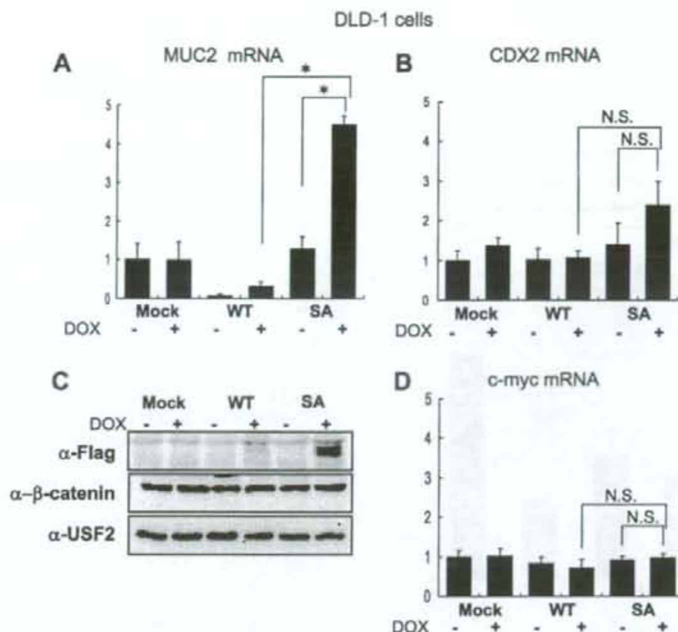

Fig. 3. Stabilization of Hath1 protein generates MUC2 mRNA independently of active Wnt signaling. (A) DLD-1 Tet-On cells were stimulated with doxycycline for 5 d. Results are expressed as the ratio of the levels of MUC2 mRNA to β-actin mRNA. $^{*}P < 0.05$. (B) (D) The same samples as in (A) were used to investigate CDX2 and c-myc mRNA expression, respectively. Results are expressed as the ratio of the levels of CDX2 and c-myc mRNA to β-actin mRNA. $^{*}P < 0.05$. (C) DLD-1 Tet-On cells were stimulated with doxycycline for 12 h. Immunoblotting was done with anti-β-catenin antibody.

(data not shown). The extent of the Hath1 contribution should be further analyzed.

Another important point of view in this study concerns the first phase of carcinogenesis. APC mutation has been revealed to promote the first stage initiation of carcinogenesis [14]. Immunohistochemical analysis of Hath1 protein expression in normal colon tissue by [6,7] suggests that APC mutation in colon cells might promote not only stable expression of β-catenin but also proteasomal degradation of Hath1 protein, leading to diminishment of Hath1 mRNA and resulting in the establishment of the undifferentiated state of cancer in the end stage. Therefore, in the early stage of carcinogenesis by APC deletion, the degradation of Hath1 protein is thought to be more essential than the diminishment of Hath1 mRNA in producing the undifferentiated state.

We finally propose that GSK3 inhibitors have potential for use in a new approach for anti-colon cancer drugs. Although many alternative therapies have been targeted to the Wnt signaling axis [15–17], development of a drug that targets Wnt signaling is difficult because of the complexity of the protein–protein interactions [15].

GSK3 is considered as a key enzyme in various neoplasms. Since many transcriptional factors, cell cycle regulators and proto-oncogenes act as substrates for GSK3, GSK3 has been considered as a suppressor of cellular neoplastic transformation [18]. Therefore, it has been suggested that GSK3 inhibitors might increase the risk of carcinogenesis [18,19]. LiCl, a relatively specific inhibitor of GSK3, has been used in therapies for bipolar disorder for many years, but the risk of cancer development in psychiatric patients treated with LiCl is even lower than in the general population, suggesting that LiCl may have a protective effect against cancer [20]. Another study indicates that the GSK3 inhibitors might not be sufficient to elevate the level of β-catenin protein in normal primary cells [21].

The benefits of GSK3 inhibitor use for colorectal cancer reduction have been described [22–24]. LiCl treatment in APC^{min} mouse does not increase the number of tumors compared with control, suggesting a low risk of cancer development [22]. Treatment with GSK3 inhibitors of mouse with subcutaneous xenografts of SW480 significantly inhibits proliferation of cancer cell xenografts, with no apparent pathological changes in other major organs [23]. Although, problematically, the molecular pathways and mechanisms involved in the anti-cancer effect of GSK3 inhibitors remain unclear [22–24], we have elucidated the molecular mechanisms of a GSK3 inhibitor in the reduction of colon cancer.

In conclusion, Hath1 protein has a potential to promote the differentiation of colon cancer cells independently of β-catenin accumulation, and stabilization of Hath1 protein

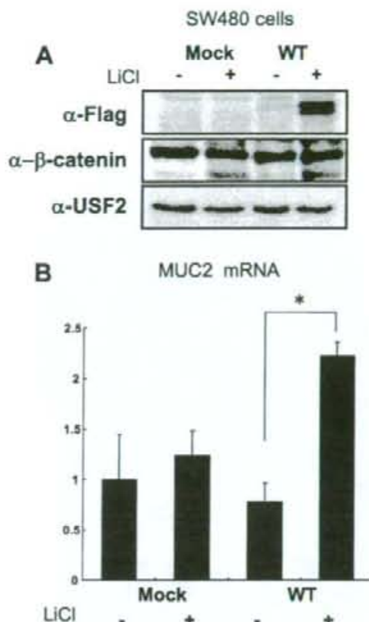

Fig. 4. The GSK3 inhibitor stabilizes Hath1 protein, leading to up-regulation of MUC2 mRNA in colon cancer cell lines. (A) SW480 cells were transiently transfected with WT Hath1 before 8 h of incubation in LiCl. Protein lysates were utilized for immunoblotting. (B) SW480 cells were transfected by the same procedure. Results of a quantitative real-time RT-PCR are expressed as the ratio of the levels of MUC2 mRNA to β-actin mRNA. $^*P < 0.05$.

by GSK3 inhibitors has intriguing therapeutic potential for colon cancer. Further detailed analyses of the switching mechanism of the GSK3β target between β-catenin and Hath1 on proteasomal degradation are required in order to introduce simple and pinpointed target therapies for colorectal cancers.

Acknowledgments

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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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the characteristics of immunosenescent colitogenic LP CD4$^+$ T cells in a murine model of chronic colitis induced by adoptive transfer of CD4$^+$CD45RB^{high} T cells [15]. The present model is useful for this purpose, because the primarily transferred CD4$^+$CD45RB^{high} T cells in the recipient can be tracked over time, thereby allowing us to exclude the impact of new naive CD4$^+$ T cells that are continuously supplied from the thymus. Furthermore, we performed sequential adoptive transfers of the colitic LP CD4$^+$ T cells after developing CD4$^+$CD45RB^{high} T cell- or colitic LP CD4$^+$ T cell-transferred colitis in SCID mice. This model is also very useful to induce the extremely rapid proliferation of colitogenic LP CD4$^+$ T cells, which presumably respond to commensal bacterial-driven or autogenous antigens by lymphopenia-driven proliferation [9]. Using this unique sequential adoptive transfer model of colitogenic LP CD4$^+$ cells in SCID mice, we assessed the characteristics of immunosenescent colitogenic LP CD4$^+$ T cells that were generated by repeated transfers into lymphopenic host mice.

Results

Incidence of colitis is gradually decreased by repeated transfers of colitic LP CD4$^+$ T cells

We previously demonstrated that LP CD4$^+$ T cells obtained from colitic SCID mice that received adoptive transfer of CD4$^+$CD45RB^{high} T cells (the first transfer) are colitogenic CD44^{high}CD62L$^-$CD4$^+$IL-7Rα^{high} effector-memory (T_{EM})-like cells [16]. SCID mice transferred with such colitic LP CD4$^+$ T cells (the second transfer) develop colitis similar to the original CD4$^+$CD45RB^{high} T cell-transferred colitis in an IL-7-dependent manner [17]. This adoptive transfer model is also characterized by the rapid proliferation of donor CD4$^+$ T cells by lymphopenia-driven proliferation [9, 17], which provides an advantageous tool to assess the longevity and change in characteristics of these colitic LP CD4$^+$ T cells during repetitive transfer into SCID mice (Fig. 1A). As a rule of the current protocol, each mouse was killed when it reached over four points of the ongoing clinical score (see *Materials and methods*) within 40 weeks from transfer. Isolated LP CD4$^+$ T cells were then transferred into new SCID mice, and the procedure was repeated until the recipient mice failed to develop colitis within 40 weeks from transfer. Recipient mice that did not develop colitis within 40 wk from transfer were judged to be non-colitic, and were sacrificed for further assessment (Fig. 1A).

Although the interval between transfers gradually lengthened with the increase in number of transfers after the second (Fig. 1B), all the recipient mice examined until the sixth transfer stably developed wasting disease with colitis within 40 weeks from transfer. After the seventh transfer, however, some mice showed no sign of colitis up to 40 weeks from transfer as assessed by the ongoing clinical score (Fig. 1C), and the incidence of colitis development decreased (Fig. 1D). To further assess whether the cell viability of the transferred cells affected the present results, we performed Annexin V/PI staining of cells directly isolated from LP of mice by flow cytometry. As shown in Fig. 1E, there were no differences in the ratio of viable Annexin$^-$/PI$^-$ cells among LP CD4$^+$ T cells obtained from original CD4$^+$CD45RB^{high} T cell-transferred colitic mice (1° colitic CD4$^+$), LP CD4$^+$ T cells obtained from colitic mice transferred with colitic LP CD4$^+$ T cells that were sequentially transferred over seven times (>7° colitic CD4$^+$), and LP CD4$^+$ T cells from non-colitic mice transferred with colitic LP CD4$^+$ T cells that were sequentially transferred over seven times (>7° non-colitic CD4$^+$).

