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Continuous generation of colitogenic CD4^{+} T cells in persistent colitis

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Inflammatory bowel diseases take chronic courses due to the expansion of colitogenic CD4^{+} cells. However, it is unclear whether the persistent disease is driven by continuous reactivation of colitogenic memory CD4^{+} cells to generate effector CD4^{+} cells or by continuous generation of effector CD4^{+} cells from naïve cells. To clarify this issue, we performed a series of sequential adoptive transfers of Ly5.2^{+} and Ly5.1^{+} CD4^{+}CD45RB^{high} cells into RAG-2$^{-/-}$ mice at different time points. We show here that the secondarily transferred CD4^{+}CD45RB^{high} cells can be converted to CD4^{+}CD44^{high}CD62L^{-}IL-7Rα^{high} effector-memory T cells even in the presence of pre-existing effector-memory CD4^{+} cells. Although the total cell numbers of CD4^{+} cells in established colitic mice were consistently equivalent irrespective of the number of primarily transferred cells, the ratio of primarily and secondarily transferred cells was dependent on the ratio of the transferred cell numbers, but not on the order of the transfer. Of note, we found that primarily transferred CD4^{+} cells produced significantly lower amounts of IFN-γ and IL-17 than CD4^{+} cells arising from secondary transfer. In conclusion, the continuous generation of colitogenic CD4^{+} cells that compensate for exhausted CD4^{+} cells may be one of the mechanisms involved in the persistence of colitis.

Key words: Colitis · Colitogenic memory T cells · Mucosal immunity

Introduction

Intestinal mucosal surfaces are continuously exposed to antigens of the intestinal flora [1]. However, the gut-associated immune system defends against systemic circulation of harmful intestinal antigens and induces systemic tolerance toward intestinal commensal antigens by various mechanisms including suppression by regulatory CD4^{+} T cells [2–7]. In contrast, inflammatory bowel disease (IBD) is associated with activation of the local and systemic immune responses due to a lack of tolerance to intestinal bacterial antigens [6, 7]. Although the etiology of IBD is uncertain, there is much evidence suggesting that the pathogenesis of IBD involves dysregulated recognition of the intestinal bacterial antigens, resulting in the generation of colitogenic CD4^{+} effector and memory T cells. Nevertheless, the nature of the colitogenic CD4^{+} T cells over time is not fully understood especially in terms of the perpetuation of chronic colitis.

In general, IBD progresses steadily or relapses after remission throughout life [8, 9]. Although it is likely that the persistent disease is caused by the activation and expansion of colitogenic CD4^{+} effector T cells, several possible mechanisms may be involved. One possibility is that the persistency is driven by the initial colitogenic CD4^{+} memory T clones acting like memory stem cells [10] throughout the entire course of disease. In this case, colitogenic CD4^{+} effector T cells would be generated from colitogenic CD4^{+} memory T cells that are established at the initial onset, but are presumably suppressed by regulatory CD4^{+} T cells in remission [5, 7]. However, this scenario has one obstacle, namely that memory T cells are believed to be generated for the first time after antigen clearance, but not in the persistent presence of

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antigens. Certainly, such is the case in models of chronic viral infections, such as lymphocytic choriomeningitis virus (LCMV) and influenza A virus infections [11]. Since the possible antigens for colitogenic CD4$^+$ T cells are derived from intestinal bacteria and/or self antigens in the intestine that are never eliminated from the body, it is doubtful whether colitogenic CD4$^+$ memory T cells could actually be established in such a situation. A second possibility is that the same or different epitope-specific colitogenic CD4$^+$ effector T cells are generated and expand after priming from newly recruited naïve CD4$^+$ T cells from the thymus. In the case of multiple sclerosis, for example, it is thought that recruitment and activation of new autoimmune T cells evoke repeated disease episodes [12]. Newly recruited T cells could recognize distinct autoantigenic epitopes on the same antigen, or even be specific for different autoantigens through the epitope-spreading cascade. In the case of persistent colitis, it is also unclear whether pre-existing colitogenic CD4$^+$ effector or memory T cells prevent or ignore the priming, expansion, and phenotypic and functional conversion of newly recruited naïve CD4$^+$ T cells to effector or memory CD4$^+$ T cells. To evaluate these unsolved, but critical, issues, we performed sequential adoptive transfers of Ly5.1$^+$ and Ly5.2$^+$ CD4$^+$CD45RB^{high} T cells into immunodeficient RAG-2$^{-/-}$ mice at different time points to induce chronic colitis.

Results

Newly transferred CD4$^+$CD45RB^{high} cells are converted into effector-memory T cells in colitic mice

To assess the possibility that continuous generation of new colitogenic CD4$^+$ T cells from naïve CD4$^+$ T cells occurs routinely in colitic mice, we performed sequential adoptive transfers of Ly5.2$^+$ and Ly5.1$^+$ CD4$^+$CD45RB^{high} T cells into RAG-2$^{-/-}$ mice at different time points. To do this, we divided RAG-2$^{-/-}$ recipient mice into three groups (Fig. 1A): Group 1, RAG-2$^{-/-}$ mice transferred with Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk; Group 2, RAG-2$^{-/-}$ mice transferred with Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk and with Ly5.1$^+$CD4$^+$CD45RB^{high} T cells at 5 wk; and Group 3, RAG-2$^{-/-}$ mice transferred with Ly5.1$^+$CD4$^+$CD45RB^{high} T cells at 5 wk. Mice were observed for 10 wk after the first transfer of Ly5.2$^+$CD4$^+$CD45RB^{high} T cells. In Group 1 and Group 2, mice developed colitis over time after the first transfer of Ly5.2$^+$CD4$^+$CD45RB^{high} T cells, and ongoing clinical scores estimated by diarrhea with increased mucus in the stool, anorectal prolapse, hunched posture, and weight loss gradually increased after transfer, reaching to the maximum score 10 wk after the first transfer regardless of whether the second transfer was made (Fig. 1B). Similarly, Group 3 mice gradually developed colitis with time after the transfer of Ly5.1$^+$CD4$^+$CD45RB^{high} T cells, and the ongoing clinical score reached a similar level to that of Groups 1 and 2 mice at 10 wk (Fig. 1B). Histological examination showed prominent epithelial hyperplasia with glandular elongation with a massive infiltration of mononuclear cells in the lamina propria (LP) of the colon from all groups of mice at 10 wk (Fig. 1C). Histological scorings revealed no significant differences between the three groups (Fig. 1D).

A further quantitative evaluation of CD4$^+$ T cell accumulation was made by isolating CD4$^+$ T cells from various sites, such as spleen (SP), peripheral blood (PB), LP, mesenteric lymph nodes (MLN), and bone marrow (BM). Consistent with the similar clinical and histological severity of colitis between the three groups, the total recovered cell numbers in all sites were equivalent among the groups at 10 wk after the first transfer (Fig. 1E). Interestingly, in Group 2 mice, the ratio of Ly5.2$^+$ and Ly5.1$^+$ CD4$^+$ T cells in all sites was almost 1:1, in accordance with the 1:1 ratio of the transferred cell numbers (Fig. 1E). Furthermore, we found that all the transferred CD4$^+$CD45RB^{high} T cells, whether Ly5.1$^+$ or Ly5.2$^+$, were converted to CD4$^+$CD44^{high} effector or memory cells both in SP and LP (Fig. 1F). In addition, substantial numbers of CD4$^+$ T cells both in SP and LP, especially in LP, expressed the activation marker CD69 (Fig. 1F). These data indicated two findings. First, secondarily transferred Ly5.1$^+$CD4$^+$CD45RB^{high} naïve T cells could be converted to CD4$^+$CD44^{high} effector or memory cells even in the presence of pre-existing Ly5.2$^+$ effector or memory cells that had previously expanded in colitic mice. Second, newly (secondarily transferred) and previously (primarily transferred) established CD4$^+$CD44^{high} effector or memory cells compete with each other and the already present CD4$^+$ T cells to occupy the space available to a constant number of CD4$^+$ T cells in established chronic colitis at 10 wk.

