × 10^5). As shown in Figure 1, the BM CD4+ T cells, as well as MLN and LP CD4+ T cells, from the colitic mice, exclusively have a phenotype of CD44^{high}CD62L^{-} cells. Furthermore, these colitic BM CD4+ T cells expressed IL-7Rα highly, indicating that the colitic BM CD4+ T cells have a characteristic of T_{EM} cells. In contrast, the BM CD4+ T cells from normal mice are composed of 3 subpopulations: CD44^{low}CD62L+ naive cells, CD44^{high}CD62L+ central-memory T cells, and CD44^{high}CD62L^{-} T_{EM} cells (Figure 1). CD69, which is associated with cell activation, was expressed by a significantly higher proportion of CD4+ T cells from colitic mice than from normal mice. Interestingly, BM CD4+ T cells from colitic mice expressed relatively, but not significantly, high levels of integrin α4β7, a homing receptor to the gut, as compared with BM CD4+ T cells from normal mice, but lower levels than did MLN CD4+ T cells from colitic mice. These data indicate that the integrin α4β7-expressing CD4+ memory T cells, which are instructed to express the molecule in MLN or Peyer's patches,[21,22] migrate to the BM.

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

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

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



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

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

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

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

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

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

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

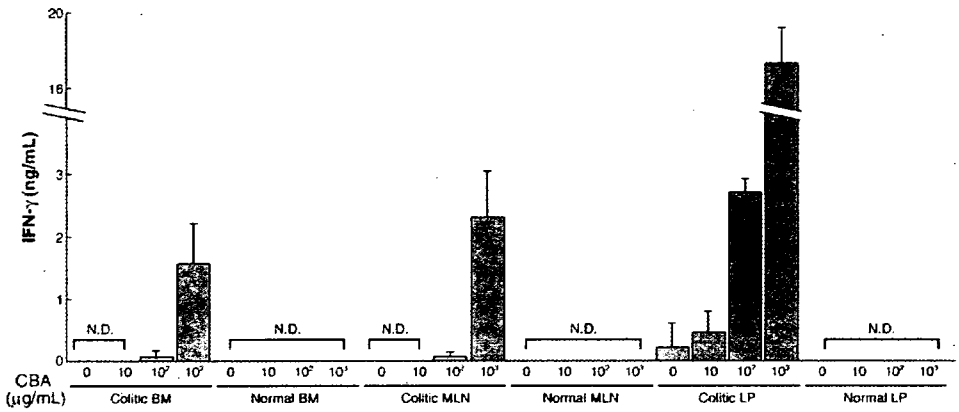


Figure 3. IFN-γ production by CD4+ T cells stimulated with APCs pulsed with CBA from colitic mice induced by adoptive transfer of CD4+CD45RB^{high} T cells. Supernatants collected on day 3 of culture were assayed for IFN-γ by ELISA. Data are indicated as the mean ± SD of 5 mice in each group. *$P < .05$.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

