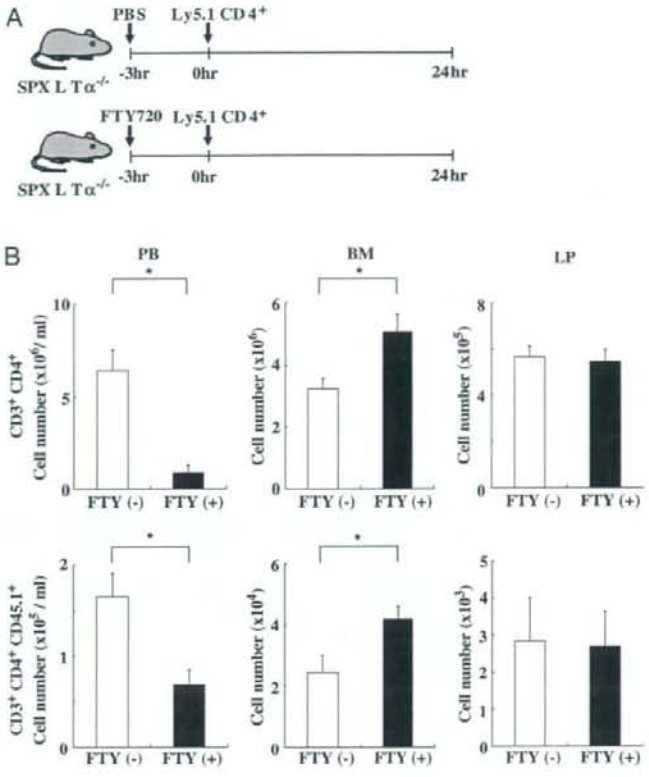

Figure 2. Transferred CD4+ T cells were preferentially accumulated in the BM of FTY720-treated LN/spleen-null mice. (A) SPX LT-$\alpha^{-/-}$ mice (Ly5.2+) were treated with FTY720 (1.0 mg/kg) or PBS at −3 h and then transferred with normal splenic CD4+ T cells (Ly5.1+) at 0 h. The changes in the absolute 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-$2^{-/-}$;

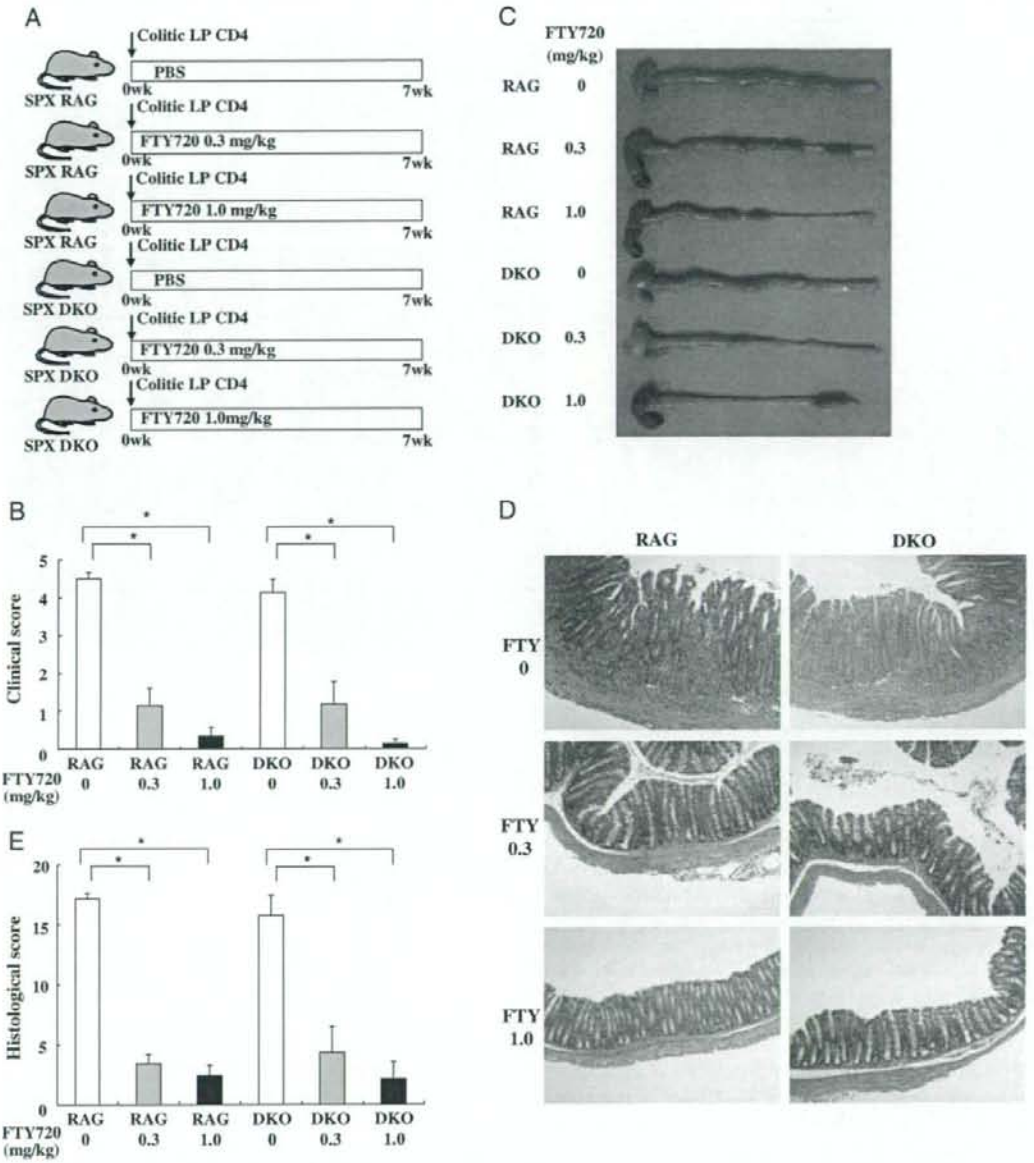

Figure 3. FTY720 prevents the development of colitogenic CD4$^+$ T_{EM}-mediated colitis in LN/spleen-null mice. (A) Colitogenic CD4$^+$ T_{EM} cells were isolated from the inflamed mucosa of colitic RAG-2$^{-/-}$ mice transferred with CD4$^+$CD45RB^{high} T cells. SPX LT-$\alpha^{+/+}$ × RAG-2$^{-/-}$ 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.

