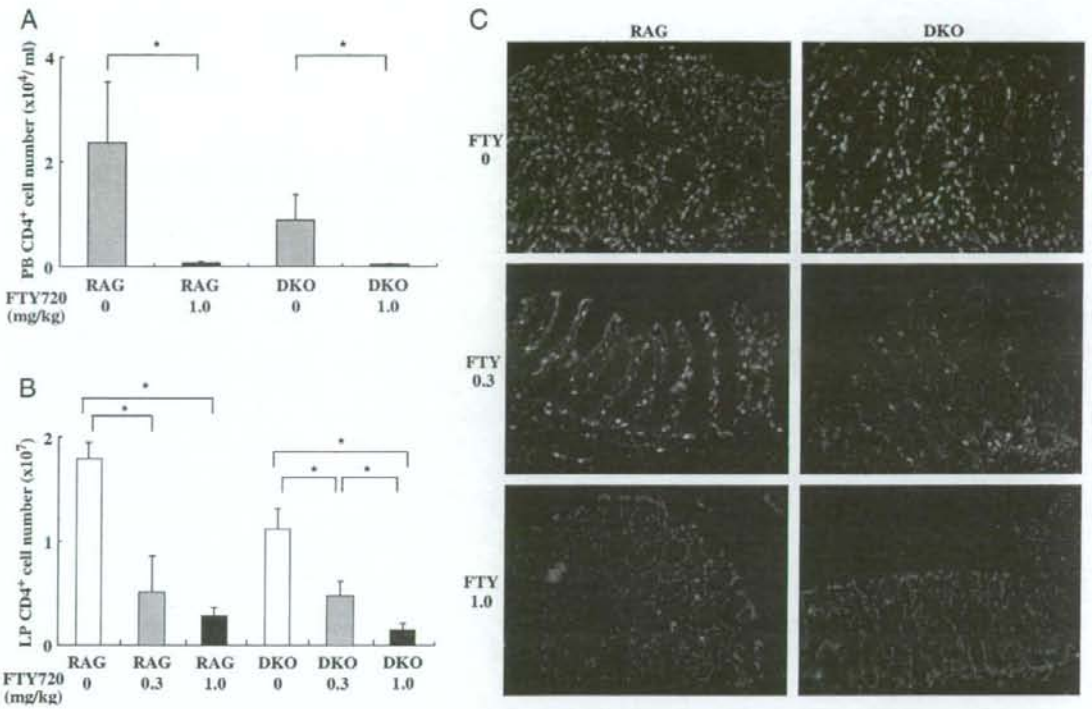

Figure 4. The number of LP CD4+ T cells was significantly decreased while PB lymphopenia was observed in FTY720-treated LN/spleen-null mice transferred with colitogenic CD4+ T_{EM} cells. (A–B) Number of CD3+CD4+ T cells in PB (A) or LP (B). Cells were stained with FITC-anti-CD3 and allophycocyanin-anti-CD4, and the total number of CD3+CD4+ cells was determined by flow cytometry. Data are indicated as the mean+SEM of nine mice in each group. *$p<0.05$. (C) CD4 immunostaining and DAPI counterstaining of the colon from Frozen sections were fixed with acetone and stained with anti-mouse CD4 mAb, followed by Alexa Fluor® 488 goat anti-rat IgG as secondary antibody and DAPI counterstaining. Representative of four separate samples in each group. Original magnification: ×100.

$1.73\pm0.15\times10^7$, SPX LT-$\alpha^{-/-}\times$RAG-$2^{-/-}$; $1.07\pm0.14\times10^7$) far exceeded the number of originally injected memory cells (3×10^5), indicating an extensive T-cell migration and/or proliferation in the inflamed colon, which did not apply to FTY720-treated mice (Fig. 4B). To clarify that the suppressed colitis in FTY720-treated mice was surely mediated by the decreased infiltration of CD4+ T cells into the LP, we next assessed the colonic infiltration of CD4+ T cells by immunohistochemistry. Figure 4C clearly demonstrated the markedly decreased infiltration of CD4+ T cells in the colon of FTY720-treated mice, regardless of LT-$\alpha^{-/-}$ or LT-$\alpha^{+/+}$ recipients. In contrast, a large number of CD4+ T cells were found in the inflamed mucosa of PBS-treated recipients (Fig. 4C).

FTY720 treatment suppresses the production of Th1 and Th2 cytokines

Production of cytokines such as IFN-γ, TNF-α, or IL-4 by LP CD4+ T cells of the control PBS- or FTY720-treated mice transferred with colitogenic LP CD4+ T_{EM} cells was assessed by ELISA. As shown in Fig. 5, LP CD4+ T cells obtained from FTY720-treated mice produced significantly less IFN-γ, TNF-α, IL-4 upon *in vitro* stimulation regardless of LT-$\alpha^{-/-}$ or LT-$\alpha^{+/+}$ recipients compared with those from the paired control PBS-treated mice.

We further asked whether FTY720 affects the cell differentiation and activation of transferred CD4+CD62L−CD44^{high} T_{EM} cell *in vivo*. However, almost all LP CD4+ T cells from PBS-treated or FTY720-treated mice retained the characteristics of CD69+CD62L−CD44^{high}IL-7Rα^{high} activated CD4+ T_{EM} cell (Supporting Information Fig. 1). Furthermore, although Daniel *et al.* recently showed a significant increase in Foxp3 mRNA expression in isolated LP CD4+ T cells of FTY720-treated mice using Th1-mediated TNBS colitis model [28], we were not able to detect any increase in Foxp3 protein in LP CD4+ T cells of our FTY720-treated mice regardless of LT-$\alpha^{-/-}$ or LT-$\alpha^{+/+}$ recipients (Supporting Information Fig. 1).

FTY720 treatment does not induce apoptosis or modulate the expression of gut-homing receptors

Given the evidence that FTY720 prevents the development of colitis induced by adoptive transfer of colitogenic LP CD4+ T_{EM}

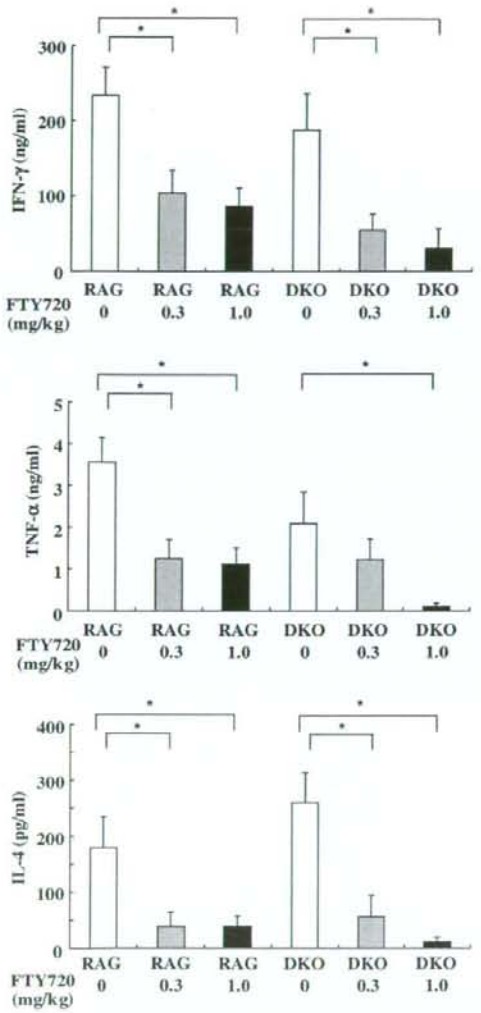

Figure 5. LP CD4$^+$ T cells in FTY720-treated LN/spleen-null mice transferred with colitogenic CD4$^+$ T_{EM} cells produce significantly less amount of Th1 and Th2 cytokines. LP CD4$^+$ T cells were isolated from mice 7 wk after the transfer of colitogenic LP CD4$^+$ T_{EM} cells and stimulated with anti-CD3 and anti-CD28 mAb for 48 h. IFN-γ, TNF-α, and IL-4 concentrations in culture supernatants were measured by specific ELISA. Data are indicated as mean+SEM of nine mice in each group. *$p<0.05$.

cells even in the absence of LN and spleen, we next investigated the mechanism by which FTY720 suppresses the development of colitis independently from such lymphoid tissues. To this end, we first checked whether FTY720 could directly induce apoptosis of colitogenic LP CD4$^+$ T_{EM} cells, contributing to the preventive effect, as suggested by other studies regarding the effect of FTY720 [29]. However, this possibility was excluded by showing that addition of various concentrations of FTY720 did not induce apoptosis of normal splenic or colitogenic LP CD4$^+$ T cells *in vitro* (Supporting Information Fig. 2A). To further assess the possibility that FTY720 might affect the gut-homing axis of CD4$^+$ T cells, we next checked the effect of FTY720 on the expression of gut-homing receptors, such as integrin α4β7 and CCR9, using a recently established *in vitro* assay of retinoic acid (RA)-induced gut-homing receptors. However, FTY720 did not skew the expression of integrin α4β7 and CCR9 on normal splenic or colitogenic LP CD4$^+$ T cells, even after 5 days of culture with RA, TGF-β, IL-2, and soluble anti-CD3 mAb [30, 31] (Supporting Information Fig. 2B).