Competition between colitic CD4$^+$ T cells is dependent on the transferred cell numbers

To further assess the mechanism of the competition between the first (old) and the second (new) transferred CD4$^+$ T cells in colitic mice, we next divided RAG-2$^{-/-}$ mice into three groups according to the number of Ly5.2$^+$CD4$^+$CD45RB^{high} T cells transferred at the first transfer (Fig. 2A): Group 1, 3×10^4 cells (named "10 ^ 4"), Group 2, 3×10^5 cells ("10 ^ 5"), and Group 3, 3×10^6 cells ("10 ^ 6"). All groups of mice were secondarily transferred with the same number (3×10^5) of Ly5.1$^+$CD4$^+$CD45RB^{high} T cells at 5 wk after the first transfer and killed at 10 wk after the first transfer. At 5 wk, the assessment of ongoing clinical scores revealed that Group 2 ("10 ^ 5") and Group 3 ("10 ^ 6") mice started to develop wasting disease and colitis, but the clinical severities of all groups were not significantly different at 5 wk after the first transfer (Fig. 2B), although the severity assessed by ongoing clinical scores of Group 1 ("10 ^ 4") tended to be low. After the second transfer, all groups of mice progressively developed wasting disease and colitis to a similar extent until 10 wk after the first transfer (Fig. 2B). Like ongoing clinical scores, histological findings at 10 wk revealed that all groups of mice developed severe colitis with a massive infiltration of mononuclear cells in the LP (Fig. 2C), and the histological scores confirmed this finding in multiple colon sections (Fig. 2D).

Interestingly, the total cell numbers recovered from various sites at 10 wk were not significantly different between the groups

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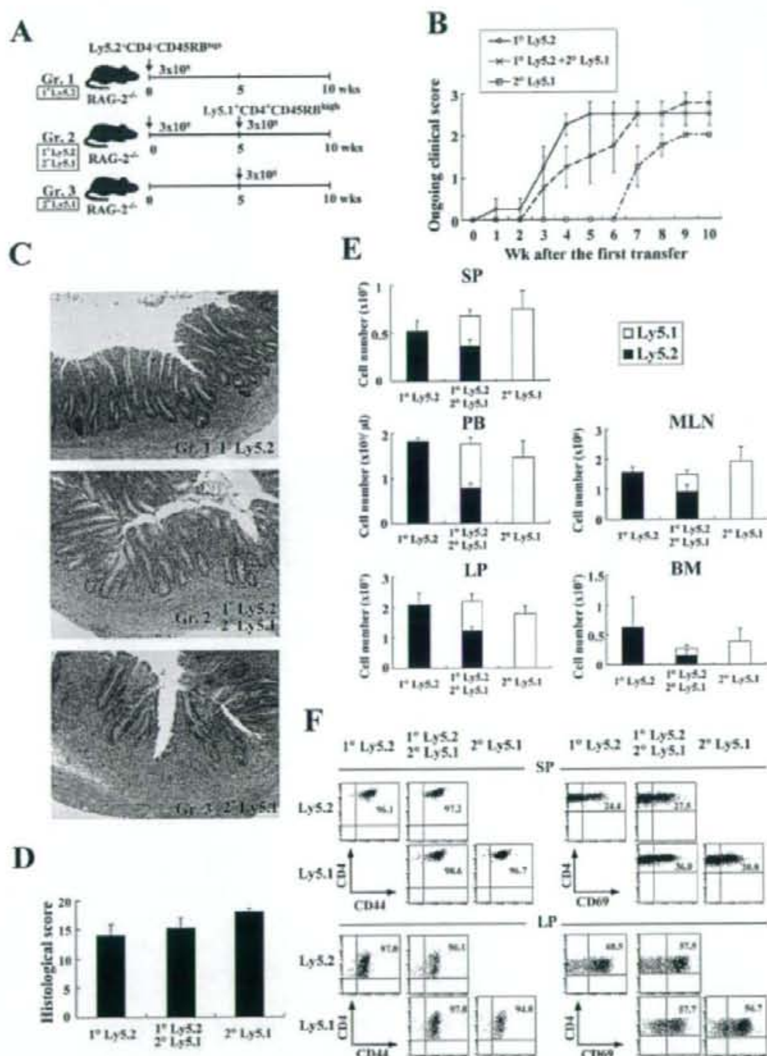

Figure 1. Newly recruited $CD4^+CD45RB^{high}$ T cells are primed and converted to $CD4^+CD44^{high}$ T cells. (A) Experimental design. C57BL/6-Ly5.2-RAG-$2^{-/-}$ mice were divided into three groups as described in the *Materials and methods*. (B) Ongoing clinical scores for the three groups were determined at the indicated times. (C) Histopathological findings of colon. Original magnification, ×100. (D) Histological scores were determined at 10 wk after the first transfer. (E) Recovered cell numbers of $CD3^+CD4^+$ T cells from SP, PB, LP, MLN, and BM. (F) Phenotypic characterization of SP and LP $CD4^+$ T cells after the transfer of $CD4^+CD45RB^{high}$ T cells. Representative results shown are from six mice per group.

in spite of the different numbers of the transferred cells in the first transfer (Fig. 2E), indicating that the space occupied by colitic $CD4^+$ T cells in each tissue of established colitic mice is equivalent. Furthermore, the ratio of Ly5.2$^+$ cells to Ly5.1$^+$ cells in colitic mice at 10 wk was dependent on the ratio of the transferred cell numbers: it was less than unity in Group 1 ("10^4") mice and greater than unity in Group 3 ("10^6"), whereas in Group 2 ("10^5") mice transferred with the same numbers of Ly5.2$^+$ cells and Ly5.1$^+$ cells at different times, the ratio of these cell types was almost 1:1 at various sites (Fig. 2F).

We next examined whether the transferred $CD4^+CD45RB^{high}$ T cells could be differentiated into effector or memory $CD4^+$ T cells in this sequential transfer experiment. As shown in Fig. 3, almost all $CD4^+$ T cells in any tissues, whether Ly5.1$^+$ or Ly5.2$^+$ cells, had

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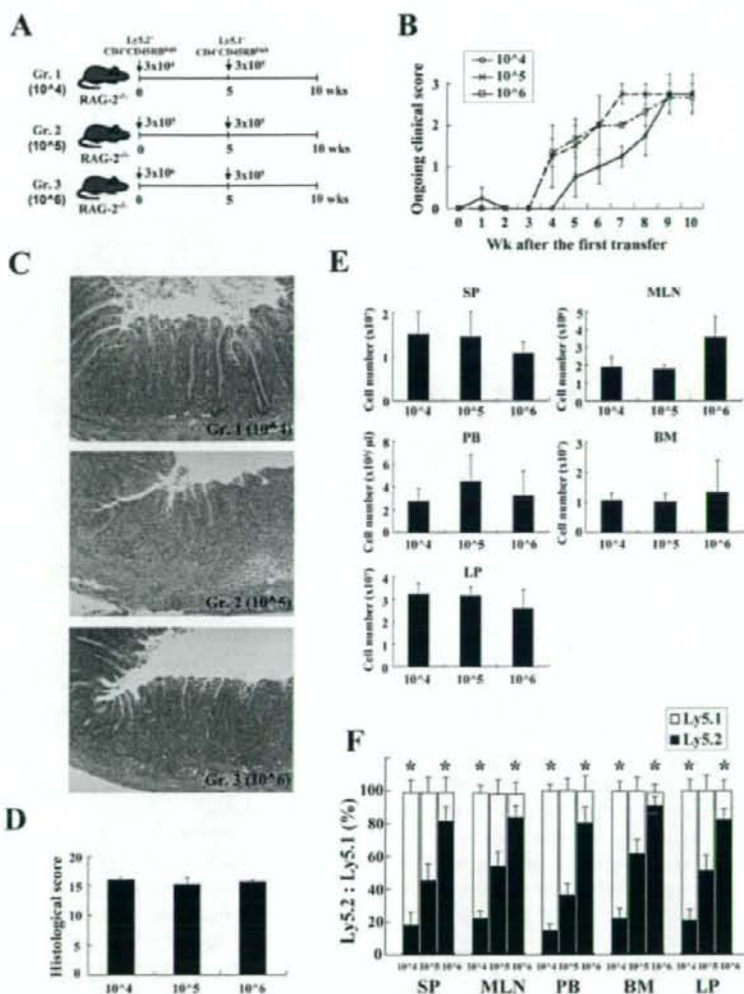