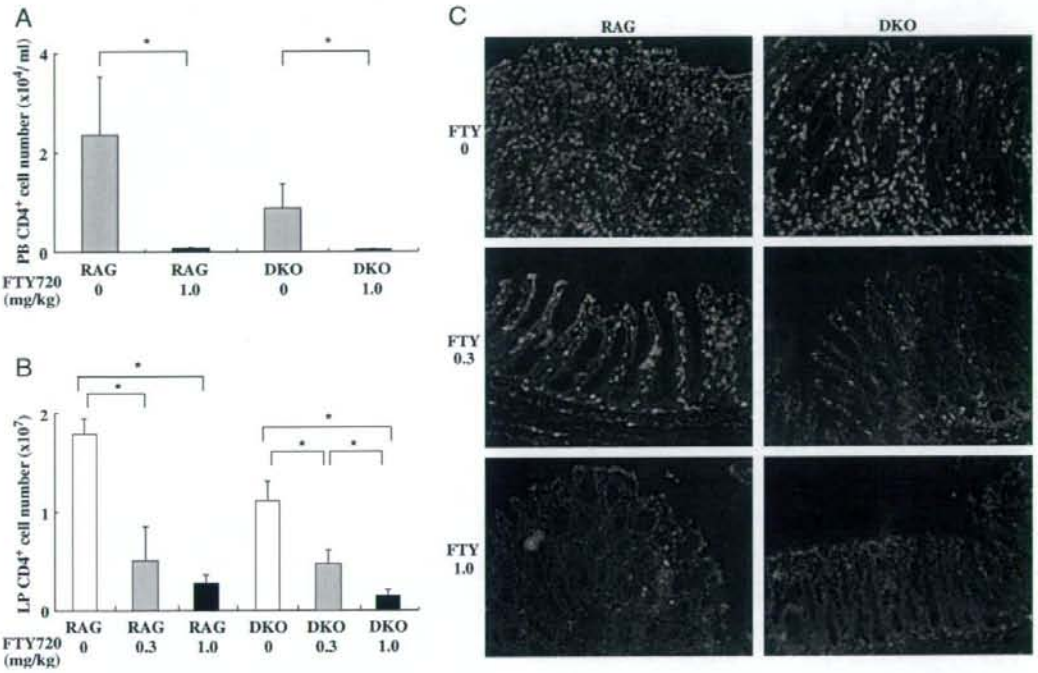

Figure 4. The number of LP CD4+ T cells was significantly decreased while PB lymphopenia was observed in FTY720-treated LN/spleen-null mice transferred with colitogenic CD4+ T_{EM} cells. (A–B) Number of CD3+CD4+ T cells in PB (A) or LP (B). Cells were stained with FITC-anti-CD3 and allophycocyanin-anti-CD4, 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.

$1.73 \pm 0.15 \times 10^7$, SPX LT-$\alpha^{-/-}$ × RAG-$2^{-/-}$; $1.07 \pm 0.14 \times 10^7$) 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-$7R\alpha^{high}$ 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}

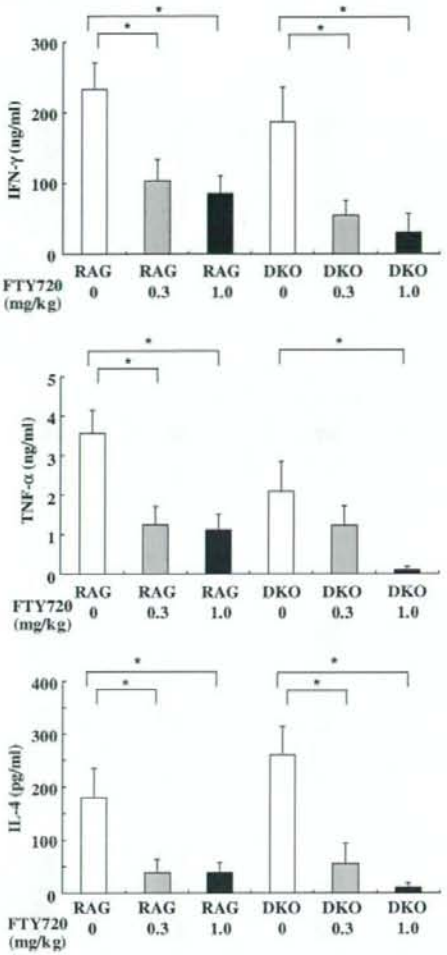

Figure 5. LP CD4$^+$ T cells in FTY720-treated LN/spleen-null mice transferred with colitogenic CD4$^+$ T_{EM} cells produce significantly less amount of Th1 and Th2 cytokines. LP CD4$^+$ T cells were isolated from mice 7 wk after the transfer of colitogenic LP CD4$^+$ T_{EM} cells and stimulated with 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