Pattern of TCR Vβ are equivalent irrespective of the number of transfer

One reason why repeated transfer of colitic LP CD4$^+$ T cells leads to delayed onset and decreased incidence of the murine colitis may be that extensively proliferating colitogenic CD4$^+$ T cell clones are selectively depleted over time. Thus, we next checked TCR Vβ repertoire patterns of the 1° colitic CD4$^+$, >7° colitic CD4$^+$, and >7° non-colitic CD4$^+$ T cells by flow cytometry. As depicted in Fig. 2, although the patterns of TCR Vβ repertoire were actually skewed into some group of TCR Vβ repertoire after both single adoptive and multiple adoptive transfers compared to those before transfer, they never integrated into a single specific TCR Vβ repertoire.

Non-colitic LP CD4$^+$ T cells generated by repeated transfer are inactivated

We next compared the immunological phenotypes of the 1° colitic, the >7° colitic, and the >7° non-colitic CD4$^+$ T cells. We first compared the phenotypic composition of these cells in the colonic LP and in the spleen (SP) of mice transferred with the corresponding cells. As shown in Fig. 3, the number of cells recovered from LP or SP was, as expected, significantly lower in mice transferred with the >7° non-colitic CD4$^+$ T cells than in mice transferred with the 1° colitic or >7° colitic CD4$^+$ T cells.

Cell surface markers of the 1° colitic, >7° colitic, and >7° non-colitic SP and LP CD4$^+$ T cells had a phenotype of CD44^{high}CD62L$^-$IL-7Rα^{high} T_{EM}-like cells (Fig. 4A). An activation marker, CD69, was expressed on approximately two thirds of the 1° colitic, >7° colitic, and >7° non-colitic LP CD4$^+$ T cells and on one third of the 1° colitic and >7° colitic SP CD4$^+$ T cells, but was markedly down-modulated on the >7° non-colitic SP CD4$^+$ T cells (Fig. 4A), indicating that >7° non-colitic SP CD4$^+$ T cells were inactivated. Since it has recently been suggested that a costimulatory molecule, PD-1, might serve as a useful marker to indicate the degree of non-functional T cell exhaustion on virus-specific CD8$^+$ and CD4$^+$ T cells [18–20], we assessed the expression of this molecule on our cells. As expected, PD-1 expression was significantly up-regulated on the >7° non-colitic CD4$^+$ T cells both in the LP and SP as compared with the paired 1° colitic and >7° colitic CD4$^+$ T cells (Fig. 4A). In contrast, no difference in CD28 expression was observed among the 1° colitic, >7° colitic, and >7° non-colitic

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$CD4^+$ T cells (Fig. 3A). These results were also confirmed by statistical analysis (Fig. 4B).

We next determined cytokine production by anti-CD3/CD28 mAb-stimulated 1° colitic, >7° colitic, and >7° non-colitic $CD4^+$ T cells. As shown in Fig. 5, the >7° $CD4^+$ T cells, whether colitic or non-colitic, produced markedly less IFN-γ, IL-2, TNF-α, IL-10, IL-4, and IL-13 than 1° colitic $CD4^+$ T cells. The production of IL-17 by the >7° colitic and >7° non-colitic $CD4^+$ T cells was significantly lower than that by the 1° colitic $CD4^+$ T cells, although it was not completely abolished. Notably, the production of IL-17 by the >7° non-colitic $CD4^+$ T cells was significantly lower than that by the >7° colitic $CD4^+$ T cells. None of the cells produced TGF-β upon the present *in vitro* stimulation. These results suggested that the >7° non-colitic $CD4^+$ T cells have a functional defect in both activation and cytokine production, presumably due to immunosenescence or exhaustion induced by lymphopenia-induced proliferation for over 2 years.

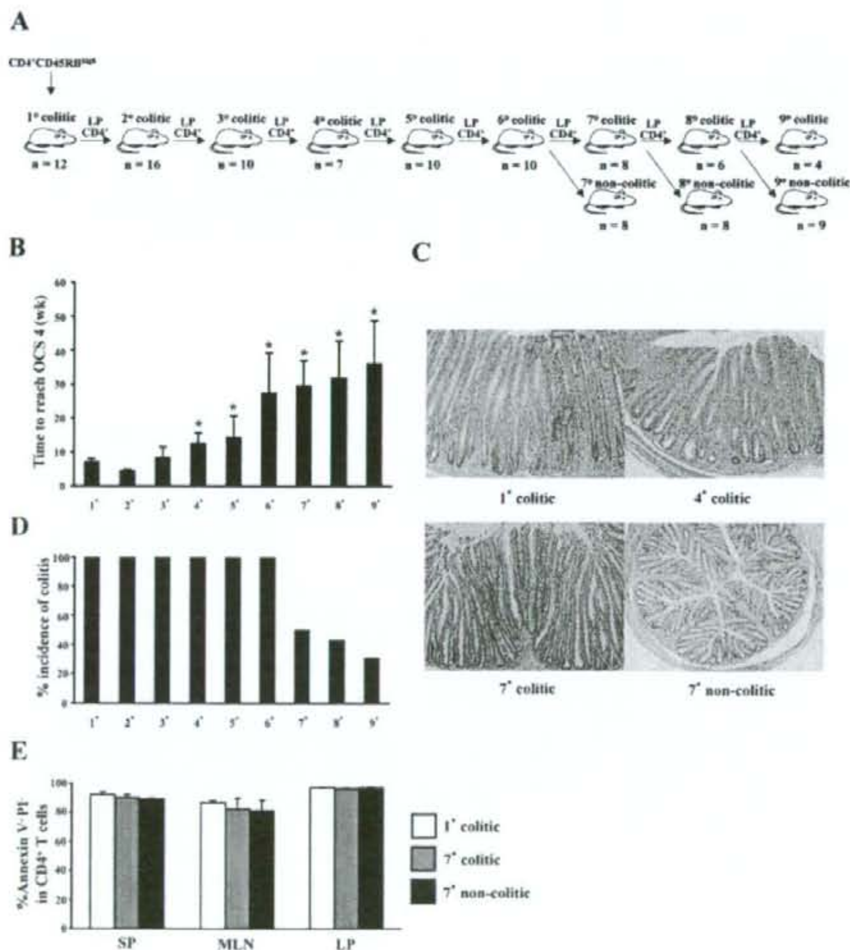

Figure 1. Incidence of colitis induced by adoptive transfer of colitic LP $CD4^+$ T cells gradually decreased as the transfers progress. (A) Schematic transfer protocol. C.B-17 SCID mice were transferred with BALB/c $CD4^+CD45RB^{high}$ T cells (1° colitic). When they reached an ongoing clinical score of four (see *Materials and methods*), LP $CD4^+$ T cells were isolated, and transferred into new SCID mice. Transfer of LP $CD4^+$ T cells was repeated up to nine times (2°–9° colitic), but was terminated when colitis did not develop within 40 weeks from transfer (7°–9° non-colitic). (B) The mean interval between transfer and establishment of colitis with an ongoing clinical score of four. Mice that did not develop colitis within 40 weeks from transfer were excluded for this index and judged to be non-colitic. OCS, ongoing clinical score. $^*p<0.05$ *vs.* the 2° transfer. (C) Histopathological findings of the colon. Original magnification, ×100. (D) The mean incidence rate of colitis in each transfer group. (E) Viability of cells directly isolated from LP of 1° colitic, >7° colitic, or >7° non-colitic mice. The number of viable cells (Annexin V^-/PI^-) was determined by a flow cytometry. Data are presented as mean ± SEM of % PI^+ Annexin V^-/PI^- cells from four mice in each group. NS, not significantly different.

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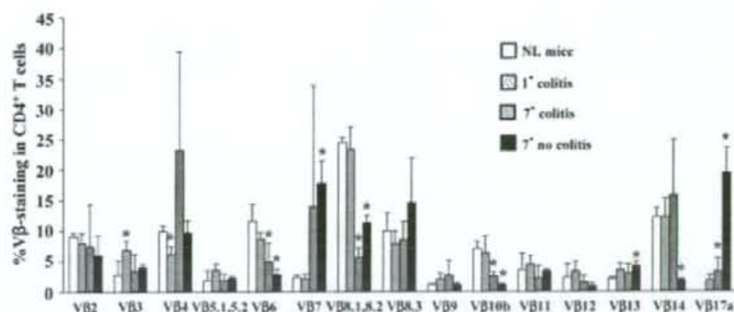

Figure 2. Flow cytometric analysis of the expression of Vβ families on the surface of the splenic normal, 1° colitic, >7° colitic, or >7° non-colitic CD4+ T cells. To analyze the TCR Vβ family repertoire, splenic cells were triple-stained with PECy5-conjugated anti-CD4 mAb and the following a panel of 15 FITC-conjugated Vβ mAb. Each percentage value indicates the frequency of each Vβ pooled from three independent experiments (each transfer; $n=6$). $^*p<0.05$ vs. normal BALB/c mice.

Non-colitic LP CD4+ T cells have no characteristics of CD4+Foxp3+ Treg cells

Vukmanvic-Stejic and colleagues [21] have recently reported that a proportion of peripheral CD4+CD25highFoxp3+ Treg cells in humans are generated from rapidly dividing memory CD4+CD45RO+ T cells in addition to thymus-derived classical CD4+CD25highFoxp3+ Treg cells. Furthermore, colitogenic CD4+ T cells in this colitis model proliferate and expand in response to foreign antigens more rapidly in immunodeficient SCID mice than do slow-dividing antigen-specific 'true' memory T cells [17]. We thus hypothesized that the colitogenic LP CD4+ T cells gradually convert after multiple transfers to cytokine-non-producing CD4+ Treg cells that have not only lost the ability to induce colitis but, conversely, gained the ability to suppress colitis. To assess this possibility, we next explored whether the >7° non-colitic CD4+ T cells retain Treg cell activity *in vitro*. Since it has been shown that resting CD4+ Treg cells express Foxp3 [22], we first analyzed the expression of Foxp3 in the 1° colitic, >7° colitic, and >7° non-colitic CD4+ T cells, with splenic CD4+CD25+ Treg cells serving as a positive control. Unexpectedly, intracellular Foxp3 expression in the >7° non-colitic CD4+ T cells was slight, and was not significantly higher than that in the 1° colitic and >7° colitic CD4+ T cells, while the splenic CD4+CD25+ Treg cells expressed Foxp3 at a high level (Fig. 6A).