To further assess the effect of long-term treatment of FTY720 on normal and colitogenic CD4$^+$ T cells *in vivo*, we conducted two adoptive transfer experiments as follows. First, SPX WT or LT-α$^{-/-}$ mice were i.p. treated with PBS or FTY720 (1.0 mg/kg) daily for 4 wk (Fig. 6A), and the changes in the number of apoptotic cells, the expression of integrin α4β7 and CCR9, and the accumulated number of CD3$^+$CD4$^+$ T cells in PB, LP, and BM were measured. As similarly shown in short-term administration of FTY720 (Fig. 1), the number of apoptotic cells (Annexin V$^+$ cells *per* total CD3$^+$CD4$^+$ T cells) (Fig. 6B) and the expression of integrin α4β7 and CCR9 (Fig. 6C) were not affected by long-term FTY720 treatment in both SPX WT and LT-α$^{-/-}$ mice. Furthermore, we found that the long-term treatment of FTY720 induced severe reduction in peripheral CD3$^+$CD4$^+$ T cells in both SPX WT and LT-α$^{-/-}$ mice, while the number of LP CD3$^+$CD4$^+$ T cells was not affected by FTY720 treatment in both SPX WT and LT-α$^{-/-}$ mice (Fig. 6D). Interestingly, the number of accumulated CD3$^+$CD4$^+$ T cells in FTY720-treated SPX WT LT-α$^{-/-}$ mice was significantly increased compared with that in PBS-treated mice, while this significant change was not observed in SPX WT mice (Fig. 6D). Similar results were also obtained in another adoptive transfer experiment of RAG-2$^{-/-}$ *versus* LT-α$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with colitogenic LP CD4$^+$ T_{EM} cells with or without FTY720 treatment for 4 wk after the transfer (Fig. 7A). Namely, the expression of gut-homing receptors (Fig. 7B) and the frequency of apoptotic CD4$^+$ T cells (Fig. 7C) were not changed by *in vivo* long-term FTY720 treatment. The number of PB and LP CD3$^+$CD4$^+$ T cells in FTY720-treated mice regardless of RAG-2$^{-/-}$ or LT-α$^{-/-}$ × RAG-2$^{-/-}$ recipients was significantly decreased compared with paired PBS-treated mice (Fig. 7D). Again, we found that the accumulated number of BM CD3$^+$CD4$^+$ T cells in FTY720-treated LN-null LT-α$^{-/-}$ × RAG-2$^{-/-}$ mice was significantly increased compared with that in PBS-treated LT-α$^{-/-}$ × RAG-2$^{-/-}$ mice (Fig. 7D).

FTY720 treatment promotes the accumulation of colitogenic CD4$^+$ T cells in BM

As FTY720 treatment induced increase in CD3$^+$CD4$^+$ T cells in BM and also lymphopenia in the PB of non-colitic SPX LT-α$^{-/-}$ mice in short-term FTY720 administration system (Figs. 1 and 2), those in long-term administration system (Fig. 6), and SPX LT-α$^{-/-}$ × RAG-2$^{-/-}$ mice transferred with colitogenic LP CD4$^+$ T_{EM} cells in long-term FTY720 administration system (Fig. 7), we

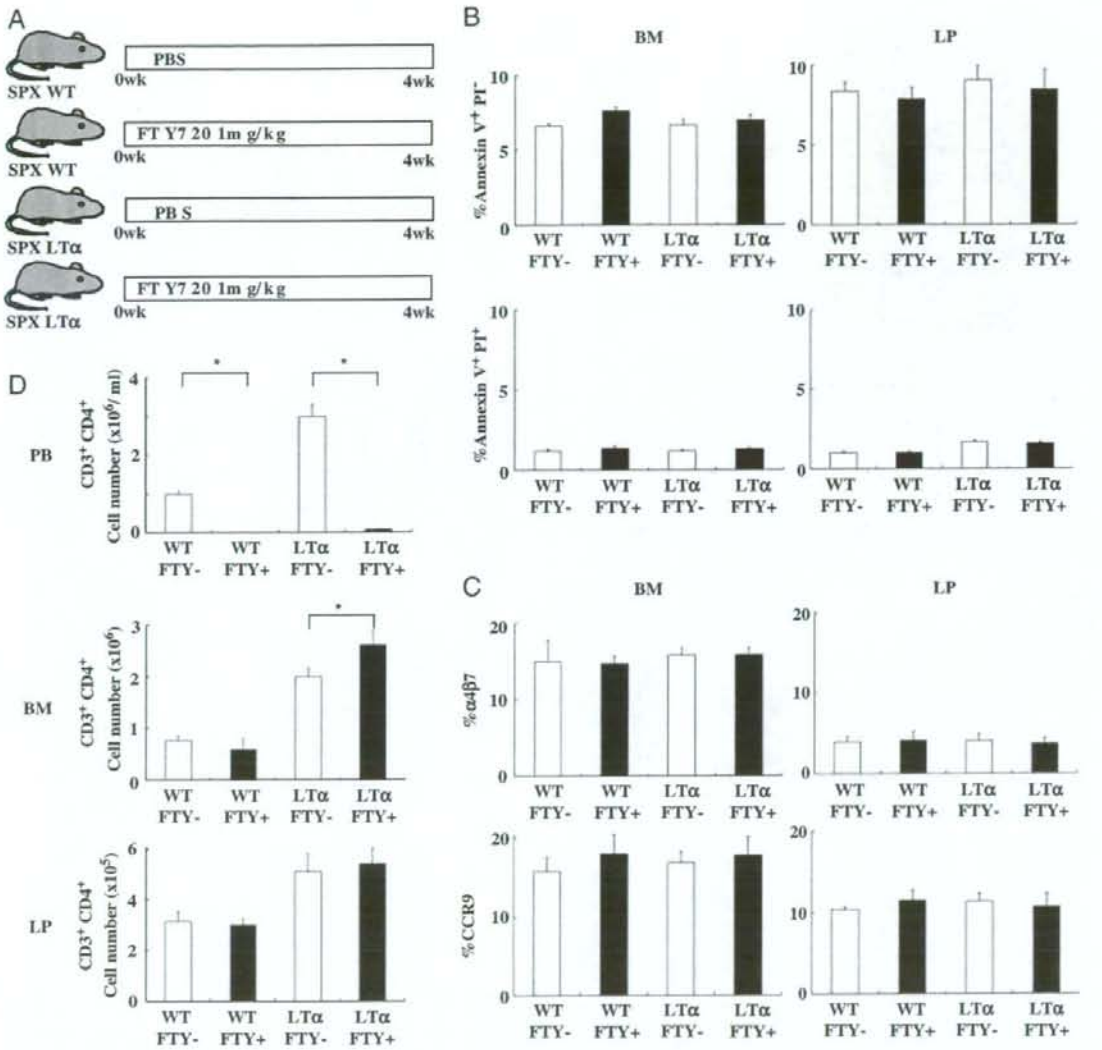

Figure 6. FTY720 treatment does not induce apoptosis or modulate the expression of gut-homing receptors of normal CD4$^+$ T cells *in vivo*. (A) SPX WT or LT-$\alpha^{-/-}$ mice were treated with FTY720 (1.0 mg/kg) or PBS for 4 wk. (B) The number of dead or apoptotic cells in the isolated BM and LP CD3$^+$CD4$^+$ T cells from each group was determined by the Annexin V-FITC/PI. Data are represented as mean ± SEM of %PI$^+$ Annexin V$^+$ (dead) cells and %PI$^-$ Annexin V$^+$ (apoptotic) cells in each group. Data are indicated as the mean ± SEM of five mice in each group. $^*p<0.05$. (C) Long-term FTY720 treatment does not modulate the expression of gut-homing receptors. Isolated BM and LP cells were stained with FITC-labeled anti-CD3, and allophycocyanin-labeled anti-CD4 or PE-labeled anti-α4β7 or PE-labeled anti-CCR9. The samples were analyzed by flow cytometry. Data are indicated as the mean+SEM of five mice in each group. (D) The absolute number of CD3$^+$CD4$^+$ T cells in PB, BM, and LP was determined at 4 wk after treatment. Freshly isolated PB, BM, and LP cells were stained with FITC-labeled anti-CD3 and allophycocyanin-labeled anti-CD4 mAb. The samples were analyzed by flow cytometry. Data are indicated as mean+SEM of five mice in each group.

finally addressed the possibility that FTY720 treatment might modulate the cell trafficking of colitogenic CD4$^+$ T_{EM} cells in BM, thereby inducing lymphopenia in the PB of FTY720-treated SPX LT-$\alpha^{-/-}$ × RAG-2$^{-/-}$ recipients, leading to inhibition of colitogenic CD4$^+$ T_{EM} cell circulation and subsequent suppression of the development of colitis. The preventive protocol of FTY720 in the current colitis model (Figs. 3–5) was not accurate to assess this possibility, as it was impossible to discriminate whether FTY720 treatment itself or the development of colitis in mice without treatment induced the increased number of colitogenic CD4$^+$ T_{EM} cells in the BM of SPX LT-$\alpha^{-/-}$ × RAG-2$^{-/-}$ recipients. Therefore, SPX LT-$\alpha^{-/-}$ × RAG-2$^{-/-}$ mice that were transferred