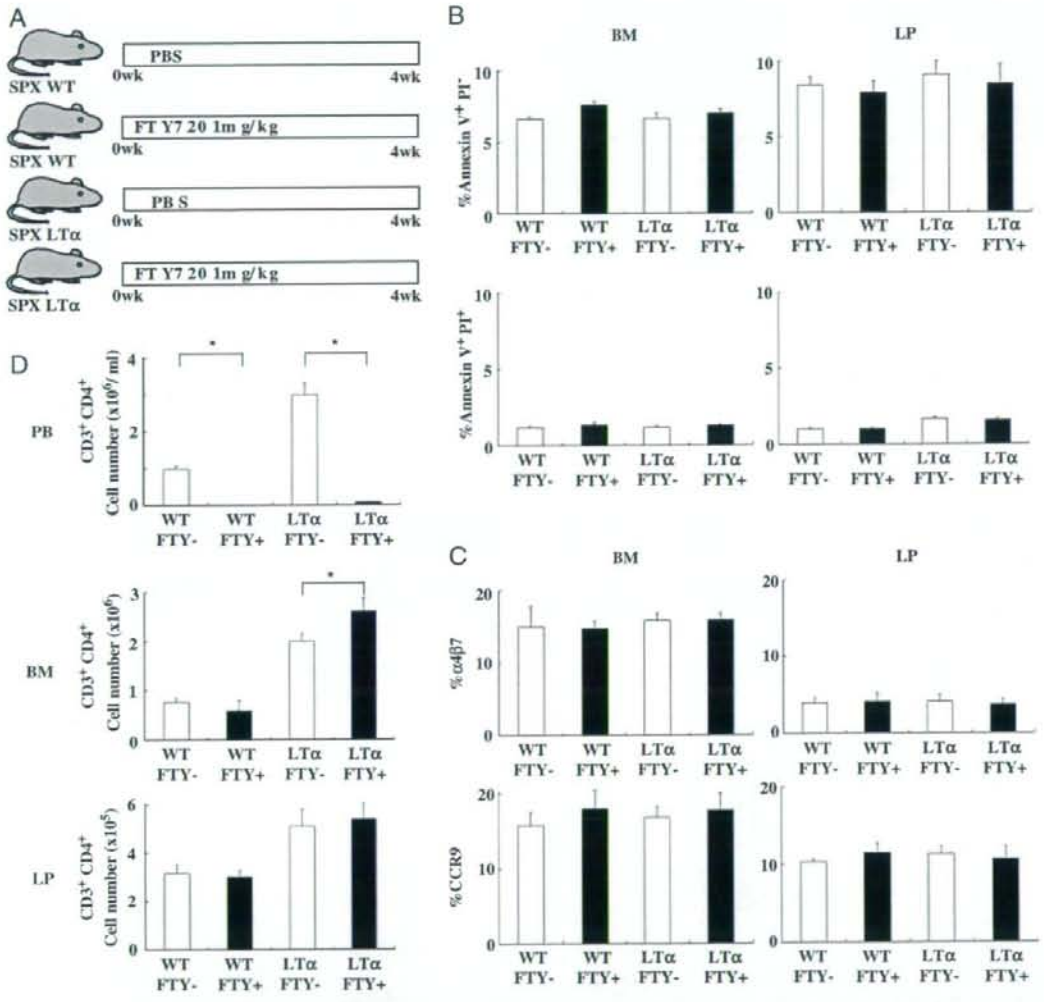

Figure 6. FTY720 treatment does not induce apoptosis or modulate the expression of gut-homing receptors of normal CD4+ T cells *in vivo*. (A) SPX WT or LT-$\alpha^{-/-}$ mice were treated with FTY720 (1.0 mg/kg) or PBS for 4 wk. (B) The number of dead or apoptotic cells in the isolated BM and LP $CD3^+CD4^+$ 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

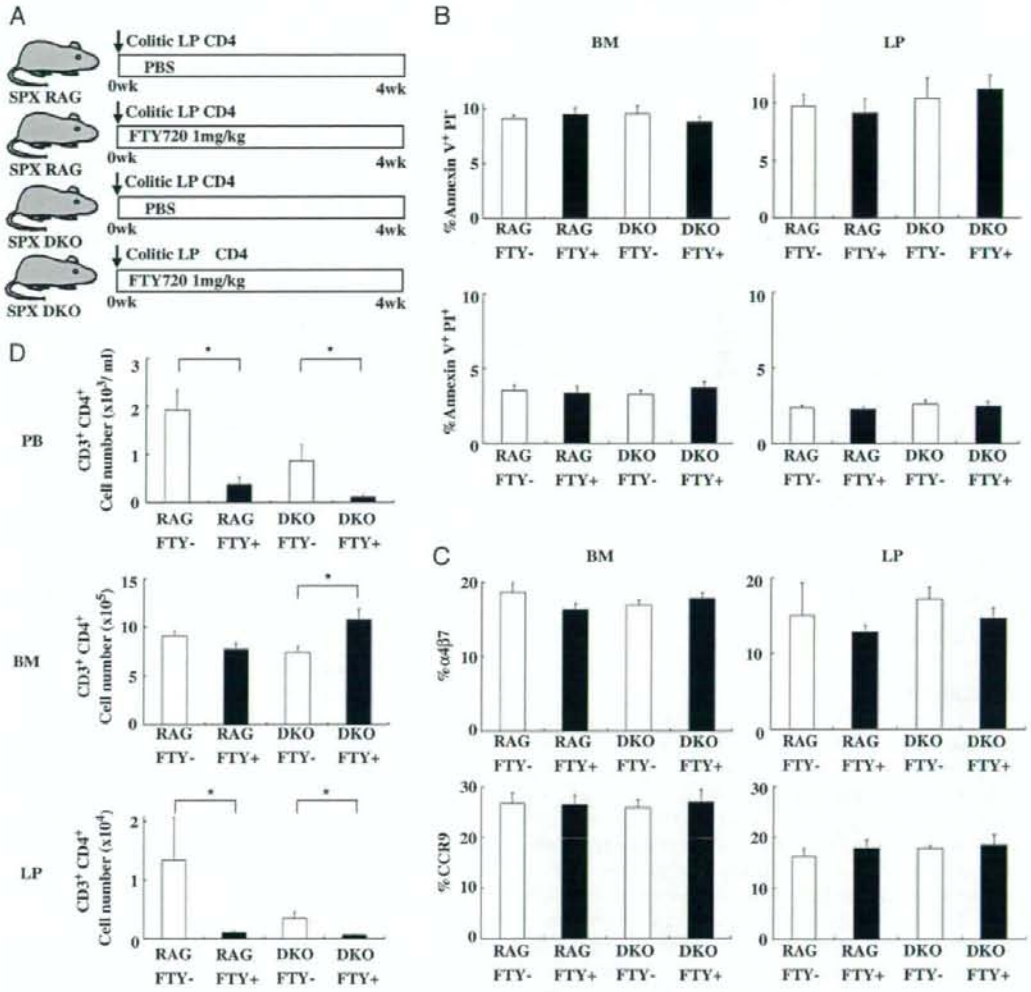

Figure 7. FTY720 treatment does not induce apoptosis or modulate expression of gut-homing receptors of CD4+ T cells in SPX RAG-2$^{-/-}$ or LT-α$^{-/-}$ mice transferred with colitogenic LP CD4$^+$T$_{EM}$ cells *in vivo*. (A) SPX RAG-2$^{-/-}$ or LT-α$^{-/-}$ mice transferred with colitogenic LP CD4$^+$T$_{EM}$ 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).