To further assess the possibility that the >7° non-colitic CD4+ T cells may function as Treg cells, we examined whether these cells could suppress the proliferation of CD4+ responder T cells in *in vitro* co-culture assay. Although splenic CD4+CD25+ Treg cells were able to suppress the proliferation of splenic CD4+CD25− responder cells at a ratio of 1:1 to 1:0.125 of responder/Treg cells in the presence of mitomycin-C (MMC)-treated CD4− APC and soluble anti-CD3 mAb, the >7° non-colitic CD4+ T cells and 1° colitic CD4+ T cells could not suppress the proliferation at any ratio (Fig. 6B). Thus, at least in *in vitro* analysis, the >7° non-colitic CD4+ T cells were a completely distinct cell population from peripherally induced CD4+Foxp3+ Treg cells, IL-10-producing Tr1 cells (Fig. 5) [23], and TGF-β-producing Th3 cells (Fig. 5) [24].

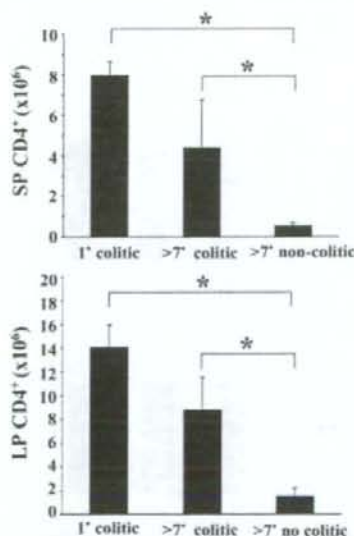

Figure 3. Expansion of CD4+ T cells in the >7° non-colitic mice was significantly decreased. SP and LP CD4+ T cells were isolated from colons when the colitic mice reached an ongoing clinical score of four, or the non-colitic mice lived up to 40 weeks post transfer. The number of CD4+ T cells was determined by flow cytometry. Data are indicated as mean ± SEM of six mice in each group. $^*p<0.05$ vs. the 1° coltic mice.

Co-transfer of non-colitic LP CD4+ T cells suppresses the development of colitis

Although the >7° non-colitic LP CD4+ T cells did not show a regulatory function in the *in vitro* co-culture assay, such assays do not always represent *in vivo* function. To assess whether the >7° non-colitic LP CD4+ T cells have characteristics of Treg cells and

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can suppress colitis *in vivo*, we performed an *in vivo* adoptive transfer experiment with four groups of SCID mice: group 1, new SCID mice transferred with $CD4^+CD45RB^{high}$ T cells alone (3×10^5) as a positive control; group 2, SCID mice transferred with $CD4^+CD45RB^{high}$ T cells (3×10^5) and $CD4^+CD25^+$ Treg cells (1×10^5) as a negative control; group 3, SCID mice

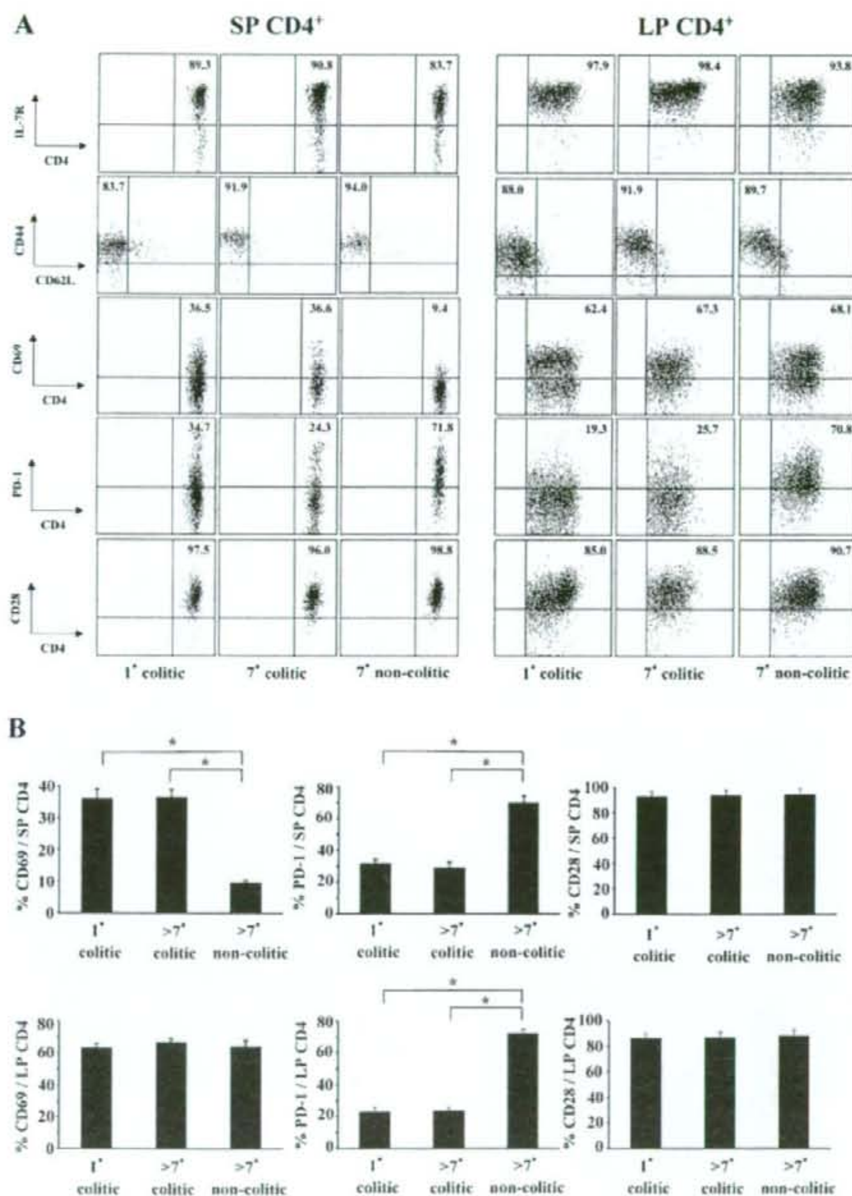

Figure 4. Expression of various cell surface markers on freshly isolated SP and LP $CD4^+$ T cells from 1° colitic, >7° colitic, and >7° non-colitic mice. (A) Representative analysis of IL-7Rα, CD62L, CD69, PD-1, and CD28 expression on SP or LP $CD4^+$ cells from 1° colitic, >7° colitic, and >7° non-colitic mice. Cells were stained with either FITC-conjugated anti-CD4, and the indicated biotinylated mAb, followed by PE-conjugated streptavidin or with fluorochrome-conjugated control Ig (not shown). (B) Percent positive cells of CD69, PD-1, and CD28 expression among SP or LP $CD4^+$ cells from 1° colitic, >7° colitic, and >7° non-colitic mice were determined by flow cytometry. Data are indicated as mean ± SEM of seven mice in each group. *$p<0.05$.

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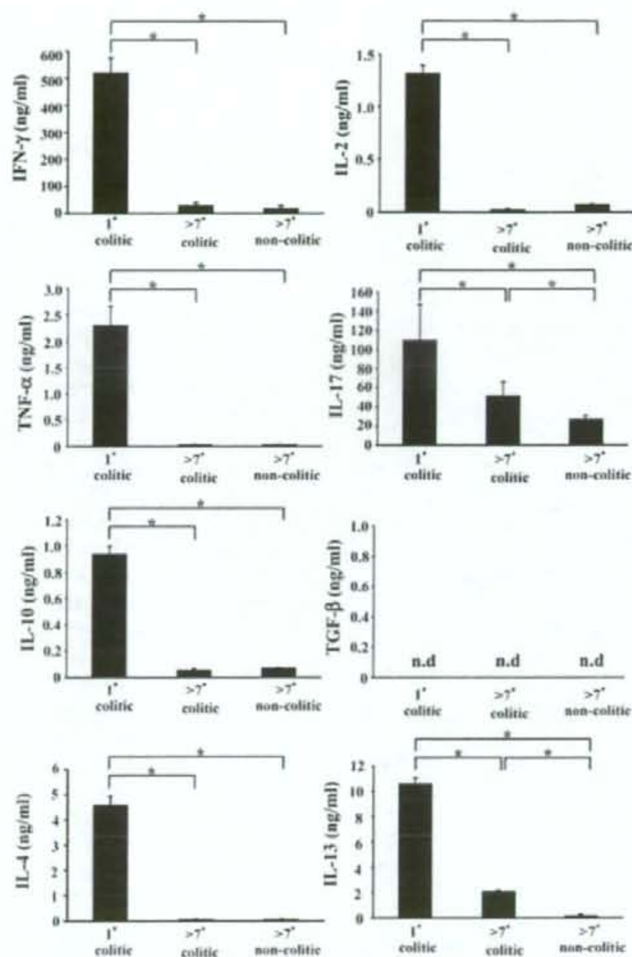

Figure 5. Cytokine production by LP CD4$^+$ T cells from 1° colitic, >7° colitic, and >7° non-colitic mice. LP CD4$^+$ T cells were isolated, and stimulated with anti-CD3/CD28 mAb for 48 h. The indicated cytokines in these supernatants were measured by ELISA. Data are shown as mean ± SD of seven mice in each group. *$p<0.05$ *vs.* the 1° colitic mice. n.d., not detected.

transferred with CD4$^+$CD45RB^{high} T cells (3 × 10^5) and 1° colitic LP CD4$^+$ T cells (1 × 10^5); and group 4, SCID mice transferred with CD4$^+$CD45RB^{high} T cells (3 × 10^5) and >7° non-colitic CD4$^+$ LP T cells (1 × 10^5) (Fig. 7A). Mice were killed 6 weeks after transfer. Surprisingly, the >7° non-colitic LP CD4$^+$ T cell fraction, like the control CD4$^+$CD25$^+$ Treg fraction, clearly showed a regulatory function toward intestinal inflammation, as these cell types both significantly inhibited the development of both wasting disease and colitis, when co-transfer with CD4$^+$CD45RB^{high} T cells (Fig. 7B–E). Colons of group 4 mice exhibited no pathological changes and were indistinguishable from the colons of group 2 mice (negative control) (Fig. 7B and D). In contrast, group 1 mice (positive control) and group 3 mice both developed wasting disease with severe colitis (Fig. 7B and D). The clinical and histological scorings also statistically confirmed these results (Fig. 7C and E).