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

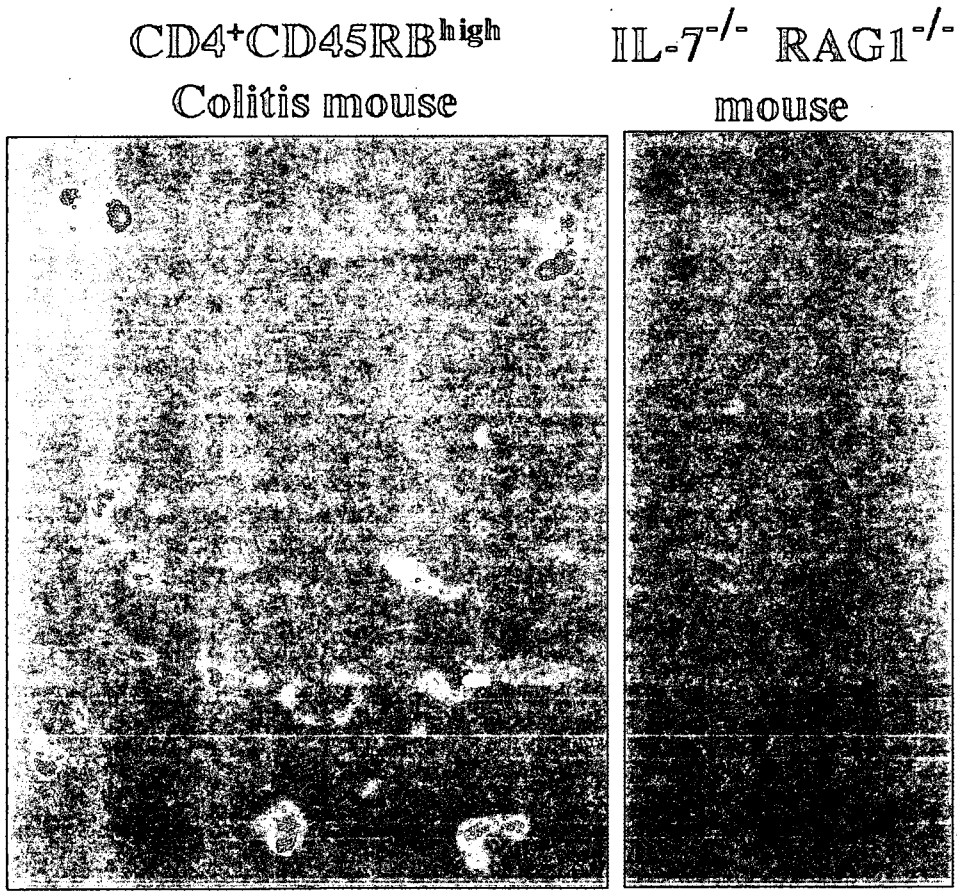


Figure 4. Cluster formation between CD4+ T cells and IL-7–expressing stromal cells within BM. Frozen sections of BM from colitic mice induced by adoptive transfer of CD4+CD45RB^{high} T cells (*left*) and untreated IL-7$^{-/-}$ × Rag-1$^{-/-}$ control mice (*right*) were stained with corresponding monoclonal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

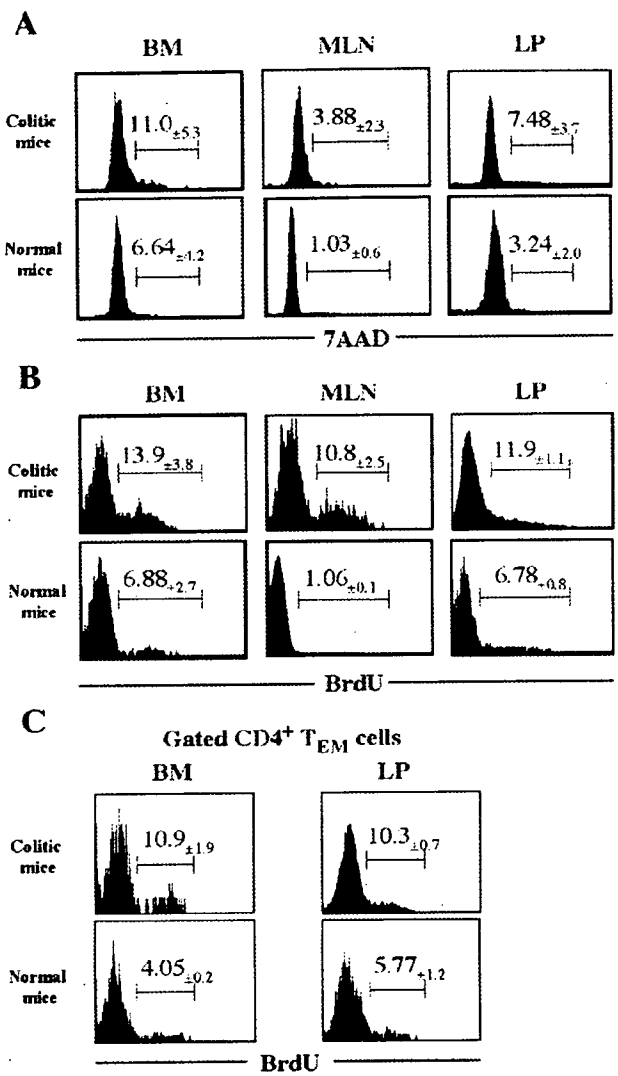


Figure 5. Colitic BM contains the actively dividing pool of memory CD4+ T cells. (*A*) BM, MLN, and LP CD4+ T cells from colitic mice or age-matched normal BALB/c mice were stained for DNA content using 7AAD. One representative mouse is shown of 5 mice analyzed. (*B*) Colitic mice and normal control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

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

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

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

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