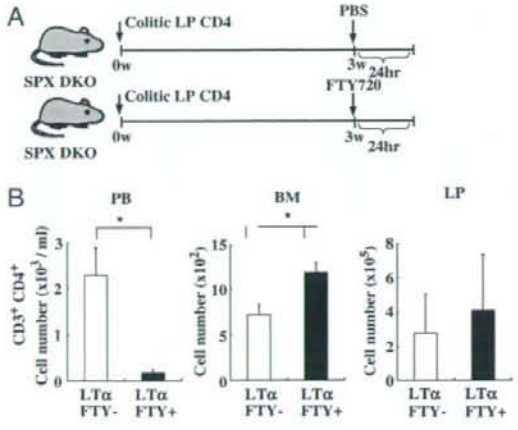

Figure 8. Colitogenic CD4$^+$ T cells were preferentially accumulated in the BM of FTY720-treated LN/spleen-null recipients transferred with colitic LP CD4$^+$ T cells. (A) SPX LT-$\alpha^{-/-}$ × RAG-2$^{-/-}$ mice were transferred with colitic LP CD4$^+$ T cells and treated with FTY720 (1.0 mg/kg) 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 400g 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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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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ELSEVIER

Effects of Immunosuppressants on the Progression of Hepatitis C in Hepatitis C Virus-Positive Renal Transplantation and the Usefulness of Interferon Therapy

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ABSTRACT

Objective. The purpose of this study was to analyze the effects of immunosuppressants on hepatitis C virus (HCV) replication to establish optimal immunosuppressive therapy in HCV-positive renal transplantation.

Materials and Methods. Cyclosporine (CsA), tacrolimus (Tac), mycophenolate acid (MPA), the active metabolite of mycophenolate mofetil (MMF), and methylprednisolone (MP) were administered to HCV replicon cells alone or in combination with interferon (IFN). HCV RNA was quantitatively determined. Of our 2064 recipients of renal transplantations between 1980 and 2005, 153 were HCV-positive. We analyzed changes in hepatic function and the efficacy of IFN therapy in these patients.

Results. Only CsA strongly inhibited the growth of HCV RNA (13.1% at 1.0 μg/mL). MPA enhanced the inhibition of the growth of HCV RNA in the presence of IFN. Tac and MP reduced, rather than enhanced, the efficacy of IFN. Progression to chronic hepatitis occurred in a significantly smaller number of patients in the CsA than the Tac group (6 vs 19; $P = .04$). Serum alanine aminotransferase (ALT) levels were comparable pretransplantation and posttransplantation in the CsA group (24.8 $\pm$ 20.5 vs 28.9 $\pm$ 28.3 IU/L, respectively, while a significant elevation was noted in the Tac group (22.2 $\pm$ 21.5 vs 32.6 $\pm$ 30.8 IU/L, respectively; $P = .024$). Two of 4 patients who underwent combination therapy with IFN and ribavirin during treatment with CsA and MMF obtained an HCV-negative status for over 24 weeks.

Conclusions. CsA effectively prevents the progression of chronic hepatitis in HCV-positive renal transplant patients. A greater response rate can be expected by concurrent administration of CsA and MMF under IFN therapy.

HEPATITIS C VIRUS (HCV) infection is a fatal disease because of progression from chronic hepatitis to liver cirrhosis and hepatocellular carcinoma after 10 years. Although a combination of interferon (IFN) and ribavirin therapy as anti-HCV therapy is prescribed for chronic hepatitis C, there is an insufficient curative effect. In contrast, renal transplant recipients show a high incidence of HCV infection. Therefore, the long-term survival of renal transplant recipients demands inhibition of the progression of chronic hepatitis C. There are many unidentified effects of immunosuppressants on the progression of HCV after renal transplantation in addition to interactions with IFN. Therefore, we undertook this study to establish the most advantageous immunosuppressive therapy to limit the progression of chronic hepatitis C among HCV-positive renal transplant recipients.

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MATERIALS AND METHODS

HCV Subgenomic Replicon Cell Culture System

HCV replicon cells, Huh7/Rep-Feo cells, which constitutively express an HCV replicon, enable the quantification of replication levels through the measurement of luciferase activity.[1,2] Huh7/Rep-Feo cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum containing 500 μg/mL G418 (Wako, Osaka, Japan) at 37°c under 5% CO_2.

Cyclosporine (CsA; Novartis Pharma AG, Basel, Switzerland) was dissolved in 50% EtOH. Tacrolimus (Tac; Astellas Pharma Inc, Tokyo, Japan) was dissolved in EtOH. Mycophenolic acid (MPA; Roche Palo Alto LLC, Calif, United States), the activated form of mycophenolate mofetil (MMF), was dissolved in DMSO. Methylprednisolone (MP) was purchased from LKT Laboratories, Inc (St Paul, Minn, United States) and recombinant human IFNα-2b was provided by Schering-Plough KK (Kenilworth, NJ, United States).

Luciferase activity was quantified using a luminometer (MicroLumat Plus LB96V; Berthold Technologies) and the Bright-Glo Luciferase Assay System (Promega) for 96-well plates. Assays were performed in triplicate and the results expressed as mean values ± SD as percentages of the controls, meaning the HCV RNA replication rate.

Renal Transplant Recipients

Among our 2064 renal tansplant recipients between 1980 and 2005, the 153 HCV-positive patients were stratified into the CsA versus the Tac group depending on the type of calcineurin inhibitor (CNI) administered. Immunosuppressants other than CNIs included MP and azathioprine or MMF. Changes in serum alanine aminotransferase (ALT) levels were analyzed between these 2 groups at 1 year posttransplantation. In addition, we compared the clinical courses of hepatic disease. IFN and ribavirin combination therapy was undertaken in 6 patients with chronic hepatitis C. Peginterferon α-2b (peg-IFNα-2b; 50–100 μg/body once a week) and ribavirin (400–800 mg/body once a day) were administered for 24 weeks. Efficacy was assessed based on whether the ALT levels normalized and whether HCV RNA disappeared as determined by the polymerase chain reaction. A sustained biochemical response (SBR) was defined as a sustained normalization of ALT activity during the 6-month follow-up after the end of treatment. A sustained virological response (SVR) was defined as sustained undetectable HCV RNA during the 6-month follow-up after the end of treatment.

RESULTS

HCV Subgenomic Replicon Cell Culture System

To assess the effects of immunosuppressants on HCV replication, subconfluent Huh7/Rep-Feo cells were cultured for 3 days with various concentrations of CsA, Tac, MPA, and MP alone or in combination with IFNα-2b (Fig 1). The luciferase activity was compared with that of untreated cells. The HCV RNA suppression rate was 13.1% at a CsA concentration of 1.0 μg/mL. Only CsA strongly inhibited the growth of HCV RNA in a dose-dependent manner. Tac, MPA, or MP failed to inhibit the growth of HCV RNA. Tac and MP reduced, rather than enhanced, the efficacy of IFNα-2b. In the presence of IFNα-2b, MPA enhanced the inhibition of the growth of HCV RNA.