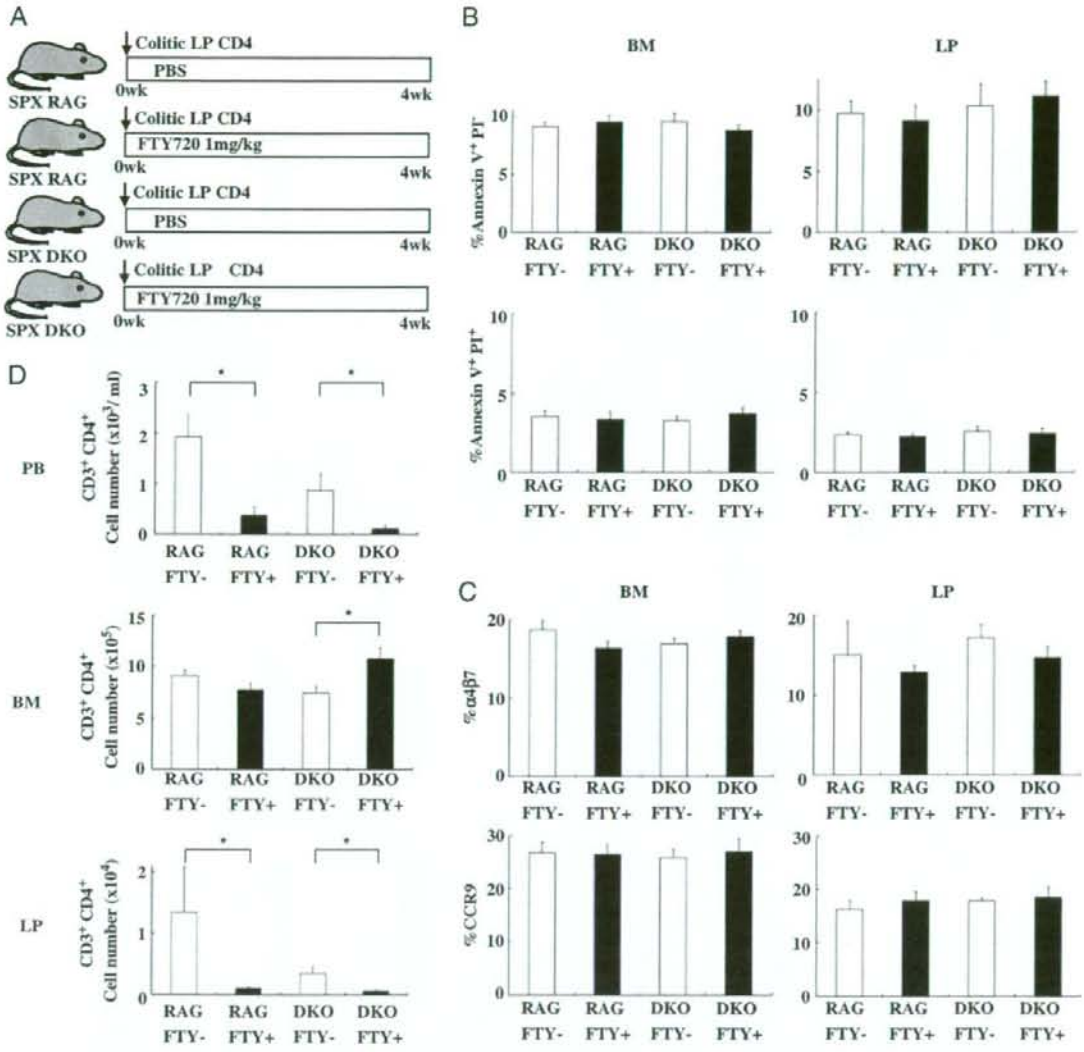

Figure 7. FTY720 treatment does not induce apoptosis or modulate expression of gut-homing receptors of CD4+ T cells in SPX RAG-2$^{-/-}$ or LT-α$^{-/-}$ mice transferred with colitogenic LP CD4+T_{EM} cells *in vivo*. (A) SPX RAG-2$^{-/-}$ or LT-α$^{-/-}$ mice transferred with colitogenic LP CD4+T_{EM} cells were treated with FTY720 (1.0 mg/kg) or PBS for 4 wk. (B) The number of dead or apoptotic cells in the isolated BM and LP CD3+CD4+ T cells from each group was determined by the Annexin V-FITC/PI. Data are represented as mean+SEM of %PI+ Annexin V+ (dead) cells and %PI- Annexin V+ (apoptotic) cells in each group. Data are indicated as the mean+SEM of five mice in each group. *$p<0.05$. (C) Long-term FTY720 treatment does not modulate the expression of gut-homing receptors. Isolated BM and LP cells were stained with FITC-labeled anti-CD3, and allophycocyanin-labeled anti-CD4 or PE-labeled anti-α4β7 or PE-labeled anti-CCR9. The samples were analyzed by flow cytometry. Data are indicated as the mean+SEM of five mice in each group. (D) The absolute number of CD3+CD4+ T cells in PB, BM, and LP were determined at 4 wk after treatment. Freshly isolated PB, BM, and LP cells were stained with FITC-labeled anti-CD3 and allophycocyanin-labeled anti-CD4 mAb. The samples were analyzed by flow cytometry. Data are indicated as mean+SEM of five mice in each group.

with colitogenic LP CD4+ T_{EM} cells were treated by single injection of FTY720 (1.0 mg/kg) at 3 wk after the transfer, and the recovered cell number of BM CD3+CD4+ T cells was measured at 24 h after treatment (Fig. 8A). The recovered cell number of CD3+CD4+ T cells was indeed significantly increased in the BM but, conversely, significantly decreased in the PB of FTY720-treated mice compared with that of PBS-treated mice (Fig. 8B), suggesting a similar mechanism with normal CD3+CD4+ T cells that FTY720 treatment induces a lymphopenia by trapping colitogenic CD4+ T_{EM} cells in BM. In addition, the number of LP CD3+CD4+ T cells was not affected by the treatment (Fig. 8B).

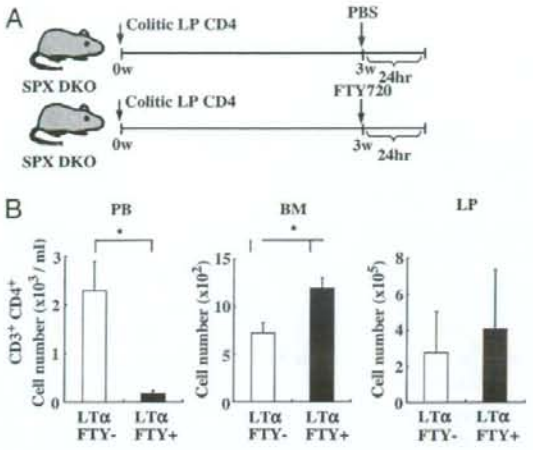

Figure 8. Colitogenic CD4$^+$ T cells were preferentially accumulated in the BM of FTY720-treated LN/spleen-null recipients transferred with colitic LP CD4$^+$ T cells. (A) SPX LT-$\alpha^{-/-}$ × RAG-2$^{-/-}$ mice were transferred with colitic LP CD4$^+$ T cells and treated with FTY720 (1.0 mg/kg) or PBS at 3 wk after the transfer. The change in absolute numbers of cells was determined at 24 h after the transfer. (B) The absolute number of CD3$^+$CD4$^+$ T cells in PB, BM, and LP was determined at 24 h after treatment. Freshly isolated PB, BM, and LP cells were stained with FITC-labeled anti-CD3 and allophycocyanin-labeled anti-CD4 mAb. The samples were analyzed by flow cytometry. Data are indicated as mean+SEM of six mice in each group. *$p<0.05$.

Discussion

In this study, we demonstrated that FTY720 is able to suppress the development of chronic colitis by modulating the trafficking of colitogenic CD4$^+$ T_{EM} cells in BM in addition to the well-known effect to control the egress and sequestration of lymphocyte in LN. Apparently, altered T-cell trafficking, sequestration, and egress in LN and spleen by FTY720 cannot be held fully accountable for the reduced disease activity in FTY-720-treated SPX LT-$\alpha^{-/-}$ × RAG-2$^{-/-}$ recipients that are transferred with colitogenic LP CD4$^+$ T cells. Importantly, since we found that FTY720 treatment guides the significant decrease in both normal CD4$^+$ T cells and colitogenic CD4$^+$ T_{EM} cells in the PB, but conversely increase in those cells in the BM of SPX LT-$\alpha^{-/-}$ × RAG-2$^{-/-}$ recipients, it is strongly suggested that FTY720 may have an effect to control the trafficking of lymphocyte in BM, resulting in accumulation of CD4$^+$ T cells in BM. Thus, the present study may provide a novel effect of FTY720 controlling the pathogenesis of IBD, by a mechanism other than the modification of trafficking of CD4$^+$ T cells toward LN.

Based on the previous preventive protocols, since it had been largely accepted that the effect of FTY720 on autoimmune diseases is owing to the promotion of sequestration of naïve T cells and the subsequent inhibition of egress of the generated effector T cells, but not those of memory T cells [1–12], it was unclear whether FTY720 could directly affect the migration of memory T cells in established autoimmune stages. To overcome this issue, we previously conducted adoptive transfer of colitogenic LP CD4$^+$ T_{EM} cells, excluded with naïve cells, into SCID mice [22]. Although almost all colitogenic LP CD4$^+$ T cells from colitic mice had the characteristics of CD44^{high}CD62L$^-$CD45RB^{low}IL-7Rα^{high} T_{EM} cells, FTY720 treatment suppressed the development of colitogenic memory T_{EM} cell-mediated colitis [22], indicating that FTY720 could surely affect the migration property of colitogenic LP CD4$^+$ T_{EM} cells. Consistently, we demonstrated that the colitogenic LP T_{EM} cells as well as normal splenic CD4$^+$CD45RB^{high} T cells did express several S1P receptors including $S1P_1$ [22]. However, we found that the number of CD4$^+$ T cells in MLN was also significantly decreased in FTY720-treated mice transferred with colitogenic LP T_{EM} cells compared with that in the control mice [22]. Consistent with this finding, since colitogenic CD4$^+$ memory T cells lack CD62L and CCR7, which naïve T cells do express [22], it was suspicious to conclude that FTY720 solely controls trafficking of colitogenic CD4$^+$ memory T cells toward LN.

We thus attempted to investigate a novel LN-independent mechanism of FTY720 using spleen/LN-null SPX LT-$\alpha^{-/-}$ × RAG-2$^{-/-}$ mice in this paper. Surprisingly, FTY720 treatment induces a severe PB lymphopenia in healthy SPX LT-$\alpha^{-/-}$ mice and colitogenic CD4$^+$ memory T-cell-transferred SPX LT-$\alpha^{-/-}$ × RAG-2$^{-/-}$ mice to a similar extent with the paired SPX LT-$\alpha^{+/+}$ mice and colitogenic CD4$^+$ memory T-cell-transferred SPX LT-$\alpha^{+/+}$ × RAG-2$^{-/-}$ recipients, respectively. This suggested the existence of as-yet-unknown reservoir other than LN and spleen, which could modulate the sequestration and/or the egress of colitogenic CD4$^+$ memory T cells. Before focusing on this possibility, we assessed other possible mechanisms based on previous literatures. The first possibility that FTY720 might directly induce apoptosis of colitogenic CD4$^+$ T cells was unlikely, because we showed that (i) the addition of various concentrations of FTY720 (10^{-6}–10^{-8} mol/L) to colitogenic CD4$^+$ memory T cells *in vitro* and *in vivo* FTY720 treatment did not induce apoptosis (Fig. 6A) and (ii) previous *in vivo* FTY720 (0.3–3.0 mg/kg) administration to normal mice did not promote apoptosis [22]. The second possibility that had been raised by others [28] was that FTY720 may induce conversion of colitogenic Th1 CD4$^+$ memory T cells into CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells. This was also unlikely, as we could not detect any increase in Foxp3 expression in LP CD4$^+$ T cells of colitogenic CD4$^+$ T-cell-transferred SPX LT-$\alpha^{-/-}$ × RAG-2$^{-/-}$ mice that were treated with FTY720 compared with those without FTY720 (Supporting Information Fig. 1). Although the previous paper demonstrated that FTY720 treatment could increase mRNA expression of CD25 and Foxp3 in LP CD4$^+$ T cells of FTY720-treated TNBS-given mice along with no development of colitis in those mice [28], it appears to be possible that FTY720 could induce regulatory T cells from naïve T cells as observed in their system, but not from established colitogenic CD4$^+$ memory T cells in our system.