A further quantitative evaluation of CD4$^+$ T cell infiltration was made by isolating the SP and LP CD4$^+$ T cells. As depicted in Fig. 7F, significantly fewer CD4$^+$ T cells were recovered from the SP and LP of mice reconstituted with CD4$^+$CD45RB^{high} and >7° non-colitic CD4$^+$ T cells (group 4) or CD4$^+$CD45RB^{high} + CD4$^+$CD25$^+$ T_R cells (group 2) as compared with mice reconstituted with CD4$^+$CD45RB^{high} cells alone (group 1) or CD4$^+$CD45RB^{high} cells + 1° colitic CD4$^+$ T cells (group 3).

To determine the effect of >7° non-colitic CD4$^+$ T cells on Th1 development, we measured IFN-γ, IL-2, and TNF-α production by anti-CD3/CD28-stimulated CD4$^+$ LP T cells. As shown in Fig. 7G, the production of IFN-γ, IL-2 and TNF-α was significantly reduced

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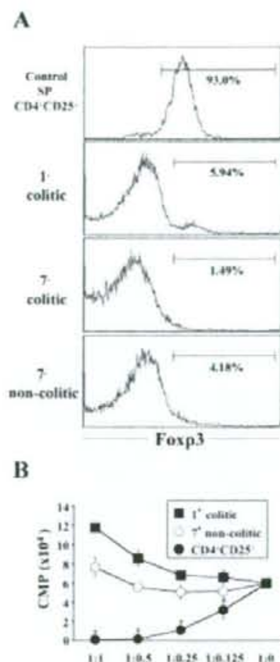

Figure 6. CD4⁺ T cells obtained from non-colitic mice after seven or more transfers did not have a regulatory character *in vitro*. (A) Expression of Foxp3 in the indicated subpopulations was determined by flow cytometry as described in *Materials and methods*. (B) Suppressive activity of the indicated subpopulations was determined at a responder/Treg ratio of 1:0, 1:0.125, 1: 0.25, 1:0.5, or 1:1. *$p<0.05$.

by the co-transfer of $CD4^+CD45RB^{high}$ and $>7^o$ non-colitic $CD4^+$ T cells (group 4) or $CD4^+CD45RB^{high}$ and $CD4^+CD25^+$ T_R cells (group 2) as compared with that of $CD4^+CD45RB^{high}$ T cells alone (group 1) or $CD4^+CD45RB^{high}$ and 1° non-colitic $CD4^+$ T cells (group 3). Collectively, these results indicated that, at least *in vivo*, the $>7^o$ non-colitic $CD4^+$ T cells act as Treg cells to suppress the development of Th1-mediated colitis in a comparable manner to the control $CD4^+CD25^+$ Treg cells.

Discussion

In the present study, we performed seven or more sequential adoptive transfers of colitogenic LP $CD4^+$ T cells obtained from colitic SCID mice into new SCID mice. The SCID mice transferred with colitic LP $CD4^+$ T cells stably developed colitis, but interestingly the interval between transfer and development of colitis gradually lengthened as the number of transfers increased. Furthermore, the incidence of colitis gradually decreased after seven sequential transfers, accompanied by markedly increased expression of PD-1 but decreased production of various cytokines by the LP $CD4^+$ T cells. Importantly, transfer of non-colitic LP $CD4^+$ T cells that were recovered after seven or more transfers suppressed the development of colitis in SCID mice, which should have been induced by the transfer of $CD4^+CD45RB^{high}$ T cells. Collectively, LP $CD4^+$ T cells that are colitogenic in origin may differentiate into $CD4^+$ Treg cells through the process of immunological exhaustion caused by lymphopenia-driven rapid proliferation [9], and gain the ability to suppress colitis. These findings have important implications for our understanding of the nature of colitogenic $CD4^+$ T cells as well as the natural course of IBD.

To exclude the possibility that changes of the bacterial flora in the examined mice affected the incidence of colitis in the present study, we routinely checked whether the examined mice might have been infected by pathological bacteria such as *Helicobacter hepaticus*, but found no evidence of contaminating bacteria throughout the experimental period for over 3 years (data not shown). In addition, we confirmed that colitis could be stably induced in SCID mice by transfer of $CD4^+CD45RB^{high}$ T cells throughout this period, including the time when colitis did not develop in some SCID mice that were co-transferred with immunosenescent LP $CD4^+$ cells. Nevertheless, further study will be needed to address this issue, since we could not evaluate the components of non-pathological commensal bacteria using a comparative 16S-rRNA-gene-sequence survey in the examined mice. Also it has recently been reported that the bacterial flora of diseased and non-diseased animals are apparently distinct, even if they appear to be in the same environment [25].

Although accumulating evidence from the models of acute virus infection suggests that memory T cells, especially $CD8^+$ memory T cells, are long living [9, 10], it remains controversial whether this is also the case with $CD4^+$ memory T cells [26]. Furthermore, it is believed that "true" memory T cells, especially $CD8^+$ memory T cells, are established after the first clearance of the corresponding antigens [27], but this is also doubtful in the case of $CD4^+$ memory T cells. Conversely, recent reports suggest that both homeostatic stimulation by IL-7 and antigenic stimulation are needed for the full maintenance of $CD4^+$ memory T cells [28]. In fact, we showed here that the colitic $CD4^+IL\text{-}7R\alpha^{high}$ T cells were stably transferable to new SCID mice, and that they continued to induce colitis in the presence of commensal bacteria through at least six transfers over a period of more than 2 years without additional supply of naive $CD4^+$ T cells.

We believe that the immunological memory of antigens is not related to the requirement of antigen clearance from the host body, because antigen-specific effector or memory T cells are inevitably separated from antigen-loading dendritic cells residing at draining lymph nodes, which leave there regardless of the presence of a corresponding antigen in the body [29]. It seems, however, that the interval before antigen-specific effector or memory T cells re-encounter the same antigen will be shorter. In fact, we previously demonstrated that colitogenic $CD4^+CD44^{high}IL\text{-}7R\alpha^{high}$ T cells reside within the SP and bone marrow [30], which lack commensal bacterial antigens. Thus, it is possible and also reasonable that separation from sites where the corresponding antigen resides, rather than complete removal of antigens, is important for the generation of memory T cells. Furthermore,

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accumulating evidence suggests that IL-7 dependency is a reliable assumption for $CD4^+$ memory T cells *in vivo* [31]. Consistently, we previously demonstrated that colitic LP $CD4^+$ T cells from colitic $CD4^+CD45RB^{high}$ T cell-transferred RAG-$2^{-/-}$ mice express representative cell surface markers of memory T cells such as IL-7Rα and CD44 at a high level, and that the IL-$7^{-/-}$ × RAG-$1^{-/-}$ recipient mice transferred with $CD4^+CD45RB^{high}$ T cells or colitic LP $CD4^+$ T cells never develop colitis [17].

However, even if the colitogenic $CD4^+$ T cells found in the $CD4^+CD45RB^{high}$ T cell-transferred colitic mice can be called memory T cells, or 'persistent antigen-specific T cells' [27], their longevity and how their characteristics change with immunosenescence through multiple rounds of cell division. To evaluate this issue, we conducted sequential adoptive transfers of colitic LP $CD4^+$ T cells into new SCID mice. Although this method may be artificial, it is quite useful to examine colitic LP $CD4^+$ T cells that