Figure 2. Newly generated CD4+CD44high T cells compete with pre-existing CD4+CD44high T cells depending on the transferred cell numbers. (A) Experimental design. C57BL/6-Ly5.2-RAG-2−/− mice were divided into three groups as described in the *Materials and methods*. (B) Ongoing clinical scores for the three groups were determined at the indicated times. (C) Histopathological findings of colon. Original magnification, ×100. (D) Histological scores were determined at 10 wk after the first transfer. (E) Recovered cell numbers of CD3+CD4+ T cells from SP, PB, LP, MLN, and BM. Cell numbers of Ly5.1+ or Ly5.2+ CD3+CD4+ T cells were determined by four-color flow cytometry. (F) Phenotypic characterization of SP, MLN, PB, BM and LP CD4+ T cells after the transfer of CD4+CD45RBhigh T cells. Results shown are from six mice per group. *$p<0.05$ *vs.* Ly5.2 ratio in same group.

a phenotype of $CD4^+CD44^{high}CD62L^-IL\text{-}7R\alpha^+$ effector-memory T (T_{EM})-like cells, in contrast to the originally transferred $CD4^+$ $CD45RB^{high}CD44^{low}$ T cells. Interestingly, in Group 3 ("10 ^ 6"), which received a high number (3×10^6 cells/mouse) of $CD4^+$ $CD45RB^{high}$ T cells at the first transfer, higher numbers of central-memory $CD4^+$ T (T_{CM})-like cells were generated in the SP and the MLN both in $Ly5.2^+$ cells and $Ly5.1^+$ populations. This agrees with a recent report that precursor numbers can impact the differentiation and the homeostasis of the resultant memory cells [13].

TCR Vβ repertoire is constant between first and second transferred cells

Although we found that the secondarily transferred $CD4^+CD45RB^{high}$ T cells could differentiate into $CD4^+$ T_{EM}-like cells *in vivo*, even in the persistent presence of previously developed $CD4^+$ T_{EM}-like cells from the first transfer (Fig. 1–3), it was unclear whether newly recruited $CD4^+$ T cells recognize the same antigenic epitopes as the previously recruited $CD4^+$ T cells at

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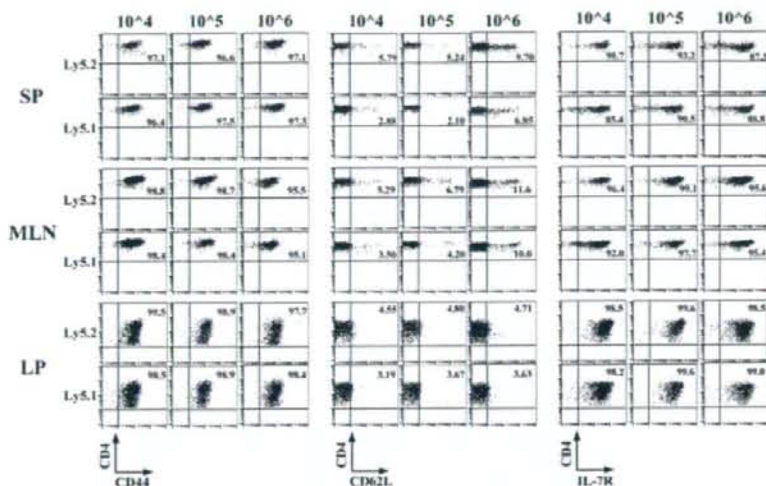

Figure 3. Phenotypic characterization of SP, MLN and LP CD4+ T cells after a sequential adoptive transfer as described in Fig. 2. Flow cytometric analysis shows that most of the transferred CD4+CD45RB^{high} T cells in Group 1 (10^4), Group 2 (10^5), and Group 3 (10^6) mice have the characteristics of CD4+CD44^{high}CD62L−IL-7Rα^{high} T_{EM} cells. Results shown are from six mice per group.

the first transfer. To clarify this issue, splenic Ly5.1$^+$ and Ly5.2$^+$ CD4$^+$ T cells from three groups were analyzed for their TCR Vβ repertoire by three-color flow cytometry. It is reasonable to use SP CD4$^+$ T cells in place of LP CD4$^+$ T cells for this assay, since we previously demonstrated that colitic SP CD4$^+$ T cells had similar characteristics in that they were CD4$^+$CD44^{high}CD62L$^-$IL-7Rα^{high} T_{EM}-like cells [14], and were also colitogenic cells by which colitis can be transferred to new recipient mice [15]. As shown in Fig. 4, a polyclonal dominant TCR Vβ repertoire with dominant Vβ2, Vβ4, Vβ8.1/8.2, and Vβ14 was almost constant between previously and newly transferred cells, regardless of the different numbers of the first transfer, indicating that colitogenic CD4$^+$ T cells recognizing the same or similar antigenic epitopes developed in accordance with the frequency or number of colitogenic antigen-specific naïve CD4$^+$ T cells. In addition, the pattern of TCR Vβ repertoire in colitic mice, whether with older Ly5.2$^+$ and newer Ly5.1$^+$ CD4$^+$ T cells, was similar to that of originally transferred CD4$^+$CD45RB^{high} T cells (Fig. 4).

Previously generated, older CD4+ T cells produce less IFN-γ and IL-17

It has recently been demonstrated that, during chronic viral infection, the functions of virus-specific CD8$^+$ T cells often become impaired and exhausted in the persistent presence of viral antigens, in contrast to the highly functional effector and memory CD8$^+$ T cells generated after virus clearance in acute infection [11, 16]. Since colitogenic CD4$^+$ T cells in colitic RAG-2$^{-/-}$ mice induced by an adoptive transfer of CD4$^+$CD45RB^{high} T cells are likely to expand by responding to resident enteric bacterial antigens that are persistently resident in the colonic lumen, it was possible that colitogenic CD4$^+$ T_{EM}-like cells gradually become exhausted over time after the transfer. To assess whether newly recruited T_{EM}-like cells that are differentiated from the secondarily transferred CD4$^+$CD45RB^{high} T cells can compete and prevail against the previously established and possibly exhausted T_{EM}-like cells in colitic mice, we next divided RAG-2$^{-/-}$ mice into two groups (Fig. 5A): Group 1, RAG-2$^{-/-}$ mice transferred with Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk, and Group 2, RAG-2$^{-/-}$ mice transferred with Ly5.2$^+$CD4$^+$CD45RB^{high} T cells at 0 wk, and secondarily transferred with Ly5.1$^+$CD4$^+$CD45RB^{high} T cells at 13 wk after fully establishing colitis by the first transfer. Mice were observed for 17 wk after the first transfer.

The assessment of clinical scores revealed that Group 1 and 2 mice developed wasting disease and severe colitis at the plateau level from approximately 6 wk after the first transfer and remained at this level until 13 wk after the first transfer without dropout by death (data not shown). At autopsy at 17 wk, the total clinical scores of both groups were not significantly different (Fig. 5B). Histological findings revealed that both groups of mice developed severe colitis with a massive infiltration of mononuclear cells in the LP (Fig. 5C), and histological scores also confirmed no differences between two groups in multiple colon sections (Fig. 5D). Interestingly, the cell numbers recovered from the SP and LP were not significantly different between the groups regardless of the second transfer (Fig. 5E), indicating again that the space occupied by colitogenic CD4$^+$ T cells in each colitic mouse is equivalent. As shown in Fig. 5F, almost all LP CD4$^+$ T cells in any tissues, whether old Ly5.2$^+$ cells or new Ly5.1$^+$ cells, had a phenotype of CD4$^+$CD44^{high}CD62L$^-$IL-7Rα$^+$ T_{EM}-like cells.