We next assessed the third possibility that FTY720 might skew the expression of gut-homing receptors on colitogenic CD4$^+$ T cells, resulting in inhibition of the migration of these cells to the inflamed mucosa of the colon. To this end, we adopted an *in vitro* assay, in which addition of RA along with TGF-β, IL-2, and anti-CD3 mAb to colitogenic LP CD4$^+$ T cells could induce the gut-homing receptors, such as integrin α4β7 and CCR9. Although such a stimulation

up-regulated the expression of integrin α4β7 and CCR9 on colitogenic LP $CD4^+$ T cells indeed, FTY720 did not modify it.

Since recent works have suggested that BM is an important secondary lymphoid tissue [32] similar to LN and spleen, we finally focused on BM as a candidate reservoir to trap colitogenic $CD4^+$ T cells, so as to explain the PB lymphopenia observed in FTY720-treated healthy SPX LT-$\alpha^{-/-}$ mice and SPX LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice transferred with colitogenic LP $CD4^+$ T cells. Expectedly, we found that the number of BM $CD4^+$ T cells was significantly increased in correlation with the decreased number of PB $CD4^+$ T cells in FTY720-treated SPX LT-$\alpha^{-/-}$ mice. Interestingly, this was not the case with FTY720-treated SPX LT-$\alpha^{+/+}$ mice, indicating that the effect of FTY720 to direct $CD4^+$ T cells toward BM may be cancelled by the presence of LN or spleen, which is the major site where lymphocytes are mainly trapped. Consistent with this hypothesis, it is interesting that the decreased number of PB $CD4^+$ T cells was approximately equivalent to the increased number of BM $CD4^+$ T cells in FTY720-treated SPX LT-$\alpha^{-/-}$ mice.

In a recent publication, Ledgerwood *et al.* reported that FTY720 inhibits the entry of T cells into afferent lymphatics under inflammatory condition in the skin [33]. Unlike their elegant system, injecting donor T cells subcutaneously into footpads of mice and monitoring the migration of cells into popliteal LN at different time points we could not detect any difference in the recovered cell number of colonic LP $CD4^+$ T cells between FTY720-treated and FTY720-untreated SPX LT-$\alpha^{-/-}$ mice, even if we adopted the Ly5.1/Ly5.2 system to surely discriminate the transferred donor cells from host cells. However, further study will be needed to address whether BM alone is a target organ of FTY720 under LN/spleen-null condition, by assessing the precise concentration of S1P and FTY720-P in various tissues, and also by establishing a more precise method to monitor cell migration from LP to the afferent lymphatics, such as direct injection of donor cells into the LP of mice. Otherwise, it might be needed to use larger animals such as sheep for this issue, since it is very difficult to inject cells into very thin wall of intestine in mice system.

Finally, it should be addressed that LT-$\alpha^{-/-}$ mice have many other defects besides the lack of lymphoid organs, although we used SPX LT-$\alpha^{-/-}$ mice and LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice as spleen/LN-null mice to evaluate the role of FTY720 in this study. For example, (i) LT-$1\alpha^{-/-}$ mice have been reported to have spontaneous infiltration of lymphocytes including $CD4^+$ T cells in multiple peripheral tissues, possibly due to defective thymic negative selection [34], (ii) the total number of $CD4^+$ T cells in the body of LT-$\alpha^{-/-}$ mice should be reduced, since spleen and LN harbor large number of lymphocytes as their reservoir, and (iii) LT-α itself, as one of the cytokines, which is critical for the cell migrations of lymphocytes, may be involved in the present results. Further studies will be needed for these points.

In conclusion, the present results indicated that FTY720 treatment could direct trafficking of $CD4^+$ T cells not only toward LN but also toward BM, thereby contributing to the prevention of chronic colitis. Of clinical importance, this study also suggests that FTY720 treatment may be applicable to control the immune response of patients who had undergone extended lymphadenectomy upon various surgeries such as intestinal resection.

Materials and methods

Animals

C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). C57BL/6-RAG-$2^{-/-}$ mice were obtained from Taconic Laboratory (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). LT-$1\alpha^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). LT-$\alpha^{-/-}$ mice were intercrossed into RAG-$2^{-/-}$ mice to generate the littermate LT-$\alpha^{+/+}$ × RAG-$2^{-/-}$ and LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice [27] in the Animal Care Facility of Tokyo Medical and Dental University. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and littermate recipients were used at 6–12 wk of age. All experiments were approved by the regional animal study committees (permission number: 2006-049) and were carried out according to the institutional guidelines and Home Office regulations.

Antibodies

The biotin-conjugated anti-mouse IL-7Rα (A7R34) and PE-conjugated anti-mouse Foxp3 (FJK-16S) mAb were obtained from eBioscience (San Diego, CA). The Fcγ (CD16/CD32)-blocking mAb (2.4G2), PE-, PerCP-, and allophycocyanin-conjugated anti-mouse CD4 (RM4–5); FITC- and PerCP-conjugated anti-mouse CD3 (145-2C11); FITC-conjugated anti-mouse CD8α (53–6.7); PE-conjugated anti-mouse CD19 (1D3); allophycocyanin-conjugated anti-mouse CD44 (IM7); FITC-conjugated anti-mouse CD62L (MEL-14); FITC-conjugated anti-mouse CD69 (H1.2F3); PE-conjugated anti-mouse α4β7 (DATK32); FITC-conjugated anti-mouse CD45RB (16A); PE-conjugated anti-mouse Ly5.1 (A20); FITC-conjugated anti-mouse Ly5.2 (104) mAb and PE-conjugated streptavidin were obtained from BD PharMingen (San Diego, CA). The PE-conjugated anti-mouse CCR9 (242503) mAb was obtained from R&D Systems (Minneapolis, MN).

Purification of T-cell subsets

$CD4^+$ T cells were isolated from normal spleen and colon using the anti-CD4 (L3T4) MACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instruction. To isolate normal 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 Hanks' balanced salt solution (BSS) containing 1 mM DTT (Sigma-Aldrich) for 45 min to remove mucus and then treated with 3.0 mg/mL collagenase (Roche Diagnostics GmbH, Germany) and 0.01% DNase (Worthington

Biomedical, Freehold, NJ) for 2 h. The cells were pelleted two times through a 40% isotonic Percoll solution and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched $CD4^+$ T cells from the spleen and the colon (spleen; 94–97% pure, colon; 80–90%, as estimated by FACS Calibur (Becton Dickinson, Sunnyvale, CA)) were then labeled with PE-conjugated anti-mouse CD4 (RM4-5) and FITC-conjugated anti-CD45RB (16A). For isolation of peripheral lymphocytes, 500 μL of 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. BM was collected from the femur by flushing with sterile PBS. Subpopulations of $CD4^+$ cells were generated by two-color sorting on a FACS Aria (Becton Dickinson). All populations were >98.0% pure on reanalysis.

FTY720 treatment

FTY720 (Novartis Pharma AG, Basel, Switzerland) was dissolved in sterile PBS. For *in vivo* treatment, FTY720 was administered i.p. at a dose of 0.3 or 1.0 mg/kg daily. All animal studies were performed according to the institutional guideline under specific pathogen-free conditions. Exp. 1: To assess the effect of FTY720 on colitogenic memory $CD4^+$ T-cell-mediated colitis *in vivo*, we prepared colitogenic LP $CD4^+$ T cells. First, colitis was induced in RAG-$2^{-/-}$ mice by adoptive transfer of syngeneic $CD4^+CD45RB^{high}$ T cells as described previously [35]. Briefly, $CD4^+$ T cells were isolated from splenocytes from normal C57BL/6 mice using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec). Enriched $CD4^+$ T cells were labeled with PE-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-CD45RB mAb and isolated $CD45RB^{high}$ (highest staining 30%) fraction on a FACS Aria. Each RAG-$2^{-/-}$ mouse was injected i.p. with syngeneic 3×10^5 $CD4^+CD45RB^{high}$ T cells. Colitic mice were sacrificed at 5–7 wk after the transfer to isolate the colitogenic LP memory $CD4^+$ T cells. SPX LT-$\alpha^{+/+}$ × RAG-$2^{-/-}$ mice and SPX LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice were then injected i.p. with 4×10^5 colitic LP $CD4^+$ T cells and were treated with PBS ($n = 9$) or FTY720 (0.3 mg/kg; $n = 9$, 1.0 mg/kg; $n = 9$) daily starting 1 day before the transfer, over a period of 4 wk. Mice after the transfer were weighed initially and then three times *per* week thereafter. They were monitored for clinical signs of illness: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. The mice were sacrificed and assessed for a clinical score, which is the sum of four 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; and an additional point was added if gross blood was noted [35]. Exp. 2: To assess the *in vivo* effect of FTY720 on hemodynamics within lymphoid-null mice, FTY720 or PBS was administered to C57BL/6 mice or LT-$\alpha^{-/-}$ mice with or without splenectomy at 0 h, and the changes in absolute numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets, and $CD19^+$ B cells at 24 h after treatment in PB, LP, and BM. Exp. 3: To exclude the influence of resident $CD3^+CD4^+$ T cells that are readily present before the treatment of FTY720, FTY720-treated splenectomized LT-$\alpha^{-/-}$ mice ($Ly5.2^+$) were transferred with $Ly5.1^+$ splenic $CD4^+$ T cells at 0 h, and the changes in the absolute number of $Ly5.1^+$ or $Ly5.2^+$ $CD3^+CD4^+$ T cells at 24 h after the transfer were examined in PB, LP, and BM. Exp. 4: To specifically assess the effect of FTY720 on the trafficking of colitogenic $CD4^+$ T_{EM} cell, SPX LT-$\alpha^{-/-}$ mice that were transferred with colitogenic $CD4^+$ T_{EM} cells were i.p. treated with PBS or FTY720 (1.0 mg/kg) at 3 wk after the transfer, and the changes in the absolute number of $CD3^+CD4^+$ T cells at 24 h after treatment in PB, LP, and BM were measured. Exp. 5: To assess the effect of long-term FTY720 treatment *in vivo*, SPX WT or LT-$\alpha^{-/-}$ mice were i.p. treated with PBS or FTY720 (1.0 mg/kg) daily for 4 wk, and the changes in the expression of integrin α4β7 and CCR9, the absolute number of $CD3^+CD4^+$ T cells in PB, BM, and LP, and the number of apoptotic cells (Annexin V^+ cells *per* total $CD3^+CD4^+$ T cells) in BM and LP were measured. Exp. 6: To assess the effect of long-term FTY720 treatment *in vivo* specifically on colitogenic $CD4^+$ T cells, SPX RAG-$2^{-/-}$ or LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice that were transferred with colitogenic LP $CD4^+$ T_{EM} cells were i.p. treated with PBS or FTY720 (1.0 mg/kg) daily for 4 wk after the transfer, and the changes in the expression of integrin α4β7 and CCR9, the absolute number of $CD3^+CD4^+$ T cells in PB, LP, and BM, and the number of apoptotic cells were measured.