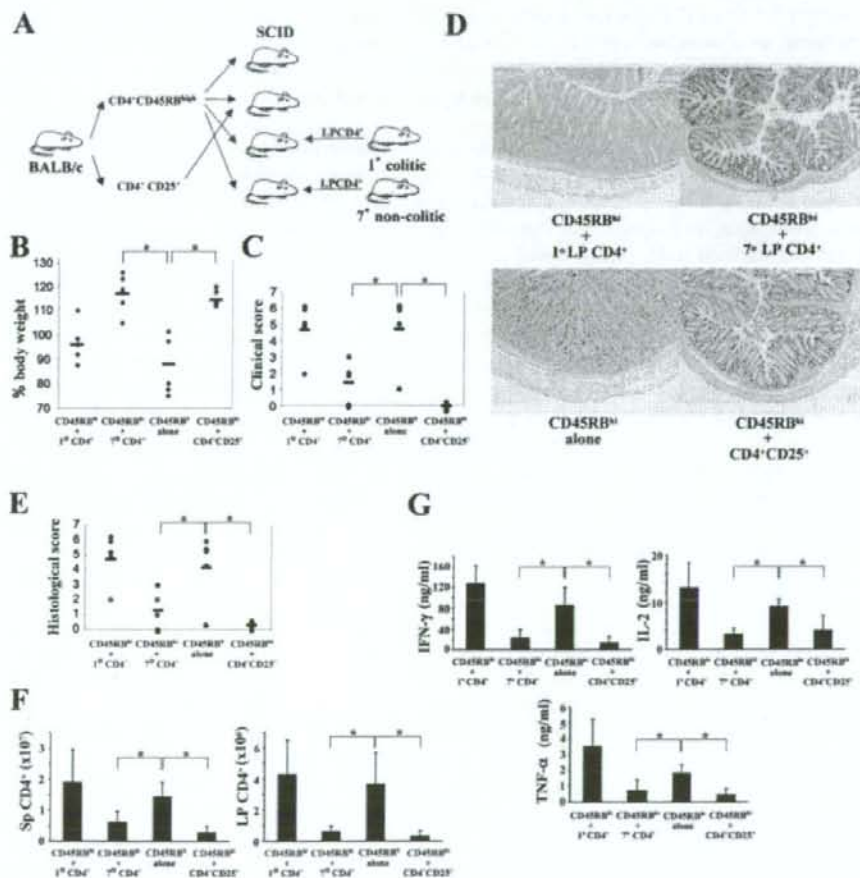

Figure 7. Cotransfer of LP $CD4^+$ T cells from >7° non-colitic mice prevents the development of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID mice. (A) New SCID mice were divided into four groups: mice transferred with $CD4^+CD45RB^{high}$ cells (3×10^5 per mouse) alone as a positive control, mice transferred with $CD4^+CD45RB^{high}$ cells (3×10^5) and $CD4^+CD25^+$ cells (1×10^5) as a negative control, mice transferred with $CD4^+CD45RB^{high}$ cells (3×10^5) and 1° colitic LP $CD4^+$ cells (1×10^5), and mice transferred with $CD4^+CD45RB^{high}$ cells (3×10^5) and 7° non-colitic LP $CD4^+$ cells (1×10^5). Mice were killed six weeks after transfer. Each experiment was performed with groups of three mice each. The data are the sum of three independent experiments (n=9). (B) Change in body weight over time is expressed as percent of the original weight. Data are represented as mean ± SEM of nine mice in each group. $^*p<0.05$ compared to mice transferred with $CD4^+CD45RB^{high}$ cells alone. (C) Clinical scores were determined at 6 weeks after transfer. Data are indicated as mean ± SEM of nine mice in each group. $^*p<0.05$ compared to mice transferred with $CD4^+CD45RB^{high}$ cells alone. (D) Histological examination of the colon from each group of mice at 6 weeks after transfer. Original magnification, ×100. (E) Histological scores were determined at 6 weeks after transfer. Data are indicated as the mean ± SEM of nine mice in each group. $^*p<0.05$ compared to mice transferred with $CD4^+CD45RB^{high}$ cells alone. (F) SP and LP mononuclear cells were isolated from the colon at 6 weeks after transfer, and the number of $CD4^+$ cells was determined by flow cytometry. Data are indicated as mean ± SEM of nine mice in each group. $^*p<0.05$ compared to mice transferred with $CD4^+CD45RB^{high}$ cells alone. (G) Cytokine production by LP $CD4^+$ T cells. LP $CD4^+$ T cells were prepared from the colons at 6 weeks after transfer and stimulated with anti-CD3/CD28 mAbs for 48 h. The indicated cytokines in these supernatants were measured by ELISA. Data are indicated as mean ± SEM of six mice in each group, which was selected from the first two *in vivo* experiments. $^*p<0.05$ compared to mice transferred with $CD4^+CD45RB^{high}$ cells alone.

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have undergone multiple rounds of cell division. In this study, we found that (i) the interval between transfer and development of colitis gradually lengthened as the number of transfers increased, (ii) a certain population of the recipient SCID mice did not develop colitis after seven or more transfers, and (iii) the production of IFN-γ, TNF-α and IL-17 by LP CD4⁺ T cells recovered after seven or more transfers was significantly impaired. These data clearly indicate that colitogenic CD4⁺ T cells are gradually exhausted over time and finally lose the ability to induce colitis. However, it remains unclear whether LP CD4⁺ T cells recovered from recipient mice and maintained for over 40 weeks post transfer are able to induce colitis, since the designation of mice that did not develop colitis within 40 weeks post transfer as non-colitic was made arbitrarily. Further study will be needed to address this issue.

Most notably, however, we also found that new SCID mice transferred with $CD4^{+}CD45RB^{high}$ T cells and LP $CD4^{+}$ T cells obtained from non-colitic SCID mice after seven transfers did not develop colitis. This finding further indicates that colitogenic $CD4^{+}$ T cells not only lose their colitogenicity over time, but also gain a regulatory function like $CD4^{+}$ Treg cells through the process of immunosenescence, and suppress colitis. It is also possible that LP $CD4^{+}$ T cells obtained from non-colitic SCID recipient mice may simply delay, but not completely suppress, the development of colitis through competition for cytokines (cytokine deprivation) [32] between newly recruited effector cells. However, our findings may also correlate with the clinical nature of IBD, as the majority of patients actually run a chronic or relapsing course, whereas patients with severe symptoms show diminishing severity of symptoms over time, presumably through the immunosenescence of colitogenic $CD4^{+}$ T cells along with the decrease of new naive T cell supply from the thymus [33].

At the moment, it is largely unknown which type of Treg cells is closely associated with the immunosenescent LP $CD4^{+}$ T cells described in the current study, which were colitogenic in origin, but acquired regulatory activity to suppress the development of colitis. A recent publication by Vukmanovic-Stejic and colleagues [21] demonstrated that a substantial proportion of peripheral human $CD4^{+}CD25^{high}Foxp3^{+}$ Treg cells is generated from rapidly dividing, highly differentiated $CD4^{+}$ memory T cells in addition to the cells of same phenotype derived from the thymus. Moreover, Liu and colleagues [34] reported that the interaction between neurons and $CD4^{+}$ T cells results in the conversion of encephalogenic $CD4^{+}$ T cells to $CD4^{+}CD25^{+}Foxp3^{+}$ T_R cells in a murine model of experimental autoimmune encephalomyelitis (EAE). Although peripherally inducible $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells appear quite similar to our immunosenescent LP $CD4^{+}$ T cells in that they are generated by continuous stimulation of antigens in the periphery, we could not detect the up-regulation of Foxp3 in the immunosenescent LP $CD4^{+}$ T cells.

It has recently been reported that PD-1 is a marker for exhausted $CD8^{+}$ and $CD4^{+}$ T cells in chronic lymphocytic choriomeningitis virus (LCMV) and HIV infections [18–20]. Consistent with this, we demonstrated that LP $CD4^{+}$ T cells obtained from non-colitic mice after over seven or more transfers expressed significantly higher levels of PD-1. In addition, we previously showed that peripheral $CD4^{+}PD\text{-}1^{+}$ T cells in normal mice possess a regulatory function both *in vivo* and *in vitro*, regardless of the expression of CD25 [35]. Thus, it is possible that the PD-1/PD-L1 pathway is required for immunosenescent LP $CD4^{+}$ T cells to function *in vivo* as Treg cells, although further study using mAb to block the PD-1/PD-L signal pathway is needed to address this issue. Apart from Treg cells, it is also noteworthy that dying exhausted cells (apoptotic cells) are frequently associated with an immunosuppressive activity against other immune cells including $CD4^{+}$ T cells [36, 37]. This mechanism involves anti-inflammatory TGF-β released by macrophages, which phagocyte the apoptotic cells. Although we could not detect decreased viability (Fig. 1) or increased TGF-β activity (Fig. 5) of the non-colitic LP $CD4^{+}$ T cells after seven transfers, it is conceivable that exhausted LP $CD4^{+}$ T cells are phagocytosed by surrounding macrophages, and the production of anti-inflammatory cytokines by such cells may be involved in the induction of immunosuppression. Further studies will be needed to address the regulatory mechanism of the immunosenescent LP $CD4^{+}$ T cells.

Finally, our results should be discussed in connection with a recent publication by Abadia-Molina and colleagues [38], which reported serial adoptive transfer of colitic $CD4^{+}$ T cells residing in the mesenteric lymph nodes (MLN) of their original model of colitis induced by the transplantation of wild-type bone marrow into adult tgε26 mice (called BM→tgε). They demonstrated that the isolated MLN $CD4^{+}$ T cells in colitic BM→tgε mice not only maintained colitogenicity with a dominant Th1 phenotype after over eight or more transfers, but also converged into a single TCR Vβ usage (Vβ8.1/2, Vβ8.3, Vβ10b or Vβ14) of up to 90% in a certain line of colitic mice, leading to a novel method for cloning colitogenic $CD4^{+}$ cells through serial adoptive transfers. In our system using LP cells as donor cells, however, we could not detect any convergence of TCR Vβ usage in Vβ8.1/2, Vβ8.3, Vβ10b and Vβ14 (Fig. 2). Furthermore, we found that the ability to reproduce colitis upon sequential transfer gradually decreased in terms of the interval between transfers and the incidence of colitis. These discrepancies would be explained by differences in the model of colitis; differences in the presence of NK and B cells, and differences in the type of donor cells, those from MLN being rich in central memory T (T_{CM}) cells, and those from LP being rich in T_{EM} cells [39]. Further study will be needed to address this issue.

In summary, we demonstrated through our unique model of sequential adoptive transfers into lymphopenic SCID mice that colitogenic $CD4^{+}$ T_{EM}-like cells in colitic mice are exhausted over time and are finally converted into cytokine-non-producing Treg cells that suppress the development of colitis. Thus, our current study may provide a new approach for the treatment of IBD by transfer of immunosenescent $CD4^{+}$ T cells generated from colitogenic $CD4^{+}$ T cells by *in vitro* acceleration of cell divisions to promote their regulatory function.

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Materials and methods

Animals

Female BALB/c and C.B-17 SCID mice were purchased from Japan Clea (Tokyo, Japan). Mice were maintained under specific pathogen-free (SPF) conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 weeks of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Antibodies

The following mAb except biotin-conjugated anti-mouse IL-7Rα (A7R34; eBioscience, San Diego) were obtained from BD Pharmingen (San Diego, CA) for purification of cell populations and flow cytometry analysis: 145–2C11, FITC-conjugated anti-mouse CD3; RM4–5, FITC- or PE-conjugated anti-mouse CD4; 16A, FITC-conjugated anti-mouse CD45RB; 7D4, FITC-conjugated anti-mouse CD25; IM7, PE-conjugated anti-mouse CD44; MEL-14, FITC- or PE-conjugated anti-mouse CD62L; H1.2F3, FITC-conjugated anti-mouse CD69; 37.51, PE-conjugated anti-mouse CD28; J43, PE-conjugated anti-mouse PD-1 and the following FITC-conjugated antibodies; Vβ2 (B20.6), Vβ3 (KJ25), Vβ4 (KT4), Vβ5 (MR9–4), Vβ6 (RR4–7), Vβ7 (TR310), Vβ8.1/2 (MR5–2), Vβ8.3 (B21.14), Vβ9 (MR10–2), Vβ10b (B21.5), Vβ11 (RR3–15), Vβ12 (MR11–1), Vβ13 (IN12.3), Vβ14 (14.2), and Vβ17 (KJ23). Biotinylated antibodies were detected with PE- or Cy-Chrome™-streptavidin (BD Pharmingen).