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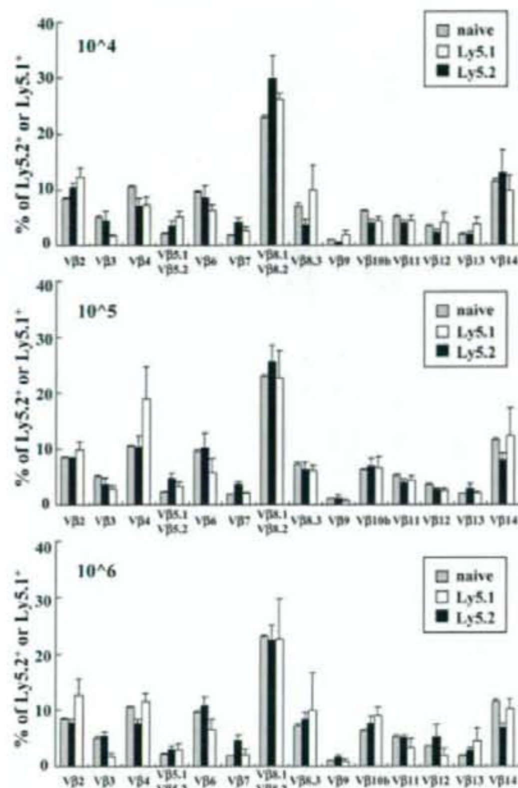

Figure 4. Flow cytometric analysis of Vβ families on the surface of the splenic CD4+ T cells in the Group 1 (10^4), Group 2 (10^5), and Group 3 (10^6) mice described in Fig. 2. To analyze the TCR Vβ family repertoire, splenic cells were four-color-stained with PerCP*-conjugated anti-CD3mAb, allophycocyanin-conjugated anti-CD4 mAb, PE-conjugate anti-Ly5.1 or Ly5.2 mAb, and the indicated mAb of a panel of 14 FITC-conjugated Vβ mAb. Each percentage value indicates the frequency of each Vβ (n=6). Naïve, $CD4^+CD45RB^{high}$ T cells serves as a control.

Of note, we found that the old LP $Ly5.2^+$ cells produced significantly less IFN-γ (Th1) and IL-17 (Th17) cytokines than the new LP $Ly5.1^+$ cells (Fig. 6A and B), indicating that the old LP $Ly5.2^+$ cells long after the first transfer had fallen into exhaustion. In addition, the ratios of IL-17^+, but not IFN-γ^+, cells in the old MLN and SP $Ly5.2^+CD4^+$ cells were significantly decreased as compared with those of new $Ly5.1^+$ cells (Fig. 6A and B), suggesting that colitogenic $CD4^+$ T cells in various sites became exhausted over time. In particular, the LP shows the greatest level of cell exhaustion, at least in the expression of IFN-γ. Since it has been reported that PD-1 is one of markers for exhausted $CD8^+$ T cells in chronic LMCV infection in mice and HIV infection in human [17–19], we also checked this molecule in old $Ly5.2^+$ cells and new $Ly5.1^+$ cells. Contrary to the above data of cytokine production and previous reports of exhausted PD-1-expressing $CD8^+$ T cells [17–19], no difference in PD-1 expression was found between old $Ly5.2^+$ cells and new $Ly5.1^+$ cells at 17 wk (Fig. 5F), indicating that PD-1 is not an appropriate marker for the exhaustion for murine $CD4^+$ T cells, at least in the present time course of the sequential adoptive transfer protocol.

Discussion

In the present study, we demonstrated that newly recruited naïve $CD4^+CD45RB^{high}$ T cells can be primed, expand and differentiate into $CD4^+CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ T_{EM}-like cells in colitic RAG-$2^{-/-}$ mice induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells in competition with previously established colitogenic T_{EM}-like $CD4^+$ T cells. Of note, the patterns of TCR Vβ repertoire were constantly similar between the first and the second transfers, indicating that a set of colitogenic polyclonal $CD4^+$ T cells compete for each clone-specific survival signal. This is most easily explained by TCR recognition of a specific and limiting antigen epitope-MHC class II ligand and homeostatic cytokines, such as IL-7 [20, 21]. Furthermore, we found that old LP $CD4^+$ T_{EM}-like cells produced less IFN-γ and IL-17 than newly developed LP $CD4^+$ T_{EM}-like cells, suggesting that old colitic LP $CD4^+$ T cells showed immunological exhaustion over time. These findings suggest that continuous generation of colitogenic $CD4^+$ T cells from newly recruited naïve $CD4^+$ T cells to compensate for the older exhausted $CD4^+$ T cells is one of the mechanisms sustaining chronic colitis.

Although IBD is thought to be caused by colitogenic effector $CD4^+$ T cells, which probably respond to intestinal bacterial antigens and damage the target intestine, the nature of the colitogenic $CD4^+$ T cells over time remains largely unclear. How are these colitogenic $CD4^+$ T cells generated and maintained in the body of patients with IBD? Are these colitogenic $CD4^+$ T cells just effector $CD4^+$ T cells, which are generated from naïve $CD4^+$ T cells or from colitogenic memory $CD4^+$ T cells acting like memory stem cells [10], and which are established at the initial attack and reside in the body throughout life? This question arises basically from the concept that 'true' memory T cells are established for the first time after antigen clearance from the body as often assessed in memory $CD8^+$ T cells in animal models of acute viral infection and vaccination [22–25]. According to this scenario, it seems that colitogenic 'true' memory $CD4^+$ T cells cannot be built up in our colitis model, since the possible target intestinal bacteria are never eliminated, but persist throughout life in chronic colitis both in animal models and human IBD. However, recent evidence suggests that maintenance of the $CD8^+$ and $CD4^+$ T cells in chronic infection is dependent on antigens [26, 27], although it seems inappropriate to call such cells memory cells. We here showed that colitic $CD4^+$ T cells of this transfer model strongly expressed CD44 and IL-7Rα, which is a reliable marker for memory, but not effector, $CD4^+$ T cells; and also we recently demonstrated that IL-7, which is an important factor for survival of memory $CD4^+$ T cells [21], is essential for the persistence of colitis by showing that IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice transferred with colitogenic LP

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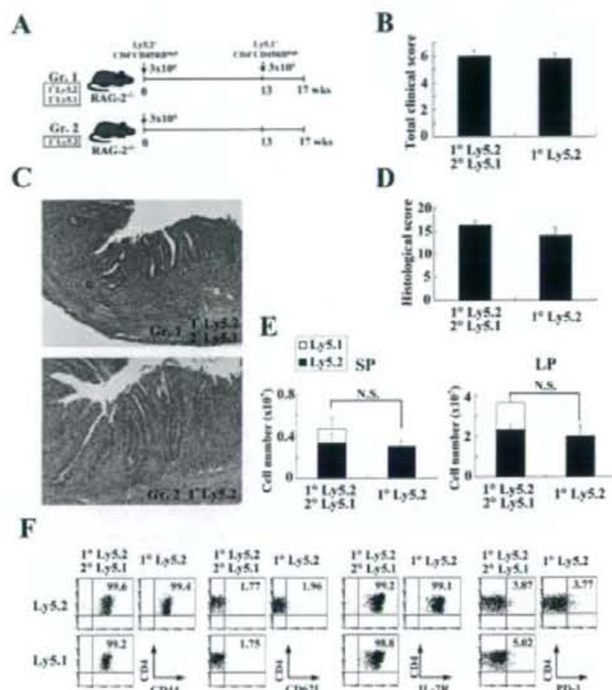

Figure 5. Pre-existing CD4$^+$CD44^{high} T cells are gradually exhausted. (A) Experimental design. C57BL/6-Ly5.2-RAG-2$^{-/-}$ mice were divided into two groups: Group 1, RAG-2$^{-/-}$ mice transferred with Ly5.2-derived CD4$^+$CD45RB^{high} T cells at 0 wk (n=6), and Group 2, RAG-2$^{-/-}$ mice transferred with Ly5.2-derived CD4$^+$CD45RB^{high} T cells at 0 wk, and again transferred with Ly5.1-derived CD4$^+$CD45RB^{high} T cells at 13 wk after the first transfer (n=6). Mice were observed for 17 wk after the first transfer. (B) Total clinical scores were determined at 17 wk after the first transfer as described in *Materials and methods*. Data are indicated as the mean ± SEM of six mice per group. (C) Histopathological findings of colon. Original magnification, ×100. (D) Histological scores were determined at 17 wk after the first transfer. Data are indicated as the mean + SEM of six mice in each group. (E) Recovered cell numbers of CD3$^+$CD4$^+$ T cells from SP and LP. The cell number of Ly5.1$^+$ or Ly5.2$^+$ CD3$^+$CD4$^+$ T cells was determined by four-color flow cytometry. Data are indicated as the mean + SEM of six mice per group. N.S., not significant. (F) Phenotypic characterization of LP CD4$^+$ T cells after the transfer of CD4$^+$CD45RB^{high} T cells. Representative results shown are from one mice per group (six mice per group were analysed).