Renal Transplant Recipients

Of the 153 HCV-positive patients, 59 and 62 continuously received only CsA or Tac, respectively. Mean ALT levels were compared before and 1 year after transplantation in the 2 groups. Significant increases were observed in the Tac group (pre: 22.2 ± 21.5 vs post 32.6 ± 30.8 IU/L; $P = .024$), but not in the CsA group (pre: 24.8 ± 20.5 vs post: 28.9 ± 28.3 IU/mL; Fig 2). To determine progression to hepatic disease the subsequent clinical course was evaluated in

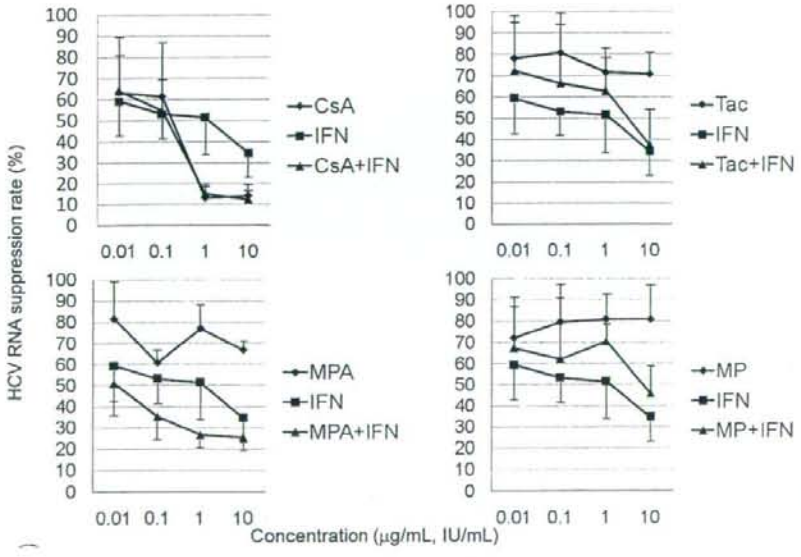

Fig 1. The direct effects of immunosuppressants and IFN on HCV RNA replication.

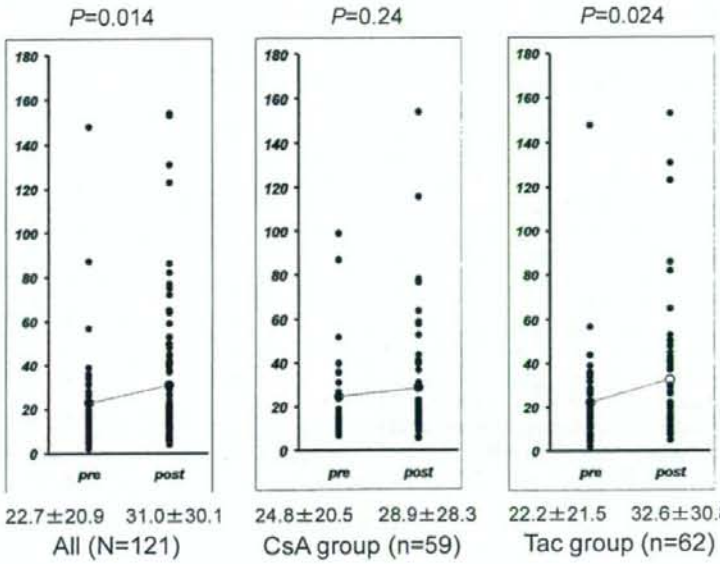

Fig 2. Changes in ALT levels pre- and post-renal transplantation.

these 2 groups. The number of patients who showed progression to chronic hepatitis was significantly smaller among the CsA (n = 6) than the Tac group (n = 19; P = .04; Table 1). The peg-INFα-2b plus ribavirin combination therapy was prescribed for 6 HCV-positive renal transplant recipients. All 6 patients received 2 (CsA and MMF) or 3 (CsA, MMF, and MP, 4 mg) immunosuppressants. All patients showed a high viral titer, namely, the pretreatment HCV RNA was over 100 KIU/mL. Four of the 6 patients completed the 24-week protocol of peg-IFNα-2b and ribavirin combination therapy. Administration of ribavirin was discontinued in the other 2 patients due to anemia. The therapeutic outcome was SBR in 4 (100%) and SVR in 2 (50%) of the 4 patients who completed the 24-week protocol of peg-IFNα-2b and ribavirin combination therapy (Table 2).

DISCUSSION

The recurrence of HCV negatively impacts graft and patient survivals in liver transplantation.[3,4] Because HCV recurrence at an early stage is inevitable after HCV-positive liver transplantation, it is necessary to inhibit the viral growth. Furthermore, because administration of immunosuppressants is essential after transplantation, the impact of the immunosuppressants must also be considered. The survival rate is low among HCV-positive of renal transplant recipients, but the impact of immunosuppressants on the progression of hepatitis has not yet been fully understood. In chimpanzees we showed that CsA suppressed the growth of HCV, which was at that time called non-A, non-B hepatitis virus.[5] Studies of HCV continued to be difficult thereafter because only humans and chimpanzees are infected with HCV. However, studies on anti-HCV drugs progressed rapidly due to the availability of HCV replicon cells,[6] which automatically replicate HCV subgenomes.

Watashi et al[7] demonstrated that CsA suppressed replication of HCV RNA in vitro. CsA is believed to inhibit HCV RNA replication as a result of its binding to the cellular target molecules, cyclophillins, resulting in generation of anti-HCV activity.

Table 1. Progression of Hepatitis C Among Patients in the CsA Vs Tac Groups

	CsA Group (n = 59)	Tac Group (n = 62)	P
Liver dysfuntion	32	44	.36
Acute hepatitis	0	1	.97
Chronic hepatitis	6	19	.04
Liver cirrhosis	1	2	.95
Death due to hepatic failure	1	3	.67
Natural improvement	23	24	.98

Table 2. Outcomes of Peg-IFNα-2b and Ribavirin Combination Therapy for Chronic Hepatitis C Post-Renal Transplantation

Characteristics	Values
Male/female	6/0
HCV genotype (1b/2b)	5/1
HCV RNA (high >100 KIU/mL/low <100 KIU/mL)	6/0
Achievement of protocol	4/6 (67%)
SBR	4/4 (100%)
Negative rate of HCV RNA	3/4 (75%)
SVR	2/4 (50%)

SBR, sustained biochemical response; SVR, sustained virological response.