T cell preparation

SP and LP $CD4^+$ T cells were isolated from mice as previously described [15]. The resultant cells contained > 94% $CD4^+$ cells when analyzed by FACSCalibur. SP $CD4^+$ T cells were then labeled with PE-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-CD45RB mAb, and then sorted to yield the $CD45RB^{high}$ (highest staining 30%) fraction by the FACS Vantage SE (Becton Dickinson, Sunnyvale, CA).

Sequential adoptive transfer experiments

Colitis was induced in C.B-17 SCID mice by adoptive transfer of $CD4^+CD45RB^{high}$ T cells [15]. Each SCID mouse was injected intraperitoneally with 3×10^5 $CD4^+CD45RB^{high}$ T cells. All the recipient mice were weighed initially, and three times per week after transfer. Mice were killed when their ongoing clinical score after transfer reached four points or more as mentioned below, and LP $CD4^+$ T cells were isolated for the next transfer. The isolated colitic LP $CD4^+$ T cells (3×10^5/mouse) were then transferred into new SCID mice [16]. After seven sequential transfers, we found that some mice failed to develop colitis within 40 weeks from transfer. To characterize these non-colitic LP $CD4^+$ T cells, we transferred $CD4^+CD45RB^{high}$ T cells (3×10^5) alone (group 1), $CD4^+CD45RB^{high}$ T cells (3×10^5) and LP $CD4^+$ T cells obtained from colitic mice originally transferred with $CD4^+CD45RB^{high}$ T cells (1×10^5) (group 2), or $CD4^+CD45RB^{high}$ T cells (3×10^5) and LP $CD4^+$ T cells obtained from the non-colitic mice after seven transfers (1×10^5) (group 3) into new SCID mice. Mice were killed 6 weeks after transfer.

Clinical and histological scorings

The recipient mice were weighed initially, then three times per week after transfer. They were observed for clinical signs of illness [40]: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. When mice were killed at a predetermined time point, their clinical score was assessed as the sum (0–8 points) of four parameters: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea); and gross blood, 0 or 1 [17]. For the sequential adoptive transfers, we monitored the clinical signs during the observation period, and the ongoing clinical score was defined as the sum (0–5 points) of the above-parameters other than colon thickening [40]. Mice were killed when their ongoing clinical score reached four points or more, and isolated LP $CD4^+$ T cells were transferred into new SCID mice. Transfers were repeated as long as the mice continued to develop colitis within 40 weeks post transfer. We judged recipient mice to be 'non-colitic' when they did not develop colitis within 40 weeks post transfer, and killed them at this time for further analysis. Tissue samples were fixed in PBS containing 10% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H&E. The mean degree of inflammation in the colon was calculated as previously described [15].

Flow cytometry

To detect surface expression of various molecules, isolated splenocytes or LP mononuclear cells (LPMC) were preincubated with an FcγR-blocking mAb (CD16/32; 2.4G2, BD PharMingen) for 15 min, then incubated with specific FITC-, PE-, PECy5- or biotin-labeled antibodies for 20 min on ice. Biotinylated antibodies were detected with PE- or Cy-Chrome™-streptavidin. Intracellular Foxp3 staining was performed with the PE-anti-mouse Foxp3 staining set (eBioscience) according to the manufacturer's instructions. Standard two- or three-color flow cytometric analyses were obtained using the FACSCalibur equipped with CellQuest software. Background fluorescence was assessed by staining of the control irrelevant isotype-matched mAb.

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Cytokine ELISA

To measure cytokine production, 1×10^5 LP CD4$^+$ T cells were cultured in 200 μL culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar, Cambridge, MA) pre-coated with 5 μg/mL hamster anti-mouse CD3ε mAb (145–2C11, BD PharMingen) and 2 μg/mL hamster anti-mouse CD28 mAb (37.51, BD PharMingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA per the manufacturer's recommendation (R&D, Minneapolis, MN).

In vitro functional analysis for Treg cells

CD4$^-$ cells were prepared from BALB/c splenocytes as APC by depleting CD4$^+$ cells with anti-CD4 MACS and treatment with 50 μg/mL MMC for 45 min at 37°C. In co-culture experiments, CD4$^+$CD25$^+$ T cells (0–1 $\times 10^4$ as Treg) or isolated LP CD4$^+$ T cells (0–1 $\times 10^4$) were cultured with CD4$^+$CD25$^-$ responder cells (1×10^4) and MMC-treated CD4$^-$ cells (5×10^5) in the presence of anti-CD3 mAb (1 μg/mL). To determine proliferation, each well was pulsed with 1 μCi [^{3}H]thymidine (NEN, Boston, MA) for the last 9 h of 72-h culture.

Statistical analysis

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

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **BM-DC:** bone marrow-derived DC · **LSEC:** liver sinusoidal endothelial cells · **MSC:** mesenchymal stem cell

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FTY720 suppresses the development of colitis in lymphoid-null mice by modulating the trafficking of colitogenic CD4$^+$ T cells in bone marrow

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2-Amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride (FTY720) suppresses T-cell egress from LN, thereby preventing pathogenic T cells from migrating toward disease sites. However, little is known about whether FTY720 could control the trafficking of T cells without the presence of lymphoid tissues. Here we demonstrate that FTY720 treatment suppresses the recirculation of CD4$^+$ T cells in splenectomized (SPX) lymphotoxin-$\alpha^{-/-}$ (LT-$\alpha^{-/-}$) mice that lack LN and spleen, as shown by peripheral blood (PB) lymphopenia in FTY720-treated SPX LT-$\alpha^{-/-}$ mice. In a short-term transfer experiment, the cell number of transferred Ly5.1$^+$CD4$^+$ T cells recovered from host FTY720-treated SPX LT-$\alpha^{-/-}$ mice (Ly5.2$^+$) was markedly decreased in PB, but conversely increased in BM. Notably, FTY720 treatment prevented the development of colitis that is otherwise induced in untreated SPX LT-$\alpha^{-/-}$ $\times$ RAG-2$^{-/-}$ mice upon transfer of colitic lamina propria CD4$^+$ T cells. In such mice, the number of CD4$^+$ T cells in PB or lamina propria of FTY720-treated SPX LT-$\alpha^{-/-}$ $\times$ RAG-2$^{-/-}$ recipients was significantly reduced, but that in the BM was significantly increased as compared with untreated control mice. Altogether, the present results indicate that FTY720 treatment may offer an additional role to direct trafficking of CD4$^+$ T cells in BM, resulting in the prevention of colitis.

Key words: Chronic colitis · Colitogenic CD4$^+$ T cells · FTY720 · Inflammatory bowel disease · Mucosal immunity

Supporting Information available online

Introduction

2-Amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol hydrochloride (FTY720) is a sphingosine-1-phosphate (S1P) receptor modulator, which induces prolonged down-modulation of the surface expression of the S1P receptor and thereby inhibits the egress of lymphocytes from thymus, LN, and Peyer's patches leading to peripheral blood (PB) lymphopenia [1–8]. From the view of clinical application, in animal models, FTY720 has been shown to prevent autoimmune diseases [9, 10], viral infection [11, 12], or graft rejection after allotransplantation [13]. Moreover, it was recently shown that FTY720 reduced the number of lesions and clinical disease activity of patients with multiple sclerosis in a phase II, placebo-controlled trial [14].

In inflammatory bowel diseases (IBD), it is believed that colitogenic memory CD4$^+$ T cells are intermittently reactivated in

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regional lymphoid organs in response to antigen-loading activated dendritic cells and thereafter return to inflammatory tissues [15–18]. We recently reported that colitogenic memory T cells survive for a long period in IL-7-dependent manner, by using a model of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID/RAG-deficient mice [19]. Although little was known about how colitogenic memory $CD4^+$ T cells in IBD are controlled by FTY720, we demonstrated that FTY720 suppresses the development of colitis induced by adoptive transfer of colitogenic lamina propria (LP) $CD4^+CD44^{high}CD62L^-$ effector-memory T (T_{EM}) cells [20, 21] that were obtained from colitic $CD4^+CD45RB^{high}$ T-cell-transferred SCID mice [22]. Furthermore, we found that FTY720 treatment induced marked lymphopenia of colitogenic $CD4^+$ T_{EM} cells in the periphery [22]. In this previous study, however, it was curious but very interesting as to why the colitogenic $CD4^+$ T_{EM} cells were controlled by FTY720 treatment, as they should preferentially reside in non-lymphoid tissues such as the gut [23, 24].

Thus, these previous results prompted us to investigate another possible effect of the FTY720 treatment, modulating a yet-known cell trafficking independent of the presence of LN and spleen. To this end, we here used splenectomized lymphotoxin-$\alpha^{-/-}$ (LT-$\alpha^{-/-}$) × RAG-$2^{-/-}$ mice lacking spleen, LN, and Payer's patches, as recipients for adoptive transfer of colitogenic $CD4^+$ T_{EM} cells and assessed the lymphoid tissue-independent effect of FTY720 treatment upon accumulation of donor T cells in the BM and prevention of colitogenic $CD4^+$ T_{EM} cell-mediated colitis.