CD4$^+$CD44^{high}IL-7Rα^{high} cells did not develop colitis [28]. Since the survival of memory, but not effector, CD4$^+$ T cells is believed to be dependent on IL-7 [21], our results suggest that colitogenic CD4$^+$CD44^{high}CD62L$^-$IL-7Rα^{high} T_{EM} cells are sustained at least in part in colitic mice even in the persistent presence of intestinal bacteria. Consistent with the present findings, we recently demonstrated that substantial numbers of colitogenic CD4$^+$CD44^{high}CD62L$^-$IL-7Rα^{high} T_{EM}-like cells reside in colitic BM, which is believed to lack intestinal bacterial antigens but produce IL-7 [28].

Naïve CD4$^+$ T cells are known to proliferate extensively, probably in response to foreign or self antigens, and are converted to memory-like CD4$^+$CD44^{high} T cells in lymphopenic immunodeficient mice, such as SCID and RAG-2$^{-/-}$ mice, in the process of 'lymphopenia-driven proliferation' [29]. Thus, it is likely that CD4$^+$CD44^{high}IL-7Rα^{high} cells in CD4$^+$CD45RB^{high} T cell-transferred colitic SCID mice represent memory-like cells, but not 'true' memory cells. Otherwise, it is also possible that the IL-7-dependent colitogenic CD4$^+$ T cells in the presence of intestinal bacterial antigens in colitic mice have unique characteristics that differentiate them from conventional effector or 'true' memory CD8$^+$ and CD4$^+$ T cells defined mainly by studies of models of acute viral infection. Further studies will be needed to address this issue.

Colitogenic CD4$^+$ T cells may also be continuously generated from naïve CD4$^+$ T cells that reside in the periphery or are newly generated from the thymus, because it is still unknown whether memory CD4$^+$ T cells, either 'true' memory or memory-like (T_{EM}-like) cells, are long-lived. Thus, it was of interest to determine whether the generation of new colitogenic CD4$^+$ T cells from naïve CD4$^+$ T cells is inhibited by the pre-existence of colitogenic CD4$^+$ T cells, and also whether it is involved in the persistence of disease, although it is known that adoptively transferred naïve CD4$^+$ T cells extensively proliferate and are converted to T_{EM}-like CD4$^+$ T cells presumably in response to intestinal bacteria under lymphopenic conditions like those in immunodeficient SCID and RAG-2$^{-/-}$ mice [25]. In a setting in the absence of antigens, it has recently been

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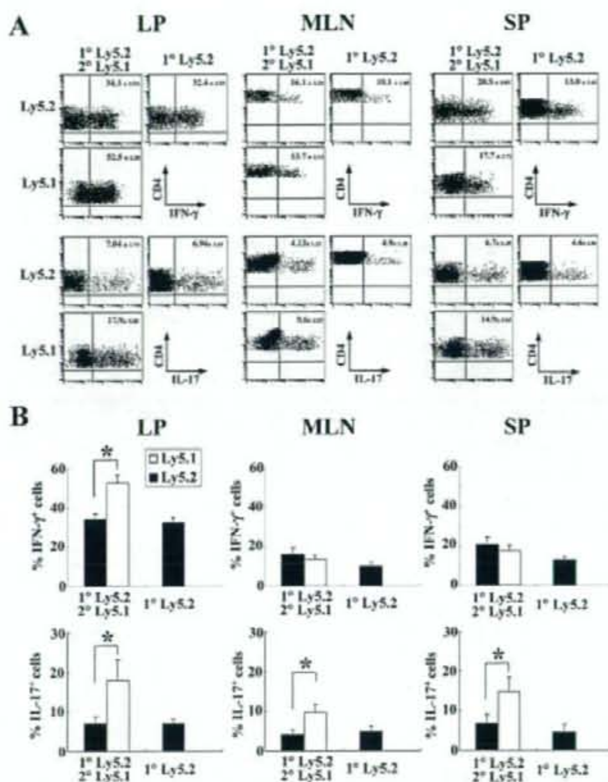

Figure 6. Preexisting CD4⁺CD44^high T cells are gradually exhausted. (A) Expression of IFN-γ and IL-17 on freshly isolated cells from LP, MLN, and SP in the Group 1 and Group 2 mice described in Fig. 5. Cells were labeled for Ly5.1, Ly5.2, CD4, and intracellular IFN-γ or IL-17. Ly5.2⁺ and Ly5.1⁺ CD4⁺ cells were gated and analyzed for the presence of CD4⁺IFN-γ⁺ cells or CD4⁺IL-17⁺. Number in upper quadrant represents the percentage of IFN-γ⁺ or IL-17⁺ cells among CD4⁺ cells. (B) The ratios of IFN-γ⁺ or IL-17⁺ cells among Ly5.1⁺ or Ly5.2⁺ cells were analyzed by gating Ly5.1 or Ly5.2 on CD4⁺ cells. Results shown are from six mice per group. *$p<0.05$.

demonstrated that pre-existing 'true' memory $CD4^+$ T cells in RAG-$2^{-/-}$ mice after adoptive transfer prevent the proliferation and conversion of newly transferred naïve $CD4^+$ T cells to the memory-like phenotype [30]. Furthermore, the same group previously demonstrated that $RAG^{-/-}$ mice transferred with a large number of naïve $CD4^+CD45RB^{high}$ T cells (12×10^6 cell/mouse) do not develop colitis [31]. This finding indicates that two mechanisms, cytokine (IL-7) competition and clonal competition, may restrain the activation and overgrowth of a small number of transferred naïve T cells in the lymphopenic condition. Nevertheless, the fact that newly recruited naïve $CD4^+CD45RB^{high}$ T cells can be activated and expand in established colitic mice, in which space available to them should already be occupied by a large number of colitogenic $CD4^+$ T cells, may suggest the older colitogenic effector or memory $CD4^+$ T cells are exhausted and permit the expansion of newly recruited colitogenic $CD4^+$ T cells in our model. By contrast, surprisingly, we found that newly recruited $CD4^+CD45RB^{high}$ T cells can be primed and expand extensively even in established colitic mice in which pre-existing colitogenic $CD4^+$ T cells were fully expanded and occupied the space of $CD4^+$ T cells. Thus, our present results may link the frequent recurrence in natural history of IBD to the continuous generation of colitogenic $CD4^+$ effector and memory T cells, because the frequency of recurrence gradually decreases in accordance with immunosenescence in patients with a decreased supply of naïve T cells by thymic involution. Interestingly, in addition, the total cell number of $CD4^+$ T cells was constant, and the ratio of pre-existing and newly recruited $CD4^+CD44^{high}$ T cells in the body was dependent on the ratio of the transferred cell numbers, indicating that both old and new $CD4^+$ T cells compete for space and a possibly constant amount of IL-7 depending on the frequency of transferred colitogenic naïve $CD4^+$ T cells.