Recently, treatment has included IFN alone or in combination with ribavirin for chronic hepatitis C,[8] which has been used to prevent and treat recurrence of HCV among patients with HCV-positive liver transplantation.[9] In the present study, we analyzed the impact on HCV RNA replication of a number of immunosuppressants used in clinical renal transplantation alone or in combination with IFN. Only CsA suppressed HCV RNA, showing strong effects alone. MPA has been suggested to have distinct anti-HCV effects in some studies.[10,11] In our study, however, MPA did not show any HCV RNA suppression when used alone, although when used in combnation with IFN, it showed potentiation.

These findings prompted us to compare changes in ALT levels after transplantation among HCV-positive renal transplant recipients treated in a CsA and a Tac group. Increases in ALT levels were significantly more suppressed among the CsA group. Progression to chronic hepatitis was significantly less marked in the CsA than in the Tac group, suggesting that CsA was effective to prevent or treat hepatitis. According to Inoue et al,[12] the IFN and CsA combination therapy produced greater SBR and SVR than IFN monotherapy among nontransplant chronic hepatitis C patients. Sugawara et al[13] reported that change of the CNI from Tac to CsA increased SVR in 5 of 8 patients who failed to respond to the IFNα-2b and ribavirin combination therapy, suggesting the clinical usefulness of CsA for patients with HCV.

We also used the peg-IFNα-2b and ribavirin combination in 6 HCV-positive renal transplant recipients. Based on the results of our in vitro study, we administered CsA, which showed direct anti-HCV effects when used alone, and MMF, a precursor of MPA, which showed potentiation when coadministered with IFN. An SBR of 100% and an SVR of 50% were obtained in the 4 patients who completed the 24-week protocol.

In conclusion, because HCV suppression is critical for HCV-positive transplant recipients, CsA, which shows direct, potent anti-HCV effects, is useful as an immunosuppressant. When IFN therapy is concurrently used, MMF, a precursor of MPA, shows potentiation of the effects.

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Original Article

Two flavonoids extracts from *Glycyrrhizae radix* inhibit *in vitro* hepatitis C virus replication

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Aim: Traditional herbal medicines have been used for several thousand years in China and other Asian countries. In this study we screened herbal drugs and their purified compounds, using the Feo replicon system, to determine their effects on *in vitro* HCV replication.

Methods: We screened herbal drugs and their purified extracts for the activities to suppress hepatitis C virus (HCV) replication using an HCV replicon system that expressed chimeric firefly luciferase reporter and neomycin phosphotransferase (Feo) genes. We tested extracts and 13 purified compounds from the following herbs: *Glycyrrhizae radix*; *Rehmanniae radix*; *Paeoniae radix*; *Artemisiae capillari spica*; and *Rhei rhizoma*.

Results: The HCV replication was significantly and dose-dependently suppressed by two purified compounds, isoliquiritigenin and glycycoumarin, which were from *Glycyrrhizae radix*. Dose-effect analyses showed that 50% effective concentrations were 6.2 ± 1.0 μg/mL and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively. The MTS assay did not show any effect on cell growth and viability at these effective concentrations, indicating that the effects of the two compounds were specific to HCV replication. These two compounds did not affect the HCV IRES-dependent translation nor did they show synergistic action with interferon-alpha.

Conclusion: Two purified herbal extracts, isoliquiritigenin and glycycoumarin, specifically suppressed *in vitro* HCV replication. Further elucidation of their mechanisms of action and evaluation of *in vivo* effects and safety might constitute a new anti-HCV therapeutics.

Key words: hepatitis C virus, herbal drugs, replicon

INTRODUCTION

HEPATITIS C VIRUS (HCV) infects 170 million people worldwide and is characterized by chronic liver inflammation and fibrogenesis leading to end-stage liver failure and hepatocellular malignancy.[1,2] The difficulty in eradicating HCV is attributable, in part, to limited treatment options against the virus. Currently, combination therapy using pegylated interferon-alpha (IFN) and ribavirin has been used worldwide.[3–5] The success rates, however, are almost half of patients treated. Furthermore, these therapies carry a significant risk of serious side effects. Thus, the development of alternative therapeutic agents against HCV is our high priority goal.

We have reported an HCV subgenomic replicon that expresses chimeric luciferase reporter "Feo" protein.[6] This Feo replicon supports stable and high levels of autonomous HCV RNA replication in transfected cells. Furthermore, the level of luciferase correlates well with levels of HCV RNA production, so that luciferase can be used as a reliable surrogate marker for HCV replication. This chimeric reporter replicon system has contributed the discovery of novel anti-HCV substances such as cyclosporins,[7–9] short interfering RNA,[10,11] interferon-gamma[12] and HMG-CoA reductase inhibitors.[13,14]

Traditional herbal drugs have been used for several thousand years in China and other Asian countries. Although these pharmacological activities are not fully

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Table 1 List of herbal drugs and their purified extracts

Herbal drug	Purified compound
Glycyrrhizae radix	Isoliquiritigenin
	Glycycoumarin
	Isoliquiritin
	Licuroside
Paeoniae radix	Paeoniflorin
	1,2,3,6-tetra-O-galloyl-β-D-glucose
Rhei Rhizoma	Rhein 8-O-β-glucoside
Rehmanniae radix	Acteoside
	Martynoside
	Isoacteoside
Artemisiae capillari spica	Demethoxycapillarisin
	3,4-di-o-galloylquinic acid
	Acteosyringone

characterized, they also have been safely used for many clinical conditions in Japan. For example, Sho-saiko-to (TJ-9; Xiao-Chae-Hu-Tang in Chinese), an oral medicine, which consists of seven herbal components (*Bupleuri radix, Pinelliae tuber, Scutellariae radix, Ginseng radix, Grycyrrhhizae radix, and Zingiberis rhizoma*),[15] has been clinically used for the treatment of chronic viral liver disease. It has been reported to regulate the cytokine production system in patients with hepatitis C[16] and to prevent the development of HCC in patients with non-B cirrhosis.[17] *Glycyrrhizin*, the major component of *Glycyrrhizae radix* (licorice), has also been used for the treatment of chronic hepatitis in Japan, known to have an alanine transaminase-lowering effect.[18,19] Despite the clinical effects of these herbal drugs, they did not suppress the HCV replication *in vitro*.[15]

In the present study, we applied the Feo replicon system to screen the herbal drugs and their purified compounds for their effects on *in vitro* HCV replication. Here, we show that two purified compounds from the herbal extracts specifically and substantially suppressed HCV replication.