Disease monitoring and clinical scoring

The recipient mice were weighed initially upon T-cell transfer and then three times *per* week thereafter. They were observed for clinical signs of illness: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. Mice were sacrificed at the indicated time point and assessed for a clinical score, which is the sum (0–8 points) of four 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); stool consistency, 0–3 (0, normal beaded stool; 1, soft stool; 2, diarrhea); and an additional point was added if gross blood was noted [35]. To monitor the clinical sign during the observed period over time, the disease activity index is defined as the sum (0–5 points) of the above-mentioned parameters except colon thickening [35].

Histological examination and immunohistology

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 [35].

Immunohistochemistry

Immunohistochemistry using purified mAb against mouse CD4 (RM4-5; BD PharMingen) was performed. Briefly, 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 antibody at 4°C overnight, followed by staining with Alexa Fluor® 488 goat anti-rat IgG (Molecular Probes) for CD4 detection at room temperature for 60 min. All slides were counterstained with DAPI (Vector) and observed under a fluorescence microscope (BioZERO BZ8000 KEYENCE, Tokyo, Japan).

Flow cytometry

To detect the surface expression of a variety of molecules, isolated splenocytes, BM, or LP mononuclear cells were preincubated with an FcγR-blocking mAb (CD16/32; 2.4G2, BD PharMingen) for 15 min followed by incubation with specific FITC-, PE-, PerCP-, allophycocyanin- or biotin-labeled antibodies for 20 min on ice. Biotinylated antibodies were detected with PE-streptavidin. To detect the dead and apoptotic cells in the cells cultured with FTY720 (0, 10^{-8}, 10^{-7}, or 10^{-6} M) *in vitro* for 48 h, they were stained with Annexin V-FITC/PI (MBL, Nagoya, Japan). Standard three- or four-color flow cytometric analyses were obtained using the FACS Calibur using CellQuest software. Background fluorescence was assessed by staining with control irrelevant isotype-matched mAb.

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 hamster 2 μg/mL anti-mouse CD28 mAb (37.51, BD PharMingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by specific ELISA as *per* the manufacturer's recommendation R&D Systems.

In vitro induction of gut-homing receptors

Cells were cultured in RPMI 1640 media supplemented with 10% FBS, HEPES, 50 μM β-ME, and penicillin/streptomycin/L-glutamine. For 96-well plate cultures, 2×10^5 cells in round-bottom plates were cultured in 200 μL of media. WT SP $CD4^+$ T cells or colitic LP $CD4^+$ T cells were activated with 1 μg/mL anti-CD3 and 2 μg/mL anti-CD28 mAb in the presence of 10 ng/mL hTGF- β1 (PeproTech), 100 U/mL hIL-2 (PeproTech), and with/without 100 nM all-*trans* RA (Sigma-Aldrich) [36]. The cells were cultured with FTY720 at a concentration of 0, 10^{-8}, 10^{-7}, or 10^{-6} M for 5 days.

Statistical analysis

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

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Abbreviations: **FTY720:** 2-amino-2-{2-[4-octylphenyl]ethyl}-1,3-propanediol hydrochloride · **IBD:** inflammatory bowel disease · **LP:** lamina propria · **LT-α:** lymphotoxin alpha · **PB:** peripheral blood · **RA:** retinoic acid · **S1P:** sphingosine-1-phosphate · **SPX:** splenectomized · **T_{EM}:** effector-memory T

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

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

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

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

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

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

Materials and Methods

Mice

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

Flow Cytometry and Immunohistochemical Procedures

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

Histologic Evaluation of Colitis

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

Real-Time Reverse-Transcription Polymerase Chain Reaction Analysis

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

Measurement of Cytokines and Chemokines by Enzyme-Linked Immunosorbent Assay

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

Chemotaxis Assay

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

Cell Transfer

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

Statistical Analysis

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

Results

Generation of TCRγ-Deficient Mice

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

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

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

Pathogenic Role of γδ T Cells in UC-Like Colitis

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

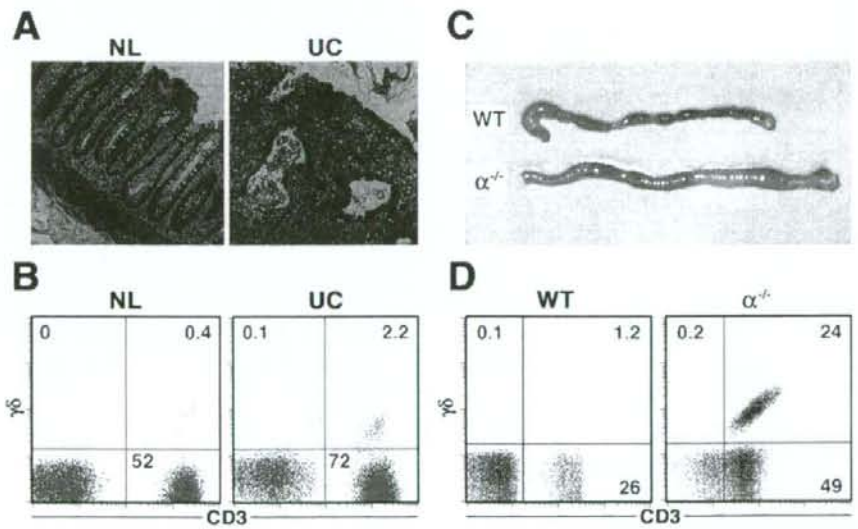

Figure 1. γδ T cells concentrate in the inflamed colonic mucosa of UC patients and colonic LP of $\alpha^{-/-}$ mice suffering from spontaneous chronic colitis. (*A*) A representative colonic tissue section from an ulcerative colitis (*UC*) patient shows a marked infiltration of lymphomyeloid cells,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

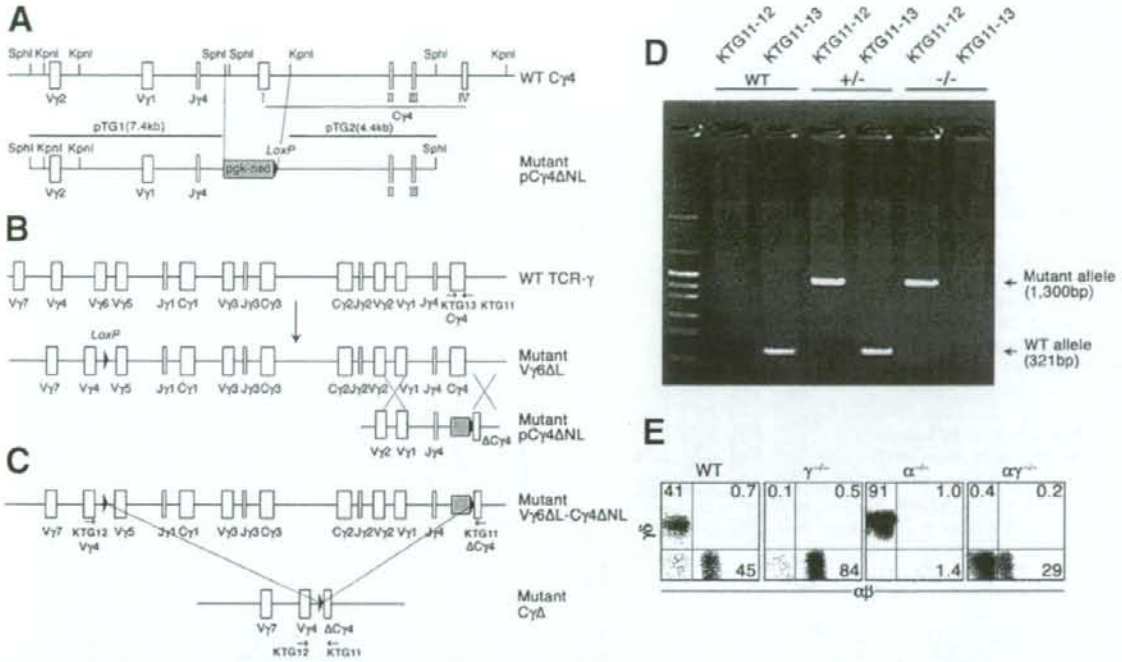

Figure 2. Generation of TCRγ-deficient mice and subsequent production of TCRαγ double mutant mice. (*A*) Schematic representation of WT and mutant (pCγ4ΔNL) genomic Cγ4 loci together with the 3 DNA fragments used to construct the mutant pCγ4ΔNL vector. The resulting targeting vector (pCγ4ΔNL) carrying a *loxP*-flanked *pgk-neo* gene cassette in place of exon 1 of Cγ4 gene used a neomycin resistance gene driven by the *pgk* promoter as positive selection marker is shown. Restriction enzyme sites, *SphI* and *KpnI* (*solid bars*), exon structures, Vγ and Jγ (*open boxes*), and *loxP* site (*solid triangle*) are indicated. (*B*) Schematic representation of the ES clone carrying WT TCRγ gene and mutant Vγ6ΔL ES clone carrying the allele in which the Vγ6 region was replaced by a single *loxP* site and mutant pCγ4ΔNL targeting vector. Exon structures, Vγ and Jγ (*open boxes*) and *loxP* site (*solid triangle*) are indicated. (*C*) Schematic representation of generation of the mutant mice that carry the TCRγ-deficient (CγΔ) allele by Cre-mediated recombination during embryonic development. Exon structures, Vγ and Jγ (*open boxes*), and *loxP* site (*solid triangle*) are indicated. (*D*) The mutant mice that carry the TCRγ-deficient (CγΔ) allele were intercrossed to produce TCRγ$^{+/+}$ (WT), TCRγ$^{+/-}$ (γ$^{+/-}$), and TCRγ$^{-/-}$ (γ$^{-/-}$) mice, and the corresponding WT and mutant alleles were typed by PCR analysis of tail DNA with each set of primers indicated. (*E*) γδ T cells are absent from the IEL compartment of γ$^{-/-}$ and αγ$^{-/-}$ mice. γ$^{-/-}$ Mice were crossed with α$^{-/-}$ mice to obtain WT (α$^{+/-}$ × γ$^{+/-}$), γ$^{-/-}$ (α$^{+/-}$ × γ$^{-/-}$), α$^{-/-}$ (α$^{-/-}$ × γ$^{+/-}$), and αγ$^{-/-}$ (α$^{-/-}$ × γ$^{-/-}$) littermate mice.