Of note, we also demonstrated that production of IFN-γ and IL-17 by older LP $CD4^+CD44^{high}$T cells was significantly less as compared with newly developed LP $CD4^+CD44^{high}$T cells (Fig. 5), indicating that these LP $CD4^+CD44^{high}IL\text{-}7R\alpha^{high}$ cells become

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exhausted over time, as seen in CD8$^+$ T cells in persistent virus infection [17, 18]. Thus, it appears that colitogenic LP CD4$^+$ T cells in colitic mice are a mixture of IL-7-dependent CD4$^+$ T_{EM}-like cells and exhaustion-facing effector cells derived from these CD4$^+$ T_{EM}-like cells after encountering intestinal bacterial antigens. Consistent with this, the most affected site of cell exhaustion as indicated by cytokine production seemed to be the LP (Fig. 6), which is thought to be the effector site. Nevertheless, it remains unknown why mice transferred secondarily with CD4$^+$CD45RB^{high} T cells did not show exacerbation of the disease as compared with mice without the second transfer in clinical and histological evaluations (Fig. 5B and D). As a clue, we recently performed over seven sequential transfers of colitic LP CD4$^+$ cells obtained from colitic CD4$^+$CD45RB^{high} cell-transferred SCID mice into new SCID mice. Although SCID mice transferred with colitic LP CD4$^+$ cells stably developed colitis over several transfers, the severity of colitis declined with the increasing number of transfers [32]. Thus, it is likely that the exhaustion of CD4$^+$ T cells requires longer to become clinically and histologically evident than does the decline in cytokine production. Further study will be needed to address this issue.

In summary, we propose that continuous generation of colitogenic CD4$^+$ T cells from naïve CD4$^+$ T cells is critically involved in the persistence of chronic colitis, suggesting that it is important not only to target the pre-existing colitogenic CD4$^+$ T cells but also to suppress and control the new generation of colitogenic CD4$^+$ T cells in developing a strategy for the treatment of IBD.

Materials and methods

Animals

C57BL/6N-Ly5.2 mice were purchased from Japan Clea (Tokyo, Japan). C57BL/6N -Ly5.1 and C57BL/6N -Ly5.2-RAG-2 deficient (RAG-2$^{-/-}$) mice were obtained from Taconic Laboratory (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). Mice were maintained under specific pathogen-free (SPF) conditions in the Animal Care Facility of Tokyo Medical and Dental University. All donors and recipients were used for adoptive transfer experiments at 6–10 wk of age. All experiments were approved by the regional animal study committees (permission number: 2006–049) and were done according to institutional guidelines and Home Office regulations.

Antibodies

The mAb other than biotin-conjugated anti-mouse IL-7Rα (A7R34; eBioscience, San Diego) were obtained from BD PharMingen (San Diego, CA) and used for purification of cell populations and flow cytometry analysis; 145–2C11, FITC-, PE- and PerCP®-conjugated anti-mouse CD3; RM4–5, PE- and allophycocyanin-conjugated anti-mouse CD4; 16A, FITC-conjugated anti-mouse CD45RB; IM7, allophycocyanin-conjugated anti-mouse CD44; MEL-14, PE-conjugated anti-mouse CD62L; H1.2F3, FITC- and PE-conjugated anti-mouse CD69; A20, FITC- and PE-conjugated anti-mouse Ly5.1 (CD45.1); 104, FITC-conjugated anti-mouse Ly5.2 (CD45.2); J43, PE-conjugated anti-mouse PD-1; XMG1.2, PE-conjugated anti-mouse IFN-γ; TC11–18H10, PE-conjugated anti-mouse IL-17; B20.6, FITC-conjugated anti-mouse Vβ2; KJ25, FITC-conjugated anti-mouse Vβ3; KT4, FITC-conjugated anti-mouse Vβ4; MR9–4, FITC-conjugated anti-mouse Vβ5.1/2; RR4–7, FITC-conjugated anti-mouse Vβ6; TR310, FITC-conjugated anti-mouse Vβ7; MR5–2, FITC-conjugated anti-mouse Vβ8.1/2; B21.14, FITC-conjugated anti-mouse Vβ8.3; MR10–2, FITC-conjugated anti-mouse Vβ9; B21.5, FITC-conjugated anti-mouse Vβ10^b; RR3–15, FITC-conjugated anti-mouse Vβ11; MR11–1, FITC-conjugated anti-mouse Vβ12; IN12.3, FITC-conjugated anti-mouse Vβ13; 14.2, FITC-conjugated anti-mouse Vβ14. Biotinylated antibodies were detected with PE-streptavidin (BD PharMingen).

T cell preparation

For isolation of peripheral lymphocytes, 600 μL PB was collected from each mouse and diluted 1:1 with PBS. The diluted blood was layered over Lymphosepar II (IBL, Gunma, Japan) and centrifuged at 400 × *g* for 30 min at room temperature. The lymphocytes were then isolated from the plasma-Ficoll interface. SP and MLN were mechanically disrupted into single cell suspensions. BM was collected from the femur by flushing with sterile PBS. For the preparation of colonic LP cells [33], colon was first flushed extensively to eliminate the lumen content, then longitudinally opened and cut into small pieces. The dissected mucosa was incubated with Ca^{2+} Mg^{2+}-free Hanks' BSS containing 1 mM DTT (Sigma-Aldrich, St. Louis, MO) for 30 min to remove mucus, then treated with 3 mg/mL collagenase (Roche Diagnostics GmbH, Germany) and 0.01% DNase (Worthington Biomedical Co., Freehold, NJ) for 2 h. After filtering through gauze, cells were pelleted two times through a 40% isotonic Percoll solution, and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched CD4$^+$ T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells contained >94% CD4$^+$ cells when analyzed by FACSCalibur.

Adoptive transfer protocols

In the first set of adoptive transfer experiments, we divided C57BL/6-Ly5.2-RAG-2$^{-/-}$ mice into three groups: Group 1, RAG-2$^{-/-}$ mice transferred with 3×10^5 Ly5.2-derived CD4$^+$CD45RB^{high} T cells at 0 wk of the starting point (n=6); Group 2, RAG-2$^{-/-}$ mice transferred with 3×10^5 Ly5.2-derived CD4$^+$CD45RB^{high} T cells at 0 wk and with 3×10^5 Ly5.1-derived CD4$^+$CD45RB^{high} T cells at 5 wk after the first transfer (n=6);

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Group 3, RAG-$2^{-/-}$ mice transferred with 3×10^5 Ly5.1-derived $CD4^+CD45RB^{high}$ T cells at 5 wk after the starting point ($n=6$). Briefly, $CD4^+$ T cells were isolated from splenocytes using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec, Auburn, CA). Enriched $CD4^+$ T cells were labeled with PE-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-CD45RB mAb, then sorted to yield the $CD45RB^{high}$ (highest staining 30%) fraction on a FACS Aria (Becton Dickinson, Sunnyvale, CA). Each mouse was injected intraperitoneally with 3×10^5 $CD4^+CD45RB^{high}$ T cells once. Mice were observed and killed at 10 wk after the starting point.

In the second set of adoptive transfer experiments, we divided C57BL/6-Ly5.2-RAG-$2^{-/-}$ mice into three groups: Group 1, RAG-$2^{-/-}$ mice transferred with 3×10^4 $Ly5.2^+CD4^+CD45RB^{high}$ T cells at 0 wk ("10^4", $n=6$); Group 2, RAG-$2^{-/-}$ mice transferred with 3×10^5 $Ly5.2^+CD4^+CD45RB^{high}$ T cells at 0 wk ("10^5", $n=6$); and Group 3, RAG-$2^{-/-}$ mice transferred with 3×10^6 $Ly5.2^+CD4^+CD45RB^{high}$ T cells at 0 wk ("10^6", $n=6$). All groups of mice were transferred with 3×10^5 $Ly5.1^+CD4^+CD45RB^{high}$ T cells at 5 wk after the first transfer. Mice were killed at 10 wk after the starting point.

In the third set of adoptive transfer experiments, we divided C57BL/6-Ly5.2-RAG-$2^{-/-}$ mice into two groups: Group 1, RAG-$2^{-/-}$ mice transferred with 3×10^5 $Ly5.2^+CD4^+CD45RB^{high}$ T cells at 0 wk of the starting point ($n=6$); and Group 2, RAG-$2^{-/-}$ mice transferred with 3×10^5 $Ly5.2^+CD4^+CD45RB^{high}$ T cells at 0 wk and with 3×10^5 $Ly5.1^+CD4^+CD45RB^{high}$ T cells at 13 wk after the first transfer ($n=6$). Mice were skilled at 17 wk after the starting point.