Results

FTY720 induces lymphopenia in LT-$\alpha^{-/-}$ mice

To first assess the alterations of systemic T-cell number or its subset composition upon FTY720 treatment without an impact of lymphoid tissues including spleen, LN, and Peyer's patches, we used splenectomized (SPX) LT-$\alpha^{-/-}$ and the control SPX LT-$\alpha^{+/+}$ littermate mice in this study (Fig. 1A). Two weeks after splenectomy, mice were i.p. administered with a single dose of FTY720 (1.0 mg/kg) or control PBS, and then the tissue distribution of lymphocytes ($CD3^+CD4^+$, $CD3^+CD8^+$, and $CD19^+$cells) in the PB, LP, and BM at 24 h after administration was analyzed. The number of total $CD3^+CD4^+$ lymphocytes in the PB of FTY720-treated SPX LT-$\alpha^{+/+}$ mice was markedly decreased compared with that of PBS-treated SPX LT-$\alpha^{+/+}$ mice (Fig. 1B), suggesting a previously recognized LN-dependent mechanism of FTY720 that promotes the sequestration of lymphocytes and inhibits the egress of lymphocytes from LN [1–4]. Unlike the decreased number of $CD3^+CD4^+$ lymphocytes in the PB of FTY720-treated SPX LT-$\alpha^{+/+}$ mice, the difference in the number of those cells in LP or BM was not significant between FTY720- and PBS-treated SPX LT-$\alpha^{+/+}$ mice. In PBS-treated SPX LT-$\alpha^{-/-}$ mice, interestingly, the cell numbers of $CD3^+CD4^+$ lymphocytes in PB, LP, and BM were significantly increased compared with those in the paired PBS-treated SPX LT-$\alpha^{+/+}$ mice presumably due to the lack of LN serving as a reservoir of lymphocytes (Fig. 1B). Surprisingly, the cell number of $CD3^+CD4^+$ lymphocytes in the PB of FTY720-treated SPX LT-$\alpha^{-/-}$ mice was also significantly decreased compared with that of the paired PBS-treated SPX LT-$\alpha^{-/-}$ mice, suggesting an existence of as-yet-unknown reservoir for $CD3^+CD4^+$ lymphocytes other than LN or spleen (Fig. 1B). Moreover, although the number of $CD3^+CD4^+$ lymphocytes in the BM of SPX LT-$\alpha^{+/+}$ mice was not affected by FTY720 treatment, the number of such cells in the BM of FTY720-treated SPX LT-$\alpha^{-/-}$ mice was significantly increased compared with that of PBS-treated SPX LT-$\alpha^{-/-}$ mice. In contrast, the number of $CD3^+CD8^+$ T cells and $CD19^+$ B cells in the BM or in the LP was not affected by FTY720 treatment in any group of mice (Fig. 1B), whereas the number of those cells in the PB of FTY720-treated SPX LT-$\alpha^{+/+}$ and SPX LT-$\alpha^{-/-}$ mice was significantly decreased compared with that of the paired PBS-treated mice.

To further assess the possible effect of FTY720 on accumulation of $CD3^+CD4^+$ T cells within the BM of SPX LT-$\alpha^{-/-}$ mice, we next performed a short-term adoptive transfer of splenic $CD4^+$ T cells that were obtained from Ly5.1-derived C57BL/6J mice into Ly5.2-derived SPX LT-$\alpha^{-/-}$mice, by treating them with or without FTY720 at 3 h before transfer. Twenty-four hours after the transfer, mice were sacrificed, and the recovered cell number of the total and $Ly5.1^+$ $CD4^+$ donor cells at various sites was analyzed (Fig. 2A). Consistent with the above-mentioned results (Fig. 1), we confirmed that the number of $CD4^+$ T cells was significantly decreased in PB in FTY720-treated SPX LT-$\alpha^{-/-}$mice regardless of the host ($Ly5.2^+$) or donor ($Ly5.1^+$), but conversely, the number of those cells in the BM was significantly increased (Fig. 2B). Interestingly, the decreased cell number of $CD4^+$ T cells in the PB of FTY720-treated SPX LT-$\alpha^{-/-}$mice ($1.33 \pm 0.80 \times 10^7$) was almost identical to the increased number of those cells in the BM ($1.45 \pm 1.47 \times 10^7$), according to the calculating formula estimating that (i) the total number of BM cells could be calculated as 7.9 times the number of cells in two femurs [25] and (ii) the total volume of PB is 2.4 mL [26].

FTY720 treatment suppresses the development of colitis in spleen and LN-null recipients

We previously reported that FTY720 treatment suppresses the development of colitis induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells [22]. This was legitimate because most $CD4^+CD45RB^{high}$ T cells were naïve $CD44^{low}CCR7^+CD62L^+$ T cells that are accessible to mesenteric LN but at the same time their egress could be inhibited by FTY720 treatment. However, we also demonstrated that FTY720 treatment suppresses the development of colitis in SCID mice that were adoptively transferred with colitogenic LP $CD4^+CD44^{high}CD62L^-IL\text{-}7R\alpha^{high}$ T_{EM} cells obtained from colitic $CD4^+CD45RB^{high}$ T-cell-transferred

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SCID mice [22]. This was wholly unexpected because colitogenic LP CD4$^+$ T cells have the characteristics of T_{EM} (CD44^{high}CD62L$^-$) cells that are believed to preferentially migrate to non-lymphoid tissues such as the gut [23, 24], but do not migrate to LN including mesenteric LN. Thus, it remained possible that the migration of colitogenic LP CD4$^+$ T cells is controlled by FTY720

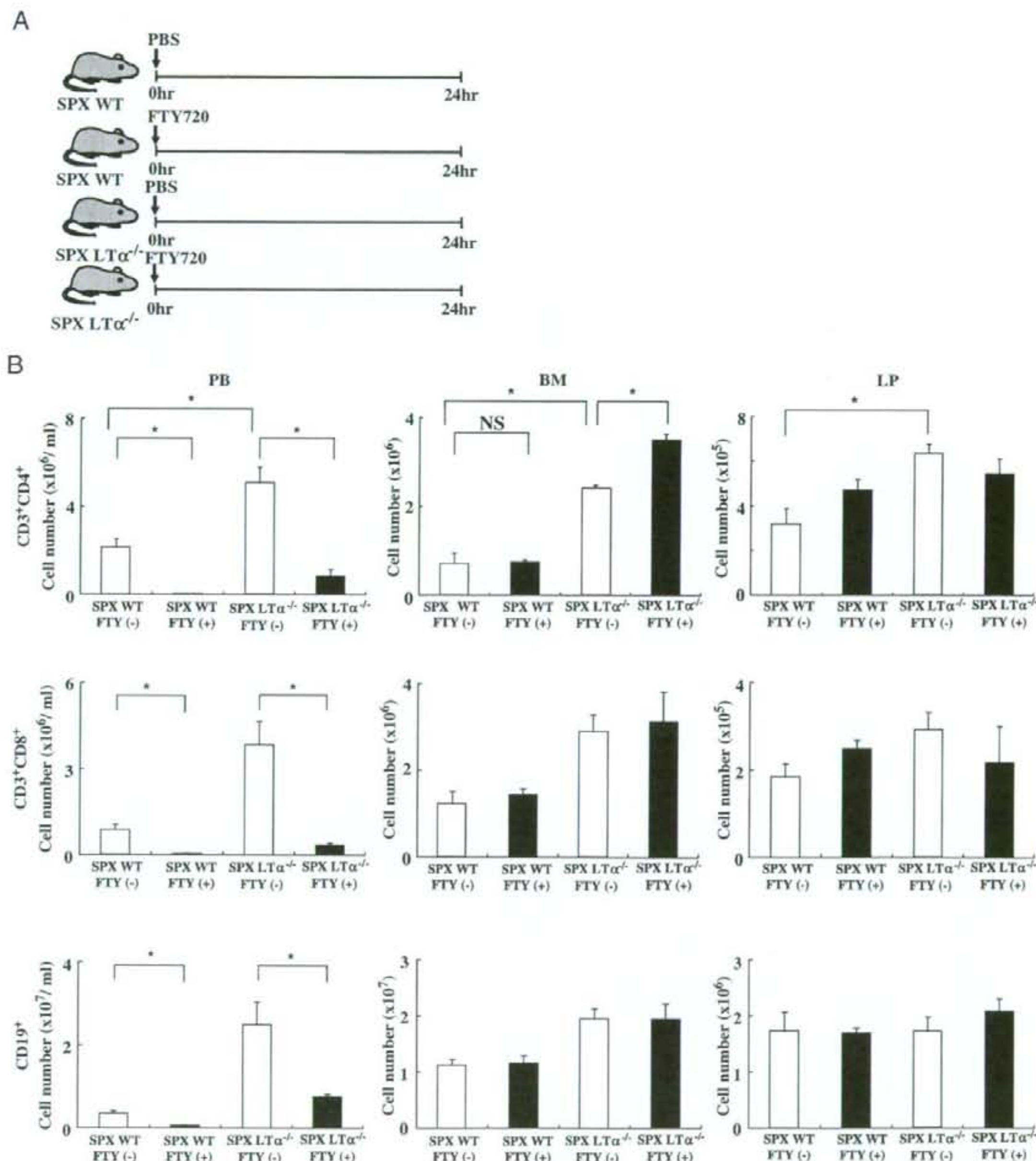

Figure 1. FTY720 treatment induces decrease in PB CD4$^+$ T cells and increase in BM CD4$^+$ T cells in LN/spleen-null mice. (A) FTY720 (1.0 mg/kg) or PBS was i.p. administered to C57BL/6 (WT) or LT-$\alpha^{-/-}$ mice with SPX, and the changes in the absolute numbers of cells were determined at 24 h after treatment. (B) The absolute number of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cell subsets, and CD19$^+$ B cells in PB, BM, and LP, was determined at 24 h after treatment using flow cytometry. Data are indicated as mean+SEM of six mice in each group. Groups of data were compared by Mann–Whitney U-test. *Indicates statistically significant at $p<0.05$. FTY, FTY720.