MATERIALS AND METHODS

Purified compounds (Table 1)

THIRTEEN COMPOUNDS WERE purified from five herbal drugs: *Glycyrrhizae radaix; Rhemanniae radix; Paeoniae radix; Artemisiae Capillari Spica;* and *Rhei Rhizoma* (Table 1; Tsumura, Tokyo, Japan). These extracts were prepared at concentrations of 5 mg/mL in dimethyl sulfoxide (DMSO), then stored at −20°C until use. Recombinant human interferon (IFN) alpha-2b was obtained from Schering-Plough (NJ, USA).

Cell culture

A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (Sigma, MO, USA) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in a medium containing 200 μg/mL G418 (Wako, Osaka, Japan).

HCV subgenomic replicon construct

An HCV subgenomic replicon plasmid, pHCV1bneo-delS,[20] was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotrasnferase (pRep-Feo) (Fig. 1a). RNA was synthesized from pRep-

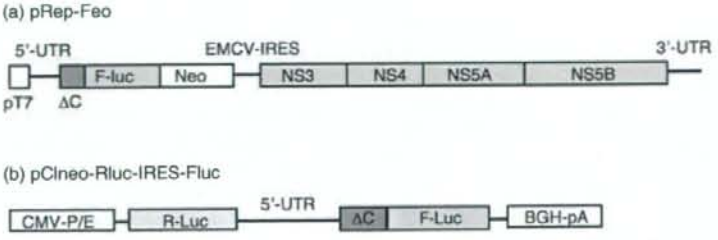

Figure 1 HCV subgenomic replicon and reporter plasmid constructs. (a) An HCV subgenomic replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene comprising the firefly luciferase (Fluc) and Neo, which we designated as "Feo"[6]. NS, nonstructural region; pT7, T7 promoter; 3' UTR, 3' untranslated region. (b) A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV-IRES-mediated translation efficiency. The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-internal ribosome entry site (IRES)-mediated initiation, was stably transfected into Huh7 cells.

Feo and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established.[10,21]

HCV-IRES reporter construct

A plasmid, pCIneo-Rluc-IRES-Fluc, was used to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (Fig. 1b).[22] The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-IRES-mediated initiation, was stably transfected into Huh7 cells. After culture in the presence of G418, Huh7/CRIF cells were established.[9] Activities of the HCV-IRES-mediated translation were measured by culture of Huh7/CRIF cells in the presence of drugs and by dual luciferase assays after 48 h.

Luciferase assays and measurements of antiviral activity

Huh7/Rep-Feo cells were cultured with various concentrations of herbal extracts or compounds. Levels of HCV replication were quantified by internal luciferase assay after 48 h of culture. Luciferase activities were quantified using a luminometer (Promega, WI, USA) and the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results were expressed as means ± SD as percentages of the controls. The 50% effective concentrations (EC50) were calculated using probit method. The determination of EC50 was performed three times, and presented as mean ± SD in each compound.

Realtime RT-PCR analysis

Total cellular RNA was extracted from cultured cells or liver tissue using ISOGEN (Nippon Gene, Tokyo, Japan). Two μg of total cellular RNA was used to generate cDNA from each sample using the SuperScript II reverse-transcriptase (Invitrogen, CA, USA). The replicon RNA expression levels were measured using the Applied Biosystems 7500 Fast Realtime PCR System (Applied Biosystems, CA, USA) and QuantiTect SYBR Green PCR Kit (QIAGEN, CA, USA). Sequences of a pair of primers has been described elsewhere.[23]

Northern blottings

Expression of HCV subgenomic RNA was detected as previously reported.[24] Total cellular RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Fifteen micrograms of the total cellular RNA was electrophoresed on a 1.0% denaturing agarose-formaldehyde gel and was transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech, Sweden). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for beta-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche, Germany) and visualized using a Fluoro-Imager (Roche).

Western blottings

Western blotting was done as reported previously.[24] Thirty micrograms of total cell lysate was separated using NuPAGE 4–12% Bis-Tris gels (Invitrogen, CA, USA) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche). The antibodies used were anti-NS5A (BioDesign, ME, USA), anti-core (provided by Dr. Wakita), and anti-beta-actin antibodies (Sigma).

MTS assays

To evaluate cell viability, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

HCV-JFH1 virus cell culture

An *in vitro* transcribed HCV-JFH1 RNA[25] was transfected into Huh7.5.1 cells.[26] Naïve Huh7.5.1 cells were subsequently infected by culture supernatant of the JFH1-RNA transfected Huh-7.5.1 cells, and subjected to culture in the presence of drugs. Culture medium was collected serially and HCV core antigen was measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen; Ortho-Clinical Diagnostics, Tokyo, Japan). Cellular virus expression was measured by the Western blotting using anti-core antibodies.[27]

Statistical analyses

Statistical analyses were performed using Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Suppression of HCV replication by purified herbal extracts, isoliquiritigenin and glycycoumarin

TO SCREEN THE herbal drugs and these purified extracts (Table 1) for their antiviral effects against HCV replication, Huh7/Rep-Feo cells were cultured with various concentrations of 5 herbal extracts; *Glycyrrhizae radaix, Rhemanniae radix, Paeoniae radix, Artemisiae capillari spica, and Rhei rhizoma*, and 13 compounds purified from these herbal extracts. Levels of HCV replication were quantified by internal luciferase assay after 48 h. None of the herbal extracts showed any effects on HCV replication (data not shown). On the other hand, among the 13 purified compounds, isoliquiritigenin and glycycoumarin, which were purified from *Glycyrrhizae radix*, suppressed replication of HCV replicon in a dose-dependent manner. The EC50s were 6.2 ± 1.0 and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively (Figs 2a,3a). The MTS assay did not show any effect on cell growth and viability (Fig. 2b), indicating that the antiviral action of the two compounds is not due to cytotoxic or antiproliferative effects. Huh7/Rep-Feo cells were cultured with various concentrations of isoliquiritigenin and glycycoumarin, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. After addition of each compounds, suppressive effect of the HCV replicon lasted for 48 h in a dose and time-dependent manner (Fig. 3b).