Disease monitoring and clinical scoring

The recipient mice after T cell transfer were weighed initially, then three times per week thereafter. They were observed for clinical signs of illness: hunched appearance, piloerection of the coat, diarrhea, and blood in the stool. Mice were killed at the indicated times and assessed for a total clinical score as the sum (0–6 points) of three parameters: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–2 (0, normal beaded stool; 1, soft stool; 2, diarrhea). To monitor clinical signs during the observation period, the ongoing clinical score is defined as the sum (0–3 points) of the two parameters other than colon thickening [34].

Histological examination

Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. The sections were analyzed without prior knowledge of the type of T cell reconstitution and recipients. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated using a modification of a previously described scoring system [33].

Flow cytometry

To detect the surface expression of a variety of molecules, isolated 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-, or biotin-labeled antibodies for 30 min on ice. Biotinylated antibodies were detected with PE-streptavidin. For intracellular staining for IFN-γ and IL-17 [35], cells were stimulated with 50 ng/mL phorbol-12-myristate-13 acetate (PMA; Calbiochem, CA) and 500 ng/mL ionomycin (Sigma-Aldrich) for 10 h, then 5 μg/mL brefeldin A (GolgiPlug; BD PharMingen) was added. Cells were first preincubated with FcγR-blocking mAb for 20 min, and then stained with PerCP-anti-CD3 mAb, allophycocyanin-anti-CD4 mAb, and FITC-anti-CD45.1 or anti-CD45.2 mAb. The stimulated cells were fixed and permeabilized with Cytofix/Cytoperm™ (BD PharMingen) at 4°C for 30 min. Staining and washing were performed in Perm/Wash Buffer™ (BD PharMingen), and cells were stained with PE-conjugated anti-IFN-γ or anti-IL-17 mAb.

Statistical analysis

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

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Abbreviations: **IBD:** inflammatory bowel disease · **LP:** lamina propria · **MLN:** mesenteric LN · **PB:** peripheral blood · **SP:** spleen · **T_{CM}:** central-memory T · **T_{EM}:** effector-memory T

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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.

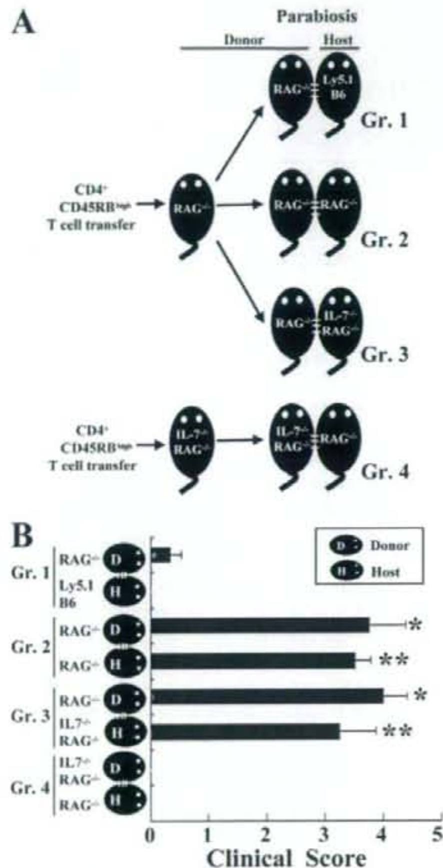

FIGURE 1. Host IL-$7^{-/-}$ × RAG-$1^{-/-}$ mice in parabionts with diseased IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice show a wasting disease and clinical signs of colitis. *A*, Parabiosis experimental design. For an adoptive transfer, splenic $CD4^+CD45RB^{high}$ T cells were isolated from C57BL/6-Ly5.2 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

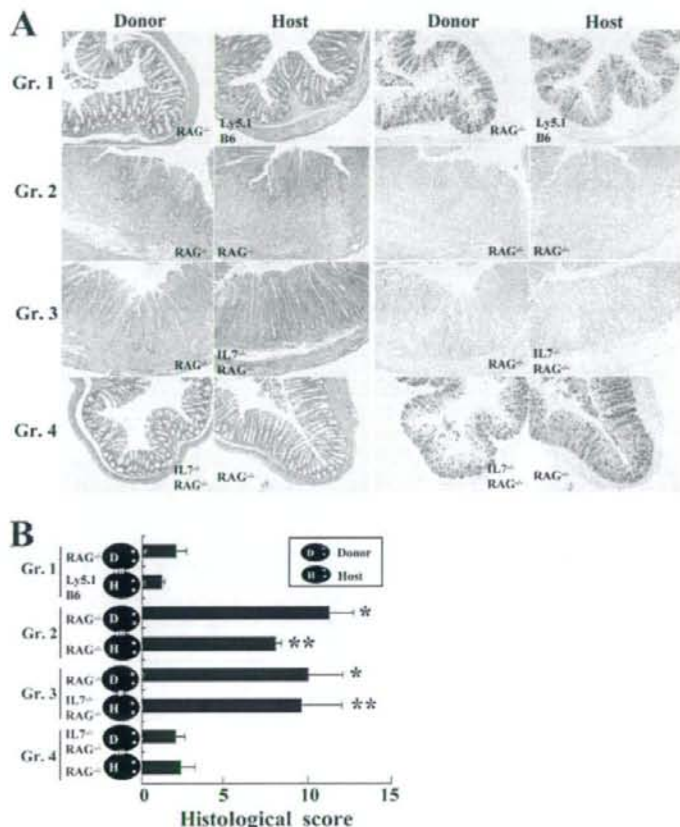

FIGURE 2. IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice in parabionts with diseased IL-$7^{+/+}$ × RAG-$1^{-/-}$ donor mice develop colitis. *A*, Histological examination by H&E staining (*left*) and Alcian blue staining (*right*) of the colon from each group at 4 wk after surgery. Representative of four separate 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

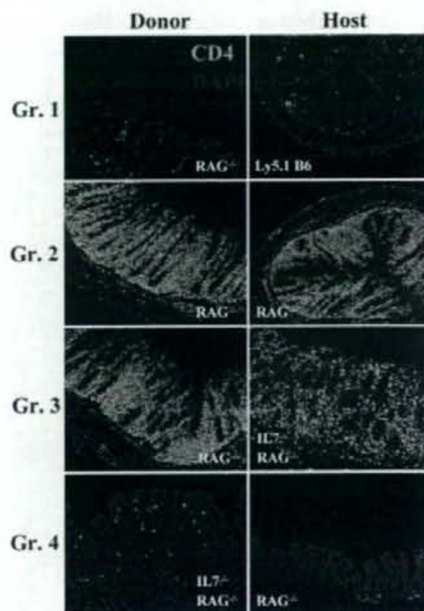

FIGURE 3. IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice in parabionts developed colitis with the marked infiltration of immigrant CD4$^+$ T cells from donor mice. CD4 immunostaining and DAPI counterstaining of the colon from Groups 1–4 at 4 wk after surgery. Frozen sections were fixed with 4% paraformaldehyde 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.

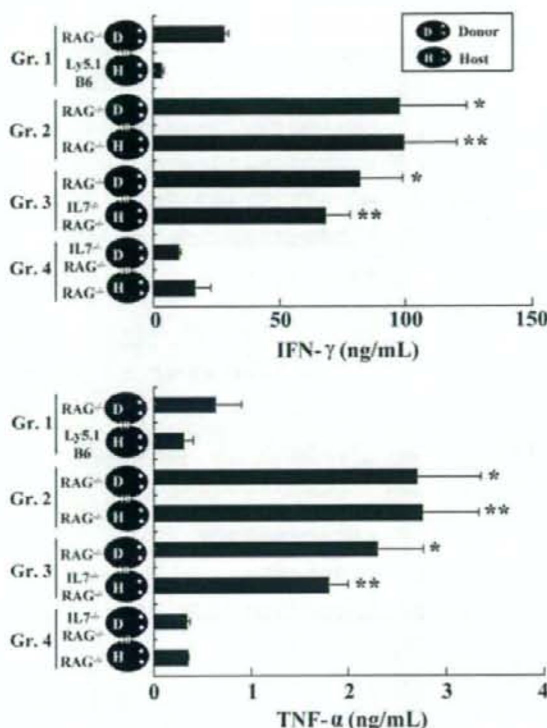

FIGURE 4. IL-$7^{-/-}$ × RAG-$1^{-/-}$ host mice in parabionts develop Th1-mediated colitis. LP CD4$^+$ T cells were prepared from colons at 4 wk after surgery and stimulated with anti-CD3 and anti-CD28 mAbs for 48 h. Concentrations of IFN-γ, and TNF-α in culture supernatants were measured by ELISA. 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