mation characterized by elongation of crypts was much milder in αγ$^{-/-}$ mice as compared with α$^{-/-}$ mice (Figure 3*A*). Although the body weight was comparable between αγ$^{-/-}$ and α$^{-/-}$ mice, it was evident that colonic weight was significantly decreased in αγ$^{-/-}$ mice as compared with α$^{-/-}$ mice (Figure 3*B*). The disease score characterized by the thickening of colonic mucosa with crypt elongation and inflammatory cell infiltration was also significantly lower in αγ$^{-/-}$ mice than that rated in α$^{-/-}$ mice (Figure 3*C*). Although approximately 20% of 20- to 60-week-old α$^{-/-}$ mice displayed anorectal prolapse (ARP), it was not discerned in any of age-matched αγ$^{-/-}$ mice (Figure 3*D*). Notably, no difference was observed in the age of onset of colitis and in the incidence of colitis (~80%) among 20- to 32-week-old αγ$^{-/-}$ and α$^{-/-}$ mice. In addition, in comparison with administration of PBS (as control), adoptive transfer of γδ T cells that were purified from α$^{-/-}$ mice did not increase the incidence of colitis in the recipient αγ$^{-/-}$ mice. However, the transfer of γδ T cells exacerbated the severity of colitis in the recipient αγ$^{-/-}$ mice. As shown in Figure 3*E*, more severe inflammatory cell infiltration was observed in the inflamed colon of the recipient αγ$^{-/-}$ mice with γδ T-cell transfer as compared with control αγ$^{-/-}$ mice. Therefore, it is possible that γδ T cells may be involved in the exacerbation, but not induction, of UC-like colitis.

Decrease in the Colonic Neutrophils and Monocytes in the Absence of γδ T Cells

The above results indicate that the spontaneous colitis in α$^{-/-}$ mice is ameliorated by the absence of γδ T cells in αγ$^{-/-}$ mice. With these findings in mind, flow cytometric analysis of colonic LP cells isolated from WT, γ$^{-/-}$, α$^{-/-}$, and αγ$^{-/-}$ littermate mice at approximately 28 weeks of age was performed, and the representative results of 5 independent experiments are presented in Figure 4*A*. In this experiment, WT, γ$^{-/-}$, α$^{-/-}$, and αγ$^{-/-}$ littermate mice yielded 5.1 × 10^5, 6.1

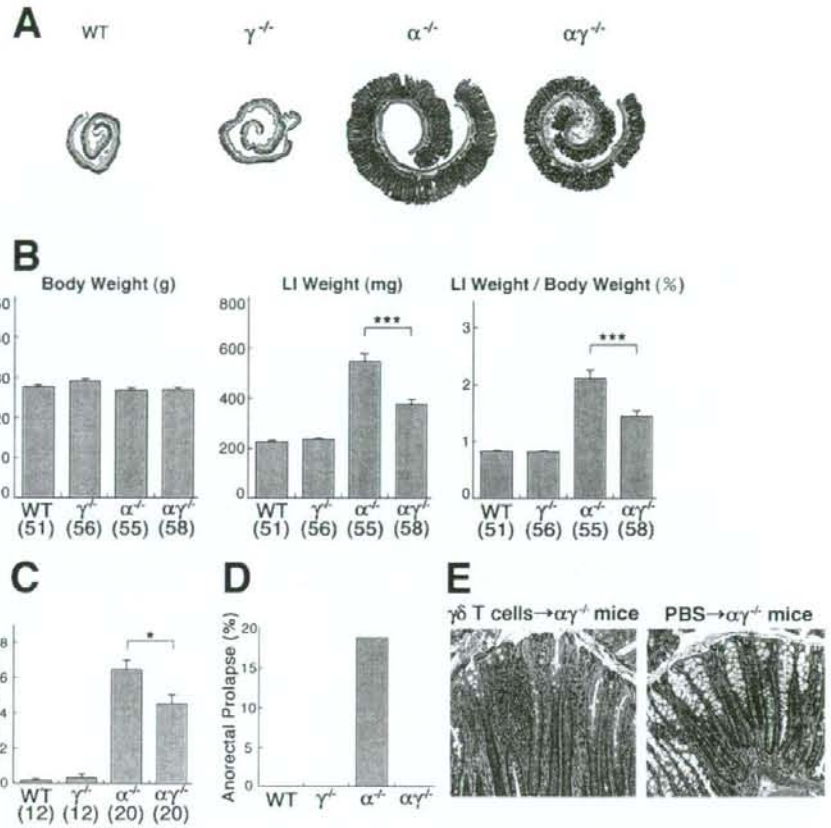

Figure 3. Colonic mucosal inflammation is milder in $\alpha\gamma^{-/-}$ mice lacking γδ T cells than in $\alpha^{-/-}$ mice possessing γδ T cells. (*A*) Representative histologic pictures of colon from WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ littermate mice. These mice were maintained in the same cages under specific pathogen-free conditions and examined at 28 weeks of age. (*B*) Body weight, wet weight of large intestine (LI weight), and ratio (%) of LI weight to body weight in WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ littermate mice at 20 to 32 weeks of age are shown. Values in parentheses are numbers of mice examined. $^{***}P < .001$. (*C*) Disease scores (0–10) of colitis in 24- to 32-week-old WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice were assessed. Values in parentheses are numbers of mice examined. $^{*}P < .05$. (*D*) Incidence of ARP in 24- to 60-week-old WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice. (*E*) The histologic findings (original magnification, ×20) of colon of recipient $\alpha\gamma^{-/-}$ mouse groups that received transfer of γδ T cells (*left panel*) and that received PBS (*right panel*).

$\times 10^5$, 32.3×10^5, and 12.0×10^5 colonic LP cells, respectively. Based on the absolute numbers of infiltrated cells and the flow cytometry results shown in Figure 4*A*, it was evident that fewer Mac-1$^+$Ly-6G$^-$ cells and Mac-1$^+$Ly-6G$^+$ cells were present in the colonic LP cell population of $\alpha\gamma^{-/-}$ mice as compared with those of $\alpha^{-/-}$ mice. Monocytes express Mac-1 but not Ly-6G, whereas neutrophils express both Mac-1 and Ly-6G.[21] Therefore, our results suggest that, in addition to monocyte infiltration (Figure 4*A*), there is a marked infiltration of neutrophils in the inflamed colonic LP of $\alpha^{-/-}$ mice. We also confirmed our previous finding[15] that a remarkable increase in γδ T cells was observed in the inflamed colonic LP of $\alpha^{-/-}$ mice (Figure 4*A*).

Immunohistochemical examination of inflamed colons from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice at approximately 28 weeks of age was performed to further confirm flow cytometric results. Consistent with the flow cytometric observations, significantly smaller numbers of Mac-1$^+$ cells and Ly-6G$^+$ cells were observed in the colonic LP of $\alpha\gamma^{-/-}$ mice as compared with those in the colonic LP of $\alpha^{-/-}$ mice (Figure 4*B*).

To investigate whether γδ T cells contribute to the generation of colonic environment for enhancing the migration of neutrophils and monocytes into the inflamed colon, we examined chemotactic activity of colonic extracts from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice to neutrophils and monocytes (Figure 5*A*). As a result, chemotactic activity to neutrophils of colonic extracts from $\alpha\gamma^{-/-}$ mice was significantly weaker than that from $\alpha^{-/-}$ mice, whereas the chemotactic activities to monocytes of both extracts were almost comparable (Figure 5*B*). The marked infiltration of neutrophils into the inflamed colonic LP of $\alpha^{-/-}$ mice is most likely mediated by some factors, such as MIP-2 (Figure 5*A*), that are enhanced in the presence of γδ T cells.

Taking all of these results together, and in conjunction with our previous findings,[22] colonic γδ T cells of $\alpha^{-/-}$ mice exert aggravating effect on the UC-like colitis by increasing primarily the influx of neutrophils into the inflamed mucosa.

Attenuation of Colonic Proinflammatory Cascades by the Absence of γδ T Cells

In view of the severe colitis, increased infiltration of Mac-1$^+$Ly-6G$^+$ and Mac-1$^+$Ly-6G$^-$ cells, and marked production of neutrophil chemotactic factor(s) in the inflamed colonic LP of $\alpha^{-/-}$ mice, quantitative real-time

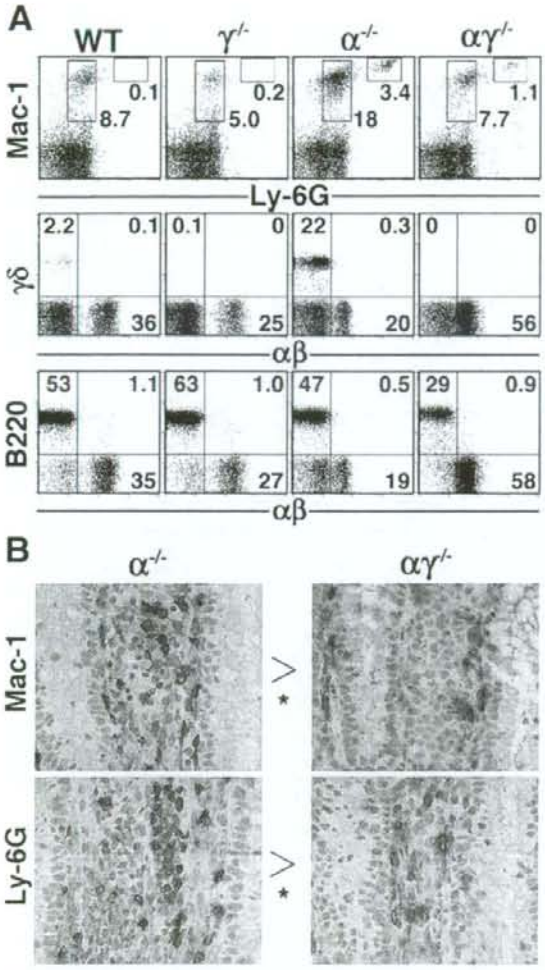

Figure 4. Decrease in colonic Mac-1$^+$Ly-6G$^-$ and Mac-1$^+$Ly-6G$^+$ cells in the absence of γδ T cells. WT (n = 3), $\gamma^{-/-}$ (n = 3), $\alpha^{-/-}$ (n = 4), and $\alpha\gamma^{-/-}$ (n = 4) mice from 28 weeks of age were examined. (*A*) Flow cytometric profiles of colonic LP cells. Absolute numbers of LP cells isolated from these WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ individuals were 5.1 × 10^5, 6.1 × 10^5, 32.3 × 10^5 and 12.0 × 10^5, respectively. (*B*) Representative immunohistochemical verification of the prominent infiltrations of Mac-1$^+$ and Ly-6G$^+$ cells into the inflamed colonic LP of $\alpha^{-/-}$ mice. Five tissue sections prepared from ascending colon to rectum per mouse, namely, 20 sections obtained from inflamed large intestines of $\alpha^{-/-}$ mice and those obtained from inflamed large intestines of $\alpha\gamma^{-/-}$ mice, were examined in a blinded fashion by 5 independent investigators, and the statistical difference in absolute numbers of Mac-1$^+$ and Ly-6G$^+$ cells between large intestinal mucosa from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice were determined by 2-sided Mann–Whitney *U* test. *$P < .05$.