Realtime-RT-PCR and Western blotting analyses

In the realtime RT-PCR analysis and Northern blot analyses, levels of the replicon RNA decreased in a dose-dependent manner following treatment with isoliquir-

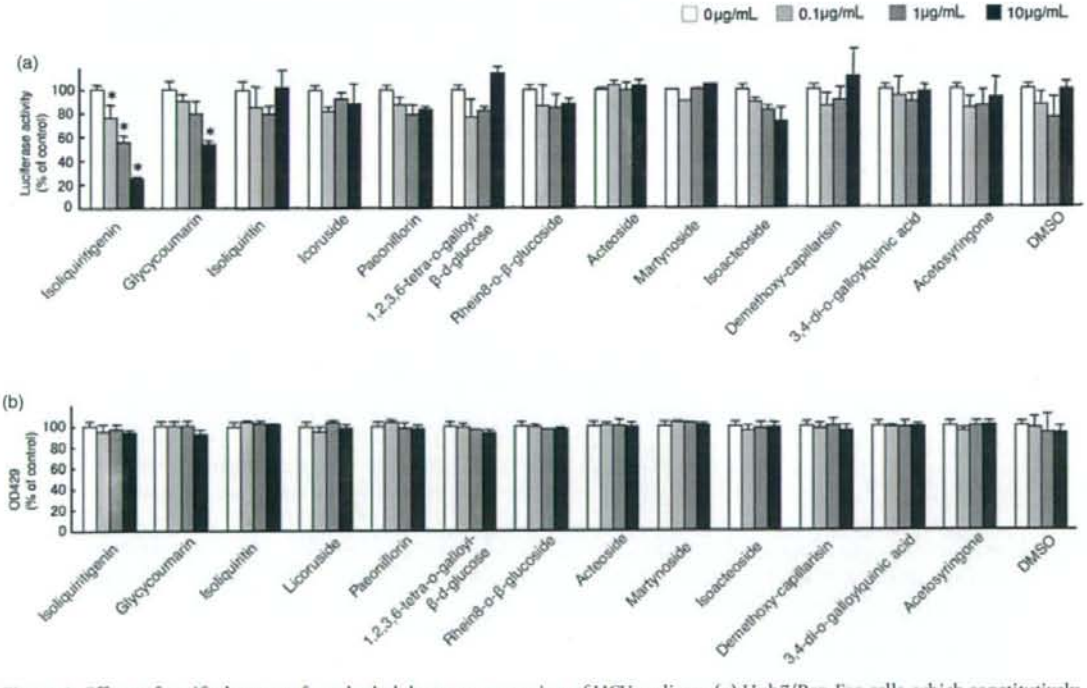

Figure 2 Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Asterisks indicate p-values of less than 0.05. (b) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of 13 compounds indicated. Error bars indicate mean ± SD.

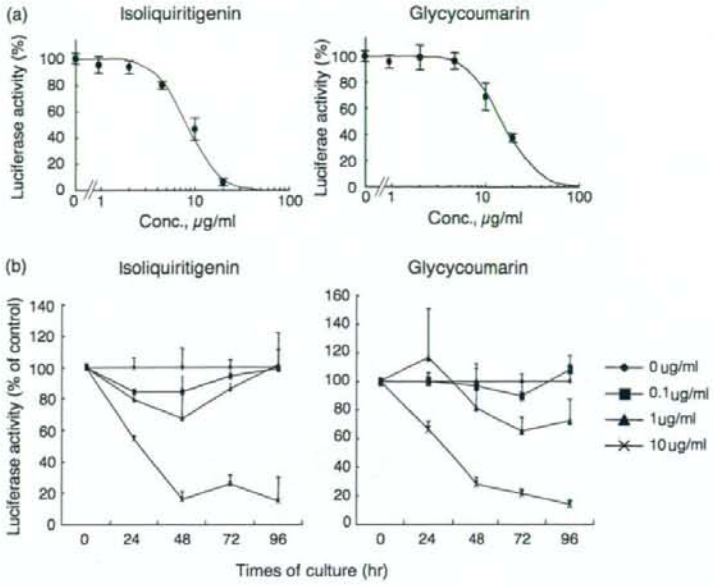

Figure 3 Dose- and time-dependent suppression of HCV replication by isoliquiritinenin and glycycoumarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycycoumarin. Error bars indicate mean ± SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo cells were cultured with the concentrations of isoliquiritigenin and glycycoumarin indicated. The internal luciferase activities were measured at times of culture indicated. Assays were performed in triplicate. Error bars indicate mean ± SD.

itigenin and glycycoumarin (Fig. 4a,b). Similarly, in Western blot analysis, the HCV non-structural protein, NS5A, which was translated from the HCV replicon, decreased by corresponding amounts in response to treatment with isoliquiritigenin and glycycoumarin (Fig. 4c). Densitometric analysis of NS5A protein showed that the intracellular levels of the virus protein in Huh7/Rep-Feo cells correlated well with the luciferase activities.

Absence of synergistic anti-HCV effects of interferon-alpha with isoliquiritigenin or glycycoumarin

To determine whether IFN and these two compounds have a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were cultured with combinations of IFNα-2b and isoliquiritigenin or glycycoumarin at various concentrations. The relative dose-inhibition curves of IFN were plotted under each fixed concentrations of isoliquiritigenin or glycycoumarin of 0, 0.1, 1, 10 μg/mL, respectively (Fig. 5). The curves did not show synergy of the two compounds and IFN against the HCV replicon. To see whether the action of isoliquiritigenin and glycycoumarin involve interferon-Jak/STAT-ISRE pathway, we conducted ISRE reporter assays. We transfected the p-55C1BLuc plasmid in Huh7 cells and cultured the cells in the presence of isoliquiritigenin or glycycoumarin. After 12 h of incubation, those drugs did not activate ISRE-promoter activities (data not shown). These results suggested that the action of the compounds on the intracellular replication of HCV replicon was independent of the IFN-ISRE pathway.

Isoliquiritigenin and glycycoumarin do not suppress the HCV IRES-dependent translation

We next determined whether these two compounds suppress HCV IRES-dependent translation, we used Huh7 cell line that had been stably transfected with pCIneo-Rluc IRES-Fluc (Huh7/CRIF; Fig. 1b). Treatment of these cells with isoliquiritigenin or glycycoumarin resulted in no significant change of the internal luciferase activities at concentrations of these two compounds that suppressed expression of the HCV replicon (Fig. 6a). The MTS assay did not show any effect on cell growth and viability at concentrations used in this assay (Fig. 6b).

Isoliquiritigenin and glycycoumarin suppress HCV-JFH1 virus cell culture

The demonstrated inhibitory effects isoliquiritigenin and glycycoumarin on HCV subgenomic replication were validated further by using HCV-JFH1 cell culture system.[25] As shown in Figure 7a, treatment of the cells with the two compounds suppressed time-dependent