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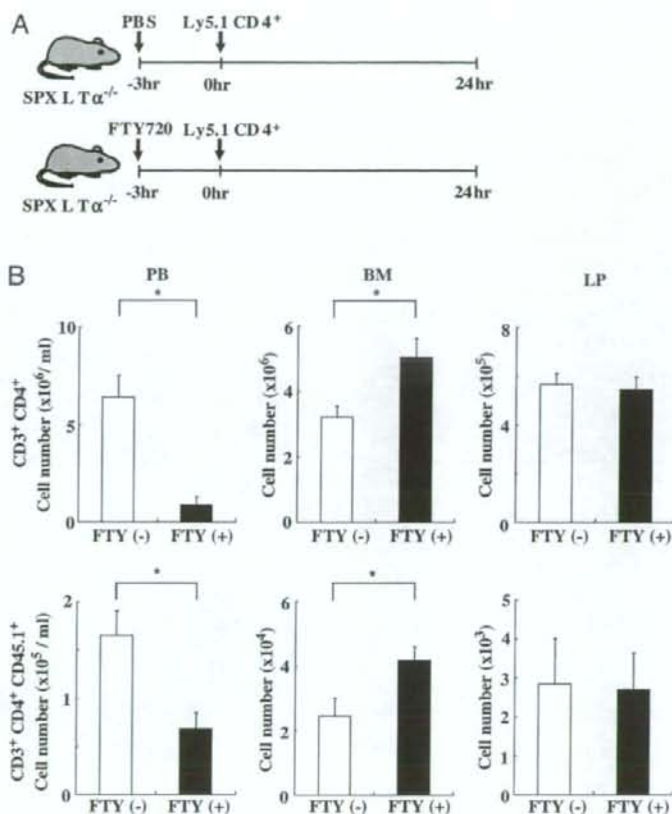

Figure 2. Transferred CD4⁺ T cells were preferentially accumulated in the BM of FTY720-treated LN/spleen-null mice. (A) SPX LT-$\alpha^{-/-}$ mice (Ly5.2⁺) were treated with FTY720 (1.0 mg/kg) or PBS at −3 h and then transferred with normal splenic CD4⁺ T cells (Ly5.1⁺) at 0 h. The changes in the absolute number of cells were determined at 24 h after the transfer. (B) The absolute number of total or transferred Ly5.1⁺ CD3⁺CD4⁺ T cells in PB, BM, and LP was determined at 24 h after the transfer using flow cytometry. Data are indicated as mean ± SEM of six mice in each group. *$p<0.05$. FTY, FTY720.

treatment in an LN-independent manner. Furthermore, we previously demonstrated that SPX LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice transferred with colitogenic LP CD4⁺ T_{EM} cells develop chronic colitis to a similar extent with the control SPX LT-$\alpha^{+/+}$ × RAG-$2^{-/-}$ recipient mice [27]. Based on such a background, we next challenged to investigate the novel role of FTY720 in suppression of colitis by a series of *in vivo* system without an impact of LN and spleen.

To this end, SPX LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ or control littermate SPX LT-$\alpha^{+/+}$ × RAG-$2^{-/-}$ mice were transferred with colitogenic LP CD4⁺ T_{EM} cells obtained from colitic CD4⁺CD45RB^{high} T-cell-transferred RAG-$2^{-/-}$ mice and treated with daily PBS or FTY720 (0.3 or 1.0 mg/kg) i.p., which was started 1 day before transfer and continued up to 7 wk (Fig. 3A). The control PBS-administered SPX LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ and SPX LT-$\alpha^{+/+}$ × RAG-$2^{-/-}$ recipients manifested progressive weight loss after 2 wk from transfer (data not shown). These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 7 wk post transfer. The clinical score of both group showed no significant difference at 7 wk post transfer (Fig. 3B). In contrast, the FTY720-treated mice appeared healthy with significantly low clinical scores, regardless of the doses (Fig. 3B). Altogether, the assessment of clinical scores showed a clear difference between control PBS-treated and FTY720-treated mice, regardless of the presence of LN (Fig. 3B). At 7 wk after the transfer, the colons from control PBS-treated mice, but not those from FTY720-treated mice, were enlarged and had a greatly thickened wall (Fig. 3C). Histological examination showed a marked inflammation in the colonic LP of control PBS-treated LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ or LT-$\alpha^{+/+}$ × RAG-$2^{-/-}$ mice (Fig. 3D). In contrast, it was mostly abrogated in the LP of FTY720-treated mice (Fig. 3D). This difference was confirmed by histological scores of multiple colon sections (Fig. 3E).

Furthermore, less number of CD4⁺ T cells was recovered from the PB of FTY720-treated mice compared with the paired control PBS-treated mice, regardless of LT-$\alpha^{-/-}$ or LT-$\alpha^{+/+}$ recipients (Fig. 4A). The number of CD4⁺ T cells recovered from the colon of control PBS-treated mice (SPX LT-$\alpha^{+/+}$ × RAG-$2^{-/-}$;

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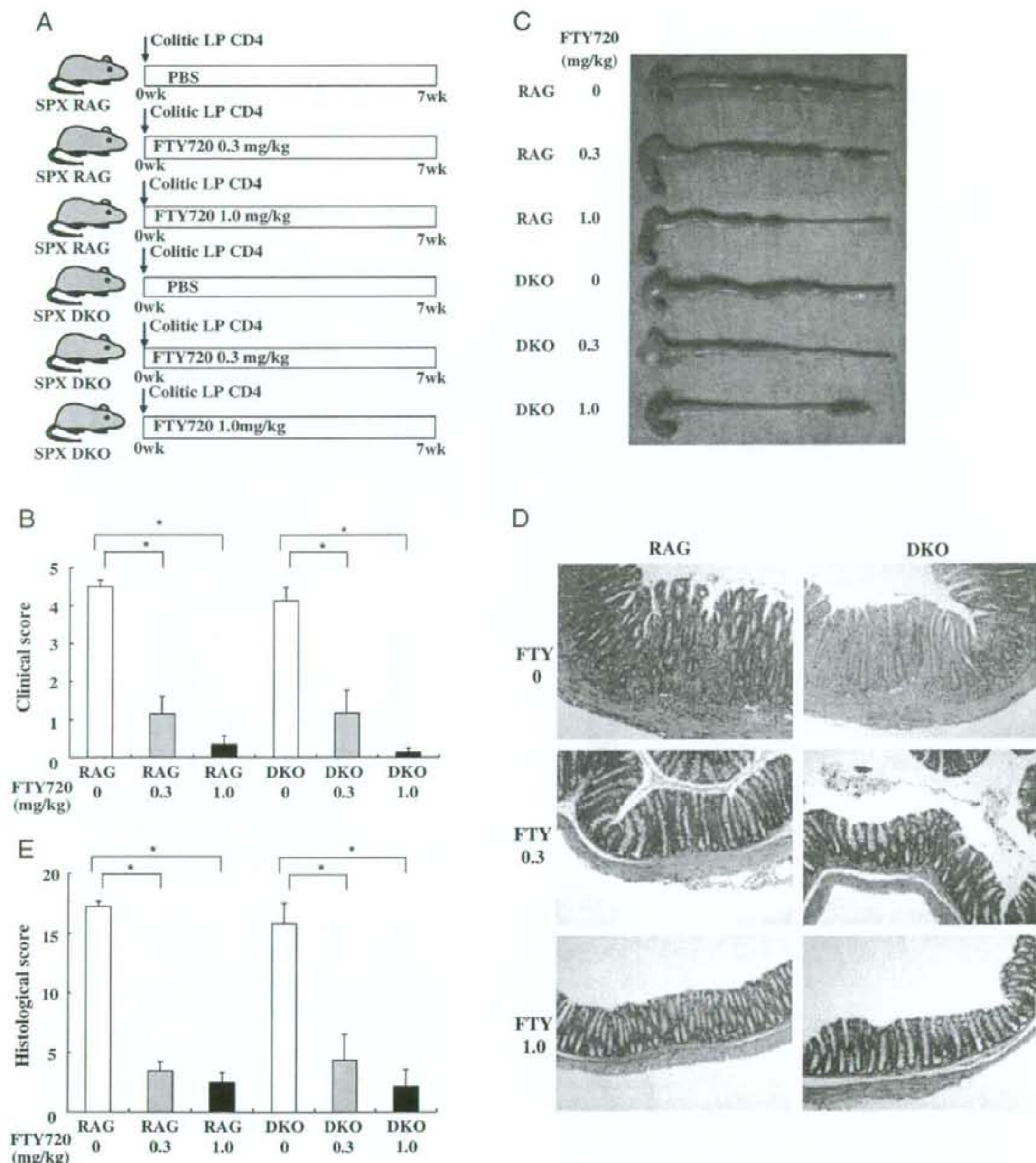

Figure 3. FTY720 prevents the development of colitogenic $CD4^+$ T_{EM}-mediated colitis in LN/spleen-null mice. (A) Colitogenic $CD4^+$ T_{EM} cells were isolated from the inflamed mucosa of colitic RAG-$2^{-/-}$ mice transferred with $CD4^+CD45RB^{high}$ T cells. SPX LT-$\alpha^{+/+}$ × RAG-$2^{-/-}$ and SPX LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice were then injected i.p. with 4×10^5 colitic LP $CD4^+$ T cells and were also treated i.p. with daily PBS ($n = 9$) or FTY720 (0.3 mg/kg; $n = 9$, 1.0 mg/kg; $n = 9$) starting 1 day before the transfer up to 7 wk. Mice were weighed initially upon transfer and then three times *per* week thereafter. (B) Clinical scores were determined at 7 wk after the transfer as described in *Materials and methods*. Data are indicated as mean+SEM of nine mice in each group. $^*p<0.05$. (C) Gross appearance of the colon at 7 wk after the transfer. (D) Histological examination of the colon at 7 wk after the transfer. Original magnification: ×100. (E) Histological scoring of colitis at 7 wk after the transfer. Data are indicated as mean+SEM of nine mice in each group. $^*p<0.05$ compared with the paired mice without FTY720 treatment.

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