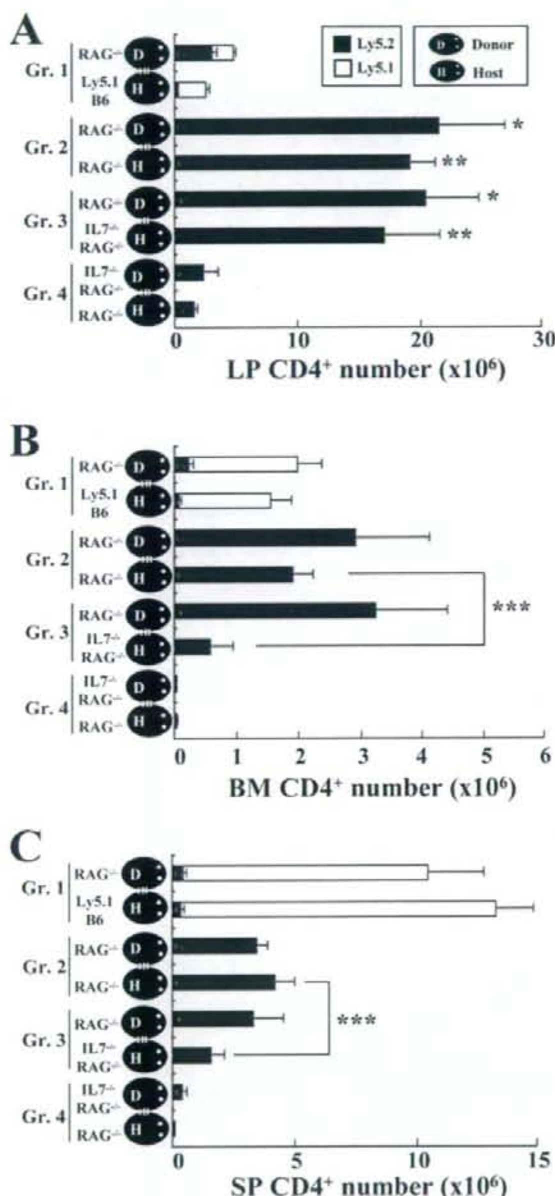

FIGURE 5. Expansion of BM and SP, but not of LP, $CD4^+$ T cells in $IL-7^{-/-} \times RAG-1^{-/-}$ host mice in parabionts is dependent on IL-7. LP (*A*), BM (*B*), and SP (*C*) $CD4^+$ T cells were isolated from each mouse of Groups 1–4 at 4 wk after surgery, and the number of $CD4^+$ cells were determined 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 $IL-7^{-/-} \times RAG-1^{-/-}$ host mice in Group 3, and was markedly decreased in colitic $IL-7^{+/+} \times RAG-1^{-/-}$ donor and host mice in Groups 2 and 3 in clear contrast to that of control C57BL/6 mice (Fig. 6*B*).

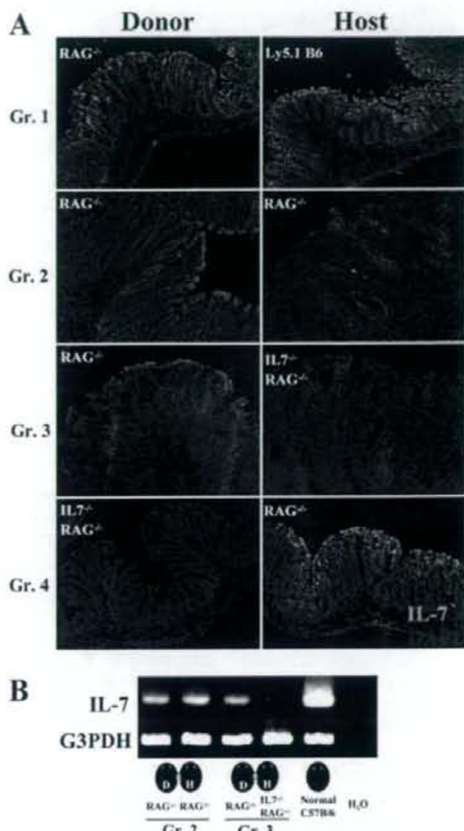

FIGURE 6. IL-7 is not detected in host $IL-7^{-/-} \times RAG-1^{-/-}$ mice in parabionts with diseased $IL-7^{+/+} \times RAG-1^{-/-}$ donor mice. *A*, Frozen sections of colon from each mouse in Groups 1–4 at 4 wk after surgery were stained with polyclonal anti-IL-7 Abs. Representative of five separate 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 $IL-7^{-/-} \times RAG-1^{-/-}$ host mice parabiosed with colitic $IL-7^{+/+} \times RAG-1^{-/-}$ 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 $IL-7^{-/-} \times RAG-1^{-/-}$ 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 $IL-7^{-/-} \times RAG-1^{-/-}$ 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

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Immunosenescent colitogenic CD4$^+$ T cells convert to regulatory cells and suppress colitis

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Inflammatory bowel diseases progress steadily by the expansion of colitogenic CD4$^+$ cells. However, it remains unknown whether colitogenic CD4$^+$ cells are long-living like memory cells or exhausted like effector cells. To assess the longevity of colitogenic lamina propria (LP) CD4$^+$ cells, we performed sequential transfers of LP CD4$^+$ cells from colitic CD4$^+$CD45RB^{high} cell-transferred SCID mice into new SCID mice. Although SCID mice transferred with colitic LP CD4$^+$ cells stably developed colitis until at least the sixth transfer, the interval to the development of colitis gradually lengthened as the number of transfers increased. The incidence of colitis gradually decreased after the seventh transfer. Furthermore, non-colitic LP CD4$^+$ cells from mice transferred over seven times expressed significantly higher levels of PD-1 and produced significantly lower amounts of IFN-γ, TNF-α, and IL-17 than colitic LP CD4$^+$ cells recovered after the first transfer. Most notably, we found that re-transfer of non-colitic LP CD4$^+$ cells recovered after multiple transfers prevented the development of colitis in SCID mice co-transferred with CD4$^+$CD45RB^{high} cells. Thus, colitogenic LP CD4$^+$ cells may be exhausted over time, become non-functional, convert to regulatory cells, and finally suppress colitis in the process of immunosenescence.

Key words: Animal models · CD4$^+$ T cells · Intestinal immunity · Mucosal immunity

Introduction

Inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis, are thought to result from the inappropriate activation and expansion of colitogenic CD4$^+$ T cells, which are driven by activated macrophages and dendritic cells. Antigens derived from the persistently present commensal bacteria continuously stimulate such cells, and this is presumably required for the induction of colitis. In general, IBD progresses steadily or with transient remissions throughout life [1, 2]. Importantly, the recurrent disease shows similar clinical features to the previous disease episode, and it is extremely uncommon that a patient with Crohn's disease relapses with another form of disease, such as ulcerative colitis [3–6]. Thus, it is conceivable that the sequential disease episodes are driven by a group of disease-specific colitogenic CD4$^+$ memory T cells, which may be designated as 'memory stem cells' [7] of the disease. In this scenario, colitogenic CD4$^+$ effector T cells established in the initial attack seem to arise repeatedly from the colitogenic CD4$^+$ memory T cells, but are presumably suppressed by regulatory T (Treg) cells during remission [8].

Although it appears that memory T cells are generally long living [9, 10] as shown by a series of successful vaccine programs, the details of their longevity are still unknown. In fact, it has been reported that the number of memory CD4$^+$ T cells declines over time in mice infected with lymphocytic choriomeningitis virus (LCMV) [11]. Furthermore, decline of the immune function due to the immunosenescence may affect the maintenance of memory CD4$^+$ T cells [12–14]. To evaluate this unsolved issue, we assessed the longevity of colitogenic lamina propria (LP) CD4$^+$ T cells and

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