RT-PCR analysis and measurement of the amounts of representative proinflammatory cytokines as well as chemokines were performed to dissect further the role of γδ T cells in the UC-like colitis in $\alpha^{-/-}$ mice.

To this end, messenger RNA (mRNA) and proteins prepared from the large intestines of WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice were examined. Inflamed colonic tissues from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice contained at least 10 times higher levels of cytokine (Table 1)- and chemokine (Table 2)-specific mRNA than those of WT and $\gamma^{-/-}$ mice except for IL-7 and IL-10 mRNA. In contrast to the mRNA from colonic tissues of $\alpha^{-/-}$ mice, those of $\alpha\gamma^{-/-}$ mice contained significantly smaller amounts of cytokine (TNF-α, IL-1β, IL-6, and TGF-β)- and chemokine (KC and MIP-2)-specific mRNA. With these observations in mind, we measured the amounts of representative cytokines as well as chemokines that had exhibited the differences in mRNA levels between the colonic tissues of $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice. First, in situ production of TNF-α, IL-1β, and IL-6 but not TGF-β proteins was significantly downregulated in the inflamed colonic mucosa of $\alpha\gamma^{-/-}$ mice as compared with that of $\alpha^{-/-}$ mice (Table 1). Second, KC and MIP-2 chemokines that are involved in the chemoattract of neutrophils and/or monocytes[23] were significantly decreased in large intestines of $\alpha\gamma^{-/-}$ mice compared with those in large intestines of $\alpha^{-/-}$ mice (Table 2).

To investigate the cell types responsible for the increases in these proinflammatory cytokines and chemokines, real-time RT-PCR analysis of mRNA present in the purified cell subsets from the inflamed colonic LP of $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice was performed (see Supplementary Figure 1 online at www.gastrojournal.org). The IL-1β and MIP-2 mRNA were expressed preferentially by Gr-1$^+$ cells, F4/80$^+$ cells, and CD11c$^+$ cells in the colon, whereas IL-6 mRNA was mainly expressed by Gr-1$^-$F4/80$^-$CD11c$^-$ cell populations. Expression levels of TNF-α- and KC-specific mRNA were comparable between all cell populations (Gr-1$^+$ cells, F4/80$^+$ cells, CD11c$^+$ cells, and Gr-1$^-$F4/80$^-$CD11c$^-$ cells) examined. Finally, the expression levels of these cytokine- and chemokine-specific mRNA in every cell subset were lower in cells from $\alpha\gamma^{-/-}$ mice than those in cells from $\alpha^{-/-}$ mice (see Supplementary Figure 1 online at www.gastrojournal.org).

Discussion

The $\alpha^{-/-}$ mice spontaneously develop colitis that shares many features with human UC.[16,24] Commensal enteric flora is required for the development of this colitis as indicated by the absence of colitis in $\alpha^{-/-}$ mice that are maintained under germ-free conditions.[22,25] The number of colonic γδ T cells drastically decreases in the $\alpha^{-/-}$ mice under germ-free conditions.[22] However, the study to identify the role of γδ T cells in the UC-like chronic colitis in $\alpha^{-/-}$ mice has been hampered by the difficulty in generating TCRαδ double mutant ($\alpha\delta^{-/-}$) mice because of the genomic organization of these TCR genes.[20] In the present study, we overcame this problem by newly generating $\gamma^{-/-}$ mice and subsequently crossing these mice with $\alpha^{-/-}$ mice to generate TCRα double mutant mice that lacked γδ T cells. By using these $\alpha\gamma^{-/-}$ mice, we herein provide a novel insight into the role of γδ T cells

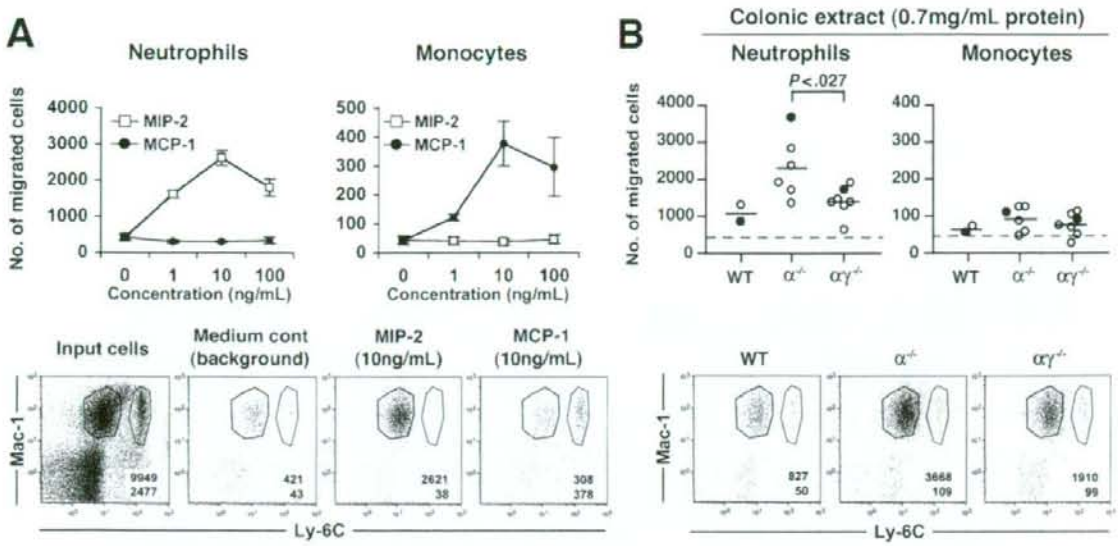

Figure 5. Chemotactic activity of colonic extracts from WT, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice to neutrophils and monocytes. (*A*) The number of neutrophils and monocytes migrated in response to the increasing concentration of MIP-2 (*open square*) and MCP-1 (*solid circle*). The representative flow cytometric profiles are shown in the *lower panels*, and *red* and *blue gates* indicate the neutrophils and monocytes, respectively. *Colored numbers* represent means of the number of cells in each gate. (*B*) Chemotactic responses of neutophils and monocytes to colonic extracts (0.7 mg/mL protein) from WT, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice, and each *circle* represents an individual mouse. *Horizontal bars* show mean values, and *dotted lines* indicate the number of migrated cells in medium alone. The representative flow cytomeric profiles of 3 individual animals indicated by the *solid red* and *blue circles* (*upper panels*) are shown in the *lower panels*, and *red* and *blue gates* indicate the neutrophils and monocytes, respectively.

that contributes to the exacerbation of UC-like colitis in $\alpha^{-/-}$ mice.

There is growing evidence supporting the fact that γδ T cells are part of the innate immune system and play an active multifaceted immunoregulatory role in the coordinated innate and acquired immune responses that maintain the integrity of many organs containing epithelia.[1,5,26,27] Nevertheless, the details of γδ T-cell functions are still not well understood as compared with those of αβ T cells. γδ T cells might play a defensive role against infections by various pathogenic microorganisms because exaggerated and severe infectious diseases occur in $\delta^{-/-}$ mice.[28-33] However, the same $\delta^{-/-}$ mice have also been demonstrated to display an increased host resistance to infection.[34,35] With regard to this, it is noteworthy that Vγ1$^+$ γδ T cells are reported to eliminate the

Table 1. Real-Time RT-PCR Analysis and ELISA Assay of Cytokines in the Colonic Tissues

	Cytokine							
Mice (n)	TNF-α	IL-1β	IL-6	TGF-β	IFN-γ	IL-7	IL-10	IL-12
RT-PCR (copies per 10^3 HPRT)								
WT (5)	11.4 ± 0.53	6.86 ± 0.55	2.45 ± 0.64	123 ± 14.6	ND	11.7 ± 1.03	2.63 ± 0.28	0.82 ± 0.21
$\gamma^{-/-}$ (5)	9.20 ± 0.76	6.01 ± 0.61	2.14 ± 0.88	106 ± 10.7	ND	10.2 ± 0.78	2.09 ± 0.31	0.33 ± 0.09
$\alpha^{-/-}$ (7)	457 ± 35.7**	260 ± 18.6***	7.06 ± 1.04*	538 ± 48.6*	64.4 ± 8.90	11.0 ± 0.89	4.99 ± 0.72	9.58 ± 1.67
$\alpha\gamma^{-/-}$ (6)	205 ± 51.8**	98.5 ± 21.4***	2.93 ± 0.73*	319 ± 57.4*	45.0 ± 15.6	9.29 ± 1.11	6.42 ± 2.11	6.63 ± 1.01
ELISA (pg/mg protein)								
$\alpha^{-/-}$ (7)	100 ± 12.2**	975 ± 70.1**	8.24 ± 1.39**	2.09 ± 0.11	8.34 ± 1.50			
$\alpha\gamma^{-/-}$ (6)	27.0 ± 8.28**	647 ± 24.6**	2.92 ± 0.52**	2.57 ± 0.31	5.92 ± 2.56			

NOTE. All results are expressed as mean ± SE.
*$P < .05$.
**$P < .01$.
***$P < .001$.
ND, not detected

Table 2. Real-Time RT-PCR Analysis and ELISA Assay of Chemokines in the Colonic Tissues

	Chemokine					
Mice (n)	KC	MIP-2	GCP-2	MCP-1	MIP-1α	MIP-1β
RT-PCR (copies per 10^3 HPRT)						
WT (5)	6.05 ± 1.38	0.23 ± 0.01	12.3 ± 4.11	1.67 ± 0.17	0.78 ± 0.07	1.36 ± 0.22
$\gamma^{-/-}$ (5)	11.8 ± 1.19	0.22 ± 0.03	4.03 ± 1.49	1.77 ± 0.26	0.80 ± 0.04	1.42 ± 0.11
$\alpha^{-/-}$ (7)	382 ± 88.6*	101 ± 12.8**	721 ± 136	23.3 ± 4.37	35.0 ± 3.01	19.8 ± 0.88
$\alpha\gamma^{-/-}$ (6)	65.2 ± 17.4*	28.0 ± 9.80**	307 ± 168	16.0 ± 4.11	23.9 ± 7.34	14.2 ± 3.97
ELISA (pg/mg protein)						
$\alpha^{-/-}$ (7)	144 ± 22.4**	113 ± 21.2*	737 ± 139			
$\alpha\gamma^{-/-}$ (6)	32.4 ± 13.4**	44.9 ± 14.0*	414 ± 219			

NOTE. All results are expressed as mean ± SE.
*$P < .05$.
**$P < .01$.

macrophages infected with *Listeria monocytogenes*, whereas γδ T cells using Vγ elements other than Vγ1 gene appear to lack the ability to control macrophages but possess the ability to protect hosts from the infection-induced tissue injury.[36,37] In contrast to the beneficial function of γδ T cells by virtue of the fact that they can maintain the homeostasis of different types of organs,[1-5,8,27] a deleterious effect of γδ T cells on the regulation of neutrophil-mediated tissue damage after thermal (postburn) injury has been reported.[38] In various chronic and/or autoimmune inflammatory diseases, such as collagen-induced arthritis in mice[39] and murine insulin-dependent diabetes,[40] γδ T cells have been shown to exert a protective effect. Conversely, γδ T cells may directly contribute to autoimmune pathology of murine experimental allergic encephalomyelitis[41] as well as lupus in MRL/lpr mice.[42] Overall, both the beneficial and detrimental roles of γδ T cells in inflammatory process are evident.[43]

In chemically induced acute intestinal inflammation models (2,4,6-trinitrobenzene sulfonic acid- or dextran sulfate sodium-induced colitis), γδ T cells have been reported to play a protective role.[11-14] Depletion of γδ T cells by administration of anti-TCRγδ mAb into $TNF^{\Delta ARE}$ mice with a high frequency of spontaneous ileitis[44] did not lead to any histologic changes of ileitis.[45] However, transfer of bone marrow-derived γδ T cells has been shown to induce CD-like colitis in the bone marrow transplanted CD3εtg colitis model.[46] Although the role of γδ T cells in spontaneous chronic colitis remains to be explored to date, the results of the present study demonstrate the exacerbating effect of γδ T cells on the UC-like chronic colitis in $\alpha^{-/-}$ mice (Figures 3 and 4 and Tables 1 and 2). Interestingly, approximately 20% of $\alpha^{-/-}$ mice during 20 to 60 weeks of age suffered from ARP, whereas none of age-matched $\alpha\gamma^{-/-}$ mice showed ARP (Figure 3*D*). Of note, there were no differences in the age of onset of colitis and in the incidence of colitis (~80%) among 20- to 32-week-old $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice, but much more severe colitis was observed in $\alpha^{-/-}$ mice as compared with $\alpha\gamma^{-/-}$ mice. Therefore, it is possible that ARP may reflect increased severity of colitis and that γδ T cells may participate in the development of ARP.

Absence of γδ T cells in $\alpha\gamma^{-/-}$ mice leads to a significantly reduced production of TNF-α, IL-1β, and IL-6 proteins in the colonic tissues. These findings are consistent with our previous results[24] showing the involvement of TNF-α, IL-1β, and IL-6 in the perpetuation of inflammatory process in $\alpha^{-/-}$ mice. These inflammatory mediators have been shown to be important for host defense and wound repair.[47] Both KC and MIP-2 attract neutrophils to inflamed sites, and, in certain microbial infection, the collection of neutrophils leads to suppuration reflecting an active and vigorous host response against microbes. We also confirmed that colonic extracts from $\alpha\gamma^{-/-}$ mice exhibited the significantly weaker chemotactic activity to neutrophils than those from $\alpha^{-/-}$ mice. KC and MIP-2 mRNA expressions were lower in all purified cell subsets ($Gr\text{-}1^+$, $F4/80^+$, $CD11c^+$, and $Gr\text{-}1^-F4/80^-CD11c^-$ cells) from $\alpha\gamma^{-/-}$ mice than those from $\alpha^{-/-}$ mice. Therefore, in the presence of γδ T cells, many types of immune cells may be triggered to produce more chemokines, followed by infiltration of neutrophils into the colonic mucosa in $\alpha^{-/-}$ mice. γδ T-cell responsiveness that is manifested by recruitment and activation of inflammatory cells in which neutrophils predominate has also been reported.[1,31] In this context, it is of importance to note that the activity and severity of UC patients with increase in γδ T cells in the inflamed mucosa[9,10] (Figure 1) can be judged by the activation state of neutrophils in circulation[48] as well as by regional accumulation of neutrophils in the colonic crypt walls (cryptitis) or in the lumen of crypts (crypt abscess).[49]

The suppressive role of B cells[50] and the aggravating role of $TCR\beta^{dim}$ T cells[22,51] in the pathogenesis of colitis in $\alpha^{-/-}$ mice have been reported. Therefore, it is possible that γδ T cells may contribute to the exacerbation of this colitis by dampening regulatory B-cell function or by cooperating the colitogenic $TCR\beta^{dim}$ T cells. The possible complicated mechanism remains to be explored in the future. Levels of TNF-α and IL-1β mRNA in $F4/80^+$ cells

are higher in $\alpha^{-/-}$ mice compared with $\alpha\gamma^{-/-}$ mice (see Supplementary Figure 1 online at www.gastrojournal.org), suggesting that γδ T cells may activate macrophages to secrete large amounts of proinflammatory cytokines.

In conclusion, although γδ T cells at the inflamed colonic LP of $\alpha^{-/-}$ mice may protect intestinal epithelial injury, proinflammatory cytokines and neutrophil- and/or monocyte-chemoattractant chemokines induced by γδ T cells may directly and/or indirectly contribute to increased severity of UC-like chronic colitis in $\alpha^{-/-}$ mice. Further understanding of the molecular mechanisms of γδ T cell-mediated exacerbation of colitis in $\alpha^{-/-}$ mice will lead us to work out better therapeutic strategies for human UC.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2007.11.056.

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BASIC—ALIMENTARY TRACT

DNA Hypermethylation Contributes to Incomplete Synthesis of Carbohydrate Determinants in Gastrointestinal Cancer

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See editorial on page 305.

Background & Aims: **It has long been known that malignant transformation is associated with abnormal expression of carbohydrate determinants. The aim of this study was to clarify the cause of cancer-associated abnormal glycosylation in gastrointestinal (GI) cancers.** ***Methods:*** **We compared the expression levels of "glyco-genes," including glycosyltransferases and glycosidases, in normal GI mucosa and in gastric and colorectal cancer cells. To examine the possibility that DNA hypermethylation contributed to the down-regulation of these genes, we treated GI cancer cells with 5-aza-2′-deoxycytidine (5-aza-dC), an inhibitor of DNA methyltransferase.** ***Results:*** **The silencing of some of these glyco-genes, but not up-regulation of certain molecules, was observed. The Sd[a] carbohydrate was abundantly expressed in the normal GI mucosa, but its expression was significantly decreased in cancer tissues. When human colon and gastric cancer cells were treated with 5-aza-dC, cell surface expression of Sd[a] and the transcription of *B4GALNT2*, which catalyzes the synthesis of the Sd[a], were induced. The promoter region of the human *B4GALNT2* gene was heavily hypermethylated in many of the GI cancer cell lines examined as well as in gastric cancer tissues (39 out of 78 cases). In addition, aberrant methylation of the *B4GALNT2* gene was strongly correlated with Epstein-Barr virus-associated gastric carcinomas and occurred coincidentally with hypermethylation of the *ST3GAL6* gene.** ***Conclusions:*** **Epigenetic changes in a group of glycosyltransferases including *B4GALNT2* and *ST3GAL6* represent a malignant phenotype of gastric cancer caused by silencing of the activity of these enzymes, which action may eventually induce aberrant glycosylation and expression of cancer-associated carbohydrate antigens.**

It has long been known that malignant transformation is associated with abnormal expression of carbohydrate determinants.[1] Many glycosyl epitopes such as sialyl Tn, Tn, T, and sialyl Lewis x/a (sLe[x/a]) have been reported to be cancer-associated antigens. Some of them show statistically significant correlations between the degree of their expression in cancer tissues and the postoperative prognosis of patients with many types of human cancers.[2–4] In addition, sLe[x/a] determinants are known to serve as ligands for E-selectin, which is inducibly expressed by endothelial cells, in hematogenous metastasis of cancers.[5,6] A long-standing debate is which is more important in understanding cancer-associated carbohydrate antigens, "neo-synthesis" or "incomplete synthesis." To verify the former hypothesis, the levels of many glycosyltransferases involved in "neo-synthesis" of tumor-related glycosyl epitopes and their mRNA expression have been studied; however, no conclusive results have been obtained to date.[7–9]

On the other hand, there is a group of carbohydrate determinants that is less expressed in cancer tissues when compared with their level in normal tissues. Because their structures are commonly more complicated, the concept of "incomplete synthesis," that the synthesis of complex carbohydrate determinants in nonmalignant cells might be impaired upon malignant transformation, has been proposed as an important cause of cancer-associated abnormal glycosylation.[10] The blood group Sd[a] carbohydrate antigen serves as a typical example among the latter

Abbreviations used in this paper: 5-aza-dC, 5-aza-2′-deoxycytidine; COBRA, combined bisulfite restriction analysis; DNMT, DNA methyltransferase; EBV, Epstein-Barr virus; FUT, fucosyltransferase; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GALNT, *N*-acetylgalactosaminyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; mAb, monoclonal antibody; HP, *Helicobacter pylori*; Sd[a]- 1, 4GalNAcT, 1,4*N*-acetylgalactosaminyltransferase, which forms Sd[a] carbohydrate determinants; ST, sialyltransferase; type II precursor, Gal 1,4GlcNAc-R.

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